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Characterization of Fluoroquinolone-Resistant Mutant Strains of *Mycobacterium tuberculosis* Selected in the Laboratory and Isolated from Patients

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To examine the mechanism of resistance to fluoroquinolones in *Mycobacterium tuberculosis*, we selected spontaneous fluoroquinolone-resistant mutants from a susceptible strain, H37Rv, and studied the susceptibilities of these mutants and two fluoroquinolone-resistant clinical isolates (A-382, A-564) to various fluoroquinolones and to isoniazid and rifampin. Furthermore, since mutations within the quinolone resistance-determining region of the structural gene encoding the A subunit of DNA gyrase are the most common mechanism of acquired resistance, we amplified this region by PCR and compared the nucleotide sequences of the fluoroquinolone-resistant strains with that of the susceptible strain. Fluoroquinolone-resistant mutants of H37Rv appeared at frequencies of 2×10^{-6} to 1×10^{-8}. For three mutants selected on ciprofloxacin, ofloxacin, and sparfloxacin, respectively, and the two clinical isolates, MICs of ciprofloxacin and ofloxacin were as high as 16 μg/ml, and those of sparfloxacin were 4 to 8 μg/ml. They displayed cross-resistance to all fluoroquinolones tested but not to isoniazid or rifampin. Sparfloxacin and FQ-A (PD 127391-0002) were the most potent fluoroquinolones. All of the fluoroquinolone-resistant strains (MICs, ≥4 μg/ml) had mutations in the quinolone resistance-determining region which led to substitution of the Asp residue at position 87 (Asp-87) by Asn or Ala or the substitution of Ala-83 by Val in the A subunit of DNA gyrase. Similar mutations have been noted in other bacterial species and recently in mycobacteria. The broad resistance to fluoroquinolones that arose readily by point mutation in the laboratory and apparently during inadequate therapy, as was the case in the clinical isolates, may ultimately lead to serious restriction of the use of these drugs in the treatment of tuberculosis.

The incidence of tuberculosis in the United States has undergone a resurgence since the mid-1980s (3, 16). Unfortunately, the AIDS epidemic has contributed significantly to this increase (2). Complicating this increasing incidence of tuberculosis have been several outbreaks of infections caused by multidrug-resistant strains of *Mycobacterium tuberculosis* (6). In New York City in April 1991, 33 percent of all patients with tuberculosis were infected with *M. tuberculosis* isolates resistant to one or more antituberculosis agents and 19 percent were infected with isolates resistant to both isoniazid and rifampin (8).

Fluoroquinolones, such as ciprofloxacin, ofloxacin, and especially sparfloxacin, have potent in vitro activities against *M. tuberculosis* (9, 17, 19, 21), including the vast majority of multidrug-resistant clinical isolates (8). Fluoroquinolones have been demonstrated to have clinical efficacy against tuberculosis in combination with other antituberculosis agents (12, 18, 27). However, resistance to ciprofloxacin and ofloxacin has appeared in clinical isolates of *M. tuberculosis*, and in some cases such resistance has been shown to emerge during treatment of patients infected with fluoroquinolone-susceptible strains (4, 12, 22, 27).

DNA gyrase, the primary target of fluoroquinolone action, consists of two A and two B subunits and catalyzes ATP-dependent supercoiling of DNA (29) that is necessary for DNA replication in vivo. High-level resistance to fluoroquinolones in various bacteria has been related to mutations in *gyrA*, the structural gene of the gyrase A subunit. These mutations are virtually always clustered within a conserved region of the *gyrA* gene referred to as the quinolone resistance-determining region (QRDR) (7, 10, 11, 20, 24, 30, 32).

We endeavored to examine the mechanism of resistance to fluoroquinolones in *M. tuberculosis* both in mutants selected from a susceptible parental strain in the presence of a fluoroquinolone and in two fluoroquinolone-resistant clinical isolates. We studied the frequency of spontaneous fluoroquinolone-resistant mutants and the susceptibilities of such mutants and the clinical isolates to approved and investigational fluoroquinolones and the standard antituberculosis drugs. Furthermore, we examined the nucleotide sequences of the QRDRs from all of these fluoroquinolone-resistant strains and compared them with that of the susceptible laboratory strain. A recent report characterized the *gyrA* and *gyrB* genes of *M. tuberculosis* and identified similar mutations within the QRDR in *gyrA* in quinolone-resistant strains of *M. tuberculosis* (25).

