Conditions required for the bactericidal activity of 4-quinolones against *Serratia marcescens*

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Summary. The conditions required to kill *Serratia marcescens* with nalidixic acid, ciprofloxacin, norfloxacin or ofloxacin were determined in nutrient broth and in phosphate-buffered saline. They were found to be similar to the conditions required for these 4-quinolones to kill *Escherichia coli*. Bacterial RNA synthesis and bacterial cell division were essential for the bactericidal activity of nalidixic acid but all three fluoroquinolones were bactericidal against non-dividing *S. marcescens*. However, as with *E. coli*, bacterial RNA synthesis was essential for the bactericidal activity of norfloxacin though this was not required to kill *S. marcescens* with ciprofloxacin or ofloxacin.

Introduction

Bacterial protein and RNA synthesis, as well as cell division, are essential for the non-fluorinated 4-quinolones such as nalidixic acid and oxolinic acid to kill *Escherichia coli*.^{1–3} Therefore, it was proposed by Smith² that these drugs possessed a single bactericidal mechanism, termed A, which required bacterial protein synthesis, RNA synthesis and bacteria capable of cell division to exert its lethal activity. Although these older 4-quinolones possess a single bactericidal mechanism, all eight of the modern fluorinated quinolones, that have been investigated, appear to exert another bactericidal mechanism, in addition to mechanism A, because they are active against non-dividing *E. coli*.

Ciprofloxacin, DR-3355, fleroxacin, ofloxacin, pefloxacin and lomefloxacin exhibit an additional mechanism, termed B, which does not require bacterial protein or RNA synthesis for its activity and is active against non-dividing bacteria.^{2–7} Even though protein synthesis and RNA synthesis are essential for the bactericidal activity of enoxacin and norfloxacin against *E. coli*,^{3,5,8} both of these drugs are, nevertheless, able to kill non-dividing *E. coli*. Therefore, it was proposed that they exhibited, as well as mechanism A, a second additional mechanism, termed C.^{3,5,8}

In in-vitro experiments, *E. coli* mutates to resistance to the older 4-quinolones at higher frequencies than those observed with the fluoroquinolones.^{9,10}

It has been suggested that this might occur because the latter drugs possess an extra bactericidal mechanism (B or C) whereas the older drugs possess only a single mechanism (A); therefore, two distinct mutations may be required to confer resistance to the fluorinated drugs whereas a single mutation may suffice to confer resistance to the older drugs.^{2,9,10}

Studies by Watanabe *et al.*¹¹ have shown that *Serratia marcescens* mutates *in vitro* to resistance to ciprofloxacin, ofloxacin and norfloxacin, at four times their MICs at higher frequencies than those observed with *E. coli*. Similarly, Smith has found that *S. marcescens* mutates to resistance to ofloxacin, at five times its MIC, at higher frequencies than those observed with *E. coli*.^{9,12} Furthermore, *in-vivo*, ofloxacin-resistant and ciprofloxacin-resistant mutants of this species have been isolated.^{13,14} Two of the five strains that developed resistance to ciprofloxacin during therapy in the phase II and III trials of the drug were *S. marcescens*.¹³

Therefore, we have investigated the bactericidal mechanisms of fluoroquinolones against *S. marcescens* to establish whether they correlate with its ability to develop resistance to the fluoroquinolones, as has been observed with *E. coli*.

Materials and methods

Bacterial strain

S. marcescens 120, a prototroph, was used throughout

this investigation. The strain was kept on drug-free nutrient-agar plates which were subcultured every 10 days. Colonies taken from drug-free nutrient agar were used to inoculate the overnight cultures used in these experiments.

Antibacterials

Ciprofloxacin (Bayer UK) and norfloxacin (Merck, Sharpe and Dohme, UK) were dissolved in sterile distilled water, and ofloxacin (Hoechst UK) and nalidixic acid (Sterling, UK) in 0.5 M NaOH before being made up to appropriate concentrations in sterile distilled water. Rifampicin (Merrel Dow, UK) was dissolved in dimethylsulphoxide.

Determination of antibacterial effects of the 4-quinolones

The killing activity of the 4-quinolones was determined in nutrient broth at 37°C over a 3-h period with various drug concentrations. Survival was estimated by serial dilution in nutrient broth followed by viable counting on nutrient agar as described by Lewin *et al.*¹⁵

The rate of kill of bacteria treated with the 4-quinolones in nutrient broth or phosphate-buffered saline (PBS) was measured at 30-min intervals over a 4-h period by viable counting on nutrient agar as described by Lewin and Smith.¹⁶

Results

Studies of the bactericidal activity of nalidixic acid, ciprofloxacin, norfloxacin or ofloxacin, separately, at various concentrations in nutrient broth over 3 h at 37°C, showed a biphasic effect with *S. marcescens* (fig. 1). The bactericidal activity of the drugs increased until a concentration known as the most, or optimum, bactericidal concentration was reached. Higher concentrations then became progressively less bactericidal. The optimum bactericidal concentration of ciprofloxacin and norfloxacin against *S. marcescens* was 1.5 mg/L of ofloxacin 5–9 mg/L and of nalidixic acid 90 mg/L. Although the optimum bactericidal concentration of ofloxacin was higher than that of the other two fluoroquinolones it was more bactericidal than either of them when compared at their optimum bactericidal concentrations (fig. 1).

