

***In vivo* pharmacodynamic efficacy of gatifloxacin against *Streptococcus pneumoniae* in an experimental model of pneumonia: impact of the low levels of fluoroquinolone resistance on the enrichment of resistant mutants**

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Objectives: To investigate the impact of low levels of fluoroquinolone resistance on the emergence of resistant mutants, we examined the mutant selection window (MSW) hypothesis in experimental pneumonia in rabbits infected with pneumococci with various susceptibility levels to fluoroquinolones and treated with gatifloxacin using a human-like regimen (equivalent to 400 mg once daily). The MSW corresponds to the range of concentrations between the minimal inhibitory concentration (MIC) and the mutant prevention concentration (MPC), which is the antibiotic concentration that prevents selection of resistant mutants.

Materials and methods: Five pneumococcal strains were tested and were defined as follows [MIC of ciprofloxacin (mg/L)/MIC of gatifloxacin (mg/L)/MPC of gatifloxacin (mg/L)/involved quinolone resistance mechanisms]: strain 16089 = 0.5/0.25/0.25/wild-type; strain MS1A = 2/0.5/1/efflux; strain MS2A = 8/1/8/*parC* S79F; strain MR3B4 = 10/1/8/*parC* S79T; strain Gyr-1207 = 6/4/4/*gyrA* S81F.

Results: A 48 h human-like treatment with gatifloxacin was significantly bactericidal on pneumonia induced by strain 16089 ($> 6 \log_{10}$ killing) as well as the efflux derivative strain MS1A ($> 5 \log_{10}$ killing). However, a small number of *parC-gyrA* mutants were recovered in 26% of the animals infected with this efflux strain. As expected, no decrease in viable bacteria counts was observed when pneumonia was induced by the *gyrA* resistant strain. In contrast, because of the enrichment of highly resistant mutants in 100% of the animals, no significant bacterial reduction was observed after treatment of pneumonia induced by the two susceptible *parC* mutated strains. A classification and regression tree (CART) analysis identified T_{MSW} (percentage of the time during which gatifloxacin serum concentrations are inside the MSW) and AUC_{MSW} (area under curve between MIC and MPC values) as the best parameters associated with the enrichment of resistant pneumococci.

Conclusions: This study shows that the acquisition of a low level of fluoroquinolone resistance (especially a *parC* mutation and to a lesser extent an efflux mechanism) is associated with a clearly lower potential for preventing resistance development. These data support the concept that resistant mutants are selectively enriched when antibiotic concentrations fall inside the mutant selection window and suggest that *in vivo* dynamic models have to be used to predict the relative abilities of quinolones to prevent mutant selection.

Keywords: *parC*, pneumococcal pneumonia, mutant selection window, mutant prevention concentration

Introduction

Streptococcus pneumoniae is the most common causative agent of community-acquired pneumonia¹ and is a leading cause of

morbidity and mortality worldwide.^{2–5} As the incidence of pneumococci exhibiting resistance to beta-lactams and macrolides has increased at an alarming rate, new fluoroquinolones have been approved as interesting candidates for the treatment of

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acute respiratory infections. However, several recent reports from Hong Kong,⁶ Canada⁷ and Spain^{8,9} have described a worrisome increase in the incidence of quinolone-resistant pneumococci, which raises concerns regarding the clinical outcome of potential cross-resistance with newer fluoroquinolones such as 8-methoxyfluoroquinolones. The development of resistance is also a concern, and an understanding of the pharmacodynamic properties of fluoroquinolones will enable better choices to be made in order to maximize bacterial killing and reduce the opportunity for resistance development.¹⁰ Fluoroquinolone-resistant mutants are usually enriched when drug concentrations fall within a range called the mutant selection window (MSW). The lower boundary of the MSW is the minimal inhibitory concentration (MIC) and the upper boundary is the mutant prevention concentration (MPC), an antibiotic concentration that allows no growth of resistant mutants.^{11–13} Such conditions have already been described in some *in vitro*^{14–16} and *in vivo* models;¹⁷ these studies confirmed the concept of MSW for staphylococcus and pneumococcus versus some fluoroquinolones. In this study, we used an experimental model of pneumococcal pneumonia in rabbits and investigated the efficacy of a 48 h gatifloxacin standard regimen (equivalent to 400 mg once daily) against five strains including one ciprofloxacin-susceptible and four ciprofloxacin-resistant strains (ciprofloxacin resistance mechanisms in these strains involved the activity of a multidrug efflux pump, a *parC* mutation or a *gyrA* mutation). We also investigated conditions in which gatifloxacin treatment was able to favour or prevent enrichment of resistant subpopulations by testing the MSW hypothesis.

