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Influence of inoculum size on the selection of resistance in *Escherichia coli* by quinolone in a mouse-thigh bacterial infection model: a PK/PD analysis

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ABSTRACT

Maintaining quinolone concentrations outside the Mutant Selection Window (MSW) between the MIC and Mutant Prevention Concentration (MPC) was suggested by in vitro and in vivo studies to prevent the selection of resistant mutants. However, selection may also depend on the presence of resistant bacterial mutants at the start of treatment that is highly dependent on the initial inoculum size. In this study, a mouse-thigh bacterial infection model was used to test the influence of different exposures to marbofloxacin on the selection of resistant bacteria after infection with a low ($10^5$ CFU) or high ($10^8$ CFU) initial inoculum of Escherichia coli. The inoculum size was shown to influence the exposure to marbofloxacin and the values of PK/PD indices. When the abilities of the indices time within the MSW ($T_{MSW}$), $AUC_{24}/MIC$ and $C_{max}/MIC$ to predict the selection of resistant bacteria were compared, only the $T_{MSW}$ appeared to be a good predictor of the prevention of resistance for values less than 30%. When the $T_{MSW}$ was higher than 34%, the selection of resistant bacteria occurred less often in thighs initially infected with the low inoculum (11/24, 46 %) than in those infected with the high inoculum (30/36, 80 %) suggesting that the selection of resistant mutants may depend on both the $T_{MSW}$ and inoculum size. The relevance of these results merits further investigation to test different strategies of antibiotic therapy depending on the expected bacterial burden at the infectious site.
INTRODUCTION

Resistances to fluoroquinolones can occur spontaneously in bacterial populations at a frequency of about $10^{-6}$ to $10^{-8}$ (4) following a stepwise process that involves mutations in genes coding for the targets, DNA gyrase and topoisomerase IV (22, 28). Consequently, if the bacterial load at the infectious site exceeds the inverse of the mutation frequency, it can be presumed that a small resistant subpopulation already coexists with a larger susceptible population, before any antimicrobial treatment is administered. Traditionally, *in vitro* antimicrobial studies and animal infection models have been used to assess the reduction in the total bacterial population at an infectious site while often ignoring the impact of drug pressure on amplification of the drug-resistant subpopulation (7). Thus, the values of pharmacokinetic/pharmacodynamic indices determined from these experiments were previously selected to predict the bacteria killing and not the selection of resistant bacteria.

These PK/PD indices: $f_{AUC}/MIC$ (area under the free-plasma concentration curve divided by the Minimal Inhibitory Concentration (MIC)), $f_{C_{max}}/MIC$ (peak free-plasma concentration divided by the MIC) and $f_{T_{>MIC}}$ (time the free concentrations are above the MIC), are all expressed as a function of the MIC, which is the pharmacodynamic parameter used to describe the susceptibility of the major drug-susceptible population. From experiments carried out with fluoroquinolones, the Mutant Prevention Concentration (MPC) has been proposed to assess the susceptibility of the resistant subpopulation (11, 23, 29). MIC and MPC then define the boundaries of the so-called Mutant Selection Window (MSW), which is the range of antibiotic concentrations that would favour the selection of first-step mutants (29). First-step mutants of *Staphylococcus aureus* and *Streptococcus pneumoniae* were selected by ciprofloxacin, levofloxacin or moxifloxacin when antibiotic concentrations fell within the MSW *in vitro* (5, 6, 16, 30). A study carried out in a rabbit lung infection model with *Streptococcus pneumoniae* showed that the selection of resistant bacteria occurred
systematically when concentrations of gatifloxacin were within the MSW ($T_{MSW}$) for more than 45% of the treatment duration (9). Another experiment in rabbits infected by *Staphylococcus aureus* also showed that drug concentrations needed to be at the bottom of the MSW (just above the MIC) for only 33% of the time to enrich mutants (10). These *in vivo* results, which suggest that fluoroquinolone concentrations need to be outside the MSW for most of the time to prevent the selection of resistant mutants, were observed with a high inoculum size (more than $10^9$ CFU). However, the bacterial burdens to be eradicated at the infectious site may be very low or null when antibiotics are used preventively as in prophylaxis of gram-negative bacteremia in immuno-compromised patients (24) or metaphylaxis in veterinary medicine. For both human and veterinary medicine, prophylaxis is the administration of antimicrobials to exposed individuals considered at risk, but before the expected onset of disease. Metaphylaxis consists of treating all animals at the herd level when there is a clinical disease in only some animals (26). In the case of prophylaxis and metaphylaxis, the bacterial inocula at the beginning of the treatment are presumably much smaller than those targeted when patients express clinical signs or are critically ill and therefore the likelihood of a mutant appearing may be low, particularly if the bacterial load is less than the inverse of the mutation rate.

