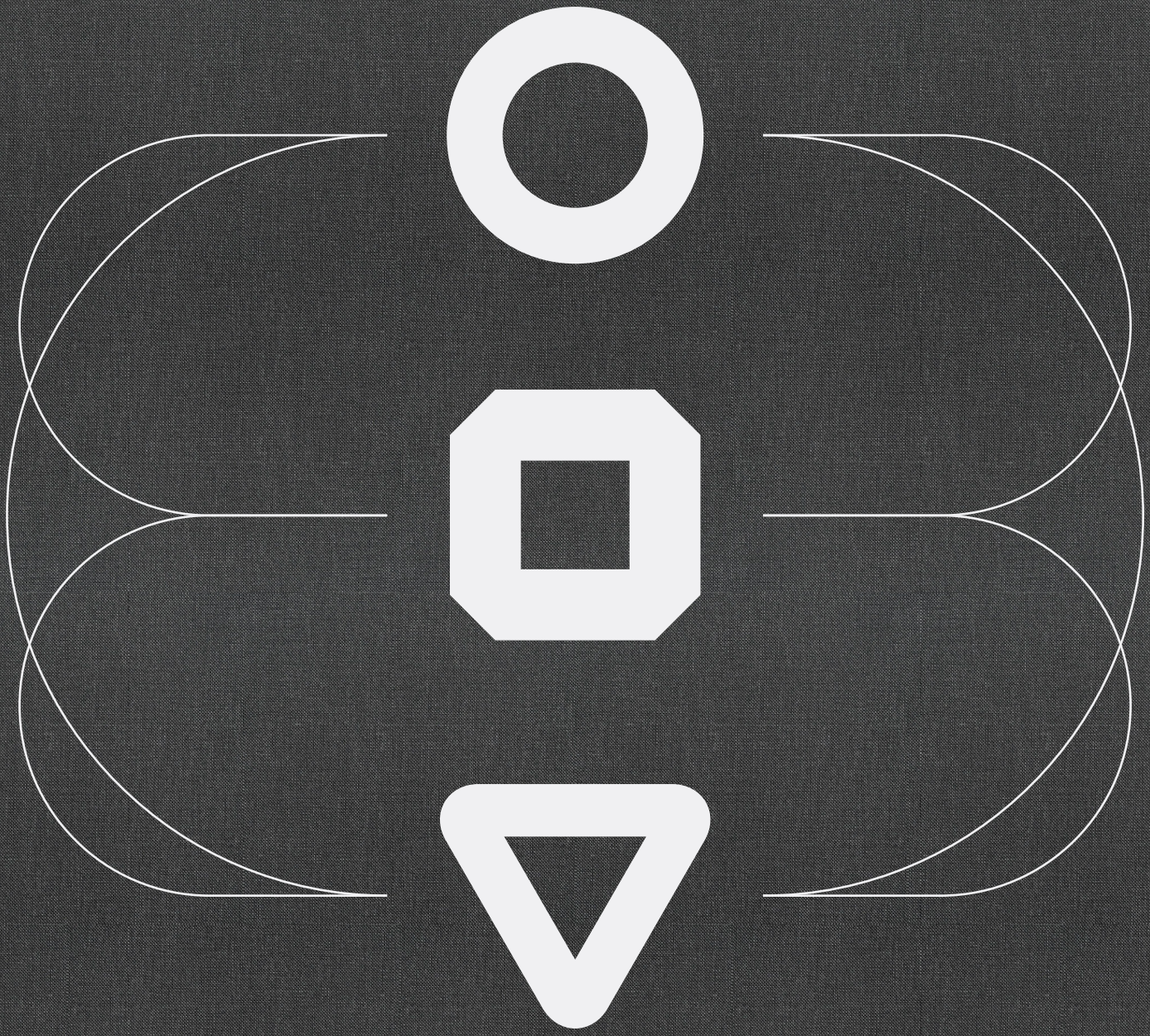
Antimicrobial resistant urinary tract infections: Pharmacodynamic profiling of oral fosfomycin.

Iain J. Abbott



Sustainable and effective antibiotic therapy is a critical, lifesaving resource. Urinary tract infections are one of the most common indications for antibiotic treatment. Yet the global rise of superbugs, which are bacteria that have acquired resistance to antibiotics, now greatly limits our treatment options.

Through the development of a novel and dynamic bladder infection laboratory model, this research rediscovers the optimised use of oral fosfomycin, an old antibiotic active against superbugs. By detailing efficacy under different simulations, we inform improved clinical practice to protect the activity of this antibiotic for the future.

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Antimicrobial resistant urinary tract infections:

Pharmacodynamic profiling of oral fosfomycin.

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A thesis submitted for the degree of Doctor of Philosophy at
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Abstract

Urinary tract infections (UTIs) are one of the most common conditions for which antimicrobials are prescribed worldwide. This, in turn, represents a significant driver for the emergence of antimicrobial resistance (AMR) in the community. Therefore, the optimisation of UTI treatment is paramount for improved clinical outcomes and the preservation of the activity of antimicrobials for the future.

Despite oral fosfomycin being recommended as a first-line agent in many international guidelines, the comprehensive pharmacodynamic profiling of fosfomycin efficacy in UTIs has never been done. Approaches to susceptibility testing and the licensed dose recommendations have remain unchanged since the 1970s.

This research program was undertaken to develop a preclinical *in vitro* model that mimics infections of the urinary bladder, simulate the dynamically changing antimicrobial concentrations and replicate the normal urodynamics of bladder filling and intermittent voiding. Furthermore, this model enables the study of pathogen response in the biomatrix of pooled human urine and the validation of a synthetic alternative.

The key findings of this research are as follows. Fosfomycin demonstrated good activity against a range of *Escherichia coli* isolates, although treatment failure and emergence of resistance was not predicted by standard susceptibility testing and current clinical breakpoints. Fosfomycin activity against *Klebsiella pneumoniae* was limited, with this species having an almost universal presence of a fosfomycin-resistant subpopulation at baseline. When comparing different dosing and urinary exposures, compared to a single dose with average exposure, there was no improvement in fosfomycin efficacy against *Enterobacterales* when urinary concentrations were high, or prolonged following multiple administered doses. The *in vitro* testing environment was found to impact upon the activity of fosfomycin, with a reduced propensity for isolates to develop emergence of resistance when comparing pooled human urine and synthetic alternatives with nutrient-rich standard laboratory media. The activity against *Pseudomonas aeruginosa* in synthetic human urine was limited, with the majority of isolates demonstrating emergence of resistance that was promoted by the administration of multiple doses over 7-days of simulated therapy. In contrast, fosfomycin activity against *Enterococcus* spp. was reasonable, albeit bacteriostatic, without any emergence of resistance when tested in synthetic human urine.

This research has contributed the *in vitro* data regarding fosfomycin susceptibility testing, spectrum of activity and clinical dosing recommendations. The development of the preclinical bladder infection model has proven to be a powerful tool for the pharmacodynamic profiling of oral fosfomycin for the treatment of UTIs. Furthermore, this research provides a strong basis for the future study of other antimicrobials to inform UTI-specific clinical breakpoints, optimised dosing schedules in human studies and for the study of new agents.

Publications during enrolment

First-author publications

Abbott IJ, Meletiadis J, Belghanch I, Wijma RA, Kanioura L, Roberts JA, Peleg AY, Mouton JW. Fosfomycin efficacy and emergence of resistance among *Enterobacteriaceae* in an *in vitro* dynamic bladder infection model. *J Antimicrob Chemother.* 2018 Mar 1;73(3):709-719.
doi: 10.1093/jac/dkx441
PMID: 29253153

Abbott IJ, Dekker J, van Gorp E, Wijma RA, Raaphorst MN, Klaassen CHW, Meletiadis J, Mouton JW, Peleg AY. Impact of bacterial species and baseline resistance on fosfomycin efficacy in urinary tract infections. *J Antimicrob Chemother.* 2020 Apr 1;75(4):988-996.
doi: 10.1093/jac/dkz519
PMID: 31873748

Abbott IJ, van Gorp E, Wijma RA, Meletiadis J, Roberts JA, Mouton JW, Peleg AY. Oral fosfomycin efficacy with variable urinary exposures following single and multiple doses against *Enterobacterales*: the importance of heteroresistance for growth outcome. *Antimicrob Agents Chemother.* 2020 Feb 21;64(3):e01982-19.
doi: 10.1128/aac.01982-19
PMID: 31907184

Abbott IJ, van Gorp E, Wijma RA, Meletiadis J, Mouton JW, Peleg AY. Evaluation of pooled human urine and synthetic alternatives in a dynamic bladder infection *in vitro* model simulating oral fosfomycin therapy. *J Microbiol Methods*. 2020 Apr;171:105861.
doi: 10.1016/j.mimet.2020.105861
PMID: 32035114

Abbott IJ, van Gorp E, Wijma RA, Dekker J, Croughs PD, Meletiadis J, Mouton JW, Peleg AY. Efficacy of single and multiple oral doses of fosfomycin against *Pseudomonas aeruginosa* urinary tract infections in a dynamic *in vitro* bladder infection model. *J Antimicrob Chemother*. 2020 Jul 1;75(7):1879-1888.
doi: 10.1093/jac/dkaa127
PMID: 32361749

Abbott IJ, van Gorp E, van der Meijden A, Wijma RA, Roberts JA, Mouton JW, Peleg AY. Oral fosfomycin treatment for enterococcal urinary tract infections in a dynamic *in vitro* model. *Antimicrob Agents Chemother*. 2020 May 21;64(6):e00342-20.
doi: 10.1128/aac.00342-20
PMID: 32253214

Abbott IJ, Roberts JA, Meletiadis J, Peleg AY. Antimicrobial pharmacokinetics and preclinical *in vitro* models to support optimized treatment approaches for uncomplicated lower urinary tract infections. *Expert Rev Anti Infect Ther*. 2020 Nov 16:1-25. Epub ahead of print.
doi: 10.1080/14787210.2020.1813567.
PMID: 32820686

Co-author publications

Gardiner BJ, Stewardson AJ, Abbott IJ, Peleg AY. Nitrofurantoin and fosfomycin for resistant urinary tract infections: old drugs for emerging problems. *Aust Prescr.* 2019 Feb;42(1):14-19.
doi: 10.18773/austprescr.2019.002.
PMID: 30765904

Wijma RA, Huttner A, van Dun S, Kloezen W, Abbott IJ, Muller AE, Koch BCP, Mouton JW. Urinary antibacterial activity of fosfomycin and nitrofurantoin at registered dosages in healthy volunteers. *Int J Antimicrob Agents.* 2019 Oct;54(4):435-441.
doi: 10.1016/j.ijantimicag.2019.07.018.
PMID: 31382030

Ten Doesschate T, Abbott IJ, Willems RJL, Top J, Rogers MRC, Bonten MM, Paganelli FL. *In vivo* acquisition of fosfomycin resistance in *Escherichia coli* by *fosA* transmission from commensal flora. *J Antimicrob Chemother*. 2019 Dec 1;74(12):3630-32.
doi: 10.1093/jac/dkz380
PMID: 31511878

Thesis including published works 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 six original research papers published in peer reviewed journals and one review article. The core theme of the thesis is antimicrobial pharmacokinetic and pharmacodynamic *in vitro* modelling. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Infectious Diseases, The Alfred Hospital and Central Clinical School, under the supervision of Prof Anton Y. 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.

For the original research chapters (chapters 2 – 7) a summary of the contribution by the co-authors to the work is as follows:

Rixt A. Wima (RW):
Fosfomycin quantification by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

Jordy Dekker (JD) and **Corné H. W. Klaassen (CW)**
Fosfomycin resistance molecular sequencing data.

Lamprini Kanioura (LK), Elke van Gorp (EG), Imane Belghanch (IB), Mere N. Raaphorst (MR) and **Aart van der Meijden (AM):**
Experimental technical assistance under my direct supervision.

Peter D. Croughs (PC):
Selection of *Pseudomonas aeruginosa* isolates.

Johan W. Mouton (JWM), Joseph Meletiadis (JM), Jason A. Roberts (JR) and **Anton Y. Peleg (AP)**
Supervision and guidance for experiment design, data analysis, interpretation and manuscript production.

In more detail, my specific contribution to all published work and that of the co-authors involved is detailed over the page.

Chapter	Publication title	Status	Student contribution (% and nature)	Co-author* contribution (% and nature)
1	Antimicrobial pharmacokinetics and preclinical *in vitro* models to support optimized treatment approaches for uncomplicated urinary tract infections	Published *Exp Rev Anti Infect Ther*	85% Planned and completed the literature review	**JR**, **JM** & **AP** (15%): Manuscript review
2	Fosfomycin efficacy and emergence of resistance among *Enterobacteriaceae* in an *in vitro* dynamic bladder infection model	Published *JAC*	72.5% *In vitro* model development, experimental work, data analysis, manuscript completion.	**JM** (5%): Model design input, data & manuscript review **IB** & **LK** (2.5%): Experimental assistance **RA** (2.5%): UPLC-MS/MS fosfomycin quantification **JR** (2.5%): Manuscript review **AP** & **JWM** (15%): Research supervision, model design, data & manuscript review
3	Impact of bacterial species and baseline resistance on fosfomycin efficacy in urinary tract infections	Published *JAC*	80% Research plan, experimental work, data analysis, manuscript completion.	**JD** & **CK** (2.5%): Molecular fosfomycin resistance data & analysis **EG** & **MR** (2.5%): Experimental assistance **RA** (2.5%): UPLC-MS/MS fosfomycin quantification **JM** (2.5%): Data & manuscript review **JWM** & **AP** (10%): Research supervision, data & manuscript review
4	Oral fosfomycin efficacy with variable urinary exposures following single and multiple doses against *Enterobacterales*: the importance of heteroresistance for growth outcome	Published *AAC*	82.5% Research plan, experimental work, data analysis, manuscript completion.	**EG** (2.5%): Experimental assistance **RA** (2.5%): UPLC-MS/MS fosfomycin quantification **JA** & **JM** (2.5%): Manuscript review **JWM** & **AP** (10%): Research supervision, data & manuscript review

Chapter	Publication title	Status	Student contribution (% and nature)	Co-author* contribution (% and nature)
5	Evaluation of pooled human urine and synthetic alternatives in a dynamic bladder infection *in vitro* model simulating oral fosfomycin therapy	Published *JMM*	82.5% Research plan, experimental work, data analysis, manuscript completion.	**EG** (2.5%): Experimental assistance **RA** (2.5%): UPLC-MS/MS fosfomycin quantification **JA** (2.5%): Manuscript review **JWM** & **AP** (10%): Research supervision, data & manuscript review
6	Efficacy of single and multiple oral doses of fosfomycin against *Pseudomonas aeruginosa* urinary tract infections in a dynamic bladder infection model	Published *JAC*	85% Research plan, experimental work, data analysis, manuscript completion.	**EG** (2.5%): Experimental assistance **RA** (2.5%): UPLC-MS/MS fosfomycin quantification **JD** (1.25%): *fosA* PCR **PC** (1.25%): isolate selection **JM** (2.5%): Manuscript review **JWM** & **AP** (5%): Research supervision, data & manuscript review
7	Oral fosfomycin treatment for enterococcal urinary tract infections in a dynamic *in vitro* model	Published *AAC*	87.5% Research plan, experimental work, data analysis, manuscript completion.	**EG** & **AM** (2.5%): Experimental assistance **RA** (2.5%): UPLC-MS/MS fosfomycin quantification **JR** (2.5%): Manuscript review **JWM** & **AP** (5%): Research supervision, data & manuscript review

** no co-authors were Monash University students*

I have not renumbered sections of submitted or published papers.

Student name: **Dr Iain J. Abbott**

Student signature: **Date:** 16/06/2020

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

Main Supervisor name: **Professor Anton Y. Peleg**

Main Supervisor signature: **Date:** 16/06/2020

Acknowledgements

This research was supported by an Australian Government Research Training Program (RTP) Scholarship (APP1114690), with laboratory expenses supported in part by the AIDA project LifeSciHealth Priority of the European Commission Seventh Framework Programme, FP7 (Preserving Old Antibiotics for the Future, grant number F3-2011-278348).

I am greatly appreciative Prof. Johan Mouton (dec. 9 July 2019) for welcoming me to Erasmus MC, Rotterdam, The Netherlands. He was an indispensable source of knowledge and his experience and insights guided the establishment of the dynamic *in vitro* PK/PD modelling research program. I will forever be indebted to Johan for the research methodology skills that he has imparted.

Special mention to Carla Roodbol de Goeij at the Department of Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre. Carla's tireless hard work and support ensured that all my laboratory requirements, no matter how strange, difficult or urgent, were met with precision, efficacy and generosity. Through every experimental up-and-down, success-and-failure, Carla was there by my side, always steadfast, loyal and dependable.

I am very thankful for the help and assistance provided by Lamprini Kanioura and A/Prof. Joseph Meletiadis, who looked after me from the moment I arrived in Rotterdam and were a continual source of support and information. My only regret is to have missed celebrating together with them their wedding in Greece, 31 August 2019.

Special thanks to Dr. Rixt Wijma, who helped me solve many problems, provided fantastic advice and was a true ally and confidante on the long road of PhD research. Be it over coffee, at a conference, going for run, or even climbing a mountain, I will forever treasure my friendship with Rixt and am forever fortunate to have known her warmth, kindness and incredible spirit for life.

Thank you to Prof. Jason Roberts for his insights, professional guidance and unending support. Always approachable, honest and open, I will continue to strive to emulate Jason's character and demeanour as I move forwards in my career.

As I end this chapter of my life and look ahead to the next one, I am indebted to my primary supervisor, Prof. Anton Peleg, who has been an insurmountable source of support and encouragement for many more years than this PhD-research program has encompassed. His drive, enthusiasm, critical insights, care and guidance has shaped the physician-scientist that I am today.

Finally, none of this would have been possible without the love and support of my partner Blaise Macdonald. While shaping the visual representation of my research, his design was intelligence made visible. Blaise positively influences the way I think and, more importantly, the way I feel about my research. He is the rock that grounds me. Thank you for following me to the other side of the world and back again.

Chapter 1

Introduction and literature review

Chapter 1

Introduction and literature review

Urinary tract infections (UTIs) are extremely common infections, experience worldwide by millions of people every year. In this introduction the following review article, invited for publication in *Expert Review of Anti-infective Therapy*, provides a background to the aetiology of UTIs, the problems faced with the emergence of antimicrobial resistance (AMR), local and international treatment guidelines and the urinary pharmacokinetics of antimicrobial agents. The second part of the review examines the history and utility of preclinical bladder infection *in vitro* models, which originally date back to the 1960s. Here, we discuss the key considerations in order to deliver robust *in vitro* data to inform antimicrobial activity in UTIs and human dosing studies.

Highlights

- Urinary tract infections (UTIs) affect millions of people every year and are a common indication of antimicrobial use in the community and a potential driver for emergence of resistance.

- Yet, how we diagnose UTIs, report antimicrobial susceptibility and provide treatment recommendation are based on practices unchanged for decades and old pharmacokinetic (PK) and pharmacodynamic (PD) data.

- Greater understanding of the specific urinary PK characteristics of recommended oral antimicrobial agents and the interaction between the host and the uropathogen, can inform optimized selection and dosing when tackling multidrug resistant (MDR) phenotypes.

- The use of dynamic *in vitro* PK/PD models allows us to explore antimicrobial spectrum of activity, dosing and duration of therapy, and the drivers of emergence of resistance in a site-specific infection model.

- This robust preclinical data can promote the rational design of antimicrobial dosing, guide laboratory susceptibility testing and translate findings into clinical trials to inform treatment guidelines.

EXPERT REVIEW OF ANTI-INFECTIVE THERAPY
https://doi.org/10.1080/14787210.2020.1813567

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REVIEW

Antimicrobial pharmacokinetics and preclinical *in vitro* models to support optimized treatment approaches for uncomplicated lower urinary tract infections

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ABSTRACT

Introduction: Urinary tract infections (UTIs) are extremely common. Millions of people, particularly healthy women, are affected worldwide every year. One-in-two women will have a recurrence within 12-months of an initial UTI. Inadequate treatment risks worsening infection leading to acute pyelonephritis, bacteremia and sepsis. In an era of increasing antimicrobial resistance, it is critical to provide optimized antimicrobial treatment.

Areas covered: Literature was searched using PubMed and Google Scholar (up to 06/2020), examining the etiology, diagnosis and oral antimicrobial therapy for uncomplicated UTIs, with emphasis on urinary antimicrobial pharmacokinetics (PK) and the application of dynamic *in vitro* models for the pharmacodynamic (PD) profiling of pathogen response.

Expert opinion: The majority of antimicrobial agents included in international guidelines were developed decades ago without well-described dose–response relationships. Microbiology laboratories still apply standard diagnostic methodology that has essentially remained unchanged for decades. Furthermore, it is uncertain how relevant standard *in vitro* susceptibility is for predicting antimicrobial efficacy in urine. In order to optimize UTI treatments, clinicians must exploit the urine-specific PK of antimicrobial agents. Dynamic *in vitro* models are valuable tools to examine the PK/PD and urodynamic variables associated with UTIs, while informing uropathogen susceptibility reporting, optimized dosing schedules, clinical trials and treatment guidelines.

ARTICLE HISTORY
Received 12 June 2020
Accepted 19 August 2020

KEYWORDS
Antimicrobial resistance; drug development; *in vitro* infection models; pharmacokinetics/pharmacodynamics; urinary tract infection

1. Introduction

Urinary tract infections (UTIs) annually affect 150 million people, with significant medical and financial implications [1–4]. More than 1-in-10 women report a UTI within the past year [5]. The incidence in premenopausal sexually active women is 0.5–0.7 cases/person-year [6]. For postmenopausal women, important risk factors are mechanical and physiological changes affecting bladder emptying [7]. Other risk factors include voiding abnormalities, diabetes, neurogenic bladder, pregnancy, obesity, renal tract calculi, prostate hypertrophy, urethral stents and indwelling catheters [8]. This review examines urinary pharmacokinetics (PK) of oral antimicrobial agents recommended for the treatment of uncomplicated UTIs in adults. We discuss how *in vitro* PK/pharmacodynamic (PD) models can be designed to inform optimized therapy (Figure 1) [9,10].

2. UTI pathogenesis

Uropathogenic *Escherichia coli* (UPEC) is the causative pathogen of UTIs in approximately 70–80% of cases [2,11]. In a retrospective study examining urinary samples collected in emergency departments in Europe (2010–2016), isolate characteristics were: *E. coli* 67.6%, *Klebsiella* spp. 8.4%, *E. faecalis* 4.5%, *Proteus* spp. 3.8%, *Pseudomonas* spp. 2.4%, *Enterobacter* spp. 2.1% and *S. saprophyticus* 1.9% [12]. Urinary pathogens often originate in the gastrointestinal tract, migrate to the periurethral area and colonize the urethra. The proximity of the urethral opening to the vaginal cavity and rectum in women allows uropathogens to reach the bladder before removal by micturition [13]. Migration relies on bacterial expression of pili, flagella and adhesins recognizing uroepithelium, and metabolic adaptations allow for replication in the harsh urinary environment. Local invasion occurs by toxin and protease production [14]. A small proportion of *E. coli* are internalized into host cells, some can go onto form intracellular bacterial communities (IBCs) [15,16]. Invasion into deeper layers of the bladder wall can also occur, forming quiescent intracellular reservoirs [17]. Uropathogen proliferation can lead to ascending infection into the ureters and renal parenchyma, with bacteremia occurring by crossing the tubular epithelial barrier into the renal vasculature.

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Article highlights

- Urinary tract infections (UTIs) affect millions of people every year and are a common indication of antimicrobial use in the community and a potential driver for emergence of resistance.
- Yet, how we diagnose UTIs, report antimicrobial susceptibility and provide treatment recommendation are based on practices unchanged for decades and old pharmacokinetic (PK) and pharmacodynamic (PD) data.
- Greater understanding of the specific urinary PK characteristics of recommended oral antimicrobial agents and the interaction between the host and the uropathogen, can inform optimized selection and dosing when tackling multidrug resistant (MDR) phenotypes.
- The use of dynamic *in vitro* PK/PD models allows us to explore antimicrobial spectrum of activity, dosing and duration of therapy, and the drivers of emergence of resistance in a site-specific infection model.
- This robust preclinical data can promote the rational design of antimicrobial dosing, guide laboratory susceptibility testing and translate findings into clinical trials to inform treatment guidelines.

Natural protection from UTI relies upon host-factors of the bladder, innate immunity, urine composition and urodynamics. In 1961, Cox and Hinman [18] published a series of *in vitro* and induced human bacteriuria experiments, demonstrating the bladder's defense to infection. Increased fluid intake dilutes bacteria in the bladder and high-volume frequent urination can assist bacterial clearance. Under these dynamics, bacterial growth rate in urine is a critical factor. Urine, however, is depleted of nutrients and the low pH, high nitrates and high urea make it naturally antimicrobial. Moreover, it is an incredibly complex biological waste product, containing over 2000 different metabolites/chemicals [19]. Specific alterations in urinary composition in different patient populations (e.g. trauma patients, elderly, diabetes) can promote uropathogen growth [20–22]. Urinary antimicrobial peptides are additional defenses to bacterial infection [23,24].

3. Initial assessment

The classification of UTIs into uncomplicated and complicated, although well established in clinical practice, may represent an over-simplification of the clinical syndrome [25,26]. In general, an uncomplicated UTI presumes infection is either confined to the bladder (uncomplicated cystitis) or an ascending infection (uncomplicated pyelonephritis) in a non-pregnant woman without factors that compromise normal host defenses [27]. A UTI in a male patient is commonly associated with anatomic/functional changes, or prostate involvement, and is often considered complicated.

UTIs are often empirically managed in the community without laboratory diagnostics. A urine culture can, however, provide confirmation of the diagnosis, organism identification and antimicrobial susceptibility [8,28]. Cultures are commonly requested only when the diagnosis is unclear or following a second UTI. An alternative approach has been to defer antimicrobials until culture and susceptibility are available, with or without the use of simple analgesics [29–33]. Studies examining such antimicrobial-sparing approaches have, however, reported increased rates of ascending infections in those not receiving antibiotics upfront [34–36].

When considering enrollment in epidemiological and interventional studies, the six symptoms of the Acute Cystitis Symptom Score (ACSS) have been shown to be strongly associated with UTI diagnosis (Table 1) [37]. European Medicines Agency (EMA) recommend that females enrolled into UTI studies should have frequency, urgency, dysuria and pyuria (≥10 WBCs/mm^3) in a midstream specimen [38]. Similarly, the US Food and Drug Administration (FDA) state that females should have evidence of pyuria and at least two of dysuria, urinary frequency, urinary urgency, and suprapubic pain [39]. In contrast, pyelonephritis is commonly associated with fever, chills, rigors, and flank pain.

4. Uropathogens and susceptibility testing

The urinary bladder is not sterile and contains its own diverse microbiome [40–42]. Asymptomatic bacteriuria can play a protective role in preventing UTI recurrences [43], and is only treated in specific situations (pregnancy, <1 month after kidney transplant, prior to invasive urological procedures) [44].

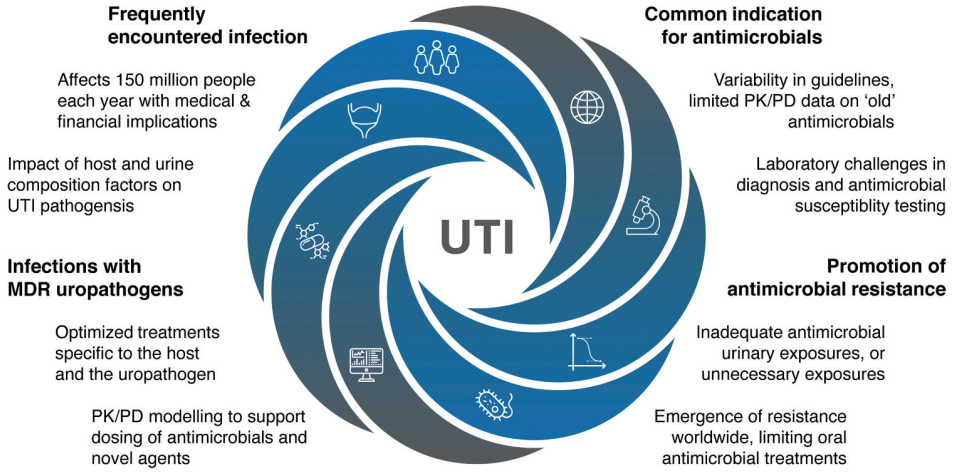

Figure 1. Overview of the challenges associated with urinary tract infections.

UTI: Urinary tract infection; PK: Pharmacokinetics; PD: Pharmacodynamics; AMR: Antimicrobial resistance; MDR, multidrug-resistant.

Table 1. Acute Cystitis Symptom Score (ACSS) Questionnaire.

Domain 1: Typical
Urinary frequency
Urgency
Dysuria
Incomplete bladder emptying
Suprapubic pain
Hematuria
Domain 2: Differential
Flank pain
Vaginal discharge
Urethral discharge
Fever
Domain 3: Quality of life
Level of discomfort
Impact on work/everyday activities
Impact on social life
Domain 4: Additional
Menstruation
Premenstrual symptoms
Menopausal symptoms
Pregnancy
Diabetes mellitus
Follow-up: Dynamics
Changes in symptoms

The ACSS contains 18 questions divided into 4 domains used at the first visit: typical acute cystitis symptoms, differential diagnosis symptoms, impact on quality of life and additional relevant questions. The first 3 domains are scored on a severity scale and totaled (0 = no, 1 = mild, 2 = moderate, 3 = severe), while the remaining are 'Yes/No' answers. The same questionnaire can also be used on follow-up. The follow-up dynamics domain details the overall impression of any changes in symptoms (0 = all symptoms resolved, 1 = majority of symptoms resolved, 2 = majority of symptoms still present, 3 = no change in symptoms, 4 = worsening of symptoms). The questionnaire has been translated into multiple different languages. Adapted from http://www.acss.world/index.html [37].

An optimally collected urine sample from a symptomatic patient is paramount for the clinical relevance of a culture result. An instructed collection of midstream urine, with prior skin cleansing preparation, can limit normal flora contamination. Samples should be collected prior to antimicrobials and should remain at room temperature for <30 min.

Standard urinary culture techniques have important limitations: failure to detect slow-growing, fastidious and non-aerobic microorganisms, inability to reliably detect microorganisms $<10^3$ cfu/mL, and difficulty differentiating pathogenic Gram-positive bacteria from normal flora [45]. Technological advancements have not been widely incorporated into practice, such as: next-generation urine point-of-care tests; urine biomarkers (differentiate between infection and colonization); flow cytometry; application of MALDI-TOF MS and molecular methods directly on urine, including Next-Generation-Sequencing [46].

The traditional urinary bacterial density threshold of $\geq 10^5$ cfu/mL to differentiate between infection and colonization is likely to be fundamentally flawed and may falsely exclude around 50% of patients with a probable diagnosis of an acute infection [37]. Lowering this threshold ($\geq 10^2$ cfu/mL) demonstrates higher sensitivity but risks over-diagnosis and unnecessary treatment [47]. Low levels of *E. coli* (10^1–10^2 cfu/mL) can represent an accurate diagnosis in symptomatic women [48]. Similarly, molecular techniques have identified *E. coli* where cultures were negative [49]. In contrast, significant quantities of *Enterococcus* spp. or Group B *Streptococcus* may still represent contaminating normal flora, highlighted where invasively collected cultures do not yield the same result as midstream collection, with the exception of *E. coli* that was consistently found in both samples [48]. It is also not infrequent to recover yeast in urine, even at high densities, but these patients seldom have a yeast UTI. An important caveat is where bacteriuria may reflect passive filtration from a hematogenous source, for example *Staphylococcus aureus* [50], *Candida* spp [51]. and *Cryptococcus* spp [52]., or represents renal parenchymal infection, as seen in *Burkholderia pseudomallei* [53,54], or evident of acute infection, or chronic carriage, with invasive *Salmonella* infections [55].

European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) report UTI breakpoints for some antimicrobials (e.g. nitrofurantoin, fosfomycin, trimethoprim, amoxicillin-clavulanate) [56,57]. In these instances, antimicrobial susceptibility results, determined by MIC or disk diffusion, relate only to uncomplicated UTIs and/or infections originating from the urinary tract. There are, however, inherent challenges in relating susceptibility testing results in a nutrient-rich *in vitro* environment to the antimicrobial activity at the site of infection [58,59]. Furthermore, individual results have variability, both biological and technical, and do not directly relate to *in vivo* antimicrobial concentrations [60,61].

5. Antimicrobial resistance

In 2018, the European Antimicrobial Resistance Surveillance Network (EARS-Net) reported population-weighted mean resistance percentages in invasive *E. coli*, finding resistance to aminopenicillins in 57.4%, followed by fluoroquinolones in 25.3%, third-generation cephalosporins (3GC) in 15.1% and aminoglycosides 11.1%. Resistance to carbapenems remained rare. For invasive *K. pneumoniae*, resistance rates were higher, with resistance to 3GC in 31.7%, followed by fluoroquinolones in 31.6%, aminoglycosides in 22.7%, and carbapenems in 7.5%. There was significant variability between countries [62]. Increasing resistance overtime has also been observed. In the US, from 2003 to 2012, ciprofloxacin-resistance in urinary *E. coli* isolates rose from 3.6% to 11.8%, and trimethoprim-sulfamethoxazole resistance from 17.2% to 22.2% [63]. Interestingly, resistance to nitrofurantoin changed only slightly (0.7 to 0.9%). Similarly, in Belgium, multidrug resistant (MDR) *E. coli* prevalence increased from 28.4 to 34.3% from 2005 to 2011–12, however, susceptibility to nitrofurantoin (90%) was maintained [64]. In Australia, over a 5-year period (2013–2017) there was a significant rise in fluoroquinolone-resistance (*E. coli*: 6.5–9.0% to 10.0–12.3%; *K. pneumoniae*: 5.1–5.3 to 6.0–7.0%) despite no increase in use [65]. A progressive rise in antimicrobial resistance among enterococcal urinary isolates has also been observed. Vancomycin-resistant *Enterococcus* (VRE) now accounts for up to 80% of *E. faecium* isolates in some hospitals [66,67].

6. Treatment guidelines

The primary goals of treatment are to ameliorate UTI symptoms and reduce the risk of progressing to severe disease. Unnecessary antimicrobials should be avoided. When indicated, antimicrobials should ideally be administered as a single dose or short course therapy (3–5 days). Prolonged courses can be poorly tolerated, promote emergence of resistance [10] and increase the risk of recurrence due to alterations in normal flora [68–72]. Longer treatment durations are recommended for ascending infections, although this assertion has been recently challenged [73–76].

Although treatment guidelines optimize care on a population level, many variations exist between different countries, societies and jurisdictions (Table 2) [27,77–101]. In a European study, 13 different antimicrobials were recommended as first-line therapy across 15 national guidelines [100,102]. Similar findings were found across different medical societies in the US [103]. The 2010 Infectious Diseases Society of America (IDSA)/European Society for Microbiology and Infectious Diseases (ESCMID) Uncomplicated Cystitis and Pyelonephritis guidelines are being updated, with an expected publication in 2022 [101]. A systematic review of randomized controlled trials of UTI treatment has challenged the durations of therapy adopted in clinical guidelines, suggesting that for some agents, shorter courses of therapy could recommended [104].

Adherence to guidelines is also suboptimal. In a US cohort of >600,000 healthy women with UTIs, over half were prescribed non-guideline-recommended antimicrobials, and three-quarters had treatment durations not consistent with the guidelines [105]. A 12-month review of US primary care clinics showed antimicrobials were optimally prescribed in only 29% of cases [106]. In Lebanon, appropriateness of prescriptions was only 21% (a composite of drug, dose and duration) [107]. In South Africa, 51.2% of errors were due to the incorrect treatment duration and 17.1% due to the incorrect drug [108]. In 2014, a European study revealed a range in adherence to guidelines, from 22.2% in Slovenia to 72.7% in the Netherlands [109]. In aged-care homes in Australia, antimicrobial selection, dose, frequency and duration was concordant with national recommendations in 22.3% of prescriptions [110]. Understanding why primary care providers make decisions is vital. A qualitative study identified areas for improvement, including awareness and familiarity with guidelines, attitudes to antimicrobial efficacy, impact of patient characteristics on choice of therapy and various other external barriers [111]. Antimicrobial package size has been linked to poor accordance with recommended treatment durations [112,113].

Table 2. Comparison of international antibiotic treatment guideline recommendations for uncomplicated UTI[a].

	FOT	NIT	PIV	TMP	SXT	QIN	AMX	AMC	1/2GC	3GC	Other	Ref.
EAU (2019)	**(1)**	**(1)**	**(1)**	(2)[c]	(2)[c]				(2)		(2) cefadroxil	[27]
International (UpToDate 2019)	**(1)**[b]	**(1)**	**(1)**	**(1)**	**(1)**	(3)		(2)	(2)	(2)		[77]
International (Sanford 2019)	**(1)**	**(1)**	(2)		**(1)**	(2)		(2)	(2)	(2)		[78]
Australia/NZ (eTG 2019)	(3)[b]	**(1)**		**(1)**	(2)	(3)	(2)	(2)	**(1)**			[79]
India (2019)	**(1)**[b]	**(1)**			(2)						(2) ertapenem, amikacin	[80]
UK (NICE 2018)	(2)	**(1)**	(2)	**(1)**								[81]
France (2018)	**(1)**	-[d]	(2)	-[e]	-[e]	-[f]						[82]
Asia (2018)	**(1)**	**(1)**			**(1)**	(2)		**(1)**	**(1)**	(2)		[83]
Korea (2018)	**(1)**	-[g]	-[h]		(2)[i]	**(1)**		(2)[i]		**(1)**		[84]
Germany (2017)	**(1)**	**(1)**	**(1)**	(2)[c]	(2)	(3)				(3)	**(1)** nitroxoline	[85]
Canada (2017)	**(1)**	**(1)**		**(1)**	**(1)**	(2)						[86]
Russia (2017)	**(1)**	**(1)**				(2)				(2)	**(1)** furazidin	[87]
Sweden (2017)		**(1)**	**(1)**	(2)[i]						(2)		[87]
Spain (2017)	**(1)**	**(1)**			-[j]	(2)	-[k]	(3)		(3)		[88]
Denmark (2016)			**(1)**	**(1)**							**(1)** sulfametizole	[89]
Norway (2016)		**(1)**	**(1)**	**(1)**		(2)						[90]
Belgium (2016)	(2)	**(1)**		(2)								[91]
Serbia (2016)	**(1)**	**(1)**			**(1)**	(2)		(2)	(2)			[92]
Japan (2015)	(2)					**(1)**		(2)	(2)	(2)	(2) faropenem	[93]
Sth Africa (2014/15)	(2)	(2)				**(1)**		(2)				[94,95]
Finland (2015)	-[l]	**(1)**	**(1)**	**(1)**		(2)	(2)	(2)		(2)		[87]
Poland (2015)	**(1)**	**(1)**		**(1)**	**(1)**	(2)		(2)			**(1)** furazidin	[87]
Croatia (2014)	**(1)**	**(1)**				(3)		(2)	(2)	(2)		[96]
Switzerland (2014)	**(1)**	**(1)**			**(1)**	(2)		(2)	(2)			[97]
Netherlands (2013)	(2)	**(1)**		(3)								[98]
Austria (2012)	**(1)**		**(1)**			**(1)**						[99,100]
IDSA/ESCMID (2010)	**(1)**	**(1)**	**(1)**		**(1)**	(2)		(2)	(2)	(2)		[101]

FOT, fosfomycin. NIT, nitrofurantoin. PIV, pivmecillinam. TMP, trimethoprim. SXT, trimethoprim-sulfamethoxazole. QIN, fluoroquinolone. AMX, amoxicillin. AMC, amoxicillin-clavulanate. 1GC, first-generation cephalosporin. 2GC, second-generation cephalosporin. 3GC, third-generation cephalosporin. EAU, European Association of Urology. [a]Recommended first-line (1, bold/green), second-line alternative (2, yellow) and third-line/reserve alternative (3, gray) agents. [b]Suggest reserving use of fosfomycin for documented MDR infections, or when other first-line agents cannot be used. [c]Only if local resistance in *E. coli* is < 20%. [d]Not recommended for regulatory reasons (very rare but risk of severe toxicity). [e]Not recommended due to resistance rates close to 20%. [f]Not recommended because of their selection pressure and preference to be saved for more severe infections. [g]Not routinely available in Korea; introduction urgently recommended as a first-line agent. [h]Not routinely available in Korea; recommended for introduction but to be used with caution. [l]Recommended only after susceptibility testing. [j]Trimethoprim/sulfamethoxazole not recommended for empiric therapy because resistance rates in *E. coli* is > 20% in Spain. [k]Ampicillin and amoxicillin not recommended given the high incidence of resistance. [i]Fosfomycin not licensed in Finland.

7. Antimicrobial urinary pharmacokinetics

High urinary antimicrobial concentrations are essential for efficacy in UTI treatment. In a rat model, systemically administered therapy only reaching the bladder tissue (and not the bladder lumen) was found to be insufficient for bacterial eradication [114]. In pyelonephritis, however, antimicrobial concentrations must also achieve adequate levels within the renal parenchyma, for which serum concentrations are used as a surrogate marker. Optimizing urinary antimicrobial exposures can restore the activity of narrow-spectrum agents. For example, a study of hospitalized elderly patients showed that a narrow-spectrum cephalosporin given intravenously (cefazolin) was non-inferior to fluoroquinolones [115]. This is despite many reports questioning the adequacy of β-lactam antibiotics [116,117] and surveillance studies reporting high resistance rates [12,118–120]. Where antimicrobial concentrations are high in urine, regardless of the susceptibility result, clinical efficacy has been reported, such as amoxicillin for resistant *Enterococcus* spp. and doxycycline for *P. aeruginosa* [121–124]. Furthermore, changes in urinary pH (acidic or alkaline) can alter antimicrobial activity [125–130]. The following details the oral antimicrobials commonly recommended for UTIs (Figure 2 [131]), highlighting the urinary drug concentrations and susceptibility testing criteria (Table 3) [56,57] and the EUCAST $MIC_{50/90}$ and epidemiological cutoff values (ECOFF) (Table 4) [132,133].

7.1. Fosfomycin

Fosfomycin is the smallest of all antimicrobials (by molecular weight) with no cross-resistance with other classes. It acts by inhibiting cell wall synthesis by irreversibly inhibiting enolpyruvyl transferase that catalyzes the first step of peptidoglycan biosynthesis. Fosfomycin trometamol (synonym: tromethamine) is the common oral form. It has a wide spectrum against Gram-negative (especially *E. coli* isolates) and Gram-positive uropathogens (not including *S. saprophyticus*). The majority of extended-spectrum β-lactamase (ESBL)-producing *E. coli* and other MDR isolates have retained fosfomycin-susceptibility [134]. Activity against *K. pneumoniae* is less certain due to heteroresistance [135–139]. Similarly, monotherapy against *P. aeruginosa* isolates appears to be inadequate [140,141]. Fosfomycin has also been used for vancomycin-resistant *Enterococcus* (VRE) [142]. A 2016 review of fosfomycin susceptibility reported high levels of susceptibility across many uropathogens [143], although this observation is complicated by poor correlations between susceptibility methods and poor prediction of efficacy [138,144–146]. Resistance is mediated by a number of different mechanisms, including: mutations in transporter genes (*glpT* and *uhpT*) and their regulators, inactivation enzymes (*fos* genes), alteration of the active binding site (*murA*) and peptidoglycan recycling pathways [147].

Fosfomycin trometamol (Monurol®, Monuril®) is licensed as a single 3 g oral dose and rapidly achieves effective urinary concentrations for >24 h. Approximately 35–50% of the oral dose is excreted unchanged in the urine at a rate approximating creatinine clearance. There are marked variabilities in urinary concentrations after a standard dose, with an approximate average (range) peak concentration between 1000–2000 mg/L (600–3500 mg/L), occurring 4–8 h after dosing, with concentrations maintained >32 mg/L for >48 h [148–150]. Single dose therapy is beneficial for patient convenience and tolerance, limits emergence of resistance and minimizes collateral damage. Acidification increases activity (2-fold lower MIC) [128,134]. Most common side effects are nausea, vomiting and diarrhea. Several different repeat dosing regimens (daily, 48- or 72-hourly dosing for 3–7 days) have been used [151–154], although lack strong evidence and are associated with more diarrhea [149,155–157]. Although historically fosfomycin was reported with clinical success rates of >90%, more recently in a 2018 randomized controlled trial found the single 3 g dose resulted in 58% clinical resolution, compared to 70% with 5-days of nitrofurantoin [158].

7.2. Nitrofurantoin

Nitrofurans are synthetic compounds, of which nitrofurantoin is most widely used. Antimicrobial activity requires intracellular bacterial nitrofuran reductase enzymes for multiple mechanisms of action including binding to bacterial ribosomes and inhibiting synthesis of DNA, RNA and other metabolic enzymes [159]. It has activity against common uropathogens (*E. coli, E. faecalis* and *S. saprophyticus*), less certain activity against *Klebsiella* spp., and intrinsic resistance in *Proteus* spp., *Pseudomonas* spp. and *E. faecium*. Emergence of resistance is rare, with resistance rates commonly <5% [12,63,119,160] or <10% in MDR *E. coli* isolates [64]. Resistance is primarily due to a loss of intracellular nitroreductase activity (chromosomal *nfsA* and *nfsB*) purported to induce a fitness cost disturbing growth kinetics [161]. Plasmid encoded efflux pump, OqxAB, is an additional resistance mechanism [162].

Nitrofurantoin is available in different formulations: microcrystals (largely no longer available), macrocrystals (Macrodantin® or Furadantin®), monohydrate/macrocrystals (Macrobid® or Furabid®) and formulations marketed as 'prolonged release' [163]. Dosing is dependent on the formulation. Macrocrystal formation is given 50–100 mg four-times-daily for 5-days. Long-acting formulations are given 100 mg twice-daily. Bioavailability is 20–30%, increasing to 40% when administered with food, and is rapidly excreted via the kidney, resulting in low serum concentrations and high urinary concentrations. Excretion is saturable, with equivalent urinary concentrations after 50 mg four-times-daily compared with 100 mg three-times-daily (macrocrystal formulation) [164]. Maximum urine concentrations are around 100 mg/L, but vary between 15–230 mg/L, occurring 3–10 h after dosing, but heavily dependent on formulation and fasting status [165]. Activity is enhanced under acidic conditions [166]. Nitrofurantoin is well tolerated with mild gastrointestinal side effects (in 5–16%). Severe toxicity (interstitial pneumonitis, liver toxicity, neurological reactions) appear to be extremely

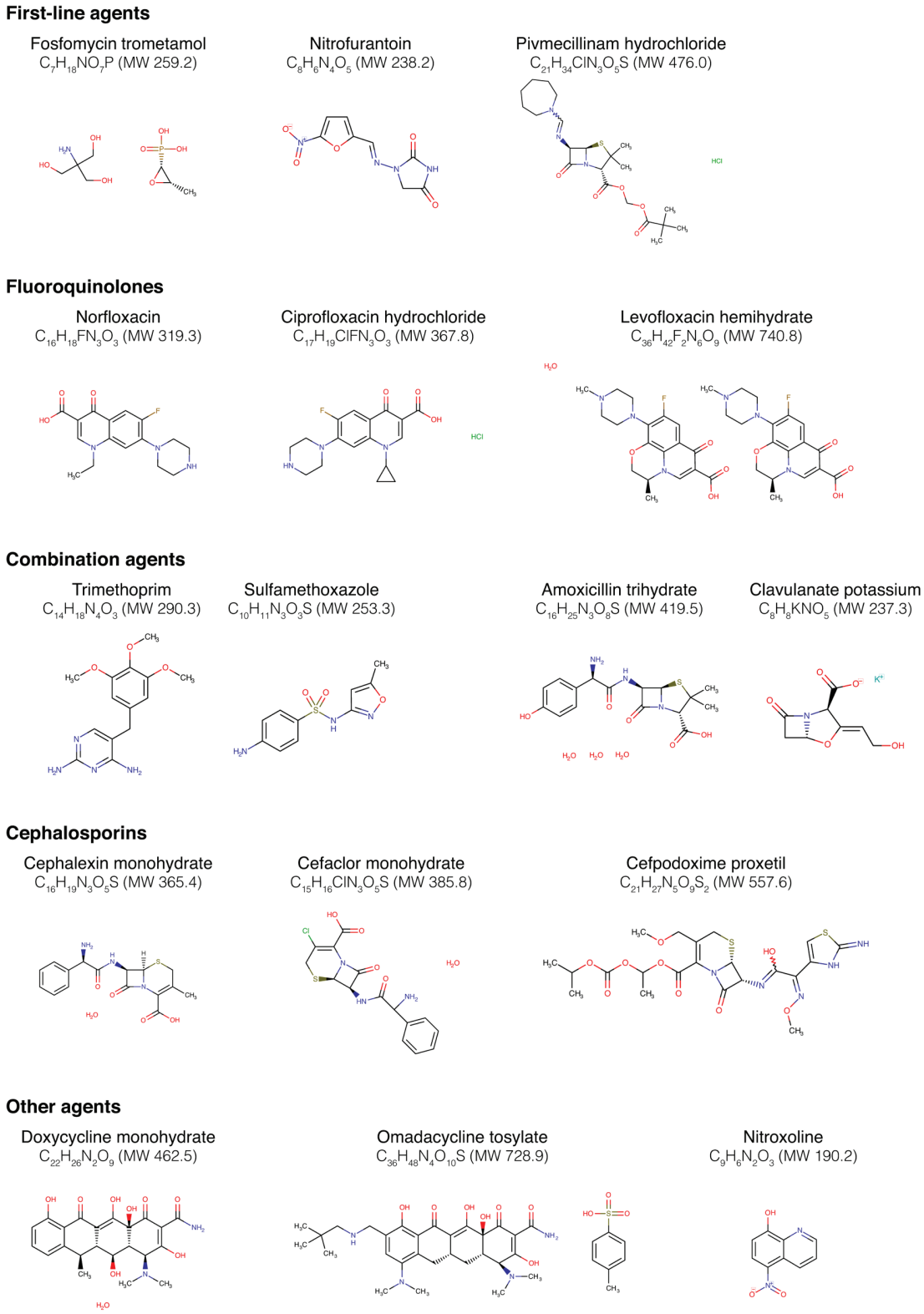

Figure 2. Oral antimicrobial agents for the treatment of urinary tract infections.
Chemical structures obtained from https://www.drugbank.ca [131]. MW, molecular weight.

rare (0.001–0.0007% of courses of therapy) and mostly associated with prolonged duration of use (>6 months) [167,168]. Nitrofurantoin should be avoided in renal failure (creatinine clearance <30 mL/min) or G6P-dehydrogenase deficiency [163,169]. Clinical cure rates vary between 70% and 92% [158,159].

Table 3. Uncomplicated UTI treatment: antimicrobial dosing, susceptibility interpretation and pharmacokinetics.

Antimicrobial	Recommended dose	Susceptibility breakpoints[a]		Pharmacokinetics (mg/L, unless otherwise stated)	Comments
		MIC (mg/L or µg/mL)	Disk diffusion diameter (mm)		
Fosfomycin					
Fosfomycin trometamol	3 g D Duration: SD	EUCAST: *Enterobacterales* [UTI]: S ≤ 32; R > 32 *Pseudomonas*: ECOFF = 125 CLSI: *E. coli* [UTI]: S ≤ 64; R ≥ 256 *E. faecalis* [UTI]: S ≤ 64; R ≥ 256	*E. coli* [UTI]: S ≥ 24; R < 24 *Pseudomonas*: ECOFF = 12 *E. coli* [UTI]: S ≥ 16; R ≤ 12 *E. faecalis* [UTI]: S ≥ 16; R ≤ 12	Plasma C_{max}: 26.1 Plasma $t_{½}$: 4.5–9 h Urine C_{max}: 1000–2000 Urine AUC_{0-24}: 8000–20,000 mg.h/L Urine recovery: 35–50% (unchanged)	*S. saprophyticus* intrinsically resistant. G6P enhances activity in most *Enterobacterales*. No enhancement with *Enterococcus* or *Pseudomonas* spp. Agar dilution with 25 mg/L G6P required for MIC. Fosfomycin 200 µg disk contains 50 µg G6P. EUCAST ignore isolated colonies with inhibition zone, CLSI read inner diameter.
Nitrofurans					
Nitrofurantoin - Macrocrystal - Monohydrate macrocrystal, or prolonged-release	50–100 mg QID (100 mg BID for prolonged-release) Duration: 5 days	EUCAST: *E. coli* [UTI]: S ≤ 64; R > 64 *E. faecalis* [UTI]: S ≤ 64; R > 64 *S. saprophyticus* [UTI]: S ≤ 64; R > 64 CLSI: *Enterobacterales* [UTI]: S ≤ 32; R ≥ 128 *Enterococcus* [UTI]: S ≤ 32; R ≥ 128 *Staphylococcus* [UTI]: S ≤ 32; R ≥ 128	*E. coli* [UTI]: S ≥ 11; R < 11 *E. faecalis* [UTI]: S ≥ 15; R < 15 *S. saprophyticus* [UTI]: S ≥ 13; R < 13 *Enterobacterales* [UTI]: S ≥ 17; R ≤ 14 *Enterococcus* [UTI]: S ≥ 17; R ≤ 14 *Staphylococcus* [UTI]: S ≥ 17; R ≤ 14	Plasma C_{max}: <2 Plasma $t_{½}$: 1.7–2.3 h Urine C_{max}: 50–250 Urine recovery: 50% (unchanged) Urine AUC_{0-24}: 900 mg.h/L	EUCAST consider *E. faecium* to have intrinsic resistance. *Proteus* and *Pseudomonas* spp. also intrinsically resistant. Absorption enhanced with food. Urinary excretion is saturable (50 mg QID = 100 mg TID). EUCAST nitrofurantoin disk content is 100 µg, CLSI uses 300 µg.
Antifolate agents					
Trimethoprim	100–200 mg BID (Alt: 300 mg D) Duration: 3–5 days	EUCAST: *Enterobacterales* [UTI]: S ≤ 4; R > 4 *Enterococcus* [UTI]: ECOFF = 1 *Staphylcoccus* [UTI]: S ≤ 4; R > 4 CLSI: *Enterobacterales* [UTI]: S ≤ 8; R ≥ 16 *Staphylococcus* [UTI]: S ≤ 8; R ≥ 16	*Enterobacterales* [UTI]: S ≥ 15; R < 15 *Enterococcus* [UTI]: ECOFF = 21 *Staphylococcus* [UTI]: S ≥ 14; R < 14 *Enterobacterales* [UTI]: S ≥ 16; R ≤ 12 *Staphylococcus* [UTI]: S ≥ 16; R ≤ 10	Trimethoprim: Plasma C_{max}: 1.5–2 (46–70% protein bound) Plasma $t_{½}$: 10–12 h Urine C_{max}: 100 Urine recovery: 40–60% (unchanged)	Activity uncertain to predict clinical outcome against *Enterococci*; CLSI report intrinsic resistance. *Pseudomonas* spp. intrinsically resistance. EUCAST test TMP/SMX in the ratio 1:19 and report TMP concentration.
Trimethoprim-sulphamethoxazole	180 + 600 mg BID Duration: 3 days	EUCAST: *Enterobacterales*: S ≤ 2; R > 4 *Enterococcus*: ECOFF MIC = 1 *Staphylococcus*: S ≤ 2; R > 4 CLSI: *Enterobacterales*: S ≤ 2/38; R ≥ 4/76 *Staphylococcus*: S ≤ 2/38; R ≥ 4/76	*Enterobacterales*: S ≥ 14; R < 11 *Enterococcus*: ECOFF = 23 *Staphylococcus*: S ≥ 17; R < 14 *Enterobacterales*: S ≥ 16; R ≤ 10 *Staphylococcus*: S ≥ 16; R ≤ 10	Sulphamethoxazole: Plasma C_{max}: 45–50 (66% protein bound) Plasma $t_{½}$: 10–12 h Urine C_{max}: 40–320 (if high dose used) Urine recovery: 46% (only 30% unchanged)	
Fluoroquinolones					
Norfloxacin	400 mg BID Duration: 3 days	EUCAST: *Enterobacterales* [UTI]: S ≤ 0.5; R > 0.5 CLSI: *Enterobacterales* [UTI]: S ≤ 4; R ≥ 16 *Pseudomonas* [UTI]: S ≤ 4; R ≥ 16 *Enterococcus* [UTI]: S ≤ 4; R ≥ 16 *Staphylococcus* [UTI]: S ≤ 4; R ≥ 16	*Enterobacterales* [UTI]: S ≥ 22; R < 22 *Enterococcus* (screen): S ≥ 12; R < 12 *Staphylococcus* (screen): S ≥ 17; R < 17 *Enterobacterales* [UTI]: S ≥ 17; R ≤ 12 *Pseudomonas* [UTI]: S ≥ 17; R ≤ 12 *Enterococcus* [UTI]: S ≥ 17; R ≤ 12 *Staphylococcus* [UTI]: S ≥ 17; R ≤ 12	Norfloxacin: Plasma C_{max}: 1.58 Plasma $t_{½}$: 3.5–5 h Urine C_{max}: 30 Urine recovery: 24–30% (unchanged)	Norfloxacin can be used as a screen for other fluoroquinolones. Note differences between EUCAST and CLSI dosing of ciprofloxacin and levofloxacin. EUCAST recommend high dose for *Pseudomonas* and *Staphylococcus* spp. CLSI recommend for levofloxacin 750 mg daily and, for *Pseudomonas* spp. ciprofloxacin 400 mg q8 intravenous
Ciprofloxacin	250–500 mg BID (HD: 750 mg BID) Duration: 3 days	EUCAST: *Enterobacterales*: S ≤ 0.25; R > 0.5 *Pseudomonas*: S ≤ 0.001; R > 0.5 *Enterococcus* [UTI]: S ≤ 4; R > 4 Coag-neg *Staph.*: S ≤ 0.001; R > 1 CLSI: *Enterobacterales* [IE]: S ≤ 0.25; R ≥ 1 *Pseudomonas* [IV]: S ≤ 0.5; R ≥ 2 *Enterococcus* [UTI]: S ≤ 1; R ≥ 4 *Staphylococcus*: S ≤ 1; R ≥ 4	*Enterobacterales*: S ≥ 25; R < 22 *Pseudomonas*: S ≥ 50; R < 26 *Enterococcus* [UTI]: use NOR screen Coag-neg *Staph.*: S ≥ 50; R < 24 *Enterobacterales* [IE]: S ≥ 26; R ≤ 21 *Pseudomonas* [IV]: S ≥ 25; R ≤ 18 *Enterococcus*: S ≥ 21; R ≤ 15 *Staphylococcus*: S ≥ 21; R ≤ 15	Ciprofloxacin (Dose: 250 mg) Plasma C_{max}: 0.8–1.9 Plasma $t_{½}$: 5–6 h Urine C_{max}: 45–69 (average at 6 - 12h) Urine recovery: 50–75% (15% as metabolites)	
Levofloxacin	250–750 mg D (HD: 500 mg BID) Duration: 3 days	EUCAST: *Enterobacterales*: S ≤ 0.5; R > 1 *Pseudomonas*: S ≤ 0.001; R > 1 *Enterococcus* [UTI]: S ≤ 4; R > 4 Coag-neg *Staph.*: S ≤ 0.001; R > 1 CLSI: *Enterobacterales* (IE): S ≤ 0.5; R ≥ 2 *Pseudomonas* (IE): S ≤ 1; R ≥ 4 *Enterococcus* [UTI]: S ≤ 2; R ≥ 8 *Staphylococcus*: S ≤ 1; R ≥ 4	*Enterobacterales*: S ≥ 23; R < 19 *Pseudomonas* (HE): S ≥ 50; R < 22 *Enterococcus* [UTI]: use NOR screen Coag-neg *Staph.*: S ≥ 50; R < 24 *Enterobacterales* (IE): S ≥ 21; R ≤ 16 *Pseudomonas* (IE): S ≥ 22; R ≤ 14 *Enterococcus*: S ≥ 17; R ≤ 13 *Staphylococcus*: S ≥ 19; R ≤ 15	Levofloxacin (Dose: 250 mg) Plasma C_{max}: 2.8 Plasma $t_{½}$: 6–8 h Urine C_{max}: 108 Urine recovery: 80% (unchanged, <5% as metabolites)	

(*Continued*)

Table 3. (Continued).

Antimicrobial	Recommended dose	Susceptibility breakpoints[a] MIC (mg/L or µg/mL)	Susceptibility breakpoints[a] Disk diffusion diameter (mm)	Pharmacokinetics (mg/L, unless otherwise stated)	Comments
Beta-lactams: Penicillins					
Pivmecillinam	400 mg TID Duration: 3 days	EUCAST: *Enterobacterales* [UTI]: S ≤ 8; R > 8 CLSI: *E. coli* [UTI]: S ≤ 8; R ≥ 32	*Enterobacterales* [UTI]: S ≥ 15; R < 15 *E. coli* [UTI]: S ≥ 15; R ≤ 11	Mecillinam: Plasma C_{max}: 2.5 Plasma $t_{½}$: 1 h Urine C_{max}: 300 Urine recovery: 30–45% (unchanged)	*Pseudomonas* spp. are intrinsically resistant. All β-lactams have optimal activity by prolonged T > MIC. Pivmecillinam is a prodrug of mecillinam with activity against ESBL-producing organisms. EUCAST report mecillinam breakpoints for *E. coli, Citrobacter* spp., *Klebsiella* spp., *Raoultella* spp., *Enterobacter* spp. and *Proteus mirabilis*. Results for ampicillin (AMP) testing can be used to predict results for amoxicillin. Oral amoxicillin dosing considered only appropriate for UTIs. EUCAST used a fixed 2 mg/L concentration for clavulanate, whereas CLSI apply a 2:1 ratio.
Amoxicillin	500 mg TID Duration: 5 days	EUCAST: *Enterobacterales* [UTI]: S ≤ 8; R > 8 *Enterococcus* [UTI]: S ≤ 4; R > 8 - CLSI: *Enterobacterales*: S ≤ 8; R ≥ 32 (AMP) *Enterococcus*: S ≤ 8; R ≥ 16 (AMP) *Staphylococcus*: S ≤ 0.12; R ≥ 0.25 (PEN)	*Enterobacterales* [UTI]: S ≥ 14; R < 14 (AMP) *Enterococcus* [UTI]: S ≥ 10; R < 8 (AMP) *S. saprophyticus*: S ≥ 18; R < 18 (AMP) *Enterobacterales*: S ≥ 17; R ≤ 13 (AMP) *Enterococcus*: S ≥ 17; R ≤ 16 (AMP) *Staphylococcus*: S ≥ 29; R ≤ 28 (PEN)	Plasma C_{max}: 8–10 Plasma $t_{½}$: 1 h Urine C_{max}: 115–1850 Urinary excretion: 60% (unchanged)	
Amoxicillin-clavulanate[b]	500 + 125 mg TID Duration: 5 days	EUCAST: *Enterobacterales* [UTI]: S ≤ 32; R > 32 *Enterococcus* [UTI]: S ≤ 4; R > 8 - CLSI: *Enterobacterales*: S ≤ 8/4; R ≥ 32/16 *Enterococcus*: S ≤ 8; R ≥ 16 (AMP) -	*Enterobacterales* [UTI]: S ≥ 16; R < 16 *Enterococcus* [UTI]: S ≥ 10; R < 8 *S. saprophyticus*: S ≥ 18; R < 18 *Enterobacterales*: S ≥ 18; R ≤ 13 *Enterococcus*: S ≥ 17; R ≤ 8 (AMP) *S. saprophyticus*: S ≥ 25; R ≤ 24 (FOX)	Clavulanate: Plasma C_{max}: 3.5 Plasma $t_{½}$: 1 h Urine recovery: 18–38% (unchanged) Relatively unstable at 37°C	
Beta-lactams: Cephalosporins					
Cephalexin 1st Gen. (1GC); Limited spectrum	500 mg BID Duration: 5 days	EUCAST: *Enterobacterales* [UTI]: S ≤ 16; R > 16 *S. saprophyticus*: R > 8 CLSI: *Enterobacterales* [UTI]: S ≤ 16; R ≥ 32 (CFZ) -	*Enterobacterales* [UTI]: S ≥ 14; R < 14 *S. saprophyticus*: S ≥ 22; R < 22 (FOX) *Enterobacterales* [UTI]: S ≥ 15; R ≤ 14 (CFZ) *S. saprophyticus*: S ≥ 25; R ≤ 24 (FOX)	Plasma C_{max}: 15–18 Plasma $t_{½}$: 1 h Urine C_{max}: 500–1000 Urinary recovery: 70–100% (unchanged)	*Enterococcus* and *Pseudomonas* spp. are intrinsically resistant. Cefadroxil is another 1GC. Cefuroxime axetil (2GC) offers limited benefit over 1GC agents for UTIs. Cefaclor (2GC) have some improved Gram-negative cover. EUCAST does not provide breakpoints for Cefaclor. Susceptibility in *S. saprophyticus* is inferred from cefoxitin. CLSI use cephazolin (CFZ) to predict susceptibility oral cephalosporins; may overcall resistance for 3GC. Other oral 3GC include: ceftibuten, cefdinir.
Cefaclor 2nd Gen. (2GC); Improved Gram-negative cover	250 mg TID Duration: 5 days (Alt. 2 g SD)	EUCAST: *S. saprophyticus*: R > 8 CLSI: *Enterobacterales* [UTI]: S ≤ 16; R ≥ 32 (CFZ) -	*S. saprophyticus*: S ≥ 22; R < 22 (FOX) *Enterobacterales* [UTI]: S ≥ 15; R ≤ 14 (CFZ) *S. saprophyticus*: S ≥ 25; R ≤ 24 (FOX)	Plasma C_{max}: 10.6 Plasma $t_{½}$: 1 h Urine C_{max}: 482 Urinary recovery: 70% (unchanged)	
Cefpodoxime 3rd Gen. (3GC); Broad spectrum	200 mg BID Duration: 3 days (Alt. 100 mg BID)	EUCAST: *Enterobacterales* [UTI]: S ≤ 1; R > 1 - CLSI: *Enterobacterales*: S ≤ 2; R ≥ 8 -	*Enterobacterales* [UTI]: S ≥ 21; R < 21 *S. saprophyticus*: S ≥ 22; R < 22 (FOX) *Enterobacterales*: S ≥ 21; R ≤ 17 *S. saprophyticus*: (FOX) S ≥ 25; R ≤ 24	Plasma C_{max}: 2–4 Plasma $t_{½}$: 2.7 h Urine C_{max}: 19.8 (200 mg) Urinary recovery: 40% (unchanged)	
Other agents					
Nitroxoline	250 mg TID Duration: 5 days	EUCAST: *E. coli* [UTI]: S ≤ 16; R > 16 *Enterococcus* [UTI]: IE *S. saprophyticus* [UTI]: IE	*E. coli* [UTI]: S ≥ 15; R < 15 *Enterococcus* [UTI]: IE *S. saprophyticus* [UTI]: IE	Plasma C_{max}: 5–9.5 (uncj.) Plasma $t_{½}$: 2 h Urine C_{max}: 0.5 (uncj.); 28 (conj. nitroxoline sulfate) Urinary recovery: 60% (99% conjugated metabolite)	*Pseudomonas* intrinsically resistant. Not widely available. CLSI do not report susceptibility.
Doxycycline	100 mg BID load, then 100 mg daily Duration: 4 days (Alt: 300 mg SD)	EUCAST: *Staphylococcus*: S ≤ 1; R > 2 CLSI: *Enterobacterales*: S ≤ 4; R ≥ 16 *Enterococcus*: S ≤ 4; R ≥ 16 *Staphylococcus*: S ≤ 4; R ≥ 16	- *Enterobacterales*: S ≥ 14; R ≤ 10 *Enterococcus*: S ≥ 16; R ≤ 12 *Staphylococcus*: S ≥ 16; R ≤ 12	Plasma C_{max}: 2.6–4.2 Plasma $t_{½}$: 14 h Urine C_{max}: 300 Urinary recovery: 35–40% (unchanged)	May still be effective against resistant uropathogens (including. *Pseudomonas* spp.) due to high urinary concentration. Very limited guidance on dosing or duration.

UTI, breakpoint related only to urinary tract infection. ECOFF, epidemiological cutoff value. MIC, minimum inhibitory concentration. SD, single dose. HD, high dose. $t_{½}$, half-life. uncj., unconjugated; conj., conjugated. 1GC, first-generation cephalosporin. 2GC, second-generation cephalosporin. 3GC, third-generation cephalosporin. AMP, ampicillin. PEN, penicillin. FOX, cefoxitin. CFZ, cefazolin. NOR, norfloxacin. TMP, trimethoprim. SMX, sulphamethoxazole. IE, insufficient evidence. MRSA, methicillin-resistant *Staphylococcus aureus*. VRE, vancomycin-resistant *Enterococcus*. Refer to the main text for all references. [a]The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) susceptibility breakpoint tables were used [56,57]. CLSI do not recommend routine testing of urine isolates of *S. saprophyticus* because infections respond to antimicrobial agents commonly used to treat acute, uncomplicated UTIs (e.g. nitrofurantoin, trimethoprim/sulphamethoxazole, or a fluoroquinolone). [b]Increased frequency dosing (three to four times daily, rather than twice daily) is more likely to achieve PK/PD targets but can be poorly tolerated. EUCAST breakpoints related to TID dosing.

Table 4 Antimicrobial MIC distributions and wild-type cutoffs for common Gram-negative uropathogens.

(a) Gram-negative uropathogens

	Enterobacterales								*Pseudomonas* spp.	
	E. coli		*K. pneumoniae*		*P. mirabilis*		*E. cloacae*		*P. aeruginosa*	
Antimicrobial	MIC 50/90	ECOFF	MIC 50/90	ECOFF	MIC 50/90	ECOFF	MIC 50/90	ECOFF	MIC 50/90	ECOFF
Fosfomycin trometamol	1/4[a]	4	16/64	ND	4/64[a]	8	16/256[e]	ND	64/128[a]	ND
Nitrofurantoin	16/32[a]	64	-	-		IR	16/64[b,c,e]	ND	IR	
Trimethoprim	0.5/64[a]	2	0.5/16[a]	ND	2/16[a]	ND	0.5/16[a]	ND	IR	
Trimeth.-sulphamethoxazole	0.125/32[a]	0.25	0.125/16[a]	0.5	0.25/16[a]	0.5	0.125/2[a]	0.5	IR	
Norfloxacin	0.064/0.125	0.25	0.125/0.25[d]	0.25	0.064/0.25[b,c]	0.25	0.064/8 [c,e]	0.25	0.5/2	2
Ciprofloxacin	0.016/1[a]	0.064	0.032/2[a]	0.125	0.032/2[a]	0.064	0.016/0.5[a]	0.125	0.25/8[a]	0.5
Levofloxacin	0.032/4[a]	0.25	0.064/2[a]	0.25	0.064/1[a]	0.25	0.064/0.5[a,e]	0.25	0.5/4[a]	2
Pivmecillinam[f]	0.125/2[a]	1	0.25/128[b]	1	2/128[b,c]	ND	2/4[b,c]	ND	IR	
Amoxicillin	8/≥ 512[a]	8	IR		1/≥ 512	2		IR	IR	
Amoxicillin-clavulanate[g]	4/16[a]	8	2/16[a]	8	2/8[a]	2		IR	IR	
Cephalexin	4/8[b]	16	4/8[b,d]	16	8/16	16		IR	IR	
Cefaclor	1/4[b]	4	0.25/2[d]	ND	1/2[b,c]	ND		IR	IR	
Cefpodoxime	0.5/4[a]	2	-	-	-	-	2/64[a]	ND	IR	
Nitroxoline[h]	4/8	16	2/4 [c]	ND	8/16	ND	8/16 [c]	ND	IR	
Doxycycline	4/32[a]	4	2/16	4	IR		2/8	8	IR	

(b) Gram-positive uropathogens

	Enterococcus spp.				*Staphylococcus* spp.			
	E. faecalis		*E. faecium*		*S. saprophyticus*		*S. aureus*	
Antimicrobial	MIC 50/90	ECOFF	MIC 50/90	ECOFF	MIC 50/90	ECOFF	MIC 50/90	ECOFF
Fosfomycin trometamol	32/64[a]	ND	64/128[b]	ND	IR		4/16[a]	32
Nitrofurantoin	8/16	32	64/256[a]	256	8/16 [c]	32	16/16[a]	32
Trimethoprim	-	-	-	-	-	-	1/8	2
Trimeth.-sulphamethoxazole	-	-	0.25/16[b]	ND	-	-	0.064/0.5[a]	0.25
Norfloxacin	4/16	8	16/64[b,c]	ND	2/4[b,c]	ND	1/32	4
Ciprofloxacin	1/2[a]	4	2/4[a]	8	0.5/0.5[a]	1	0.5/2[a]	1
Levofloxacin	2/32[a]	4	4/64[a]	4	16/≥512	0.5	0.25/4[a]	0.5
Pivmecillinam[f]	-	-	-	-	16/32[b,c]	ND	-	-
Amoxicillin	-	-	IR		IR		IR	
Amoxicillin-clavulanate[g]	2/2[a]	4	32/32[b]	4	2/16[b]	ND	0.5/8[a]	2
Cephalexin	IR		IR		4/8[b,c]	ND	2/128[b]	8
Cefaclor	IR		IR		-	-	4/128[b]	8
Cefpodoxime	IR		IR		-	-	2/32[a]	4
Nitroxoline[h]	16/32	ND	8/8 [c]	ND	8/8 [c]	ND	8/8	ND
Doxycycline	8/32[a]	0.5	16/32[a]	0.5	0.125/0.25[b,c]	ND	0.125/2[a]	0.5

Data from the EUCAST MIC distribution website http://www.eucast.org (last accessed 17 August 2020) [132], which define the epidemiological cutoff values (ECOFF) and give an indication of the MICs for organisms with acquired resistance mechanisms. The distributions should not infer resistance rates since the data are aggregated from many time periods and many countries. IR, intrinsic resistance. ND, not determined. -, indicates data not available. [a]>1 data source and >1000 observations. [b]Single data source only. [c]< 100 observations. [d]Refers to *Klebsiella* spp. [e]Refers to *Enterobacter* spp. [f]Refers to mecillinam MIC. [g]amoxicillin-clavulanate as a ratio. [h]Data from EUCAST nitroxoline rationale document (version 1.0, 2016) [133], the number of contributing data sources not documented.

