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Influence of Inoculum Size and Marbofloxacin Plasma Exposure on the Amplification of Resistant Subpopulations of *Klebsiella pneumoniae* in a Rat Lung Infection Model.

Anne-Sylvie Kesteman\(^1,2\), Aude A. Ferran\(^1\), Agnès Perrin-Guyomard\(^2\), Michel Laurentie\(^2\), Pascal Sanders\(^2\), Pierre-Louis Toutain\(^1\) and Alain Bousquet-Mélou\(^1\)*

\(^1\) UMR181 Physiopathologie et Toxicologie Expérimentales, INRA, ENVT, Ecole Nationale Vétérinaire de Toulouse, 23 chemin des Capelles, BP 87614, 31076 Toulouse Cedex 3, France.

\(^2\) Laboratory for the Research and Investigation of Veterinary Drugs and Disinfectants, AFSSA Fougères, La Haute Marche, BP 90203, Javené 35302 Fougères, France

* Corresponding author. Mailing address: UMR181 Physiopathologie et Toxicologie Expérimentales, INRA, ENVT, Ecole Nationale Vétérinaire de Toulouse, 23 chemin des Capelles, BP 87 614, 31 076 Toulouse Cedex 3, France. Phone: +33 (0)561193925. Fax: +33 (0)561193917. E-mail: a.bousquet-melou@envt.fr
ABSTRACT

We tested the hypothesis that the bacterial load at the infection site could impact considerably on the pharmacokinetic-pharmacodynamic parameters of fluoroquinolones. Using a rat lung infection model we measured the influence of different marbofloxacin dosage regimens on the selection of resistant bacteria after infection with a low ($10^5$ CFU) or a high ($10^9$ CFU) inoculum of *Klebsiella pneumoniae*. For daily fractionated doses of marbofloxacin, the prevention of resistance occurred for an AUC/MIC ratio of 189h for the low inoculum whereas for the high inoculum, resistant subpopulation enrichment occurred for AUC/MIC values up to 756h. For the high inoculum infected rats, the AUC/MIC, $C_{\text{max}}$/MIC and time within the Mutant Selection Window (T$_{\text{MSW}}$) were not effective predictors of resistance prevention when comparing fractionated and single administrations. An index corresponding to the ratio of the time the drug concentrations were above the MPC and within the MSW (T$_{>\text{MPC}}$/T$_{\text{MSW}}$) was the best predictor of the emergence of resistance: a T$_{>\text{MPC}}$/T$_{\text{MSW}}$ of 0.54 was associated with the prevention of resistance for both fractionated and single administrations. These results suggest that the enrichment of resistant bacteria depends heavily on the inoculum size at the start of an antimicrobial treatment, and that classical PK/PD parameters cannot adequately describe the impact of different dosage regimens on resistant bacteria enrichment. We propose an original index, the ratio T$_{>\text{MPC}}$/T$_{\text{MSW}}$, that reflects the proportion of time the less susceptible bacterial subpopulation is killed over the time it is selected, as a potentially powerful indicator of the prevention of resistant bacteria enrichment. This ratio is valid only if plasma concentrations achieved the MPC.
INTRODUCTION

In infections such as pneumonia, the burden of microorganisms can become quite high and may frequently exceed the inverse of the frequency of mutations, leading to the development of a resistant subpopulation. This leads to the presence of a small subpopulation of resistant organisms at the time that antimicrobial therapy is initiated. Under these conditions, a drug exposure that will kill only the susceptible dominant population may allow amplification of the resistant mutant subpopulation that is present before treatment, resulting in the emergence of resistance during therapy (24, 38).

Resistance to fluoroquinolones can occur spontaneously in bacterial populations at a frequency of about $10^{-6}$ to $10^{-8}$ (6) following a stepwise process that involves mutations in the genes coding for the targets, DNA gyrase and topoisomerase IV (21, 32). Therefore, we can presume that an increase in the bacterial load at the infection site could be associated with an increase in the likelihood of a resistant mutant subpopulation being present before any fluoroquinolone treatment is administered.

Until recently, optimization and individualisation of antimicrobial dosing regimens have been primarily based on the pharmacokinetic/pharmacodynamic (PK/PD) indices that describe both the optimal efficacy and/or prevention of toxicity (27). However, the increasing problem of emergence of resistance under the influence of antibiotic selection pressure led to the identification of PK/PD indices that best correlate with the prevention of antimicrobial resistance (2). An approach to prevent the emergence of antimicrobial resistance has been proposed, consisting of administering the drug at doses that produce plasma concentrations that continuously exceed the threshold of spontaneous drug-resistant mutants susceptibility and thereby prevent the selective amplification of any mutant subpopulation (12, 37, 38). The drug concentration capable of inhibiting the growth of the least-susceptible single-step mutant subpopulation has been called the “Mutant Prevention Concentration” (MPC). In addition, it
has been suggested that selective amplification of spontaneous drug-resistant mutants is more pronounced within the range of antimicrobial plasma concentrations between the MIC of the wild bacterial population and the MPC, defined as the “Mutant Selection Window” (MSW) (6, 23, 36, 37). The concept of the MSW has been characterized in vitro (15, 18) and in vivo, (16) and has been shown to be an association between the time a pathogen population is subjected to antimicrobial exposure within the MSW (i.e. the so called T\textsubscript{MSW}) and the mutant enrichment. On the other hand, this concept has been challenged and debated in the literature (31, 35). More recently, publications have highlighted that the actual antimicrobial concentration within the MSW, at the bottom or at the top of the MSW boundaries could also play an important role in the enrichment of a mutant subpopulation (10, 17).