Materials and methods

Bacterial strains, growth conditions and antimicrobial agents

Five penicillin-resistant pneumococcal strains¹⁸ were used in this study (Table 1). They included one parent ciprofloxacin-susceptible strain (strain 16089) and four strains with different ciprofloxacin resistance phenotypes (efflux, *parC* and *gyrA*). Bacteria were grown either in brain heart infusion (BHI) broth (bioMérieux, Marcy l'Étoile, France) or on sheep blood agar plates (bioMérieux) in 5% CO₂. Bacterial stocks were kept frozen at –70°C in a 15% (v/v) glycerol-supplemented BHI. Ciprofloxacin was provided by Sigma–Aldrich (Steinheim, Switzerland) and gatifloxacin was kindly supplied by Grünenthal (Levallois Perret, France). The drugs were reconstituted according to the manufacturers' instructions. Stock solutions were used as fresh preparations.

In vitro susceptibility testing

All determinations were done in triplicate and the results were identical.

Determination of the MICs. MICs were determined by the standard serial two-fold dilutions method in agar, with an inoculum of 5×10^5 cfu/mL.¹⁹ Results were recorded after 18 h of incubation in 5% CO₂ at 37°C.

Determination of the MPCs. MPCs were determined by plating the bacterial suspension (10^{10} cfu/mL) on tryptic soy agar plates containing 5% of defibrinated sheep blood and various concentrations of gatifloxacin. MPCs were recorded as the lowest concentration of quinolone that allowed no growth of mutant strains after 72 h of incubation in 5% CO₂ at 37°C.¹³

Time–kill curves. The *in vitro* bactericidal activity of gatifloxacin on the different strains was evaluated as described previously.²⁰ The bacterial growth in test and control tubes was counted at 0, 3, 6, 12 and 24 h after incubation at 37°C. The initial inoculum size was 5×10^5 cfu/mL. The concentrations of gatifloxacin corresponded approximately to the maximal blood concentration observed in humans.

Determination of quinolone resistance mechanisms

PCR amplification of quinolone resistance-determining regions (QRDRs) and DNA sequencing. PCR was used to amplify *parC*, *parE*, *gyrA* and *gyrB* genes²¹ with primers that were those previously reported.²² After amplification, PCR products were sequenced (Génôme Express S.A., Grenoble, France) with an automated sequencer (ABI Prism 377 DNA sequencer).

Presence of quinolone efflux mechanism. MICs were determined in the presence and absence of 10 µg/mL of reserpine (Sigma Chemicals, St Louis, MO, USA) as described previously.^{23–25} By definition, an efflux mechanism existed when there was at least a four-fold reduction in MIC in the presence of reserpine.

Production and human-like treatment of experimental pneumococcal pneumonia in rabbits

Animals. Male immunocompetent New Zealand rabbits (weight, 2.7–3.0 kg; Elevage Scientifique des Dombes, Romans, France) were used for all studies. Animals were kept in accordance with current recommendations.

Table 1. *In vitro* susceptibilities to ciprofloxacin and gatifloxacin, and quinolone resistance mechanisms of the pneumococcal strains tested

Strain	Ciprofloxacin MIC (mg/L)	Gatifloxacin			
		MIC (mg/L)	MPC (mg/L)	Efflux ^a	QRDR ^b genotype
16089	0.5	0.25	0.25	–	
MS1A	2	0.5	1	+	
MS2A	8	1	8	+	<i>parC</i> Ser-79→Phe
MR3B4	10	1	8	+	<i>parC</i> Ser-79→Tyr
Gyr-1207	6	4	4	–	<i>gyrA</i> Ser-81→Phe

^aEfflux reserpine-ciprofloxacin (see Materials and methods).

^bQuinolone resistance determining region.

Lung infection model. The production of pneumonia in immunocompetent rabbits and the installation of the central venous catheters were carried out as previously described.^{18,26} Briefly, 24 h after jugular catheterization, bacterial pneumonia was induced by endobronchial challenge of the animals with 0.5 mL of saline containing 10^{10} cfu/mL of either tested strain. Human-like treatment with gatifloxacin was started 5 h after bacterial challenge and lasted for 2 days. Gatifloxacin was delivered through the first central venous catheter at changing infusion rates obtained by a computer-controlled electric pump and at doses that simulated antibiotic kinetics observed in human serum after a standard dose of 400 mg once a day.

