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Correlation of Quinolone MIC and Inhibition of DNA, RNA, and Protein Synthesis and Induction of the SOS Response in *Escherichia coli*

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The effects of nalidixic acid and four fluoroquinolones on DNA, RNA, and protein synthesis in the presence and absence of 20 mg of chloramphenicol per liter were examined by comparing the killing kinetics, MIC, morphological response, and maximum concentration to induce *recA* in *Escherichia coli*. All agents demonstrated paradoxical killing kinetics, in that above an optimum concentration the rate of bactericidal action was slower. Filamentation of *E. coli* AB1157 was observed with all quinolones up to the optimum bactericidal concentration. Addition of chloramphenicol reduced the bactericidal activity, inhibited filamentation, and abolished *recA* induction, but it had no effect on DNA synthesis inhibition by any of the agents. Excellent correlation was obtained between the concentration required to inhibit DNA synthesis by 50%, the MIC, the maximum concentration to induce *recA*, and the optimum bactericidal concentration. Evidence from this study and previously published data suggest that the primary mechanism of action of quinolones is independent of the SOS response and does not require active protein synthesis; however, induction of *recA* and SOS responses is consequential and enhances cell death.

The precise mechanisms of action of quinolone (including fluoroquinolone) antimicrobial agents have yet to be determined; however, one of the major interactions of quinolones is that with the enzyme DNA gyrase (topoisomerase II). It has been shown by two assays (one that measures the conversion of relaxed plasmid DNA to its native supercoiled form and the other that measures the production of linear DNA in a cleavage reaction) that quinolones inhibit the supercoiling activity of DNA gyrase (10, 15, 32, 36). It has been postulated that nalidixic acid inhibits the resealing of DNA that occurs at the replication fork catalyzed by DNA gyrase, thereby preventing supercoiling (11). It is proposed that the complex of quinolone plus DNA gyrase is bound to the DNA, forming a replication fork barrier and allowing the accumulation of gapped or single-stranded DNA (25), which has been shown to accumulate in nalidixic acid-treated *Escherichia coli* (7). Quinolones induce the SOS response (DNA repair mechanism [4, 14, 22, 23]), the inducing signal for which is thought to be gapped DNA (25). While there is a large body of evidence that DNA gyrase is the major target of quinolones, measurements of the concentration of a quinolone required to inhibit in vitro supercoiling activity often produce values that are 10-fold higher than the MIC (10, 16, 27). The poor correlation is unlikely to be due to a partially denatured DNA gyrase preparation (A. Maxwell, personal communication).

The concentration of a quinolone needed to inhibit in vivo DNA synthesis by 50% (IC_{50}) and quinolone MICs show an excellent correlation (2; J. M. Diver, L. J. V. Piddock, and R. Wise, Proc. 15th Int. Congr. Chemother., abstr. 985, 1987), and it has therefore been hypothesized that the initial reaction in the cascade of events causing quinolone-induced cell death is DNA gyrase-mediated inhibition of DNA synthesis independent of supercoiling (2).

It has been shown that quinolones are rapidly bactericidal up to a specific concentration, above which there is a reduced rate of kill (6, 29). The numbers of organisms surviving the optimum bactericidal concentration (OBC), sometimes called the MBC (29), are quinolone and time dependent. For ciprofloxacin, 0.0001% of the original inoculum may survive the OBC; however, for nalidixic acid, up to 10% of the original inoculum may survive (9). In actual numbers this would be equivalent to thousands of surviving bacterial cells. For nalidixic acid, the slower rate of kill at higher concentrations has been shown to be associated with the inhibition of RNA and protein synthesis (8). It has also been shown that at concentrations up to the OBC, the bacterial cells are filamented, and it is proposed that this is a consequence of SOS induction (9, 23; Diver et al., 15th Int. Congr. Chemother.).

In this study we examined the inhibition of DNA, RNA, and protein synthesis by quinolones, the induction of the SOS response, and the morphological changes exerted by these agents to determine the role of the DNA repair processes (and their consequent inhibition of cell division) in the mechanism of quinolone-induced cell death.

(This study was presented in part at the 15th International Congress on Chemotherapy [Diver et al., 15th Congr. Chemother.].)

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 AB1157 (*thr leu oriA his orgE aro lac gal mtl xyl thl rpsl tsx supE*) and its derivative *E. coli* GC 2241 containing a *recA*::*lac* fusion were obtained from S. Casaregola and were used throughout this study.

