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

3

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18 **ABSTRACT**

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

39 INTRODUCTION

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

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

53 Until recently, optimization and individualisation of antimicrobial dosing regimens
54 have been primarily based on the pharmacokinetic/pharmacodynamic (PK/PD) indices that
55 describe both the optimal efficacy and/or prevention of toxicity (27). However, the increasing
56 problem of emergence of resistance under the influence of antibiotic selection pressure led to
57 the identification of PK/PD indices that best correlate with the prevention of antimicrobial
58 resistance (2). An approach to prevent the emergence of antimicrobial resistance has been
59 proposed, consisting of administering the drug at doses that produce plasma concentrations
60 that continuously exceed the threshold of spontaneous drug-resistant mutants susceptibility
61 and thereby prevent the selective amplification of any mutant subpopulation (12, 37, 38). The
62 drug concentration capable of inhibiting the growth of the least-susceptible single-step mutant
63 subpopulation has been called the “Mutant Prevention Concentration” (MPC). In addition, it

64 has been suggested that selective amplification of spontaneous drug-resistant mutants is more
65 pronounced within the range of antimicrobial plasma concentrations between the MIC of the
66 wild bacterial population and the MPC, defined as the “Mutant Selection Window” (MSW)
67 (6, 23, 36, 37). The concept of the MSW has been characterized *in vitro* (15, 18) and *in vivo*,
68 (16) and has been shown to be an association between the time a pathogen population is
69 subjected to antimicrobial exposure within the MSW (*i.e.* the so called T_{MSW}) and the mutant
70 enrichment. On the other hand, this concept has been challenged and debated in the literature
71 (31, 35). More recently, publications have highlighted that the actual antimicrobial
72 concentration within the MSW, at the bottom or at the top of the MSW boundaries could also
73 play an important role in the enrichment of a mutant subpopulation (10, 17).

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

83 **MATERIALS AND METHODS.**

84 **Bacterial strains and antibiotics.**

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

88 **In vitro susceptibility testing.**

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

91 **(ii) MPC determination.** The MPC was determined as described previously (6). Briefly, an
92 overnight culture of the tested bacteria in Mueller Hinton (MH) broth was concentrated 100
93 times in NaCl 0.9% to obtain a suspension containing 10^{10} CFU/mL (Colony Forming
94 Unit/mL). One hundred microliters of this suspension were then plated on MH agar
95 containing various concentrations of marbofloxacin obtained by successive two-fold dilutions.
96 The MPC was the lowest marbofloxacin concentration preventing the growth of bacterial
97 colonies after incubation for 72 hours at 37°C. Determinations were done in triplicate.

98 **Animals.**

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

103 All animal procedures were conducted in accordance with accepted humane standards of
104 animal care under the agreement number A 31909 for animal experimentation from the
105 French Ministry of Agriculture.

106 ***Klebsiella pneumoniae* lung infection.**

107 Experimental lung infection was produced as previously described (4). In brief, rats were
108 anesthetized with ketamine/medetomidine (ImalgeneND 1000, Merial SAS, Villeurbanne,
109 France / DomitorND, Pfizer, Paris, France). The trachea was cannulated and the lungs were
110 inoculated with 0.05mL of a saline suspension of *KP* containing 2×10^6 (Group A) or 2×10^{10}
111 (Group B) CFU/mL. For each inoculum size, a control group of 4 rats did not receive any
112 antimicrobial treatment and a group of 3 rats were sacrificed just prior to the start therapy, for
113 baseline quantitative cultures of lung homogenates.

114 **Antimicrobial treatment.**

115 Marbofloxacin treatment (MarbocylND, Vetoquinol, Lure, France) was started 4 hours (Group
116 A) or 24 hours (Group B) after the inoculation of the lungs with *KP*. Marbofloxacin was
117 administered subcutaneously in a volume of 0.3 mL. There were two modalities of treatment.
118 The marbofloxacin doses were administered according to the following time schedule: either
119 in one single administration or the same total dose was fractionated in 4 daily administrations
120 over 4 days. For group A, a single total marbofloxacin dose (16 mg/kg) was tested according
121 to the time schedule. For group B, three total marbofloxacin doses were tested: *i.e.* 16, 64 and
122 100 mg/kg according to the time schedule. Animals were sacrificed 96 hours after the first
123 marbofloxacin administration by an intraperitoneal injection of pentobarbital sodium
124 (DolethalND, Vetoquinol, France). The lungs were aseptically removed and homogenized in
125 10mL of NaCl 0.9%. The homogenates were centrifuged at 3000g for 10 minutes and washed
126 twice in 10mL of NaCl 0.9% to prevent drug carry-over. Ten microliters of successive 10-fold
127 dilutions of the homogenates were then plated in triplicate on MH drug-free agar plate
128 containing 10% activated charcoal and 10% MgSO₄ and 100μL of the homogenates were
129 plated on Mac Conkey agar supplemented with 0.064μg/mL and 0.256μg/mL marbofloxacin.
130 Colonies were counted after overnight incubation at 37°C. If the colonies were too small,

131 incubation was continued for a further 24 hours. The lowest level of detection was
132 100 CFU/lung and bacteria were considered eradicated below this level. Ten to twelve rats
133 were included per treatment group. The proportion of resistant bacteria was calculated as the
134 ratio of bacterial counts in the presence of 0.064 or 0.256 $\mu\text{g/mL}$ marbofloxacin over the total
135 bacterial counts on plates without marbofloxacin.

