

## Mechanism of Differential Activities of Ofloxacin Enantiomers†

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**Ofloxacin, a potent quinolone antibacterial agent, has a tricyclic ring structure with a methyl group attached to the asymmetric carbon at the C-3 position on the oxazine ring. The *S* isomer (DR-3355) of ofloxacin has antibacterial activity up to 2 orders of magnitude greater than that of the *R* isomer (DR-3354). This differential antibacterial activity was not due to different drug transport mechanisms of the two isomers but was found to be derived from the inhibitory activity against the target enzyme, DNA gyrase. Previous mechanistic studies have suggested that the bactericidal effect of the drug is mediated through the stabilization of a cleavable complex via a cooperative drug binding process to a partially denatured DNA pocket created by DNA gyrase. The drug binds to supercoiled DNA in a manner similar to that to which it binds to the enzyme-DNA complex. In the present studies, we first examined the binding of the two radiolabeled ofloxacin enantiomers to supercoiled pUC9 plasmid DNA. Surprisingly, the two enantiomers possessed similar apparent binding affinities and binding cooperativities. The major difference in binding between the two stereoisomers was the molar binding ratio: 4 for the more active *S* isomer versus 2 for the less active *R* isomer. We next examined the relative binding potencies of the stereoisomers to the DNA-DNA gyrase complex. The results of a competition assay showed that (*S*)-ofloxacin binds 12-fold better to the complex than (*R*)-ofloxacin. The binding potencies of the two enantiomers and two other quinolones correlated well with their respective concentrations causing 50% inhibition against DNA gyrase. The results are interpreted by a stacking model by using the concept of the cooperative drug-DNA binding mechanism, indicating that the potencies of quinolones cannot be determined solely by the DNA binding affinity and cooperativity but can also be determined by their capability in maximally saturating the binding site. The capability of the drug in saturating the binding pocket manifests itself in an increased efficacy at inhibiting the enzyme through a direct interaction between the drug and the enzyme. The results augment the previous suggestion that the binding pocket in the enzyme-DNA complex involves multiple receptor groups including not only DNA bases but also a gyrase subunit. The higher level of potency of (*S*)-ofloxacin is proposed to derive from the fact that a greater number of molecules are assembled in the pocket. This greater number of molecules optimizes the interaction between the drug and the enzyme, possibly through a contact between the C-7 substituent and the quinolone pocket on the B subunit of DNA gyrase.**

Four decades after the discovery of the first member of the quinolone antibacterial family, nalidixic acid (21), this class of synthetic antibacterial agents has become widely used clinically. These drugs, which possess broad antibacterial spectra and high degrees of potency against gram-negative and gram-positive bacteria, are found to be specific inhibitors of DNA gyrase (5, 8). At least two factors determine the efficacies of these drugs against bacteria: the transport of the drug into the cell and the inhibition of the target enzyme, DNA gyrase. In *Escherichia coli*, hydrophilic fluoroquinolones are thought to cross the outer membrane, mainly via the OmpF porin (11). These drugs, particularly those with higher lipophilicities, also penetrate the lipid bilayer of the inner membrane (3, 4, 6). At the enzyme inhibition level, it is proposed that quinolones bind to a specific site on DNA in the DNA-DNA gyrase complex

(28, 30-32). According to this model, differences in enzyme inhibitory potency are mainly determined by the binding strength of the drug to a DNA receptor site on the enzyme-substrate complex, while the interaction of the C-7 substituent with the enzyme plays a supporting role.

Ofloxacin (26) possesses an asymmetric carbon at the C-3 position in the oxazine ring, thus having two optically active isomers (Fig. 1). Interestingly, the antibacterial activity of the *S* isomer (DR-3355) against laboratory strains is 8 to 128 times more potent than that of the *R* isomer (DR-3354) (10, 33). The inhibitory activities of these two isomers against DNA gyrase also differ in the same proportion (14, 16), indicating that the differential potencies probably result from effects at the enzyme inhibition level, perhaps determined by the mode of drug interaction with the receptor site on DNA, as suggested by a current model (31).

In the study described here, we examined the permeation properties of the ofloxacin enantiomers with *E. coli*, the inhibitory potency of ofloxacin against DNA gyrase using pUC9 DNA, and the binding properties of these isomers to the DNA substrate and to the DNA-DNA gyrase complex. By using computer model systems, a model interpreting the chiral selectivity phenomenon of these optically active isomers of ofloxacin at the enzyme inhibition level is proposed.

(This is a Daiichi-Abbott joint publication, with the original data provided by Daiichi. Abbott authors provided data analysis, molecular graphics, and additional molecular modeling

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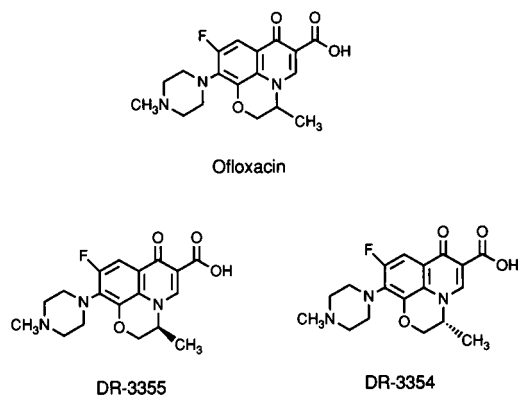


FIG. 1. Structures of ofloxacin and its enantiomers, DR-3355 (*S* isomer) and DR-3354 (*R* isomer).

investigations. Some preliminary data have been reported by the Daiichi authors at the Third Conference on DNA Topoisomerases in Therapy, New York, N.Y., October 1990 [15].)

#### MATERIALS AND METHODS

**Chemicals.** Ofloxacin, DR-3355 [(*S*)-ofloxacin and levofloxacin], and DR-3354 [(*R*)-ofloxacin] were synthesized at Daiichi. [ $^{14}\text{C}$ ]DR-3355 and [ $^{14}\text{C}$ ]DR-3354 were synthesized by New England Nuclear and had specific radioactivities of 24.8 and 22.9 mCi/mmol, respectively. [ $^3\text{H}$ ](*S*)-ofloxacin was synthesized by Tokai Research Laboratories (Ibaraki, Japan) and had a specific radioactivity of 66.4 Ci/mmol. All other reagents were purchased from commercial sources and were at least of analytical grade.

**Bacterial strains.** *E. coli* KL-16 (25), *E. coli* RW1053 (23) *E. coli* CS109, and the *OmpF* porin-deficient mutant of *E. coli* CS109, *E. coli* CS197 (24), were used in the study.

**MIC determinations.** MICs were determined by an agar dilution technique and were the lowest concentrations that completely prevented visible growth of the bacteria. The inoculum size was approximately  $5 \times 10^4$  CFU per spot.

**Nucleic acids and enzymes.** Supercoiled pUC9 plasmid DNA was prepared by the cesium chloride density gradient method (22), and the relaxed form of the DNA was prepared by using calf thymus topoisomerase I (obtained from Bethesda Research Laboratories). DNA gyrase was isolated from *E. coli* KL-16 by the method described previously (16) or was isolated from *E. coli* RW1053 transformed with plasmids pMK90 or pYK512 (gifts from J. Kato, University of Tokyo), which allow for the overproduction of the GyrA or the GyrB protein, respectively. One unit of DNA gyrase was defined as that amount of DNA gyrase required to fully supercoil 0.2  $\mu\text{g}$  of relaxed pUC9 DNA in 1 h at 37°C.

