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Pharmacodynamics and differential activity of nitrofurantoin against ESBL-positive pathogens involved in urinary tract infections

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Background: Although nitrofurantoin has been used for >60 years for the treatment of uncomplicated urinary tract infections, its pharmacodynamic properties are not fully explored. Use is increasing because of increasing resistance to other antimicrobials due to ESBLs.

Methods: We tested nine ESBL+ and two ESBL− strains in time–kill assays. Bactericidal activity and regrowth were assessed for all species and concentrations. Early-phase pharmacodynamics was analysed with a sigmoidal E_{max} model and the maximal killing rate, slope and EC_{50}/MIC ratio were determined for each species.

Results: A bactericidal effect was found at ≥2× MIC for *Enterobacter cloacae* after 4–8 h, for *Klebsiella pneumoniae* after 8–10 h and for *Escherichia coli* after 12–16 h. Overall, no killing was observed at low sub-MIC concentrations, whereas regrowth was found at 0.5–1× MIC after a short decline in cfu. The lowest maximal killing rates were observed for *E. coli* ($0.21 \pm 0.05\ h^{-1}$), followed by *K. pneumoniae* ($0.37 \pm 0.09\ h^{-1}$) and *E. cloacae* ($0.87 \pm 0.01\ h^{-1}$). Surprisingly, the Hill slopes for these three species were significantly different (10.45 ± 9.37, 2.68 ± 0.64 and 1.01 ± 0.06, respectively), indicating a strong concentration-dependent early-phase antibacterial activity against *E. cloacae*. EC_{50}/MIC ratios were significantly lower for *E. coli* (0.24 ± 0.08 mg/L) and *K. pneumoniae* (0.27 ± 0.03 mg/L) as compared with *E. cloacae* (0.77 ± 0.18 mg/L).

Conclusions: Nitrofurantoin was bactericidal against all species, demonstrating an unusual differential pattern of activity with concentration-dependent-type killing behaviour against *E. cloacae* and time-dependent killing behaviour against *E. coli*, which may have significant consequences on species-dependent dosing regimens. The results also demonstrate that the pharmacodynamic properties of some drugs cannot be generalized within a family, here the Enterobacteriaceae.

Introduction

One of the most common human infections is urinary tract infection (UTI). The treatment of these infections is increasingly complicated by resistance to commonly used antibiotics, such as fluoroquinolones and second- and third-generation cephalosporins.[1,2] The increase in antibiotic resistance in Gram-negative bacteria and the unavailability of new antibiotics has increased interest in and a revival of old antibiotics, including nitrofurantoin.[3] Although viewed as a drug to be used against *Escherichia coli*,[4,5] nitrofurantoin is currently primarily used to treat uncomplicated UTIs caused by susceptible Enterobacteriaceae, such as *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. However, susceptibility of Enterobacteriaceae varies among species, whereas *Proteus* spp., *Pseudomonas aeruginosa* and *Streptococcus faecalis* are usually resistant to nitrofurantoin.[6] Despite being used for >60 years, evidence of clinical efficacy is still meagre.[7]

Nitrofurantoin was shown to be bactericidal in urine at therapeutic doses. Currently, the standard therapeutic dosages of nitrofurantoin for UTIs are 50 mg three to four times daily or 100 mg two or four times daily.[4] Since ESBL-producing bacteria have been progressively increasing in recent years, re-evaluation of 'old' antibiotics is needed in terms of dose optimization, duration of therapy and understanding the pharmacokinetic/pharmacodynamic relationship.[8] Limited pharmacodynamic information is available for nitrofurantoin, as is the case for many older antibiotics.[9,10]

Since the pharmacokinetic/pharmacodynamic properties of nitrofurantoin are still largely unknown, including for ESBL+ uropathogens, we determined the basic pharmacodynamic properties of this antibiotic using *in vitro* time–kill assays and analysing in depth the early-phase pharmacodynamics against common uropathogens, namely *E. coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae*.