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MATERIALS AND METHODS

Strains. H37Rv served as the wild-type strain of *M. tuberculosis* susceptible to all antituberculosis drugs including fluoroquinolones. Two clinical isolates of *M.*

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tuberculosis resistant to ciprofloxacin (MICs >2 μg/ml), strains A-382 and A-564, were as described below. All strains were grown in Middlebrook 7H9 broth and on Middlebrook 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; Difco, Detroit, MI) in 5% CO_2 at 37°C.

Clinical isolates. (i) Isolate A-382. A 48-year-old male with AIDS and cervical lymphadenitis was found to have caseation necrosis and acid-fast bacilli in a lymph node biopsy specimen. He was treated with rifampin, ethambutol, ciprofloxacin, and clofazimine for presumed *Mycobacterium avium* complex disease while awaiting the culture result. One month later the cultures of the lymph node as well as of sputum yielded *M. tuberculosis*, and his treatment regimen was changed to isoniazid, rifampin, and ethambutol. The patient was noncompliant, and his sputum remained smear positive for acid-fast bacilli 3 months later. A year later culture of another lymph node biopsy specimen yielded *M. tuberculosis* resistant to rifampin. His therapy was changed to isoniazid, ethambutol, pyrazinamide, and ofloxacin. He continued to be noncompliant and was lost to follow-up. He presented after 14 months with fever and respiratory distress and died 2 weeks later. At this time his sputum (from which isolate A-382 was obtained) and blood cultures were positive for *M. tuberculosis*. These isolates were resistant to rifampin, isoniazid, and ciprofloxacin.

(ii) Isolate A-564. An 88-year-old woman with symptoms of painful cystitis and pyuria was treated unsuccessfully with several courses of antibiotics, including at least five courses of ciprofloxacin, over a period of 1 year. Subsequently, a urine culture yielded *M. tuberculosis* A-564, which was resistant to rifampin, isoniazid, and ciprofloxacin. She was treated with isoniazid, rifampin, and pyrazinamide for 18 months with significant resolution of her symptoms. All of her subsequent urine cultures remained negative.

Quinolones. Ciprofloxacin was a gift from Miles, Inc.; ofloxacin was a gift from Ortho Pharmaceuticals; sparfloxacin and five investigational fluoroquinolones, PD127391-0002 (FQ-A), PD117596-0002 (FQ-B), PD117558-0073 (FQ-C), PD138312-0002 (FQ-D), and PD131628-00028 (FQ-E), were gifts from Parke-Davis/Warner-Lambert.

Selection of fluoroquinolone-resistant mutants. Cells of H37Rv were plated onto Middlebrook 7H10 agar containing serially twofold diluted concentrations of ciprofloxacin, ofloxacin, and sparfloxacin ranging from 0.125 to 32 μg/ml. The plates were incubated at 37°C in 5% CO_2. The inoculum titer was determined by plating diluted aliquots onto drug-free agar and counting the colonies that grew. The frequency of occurrence of resistant mutants in the culture population was calculated from the numbers of colonies on fluoroquinolone-containing agar plates and the original inoculum titer.

Determination of MICs. Each strain was suspended in saline to the turbidity of a McFarland no. 1 standard and was further diluted 10^3 and 10^6 times. Three drops (~100 μl) of each suspension was inoculated onto a quadrant of a Middlebrook 7H10 agar plate with no antibiotic and with serial twofold dilutions of each antibiotic. The H37Rv strain served as a control. The inoculated plates were incubated for 15 days in 5% CO_2 at 37°C. The MIC was defined as the lowest concentration of each drug at which ≤1% of the plated cells grew as colonies (28). Full susceptibility testing was carried out twice.

Isolation of DNA. Chromosomal DNA was isolated by the physical disruption method with a mini-bead beater cell disrupter (15). Briefly, 100-ml cultures of the *M. tuberculosis* strains were grown for 7 to 9 days in 7H9 broth, and the cells were harvested by centrifugation at 3,000 × *g* for 10 min at 4°C. The pellet was resuspended in 0.5 ml of homogenization buffer (0.3M Tris [pH 8.0], 0.1 M NaCl 6 mM EDTA). The cell suspensions were transferred to vials containing 0.5-mm-diameter glass beads and were disrupted with a mini-bead beater cell disrupter (Biospec Products, Bartlesville, Okla.). The DNA was extracted with a phenol-chloroform (1:1) solution; this was followed by chloroform-isoamyl alcohol (24:1) extraction and ethanol precipitation. The precipitate was collected by centrifugation and was resuspended in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA). RNA was removed by treatment with RNase A at 100 μg/ml at 37°C for 30 min; this was followed by phenol extraction and ethanol precipitation as described above, and the DNA was resuspended in TE buffer.