Media and antibiotics. Cultures were grown in Davis minimal broth (Difco) supplemented with 10 mg of thiamine per liter (Sigma)–0.2% glucose (BDH)–0.4% Casamino Acids

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TABLE 1. Concentration of quinolone required to inhibit growth and DNA, RNA, and protein synthesis and to induce RecA in *E. coli* AB1157

Quinolone	Concn (mg/liter)							
	Minimum inhibitory	DNA synthesis inhibition		RNA synthesis inhibition	Protein synthesis inhibition	RecA protein induction		Optimal bactericidal
		IC_{50}	IC_{90}			Minimum	Maximum	
Nalidixic acid	4	4.5	100	200	56	1.02	10	20
Ciprofloxacin	0.014	0.016	0.074	1.7	0.56	0.0019	0.01	1
Fleroxacin	0.100	0.0500	0.1	3.8	3.0	0.0095	0.075	10
Norfloxacin	0.120	0.058	0.5	4.3	1.4	0.0023	0.250	10
Enoxacin	0.160	0.126	0.27	8.5	2	0.0160	0.250	10

(Difco). Antimicrobial agents were kindly provided as gifts, as follows: nalidixic acid, Sterling-Winthrop; ciprofloxacin, Bayer AG; enoxacin and chloramphenicol, Parke-Davis-Warner-Lambert; norfloxacin, Merck Sharpe & Dohme; fleroxacin, Hoffmann-La Roche.

Susceptibility testing. MICs were determined in Davis minimal broth by a microtiter broth dilution procedure with an inoculum of 10^5 CFU per well. MICs were recorded after 18 h of incubation under aerobic conditions at 37°C.

Killing kinetics and morphological response of quinolones. A quinolone or a quinolone plus 20 mg of chloramphenicol per liter was added to parallel flasks containing 100 ml of AB1157 at the mid-exponential growth phase (at approximately 10^7 CFU/ml to correspond to other assays), and the flasks were incubated with shaking at 37°C. Samples were withdrawn at timed intervals, examined by differential interference microscopy (Carl Zeiss), diluted in phosphate-buffered saline, and cultured on Iso-Sensitest agar to determine the viable count.

Determination of DNA, RNA, and protein synthesis. The DNA, RNA, and protein synthesis assays were performed essentially as described previously (3, 24, 26). The incorporation of radiolabeled nucleic acids into ice-cold 5% trichloroacetic acid-precipitable material of *E. coli* AB1157 was measured over 30 min, precipitates were collected by vacuum filtration through glass pore filters, and the radioactivity was measured by scintillation counting. Inhibition of early DNA synthesis was also measured by the methods of Drlica et al. (12) and a modification of the method described by Schubach et al. (26). The incorporation of [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine (DNA, RNA, and protein synthesis assays, respectively) was measured in the presence of logarithmic dilutions of quinolone. DNA synthesis inhibition was measured with and without the presence of 20 mg of chloramphenicol per liter. The concentration of quinolone that inhibited 50 or 90% of the organisms (IC_{50} or IC_{90}, respectively) was calculated from the linear portion of the sigmoidal curves.

Quantitative estimation of RecA protein. The quantitative estimation of the RecA protein was performed essentially as described previously (1). Briefly, parallel flasks containing 100 ml of GC 2241 cells in the mid-exponential growth phase were inoculated with test antibiotic at logarithmic dilutions (0.001 to 1,000 mg/liter) and incubated with shaking at 37°C. Samples were withdrawn at 15-min intervals (up to 2 h), and assayed for β-galactosidase (21). Enzyme concentrations (units per milliliter) were calculated from the formula given by Casaregola et al. (1). An arbitrary value of 60 U/ml was chosen to estimate the minimum inducing concentration of quinolone (23).

