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In Vitro Cleavable-Complex Assay To Monitor Antimicrobial Potency of Quinolones

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Seven quinolones were evaluated to determine whether their ability to generate the DNA gyrase-mediated cleavable complex correlated with their ability to inhibit the catalytic activity of purified DNA gyrase and inhibit the growth of *Escherichia coli*. The rank order of potency of these drugs in the cleavable-complex assay was essentially the same as in the DNA supercoiling-inhibition assay. It required 2- to 10-fold-lower drug concentrations to generate the cleavable complex than to inhibit *E. coli* DNA gyrase. With the newer fluoroquinolones, a 25- to 100-fold-greater concentration was required for DNA gyrase inhibition than for cell growth inhibition, suggesting a more subtle interaction between these inhibitors and DNA gyrase than mere enzyme inhibition.

Topoisomerases are enzymes that regulate the superhelical density of DNA by transiently nicking either one (type I) or both (type II) strands of the DNA helix (reviewed in reference 30). DNA gyrase is a type II topoisomerase which catalyzes the ATP-dependent negative supercoiling of closed circular duplex DNA and is an essential enzyme in *Escherichia coli* (9). It actively maintains the supercoiled state of bacterial DNA and is involved in DNA replication, transcription, and recombination (2). *E. coli* DNA gyrase is an A_2B_2 tetramer; the A subunits mediate DNA breakage and rejoining, while the B subunits bind ATP and participate in energy transduction (9). The subunit A protein is the target of the quinolone family of antibacterial agents, and the newer fluoroquinolones, such as norfloxacin and ciprofloxacin, strongly inhibit the catalytic (strand-passing) activity of DNA gyrase (K. Sato, Y. Inoue, S. Yamashita, M. Inoue, and S. Mitsuhashi, Proc. Workshop 14th Int. Congr. Chemother., p. 21–25, 1986). In addition, nalidixic acid has been shown to stabilize a gyrase-DNA complex, which upon protein-denaturant treatment results in DNA double-strand breaks and the covalent linking of the *gyrA* subunit to the 5′-phosphoryl end of the broken DNA (6, 19). Similarly, certain intercalative antitumor drugs [e.g., 4′-(9-acridinylamino)methanesulfon-*m*-aniside and Adriamycin] are thought to interfere with the normal breakage-reunion reaction of mammalian DNA topoisomerase II by stabilizing a reversible enzyme-DNA complex which has been referred to as the cleavable complex (20).

Domagala and co-workers (4) were the first to systematically compare a variety of quinolones with respect to their potency against DNA gyrase versus their MICs against intact microorganisms. They monitored the ability of drugs to inhibit the gyrase-catalyzed conversion of relaxed plasmid DNA to its native supercoiled form and their ability to linearize supercoiled plasmid DNA. In this study, we sought to determine whether the potency of various quinolones could be assessed by using the cleavable-complex assay, an in vitro technique which uses uniquely 3′-end-labeled, linear DNA as substrate (16, 20). The potency of selected quinolones to generate the cleavable complex was correlated with their ability to inhibit negative supercoiling of DNA and to inhibit the growth of intact microorganisms.

Bacterial strains. The bacterial strains used are listed in Table 1.

Compounds. The compounds tested are shown in Table 2. BW A855U is 6-[(2,4-diamino-5-pyrimidinyl)-methyl]-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid-hydrochloride.

Susceptibility testing. The MICs were determined by using a semiautomated broth microdilution system (Dynatech Laboratories, Inc., Alexandria, Va.) with Wellcotest broth, a thymidine-free, all-purpose bacteriological growth medium.

Enzymes. (i) DNA gyrase. The A subunit of DNA gyrase was prepared by using *E. coli* RW1053(pMK90), and the GyrB subunit was prepared by using *E. coli* RW1053 (pMK47), as previously described (18). On the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the A and B subunits were estimated to be 90 to 95% pure. Functional DNA gyrase was reconstituted by adding 5 μl of GyrA protein and 10 μl of GyrB protein to 25 μl of preincubation buffer as described by Mizuuchi et al. (18).