7.3. Pivmecillinam

-Pivmecillinam is an amidinopenicillin, hydrolyzed by gut esterases to the active drug, mecillinam. It acts on the bacterial cell wall binding to penicillin-binding-protein (PBP)-2. Mecillinam is active against *E. coli, Klebsiella* spp. and *P. mirabilis*, including ESBL-producing strains, without activity against *Pseudomonas* spp. or Gram-positive uropathogens [170]. Although, *in vivo* activity has been demonstrated against *S. saprophyticus* [171]. Despite 95% susceptibility in ESBL-producing urinary isolates [172], treatment failure has been reported in susceptible strains (44% treatment failure in ESBL; 14% in non-ESBL) [173]. Resistance can arise following permeability changes or β-lactamase enzymes. Pivmecillinam is not widely available outside of Scandinavia, Austria and Germany.

Dosing ranges from 200 mg twice-daily to 400 mg three-times-daily, with insufficient evidence to support the optima combination of dose, frequency and duration [174]. Reports demonstrate similar cure rates to nitrofurantoin [175] and non-inferiority of 3-days of therapy compared to 5-days (73% versus 76% clinical success, respectively) using 400 mg three-times-daily [176]. Pivmecillinam has also been used successfully (>90%) to treat UTI in men [177]. Mecillinam is actively excreted by kidney tubules. The 12–24 h urinary recovery of unchanged mecillinam after 400 mg is 30–45% [178]. Maximum peak urinary concentration of 300 mg/L occur after 0–3 h, rapidly declines to 50 mg/L by 6 h and <5 mg beyond 12 h [179,180]. Adverse effects are commonly rash and gastrointestinal. Gastrointestinal side effects are more common (24%) at the higher dose.

7.4. Trimethoprim/Trimethoprim-sulfamethoxazole

Trimethoprim is a synthetic diaminopyrimidine agent, acting as a competitive inhibitor of dihydrofolate reductase (DHFR). Sulfamethoxazole, a sulfonamide agent, is a competitive inhibitor of dihydropteroate synthetase and enables synergistic activity by inhibiting different steps in tetrahydrofolic acid synthesis. These agents are active against *Enterobacterales* and *S. saprophyticus* isolates. There is uncertain activity against *Enterococcus* spp. and intrinsic resistance in *Pseudomonas* spp. Increasing resistance in *Enterobacterales* has limited empirical use [181,182]. Co-trimoxazole resistance in urinary isolates is around 20–40%, but can be greater in developing countries and carbapenem-resistant isolates [1,12,63,119,183–185]. Among *Enterobacterales*, the addition of sulfamethoxazole may not improve bacterial kill over trimethoprim alone, representing an unnecessary risk to many patients [186,187]. Resistance occurs by over-production or modification of target enzymes, reduced permeability and/or efflux pumps, and different *dfr*-genes encoding dihydrofolate reductase enzymes. Sulfamethoxazole-resistance is conferred by sulfonamide resistance genes (*sul*) acting as competitive inhibitors of dihydropteroate synthase. In *Enterobacterales*, resistance genes are mainly spread horizontally on integrons, commonly associated with co-resistance to β-lactams and fluoroquinolones.

Dosing of trimethoprim varies internationally from 100 to 200 mg twice-daily to 300 mg daily. Co-trimoxazole dosing is one 'double-strength' tablet/capsule (trimethoprim/sulfamethoxazole 160/800 mg) twice-daily. The 24 h urine excretion of trimethoprim corresponds to 61% of the total oral dose (200 mg). Of the excreted drug, around 90% is unchanged, the remainder as metabolites. Mean (±SD) urinary concentration of the unchanged drug is 36.7 mg/L (±21.9 mg/L) from 0–4 h and 38.6 mg/L (±16.9 mg/L) from 4 to 8 h [188]. Sulfamethoxazole is also mainly excreted in the urine, but only 30% is unchanged. The impact of pH is mixed, with trimethoprim activity enhanced in an alkaline environment, but with a concurrent reduction in urinary excretion [189]. Whereas an alkaline environment enhances sulfamethoxazole excretion. Therefore, the final ratio of trimethoprim and sulfamethoxazole can range from 1:1 in acid urine to 1:5 in alkaline urine [190]. Co-trimoxazole is associated with some severe adverse effects, including neurologic changes, decreased oxygen-carrying capacity and other hematologic effects, toxic epidermal necrolysis and other drug hypersensitivity reactions, reproductive abnormalities and hypoglycemia [191]. Hyperkalemia and acute kidney injury are seen more commonly in the elderly and in preexisting renal impairment (creatinine clearance <60 mL/min) [192,193]. Cardiac arrythmias have been reported with concurrent use with drugs that block the renin-angiotensin system. Trimethoprim alone appears better tolerated, but acute kidney injury and hyperkalemia are still reported in patients aged >65 years [194].

7.5. Fluoroquinolones

Although highly efficacious, with reports of improved clinical outcomes compared to other agents [195,196], concerns regarding emergence of resistance and rare but serious side effects have seen fluoroquinolones commonly relegated to second-line, or reserve agents. Most treatment guidelines include norfloxacin, ciprofloxacin and levofloxacin, with newer agents less commonly available. Fluoroquinolones are derived from nalidixic acid and act by direct inhibitors of DNA synthesis, inhibiting DNA gyrase and topoisomerase IV. Emergence of resistance is primarily due to stepwise mutations in the quinolone resistance-determining region (QRDR) of chromosomal *gyr* and *par* genes, efflux pumps, Qnr proteins (protecting DNA gyrase) and inactivating enzymes. Resistance to fluoroquinolones among *Enterobacterales* has steadily increased overtime. The 2018 ECDC report showed 25.3% of invasive *E. coli* were resistant (7.2% in Iceland, up to 44.5% in Italy) and 31.3% in *K. pneumoniae* (0.3% in Iceland, up to 64.7% in Greece) [62]. A European UTI study reported resistance rates >20% [12,119] and 34% resistance among *E. coli* uropathogens in the US [185].

Recommended dosing of norfloxacin is 400 mg twice-daily. For ciprofloxacin and levofloxacin dosing varies from 250 to 750 mg twice-daily, with lower doses tended to be relied upon for UTI treatment, and higher doses for complicated infections or treatment of *Pseudomonas* spp. Three-day duration of therapy is commonly recommended, although for third- and fourth-generation agents, single dose therapy has been reported to be as equally effective [104]. Fluoroquinolones are predominately renally excreted by glomerular filtration and tubular secretion. For norfloxacin, 30% is excreted unchanged in the urine, with average peak urine concentrations of 30 mg/L occurring 1–2 h after administration. For ciprofloxacin, 50–75% is excreted unchanged in urine (15% as metabolites of limited activity), with >50% occurring in the first 4 h, and urinary concentrations at 6–12 h following 250 mg of around 45–69 mg/L, and after 500 mg peak urine concentration of 200 mg/L. For levofloxacin, 80% of dose is recovered in urine after 24 h (metabolites <5%) and mean urinary concentrations after a 250 mg dose were 108 mg/L (0–12 h) and 63 mg/L (12–24 h). After a single 500 mg dose peak urine concentrations were 521–771 mg/L [192,197]. Most adverse events are mild and reversible, such as diarrhea, nausea and headaches, but serious adverse events and their low barrier to resistance, have promoted a Black Box warning [198] of collagen-associated adverse effects include aortic rupture, tendinitis and tendon rupture and retinal detachment (odds ratio: 2.2, 1.89 and 1.3, respectively) [199]. Other serious adverse events are seizures, depression, hallucinations, dysglycemia, hepatic toxicity, phototoxicity, renal impairment and QT prolongation [200].

7.6. Oral aminopenicillins

Ampicillin and amoxicillin are narrow-spectrum penicillins. Amoxicillin is preferred due to its better absorption. The addition of the β-lactamase inhibitor (BLI), clavulanate, increases the spectrum of activity by inhibiting some intrinsic and acquired narrow-spectrum β-lactamase enzymes. *E. faecalis* are commonly susceptible, whereas *E. faecium* are considered intrinsically resistant, with or without the addition of clavulanate, due to the production of PBP-5. *Pseudomonas* spp. are also intrinsically resistant. Acquired resistance among

Enterobacterales is commonly due to β-lactamase enzymes. Although amoxicillin resistance is higher than amoxicillin-clavulanate, the fraction of amoxicillin-clavulanate susceptible strains that remain susceptible to amoxicillin alone can be >50% in *E. coli* urinary isolates, thereby limiting the need for clavulanate [201].

The usual adult oral dosage of amoxicillin is 250–500 mg, given three- to four-times daily, although PK/PD data would suggest that 500 mg given 8-hourly for 4-days would be the optimal dose for UTIs [202]. Amoxicillin-clavulanate is often dosed as a 4:1 ratio (500/125 mg), given twice or three-times daily for UTI treatment. An alternate oral formulation contains a greater amount of amoxicillin, at a 7:1 ratio (875/125 mg). A recent review suggested that using the formulation with a narrower ratio (e.g., 4:1) and with more frequent dosing (three or four-times daily) is preferable, although the clavulanate component is dose-limiting due to intolerance [201]. Following oral administration, high amoxicillin urinary levels are found, with 60% of the dose excreted unchanged in urine in the first 6 h. Absorption is saturable, supporting more frequent dosing schedules, with no additional benefit of doses >750 mg per administration [201,203]. In healthy adults, peak urinary concentrations are 306–856 mg/L after 250 mg, and after 500 mg between 115–1850 mg/L [204,205]. Clavulanate has highly variable absorption. Only 28% (18–-38%) of the dose is excreted unchanged in urine by 6 h, with hepatic clearance accounting for 50% of the absorbed dose and 30% protein-binding in serum [206,207]. Therefore, the ratio of amoxicillin to clavulanate in urine is different to that found systemically. Amoxicillin activity is largely unchanged in acidic conditions [208]. Side effects are mostly nausea, vomiting and diarrhea (2–5%) and eosinophilia (2%). Greater rates of side effects are found with the addition of clavulanate, especially diarrhea (9%) and increased hepatotoxicity. There is also greater microbiome impact with amoxicillin-clavulanate and higher risk of *C. difficile* [201].

7.7. Oral cephalosporins

Multiple different oral agents exist, although activity is increasingly limited due to resistance. Acquired resistance is essentially the same as the aminopenicillins, and all are hydrolyzed by broad-spectrum ESBLs (e.g. SHV-2 and CTX-M) and AmpC hyperproducers. All agents have no activity against *Enterococcus* spp. and *Pseudomonas* spp. Assessment of clinical activity has demonstrated variable treatment responses when compared to comparator agents, although in older trials, clinical cure rates have been reported >70% [117,209–211]. Activity is enhanced under acidic conditions [208] and gastrointestinal disturbances are the most common adverse events.

Cephalexin is a limited-spectrum agent (first-generation cephalosporin, 1GC) and are more readily inactivated by narrow-spectrum TEM-1 β-lactamases. Cephalexin is commonly dosed at 500 mg twice-daily for UTI, however more frequent dosing would be more efficacious. Cephalexin is not metabolized and excreted in the urine unchanged by glomerular filtration and tubular secretion, such that 70–100% of the dose is found in the urine by 6–8 h. Urine concentrations are 500–1000 mg/L following 250–500 mg dose [212].

Second-generation cephalosporins (2GC), such as cefaclor, have increased activity against wild-type Gram-negative bacteria and are structurally similar to cephalexin with a chlorine atom replacing the methyl group. Cefaclor, commonly dosed 250 mg 8-hourly [213–215], or as a 2 g single-dose [216], achieves a mean peak urinary concentration of 482–684 mg/L after a 250 mg dose, and 1174–1533 mg/L after 500 mg, with 50–70% of the dose recovered in the urine by 4–6 h [217,218]. More recently, a 'modified release' formulation has been marketed [219].

Cefpodoxime, an oral 3GC, primarily targets PBP-3 and is characterized by stability against some acquired β-lactamase enzymes (including TEM-2 and SHV-1 enzymes). Reported resistance in urinary isolates is dependent on location (commonly 5–16%) [12,65,119,185]. In a EU-wide surveillance of invasive isolates, resistance in *E. coli* was 15.2% (range 5.7–-35.5%) and in *K. pneumoniae* 31.2% (range 3.6–69.8%) [62]. Cefpodoxime is given as the pro-drug cefpodoxime proxetil and is commonly dosed between 100–200 mg twice-daily and has been found to be non-inferior to ciprofloxacin [117]. It is de-esterified by the intestinal mucosa, with 50% bioavailability and around 80% of the absorbed dose excreted unchanged in the urine [220]. Peak urine concentration range from 49 mg/L (50 mg dose) to 196 mg/L (800 mg dose) [221]. Following 200 mg, the mean (±SD) urine concentration was 19.8 mg/L (± 11.5 mg/L) in the 8–12 h time period and 3.9 mg/L after 12–24 h [222].

7.8. Nitroxoline

Nitroxoline is an old oral antimicrobial, although not widely available. It has broad activity against MDR uropathogens [223]. With a structurally distinct chemical structure, it is unrelated to other antimicrobial classes. Activity is mediated via multiple targets inducing chelation of metallic bivalent cations required for bacterial RNA polymerase and adhesion to bladder epithelial cells. Spectrum covers *Enterobacterales*, including MDR strains, and atypical uropathogens including *Mycoplasma hominis* and *Ureaplasma urealyticum*. Nitroxoline also has activity against *Candida* spp., while *Pseudomonas* spp. are intrinsically resistant. There is limited effect on the fecal flora [224]. Antibacterial activity appears to be static, and concerns about the inability to eradicate bacteriuria in a geriatric patient population has been reported [225]. Susceptibility of >3000 clinical UTI isolates from Germany between 2009–2012 showed >90% susceptibility in *E. coli, K. pneumoniae, P. mirabilis, Enterobacter* spp., *S. saprophyticus* and *Enterococcus* spp [226].

Standard dosing of nitroxoline is 250 mg three-times per day for 5-days. Approximately 60% of the administered dose is eliminated in the urine, 99% as conjugated metabolites (mainly nitroxoline sulfate and nitroxoline glucuronide), which are considered to have antimicrobial activity [188]. After a single 250 mg dose, mean (± SD) peak urinary concentrations (at 0–4 h) of nitroxoline are 0.5 mg/L (± 0.37 mg/L) and of nitroxoline sulfate are 27.8 (± 7.4 mg/L) [188]. In a geriatric

population, urinary concentrations of nitroxoline and nitroxoline sulfate were 0.1–5.4 mg/L and 0.8–210.6 mg/L, respectively [225]. Activity is enhanced in an acidic environment. Side effects are reported in 9.4% of patients, mainly mild gastrointestinal [224]. Efficacy in a meta-analysis of clinical data is reported at >90% and non-inferiority to co-trimoxazole and norfloxacin [224].

7.9. Tetracyclines

Although not included in most treatment guidelines, doxycycline is a therapeutic option for MDR uropathogens. The same is not true for other tetracycline agents, such as oral minocycline, oral eravacycline and intravenous tigecycline, all of which have minimal urinary excretion. Eravacycline was found to be inferior to levofloxacin in complicated UTIs, attributed to low bioavailability (28%) and a significant food effect limiting absorption [227]. Slightly more promising is oral omadacycline, a semisynthetic tetracycline derivative [228]. Tetracyclines inhibit microbial protein synthesis through interaction with 30S ribosomal subunit. Tetracyclines have a broad-spectrum of activity, including intracellular bacteria. Resistance is commonly associated with the acquisition of *tet* and *otr* genes encoding for efflux pumps or ribosomal protection proteins. Clinical and urinary *in vitro* activity has been reported against tetracycline-resistance bacteria, including *Pseudomonas* spp. that are considered intrinsically resistant [122,229].

Doxycycline is classically given as a loading dose of 100 mg twice-daily, then continued 100 mg daily. Limited guidance is provided for UTI treatment, but has been given for a duration of 4-days [230], or as a single 300 mg dose [231]. Concentration in serum is 4 mg/L, compared to >150 mg/L in urine [122]. Renal excretion accounts for 30–65% of the oral dose, which is reduced in renal impairment. Doxycycline has a prolonged serum half-life and activity is enhanced in acidic urine. Side-effects include gastrointestinal (including esophagitis) and photosensitivity. Omadacycline is given as a loading dose (300 mg or 450 mg twice-daily) and then continued daily (300 mg or 450 mg, respectively) for 5-days. Bioavailability is 35%. The estimated fraction excreted in urine over 24 h is 34% of the absorbed dose (equivalent to approximately 12% after oral dosing) [228]. Urinary concentrations (18–48 mg/L) may cover the omadacycline MIC_{90} for common uropathogens.

8. *In vitro* PK/PD bladder infection models

Translating PK/PD data from the bench to the bedside to optimize patient outcomes is now an established pathway for antimicrobial research and development 232–237]. PK/PD analyzes can inform antimicrobial targets, susceptibility breakpoints, optimized dosing regimens and describe exposures associated with emergence of resistance [238–241]. Guidance is now provided on the approach for generating robust PK/PD data [242]. *In vitro* models have the advantage of directly mimicking human PK exposures to directly elucidate exposure-response relationships [243]. In contrast, animal models require sophisticated scaling in relation to dosing, PK and elimination [244,245].

In vitro PK/PD models can be classified according to whether antimicrobial concentrations change over time ('static' versus 'dynamic') and whether there is bacterial loss in the system (Figure 3) [243]. Usually, bacterial loss is unintended, or a source of bias. This was overcome by the hollow-fiber infection model (HFIM), which uses a separating capillary membrane to allow media and antibiotics to flow through central fibers and diffuse into the extra-capillary space where the microorganisms are trapped [242]. When investigating UTIs, however, normal urodynamics must be also considered. The dilution of bacteria during bladder filling and loss through voiding are important experimental elements unique to UTIs.

The first dynamic UTI *in vitro* model, designed in 1966 by O'Grady and Pennington (Figure 4(a)) [246], used a vertical glass vessel, with a bacterial culture diluted over time with inflowing broth at a rate of 1 mL/min during the day and slowed overnight. At pre-set intervals, the vessel was emptied, leaving a residual volume. Turbidity measurements were taken to reflect bacterial density. The media used contained casitone pancreatic digest, yeast extract, glucose, K_2HPO_4, KH_2PO_4 and NaCl. Subsequently, the phosphate buffer was replaced with Tris buffer, while in later experiments Eugon broth was used. This model was updated (Figure 4(b)) and used through to the 1990s, enabling the study of β-lactams, trimethoprim, co-trimoxazole, fluoroquinolones and fosfomycin [187,247–258].

In 1969, Rowe and Morozowich [259], applied drug distribution equations with consecutive first-order processes, in order to simulate dynamic drug concentration changes. The variables were the starting drug dose, *in vitro* flow rates and compartment volumes. This principle was used by Grasso et al. in 1978 (Figure 4(c)) [260], with an open one compartment model simulating plasma cephalosporin concentrations after both intravenous and oral (or intramuscular) administration. A decade later, Satta et al. (Figure 4(d)) [261] used a similar model with human urine as the test medium, examining the activity of ampicillin, ceftriaxone, aztreonam and gentamicin against *E. coli*. Around the same time, urine was used in a one compartment model examining the activity of ampicillin, ciprofloxacin and co-trimoxazole compared to laboratory media [262]. Two decades later, the same model set-up was used again to examine fosfomycin activity against *E. coli* in standard laboratory media [263]. These models, however, all lacked the bladder emptying kinetics integral to earlier models.

In the late 1990s and early 2000s, a Japanese research group used a multicompartment dilution model of a 'complicated' bladder infection (Figure 4(e)) [264,265]. This design incorporated intermittent bladder voiding every 2 h during the day and a 10 h 'night phase' without voiding. A relatively large post-void residual volume (10 mL) remained after each void. The activity of levofloxacin and gatifloxacin against *P. aeruginosa* and *E. faecalis* was investigated. Their model ran at 0.5 mL/min with Antibiotic Medium #3. In other iterations, glass beads were included within the bladder compartment to assess activity against biofilms (ofloxacin against *E. coli*; clarithromycin and fluoroquinolones against

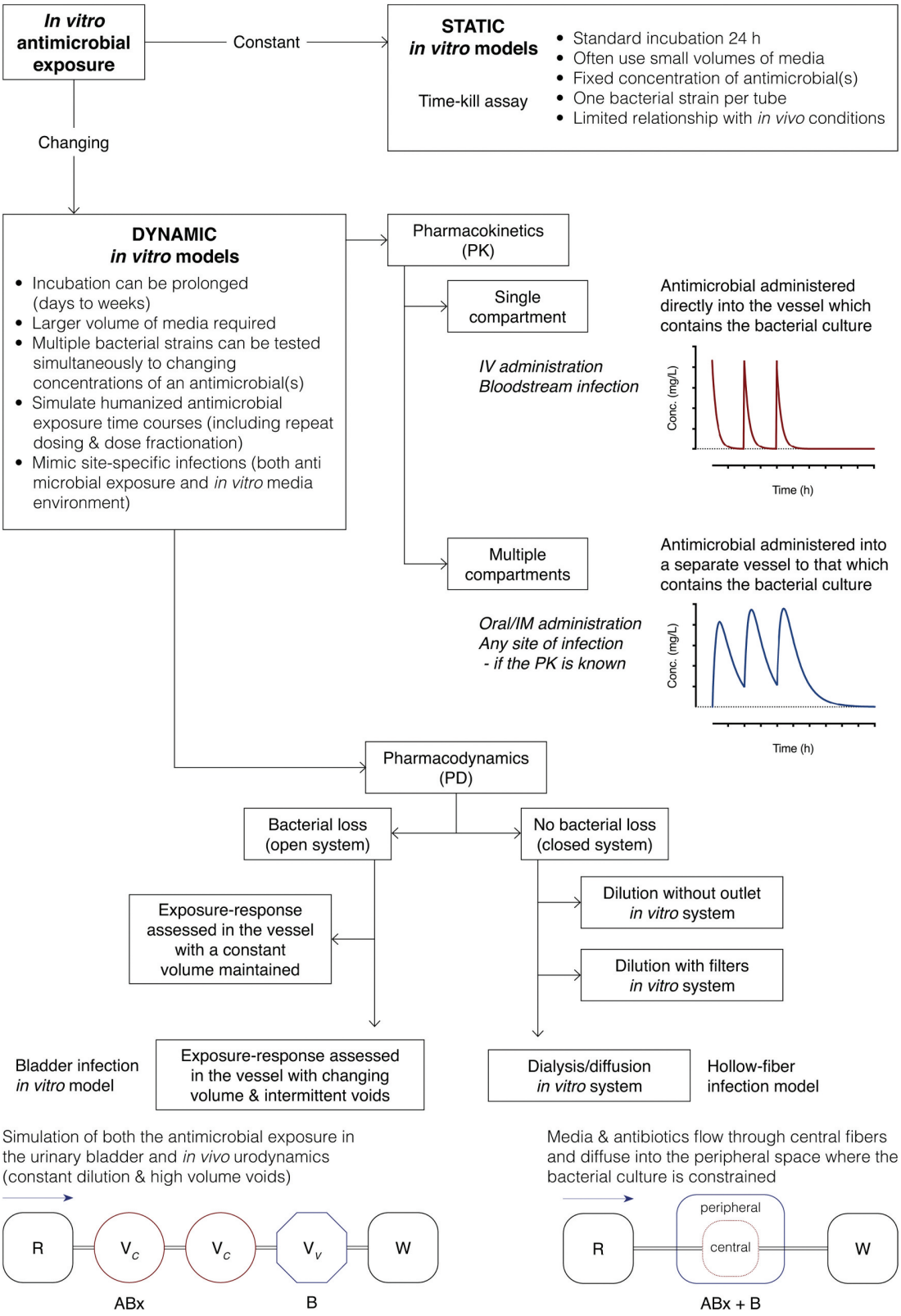

Figure 3. Generalized overview of PK/PD *in vitro* models.

IV: intravenous. IM: intramuscular. R: reservoir. V_C: constant volume compartment. V_V: variable volume compartment. W: waste. ABx: antimicrobial. B: bacterial culture.

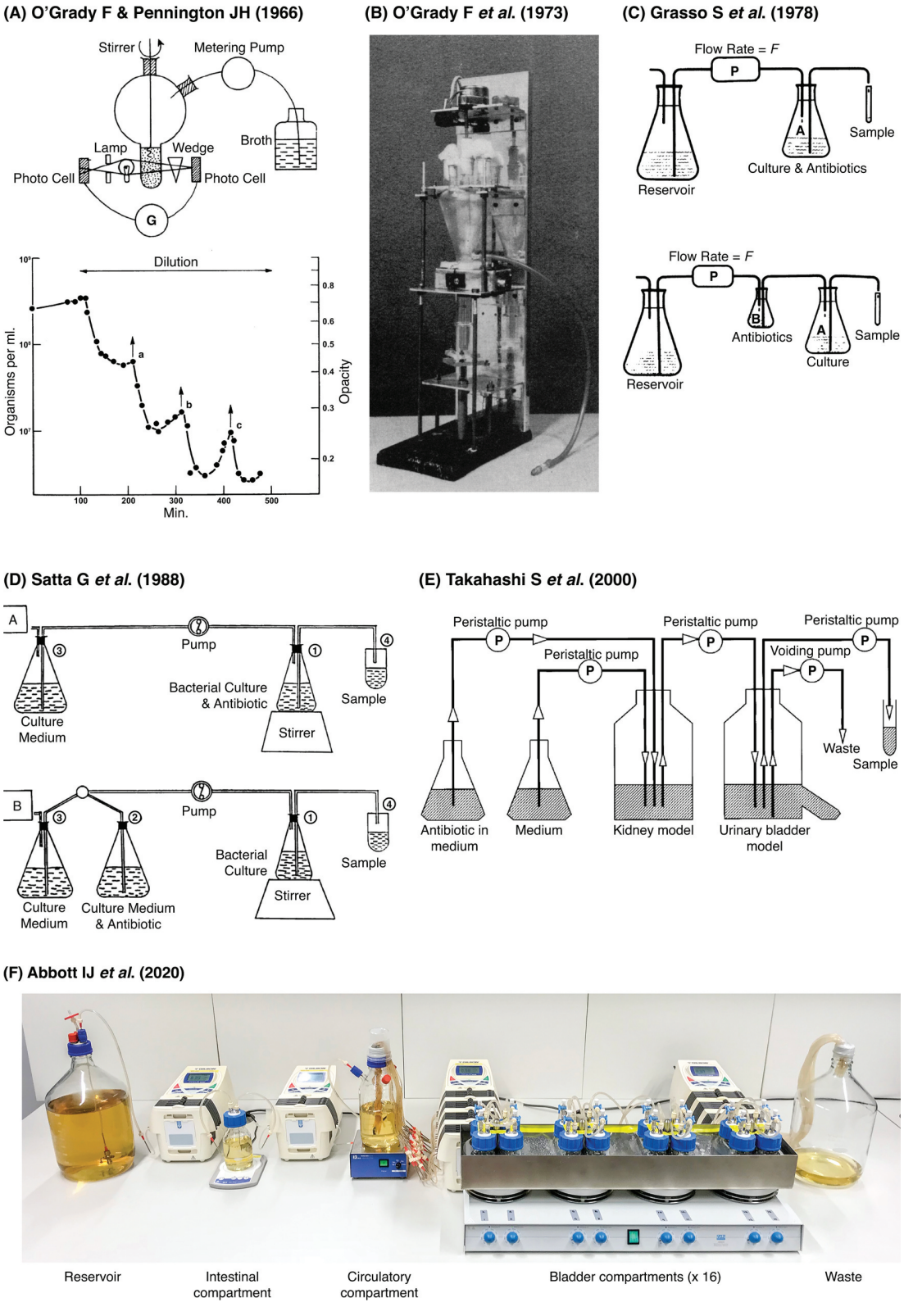

Figure 4. Bladder infection *in vitro* models.

(a) A 400 mL glass vessel with a tubular prolongation at the base enclosed in a water bath maintained at 37°C, with a stirrer. The tubular base is fixed in the light path of a photometer. The graph shows the effect of adding fresh broth at 1 mL/min, while at a, b and c the volume of the culture was reduced to 30 mL. Copyright © Blackwell Publishing LTD. Reproduced with permission [246]. (b) Updated designed from the previous model to overcome imperfect mixing and progressive occlusion of the light path of the photometer. The bladder as an inverted conical flask with tubulures set into the base, a drainage tube at the side and a glass syringe welded to the neck. The stirrer motor sits above, the photometer box at the waist and the piston at the base. The piston is activated every 5-minutes to clear the light path of the photometer. Copyright © Blackwell Publishing LTD. Reproduced with permission [247]. (c) Apparatus for simulation of mono-exponential decreases in antibiotic concentration and for simulation of biexponential time curves of antibiotic concentrations, such as those observed in serum after oral or intramuscular administration of drug. Copyright © American Society for Microbiology. Reproduced with permission [260]. (d) *In vitro* set-up simulating antibiotic concentrations in blood (apparatus A) and urine (apparatus B). Copyright © American Society for Microbiology. Reproduced with permission [261]. (e) Model used to simulate urinary concentrations of fluoroquinolones. Changing urinary antimicrobial concentrations simulated by a flow of media at 0.5 mL/min into the bladder, that was voided every 2 h during the day, withdrawing the entire volume except for 10 ml in the side arm. Overnight the bladder was not voided for 10 h. Copyright © Karger Publishers. Reproduced with permission [265]. (f) Media continuously pumped through three sequentially arranged peristaltic pumps from the fresh medium reservoir. Fosfomycin was administered into the intestinal compartment, simulating absorption, distribution and elimination into the 16 bladder compartments run in parallel. Automated and timed bladder voiding was controlled by a fourth peristaltic pump. Copyright © American Society for Microbiology. Reproduced with permission [272].

P. aeruginosa; clarithromycin against methicillin-resistant S. *aureus*) [266–269].

An alternative multicompartment infection model (Figure 4 (f)), applies a continuous dilution system that simulates oral antimicrobial absorption and elimination into 16 bladder compartments. This design enabled a higher throughput of bacterial strains to provide PK/PD data examining the efficacy of oral fosfomycin against different uropathogens (*E. coli, K. pneumoniae, E. cloacae, P. aeruginosa, E. faecalis, E. faecium*) [135,136,270,271] and following single and multiple doses [272]. The model was run with standard laboratory media, human urine and synthetic urine alternatives [273].

Most recently, a dynamic UTI 'micromodel' has been used to analyze the impact of urinary flow on persistence of *E. coli* colonization [274]. This model uses transitional epithelial cells and type IV collagen. By simulating urinary tract shear stresses and flow velocities, they have examined the dynamics of *E. coli* cell adhesion, reporting a phenomenon of epithelial cell 'rolling-shedding' that promotes bacterial attachment into deeper layers of epithelial cells.

Although *in vitro* UTI models mimic, as closely as possible, the conditions at the site of infection, important limitations apply to the translation of results to humans [58,275–277]. Immunological factors, host-pathogen interactions, pathological reactions to infection, tissue architecture, bacterial gene expression, virulence and metabolic changes are not easily simulated [11,24,278–280]. Equally, despite urinary bladder containing a relatively low oxygen content (urinary PO_2 approximately 40 mmHg) [281–285], *in vitro* models are commonly held at normal atmospheric conditions.

8.1. Media

The environment in which bacteria are challenged with an antimicrobial is critical when considering their response. In a nutrient-rich environment, there is an evolutionary drive for bacteria to develop resistance. In contrast, in a nutrient-deficit environment, such as urine, there is greater propensity to alter metabolic pathways leading to persistence [286,287]. Collecting and using human urine in *in vitro* models is logistically challenging. Considerations include: collection method (midstream versus 24 h urine-collection); source (gender, age, dietary and fluid intake, number of volunteers, exclusion criteria); sterilization (autoclave, filtration, gamma-irradiation); storage (uncertain shelf life refrigerated or frozen); and reproducibility (variability between collections). Different chemical recipes for artificial alternatives have been suggested [288–295]. These media provide a reproducible way to examine growth kinetics and antimicrobial activity. Synthetic human urine (SHU) is the most recently developed medium [14].

8.2. Antimicrobial exposure

In humans, urinary antimicrobial concentrations are greatly impacted upon behavioral factors, such as fluid intake, urine output and voiding pattern. As such, most PK studies demonstrate marked inter-/intrapersonal variation. Considerations should be made to simulate high and low extremes. The free, unchanged, active drug present in urine should be simulated. Where active metabolites are also excreted, their contribution to the overall bacterial killing should be evaluated. Dose fractionation studies can be performed to examine the PK/PD index important for bacterial clearance. Studies performed over only 24 h may provide insufficient time for the amplification of a resistance subpopulation. Ideally, simulated treatment durations should mimic the therapy intended in the clinical indication.

8.3. Quantification of antimicrobial concentrations

In vitro antimicrobial concentrations should be measured to confirm that observed values match the simulation, while also providing data for analysis. Drug concentrations should be quantified multiple times during each dosing interval to detail the peak concentration, rate of decline and trough measurements. The method of quantification will depend on availability of resources. Direct quantification using a HPLC or LCMS method is preferable [296]. Biological assays using inhibition zones of an indicator organism on solid agar may also be used [297,298]. Drug stability should be confirmed within the conditions of the *in vitro* model, or appropriate dose adjustments made.

8.4. Strain selection and starting inoculum

The selection of test isolates is paramount for analyzing experimental data to answer clinically relevant research questions. Multiple strains of the same, or different species, should be selected, based on the full range of susceptibility profiles to the test antimicrobial, including fully susceptible, low-level and high-level resistant isolates. Inclusion of clinical UTI strains in preferable, together with a reference control strain. To test resistance suppression, the number of bacteria added to the *in vitro* model is required to be 1 $\log_{10}$ CFU higher than the inverse of the mutant frequency [299,300]. The starting inoculum should be in log-growth phase prior to exposure to antimicrobials, therefore an initial period of drug-free incubation within the *in vitro* model should be observed.

8.5. Quantifying bacterial density and emergence of resistance

The bacterial response to antimicrobial exposure should be assessed at multiple timepoints. The standard method is quantitative cultures on antibiotic-free agar. Antibiotic carry-over should be addressed by serial dilution [263], repeat washing and centrifuge steps [301] and/or antimicrobial inactivation [302]. Other methods of bacterial density quantification for growth curve analysis include: turbidimetry, impedance, bioluminescence, phase-contrast microscopy, fluorimetric assays, microcalorimetry, and flow cytometry [243,303–305]. Molecular techniques include, quantitative PCR (qPCR) using primers and probes targeting *hlyD* [306], bacterial growth assessments measuring plasmid segregation (pGTR902) and measuring chromosomal replication [307,308]. Emergence of

resistance can be assessed by quantitative growth on agar supplemented with critical antimicrobial concentrations, incubated for 48–72 h [242]. Re-assessment of antimicrobial susceptibility can also be performed on the re-growth of bacteria over time. Whole genome sequencing of paired isolates (pre and post-exposure), quantitative gene expression and assessment of changes in metabolic pathways can also provide insights into the drivers of antimicrobial failure. Bacterial persistence and tolerance are other important factors to consider in the re-growth population [309,310].

9. Conclusions

To optimize UTI treatment, the correct antimicrobial, given at the right dose and for the shortest effective duration, should be individualized to the patient and the infecting uropathogen. Future advances could incorporate the presence of macrophages and bladder epithelial cell lines into existing *in vitro* models [311–315]. With a greater appreciation of antimicrobial urinary PK and uropathogen susceptibility, bladder infection models can help establish robust drug-bug targets, inform UTI-specific breakpoints, define PK/PD targets for bactericidal activity and support dose-optimization in patients. Furthermore, the adequacy of current antimicrobial dosing and reported clinical success rates should be re-assessed, applying modern laboratory diagnostics and detailing activity in antimicrobial-resistant uropathogens.

10. Expert opinion

Understanding the exposure-response relationship at the site of infection, and the drivers that promote emergence of resistance, is crucial to prevent modern medicine slipping into a 'post-antibiotic' era. UTIs are a common indication for an antimicrobial. By optimizing therapy in this setting, we can benefit a large number of patients and reduce a major driver for the emergence of resistance. However, our understanding of the relationship between the host, urine composition, uropathogen growth, metabolism and virulence remains limited.

Novel antimicrobial agents hold some promise for the future, although clinical trials are needed. In 2020, the WHO published a target product profile to guide the urgent development of new oral antimicrobial agents for UTIs, which, in turn, would benefit from assessment within a dynamic bladder infection PK/PD *in vitro* model [316]. Novel oral β-lactamase inhibitor combinations can expand the antimicrobial activity against ESBL-producing uropathogens [209]. 3GC agents (cefpodoxime and ceftibuten) have been paired with β-lactamase inhibitors such as QPX7728, ETX0282 and VNRX7145, while ceftibuten has been paired with clavulanate [317–320]. In addition, an orally absorbed derivative of avibactam has been developed [321], and oral carbapenems, sulopenem and tebipenem, are under investigation [322,323].

The management of recurrent UTIs has attracted novel therapeutic approaches, such as behavior and dietary interventions, probiotics, phytotherapy, D-mannose, methenamine hippurate, vaginal estrogens and intravesical glycosaminoglycans [324]. Of particular interest are the studies into immunotherapies that stimulate the host's immune response (e.g. bacterial lysates, oral immunostimulants and vaccines) [325,326], bacterial interference by the deliberate colonization of the bladder with an asymptomatic bacteriuria strain [327,328], fecal microbiota transplantation [329–332] and bacteriophage therapy [333–335].

More robust and contemporary bladder infection *in vitro* models will continue to inform antimicrobial PD profiling and the setting of urine-specific susceptibility breakpoints. In the future, a symptomatic patient will have access to a rapid diagnosis that differentiates infection from colonization and provides a risk profile for ascending infection. Uropathogens will have an antimicrobial susceptibility profile specific to the urinary tract. Updated international guidelines will provide antimicrobial dosing and duration recommendations that consider urinary PK, while minimizing emergence of resistance and microbiome disruption. With AMR forcing reliance on broad-spectrum antimicrobials, novel approaches targeting the host–pathogen interface, such as bacterial virulence, antimetabolites and alterations to urine composition, will be valuable antimicrobial-sparing tools.

Acknowledgments

We thank Professor Johan W. Mouton (3 November 1956 - 9 July 2019) for his insights, expertise and guidance in establishing and supporting the pharmacodynamic profiling of antimicrobial agents through the use of dynamic *in vitro* modelling. I.J.A. was funded by an Australian Government Research Training Program Scholarship (APP1114690) from the National Health and Medical Research Council of Australia. A.Y.P. and J.A.R. are part funded through an Australian National Health and Medical Research Council Practitioner Fellowships (APP1117940 and APP1117065, respectively). J.A.R. would like to acknowledge funding for a NHMRC Centre of Research Excellence (APP1099452).

Funding

This paper was not funded.

Declaration of interest

JA Roberts discloses consultancies/advisory boards for MSD (2019); QPEX (2019); Discuva Ltd (2019); Accelerate Diagnostics (2017); Bayer (2017); Biomerieux (2016). Speaking fees for MSD (2018); Biomerieux (2018), Pfizer (2019). Industry grants from MSD (2017); The Medicines Company (2017); Cardeas Pharma (2016); Biomerieux (2019); QPEX (2019); Pfizer (2019). J Meletiadis discloses research grants from Pfizer, Gilead, Astellas, MSD, F2G and VenatoRx. A Peleg discloses research funding from MSD through an investigator-initiated research project. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

Author contributions

All authors were involved in the content development of the manuscript and approved the final version. The authors take full responsibility for the content of this article.

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Chapter 2

Dynamic bladder infection *in vitro* model

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Fosfomycin is an old, off-patent, antimicrobial agent, that never underwent extensive pharmacokinetic (PK) / pharmacodynamic (PD) profiling when licensed in the 1970s. Contextually for the Australian setting, oral fosfomycin (Monurol®) has only recently be approved for unrestricted use by the TGA (2017), although clinically it has been recommended as an agent to be held in reserve for when antimicrobial resistance is encountered.

Following on from the introduction, this first original research article, published in the *Journal of Antimicrobial Chemotherapy*, describes the development and application of a novel, multi-compartment, bladder infection *in vitro* model and the simulation of oral fosfomycin in the treatment of UTIs caused by different *Enterobacterales*. This paper serves to validate design of the dynamic *in vitro* model, while providing initial PD profiling for fosfomycin efficacy.

Highlights

- Proof of concept that the multi-compartment *in vitro* model was able to accurately simulate dynamically changing *in vitro* fosfomycin concentrations mimicking historical human urinary fosfomycin PK data after a single 3 g oral dose.
- PK/PD analysis suggested that *E. coli* and *E. cloacae* isolates with a baseline MIC > 16 mg/L would not be adequately treated with a single dose of fosfomycin.
- In contrast, the majority of *K. pneumoniae* isolates re-grew regardless of baseline fosfomycin MIC value.
- The detection of a baseline resistance subpopulation was associated with emergence of fosfomycin resistance in the post-exposure re-growth.

J Antimicrob Chemother 2018; **73**: 709–719
doi:10.1093/jac/dkx441 Advance Access publication 14 December 2017

Journal of Antimicrobial Chemotherapy

Fosfomycin efficacy and emergence of resistance among Enterobacteriaceae in an *in vitro* dynamic bladder infection model

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Received 26 September 2017; returned 20 October 2017; revised 30 October 2017; accepted 31 October 2017

Background: Urinary tract infections (UTIs) are among the most common bacterial infections and a frequent indication for antibiotic use. Fosfomycin, an important oral antibiotic for outpatient UTIs, remains a viable option for MDR uropathogens. We aimed to perform pharmacodynamic profiling simulating urinary concentrations to assess the adequacy of the current dosing regimen.

Methods: A dynamic *in vitro* bladder infection model was developed, replicating urinary fosfomycin concentrations after gastrointestinal absorption, systemic distribution and urinary elimination. Concentrations were measured by LC-MS/MS. Twenty-four Enterobacteriaceae strains (*Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae*; MIC range 0.25–64 mg/L) were examined. Pathogen kill and emergence of resistance was assessed over 72 h.

Results: Observed *in vitro* fosfomycin concentrations accurately simulated urinary fosfomycin exposures (T_{max} 3.8±0.5 h; C_{max} 2630.1±245.7 mg/L; $AUC_{0\text{-}24}$ 33932.5±1964.2 mg·h/L). Fifteen of 24 isolates regrew, with significant rises in fosfomycin MIC (total population MIC_{50} 4 to 64 mg/L, MIC_{90} 64 to >1024 mg/L, $P = 0.0039$; resistant subpopulation MIC_{50} 128 to >1024 mg/L, MIC_{90} >1024 mg/L, $P = 0.0020$). *E. coli* and *E. cloacae* isolates were killed with pharmacokinetic/pharmacodynamic EI_{50} of $fAUC_{0\text{-}24}/MIC = 1922$, $fC_{max}/MIC = 149$ and $f\text{Time}>4\times MIC = 44$ h. In contrast, *K. pneumoniae* isolates were not reliably killed.

Conclusions: Using dynamic *in vitro* simulations of urinary fosfomycin exposures, *E. coli* and *E. cloacae* isolates with MIC >16 mg/L, and all *K. pneumoniae* isolates, were not reliably killed. Emergence of resistance was significant. This challenges fosfomycin dosing and clinical breakpoints, and questions the utility of fosfomycin against *K. pneumoniae*. Further work on *in vitro* dose optimization is required.

Introduction

Urinary tract infections (UTIs) are a frequent indication for antibiotic use and are among the most commonly encountered bacterial infections.[1] Incorrect outpatient use of antibiotics can serve as a potentially large breeding ground for antibiotic resistance in the wider community.[2,3] Emergence of MDR uropathogens is an increasing problem,[4] challenging current oral antibiotic treatment options. Limited data are available to guide dosing in MDR or complicated UTIs.

Fosfomycin is an old, off-patent antibiotic that remains active against many MDR uropathogens[5] and is recommended by IDSA and ESCMID as one of the first-line oral agents for the treatment of uncomplicated UTIs.[6–9] Limited evidence, however, supports the current dosing and clinical breakpoints. Further still, Enterobacteriaceae susceptibility classification differs between advisory bodies. CLSI criteria report susceptibility (S) ≤64 mg/L and resistance (R) ≥256 mg/L for uncomplicated UTIs,[10] whereas EUCAST report S ≤32 mg/L and R >32 mg/L.[11]

Oral fosfomycin tromethamine does not undergo metabolism and is primarily excreted unchanged in the urine by glomerular filtration, with little tubular secretion and reabsorption.[12,13] A compartmental model of these processes can describe the processes of gastrointestinal absorption, distribution into systemic circulation

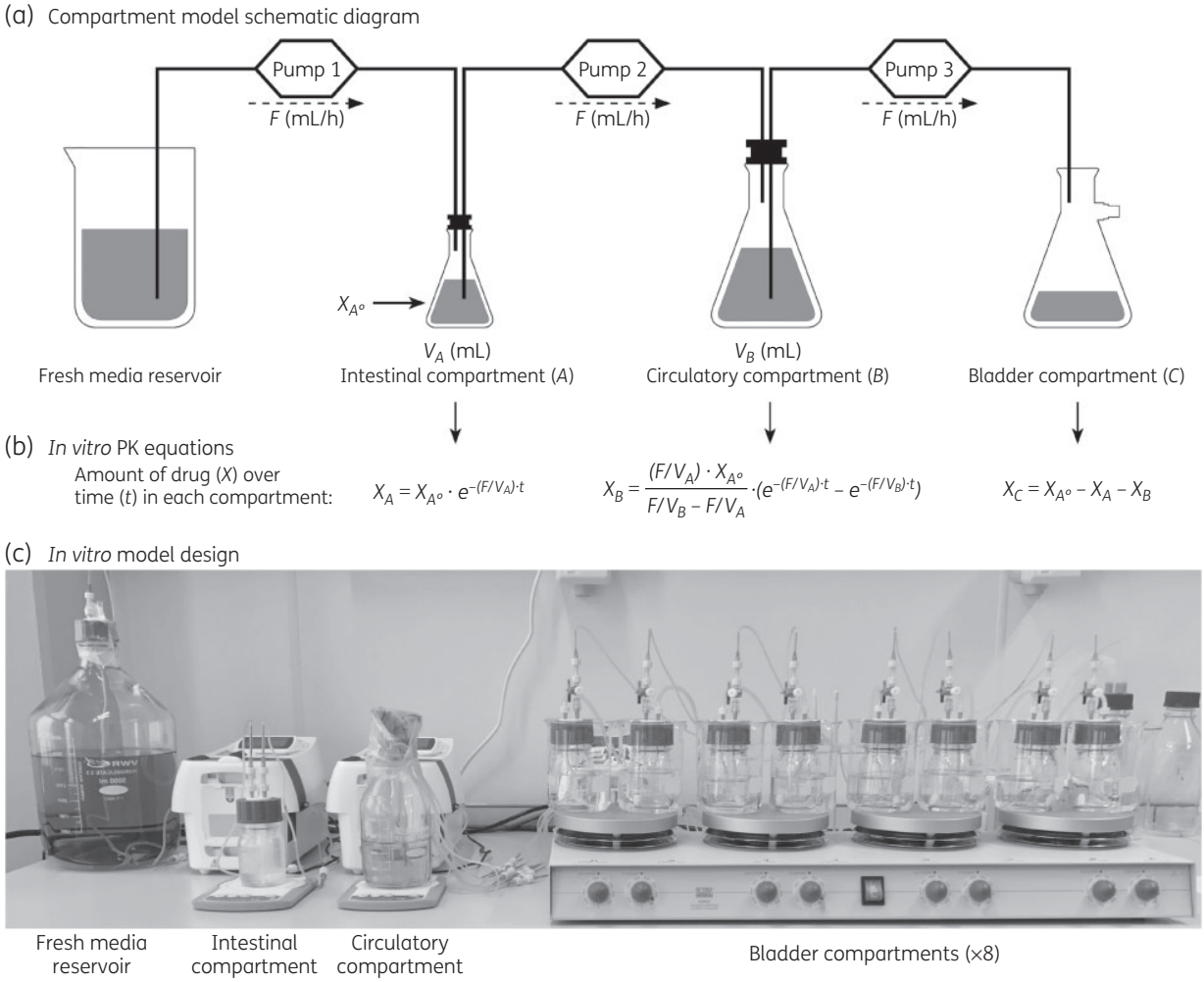

Figure 1. Drug distribution in an *in vitro* compartmental model. (a) Schematic model for first-order absorption in a two-compartment model with first-order elimination. Tandem first-order processes are simulated by exponential changes in antibiotic concentration undergoing dilution at constant volume. The volumes in the intestinal (V_A) and circulatory (V_B) compartments are kept static, while the volume in the bladder compartment is allowed to increase over time, followed by intermittent voiding, akin to normal urination. The flow rate (F) is maintained constant throughout. X_{A° represents the amount of fosfomycin in the gastrointestinal tract at time zero. Note that this does not consider any drug that, *in vivo*, would never reach the systemic circulation. Once fosfomycin is added to the intestinal compartment (*A*), rapid absorption into the circulatory compartment (*B*) and elimination into the bladder compartment (*C*) can be simulated. (b) Mathematical equations that describe the changes in the amount of drug present in each compartment over time. (c) Laboratory set-up of the *in vitro* model. Eight bladder compartments are run in parallel and placed on magnetic stirring and heating elements to ensure both adequate mixing within the compartment and maintenance of the surrounding water-bath at a temperature between 36 and 38 °C.

and excretion into the bladder.[14] *In vivo* pharmacokinetic (PK) drug distribution equations can then be used to simulate the amount of antibiotic present in each theoretical compartment as it changes over time.[15] By using dynamic *in vitro* modelling techniques, these *in vivo* PK equations can be integrated into a mathematical model that incorporates two consecutive first-order processes, with the antibiotic dose, the flow rate and compartment volumes used as the variables (Figure 1).[15] Here we used the mathematical model to construct an *in vitro* model to allow pharmacodynamic (PD) profiling of fosfomycin concentrations in bladder compartments during a simulated uncomplicated UTI. The aim of the dynamic *in vitro* model is to provide a means to demonstrate the relationship between urinary fosfomycin exposures and the microbiological effect, as well as detailing the emergence of fosfomycin resistance.

Materials and methods

Dynamic bladder infection in vitro model

The *in vitro* model was constructed to reflect normal human urodynamics on a 1:15 scale. Autoclavable PVC tubing (Gilson Inc., Middleton, WI, USA) and glassware (VWR International, Radnor, PA, USA; DURAN Group GmbH, Germany) were connected by peristaltic pumps (Gilson Inc.), which enabled eight individual bladder compartments (set within water-baths maintained

Table 1. Baseline bacterial strain characteristics and dose–response

		Fosfomycin susceptibility			Other oral antibiotic susceptibility (VITEK 2 AST-N344)					
Species	Strain no.	MIC (mg/L)	interpretation	ESBL	AMC	CXM	CIP	NIT	SXT	Dose–response at 72 h (log_{10} cfu/mL)
E. coli	11	0.5	S	yes	R	R	S	S	S	NG
	39	0.5	S	yes	R	R	S	S	S	NG
	41	0.25	S	yes	R	R	S	S	R	NG
	51	1.0	S	yes	R	R	R	S	R	3.70
	1016	16.0	S	yes	R	R	R	R	R	NG
	1231	16.0	S	yes	R	R	R	R	R	8.70
	4757	64.0	R	yes	R	R	R	S	S	8.00
	4807	32.0	S	yes	R	R	R	S	R	8.40
	12620	2.0	S	yes	R	R	R	S	S	NG
K. pneumoniae	6	4.0	S	yes	R	R	R	NR	R	4.40
	17	4.0	S	yes	R	R	R	NR	R	9.18
	50	8.0	S	yes	R	R	R	NR	R	8.85
	52	16.0	S	yes	R	R	R	NR	R	NG
	55	4.0	S	yes	R	R	S	NR	R	9.11
	892	4.0	S	yes	R	R	I	NR	R	6.85
	31865	2.0	S	no	R	R	R	NR	R	9.06
	34672	1.0	S	no	S	S	S	NR	R	9.40
E. cloacae	9	32.0	S	yes	R	R	R	NR	R	9.88
	10	64.0	R	yes	R	R	R	NR	R	9.60
	21	8.0	S	yes	R	R	S	NR	S	NG
	32	32.0	S	yes	R	R	S	NR	R	10.78
	94	1.0	S	yes	R	R	S	NR	S	NG
	35166	0.5	S	no	R	R	S	NR	S	NG
	36837	2.0	S	no	R	R	S	NR	S	7.18

R, resistant; S, susceptible; I, intermediate; NR, not reported; AMC, amoxicillin/clavulanate; CXM, cefuroxime; CIP, ciprofloxacin; NIT, nitrofurantoin; SXT, trimethoprim/sulfamethoxazole; NG, no growth.
Fosfomycin MIC determined by agar dilution. Other oral antibiotic susceptibility testing performed by VITEK 2 (bioMérieux) using the AST-N344 card. Interpretation of MIC results based on EUCAST clinical breakpoints. ESBL phenotype determined by VITEK 2 advanced expert system (bioMérieux).

at 36–38 °C) to be run in parallel (Figure 1c). Mathematical simulation applying drug distribution PK equations instructed the fosfomycin dose, volumes and flow rates to obtain the dynamic changes in fosfomycin concentrations required. Normal human PK parameters following administration of a single dose of 3 g of oral fosfomycin tromethamine were targeted. This included a serum elimination half-life of 5.7 h and peak urinary concentration between 1053 and 4415 mg/L, occurring within 4 h.[12] Simulated urination was performed four times each day, leaving a post-void residual volume of 1.5–3.0 mL (equivalent to 22.5–45 mL on the human scale). Individual test pathogens were added to each bladder compartment, at an inoculum of 10^7 cfu, providing an equivalent total number of bacteria expected in human infections (i.e. 10^5 cfu/mL in an average 250 mL void).[16]

Antibiotic and media

Stock solution of fosfomycin ('Fomicyt', InfectoPharm, Germany) was used for the *in vitro* model and media production, reconstituted to a stock concentration of 50000 mg/L. Mueller–Hinton broth (MHB) (Becton Dickinson, Sparks, MD, USA) supplemented with glucose-6-phosphate (G7879-5G, Sigma–Aldrich, St Louis, MO, USA) at a concentration of 25 mg/L, was used within the *in vitro* model. Trypticase soy agar containing 5% sheep blood (TSA) (Becton Dickinson) was used to subculture isolates from the freezer stock. Unsupplemented Mueller–Hinton II agar (MHA) (Becton Dickinson) was used for quantitative growth cultures. Fosfomycin was incorporated into MHA (supplemented with glucose-6-phosphate at a final concentration of 25 mg/L) for both agar dilution susceptibility testing and for quantitative growth cultures of any resistant subpopulation.

Bacterial strains and in vitro susceptibility studies

Twenty-four Enterobacteriaceae clinical isolates were selected for testing, including nine *Escherichia coli*, eight *Klebsiella pneumoniae* and seven *Enterobacter cloacae*. The characteristics of the strains are shown in Table 1. Species identification was confirmed by MALDI-TOF MS (Bruker Daltonik GmbH, Germany). The clinical isolates originated from the Netherlands and were selected to provide a representative range of MIC values with a baseline MIC $\leq$64 mg/L (Figure 2). Fosfomycin susceptibility testing was performed by agar dilution following ISO standards.[17,18] *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms. All isolates also underwent VITEK 2 (bioMérieux, France) Gram-negative antimicrobial susceptibility testing (AST-N344 card). ESBL phenotype was determined using a VITEK 2 advanced expert system (bioMérieux).

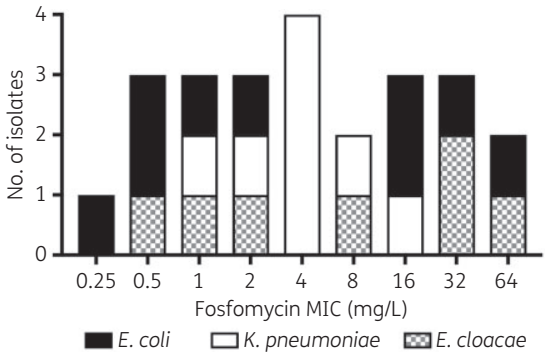

Figure 2. Baseline fosfomycin MIC distribution. MIC testing performed by agar dilution. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control organisms and returned MIC values within range.

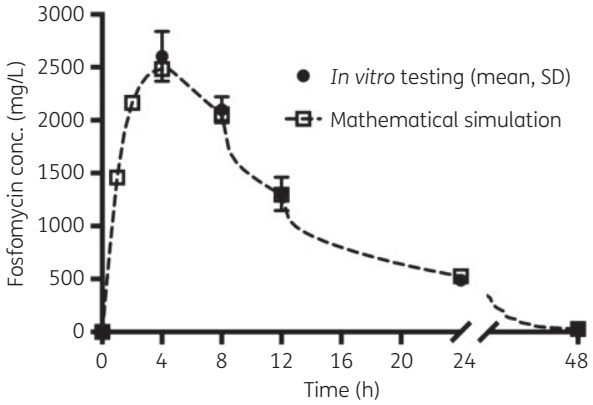

Figure 3. Average fosfomycin concentration changes over time within the *in vitro* bladder compartment following a single dose of fosfomycin. The broken line represents the concentration–time curve generated from values derived from the mathematical simulation. Open squares highlight the concentrations expected at bladder voiding timepoints. Filled circles represent the average *in vitro* fosfomycin concentrations measured by LC-MS/MS, with error bars representing the SD.

In vitro sample processing

Samples for PK and PD assessment were taken directly from each bladder compartment, collected at every simulated bladder void over each 72 h experiment. Samples for fosfomycin concentration quantification were immediately frozen at −80 °C until testing. Quantitative cultures for PD assessments were processed immediately, with cfu/mL calculated at each timepoint. Specifically, collected samples underwent a series of 10-fold dilutions, of which 20 μL from each dilution was plated onto MHA. The lower limit of detection was 25 cfu/mL. Repeated washing and centrifugation of the samples was not performed as previous reports have demonstrated no difference in antibiotic carryover between dilution and washing.[19] In order to confirm successful pathogen kill at 72 h, any bladder compartment without visible growth was confirmed as 'no growth' by culturing the centrifuged sediment from the total volume of the final void (∼50 mL) onto TSA. All plates were incubated aerobically at 37 °C for 18–24 h.

Resistant subpopulation studies

A quantitative culture of the resistant subpopulation was performed by plating the sample onto MHA containing fosfomycin at two concentrations (32 and 512 mg/L; supplemented with 25 mg/L glucose-6-phosphate) in parallel with that plated on unsupplemented MHA. This assessment was performed every 12 h. In order to increase the limit of detection of the resistant subpopulation from cultures performed at baseline and at 72 h (where regrowth occurred), subcultures were made and then, using a heavy starting inoculum, plated on both unsupplemented MHA and MHA containing 32 mg/L fosfomycin.

Measurement of fosfomycin concentrations

An LC-MS/MS method was used for the quantification of fosfomycin from PK samples collected at the time of bladder compartment voiding.[20] The method was validated for urine and plasma samples of fosfomycin, but additional tests confirmed its applicability for fosfomycin in MHB samples. The method was validated according to the FDA guidelines for bioanalytical method validations[21] over a range of 0.75–375 mg/L ($R^2 = 0.9998$). The lower limit of quantification was 0.75 mg/L and the lower limit of detection was 0.70 mg/L. The method was found to be accurate and precise with a maximum deviation of 5.0%. Prior to testing, samples were defrosted, vortexed and diluted 1:10 with saline. Stability of fosfomycin at −80 °C for at least 6 months was confirmed during the method validation.

Statistical and PK/PD analyses

Statistical comparison between MIC_{50} and MIC_{90} values, before and after exposure to fosfomycin, was performed using a Wilcoxon matched-pairs signed rank test. The resistant subpopulation percentage of the total population was determined by dividing the quantitative growth on MHA containing 32 mg/L fosfomycin by that of the growth on unsupplemented MHA. Concentration–time curves were evaluated using non-linear least-square regression. The interpolated PK parameters (C_{max} and AUC_{0-24}) and Time>4×MIC were then used for the PK/PD analysis using a four-parameter dose–response curve. The non-linear regression line was weighted by growth control values as appropriate. The relationships between the outcome variables at 72 h of the (i) total growth (log_{10} cfu/mL on unsupplemented MHA) and (ii) emergence of fosfomycin resistance (resistant subpopulation proportion) were evaluated against the three PK/PD indices and the baseline resistant subpopulation proportion. The PK/PD indices included the ratio of the free-drug AUC_{0-24} to the pathogen MIC ($fAUC_{0-24}$/MIC), the ratio of the maximal free-drug concentration to the pathogen MIC (fC_{max}/MIC) and the time that the free-drug concentrations exceeded four times the pathogen MIC (fTime>4×MIC). All analyses were performed with GraphPad Prism (version 7.0b, MAC OS X). Data are presented as means (±SD).

Results

PK validation of the dynamic in vitro model

The observed *in vitro* concentrations closely matched the concentration–time curve predicted by the mathematical simulation detailing fosfomycin exposures reported in humans following a single 3 g dose of oral fosfomycin tromethamine (Figure 3).[12] There was minimal inter-bladder compartment variation. Across all tested bladder compartments, using non-linear regression interpolated values, the mean T_{max} was 3.8 h (±0.5), the mean C_{max} was 2630.1 mg/L (±245.7) and the mean AUC_{0-24} was 33932.5 mg·h/L (±1964.2).

Dose–response

Following the administration of a single dose of fosfomycin, 9 out of 24 isolates were killed, determined by no growth from the total

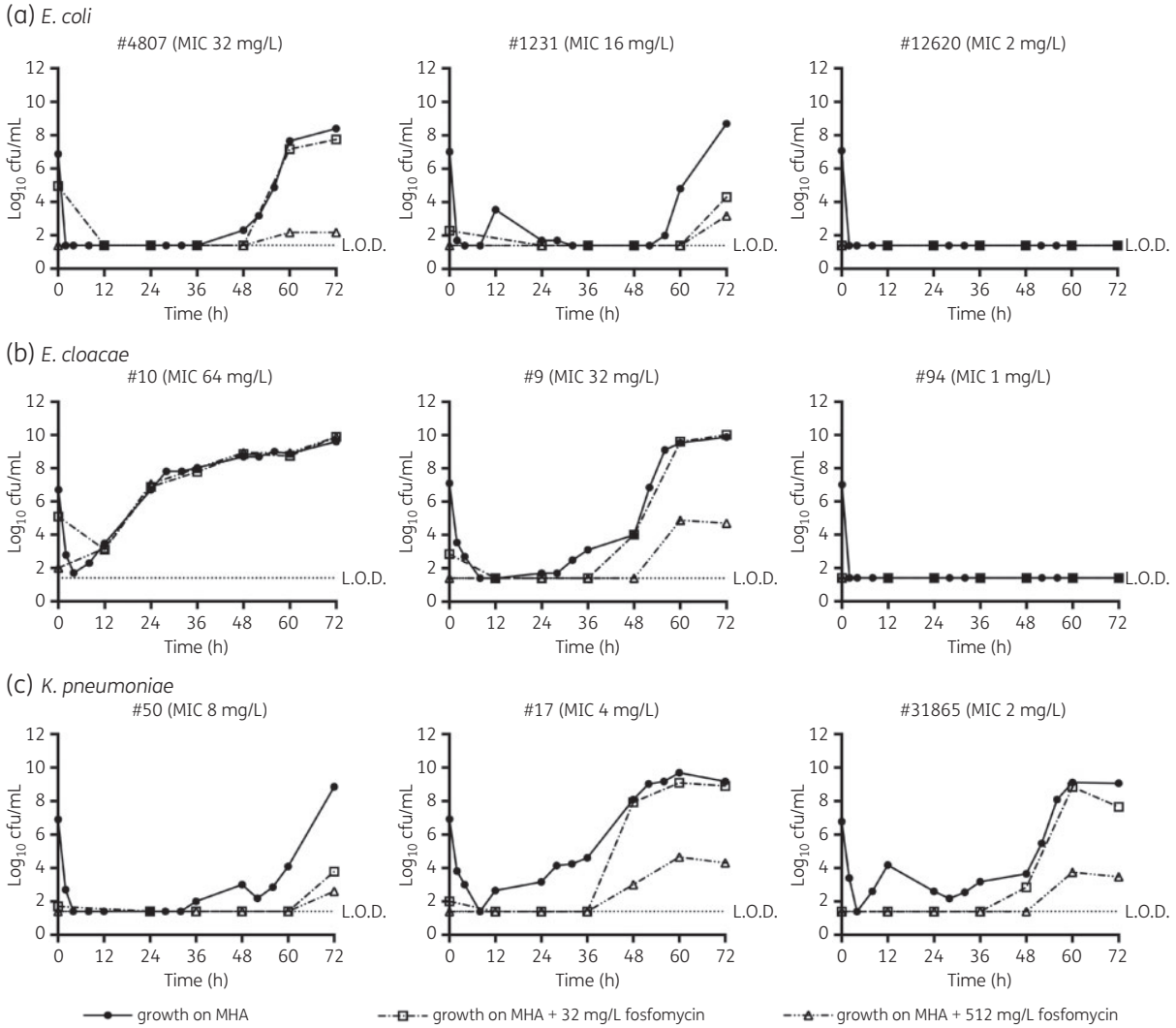

Figure 4. Examples of the quantitative PD assessment following a single dose of fosfomycin. Quantitative cultures performed at every simulated bladder compartment void. Filled circles represent total population growth, open squares represent low-level fosfomycin resistance and open triangles represent high-level fosfomycin resistance. L.O.D., limit of detection.

volume of the final void at 72 h (Table 1). This accounted for around half of the *E. coli* (5/9) and *E. cloacae* (3/7) isolates, but only one *K. pneumoniae* isolate was killed. The remaining 15 isolates regrew with variable degrees of fosfomycin resistance. Examples of the dose–response curve of isolates with different baseline fosfomycin MICs are presented in Figure 4. Here the PD response is that of effective kill, regrowth where the total population is predominantly replaced by a 'low-level' resistant population (i.e. similar quantity of growth on MHA containing 32 mg/L fosfomycin compared with unsupplemented MHA) or regrowth with complete population substitution for the high-level resistant population (i.e. similar quantity of growth on MHA containing 512 mg/L fosfomycin compared with unsupplemented MHA). Some isolates demonstrated detectable fosfomycin-resistant growth only at the final 72 h assessment, despite total population regrowth detected from an earlier timepoint.

In vitro susceptibility and resistant subpopulation studies

The change in MIC for the test isolates, before and after fosfomycin exposure, is presented in Figure 5. The total population and resistant subpopulation are shown in Figure 5(a) and Figure 5(b), respectively. Following exposure to fosfomycin, there was a significant increase in the fosfomycin MIC for the total population for the 15 isolates that regrew (MIC_{50} 4 mg/L and MIC_{90} 64 mg/L at baseline compared with MIC_{50} 64 mg/L and MIC_{90} >1024 mg/L at 72 h, $P = 0.0039$). There was also a significant rise in the resistant subpopulation MIC compared with baseline ($MIC_{50} = 128$ mg/L and MIC_{90} >1024 mg/L at baseline compared with MIC_{50} >1024 mg/L and MIC_{90} >1024 mg/L at 72 h, $P = 0.0020$). All the isolates that regrew had a detectable resistant subpopulation at 72 h, including two isolates for which this was not detected at baseline.

The proportion of the total population that the resistant subpopulation was detected varied between isolates. At baseline, more

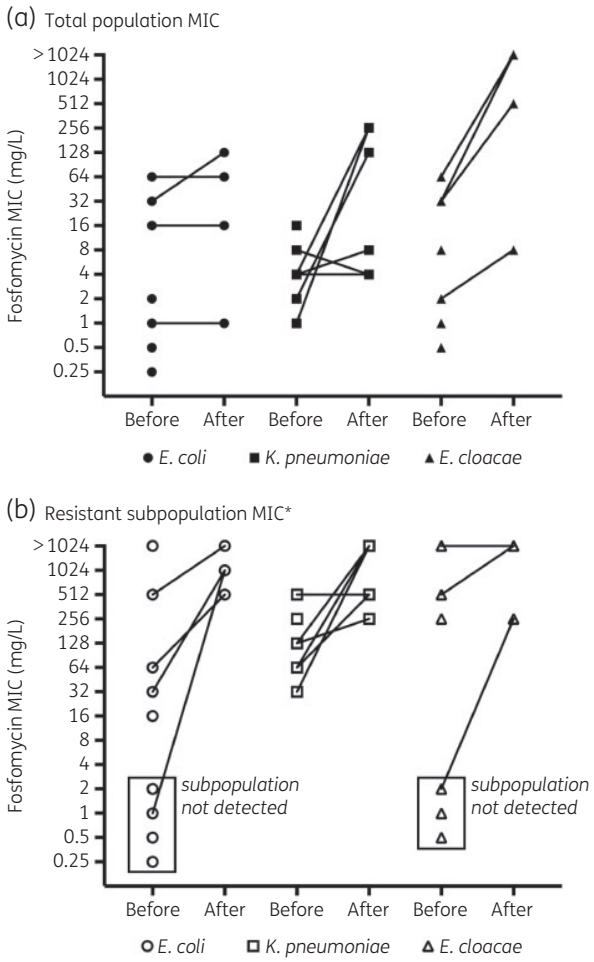

Figure 5. Changes in fosfomycin MIC before and after exposure to a single dose of fosfomycin. *Resistant subpopulation testing was performed from a subculture of growth from MHA containing fosfomycin. MIC testing performed by agar dilution. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control organisms and returned MIC values within range.

than half of all the isolates had a detectable resistant subpopulation: four out of nine *E. coli* isolates (0.002%–3.7%); six out of eight *K. pneumoniae* (0.0006%–0.001%); and four out of seven *E. cloacae* isolates (0.003%–2.5%). Using a heavy inoculum from a subculture from the initial *in vitro* growth, as described previously, an additional three isolates (one *E. coli* and two *K. pneumoniae*) also had a resistant subpopulation detected at baseline. After exposure to fosfomycin, the resistant subpopulation proportions were higher than that seen at baseline. In three isolates (one *K. pneumoniae* and two *E. cloacae*) the total population was completely replaced by the resistant population. For the remaining isolates that regrew, the proportion of the total population that the resistant subpopulation made up were as follows: >20% in four isolates (two *E. coli*, one *K. pneumoniae* and one *E. cloacae*); ≥1% in two isolates (one *K. pneumoniae* and one *E. cloacae*); and between 0.0008% and 0.01% in four isolates (one *E. coli* and three *K. pneumoniae*). The remaining isolates (one *E. coli* and one *K. pneumoniae*) had a detectable resistant subpopulation below the limit of detection during the *in vitro* PD sampling and culture, and were detected using a heavy inoculum from a subculture of the total population growth. The relationship between the baseline resistant subpopulation proportion and the microbiological outcomes after exposure to fosfomycin was assessed (Figures 6 and 7). *E. coli* and *E. cloacae* isolates with a greater resistant subpopulation proportion at baseline had a greater propensity for the emergence of resistance at 72 h (EC_{50} = 0.003%; R^2 = 0.8036) (Figure 7a). In contrast, for *K. pneumoniae* isolates, this relationship was not demonstrated, but instead an inverse, or paradoxical, relationship tended to be observed (Figure 7b).

PK/PD analysis

There were distinct differences in the response to fosfomycin between the different species of Enterobacteriaceae. *E. coli* and *E. cloacae* demonstrated similar PD responses to fosfomycin exposure and were therefore analysed together. In contrast, *K. pneumoniae* isolates differed greatly in their response and were analysed separately. Following the administration of a single dose of fosfomycin, the effective killing of *E. coli* and *E. cloacae* isolates was described by PK/PD EI_{50} of $fAUC_{0-24}$/MIC = 1922 (R^2 = 0.7115), fC_{max}/MIC = 149 (R^2 = 0.7042) and fTime>4×MIC = 44 h (R^2 = 0.7045) (Figure 6a). The emergence of fosfomycin resistance was similarly described by PK/PD EI_{50} of $fAUC_{0-24}$/MIC = 1805 (R^2 = 0.8256), fC_{max}/MIC = 139.8 (R^2 = 0.8218) and fTime>4×MIC = 40 h (R^2 = 0.8150) (Figure 7a). The individual importance of concentration- or time-dependent drug activity for pathogen kill and the suppression of the emergence of resistance could not be established given that all three PK/PD indices are intrinsically linked following the administration of a single dose of fosfomycin.

K. pneumoniae isolates, in contrast, demonstrated a paradoxical response to fosfomycin exposure. These isolates tended to display a greater propensity for regrowth and emergence of fosfomycin resistance in the setting of higher PK/PD indices ($fAUC_{0-24}$/MIC, fC_{max}/MIC and fTime>4×MIC) (Figures 6b and 7b).

Discussion

Fosfomycin-susceptible Enterobacteriaceae tested within the dynamic bladder infection *in vitro* model demonstrate significant rates of regrowth following a single dose of fosfomycin. This is in contrast to a recent study that examined fosfomycin urinary concentrations against 11 *E. coli* isolates in an *in vitro* PD model, which afforded pathogen kill in all cases.[19] This is not an unexpected finding given that all isolates tested had a fosfomycin MIC ≤4 mg/L and were exposed to a simulated peak fosfomycin concentration of 4000 mg/L.