We previously observed in vitro and in vivo, in neutropenic mice, that both the time of antimicrobial concentrations within the MSW and the bacterial inoculum size at the start of the antimicrobial treatment play an important role in the enrichment of mutant resistant subpopulation (15, 16). The aim of the present study was to use a rat model of Klebsiella pneumoniae lung infection to investigate i) whether different bacterial inoculum sizes at the infection site at the beginning of antimicrobial treatment could influence the selection of resistant mutants in immunocompetent animals and ii) the PK/PD indices that are best associated with the prevention of resistant mutant enrichment after different dosage regimens of marbofloxacin, a fluoroquinolone of veterinary interest.
MATERIALS AND METHODS.

Bacterial strains and antibiotics.

*Klebsiella pneumoniae* ATCC 43 816 (*KP*) was used for establishment of the lung infection throughout all the experiments. Marbofloxacin powder, a third generation quinolone, was kindly provided by Vetoquinol, Lure, France.

In vitro susceptibility testing.

(i) MIC determination. The MIC was determined in triplicate for the bacteria by a broth micro dilution method according to CLSI reference methods.

(ii) MPC determination. The MPC was determined as described previously (6). Briefly, an overnight culture of the tested bacteria in Mueller Hinton (MH) broth was concentrated 100 times in NaCl 0.9% to obtain a suspension containing $10^{10}$ CFU/mL (Colony Forming Unit/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.

Animals.

OFA male rats (Charles River, L’arbresle, France), weighing 200-270g, were used for all the studies. The rats were housed 2 to 3 per cage at room temperature with a 12 h light/dark cycle and were acclimatised for at least 2 weeks before the beginning of the experiments. The rats had free access to food (Harlan, T2014, Gannat, France) and tap water.

All animal procedures were conducted in accordance with accepted humane standards of animal care under the agreement number A 31909 for animal experimentation from the French Ministry of Agriculture.
Klebsiella pneumoniae lung infection.

Experimental lung infection was produced as previously described (4). In brief, rats were anesthetized with ketamine/medetomidine (Imalgene<sup>ND</sup> 1000, Merial SAS, Villeurbanne, France / Domitor<sup>ND</sup>, Pfizer, Paris, France). The trachea was cannulated and the lungs were inoculated with 0.05mL of a saline suspension of KP containing $2 \times 10^6$ (Group A) or $2 \times 10^{10}$ (Group B) CFU/mL. For each inoculum size, a control group of 4 rats did not receive any antimicrobial treatment and a group of 3 rats were sacrificed just prior to the start therapy, for baseline quantitative cultures of lung homogenates.

Antimicrobial treatment.

Marbofloxacin treatment (Marbocyl<sup>ND</sup>, Vetoquinol, Lure, France) was started 4 hours (Group A) or 24 hours (Group B) after the inoculation of the lungs with KP. Marbofloxacin was administered subcutaneously in a volume of 0.3 mL. There were two modalities of treatment. The marbofloxacin doses were administered according to the following time schedule: either in one single administration or the same total dose was fractionated in 4 daily administrations over 4 days. For group A, a single total marbofloxacin dose (16 mg/kg) was tested according to the time schedule. For group B, three total marbofloxacin doses were tested: i.e. 16, 64 and 100 mg/kg according to the time schedule. Animals were sacrificed 96 hours after the first marbofloxacin administration by an intraperitoneal injection of pentobarbital sodium (Dolethal<sup>ND</sup>, Vetoquinol, France). The lungs were aseptically removed and homogenized in 10mL of NaCl 0.9%. The homogenates were centrifuged at 3000g for 10 minutes and washed twice in 10mL of NaCl 0.9% to prevent drug carry-over. Ten microliters of successive 10-fold dilutions of the homogenates were then plated in triplicate on MH drug-free agar plate containing 10% activated charcoal and 10% MgSO<sub>4</sub> and 100µL of the homogenates were plated on Mac Conkey agar supplemented with 0.064µg/mL and 0.256µg/mL marbofloxacin. Colonies were counted after overnight incubation at 37°C. If the colonies were too small,
incubation was continued for a further 24 hours. The lowest level of detection was 100 CFU/lung and bacteria were considered eradicated below this level. Ten to twelve rats were included per treatment group. The proportion of resistant bacteria was calculated as the ratio of bacterial counts in the presence of 0.064 or 0.256 µg/mL marbofloxacin over the total bacterial counts on plates without marbofloxacin.

