Shape does matter: Short high-concentration exposure minimises resistance emergence for fluoroquinolones

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Synopsis

Objectives: For fluoroquinolones, the area under the free plasma concentration-time curve divided by the MIC (*f*AUC/MIC) best predicts bacterial killing in mice and outcomes in patients. However, it is unknown whether the shape of the antibiotic concentration profile affects resistance emergence. Our objective was to compare killing and resistance between ciprofloxacin concentration profiles with different shapes at the same *f*AUC/MIC and identify the durations of ciprofloxacin exposure that minimise resistance.

Methods: Static time-kill studies over 24h using *P. aeruginosa* ATCC 27853 assessed *f*AUC/MIC of 44 and 132 for ciprofloxacin (MIC_{CIP}: 0.25mg/L) and *f*AUC/MIC of 22, 44 and 132 for ciprofloxacin plus an efflux pump inhibitor ($MIC_{CIP+EPI}$: 0.031mg/L) at initial inocula of 10^4, 10^5 and 10^6 CFU/mL, in duplicate. Ciprofloxacin was added at 0h and rapidly removed at 1, 4, 10, 16 or 24h. Mutation frequencies and real-time MICs were determined at 24h.

Results: High ciprofloxacin concentrations over 1 to 10h yielded more rapid and extensive initial killing compared to 16 and 24h exposures at the same *f*AUC/MIC. No resistance emerged for 1 to 10h exposures, although regrowth of susceptible bacteria was extensive. The 24h exposure yielded less regrowth, but ciprofloxacin-resistant bacteria at 5xMIC amplified by over 5-$\log_{10}$ and almost completely replaced the susceptible bacteria by 24h; MICs increased 4- to 8-fold. Resistance also emerged on 3xMIC plates while efflux was inhibited.

Conclusions: Pre-existing resistant mutants amplified extensively for 24 and 16h durations of exposure but not for shorter durations. Shape of the ciprofloxacin concentration profile was critical to minimise resistance.

Introduction

Pseudomonas aeruginosa and other Gram-negative pathogens are causing a global health crisis which is exacerbated by a severe shortage of effective antibiotics.[1-3] *P. aeruginosa* causes life-threatening infections in hospitalized patients and has an exceptional potential to become resistant during antibiotic therapy.[4-6] The most important resistance mechanisms for fluoroquinolones in *P. aeruginosa* include Mex-efflux pumps[7, 8] and target site mutations of *gyrA* and *parC*.[9]

Over the last few decades, extensive pharmacokinetic / pharmacodynamic (PK/PD) studies on fluoroquinolones have shown that the clinical success and in mice the bacterial killing at 24 h are best predicted by the area under the free plasma concentration-time curve divided by the MIC (*f*AUC/MIC)[10-14]. Forrest *et al.*[15] showed that a ciprofloxacin AUC/MIC above 125 (equivalent to an *f*AUC/MIC of 87.5) is correlated with clinical success in acutely ill patients with bacterial infections; however, this *f*AUC/MIC target was often not reached for strains with MICs of 1 mg/L and higher[16].

Dose fractionation studies are commonly performed to identify the PK/PD index which best predicts bacterial killing in mice and dynamic *in vitro* models. These studies divide a range of daily doses into one or multiple dose(s) using different dosing intervals. In dose fractionation studies, dosing continues throughout the 24 h period (except for once daily dosing) and therefore antibiotic concentrations are present throughout the entire treatment period. Thus, dose fractionation studies do not identify the durations of antibiotic exposure which lead to resistance emergence at a given *f*AUC/MIC.

The *f*AUC and the free peak concentration (*f*Cmax) are often correlated in patients and the *f*Cmax/MIC usually also predicts bacterial killing in mice and clinical success of fluoroquinolones reasonably well.[12, 14, 17] Earlier studies suggested the need

93 for future studies that identify whether dosage regimens with high fluoroquinolone peak
94 concentrations better prevent clinical emergence of resistance than dosage regimens
95 with lower peaks.[18, 19] However, it is still not known whether high fluoroquinolone
96 concentrations applied over a short period yield more or less resistance than lower
97 concentrations over a longer period at the same overall exposure (i.e. at the same
98 *f*AUC/MIC). A few studies determined the *f*AUC/MIC value which is associated with
99 prevention of resistance for fluoroquinolones in *P. aeruginosa*[20-23]. However, it is
100 unknown for fluoroquinolones which exposure durations lead to resistance at a given
101 *f*AUC/MIC.

102 The primary objective of this study was to compare bacterial killing and resistance
103 between ciprofloxacin concentration profiles at the same *f*AUC/MIC but with different
104 shapes and to identify the durations of ciprofloxacin exposure that minimise resistance.
105 Our second objective was to assess whether resistance emergence for the tested
106 ciprofloxacin exposure profiles was dependent on efflux mechanisms. To achieve these
107 objectives we developed an efficient study design based on static *in vitro* time-kill
108 studies to evaluate resistance for different exposure profiles. Studies were performed at
109 an initial inoculum of 10^6 colony forming units / mL which most likely contained pre-
110 existing resistant mutants as well as at a low initial inoculum (10^4 colony forming units /
111 mL) which most likely lacked such pre-existing mutants.

Materials and methods

Bacterial strains and media. The *P. aeruginosa* ATCC 27853 strain was used in all experiments. All susceptibility and time-kill studies were performed in cation-adjusted Mueller Hinton broth (CAMHB; containing 20 to 25 mg/L Ca^{2+} and 10 to 12.5 mg/L Mg^{2+}; BD, Sparks, MD, USA). Viable counting was performed on cation-adjusted Mueller Hinton agar (CAMHA; containing 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+}; Medium Preparation Unit, The University of Melbourne, Parkville, Australia). Drug-containing agar plates were prepared using CAMHA (BD, Sparks, MD, USA) supplemented with the appropriate amount of ciprofloxacin.