RESULTS

Susceptibility, killing kinetics, and morphology. The MIC of each agent is given in Table 1; the MICs were typical of those for a normal susceptible strain of *E. coli*, with ciprofloxacin being the most active agent and nalidixic acid being the least active. Fleroxacin, norfloxacin, and enoxacin displayed similar activities. The killing kinetics of three of the test agents at the OBC are shown in Fig. 1. The four fluoroquinolones killed *E. coli* AB1157 more rapidly than nalidixic acid did, with the most active agent also being the agent that killed quickest, ciprofloxacin. Figure 2 demonstrates the dose-response curves of strain AB1157 at 1.5 h for the fluoroquinolones and 3 h for nalidixic acid. All the agents killed the organism at different rates; however, to compare the effect of an increase in the concentration of each agent with the bactericidal activity, i.e., a dose response, data from a single time period were usually compared. It was found that for all agents except nalidixic acid, data obtained after 1.5 h of exposure could be compared. After 1.5 h of exposure to nalidixic acid, little killing of strain AB1157 was observed, and so data from 3 h of exposure are shown in Fig. 2. After 3 h of exposure to fluoroquinolones, high concentrations killed all of the culture (data not shown). All five agents demonstrated a paradoxical response; i.e., with an increase in antibiotic concentration there was an increase in bactericidal action up to a certain concentration, and above this OBC, a decrease in bactericidal activity was observed. This response was time dependent and reflected the rate of kill (Fig. 1), and so after 8 h of exposure no paradoxical effect was observed for any of the test agents.

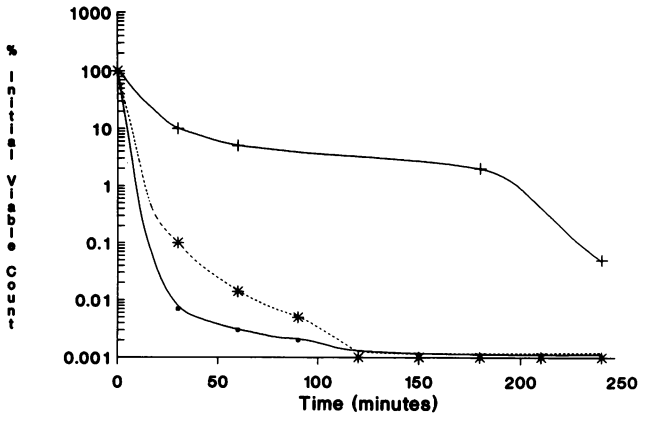

FIG. 1. Killing kinetics of *E. coli* AB1157 by ciprofloxacin at 1 mg/liter (■), nalidixic acid at 10 mg/liter (+), and fleroxacin at 10 mg/liter (*).

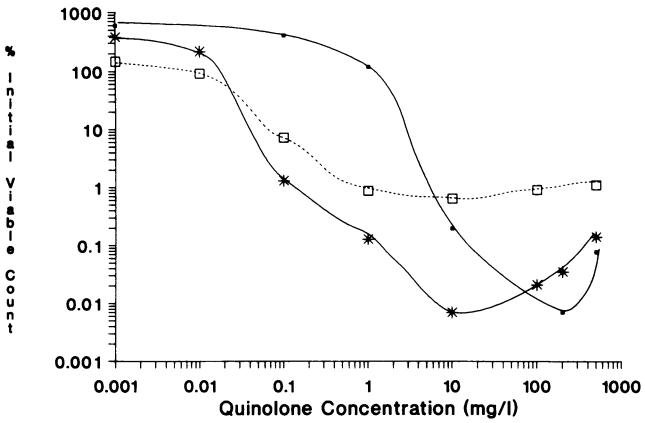

FIG. 2. Dose response of *E. coli* AB1157 to nalidixic acid after 3 h of incubation (■), fleroxacin after 1.5 h of incubation (*), and fleroxacin and 20 mg of chloramphenicol per liter after 1.5 h of incubation (□).

The typical morphological response of the bacteria observed after 1.5 h of exposure to the quinolones was the same as that described previously after exposure to ciprofloxacin (9). With an increase in the quinolone concentration, the cells elongated, and at the OBC maximum filamentation and vacuolation were observed (data not shown). Above the OBC little filamentation was seen, and the bacteria were either a normal rod shape or slightly ovoid.

Addition of 20 mg of chloramphenicol per liter (which was shown to be sufficient to inhibit protein synthesis in *E. coli* AB1157 without being bactericidal; data not shown) concurrently with any of the test quinolones to the bacterial culture significantly reduced but did not completely inhibit the filamentation and bactericidal actions of the fluoroquinolones. The lengths of the cells exposed to fluoroquinolone plus chloramphenicol were approximately double those of antibiotic-free controls; however, no filaments were observed.

Inhibition of DNA, RNA, and protein synthesis. DNA synthesis was inhibited by all quinolones tested (Fig. 3 and Table 1), with ciprofloxacin having the lowest IC_{50} and IC_{90} and nalidixic acid having the highest.