136 **Pharmacokinetics.**

137 Two satellite groups (Group C and D) of male OFA rats (Charles River, L'arbresle, France),
138 weight 250-270g, were catheterized in the femoral vein. After three days, the rats' lungs were
139 inoculated with 0.05 mL of an inoculum of 2×10^6 CFU/mL (Group C) or 2×10^{10} CFU/mL
140 (Group D) of *KP*. Four hours after the inoculation group C was given a single subcutaneous
141 dose of marbofloxacin. For the group D, marbofloxacin administration was 24 hours after the
142 *KP* inoculation. The doses were 4 and 16 mg/kg for group C, and 4, 16 and 100 mg/kg for
143 group D. Blood samples (200 μL) were collected at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 hours
144 after dosing. Two to four rats were included per treatment group. After each serial blood
145 sample, a volume of physiological saline equivalent to the collected blood volume was
146 administered. Blood samples were centrifuged at 7000 g for 10 min at 4 °C and plasma was
147 stored at -20 °C until assay. A high performance liquid chromatography method, with
148 fluorescence detection ($\lambda_{\text{exc}} = 295$ nm, $\lambda_{\text{em}} = 500$ nm) (Agilent 1100) was adapted from
149 Schneider et al. (30) to determine the marbofloxacin concentrations in the rat plasma. Briefly,
150 marbofloxacin was obtained by liquid-liquid extraction: 0.1 mL plasma was added to 1 mL of
151 dichloromethane and mixed for 10 seconds. 0.2 mL of a mixture of MeOH (2% HCl)/ H_2O
152 (90:10) was added to the organic layer and 100 μL of the supernatant were injected into a
153 C18e (Lichrospher, Merck, 5 μm 125x4 mm) column and with a phosphoric acid (0.01M)-
154 triethylamine(0.004M) (pH = 2)/Acetonitrile gradient elution. The calibration curve of
155 marbofloxacin was established over the concentration range from 20 to 500 ng/mL with a

156 linear regression model. The plasma samples were diluted to ensure that the concentrations
157 were within the range of the calibration curve. The accuracy varied from 104.1 to 107.5%
158 and the intra-day and inter-day precision were lower than 6.2% and 8.2% respectively. The
159 limit of quantification was 20 ng/mL.

160 **Pharmacokinetic analysis and PK/PD indices.**

161 Pooled pharmacokinetic data were analysed using software dedicated to population
162 pharmacokinetic analysis (MONOLIX) (22), an approach that is consistent with properly
163 analysing sparse data as described by Burtin *et al.* (7). Estimations were performed using
164 MONOLIX version 2.1 with the SAEM (Stochastic Approximation version of the Expectation
165 Maximization algorithm) method. Marbofloxacin pharmacokinetic parameters were obtained
166 with a two compartment model with extravascular input, without lag time. These parameters
167 were then used to calculate PK/PD indices for each dosing regimen. The derived PK/PD
168 indices selected were the ratio of Area Under the plasma Concentration curve (AUC) over the
169 MIC (AUC/MIC), the ratio of the peak of plasma concentration over MIC (C_{max}/MIC), time
170 the concentrations are above the Mutant Prevention Concentration ($T_{>MPC}$) and time the
171 concentrations were within the Mutant Selection Window (T_{MSW}). The AUC/MIC was
172 calculated using the area under the concentration-time curve at steady-state over 24h for the
173 fractionated administrations as defined by Mouton *et al.* (28) and using $AUC_{0-\infty}$ for the single
174 administrations as proposed by Toutain *et al.* (33). The T_{MSW} and $T_{>MPC}$ were calculated in the
175 same way, over 24h for the fractionated administrations and over 96h for the single
176 administrations. As the plasma protein binding of marbofloxacin is lower than 10 % in the rat
177 (14), the PK/PD indices were determined from total plasma concentrations.

178 The sigmoid inhibitory Emax models describing the relationships between PK/PD indices and
179 the proportion of resistant bacteria for the high inoculum 96h after the start of marbofloxacin

180 treatment were delineated with WinNonlin version 5.2 (Pharsight Corporation, Mountain
181 View, CA).