Ciprofloxacin was purchased from Sigma-Aldrich (Shanghai, China) and the efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN) from Bachem (Bubendorf, Switzerland). Antibiotic stock solutions were prepared in Milli-Q water and subsequently filter-sterilized using a 0.22 μm PVDF syringe filter (Merck Millipore, Cork, Ireland).

Time-kill experiments. Static time-kill experiments were performed to assess bacterial killing and emergence of resistance for different ciprofloxacin exposure profiles with and without PAβN. Bacteria were grown on CAMHA and incubated at 35 °C for approximately 20 h. Bacteria were then transferred into sterile CAMHB and incubated for 60 min in a shaking waterbath at 35 °C. The optical density of this bacterial suspension was measured via a spectrophotometer to appropriately dilute this suspension to achieve the targeted initial inocula in 20 mL of fresh, pre-warmed, sterile CAMHB. The diluted bacterial suspensions were incubated in a shaking waterbath at 35 °C for approximately 15 min before the addition of ciprofloxacin, PAβN or both.

We studied ciprofloxacin *f*AUC/MIC of 44 and 132 at initial inocula of 10^6, 10^5 and 10^4 CFU/mL. These exposures were achieved by the appropriate ciprofloxacin

concentration applied over 1, 4, 10, 16 and 24 h. Ciprofloxacin was dosed at 0 h and then rapidly removed at the respective time point (removal procedure described below).

For studies of ciprofloxacin in combination with 60 mg/L PAβN, we decreased the ciprofloxacin concentrations according to the MIC ratio with and without the efflux pump inhibitor. Thus, lower ciprofloxacin concentrations were used to achieve the targeted *f*AUC/MIC. Ciprofloxacin *f*AUC/MIC of 22, 44 and 132 in combination with 60 mg/L PAβN were studied at initial inocula of 10^6 and 10^4 CFU/mL. The ciprofloxacin exposure durations were 1, 4, 10, 16 and 24 h. All studies included a growth control and a 60 mg/L PAβN control without ciprofloxacin.

Ciprofloxacin or ciprofloxacin and PAβN were rapidly removed by multiple sequential centrifugation and resuspension steps. For vials with ciprofloxacin concentrations of 8x MIC or higher, three sequential centrifugation and resuspension processes were used; for lower concentrations two sequential steps were applied. The conicals containing the bacterial cultures in broth were centrifuged at 3,220 *g* for 10 min at 35 °C, the supernatant removed and the bacteria resuspended in fresh, pre-warmed, drug-free CAMHB. The overall drug dilution factor was approximately 400-fold for two sequential centrifugation and resuspension processes and 8000-fold for three processes. This method assured that the ciprofloxacin concentrations were negligible (<0.004 x MIC) after drug removal.

***Viable counting*.** For all experimental arms, counts of viable bacteria were determined within 5 min prior to dosing and at 1, 2, 3.5 or 3.9, 6, 12, 16.5 and 24 h after dosing. Bacterial numbers were also determined 5 min prior to drug removal and 10 min after the final bacterial resuspension in fresh broth to assure minimal loss of bacteria during drug removal. All viable count samples were washed twice in sterile saline to

effectively minimize antibiotic carryover. Colony counts on CAMHA were determined by manual plating of 100 µL of the undiluted or diluted bacterial suspensions in saline. This plating method yielded a limit of counting of 1.0 $\log_{10}$ CFU/mL (equivalent to 1 colony per plate). Agar plates were incubated at 35 °C for 48 h.

Emergence of resistance. Mutation frequencies (MF) were determined at 0 h (*i.e*. before treatment) and at 24 h. Real-time MICs were determined at 24 h by spectrophotometrically adjusting the bacterial suspensions (*i.e*. dilution in fresh, pre-warmed, sterile CAMHB) to an inoculum of 10^6 CFU/mL, if the bacterial suspension was above 10^6 CFU/mL. For experimental arms with less than 10^6 CFU/mL at 24 h, undiluted bacterial suspensions were used for MIC testing at 24 h. Ciprofloxacin was removed from all arms before testing emergence of resistance.

Agar plates containing 3x or 5x the ciprofloxacin MIC were used for studies on ciprofloxacin monotherapy. For studies on ciprofloxacin with 60 mg/L PAβN, the ciprofloxacin concentration in agar was calculated based on the lower MIC in the presence of PAβN and drug plates contained 3x or 5x this lower ciprofloxacin MIC plus 60 mg/L PAβN. Antibiotic-containing agar plates were incubated for 3 days and MF calculated as the difference between the $\log_{10}$ CFU/mL on antibiotic-containing agar plates and the $\log_{10}$ CFU/mL on drug-free plates at the same observation time.

Some of the viable counts at 24 h were too low to enable quantifying colonies on antibiotic-containing agar plates. These arms still provided information on the upper limit of the $\log_{10}$ MF (such as $\log_{10}$ MF of ≤ -6). To include these data in the summary statistics, we used the following reporting rules. If the calculated MF was not quantifiable but the upper limit was within 1.1 $\log_{10}$ of the MF for the growth control, we assumed the MF was unchanged and used the value of the growth control. If the MF was not

quantifiable and the upper limit was more than 1.1 $\log_{10}$ higher than the MF for the growth control, the MF of this arm was reported as missing.