**Pharmacokinetics.**

Two satellite groups (Group C and D) of male OFA rats (Charles River, L’arbresle, France), weight 250-270g, were catheterized in the femoral vein. After three days, the rats’ lungs were inoculated with 0.05 mL of an inoculum of $2 \times 10^6$ CFU/mL (Group C) or $2 \times 10^{10}$ CFU/mL (Group D) of *KP*. Four hours after the inoculation group C was given a single subcutaneous dose of marbofloxacin. For the group D, marbofloxacin administration was 24 hours after the *KP* inoculation. The doses were 4 and 16 mg/kg for group C, and 4, 16 and 100 mg/kg for group D. Blood samples (200µL) were collected at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 hours after dosing. Two to four rats were included per treatment group. After each serial blood sample, a volume of physiological saline equivalent to the collected blood volume was administered. Blood samples were centrifuged at 7000 g for 10 min at 4 °C and plasma was stored at -20 °C until assay. A high performance liquid chromatography method, with fluorescence detection ($\lambda_{\text{exc}} = 295$ nm, $\lambda_{\text{em}} = 500$ nm) (Agilent 1100) was adapted from Schneider et al. (30) to determine the marbofloxacin concentrations in the rat plasma. Briefly, marbofloxacin was obtained by liquid-liquid extraction: 0.1 mL plasma was added to 1 mL of dichloromethane and mixed for 10 seconds. 0.2 mL of a mixture of MeOH (2%HCl)/H$_2$O (90:10) was added to the organic layer and 100 µL of the supernatant were injected into a C18e (Lichrospher, Merck, 5 µm 125x4 mm) column and with a phosphoric acid (0.01M)-triethylamine(0.004M) (pH = 2)/Acetonitrile gradient elution. The calibration curve of marbofloxacin was established over the concentration range from 20 to 500 ng/mL with a
linear regression model. The plasma samples were diluted to ensure that the concentrations
where within the range of the calibration curve. The accuracy varied from 104.1 to 107.5%
and the intra-day and inter-day precision were lower than 6.2% and 8.2% respectively. The
limit of quantification was 20 ng/mL.

Pharmacokinetic analysis and PK/PD indices.

Pooled pharmacokinetic data were analysed using software dedicated to population
pharmacokinetic analysis (MONOLIX) (22), an approach that is consistent with properly
analysing sparse data as described by Burtin et al. (7). Estimations were performed using
MONOLIX version 2.1 with the SAEM (Stochastic Approximation version of the Expectation
Maximization algorithm) method. Marbofloxacin pharmacokinetic parameters were obtained
with a two compartment model with extravascular input, without lag time. These parameters
were then used to calculate PK/PD indices for each dosing regimen. The derived PK/PD
indices selected were the ratio of Area Under the plasma Concentration curve (AUC) over the
MIC (AUC/MIC), the ratio of the peak of plasma concentration over MIC (C_{max}/MIC), time
the concentrations are above the Mutant Prevention Concentration (T_{>MPC}) and time the
concentrations were within the Mutant Selection Window (T_{MSW}). The AUC/MIC was
calculated using the area under the concentration-time curve at steady-state over 24h for the
fractionated administrations as defined by Mouton et al. (28) and using AUC_{0-\infty} for the single
administrations as proposed by Toutain et al. (33). The T_{MSW} and T_{>MPC} were calculated in the
same way, over 24h for the fractionated administrations and over 96h for the single
administrations. As the plasma protein binding of marbofloxacin is lower than 10 % in the rat
(14), the PK/PD indices were determined from total plasma concentrations.
The sigmoid inhibitory Emax models describing the relationships between PK/PD indices and
the proportion of resistant bacteria for the high inoculum 96h after the start of marbofloxacin
treatment were delineated with WinNonlin version 5.2 (Pharsight Corporation, Mountain View, CA).
RESULTS

Susceptibility studies.

The MIC of marbofloxacin was 0.032µg/mL for *Klebsiella pneumoniae* (ATCC43816) and the MPC was 0.512µg/mL. The term “R-2×MIC” in the present paper should be understood as bacteria growing on 0.064 µg/ml marbofloxacin (2 × MIC), and the term “R-8×MIC” should be understood as bacteria growing on 0.256 µg/mL marbofloxacin (8 × MIC).

*K. pneumoniae* lung infection model.

Low inocula (2 × 10⁶ CFU/mL) never contained bacteria resistant to 0.064 or 0.256 µg/mL of marbofloxacin. High inocula (2 × 10¹⁰ CFU/mL) contained between 2.1 to 5.0 log₁₀ CFU/mL of bacteria resistant to 0.064 µg/mL marbofloxacin and 1.1 to 2.2 log₁₀ CFU/mL of bacteria resistant to 0.256 µg/mL marbofloxacin.

At the start of the marbofloxacin treatment, the bacterial counts in the lungs were 5.4 ± 0.1 log₁₀ CFU/lungs for group A (4 hours after infection) and 10 ± 0.4 log₁₀ CFU/lungs for group B (24 hours after infection). For group A, 4 hours after the inoculation we never found any bacteria resistant to 0.064 or 0.256 µg/kg marbofloxacin. For group B, 24 hours after the inoculation, there was between 3.2 to 3.4 log₁₀ CFU/lungs bacteria resistant to 0.064 µg/mL marbofloxacin (R-2×MIC) and 2.75 to 3.06 log₁₀ CFU/lungs bacteria resistant to 0.256 µg/mL (R-8×MIC) marbofloxacin. Taking into account both total bacterial counts and resistant subpopulation counts (R-2×MIC and R-8×MIC), for the high inoculum, at the beginning of the marbofloxacin treatment we calculated that the initial proportion of both resistant subpopulations was about 10⁻⁷-10⁻⁸.