Materials and methods

Bacterial strains

Nine ESBL+ strains (six *E. coli*, two *K. pneumoniae* and one *E. cloacae*) and two ESBL− strains (one *E. cloacae* and one *K. pneumoniae*) that were collected and analysed during a prevalence study were included in the study. Five out of the 11 strains were isolated either from urine (strains 41 and 58) or from a urinary catheter (strains 3, 11 and 39). Details are described elsewhere.[11] Briefly, PCR experiments were performed to detect the bla_{TEM}, bla_{SHV}, bla_{OXA} and $bla_{CTX\text{-}M}$ genes. PCR products were sequenced by Leiden Genome Technology Center (the Netherlands) and sequence analysis was performed with BioNumerics version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). Characteristics of the strains are shown in Table 1. Most isolates harboured CTX-M-type β-lactamases together with other resistance genes (SHV, OXA and TEM).

Bacterial suspensions were prepared in 2 mL of CAMHB (BD Bioscience, Erembodegem, Belgium) from 16–24 h old cultures on blood agar and adjusted to a turbidity equivalent to that of a 0.5 McFarland standard using a spectrophotometer. A working suspension was prepared after 1/20 dilution in CAMHB followed by 1/10 dilution in the bottle of the time–kill assays in order to obtain a final inoculum of 5×10^5cfu/mL.

Antibiotics and susceptibility testing

Nitrofurantoin was obtained from Molekula (Munich, Germany). Stock solution was prepared freshly on the day of each experiment by dissolving 0.229 g of nitrofurantoin (potency 99.36%) in 10 mL of *N*,*N*-dimethylformamide (DMF). The desired working concentrations were obtained after appropriate dilution in pre-warmed CAMHB. The MIC of nitrofurantoin for each of the strains was determined by broth microdilution, according to ISO guidelines.[12] The MIC was defined as the lowest concentration that completely inhibited visible growth. MIC determinations were made in duplicate on different days.

Time–kill assays

Glass bottles of 18 mL of CAMHB (pH 7.3 ± 0.1) containing 2- or 4-fold increasing concentrations of nitrofurantoin ranging from 0.0625× or 0.125× up to 16× MIC were prepared and kept in darkness until inoculation with 2 mL of bacterial suspension. The bottles were then incubated in darkness at 37°C under shaking conditions (260 rpm) for 24 h. For each experiment, a drug-free growth control and sterile inoculum-free control were included.

To assess the effect of nitrofurantoin on bacterial growth, 1 mL samples were taken from each bottle at selected time intervals (0, 1, 2, 3, 4, 6, 8, 16 and 24 h after the start of the experiment) and serial 10-fold dilutions in 0.9% saline solution were prepared. Ten microlitres from each dilution and an undiluted sample were plated in triplicate onto Mueller–Hinton agar plates (BD Bioscience, Erembodegem, Belgium). The numbers of cfu were counted after incubation for 20–24 h at 37°C. The lower limit of detection was 33.3 cfu/mL per plate, corresponding to 1.52 $\log_{10}$ cfu/mL. Absence of growth after 24 h was regarded as complete kill. Reproducibility of the time–kill assays was assessed by testing on different days for selected concentrations. The differences among replicates were <0.5 $\log_{10}$ cfu/mL.

To reduce the effect of carryover, particularly at concentrations ≥4× MIC, bacterial counts were calculated from at least the 10^{-1} diluted samples (if colonies were present), which yielded an antibiotic concentration below the MICs for strains. Furthermore, in order to exclude in advance any bias that might occur due to the potential antibacterial effect of the solvent DMF, time–kill curves in the presence of serial dilutions from 5% to 0.15% DMF were compared with solvent-free control and no differences were found at the concentrations tested in the present study (maximum 1.25% DMF) (results not shown).

