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International Journal of Pharmaceutics 227 (2001) 149–156

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Investigation of Smith's quinolone killing mechanisms during the PAE of ciprofloxacin on *Escherichia coli*

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Received 10 January 2001; received in revised form 14 March 2001; accepted 17 March 2001

Abstract

Quinolone antibacterials interact with the DNA–DNA gyrase complex, but subsequent events that lead to cell death are unresolved. Three distinct mechanisms of quinolone lethality have been identified by Smith and co-workers: Mechanism A, which requires RNA and protein synthesis and cell division for expression; Mechanism B, which remains active when these functions are precluded; and Mechanism C, which is active on non-dividing cells. Exposure to 4 × MIC ciprofloxacin (Cip) in nutrient broth (NB) for 3 h reduced the viability of *Escherichia coli* AB1157 to 0.25%. Addition of rifampicin (Rif) or chloramphenicol (Cm), to inhibit RNA or protein synthesis, respectively, increased survival 70-fold. Treatment of cells with Cip in phosphate-buffered saline (PBS), to inhibit cell division, increased survival 20-fold. No further cell death occurred once the various drug combinations or PBS had been washed out and cells resuspended in drug-free nutrient broth. These latter conditions allow expression of the post-antibiotic effect (PAE). PAE was lengthened in cells exposed to Cip in the presence of Rif or Cm, probably as a result of delay in the initiation of inducible DNA repair.

Keywords: Antibacterials; Quinolones; Post antibiotic effect; Ciprofloxacin; Mechanisms of action

1. Introduction

Quinolone antibacterial agents bind to the bacterial DNA gyrase–DNA complex, arresting enzyme activity and causing rapid lethality (Shen et al., 1989a,b,c). However, the chain of events that leads to cell death remains a focus of debate. Induction of error-prone ('SOS') DNA repair is a characteristic response to quinolone treatment (Phillips et al., 1987; Piddock et al., 1990; Piddock and Walters 1992), but whether SOS processing of quinolone-induced lesions is beneficial to the cell or contributes to lethality remains unclear. Protein synthesis and active cell division are prerequisites for at least part of the lethal effect (Smith, 1984; Ratcliffe and Smith, 1985; Lewin and Smith 1986), conditions which Howard et al. (1993a) have argued reflect a requirement for the induction of SOS activity in the bactericidal response. Noting that inhibition of protein synthesis or cell division affected quinolone activity in a

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0378-5173/01/$ - see front matter
PII: S0378-5173(01)00793-1

predictable way, Smith proposed three mechanisms for quinolone killing: Mechanism A requires both RNA and protein synthesis, and is only active on dividing cells, Mechanism B kills in the absence of RNA or protein synthesis, or of cell division, and Mechanism C functions when RNA and protein synthesis are occurring but cell division is inhibited (Smith 1984; Ratcliffe and Smith, 1985; Lewin and Smith, 1986; Howard et al., 1993a).

In this study, selective expression of Smith's lethality mechanisms was achieved by manipulating the conditions under which *Escherichia coli* was exposed to ciprofloxacin. The quinolone-damaged cells were then removed to a drug-free growth medium and their recovery and re-growth studied during, and after, the period of post antibiotic effect (PAE). PAE is a widely demonstrated phenomenon (for reviews, see Craig and Gudmundsson, 1991; MacKenzie and Gould, 1993), whereby bacteria previously treated with an antibacterial undergo a period of growth inhibition following removal of the antibacterial. The clinical value of PAEs lies in their potential to lengthen dosage intervals, but they are also significant to the investigation of antimicrobial lethality mechanisms: the PAE period may be viewed as an expression of the time required for repair before cell division can recommence.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli AB1157 *thr-1 leuB6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 hisG4 argE3 rpsL31 tsx-33 supE44* λ^- F^- (Howard-Flanders et al., 1964) was used throughout. The strain is wild type with respect to ciprofloxacin sensitivity.

