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 EC50/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 ± 0.05 h⁻¹), followed by K. pneumoniae (0.37 ± 0.09 h⁻¹) and E. cloacae (0.87 ± 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. EC50/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.8 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.9 Limited pharmacodynamic information is available for nitrofurantoin, as is the case for many other 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-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 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).

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 and was defined as the lowest concentration corresponding to 50% of initial inoculum (MIC_{50}) or 90% (MIC_{90}). The MIC of nitrofurantoin was determined from at least the 10^{-1} diluted sample, 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).

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× to 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.

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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·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}) and the concentration corresponding to 50% of E_{max} (EC_{50}) were determined for the MIC (EC_{50}/MIC), the concentration corresponding to stasis (no cfu reduction compared with initial inoculum) and the Hill slope (V) 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

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Resistance phenotype</th>
<th>MIC (mg/L)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NIT</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>32</td>
<td>ESBL (CTX-M-9, SHV-12)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>non-ESBL (unknown)</td>
<td>16</td>
</tr>
<tr>
<td>E. coli</td>
<td>3</td>
<td>ESBL (CTX-M-15, TEM-84)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>ESBL (CTX-M-9, OXA-1)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>ESBL (CTX-M-15, OXA-1, SHV-12)</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>ESBL (CTX-M-2, TEM-1)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>ESBL (CTX-M-15, OXA-1)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>ESBL (CTX-M-14, TEM-1)</td>
<td>16</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>4</td>
<td>ESBL (CTX-M-15, OXA-1, SHV-1)</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>non-ESBL (TEM-1)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>SHV-11, TEM-84</td>
<td>32</td>
</tr>
</tbody>
</table>

NIT, nitrofurantoin; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; MEM, meropenem; SXT, trimethoprim/sulfamethoxazole.
within 6 h) corresponded to a kill rate of 0.6 log₁₀ cfu/mL × h⁻¹. Goodness of fit of both log-linear and E_max model was assessed using R² 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 × 10⁹ 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 log₁₀ cfu/mL × h⁻¹). These findings indicate no significant

![Figure 1](http://jac.oxfordjournals.org/)
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 log₁₀ cfu/mL reduction) within the first 6 h of incubation compared with *E. cloacae* and *K. pneumoniae*. Nitrofurantoin was bactericidal (3 log₁₀ 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 ≤0.5–1 × 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 6–8 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 $E_{\text{max}}$ 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](image_url). Early-phase pharmacodynamics of nitrofurantoin. Concentration–kill rate data and best-fitted sigmoid curves obtained from the sigmoid maximum effect ($E_{\text{max}}$) 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 log₁₀ cfu/mL reduction) corresponded to a kill rate of 0.6 and was achieved only for *E. cloacae* strains. Species-specific mean (95% CI) $E_{\text{max}}$ model parameters are shown in each graph after analysis of all strains per species together.
In vitro pharmacodynamics of nitrofurantoin

Table 2. Pharmacodynamic parameter estimates of nitrofurantoin against different uropathogens

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Growth rate</th>
<th>Maximal kill rate (h⁻¹)</th>
<th>Hill slope (Y)</th>
<th>EC₅₀ (mg/L)</th>
<th>EC₅₀/MIC</th>
<th>Stasis (mg/L)</th>
<th>R²</th>
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<tr>
<td>E. cloacae</td>
<td>32</td>
<td>0.604</td>
<td>0.88</td>
<td>1.05</td>
<td>10.25</td>
<td>0.64</td>
<td>7.33</td>
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<td>94</td>
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<td>10.13</td>
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<td>E. coli</td>
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<td>0.589</td>
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<td>3.40±1.059b</td>
<td>0.24±0.082b</td>
<td>4.14±1.10b</td>
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</table>

aP<0.05 for E. cloacae versus K. pneumoniae.
bP<0.05 for E. cloacae versus E. coli.
cP<0.05 for K. pneumoniae versus E. coli.

Eₘₐₓ, EC₅₀, EC₅₀/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 EC₅₀ + EC₅₀/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 Y. 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.14; in that in vitro model, the T₅₀/MIC correlated better to both outcome indices delta cfu₀–⁴ and AUCB (R² > 0.82 and 0.67) as compared with other pharmacokinetic/pharmacodynamic indices log (AUC/MIC) (R² > 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 Eₘₐₓ 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., where the bactericidal effect was achieved within 4–8 h for E. coli for concentrations of 8–32× MIC irrespective of the ESBL+ or ESBL− status. In the study of Komp Lindgren et al., bacterial cells were washed and centrifuged before plating. However, in the study of Pembray et al., centrifugation at 15 000 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 effectiveness between ESBL+ and ESBL− strains in agreement with Komp Lindgren et al., 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 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. Urine concentrations may reach 250 mg/L at doses up to 400 mg every 6 h, but these doses may not be feasible clinically. 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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**References**