182 **RESULTS**

183 **Susceptibility studies.**

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

188 ***K. pneumoniae* lung infection model.**

189 Low inocula (2 \times 10⁶ CFU/mL) never contained bacteria resistant to 0.064 or 0.256 μ g/mL of
190 marbofloxacin. High inocula (2 \times 10¹⁰ CFU/mL) contained between 2.1 to 5.0 log₁₀ CFU/mL
191 of bacteria resistant to 0.064 μ g/mL marbofloxacin and 1.1 to 2.2 log₁₀ CFU/mL of bacteria
192 resistant to 0.256 μ g/mL marbofloxacin.

193 At the start of the marbofloxacin treatment, the bacterial counts in the lungs were
194 5.4 \pm 0.1 log₁₀ CFU/lungs for group A (4 hours after infection) and 10 \pm 0.4 log₁₀ CFU/lungs
195 for group B (24 hours after infection). For group A, 4 hours after the inoculation we never
196 found any bacteria resistant to 0.064 or 0.256 μ g/kg marbofloxacin. For group B, 24 hours
197 after the inoculation, there was between 3.2 to 3.4 log₁₀ CFU/lungs bacteria resistant to
198 0.064 μ g/mL marbofloxacin (R-2 \times MIC) and 2.75 to 3.06 log₁₀ CFU/lungs bacteria resistant to
199 0.256 μ g/mL (R-8 \times MIC) marbofloxacin. Taking into account both total bacterial counts and
200 resistant subpopulation counts (R-2 \times MIC and R-8 \times MIC), for the high inoculum, at the
201 beginning of the marbofloxacin treatment we calculated that the initial proportion of both
202 resistant subpopulations was about 10⁻⁷-10⁻⁸.

203 For the low inoculum challenge (Group A), bacterial counts from untreated animals were 4.2
204 to 11.2 log₁₀ CFU/lungs 4 days after the infection. This large range of bacterial load in the
205 lungs might be due to the immunocompetent status of the animals, and reflect the inter-
206 individual variability in the evolution of this infection. However this did not impact our study

207 since the antimicrobial treatment started 4 hours after the *KP* inoculation and, at this time, the
208 bacterial load was rather homogeneous (see below).

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

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

212 The total bacterial population 96 hours after the start of marbofloxacin treatment is reported
213 for each inoculum size and each marbofloxacin dosing regimen in Table 1. For the low
214 inoculum the marbofloxacin dose of 16 mg/kg, fractionated or not, led to a significant
215 decrease in *KP* in the lungs. For the high inoculum, the three marbofloxacin doses,
216 fractionated or not, reduced the bacterial population by at least two log, but no difference was
217 detected between marbofloxacin dosing regimens. With the lower marbofloxacin dose
218 (16 mg/kg) administered as a single administration, 4 out of 10 rats died between 72 and 96
219 hours after marbofloxacin whereas for the fractionated administration, all the animals
220 survived. With the higher marbofloxacin dose (100 mg/kg) administered in a single
221 administration 3 out of 12 rats died during the first 24 hours after marbofloxacin
222 administration. The death of animals in group B treated with 100 mg/kg in a single
223 administration occurred earlier during the experiment compared to animals of group B treated
224 with 16 mg/kg of marbofloxacin in a single administration or untreated animals (i.e. 24h vs.
225 72-96h respectively). Despite the lack of observed adverse effects such as convulsions, the 3
226 deaths were probably due to marbofloxacin toxicity in ill animals.

227 **Effect of different marbofloxacin dosage regimens on R-2×MIC and R-8×MIC** 228 **subpopulation enrichment.**

229 The percentages of animals with R-2×MIC and R-8×MIC bacteria in their lungs 96 hours
230 after the start of marbofloxacin treatment are shown for each inoculum size and each dosing
231 regimen in Table 1. In rats infected with the low inoculum and treated with 16 mg/kg of

232 marbofloxacin, neither R-2×MIC nor R-8×MIC bacteria were detected at the end of the
233 experiment. By contrast, rats infected with the high inoculum had both R-2×MIC and R-
234 8×MIC bacteria in their lungs. The frequency of rats harbouring these two *KP* subpopulations
235 was negatively associated with the marbofloxacin doses. Moreover, for the same total
236 marbofloxacin dose, fractionated administration seemed to be associated with a lower
237 presence of resistant subpopulations than the single dose administration.

238 **Pharmacokinetic study.**

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

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

248 The PK/PD indices for the different marbofloxacin dosage regimens are shown in Table 2.
249 The proportions of resistant *KP* subpopulations after the different dosage regimens in rats
250 infected with the high inoculum are shown in Figure 2. In all animals carrying resistant
251 bacteria, the proportion of both R-2×MIC and R-8×MIC bacteria was higher than 10^{-6} ,
252 indicating an enrichment compared with the initial inoculated *KP* population (proportion $< 10^{-6}$).
253 $^{-6}$).