We previously observed *in vitro* that emergence of resistance was more frequent when both the time within MSW ($T_{MSW}$) and the bacterial inoculum size increased (14) and the aim of this study was to test, *in vivo*, the ability of $T_{MSW}$ and also of AUC$_{24}$/MIC and $C_{max}$/MIC indices to predict the selection of resistant bacteria in a low and a high inoculum. We assessed the selection of a pre-existing resistant subpopulation of *Escherichia coli* in mouse thighs infected with the two bacterial inoculum sizes, after different exposures to marbofloxacin, a fluoroquinolone extensively used in veterinary medicine.
MATERIALS AND METHODS.

Bacteria and antibiotic.

*Escherichia coli* ATCC25922 was used as the susceptible bacteria and an *Escherichia coli* isolated from a previous thigh infected by *E. coli* ATCC25922, treated with marbofloxacin and carrying a S83L mutation on GyrA was used as the resistant bacteria. Marbofloxacin, a third generation quinolone, was kindly provided by Vetoquinol, Lure, France.

In vitro susceptibility testing. (i) MIC determination.

MICs were determined in triplicate for the susceptible and mutant bacteria by a broth microdilution method according to CLSI reference methods.

(ii) MPC determination. The MPC was determined as previously described (4). Briefly, an overnight culture of the tested bacteria in Mueller Hinton broth (MHB) was concentrated 100 times in NaCl 0.9% to obtain a suspension containing $10^{10}$ CFU/mL. One hundred microliters of this suspension were then plated on MH agar containing various concentrations of marbofloxacin obtained by successive two-fold dilutions. The MPC was the lowest marbofloxacin concentration preventing the growth of bacterial colonies after incubation for 72 hours at 37°C. Determinations were done in triplicate.

PCR amplification of quinolone resistance-determining regions (QRDRs) and DNA sequencing.

Genomic DNA was isolated from bacterial strains and used as the template in PCR amplification of the QRDRs of gyrA and parC genes. PCR amplification and nucleotide sequencing were carried out with previously described primers (13).

Inoculum preparation.

A few colonies from an overnight culture of susceptible (ATCC25922) and resistant (mutant) *Escherichia coli* were grown at 37°C for 18 h in 50 mL of MHB and in 10 mL of MHB supplemented with 0.128 µg/mL marbofloxacin (the first concentration below the MIC of the
resistant bacteria) respectively. Bacteria were then collected by centrifugation at 3000 g for 10 min and resuspended in NaCl 0.9% to obtain a final suspension containing $10^9$ CFU/mL of susceptible bacteria and $10^3$ CFU/mL of resistant bacteria. This suspension, which corresponded to the high inoculum, was then diluted 1000 times to obtain the low inoculum. This method to prepare the bacterial inoculum implied that the low inoculum ($6.6\pm0.21 \log_{10}$ CFU/mL) may have contained resistant mutants in about 40% of the experiments depending on the sampling.

**Animals.**

Female Swiss mice (Charles River Laboratories, L’arbresle, France) were used for all studies. All animal procedures were conducted in accordance with accepted human standards of animal care under the agreement number A 31909 for animal experimentation from the French Ministry of Agriculture.

**Neutropenic mouse thigh infection model.**

The model used was previously described by Andes and Craig (2). Briefly, female Swiss mice were made neutropenic by intraperitoneal injection of cyclophosphamide (Sigma-Aldrich, Saint Quentin Fallavier, France) at rates of 150 mg/kg and 100 mg/kg of body weight on days 1 and 4 respectively. On day 5, the mice were infected by intramuscular injection of 100 µL of the high or low inoculum in each thigh (six thighs per dose, dosing schedule and inoculum size). Marbofloxacin was administered intraperitoneally beginning 2 hours after infection. The total doses were 0, 2, 5, 10 and 20 mg/kg marbofloxacin for mice sacrificed at 24 hours and 0, 4, 10, 20 and 40 mg/kg marbofloxacin for mice sacrificed at 48 hours after infection. For each dose of marbofloxacin, one group of mice received the total dose in a single administration two hours after infection and another group received the same total dose divided into two or four administrations for mice sacrificed 24 or 48 hours respectively after infection (that corresponded to an administration twice a day). The mice were sacrificed by an
intraperitoneal injection of pentobarbital (Dolethal\textsuperscript{ND}, Vetoquinol, France) 2 hours (non-infected mice), 24 or 48 hours after the infection. Both thighs were aseptically removed and homogenized in 10 mL of NaCl 0.9%. The homogenates were centrifuged at 3000 g for 10 minutes and washed twice in 10 mL of NaCl 0.9%. Ten microliters of successive 10-fold dilutions of homogenates were then plated in triplicate on MH drug-free agar plates or on MH plates supplemented with 0.128 µg/mL of marbofloxacin. The colonies were counted after overnight incubation at 37°C. If the bacterial counts were less than 1000 CFU/thigh, 100 µL of the homogenates were plated on agar. If the colonies were too small, incubation was continued for a further 24 hours. The lowest level of detection was 100 CFU/thigh and bacteria were considered eradicated below this level.