Pharmacokinetic (PK) analysis. For each animal, the concentrations of gatifloxacin in the serum were determined on iterative blood samples, obtained through the second central catheter by a standard microbiological assay method with antibiotic diffusion medium II (Difco Laboratories) and *Escherichia coli* NIJHC2 as the indicator organism.²⁷ The level of protein binding in the serum of infected rabbits was determined *ex vivo* by a membrane ultrafiltration method.²⁸ The degree of binding was measured by using gatifloxacin concentrations of 0.5–6 mg/L. Pharmacokinetics data were analysed using Kinetica software (Innaphase, Philadelphia, PA, USA).

Evaluation of infection. The rabbits were anaesthetized and killed 2 h after the end of the antibiotic infusion. Untreated control rabbits were killed just before treatment or after 48 h. The assessment of primary efficacy was based on the analysis of the spleen and each pulmonary lobe, which were removed and processed to determine the numbers of cfu/g. Then residual bacteria were examined for fluoroquinolone susceptibility on agar plates containing gatifloxacin at $2\times$ and $4\times$ MIC. Resistant mutants were examined for their drug susceptibility and their gene mutations in the QRDR.

Pharmacokinetic–pharmacodynamic (PK–PD) analysis. By using the individual pharmacokinetics data obtained for each animal, the relationship between drug exposure and bacterial response was expressed according to the following parameters (expressed as the unbound fraction): maximal concentration over MIC (C_{\max}/MIC) or MPC (C_{\max}/MPC), area under the curve of serum concentration versus time divided by MIC ($\text{AUC}_{0-24}/\text{MIC}$) or MPC ($\text{AUC}_{0-24}/\text{MPC}$), time of serum concentration above MIC ($T > \text{MIC}$) or MPC ($T > \text{MPC}$), time of serum concentration within MIC and MPC (T_{MSW}) and area of serum concentration within MIC and MPC versus time (AUC_{MSW}). E_{\max} , sigmoid and peak formulas were obtained from Sigma Plot Software (Version 7.101).

Statistical analysis

The results were expressed as the mean \pm S.D. Quantitative variables were compared with Mann–Whitney or analysis of variance (ANOVA), eventually completed by a *post-hoc* analysis using Bonferroni's test. Percentages were compared using the χ^2 test with Yates correction or with the Fisher exact test. The correlation between antimicrobial efficacy and each of the PK–PD parameters (C_{\max}/MIC , C_{\max}/MPC , $\text{AUC}_{0-24}/\text{MIC}$, $\text{AUC}_{0-24}/\text{MPC}$, $T > \text{MIC}$, $T > \text{MPC}$, T_{MSW} , AUC_{MSW}) was determined by non-linear least-squares regression (Sigma Plot Software, Version 7.101). To define the PK–PD zones associated with the occurrence of mutants, a qualitative analysis was carried out using the classification and regression tree (CART) methodology.^{29,30} For all the tests, a P value < 0.05 was considered indicative of statistical significance.

Results

In vitro susceptibility testing

The MICs of ciprofloxacin and gatifloxacin, and MPCs of gatifloxacin for the five strains studied are shown in Table 1. All these strains, except for strain 16089, were resistant to ciprofloxacin. As expected, gatifloxacin retained a better *in vitro* activity than ciprofloxacin against all strains tested. All strains except strain Gyr-1207 (MIC = 4 mg/L) were susceptible to gatifloxacin. The presence of a *gyrA* mutation (strain Gyr-1207) was associated with a 16-fold increase in the MIC of gatifloxacin. However, the presence of only one ciprofloxacin efflux mechanism (strain MS1A) or of a *parC* mutation (strains MS2A and MR3B4) slightly increased the MICs of gatifloxacin. In contrast, the presence of a *parC* mutation (strains MS2A and MR3B4), and to a lesser extent an efflux mechanism (strain MS1A), was clearly associated with an increase in MPC values, thus these strains exhibited an *in vitro* mutant selection window since their MPCs were higher than their MICs.

