Protein- and RNA-synthesis independent bactericidal activity of ciprofloxacin that involves the A subunit of DNA gyrase

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Summary. Ciprofloxacin, unlike nalidixic acid, can kill *Escherichia coli* cells in the absence of synthesis of protein or RNA. Hence, chloramphenicol or rifampicin do not abolish the bactericidal activity of ciprofloxacin against wild-type *E. coli*. Protein and RNA synthesis were not required for the bactericidal activity of ciprofloxacin against *nalB*, *nalC* and *nalD* mutants of *E. coli*. However, the addition of chloramphenicol or rifampicin abolished the bactericidal activity of ciprofloxacin against a *nalA* mutant in nutrient broth. It is concluded that the ability of ciprofloxacin to kill *E. coli* in the absence of protein or RNA synthesis involves the A subunit of DNA gyrase.

Introduction

The 4-quinolones appear to act on DNA gyrase, the bacterial enzyme that introduces negative supercoils into DNA.¹ Mutations affecting either the A or B subunits of this enzyme alter bacterial susceptibility to the 4-quinolones.² Shen *et al.*³ have proposed that, rather than acting directly on DNA gyrase, the drugs bind to saturable sites on supercoiled DNA. This binding is suggested to be facilitated by DNA gyrase.

The molecular events leading to the death of bacteria exposed to the 4-quinolones are not known. However, differences have been observed amongst these drugs in the conditions required for their lethal action. Protein and RNA synthesis are essential for the bactericidal activity of the older 4-quinolones, such as nalidixic acid.^{4,5} On the other hand, some fluoroquinolones kill bacteria in the absence of protein or RNA synthesis.^{6–9} This observation has led to the suggestion that these fluoroquinolones exert an extra bactericidal mechanism, termed B, in addition to that termed A, exhibited by nalidixic acid. Mechanism A requires protein and RNA synthesis as well as cell division^{4,5} but mechanism B, which is possessed by ciprofloxacin,⁶ DR-3355,⁷ fleroxacin,⁸ lomefloxacin,⁹ ofloxacin⁶ and pefloxacin⁸ does not require these activities, and allows the killing of non-dividing bacteria. The basis of mechanism B is unknown but has been suggested by Dalhoff¹⁰ to involve the bacterial membrane, rather than DNA gyrase.

In the present study, the bactericidal mechanisms of ciprofloxacin were determined for various mutants of *E. coli* KL16 that had altered susceptibility to 4-quinolones. The aim was to determine whether

mechanism B could be abolished by specific mutations. Four different mutations were investigated: the *nalA* (*gyrA*) mutation, which causes an alteration in the A subunit of DNA gyrase;¹¹ the *nalB* mutation, which causes a reduction in permeability;¹¹ the *nalC* mutation, which alters the B subunit of DNA gyrase;¹² and the *nalD* mutation, which appears to alter both the B subunit and permeability.¹³

Materials and methods

Bacterial strains

E. coli KL16, a prototroph,⁵ and its *nalA*,¹¹ *nalB*,¹¹ *nalC* (*nal 31*)¹² and *nalD* (*nal 24*)¹³ mutants were used. All the mutants were resistant to nalidixic acid. The *nalA*, *nalB* and *nalD* mutations increased the MIC of ciprofloxacin, whereas the *nalC* mutation reduced the MIC of the drug (table). The strains were kept on nutrient-agar plates, which were subcultured every 10 days. Colonies taken from these plates were used to seed the overnight cultures for the experiments described below.

Table. MICs of ciprofloxacin for *E. coli* strain KL16 and mutants

<i>E. coli</i> strain or mutant	MIC (mg/L)
KL16	0.0075
<i>nalA</i>	0.05
<i>nalB</i>	0.01
<i>nalC</i>	<0.002
<i>nalD</i>	0.02

Fig. 1. (a) Killing of *E. coli* KL16 $\bullet$ — $\bullet$, *nalC* $\blacksquare$ — $\blacksquare$ and *nalD* $\blacktriangle$ — $\blacktriangle$ during exposure to various concentrations of ciprofloxacin in nutrient broth for 3 h at 37°C. (b) Killing of *E. coli* *nalA* $\blacksquare$ — $\blacksquare$ and *nalB* $\blacktriangle$ — $\blacktriangle$ during exposure to various concentrations of ciprofloxacin in nutrient broth for 3 h at 37°C.

Antibacterial preparation

Ciprofloxacin (Bayer, UK) and chloramphenicol (Parke-Davis, UK) were dissolved in sterile distilled water. Ofloxacin (Hoechst UK) was dissolved in 0.5 M NaOH (0.02 ml/mg) before dilution in sterile distilled water. Rifampicin (Merrell Dow, UK) was dissolved in dimethylsulphoxide.

Antibacterial activity of the 4-quinolones

The bactericidal activity of ciprofloxacin (0.15–

150 mg/L) alone, or in combination with chloramphenicol, was determined in nutrient broth at 37°C after a 3-h exposure period. Bacteria were enumerated by serial dilution in nutrient broth followed by viable counting on nutrient agar, as described by Lewin *et al.*¹⁴

The rate of kill of bacteria treated with ciprofloxacin and ofloxacin alone, or in combination with rifampicin, in nutrient broth was derived from sequential viable counts determined at 30-min intervals over a 4-h period, as described by Lewin and Smith.¹⁵

Fig. 2. Killing of *E. coli* KL16 $\bullet$ — $\bullet$, *nalA* $\blacksquare$ — $\blacksquare$, *nalB* $\square$ — $\square$, *nalC* $\times$ — $\times$ and *nalD* $\circ$ — $\circ$ during exposure to various concentrations of ciprofloxacin in the presence of chloramphenicol 20 mg/L in nutrient broth for 3 h at 37°C.