2.2. Media and antimicrobials

Cultures were grown overnight at 37 °C in Oxoid No. 2 nutrient broth (NB) (Unipath Ltd, Basingstoke, UK), which was solidified with 1.5% Lab M agar (Lab M, Bury, UK) to make nutrient agar (NA). Also 0.5 M phosphate-buffer was produced by the addition of 80 ml 0.5 M disodium hydrogen orthophosphate dodecahydrate solution to 20 ml 0.5 M sodium hydrogen orthophosphate solution, and the resultant solution adjusted to pH 7.4 by the dropwise addition of 0.5 M sodium hydroxide. The 0.5 M phosphate buffer was diluted to 0.025 M and 0.9 g sodium chloride added per 100 ml to give phosphate-buffered saline (PBS). Ciprofloxacin (Cip) was generously donated by Bayer (Newbury, UK). Rifampicin (Rif) (Merrell Dow, Uxbridge, UK) and chloramphenicol (Cm) (Sigma, Poole, UK) were purchased from their respective suppliers. Antimicrobial solutions were freshly prepared in sterile distilled water. Rifampicin was initially dissolved at 0.02 ml/mg in dimethyl sulphoxide, before further dilution in sterile distilled water.

2.3. Drug exposure

Overnight cultures, grown in NB, were diluted 1 in 50 into 9.8 ml NB containing the required concentrations of antimicrobial agent, or into drug-free broth as control. Alternatively, overnight NB-grown cultures were washed in PBS and diluted into PBS, with or without drug as required. All incubation and exposure was at 37 °C. Mixtures were sampled immediately, diluted in NB, and 20 μl aliquots plated in triplicate on NA to obtain viable counts. Drug exposure was then continued for 180 min, after which a further viable count was performed and the drug solution removed by filtration.

2.4. Filtration and recovery

After 180 min incubation, each drug-exposed or control culture was filtered through a 0.45 μm Millipore HA filter disc (25 mm diameter) (Millipore, Watford, UK) and the filter washed through with two 10 ml portions of warm NB. The filter disc was then placed in 10 ml warm NB and vortexed for 1 min to resuspend the cells, before removal of the filter disc using aseptic technique. A viable count was performed immediately on re-suspension in drug-free broth, and at hourly intervals during incubation at 37 °C. The viable counts of control cultures increased to ap-

proximately 5×10^8 cfu/ml during the 180 min period in drug-free NB. They were, therefore, diluted 1 in 1000 after filtration to reduce their counts to approximate those of drug-exposed cultures.

2.5. Estimation of post-antibiotic effect

Plots of log viable count against time were used to determine PAE as described by Craig and Gudmundsson (1991), using the formula $PAE = T - C$, where T is the time taken for the viable count of a drug-exposed culture to increase by one log cycle, and C is the equivalent period for the untreated control.

3. Results

3.1. Lethality

The minimum inhibitory concentration (MIC) of ciprofloxacin against *E. coli* AB1157 was found to be 0.005 μg/ml. PAEs were determined after cells had been exposed to 4 × this MIC for 3 h. Cells were also treated with ciprofloxacin in the presence of 20 μg/ml chloramphenicol, to inhibit protein synthesis, or 160 μg/ml rifampicin, to inhibit RNA synthesis. Other cultures were exposed to ciprofloxacin in PBS to inhibit cell division. Chloramphenicol at 20 μg/ml was bacteriostatic, with rifampicin at 160 μg/ml reducing viability by about 30%. Cell viability was unaltered after suspension in PBS for 3 h (Table 1). These conditions are as used by Smith et al. (Smith, 1984; Ratcliffe and Smith, 1985; Lewin and Smith, 1986; Howard et al., 1993b) in their experiments on quinolone mechanisms A, B and C. Survival levels after 3 h exposure to the various combinations of drugs are shown in Table 1. As expected, ciprofloxacin in NB gave the highest level of kill, reducing viability by over 99%. Addition of either chloramphenicol or rifampicin, which abolish quinolone lethality Mechanisms A and C, increased survival by an average of 70-fold. Treatment in PBS, which abolishes Mechanism A only, increased survival by 20-fold (Table 1).