Analysis

Viable bacterial count (cfu/mL) versus time curves were constructed for each strain. Bactericidal effects (≥3 $\log_{10}$ cfu/mL reduction from initial inocula) and regrowth (increased growth after an initial cfu reduction) were assessed by visual inspection of time–kill curves for each strain and concentration. Since nitrofurantoin is administered every 6–8 h for UTIs, we studied in depth the early-phase (within 6 h) pharmacodynamics. Thus, the kill rate ($\log_{10}$ cfu/mL$\times h^{-1}$) observed after drug addition was determined at each concentration as the slope of the log-linear regression analysis of 1–6 h time–kill curves. Kill rates were then plotted against each $\log_{10}$-transformed concentration and analysed with non-linear regression analysis using a sigmoidal E_{max} model with variable slope. The maximal killing rate (E_{max}), the concentration corresponding to 50% of E_{max} (EC_{50}), the EC_{50} corrected for the MIC (EC_{50}/MIC), the concentration corresponding to stasis (no cfu reduction compared with initial inoculum) and the Hill slope (γ) were determined for each isolate. An early-phase bactericidal effect (3 $\log_{10}$ cfu/mL reduction

Table 1. Nitrofurantoin MICs determined by broth microdilution in CAMHB and other (susceptibility) characteristics for *E. cloacae*, *E. coli* and *K. pneumoniae*

Species	Strain	Resistance phenotype	MIC (mg/L)					
			NIT	CAZ	CIP	CRO	MEM	SXT
E. cloacae	32	ESBL (CTX-M-9, SHV-12)	16	128	0.094	256	0.094	32
	94	non-ESBL (unknown)	16	48	0.023	96	0.047	0.064
E. coli	3	ESBL (CTX-M-15, TEM-84)	8	32	32	256	0.047	32
	5	ESBL (CTX-M-9, OXA-1)	8	0.5	0.012	16	0.023	0.19
	11	ESBL (CTX-M-15, OXA-1, SHV-12)	32	256	32	256	0.094	32
	41	ESBL (CTX-M-2, TEM-1)	16	6	0.012	0	0.047	0
	51	ESBL (CTX-M-15, OXA-1)	16	16	32	256	0.032	32
	82	ESBL (CTX-M-14, TEM-1)	16	3	32	256	0.047	32
K. pneumoniae	4	ESBL (CTX-M-15, OXA-1, SHV-1)	32	256	32	256	0.125	32
	39	non-ESBL (TEM-1)	16	4	0.008	0.25	0.023	0.38
	58	ESBL (SHV-11, TEM-84)	32	48	0.75	4	0.064	0.094

NIT, nitrofurantoin; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; MEM, meropenem; SXT, trimethoprim/sulfamethoxazole.

within 6 h) corresponded to a kill rate of 0.6 $\log_{10}$ cfu/mL×h^{-1}. Goodness of fit of both log-linear and E_{max} model was assessed using R^2 and post-run test. Differences in pharmacodynamic parameters among the three species were assessed with analysis of variance followed by Tukey multiple comparison tests. All analyses were performed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA).

Results

Susceptibility

The MICs of nitrofurantoin and other drugs together with the resistance phenotypes for each strain are presented in Table 1. The MICs of nitrofurantoin for *E. cloacae*, *E. coli* and *K. pneumoniae* were 16, 8–32 and 16–32 mg/L, respectively.

Time–kill assays

Representative time–kill curves for *E. coli*, *K. pneumoniae* and *E. cloacae* strains at different concentrations of nitrofurantoin are shown in Figure 1. Maximum growth in drug-free controls was observed within 8 h for all strains and was similar at ~2.0×10^9 cfu/mL. Log-linear growth rates in the drug-free control as determined over the first 6 h were also similar for all strains (0.6 $\log_{10}$ cfu/mL×h^{-1}). These findings indicate no significant

