Pharmacodynamics of moxifloxacin against a high inoculum of Escherichia coli in an in vitro infection model

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Objectives: Escherichia coli is the leading bacterial species implicated in intra-abdominal infections. In these infections a high bacterial burden with pre-existing resistant mutants are likely to be encountered and resistance could be amplified with suboptimal dosing. Our objective was to investigate the pharmacodynamics of moxifloxacin against a high inoculum of E. coli using an in vitro hollow fibre infection model (HFIM).

Methods: Three wild-type strains of E. coli (ATCC 25922, MG1655 and EC28044) were studied by exposing ~2 × 10^8 cfu/mL (20 mL) to escalating dosing regimens of moxifloxacin (ranging from 30 to 400 mg, once daily). Serial samples were obtained from HFIM over 120 h to enumerate the total and resistant subpopulation. Quinolone resistance-determining regions of gyrA and parC of resistant isolates were sequenced to confirm the mechanism of resistance.

Results: The pre-exposure MIC of the three wild-type strains was 0.0625 mg/L. Simulated moxifloxacin concentration profiles in HFIM were satisfactory (r² ≥ 0.94). Placebo experiments revealed natural mutants, but no resistance amplification. Regrowth and resistance amplification was observed between 30 mg/day (AUC/MIC = 47) and 80 mg/day dose (AUC/MIC = 117). Sustained bacterial suppression was achieved at ≥120 mg/day dose (AUC/MIC = 180). Point mutations in gyrA (D87G or S83L) were detected in resistant isolates.

Conclusions: Our results suggest that suboptimal dosing may facilitate resistance amplification in a high inoculum of E. coli. The clinical dose of moxifloxacin (400 mg/day) was adequate to suppress resistance development in three wild-type strains. Clinical relevance of these findings warrants further in vivo investigation.

Keywords: quinolones, E. coli, resistance

Introduction

Moxifloxacin is a broad-spectrum fluoroquinolone used to treat a variety of infections, including complicated intra-abdominal infections (approved in the USA), community-acquired pneumonia and acute bacterial sinusitis. However, the recent increase in resistance to fluoroquinolones is a major concern and warrants immediate steps to be taken to suppress (or delay) emergence of resistance.1,2 One of the strategies to maximize microbial kill and to suppress emergence of resistance is pharmacodynamic-based dosing. It is widely accepted that a dense bacterial population of sufficient size consists of two subpopulations—susceptible and resistant.3 Considering the mutational frequency of bacteria, a high inoculum of bacteria (more than the inverse of mutational frequency to resistance) is likely to harbour resistant mutants at the start of therapy and a suboptimal dose exposure would selectively amplify the resistant subpopulation. On the other hand, an optimal dose exposure could lead to suppression of both the susceptible and resistant subpopulation.

Escherichia coli is a common pathogen implicated in intra-abdominal infections,4 which are often associated with high bacterial inocula.5 Pre-existing resistant mutants are likely
present in these infections and a suboptimal dose exposure will lead to emergence of resistance. Several studies have examined the pharmacodynamics of fluoroquinolones (e.g. ciprofloxacin,7–9 levofloxacin7–9 and moxifloxacin8,9), but none has investigated the propensity of resistance suppression by moxifloxacin against a high inoculum of E. coli.

Resistance to fluoroquinolones could be caused by multiple mechanisms. Chromosomally mediated resistance is more common and may occur through alterations in the genes encoding DNA gyrase (subunits gyrA and gyrB) and topoisomerase IV (subunits parC and parE), where the target binding sites of fluoroquinolones are located.10,11 Mutations are often detected in discrete regions of DNA gyrase and topoisomerase IV known as quinoline resistance-determining regions (QRDRs).10 Resistance can also occur by overexpression of efflux pumps, such as AcrAB,10,11 Lately, plasmid-mediated quinolone resistance (such as qnrA) is also known to be implicated.11,12

In this study, we utilized an in vitro hollow fibre infection model (HFIM) to study the pharmacodynamics of moxifloxacin against a high inoculum of E. coli. Specifically, the relationship between drug exposure and resistance amplification was examined. Additionally, we also attempted to characterize the mechanism of moxifloxacin resistance developed as a consequence of suboptimal pharmacodynamic exposure.