For the low inoculum challenge (Group A), bacterial counts from untreated animals were 4.2 to 11.2 log₁₀ CFU/lungs 4 days after the infection. This large range of bacterial load in the lungs might be due to the immunocompetent status of the animals, and reflect the inter-individual variability in the evolution of this infection. However this did not impact our study.
since the antimicrobial treatment started 4 hours after the *KP* inoculation and, at this time, the bacterial load was rather homogeneous (see below).

For the high inoculum challenge (Group B), all untreated animals were dead 72 hours after infection.

**Effect of different marbofloxacin dosage regimens on the total bacterial population.**

The total bacterial population 96 hours after the start of marbofloxacin treatment is reported for each inoculum size and each marbofloxacin dosing regimen in Table 1. For the low inoculum the marbofloxacin dose of 16 mg/kg, fractionated or not, led to a significant decrease in *KP* in the lungs. For the high inoculum, the three marbofloxacin doses, fractionated or not, reduced the bacterial population by at least two log, but no difference was detected between marbofloxacin dosing regimens. With the lower marbofloxacin dose (16 mg/kg) administered as a single administration, 4 out of 10 rats died between 72 and 96 hours after marbofloxacin whereas for the fractionated administration, all the animals survived. With the higher marbofloxacin dose (100 mg/kg) administered in a single administration 3 out of 12 rats died during the first 24 hours after marbofloxacin administration. The death of animals in group B treated with 100 mg/kg in a single administration occurred earlier during the experiment compared to animals of group B treated with 16 mg/kg of marbofloxacin in a single administration or untreated animals (i.e. 24h vs. 72-96h respectively). Despite the lack of observed adverse effects such as convulsions, the 3 deaths were probably due to marbofloxacin toxicity in ill animals.

**Effect of different marbofloxacin dosage regimens on R-2×MIC and R-8×MIC subpopulation enrichment.**

The percentages of animals with R-2×MIC and R-8×MIC bacteria in their lungs 96 hours after the start of marbofloxacin treatment are shown for each inoculum size and each dosing regimen in Table 1. In rats infected with the low inoculum and treated with 16 mg/kg of
marbofloxacin, neither R-2×MIC nor R-8×MIC bacteria were detected at the end of the experiment. By contrast, rats infected with the high inoculum had both R-2×MIC and R-8×MIC bacteria in their lungs. The frequency of rats harbouring these two *KP* subpopulations was negatively associated with the marbofloxacin doses. Moreover, for the same total marbofloxacin dose, fractionated administration seemed to be associated with a lower presence of resistant subpopulations than the single dose administration.

**Pharmacokinetic study.**

The pooled pharmacokinetic data obtained with different marbofloxacin doses were successfully analysed using the same compartmental model, indicating dose-proportionality of marbofloxacin kinetics within both the low and high inoculum groups. This proportionality enabled PK/PD indices to be calculated for the different marbofloxacin doses. Moreover, no significant difference in marbofloxacin pharmacokinetic parameters was observed between the low and high inoculum groups receiving the same doses (data not shown). The predicted concentrations versus time profiles of marbofloxacin for the different dosage regimens are given in Figure 1.

**PK/PD indices and R-2×MIC and R-8×MIC subpopulation enrichment.**

The PK/PD indices for the different marbofloxacin dosage regimens are shown in Table 2. The proportions of resistant *KP* subpopulations after the different dosage regimens in rats infected with the high inoculum are shown in Figure 2. In all animals carrying resistant bacteria, the proportion of both R-2×MIC and R-8×MIC bacteria was higher than 10⁻⁶, indicating an enrichment compared with the initial inoculated *KP* population (proportion < 10⁻⁶).

(i) **AUC/MIC.** For the lowest dose of 16 mg/kg, the AUC/MIC values were 4×189 and 1×756 hours for the fractionated and single dose administrations respectively. These ratios were associated with an enrichment of R-2×MIC and R-8×MIC subpopulations in animals infected
with the high inoculum whereas there was no enrichment for animals infected with the low inoculum (Table 1). In animals infected with the high inoculum, AUC/MIC values of $4 \times 756$ and $1 \times 3026$ hours, for the fractionated and single 64 mg/kg administration respectively, were also associated with R-2×MIC and R-8×MIC subpopulations whereas the ratios of $4 \times 1182$ and $1 \times 4728$ hours corresponding to the 100 mg/kg total dose were associated with the absence of detection of R-8×MIC subpopulation (Table 1 and 2).