**Inhibitory activities of quinolones against DNA gyrase.** The inhibitory activity of quinolones against DNA gyrase was expressed as the drug concentration inhibiting 50% of DNA supercoiling activity ( $\text{IC}_{50}$ ). The method used was the same as that described previously (16), except that pUC9 DNA was used as the substrate.

**Quinolone accumulation in *E. coli* cells.** A fluorometric method (3) was used for determination of the uptake of the quinolones by *E. coli* CS109 and its porin-deficient mutant. Bacteria were grown in Penassay broth (Difco Laboratories) to an  $A_{660}$  reading of 0.4. The harvested cells were washed with sodium phosphate buffer (50 mM; pH 7.2) and were then suspended in the same buffer to 40 mg (wet weight) of cells per ml. The drugs were added to the suspension to a final concentration of 10  $\mu\text{g}/\text{ml}$ , and a portion of the suspension was removed at an appropriate time. The samples were washed once and were treated with 0.1 M glycine hydrochloride (pH 3.0). The samples were centrifuged ( $5,600 \times g$  for 2 min), and the fluorescence intensity (at 501 nm) of the supernatant was determined with excitation at 298 nm.

**DNA binding assay with membrane filtration.** DNA binding studies with the quinolone compounds were performed by the method reported previously (28, 32) by using Centrifree micropartition devices (MPS-3; Amicon). The reaction mixtures consisted of 400  $\mu\text{l}$  of reaction mixture which contained  $\approx 30$  pmol of DNA, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4), 20 mM KCl, 5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1 mM dithiothreitol, and various amounts of the labeled compound. After 45 min of incubation at 33°C, the mixture was filtrated with a Centrifree micropartition device by centrifuging at  $3,000 \times g$  for 45 min. The amount of ligand bound to the DNA was calculated by subtracting the free ligand concentration in the reaction mixture from the initial ligand concentration, with both ligand concentrations being determined from the radioactivity counts of the corresponding filtrates with and without the receptors.

**Molecular modeling.** In the present study, the model of enantiomers of ofloxa-

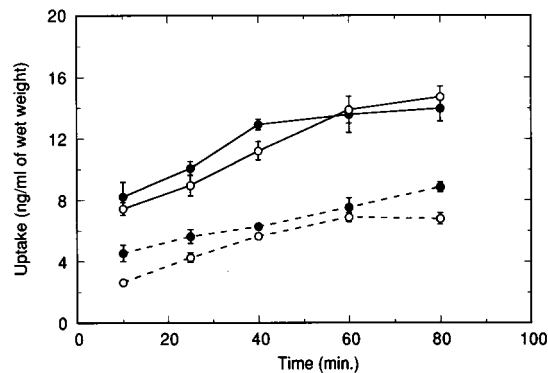


FIG. 2. Accumulation of (*S*)-ofloxacin (●) and (*R*)-ofloxacin (○) by *E. coli* cells. The levels of uptake of ofloxacin isomers by strains CS109 (solid line) and its mutant strain CS197 (dotted line) were assayed at 37°C after the addition of the isomers at time zero.

cin binding to DNA was developed by using two molecular graphics programs, SWAMI and InsightII. SWAMI (Structural Wisdom and Modeling Integration) is a molecular graphics and modeling program developed and used at Abbott Laboratories. InsightII is a molecular graphics, modeling, and simulation software package developed and distributed by Biosym Technologies (San Diego, Calif.). The intermolecular interactions in this complex were also investigated by using SWAMI, InsightII, and simple empirical energy functions reported in the literature (12).

**DNA-DNA gyrase complex binding assay.** The incubation conditions used were essentially the same as those of Shen et al. (30) and Yoshida et al. (36). A total of 30  $\mu\text{l}$  of binding buffer (50 mM Tris-HCl [pH 7.5], 20 mM KCl, 6 mM  $\text{MgCl}_2$ , 1 mM ATP, 1 mM dithiothreitol, 25% [vol/vol] glycerol) containing 0.1 pmol of *EcoRI*-cleaved pUC9 DNA and DNA gyrase (10 supercoiling units) was preincubated for 60 min at 37°C, and then [ $^3\text{H}$ ](*S*)-ofloxacin in binding buffer was added at various concentrations. The mixture was further incubated for 120 min and was then applied to a Sephadex G-50 NICK spin column (Pharmacia) according to the manufacturer's instructions. The radioactivity that passed through the column was used to calculate the amount of [ $^3\text{H}$ ](*S*)-ofloxacin bound to DNA-DNA gyrase complexes. For competition assays with nonlabeled quinolones, the competitor drug was added simultaneously with the [ $^3\text{H}$ ](*S*)-ofloxacin.

#### RESULTS

**Permeation properties of the optically active isomers of ofloxacin.** The levels of accumulation of DR-3355 (*S* isomer) and DR-3354 (*R* isomer) by *E. coli* CS109 and its porin-deficient mutant (CS197) over an 80-min time period are provided in Fig. 2. The accumulation kinetics of the isomers were quite similar, and the concentrations of both compounds in the cell increased gradually until 60 min after incubation. The level of accumulation of these isomers by the porin-deficient mutant was approximately half that of the parent strain. These results indicate that these isomers do not differ in their levels of permeation through the outer membrane.

**Correlation between the antibacterial potencies and the anti-gyrase activities of the isomers.** The antibacterial potencies (expressed by the MICs) and the inhibitory potencies against DNA gyrase determined by using pUC9 DNA as the substrate are given in Table 1. The antibacterial activity of (*S*)-ofloxacin against *E. coli* KL-16 is twice that of ofloxacin (racemic mixture) and 32 times that of (*R*)-ofloxacin. Similarly, the inhibitory potency of (*S*)-ofloxacin against DNA gyrase is about twice that of ofloxacin, and (*S*)-ofloxacin is 50 times more active than (*R*)-ofloxacin. The fact that with these two isomers the inhibitory activity against DNA gyrase parallels the antimicrobial activity suggests that the differential potencies of these drugs results from differences in the inhibition of DNA gyrase.

