Emergence of resistant *Streptococcus pneumoniae* in an in vitro dynamic model that simulates moxifloxacin concentrations inside and outside the mutant selection window: related changes in susceptibility, resistance frequency and bacterial killing

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**Objectives:** According to the mutant selection window (MSW) hypothesis, resistant mutants are selected or enriched at antibiotic concentrations above the MIC but below the mutant prevention concentration (MPC). To test this hypothesis, *Streptococcus pneumoniae* ATCC 49619 (MIC 0.1 mg/L; MPC 0.5 mg/L) was exposed to moxifloxacin concentrations below the MIC, above the MPC and between the MIC and MPC, i.e. within the MSW.

**Methods:** Daily administration of moxifloxacin for 3 consecutive days was mimicked using a two-compartment dynamic model with peripheral units containing a starting inoculum of $10^8$ cfu/mL *S. pneumoniae*. Changes in susceptibility were examined by repeated MIC determinations and by plating the specimens on agar containing zero, $2 \times$ MIC, $4 \times$ MIC and $8 \times$ MIC of moxifloxacin.

**Results:** Both in terms of the MIC and resistance frequency, *S. pneumoniae* resistance developed at concentrations that fell inside the MSW [ratios of 24 h area under the curve (AUC24) to MIC between 24 and 47 h]. A Gaussian-like function fitted the AUC24/MIC-dependent increases in MIC and resistance frequency with central points at AUC24/MICs of 38 and 42 h, respectively, where resistant mutants are enriched selectively. Selective enrichment of resistant mutants was not seen at AUC24/MICs <10 h or >100 h.

**Conclusions:** These data suggest that AUC24/MICs >100 h may protect against the selection of resistant *S. pneumoniae* mutants. Since the usual 400 mg dose of moxifloxacin provides much higher AUC24/MIC (270 h) than the MICs, it is expected to prevent mutant selection at clinically achievable concentrations. Also, these data provide further support for the MSW hypothesis.

**Introduction**

According to a recently proposed hypothesis,¹ resistant mutants are selected at antibiotic concentrations above the MIC and below the mutant prevention concentration (MPC), i.e. within the mutant selection window (MSW). This hypothesis has been tested successfully in our in vitro study with quinolone-exposed *Staphylococcus aureus*.² Significant increases in MICs of ciprofloxacin, gatifloxacin, levofloxacin and moxifloxacin were observed with 3 day treatments at concentrations that fall inside the MSW, without losses in susceptibility at concentrations below the MICs or above the MPCs. A quinolone-independent relationship of resistance (ratio of the post-treatment MIC to the pre-treatment MIC) to the ratio of 24 h area under the curve (AUC24) to MIC was reflected by a bell-shaped curve with a maximum at an AUC24/MIC ratio of 43 h.²

The present study is aimed at further examination of the MSW hypothesis with moxifloxacin-exposed *Streptococcus pneumoniae*. To express *S. pneumoniae* resistance better, a population analysis of resistance frequency is provided, along with time–kill dynamics and MIC time courses.

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Moxifloxacin pharmacodynamics and pneumococcal resistance

Materials and methods
Antimicrobial agent and bacterial strain
Moxifloxacin powder was provided by Bayer Corporation (West Haven, CT, USA). S. pneumoniae ATCC-49619 (MIC 0.1 mg/L; MBC 0.5 mg/L)3 was selected for the study.

Susceptibility testing was performed in duplicate on bacteria obtained before and 24 h after each moxifloxacin dose (0, 24 and 48 h). The MICs were determined using broth microdilution techniques with S. pneumoniae grown in Mueller–Hinton broth (MHB) supplemented with lysed horse blood (2% v/v). The inoculum size was ~105 cfu/mL.

MPC was determined as described elsewhere. Briefly, the tested microorganisms were cultured in MHB and incubated for 24 h. The suspension was centrifuged (4000g for 10 min) and re-suspended in MHB to yield a concentration of 109 cfu/mL. A series of agar plates containing known moxifloxacin concentrations was then inoculated with ~108 cfu of S. pneumoniae. The inoculated plates were incubated for 48 h at 37°C and scored visually for growth. To estimate MPC, logarithms of bacterial numbers were plotted against moxifloxacin concentrations. MPC was taken as the point where the plot intersected the x-axis, i.e. the lowest fluoroquinolone concentration that inhibited growth completely.