254 (i) **AUC/MIC.** For the lowest dose of 16 mg/kg, the AUC/MIC values were 4×189 and 1×756
255 hours for the fractionated and single dose administrations respectively. These ratios were
256 associated with an enrichment of R-2×MIC and R-8×MIC subpopulations in animals infected

257 with the high inoculum whereas there was no enrichment for animals infected with the low
258 inoculum (Table 1). In animals infected with the high inoculum, AUC/MIC values of 4×756
259 and 1×3026 hours, for the fractionated and single 64 mg/kg administration respectively, were
260 also associated with R-2×MIC and R-8×MIC subpopulations whereas the ratios of 4×1182
261 and 1×4728 hours corresponding to the 100 mg/kg total dose were associated with the
262 absence of detection of R-8×MIC subpopulation (Table 1 and 2).

263 **(ii) C_{\max}/MIC .** In animals infected with the low inoculum (Group A), the C_{\max}/MIC value of
264 4×54 and 1×217 for fractionated and single 16 mg/kg administration respectively, were
265 associated with the absence of R-2×MIC or R-8×MIC subpopulation enrichment (Table 1 and
266 2). In animals infected with the high inoculum (Group B), the higher the C_{\max}/MIC value, the
267 less the R-2×MIC and R-8×MIC subpopulation enrichment occurred for each dosing regimen
268 (Table 1 and 2). The C_{\max}/MIC values that were associated with the absence of R-8×MIC
269 bacteria enrichment were 4×339 and 1×1357, for the fractionated and single 100 mg/kg
270 administrations respectively. The 4×339 ratio value for the fractionated administration was
271 also associated with the lower frequency of R-2×MIC subpopulation enrichment (Table 1 and
272 2).

273 **(iii) T_{MSW} and $T_{>MPC}$.** The dosage regimen had an important impact on T_{MSW} . For the single
274 administration, marbofloxacin plasma concentrations were 28, 40 and 41 % of the time (over
275 96 hours) within the MSW for 16, 64 and 100 mg/kg marbofloxacin doses respectively (Table
276 2) and for the fractionated administration, T_{MSW} was 50, 67 and 60 % of the time (over
277 24 hours) (Table 2). The T_{MSW} was lower for the 100 mg/kg dose compared to 64 mg/kg dose
278 as it was calculated over 24h and that the $T_{>MPC}$ was higher for the 100 mg/kg dose than for
279 the 64 mg/kg (*i.e.* 40% vs. 33% respectively) (Table 2). The relationships between time
280 indices and the proportion of R-2×MIC and R-8×MIC bacteria after the different
281 marbofloxacin dosage regimens for animals infected with the high inoculum are presented in

282 Figure 2. No clear relationship existed between T_{MSW} and the R-2×MIC and R-8×MIC
283 subpopulation enrichment (Fig. 2, panel A1 and A2). For $T_{>MPC}$, there was a relationship
284 between $T_{>MPC}$ and the R-2×MIC or R-8×MIC subpopulation enrichment, only when each
285 dosage regimen was considered separately (fractionated or single) (Fig. 2, panel B1 and B2).
286 In fact, there was a relationship between R-2×MIC or R-8×MIC subpopulation enrichment
287 and the ratio of $T_{>MPC}/T_{MSW}$ (Fig. 2, panels C1 and C2). Irrespective of the dosage regimen,
288 the closer the $T_{>MPC}/T_{MSW}$ ratio came up to 1, the more the R-2×MIC or R-8×MIC
289 subpopulation enrichment was limited. These observations were confirmed by the fitting of
290 the percentages of rats with resistant bacteria in the lungs *versus* T_{MSW} , $T_{>MPC}$ and
291 $T_{>MPC}/T_{MSW}$ using a sigmoid E_{max} model. Indeed, the best fit was obtained between the
292 percentages of rats with resistant bacteria in the lungs *versus* $T_{>MPC}/T_{MSW}$ with R^2 values of
293 0.99 and 0.97 for R-2×MIC and R-8×MIC respectively (Figure 3, panel B1 and B2) whereas
294 the R^2 values were 0.87 and 0.93 for $T_{>MPC}$ with a misfit at the end of the curve (Figure 3,
295 panel B1 and B2) and 0.54 and 0.60 for T_{MSW} (data not shown).