In vitro bactericidal activity of gatifloxacin

Time–kill curves for the five pneumococcal strains tested for gatifloxacin at the simulated serum concentration after oral dosing of 400 mg are shown in Figure 1. Gatifloxacin rapidly reduced bacteria below the limit of detection within 6 h after exposure for strains 16089 (wild-type) and MS1A (efflux) ($P \leq 0.01$ compared to the other strains). In contrast, for strains MS2A (*parC*), MR3B4 (*parC*) and Gyr-1207 (*gyrA*), decreases in viable bacterial counts slowed (18 to 24 h) ($P < 0.01$ compared to strains 16089 and MS1A) and gatifloxacin showed a minimum bactericidal activity at 24 h for the resistant strain Gyr-1207 (*gyrA*).

Simulated human gatifloxacin treatment and pharmacokinetic parameters

Figure 2 shows that total serum concentrations observed in rabbits ($n = 51$) mirrored the human serum concentration curve observed after the administration of 400 mg of gatifloxacin. The level of gatifloxacin protein binding in infected rabbits was 25% at drug concentrations from 0.5 to 6 mg/L. This is similar to the low degree of binding reported for gatifloxacin in other animal species and in human serum.^{28,31,32} Free drug levels were considered in pharmacokinetic calculations throughout the study.

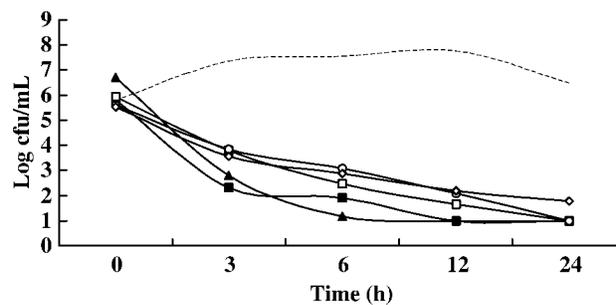


Figure 1. *In vitro* bacterial killing curves of gatifloxacin (5 mg/L) against the five pneumococcal strains tested (growth control, dashed line; 16089 wild-type, filled squares; MS1A efflux, filled triangles; MS2A *parC*, open squares; MR3B4 *parC*, open circles; Gyr-1207 *gyrA*, open diamonds).

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The pharmacokinetic parameters obtained in rabbits were as follows (expressed as the free fraction): AUC_{0-24} , 29.01 ± 8.5 mg·h/L; C_{max} , 2.66 ± 0.5 mg/L; C_{min24} , 0.46 ± 0.28 mg/L; C_{min48} , 0.56 ± 0.35 mg/L. The exposure to gatifloxacin (AUC_{0-24}) was similar for the groups as defined by strains ($P > 0.5$).

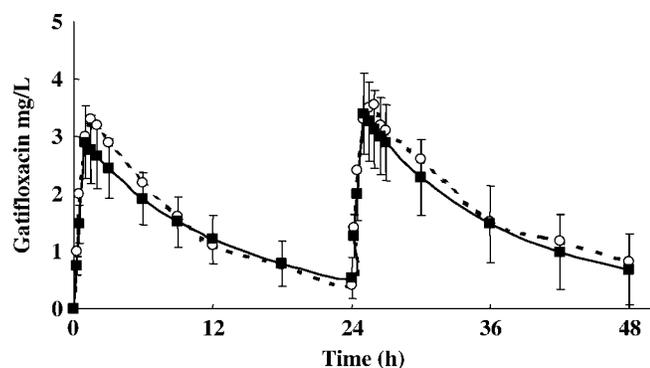


Figure 2. Human-like pharmacokinetics of total fraction of gatifloxacin in rabbit (equivalent in human to 400 mg once a day). Desired human concentrations (broken line); obtained concentrations in rabbits (continuous line).

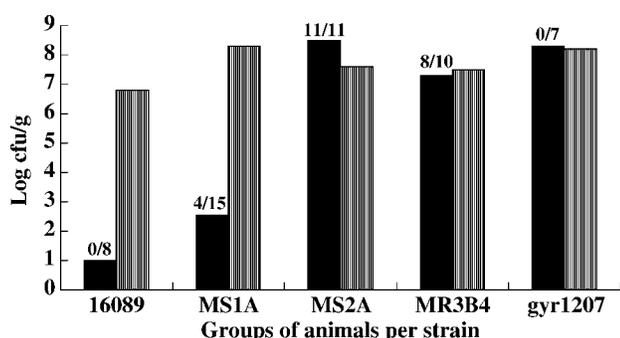


Figure 3. Impact of a 48 h gatifloxacin human-like treatment on *in vivo* efficacy against pneumococcal pneumonia induced by five pneumococcal strains with various susceptibilities to fluoroquinolones. Black bars, gatifloxacin-treated animals; vertically striped bars, animals without treatment. Numbers indicate the number of animals harbouring mutant pneumococci in lungs over the total number of treated rabbits. * $P < 0.05$ compared to control animals without treatment and $P < 0.01$ between 16089 and MS1A groups (Mann–Whitney test).

