J. Pharm. Pharmacol. 1996, 48: 985–987
Received November 21, 1995
Accepted March 22, 1996

© 1996 J. Pharm. Pharmacol.

Transient Relaxation of Plasmid DNA in *Escherichia coli* by Fluoroquinolones

MIKI MATSUO, YOSHIHISA OHTSUKA, KAZUHIRO KATAOKA, TOHRU MIZUSHIMA AND KAZUHISA SEKIMIZU

Department of Microbiology, Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-82, Japan

Abstract

We examined the influence of fluoroquinolones (norfloxacin, enoxacin, ofloxacin, levofloxacin, and sparfloxacin) on DNA supercoiling of plasmids in *Escherichia coli* cells by analysis with agarose gel electrophoresis in the presence of chloroquine.

All the fluoroquinolones tested immediately induced DNA relaxation. The relaxed DNA was re-supercoiled, and the process was sensitive to chloramphenicol, suggesting that newly synthesized proteins participate in the reaction. The concentrations of fluoroquinolones required for DNA relaxation were much higher than those required for cell killing.

The bactericidal effect of fluoroquinolones is apparently related to mechanisms other than DNA relaxation.

Fluoroquinolones have broad spectra against Gram-positive and -negative bacteria. Biochemical and genetic studies show that DNA gyrase is a target of fluoroquinolones (Cambau & Gutmann 1993; Hooper 1993; Drlica & Kreiswirth 1994; Shen 1994). However, the precise mechanism by which fluoroquinolones kill microorganisms remains to be elucidated.

Generally, chromosomal and plasmid DNA are negatively supercoiled. In *Escherichia coli* cells, DNA gyrase catalyses the formation of negative supercoiling of DNA, in an ATP-dependent manner (Gellert et al 1976). Therefore, inhibitors of DNA gyrase would be expected to relax DNA in cells. Nalidixic acid and novobiocin, specific inhibitors of DNA gyrase, relax plasmid DNA in *Escherichia coli* cells (Mizushima et al 1993). Some fluoroquinolones also relax plasmid DNA in *Escherichia coli* cells (Bliska & Cozzarelli 1987; Aleixandre et al 1991).

We have systematically examined the influence of various fluoroquinolones, which induce transient DNA relaxation, on DNA supercoiling in *Escherichia coli* cells.

Materials and Methods

Materials

Norfloxacin was provided by Kyorin Pharm. Co. Enoxacin and sparfloxacin were provided by Dainippon Pharm. Co. Ofloxacin and levofloxacin were provided by Daiichi Pharm. Co.

Bacterial strains

The bacterial strains used were derivatives of *Escherichia coli* K-12 (Bachmann 1972). W3110 was from our laboratory stock.

Analysis of the supercoiling of plasmid DNA in cells

We used methods described elsewhere for the analyses of the supercoiling of plasmid DNA (Pruss 1985; Mizushima et al 1992, 1993; Ogata et al 1994). Briefly, exponentially growing (37°C) *Escherichia coli* cells (W3110 or *nal*A26 mutant) harbouring pUC118 in LB medium containing 1% NaCl were further incubated with quinolones in the same medium. Cultures were chilled on ice and then cells were harvested by centrifugation at 4°C for 5 min followed by washing with 0·9% NaCl (saline). Plasmid DNA was extracted and purified by the alkaline method (Sambrook et al 1989). The samples were analysed by agarose gel electrophoresis in the presence of chloroquine. Electrophoresis was carried out in 100 mM Tris-borate, 2 mM EDTA buffer (Maniatis et al 1989) at 60 V for 16 h. Gels were rinsed in deionized water for 5 h to remove chloroquine and DNA was stained with 1 μg mL^{-1} ethidium bromide. DNA bands were visualized by UV and photographed.

P1 transduction

H1515 (*nal*A26) (Ikeda et al 1981) was kindly provided by Dr H. Ikeda (University of Tokyo). P1 transduction of *nal*A26 mutation in H1515 to W3110 was as described previously (Mizushima et al 1994; Shinpuku et al 1995). P1 phages were grown in the H1515 cells, transduced into W3110 cells and the transductants were selected on LB agar plates containing 25 μg mL^{-1} tetracycline. Sensitivity of transductant to nalidixic acid (25 μg mL^{-1}) was examined to select the *nal*A26 mutant with the genetic background of W3110. We did not use plates containing both tetracycline and nalidixic acid to avoid appearance of spontaneous mutation resistant to nalidixic acid.

Determination of IC50 values for fluoroquinolones

Exponentially growing *Escherichia coli* cells (optical density at 650 nm = 0·5) harbouring pUC118 in LB medium containing 1% NaCl were incubated with various concentrations of fluoroquinolones in the same medium for 1 h at 37°C. The cultures were appropriately diluted and spread on LB agar plates and incubated at 37°C for 12 h. The numbers did not increase when the plates were further incubated at 37°C for 12 h. The numbers of colonies were counted and concentrations of drugs necessary for 50% inhibition of colony formation were determined.