Results

The MIC of *P. aeruginosa* ATCC 27853 was 0.25 mg/L for ciprofloxacin. The $\log_{10}$ MF before treatment was -6.2 on 3x MIC ciprofloxacin plates and -7.1 on 5x MIC ciprofloxacin plates. As expected, the extent of killing of *P. aeruginosa* increased with the ciprofloxacin exposure *(f*AUC/MIC). Ciprofloxacin without PAβN at an *f*AUC/MIC of 44 and 132 yielded ~2 to >5 $\log_{10}$ killing (Figure 1). At the *f*AUC/MIC of 44, initial killing was noticeably slower for the 16 and 24 h durations of exposure than for the shorter durations. Initial killing was rapid for all durations of exposure at the *f*AUC/MIC of 132 with minimum viable counts occurring within the first 4 h.

Considerable regrowth at 24 h was observed for the vast majority of ciprofloxacin profiles, in particular at the 10^5 and 10^6 CFU/mL inocula. Complete killing with no regrowth was observed for the 4 h duration of exposure at the *f*AUC/MIC of 132 for the 10^4 CFU/mL inoculum. Based on our MF results, the 10^4 CFU/mL inoculum had a probability of approximately 1.2% to harbour at least one pre-existing mutant cell resistant at 5x MIC; the probability was approximately 10% at 3x MIC. In contrast, the 10^6 CFU/mL inoculum had a probability above 99.99% to contain at least one pre-existing resistant mutant at 3x MIC and a probability of approximately 80% to contain a resistant mutant at 5x MIC. Overall regrowth was slower and viable counts at 24 h tended to be lower for the 24 h duration of exposure compared to the shorter durations (Figure 1).

207 The extensive regrowth at 24 h for almost all viable count profiles posed the
208 important question of whether these bacteria were resistant to ciprofloxacin.
209 Interestingly, for the 24 h ciprofloxacin exposure the MF on 3x MIC plates was
210 approximately 6.1 $\log_{10}$ (at the *f*AUC/MIC of 44) and 5.6 $\log_{10}$ (at the *f*AUC/MIC of 132)
211 higher than the MF of the growth control at 24 h (Figure 2). Likewise, a 5.9 $\log_{10}$ (at the
212 *f*AUC/MIC of 44) and 7.3 $\log_{10}$ (at the *f*AUC/MIC of 132) higher MF on 5x MIC plates
213 was observed. This trend was clearly present for both the 10^6 and 10^5 CFU/mL inocula.
214 Ciprofloxacin over a 16 h exposure duration at the *f*AUC/MIC of 44 yielded up to 6.5
215 $\log_{10}$ increased MF on 3x and 5x MIC plates, whereas the MF was increased by up to
216 2.6 $\log_{10}$ for the *f*AUC/MIC of 132 at the 10^6 CFU/mL inoculum.
217 Emergence of resistance however did not occur or was substantially less for the
218 shorter durations of ciprofloxacin exposure (1, 4 and 10 h). The MF for these durations
219 of exposure was comparable to the MF of the growth control at 24 h (only up to 1.9 $\log_{10}$
220 higher) for all studied inocula on 3x and 5x MIC plates (Figure 2).
221 The higher MF correlated well with the increased real-time MICs at 24 h (Table 1).
222 The MICs remained relatively unchanged for 1 to 10 h durations of exposure, whereas
223 the MICs increased up to 8-fold at the 10^6 and 10^5 CFU/mL inocula for the 16 and 24 h
224 durations of exposure (Table 1). Emergence of resistance at the 10^4 CFU/mL inoculum
225 was much less compared to the 10^6 and 10^5 CFU/mL inocula (Figure 2 and Table 1).
226 To explore whether efflux was the primary cause for emergence of resistance, we
227 carried out time-kill experiments in the presence of 60 mg/L PAβN (Figure 3). The
228 ciprofloxacin MIC was 0.031 mg/L in the presence of 60 mg/L PAβN. Thus, 8-fold lower
229 ciprofloxacin concentrations were used to account for the lower MIC in the presence of
230 PAβN. Viable count profiles for 60 mg/L PAβN largely paralleled the growth control. In

231 the presence of PAβN, all experimental arms achieved >5 log_{10} killing at the
232 ciprofloxacin *f*AUC/MIC of 132. At the *f*AUC/MIC of 22 and 44, the maximum extent of
233 killing was larger and minimum viable counts occurred earlier for the shorter compared
234 to the longer durations of exposure (Figure 3). Even in the presence of 60 mg/L PAβN,
235 ciprofloxacin could not eradicate *P. aeruginosa*. Regrowth was limited or absent for the
236 24 h duration of exposure at all studied *f*AUC/MIC, but regrowth occurred for the shorter
237 durations of exposure.

238 For ciprofloxacin combined with 60 mg/L PAβN, 10 and 16 h durations of
239 exposure led in general to considerably increased MF on 3x MIC plates (Figure 4),
240 whereas resistance emergence was absent or much less for the 1 and 4 h durations of
241 exposure. Viable counts for the 24 h duration of exposure were low at 24 h and the MF
242 was usually below the quantification limit for these arms. Resistance on 3x MIC plates
243 was similar for ciprofloxacin with and without the efflux pump inhibitor (Figure 2); except
244 for the 10 h duration of exposure for the *f*AUC/MIC of 132 that showed an approximately
245 6.4 log_{10} increase in MF in the presence of PAβN (Figure 4). However, resistance on 5x
246 MIC plates was less in the presence compared to absence of PAβN. Emergence of
247 resistance for the 16 and 24 h durations of exposure also occurred for an *f*AUC/MIC of
248 22, but was less pronounced at this low ciprofloxacin *f*AUC/MIC.

249 The MF corresponded with the MICs for the combination of ciprofloxacin with
250 60 mg/L PAβN (Table 2). MICs were relatively unchanged except for the 16 h duration of
251 exposure which led to a 5- to 10-fold increased MIC for an *f*AUC/MIC of 44 or 132 at the
252 10^6 CFU/mL inoculum (Table 2). The real-time MICs at 24 h could not be determined for
253 the 24 h duration of exposure for *f*AUC/MIC of 44 and 132 likely due to the low viable
254 counts.