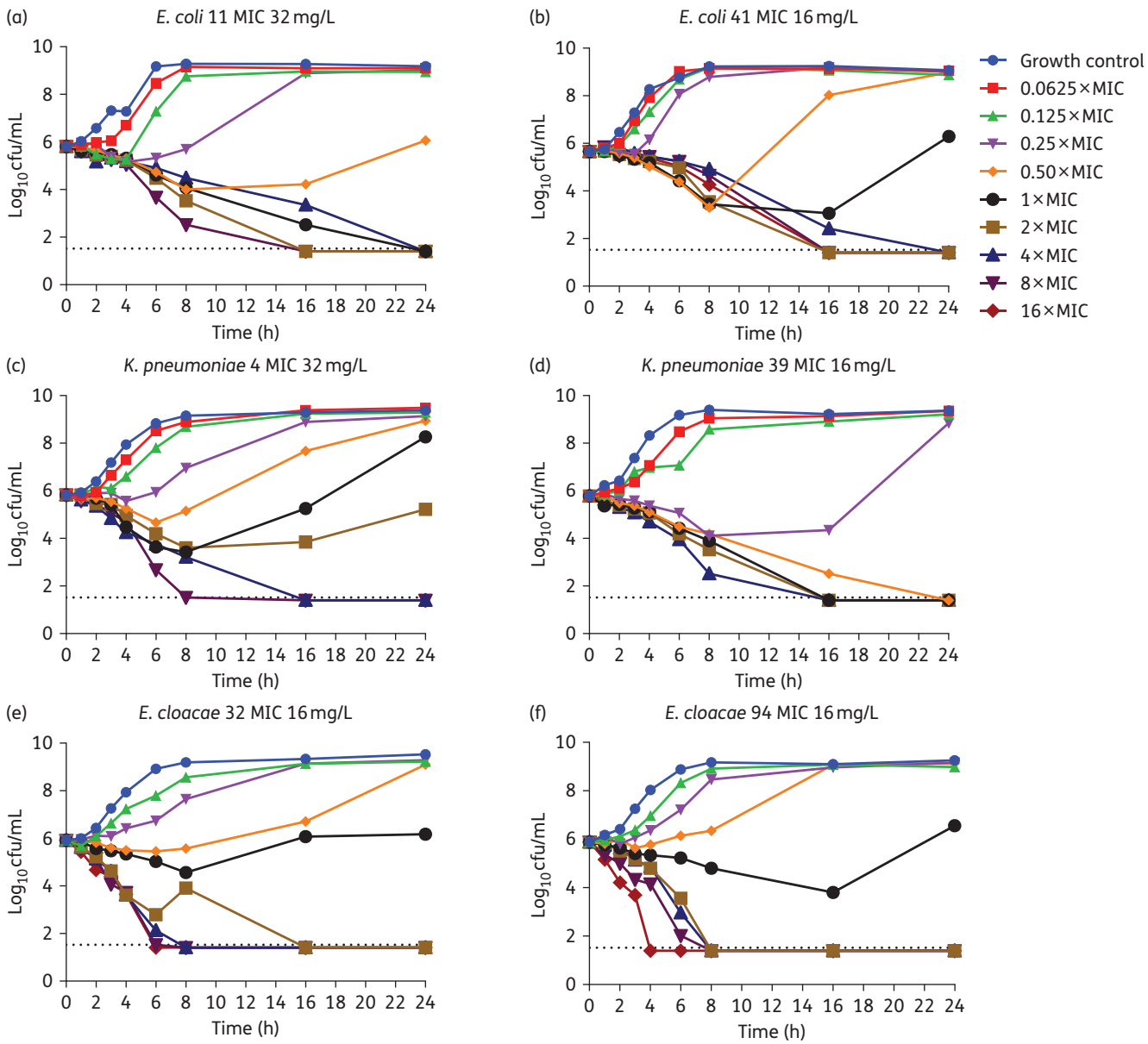

Figure 1. Typical examples of growth curves of nitrofurantoin against various strains of *E. coli* (a and b), *K. pneumoniae* (c and d) and *E. cloacae* (e and f). Cell viability ($\log_{10}$ cfu/mL) is plotted for cultures grown at different concentrations of nitrofurantoin relative to strain-specific MICs. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

differences in growth characteristics between the strains and the species examined in the absence of antibiotic.