Correspondence: K. Sekimizu, Department of Microbiology, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-82, Japan.

Results and Discussion

DNA relaxation by fluoroquinolones in Escherichia coli cells

We first determined concentrations of fluoroquinolones necessary for 50% inhibition of cell viability (IC50), under the same conditions as those where the extent of DNA supercoiling was examined in the following sections. IC50 values of enoxacin, norfloxacin, ofloxacin, levofloxacin, and sparfloxacin were 0·2, 0·06, 0·06, 0·04 and 0·02, respectively, consistent with literature values.

DNA supercoiling in cells treated with fluoroquinolones was then analysed. pUC118 was used as a reporter for analysis of the DNA supercoiling. The plasmids were extracted using alkaline methods (Maniatis et al 1989) and the extent of supercoiling was analysed with agarose gel electrophoresis in the presence of chloroquine. When we examined the effects of the fluoroquinolones on DNA supercoiling at concentrations much the same as the IC50, no significant alteration was seen by treatment with the drugs (data not shown). Next, we examined the effects of fluoroquinolones on DNA supercoiling at concentrations greater than the IC50 value. Fig. 1 shows that sparfloxacin altered the extent of DNA supercoiling, in a dose-dependent manner. In the agarose gel containing 20 μg mL^{-1} chloroquine, the more relaxed molecules migrate faster (Higgins et al 1988). Therefore, sparfloxacin relaxes the plasmids in cells. The concentration of sparfloxacin required to detect DNA relaxation was 0·8 μg mL^{-1}, that is 40 times the IC50 value.

We recently found that relaxation of plasmid DNA by nalidixic acid is transient; the extent of DNA supercoiling reverts to the original level within 60 min, after the addition of nalidixic acid (Ohtsuka et al unpublished results). In this study, we examined the time course of DNA relaxation reaction caused by fluoroquinolones. As shown in Fig. 1, DNA relaxed 10 min after exposure to the drug and re-supercoiled 60 min later. Thus, the DNA relaxation caused by sparfloxacin was transient. We also examined the extent of DNA supercoiling by other fluoroquinolones, enoxacin, norfloxacin, ofloxacin, and levofloxacin, at higher concentrations than the IC50 of each (Fig. 2). All these fluoroquinolones transiently relaxed DNA in cells as did sparfloxacin. The concentrations of enoxacin, norfloxacin, ofloxacin, and levofloxacin required for DNA relaxation in cells were 1·6, 0·6, 0·6 and 0·4 μg mL^{-1}, respectively and these values are much higher than those required for bactericidal effects. Thus, the bactericidal actions of fluoroquinolones do not seem to be related to the DNA relaxation caused by these drugs.

Influence of fluoroquinolones on DNA supercoiling in a nalA26 mutant

Some mutations in the *gyrA* gene, including the *nal*A26 mutation, cause a phenotype resistant to fluoroquinolones (Cambau & Gutmann 1993). This means that DNA gyrase is a target molecule of fluoroquinolones for their bactericidal actions. The IC50 value for sparfloxacin was determined to be 0·92 μg mL^{-1} for the *nal*A26 mutant, being 45 times that for the wild type cells. We examined the influence of sparfloxacin on DNA supercoiling in the *nal*A26 mutant (Fig. 3). The concentration of sparfloxacin required to relax DNA in the *nal*A26 mutant was 72 μg mL^{-1}, that is 90 times that required for the wild type cells. Similar results were obtained with other