Discussion

Many PK/PD studies on fluoroquinolones have found the *f*AUC/MIC to be the PK/PD index that best predicts bacterial killing in mice and therapeutic success in patients.[11, 13-15] From such studies conducted during drug development, clinical dosing of fluoroquinolones is now guided by the *f*AUC/MIC. However, only a few studies assessed prevention of resistance to fluoroquinolones against Gram-negative pathogens.[20-23] While the design of these studies implicitly assumed that the *f*AUC/MIC best predicts prevention of resistance for fluoroquinolones, it is unknown which PK/PD index is relevant for this endpoint. In fact, for rifampicin and linezolid, different PK/PD indices predict bacterial killing (*f*AUC/MIC) and prevention of resistance (*f*Cmax/MIC).[24, 25]

In the present study we demonstrated a greater than 5 $\log_{10}$ increase in emergence of resistant bacteria at the 10^6 CFU/mL inoculum for 16 and 24 h durations of exposure compared to 1 to 10 h durations of exposure at the same *f*AUC/MIC (Figure 2). For the 16 and 24 h durations of exposure at an *f*AUC/MIC of 44 and 132, resistance was extensive both on 3x and 5x MIC agar plates and MICs were 4- to 8-fold higher compared to pre-treatment (Table 1). As expected, profiles with a short duration of exposure yielded more extensive killing during the first 2 to 4 h compared to long durations of exposure at the same *f*AUC/MIC (Figure 1 and Figure **3**).

Emergence of resistance was absent or much less pronounced and MICs were not elevated at the 10^4 CFU/mL inoculum which likely lacked pre-existing resistant mutants (Figure 2 and Table 1). It seems possible that the high ciprofloxacin concentrations for the 1 to 10 h durations of exposure killed both susceptible and resistant bacteria to a comparable extent and therefore did not give rise to emergence of resistance. In contrast, the 24 h duration of exposure likely provided a growth advantage

279 for resistant mutants. Alternatively, adaptive resistance, if present, might require a
280 duration of antibiotic exposure of longer than 10 h to be (fully) up-regulated, or adaptive
281 resistance may revert back to baseline between 10 and 24 h. The latter two alternatives
282 seem less likely, as efflux of levofloxacin in *P. aeruginosa* was extensively up-regulated
283 within 1 h and did not revert back to baseline between 6 and 24 h; *i.e.* when levofloxacin
284 concentrations were negligible due to a short half-life and a 24 h dosing interval in
285 mice[22]. Ultimately, molecular studies combined with a full mechanism-based modelling
286 analysis would be highly valuable to elucidate these mechanistic details.

287 We explored the role of efflux in the rapid and extensive emergence of resistance
288 described above. In the presence of the broad-spectrum efflux pump inhibitor PAβN,
289 considerable emergence of resistance occurred for *f*AUC/MIC of 44 and 132 (Figure 4)
290 and the MICs at 24 h were up to 10-fold increased for the 16 h duration of exposure
291 (Table 2). Emergence of resistance was less pronounced at the *f*AUC/MIC of 22 likely
292 because this drug exposure did not provide a sufficient growth advantage for less
293 susceptible bacteria, in agreement with the inverted U principle.[20, 21] As emergence of
294 resistance on 5x MIC plates in the presence of PAβN was considerably less (Figure 4)
295 than in the absence of PAβN (Figure 2), efflux played a role in the development of high
296 level resistance.

297 The extensive contribution of efflux pumps to fluoroquinolone resistance was
298 studied in detail in a 48 h mouse infection model at a high bacterial inoculum; an
299 *f*AUC/MIC of 110 for levofloxacin (equivalent to a total drug AUC/MIC of 157) prevented
300 amplification of resistant mutants whereas an *f*AUC/MIC of 37 led to amplification of
301 resistant mutants.[22] Two hollow fibre *in vitro* infection model studies determined the
302 *f*AUC/MIC of garenoxacin that prevented resistance;[20, 21] for *P. aeruginosa*, an

303 *f*AUC/MIC of 190 prevented amplification of pre-existing resistant mutants at 48 h.[21]
304 Another hollow fibre study found extensive emergence of resistance to ciprofloxacin by
305 48 to 72 h using a ciprofloxacin *f*AUC/MIC of 180 against three *P. aeruginosa* strains.[23]
306 The studies mentioned above[20-23] assessed emergence of resistance for different dose
307 levels and thus for different *f*AUC/MIC. These studies neither varied the fluoroquinolone
308 half-life nor the dosing interval and therefore kept the shape of the antibiotic
309 concentration-time profile constant.

310 An earlier dose fractionation study in the hollow fibre model with ciprofloxacin
311 against two *P. aeruginosa* isolates found extensive resistance emergence for 400 mg
312 ciprofloxacin dosed every 8 h and 600 mg every 12 h (equivalent to an *f*AUC/MIC of
313 approximately 60).[19] Resistance emergence at 24 h was less extensive for one isolate
314 and absent for a second *P. aeruginosa* isolate for 1200 mg ciprofloxacin every 24 h, but
315 this regimen was subject to regrowth of mostly susceptible *P. aeruginosa* at 24 h.[19]
316 Another *in vitro* study gave the same daily enoxacin dose (equivalent to an *f*AUC/MIC of
317 approximately 50) every 12 or 24 h and found more killing for dosing every 24 h.
318 However, extensive *P. aeruginosa* resistance occurred at 12 to 24 h for both regimens.[26]
319 These dose fractionation studies[19, 26] had high or low fluoroquinolone concentrations
320 present throughout the entire treatment period. Therefore, the durations of
321 fluoroquinolone exposure that lead to resistance have not been assessed previously.[19-
322 23, 26]

