

Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: The target of the drugs is DNA

(norfloxacin/quinolone antibiotic/DNA supercoiling/topoisomerase)

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ABSTRACT Norfloxacin is a nalidixic acid analogue and one of the most potent DNA gyrase inhibitors. To study the mechanism of this important class of inhibitors, the binding of [³H]norfloxacin to gyrase and substrate DNA was measured. We found that, contrary to prior belief, norfloxacin does not bind to gyrase but instead binds to DNA. This was demonstrated by both equilibrium dialysis and membrane filtration techniques. Binding to ColE1 and pBR322 plasmids showed a primary process that is saturated at a norfloxacin concentration about equal to its supercoiling K_i (1.8×10^{-6} M) and is followed by weaker secondary binding. The apparent K_d values are 1×10^{-6} M for both plasmids. The molar binding ratio at this initial saturation point is extremely low: only 4×10^{-4} norfloxacin per nucleotide for both plasmids. The binding of norfloxacin to DNA plasmids is nonintercalative, as shown by the fact that the drug binds preferentially to single-stranded DNA rather than to double-stranded DNA. The binding is reduced at high salt concentration, has a pH optimum between 4.5 and 6.5, and does not require divalent ions. The binding affinities of other nalidixic acid analogues were estimated by an indirect competition method. The calculated apparent K_d values of these analogues correlate well with their K_i values, providing strong evidence that the binding affinity of the drug to DNA determines biological potency.

Nalidixic acid was the first member of the quinolone family of antibacterial agents synthesized (1). Subsequent developments have led to increasingly potent derivatives: pipemedic acid, oxolinic acid, and norfloxacin. The minimum inhibitory concentration of norfloxacin of 0.2 μ M against *Escherichia coli* is 2 orders of magnitude lower than that of nalidixic acid, and norfloxacin has been shown effective in treatment of urinary tract infections. Its potency makes it a suitable choice for studying the mechanism of action of quinolone antibiotics.

The antibacterial activity of this drug family is due to inhibition of DNA synthesis. The drugs inhibit DNA gyrase, a topoisomerase that negatively supercoils DNA in a reaction driven by ATP hydrolysis (for review, see refs. 2 and 3). The enzyme has two subunits, A and B, and it is believed that subunit A is the direct target for the drug. There are three lines of evidence for this conclusion. (i) Quinolone antibacterials are highly specific inhibitors of DNA gyrase. Even other topoisomerases are not inhibited at all or only at orders of magnitude higher concentrations (4-6). (ii) Mutations leading to high-level drug resistance are exclusively in *gyrA*, the structural gene for subunit A (7). Subunit A purified from the mutant plus wild-type subunit B reconstitutes quinolone-resistant gyrase. Because the minimum inhibitory concentration is raised up to 100-fold in *gyrA* mutants, no other vital target in *E. coli* can be important. (iii) Quinolone antibiotics inhibit reactions of DNA gyrase such as supercoiling and re-

laxation that require DNA breakage and reunion, the active site of which is in subunit A. The key evidence is that the drugs uncouple DNA breakage and reunion and lead to a complex that on addition of a protein denaturant yields a double-strand break in DNA with subunit A protomers attached covalently to the revealed 5' ends. The site of cleavage induced by quinolone drugs is in the center of the region bound by DNA gyrase (8). The nalidixic acid-resistant *gyrA* mutants such as KNK437 are also resistant to norfloxacin and other analogues such as ciprofloxacin (unpublished results), indicating that these drugs share a common target. Quinolone antibiotics contrast nicely with novobiocin type inhibitors. These latter drugs bind to subunit B and competitively block ATP binding and thereby inhibit reactions of gyrase requiring energy coupling such as supercoiling but not relaxation.

When we began to study the mechanism of action of quinolone antibacterial agents by having [³H]norfloxacin synthesized, we anticipated that the labeled drug would bind selectively and tightly to gyrase subunit A. Surprisingly, we found that it did not but instead bound to pure DNA. We describe here the characterization of this binding and present evidence that it is directly relevant to the mechanism of action of the drug. We suggest that the proximal inhibitor of DNA gyrase-mediated breakage and reunion is the drug-DNA complex.

