**Mycobacterium tuberculosis** DNA Gyrase: Interaction with Quinolones and Correlation with Antimycobacterial Drug Activity

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Genome studies suggest that DNA gyrase is the sole type II topoisomerase and likely the unique target of quinolones in *Mycobacterium tuberculosis*. Despite the emerging importance of quinolones in the treatment of mycobacterial disease, the slow growth and high pathogenicity of *M. tuberculosis* have precluded direct purification of its gyrase and detailed analysis of quinolone action. To address these issues, we separately overexpressed the *M. tuberculosis* DNA gyrase GyrA and GyrB subunits as His-tagged proteins in *Escherichia coli* from pET plasmids carrying gyrA and gyrB genes. The soluble 97-kDa GyrA and 72-kDa GyrB subunits were purified by nickel chelate chromatography and shown to reconstitute an ATP-dependent DNA supercoiling activity. The drug concentration that inhibited DNA supercoiling by 50% (IC50) was measured for 22 different quinolones, and values ranged from 2 to 3 μg/ml (sparfloxacin, sitafloxacin, clinafloxacin, and gatifloxacin) to >1,000 μg/ml (pipemidic acid and nalidixic acid). By comparison, MICs measured against *M. tuberculosis* ranged from 0.12 μg/ml (for gatifloxacin) to 128 μg/ml (both pipemidic acid and nalidixic acid) and correlated well with the gyrase IC50s (R2 = 0.9). Quinolones promoted gyrase-mediated cleavage of plasmid pBR322 DNA due to stabilization of the cleavage complex, which is thought to be the lethal lesion. Surprisingly, the measured concentrations of drug inducing 50% plasmid linearization correlated less well with the MICs (R2 = 0.7). These findings suggest that the DNA supercoiling inhibition assay may be a useful screening test in identifying quinolones with promising activity against *M. tuberculosis*. The quinolone structure-activity relationship demonstrated here shows that C-8, the C-7 ring, the C-6 fluorene, and the N1 cyclopropyl substituents are desirable structural features in targeting *M. tuberculosis* gyrase.

Fluoroquinolones are active against *Mycobacterium tuberculosis* and are the first new antimycobacterial drugs to be available since the discovery of rifampin (7, 13, 40). Fluoroquinolones are part of the drug regimens now recommended for treating rifampin-resistant tuberculosis (6, 10). Ofloxacin and ciprofloxacin have a bacteriostatic antimycobacterial activity (13, 20, 40), but several new fluoroquinolones, such as sparfloxacin and moxifloxacin, show high bactericidal activity against *M. tuberculosis* mycobacterial disease, the slow growth and high pathogenicity of *M. tuberculosis* have precluded direct purification of its gyrase and detailed analysis of quinolone action. To address these issues, we separately overexpressed the *M. tuberculosis* DNA gyrase GyrA and GyrB subunits as His-tagged proteins in *Escherichia coli* from pET plasmids carrying gyrA and gyrB genes. The soluble 97-kDa GyrA and 72-kDa GyrB subunits were purified by nickel chelate chromatography and shown to reconstitute an ATP-dependent DNA supercoiling activity. The drug concentration that inhibited DNA supercoiling by 50% (IC50) was measured for 22 different quinolones, and values ranged from 2 to 3 μg/ml (sparfloxacin, sitafloxacin, clinafloxacin, and gatifloxacin) to >1,000 μg/ml (pipemidic acid and nalidixic acid). By comparison, MICs measured against *M. tuberculosis* ranged from 0.12 μg/ml (for gatifloxacin) to 128 μg/ml (both pipemidic acid and nalidixic acid) and correlated well with the gyrase IC50s (R2 = 0.9). Quinolones promoted gyrase-mediated cleavage of plasmid pBR322 DNA due to stabilization of the cleavage complex, which is thought to be the lethal lesion. Surprisingly, the measured concentrations of drug inducing 50% plasmid linearization correlated less well with the MICs (R2 = 0.7). These findings suggest that the DNA supercoiling inhibition assay may be a useful screening test in identifying quinolones with promising activity against *M. tuberculosis*. The quinolone structure-activity relationship demonstrated here shows that C-8, the C-7 ring, the C-6 fluorene, and the N1 cyclopropyl substituents are desirable structural features in targeting *M. tuberculosis* gyrase.