In vitro dynamic model and simulated pharmacokinetic profiles
The in vitro dynamic model used in this study has been described elsewhere. For all experiments, the bacterial inoculum was prepared from previously frozen inocula by thawing, diluting with an equal part of fresh MHB supplemented with lysed horse blood (LHB; 2% v/v) and incubating for 90 min at 37°C to bring the organisms into growth phase. This mixture was then inoculated into each peripheral compartment, which also contained MHB/LHB 2%, via an entry port, and incubated to a density of ~106 cfu/mL, at which time the antibiotic was introduced into the central compartment (time zero). Given a 20 mL volume of the peripheral compartment, the total number of organisms in the starting inoculum reached ~2 × 1010 cfu. Antibiotic-free, sterile MHB (no horse serum was added to the MHB) was infused and eliminated at flow rates selected to mimic the half-life of moxifloxacin (12 h) that corresponds to values reported in humans: 11–14 h. All dynamic model experiments were performed in triplicate.

A series of monoexponential profiles that mimicked daily administration of moxifloxacin for 3 consecutive days was simulated over a 32-fold range of the AUC24/MIC ratio, from 8 to 256 h. At the end of a 60 min infusion, the drug concentration reached a maximum, analogous to peak concentrations that are reached after oral administration of the quinolone. As the antimicrobial effect depends on quinolone concentration in peripheral compartments (where the organisms contact antibiotic), peripheral compartments were sampled to determine moxifloxacin concentrations by bioassay using well plates seeded with BBL Bacillus subtilis spore suspension, origin ATCC 6633.

Quantification of the time–kill curves and antimicrobial effect
In each experiment, the peripheral compartments were sampled to determine bacterial concentrations. To determine the number of surviving organisms, a sample was serially diluted in cold sterile saline and 20 µL was inoculated in triplicate onto Mueller–Hinton agar (MHA) supplemented with 5% sheep blood (SB). A small number of bacteria were cultured in MHB and incubated for 24 h. The suspension was centrifuged (4000g for 10 min) and re-suspended with an equal part of fresh MHB supplemented with lysed horse blood (2% v/v) and incubated to a density of ~106 cfu/mL. Antibiotic-free, sterile MHB (no horse serum was added to the MHB) was infused and eliminated at flow rates selected to mimic the half-life of moxifloxacin (12 h) that corresponds to values reported in humans: 11–14 h. All dynamic model experiments were performed in triplicate.

To reveal changes in susceptibility, moxifloxacin MICs for bacterial cultures sampled from the model were determined 24, 48 and 72 h after beginning treatment and at the end of the observation period if it was longer than 72 h. The final MIC (MICfinal) was then related to the initial value (MICinitial). The stability of resistance in each of these specimens was determined by consecutive passaging of S. pneumoniae onto antibiotic-free agar plates for 3–10 consecutive days. MICs were determined frequently during this time as described above.

To determine resistance frequency (f) in experiments where bacterial regrowth occurred, each sample was plated onto agar plates containing 2 × MIC, 4 × MIC and 8 × MIC of moxifloxacin (detection limit 2 × 102 cfu/mL). At a given time, f was expressed by the ratio of bacterial number observed in the presence of antibiotic to that in the absence of antibiotic (fexp = fexp/MICfinal/MICinitial). Then, the respective ratios of the final f(final) to the initial value (finitial) were calculated.

To relate the increase in the MIC and f to the simulated AUC24/MICs, a Gaussian type function was used:

\[ Y = Y_0 + a \exp \left\{ -(x-x_c)^2/b \right\} \]  

(Equation 1)

where Y is the MICfinal/MICinitial or ffinal/finitial ratio, Y₀ is the minimal value of Y, x is log₁₀ AUC24/MIC, x_c is log₁₀ AUC24/MIC that corresponds to the maximal value of MICfinal/MICinitial or ffinal/finitial, and a and b are parameters.
Figure 2. Effect of dosing regimen on bacterial survival and enrichment of mutants. The left column of panels shows the simulated pharmacokinetics at various values of AUC_{24}/MIC, as indicated in the boxes in the upper right portion of each panel. Circles indicate moxifloxacin concentration determined by bioassay. Arrows indicate the time of moxifloxacin addition. MSW is indicated by the shaded regions. The central column of panels shows the effect of each moxifloxacin treatment regimen on bacterial survival. Samples were taken at the indicated times and plated on drug-free agar to determine viable cells. The right column of panels shows the effect of each moxifloxacin regimen on the susceptibility of bacteria recovered from the dynamic model at the indicated times.
Moxifloxacin pharmacodynamics and pneumococcal resistance

Figure 3. Effect of AUC24/MIC on the susceptibility of S. pneumoniae exposed to moxifloxacin. Equation (1): \( y = 1, a = 9.8, b = 81.4, x = 1.60. \)

Results

Moxifloxacin concentrations determined by bioassay were close to the target values. The overall range of the determined AUC24/MICs (average of values reached during the first, second and third days) that reflects different doses of moxifloxacin was 6–224 h, with a half-life estimated of 14 h. Representative pharmacokinetic profiles observed in the peripheral compartments of the model at different AUC24/MIC ratios are shown in the left panel of Figure 2.