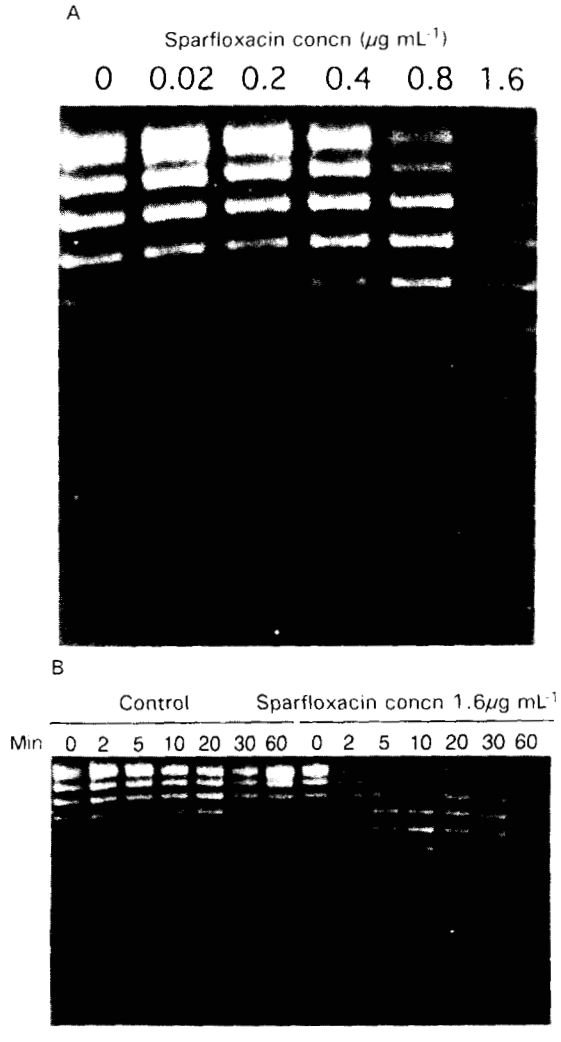

FIG. 1. DNA relaxation by sparfloxacin. Exponentially growing cells harboring pUC118 at 37°C were incubated with indicated concentrations of sparfloxacin for 10 min (A) or with 1·6 μg mL^{-1} sparfloxacin for the indicated period (B). pUC118 was extracted and analysed by 1% agarose gel electrophoresis in the presence of 20 μg mL^{-1} chloroquine.

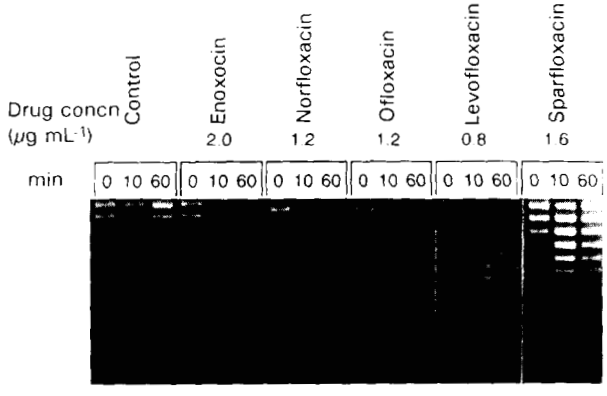

FIG. 2. DNA relaxation and re-supercoiling reactions with various fluoroquinolones. Exponentially growing cells harboring pUC118 were incubated with enoxacin, norfloxacin, ofloxacin, levofloxacin and sparfloxacin for 10 or 60 min. pUC118 was extracted and analysed by 1% agarose gel electrophoresis in the presence of 15 μg mL^{-1} chloroquine.

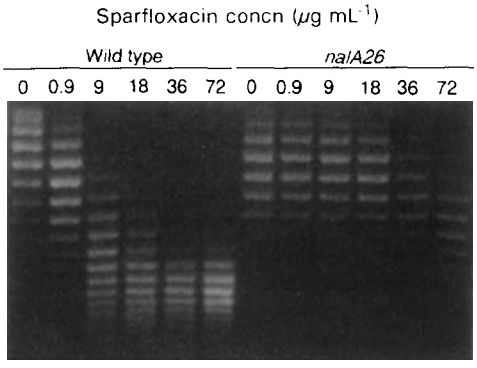

FIG. 3. Influence of the *nal*A26 mutation on DNA relaxation by sparfloxacin. Exponentially growing *nal*A26 mutants harboring pUC 118 were incubated with indicated concentrations of sparfloxacin for 10 min. pUC118 was extracted and analysed by 1% agarose gel electrophoresis in the presence of 20 μg mL^{-1} chloroquine.

fluoroquinolones, enoxacin, norfloxacin, ofloxacin, and levofloxacin (data not shown). These data strongly suggest that fluoroquinolones relax DNA through inhibition of the supercoiling activity of DNA gyrase.

Requirement of protein synthesis for re-supercoiling reaction in the presence of fluoroquinolones

We examined the influence of an inhibitor of protein synthesis on relaxation and re-supercoiling of DNA in cells after exposure to fluoroquinolones. Cells were preincubated with 100 μg mL^{-1} chloramphenicol for 10 min and then further incubated with sparfloxacin. As shown in Fig. 4, preincubation with chloramphenicol caused an excessive and continuous DNA relaxation by sparfloxacin; the re-supercoiling reaction of relaxed DNA was completely inhibited by the chloramphenicol treatment. Chloramphenicol itself did not alter the extent of DNA supercoiling. Similar results were obtained with other fluoroquinolones (data not shown). These results suggest that newly synthesized proteins after exposure to fluoroquinolones are required for the re-supercoiling reaction but not for the relaxation reaction with fluoroquinolones. The increased expression of *gyr*A and *gyr*B genes, encoding A and B subunits of DNA gyrase, by relaxation of DNA has been reported (Menzel & Gellert 1983). Thus, the overproduction of DNA gyrase that is sensitive to chloramphenicol, may account for the re-supercoiling reaction in the presence of fluoroquinolones.