323 The present study systematically explored whether the shape of the ciprofloxacin
324 concentration-time profile affects bacterial killing and resistance emergence. We
325 developed an efficient *in vitro* study design which delivered the same *f*AUC/MIC by
326 varying the duration of exposure and ciprofloxacin concentration accordingly. Our

studies were performed in the presence and absence of an efflux pump inhibitor to assess the contribution of efflux to resistance emergence. Most available studies on emergence of fluoroquinolone resistance in dynamic *in vitro* or animal models were performed at high inocula that contained pre-existing resistant mutants.[20-22] We extended these studies by assessing a low inoculum (10^4 CFU/mL) in duplicate that most likely (probability ≥90%) lacked pre-existing resistant mutants.

Our study design was efficient to achieve the objectives of this work; a potential limitation is the use of static antibiotic concentrations. Therefore, future studies in dynamic *in vitro* and animal infection models are warranted to further confirm that short durations of exposure provide killing with no or limited resistance. Our study lacked the effect of the immune system and used standard, nutrient-rich broth medium. These factors likely resulted in more rapid (re-)growth of bacteria compared to an *in vivo* infection, in agreement with the relatively slow regrowth of *P. aeruginosa* in mice.[22]

Overall, the observed differences in emergence of resistance for ciprofloxacin profiles with a short and long duration at the same *f*AUC/MIC were dramatic (Figure 2 and Figure **4**). However, regrowth of susceptible *P. aeruginosa* in our *in vitro* study was extensive for the short durations of exposure, indicating that the post-antibiotic effect of ciprofloxacin was short, as reported previously.[27] This suggests that clinical regimens with a short-term exposure of high ciprofloxacin concentrations can yield extensive and rapid bacterial killing with no or limited resistance. However, such ciprofloxacin concentration profiles would be expected to be best used in combination regimens with a second antibiotic that prevents the regrowth of ciprofloxacin susceptible bacteria.

In summary, we found that delivering the same *f*AUC/MIC over short durations of exposure (*i.e.* 1, 4 or 10 h) achieved more rapid killing with no or very limited emergence

of resistance, whereas longer durations of exposure over 16 and 24 h led to a 5 $\log_{10}$ increase in the concentration of resistant bacteria. Therefore, the shape of the concentration-time profile had a pronounced effect on prevention of resistance emergence. Pre-existing resistant mutants likely caused emergence of resistance. Efflux was important for the development of high-level resistance (at 5x MIC), but was not required for the development of low-level resistance on 3x MIC plates. Regrowth of *P. aeruginosa* was extensive for most regimens with durations of exposure of 16 h or shorter. Therefore, regimens with high-intensity, short exposure durations may be promising as part of a combination regimen with a second antibiotic that prevents regrowth. Studies in dynamic *in vitro* and animal infection models are warranted to optimally translate our results to future studies in patients. The present study highlights the potential to greatly minimise emergence of resistance by innovative fluoroquinolone dosage regimens with an optimised shape of the plasma concentration-time profiles.

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Transparency declarations

All authors: None to declare.

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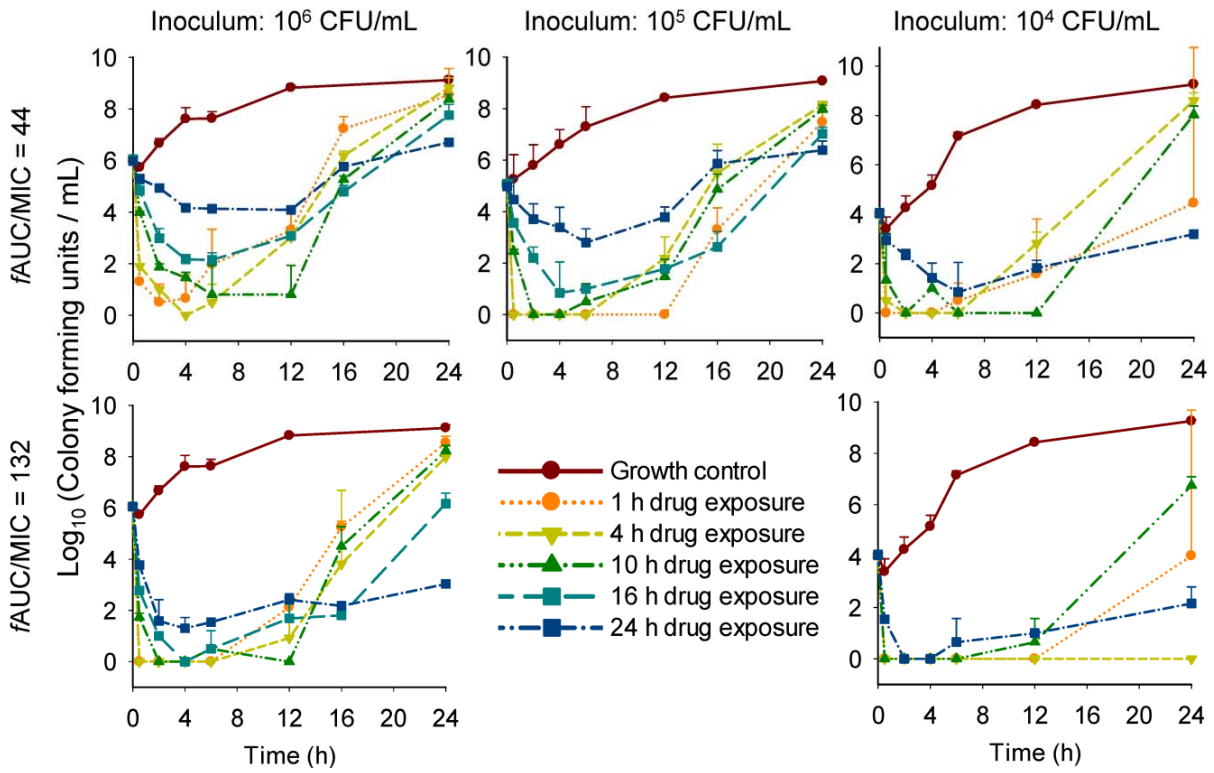