Materials and methods

Antimicrobial agent

Moxifloxacin powder was a gift from Bayer Pharmaceuticals (West Haven, CT, USA). A stock solution of moxifloxacin in sterile water was prepared, aliquotted and stored at −70°C. Prior to each susceptibility testing, an aliquot of the drug was thawed and diluted to the desired concentrations with cation-adjusted Mueller–Hinton broth (Ca-MHB; BBL, Sparks, MD, USA) or sterile water, as required.

Microorganisms

Three wild-type strains of E. coli—American type culture collection (ATCC) (Manassas, VA, USA) 25922, MG1655 and EC28044—were used in this study. MG1655 is a molecular standard strain, for which the whole genome has been sequenced.13 EC28044 is a urinary (pathogenic) isolate obtained from a patient in Houston, TX, USA. These isolates were stored at −70°C in Protect® storage vials (Key Scientific Products, Round Rock, TX, USA). Fresh isolates were subcultured at least twice on 5% blood agar plates (Hardy Diagnostics, Santa Maria, CA, USA) for 24 h at 35°C prior to each experiment.

Susceptibility studies

MICs/MBCs were determined in Ca-MHB using the broth macrodilution method as described by the CLSI.14 The final concentration of bacteria in each broth macrodilution tube was ~5×10⁷ cfu/mL of Ca-MHB. Serial 2-fold dilutions of moxifloxacin were prepared. The MIC was defined as the lowest concentration of drug that resulted in ≥99.9% kill of the initial inoculum. Drug carryover effect was assessed by visual inspection of the distribution of colonies on MHA plates. The experiment was repeated at least twice on separate days.

In vitro HFIM

The basic design of the system has been described previously.15 The human non-protein-bound serum pharmacokinetic exposures of moxifloxacin were simulated over 5 days, with repeated doses given once daily. The targeted elimination half-life was 12 h (reported range 7–14 h).16–18 Various dose exposures, ranging from 30 to 400 mg given once daily, were simulated.

On the day of experiment, overnight culture of the isolate was diluted with pre-warmed Ca-MHB and incubated further at 35°C until reaching late log-phase growth. The targeted inoculum was calculated based on absorbance at 630 nm and 20 mL of ~2×10⁷ cfu/mL bacteria (total population ~4×10⁹ cfu) were introduced to the extracapillary space of the hollow fibre cartridge (Fibercell Systems, Inc., Frederick, MD, USA). The experimental set-up was maintained at 35°C in a humidified incubator for 120 h. Bacterial samples (500 µL) were taken at various time points (0, 4, 8, 24, 48, 72, 96 and 120 h; pre-dose where appropriate) in duplicate from the sampling ports. Before plating, the samples were centrifuged at 10000 g for 15 min at 4°C. Supernatant was discarded and the pellet was resuspended with saline to minimize the drug carryover effect. These samples (50 µL) were spirally plated (Spiral Biotech, Bethesda, MD, USA) on drug-free MHA plates and moxifloxacin-supplemented MHA plates. Plating on drug-free MHA plates was to quantify the total bacterial population and the moxifloxacin-supplemented plates (3×MIC) were used to ascertain the bacterial population with reduced susceptibility (resistance). Considering the fact that a 2-fold difference in the MIC value is a generally accepted interday deviation for MIC testing, a 3×MIC supplemented plate would allow reliable detection of the resistant population. Drug-free plates were incubated for 24 h and moxifloxacin-supplemented plates were incubated for ≤72 h (if required) at 35°C before counting. Bacterial density was quantified by an automated colony counter (IUL, Farmingdale, NY, USA). The theoretical lower limit of detection was 400 cfu/mL. Placebo HFIM experiments were performed for the three wild-type strains. Samples (500 µL) were also collected in duplicate on alternate days at various time points to ascertain the simulated drug exposure. These samples were stored (<1 month) at −20°C until analysed by a validated HPLC assay as described below. A one-compartment linear model was fitted to the observed concentration–time profiles using the ADAPT II program.19