The IC_{50} and IC_{90} data correlated well with the MIC;

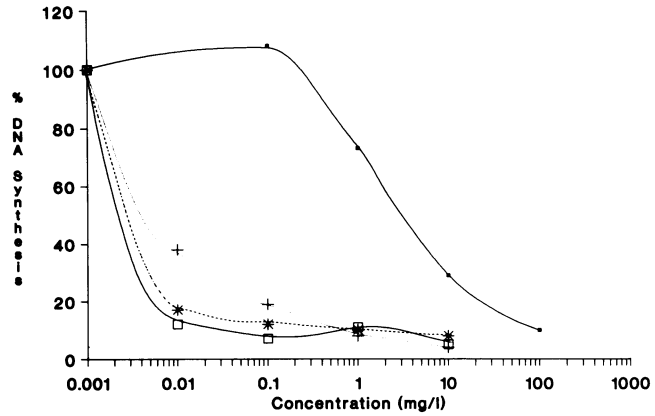

FIG. 3. Inhibition of DNA synthesis in *E. coli* AB1157 after 30 min of exposure to nalidixic acid (■), ciprofloxacin (*), norfloxacin (+), and ciprofloxacin and 20 mg of chloramphenicol per liter (□).

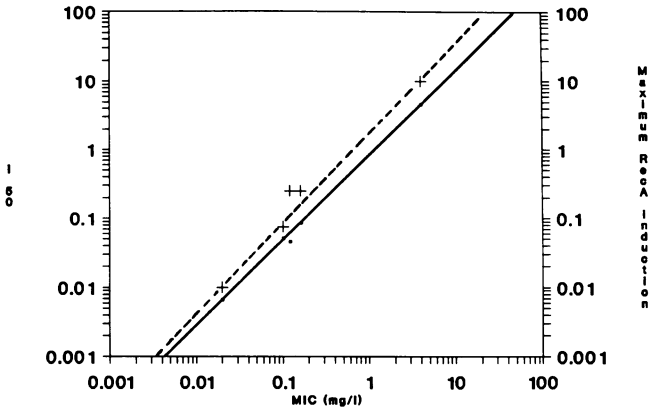

FIG. 4. Correlation of MIC with the IC_{50} for DNA synthesis (+) and the maximum *recA*-inducing concentration (■).

however, in this study a consistent and reproducible finding was that, numerically, the IC_{50} correlated better with the MIC than it did with the IC_{90}. Using nalidixic acid, ciprofloxacin, and fleroxacin and the method of Drlica et al. (12), we attempted to measure numerous times (n = 7) an initial early phase of DNA synthesis inhibition. In each experiment there was a decrease in DNA synthesis similar to those described by Drlica et al. (12) and Chow et al. (2); however, this decrease was also seen in the control. We believe that this decrease is an artifact of the experimental procedure. Measurement of DNA synthesis at 10, 30, 60, 90, and 120 min after exposure to quinolones yielded similar IC_{50} data, 0.02, 0.01, 0.008, and 0.006 μg of ciprofloxacin per ml and 1, 4.5, 2, and 1.5 μg of nalidixic acid per ml, respectively, and therefore, we reported inhibition data for quinolone exposure times of 30 min. The IC_{50}s of fleroxacin and norfloxacin were very similar; however, the IC_{50} of enoxacin was nearly double those of fleroxacin and norfloxacin. There was a good correlation between the MIC and the IC_{50} (Fig. 4) (correlation coefficient, 0.99).

All the agents also inhibited RNA and protein synthesis but at far higher concentrations than were required to inhibit DNA synthesis (Table 1). The data from these experiments for fleroxacin and enoxacin were similar to those for norfloxacin. Approximately one-quarter of the concentration of quinolone required to inhibit RNA synthesis by 50% was required to inhibit protein synthesis by 50%. One hundredfold higher levels of fleroxacin, enoxacin, and norfloxacin were required to inhibit RNA synthesis by 50% compared with those required to inhibit DNA synthesis; for nalidixic acid and ciprofloxacin, 200-fold higher levels were required. The rank order of quinolones and their activity and inhibition of DNA and RNA synthesis were the same. In this study, chloramphenicol was shown to partially inhibit the bactericidal activity of the quinolones; however, addition of 20 mg of chloramphenicol per liter did not affect the inhibition of DNA synthesis by any of the test agents.