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464 **Figure 1:** Observed viable counts (mean ± SD) for *P. aeruginosa* ATCC
465 27853 exposed to ciprofloxacin at an *f*AUC/MIC of 44 (top) or 132
466 (bottom). The same *f*AUC/MIC (44 or 132) for ciprofloxacin were
467 delivered by varying the duration of exposure over 1, 4, 10, 16 or
468 24 h at inocula of 10^6 (left), 10^5 (middle) and 10^4 CFU/mL (right).
469 The 16h exposure was not studied at the 10^4 CFU/mL inoculum.
470 None of these treatment arms contained PAβN.

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473 print version of JAC.

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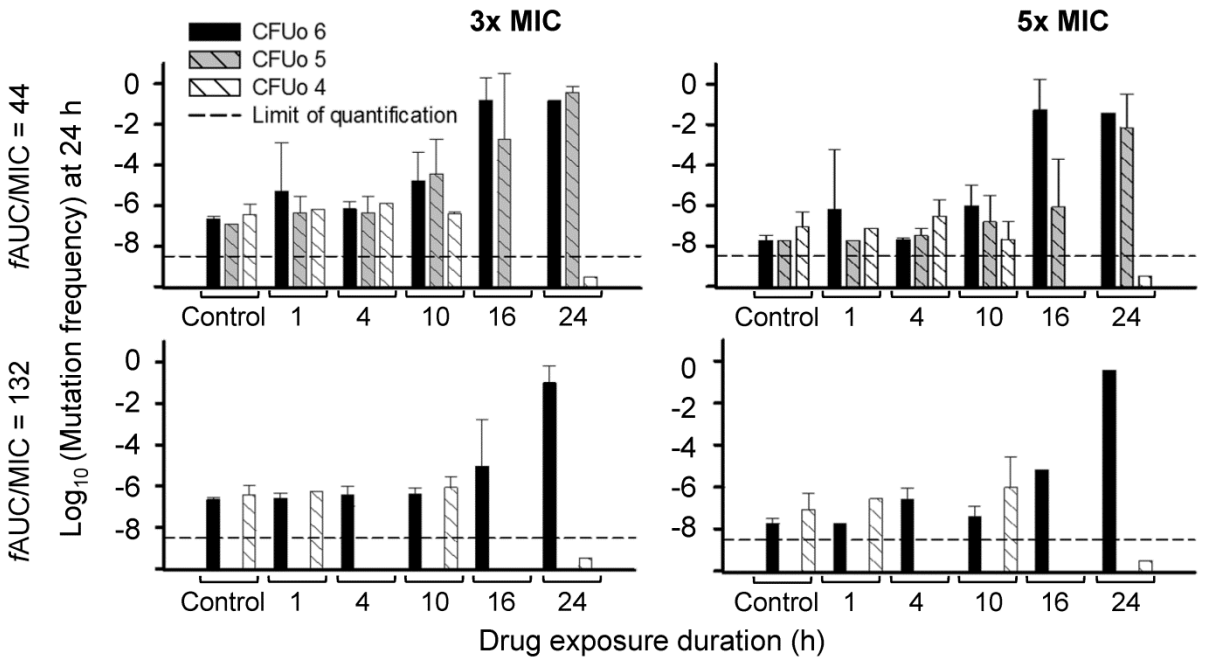
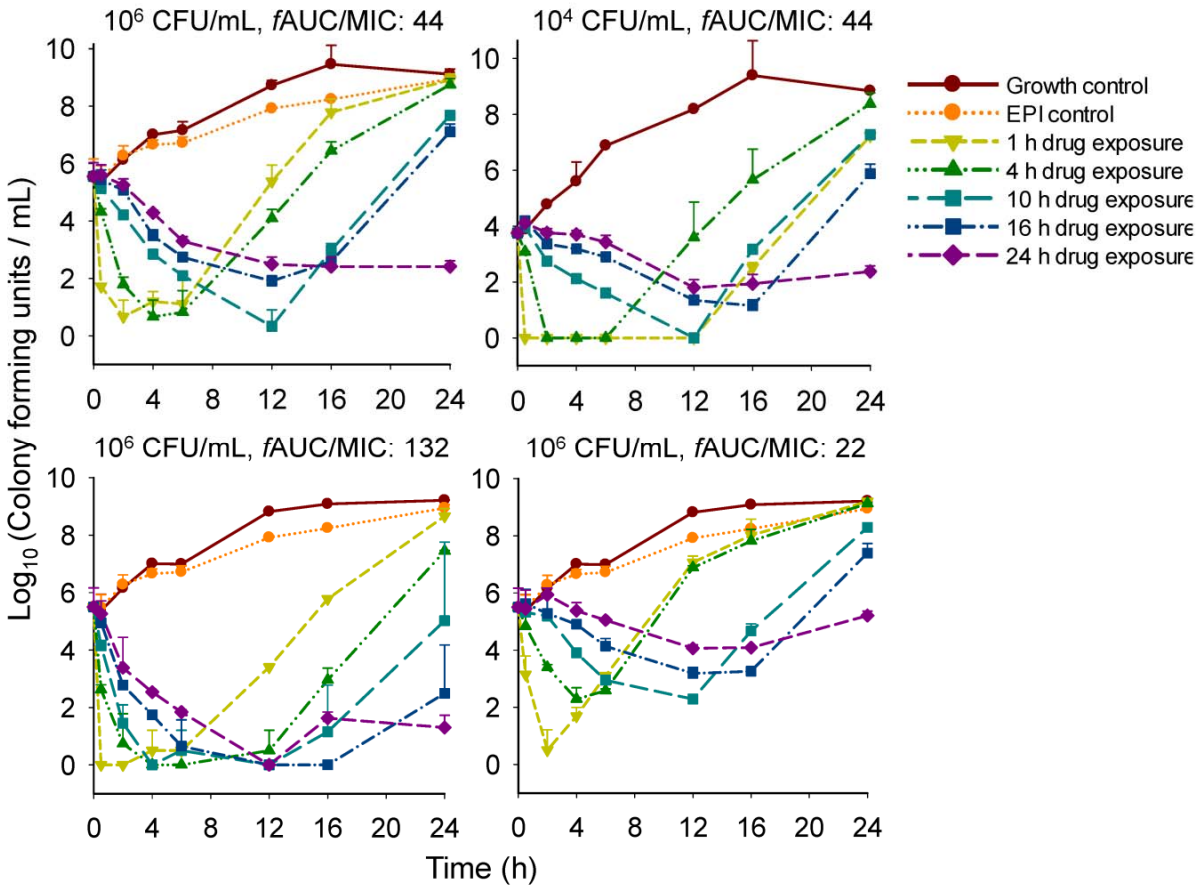