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applied to gyrase from *Mycobacterium smegmatis*, a nonpathogenic, rapidly growing (3-day culture) mycobacterium (34). Gyrase was also purified from the low-pathogenicity opportunistic agents *M. fortuitum*, a rapid grower, and *M. avium*, a slow grower (14-day culture) (15). However, because *M. tuberculosis* grows slowly (21-day culture) and is highly pathogenic, the purification of its DNA gyrase requires recombinant methods that do not rely on bulk culture of the organism. In a previous study (28), maltose-binding protein fusions were used to produce *M. tuberculosis* gyrase subunits and to perform some preliminary assays of gyrase inhibition by quinolones. There are two potential limitations in the earlier work. First, it is known that gyrase subunits expressed as fusion proteins can have very low specific activities, unlike their recombinant His-tagged counterparts (28). Second, only four quinolones were examined, with inhibition of DNA supercoiling as the sole assay.

To investigate the interaction of *M. tuberculosis* gyrase with quinolones, particularly the newer, highly potent agents, we have developed recombinant plasmid clones that allow the production in *E. coli* and purification of recombinant *M. tuberculosis* GyrA and GyrB subunits carrying His tags. This alternative strategy provided *M. tuberculosis* DNA gyrase subunits safely and in large quantities. The recombinant GyrA and GyrB subunits were stable and reconstituted a functional DNA gyrase activity. We investigated the interaction of the enzyme with a large panel of quinolones with two complementary assays: inhibition of DNA supercoiling and induction of DNA cleavage arising from stabilization of the cleavage complex, which is thought to be the cytotoxic lesion. The results of this systematic study allowed us to establish a quinolone structure-activity relationship in which inhibition of supercoiling activity by 50% (IC50) correlated well (better than DNA cleavage) with growth (as measured by the MIC).

**Materials and Methods**

Reagents. Ofloxacin and levofloxacin (Aventis, Paris, France), gatifloxacin (Grünenthal, Levallois-Perret, France), ciprofloxacin and moxifloxacin (Bayer Pharma, Puteaux, France), sparfloxacin and pefloxacin (Rhône Poulenc Rorer, Vitry sur Seine, France), nalidixic acid, novobiocin, oxolinic acid, and pipemidic acid (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France), toluuidoxacin (Lederle, Paris La Défense, France), garenoxacin (Bristol-Meyers Squibb, Saint Avre, France), nalidixic acid, and pipemidic acid (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France), toluuidoxacin (Lederle, Paris La Défense, France), garenoxacin (Bristol-Meyers Squibb, Saint Avre, France), nalidixic acid, and pipemidic acid (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France), toluuidoxacin (Lederle, Paris La Défense, France). Plasmid DNA was isolated from Escherichia coli DH5α using the MID Bacterial Miniprep kit (GE Healthcare, Little Chalfont, Bucks, UK). DNA was eluted from the 2.1-kb plasmid DNA fragments using the MID Dnaseq kit (GE Healthcare, Little Chalfont, Bucks, UK). DNA was dissolved in distilled water and stored at −20°C.

**Drug susceptibility.** *M. tuberculosis* H37Rv was grown on Löwenstein-Jensen medium. MICs were determined by the proportion method as described previously (14). Briefly, 104 and 105 CFU were inoculated onto 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase and containing serial twofold dilutions of the quinolone. Colonies were enumerated after 21 days of incubation at 37°C. The MIC was defined as the drug concentration at which the bacterial growth was reduced to 1% or less of that of the drug-free control culture (18). For five quinolones (sitafoxacin, norfloxacin, temafloxacin, fleroxacin, and enoxacin), the MICs were taken from the literature (11, 12, 36, 39, 42).

**Construction of GyrA and GyrB expression vectors.** The gyrA and gyrB genes of *M. tuberculosis* were amplified from cosmid T776, cloned into plasmids pET-29a and pET-19b, respectively. Three different BL21 clones were grown separately. Into each of the forward primers used for the amplification step, Ndel sites (CA/TATG) were engineered, overlapping the ATG initiation codons of gyrA and gyrB. An Xhol site was engineered before the stop codon for the gyrA primer and after the stop codon for gyrB in each of the reverse primers. The gyrA gene was amplified with forward primer GYRATB1 (5'-GCAAACGCGGAAATATGACAGACAC; the Ndel site overlapping the ATG initiation codon is italic) and reverse primer GYRATB2 (5'-CGACCTGTATTCGAGCGCTTGTG; the Xhol site is italic). The gyrB gene was amplified with the forward primer GYRBTB1 (5'-GGCCGCGCTATAGTGATCAGTG; the Ndel site overlapping the ATG initiation codon is italic) and reverse primer GYRBTF2 (5'-CGAAGCGACGGTCTAGTACAC; the Xhol site overlaps the ATG initiation codon is italic). The PCR products corresponding to the 2.5-kb gyrA and 2.1-kb gyrB fragments were ligated into the pMOS Blue plasmid, transformed into MosBlue competent cells, and plated on Luria-Bertani (LB) agar containing ampicillin (100 μg/ml), 3-bromo-4-chloro-5-indolyl-p-D-galactopyranoside (X-Gal), and isopropylthiogalactopyranoside (IPTG). Recombinant plasmids were recovered from the white colonies and digested with Ndel and Xhol. The DNA fragment corresponding to gyrA was ligated into Ndel- and Xhol-cut pET-29a, and that corresponding to gyrB was ligated into Ndel- and Xhol-cut pET-19b, and both were transformed into *E. coli* DH5α. Recombinant clones were selected from the resistant colonies selected on plates containing kanamycin (50 μg/ml) for recombinant pET-29a or ampicillin (100 μg/ml) for recombinant pET-19b.