(ii) Nicking-closing enzyme. Nicking-closing extracts (DNA topoismerase I) were prepared from duck reticulocytes according to published procedures (21). Reticulocytes were obtained from the blood of ducks treated with 1-acetyl-2-phenylhydrazine (supplied by Granite Diagnostics, Burlington, N.C.).

Nucleic acids. (i) Plasmid DNA. pUC19 and pBR322 plasmid DNAs were isolated from *E. coli* strains (pUC19) by using a discontinuous, two-step CsCl-ethidium bromide gradient technique described by Garger et al. (8). Relaxed, covalently closed, circular plasmid DNA was obtained by treating purified plasmid DNA with nicking-closing extract (DNA topoisomerase I) as previously described (21).

(ii) End-labeled [^{32}P]ATP pBR322 DNA. The procedure for 3′-end labeling *Eco*RI-digested pBR322 DNA was done as previously described (16).

Supercoiling-inhibition assay. Relaxed, covalently closed circular pUC19 and pBR322 DNAs were used to assay for the ability of selected quinolones to inhibit the strand-passing activity of purified *E. coli* DNA gyrase. The reaction conditions were described previously (10). The reaction

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TABLE 1. *E. coli* strains used in this study

Strain	Source[a]	Comment
RW1053(pMK90)	M. Gellert (NIH)	Plasmid pMK90 contains the *gyrA* gene (12)
RW1053(pMK47)	M. Gellert (NIH)	Plasmid pMK47 contains the *gyrB* gene (12)
CN314	BW culture collection	Clinical isolate
CN348	BW culture collection	Clinical isolate
P855	BW culture collection	Clinical isolate
P148	BW culture collection	Clinical isolate
P39	BW culture collection	Clinical isolate
P27	BW culture collection	Clinical isolate

[a] NIH, National Institutes of Health; BW, Burroughs Wellcome Co.

mixture containing 1 U of enzyme (in preliminary experiments, 1 U of enzyme was found to be the smallest amount of enzyme that catalyzes the conversion of 0.25 μg of relaxed DNA to the fully supercoiled form in 30 min at 37°C under our assay conditions) (7), 0.25 μg of relaxed pUC19 or pBR322 DNA, and drug solution was incubated at 37°C for 30 min. Sodium dodecyl sulfate (1.3% final concentration) was added to stop the reaction. Relaxed and supercoiled plasmid DNAs were separated by using 0.8% agarose gel electrophoresis. Gels were stained with ethidium bromide and photographed by using UV light.

Cleavable-complex assay. Assays for *E. coli* gyrase-mediated DNA cleavage were performed with reaction mixtures (20 μl) containing 35 mM Tris hydrochloride (pH 7.5), 8 mM $MgCl_2$, 24 mM KCl, 1.8 mM spermidine hydrochloride, 0.14 mM EDTA, 5 mM dithiothreitol, 6.5% (wt/vol) glycerol, 1.4 mM ATP, 9 μg of *E. coli* tRNA per ml, 0.36 mg of bovine serum albumin per ml, 3'-end-labeled ^{32}P-pBR322 DNA (approximately 30 ng), *E. coli* DNA gyrase (approximately 20 U of enzyme), and drugs as indicated (see Fig. 2). Reactions were incubated at 37°C for 30 min and then treated with proteinase K (1.5 mg/ml; 2 μl) and 10% sodium dodecyl sulfate (2 μl) and incubated at 50°C for an additional 30 min. Reaction products were analyzed on a horizontal, 1% agarose gel in TBE buffer (89 mM Tris borate [pH 8.3], 2.0 mM EDTA) electrophoresed for 16 h at 40 V. DNA was transferred onto nitrocellulose membrane filters (Schleicher & Schuell, Inc., Keene, N.H.) as previously described (26). Filters were autoradiographed at −80°C by using Kodak XAR-5 film plus intensifying screens.