Induction of RecA protein. All agents induced RecA protein expression, which was time and concentration dependent. With an increase in quinolone concentration there was an increase in the quantity of RecA protein, until a maximum quinolone concentration was achieved, after which the expression decreased with an increase in quinolone concentration (Fig. 5). All agents induced the expression of *recA* and, therefore, production of RecA protein below the MIC (Table 1); however, there was no clear correlation between the minimum inducing concentration and the MIC. Replicate

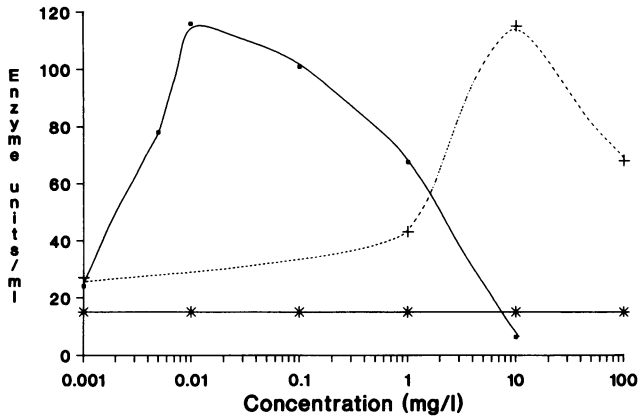

FIG. 5. Induction of *recA* protein in *E. coli* GC2241 by nalidixic acid (+), ciprofloxacin (■), and 20 mg of chloramphenicol per liter (*)

experiments showed that the values obtained for the maximum *recA*-inducing concentration and the MIC were very similar (Fig. 4) (correlation coefficient, 0.99). Chloramphenicol at 20 mg/liter abolished induction.

DISCUSSION

The paradoxical dose-response curves obtained for the test agents in this study are typical of those for quinolones. This phenomenon has been described previously for nalidixic acid (6, 9, 29, 30, 31), norfloxacin (5, 29), and ciprofloxacin (9, 30). Crumplin and Smith (6) noted that extension of the incubation time of *E. coli* with nalidixic acid decreased the percentage of surviving cells, although 50 to 100 mg/liter remained the most active concentration. In this study the paradoxical response for all the agents examined was time dependent. Cells grown at concentrations above the OBC died more slowly than cells grown at concentrations below the OBC did, so at early times (e.g., 30, 60, and 90 min) a paradoxical response was seen. However, at high concentrations after prolonged exposure, surviving viable bacteria were rarely detected. Chloramphenicol has been shown to inhibit the bactericidal actions of nalidixic acid (6, 9, 29), norfloxacin (30), and ciprofloxacin (9, 29), and in this study we obtained similar data for these agents, as well as for enoxacin and fleroxacin. However, the incomplete inhibition of killing by rifampin and chloramphenicol has also been observed for ciprofloxacin and ofloxacin, and so two mechanisms of quinolone killing have been proposed (30; J. T. Smith, personal communication): mechanism A, which is common to all quinolones and which can be abolished by the inhibitors of protein and RNA synthesis, and mechanism B, which is an additional mechanism unique to ciprofloxacin and ofloxacin and is independent of protein and RNA synthesis. As shown by ourselves and Chow et al. (2) and extended in this study, there is an excellent correlation between the inhibition of DNA synthesis by quinolones and their respective MICs, unlike the data derived from supercoiling assays. The IC_{50}s for DNA synthesis that were obtained for nalidixic acid in this study are very similar to those obtained for *E. coli* by Winshell and Rosenkrantz (35) and by Stevens (31). The IC_{50}s of ciprofloxacin and norfloxacin for DNA synthesis obtained in this study were very similar to those obtained by Chow et al. (2), even though the exposure time of the cells to quinolone was longer in this study. Chloramphenicol at 20 mg/liter had no effect on the inhibition of DNA synthesis by any of the agents studied; a similar observation was made by Winshell and Rosenkrantz (35) for nalidixic acid.

The values obtained for quinolone inhibition of protein and RNA synthesis were greater than the MIC, as has previously been shown for nalidixic acid and norfloxacin (5, 6, 31). The value for the inhibition of protein synthesis (IC_{50}) by nalidixic acid was lower than values published previously (6, 31). In both previous studies (6, 31), the concentrations of quinolone needed to inhibit RNA and protein synthesis were similar, and extrapolation from the graphical data would give IC_{50}s of approximately 350 mg/liter (6) and 250 mg/liter (31). It cannot be explained why the results were different from those found in this study; however, the pattern that the IC_{50} for protein synthesis was slightly lower than that for RNA synthesis was a consistent finding for all the agents examined in this study.