296 **DISCUSSION**

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

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

314 Previous *in vitro* studies on *E. coli*, *S. aureus* and *P. aeruginosa* (15, 26) and an *in vivo*
315 study on *E. coli* in immunocompromized mice (16) showed an impact of inoculum size on the
316 enrichment of resistant mutants. The present study confirms these results and clearly shows
317 this impact in immunocompetent animals. Indeed, with an early antimicrobial treatment on an
318 initial small bacterial population at the infection site (Group A), we never observed a R-
319 2×MIC or R-8×MIC subpopulation enrichment. In addition, the total bacterial load decreased
320 dramatically. On the contrary, with a delayed start of the antimicrobial treatment on an initial

321 large bacterial population (Group B), we observed a limited decrease in the total bacterial
322 population, accompanied by an enrichment of R-2×MIC and R-8×MIC subpopulations,
323 depending on the marbofloxacin dosing regimen. Moreover, for the smaller marbofloxacin
324 dose (16 mg/kg) administered as a single administration, 4 out of 10 rats died between 72 and
325 96h after the start of marbofloxacin treatment. These deaths were attributed to the infection,
326 based upon observed clinical signs of infection in these rats. On the contrary, the 3 deaths
327 observed with the highest single marbofloxacin dose (100 mg/kg) were unlikely to have been
328 due to infection because they occurred in the first 48h after the inoculation while in non-
329 treated animals death always occurred after 72h . These early deaths could be attributed to the
330 marbofloxacin toxicity in ill animals.

331 The ability of PK/PD indices such as AUC/MIC, C_{max}/MIC , $T_{>MPC}$ and T_{MSW} to
332 predict resistant subpopulation enrichment has already been studied. Previous studies
333 suggested that AUC/MIC was the PK/PD index that best correlated with efficacy of
334 fluoroquinolones in both neutropenic and non-neutropenic murine thigh and lung infection
335 models (3, 5, 13, 34). In our study, for the low inoculum (Group A) with the lowest
336 marbofloxacin dose (16 mg/kg), the AUC/MIC value of 4×189 hours or 1×756 hours for
337 fractionated or single dose administration respectively, was associated with the prevention of
338 the emergence of any resistant subpopulation after 96 hours of treatment. For the high
339 inoculum (Group B), the AUC/MIC values required to prevent R-8×MIC subpopulation
340 enrichment were higher than for the low inoculum, *i.e.* 4×1,182 hours and 1×4,728 hours for
341 fractionated and single administration respectively. However, considering the MPC, which is
342 linked to the MIC of the R-8×MIC bacteria, to calculate the corresponding PK/PD indices, it
343 appears that the AUC/MPC ratio which was associated with the prevention of the enrichment
344 of an R-8×MIC subpopulation is now 4×74 hours or 1×295 hours for fractionated or single
345 administration respectively. These AUC/MPC values are of the same order of magnitude as

346 the AUC/MIC values obtained to prevent an R-8×MIC subpopulation enrichment from a low
347 initial inoculum. In other words and as previously suggested (38), to be predictive, the
348 AUC/MIC ratio should take into account the MIC of the subpopulation having the highest
349 MIC value and not the MIC of the dominant population. Moreover, the differences observed
350 between R-8×MIC enrichment for fractionated and single dose administrations indicated that,
351 for a same total exposure (over 96h), the effectiveness of the fractionated dosage regimen is
352 greater than that of a single dose administration; this suggest that enrichment of resistant
353 subpopulations is co-dependent on both the total exposure and the time above some critical
354 concentration (see later).

355 The time the fluoroquinolone concentrations were within the Mutant Selection
356 Window has previously been shown to be associated with a promotion of resistant bacteria
357 subpopulation enrichment *in vitro* (1, 9, 10, 15) and *in vivo* (16). However, we did not
358 observe in our experiment such a relationship between T_{MSW} and R-8×MIC subpopulation
359 enrichment (Fig. 2, panels A1 and A2), which is in agreement with a previous *in vitro* study
360 with *Staphylococcus aureus* (8) although it is important to note that the target of mutation
361 resistance differs between gram positive and gram negative bacteria (11). Recently, it was
362 suggested that the apparent inability of T_{MSW} to predict mutant resistant enrichment may be
363 explained by the confounding influence of the actual antimicrobial concentrations at the edges
364 of the selection window (10, 17). In other words, for a given T_{MSW} , situations are not
365 equivalent when time outside the MSW is under the MIC or above the MPC. In the present
366 study, for the fractionated 16 mg/kg dose, the half-life of marbofloxacin of 2.41h (Fig. 1) led
367 plasma concentrations to decay below the MSW whereas for the 2 other doses (64 and
368 100 mg/kg), most of the exposure to marbofloxacin was at the top of the MSW. This could
369 explain the absence of a relationship between T_{MSW} and R-8×MIC enrichment. For the single
370 dose administration, the pharmacokinetic profiles showed that for the two highest doses (64

