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Inhibition of *Micrococcus luteus* DNA Gyrase by Norfloxacin and 10 Other Quinolone Carboxylic Acids

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The ability of norfloxacin, amifloxacin, cinoxacin, ciprofloxacin, flumequine, nalidixic acid, ofloxacin (OFL), oxolinic acid, perfloxacin, pipemidic acid, and rosoxacin to inhibit the in vitro supercoiling activity of *Micrococcus luteus* DNA gyrase was compared with the ability of each drug to inhibit the growth of the *M. luteus* strain from which the gyrase was purified. The potency of the quinolones as DNA gyrase inhibitors did not always correlate with antimicrobial potency. For example, OFL was a less potent inhibitor of gyrase than rosoxacin, yet the MIC of OFL was 16-fold lower than that of rosoxacin. Similarly, the MICs of norfloxacin and ciprofloxacin (the most potent of the antibiotics tested in these assays) were several hundredfold lower than the MIC of nalidixic acid (the least potent of these antibiotics), but the inhibition of purified gyrase by these two quinolones was only 8- to 16-fold lower than that of nalidixic acid. These results suggest that factors in addition to inhibition of gyrase supercoiling activity are important in determining the potency of these drugs. Further studies indicated that the uptake of norfloxacin, OFL, and amifloxacin by *M. luteus* cells may not account for the large differences in MICs observed for these drugs (MICs of 0.8, 2.0, and 128 μg/ml, respectively).

Many of the new quinolone carboxylic acids (e.g., norfloxacin [NOR]) possess a broader antibacterial spectrum and are more potent than their structurally related predecessors (e.g., oxolinic acid [OXO] and nalidixic acid [NAL]) against both gram-positive and gram-negative bacteria. The MIC of the newer drugs is often several hundredfold less than the MIC of NAL (25). Genetic and biochemical studies have provided convincing evidence that the intracellular target of NAL is DNA gyrase. In *Escherichia coli*, resistance to NAL is conferred by the *gyrA* locus which encodes the A subunit of DNA gyrase. Gyrase purified from *gyrA* (Nal^r) cells is resistant to inhibition by both NAL and OXO (1, 4, 6, 7, 23, 24). Although NOR and the other new quinolones have not been studied as extensively as NAL and OXO, they are also believed to act by inhibition of gyrase (2).

In this study, we have compared NOR and 10 other quinolone antibiotics for their ability to inhibit the supercoiling activity of DNA gyrase in vitro and to inhibit the growth of the bacterial strain from which the gyrase was purified. *Micrococcus luteus* DNA gyrase, an enzyme known to be quite resistant to NAL and OXO, was used as a model system (12, 14). Our results indicate that many newer quinolones are better inhibitors of DNA gyrase than NAL and OXO and that better inhibition of gyrase often correlates with better inhibition of bacterial growth. In some instances, however, improved inhibition of gyrase did not lead to improved MICs, suggesting that factors other than inhibition of DNA gyrase may be important in determining the potency of the quinolone antibiotics.

MATERIALS AND METHODS

Strains. *M. luteus* MB1784 (ATCC 4698), and *Klebsiella pneumoniae* MB480 (ATCC 10031) were maintained on brain heart infusion (Difco Laboratories) agar slants at room temperature with weekly transfers.

MIC. The MIC of each quinolone for *M. luteus* MB1784 was determined by broth dilution. An overnight culture of MB1784 was diluted to 10^4 cells per ml in tryptic soy broth (Difco), and 0.9 ml of the diluted culture was added to 100 μl of a twofold dilution series of each drug. The tubes were incubated at 37°C and scored for visible turbidity at 24 h. The MIC was defined as the lowest dilution of drug which resulted in no visible growth. MICs were also determined by using a log-phase culture of *M. luteus* MB1784. An overnight culture was diluted 1:50 in tryptic soy broth, incubated with shaking for 4 h, diluted to 10^4 cells per ml in tryptic soy broth, and added to each drug as described above.

