Faculty of Health Sciences, Department of Medical Biology

Old antibiotics as alternative treatment options for urinary tract infections caused by ESBL-, AmpC- and carbapenemase-producing *Escherichia coli*

Studies with fosfomycin, mecillinam, nitrofurantoin, and temocillin

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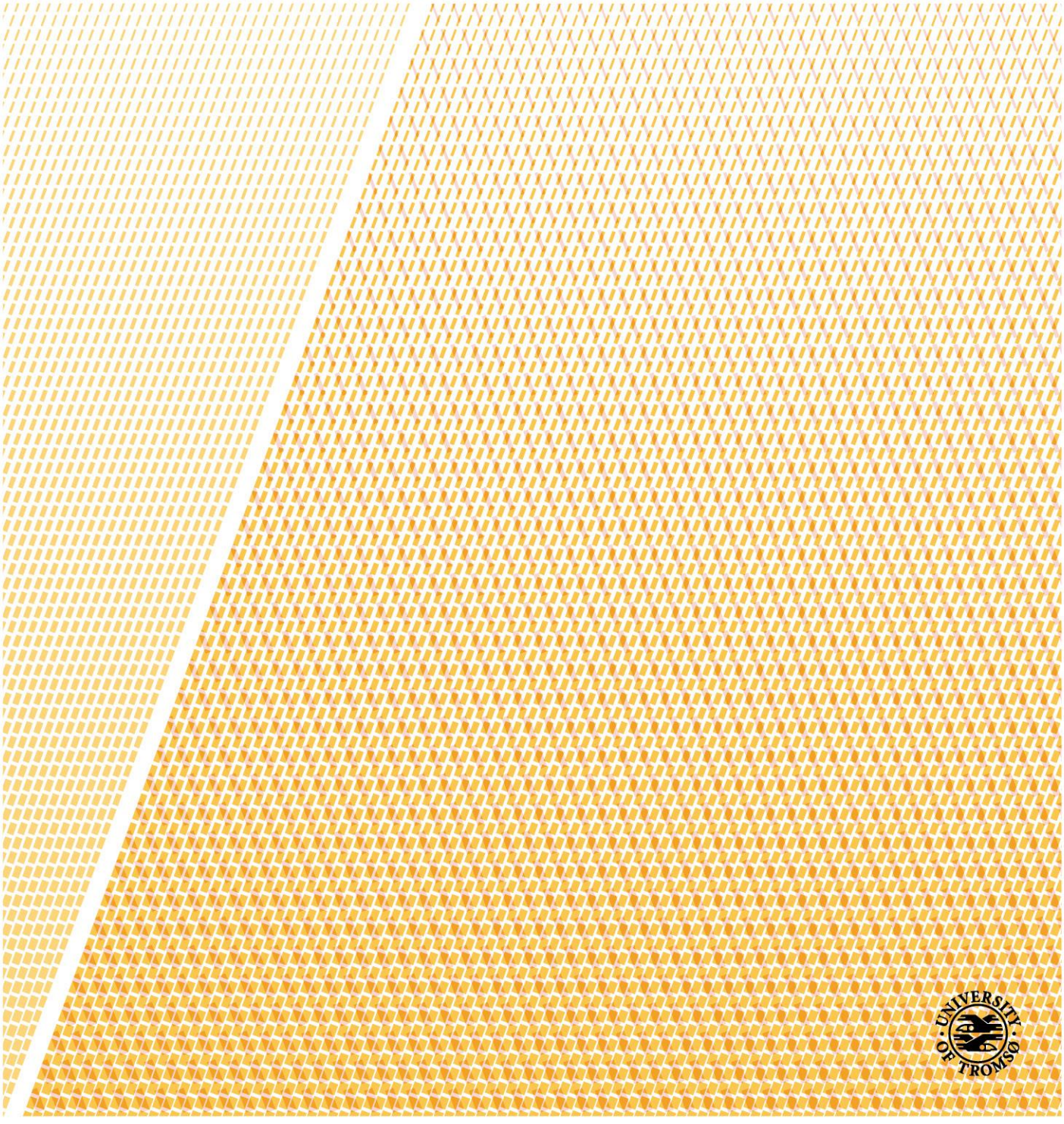
Ilya Nikolaevich Zykov

A dissertation for the degree of Philosophiae Doctor - 2020



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Studies with fosfomycin, mecillinam, nitrofurantoin, and temocillin

ILYA NIKOLAEVICH ZYKOV

UiT The Arctic University of Norway

UNIVERSITETSSYKEHUSET NORD-NORGE
UNIVERSITY HOSPITAL OF NORTH NORWAY

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Dedicated to the memory of Tamara Perova

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List of papers

Paper 1.

The antimicrobial activity of mecillinam, nitrofurantoin, temocillin and fosfomycin and comparative analysis of resistance patterns in a nationwide collection of ESBL-producing *Escherichia coli* in Norway 2010–2011

Ilya Nikolaevich Zykov, Arnfinn Sundsfjord, Lars Småbrekke & Ørjan Samuelsen

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Paper 2.

Pharmacokinetics and pharmacodynamics of fosfomycin and its activity against ESBL-, plasmid-mediated AmpC-and carbapenemase-producing *Escherichia coli* in a murine urinary tract infection model.

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Paper 3.

Efficacy of mecillinam against clinical multidrug-resistant *Escherichia coli* in a murine urinary tract infection model.

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Abbreviations

ADME	absorption, distribution, metabolism, and excretion
AIEC	adherent-invasive *E. coli*
AmpC	ampicillinace C (Ambler class C β-lactamase)
Asn	asparagine
Asp	aspartic acid
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AUC	area under the total drug concentration-time curve
$AUC/MIC_{0\text{-}72h}$	area under the total drug concentration-time curve over 72 h divided by MIC
$AUC24/MIC_{24h}$	area under the total drug concentration-time curve over 24 h divided by MIC
BID	*bis in die*, dosing frequency two times daily
bla	gene encoding β-lactamase
BSAC	The British Society for Antimicrobial Chemotherapy
C	concentration
C(t)	drug concentration over time
C_0	initial concentration
C_{max}	the maximum concentration of a drug in a specific compartment after dosing
cAMP	cyclic adenosine monophosphate
CARB-X	Combating Antimicrobial Resistant Bacteria Biopharmaceutical Accelerator
CDEC	cell-detaching *E. coli*
CFU	colony-forming unit
CFU/ml	colony-forming units per milliliter
CLSI	Clinical and Laboratory Standards Institute
Cmax	maximum concentration
CMY	cefamycin β-lactamase
CTX-M	cefotaximase-Munich β-lactamase
Cys	cysteine
DHA	Dhahran hospital β-lactamase
DNA	deoxyribonucleic acid
E	effect
E.	*Escherichia* OR *Enterococcus* OR *Enterobacter*
E_0	effect observed without the exposure
EARS-NET	Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
ECOFF	epidemiological cut-off value
E_{max}	maximal effect
ESBL	extended-spectrum β-lactamase

EU	The European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EX_{50}	the value of PK characteristic, at which 50% of the effect is observed
ExPEC	extraintestinal pathogenic *E. coli*
f	unbound ('free') fraction
*f*AUC/MIC	area under the unbound drug concentration-time curve divided by MIC
fC_{max}/MIC	maximal unbound drug concentration divided by MIC
FDA	Food and Drug Administration
$fT_{>MIC}$	time period that unbound drug concentration exceeds the MIC
GDP	total gross domestic product
GES	Guiana extended-spectrum β-lactamase
GIM	German imipenemase
HGT	horizontal gene transfer
i.v.	intravenous
Ile	isoleucine
IMP	imipenemase
IPEC	intestinal pathogenic *E. coli*
IS	insertion sequence
K.	*Klebsiella*
k_{el}	elimination constant
K_m	Michaelis-Menten constant
KPC	*Klebsiella pneumoniae* carbapenemase
K-res	Norwegian National Advisory Unit on Detection for Antimicrobial Resistance
Leu	leucine
LOD	limit of detection
MBC	minimal bactericidal concentration
MDR	multi-drug resistance OR multi-drug resistant
MIC	minimal inhibitory concentration
MIC_{50}	MIC, which inhibits 50% of isolates in a tested sample
MIC_{90}	MIC, which inhibits 90% of isolates in a tested sample
MNEC	meningitis/sepsis-associated *E. coli*
MPC	the mutant prevention concentration
NDM	New Delhi metallo-β-lactamase
NORM	Usage of Antimicrobial Agents and Occurrence of Antibiotic Resistance in Norway
NTEC	necrotoxic *E. coli*
OF-1	Oncins France 1, mice strain
OXA	oxacillinase
p	probability value
p.o.	*per os*
PAE	post-antimicrobial effect
pAmpC	plasmid-mediated AmpC
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PD	pharmacodynamics / pharmacodynamic

PD target	pharmacodynamic target
PK	pharmacokinetics / pharmacokinethic
q12h	*quaque* 12 hours, dosing frequency every 12 hours
q36h	*quaque* 36 hours, dosing frequency every 36 hours
q6h	*quaque* 6 hours, dosing frequency every 6 hours
R^2	the coefficient of determination
RIC	resistance inhibiting concentration
RNA	ribonucleic acid
S	substrate
s.c.	subcutaneous
SHV	sulfhydryl variable β-Lactamase
SPM	Sao Paulo metallo-β-lactamase
spp.	*species pluralis*
ST	sequence type
t	time
$T_{>C}$	time of the concentration above a certain concentration threshold
$T_{>MIC}$	time period that total drug concentration exceeds the MIC
$T_{>RIC}$	time period that total drug concentration exceeds the RIC
$t_{1/2}$	half-life
TEM	Temoniera β-Lactamase
TID	*ter in die*, dosing frequency three times daily
TLR-4	toll-like receptor
UDP-GlcNac	uridine 5′-diphospho-N-acetylglucosamine
UPEC	uropathogenic *E. coli*
US	The United States
UTI	urinary tract infection
VIM	Verona Integron-encoded Metallo-β-Lactamase
V_{max}	the maximal rate of elimination
WGS	whole-genome sequencing
WHO	World Health Organization
WT	wild-type
γ	coefficient determining the steepness of the Hill curve

Abstract

Background The emergence of multidrug-resistant (MDR) bacteria is acknowledged as one of the most significant global health threats. Among the MDR bacteria, *Escherichiae coli*, producing extended-spectrum β-lactamases (ESBLs), pAmpCs, or carbapenemases, are of great concern. *E. coli* is a prevalent commensal member of the human microbiota capable of causing a wide range of infections, including urinary tract infections (UTIs). A high prevalence of co-resistance also to non-β-lactam antimicrobial classes among the ESBL-, pAmpC-, or carbapenemase-producing *E.coli*, along with the shortage of new antibiotics in the development pipeline can lead to an increase in the use of reserve antimicrobials. Re-introducing "old antimicrobials" could offer a timely solution.

Objectives The aim of this work was to evaluate the role of fosfomycin, mecillinam, temocillin, and nitrofurantoin as the treatment options for UTIs caused by MDR ESBL-, pAmpC-, and carbapenemase-producing *E. coli*. Our specific objectives were: (i) Analyze the resistance patterns among a nationwide strain collection of ESBL-producing *E. coli*; (ii) to determine the antimicrobial activity of fosfomycin, mecillinam, temocillin and nitrofurantoin in Norwegian ESBL-producing *E. coli*; (iii) to evaluate the in vivo efficacy of fosfomycin and mecillinam in the treatment of UTI caused by MDR *E. coli*; (iv) to determine the optimal pharmacokinetic/pharmacodynamic (PK/PD) indices for fosfomycin in UTI; and (v) to determine the optimal dosing (200 vs. 400 mg TID) of mecillinam in UTI;

Methods We have used several microbiological and molecular *in vitro* methods: susceptibility testing of bacterial strains using broth microdilution, agar dilution, gradient test strips, and VITEK2; detection antibiotic resistance genes by polymerase chain reaction and microarray. Additionally, whole-genome sequencing was used to determine the presence of virulence genes, resistance genes, ST-type, serotype, and FimH variant in clinical strains. The expression of type-1 fimbriae was evaluated in a yeast agglutination assay. Fosfomycin activity was assessed *in vitro* using the time-kill kinetics test; its PK was determined in the OF-1 mice using bioassay, *in vivo* PK/PD was described with Hill function in a murine UTI model. The efficacy of fosfomycin and mecillinam against clinical MDR *E. coli* strains was evaluated *in vivo* in the murine UTI model.

Results In **paper 1**, we investigated the susceptibility patterns among a Norwegian nationwide collection of ESBL-producing *E. coli* from 2010-2011. A high proportion of isolates (91-100%) was sensitive to fosfomycin, mecillinam, temocillin, and nitrofurantoin and had low co-resistance. This is comparable to amikacin and carbapenems (95-100%). In contrast, high levels of resistance were observed to broad-spectrum β-lactams such as 3rd generation cephalosporins and aztreonam (67-100%). Moreover, we found a high proportion of resistance for trimethoprim-sulfamethoxazole (71%), gentamicin (40%), tobramycin (50%), and ciprofloxacin (74%) and co-resistance among them (36% for three and 40% for two drug classes). In **paper 2** and **paper 3**, we further studied fosfomycin and mecillinam, respectively, in a murine UTI model. For fosfomycin, we performed the pharmacokinetic/pharmacodynamic (PK/PD) analysis and tested *in vivo* efficacy against MDR plasmid-mediated pAmpC-/ESBL-/carbapenemase-producing clinical *E. coli* isolates. The optimal PK/PD index, based on fosfomycin bloodstream levels, was Cmax, followed by AUC/MIC0–72. Fosfomycin reduced the CFU/ml in urine, bladder, and kidneys of all susceptible MDR strains, except for one harboring *fos*A. In **paper 3**, two mecillinam dosing regimens were calculated. We aimed to mimic human PK for pivmecillinam dosing regimens of 200 mg and 400 mg TID. For both doses, mecillinam reduced the urinary CFU-counts for all strains except one ESBL-producer at 400 mg TID. Efficacy was shown against carbapenemase-producers, including NDM-1 (mecillinam MIC 2 mg/L) and VIM-29 (mecillinam MIC 64 mg/L).

Conclusion The present works suggest old drugs to be promising alternatives to reserve drugs against UTIs caused by ESBL-, pAmpC-, or carbapenemases-producing MDR *E. coli*.

1 INTRODUCTION

1.1 The problem of antimicrobial resistance

Antimicrobials, improved sanitary conditions, vaccines, and access to clean water, were essential factors for improved health and life expectancy in the 20th century [(1)]. The effectiveness of currently available antimicrobials is declining worldwide due to increasing antimicrobial resistance [(2)]. Antimicrobial resistance defines as the ability of microbes to withstand the effects of antimicrobial drugs.

The medical burden of antimicrobial resistance includes increased morbidity and mortality, as well as prolonged hospital stays, additional visits in the outpatient settings, delays to the clinical decision-making, use of more expensive treatment options, a higher chance of complications and side effects of the treatment, temporary and permanent disability [(3)]. A population-level modeling analysis, performed using data from the European Antimicrobial Resistance Surveillance Network (EARS-NET), estimated 672000 infections with antimicrobial-resistant bacteria to occur in EU [(4)]. Of which 64% were associated with health care and contributed to 33000 attributable deaths and 875000 disability-adjusted life-years in 2015 [(4)]. The number of attributable deaths was highest for 3rd generation cephalosporin-resistant *E. coli* [(4)]. The burden was increasing with increased age. In young-adults and adults, the highest burden was associated with carbapenem- and colistin-resistant microorganisms [(4)].

Attempts to estimate the total economic burden of antimicrobial resistance suggest considerable costs. For instance, in 2014, Gandra *et al.* estimated the cost to be minimum 1.5 billion Euro for Europe (in 2007) and minimum 55 billion US Dollars for the United States (in 2000) [(5)]. Decreased productivity due to fewer healthy individuals in the labour market would lead to increased cost of goods and services, rising prices, and eventually decrease the total gross domestic product (GDP) by 3.1% of total global output [(6)].

1.1.1 Future perspectives on antimicrobial resistance and antimicrobial use

According to the current increasing trends of antimicrobial consumption and antimicrobial resistance, antimicrobials will become less effective in the future, and the burden of resistance is likely to increase [(7)].

In 2014, the attempt to estimate future scenarios of antimicrobial resistance was reported by O'Neill [8]. The increase in mortality due to antimicrobial-resistant bacteria was estimated to reach 10 million yearly by 2050, exceeding the cancer mortality today. The global economic burden could reach a 7% loss of GDP (including indirect costs) or over 210 trillion US dollars over the next 35 years [8, 9]. This approach was criticized for being rather crude due to surveillance data limitations and a somewhat arbitrary choice of endpoint resistance levels [10].

Another example of estimating the future consequences of antimicrobial resistance was performed on a smaller scale – with the only focus at carbapenem-resistant *E. coli* [11]. According to one of the proposed scenarios, 40 000 people would die annually in the EU, and excess hospital stay would reach 1.7 million days [11]. In this situation, a new drug combined with a rapid diagnostic test would prevent 15 000 deaths and decrease excess hospital stays by 650 000 days per year [11].

Overall, antimicrobial resistance is a global problem. If no action is taken, we risk facing the post-antimicrobial era, where the majority of the world population would not be able to afford newer drugs.

1.1.2 Current status of drug development

According to a recent World Health Organization (WHO) report [12], a total of 51 including combination drugs, along with 11 biologic substances, have reached the clinical stage of the drug development pipeline by May 2017. In total, there are only 14 drugs against *Enterobacterales* (nine in phase one and five in phase three of clinical development) [12]. Although trends among newly submitted potential drug candidates show increased attention to the gram-negative pathogens, most of the current candidates are modifications of existing drugs, aimed to avoid specific resistance mechanisms. Additionally, few agents can target more than one group of pathogens, limiting their empiric use, and only two (cephalosporins combined with siderophores) are active against all critical priority pathogens. Recently, cefiderocol (siderophore cephalosporin) has been approved for use in complicated UTIs [13]. Oral formulations, suitable for targeting community treated infections, such as UTIs, are available only for three drugs [12].

According to the reported average success rates and development times for drug development [14], ten new approvals can be awaited in the next five years [12]. For the nine drugs in phase one, only two are expected to reach the market in the next seven years [12]. An increase in innovative projects in early preclinical development has been observed, according to the

Combating Antimicrobial Resistant Bacteria Biopharmaceutical Accelerator (CARB-X), where 368 applications have been submitted [15], however the time from preclinical projects to the market clearance could take 15-20 years.

As for now, the available antimicrobials in the pipeline are not sufficient to combat the antimicrobial resistant pathogens, and treatment options will remain scarce [12, 15]. This again underlines that alternative strategies are urgently needed to be implemented for tackling the current situation with antimicrobial resistance.

1.2 Definitions of antimicrobial resistance

Antimicrobial resistance can broadly be defined into two categories – clinical resistance and microbiological resistance [16, 17]. Both definitions are based on interpreting the susceptibility testing results obtained using various laboratory tests. However, all susceptibility testing methods have a common basis –either the direct measurement of the minimal inhibitory concentration (MIC) or correlating to it [18].

Clinical resistance is based on the susceptibility of bacteria in relation to the likelihood of therapeutic success in patients taking into account factors such as the dose, dosing scheme, pharmacokinetics (PK), pharmacodynamics (PD), and infection site [16].

Based on this, antimicrobial-bacteria combinations are classified into three categories, "susceptible, standard dosing regimen (S), susceptible, increased exposure (I) and resistant (R)" [16] with the following definitions as set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST):

- "S: a microorganism is categorized as **Susceptible, standard dosing regimen** when there is a high likelihood of therapeutic success using a standard dosing regimen of the agent.
- I: a microorganism is categorized as **Susceptible, increased exposure** when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection.
- R: a microorganism is categorized as **Resistant** when there is a high likelihood of therapeutic failure even when there is increased exposure" [16].

Microbiological definition. The microbiological definition of antimicrobial resistance is based on the concept of wild-type (WT) and non-wild type (non-WT). A WT microorganism

is defined as a microorganism devoid of acquired and mutational resistance mechanisms [17, 18]. Consequently, non-WT microorganism possesses acquired and/or mutational resistance mechanisms reducing the susceptibility to a specific antimicrobial. Based on the determination of susceptibility distributions to an antimicrobial, WT and non-WT can be distinguished by the epidemiological cut-off value (ECOFF) [17].

1.3 General mechanisms of resistance

Molecular mechanisms of antimicrobial resistance include mutations or modifications in the target molecule for the antimicrobial drug, enzymatic inactivation of the antimicrobial, active efflux, and prevention of access to the target [19, 20]. Antimicrobial resistance can be intrinsic (as the results of the inherent structural of functional characteristics) or acquired through horizontal gene transfer (HGT) or mutations [19].

1.3.1 Prevention of access to the target

Porin loss. Cell walls of gram-negative bacteria are intrinsically less permeable to many antimicrobials due to the presence of an outer membrane [20]. Outer-membrane porins are channels allowing hydrophilic molecules (incl. antimicrobials) to penetrate the outer membrane [21, 22]. Loss of such channels can, therefore, serve as a resistance mechanism by limiting the diffusion of antimicrobials into the cell. This mechanism is often observed in gram-negatives [19], for example, in resistance to carbapenems in *E. coli* due to changes in porins (e.g., OmpF and OmpC) [23].

Increased efflux. Antimicrobials can be actively pumped out of the bacterial cell via efflux pumps. Efflux pumps are often the cause of intrinsic resistance [19]. Moreover, overexpression of efflux pumps can lead to acquired resistance - for instance, the overexpression of AcrAB and MdfA efflux pumps leads to resistance to fluoroquinolones in *E. coli* [24]. Overexpression of efflux pumps is often induced by specific molecules (e.g., acrAB induction by indole [25]), acquired via HGT [26], or caused by mutations in regulatory genes. According to substrate specificity, efflux pumps can be narrow-spectrum or transport a wide variety of substances with different chemical structures (known as multidrug efflux pumps) [19].

1.3.2 Changes in antimicrobial target

Another strategy to overcome the action of antimicrobials is by alteration of the target molecule leading to reduced affinity to antimicrobials [19]. Changes in the target genetic

sequence can be acquired by mutation or HGT [19]. Target modification (protection) can also be possible without changing the protein sequence of the target (e.g., methylation) [27, 28]. Specific examples of such strategies include changes in DNA gyrase (GyrA) and topoisomerase (ParC) conferring quinolone resistance [29], 16S rRNA methylases (ArmA, RmtA, RmtB, RmtC, RmtD, and NpmA) conferring aminoglycoside resistance [27], or alterations in penicillin-binding proteins (PBPs), conferring β-lactam resistance [30].

1.3.3 Direct modification of antimicrobials

Direct inactivation or modification of antimicrobials is arguably the most important mechanism of resistance [19, 20]. These mechanisms can also have a collateral effect by decreasing the concentration of active antimicrobial in the immediate environment, thus protecting otherwise sensitive microbes [31]. Examples of inactivation or modification of antimicrobials include β-lactamases, which inactivate β-lactams by hydrolysis of the β-lactam ring [32-35], aminoglycoside modifying enzymes [36], and chloramphenicol-acetyl-transferases [30] which modify parts of the antimicrobials reducing the affinity to the target.

Modification of antimicrobials could also occur through reduction or oxidation (e.g., through cytochrome systems in animals); however, this strategy is rarely seen in bacteria [20]. The relevant examples are nitroreductases (NfsA or NfsB), conferring resistance to nitrofurantoin [37, 38].

1.3.4 The mechanisms of spread of antimicrobial resistance

Vertical transfer

As soon as a spontaneous mutation occurs, it is passed directly to further generations through DNA replication and cell division [2]. The importance of the emergence of *de novo* resistance mutations have been described in patients undergoing antimicrobial treatment. Resistance to ertapenem in a patient occurring under treatment for a respiratory infection caused by *K. pneumoniae* is a relevant example [39]. Further, it has been suggested that hypermutability can facilitate this process, promoting faster selection for resistance [40].

Horizontal transfer

In addition to vertical transfer, genes, including those encoding antimicrobial resistance, can be transferred horizontally between organisms through HGT [2]. HGT can occur across various strains and species and even genera [41, 42]. Genes encoding antimicrobial resistance localized on the same mobile genetic element are very likely to be transferred simultaneously.

This accelerates the spread of multi-drug resistance (MDR) [42]. Antimicrobials in the environment, in addition to the positive selective pressure towards resistance, can increase the rate of HGT through induction of SOS-response in bacterial cells [43].

Several mechanisms of HGT have been identified, but the major mechanisms include transformation, transduction, and conjugation [42]. Transformation or natural transformation is the process of uptake, integration, and expression of the genetic material from the environment, usually in the form of fragments of extracellular DNA [44]. This was first demonstrated in 1928 for *S. pneumoniae* [45] and, subsequently, in 1951, shown that previously sensitive *S. pneumoniae* can "learn" penicillin resistance by exposure to the fragments of extracellular DNA [46]. In *E. coli*, transformation is considered quite unlikely to occur [47].

Transduction is the transmission of genetic material between two hosts, involving a bacteriophage as a vector [42, 48]. DNA transferred by phages can be of different origins: phage DNA itself, chromosomal DNA, or mobile genetic elements, including genomic islands, transposons, and plasmids [49]. The mobilization and transduction of antimicrobial resistance genes conferring resistance to various antimicrobials, including erythromycin, tetracycline, vancomycin, clindamycin, and chloramphenicol by phages has been observed [48]. For *E. coli*, the RCS47 mobile genetic element, carrying $bla_{\text{SHV-2,}}$ was found to be a 99.9% identical sequence to the bacteriophage AF234172 [50]. Other β-lactam resistance-determinants such as bla_{TEM} and $bla_{\text{CTX-M}}$ have been identified in bacteriophages from sewage water samples and have been successfully experimentally transferred into previously sensitive *E. coli*, rendering it resistant [51].

Conjugation is the stepwise process of transfer of genetic material between bacterial cells through a connecting channel [42, 52]. The process is facilitated by a type 4 secretion system and includes forming a pilus and surface adhesion, transfer of single-stranded DNA, and replication of the DNA in both cells [53]. Conjugative machinery can be encoded on either the chromosome or plasmids [53]. This machinery may also enable other mobile genetic elements such as non-conjugative plasmids and transposons to be transferred [52]. Conjugation is considered an important driver for the spread of resistance in gram-negative bacteria, including the transfer of plasmids encoding resistance genes for a wide range of antimicrobials [54, 55].

More recently, other processes of HGT have been identified. These mechanisms include temporary cell fusion supporting chromosomal recombination [56]; nanotubes [57]; emitting of

membrane microvesicles containing DNA [58]; phage-like transduction involving gene transfer agents (GTAs) [59]; and, lastly, one combining both emission vesicle-like particles and further phage-like transduction [60]. All these modes of gene exchange can also potentially contribute to the spread of antimicrobial resistance.

1.4 *E. coli* as a human pathogen

1.4.1 General properties of *E. coli*

E. coli is a gram-negative, facultative anaerobic bacterium, which does not sporulate. *E. coli* can switch metabolism from anaerobic respiration (fermentation) if oxygen is absent to aerobic respiration (producing ATP) when oxygen is present. *E. coli* cells have a rod shape of 0.25-1.0 μm in diameter, with the cell volume of 0.6-0.7 μm^3 [61]. Strains that possess flagella are motile. *E. coli* is found populating the mucosa of the gastrointestinal tract of mammals, birds, and reptiles [62] and is the predominant facultative anaerobe in the intestines [63], outnumbered only by obligatory anaerobes [62]. *E. coli* can exit the host organism, often through the fecal route, and can survive days in the environment outside the host [64]. It has also been shown that *E. coli* can reside transiently in the environment, and some strains (saprophytic variants) are also capable of growing outside of the host [62, 64].

Taxonomy

E. coli belongs to the genus *Escherichia* of the family *Enterobacteriaceae*, which was recently classified as a part of '*Enterobacterales'*, a large and diverse group of rod-shaped, non-sporulating, facultatively anaerobic gram-negative bacteria within the class *Gammaproteobacteria* [65].

Initially, the population structure of *E. coli* was believed to be the clonal, according to serotyping analysis [66]. However, with the advancement of DNA sequencing, including whole-genome sequencing (WGS), studies began demonstrating genetic diversity (recombination) within distinctive isolates, with phylogenetic trees constructed from individual genes being inconclusive [62]. In *E. coli*, the core genome is believed to contain ~2000 genes, out of total ~4700 genes [67], which are conserved among all strains. However, the more isolates that are being sequenced, the more core genome will be reduced [62, 68, 69]. Figure 1 describes a relatively conform phylogeny of *E. coli*.

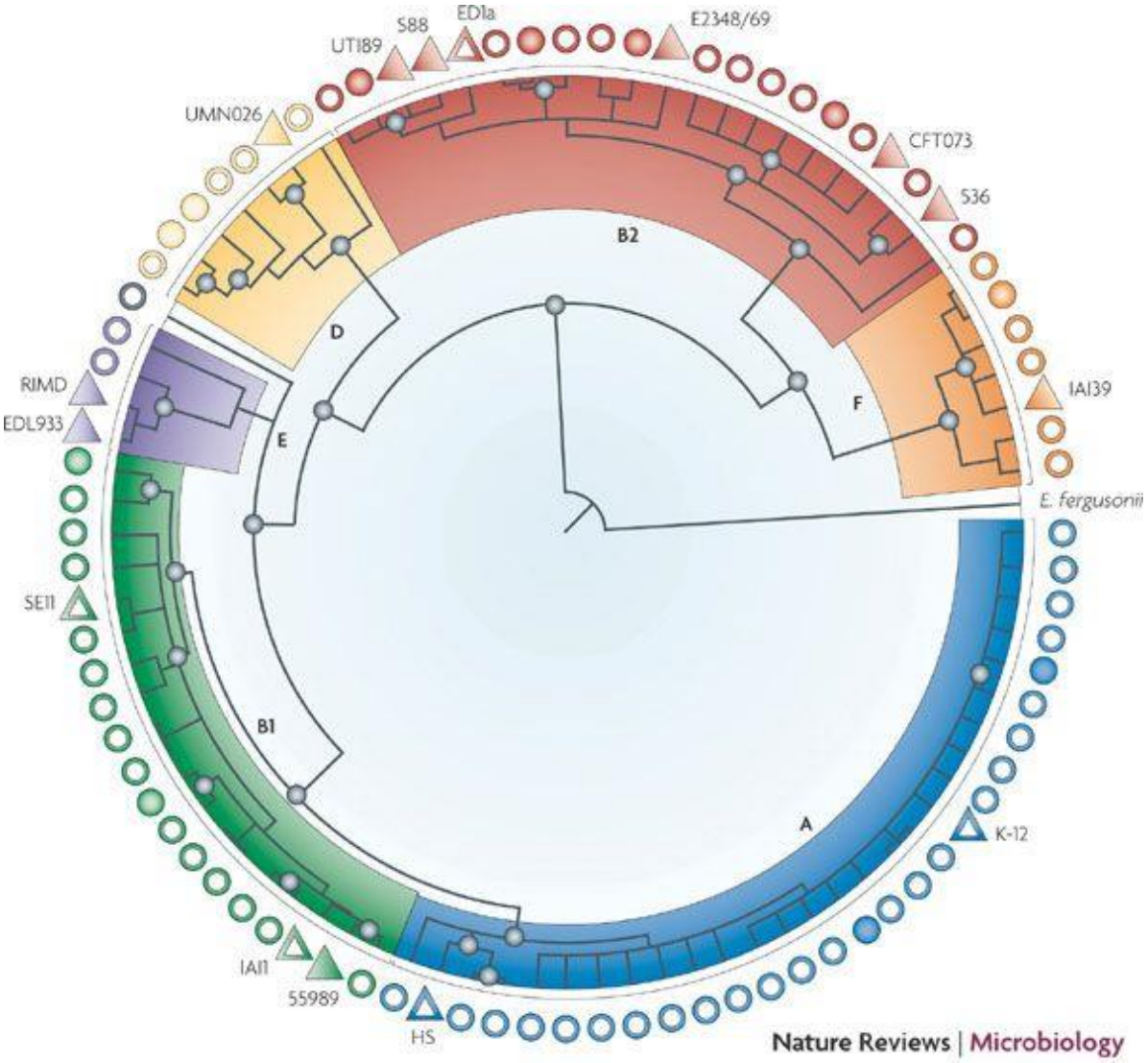

Figure 1. Phylogenetic tree of *Escherichia coli*. Reused with permission from Nature Reviews Microbiology (Tenaillon *et al.*) [(62)]. The clonal genealogy of 87 *E.coli* strains is built on the basis of nucleotide sequences of 8 housekeeping genes using the ClonalFrame analysis, which takes into account the recombination events within distinctive isolates. Open symbols indicate commensal strains, while full symbols indicate pathogenic strains. Circles indicate strains from reference collection (ECOR); triangles indicate genome reference strain. Different phylogenetic groups are highlighted with color. The tree is routed on *E. fergusonii*, a closely related species.

The *E. coli* population can be classified further into different phylogroups [(62)]. The B2 group is subject to the highest diversity between strains. Group A strains and group D strains are more likely to be carriers of antimicrobial resistance, while B2 group strains are, in general, less often associated with resistance [(62, 70)]. Groups A and B1 mostly consist of commensal strains possessing few virulence factors. Groups B2 and D usually carry virulence determinants, enhancing intestinal colonization and attachment to uroepithelium [(71)]. MDR *E. coli* isolates are often associated with high-risk clones (e.g., ST38, ST69, ST131, ST155,

ST393, ST40, ST10, ST405) [72, 73]. Interestingly, one of the most successful MDR ST-types (ST131) belongs to the B2 group [74, 75].

Virulence and pathogenicity factors

E. coli colonizes the human gastrointestinal tract soon after birth and normally causes no harm to the host [71]. Commensal strains can cause infection if bacteria are outside the common habitat (e.g., peritonitis) or if the host is immunocompromised [76]. These bacteria have evolved different survival strategies [77], and it has been suggested that virulence genes have evolved for other purposes than infection, including the colonization of different hosts, prevention against predation from protozoa, or prevention of bacteriophage attacks [62]. However, *E. coli* is equipped with virulence factors that can cause a notable range of diseases. Selected *E. coli* virulence and pathogenicity factors are summarized in Table 1.

