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

Iain J. Abbott[1,2], Joseph Meletiadis[2,3], Imane Belghanch[2], Rixt A. Wijma[2], Lamprini Kanioura[2], Jason A. Roberts[4,5], Anton Y. Peleg[1,6] and Johan W. Mouton[2]*

[1]*Department of Infectious Diseases, Alfred Hospital and Central Clinical School, Monash University, Melbourne, Victoria, Australia;* [2]*Department of Medical Microbiology and Infectious Diseases, Research and Development Unit, Erasmus Medical Centre, Rotterdam, The Netherlands;* [3]*Clinical Microbiology Laboratory, Attikon University Hospital, Medical School, National and Kapodistrian University of Athens, Haidari, Athens, Greece;* [4]*Faculty of Medicine and School of Pharmacy, The University of Queensland, Brisbane, Queensland, Australia;* [5]*Royal Brisbane and Women's Hospital, Herston, Queensland, Australia;* [6]*Biomedicine Discovery Institute, Department of Microbiology, Monash University, Clayton, Victoria, Australia*

*Corresponding author. Tel: +31-10-70-33511; Fax: +31-10-70-33875; E-mail: jwmouton@gmail.com

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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*Time>4×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

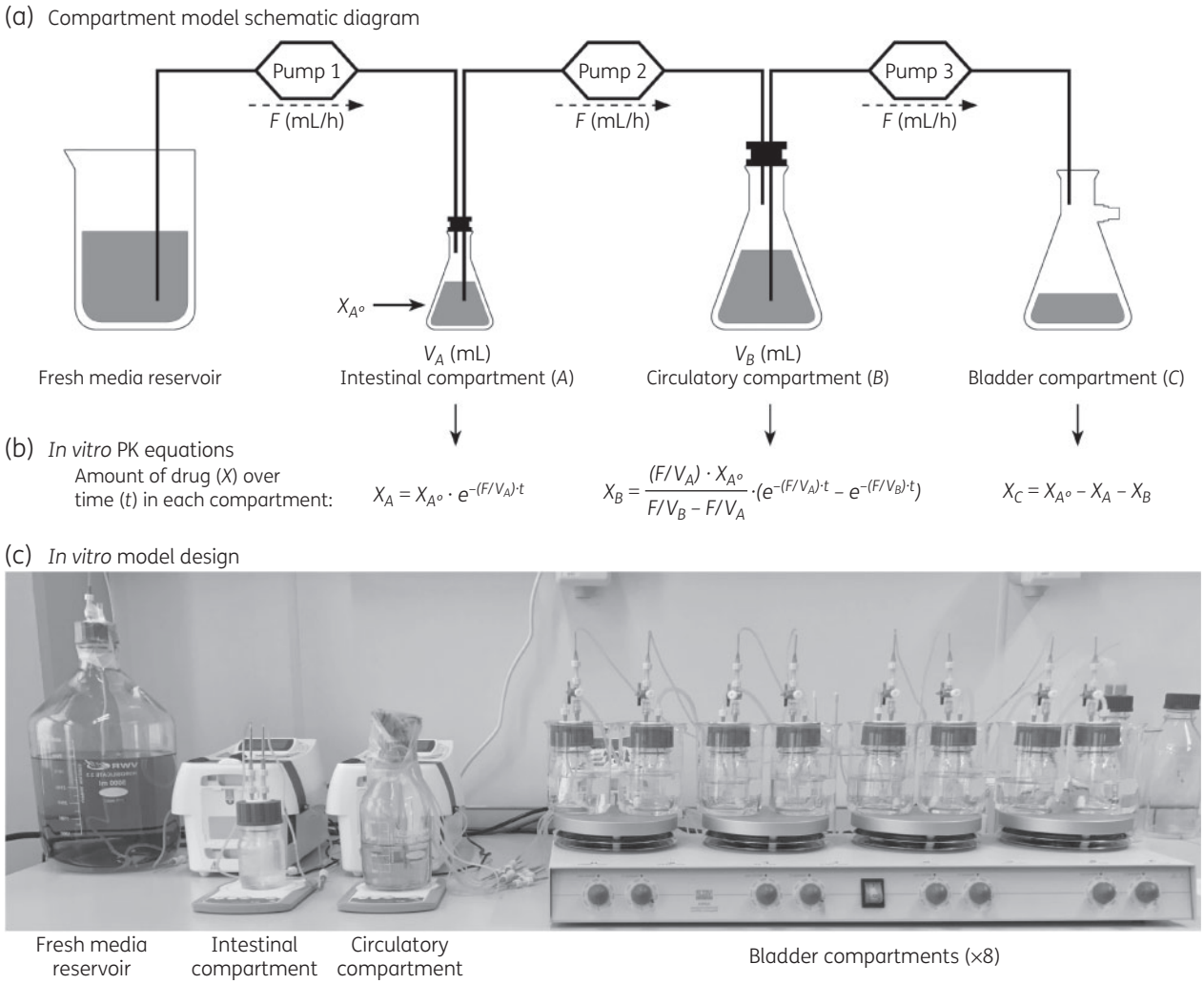

Figure 1. Drug distribution in an *in vitro* compartmental model. (a) Schematic model for first-order absorption in a two-compartment model with first-order elimination. Tandem first-order processes are simulated by exponential changes in antibiotic concentration undergoing dilution at constant volume. 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).

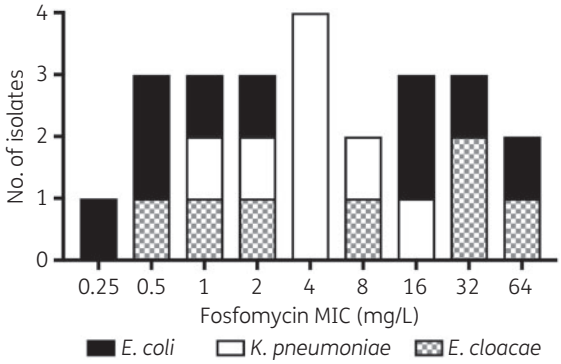

Figure 2. Baseline fosfomycin MIC distribution. MIC testing performed by agar dilution. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control organisms and returned MIC values within range.

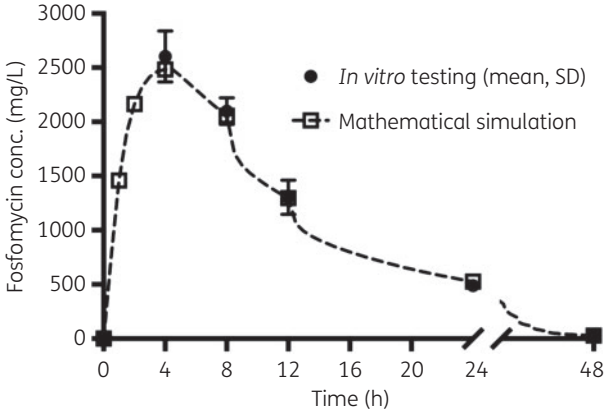

Figure 3. Average fosfomycin concentration changes over time within the *in vitro* bladder compartment following a single dose of fosfomycin. The broken line represents the concentration–time curve generated from values derived from the mathematical simulation. Open squares highlight the concentrations 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 (*f*Time>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