**Protein overexpression and purification of GyrA and GyrB subunits.** The recombinant plasmid carrying the gyrA gene of *M. tuberculosis* (pATB) and that carrying the gyrB gene of *M. tuberculosis* (pPTB) were separately transformed by electroporation into *E. coli* BL21(DE3) pLysS. GyrA and GyrB proteins were purified by the same procedure. Three different BL21 clones were grown separately at 37°C in 4 ml of LB medium containing the selective antibiotic until the optical density at 600 nm reached 0.8 to 1.0. The clone subcultures (1 ml) were then mixed and used to inoculate 50 ml of LB medium containing the selective antibiotic. Cells were grown at 37°C with shaking for 10 min to 1 h. The pellet was washed twice with 1 ml of LB medium and then resuspended in 1 ml of binding buffer (100 mM Tris-HCl [pH 7.9], 500 mM NaCl, and 250 mM imidazole [Novagen]) with 0.8 to 1.0. Bacteria were harvested by centrifugation at 3,000 × g for 10 min at 4°C. The pellet was suspended in 20 ml of LB and used to inoculate 50 ml of LB medium containing the selective antibiotic. Cells were grown at 37°C with shaking for 10 min to 1 h. The pellet was washed with 1 ml of binding buffer and then washed with 5 ml of wash buffer (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, and 5 mM imidazole [Novagen]). The histidine-tagged GyrA and GyrB proteins were eluted with 2 ml of elution buffer (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, and 250 mM imidazole). The total volume of the elution fraction (2 to 3 ml) was spun at 15,000 × g for 30 min at 4°C and then dialyzed overnight at 4°C against 2.5 liters of 0.05 M Tris-HCl (pH 7.9) and 0.05 M sodium glycerol. Derivatized GYRA and GYRB were added to eluate to obtain the final concentrations of 1 mM each. The GyrA and GyrB proteins were then flash frozen in aliquots in liquid nitrogen and stored at −80°C.
fractons were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

DNA supercoiling assays. DNA supercoiling activity was tested with various ratios of purified M. tuberculosis GyrA and GyrB subunits. The reaction mixture (total volume, 30 μl) contained DNA gyrase assay buffer (40 mM Tris-HCl [pH 7.5], 25 mM KCl, 6 mM magnesium acetate, 2 mM spermidine, 4 mM dithiothreitol, 0.1 mg of E. coli tRNA per ml, bovine serum albumin [0.36 mg/ml], 100 mM potassium glutamate, 1 mM ATP) (pH 8.0) and relaxed pBR322 DNA (0.4 μg) as the substrate. Gyrase proteins were added, and the reaction mixtures were incubated at 37°C for 1 h. Reactions were terminated by the addition of 50% glycerol containing 0.25% bromophenol blue, and the total reaction mixture was subjected to electrophoresis in a 1% agarose gel in 0.5× TBE (Tris-borate-EDTA, pH 8.3) buffer. After running for 5.5 h at 50 V, the gel was stained with ethidium bromide (0.7 μg/ml). One unit of enzyme activity was defined as the amount of DNA gyrase that converted 400 ng of relaxed pBR322 to the supercoiled form in 1 h at 37°C.

DNA gyrase of E. coli (John Innes Enterprises Ltd.) was used as a positive control for the assay procedures and buffer. Inhibition of supercoiling activity of the recombinant DNA gyrase was performed by the method described previously (29). In brief, a reaction mixture containing 1 U of purified DNA gyrase and increasing concentrations of quinolones was incubated as described above. The inhibitory effect of quinolones on DNA gyrase was assessed by determining the concentration of drug required to inhibit the supercoiling activity of the enzyme by 50% (IC50). Supercoiling activity was assessed by tracing the brightness of the bands corresponding to the supercoiled pBR322 DNA with Molecular Analyst software (Bio-Rad).