Adhesion/colonization

Various adherence factors help *E. coli* to inhabit and colonize environments [78]. These factors can be divided into different classes, but are most often taking the form of rod-like structures. Fimbriae (or pili) are structures 5-10 nm in diameter and fibrillae are rod-like structures 2-4 nm in diameter, that can take various forms (long and wiry or curly and flexible) [79]. Flagella is a relatively longer filament (5-10 μm), approximately 20 nm in diameter, which is capable of motoric function, allowing *E. coli* cells to be motile [80]. Some adhesion factors are non-fimbrial (intimin, Iha), which is expressed as an outer-membrane protein [81].

Several bacterial surface structures (e.g., Df adhesins, IcsA) cause activation of signal transduction pathways or cytoskeletal rearrangement, which helps *E. coli* to internalize into the host cells [78, 82]. Some of the surface structures, present even in commensal strains, can, in certain situations, be recognized by host receptors, inducing the inflammatory response and, thus, serve as virulence factors. For instance, lipopolysaccharide activates the toll-like receptor 4 (TLR-4), while flagellin (which is present in the flagella) can bind to TLR-5. Binding to the TLRs causes a cytokine release cascade, which can eventually result in septic shock [78].

Toxins

In addition to surface factors that can trigger the inflammatory response, pathogenic strains of *E. coli* secrete numerous toxins and effector proteins (Table 1). These molecules are capable of killing epithelial and endothelial cells, resulting in disruption of the host barriers and

enable the further spread of infection or helping the internalized *E. coli* to exit the host cell when needed [78].

Table 1. *Escherichia coli* ExPEC-associated virulence factors and toxins. Adapted with permission from Nature Reviews Microbiology (Kaper *et al.*) [78].

Factor	Pathotype	Class/activity/effect
Virulence factors		
Chu (Shu)	IPEC, UPEC, MNEC	Iron acquisition, haem transport
Curli	Various	Adhesin; binds to fibronectin
Dr adhesins	IPEC, UPEC	Adhesin, binds to decay-accelerating factor (DAF) ; >10 Dr adhesins described
F1C fimbriae	UPEC	Adhesin
Flagellin	All	Motility; induces cytokine expression through TLR5; >50 flagella (H) serotypes
IreA	UPEC	Iron acquisition, siderophore receptor
IroN	UPEC	Iron acquisition, siderophore receptor
Iss	ExPEC	Increased serum survival factor
Lipopolysaccharide	All	Induces cytokine expression through TLR4; >180 O types
Long polar (LpfA) fimbriae	ExPEC, IPEC	Adhesin
P (Pap) fimbriae	UPEC	Adhesin; includes cytokine expression
S fimbriae	UPEC, MNEC	Adhesin
Type-1 fimbriae	All	UPEC adhesin; binds to uroplakin
Yersiniabactin	Various	Iron acquisition, siderophore
Toxins and effectors		
Cytotoxic necrotizing factors (CNF-1,-2)	MNEC, UPEC, NTEC	Targets RhoA, Cdc42 and Rac – causes altered cytoskeleton, necrosis
Hemoglobin-binding protease (Tsh)	ExPEC	Autotransporter; targets haem – degrades hemoglobin to release haem/iron
HlyA	UPEC	RTX toxins; targets erythrocytes and leukocytes – causes cell lysis
Pic	UPEC, IPEC	Autotransporter – protease, mucinase,
Sat	UPEC	Secreted autotransporter toxin, protease
Vat	UPEC	Autotransporter – vacuolation

TLR, Toll-like receptor.

1.4.2 Infections caused by *E. coli*

Highly adapted *E. coli* variants, capable of inducing certain types of infections can be classified as "pathotypes" [78]. In general, *E. coli* can cause three main clinical syndromes in humans [78]: enteric/diarrheal disease, UTIs, and systemic infections. *E. coli* is one of the leading causative agents for bacteremia [83, 84], and its prevalence is increasing [85]. *E. coli* can be divided into two pathotypes: intestinal pathogenic *E. coli* (IPEC), which is an obligatory pathogen, and extraintestinal pathogenic *E. coli* (ExPEC), which may be present in a

microbiome but not necessarily causing disease. [78]. ExPEC can be further divided into two main types: uropathogenic (UPEC) and meningitis/sepsis-associated *E. coli* (MNEC) [78, 86].

ExPEC causes manifold of diseases in humans, including upper/lower UTIs and bacteremia, as well as cellulitis, cholangitis, cholecystitis, infectious arthritis, osteomyelitis, peritonitis, pneumonia, and neonatal meningitis [78, 87].

Other pathotypes, such as adherent-invasive *E. coli* (AIEC), necrotoxic *E. coli* (NTEC), cell-detaching *E. coli* (CDEC), have been described, but are not yet clearly established and require further epidemiologic investigations [78].

UTIs and urosepsis

UTIs are extremely common infections, yearly affecting 150 million people worldwide [88]. This results in a significant economic burden; for instance, in the US, UTIs have caused economic losses of 3.5 billion USD annually [89]. The disease prevalence is higher in women due to the smaller distance between urethral orifice and anus, and shorter urethra compared to men [89]. UTIs can be categorized as complicated and uncomplicated. Uncomplicated UTIs affect otherwise healthy people, with no anatomical abnormalities, comorbidities, and are caused by non-MDR strains [89]. UTIs can be further classified into lower (cystitis) and upper (pyelonephritis) (Figure 2). In cystitis, it is generally assumed that infection is limited to bladder and kidneys are not affected, while in pyelonephritis, the infection reaches the kidneys [90]. UTIs can have significant complications and consequences, including recurrences, bloodstream infections, renal damage, and pre-term birth [89].

UPEC is a primary etiologic agent for UTIs, responsible for approximately half of the hospital-acquired cases and 70–95% of community-acquired UTIs [86]. *E. coli*, initially present in fecal flora, can spread and colonize the urinary tract through the fecal-perineal-urethral route [91]. Uropathogenic strains are also found in other environments, for example, production animals and meat [92] and can be shared between sexual partners [93]. Phylogenetic groups often responsible for the development of UTI are B2 and D [94], and clonal groups, as well as endemic strains, have been identified [95]. However, no distinctive phenotypic profile that could be deemed as obligatory for establishing a UTI has been found. Still, a combination of specific virulence factors is often found in the commensal strains as large and small pathogenicity islands. These factors seem to be necessary for successful colonization of the urinary tract as well as other environments [94] (Figure 2, Figure 3, and Table 1).

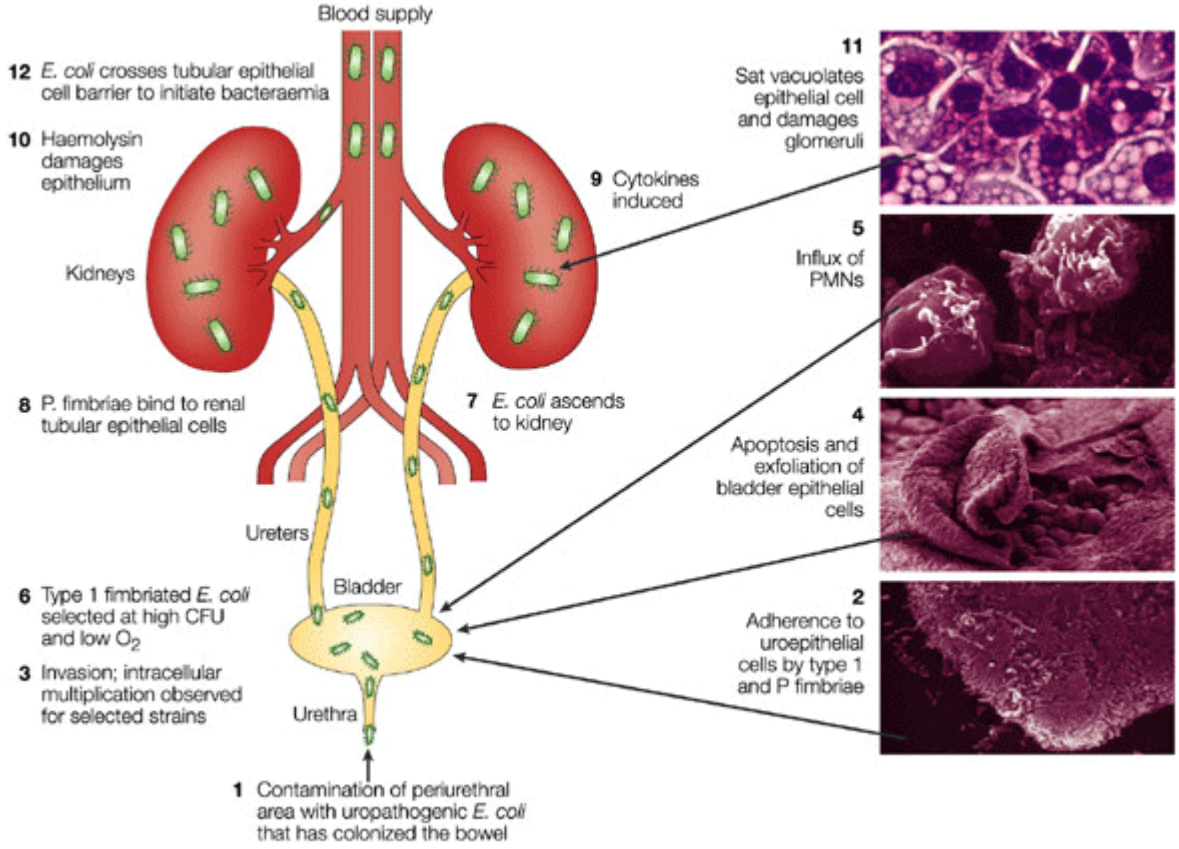

Figure 2. The pathogenesis of urinary tract infection and bacteremia. Reused with permission from Nature Reviews Microbiology (Kaper *et al.*) [(78)]. PMNs - polymorphonuclear leukocytes

Among the most important virulence traits for causing UTI is the ability to attach (via adhesins) to the mucosal or urothelial cells, and thus, colonize the gut, perineum, and urinary tract, including renal interstitium [(96)]. Strains that are unable to attach are more likely flushed by the flow of urine [(97)].

Type-1 fimbriae have a significant role early in the UTI pathogenesis (Figure 3) [(78)]. After the bacteria have disseminated from the gut to the perineal area and ascended to the urethra, the new environment stimulates the expression of type-1 fimbriae, which typically occurs within 4-24 hours [(98)]. Type-1 fimbriae attach to the mannose component of uroplakin [(99)], which covers the transitional epithelium of the urinary tract. Attachment helps bacteria to resist the urine flow dynamics, and facilitates further ascending to the bladder [(100)], triggers apoptosis and exfoliation of urothelial cells [(78)], and causes an inflammatory response [(101)]. Additionally, it is argued that type-1 fimbriae promote biofilm formation, entrapping the bacteria, by surrounding with urothelium/bladder epithelium, holding them in the nutrient-rich matrix [(97)] and promoting their survival inside macrophages if phagocytosed [(102)]. This could help bacteria to evade antimicrobials and form a reservoir for recurrent infections [(102)]. When the

infection is limited to the bladder, type-1-fimbriae are expressed continuously. However, in pyelonephritis, the expression of type-1 fimbriae is switched off [98]. It has been proposed that it helps to release the attached *E. coli* allowing it to spread further to kidneys, using pap-fimbriae to attach to the kidney epithelium and erythrocytes [98]. At this stage of infection, the renal epithelium could be damaged with toxins: Sat vacuolating cytotoxin[82], cytotoxic necrotizing factor, and haemolysin [78]. This damages the glomeruli and proximal tubules and can, in some cases, lead to the escape of *E. coli* into the bloodstream, causing bacteremia [78]. LPS, haemolysin, along with *E. coli* virulence factors induce the systemic inflammatory response leading to sepsis [78, 103].

The pathogenesis of UTI involves several other factors [78], such as diverse fimbriae and adhesins, biofilm formation, flagellae, aerobactin (iron acquisition), resistance to plasma bactericidal effects, and others (Figure 3, Table 1). Interaction with vaginal microbiota could also play a role, as seen in recurrence of UTI, preceded by a short-term exposure to *Gardnerella vaginalis* (which is considered non-uropathogenic) [104].

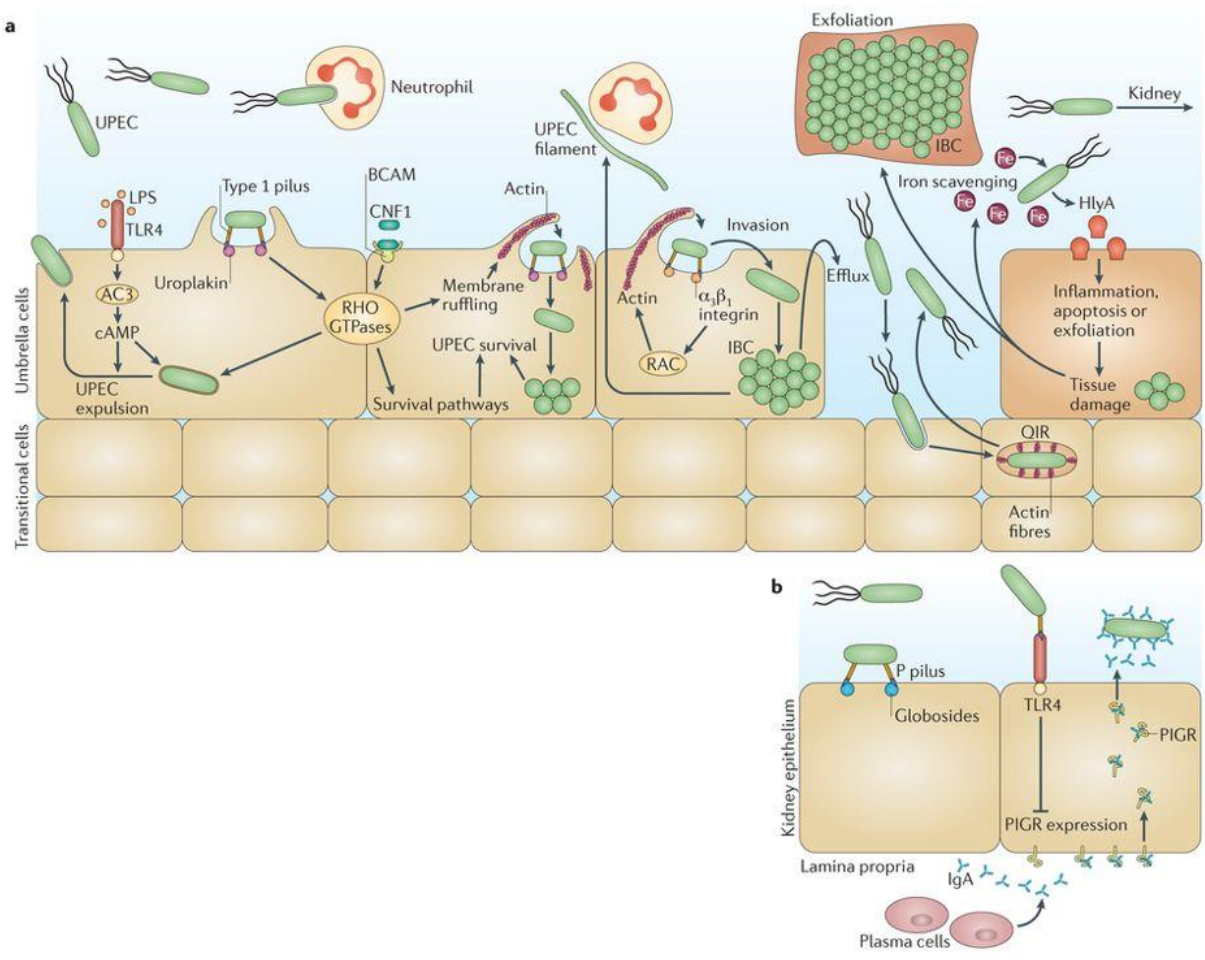

Figure 3. *Escherichia coli* virulence factors in the pathogenesis of urinary tract infections. Reused with permission from Nature Reviews Microbiology (Flores-Mireles *et al.*) [89]

1.5 Challenging resistance mechanisms in *E. coli*

According to EUCAST, *Enterobacterales*, including *E. coli*, are "intrinsically resistant to benzylpenicillin, glycopeptides, fusidic acid, macrolides, lincosamides, streptogramins, rifampicin, daptomycin, and linezolid" [105]. The potentially active classes for the treatment of *E. coli* infections include penicillins (ampicillin), penicillins combined with β-lactamase inhibitors, cephalosporins, folate pathway inhibitors, monobactams, carbapenems, aminoglycosides, fluoroquinolones, phenicols, glycylcyclines, polymyxins, phosphonic acids and tetracyclines [106]. In addition to the intrinsic resistance, acquired resistance to major antimicrobials used to treat *E. coli* infections such as β-lactams including β-lactam-β-lactamase inhibitors, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole is increasing in prevalence [86, 107].

1.6 Resistance to β-lactams

Major concerns in terms of resistance in *E. coli* have been the rise of resistance to β-lactams and, in particular, to 3rd generation cephalosporins and carbapenems [108]. Resistance to β-lactams is the result of an interplay of different mechanisms; however, hydrolysis by β-lactamases is suggested to be the leading cause [109]. *E. coli* intrinsically harbor a chromosomal AmpC type β-lactamase, but the expression is typically low due to the weak promotor and a strong attenuator system [110]. Although various mechanisms, including promoter mutations and insertion of IS-elements, can lead to hyperproduction of the chromosomal AmpC, acquired β-lactamases are the main mechanisms of β-lactam resistance. Today narrow-spectrum β-lactamases such as TEM-1 with activity limited to penicillins such as ampicillin, 1st, and 2nd generation cephalosporins are widely present in *E. coli* [32]. However, the main concern is related to broad-spectrum β-lactamases such as plasmid-mediated AmpC β-lactamases (pAmpCs), extended-spectrum β-lactamases (ESBLs) and carbapenemases [86]. Examples of pAmpC, ESBLs, and carbapenemases identified in *E. coli* and their properties are shown in Table 2. The rapid spread of ESBLs and carbapenemases is a worrying trend [108], and carbapenem-resistant/ESBL-producing *Enterobacteriaceae (Enterobacterales)* have been listed by the WHO among the top three pathogen groups, where lack of treatment alternatives is the most critical [111].

Table 2. Important classes of acquired β-lactamases identified in *Escherichia coli* (based on [32, 33, 35]

	Ambler class	Examples	Spectrum of activity
Plasmid-mediated AmpC	C	CMY, DHA	Penicillins, cephalosporins (except 4th generation)
ESBLs	A	CTX-M, TEM-ESBLs, SHV-ESBLs	Penicillins, cephalosporins (except cephamycins), monobactams
Carbapenemases	A	KPC	Penicillins, cephalosporins, monobactam, carbapenems
	B	NDM, VIM, IMP	Penicillins, cephalosporins, carbapenems
	D	OXA-48, OXA-181	Penicillins, carbapenems

1.6.1 Resistance mechanisms to other antimicrobials in *E. coli*

In addition to β-lactams, the folate pathway inhibitors, aminoglycosides, and fluoroquinolones are essential antimicrobials in the treatment of *E. coli* infections [112]. For both aminoglycosides and trimethoprim-sulfamethoxazole, several acquired plasmid-mediated resistance mechanisms have been described [113-115]. For aminoglycosides, these include 16S rRNA methylases and aminoglycoside modifying enzymes [36]. The aminoglycoside modifying enzymes can be divided into three main groups: acetyltransferases, nucleotidyltransferases, and phosphotransferases [27, 36]. A wide variety of aminoglycoside modifying enzymes was identified in *E. coli*, and the spectrum of activity is variable [36]. The 16S rRNA methylases, modify the binding site of aminoglycosides on the ribosome conferring broad-spectrum high-level aminoglycoside resistance [114]. Plasmid-mediated trimethoprim and sulfamethoxazole resistance mechanisms include the *dfr* and *sul* genes, respectively [115], which are widely prevalent in *E. coli*.

In terms of fluoroquinolone resistance, this is mainly caused by mutations in *gyrA* and *parC*, which alters the binding affinity of fluoroquinolones to the target molecules GyrA and ParC [29, 116]. The number of mutations and location of mutations determine the level of resistance, and double mutations are often required for clinical resistance. However, several plasmid-mediated quinolone resistance mechanisms have been identified [117]. This includes the aminoglycoside modifying enzyme AAC(6')-Ib-cr, which can modify ciprofloxacin and other fluoroquinolones with amino nitrogen on the piperazinyl ring, Qnr proteins [28], and efflux pumps such as QepA and OqxAB [117, 118].

The above mentioned plasmid-mediated resistance mechanisms are frequently co-located on the same plasmid resulting in MDR phenotypes, thus limiting treatment options and facilitating the spread of MDR [114, 119, 120].

1.6.2 Challenges with possible antimicrobial treatment options for MDR *E. coli* UTIs

The rates of ESBL-producing UPEC are steadily increasing [121]. In Europe, UPEC isolates collected in 2014, were, on average, 12% resistant to third-generation cephalosporins and 22% for fluoroquinolones [122]. In the US, the proportion of fluoroquinolone-resistant UPEC was 31% already in 2007-2010 [123]. ESBL-producing *E. coli* is often co-resistant to other antimicrobial classes, and according to the recent EARS-net surveillance report [124], a rate of co-resistance to fluoroquinolones, 3rd generation cephalosporins and aminoglycosides in European countries averages 4.8% (with minimum registered in Iceland 1.4% and Norway 2.0%, and maximum in Slovakia and Bulgaria with 17.1 and 19.7 % respectively). The rates of nosocomial ESBL-producing isolates in Southeast and East Asia are 20-40% and 60-70%, respectively [125, 126]. According to a recent meta-analysis, ESBL-producers in the long-term medical care units are detected with a rate of 10-60% in Europe and 50% in China [127]. Fecal ESBL colonization in the community is 10% in European countries and North America, but as high as 50% in Asia, resulting in a global average of 14% [128]. Low susceptibility of UPEC isolates in pregnant women with a recurrent UTI in the anamnesis has been reported against ampicillin (4%), tetracycline (15%), amikacin (29%), ciprofloxacin (33%), and gentamicin (42%) [129]. Taking this, along with possible uncertainty in distinguishing complicated from uncomplicated UTI, into account, physicians could be left with no options, but using the last line drugs (such as carbapenems, tigecycline or colistin). Notably, the first oral carbapenem indicated for treating the complicated UTIs has been recently approved [130]. Resistance to carbapenems is still uncommon in Europe, with most of the countries reporting less than 0.1%, but no trends towards the decrease of prevalence were observed for any country [124]. Although carbapenemase-producing isolates are still uncommon in community settings, recent reports are worrisome: 8–30% globally and 6–11% in the US. The numbers were generally higher in Asian countries, especially in Taiwan and India [131]. Additionally, it is worth mentioning that the surveillance data was based only on invasive isolates, reported by laboratories, and this could only represent a tip of the iceberg, since community cases of carbapenem-resistant *Enterobacterales* carriage have been reported [132].

In the elderly, UTIs are likely the number one cause of gram-negative bacteremia and a common cause of UTI in the general population [133]. The number of new antimicrobial agents has dramatically declined in recent years; it is thus crucial to spare carbapenems when targeting lower UTIs [134]. If MDR ESBL-producer is suspected, some guidelines recommend against the empiric use of cephalosporins, quinolones, and amoxicillin-clavulanic acid [135]. The same guidelines also underline that the use of piperacillin-tazobactam and gentamicin in the areas, where ESBLs are frequent would not be optimal [135]. These considerations are based on the high chance of co-resistance. Trimethoprim-sulfamethoxazole was long considered a feasible alternative and a first-choice option in numerous guidelines [136-139]. However, studies report a high prevalence of trimethoprim resistance, especially among MDR isolates, including ESBL-producers [113, 140, 141]. It has been previously recommended against the empiric use of a certain drug for UTIs when the local prevalence of resistance exceeds 20% [138, 142]. Therefore, the use of trimethoprim could result in selection for MDR isolates [135]. In some regions, as in Kronoberg, Sweden, interventions for withholding its use were attempted [113]. Today, it is not recommended to use trimethoprim, when risk factors for ESBL-producers are present unless isolates are tested to be susceptible. [141]. The use of colistin on such large populations such as UTI patients is limited by its toxicity and potentially last-resort status for severe infections.

Limited treatment options against MDR ESBL-/carbapenemase-producing *E. coli*, as well as the potential for expansion due to the co-resistance, are well shown on the example of ST131, which is often associated with an MDR virulent *E. coli* phenotype and ESBL-production [74, 75, 140, 143]. The review by Zubair *et al.* [143] summarized the susceptibility rates for ST131 (Table 3). It illustrates the need for alternative options for treating the prevalent and less non-severe infections, such as UTIs. The ideal strategies would allow sparing the last-resort antimicrobials for severe infections and putting less pressure towards resistance.

Table 3. The analysis of available treatment options for multidrug-resistant *Escherichia coli* sequence type 131 infections. Adapted with permission from Expert Review of Anti-Infective Therapy (Zubair *et al.*) [143].

Antimicrobials	Route of administration	Susceptibility rates (range, %)	Comments
β-Lactam agents			
Amoxicillin-clavulanic acid	Peroral/ intravenous	42-67	Resistance is common. For non-ESBL-producing isolates, iv. formulation available in some countries
Carbapenems (imipenem, meropenem, ertapenem, doripenem)	intravenous	95-100	First-choice for severe infections caused by ESBL-producing isolates. Resistance is emerging
Expanded-cephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefepime)	intravenous	90	For non-ESBL-producing isolates Cefepime is stable against some ESBLs but is hydrolyzed by CTX-Ms, especially CTX-M-15
Piperacillin-tazobactam	intravenous	94-98 Non-ESBL 40-91 ESBL	Various susceptibility rates for ESBL-producing isolates
Non-β-lactam agents			
Amikacin	intravenous	3-10	Nephrotoxic. Reserved for invasive infections
Ciprofloxacin	Peroral/ intravenous	25-65 Non-ESBL 6-15 ESBL	Resistance common, especially among fimH30 core subclone; not for empiric use
Gentamicin	intravenous	30-50	Nephrotoxic. Reserved for invasive infections
Polymyxins (colistimethate, polymyxin b)	intravenous	99-100	Nephrotoxic. Reserved for invasive infections caused by carbapenem-resistant isolates
Tetracyclines (doxycycline, minocycline)	Peroral/ intravenous	60-75	Reasonable susceptibility but clinical experience lacking
Tigecycline	intravenous	98-100	Clinical role likely limited (e.g., intra-abdominal infections by ESBL-producers)
Trimethoprim-sulfamethoxazole	Peroral/ intravenous	30-60	Resistance is common; not for empiric use

ESBL: Extended Spectrum β-lactamase

Accordingly, to spare the potent antimicrobials for serious infections, it has been suggested to "reuse" old antimicrobials, which have been used on a limited scale previously. For the uncomplicated lower UTIs caused by MDR UPEC, old drugs such as nitrofurantoin, fosfomycin, and mecillinam are the first choice treatment options in the recent guidelines [90, 136-139].

High recurrence rates are often observed for UTIs [144], and the contemporary trends for an increase in antimicrobial resistance have led to reconsidering these simple infections as a serious public health problem [89]. On the one hand, there is the risk of complications and possible progress to bloodstream infections if such common infections as UTIs are not treated. On the other hand, potent antimicrobials should be reserved for less frequent but serious and life-threatening conditions [145]. Ideally, we should establish alternative strategies allowing us to spare antimicrobials (or at least to reserve potent antimicrobials to be used for severe infections). Despite the greater public awareness of the problem of antimicrobial resistance, broad-spectrum antimicrobials, such as fluoroquinolones and cephalosporins, are still frequently prescribed for UTIs [89].

1.6.3 Alternative non-antimicrobial treatment options for UTI

Apart from antimicrobial therapy, several promising non-antimicrobial approaches are in the pipeline for development [122, 146, 147]. The basic concept is to neutralize pathogens or prevent the development of infection, by targeting different pathways; ideally, without altering the gut commensal microbiota or promoting antimicrobial resistance [122, 146]. These approaches include vaccines against UPEC [148]; probiotics, e.g., vaginal with *Lactobacillus* spp. [149]; anti-virulence therapies – for instance, pilicides/curlicides [150, 151] or D-mannose/mannose-derived FimH antagonists [152, 153]; antiseptics such as methenamine [154], phenols and polyphenols [122]; and supplementation with vitamin D [146] and estrogens [155]. In addition, a wide variety of potentially active natural extracts is under investigation. In many cases, the mechanisms of action are unclear, and acting chemical substances are still unidentified [122, 146, 147]. For now, most of the abovementioned non-antimicrobial approaches are still unavailable for clinical use or have limited indications for use [122, 146].

Antimicrobial-sparing approaches for uncomplicated lower UTIs such as delayed treatment and anti-inflammatory drugs instead of antimicrobials are gaining increasing interest [156]. Currently, taking into account the risk of complications, anti-inflammatory therapy alone as an initial approach treat uncomplicated lower UTIs is either not recommended [90] or may be considered only if the patient accepts to delay antimicrobial prescription [136]. For older patients, delayed antimicrobial treatment is correlated with higher rates of bloodstream infection and a significant increase in mortality [157].

1.7 Pharmacometrics

Pharmacometrics can be described as the bridging discipline aimed at analyzing, translating, and communicating complex interactions between xenobiotics and living organisms in a quantitative manner [158]. Mathematical models are applied to explain the processes in the biological data, by separating the systematic component from "noise" (errors/unexplained variation), resulting in describing the data with equations [159]. This can be further used to make predictions by changing the values in the equation variables [159].

Pharmacometrics can thus be defined as "the science of developing and applying mathematical and statistical models characterize, understand, and predict a drug's pharmacokinetic (PK), pharmacodynamic (PD), and biomarker-outcome behavior." (Figure 4) [158].

The PK/PD relationship has allowed analysis of doses, dosing regimens and compliance patterns partly outside the historical patient expertise, adding the benefit of safely exploring exposure-response-efficacy relationship [160] and provide guidance and decision support for different treatment strategies, trial design and making assumptions for treatment efficacy [158, 161, 162]. Moreover, the information derived through the pharmacometrics analysis can support regulatory decisions [163], improve clinical care, and reduce the cost and development time of the drugs [162]. Antimicrobial agents can also be studied using pharmacometrics by exploring the interrelationship between the drug, microorganisms, and a host (patient). Today, the application of pharmacometrics is strongly encouraged by regulatory agencies such as the FDA [164], and its use is increasing in both industry and academia [162]. Pharmacometrics is crucial for the development of new antimicrobials [162], as well as for dose selection and risk-benefit evaluations for the drugs, already in current clinical use [158, 161, 162].

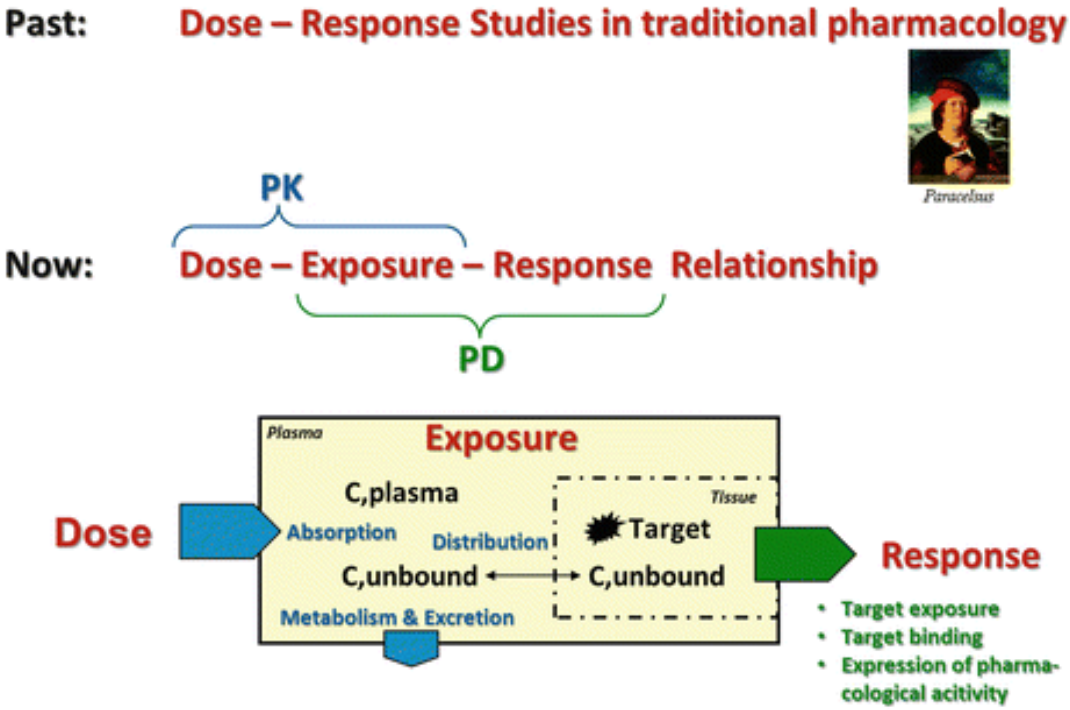

Figure 4. The concept of the dose-response relationship. In the current paradigm, exposure is a sum of factors providing the link between the dose and the drug concentration profile. Different measures of pharmacologic response could be correlated to the exposure. The pharmacokinetic (PK) processes and the pharmacodynamic (PD) processes are highlighted in the blue and green colors, respectively. Reused with permission from the New Approaches to Drug Discovery (Reichel *et al.*) [160]

1.7.1 Pharmacokinetics

PK is one of the main branches of modern pharmacology. It characterizes "the relationship between drug dosing and the drug concentration-time profile in the body" [161]. Most often, the focus is on the drug concentration in plasma and its change over time. The fate of the drug in the body (drug disposition) is classically described by a consequence of phases, abbreviated as ADME: Absorption, Distribution, Metabolism, and Excretion [165]. Numerous underlying factors influence the dynamics of drug disposition such as drug molecule size, solubility, hydrophobicity, electrical charge as well as the means of transport of the drug inside and outside of the body (active, passive), the volume of distribution, plasma protein binding, glomerular filtration rate (kidney function), liver metabolism (and enzyme induction) and others[163, 166]. Exogenous factors such as patient's sex, age, genetics, nutrition, as well as the time of the day and route of administration are also important [163, 166].