Table 1
Survival levels, as percentages of initial viable counts, of *E. coli* AB1157 exposed to ciprofloxacin under conditions allowing, or inhibiting, quinolone killing Mechanisms A, B or C

Exposure conditions	Percentage survival after 3 h exposure at 37 °C	
	Control, no Cip	Plus Cip 0.02 μg/ml
NB	1200	0.25
Cm 20 μg/ml in NB	100	20
Rif 160 μg/ml in NB	72	17
PBS	98	5.6

3.2. Viable counts after drug removal

Bacterial viability after drug removal is illustrated in Figs. 1 and 2, where zero on the time axes represents the point at which cells were resuspended in drug-free nutrient broth. Recovery

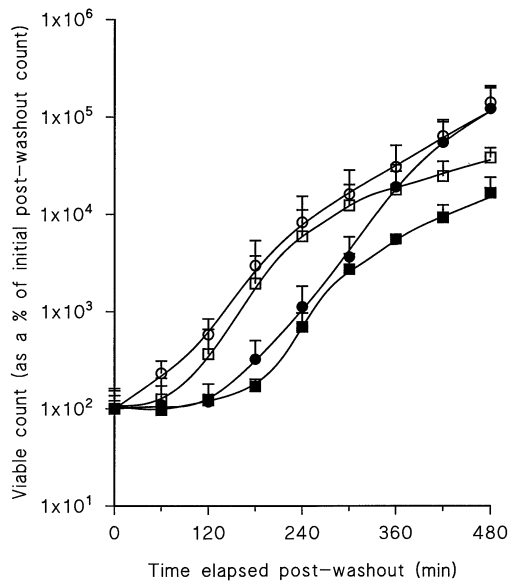

Fig. 1. Viability after drug washout of *Escherichia coli* AB1157 previously exposed to 4 × MIC ciprofloxacin for 3 h in NB (●), or in PBS (■), and of control cultures previously incubated for 3 h in drug-free NB (○) or drug-free PBS (□). Zero on the *X* axis represents the point at which cells were re-suspended in drug-free NB. Results are the means of three experiments conducted on separate days.

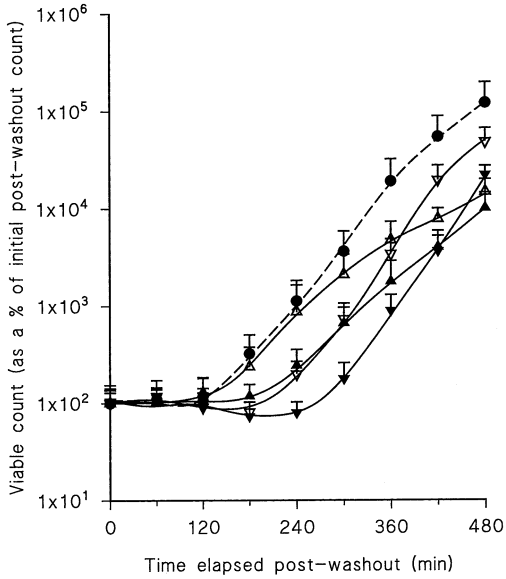

Fig. 2. Recovery of *E. coli* AB1157 previously exposed to 4 × MIC ciprofloxacin for 3 h in NB (●) and in NB containing either 20 μg/ml chloramphenicol (▲) or 160 μg/ml rifampicin (▼). Recovery of control cultures incubated in NB containing either 20 μg/ml chloramphenicol (△) or 160 μg/ml rifampicin (▽) is also shown. Other conditions are as in Fig. 1.

of the control cultures previously suspended for 3 h in drug-free PBS closely followed that of the NB-incubated controls (Fig. 1), with the viable counts of the PBS controls taking an average of 9 min longer to increase 10-fold, compared with the broth-grown cells. Siegele and Guynn (1996) have argued that such a delay might reflect the time taken for cells to revert from a less active starvation state to normal metabolism. However, the difference shown in Fig. 1 is statistically insignificant ($P = < 0.05$).