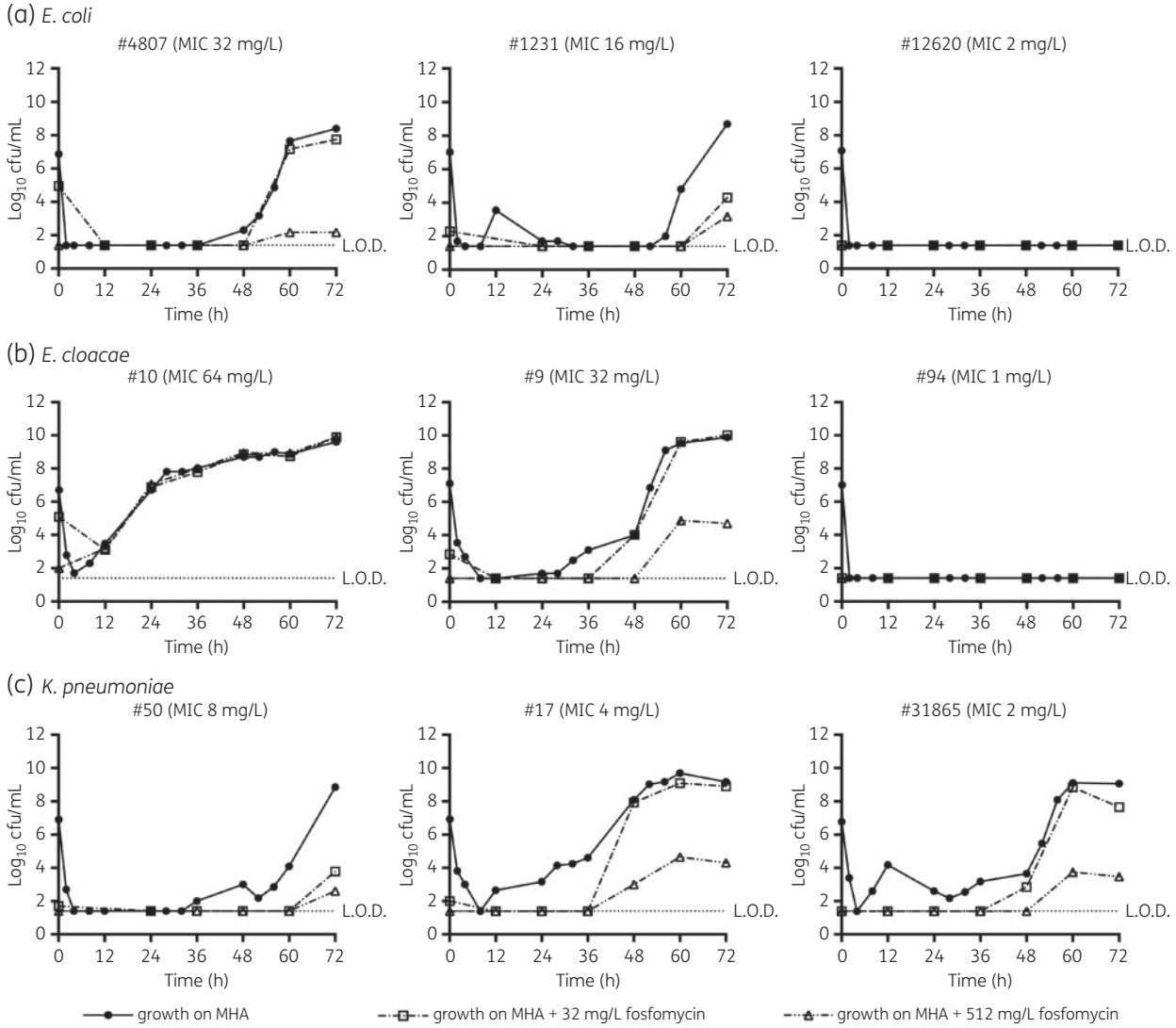

Figure 4. Examples of the quantitative PD assessment following a single dose of fosfomycin. Quantitative cultures performed at every simulated bladder compartment void. Filled circles represent total population growth, open squares represent low-level fosfomycin resistance and open triangles represent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

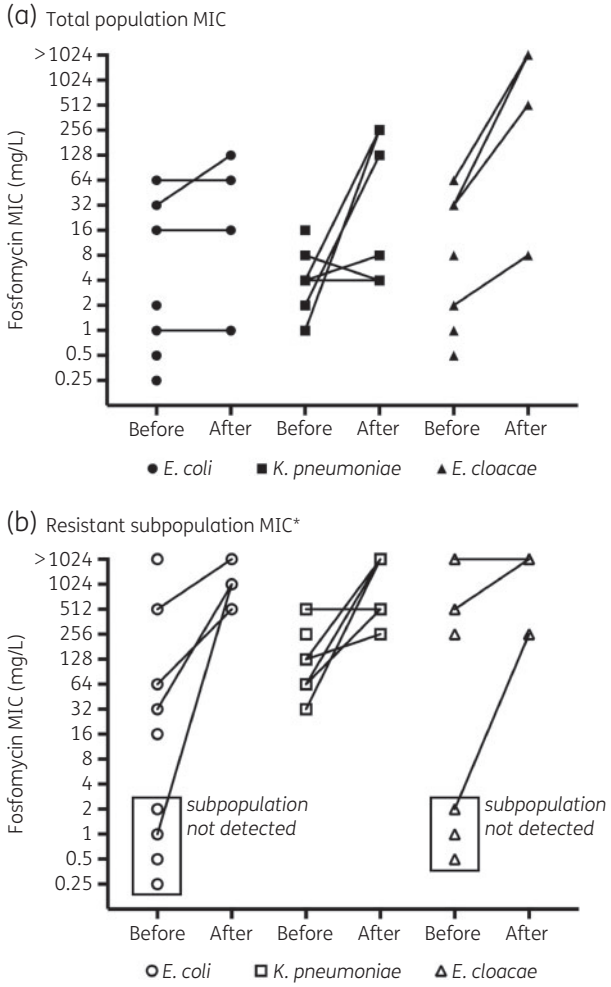

Figure 5. Changes in fosfomycin MIC before and after exposure to a single dose of fosfomycin. *Resistant subpopulation testing was performed from a subculture of growth from MHA containing fosfomycin. MIC testing performed by agar dilution. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used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