As seen in the middle column of panels in Figure 2, the experiments in which AUC24/MICs were relatively low (at 9 and 24 h where peak concentrations were close to the MIC—see left column of panels) provided little reduction in the starting inoculum. At higher AUC24/MICs (≥39 h), killing of S. pneumoniae was more pronounced: the greater the AUC24/MICs, the greater the antimicrobial effect. Regardless of the extent of bacterial killing, gradual increases in MIC, most pronounced after the third dose, were observed at moxifloxacin concentrations that fell inside the MSW (AUC24/MICs 24–46 h; right column of panels in Figure 2). Serial passage on antibiotic-free plates of resistant isolates obtained after 72–96 h of fluoroquinolone exposure revealed minimal or no changes in the elevated MICs (data not shown). Thus, resistance was stable after the multiple passages. No loss of S. pneumoniae susceptibility was associated with concentrations below the MIC (AUC24/MIC 9 h) or above the MPC (AUC24/MICs 218 h). Thus, changes in the susceptibility of moxifloxacin-exposed S. pneumoniae were dependent on the simulated AUC24/MIC ratio, at least with these distinctly different AUC24/MICs.

To relate increases in MIC to AUC24/MIC for the entire data set (18 AUC24/MIC values), the MICs observed at the end of each treatment were normalized to their respective initial MIC values. As seen in Figure 3, a Gaussian-like function [Equation (1)] fitted the MICfinal/MICinitial versus log AUC24/MIC relationship (\( r^2 = 0.90 \)). The central point was at an AUC24/MIC of 38 h, where the loss in pneumococcal susceptibility was maximal. No resistance was observed at AUC24/MICs < 10 h or AUC24/MICs > 100 h.

Changes in moxifloxacin susceptibility of S. pneumoniae or the lack thereof were consistent with the selection of resistant mutants (Figure 4). At the lower AUC24/MICs (7 and 10 h) and the highest AUC24/MIC (107 h), no viable counts were recorded on the plates containing 4 × MIC and 8 × MIC of moxifloxacin. At the intermediate AUC24/MICs, 72 h samples contained organisms resistant to 4 × MIC (AUC24/MIC 24, 38 and 47 h) and 8 × MIC of moxifloxacin (AUC24/MIC 24 and 38 h). Although the relationship between AUC24/MIC of resistance inherent in organisms that survived in the presence of 2 × MIC of moxifloxacin was less clear, the total number of surviving organisms exposed to moxifloxacin AUC24/MICs of 38 and 47 h was distinctly higher than at AUC24/MICs of 7–10 and 107 h. As seen in Figure 5, resistance frequencies at the simulated AUC24/MICs were very similar for organisms grown on agar plates that contain 4 × MIC or 8 × MIC of moxifloxacin. This allows further analysis of combined data. Like the MICfinal/MICinitial ratio, Equation (1) fitted the AUC24/MIC-dependent ffinal/ffinal ratio, with the central point at AUC24/MIC of 42 h. No selection was seen at AUC24/MICs < 10 h and AUC24/MICs > 100 h (Figure 6).

The selective enrichment of resistant mutants by moxifloxacin concentrations inside the MSW was consistent with the antimicrobial effects observed within each dosing interval, i.e. ABBC1, ABBC2 and ABBC3. As shown in Figure 7, the effects observed during the first dosing interval were always less than those during the second and third intervals. At the lower AUC24/MIC ratios (7–10 h) and at the higher AUC24/MICs (75–220 h), similar ABBCs were observed during the second and third dosing intervals. At the intermediate AUC24/MICs (24–46 h), the effects of the third doses were less pronounced than the second doses of moxifloxacin. The erosion of the antimicrobial effect (ABBC3/ABBC2 < 1) is directly associated with the AUC24/MICs (Equation (2)).

Discussion

Bacterial resistance and its relationship to the AUC24/MIC or the ratio of peak concentration to MIC have been assessed in recent studies using in vitro dynamic models, but most of those studies were unable to relate resistance to simulated AUC/MICs or peak-to-MIC ratios. The reasons for these failures have been discussed in detail elsewhere and include a lack of adequate quantitative resistance data, short-term treatment, low inocula, use of inappropriate fitting procedures, etc. In fact, the general pattern of the relation of AUC/MIC to resistance (elevated MICs) was first delineated in our recent in vitro study with quinolone-exposed S. aureus.