In our study, and similar to reports from other *in vitro* studies that simulate plasma concentrations,[22–24] the emergence of resistance following exposure to fosfomycin appears to be due to the amplification of bacterial subpopulations. Our data also demonstrate significant increases in the total and subpopulation fosfomycin MIC values for isolates that regrow. Based on the PK/PD analysis, *E. coli* and *E. cloacae* isolates with MIC values >16 mg/L would not be reliably killed. If urinary fosfomycin exposure was reduced in the setting of normal human PK variation (reported C_{max} normal range from 1053 to 4415 mg/L[12]), then *E. coli* and

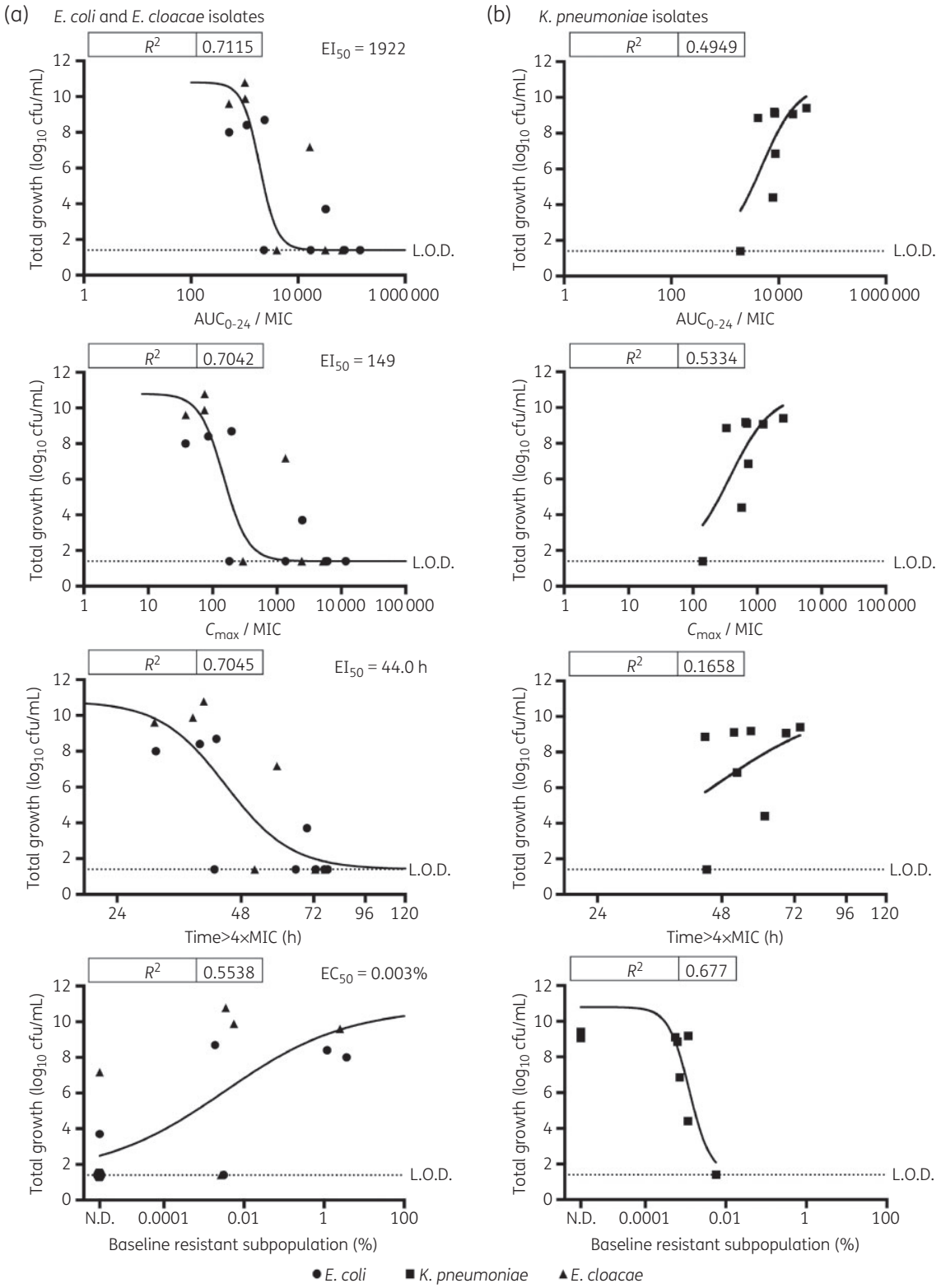

Figure 6. Relationship between drug exposure and baseline resistance with effective pathogen kill. Relationships between free-drug fosfomycin *f*AUC$_{0-24}$/MIC ratio, *fC*$_{max}$/MIC ratio, *f*Time>4×MIC and baseline resistant subpopulation proportion and the total growth at 72 h of *E. coli*, *E. cloacae* and *K. pneumoniae* isolates. In the bottom left-hand graph, the large filled hexagon represents multiple isolates (four *E. coli* and two *E. cloacae*) that did not have a resistant subpopulation detected at baseline and were then effectively killed after exposure to fosfomycin. The broken lines represent the limit of detection (L.O.D.). N.D., not detected.

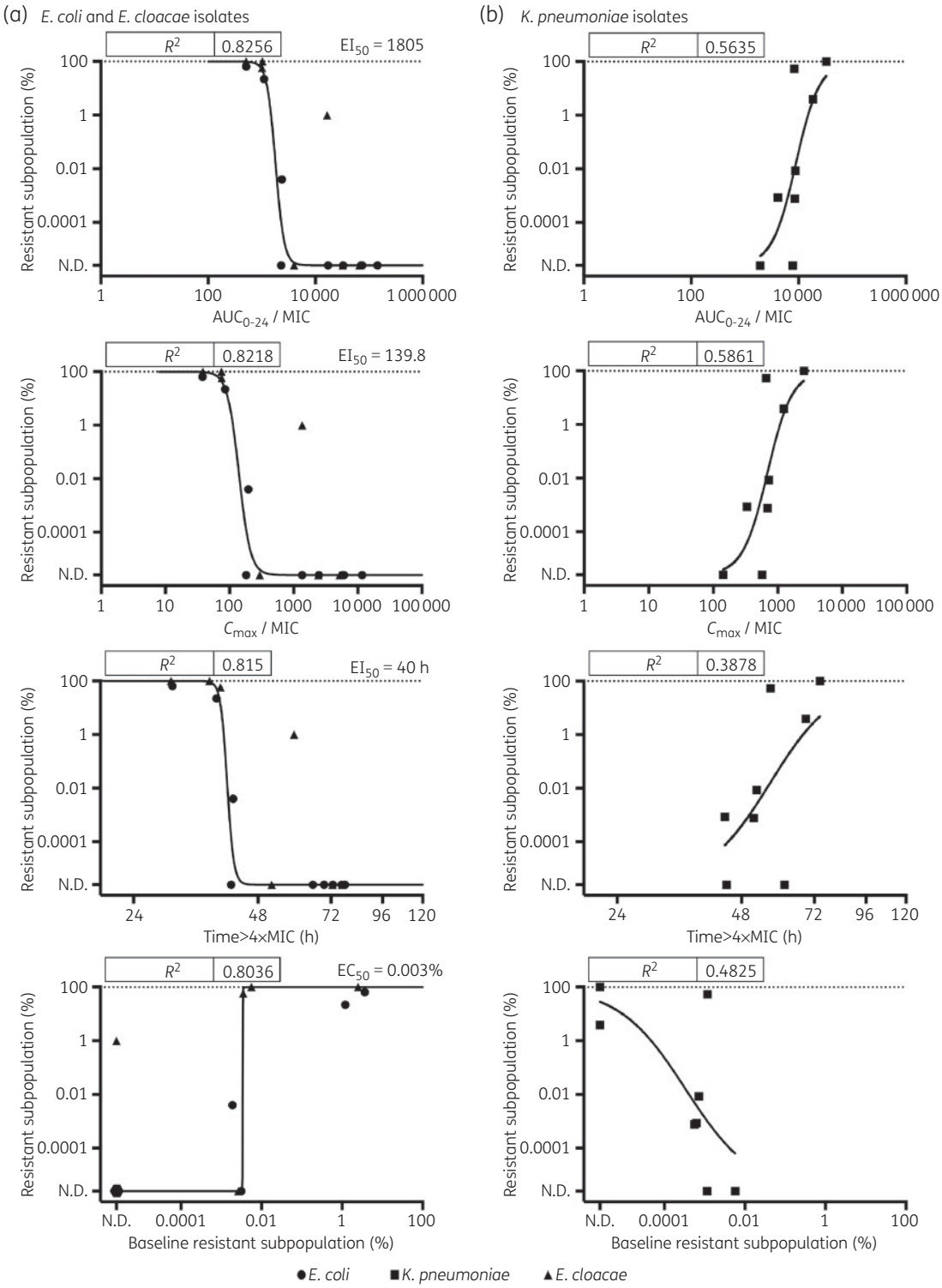

Figure 7. Relationship between drug exposure and baseline resistance with the emergence of fosfomycin resistance. Relationships between free-drug fosfomycin $fAUC_{0-24}$/MIC ratio, fC_{max}/MIC ratio and fTime>4×MIC and baseline resistant subpopulation proportion and the resistance subpopulation proportion at 72 h of *E. coli*, *E. cloacae* and *K. pneumoniae* isolates. In the bottom left-hand graph, the large filled hexagon represents multiple isolates (four *E. coli* and two *E. cloacae*) that did not have a resistant subpopulation detected at baseline and were then effectively killed after exposure to fosfomycin. One additional *E. coli* isolate, also represented by this data point, did regrow at 72 h, but the resistant subpopulation of the regrowth was below the lower limit of detection during *in vitro* testing. The broken lines represent where the resistant subpopulation has completely replaced the susceptible population. N.D., not detected.

E. cloacae isolates with MIC >4 mg/L would also not be reliably killed. These *in vitro* data challenge the current clinical breakpoints set by both EUCAST and CLSI (S ≤32 mg/L and S ≤64 mg/L, respectively).[10,11]

A strength of our study is the design of the dynamic *in vitro* bladder infection model, adapted from previous *in vitro* designs,[25–31] which simulates the entire drug distribution PK of oral fosfomycin, including gastrointestinal absorption, distribution into the systemic circulation and elimination into the bladder. This provides a more accurate physiological simulation compared with other one-compartment models.[19] LC-MS/MS quantification of fosfomycin concentrations from PK samples returned accurate measurements within the error margin and standard deviation allowed according to the FDA guidelines[21] and closely matched that of the mathematical simulation that applies theoretical PK drug distribution equations.[15] Our data demonstrate that the *in vitro* model can accurately simulate dynamic urinary fosfomycin exposures expected in humans following the administration of a single 3 g oral dose of fosfomycin tromethamine. This enables PD profiling of test pathogens exposed to urinary concentrations of fosfomycin.

A novel finding of this research is the behaviour of the *K. pneumoniae* isolates within the *in vitro* model. Regardless of the baseline MIC, *K. pneumoniae* isolates are not reliably killed when exposed to normal urinary fosfomycin concentrations and, in fact, demonstrate a paradoxical response. How this relates to clinical outcomes is uncertain, although some clinical data indicate that *Klebsiella* UTIs treated with oral fosfomycin are more likely to fail compared with *E. coli*.[32] This suggests that fosfomycin may not be adequate as a single agent for *K. pneumoniae* UTIs.

The ability to accurately predict treatment success of fosfomycin when an isolate is cultured is vital. However, discrepant results between the gold standard susceptibility testing method (i.e. agar dilution) and other methods, such as VITEK 2 (bioMérieux) and gradient concentration strips, e.g. Etest (bioMérieux) and MIC Strip (MIC Test Strip, Liofilchem, Italy), remain problematic.[33–37] Treatment outcome may be clearly predictable for Enterobacteriaceae with fosfomycin MIC values at the extremes (i.e. ≤0.5 and ≥64.0 mg/L); however, for a number of isolates that have MIC values that fall within this range, the treatment outcome may be less certain. For *K. pneumoniae* isolates, even less is certain when considering baseline MIC and response to therapy. An individual isolate's fosfomycin MIC value may in fact not be the only important predictor for treatment success.[38] Our data demonstrate that for *E. coli* and *E. cloacae* isolates, the proportion of the baseline resistant subpopulation is also an important factor for the emergence of resistance, where a baseline resistant subpopulation percentage of >0.003% was predictive of regrowth. This may also suggest that a specific gene mutation, or combination of mutations, might be an important factor for treatment failure.[39–41]

To address these issues, more urinary PK/PD profiling of fosfomycin is required. Both dose fractionation and optimization strategies, including repeat dosing schedules, should be investigated. Furthermore, molecular analysis of the mechanism of fosfomycin resistance, both at baseline and following exposure to fosfomycin with *in vitro* and *in vivo* isolates, would help ascertain the importance of specific mutations to the microbiological outcome. Comparative growth kinetics of the test isolates, before and after exposure to fosfomycin, could also provide pathogen-specific information important for treatment success and identify any fitness cost due to the emergence of fosfomycin resistance.

Given the nature of the *in vitro* model, a number of other limitations should be highlighted. Firstly, conclusions drawn from this analysis do not take into consideration the important effects of both the tissue anatomy of the human bladder and the role of the innate and adaptive immune responses, and the importance of the local microbiome.[42] Nor does the model simulate the normal diurnal variation in urine output under the control of neurohormonal factors. Similarly, the effect of urine as the culture medium, which would impact both isolate growth and fosfomycin activity, has not been assessed. In addition, the use of glucose-6-phosphate in the liquid medium may in fact preferably select for mutants in the hexose phosphate transport system rather than others.[38] Future work will focus upon using pooled human urine, or an artificial urine, as the growth medium for the test organisms.[43,44] Finally, the assessment of the resistant subpopulation does not account for any fitness cost that fosfomycin resistance may cause in the growth of these strains.

The strengths of our results lie in the accurate and dynamic simulation of urinary fosfomycin exposure tested across 24 isolates, including different species of Enterobacteriaceae, with a range of baseline fosfomycin MICs. Although individual pathogen responses may differ, our data establish patterns in PD effects across a broad spectrum of isolates, rather than testing fewer isolates in multiple replicates. Further validation of the *in vitro* model would require the PK/PD assessment of a different antibiotic, such as ciprofloxacin, that has well described *in vitro* and *in vivo* efficacy at approved doses.[45,46]

Given that fosfomycin remains one of the few oral antibiotics with activity against MDR uropathogens, it is vital to preserve its activity for the future. Suboptimal dosing can drive the emergence of resistance and ultimately contribute to the loss of activity. This is further compounded by high inter-individual variability in urinary fosfomycin concentrations seen in humans, which thereby affects antibiotic exposure on uropathogens.[12,47,48] Therefore, further work is required to confirm the scientific basis behind the current fosfomycin dosing schedules and laboratory clinical breakpoints. Dose optimization strategies, such as administering one or multiple repeat doses at 48 or 24 h intervals, should be investigated to help support, or caution against, such clinical approaches.

Acknowledgements

We would like to acknowledge the laboratory assistance of Carla Roodbol de Goeij (Department of Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre) during the *in vitro* model development and testing.

Funding

This work was supported by an Australian Government Research Training Program Scholarship (APP1114690) and the AIDA project LifeSciHealth Priority of the European Commission Seventh Framework Programme, FP7 (Preserving Old Antibiotics for the Future, grant number F3-2011-278348).

J. A. R. wishes to recognize funding from the Australian National Health and Medical Research Council for a Centre of Research Excellence (APP1099452). J. A. R. is funded in part by a Practitioner Fellowship

(APP1117065) from the National Health and Medical Research Council of Australia.

A. Y. P. was part funded through an Australian National Health and Medical Research Council Practitioner Fellowship (APP1117940).

Transparency declarations

J. A. R. has received investigator-initiated grants from, or has consulted for, bioMérieux, Astellas, MSD, The Medicines Company and Cardeas Pharma. J. W. M. has received research funding from Adenium, AstraZeneca, Basilea, Cubist, Polyphor, Roche, Eumedica, Basilea, VenatorX, AiCuris, Gilead and Wockhardt. All other authors: none to declare.

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Chapter 3

Fosfomycin efficacy against common uropathogens

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Fosfomycin efficacy against common uropathogens

The previous paper demonstrated that dynamic changes in urinary fosfomycin concentration can be accurately simulated. A key finding from this work questioned the efficacy of fosfomycin against *K. pneumoniae* species.

This next paper, published in *Journal of Antimicrobial Chemotherapy*, expanded upon this initial testing and further examined the efficacy of fosfomycin against 24 *E. coli* and 20 *K. pneumoniae* isolates. This series of experiments applied modern fosfomycin urinary PK data and a modified *in vitro* model design, in which sixteen individual bladder compartments were run concurrently and the voiding schedule was controlled by a bespoke computer software program that controlled a fourth peristaltic pump. An in-depth analysis of the baseline predictors for treatment response was examined, including molecular analysis of fosfomycin resistance genes.

Highlights

- With an expanded selection of *E. coli* and *K. pneumoniae* isolates, discrepant responses to a single fosfomycin dose was confirmed, regardless of baseline susceptibility.

- Whole genome sequencing failed to identify a baseline mutation in fosfomycin resistance genes that was able to predict treatment failure.

- For *E. coli* isolates, fosfomycin demonstrated good activity against isolates with a range of MIC values, and consistently effective in isolates with an MIC ≤ 2 mg/L.
    - However, failure was related to high-level heteroresistance, in isolates that were classified as susceptible by agar dilution MIC.

- For *K. pneumoniae* isolates, fosfomycin was largely ineffective, regardless of baseline MIC.
    - The majority of isolates have a functionally-fit resistant subpopulation.
    - All have a *fosA* gene, although without evidence of *fosA* upregulation post-exposure.

- Overall, fosfomycin MIC appears to be a poor predictor for efficacy, thereby challenging the application of clinical breakpoint set for all *Enterobacterales* by EUCAST.

- Screening for baseline fosfomycin heteroresistance may be more informative, especially for *E. coli* isolates with a fosfomycin MIC between 4 – 128 mg/L.

J Antimicrob Chemother
doi:10.1093/jac/dkz519

Journal of Antimicrobial Chemotherapy

Impact of bacterial species and baseline resistance on fosfomycin efficacy in urinary tract infections

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Received 5 September 2019; returned 28 October 2019; revised 17 November 2019; accepted 22 November 2019

Objectives: To assess the antibacterial effects of a single 3 g oral fosfomycin dose on *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates within a dynamic bladder infection model.

Methods: An *in vitro* model simulating dynamic urinary fosfomycin concentrations was used. Target fosfomycin exposure (C_{max} = 1984 mg/L and T_{max} = 7.5 h) was validated by LC-MS/MS. Pharmacodynamic responses of 24 *E. coli* and 20 *K. pneumoniae* clinical isolates were examined (fosfomycin MIC ≤0.25–128 mg/L). Mutant prevention concentration (MPC), fosfomycin heteroresistance, fosfomycin resistance genes and *fosA* expression were examined. Pathogen kill and emergence of high-level resistance (HLR; MIC >1024 mg/L) were quantified.

Results: Following fosfomycin exposure, 20 of 24 *E. coli* exhibited reductions in bacterial counts below the lower limit of quantification without regrowth, despite baseline fosfomycin MICs up to 128 mg/L. Four *E. coli* regrew (MIC = 4–32 mg/L) with HLR population replacement. At baseline, these isolates had detectable HLR subpopulations and MPC >1024 mg/L. All *E. coli* isolates were *fosA* negative. In contrast, 17 of 20 *K. pneumoniae* regrew post exposure, 6 with emergence of HLR (proportion = 0.01%–100%). The three isolates without regrowth did not have a detectable HLR subpopulation after dynamic drug-free incubation. All *K. pneumoniae* had MPC >1024 mg/L and were *fosA* positive. WGS analysis and *fosA* expression failed to predict fosfomycin efficacy.

Conclusions: *E. coli* and *K. pneumoniae* isolates demonstrate discrepant responses to a single fosfomycin dose in a dynamic bladder infection *in vitro* model. Treatment failure against *E. coli* was related to an HLR subpopulation, not identified by standard MIC testing. Activity against *K. pneumoniae* appeared limited, regardless of MIC testing, due to universal baseline heteroresistance.

Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections, affecting millions of people every year.[1] Almost half of all women will be affected in their lifetime.[2,3] The significant cost and morbidity of these infections are experienced worldwide, worsened by the increase in antimicrobial resistance limiting treatment options.[4–9]

Oral fosfomycin, an old, off-patent antimicrobial, licensed as a single 3 g oral dose, is a recommended first-line therapeutic option in the USA and Europe.[10,11] In Australia it is a second-line agent, together with quinolone antimicrobials, reserved for when resistance is confirmed to first-line options.[12] Fosfomycin is well tolerated and has few reported side effects.[13] Single-dose administration has benefits for patient compliance and acceptability, while limiting the collateral damage on the microbiome. However, reduced clinical efficacy compared with comparator agents has been reported.[14]

Escherichia coli and *Klebsiella* spp. are the most commonly reported uropathogens.[1] Fosfomycin clinical breakpoints, however, differ between reference guidelines. CLSI reports MIC and disc diffusion breakpoints for *E. coli* only (susceptible: ≤64 mg/L and >16 mm),[15] whereas EUCAST reports MIC breakpoints for all Enterobacterales (susceptible: ≤32 mg/L) and disc diffusion for *E. coli* only (susceptible: >24 mm).[16] Agar dilution is the only approved MIC method for fosfomycin susceptibility testing, which

is neither widely available nor practical for diagnostic laboratories. Other susceptibility methods have performed poorly in comparative studies.[17,18]

To investigate the observed discrepancies between the reduced clinical success rate of fosfomycin and the high *in vitro* susceptibility rates, we examined oral fosfomycin efficacy against *E. coli* and *Klebsiella pneumoniae* clinical isolates, including ESBL-producing strains, to determine baseline characteristics predictive for fosfomycin efficacy in a dynamic bladder infection *in vitro* model.

Materials and methods

Antibiotic and media

Reconstituted stock solution of fosfomycin ('Fomicyt', InfectoPharm GmbH, Germany) at a concentration of 50 000 mg/L was used for the *in vitro* model and media production. CAMHB (Becton Dickinson, MD, USA) supplemented with glucose-6-phosphate (G6P) (G7879-5G, Sigma–Aldrich, MO, USA) at a final concentration of 25 mg/L was used as the liquid medium within the *in vitro* model. Solid media used included: Trypticase soy agar containing 5% sheep blood (TSA) (Becton Dickinson) and Mueller–Hinton II agar (MHA) (Becton Dickinson). All MHA to which fosfomycin was added also contained 25 mg/L G6P.

Bacterial strains, fosfomycin susceptibility and heteroresistance

Forty-four clinical isolates were included (24 *E. coli* and 20 *K. pneumoniae*). Species identification was confirmed by MALDI-TOF MS (Bruker Daltonik GmbH, Germany). The isolates originated from the Netherlands and were selected to provide a representative range of MIC values.[17,19] Fosfomycin susceptibility was determined by agar dilution following the reference standard,[20,21] disc diffusion using FOT200 discs (Oxoid Ltd/Thermo Fisher Scientific, UK), VITEK 2 using the AST-N344 card (bioMérieux, France) and broth microdilution (BMD; in CAMHB with and without G6P).[20,21] MIC values determined by agar dilution and BMD were performed in triplicate, presented as the median value. Baseline heteroresistance was assessed by the mutant prevention concentration (MPC)[22] and a modified disc-elution screening test (Figure S1, available as Supplementary data at *JAC* Online).[23,24] Details of both methods are available as Supplementary data. *E. coli* ATCC 25922 was used for quality control in all testing.

Dynamic bladder infection in vitro model

The bladder infection model was adapted from previous studies.[25] Drug distribution pharmacokinetic (PK) equations (Figure S2)[26] were used in a mathematical model to inform initial fosfomycin dose, compartment volumes and flow rates in order to simulate fosfomycin concentrations expected in the human bladder following a single 3 g oral dose,[27] while simulating normal urodynamics, with a urine output of 1 mL/min, 6 voids/day and a post-void residual volume <50 mL. The *in vitro* model was then constructed on a 1:16 scale, enabling dynamic drug distribution to 16 independent bladder compartments, each inoculated with a different bacterial isolate (Figure 1). Test isolates were added to each bladder compartment (inoculum of 10^7 cfu) to provide an equivalent number of bacteria expected in human infections (i.e. 10^5 cfu/mL in an average 250 mL void). The model was run without fosfomycin for 18 h to determine growth capacity and quantification of any low-level resistant (LLR) and high-level resistant (HLR) subpopulations. After fosfomycin administration, the model was run for 72 h, with regular PK and pharmacodynamic (PD) sampling directly from each bladder compartment. Quantitative cultures (cfu/mL) were performed using colony counts on drug-free MHA and MHA with 64 and 512 mg/L fosfomycin (with 25 mg/L G6P). All agar plates were incubated at 37°C in ambient air for 16–20 h. Plates supplemented with fosfomycin were re-incubated for a further 24 h to confirm colony counts. Species identification was confirmed by MALDI-TOF MS (Bruker Daltonik GmbH, Germany) to exclude contamination at the final PD assessment. The post-exposure fosfomycin MIC for the total population, and any resistant subpopulation, was determined by agar dilution.

Measurement of fosfomycin concentrations

PK samples for fosfomycin quantification were diluted 1:10 with saline and immediately frozen at −80°C until testing using an ultraperformance LC-MS/MS method. All bladder compartments were sampled at the C_{max} timepoint (7.5 h), providing an assessment of intercompartment variation and accuracy. At the remaining timepoints (2.5, 5, 12 and 24 h), three bladder compartments were sampled to represent the concentration changes expected in all bladder compartments. Measured concentrations were compared with the mathematical simulation. The LC-MS/MS method was validated according to FDA guidelines[28] over a range of 0.75–375 mg/L ($R^2 = 0.9998$) for urine and plasma samples of fosfomycin, with additional tests confirming its applicability for CAMHB samples. The method was accurate and precise with a maximum deviation of 5.0%; lower limit of quantification was 0.75 mg/L and lower limit of detection was 0.70 mg/L.[29] The stability of fosfomycin in CAMHB at 37°C for 72 h, and stored at −80°C for at least 6 months, was confirmed.

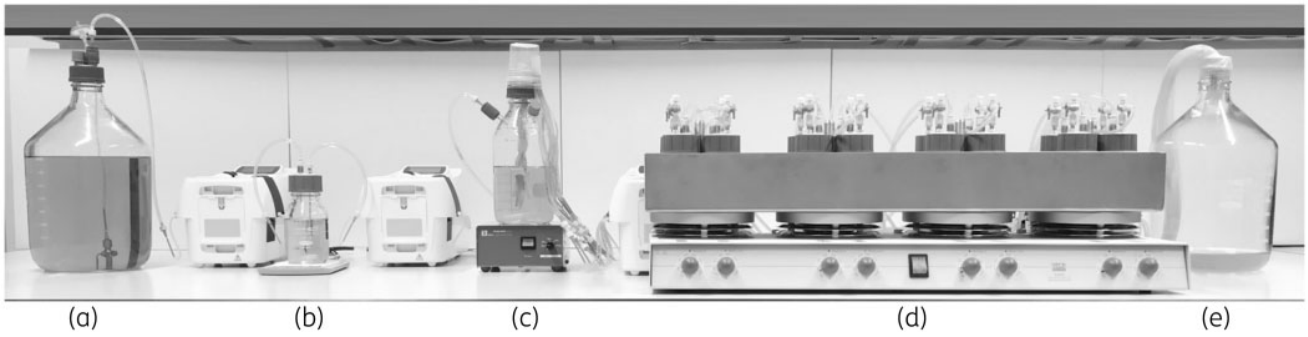

Figure 1. Dynamic bladder infection *in vitro* model. The *in vitro* model consists of autoclavable 1.01 mm PVC tubing (Gilson, UK) run through sequentially arranged peristaltic pumps (Gilson, UK) delivering matching flow rates from the fresh medium reservoir (a) to the gastrointestinal compartment (b), into which fosfomycin was administered, the circulatory compartment (c) and into the 16 bladder compartments (d) run in parallel within a water bath at 37±1°C. A fourth peristaltic pump facilitated automated and timed intermittent bladder-compartment voiding to the waste container (e).

Bacterial DNA and RNA isolation

Pure cultures of the test isolates were grown from −80°C freezer stock onto a TSB plate incubated overnight at 37°C. Colonies were selected and resuspended in 250 μL of nuclease-free PBS. DNA was isolated for qualitative *fosA* detection by boiling at 100°C for 10 min, spun down and supernatant collected. For all other molecular testing, DNA and RNA were isolated using the MagNA Pure 96 system (Roche Diagnostics, Switzerland) and the MagNA Pure 96 DNA and Viral NA Small Volume kit (Roche Diagnostics, Switzerland). Isolated DNA/RNA samples were stored at −80°C.

fosA detection and quantitative expression

Qualitative detection of the *fosA* gene was performed on all isolates before exposure to fosfomycin and after exposure where regrowth occurred and the baseline result was negative. Testing was performed using PCR amplification with forward and reverse primers, shown in Table S1. Two *fosA*-positive isolates (*Klebsiella variicola* and *E. coli*)[30] and a *fosA*-negative *E. coli* were used as controls. Amplified PCR products were visualized by gel electrophoresis. Expression of *fosA* was assessed using quantitative PCR amplification (RT–qPCR) with primers and probe designed to match *K. pneumoniae fosA* (Table S1). 16S rRNA was used for data normalization. RT–qPCR was performed using a QuantiTect Multiplex PCR NoROX kit (Qiagen, Germany). All samples were tested in duplicate, with sterile water run as a negative control. Baseline relative *fosA* mRNA expression was determined using the $2^{-\Delta CT}$ method and assessment of the fold change in expression post exposure by the $2^{-\Delta\Delta CT}$ method.[31]

WGS

The isolated DNA samples were sequenced using Illumina chemistry generating 2×150 bp paired-end sequence data, processed using CLC genomic workbench 12 (Qiagen, Germany). Core-genome MLST (cgMLST) using SeqSphere 5.1.0 (Ridom, Germany) was performed and distance between strains uploaded to the Interactive Tree of Life (iTOL) for phylogenetic analysis.[32] The coding regions of fosfomycin resistance genes, including those for the binding site (*mur*A), transporters (*glpT* and *uhpT*), *uhpT* regulators (*uhpA*, *uhpB*, *uhpC*), the *glpT* repressor (*glpR*), cAMP regulators (*cyaA*, *ptsI*) and the inactivating enzyme (*fosA*), were extracted using BioNumerics (version 7.6.3, Applied Maths, Belgium) and analysed for gene deletions, insertions, nonsense, missense and silent mutations in reference to the sequenced *E. coli* ATCC 25922 strain and the downloaded sequence of *K. pneumoniae* strain Kp52.145 (GenBank: FO834906.1). The presence of other antimicrobial resistance genes was investigated using ResFinder (www.genomicepidemiology.org).[33]

Statistical and PK/PD analyses

Linear regression and Bland–Altman plots were used to determine the precision and bias of the observed fosfomycin concentrations compared with the target derived from the mathematical simulation. Fisher's exact test with two-sided *P* value was used to compare associations with post-exposure growth outcome with baseline susceptibility and heteroresistance characteristics. The PK/PD relationship between fosfomycin exposure and isolate growth is presented as the ratio of the free-drug AUC to the pathogen MIC ($fAUC_{0-72}$/MIC), the ratio of the maximal free-drug concentration to the pathogen MIC (fC_{max}/MIC) and the time (0–72 h) that the free-drug concentrations exceeded the pathogen MIC or 4× MIC ($f\%T_{>MIC/4\times MIC}$). Agar dilution MIC values were used. Non-linear regression was performed using a variable slope E_{max} model with the top parameter corresponding to the maximal bacterial counts following dynamic drug-free control growth and the bottom to the lower limit of bacterial count quantification. Where appropriate, data are presented as mean ± SD. All analyses were performed with GraphPad Prism (version 7.0b, MAC OS X).

Results

Bacterial isolates and in vitro susceptibility

Of the 44 isolates, 38 (86%) were originally from a urinary source and the majority (84%) were ESBL (i.e. bla_{CTX-M})-producing strains (Table S2). All 20 *K. pneumoniae* isolates, and 21 of 24 *E. coli* isolates, had a fosfomycin MIC ≤32 mg/L by agar dilution (susceptible by EUCAST). Only 2 *E. coli* isolates had disc inhibition zone diameters <24 mm (resistant by EUCAST) compared with 15 of 20 *K. pneumoniae* (Figure 2). The results of VITEK 2 and BMD susceptibility (with and without G6P) are presented in Table 1.

Fosfomycin exposure in the bladder infection in vitro model

Observed *in vitro* concentrations closely matched the simulation with a bias of 11.0%±7.5%, with the slope of a linear regression line equal to 1.2 ($R^2 = 0.94$) and the relative standard deviation of the measured values 6.3%±1.4% (Figure 3 and Figure S3). There was minimal intercompartmental variation when all bladder compartments were measured at the peak concentration, with an average C_{max} of 2249.6±182.1 mg/L.

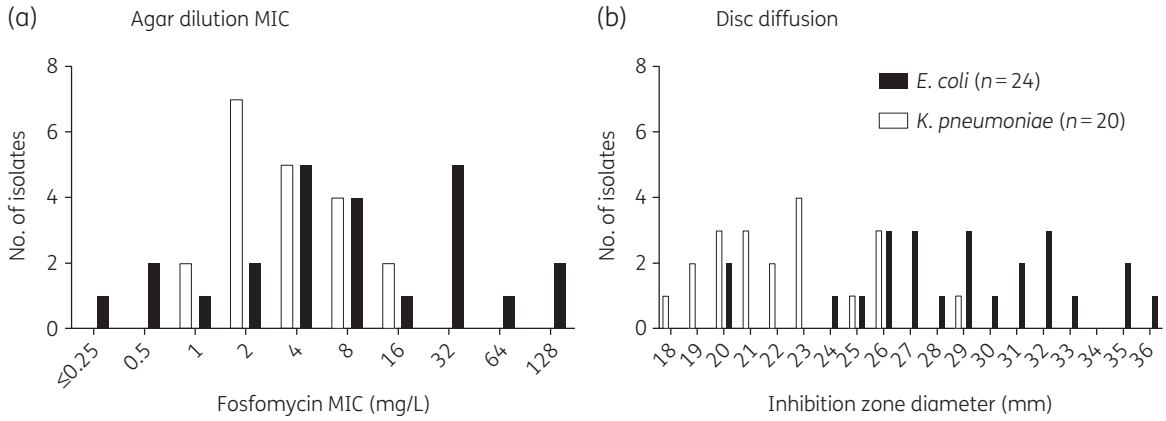

Figure 2. Baseline fosfomycin susceptibility of test isolates. MIC testing was performed in triplicate by agar dilution (a). Disc diffusion (b) was performed using FOT200 discs (Oxoid Ltd/Thermo Fisher Scientific, UK). *E. coli* ATCC 25922 was used as a quality control organism.

Table 1. Fosfomycin susceptibility, heteroresistance and response to fosfomycin therapy

	Pre-exposure									Post-exposure *in vitro* model outcome	
	fosfomycin susceptibility testing					heteroresistance					
				BMD MIC, mg/L							
Strain	MIC[a], mg/L	disc diffusion zone, mm	VITEK 2 MIC, mg/L	CAMHB + G6P	CAMHB only	MPC, mg/L	dynamic incubation, HLR %	disc elution, HLR	*fosA* PCR	bacterial count, log_{10} cfu/mL (HLR %)	MIC[a], mg/L
E. coli isolates											
41	≤0.25	33	≤16	4	32	64	—	—	—	—	—
11	0.5	36	≤16	1	16	32	—	—	—	—	—
39	0.5	32	≤16	2	32	32	—	—	—	—	—
472	1	31	≤16	8	64	32	—	—	—	—	—
12620	2	26	≤16	4	16	32	—	—	—	—	—
583	2	27	≤16	4	256	256	—	—	—	—	—
143[b]	4	32	≤16	8	128	64	—	—	—	—	—
629	4	32	≤16	16	128	512	—	—	—	—	—
692	4	29	≤16	16	1024	512	—	—	—	—	—
255	4	35	≤16	32	256	512	—	—	—	—	—
745[b]	4	25	≤16	64	512	>1024	3E−04	+	—	9.5 (+++)	1024
381	8	35	≤16	32	512	128	—	—	—	—	—
010	8	31	≤16	16	512	512	—	—	—	—	—
030	8	30	≤16	256	512	512	—	—	—	—	—
214	8	24	≤16	16	1024	512	—	—	—	—	—
1016[b]	16	20	32	64	>1024	>1024	2E−04	+	—	9.5 (+++)	256
1231[b]	32	20	32	1024	>1024	>1024	3E−04	+	—	9.5 (+++)	>1024
574[b]	32	29	32	32	32	256	—	—	—	—	—
672[b]	32	27	≤16	256	>1024	>1024	5E−03	+	—	7.0 (+++)	1024
123[b]	32	28	≤16	128	>1024	1024	—	—	—	—	—
4807	32	29	32	64	64	256	—	—	—	—	—
4757	64	26	64	128	128	1024	—	—	—	—	—
746[b]	128	26	≤16	128	256	1024	3E−05[c]	—	—	—	—
202	128	27	≤16	256	512	512	—	—	—	—	—
K. pneumoniae isolates											
787[b]	1	29	≤16	2	128	>1024	—	—	+	—	—
790	1	26	≤16	1	64	>1024	—	—	+	—	—
864	2	26	≤16	8	64	>1024	8E−05[c]	—	+	2.0	1
976	2	26	≤16	4	64	>1024	6E−05	+[d]	+	3.8	2
855	2	25	≤16	16	128	>1024	4E−05	—	+	2.8	4
874[b]	2	23	≤16	16	256	>1024	—	—	+	—	—
862	2	23	≤16	8	128	>1024	8E−05	—	+	5.0	2
34672	2	23	≤16	8	512	>1024	4E−05	+	+	9.3 (+++)	>1024
31865	2	22	≤16	8	256	>1024	8E−05	+	+	9.4 (++)	256
972	4	23	≤16	8	512	>1024	1E−04	+	+	2.7	4
803	4	22	≤16	16	512	>1024	1E−04	—	+	5.5	4
856	4	20	≤16	16	512	>1024	2E−04[c]	+	+	6.5	4
55	4	19	≤16	16	1024	>1024	4E−05	+	+	6.9	4
830	4	19	≤16	32	256	>1024	3E−05	+	+	7.0	4
915[b]	8	21	≤16	16	512	>1024	3E−05	—	+	6.6	8
820[b]	8	21	≤16	32	512	>1024	2E−05[c]	+	+	9.7 (++)	512
899[b]	8	20	≤16	16	>1024	>1024	6E−04	+	+	9.2 (+++)	>1024
956[b]	8	20	≤16	64	>1024	>1024	2E−04	+	+	9.3 (++)	512
52[b]	16	21	≤16	32	>1024	>1024	3E−04	+	+	9.2 (+++)	>1024

Continued

Table 1. *Continued*

	Pre-exposure									Post-exposure *in vitro* model outcome	
	fosfomycin susceptibility testing					heteroresistance					
				BMD MIC, mg/L							
Strain	MIC[a], mg/L	disc diffusion zone, mm	VITEK 2 MIC, mg/L	CAMHB + G6P	CAMHB only	MPC, mg/L	dynamic incubation, HLR %	disc elution, HLR	*fosA* PCR	bacterial count, log_{10} cfu/mL (HLR %)	MIC[a], mg/L
891[b]	16	18	32	128	512	>1024	6E−05[c]	+	+	3.8	8
E. coli ATCC (not run in the *in vitro* model)											
25922	1	30	≤16	1	64	64	not tested	—	—	not tested	

—, not detected or no growth.
HLR percentage in post-exposure regrowth classified as greater than 1% (+++), between 0.01% and 1% (++) and less than 0.01% (+).
[a]MIC determined by agar dilution.
[b]Isolates tested in duplicate in the *in vitro* model (Table S3).
[c]HLR subpopulation without an MIC >1024 mg/L.
[d]Disc elution was negative at 48 h and became positive at 72 h of incubation.

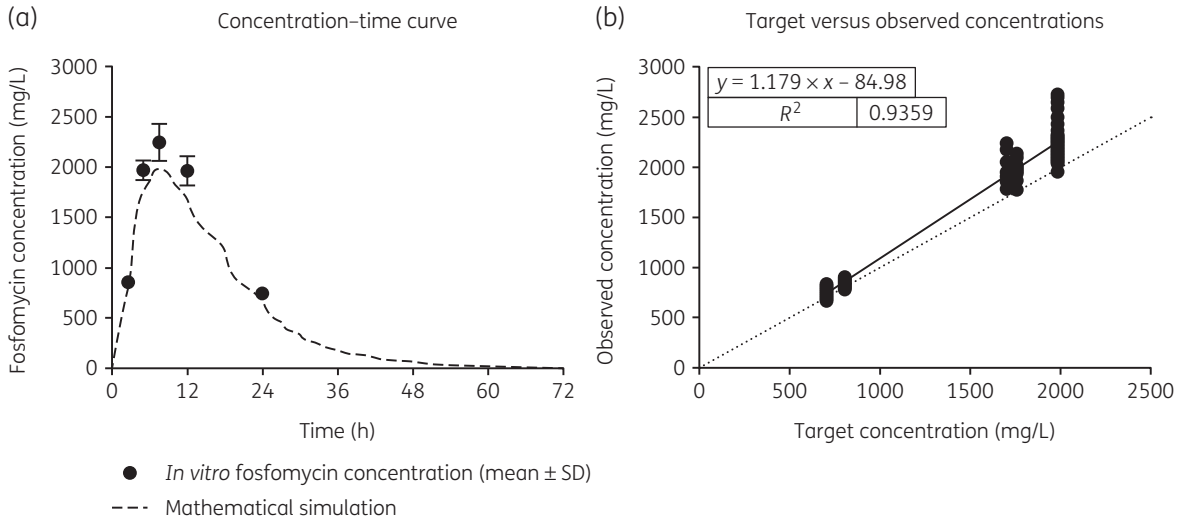

Figure 3. Observed fosfomycin concentrations from *in vitro* bladder compartments. (a) Filled circles represent mean ± SD measured fosfomycin concentrations overlaid on the target concentration curve (dashed line). (b) Accuracy of the observed fosfomycin concentrations compared with the target values from the mathematical simulation with a linear regression (continuous line) and $y = x$ (dotted line).

Dose–response outcome in the bladder infection in vitro model

Following urinary exposure to fosfomycin after a simulated 3 g oral dose, 20 of 24 *E. coli* exhibited reductions in bacterial counts below the lower limit of quantification compared with only 3 of 20 *K. pneumoniae* isolates (Table 1 and Figure 4). To ensure reproducibility of the results, 16 isolates were re-run in the *in vitro* model, which returned concordant final growth results (Table S3).

Of the four *E. coli* isolates that regrew, all had a rise in the total population MIC from baseline. These isolates also had a baseline MPC >1024 mg/L, a detectable HLR subpopulation following the dynamic drug-free incubation and a positive disc-elution HLR screen (Table S4). Due to the lower bacterial density used for the starting inoculum added to the model (10^7 cfu/mL), baseline HLR was only detected in *E. coli* 672 and LLR was detected in *E. coli* 1231 from the initial culture. Following fosfomycin exposure, emergence of resistance was detected as early as 6 h after fosfomycin administration and achieved maximal growth capacity (Figure 5). In *E. coli* that did not regrow, despite having MIC values up to 128 mg/L, all had MPC ≤1024 mg/L and a negative disc-elution HLR screen. Only one isolate had a detectable HLR subpopulation after dynamic drug-free incubation; however, the MIC for this subpopulation was equal to 1024 mg/L, whereas the HLR subpopulations of the *E. coli* isolates that regrew had MIC >1024 mg/L. From the starting inoculum added to the model, 6 out of 20 had LLR detected from the initial culture timepoint.

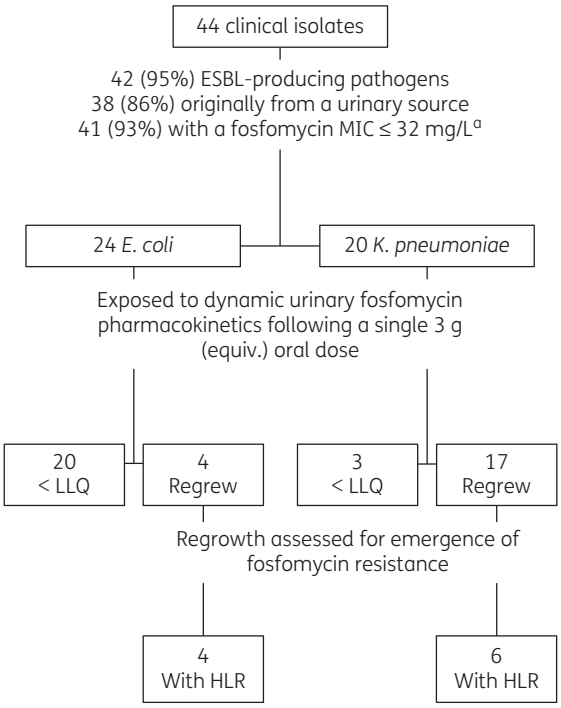

Figure 4. Dose–response outcome following dynamic urinary fosfomycin exposure. [a]Fosfomycin MIC determined by agar dilution. HLR determined by growth on MHA with 512 mg/L fosfomycin. LLQ, lower limit of quantification.

Of the 17 *K. pneumoniae* isolates that regrew, the timing of regrowth varied between isolates. In nine isolates, regrowth was detected only at the final timepoint. All isolates that regrew had a detectable HLR subpopulation after dynamic drug-free incubation. Susceptibility testing of this subpopulation confirmed an MIC of >1024 mg/L for 13 of 17 isolates. At the initial culture timepoint, from starting inoculum being added to the model, only two isolates (strains 899 and 52) had HLR detected and only four strains (strains 31865, 864, 956 and 856) had LLR detected. Post exposure, emergence of resistance was variable, with HLR and rise in total population fosfomycin MIC detected for only six isolates (Figure 5). These isolates all screened positive for HLR by disc elution (Table S4). The three isolates that did not regrow in the model were the only *K. pneumoniae* isolates to not have an HLR subpopulation detected following the dynamic drug-free incubation. These isolates also had negative disc-elution HLR screening results, although five isolates that did regrow in the model also had negative disc-elution HLR results. All *K. pneumoniae* isolates had a baseline MPC >1024 mg/L, including the isolates that had reductions in their bacterial counts below the lower limit of quantification without regrowth.

Overall, there was no association between isolate MIC susceptibility (≤32 mg/L) and growth outcome (agar dilution MIC: $P = 0.2341$; BMD MIC in MHB + G6P: $P > 0.9999$). In contrast, disc diffusion (susceptible: ≥24 mm) had a better association with growth outcome [sensitivity 0.76 (95% CI = 0.55–0.89), specificity 0.96 (95% CI = 0.79–1), $P < 0.0001$], which improved if the inhibition zone cut-off was increased to ≥28 mm [sensitivity 1 (95% CI = 0.85–1), specificity 0.65 (95% CI = 0.45–0.81), $P < 0.0001$]. Growth outcome was most significantly associated with baseline heteroresistance, an MPC >1024 mg/L [sensitivity 1 (95% CI = 0.85–1), specificity 0.87 (95% CI = 0.68–0.95), $P < 0.0001$], a positive disc-elution HLR screen [sensitivity 0.76 (95% CI = 0.55–0.89), specificity 1 (95% CI = 0.86–1), $P < 0.0001$] and HLR detected after dynamic drug-free incubation [sensitivity 1 (95% CI = 0.85–1), specificity 0.96 (95% CI = 0.79–1), $P < 0.0001$].

PK/PD analysis

Non-linear regression using a variable slope E_{max} model failed to provide reliable relationships to infer EC_{50} values for the relationship between drug exposure ($fAUC_{0-72}$/MIC, fC_{max}/MIC and $f\%T_{>MIC/4\times MIC}$) and isolate growth and emergence of resistance 72 h after fosfomycin administration (Figure S4). For *E. coli* isolates, fosfomycin exposure relative to MIC was a poor predictor for outcome and emergence of resistance. For *K. pneumoniae* isolates, fosfomycin exposure performed slightly better to predict antimicrobial efficacy to suppress regrowth, but did not predict which isolates would regrow with emergence of resistance.

Molecular assessment of fosfomycin resistance

fosA was not detected in any *E. coli* isolates, either at baseline or in the four isolates that regrew after exposure to fosfomycin. In contrast, all *K. pneumoniae* isolates had a detectable *fosA* gene. However, gene expression did not predict which isolates would regrow, with or without HLR, or identify those isolates where bacterial counts were maintained below the lower limit of quantification post-fosfomycin exposure. There was also no up-regulation of *fosA* expression after exposure to fosfomycin compared with baseline (Figure 6).

There was a wide variation of STs found in the test isolates (Figure S5). The 24 *E. coli* isolates came from 12 different STs, whereas the 20 *K. pneumoniae* isolates came from 17 different STs. There was no association between ST and fosfomycin efficacy in the *in vitro* model. Furthermore, WGS assessment of sequence variations in the fosfomycin resistance genes failed to identify any baseline mutations that predicted the treatment response in the *in vitro* model (Table S5 and Table S6). Despite a wide number of different insertion–deletion, nonsense and missense mutations identified in all *E. coli* isolates prior to fosfomycin exposure, the only unique mutation that predicted regrowth was detected in strains 1016 and 1231, which had an R269C mutation in the *glpT* transporter gene. In the four *E. coli* isolates that regrew, three demonstrated new sequence variations in the post-exposure strains. Strains 1016 and 1231 both had a three amino acid deletion in *uhpA* (G42_P44del and A41_L43del, respectively). In strain 672, there was the entire loss of the G6P transporter and regulator genes, and a new mutation in the G3P transporter gene. In the six *E. coli* isolates where BMD susceptibility testing demonstrated a lack of G6P potentiation of fosfomycin activity, no common mutation was identified in the G6P transporter gene (*uhpT*) or its regulators (*uhpA, uhpB, uhpC*).

For the *K. pneumoniae* isolates, there were fewer sequence variations identified in the fosfomycin resistance genes. All isolates were confirmed to carry the *fosA* gene. The three isolates that did not regrow in the model carried unique mutations in the *fosA* gene

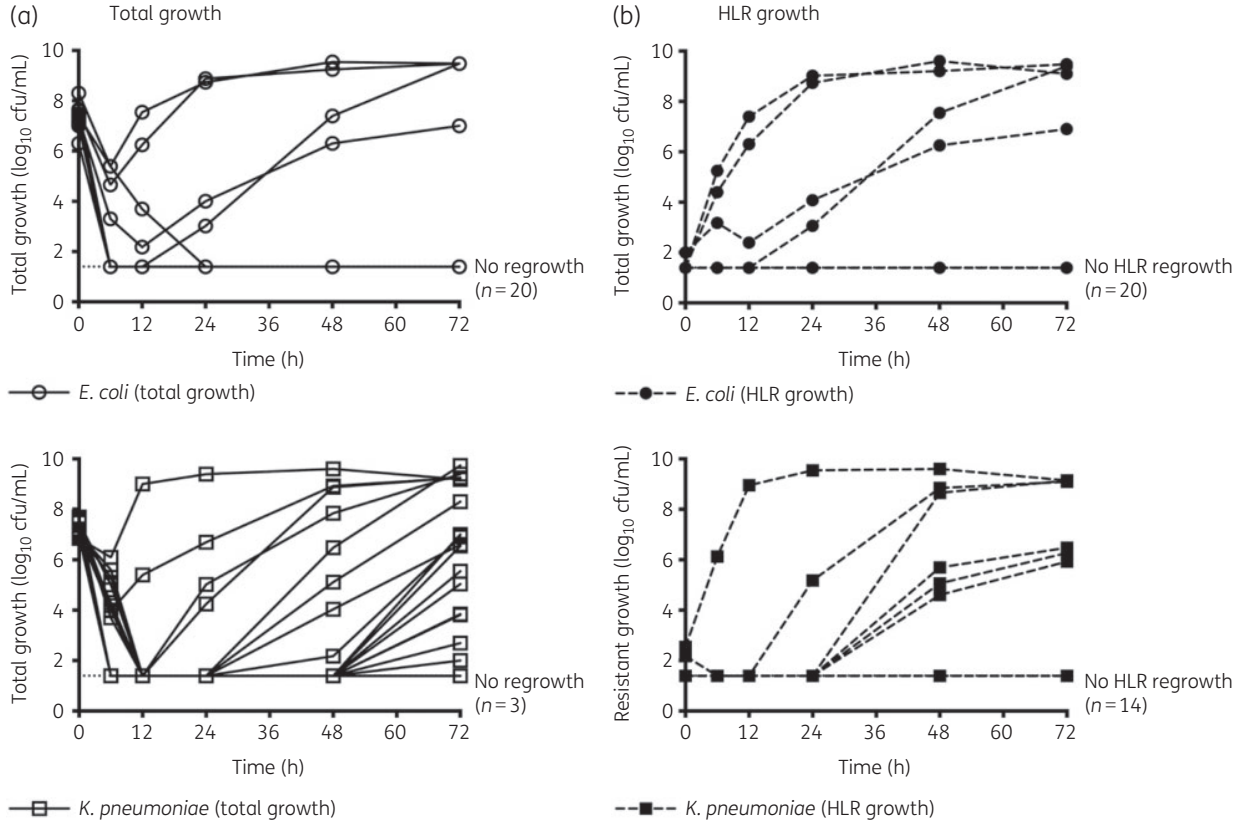

Figure 5. Time–kill curves following fosfomycin exposure. Total bacterial counts (a) are represented by open circles (*E. coli*) and open squares (*K. pneumoniae*). HLR bacterial counts (b) are represented by filled circles (*E. coli*) and filled squares (*K. pneumoniae*). The limit of detection (dotted line) was considered to be 1.4 $\log_{10}$ cfu/mL.

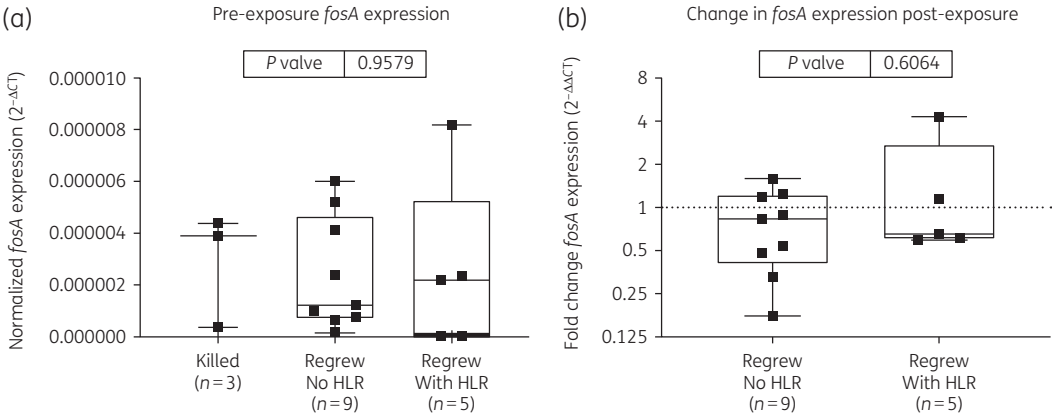

Figure 6. Inactivating enzyme (*fosA*) expression in *K. pneumoniae* isolates. Normalized *fosA* expression for each isolate before fosfomycin exposure (a) compared by growth outcome in the *in vitro* model by one-way ANOVA. Post-exposure fold change in *fosA* expression (b) with comparison between isolates that regrew without and with HLR by Mann–Whitney test. Box and whisker plots represent the median, 25th to 75th percentiles and maximum and minimum values. Results exclude three isolates (strains 855, 864 and 34672) that failed to amplify *fosA* due to mutations in the region of the reverse primer.

(S18G and M134I) compared with other isolates. Isolates that regrew with emergence of HLR did not have common baseline sequence variations. Following fosfomycin exposure, *K. pneumoniae* 52 demonstrated no new mutations compared with baseline, whereas *K. pneumoniae* 899, which at baseline had a complete deletion of the G3P transporter gene, had an additional deletion

and frameshift mutation in the G6P transporter gene. *K. pneumoniae* 34672 acquired a new mutation in *uhpB* (P153L).

Discussion

We demonstrated a clear disparity between *E. coli* and *K. pneumoniae* isolates in their response to dynamic urinary concentrations of fosfomycin following a simulated single 3 g oral dose. Approved fosfomycin MIC testing by agar dilution appears to be a poor predictor for efficacy. Fosfomycin has limited sustained activity against *K. pneumoniae* isolates. The detection of a pre-existing, high-level fosfomycin-resistant subpopulation was most predictive for treatment failure. For *E. coli* isolates, screening for heteroresistance by a disc-elution method provided a simple method to detect heteroresistance and could complement an existing susceptibility result, especially when that result is >2 mg/L.

For *E. coli* isolates, fosfomycin demonstrated good activity against isolates with a wide range of MIC values, even up to 128 mg/L. However, when failure did occur, there was rapid emergence of HLR that was not predicted by the baseline MIC. In contrast, for *K. pneumoniae* isolates fosfomycin was shown to be largely ineffective, regardless of baseline MIC, although emergence of resistance was not uniform in the post-exposure population. We demonstrated that *K. pneumoniae* isolates all have fosfomycin heteroresistance, evidenced by an MPC >1024 mg/L for all isolates. In the majority of isolates, an HLR subpopulation was also detected after dynamic, drug-free incubation within the *in vitro* model. All *K. pneumoniae* isolates had a *fosA* gene; however, no sustained up-regulation of gene expression was demonstrated to explain regrowth or emergence of resistance.

WGS and analysis of the genes associated with fosfomycin resistance did not demonstrate any single mutation that could predict treatment failure or any common *de novo* mutations post exposure. However, it would seem that mutations in both transporter genes and/or regulators are required to promote HLR, especially in the absence of changes at the binding site (MurA) or enzymatic inactivation (FosA).

Our results challenge the current clinical breakpoint set for fosfomycin. For *E. coli* isolates, if the susceptible MIC breakpoint was reduced to closer to the epidemiological cut-off (ECOFF) value (i.e. MIC 2 mg/L),[17] this would reduce the risk of classifying an isolate as susceptible, when in fact it could harbour a resistant subpopulation. In our collection, no *E. coli* isolate with an MIC $\leq$2 mg/L had an HLR subpopulation. *E. coli* isolates with an MIC between 4 and 128 mg/L may represent an 'area of technical uncertainty' for susceptibility classification and a heteroresistance screening test may be helpful in order to predict treatment efficacy. For *K. pneumoniae* isolates (fosfomycin ECOFF of 64 mg/L),[17] any reduction in the clinical breakpoint would split the WT population. This also highlights that *K. pneumoniae* isolates with low fosfomycin MICs (i.e. MIC $\leq$2 mg/L) would appear to be exceedingly uncommon. Fosfomycin does, however, initially inhibit the growth of *K. pneumoniae* isolates. Therefore, treatment *in vivo* could still promote clinical cure in the presence of a functioning immune system, a low inoculum infection and healthy urodynamics. The benefit of repeat oral fosfomycin doses in this scenario is uncertain, although the timing of the repeat doses should be before the emergence of resistance. The use of fosfomycin in combination with another oral antimicrobial agent, such as amoxicillin/clavulanate or pivmecillinam, may also warrant further study.

Despite applying normal urodynamics and dynamic urinary fosfomycin exposures, the *in vitro* model lacks the tissue architecture of the bladder and host factors such as the immune system. This is therefore a limiting factor in translating these results to the treatment of human infections. Similarly, our exposure–response analysis was limited by the examination of only a single-dose exposure for all isolates. However, given the large number of isolates examined with a wide range of baseline susceptibility to fosfomycin, a dose–response relationship relative to MIC susceptibility would have been expected to have been identified. Importantly, the use of standard laboratory medium, namely CAMHB supplemented with G6P, does not reflect the biomatrix of urine. It is well recognized that nutritional factors, which are less abundant *in vivo*, are important to growth rates and antimicrobial kill rates.[34] Examining the effect of the media on fosfomycin efficacy is an important consideration for translating these *in vitro* results to clinical UTIs. Furthermore, there are wide variations in reported urinary concentrations of fosfomycin between individuals. This research applied PK data from a single, healthy volunteer study,[27] which demonstrated relatively high urinary fosfomycin concentrations compared with other recent studies.[13,35] The impact on pathogen response with extremes in urinary fosfomycin exposure would benefit from additional research.

By applying novel and dynamic PK/PD *in vitro* modelling techniques, we highlight the challenges of applying current fosfomycin susceptibility results in order to predict the likelihood for treatment success. We also caution against the use of fosfomycin for infections caused by *K. pneumoniae*.

Acknowledgements

We would like to acknowledge the laboratory assistance of Carla Roodbol de Goeij (Department of Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus MC) and Alex Brouwer and Geert Springeling (Department of Experimental Medical Instrumentation, Erasmus MC) for their assistance in the *in vitro* equipment design and production.

Funding

This work was supported in part by the AIDA project LifeSciHealth Priority of the European Commission Seventh Framework Programme, FP7 (Preserving Old Antibiotics for the Future, grant number F3-2011-278348). I.J.A. is funded by an Australian Government Research Training Program Scholarship (APP1114690) from the National Health and Medical Research Council of Australia. A.Y.P. was part funded through an Australian National Health and Medical Research Council Practitioner Fellowship (APP1117940).

Transparency declarations

J.W.M. has received research funding from Adenium, AstraZeneca, Basilea, Cubist, Polyphor, Roche, Eumedica, Basilea, VenatorX, AiCuris, Gilead and Wockhardt. All other authors: none to declare.

Supplementary data

Supplementary data, including Figures S1 to S5 and Tables S1 to S6, are available at *JAC* Online.

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SUPPLEMENTARY DATA

METHODS

Mutant prevention concentration (MPC)

The method used was as follows: pure isolate cultures were grown from freezer stock onto TSA; all growth was transferred to 100 mL MHB and incubated overnight with vigorous shaking; the turbid broth was spun down and the pellet re-suspended to a volume of 5 mL; quantitative culture confirmed a high-density bacterial inocula greater than 5×10^{10} cfu/mL; 200 µL was spread evenly across the agar surface of each MHA plate (incorporated with 25 mg/L G6P) containing doubling concentrations of fosfomycin up to 1024 mg/L; plates were incubated for 24 h and read for viable growth (i.e. disregarding point-point colonies or haze). The MPC was recorded was the lowest concentration of fosfomycin that allowed no bacterial growth.

Disk elution heteroresistance screen

A modified disk elution test was used as a screening test for the presence of low-level and high-level heteroresistance. In short, three tubes with 1.9 ml MHB were used per isolate, into which 0, 2, and 4 fosfomycin disks (FOT200, Oxoid, UK) were added. Each disk contains 200 µg fosfomycin and 50 µg G6P. Tubes were kept at room temperature for at least 90 min to allow the fosfomycin and G6P to elute from the disks into the MHB. Inocula were prepared by suspending fresh colonies from an overnight incubation on TSB in normal saline to a 0.5 McF turbidity. A 0.1 mL aliquot of the suspension was added to each tube, producing a final bacterial density of approximately 7.5×10^6 cfu/mL. Turbidity of the broth was read visually, initially after a 16 – 20 h incubation at 37°C in ambient air, then read again after re-incubation on day 2 and day 3. After 3-days of incubation all non-turbid tubes were sub-cultured onto TSB and assessed for viable growth (> 1 colony) that was not visualised in the tube. Bacterial-free negative control tubes with FOT200 disks added were set-up and incubated. *E. coli* ATCC 25922 was run as a negative growth control for heteroresistance. A *fosA*-positive *E. coli* (fosfomycin MIC > 1024 mg/L) was run as a positive control. Quantification of fosfomycin eluted from the disks was performed by LC-MC/MS after an initial 30- and 90-minutes elution time at room temperature prior to inoculation, and then after incubation, measured on daily for the 3-day incubation period (Fig. S1).

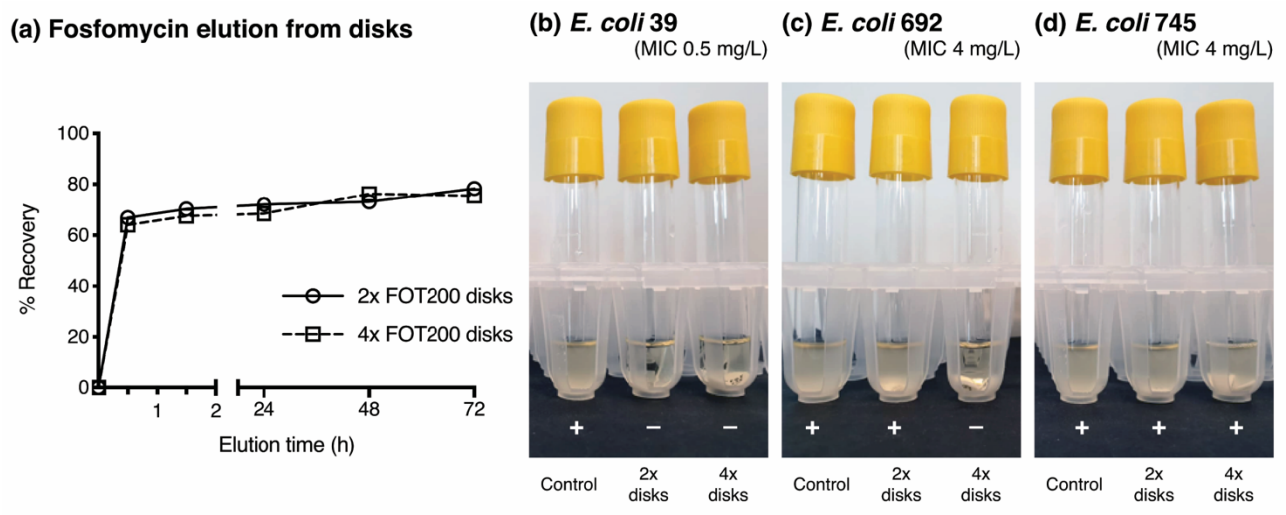

Figure S1. Disk elution heteroresistance screening test.
(a) Quantification of fosfomycin from the disk elution tubes; 68 – 70% of fosfomycin was eluted from the FOT200 disks after 90 min (2-disks – 140.6 mg/L; 4-disks – 270.2 mg/L), rising to 76 – 78% by 72 h (2-disks – 156.6 mg/L; 4-disks – 301.8 mg/L). (b - d) Examples of the results. Photo (a) presents a negative result. Photo (b) shows low-level resistance. Photo (c) shows high-level resistance.

(a) *In vivo* equations

Drug in G.I. tract:

$$X_{GI} = X_{dose} \cdot e^{-k_1 \cdot t}$$

Drug in blood:

$$X_{blood} = \frac{k_1 \cdot X_{dose}}{k_2 - k_1} \cdot \left(e^{-k_1 \cdot t} - e^{-k_2 \cdot t} \right)$$

Excreted drug:

$$X_{bladder} = X_{dose} - X_{GI} - X_{blood}$$

(b) *In vitro* equations

Drug in first constant volume compartment:

$$X_A = X_{A^\circ} \cdot e^{-(F/V_A) \cdot t}$$

Drug in second constant volume compartment:

$$X_B = \frac{(F/V_A) \cdot X_{A^\circ}}{F/V_B - F/V_A} \cdot \left(e^{-(F/V_A) \cdot t} - e^{-(F/V_B) \cdot t} \right)$$

Cumulative drug in third compartment:

$$X_C = X_{A^\circ} - X_A - X_B$$

Figure S2. Drug distribution equations following oral fosfomycin administration.
The dynamic amount of drug (X mg) in each respective compartment at time t (h) as a function of the first-order rate constants (absorption k_1; elimination k_2). The initial dose of fosfomycin (mg) is indicated by X_{dose} or X_{A°. In the *in vitro* equations the fluid volumes (V mL) in the respective compartments and flow rate of fluid (F mL/h) are variables.

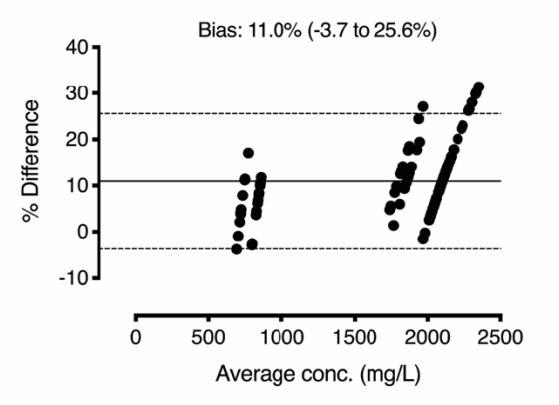

Figure S3. Accuracy and bias of observed fosfomycin PK.
Measured fosfomycin concentrations compared to target from the mathematical simulation. Bland-Altman plot of the percentage difference of the observed and target measurements (*y*-axis) and the average of the two measurements (*x*-axis) presented with the bias (solid line) and 95% limits of agreement (dotted lines).

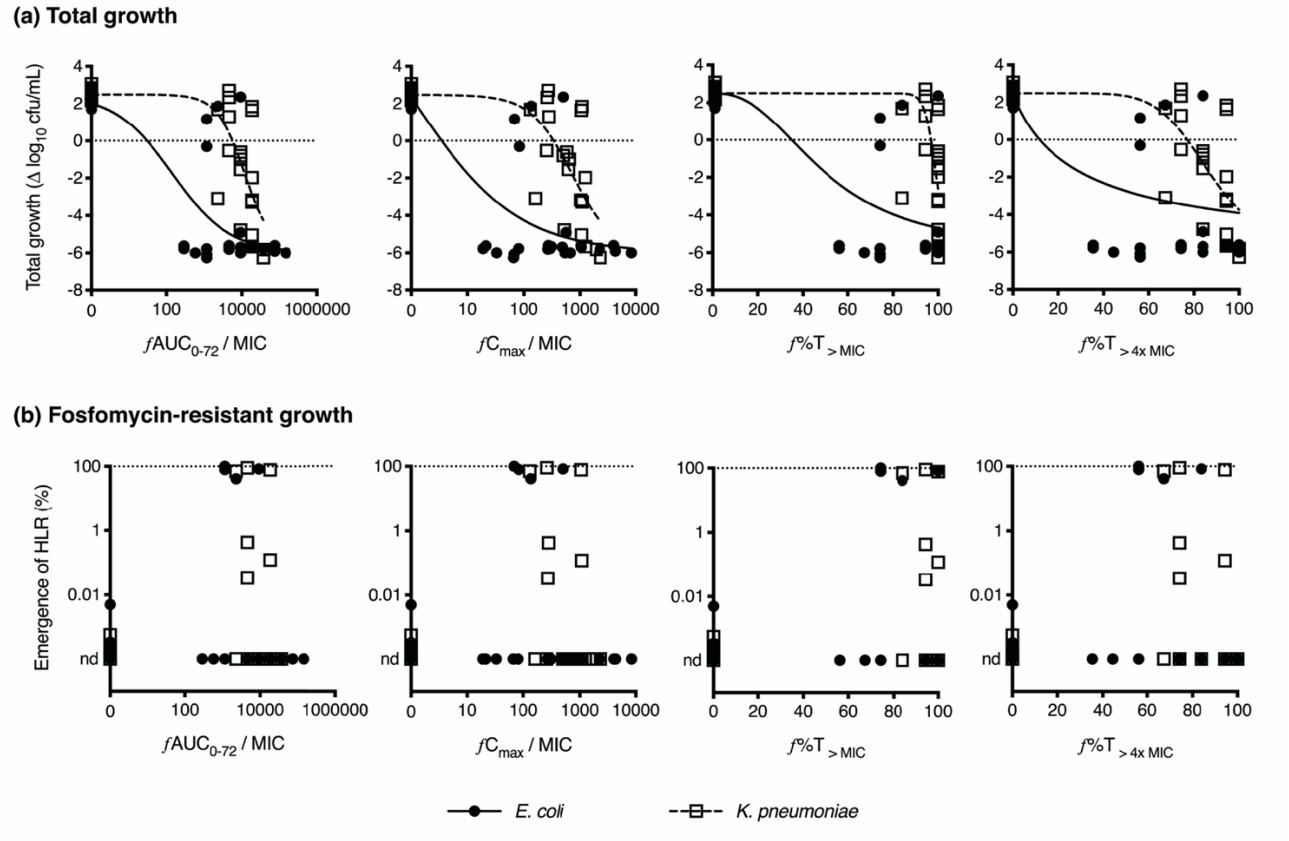

Figure S4. Relationship between drug exposure and isolate growth.
E. coli, solid circles. *K. pneumoniae*, open squares. nd, not detected. Isolate susceptibility measured by agar dilution. Fosfomycin exposure presented as the ratio of $fAUC_{0–72}$, fC_{max}/MIC and $f\%T_{>MIC/4xMIC}$ (0 – 72 h) to isolate susceptibility. Antimicrobial effect is presented as: (a) change in bacterial density, and (b) emergence of resistance (% high-level resistance (HLR) in the re-growth).