DNA cleavage assays. DNA cleavage assays were carried out in the same buffer as for DNA supercoiling except that relaxed pBR322 DNA was used instead of supercoiled pBR322 DNA. These assays were performed in the absence and presence of 1 mM ATP (31).

Various DNA gyrase amounts from 0.1 to 10 U were tested to determine the optimal amount of gyrase that had to be incubated with DNA in the presence of increasing concentrations of quinolones for 1 h at 25°C in order to produce the maximum cleaved band. Three microliters of 2% SDS and 3 μl of a 1-mg/ml solution of proteinase K were added, and incubation was continued for 30 min at 37°C. The reactions were stopped as for supercoiling. After electrophoresis for 5.5 h at 50 V, the 1% agarose gel was stained with ethidium bromide (0.7 μg/ml) and photographed under UV transillumination. The extent of DNA cleavage was quantified with the Molecular Analyst software. The concentration of quinolone that induced 50% of the maximum DNA cleavage (CC50) was determined. Plasmid pBR322 linearized by BamHI digestion was used as a marker for cleaved DNA. E. coli gyrase was used as a positive control for the assay procedures and buffer.

Correlation between MICs and IC50s against M. tuberculosis gyrase. The relationships between the MICs and IC50s or CC50s were assessed by estimating a linear regression between two components, both first translated on the log10 scale. The strength of this relationship was quantified by the R2 coefficient and displayed graphically by the regression line and the two curves defining the 95% confidence interval for this regression.

RESULTS

Construction of expression vectors for M. tuberculosis DNA gyrase genes and purification of recombinant His-tagged GyrA and GyrB proteins. The M. tuberculosis gyrA and gyrB genes were each amplified from cosmid T776, which contains the gyrB-gyrA contig of M. tuberculosis (8). Taq polymerase was mixed with Pwo polymerase to minimize the introduction of polymerase errors, because Pwo polymerase has proofreading activity. The entire amplified gyrA and gyrB genes were inserted separately in-frame downstream of a T7 promoter in pET expression vectors to yield recombinant plasmids pATB (gyrA in pET-29a) and pPTB (gyrB in pET-19b). Expression of the gyrA and gyrB genes in E. coli BL21 (DE3) lysS by induction with IPTG and subsequent purification by nickel chelate chromatography resulted in 2.5 mg and 10 mg of soluble His-tagged 97-kDa and 72-kDa proteins, respectively, from 500-ml cultures of induced cells (Fig. 1). The recombinant GyrA and

FIG. 1. SDS-PAGE analysis of purified M. tuberculosis GyrA and GyrB proteins. The His-tagged proteins were overexpressed in E. coli and purified by nickel resin chromatography, and approximately 16 μl of each protein sample was loaded on an SDS–9% polyacrylamide gel. Following electrophoresis, proteins were revealed by staining with Coomassie blue. Lane M, size markers (sizes are indicated to the left in kilodaltons).

GyrB proteins carried hexa- or decahistidine tags, respectively, at the C-terminal and N-terminal ends, respectively.

M. tuberculosis DNA gyrase is catalytically efficient for DNA supercoiling. The combination of GyrA and GyrB subunits was tested for DNA supercoiling activity with relaxed pBR322 DNA as a substrate in the absence and presence of ATP (Fig. 2). One unit of GyrA was measured in the presence of excess amounts of GyrB, and conversely, 1 U of GyrB was measured in the presence of excess amounts of GyrA. One unit was defined as the amount that was sufficient to convert 100% of 0.4 μg of relaxed plasmid pBR322 DNA to the supercoiled form. Neither subunit alone induced DNA supercoiling. The combination of GyrA and GyrB led to plasmid supercoiling in the presence of ATP, which demonstrated that they reconstituted a functional DNA gyrase. No supercoiling was observed when ATP was omitted (Fig. 2). Two units of GyrA plus 2 U of GyrB were used subsequently for all the DNA supercoiling experiments. The specific activity of recombinant M. tuberculosis DNA gyrase was tested for DNA supercoiling activity with relaxed pBR322 DNA. These assays were performed in the absence and presence of 1 mM ATP (31).