Table 2. Pharmacodynamic parameters (unbound fraction) obtained in rabbits with experimental pneumococcal pneumonia induced by strains with various quinolone resistance mechanisms after a 48-h gatifloxacin standard regimen (equivalent to 400 mg once daily in human)

	Groups of treated animals by strain				
	16089 (wild-type)	MS1A (efflux)	MS2A (<i>parC</i>)	MR3B4 (<i>parC</i>)	Gyr-1207 (<i>gyrA</i>)
<i>n</i>	8	15	11	10	7
C_{max}/MIC	9.8 ± 2.1	5.2 ± 1.0	2.8 ± 0.5	2.5 ± 0.4	0.7 ± 0.1
C_{max}/MPC	9.8 ± 2.1	2.6 ± 0.5	0.3 ± 0.06	0.3 ± 0.05	0.7 ± 0.1
AUC_{0-24}/MIC	93.2 ± 16	58.3 ± 21.2	32.7 ± 8.5	27.2 ± 5.7	7.8 ± 1.05
AUC_{0-24}/MPC	93.2 ± 16	29.15 ± 10.6	4.1 ± 1.1	3.4 ± 0.7	7.8 ± 1.05
$T > MIC$ (%)	92.8 ± 9.9	82.35 ± 21.05	65.45 ± 21.75	40.0 ± 15.5	0
$T > MPC$ (%)	92.8 ± 9.9	54.6 ± 25.8	0	0	0
T_{MSW} (%)	0	27.8 ± 15.3	65.45 ± 21.75	49 ± 15.5	0
AUC_{MSW}	0	5.1 ± 2.3	27.7 ± 10.65	20.2 ± 6.7	0

Antimicrobial effects of a gatifloxacin-simulated human-like treatment on experimental pneumococcal pneumonia

Bacterial reduction after treatment. At the start of therapy, the mean pulmonary bacterial concentration was $8.5 \pm 0.54 \log_{10}$ cfu/g with no difference between groups of animals. As shown in Figure 3, gatifloxacin, at a dose equivalent to the standard regimen of 400 mg once a day in humans, was associated with a sharp decrease in viable bacterial counts in lungs when pneumonia was induced by strain 16089 ($1.00 \pm 0.00 \log_{10}$ cfu/g, $P < 0.0001$) and its derivative efflux strain MS1A ($2.46 \pm 1.50 \log_{10}$ cfu/g, $P < 0.0001$) after 48 h of drug exposure, compared to untreated controls. A significant difference in efficacy was found between these two groups ($P = 0.01$, Mann–Whitney test).

In contrast, no decrease in viable bacterial counts in lungs was observed when pneumonia was induced by either the Gyr-1207 resistant strain, or the two *parC* mutated strains (MS2A and MR3B4). Similar results were obtained in the spleen (data not shown).

Emergence *in vivo* of resistant mutants. No gatifloxacin-resistant mutants were recovered after 48 h of antibiotic exposure when the experimental pneumonia was induced by either the ciprofloxacin-susceptible strain 16089 or the gatifloxacin-resistant *gyrA* mutated strain Gyr-1207. In contrast, resistant mutants (MIC for gatifloxacin = 4 mg/L), which had acquired an additional *gyrA* mutation (*gyrA* Ser-81 → Phe), were recovered in 100% and 80% of the animals infected by the two *parC* mutated strains MS2A and MR3B4, respectively. The mean pulmonary concentrations of resistant mutants for these two strains were $8.31 \pm 0.43 \log_{10}$ cfu/g and $7.42 \pm 1.11 \log_{10}$ cfu/g, respectively. Gatifloxacin standard regimen was also associated with the emergence of double mutated *gyrA-parC* resistant mutants when pneumonia was induced by the efflux strain (MS1A), but in only four of 15 treated rabbits (26%) and at a very low concentration ($1.1 \pm 0.8 \log_{10}$ cfu/g).