MATERIALS AND METHODS

Chemical and Reagents. Agarose (type II), Tris, EDTA, and Hepes were from Sigma. *EcoRI* was a product of Boehringer Mannheim. Nalidixic acid and rosoxacin were obtained from Sterling-Winthrop Laboratories. Oxolinic acid was from the Warner-Lambert Research Institute. Norfloxacin and other analogues were synthesized at Abbott; stock solutions of 1-10 mg/ml were stored at pH 10 at 4°C. DNA gyrase holoenzyme was isolated from *E. coli* strain H560 cells as described (9) except that a heparin-Sepharose affinity column (10) was used in place of the DEAE-cellulose column. DNA gyrase subunits A and B with purities >90% were provided by R. Otter and N. Cozzarelli of the University of California (Berkeley). ColE1 DNA was used in all experiments, unless otherwise mentioned. ColE1 and pBR322 DNA were isolated from *E. coli* strains JC411 and HB101, respectively. The isolated DNA contained \approx 95% supercoiled and 5% nicked forms. The preparation of relaxed DNA for the gyrase supercoiling assay was as described (10). The amount of supercoiled DNA formed was determined quantitatively from densitometer traces obtained with an LKB Ultrosan. The inhibition of DNA gyrase by nalidixic acid analogues is noncompetitive; the K_i value was calculated from a Dixon plot of reciprocal velocity versus inhibitor concentration (11). K_i values thus determined for norfloxacin and nalidixic acid were 1.8×10^{-6} and 110×10^{-6} M, respectively. DNA was linearized using *EcoRI*.

Abbreviation: *r*, molar binding ratio.

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[³H]Norfloxacin. [³H]Norfloxacin was synthesized by New England Nuclear using a two-step process designed at Abbott. [³H]Piperazine was prepared by tritiation of pyrazine and treated with 7-chloro-1-ethyl-6-fluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid in *N*-methyl-2-pyrrolidinone overnight at 145°C. The labeled drug has a specific radioactivity of 390 mCi/mmol (1 Ci = 37 GBq). It has the same antibacterial activity, identical mobility on TLC and HPLC, and isoelectric point as authentic norfloxacin.

Binding Experiments. Norfloxacin binding was determined by a membrane filtration method using Centrifree micropartition devices (Amicon, no. 4103) that have thin membranes (YM-T) with a M_r cutoff of 30,000. Each sample consisted of a 400- μ l reaction mixture containing \approx 5 pmol of DNA and various amounts of radioactive ligand in standard buffer (50 mM HEPES, pH 7.4/20 mM KCl/5 mM MgCl₂/1 mM EDTA/1 mM dithiothreitol). After 60 min at 33°C, the mixture was transferred to the Centrifree devices. Controls containing the same amounts of ligand but no DNA were run in parallel to determine the background radioactivity due to free ligand retained in the membrane (\approx 4% of total radioactivity).^{*} Centrifugation at 3000 \times *g* was carried out in a Beckman J-6B centrifuge equipped with a swinging bucket rotor for 45 min, a time longer than necessary for radioactivity on the filter to reach a minimum value. The filtrates were collected in the reservoir on the lower end of the device. The membranes were removed and radioactivity was determined. Bound ligand (mol) was calculated after subtracting background radioactivity. The apparent K_d of norfloxacin is the drug concentration at the midpoint of the saturation curve (12).

The K_d values for a series of nonlabeled nalidixic acid analogues were determined using an indirect competition method. r , the molar binding ratio of drug to DNA, for a constant amount of [³H]norfloxacin was plotted against the concentration of competing analogue. The apparent K_d for a competitive inhibitor can be evaluated from its IC₅₀ value by the equation (13) $K_d = IC_{50}/(1 + S/K_m)$, where K_d and K_m are the dissociation constants for the analogue and [³H]norfloxacin, respectively, and S is the concentration of free [³H]norfloxacin. Because of the biphasic binding of norfloxacin to DNA (Fig. 2A), the inhibition of binding by these analogues was also biphasic: as analogue concentration increased an initial decrease of [³H]norfloxacin binding was followed by an increase due to the gradual taking-over of the nonsaturable second binding phase. Regression analysis of the data points in the first inhibition phase was used to extrapolate to the IC₅₀ values.