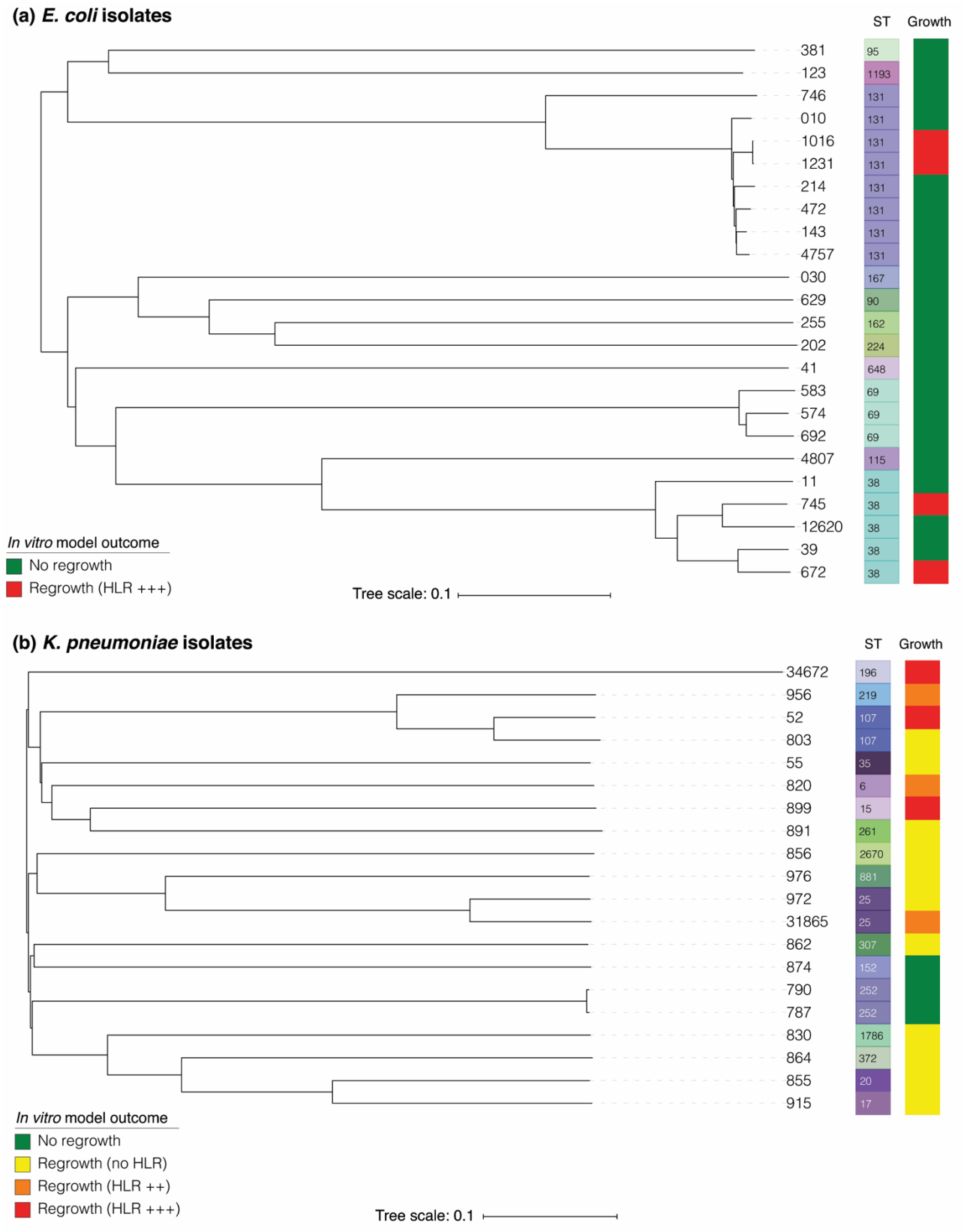

Figure S5. Strain relatedness, sequence type and growth outcome in the *in vitro* model.
Pathogen outcome in the *in vitro* model classified as no regrowth (green), regrowth without HLR (yellow), regrowth with HLR between 0.01 to 1% of total population (orange), and regrowth with HLR >1% of total population (red). Each ST allocated a different colour. *E. coli* ST according to the Warwick scheme. An outlying isolate, strain 34672, was identified as *K. quasipneumoniae* subsp. *quasipneumoniae*, part of the *K. pneumoniae* complex, but not differentiated by MALDI-TOF MS (Bruker Daltonik GmbH, Germany).

Table S1. Forward, reverse primer and probe sequences for the assessment of *fosA* gene.

Test	Gene	Primer sequence (5' – 3')	Probe sequence (R-5' – 3'-Q)
PCR	*fosA*	F: GAGCGTGGCGTTTTATCAGC R: GAGCGTGGCGTTTTATCAGC	-
RT-qPCR	*fosA*	F: GATYTGTGGCTGTGCCTGTC R: CGGCGAAGCTAGCRAAAT	Cy5-ACGCATAATGGGTGTAGTCGCTCTC-BHQ2
	16s rRNA	F: GGGRCCCGCACAA R: GGGTTGCGCTCGTT	6-FAM -TGACGACARCCATGCA-MGB-EDQ

Table S2. Detection of relevant antibiotic resistance genes.

	Beta-lactam	Amino-glycoside	Colistin	Fluoro-quinolone	MLS	Pheni-col	Sulpho-namide	Tetra-cycline	Trime-thoprim
***E. coli* isolates**									
010	TEM-1B CTX-M-14	aac(3)-IId aadA5	-	-	mph(A)	-	sul1 su2	tet(A)	dfrA17
11	CTX-M-14 OXA-1	aac(3)-IIa aac(6')-Ib-cr	-	aac(6')-Ib-cr	mdf(A)	catB3	-	tet(A)	-
30	TEM-1B	aph(3')-Ia aph(3'')-Ib aph(6)-Id	-	-	mdf(A) mph(B)	-	sul1	-	dfrA1
39	TEM-1B	aph(3'')-Ib aph(6)-Id	-	-	mdf(A)	catA1	sul2	tet(D)	-
41	TEM-1B CTX-M-2	aadA1 aph(3')-Ia aph(6)-Id	-	-	mdf(A)	-	sul1 sul2	tet(A)	dfrA1
123	CTX-M-27	-	-	-	-	-	-	-	-
143	CTX-M-15 OXA-1	aadA5 aac(6')-Ib-cr	-	aac(6')-Ib-cr	mph(A)	catB3	sul1	tet(A)	dfrA17
202	CTX-M-32	aph(3')-Ia	-		mdf(A)	-	-	tet(A)	-
214	CTX-M-15 OXA-1	aac(6')-Ib-cr	-	aac(6')-Ib-cr	-	catB3	-	tet(A)	-
255	CTX-M-3	aac(3)-IIa	-	-	mdf(A) mph(A)	-	-	-	-
381	CTX-M-1	-	-	-	-	-	-	-	-
472	TEM-1B CTX-M-15	aac(3)-IId aadA5	-	-	mph(A)	-	sul1	tet(A)	dfrA17
574	TEM-1B CTX-M-15	aadA5 aac(3)-IId	-	-	mph(A)	-	sul1	tet(B)	dfrA17
583	CTX-M-15	aadA5	-	-	mph(A)	-	sul1		dfrA17
629	TEM-1B CTX-M-15	aac(3)-IIa aac(3)-IIa aph(3'')-Ib aadA5 aph(6)-Id	-	aac(6')-Ib-cr	mdf(A) mph(A)	catB3	sul1 sul2	tet(B)	dfrA17
672	CTX-M-15 TEM-33	-	-	-	mdf(A)	-	sul2	-	-
692	CTX-M-15	-	-	-	mph(A)	-		tet(B)	dfrA14
745	CTX-M-14	aph(3'')-Ib aph(6)-Id	-	-	mph(A) mdf(A) erm(B)	-	sul1 sul2	tet(A)	dfrA17
746	CTX-M-27	aph(3'')-Ib aadA5 aph(6)-Id	-	-	mph(A)	-	sul1 sul2	tet(A)	dfrA17
1016	TEM-1B CTX-M-15	aac(3)-IId aph(3'')-Ib aadA5 aph(6)-Id	-	-	mph(A)	-	sul2	tet(A)	dfrA17
1231	CTX-M-15	aac(3)-IId aadA5 aph(6)-Id	-	-	mph(A)	-	sul1 sul2	tet(A)	dfrA17
4757	TEM-1B CTX-M-15	aac(6')-Ib-cr	-	aac(6')-Ib-cr	-	catB3	sul1	tet(A)	dfrA16
4807	CTX-M-14b	aadA2	-	-	-	-	-	-	-
12620	CMY-2	aadA1 ant(2'')-Ia aac(6')-Ib-cr	-	aac(6')-Ib-cr	mdf(A) mph(A)	cmlA1 catB3	sul1	tet(B)	-
***K. pneumoniae* isolates**									
52	SHV-12 OXA-1	aadA1 aph(3'')-Ib aac(6')-Ib3 aph(6)-Id aac(6')-Ib-cr	-	aac(6')-Ib-cr oqxA oqxB	-	catB3	sul2	tet(A)	dfrA14

	Beta-lactam	Amino-glycoside	Colistin	Fluoro-quinolone	MLS	Pheni-col	Sulpho-namide	Tetra-cycline	Trime-thoprim
55	TEM-1B SHV-33	aadA1 aac(3)-IIa aph(3'')-Ib aph(6)-Id	-	oqxA oqxB	-	catA1	sul1 sul2	tet(A)	dfrA1
787	CTX-M-15 SHV-1 OXA-1	aac(3)-IIa aph(3'')-Ib aph(6)-Id	-	aac(6')-Ib-cr oqxA oqxB qnrB1	-	catB3	sul2	-	dfrA14
790	CTX-M-15 SHV-1 OXA-1	aadA1 aph(3'')-Ib aph(6)-Id aadA24	-	aac(6')-Ib-cr oqxA oqxB qnrB1	-	catA1	sul2	-	dfrA14
803	CTX-M-15 SHV-36 OXA-1	aadA1 aph(3'')-Ib aph(6)-Id	-	oqxA oqxB	-	-	sul1	tet(B)	-
820	CTX-M-15 TEM-1B SHV-26	aph(3'')-Ib aph(6)-Id	-	oqxA oqxB qnrB1	-	-	sul2	tet(A)	dfrA14
830	TEN-1B CTX-M-14 SHV-36	aph(3'')-Ib aph(6)-Id	-	oqxA oqxB qnrS1	-	-	sul1 sul2	tet(A) tet(D)	dfrA1
855	TEM-1B CTX-M-15 SHV-26 OXA-1	aph(3'')-Ib aph(6)-Id aac(6')-Ib-cr	-	aac(6')-Ib-cr oqxA oqxB	-	catB3	sul2	-	dfrA14
856	TEM-1B CTX-M-15 SHV-27 OXA-1	aac(3)-IIa aph(3'')-Ib aph(6)-Id	-	aac(6')-Ib-cr oqxA oqxB qnrB1	-	catB3	sul2	tet(A)	dfrA14
862	TEM-1N CTX-M-15 SHV-28 OXA-1	aph(3'')-Ib aph(6)-Id	-	aac(6')-Ib-cr oqxA oqxB qnrB1	-	catB3	sul2	tet(A)	dfrA14
864	TEM-1B CTX-M-15 SHV-26	aph(3'')-Ib aph(6)-Id	-	oqxA oqxB qnrB1	-	-	sul2	-	dfrA14
874	CTX-M-15 SHV-187	aph(3'')-Ib aac(6')-Ib-cr aph(6)-Id	-	aac(6')-Ib-cr) oqxA oqxB qnrB1	-	catB3	sul2	tet(A)	dfrA14
891	CTX-M-14 SHV-142 GES-1	aac(3)-IId	-	aac(6')-Ib-cr) qnrS1	-	-	sul1	tet(A) tet(D)	dfrA1
899	TEM-1B CTX-M-15 SHV-28	aadA2 aac(6')-Ib-cr	-	aac(6')-Ib-cr oqxA oqxB	mph(A)	catB3	sul1	-	dfrA12
915	CTX-M-15 SHV-40	-	-	oqxA oqxB	-	-	sul2	-	-
956	CTX-M-15 SHV-26	aadA2	-	oqxA oqxB qnrS1	mph(A)	-	sul1 sul2	tet(A)	dfrA12
972	TEM-1B CTX-M-15 SHV-81 OXA-1	aph(3'')-Ib aph(6)-Id aac(6')-Ib-cr	-	aac(6')-Ib-cr oqxA oqxB qnrS1	-	catB3	sul2	tet(A)	dfrA14
976	CTX-M-14 SHV-27	aac(3)-IId aph(3'')-Ib aph(6)-Id	-	oqxA oqxB qnrB1	-	-	sul1 sul2	tet(A)	dfrA1
31865	TEM-1B SHV-81	-	-	oqxA oqxB	-	-	sul1	tet(D)	dfrA5
34672	OKP-A-5	aadA1	-	-	-	-	sul1	-	dfrA1

Minimum percentage of nucleotides identical between sequence in genome and matching resistance gene was stated at 98%.

Table S3. Results of the duplicate testing in the bladder infection *in vitro* model.

Strain no.	Pre-exposure fosfomycin MIC (mg/L)	Post-exposure outcome in the *in vitro* model			
		Initial testing		Repeat testing	
		Bacterial growth, log_{10} cfu/mL (HLR %)	MIC (mg/L)	Bacterial growth, log_{10} cfu/mL (HLR %)	MIC (mg/L)
***E. coli* isolates**					
143	4	-	-	-	-
745	4	9.5 (HLR +++)	1024	4.0	8
1016	16	9.5 (HLR +++)	256	9.4 (HLR +++)	1024
1231	32	9.5 (HLR +++)	>1024	9.1 (HLR +++)	>1024
574	32	-	-	-	-
672	32	7.0 (HLR +++)	1024	9.1 (HLR +++)	>1024
123	32	-	-	-	-
746	128	-	-	-	-
***K. pneumoniae* isolates**					
787	1	-	-	-	-
874	2	-	-	-	-
915	8	6.6	8	8.7 (HLR ++)	512
820	8	9.7 (HLR ++)	512	7.0 (HLR ++)	512
899	8	9.2 (HLR +++)	>1024	9.4 (HLR +++)	>1024
956	8	9.3 (HLR ++)	512	9.0 (HLR ++)	512
52	16	9.2 (HLR +++)	>1024	9.0 (HLR +++)	>1024
891	16	3.8	8	6.5	32

MIC testing performed by agar dilution. Where re-growth occurred, the proportion of the total population that demonstrated high level resistance (HLR) was classified as greater than 1% (+++), between 0.01 to 1% (++), and less than 0.01% (+). MIC testing performed on the HLR subpopulation confirmed fosfomycin MIC ≥ 1024 mg/L.

Table S4. Disk elution heteroresistance screen.

	2x FOT200 disks			4x FOT200 disks		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
E. coli						
41	-	-	-	-	-	-
11	-	-	-	-	-	-
39	-	-	-	-	-	-
472	-	-	-	-	-	-
12620	-	-	-	-	-	-
583	-	-	-	-	-	-
143	-	-	-	-	-	-
629	-	-	-	-	-	-
692	-	+	+	-	-	-
255	-	-	-	-	-	-
745	+	+	+	-	+	+
381	-	-	-	-	-	-
010	-	-	-	-	-	-
030	-	-	+	-	-	-
214	-	+	+	-	-	-
1016	+	+	+	+	+	+
1231	+	+	+	+	+	+
574	-	+	+	-	-	-
672	+	+	+	-	+	+
123	-	+	+	-	-	-
4807	-	+	+	-	-	-
4757	-	+	+	-	-	-
746	-	-	-	-	-	-
202	-	+	+	-	-	-
K. pneumoniae						
787	-	-	-	-	-	-
790	-	-	-	-	-	-
864	-	-	-	-	-	-
976	-	-	+	-	-	+
855	-	-	-	-	-	-
874	-	-	-	-	-	-
862	-	-	+	-	-	-
34672	+	+	+	-	+	+
31865	-	+	+	-	+	+
972	-	+	+	-	+	+
803	-	+	+	-	-	-
856	+	+	+	-	+	+
55	-	+	+	-	+	+
830	-	+	+	-	+	+
915	-	+	+	-	-	-
820	-	+	+	-	+	+
899	+	+	+	+	+	+
956	+	+	+	+	+	+
52	-	+	+	-	+	+
891	-	+	+	-	+	+
Controls						
NC	-	-	-	-	-	-
25922	-	-	-	-	-	-
PC	+	+	+	+	+	+

Broth turbidity (+) is highlighted in grey. Clear broth is indicated by a dash (-), which were confirmed as no growth after subculture to blood agar. All isolate growth control tubes (without FOT200 disks added) were turbid from day 1 of incubation. Bacteria-free negative control (NC) tubes remained clear for the entire incubation period. *E. coli* ATCC 25922 was run as a negative heteroresistance control. The positive control (PC) was a *fosA*-positive *E. coli* (fosfomycin MIC > 1024 mg/L), which had growth in all tubes from day 1 of incubation.

Table S5. Sequence variations in genes related to fosfomycin-resistance in *E. coli*.

Strain	Binding site	Transporters		*uhpT* regulators			*glpT* repressor	cAMP regulators		Enzyme
	murA	*glpT* (G3P)	*uhpT* (G6P)	*uhpA*	*uhpB*	*uhpC*	*glpR*	*cyaA*	*ptsI*	*fosA*
***Pre-exposure*: Isolates killed in the *in vitro* model**										
41	-	L297F E443Q Q444E	-	-	L5F T166I P252S H442Q D459G H464Q	S177A	S17E T249A M250P A253V K260T	S352T V514I E837D A840T	T306A	n.d.
11	-	L297F E443Q Q444E	-	-	M75T T166I P252S H442Q D459G H464Q	S177A A417S	S17* T249A M250P A253V K260T	S352T V514I E837D A840T	T306A	n.d.
39	-	L297F E443Q Q444E	-	-	M75T T166I P252S D459G H442Q H464Q	S177A A417S	S17* T249A M250P A253V K260T	S352T V514I E837D A840T	T306A	n.d.
472	-	-	E350Q	-	-	A51S	-	E349A K356S E359G D362E V514I	V25I T306A	n.d.
12620	-	L297F E443Q Q444E	-	-	M75T T166I P252S H442Q D459G H464Q	S177A A417S	S17* T249A M250P A253V K260T	S352T V514I E837D A840T	T306A	n.d.
583	-	L297F T348N E443Q Q444E	-	A110S	M75T D205A H442Q D459G H464Q T482H	S177A G282D A417S T435A	S17* T249A M250P A253V K260T	L38Q S352T V514I E837D A840T	T306A	n.d.
143	-	-	E350Q	-	-	A51S	-	E349A K356S E359G D362E V514I	V25I T306A	n.d.
629	-	-	-	-	M75T S84P H442Q D459G H464Q T482H	H18Y S177A A417S	S17* T249A M250P A253V K260T	E349A K356S E359G D362E V514I E837D A840T	T306A	n.d.
692	-	L297F T348N E443Q Q444E	-	A110S	M75T D205A H442Q D459G H464Q T482H	S177A G282D A417S T435A	S17* T249A M250P A253V K260T	S352T V514I E837D A840T	T306A	n.d.
255	-	-	-	-	M75T S84P T374S H442Q D459G H464Q T482H	S177A A417S T435A	S17* T249A M250P A253V K260T	G222S E349A K356S E359G D362E V514I E837D A840T	T306A	n.d.
381	-	A16T	-	R46C	H442Q	T280M A401V	-	V514I	-	n.d.

Strain	Binding site	Transporters		*uhpT* regulators			*glpT* repressor	cAMP regulators		Enzyme
	murA	*glpT* (G3P)	*uhpT* (G6P)	*uhpA*	*uhpB*	*uhpC*	*glpR*	*cyaA*	*ptsI*	*fosA*
010	-	-	E350Q	-	-	A51S	-	E349A K356S E359G D362E V514I	V25I T306A	n.d.
214	-	-	E350Q	-	-	A51S	-	E349A K356S E359G D362E V514I	V25I T306A	n.d.
123	-	K448E	E350Q	-	M75T	-	S17E T249A M250P A253V K260T	A363S V514I	T306A	n.d.
***Pre-exposure*: Isolates killed in the *in vitro* model (with non-potentiating effect of G6P on baseline BMD MIC)[a]**										
574	-	L297F T348N E443Q Q444E	-	A110S	M75T D205A A223V H442Q D459G H464Q T482H	S177A G282D A417S T435A	S17* T249A M250P A253V K260T	S352T V514I E837D A840T	T306A	n.d.
4807	-	L297F E443Q Q444E	-	-	M75T M1_D3-del T166I P252S H442Q D459G H464Q	S177A	S17* T249A M250P A253V K260T	S352T V514I	T306A	n.d.
4757	-	-	E350Q	-	-	A51S W407* A417S	S17* T249A M250P A253V K260T	E349A K356S E359G D362E V514I	V25I T306A	n.d.
030	P99S	K448E	T65M	-	M75T S84P H442Q D459G H464Q T482H	H18Y S177A A417S	S17* T249A M250P A253V K260T	S142N E349A K356S E359G D362E V514I E837D A840T	T306A K367R	n.d.
746	-	W256*	E350Q	-	-	A51S	-	E349A K356S E359G D362E V514I	V25I T306A	n.d.
202	-	W132*	E350Q	-	M75T S84P H442Q D459G H464Q T482H	H18Y S177A A417S T435A	S17* T249A M250P A253V K260T	E349A K356S E359G D362E V514I E837D A840T	T306A	n.d.
***Pre-exposure*: Isolates that re-grew in the *in vitro* model (all re-grew with HLR population replacement)**										
745	-	W256* L297F E443Q Q444E	-	-	M75T T166I P252S H442Q D459G H464Q	S177A A417S	S17* T249A M250P A253V K260T	S352T V514I E837D A840T	T306A	n.d.

Strain	Binding site	Transporters		*uhpT* regulators			*glpT* repressor	cAMP regulators		Enzyme
	murA	***glpT* (G3P)**	***uhpT* (G6P)**	***uhpA***	***uhpB***	***uhpC***	***glpR***	***cyaA***	***ptsI***	***fosA***
1016	-	R269C	E350Q	-	-	A51S	-	E349A K356S E359G D362E V514I	V25I T306A	n.d.
1231	-	R269C	E350Q	-	-	A51S	-	E349A K356S E359G D362E V514I	V25I T306A	n.d.
672	-	L297F E443Q Q444E	-	-	M75T T166I P252S H442Q D459G H464Q	S177A A417S	S17* T249A M250P A253V K260T	S352T V514I E837D A840T	T306A	n.d.
***Post-exposure*: Isolates that re-grew in the *in vitro* model (all re-grew with HLR population replacement)**										
745	-	W256* L297F E443Q Q444E	-	-	M75T T166I P252S H442Q D459G H464Q	S177A A417S	S17* T249A M250P A253V K260T	S352T V514I E837D A840T	T306A	n.d.
1016	-	R269C	E350Q	**G42-P44-del**	-	A51S	-	E349A K356S E359G D362E V514I	V25I T306A	n.d.
1231	-	R269C	E350Q	**A41-L43-del**	-	A51S	-	E349A K356S E359G D362E V514I	V25I T306A	n.d.
672	-	L297F **T330S** E443Q Q444E **V327_M329del**	**del.**	**del.**	**del.**	**del.**	S17* T249A M250P A253V K260T	S352T V514I E837D A840T	T306A	n.d.

Isolates are grouped based on their growth outcome from testing in the *in vitro* model. *E. coli* ATCC 25922 was sequenced and used as the reference strain. Sequence variations in bold highlight new changes in the post-exposure strain compared to baseline. [a], comparison of broth microdilution (BMD) MIC results in Mueller-Hinton broth (MHB) with and without glucose-6-phosphate (G6P). n.d., not detected. *, premature stop codon.

Table S6. Sequence variations in genes related to fosfomycin-resistance in *K. pneumoniae*.

Strain	Binding site	Transporter genes		Transporter (*uhpT*) regulators			*glpT* repressor	cAMP regulators		Enzyme
	murA	*glpT* (G3P)	*uhpT* (G6P)	*uhpA*	*uhpB*	*uhpC*	*glpR*	*cyaA*	*ptsI*	*fosA*
***Pre-exposure*: Isolates that were killed in the *in vitro* model**										
787	-	-	-	-	-	-	-	-	-	S18G I57V A86V I91V
790	-	-	-	-	-	-	-	-	-	S18G I57V A86V I91V
874	T17I	-	-	-	-	-	-	-	L561I	I57V M134I Q139E
***Pre-exposure*: Isolates that re-grew in the *in vitro* model without emergence of HLR**										
864	-	-	-	-	-	-	-	-	-	L25Q I57V S79R A86V I91V D138_ 139EinsD
976	-	-	-	-	L72F	-	-	-	K174N	P53T I57V
855	-	-	-	-	-	-	-	-	-	L25Q I57V S79R A86V I91V D138_ 139EinsD
862	-	-	-	-	L72F	-	-	-	-	I57V
972	-	-	-	-	L72F	-	-	-	-	P53T I57V D138E
803	-	-	-	-	L72S	-	-	-	-	I57V A86V I91V Q139E
856	-	-	-	-	-	-	-	A841P	-	L25Q I57V I91V
55	-	-	-	-	L72F	-	-	-	-	I57V
830	-	-	-	S183N	C55S G58S L72F	T188I	-	-	-	I57V D138E
915	-	S119N	-	-	-	-	-	-	-	I57V D138E E138_139Ei nsD
891	-	-	-	-	L72S	M404I	-	-	-	I57V
***Pre-exposure*: Isolates that re-grew in the *in vitro* model with HLR**										
31865	-	-	-	-	L72F	-	-	-	-	I57V P53T D138E
820	-	-	-	-	L72S	L336M	-	-	-	I57V A120S Q139E
899	-	del.	-	-	L72F	L336M	-	-	-	I57V
956	-	-	-	-	L72S	-	-	-	-	I57V Q139E
52	-	-	-	-	L72S	-	-	-	-	I57V A86V I91V Q139E

Strain	Binding site	Transporter genes		Transporter (*uhpT*) regulators			*glpT* repressor	cAMP regulators		Enzyme
	murA	***glpT* (G3P)**	***uhpT* (G6P)**	***uhpA***	***uhpB***	***uhpC***	***glpR***	***cyaA***	***ptsI***	***fosA***
***Post-exposure*: Isolates that re-grew with HLR replacement (i.e. >1% of total population)**										
899	-	del.	**T340Ifs Ter57**	-	L72F	L336M	-	-	-	I57V
52	-	-	-	-	L72S	-	-	-	-	I57V A86V I91V Q139E
K. pneumoniae* 34672 (*K. quasipneumoniae* subsp. *quasipneumoniae*); *Pre-exposure										
34672*	S1483 T206S S210T N253H K358E	K234E E237Q I260V I429V	V434I	A25T T41A I87V V169I	A3T V4L V5L S16P T66S L72F A75T L138F A173S E295D Q349H D442E N461S L462A	E55D T188I C192G M236L S237T A240E V415A T438A	-	-	S241N	D35E S79C A86V I91V S118N
***K. pneumoniae* 34672 (*K. quasipneumoniae* subsp. *quasipneumoniae*); *Post-exposure* re-grew with HLR**										
34672	S1483 T206S S210T N253H K358E	K234E E237Q I260V I429V	V434I	A25T T41A I87V V169I	A3T V4L V5L S16P T66S L72F A75T L138F **P153L** A173S E295D Q349H D442E N461S L462A	E55D T188I C192G M236L S237T A240E V415A T438A	-	-	S241N	D35E S79C A86V I91V S118N

Isolates grouped based on growth outcome from testing in *in vitro* model. Coding regions of fosfomycin resistance genes of str. Kp52.145 (GenBank FO834906.1) were downloaded from GenBank and used as the reference. Sequence variations in bold highlight new changes in the post-exposure strain compared to baseline. *K. pneumoniae* 34672, presented separately at the end of the table, was further identified as *K. quasipneumoniae* subsp. *quasipneumoniae*, part of the *K. pneumoniae* complex, but distinctly different from the other *K. pneumoniae* isolates tested (Fig. S3).

Chapter 4

Impact of different urinary fosfomycin exposures

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Impact of different urinary fosfomycin exposures

The previous paper confirmed the discrepant response to a single dose of fosfomycin between *E. coli* and *K. pneumoniae* isolates. Fosfomycin susceptibility testing, performed by agar dilution, failed to predict treatment response and did not detect the presence of a high-level resistant subpopulation that was implicated in treatment failure. Interestingly, the molecular characteristics of the isolates failed to identify a common gene mutation that predicted outcome. A hypothesis generated from this paper questioned whether increasing the fosfomycin exposure could overcome the observed re-growth and emergence of resistance.

This next paper, published in *Antimicrobial Agents and Chemotherapy*, simulated whether extremes in urinary peak concentrations following a single dose, or exposures following three doses, given either 72-hourly, 48-hourly or 24-hourly could enhance fosfomycin activity.

Highlights

- The commonly adopted, off-label practice of prescribing multiple doses of oral fosfomycin is without strong *in vitro* or clinical evidence.

- There is uncertainty regarding the impact of the interpersonal variability in urinary fosfomycin exposure, which is largely due to behavioural practices such as time of administration, fluid intake, urine output and voiding practices.

- This study demonstrated the flexibility of the *in vitro* model to simulate different urinary exposure targets, the administration of multiple fosfomycin doses and prolonged experimental run-times up to 9-days.

- Simulation of normal variability in urinary exposures following a single fosfomycin dose did not correlate with any clear change in efficacy.
    - Although, low exposure led to less resistance in *K. pneumoniae* isolates.

- Increasing the total drug exposure by administering multiple doses failed to provide any additional suppression of re-growth in the majority of isolates compared to standard single dose therapy.
    - Emergence of resistance often occurs within the first 24 h.
    - Limited role in delaying repeat doses 48- or 72 h, which is recommended in some guidelines.

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CLINICAL THERAPEUTICS

Oral Fosfomycin Efficacy with Variable Urinary Exposures following Single and Multiple Doses against *Enterobacterales*: the Importance of Heteroresistance for Growth Outcome

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ABSTRACT Oral fosfomycin trometamol is licensed as a single oral dose for the treatment of uncomplicated urinary tract infections, with activity against multidrug-resistant uropathogens. The impact of interindividual variability in urinary concentrations on antimicrobial efficacy, and any benefit of giving multiple doses, is uncertain. We therefore performed pharmacodynamic profiling of oral fosfomycin, using a dynamic bladder infection *in vitro* model, to assess high and low urinary exposures following a single oral dose and three repeat doses given every 72 h, 48 h, and 24 h against 16 clinical isolates with various MICs of fosfomycin (8 *Escherichia coli*, 4 *Enterobacter cloacae*, and 4 *Klebsiella pneumoniae* isolates). Baseline fosfomycin high-level-resistant (HLR) subpopulations were detected prior to drug exposure in half of the isolates (2 *E. coli*, 2 *E. cloacae*, and 4 *K. pneumoniae* isolates; proportion, 1×10^{-5} to 5×10^{-4}% of the total population). Fosfomycin exposures were accurately reproduced compared to mathematical modeling (linear regression slope, 1.1; R^2, 0.99), with a bias of 3.8% ± 5.7%. All 5/5 isolates with MICs of ≤1 μg/ml had no HLR and were killed, whereas 8/11 isolates with higher MICs regrew regardless of exposure to high or low urinary concentrations. A disk diffusion zone of <24 mm was a better predictor for baseline HLR and regrowth. Administering 3 doses with average exposures provided very limited additional kill. These results suggest that baseline heteroresistance is important for treatment response, while increased drug exposure and administering multiple doses may not be better than standard single-dose fosfomycin therapy.

KEYWORDS PK/PD, antimicrobial resistance, fosfomycin, *in vitro* model, urinary tract infection

Fosfomycin trometamol is an old, off-patent oral antibiotic, recommended as a single 3-g dose as a first-line treatment in international guidelines for the treatment of uncomplicated urinary tract infections (uUTIs) (1–3). Surveillance reports demonstrate high rates of fosfomycin susceptibility, even among emergent multidrug-resistant (MDR) uropathogens (4–8). In the era of rising antimicrobial resistance seen globally, there is renewed interest in fosfomycin as an attractive therapeutic option (9–14).

Citation Abbott IJ, van Gorp E, Wijma RA, Meletiadis J, Roberts JA, Mouton JW, Peleg AY. 2020. Oral fosfomycin efficacy with variable urinary exposures following single and multiple doses against *Enterobacterales*: the importance of heteroresistance for growth outcome. Antimicrob Agents Chemother 64:e01982-19. https://doi.org/10.1128/AAC.01982-19.

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Received 1 October 2019
Returned for modification 26 October 2019
Accepted 24 November 2019
Accepted manuscript posted online 6 January 2020
Published 21 February 2020

High rates of fosfomycin susceptibility have been reported in many countries, with a 2016 systematic review demonstrating fosfomycin susceptibilities among extended-spectrum-β-lactamase (ESBL)-producing isolates of 95.1% for *Escherichia coli* and 83.8% for *Klebsiella pneumoniae* (15). Clinical efficacy, however, has been more variable. Although earlier clinical trials reported a treatment efficacy for uUTIs ranging from 77.2% to 95% (16), more recently, a retrospective study (17) found a microbiological cure rate of only 59% in the treatment of MDR uropathogens. Moreover, in a prospective randomized clinical trial (18), clinical resolution was achieved in 58% of patients receiving fosfomycin, compared to 70% receiving nitrofurantoin. This latest study raised doubts about whether a single 3-g dose reached adequately durable urine concentrations and the role of alternative fosfomycin prescribing practices, such as multiple doses given every 3 days (19, 20).

The original dosing studies for oral fosfomycin lacked the modern methods used today for the licensing of new antimicrobial agents. Supporting evidence for the efficacy of single-dose therapy was largely based upon pharmacokinetic (PK) reports of urinary concentrations of fosfomycin remaining greater than 128 mg/liter for 24 to 48 h (21). Several off-label dosing practices have since emerged, with some publications recommending giving 3 doses every 2 to 3 days and other studies recommending daily dosing, with emphasis on infections caused by MDR uropathogens and a longer duration of therapy following renal transplantation (17, 22–29). The efficacy of multidose oral fosfomycin has also been examined by population PK modeling (30). Repeat daily dosing of oral fosfomycin, however, has been associated with higher rates of diarrhea (31) and lacks detailed microbiological or clinical evidence for superiority over single-dose therapy.

Many studies evaluating the urine concentrations of fosfomycin after oral dosing have demonstrated significant variability between subjects (21, 31–37). Fosfomycin is primarily eliminated by the kidneys, with clearance approximating glomerular filtration. However, the resulting urinary concentrations in healthy populations have a wide range of values. As such, key urinary PK parameters, such as the peak urinary concentration (C_{max}), the time of C_{max} (t_{max}), and the duration of time that urinary concentrations remain above the MIC of the uropathogen, can be dramatically different. This ultimately creates uncertainty regarding what PK/pharmacodynamic (PD) index can best predict treatment efficacy.

To help address the uncertainty around fosfomycin efficacy in the context of varying urinary concentrations and multiple doses, we performed PD profiling of fosfomycin using a dynamic bladder infection *in vitro* model against common uropathogens.

RESULTS

Fosfomycin susceptibility of bacterial strains. A total of 16 contemporary, Gram-negative uropathogens with varying MICs for fosfomycin were included in this study. Using agar dilution, the MIC values for eight *E. coli* isolates ranged from $\leq$0.25 to 64 μg/ml, those for four *Enterobacter cloacae* isolates ranged from 0.5 to 32 μg/ml, and those for four *K. pneumoniae* isolates ranged from 2 to 16 μg/ml (Fig. 1A). Using the disk diffusion susceptibility method, inhibition zone diameters ranged from 20 to 36 mm for *E. coli*, 17 to 40 mm for *E. cloacae*, and 19 to 23 mm for *K. pneumoniae* (Fig. 1B). All *E. coli* isolates were classified as susceptible by applying Clinical and Laboratory Standards Institute (CLSI) breakpoints (38), with no interpretations provided for *E. cloacae* and *K. pneumoniae*. Applying the European Committee on Antimicrobial Susceptibility Testing (EUCAST) *Enterobacterales* breakpoints (39), only one *E. coli* isolate was classified as resistant by MIC testing. The EUCAST provides disk diffusion susceptibility breakpoints for *E. coli* only (susceptible [S] at $\geq$24 mm), by which two isolates were classified as resistant. For the other species, all *K. pneumoniae* isolates and 2 of 4 *E. cloacae* isolates had inhibition diameters of less than 24 mm, without provided interpretation. We also assessed for baseline fosfomycin heteroresistance and found that two *E. coli*, two *E. cloacae*, and all *K. pneumoniae* isolates had evidence of high-level-resistant (HLR) subpopulations at a percentage of the total population from 1×10^{-5} to 5×10^{-4}%

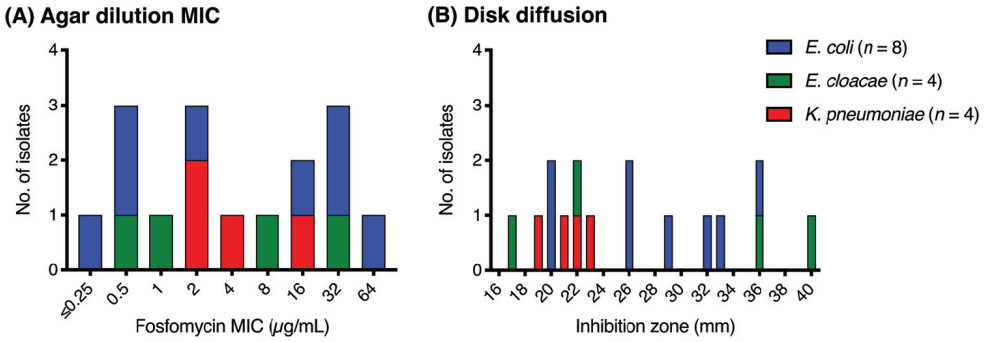

FIG 1 Baseline fosfomycin susceptibility of test isolates. (A) MIC testing was performed in triplicate by agar dilution. (B) Disk diffusion was performed using a FOT200 disk (Oxoid Ltd./Thermo Fisher Scientific, UK). *E. coli* ATCC 25922 was used as a quality control organism.

(Table 1). None of the isolates with an agar dilution MIC of ≤1 μg/ml or a disk diffusion inhibition zone of >24 mm had a detectable HLR subpopulation at baseline. Of the isolates with inhibition zones of <24 mm, 8/8 had an HLR subpopulation, whereas of isolates with MICs of >1 μg/ml, 8/11 had an HLR subpopulation (2 *E. coli*, 2 *E. cloacae*, and 4 *K. pneumoniae* isolates). The 3 isolates with MICs of >1 μg/ml without HLR (MICs of 2, 32, and 64 μg/ml) were all *E. coli* isolates. Note that 4/4 *K. pneumoniae* isolates had MICs of ≥2 μg/ml, and all had detectable HLR.

Fosfomycin exposure in the bladder infection *in vitro* model. We used an adaptation of a previously described *in vitro* model (40). Observed *in vitro* concentrations closely matched the target concentration of each of the single and multidose fosfomycin exposure simulations. The slope of the linear regression line was equal to 1.1 (R^2, 0.99), with a bias of 3.8% ± 5.7% (Fig. 2). There was minimal intercompartment variation, with an average relative standard deviation of 3.1% ± 1.9%. Following a single fosfomycin dose, targeting an average urinary exposure, the measured C_{max} was 2,122.2 ± 46.0 mg/liter, whereas for the low-C_{max} exposure, it was 975.7 ± 34.8 mg/liter, and for the high-C_{max} exposure, it was 3,628.2 ± 218.4 mg/liter (Fig. 3). Following the multidose experiments, fosfomycin concentrations were accurately reproduced following dosing schedules every 72, 48, and 24 h (Fig. 4).

Impact of variable urinary fosfomycin concentrations on treatment response. Given the variability observed in urinary fosfomycin concentrations in patients after a single oral dose, we tested the treatment efficacy of average, low, and high urinary fosfomycin C_{max} values using our established dynamic bladder infection model (Fig. 5 and Table 1). Irrespective of the urinary fosfomycin concentration, the same bacterial isolates regrew over a 72-h period (two *E. coli*, two *E. cloacae*, and all *K. pneumoniae* isolates). For the majority of clinical isolates, following exposure to different urinary concentrations, regrowth was associated with baseline heteroresistance identified within the starting bacterial population (Table 1). Low-level-resistant (LLR) regrowth (growth on Mueller-Hinton II agar [MHA] with 64 mg/liter) coincided with the detection of high-level-resistant (HLR) growth (growth on MHA with 512 mg/liter). All 5/5 isolates with MICs of ≤1 μg/ml were killed, whereas isolates with higher MICs had mixed behavior depending on the presence of baseline HLR. Only one isolate with an inhibition zone of >24 mm (*E. coli* 4757) regrew, and this occurred only following high-C_{max} exposure, without the emergence of HLR. All *K. pneumoniae* isolates regrew after all exposures, although the emergence of resistance during regrowth was lowest following exposure to a low C_{max}.

Impact of multiple doses of fosfomycin on treatment response. When 3 doses of fosfomycin were administered at different frequencies (every 72 h [q72], q48, and q24), pathogen kill was not uniformly enhanced (Table 1 and Fig. 6). Across all exposure experiments, the same five *E. coli* and two *E. cloacae* isolates were killed. *E. coli* 4757 (baseline MIC of 64 μg/ml) had regrowth detected at the final sampling time point,

TABLE 1 Isolate characteristics and response to urinary fosfomycin exposures[b]

Strain	Fosfomycin susceptibility		Growth control (baseline HLR [%])	Postexposure outcome[a] (bacterial count [$\log_{10}$ CFU/ml]/MIC [μg/ml]; HLR proportion)					
				Single dose			3 doses		
	MIC (μg/ml)	Disk diffusion (mm)		Avg C_{max}	Low C_{max}	High C_{max}	Every 72 h	Every 48 h	Every 24 h
E. coli									
41	≤0.25	33	−	−	−	−	−	−	−
11	0.5	36	−	−	−	−	−	−	−
39	0.5	32	−	−	−	−	−	−	−
12620	2	26	−	−	−	−	−	−	−
1016	16	20	2E−04	9.5/**256**; HLR +++	9.0/**>1,024**; HLR +++	9.0/**>1,024**; HLR +++	9.6/**>1,024**; HLR +++	9.5/**>1,024**; HLR +++	9.6/**>1,024**; HLR +++
1231	32	20	2E−04	9.5/**>1,024**; HLR +++	9.1/**>1,024**; HLR +++	9.3/**>1,024**; HLR +++	9.5/**>1,024**; HLR +++	9.3/**>1,024**; HLR +++	9.3/**>1,024**; HLR +++
4807	32	29	−	−	−	−	−	−	−
4757	64	26	−	−	−	6.3/**64**; no HLR	−	7.5/**64**; HLR +	−
E. cloacae									
35166	0.5	36	−	−	−	−	−	−	−
94	1	40	−	−	−	−	−	−	−
21	8	22	2E−05	9.5/**>1,024**; HLR +++	8.4/**8**; HLR +++	9.5/**32**; HLR ++	8.9/**16**; HLR +++	8.6/**>1,024**; HLR +++	6.6/**16**; no HLR
32	32	17	5E−04	9.5/**>1,024**; HLR +++	9.4/**>1,024**; HLR +++	9.9/**>1,024**; HLR +++	9.7/**>1,024**; HLR +++	9.5/**>1,024**; HLR +++	9.4/**>1,024**; HLR +++
K. pneumoniae									
34672	2	23	1E−05	9.3/**>1,024**; HLR +++	7.3/**4**; HLR +++	9.6/**1,024**; HLR +	9.5/**>1,024**; HLR +++	8.8/**512**; HLR ++	−
31865	2	22	2E−04	9.4/**256**; HLR ++	3.9/**2**; no HLR	7.5/**32**; no HLR	−	6.8/**2**; no HLR	5.9/**2**; no HLR
55	4	19	1E−04	6.9/**4**; no HLR	4.6/**8**; no HLR	9.6/**>1,024**; HLR +	9.4/**>1,024**; HLR +++	9.5/**512**; HLR +++	9.5/**512**; HLR +
52	16	21	5E−04	9.2/**>1,024**; HLR +++	6.8/**16**; no HLR	2.8/**256**; no HLR	9.6/**16**; HLR ++	5.2/**16**; no HLR	9.2/**1,024**; HLR ++

[a]Postexposure outcome was determined as no growth or the bacterial count measured at the final time point (72 h after the last administered fosfomycin dose). The fosfomycin MIC was reassessed on the total population regrowth (values presented in boldface type); dark gray shading highlights a >2-$\log_2$ rise in the MIC, and light gray shading highlights growth without a significant rise in MIC. The HLR proportion of the total population is presented as greater than 1% (+++), between 0.01 and 1% (++), or less than 0.01% (+). MIC testing of all HLR subpopulations confirmed MICs of ≥1,024 μg/ml after subculturing on TSB.

[b]− indicates not detected or no growth.

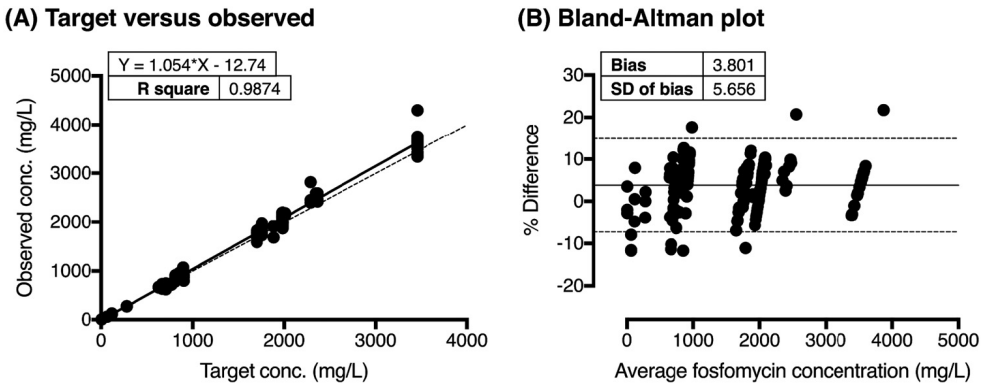

FIG 2 Relationship between the observed and target fosfomycin concentrations. (A) Accuracy of observed fosfomycin concentrations compared with the target with linear regression (solid line) and *y* equal to *x* (dashed line). (B) Bland-Altman plot of the percent differences of the observed and target measurements (*y* axis) and the averages of the two measurements (*x* axis), presented with the bias (solid line) and 95% limits of agreement (dotted lines).

after 3 doses given q48, without any rise in the postexposure total population MIC. *E. coli* strains 1016 and 1231 and *E. cloacae* strain 32 regrew with emergence of HLR in all exposure experiments. Similarly, *E. cloacae* 21 also regrew following all exposures but had a variable emergence of HLR, with and without rises in the postexposure fosfomycin MIC. All 4 *K. pneumoniae* isolates tended to regrow following all exposure experiments (except *K. pneumoniae* 31865 after 3 doses q72 and *K. pneumoniae* 34672 after 3 doses q24). LLR regrowth coincided with HLR. Similar to that observed following varying urinary concentrations after a single dose, isolates with a preexposure MIC of >1 μg/ml had variable regrowth, whereas a disk diffusion inhibition zone of <24 mm and the presence of an HLR subpopulation at baseline better predicted regrowth.

DISCUSSION

Understanding how to optimize oral fosfomycin therapy, especially when other antimicrobial options are limited due to resistance or unfavorable safety profiles, is

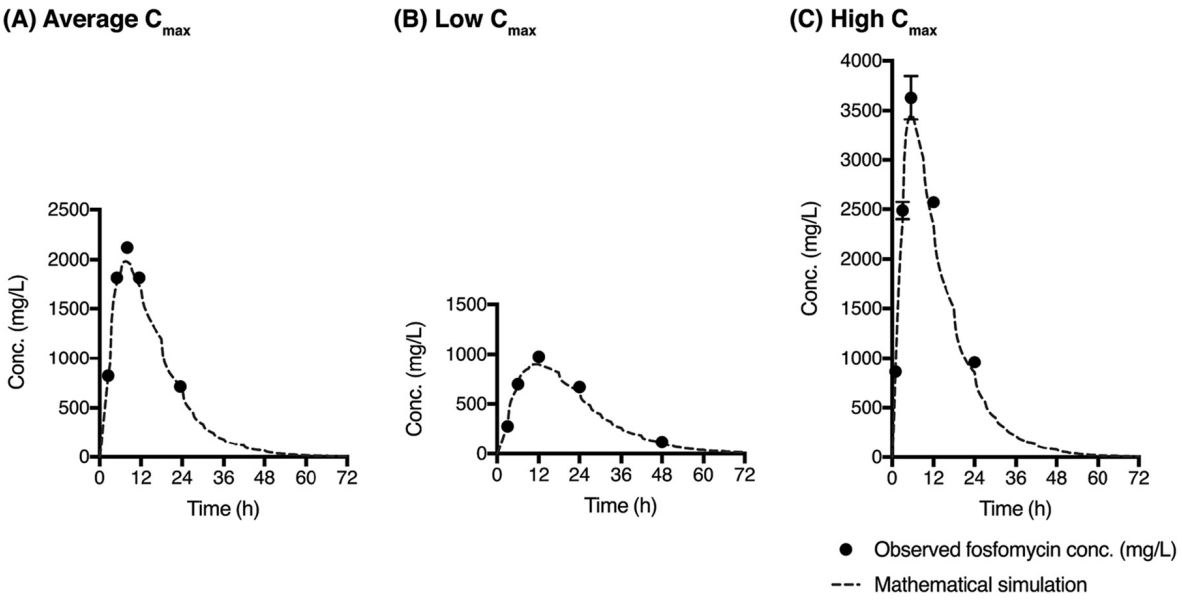

FIG 3 Single-dose fosfomycin urinary concentrations. Fosfomycin was administered as a single dose, simulating an oral 3-g dose with average exposure (A), low urinary C_{max} (B), and high urinary C_{max} (C). The average measured fosfomycin concentrations are overlaid on the target concentration-time curves (dashed line). Note that the mathematically simulated urinary concentration curves do not demonstrate a smooth drug elimination phase due to the dynamic fluid shifts that occur after each voiding cycle of the bladder compartment.

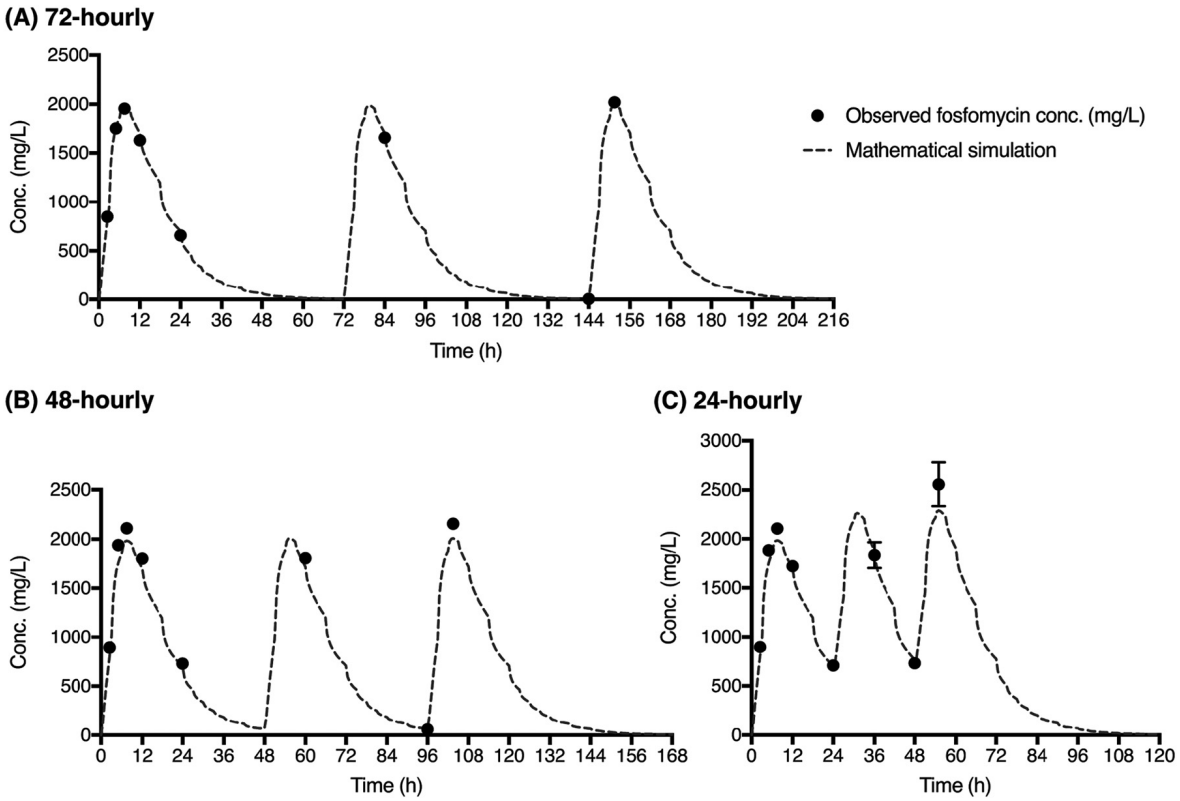

FIG 4 Multidose fosfomycin urinary concentrations. Shown are data for simulations of three 3-g oral doses of fosfomycin administered every 72 h (A), 48 h (B), and 24 h (C). The average measured fosfomycin concentrations are overlaid on the target concentration-time curve (dashed lines). Note that the mathematically simulated urinary concentration curves do not demonstrate a smooth drug elimination phase due to the dynamic fluid shifts that occur after each voiding cycle of the bladder compartment.

paramount for improving clinical efficacy. However, the impact on treatment efficacy of interpersonal variability in fosfomycin urinary concentrations and the benefit of off-label, multidose prescribing are uncertain. Here, we showed that variability in urinary exposures following a single 3-g oral dose did not correlate with any clear change in efficacy. Furthermore, increasing the total drug exposure by administering multiple doses failed to provide any additional suppression of regrowth in the majority of isolates compared to standard single-dose therapy. In our study, the efficacy of fosfomycin appeared to be influenced more by the baseline characteristics of the infecting pathogen than by variations in urinary drug exposure or dosing frequency. Baseline high-level heteroresistance and bacterial species were found to be more predictive of regrowth. Although isolates with an agar dilution MIC of ≤ 1 μg/ml were reliably killed, isolates with higher MICs demonstrated mixed behavior depending on the presence of HLR, in particular for *E. coli* isolates that were killed despite high MICs of up to 64 μg/ml. A disk diffusion zone of <24 mm, however, better predicted the presence of baseline HLR and regrowth after fosfomycin exposure. In addition, emergence of resistance was often detected early, within the first 24 h, such that repeat doses of fosfomycin given after that time had very little impact on the overall bacterial density. This rapid emergence of resistance negated the benefit of giving multiple doses and particularly questions the role of delaying repeat doses by 48 or 72 h. Overall, the efficacy of fosfomycin against our *K. pneumoniae* isolates appeared limited, regardless of the baseline MIC or drug exposure, a finding supported by other studies (40–42).

Treating physicians may look to increase urinary fosfomycin exposure for a number of reasons: in anticipation of the variability in urinary drug concentrations, limited alternative antimicrobial options, vulnerable patient groups (such as after kidney transplantation) (43, 44), urinary tract infections (UTIs) in male patients, *E. coli* isolates with elevated fosfomycin MIC values, and for the treatment of other species of

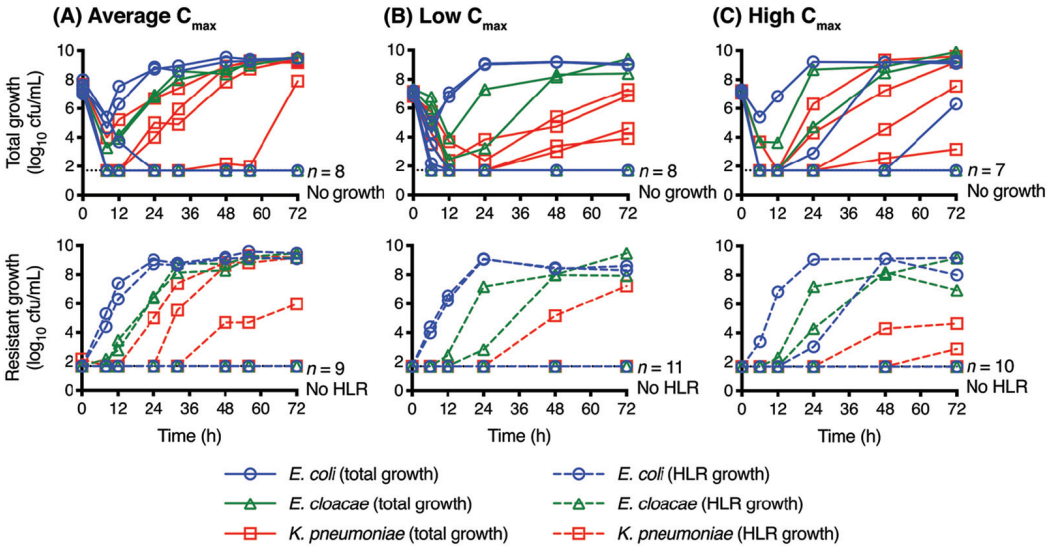

FIG 5 Growth outcome following a single fosfomycin dose. Shown are data for simulated 3-g oral doses of fosfomycin with average exposure (A), low C_{max} (B), and high C_{max} (C). Total growth and high-level-resistant (HLR) growth are presented for all 16 isolates: 8 *E. coli* isolates, 4 *E. cloacae* isolates, and 4 *K. pneumoniae* isolates. The limit of detection was 50 CFU/ml. The number of isolates at the final time point without growth or emergence of HLR is indicated. See Table 1 for isolate-specific details.

Gram-negative uropathogens. Concerningly, discrepancies have been reported between different fosfomycin susceptibility methods (45–48), and the gold-standard MIC method by agar dilution may not be the best predictor of clinical efficacy (49). Furthermore, in one study, patients with UTIs with carbapenem-resistant *K. pneumoniae* had a microbiological cure rate of only 46% when treated with fosfomycin, despite an *in vitro* susceptibility of 92% (17). Our data suggest that baseline fosfomycin heteroresistance was more predictive of the treatment response in our bladder infection model, and if present, regrowth with resistant populations was almost universal irrespective of the modeled urinary concentrations or 3-dose frequency. This finding was supported by a clinical review of fosfomycin treatment in MDR UTIs, which found no association of treatment outcomes with the MIC of fosfomycin or the number of doses received (14).

Following a single-fosfomycin-dose exposure, there was no progressive improvement in efficacy from low to high urinary fosfomycin concentrations. Given that baseline resistant subpopulations had MICs of fosfomycin of $>$1,024 μg/ml, even with high urinary C_{max} exposures, there would likely be a minimal time that concentrations were maintained above the mutant prevention concentration (MPC) in order to suppress growth. Similarly, the reduced emergence of resistance seen among the *Klebsiella* strains following low urinary concentrations may highlight the left side of the inverted U-shaped pattern that, in general, describes the relationship between exposure and emergence of resistance. In clinical practice, the inoculum size, duration of therapy, and activity of the immune system would impact the shape of this curve (50). In this setting, a prolonged treatment duration can make it increasingly difficult to suppress the amplification of the resistant subpopulation.

Compared to serum antimicrobial concentration measurements, the assessment of urinary concentrations has greater complexity. Although cumulative urinary measurements of an antimicrobial provide an assessment of the urinary recovery of the administered dose, the actual concentration measured in a voided urine sample at any one time is greatly impacted by individual behaviors, such as fluid intake, urine output, and voiding pattern. Because of these variabilities in observations, there can be uncertainty regarding which urinary PK/PD targets are important for clinical efficacy. Oral fosfomycin achieves urinary concentrations that are 100 to 1,000 times higher than the serum concentrations (21, 31–33). Such high urinary antimicrobial concentrations are essential for efficacy. In a mouse model, systemically administered therapy reaching

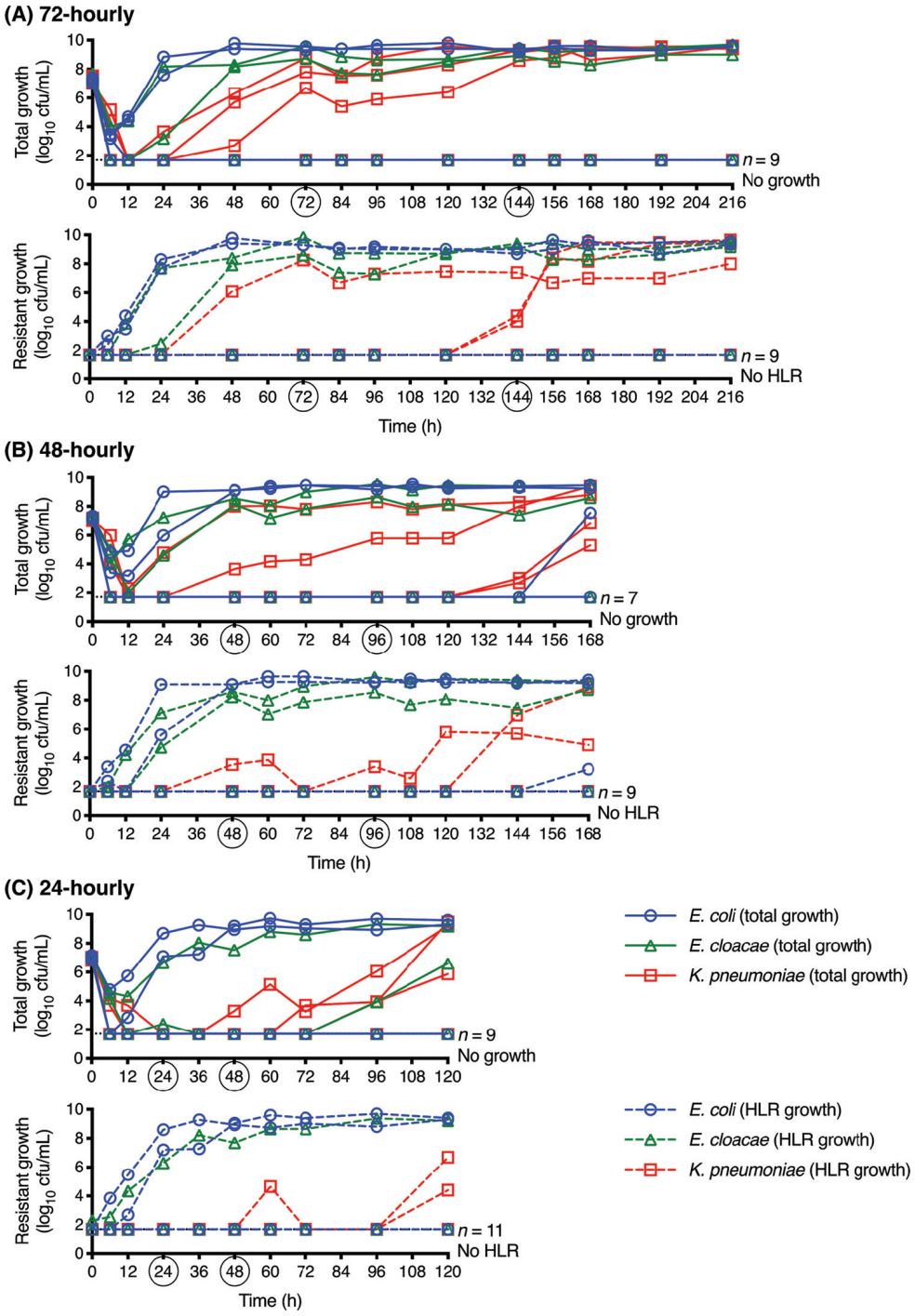

FIG 6 Growth outcome following multiple fosfomycin doses. Shown are data for three simulated 3-g oral doses of fosfomycin given every 72 h (A), 48 h (B), and 24 h (C). Total growth and high-level-resistant (HLR) growth are presented for all 16 isolates: 8 *E. coli* isolates, 4 *E. cloacae* isolates, and 4 *K. pneumoniae* isolates. The limit of detection was 50 CFU/ml. The number of isolates at the final time point without growth or emergence of HLR is indicated. The timing of the second and third doses of fosfomycin is indicated by circles on the *x* axis. See Table 1 for isolate-specific details.

only the bladder tissue (and not the bladder lumen) was found to be insufficient for bacterial eradication (51). However, optimizing clinical cure of UTIs by targeting only high antimicrobial urinary concentrations negates the other important nonantimicrobial factors that can also assist in bacterial clearance, such as increased fluid intake to promote increased urine output and dilution of bacteria in the bladder and high-

volume and frequent urination to assist in bacterial clearance. However, such urokinetic strategies would concurrently reduce antimicrobial concentrations in the bladder.

An advantage of the bladder infection *in vitro* model used in this study is the application of a dynamic UTI simulation, which applies normal urodynamics and accurate urinary fosfomycin exposures, in which experiments with multiple different pathogens can be run for prolonged periods of time to reflect clinical dosing regimens. Although this study applied PK data from a single healthy-volunteer study (32), which reported relatively high urinary concentrations compared to those in other recent studies (31, 33), our single-dose experiments examined 1 standard deviation above and below the reported average to account for the observed interpersonal variability. The main limitation of the *in vitro* model, as with all *in vitro* models, is whether the pathogen response demonstrated can be translated to, and be predictive of, the real *in vivo* situation. Importantly, the *in vitro* model lacks bladder tissue architecture and host immune responses. The use of standard laboratory medium, Mueller-Hinton II broth (MHB) supplemented with glucose-6-phosphate (G6P), may also not reflect fosfomycin activity and pathogen growth in urine. Furthermore, bacterial density measurements collected at the time of the peak *in vitro* fosfomycin concentration may underestimate growth due to antibiotic carryover being addressed by serial dilutions only, although previous reports have demonstrated no difference between dilution and washing (52).

Fosfomycin is a very useful antimicrobial for uUTIs. Reassuringly, variabilities in individual urinary fosfomycin exposures seem to have a minimal impact. Given the current limitations of fosfomycin susceptibility methods and clinical breakpoints, more work is required to accurately identify isolates with a high likelihood of clinical success. Our *in vitro* data, however, provide caution for the off-label practice of administering multiple oral doses of fosfomycin. It is uncertain, however, whether repeat doses of fosfomycin could be beneficial in more complex infection syndromes, such as an ascending infection leading to pyelonephritis (53), or infections in difficult sites, such as the prostate and in biofilms (54, 55). We also await the results of the FORECAST study (56), which will examine the treatment of complicated UTIs with an intravenous (i.v.)-to-oral switch, comparing ciprofloxacin with fosfomycin administered daily to complete a total of 10 days of therapy. Furthermore, to build upon the clinical study by Huttner et al. (18), a randomized controlled trial examining the treatment of uUTI comparing nitrofurantoin at 100 mg twice daily for 5 days (macrocrystal-monohydrate formulation) versus 3 to 5 daily doses of 3 g fosfomycin would be a valuable clinical study to guide treatment recommendations. An assessment of baseline fosfomycin heteroresistance in the identified uropathogens would also be an important adjunct to standard susceptibility testing.

MATERIALS AND METHODS

Antibiotic and media. Fosfomycin (Fomicyt; InfectoPharm, Germany) was reconstituted to a concentration of 50,000 mg/liter and used in the bladder infection *in vitro* model and for medium production. Cation-adjusted MHB (Becton, Dickinson [BD], USA) supplemented with G6P (catalog number G7879-5G; Sigma-Aldrich, USA) at a concentration of 25 mg/liter was used as the liquid medium in the *in vitro* model. Trypticase soy agar (TSA) containing 5% sheep blood (BD) was used for subculturing isolates from a freezer stock. Mueller-Hinton II agar (MHA) (BD) was used for the quantification of bacterial density. Emergence of resistance was assessed by plating on MHA with 64 mg/liter (low-level resistance [LLR]) and 512 mg/liter (high-level resistance [HLR]) of fosfomycin. Fosfomycin was also added to MHA (0.25 to 1,024 mg/liter) for agar dilution susceptibility testing. All media to which fosfomycin was added also contained 25 mg/liter G6P.

Bacterial strains and *in vitro* susceptibility testing. Sixteen clinical isolates were selected to reflect a range of baseline fosfomycin MIC values, originating from The Netherlands (57). Isolates included 8 *E. coli*, 4 *E. cloacae*, and 4 *K. pneumoniae* isolates. Species identification was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Germany). Fosfomycin susceptibility was determined by agar dilution (in triplicate, presented as the median value) according to the reference methodology (58) and disk diffusion using a FOT200 disk (Oxoid Ltd./Thermo Fisher Scientific, UK). *E. coli* ATCC 25922 was used as the quality control organism. The baseline proportion of an HLR subpopulation was assessed from a culture grown overnight in drug-free MHB with G6P and plated onto MHA containing 512 mg/liter of fosfomycin, with the HLR bacterial density divided by the total-growth density on drug-free MHA.

Dynamic bladder infection *in vitro* model. In short, the dynamic bladder infection *in vitro* model simulates dynamic urinary fosfomycin exposure, on a 1:16 scale to *in vivo*, to 16 independent bladder compartments (see Fig. S1 in the supplemental material). Fosfomycin is administered into the intestinal compartment, absorbed into the circulatory compartment, and eliminated into each bladder compartment in parallel. By applying drug distribution PK equations (Fig. S2) (59), the variables of the initial fosfomycin dose, compartment volumes, and flow rates were modified in order to simulate different urinary exposures following a single oral dose and the dynamic cumulative exposure following multiple doses. Test isolates were added to each bladder compartment, at an inoculum of 10^7 CFU, to provide a total number of bacteria equivalent to that expected in human infections (i.e., $>10^5$ CFU/ml in an average 250-ml void). The remainder of the starting inoculum was incubated overnight as a drug-free growth control from which an assessment of baseline HLR was performed, as described previously. Following exposure to fosfomycin within the *in vitro* model, pathogen kill and emergence of resistance were assessed by quantitative cultures on drug-free and fosfomycin-containing MHA (64 mg/liter and 512 mg/liter). Single-dose experiments were run for 72 h. Multidose experiments were run for an additional 72 h after the third dose of fosfomycin.

Fosfomycin dosing schedules and simulated urinary exposures. Given the large recognized inter- and intrasubject variability in urinary fosfomycin concentrations following a single 3-g oral dose, average-, low-, and high-exposure situations were simulated, applying data from a healthy-volunteer PK study, which reported an average urinary fosfomycin C_{max} of 1,982.0 ± 1,257.4 mg/liter (32). When targeting this average urinary exposure, the model was designed to deliver a blood elimination half-life of 6.9 h, urinary C_{max} at 7.5 h, and urinary concentrations maintained at >128 mg/liter for at least 40 h. In order to simulate low urinary exposure, which could occur *in vivo* in the setting of increased fluid intake and increased urine output, a C_{max} of 898 mg/liter was targeted, representing 1 standard deviation below the mean reported value. The same blood elimination half-life was targeted, with the *in vitro* flow rate and volumes of the gastrointestinal and circulatory compartments increased. For a high-urinary-exposure simulation, which could occur in the setting of reduced fluid intake and a decreased urine output volume, a urinary C_{max} of 3,454 mg/liter at 5.5 h was targeted, representing 1 standard deviation above the mean. The same blood elimination half-life was again targeted, with the *in vitro* flow rate and volumes of the gastrointestinal and circulatory compartments reduced. The targeted total urinary fosfomycin exposure for the single-dose experiments were as follows: an average-exposure area under the concentration-time curve from 0 to 72 h (AUC_{0-72}) of 36,941 mg · h/liter, reduced by 65.7% for the low-exposure experiment (24,284 mg · h/liter) and increased by 150.1% for the high-exposure experiment (55,457 mg · h/liter). To reflect different off-label oral dosing schedules commonly employed, 3 doses were administered either every 72, 48, or 24 h. All multidose experiments applied an average urinary exposure.

***In vitro* sample processing.** Samples for PK and PD assessments were taken directly from each bladder compartment at predetermined time points. Samples for fosfomycin concentration quantification, initially diluted 1:10 with saline when expected to fall outside the validated concentration range of the assay, were immediately frozen at −80°C until testing. Quantitative cultures for PD assessments were processed immediately, with bacterial density (CFU per milliliter) calculated at each time point. Specifically, medium from within each bladder compartment was sampled via a 3-way stopcock (BD) connected to the outflow tract and underwent serial 10-fold dilutions, of which 20 μl from each dilution was plated onto drug-free MHA and MHA containing 64 and 512 mg/liter of fosfomycin. The lower limit of detection was considered to be 50 CFU/ml, discounting nonviable growth such as pinpoint colonies or haze. All plates were incubated aerobically at 37°C for 16 to 20 h. Plates supplemented with fosfomycin were reincubated for a further 24 h.

Measurement of fosfomycin concentrations. An ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method was used for the quantification of fosfomycin from PK samples collected from bladder compartments during each experiment. All bladder compartments were sampled during each experiment at the initial C_{max} time point, providing an assessment of intercompartment variation. On all other occasions, three bladder compartments were sampled to provide representative concentration changes over time. For the single-dose experiments, samples were collected twice before and twice after the C_{max} time point. Additionally, for the multidose experiments, samples were also collected 12 h after the second dose, and trough and peak samples were collected at around the 3rd dose. Observed concentrations were compared to target concentrations determined by the drug distribution equations (Fig. S2). Linear regression and Bland-Altman analysis were used to determine the accuracy of the observed concentrations compared to the target in terms of bias and precision. The UPLC-MS/MS method was validated for urine and plasma samples of fosfomycin (60), but additional tests confirmed its applicability for fosfomycin in MHB samples. The method was validated according to FDA guidelines (61) over a range of 0.75 to 375 mg/liter (R^2, 0.9998). The lower limit of quantification was 0.75 mg/liter, and the lower limit of detection was 0.70 mg/liter. The method was found to be accurate and precise, with a maximum deviation of 5.0%. The stability of fosfomycin in MHB at 37°C for 72 h and stored at −80°C for at least 6 months was confirmed.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

ACKNOWLEDGMENTS

We acknowledge the laboratory assistance of Carla Roodbol de Goeij (Department of Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre) and of Alex Brouwer and Geert Springeling (Department of Experimental Medical Instrumentation, Erasmus Medical Centre) for their assistance in the *in vitro* equipment design and production.

This work was supported in part by the AIDA project LifeSciHealth Priority of the European Commission Seventh Framework Programme, FP7 (Preserving Old Antibiotics for the Future, grant number F3-2011-278348). I.J.A. was funded by an Australian Government Research Training Program scholarship (APP1114690) from the National Health and Medical Research Council of Australia. A.Y.P. and J.A.R. are in part funded through Australian National Health and Medical Research Council practitioner fellowships (APP1117940 and APP1117065, respectively).

J.W.M. has received research funding from Adenium, AstraZeneca, Basilea, Cubist, Polyphor, Roche, Eumedica, Basilea, VenatorX, AiCuris, Gilead, and Wockhardt. A.Y.P. has received research funding from MSD through an investigator-initiated research project. All other authors have no conflicts of interest to declare.