HPLC assay

A validated HPLC method (Waters 2695 separations module) was used to determine the concentration of moxifloxacin in samples obtained from HFIM. The column used was NovaPak C18 [3.9×15 mm, particle size 4 µm (Waters Corporation, Milford, MA, USA)]. A C18 guard column (Waters) was also used. Levofoxacin (100 mg/L) was utilized as an internal standard. The mobile phase included acetonitrile, 0.1 M phosphoric acid (adjusted to pH 3) and 0.01 M n-octylamine (adjusted to pH 3). A gradient elution was performed. Detection was done at 290 nm by a UV detector (Waters 2487 UV detector). Stock solutions (1 g/L) of moxifloxacin and levofloxacin were prepared in HPLC-grade water and stored at −20°C until used. A working solution (100 mg/L) of moxifloxacin was prepared by spiking the stock solution into Ca-MHB. Aliquots of
Confirmation of moxifloxacin resistance mechanism

Six random isolates were recovered from the moxifloxacin-supplemented plates at the end of the each HFIM experiment and subcultured on 5% blood agar plates. Susceptibility testing of these recovered isolates to moxifloxacin were repeated to confirm the emergence of resistance. In addition, Etest (AB Biodisk, Piscataway, NJ, USA) was also performed to evaluate cross-resistance to ciprofloxacin and levofloxacin. Subsequently, the QRDRs of the gyrA and parC genes of resistant isolates (and their respective parent strains) were amplified by PCR, to provide molecular evidence of moxifloxacin resistance. The GenBank accession numbers and sequences of primers used are listed in Table 1. Amplification was performed in a thermal cycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturing step of 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and final extension at 72°C for 10 min. No template (negative) controls were included and the reactions were evaluated by electrophoresis in 2% agarose gel. The PCR products were sequenced by Lonestar Laboratories, Houston, TX, USA.

Results

Susceptibility studies

The pre-exposure MIC for the three wild-type strains of E. coli was 0.0625 mg/L. The pre-exposure MBC for ATCC 25922 and MG1655 was also 0.0625 mg/L, while that for EC28044 was 0.125 mg/L. MG1655 was also 0.0625 mg/L, while that for EC28044 was 0.0625 mg/L. The pre-exposure MBC for ATCC 25922 and EC28044 was 0.0625 mg/L. The pre-exposure MIC for the three wild-type strains of E. coli was also 0.0625 mg/L. The pre-exposure MBC for ATCC 25922 and EC28044 was 0.0625 mg/L. The pre-exposure MIC for the three wild-type strains (data not shown). The microbial populations could not be controlled after 24 h despite repeated dosing, as the dose exposure was insufficient to prevent the resistant subpopulation from proliferating. For MG1655 (Figure 3b), regrowth was apparent at 48 h with a suboptimal dose of 80 mg once daily (AUC/MIC=117). In all cases, the resistant subpopulation almost completely replaced the susceptible population over time. Further increasing the dose to 80 mg/day (AUC/MIC=117) for ATCC 25922 (Figure 2c) and EC28044 (Figure 4c) resulted in suppression of resistance. Similarly, a dose of 120 mg/day (AUC/MIC=180) for MG1655 (Figure 3c) led to resistance suppression. The clinical dose of moxifloxacin (400 mg/day, equivalent to AUC/MIC=627) was sufficient to suppress the resistance development in all three wild-type strains of E. coli (data not shown). The microbial responses observed with various pharmacodynamic exposures are summarized in Table 2.

In vitro HFIM

The changes in bacterial burden over time with different dosing regimens of moxifloxacin are shown in Figures 2–4. Placebo HFIM experiments for the three wild-type strains revealed that a very small proportion of pre-existing natural mutants were present. However, the relative proportion of the pre-existing resistant subpopulation to the total population remained unchanged over time. Placebo did not exert any selective pressure on the heterogeneous bacterial populations; therefore, no resistance amplification was observed (Figures 2a, 3a and 4a).

On the other hand, in the presence of a selective pressure exerted by a suboptimal dose of 30 mg once daily (AUC/MIC=47), amplification of resistant mutants was observed for ATCC 25922 and EC28044. As shown in Figures 2(b) and 4(b), a considerable reduction in total bacterial burden was observed at 4 h for both strains. This reduction was likely due to the rapid and preferential killing of the susceptible subpopulation. However, the bacterial populations could not be controlled after 24 h despite repeated dosing, as the dose exposure was insufficient to prevent the resistant subpopulation from proliferating.