FIG. 2. M. tuberculosis GyrA and GyrB proteins generate an ATP-dependent DNA supercoiling activity. Relaxed pBR322 (0.4 μg) was incubated with DNA gyrase reconstituted from GyrA (1 U) and GyrB (1 U) in the absence and presence of 1 mM ATP. The reactions were stopped, and the DNA products were separated by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide and photographed under UV illumination. Lanes: a, supercoiled pBR322 DNA; b, relaxed pBR322 DNA; A, relaxed pBR322 DNA and GyrA (1 U) protein; B, relaxed pBR322 DNA and GyrB (1 U) protein; AB, relaxed pBR322 DNA and both GyrA (1 U) and GyrB (1 U). R and S, relaxed and supercoiled DNA, respectively.
to their IC50s. The quinolones in the
The 22 quinolones tested clustered into three groups according
shown in rank order of potency from sita
and representative data are shown in Table 1. IC50 values are
each experiment was done at least twice with similar results,
and supercoiled pBR322 DNA, respectively. N, R, and S, nicked,
DNA gyrase was compared. An example of representative data is
22 quinolones to inhibit DNA supercoiling by
M. tuberculosis
5
The ability of
Inhibition of DNA supercoiling by quinolones. The ability of
22 quinolones to inhibit DNA supercoiling by M. tuberculosis
gyrase was compared. An example of representative data is
shown for levofloxacin (LVX). The concentration of levo
fl
oxacin that inhibited DNA supercoiling by 50% was calculated as
5 µg/ml. Each of the quinolones tested showed dose-depen-
dent inhibition, and their IC50 data are summarized in Table 1. IC50 values are
shown in rank order of potency from sitafloxacin and spar-
fl
oxacin, the
fl
oxacin, as expected. All the compounds in the third
group lacked at least one of the other features, since gemifloxacin is
not a C-8 quinolone but has an N-8, garenoxacin is not fluor-
inated at C-6, and grepafloxacin has an additional group at
C-5. Ofloxacin, which is a racemic mixture of an L-isomer,
levofloxacin, and an inactive R-isomer, was twofold less active
than levofloxacin, as expected. All the compounds in the third
group lacked fluoreine at the C-6 position except fluoreine,
which had no substituent at C-7.
Activity of quinolones against M. tuberculosis and compar-
ison with gyrase inhibition. The MICs of the 22 quinolones
tested ranged from 0.25 to 128 µg/ml (Table 1). The quinolone
IC50 values correlated well with the MICs (R2 = 0.9), as shown in
Fig. 4. However for some quinolones, such as trovafloxacin,
 gemifloxacin, tosufloxacin, norfloxacin, and ciprofloxacin, the
MICS were higher than expected from the IC50s, and con-

TABLE 1. Structural features and concentrations of quinolones that inhibit M. tuberculosis DNA gyrase activity and M. tuberculosis growth*

<table>
<thead>
<tr>
<th>Quinolone</th>
<th>Substituent</th>
<th>Conc, µg/ml (reference)</th>
</tr>
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<tbody>
<tr>
<td>Sparfloxacin</td>
<td>Cyclopropyl</td>
<td>NH2 F Piperazine C-F</td>
</tr>
<tr>
<td>Sitafloxacin</td>
<td>Fluorinated cyclopropyl H F Pyrrolidine C-Cl</td>
<td>2.5 2 0.25 (36)</td>
</tr>
<tr>
<td>Clinafloxacin</td>
<td>Cyclopropyl H F Pyrrolidine C-Cl</td>
<td>2.5 5 0.5</td>
</tr>
<tr>
<td>Gatifloxacin</td>
<td>Cyclopropyl H F Piperazine C-OCH3</td>
<td>3 4 0.12</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Cyclopropyl H F Piperazine C-OCH3</td>
<td>3.5 6 0.5</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>Cyclopropyl H F Azabicyclo C-OCH3</td>
<td>4.5 4 0.5</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>Bridge C1-C8 H F Bridge C1-C8 C-H</td>
<td>5 12 0.5</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>Bridge C1-C8 H F Bridge C1-C8 C-H</td>
<td>10 20 1</td>
</tr>
<tr>
<td>Gemifloxacin</td>
<td>Cyclopropyl H F Pyrrolidine N</td>
<td>11 6 4</td>
</tr>
<tr>
<td>Garenoxacin</td>
<td>Cyclopropyl H H Azabicyclo C-OCH2</td>
<td>13 15 2</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>Ethyl H F Piperazine C-H</td>
<td>14 40 4</td>
</tr>
<tr>
<td>Trovafloxacin</td>
<td>Difluorophenyl H F Bicyclique N</td>
<td>15 25 16</td>
</tr>
<tr>
<td>Grepafloxacin</td>
<td>Cyclopropyl CH3 F Piperazine C-H</td>
<td>16 15 1</td>
</tr>
<tr>
<td>Pefloxacin</td>
<td>Ethyl H F Piperazine C-H</td>
<td>37 40 8</td>
</tr>
<tr>
<td>Tosufloxacin</td>
<td>Difluorophenyl H F Pyrrolidine N</td>
<td>37 25 16</td>
</tr>
<tr>
<td>Temafloxacin</td>
<td>Difluorophenyl H F Piperazine C-H</td>
<td>40 35 4 (42)</td>
</tr>
<tr>
<td>Fleroxacin</td>
<td>Fluorooethyl H F Piperazine C-H</td>
<td>45 50 6.25 (39)</td>
</tr>
<tr>
<td>Enoxacin</td>
<td>Ethyl H F Piperazine N</td>
<td>50 25 8</td>
</tr>
<tr>
<td>Oxolinic acid</td>
<td>Ethyl H H Bridge C6-C7 C-H</td>
<td>300 NC 32</td>
</tr>
<tr>
<td>Flumequin</td>
<td>Bridge C1-C8 H F H Bridge C1-C8</td>
<td>500 NC 64</td>
</tr>
<tr>
<td>Pipemidic acid</td>
<td>Ethyl H H Piperazine N</td>
<td>1,000 NC 128</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>Ethyl H H CH3 N</td>
<td>1,100 NC 128</td>
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* NC, no or weak cleavage observed.
relaxation was inhibited by the quinolones in a dose-dependent fashion. At higher quinolone levels, DNA cleavage led to linearization of the plasmid DNA. However, the relaxation activity hampered visualization of the cleaved band (Fig. 5). When ATP was added to the assay, it inhibited the relaxation activity and allowed better quantitation of the cleaved band.