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SUPPLEMENTARY MATERIAL

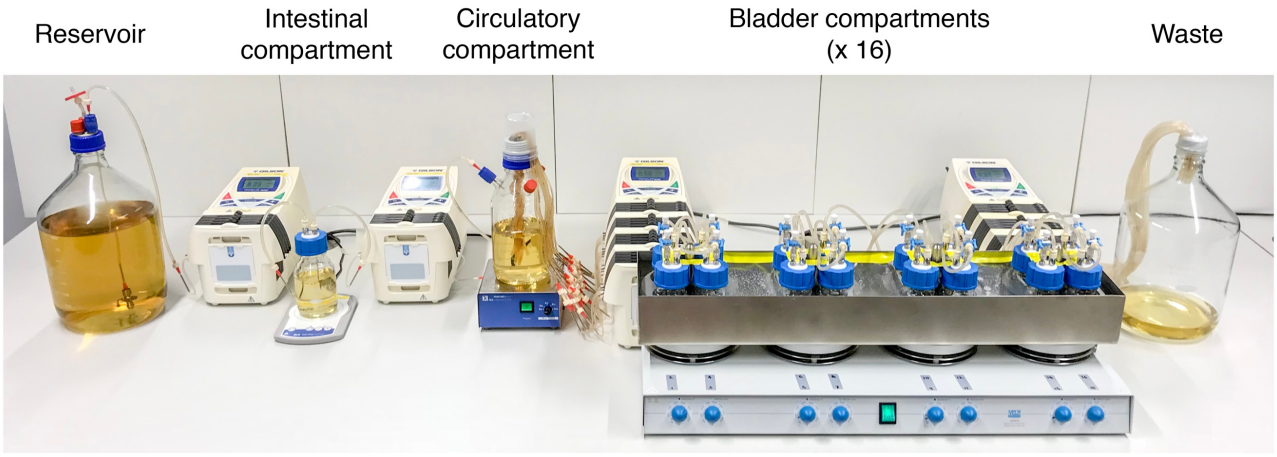

Figure S1. Bladder infection *in vitro* model design
Media pumped via autoclavable 1.01 mm PVC tubing (Gilson, UK) run through three sequentially arranged peristaltic pumps (Gilson, UK) delivering fresh media from the reservoir to the intestinal compartment, into which fosfomycin was administered, through to the circulatory compartment, and eliminated into the sixteen bladder compartments, which were run in parallel and held within a water-bath at 37°C ± 1°C. Automated and timed intermittent bladder compartment voiding was controlled by a fourth peristaltic pump.

(A) *In vivo* equations

Drug in G.I. tract:

$$X_{GI} = X_{dose} \cdot e^{-k_1 \cdot t}$$

Drug in blood:

$$X_{blood} = \frac{k_1 \cdot X_{dose}}{k_2 - k_1} \cdot \left(e^{-k_1 \cdot t} - e^{-k_2 \cdot t}\right)$$

Excreted drug:

$$X_{bladder} = X_{dose} - X_{GI} - X_{blood}$$

(B) *In vitro* equations

Drug in first compartment:

$$X_A = X_{A^\circ} \cdot e^{-(F/V_A)t}$$

Drug in second compartment:

$$X_B = \frac{(F/V_A) \cdot X_{A^\circ}}{F/V_B - F/V_A} \cdot \left(e^{-(F/V_A)t} - e^{-(F/V_B)t}\right)$$

Cumulative drug in third compartment:

$$X_C = X_{A^\circ} - X_A - X_B$$

Figure S2. Drug distribution equations informing target urinary fosfomycin concentrations
The dynamic amount of drug (X mg) in each respective compartment at time t (h) as a function of the first-order rate constants (absorption k_1; elimination k_2). The initial dose of fosfomycin (mg) is indicated by X_{dose} or X_{A°. In the *in vitro* equations the fluid volumes (V mL) in the respective compartments and flow rate of fluid (F mL/h) are the variables.

Chapter 5

Fosfomycin activity in different *in vitro* media

Chapter 5

Fosfomycin activity in different *in vitro* media

By demonstrating the adaptability of the *in vitro* model to enable the different urinary fosfomycin exposures presented in the previous paper, a key message was that the baseline characteristics of the uropathogen and the presence of a resistant subpopulation was most important in predicting treatment response, and that increase exposure, or multiple doses, did not improve treatment response. However, the use of standard laboratory media, supplemented with glucose-6-phosphate, may be presenting an unrealistic representation of fosfomycin activity, where altered pathogen growth and antimicrobial activity occurs in urine *in vivo*.

This next paper, published in *Journal of Microbiological Methods*, examined fosfomycin activity and emergence of resistance in standard laboratory media and pooled human urine. Furthermore, this paper examined synthetic media alternatives for urine.

Highlights

- The translation of data from *in vitro* preclinical infection models to the clinical setting relies on an accurate simulation of pathogen growth and antimicrobial activity at the site of infection.

- We demonstrated that fosfomycin activity is affected by the *in vitro* environment, when comparing standard laboratory media, pooled human urine and synthetic alternatives.

- In the dynamic bladder infection *in vitro* model, synthetic human urine was showed to be a good surrogate for human urine in the pharmacodynamic profiling of oral fosfomycin against common uropathogens.
    - Although, reduced rates of post-exposure fosfomycin resistance in the synthetic media suggests that antimicrobial exposure in a nutrient-deplete environment can alter bacterial evolutionary pathway – leading to persistence rather than resistance.

Journal of Microbiological Methods 171 (2020) 105861

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Evaluation of pooled human urine and synthetic alternatives in a dynamic bladder infection *in vitro* model simulating oral fosfomycin therapy

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ARTICLE INFO

Keywords:
Urinary tract infection
in vitro model
Urine
Synthetic human urine
Fosfomycin
Pharmacokinetics
Pharmacodynamics

ABSTRACT

The impact of the bladder environment on fosfomycin activity and treatment response is uncertain. Standard laboratory media does not reflect the biomatrix of urine, where limited nutritional factors are important for growth and antimicrobial kill rates. We compared fosfomycin activity against *Enterobacteriaceae* in laboratory media, human urine and synthetic alternatives. Sixteen clinical isolates (8-*Escherichia coli*, 4-*Enterobacter cloacae*, 4-*Klebsiella pneumoniae*) were studied with broth microdilution (BMD) susceptibility, static time-kill assays and dynamic testing in a bladder infection model simulating a 3 g oral fosfomycin dose. Mueller-Hinton broth (MHB) with and without 25 mg/L glucose-6-phosphate (G6P), pooled midstream urine (MSU), pooled 24 h urine collection (24 U), artificial urine medium (AUM) and synthetic human urine (SHU) were compared. BMD susceptibility, bacterial growth and response to static fosfomycin concentrations in urine were best matched with SHU and were distinctly different when tested in MHB with G6P. Fosfomycin exposure in the bladder infection model was accurately reproduced (bias 4.7 ± 6.2%). Under all media conditions, 8 isolates (2-*E. coli*, 2-*E. cloacae*, 4-*K. pneumoniae*) re-grew and 4 isolates (4-*E. coli*) were killed. The remaining isolates (2-*E. coli*, 2-*E. cloacae*) re-grew variably in urine and synthetic media. Agar dilution MIC failed to predict re-growth, whereas BMD MIC in media without G6P performed better. Emergence of resistance was restricted in synthetic media. Overall, SHU provided the best substitute for urine for *in vitro* modelling of antimicrobial treatment of uropathogens, and these data have broader utility for improved preclinical testing of antimicrobials for urinary tract infections.

1. Introduction

Nutritional factors required for bacterial growth are well recognised to be less abundantly available *in vivo* compared to the standard laboratory media used in *in vitro* experiments (Mouton, 2018). Human urine has a diverse distribution of chemical constituents, with investigations into the urine metabolome identifying a total of 2651 metabolites or metabolite species (Bouatra et al., 2013). As a biological waste product, urine contains metabolic breakdown products, endogenous waste metabolites and bacterial by-products. It is nutritionally deplete and naturally antimicrobial with hypertonicity, low pH, relatively low oxygen content and high concentrations of nitrites and urea that would inhibit most bacteria (Ipe et al., 2016). Specific bacterial species, however, have unique adaptions in metabolism to promote their growth in human urine. Important traits in *Enterobacteriaceae* include upregulation of iron acquisition and osmoadaptive systems, intracellular accumulation of antioxidant defence mechanisms, the ability to use urine for the catabolism of amino acids to form TCA cycle intermediates and gluconeogenic substrates, and the synthesis of guanine-dependent products critical for bacterial survival in urine (Alteri et al., 2009; Ipe et al., 2016).

Given the importance of human urine to host-pathogen interactions and its potential impact on antimicrobial activity, ideally, human urine should be used for *in vitro* studies assessing the efficacy of antimicrobials against uropathogens. However, human urine is an impractical laboratory media due to logistical challenges in its collection, safe handling and sterilisation, whilst also having a highly variable chemical make-up between different samples and a short shelf-life. The

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https://doi.org/10.1016/j.mimet.2020.105861
Received 25 October 2019; Received in revised form 3 February 2020; Accepted 3 February 2020
Available online 05 February 2020
0167-7012/

impact of variability in urine composition between healthy people and those with underlying disease states is also uncertain. Here, we applied our dynamic *in vitro* bladder infection model to assess synthetic media alternatives that best mimic the impact of human urine on oral fosfomycin pharmacokinetics (PK) and pharmacodynamics (PD). This work has important implications for future *in vitro* efficacy testing of antimicrobials in the context of UTI therapeutics.

2. Materials and methods

2.1. Antibiotic and standard laboratory media

Fosfomycin ('Fomicyt', InfectoPharm, Germany) was reconstituted in water to a concentration of 50,000 mg/L and used for media production for susceptibility testing, time-kill assays and within the bladder infection model. Trypticase soy agar containing 5% sheep blood (TSA) (Becton Dickinson, USA) was used for sub-culturing isolates from freezer stocks. Mueller–Hinton II agar (MHA) (BD) was used to quantify bacterial counts, and, with fosfomycin added, used for agar dilution susceptibility testing and screening for fosfomycin resistance from bacterial density quantification samples. All solid media to which fosfomycin was added also contained 25 mg/L glucose-6-phosphate (G6P) (G7879-5G, Sigma-Aldrich, USA). Cation-adjusted Mueller–Hinton II broth (MHB) (BD), with or without 25 mg/L G6P, was used as the standard liquid laboratory media for all testing.

2.2. Pooled human urine

Pooled human urine was collected from healthy female volunteers following an approved ethical consent process (Medisch Ethische Toetsings Commissie, METC 2018-1186). Volunteers were not taking antibiotics (either currently, or in the last month), were free from any symptoms of a UTI, were not pregnant, and did not report a medical history of diabetes, kidney disease or nephrolithiasis. Urine was collected by two methods: a randomly timed, midstream urine collection (MSU), from which the entire volume from anonymously donated urine was pooled; and a 24 h urine collection (24 U) from 12 volunteers, which was pooled equally by volume (1000 mL from each volunteer). Pooled urine underwent a stepwise filtration process, using filter paper of increasing grade (Whatman filters, Sigma-Aldrich, USA) with particle filtration size of 25 μm, 7 μm and 3 μm, prior to filter sterilisation through a 0.22 μm filter system (Nalgene Rapid-Flow disposable filters, Thermo-Fisher Scientific, USA). The final pooled urine samples underwent basic biochemical testing including pH (Mettler Toledo MA235 pH/ion analyser), osmolality (Osmo Station OM-6050) and D-glucose (Roche Cobas 8000) measurements. Creatinine and urea (Roche Cobas 8000) were additionally measured on the 24 U sample, together with quantification of G6P using a high sensitivity enzymatic assay with a detection range 10–500 pmoles (MAK021, Sigma-Aldrich, USA). The pooled MSU sample was more dilute than the 24 U with a pH 7.0, osmolality 260 mOsm and glucose < 0.1 mmol/L in the MSU sample compared to a pH 6.5, osmolality 468 mOsm and glucose 0.2 mmol/L in the 24 U sample. Additionally, the 24 U sample demonstrated a creatinine of 7.5 mmol/L, urea 196.1 mmol/L and negligible amounts of G6P (0.2 mg/L).

2.3. Synthetic media alternatives

Two different synthetic urine media were tested (Brooks and Keevil, 1997; Ipe et al., 2016; Ipe and Ulett, 2016). The chemical ingredients for the artificial urine medium (AUM) (Brooks and Keevil, 1997) and the synthetic human urine (SHU) (Ipe and Ulett, 2016) are outlined in Table 1. D-glucose (18 mg/L) was added to the final SHU recipe to match the concentration found in the urine samples. The pH was unadjusted in the AUM (pH 6.5), whereas it was adjusted to pH 5.6 in the SHU. Precipitation of the AUM was noted after 48 h incubation, which was similarly found with the SHU if the pH was unadjusted.

Table 1
Chemical ingredients for synthetic human urine and artificial urine medium.

Chemical		g/L	
		SHU[a]	AUM[b]
Sodium chloride	NaCl	5.844	5.2
Sodium sulphate	Na_2SO_4	2.4147	3.2 (decahydrate)
Urea	Urea	16.8168	10
Potassium chloride	KCl	2.8329	–[c]
Calcium chloride	$CaCl_2$	0.4439	0.37 (dihydrate)
Creatinine	Creatinine	1.0181	0.8
Citric acid trisodium salt dihydrate	$Na_3C_6H_5O_7$	1.9999	–
Ammonium chloride	NH_4Cl	1.0698	1.3
Magnesium sulphate	$MgSO_4$	0.3852	0.49 (heptahydrate)
Sodium oxalate	$Na_2C_2O_4$	0.0241	–
Sodium phosphate monobasic	NaH_2PO_4	0.5616	–
Sodium phosphate dibasic	Na_2HPO_4	0.9227	–
Potassium dihydrogen phosphate	KH_2PO_4	2.1774	0.95
Uric acid	$C_5H_4N_4O_3$	0.1009	0.07
Sodium bicarbonate	$NaHCO_3$	1.1341	2.1
Magnesium chloride hexahydrate	$MgCl_2{\cdot}6H_2O$	0.6506	–
Lactic acid	$C_3H_6O_3$	0.0991	1.0
Ferrous sulfate heptahydrate	$FeSO_4{\cdot}7H_2O$	0.0014	0.0012
20% (*w/v*) casamino acids	–	0.1% (*v/v*)	–
Citric acid	$C_6H_8O_7$	–	0.4
Di-potassium hydrogen phosphate	HK_2PO_4	–	1.2
Yeast extract	–	–	0.005
Peptone L37	–	–	1.0

[a] Synthetic human urine (SHU) adjusted to pH 5.6. D-glucose (18 mg/L) was added to match the pooled 24 h urine sample.
[b] Artificial urine medium (AUM), pH 6.5.
[c] – indicates not included in that media recipe.

2.4. Bacterial strains and in vitro susceptibility

Sixteen clinical isolates were chosen to represent different uropathogen species with a range of baseline fosfomycin MIC values, originating from the Netherlands (Mouton et al., 2007). Isolates included 8 *Escherichia coli*, 4 *Enterobacter cloacae* and 4 *Klebsiella pneumoniae*. Species identification was confirmed by MALDI-TOF MS (Bruker Daltonik GmbH, Germany). Fosfomycin susceptibility was determined by agar dilution following the reference method (CLSI, 2012), by plating 10 μL of a freshly prepared bacterial suspension (containing 10^4 cfu/drop) onto MHA with doubling concentrations of fosfomycin from 0.25 to 1024 mg/L. Broth microdilution (BMD) was performed following reference methodology applicable to other antimicrobial agents (CLSI, 2012) with testing performed in MHB with and without G6P, 24 U and SHU. In short, a 96-well plate was filled with the different media and a fosfomycin concentration gradient of 0.125–1024 mg/L. Bacterial inocula were prepared to achieve a final inoculum of 5×10^5 cfu/mL in each well. MICs were read visually after 16–20 h incubation at 37 °C. All MIC testing was performed in triplicate. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 25983 were run as quality control organisms to ensure reproducibility between tests.

2.5. Static time-kill assay

In order to establish if SHU supported similar fosfomycin activity in urine, half of the test isolates (4 *E. coli*, 2 *E. cloacae*, *2 K. pneumoniae*) underwent static time-kill testing in 24 U and SHU, compared also to testing in MHB with G6P. Bacterial inocula were prepared from a fresh overnight culture, and a starting inoculum of approximately 4×10^5 cfu/mL in 10 mL of media was used. Fosfomycin was tested at

four different concentrations (8, 32, 128 and 512 mg/L) and a drug-free control tube. Time-kill assay (TKA) tubes were incubated at 37 °C with vigorous shaking (200 rpm). Samples for bacterial density were collected at 1, 3, 6 and 24 h incubation. To negate the effect of antibiotic carry-over (Eng et al., 1991), samples (500 μL) underwent centrifugation (5 min 12,500 ×*g*), removal supernatant and the pellet re-suspended with phosphate-buffered saline to the original pre-centrifuge volume. This centrifuge and washing process was performed twice prior to plating.

2.6. Dynamic bladder infection model

The *in vitro* bladder infection model was adapted from previous testing (Abbott et al., 2018). In short, the model enables the dynamic simulation of urinary fosfomycin exposure, on a 1:16 scale to *in vivo*, allowing the testing of sixteen independent bladder compartments in parallel. Supplied by a constant flow of fresh media, fosfomycin was administered into the first constant-volume compartment (simulating the gastrointestinal tract), from which media flows into the second constant-volume compartment (simulating systemic circulation) and then into each bladder compartment, simulating renal elimination of fosfomycin into the bladder. By applying drug distribution equations (Fig. A.1) (Rowe and Morozowich, 1969) and normal human urodynamics, dynamic urinary fosfomycin exposures following a single 3 g oral dose were simulated. Urinary fosfomycin PK targets were informed by healthy human data (C_{max} 1984 mg/L, T_{max} 7.5 h, and concentration > 128 mg/L for at least 40 h) (Wijma et al., 2018). Test isolates were added to each bladder compartment, at an inoculum of 10^7 cfu to provide an equivalent total number of bacteria expected in human infections (*i.e.* 10^5 cfu/mL in an average 250 mL void). A drug-free growth control was performed in each media from the starting inoculum and incubated under static conditions at 37 °C for 24 h. Post-fosfomycin exposure growth outcomes were assessed in standard laboratory media, pooled human urine and synthetic alternatives. Pathogen kill and emergence of resistance were determined over 72 h. Samples for bacterial counts were taken directly from each bladder compartment at regular timepoints and were plated onto drug-free MHA, and MHA with 64 mg/L fosfomycin (low-level resistance, LLR) and 512 mg/L of fosfomycin (high-level resistance). The lower limit of detection was considered to be 50 cfu/mL. All plates were incubated aerobically at 37 °C for 16–20 h. Plates supplemented with fosfomycin were re-incubated for a further 24 h to confirm colony counts.

2.7. Measurement of fosfomycin concentration

Samples for fosfomycin quantification were assessed using an ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method (Wijma et al., 2017). Samples were taken directly from all bladder compartments at the peak concentration (C_{max}) timepoint and from three representative bladder compartments at two timepoints prior to, and after the $C_{max,}$ providing measured PK parameters for exposure-response analyses and an assessment of inter-compartment variation. Observed fosfomycin concentrations were compared to the target as determined by the mathematical model (Appendix Fig. A.1). The UPLC-MS/MS method was validated for urine and plasma samples of fosfomycin, but additional tests confirmed its applicability in MHB, AUM and SHU samples. The method was validated according to FDA guidelines for bioanalytical method validations (FDA, 2013), over a range of 0.75–375 mg/L (R^2 0.9998). The lower limit of quantification was 0.75 mg/L and the lower limit of detection was 0.70 mg/L. The method was found to be accurate and precise with a maximum deviation of 5.0%. The stability of fosfomycin at 37 °C for 72 h, and stored at −80 °C for at least 6 months, was confirmed.

2.8. Statistical and PK/PD analyses

Bias between MIC susceptibility measurements by BMD compared to agar dilution was calculated using the method described by Bland and Altman (1995) on log_2-transformed MIC data. For the liquid media used in BMD the bias was calculated as the difference against the average of the log_2 MIC measurements by BMD and agar dilution. A positive bias value quantified the degree that the BMD method overestimated the MIC compared to agar dilution with a 95% confidence interval. Fosfomycin activity from the static time-kill curves was assessed by the area under the time-kill curve ($AUTKC_{0–24}$) plotted against the ratio of the fosfomycin concentration and the agar dilution MIC, with a non-linear regression analyses using a variable slope E_{max} model and curves compared statistically using the F test. Quantified dynamic fosfomycin concentrations in the bladder infection model was assessed by linear regression and Bland-Altman plots in terms of bias and precision compared to the target values. The exposure-response relationship was assessed by the ratio of the measured peak free-drug concentration to the pathogen MIC (??C_{max}/MIC) and the change in bacterial count. In this PK/PD analysis, MIC measurements by agar dilution were compared to measurements obtained by BMD performed in either MHB, MHB with G6P, 24 U or SHU. Analysis of the dynamic testing was performed by non-linear regression using a variable slope E_{max} model with the top parameter corresponding to the maximal bacteria counts in drug-free control. Where appropriate data were presented as means (± SD). All analyses were performed with GraphPad Prism (version 7.0b, MAC OS X).

3. Results

3.1. In vitro susceptibility and static time-kill assay

For the 16 bacterial isolates used in this study, the agar dilution MIC values ranged from ≤0.25 to 64 mg/L (Table 2). When tested by BMD in MHB (with and without G6P), pooled urine (24 U) and SHU, values were all generally higher compared to agar dilution (Fig. 1). Without G6P supplementation, MIC values increased further in all isolates, except two *E. coli* isolates (strains 4807 and 4757) where there was no potentiation in fosfomycin activity. Susceptibility results in urine were best matched with testing in SHU, with an average bias of one MIC dilution higher in SHU when compared to testing in urine (95% CI: −1.0 to 3.1), excluding *E. coli* 4807 that displayed insufficient growth in SHU to allow visual assessment of the MIC.

Fosfomycin activity using static time-kill assays was significantly different in 24 U compared to MHB with G6P for the *E. cloacae* and *K. pneumoniae* isolates tested (Fig. 2). Exposure-response curves were shifted to the right, with higher EC_{50} values demonstrated in 24 U compared to MHB with G6P (*E. cloacae*: EC_{50} 22.9 in 24 U, 2.7 in MHB with G6P, $p \leq .0001$; *K. pneumoniae*: EC_{50} 70.0 in 24 U, 4.1 in MHB with G6P, $p = .0001$). Testing in SHU closely matched the results in 24 U, without significant differences between the non-linear regression curves. In contrast, testing in *E. coli* isolates did not demonstrate significant differences between the three media types, and non-linear regression curves were unable to elicit well-matched exposure-response relationships for testing in urine and SHU ($R^2 \leq 0.6$), limiting comparative analyses.

3.2. Growth response in the bladder infection in vitro model

Dynamic fosfomycin concentrations in the bladder infection model closely matched the simulation with a bias of 4.7% (95% CI: −7.4 to 16.8%) and the slope of the linear regression line equal to 1.1 (R^2 0.96, Fig. 3). There was minimal variation of fosfomycin concentrations between bladder compartments with an average relative standard deviation of 4.4 ± 3.0%. Following fosfomycin exposure in the six different media conditions, the same 8-isolates (2 *E. coli*, 2 *E. cloacae*, 4 *K.*

Table 2
Baseline fosfomycin susceptibility.

Strain	Pre-exposure fosfomycin MIC (mg/L)[a]				
	Agar dilution	Broth microdilution			
		MHB+G6P	MHB	24U	SHU
E. coli					
41	≤0.25 (≤0.25 – 0.5)	0.5 (0.5 – 4)	32 (4 – 64)	16 (16)	16 (16 – 32)
11	0.5 (0.5)	1 (1 – 2)	16 (8 – 16)	8 (8 – 16)	16 (16)
39	0.5 (0.5)	2 (2 – 8)	32 (32 – 64)	8 (8 – 16)	64 (32 – 64)
12620	2 (2 – 4)	4 (4 – 8)	16 (16 – 32)	4 (4 – 8)	16 (16)
1016	16 (16)	64 (64 – 128)	>1024 (>1024)	512 (256 – 1024)	512 (512 – 1024)
1231	32 (16 – 32)	1024 (256 – >1024)	>1024 (>1024)	256 (128 – 512)	1024 (512 – 1024)
4807	32 (32)	64 (32 – 64)	64 (64 – 128)	16 (8 – 16)	-[b]
4757	64 (64)	128 (128)	128 (128 – 256)	16 (16)	32 (32)
E. cloacae					
35166	0.5 (0.5 – 2)	1 (1 – 2)	32 (32)	8 (8 – 16)	16 (16 – 32)
94	1 (1)	2 (2 – 4)	8 (8 – 16)	4 (4)	4 (4)
21	8 (8)	32 (32 – 128)	256 (256)	256 (256)	256 (256)
32	32 (16 – 32)	64 (32 – 64)	512 (512 – 1024)	1024 (512 – 1024)	512 (512)
K. pneumoniae					
34672	2 (1 – 8)	8 (8 – 16)	512 (256 – 1024)	256 (128 – 256)	512 (256 – 512)
31865	2 (2)	8 (4 – 8)	256 (256)	64 (64)	256 (256)
55	4 (4 – 8)	16 (8 – 32)	1024 (1024 – >1024)	128 (128 – 256)	256 (256 – 512)
52	16 (16)	32 (32)	> 1024 (512 – >1024)	256 (256 – 512)	1024 (1024)
Control organism: *E. coli* ATCC					
25922	0.5 (0.5 – 1)	1 (0.5 – 1)	64 (32 – 64)	8 (8 – 16)	32 (32 – 64)

[a]All MIC measurements determined in triplicate, median (range). Values > 32 mg/L are highlighted in grey.
[b]Insufficient growth in BMD plate resulted in an unrecordable an MIC value.

pneumoniae) re-grew and the same 4-isolates (4 *E. coli*) were eradicated (Table 2 and Fig. 4). The remaining 4-isolates (2 *E. coli*, 2 *E. cloacae*) had variable low-level re-growth (≤ 4.7 $\log_{10}$ cfu/mL) in urine and the synthetic media. Despite similar total population two-$\log_{10}$ kill kinetics, the emergence of fosfomycin resistance varied between the different media. For *E. coli* 1016 and 1231, post-exposure HLR population replacement occurred when tested in MHB with G6P and both pooled urine samples. However, testing in MHB without G6P and both synthetic media did not support the same degree of emergence of HLR (Table 2; Fig. 4). *E. cloacae* isolates only displayed population replacement with HLR when tested in MHB (with or without G6P). For *K. pneumoniae* isolates, emergence of resistance was lowest when tested in the synthetic media, restricted in the urine samples, and most apparent in MHB without G6P (Table 2; Fig. 4). A rise of the fosfomycin MIC in

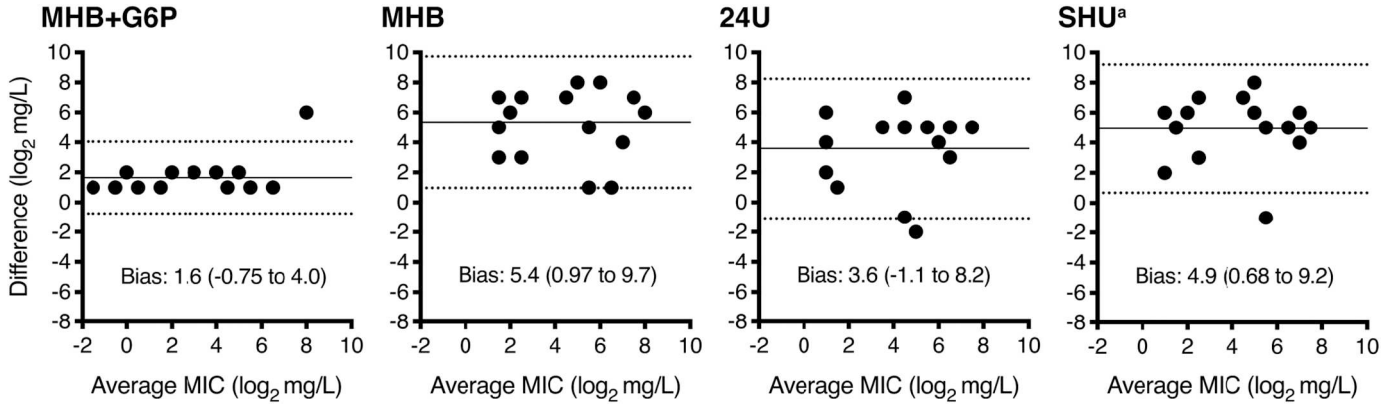
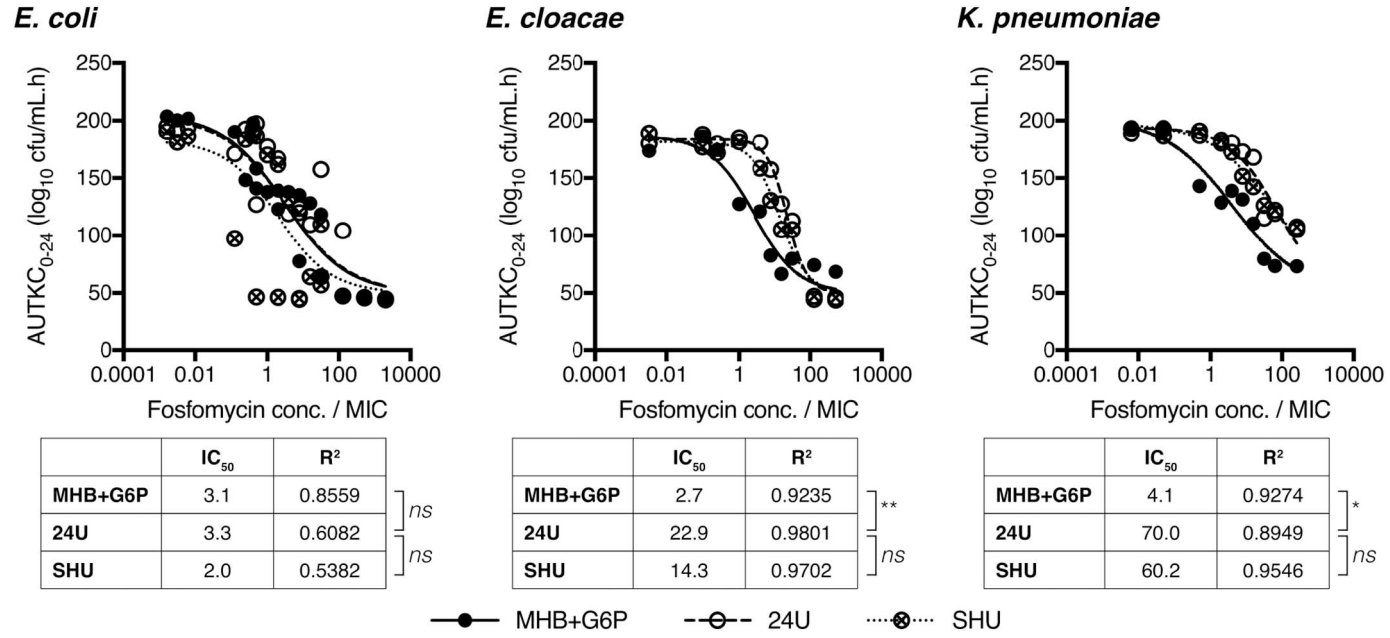


Fig. 1. Agar dilution fosfomycin MIC compared to broth microdilution performed in different media.
Bland-Altman plots of the difference in MIC between the two methods on the *y*-axis, and the average of the measurements from the two methods on the *x*-axis, with the bias presented as a solid line and the 95% confidence intervals as dotted lines. Testing in Mueller-Hinton broth (MHB) with and without 25 mg/L glucose-6-phosphate (G6P), 24 h pooled urine (24 U) and synthetic human urine (SHU).
[a]Excluding *E. coli* 4807 that failed to grow sufficiently in SHU to measure a MIC.



	IC50	R²	
MHB+G6P	3.1	0.8559	ns
24U	3.3	0.6082	ns
SHU	2.0	0.5382	

	IC50	R²	
MHB+G6P	2.7	0.9235	**
24U	22.9	0.9801	ns
SHU	14.3	0.9702	

	IC50	R²	
MHB+G6P	4.1	0.9274	*
24U	70.0	0.8949	ns
SHU	60.2	0.9546	

Fig. 2. Fosfomycin exposure-response in different media from static time-kill assays.
Graphs relate to the area under the time-kill curve ($AUTKC_{0–24}$) compared to the fosfomycin exposure, normalised to the agar dilution MIC of the pathogen. Mueller-Hinton broth with glucose-6-phosphate (MHB + G6P, solid circles), pooled 24 h female urine (24 U, open circles) and synthetic human urine (SHU, crossed circles). Time-kill curves are presented in appendices Fig. A.2. *ns*, not significant. $^{*}p = .0001$. $^{**}p < .0001$. Note that the equally poor E_{max} non-linear regression curves derived from the *E. coli* analyses in SHU and 24 U limit the comparative analyses.

the post-exposure total population re-growth was also reduced in the synthetic media.

3.3. Dynamic exposure-response relationship

MIC testing with G6P supplementation, either by agar dilution or BMD, failed to predict outcome in the *in vitro* model (Fig. 5). When applying agar dilution MIC values, fosfomycin exposure (C_{max}/MIC) was unrelated to the change in bacterial count when the model was run with all six different media (R^2 ranged from 0.11 to 0.38). Notably, all *K. pneumoniae* isolates re-grew despite having low agar dilution MIC values (Table 3). Similarly, BMD MIC values when measured in MHB with G6P failed to correlate exposure to outcome when the model was run in MHB with G6P (R^2 0.30). In contrast, for the remaining media conditions, MIC testing by BMD in the same media that the *in vitro* model was run with, was able to better predict the exposure-response relationship (R^2 range 0.85–0.98). Assuming a urinary C_{max} 2000 mg/L is achieved, applying the calculated EC_{50} values, post-exposure isolate re-growth was predicted in isolates with a BMD MIC in MHB > 128 mg/L, MIC in 24 U > 32 mg/L, and MIC in SHU > 64 mg/L.

4. Discussion

The translation of *in vitro* results to clinical infections is of paramount importance to guide rational treatment recommendations. We demonstrate that fosfomycin susceptibility and antimicrobial activity

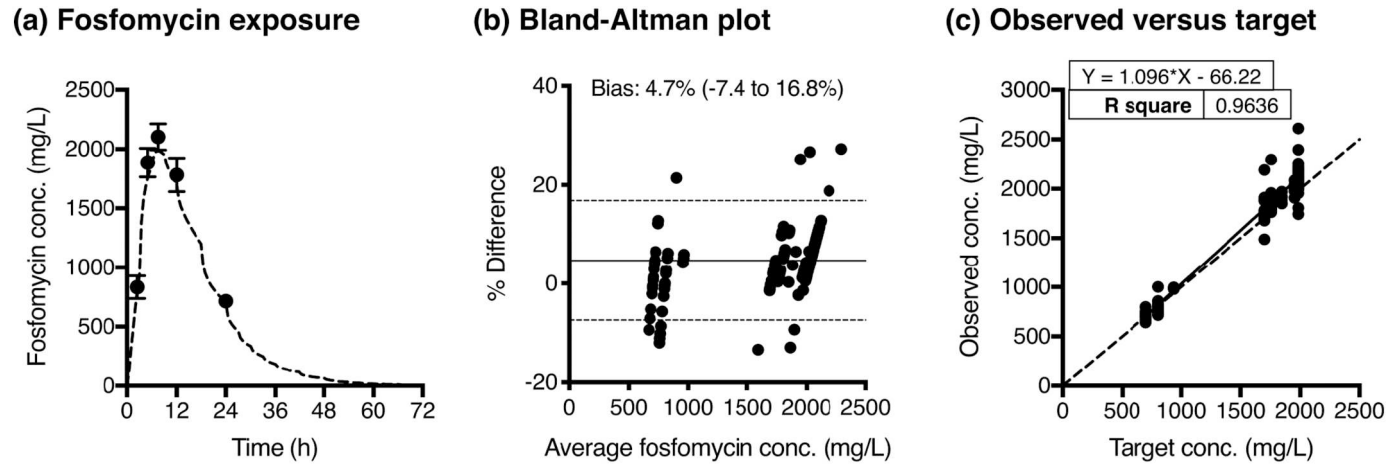


Fig. 3. Observed fosfomycin concentrations in the *in vitro* bladder compartments following a single dose of fosfomycin.
(a) Average fosfomycin concentrations measured from all PK samples overlaid on the target concentration curve (dashed line). (b) Bland-Altman plot of the percentage difference of the observed and target fosfomycin concentration measurements (y-axis) and the average of the two measurements (x-axis) presented with the bias (solid line) and 95% limits of agreement (dotted lines). (c) Accuracy of observed fosfomycin concentrations compared with the target with linear regression (solid line) and $y = x$ (dashed line). Note: the mathematically simulated urinary concentration curve in (a) do not demonstrate a smooth drug elimination phase due to the dynamic fluid shifts that occur after each voiding cycle of the bladder compartment.

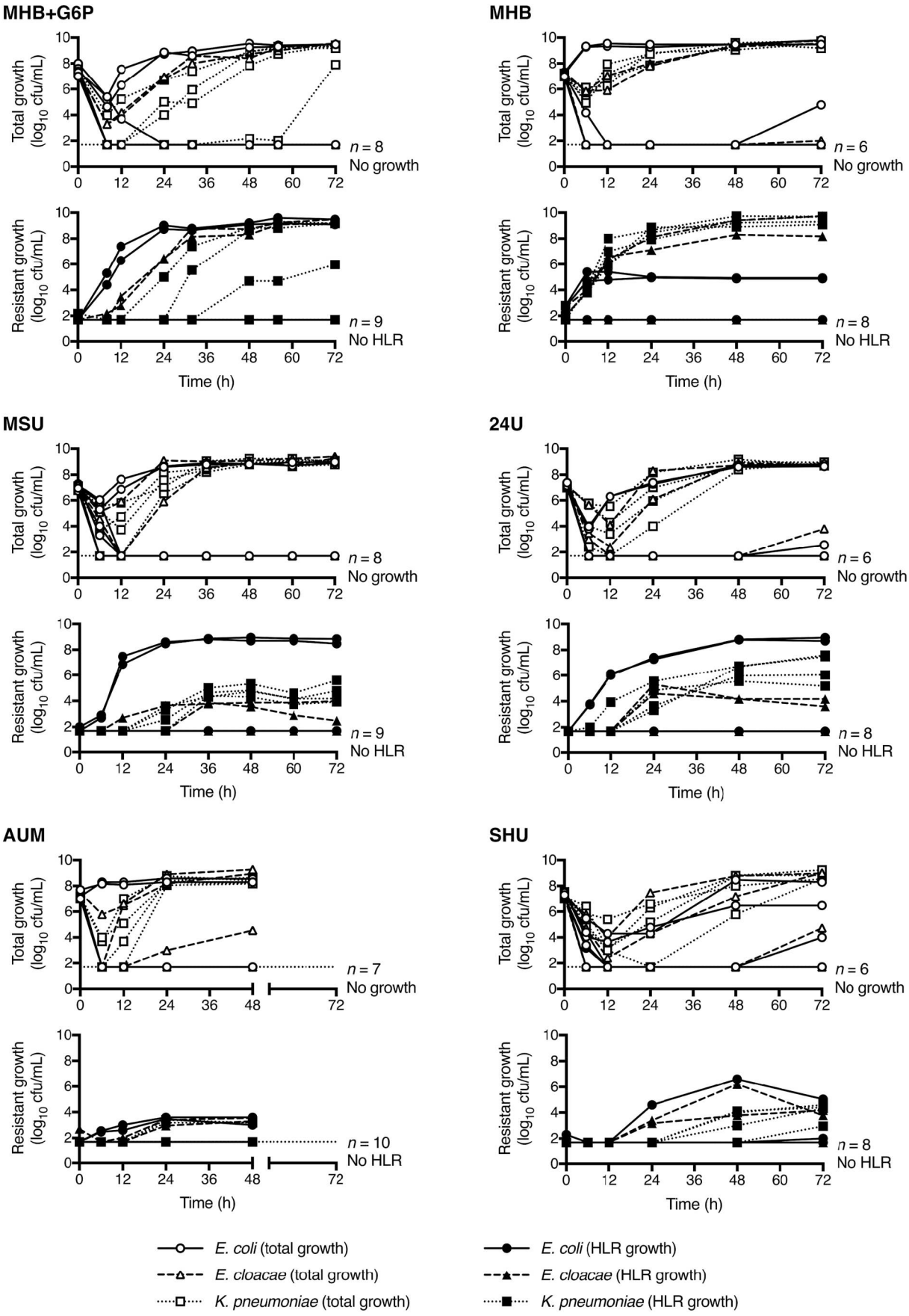


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Fig. 4. Growth response in different media following fosfomycin exposure in the bladder infection *in vitro* model.
Limit of detection (dotted line) was considered 50 cfu/mL. MHB + G6P, Mueller-Hinton broth with 25 mg/L glucose-6-phosphate. MSU, pooled mid-stream urine collection. 24 U, pooled 24 h urine collection. AUM, artificial urine medium. SHU, synthetic human urine. Note that testing in AUM was complicated by media precipitation interrupting media flow, so bacterial counts were assessed only up to at 48 h.

are greatly affected by the media environment, including within the biomatrix of urine that is most relevant for UTIs. Compared to nutrient-rich laboratory media, bacterial growth capacity in pooled female urine was restricted and fosfomycin activity was reduced. Synthetic alternatives supported similar growth capacity and reflected comparable fosfomycin activity to that found in urine. Post-exposure emergence of resistance was, however, restricted in the synthetic urine alternatives. Overall, SHU appears to be a reasonable substitute for human urine for UTI *in vitro* modelling.

The assessment of antimicrobial activity and the prediction of likelihood of clinical success is a complex process involving the characteristics of the microorganism, bacterial adaptations, antimicrobial chemistry, and dynamic pharmacokinetics and host factors at the *in vivo* site of infection. In a recent clinical study, a clinical failure rate of oral fosfomycin for the treatment of uUTIs was reported at 42%, despite minimal fosfomycin resistance being reported among the cultured urinary isolates (Huttner et al., 2018). Despite the MIC of an isolate being a highly standardised, reference value for susceptibility of bacteria to an antimicrobial agent, the MIC is not an intrinsic property of a microorganism, but rather an artificially generated value in a nutritionally rich *in vitro* environment (Mouton, 2018). This value factors in the antimicrobial kill rate, the isolate growth rate and the starting inoculum (Mouton et al., 2018a, 2018b; Mouton and Vinks, 2005). Given a favourable *in vitro* environment, an MIC could underestimate the effect *in vivo* where conditions could be either disadvantageous for the pathogen.

Importantly, the only recommended methodology for fosfomycin MIC determination requires testing by agar dilution with the addition of 25 mg/L G6P (Greenwood et al., 1986). Although, more recent reports have demonstrated a poor correlation with agar dilution fosfomycin

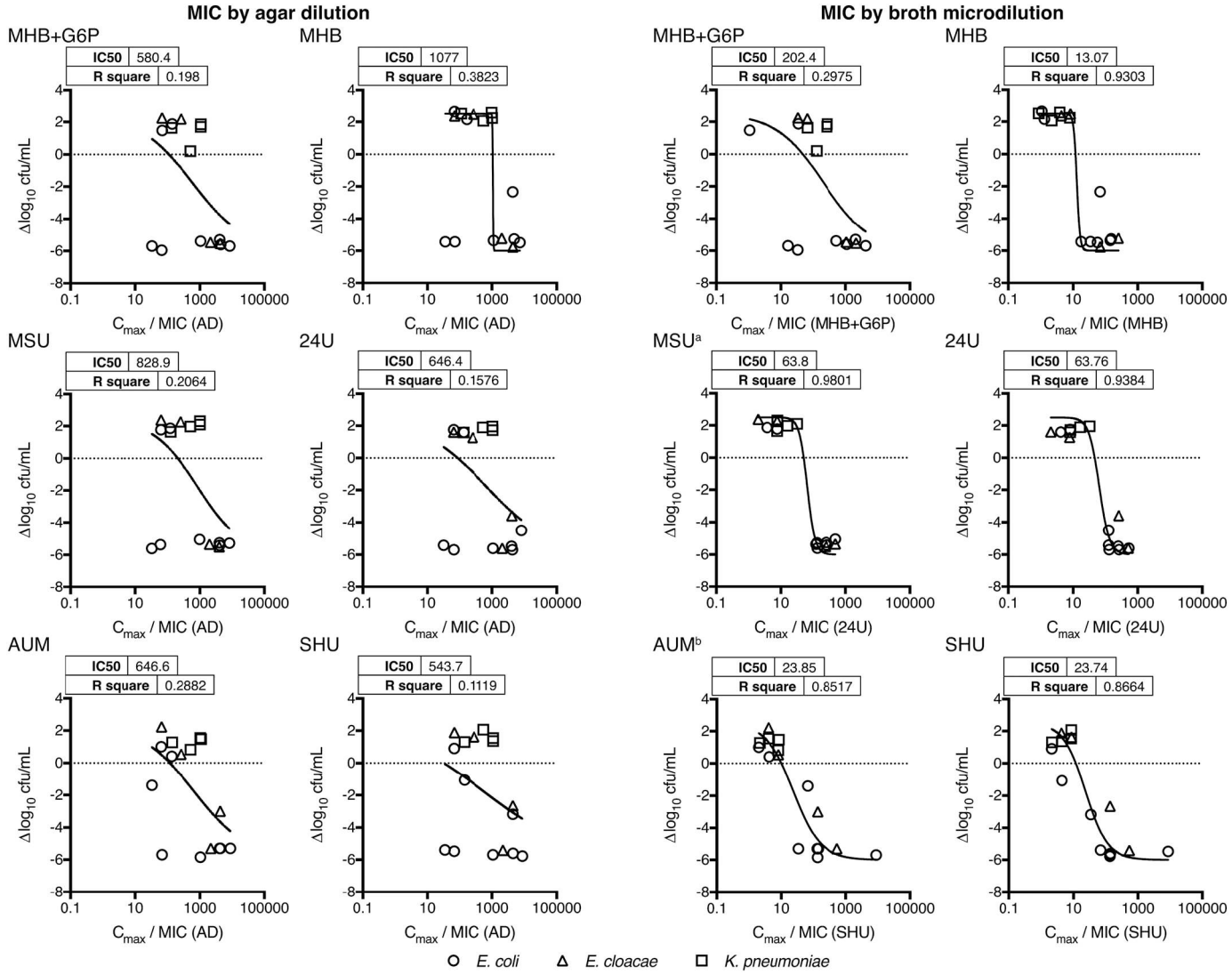

Fig. 5. Exposure-response relationship.
Change in bacterial counts of *E. coli* (circles), *E. cloacae* (triangles) and *K. pneumoniae* (squares), assessed 72 h after fosfomycin administration in the bladder infection *in vitro* model run with different media conditions: Mueller-Hinton broth (MHB) with and without 25 mg/L glucose-6-phosphate (G6P), pooled mid-stream urine collection (MSU), pooled 24 h urine collection (24 U), artificial urine medium (AUM), and synthetic human urine (SHU). [a]BMD MIC in 24 U values used. [b]BMD MIC in SHU values used. Note that testing in AUM was complicated by media precipitation interrupting media flow, so final bacterial counts were assessed at 48 h.

Table 3
Growth outcomes in the dynamic bladder infection *in vitro* model.

Strain	Post-exposure outcome[a] Bacterial count (log_{10} cfu/mL); HLR proportion (%)[b]; MIC (mg/L)					
	Laboratory media		Pooled human urine		Synthetic urine alternative	
	MHB + G6P	MHB	MSU	24U	AUM[c]	SHU
E. coli						
41	-[d]	-	-	2.5 / **0.5**	-	-
11	-	-	-	-	-	-
39	-	4.7 / **0.5**	-	-	-	4.0 / **0.5**
12620	-	-	-	-	-	-
1016	9.5^{+++} / **256**	9.5^{+} / **16**	9.0^{+++} / **>1024**	8.7^{+++} / **>1024**	8.3^{+} / **16**	6.5^{+} / **32**
1231	9.5^{+++} / **>1024**	9.3^{+} / **16**	8.9^{+++} / **>1024**	8.8^{+++} / **>1024**	8.5^{+} / **16**	8.3^{++} / **32**
4807	-	-	-	-	-	-
4757	-	-	-	-	-	-
E. cloacae						
35166	-	-	-	3.8 / **0.5**	4.5 / **256**	4.7 / **1**
94	-	2.0 / **1**	-	-	-	-
21	9.5^{+++} / **>1024**	9.8^{+++} / **>1024**	9.3^{+} / **16**	8.7^{+} / **32**	9.3^{+} / **8**	9.0^{+} / **16**
32	9.5^{+++} / **>1024**	9.5^{+++} / **>1024**	9.4^{+} / **64**	8.8^{+} / **32**	9.0^{+} / **32**	9.0^{+} / **32**
K. pneumoniae						
34672	9.3^{+++} / **>1024**	9.5^{+++} / **64**	9.1^{+} / **128**	8.7^{+} / **128**	8.7 / **2**	8.5^{+} / **128**
31865	9.4^{++} / **256**	9.5^{+++} / **>1024**	8.9^{+} / **128**	8.9^{+++} / **256**	8.2 / **64**	8.5^{+} / **8**
55	6.9 / **4**	9.5^{+++} / **1024**	9.0^{++} / **256**	9.0^{++} / **128**	8.4^{+} / **4**	9.2^{+} / **64**
52	9.2^{+++} / **>1024**	9.7^{+++} / **>1024**	8.8^{++} / **128**	8.7^{++} / **512**	8.2^{+} / **32**	8.8^{+} / **16**

[a]Post-exposure outcome determined as growth, or no growth, 72 h after fosfomycin administration. Dark grey highlights where regrowth occurred with a rise in the total population MIC (value presented in bold). Light grey highlights where regrowth occurred without a significant rise in MIC.
[b]The HLR proportion of the total population is presented as greater than 1% (+++), between 0.01 and 1% (++), or less than 0.01% (+).
[c]Growth outcome assessed after 48 h due to media precipitation interrupting media flow.
[d]– indicates no growth detected.

MIC and efficacy (Abbott et al., 2019; Ballestero-Tellez et al., 2017a, 2017b; Seroy et al., 2016). G6P acts as an inducer of the hexose phosphate transport pathway to increase the intracellular concentration of fosfomycin in *Enterobacteriaceae*, but not in *Pseudomonas aeruginosa* or *Enterococcus* spp., both of which lack the UhpT transporter (Castaneda-Garcia et al., 2009; Silver, 2017). There are no physiological reasons for the addition of G6P to the media, and the potentiation of G6P on fosfomycin activity appears to vary between different bacterial strains (Greenwood, 1990; Greenwood et al., 1987; Greenwood et al., 1986). We documented negligible amounts of G6P found in human urine. Furthermore, we demonstrated variable changes in fosfomycin MIC in liquid media without G6P supplementation, and results in 24 U and SHU demonstrated greater variability between isolates compared to agar dilution, reflecting distinctive pathogen responses within these humanised media environments. Interestingly, exposure-response relationships in the bladder infection model were improved when applying MIC values obtained in the same media (without G6P supplementation) in which the model was run. These findings question the utility of the reference fosfomycin susceptibility method, with the additional of G6P, for predicting fosfomycin efficacy in UTIs.

Fosfomycin activity is negatively affected by a high inoculum (Greenwood et al., 1986; Martin-Gutierrez et al., 2018), and the higher inoculum used in BMD MIC testing has been suggested as the reason for MIC discrepancies with respect to agar dilution, due in part due to the enrichment of the starting inoculum with resistant subpopulations (Ballestero-Tellez et al., 2017b). Similarly, the initial bacterial inoculum added to the bladder infection model is higher than that used for susceptibility testing, relevant to the clinical syndrome of UTIs. The total number of bacteria added to the *in vitro* model is vital for the assessment of the suppression of the amplification of a pre-existing less susceptible population (Bulitta et al., 2019). Interesting, despite adding the same number of bacteria into the bladder infection model with each experiment, following the same fosfomycin exposure, emergence of resistance was variable dependent on the media, despite largely concordant total population re-growth kinetics. The synthetic media supported the least amount of resistance. This discrepant bacterial response to fosfomycin exposure, while exposed in a nutrient-restricted environment, may be uncovering a fitness cost limiting the proliferation of the fosfomycin-resistant subpopulation. Alternatively, this response may reflect a bacterial evolutionary drive towards persistence, instead of emergence of resistance, which has been reported as an important alternative explanation for antimicrobial failure (Balaban et al., 2019; Brauner et al., 2016; Gutierrez et al., 2017; Levin-Reisman et al., 2019; Wilmaerts et al., 2019). Supporting this theory, in a zebrafish larva infection model, clinical *E. coli* urinary isolates have been shown to be able to transition to a cell-wall deficient form (L-form) when exposed to

fosfomycin in urine, and then transition back to a walled state following antibiotic withdrawal (Mickiewicz et al., 2019).

Fosfomycin activity has been reported to be enhanced in an acidic environment (Fedrigo et al., 2017; Martin-Gutierrez et al., 2018). In this study, the pH of the collected pooled urine was between 6.5 and 7.0, compared to a pH of 5.6 in the SHU. We did not, however, demonstrate enhanced fosfomycin activity in SHU compared to urine. Interestingly, the high concentrations of phosphate buffers and sodium chloride included in the synthetic media may have had additional chemical influences limiting fosfomycin activity. Fosfomycin activity has also been reported to be enhanced under anaerobic culture conditions (Martin-Gutierrez et al., 2018). Urine contained within the human bladder contains a relatively low dissolved oxygen content (urinary PO_2 approximately 40 mmHg) (Aukland and Krog, 1960; Evans et al., 2014; Giannakopoulos et al., 1997; Leonhardt and Landes, 1963; Sgouralis et al., 2016), although the impact on fosfomycin activity and pathogen outcome by running the *in vitro* model at atmospheric aerobic conditions is uncertain. Therefore, the current set-up of the model represents a limitation in its design to accurately reflect the reduced oxygen environment *in vivo*.

The strengths of this study are the use of multiple media types and the application of a dynamic UTI simulation, applying normal urodynamics and dynamic urinary fosfomycin exposures. However, there are limiting factors in translating our results to the actual *in vivo* environment. The *in vitro* model lacks the tissue structure of the bladder and host factors such as the immune system. Also, the pooled urine samples were required to be filter sterilised prior to used. This process would likely remove some of the important innate antimicrobial properties of urine. Alternative methods for sterilisation proved suboptimal. Sterilisation by autoclaving resulted in precipitation, and sterilisation by gamma-irradiation resulted in delays without refrigeration and fouling of the urine that resulted alkalisation and failure of the urine to support bacterial growth. Furthermore, use of urine donated by healthy volunteers may not be reflective of those patients who are unwell.

This research highlights the importance of the media environment in the assessment of antimicrobial activity in PK/PD experiments for UTIs. We demonstrate that SHU is a good surrogate for human urine, providing a nutritionally-deplete media in which bacterial growth kinetics and fosfomycin activity was studied. Further work would be required to support the use of SHU with other uropathogens, and its applicability to the study of other antimicrobial agents used in the treatment of UTIs.

Funding

This work was supported in part by the AIDA project LifeSciHealth Priority of the European Commission Seventh Framework Programme, FP7 (Preserving Old Antibiotics for the Future, grant number F3-2011-278348). I. J. A. was funded by an Australian Government Research Training Program Scholarship (APP1114690) from the National Health and Medical Research Council of Australia. A. Y. P. was part funded through an Australian National Health and Medical Research Council Practitioner Fellowship (APP1117940).

Acknowledgements

We would like to acknowledge the laboratory assistance of Carla Roodbol de Goeij (Department of Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre) and Alex Brouwer and Geert Springeling (Department of Experimental Medical Instrumentation, Erasmus Medical Centre) for their assistance in the *in vitro* equipment design and production.

Transparency declarations

J. W. M. has received research funding from Adenium, AstraZeneca, Basilea, Cubist, Polyphor, Roche, Eumedica, Basilea, VenatorX, AiCuris, Gilead and Wockhardt. A.Y·P has received research funding from MSD through an investigator-initiated research project. All other authors: none to declare.

Appendix

(a) *In vivo*

Drug in G.I. tract:

$$X_{GI} = X_{dose} \cdot e^{-k_1 \cdot t}$$

Drug in blood:

$$X_{blood} = \frac{k_1 \cdot X_{dose}}{k_2 - k_1} \cdot \left(e^{-k_1 \cdot t} - e^{-k_2 \cdot t} \right)$$

Excreted drug:

$$X_{bladder} = X_{dose} - X_{GI} - X_{blood}$$

(b) *In vitro*

Drug in first compartment:

$$X_A = X_{A^\circ} \cdot e^{-(F/V_A)t}$$

Drug in second compartment:

$$X_B = \frac{(F/V_A) \cdot X_{A^\circ}}{F/V_B - F/V_A} \cdot \left(e^{-(F/V_A)t} - e^{-(F/V_B)t} \right)$$

Cumulative drug in third compartment:

$$X_C = X_{A^\circ} - X_A - X_B$$

Fig. A.1. Drug distribution equations informing target urinary fosfomycin concentrations.
The dynamic amount of drug (X mg) in each respective compartment at time t (h) as a function of the first-order rate constants (absorption k_1; elimination k_2). The initial dose of fosfomycin (mg) is indicated by X_{dose} or X_{A°. In the *in vitro* equations the fluid volumes (V mL) in the respective compartments and flow rate of fluid (F mL/h) are variables.

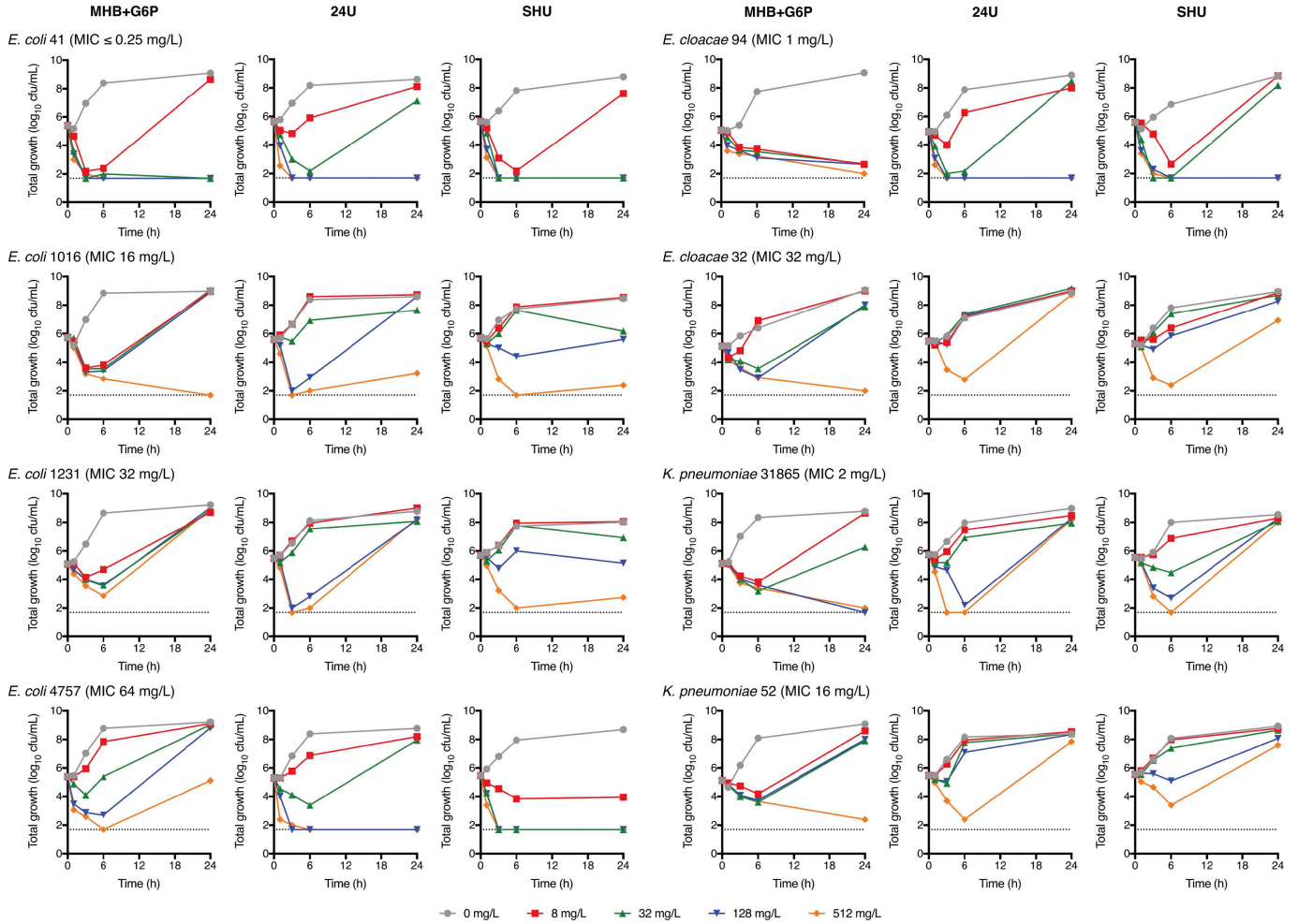

Fig. A.2. Static time-kill assays.
Static time-kill assays in Mueller-Hinton broth with glucose-6-phosphate (MHB + G6P), pooled 24 h female urine (24 U) and synthetic human urine (SHU). Each isolate was exposure to the same fosfomycin concentrations, namely drug free control (grey circle), 8 mg/L (red square), 32 mg/L (green upward triangle), 128 mg/L (blue downward triangle), 512 mg/L (orange diamond). The limit of detection was considered 50 cfu/mL (dotted line).

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Chapter 6

Fosfomycin efficacy against *Pseudomonas aeruginosa*

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The translatability from preclinical *in vitro* models to humans relies on creating the most accurate simulation possible of pathogen growth and antimicrobial activity under a humanised situation. The previous paper highlighted an important finding that fosfomycin activity is affected by the *in vitro* environment. Synthetic human urine (SHU) was shown to be a good substitute for human urine.

In this next paper, published in *Journal of Antimicrobial Chemotherapy*, fosfomycin efficacy is examined against *Pseudomonas aeruginosa* urinary isolates. Here the bladder infection model in run with SHU and sixteen clinical *P. aeruginosa* isolates examined. Both single dose and seven, daily doses of oral fosfomycin was simulated.

Highlights

- With the lack of available oral antimicrobials active against *P. aeruginosa*, beyond that of the fluoroquinolone class, oral fosfomycin is a potentially attractive therapeutic option.

- The bladder infection *in vitro* model was able to run continuously for 9-days of testing and successful in simulating of 7 doses of fosfomycin given daily.

- Prior to exposure, all *P. aeruginosa* isolates had a *fosA* gene detected and the majority had a detectable resistant subpopulation.

- Following administration, fosfomycin was ineffective at eradicating *P. aeruginosa* isolates.
    - Baseline MIC ≥ 8 mg/L with a high-level resistant subpopulation was predictive of post-exposure emergence of resistance, even following multiple doses.

- Extending drug exposure over 7-days did not improve efficacy, and in fact worsened the emergence of fosfomycin-resistance.

J Antimicrob Chemother 2020; **75**: 1879–1888
doi:10.1093/jac/dkaa127 Advance Access publication 3 May 2020

Journal of Antimicrobial Chemotherapy

Efficacy of single and multiple oral doses of fosfomycin against *Pseudomonas aeruginosa* urinary tract infections in a dynamic *in vitro* bladder infection model

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Received 6 January 2020; returned 25 February 2020; revised 7 March 2020; accepted 11 March 2020

Objectives: We used a dynamic bladder infection *in vitro* model with synthetic human urine (SHU) to examine fosfomycin exposures to effectively kill, or prevent emergence of resistance, among *Pseudomonas aeruginosa* isolates.

Methods: Dynamic urinary fosfomycin concentrations after 3 g oral fosfomycin were simulated, comparing single and multiple (daily for 7 days) doses. Pharmacodynamic response of 16 *P. aeruginosa* (MIC range 1 to >1024 mg/L) were examined. Baseline disc diffusion susceptibility, broth microdilution MIC and detection of heteroresistance were assessed. Pathogen kill and emergence of resistance over 72 h following a single dose, and over 216 h following daily dosing for 7 days, were investigated. The *f*AUC$_{0-24}$/MIC associated with stasis and 1, 2 and 3 $\log_{10}$ kill were determined.

Results: Pre-exposure high-level resistant (HLR) subpopulations were detected in 11/16 isolates after drug-free incubation in the bladder infection model. Five of 16 isolates had >2 $\log_{10}$ kill after single dose, reducing to 2/16 after seven doses. Post-exposure HLR amplification occurred in 8/16 isolates following a single dose and in 11/16 isolates after seven doses. Baseline MIC $\geq$8 mg/L with an HLR subpopulation predicted post-exposure emergence of resistance following the multiple doses. A PK/PD target of *f*AUC$_{0-24}$/MIC >5000 was associated with 3 $\log_{10}$ kill at 72 h and 7 day-stasis.

Conclusions: Simulated treatment of *P. aeruginosa* urinary tract infections with oral fosfomycin was ineffective, despite exposure to high urinary concentrations and repeated daily doses for 7 days. Emergence of resistance was observed in the majority of isolates and worsened following prolonged therapy. Detection of a baseline resistant subpopulation predicted treatment failure.

Introduction

Fosfomycin trometamol is a recommended first-line agent for uncomplicated urinary tract infections (uUTIs) as a single 3 g oral dose,[1] active against MDR uropathogens, including *Pseudomonas* spp.[2] Although uUTIs are commonly caused by *Escherichia coli* (in approximately 75% of cases), *Pseudomonas* spp. are seen in 2%–4% of cases, with a higher proportion in complicated UTIs, hospital-acquired UTIs and those associated with indwelling urinary catheter use.[3–5] Given the intrinsic and acquired antimicrobial resistance mechanisms that *Pseudomonas* spp. express, and increasing rates of MDR isolates (15%–30%),[6] these infections represent challenging clinical cases with limited treatment options. Fluoroquinolones are the only orally active antipseudomonal agents that are readily available. When resistance is identified, parenteral therapy is often relied upon. Oral fosfomycin, therefore, is an attractive alternative agent.

Fosfomycin has a unique chemical structure, lacks cross-resistance to other antimicrobials, and its small molecular mass (138 Da) and polarity mean it can readily cross the outer membrane of Gram-negative bacteria through porins. High urinary concentrations of fosfomycin are observed after a 3 g oral dose, with peak concentrations of 1000–2000 mg/L.[7,8] There is

limited evidence for the treatment of MDR uropathogens, including the off-label clinical practice of giving multiple repeat oral doses.[9–15] The pharmacokinetics (PK), safety and tolerability of giving 3 g oral fosfomycin every day for seven doses showed adverse events in 89% of subjects.[16]

We examined the efficacy of oral fosfomycin against *P. aeruginosa* urinary isolates in a dynamic bladder infection *in vitro* model. The model was run using synthetic human urine (SHU), with the impact on pathogen kill and emergence of resistance assessed following fosfomycin administered as a single 3 g oral dose, and as 3 g given daily for 7 days.

Materials and methods

Antibiotic and media

Fosfomycin ('Fomicyt', InfectoPharm, Germany), reconstituted to 50000 mg/L, was used in the bladder infection model and incorporated into solid media for susceptibility testing. SHU was used as a substitute for human urine. The chemical components of SHU are outlined in Table 1.[17] CAMHB (Becton Dickinson, USA) with and without supplementation with 25 mg/L glucose-6-phosphate (G6P, Sigma–Aldrich, USA) was used for broth microdilution (BMD) MIC testing. Trypticase soy agar with 5% sheep blood (TSA, Becton Dickinson) was used for subculturing isolates from freezer stock. Mueller–Hinton II agar (MHA, Becton Dickinson) was used for bacterial count quantification. Screening for fosfomycin-resistant subpopulations was performed by quantifying growth on media containing 64 mg/L (low-level resistance, LLR) and 512 mg/L fosfomycin (high-level resistance, HLR) compared with the total growth determined on drug-free MHA. All solid media to which fosfomycin was added also contained 25 mg/L G6P.

Table 1. Synthetic human urine (SHU) chemical constituents

Chemical		Concentration (g/L)[a]
Sodium chloride	NaCl	5.844
Sodium sulphate	Na_2SO_4	2.4147
Urea	Urea	16.8168
Potassium chloride	KCl	2.8329
Calcium chloride	$CaCl_2$	0.4439
Creatinine	Creatinine	1.0181
Citric acid trisodium salt dihydrate	$Na_3C_6H_5O_7$	1.9999
Ammonium chloride	NH_4Cl	1.0698
Magnesium sulphate	$MgSO_4$	0.3852
Sodium oxalate	$Na_2C_2O_4$	0.0241
Sodium phosphate monobasic	NaH_2PO_4	0.5616
Sodium phosphate dibasic	Na_2HPO_4	0.9227
Potassium dihydrogen phosphate	KH_2PO_4	2.1774
Uric acid	$C_5H_4N_4O_3$	0.1009
Sodium bicarbonate	$NaHCO_3$	1.1341
Magnesium chloride hexahydrate	$MgCl_2{\cdot}6H_2O$	0.6506
Lactic acid	$C_3H_6O_3$	0.0991
Ferrous sulphate heptahydrate	$FeSO_4{\cdot}7H_2O$	0.0014
20% (w/v) casamino acids	–	0.1% (v/v)

[a]SHU was made up in 3 L batches with pH adjusted to 5.6 using 1 M NaOH and 5 M HCl.

Bacterial isolates and in vitro susceptibility

Clinical *P. aeruginosa* isolates from a urinary source were selected from a surveillance collection from throughout the Netherlands, together with reference and clinical isolates from previous *in vivo* experiments.[18] All isolates underwent fosfomycin susceptibility testing by agar dilution following standard reference methodology.[19] Sixteen isolates were selected, reflecting the range of MIC values, and underwent additional testing, including disc diffusion (FOT200 discs, Oxoid Ltd/Thermo Fisher Scientific, UK), in-house BMD testing using reference methodology,[19] Vitek-2 (AST-N344, BioMérieux, France) and Sensititre (Thermo-Fisher Scientific, UK) using a custom-designed plate. In-house BMD testing was performed in triplicate (median value reported) in CAMHB, CAMHB with G6P, and SHU. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 25983 were run as quality controls to ensure reproducibility. Species identification was confirmed by MALDI-TOF MS (Bruker Daltonik GmbH, Germany).

fosA^{PA} gene PCR

Bacterial DNA was extracted from the 16 isolates by boiling at 100°C for 10 min. Qualitative PCR detection of the *fosA*^{PA} gene was performed using in-house forward primer 5′-CGGGTCGAGGAAGTAGAACG-3′ and reverse primer 5′-TGCTCACCGGTCTCAATCAC-3′.[20] The PCR amplification resulted in a 311 bp amplicon size, visualized by gel electrophoresis run at 160 V for 60 min. *P. aeruginosa* ATCC 25983 and sterile water were used as positive and negative controls, respectively.

Dynamic bladder infection in vitro model

The *in vitro* model design was modified from a previous publication.[21] In brief, applying two consecutive first-order processes, drug distribution PK equations[22] informed fosfomycin dose, compartment volumes and flow rates to generate dynamic changes in fosfomycin concentrations following oral absorption, applying normal urodynamics, including urine output 1 mL/min, six voids/day, and a post-void residual volume <50 mL. The *in vitro* model was then constructed on a 1:16 scale, with 16 bladder compartments run concurrently. Urinary fosfomycin concentrations following a 3 g oral dose were simulated, with a urinary C_{max} of 1984 mg/L at 7.5 h and blood elimination half-life 6.9 h, giving an *in vitro* AUC of approximately 36000 mg·h/L.[7] Isolates were added to bladder compartments at a 10^7 cfu inoculum, providing an equivalent total number of bacteria expected in human infections (10^5 cfu/mL in 250 mL void). In SHU, growth capacity was determined and fosfomycin-resistant subpopulations were quantified following an 18 h drug-free dynamic incubation within the *in vitro* model. Fosfomycin was then administered as a single dose, and as daily dosing for 7 days. Pharmacodynamic (PD) response (pathogen kill and emergence of resistance) was assessed over the time course of each experiment. The post-exposure fosfomycin MIC of any regrowth was rechecked by agar dilution after subculture to TSA.

In vitro quantitative bacterial cultures

Samples for bacterial quantification were taken directly from each bladder compartment at predetermined timepoints, undergoing serial 10-fold dilutions, of which 20 µL was plated from each dilution onto drug-free MHA (total growth quantification), MHA with 64 mg/L fosfomycin (LLR quantification) and MHA with 512 mg/L fosfomycin (HLR quantification). The lowest limit of detection was considered to be 50 cfu/mL. All plates were incubated aerobically at 37°C for 16–20 h. Plates supplemented with fosfomycin were re-incubated for an additional 24 h.

Measurement of fosfomycin concentrations

An ultra-performance (UP) LC-MS/MS method was used for fosfomycin quantification, validated for urine and plasma samples,[23] with additional

tests confirming its applicability in SHU; stability was confirmed at 37°C for 72 h. The method was validated according to FDA guidelines[24] over a range of 0.75–375 mg/L ($R^2 = 0.9998$) and a maximum deviation of 5.0%. All 16 bladder compartments were sampled at the C_{max} after a single fosfomycin dose, and trough (at 144 h) and C_{max} (at 151 h) of the seventh dose during the multidose experiment. At all other timepoints, representative PK samples were collected from three bladder compartments, including before and after initial C_{max}, trough and peak concentrations with each administered dose, and 24, 48 and 72 h after the last dose.