To functionally describe the pharmacokinetic processes, models are divided into compartments. Compartments can represent an actual region of the body (e.g., central blood flow, urine, peripheral tissues) or can be abstract concepts, not representing any particular region [167, 168]. The number of compartments can vary from an extreme example of Physiologically Based Pharmacokinetic Modelling (most actual parts of the body are

represented) [168] to non-compartmental analysis, where only sampled drug concentrations are used, and no physiologic mechanisms are modelled [161]. It is also possible to simplify models, still allowing for predictions. For instance, ADME phases could be simplified to the Input-Output Models [169]. Simplification of models could reduce the time and complexity of calculations [170]. The choice of model depends on its explanatory power and aims of the research. The simplest model, which allows explaining most of the variance in the data, is often preferred.

In the one-compartment model, the simplest case of a compartmental PK models, drug excretion course (C) can be explained by a declining exponential function, with initial concentration (C_0), time (t) and elimination constant (k_{el}) [161, 168]:

$$C = C_0 \times e^{-k_{el} \times t}.$$

Here, the elimination rate at any moment is proportional to the concentration of drugs present in the system at the given time point [161, 168].

The half-life ($t_{1/2}$) describes the time needed for the drug concentration to decrease to half its initial value. It can be calculated, knowing the elimination constant [161, 168]:

$$t_{1/2} = \frac{ln(2)}{k_{el}}.$$

Once this constant is known, the model can predict drug concentration for any time point for a given C_0. In this model, the shape of the drug concentration curve will then be only influenced by the factors prior to the elimination (the dose, dosing frequency, route of administration, and drug disposition) [161, 168].

Some pharmacokinetic processes (for instance, enzymatic modification, saturable absorption or elimination), can be non-linear and depend on the saturation of enzymes. At high concentrations, with 100% enzyme saturation, the kinetics would be zero-order (constant rate, not influenced by the concentration), while at low concentrations increase in substrate concentration would increase the speed of process (first-order kinetics). This can be described by the Michaelis-Menten function [161]:

$$V = \frac{V_{max} \times S}{K_m + S},$$

where S is the substrate, V_{max} is the maximum rate of elimination, and K_m is the concentration required to achieve 50% of this maximum elimination rate.

1.7.2 Pharmacodynamics

PD describes the response to the drug (including both the therapeutic effect and side effects). The endpoint for the effect variable may be a direct measurement (e.g., change in bacterial CFU) or a composite outcome (treatment failure/success, mortality) or a time to an event (time to cure, time to death) [161, 163, 168]. The endpoint could also be surrogate (indirect measurement, for instance, a decline in C-reactive protein, symptoms resolution) [171]. The observed effect is empirically found to be dependent on the exposure in the following way: when exposure is low, we typically observe minimal effect; the effect starts to increase rapidly when the exposure reaches the certain point; however, when the effect reaches maximum, further increase in the exposure will not increase the effect (Figure 5). The link between the concentration and effect is usually described with a sigmoid response function (Hill's equation) in the PK/PD analysis: [161, 168]

$$E = E_0 - \frac{E_{max} \times X^{\gamma}}{EX_{50}^{\gamma} + X^{\gamma}},$$

where E is the effect; E_0 is the effect observed without the exposure; E_{max} is the maximal effect in relation to E_0; X is one of the pharmacokinetic characteristics (in the simplest case, the concentration of the drug under steady-state conditions); EX_{50} is the value of PK characteristic, at which we observe 50% of effect, and γ is the steepness of Hill curve. As γ increases, the steepness of the curve increases, tending towards the all-or-none effect in the extreme case [161].

The popularity of the E_{max} model can be explained as it effectively models the upper limit for drug efficacy [161]. However, more data or more knowledge about the mechanisms of the underlying system could lead to the use of more complex models [163].

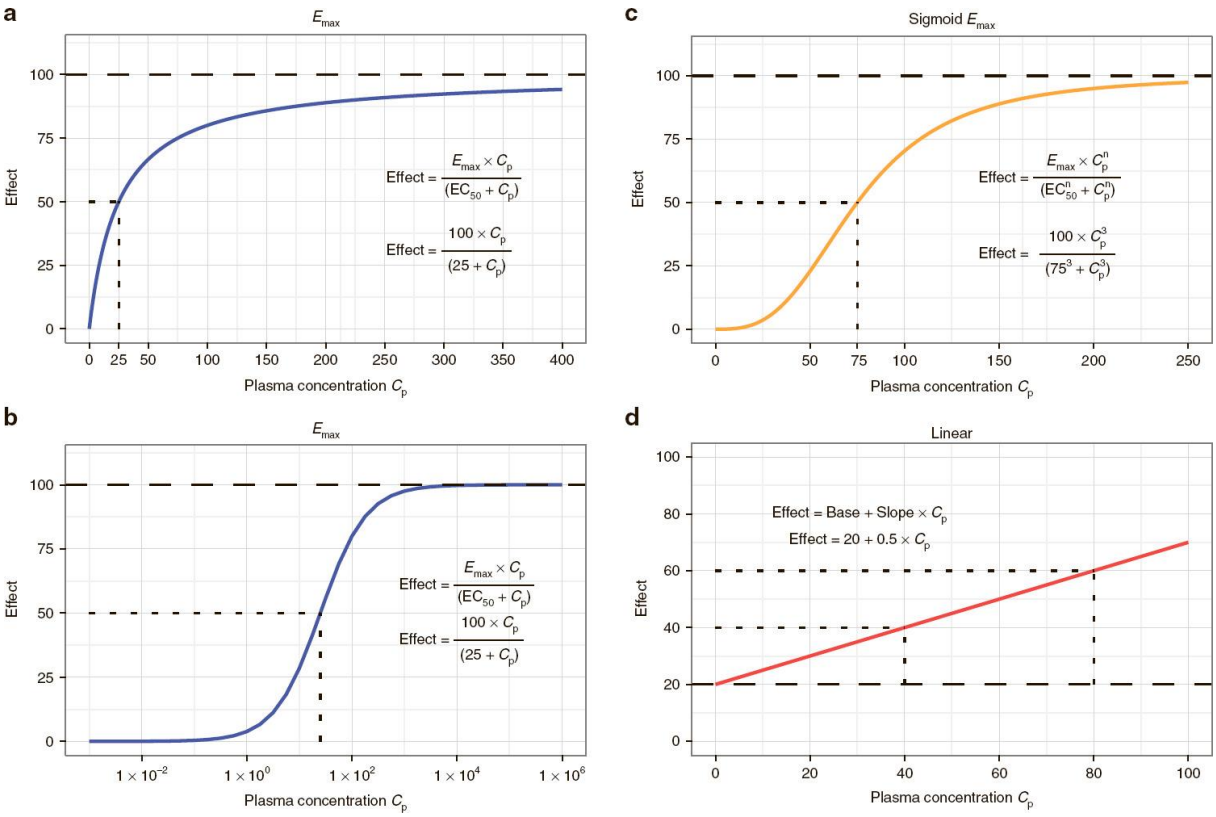

Figure 5. The concentration–effect relationships. (a) The E_{max} model originates from the single ligand-receptor theory. Fifty percent of the maximum effect is achieved at the EC_{50} concentration. (b) The same relationship using the log-concentration scale, which allows plotting a broader range of concentration. (c) The sigmoid E_{max} concentration–effect relationship where "Hill factor" n influences the curve steepness, allowing to account for allosteric interaction. (d) A linear concentration–effect relationship. Although sometimes considered as semi-empirical, it could be a useful model in cases when the observed drug effect considerably lower than $E_{max,}$ and a concentration is rather narrow. Reused with permission from CPT: Pharmacometrics & Systems Pharmacology (Upton *et al.*[167])

1.7.3 PK/PD relationships

PK/PD analysis provides the link between the dose-concentration relationships and concentration-effect relationships [161]. For the antimicrobial drugs, a system consists of three units (host, pathogen, and drug), resulting in a triangular relationship (Figure 6) [172, 173].

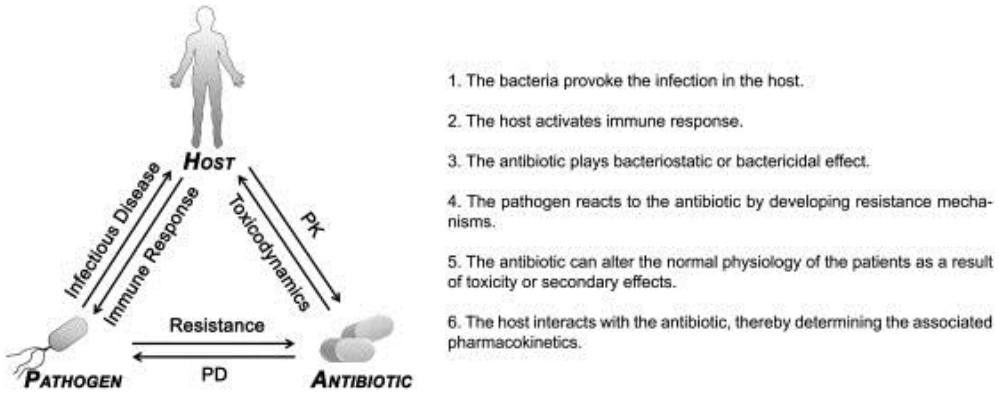

Figure 6. Relationship triad between the antimicrobial, pathogen, and the host. Reused with permission from Journal of Infection and Chemotherapy (Asín-Prieto *et al.*) [173].

In the recent two decades, the knowledge on the mode of action of antimicrobials has expanded, and three main PK/PD parameters (indices) correlating with the efficacy have been distinguished [174, 175]. These indices incorporate information on both the pharmacokinetics including simplified mathematical characteristics of non-linear concentration-dynamics function, such as Area Under the total drug concentration-time Curve (AUC), Time of the concentration above a certain concentration threshold ($T_{>C}$), or peak Concentration values (C_{max}) and pharmacodynamic aspects such as the MIC of the pathogen [174, 176]. Determining the PK and PD relationship is crucial for modern drug development [166, 167]. Once PK and the PK/PD relationship are characterized (i.e., the parameter correlating best with the efficacy is known, and its threshold value for the efficacy is computed), the concentration-profiles leading to the maximized desired and minimized undesired effects can be found. The threshold associated with desirable efficacy is defined as the pharmacodynamic target (PD target) [161]. The trade-off between the best efficacy and minimal side effects is the key to optimize the dosing regimen [161, 172].

The three classical parameters (Figure *7*), associated with efficacy are the $T_{>MIC}$, AUC/MIC, and C_{max}/MIC [161, 172]. As only the free fraction of the drug exhibits the effect, for highly protein-bound antimicrobials, the indices calculated based on free concentrations are used: $fT_{>MIC}$, *f*AUC/MIC, and fC_{max}/MIC. According to the abovementioned indices, drugs are characterized into the time-dependent drugs ($T_{>MIC}$, e.g., β-lactams), concentrations dependent drugs (C_{max}, e.g., aminoglycosides), or AUC dependent (cumulative dose affects the efficacy, e.g., daptomycin). For every group, the respective PK/PD index must be maximized in order to maximize efficacy [168, 172].

Sometimes, the PK parameters that affect the microbial killing rate are different from the PK parameters leading to the suppression of the emergence of resistance. In most cases, the exposure associated with resistance suppression is substantially higher than that needed to optimize bacterial killing. The optimal dose should be ideally able to prevent the emergence of resistance and produce minimal toxicity. Similar PK/PD models could be applied for such cases, with additional indices introduced. For instance, the MIC as the threshold for the PK/PD indices can be substituted to MPC - the mutant prevention concentration [176], or RIC - resistance inhibiting concentration [177].

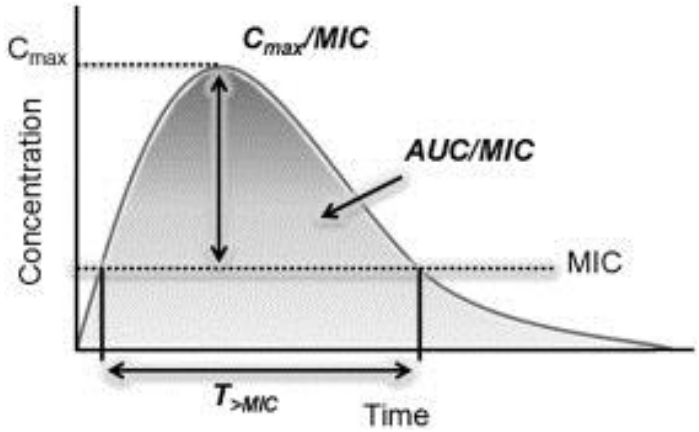

Figure 7. Typical PK/PD parameters. Reused with permission from Journal of Infection and Chemotherapy (Asín-Prieto *et al.*) [173].

1.8 Old drugs for MDR infections: challenges

Reducing the consumption of antimicrobials is crucial to reduce the expansion of antimicrobial resistance [1]. Nevertheless, new antimicrobials are still needed, regardless of the success of efforts to reduce antimicrobial demand [1]. It is critical to rationalize antimicrobial use and spare the broad-spectrum agents, especially considering a long time from the drug discovery to marketing approval.

Repurposing of forgotten or underused antimicrobials is a promising strategy. This approach has resulted in the revival of colistin and fosfomycin [2]. For the drugs achieving high urinary concentrations, significantly exceeding the MICs of the pathogens, it could be argued that PK/PD analysis is unnecessary to guarantee the treatment success in this case [178]. However, taking into account the rise in the antimicrobial resistance, it is extremely important to use the available antimicrobials as effective as possible [178, 179], and reconsider the theoretical urinary PK/PD thresholds even for uncomplicated UTIs [178, 179]. Knowledge about the dose-effect relationships could lead to reappraising current clinical breakpoints with respect to current bacterial populations (where MIC distribution could be shifted) and may change the currently used dosing regimen [134, 172]. However, the appropriate data for dose optimization of old antimicrobials is often scarce [134, 180]. Many PK and PD studies with the old drugs have been performed in the 1950s - 1960s and should be validated with modern methods and against the contemporary strains [180]. This type of research is currently carried out to a limited extent, limiting the possibility for evaluation by the regulatory agencies. A study conducted in 2012, has shown that more than half of antimicrobials are unavailable for clinical use in 38 high-income countries [181], making it clear that urgent measures are needed.

To revive old antimicrobials, three main questions are needed to be answered [134, 180]:

- "What is the MIC distribution of interest?" [180]

  The pathogens are continuously evolving, and disease pathogenesis is changing. Since the discovery of old antimicrobials typically happened a half-century ago, new resistance mechanisms have appeared. Before entering clinic use, especially when empirical therapy is in focus, the knowledge of MIC distribution is essential. In addition to this, MIC is an important factor for carrying out the PK/PD analysis and choosing the optimal dose.

- "What are the exposures (PK profiles) for various doses and patient populations?" [180]

  Due to the advances in methods determining the drug in biological samples, along with increased knowledge about human physiology, a *de novo* PK analysis would provide useful information.

- "What are the exposure-response relationship and PD target?" [180]

  The therapeutic efficacy and toxicity relationships are important for optimal dose selection and establishing new indications for use.

These three questions are finally aimed at re-setting clinical breakpoints and choosing the right dose [180] (Figure 8).

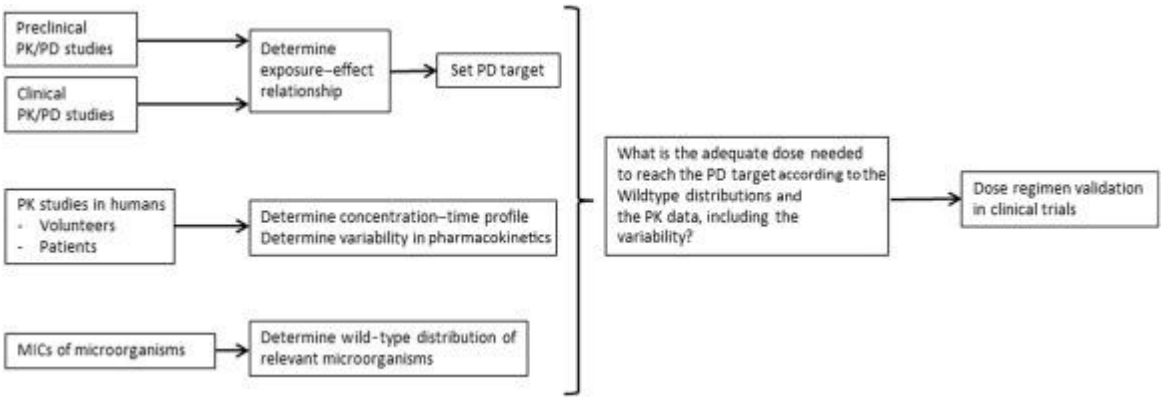

Figure 8. Schematic outline of the process of re-introducing the old antimicrobials. PD, pharmacodynamic; PK, pharmacokinetic. Reused with permission from Clinical Microbiology and Infection (Muller *et al.)* [180].

1.9 Fosfomycin, nitrofurantoin, mecillinam, and temocillin as treatment options for MDR *E. coli* UTIs

For lower UTIs, certain optimal characteristics for potential treatment options have been summarized in Table 4 [182, 183].

Table 4. Characteristics of an ideal agent for treatment of urinary tract infections. Adapted with permission from Infection (Neu *et al.*) [182] and Journal of Chemotherapy (Novelli *et al.*) [183]

- Activity against the major pathogens
- Minimum percentage of resistance (<20% for empiric use)
- Low potential for the development of resistance
- Little cross-resistance to other agents
- Tolerance and acceptability to patients
- Optimal PK characteristics, including excellent urinary levels for an adequately prolonged period to eliminate organisms rapidly
- Effective in inhibiting bacterial adherence
- Activity unaffected by pH, acid or alkaline, and not decreased by cations
- Lack of significant alteration of intestinal flora
- Does not cause perineal fungal colonization/infection or local irritation
- Treatment results in prolonged period between recurrences
- Repeated exposure fails to provoke allergic reactions

1.9.1 Fosfomycin

General properties

Fosfomycin was first discovered in 1969 in Spain, where it was isolated from a *Streptomyces fradiae* strain from soil samples [184]. Fosfomycin has a low molecular weight (138 g/mol) and is highly polar [185]. Fosfomycin, fosmidomycin, and alafosfalin represent a distinct antimicrobial class of phosphonic acid derivatives [185]. Fosfomycin has a unique mode of action: it irreversibly blocks bacterial cell wall synthesis at an early stage. Upon reaching the cytoplasm, fosfomycin, by mimicking phosphoenolpyruvate, permanently binds to UDP-GlcNAc enopyruvil transferase (MurA), thereby blocking the formation of UDP-GlcNac-3-O-enolpyruvate in the early step of peptidoglycan biosynthesis [184]. Fosfomycin has a broad spectrum of activity, including both gram-positive and gram-negative pathogens (including *E. coli*, *Proteus mirabilis*, *K. pneumoniae*, *Enterobacter spp.*, *Citrobacter spp.*, and *Salmonella typhi*) [185]. It is also active in combination therapy against *P. aeruginosa*, while *Acinetobacter baumannii, Vibrio fischeri, Chlamydia trachomatis,* and *Bacteroides* isolates are intrinsically resistant [185, 186]. Multiple studies (mostly *in vitro*) report fosfomycin to retain good activity against MDR strains, both gram-positive (including vancomycin-resistant *Enterococcus* and methicillin-resistant *S. aureus* and) [184, 187] and gram-negative (including ESBL- and carbapenemase-producing *Enterobacterales*) [186-188]. In the US, fosfomycin is only approved

for uncomplicated lower UTIs as a single oral dose [189]. However, in some European countries such as Spain, France, Germany, the United Kingdom, the Netherlands, Austria, and Greece, fosfomycin is also available for the treatment of soft-tissue infections and sepsis by intravenous administration [185]. Numerous other applications of fosfomycin are considered as it penetrates well into various tissues, achieving biologically relevant concentrations in kidneys, urine bladder, prostate gland, lungs, bone, heart valves as well as into inflamed tissues and abscesses [183, 185]. It crosses the blood-brain barrier and is detected in the cerebrospinal fluid [183, 185].

Pharmacokinetics and Pharmacodynamics

Fosfomycin is prone to hydrolysis in the gastric acid environment if administered orally. Tromethamine salt of fosfomycin is used in oral formulations to elevate the pH and slow down the rate of hydrolysis [190]. The acidity of the environment, as well as the gastric emptying rate, can affect fosfomycin bioavailability, which ranges from 33% to 58% for fosfomycin tromethamine [190-195]. In the intestines, two ways of absorption of orally administered fosfomycin have been described: saturable absorption via phosphate transport system and ordinary diffusion with first-order kinetics [191]. After the administration of a standard oral dose of fosfomycin (3g), peak plasma concentrations of 22-32 mg/L occur within 2.5-3 hours, with trough concentrations at 24h of 3 mg/L, resulting in AUC of approximately 145-228 mg×h/L [183, 190-195]. Plasma protein binding is negligible [196]. The approximate volume of distribution (for orally administered fosfomycin) is 100-170 L [185]. Fosfomycin is eliminated unmetabolized through glomerular filtration, 11-60% of the drug (depending on age, renal function, and food intake) is found in urine within 24h after administration [183, 185]. Fosfomycin has been detected in bile (approximately 20% of corresponding serum concentrations), suggesting hepatobiliary recirculation [185, 193]. The total clearance rate is estimated to 5-10 L/h, while renal clearance is in the range of 6-8 L/h [185]. The half-life for an oral dose of fosfomycin tromethamine is 3.6 to 8.3 h [190, 195, 197], however in renal failure patients or patients receiving hemodialysis $t_{1/2}$ could reach 50 h [198]. Thus, fosfomycin achieves significant concentrations in urine (1000-5000 mg/L), which remain at the level above 100 mg/L for at least 30-48 h [178, 183].

Parenteral administration results in improved bioavailability, achieving higher C_{max} (276–370 mg/L), and AUC (405–448 mg×h/L) [185, 190, 193, 195]. Due to the higher bioavailability, different volume of distribution of 9-30 L at steady state has been reported [185, 190, 193, 195]. The

pharmacokinetic curve of i.v. fosfomycin follows a bi-exponential pattern, with serum distribution half-life ($t_{1/2\alpha}$) of 0.18–0.38 h and elimination half-life ($t_{1/2\beta}$) of 1.9–3.9 h [185].

Fosfomycin is generally well tolerated with minimal side effects. The most common side effects include mild diarrhea and nausea, occurring in 2-3% [199]. The high drug levels achieved and sustained in urine for a considerable amount of time suggest that the majority of common uropathogens are covered based on the current clinical breakpoint (R> 32 mg/L) [183]. Treatment success after a single dose of fosfomycin trometamol in lower UTIs is reported [183, 186]. However, studies characterizing fosfomycin PK/PD properties are relatively scarce, and data on optimal PK/PD parameter for fosfomycin is somewhat inconsistent results. Some studies performed on gram-positive bacteria (*S. aureus* and *S. pyogenes*) suggest fosfomycin to act time-dependent [200, 201], while other studies in *E. coli* and *P. mirabilis* identify fosfomycin to act concentration-dependent [178, 202, 203]. A concentration-dependent post-antimicrobial effect (PAE) of 3.2–3.4 h at 0.25×MIC and 3.5–4.7 h at 8×MIC was observed *in vitro*. It has also been suggested that a threshold other than MIC may be predictive of fosfomycin activity, a concept similar to MPC for fluoroquinolones. For instance, Van-Scoy *et al*., explored the value of $T_{>RIC}$ (resistance inhibiting concentration) as a better predictor for fosfomycin activity [202, 203]. Emergence of resistance under the fosfomycin therapy is reported in numerous *in vitro* and *in vivo* studies [202, 204, 205] and poses a challenging problem for both susceptibility testing [206, 207], clinical breakpoint setting [185, 196] and widening the range of indications for clinical use [196], especially as a monotherapy agent [185]. Therefore, the optimal PD target for fosfomycin remains unknown and has to be further studied in order to optimize efficacy. The clinical breakpoint for susceptibility is currently ≤32 mg/L, according to the EUCAST [208] and ≤32 mg/L: sensitive and ≥128 mg/L: resistant, according to Clinical and Laboratory Standards Institute (CLSI) [209]. Thus, no universally accepted breakpoints exist to date, due to the lack of data [185]. Overall, fosfomycin trometamol is a promising treatment alternative for MDR isolates [183].

Resistance mechanisms

Several bacterial species are inherently resistant to fosfomycin, including *M. tuberculosis*, *V. fischeri*, and *Chlamydia* spp. [187, 205].

Enterobacterales and *E. coli*, in particular, can acquire resistance to fosfomycin either through chromosomal mutations or through HGT [187, 205, 210]. Chromosomally acquired fosfomycin resistance can be achieved through mutations in structural genes, that encode

membrane transport systems or their local regulators [211]: GlpT and UhpT, that transport glucose-6-phosphate glycerol-3-phosphate and other carbohydrates. The same systems are utilized for the uptake of fosfomycin into the bacterial cells [187]. Mutations can occur in the *uhpA* gene (response regulating protein, necessary for activating the expression of *uhpT*) [212], *ptsI* [213], and *cyaA* [214] (reduction of cAMP levels, leading to lower expression of GlpT and UhpT [215]). Such mutations can decrease the growth rates of *E.coli* [215, 216] as well as decrease the production of pili [205, 215], both possibly explaining lower virulence observed in fosfomycin resistant strains [217]. However, a strategy to alleviate fitness costs by controlling the expression of *uhpT* and/or *glpT* and through two-component signal transduction systems (CpxAR or TorSRT), has been described [216]. Another mechanism of mutational resistance to fosfomycin is target modification [212]. Several substitutions in MurA result in fosfomycin resistance. For instance, Cys115Asp (active enzyme but insusceptible to fosfomycin [218]) or Asp369Asn and Leu370lle have been shown to confer resistance [187, 212]. Isolates with decreased fosfomycin sensitivity due to target modification are rare in the clinical setting [210]. Increased MurA production, leading to decreased susceptibility with lower fitness costs, has also been described [219].

Fosfomycin resistance through direct antimicrobial modification, mediated by metalloenzymes, which cleaves the epoxide structure, has also been observed [220]. At least ten subtypes of metalloenzymes encoded by *fos* genes have been identified [220, 221]. The most prevalent include FosA (glutathione as a nucleophile), FosB (nucleophile and other thiols as nucleophiles), and FosX (water as a nucleophile). FosB-enzymes were identified on both chromosome and plasmids in *Staphylococcus spp.*, *Enterococcus spp.* or *Bacillus* spp. and *E. faecium* as well as in *Enterobacterales* [220]. FosX is present in *Listeria monocytogenes* [221]. In *Enterobacterales*, *Pseudomonas* spp. and *Acinetobacter* spp. *fosA* is the most prevalent gene [222]. Different subtypes of *fosA* have been described in *Enterobacterales* in the past decade: *fosA* and related *fosA2* through *fosA9* [220, 223]. *FosA2* through *fosA5* share approximately 70% identity with *fosA* [187]. Similarly to the *fosA* gene [220], *fosC2* encodes an enzyme that modifies fosfomycin through glutathione-S-transferase activity, but is evolutionary more distant, being 56% similar to *fosA* and 72% similar to *fosG* [220, 224].

Lastly, enzymes catalyzing the phosphorylation of fosfomycin have been identified in fosfomycin producing bacteria such as *Streptomyces wedmorensis* and *Streptomyces fradiae* [225]. The *fom*A, *fom*B, and *fom*C kinases convert fosfomycin into the diphosphate, triphosphate, and monophosphate states, respectively [225].

1.9.2 Mecillinam

General properties

Mecillinam (also known as amdinocillin) was introduced in 1972 as a member of a new group within the β-lactam class of antimicrobials, the amidine derivatives of penicillin [226]. It has been used against UTIs in Scandinavian countries since the 1980s. The safety profile, as well as good efficacy to treat lower UTIs, is well documented [227-229]. Moreover, its possible role in the treatment of pyelonephritis is discussed [230]. Mecillinam has a narrow-spectrum of activity, being active against gram-negative organisms such as *E. coli*, *P. mirabilis*, *Klebsiella* spp., *Salmonella*, and *Shigella* [231]. At the same time, *Pseudomonas* spp., *Enterococcus faecalis*, and *S. aureus* are resistant to mecillinam [228]. Mecillinam binds to penicillin-binding proteins (PBPs), showing a higher affinity towards PBP-2, in contrast to other common β-lactams, which could possibly allow for combination therapy [227, 232]. Inhibition of PBP-2 results in changes in the shape of the bacterial cell and the formation of enlarged spherical cells, which eventually lyse due to impaired elongation [233]. However, the precise mechanism of action is not fully understood [228, 232]. The effect of pivmecillinam on the intestinal and vaginal microflora is minimal [234, 235].

In *E. coli*, low levels of resistance (1.9-6.5%) are reported [107, 236]. Moreover, it has retained excellent activity against ESBL-producing organisms, where resistance prevalence does not substantially differ from the non-ESBL population [237-239]. The data on *in vivo* and clinical efficacy of mecillinam against ESBL-producing *E. coli* is scarce [228]. Søraas *et al.* [240] found clinical failure rates of 44% and 14% at 200 mg TID when treating community-acquired UTI (no distinguishing between upper and lower UTI) for ESBL- and non-ESBL-producing *E. coli*, respectively. This finding was also supported by a prospective, multicenter, observational cohort study by Bollestad *et al*., where 200 mg TID schedule for the UTIs caused by ESBL-producing *E. coli* was associated with treatment failure [241]. A good clinical response (100%) but a low proportion of bacteriological cure (25%) for lower UTIs were reported by Titelman *et al*. [242]. This led to the uncertainty of whether mecillinam is effective for infections caused by ESBL-producing strains [240, 243]. In contrast, Jansåker *et al.* reported 80% of the bacteriological cure for lower UTIs, treated with 400 mg pivmecillinam TID [243]. In addition, the study by Bollestad *et al.*, mentioned above, shows that 400 mg TID regimen to produce comparable clinical and bacteriological cure rates irrespective of ESBL-production [241]. Mecillinam has also been shown to have *in vitro* activity against some carbapenemase-

producers (NDM-1 [244, 245] or OXA-48-like[245]), but no *in vivo* studies have investigated that yet.

Pharmacokinetics and Pharmacodynamics

Pivmecillinam (the pivaloyloxymethyl-ester of mecillinam, serving as a prodrug), is used as the peroral treatment of acute lower UTI [228]. Pivmecillinam is well absorbed after oral administration and converted to mecillinam by non-specific esterases in blood, gastrointestinal mucosa, and other tissues [246]. Its bioavailability is 60–75% [234]. Food intake does not alter the bioavailability [228, 246]. Following oral administration of 200 mg and 400 mg pivmecillinam, peak mecillinam concentrations of approximately 1.4 μg/mL and 3.2 μg/mL, respectively, are attained within 1-1½ hours after dosing [246-248]. In human kidneys, the concentrations of mecillinam were reported to be 1.4 fold higher than those in serum [249]. The elimination half-life of mecillinam is approximately 1 hour [246-248]. It is excreted primarily through kidneys, by filtration and active tubular secretion [246-248], and to some extent through biliary excretion [228, 246]. Approximately 45% of the fosfomycin is excreted in the urine. For 400 mg dose, the mean peak urinary concentration ranges from 176 mg⁄L to 1324 mg⁄L, usually within the first 3h period [250]. At 6h and 12h after administration, the concentration drops to 12-57 and ≤5 mg/L, respectively [228, 250, 251]. Probenecid can inhibit the elimination of mecillinam by repressing tubular secretion [246]. The elimination rate constant correlates linearly with creatinine clearance [252]. In subjects with creatinine clearances > 50 ml/min, the elimination half-life remains relatively constant; however, a decrease in creatinine clearance results in increased half-life [252].

Adverse effects for mecillinam are generally low compared to other antimicrobials (<10%), and mecillinam is safe for use in pregnant women [251]. Nausea and rash have been reported as the most common side effects [229]. Data regarding the optimal PK/PD index for mecillinam is limited. As a β-lactam, the optimal PK/PD index is expected to be $T_{>MIC}$. Data from a murine UTI study by Kerrn *et al.* [253] supports this. However, $T_{>MIC}$ could not explain the pronounced effects mecillinam on kidney CFU counts of mice infected with resistant strains. Selecting an optimal dosage regimen for UTI is also challenging. Studies have found that low doses of 200 mg BID for seven days are equally efficient to higher doses [254]. However, according to a recent meta-analysis, there was insufficient evidence to support one or another dosing regimen, although higher doses with shorter duration (e.g., 400 mg BID for three days or 400 mg TID for three days) were suggested to reduce the emergence of resistance [255]. At the same time, a higher chance of adverse effects with higher dosages (2900-16800 mg total

dose) have been observed [255]. A recent literature review by Jansåker *et al.* [230] suggests that mecillinam could be a treatment alternative for uncomplicated pyelonephritis. It also found 400 mg-based dosing regimens to be more effective for treating pyelonephritis and UTI caused by ESBL-producers [230]. Currently, evidence for the use of mecillinam for bacteremia is insufficient [230].

Resistance mechanisms

Mutational acquired resistance to mecillinam is easily acquired *in vitro* under the selective pressure [256]. More than 60 genes involved in mecillinam resistance have been identified, including genes involved in cell division and elongation (*mrdA*, *mrdB*, *mreB*, *mreC* and *mreD* and related genes such as *nlpI*, *pgsA*, and *sppA*), RNA synthesis and processing function (*rcsD* and *rpoB*), stress response (*rcsB*- and *relA*-dependent loci), cysteine biosynthesis (*cysB* and genes related to the cysteine pathway) and others [232, 256-258]. Notably, despite the high frequency of resistance *in vitro*, the prevalence of clinical isolates identified with these resistance mechanisms remains limited [232, 256]. This is most likely caused by a high associated fitness cost [256, 258]. High urine concentrations (176–1324 mg/L) during treatment combined with relatively low MICs (8–64 mg/L) of mecillinam among uropathogens or, perhaps, other factors could also explain the limited observations of resistance [259]. Among the resistance mechanisms, the most common [238], and arguably, most important due to the relatively lower fitness cost are the *cysB* mutations [256, 258]. Interestingly, the resistance caused by this mechanism could be reversed in urine (likely due to the presence of cysteine) [260]. The efficacy of mecillinam for UTI could be partly explained due to this [231, 258, 260]. There is an ongoing discussion of whether the media for mecillinam susceptibility testing should be enriched with cysteine to produce more accurate results (similarly as for fosfomycin and glucose-6-phosphate) [231].