Amplification of QRDR by PCR. Amplification of the QRDR of *gyrA* was initially carried out with primers GYR-1 (5′-GATGGCCTGAAGCCGGTAC AC) and GYR-2 (5′-TGCCATACCTACGGCGATACC) designed from the consensus sequences of known *gyrA* genes from various bacteria (13, 14). Later, when the sequence of the *gyrA* gene of *M. tuberculosis* became available (25), new primers flanking its QRDR were used: TBGYR-1 (5′-CAGCTACATCGACT ATGCG) and TBGYR-2 (5′-GGGCTTCGGTGTGTACCTCAT). PCR amplification was performed in a final volume of 100 μl. The mixture contained approximately 1 ng of DNA template, final concentrations of 1.0 μM (each) primer (TBGYR-1, TBGYR-2), 200 μM (each) deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP; Pharmacia), 10 μl of *Taq* buffer (Gibco-Bethesda Research Laboratories), and 5.0 U of *Taq* polymerase (Gibco-Bethesda Research Laboratories). Amplification was performed for 40 cycles (1 min at 94°C, 1 min at 60°C, and 2 min at 72°C) for an expected product of 280 bp. The QRDR of each strain was amplified in two independent reactions.

Sequencing of the PCR product. The DNA product obtained from PCR was purified by passage through resin by using the Wizard DNA clean-up system (Promega). Sequencing of both DNA strands was carried out with T7 DNA polymerase (Sequenase 2.0; United States Biochemicals) by using TBGYR-1 and TBGYR-2 primers. Analyses of nucleotide and derived amino acid sequences were performed by using the PC Gene software (IntelliGenetics, Inc.). Numbering of the amino acid residues was based on the *Escherichia coli* DNA gyrase A subunit and its *gyrA* gene.

TABLE 1. Frequencies of fluoroquinolone-resistant mutants of *M. tuberculosis* H37Rv growing at various concentrations of each fluoroquinolone

Fluoroquinolone[a]	MIC (μg/ml) for H37Rv	Frequency of resistant mutants of *M. tuberculosis* H37Rv obtained at the following fluoroquinolone concn (μg/ml):		
		2.0	4.0	8.0
CIP	0.5	2×10^{-6}	1×10^{-7}	2×10^{-8}
OFL	2.0	2×10^{-6}	1×10^{-7}	2×10^{-8}
SPL	0.5	1×10^{-6}	1×10^{-8}	$<1 \times 10^{-9}$

[a] CIP, ciprofloxacin; OFL, ofloxacin; SPL, sparfloxacin.

RESULTS

Selection of quinolone-resistant mutants of *M. tuberculosis*. Mutants of parental strain H37Rv resistant to ciprofloxacin, ofloxacin, and sparfloxacin were obtained on 7H10 agar containing 2, 4, and 8 μg of each fluoroquinolone per ml. Spontaneous mutants appeared at frequencies of 2×10^{-6} to 1×10^{-8} (Table 1). The highest concentration of each fluoroquinolone at which such spontaneous mutants were obtained was 8 μg of ciprofloxacin and ofloxacin per ml and 4 μg of sparfloxacin per ml. Single spontaneous quinolone-resistant mutants, mutants CIP-1, OFL-1, and SPL-1, obtained by selection on ciprofloxacin, ofloxacin, and sparfloxacin, respectively, were chosen for further studies.