Antibiotics. NOR (Merck & Co., Inc.), ciprofloxacin (CIP; Miles Laboratories), rosoxacin and amifloxacin (AMI) (Winthrop-Breon Laboratories), ofloxacin (OFL; Ortho Pharmaceuticals); pefloxacin (PEF; Merck), cinoxacin (Eli Lilly & Co.); OXO (Parke, Davis & Co.), flumequine (Riker Laboratories), pipemidic acid (Dainippon Pharmaceuticals), NAL (Aldrich Chemical Co., Inc.), and novobiocin (NOV; Sigma Chemical Co.) were prepared as 10-mg/ml solutions in 0.05 N NaOH and stored at −20°C. The same lot of drug was used for both MIC determinations and gyrase inhibition assays.

Assay of gyrase supercoiling activity. Plasmid pBR322 was purified from *E. coli* as described before (15, 16). ϕX174 DNA (Bethesda Research Laboratories) was converted to the relaxed circular form with calf thymus topoisomerase I (Bethesda Research Laboratories) and purified by extraction with phenol saturated with 10 mM Tris hydrochloride (pH 7.5), followed by extraction with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with ethanol and stored at 4°C in 10 mM Tris–1 mM EDTA (pH 7.5). The preparations contained approximately 25% nicked DNA.

M. luteus DNA gyrase purified from strain ATCC 4698 (12) was obtained from Bethesda Research Laboratories (20,000 U/mg; ratio of A/B subunits, 1:1). One unit of gyrase is defined as that amount of enzyme which catalyzes the conversion of 0.5 μg of relaxed plasmid DNA to the supercoiled form in 30 min at 37°C. The enzyme was assayed in a reaction mixture containing 35 mM Tris hydrochloride (pH 7.5), 20 mM $MgCl_2$, 20 mM KCl, 0.1 mM disodium EDTA, 10 mM 2-mercaptoethanol, 20 μg of bovine serum albumin per ml, 2 mM spermidine, 10% (vol/vol) glycerol,

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1.0 μg of relaxed φX174 DNA, and 0.5 U of gyrase. Appropriate dilutions of each drug were added to the assay tubes, and reactions were started by the addition of ATP (1 mM final concentration) in a final volume of 40 μl. The addition of drug and ATP was completed in less than 2 min. After 30 min of incubation at 37°C, the tubes were transferred to an ice bath, and the reaction was terminated by the addition of 40 μl of 10 mM EDTA containing 10% Ficoll and 0.05% bromophenol blue. Under these conditions, approximately 50% of the DNA substrate was converted to the completely supercoiled form. Reaction products were analyzed by 1% agarose gel electrophoresis, using Tris-phosphate buffer (0.08 M Tris-phosphate, 0.008 M EDTA, pH 8.0). The gels were electrophoresed for 18 h at 30 V, stained with ethidium bromide, and photographed.

Drug uptake by *M. luteus*. Uptake of NOR, OFL, and AMI was measured essentially as described by Hirai et al. (8). *M. luteus* cells were grown to late log phase in LB broth (15). OFL, NOR, and AMI were added to 3.0 ml of cells to final concentrations of 25 and 250 μg/ml. The number of cells in the suspension was determined by triplicate dry-weight measurements of 10 ml of culture. Following 30 min of incubation at 37°C, the cells were rapidly chilled and washed twice with 3.0 ml of ice-cold saline (0.085% NaCl), and the final pellet was suspended in 50 μl of potassium phosphate buffer (pH 8.0). Cell-associated drug was extracted by heating the cells in a boiling-water bath for 7 min. Cells were removed by centrifugation, and the concentration of drug in the supernatant fluid was quantitated by bioassay, using *K. pneumoniae* MB480 as described below. Results were expressed as the amount (micrograms) of drug released into the final supernatant per milligram of cells.

"Cell associated" drug concentration was also determined by a second method. *M. luteus* cells were grown as above, collected by centrifugation, and suspended in 0.2 volume of LB broth. The concentrated cells (3.0 ml) were added to tubes containing OFL, AMI, or NOR (final concentration, 25 μg/ml). Following 30-min incubation at 37°C, the cells were filtered onto 0.45-μm membrane filter disks (Millipore Corp.) and washed by filtration twice with 5 ml of ice-cold 0.9% saline. The filters were carefully placed in screw-capped vials containing 4,000 U of lysozyme (Sigma) and 135 U of DNase (Sigma) in a total volume of 500 μl. The vials were incubated with rocking at 37°C for 30 min. The concentration of drug in the lysate was determined by bioassay as described below and expressed as micrograms per milligram of cells.