**Binding of ofloxacin enantiomers to pUC9 plasmid DNA.** A current quinolone inhibition model of DNA gyrase (31) sug-

TABLE 1. Inhibition effects of ofloxacin and its stereoisomers against *E. coli* KL-16 cells and *E. coli* DNA gyrase

Compound	MIC ( $\mu\text{g/ml}$ ) for <i>E. coli</i> KL-16	IC <sub>50</sub> ( $\mu\text{g/ml}$ ) for DNA gyrase
DR-3355 [( <i>S</i> )-ofloxacin]	0.05 (1) <sup>a</sup>	1.5 (1)
DR-3354 [( <i>R</i> )-ofloxacin]	1.6 (32)	75 (50)
Ofloxacin (racemic mixture)	0.10 (2)	2.6 (1.7)

<sup>a</sup> Numbers in parentheses are ratios of the value relative to the value for (*S*)-ofloxacin.

gests that the inhibition potencies of the quinolones are primarily determined by the binding of the drug to a DNA site in the DNA-enzyme complex. Previous results obtained by using [<sup>3</sup>H]norfloxacin also show that the binding of the drug to supercoiled DNA mimics the binding to the DNA-DNA gyrase complex (28, 30). In our current investigation, we first examined the levels of binding of the two ofloxacin enantiomers to supercoiled pUC9 DNA, and the results are provided in Fig. 3A. The striking fact gleaned from the results is that both enantiomers showed significant levels of binding to the DNA in the concentration range spanning either side of the IC<sub>50</sub> (~4  $\mu\text{M}$ ) of (*S*)-ofloxacin. Both binding curves were very similar in shape, but the magnitude of binding was distinctly different. Twice as much of the more potent (*S*)-ofloxacin was seen to bind to DNA compared with the level of binding of the less potent (*R*)-ofloxacin over the same concentration range. By using a more quantitative computer data analysis method (28), the dissociation constants ( $K_d$ ) and binding cooperativities were calculated for both isomers. Using the partial binding plateau near 5.5  $\mu\text{M}$  as the saturation point for this initial phase of binding,  $K_d$  values of 2.76 and 2.97  $\mu\text{M}$  were obtained for (*S*)- and (*R*)-ofloxacin, respectively (Fig. 3B). Similar binding cooperativities were found for both isomers, with Hill constants of about 3. The only major difference between the two isomers was the maximum molar binding ratio, which was about 4 per DNA molecule for (*S*)-ofloxacin and about 2 for (*R*)-ofloxacin. The results suggest that these isomers differ in their abilities to saturate the proposed binding pocket on supercoiled pUC9 DNA, although they have a similar apparent binding affinities and binding cooperativities.

**Molecular modeling of the ofloxacin stereoisomer binding.** Computer modeling studies (29) have been used to investigate the effect of the steric configuration of the methyl group associated with the ofloxacin stereoisomers on molecular stacking. The rationale behind such an investigation was based on a DNA gyrase inhibition model (Fig. 4) originally proposed by Shen et al. (31). To facilitate the description of this model for the current interpretation of the chiral discrimination between the ofloxacin enantiomers, the model is reconstructed and presented in Fig. 5 to illustrate alternatively the proposed modes of interaction among the drug, DNA, and the enzyme. As illustrated in the modeling cartoon in Fig. 5, quinolones bind cooperatively to a DNA pocket formed by the action of DNA gyrase, and the R7 groups (substituent at C-7 of the quinolone ring) on the drug supermolecule interact with the enzyme subunit to further strengthen the binding. This new model presentation will be used to further illustrate our interpretation of the differential activities of ofloxacin stereoisomers. Figure 6A shows the result from our previous modeling studies (29) that the two ofloxacin enantiomers can pair only when they are positioned in an opposed orientation because of the steric hindrance of the chiral methyl group; i.e., the methyl groups need to be located outside of the stacked complex to promote a closer contact. The most favorable configuration of

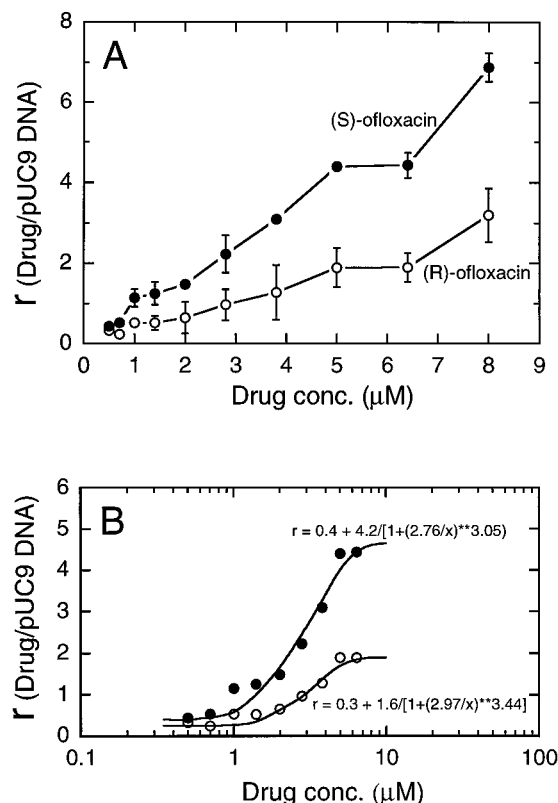


FIG. 3. (A) Binding of [<sup>14</sup>C](*S*)-ofloxacin (●) and [<sup>14</sup>C](*R*)-ofloxacin (○) to supercoiled pUC9 DNA. Each datum point represents the results of three binding experiments, and the vertical bars denote the standard deviations. Points without vertical bars are values with standard deviations less than the diameter of the symbol. (B) Curve fitting by the methods described by Shen et al. (28). The same binding data were analyzed by using Klotz plots (20), and the binding cooperativity was assessed by a semiempirical approach. The calculation was made by using a nonlinear least-squares computer program, RS/1, to fit the binding data with the equation  $r = r_m/[1 + (K_d/D)^n]$ , where  $r$ ,  $r_m$ ,  $K_d$ , and  $D$  represent the molar binding ratio, the maximum molar binding ratio, apparent dissociation constant, and free ligand concentration, respectively, while  $n$  is the Hill constant. Since the amount of bound ligand is less than 5% of the total ligand used in one experiment, the initial ligand concentration is thus used as the value for the free ligand concentration ( $D$ ) in the plot.  $r_m$  was chosen as the value at the partial saturation plateau at drug concentrations near 5.5  $\mu\text{M}$ . The  $x$  and double asterisk in the equation denote the free drug concentration and the exponentiation operator, respectively.

the stacked pairs of the *S* and *R* isomers are in a mirror-image relationship; i.e., the pair on the left and the pair on the right represent two asymmetric supermolecular complexes with their functional groups (C=O) located in chiral positions. This process demonstrates how such a self-assembly process can transfer the chirality from the nonfunctional methyl groups to the functional (binding) groups. Furthermore, from Fig. 6B, we demonstrate that four molecules of the *S* isomer can stack in a way to allow all of the hydrogen bond acceptors to be positioned in a correct and asymmetric orientation that allows bond pairing, while the *R* isomers can only stack to have hydrogen bond acceptors positioned in a mirror-image configuration compared with the configuration of the *S* isomers, thus producing an unfitted orientation. As shown in Fig. 6C, the less active *R*-isomer molecules, however, can still bind to the proposed site through the hydrophobic tail-tail interactions in the absence of any ring-ring stacking, thus limiting the binding of the drug to two molecules per site. This proposed model accounts for the experimental observation that the maximum

