Mechanism of Differential Activities of Ofloxacin Enantiomers†

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Received 12 February 1996/Returned for modification 7 March 1996/Accepted 23 April 1996

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 by 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. 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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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. [14C]DR-3355 and [14C]DR-3354 were synthesized by New England Nuclear and had specific radioactivities of 24.6 and 22.9 mCi/mmol, respectively. [3H]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.

Nucleic acids and enzymes. Supercoiled pUC9 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 Becton Dickinson) to an amount of approximately 5 × 10^8 CFU per spot.

Bacterial strains, E. coli KL-16 (25), E. coli RW1053 (23) E. coli CS109, and the Omph 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 × 10^8 CFU per spot.

Molecular modeling. In the present study, the model of enantiomers of ofloxacin 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 µl of binding buffer (50 mM Tris-HCl [pH 7.5], 20 mM KCl, 6 mM MgCl₂, 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 [3H]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 [3H]S-ofloxacin bound to DNA-DNA gyrase complexes. For competition assays with nonlabeled quinolones, the competitor drug was added simultaneously with the [3H]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 antibiotic 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 antibacterial 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-
suggests 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 \(^{[3]H}\)norfloxacins 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 \(IC_{50}\) (~4 \(\mu M\)) 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 \(\mu M\) as the saturation point for this initial phase of binding, \(K_d\) values of 2.76 and 2.97 \(\mu M\) 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 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

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC ((\mu g/ml)) for E. coli KL-16</th>
<th>IC(_{50}) ((\mu g/ml)) for DNA gyrase</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR-3355 [(S)-ofloxacin]</td>
<td>0.05 (1)*</td>
<td>1.5 (1)</td>
</tr>
<tr>
<td>DR-3354 [(R)-ofloxacin]</td>
<td>1.6 (32)</td>
<td>75 (50)</td>
</tr>
<tr>
<td>Ofloxacin (racemic mixture)</td>
<td>0.10 (2)</td>
<td>2.6 (1.7)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are ratios of the value relative to the value for (S)-ofloxacin.
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 interactions 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)_4$$- d(C)_4$ was chosen initially as the DNA sequence in the binding site, and the oligo-
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)_4 were eliminated, and then two (S)-ofloxacin molecules were used to replace the eliminated d(C)_2 for modeling the pairing with the unpaired bases of d(G)_4 (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)_4 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 [3H](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 [3H](S)-ofloxacin binding, the ex-
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 ColEl 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 a unfavorably 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
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

![Image of stereo views of possible mode of interaction with DNA](http://aac.asm.org/...)

**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)₂-d(G)₄]. (Top panel) Side view of two (S)-ofloxacin molecules bound to the two unpaired guanine bases in d(C)₂-d(G)₄. (Bottom panel) Top view of the modeled complex, with the paired d(C)₂-d(G)₂ portion eliminated for clarity.
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 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, [3H](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.

FIG. 9. Correlation between DNA gyrase supercoiling IC50 and BIC50. Supercoiling IC50 data are from previous studies (13, 17). The correlation coefficient was 0.992. NA, nalidixic acid.

FIG. 8. Competitive assay between nonradiolabeled quinolones and [3H](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.
with pMK00 and pYK512 plasmids and information on DNA gyrase purification.

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