Bioassay procedure. *K. pneumoniae* MB480 was grown overnight at 35°C in nutrient broth (Difco) supplemented with 0.2% yeast extract (Difco). Cells were diluted in molten (50°C) tryptic soy agar (Difco) to a final concentration of 10^5 cells per ml. The seeded agar was dispensed into 100-mm petri dishes (14.0 ml per dish) and allowed to harden. Wells 4 mm in diameter were punched in the agar, and 20 μl of supernatant fluid or lysate was pipetted into each well. Zone sizes were determined following overnight incubation at 35°C. Standard curves to determine drug concentration were developed for each drug (NOR, OFL, and AMI). Changes in the potency of each drug were also determined after heating in a boiling-water bath for 7 min and in the presence of cell lysate. All bioassays were performed in quadruplicate.

RESULTS

The MICs of the 11 quinolones for *M. luteus* ATCC 4698 are shown in Table 1. NOR and CIP were the most potent of the quinolones tested, and each inhibited the growth of this strain at concentrations several hundredfold lower than NAL. In these experiments, MICs were the same when either stationary-phase or log-phase cells were used as the inoculum.

TABLE 1. Antibacterial activity (MIC) against *M. luteus* MB1784 versus inhibition of purified *M. luteus* gyrase (I_P)

Drug	MIC (μg/ml)	I_P (μg/ml)	Relative inhibitory potency[a]	
			MIC_R[b]	I_R[c]
NOR	0.8	39	320	16
CIP	0.8	78	320	8
Rosoxacin	32	78	8	8
OFL	2	156	128	4
AMI	128	312	2	2
PEF	16	312	16	2
Cinoxacin	>1,000	312	<0.25	2
OXO	128	625	2	2
Flumequine	128	625	2	1
Pipemidic acid	128	625	2	1
NAL	256	625	1	1

[a] The MIC and I_P of each quinolone were compared with those of NAL.
[b] Relative MIC. The MIC of NAL was divided by the MIC of each quinolone. (e.g., NOR is 320-fold more potent than NAL.)
[c] Relative I_P. The I_P of NAL was divided by the I_P of each quinolone. (e.g., NOR is 16-fold more potent than NAL.)

The gyrase inhibition assays were performed with twofold dilution series of each drug to facilitate comparison with the MICs. NOV (an inhibitor of the gyrase B subunit) was included as a positive control. Figure 1 illustrates the gel electrophoretic pattern produced when gyrase assays were performed with PEF, NOR, and NOV. In these gels, the upper band is relaxed circular DNA and the lower band is fully supercoiled DNA. Because the magnitude of supercoiling is difficult to quantitate (10, 20), the gyrase inhibition assays were scored as follows: complete inhibition (no supercoil band remained; e.g., lanes 2, 9, and 10), partial inhibition (the intensity of the supercoil band was significantly diminished; e.g., lanes 5 and 11), and no inhibition (the intensity of the supercoil band was not diminished relative to an uninhibited control). Similar experiments were performed with each drug (Table 1). NAL was the least potent inhibitor of DNA gyrase in vitro (NAL partially inhibited supercoiling at both 1,250 and 650 μg/ml, whereas OXO, flumequine, and pipemidic acid completely inhibited supercoiling at 1,250 μg/ml). NOR and CIP were the most potent of the quinolones tested and partially inhibited gyrase activity at concentrations 8- to 16-fold lower than NAL. NOV partially inhibited gyrase activity in this assay at 0.7 μg/ml (1. 2 μM), a value consistent with studies involving other DNA gyrase preparations (10).

When relaxed circular pBR322 was used as substrate, or when a higher concentration of φX174 DNA was used, the concentration of drug that yielded partial inhibition of gyrase activity was unchanged.