(ii) C_{max}/MIC. In animals infected with the low inoculum (Group A), the C_{max}/MIC value of $4 \times 54$ and $1 \times 217$ for fractionated and single 16 mg/kg administration respectively, were associated with the absence of R-2×MIC or R-8×MIC subpopulation enrichment (Table 1 and 2). In animals infected with the high inoculum (Group B), the higher the C_{max}/MIC value, the less the R-2×MIC and R-8×MIC subpopulation enrichment occurred for each dosing regimen (Table 1 and 2). The C_{max}/MIC values that were associated with the absence of R-8×MIC bacteria enrichment were $4 \times 339$ and $1 \times 1357$, for the fractionated and single 100 mg/kg administrations respectively. The $4 \times 339$ ratio value for the fractionated administration was also associated with the lower frequency of R-2×MIC subpopulation enrichment (Table 1 and 2).

(iii) T_{MSW} and T_{>MPC}. The dosage regimen had an important impact on T_{MSW}. For the single administration, marbofloxacin plasma concentrations were 28, 40 and 41 % of the time (over 96 hours) within the MSW for 16, 64 and 100 mg/kg marbofloxacin doses respectively (Table 2) and for the fractionated administration, T_{MSW} was 50, 67 and 60 % of the time (over 24 hours) (Table 2). The T_{MSW} was lower for the 100 mg/kg dose compared to 64 mg/kg dose as it was calculated over 24h and that the T_{>MPC} was higher for the 100 mg/kg dose than for the 64 mg/kg (i.e. 40% vs. 33% respectively) (Table 2). The relationships between time indices and the proportion of R-2×MIC and R-8×MIC bacteria after the different marbofloxacin dosage regimens for animals infected with the high inoculum are presented in
No clear relationship existed between $T_{MSW}$ and the R-2×MIC and R-8×MIC subpopulation enrichment (Fig. 2, panel A1 and A2). For $T_{>MPC}$, there was a relationship between $T_{>MPC}$ and the R-2×MIC or R-8×MIC subpopulation enrichment, only when each dosage regimen was considered separately (fractionated or single) (Fig. 2, panel B1 and B2).

In fact, there was a relationship between R-2×MIC or R-8×MIC subpopulation enrichment and the ratio of $T_{>MPC}/T_{MSW}$ (Fig. 2, panels C1 and C2). Irrespective of the dosage regimen, the closer the $T_{>MPC}/T_{MSW}$ ratio came up to 1, the more the R-2×MIC or R-8×MIC subpopulation enrichment was limited. These observations were confirmed by the fitting of the percentages of rats with resistant bacteria in the lungs versus $T_{MSW}$, $T_{>MPC}$ and $T_{>MPC}/T_{MSW}$ using a sigmoid $E_{max}$ model. Indeed, the best fit was obtained between the percentages of rats with resistant bacteria in the lungs versus $T_{>MPC}/T_{MSW}$ with R² values of 0.99 and 0.97 for R-2×MIC and R-8×MIC respectively (Figure 3, panel B1 and B2) whereas the R² values were 0.87 and 0.93 for $T_{>MPC}$ with a misfit at the end of the curve (Figure 3, panel B1 and B2) and 0.54 and 0.60 for $T_{MSW}$ (data not shown).
DISCUSSION

The aim of this study was to investigate the influence of bacterial load at the start of a quinolone treatment on resistant subpopulation enrichment and the ability of PK/PD parameters (AUC/MIC, $C_{\text{max}}$/MIC, $T_{>\text{MPC}}$ and $T_{\text{MSW}}$) to predict resistant subpopulation enrichment in a lung infection model in immunocompetent rats.

In order to fully characterize the model of KP lung infection in immunocompetent rats, we evaluated the pharmacokinetics of marbofloxacin in infected animals. Indeed, bacterial infections have been shown to alter the pharmacokinetics of drugs (19), including fluoroquinolone antimicrobials, such as marbofloxacin (20, 29). Moreover, a similar investigation by our group on an Escherichia coli thigh infection model in neutropenic mice indicated that marbofloxacin pharmacokinetics was altered by infection. There were considerable differences in marbofloxacin exposure between animals infected by a low or a high bacterial inoculum leading to a large difference in the PK/PD parameters for the same marbofloxacin dose (16). In the present study, we did not observe any difference in the pharmacokinetic parameters between animals infected with the low (Group A) or the high (Group B) KP inoculum. The characteristics of these two infectious model (lungs vs. thigh and KP vs. E. coli) or/and a possible difference in the level of inflammation, might explain these discrepancies.

Previous in vitro studies on E. coli, S. aureus and P. aeruginosa (15, 26) and an in vivo study on E. coli in immunocompromized mice (16) showed an impact of inoculum size on the enrichment of resistant mutants. The present study confirms these results and clearly shows this impact in immunocompetent animals. Indeed, with an early antimicrobial treatment on an initial small bacterial population at the infection site (Group A), we never observed a R-$2\times$MIC or R-$8\times$MIC subpopulation enrichment. In addition, the total bacterial load decreased dramatically. On the contrary, with a delayed start of the antimicrobial treatment on an initial
large bacterial population (Group B), we observed a limited decrease in the total bacterial population, accompanied by an enrichment of R-2×MIC and R-8×MIC subpopulations, depending on the marbofloxacin dosing regimen. Moreover, for the smaller marbofloxacin dose (16 mg/kg) administered as a single administration, 4 out of 10 rats died between 72 and 96h after the start of marbofloxacin treatment. These deaths were attributed to the infection, based upon observed clinical signs of infection in these rats. On the contrary, the 3 deaths observed with the highest single marbofloxacin dose (100 mg/kg) were unlikely to have been due to infection because they occurred in the first 48h after the inoculation while in non-treated animals death always occurred after 72h. These early deaths could be attributed to the marbofloxacin toxicity in ill animals.