**Pharmacokinetics.**

Female Swiss mice, satellites from the pharmacodynamic assay, were treated by cyclophosphamide as described above. On the fifth day, the mice were infected with either the high or low inoculum in each thigh and two hours later were administered a single intraperitoneal dose of marbofloxacin. The doses tested were 1 and 5 mg/kg marbofloxacin for the low inoculum and 5 and 20 mg/kg marbofloxacin for the high inoculum. Groups of 3 or 4 mice each were anesthetized by intraperitoneal injection of pentobarbital (Dolethal\textsuperscript{ND}, Vetoquinol, France) 0.25, 0.5, 1, 2, 4, 6, 8 or 24 hours after dosing. Blood samples (one sample from each animal) were collected by puncture of the caudal vena cava. Protein binding was assessed by ultrafiltration as previously described (20). Briefly, plasma was spiked with 0.05, 0.1, 0.5, 1, 5 or 10 µg/mL marbofloxacin and was incubated for 30 min at 37°C. Plasma was then transferred to Centrifree devices (Millipore Corporation, Billerica, MA, USA) and centrifuged at ambient temperature for 30 min at 1200 g. The volumes of the ultrafiltrates were at least 200 µL. A simple and sensitive high performance liquid chromatography method, using ultraviolet detection at 295 nm to determine the marbofloxacin concentrations.
in plasma and ultrafiltrates, was adapted from Schneider et al. (27). Briefly, samples were extracted by solid phase extraction on a C8 100 mg cartridge. Marbofloxacin and the internal standard, ofloxacin, were separated on a reverse-phase C18 Inertsil ODS-3 column and eluted with 25 mM citrate buffer (pH=3.0) and acetonitrile in an 85:15 ratio. The standard calibration curve for marbofloxacin, using a weighted linear regression model, was linear for concentrations ranging from 0.01 to 2 µg/mL. The intra-day and inter-day precision ranged from 4.58 to 9.27% and from 4.85 to 9.46% respectively. The accuracy varied from 91 to 108%.

**PK-PD analysis.**

The data obtained with a single intraperitoneal marbofloxacin injection of 5 mg/kg were analysed separately for each inoculum size, using WinNonlin version 5.2 (Pharsight Corporation, Mountain View, CA, USA). For the two inocula tested (low and high), a naïve pooling approach was used to fit the marbofloxacin data with a bi-exponential model. The AUC\textsubscript{tot}, AUC\textsubscript{24} and AUC\textsubscript{48} were calculated by integrating the equation used to fit the data from zero to infinity, from zero to 24 hours and from zero to 48 hours, respectively (17). The terminal half-life (T\textsubscript{1/2elim}) was calculated as the naperian logarithm of 2 divided by the elimination rate constant. Dose-proportionality was assessed from the kinetic data obtained with 1 mg/kg marbofloxacin for the low and with 20 mg/kg marbofloxacin for the high inoculum. Marbofloxacin pharmacokinetic parameters obtained from mice treated with 5 mg/kg were then used to estimate the T\textsubscript{MSW}, AUC\textsubscript{24}/MIC and C\textsubscript{max}/MIC for each tested marbofloxacin dosing regimen (single and fractionated doses) and bacterial inoculum size. The AUC\textsubscript{24}/MIC for mice sacrificed 48 hours after the infection were determined by dividing the AUC\textsubscript{48}/MIC by 2 to obtain a mean AUC\textsubscript{24}/MIC for each day.
The sigmoid inhibitory $E_{\text{max}}$ models describing the dose-response relationships for the low and high inoculum after 24 hours of exposure to marbofloxacin were delineated with WinNonlin version 5.2 (Pharsight Corporation, Mountain View, CA, USA).
RESULTS

Susceptibility studies. The MICs of marbofloxacin were 0.008 and 0.256 µg/mL for the susceptible and mutant strains of *Escherichia coli* respectively. The marbofloxacin MPC was 0.256 µg/mL for the susceptible bacteria and corresponded to the MIC of the mutant strain. The Mutant Selection Window (MSW) for the susceptible bacteria was therefore the range of marbofloxacin concentrations between the MIC (0.008 µg/mL) and the MPC (0.256 µg/mL). The term “resistant bacteria” in the present paper should be understood as bacteria growing on 0.128 µg/mL marbofloxacin.