Figure 6 shows the results for sparfloxacin in the absence and presence of ATP. Table 1 summarizes the results obtained for the 22 quinolones. The drug concentration producing 50% of the maximum DNA cleavage (CC50) for sitafloxacin, gatifloxacin, sparfloxacin, moxifloxin, clinafloxacin, and gemifloxin was below 10 μg/ml; the CC50s of levofloxacin, ofloxacin, garenoxacin, grepafloxacin, trovafloxacin, tosufloxacin, pefloxacin, norfloxacin, temafloxacin, enoxacin, and fleroxacin were between 10 and 100 μg/ml. Little or no DNA cleavage was observed when the assay was performed in the presence of nalidixic acid, oxolinic acid, pipemidic acid, or flumequine.

The CC50s grouped the quinolones similarly to the IC50s derived from the DNA supercoiling test. Two exceptions were gemifloxin, which was more effective in DNA cleavage induction than in inhibition of DNA supercoiling, and levofloxacin, which was less effective in cleavage than in supercoiling inhibition. From quinolone structure-activity considerations, the presence of C or N at position 8 (gemifloxin has N-8) is probably less important for DNA cleavage than the N-1 cyclopropyl, which is not present in levofloxacin. The quinolones that were inefficient in stimulating DNA cleavage, i.e., the classical quinolones, also lacked a fluorine at position 6 or a piperazinyl ring at C-7. Overall, the CC50s for DNA cleavage showed a poorer correlation with the MICs (R² = 0.7) than the IC50s did (Table 1).

**DISCUSSION**

The slow growth and high pathogenicity of *M. tuberculosis* preclude classical purification methods that have yielded native gyrase from the less pathogenic mycobacteria *M. smegmatis*, *M. fortuitum*, and *M. avium* (15, 25). To circumvent these difficulties, *M. tuberculosis* DNA gyrase was produced as His-tagged A and B subunits. Its interaction with quinolones was evaluated with two assays, DNA supercoiling and quinolone-mediated DNA cleavage (4).

The use of His tags facilitated safe and rapid purification and yielded products that were free of host (E. coli) proteins. Although results with native *M. tuberculosis* gyrase are not available, it does not seem that the His tags hampered the function of *M. tuberculosis* DNA gyrase or its interaction with quinolones. Indeed, the data obtained compared with those observed for the native *M. smegmatis* gyrase (15, 25, 34). Maltose-binding protein fusions with the GyrA and GyrB subunits have been used to obtain recombinant *M. tuberculosis* DNA gyrase, which has been tested against four quinolones (28). Of the IC50s of these four quinolones measured, only that of sitafloxacin agreed with our data. Although the DNA supercoiling assay was performed under the same conditions, the earlier IC50s for levofloxacin, ciprofloxacin, and sparfloxacin were two- to fourfold higher than those we measured. Maltose-binding protein fusion could result in misfolding compared to the native form. His-tagged gyrase have been shown to display