In *E. coli*, the main mechanism of resistance to β-lactams (including mecillinam) is the acquisition and production of a β-lactamases, such as ESBLs, AmpCs, and carbapenemases [232]. Mecillinam is generally considered to have higher stability against ESBLs and AmpCs compared to other β-lactams, except OXA-3 [261]. However, *in vitro* studies by Thomas *et al.* [262], have shown a marked inoculum effect, resulting in higher MICs to mecillinam in isolates producing β-lactamases (TEM-1, SHV-2, -4 and -5, and to a lesser extent, CTX-Ms). This, along with the time-kill studies [227, 237, 263], suggests mecillinam being prone to hydrolysis by these enzymes. The overproduction of TEM-1 confers high-level resistance to mecillinam [264]. In contrast to that, Livermore *et al.* have shown MICs for most ESBL- and AmpC-

producing organisms to still be within susceptible range (≤1 mg/L), even at a high inoculum [265, 266]. Mecillinam is inactivated by most of the carbapenemases [231, 245]. However, some carbapenemase-producing *E. coli* (notably NDM-1 and OXA-48-like-producers) have also been shown to be *in vitro* susceptible to mecillinam [244, 245].

1.9.3 Nitrofurantoin

General properties

Nitrofurantoin, a synthetic nitrofuran compound, discovered in the 1940s, has been available for the treatment of UTIs since 1952 [267, 268]. Since the 1970s, when other antimicrobials came to market, its use became limited [269]. However, in the past decade, due to the increase of antimicrobial resistance, it has regained attention as the first-line drug against UTIs [90, 267]. Nitrofurantoin is indicated against uncomplicated UTIs caused by *E. coli* and *E. faecalis* [208]. Similar *in vitro* effectiveness against ESBL and non-ESBL *E. coli* strains has been observed in time-kill studies [270, 271]. Among ESBL-producers, susceptibility proportions for nitrofurantoin are varying from 71-100%, depending on study location [188, 272-276]. According to meta-analyses, nitrofurantoin's clinical efficacy for treating lower UTIs is high and comparable to that of other antimicrobials [267, 269].

The mechanisms of action of nitrofuran drugs are not entirely understood [271]. Studies performed in the 1970s suggest reduced forms of the drug are responsible for the effect [271]. Bacterial nitroreductases convert nitrofurantoin to highly reactive electrophilic forms, that non-specifically attack bacterial proteins in the ribosomes, completely ceasing protein, cell wall, RNA, and DNA synthesis; impair carbohydrate metabolism, and cause single-strand breaks in DNA [268, 277-279]. Bacterial sensitivity to nitrofurantoin is correlated with the presence of nitroreductases in bacteria [279]. However, some antibacterial activity has also been shown with inhibited nitroreductase activity, suggesting that nitrofurantoin may partly act without the reduction to active forms [280].

Pharmacokinetics and Pharmacodynamics

Current data on the PK of nitrofurantoin is mostly based on older studies [267]. After the administration of a 100 mg oral dose, nitrofurantoin is absorbed with a T_{max} of 2.0–2.3 h [281]. The bioavailability of nitrofurantoin is about 90% [282], which could be affected by food, drug particle size (the macrocrystalline form is absorbed and excreted slower compared to the microcrystalline formulation) and pH [268]. Despite the high bioavailability, plasma C_{max} is low, 0.51-1.11 mg/L after administering 100 mg *per os* [281, 283]. Furthermore, nitrofurantoin

does not distribute into cerebrospinal, prostate and amniotic fluids, and the aqueous humor, or umbilical cord serum. However, it is found in urine, medullary tubular lumen, interstitial space, and renal lymph [268]. Nitrofurantoin is actively transported into human milk, achieving concentrations of 1.3 mg/L [284]. The elimination half-life is 0.72-1.7 h, corresponding an AUC of 1.05-2.62 mg×h×ml^{-1} [281, 283]. Nitrofurantoin is excreted in urine and bile [268], and approximately 27–50% of the drug is eliminated via kidneys in unchanged form, with the clearance rates of 16.7-19.4 L×h^{-1} [183, 268, 281]. Urinary excretion of nitrofurantoin is a result of glomerular filtration, tubular secretion and reabsorption; the latter is pH-dependent [285]. Nitrofurantoin reaches high urinary levels of 50–300 mg/L, which are achieved within 30 minutes and maintained for 4-5 hours [268].

Nitrofurantoin acts bactericidal against susceptible organisms [270, 271]. Data regarding optimal PK/PD parameters of nitrofurantoin are scarce, with existing studies suggesting that optimal PK/PD parameters differ across species, with time-dependent behavior against *E. coli* and concentration-dependent against *E. cloacae*. The acidity of the environment affects killing rates, which increases with a lower pH [286]. Currently, there is not enough data to establish confident clinical breakpoints and choose the optimal dosing regimen of nitrofurantoin [283].

Nitrofurantoin has been reported to cause various adverse reactions, which are uncommon (<0.001% of total courses of therapy) and generally occur after long-term treatment [268, 285]. Among side effects, gastrointestinal disturbances (nausea, vomiting, and anorexia) are most common. The macrocrystalline form of nitrofurantoin reduces these side effects due to slower absorption [268]. No significant impact on microbiota, except for a temporary increase in the prevalence of beneficial *Bifidobacterium* genus, was observed after nitrofurantoin treatment [287]. Other side effects generally occur after long-term treatment and include skin eruptions, hematologic disorders (anemia in patients deficient in glucose-6-phosphate dehydrogenase), acute and chronic pulmonary reactions (infiltrates/eosinophilia and interstitial lung disease, respectively) and chronic hepatitis, which [268, 285, 288]. Nitrofurantoin can cause neurotoxicity (peripheral neuropathy, dizziness, vertigo, diplopia, and cerebellar dysfunction) and benign intracranial hypertension. Neurological side effects are possibly caused by the axon loss and are more common in women and elderly patients [268, 289]. The teratogenic risk of nitrofurantoin is uncertain, with conflicting results among existing studies [285]. The carcinogenic effect of nitrofurantoin was observed in animals, which has led to the ban of its animal use in the EU [290]. However, current human data, despite >50 years of use, seems not to support such observations, highlighting the need for further studies [291]. Finally,

nitrofurantoin urine levels tend to decrease below the therapeutic range, and the serum levels simultaneously rise, causing toxicity in a patient with impaired renal function. Nitrofurantoin should be, therefore, avoided in patients with creatinine clearance <30 mL/min [292]. Overall, nitrofurantoin is considered as a safe drug, with the incidence of side effect comparable to other antimicrobials [285].

Resistance mechanisms

The mechanism of resistance to nitrofurantoin seems to be mainly caused by mutations in *nfsA* or *nfsB,* encoding oxygen-insensitive and oxygen-sensitive nitroreductases, respectively [37, 38]. Mutations affecting the function of these enzymes lead to the reduction of nitrofurantoin, ceasing the formation of toxic intermediate metabolites [293]. According to the analysis of clinical isolates, mutations in *nfsA* and *nfsB* were present in 88.9% and 66.7% of nitrofurantoin-resistant isolates, respectively [38]. Recently, mutations in *ribE*, encoding lumazine synthase involved in the biosynthesis of riboflavin, have also been shown to increase nitrofurantoin MIC levels in laboratory mutants [293], and in clinical isolates [294]. Finally, plasmid-mediated efflux pumps *oqxA* and *oqxB* have been shown to play an important role in nitrofurantoin resistance, especially in combination with other mechanisms mentioned above [294]. Resistance to nitrofurantoin seems not to be a clonal phenomenon [293, 295]. Low levels of resistance to nitrofurantoin, despite its extensive use in the last 50 years [296], could be explained by the multiple mechanisms of action, along with the high fitness costs of alterations in such enzymes [279, 293].

1.9.4 Temocillin

General properties

Temocillin is a β-lactam antimicrobial, developed in the 1980s as a 6-α-methoxy derivative of ticarcillin [297, 298]. Temocillin has a molecular mass of 414.453 g/mol and high water solubility [297]. Compared to other penicillins, temocillin is more stable against β-lactamases, including AmpC and ESBL types [299]. Temocillin has a narrow-spectrum of activity, limited to gram-negative bacteria including *E. coli, K. pneumoniae, Citrobacter, Proteus, Providencia, Salmonella* and *Shigella*, *Haemophilus influenza, Neisseria gonorrhoea,* and some strains of *B. cepacia* but not *P. aeruginosa* or *A. baumannii,* gram-positive or anaerobic organisms [297, 298, 300-302]. Due to its narrow spectrum, temocillin has been considered an inferior alternative to more broad-spectrum drugs for the treatment of serious infections [298], thus limiting the use [297]. However, the interest in temocillin has been renewed decades later

[299], due to the increasing prevalence of ESBL-producing *Enterobacterales*. Temocillin is now available in the UK, Belgium, Luxemburg, and France for the treatment of bloodstream infections, UTIs (including ESBL-producers), and lower respiratory infections [297]. The narrow spectrum of activity, along with its resistance to hydrolysis by β-lactamases, are now considered as important ecological and bacteriological advantages of the drug [297]. Temocillin has shown good efficacy (both clinical and microbiological) for UTIs, bloodstream infections, and healthcare-acquired pneumonia, with 91-97% success rates, using a dose of 2g BID (or renal-adjusted equivalent), irrespective of ESBL/depressed-AmpC strain status [303].

Pharmacokinetics and Pharmacodynamics

The oral formulation is unavailable [297], and currently, temocillin is administrated intramuscularly or intravenously as disodium salt [297], containing R- and S-epimers, in an approximate ratio of 65/35, respectively [304]. Pharmacokinetics of R- and S-epimers differ, with the R-epimer having 2× higher elimination rate from the bloodstream and a 23% higher volume of distribution [304], but no difference in their antibacterial activity [298]. Temocillin is highly protein-bound, approximately 70-85% [299, 302], with S-epimer being more protein-bound [298]. Moreover, protein binding is considered to be concentration-dependent as increasing doses of temocillin from 500 mg to 2 g results in a decrease in protein binding from 85% to 63%, respectively [297]. Saturable protein binding occurs likely because only one binding site exists on each albumin molecule [305]. Protein-binding saturation results in increase of the volume of distribution for higher temocillin doses, from 0.15-0.2 L/kg after an increase in dose from 500 mg to 2 g [306, 307]. Serum C_{max} ranges from 78 to 263 mg/L and the corresponding AUC from 344.1 to 784.5 mg×h×ml^{-1} [299, 305, 306]. Temocillin is found in prostate (38 and 27 mg/kg in peripheral and central prostate tissues, respectively), bile (8-10× corresponding serum concentrations), accumulates in peritoneal fluid (0.6-1.7× of AUC_{0-24h} in plasma), lung tissue (0.26× of AUC_{0-24h} in serum), cerebrospinal fluid (0.1× of serum concentrations) [297]. The urinary concentration of temocillin after 500 mg BID is approximately 500 mg/L [308]. Temocillin has an elimination half-life (intravenous infusion) of approximately 3-5 h, regardless of dose infusion (range 500 mg - 2g) [297, 309]. The S-epimer has a 3×fold higher half-life compared to the R-epimer [298]. Temocillin clearance in healthy patients ranges from 18-45 ml/min and is mainly renal [305, 307, 310]. Temocillin is eliminated unchanged in urine [298]; the reported urinary recovery rates are high, ranging from 66 to 82% [306, 307, 311]. Renal clearance of temocillin increases with higher doses due to the saturable serum protein binding. However, the clearance of free temocillin is not affected by dose

variation [297, 305]. Glomerular filtration has been considered to be the most important mechanism, as blocking of tubular secretion only slightly affects renal clearance [297, 305]. Temocillin reaches high concentrations in urine, ranging from 490-605 mg/L after 500mg dose [311]. In critically ill patients, patients with kidney dysfunction or undergoing dialysis, an adjustment in dose is required [297].

Due to the presence of the α-methoxy moiety on the ticarcillin backbone, temocillin affinity to PBPs is changed (no affinity to PBP2, a lower affinity for PBP1a, 1bs, and 3, and increased affinity to PBP4 and 5), leading to different pharmacodynamics [297]. The degree of affinity to different PBPs varies with experimental conditions, with PBPs 1, 3, and 4-6 showing various degrees of affinity, but not PBP2 [312, 313]. Authors have suggested that the formation of an unstable complex with PBP-3 to be the primary mechanism in *E. coli*, according to low-temperature labeling studies and the typical response of exposed cells (filamentation) [312-314]. However, *in vitro* time-kill studies have shown that bacteria started to grow in filaments, at "relatively lower" supra-MIC (2-8×MIC) concentrations, followed by eventual regrowth at 24h [300, 315, 316]. At higher concentrations, lysis was observed. Yourassowsky *et al.* [316] suggested that this could be due to the target switch from PBP3 to PBP1 (lysis). Accordingly, temocillin acts not rapidly bactericidal, and the effect is concentration-dependent [300]. The optimal PK/PD index for β-lactam drugs is considered to be $T_{>MIC}$, and the same is empirically applied to temocillin; however, no *in vivo* study, aiming specifically at determining the optimal index has been performed [297]. However, several studies, although not comparing different indices, have found an association of $T_{>MIC}$ of 40-50% with treatment efficacy [317-319].

Several studies in severely ill patients performing Monte-Carlo simulations have been made [319-321]; however, the controversy regarding optimal dosing and breakpoints still exists [322]. EUCAS breakpoints for *Enterobacterales* are still under consideration [208]. The British Society for Antimicrobial Chemotherapy (BSAC) has previously recommended breakpoint UTI breakpoint for >32 mg/L, and systemic breakpoint of >8 mg/L[323]. The breakpoint is generally considered difficult to reach except for UTIs; however, newer studies conclude a dose of 2 g BID appropriate to adequately cover pathogens with MICs up to 16 mg/L [297, 324]. Kandil *et al.* [322] suggest increasing the currently recommended dose of temocillin, as it increases clinical success rates, and is not associated with increased adverse effects [303, 322].

According to retrospective studies, temocillin has minimal adverse effects on the intestinal flora, including the minimal risk of *C. difficile* infection [297]. It is generally considered very

safe in terms of hematological, hepatic, and renal adverse effects [299, 325]. However, adverse effects common to β-lactams such as allergy, have been reported [299].

Resistance mechanisms

In vitro, spontaneous mutations towards temocillin resistance are reported at a low frequency (10^{-8}- 10^{-10}, only after 6-8 repeated cultures), and are not affected by the presence of β-lactamase production [297, 311, 319, 326]. A minimal inoculum effect has been observed for temocillin MIC for *E. coli* [301], besides a modest inoculum effect for minimal bactericidal concentration (MBC) in AmpC-producers and MIC in CTX-M-15-/KPC-producing strains [297, 301, 317, 319]. In contrast, a significant degree of resistance development has been observed *in vitro*, but not *in vivo* (murine UTI model) [315]. These data are further confirmed by the observation of regrowth at lower concentrations *in vitro* time-kill studies [300, 316], and by no mutant selection *in vivo,* after treatment for 24h [317, 319]. To elucidate the exact mechanisms of such resistance due to spontaneous mutations require further studies [297].

The α-methoxy radical of temocillin prevents entry of a water molecule into the active site of serine-dependent ß-lactamases [327], thereby conferring resistance to hydrolysis by Ambler classes A and C ß-lactamases (such as AmpCs, ESBLs, and KPC). However, temocillin is liable to hydrolysis by class B metalloenzymes or some class D enzymes such as VIM, NDM, IMP, and OXA-48 [297, 299, 324, 328]. Despite this, the resistance levels are still higher in ESBL- and AmpC-producers than in WT isolates (13% among ESBL-/pAmpC-producing *E. coli*, vs. 2.5% in WT) [329]. In two Swedish studies of ESBL-producing *E. coli* and *K. pneumoniae*, overall resistance to temocillin was 17% to 24% [239, 330]. A significant proportion of ESBL-producers had MIC one dilution step above the sensitivity breakpoint, suggesting those strains could still be covered using higher doses [303, 331].

Other evidence for reported mechanisms for temocillin resistance is scarce. Mutations in MexAB-OprM efflux system (*mexA* and *mexB*) contributing to the intrinsic resistance of *P. aeruginosa* [332]. Overexpression of efflux pumps (AcrAB) in combination with the presence of DHA-1 or CTX-M-1, resulting in the non-susceptibility to temocillin in *K. pneumoniae* [333].

2 AIMS

The overall aim of the studies in this thesis was to evaluate the role of old antimicrobials as treatment options for UTIs caused by MDR *E. coli*.

The specific objectives were:

- Determine the antimicrobial activity of fosfomycin, mecillinam, temocillin and nitrofurantoin in Norwegian ESBL-producing *E. coli*;
- Analyze the resistance patterns among a nationwide strain collection of ESBL-producing *E. coli*;
- Determine the optimal PK/PD indices for fosfomycin in UTI;
- Determine the optimal dosing (200 vs. 400 mg TID) of mecillinam in UTI;
- Evaluate the *in vivo* efficacy of fosfomycin and mecillinam in the treatment of UTI caused by MDR *E. coli*.

3 MATERIALS AND METHODS

This section provides a brief summary of the strain collections and main methods used in the studies. A more detailed description is presented in the papers.

3.1 Strain material

In paper 1, the strain material was based on 2010-11 data from the Norwegian Surveillance System for Antimicrobial Resistance (NORM). Initially, 111 clinical isolates of *E. coli* were selected based on reduced susceptibility (disk diffusion) to cefotaxime and/or ceftazidime, which were further investigated for the presence of ESBL genes by molecular methods (see below). In total, 105 strains (81 and 24 from blood culture and urine, respectively) were identified as ESBL-producers and included in the study.

In paper 2, the fosfomycin-susceptible clinical strain *E. coli* NU14 was used to characterize the PK/PD properties of fosfomycin. The strain is acknowledged as an archetypal UPEC [334] and has been used in multiple studies on UPEC and UTI pathogenesis [99, 335-338] as well as in the same murine UTI model [339]. Further, three isogenic NU14 derivatives with different levels of fosfomycin susceptibility, DA6313, DA6328, and DA6401, were used to evaluate the selected dose in the UTI model. In these strains, chromosomal mutations/deletions [215], including deletion in ptsI (DA6313), a missense mutation in *glpT* (DA6328) and *uhpT* (DA6401), caused an increase in fosfomycin MICs in these strains. Apart from NU14 and its derivatives, five fosfomycin susceptible and one fosfomycin resistant contemporary clinical ESBL-, pAmpC-, and/or carbapenemase-producing *E. coli* strains were used.

In paper 3, similarly to **paper 2**, four contemporary clinical strains of ESBL-, pAmpC-, and/or carbapenemase-producing *E. coli* were used to investigate the efficacy of two mimicked clinical doses of mecillinam against MDR isolates. To make the strain collection diverse in mecillinam MIC, a pan susceptible strain 24623884-114 and mecillinam resistant TEM-1B-producer 21773360-98 were added to the collection.

3.2 Susceptibility testing methods

For all studies, the MIC value was determined as this is considered predictive of bacterial eradication in relation to PK/PD parameters (AUC/MIC, $T_{>MIC}$, or C_{max}/MIC) [175, 340]. In **paper 1,** the susceptibility testing was performed using MIC gradient tests for fosfomycin,

mecillinam, temocillin, nitrofurantoin, and amikacin, and VITEK2 for other antimicrobial agents. The MIC gradient tests were also used to determine mecillinam MIC in **paper 3**. In **paper 2**, agar dilution was used for MIC determination of fosfomycin. Additionally, in **paper 2** and **paper 3**, microbroth dilution was performed as the MIC method for other antimicrobial agents. The results were interpreted according to EUCAST clinical breakpoints, valid at the time of the study (http://www.eucast.org/clinical_breakpoints/).

3.3 Molecular methods for detection of antimicrobial resistance

Three different methods were used to molecularly determine the resistance mechanisms in the strain collections with the main focus on the presence of β-lactamases. In **paper 1**, two different methods were used to identify ESBLs in the phenotypic ESBL-positive isolates molecularly. The PCRs for *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV,}}$ and *bla*$_{\text{TEM}}$ were performed on isolates from 2010, according to Tofteland *et al.* [341]. The Check-MDR CT101 microarray, detecting *bla*$_{\text{CTX-M}}$, the most common *bla*$_{\text{SHV}}$/*bla*$_{\text{TEM}}$ ESBL variants, pAmpC genes, and the carbapenemase genes *bla*$_{\text{KPC}}$ and *bla*$_{\text{NDM}}$ was used on isolates from 2011. **In paper 2**, the strain collection was initially tested with Check-MDR CT103, which has extended coverage over carbapenemases-producing isolates (KPC, NDM, VIM, IMP, OXA-48-like, GES, GIM, SPM, OXA-23-like, OXA-24/40-like, OXA-58-like) in addition to the most common types of TEM, SHV, and CTX-M ESBLs and pAmpCs. Finally, in **paper 2** and **paper 3,** whole-genome sequencing (WGS) using the Illumina technology was applied.

3.4 *In vitro* time-kill studies

In **paper 2,** we used *in vitro* time-kill studies to determine the effect of different fosfomycin concentrations on target bacteria under controlled conditions. The time-kill studies were performed as previously described [342-344]. However, due to the high MICs of fosfomycin resistant isolates in the study, we modified the protocol: instead of adding the small volume of concentrated fosfomycin solution to the tubes with bacterial suspension, the concentrated bacterial suspension was added to tubes with fosfomycin. This was performed in order not to exceed the solubility limit provided by the supplier.

3.5 *In vivo* modelling – murine urinary tract infection model

To explore the PK/PD and efficacy of fosfomycin (**paper 2**) and the efficacy of different doses of mecillinam *in vivo* (**paper 3**), we chose to use a murine UTI model [345]. The model is well established and has been used in numerous studies [253, 319, 342, 345, 346]. Furthermore, mice seem to be the species offering an optimal balance due to similar physiology (similar urinary tract anatomy compared to non-mammals [347]), small size (compared to bigger mammals [347]), and a greater similarity in UTI pathogenesis compared to other rodents. Mice also lack vesicourinary reflux like humans [345], and in contrast to rats contain galα(1-4)galβ glycolipid in renal tissue, necessary for successful modelling of pyelonephritis [348]. In mice, bacteria attach to the urinary tract surfaces via type-1 fimbriae and pap-fimbriae; intracellular bacterial communities/biofilm in the bladder are observed in both mice [349] and humans [350]. The use of inbred mice also offers an advantage of similar genetic background, which is impossible to achieve in studies using human volunteers [347].

3.5.1 General description of the model

In both **paper 2** and **paper 3,** we used immunocompetent outbred albino female mice (OF-1; Charles Rivers Laboratories, Chatillon-sur-Chalaronne, France). On the inoculation day, mice were anesthetized and inoculated with a bacterial suspension containing approximately 10^9 CFU/ml into the urine bladders. Urine was collected twice: on day two to control for the establishment of infection and day five to control for treatment effect. During three days of treatment (with either antimicrobial or vehicle), mice were observed for any signs of pain and given a subcutaneous injection of either saline or test drug according to the dosing schedule. On day five, after the collection of urine, the mice were euthanized by cervical dislocation, and the emptied bladder and both kidneys were removed aseptically. The CFU-counts in the urine samples and homogenized organs were assessed using a spot dilution method.

3.5.2 PK studies

In **paper 2**, for PK studies of fosfomycin, the same breed of mice was used. Mice (weight, ~30 g) were given a single subcutaneous (s.c.) doses of 0.75, 7.5, and 30 mg fosfomycin per mouse, respectively. Blood and urine were sampled at multiple time points (15, 30, 60, 120, 180, and 240 min after dosing). Fosfomycin concentrations were measured by a bioassay with *E. coli* ATCC 25922 strain (fosfomycin susceptible). Standard fosfomycin concentrations were used as the control, with a day-to-day variation of <10%.

3.5.3 PK/PD studies

In **paper 2**, the PK data was further inter-/extrapolated and used to design 6 dosing regimens: 30 and 7.5 mg/mouse in a single dose, 15 mg/mouse twice (every 36 h [q36h]), 1.88 and 0.47 mg/mouse every 6 h, and 0.47 mg/mouse every 12h (q12h). These dosing regimens were aimed at maximizing the variation in the PK/PD indices. $T_{>MIC}$, $C_{max,}$ and $AUC/MIC_{0\text{-}72h}$ were calculated using the trapezoid rule using the measured values, when possible, and extrapolated values for the further time points.

Infection with NU14 was induced in mice as described above, and treatment was initiated at 24h post-infection. The CFU-counts to assess the treatment effect were performed similarly, as described above. The effect of treatment was measured as the percentage of negative cultures (below the limit of detection) for urine and kidneys, and as the proportion of CFU-counts that were lower than minimal counts for the control group (approximately 10^4 CFU/ml).

The relationship between the resulting treatment effect estimates was analyzed according to the dose-response model with sigmoidal Hill function [(161)]. The indices correlating best with treatment effect were determined, and the dosing regimen aimed at corresponding with both efficacy and human PK parameters was chosen to be further evaluated in the treatment of isolates with diverse genetic background and fosfomycin susceptibility.

3.5.4 *In vivo* treatment studies

Treatment studies (**paper 2** and **3**) were performed similarly according to the general model description described in paragraph 3.5.1.

3.5.5 Statistical analysis for PK, PK/PD and treatment studies

Statistical analysis and graphical representation of the data was carried out using IBM SPSS Statistics Ver 24 (IBM, Armonk, NY, USA) and GraphPad Prism 7 (GraphPad Software, CA, USA).

The determination of the relationship between the PK/PD index and effect (PD endpoint) is performed mathematically using the E_{max} model or Hill equation using the regression analysis [(351)]. The goodness of fit was assessed as the R^2 coefficient.

The calculation of PK/PD indices was performed by the trapezoidal rule for the doses where actual experimental data was available (15-240 min after s.c. dose) and by inter- and extrapolation of the PK data using the exponential equation (single-compartment model).

The median CFU/ml counts between the treated and vehicle groups were compared using the Mann-Whitney U test at a significance threshold of 0.05. A comparison of binomial data (treatment success/failure or susceptible/resistant) was performed using Fischer's exact test U with a significance level of 0.05. Corrections for multiple hypotheses testing were not performed, taking into account already small groups of comparison in animal studies [352].

3.5.6 Ethical approvals

All experimental protocols regarding the animal keeping and handling at Statens Serum Institute were according to animal welfare standards and approved by both veterinarians at the institution and by the Danish Animal Experimentation Inspectorate (no. 2014-15-0201-00204).

4 SUMMARY OF MAIN RESULTS

4.1 Paper 1. The antimicrobial activity of mecillinam, nitrofurantoin, temocillin and fosfomycin and comparative analysis of resistance patterns in a nationwide collection of ESBL-producing *Escherichia coli* in Norway 2010–2011

In paper 1, we found that the majority of ESBL-producing *E. coli* in Norway in 2010 and 2011 were CTX-M-producers, belonging to group 1 (70%) and group 9 (26%). SHV-ESBL- and TEM-ESBL-producers only represented 2% and 1 %, respectively. The proportion of isolates sensitive to fosfomycin, mecillinam, temocillin, and nitrofurantoin was 100%, 94%, 100%, and 91%, respectively indicating that these drugs should be considered as good options for treatment of uncomplicated UTI caused by ESBL-producing *E. coli* in Norway. Moreover, a high degree of susceptibility to amikacin (95%) was observed. In contrast, high levels of resistance to β-lactams such as ampicillin (100%), cefuroxime (97%), cefpodoxime (98%), cefotaxime (94%), ceftazidime (77%), and aztreonam (67%) were observed. All isolates were susceptible to meropenem, and 53% were non-susceptible (both Resistant and Intermediate) to amoxicillin-clavulanic acid. A high proportion of resistance to non-β-lactams was also observed, including trimethoprim-sulfamethoxazole (71%), gentamicin (40%), tobramycin

(50%), and ciprofloxacin (74%). Co-resistance was also common with resistance to all three drug-classes observed in 36% and for two drug classes in 40% of the isolates. In contrast, co-resistance to both mecillinam and nitrofurantoin were rare (2%), with the most common pattern being susceptibility to both drugs (88%). Mecillinam MIC among the strains in our study had higher MIC values compared with EUCAST MIC distributions (containing both ESBL-producing and non-ESBL-producing strains). The high proportion of co-resistance to several classes of antimicrobials observed among ESBL-producing *E. coli* limits empiric treatment options. At the same time, old drugs seem to remain *in vitro* active against such strains.

4.2 Paper 2. Pharmacokinetics and pharmacodynamics of fosfomycin and its activity against extended-spectrum β-lactamase-, plasmid-mediated AmpC-, and carbapenemase-producing *Escherichia coli* in a murine urinary tract infection model.

In paper 2, we have addressed three questions: "What are the exposures (PK profiles) for various doses" and "What are the exposure-response relationship and PD target" for fosfomycin as well as its *in vivo* efficacy against ESBL-, pAmpC-, and carbapenemase-producing clinical isolates. In the PK studies, fosfomycin plasma and urine concentrations were determined after administering single subcutaneous injections of fosfomycin in the doses of 0.75, 7.5, and 30 mg/mouse. This resulted in mean peak plasma and urine concentrations of 36, 280, and 750 mg/liter and 1 100, 33 400, and 70 000 mg/liter, respectively. Urine concentrations were expected to sustain above 1 mg/liter for 5, 8, and 9.5 h, respectively.

The effect of different fosfomycin concentrations on the clinical isolates was evaluated in the time-kill assay, showing a concentration-dependent killing pattern. Initial rapid bactericidal effect (<2h), was followed by regrowth, observed for all tested concentrations below 16× to 32× MIC. Further increase in concentration prevented the regrowth. For the resistant strain (*fosA*), transient killing (≤ 2 $\log_{10}$) was followed by the regrowth, irrespective concentration (including the maximal concentration of 16384 mg/L).

Based on the obtained PK profiles, we designed six dose regimens in order to produce variation in the $T_{>MIC}$ and AUC/MIC_{0-72} and C_{max}. These dosing regimens were further applied against the susceptible strain (fosfomycin MIC 1 mg/L) in a murine UTI model. The

optimal index, according to the PK/PD analysis based on the bloodstream concentrations, was $C_{max,}$ followed by $AUC/MIC_{0–72}$.

A dose of 15 mg/mouse administered twice (q36h) was further tested against isogenic strains (n=3) with decreased fosfomycin susceptibility and a diverse collection of MDR clinical isolates (n=6) including ESBL-, pAmpC, carbapenemase- (VIM-29 and NDM-1) and FosA-producers. For the isogenic strains, the decrease in effect was proportional to MIC. For the clinical isolates, fosfomycin significantly reduced the urinary CFU-counts for all susceptible strains (including clinical MDR strains and carbapenemase-producers), but not for the fosfomycin resistant isolate harboring *fosA*.

4.3 Paper 3. Efficacy of mecillinam against clinical multidrug-resistant *Escherichia coli* in a murine urinary tract infection model

Six clinical MDR *E. coli* strains obtained from patients with UTI (n=4), bacteremia (n=1), or wound infection (n=1) were used in the study. The strain collection was in the genetic background and included strains producing ESBLs (CTX-M-14 and -15), pAmpC (CMY-6 and -4), and carbapenemases (VIM-29 and NDM-1). Some of the strains harbored additional β-lactamases such as TEM-1B and OXA-1. Isolates were diverse with respect to the mecillinam MIC, which ranged from 0.5 to 64 mg/L.

According to the previously obtained PK data for mecillinam [250, 253], two dosing regimens were calculated to mimic the PK profiles observed in humans after two different clinical doses of pivmecillinam: 200 mg and 400 mg TID. The calculated dosing regimens were applied in the murine UTI model. Both mecillinam dosing regimens significantly reduced the number of CFU per milliliter in urine, also including both carbapenemase-producing isolates. A variable degree of reduction was observed for the bladder and kidneys. A significant decrease in bacterial of CFU-counts for urine, bladder, and kidneys, was observed even for all strains, including mecillinam-resistant.

Determining a superior dose was impossible, as both doses effectively reduced the urinary CFU counts for most isolates. The “400 mg” dose resulted in a slightly more statistically significant reduction of CFU-counts in kidneys. When combining the results for all six strains with 30 – 40 mice per group, a significant effect of mecillinam treatment in both doses was obvious at all sites. Mecillinam has reduced the urinary CFU-counts for NDM-1 (mecillinam MIC 2 mg/L) and VIM-29 (mecillinam MIC 64 mg/L) producers.

5 GENERAL DISCUSSION

The overall aim of this study was to evaluate the role of fosfomycin, mecillinam, temocillin, and nitrofurantoin (for simplicity, we would refer to them as "old antimicrobials") as treatment options for UTIs caused by MDR pAmpC-/ESBL-/carbapenemase-producing *E. coli*. In this context, the following questions have to be addressed:

1. What is the MIC distribution of interest?
2. What are the exposures (PK profiles) for various doses and patient populations?
3. What are the exposure-response relationship and PD target?

Answering these questions helps to re-evaluate the properties and applicability of these drugs in the current situation of increasing antimicrobial resistance.

5.1 Methodological considerations and limitations

5.1.1 Strain material

In **paper 1,** the use of strains sampled via the national surveillance system assured a collection that represented the whole country. However, the limited number of isolates could influence the results due to local outbreaks or local expansion of clonal ESBL-producing isolates. In **paper 1**, we have not performed molecular strain typing. However, the previous nationwide Norwegian ESBL study in 2003, has revealed a rather heterogeneous population structure [(353)]. According to the multilocus sequence typing, 19 different sequence types were identified among 45 isolates. In **paper 1,** we have observed heterogeneous susceptibility patterns among the isolates. This could also serve as the argument that a diverse clonal population structure is likely.

In **paper 2** for the PK/PD modelling, we chose the archetypal uropathogenic strain (NU14) [(334)], previously used in numerous UTI models [(99, 335-338)]. This avoided any influence of the genetic background on the PK/PD calculations, as fosfomycin resistance by chromosomal mutations is known to affect the strain fitness [(187, 198, 202, 205, 215)]. For the treatment studies, isogenic derivatives with defined genotypes and variable fosfomycin MIC were selected [(215)]. This avoided any influence of the genetic background on the PK calculations. For the treatment studies in **paper 2** and **paper 3,** well-characterized clinical isolates were selected to represent contemporary isolates harboring the clinically most relevant β-lactamases. These isolates were also selected to represent a diversity of fosfomycin and mecillinam MICs.

