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Activity of the New Fluoroquinolone Trovafloxacin (CP-99,219) against DNA Gyrase and Topoisomerase IV Mutants of *Streptococcus pneumoniae* Selected In Vitro

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The MICs of trovafloxacin, ciprofloxacin, ofloxacin, and sparfloxacin at which 90% of isolates are inhibited for 55 isolates of pneumococci were 0.125, 1, 4, and 0.5 μg/ml, respectively. Resistant mutants of two susceptible isolates were selected in a stepwise fashion on agar containing ciprofloxacin at 2 to 10 times the MIC. While no mutants were obtained at the highest concentration tested, mutants were obtained at four times the MIC of ciprofloxacin (4 μg/ml) at a frequency of 1.0×10^{-9}. Ciprofloxacin MICs for these first-step mutants ranged from 4 to 8 μg/ml, whereas trovafloxacin MICs were 0.25 to 0.5 μg/ml. Amplification of the quinolone resistance-determining region of the *grlA* (*parC*; topoisomerase IV) and *gyrA* (DNA gyrase) genes of the parents and mutants revealed that changes of the serine at position 80 (Ser80) to Phe or Tyr (*Staphylococcus aureus* coordinates) in GrlA were associated with resistance to ciprofloxacin. Second-step mutants of these isolates were selected by plating the isolates on medium containing ciprofloxacin at 32 μg/ml. Mutants for which ciprofloxacin MICs were 32 to 256 μg/ml and trovafloxacin MICs were 4 to 16 μg/ml were obtained at a frequency of 1.0×10^{-9}. Second-step mutants also had a change in GyrA corresponding to a substitution in Ser84 to Tyr or Phe or in Glu88 to Lys. Trovafloxacin protected from infection mice whose lungs were inoculated with lethal doses of either the parent strain or the first-step mutant. These results indicate that resistance to fluoroquinolones in *S. pneumoniae* occurs in vitro at a low frequency, involving sequential mutations in topoisomerase IV and DNA gyrase. Trovafloxacin MICs for wild-type and first-step mutants are within clinically achievable levels in the blood and lungs of humans.

Streptococcus pneumoniae is the leading cause of bacterial respiratory tract infections in humans. The pathogenic potential of this organism is great, ranging from bronchitis to life-threatening pneumonia, bacteremia, and meningitis. Ciprofloxacin and other marketed quinolones have only modest activity against this pathogen, limiting their use in patients with serious respiratory tract infections (4, 5, 22). In addition to the limited utility of fluoroquinolones against this organism, pneumococci for which MICs of beta-lactams and macrolides are elevated are becoming more prevalent throughout the world (1, 2).

Trovafloxacin is a new fluoroquinolone with potent activity in vitro against gram-positive, gram-negative, and anaerobic organisms (5, 10, 23). Trovafloxacin is equally active against both penicillin-susceptible and -resistant pneumococci, with MICs of 0.06 to 0.25 μg/ml reported for more than 700 isolates tested from around the world (10, 16). A recently published study indicates that trovafloxacin is more efficacious than ciprofloxacin or temafloxacin in treating lethal lung infections with *S. pneumoniae* in mice (9). Trovafloxacin is well absorbed following oral dosing in humans and has an elimination half-life of approximately 10 h (26), allowing for once-daily dosing for many indications. The potent activity of trovafloxacin against pneumococci and other respiratory tract pathogens has encouraged its use in clinical trials of lung infections not normally treated with earlier fluoroquinolones such as ciprofloxacin (20).

Studies with other gram-positive organisms indicate that mutations in at least three different types of genes can result in clinically relevant levels of quinolone resistance. One mechanism involves the efflux of drug, such as with the NorA transporter in *Staphylococcus aureus* (14, 28). The other targets represent the most frequent sites of mutation (11, 12, 21) and involve changes in the A subunit of DNA gyrase (GyrA) and its homolog, topoisomerase IV (GrlA; also designated ParC [21, 24]). While topoisomerase IV appears to be a secondary target for inhibition by fluoroquinolones in *Escherichia coli* (13, 15), recent studies have revealed that initial mutation to quinolone resistance in *S. aureus* involves a change in topoisomerase IV (7). Studies by Ferrero et al. (6) indicate that mutations occur first in *grlA*, leading to moderate levels of resistance to fluoroquinolones (MIC, 8 μg/ml); this is followed by a second mutation in *gyrA* which leads to high-level resistance. Such *S. aureus* double mutants demonstrate high-level resistance, and the MICs of ciprofloxacin for these mutants are ≥64 μg/ml (6).