Confirmation of moxifloxacin resistance mechanism

Isolates recovered from moxifloxacin-supplemented plates were subcultured at least three times on blood agar plates to confirm
the resistant phenotype was stable. Susceptibility studies performed on these isolates revealed resistance to moxifloxacin with an 8- to 32-fold increase in MIC (data not shown). Cross-resistance was also observed with ciprofloxacin and levofloxacin. Sequencing the QRDR region of \textit{gyrA} and \textit{parC} revealed that all the resistant isolates had one amino acid substitution in \textit{gyrA}. While all the resistant isolates derived from MG1655 had an S83L substitution, there was a D87G substitution in resistant isolates derived from ATCC 25922 and EC28044.

**Discussion**

Enterobacteriaceae, especially \textit{E. coli}, remain one of the major pathogens implicated in intra-abdominal infections.\textsuperscript{4} These infections often harbour a high inoculum of bacteria and resistance is likely to develop subsequent to suboptimal dosing. Hence, knowledge of the pharmacodynamics of antibacterial agents at a high inoculum is indispensable in order to formulate treatment strategies to suppress resistance.

Recently, emphasis has been placed on understanding the pharmacodynamics of antimicrobial agents (especially fluoroquinolones) to improve clinical outcomes. Studies by Forrest \textit{et al.}\textsuperscript{20} and Drusano \textit{et al.}\textsuperscript{21} were pioneering in this regard. In a retrospective analysis using patients receiving ciprofloxacin for nosocomial infections, Forrest \textit{et al.}\textsuperscript{20} characterized the drug exposure determinants of treatment outcome. They demonstrated that the probability of a good clinical outcome was related to the AUC/MIC ratio. Their data suggested that the clinical and bacteriological outcomes could be significantly improved by optimizing the dosing exposure. In another study, Drusano \textit{et al.}\textsuperscript{21}
investigated the factors affecting the likelihood of a good microbiological or clinical outcome in patients with nosocomial pneumonia. They advocated that only the age of patients and the achievement of an AUC/MIC ratio of $\geq 87$ had a significant impact on pathogen eradication. Differences in clinical outcomes with age were probably related to underlying co-morbidities and the impact of the immune system in elderly individuals.

Understanding fluoroquinolone pharmacodynamics has also been targeted towards suppression of resistance. In cases where resistance is due to selective amplification of pre-existing resistant mutants, the knowledge of pharmacodynamics can be utilized to suppress resistance development by optimizing the drug exposure. Different pathogens and various experimental set-ups have been used to identify the drug exposure with the highest probability of achieving this goal. Using a non-neutropenic mouse model to examine levofloxacin exposure and resistance development in Pseudomonas aeruginosa, Jumbe et al. put forward a mathematical model to describe the target exposure required to suppress the emergence of a resistant bacterial subpopulation. This model was extended and validated in two separate in vitro studies by Tam et al., where they investigated the impact of garenoxacin exposure intensity on resistance emergence in Staphylococcus aureus and P. aeruginosa. The authors suggested an optimal drug exposure could be used in suppressing resistance in S. aureus and P. aeruginosa. Other pathogens, such as Mycobacterium tuberculosis, have also been examined for the propensity of in vitro resistance suppression.

A second approach to suppress resistance has been put forward by Hansen et al., which is based on the concept that mutations in QRDR are likely to be sequential instead of simultaneous. Thus, a drug concentration (mutant prevention concentration) that prevents the amplification of a first-step mutation would also prevent subsequent mutations. The concept has evolved into minimizing the time in which the drug concentration is in the range that selectively enriches the resistant subpopulation (mutant selection window).

Moxifloxacin pharmacodynamics has been investigated by many authors, but mostly in Gram-positive microorganisms such as S. aureus, Streptococcus pneumoniae, and Streptococcus pyogenes. Anaerobes and M. tuberculosis have also been examined in this context. In Gram-negative microorganisms, the pharmacodynamics of moxifloxacin have been investigated against P. aeruginosa and E. coli. In particular, an in vitro study by Hermens et al. compared the activity of levofloxacin plus metronidazole versus moxifloxacin in a mixed-infection model of E. coli and Bacteroides fragilis. Only one clinical isolate of E. coli and B. fragilis, and one dose exposure was studied for 24 h. Although this study aimed to compare combination versus monotherapy for intra-abdominal infections, the inoculum used in the study was $10^6$ cfu/mL, which might not be reflective of the bacterial burden.