5.2 Susceptibility testing methods

For all studies, the MIC values were determined as this is considered predictive of antimicrobial efficacy in relation to PK/PD parameters (AUC/MIC, $T_{>MIC}$, or C_{max}/MIC) [175, 340].

In **all papers**, susceptibility testing was performed following the EUCAST/CLSI methodology and manufacturer instructions. The choice of the particular method was made by balancing the optimal reliability of results with the complexity and speed of the test procedure. For the smaller group of isolates, the "gold-standard" procedures such as broth microdilution (BMD) were preferred. When testing a large number of isolates for a significant number of antimicrobials, the choice of methods was shifted towards less labor-demanding techniques, which have an acceptable agreement with the reference methodology [354, 355] and are commonly used in the clinical laboratories [356]. There are, however, some exceptions for specific organism/drug combinations [355], which are discussed below.

Susceptibility testing to fosfomycin is challenging [357], as fosfomycin has a high frequency of mutational resistance [215]. According to EUCAST guidelines [208], one has to ignore isolated colonies within the inhibition zone when using diffusion-based tests for fosfomycin. Spontaneous mutations could potentially lead to overestimation of resistance due to the higher inoculum and/or regrowth of the single emerging mutant, especially in broth [357]. Fosfomycin susceptibility testing is generally considered as error-prone [358]. Discordances are reported for multiple susceptibility testing methods, including gradient tests [358]. For the strains used in **paper 2** and some selected strains from **paper 1**, we compared the results of susceptibility testing with multiple methods (BMD, gradient test strips, and agar dilution). Agar dilution, followed by the gradient test strips produced results with minimal variation and maximal agreement to the results of time-kill studies (data not shown). This is in accordance with other studies involving susceptibility testing [206, 207, 359]. A commonly accepted approach to produce more clinically relevant results is to supplement the media with 25µg/ml glucose-6-phosphate [308, 360]. Glucose-6-phosphate causes activation of UhpT, which is one out of two transporting mechanisms of fosfomycin into the bacterial cell [211, 308]. Susceptibility testing with the addition of glucose-6-phosphate usually results in lower MIC values and is generally accepted to produce more clinically relevant results, as glucose-6-phosphate is found in abundance in human tissues [308, 360]. All susceptibility testing for fosfomycin (in **papers 1**, **2,** and **3**) along with the time-kill studies in **paper 2** was performed with the addition of 25µg/ml glucose-6-

phosphate. For fosfomycin, agar dilution is considered as the gold-standard method by both EUCAST [208] and CLSI [209]. Agar dilution was applied to the smaller number of isolates, used in **paper 2**.

Mecillinam has been associated with errors when tested for susceptibility with BMD [209, 244, 262, 361]. This is likely to its mechanism of action: mecillinam binds to PBP 2, which leads to the formation of spherical-shaped cells [233]. This increases density and can be misinterpreted as growth. Diffusion techniques are preferred for mecillinam susceptibility testing because the results of dilution-based tests are known to be inoculum-dependent [244, 361]. In **paper 1** and **paper 3**, gradient test strips were used to determine the susceptibility to mecillinam.

Temocillin powder/stock solution was not easily available commercially at the time of the study (**paper 1**), limiting our options to diffusion methods. Several studies have also shown good agreement between gradient tests and BMD [362, 363]. We, therefore, decided not to repeat the tests with a different technique.

For VITEK-2, differences in S-I-R categorization between VITEK2 and disc diffusion was observed for some antimicrobials. For instance, in **paper 1**, 6% of ESBL-confirmed strains were susceptible to cefotaxime and 2% to cefpodoxime. Half of these isolates were also susceptible to ceftazidime. However, all of these strains had reduced susceptibility to cefotaxime and/or ceftazidime according to disc diffusion. In 2010, the piperacillin-tazobactam card for VITEK2 was recalled due to incorrect performance. The test could overestimate the susceptibility, potentially resulting in treatment failure, especially with CTX-M producing strains [364].

There are some general limitations for MIC determination as a method for determining the clinical efficacy of when facing a relatively novel resistance determinant. The MIC is inoculum dependent, is measured at a fixed time point, relates not to a certain drug concentration but to a certain range within 2-fold steps (which is often measured by the naked eye and potentially subject to subjective errors). It is impossible to identify definite mechanisms of resistance based exclusively on the results of MIC. Another major limitation is that MIC measured *in vitro* might not correlate exactly to the therapeutic outcome, especially for isolates with relatively novel resistance determinants. MIC is often mistakenly interpreted as "*no bacterial killing occurs below a certain threshold concentration*". In reality, MIC is a net effect of bacterial killing by antimicrobial versus bacterial growth over time, evaluated at a certain time-point (16-20 hours). It is thus a quite relative, mono-dimensional estimation of antimicrobial effect, not taking into account the changes in growth

rates and the degree of susceptibility throughout the test period [161]. The complexity of factors such as strain differences in growth and kill rates, environmental conditions, different dosing regimens are not controlled for with standard susceptibility testing. This would result in a discrepancy between *in vivo* efficacy and MIC *in vitro.* Changes in mecillinam MIC, measured in urine and standard media containing little cysteine [260], could be a relevant example. This underlines the need for correlating the results of susceptibility testing (**paper 1**) with data from animal models (**paper 2** and **paper 3**) and clinical studies. Still, MIC in a clinical setting, it is a convenient metric, routinely used to guide treatment decisions [365].

5.3 Molecular characterization of isolates

Susceptibility testing detects the resistance phenotype; however, it provides limited information on what are the causes of the resistance. Molecular methods are generally used to identify the genes causing the resistant phenotype. Nucleic acid-based detection systems have high sensitivity to detect the presence of resistance genes and give rapid results. This information is useful from both clinical and epidemiological point of view.

A major limitation of PCR is that we need information about the target sequence before generating the primers to allow for successful selective amplification. In **paper 1**, $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} PCRs were performed on all phenotypic ESBL-positive isolates, according to Tofteland *et al.* [341]. In **paper 2**, real-time PCR was used to confirm β-lactamase presence in *E. coli* isolated from mice in post-treatment studies.

Microarray, based on DNA hybridization, allows testing for multiple gene-combinations simultaneously. In **paper 1** and **paper 2** microarray method was implemented. The microarray screening results for **paper 2** were confirmed/supplemented with the whole genome sequencing (WGS). DNA sequencing is the process of determining the order of nucleotides in DNA. WGS determines the order of nucleotides in the whole genome. Compared to the PCR, this technology does not imply prior knowledge of the target genes. However, this method is costly (for now) and thus unsuitable for testing large strain collections. In **paper 1,** BigDye 3.1 technology (Life Technologies, Carlsbad, CA, USA) was used to sequence the bla_{SHV} and bla_{TEM} PCR products in order to confirm ESBL-variants**. In papers 2** and **3,** all isolates underwent WGS (Illumina, San-Diego, CA, USA) and further analysis of the sequences at the Centre for Genomic Epidemiology (https:/cge.cbs.dtu.dk/services/CGEpipeline-1.1) in order to extensively characterize the isolates.

5.4 *In vitro* time-kill studies

Currently, time-kill assays require extensive labour input and are seldom used to guide chemotherapy in the individual patient, but are excellent tools for scientific purposes. Both concentration-dependent and time-dependent bactericidal activities of antimicrobial agents can be studied using time-kill curves. Time-kill serves as a valuable aid, but it does not substitute the PK/PD studies with dynamic drug exposure. Despite the flexibility and robustness, the complex *in vivo* situation may not be fully represented. Time-kill studies do not allow for a true PK/PD index determination as all three of the PK/PD indices will increase simultaneously and in the same proportion (i.e., interdependent) [161]. According to the patterns of the time-kill plot, certain predictions on the drug mode of action could be made (faster and concentration-dependent decline in CFU versus the same effect irrespective of the increase in concentrations). A dose fractionation study (*in vitro* or *in vivo*) is needed to isolate single PK/PD indices. A modified time-kill study aimed at isolating the indices involving washing out the antimicrobial has been reported [366] as well as one compartment *in vitro* models [367]; however, we have chosen not to perform such due to the complexity.

Another possibility to mimic the PK *in vitro* could be to imply the hollow fiber model. In this model, bacteria are contained in a compartment between fiber microtubes with pore size chosen to trap the organisms inside while allowing to change the drug concentration [367]. We have chosen to perform *in vivo* models to approach the existing knowledge gaps, as a hollow fiber studies with fosfomycin do not take into account the host factors such as biochemical contents of host environment, bacteria wash-out with urine flow, the role of immune system and the *in vivo* fitness of the resistant subpopulations [215, 216]. In **paper 2**, we used *in vitro* time-kill studies to determine the effect of different fosfomycin concentrations on target bacteria under controlled conditions.

Due to the high MICs of fosfomycin resistant isolates in the study, we modified the originally reported time-kill protocol [342-344]. The modification did not affect the final fosfomycin concentrations or bacterial density in the test tubes. Both the original protocol and the modified protocol were experimentally tested on the same fosfomycin-sensitive isolates and have produced similar time-kill curves.

5.5 *In vivo* modelling – murine urinary tract infection model

Although animal models are one step closer to the clinical setting, one can always question the relevance of the results of a particular animal model. The results of a modelling performed on other species do not necessarily extrapolate well to the processes in the human body. The choice of the model must accurate, and results should be interpreted with caution. We have focused only on female mice in the studies, as the model is well established [92, 253, 345, 368, 369] and because the incidence of UTIs in women is higher [89]. Despite many similarities discussed in the introduction, several differences from the human UTI progression for our model can be noted. The induction of a UTI in mice is performed by introducing a large number of bacteria directly into the bladder, which is usually not the way of natural infection ascension from the urethra to the bladder. Moreover, to ensure colonization, a high inoculum of $\geq 10^7$ CFU is used. It is likely to exceed those in the real setting. It is important to note, however, that in infectious disease models, the PD target is not located in the host animal, but is associated with the bacteria that produce infection. The PK/PD data obtained in animal models generally correlates with clinical outcomes [370]. The murine model has been successfully used to study the therapeutic effects of antimicrobials [319, 342, 346, 368].

Despite the mentioned challenges, we believe that the murine UTI model is a useful tool to investigate the UTI pathogenesis and validate the efficacy and safety of antimicrobial treatments [347, 371]. The animal PK/PD data is known to be remarkably consistent, despite the significant variety of techniques and models used [174].

5.5.1 PK studies

The relationship of studied dosing regimens in mice to human PK is discussed in paragraph 5.7. A general limitation of animal models is the difference in PK, as shorter half-lives are observed in small animals such as mice [372]. Common attempts to humanize the PK data include a more frequent dosing or using agents such as uranyl nitrate (decreases glomerular filtration rate and produces tubular necrosis [373]) or probenecid (blocks the tubular secretion of penicillins and cephalosporins) to alter the kidney function [351, 373]. For most antimicrobials, urinary drug concentrations are the most relevant predictor for concentrations in the bladder lumen and, perhaps, inside the renal calyx and renal pelvis. At the same time, serum/plasma PK correlates with the drug concentrations in the bladder wall and kidney tissues [374]. In **paper 2,** both urine and serum antimicrobial concentrations were important.

Therefore, we chose not to alter the kidney function, but to use different dosing and frequency to produce the variation in the PK/PD indices at different sites of infection.

However, a **classical dose-fractionation scheme** with the same total dose, fractioned at different time intervals, appeared to be insufficient in our case. As the rate of elimination for fosfomycin was high, significant drug concentrations were observed in urine even for the middle-range doses (e.g., 7.5 mg/mouse). Therefore, if we kept the total dose constant, several challenges could be met:

- A high total dose would produce high concentrations in urine and, possibly, accumulation of fosfomycin in serum;
- A total daily dose in the middle range would achieve the variation in C_{max}, but $T_{>MIC}$ and AUC/MIC would likely be high for most of the doses used (in addition to a high chance of drug accumulation if the dosing intervals are too short);
- A total low dose would achieve good variation in $T_{>MIC}$, but minimal variation in C_{max}. The fosfomycin MIC of the test strain (1 mg/L) was much lower compared to the concentrations achieved by the doses used in our study.

Moreover, we decided against including strains with different fosfomycin MIC in the PK study, due to possible influence of varying growth rates of fosfomycin resistant mutants [(215)], and due to difficulties in finding clinical strains with elevated fosfomycin MIC (but below the resistance threshold) in our collections. As the initial time-kill studies showed that fosfomycin is expected to act concentration-dependent, we would most likely risk either to not observe the change in efficacy for different doses or to be unable to produce sufficient variation in both urine/plasma concentrations and the PK/PD indices by keeping the total daily dose constant.

We had, therefore, to balance between producing maximal variation and keeping the total daily dose constant. Therefore, we used the dosing schemes that mimicked different aspects of human PK (discussed in paragraph 5.1). We also aimed to achieve maximal variation (and minimal co-variation) in all the indices, with a special focus on C_{max}.

Another limitation of our methods is the use of bioassay as a proxy for the fosfomycin concentration. LCMS-MS is most likely to offer better precision; however, bioassay, as such, is the original standard method against which all modern methods are compared. We chose to use the bioassay method because LCMS-MS was unavailable at the institution at the start of the project, and since initial testing showed, that fosfomycin concentrations could be

validated both in serum and urine as compared with spiked samples with less than 10% variation.

5.5.2 PK/PD studies, treatment studies, and the efficacy breakpoint

Another limitation is the choice of efficacy endpoint (PD endpoint). Ethical considerations restrict using large numbers of mice, frequent sampling, and observation for an extended time. The PD endpoint, commonly used for the efficacy assessment in the early studies with antimicrobials, was "animal survival" [174, 375, 376]. Today, using CFU-counts in the infected tissues or fluids is more common [161]. We used CFU-counts in our efficacy studies with the isogenic isolates in **paper 2** and clinical isolates in **paper 2** and **paper 3.** However, the robustness of this approach for the PK/PD analysis in association with the regression model (**paper 2**) was limited due to:

1) The high proportion of spontaneous "effect" observed in the control group, making it challenging to statistically differentiate the observed effect of treatment from its absence. This is possibly caused by the additive effect of the immune system or unsuccessful infection induction in the control group (Figure 9).

2) A high total proportion of the values below the limit of detection (LOD) in the treatment group, which was, however, still much higher than in the control group (Figure 9). A significant effect of all doses on bacterial CFUs (presumably due to high renal clearance, and thus, high concentrations of fosfomycin observed in urine even for low doses) could explain this.

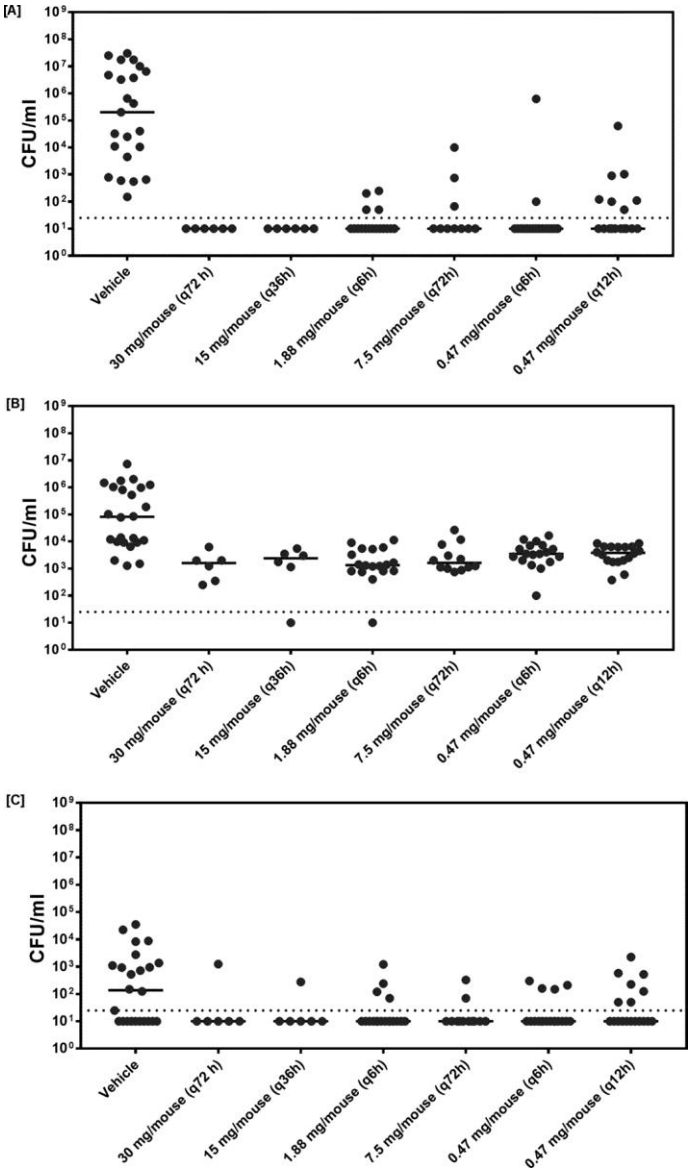

Figure 9 Effect of six fosfomycin dosing regimens in murine UTI model. The CFU/ml counts for each animal in urine (A), homogenized urine bladder (B), and homogenized kidney (C) are depicted as black dots. *E. coli* NU14 with fosfomycin MIC of 1mg/L is used as a test strain. Note the substantial proportion of CFU below the limit of detection (LOD) for urine (for treated mice) and kidneys (both for treated groups and vehicle). Reprinted with permission from Antimicrobial Agents and Chemotherapy (Zykov *et al.,* 2018) [(377)].

The substantial amount of observations clustering at the LOD forms "the system". If we perform the regression analysis using the ordinary least squares algorithm, the curve is going to be fitted through the points which produce the least deviation the predicted and observed values (most below LOD). All CFUs higher then this point will then be treated by the algorithm as "outliers", and thus not explained by the model. This would result in the same shape of the graph for all indices (where all the different doses fall onto the "plateau" part of the sigmoid, giving a false insight that changing the dose does not make sense) and low goodness-of-fit.

To account for both spontaneous treatment success proportion in the control group and overall high efficacy of all doses, we chose to set the PD endpoint to "treatment success or microbiological cure", in our case - the percent of CFU reduction below the LOD. A similar PD endpoint is often used in human studies [378-380]. A limitation of this approach is that the information on the magnitude of CFU-reduction is inevitably lost. For bladders, this approach was even more complicated as we could never observe 100% cleared bladders in this model (Figure 9). This is a well-known limitation of the murine UTI model [345], probably due to the intracellular bacterial communities or the high initial inoculum in the bladder when inducing the infection. We could exclude bladders from PK/PD analysis (since we see no data for CFU counts below LOD) but decided to try using the proportion of CFU counts below the minimum counts observed in the control group. The results were correlated with the findings at the other sites.

The **non-sterile bladder** is also a limitation generally observed in our model. The fact that bladder counts are difficult to change with antimicrobial therapy is well known from previous studies [345]. The most likely reason is the intracellular bacterial reservoir. *E. coli* is taken up by bladder epithelial cells, where the bacteria proliferate intracellularly until the cells apoptose and release bacteria into the bladder lumen and re-infect the bladder epithelium. The bacteria grow in a sort of biological biofilm, which is tolerant towards most antimicrobials [253, 374]. The discrepancy between the CFU counts in the urine and the homogenized bladders, as well as the microscopy results from other studies [368], may be a good reflection of this phenomenon.

Spontaneous treatments/no infection induction (especially for kidney CFU-counts) is also a well-known limitation of this model. Lower p-values for efficacy in kidneys are observed, to our knowledge, for all antimicrobials tested, even those aimed at treating pyelonephritis [253, 342, 345, 368, 374]. We believe this phenomenon is worth further studying, for example, to determine the influence of prolonged treatment regimens on bladder CFU counts.

5.5.3 Statistical analysis for PK, PK/PD and treatment studies

The determination of the relationship between the PK/PD index and effect (PD endpoint) was performed mathematically using the E_{max} model or Hill equation [351]. The goodness of fit is typically assessed as the R^2 coefficient. PD endpoints can vary from net stasis, x-log kill, or as % of clinical or microbiological cure [351, 378-380].

In **paper 2**, we used plasma PK data to calculate indices, as the concentration of fosfomycin in urine may be subject to more variation (however, the urine PK levels still depends on plasma PK), and the plasma PK is a more accurate proxy for drug concentration and, thus, treatment activity in kidney and bladder tissues [374]. The calculation of PK/PD indices was performed by the trapezoidal rule for the doses where actual experimental data was available (15-240 min after s.c. dose) and by inter- and extrapolation of the PK data using the exponential equation. Since the highest drug concentrations in plasma were observed already at the first measured time point (15 minutes after injection), a concentration peak could have occurred in the period between 0-15 min. To address this, we performed PK/PD analysis of both the actual peaks observed experimentally along with extrapolation-based "$T_{>MIC}$ (h) after the first dose". The second parameter is logically connected with C_{max} (i.e., the longer drug concentrations persist, the higher starting concentration should have been observed). Both approaches yielded similar results. Since the number of doses administered differed (both time between administrations and the total number of administrations), "$T_{>MIC}$ (h) after the first dose" did not correlate with total fraction of "$T_{>MIC}$ (%) in the 72h period".

In paper 3, the mecillinam dose calculations were performed by inter- and extrapolation of the data from previous PK studies in mice [250, 253] and were adjusted to match the concentrations of mecillinam in urine observed in humans [250, 253]. Doses were calculated based on AUC in urine, which for a 400 mg dose in humans reaches approximately a mean of 900 mg/L/h, reflecting a dose of 50 mg/kg in mice [369].

For the treatment studies in **paper 2** and **paper 3**, the relatively high p-values when comparing separate (non-pooled) mice groups (Mann-Whitney test/Fisher's exact test) are observed. This is due to a rather low number of mice in the groups and the abovementioned problems with **non-infected mice and non-sterile bladders.**

A **non-infected mice** problem could especially affect the statistical test-performance for the high doses of antimicrobials when a high eradication rate in the treatment group is compared to the control group. This can lead to underestimating the effect of the antimicrobial and could be theoretically corrected if we were able to find a reliable early predictor for infection development. Unfortunately, CFU counts for the control group on day 1 after inoculation did not correlate well with the infection development on day 3 (e.g., mice with negative counts on day 1 could show infection on day 3). As we could not separate non-infected mice from spontaneous treatment effects, CFU-counts on day 1 could not be used as the "exclusion" parameter.

The **non-sterile bladders** problem caused a smaller difference in medians and, combined with the relatively small animal groups, resulted in higher p-values. This affected the results of the treatment studies in **paper 2** and **paper 3.**

The effect of both abovementioned phenomena could be especially problematic when using the Mann-Whitney test. We have, therefore, supplemented the Mann-Whitney test with Fisher's exact test by converting to binomial data, using the thresholds discussed above (the choice of PD endpoint in paragraph 5.6.2). In **paper 3**, we have also used the pooled data in order to increase the sample size, which produced good results. We have not used the data for the virulence studies to supplement the control group as we considered it as a separate study. The use of such data would reduce experimental purity. Firstly, no vehicle was used. Secondly, despite the technical experiment parameters were alike (e.g., mice breed, inoculum size, sampling technique, and sample processing), the experiments were not performed at the same time. We have chosen a one-sided test since it would be natural to assume that administering the antimicrobial agent would not increase CFU-counts. In the pessimistic scenario, when bacteria are resistant, it would result in the same CFU-counts compared to the vehicle group.

5.5.4 Ethical considerations

In addition to obtaining approval for the animal experiments, we adhered to the basic principles for the ethical use of laboratory animals formulated by Russel [(381)] in the form of 3 "Rs": Replacement, Reduction, and Refinement.

Replacement is defined as the methods aimed to avoid animal use by replacing animal models by other methods. Replacement methods could be classified into absolute (e.g., *in silico* and *in vitro* modelling or human volunteers) or relative (e.g., using animals with less developed central nervous system, rodents vs. larger mammals and primates or invertebrates vs. vertebrates). We have chosen to perform the time-kill studies prior to the animal models as a replacement. However, to study the fosfomycin pharmacokinetics, we have not implemented the hollow fiber models, because they do not take into account factors such as the immune system and environmental differences. Moreover, hollow fiber models implemented in previous studies have shown the emergence of resistance [(202)] similarly to time-kill studies, which may not occur *in vivo*. In the *in vivo* studies, mice were chosen as the lowest possible taxa, as the use of animals with urinary tract anatomy much different from humans would compromise the interpretation. Additionally, we tried to gain as much information about the

drug as possible from *in vitro* experiments before proceeding to animal studies. For instance, we did not extend the available set of doses to further increase $T_{>MIC}$ (maximal value of $T_{>MIC}$ was 42% in our study), as the increase in efficacy correlated with concentration-dependent indices according to the time-kill studies and PK/PD analysis. Low or middle-range $T_{>MIC}$ but high C_{max} or AUC/MIC was already associated with maximal efficacy, logically eliminating the need to further experiment with increasing $T_{>MIC}$.

Reduction refers to the use of methods, obtaining comparable levels of information from fewer animals (e.g., improvements in the experimental design, using modern research methods, using shared or open-access data). Prior to animal studies, the isolates were checked to express type-1 fimbriae to reduce the unnecessary use of non-virulent strains. *In vivo* virulence studies (using fewer mice) preceded the treatment studies (using more mice due to both control and treatment group) in order to exclude strains that fail to induce sufficient infection to other reasons than type-1 fimbriae. Additionally, in order to reduce the number of *in vivo* virulence studies, we often re-used the strains already proven effective from other studies with the same model (NU14 and derivatives in **paper 2**, 24623884-114 and 21773360-98 in **paper 3**). Moreover, K5-08, K4-40, K71-77, and 50639799 were included in both **paper 2** and **paper 3**, so that *in vivo* virulence testing was needed to be performed only once. In **paper 2** and **paper 3,** we had to sacrifice a high level of statistical significance by using the smallest possible groups of mice.

Refinement refers to improvements concerning animal handling and keeping conditions. It is achieved by implementing procedures, which minimize distress, pain, suffering, or long-lasting damage and improve animal well-being (e.g., a better environment and keeping conditions, pain management and anesthesia, minimum-invasive methods). In **paper 2** and **paper 3**, inoculations were performed on anesthetized mice. Mice had an unlimited supply of food and drinking water and were checked three times daily for any observable signs of suffering. If mice showed any signs of severe illness or suffering, the animal was immediately euthanized. According to the EU directive from 2010, "the methods selected should avoid, as far as possible, death as an end-point due to the severe suffering experienced during the period before death" [(382)]. In our UTI model, the mice were sacrificed the next day after treatment cessation, and changes in CFU counts were used as the PD endpoint.

5.6 Fosfomycin

What is the MIC distribution of fosfomycin in contemporary isolates? In 2010 and 2011, fosfomycin MIC values have not been routinely tested as a part of the NORM surveillance program. In **paper 1**, we showed that fosfomycin was active against all 105 ESBL-producing isolates. The MIC50 and MIC90 among these isolates were both 2µg/L, below the susceptible breakpoint and the ECOFF. Moreover, carbapenemase-producing strains from **paper 2** had a MIC of 2 mg/L to fosfomycin. In both **paper 1** and **paper 2**, none of the isolates had MIC above the ECOFF, except for P14-63, which harbored *fosA* in addition to $bla_{\text{CTX-M-3}}$ and $bla_{\text{TEM-1B}}$.

What are the PK profiles for various doses? In order to carry out the PK/PD studies (**paper 2**), we have determined fosfomycin PK in mice. The PK in smaller rodents, such as mice, generally differs from humans [(351)]. Mice metabolize drugs faster and have a shorter elimination time, resulting in lower $T_{1/2}$. Since the murine and human PK differ, it was impossible to mimic all parameters in one dose, especially taking into account that both plasma and urine PK are assessed. Mazzei *et al*. [(178)] have reported high fosfomycin urinary concentrations (1000-4000 mg/L) to be achieved and sustained at ≥100 mg/L for at least 30-48h. This served as the PK basis for choosing the 3g single-dose oral regimen. All doses used in the PK studies, performed in **paper 2,** resulted in plasma peak concentrations of 36, 280, and 750 mg/L for the 0.75, 7.5, and 30 mg/mouse, respectively. The protein binding of fosfomycin was negligible in humans and various animal species [(383)]. In our study, we assume protein binding to be 0%, as done in similar studies [(177)]. The mean elimination half-life was shorter than in humans: 28 min against 2.7 hours (3g intravenous dose) [(384)]. In urine, peak fosfomycin concentrations of 1100, 33400, and 70000 mg/L were observed**.** In humans, a peak concentration of 6353 mg/L was observed after given the 3g intravenous dose [(384)]. In the PK/PD part of the **paper 2,** we have the extended set of doses, which appeared correspond to the standard human fosfomycin-trometamol 3g single dose in different ways (for instance, 0.47 mg q6h would produce comparable plasma AUC; 0.47 mg q12h would have comparable plasma C_{max}; 1.88 mg q6h would result in comparable urine C_{max} according to the serum or urine C_{max}, maximal plasma $T_{>MIC}$ (42%) could be achieved with 1.88 g q6h) [(178, 185, 384)]. Due to the rapid drug clearance, there was no need to account for drug accumulation. A dose of 15 mg q36h resulted in relatively lower serum PK parameters but produced sustained high concentrations of fosfomycin in urine (>18h). Moreover, this dose is based on a surface area of mice of ≈70 cm^2 as related to a 17200 cm^2 surface area of a 70 kg human person. The

calculation results in a human dose of 3.6 g, which is close to the standard dose of 3g fosfomycin (Frimodt-Møller, personal communication).

What are the exposure-response relationship and PD target? As discussed in the introduction, the optimal index for fosfomycin remains a matter of debate. According to the time-kill studies in **paper 2**, fosfomycin acts concentration-dependent, with rapid initial killing at concentrations exceeding 1×MIC, and high concentrations (32-64×MIC) preventing regrowth after 24h. As the time-kill patterns did not substantially differ between the genetically diverse isolates tested, the concentration threshold of 64 mg/L (32-64×MIC) would potentially cover all *E. coli* isolates in the NORM-ESBL collection in **paper 1** and the isolates in used **paper 3,** according to their reported MICs.

To confirm the *in vitro* time-kill results, we performed *in vivo* PK/PD studies in the murine UTI model (**paper 2**). After investigating the PK/PD relationships, we found that C_{max} is a good predictor for drug efficacy despite the very low $T_{>MIC}$ values. Since we have already observed the maximal effect with low $T_{>MIC}$ and high C_{max}, we decided it sufficient and have not extended the dosage. As fosfomycin is known for a high proportion of resistance development, it would be interesting to study the impact of resistance inhibiting concentration (RIC), which might be a better predictor, as shown by Van-Scoy *et al.* [(203)], and elucidate the possible role of $T_{>RIC}$ *in vivo*. This, however, requires rigorous investigation and is better performed as a separate study.

A dose of 15mg q36h was further tested against clinical isolates and NU14 derivatives with decreasing fosfomycin susceptibility (chromosomal mutations) in **paper 2**. The applied regimen resulted in a significant CFU decrease for most susceptible isolates at all infection sites. For the isolates with decreased fosfomycin susceptibility, the decrease in efficacy was relative to strain MIC. However, isolates with fosfomycin resistance due to the chromosomal mutations were shown to have decreased growth rates, potentially compromising the effective infection establishment [(215)]. The CFU counts for the isolate harboring *fosA* were not significantly reduced at any site. We, along with other authors [(385)], have observed the significant reduction of bacterial loads in kidneys in a murine model. However, we have not observed the same significant effect on kidney CFU-counts for fosfomycin resistant isolates reported by Pourbaix *et al.* [(385)]. Moreover, some experts place fosfomycin among the drugs, reserved for treating carbapenemase producers (e.g., polymyxins and tigecycline) [(145)]. Further studies, including clinical trials, are necessary to elucidate the role of fosfomycin in treating UTI, pyelonephritis, and other infections [(145)].

Overall, **paper 2** shows fosfomycin to be an effective treatment option for treating the upper UTIs caused by susceptible isolates, irrespective of the ESBL, pAmpC, or carbapenemase production. The epidemiological data provided in **paper 1** (Nationwide ESBL-collection, 2010-2011), study by Samuelsen *et al.* [245] (nationwide collection of all carbapenemase-producers in Norway 2007-2014), along with the MICs for the isolates reported in **paper 2** suggests fosfomycin to be an effective empirical option when targeting lower UTIs, caused by ESBL-producing *E. coli*.

5.7 Mecillinam

What is the MIC distribution of interest to mecillinam in contemporary isolates? High mecillinam use is traditional in Scandinavian countries [228, 229]. Despite the widespread use, a low prevalence of resistance is routinely reported as a part of the NORM program [386, 387]: 4.4%, 6%, and 4.4% (in 2010, 2017 and 2018, respectively). In **paper 1**, we have shown mecillinam to be active against a major proportion (94%) of 105 ESBL-producing isolates. However, 32% of the *E. coli* isolates had mecillinam MIC > ECOFF, indicating a decreased susceptibility among the ESBL population. This observation is expected as mecillinam despite some degree of stability, is liable to β-lactamases [261, 262, 264, 265]. Still, despite the relatively higher MICs among the β-lactamase producers, the proportions of isolates *in vitro* sensitive to mecillinam remain high. The majority of isolates with elevated mecillinam MIC (28 of 34) carried a variant of bla_{TEM}. The possible role of TEM hyperproduction has to be evaluated, as it has been reported as one of the causes of mecillinam resistance [264]. As resistance to mecillinam might additionally involve mutations in multiple chromosomal genes [232, 256-258], we cannot draw a firm conclusion on what is the definitive cause of the MIC shift. Susceptibility testing in media supplemented with cysteine could potentially provide additional information [256, 260] and could be the direction of further studies. Interestingly, mecillinam resistance was also associated with amoxicillin-clavulanic acid resistance, which could also suggest further directions in research. In our ESBL collection in **paper 1,** the mecillinam MIC_{50} lies on the border MIC of the "wild-type" (1 mg/L), while MIC90 is 4 mg/L. An interesting observations is that the proportion of resistance to mecillinam, was significantly higher in strains from urine (n=24, 17% ressitant) compared to the strains from bloodstream (n=81, 3% resistant) isolates. This could suggest some degree of selective pressure and previous exposure, as mecillinam is frequently used in the treatment of uncomplicated UTIs in Norway. Moreover, isolates with mecillinam MIC>ECOFF were more

resistant to nitrofurantoin (also UTI-only drug), trimethoprim-sulfamethoxazole, amoxicillin-clavulanic acid, and gentamicin.