Binding was also measured by equilibrium dialysis carried out in a 1-ml Lucite dialysis cell (Bel-Art Products, Pequannock, NJ). Spectrapor 2 membranes (Spectrum Medical Industries, Los Angeles) were used. The filled cell assembly was rotated at 30 rpm at 33°C. The equilibrium time was \approx 2 hr. Binding experiments were carried out with initial ligand concentrations near equilibrium and samples were removed using a Hamilton syringe after equilibrium was attained.

RESULTS

Norfloxacin Does Not Bind to DNA Gyrase But Does Bind to the Substrate DNA. The binding of [³H]norfloxacin to DNA gyrase or its subunits was negligible (Table 1). With 240 pmol of gyrase or subunit B or 1400 pmol of subunit A, only 1–4

Table 1. Binding of [³H]norfloxacin to DNA gyrase, DNA gyrase subunits A and B, and DNA

Sample (pmol)	Ligand bound, pmol	r	Free ligand, μ M
Equilibrium dialysis			
Gyrase (240)	2.58 \pm 2.06	0.0110	1.7
Subunit A (1400)	0.73 \pm 3.64	0.0005	1.7
Subunit B (240)	3.53 \pm 1.29	0.0147	1.7
ColE1 (25)	121.4 \pm 3.1	4.84	1.6
Membrane filtration			
Gyrase (10)	-0.31 \pm 0.80	-0.03	1.9
Subunit A (10)	-0.21 \pm 0.46	-0.02	1.9
Subunit B (10)	-0.33 \pm 0.74	-0.03	1.9
ColE1 (3.4)	19.1 \pm 0.62	5.71	1.8
Magnesium-free ColE1 (10.3)	41.9 \pm 0.84	4.07	1.4
Magnesium-free ColE1 (10.3)/5 mM Mg ²⁺	42.09 \pm 1.21	4.08	1.4

Gyrase holoenzyme was reconstituted by using subunits A and B having purity >90%. The magnesium-free ColE1 DNA used (600 μ g in 1 ml) had been dialyzed against 400 ml of 20 mM EDTA/20 mM Tris pH 7.4/100 mM NaCl, for 3 days with two changes of buffer and then against 200 ml of standard binding buffer for 1 day with one change of buffer.

pmol of bound drug was detected by equilibrium dialysis. These amounts are near the detection limit of the method and thus insignificant. Similarly, negative results were obtained with the membrane filtration technique. The gyrase subunit A and B preparations used in these experiments had specific activities of 8×10^5 and 9×10^4 units/mg of protein, respectively, values comparable with the published values for pure gyrase components (7, 14) and indicating that the species used in these binding experiments were indeed fully active. Under the same experimental conditions, however, norfloxacin bound strongly to DNA in the absence of gyrase. Both the equilibrium dialysis and the membrane filtration methods gave a similar r value (\approx 5) for norfloxacin binding to supercoiled ColE1 DNA. The amount of drug bound increased linearly with DNA concentration (Fig. 1). This ensured the reliability of the method by showing that concentrating the DNA on a filtration membrane did not influence the binding equilibrium. The binding was rapid, the reaction was complete after mixing, and a plateau in binding was maintained for at least 3 hr.

The Initial Binding Phase Takes Place Near the Supercoiling K_t . The binding of norfloxacin to both ColE1 and pBR322 plasmids shows two phases (Fig. 2), a primary saturation phase and a secondary progressive binding phase at higher norfloxacin concentrations. For both plasmids, the primary

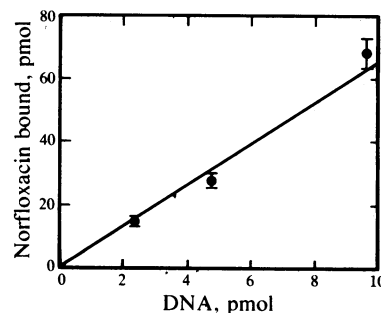


FIG. 1. Dependence of binding of norfloxacin on DNA concentration. Binding of [³H]norfloxacin to ColE1 DNA was determined by the membrane filtration technique. The initial norfloxacin concentration was 3.0 μ M.