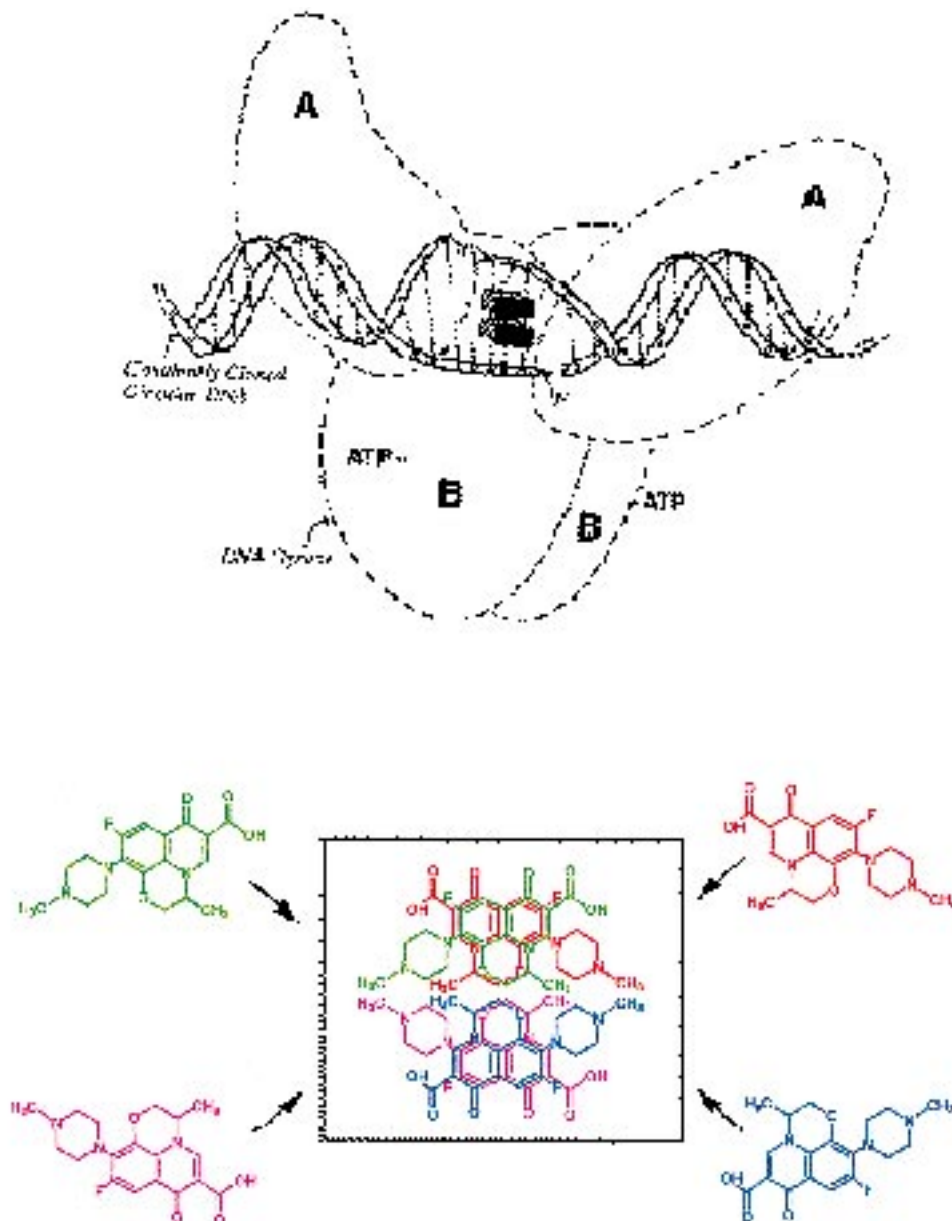


FIG. 4. (Upper panel) Cooperative quinolone-DNA binding model for the inhibition of DNA gyrase as originally published by Shen et al. (31). Quinolone molecules (filled and slashed rectangles) bind to a gyrase-induced DNA site during the intermediate gate-opening step of the DNA supercoiling process via hydrogen bonds (dotted lines) to the unpaired bases. Gyrase A subunits form covalent bonds between tyrosine 122 and the 5' end of the DNA chain, and the subsequent opening of the DNA chains along the 4-bp staggered cuts results in a locally denatured DNA bubble which is proposed to be the site to which the drug binds. When relaxed DNA substrate (represented by the short DNA segment in the diagram) is used, ATP is required for the induction of the drug binding site. Dashed curves mimic the shape of the DNA gyrase. (Lower panel) More details of how four molecules of quinolones (ofloxacin) are self-associated to form a micelle-like (a spherical structure consists of a hydrophilic surface and a hydrophobic core) supermolecular drug complex when occupying the binding pocket, as shown in the upper panel. The modeling results shown in Figure 7 indicate that the actual mode of stacking between the two quinolone rings when pairing with DNA bases are not perfectly oriented as shown in this diagram.

binding of the *R* isomer is limited to only two drug molecules per DNA molecule, whereas the *S* isomer can bind four drug molecules.

The X-ray crystal structure of ofloxacin perchlorate has been investigated (37), and molecular orbital calculations performed by *ab initio* (14) and semiempirical (AM1) methods have been used to determine the relative energies of the conformations of the ofloxacin enantiomers. Simple energy calculations performed by using empirical energy functions (12) were used in the present study to evaluate intermolecular in-

teractions between two (*S*)-ofloxacin molecules and between stacked (*S*)-ofloxacin dimers and DNA. The results of this work, with the aid of molecular graphics and modeling (InsightII), were used to build a DNA binding model of two stacked (*S*)-ofloxacin molecules. This DNA-drug binding interaction was modeled by considering the binding of two stacked ofloxacin molecules to two adjacent base pairs in a segment of B-DNA. During the process of building this quinolone-DNA interaction model, d(G)<sub>4</sub>-d(C)<sub>4</sub> was chosen initially as the DNA sequence in the binding site, and the oligo-