All isolates used in **paper 3** (including K71-77, NDM-1 producer) were susceptible to mecillinam, except for 21773360-98 (TEM-1B producer) and 50639799 (VIM-29 producer). Interestingly, some clinically important carbapenemase-producers (such as NDM-1 and OXA-48) are *in vitro* susceptible to mecillinam [244, 245, 388]. For Norway, all OXA-48-like-producing and 83% of NDM-producing *E. coli* isolates in 2007-2014 were *in vitro* susceptible to mecillinam [245].

What are the exposure-response relationship and PD target? The data on optimal PK/PD index for mecillinam is scarce, but one study suggests $T_{>MIC}$ to be the optimal index [253]. The data on optimal dosing regimen remains inconclusive: some studies find low doses of 200 mg BID for 7 days equally efficient to higher doses [254], other authors tend to support higher doses with shorter duration (e.g., 400 mg BID for 3 days or 400 mg TID for 3 days) to reduce emergence of resistance [255]. Moreover, it remains an open question of whether (and in which dose) mecillinam is a suitable *in vivo* option for treating ESBL-producers. Søraas *et al.* [240], reported clinical failure rates of 44% and 14% at 200 mg TID, when treating community-acquired UTI for ESBL- and non-ESBL-producing *E. coli*, respectively. However, they were not able to distinguish between upper and lower UTIs. In contrast, Jansåker *et al.* [243] reported an 80% bacteriological cure for lower UTIs caused by ESBL-producing *E. coli* and *K. pneumoniae*, treated with 400 mg pivmecillinam TID. Similar results (good clinical response (100%) and a low proportion of bacteriological cure (25%)) were reported by Titelman *et al.* for lower UTIs [242]. To address the current uncertainty of whether mecillinam is suitable for the treatment of UTIs caused by ESBL-producing isolates [240, 243], we performed an *in vivo* comparison of two mimicked standard mecillinam doses (200 mg and 400 mg) in **paper 3**. The following doses were applied to both susceptible wild-type *E. coli* strains, ESBL-, pAmpC- and certain carbapenemase-producers, as well as against resistant isolates. Our results show that both mecillinam dosing regimens significantly decreased the urine CFU/ml for both carbapenemase-producing isolates, and even for isolates resistant according to the current clinical breakpoint (MIC range 0.25-64 mg/L). A variable degree of reduction was observed for the bladder and kidneys. We could not identify which dose is more suitable as both doses resulted in a significant eradication. A "400 mg" dose seemed to have a slightly higher proportion of CFU-reduction, but the difference was insufficient to make firm conclusions. Analysis based on pulled data from all six strains could not identify any

significant differences in effect between the doses at any infection site. Thus, we could not identify a superior dosing regimen. However, both doses produced statistically significant effects for urine, kidney, and bladder, compared to placebo. In line with our results, a recent literature review by Jansåker *et al.* [(230)], suggests mecillinam as a treatment alternative for uncomplicated pyelonephritis. According to the results of **paper 3**, we could not identify the superior dosing regimen, despite minor differences in the efficacy in kidneys. According to the literature review, 400 mg-based dosing regimens are more effective for treating pyelonephritis, and UTI caused ESBL-producers [(230)]. However, Bollestad et al. [(389)], in a prospective study comparing both 200 mg TID and 400 mg TID pivmecillinam doses against ESBL-producer and non-ESBL controls. As in **paper 1**, the resistance to mecillinam among ESBL-producers was low, but MICs were higher compared to non-ESBLS. The 200 mg doses were associated with an increased odds ratio for treatment failure when applied against ESBL-producer, according to Bollestad *et al.* [(389)]. In contrast, 400 mg dose had similar in effect both ESBL- and non-ESBL-producers [(389)]. Overall, the authors conclude that 400 mg TID for >5 days is a reasonable treatment option for ESBL-producing *E. coli* causing community-acquired lower UTI.

Despite a long history of widespread use in the Scandinavian countries, mecillinam remains a suitable option for treating UTIs, even for the cases when ESBL-producers are suspected. Its use against certain carbapenemase-producers (OXA-48 and NDM-1) is promising but requires further studies. Further studies aimed at determining the optimal dosing regimen are also needed.

5.8 Nitrofurantoin

What is the MIC distribution of interest to nitrofurantoin in contemporary isolates? At the beginning of the 2000s, nitrofurantoin was reintroduced into clinical guidelines [(267)]. Its MIC values for the general *E. coli* population causing UTIs are also routinely tested as a part of the NORM surveillance program [(386, 387)]. As with mecillinam, a low prevalence of resistance is constantly reported among urinary *E. coli* isolates: 2.3%, 1.4%, and 1% (in 2010, 2017 and 2018, respectively). In **paper 1,** we reported 9% nitrofurantoin resistance among the nationwide collection of ESBL-producing *E. coli,* with MIC_{50} and MIC_{90} being within the susceptibility range. All isolates used in the **paper 2** and **paper 3** were *in vitro* susceptible to nitrofurantoin. According to the meta analyses, nitrofurantoin's clinical efficacy for treating

lower UTIs is high and comparable to that of other antimicrobials, including for ESBL-producing isolates [267, 269].

5.9 Temocillin

Today, temocillin approved for marketing in a limited number of countries [297]. The narrow-spectrum of activity, along with its resistance to hydrolysis by β-lactamases, are now considered important ecological and bacteriological advantages of the drug [297]. What is the MIC distribution of interest to temocillin? In **paper 1,** all isolates were susceptible to temocillin according to the UTI breakpoint defined by BSAC [323]. However, according to the BSAC breakpoint for systemic infections [323], 29% of the isolates were resistant. Temocillin resistance was statistically associated with resistance to amoxicillin-clavulanic acid, aztreonam, ceftazidime, gentamicin, tobramycin, and cefoxitin. However, further phenotypic testing of the isolates has suggested that AmpC hyperproduction might not be involved in elevating temocillin MICs. Most of the isolates used in **paper 2** and **paper 3** were *in vitro* sensitive to temocillin using UTI breakpoint. However, K26-07 (*bla*$_{\text{CMY-2}}$; MIC 16 mg/L) and K71-77 (*bla*$_{\text{NDM-1}}$, *bla*$_{\text{CMY-6}}$, *bla*$_{\text{OXA-1;}}$ MIC 32 mg/L) were resistant according to the systemic breakpoint, while 50639699 (*bla*$_{\text{VIM-29}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CMY-4}}$, *bla*$_{\text{OXA-1}}$*;* MIC 256 mg/L*)* was resistant according to both systemic and UTI breakpoints. The data on MIC distributions to temocillin among the Norwegian carbapenemase-producers is currently not reported. However, it is generally accepted that temocillin is stable against Ambler classes A and C β-lactamases (such as AmpCs, ESBLs, and KPC) [327, 390]. However, temocillin is hydrolyzed by class B metalloenzymes or some class D enzymes such as VIM, NDM, IMP, and OXA-48 [297, 299, 324, 328]. Moreover, temocillin has shown to be a sensitive phenotypic indicator for the presence of OXA-48-like enzymes [245, 391], which along with other confirmatory tests improving specificity, can serve as a diagnostic aid to clinicians.

Despite the higher resistance to temocillin among ESBL and AmpC producers compared to wild-type isolates [329], a significant proportion is still sensitive to temocillin, as shown in **paper 1** and other studies [239, 330]. This suggests that isolates causing the lower UTIs could still be covered using higher doses [303, 331]. Temocillin has shown good clinical and microbiological efficacy for UTIs, bloodstream infections, and healthcare-acquired pneumonia, in a dose of 2 g BID (or renally-adjusted equivalent), irrespective of ESBL/depressed-AmpC strain status [303]. However, since most carbapenemases affect temocillin susceptibility, its use could bear the potential for selective pressure towards

carbapenemase-producers. Still given the ecological advantages such as a narrow spectrum of activity, this drug can be considered an alternative to carbapenems in the treatment of UTIs, caused by ESBL- and AmpC-producing *E.coli*.

5.10 Is there a need to use the old antimicrobials in Norway?

MDR *E. coli* is one of the greatest threats among the MDR pathogens and is escalating the use of carbapenems. Old drugs may offer a timely solution to this problem. However, are these drugs needed in Norway, a country with a relatively low prevalence of antimicrobial resistance? Surveillance data could provide answers to this question. *In vitro* surveillance is an important strategy not only to monitor the trends and changes in antimicrobial resistance over time but can also be used to guide appropriate empirical therapy, detect novel resistance mechanisms or identify the need to introduce new agents into the guidelines [(392)]. Networks as European Surveillance of Antimicrobial Consumption Network (ESAC-Net) by the European Center for Disease Control (ECDC) for the EU [(393)] and ResistanceMap by the Center for Disease Dynamics, Economics & Policy (CDDEP) in the USA [(394)] are essential for understanding the resistance patterns in current bacterial populations both locally and globally. Combined with knowledge on PK and PD of antimicrobials in question, surveillance data can also be used to select/revise the optimal dose for antimicrobials, by modelling target attainment in Monte Carlo simulations [(395)].

The majority of experts agree that for community-acquired lower UTIs, the selected empirical antimicrobial therapy is appropriate if local resistance prevalence does not exceed 10 to 20 % [(90, 396)]. According to the NORM surveillance program, overall resistance to commonly used antimicrobials among the Norwegian urinary tract *E. coli* isolates is less than 10% for cephalosporins, gentamicin, nitrofurantoin and mecillinam [(386, 387, 397)]. However, for ampicillin, trimethoprim-sulfamethoxazole, and ciprofloxacin, the resistance prevalence is higher [(386, 387, 397)]. However, does the same apply for ESBL- or carbapenemase-producing *E. coli*? ESBL-producing isolates are often co-resistant to other antimicrobial classes. The overall prevalence of *E. coli* with ESBL-phenotype in Norway is relatively low, but increasing from 1.5% to 6.5%, from 2010 to 2018, respectively; the prevalence of ESBL-producers in urine isolates is approximately 3,7% [(386, 387)].

In **paper 1**, we evaluated the role of the old antimicrobials (fosfomycin, mecillinam, nitrofurantoin, and temocillin) against the nationwide collection of ESBL-producing *E. coli*. The prevalence of resistance to conventionally used antimicrobials was much higher. As

expected, resistance to non-carbapenem β-lactams was close to 100% for most drugs except ceftazidime (77%), aztreonam (67% resistant), and amoxicillin-clavulanic acid (53% using systemic breakpoint of 8 mg/L). Resistance to conventionally used non-β-lactams was >70% for trimethoprim-sulfamethoxazole and ciprofloxacin and >40% to gentamicin and tobramycin. Low levels of resistance were observed only to meropenem (0%) and amikacin (1%). These drugs do not meet the 10 to 20 % threshold for the empirical therapy for lower UTI if ESBL-producing *E. coli* is suspected. Meropenem and amikacin should, ideally, be reserved for treating severe infections. According to a study on an Eastern-Norwegian patient population [(398)], the risk factors for UTI caused by the ESBL-producing *E.coli* are travel to Asia, Middle East, or Africa in the past six weeks to two years, recent treatment with fluoroquinolones and β-lactams and recreational freshwater swimming. Interestingly, the recent use of mecillinam was not associated with the ESBL risk.

In **paper 2** and **paper 3,** in addition to the ESBL-producing strains, we have used the carbapenemase-producing isolates K71-77 (NDM-1) and 50639799 (VIM-29). Both strains carried resistance determinants to aminoglycosides, fluoroquinolones, phenicols, and sulphonamides. VIM-29 isolate had, in addition, resistant determinants to tetracycline and trimethoprim.

In a study by Samuelsen *et al.* [(245)], all carbapenemase-producing isolates (including *E. coli*) in Norway (2007 to 2014) underwent genotypic and phenotypic characterization. Again, high levels of co-resistance among these strains were found to gentamicin, 51%; amikacin, 63%; and tobramycin, 83% (aminoglycosides), tigecycline (58%) and ciprofloxacin (83%). All these observations lead to the conclusion that there is a need to use other empiric alternatives than the abovementioned drugs, especially for in lower UTIs. Otherwise, there is a potential risk for the selection of ESBL-producers. Old drugs could serve as such alternative.

In **paper 1**, all isolates were susceptible to fosfomycin and temocillin (UTI breakpoint). When systemic breakpoint was applied, 29% of isolates were temocillin resistant. Resistance to mecillinam and nitrofurantoin was also low (6% and 9%, respectively). Among the general Norwegian *E. coli* population, resistance to mecillinam and nitrofurantoin is annually reported. In 2010 and 2011, 4.4% and 8.7% of all *E. coli* isolates were resistant to mecillinam, and 2.3% and 1.5% were resistant to nitrofurantoin.

6 CONCLUSIONS AND FUTURE REMARKS

Old drugs are unlikely to offer a permanent solution to the problem of increasing antimicrobial resistance. However, it is significantly easier and less time-consuming to reintroduce old drugs compared to developing new ones [134]. We have evaluated the role of fosfomycin, mecillinam, nitrofurantoin, and temocillin against MDR pAmpC-, ESBL- and carbapenemase-producers. In a nationwide collection of ESBL-producing *E. coli* (2010-2011), these old drugs were associated with a low prevalence of resistance, minimal co-resistance with conventionally used drugs, and among each other. *In vivo* studies of fosfomycin and mecillinam confirm the assumptions on efficacy based on the *in vitro* susceptibility of MDR pAmpC-, ESBL- and carbapenemase-producers**.** Together, these findings suggest the old drugs to be valuable alternatives to the use of carbapenems. Given the current resistance proportions in Norway, these drugs could be used as the empiric alternatives for lower UTIs caused by both ESBL and non-ESBL producers. The pharmacokinetic and pharmacodynamic data on fosfomycin provides additional information, which is valuable for further dose-optimization of fosfomycin. As knowledge gaps still exist for the optimal use of these drugs, further research in this field is needed promptly [399].

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8 PAPERS

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PHARMACOLOGY

Pharmacokinetics and Pharmacodynamics of Fosfomycin and Its Activity against Extended-Spectrum-β-Lactamase-, Plasmid-Mediated AmpC-, and Carbapenemase-Producing *Escherichia coli* in a Murine Urinary Tract Infection Model

Ilya Nikolaevich Zykov,[a,b] Ørjan Samuelsen,[a,c] Lotte Jakobsen,[d] Lars Småbrekke,[e] Dan I. Andersson,[f] Arnfinn Sundsfjord,[a,b] Niels Frimodt-Møller[g]

[a]Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway

[b]Research Group for Host-Microbe Interactions, Department of Medical Microbiology, Faculty of Health Sciences, UiT—The Arctic University of Norway, Tromsø, Norway

[c]Microbial Pharmacology and Population Biology Research Group, Department of Pharmacy, Faculty of Health Sciences, UiT—The Arctic University of Norway, Tromsø, Norway

[d]Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark

[e]Clinical Pharmacy and Pharmacoepidemiology (IPSUM), Department of Pharmacy, Faculty of Health Sciences, UiT—The Arctic University of Norway, Tromsø, Norway

[f]Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

[g]Department of Clinical Microbiology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark

ABSTRACT Fosfomycin has become an attractive treatment alternative for urinary tract infections (UTIs) due to increasing multidrug resistance (MDR) in *Escherichia coli*. In this study, we evaluated the pharmacokinetic (PK) and pharmacodynamic (PD) indices of fosfomycin and its *in vivo* activity in an experimental murine model of ascending UTI. Subcutaneous administration of fosfomycin showed that the mean peak plasma concentrations of fosfomycin were 36, 280, and 750 mg/liter following administration of a single dose of 0.75, 7.5, and 30 mg/mouse, respectively, with an elimination half-life of 28 min, and urine peak concentrations of 1,100, 33,400, and 70,000 mg/liter expected to be sustained above 1 mg/liter (MIC of the test strain, NU14) for 5, 8, and 9.5 h, respectively. The optimal PK/PD indices for reducing urine colony counts (number of CFU per milliliter) were determined to be the area under the concentration-time curve/MIC from 0 to 72 h and the maximum concentration/MIC on the basis of the dose-dependent bloodstream PK and the results of an evaluation of six dosing regimens. With a dosing regimen of 15 mg/mouse twice (every 36 h), fosfomycin significantly reduced the number of CFU per milliliter of all susceptible strains in urine, including clinical MDR strains, except for one clinical strain ($P = 0.062$). Variable degrees of reduction were observed in the bladder and kidneys. No significant reductions in the number of CFU per milliliter were observed with the resistant strains. In conclusion, fosfomycin shows concentration-dependent *in vivo* activity, and the results suggest that fosfomycin is an effective alternative to carbapenems in treating MDR *E. coli* in uncomplicated UTIs. The data on the effectiveness of fosfomycin against the MDR isolates along with the results of PK/PD modeling should facilitate the further development of improved recommendations for its clinical use.

KEYWORDS reviving old drugs, CTX-M, VIM, NDM, multidrug resistant, *in vivo*, time-kill, PK/PD, UTI, UTI model, fosfomycin, *in vivo* model

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Address correspondence to Ilya Nikolaevich Zykov, i.n.zykov@gmail.com.

Urinary tract infections (UTIs) may progress to bloodstream infections (1), and they account for ~40% of hospital-acquired cases of sepsis (2, 3). *Escherichia coli* is responsible for 75 to 90% cases of community-acquired UTIs (4–7). The increase in antimicrobial resistance and multidrug resistance (MDR) among *E. coli* isolates (i.e., extended-spectrum-β-lactamase [ESBL]-producing *E. coli* isolates) is currently limiting treatment options for UTIs (8). This could lead to the more extensive use of carbapenems, which are reserved for use against other complicated infections (9). In addition, carbapenemase-producing organisms are now spreading worldwide (10, 11). The lack of effective antimicrobials due to the emergence of antimicrobial resistance inflates the use of last-resort antimicrobials for common infections, such as UTIs. Alternative therapeutic options for UTIs are therefore urgently needed (12).

As there are a very limited number of new antimicrobials in the pipeline, it has been suggested that an alternative approach is to reevaluate the efficacy of old antimicrobials to extend the set of drugs available for the treatment of MDR infections (13–16). One such agent is fosfomycin, a broad-spectrum bactericidal agent that has been suggested to be an alternative treatment option for infections caused by MDR Gram-negative bacteria (17). Fosfomycin acts on the cell wall by inactivating enolpyruvate transferase, thereby blocking the condensation of UDP-*N*-acetylglucosamine with *p*-enolpyruvate (18).

Recent studies have shown that fosfomycin exhibits potent *in vitro* activity against both non-MDR and MDR *Enterobacteriaceae*, including ESBL- and carbapenemase-producing isolates (18–20). However, increasing frequencies of fosfomycin resistance have been observed in some countries where fosfomycin is used (18, 20). The emergence of resistance during fosfomycin monotherapy occurs rapidly *in vitro* but is rarely observed *in vivo* (21). Data on the pharmacokinetic (PK) and the pharmacodynamic (PD) behavior of fosfomycin are somewhat conflicting among existing studies (22–26). Docobo-Perez et al. (22) suggest insufficient evidence on efficacy to be among the factors discouraging the use of fosfomycin as a treatment option. Therefore, reevaluation of the *in vivo* activity and PK/PD properties of fosfomycin is required to develop an effective dosing regimen that complies with current standards and that is applicable to the current bacterial population (15, 16, 21). To our knowledge, no *in vivo* studies have investigated the PK/PD of fosfomycin in UTIs during the past 2 decades.

Thus, the objectives of this study were to elucidate the predictive PK/PD index for fosfomycin in an experimental model of ascending UTI, identify the dose that targets appropriate exposure toward *E. coli* strains with decreased susceptibility to fosfomycin, and investigate the *in vivo* activity of fosfomycin against MDR ESBL-, plasmid-mediated AmpC-, and/or carbapenemase-producing *E. coli in vivo*.

RESULTS

Bacterial strain characteristics. The characteristics of the strains used in this study are summarized in Table 1. On the basis of the results of whole-genome sequencing (WGS) analysis, no resistance determinants were identified in the NU14 strain. The sequence type (ST) of NU14 was determined to be ST1231. The MICs of fosfomycin for NU14 and NU14-derived strains DA6313, DA6328, and DA6401 were determined to be 1, 2, 128, and >1,024 mg/liter, respectively.

Five of the clinical MDR *E. coli* isolates selected for the *in vivo* activity studies were susceptible to fosfomycin with MICs of 0.5 to 2 mg/liter, while isolate P14-63 was resistant with an MIC of 512 mg/liter. Two of the clinical isolates were carbapenemase producers harboring bla_{NDM-1} (strain K71-77) or bla_{VIM-29} (strain 50639799). Three isolates were ESBL producers harboring $bla_{CTX-M-15}$, $bla_{CTX-M-14}$, or $bla_{CTX-M-3}$, while the isolate with a plasmid-mediated AmpC harbored bla_{CMY-2}. The fosfomycin-resistant P14-63 isolate harbored the *fosA* gene. Multilocus sequence typing (MLST) analysis showed that the isolates were diverse with respect to sequence types, with the sequence types of the isolates including ST167, ST2016, ST420, ST410, ST6355, and ST131.

TABLE 1 Characteristics of the *E. coli* strains used in the study

Strain	Specimen or origin	Fosfomycin MIC (mg/liter)	Resistance gene profile	MLST type
NU14	Urine	1		ST1231
DA6313	NU14 derivative	2	*ptsI* deletion	ST1231
DA6328	NU14 derivative	128	*glpT* missense mutation	ST1231
DA6401	NU14 derivative	>1,024	*uhpT* missense mutation	ST1231
K4-40	Wound	2	*aadA1*, *aac(6′)Ib-cr*, $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{TEM-1B}, *catB3*, *dfrA1*, *erm*(B), *mph*(A), *strA*, *strB*, *sul2*, *tet*(A)	ST167
K5-08	Urine	0.5	*aadA5*, $bla_{CTX-M-14}$, *dfrA17*, *sul2*, *tet*(A)	ST2016
K26-07	Urine	2	bla_{CMY-2}	ST420
K71-77	Blood culture	2	*aac(3)-IId*, *aac(6′)Ib-cr*, *aacA4*, bla_{CMY-6}, bla_{NDM-1}, bla_{OXA-1}, *catB3*, *rmtC*, *sul1*	ST410
50639799	Urine	0.5	*aac(3)-IIa*, *aac(6′)Ib-cr*, *aadA24*, bla_{CMY-4}, $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{VIM-29}, *catB3*, *dfrA1*, *floR*, *strA*, *strB*, *sul2*, *tet*(A)	ST6355
P14-63	Urine	512	$bla_{CTX-M-3}$, bla_{TEM-1B}, *fosA*	ST131

***In vitro* time-kill studies.** *In vitro* time-kill studies with NU14 (Fig. 1) and the susceptible clinical strains (Fig. 2A to E) at concentrations of 1× to 8× MIC showed an initial rapid bactericidal effect up to 2 h, followed by regrowth at 24 h. At concentrations of 16× to 32× MIC (64× MIC and higher for NU14), bacterial counts reached levels below the limit of detection (LOD; ≥50 CFU/ml) at 2 to 4 h. No regrowth was observed at 24 h for any of the susceptible strains, except for K5-08 and 50639799. For NU14, MIC testing of the subsequently isolated colonies (at time points of 0 h, 4 h, and 24 h) showed 8- to 32-fold increases in the MIC at time points of 4 h and 24 h, whereas there was no increase in the MIC for colonies from the control tube. The MICs for the isolated colonies with an increased MIC remained stable after five passages on nonselective Mueller-Hinton (MH) agar (data not shown).

For the resistant clinical strain P14-63 (Fig. 2F), transient killing was soon followed by regrowth. After 24 h, regrowth was observed irrespective of the fosfomycin concentration. For this strain, no dependence between the fosfomycin concentration and the

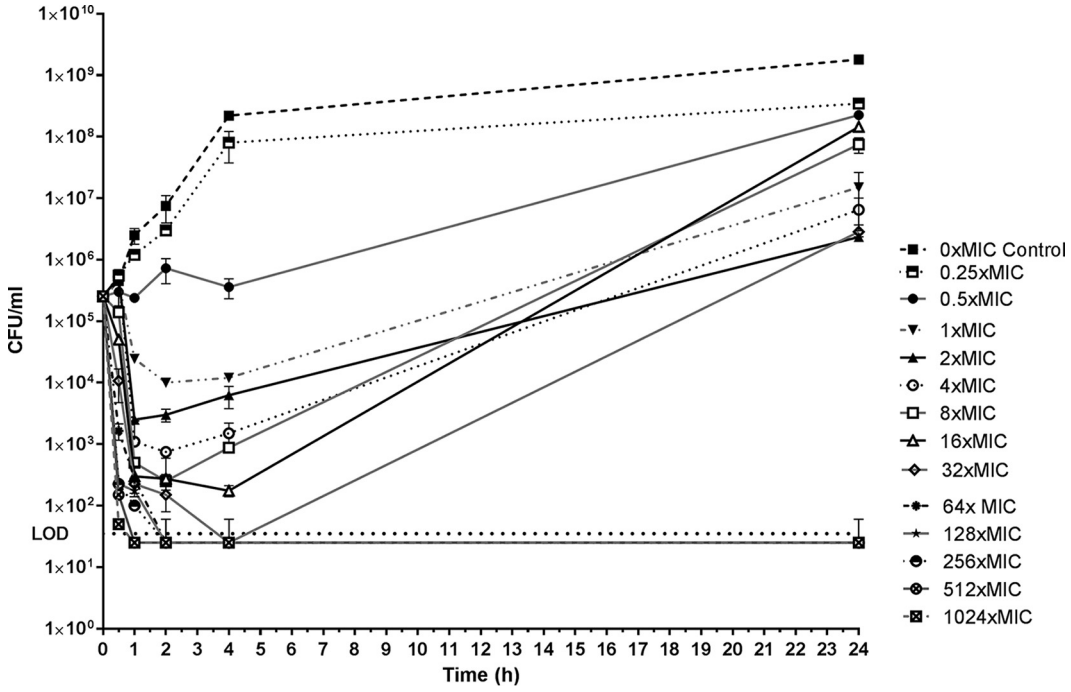

FIG 1 *In vitro* time-kill curves with fosfomycin against fosfomycin-susceptible *E. coli* NU14 (MIC, 1 mg/liter). The graph shows the viable counts as the $\log_{10}$ number of CFU per milliliter at time points of 0 h, 30 min, 1 h, 2 h, 4 h, and 24 h. The horizontal dotted line shows the limit of detection (LOD).

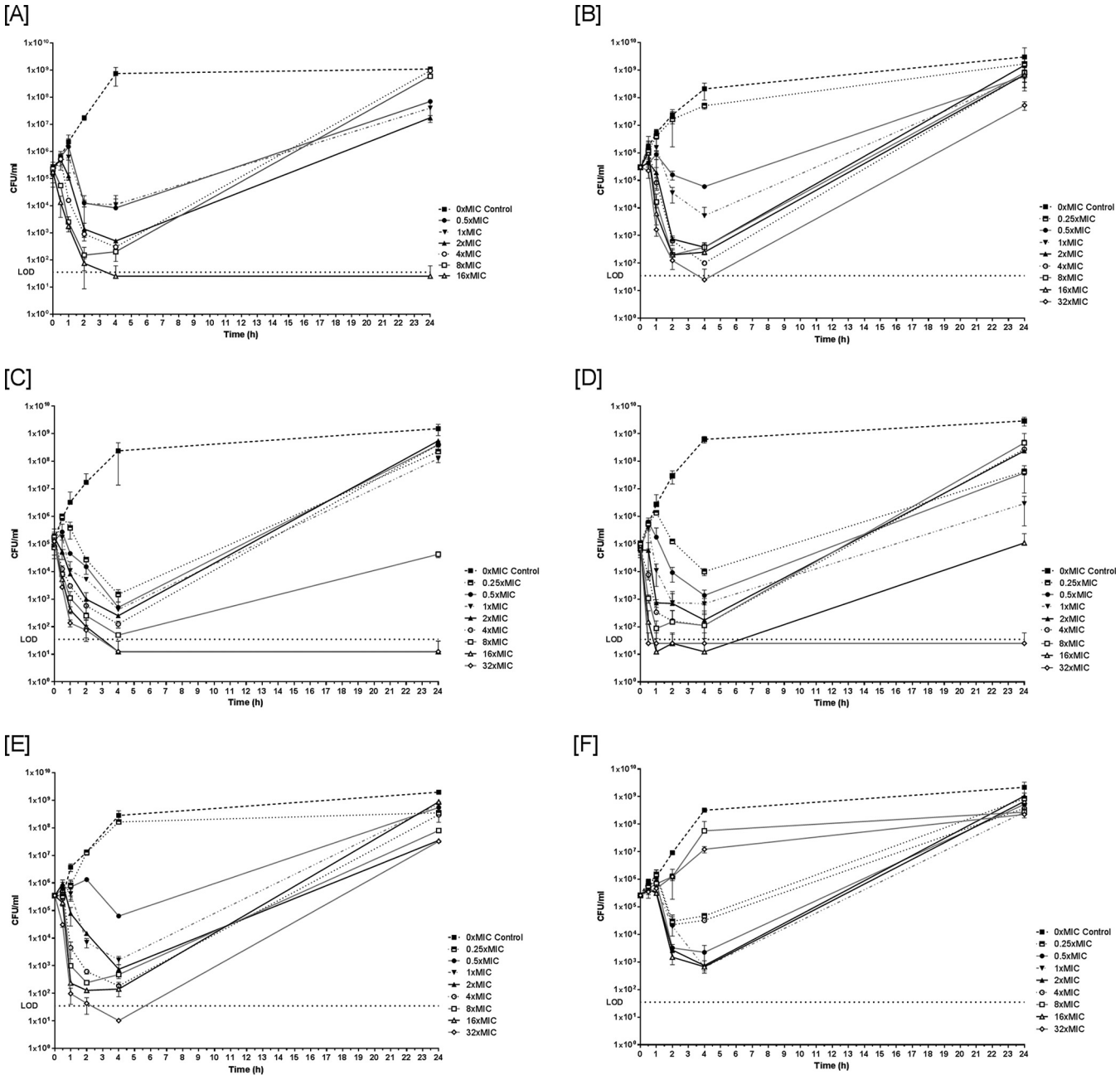

FIG 2 *In vitro* time-kill curves with fosfomycin against *E. coli* clinical isolates K4-40 (MIC, 2 mg/liter) (A), K5-08 (MIC, 0.5 mg/liter) (B), K26-07 (MIC, 2 mg/liter) (C), K71-77 (MIC, 2 mg/liter) (D), 50639799 (MIC, 0.5 mg/liter) (E), and P14-63 (MIC, 512 mg/liter) (F). The graphs show viable counts as the $\log_{10}$ number of CFU per milliliter at time points of 0 h, 30 min, 1 h, 2 h, 4 h, and 24 h. The horizontal dotted lines show the limit of detection (LOD).

rate of killing was found (i.e., lower concentrations could result in killing rates initially higher than those achieved with higher concentrations of fosfomycin; in the case of 8× MIC and 32× MIC, the growth rates were close to the rates observed for the control). The maximal bactericidal effect did not exceed >2 $\log_{10}$ CFU/ml of the initial number of CFU per milliliter.

PK/PD of fosfomycin. (i) Pharmacokinetics. The plasma and urine concentrations of fosfomycin were measured in mice after single subcutaneous (s.c.) doses of 0.75, 7.5, and 30 mg/mouse. Peak fosfomycin plasma concentrations were 36, 280, and 750 mg/liter for the respective doses (Fig. 3A). The mean elimination half-life was 28 min. In urine, peak fosfomycin concentrations of 33,400 and 70,000 mg/liter were reached

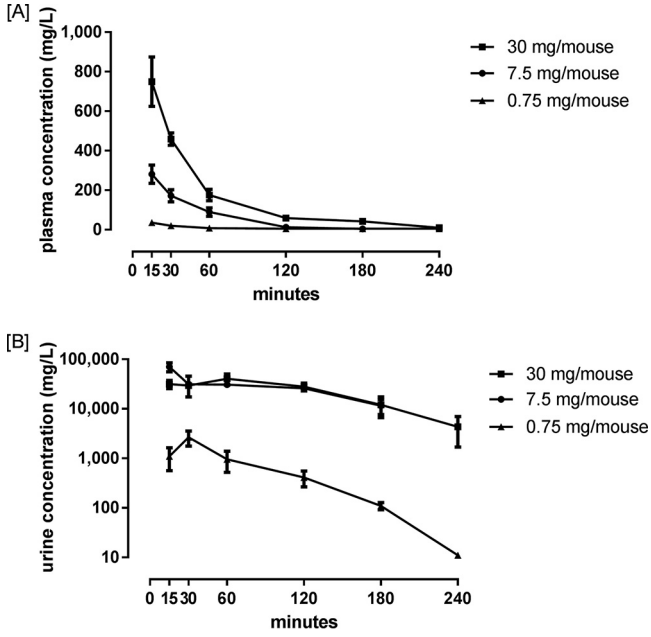

FIG 3 Fosfomycin concentrations (in milligrams per liter) in plasma (A) and urine (B) in OF-1 mice following subcutaneous administration of single doses of 30, 7.5, and 0.75 mg/mouse. The data are presented as the mean for three mice at each time point. Error bars represent SDs.

after 15 min with the 7.5- and 30-mg/liter doses, respectively (Fig. 3B). After 15 min, the measured concentrations of fosfomycin for the 7.5- and 30-mg/liter doses were similar, and the two doses followed the same elimination pattern. For the 0.75-mg dose, a peak urine concentration of 1,100 mg/liter was reached after 30 min.

(ii) Dose fractionation and calculation of PK/PD indices. On the basis of the results of the PK analysis, six dose regimens were designed (Table 2), in order to produce variations in bloodstream PK/PD indices: 30 and 7.5 mg/mouse in a single dose, 15 mg/mouse twice (every 36 h [q36h]), 1.88 and 0.47 mg/mouse every 6 h, and 0.47 mg/mouse every 12 h (q12h). Treatment was initiated at 24 h postinfection, and the treatment period was 72 h for all doses used. PK/PD indices for the NU14 strain were calculated using the systemic drug concentrations. The cumulative percentage of a 72-h period that the drug concentration exceeded the MIC (percent $T_{>MIC}$) ranged from 4 to 42%, the area under the concentration-time curve (AUC)/MIC ratios ranged from 607 to 79 h^{-1}, and the maximum concentration (C_{max})/MIC (for doses of 30 mg and 7.5 mg, the actual measured values were used to calculate C_{max}/MIC) ratios ranged from 750 to 22 (Table 2).