Given the potential for trovafloxacin in the therapy of infections caused by *S. pneumoniae*, we have studied the mutation frequency to resistance for trovafloxacin compared with that for ciprofloxacin and characterized mutations in the structural genes for topoisomerase IV and DNA gyrase A subunits. We studied the activity of trovafloxacin against representative pneumococcal mutants with alterations in both genes. The results indicate that mutation to trovafloxacin resistance in *S. pneumoniae* occurs at a low frequency and that first-step mu-

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tants with a change in the topoisomerase IV A subunit are still inhibited by clinically achievable levels of trovafloxacin.

MATERIALS AND METHODS

Susceptibility studies with *S. pneumoniae*. The in vitro activities of trovafloxacin and other antimicrobial agents against 53 clinical isolates and 2 control strains were determined by agar dilution tests. Strains from Europe, South Africa, and the United States were included. The strains were selected in part for their resistance to penicillin G and macrolides.

The MICs were determined by using twofold dilutions of antibiotics prepared in cation-supplemented Mueller-Hinton agar containing 5% sheep's blood with a final inoculum of 1×10^4 to 4×10^4 CFU per spot. MBCs for selected parent and mutant strains were determined in cation-supplemented Mueller-Hinton broth containing 5% lysed horse blood with an inoculum of 5×10^5 CFU/ml. Subcultures (0.01 ml) were sampled from each tube without visible growth in the MIC test and were inoculated onto blood agar plates.

Selection of resistant mutants. Two types of selection were applied. First, representative clinical strains were grown overnight on agar and a cell suspension (more than 10^9 cells) was plated in duplicate on medium containing ciprofloxacin or trovafloxacin at concentrations achievable in the blood of humans (0.25, 0.5, 1, 2, and 4 μg/ml). After 48 h of incubation at 35°C, the colonies were counted and the frequency of clones growing at each concentration was determined relative to the total viable count plated. Second, cells prepared in a manner identical to that described above were plated onto agar containing from 2 to 10 times the MIC of ciprofloxacin. After 48 h of incubation, the clones were passed onto drug-free agar plates, tested for their susceptibilities to antibacterial agents, and stored at −70°C. Several first-step mutants for which ciprofloxacin MICs were elevated were used in a second round of selection by plating more than 10^9 cells on agar containing 32 μg of ciprofloxacin per ml. The second-step mutants were counted, transferred onto drug-free agar plates, and tested for their susceptibilities to various antimicrobial agents.

Amplification of QRDRs of *grlA* and *gyrA* genes. Mutants derived by plating two parental strains on medium containing multiples of the MIC of ciprofloxacin were examined by PCR for changes in the quinolone resistance-determining regions (QRDRs) of *gyrA* and *grlA*. The primer sequences for *S. pneumoniae grlA* (24) from positions 109 to 362 (*S. aureus* coordinates) were as follows: sense primer I, 5′-GGNTTRAARCCNGTNCAG-3′, and antisense primer II, 5′-TC WGTRTAWCKCATWGC-3′ (N is A, C, G, or T; W is A or T; R is A or G; and K is G or T). The amplification mixture contained, in 100 μl, 150 pmol of each primer, 500 μM (each) deoxynucleoside triphosphate (dNTP), 5 mM $MgCl_2$, and 2 U of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.). PCR conditions were a first denaturation for 4 min at 94°C and a first hybridization for 3 min at 51°C; these were followed by 30 cycles of elongation for 30 s at 72°C, 30 s of denaturation at 94°C, and hybridization for 30 s at 51°C; the final elongation was for 5 min at 72°C. The size of the amplified *grlA* fragment was 254 bp.

