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Inhibition of DNA Gyrase by Optically Active Ofloxacin

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Inhibition of DNA gyrase activity by optically active ofloxacins was studied and compared with the inhibition of norfloxacin and ciprofloxacin. The (−)-isomer of ofloxacin inhibited the supercoiling activity of gyrase from *Micrococcus luteus* more effectively than did the (+)-isomer. The 50% inhibitory concentrations of (−)-, (±)-, and (+)-ofloxacin; norfloxacin; and ciprofloxacin for gyrase from *Escherichia coli* were 0.78, 0.98, 7.24, 0.78, and 1.15 μg/ml, respectively. These values correlated well with the antibacterial activity of each compound against intact bacterial cells.

Ofloxacin (OFLX; Fig. 1) has been developed as a highly active quinolone compound against both gram-positive and gram-negative pathogens (8). Chemically, it is characterized by a tricyclic structure with a methyl group at the C-3 position in the oxazine ring, thus providing an asymmetric center at this position. We have succeeded in preparing optically active OFLX isomers by a method using high-performance liquid chromatography and found that (−)-OFLX, the configuration of which is the S-form (S. Atarashi, S. Yokohama, K. Yamazaki, K. Sakano, M. Imamura, and I. Hayakawa, submitted for publication), was 8 to 128 times more potent than (+)-OFLX against gram-positive and gram-negative bacteria (4). These results indicate that the configuration of the methyl group at the C-3 position in the oxazine ring has significant effects on the antibacterial activity of OFLX. Quinolone antibacterial agents have been shown to inhibit bacterial DNA gyrase, and their antibacterial activities seem to depend on this inhibition (2, 5, 11). It was therefore of great interest to examine whether the difference in antibacterial activity among the isomers is attributable to the degree of inhibition of the target enzyme by them.

OFLX, norfloxacin (NFLX), and ciprofloxacin (CPFX) were synthesized in our laboratory. Optically active (+)- and (−)-OFLX were prepared by use of their optically resolved synthetic intermediates (4). DNA gyrase from *Micrococcus luteus* ATCC 4698 was a commercial product (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). pBR322 DNA was prepared from *Escherichia coli* MC1061 by the methods described by Sakakibara and Tomizawa (7) and rendered in the relaxed form with topoisomerase I (Bethesda Research Laboratories) (10). DNA gyrase was extracted from a 4-liter broth culture of *E. coli* K-12 KL-16 and purified partially by a modification of the methods described by Gellert et al. (3) and Staudenbauer and Orr (10). The cells were lysed with lysozyme and Brij 58, and the nucleic acids in the lysate were removed by precipitation with streptomycin (1). The 0 to 42% (wt/vol) ammonium sulfate fraction was dialyzed against TGED buffer (50 mM Tris hydrochloride [pH 8.0], 10% [wt/vol] glycerol, 1 mM EDTA, 1 mM dithiothreitol) and loaded onto a novobiocin-Sepharose column for chromatography (10). After the column was washed with several volumes of TGED buffer, DNA gyrase was eluted with TGED buffer supplemented with 5 M urea. Active fractions were pooled and dialyzed against 2 liters of TGED buffer for 4 h, mixed with glycerol at a final concentration of 50% (vol/vol), and stored at −20°C. The inhibitory effects of the OFLX isomers and other quinolones against the DNA gyrase supercoiling activities were measured by electrophoretic assays. Samples of 30 μl of reaction mixtures supplemented with 11 μl of partially purified *E. coli* DNA gyrase (1.2 U), 0.35 μg of relaxed DNA, 1.7 mM ATP, 40 mM Tris hydrochloride (pH 8.0), 40 mM KCl, 8 mM $MgCl_2$, 1.5 mM spermidine, 4 mM dithiothreitol, 40 μg of tRNA (*E. coli* strain W; Sigma Chemical Co., St. Louis, Mo.) per ml, and several concentrations of quinolones were incubated for 2 h at 37°C and then cooled to 0°C. A total of 10 μl of sample buffer (50% glycerol, 50 mM Tris hydrochloride [pH 7.4], 10 mM EDTA) was added to the samples, and they were subjected to electrophoresis on 0.9% agarose in TAE buffer (40 mM Tris acetate, 2 mM EDTA) at a constant voltage of 40 V for 16 h at 4°C. After the agarose plate was stained with 500 ml of 0.5 μg of ethidium bromide per ml for 2 h, the amount of supercoiled DNA was measured by densitometric analysis. One unit of the gyrase is defined as the amount of activity that supercoils 0.35 μg of relaxed pBR322 DNA in 2 h at 37°C. The assay conditions with the *M. luteus* gyrase were as follows. A total of 12 μl of reaction mixture containing 0.35 μg of relaxed DNA, 1.5 mM ATP, 1.5 U DNA gyrase (20,000 U/mg; ratio of A/B subunits, 1:1), 35 mM Tris hydrochloride (pH 7.5), 20 mM $MgCl_2$, 20 mM KCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 2 mM spermidine, 0.4 μg of bovine serum albumin, 10% (vol/vol) glycerol, and several concentrations of quinolones were incubated for 30 min at 37°C. One unit of *M. luteus* gyrase is defined as that amount of enzyme that catalyzes the conversion of 0.5 μg of relaxed pBR322 DNA to the supercoiled form in 30 min at 37°C (12). The concentrations of the quinolones used for the test with the gyrase of *E. coli* and *M.*