Susceptibilities of fluoroquinolone-resistant spontaneous mutants and clinical isolates. Susceptibility testing of CIP-1, OFL-1, and SPL-1; their parental strain, H37Rv; and the two clinical isolates of *M. tuberculosis*, A-382 and A-564, was performed on agar containing isoniazid, rifampin, ciprofloxacin, ofloxacin, sparfloxacin, and the five investigational fluoroquinolones. In comparing the MICs obtained from the two successive susceptibility determinations, in no case did comparable MICs differ by more than 1 dilution. In cases of twofold differences, the lower value is reported in Table 2. Parental strain H37Rv was susceptible to all fluoroquinolones tested. Ciprofloxacin, sparfloxacin, FQ-A, and FQ-C were the most potent (MICs, 0.25 to 0.5 μg/ml) fluoroquinolones against this strain. For all of the fluoroquinolone-resistant strains except SPL-1, ciprofloxacin and ofloxacin MICs were 16 μg/ml and sparfloxacin MICs were 4 to 8 μg/ml. Strain SPL-1 was marginally less resistant. All of the fluoroquinolone-resistant strains were also cross-resistant to all of the investigational fluoroquinolones (MICs, ≥4 μg/ml). In general, however, sparfloxacin and FQ-A exhibited the most potent activities against the panel of five fluoroquinolone-resistant strains. None of the spontaneous fluoroquinolone-resistant mutants displayed resistance to isoniazid or rifampin. The two clinical isolates, however, were resistant to these first-line antituberculosis drugs.

PCR amplification and sequence analysis of the QRDR of the *gyrA* gene. A 320-bp region of the chromosomal DNA which includes the QRDR of the *gyrA* gene was amplified from the parental strain and the fluoroquinolone-resistant strains by PCR with primers TBGYR-1 and TBGYR-2. The nucleotide sequences and the deduced amino acid sequences were compared, and the results are summarized in Table 3. Comparison of the QRDRs of the *gyrA* genes from the fluoroquinolone-resistant mutants with those of their susceptible parental strains revealed single base changes leading to replacements of

TABLE 2. Susceptibilities of fluoroquinolone-resistant spontaneous mutants and clinical isolates of *M. tuberculosis*[a]

Strain	MIC (μg/ml)									
	INH	RIF	CIP	OFL	SPL	FQ-A	FQ-B	FQ-C	FQ-D	FQ-E
H37Rv (parental)	≤0.05	≤0.5	0.5	2.0	0.5	0.25	1	0.5	1	1
CIP-1 (selected on CIP, 8 μg/ml)	≤0.05	≤0.5	16	16	8	4	16	8	>16	8
OFL-1 (selected on OFL, 8 μg/ml)	≤0.05	≤0.5	16	16	8	4	16	8	>16	16
SPL-1 (selected on SPL, 4 μg/ml)	≤0.05	≤0.5	4	8	4	4	8	16	16	8
A-382 (clinical isolate)	>4	>8	16	16	4	8	16	>16	16	8
A-564 (clinical isolate)	>4	>8	16	16	8	16	16	>16	16	>16

[a] INH, isoniazid; RIF, rifampin; CIP, ciprofloxacin; OFL, ofloxacin; SPL, sparfloxacin; FQ-A, PD127391-0002; FQ-B, PD117596-0002; FQ-C, PD117558-0073; FQ-D, PD138312-0002; FQ-E, PD131628-00028.

the Asp residue at position 87 (Asp-87) with Asn or Ala-83 with Val in the A subunit of the mutant DNA gyrase. Mutations at codon 87 leading to Asp→Asn or Asp→Ala replacements were noted in the two fluoroquinolone-resistant clinical isolates. H37Rv and its spontaneous resistant mutants had serine residues at codon 88, whereas the two clinical isolates had threonine residues at that position.

DISCUSSION

Spontaneous fluoroquinolone-resistant mutants of *M. tuberculosis* H37Rv appeared at frequencies of 2×10^{-6} to 1×10^{-8}. Similar frequencies of fluoroquinolone-resistant mutants have been observed for *M. tuberculosis* (25, 26) and *Mycobacterium smegmatis* (23). Since the MIC of ofloxacin for H37Rv is four times that of ciprofloxacin, the selecting concentrations of ofloxacin are 1, 2, and 4 times the MIC, in comparison with 4, 8, and 16 times the MIC of ciprofloxacin. Therefore, it is remarkable that the frequencies of resistant mutants were no higher with ofloxacin than with ciprofloxacin at each of the three concentrations. With sparfloxacin, it is noteworthy that at 8 μg/ml (16 times the MIC) we could detect no resistant mutants.