Figure 1 shows the inhibition of supercoiling activity of DNA gyrase by the seven quinolones. Each compound is represented by two lanes; the concentration in the left lane represents approximately the lowest concentration that inhibited DNA supercoiling, whereas the concentration on the right is essentially a "no-effect" concentration for that particular compound or drug. The experiment whose results are shown in Fig. 1 was done with the plasmid pUC19. Similar experiments were done with relaxed, covalently closed, circular pBR322 plasmid DNA as a substrate with identical results (data not shown). The precise endpoints for these compounds in the supercoiling-inhibition assay are shown in Table 2. An examination of Table 2 shows that there is wide range of potency within this series of compounds. For example, ciprofloxacin and norfloxacin were 100 times more potent than nalidixic acid in inhibiting the supercoiling activity of *E. coli* DNA gyrase.

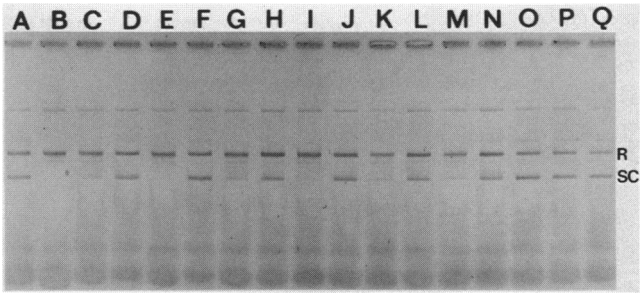

FIG. 1. Agarose gel electrophoresis assay of inhibition of DNA gyrase supercoiling activity. The positions of relaxed (R) and supercoiled (SC) pUC19 DNA are indicated. The reactions were performed as described in the text. Lanes: A, enzyme control (DNA gyrase plus DNA; no inhibitor); B, relaxed DNA control (no enzyme and no inhibitor). Incubation of DNA gyrase plus relaxed DNA was done in the presence of various quinolones at the concentrations indicated. Lanes: C and D, nalidixic acid at 100 and 50 μM, respectively; E and F, oxolinic acid at 20 and 10 μM, respectively; G and H, norfloxacin at 0.5 and 0.1 μM, respectively; I and J, ciprofloxacin at 1.0 and 0.2 μM, respectively; K and L, ofloxacin at 2.0 and 0.5 μM, respectively; M and N, CI-934 at 2.0 and 0.5 μM, respectively; O, P, and Q, BW A855U at 1,000, 250, and 50 μM, respectively.

The results of a similar analysis using the cleavable-complex assay are presented in Fig. 2. Similar to Fig. 1, each drug is represented by two lanes; the concentration in the left lane represents approximately the lowest concentration of each drug that generated a cleavable complex, whereas the concentration on the right is essentially a no-effect concentration for that particular drug. Ethidium bromide, an avid DNA intercalator, does not stimulate mammalian DNA topoisomerase II-mediated DNA cleavage (28). As can be seen in Fig. 2, it did not generate DNA gyrase-mediated DNA cleavage either. It is also of interest to note that the cleavage patterns for the six active quinolones are very

TABLE 2. Comparison of DNA gyrase results with growth inhibition against six clinical isolates of *E. coli*

Compound or drug	Inhibitory concn (μM)		Avg MIC[a]	
	Supercoiling-inhibition assay	Cleavable-complex assay	μM	μg/ml (range)
BW A855U	>2,500	>1,000	>240	>100
Nalidixic acid	100	50	13	3.0 (1.0–10.0)
Oxolinic acid	20	10	1.3	0.3 (0.1–0.6)
CI-934	2.0	0.2	0.10	0.04 (0.03–0.10)
Ofloxacin	2.0	0.2	0.08	0.03
Norfloxacin	1.0	0.2	0.06	0.02 (0.01–0.03)
Ciprofloxacin	1.0	0.1	0.009	0.003 (0.001–0.004)

[a] MIC results for six *E. coli* clinical isolates were simply averaged.