Cells previously exposed to ciprofloxacin for 3 h in NB gave an average PAE of 96 min (Fig. 1, Table 2). The average PAEs for cells exposed to ciprofloxacin in PBS were calculated to be 113 min, when data from NB-grown cultures were used as control, or 104 min with reference to PBS-held control cultures (Fig. 1, Table 2). This latter value was little different from the length of PAE produced by ciprofloxacin in NB (Table 2).

Fig. 2 shows the re-growth curves for cultures exposed in NB to chloramphenicol or rifampicin, in the presence or absence of 4 × MIC ciprofloxacin. Re-growth of the chloramphenicol-exposed culture was delayed relative to the NB control culture, with the average PAE of chloramphenicol calculated to be 100 min (Table 2). The PAE generated when cells were exposed to chloramphenicol and ciprofloxacin in combination was 183 min, roughly the sum of the individual PAEs (Fig. 2, Table 2). Cell division in the rifampicin-exposed culture was inhibited during the first 4 h following drug washout; exponential growth then commenced, giving a calculated average PAE of 166 min. This was extended to 224 min on the addition of ciprofloxacin, again giving a co-exposure PAE that approximated to the sum of the PAEs of the individual drugs (Fig. 2, Table 2).

It is apparent from Figs. 1 and 2 that no further cell death occurred after any drug treatment, once the bacteria had been re-suspended in drug-free broth. Damage sustained during ciprofloxacin treatment did not, therefore, appear to exert a lethal effect once the drug had been removed.

3.3. Effects of ciprofloxacin concentration on length of PAE

The bactericidal activity of a quinolone increases with concentration up to its maximal bactericidal concentration, above which activity declines (Crumplin and Smith, 1975; Smith, 1984). This phenomenon, known as the paradoxical re-

Table 2
Effects of chloramphenicol, rifampicin and PBS on PAE values after 3 h exposure to 4 × MIC ciprofloxacin

Exposure conditions	PAE value (min)	PAE value when 4 × MIC Cip added (min)
NB	*147 − 147* = 0	*147 − 147* = 96
NB + Cm	*247 − 147* = 100	*147 − 147* = 183
NB + Rif	*313 − 147* = 166	*147 − 147* = 224
PBS	*156 − 147* = 9	*147 − 147* = 113
	156 − 156[a] = 0	*147 − 156*[a] = 104

Values in italics are those used in the calculation of PAEs ($T - C$ = PAE, see Section 2). All data are averages from a minimum of three experiments, conducted on separate days.

[a] PAEs calculated using data from drug-free PBS controls.

Table 3
Survival of, and PAE values for, *E. coil* AB1157 following 3 h exposure to 0.02 μg/ml (4 × MIC), 0.2 μg/ml (maximal bactericidal concentration) or 10 μg/ml ciprofloxacin

Exposure conditions	Survival after 3 h exposure (%)	Calculated PAE (min)
NB	1200	0
NB + 0.02 μg/ml Cip	0.25	96
NB + 0.2 μg/ml Cip	0.07	216
NB + 10 μg/ml Cip	0.7	>330

sponse, has been attributed to a quinolone-induced secondary inhibition of protein or RNA synthesis, which becomes apparent at high quinolone concentrations (Crumplin and Smith, 1975; Smith, 1984). If this is indeed the explanation, then the PAEs resulting from exposure to high ciprofloxacin concentrations might be expected to mimic those seen after treatment with the quinolone in the presence of chloramphenicol or rifampicin.