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**FIG. 4.** Correlation between quinolone inhibition of *M. tuberculosis* gyrase (IC50 for DNA supercoiling) and quinolone MICs for *M. tuberculosis*. Dotted lines represent the confidence interval for 95% of the regression. R² is the correlation coefficient. CIP, ciprofloxacin; CLX, clinafloxacin; ENX, enoxacin; FLE, fleroxacin; FLU, flumequine; GAR, garenoxacin; GAT, gatifloxacin; GEM, gemifloxin, GRX, grepafloxacin; LVX, levofloxacin; MXF, moxifloxin; NAL, nalidixic acid; NOR, norfloxacin, OFX, ofloxacin; OXO, oxolinic acid; PEF, pefloxacin; PIP, pipemidic acid; SIT, sitafloxacin; SPX, sparfloxacin; TEM, temafloxacin; TOS, tosufloxacin; TVA, trovafloxacin.

**FIG. 5.** Levofloxacin-mediated DNA cleavage by *M. tuberculosis* DNA gyrase. Supercoiled pBR322 DNA (0.4 μg) was incubated with *M. tuberculosis* GyrA (2 U) and GyrB (2 U) proteins in the absence of ATP and in the presence of levofloxacin (LVX) at the concentrations indicated on the figure. After addition of SDS and proteinase K, DNA samples were analyzed by electrophoresis in 1% agarose. Lanes a and b, supercoiled pBR322 DNA and BamHI-linearized pBR322. N, L, and S, nicked, linear, and supercoiled DNA, respectively.
interactions with quinolones comparable to those of the native bacterial gyrases for bacteria other than mycobacteria (5, 29, 35).

The ability to reconstitute *M. tuberculosis* gyrase activity allowed us to examine and compare the inhibitory effects of a large panel of quinolones, including recently developed agents. Twenty-two quinolones were evaluated for the ability to inhibit DNA supercoiling and to stimulate gyrase-mediated DNA cleavage. The quinolones inhibited the DNA supercoiling of *M. tuberculosis* gyrase in a dose-dependent manner, as observed in bacteria other than mycobacteria (5, 17, 30). The potency of the quinolones was demonstrated in both assays.

From the inhibition results, seven quinolones showed high inhibitory activity against *M. tuberculosis* DNA gyrase, with IC$_{50}$ below 10 μg/ml. Analysis of the quinolone structure-activity relationship showed that these seven compounds shared certain structural features (C-8 with or lacking a substituent, N-1 cyclopropyl group, a ring at C-7, and a fluorine at C-6) (see Table 1). Quinolone structure-activity relationship analyses done to date for quinolones and mycobacteria (21, 24, 32, 38) were not based on target inhibition, as in our study, but on MICs, which are the result of target inhibition, as well as many other factors of activity, such as cell wall permeability and efflux (19). Moreover, these analyses were done with nontuberculous mycobacteria (*M. smegmatis*, *M. avium*, and *M. fortuitum*) and not with *M. tuberculosis*. Although cyclopropyl at N-1 was shown to be a key feature (24, 32, 33), these studies often attributed added importance to the substituent at C-8.

There was no relationship between quinolone activity against *M. tuberculosis* gyrase and activity against other bacteria classified according to gram-positive and gram-negative status (16). The quinolones that were highly active against *M. tuberculosis* gyrase included compounds that are highly active against gram-positive bacteria and indeed were developed especially for pneumococci (sitafloxacin, sparflloxacin, clinafl oxacin, moxifloxacin, and gatifloxacin). Conversely, four compounds (grepafloxacin, gemifloxacin, trovafloxacin, and the des[6]fluoroquinolone garenoxacin) with high activity against pneumococci showed only moderate activity against *M. tuberculosis* gyrase. Most of the classical fluoroquinolones developed for their activity against gram-negative bacteria (norfloxacin, pefloxacin, enoxacin, fleroxacin, ofloxacin, temafloxacin, and tosufloxacin) had moderate IC$_{50}$ except for levofloxacin and ciprofloxacin, which had low IC$_{50}$ against *M. tuberculosis* gyrase. Contrary to its effects against pneumococci, the presence of a group at C-5 (27) or a substituent in the 7-piperazinyl ring (1) does not seem to improve gyrase affinity. Moreover, the presence of a naphthyridone core (N-8) in gemifloxacin, which has the lowest MIC against gram-positive bacteria, seems unfavorable for a tight interaction with *M. tuberculosis* gyrase. Similarly, the naphthyridones, tosufloxacin and enoxacin, were only moderately active (Table 1).