In this study the OBC was found to be similar to the IC_{50} for protein and RNA synthesis, and therefore, these values coincide with the section of the dose-response curve indicating the point at which bacteria die at a slower rate. This confirms previous observations that active protein and RNA syntheses are required, in part, for the bactericidal action of quinolones (6, 30, 31). However, direct inhibition of RNA and protein synthesis by quinolones per se is not the primary mechanism of action for this class of agents. The IC_{50} for RNA and protein synthesis also correlated with the concentration at which maximum *recA* induction was observed.

DNA damage to *E. coli* induces a highly pleiotropic cellular response known as the SOS response or DNA repair mechanism. Induction of this response is thought to be triggered by the accumulation of nicked DNA, although the precise identity of the signal is still unknown (25). The inducing signal activates RecA (the primary protein in this response) to its proteolytic form, which promotes cleavage of the LexA protein, which is the repressor of at least 17 unlinked genes. The proteins produced during the SOS response enhance cell survival and allow DNA repair (33). A consequence of SOS induction is the inhibition of cell division, so that no daughter cells with damaged DNA are produced. The *sulA* (*sfiA*) gene has been shown to be under the control of the *lexA* gene (18), and the SulA protein binds to the product of *sulB* (*ftsZ*), which is an essential protein in cell division; therefore, inhibition causes filamented bacteria (19).

Quinolones have been shown to induce the production of RecA and SulA (22, 23), and therefore, the filamentation that has been observed to occur when gram-negative bacteria are exposed to quinolones is presumably due to the induction of the SOS response via DNA damage. Induction of *recA* requires active protein synthesis (25), and so the observation in this study that filamentation is inhibited at concentrations above the OBC (or when 20 mg of chloramphenicol per liter is added) further suggests that quinolone-induced filamentation is a product of the SOS response. Therefore, the inhibition of cell division via induction of the SOS response owing to DNA damage fits the criteria of mechanism A proposed by J. T. Smith (30). Mechanism A, the involvement of the SOS response, is only one component in a complex process resulting in quinolone-induced bacterial killing, because quinolones are still bactericidal against mutants that have defective SOS responses (17, 20, 34), albeit at different rates (34), presumably via mechanism B. This study also demonstrated an excellent correlation (r = 0.99) among the maximum concentration needed to induce

recA, the concentration needed to inhibit DNA synthesis, and MICs, which would suggest direct relationships among these parameters. However, inhibition of protein synthesis had no detectable effect on inhibition of DNA synthesis by quinolones. Mechanism B could be the protein synthesis-independent inhibition of DNA synthesis, which could occur by direct action of the quinolone upon either DNA (27, 28) or the DNA gyrase complex, which results in gapped or nicked DNA. Gapped DNA has been shown to accumulate in *E. coli* cells after treatment with nalidixic acid (6) and is proposed to be the SOS response-inducing signal (19).

The role of the SOS response in the bactericidal action of quinolones has been investigated. Lewin et al. (17) examined the killing kinetics of 50 μg of nalidixic acid per ml on *E. coli* AB1157 compared with those on six strains of *E. coli* containing mutations in genes involved in the SOS response. In parallel, we performed similar studies (34) using four quinolones at a range of concentrations on six strains of *E. coli*, including some of those studied by Lewin et al. (17). Similar data were obtained for *E. coli recB21* but not for *E. coli recA430*. We demonstrated that there are clear differences between the responses of the mutants and different agents that are also time and concentration dependent. In the absence of SOS induction, e.g., in *E. coli recA430*, the rate of killing by ciprofloxacin is increased (34), and mutants which lack DNA repair (*E. coli recBC21*) are hypersusceptible to quinolones (17, 34). There was little correlation between the data for nalidixic acid and those for the other agents. Further studies in which we are investigating quinolone killing kinetics and mutants that are defective in components of the SOS response and cell division pathways are under way in our laboratory.

In this study we examined various parameters to study the mechanism of action of quinolones and combined these data with those from previously published studies of killing kinetics of mutants defective in components of the SOS response (17, 34) to suggest a role for the SOS response in the bactericidal actions of these agents.

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