371 and 100 mg/kg), marbofloxacin concentrations reside almost the same time within the MSW
372 while the residence time within the MSW was shorter for the lower dose (16mg/kg). There
373 was thus no relationship between the T_{MSW} and R-8×MIC subpopulation enrichment. By
374 contrast, there was a relationship between both R-2×MIC and R-8×MIC subpopulation
375 enrichment and the ratio $T_{>MPC}/T_{MSW}$ (Fig. 2, panels C1 and C2, Fig. 3, panels B1 and B2).
376 With this ratio, it is now possible to discriminate between situations characterized by the same
377 T_{MSW} but with different levels of antimicrobial concentrations within this MSW, and the
378 higher is this ratio, the lower the risk of R-8×MIC subpopulation enrichment. The ability of
379 this new index to predict enrichment of resistant mutants is due to the fact that it consists of
380 two terms reflecting two antagonistic and sequential processes: $T_{>MPC}$, that reflects the time
381 during which the antibiotic eliminates all pathogens including resistant mutants, and T_{MSW} ,
382 that reflects the time during which the antibiotic produces selection of mutant resistant
383 subpopulations. It is noteworthy that this ratio allows an unbiased comparison of fractionated
384 and single dose administrations given that for fractionated administration, the ratio is the same
385 when calculated over 24h or the total duration of the treatment (96h). Cut-off values of
386 $T_{>MPC}/T_{MSW}$ associated with the prevention of resistant subpopulation enrichment need to be
387 known; as shown in the present experiment, the ratio is influenced by the likelihood of having
388 resistant mutants already present at the start of antimicrobial treatment. With the low
389 inoculum (Group A), $T_{>MPC}/T_{MSW}$ values of 0.31 and 0.30, for fractionated and single dose
390 administration respectively, were required to prevent mutant resistant enrichment. By contrast
391 for the high inoculum (Group B) the corresponding values were 0.67 and 0.54 respectively.
392 From our results, it appears that the most desirable situation to carry out an antimicrobial
393 therapy that does not simultaneously promote antimicrobial resistance is one in which the
394 inoculum size at the initiation of the treatment is low or null (metaphylaxis, prophylaxis). In a
395 curative setting, characterized by a greater likelihood of the antimicrobial facing a high

396 bacterial load, our results suggest that the best strategy would be to immediately achieve
397 plasma concentrations above the MPC. Besides such intuitive findings, the present study
398 offers the first evidence of the rational PK/PD approach to select the antimicrobial dosage
399 regimens adapted to either preventive or curative settings. In addition, as shown by our
400 results, the $T_{>MPC}$ needs to be long enough to produce an early reduction of the inoculum load
401 to be in the same situation as with an initial low inoculum load. In the present experiment, it
402 appears that with the single marbofloxacin dose (mimicking the so called “one shot”
403 treatment that is usually recommended in veterinary medicine), $T_{>MPC}$ is not long enough to
404 achieve this goal. However, it is important to note that in our study, the tested doses always
405 achieved concentrations above the MPC for fractionated administration but the same total
406 fluoroquinolone dose which achieves a concentration above the MPC for a single but not for a
407 fractionated administration would have been better in a single than in a fractionated
408 administration for mutant prevention. Secondly, the terminal half life of marbofloxacin in
409 domestic animal species may be much longer (up to 13 hours) than in rats (2.41 hours in our
410 study) and a single dose administration in large animal species may give a longer $T_{>MPC}$ than
411 in rats.

412 In addition to R-8×MIC subpopulation enrichment, we also studied the R-2×MIC
413 enrichment which could be attributed to efflux pump over-expression in bacteria (25). In
414 some animals we observed an enrichment of the R-2×MIC subpopulation without
415 simultaneous R-8×MIC subpopulation enrichment. For the two highest marbofloxacin doses
416 (64 and 100 mg/kg), the R-2×MIC subpopulation enrichment was greater for the single
417 administration than the fractionated one (Table 1). This could in turn explain the higher
418 percentage of animals harbouring R-8×MIC bacteria in their lungs with a single rather than a
419 fractionated administration of marbofloxacin at 64 mg/kg. Indeed, if the R-2×MIC
420 subpopulation enrichment is promoted, the ability of these bacteria to survive antimicrobial

421 concentrations closer to the MIC favours the possibility of generating a new and more
422 resistant subpopulation (R-8×MIC bacteria), as has been suggested by Louie *et al.* (25). Thus
423 despite the same impact on the total bacterial population, in our model the single
424 antimicrobial administration seems to be less beneficial than the fractionated administration in
425 preventing resistant mutant enrichment.

426 In conclusion, our results show that the bacterial load at the start of antimicrobial
427 treatment plays a critical role on the pattern of selection of *KP* resistant mutants. A low initial
428 bacterial load limits resistant mutant enrichment due to the lower likelihood of having
429 resistant mutant subpopulation already present at the beginning of the treatment. With a high
430 bacterial load at the start of treatment, we have shown the ability of the $T_{>MPC}/T_{MSW}$ ratio to
431 define the relevant features of antibiotic exposure to prevent mutant enrichment. This PK/PD
432 index might contribute to the rational selection of more adapted antimicrobial dosage
433 regimens. Nevertheless, further investigations in different infectious and animal models are
434 needed to confirm what we observed with our trials and to quantify the cut-off value for
435 $T_{>MPC}/T_{MSW}$ in target patients (man and domestic animal species).