^{*}Some newer lots of the Centrifree devices (such as lots MU0068, -0185, and -0186) had thicker and irregular membrane backing material. These membrane discs gave higher background radioactivities and higher standard errors. It is recommended that the membrane lots be tested prior to use. The background radioactivity should be less than that equivalent to 17 μ l of the testing solution and the standard error of the background should be less than \pm 5%.

binding is saturated at a norfloxacin concentration very close to its K_i of 1.8×10^{-6} M. The presence of these narrow saturation plateaus near the K_i is reproducible. If the binding represents the saturation of a single class of binding sites, the apparent K_d is the midpoint of the curve: both plasmids give a K_d of $\approx 1 \times 10^{-6}$ M. The second (weaker) binding phase could perhaps be due to stacking of the dye aggregates on the outside of the DNA helix as has been shown for acridine orange (15). The r value (drug bound per nucleotide) at saturation of the primary event is $\approx 4 \times 10^{-4}$ for both plasmids, an extremely low value.

The Binding of Norfloxacin to DNA Is Dependent on pH and Ionic Strength. The binding of norfloxacin to plasmid DNA has a pH optimum between 4.5 or 6.5 and decreases sharply when the pH is either increased or decreased (Fig. 3A). The midpoints of the decreases occur at pH values of 3.5 and 8.5. The binding of norfloxacin is inhibited by increasing salt concentration (Fig. 3B). At 100 mM KCl, $\approx 50\%$ of the binding was eliminated and, at KCl concentrations $>500 \mu\text{M}$, binding was essentially abolished. Nalidixic acid and its analogues can chelate divalent metal ions (16). To test the possible effect of metal chelate complexes, binding experiments were carried out using DNA that had been extensively dialyzed against EDTA. The presence or absence of magnesium did not affect binding (Table 1).

The Drug Binds Preferentially to Single-Stranded Regions of DNA. As shown in Fig. 2, the r value of norfloxacin to DNA at the K_i is ≈ 5 for ColE1 plasmids and 3 for pBR322. These low values indicate that the target of norfloxacin may be quite specific. The binding of norfloxacin to relaxed, supercoiled, linearized, partially denatured, and denatured ColE1 DNA is shown in Fig. 4. The binding is lowest to the linear and relaxed species and both are $1/4$ th of that of supercoiled DNA. The binding to denatured DNA is 10- to 20-fold greater than that to relaxed and linear DNA, and the amount of binding is proportional to the extent of denaturation. Because of the low r values, distinct saturation plateaus of norfloxacin binding to relaxed and linear DNA are not demonstrable with the sensitivity of the present binding method. However, as shown in Fig. 4, there are about two molecules of norfloxacin bound to each relaxed DNA at the supercoiling inhibition concentration. The presence of gyrase, which presumably is tightly bound to its substrate relaxed DNA, does not change the binding pattern from that of

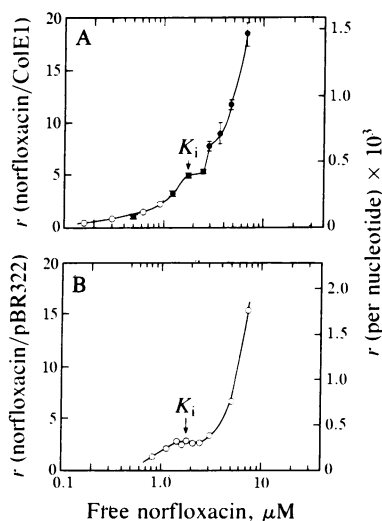


FIG. 2. Binding of norfloxacin to plasmid DNA. Binding mixtures contained 4.7 pmol of ColE1 (A) or pBR322 (B) DNA and the indicated amounts of $[^3\text{H}]$ norfloxacin. Vertical bars represent SDs. Different symbols indicate results obtained from different experiments. Supercoiling K_i values are shown for comparison.