Figure 2: Log_{10} mutation frequency (mean ± SD) at 24 h on 3x (left) and 5x MIC (right) agar plates for ciprofloxacin in the absence of PAβN at an *f*AUC/MIC of 44 (top) and 132 (bottom) delivered over differing durations of exposure. Inocula (CFUo) of 10^6, 10^5 and 10^4 CFU/mL were studied for the *f*AUC/MIC of 44 and inocula of 10^6 and 10^4 CFU/mL for the *f*AUC/MIC of 132. The *f*AUC/MIC of 132 yielded eradication for the 4 h exposure duration at the 10^4 CFU/mL inoculum and thus no mutation frequency could be determined. The 16 h duration of exposure was not studied at the 10^4 CFU/mL inoculum. The mutation frequency for the 24 h exposure at the 10^4 CFU/mL inoculum was arbitrarily drawn at -9.5 log_{10} (*i.e.* below the limit of quantification); these two experimental arms had less than $10^{3.3}$ CFU/mL for the total population at 24 h and no colonies grew on antibiotic-containing agar plates.



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492 **Figure 3:** Observed viable counts (mean ± SD) for *P. aeruginosa* ATCC
493 27853 exposed to ciprofloxacin, *f*AUC/MIC of 22, 44 or 132, in
494 combination with 60 mg/L PAβN. The same *f*AUC/MIC (22, 44 or
495 132) for ciprofloxacin were delivered by varying the duration of
496 exposure over 1, 4, 10, 16 or 24 h at initial inocula of 10^6 and 10^4
497 CFU/mL.

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499 This figure appears in colour in the online version of JAC and in black and white in the
500 print version of JAC.