Pharmacodynamic analysis

Table 2 lists all mean MIC- and MPC-related parameters (unbound fraction) obtained for each group of animals as defined by strains. All these values decreased as the MIC of the infecting strain increased; for example, AUC_{0-24}/MIC ratios ranged from

7.8 ± 1.05 to 93.2 ± 16 . The relationships between the microbiological effect in animals and each of the MIC- and MPC-related parameters were studied. Thus, the residual bacterial population in the lungs after 48 h of antibiotic exposure was split into the

population with and the population without an increase in MIC to gatifloxacin (Figure 4a–g).

In these conditions, for the non-mutated bacterial population, the drug exposure–effect relationships are represented as an

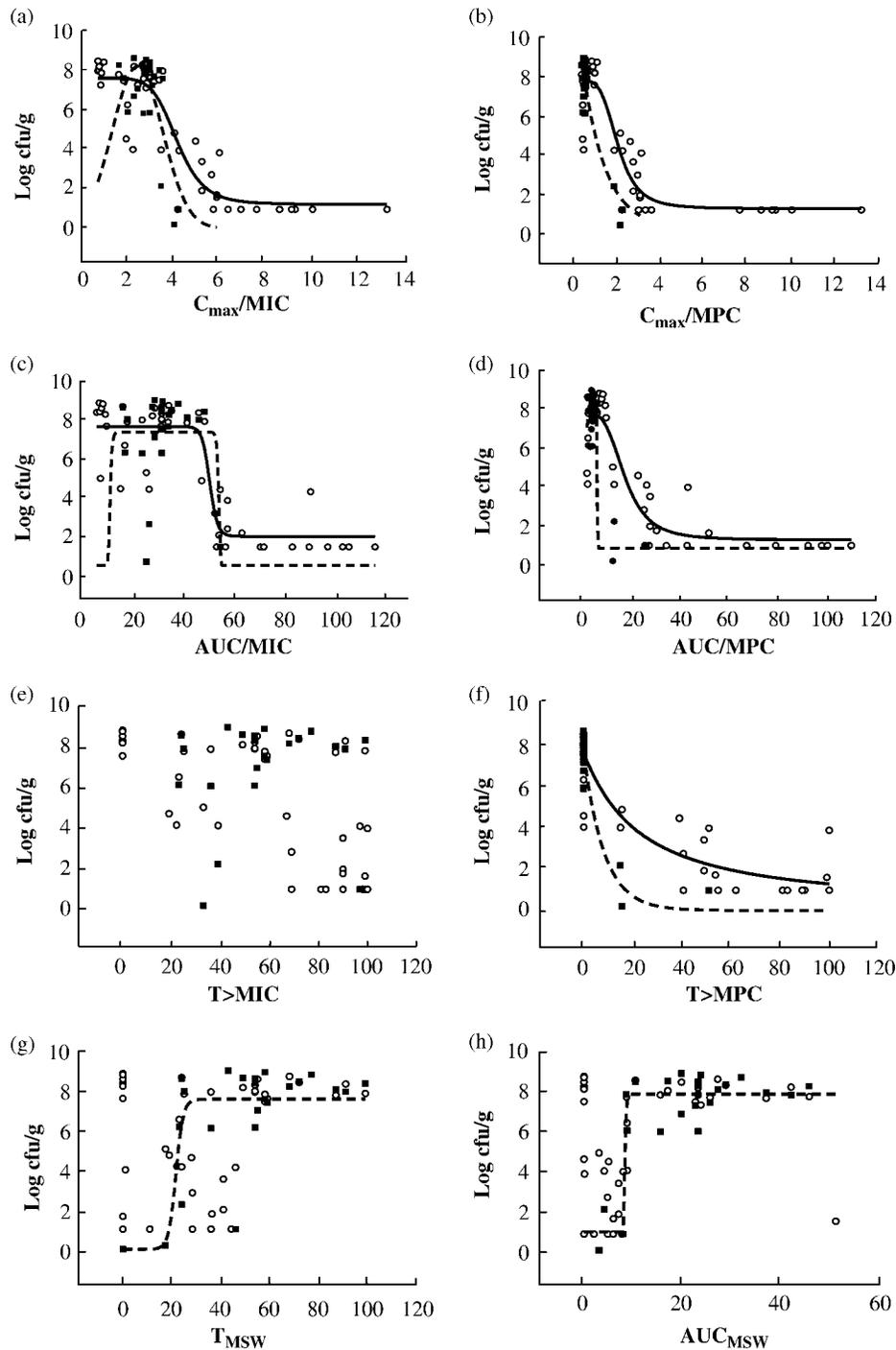


Figure 4. Relationship between the MIC- and MPC-related parameters (unbound fraction) obtained in infected rabbits after a 48 h human-like gatifloxacin regimen with the residual bacterial pulmonary concentration (\log_{10} cfu/g). Open circles symbolize animals with bacterial population without increase in MIC after treatment; filled squares symbolize bacterial population with increase in MIC after treatment. For the non-mutated bacterial population, the drug exposure–effect relationships are represented as an E_{\max} curve for the following parameters: C_{\max}/MIC (panel a; $R^2=0.83$); C_{\max}/MPC (panel b; $R^2=0.87$); AUC/MIC (panel c; $R^2=0.81$); AUC/MPC (panel d; $R^2=0.86$) and $T \geq \text{PC}$ (panel f; $R^2=0.87$). For the mutated bacterial population, the dose exposure–effect relationships are represented as a peak curve for the following parameters: C_{\max}/MIC (panel a; $R^2=0.75$), AUC/MIC (panel c, $R^2=0.73$), AUC/MPC (panel d; $R^2=0.8$), an exponential decay curve for C_{\max}/MPC (panel b; $R^2=0.82$) and $T \geq \text{MPC}$ (panel f; $R^2=0.86$) and a sigmoid curve for T_{MSW} (panel g; $R^2=0.63$) and AUC_{MSW} (panel h; $R^2=0.87$).

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Table 3. Classification and regression tree (CART) analysis for pharmacodynamic parameters versus emerging mutants in rabbits with experimental pneumococcal pneumonia induced by strains with various quinolone resistance mechanisms after a 48-h standard gatifloxacin regimen (equivalent to 400 mg once daily in humans)