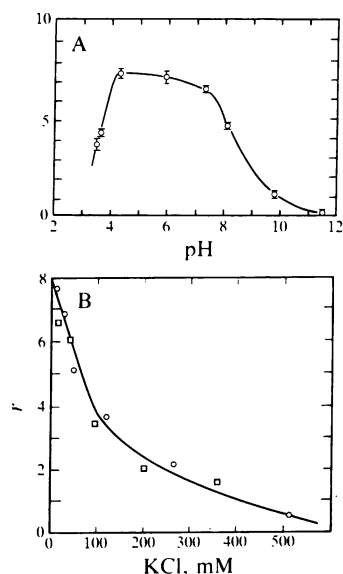


FIG. 3. Effect of pH (A) and salt concentration (B) on binding of norfloxacin to plasmid DNA. The free ligand concentration was $1.8 \mu\text{M}$. (A) HEPES and borate buffers were used for pH values lower and higher than 8.5, respectively. The abscissa represents the pH in sample filtrates. (B) Reaction mixtures contained 3.5 pmol of DNA and the indicated amounts of KCl. Different symbols indicate results obtained from different experiments.

relaxed DNA alone. The preferential binding of norfloxacin to single-stranded rather than to double-stranded DNA and the extremely low molar binding ratio make it less likely that the binding is intercalative. We have performed a series of agarose gel electrophoresis experiments to examine the possible electrophoretic mobility change of covalently closed circular DNA in the presence of the drug. Chloroquine, a weak intercalating agent (27), changes the electrophoretic mobility of supercoiled and relaxed DNA at concentrations as low as $0.3 \mu\text{M}$, whereas norfloxacin did not show any effect at concentrations up to $60 \mu\text{M}$, at which the r value is ≈ 50 (data not shown). The technique, however, is not sensitive enough to test the possible unwinding effect of the very

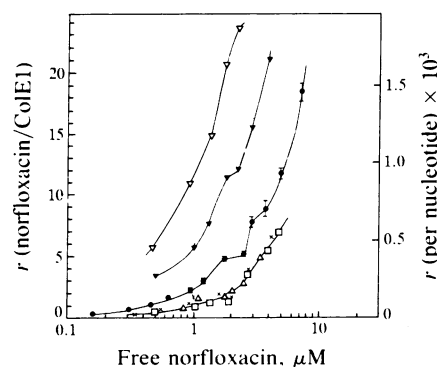


FIG. 4. Binding of norfloxacin to linear (\square), relaxed (Δ), supercoiled (\bullet), and partially (∇) and completely (∇) denatured ColE1 DNA and to gyrase-relaxed DNA complex (\times). Reaction mixtures contained 3.5 pmol of DNA. Partially denatured DNA was prepared by heating the linearized DNA to 90°C for 10 min and then rapidly cooling it to 25°C . Completely denatured DNA was prepared by heating at 98°C for 20 min. Thermal denaturation gave 5% (∇) and 25% (∇) increases in absorbance at 260 nm. The gyrase-relaxed DNA complex was prepared by incubating 3.5 pmol of relaxed DNA with 10 pmol of gyrase holoenzyme in the absence of ATP in $100 \mu\text{l}$ of binding buffer for 60 min before addition of $[^3\text{H}]$ norfloxacin. The curve for supercoiled DNA is the same as that shown in Fig. 2A.

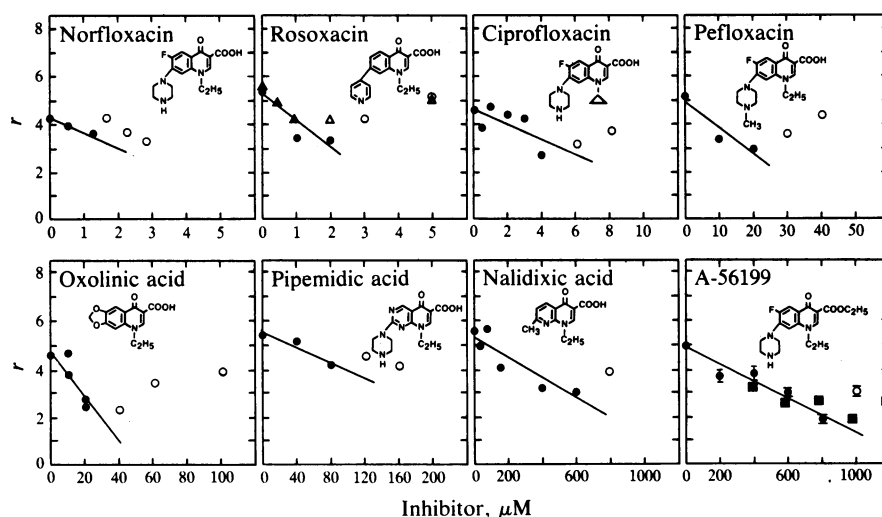


FIG. 5. Competition of [^3H]norfloxacin binding to ColE1 DNA and unlabeled gyrase inhibitors. The data points for the initial inhibition phase (solid symbols) were used to extrapolate to IC_{50} values by a linear regression method. The initial [^3H]norfloxacin concentration was $1.6 \mu\text{M}$.

few norfloxacin molecules (fewer than five) bound to the first strong-binding sites.