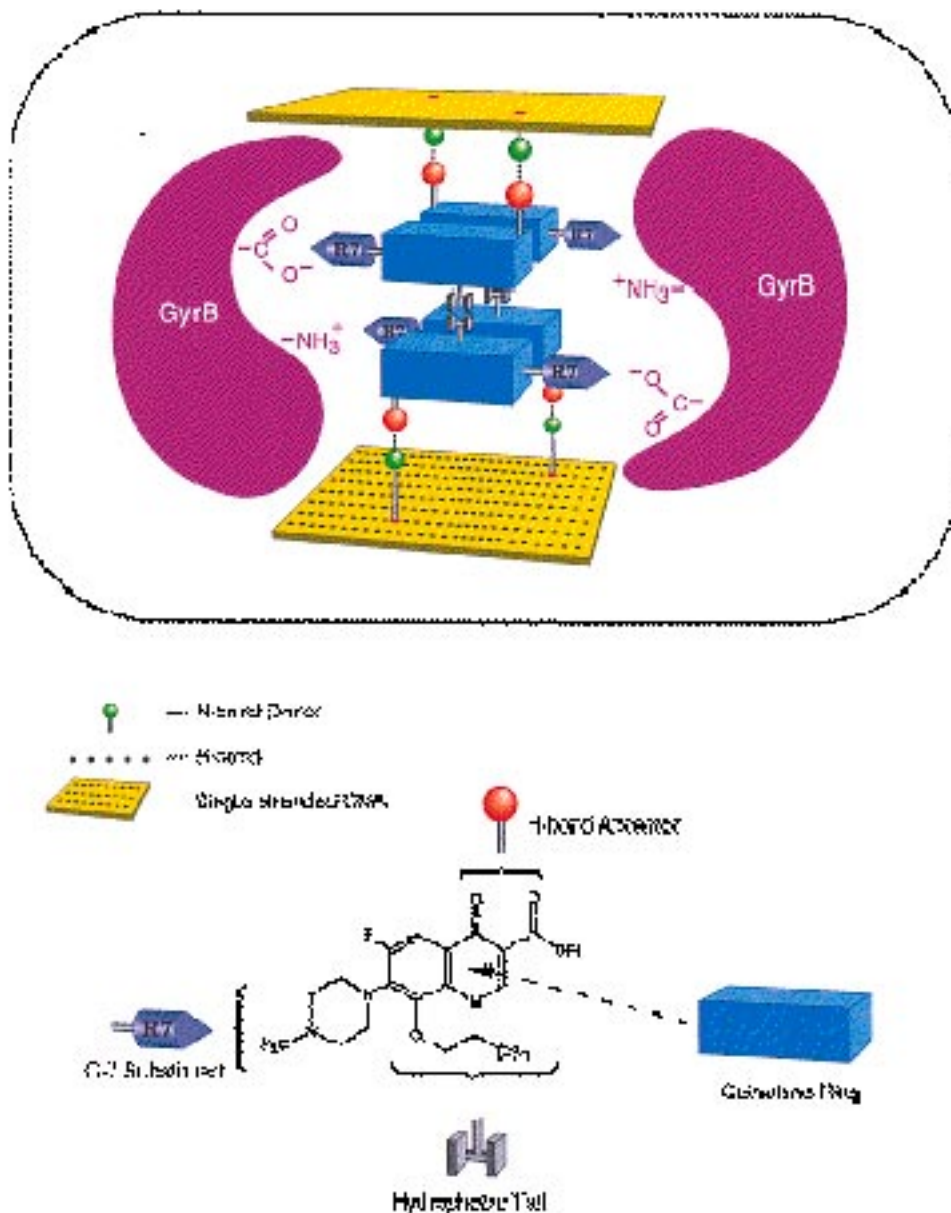


FIG. 5. The model described in Fig. 4 is further depicted by a brick-stacking schematic presentation, with the symbols explained in the lower panel.

nucleotide was assembled by using standard parameters for a B-form double helix (1). Subsequently, two cytosine residues at the 5' end of the d(C)<sub>4</sub> were eliminated, and then two (*S*)-ofloxacin molecules were used to replace the eliminated d(C)<sub>2</sub> for modeling the pairing with the unpaired bases of d(G)<sub>4</sub> (Fig. 7, upper panel). This modeling result suggests that it is necessary to rotate one of the two (*S*)-ofloxacin molecules relative to the other in order to accommodate an optimal hydrogen-bonding arrangement with the adjacent DNA base pairs. Thus, in this quinolone-DNA interaction model, the quinolone rings are not stacked directly on top of each other, as reported in our preliminary stacking model for (*S*)-ofloxacin shown in Fig. 6A or as shown ideally in Fig. 4 (lower panel). Rather, one (*S*)-ofloxacin molecule is rotated and translated slightly (in the plane of the quinolone ring) relative to the other (*S*)-ofloxacin molecule in order to achieve the best possible hydrogen-bonding interactions with the DNA bases. The drug-DNA pairing

shown in Fig. 7 is in its optimal configuration; rotation of the d(G)<sub>4</sub> backbone such that it is deviated from the B form of the double helix would only worsen the stacking configuration of the drug pair. In conclusion, our current modeling result implies that ring stacking may not be as crucial as we first anticipated for contributing to the overall binding strength and cooperativity.

**Binding of ofloxacin enantiomers to the DNA-DNA gyrase complex.** We used a spin column technique, a convenient method for determining tightly bound drugs to receptors, to investigate the binding of quinolones to DNA-DNA gyrase complex. The binding of [<sup>3</sup>H](*S*)-ofloxacin to complexes of DNA-DNA gyrase was found to be proportional to both the DNA and the DNA gyrase concentrations (data not shown). This phenomenon has been shown previously with other quinolones (32, 36). When nonradiolabeled quinolones were added to compete with the [<sup>3</sup>H](*S*)-ofloxacin binding, the ex-



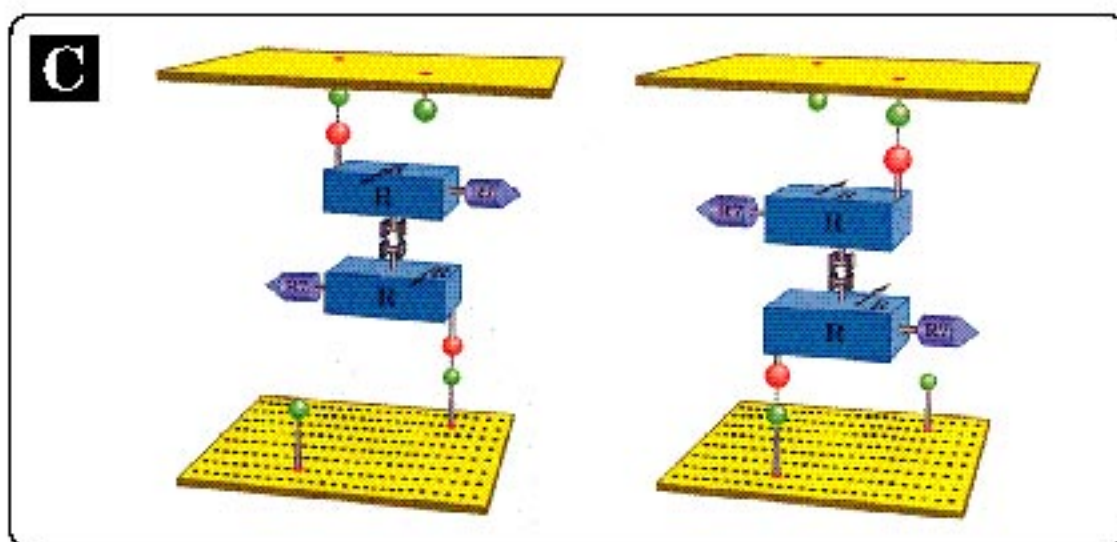
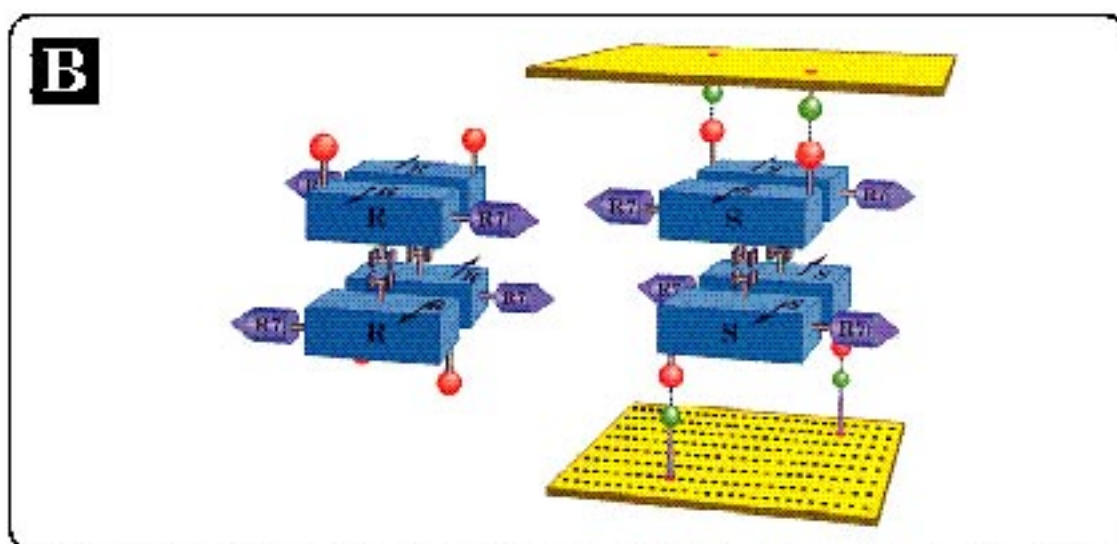
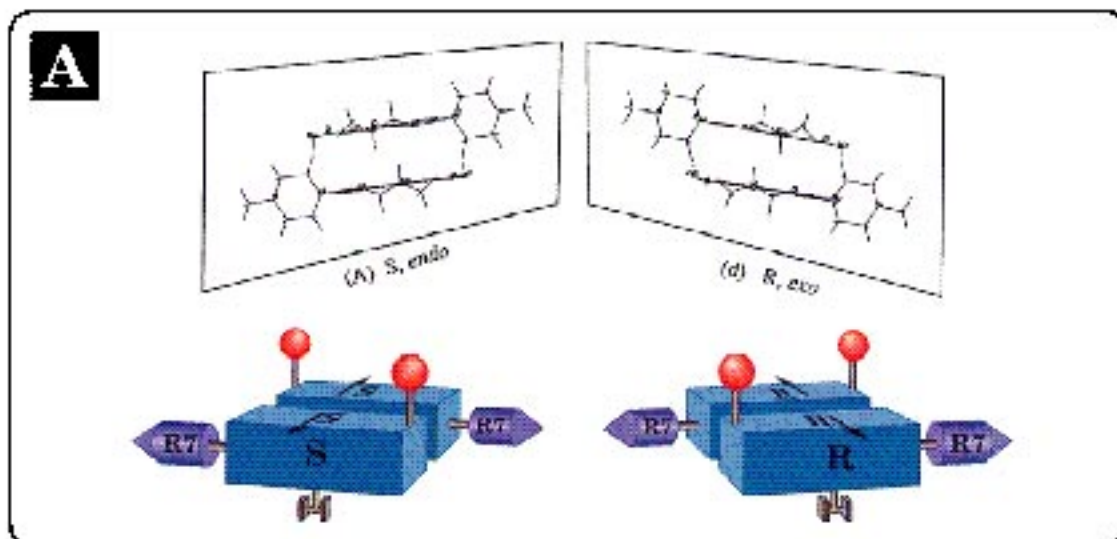