The present study demonstrates good concordance between S. pneumoniae resistance expressed by susceptibility testing and by population analysis. The selective enrichment of resistant S. pneumoniae exposed to moxifloxacin occurred at similar AUC24/MIC ratios, both in terms of loss in the susceptibility and increases in resistance frequency. Moreover, both methods showed similar AUC24/MIC relationships of resistance that were reflected by bell-shaped curves having a maximum at similar AUC24/MICs (38 h with the MIC data and 42 h with the resistance frequency data). A similar relationship between AUC24/MIC and MICfinal/MICinitial was delineated in our recent study with quinolone-exposed S. aureus. In that case a maximum was also seen at an AUC24/MIC of 43 h. Further studies are required to determine whether this pattern of AUC24/MIC resistance relationship occurs with other antibiotic–pathogen pairs.

Like S. aureus exposed to four quinolones including moxifloxacin, selection of resistant S. pneumoniae occurred when moxifloxacin concentrations were inside the MSW (TREF ≥ 20% of the dosing interval (AUC24/MICs 6–100 h). No selection was seen
with shorter times inside the MSW ($T_{MSW} < 20\%$ of the dosing interval), i.e. at AUC$_{24}$/MICs < 10 h and > 100 h.

Changes in resistance frequency and susceptibility of moxifloxacin-exposed \textit{S. pneumoniae} were accompanied by respective changes in the anti-pneumococcal effect. Its loss during treatment (ABBC$_1 < ABBC_2$) was observed at moxifloxacin concentrations that fall inside the MSW (AUC$_{24}$/MIC 24–47 h), where selection of resistant mutants occurred. No erosion in effectiveness was seen at concentrations that were outside the MSW. Similar correlations were reported in our study with moxifloxacin- and levofloxacin-exposed \textit{S. aureus}.\cite{17}

There the loss of the antimicrobial effect was observed at AUC$_{24}$/MICs of 28 h (moxifloxacin) and 31–61 h (levofloxacin).

As with \textit{S. aureus}, the most pronounced losses in \textit{S. pneumoniae} susceptibility and the highest resistance frequencies were observed at moxifloxacin concentrations that fell inside the MSW (AUC$_{24}$/MIC from 24 to 47 h). At concentrations <MIC or >MPC (AUC$_{24}$/MIC < 10 h or > 100 h), no resistance was observed. Based on these data, an AUC$_{24}$/MIC ratio of 100 h might protect against pneumococcal resistance. This value is readily achievable in patients treated with moxifloxacin: much higher ratios of AUC (33 mg × h/L)$^5$ to MIC$_{50}$ (0.125 mg/L)$^18$, i.e. 33/0.125 = 270 h, are provided by its usual 400 mg clinical dose.

Overall, these findings suggest that selection of resistant mutants can be observed using \textit{in vitro} pharmacokinetic simulations in which resistance may be monitored by either the frequency of mutations or increases in MICs. Also, these data support the MSW hypothesis\cite{1} that predicts selection of resistant mutants at antibiotic concentrations >MIC and <MPC.

By supporting the MSW hypothesis, the data presented above challenge a central assumption of antimicrobial therapy, that resistant mutants are enriched selectively when antimicrobial concentrations

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**Figure 4.** Effect of AUC$_{24}$/MIC on survival of moxifloxacin-exposed \textit{S. pneumoniae} (selected data). The AUC$_{24}$/MICs are shown in the bottom right corner of each plot. Bacteria were recovered at the indicated times and survival was determined by plating on agar containing zero moxifloxacin (triangles), 2 × MIC moxifloxacin (inverted triangles), 4 × MIC (squares), or 8 × MIC (diamonds).
Moxifloxacin pharmacodynamics and pneumococcal resistance

are <MIC. This assumption may lead to dosing recommendations, such as achieving an AUC/MIC of 30–6016,19,20 that place antimicrobial concentrations inside the MSW. According to the MSW hypothesis, many traditional dosing regimens may constitute misuse of antimicrobial agents.

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References


Figure 5. Effect of AUC24/MIC on the frequency at which moxifloxacin-resistant mutants are recovered. The frequency at which resistant mutants were recovered (black bars) was determined by plating on moxifloxacin at either 4 × MIC (panel a) or 8 × MIC (panel b). White bars indicate the frequency at which mutants were recovered from cells prior to moxifloxacin exposure.

Figure 6. Effect of AUC24/MIC on the increase in frequency of recovery of resistant mutants. Agar plates used to detect mutants contained either 4 × MIC (squares) or 8 × MIC (diamonds). Equation (1): Y = 1, a = 5.9, b = 39.2, x = 1.62.

Figure 7. Effect of AUC24/MIC on the loss of antimicrobial action during the treatment of S. pneumoniae with moxifloxacin: ABBC1 (white bars), ABBC2 (grey bars), ABBC3 (black bars).