The lowest concentration of drug that resulted in partial inhibition of gyrase activity was termed the I_P, and these values were compared with the MIC for each drug. To further facilitate comparison, the I_P and MIC of each drug were calculated relative to the I_P and MIC of NAL (Table 1). This comparison suggested that other factors contribute to the ability of each of these drugs to inhibit bacterial growth. For example, the MICs of NOR and CIP were several hundredfold lower than the MIC of NAL for this *M. luteus* strain, but the concentration required to partially inhibit purified gyrase by these two quinolones was only 8- to

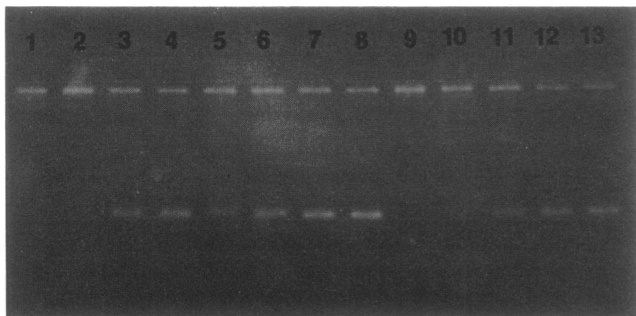

FIG. 1. Inhibition of the supercoiling activity of *M. luteus* DNA gyrase by PEF, NOR, and NOV. Gyrase assays were performed as described in the text. Reaction mixtures contained no enzyme (lane 1) or: PEF at 625 (2), 312 (3), and 156 (4) μg/ml; NOR at 38 (5), 19 (6), 9 (7), and 4.5 (8) μg/ml; or NOV at 2.8 (9), 1.4 (10), 0.7 (11), 0.35 (12), and 0.18 (13) μg/ml.

16-fold lower than that of NAL. Similarly, OFL was a less potent inhibitor of gyrase than rosoxacin, yet the MIC of OFL was 16-fold lower than that of rosoxacin.

Because penetration into the bacterial cell is an important factor in drug potency, the uptake of three of the quinolones by *M. luteus* cells was examined according to the method of Hirai et al. (8). *M. luteus* cells were incubated with OFL, AMI, or NOR at two different concentrations (25 and 250 μg/ml) for 30 min; extracellular drug was removed by centrifugation. The intracellular (or cell-associated) drug was extracted by boiling, and the final concentration of each drug was determined by bioassay. Under the conditions of these experiments, the cell-associated concentration was very similar for each of these three drugs. When cells were incubated with 25 μg of drug per ml, the final concentrations of NOR, AMI, and OFL were 0.1 to 0.2 μg per mg of cells, and when cells were incubated with 250 μg/ml the final concentrations were 0.4 to 0.8 μg per mg of cells. In these experiments, it was noted that the potency of the drugs decreased by 20 to 30% when the drugs were boiled. This factor was taken into account when the cell-associated concentration of these drugs was calculated.

To avoid the adverse effects of boiling, a second method was developed to measure drug uptake. Intracellular (or cell-associated) drug was released by lysozyme treatment rather than by boiling the cells. The results supported the observations described above. When cells were incubated with 25 μg of NOR, AMI, or OFL per ml, the cell-associated drug concentration did not vary by more than twofold and was very similar to the concentration determined by the first method (0.15 to 0.3 μg per mg of cells). These small differences do not account for the large differences in the MICs of the three drugs.

DISCUSSION

In this study, we have shown that many of the newer quinolones are better inhibitors of the supercoiling activity of DNA gyrase in vitro than their structurally related predecessors. However, our results also suggest that the potency of these quinolones as DNA gyrase inhibitors does not always correlate with antimicrobial potency. For example, OFL and AMI differ only twofold in ability to inhibit *M. luteus* gyrase, yet the MIC of OFL is 60-fold better than that of AMI (2 versus 128 μg/ml). Apparently, other factors contribute to the antibacterial activity of these drugs.

Potency differences among members of certain classes of antibiotics are often related to differences in cell penetration. To determine whether penetration differences could account for the potency differences described in this report, the uptake of three quinolones, NOR, OFL, and AMI, by *M. luteus* cells was estimated by two methods. In these experiments, the final cell-associated concentration of the three drugs was not sufficiently different to account for the wide variation in MIC (0.8, 2, and 128 μg/ml, respectively). While these experiments suggest that more complex factors are involved in quinolone potency, it is possible that certain properties of the quinolones could generate anomalous results in these types of experiment. For example, more potent quinolones may bind more strongly to cell constituents and their apparent concentration may be underestimated by bioassay. Alternatively, the antibacterial activity of these drugs may change the cell permeability barrier and affect the uptake or efflux, or both, of the drugs. Short-term uptake experiments with radiolabeled drugs would help to resolve these issues.