FIG. 6. Brick-stacking model for the interpretation of the chiral discrimination phenomenon of ofloxacin enantiomers. (A) The most favorable stacking pairs for (*S*)-ofloxacin (left) and (*R*)-ofloxacin (right). Both dimeric drug complexes are asymmetric, and they are in a mirror-image configuration. The computer graphics of the molecular pairs in the boxes are retrieved from Shen et al. (29) with permission. In this brick-stacking model, an arrow on the upper surface of the brick is assigned to indicate the direction of the protruding methyl group from the plane of the quinolone ring. According to the our modeling result (see text), for a best fit two molecules can stack only when the arrows are pointing away from each other, but not when they are pointing against each other or in the same direction. (B) The two asymmetric tetrameric supermolecules assembled by (*S*)-ofloxacin and by (*R*)-ofloxacin are in a mirror-image configuration, with the hydrogen acceptors distributed in chiral positions. They are incompatible in terms of their capability of fitting into the proposed asymmetrical DNA binding site. It should be emphasized that the mode of stacking for the enantiomer is determined by the direction of the arrows on the bricks. (C) (*R*)-Ofloxacin can still bind to the asymmetrical site; however, it binds not through the stacking but through hydrophobic tail-tail interactions, thus limiting the binding to only two molecules per site.

tent of [ $^3\text{H}$ ](*S*)-ofloxacin binding decreased as expected (Fig. 8). It can be seen that the quinolones tested varied considerably in their antagonizing effects on [ $^3\text{H}$ ](*S*)-ofloxacin binding. (*R*)-Ofloxacin and nalidixic acid inhibited [ $^3\text{H}$ ](*S*)-ofloxacin binding poorly, whereas (*S*)-ofloxacin and DU-6859a were more potent antagonists of [ $^3\text{H}$ ](*S*)-ofloxacin binding. If it is assumed, and it is more likely to be so, that inhibition of [ $^3\text{H}$ ](*S*)-ofloxacin binding is relative to the binding potency of the competing quinolone, it would appear that (*S*)-ofloxacin and (*R*)-ofloxacin vary considerably in their abilities to bind to DNA-DNA gyrase complexes. The concentration of each quinolone required to inhibit [ $^3\text{H}$ ](*S*)-ofloxacin binding by 50% ( $\text{BIC}_{50}$ ) were interpolated from Fig. 8 and are plotted against the  $\text{IC}_{50}$ s of the drugs for DNA gyrase (Fig. 9). It can be seen that there was good correlation between  $\text{BIC}_{50}$ s and  $\text{IC}_{50}$ s for DNA gyrase. Hence, quinolone anti-gyrase, and antibacterial activity as well, is related to the ability to bind to the DNA-DNA gyrase complex.

## DISCUSSION

The differences in antibacterial activities between enantiomers of tricyclic quinolone compounds (flumequine and ofloxacin) have been reported previously (9, 10). In general, the *S* isomers of either compound are up to 2 orders of magnitude more potent against *E. coli* strains than their corresponding *R* isomers. The phenomenon is surprising, since the difference in structure between the isomers is only the steric configuration of a presumably nonfunctional methyl group relative to the ring plane. In the present study we attempted to clarify the mechanism of the differential potencies of the optically active isomers of ofloxacin. First, the permeation properties of these isomers were compared. The results indicated that both isomers can accumulate inside the cell to the same extent at an equal rate, thus eliminating the possibility that the difference in antibacterial activity is due to their different capabilities of penetrating into the cell. Second, we compared the inhibitory activities against *E. coli* DNA gyrase. The  $\text{IC}_{50}$ s for the enzyme were found to correlate well with the antibacterial potencies of the isomers, suggesting that the difference in the antibacterial activities of the isomers results from the difference in the inhibitory activities against the target enzyme. The  $\text{IC}_{50}$ s obtained in the present study were higher than those published previously (14, 18), and this was probably due to the use of pUC9 DNA as opposed to the pBR322 DNA used in the previous studies. The former plasmid DNA was chosen because it was also used in the current binding studies.