Pharmacokinetics. The observed (total) and the predicted (total) plasma concentrations of marbofloxacin following single intraperitoneal doses of 1 and 5 mg/kg in neutropenic mice infected with the low inoculum and doses of 5 and 20 mg/kg in neutropenic mice infected with the high inoculum are shown in Figure 1. The values for the marbofloxacin pharmacokinetic parameters obtained for each dose are shown in Table 1. Marbofloxacin kinetics in mice differed considerably between the two inoculum sizes, the AUC (exposure) in mice subjected to the high inoculum being two times greater after the same dose of 5 mg/kg. Dose-proportionality was assessed with marbofloxacin doses of 1 mg/kg for the low and 20 mg/kg for the high inoculum, respectively. The dose/AUC ratios with both doses were similar to those of mice infected with the same inoculum size and treated with 5 mg/kg marbofloxacin, suggesting dose-proportionality for the range of concentrations tested. The binding of marbofloxacin to mice plasma proteins, between 0.05 µg/mL and 10 µg/mL, was less than 10 % and the PK/PD indices were determined from the total plasma concentrations. The time within the MSW ($T_{\text{MSW}}$), and the $AUC_{24}/\text{MIC}$ and $C_{\text{max}}/\text{MIC}$ values, obtained after the administration of each marbofloxacin dosing regimen to mice sacrificed 48 hours after infection with the low or high inoculum, are presented in Table 2. Assuming dose-proportionality, the calculated $AUC_{24}$ values were directly proportional to the total dose of
marbofloxacin for a given inoculum size and were independent of dose fractionation. The
$C_{\text{max}}$ values were also linked to the total dose but the values were four times less after
fractionation of the doses into four administrations (twice a day for 2 days) than after the
administration of single doses. The influence of marbofloxacin dosage regimen (both total
dose and fractionation) and inoculum size on the $T_{\text{MSW}}$ indice was much more complex and
resulted in an overlap of $T_{\text{MSW}}$ values between the dosage regimens.

PK-PD analysis.

(i) total bacterial populations. The total bacterial populations after 24 hours of exposure to
marbofloxacin are shown for each inoculum size and each dosing regimen in Table 3. The
lowest tested dose of 2 mg/kg marbofloxacin, corresponding to $\text{AUC}_{24}/\text{MIC}$ values of 175 and
387 h for the low and high inoculum respectively, was bacteriostatic. The dose of 5 mg/kg
marbofloxacin, corresponding to $\text{AUC}_{24}/\text{MIC}$ values of 450 and 962 h for the low and high
inoculum respectively, reduced the bacterial population by more than one log for both
inocula. Figure 2 shows that the dose-response relationship was best described by inhibitory
$E_{\text{max}}$ curves for the low (equation 1) and the high inoculum (equation 2).

$$\log_{10} \text{CFU/thigh} = 9.58 - \left(\frac{6.28 \times \text{dose}}{\text{dose} + 1.27}\right)$$

$$\log_{10} \text{CFU/thigh} = 10.02 - \left(\frac{5.70 \times \text{dose}}{\text{dose} + 3.92}\right)$$

By assuming dose-proportionality for a given inoculum, the relationships between the dose or
$\text{AUC}_{24}/\text{MIC}$ and the reduction of the total bacterial population had exactly the same shape.
After 24 hours, we observed the same reduction in the total bacterial population whatever the
dose fractionation even though $C_{\text{max}}$ were four times lower and $T_{>\text{MIC}}$ always longer after
fractionated doses than after single doses. As a consequence, no relationship could be found
between $C_{\text{max}}/\text{MIC}$ or $T_{>\text{MIC}}$ and the reduction of the total bacterial population.

The bacterial populations after 48 hours of exposure to marbofloxacin are shown in Table 4
for each inoculum size and each dosing regimen. All control mice (dose 0) and all mice
infected by the high inoculum and treated with a total dose of 4 mg/kg marbofloxacin, corresponding to AUC$_{24}$/MIC and AUC$_{24}$/MPC values of 387 and 12 h respectively, were dead within 48 hours post-inoculation. At the higher doses and also for the low inoculum, the bacterial population was eradicated in some thighs, the frequency of eradication being greater the higher the total dose. However, no clear relationship between reduction of the bacterial populations after 48 hours of exposure to marbofloxacin and the total dose or AUC$_{24}$/MIC was apparent. After single doses associated with higher C$_{max}$/MIC, the bacterial populations were lower when the initial inoculum was low but higher when the initial inoculum was high than after the same fractionated doses. This strongly suggested that there was no relationship between C$_{max}$/MIC and the reduction of the total bacterial population.