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555

556 **TABLE 1.**

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

Total marbofloxacin dose (mg/kg)	Untreated controls		Fractionated			Single		
	At the start of treatment	96h after the start of treatment	16	64	100	16	64	100
Low inoculum								
<i>n</i>	3	4	10	nd	nd	10	nd	nd
Dead animal	0/3	0/4	0/10	nd	nd	0/10	nd	nd
log ₁₀ cfu/lungs (mean ± SD)	5.4±0.1	9.46±3.8	0.9±1.4 [†]	nd	nd	0.3±1.0 [†]	nd	nd
R-2×MIC	0%	75%	0%	nd	nd	0%	nd	nd
R-8×MIC	0%	50%	0%	nd	nd	0%	nd	nd
High inoculum								
<i>n</i>	3	4	11	11	10	10	12	9 ^c
Dead animal	0/3	4/4	0/11	0/11	0/10	4/10 ^b	0/12	3/12 ^c
log ₁₀ cfu/lungs (mean ± SD)	10±0.4	nd ^a	7.3±0.7	6.8±0.8	6.2±0.4	7.5±1.3	7.5±0.7	6.6±0.6 ^c
R-2×MIC	100%	nd ^a	100%	55%	10%	90% ^b	92%	42% ^c
R-8×MIC	100%	nd ^a	55%	27%	0%	70% ^b	50%	0% ^c

560

561 [†] For these means calculation, we assigned the value 0 log₁₀ CFU to lungs for which bacteria
 562 were undetectable.

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

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

567 ^{**} The death of these 3 rats occurred during the 24 first hours after the drug administration.
 568 The 3 animals that died 24h hour after marbofloxacin administration were considered as
 569 dying because of toxicity of the high dose of marbofloxacin administered in single-injection
 570 and are not included in the percentages.

571 nd: not determined

572 Dead rats are not taken into consideration to calculate the total bacterial population in lungs.

573 **TABLE 2.**

574 Simulated PK/PD Parameters after administration of 16, 64 or 100 mg/kg marbofloxacin in

575 fractionated or single administrations.

Total marbofloxacin dose (mg/kg)	T_{MSW} (%)	$T_{>MPC}$ (%)	$T_{>MPC}/T_{MSW}$	AUC/MIC (h)	AUC/MPC (h)	C_{max}/MIC
Fractionated						
16	50	16	0.31	4×189	4×12	4×54
64	67	33	0.49	4×756	4×47	4×217
100	60	40	0.67	4×1,182	4×74	4×339
Single						
16	28	8	0.30	1×756	1×47	1×217
64	40	16	0.41	1×3,026	1×189	1×868
100	41	22	0.54	1×4,728	1×295	1×1,357

576 **LEGENDS**

577

578 **FIGURE 1:** Pharmacokinetic profiles of marbofloxacin simulated for 3 marbofloxacin doses
579 (16, 64 and 100 mg/kg) with two dosing regimens: fractionated (A) or single (B)
580 administration. The elimination half-life ($T_{1/2elim}$) was 2.41 hours.