Although ample evidence indicates that DNA gyrase is the target of the quinolone antibiotics, the mechanism of inhibition is not yet clear. Recent evidence by Shen et al. has suggested that NOR, and other quinolones, bind to DNA, but not to gyrase (21). In their studies, the affinity of each drug for DNA was correlated with its ability to inhibit the supercoiling activity of purified DNA gyrase in vitro. It is interesting to note that the drugs did not display a higher affinity for DNA-gyrase complexes than for DNA alone, and yet studies in this lab and in others (14, 23) have indicated that gyrases purified from different bacteria vary in their susceptibility to these drugs (e.g., the *M. luteus* gyrase is much more resistant to inhibition than the *E. coli* enzyme is). Shen et al. (21) have shown further that the quinolones bind preferentially to single-stranded DNA rather than to double-stranded DNA. It is possible that the binding of gyrase to DNA induces changes in the secondary structure of the nucleic acid, thereby changing its binding affinity for the drug. Enzymes from different organisms may vary in their effects on the DNA.

It is also not yet clear how the inhibition of DNA gyrase by the quinolones leads to the death of the bacterial cell. In experiments with bacteriophage T7, it was shown that the action of NAL is not strictly equivalent to inhibition of gyrase. NAL inhibits the replication of T7, while the phage continues to replicate at the nonpermissive temperature in cells bearing a temperature-sensitive gyrase A subunit (13). Deitz et al. (3) and, more recently, Crumplin et al. (2) have shown that the bactericidal effects of NOR require competent RNA and protein synthesis, suggesting an active process rather than a simple irreversible inhibition of an essential cell enzyme.

In several other studies, it has been shown that the concentration of quinolones required to inhibit the supercoiling activity of gyrase in vitro is substantially higher than that required to inhibit the growth of the bacterial cell from which the gyrase was purified. For example, for *Pseudomonas* spp., the concentration of NAL necessary to inhibit 50% of purified gyrase activity in vitro was 600 μg/ml, threefold higher than the MIC for the same strain (17). *E. coli* gyrase activity is inhibited to 50% by NAL at 200 μg/ml, a concentration 50-fold higher than the MIC for the same strain (17, 23), and in this study I_P concentrations ranged 2- to 100-fold higher than the corresponding MICs. While this phenomenon may be due to active uptake of the quinolones by bacterial cells, it is also possible that it is related to the mechanisms by which inhibition of gyrase leads to cell death. Gellert et al. (6) and Yamagishi et al. (24) have shown

that the supercoiling of intracellular bacteriophage lambda DNA is only partially inhibited by OXO even at levels well above the MIC, suggesting that concentration of the drug by the cell does not explain the I_P-MIC disparity. Engle et al. (5) have suggested that the quinolones form a drug-gyrase-DNA complex that blocks migration of the replication fork. It is also known that, under appropriate conditions, the interaction of quinolones with gyrase may cause double-stranded breaks in the DNA (6, 11, 18, 19, 22, 23). By either of these mechanisms, cell death would require only a "single hit" event.

Hogberg et al. have recently synthesized a series of OXO analogs to evaluate the role of the N-1 atom in the mode of action of these antibiotics (9). Interestingly, of 25 compounds tested, only the unsubstituted parent compound had any antimicrobial activity, yet this compound did not inhibit the supercoiling activity of *E. coli* gyrase in vitro. It is not known whether or not this compound inhibits gyrase in the cell. The uncertainty surrounding the actual mode of action of the quinolones suggests that inhibition of supercoiling may not be the most valid criterion for predicting the potency of these drugs. Perhaps other assays (strand breakage, DNA replication, or phage replication) would provide a more direct indication of the effects of these drugs on critical intracellular events. Further studies that compare the biological effects of the quinolones with their behavior in other assays may help to resolve the complexities of their mode of action.

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