### Table 2. Summary of bacterial response observed with the simulated exposures of moxifloxacin

<table>
<thead>
<tr>
<th>AUC/MIC</th>
<th>47</th>
<th>117</th>
<th>145</th>
<th>180</th>
<th>627</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25922</td>
<td>regrowth</td>
<td>suppression</td>
<td>NT</td>
<td>suppression</td>
<td>NT</td>
</tr>
<tr>
<td>MG1655</td>
<td>NT</td>
<td>regrowth</td>
<td>NT</td>
<td>suppression</td>
<td>suppression</td>
</tr>
<tr>
<td>EC28044</td>
<td>regrowth</td>
<td>suppression</td>
<td>NT</td>
<td>NT</td>
<td>suppression</td>
</tr>
</tbody>
</table>

NT, not tested.
Pharmacodynamics of moxifloxacin

encountered clinically. The study also did not delineate the drug exposure necessary for resistance suppression.

Another study by Odenholt et al.31 compared the pharmacodynamics of moxifloxacin and levofloxacin against *S. pneumoniae*, *S. aureus*, *Klebsiella pneumoniae* and *E. coli* isolates with differing susceptibility in an *in vitro* kinetic model. The authors suggested that a maximal antibacterial effect was observed when the AUC/MIC exceeded 100. However, a low inoculum of 5×10⁸ cfu/mL and a 24 h time course was studied, which may not closely represent the pathophysiology of intra-abdominal infections and clinical treatment. As such, the pharmacodynamics of moxifloxacin to suppress resistance in *E. coli* has not been thoroughly studied.

Results from our current study provide additional insights to our understanding of the pharmacodynamics of moxifloxacin. First, in terms of the drug exposure necessary to counterelect select resistance, three wild-type strains from different backgrounds were used to impart greater generalizability to our hypothesis. Our data suggested an AUC/MIC ratio of 117–180 would be needed. These results are in general agreement with Odenholt et al.31 and the difference could be due to variations in the strains examined. Second, the *in vitro* hollow fibre system provided flexibility to test our hypothesis. This *in vitro* system enabled us to study high inocula of bacteria, which might be difficult in animal models since such high inocula could be associated with unacceptable mortality. It also allowed us to investigate a longer drug exposure duration, which has not been commonly reported in other infection models. Third, our observation of one *gyrA* mutation in all resistant isolates was consistent with the findings of Morgan-Linnell et al.32 However, unlike their observation of an additional *parC* mutation in 85% of isolates, we found no *parC* mutation in any of the resistant isolates. One of the reasons for having these *parC* mutations could be attributed to the fact that most of the isolates had several-fold higher MICs than the randomly selected resistant isolates in this study. A higher selection threshold (e.g. 8–12×MIC) might be needed to detect these high-level resistant mutants reliably. A second reason could be that moxifloxacin possesses greater ability to prevent resistance development, which was not extensively used in the previous study.32

It should be noted that the results of this study are conservative, as the *in vitro* infection system lacked an immune response and represented only the direct relationship between drug exposure and microorganism. However, this allowed us to delineate an unambiguous pharmacodynamic response for testing our hypothesis. Resistance to fluoroquinolones could be a consequence of one or a combination of multiple mechanisms (e.g. mutations in QRDR, efflux pump overexpression or mediated via qnrA). Only QRDR of *gyrA* and *parC* were screened for mutation as these are the primary targets11 and mutations in *gyrB* or *parE* do not consistently contribute to a significant increase in MIC.32 However, the possibility of *gyrB* or *parE* mutation(s) and efflux pump involvement in conferring resistance cannot be completely ruled out. This study was not designed to delineate the role of plasmid-mediated quinolone resistance.

In summary, our data suggest that a suboptimal dose exposure of moxifloxacin may facilitate resistance amplification in a high inoculum of *E. coli*. The clinical dose of moxifloxacin of 400 mg/day was likely adequate in suppressing the emergence of resistance. The clinical relevance of this study needs further *in vivo* investigation.

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Transparency declarations

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