According to a model proposed by Shen and coworkers (31), the inhibitory activities of quinolones against DNA gyrase are primarily determined by their capabilities of binding specifically to a DNA site created by the enzyme during catalysis, and the interaction between R7 and the enzyme plays a supporting role for further strengthening the binding. The existence of a cooperative drug binding site was demonstrated with the enzyme-substrate complex as well as with the supercoiled DNA molecule (28, 30). Taking this into account, the properties of

binding of the two ofloxacin stereoisomers to pUC9 DNA were evaluated to determine any differences in the levels of DNA binding between (*S*)-ofloxacin (the more active isomer) and (*R*)-ofloxacin (the less active one). The interesting, but somewhat unexpected, finding from those studies was that both enantiomers produced a significant amount of binding to supercoiled DNA, with a striking similarity in the shapes of their binding curves. This occurred even though there were large differences in their inhibitory activities against DNA gyrase and against intact bacteria. The shapes of the binding curves for the binding of the stereoisomers to supercoiled DNA implies that the two isomers have similar binding affinities and binding cooperativities, as indicated by the similar binding transition midpoints and the steepnesses of the curves. The two binding curves both showed biphasic binding characteristics, which closely resembled the binding of norfloxacin to supercoiled ColE1 DNA reported previously (28). However, the maximum molar binding ratio for (*S*)-ofloxacin binding was approximately twice that for (*R*)-ofloxacin. The results indicate that four molecules of (*S*)-ofloxacin can bind to the DNA site, whereas only two molecules of (*R*)-ofloxacin can bind to the DNA site. As suggested previously (31), such a binding site is presumably a partially denatured DNA pocket promoted by DNA supercoiling. It is conceivable that different supercoiled DNA substrates may possess different numbers of DNA pockets of various sizes; this is particularly eminent if the drug-targeted DNA is the large bacterial chromosome with an enormously complex structure. Under this circumstance, the number of drug molecules bound per site or per molecule will certainly differ from the numbers of two and four for the much smaller pUC9. The important information obtained from the current study by using pUC9 as a model is that the two ofloxacin enantiomers show at least a twofold difference in their capabilities of saturating such a potential DNA binding pocket.

The DNA binding results for the two stereoisomers, however, are rather surprising since they demonstrate that a subtle difference in the steric configuration of a nonfunctional group causes a twofold difference in terms of the number of drug molecules bound to the receptor site. An essential feature of the current inhibition model is that self-assembly of the drug molecules promotes cooperative drug binding, stabilizes the transient DNA pocket, and then results in the trapping of the enzyme-DNA-cleavable complex. Our computer modeling data show that only two (*R*)-ofloxacin molecules, because of the methyl group of (*R*)-ofloxacin protruding into an unfavorable position, can fit into the proposed DNA binding site due to an improper positioning of the essential hydrogen bond acceptors involved in DNA binding. With (*S*)-ofloxacin, however, the positioning of the carbonyl groups allows four (*S*)-ofloxacin molecules to bind. Thus, the computer modeling data complement the physical DNA binding data, in which the maximum molar binding ratios for (*R*)-ofloxacin and (*S*)-ofloxacin were found to be 2 and 4, respectively. It is evident that the activity of a quinolone drug may be determined not only by its

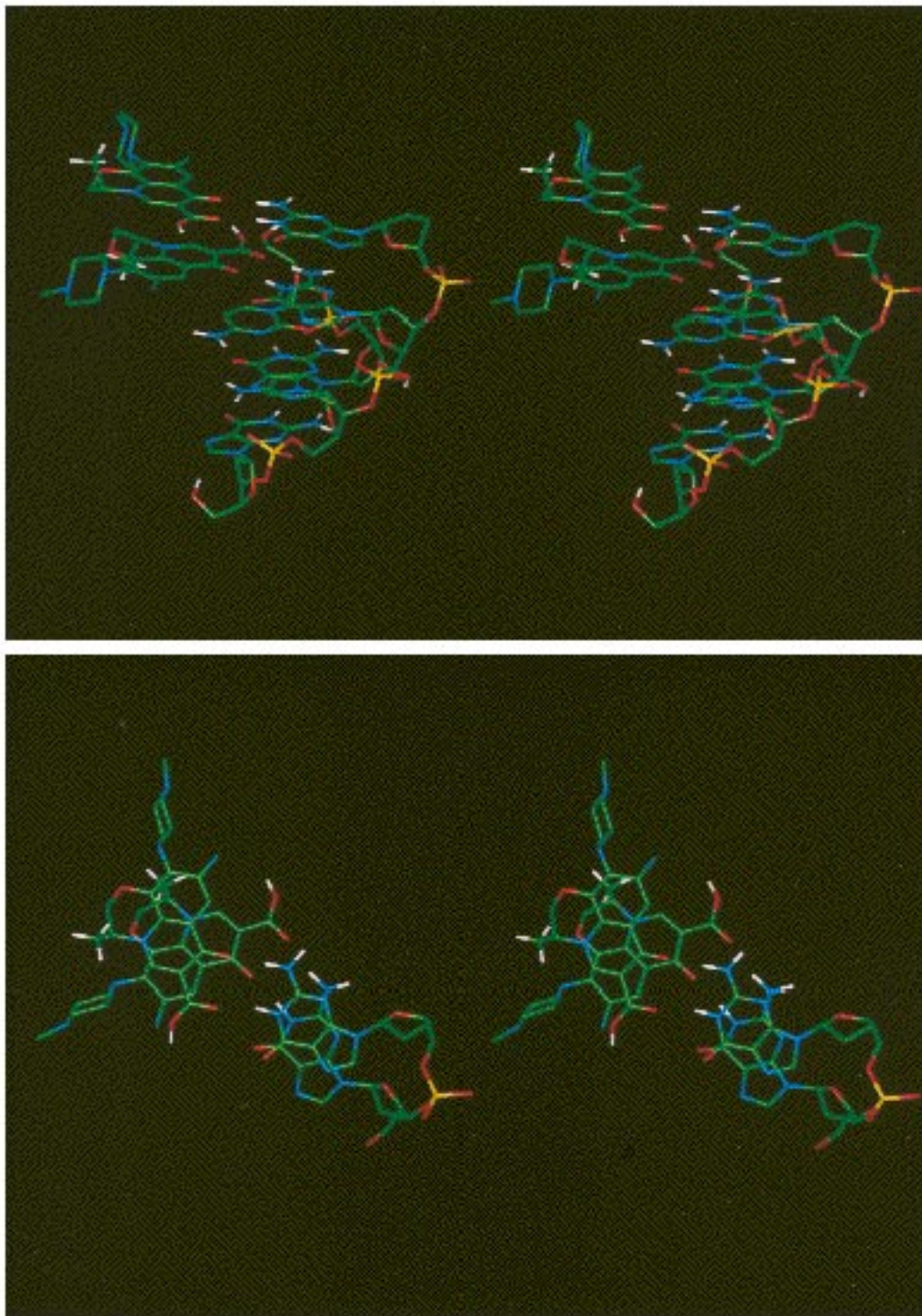


FIG. 7. Stereo views of a possible mode of interaction between the stacked dimer of (*S*)-ofloxacin and guanine bases in B-form DNA [d(C)<sub>2</sub>-d(G)<sub>4</sub>]. (Top panel) Side view of two (*S*)-ofloxacin molecules bound to the two unpaired guanine bases in d(C)<sub>2</sub>-d(G)<sub>4</sub>. (Bottom panel) Top view of the modeled complex, with the paired d(C)<sub>2</sub>-d(G)<sub>2</sub> portion eliminated for clarity.

intrinsic DNA binding affinity but also, more importantly, by its ability to saturate the quinolone binding pocket.