The ability of PK/PD indices such as AUC/MIC, $C_{\text{max}}$/MIC, $T_{>\text{MPC}}$ and $T_{\text{MSW}}$ to predict resistant subpopulation enrichment has already been studied. Previous studies suggested that AUC/MIC was the PK/PD index that best correlated with efficacy of fluoroquinolones in both neutropenic and non-neutropenic murine thigh and lung infection models (3, 5, 13, 34). In our study, for the low inoculum (Group A) with the lowest marbofloxacin dose (16 mg/kg), the AUC/MIC value of 4×189 hours or 1×756 hours for fractionated or single dose administration respectively, was associated with the prevention of the emergence of any resistant subpopulation after 96 hours of treatment. For the high inoculum (Group B), the AUC/MIC values required to prevent R-8×MIC subpopulation enrichment were higher than for the low inoculum, i.e. 4×1,182 hours and 1×4,728 hours for fractionated and single administration respectively. However, considering the MPC, which is linked to the MIC of the R-8×MIC bacteria, to calculate the corresponding PK/PD indices, it appears that the AUC/MPC ratio which was associated with the prevention of the enrichment of an R-8×MIC subpopulation is now 4×74 hours or 1×295 hours for fractionated or single administration respectively. These AUC/MPC values are of the same order of magnitude as
the AUC/MIC values obtained to prevent an R-8\times MIC subpopulation enrichment from a low initial inoculum. In other words and as previously suggested (38), to be predictive, the AUC/MIC ratio should take into account the MIC of the subpopulation having the highest MIC value and not the MIC of the dominant population. Moreover, the differences observed between R-8\times MIC enrichment for fractionated and single dose administrations indicated that, for a same total exposure (over 96h), the effectiveness of the fractionated dosage regimen is greater than that of a single dose administration; this suggest that enrichment of resistant subpopulations is co-dependent on both the total exposure and the time above some critical concentration (see later).

The time the fluoroquinolone concentrations were within the Mutant Selection Window has previously been shown to be associated with a promotion of resistant bacteria subpopulation enrichment \textit{in vitro} (1, 9, 10, 15) and \textit{in vivo} (16). However, we did not observe in our experiment such a relationship between T_{MSW} and R-8\times MIC subpopulation enrichment (Fig. 2, panels A1 and A2), which is in agreement with a previous \textit{in vitro} study with \textit{Staphylococcus aureus} (8) although it is important to note that the target of mutation resistance differs between gram positive and gram negative bacteria (11). Recently, it was suggested that the apparent inability of T_{MSW} to predict mutant resistant enrichment may be explained by the confounding influence of the actual antimicrobial concentrations at the edges of the selection window (10, 17). In other words, for a given T_{MSW}, situations are not equivalent when time outside the MSW is under the MIC or above the MPC. In the present study, for the fractionated 16 mg/kg dose, the half-life of marbofloxacin of 2.41h (Fig. 1) led plasma concentrations to decay below the MSW whereas for the 2 other doses (64 and 100 mg/kg), most of the exposure to marbofloxacin was at the top of the MSW. This could explain the absence of a relationship between T_{MSW} and R-8\times MIC enrichment. For the single dose administration, the pharmacokinetic profiles showed that for the two highest doses (64
and 100 mg/kg), marbofloxacin concentrations reside almost the same time within the MSW while the residence time within the MSW was shorter for the lower dose (16mg/kg). There was thus no relationship between the T_{MSW} and R-8×MIC subpopulation enrichment. By contrast, there was a relationship between both R-2×MIC and R-8×MIC subpopulation enrichment and the ratio T_{>MPC}/T_{MSW} (Fig. 2, panels C1 and C2, Fig. 3, panels B1 and B2). With this ratio, it is now possible to discriminate between situations characterized by the same T_{MSW} but with different levels of antimicrobial concentrations within this MSW, and the higher is this ratio, the lower the risk of R-8×MIC subpopulation enrichment. The ability of this new index to predict enrichment of resistant mutants is due to the fact that it consists of two terms reflecting two antagonistic and sequential processes: T_{>MPC}, that reflects the time during which the antibiotic eliminates all pathogens including resistant mutants, and T_{MSW}, that reflects the time during which the antibiotic produces selection of mutant resistant subpopulations. It is noteworthy that this ratio allows an unbiased comparison of fractionated and single dose administrations given that for fractionated administration, the ratio is the same when calculated over 24h or the total duration of the treatment (96h). Cut-off values of T_{>MPC}/T_{MSW} associated with the prevention of resistant subpopulation enrichment need to be known; as shown in the present experiment, the ratio is influenced by the likelihood of having resistant mutants already present at the start of antimicrobial treatment. With the low inoculum (Group A), T_{>MPC}/T_{MSW} values of 0.31 and 0.30, for fractionated and single dose administration respectively, were required to prevent mutant resistant enrichment. By contrast for the high inoculum (Group B) the corresponding values were 0.67 and 0.54 respectively. From our results, it appears that the most desirable situation to carry out an antimicrobial therapy that does not simultaneously promote antimicrobial resistance is one in which the inoculum size at the initiation of the treatment is low or null (metaphylaxis, prophylaxis). In a curative setting, characterized by a greater likelihood of the antimicrobial facing a high
bacterial load, our results suggest that the best strategy would be to immediately achieve plasma concentrations above the MPC. Besides such intuitive findings, the present study offers the first evidence of the rational PK/PD approach to select the antimicrobial dosage regimens adapted to either preventive or curative settings. In addition, as shown by our results, the $T_{\text{MPC}}$ needs to be long enough to produce an early reduction of the inoculum load to be in the same situation as with an initial low inoculum load. In the present experiment, it appears that with the single marbofloxacin dose (mimicking the so called “one shot” treatment that is usually recommended in veterinary medicine), $T_{\text{MPC}}$ is not long enough to achieve this goal. However, it is important to note that in our study, the tested doses always achieved concentrations above the MPC for fractionated administration but the same total fluoroquinolone dose which achieves a concentration above the MPC for a single but not for a fractionated administration would have been be better in a single than in a fractionated administration for mutant prevention. Secondly, the terminal half life of marbofloxacin in domestic animal species may be much longer (up to 13 hours) than in rats (2.41 hours in our study) and a single dose administration in large animal species may give a longer $T_{\text{MPC}}$ than in rats.