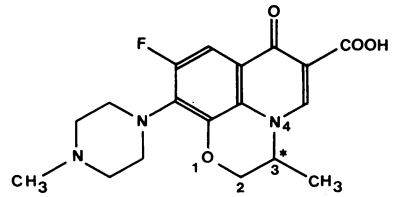

FIG. 1. Chemical structure of OFLX: (±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]-benzoxazine-6-carboxylic acid. *, the asymmetric carbon at the C-3 position.

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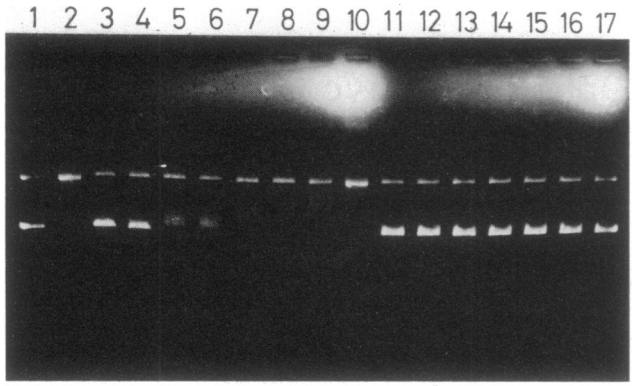

FIG. 2. The electrophoresis pattern of pBR322 DNA treated with *M. luteus* DNA gyrase. After incubation at 37°C for 30 min, the reaction mixtures (see text) with or without the test drug were subjected to agarose gel electrophoresis. Lane 1, A sample without enzyme representing a pattern of supercoiled pBR322 DNA and a small amount of open circular DNA; lane 2, closed circular DNA (the substrate for DNA gyrase) relaxed with calf thymus topoisomerase I at 37°C for 2 h; lanes 4 to 17, inhibitory patterns of supercoiling DNA with several concentrations of (−)- and (+)-OFLX. The concentrations of (−)-OFLX in lanes 4, 5, 6, 7, 8, 9, and 10 are 200, 400, 600, 800, 1,000, 1,600, and 2,400 μg/ml, respectively. The same concentrations of (+)-OFLX were added to lanes 11 to 17, respectively. Lane 3, a sample with the enzyme but without drug, shows pBR322 supercoiled by DNA gyrase (drug-free control).

luteus ranged from 0.0938 to 24 μg/ml and from 200 to 2,400 μg/ml, respectively.