Statistical analyses

Comparison of MIC values measured by agar dilution and BMD (in CAMHB and SHU) was performed using the Bland–Altman method, where the mean MIC value of compared methods (*x*-axis) is plotted against the difference in MIC (*y*-axis).[25] Agar dilution and disc diffusion were compared by linear regression. Accuracy and bias of observed fosfomycin concentrations in the bladder infection model were compared with the target by linear regression and Bland–Altman plot. Pre- and post-exposure fosfomycin MICs were compared by the Wilcoxon matched-pairs signed rank test. The exposure–response relationship of $fAUC_{0-24}$/MIC and bacterial response (change in total bacterial count and emergence of HLR) was assessed at 72 h for both dosing schedules, and at the end of treatment (EOT) after the multidose experiment (at 216 h). Standard slope E_{max} non-linear regression was performed, with the top parameter corresponding to maximal bacterial counts and the bottom parameter as the lower limit of bacterial count quantification. Drug exposures associated with stasis and 1, 2 and 3 $\log_{10}$ kill were determined, with growth outcomes at 24, 48 and 72 h compared. Non-linear regression curves were compared statistically using the f-test. Isolate growth at 72 h was compared with EOT following the multidose experiment by linear regression and Pearson's correlation coefficient (two-tailed *P* value). Where appropriate, data are presented as means (± SD). All analyses were performed with GraphPad Prism (version 8.3, Mac OS).

Results

Baseline in vitro susceptibility

Fifty-three *P. aeruginosa* isolates underwent fosfomycin susceptibility testing by agar dilution. The majority were from a urinary source (38, 71.7%). $MIC_{50/90}$ was 32/64 mg/L (Figure 1). Sixteen isolates selected for testing in the bladder infection model had MICs ranging from 1 to >1024 mg/L; 12 of these 16 isolates had MICs from 8 to 64 mg/L (Table S1, available as Supplementary data at *JAC* Online). Compared with agar dilution, MICs determined by BMD in CAMHB were higher, with a bias of 1.3±0.9 doubling dilutions (Figure S1a). Testing in CAMHB with G6P supplementation did not potentiate the activity of fosfomycin (Table S1). Testing in SHU also resulted in MIC values higher than agar dilution (bias 1.6±2.1) (Figure S1b). Two isolates (R005 and H011), however, demonstrated reductions in their MICs when tested in SHU, despite adequately growing in drug-free medium. Vitek-2 and Sensititre returned values comparable with each other and with the in-house BMD in CAMHB (Table S1). Disc diffusion zone diameters showed reasonable linear correlation to agar dilution MIC values ($R^2 = 0.80$, Figure S1c). Susceptibility to other antimicrobials is presented in Table S2. $fosA^{PA}$ was detected in all isolates.

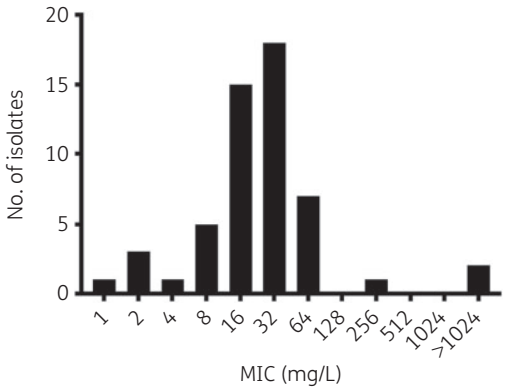

Figure 1. Fosfomycin MIC distribution of 53 *P. aeruginosa* isolates that underwent screening for fosfomycin susceptibility. MIC testing was performed by agar dilution using Mueller–Hinton agar supplemented with 25 mg/L G6P. $MIC_{50} = 32$ mg/L; $MIC_{90} = 64$ mg/L.

Fosfomycin exposure within the bladder infection model

Observed dynamic *in vitro* fosfomycin concentrations closely matched the target values, with the slope of the linear regression line equal to 1.1 ($R^2 = 0.97$; Figure S2a) with a bias of 4.8% (95% CI −15.0% to 24.7%) (Figure S2b). There was minimal inter-compartmental variation, with an average relative standard deviation of 4.3±1.5% when all bladder compartments were sampled at the same time (Figures 2a and 3a).

Bacterial growth in the bladder infection model

Pre-exposure characteristics and growth in drug-free media

After 18 h drug-free incubation in the bladder infection model, isolates increased their density from an average starting inoculum (± SD) of 6.9±0.2 to 8.5±0.6 $\log_{10}$ cfu/mL. Only one isolate (D001) demonstrated growth restriction, from a starting inoculum of 7.2 $\log_{10}$ cfu/mL that reduced to 6.8 $\log_{10}$ cfu/mL. After incubation, HLR subpopulations were detected in 11 out of 16 isolates. Only isolate H011 had an HLR subpopulation detected in the starting inoculum prior to incubation. Despite this isolate having a baseline MIC >1024 mg/L, the HLR population only accounted for 4×10^{-4} of the total population. For the remaining isolates in which an HLR subpopulation was detected, the proportion the HLR subpopulation made up of the total population ranged from 3×10^{-7} to 9×10^{-6} (Table 2). In the isolates that did not have an HLR subpopulation detected, three of five had LLR detected. Note that isolate R005 (baseline MIC 256 mg/L) grew equally on drug-free MHA and MHA with 64 mg/L fosfomycin. Isolates A934 and D001 did not grow on either of the fosfomycin-containing media. Agar dilution MIC of isolates without a detected HLR subpopulation was ≤8 mg/L in four of five isolates, and ≤32 mg/L when tested by BMD in SHU in five of five isolates.

Fosfomycin activity

Following exposure to fosfomycin, all isolates regrew, regardless of whether fosfomycin was administered as a single dose (Figure 2) or seven once-daily doses (Figure 3). Bacterial counts at the final timepoint ranged from 3.3 to 9.2 $\log_{10}$ cfu/mL, with variable emergence of resistance (Table 2). Following a single dose, >2 $\log_{10}$ kill at 72 h was observed in 5 of 16 isolates (A394, A24354, R006, D001 and R005), without emergence of resistance, and all had

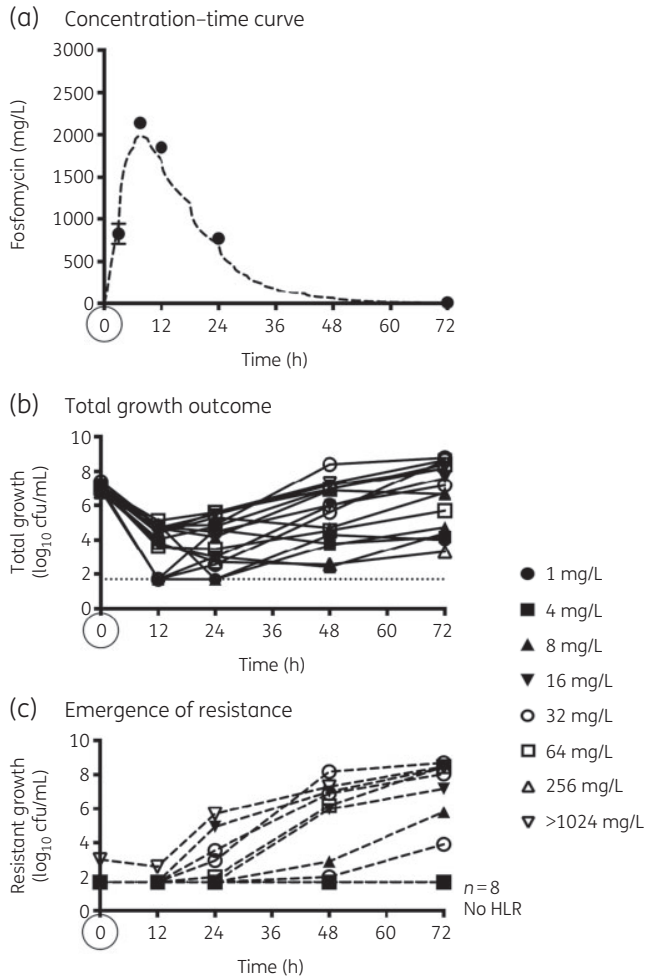

Figure 2. Fosfomycin exposure and growth outcome following a single fosfomycin dose. (a) Solid circles represent the measured mean (± SD) fosfomycin concentration from samples collected from the *in vitro* bladder compartments over time, overlaid on the target concentration curve (dashed line). Total growth (b) and HLR growth (c) after fosfomycin exposure. Circle on the *x*-axis highlights the timing of the fosfomycin dose. Limit of detection (dotted line) was considered to be 50 cfu/mL (1.7 $\log_{10}$ cfu/mL).

baseline MICs 1–8 mg/L, except isolate R005 (MIC 256 mg/L). Amplification (10^2- to 10^6-fold) of the HLR subpopulation occurred in 8 of 16 isolates, of which 5 (H004, F020, F002, I005 and H008) had an increase in the total population MIC to ≥1024 mg/L and 1 isolate (H011) maintained its MIC of >1024 mg/L. The remaining two (H002, I002) did not demonstrate a rise in the total population (MIC 16 and 32 mg/L, respectively), although the HLR subpopulation had an MIC >1024 mg/L when tested separately.

Following seven once-daily doses of fosfomycin, a reduction in bacterial counts at the EOT was observed in 5 of 16 isolates (all without detectable pre-exposure HLR), with 2 isolates (A934 and A24354) demonstrating >2 $\log_{10}$ kill (baseline MICs 1–4 mg/l) and 3 isolates (R006, D019 and R005) with 0.7–1.2 $\log_{10}$ kill. The remaining 11 of 16 isolates (all with detected pre-exposure HLR) regrew with amplification (10^2- to 10^6-fold) of the HLR subpopulation, which occurred within 72 h for 5 isolates, and after 132 h for the remaining 6 isolates. All of the latter isolates demonstrated an initial ≥1.5 $\log_{10}$ kill up to 72 h, but with subsequent regrowth despite continued daily fosfomycin administration. Of the 11 isolates that regrew with HLR amplification, 9 had a rise in the MIC of the total population to >1024 mg/L. Isolate H011 maintained its baseline MIC of >1024 mg/L. The remaining isolate (H002) regrew without a rise in the total population MIC (16 mg/L), but with amplification of the HLR subpopulation (MIC >1024 mg/L) to a proportion of 2×10^{-3}. There was a significant rise in the post-exposure MIC of the total population when compared with baseline following seven once-daily doses of fosfomycin ($P = 0.0039$; Figure 4).

PK/PD analysis

Two outlying isolates were excluded from the PK/PD analysis. Namely, R005, which had an MIC four dilutions lower when tested by BMD in SHU (Figure S1), and D001, which demonstrated an inability to increase its bacterial density in the bladder infection model when run with drug-free SHU. The multiple dosing regimen had a greater response at 72 h compared with a single dose, although this additional kill was lost by EOT (Figure 5a). Applying an E_{max} curve for the 72 h PD outcomes following the single dose ($R^2 = 0.7287$) and multiple doses ($R^2 = 0.8307$), the mean fAUC$_{0-24}$/MIC (95% CI) associated with stasis and 1, 2 and 3 $\log_{10}$ kill was 2904 (2034–4471), 5685 (3920–8518), 10152 (6867–14886) and 18502 (12225–26657), compared with 677 (472–1048), 1325 (909–1995), 2366 (1593–3486) and 4312 (2834–6239), respectively, with the latter target achieved only with the multiple dosing regimen (Table 3). The EOT growth outcome after seven daily doses demonstrated an additional shift in the E_{max} curve to the right ($R^2 = 0.7148$). Stasis at EOT following seven once-daily doses was associated with a mean fAUC$_{0-24}$/MIC of approximately 5000, which correlated with an initial 3 $\log_{10}$ kill at 72 h (Pearson's $r = 0.75$; $P = 0.0008$) (Table 3 and Figure S3). When comparing outcomes at 24, 48 and 72 h following both dosing schedules, each exposure–response curve is progressively shifted to the right (Figure 6). Importantly, the margin between curves is greater following the single dose compared with the response following multiple doses, indicating that multiple doses keep the initial bacterial counts at a level lower than the single dose. The relationship relating to emergence of fosfomycin resistance was less clear. Increased exposure with multiple doses, however, failed to provide additional suppression of resistance, and, in fact, was associated with greater HLR growth at the final timepoint. Similar to the PK/PD target for an initial 3 $\log_{10}$ kill and EOT stasis following multiple doses, HLR growth was not detected with fAUC$_{0-24}$/MIC >5000 (Figure 5b). The same PK/PD analyses with all isolates included demonstrated a similar pattern of results (Figure S4).

Discussion

We demonstrated that standard-dose (3 g) oral fosfomycin for the treatment of *P. aeruginosa* UTIs in a dynamic *in vitro* model was unable to achieve complete bacterial eradication. This was despite high urinary fosfomycin concentrations after a single dose and seven once-daily doses. The degree of bacterial kill and emergence of HLR during treatment was related to baseline fosfomycin MIC.

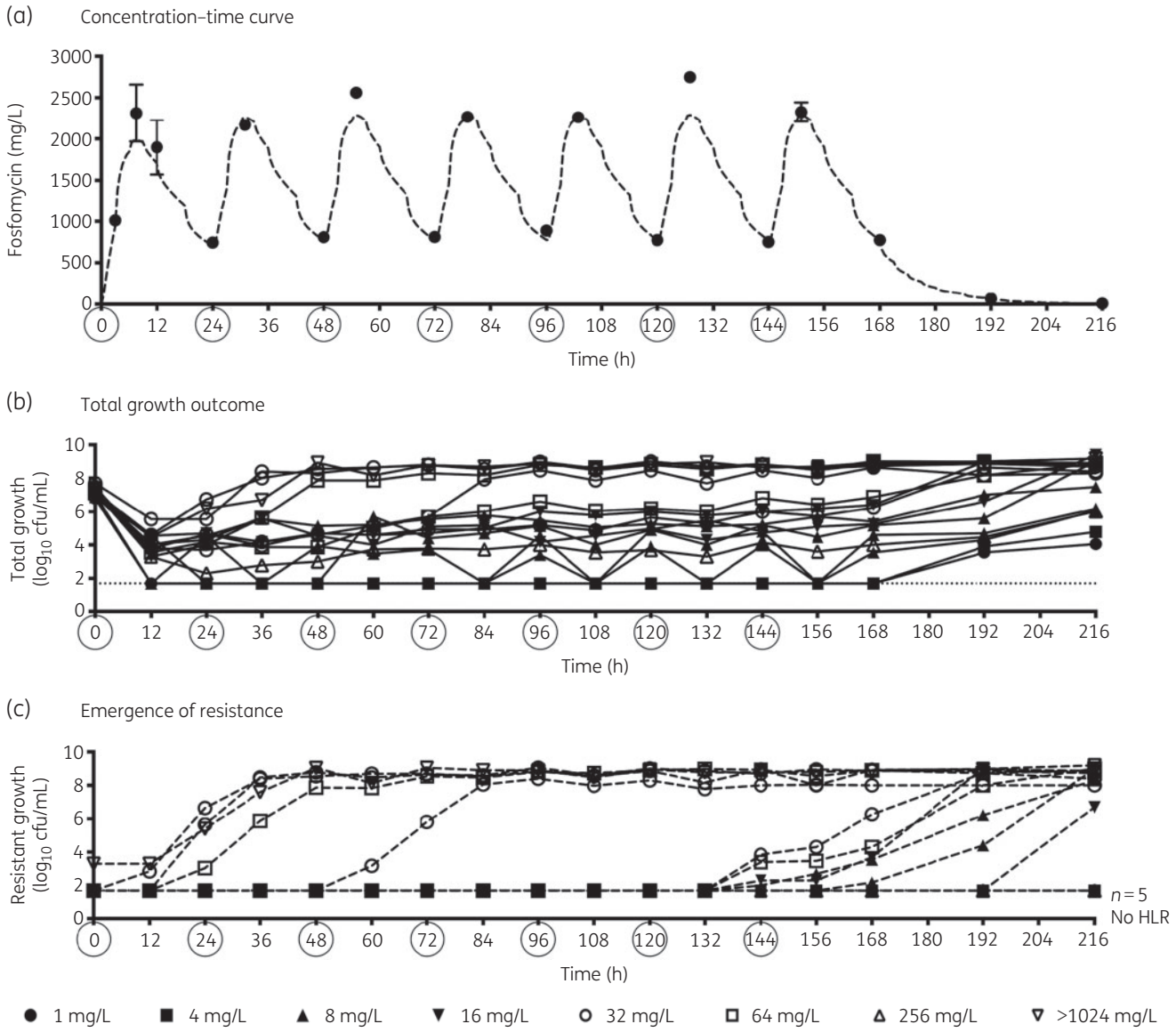

Figure 3. Fosfomycin exposure and growth outcome following seven fosfomycin doses given daily. (a) Solid circles represent the measured mean (± SD) fosfomycin concentration from samples collected from the *in vitro* bladder compartments over time, overlaid on the target concentration curve (dashed line). Total growth (b) and HLR growth (c) after fosfomycin exposure. Circles on the *x*-axis highlight the timing of the fosfomycin dosing. Limit of detection (dotted line) was considered to be 50 cfu/mL (1.7 $\log_{10}$ cfu/mL).

The majority of isolates with an MIC >8 mg/L regrew with HLR. However, isolates with an MIC of 1–4 mg/L did not have detectable HLR emergence and demonstrated >2 $\log_{10}$ kill in single-dose and multidose experiments. Isolates with an MIC of 8 mg/L without an HLR subpopulation showed 1 and 2 $\log_{10}$ kill, whereas isolates with an MIC of 8 mg/L with an HLR subpopulation showed no kill and regrew with a rise in fosfomycin MIC. Thus, fosfomycin activity was predicted by baseline MIC and the detection of an HLR subpopulation.

In *Pseudomonas* spp., fosfomycin is actively transported into the cell by the glycerol-3-phosphate transporter GlpT and lacks the hexose phosphate uptake transporter UhpT present in most Enterobacteriaceae.[26] Concordant MIC values when tested with and without G6P in this study support the absence of the UhpT transporter, questioning the requirement for G6P media supplementation for susceptibility testing.[27] *In vitro* studies report the WT population for *Pseudomonas* as having a fosfomycin MIC ≤128 mg/L.[27–29] Our findings support this, with our isolates having a fosfomycin $MIC_{50/90}$ of 32/64 mg/L. However, fosfomycin clinical breakpoints are not provided by EUCAST or CLSI for *Pseudomonas* spp. and therefore MIC values can only discriminate between WT and non-WT isolates, rather than supporting clinical efficacy.[30,31]

Similar to other reports, we detected the gene encoding the inactivating enzyme FosA in all test isolates. The presence of a

Table 2. Pre- and post-exposure MICs, HLR and growth outcomes in the dynamic bladder infection *in vitro* model

	Pre-exposure		Post-exposure					
			change in bacterial count; emergence of HLR; fosfomycin MIC[a]					
			single dose			daily dosing (7 days)		
Strain no.	fosfomycin MIC (mg/L)[a]	HLR subpopulation proportion[b]	Δ $\log_{10}$ cfu/mL	HLR[c]	MIC (mg/L)[d]	Δ $\log_{10}$ cfu/mL	HLR[c]	MIC (mg/L)[d]
A934	1	–[e]	−3.0	–	1	−2.7	–	1
A24354	4	–[f]	−2.9	–	2	−2.2	–	2
R006	8	–[f]	−2.0	–	8	−0.7	–	8
D019	8	3E−07	0.0	–	4	0.5	1.0	>1024
H004	8	2E−06	−0.2	1E−01	>1024	2.0	9E−01	>1024
D001[g]	8	–[e]	−2.6	–	8	−0.9	–	8
F020	16	1E−06	1.5	1.0	1024	1.7	1.0	>1024
H002	16	8E−07	0.5	4E−01	16[h]	2.1	2E−03	16[h]
I002	32	3E−07	0.0	6E−04	32[h]	0.9	1.0	>1024
A019	32	4E−07	1.8	–	32	1.3	5E−01	>1024
F002	32	3E−06	1.4	8E−01	>1024	1.0	1.0	>1024
I005	32	1E−06	1.3	7E−01	>1024	1.7	9E−01	>1024
H008	64	2E−06	1.4	1.0	>1024	1.8	1.0	>1024
D004	64	9E−06	−1.3	–	64	1.6	1.0	>1024
R005	256	–[f]	−3.5	–	256	−1.2	–	256
H011	>1024	4E−04	1.5	7E−01	>1024	2.2	1.0	>1024

[a]Fosfomycin MIC was determined by agar dilution.
[b]HLR growth (i.e. growth on agar with 512 mg/L fosfomycin) as a proportion of the total growth after 18 h drug-free dynamic incubation in the bladder infection model.
[c]Proportion of the regrowth that was HLR, where a proportion of 1.0 indicates complete population replacement with HLR growth after fosfomycin exposure.
[d]Fosfomycin MIC of the regrowth determined after subculture on non-selective agar.
[e]Neither LLR (i.e. growth on agar with 64 mg/L) nor HLR subpopulation detected.
[f]LLR subpopulation detected only.
[g]Isolate D001 demonstrated growth restriction after the 18 h drug-free dynamic incubation in the bladder infection model.
[h]Fosfomycin agar dilution MIC of the HLR subpopulation growth off selective agar had MIC >1024 mg/L after subculture on non-selective agar.

chromosomal *fosA*^{PA} gene has been found to be essentially uniformly present in *P. aeruginosa* isolates.[32] Mutations in GlpT have also been reported to be responsible for fosfomycin resistance in *P. aeruginosa*,[33] and the amplification of pre-existing fosfomycin-resistant subpopulations has been implicated in treatment failures.[34,35] *Pseudomonas* spp. have been commonly found to have an elevated mutant prevention concentration (≥2048 mg/L).[33] In our bladder infection model, amplification of the HLR subpopulation was detected in the majority of isolates and predicted treatment failure and a rise in fosfomycin MIC. Importantly, once emergence of HLR was detected, subsequent repeat fosfomycin doses had little impact on the total bacterial density. Despite the *fosA*^{PA} gene being detected in all isolates, eradication was found for isolates with low MICs, suggesting a dose–effect relationship for FosA enzymatic activity. When an *E. coli* is transformed with a recombinant plasmid containing the pseudomonal *fosA*^{PA} gene (pFosA^{PA}), the fosfomycin MIC was found to only rise from 1 to 16 mg/L, whereas recombinant plasmids from other species (*Klebsiella pneumoniae*, *Enterobacter cloacae*, *Serratia marcescens*, *Morganella morganii*, *Klebsiella oxytoca* and *Providencia stuartii*) resulted in MIC rises to ≥1024 mg/L.[32]

An additional determinant of fosfomycin resistance in *Pseudomonas* spp. is the ability to undertake anabolic peptidoglycan (PG) recycling of the cell wall. The enzyme MupP has been shown to have an important role in bypassing the *de novo* biosynthesis of uridyldiphosphate (UDP)-*N*-acetylmuramic acid (MurNAc). In turn, this also bypasses the activity of fosfomycin, which acts by binding to the MurA enzyme, which prevents the first step of bacterial cell wall biosynthesis [specifically, UDP-*N*-acetylglucosamine (UDP-GlcNAc) to UDP-MurNAc].[36,37] Interestingly, a small-molecule inhibitor of PG recycling has been purported to increase isolate susceptibility to fosfomycin, while also overcoming AmpC-driven β-lactam resistance.[38] Similarly, a small-molecule active-site inhibitor of FosA has been reported to restore fosfomycin activity in bacterial strains that are resistant.[39]

Bacterial killing of *P. aeruginosa* by fosfomycin has been most strongly linked with the *f*AUC/MIC index.[40,41] The time-course of the response to fosfomycin in our model, for the most part, resulted in an initial >2 $\log_{10}$ kill and then subsequent regrowth. A PK/PD target of $fAUC_{0-24}$/MIC >5000 predicted initial kill and subsequent bacterial stasis, without emergence of HLR at EOT following multiple doses. The clinical translation of this value, however, is challenging given the significant variability reported in urinary

fosfomycin concentrations after a single 3 g oral dose. Our model simulated higher than average urinary fosfomycin exposure. Recent fosfomycin PK data in urine report average peak urinary concentrations (and approximate $fAUC_{0-24}$) as 1982 mg/L (20000 mg·h/L),[7] 1049 mg/L (14000 mg·h/L)[8] and 600 mg/L (8000 mg·h/L).[16] Lower urinary exposures could occur with greater fluid intake, increased urine output and fewer bladder voids, resulting in reduced $fAUC_{0-24}$ as low as 4800 mg·h/L.[16] Therefore, the $fAUC_{0-24}$/MIC target of 5000 may only be reliably achieved, following standard dosing, against isolates with MICs $\leq$2 mg/L. Furthermore, although suppression of fosfomycin resistance has previously been linked to the time concentrations remain above the MIC,[42] in our model emergence of resistance still occurred despite fosfomycin concentrations maintained >128 mg/L for 186 h during the multiple dosing schedule. Alternative fosfomycin dosing strategies, such as administering multiple 3 g oral sachets, two or three times per day, could target an increased urinary *f*AUC, but would risk patient intolerance, and delays in absorption, due to flip-flop kinetics, would limit urinary excretion. Alternatively, the use of fosfomycin in combination with other antimicrobial agents, for which several *in vitro* studies have reported synergy,[34,38,43–47] may provide a means to increase fosfomycin activity against those *Pseudomonas* isolates with baseline MIC >2 mg/L.

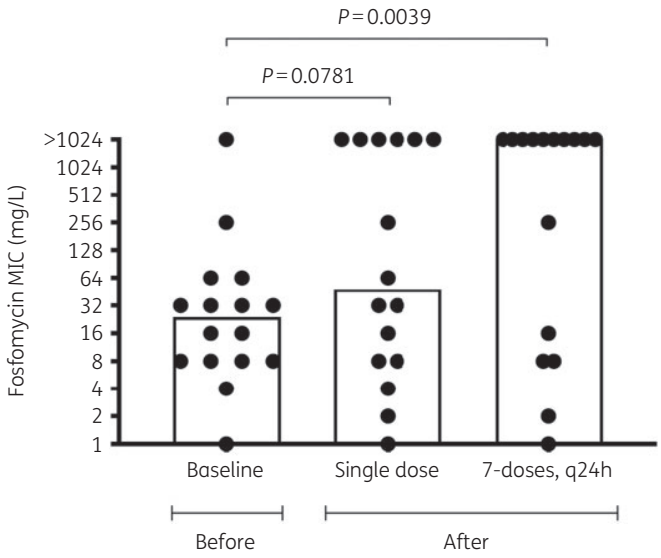

Figure 4. Pre- and post-exposure fosfomycin MIC changes. MICs were determined by agar dilution before and after exposure to fosfomycin, following administration either as a single dose or seven doses given daily. Columns highlight the MIC_{50} values. Comparison with baseline by Wilcoxon matched-pairs signed rank test.

An important limitation for the clinical translation of the bladder infection model relates to the aerobic *in vitro* environment, which lacks both bladder tissue architecture and host immune responses. In fact, in a mouse pseudomonal sepsis model, fosfomycin was shown to promote a beneficial immunomodulatory effect.[48] Furthermore, repeated dosing of fosfomycin may have greater efficacy *in vivo* by maintaining bacterial counts at a constant lower level for longer, compared with a single dose, thereby allowing more time for effective phagocytosis and bacterial eradication from the bladder. When considering the EOT bacterial response in our *in vitro* model, measured at 72 h after the last dose of fosfomycin and when *in vitro* concentrations had fallen to 8 mg/L, we are assessing the bactericidal activity of fosfomycin. Alternatively, if the response was static in nature, in the absence of an immune system regrowth would be expected to be observed once concentrations fell below the baseline MIC value and could skew the PK/PD analysis towards suggesting higher exposures. Conversely, however, when compared with single-dose exposure, we demonstrated that by increasing the duration of exposure

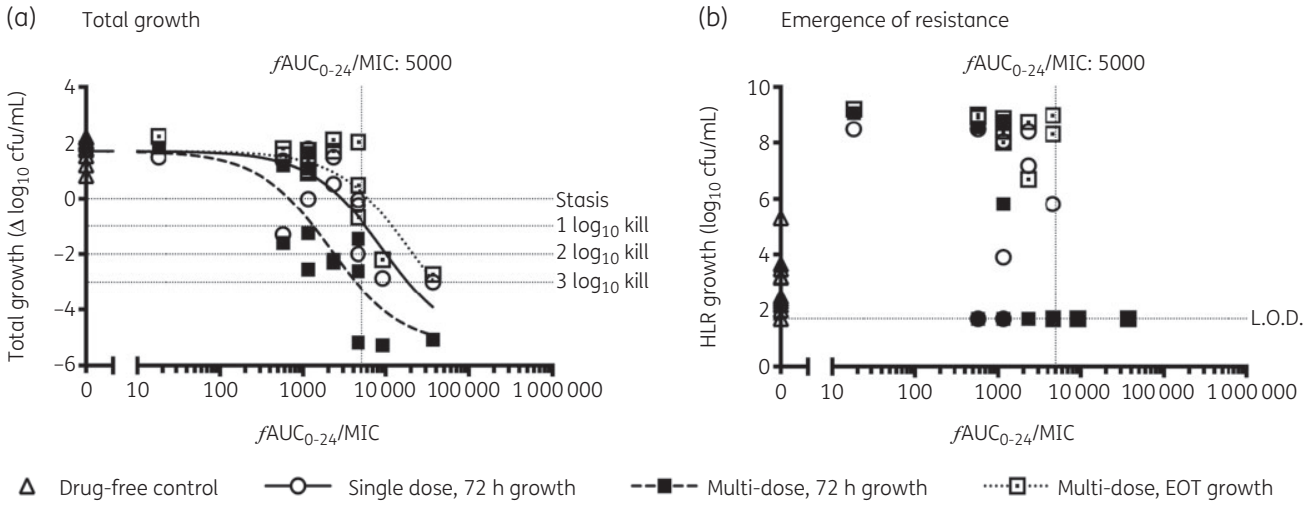

Figure 5. Exposure–response relationship. Total growth (a) and emergence of resistance (b) assessed in relation to the simulated urinary fosfomycin exposure ($fAUC_{0-24}$/MIC) after a single dose (open circles) and after multiple doses [solid squares, 72 h growth; open squares with central dot, end of treatment (EOT) growth]. Open triangles represent dynamic drug-free control growth. Isolates D001 and R005 are not included (see Figure S4 for inclusion of all isolates). Limit of detection (L.O.D.) was considered to be 50 cfu/mL (1.7 log_{10} cfu/mL). For PK/PD parameters, refer to Table 3.

Table 3. Mean PK/PD parameters

	$fAUC_{0-24}$/MIC (95% CI)					
Dosing	IC_{50}	stasis	1 log_{10} kill	2 log_{10} kill	3 log_{10} kill	R^2
Single dose						
24 h	743 (381–1326)	238 (150–432)	466 (286–804)	833 (495–1384)	1518 (865–2450)	0.7659
48 h	3814 (2348–6422)	1223 (820–2010)	2395 (1574–3796)	4276 (2744–6595)	7793 (4852–11758)	0.6363
72 h	9054 (6209–13 903)	2904 (2034–4471)	5685 (3920–8518)	10152 (6867–14886)	18502 (12225–26657)	0.7287
Seven doses, q24h						
24 h	692 (425–1064)	222 (152–352)	435 (293–668)	776 (513–1164)	1415 (910–2081)	0.8370
48 h	1397 (873–2197)	448 (298–744)	877 (572–1404)	1566 (996–2436)	2854 (1759–4340)	0.8175
72 h	2110 (1425–3136)	677 (472–1048)	1325 (909–1995)	2366 (1593–3486)	4312 (2834–6239)	0.8307
216 h	19070 (13019–29769)	6117 (4201–9693)	11974 (8081–18396)	21382 (14129–32069)	–[a]	0.7250

[a]Value not presented as no isolate achieved a 3 log_{10} kill.

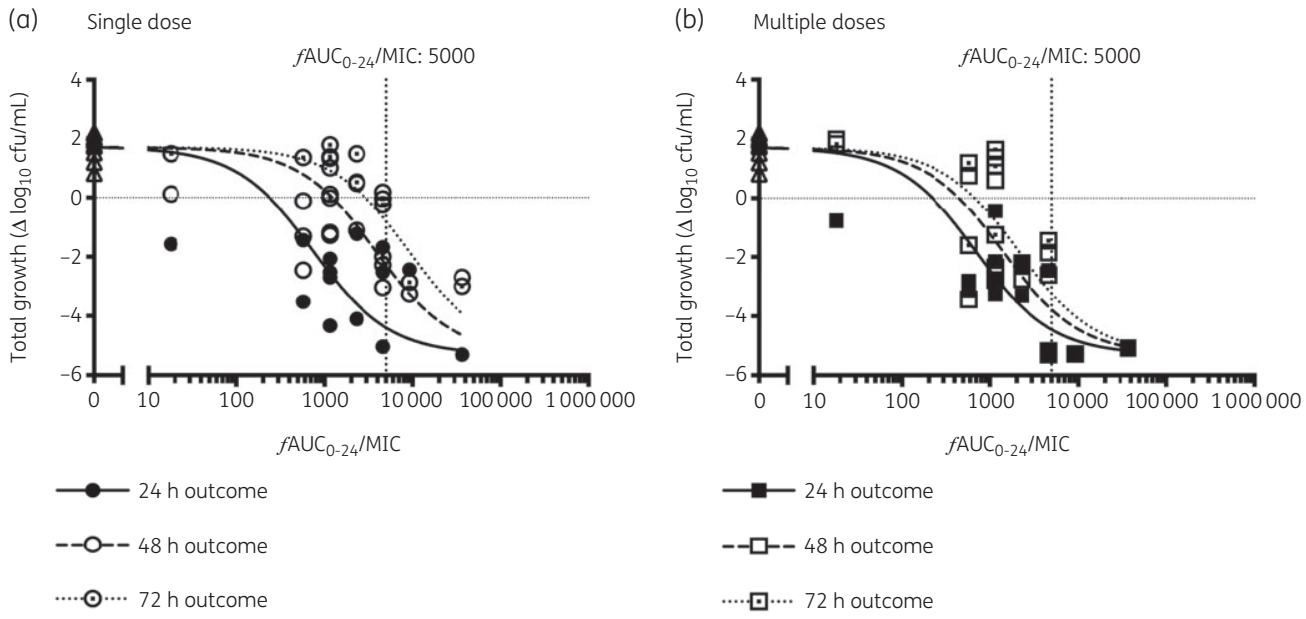

Figure 6. Single-dose (a) compared with multiple-dose (b) 24, 48 and 72 h growth outcome with the mean $fAUC_{0-24}$/MIC IC_{50} (95% CI) presented. Open triangles represent dynamic drug-free control growth. Isolates D001 and R005 are not included (see Figure S4 for inclusion of all isolates). Limit of detection was considered to be 50 cfu/mL (1.7 log_{10} cfu/mL). For PK/PD parameters, refer to Table 3.

there was greater amplification of the resistant subpopulation and post-exposure emergence of resistance. In order to further validate the outputs from the *in vitro* model, additional PD profiling of a different antibiotic, such as ciprofloxacin, that has well-described *in vitro* and *in vivo* efficacy at approved doses (750 mg q12h), would help clarify whether our observations are a general phenomenon with *P. aeruginosa* or something unique to fosfomycin. Finally, given the wide variability reported in human fosfomycin urinary concentrations,[7,8,16] additional studies with lower urinary exposure may generate different results.

With rising antibiotic resistance, and widespread reports of fluoroquinolone resistance among *Pseudomonas* isolates, an alternative oral antimicrobial is desperately required. Our results, however, suggest that monotherapy with oral fosfomycin may be limited in the treatment of pseudomonal UTIs. Furthermore, despite extending fosfomycin exposure over 7 days, there was no improvement in efficacy, and, in fact, there was worsening of fosfomycin resistance. The benefit of fosfomycin in combination with another oral antimicrobial, such as ciprofloxacin, warrants further investigation.

Acknowledgements

We acknowledge the laboratory assistance of Carla Roodbol de Goeij (Department of Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre).

Funding

This study was supported by internal funding. I.J.A. was funded by an Australian Government Research Training Program Scholarship (APP1114690) from the National Health and Medical Research Council of Australia. A.Y.P. was part funded through an Australian National Health and Medical Research Council Practitioner Fellowship (APP1117940).

Transparency declarations

J.W.M. has received research funding from Adenium, AstraZeneca, Basilea, Cubist, Polyphor, Roche, Eumedica, Basilea, VenatorX, AiCuris, Gilead and Wockhardt. A.Y.P has received research funding from MSD through an investigator-initiated research project. All other authors: none to declare.

Supplementary data

Tables S1 to S3 and Tables S1 to S4 are available as Supplementary data at *JAC* Online.

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SUPPLEMENTARY MATERIAL

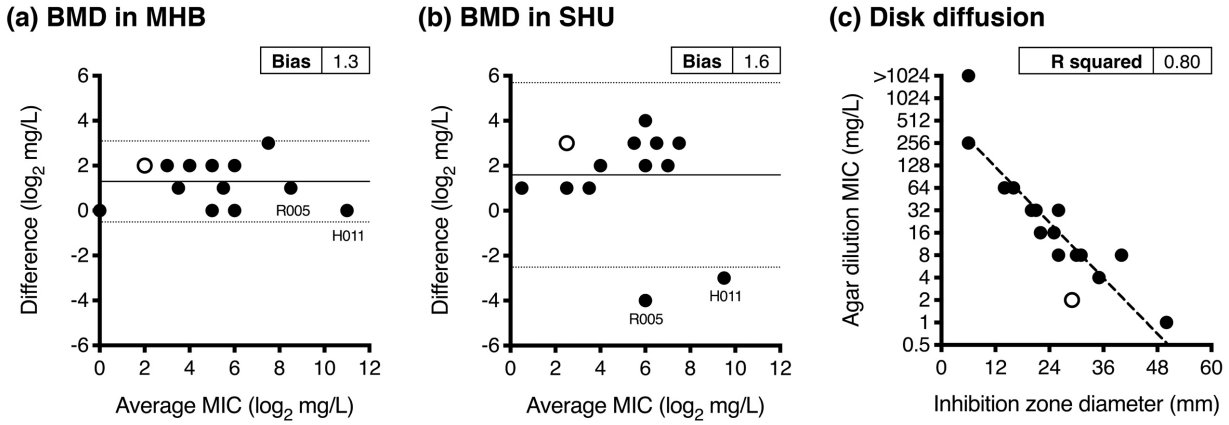

Figure S1. Comparison of fosfomycin susceptibility methods. Bland-Altman plots demonstrate the comparison between MIC values tested by agar dilution and broth microdilution (BMD) in MHB (a) and SHU (b), with the difference between the two MIC measurements on the *y*-axis, and the average of the two measurements on the *x*-axis, with the bias (solid line) and 95% limits of agreement (dotted lines) presented. Disk diffusion results (c) are compared to agar dilution MIC values, presented as scattergram and analysed by linear regression (dashed line). *P. aeruginosa* ATCC 27853 is represented as the open circle data point. Isolates R005 and H011 are highlighted as outliers in the BMD in SHU MIC comparison; when these isolates are removed from the comparison analysis, the MIC bias is 2.3 doubling dilutions (95% CI: 0.53 to 4.0).

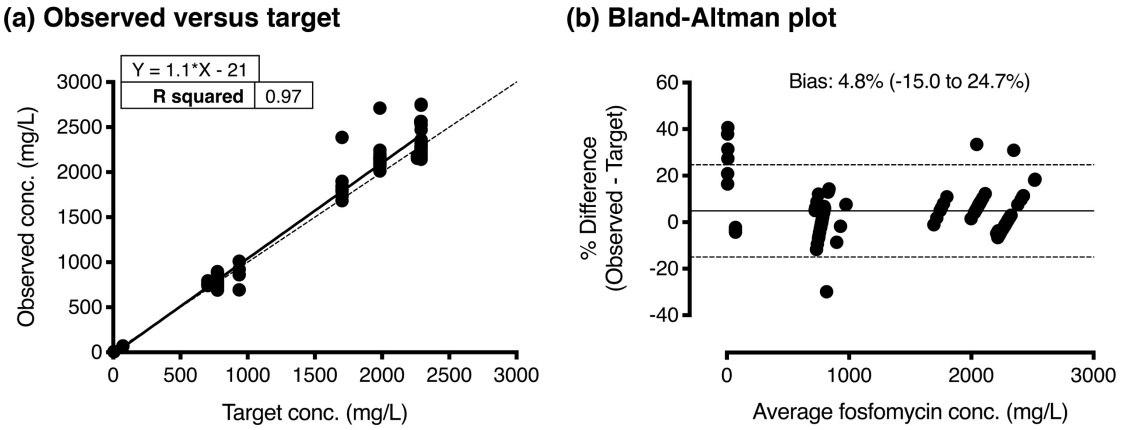

Figure S2. Relationship between the observed and targeted fosfomycin concentrations. (a) Observed fosfomycin concentrations compared to target, linear regression (solid line) and $y = x$ (dashed line). (b) Bland-Altman plot of the percentage difference of the observed and target measurements (*y*-axis) and the average of the two measurements (*x*-axis) presented with the bias (solid line) and 95% limits of agreement (dotted lines).

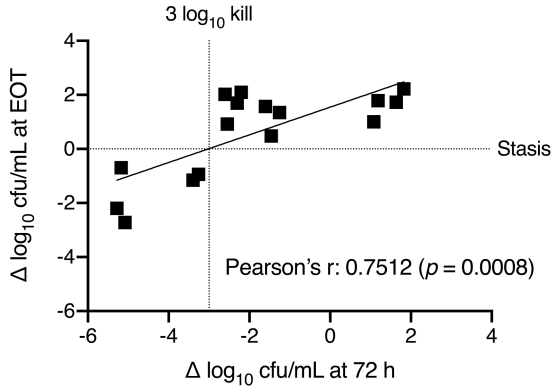

Figure S3. Correlation between 72 h growth and the end of treatment (EOT) outcome following multiple fosfomycin doses.

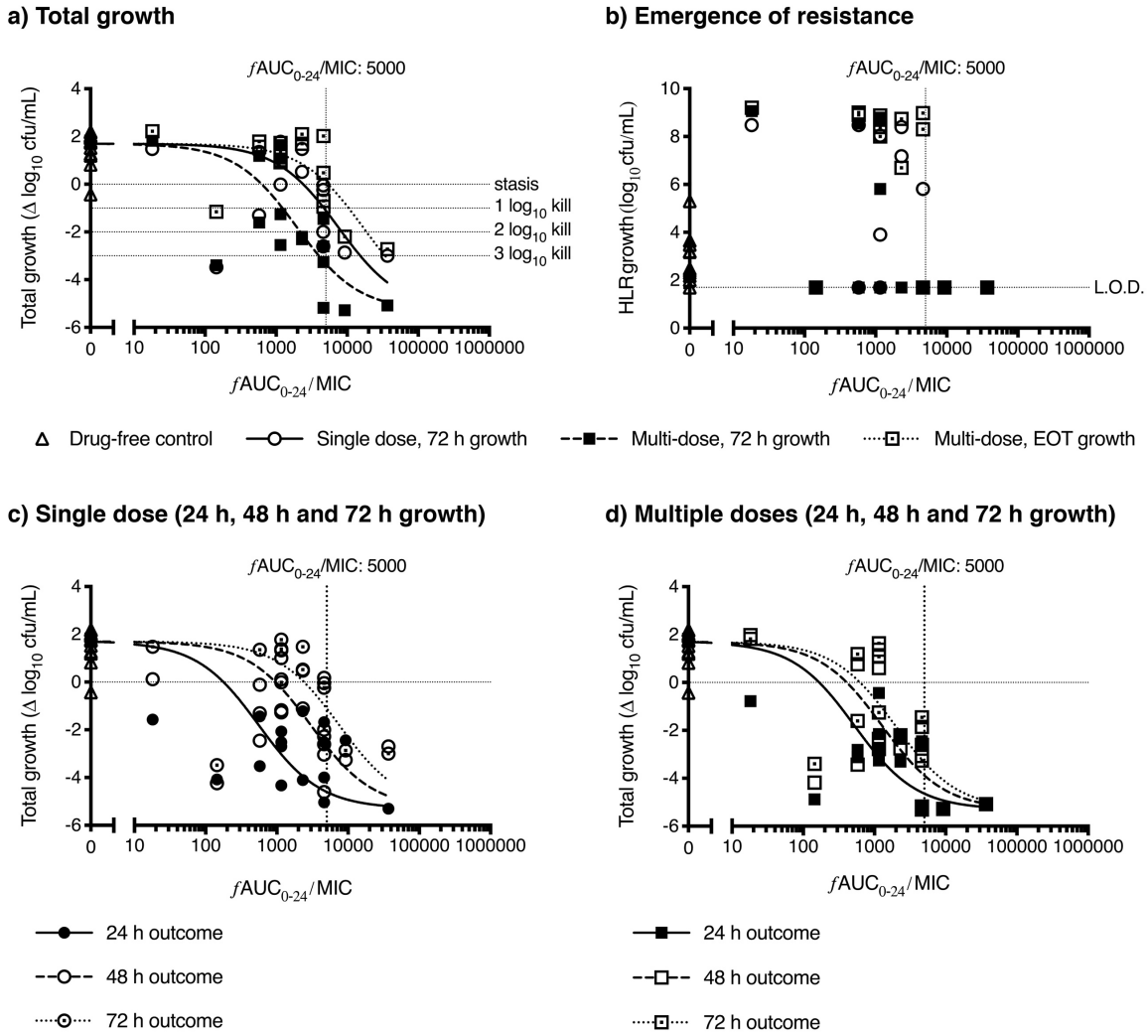

Figure S4. Exposure-response relationship, including all test isolates (i.e. outliers not excluded). Total growth (a) and emergence of resistance (b) assessed in relation to the simulated urinary fosfomycin exposure ($fAUC_{0-24}$/MIC) after a single dose (open circles) and after multiple doses (solid squares: 72 h growth; open squares with central dot: end of treatment (EOT) growth). Single dose (c) and multiple dose (d) 24 h, 48 h and 72 h growth outcome. Open triangles represent dynamic drug-free control growth. Limit of detection was considered 50 cfu/mL (1.7 log_{10} cfu/mL). *ns*, not significant. For PK/PD parameters refer to Table S3.

Table S1. Baseline fosfomycin susceptibility by different methods.

Strain no.	Source	AD MIC (mg/L)[a]	Disc diffusion (mm)[b]	Vitek-2 MIC (mg/L)[c]	Sensititre MIC (mg/L)[d]	BMD MIC (mg/L)[e]		
						MHB + G6P	MHB	SHU
A934	Blood	1	50	≤ 16	≤ 16	1	1	2
A24354	Unknown	4	35	≤ 16	≤ 16	16	16	8
R006	Urine	8	40	≤ 16	≤ 16	16	16	16
D019	Urine	8	31	≤ 16	≤ 16	16	16	32
H004	Urine	8	30	≤ 16	32	32	32	32
D001	Urine	8	26	32	≤ 16	16	32	32
F020	Urine	16	25	64	64	64	64	256
H002	Urine	16	22	64	32	64	64	128
I002	Urine	32	26	64	32	32	32	128
A019	Urine	32	20	128	64	128	128	256
F002	Urine	32	20	64	64	128	64	128
I005	Urine	32	21	64	64	64	64	256
H008	Urine	64	16	≥ 256	64	64	64	256
D004	Urine	64	14	≥ 256	≥ 256	512	512	512
R005	Urine	256	6	≥ 256	≥ 256	512	512	16
H011	Urine	>1024	6	≥ 256	≥ 256	>1024	>1024	256
27853[f]	ATCC	2	29	≤ 16	≤ 16	8	8	16

[a] Agar dilution (AD) MIC testing was performed on Mueller-Hinton agar (MHA) supplemented with 25 mg/L glucose-6-phosphate, performed in triplicate. [b] Disk diffusion performed using FOT200 disks (Oxoid Ltd/Thermo Fisher Scientific, UK). [c] Vitek-2 (BioMérieux, France) automated susceptibility was performed using the AST-N344 card. [d] Semi-automated susceptibility testing was performed using a Sensititre (Thermo Fisher Scientific, UK) customised plate and reader. [e] Broth microdilution (BMD) MIC testing was performed in Mueller-Hinton broth (MHB), with and without 25 mg/L glucose-6-phophate (G6P) supplementation, and in synthetic human urine (SHU). Testing in MHB and SHU was performed in triplicate. [f] *P. aeruginosa* ATCC 27853 was included in susceptibility testing, but not assessed in the dynamic bladder infection *in vitro* model. Note: MIC values > 128 mg/L are highlighted in grey.

Table S2. Antibiogram of test isolates[a]

Strain	AMK	ATM	FEP	CAZ	CIP	CST	GEN	IPM	MEM	TZP	TOB
A934	>32[b]	>8	>8	>8	4	2	>8	>8	16	>128	>8
A24354	16	>8	>8	>8	>4	2	>8	>8	16	>128	>8
R006	>32	>8	>8	>8	>4	2	>8	>8	>32	>128	>8
D019	≤ 2	8	2	2	0.12	2	1	8	0.5	≤ 2	≤ 0.5
H004	≤ 2	2	2	2	0.12	2	1	8	2	4	≤ 0.5
D001	≤ 2	4	1	4	≤ 0.06	2	2	2	0.5	4	≤ 0.5
F020	8	2	8	2	0.25	2	2	4	2	≤ 2	≤ 0.5
H002	≤ 2	2	1	2	≤ 0.06	2	1	2	0.25	8	≤ 0.5
I002	≤ 2	4	1	2	0.12	2	1	2	1	4	≤ 0.5
A019	4	4	4	8	0.12	2	2	8	4	8	≤ 0.5
F002	≤ 2	2	1	1	0.12	2	1	4	0.5	4	≤ 0.5
I005	4	4	2	2	0.12	2	2	4	1	4	≤ 0.5
H008	≤ 2	8	2	2	0.12	2	1	4	2	4	≤ 0.5
D004	≤ 2	2	1	1	0.25	2	≤ 0.5	4	0.25	≤ 2	≤ 0.5
R005	>32	8	>8	>8	>4	1	>8	>8	>32	>128	>8
H011	≤ 2	8	4	4	0.5	2	1	4	2	32	≤ 0.5

[a] Susceptibility performed by Sensititre (Thermo Fisher Scientific, UK) using custom-designed Gram-negative antimicrobial susceptibility plate. [b] Grey shading reflects resistant MICs applying EUCAST clinical breakpoints. Abbreviations: Amikacin (AMK), aztreonam (ATM), cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (CST), gentamicin (GEN), imipenem (IPM), meropenem (MEM), piperacillin-tazobactam (TZP, concentration of tazobactam is fixed at 4 mg/L), tobramycin (TOB).

Table S3. Mean PK/PD parameters, including all test isolates.

	*f*AUC$_{0\text{-}24}$/MIC (95% CI)					R^2
	IC_{50}	**Stasis**	**1 log_{10} kill**	**2 log_{10} kill**	**3 log_{10} kill**	
Single dose						
24 h	556 (182 – 1188)	178 (103 – 369)	349 (196 – 671)	623 (334 – 1139)	1135 (575 – 1997)	0.6748
48 h	2985 (1564 – 5811)	958 (581 – 1837)	1875 (1105 – 3388)	3347 (1903 – 5795)	6101 (3305 – 10219)	0.4431
72 h	7495 (4386 - 14096)	2404 (1482 – 4498)	4706 (2823 – 8329)	8404 (4871 – 14286)	15317 (8485 – 25237)	0.4566
7-doses, q24						
24 h	523 (167 – 1082)	168 (99 – 339)	328 (187 – 620)	586 (321 – 1054)	1069 (553 – 1852)	0.7367
48 h	1276 (670 – 2288)	409 (251 – 771)	801 (478 – 1426)	1431 (825 – 2444)	2607 (1435 – 4316)	0.6967
72 h	1958 (1176 - 3228)	628 (407 – 1085)	1229 (779 – 2034)	2195 (1354 – 3518)	4001 (2380 – 6250)	0.7137
216 h	16721 (10425 – 29907)	5363 (3406 - 9575)	10499 (6506 – 17863)	18747 (11268 – 30786)	-[a]	0.5323

[a] value not presented as no isolate achieved a 3 log_{10} kill.

Chapter 7

Fosfomycin efficacy against *Enterococcus* spp.

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Fosfomycin efficacy against *Enterococcus* spp.

Oral antimicrobial options for multidrug-resistant (MDR) Gram-negative uropathogens are limited. The previous paper demonstrated that oral fosfomycin for *P. aeruginosa* was unlikely to be a successful, even when multiple daily doses were administered. The amplification of a pre-existing resistance subpopulation was not only evident in the re-growth but was promoted following 7-doses given daily. This paper cautions against the use of fosfomycin monotherapy for *P. aeruginosa* UTI treatment.

This next paper, published in *Antimicrobial Agents and Chemotherapy*, examines the efficacy of fosfomycin against *Enterococcus* spp.. Fosfomycin has been shown to have good *in vitro* susceptibility against both *E. faecalis* and *E. faecium* clinical isolates, including vancomycin-resistance enterococci (VRE). Both high and low urinary fosfomycin exposures were simulated, in recognition of the large interpersonal variability seen in urinary fosfomycin concentrations.

Highlights

- The *in vitro* model was able to accurately simulate different urinary fosfomycin exposures, reflecting the wide variability in reported human urinary concentrations.

- Bacterial kill (>3 $\log_{10}$ reduction in bacterial density) and suppression of regrowth was promoted in the majority of isolates following high urinary exposures after a single dose, and after two-daily doses in the setting of lower urinary exposures.

- Low-level and sustained re-growth post-fosfomycin exposure was related to isolate persistence, rather than emergence of resistance, supporting the reported bacteriostatic activity of fosfomycin against enterococci.

- Where reduced urinary fosfomycin exposure was simulated, a second dose given at 24 h resulted in comparative results to a single dose with high urinary exposure.

- Overall, oral fosfomycin displayed reasonable activity against *Enterococcus* spp. and may represent an attractive alternative treatment option.

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PHARMACOLOGY

Oral Fosfomycin Treatment for Enterococcal Urinary Tract Infections in a Dynamic *In Vitro* Model

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ABSTRACT There are limited treatment options for enterococcal urinary tract infections, especially vancomycin-resistant *Enterococcus* (VRE). Oral fosfomycin is a potential option, although limited data are available guiding dosing and susceptibility. We undertook pharmacodynamic profiling of fosfomycin against *E. faecalis* and *E. faecium* isolates using a dynamic *in vitro* bladder infection model. Eighty-four isolates underwent fosfomycin agar dilution susceptibility testing (*E. faecalis* $MIC_{50/90}$ 32/64 μg/ml; *E. faecium* $MIC_{50/90}$ 64/128 μg/ml). Sixteen isolates (including *E. faecalis* ATCC 29212 and *E. faecium* ATCC 35667) were chosen to reflect the MIC range and tested in the bladder infection model with synthetic human urine (SHU). Under drug-free conditions, *E. faecium* demonstrated greater growth restriction in SHU compared to *E. faecalis* (*E. faecium* maximal growth 5.8 ± 0.6 $\log_{10}$ CFU/ml; *E. faecalis* 8.0 ± 1.0 $\log_{10}$ CFU/ml). Isolates were exposed to high and low fosfomycin urinary concentrations after a single dose, and after two doses given over two days with low urinary concentration exposure. Simulated concentrations closely matched the target (bias 2.3%). *E. faecalis* isolates required greater fosfomycin exposure for 3 $\log_{10}$ kill from the starting inoculum compared with *E. faecium*. The $fAUC_{0\text{-}72}$/MIC and $f\%T > MIC_{0\text{-}72}$ for *E. faecalis* were 672 and 70%, compared to 216 and 51% for *E. faecium*, respectively. There was no rise in fosfomycin MIC postexposure. Two doses of fosfomycin with low urinary concentrations resulted in equivalent growth inhibition to a single dose with high urinary concentrations. With this urinary exposure, fosfomycin was effective in promoting suppression of regrowth (>3 $\log_{10}$ kill) in the majority of isolates.

KEYWORDS *Enterococcus*, fosfomycin, in vitro modelling, pharmacodynamics, pharmacokinetics, synthetic human urine, urinary tract infection, vancomycin resistance

Urinary tract infections (UTIs) are among the most common infections experienced worldwide (1, 2). UTIs caused by *Enterococcus* spp. are responsible for approximately 5% of community-acquired infections, the third most leading cause of hospital-acquired UTIs, and implicated in 30% of catheter-associated UTIs (3). The most prevalent species is *E. faecalis*, which is more virulent than *E. faecium* but with less intrinsic and acquired antimicrobial resistance. Enterococci are well-adapted pathogens and are

Citation Abbott IJ, van Gorp E, van der Meijden A, Wijma RA, Meletiadis J, Roberts JA, Mouton JW, Peleg AY. 2020. Oral fosfomycin treatment for enterococcal urinary tract infections in a dynamic *in vitro* model. Antimicrob Agents Chemother 64:e00342-20. https://doi.org/10.1128/AAC.00342-20.

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Received 20 February 2020
Returned for modification 19 March 2020
Accepted 26 March 2020
Accepted manuscript posted online 6 April 2020
Published 21 May 2020

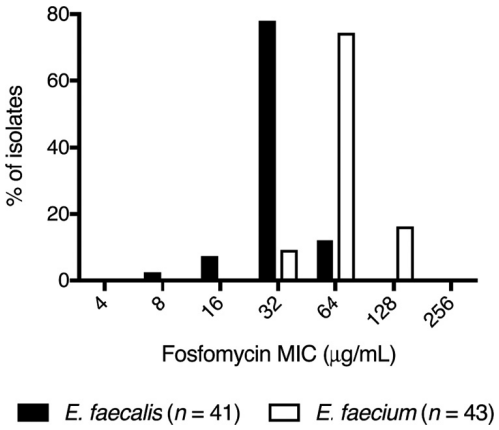

FIG 1 Enterococcal fosfomycin MIC distribution. *E. faecalis* ($n = 41$; black bars) and *E. faecium* ($n = 43$; white bars) isolates screened for susceptibility by agar dilution. *E. faecalis* $MIC_{50}/MIC_{90} = 32/64$ μg/ml, *E. faecium* $MIC_{50}/MIC_{90} = 64/128$ μg/ml.

able to survive in harsh conditions, with the capacity to attach to both host cells and inert materials and form biofilms (4). They have a broad spectrum of intrinsic resistance and tolerance to the bactericidal activity of many agents and can readily acquire new resistance to antimicrobials. Acquired resistance rates have progressively risen over time (5). Of particular concern is the increase in vancomycin-resistant *Enterococcus* (VRE) strains, now accounting for up to 80% of *E. faecium* isolates in some hospitals (6).

Fosfomycin demonstrates good *in vitro* activity against enterococci, including VRE strains (7–10), and is licensed for the treatment of uncomplicated UTIs as a single 3 g oral dose, which is well tolerated and achieves high concentrations in the urine (11–14). With a unique chemical structure and without cross-resistance with other agents, oral fosfomycin is gaining interest in its expanding role for the treatment of multidrug-resistant UTIs (15–17), and as a therapeutic option for enterococcal UTIs (18, 19). Clinical breakpoints for fosfomycin susceptibility is provided for *E. faecalis* urinary isolates by the Clinical and Laboratory Standards Institute (CLSI) (20). This breakpoint (agar dilution susceptible MIC $\leq$64 μg/ml) is the same as that provided for *Escherichia coli* urinary isolates, with the same requirement for the addition of 25 mg/liter glucose-6-phosphate (G6P) to the media for agar dilution susceptibility testing. Broth microdilution testing is not recommended. Similarly, for disk diffusion susceptibility testing, fosfomycin 200-μg disks also contain 50 μg of G6P, with the CLSI susceptible inhibition zone diameter $\geq$16 mm. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) does not provide fosfomycin breakpoints for *Enterococcus* spp. (21).

We examined fosfomycin susceptibility among clinical enterococcal isolates and assessed the efficacy of oral fosfomycin therapy in a dynamic bladder infection *in vitro* model against eight *E. faecalis* and eight *E. faecium* isolates in synthetic human urine (SHU). To reflect the significant variability in fosfomycin urine concentrations among healthy individuals after oral dosing (11, 12), urinary exposures following a single simulated 3-g oral fosfomycin dose with high and low urinary exposures, and two doses given over two days with low urinary exposure, were simulated. Inhibition of regrowth and emergence of resistance were assessed.

RESULTS

***In vitro* susceptibility studies.** In total, 84 enterococci were screened for fosfomycin susceptibility using agar dilution, including 41 *E. faecalis* and 43 *E. faecium*. Forty-one (49%) isolates originated from blood cultures, 17 (20%) from wound cultures, and 15 (18%) from a urinary source. Isolates cultured from screening surveillance swabs were not assessed. Both species demonstrated relatively narrow MIC distributions with $MIC_{50/90}$ of 32/64 μg/ml for *E. faecalis* and 64/128 μg/ml for *E. faecium* (Fig. 1). Sixteen isolates that represented the range of fosfomycin MIC values were selected for testing

TABLE 1 Baseline fosfomycin susceptibility and growth outcomes in the bladder infection *in vitro* mode[a]

			Baseline fosfomycin susceptibility testing					Change in bacterial counts (Δ $\log_{10}$ CFU/ml)[c]			
				BMD MIC (μg/ml)					High exposure	Low exposure	
Strain	Source	*Van* gene[b]	AD MIC (μg/ml)	MHB	MHB + G6P	SHU	DD (mm)	Drug-free	Single dose	Single dose	Two doses, q24
E. faecalis											
42601	Urine	-	8	4	8	8	23	−1.2	-	−1.7	-
36361	Blood	-	16	8	8	8	23	0.9	−4.3	−2.1	−4.7
47130	Urine	-	32	64	64	16	23	1.7	−3.7	1.4	−4.9
16313	Urine	-	32	64	32	16	18	1.1	−4.1	0.1	-
29212	ATCC	-	32	32	32	16	17	1.2	−3.7	1.6	−4.9
46182	Blood	-	64	32	64	32	19	1.6	−2.7	0.7	−4.8
46639	Blood	-	64	64	64	16	15	1.6	−4.2	−1.8	−4.7
46222	Blood	-	64	64	64	32	12	1.6	−3.2	2.0	−3.6
E. faecium											
44131	Aspirate	A	32	16	16	16	17	−0.6	-	-	-
20143	Blood	A	32	32	32	32	18	−1.0	-	−3.9	-
12818	Urine	A	32	64	128	32	19	−0.9	-	−1.9	-
35667	ATCC	-	64	64	64	32	14	0.2	−5.0	−0.7	-
01976	Urine	A	64	32	32	16	19	−1.6	-	−1.7	-
20292	Urine	B	64	64	64	64	13	−0.7	-	−1.8	−4.7
08582	Urine	A	64	128	64	32	14	−1.2	−4.7	−1.1	−4.1
14242	Blood	A	128	64	64	32	15	−1.5	-	-	-

[a]AD, agar dilution; BMD, broth microdilution; MHB, Mueller-Hinton broth; G6P, glucose-6-phosphate; SHU, synthetic human urine; DD, disk diffusion.
[b]Symbol -, *vanA* and *vanB* gene not detected.
[c]Change in total bacterial density from a starting inoculum of approximately 7.0 $\log_{10}$ CFU/ml after an 18 h drug free incubation, or 72 h incubation following fosfomycin exposure. Symbol -, no growth detected after 72 h incubation.

in the bladder infection model, including 14 clinical strains and 2 ATCC isolates (Table 1). The agreement between agar dilution and broth microdilution (BMD) MIC methods were examined, including the effect of alterations in the BMD liquid medium. MIC values were largely concordant between agar dilution and broth microdilution (BMD) performed in Mueller-Hinton broth (MHB) with 25 mg/liter G6P (Fig. 2a). There was no significant difference in MIC measurements when tested without G6P (Fig. 2b). MIC values were, on average, one dilution lower compared to agar dilution when tested in modified SHU (Table 2) (bias −0.9, 95% confidence interval [CI] −2.3 to 0.4), suggestive of an interplay between growth restriction and increased fosfomycin antimicrobial activity in this nutritionally deplete, acidic medium (Fig. 2c). Disk diffusion inhibition diameters were less than 24 mm in all isolates, and 4 isolates had zone sizes less than 16 mm (CLSI breakpoint: susceptible [S] at ≥16 mm) (Table 1). There was relatively poor linear correlation between inhibition diameter and agar dilution MIC (R^2 0.5537) (Fig. 2d). No differences were found between the two species. All clinical *E. faecium* isolates were vancomycin resistant and contained either a *vanA* or *vanB* gene.

Comparative growth in urine and SHU. Isolate growth capacity was calculated by quantifying the maximal bacterial density after overnight incubation in modified SHU and pooled female urine (FU) and compared to establish how well the synthetic medium matched the enterococcal growth kinetics in a urinary environment. Following 24 h static incubation in modified SHU, the eight selected *E. faecalis* isolates were able to increase their bacterial density from 5.0 ± 0.2 $\log_{10}$ CFU/ml to 7.0 ± 1.2 $\log_{10}$ CFU/ml, and the eight *E. faecium* isolates from 4.9 ± 0.1 $\log_{10}$ CFU/ml to 6.4 ± 0.8 $\log_{10}$ CFU/ml. This represented a greater growth restriction compared to incubation in FU, where *E. faecalis* isolates achieved a 24-h bacterial density of 7.8 ± 0.7 $\log_{10}$ CFU/ml and *E. faecium* isolates achieved 7.7 ± 0.4 $\log_{10}$ CFU/ml (Fig. 3a). There was also a greater variation in growth capacity between strains in modified SHU compared to FU (coefficient of variation [CV] of 13 to 17% versus 5 to 9%, respectively). When tested in fosfomycin time-kill assays, the *E. faecalis* and *E. faecium* ATCC strains demonstrated comparable fosfomycin activity in modified SHU compared with FU (Fig. 3b). Whereas, testing performed in MHB demonstrated greater drug-free growth capacity and reduced fosfomycin kill when exposed to 32 mg/liter. Given that the large media volume requirements for testing in the bladder infection model precluded the use of FU, which

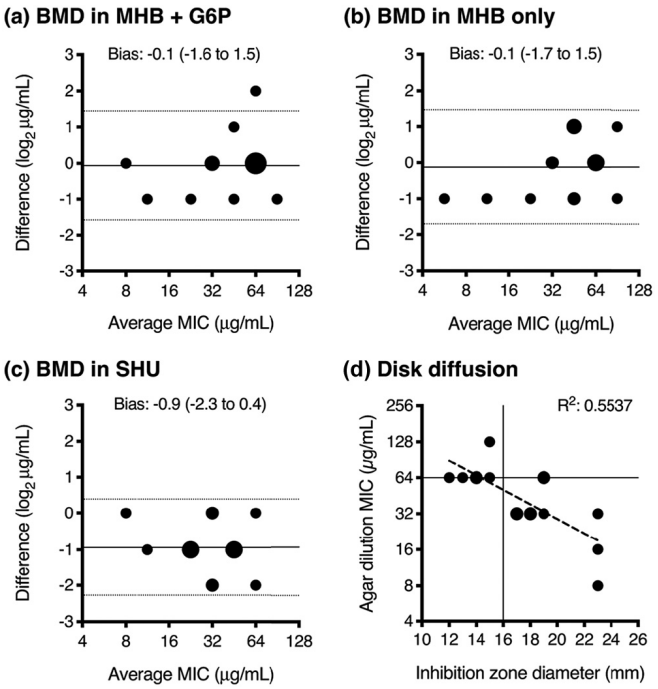

FIG 2 Comparison of fosfomycin susceptibility methods. Bland-Altman plots demonstrate the comparison between MIC values tested by agar dilution (AD) and broth microdilution (BMD) in MHB glucose-6-phosphate (G6P) (a), MHB alone (b), and synthetic human urine (SHU) (c), with the difference between the two MIC measurements (by AD and BMD) on the *y* axis, and the average of the two MIC measurements on the *x* axis. The bias (solid line) and 95% limits of agreement (dotted lines) are presented. Disk diffusion results (d) are compared to agar dilution MIC values, presented as a scattergram and analyzed by linear regression (dashed line), with the CLSI *E. faecalis* breakpoints highlighted (susceptible agar dilution MIC $\leq$64 μg/ml, disk diffusion inhibition zone diameter $\geq$16 mm). The larger circle sizes indicate a greater number of superimposed data points.

would require ethically approved collections, filter sterilization, and use within several days of collection, modified SHU was used in preference to standard laboratory media in order to better mimic the urinary environment.

Fosfomycin exposure in the bladder infection *in vitro* model. The bladder infection *in vitro* model provides a dynamic simulation of urinary fosfomycin exposure, enabling the assessment of the response of bacterial pathogens inoculated within sixteen independent bladder compartments. Observed *in vitro* fosfomycin concentrations accurately reproduced the expected urinary exposures following a single 3-g oral dose with both high and low urinary concentrations, and following two doses given over two days with low urinary concentrations. The slope of the linear regression line was equal to 1.0 (R^2 0.99) with a bias of 2.9% (95% CI −20.9 to 26.7%) (Fig. 4). Percentage variations were most apparent when target concentrations were at their lowest (i.e., $<$15 mg/liter). During each experiment, when all bladder compartments were sampled at the time of peak concentration, there was minimal intercompartment variability, with an average relative standard deviation of 4.4 $\pm$ 2.3%.

Growth outcome in the bladder infection *in vitro* model. Following drug-free dynamic incubation for 18 h in the bladder infection model, in modified SHU, all *E. faecalis* isolates demonstrated an increase in bacterial density from an average starting inoculum of 6.9 $\pm$ 0.1 $\log_{10}$ CFU/ml to 8.0 $\pm$ 1.0 $\log_{10}$ CFU/ml, except one strain (*E. faecalis* 42601) that had a reduction in bacterial density to 5.6 $\log_{10}$ CFU/ml (Fig. 5 and Table 1). In contrast, all *E. faecium* isolates had, on average, a reduction in their bacterial density from 6.7 $\pm$ 0.2 $\log_{10}$ CFU/ml to 5.8 $\pm$ 0.6 $\log_{10}$ CFU/ml (Fig. 5 and Table 1). Compared to static incubation, which had a lower starting inoculum (4.9 $\pm$ 0.2 $\log_{10}$ CFU/ml), the growth capacity (maximal bacterial density achieved) under dynamic conditions was higher for *E. faecalis* (static growth capacity: 7.0 $\pm$ 1.2 $\log_{10}$ CFU/ml), but lower for *E. faecium* (static growth capacity: 6.4 $\pm$ 0.8 $\log_{10}$ CFU/ml).

TABLE 2 Modified synthetic human urine (SHU) chemical constituents

Chemical name	Chemical formula	g/liter
Sodium chloride	NaCl	5.844
Sodium sulphate	Na_2SO_4	2.4147
Urea	Urea	16.8168
Potassium chloride	KCl	2.8329
Calcium chloride	$CaCl_2$	0.4439
Creatinine	Creatinine	1.0181
Citric acid trisodium salt dihydrate	$Na_3C_6H_5O_7$	1.9999
Ammonium chloride	NH_4Cl	1.0698
Magnesium sulphate	$MgSO_4$	0.3852
Sodium oxalate	$Na_2C_2O_4$	0.0241
Sodium phosphate monobasic	NaH_2PO_4	0.5616
Sodium phosphate dibasic	Na^2HPO_4	0.9227
Potassium dihydrogen phosphate	KH_2PO_4	2.1774
Uric acid	$C_5H_4N_4O_3$	0.1009
Sodium bicarbonate	$NaHCO_3$	1.1341
Magnesium chloride hexahydrate	$MgCl_2{\cdot}6H_2O$	0.6506
Lactic acid	$C_3H_6O_3$	0.0991
Ferrous sulfate heptahydrate	$FeSO4{\cdot}7H_2O$	0.0014
20% (wt/vol) casamino acids		0.1 % (vol/vol)
10% (wt/vol) yeast extract		0.2 % (vol/vol)
Proteose peptone no. 3[a]		1.0

[a]An additional component added to the published SHU recipe in order to additionally support *E. faecium* growth. Final pH adjusted to 5.6.

After a single fosfomycin dose mimicking high urinary exposure, at 72 h all isolates had $\geq$3 $\log_{10}$ kill, with low-level regrowth (bacterial counts $\leq$3.6 $\log_{10}$ CFU/ml) detected in 7 of 8 *E. faecalis* and 2 of 8 *E. faecium* isolates (Fig. 5 and Table 1). However, when urinary exposures were reduced to mimic low urinary concentrations, $\geq$3 $\log_{10}$ kill was only detected in 2 *E. faecalis* and 1 *E. faecium* (Fig. 5 and Table 1). All but one *E. faecium* isolate had detectable regrowth. Bacterial counts at 72 h were within 0.5 $\log_{10}$ of the growth measured in the drug-free control in 4 of 8 *E. faecalis* and 2 of 7 *E. faecium*. Notably, administering a second fosfomycin dose at 24 h, with the same low urinary exposure profile, afforded similar pharmacodynamic (PD) results as the single dose with high exposure, with all isolates having a $\geq$3 $\log_{10}$ kill (Fig. 5 and Table 1). Low-level regrowth (bacterial counts of $\leq$3.2 $\log_{10}$ CFU/ml) was detected in 6 of 8 *E. faecalis* and 2 of 8 *E. faecium* (Fig. 5 and Table 1).

Emergence of high-level resistance (growth on Mueller–Hinton II agar [MHA] with 1,024 mg/liter fosfomycin) was not detected in any isolate and there was no rise in fosfomycin MIC in any postexposure regrowth. A postexposure, low-level resistant subpopulation (growth on MHA with 256 mg/liter fosfomycin) was detected in only two *E. faecalis* isolates (strains 46222 and 46182) following the single fosfomycin dose with low exposure. The density of this resistant growth was minimal (approximately 10^2 CFU/ml). The fosfomycin MIC of these subpopulations were elevated (MIC 512 μg/ml) when testing was performed by agar dilution after subculture on drug-free blood agar.