(ii) resistant bacterial populations. When the treatment was started, no resistant bacteria were detected in mice infected with the low inoculum while 2.74±0.41 log$_{10}$ CFU of resistant bacteria were present in mice infected with the high inoculum. Twenty-four hours after infection, no resistant bacteria were recovered from the control mice infected with the low inoculum. The proportions of resistant bacteria after 24 hours and 48 hours versus dose are shown in Figure 3. After 24 hours, with the low inoculum, resistant bacteria in a proportion of 3.10$^{-2}$ of the total bacteria count were only found in one thigh of a mouse treated with the fractionated dose of 2 mg/kg marbofloxacin (Figure 3A). With the high inoculum, the proportion of resistant bacteria in all thighs was always greater than 10$^{-5}$ except for four thighs of mice treated with a total dose of 20 mg/kg marbofloxacin in which all resistant bacteria had been eradicated (Figure 3C).

After 48 hours, no resistant bacteria were detected in mice infected with the low inoculum and treated with single doses of marbofloxacin whereas resistant bacteria were recovered in 46 % of the thighs treated with fractionated doses. The proportion of resistant bacteria ranged from 10$^{-2}$ to 1 in these thighs (Figure 3B). For the high inoculum, resistant bacteria were recovered
in all thighs after single doses whereas resistant bacteria were only recovered in 33% of the
thighs treated with fractionated doses. The proportions of resistant bacteria were always
greater than $10^{-2}$ after single doses and ranged from $10^{-6}$ to $10^{-2}$ after the administration of
fractionated doses (Figure 3D). As these results indicated a net difference in the selection of
resistant bacteria after administrations of single or fractionated doses, it can be presumed that
no simple relationship existed between the total dose and the selection of resistant bacteria.
We then investigated the ability of $T_{MSW}$ and also $AUC_{24}/MIC$ and $C_{max}/MIC$ indices, that
reflected the overall exposure, to predict the selection of resistant bacteria. The proportions of
resistant bacteria after 48 hours of treatment are shown against the PK/PD indices in Figure 4.
The relationship between the proportion of resistant bacteria and the $AUC_{24}/MIC$ and
$C_{max}/MIC$ indices was poor whereas a clear relationship existed between $T_{MSW}$ and the
prevention of resistance. Indeed, after 48 hours of treatment, the marbofloxacin dosing
regimen associated with a $T_{MSW}$ of 34 to 82% led to the selection of resistant bacteria in 46%
of the thighs with the low inoculum and in 80% with the high inoculum. By contrast no
selection of resistant bacteria occurred when $T_{MSW}$ was less than 30%, whatever the inoculum
size.
DISCUSSION

Maintaining drug concentrations outside the MSW, which is the range of antibiotic concentrations favouring the selection of resistant mutants, has been suggested to restrict mutant outgrowth (12, 29). Previous in vivo studies that showed an enrichment of mutants when drug concentrations were most of the time within the MSW tested only one inoculum size (1, 8-10, 18). However, the bacterial load at the site of infection, against which antibiotic drugs are prescribed, can vary considerably whether the treatment is prophylactic or curative.

The aim of the present study was to investigate the ability of $T_{\text{MSW}}$ and also of AUC$_{24}$/MIC and C$_{\text{max}}$/MIC indices to predict the selection of resistant bacteria in a mouse-thigh infection model. Mice infected with a low or high inoculum of *Escherichia coli*, already containing a proportion of $10^{-6}$ resistant mutants, were treated with different regimens of marbofloxacin to assess the influence of dose, actual exposure (as determined by the AUC$_{24}$/MIC, C$_{\text{max}}$/MIC and $T_{\text{MSW}}$) and inoculum size on the selection of resistant bacteria.

Pharmacokinetic studies in which 5 mg/kg marbofloxacin was administered to infected animals revealed a large difference between the rates of elimination of marbofloxacin with the two tested inoculum sizes. Higher elimination half-lives and decreased systemic clearance were previously observed in infected calves (20) and horses (25) that were treated with marbofloxacin. Explanations for alteration of the drug elimination pathways may be the production by gram-negative bacteria of endotoxins that were shown to decrease the activity of hepatic metabolism, the renal blood flow and glomerular filtration rate (19).