(iii) PK/PD analysis. The *in vivo* activity of the six dose regimens against the NU14 strain was further investigated to estimate the predictive value of the PK/PD indices for

TABLE 2 Fosfomycin dosing regimens, based on bloodstream PK data, applied in the PK/PD study in the experimental UTI model[a]

Dose (mg/mouse)	Dosing interval (h)	No. of doses per 72-h treatment interval	Total dose (mg)	Value of the following PK/PD index:		
				$T_{>MIC}$ (%)	AUC/MIC (h^{-1})	C_{max}/MIC
30	72	1	30	9	607	750
15	36	2	30	14	727	468
7.5	72	1	7.5	4	212	281
1.88	6	12	22.56	42	635	78
0.47	6	12	5.64	30	158	22
0.47	12	6	2.82	15	79	22

[a]Treatment was initiated at 24 h postinfection, and the treatment period was 72 h.

a bactericidal effect. For all doses tested, the median number of CFU per milliliter in urine and kidneys was reduced to below the LOD (50 CFU/ml) (Fig. 4A and C). However, for some mice and on the basis of the dose, counts (number of CFU per milliliter) above the LOD were observed in the urine of a fraction of the mice in each separate experiment, with the fractions ranging from 0% (for a single dose of 30 mg/mouse and two doses of 15 mg/mouse [q36h]) to 55.6% (for a dose of 0.47 mg/mouse twice a day [q12h]) of the mice (Fig. 4A). This was also observed for the counts (number of CFU per milliliter) in the kidneys, in which the fraction of mice with counts (number of CFU per milliliter) above the LOD ranged from 16.6% (for doses of 7.5 and 30 mg/mouse) to 38.8% (for a dose of 0.47 mg/mouse q12h) (Fig. 4C). In the bladders, none of the median counts (number of CFU per milliliter) fell below the LOD, but a reduction in median counts (number of CFU per milliliter) of up to ~2 $\log_{10}$ compared to the counts for the control was observed for all doses tested (Fig. 4B).

For all infection sites, the PD indices with the best correlation with *in vivo* activity were the AUC/MIC from 0 to 72 h (AUC/MIC_{0-72}) and C_{max}/MIC (Fig. 5). The percent $T_{>MIC}$ for 72 h had minimal, if any, influence on the *in vivo* activity ($R^2 = 0.74$, 0.36, and 0.7 for urine, bladder, and kidneys, respectively). However, the amount of time that the concentration exceeded the MIC ($T_{>MIC}$ in hours) for the first injection (which could also serve as a surrogate for C_{max}/MIC) also correlated well with the bactericidal effect. The optimal AUC/MIC_{0-72} ratio appeared to be >600 h^{-1} for urine ($R^2 = 0.91$) and >200 h^{-1} for the bladder and kidneys ($R^2 = 0.91$ and 0.97, respectively). The optimal values of C_{max}/MIC were >450 for urine ($R^2 = 0.88$) and >280 for kidneys and bladder ($R^2 = 0.91$ and 0.98, respectively). On the basis of this finding, the treatment associated with the best *in vivo* activity (15 mg of fosfomycin per mouse twice [q36h]) was selected for further studies with clinical strains. This dose was calculated on the basis of a surface area of a mouse to be 70 cm^2, which correlates with a surface area of 17,200 cm^2 in a 70-kg human. The calculation results in a human dose of 3.6 g, which is close to the standard dose of 3 g fosfomycin used for treating UTIs in most clinical studies (27, 28).

***In vivo* activity studies.** Both the NU14-derived isogenic and clinical MDR strains were confirmed to be type 1 fimbria positive and virulent in the murine UTI model (data not shown).

(i) NU14 and isogenic NU14-derived strains. Using the dose of 15 mg/mouse twice (q36h), a reduction in the median number of CFU per milliliter was observed only with fosfomycin-susceptible strain NU14 in urine (5.3-log reduction, $P < 0.0001$), bladder (4.9-log reduction, $P < 0.0006$), and kidneys (2.13-log reduction, $P = 0.063$) and with fosfomycin-susceptible strain DA6313 in urine (5.8-log reduction, $P = 0.1326$) (Fig. 6A) and bladder (1.7-log reduction, $P = 0.014$) (Fig. 6B). No reduction was observed in kidneys (Fig. 6C). For fosfomycin-resistant strains DA6328 and DA6401, no significant reduction in the median counts (number of CFU per milliliter) was observed at any infection site, except that a significant reduction in the number of CFU per milliliter was observed in the kidneys for DA6328 (1.16 log reduction, $P = 0.041$) (Fig. 6C).

(ii) Clinical MDR strains. The applied treatment regimen significantly reduced the counts (number of CFU per milliliter) in urine compared to those achieved with the vehicle for all fosfomycin-susceptible MDR clinical *E. coli* strains except one (strain K71-77; $P = 0.062$) (Fig. 7A). In the bladder and kidneys, the counts (number of CFU per milliliter) were significantly reduced for 3/5 and 1/5 of the fosfomycin-susceptible strains, respectively (Fig. 7B and C). No significant difference in the counts (number of CFU per milliliter) between the treated and the vehicle groups was observed for the fosfomycin-resistant strain (P14-63) at all infection sites (Fig. 7A to C).

Posttreatment studies. No significant changes in the MIC of fosfomycin for colonies of selected isolates on day 2 and day 5 ($n = 71$) from any infection site of either the treatment or the vehicle group randomly picked from nonselective agar were observed. All strains retained their expected determinants of resistance to third-generation cephalosporins and/or carbapenems.

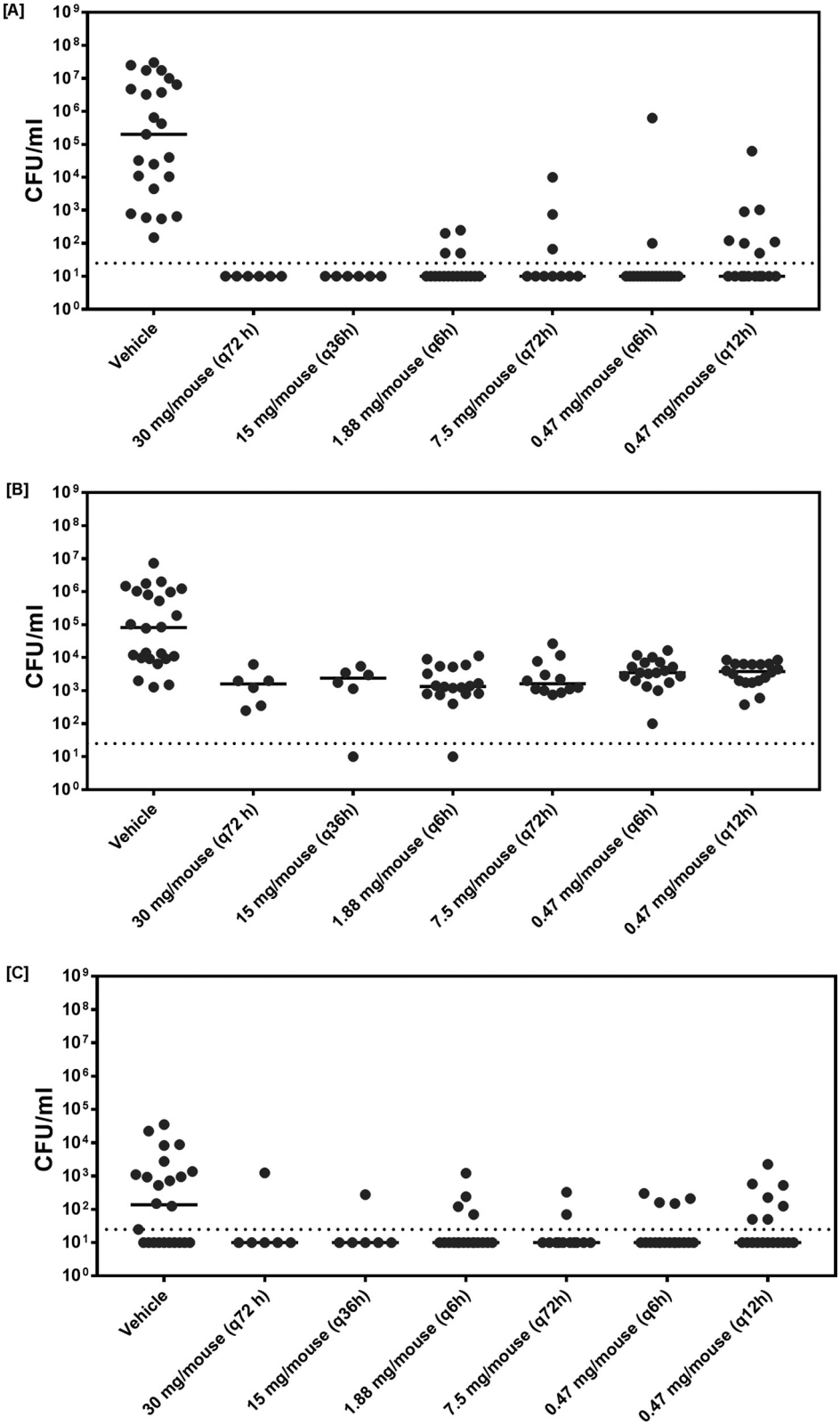

FIG 4 Study of the effect of treatment with six fosfomycin dosing regimens (milligrams per mouse) against *E. coli* NU14 (MIC, 1 mg/liter). The bacterial counts (number of CFU per milliliter) from urine (A), homogenized urine bladder (B), and homogenized kidneys (C) in mice with UTIs at day 5 after the inoculation are shown. Each point indicates the number of CFU per milliliter in a single animal. Solid horizontal lines represent the median bacterial count for each group, and horizontal dotted lines represent the LOD.

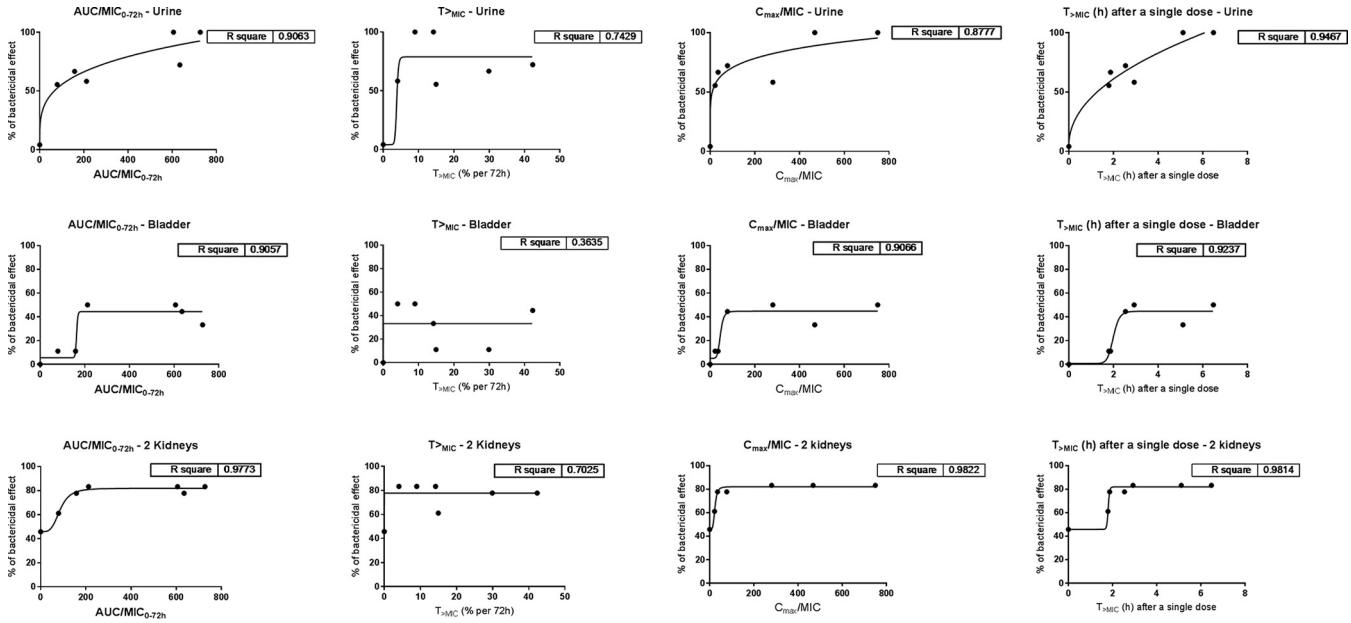

FIG 5 Relationship between AUC/MIC_{0-72}, percent $T_{>MIC}$ (percent per 72 h), C_{max}/MIC, and T_{MIC} (in hours) after a single dose, based on plasma drug concentrations (protein binding is assumed to be 0%) and fosfomycin efficacy against *E. coli* NU14 in the experimental UTI model. The dosing regimens applied for the efficacy study are listed in Table 2. Six to 18 mice were used for the investigation of each dose. R^2 represents the goodness of fit, as calculated in GraphPad Prism software.

DISCUSSION

Pharmacokinetics/pharmacodynamics. Our first objective was to perform PK/PD studies to find the predictive index for fosfomycin. In the mouse model, fosfomycin was rapidly absorbed after s.c. injection. After an almost negligible distribution phase, the drug was eliminated with a mean half-life of 28 min in plasma (Fig. 3). The elimination rate in mice was almost 10 times faster than that observed in humans, as is usually seen for drug kinetics in mice. No accumulation of the drug in serum is expected for the observed concentrations due to its short half-life in mice. Due to a significant variation in urinary drug concentrations and because it was not possible to measure the total mass of excreted drug, we did not use these data in the PK/PD analysis. Interestingly, the urine concentration curves of fosfomycin were similar for two different doses (30 mg and 7.5 mg), except at the time point of 15 min, where an approximately 4-fold difference in peak urine concentrations was observed (Fig. 3B). This could suggest saturable elimination in combination with first-order elimination. Other studies, in both humans and animal models, suggest that fosfomycin is eliminated in kidneys exclusively by glomerular filtration and is neither protein bound nor metabolized (29–35). However, there are some examples of similar findings in human studies, where a ceiling effect on excretion has been observed (36, 37). We believe that this phenomenon deserves further studies, especially with respect to the optimal dosing regimen in humans.

According to the results of time-kill studies, the bactericidal effect of fosfomycin was rapid (<2 h) and concentration dependent (Fig. 1 and 2). Regrowth after 24 h was also shown to be concentration dependent and was observed for all the concentrations below 16× to 32× MIC. Moreover, the MICs for the survivors increased 8- to 32-fold and were stable, indicating the development of resistance. This is in contrast to the lack of an increase in the MIC for randomly selected colonies posttreatment *in vivo*. Although no firm conclusions can be drawn with respect to the *in vivo* emergence of fosfomycin resistance in our experimental setup, the findings of the present study support previous findings indicating differences between the *in vitro* and *in vivo* emergence of fosfomycin resistance (38).

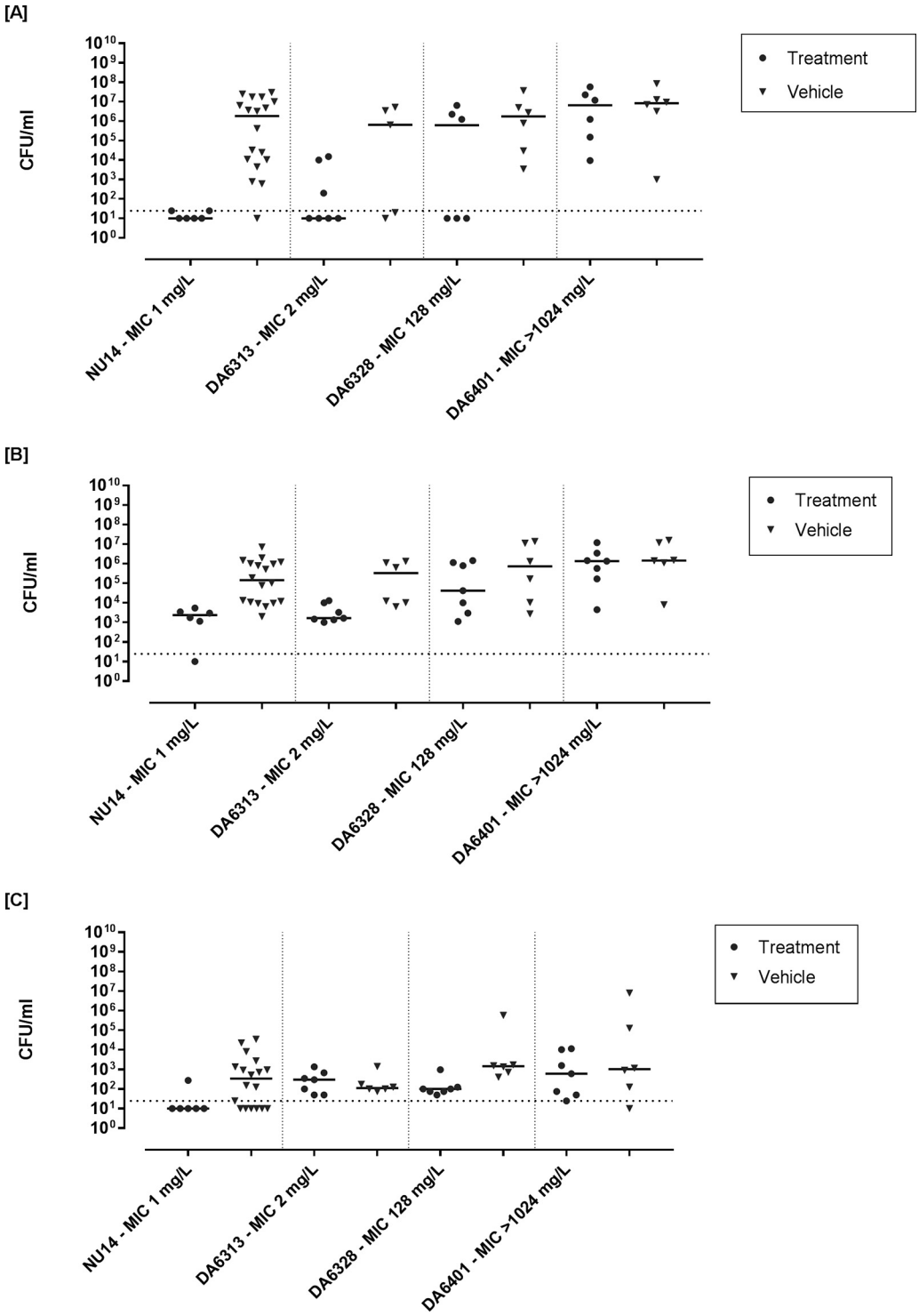

FIG 6 Bacterial counts (number of CFU per milliliter) from urine (A), homogenized urine bladder (B), and homogenized kidneys (C) of OF-1 mice treated for 3 days at 15 mg/mouse twice (q36h) or saline (control) after infection with isogenic *E. coli* strains with decreasing susceptibility to fosfomycin. Solid horizontal lines represent the median bacterial count for each group, and horizontal dotted lines represent the LOD.

We used bloodstream drug concentrations in the PK/PD analysis. According to Frimodt-Møller (39), the serum PK/PD indices represent a more accurate predictor of drug levels and treatment activity in kidneys. For activity in bladder, a combination of urinary (lumen) and serum (bladder tissue) PK could be important. Our dosing regimens

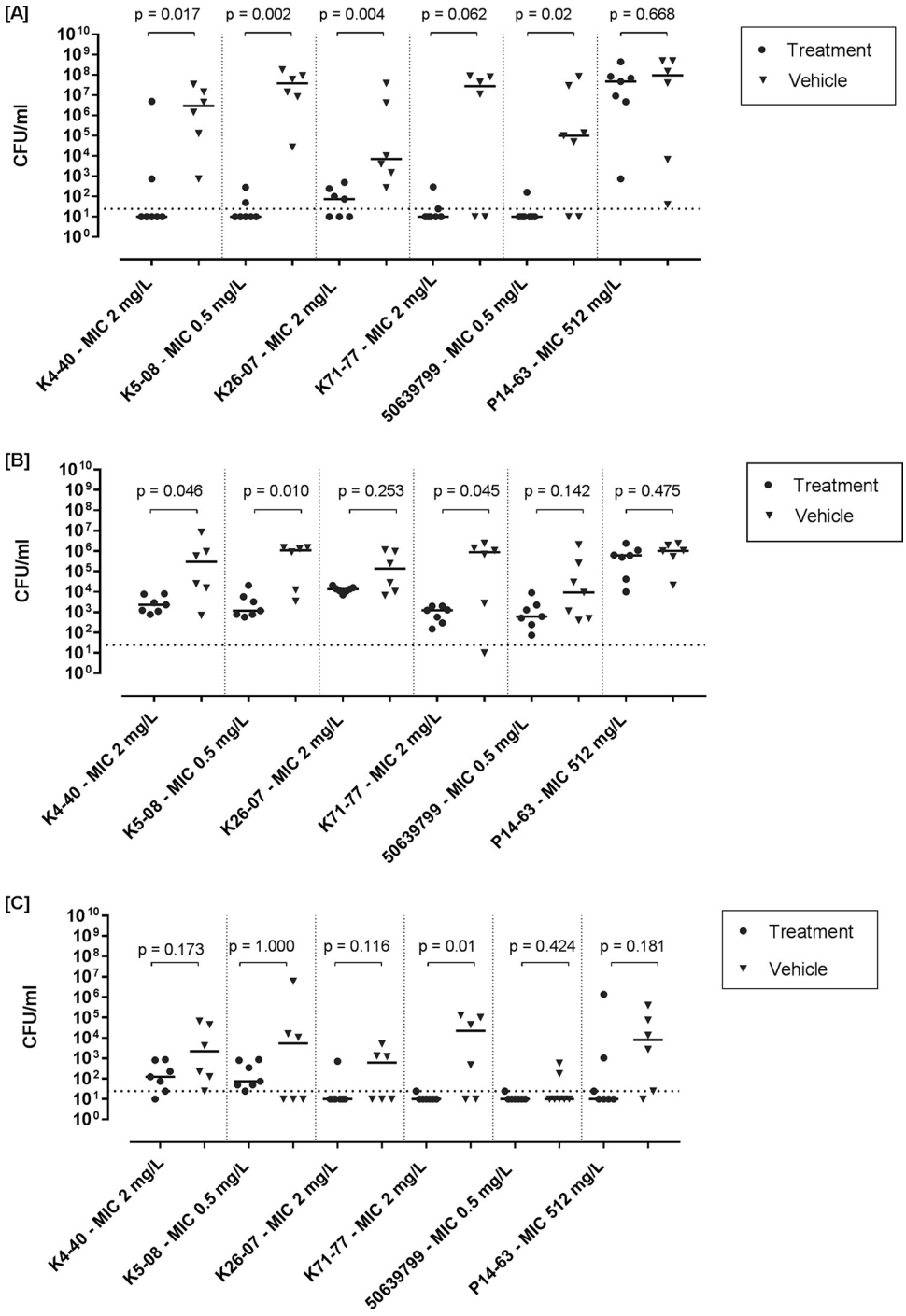

FIG 7 Bacterial counts (number of CFU per milliliter) from urine (A), homogenized urine bladder (B), and homogenized kidneys (C) of OF-1 mice treated for 3 days at 15 mg/mouse twice (q36h) or saline (control) after infection with virulent clinical *E. coli* isolates with various degrees of fosfomycin susceptibility. Solid horizontal lines represent the median bacterial count for each group, and horizontal dotted lines represent the LOD.

allowed variations in the magnitudes of the PK/PD indices. However, due to a high renal clearance in mice, the $T_{>MIC}$ did not exceed 42% for the strain used in the study. We observed overall good activity for all dosing regimens, despite the relatively low percent $T_{>MIC}$ for some doses (Fig. 4). Due to the differences in pharmacokinetics between mice and humans, we could not mimic all the parameters with one dose, especially when the fact that we were assessing both plasma and urine concentrations

is taken into account. Mazzei et al. have reported that high fosfomycin urine concentrations (1,000 to 4,000 mg/liter) are achieved and remain at 100 mg/liter for at least 30 to 48 h (40), which is the pharmacokinetic basis for the oral 3-g single-dose regimen. In our dose fractionation study, we used a set of doses which appeared to be related to the standard human oral 3-g dose in different ways. In comparison with the PK of the standard human oral 3-g dose, a dose of 0.47 mg/mouse had a comparable plasma C_{max} of 22 mg/liter (41), a dose of 7.5 mg/mouse had a comparable plasma AUC of 212 h^{-1} (41), doses of 1.88 to 0.47 mg/mouse had comparable peaks in urine concentrations (40) (however, the concentrations declined faster), and doses of 15 to 30 mg/liter allowed the urine concentrations of fosfomycin to be retained at >100 mg/liter for the longest time compared to the other doses used in this study, while plasma C_{max} levels were comparable to those obtained with intravenous (i.v.) bolus doses in humans (42). The differences in PK parameters at different sites and for different doses can also be seen to be an advantage, as it would allow us to isolate the PK/PD parameters which are important for the successful treatment of UTIs with fosfomycin.

We used a proportion of the bactericidal effect approach, which allowed us to account for both noninfected/self-recovered kidneys and the good overall activity seen for all doses (Fig. 4). For urine and kidneys, the bactericidal effect was defined as the number of CFU per milliliter below the LOD, while for the bladder, the bactericidal effect was defined as the number of CFU per milliliter below the minimum count observed in the control group. Similar results for the number of CFU per milliliter in the bladder were previously observed in the same animal model with other antimicrobials (43). For all infection sites, the optimal PK/PD indices were AUC/MIC_{0-72} and C_{max}/MIC.

Published data regarding the appropriate PK/PD index for fosfomycin are somewhat inconsistent. Some authors (23, 24) consider $T_{>MIC}$ to be an appropriate index; however we, along with others (22, 26), found the AUC/MIC and/or C_{max}/MIC to be more appropriate. This may be because for most of the strains, the dose allowed the fosfomycin concentrations to remain above the MIC for a substantial amount of time. Treatment failures may happen due to the emergence of an inherently resistant subpopulation. VanScoy et al. (25) showed *in vitro* that the time above the inherent resistance inhibitory concentration (32× to 64× MIC, in their case) appeared to be the optimal PK/PD index; our results do not contradict this hypothesis.

***In vivo* activity studies.** In order to balance comparable serum concentrations and prolonged fosfomycin concentrations in urine, a dose of 15 mg/mouse administered twice (q36h) was considered to be the most effective and, in terms of surface area and as discussed above, comparable to the standard human dose of 3 g fosfomycin used for the treatment of UTIs. This dose is also expected to sustain fosfomycin concentrations in urine of >100 mg/liter for the longest possible time (≈10 h) and was further used for the treatment studies. The decrease in bactericidal effect was relative to the MICs for the strains. For the isogenic strain NU14 derivative (DA6328) with an MIC of 128 mg/liter, the dose resulted in a reduction in the number of CFU per milliliter in urine for some mice (3/6), which was, overall, statistically nonsignificant. For the same strain, no reduction in the number of CFU per milliliter in the kidneys and bladder was observed (Fig. 6).

The second objective of the study was to evaluate the activity of fosfomycin against MDR *E. coli in vivo*. Fosfomycin significantly reduced the number of CFU per milliliter in the urine and bladder for most of the isolates. Although not all the isolates showed statistically significant reductions in the number of CFU per milliliter in the bladder and kidneys, nonsignificant tendencies toward reductions could still be observed (Fig. 7). For cases with low statistical significance, the results could be explained by the lower total number of CFU per milliliter per organ compared to that in urine, and thus, the difference in the reduction (between the treatment and vehicle-treated groups) was smaller. The high proportion of low counts (number of CFU per milliliter) of some strains in the kidneys in the vehicle-treated group (which could be interpreted as indicating either that the animals were not infected or self-recovered) is considered a

limitation of the model. We believe that this situation causes a lower level of statistical significance when the two-tailed Mann-Whitney test for the counts (number of CFU per milliliter) in kidneys is used. These results are in concordance with those of other studies (43, 44) implementing the same animal model but using different antimicrobials and, thus, can be considered to be due to the limitations of the model and not the action of fosfomycin itself. The results indicate that fosfomycin might have a potential for use in the treatment of upper UTIs (for strains with lower MICs and likely by use of a dose or route of administration different from the standard 3 g oral single dose used in the clinical setting), but this requires further studies.

The colony counts of the resistant clinical isolate harboring *fosA* (P14-63) were not significantly reduced *in vivo* in this study, despite the high plasma and urinary concentrations (Fig. 7). In accordance with this finding, the time-kill studies also showed that the strain was not inhibited even by concentrations exceeding 32× MIC (>16,384 mg/liter). The same time-kill pattern for isolates with plasmid-mediated fosfomycin resistance has also been previously observed (45). Examples of colinked *fosA* and ESBL determinants have already been reported (46, 47). However, *ad interim*, the global rates of susceptibility to fosfomycin remain high, including for ESBL- and carbapenemase-producing *Enterobacteriaceae* (48, 49).

In conclusion, our observations support the notion that fosfomycin is a promising option for the treatment of uncomplicated UTIs caused by MDR *E. coli*. The proportions of susceptible isolates among the subgroup of ESBL or carbapenemase producers remain high globally (19, 48, 49). The optimal PK/PD indices included AUC/MIC and C_{max}/MIC. The dose of 15 mg/mouse twice (q36h) demonstrated a good effect against clinical isolates. To our knowledge, this is the first *in vivo* study reporting the successful treatment of UTIs caused by carbapenemase-producing *E. coli* with fosfomycin.

MATERIALS AND METHODS

Bacterial strains and chemicals. The fosfomycin-susceptible clinical uropathogenic *E. coli* strain (UPEC) NU14 (38) was used to evaluate the effect of different doses of fosfomycin for the PK/PD study. The strain has been used in a number of studies of UPEC (50) as well as in the UTI model (51).

Three isogenic *E. coli* strains with decreasing fosfomycin susceptibility, DA6313, DA6328, and DA6401, derived from NU14 (38), were included for evaluation of the selected doses in the UTI model. These strains have increased MICs against fosfomycin due to chromosomal mutations/deletions (38), including a deletion in *ptsI* (DA6313), a *glpT* missense mutation (DA6328), and a missense mutation in *uhpT* (DA6401). Further, one fosfomycin-resistant and five fosfomycin-susceptible clinical ESBL-, plasmid-mediated AmpC-, and/or carbapenemase-producing *E. coli* isolates were used to further evaluate the efficacy of the selected doses. All isolates used in the study are listed in Table 1.

The MIC of fosfomycin was determined by agar dilution using Mueller-Hinton (MH) agar (Oxoid, Waltham, MA) with the addition of 25 mg/liter glucose-6-phosphate (G6P; Sigma-Aldrich, St. Lois, MO), as recommended by EUCAST (52) and by CLSI guidelines (53). Fosfomycin powder (fosfomycin disodium; batch no. 20120323) was supplied by Ningbo Honor Chemtech Co., Ltd., Ningbo, China. Fosfomycin disodium solution has been reported to be stable for both a short period (24 h) and longer periods of up to 14 days (54, 55) and to have a long shelf-life (2.9×10^7 h) when stored in dry powder form (56). Moreover, the potency of the powder was regularly reevaluated during the study by agar dilution MIC testing of both a fresh solution and a solution that had been stored overnight at 4°C, and it remained stable. A stock solution was prepared from dry powder prior to each experiment. *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA) was used as a quality control organism in the susceptibility testing and for the bioassay evaluating fosfomycin concentrations in the PK studies.

For the isolates used in the treatment study, the multilocus sequence type and resistance determinants were explored by whole-genome sequencing (Illumina, San Diego, CA) and analysis of the sequence at the Centre for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/CGEpipeline-1.1).

Type 1 fimbria production, essential for establishment of a successful murine UTI (44), was confirmed for all clinical MDR strains using a mannose-sensitive agglutination of yeast cells (*Saccharomyces cerevisiae*, lot BAD0641-2; Idun Industri, Norway) as described before (57, 58).

***In vitro* time-kill studies.** Time-kill studies were performed for all isolates as described previously (59), with one minor modification. The bacterial suspension was added to tubes with fosfomycin instead of addition of fosfomycin to the bacterial suspension. This reverse order was introduced to avoid possible problems with fosfomycin solubility in the concentrated stock solution. The modification did not result in a change of the final bacterial density or the fosfomycin concentration in the test tube. Comparison of the time-kill curves obtained with the original protocol and the modified protocol did not show any difference with the fosfomycin-sensitive isolates (data not shown). Briefly, colonies from an overnight culture were suspended in 0.9% saline to an optical density at 546 nm of 0.13. One milliliter of the bacterial suspension was added to a tube containing 9 ml MH broth (Mueller-Hinton II broth; catalog number BBL 212322; Becton Dickinson, Franklin Lakes, NJ) with 25 μg/ml G6P (Sigma-Aldrich), resulting

in a bacterial density of 1×10^7 CFU/ml. The bacterial suspension was incubated at 37°C with shaking (140 rpm) for 25 min, and 1 ml was added to tubes containing 19 ml of fosfomycin at different concentrations proportional to the MIC for each strain in MH broth with 25 mg/liter G6P (Sigma-Aldrich), resulting in a bacterial density of approximately 5×10^5 CFU/ml. Viable counts were determined at time points of 0, 0.5, 1, 2, 4, and 24 h after the start of antimicrobial exposure using spot serial dilution (60).

Single NU14 colonies appearing on the MH agar plates used for determination of the number of CFU (for each fosfomycin concentration, including a negative control, at time points of 0 h, 4 h, and 24 h) were resuspended and tested for a change in the fosfomycin MIC by agar dilution, as described above. The isolated subpopulations were further passaged five times on MH agar medium (Oxoid, Waltham, MA) to evaluate the stability of the fosfomycin MIC.