The fact that the quinolone structure-activity relationship against *M. tuberculosis* does not follow those established for other gram-positive organisms (*Staphylococcus aureus* and *Streptococcus pneumoniae*) may arise from two unique characteristics of the *M. tuberculosis* DNA gyrase: (i) it is the sole type II topoisomerase in its host, and therefore there is no dual activity on DNA gyrase and on topoisomerase IV, and (ii) the peptidic structure of the quinolone resistance-determining region in the A and B subunits is unique, as described previously (14). Ser-83 of *E. coli* GyrA is the key residue for interaction with quinolones (3) and is conserved in the GyrA proteins of many bacterial species, such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. The equivalent residue (position 90) in *M. tuberculosis* GyrA is an alanine, a difference that may have key importance for quinolone interactions.

The concentrations of quinolones that inhibited 50% of the DNA supercoiling activity of the *M. tuberculosis* DNA gyrase correlated well with the MICs, i.e., their ability to inhibit the growth of *M. tuberculosis*. However, the IC$_{50}$s and MICs were not always proportional; for example, grepafloxacin and trovafloxacin were equipotent in the gyrase assay, and yet the trovafloxacin MIC was about 16-fold higher than that of grepafloxacin against *M. tuberculosis*. This nonproportionality has been noted by others (41) and presumably reflects basic differences in the cell-permeating properties and accumulation of the different quinolones (19). Penetration of the *M. tuberculosis* cell wall by quinolones has not been evaluated yet because the study of the mycobacterial cell wall is still a difficult and uncertain task (19). However, penetration of the *M. tuberculosis* cell wall seems to be at least 100-fold less efficient than that of...
E. coli (9). In the present study, as in studies on gyrases from other bacteria, it has been shown that the concentration of quinolones required to inhibit DNA supercoiling by gyrase is substantially higher than that required to inhibit growth. This fact has been attributed to the poisoning effect of quinolones interacting with the topoisomerases (22).

DNA cleavage assays have been proposed as a more relevant test than supercoiling inhibition to correlate inhibition of gyrase with antibacterial activity (4, 41). Because of the high relaxation activity of M. tuberculosis DNA gyrase, cleavage data needed to be evaluated in the presence of ATP. Unlike the gyrases of E. coli and Streptococcus pneumoniae, M. tuberculosis gyrase exhibited overall a low level of DNA cleavage activity in the presence of quinolones. This has been described previously for gyrA mutants of Streptococcus pneumoniae (31). Moreover, classical quinolones (nalidixic acid, pipemidic acid, oxolinic acid, and flumequine), which lack F-6 or a 7-piperazinyl ring, were very poor inducers of DNA cleavage. Low DNA cleavage stimulation suggests that quinolones may interact less avidly with M. tuberculosis DNA gyrase than with gyrases from other bacterial species (4, 5). This may be due in part to the presence of an alanine at residue 90 of M. tuberculosis GyrA. A Ser83Ala change in E. coli GyrA confers a degree of resistance to quinolones.

In the DNA cleavage assay, the effective quinolone concentrations were slightly different from those inhibiting supercoiling and less correlated with those inhibiting M. tuberculosis growth. The DNA gyrase supercoiling inhibition assay and DNA gyrase cleavable-complex assay are distinct in that the former is a measure of catalytic inhibition, whereas the latter probes an established equilibrium between the ternary DNA-DNA gyrase cleavable-complex assay are distinct in that the former is a measure of catalytic inhibition, whereas the latter probes an established equilibrium between the ternary DNA-DNA gyrase complex (37). The DNA cleavage results might be better correlated to bactericidal activity. Although the in vitro bactericidal activity of quinolones has not been widely explored for mycobacteria, in animal models moxifloxacin showed much higher bactericidal activity than levofloxacin, which is concordant with the CC_{50} data (20).

The study of quinolone interaction with M. tuberculosis DNA gyrase represents a crucial step in investigating quinolone structure-activity relationships and in developing compounds with good activity against tubercle bacilli. The inhibition of DNA supercoiling by M. tuberculosis gyrase could be a safe and quick test for screening drugs with promising anti- tuberculosis activity. Quinolones with low IC_{50} (below 10 μg/ml) will potentially be active against M. tuberculosis and will justify testing in experimental in vivo models of tuberculosis. By contrast, quinolones with high IC_{50} in the enzyme assay will likely not be suitable for further evaluation as antimycobacterial drugs.

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