The primer sequences for the QRDR of *gyrA* from positions 70 to 354 (*S. aureus* coordinates) included sense primer I of 5′-GAITAYGCIATGAGYGT-3′, where Y is C or T (24). The antisense primer II was 5′-AGCACTATCTCC ATCCATGGA-3′. The amplification conditions were as described above, except that 100 pmol of primer I, 20 pmol of primer II, and 0.25 mM (each) dNTP were used. The size of the amplified *gyrA* fragment obtained was 285 bp.

The PCR fragments generated in two separate experiments were purified, and the sequences of both strands were determined directly.

Mouse lung infection. *S. pneumoniae* pneumonia was induced in mice by intranasal challenge with 10^6 CFU of strain 02J1016 (9). Therapy was initiated 18 h after the challenge, and oral doses of trovafloxacin were given twice per day for 3 days. The number of CFU per lung was approximately 10^5 at the start of therapy. The numbers of survivors were recorded daily over a 10-day period, after which the 50% protective dose (PD_{50}) was calculated from the dose titration. Comparative tests were also performed with one mutant with an alteration in GrlA (02J1016-30). The 50% lethal doses for the parental strain and its derivative were indistinguishable.

Determination of trovafloxacin levels in mouse serum and lung samples. The levels of trovafloxacin in serum were determined as described previously (25). In brief, samples were acidified and a methylated analog of trovafloxacin was added as an internal standard. The mixture was eluted from Polysorb C-18 MP-1 extraction columns (Interaction Chromatography, San Jose, Calif.) with methanol. The extract was evaporated and redissolved in 0.5 ml of mobile phase (83% 0.04 M H_3PO_4, 15.5% acetonitrile, 0.5% tetrabutylammonium hydroxide [40%; wt/wt; Sigma Chemicals, St. Louis, Mo.], and 1.0% dibutylamine phosphate). The samples were mixed, and 50 μl was injected onto a high-pressure liquid chromatography (HPLC) column (3.9 by 150 mm; Nova-pak C-18; Waters, Milford, Mass.). The elution of trovafloxacin was monitored by UV detection at 275 nm.

Lung samples (200 mg) were added to 5 ml of extraction buffer (0.15 M $HClO_3$, 0.15 M H_3PO_4 in distilled H_2O–CH_3OH; 50/50; vol/vol). The internal standard was added to each sample, and the tissue was homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, N.Y.). The samples were centrifuged, and the resulting supernatant was evaporated at 55°C. The residue was dissolved in 2 ml of 0.025 M KH_2PO_4 (pH 3.0) and extracted twice with 5 ml of ethyl acetate. The ethyl acetate was evaporated, and the residue was resuspended in 0.25 ml of mobile phase and washed with 1 ml of hexane. The hexane was aspirated off, and 100-μl aliquots were injected onto the HPLC column.

RESULTS

Antimicrobial susceptibility tests. Fifty-three clinical isolates and two control strains of *S. pneumoniae* were tested for their susceptibilities to trovafloxacin and nine other antimicrobial agents (Table 1). Trovafloxacin was the most potent fluoroquinolone, with an MIC at which 90% of isolates are inhibited (MIC_{90}) of 0.125 μg/ml, whereas ciprofloxacin, sparfloxacin, and ofloxacin had MIC_{90}s of 1.0, 0.5, and 4 μg/ml, respectively. This collection of pneumococci contained organisms resistant to other clinically important antimicrobial agents for which the MIC_{90}s of penicillin G and ceftriaxone were 2 μg/ml. The MIC_{90}s were out of the susceptible range for erythromycin and tetracycline. The strains were susceptible to vancomycin (MIC_{90}, 0.5 μg/ml). Broth dilution MICs and MBCs were determined for a selected number of parent and mutant isolates. The MICs were comparable to those determined by the agar dilution method, and the MBCs were no more than fourfold higher than the MICs for parent and mutant strains alike (data not shown).