For the spontaneous fluoroquinolone-resistant mutants obtained in the one-step selection on agar medium containing various concentrations of fluoroquinolones, ciprofloxacin and ofloxacin MICs were as high as 16 μg/ml and sparfloxacin MICs were 4 to 8 μg/ml. Single-step mutants of *M. smegmatis* for which MICs are similar have been described previously (23), although sequential serial-step selection was needed to obtain *M. tuberculosis* mutants with such levels of fluoroquinolone resistance (25). Clinical studies with either ofloxacin alone or ofloxacin with other second-line antituberculosis agents in the treatment of tuberculosis resulted in the ready selection of ofloxacin-resistant mutants (4, 12, 22, 27). This also appears to be the case in the treatment of the two patients from whom our fluoroquinolone-resistant clinical isolates of *M. tuberculosis* were obtained. Both of these patients had been exposed to a fluoroquinolone as part of their therapy prior to the development of resistance.

TABLE 3. Mutations in *gyrA* associated with fluoroquinolone resistance in the *M. tuberculosis* strains in the present study

Strains	Phenotype (MIC [μg/ml])[a]			Mutation[b]	Residue at position 88
	CIP	OFL	SPL		
H37Rv	0.5	2.0	0.5		Ser
CIP-1	16	16	8	Asp-87→Asn (GAC→AAC)	Ser
OFL-1	16	16	8	Asp-87→Asn (GAC→AAC)	Ser
SPL-1	4	8	4	Ala-83→Val (GCG→GTG)	Ser
A-382	16	16	4	Asp-87→Ala (GAC→GCC)	Thr
A-564	16	16	8	Asp-87→Asn (GAC→AAC)	Thr

[a] CIP, ciprofloxacin; OFL, ofloxacin; SPL, sparfloxacin.
[b] Numbering of residues is based on the *E. coli* DNA gyrase A subunit.

Our fluoroquinolone-resistant mutants and clinical isolates displayed cross-resistance to all of the fluoroquinolones tested, with sparfloxacin and one of the investigational fluoroquinolones, FQ-A (PD127391-0002), being the most potent. However, no concomitant development of resistance to isoniazid or rifampin was found.

Analysis of the QRDRs of *gyrA* genes from all of the fluoroquinolone-resistant strains showed mutations resulting in a substitution of Ala-83 or Asp-87. These are two of the positions at which mutations have been most frequently associated with resistance to fluoroquinolones in other bacterial species (11, 14, 30). In the strains of *M. tuberculosis* that we examined, substitution at position Asp-87 (Asp-94 elsewhere [25]) was the most common, as reported previously for fluoroquinolone-resistant strains of *M. tuberculosis* (25). The substitutions observed at this position, asparagine or alanine, were among those noted in fluoroquinolone-resistant strains of *M. tuberculosis* (4, 25) and *M. smegmatis* (23).

M. tuberculosis, *M. smegmatis*, and *M. avium* have alanine residues at position 83 in the region of the A subunit of DNA gyrase corresponding to the QRDR (5, 23, 25) rather than serine or threonine, which are present at this site in the majority of reported sequences (14). It has been suggested that the presence of Ala-83 makes an organism moderately less susceptible to ciprofloxacin (14), although other differences among bacterial species may contribute to differential susceptibilities. Certain mutational replacements at position 83 have often been reported in fluoroquinolone-resistant mutants of various bacteria (14, 30), and such is the case with *M. tuberculosis*, *M. smegmatis*, and *M. avium* (5, 23, 25). It is of interest that the Ala-83→Val mutation in *M. tuberculosis* resulted in a somewhat lower level of fluoroquinolone resistance than that observed with mutations at position 87.

The wild-type parental strain and the spontaneous mutants of H37Rv displayed serine residues at position 88, whereas the clinical isolates had threonine residues at this position. This may represent a naturally occurring nonfunctional polymorphism (25).

It is unlikely that mechanisms of resistance other than those from the observed mutations could explain the development of resistance in the mutants obtained from a single-step selection. However, the observed fluoroquinolone resistance in the two clinical isolates may be due not only to mutations in the QRDR of *gyrA* but perhaps also to other mutations affecting DNA gyrase or the permeabilities of the cells to fluoroquinolones (31).

In conclusion, broad resistance to fluoroquinolones arose readily in *M. tuberculosis* H37Rv. Although resistance to fluoroquinolones so far is infrequent in clinical isolates of *M. tuberculosis* (8), the ease with which resistance arises in this organism may have serious consequences because these agents are increasingly used in the treatment of multidrug-resistant tuberculosis or in the event of treatment failure. Furthermore, the potential selective pressure for fluoroquinolone resistance is considerable in developing countries, where tuberculosis is highly endemic and patients are frequently treated with fluoroquinolones for enteric infections.

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