Whatever the regimen, a given total dose of marbofloxacin produced a similar reduction in bacterial population size after 24 hours of treatment, thereby suggesting that the AUC$_{24}$/MIC indice, which was proportional to the total dose, was an appropriate predictor of bacterial killing. In addition, after 48 hours of exposure to marbofloxacin, the only PK/PD indice that was identical for all mice that died after infection with the high inoculum and a
treatment with 4 mg/kg marbofloxacin for 48 hours, was also the $AUC_{24}/MIC$ since the $C_{\text{max}}/MIC$ and $T_{\text{MSW}}$ indices differed between mice treated with single doses and mice treated with fractionated doses. In a previous study, an $AUC/MIC$ value of 70 h for gatifloxacin was suggested to have a bacteriostatic effect in a thigh infection of *Klebsiella pneumoniae* in neutropenic mice (3). In our study, the value of $AUC_{24}/MPC$ (corresponding to the $AUC_{24}/MIC$ for the resistant bacteria) was 12.1 h for a dose of 4 mg/kg marbofloxacin, which would explain the inability of this dose to slow the growth of the resistant subpopulation enough to prevent death. For the susceptible bacteria, the lowest $AUC_{24}/MIC$ observed in our study, i.e. 175 and 387 h for the low and high inoculum respectively, were always sufficient to prevent bacterial growth for 24 hours. For the low inoculum, in which the initial resistant population was assumed to be very small (the probability of the presence of at least one resistant mutant was 0.4), an $AUC/MIC$ value of 175 h was associated with the prevention of the emergence of a resistant population for 24 hours in 11 thighs out of 12. This is in agreement with the $AUC/MIC$ value of 157 h found by Jumbe *et al.* (21) to prevent the amplification of mutant strains in a large population of *Pseudomonas aeruginosa* treated with levofloxacin. However, after 48 hours of treatment, the $AUC_{24}/MIC$ became a poor predictor of the selection of resistant bacteria. For example, no resistant bacteria were selected in the low inoculum after a single dose of marbofloxacin corresponding to an $AUC_{24}/MIC$ value of 175 h whereas resistant bacteria were selected in four thighs out of six after fractionated doses corresponding to the same $AUC_{24}/MIC$ value. With the high inoculum challenge, the opposite was observed. Indeed, for the same $AUC_{24}/MIC$ and $AUC_{24}/MPC$ values of 3850 and 120 h respectively, resistant bacteria were only selected after single doses. This could not be explained by the PK/PD parameter $C_{\text{max}}/MIC$ because the value of $C_{\text{max}}$ that prevented the selection of resistant bacteria after dose fractionation was 4 times lower than after a single dose which did lead to the selection of resistant bacteria. So in our study the lack of
consistency in the selection of resistance, regarding inoculum size and the dosing regimen after 48 hours of treatment, could not be explained by the AUC$_{24}$/MIC and C$_{\text{max}}$/MIC indices. Indeed, no relationship was found in these cases between AUC$_{24}$/MIC or C$_{\text{max}}$/MIC and prevention of the selection of resistant bacteria.

By contrast, when the marbofloxacin exposures expressed as T$_{\text{MSW}}$ for each inoculum size were examined, a T$_{\text{MSW}}$ of less than 30% was found to predict the prevention of resistance selection for both inoculum sizes. This result suggested that T$_{\text{MSW}}$ could be a good predictor of resistance prevention in vivo although a previous in vitro model with *Staphylococcus aureus* showed no correlation between T$_{\text{MSW}}$ and selection of resistant bacteria (5). However, it was recently suggested that the discrepancies observed in the T$_{\text{MSW}}$ ability to predict selection may be explained by an additional influence of the position of antimicrobial concentrations within the MSW on the selection of resistant bacteria (10, 15). Indeed, in vivo, it was shown in a local infection of rabbits with *Staphylococcus aureus* that the T$_{\text{MSW}}$ leading to the emergence of resistance could be 30 % or 80 % depending on the area of the MSW in which drug concentrations fluctuated (10). In our study, we found the same minimal time of 30 % associated with the selection of resistance. By relating marbofloxacin exposure to the T$_{\text{MSW}}$, we clearly showed that all mice in which a selection of resistant bacteria occurred after 48 hours of marbofloxacin treatment were exposed to a T$_{\text{MSW}}$ greater than 34 % for 48 hours irrespective of the inoculum size. Similar results were also reported in a rabbit model of human therapy with *Streptococcus pneumoniae* and moxifloxacin or gatifloxacin in which selection occurred when the T$_{\text{MSW}}$ ranged from 72.5 to 93.5% or when T$_{\text{MSW}}$ was greater than 45% (8, 9). However, in these latter experiments, the rabbits were infected with a high inoculum containing more than $10^9$ CFU/lung. By testing $10^6$ and $10^9$ CFU/thigh we were able to show that resistance was less frequently selected in the low inoculum than in the high inoculum. After 48 hours of exposure to marbofloxacin, we showed that for a T$_{\text{MSW}}$ of 34 %
or more, resistant bacteria were selected in 80% (30/36) of the thighs exposed to the high inoculum but in only 46% of thighs (11/24) in mice infected by the low inoculum. Interestingly, this percentage of 46% corresponded to the probability of resistant bacteria being present at the start of treatment with our method of inoculum preparation. The less frequent selection of resistant bacteria in the low inoculum, which remained within the MSW for a long time, seemed to be due to the fact that these bacteria were rarer from the start in the low inoculum.