In addition to $R-8\times\text{MIC}$ subpopulation enrichment, we also studied the $R-2\times\text{MIC}$ enrichment which could be attributed to efflux pump over-expression in bacteria (25). In some animals we observed an enrichment of the $R-2\times\text{MIC}$ subpopulation without simultaneous $R-8\times\text{MIC}$ subpopulation enrichment. For the two highest marbofloxacin doses (64 and 100 mg/kg), the $R-2\times\text{MIC}$ subpopulation enrichment was greater for the single administration than the fractionated one (Table 1). This could in turn explain the higher percentage of animals harbouring $R-8\times\text{MIC}$ bacteria in their lungs with a single rather than a fractionated administration of marbofloxacin at 64 mg/kg. Indeed, if the $R-2\times\text{MIC}$ subpopulation enrichment is promoted, the ability of these bacteria to survive antimicrobial
concentrations closer to the MIC favours the possibility of generating a new and more resistant subpopulation (R-8×MIC bacteria), as has been suggested by Louie et al. (25). Thus despite the same impact on the total bacterial population, in our model the single antimicrobial administration seems to be less beneficial than the fractionated administration in preventing resistant mutant enrichment.

In conclusion, our results show that the bacterial load at the start of antimicrobial treatment plays a critical role on the pattern of selection of KP resistant mutants. A low initial bacterial load limits resistant mutant enrichment due to the lower likelihood of having resistant mutant subpopulation already present at the beginning of the treatment. With a high bacterial load at the start of treatment, we have shown the ability of the $T_{>MPC}/T_{MSW}$ ratio to define the relevant features of antibiotic exposure to prevent mutant enrichment. This PK/PD index might contribute to the rational selection of more adapted antimicrobial dosage regimens. Nevertheless, further investigations in different infectious and animal models are needed to confirm what we observed with our trials and to quantify the cut-off value for $T_{>MPC}/T_{MSW}$ in target patients (man and domestic animal species).
ACKNOWLEDGMENTS

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REFERENCES


**TABLE 1.**

Total bacterial population (mean ± SD), and percentages of rats with R-2×MIC and R-8×MIC in their lungs 96 hours after the start of marbofloxacin treatment for the initial low and high inocula.

<table>
<thead>
<tr>
<th>Total marbofloxacin dose (mg/kg)</th>
<th>Untreated controls</th>
<th>Fractionated</th>
<th>Single</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At the start of treatment</td>
<td>96h after the start of treatment</td>
<td></td>
</tr>
<tr>
<td>Low inoculum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>3/3</td>
<td>10 nd</td>
<td>10 nd</td>
</tr>
<tr>
<td>Dead animal</td>
<td>0/3</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>log_{10} cfu/lungs (mean ± SD)</td>
<td>5.4±0.1</td>
<td>0.9±1.4†</td>
<td>0.3±1.0†</td>
</tr>
<tr>
<td>R-2×MIC</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>R-8×MIC</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>High inoculum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>3/3</td>
<td>11 11</td>
<td>10</td>
</tr>
<tr>
<td>Dead animal</td>
<td>0/3</td>
<td>0/11</td>
<td>4/10 b</td>
</tr>
<tr>
<td>log_{10} cfu/lungs (mean ± SD)</td>
<td>10±0.4</td>
<td>6.3±0.7</td>
<td>7.5±1.3</td>
</tr>
<tr>
<td>R-2×MIC</td>
<td>100%</td>
<td>100%</td>
<td>90% b</td>
</tr>
<tr>
<td>R-8×MIC</td>
<td>100%</td>
<td>55%</td>
<td>70% b</td>
</tr>
</tbody>
</table>

† For these means calculation, we assigned the value 0 log_{10} CFU to lungs for which bacteria were undetectable.

a The 4 animals died 72 hours after the inoculation of KP

b The 4 animals that died between 72 and 96 hours were considered as carriers of resistant bacteria and were included in the percentage of animals with R-2×MIC and R-8×MIC in their lungs.