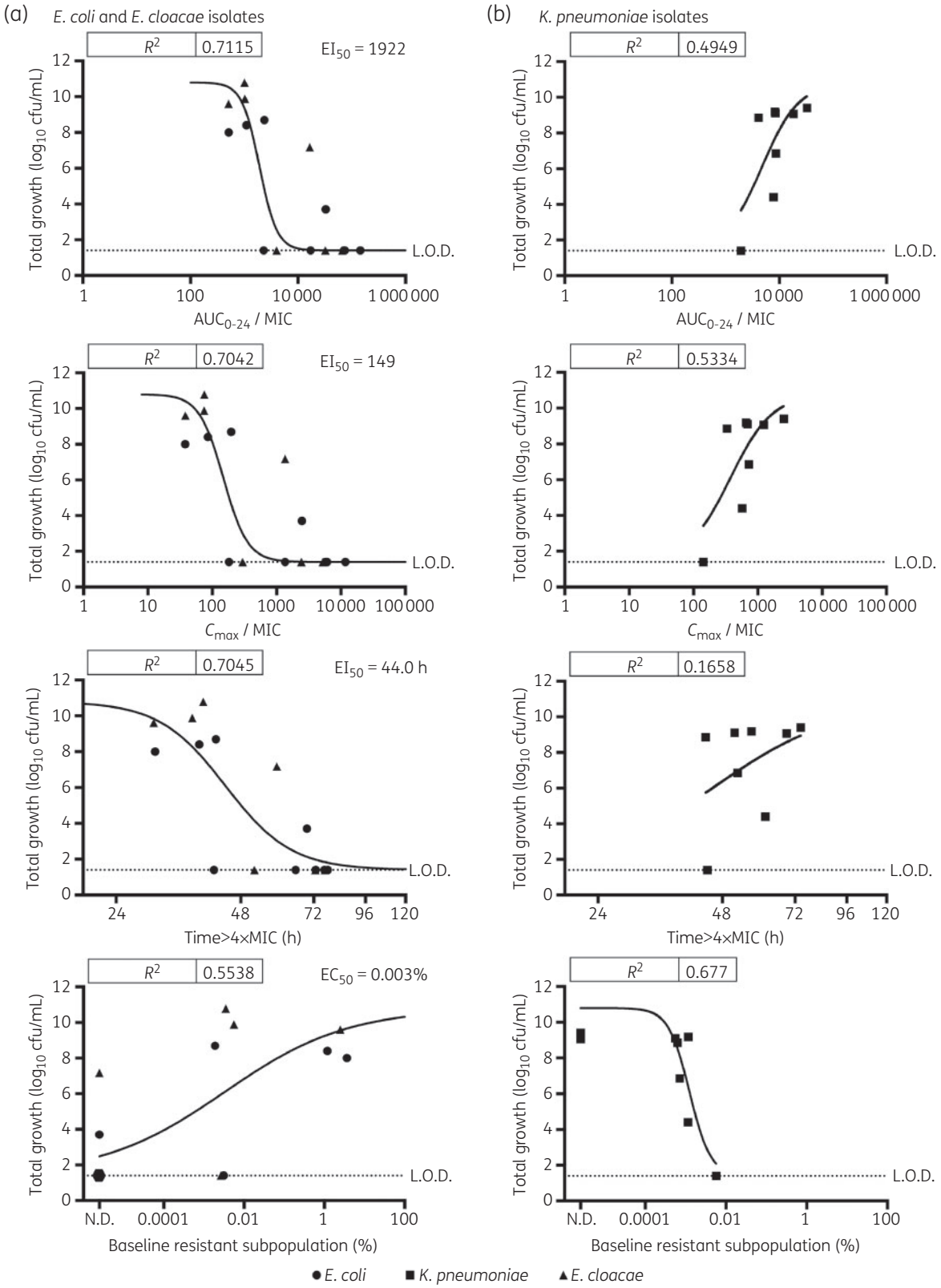

Figure 6. Relationship between drug exposure and baseline resistance with effective pathogen kill. Relationships between free-drug fosfomycin fAUC$_{0-24}$/MIC ratio, fC_{max}/MIC ratio, fTime>4×MIC and baseline resistant subpopulation proportion and the total growth at 72 h of *E. coli*, *E. cloacae* 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.