The maximal bactericidal concentration of ciprofloxacin against *E. coli* AB1157 was determined to be between 0.05 and 0.5 μg/ml (data not shown), which is in keeping with estimates that such concentrations for quinolones are usually 30–60 times their MICs (Wolfson and Hooper, 1990; Howard et al., 1993b). Three concentrations of ciprofloxacin were, therefore, selected for comparison: 0.02 μg/ml (4 × MIC), 0.2 μg/ml, which approximates to the maximal bactericidal concentration found by Howard et al. (1993b) and is in agreement with the estimate of Wolfson and Hooper (1990), and 10 μg/ml, a concentration above the maximal bactericidal concentration giving post-exposure survival similar to that achieved with 0.02 μg/ml (Table 3).

As predicted, both 0.02 and 10 μg/ml ciprofloxacin were less bactericidal than the maximal bactericidal concentration of 0.2 μg/ml (Table 3). PAEs of 98 and 205 min were calculated following treatment with 0.02 and 0.2 μg/ml, respectively (Fig. 3, Table 3), whilst cells previously exposed to 10 μg/ml ciprofloxacin re-grew so slowly that a PAE could not be determined from viable counts obtained during an 8 h sampling period post-washout: after 8 h the viable count had doubled, but the growth rate remained very slow (Fig. 3).

4. Discussion

One objective in carrying out this series of experiments was to investigate the action of inducible SOS DNA repair in quinolone-damaged cells. The presence of chloramphenicol or rifampicin in combination with ciprofloxacin inhibits SOS induction (Piddock et al., 1990), and it was of interest to determine whether cell death continued once these drug combinations had been washed from the cultures. This was found not to be the case (Fig. 2), which suggests the SOS process is fulfilling its repair function, rather than contributing to lethality as previously suggested (Phillips et al., 1987; Howard et al., 1993a).

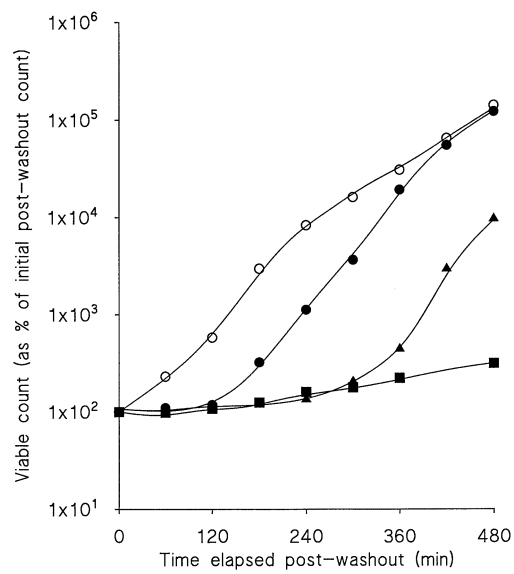

Fig. 3. The lengthening of PAE produced by increasing ciprofloxacin concentration. Cells were exposed for 3 h to ciprofloxacin in NB at 0.02 μg/ml (●), 0.2 μg/ml (▲) or 10 μg/ml (■) before re-suspension in drug-free broth. A control culture (○) was incubated in drug-free NB for 3 h. Other conditions were as in Fig. 1. Reasons for the choice of concentrations are discussed in the text.

The killing activities of some antibacterial combinations may be predicted from whether the PAEs of the individual drugs are additive, synergic or indifferent (Fuursted, 1988; Gudmundsson et al., 1991). This was clearly not the case when ciprofloxacin was combined with chloramphenicol or rifampicin. Even though dramatic reductions in bactericidal activity were observed (Table 1), the PAEs of the drug combinations were additive (Table 2). A similar lack of correlation was found at high drug concentrations: 10 μg/ml ciprofloxacin was less bactericidal than 0.2 μg/ml, but gave a PAE too long to be determined accurately. Such findings are of relevance when drug combinations are used clinically, as an increase in PAE may be beneficial therapeutically, even though it is achieved at the expense of a reduction in kill. The data in Table 2 differ from those reported from similar experiments with *Pseudomonas aeruginosa*, where a combination of ciprofloxacin with rifampicin gave a PAE no longer than that of ciprofloxacin alone (Gould et al., 1989; Gudmundsson et al. 1991). However, rifampicin has been shown to prolong the PAEs of some other drugs without enhancing their killing effect (Gudmundsson et al., 1991).