In conclusion, our results fully support the concept of MSW in vivo in a mouse-thigh model since we showed that maintaining the antibiotic concentrations outside the MSW prevented the selection of resistant mutants. We also showed in our pharmacokinetic study in animals infected with either a high or low inoculum, that the size of the bacterial population at the start of treatment could influence both the exposure to the antibiotic and the selection of resistance. Indeed, the PK/PD study indicated that, for approximately the same T_{MSW}, the selection of resistant bacteria occurred less often with the low inoculum than with the high inoculum probably due to the less frequent presence of mutants in the low inoculum. Finally, this study highlights the importance of assessing antibiotic exposure in critically ill patients or animal models when establishing fluoroquinolone-dosing strategies in relation to the MSW concept. The relevance of these results merits further investigation with pathogens of interest and in a clinical context because of the common use of fluoroquinolones to treat a variety of bacterial infections including urinary bacterial infection and to prevent febrile neutropenia in cancer patients.
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REFERENCES


FIGURE 1: Plasma marbofloxacin concentrations versus time after administration of a single intraperitoneal dose of 1 (□) and 5 (○) mg/kg in mice infected with a low inoculum and doses of 5 (●) and 20 (■) mg/kg in mice infected with a high inoculum. Fitted concentration-time curves for the low (thin line) and the high (bold line) inoculum are also represented.

FIGURE 2: Total bacterial population in each thigh infected with a low (○) or a high (■) inoculum twenty-four hours after different total doses of marbofloxacin. Fitted curves for the dose-effect relationship are shown for the low (dashed line) and the high (continuous line) inocula. The bacterial populations were assumed to be $10^{10}$ CFU/thigh for the dead mice.

FIGURE 3: Proportions of resistant bacteria in each thigh infected with a low (A-B) or a high (C-D) inoculum twenty-four (A-C) or forty-eight (B-D) hours after different total doses of marbofloxacin. Proportions obtained after single doses (●) and after fractionated doses (□) are represented. Six thighs were tested per time, inoculum size and dosing regimen.

FIGURE 4: Proportions of resistant bacteria in each thigh infected with low (circles) or high (squares) inoculum after forty-eight hours of exposure to marbofloxacin versus AUC$_{24}$/MIC (A), $C_{\text{max}}$/MIC (B) and T$_{\text{MSW}}$ (C). Proportions are represented by empty symbols when they were obtained after fractionated doses of marbofloxacin and by full symbols after single doses of marbofloxacin.
Marbofloxacin pharmacokinetic parameters (elimination half-life ($T_{1/2\text{elim}}$), area under the curve from 0 to infinity ($AUC_{\text{tot}}$), and the dose/AUC ratio) for mice infected with a low or a high bacterial inoculum and treated with single intraperitoneal doses of 1, 5 or 20 mg/kg.

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</tr>
<tr>
<td>$AUC_{\text{tot}}$ (µg.h.mL$^{-1}$)</td>
<td>0.78</td>
<td>3.59</td>
</tr>
<tr>
<td>Dose/AUC (mL.h$^{-1}$.kg$^{-1}$)</td>
<td>1282</td>
<td>1393</td>
</tr>
</tbody>
</table>
The estimated times within the Mutant Selection Window ($T_{MSW}$), AUC$_{24}$/MIC and $C_{max}$/MIC values after administration of 4, 10, 20 or 40 mg/kg marbofloxacin for mice sacrificed after 48 hours of infection with the low or high inoculum. The values are given for each dosing regimen (total dose administration in a single dose two hours after the infection or fractionated twice a day).