Exposure-response PK/PD analysis. Exposure-response curves, assessing fosfomycin exposure over 72 h, demonstrated that $f\%T_{0\text{-}72} >$ MIC provided the most reliable pharmacokinetic/pharmacodynamic (PK/PD) index for the suppression of regrowth, followed by $f\text{AUC}_{0\text{-}72}$/MIC (Fig. 6). The fC_{max}/MIC index generated the weakest relationship. There were significant differences between the two species, with *E. faecalis* isolates requiring greater drug exposures compared to *E. faecium* for a $>$3 $\log_{10}$ kill from the starting inoculum. The mean (95% CI) $f\text{AUC}_{0\text{-}72}$/MIC and $f\%T_{0\text{-}72} >$ MIC for 3 $\log_{10}$ kill for *E. faecalis* was 672 (462 to 1,081, R^2 0.63) and 70% (66 to 73%, R^2 0.89), compared to 216 (107 to 307, R^2 0.67) and 51% (46 to 56%, R^2 0.78) for *E. faecium*, respectively (Fig. 6a). These relationships and values were essentially unchanged when MIC measurements by BMD in MHB, or MHB with G6P, were used in the analysis (data not shown). When MIC measurements by BMD in modified SHU were used (Fig. 6b), the R^2 values were all improved and the mean (95% CI) $f\text{AUC}_{0\text{-}72}$/MIC and $f\%T_{0\text{-}72} >$ MIC

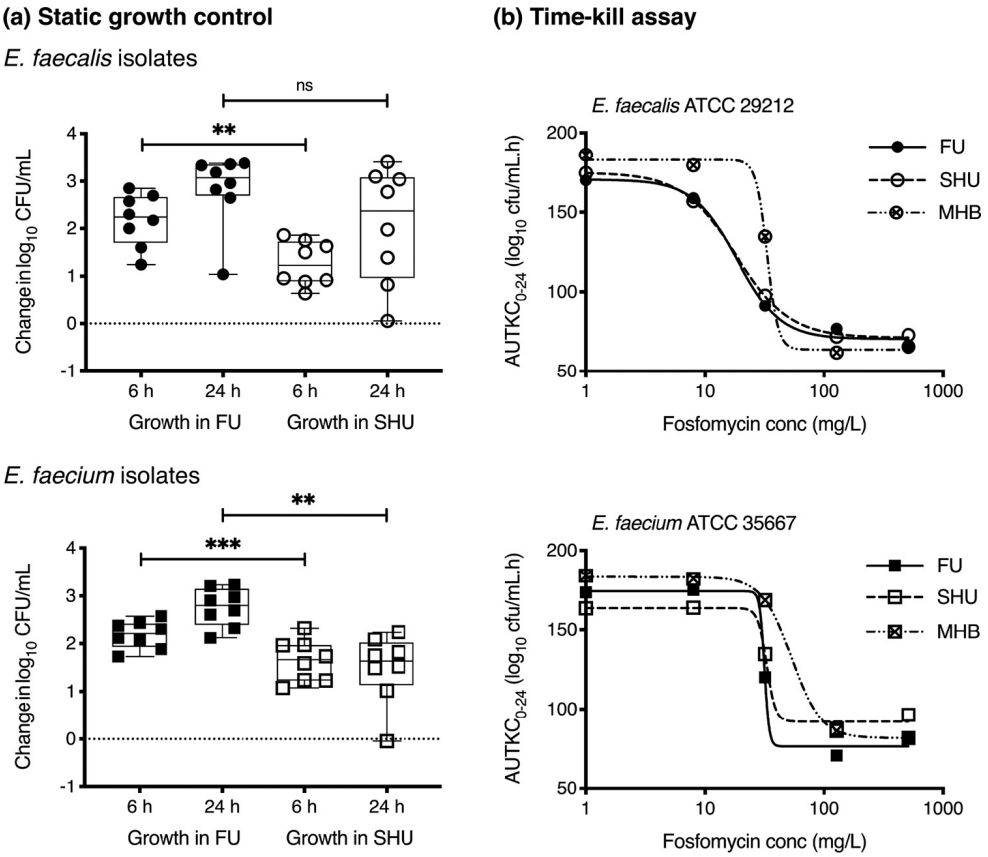

FIG 3 Impact of the medium on drug-free growth capacity and static time-kill assay. (a) Growth capacity of 8 *E. faecalis* (circles) and 8 *E. faecium* (squares) in pooled female urine (FU) and modified synthetic human urine (SHU). The average starting inoculum was 4.9 ± 0.2 $\log_{10}$ CFU/ml. Change in bacterial density was determined after 6 and 24 h of incubation. *ns*, not significant; **, $P \leq 0.01$; ***, $P \leq 0.001$. (b) Comparison of exposure-response curves of *E. faecalis* ATCC 29212 (circles) and *E. faecium* ATCC 35667 (squares) from the static time-kill assays performed in FU, modified SHU, and Mueller-Hinton broth (MHB). R^2 >0.99 for all variable-slope E_{max} nonlinear regression lines.

for 3 $\log_{10}$ kill for *E. faecalis* was 1,403 (1,108 to 1,832, R^2 0.73) and 78% (75 to 81%, R^2 0.92), compared to 425 (264 to 552, R^2 0.73) and 56% (49 to 61%, R^2 0.82) for *E. faecium*. In contrast, when assessing the fosfomycin exposure required to produce a 3 $\log_{10}$ kill from the drug-free growth capacity in the bladder infection model of each isolate, both *E. faecalis* and *E. faecium* returned similar responses. Analyzed together, the mean (95%

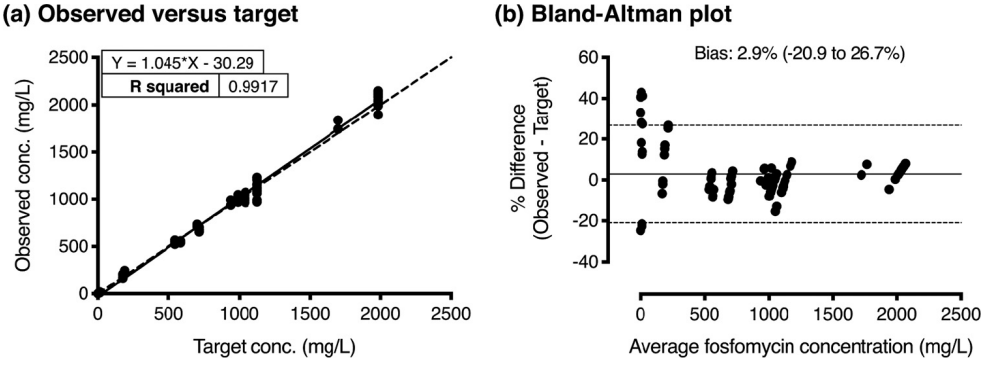

FIG 4 Relationship between the observed and targeted fosfomycin concentrations in the bladder infection model. (a) Scattergram of observed fosfomycin concentrations compared to target values, with linear regression (solid line) and $y = x$ (dashed line). (b) Bland-Altman plot of the percentage difference of the observed and target measurements (y axis) and the average of the two measurements (x axis) presented with the bias (solid line) and 95% limits of agreement (dotted lines).

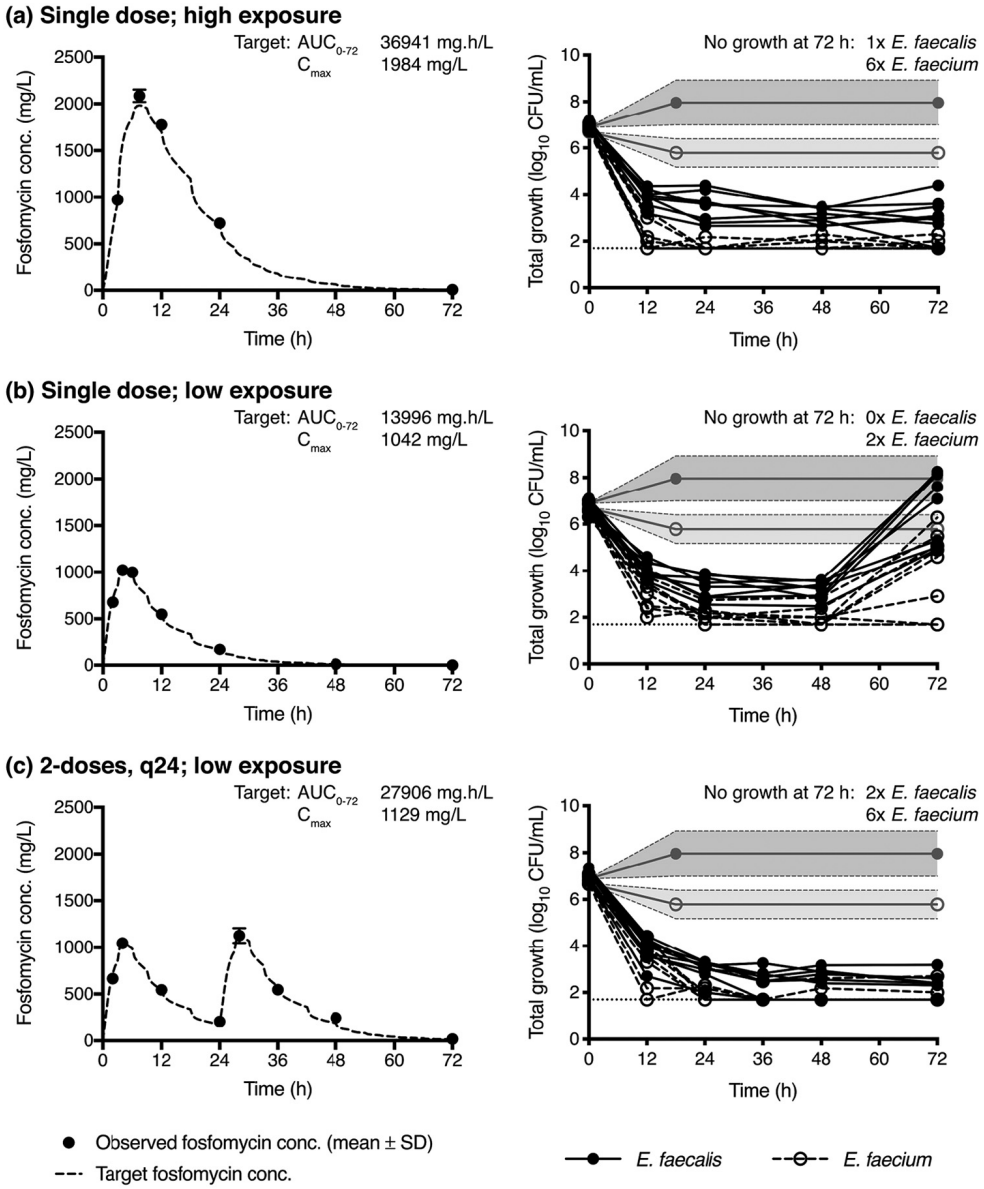

FIG 5 Fosfomycin exposure and growth outcome in the bladder infection model. Fosfomycin was administered as a single dose with high urinary exposure (a), a single dose with low urinary exposure (b), or 2 doses given daily with low urinary exposure (c). Concentration time-curves present the average measured fosfomycin concentration (solid circles), overlaid on the target concentration curve (dashed line). Note that the target urinary concentration curves in (a) to (c) do not demonstrate a smooth drug elimination phase due to the dynamic fluid shifts that occur after each voiding cycle of the bladder compartment. The corresponding growth curves present the total growth of *E. faecalis* (solid circles) and *E. faecium* (open circles) isolates over time. Limit of detection (dotted line) was considered 50 CFU/ml. The gray lines and shading correspond to the average (±SD) growth in SHU of both *E. faecalis* and *E. faecium* isolates after 18 h of incubation in the bladder infection model without fosfomycin.

CI) $fAUC_{0\text{-}72}$/MIC and $f\%T_{0\text{-}72} >$ MIC for 3 $\log_{10}$ kill from the growth capacity in SHU was 357 (276 to 458, R^2 0.63) and 59% (54 to 64%, R^2 0.62), applying agar dilution MIC measurements.

DISCUSSION

Following the dynamic simulation of oral fosfomycin treatment for enterococcal UTIs, bacterial kill (>3 $\log_{10}$) and suppression of regrowth was promoted in the majority of isolates following high urinary exposures after a single dose, and after two doses given over two days in the setting of lower urinary exposures. Low-level and sustained regrowth post-fosfomycin exposure was related to isolate persistence, rather than

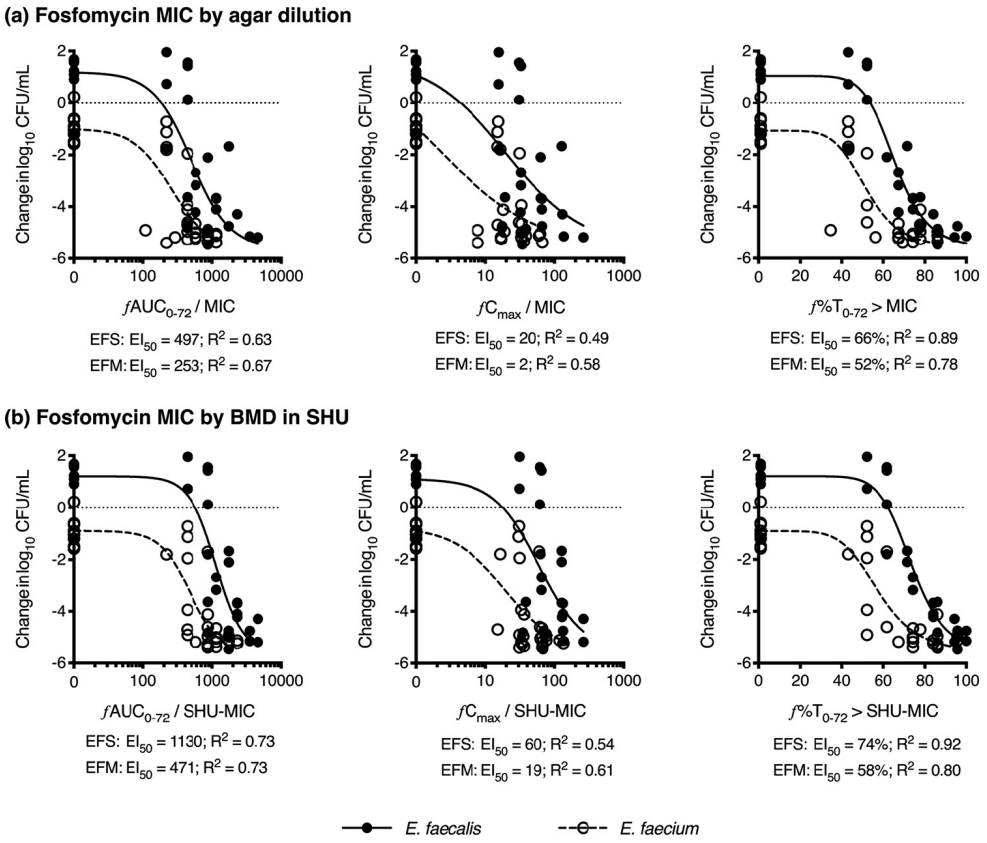

FIG 6 Exposure-response relationship in the bladder infection model. Fosfomycin exposure assessed by $fAUC_{0-72}$/MIC, fC_{max}/MIC and $f\%T_{0-72} >$ MIC. The data are presented with agar dilution MIC values (a) and MIC values by broth microdilution (BMD) performed in modified synthetic human urine (SHU) (b). Variable slope E_{max} nonlinear regression lines are shown for *E. faecalis* (EFS, solid circles and solid lines) and *E. faecium* (EFM, open circles and dashed lines). All nonlinear regression lines were compared by F test and found to be significantly different ($P \leq 0.001$).

emergence of resistance, supporting the reported bacteriostatic activity of fosfomycin against enterococci (22). Overall, *E. faecalis* isolates required greater fosfomycin exposure, relative to MIC, to promote kill compared with *E. faecium* (672 versus 216 $fAUC_{0-72}$/MIC, respectively). However, the greater growth restriction in modified SHU seen for the *E. faecium* isolates (1 $\log_{10}$ kill in drug-free SHU) would impact upon their kill/growth outcomes. Therefore, the lower fosfomycin exposure index required for 3 $\log_{10}$ kill from the starting inoculum in *E. faecium* is likely due to both the additional killing effect of the modified SHU medium itself and the reduced growth capacity under dynamic conditions, thereby shifting the *E. faecium* drug-exposure-effect curves to the left of those of *E. faecalis*.

The impact of the laboratory medium on bacterial growth kinetics and antimicrobial activity is an important factor when translating *in vitro* results to humans. The availability of nutritional factors has been demonstrated to impact upon the MIC and exposure-response values (23). Compared to standard laboratory media, nutritional factors can be reduced *in vivo*, and cannot only promote a slower growth but can also result in greater antimicrobial kill. This is especially relevant for infections involving the urinary tract, where normal urodynamics of the constant bladder filling and intermittent voiding has an important role in bacterial clearance. Infecting bacteria within the bladder must maintain a sufficient growth rate in the urine biomatrix to maintain a sufficient bacterial density to promote clinical infection, despite the constant dilution and elimination by urination. We demonstrate that enterococcal growth, especially that of *E. faecium*, is restricted in modified SHU, which is a nutrient-deplete medium specifically designed to mimic human urine (24, 25). This suggests that the use of SHU

to truly reflect *E. faecium* bacteriuric potential and response to antimicrobial therapy may be limited. The use of this medium, however, still offers distinct advantages over standard nutrient-rich laboratory media when simulating UTIs.

Molecular mechanisms for fosfomycin resistance among enterococci include the plasmid enzymatic inactivation gene (*fosB*) and acquired mutations in the active site of UDP-*N*-acetylglucosamine enolpyruyl transferase (Cys119Asp), both of which result in a rise in MIC to >1,024 mg/liter (26–28). In our model, however, despite the detection of postexposure regrowth, there was no rise in fosfomycin MIC. This is in contrast to other studies that have examined fosfomycin efficacy against *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, where fosfomycin efficacy was greatly impacted by baseline heteroresistance and demonstrated postexposure emergence of resistance (29–32). It is uncertain, however, if the lack of resistance observed in our study was affected by the medium environment (24), or if this is reflective of the bacteriostatic activity of fosfomycin reported for enterococci (22). The adaptation of enterococci during antibiotic exposure in this nutrient-restricted media may induce a fitness cost that limits the growth rate. Alternatively, the environment could induce a structural or metabolic change promoting persistence over the emergence of resistance (33, 34). Furthermore, enterococcal biofilm production within the *in vitro* model could shield a subpopulation of the bacterial inoculum to the effects of fosfomycin, and thereby seed the regrowth population when fosfomycin concentrations fall below the MIC of the isolate.

In clinical practice, the identification of an *Enterococcus* spp. from a urine culture may not reflect a true infection and may represent contaminating normal flora (35). When treatment is indicated, oral antimicrobial options active against *Enterococcus* spp. are limited. Similar to fosfomycin, aminopenicillins achieve high urinary concentrations and have been reported to overcome the resistance mechanisms in enterococci and provide a therapeutic option, despite a laboratory report of nonsusceptibility (36, 37). Nitrofurantoin is another active oral antimicrobial option, although activity is largely limited to *E. faecalis* isolates (38–40). Other oral options, albeit with limitations, include linezolid (toxicity risk), quinupristin-dalfopristin (*E. faecium* only), nitroxoline (not widely available), newer fluoroquinolones (moxifloxacin, gatifloxacin, gemifloxacin; toxicity risk) and doxycycline (effective in the setting of high urinary concentrations; synergy reported in combination with fosfomycin) (19, 39, 41–47).

A strength of this research is the use of a dynamic *in vitro* model to accurately simulate a range of urinary fosfomycin exposures while mimicking human urodynamics. Given the wide variability reported in human fosfomycin urinary concentrations, establishing the most relevant dynamic concentration to simulate poses a great challenge. It is unclear how differences in urinary fosfomycin exposure may impact antimicrobial efficacy. Much of this variation is likely due to individual behaviors, such as bladder emptying kinetics, fluid intake, urinary output, and the timing of the oral fosfomycin dose. In this study, two different exposure parameters were targeted from published healthy human data (11, 12). Wenzler et al. have subsequently published a phase 1 study of daily and alternate-daily dosing of oral fosfomycin (13). From these data the authors report that oral fosfomycin undergoes flip-flop kinetics, where excretion is limited by a slow absorption half-life. They also report urine fosfomycin concentrations at 48 h to be 45.2 ± 84.2 mg/liter after a single dose, and 312.7 ± 263.3 mg/liter after two daily doses. The wide range in standard deviations indicates large intersubject variability. In our model, the simulated fosfomycin concentrations at 48 h were as follows: high urine exposure (single dose) 65.5 mg/liter; low urine exposure (single dose) 12.6 mg/liter; low urine exposure (two daily doses) 188.8 mg/liter. Therefore, by simulating a wide range of different urinary exposures, we would expect to cover the majority of fosfomycin exposures expected to be seen in healthy individuals.

The important PK/PD index for fosfomycin efficacy in *Enterobacterales* has been reported to be *f*UC/MIC (48), and similarly for *P. aeruginosa*, where *f*AUC/MIC was pharmacodynamically linked as the driver for bacterial cell kill but with a time-dependence as the driver for resistance suppression (49). In our model, the PK/PD index

associated with 3 $\log_{10}$ kill was best described by maintaining urinary fosfomycin concentrations above the MIC for approximately 36 h (*E. faecium*) to 48 h (*E. faecalis*). Similarly, this was achieved by an $fAUC_{0\text{-}72}$/MIC between 216 (*E. faecium*) and 672 (*E. faecalis*). However, our data were not designed as a dose fractionation study, but as a reflection of real-life prescribing practices. This is particularly relevant given that two of the three fosfomycin dosing regimens studied were as a single dose, which causes all PK/PD indices to be intrinsically linked with each other. In order to extrapolate these data to humans, urinary fosfomycin PK following standard dosing should be considered, together with the expected fosfomycin susceptibility among enterococcal isolates. Given that the average peak urinary concentrations ($fAUC_{0\text{-}24}$) can range between 600 mg/liter (8,000 mg · h/liter) (13) and 1,982 mg/liter (20,000 mg · h/liter) (11), while urinary concentrations are maintained at >32 mg/liter for 48 h in the majority of subjects, one would expect that many *E. faecalis* and *E. faecium* isolates (reported MIC_{50} 32 and 64 μg/ml, respectively) (7, 8, 10, 26, 50, 51) would be adequately treated following standard oral fosfomycin therapy in an immunocompetent individual. However, where lower urinary fosfomycin exposures are expected, such as in the setting of increased fluid intake and high urinary volume output, or with an isolate with a higher MIC value (reported MIC_{90} 64 and 128 μg/ml for *E. faecalis* and *E. faecium* isolates), administration of a repeated daily dose (or multiple daily dosing), could better ensure PK/PD targets would be achieved. Given the known variability in urinary fosfomycin PK, and that lowering the current clinical breakpoint (CLSI: susceptible MIC ≤64 μg/ml for *E. faecalis*) would risk splitting the wild-type population of both *Enterococcal* spp., changing the dosing recommendations to support repeated daily doses may increase the likelihood that a susceptible result from the diagnostic laboratory would confidently predict clinical efficacy.

This dynamic bladder infection model is limited by the lack of an immune system and the tissue structure found in the human bladder. Given the impracticalities of using pooled human urine for the *in vitro* media, using a well-characterized synthetic alternative was chosen over the use of a nutrient-rich laboratory media. However, the chemical components included in the modified SHU used in these experiments may still not reflect the true *in vivo* situation and enterococcal growth kinetics. Further work would benefit learning how best to match enterococcal growth kinetics in human urine with specific variations in the chemical components in a further modified SHU recipe. It is also uncertain what would be the impact of the changes observed in the chemical composition of urine in patients with, or at risk of, UTIs, and how to best simulate these situations. Finally, our PK/PD analysis is limited by the fosfomycin exposures tested, and further studies would be required to fully support the quantitative targets recommended.

Overall, oral fosfomycin appears to display reasonable activity against *Enterococcus* spp. in a bladder infection model. Similar rates of regrowth suppression were found following a single 3-g dose with high fosfomycin urinary concentrations as followed two doses with lower urinary concentrations. Future *in vitro* research should examine any benefit of administering several daily doses of fosfomycin. The role of combination therapy of fosfomycin with amoxicillin, or fosfomycin with doxycycline, given that *in vitro* synergy has been reported (22, 47), would be of further interest.

MATERIALS AND METHODS

Antibiotics and media. Fosfomycin (Fomicyt, InfectoPharm, Germany) was used in the *in vitro* model and for medium production, reconstituted to a concentration of 50,000 mg/liter and frozen at −80°C. Trypticase soy agar containing 5% sheep blood (TSA) (Becton, Dickinson, USA) was used for subculturing isolates from freezer stock. Mueller–Hinton II agar (MHA) (BD) was used for quantification of bacterial density. MHA with fosfomycin added was used for agar dilution susceptibility testing and screening for fosfomycin resistance by quantifying growth on medium containing 256 mg/liter fosfomycin (low-level resistance, LLR) or with 1,024 mg/liter fosfomycin (high-level resistance, HLR). All media to which fosfomycin was added contained 25 mg/liter glucose-6-phosphate (G6P) (G7879-5G, Sigma–Aldrich, USA).

A modified recipe for synthetic human urine (SHU) (25) was used (Table 2) as the liquid medium for broth microdilution (BMD) susceptibility testing, growth control experiments, and as the substitute for

human urine in the bladder infection *in vitro* model. This medium provided advantages over donated pooled human urine, given the large volumes of liquid medium required, method of preparation, reproducible chemical composition between batches, and longer shelf-life. The chemical components of SHU have been previously published with casamino acids and yeast extract added to support the growth of *E. faecalis* isolates (52). However, *E. faecium* isolates failed to grow in this supplemented SHU and required further modification by the addition of proteose peptone, a supplement used in an alternate published synthetic urine medium termed artificial urine medium (AUM) (53). The final constituents of the modified SHU used for all experiments included 20% (wt/vol) casamino acids (BD), 10% (wt/vol) yeast extract (BD), and 1 g/liter proteose peptone no. 3 (BD), with the final pH adjusted to 5.6. Pooled female urine (FU) was used as a comparison for static growth and time-kill assays. Urine was collected from 12 healthy female volunteers after ethical committee approval (Medisch Ethische Toetsings Commissie, METC-2018-1186), pooled equally by volume, filter sterilized through a 0.22-μm filter system (Thermo Fisher Scientific Nalgene Rapid-Flow disposable filters, USA) and kept at −20°C until use.

Bacterial strains and *in vitro* susceptibility. Nonconsecutive, clinical *E. faecalis* and *E. faecium* isolates from patient samples were identified from the hospital diagnostic database with ethical committee approval (METC-2015-206). Rectal screening isolates were not included. Isolates underwent fosfomycin susceptibility by agar dilution MIC testing following the reference method (54). *E. coli* ATCC 25922 was used as the quality control organism. A selection of sixteen isolates (including 2 ATCC strains, *E. faecalis* ATCC 29212 and *E. faecium* ATCC 35667) were chosen to be tested in the bladder infection *in vitro* model, representing the range of baseline fosfomycin MIC values. Species identification was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS; Bruker Daltonik GmbH, Germany). Additional fosfomycin susceptibility testing was performed on the 16 selected isolates, including disk diffusion using FOT200 disk (Oxoid Ltd./Thermo Fisher Scientific, UK) and BMD MIC testing following reference methodology applicable to other antimicrobial agents (54), performed in MHB, MHB with G6P, and in modified SHU. MIC values determined by agar dilution were performed in triplicate, with the median result reported. Isolate DNA was extracted using the MagNA Pure 96 system, and the Roche LightCycler 480 instrument was used to identify the presence of *vanA* and *vanB* genes using in-house primer and probe sequences. In each run, positive controls (VanA *E. faecium*, VanB *E. faecalis*), a negative control (sterile saline), and an internal control (Phocine herpesvirus, PhHV) were included.

Static growth and time-kill assay. The sixteen selected isolates underwent assessment of drug-free growth capacity in modified SHU compared with FU. The growth capacity was considered the maximal bacterial density achieved after overnight incubation in drug-free media. Bacterial inocula were prepared from a fresh overnight culture from freezer stock onto TSB, from which a 0.5 McFarland suspension was serially diluted to a starting inoculum of 10^5 CFU/ml. Each isolate was incubated in 10 ml of both FU and modified SHU at 37°C with shaking (200 rpm). Assessment of bacterial growth was performed at 6 and 24 h. *E. faecalis* ATCC 29212 and *E. faecium* ATCC 35667 were additionally assessed in static fosfomycin time-kill assays (TKA), comparing the activity of fosfomycin in FU, modified SHU, and MHB. Bacterial inocula were prepared by the same method described above and were exposed to fosfomycin at four different concentrations (8, 32, 128, and 512 mg/liter) together with a drug-free control tube. TKA tubes were incubated at 37°C with shaking (200 rpm). Bacterial density was assessed after 1, 3, 6, and 24 h of incubation. To account for antibiotic carryover, all samples (500 μl) underwent centrifugation (5 min 12,500 $\times$ *g*), removal of the supernatant, and addition of phosphate-buffered saline. This centrifuge and washing process was performed twice prior to plating.

Dynamic bladder infection *in vitro* model. The dynamic bladder *in vitro* model was used to simulate urinary antimicrobial concentration changes by modeling tandem first-order processes and applying drug distribution equations, while concurrently simulating normal human urodynamics. This model has been previously applied to fosfomycin efficacy against *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Pseudomonas aeruginosa* pathogens (30, 55), the examination of multiple repeat doses and different urinary exposures (56), and the effect of running the model with pooled human urine and synthetic alternatives (24). The *in vitro* setup consists of a reservoir that delivers fresh medium at a constant rate into the gastrointestinal compartment, in which fosfomycin is administered. Peristaltic pumps control matching flow rates to simulate rapid fosfomycin absorption, systemic distribution, and elimination into sixteen bladder compartments run in parallel. Bladder compartments were voided six-times per day by a timed peristaltic pump program, reducing the contents within each compartment (medium and bacteria) to a postvoid residual volume of approximately 3 ml. Dynamic urinary fosfomycin exposures were simulated following a 3-g oral dose. Test isolates were added to each bladder compartment at an inoculum of 10^7 CFU/ml to provide an equivalent total number of bacteria expected in human infections (i.e., $>10^5$ CFU/ml in an average 250 ml void). Drug-free growth capacity was determined following 18 h of incubation within the bladder infection model without the addition of fosfomycin. Subsequently, pathogen growth and emergence of resistance was assessed in response to different simulated urinary fosfomycin exposures over 72 h by quantitative cultures on drug-free and fosfomycin-containing (256 and 1,024 mg/liter) MHA. Given the large inter- and intrasubject variability reported in urinary fosfomycin concentrations, two different PK exposures following a single 3-g oral dose were simulated, namely, a peak urinary concentration of 1,982.0 mg/liter at 7.5 h (11), compared to a peak of 1,040 mg/liter at 4 h (12). The impact of administering a second 3-g dose of fosfomycin at 24 h in the setting of the lower urinary exposure was also examined.

***In vitro* sample processing.** Samples were taken directly from each bladder compartment at the predetermined time points. Quantitative cultures for PD assessments were processed immediately, with bacterial density (CFU/ml) calculated at each time point. Specifically, medium from the outflow tract of

each bladder compartment was sampled and underwent serial 10-fold dilutions, from which 20 μl of each dilution was plated onto drug-free MHA, or MHA with 256 or 1,024 mg/liter of fosfomycin. The lower limit of detection was considered to be 50 CFU/ml. All plates were incubated aerobically at 37°C for 16 to 20 h. Plates supplemented with fosfomycin were reincubated for a further 24 h to confirm colony counts. The fosfomycin MIC of any regrowth at 72 h was rechecked by agar dilution after subculture to TSB.

Samples for fosfomycin concentration quantification, which first underwent a 1:10 dilution with saline when concentrations were expected to be above the upper limit of the assay, were frozen at −80°C until testing. An ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS) method was used (57). The UPLC-MS-MS method was validated for urine and plasma samples of fosfomycin, but additional tests confirmed its applicability for fosfomycin in SHU samples. The stability of fosfomycin in SHU at 37°C for 72 h, and stored at −80°C for at least 6 months, was confirmed (data not shown). The method was validated according to FDA guidelines for bioanalytical method validations (58), over a range of 0.75 to 375 mg/liter (R^2 0.9998). The lower limit of quantification was 0.75 mg/liter and the lower limit of detection was 0.70 mg/liter. The method was found to be accurate and precise with a maximum deviation of 5.0%. During experimentation, all bladder compartments were sampled at the peak concentration time point, providing an assessment accuracy and intercompartment variation. At the remaining time points, three bladder compartments were sampled before and after the peak concentration, and at 24, 48, and 72 h, thereby providing representative concentrations changes over time. All measured concentrations were compared to the target determined by PK drug distribution equations (59).

Statistical and PK/PD analyses. (i) MIC comparisons. The bias between MIC susceptibility measurements by BMD compared to agar dilution was calculated using the method described by Bland and Altman on $\log_2$-transformed MIC data (60), with a negative bias value representing the degree that BMD measurements underestimate the MIC compared to agar dilution, with a 95% confidence interval.

(ii) Static time-kill curves. Growth capacity in FU and modified SHU at 6 h and 24 h were compared with Student's paired *t* test for each species. Fosfomycin concentration–effect curves were analyzed with nonlinear regression analysis using the variable slope sigmoid E_{max} model, with the line parameters (top, bottom, EC_{50}, and Hill slope) in MHB, FU, and SHU compared with the F tests.

(iii) Dynamic time-kill curves. In the bladder infection *in vitro* model, the accuracy of the observed fosfomycin concentrations was determined by linear regression and Bland-Altman analysis in terms of bias and precision. Exposure-response relationships were analyzed using the total predicted free drug area-under-concentration-time curve ($\mathit{f}AUC_{0-72}$), the measured free drug maximal concentration ($\mathit{f}C_{max}$) and the predicted percentage of time that free drug concentrations were maintained above the MIC of the isolate ($\mathit{f}\%T_{0\text{-}72\ >\ MIC}$). Relationships were compared using MIC measurements obtained by agar dilution and BMD. Nonlinear regression was performed using a variable slope E_{max} model, assessing the bacteria counts in the drug-free control and following the different fosfomycin exposures. Curves were constrained by the lower limit of bacterial count quantification as the bottom parameter and compared statistically using the F test. The 50% effective PK/PD index (EI_{50}) and drug exposures associated with 3 $\log_{10}$ kill were determined. Where appropriate the data are presented as means ± SD. All analyses were performed with GraphPad Prism (version 7.0b, MAC OS X).

ACKNOWLEDGMENTS

We acknowledge the laboratory assistance of Carla Roodbol de Goeij (Department of Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre).

I.J.A. was funded by an Australian Government Research Training Program Scholarship (APP1114690) from the National Health and Medical Research Council of Australia. A.Y.P. and J.A.R. are funded partly through Australian National Health and Medical Research Council Practitioner Fellowships (APP1117940 and APP1117065, respectively).

J.W.M. has received research funding from Adenium, AstraZeneca, Basilea, Cubist, Polyphor, Roche, Eumedica, Basilea, VenatorX, AiCuris, Gilead, and Wockhardt. A.Y.P. has received research funding from MSD through an investigator-initiated research project. J.A.R. has received funding from MSD, Accelerate Diagnostics, bioMérieux, Pfizer, and The Medicines Company. All other authors have no conflicts to declare.

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Chapter 8

Conclusion and future perspectives

Chapter 8.1

Conclusions

Fosfomycin is a key antimicrobial in our fight against antimicrobial resistance (AMR). However, robust evidence supporting susceptibility testing, antimicrobial spectrum of activity and clinical dosing is limited. Across the six original research papers presented in this thesis, the use of a dynamic bladder infection model has facilitated an in-depth pharmacodynamic profiling of oral fosfomycin for the treatment of uncomplicated UTIs. Informed by drug distribution mathematical equations, this *in vitro* model has provided a robust, durable and adaptable method to accurately simulate different antimicrobial exposures in the bladder. A particular strength of the research is the ability to not only alter the media in which the experiments to best simulate the urinary environment, but also to run experiments for prolonged durations and under constant urodynamic kinetics.

Papers 1 and 2 have demonstrated how fosfomycin appeared to eradicate the majority of *E. coli* isolates following a single dose, but, in contrast, had a distinct lack of activity against *K. pneumoniae* isolates. Despite the good activity against *E. coli*, a significant limitation identified was that standard susceptibility testing did not predict which *E. coli* would respond and those that would regrow with amplification of the resistant subpopulation. This research suggests that current clinical breakpoints should be revised to ensure the accurate differentiation between isolates with a high likelihood of treatment success, and those in which emergence of resistance would limit antimicrobial activity.

In paper 3, we showed that the baseline characteristic of the pathogen was more important for treatment outcome than either the drug exposure after a single dose or following multiple doses. Therefore, we do not provide supporting *in vitro* evidence for the off-label practice of giving of repeat doses of fosfomycin. In paper 4, we showed that the biomatrix of urine impacted upon pathogen growth and fosfomycin activity, which was then able to be mimicked by the use of a synthetic alternative. Our findings question the validity of adding glucose-6-phosphate to the media for standard fosfomycin susceptibility testing for UTIs.

In the final two research papers, we demonstrated poor fosfomycin activity against *P. aeruginosa*, with the emergence of resistance promoted by multiple administered doses. While in *Enterococcus* spp., we showed reasonable bacteriostatic activity without any emergence of resistance.

The key findings of this research and recommendations include:

- Oral fosfomycin appears to have good activity against *E. coli* and enterococcal isolates.

- Treatment success, however, is significantly impacted upon by the presence of a pre-existing high-level resistant subpopulation among Gram-negative uropathogens, which is not identified by susceptibility testing by the standard method and the application of current clinical breakpoints.

- Heteroresistance appears to be almost universally present among *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolates, where fosfomycin activity is limited, even when isolates are exposed to high peak concentrations, or following prolonged exposures with administration of multiple doses.

- Where agar dilution remains the only accepted MIC susceptibility method, with the addition of glucose-6-phosphate, clinical breakpoints for *Enterobacterales* (EUCAST) and *E. coli* (CLSI) could benefit by reducing the susceptible category to around ≤ 2 mg/L. This would accurately identify *E. coli* isolates that do not have a resistant subpopulation, while classifying the majority of wild-type *K. pneumoniae* as non-susceptible.

- *E. coli* isolates with an agar dilution MIC 4 – 32 mg/L may, or may not, have a resistant subpopulation. In these isolates, an additional heteroresistance screen could help identify isolates that could still have a high likelihood of responding to therapy.

- Disk diffusion susceptibility testing for *Enterobacterales* appears to perform better at separating isolates with a high likelihood of treatment response and may provide a useful alternative. Other susceptibility methods, such as broth microdilution, with and without glucose-6-phosphate, require further investigation.

- Overall, this body of research provides important *in vitro* data in a humanised preclinical model that can inform antimicrobial susceptibility criteria and promote a considered and optimised approach to therapeutic choice and dosing.

Chapter 8.2

Future perspectives

The use and application of preclinical *in vitro* models in the examination and design of antimicrobial dosing regimens has increased overtime and are now important elements of regulatory submissions for new antimicrobials. Modern PK/PD *in vitro* models can characterise the entire time course of antimicrobial exposure, the killing effect and the impact on emergence of resistance. These models can, in turn, be used to inform experimental and clinical study design, as well as in the development of dosing guidelines customised to specific patient populations.

As presented in this thesis, the bladder infection *in vitro* model was developed to be adaptable to test different bacterial species, different antimicrobial dosing schedules, while also reflecting the interpersonal variations in urodynamics and antimicrobial exposures. Furthermore, the media in which the testing was performed was designed to reflect the site of infection. However, it still represents an over-simplification of a much more complex biological system. The host defence mechanisms, cell invasion, pathology of infection and pathogen virulence are not addressed. Furthermore, there is no assessment of antimicrobial toxicity relating to the exposures examined. However, a benefit of the *in vitro* model lies in the flexible design and descriptive results, while having distinct ethical advantages over animal studies. Through careful selection and planning of the experiments conducted, including the frequency and timing of samples, future research can be expanded at minimal cost. The *in vitro* design can be informed by antimicrobial exposure profiles in different patient populations and apply clinically relevant dosing schedules.

In order to increase testing capacity, it is necessary to make these methods less labour-intensive by the incorporation of standardisation and automation. Such methodological refinements would allow for the future evaluation of multiple old and new antimicrobial agents against a range of both common and rarer pathogens. Furthermore, the addition of macrophages to the *in vitro* model could further advance the clinical translatability of the results by simulating the host's immune response.

Ultimately, the work presented here represents the foundation for further preclinical *in vitro* testing of antimicrobials for UTIs. With particular emphasis on observations relating to emergence of resistance, the future use will inform antimicrobial choice, provide supporting evidence for site-specific clinical breakpoints and promote optimised dosing regimens that will preserve antimicrobial activity for the future.

Appendices

Co-author publications

- Gardiner BJ, Stewardson AJ, Abbott IJ, Peleg AY. Nitrofurantoin and fosfomycin for resistant urinary tract infections: old drugs for emerging problems. *Aust Prescr.* 2019;42(1):14-9. PMID: 30765904.

- Wijma RA, Huttner A, van Dun S, Kloezen W, Abbott IJ, Muller AE, *et al.* Urinary antibacterial activity of fosfomycin and nitrofurantoin at registered dosages in healthy volunteers. *Int J Antimicrob Agents.* 2019;54(4):435-41. PMID: 31382030.

- Ten Doesschate T, Abbott IJ, Willems RJL, Top J, Rogers MRC, Bonten MM, *et al. In vivo* acquisition of fosfomycin resistance in *Escherichia coli* by *fosA* transmission from commensal flora. *J Antimicrob Chemother.* 2019;74(12):3630-2. PMID: 31511878.

Australian Prescriber
VOLUME 42 : NUMBER 1 : FEBRUARY 2019

ARTICLE

Nitrofurantoin and fosfomycin for resistant urinary tract infections: old drugs for emerging problems

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Keywords
antibiotic resistance, fosfomycin, nitrofurantoin, urinary tract infection

Aust Prescr 2019;42:14–9
https://doi.org/10.18773/austprescr.2019.002

SUMMARY

Uncomplicated urinary tract infection is one of the most common indications for antibiotic use in the community. However, the Gram-negative organisms that can cause the infection are becoming more resistant to antibiotics.

Many multidrug resistant organisms retain susceptibility to two old antibiotics, nitrofurantoin and fosfomycin. Advantages over newer drugs include their high urinary concentrations and minimal toxicity.

Fosfomycin is a potential treatment option for patients with uncomplicated urinary tract infection due to resistant organisms. Nitrofurantoin may be more effective and can be used for urinary infections in pregnant women.

Introduction

Antimicrobial resistance is increasing worldwide, resulting in infections that are more difficult to treat and associated with higher mortality, morbidity and cost.[1-3] In Australia, multidrug resistant Gram-negative bacilli are responsible for a rising proportion of community-acquired uncomplicated urinary tract infections. Consequently, empiric therapy is more likely to fail. This has resulted in increasing numbers of patients with uncomplicated urinary tract infections requiring hospitalisation for intravenous antibiotics because there are no oral treatment options.

Limited Australian data are available for antimicrobial resistance rates in community-onset urinary tract infections.[4,5] One large national survey of urinary isolates from 2015 found resistance rates in *Escherichia coli* of 43% for ampicillin, 9% for amoxicillin with clavulanic acid, 16% for cefazolin, 22% for trimethoprim, and 7% for ciprofloxacin.[6] It is likely that resistance rates have continued to rise since then.

There are few new antibiotics on the horizon and those that have been recently approved are mostly for intravenous use, so older 'forgotten' drugs are being re-explored for the treatment of cystitis.[7-10] Nitrofurantoin and fosfomycin are old antibiotics. They share some important properties including high concentrations in the urinary tract, a minimal impact on gastrointestinal flora and a low propensity for resistance (Table).

Nitrofurantoin

Nitrofurantoin has been available since 1953, and in Australia since the 1970s. Its exact mechanism of action is not well understood and presumably multifactorial. Nitrofurantoin requires reduction by bacterial enzymes producing 'highly reactive electrophilic' metabolites. These then inhibit protein synthesis by interfering with bacterial ribosomal proteins.[11]

Nitrofurantoin has 80% oral bioavailability, and approximately 25% is excreted unchanged in the urine, with only a small portion reaching the colon.[12] Like fosfomycin, therapeutic concentrations are only reached in the urinary tract,[13] so the clinical use of nitrofurantoin is limited to the treatment of uncomplicated urinary tract infection in women. Administration with food results in higher urinary concentrations and fewer gastrointestinal adverse effects.

Antimicrobial activity

Nitrofurantoin is active against common causes of urinary tract infection including *E. coli*, *Citrobacter* and *Enterococcus*. *Klebsiella* and *Enterobacter* are less reliably susceptible. *Serratia*, *Acinetobacter*, *Morganella*, *Proteus* and *Pseudomonas* are usually resistant.[14] Overall, resistance to nitrofurantoin is uncommon and many multidrug resistant organisms retain susceptibility.[15-17] Australian data are limited, but studies suggest resistance rates in *E. coli* of 1–2%.[4,6]

Table **Features of nitrofurantoin and fosfomycin**

Characteristic	Nitrofurantoin	Fosfomycin
Year of discovery	1953	1969
Formulations	Nitrofurantoin macrocrystal 50 mg, 100 mg capsules Slow-release formulation not available in Australia Older microcrystal formulation less available now (more adverse effects)	Fosfomycin trometamol 3 g sachet containing granules to be dissolved in water Intravenous formulation available but for specialised use only
Pharmacokinetics	High urinary concentrations Serum concentrations negligible	Long half-life with high urinary concentrations Serum concentrations inadequate for treatment of systemic infection
Mechanism of action	Not well understood, multifactorial, inhibits ribosomal protein synthesis	Inhibits pyruvyl transferase and therefore cell wall synthesis
Spectrum of activity	Mostly susceptible: *E. coli*, *Enterococcus* Variably susceptible: *Klebsiella*, *Enterobacter*, *Citrobacter* and *Providencia* Typically resistant: *Proteus*, *Serratia*, *Acinetobacter*, *Morganella* and *Pseudomonas*	Mostly susceptible: *E. coli* Variably susceptible: *Klebsiella*, *Proteus*, *Citrobacter*, *Enterobacter*, *Pseudomonas* and *Enterococcus* Typically resistant: *Morganella* and *Acinetobacter*
Resistance	Uncommon	Uncommon
Indications	Uncomplicated urinary tract infection in women	Uncomplicated urinary tract infection in women
Dosing	50–100 mg 4 times a day for 5 days	Single 3 g oral dose
Adverse events	Infrequent, mainly gastrointestinal Rare reports of pulmonary or liver toxicity, peripheral neuropathy	Infrequent, mainly gastrointestinal (9% diarrhoea, 4% nausea)
Pregnancy and breastfeeding	Category A, although not recommended beyond 38 weeks gestation due to risk of haemolytic anaemia in neonates. For this reason it is also best to avoid during the first month of breastfeeding	Category B2, small amounts excreted in breast milk so not recommended in breastfeeding
Children	Avoid <1 month of age	Avoid <12 years of age
Interactions	Few significant drug interactions	Co-administration with metoclopramide can lower serum and urine concentrations
Renal impairment	Contraindicated if CrCl <30 mL/min Cautious use between CrCl 30–60 mL/min if benefits outweigh risks	Dose reduction required if CrCl <50 mL/min

CrCl creatinine clearance

Efficacy and safety

A meta-analysis of 27 older controlled trials (4807 patients) found clinical cure rates of 79–92%, similar to comparator antibiotics. Only mild toxicities (most commonly gastrointestinal) and no cases of pulmonary fibrosis or hepatotoxicity were reported.[18] Dosing recommendations for the standard formulation are 50–100 mg four times daily. There is a long-acting formulation available overseas, but not in Australia, which can be dosed twice daily. This slow-release formulation (100 mg three times daily) was used in a recent open-label comparison with fosfomycin. The cure rate was 70% in the nitrofurantoin group.[19]

Historically nitrofurantoin was thought to be contraindicated if the creatinine clearance was less than 60 mL/minute due to an increased risk of toxicity. However, recommendations have been changing to allow cautious, short-term use in patients with mild renal impairment (30–60 mL/min) if there are no alternative antibiotics.[20,21] Nitrofurantoin can be used to treat cystitis in pregnancy (although not beyond 38 weeks gestation due to the risk of haemolytic anaemia in the neonate).

Nitrofurantoin became a preferred drug in the international consensus guidelines for urinary tract infection in 2010.[22] These emphasised the lower rates of 'collateral damage' on gastrointestinal flora.[23-24]

It remains to be seen if resistance rates increase as a consequence of this recommendation and the subsequent rise in nitrofurantoin prescribing. The true incidence of major hepatic and pulmonary toxicity is unclear, but this appears to be more common with long-term use in the elderly.[14] For the short-term treatment of uncomplicated urinary tract infection in otherwise healthy young women, nitrofurantoin is a safe and effective choice, and overall efficacy and rates of adverse events appear similar to comparator antibiotics. In patients with infections due to multidrug resistant organisms and therefore few alternative treatment options, we recommend using 100 mg four times daily for five days, administered with food to optimise absorption and efficacy.

Fosfomycin

Fosfomycin was first isolated in Spain in 1969, and was introduced in Europe throughout the 1970s.[25] It is a small molecule from a unique drug class that acts by inhibiting pyruvyl transferase. This enzyme is responsible for synthesising the precursors of peptidoglycan, the key component of the bacterial cell wall. Uptake in the USA was initially limited due to problems with susceptibility testing, but this was standardised in 1983.

Fosfomycin trometamol, an oral formulation that can be taken as a single 3 g dose, was introduced in 1995. In many countries it is now a first-line treatment option for uncomplicated urinary tract infection in women.[22] This single-dose regimen is attractive due to better adherence and is generally well tolerated. While transient gastrointestinal disturbance can occur, serious adverse events are rare.[26]

In Australia, fosfomycin was only previously available via the Special Access Scheme. The Therapeutic Goods Administration has now approved it for acute uncomplicated lower urinary tract infection, in females more than 12 years of age, caused by susceptible organisms (Enterobacteriaceae including *E. coli*, and *Enterococcus faecalis*).

Antimicrobial activity

Susceptibility testing for fosfomycin is available, but can be complicated and is not necessarily routine in Australian microbiology laboratories. Fosfomycin is most active against *E. coli*, and minimum inhibitory concentrations are typically low.[27-29] Other urinary pathogens such as *Klebsiella*, *Proteus*, *Citrobacter*, *Enterobacter*, *Pseudomonas* and *Enterococcus* have variable susceptibility.[30-32] *Morganella morganii* and *Acinetobacter* are typically resistant.[28] Urinary concentrations following a single 3 g dose are generally sufficient to treat patients infected with susceptible organisms, although some recent data suggest more variability in urinary concentrations than previously thought.[33,34]

As fosfomycin has a unique structure there is minimal cross-resistance with other antibiotics. At present, many multidrug resistant isolates remain susceptible to fosfomycin, even in geographic regions where there has been widespread use of the drug.[35,36] No comprehensive studies examining fosfomycin susceptibility have been conducted in Australia.

While resistant subpopulations of bacteria may develop with fosfomycin exposure, resistant strains do not seem to easily survive in vivo.[32,37-40] However, there are multiple resistance mechanisms and there are reports of increasing resistance correlating with higher fosfomycin usage in Spain.[32,41-43] Plasmid-mediated resistance, which could disseminate more readily, has been described in Japan,[44] and among livestock[45] and pets[46] in China.

Efficacy and safety

Historically, the clinical efficacy of fosfomycin was thought to be similar to antibiotics such as trimethoprim, trimethoprim/sulfamethoxazole, fluoroquinolones, beta-lactams and nitrofurantoin, with reported cure rates of 75–90%.[47-51] However, methodological flaws in the older studies may have resulted in clinical efficacy being overestimated. A recent large randomised trial found a lower clinical cure rate with fosfomycin compared with nitrofurantoin (58% vs 70%, p=0.004).[19] While some recent observational studies have demonstrated fosfomycin efficacy in uncomplicated urinary tract infection caused by resistant organisms,[52-56] including non-inferiority to carbapenems,[57,58] there are reports of treatment failures particularly with *Klebsiella*.[59]

As low serum concentrations lead to treatment failures, fosfomycin is not appropriate for patients with bacteraemia or upper urinary tract infections such as pyelonephritis. Occasionally, longer courses have been used to treat complicated urinary tract infection, for example as completion therapy when there are no oral alternatives to intravenous antibiotics.[57] There is also an emerging role in prostatitis and perioperative prophylaxis for urological procedures in men.[60-62] Specialist infectious diseases input should be sought for these complex cases if off-label use or prolonged courses of therapy are being considered.

Fosfomycin is generally well tolerated, with adverse events rare and usually transient. Gastrointestinal events (9% diarrhoea, 4% nausea) have been most commonly reported with rare reports of other more serious problems.[26] Co-administration with metoclopramide can lower serum and urinary concentrations and should be avoided, but there are few other problematic drug

interactions. Fosfomycin is classified in pregnancy category B2. It is not recommended in breastfeeding as small amounts are excreted in breast milk. Given there are minimal data on use in children under 12 years of age, it is not advised for this group.

In Australia, we currently recommend reserving fosfomycin for the treatment of uncomplicated urinary tract infection in patients when the standard first-line drugs are not an option. Part of the rationale behind this is to minimise the emergence of resistance and prolong the usefulness of fosfomycin for patients without alternative options.[35] As resistance to other drugs inevitably rises and local experience increases, fosfomycin may become a first-line option in the future.

Antibiotic resistance

While re-exploring older 'forgotten' drugs like nitrofurantoin and fosfomycin is a useful strategy, it represents only part of the multifaceted response required to tackle the complex problem of antimicrobial resistance and 'preserve the miracle' of antimicrobials over the coming decades.[63] As we have seen historically with virtually all other antibiotics, resistance is likely to emerge as usage increases. It remains to be seen how long this will take, to what extent it will occur and whether it will be via dissemination of existing resistance mechanisms or evolution of new ones. The increasing failure of standard empirical therapy for urinary tract infection is foreseeable, and it is likely that more patients will require microbiological testing before starting antibiotics, not only for individualised patient management but also for broader epidemiological surveillance to inform guideline recommendations.

Consultation with an infectious diseases specialist can assist with the management of patients with multidrug resistant infections and leads to better outcomes.[64] Other important strategies include the development of new antimicrobial drugs, preserving those currently available by judicious use, implementation of comprehensive antimicrobial stewardship programs and stringent infection control practices worldwide to reduce the spread of resistant organisms.

Conclusion

Nitrofurantoin is suitable for uncomplicated lower urinary tract infections. Bacterial resistance is uncommon.

Fosfomycin is a safe and effective antibacterial drug for urinary tract infections, but its use should be limited to delay the development of resistance. It will prove to be a useful treatment option for community-based treatment of patients with resistant organisms. ≺

Bradley Gardiner and Iain Abbott are supported by Australian Government National Health and Medical Research Council (NHMRC) Research Training Program Scholarships (APP1150351 and APP1114690). Andrew Stewardson is supported by an NHMRC Fellowship (APP1141398). Anton Peleg is part funded through an NHMRC Practitioner Fellowship (APP1117940) and is the recipient of an investigator-initiated research grant from Merck, Sharp & Dohme.

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International Journal of Antimicrobial Agents 54 (2019) 435–441

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Urinary antibacterial activity of fosfomycin and nitrofurantoin at registered dosages in healthy volunteers

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ARTICLE INFO

Article history:
Received 14 May 2019
Accepted 21 July 2019

Editor: Matthew Falagas

KEYWORDS:
Fosfomycin
Nitrofurantoin
Urinary tract infection
Pharmacokinetics
Pharmacodynamics
Healthy volunteers

ABSTRACT

Given emerging uropathogen resistance to more recent antibiotics, old antibiotics used for uncomplicated urinary tract infection (UTI) warrant re-examination. In this study, the urinary antibacterial activities of fosfomycin and nitrofurantoin were investigated by determining the urinary inhibitory titre and urinary bactericidal titre against uropathogens in urine samples from female volunteers following administration of single-dose fosfomycin (3 g) or nitrofurantoin (50 mg q6h or 100 mg q8h). Urine samples were collected over 48 h (fosfomycin) or 6 or 8 h (nitrofurantoin), with drug levels quantified with every void. Fosfomycin concentrations ranged from <0.75 mg/L [lower limit of quantification (LLOQ)] to 5729.9 mg/L and nitrofurantoin concentrations ranged from <4 mg/L (LLOQ) to 176.3 mg/L (50 mg q6h) or 209.4 mg/L (100 mg q8h). There was discrepancy in the response to fosfomycin between *Escherichia coli* and *Klebsiella pneumoniae*, with fosfomycin displaying strong bactericidal activity for 48 h against *E. coli* but moderate bactericidal activity for 18 h against *K. pneumoniae*. This effect was not related to the strain's baseline minimum inhibitory concentration but rather to the presence of a resistant subpopulation. Maximum titres of nitrofurantoin were obtained during the first 2 h, but no antibacterial effect was found in most samples regardless of the dose. In the rare samples in which antibacterial activity was detectable, titres were comparable for both species tested. These findings confirm doubts regarding fosfomycin administration in UTIs caused by *K. pneumoniae* and reveal a discrepancy between nitrofurantoin's measurable ex vivo activity and its clinical effect over multiple dosing intervals.

1. Introduction

Fosfomycin and nitrofurantoin are recommended first-line antimicrobial agents for urinary tract infections (UTIs), the most common bacterial infection among otherwise healthy women [1]. Although antimicrobial resistance among uropathogens is increasing, it remains relatively low to fosfomycin and nitrofurantoin [2–4]. Despite their use over several decades, the pharmacokinetic (PK) and pharmacodynamic (PD) properties of these antibiotics remain poorly defined, although such information is essential for therapy optimisation and for the prevention of resistance emergence [5,6]. Whilst new data are beginning to emerge on the PK [7–11] and PD properties [12,13] of both drugs, most in vitro PD studies have been conducted in a non-biological matrix and/or did not take into account drug concentration changes over time ex vivo, thus limiting the clinical translation of these results.

A method to address these limitations is the determination of the urinary antibacterial activity of antimicrobial agents in which ex vivo PK data are used within a static in vitro model [14–16]. The urinary antibacterial activity of an antimicrobial agent is described by the urinary inhibitory titre (UIT) and urinary bactericidal titre (UBT). These are measures of antibacterial activity over time in urine, the relevant biological matrix, thus providing in vitro data that more closely reflects the clinical scenario by describing antibiotic activity against the pathogen within the host's environment.

In this study, the urinary antibacterial activities of fosfomycin and nitrofurantoin were determined against common uropathogens

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https://doi.org/10.1016/j.ijantimicag.2019.07.018
0924-8579/

following administration of registered doses for the treatment of UTI in order to evaluate the effectiveness of these drugs.

2. Materials and methods

2.1. Study design, subjects, drug administration and sample collection

Urine samples to determine the UIT and UBT were obtained in two previous studies evaluating the PK properties of both fosfomycin and nitrofurantoin [7,8]. Briefly, the fosfomycin urinary PK study was a single-centre study examining the urinary pharmacokinetics following a single oral 3 g dose of fosfomycin trometamol (Monuril®; Zambon Nederland B.V., Amersfoort, the Netherlands) in 40 healthy female volunteers [7]. Fosfomycin was administered under supervision of one of the researchers. Urine samples were collected in a home setting over 48 h with every void and then two times daily from 48 h until 7 days after administration. For the present study, only samples collected in the first 48 h were used. There were no dietary restrictions prior to or after drug administration. Samples were kept in home freezers until handed to investigators. The nitrofurantoin PK study was a single-centre study in which macrocrystalline nitrofurantoin was administered at either 50 mg every 6 h (q6h) (Furadantine® MC; Mercury Pharma Ltd., Croydon UK) or 100 mg every 8 h (q8h) (Furadantine® retard; Mercury Pharma Ltd.) in a crossover design to 12 healthy female volunteers [8]. The drug was administered with food and administration began in a home setting 24 h prior to sample collection in order to achieve steady-state. The last dose was administered in the hospital at the start of an 8-h visit during which urine samples were collected for 6 h or 8 h depending on the assigned dosing interval. Volunteers were instructed to protect the nitrofurantoin samples from daylight using aluminium foil to avoid photodegradation of the drug.

Total volume, pH and time of each sample were recorded both for fosfomycin and nitrofurantoin samples prior to storage at –80 °C. The stability of the samples under these conditions was confirmed during validation of the analytical methods [17,18]. Drug levels were quantified using ultrahigh performance liquid chromatography (UHPLC) with tandem mass spectrometry (MS/MS) detection for fosfomycin or using ultraviolet (UV) detection for nitrofurantoin. Both methods were validated according to US Food and Drug Administration (FDA) guidelines as described elsewhere [17,18].

2.2. Test organisms and minimum inhibitory concentrations (MICs)

Isolates were obtained from clinical sources (except the *Escherichia coli* ATCC reference strain) and were selected with a range of fosfomycin and nitrofurantoin MICs (Table 1). Fosfomycin susceptibility was determined by agar dilution using 10^4 CFU/spot of each isolate inoculated on Mueller–Hinton II agar (BD Diagnostics, Franklin Lakes, NJ, USA) containing 25 mg/L glucose-6-phosphate (G6P) (Sigma, Taufkirchen, Germany) and fosfomycin (InfectoPharm, Heppenheim, Germany) following Clinical and Laboratory Standards Institute (CLSI) recommendations at a concentration range of 0.25–1024 mg/L. Isolates were tested in triplicate. Nitrofurantoin susceptibility was determined by broth microdilution according to ISO guidelines [19].

Fosfomycin-containing urine samples from volunteers were divided into two sets to allow for the limited volume of material. Set 1 consisted of samples from the initial 20 volunteers and the second set consisted of those from the remaining 20 volunteers. Both sets were tested against two *E. coli* strains, two *Klebsiella pneumoniae* strains and the ATCC strain (Table 1). All strains were used for testing the nitrofurantoin samples.

Table 1
Minimum inhibitory concentrations (MICs) of fosfomycin and nitrofurantoin

Test strain	Source	MIC (mg/L)[a]	
		Fosfomycin	Nitrofurantoin
Escherichia coli			
ATCC 25922	Laboratory strain	1	16
51[b]	Blood	2	32
03[b]	Urine	0.25	16
1231	Urine	16	512
4807	Rectal swab	32	16
Klebsiella pneumoniae			
58[b]	Urine	8	64
20[b]	Rectal swab	32	256
31865	Blood	2	128
55	Sputum	4	256

[a] The MIC represents the modal value based on the results of agar dilution (fosfomycin) or microdilution (nitrofurantoin) performed in triplicate.
[b] Strains used for set 1 of the urine samples from volunteers 1–40 in the fosfomycin study.

2.3. Determination of urinary inhibitory titres and urinary bactericidal titres

All urine samples were filtered before analysis by centrifugation (10 min at 13 000 rpm) using an Amicon® Ultra-0.5 Centrifugal Filter Unit with a 10 kDa cut-off Ultracel-10 membrane (UFC5010BK; Merck, Amsterdam, the Netherlands). The large volumes of antibiotic-free urine were filtered over 0.2 µm bottle-top vacuum filters (CLS430756; Corning, Taufkirchen, Germany). UITs and UBTs were determined by microdilution. Urine samples underwent serial two-fold dilution in antibiotic-free urine from healthy volunteers such that the first well of the microtitre plate contained a 2-times diluted sample. The final bacterial inoculum within the microtitre tray was approximately 2.5×10^5 CFU/mL. Inoculated plates were incubated for 18 ± 2 h at 35 ± 2 °C, after which every well was checked visually for growth. The UIT represents the bacteriostatic activity and was defined as the highest dilution that inhibited visible growth. The UBT represents the bactericidal activity and was defined as the absence of bacterial growth following subculture from the microtitre tray onto an antibiotic-free tryptic soy agar (TSA) plate supplemented with 5% sheep blood (254087; Becton Dickinson, Franklin Lakes, NJ, USA). The limit of detection was 50 CFU/mL. TSA plates were incubated for 18 ± 2 h at 35 ± 2 °C. The UBT was defined as the highest dilution of the sample that still exhibited bactericidal activity. Comparable UITs and UBTs reflect antibiotic bactericidal activity, whilst a UIT exceeding the UBT reflects bacteriostatic activity. UITs and UBTs are presented as reciprocal values of the titres and could therefore range from <2 (no antibacterial activity observed) to 1024, with higher titres indicating greater antibacterial activity.

2.4. Determination of fosfomycin-resistant subpopulations

To determine the presence of fosfomycin low-level resistant or high-level resistant (HLR) subpopulations, isolates were cultured overnight both in Mueller–Hinton broth and in antibiotic-free urine using a starting inoculum of 2.5×10^5 CFU/mL. Quantitative cultures were then performed in parallel on antibiotic-free Mueller–Hinton agar (MHA) and MHA supplemented with 25 mg/L G6P together with 64 mg/L or 512 mg/L fosfomycin. Total bacterial density as well as the comparative density of any growth on the fosfomycin-containing media was determined by plating 20 µL from a serial 10-fold dilution of the incubated liquid medium. Growth capacity and resistant subpopulation proportions were compared between Mueller–Hinton broth and urine.

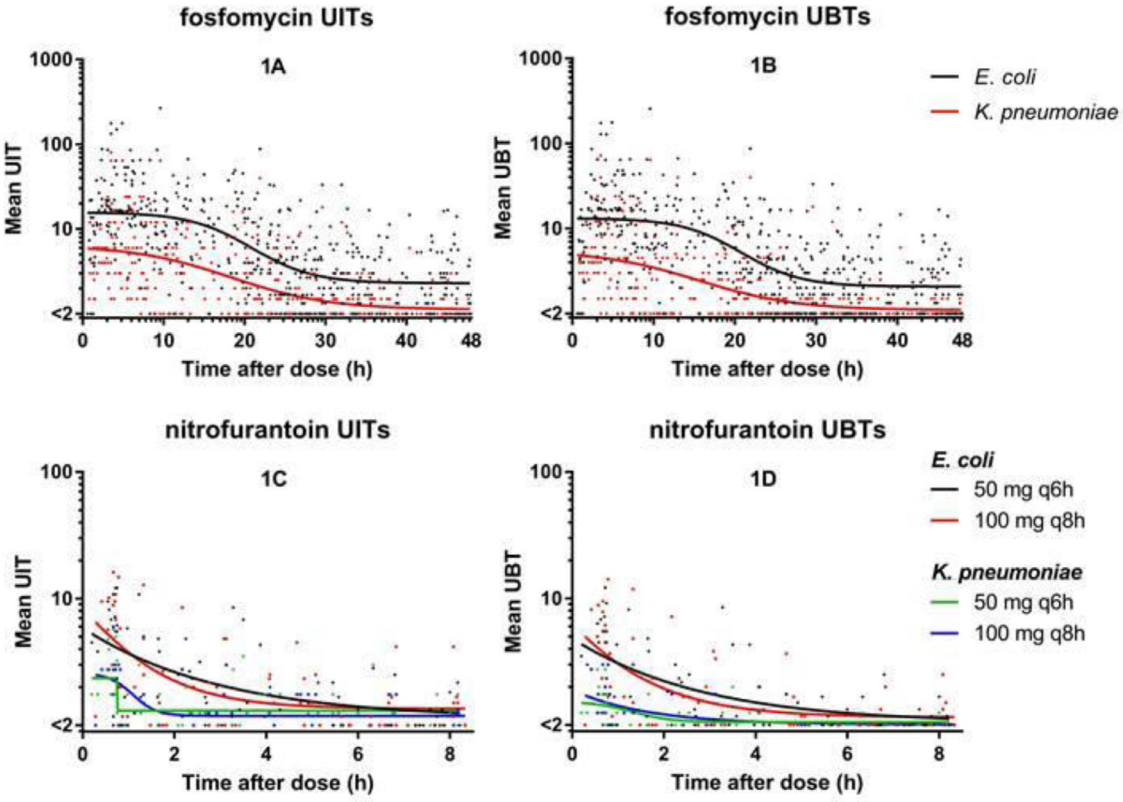

Fig. 1. (A) Urinary inhibitory titres (UITs) and (B) urinary bactericidal titres (UBTs) of fosfomycin for *Escherichia coli* and *Klebsiella pneumoniae* for all samples. (C) UITs and (D) UBTs of nitrofurantoin for both dosing regimens for *E. coli* and *K. pneumoniae*. Each dot represents the mean UIT or UBT for each sample for the *E. coli* and *K. pneumoniae* strains, respectively.

MHA plates were incubated overnight at 35 ± 2 °C. The limit of detection was considered to be 1.4 $\log_{10}$ CFU/mL. This additional analysis was performed for fosfomycin based on previous studies in which a resistant subpopulation was identified in susceptible Enterobacteriales [9,20].

2.5. Statistical analysis

Microsoft Excel 2013 (Microsoft Corp., Redmond, WA, USA), IBM SPSS Statistics v.24 (IBM Corp., Armonk, NY, USA) and GraphPad Prism v.7.0 (GraphPad Software Inc., San Diego, CA, USA) were used for processing the data. Fosfomycin samples were grouped in 6-h time intervals, and nitrofurantoin samples were grouped in 2-h time intervals. The median and range of UITs and UBTs were calculated for each interval. The area under the inhibitory titre–time curve (AUIT) and the area under the bactericidal titre–time curve (AUBT) were calculated to give an indication of the inhibitory and bactericidal activity for each strain using the trapezoidal rule [14]. A period of 48 h was considered for fosfomycin and 6 h or 8 h for nitrofurantoin for the 50 mg q6h and 100 mg q8h dosing regimens, respectively. Titre values were compared using a two-sided Wilcoxon matched-pairs rank test ($P < 0.0001$) to compare the titres of the two species, and a one-sided Wilcoxon matched-pairs rank test ($P < 0.0001$) was used to compare the UIT values and the UBT values per time interval. The d'Agostino–Pearson test was used to check the normal distribution of the data. Untransformed data were used for statistical analysis. Titre values of <2 were transformed into 1 for statistical analysis. The UBT in the most concentrated sample was used to calculate the percentage of volunteers in whom bactericidal activity (UBT ≥ 2) could be measured.

3. Results

3.1. Subjects and urine samples

Volunteers in both studies were Caucasian females with a mean ± standard deviation (S.D.) age of 24.3 ± 7.9 years and 28.5 ± 7.9 years in the fosfomycin and nitrofurantoin groups, respectively. A more detailed overview of volunteer characteristics can be found in the original studies [7,8]. The number of samples collected by the volunteers varied from 6–19 for fosfomycin and 3–9 for nitrofurantoin because they were not instructed to follow a voiding schedule. Fosfomycin urinary concentrations ranged from <0.75 mg/L [lower limit of quantification (LLOQ)] to 5729.9 mg/L and did not differ significantly between the two sets ($P < 0.05$; Supplementary Table S1). Nitrofurantoin concentrations ranged from <4 mg/L (LLOQ) to 176.3 mg/L (nitrofurantoin 50 mg q6h) and from <4 mg/L to 209.4 mg/L (nitrofurantoin 100 mg q8h) (Supplementary Table S1). Nitrofurantoin concentrations were slightly higher for the 100 mg dose but peak concentrations (C_{max}) were almost equal (mean ± S.D. C_{max} of 94.4 ± 47.8 mg/L for 50 mg q6h vs. 94.1 ± 49.9 mg/L for 100 mg q8h).

3.2. (A)UITs and (A)UBTs

3.2.1. Fosfomycin

The high interindividual variability in urinary drug concentrations was reflected by the wide range in UITs and UBTs [7]. For *E. coli*, fosfomycin UITs ranged from <2 to 256 and maximum titres were obtained during the first 12 h after dosing (Fig. 1A; Supplementary Table S2). Likewise, UBTs ranged from <2 to 512 and were comparable with the UITs for *E. coli*. Thus, fosfomycin was bactericidal against *E. coli* (Fig. 1B; Table 2). There was still reasonable

Table 2
UBTs and $AUBT_{0-48h}$ values for fosfomycin over time for each strain

Strain (MIC in mg/L)	UBT [median (range)] for the indicated time period								$AUBT_{0-48h}$ [median (range)]
	0–6 h	6–12 h	12–18 h	18–24 h	24–30 h	30–36 h	36–42 h	42–48 h	
Escherichia coli									
ATCC 25922 (1)	16 (<2–256)	16 (<2–512)	16 (<2–128)	4 (<2–128)	4 (<2–64)	2 (<2–64)	3 (<2–32)	2 (<2–32)	152 (97–303)
51 (2)	8 (<2–64)	4 (<2–32)	4 (<2–16)	4 (<2–16)	2 (<2–16)	2 (<2–8)	2 (<2–8)	2 (<2–4)	115 (53–163)
03 (0.25)	16 (<2–64)	16 (4–64)	16 (2–32)	8 (<2–32)	3 (<2–16)	2 (<2–16)	2 (<2–8)	2 (<2–8)	143 (101–192)
1231 (16)	2 (<2–16)	2 (<2–8)	<2 (<2–8)	<2 (<2–8)	<2 (<2–4)	<2 (<2–4)	<2 (<2–4)	<2 (<2–2)	63 (46–112)
4807 (32)	16 (<2–256)	16 (<2–256)	16 (<2–64)	6 (<2–128)	3 (<2–32)	2 (<2–32)	3 (<2–16)	2 (<2–16)	162 (88–275)
Klebsiella pneumoniae									
58 (8)	2 (<2–8)	<2 (<2–16)	<2 (<2–2)	<2 (<2–4)	<2 (<2–2)	<2 (<2–2)	<2 (<2–4)	<2 (<2–2)	47 (39–102)
20 (32)	4 (<2–32)	2 (<2–32)	<2 (<2–8)	2 (<2–16)	<2 (<2–2)	<2 (<2–2)	<2 (<2–2)	<2 (<2–2)	69 (44–114)
31865 (2)	8 (<2–128)	8 (<2–64)	4 (<2–64)	2 (<2–64)	<2 (<2–32)	<2 (<2–16)	<2 (<2–8)	<2 (<2–4)	106 (74–218)
55 (4)	4 (<2–16)	2 (<2–64)	<2 (<2–16)	<2 (<2–16)	<2 (<2–4)	<2 (<2–8)	<2 (<2–4)	<2 (<2–2)	68 (44–153)

UBT, urinary bactericidal titre; $AUBT_{0-48h}$, area under the bactericidal titre–time curve from 0–48 h; MIC, minimum inhibitory concentration.

bactericidal activity after 48 h for *E. coli* because UBTs were ≥2 in the majority (95%) of samples. The only exception was *E. coli* strain 1231 (MIC = 16 mg/L) where UITs and UBTs did not exceed 2 for the full 48 h. The AUIT from 0–48 h ($AUIT_{0-48h}$) values between the five *E. coli* strains were comparable, again with the exception of *E. coli* 1231 (Supplementary Table S2). The same is true for the AUBT from 0–48 h ($AUBT_{0-48h}$) values (Table 2). The difference in $AUIT_{0-48h}$ and $AUBT_{0-48h}$ values between the *E. coli* strains did not reflect their varying baseline MICs to fosfomycin (Table 1).