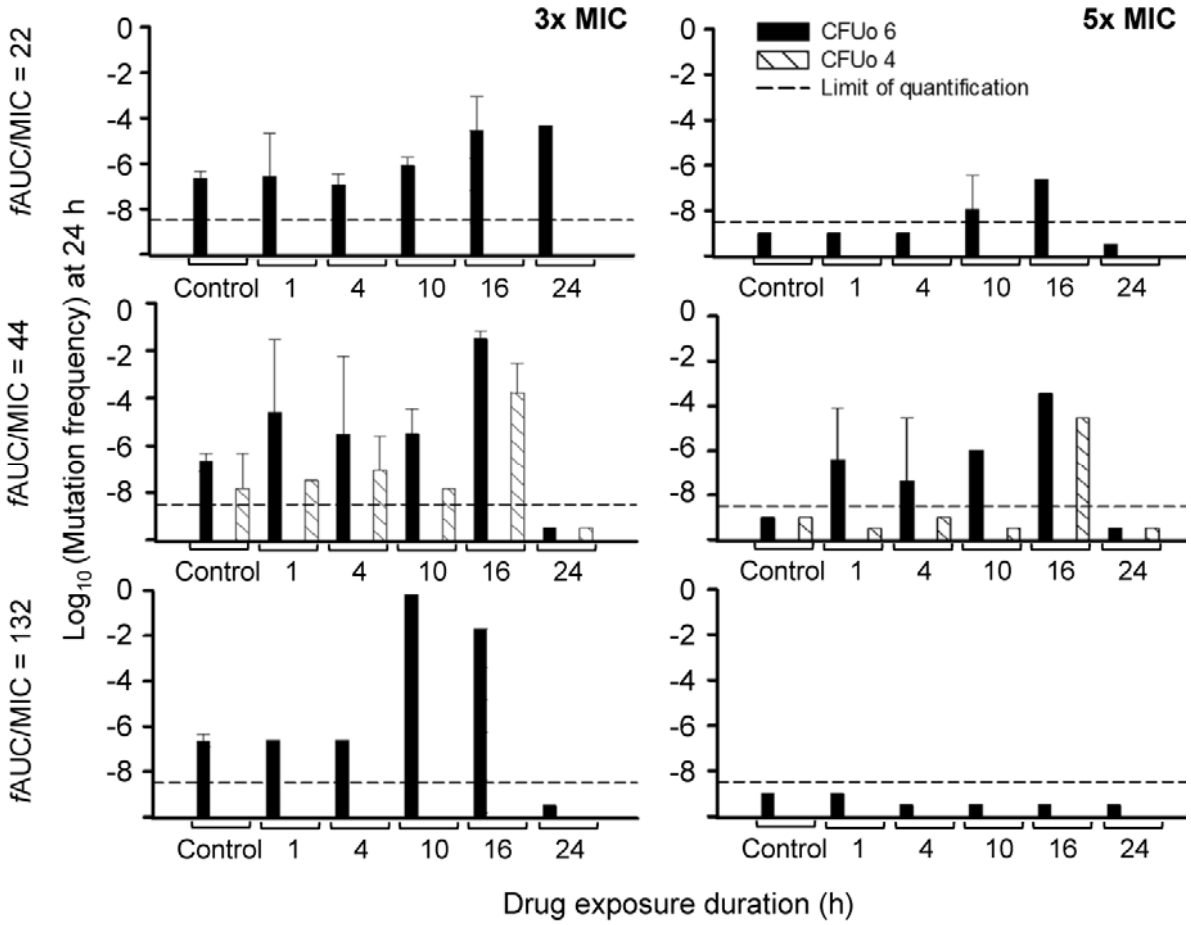

Figure 4: Log_{10} mutation frequency (mean $\pm$ SD) at 24 h on 3x (left) and 5x MIC (right) plates for ciprofloxacin in the presence of 60 mg/L PAβN at an *f*AUC/MIC of 22 (top), 44 (middle) and 132 (bottom) delivered over differing durations of exposure. Inocula (CFUo) of 10^6 and 10^4 CFU/mL were studied for the *f*AUC/MIC of 44 and an inoculum of 10^6 CFU/mL for the *f*AUC/MIC of 22 and 132. The growth controls in the presence of 60 mg/L PAβN did not yield colonies on agar plates with 5x MIC; given their total viable counts of approximately 10^9 CFU/mL, the log_{10} mutation frequency of the growth controls for 5x MIC is drawn as -9 (below the limit of quantification). For all experimental arms that had no colonies on agar plates containing 5x MIC ciprofloxacin and that had a viable count of at least $10^{7.9}$ CFU/mL on antibiotic-free agar plates, the log_{10} mutation frequency was drawn as -9. For experimental arms that showed no colonies on antibiotic-containing agar plates that had total populations of less than $10^{7.9}$ CFU/mL on antibiotic-free agar plates, the log_{10} mutation frequency was arbitrarily drawn at -9.5 (below the limit of quantification).

520 **Table 1:** The MICs (mg/L) at 24 h (geometric mean [range]) for ciprofloxacin
521 *f*AUC/MIC of 44 or 132 delivered over various durations of
522 exposure and initial inocula. MICs are bolded, if they were at least
523 4-fold above baseline. No range is provided, if only one replicate
524 was available.

525

Drug exposure duration (h)	*f*AUC/MIC: 44			*f*AUC/MIC: 132	
	CFUo 1 x 10^6	CFUo 1 x 10^5	CFUo 1 x 10^4	CFUo 1 x 10^6	CFUo 1 x 10^4
Control	0.25 [0.25 - 0.25]	0.50	0.25 [0.25 - 0.25]	0.25 [0.25 - 0.25]	0.25 [0.25 - 0.25]
1	0.50 [0.25 - **1.00**]	0.35 [0.25 - 0.50]	0.25	0.25 [0.25 - 0.25]	0.50
4	0.25 [0.25 - 0.25]	0.35 [0.25 - 0.50]	0.25 [0.25 - 0.25]	0.25 [0.25 - 0.25]	0.25
10	0.50 [0.25 - **1.00**]	0.71 [0.50 - **1.00**]	0.25 [0.25 - 0.25]	0.25 [0.25 - 0.25]	0.35 [0.25 - 0.50]
16	**2.00 [2.00 - 2.00]**	0.71 [0.50 - **1.00**]	(not studied)	0.50 [0.25 - **1.00**]	(not studied)
24	**1.41 [1.00 - 2.00]**	**1.00 [1.00 - 1.00]**	0.18 [0.125 - 0.25]	**1.41 [1.00 - 2.00]**	0.25

526

Table 2: The MICs (mg/L) at 24 h (geometric mean [range]) for ciprofloxacin *f*AUC/MIC of 22, 44 or 132 delivered over various durations of exposure and initial inocula in the presence of 60 mg/L PAβN. MICs are bolded, if they were at least 4-fold above baseline. No range is provided, if only one replicate was available.

Drug exposure duration (h)	*f*AUC/MIC: 22	*f*AUC/MIC: 44		*f*AUC/MIC: 132
	CFUo 1 x 10^6	CFUo 1 x 10^6	CFUo 1 x 10^4	CFUo 1 x 10^6
Control	0.025 [0.016 - 0.031]	0.025 [0.016 - 0.031]	0.031 [0.031 - 0.031]	0.025 [0.016 - 0.031]
PAβN control	0.022 [0.016 - 0.031]	0.022 [0.016 - 0.031]	(not studied)	0.022 [0.016 - 0.031]
1	0.031 [0.031 - 0.031]	0.063 [0.031 - **0.25**]	0.044 [0.031 - 0.063]	0.022 [0.016 - 0.031]
4	0.031 [0.031 - 0.031]	0.050 [0.031 - **0.125**]	0.044 [0.031 - 0.063]	0.031 [0.031 - 0.031]
10	0.022 [0.016 - 0.031]	0.031 [0.031 - 0.031]	0.031	0.062 [0.016 - **0.25**]
16	0.022 [0.016 - 0.031]	**0.25 [0.25 - 0.25]**	0.063	**0.125**
24	0.063 [0.063 - 0.063]			