It would still appear puzzling why just a twofold difference in the number of bound drug molecules should cause such marked differences in enzyme inhibitory potencies between the

stereoisomers. Similarly, when binding to the DNA-DNA gyrase complex was measured a profound difference was observed between the two stereoisomers, which was in stark contrast to data on binding to DNA alone. The relative binding affinities of the stereoisomers and the other quinolones to the



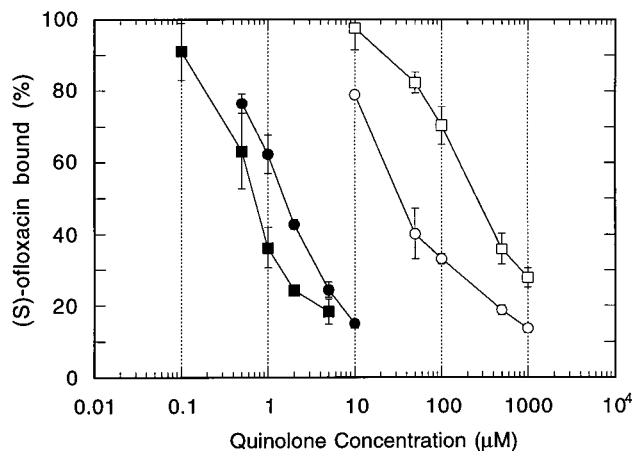


FIG. 8. Competitive assay between nonradiolabeled quinolones and [ $^3\text{H}$ ](*S*)-ofloxacin ■, DU-6859a; ●, (*S*)-ofloxacin; ○, (*R*)-ofloxacin; □, nalidixic acid. The results are the averages of three experiments, with the standard errors indicated by vertical bars.

DNA-DNA gyrase complex correlate well with their inhibitory potencies. Therefore, the difference in enzyme inhibitory potencies between these stereoisomers would appear to be due to their ability to bind to the DNA-DNA gyrase complex, which from the modeling discussed above is due to the ability to saturate the quinolone binding pocket on the DNA-DNA gyrase complex. It is highly probable that the missing link between the DNA binding data and the DNA-enzyme binding results is the potential interaction of the quinolone R7 substituent with the enzyme. Yoshida et al. (35) have provided evidence showing that R7 is in close contact with the so-called quinolone pocket in GyrB (DNA gyrase subunit B), and it was speculated that such an interaction is important in strengthening the drug binding (27). It is plausible to propose that the number of drug molecules assembled in the binding pocket must have a profound effect on promoting such a drug-enzyme interaction. A failure to saturate the binding site, producing a

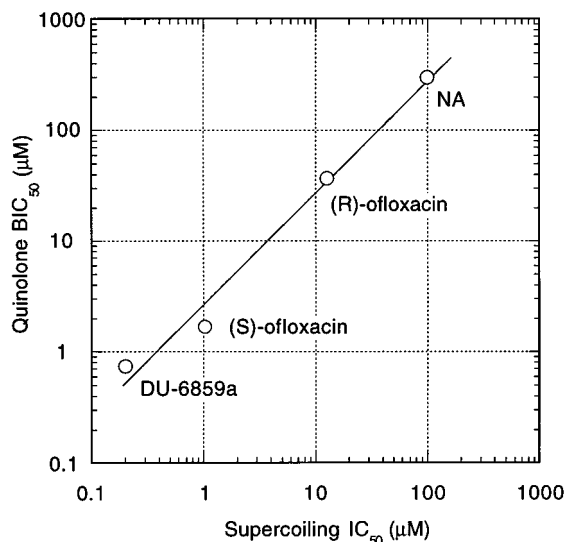


FIG. 9. Correlation between DNA gyrase supercoiling  $\text{IC}_{50}$  and  $\text{BIC}_{50}$ . Supercoiling  $\text{IC}_{50}$  data are from previous studies (13, 17). The correlation coefficient was 0.992. NA, nalidixic acid.

reduced number of molecules in the binding pocket, thus greatly reduces the probability of enhancing drug binding through the R7-GyrB interaction, as is the case with (*R*)-ofloxacin. The model also implies that the chiral methyl group, while in a noninterfering steric configuration, is important for maintaining good activity through the hydrophobic tail-tail interaction since omission of this group, i.e., desmethyl quinolones, renders poor inhibitory potencies on these quinolones. The proposed stacking model, however, does not explain satisfactorily the structure and activity relationship of the *N-tert*-butyl analogs, which have a bulky N-1 substituent. By necessity, at least one of the methyl groups must protrude at least partially into the forbidden space necessary for stacking when associated in the way proposed here. However, these agents are less active against gram-negative bacteria than against gram-positive bacteria, for which enzyme studies have shown that there is a larger or different binding pocket configuration (31). A more thorough investigation of their uptake mechanisms and inhibitory activities against various species of gyrase and other bacterial topoisomerases, especially topoisomerase IV (2, 7, 13, 19), is needed to better understand such a discrepancy.

Willmott and Maxwell (34) have found that a mutated GyrA protein can greatly reduce the level of binding to the DNA-DNA gyrase complex, and thus that a direct interaction must occur between quinolones and the DNA-DNA gyrase complex. We believe that the effect of a mutation in GyrA is indirect and affects the conformation of the binding pocket. In the present study we focused on the interaction between quinolone molecules and staggered DNA strands in the pocket to explain the chiral discrimination phenomenon of optical isomers of ofloxacin. However, we propose that the interaction between quinolones and DNA is crucial in the overall inhibition of DNA gyrase, while the subtle interaction with DNA gyrase in the complex is also important and cannot be neglected. Unfortunately, [ $^3\text{H}$ ](*R*)-ofloxacin was not available to allow for a direct comparison of the levels of binding to the DNA-DNA gyrase complex. The competition assay, however, provided convincing evidence that the binding affinities of the two stereoisomers to the complex are indeed drastically different.

From the drug design point of view, the implications of the current studies are at least fourfold. (i) The stacking orientation between the two quinolone molecules needs to be at least partially maintained. In fact, the molecular modeling results shown in Fig. 7 (lower panel) indicate that (*S*)-ofloxacin has a less-than-perfect orientation of stacking in the actual mode of binding. If any unfavorable structural feature that interferes with a proper assembly, such as the methyl group on (*R*)-ofloxacin, is introduced, then a detrimental effect is imposed. (ii) In view of the fact that the binding cooperativities and affinities of the ofloxacin *S*- and *R*-isomers are nearly equal and that the latter isomer cannot stack properly at the binding site according to our model, the hydrophobic tail-tail interaction is crucial and can act alone in contributing to the binding affinity and cooperativity. (iii) The potencies of ofloxacin and other quinolone drugs can be further improved by modification of the ring structure that maintains a good fit to the configuration of the H-bond donors in the binding pocket and at the same time allows for optimal stacking between the drug molecules. (iv) It is crucial that sufficient R7 substituents are in correct positions to maintain an interaction with a DNA gyrase subunit, presumably GyrB.

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