UITs for *K. pneumoniae* ranged from <2 to 128 and maximum titres were found during the first 6-h time period (Fig. 1A; Supplementary Table S2). UITs and UBTs were comparable, reflecting the bactericidal activity of fosfomycin in *K. pneumoniae* (Fig. 1B; Table 2). In contrast to *E. coli*, no antibacterial activity of fosfomycin in *K. pneumoniae* was observed in the majority (86%) of samples throughout the complete 48 h. Where an antibacterial effect was detected, it was bactericidal in the majority (90%) of samples but was present only during the first 18 h after administration. UITs and UBTs declined dramatically after that 18-h time point. UITs and UBTs for *K. pneumoniae* were significantly lower than those for *E. coli* ($P < 0.0001$ for all time intervals) (Fig. 1A,B). $AUIT_{0-48h}$ and $AUBT_{0-48h}$ values ranged from 47–110 and were independent of the strain's baseline MIC for fosfomycin (Table 2; Supplementary Table S2).

3.2.2. Nitrofurantoin

For *E. coli*, nitrofurantoin UITs ranged from <2 to 16 for the 50 mg q6h regimen and from <2 to 32 for the 100 mg q8h regimen and were generally within the same range for both dosing regimens (Fig. 1C; Supplementary Table S3). Maximum titres were obtained within the first 2 h after administration. UBTs for *E. coli* were comparable with the UIT values, demonstrating bactericidal activity of nitrofurantoin against *E. coli* (Fig. 1D; Table 3). After 2 h, no detectable antibacterial activity was found in the majority of samples (titres of <2).

For *K. pneumoniae*, nitrofurantoin UITs ranged from <2 to 16 for both dosing regimens and maximum titres were found in the first 2 h after administration (Fig. 1C; Supplementary Table S3). UBTs ranged from <2 to 8 and did not differ between dosing regimens (Fig. 1D; Table 3). UITs and UBTs were comparable in these first two 2 h, again reflecting the bactericidal activity of nitrofurantoin in the few samples in which antibacterial activity was detectable.

The UITs and UBTs were higher for *E. coli* compared with those for *K. pneumoniae* for both dosing regimens (Fig. 1C,D). Similar to fosfomycin activity, the AUIT and AUBT values were found to be independent of the baseline nitrofurantoin MICs of the isolates (Table 3; Supplementary Table S3). This is true for both dosing regimens.

3.2.3. Bactericidal effect in the samples and in volunteers

To correlate with clinical antibiotic effectiveness for UTI treatment, the percentage of volunteers in which bactericidal activity was found was calculated for sequential time intervals for the five *E. coli* and four *K. pneumoniae* strains. Fig. 2 demonstrates these percentages over time; a higher percentage reflects a more effective treatment.

Considering fosfomycin, the percentages for *E. coli* were higher than for *K. pneumoniae*. Bactericidal activity against *E. coli* was found in a mean of 90% of the volunteers during 24 h, but this declined to <60% thereafter. This applied to all *E. coli* strains with the exception of *E. coli* strain 1231 against which fosfomycin was not bactericidal in the least diluted sample in 50% of volunteers (Fig. 2A). This finding was supported by the detection of a resistant subpopulation in this isolate (Section 3.3). Against *K. pneumoniae*, bactericidal activity of fosfomycin was found in only a mean of 60% of the volunteers during the first 18 h after administration (Fig. 2A). Percentages declined quickly thereafter to <20% beyond 24 h after dosing. Thus, fosfomycin remained bactericidal against *K. pneumoniae* isolates after 24 h in a very small number of volunteers.

Nitrofurantoin was bactericidal in *E. coli* ATCC 25922, *E. coli* 51 and *E. coli* 03 regardless of the administered dose (Fig. 2B,C). However, this bactericidal activity was only found in 0–50% of volunteers. Percentages of >60% were found only in the first 2 h after administration. In *K. pneumoniae*, percentages never exceeded 17% in strains 58, 20 and 31865, independent of the administered dose. Only in *K. pneumoniae* 55 was bactericidal activity found in approximately 40% (100 mg q8h) and 60% (50 mg q6h) of volunteers. These percentages remained consistent over the 8-h urine collection time period for this strain.

3.3. Fosfomycin-resistant subpopulation

Only one of five *E. coli* isolates had a detectable fosfomycin-resistant subpopulation when grown both in standard laboratory medium and in human urine, whereas all of the *K. pneumoniae* isolates had a detected resistant subpopulation (Table 4). The resistant subpopulation detected in *E. coli* 1231 and in the *K. pneumoniae* isolates had fosfomycin MICs of >1024 mg/L after subculturing off the fosfomycin-containing MHA onto TSA. This result is consistent with the low antibacterial activity of fosfomycin in these strains.

4. Discussion

Whilst fosfomycin exhibited bactericidal activity for ≥48 h against *E. coli*, no antibacterial activity was detected in the majority of *K. pneumoniae* samples. In contrast to fosfomycin, nitrofurantoin showed low antibacterial activity in both species

Table 3
UBTs and $AUBT_{0-6h}$ or $AUBT_{0-8h}$ values for nitrofurantoin over time for each strain

Dose/strain (MIC in mg/L)	UBT [median (range)] for the indicated time period				$AUBT_{0-6h}$ or $AUBT_{0-8h}$ [median (range)]
	0–2 h	2–4 h	4–6 h	6–8 h	
Nitrofurantoin 50 mg q6h					
Escherichia coli					
ATCC 25922 (16)	8 (<2–16)	2 (<2–16)	<2 (<2–4)	<2 (<2–4)	18 (10–31)
51 (32)	4 (<2–16)	<2 (<2–8)	<2 (<2–2)	<2 (<2–2)	14 (9–25)
03 (16)	4 (<2–16)	<2 (<2–8)	<2 (<2–2)	<2 (<2–2)	15 (9–25)
1231 (512)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (9–22)
4807 (16)	2 (<2–8)	<2 (<2–4)	<2 (<2–<2)	<2 (<2–2)	12 (8–11)
Klebsiella pneumoniae					
58 (64)	2 (<2–8)	<2 (<2–4)	<2 (<2–8)	<2 (<2–2)	11 (7–9)
20 (256)	<2 (<2–2)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–<2)	9 (9–17)
31865 (128)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (7–9)
55 (256)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (7–11)
Nitrofurantoin 100 mg q8h					
E. coli					
ATCC 25922 (16)	4 (<2–32)	2 (<2–16)	<2 (<2–8)	<2 (<2–4)	15 (10–25)
51 (32)	2 (<2–16)	<2 (<2–8)	<2 (<2–2)	<2 (<2–2)	12 (8–21)
03 (16)	2 (<2–16)	<2 (<2–8)	<2 (<2–2)	<2 (<2–4)	13 (11–24)
1231 (512)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (8–21)
4807 (16)	<2 (<2–8)	<2 (<2–4)	<2 (<2–2)	<2 (<2–2)	11 (7–11)
K. pneumoniae					
58 (64)	2 (<2–8)	<2 (<2–4)	<2 (<2–4)	<2 (<2–<2)	11 (7–9)
20 (256)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (7–20)
31865 (128)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (7–10)
55 (256)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (7–10)

UBT, urinary bactericidal titre; $AUBT_{0-6h}$, area under the bactericidal titre–time curve from 0–6 h; $AUBT_{0-8h}$, area under the bactericidal titre–time curve from 0–8 h; MIC, minimum inhibitory concentration; q6h, every 6 h; q8h, every 8 h.

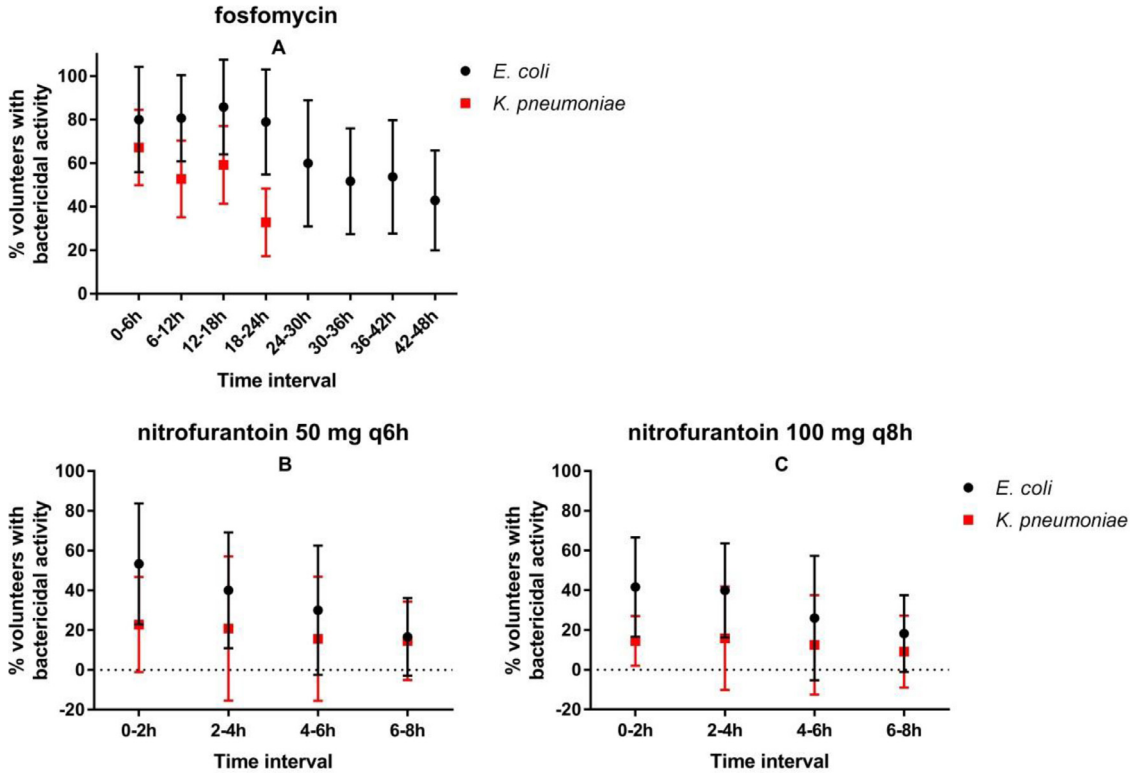

Fig. 2. Percentage of volunteers where a bactericidal effect was found for (A) fosfomycin during 48 h and (B,C) nitrofurantoin 50 mg q6h during 6 h (B) or nitrofurantoin 100 mg q8h during 8 h (C) in *Escherichia coli* and *Klebsiella pneumoniae*. Data are the mean ± standard deviation percentage for both species (*y*-axis) of the total number of volunteers that produced urine samples in the considered time interval (*x*-axis). Because bactericidal activity in *K. pneumoniae* was found in only a small number of volunteers (range 1–3 volunteers) in the time intervals after 24 h, these percentages are negligibly small and are therefore not presented in part (A). q6h, every 6 h; q8h, every 8 h.

Table 4
Presence of a fosfomycin low-level resistant (LLR) and/or high-level resistant (HLR) subpopulation of the strains in urine or Mueller–Hinton broth (MHB)

Strain (MIC in mg/L)	LLR/HLR subpopulation present	
	Urine	MHB
Escherichia coli		
ATCC 25922 (1)	No	No
51 (2)	No	No
03 (0.25)	LLR	LLR
1231 (16)	HLR	HLR
4807 (32)	LLR	LLR
Klebsiella pneumoniae		
58 (8)	HLR	HLR
20 (32)	LLR	HLR
31865 (2)	HLR	HLR
55 (4)	HLR	HLR

MIC, minimum inhibitory concentration.

regardless of the administered dose, although only one dose interval was examined among the many intervals intended with a course of nitrofurantoin.

In general, fosfomycin exhibited bactericidal activity as demonstrated by comparable UIT and UBT values. The duration of activity was strongly species-dependent, with ≥48 h for *E. coli* and only 18 h for *K. pneumoniae*. Indeed, 48-h antibacterial activity against *K. pneumoniae* could be demonstrated in only a small subset. These findings are supported by earlier in vitro research demonstrating that fosfomycin was not able to reliably kill *K. pneumoniae* isolates [9,20].

It was suggested that fosfomycin is able to kill (or at least inhibit the growth of) *E. coli*, but re-growth occurs thereafter. The extent of re-growth depends on the presence of a resistant subpopulation and this is not predicted based on the baseline fosfomycin MIC for the strain [21]. This is corroborated by the finding in the current study that bactericidal activity against *E. coli* over 24 h was found in approximately 90% of volunteers, but quickly fell below 60% thereafter. This was true for all *E. coli* strains, with the exception of *E. coli* 1231, the strain harbouring a HLR subpopulation. For *K. pneumoniae*, moderate (or almost totally absent) antibacterial activity of fosfomycin was found in the majority of samples, confirming other reports [9,10,22]. All *K. pneumoniae* strains had a HLR subpopulation. This may be more a matter of intrinsic rather than acquired resistance following antibiotic exposure [9,23]. These findings suggest that a single 3 g fosfomycin dose may be sufficient for UTIs caused by *E. coli* without HLR subpopulations, and that fosfomycin is inappropriate for UTIs caused by *K. pneumoniae* regardless of the MIC of the strain and the fosfomycin dose.

Maximum UITs and UBTs of nitrofurantoin were obtained in the first 2-h time interval. Titres were low but comparable for both species tested, demonstrating reasonable bactericidal activity of nitrofurantoin only in the first 2 h, confirming a previous report describing early activity against extended-spectrum β-lactamase (ESBL)-producing pathogens such as *E. coli* and *K. pneumoniae* [12]. No significant differences in antibacterial activity between the two dosing regimens were found; the slightly higher urinary concentrations of nitrofurantoin after 100 mg versus 50 mg did not result in more antibacterial activity in our experiment [8].

The major advantage of the method used here is that it is an ex vivo model combining patient-related PK properties of a drug with its PD effect. The ex vivo results obtained with this method may therefore reflect the antimicrobial clinical effectiveness against uropathogens better than most other ex vivo/in vitro methods. This is important as bacterial growth ex vivo/in vitro can be different from that in humans [24]. Yet we found bactericidal activity of nitrofurantoin in <50% of volunteer samples, and only for a short period of time. This contrasts with the bactericidal activity of fosfomycin, which was detected in 90% of volunteer samples. These results are in conflict with what was found in a recent randomised clinical trial comparing 5 days of nitrofurantoin (100 mg q8h) with single-dose fosfomycin (3 g) for acute lower UTI [25] in which 70% of those receiving nitrofurantoin had clinical success versus only 58% of those receiving fosfomycin. Microbiological resolution was achieved in 74% versus 63%, respectively. There is thus discrepancy between the ex vivo activity of nitrofurantoin in a single dosing interval (and also, but to a smaller extent, of fosfomycin) and its clinical efficacy.

There are several possible factors that could explain this discrepancy. Fosfomycin requires G6P to enter bacterial cells to exert its antibacterial activity, therefore it is standard practice to add 25 mg/L G6P to the laboratory medium when performing in vitro experiments with fosfomycin [26]. Because human urine normally does not contain G6P in significant amounts, the ex vivo antibacterial activity was measured without adding G6P. It should be noted, however, that the baseline MICs for fosfomycin were measured in the presence of G6P, as per the reference standard for fosfomycin susceptibility testing [27]. This could party explain the discrepancy between the fosfomycin MICs at baseline and the urinary antibacterial activity. For nitrofurantoin, its activity was investigated during only one dosing interval of a drug intended to be administered over ≥5 days. It would therefore seem likely that the short period of antibacterial activity found would be sufficient to achieve clinical success in the majority of patients when administered as a course of multiple oral doses. The cumulative effect of the full nitrofurantoin course after repetitive dosing has not been investigated, such that the current results would underestimate the effect of the antimicrobial agent. Second, the bactericidal activity was considered only when calculating the percentages of bactericidal success, but whether pathogen killing is needed to achieve clinical success is questionable. Bacteriostatic activity, or a bactericidal effect during a short period of time (e.g. <2 h), might be sufficient to promote clinical success, in particular because of the natural urodynamics of regularly voiding episodes during which uropathogens are flushed out together with the urine. Finally, the percentage of bactericidal success gives an underestimation of daily clinical practice since we were not able to measure the antibacterial activity in the undiluted sample owing to limited sample volumes.

5. Conclusion

Strong bactericidal activity of fosfomycin against *E. coli* over ≥48 h after administration and moderate bactericidal activity against *K. pneumoniae* over 18 h was found. High-level resistant subpopulations were found in all *K. pneumoniae* strains and in one *E. coli* strain, a finding that further supports the likelihood of intrinsic resistance of *K. pneumoniae* against fosfomycin and highlights that MIC measurements might not be the best measure for predicting the ex vivo activity of fosfomycin. Titres of nitrofurantoin were comparable both for *E. coli* and *K. pneumoniae*, demonstrating moderate bactericidal activity in the first 2 h after dosing. In the majority of subsequent samples, however, no antibacterial activity was detected regardless of the administered dose. This finding is in contrast to the well-observed clinical effects of nitrofurantoin over multiple dosing intervals. The current findings reveal a discrepancy between nitrofurantoin's measurable ex vivo activity in a single dosing interval time period and its clinical effectiveness. For fosfomycin, the current findings suggest that the current single-dose approach to fosfomycin administration in UTIs caused by *E. coli* without HLR may be sufficient, but confirm doubts on the use of fosfomycin in general in UTIs caused by *K. pneumoniae*.

Funding

This work was supported in part by the European Commission's FP7 AIDA project [Preserving old antibiotics for the future, Health-F3-2011- 278348].

Competing interests

None declared.

Ethical approval

Ethical approval was given by the local ethical committees [Erasmus Medical Center (MEC-2016-121) and Geneva University Hospital (13-036)] as mentioned in the papers of the original publications of both healthy volunteer studies [7,8].

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.07.018.

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SUPPLEMENTARY MATERIAL

Table S1. Characteristics of the fosfomycin and nitrofurantoin samples

Collection period (h)	Drug concentration (mg/L) [median (range)] [a]	No. of samples	pH	
Fosfomycin				
0–6	808.1 (19.9–5729.9)	77	5	5–7
6–12	744.4 (90–4375.9)	68	5.5	5–7
12–18	512.7 (145.1–1866.6)	33	6	5–7
18–24	348.3 (51.5–2189.1)	67	5	5–7
24–30	124 (<0.75–947.9)	82	5	5–7
30–36	78.5 (9.1–707.9)	64	5	5–7
36–42	80.8 (<0.75–454.8)	32	5	5–7
42–48	62.3 (<0.75–495.7)	61	5	5–7
Nitrofurantoin (50 mg every 6 h)				
0–2	53.6 (4–176.3)	23	5.6	5–6
2–4	26.8 (<4–106.7)	17	5.8	5–6
4–6	19.7 (5.3–54.4)	9	6	6–6
6–8	15.7 (<4–37.6)	19	5.8	5–6
Nitrofurantoin (100 mg every 8 h)				
0–2	32.6 (4–209.4)	25	5.7	5–7
2–4	21.3 (13.4–97.9)	11	5.6	5–6
4–6	17 (5.2–79.3)	16	5.8	5–6
6–8	10 (<4–56.4)	20	5.8	5–6

[a] The lower limit of quantification (LLOQ) of the analytical method was 0.75 mg/L for fosfomycin and 4 mg/L for nitrofurantoin.

Table S2. UITs and $AUIT_{0\text{-}48h}$ values of fosfomycin over time for each strain

Strain (MIC in mg/L)	UIT [median (range)] for the indicated time period								$AUIT_{0–48h}$
	0–6 h	6–12 h	12–18 h	18–24 h	24–30 h	30–36 h	36–42 h	42–48 h	
Escherichia coli									
ATCC 25922 (1)	16 (<2–256)	16 (<2–256)	16 (<2–128)	8 (<2–128)	4 (<2–64)	2 (<2–64)	4 (<2–32)	2 (<2–32)	164 (98–309)
51 (2)	16 (<2–128)	8 (2–32)	4 (<2–16)	4 (<2–16)	2 (<2–16)	2 (<2–8)	2 (<2–8)	<2 (<2–8)	117 (74–164)
03 (0.25)	16 (<2–64)	16 (4–64)	16 (2–32)	4 (<2–64)	2 (<2–16)	2 (<2–16)	2 (<2–8)	<2 (<2–8)	150 (96–199)
1231 (16)	2 (<2–16)	2 (<2–32)	<2 (<2–8)	<2 (<2–8)	<2 (<2–4)	<2 (<2–4)	<2 (<2–4)	<2 (<2–2)	69 (46–144)
4807 (32)	32 (<2–256)	16 (<2–256)	16 (<2–64)	8 (<2–128)	3 (<2–32)	4 (<2–32)	4 (<2–16)	2 (<2–16)	171 (91–279)
Klebsiella pneumoniae									
58 (8)	4 (<2–16)	2 (<2–16)	<2 (<2–8)	<2 (<2–8)	<2 (<2–2)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–2)	57 (45–100)
20 (32)	4 (<2–32)	4 (<2–64)	2 (<2–8)	2 (<2–16)	<2 (<2–4)	<2 (<2–4)	<2 (<2–2)	<2 (<2–<2)	79 (41–129)
31865 (2)	8 (<2–128)	8 (<2–64)	8 (<2–64)	3 (<2–64)	<2 (<2–32)	<2 (<2–16)	<2 (<2–8)	<2 (<2–4)	110 (74–224)
55 (4)	4 (<2–64)	4 (<2–64)	4 (<2–16)	2 (<2–16)	<2 (<2–8)	<2 (<2–8)	<2 (<2–4)	<2 (<2–4)	90 (58–174)

UIT, urinary inhibitory titre; $AUIT_{0–48h}$, area under the inhibitory titre–time curve from 0–48 h; MIC, minimum inhibitory concentration.

Table S3. Urinary inhibitory titres (UITs) and $AUIT_{0\text{-}6h}$ or $AUIT_{0\text{-}8h}$ values of nitrofurantoin over time for each strain

Dose, strain (MIC in mg/L)	UIT [median (range)] for the indicated time period				$AUIT_{0–6h}$ or $AUIT_{0–8h}$
	0–2 h	2–4 h	4–6 h	6–8 h	
Nitrofurantoin 50 mg every 6 h					
Escherichia coli					
ATCC 25922 (16)	8 (<2–16)	2 (<2–16)	1 (<2–4)	<2 (<2–4)	20 (12–31)
51 (32)	4 (<2–16)	2 (<2–8)	<2 (<2–4)	<2 (<2–4)	17 (10–26)
03 (16)	4 (<2–16)	2 (<2–8)	2 (<2–2)	<2 (<2–2)	18 (12–26)
1231 (512)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (11–32)
4807 (16)	2 (<2–8)	<2 (<2–8)	<2 (<2–<2)	<2 (<2–2)	13 (8–15)
Klebsiella pneumoniae					
58 (64)	4 (<2–16)	2 (<2–8)	2 (<2–4)	<2 (<2–8)	18 (7–9)
20 (256)	<2 (<2–4)	<2 (<2–4)	<2 (<2–<2)	<2 (<2–8)	10 (9–24)
31865 (128)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (7–11)
55 (256)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (7–11)
Nitrofurantoin 100 mg every 8 h					
E. coli					
ATCC 25922 (16)	4 (<2–32)	2 (<2–16)	1 (<2–8)	<2 (<2–8)	18 (13–30)
51 (32)	4 (<2–16)	2 (<2–8)	2 (<2–2)	<2 (<2–4)	15 (10–23)
03 (16)	4 (<2–16)	2 (<2–8)	1 (<2–4)	<2 (<2–4)	16 (11–26)
1231 (512)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (11–30)
4807 (16)	<2 (<2–16)	<2 (<2–4)	<2 (<2–4)	<2 (<2–4)	12 (7–14)
K. pneumoniae					
58 (64)	4 (<2–16)	2 (<2–8)	<2 (<2–4)	<2 (<2–4)	14 (7–9)
20 (256)	<2 (<2–4)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–2)	9 (7–24)
31865 (128)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (7–11)
55 (256)	<2 (<2–2)	<2 (<2–<2)	<2 (<2–<2)	<2 (<2–<2)	9 (7–11)

UIT, urinary inhibitory titre; $AUIT_{0–6h}$, area under the inhibitory titre–time curve from 0–6 h; UIT, urinary inhibitory titre; $AUIT_{0–8h}$, area under the inhibitory titre–time curve from 0–8 h; MIC, minimum inhibitory concentration.

J Antimicrob Chemother 2019; **74**: 3630–3632
doi:10.1093/jac/dkz380
Advance Access publication 11 September 2019

In vivo acquisition of fosfomycin resistance in *Escherichia coli* by *fosA* transmission from commensal flora

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Sir,

Fosfomycin is increasingly used to treat infections caused by MDR bacteria.[1] Fosfomycin acts by inhibiting UDP-*N*-acetylglucosamine enolpyruvyl transferase (*murA*), which prevents the formation of *N*-acetylmuramic acid, an essential component of peptidoglycan.[1] Although resistance to fosfomycin is still low in *Escherichia coli*, the acquisition of *fosA* may reduce future activity of fosfomycin to treat infections caused by *E. coli*.[2] FosA is a glutathione transferase that inactivates fosfomycin through catalysing the addition of glutathione. *fosA* genes are often present in the chromosome of *Klebsiella pneumoniae*, but not in the chromosome of *E. coli*.[2,3] *Klebsiella variicola* is closely related and often misidentified as *K. pneumoniae*.[4] While horizontal spread of *fosA* has been demonstrated *in vitro*,[5] we here provide evidence for *in vivo fosA* transmission from *K. varii-cola* to *E. coli*, resulting in development of fosfomycin resistance.

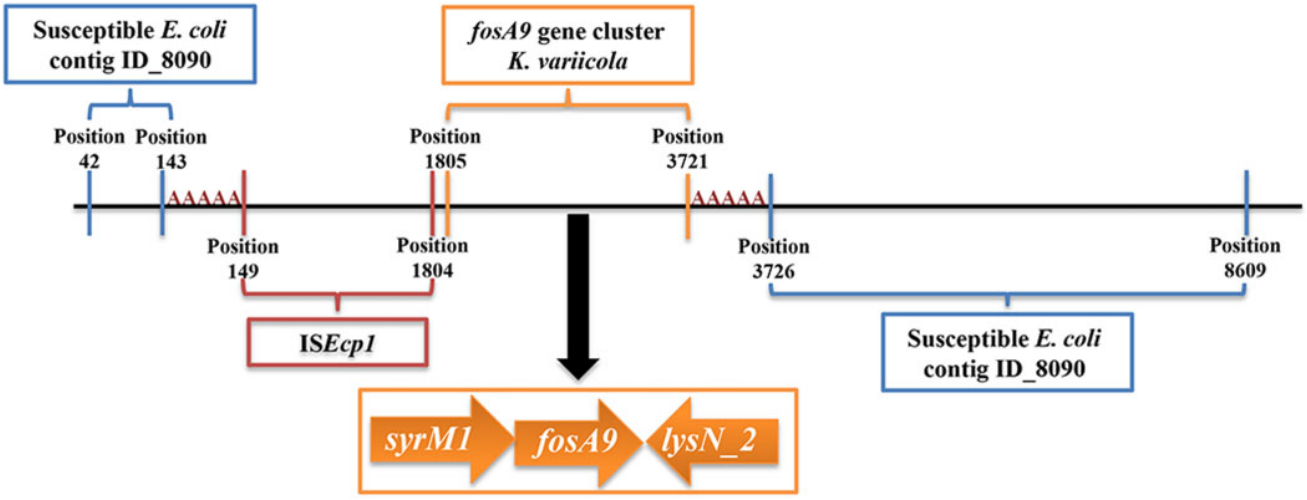

Figure 1. Schematic representation of the contig (ECO-BAB-IMI-103297_P-ACH-BAB-IMI-103242_1528359160_131_length_8653_cov_18.1163_ID_8928, 8653 bp) in the fosfomycin-resistant *E. coli* isolate containing a *fosA9* gene cluster originating from a *K. variicola* isolate. The IS*Ecp1-syrM1-fosA9-lysN2* region is flanked by 5 bp DRs (AAAAA), suggesting mobilization from *K. variicola* by IS*Ecp1*. Upstream and downstream sequences of the insertion region align to contig ECO-BAB-IMI-103298_P-ACH-BAB-IMI-103242_1528359160_92_length_16411_cov_29.2905_ID_8090 from the first susceptible *E. coli* isolate. Sequence information of complete genomes of all isolates and separate sequences of the relevant contigs (containing *fosA9* in *E. coli* and *K. variicola*, and ECO-BAB-IMI-103298_P-ACH-BAB-IMI-103242_1528359160_92_length_16411_cov_29.2905_ID_8090 from the susceptible *E. coli*) have been deposited in the ENA under project number PRJEB32329. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

The Medical Research Ethics Committee of the University Medical Center Utrecht confirmed that the Medical Research Involving Human Subjects Act does not apply to this study (reference number WAG/mb/18/027282). We were not able to obtain informed consent because the patient died a few years ago. All information including gender, age, dates and medical history that was not directly clinically relevant has been omitted to protect the privacy of the patient.

An aged patient had a suspicion of chronic endovascular infection of their aortic bifurcation graft, which the patient received after an acute aortic aneurysm 22 years earlier. The patient had suffered from recurrent episodes of sepsis, with blood cultures yielding *Propionibacterium* spp., *K. variicola*, *Citrobacter koseri* and *Pseudomonas aeruginosa*, as determined by MALDI-TOF MS. Positron emission tomography (PET)-CT findings were compatible with prosthetic graft infection. The patient subsequently developed septic shock with *E. coli* bacteraemia without a clear source of infection that was treated successfully with intravenous ceftriaxone. The isolate was resistant to amoxicillin/clavulanic acid and ciprofloxacin that had been used to suppress chronic infection, prompting the addition of oral fosfomycin at 3 g every 48 h. Seven months later, while still using fosfomycin, the patient developed spondylodiscitis. Blood cultures drawn at the time isolated *E. coli* with an identical resistance pattern, except being resistant to fosfomycin. Fosfomycin was discontinued and the patient received a prolonged course of ceftriaxone.

Fosfomycin susceptibility, determined by agar dilution according to CSLI guidelines,[6] demonstrated a rise in the MIC from 2 mg/L for the initial *E. coli* isolate to >1024 mg/L for the second *E. coli* isolate. WGS revealed five SNP differences between *E. coli* isolates in the core genome, based on core genome MLST (cgMLST) analysis.[7] Yet, the second *E. coli* isolate has a 3573 bp insertion consisting of IS*Ecp1*, a *fosA* gene we named *fosA9* as the next available number according to NCBI, *syrM1* and *lysN2*. The insertion is flanked by 5 bp DRs (AAAAA) suggesting mobilization of this *fosA*9 gene cluster by IS*Ecp1* (Figure 1).[8] Genes other than *fosA9* responsible for fosfomycin resistance were not found. At the time of the first *E. coli* sepsis episode, six *K. variicola* had been isolated from rectum swabs and blood cultures over a period of 20 months (Table S1, available as Supplementary data at *JAC* Online). cgMLST analysis revealed a maximum of 16 SNP differences between *K. variicola* isolates.[7] The same cluster as above containing *fosA*9, without the mobile genetic element IS*Ecp1*, was identified in the *K. variicola* isolates, suggesting *K. variicola* to be the source of *fosA9* acquired by *E. coli* (Figure 1). *fosA* genes were not identified in other clinical isolates from this patient. Sequence information of all isolates has been deposited in the European Nucleotide Archive (ENA) under project number PRJEB32329.

fosA transfer from *Klebsiella* spp. to *E. coli*, leading to fosfomycin resistance, has been demonstrated *in vitro*.[3] Based on publicly available genomes, *fosA* and adjacent genes are well conserved in *K. variicola* (minimum 98% identity to *fosA9*) and *K. pneumoniae* (minimum 94% identity to *fosA9*) isolates. According to mlplasmids, PlasmidFinder and contig coverage, *fosA9* was predicted to be located in the chromosome of the second *E. coli* and all *K. variicola* isolates.[9,10] However, based on BLASTn, the contig containing *fosA9* aligns to plasmid sequences. The localization of *fosA9* in *E. coli* can thus only be confirmed by completely assembling its genome using long-read sequencing, as the mobilization of the *fosA9* gene cluster by an IS element might switch its genomic background. We postulate that *fosA9* transfer from *K. variicola* to *E. coli* occurred in the gastrointestinal tract, as *K. variicola* was not co-cultured in the blood at the time of *E. coli* bacteraemia. We hypothesize that fosfomycin pressure played a role in this transfer; however, this

has to be confirmed with further experiments *in vitro*. Acquisition of *fosA9* was associated with an 8-fold increase in the MIC for *E. coli* (from 2 to 1024 mg/L) while, despite the presence of *fosA9* in the chromosome of the *K. variicola* isolates, the fosfomycin MICs were below the EUCAST susceptibility breakpoint of ≤32 mg/L (Table S1).[6] This could suggest either higher dependency of *E. coli* growth on glutathione or a difference in *fosA9* expression or metabolism, i.e. higher expression by the IS*Ecp1* promoter present upstream of the *fosA9* gene cluster.[8]

In conclusion, our case illustrates the potential of long-term use of oral fosfomycin to promote horizontal gene transfer of *fosA9* from commensal gut flora to potential pathogenic microorganisms, such as *E. coli*.

Acknowledgements

We are grateful to Dr Ad C. Fluit and Dr Anita Schurch from the University Medical Center Utrecht for the critical appraisal of this case report prior to submission.

Funding

This study was carried out as part of our routine work.

Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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SUPPLEMENTARY DATA

Table S1. Characteristics of the isolates, including the species, source, molecular characteristics, susceptibility and exposure to fosfomycin

Month	Species	Source	Strain identity*	*fosA* presence	MIC (AD, mg/l)	Oral fosfomycin exposure**
1	*Klebsiella variicola*	Blood	16	Yes	2	No
18	*Klebsiella variicola*	Rectum	7	Yes	16	No
18	*Klebsiella variicola*	Rectum	5	Yes	32	No
19	*Klebsiella variicola*	Rectum	4	Yes	8	No
20	*Klebsiella variicola*	Rectum	4	Yes	16	No
22	*Escherichia coli*	Blood	5	No	2	Started 3gr/48h
22	*Klebsiella variicola*	Rectum	15	Yes	2	3gr/48h
28	*Escherichia coli*	Blood	5	Yes	>1024	Stopped

AD: Agar dilution. * Difference in SNPs within the same species based on core MLST.[7] ** Fosfomycin-tromethamine (oral).

Conference abstracts: Oral presentations

- ASA 2020 (EUCAST Workshop). Fosfomycin frustrations. [Invited speaker].
- ECCMID 2019. Species and baseline resistance are more predictive than fosfomycin MIC for therapeutic success in urinary tract infections.
- ASA 2019. Oral fosfomycin ineffective against *K. pneumoniae* uropathogens in a dynamic bladder infection *in vitro* model.
- ISAP 2018. Efficacy of 3-doses of oral fosfomycin in a dynamic bladder infection *in vitro* model.
- ECCMID 2018. Impact of urine on fosfomycin PK/PD activity in a dynamic bladder infection *in vitro* model.
- ECCMID 2017. Emergence of fosfomycin resistance among susceptible *Enterobacteriaceae* in a novel bladder infection *in vitro* model.

Title: Fosfomycin frustrations (EUCAST workshop; Oral presentation)

Author: I.J. Abbott[1]

Institution: 1. Dept. Infectious Diseases, Alfred Hospital and Central Clinical School, Monash University, Melbourne, Victoria Australia.

Objectives: Outline the current limitations and challenges associated with fosfomycin susceptibility testing, with particular emphasis on updated pharmacokinetic and pharmacodynamic data to inform susceptibility testing, target pathogens and dosing recommendations.

Questions to address:

(1) What are the target pathogens?
   a. Gram-negative: *E. coli* only? All Enterobacterales? *Pseudomonas aeruginosa*?
   b. Gram-positive: *Enterococcus faecalis* & *E. faecium* (inc. VRE)? *Staphylococcus aureus*?

(2) What are the clinical indications?
   a. Strictly only for "uncomplicated UTIs"?
   b. Include "infections originating from the urinary tract" (including pyelonephritis and BSIs)?
   c. Infections outside of the urinary tract (e.g. prostatitis, MDR infections)?

(3) Oral and intravenous formulations?
   a. Oral: Is a single 3g dose sufficient? Should multiple doses be given? What dosing frequency?
   b. IV: When to use? What dose? Monotherapy vs. combination?

(4) How can the diagnostic laboratory confidently report susceptibility?
   a. Highlight the differences between EUCAST and CLSI
   b. Should clinical breakpoints change?
   c. Is the reference standard MIC measurement the best predictor for clinical success?

Key messages: Single dose oral fosfomycin remains an attractive and efficacious option for *E. coli* uUTIs and has good bacteriostatic activity against *Enterococcus* spp. There is much less certainty of activity against other *Enterobacterales* and *Pseudomonas aeruginosa*. Multi-dose oral regimens promote emergence of resistance when heteroresistance was present at baseline. Agar dilution MIC testing (with 25 mg/L glucose-6-phosphate, G6P) appears to be a poor reference standard MIC method to predict efficacy and fails to identify isolates with a resistant subpopulation important in treatment failure. Clinical breakpoints may benefit from being reduced closer to the *E. coli* epidemiological cut-off value. EUCAST plan to do Monte-Carlo simulations to account for PK variability and extrapolate to UTIs. CLSI have no plans to change current advice but will review all data about G6P and await PK-PD/animal data for non-*E. coli* species. Finally, we are awaiting the outcome data from clinical trials: FORECAST - cUTI, iv to oral switch, ciprofloxacin vs. fosfomycin, daily to complete 10 days; FOREST - iv fosfomycin vs. meropenem bacteraemic UTI caused by ESBL-*E. coli*.

Title: Species and baseline resistance are more predictive than fosfomycin MIC for therapeutic success in urinary tract infections (Oral presentation)

Authors: Iain J. Abbott,[1,2] Elke van Gorp,[2] Jordy Dekker,[2] Rixt A. Wijma,[2,3] Brenda C. M. de Winter,[3] Anton Y. Peleg, [1] Johan W. Mouton.[2]

Institution: 1. Department of Infectious Diseases, Alfred Hospital and Central Clinical School, Monash University, Melbourne, VIC Australia. 2. Department Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre, Rotterdam, The Netherlands. 3. Department of Hospital Pharmacy, Erasmus University Medical Centre, Rotterdam, The Netherlands

Background: Oral fosfomycin is a first-line antibiotic for uncomplicated urinary tract infections, with activity against MDR-uropathogens. However, baseline MIC testing may not adequately identify isolates with high likelihood of therapeutic success using standard dosing. We simulate urinary fosfomycin pharmacokinetics following a single 3g dose within a dynamic bladder-infection *in-vitro* model to assess antibacterial effects against ESBL-positive *E. coli* and *K. pneumoniae* uropathogens.

Materials/methods: A bladder-infection *in-vitro* model simulating urinary fosfomycin concentrations after gastrointestinal absorption was used using Mueller-Hinton broth (MHB) with 25mg/L glucose-6-phosphate(G6P). Target fosfomycin exposure (C_{max}:1984mg/L, T_{max}:7.5h, AUC_{0-24}:30938mg.h/L) was validated by LC-MS/MS. Pharmacodynamic (PD) response of 24-*E. coli* and 20-*K. pneumoniae* clinical isolates were examined (agar dilution MIC ≤0.25–128mg/L; 42/44 ESBL-positive). Additional baseline characteristics were assessed: mutant prevention concentration (MPC), disk diffusion diameter, broth microdilution (BMD) MIC (in MHB ±G6P), fosfomycin heteroresistance and *fosA* PCR. Dynamic pathogen kill and high-level resistance (HLR, MIC >1024mg/L) was assessed over 72h by quantitative cultures on drug-free and fosfomycin-containing Mueller-Hinton agar (64mg/L, 512mg/L).

Results: Observed *in-vitro* fosfomycin concentrations matched the simulation (accuracy: 10.6% ±4.4%), with minimal variation (relative: SD 6.3% ±1.4%). Twenty of 24-*E. coli* were killed, despite baseline fosfomycin MICs up to 128mg/L. The 4 *E. coli* that re-grew (MICs 4–32mg/L) had population replacement with HLR. At baseline, these isolates all had detectable high-level heteroresistance and MPC >1024mg/L. Only one-*E. coli* that was killed also had high-level heteroresistance at baseline and none had an MPC >1024mg/L. All *E. coli* isolates were *fosA*-negative. In contrast, at baseline, all *K. pneumoniae* isolates had detectable HLR heteroresistance, MPC >1024mg and were all *fosA*-positive. Only 3 of 20-*K. pneumoniae* isolates were killed, despite all having an MIC ≤16mg/L. Six isolates re-grew with emergence of HLR (proportion: 0.01%–100%), while 11 re-grew with HLR below the limit-of-detection.

Conclusions: *E. coli* and *K. pneumoniae* isolates demonstrate clear differences in their response to fosfomycin. Treatment failure in *E coli* appears to be related to the presence of high-level heteroresistance, not identified on standard MIC testing. Activity against *K. pneumoniae* is limited and challenge the applicability of oral fosfomycin for *K. pneumoniae* UTI treatment, regardless of susceptibility testing.

Title: Oral fosfomycin ineffective against *K. pneumoniae* uropathogens in a dynamic bladder infection *in vitro* model (Oral presentation)

Authors: IJ. Abbott*[1,2], E. van Gorp[2], J. Dekker[2], RA. Wijma[2,3], BCM. de Winter[3], AY. Peleg[1], JW. Mouton[2]

Institution: 1. Department of Infectious Diseases, Alfred Hospital and Central Clinical School, Monash University, Melbourne, VIC Australia. 2. Department Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre, Rotterdam, The Netherlands. 3. Department of Hospital Pharmacy, Erasmus University Medical Centre, Rotterdam, The Netherlands

Objectives: Oral fosfomycin is a first-line antibiotic for uncomplicated urinary tract infections, with activity against MDR-uropathogens. However, baseline MIC testing may not adequately identify isolates with high likelihood of therapeutic success using standard dosing. We simulate urinary fosfomycin pharmacokinetics following a single 3g dose within a dynamic bladder-infection *in-vitro* model to compare antibacterial effects against ESBL-positive *E. coli* and *K. pneumoniae* uropathogens.

Methods: A bladder-infection *in-vitro* model simulating urinary fosfomycin concentrations after gastrointestinal absorption was used using Mueller-Hinton broth (MHB) with 25mg/L glucose-6-phosphate (G6P). Target fosfomycin exposure (C_{max}:1984mg/L, T_{max}:7.5h, AUC_{0-24}:30938mg.h/L) was validated by LC-MS/MS. Pharmacodynamic (PD) response of 24-*E. coli* and 20-*K. pneumoniae* clinical isolates were examined (agar dilution MIC ≤0.25–128mg/L; 42/44 ESBL-positive). Additional baseline characteristics were assessed: mutant prevention concentration (MPC), disk diffusion diameter, broth microdilution (BMD) MIC (in MHB ±G6P), fosfomycin heteroresistance and *fosA* PCR. Dynamic pathogen kill and high-level resistance (HLR, MIC >1024mg/L) was assessed over 72h by quantitative cultures on drug-free and fosfomycin-containing Mueller-Hinton agar (64mg/L, 512mg/L).

Results: Observed *in-vitro* fosfomycin concentrations matched the simulation (accuracy: 10.6% ±4.4%), with minimal variation (relative: SD 6.3% ±1.4%). Twenty of 24-*E. coli* were killed, despite baseline fosfomycin MICs up to 128mg/L. The 4 *E. coli* that re-grew (MICs 4–32mg/L) had population replacement with HLR. At baseline, these isolates all had detectable high-level heteroresistance and MPC >1024mg/L. Only one-*E. coli* that was killed also had high-level heteroresistance at baseline and none had an MPC >1024mg/L. All *E. coli* isolates were *fosA*-negative. In contrast, at baseline, all *K. pneumoniae* isolates had detectable HLR heteroresistance, MPC >1024mg and were all *fosA*-positive. Only 3 of 20-*K. pneumoniae* isolates were killed, despite all having an MIC ≤16mg/L. Six isolates re-grew with emergence of HLR (proportion: 0.01%–100%), while 11 re-grew with HLR below limit-of-detection.

Conclusions: *E. coli* and *K. pneumoniae* isolates demonstrate clear differences in their response to fosfomycin. Treatment failure in *E coli* appears to be related to the presence of high-level heteroresistance, not identified on standard MIC testing. Fosfomycin activity against *K. pneumoniae* is limited and challenge the applicability of oral fosfomycin for *K. pneumoniae* UTI treatment, regardless of susceptibility testing.

Title: Efficacy of 3-doses of oral fosfomycin in a dynamic bladder infection *in vitro* model (Oral presentation)

Authors: Iain J. Abbott,[1,2] Rixt A. Wijma,[2] Nick R.J.F. Broos,[2] Anton Y. Peleg,[1] Johan W. Mouton.[2]

Institution: 1. Department of Infectious Diseases, Alfred Hospital and Central Clinical School, Monash University, Melbourne, VIC Australia. 2. Department Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre, Rotterdam, The Netherlands.

Background: Oral fosfomycin is a first-line antibiotic for uncomplicated UTIs, with activity against MDR-uropathogens. Despite off-label administration of 3-doses every 2-3 days, limited data are available to support such approaches. We performed pharmacodynamic profiling using a dynamic bladder infection *in-vitro* model to assess the adequacy of administering 3-doses of fosfomycin compared to single-dose.

Methods: A bladder infection *in-vitro* model simulating urinary fosfomycin concentrations after oral absorption of an equivalent 3g dose was used. Fosfomycin exposure (target: C_{max} 1984mg/L, T_{max} 7.5h, AUC_{0-24} 30938mg.h/L) was validated by LC-MS/MS measurements. Pharmacodynamic response of 16-Enterobacteriaceae clinical strains were examined (8 *E. coli*, 4 *E. cloacae*, 4 *K. pneumoniae*; agar dilution MIC 0.25–64mg/L). Isolates were exposed to single-dose fosfomycin in Mueller-Hinton broth (with 25mg/L glucose-6-phosphate, MHB) and in pooled urine from healthy female volunteers (FU), and 3-doses, given every 3-days, in MHB. Pathogen kill and resistance was examined by quantitative cultures on drug-free and fosfomycin-containing Mueller-Hinton agar (64mg/L, 512mg/L). Outcome was assessed 72h after the last fosfomycin dose.

Results: Observed *in-vitro* fosfomycin concentrations closely matched the simulation (see figure). After a single-dose, the same 8-isolates were killed in both MHB and FU. All *K. pneumoniae* isolates re-grew. For *E. coli* and *E. cloacae* isolates, PK/PD EI_{50} for effective kill (change in log_{10}cfu/mL at 72h) was similar in MHB and FU: $fAUC_{0-24}$/MIC 8251 (Hill-slope -3.2, R^2 0.9805) in MHB, compared to 8915 (Hill-slope -3.0, R^2 0.9774) in FU. Following 3-doses given every 3-days, 9-isolates were killed. One additional *K. pneumoniae* isolate was killed compared to single-dose. Outcome of all other isolates was unchanged. Amplification of the high-level-resistant subpopulation was most pronounced following 3-doses (median proportion 70%, range 2.5-100%), compared to single dose in MHB (59%, <0.00001-100%), and in FU (0.06%, 0.00002-88%).

Conclusions: In MHB and FU, *E. coli* and *E. cloacae* isolates with MIC >4mg/L are not reliably killed following a single 3g oral dose of fosfomycin. All *K. pneumoniae* isolates re-grew. Exposure to 3- doses did not alter the outcome in majority of isolates. These results challenge fosfomycin clinical breakpoints and suggest off-label practice of giving 3-doses every 3-days is unlikely to be an effective treatment strategy, and may promote the emergence of resistance.

Young Investigator Award

Title: Impact of urine on fosfomycin PK/PD activity in a dynamic bladder infection *in vitro* model (Oral presentation)

Authors: Iain J. Abbott,[1,2] Rixt A. Wijma,[2] Nick R.J.F. Broos,[2] Joseph Meletiadis,[2,3] Anton Y. Peleg, [1] Johan W. Mouton.[2]

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Background: Oral fosfomycin is a first-line antibiotic for uncomplicated urinary tract infections, with good activity against MDR-uropathogens. Little is known of the impact of urine on fosfomycin activity. We simulate urinary fosfomycin pharmacokinetics, using drug-free urine, within a dynamic bladder-infection *in-vitro* model to assess the antibacterial effects.

Materials/methods: A bladder-infection *in-vitro* model simulating urinary fosfomycin concentrations after gastrointestinal absorption of a 3g dose was used using pooled, drug-free urine, pH 7.0, from healthy female volunteers, filtered prior to use. Fosfomycin exposure (PK simulation: C_{max} 1984mg/L, T_{max} 7.5h, AUC_{0-24} 30938mg.h/L) was validated by LC-MS/MS measurements from bladder compartments during voiding time-points. Pharmacodynamic response of 16-Enterobacteriaceae strains were examined (8 *E. coli*, 4 *E. cloacae*, 4 *K. pneumoniae*; agar dilution MIC 0.25–64mg/L). Pathogen kill and resistance was assessed over 72h by quantitative cultures on drug-free and fosfomycin-containing Mueller-Hinton agar (64mg/L, 512mg/L).

Results: Observed *in-vitro* fosfomycin concentrations closely matched the simulation (see figure). Eight-isolates were killed. Isolates that re-grew had significant rise in total population fosfomycin MIC (MIC_{50} 12mg/L, MIC_{90} 16mg/L; to MIC_{50} 128mg/L, MIC_{90} >1024mg/L, p=0.0078). All *K. pneumoniae* isolates re-grew regardless of MIC. For *E. coli* and *E. cloacae* isolates, PK/PD EI_{50} for effective kill (72h log_{10}cfu/mL) were: *f*AUC_{0-24}/MIC 6777, *f*C_{max}/MIC 435 (Hill-slope -7.2, R^2 0.997 for both), *f*Time >4xMIC 52h (Hill-slope -44.9, R^2 0.997). Area-under-time-kill-curve demonstrated similar results: *f*AUC_{0-24}/MIC 5744, *f*C_{max}/MIC 368 (Hill-slope -3.1, R^2 0.996 for both), *f*Time >4xMIC 51h (Hill-slope -17.9, R^2 0.995). The exposure-response curves were steep. The proportion of resistant sub-population at baseline was also related to effective kill (EC_{50} 0.0001%, Hill-slope 2.1, R^2 0.998) and area-under-time-kill-curve (EC_{50} 0.0001%, Hill-slope 1.3, R^2 0.992). Two-*E. coli* isolates identified as outliers in non-linear regression analysis, were killed despite baseline fosfomycin MIC 32 and 64mg/L, but lacked a detectable sub-population.

Conclusions: Compared to Mueller-Hinton broth with glucose-6-phosphate, human urine impedes fosfomycin activity, despite concurrent limitations on uropathogen growth. Of clinical significance, *E. coli* and *E. cloacae* isolates with MIC >4mg/L are not reliably killed in urine, together with all *K. pneumoniae* isolates. Emergence of resistance was significant. These results challenge oral fosfomycin dosing and clinical breakpoints for UTIs.

Title: Emergence of fosfomycin resistance among susceptible *Enterobacteriaceae* in a novel bladder infection in-vitro model (Oral presentation)

Authors: Iain J. Abbott,[1,2] Lamprini Kanioura,[2] Anton Y. Peleg,[1] Johan W. Mouton,[2] Joseph Meletiadis.[2]

Institution: 1. Department of Infectious Diseases, the Alfred Hospital and Central Clinical School, Monash University, Melbourne, Australia. 2. Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands.

Background: Urinary tract infections (UTIs) are a frequent indication for antimicrobial use and are among the most commonly encountered bacterial infections. Oral fosfomycin remains one of the most active antimicrobials for outpatient UTI treatment and a viable option for multidrug resistant uropathogens. Despite longstanding use, limited data are available to guide dosing in complicated or multidrug resistant UTIs. We used an in-vitro bladder model to demonstrate drug exposures that can either effectively kill, or select for resistance, among initially susceptible *Enterobacteriaceae*.

Material/methods: A novel in-vitro bladder infection model simulating the urinary pharmacokinetics of a 3g dose of oral fosfomycin tromethamine undergoing first-order absorption in a two-compartment model with first-order elimination was used. Using a 1:15 scaled version of normal human urodynamics, the in-vitro model uses exponential changes in fosfomycin concentrations undergoing dilution at constant volumes and elimination into eight bladder compartments at a flow rate of 3.95ml/h, voided 4-hourly during the day and 12-hour interval overnight, with 1ml post-void-residual volume. Eight clinical isolates, fosfomycin-susceptible, ESBL-producing *Enterobacteriaceae* (4-*Escherichia coli*, 4-*Klebsiella pneumoniae*), with baseline MIC 0.5–16.0mg/L by agar dilution, were introduced into the bladder compartment of the model and run for 72-hours. Starting inoculum was 10^7 CFU, providing a total number of bacteria expected in human infections (10^5 CFU/ml in average 250ml void). Fosfomycin concentrations were measured with a microbiological assay. Quantitative growth was assessed by serial dilutions on Mueller-Hinton agar (MHA) and resistant subpopulations on MHA containing 32 and 512mg/L fosfomycin (supplemented with 25mg/L glucose-6-phosphate).

Results: Simulated urinary pharmacokinetic profiles were attained, with an average excretion half-life of 5.5-hours (range 4.4–6.4-hours) and peak concentration of 2142mg/L (range 1271.9–3347.9mg/L) measured at 4-hours. All 8-isolates were initially rapidly killed. Four-isolates (baseline MIC 4.0–16.0mg/L) had detectable re-growth from 28 to 52-hours, and by 72-hours had re-grown exceeding the starting inoculum. Emergence of resistance was demonstrated. Three out of 4-isolates re-grew (10^3–10^4 CFU/ml) on MHA with 512mg/L fosfomycin. The fourth isolate re-grew (10^3 CFU/ml) on MHA with 32mg/L fosfomycin only. Prior to fosfomycin exposure, these isolates all initially had a detectable resistant sub-population (10^2 CFU/mL on MHA with 32mg/L fosfomycin). Three isolates killed in the model had baseline MIC ≤1.0mg/L, without an initial resistant subpopulation detected. The fourth isolate killed had a baseline MIC 16.0mg/L with a detectable (10^3 CFU/ml) resistant sub-population, but was exposed to the highest C_{max} at 4-hours (>3300mg/L).

Conclusions: Using a novel and dynamic in-vitro bladder infection model, when exposed to urinary fosfomycin concentrations expected after a 3g oral stat dose, fosfomycin-susceptible *Enterobacteriaceae* uropathogens that were more likely to re-grow and to select for resistance were those with a higher baseline fosfomycin MIC, a detectable resistant sub-population, and those exposed to a lower C_{max}/MIC ratio.

Conference abstracts: Poster presentations

- ASA 2020. Oral fosfomycin treatment for enterococcal urinary tract infections in a dynamic *in vitro* model.
- ECCMID 2019. Efficacy of single and multiple oral fosfomycin dosing for *Pseudomonas* urinary tract infections.
- ECCMID 2019. PK/PD assessment of fosfomycin in synthetic human urine compared to pooled human urine in a dynamic *in vitro* bladder infection model.
- ID Week 2018. Efficacy of repeat dosing of oral fosfomycin in a dynamic bladder infection *in vitro* model.
- ECCMID 2018. Efficacy of 48-hour and 24-hour repeat dosing of fosfomycin in a dynamic bladder infection *in vitro* model.
- ECCMID 2017. Development and validation of a novel *in vitro* bladder infection model simulating urinary fosfomycin pharmacokinetics.

Title: Oral Fosfomycin Treatment for Enterococcal Urinary Tract Infections in a Dynamic *In Vitro* Model (Poster presentation)

Authors: I.J. Abbott*[1,2], E. van Gorp[2], A. van der Meijden,[2] R.A. Wijma,[2,3] J. Meletiadis,[4] J.A. Roberts,[5,6,7] J.W. Mouton,[2] A.Y. Peleg.[1,8]

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Objectives: Limited data are available to guide oral fosfomycin dosing in enterococcal urinary tract infections, including vancomycin-resistant *Enterococcus* (VRE).

Methods: Eighty-four enterococcal isolates underwent fosfomycin susceptibility testing by agar dilution. Sixteen isolates (including *E. faecalis* ATCC 29212 and *E. faecium* ATCC 35667) were selected for testing in a bladder infection *in vitro* model, run with synthetic human urine (SHU), simulating different dynamic changing urinary fosfomycin concentrations after oral administration.

Results: Fosfomycin $MIC_{50/90}$ for the 84-isolates were 32/64 mg/L for *E. faecalis* and 64/128 mg/L for *E. faecium*. The 16-isolates selected for testing in the bladder infection model represented the range of baseline fosfomycin MIC values. In the selected isolates, broth microdilution MIC results in Mueller-Hinton broth (with and without glucose-6-phosphate) were concordant with agar dilution, while testing in SHU returned values one dilution lower. Under drug-free conditions in the bladder infection model, *E. faecium* demonstrated greater growth restriction in SHU compared to *E. faecalis* (*E. faecium* growth capacity 5.8 ± 0.6 $\log_{10}$ CFU/mL; *E. faecalis* 8.0 ± 1.0 $\log_{10}$ CFU/mL). Simulated fosfomycin concentrations closely matched the target (bias 2.3%) following high and low fosfomycin urinary concentrations after a single dose, and after two-doses given daily with low urinary exposure. Initial bacterial kill (> 3 $\log_{10}$) and suppression of regrowth was promoted in the majority of isolates following high urinary exposure after a single dose, and after two-daily doses with low urinary exposure. Low-level re-growth post-fosfomycin exposure was related to isolate persistence, without any emergence of resistance or rise in fosfomycin MIC. *E. faecalis* isolates required greater fosfomycin exposure to inhibit growth compared with *E. faecium* (*E. faecalis*: $fAUC_{0\text{-}72}$/MIC EC_{50} 497, $f\%T > MIC_{0\text{-}72}$ EC_{50} 66%; *E. faecium* $fAUC_{0\text{-}72}$/MIC EC_{50} 253, $f\%T > MIC_{0\text{-}72}$ EC_{50} 52%).

Conclusions: Administration of two-doses of fosfomycin with low urinary concentrations resulted in equivalent growth inhibition to a single dose with high urinary concentrations. With this urinary exposure, fosfomycin was effective in promoting suppression of regrowth (>3 $\log_{10}$ kill) in the majority of isolates.

Title: Efficacy of single and multiple oral fosfomycin dosing for *Pseudomonas* urinary tract infections (Poster presentation)

Authors: Iain J. Abbott,[1,2] Elke van Gorp,[2] Rixt A. Wijma,[2,3] Brenda C. M. de Winter,[3] Anton Y. Peleg,[1] Johan W. Mouton.[2]

Institution: 1. Department of Infectious Diseases, Alfred Hospital and Central Clinical School, Monash University, Melbourne, VIC Australia. 2. Department Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre, Rotterdam, The Netherlands. 3. Department of Hospital Pharmacy, Erasmus University Medical Centre, Rotterdam, The Netherlands.

Background: Oral fosfomycin is indicated for uncomplicated urinary tract infections, with activity against MDR-uropathogens. Limited data are available to guide dosing in complicated, antibiotic-resistant UTIs. We use a dynamic *in-vitro* bladder model with synthetic human urine (SHU) to demonstrate drug exposures that can effectively kill, or select for resistance, among *Pseudomonas aeruginosa* urinary isolates.

Materials/methods: A bladder-infection *in-vitro* model was used, simulating dynamic urinary fosfomycin concentrations in SHU after absorption of 3g oral fosfomycin. Single dose was compared to daily-dosing for 7-days. Target fosfomycin exposure (C_{max}:1984mg/L, T_{max}:7.5h, AUC_{0-24}:30938mg.h/L) was validated by LC-MS/MS. Pharmacodynamic (PD) response of 16-*P. aeruginosa* isolates were examined (agar dilution MIC 1–>1024mg/L). Additional baseline fosfomycin susceptibility was assessed; disk diffusion, broth microdilution (BMD) MIC, Vitek-2 and heteroresistance. Dynamic pathogen kill/resistance assessed over 72h following a single dose, and over 216h following daily dosing for 7-days. Quantitative cultures were performed on drug-free and fosfomycin-containing Mueller-Hinton agar (64mg/L, 512mg/L).

Results: At baseline, 11/16 isolates had high-level heteroresistance detected. Compared to agar dilution, baseline BMD MIC values were 1-2 dilutions higher in MHB, and a further dilution higher in SHU. No difference was noted when tested with or without glucose-6-phosphate. *In-vitro* flow rates were accurately reproduced to ensure fosfomycin exposure matched the simulation, confirmed by LC-MS/MS. Following exposure to both single and 7-doses of fosfomycin, all isolates re-grew. Emergence of high-level fosfomycin resistance (MIC >1024mg/L) occurred in 6/16 isolates following the single dose. Whereas after 7-doses, 10/16 isolates had emergence of high-level resistance. One isolate (MIC 256mg/L) had limited re-growth without emergence of resistance following both dosing schedules (3.3 log_{10} and 6.0 log_{10}CFU/mL respectively). In contrast to other isolates, BMD MIC was lower in SHU (MIC 16mg/L), suggesting a specific growth restriction in SHU impacting upon the PD outcome.

Conclusions: *P. aeruginosa* isolates are ineffectively killed following urinary fosfomycin exposures following single or multiple oral doses. Emergence of resistance was significant, and worsened by prolonged therapy. These results should caution against the off-label use of multiple doses of oral fosfomycin monotherapy for *P. aeruginosa* UTIs, regardless of baseline susceptibility testing.

Title: PK/PD assessment of fosfomycin in synthetic human urine compared to pooled human urine in a dynamic *in vitro* bladder infection model (Poster presentation)

Authors: Iain J. Abbott,[1,2] Elke van Gorp,[2] Rixt A. Wijma,[2,3] Brenda C. M. de Winter,[3] Anton Y. Peleg,[1] Johan W. Mouton.[2]

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Background: Little is known of the impact of the bladder environment on fosfomycin activity, nor how to best simulate this *in-vitro*. In a dynamic bladder infection *in-vitro* model, we compare laboratory media to pooled human urine and synthetic alternatives to test which best resembles *in-vivo*.

Materials/methods: Urinary fosfomycin concentrations after absorption of a 3g oral dose were simulated with different media: Mueller-Hinton-broth (MHB); MHB with glucose-6-phosphate (MHB+G6P, 25mg/L); female midstream urine (MSU, randomly pooled); female 24h-collected urine (24U, pooled equal volume); artificial urine medium (AUM, Brooks *et al.*1997); synthetic human urine (SHU, Ipe *et al.*2016). Target fosfomycin exposure (C_{max}:1984mg/L, T_{max}:7.5h, AUC_{0-24}:30938mg.h/L) was validated by LC-MS/MS. Pharmacodynamic response of 16-Enterobacteriaceae were examined (8-*E. coli*, 4-*E. cloacae*, 4-*K. pneumoniae*; agar dilution MIC ≤0.25–64mg/L). Broth microdilution (BMD) MIC was performed in MHB, MHB+G6P, 24U and SHU. Pathogen kill/resistance was assessed over 72h by quantitative cultures on drug-free and fosfomycin-containing Mueller-Hinton agar (64mg/L, 512mg/L).

Results: MSU was more dilute than 24U (pH 7.0, osmolality 260mOsm, glucose <0.1mmol/L; compared to pH 6.5, osmolality 468mOsm, glucose 0.2mmol/L). Neither had detectable G6P (<2nmoles). Synthetic urine alternatives differed slightly in chemical composition and pH (AUM pH 6.5; SHU pH 5.6), however, AUM precipitation limited its used. BMD in MHB+G6P demonstrated ≥1-dilution higher MIC compared to agar dilution. Without G6P, MICs were ≥4-fold higher, except two *E. coli* (MIC 32 & 64mg/L) where MIC was unchanged, and were killed in the model in all media. Overall, the same 8-isolates (2 *E. coli*, 2 *E. cloacae*, 4 *K. pneumoniae*) re-grew and 4-isolates (4 *E. coli*) killed in all media. Remaining 4-isolates (2 *E. coli*, 2 *E. cloacae*) re-grew variably in urine and synthetic media. Emergence high-level resistance (proportion >0.01%) depended on media (7/8 MHB+G6P; 6/8 MHB, 4/8 MSU; 5/8 24U; 0/8 AUM; 1/8 SHU). Dynamic *in-vitro* fosfomycin concentrations matched simulation (accuracy: 4.7% ±2.7%), with minimal variation (relative: SD 4.4% ±3.0%).

Conclusions: The media in which fosfomycin susceptibility testing and PK/PD experiments are performed impacts upon results obtained. By using SHU, a more accurate representation of the *in-vivo* PK/PD activity of fosfomycin can be reproduced, although emergence of resistance appears to be restricted.

Title: Efficacy of Repeat Dosing of Oral Fosfomycin in a Dynamic Bladder Infection *In Vitro* Model (Poster presentation)

Subject category: A2. PK/PD studies. C8. UTIs

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Background: Oral fosfomycin is indicated for uncomplicated urinary tract infections with activity against MDR-uropathogens. Despite off-label use of giving 3 doses every 2-3 days, limited supporting data are available. We performed pharmacodynamic profiling using a dynamic bladder infection *in vitro* model to assess adequacy of repeat doses of fosfomycin.

Methods: A bladder infection *in vitro* model simulating urinary fosfomycin concentrations after 3g (equiv.) oral doses was used with Mueller-Hinton broth (MHB) with 25mg/L glucose-6-phosphate. Fosfomycin exposures were validated by LC-MS/MS measurements. Pharmacodynamic response of 16 clinical Enterobacteriaceae were examined (8 *E. coli*, 4 *E. cloacae*, 4 *K. pneumoniae*; agar dilution MIC 0.25–64mg/L) following 3 doses of fosfomycin given every 72h, 48h or 24h, compared to single dose therapy. Pathogen kill and resistance was assessed by quantitative cultures on drug-free and fosfomycin-containing Mueller-Hinton agar (MHA +64mg/L, +512mg/L).

Results: Fosfomycin exposure following single and multiple doses were accurately reproduced (mean deviation from target 5.0% ±3.4%, max 11.8%) with minimal variability (mean relative SD 2.7% ±1.7%, max 8.8%). Fosfomycin high-level heteroresistance was detected prior to drug exposure in 8/16 isolates (proportion 0.00002-0.001% of total population). All isolates with high-level heteroresistance regrew following single dose fosfomycin. Following 3-doses given every 72h, one additional *K. pneumoniae* isolate was killed. All other isolates regrew with amplification of HLR subpopulation (median proportion: 71.4%, IQR 57.5-100%). Despite dosing 48- and 24-hourly, the same isolates regrew, although HLR subpopulation amplification was reduced (48h dosing: 32.0%, IQR 0.005-83.3%; 24h dosing: 0.3%, IQR 0.0004-81.3%).

Conclusions: Dynamic *in vitro* modelling of multiple doses of oral fosfomycin fails to additionally suppress regrowth in majority of isolates compared to single dose therapy. Baseline high-level heteroresistance is an important predictor for regrowth. These results suggest that more fosfomycin is not necessarily better than standard single dose therapy. Earlier timing of repeat doses may help suppression of resistance.

Title: Efficacy of 48-hour and 24-hour repeat dosing of fosfomycin in a dynamic bladder infection *in vitro* model (Poster presentation)

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Background: Urinary tract infections are a common indication for antibiotics. Oral fosfomycin remains one of the most active antibiotics for MDR-uropathogens. Despite clinical practice of administering repeat oral doses, limited data are available supporting such approaches. We performed pharmacodynamic profiling using a dynamic bladder-infection *in-vitro* model to assess the adequacy of administering a repeat dose (RD) of fosfomycin.

Materials/methods: A bladder-infection *in-vitro* model simulating urinary fosfomycin concentrations after gastrointestinal absorption of repeat 3g dosages was used with Mueller-Hinton broth supplemented with 25mg/L glucose-6-phosphate. Simulated *in-vitro* fosfomycin concentrations were validated by LC-MS/MS measurements. Eight-Enterobacteriaceae isolates that had repeatedly re-grown following a single dose (SD) of fosfomycin were tested (2 *E. coli*, 3 *E. cloacae*, 3 *K. pneumoniae*; baseline MIC 2–64 mg/L). Isolates were exposed to a RD of fosfomycin at 48h and, if re-growth occurred, re-tested with RD given at 24h. Pathogen kill and emergence of resistance was assessed for 72h after RD by quantitative cultures on drug-free and fosfomycin-containing Mueller-Hinton agar (64 mg/L, 512 mg/L).

Results: Observed *in-vitro* fosfomycin concentrations simulated the expected urinary exposures following each dose (average ±SD: T_{max} 3.7 ±0.8h, C_{max} 2565.2 ±375.9mg/L, AUC_{0-24} 36298.3 ±5960.2mg.h/L). *E. coli* isolates were killed following the 48h RD of fosfomycin (baseline MIC 16 and 64mg/L; HLR sub-population 0.0003 and 0.0002% after SD). Six-isolates that re-grew (3 *E. cloacae*, 3 *K. pneumoniae*) were re-tested with RD administered at 24h. Two *K. pneumoniae* isolates were killed (baseline MIC 2 and 4mg/L; HLR subpopulation 0.0001 and 0.0003% after SD). The remaining *K. pneumoniae* isolate (MIC 4mg/L; HLR subpopulation 0.002% after SD) and 3 *E. cloacae* isolates (MIC 32–64mg/L; HLR subpopulation 11.6–100% after SD) re-grew.

Conclusions: Repeat dosing of fosfomycin is most effective in *E. coli* isolates. Reducing the time to the second dose to 24h provided additional kill. The second dose of fosfomycin failed in half of tested isolates. Failure appears to be related to the emergent HLR subpopulation, selected for after the initial dose. These results demonstrate that a single repeat dose will not provide adequate treatment in all cases.

Title: Development and validation of a novel in-vitro bladder infection model simulating urinary fosfomycin pharmacokinetics (Poster presentation)

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Background: Urinary tract infections (UTIs) are among the most commonly encountered bacterial infections and a frequent indication for antimicrobials. Despite longstanding use of single dose oral fosfomycin, limited data are available to support current dosing and clinical breakpoints. Establishing supporting evidence for optimal dosing schedules that promote uropathogen kill and prevent emergence of resistance is vital. Accurate simulations of urinary exposure are required to assess antibacterial effects. We have developed a novel in-vitro bladder two-compartment infection model simulating urinary fosfomycin pharmacokinetics after oral administration.

Material/methods: Exponential changes in fosfomycin concentrations undergoing dilution at constant volumes and elimination into the eight in-vitro bladder compartments was controlled by two peristaltic pumps and connecting tubing, simulating a 3g oral dose of fosfomycin tromethamine. Mathematical equations describing antibiotic concentrations over time were applied in a two-compartment model with first-order absorption and elimination targeting normal urinary pharmacokinetics (elimination half-life 2.4–7.3-hours, urine C_{max} 1053–4415mg/L within 4-hours, 85–95% excreted within 24-hours, and urinary concentration >100mg/L for 30–48-hours [Patel *et al.* Drugs 1997]). Normal human urodynamics (24-hour void volume 1500ml; post void residual <30ml [Haylen *et al.* Neurourol. Urodynam. 2010]) was mimicked with a 1:15 scale. Bladder compartments increased in volume, voided 4-hourly during the day and a 12-hour interval overnight, with 1ml returned simulating a low-normal post-void residual volume. Fosfomycin concentrations were determined by a microbiological bioassay. A standard curve was generated from inhibition diameters from a susceptible *Escherichia coli*. The validity of the model was confirmed by computer simulation, reproducing the expected time course of fosfomycin concentrations.

Results: A flow rate of 31.6ml/h (3.95ml/h to each bladder compartment), with maintained volumes of 20.0ml and 260.0ml in the first two compartments (A and B), simulated absorption and excretion half-lives of 0.4 and 5.7-hours respectively, peak urinary concentrations occurred at 4-hours, measuring 2484.5mg/L. Concentrations remained >100mg/L for 32-hours, and 94.1% of total dose was excreted after 24-hours. The in-vitro model was constructed and despite initially generating faster flow-rates, absorption and excretion half-lives were 0.3 and 3.0-hours respectively, and peak bladder concentrations ranged from 1931.8 to 2105.3mg/L at 3 hours. Subsequent testing better matched the simulation, generating an average peak bladder concentration of 2142mg/L (range 1271.9–3347.9mg/L) at 4 hours, with a calculated excretion half-life of 5.5 hours (range 4.4–6.4-hours). Standard curve for the bioassay was logarithmic (R^2=0.993-0.998).

Conclusions: This novel in-vitro bladder infection two compartment model, incorporating first-order absorption and bladder elimination, is a valid method to accurately simulate urine pharmacokinetics following an oral dose of fosfomycin tromethamine. Further use of this model will enable the pharmacokinetic and pharmacodynamic assessment of uropathogens exposed to fosfomycin and thereby provide updated evidence for clinical breakpoints and dosing schedules.

Image courtesy of Bob den Outer