E. coli

When exposed to nitrofurantoin, *E. coli* showed smaller decreases in the bacterial population size (<2 $\log_{10}$ cfu/mL reduction) within the first 6 h of incubation compared with *E. cloacae* and *K. pneumoniae*. Nitrofurantoin was bactericidal (3 $\log_{10}$ cfu/mL reduction) against the *E. coli* strains within 16–24 h at 2–16× MIC (equivalent to 16–256 mg/L), with the exception of strains 11 and 82 where 1× MIC was also bactericidal. For *E. coli* strains 5 and 51, complete kill was observed for 16× MIC within 8 h (data not shown).

In all *E. coli* strains, the killing was not increased at higher concentrations, but lasted for a long time with no regrowth up to 24 h, indicating a concentration-independent killing effect (Figure 1a and b). Growth was observed at concentrations ≤1× MIC after 2–4 h (0.0625–0.25× MIC), 6–8 h (0.25–0.5× MIC) and 16 h (0.5–1× MIC) for all strains.

K. pneumoniae

In the three *K. pneumoniae* strains, a bactericidal effect was observed at nitrofurantoin concentrations ≥8× MIC (equivalent to 128–256 mg/L) within 8 h and at 4× MIC within 16 h. A bactericidal effect at 2× MIC was observed only for strain 58 (data not shown). For the non-ESBL *K. pneumoniae* strain 39, a bactericidal effect was also observed at concentrations of 1–2× MIC. A less pronounced concentration-independent effect was observed for *K. pneumoniae* strains (Figure 1c and d). Growth was observed at concentrations ≤0.5–1× MIC (growth was also observed at 2× MIC in *K. pneumoniae* strain 4) after 2–4 h (0.125× MIC), 4–6 h (0.25× MIC) and 8–16 h (0.5–1× MIC).

E. cloacae

In the experiments with *E. cloacae* strains 94 and 32 (Figure 1e and f), nitrofurantoin concentrations ≥2× MIC (equivalent to 64 mg/L) were bactericidal within 6–8 h. In the non-ESBL strain, this effect was observed at 4 h with 16× MIC. The effect of nitrofurantoin increased at higher concentrations, indicating a concentration-dependent bactericidal activity against *E. cloacae*.

Growth was observed at concentrations ≤1× MIC after 2 h (0.125× MIC), 4 h (0.25× MIC), 6–8 h (0.5× MIC) and 16 h (1× MIC).

Early-phase pharmacodynamic modelling

The sigmoidal E_{max} model with variable slope fitted well to early-phase concentration–kill rate data ($R^2 > 0.96$), as shown in Figure 2 for each strain. The pharmacodynamic parameters