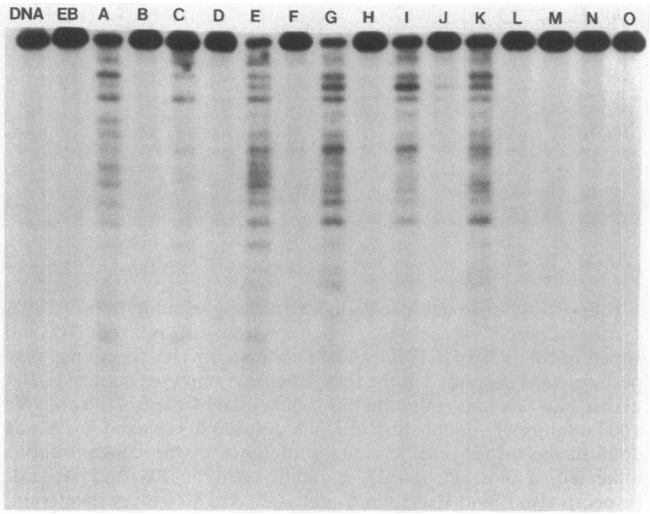

FIG. 2. DNA breakage by various quinolones mediated by *E. coli* DNA gyrase. Assays for topoisomerase II-mediated DNA cleavage were performed as described in the text. Lane DNA was the enzyme control (DNA gyrase plus end-labeled pBR322 DNA; no drug). Incubation of DNA gyrase plus end-labeled pBR322 DNA was done in the presence of various compounds at the concentrations indicated. Lanes: EB, ethidium bromide at 100 μM; A and B, nalidixic acid at 50 and 20 μM, respectively; C and D, oxolinic acid at 10 and 2 μM, respectively; E and F, norfloxacin at 0.5 and 0.1 μM, respectively; G and H, ciprofloxacin at 0.1 and 0.02 μM, respectively; I and J, ofloxacin at 0.2 and 0.05 μM, respectively; K and L, CI-934 at 0.2 and 0.05 μM, respectively; M, N, and O, BW A855U at 1,000, 250, and 50 μM, respectively.

similar. This is consistent with previous studies on the interaction of certain intercalative antitumor drugs with mammalian DNA topoisomerase II in which drugs of the same chemical class were shown to stimulate cleavage at similar sites while drugs of different chemical classes showed strikingly different cleavage patterns (28). Table 2 indicates that the rank order of potency for these particular drugs in the cleavable-complex assay is essentially the same as that in the supercoiling-inhibition assay. The main difference is one of sensitivity in that it appears to require 2- to 10-fold-lower drug concentrations to generate the cleavable complex than to inhibit the strand-passing activity of DNA gyrase.

Table 2 also compares the minimum drug concentrations necessary to elicit an effect in the two in vitro DNA gyrase assays and those required to inhibit cell growth. Of the seven quinolones tested, ciprofloxacin and norfloxacin were the most active in the enzyme assays and were the most potent inhibitors of cell growth. This generality appears to hold throughout this series, including the inactive compound, BW A855U. However, these values are not always directly proportional; for example, norfloxacin and ciprofloxacin were essentially equipotent in the gyrase assays and yet ciprofloxacin was about sevenfold more active against the *E. coli* strains tested in this study. This nonproportionality has been noted by others (4, 32) and presumably reflects basic differences in the cell permeation properties of different quinolones.