The bactericidal mechanisms of these 4-quinolones against *S. marcescens* were then investigated. Fig. 2 shows that the addition of a bacteriostatic concentration of rifampicin (300 mg/L), an inhibitor of RNA synthesis, completely abolished the bactericidal activity of nalidixic acid 90 mg/L or norfloxacin 1.5 mg/L against *S. marcescens* in nutrient broth. Nalidixic acid 90 mg/L was also shown

Fig. 1. Survival of *S. marcescens* after exposure for 3 h to ciprofloxacin (●—●), ofloxacin (×—×), nalidixic acid (○—○) or norfloxacin (□—□) in nutrient broth at 37°C.

to be unable to kill non-dividing *S. marcescens* because it was not bactericidal in PBS (fig. 3). Therefore, nalidixic acid possesses only mechanism A against *S. marcescens*. In contrast, norfloxacin was weakly bactericidal against *S. marcescens* in PBS—only 30% of the bacteria survived after exposure to the drug for 4 h (compared with ≥90% that survived exposure to nalidixic acid for 4 h) (fig. 3). Hence, in addition to mechanism A, norfloxacin appears to possess mechanism C, which requires bacterial RNA synthesis for its activity

Fig. 2. Survival of *S. marcescens* in nutrient broth at 37°C with nalidixic acid 90 mg/L (△—△), norfloxacin 1.5 mg/L (▲—▲), rifampicin 300 mg/L (□—□), nalidixic acid 90 mg/L + rifampicin 300 mg/L (○—○) or norfloxacin 1.5 mg/L + rifampicin 300 mg/L (●—●).

Fig. 3. Survival of *S. marcescens* in PBS at 37°C alone (control, ○—○) or with nalidixic acid 90 mg/L (●—●) or norfloxacin 1.5 mg/L (□—□).

but can kill non-dividing bacteria, against *S. marcescens*.

Fig. 4 shows that the addition of rifampicin 300 mg/L reduced, but did not completely abolish, the bactericidal activity of ciprofloxacin 1.5 mg/L or ofloxacin 9 mg/L against *S. marcescens* in nutrient broth. Hence, ciprofloxacin and ofloxacin exert mechanism B which does not require bacterial

Fig. 4. Survival of *S. marcescens* in nutrient broth at 37°C with ciprofloxacin 1.5 mg/L (▲—▲), ofloxacin 9 mg/L (△—△), rifampicin 300 mg/L (○—○), ciprofloxacin 1.5 mg/L + rifampicin 300 mg/L (●—●) or ofloxacin 9 mg/L + rifampicin 300 mg/L (□—□).

RNA synthesis for its activity against this species. Furthermore, the reduction in the bactericidal activity of ciprofloxacin and ofloxacin confirms the presence of mechanism A as RNA synthesis is required for its lethal action. When the bactericidal activity of ciprofloxacin 1.5 mg/L and ofloxacin 9 mg/L against *S. marcescens* were investigated in PBS, the fluoroquinolones were found to be bactericidal, confirming the presence of mechanism B (fig. 5). The mechanism B possessed by ofloxacin against *S. marcescens* would appear to be more powerful than that of ciprofloxacin, as ofloxacin was significantly more bactericidal than ciprofloxacin in nutrient broth even when rifampicin 300 mg/L was present or in PBS without rifampicin (figs 4 and 5). This suggests that mechanism B has a more significant role in the bactericidal activity of ofloxacin than in the activity of ciprofloxacin.

Discussion

All of the 4-quinolones investigated showed a biphasic effect against *S. marcescens* 120 in nutrient broth. The conditions required for the lethal activity of ciprofloxacin, ofloxacin, norfloxacin and nalidixic acid against *S. marcescens* were identical to those required by these drugs against *E. coli*.^{2,3,8} Nalidixic acid exhibited mechanism A only; therefore, RNA synthesis and bacterial cell division are essential for this drug to be bactericidal against *S. marcescens* as well as against *E. coli*. Norfloxacin exerted mechanism C in addition to mechanism A

Fig. 5. Survival of *S. marcescens* in PBS at 37°C alone control, ○—○) or with ciprofloxacin 1.5 mg/L (●—●) or ofloxacin 9 mg/L (□—□).

and was able to kill non-dividing *S. marcescens* although RNA synthesis was a pre-requisite for its lethal activity against this species. However, RNA synthesis was not required for the bactericidal activities of ciprofloxacin and ofloxacin against *S. marcescens*; therefore, these two 4-quinolones possessed mechanism B as well as mechanism A against this species. The mechanism B possessed by ofloxacin against *S. marcescens* was more potent than that of ciprofloxacin. This may explain why, despite the lower optimum bactericidal concentration of ciprofloxacin than of ofloxacin against *S. marcescens*, ofloxacin was more bactericidal at this concentration. In this context, it is interesting to note that ofloxacin displays mechanism B against staphylococci while ciprofloxacin does not.¹⁶ Hence the mechanism B of ofloxacin may differ from that of ciprofloxacin.

All three fluoroquinolones investigated exerted two bactericidal mechanisms against *S. marcescens*. However, although the mechanisms were the same

two as those exhibited against *E. coli*, mutational resistance to the fluoroquinolones at four times their MICs could be detected at significantly higher frequencies in *S. marcescens*. Furthermore, despite both ciprofloxacin and ofloxacin displaying similar bactericidal mechanisms against *S. marcescens*, mutants resistant to ofloxacin but not to ciprofloxacin could be detected at five times their MICs.¹² Hence the possession of a second bactericidal mechanism by a fluoroquinolone does not necessarily reduce the frequency with which bacteria can mutate to resistance. Thus the suggestion^{3,9,10} that the low frequency of resistance to fluoroquinolones, compared to the non-fluorinated quinolones, in *E. coli* may result from the possession of an additional bactericidal mechanism by the fluoroquinolones may not be correct. However, the possession of an additional bactericidal mechanism would still seem to be beneficial as the fluoroquinolones, unlike the 4-quinolones, are able to kill non-dividing bacteria.

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