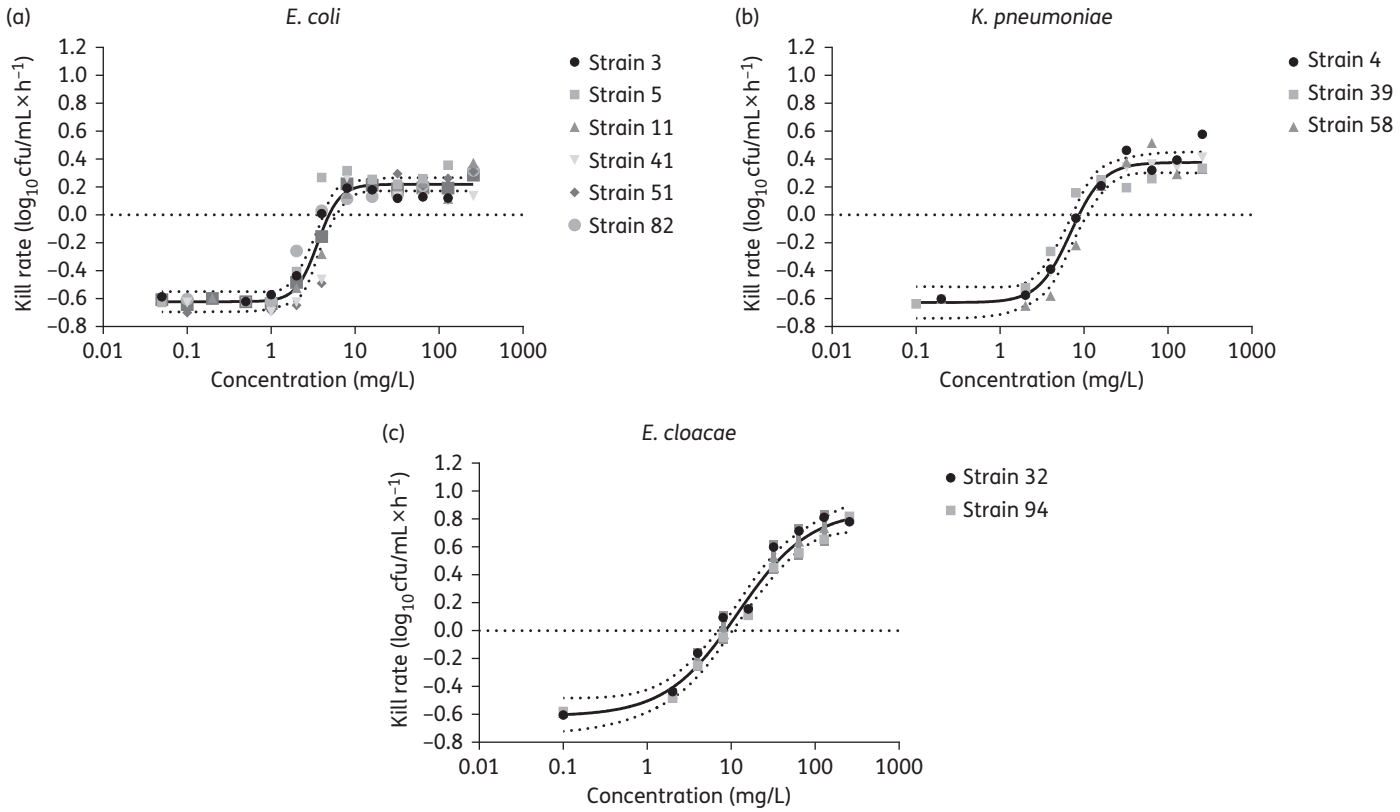

Figure 2. Early-phase pharmacodynamics of nitrofurantoin. Concentration–kill rate data and best-fitted sigmoid curves obtained from the sigmoid maximum effect (E_{max}) model for all *E. coli*, *K. pneumoniae* and *E. cloacae* strains after exposure to nitrofurantoin for 6 h. The 95% confidence band of the best-fitted curve is also plotted. The horizontal dotted line represents stasis, i.e. no cfu reduction compared with the initial inoculum. An early-phase bactericidal effect (3 $\log_{10}$ cfu/mL reduction) corresponded to a kill rate of 0.6 and was achieved only for *E. cloacae* strains. Species-specific mean (95% CI) E_{max} model parameters are shown in each graph after analysis of all strains per species together.

Table 2. Pharmacodynamic parameter estimates of nitrofurantoin against different uropathogens

Species	Strain	Growth rate	Maximal kill rate (h^{-1})	Hill slope (γ)	EC_{50} (mg/L)	EC_{50}/MIC	Stasis (mg/L)	R^2
E. cloacae	32	0.604	0.88	1.05	10.25	0.64	7.33	0.984
	94	0.579	0.86	0.97	14.44	0.90	10.13	0.992
	all (mean ± SD)	0.592 ± 0.018	0.87 ± 0.014	1.01 ± 0.056	12.35 ± 2.96	0.77 ± 0.18	8.73 ± 1.98	
K. pneumoniae	4	0.592	0.45	1.95	7.51	0.23	8.87	0.976
	39	0.639	0.27	2.97	4.43	0.28	5.89	0.990
	58	0.604	0.38	3.13	9.15	0.29	10.81	0.982
	all (mean ± SD)	0.612 ± 0.024	0.37 ± 0.091[a]	2.68 ± 0.64	7.03 ± 2.40[a]	0.27 ± 0.032[a]	8.52 ± 2.48	
E. coli	3	0.589	0.15	4.11	2.75	0.34	3.86	0.994
	5	0.627	0.28	7.60	2.33	0.29	2.59	0.993
	11	0.600	0.23	3.69	4.56	0.14	5.89	0.966
	41	0.627	0.17	23.37	4.21	0.26	4.46	0.982
	51	0.701	0.27	21.27	4.27	0.27	4.46	0.996
	82	0.604	0.19	2.67	2.27	0.14	3.58	0.968
	all (mean ± SD)	0.625 ± 0.040	0.21 ± 0.054[b,c]	10.45 ± 9.37	3.40 ± 1.059[b]	0.24 ± 0.082[b]	4.14 ± 1.10[b,c]	