Competition by Nalidixic Acid Analogues. Although the similarity of K_d and K_i for norfloxacin suggested that the DNA binding might be directly related to the mechanism of the action of the drug, it could have been coincidental. Therefore, we tested the biological significance of the binding of norfloxacin to DNA by calculating K_d values of a series of unlabeled analogues using their ability to compete with [^3H]norfloxacin binding to DNA at a point near the saturation plateau. It seems more relevant to the test to use relaxed DNA for these experiments. However, because of the low r values for relaxed DNA, we used native supercoiled ColE1 DNA, which gives higher r values and displays a more distinct saturation plateau, and the results were satisfactory. The results of eight competition experiments are shown in Fig. 5. K_d values were calculated from the concentration that halves [^3H]norfloxacin binding, and a log-log plot of those values versus the supercoiling K_i is shown in Fig. 6. It is clear that the K_d values are proportional to their K_i values. This provides strong evidence that the binding of these quinolone drugs to DNA is relevant to their inhibitory potencies. The correlation line in Fig. 6 has slope nearly equal to 1. This further indicates that the binding affinity of

these drugs to DNA may be the sole determinant of their activities.

DISCUSSION

We have shown that norfloxacin, a nalidixic acid analogue, inhibits DNA gyrase by interacting with the substrate DNA. The binding of the drug to DNA was demonstrated by two techniques—equilibrium dialysis and membrane filtration—and they gave comparable results (Table 1). A previous attempt using equilibrium dialysis (17) failed to detect binding of [^3H]nalidixic acid to calf thymus DNA, probably because the concentration of nalidixic acid tested ($1 \mu\text{M}$) is $\approx 1/100$ th of its K_i while the binding of norfloxacin takes place at ligand concentrations near its K_i of $1.8 \times 10^{-6} \text{ M}$ (Fig. 3). Some preliminary reports (4, 18) have also indicated that nalidixic acid does not bind to double-stranded or supercoiled DNA and binds to single-stranded DNA only in the presence of an excess amount of copper ions. These results are not quite consistent with our present finding with norfloxacin, which, to different extents, binds to all these DNA species and does not require divalent ions for its binding (Table 1). The failure to detect nalidixic acid binding to DNA reported in refs. 4 and 18 may arise from the low sensitivity of the UV spectroscopic method used for those studies.

Several pieces of evidence show that the binding to DNA is of biological significance. (i) The drug binds selectively to DNA rather than to gyrase. The presence of the enzyme does not increase the drug binding affinity. The lack of any interaction between the drug and gyrase suggests that the enzyme plays a passive role in the inhibition mechanism. The inhibitory effect of norfloxacin on gyrase is therefore likely to be transferred from an effect generated by the interaction of the drug with DNA. (ii) Norfloxacin binding to DNA displays a saturation plateau at a position corresponding to its K_i (Fig. 3), and both ColE1 and pBR322 plasmids show such a primary binding step. This suggests that the inhibition of gyrase supercoiling activity is associated with occupation of the strong-binding sites. (iii) The most direct evidence comes from the results of competition experiments showing a good correlation between the DNA binding affinities and the DNA supercoiling inhibitory potencies of various nalidixic acid analogues (Fig. 6). The success in the use of the competition method further verifies the existence of the primary saturation process.