PK studies. Three studies of the pharmacokinetics (PK) of fosfomycin in the bloodstream and urine were performed in outbred female albino OF-1 mice (weight, ~30 g; Charles River Laboratories, Chatillon-sur-Chalaronne, France) given a single subcutaneous (s.c.) dose of 0.75, 7.5, and 30 mg fosfomycin per mouse, respectively. Blood was sampled by periorbital cut-down, and urine was collected directly in an Eppendorf tube by placing the tube over the orifice and gently tapping the mouse on the abdomen. Samples were drawn at 15, 30, 60, 120, 180, and 240 min after dosing. Three mice were sampled at each time point. Blood was sampled in EDTA-coated Eppendorf tubes (Eppendorf, Hamburg, Germany), the tubes were centrifuged at $1,800 \times g$, and plasma was transferred to fresh Eppendorf tubes and stored at −80°C. Urine was also sampled in Eppendorf tubes and stored at −80°C. Fosfomycin concentrations were measured by a bioassay using the fosfomycin-susceptible *E. coli* ATCC 25922 strain. A bacterial suspension (10^6 CFU/ml) was floated on MH agar plates (Oxoid), and paper discs (Oxoid) were placed on the inoculated agar. Twenty microliters of fosfomycin standards (1.1, 3.3, 11, 33, and 100 mg/liter) spiked in pooled mouse plasma or urine from untreated mice was pipetted onto each disc. The same procedures were performed with triplicate samples from plasma and urine from treated mice. After overnight incubation, the inhibition zone diameters were measured and the concentrations were calculated from standard curves using regression analysis. For concentrations higher than 100 mg/liter, samples were diluted in plasma or urine until measured values below the maximum standard were obtained. The standard concentrations showed a day-to-day variation of <10%.

Calculation of dosing regimens. Doses for the *in vivo* activity study were designed to vary the $T_{>MIC}$ and AUC/MIC_{0-72}. Through interpolation and extrapolation of the PK data, the exponential equation describing the concentration curve was estimated. Dose-dependent PK indices (AUC/MIC_{0-72}, $T_{>MIC}$, C_{max}/MIC) were computed on the basis of the total drug concentrations. $T_{>MIC}$ (percent per 72 h) was calculated as the percentage of time that the drug concentration was above the MIC for the test strain (NU14; MIC, 1 mg/liter) during the treatment period (72 h); indices related to concentration dependence (C_{max}/MIC) were calculated on the basis of the highest concentrations observed experimentally 15 to 240 min after the s.c. dose (for the doses of 30 mg and 7.5 mg) or through interpolation and extrapolation (for the other doses). Since the maximal drug concentrations in plasma were registered at the first measurement time point (15 min after injection), leading to the assumption that the real peak in the fosfomycin concentration might have occurred before the first measurement, we additionally used the "$T_{>MIC}$ (in hours) after the first dose" as a surrogate marker for C_{max}/MIC after the single dose as an index by assuming that the longer the $T_{>MIC}$ (in hours) after the first dose is, the higher the C_{max}/MIC is. As the total number of doses administered within 72 h varied significantly (1 to 12 doses per 72 h; Table 2), the $T_{>MIC}$ (in hours) after the first dose did not show a linear relationship with the $T_{>MIC}$ (percent per 72 h).

Indices considering both time and concentration (AUC/MIC_{0-72}) were calculated as the size of the area under the concentration-time curve divided by the MIC using the trapezoidal rule (regular). All calculations were performed in GraphPad Prism (version 7) software (GraphPad Software, San Diego, CA). The relevant PK indices for the applied dosages are listed in Table 2.

PK/PD, virulence, and *in vivo* activity studies. The virulence of the strains was confirmed *in vivo* in the murine UTI model before proceeding to the treatment studies, as previously described (43, 44). Briefly, immunocompetent outbred albino female mice (OF-1; Charles Rivers Laboratories, Chatillon-sur-Chalaronne, France) were used. Three days prior to inoculation, the drinking water was replaced with 5% glucose solution (Sigma-Aldrich). On the inoculation day, mice were given ibuprofen (Nurofen Junior; Novartis, Basel, Switzerland) orally and tiletamine-zolazepam (Zoletil; Virbac SA, Carros, France) plus butorphanol tartrate (Torbugesic; Fort Dodge Laboratories, IA, USA) subcutaneously. The bladders of anesthetized mice were inoculated with 50 μl a bacterial suspension containing approximately 10^9 CFU/ml. Transurethral inoculation was performed with a sterilized plastic catheter (Becton Dickinson, NC, USA), which was further retracted. Urine was collected from day 2 to control for the establishment of infection. Mice were observed for any signs of pain or illness during the next 3 days. On day 5, urine was collected from the mice by gently pressing on the abdomen. The mice were then euthanized by cervical dislocation and the remaining urine was added to the tubes. Subsequently, the emptied bladder and both kidneys were aseptically removed. The urine samples were processed on the same day by spotting (20 ml) of a series of 10-fold dilutions in duplicate (spot dilution technique) on bromothymol blue agar plates (Statens Serum Institut, Copenhagen, Denmark). The organs were homogenized in a Tissue Lyser apparatus (Qiagen, Ballerup, Denmark); organ homogenizing was performed by adding 0.9% saline to the organs until the total volume of 500 μl for bladders and 100 μl for two kidneys was reached. Tissue homogenates were stored frozen and used to determine viable bacterial counts on the next day as described above for urine. Colony counts on plates were performed after 18 to 24 h of incubation at 37°C in ambient atmosphere.

The NU14 strain was used for performing the PK/PD study, where the effect of the calculated fractioned doses was evaluated. Infection in the murine model was initiated as described above. Six to

18 mice per group were used for investigation of each dose (43, 44). At 24 h postinoculation (day 2), after the collection of urine, mice were treated subcutaneously with fosfomycin (total doses of 2.82 to 30 mg per mouse for a treatment period of 72 h at a dosing frequency ranging from a single dose to dosing q6h; Table 2) or saline on days 2 to 4. On day 5, the colony counts in the urine, bladders, and kidneys were determined as described above.

To evaluate the *in vivo* activity of a selected dose on the basis of the results of the PK/PD study, the effect of treatment against three isogenic strains of NU14 and clinical MDR *E. coli* strains with different fosfomycin MICs was tested (Table 1). The *in vivo* activity studies were performed as described above. For each strain, animal groups were treated with either 15 mg fosfomycin q36h per mouse (7 mice) or vehicle (6 or 7 mice).

For the clinical isolates, bacterial populations that survived during the *in vivo* fosfomycin treatment were collected. Two colonies from both the treatment and vehicle groups and, when possible, from the same mouse were collected from plates on which urine was seeded on day 2 and from plates for the colony counts for every site of infection (urine, bladder, and kidneys) on day 5. Further, the fosfomycin MICs for these colonies were compared to the MICs for strains isolated from the same mouse on day 2. PCR was used to confirm the presence of ESBL, plasmid-mediated AmpC, and carbapenemase genes as described before (61–63). The fosfomycin MIC for the surviving strains was determined by agar dilution, as described above.

Statistical analysis. GraphPad Prism (version 7) software (GraphPad Software, San Diego, CA, USA) was used for the PK/PD analysis. The relationship between the effect and PK/PD indices was analyzed according to a sigmoidal Hill function (four-parameter dose-response curve). For each PK/PD index, the data were fitted simultaneously for all distinct doses using nonlinear regression with the ordinary least-squares (OLS) algorithm. Due to the high proportion of mice in all dose groups with reductions in the number of CFU per milliliter below the LOD, we used the cumulative effect, measured in percent (defined as the bactericidal effect), as the PD endpoint (64). This approach accounted for the high proportion of cases with colony counts below the LOD and the 40% proportion of nonaffected/self-recovered kidneys in the control group. For urine and kidneys, the bactericidal effect was defined as the counts (number of CFU per milliliter) below the LOD, while for bladder counts (number of CFU per milliliter), the bactericidal effect was defined as the proportion of counts (number of CFU per milliliter) that were lower than that for the control group.

In the *in vivo* activity study with clinical strains, the median counts (number of CFU per milliliter) were compared pairwise (between the treatment and vehicle-treated groups, separately for each strain) using the Mann-Whitney test (two-tailed) with a significance level of a P value of ≤ 0.05 (GraphPad Prism [version 7] software). For each strain, a separate control group treated with vehicle was used. We chose not to perform the correction for multiple comparisons (65).

Ethical considerations. The animal experiments were carried out at the animal facility at the Statens Serum Institute, Copenhagen, Denmark, and approved by the Danish Animal Experimentation Inspectorate (no. 2014-15-0201-00204).

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Efficacy of mecillinam against clinical multidrug-resistant *Escherichia coli* in a murine urinary tract infection model

Ilya Nikolaevich Zykov[a,b], Niels Frimodt-Møller[c], Lars Småbrekke[d], Arnfinn Sundsfjord[a,b], Ørjan Samuelsen[a,d,*]

[a] Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway
[b] Department of Medical Biology, Faculty of Health Sciences, UiT–The Arctic University of Norway, Tromsø, Norway
[c] Department of Clinical Microbiology, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark
[d] Department of Pharmacy, Faculty of Health Sciences, UiT–The Arctic University of Norway, Tromsø, Norway

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ABSTRACT

Pivmecillinam, a pro-drug of mecillinam, has been used extensively in Scandinavia for the treatment of acute lower urinary tract infections (UTIs) caused by Enterobacterales. It is still an attractive first-line drug for the empirical treatment of UTIs owing to the low prevalence of resistance as well as its favourable impact on the intestinal microbiota as a pro-drug and good in vitro efficacy against extended-spectrum β-lactamase (ESBL)- and plasmid-mediated AmpC β-lactamase-producing *Escherichia coli*. However, optimal dosing of pivmecillinam as well as its in vivo efficacy against UTIs caused by multidrug-resistant (MDR) broad-spectrum β-lactamase-producing *E. coli* has not been thoroughly studied. In this study, the efficacy of two mimicked human dosing regimens of pivmecillinam (200 mg and 400 mg three times daily) against clinical *E. coli* strains, including isolates producing ESBLs (CTX-M-14 and CTX-M-15), plasmid-mediated AmpCs (CMY-4 and CMY-6) and carbapenemases (NDM-1 and VIM-29), in a murine UTI model was compared. Both dosing regimens reduced the number of CFU/mL in urine for all strains, including mecillinam-resistant strains. Combining the effect for all six strains showed no significant differences in effect between doses for all three fluids/organs, but for each dose there was a highly significant effect in urine, kidney and bladder compared with vehicle-treated mice. Overall, this highlights the need for further studies to elucidate the role of mecillinam in the treatment of infections caused by MDR *E. coli* producing broad-spectrum β-lactamases, including specific carbapenemases.

1. Introduction

The global increase in multidrug-resistant (MDR) Enterobacterales owing to the dissemination of extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases and carbapenemases is of concern [1–3]. Moreover, MDR Enterobacterales strains frequently express co-resistance to fluoroquinolones, trimethoprim/sulfamethoxazole, aminoglycosides and, increasingly, also to colistin.

Mecillinam, in the form of the pro-drug pivmecillinam, is part of the international clinical practice recommendations for uncomplicated urinary tract infections (UTIs) [4]. The drug reaches high concentrations in urine [5], is well tolerated and has a minimal effect on the intestinal and vaginal microbiota [6,7]. Mecillinam targets penicillin-binding protein 2 (PBP2), and the prevalence of resistance remains low in the majority of European countries, including in Scandinavia where it has been extensively used for more than 30 years [7–12].

Mecillinam is considered more resistant to hydrolysis compared with other penicillins [13–16] and has good in vitro activity against ESBL-producing *Escherichia coli* and NDM/OXA-48 carbapenemase-producing *E. coli* [12–14,17–20]. Furthermore, in vitro resistance to mecillinam reported by conventional laboratory methods can, in some cases, be reverted when bacteria are grown in host urine

* Corresponding author. Present address: Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, 9038 Tromsø, Norway. Tel.: +47 776 27043.
E-mail address: orjan.samuelsen@unn.no (Ø. Samuelsen).

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[21]. Despite a high resistance mutation frequency in vitro, the relatively low prevalence of resistance is probably related to a high fitness cost of the majority of mutations [22] as well as the high concentration of mecillinam in the bladder during treatment [5].

In Scandinavia, the recommended dosing of pivmecillinam for uncomplicated UTI varies and includes either 200 mg or 400 mg three times daily (TID) for 3 days or 5–7 days [23–25]. Dosing differences could explain observed differences in the clinical efficacy of treatment of UTIs caused by ESBL-producing Enterobacterales. Jansåker et al. reported a similar bacteriological cure rate for 200 mg TID (78%) and 400 mg TID (80%) for the treatment of UTI caused by ESBL-producing *E. coli* or *Klebsiella pneumoniae* [26]. Moreover, a good clinical response (100%), but a lower proportion of bacteriological cure (25%), was identified in a study by Titelman et al. using 200 mg twice daily or TID for the treatment of lower UTI [27]. In contrast, Søraas et al. found clinical failure rates of 44% and 14% when treating community-acquired UTI with 200 mg TID caused by ESBL- versus non-ESBL-producing *E. coli*, respectively [28]. This is supported by a prospective, multicentre, observational cohort study where 200 mg TID was associated with treatment failure in patients with UTI caused by ESBL-producing *E. coli* [29]. In contrast, the same study shows that 400 mg TID gave comparable clinical and bacteriological cure rates irrespective of ESBL production [29].

To evaluate the current dosing regimens and the role of pivmecillinam in the treatment of UTIs caused by MDR *E. coli*, the current study investigated the efficacy of mimicked pivmecillinam 200 mg TID and 400 mg TID dosing for the treatment of ESBL-, plasmid-mediated AmpC- and carbapenemase-producing human clinical strains of *E. coli* in a murine UTI model.

2. Materials and methods

2.1. Strain collection

Six clinical *E. coli* strains (Table 1) obtained from patients with UTI ($n = 4$), bacteraemia ($n = 1$) and wound infection ($n = 1$) were used in this study. All strains expressed type 1 fimbriae and were able to establish infection in the UTI model [30,31]. Whole-genome sequencing (WGS) of isolates K5-08, K4-40, K71-77 and 50639799 had been performed previously [20,31]. Isolates 24623884-114 and 21773360-98 were examined by WGS as a part of the current study using a MiSeq System (Illumina Inc., San Diego, CA, USA) as described previously [20]. WGS data were analysed with respect to resistance determinants, multilocus sequence typing (MLST), virulence genes, serotype and *fimH* variant using the ResFinder v.3.1, MLST v.2.0, VirulenceFinder v.2.0, SerotypeFinder v.2.0 and FimTyper 1.0 tools at the Centre for Genomic Epidemiology (http://www.genomicepidemiology.org/) [32]. The minimum inhibitory concentration (MIC) of mecillinam was determined using Liofilchem® MIC Test Strips (Liofilchem, Roseto degli Abruzzi, Italy). For other antimicrobials, MIC determination was performed by the broth microdilution method (Thermo Fisher Scientific, East Grinstead, UK). The results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints v.9.0 (http://www.eucast.org).

2.2. Dose calculation

Two dosing regimens in mice were calculated in order to mimic human concentrations in serum and urine following oral administration of 200 mg or 400 mg of pivmecillinam. Calculations were performed by interpolation and extrapolation of data from previous studies in mice [5,30], and doses were adjusted to match the concentrations of mecillinam in urine observed in human volunteers following ingestion of pivmecillinam [5,30].

Table 1
Characteristics of the strains included in the study ($n = 6$).

Strain	Specimen	MLST	Virulence gene(s)	Serotype	FimH variant	Acquired β-lactamase gene(s)	MIC (mg/L) to β-lactams										
							MEC	AMC	TZP	TEM	CAZ	CTV	CTX	FOX	FEP	ATM	MEM	IPM	ETP
24623884-114	Urine	ST73	*cnf1, gad, iroN, iss, mchB, mchC, pic, vat*	O6:H1	H70	None	0.5	≤4	≤1	≤4	≤0.12	≤0.03	≤0.06	≤2	≤0.06	≤0.12	≤0.015	≤0.06	≤0.015
K5-08	Urine	ST2016	*lpfA*	O100:H25	H32	$bla_{CTX\text{-}M\text{-}14}$	0.25	16	2	≤4	1	0.12	≥16	8	2	2	≤0.015	≤0.06	≤0.015
K4-40	Wound	ST167	*iss*	Novel	Novel	$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, $bla_{OXA\text{-}1}$	1	32	4	≤4	8	0.06	≥16	4	8	≥16	≤0.015	≤0.06	≤0.015
K71-77	Blood culture	ST410	*cnf1, lpfA*	O8:H9	H24	$bla_{NDM\text{-}1}$, $bla_{CMY\text{-}6}$, $bla_{OXA\text{-}1}$	2	≥128	≥64	32	≥32	≥32	≥16	≥32	≥16	4	1	1	2
21773360-98	Urine	ST88	*iroN, iss, lpfA, mchF*	O8:H9	H39	$bla_{TEM\text{-}1B}$	16	128	64	≤4	0.25	0.12	≤0.06	8	0.12	≤0.12	≤0.015	0.12	≤0.015
50639799	Urine	ST6355	*cnf1, iha, iroN, iss, mchB, mchC, mchF, mcmA, sat*	O18/O18ac:H5	H106	$bla_{VIM\text{-}29}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CMY\text{-}4}$, $bla_{OXA\text{-}1}$	64	≥128	≥64	256	≥32	≥32	≥16	≥32	≥16	≥16	1	4	0.5

MLST, multilocus sequence typing; MIC, minimum inhibitory concentration; MEC, mecillinam; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; TEM, temocillin; CAZ, ceftazidime; CTV, ceftazidime/avibactam; CTX, cefotaxime; FOX, cefoxitin; FEP, cefepime; ATM, aztreonam; MEM, meropenem; IPM, imipenem; ETP, ertapenem.

Doses were calculated on the basis of the area under the curve (AUC) in urine. A 400 mg oral dose of pivmecillinam in humans reaches a mean AUC of ~900 mg/L/h mecillinam, corresponding to a dose in mice of 50 mg/kg [33]. Mice weighing 20 g were therefore given subcutaneous injections of 0.5 mg or 1 mg mecillinam (Mecillinam for intravenous administration; LEO Pharma A/S, Copenhagen, Denmark) TID, mimicking oral human pivmecillinam doses of 200 mg TID and 400 mg TID, respectively. Mecillinam was dissolved in sterile 0.9% NaCl and was prepared fresh for each experiment.

2.3. Treatment study

The treatment study was performed as previously described [30,31]. Briefly, outbred albino female OF1 mice (Charles Rivers Laboratories, Chatillon-sur-Chalaronne, France) were used. Three days prior to inoculation, drinking water was substituted with 5% glucose solution (Sigma, St Louis, MO, USA). On the inoculation day, mice were given Nurofen Junior (Novartis, Basel, Switzerland) orally and Zoletil (Virbac SA, Carros, France) plus Torbugesic (Fort Dodge Laboratories, Overland Park, KS, USA) subcutaneously. Anaesthetised mice were inoculated in the bladder with 50 µL of bacterial suspension containing ~10^9 CFU/mL using a sterilised plastic catheter (Becton Dickinson, Durham, NC, USA), which was further retracted. Urine was collected on Day 1 (after 24 h) to verify infection and then treatment was subsequently initiated. Mice (n = 105; 4–7 animals per group) were given 0.5 mg mecillinam/mouse TID, 1 mg mecillinam/mouse TID or vehicle (0.9% NaCl solution) as 0.2 mL subcutaneous injections. On Day 4, urine was collected from mice by gently pressing on the abdomen. The mice were then euthanised by cervical dislocation. The remaining urine was added to tubes and the bladder and both kidneys were aseptically removed. All samples were stored in Eppendorf tubes, with 0.9% saline added to the tubes to a total volume of 500 µL for bladders and 1000 µL for two kidneys. Homogenisation was performed in a Tissue Lyser apparatus (QIAGEN, Ballerup, Denmark). Urine samples were processed the same day by spotting 20 µL of 10-fold dilutions in duplicate (spot dilution technique) on bromothymol blue agar plates (Statens Serum Institut, Copenhagen, Denmark). Tissue homogenates were stored frozen at −80 °C and were processed similarly on the next day. Tissue homogenates were used to determine viable bacterial counts. Colony counts on plates were performed after 18–24 h of incubation at 37 °C in an ambient atmosphere.

2.4. Data analysis

Median colony counts (CFU/mL) across the groups were compared using Mann–Whitney U-test (one-tailed, as it would be natural to expect CFU counts in the antibiotic treatment group to be at least not higher compared with the vehicle group) with a significance level of $P \leq 0.05$. Corrections for multiple hypotheses testing were not performed, taking into account already small groups of comparison in animal studies [34]. Comparison of binomial (pooled and individual data) was performed using Fisher's exact test. Statistical analysis and graphical representation of the data were performed using IBM SPSS Statistics v.24 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

3. Results and discussion

Relevant characteristics of the bacterial strains are presented in Table 1. The strains represent a genetically diverse collection of clinical *E. coli* strains with a mecillinam MIC range (0.25–64 mg/L) covering the epidemiological cutoff (ECOFF) value (≤1 mg/L) as well as the EUCAST clinical susceptibility (≤8 mg/L) and resistance breakpoints (>8 mg/L). Four strains, including three strains producing ESBLs (CTX-M-14 or CTX-M-15) and carbapenemase (NDM-1), were susceptible to mecillinam. Two strains were mecillinam-resistant: the VIM-29 positive strain (MIC = 64 mg/L) and one strain harbouring TEM-1B (MIC = 16 mg/L). The strains also showed a diverse set of virulence genes and variability in terms of type 1 fimbriae and serotype (Table 1).

CFU counts for the treatment study are shown in Fig. 1, and the median log CFU/mL changes are given in Table 2. The results are depicted as the number of positive or negative cultures for urine and kidneys in Table 3, whilst ≥10^4 CFU/mL was set as a threshold for a positive culture from bladder. Although a number of urine cultures are negative for most strains, the bladder counts depict that infection was induced in all mice except one for the β-lactamase-negative wild-type 24623884-114 strain (Fig. 1). Combining the effect for all six strains, there were no significant differences in effect between the dosing regimens for all three fluids/organs, respectively (Table 4), but for each dose there was a highly significant effect for urine, kidney and bladder compared with vehicle-treated mice. Both dose regimens resulted in a statistically significant reduction in the median log CFU/mL counts in urine for all strains except for the NDM-1-producing strain (K71-77, mecillinam MIC = 2 mg/L; P = 0.09) with the higher dose (Table 2). In the bladder, a significant reduction was observed for 4/6 strains for both doses. However, the cases of a non-significant reduction varied between the doses, except for mecillinam-resistant strain 21773360-98 (MIC = 16 mg/L) where the reduction of median log CFU/mL counts were non-significant for both doses. A similar pattern was also observed in the kidneys where 1/6 strains and 2/6 strains showed a significant reduction in median log CFU/mL counts for the mimicked 200 mg and 400 mg TID doses, respectively. Apparently, the results reveal that the 200 mg mimicking dose being equal in effect to the 400 mg mimicking dose is sufficient to treat UTI almost irrespective of the MIC (up to MIC of 64 mg/L) of the infecting strain.

For urine, these findings may be explained by the sustained high drug concentrations in urine even at low doses, presumably due to active tubular secretion of mecillinam [35]. The absence of total eradication (CFU reduction below the limit of detection) in the bladder is a known phenomenon for this infection model also observed for other antimicrobials [30,31,36–38]. This may be explained by the intracellular reservoir of *E. coli* [38–40], i.e. bacteria that persist in the bladder ≥4 weeks even after the clearance from other sites [36]. Whether a similar intracellular reservoir in the bladder is present during UTI in humans has not been fully substantiated, and its importance for the effect of antibiotic treatment of UTI in humans is unknown. So far, clearance of or a significant reduction in bacteriuria has shown excellent correlation with clinical cure in studies of antibiotic treatment of uncomplicated UTIs [4,7,26–30]. The low statistical significance associated with the treatment results in kidneys is due to the fact that in most cases only one-half of the mice experience renal infection. Therefore, in order to show an effect of antibiotic treatment in this mouse model more mice should be included; this is clear from the result of combining the results for all six strains (Table 4), i.e. with 30–40 mice per group a significant effect of treatment is likely. Thus, more data are required to evaluate the use of pivmecillinam for the treatment of pyelonephritis.

The use of strains with diverse genetic backgrounds and multiple β-lactamases did not allow for specific evaluation of the isolated effect of specific β-lactamase variants on mecillinam treatment. However, the data show that mecillinam significantly reduced the bacterial load in urine, bladder and kidneys of all strains at least when combining results from groups irrespective of β-lactamase profile, indicating the efficacy of mecillinam for

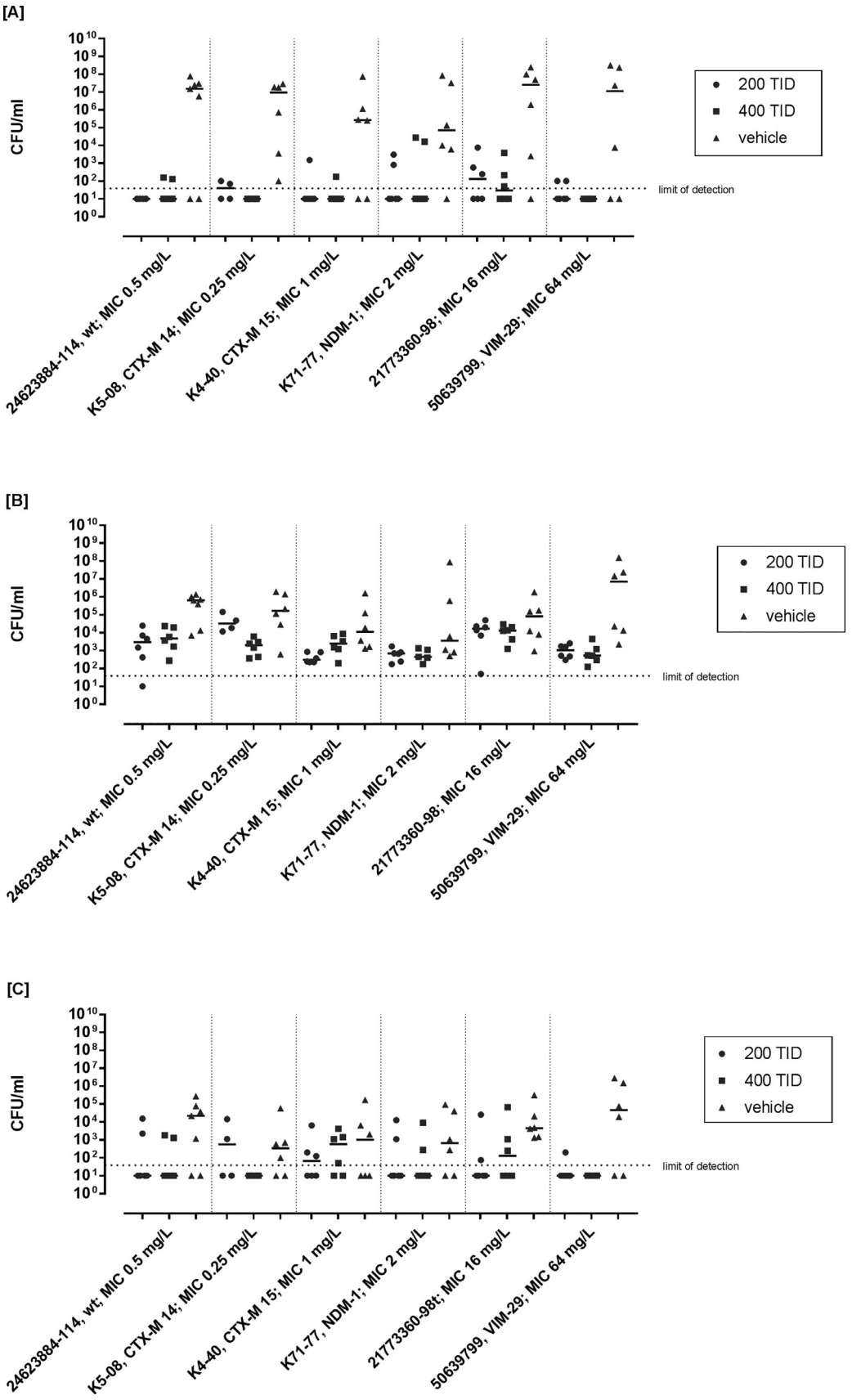

Fig. 1. Bacterial counts from [A] urine, [B] homogenised bladder and [C] homogenised kidneys of OF1 mice treated with mimicked human doses of pivmecillinam (200 mg TID and 400 mg TID) or vehicle. Symbols represent individual colony counts and the small solid horizontal lines represent the median bacterial count for each group. The dotted horizontal line indicates the limit of detection (≥50 CFU/mL). TID, three times daily; wt, wild-type; MIC, minimum inhibitory concentration.

Table 2
Changes in bacterial colony counts in urine, bladder and kidneys of mimicked human 200 mg and 400 mg TID pivmecillinam doses in a murine infection model compared with the vehicle control.

Strain	Median log CFU/mL change (*P*-value)					
	200 mg TID			400 mg TID		
	Urine	Bladder	Kidneys	Urine	Bladder	Kidneys
24623884-114	−7.19 ($P = 0.02$*)	−4.35 ($P < 0.01$*)	−4.35 ($P = 0.06$)	−7.19 ($P = 0.03$*)	−2.14 ($P < 0.01$*)	−4.35 ($P = 0.06$)
K5-08	−5.38 ($P = 0.01$*)	−0.71 ($P = 0.26$)	+0.23 ($P = 0.5$)	−6.98 ($P < 0.01$*)	−1.93 ($P = 0.01$*)	−2.53 ($P = 0.03$*)
K4-40	−5.43 ($P = 0.03$*)	−1.55 ($P < 0.01$*)	−1.18 ($P = 0.32$)	−5.43 ($P = 0.03$*)	−0.65 ($P = 0.09$)	−0.25 ($P = 0.40$)
K71-77	−4.86 ($P = 0.01$*)	−0.71 ($P = 0.03$*)	−2.82 ($P = 0.19$)	−4.86 ($P = 0.09$)	−0.90 ($P = 0.04$*)	−2.82 ($P = 0.18$)
21773360-98	−5.30 ($P = 0.02$*)	−0.68 ($P = 0.24$)	−3.65 ($P = 0.02$*)	−5.94 ($P = 0.02$*)	−0.79 ($P = 0.29$)	−1.54 ($P = 0.02$*)
50639799	−7.06 ($P = 0.05$*)	−4.67 ($P = 0.01$*)	−4.67 ($P = 0.03$*)	−7.06 ($P = 0.05$*)	−4.13 ($P < 0.01$*)	−4.67 ($P = 0.03$*)

TID, three times daily.
* Statistically significant difference compared with the vehicle control ($P \leq 0.05$), Mann–Whitney *U*-test, one-tailed.

Table 3
Results of mecillinam treatment according to positive or negative cultures for urine, bladder and kidneys.

Strain	Positive/negative cultures											
	Urine				Bladder[a]				Kidneys			
	200 mg TID	400 mg TID	Veh.	*P*-value[b]	200 mg TID	400 mg TID	Veh.	*P*-value[b]	200 mg TID	400 mg TID	Veh.	*P*-value[b]
24623884-114	0/6	2/4	5/2	0.03*	1/5	2/4	6/1	0.02*	2/4	2/4	5/2	0.13
K5-08	2/2	0/6	6/0	<0.01*	4/0	0/6	5/1	0.12	2/2	0/6	4/2	0.09
K4-40	1/5	1/5	4/2	0.06	0/6	0/6	3/3	0.03*	3/3	4/2	3/3	0.5
K71-77	2/4	2/3	5/1	0.09	0/6	0/5	2/4	0.11	2/4	2/3	4/2	0.25
21773360-98	3/3	3/3	5/1	0.20	4/2	4/2	4/2	0.71	2/4	3/3	6/0	0.03*
50639799	2/4	0/5	4/2	0.07	0/6	0/5	5/1	<0.01*	1/5	0/5	4/2	0.03*

TID, three times daily; Veh, vehicle.
[a] For the bladder, a threshold of $\geq 10^4$ CFU was set as positive culture and $<10^4$ CFU as negative culture.
[b] *P*-value for comparison of 200 mg TID and 400 mg TID versus vehicle, Fisher's exact test, one-tailed.
* Statistically significant ($P \leq 0.05$).

Table 4
Statistical comparison of total positive/negative cultures in urine, bladder and kidneys including all strains

Organ	Dosage	No. of positive cultures	No. of negative cultures	*P*-value[a]		
				Vehicle vs. 200 mg TID	Vehicle vs. 400 mg TID	200 mg TID vs. 400 mg TID
Urine	Vehicle	29	8	<0.01*	<0.01*	0.39
	200 mg TID	10	24			
	400 mg TID	8	26			
Bladder[b]	Vehicle	25	12	<0.01*	<0.01*	0.28
	200 mg TID	9	25			
	400 mg TID	6	28			
Kidneys	Vehicle	26	11	<0.01*	<0.01*	0.5
	200 mg TID	12	22			
	400 mg TID	11	23			

TID, three times daily.
[a] Fisher's exact test.
[b] For the bladder, a threshold of $\geq 10^4$ CFU was set as positive culture and $<10^4$ CFU as negative culture.
* Statistically significant ($P \leq 0.05$).

the treatment of UTI caused by broad-spectrum β-lactamase-producing *E. coli*. Although mecillinam is liable to hydrolysis by TEM-1 [13], the presence of TEM-1 in the two strains used in this study (K4-40 and 21773360-98) resulted in different in vitro susceptibility to mecillinam but almost similar significant in vivo efficacy. We have not investigated the underlying mechanisms in the present study, but it could be potentially explained by additional mechanisms such as TEM-1 overproduction (induced by the *Pa*/*Pb* promoter) [41] or *cysB* mutations [21,22]. The lack of correlation between efficacy and mecillinam MIC was also shown in a retrospective study where bacteriological cure rates were similar irrespective of whether the isolates were mecillinam-susceptible or -resistant at inclusion [42]. The diversity of strains, including the variable virulence profiles, could have influenced the results. However, separate control groups for each strain were included to control for this.

In conclusion, these data suggest that pivmecillinam is a promising option for the treatment of UTI caused by *E. coli* producing broad-spectrum β-lactamases, including NDM-1-producing *E. coli*. However, further research is required to establish the role of pivmecillinam in the treatment of infections caused by *E. coli* with other carbapenemases.

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