[a]$P < 0.05$ for *E. cloacae* versus *K. pneumoniae*.
[b]$P < 0.05$ for *E. cloacae* versus *E. coli*.
[c]$P < 0.05$ for *K. pneumoniae* versus *E. coli*.

E_{max}, EC_{50}, EC_{50}/MIC, static concentrations and Hill slope are shown in Table 2 for each species.

The lowest maximal killing rates were observed in *E. coli* strains (0.21 ± 0.05 h^{-1}) followed by *K. pneumoniae* (0.37 ± 0.09 h^{-1}) and *E. cloacae* (0.87 ± 0.01 h^{-1}). An early-phase bactericidal effect was observed only for *E. cloacae*. The species mean ± SD Hill coefficients for *E. coli*, *K. pneumoniae* and *E. cloacae* were 10.45 ± 9.37, 2.68 ± 0.64 and 1.01 ± 0.06, respectively, indicating a strong concentration-dependent early-phase antibacterial activity against *E. cloacae* (Table 2).

The EC_{50} + EC_{50}/MIC of nitrofurantoin were significantly lower for *E. coli* and *K. pneumoniae* strains (3.40 ± 1.06 mg/L + 0.24 ± 0.08 and 7.03 ± 2.40 mg/L + 0.27 ± 0.03, respectively) as compared with *E. cloacae* (12.35 ± 2.96 mg/L + 0.77 ± 0.18), indicating that a lower nitrofurantoin concentration is needed in *E. coli* and *K. pneumoniae* to reach 50% of the maximum effect.

The mean ± SD static concentrations were 8.73 ± 1.98, 8.52 ± 2.48 and 4.14 ± 1.10 mg/L for *E. cloacae*, *K. pneumoniae* and *E. coli*, respectively, indicating that the same antibacterial effect was attained with lower nitrofurantoin concentrations for *E. coli* (Table 2).

Discussion

The main purpose of this study was to investigate the time–kill effects of nitrofurantoin and describe the early-phase pharmacodynamic relationships of nitrofurantoin against common uropathogens with various *in vitro* susceptibilities. Nitrofurantoin was bactericidal at ≥2× MIC after 4–8 h against *E. cloacae*, whereas a late bactericidal effect was found for *K. pneumoniae* after 8–10 h and for *E. coli* after 12–16 h. Overall, no killing was observed at low sub-MIC concentrations, whereas regrowth was found at 0.5–1× MIC after a small decline in cfu. Early-phase (0–6 h) pharmacodynamics was remarkably different among the three species with concentration-dependent bactericidal activity observed only for *E. cloacae* and a lower concentration required for stasis for *E. coli*.

Early-phase pharmacodynamic analysis showed high maximal killing rates for *E. cloacae* followed by *K. pneumoniae* and *E. coli*. In the various *E. coli* strains, the killing behaviour appeared to be relatively concentration independent. When there is concentration-independent killing, the concentration–effect relationship is steep, which is represented by a high Hill slope factor γ. This was indeed the case for *E. coli* with an average slope factor of 10.45, besides also the concentration range for maximal killing was narrow, and therefore resembles a β-lactam antimicrobial type of killing behaviour, such as meropenem.[13] This is in agreement with the findings of Komp Lindgren *et al.*[10]; in that *in vitro* model, the $T_{>MIC}$ correlated better to both outcome indices delta cfu_{0-24} and AUCB ($R^2 > 0.82$ and 0.67) as compared with other pharmacokinetic/pharmacodynamic indices log (AUC/MIC) ($R^2 > 0.38$ and 0.52).