The binding saturation at K_i gives an r value of $\approx 10^{-4}$ per nucleotide for relaxed DNA. In other words, a maximum of

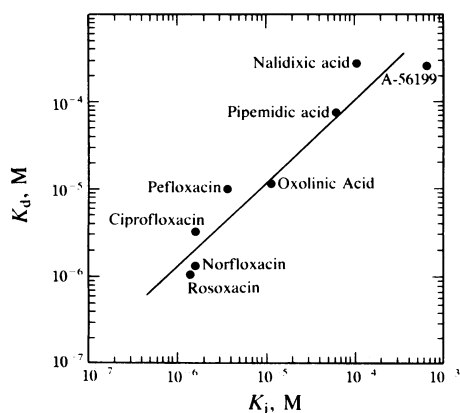


FIG. 6. Log-log plot of apparent K_d values versus supercoiling K_i values of eight DNA gyrase inhibitors. K_d values were obtained using the IC_{50} values determined in Fig. 5. Correlation, 0.963; slope, 0.93.

two drug molecules bound per substrate DNA inhibits supercoiling by 50%. This extreme efficiency implies the existence of specific drug binding sites that, when occupied, interact directly or indirectly with gyrase. The possibilities include that (i) the drug binds to the site at which the enzyme binds on its own, (ii) the presence of a drug molecule creates a high-affinity site for the enzyme, and (iii) the drug or enzyme translocates along the DNA until a ternary complex is formed. To evaluate these three possibilities, we need first to review the nature of gyrase binding to DNA. The binding of gyrase holoenzyme to DNA is extremely tight, with a K_d equal to 10^{-10} M (19), and there are about 140 base pairs in near contact with gyrase as indicated by gyrase's protection effect from nuclease attack (8). Although gyrase interacts at specific sites on a given DNA molecule, it shows no unique nucleotide recognition sequence (20, 21). Moreover, Lothar *et al.* (22) found that gyrase binding sites on minichromosomal DNA containing the origin of replication are scattered around the whole DNA molecule. The most prominent binding site was in fact abolished by oxolinic acid but the randomness of the gyrase binding was not changed. Therefore, possibilities *i* and *ii* seem unlikely. It is probable that gyrase translocates along DNA or DNA segments translocate through gyrase, probably during the first few runs of the supercoiling process, to form a ternary complex with the inhibitor and that this ternary complex is more stable than the binary gyrase-DNA complex.

Information on the chemical nature of the drug binding site is lacking, but one candidate is the single-stranded regions of the DNA. Norfloxacin binds poorest to relaxed circular DNA and linear DNA. The binding is increased by DNA supercoiling, which is known to promote DNA strand separation. The large enhancement of norfloxacin binding to denatured DNA (Fig. 4) supports this hypothesis.

Since the target of the drug is DNA, which is the substrate for numerous enzymes, the question concerning the specificity to DNA gyrase must be answered. The specificity of these drugs may well be explained by the unique functional activities of the enzyme: the transient double-strand breakage, subunit movement to pass a DNA through the break, and the resealing of the break. We speculate that the binding of the drug to the substrate DNA creates a hindrance or blockade to functions unique to DNA gyrase. The binding may not be either strong enough or specific enough to inhibit other enzymes. The drug-resistance mutations in *gyrA* may result in an altered DNA gyrase subunit A structure that is capable of ignoring or overcoming such drug effects.

The conclusion reported here parallels in some ways those of an earlier study on the inhibition of urokinase-induced human plasma clot lysis by L-lysine and its analogues. Those ligands bind not to the enzyme (urokinase) but instead to plasminogen, the substrate. The binding curves are biphasic: initial saturation of a single strong-binding site in the micromolar concentration range followed by saturation of four or five weaker-binding sites in the millimolar range (23). Shen *et al.* (24) have shown that the inhibition of clot lysis by these ligands is associated with saturation of the first, strong-binding site whereas saturation of the second, weaker-binding sites results in conformational changes of the substrate. The low *r* value and the absence of a major conformational change at the IC_{50} value demonstrate the high specificity and efficiency of the inhibitory effect on the enzyme imposed by the ligand-substrate interaction. Recently, Pedrini and Ciarrocchi (25) reported that UV irradiation inhibits the relax-

ation activity of DNA topoisomerase I by formation of photoproducts that change the supercoil conformation. Furthermore, the inhibitory effect of many DNA-binding antitumor drugs on mammalian DNA topoisomerase II (26) is a remarkable analogy to the effect of nalidixic acid on DNA gyrase, in that the two agents similarly stabilize cleavable protein-DNA complexes. The analogous mechanism has been complicated by the general belief that nalidixic acid does not bind to DNA. The finding reported in this paper evidently has solved such a complication.

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