PK–PD parameter	CART analysis				
	PK–PD boundaries		<i>in vivo</i> mutation ^a	statistics	
	lower	upper		univariate <i>P</i> value	multivariate <i>P</i> value
$T > \text{MIC}$ (%)				0.18	
$T > \text{MPC}$ (%)				0.2	
T_{MSW} (%)	>45		100	0.03	
$C_{\text{max}}/\text{MIC}$	<1.2	>6	0	0.09	
AUC/MIC	<48	>90	0	0.07	
AUC > MIC				0.38	
AUC > MPC				0.16	
AUC _{MSW}	>7.83		91	0.02	0.02

^a Percentage of treated animals with emerging resistant mutants.

E_{max} curve for the following parameters: $C_{\text{max}}/\text{MIC}$ (panel (a); $R^2=0.83$), $C_{\text{max}}/\text{MPC}$ (panel (b); $R^2=0.87$), AUC/MIC (panel (c); $R^2=0.81$), AUC/MPC (panel (d); $R^2=0.86$) and $T > \text{MPC}$ (panel (f); $R^2=0.87$). For the mutated bacterial population, the drug exposure–effect relationships are represented as (1) a peak curve for the following parameters: $C_{\text{max}}/\text{MIC}$ (panel (a); $R^2=0.75$), AUC/MIC (panel (c), $R^2=0.73$), AUC/MPC (panel (d); $R^2=0.8$); in other words a window, in which bacteria with increased MIC were found, can be observed for these parameters; (2) an exponential decay curve for $C_{\text{max}}/\text{MPC}$ (panel (b); $R^2=0.82$) and $T > \text{MPC}$ (panel (f); $R^2=0.86$) and (3) a sigmoid curve for T_{MSW} (panel (g); $R^2=0.62$) and AUC_{MSW} (panel (h); $R^2=0.90$); in other words, the concentration of bacteria with increased MIC increased as both T_{MSW} and AUC_{MSW} increased.

Thresholds of the mutant selection window (MSW) (Table 3). In order to approach the limits within which mutants appear *in vivo*, a classification and regression tree (CART) analysis was tested for each PK–PD parameter. This analysis identified T_{MSW} (percentage of the time during which gatifloxacin serum concentrations are inside the MSW) and AUC_{MSW} (area under curve between MIC and MPC values) as the best parameters statistically associated with the emergence of resistant mutants; however, only AUC_{MSW} was significant in the multivariate analysis. When this analysis was done only for rabbits infected with *parC* strains, the selection window was narrow and again only AUC_{MSW} was found significantly associated with the *in vivo* emergence of mutants (not shown).