Remarkably, a completely different pattern of kill was observed in *E. cloacae*. In this species, the killing increased significantly over a wide concentration range at higher concentrations, which resulted in a shallower S-curve and higher maximum kill rate. This is represented by slope factors ∼1.0 (1.05 and 0.97 in our study), indicating a shallow S-curve, which resembles the pharmacodynamic efficacy of the aminoglycoside tobramycin.[13,14] No difference in kill pattern was observed between ESBL+ and ESBL− pathogens or between strains of urogenic and non-urogenic origin. To the best of our knowledge, this is the first occasion where significantly different killing characteristics are described for the same drug for closely related species.

Some variation (20%–30%) in the pharmacodynamic parameters E_{max} and static concentrations was present among the used isolates. The drug-specific characteristics and the (un)availability of bacterial nitroreductases also might explain part of the observed inter- and intraspecies differences.[15]

E. coli strains required a longer period (12–16 h) for concentrations of 2–16× MIC to reach a bactericidal effect as compared with the study of Komp Lindgren *et al.*,[10] where the bactericidal effect was achieved within 4–8 h for *E. coli* for concentrations of 8–32× MIC irrespective of the EBSL+ or ESBL− status. In the study of Komp Lindgren *et al.*,[10] bacterial cells were washed and centrifuged before plating. However, in the study of Pembrey *et al.*,[16] centrifugation at 15000 **g** caused significant reductions of up to 36% in *E. coli* viability as compared with centrifugation at 5000 **g**; it is possible that some of the bacteria were killed during the procedure and this might have influenced the results and led to underestimation of the time needed to reach a bactericidal effect of nitrofurantoin in that study.

Although only two ESBL− strains were used in the present study, we did not find differences on nitrofurantoin effectivity between ESBL+ and ESBL− strains in agreement with Komp Lindgren *et al.*,[10] where the effectiveness of nitrofurantoin treatment did not differ between the ESBL-producing *E. coli* and the non-ESBL-producing *E. coli* strains.

The clinical implications of these findings are related to the concentration-dependent activity of nitrofurantoin against *E. cloacae* and to the finding that a bactericidal effect occurred at different exposure times and concentrations. Increased urine concentrations may enhance killing against *E. cloacae*, but not the other species, whereas nitrofurantoin concentrations should remain higher than the MIC for the pathogen for a longer time for *E. coli* than the other species. Dosing regimens should target urine concentrations ≥2× MIC (i.e. ≥64 mg/L for the isolates of the present study) for ≥4 h for *E. cloacae*, 8 h for *K. pneumoniae* and 12 h for *E. coli*. The dose of 100 mg given every 8 h resulted in urine concentrations <16 mg/L, whereas when 100 mg was given every 6 h urine concentrations were always >16 mg/L.[17] Urine concentrations may reach 250 mg/L at doses up to 400 mg every 6 h, but these doses may not be feasible clinically.[18] Based on the present findings, dosing regimens may have to be adjusted depending on the microorganism cultured, with a more frequently administered standard dosing regimen for *E. coli* and *K. pneumoniae*. A limitation is the lack of recent urinary pharmacokinetic data, which limits the extrapolation of our results. In addition, the differential killing activity against *E. coli*, *K. pneumoniae* and *E. cloacae* found in this study might be different from the human (*in vivo*) situation where during UTI an inflammatory response is induced and neutrophils are activated. Since we did not mimic the innate immune response in these experiments, we can only speculate on the impact of that on a possible dosing regimen and the influence of the immune system on the pharmacodynamics of nitrofurantoin during UTI should be investigated further.

In summary, our findings show that nitrofurantoin was bactericidal at different exposure times against all species, but showed distinctly different patterns of kill against different species irrespective of their ESBL status. This phenomenon is highly unusual and not observed for other drugs. The differential pattern of activity may have significant consequences for dosing depending on the pathogen and this should be explored further.

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Transparency declarations

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