Results

Ciprofloxacin, at concentrations above the MIC, was bactericidal against the *nalA*, *nalB*, *nalC* and *nalD* mutants (figs. 1a and b). Addition of a bacteriostatic concentration of the protein-synthesis inhibitor chloramphenicol 20 mg/L reduced, but did not completely abolish, this activity against *E. coli* KL16, and the *nalB*, *nalC* and *nalD* mutants (fig. 2). On the other hand, chloramphenicol 20 mg/L completely abolished the bactericidal activity of ciprofloxacin against the *nalA* mutant at concentrations up to 150 mg/L (fig. 2). Thus, ciprofloxacin was not bactericidal against the *nalA* mutant in the absence of protein synthesis.

The bactericidal activity of ciprofloxacin at its optimum bactericidal concentration (OBC, defined as the drug concentration giving the most rapid killing of the bacteria) was examined alone and in the presence of rifampicin, an inhibitor of RNA synthesis. At its OBC, ciprofloxacin was bactericidal against the parent *E. coli* strains and the mutants (figs. 3–4). Addition of a bacteriostatic concentration of rifampicin (160 mg/L) reduced, but failed to abolish, the bactericidal activity of ciprofloxacin against the *nalB*, *nalC* and *nalD* mutants, confirming that the 4-quinolone continued to exhibit mechanism B against these organisms (fig. 4 and data not shown). However, addition of a bacteriostatic concentration of rifampicin completely abolished the bactericidal activity of ciprofloxacin against the *nalA* mutant (fig. 3).

The bactericidal activity of ofloxacin, which also exerts mechanism B, was investigated against the four mutants. Rifampicin abolished the bactericidal activity of ofloxacin at its OBC against the *nalA* mutant but

Fig. 3. Killing of *E. coli* KL16 during exposure to ciprofloxacin 0.15 mg/L alone □—□ and plus rifampicin 160 mg/L ●—● in nutrient broth at 37°C, and of *E. coli nalA* during exposure to ciprofloxacin 0.9 mg/L alone ■—■ and plus rifampicin 160 mg/L ▲—▲ in nutrient broth at 37°C.

Fig. 4. Killing of *E. coli* KL16 during exposure to ciprofloxacin 0.15 mg/L alone □—□ and plus rifampicin 160 mg/L ●—● and of the *nalB* mutant by ciprofloxacin 0.15 mg/L alone ■—■ and plus rifampicin 160 mg/L ▲—▲. All experiments were performed at 37°C in nutrient broth.

not against the *nalB*, *nalC* or *nalD* mutants. Hence, as with ciprofloxacin, ofloxacin could not kill the *nalA* mutant in the absence of RNA synthesis.

Discussion

Protein and RNA synthesis were found to be prerequisites for the mechanism B-dependent bactericidal activity of ciprofloxacin or ofloxacin against a *nalA* *E. coli* mutant. Mutations which affected the outer membrane (*nalB*) or the B subunit of DNA gyrase (*nalC* and *nalD*) and which altered bacterial sensitivity to the fluoroquinolones had no effect on the occurrence of mechanism B. Thus, the fluoroquinolones were bactericidal against *nalB*, *nalC* and *nalD* mutants, even when protein or RNA synthesis were inhibited. These data suggest that mechanism B involves the A subunit of DNA gyrase rather than the bacterial membrane, as has been suggested previously.¹⁰

It has been suggested¹⁶ that the possession of mechanism B explains why the frequencies of bacterial mutation to resistance to the fluorinated quinolones is much lower than to the non-fluorinated drugs such as nalidixic and oxolinic acids.^{2,16–19} It was proposed¹⁶ that two distinct mutations might be required to confer resistance to the fluoroquinolones whereas a single mutation would suffice for the older non-fluorinated drugs. However, the present data indicate that the *nalA* mutation abolishes mechanism B. In this context, it is interesting to note that although ofloxacin and ciprofloxacin both exhibit mechanisms A and B against *S. marcescens*,²⁰ the frequency of mutation to resistance to the latter drug was higher than to the

former.^{21,22} Hence, a low frequency of mutation resistance does not appear to be an inevitable correlate of possession of mechanism B by a drug. However, the possession of this additional bactericidal mechanism by the fluoroquinolones should still be advantageous as it enables these drugs to kill non-dividing bacteria.⁶⁻⁹

Finally, we have recently reported that, in the absence of protein synthesis, ciprofloxacin is no longer able to kill *E. coli* strains that have acquired 4-quinolone resistance during therapy.²³ Thus, these isolates appear to have lost sensitivity to killing by mechanism B.²³ As with the *nalA* mutant studied here, such isolates may have an alteration in the A

subunit of DNA gyrase. The loss of sensitivity to mechanism B-dependent killing might be exploited to identify *gyrA* (*nalA*) mutants. At present, although a high MIC for fluoroquinolones strongly suggests a *gyrA* phenotype, the only method of conclusively identifying the *gyrA* gene as the site of mutation requires the purification of DNA gyrase.² This is laborious and is not yet feasible for large numbers of isolates. The present results suggest that testing 4-quinolone-resistant isolates for loss of mechanism B may provide a rapid screening method of identifying whether resistance could be due to a mutation in the *gyrA* gene. Further investigation with different strains will be required to confirm this proposal.

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