Discussion

Our animal study is in concordance with the results of clinical trials on the efficacy of gatifloxacin in the treatment of pneumococcal respiratory tract infections^{32–37} but also brings information concerning the occurrence of mutations particularly through the concept of the mutant selection window.^{11,13,14,38}

In this human-like animal model, a standard gatifloxacin treatment is associated with a sharp bacterial reduction when the animals were infected by the wild-type pneumococcal strain (strain 16089) and its efflux derivative (strain MS1A). This efficacy was associated with a large drug exposure as the AUC/MIC ratio was on average 90 and 58, respectively (Table 2). However, a few resistant mutants were detected (26% of the treated animals) when the pneumonia was induced with the efflux derivative strain. This result was not anticipated by the *in vitro* killing curve or the *in vitro* model³⁹ and also gatifloxacin is considered insensitive to efflux by clinical strains.⁴⁰ However, for this specific strain, a mutant selection window did exist since its MPC was twice the MIC. *In vivo*, gatifloxacin concentration was within this window for approximately 30% of the time compared to zero for the wild-type strain infection (Table 2). Although this finding could confirm the MSW concept *in vivo*, only one efflux strain was tested in this study; so we are unable to anticipate the real role of efflux on the eventual emergence of fluoroquinolone resistance. Of note, the residual concentration of these mutants after treatment was very low.

As expected, no decrease in viable bacterial counts was observed when pneumonia was induced by the *gyrA* resistant strain which thus validates our humanized animal model. Furthermore, no mutants were detected in any animals; of note this strain was not only resistant to gatifloxacin but also did not exhibit any mutation selection window since its MPC and MIC were equal.

Concerning *parC* induced pneumonia, the occurrence of resistant mutants was very high since 80–100% of the treated animals harboured such double *parC*–*gyrA* mutants in their lungs. Furthermore, the proportion of the mutated bacterial population was also high (Figure 4); these *in vivo* findings are concordant with the increased numbers of a second mutation found *in vitro* in *parC*-harbouring pneumococci.^{41,42}

This finding also confirms the MSW concept; indeed the MPC of the two *parC* strains were eight times the MIC and consequently, in animals, the percentage of time gatifloxacin concentration was between these two concentrations (T_{MSW}) and AUC_{MSW} was high. The CART analysis revealed the boundaries

of this *in vivo* mutant selection window (Table 3); clearly, when T_{MSW} is above 45%, the risk of mutation is 100%. Of note, no mutant appeared when the C_{max}/MIC ratio was above 6. This result is close to other experimental findings;²⁸ however, the corresponding threshold for the AUC/MIC ratio was 90; this value is higher than both the usually considered range of 30–50 for the efficacy of fluoroquinolones on pneumococcal infections²⁸ and 33 for clinical pneumococcal eradication in humans.³¹ To explain this result, it is important to point out that our study focused on mutant occurrence using several strains with various fluoroquinolone susceptibility profiles and not only on the global concentration–effect relationship. The other explanation could be that the PK–PD values could be somewhat specific to a bug–drug couple in a particular model.⁴³ However, these results are of the same order of magnitude as those obtained in previous studies.^{14,16,17}

Even considering these limitations, our study showed that an AUC/MIC ratio of 50 is associated with a 100% bacterial reduction in lungs (in fact, a reduction of more than $6 \log_{10}$ cfu), which is probably equivalent to clinical cure with eradication, if the initial causative pneumococcus was fully susceptible to fluoroquinolones. This is concordant with pharmacodynamic estimations for gatifloxacin.⁴⁴ On the other hand, if the initial causative pneumococcus harboured a low level of resistance to gatifloxacin (*parC* mutation and to a lesser extent efflux), the risk of both emerging resistance and failure to decrease the infecting burden in the lung with a standard regimen of gatifloxacin 400 mg per day is high. Since the current frequency of infection with *parC* pneumococci remains low,⁴⁵ this latter risk is also low. However, from a clinical point of view, this risk is much higher when patients have previously received a second generation fluoroquinolone treatment.⁴⁶

In conclusion, our findings have shown the excellent *in vivo* efficacy of a human-like regimen of 400 mg gatifloxacin on experimental pneumococcal pneumonia due to a ciprofloxacin-susceptible strain. However, mutants emerged when the pneumonia was induced by pneumococci with a low level of resistance to fluoroquinolones, especially for *parC* strains. These findings could be anticipated using the *in vitro* mutation selection window concept that our results translate *in vivo*.

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