Exposure to ciprofloxacin in PBS, which inhibited cell division but allowed protein and RNA synthesis to continue, reduced ciprofloxacin kill (Table 1) but did not significantly alter the length of the ciprofloxacin PAE from that determined for cultures exposed to the drug in NB (Table 2, Fig. 1). This was in contrast to the effects of chloramphenicol and rifampicin, which were found both to reduce the killing affect of ciprofloxacin in NB and to extend its PAE (Table 1, Fig. 2).

The additive nature of the ciprofloxacin–chloramphenicol and ciprofloxacin–rifampicin PAEs (Table 2, Fig. 2) suggest that the lengths of such PAEs are determined by a two-phase recovery process, during which dissociation of the inhibitory drug (chloramphenicol or rifampicin) is a precursor to repair of ciprofloxacin-induced damage. The PAE would, therefore, represent the time taken for chloramphenicol or rifampicin to dissociate from its target site, permitting the induction of DNA repair, plus the time taken for this repair of the quinolone-induced lesions before cell division resumed. The similarity of the PAEs determined for cells previously exposed to ciprofloxacin in broth or in PBS (Table 2) implies that the time taken to repair quinolone-induced lesions are similar under the two sets of conditions. In both cases, there is no additional inhibitory drug to dissociate before repair commences. Repair functions would be induced immediately ciprofloxacin was removed and the cells suspended in drug-free broth.

An increase in PAE up to the maximal bactericidal concentration of ciprofloxacin was expected: previous work has shown the length of PAE to correlate well with drug concentration (Bundtzen et al., 1981; Craig and Ebert, 1991; Minguez et al., 1991; Li et al., 1997). Since quinolones display the unusual quality of decreased activity at concentrations above the maximal bactericidal concentration (Crumplin and Smith, 1975), a superficial prediction might have been that PAEs would actually decrease at these high drug concentrations. This was not the case, with the PAE of a supra-maximal bactericidal concentration of ciprofloxacin, equivalent to 500 × MIC, being too long to calculate accurately. This elongated PAE may have resulted from intracellular persistence of the drug, but it was not reduced by a second wash of the culture before the cells were re-suspended in drug-free broth (data not shown). A more likely explanation is to be found in the origins of the paradoxical response: reduced lethality at high concentrations has been attributed to secondary quinolone-induced inhibition of protein and RNA synthesis (Crumplin and Smith, 1975). The extended PAE seen at the highest ciprofloxacin concentration is, therefore, a representation of the dual-phase recovery process, which results from inhibition of protein or RNA synthesis during quinolone exposure.

In the presence of chloramphenicol or rifampicin, quinolone lethality is limited to Mechanism B, whereas when cells are treated in PBS instead of NB, both Mechanisms B and C are active (Smith, 1984; Lewin and Smith, 1986). This explains why ciprofloxacin lethality is reduced 100-fold under the former conditions, but by only 25-fold under the latter (Table 1). Accepting that

the PAEs produced by two drugs in combination are approximately equal to the sums of their individual PAEs (Table 2), the contribution of the damage-repair stage to total PAE length is approximately constant, at around 90 min. It thus appears that ciprofloxacin engenders the same PAE whatever the exposure conditions and whichever of its three Mechanisms are active. This implies that the length of PAE caused by the protein synthesis-independent, cell division-independent Mechanism B approximates to that of all three Mechanisms acting in concert: perhaps the damage caused by Mechanism B is repairable, whilst that caused by Mechanisms A or C is invariably lethal. Alternatively, expression of Mechanism B may overwhelm the effects of the other Mechanisms.

Acknowledgements

The authors would like to thank Professor J.M. Newton for his interest and support. This work was partially funded by the award of a University of London post-graduate studentship to Hayley J. Wickens. We are also grateful to the Newby Trust and to The School of Pharmacy for support.

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