Figure 2 shows electrophoretograms of the DNA bands that were treated with *M. luteus* gyrase and the OFLX isomers. The relaxed DNA treated with the gyrase but without OFLX isomers became superhelical form I DNA (lane 3). Under the same conditions, (−)-OFLX inhibited the supercoiling activities of gyrase in a dose-dependent manner (lanes 4 to 10). However, (+)-OFLX (lanes 11 to 17) did not inhibit the DNA gyrase even at a maximum concentration of 2,400 μg/ml (lane 17). The MICs of (+)-, (−)-, and (±)-OFLX for *M. luteus* ATCC 4698, as determined by the twofold broth dilution method with 10^5 CFU/ml, were 200, 0.78, and 1.56 μg/ml, respectively.

Figure 3 shows the percent inhibition of DNA gyrase from *E. coli* by (−)-, (+)-, and (±)-OFLX. (±)-OFLX, the racemic compound containing an equal amount of (−)- and (+)-OFLX, also reduced DNA gyrase activities; it was 1.3 times less active than (−)-OFLX and 7.4 times more active than (+)-OFLX. The concentrations of CPFX and NFLX required to inhibit 50% DNA gyrase from *E. coli* (IC_{50}s) were

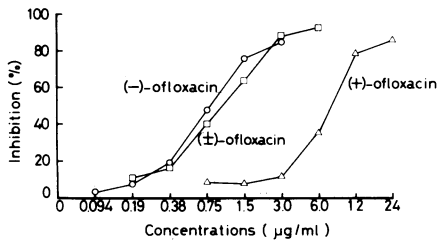

FIG. 3. The inhibitory effects of (−)-, (+)-, and (±)-OFLX on the supercoiling activity of gyrase from *E. coli* KL-16. The amount of the supercoiled DNA was measured densitometrically. Symbols: ○, (−)-OFLX; △, (+)-OFLX; □, (±)-OFLX.

TABLE 1. Inhibitory activities of OFLX derivatives, NFLX, and CPFX against growth of *E. coli* KL-16 and its DNA gyrase

Compound	IC_{50} for supercoiling of pBR322 DNA (μg/ml)[a]	MIC (μg/ml)[b]
(−)-OFLX	0.78	0.025
(+)-OFLX	7.24	1.56
(±)-OFLX	0.98	0.05
NFLX	1.15	0.05
CPFX	0.78	0.013

[a] Supercoiling IC_{50} values were calculated by the probit method described by Litchfield and Wilcoxon (6).

[b] MICs were measured by the twofold broth dilution method, with an inoculum size of 10^5 CFU/ml, against *E. coli* KL-16.

almost equal to those of (−)-OFLX and (±)-OFLX, respectively; the IC_{50}s of CPFX, (−)-OFLX, NFLX, and (±)-OFLX were 0.78, 0.78, 1.15, and 0.98 μg/ml, respectively (Table 1). (+)-OFLX was the least active (IC_{50}, 7.24 μg/ml). The MICs of these drugs for *E. coli* KL-16 correlated well with the IC_{50}s of gyrase inhibition.

Although the mode of action of 4-quinolone antibacterial agents has not yet been clarified fully, their antimicrobial activities seem dependent on their anti-DNA gyrase activities. Thus, the difference in antibacterial activities between these two compounds may be largely attributed to their anti-DNA gyrase activities; that is, the configuration of the C-3 methyl group of the isomers is probably responsible for the activities. In general, the N-1 substituents of the 1,4-dihydro-4-oxopyridine-3-carboxylic acid moiety of quinolones, e.g., the ethyl group in NFLX and the cyclopropyl group in CPFX, rotate freely along the carbon-nitrogen bond; and a certain conformation must be responsible for their antimicrobial activities. On the other hand, the N-4 substituent in OFLX is fixed with the oxazine ring, thus resulting in the formation of the enantiomers (−)- and (+)-OFLX. The C-3 methyl group in the oxazine ring of each isomer swings symmetrically through an angle of about 90 degrees following the inversion of the ring, thus indicating that the steric configuration of the methyl group of (−)-OFLX is optimal for the antimicrobial activity of OFLX.

Shen and Pernet (9) stated that quinolones bind preferentially to bacterial plasmid DNA and, consequently, interfere with gyrase activity. In this study the possibility that OFLX bound preferentially to plasmid DNA, the substrate for gyrase, could not be excluded. We are therefore studying the differences between these isomers in DNA binding and affinity to purified DNA gyrase.

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