<table>
<thead>
<tr>
<th>Total dose (mg/kg)</th>
<th>Low inoculum</th>
<th>High inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td><strong>Fractionated Dose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{MSW}$ (%)</td>
<td>57.7</td>
<td>66.6</td>
</tr>
<tr>
<td>AUC$_{24}$/MIC (h)</td>
<td>175</td>
<td>450</td>
</tr>
<tr>
<td>$C_{max}$/MIC</td>
<td>95</td>
<td>237</td>
</tr>
<tr>
<td><strong>Single Dose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{MSW}$ (%)</td>
<td>18.9</td>
<td>19.7</td>
</tr>
<tr>
<td>AUC$_{24}$/MIC (h)</td>
<td>175</td>
<td>450</td>
</tr>
<tr>
<td>$C_{max}$/MIC</td>
<td>375</td>
<td>950</td>
</tr>
</tbody>
</table>

The indices were estimated from kinetic studies performed in mice subjected to a low or high inoculum and treated with 5mg/kg marbofloxacin.
TABLE 3.

Total bacterial populations (mean±SD) after 24 hours of exposure to different total doses of marbofloxacin for the initial low and high inocula.

<table>
<thead>
<tr>
<th>Total dose (mg/kg)</th>
<th>Start&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low inoculum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single doses</td>
<td>6.15±0.48</td>
<td>9.52±0.35 *</td>
<td>6.17±1.12</td>
<td>4.36±1.36</td>
<td>4.31±1.45</td>
<td>4.06±0.83 (1)</td>
</tr>
<tr>
<td>Fractionated doses</td>
<td>5.07±1.62</td>
<td>4.77±1.16</td>
<td>4.51±1.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>High inoculum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single doses</td>
<td>8.65±0.17</td>
<td>**</td>
<td>8.66±0.61</td>
<td>6.86±0.58</td>
<td>6.39±1.37</td>
<td>5.54±1.28</td>
</tr>
<tr>
<td>Fractionated doses</td>
<td>7.86±0.61</td>
<td>6.03±0.77</td>
<td>6.21±0.55</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Six thighs were tested for each inoculum, each dose and each dosing regimen. For start and at dose 0 twelve thighs were tested per inoculum size. Numbers in brackets correspond to the number of thighs in which bacteria were eradicated.

<sup>a</sup> Total bacterial population two hours after the time of infection

* one mouse out of six was dead

** all six mice were dead
TABLE 4.

Total bacterial populations (mean±SD) after 48 hours of exposure to different total doses of marbofloxacin administered in single or fractionated doses for initial low and high inocula.

<table>
<thead>
<tr>
<th>Total dose (mg/kg)</th>
<th>0</th>
<th>4</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low inoculum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single doses</td>
<td>*</td>
<td>3.63±1.65</td>
<td>4.92±1.79</td>
<td>2.75±0.37</td>
<td>4.25±1.59 (4)</td>
</tr>
<tr>
<td>Fractionated doses</td>
<td>*</td>
<td>6.24±1.57 (2)</td>
<td>5.23±1.38 (3)</td>
<td>5.61±0.44 (4)</td>
<td>5.21±0.12 (4)</td>
</tr>
<tr>
<td><strong>High inoculum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single doses</td>
<td>*</td>
<td>*</td>
<td>7.93±1.50</td>
<td>8.28±0.92</td>
<td>5.89±0.98</td>
</tr>
<tr>
<td>Fractionated doses</td>
<td>*</td>
<td>*</td>
<td>6.23±0.86</td>
<td>4.72±1.37</td>
<td>3.67±1.16 (1)</td>
</tr>
</tbody>
</table>

Six thighs were tested for each inoculum, each dose and each dosing regimen. Numbers in brackets correspond to the number of thighs in which bacteria were eradicated. There was no clear relationship between the dose and the reduction of the total bacterial population.

* all three mice were dead
FIGURE 1

Marbofloxacin plasma concentrations (µg/mL)

MPC

MIC

Time (h)
FIGURE 2:

![Graph showing the relationship between Marbofloxacin total dose (mg/kg) and total bacterial population (log₁₀ CFU/thigh).]
PROPORTIONS OF RESISTANT BACTERIA PER THIGH

MARBOFLOXACIN TOTAL DOSES (MG/KG)

24 HOURS  

LOW INOCULUM

24 HOURS

HIGH INOCULUM

48 HOURS

NO RESISTANT BACTERIA

MARBOFLOXACIN TOTAL DOSES (MG/KG)
FIGURE 4

Proportions of resistant bacteria per thigh

A

B

C

No resistant bacteria

$AUC_{24}/MIC$ (h)

$C_{max}/MIC$

$T_{MSW}$ (%)