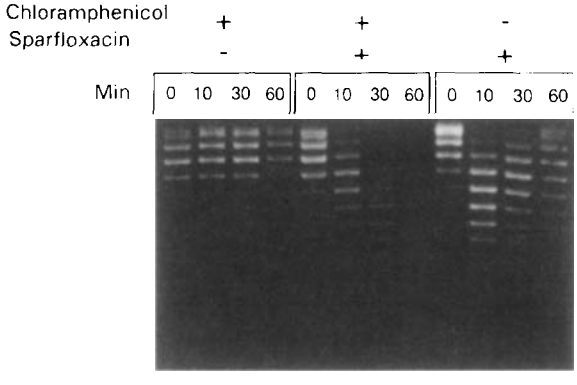

FIG. 4. Influence of chloramphenicol on the topological change of DNA after addition of sparfloxacin. Exponentially growing cells harbouring pUC118 were preincubated with or without 100 μg mL^{-1} chloramphenicol for 10 min, followed by incubation with or without 1·6 μg mL^{-1} sparfloxacin for the indicated period. pUC118 was extracted and analysed by 1% agarose gel electrophoresis in the presence of 20 μg mL^{-1} chloroquine.

Acknowledgments

We thank Drs S. Higuchi (Kyushu University) and K. Ohtsubo (Tottori University) for helpful discussion and M. Ohara for comments on the manuscript. This work was supported in part by Grants in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

References

Aleixandre, V., Herrera, G., Urios, A., Blanco, M. (1991) Effects of ciprofloxacin on plasmid DNA supercoiling of *Escherichia coli* topoisomerase I and gyrase mutants. Antimicrob. Agents Chemother. 35: 20–23

Bachmann, B. J. (1972) Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. 36: 525–557

Bliska, J. B., Cozzarelli, N. R. (1987) Use of site-specific recombination as a probe of DNA structure and metabolism in vivo. J. Mol. Biol. 194: 205–218

Cambau, E., Gutmann, L. (1993) Mechanisms of resistance to quinolones. Drugs 45 (Suppl. 3): 15–23

Drlica, K., Kreiswirth, B. (1994) 4-Quinolones and the physiology of DNA gyrase. Adv. Pharmacol. 29A: 263–283

Gellert, M., Mizuuchi, K., O'Dea, M. H., Nash, H. A. (1976) DNA gyrase: An enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA 73: 3872–3876

Higgins, C. F., Dorman C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G., Bremer, E. (1988) A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. Cell 52: 569–584

Hooper, D. C. (1993) Quinolone mode of action—new aspects. Drugs 45 (Suppl. 3): 8–14

Ikeda, H., Moriya, K., Matsumoto, T. (1981) In vitro study of illegitimate recombination: involvement of DNA gyrase. Cold Spring Harbor Symp. Quant. Biol. 46: 399–408

Maniatis, T., Fritsch, E. F., Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Menzel, R., Gellert, M. (1983) Regulation of the genes for *E. coli* DNA gyrase: homeostatic control of DNA supercoiling. Cell 34: 105–113

Mizushima, T., Natori, S., Sekimizu, K. (1992) Inhibition of *Escherichia coli* DNA topoisomerase I activity by phospholipids. Biochem. J. 285: 503–506

Mizushima, T., Natori, S., Sekimizu, K. (1993) Relaxation of supercoiled DNA associated with induction of heat shock proteins in *Escherichia coli*. Mol. Gen. Genet. 238: 1–5

Mizushima, T., Tomura, A., Shinpuku, T., Miki, T., Sekimizu, K. (1994) Loss of flagellation in dnaa mutants of *Escherichia coli*. J. Bacteriol. 176: 5544–5546

Ogata, Y., Mizushima, T., Kataoka, K., Miki, T., Sekimizu, K. (1994) Identification of DNA topoisomerases involved in immediate and transient DNA relaxation induced by heat shock in *Escherichia coli*. Mol. Gen. Genet. 244: 451–455

Pruss, G. J. (1985) DNA topoisomerase I mutants increased heterogeneity in linking number and other replicon-dependent changes in DNA supercoiling. J. Mol. Biol. 185: 51–63

Shen, L. L. (1994) Molecular mechanisms of DNA gyrase inhibition by quinolone antibacterials. Adv. Pharmacol. 29A: 285–304

Shinpuku, T., Mizushima, T., Guo, L., Miki, T., Sekimizu, K. (1995) Phenotypes of *dna*A mutants of *Escherichia coli* sensitive to detergents and organic solvents. Biochem. Biophys. Res. Commun. 212: 84–89