The most notable nonproportionality can be seen between the inhibitory concentrations for gyrase activity and cell growth with some of the fluoroquinolone antibacterial agents tested. For example, there are 25- and 100-fold-greater concentration requirements for gyrase inhibition than for cell growth inhibition for ofloxacin and ciprofloxacin, respectively (Table 2). In several other studies, it has been shown that the concentration of quinolones required to inhibit the in vitro catalytic activity of gyrase is substantially higher than that required to inhibit the growth of the organism from which the gyrase was purified, including *Pseudomonas* spp. (17), *Micrococcus luteus* (7, 32), and *E. coli* (25, 27). Gellert et al. (11) reported that it required 50 times the concentration of oxolinic acid to inhibit the DNA gyrase-mediated supercoiling reaction than to inhibit cell growth. They suggested the possibility of a more subtle interaction between these inhibitors and DNA gyrase than mere enzyme inhibition. The possible involvement of the SOS response in the bactericidal effect of nalidixic acid and its active congeners has been postulated by several investigators (1, 22, 23). The SOS response is an inducible mechanism for processing DNA damage which, in *E. coli*, involves a number of diverse functions, including increased recombination and DNA repair, enhanced mutagenesis, prophage induction, and filamentation (reviewed in references 15 and 29). Genetic studies have shown that nalidixic acid and the newer fluoroquinolone antibacterial agents are potent inducers of SOS repair functions (22, 23, 30, 31). Based on these observations, it has been suggested that the basis of lethality for the quinolone antibacterial agents may involve the generation of a critical number of DNA double-strand breaks, via the cleavable complex, which in turn results in the induction of the SOS response (3, 5, 23). Presumably, much less drug would be required to generate a crucial threshold of DNA damage than would be needed to titrate all of the DNA gyrase of the cell. This would help explain the large discrepancy between the concentration of drug required to inhibit the DNA gyrase-mediated DNA supercoiling reaction and that required to inhibit intact microorganisms. Adding to the apparent complexity of the molecular nature of the interaction of quinolones with their target is the finding of Hooper and Wolfson (13), who showed that low levels of inhibition of DNA supercoiling occur at quinolone concentrations approaching those inhibiting bacterial growth. Thus, it is possible that, within the cell, only minimal levels of supercoiling inhibition or inhibition of supercoiling within specific domains of DNA are critical (13).

Bacterial diploids formed from the hybridization of nalidixic acid-resistant gyrase mutants and drug-susceptible wild-type cells retain susceptibility to the drug (12). This finding is most easily explained by a mechanism which requires the formation of the cleavable complex as a prerequisite for lethality rather than one which requires substantial inhibition of the catalytic activity of all the available DNA gyrase. With respect to this possibility, Kreuzer and Cozzarelli (14) have proposed that nalidixic acid-mediated inhibition of bacteriophage T7 growth and replication results from a "poisoning" rather than simple elimination of the target, DNA gyrase. Thus, the trapped ternary complex of drug, DNA gyrase, and DNA, postulated as the crucial event in nalidixic acid-mediated T7 growth inhibition, might be analogous to the cleavable complex (14).

A couple of cautionary notes regarding these experiments are in order. In this study we used and compared the results generated by three substantially different assays. One consists of purified gyrase and uniquely 3′-end-labeled linear DNA and is noncatalytic (cleavable-complex assay); the second assay uses purified enzyme and relaxed, covalently closed, circular plasmid DNA and is catalytic (supercoiling-inhibition assay); and the third assay involves living cells (inhibition of growth). Although the rank orders of potency of the seven quinolones tested in this study were essentially

the same in all three assays, there is no guarantee that comparisons of different kinds of compounds will be nearly so straightforward or as apparently easy to interpret. The second caveat is that we used clinical isolates of *E. coli* in this survey and these strains were not characterized with respect to their competence or incompetence to mount an SOS response. It might be useful, therefore, to expand this type of survey to include mutants defective in certain components of the SOS system (e.g., Lon protease) and determine whether susceptibility to quinolones increases or decreases in these mutant organisms.

The accumulating body of evidence with respect to the mode of action of the quinolones against DNA gyrase and the epipodophyllotoxins against mammalian DNA topoisomerase II (24) suggests that inhibition of supercoiling may not be the most valid criterion for predicting the potency of either class of drugs. It has been suggested that other assays, e.g., phage replication, DNA replication, or strand breakage, might provide a more direct indication of the effects of these particular drugs on critical intracellular events (32). The results of this study suggest that the in vitro cleavable-complex assay may be a useful and predictive technique to monitor the antibacterial potency of quinolones.

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