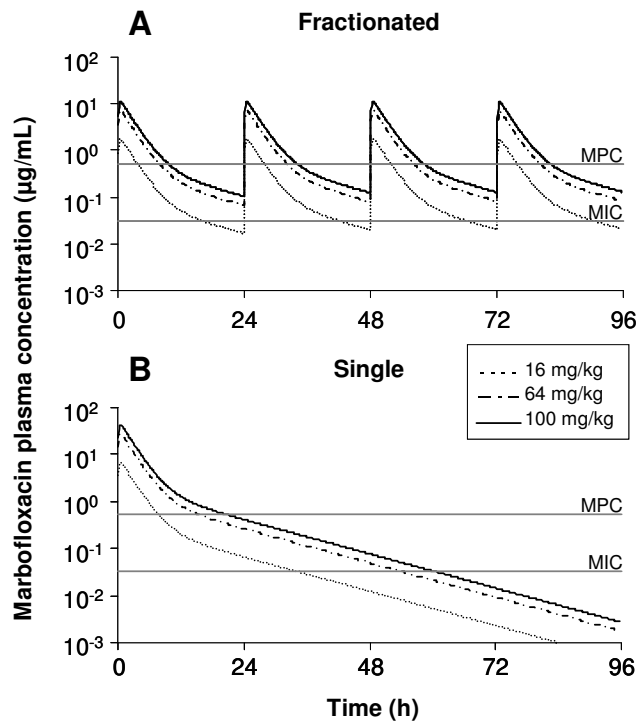
581

582 **FIGURE 2:** Proportion of R-2×MIC resistant to 0.064 µg/mL (A1, B1, C1) and R-8×MIC
583 resistant to 0.256 µg/mL (A2, B2, C2) in the lungs of rats infected with the high inoculum
584 (10^9 CFU) of *KP* versus T_{MSW} (A1, A2), $T_{>MPC}$ (B1, B2) and the ratio $T_{>MPC}/T_{MSW}$ (C1, C2).
585 Proportions are represented by full symbols when they were obtained after the fractionated
586 administration and by empty symbols after the single administration of marbofloxacin.
587 Symbols under the dotted line represent animals without detectable R-2×MIC and R-8×MIC
588 bacteria in their lungs. T_{MSW} and the $T_{>MPC}$ were calculated over 24h for fractionated
589 administrations and over 96h for single administrations.

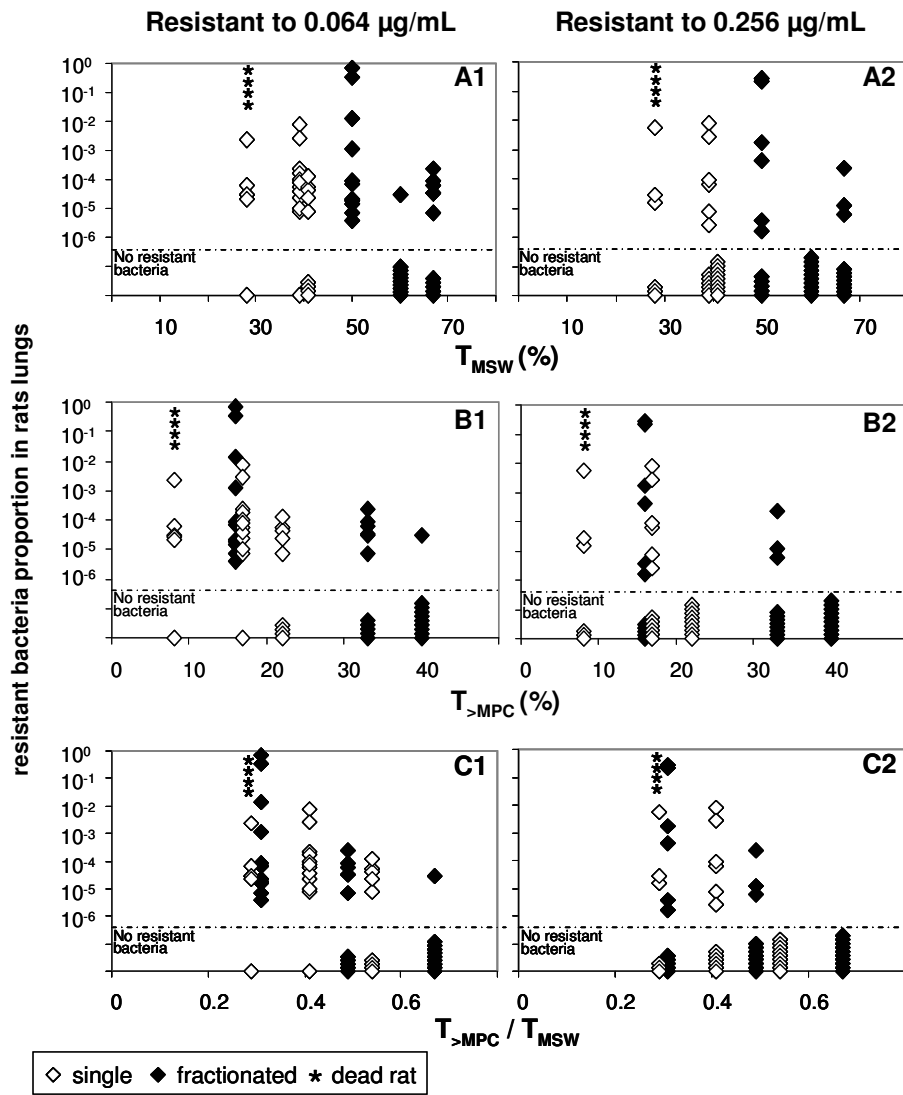
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591 **FIGURE 3:** Relationships of the $T_{>MPC}$ (Panel A1 and A2) and the ratio $T_{>MPC}/T_{MSW}$ (Panel
592 B1 and B2) with the percentage of animals with R-2×MIC (Panel A1 and B1) and R-8×MIC
593 (Panel A2 and B2) bacteria 96h after the start of antimicrobial treatment for the animals
594 infected by the large inoculum (Group B).

595 **FIGURE 1:**



596



599 **FIGURE 3:**

600

601

602