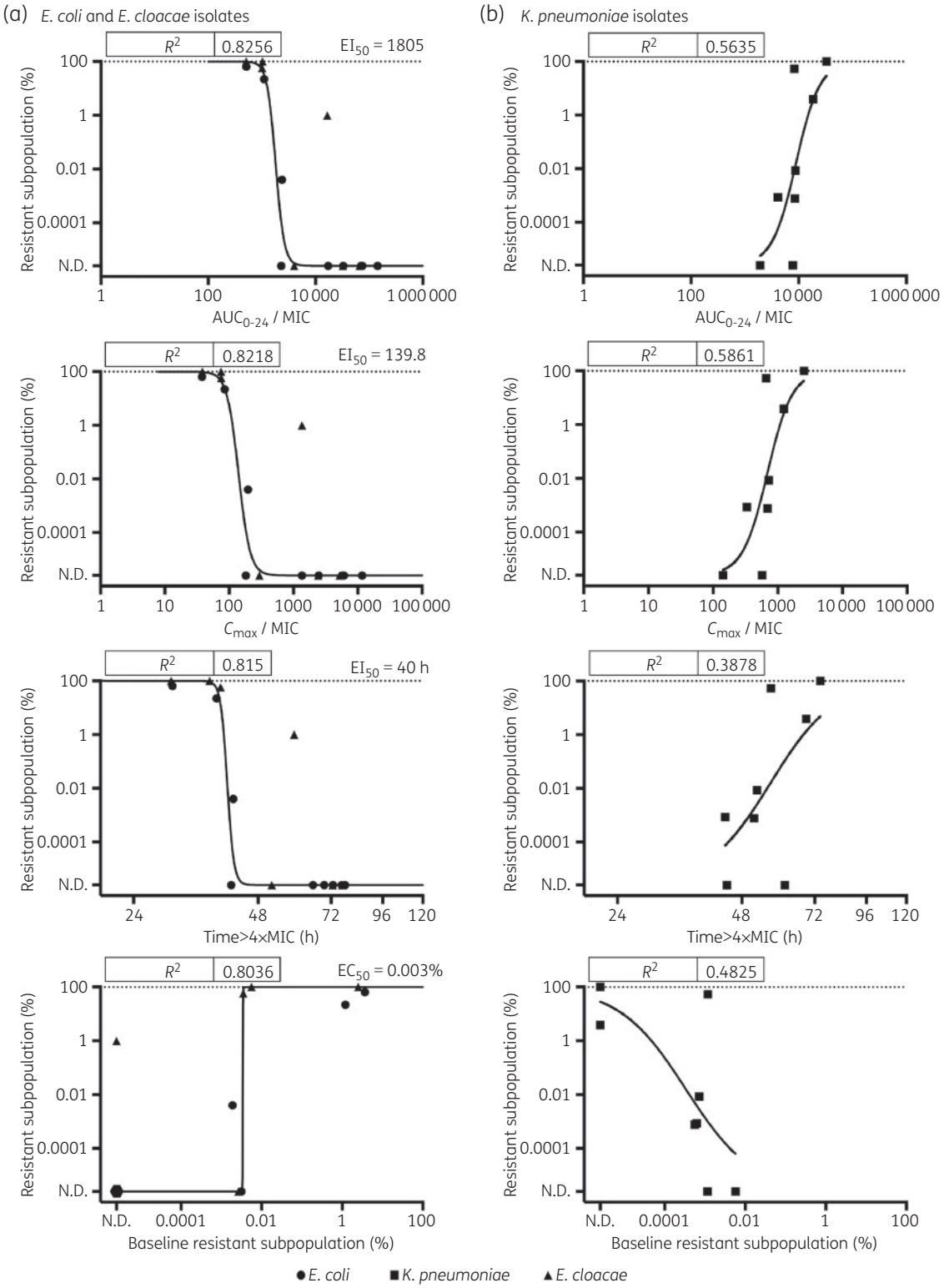

Figure 7. Relationship between drug exposure and baseline resistance with the emergence of fosfomycin resistance. Relationships between free-drug fosfomycin $fAUC_{0-24}$/MIC ratio, fC_{max}/MIC ratio and *f*Time>4×MIC and baseline resistant subpopulation proportion and the resistance subpopulation proportion 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.

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