** The death of these 3 rats occurred during the 24 first hours after the drug administration.

The 3 animals that died 24h hour after marbofloxacin administration were considered as dying because of toxicity of the high dose of marbofloxacin administered in single-injection and are not included in the percentages.

nd: not determined

Dead rats are not taken into consideration to calculate the total bacterial population in lungs.
TABLE 2.
Simulated PK/PD Parameters after administration of 16, 64 or 100 mg/kg marbofloxacin in fractionated or single administrations.

<table>
<thead>
<tr>
<th>Total marbofloxacin dose (mg/kg)</th>
<th>$T_{\text{MSW}}$ (%)</th>
<th>$T_{\text{MPC}}$ (%)</th>
<th>$T_{\text{MPC}}/T_{\text{MSW}}$</th>
<th>AUC/MIC (h)</th>
<th>AUC/MPC (h)</th>
<th>Cmax/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fractionated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>50</td>
<td>16</td>
<td>0.31</td>
<td>4×189</td>
<td>4×12</td>
<td>4×54</td>
</tr>
<tr>
<td>64</td>
<td>67</td>
<td>33</td>
<td>0.49</td>
<td>4×756</td>
<td>4×47</td>
<td>4×217</td>
</tr>
<tr>
<td>100</td>
<td>60</td>
<td>40</td>
<td>0.67</td>
<td>4×1,182</td>
<td>4×74</td>
<td>4×339</td>
</tr>
<tr>
<td><strong>Single</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>28</td>
<td>8</td>
<td>0.30</td>
<td>1×756</td>
<td>1×47</td>
<td>1×217</td>
</tr>
<tr>
<td>64</td>
<td>40</td>
<td>16</td>
<td>0.41</td>
<td>1×3,026</td>
<td>1×189</td>
<td>1×868</td>
</tr>
<tr>
<td>100</td>
<td>41</td>
<td>22</td>
<td>0.54</td>
<td>1×4,728</td>
<td>1×295</td>
<td>1×1,357</td>
</tr>
</tbody>
</table>
FIGURE 1: Pharmacokinetic profiles of marbofloxacin simulated for 3 marbofloxacin doses (16, 64 and 100 mg/kg) with two dosing regimens: fractionated (A) or single (B) administration. The elimination half-life ($T_{1/2\text{elim}}$) was 2.41 hours.

FIGURE 2: Proportion of R-2×MIC resistant to 0.064 µg/mL (A1, B1, C1) and R-8×MIC resistant to 0.256 µg/mL (A2, B2, C2) in the lungs of rats infected with the high inoculum ($10^9$ CFU) of KP versus TMSW (A1, A2), T>MPC (B1, B2) and the ratio T>MPC/TMSW (C1, C2). Proportions are represented by full symbols when they were obtained after the fractionated administration and by empty symbols after the single administration of marbofloxacin. Symbols under the dotted line represent animals without detectable R-2×MIC and R-8×MIC bacteria in their lungs. TMSW and the T>MPC were calculated over 24h for fractionated administrations and over 96h for single administrations.

FIGURE 3: Relationships of the T>MPC (Panel A1 and A2) and the ratio T>MPC/TMSW (Panel B1 and B2) with the percentage of animals with R-2×MIC (Panel A1 and B1) and R-8×MIC (Panel A2 and B2) bacteria 96h after the start of antimicrobial treatment for the animals infected by the large inoculum (Group B).
FIGURE 1:

A. Fractionated

B. Single

Marbofloxacin plasma concentration (µg/mL)

Time (h)
FIGURE 2:

- Resistant to 0.064 µg/mL
- Resistant to 0.256 µg/mL

**TMSW (%)**

**Resistant bacteria proportion in rats lungs**

- **A1**
- **A2**

**T > MPC (%)**

**Resistant bacteria proportion in rats lungs**

- **B1**
- **B2**

**C1**

**C2**

- **single**
- **fractionated**
- **dead rat**
FIGURE 3:

Resistant to 0.064 µg/mL

Resistant to 0.256 µg/mL

Fractionated

Single

R² = 0.87

R² = 0.93

R² = 0.99

R² = 0.97
Resistant bacteria proportion in rats lungs

Resistant to 0.064 µg/mL

Resistant to 0.256 µg/mL

A1 A2

B1 B2

C1 C2

T_{MSW}(\%) 10 30 50 70

T_{>MPC}(\%) 0 10 20 30 40

T_{>MPC} / T_{MSW} 0 0.2 0.4 0.6

No resistant bacteria

single • fractionated ★ dead rat
Resistant to 0.064 µg/mL

Resistant to 0.256 µg/mL

A1
Resistant to 0.064 µg/mL

A2
Resistant to 0.256 µg/mL

B1
Resistant to 0.064 µg/mL

B2
Resistant to 0.256 µg/mL

R² = 0.87

R² = 0.93

R² = 0.99

R² = 0.97

- **Fractionated**
- **Single**