Since pneumococcal resistance to beta-lactams is increasing worldwide, we stratified our susceptibility data on the basis of strain susceptibility or resistance to these agents. As indicated in Table 1, trovafloxacin was as active against the subgroup of 25 penicillin-resistant isolates, as it was against 22 penicillin-intermediate or 8 penicillin-susceptible strains.

In vitro selection of resistance. Two penicillin G- and macrolide-resistant strains (02J1054 and 02J1056) and three penicillin G- and macrolide-susceptible strains (02J1016, 02J1010, and ATCC 6303) were chosen for in vitro selection of resistance studies.

In the first set of studies, the frequencies of mutation to resistance were determined by plating $>1.0 \times 10^9$ parent cells onto agar containing concentrations of ciprofloxacin or trovafloxacin that reflect the levels achievable in the blood of humans after oral dosing. Following the administration of a 200-mg dose of trovafloxacin, peak levels in the blood of humans are approximately 2.0 μg/ml (17). The MIC_{90}s of trovafloxacin and ciprofloxacin for our collection of pneumococci were 0.125 and 1.0 μg/ml, respectively. The data in Table 2 indicate that at ciprofloxacin levels of from 1 to 4 μg/ml, resistance selection occurred at frequencies of 1.8×10^{-5} to $<1.0 \times 10^{-9}$. The selection of resistance with trovafloxacin at these concentrations was uniformly less frequent ($\leq 8.9 \times 10^{-9}$). It is probable that the survivors found at higher frequencies among cells plated with both drugs at their MICs represent surviving parent cells and not true mutants.

Given the observed mutation frequency data, a second set of selection experiments was performed by plating at least 1.0×10^9 cells from parent strains 02J1016 and 02J1056 onto agar containing 2 to 10 times the MIC of ciprofloxacin. The mutants were counted, and clones were used in a second step of selection with ciprofloxacin (four times the MIC, or 32 μg/ml). The first- and second-step mutants were transferred onto drug-free medium and were tested for their susceptibilities to antimicrobial agents.

In the first selection with parental strain 02J1016, 100 colonies were studied: for 55 clones ciprofloxacin MICs were 4 μg/ml and for 45 clones ciprofloxacin MICs were 8 μg/ml. In the second selection, 100 colonies were studied: for 27 clones ciprofloxacin MICs were 32 μg/ml, for 69 clones ciprofloxacin MICs were 64 μg/ml, and for 4 clones ciprofloxacin MICs were 128 μg/ml.

TABLE 1. Susceptibility of *S. pneumoniae* to 10 antimicrobial agents

Organism group (no. of isolates)	Antimicrobial agent	MIC (μg/ml)		
		50%	90%	Range
S. pneumoniae (55)	Trovafloxacin	0.125	0.125	0.063–0.25
	Ciprofloxacin	0.5	1.0	0.25–2.0
	Sparfloxacin	0.25	0.5	0.063–1.0
	Ofloxacin	2.0	4.0	2.0–4.0
	Penicillin G	1.0	2.0	≤0.008–4.0
	Ceftriaxone	1.0	2.0	≤0.008–4.0
	Erythromycin	0.5	8.0	≤0.008–≥16
	Clarithromycin	0.063	0.5	≤0.008–≥16
	Tetracycline	0.5	>16	0.125–>16
	Vancomycin	0.5	0.5	0.063–0.5
S. pneumoniae, penicillin resistant (25)	Trovafloxacin	0.125	0.125	0.063–0.125
	Ciprofloxacin	0.5	1.0	0.5–2.0
	Sparfloxacin	0.25	0.5	0.25–0.5
	Ofloxacin	2.0	4.0	2.0–4.0
	Penicillin G	2.0	4.0	2.0–4.0
	Ceftriaxone	1.0	2.0	0.5–4.0
	Erythromycin	2.0	8.0	0.015–>16
	Clarithromycin	0.063	0.5	≤0.008–>16
	Tetracycline	8.0	16	0.25–>16
	Vancomycin	0.5	0.5	0.25–0.5
S. pneumoniae, penicillin intermediate (22)	Trovafloxacin	0.125	0.125	0.063–0.25
	Ciprofloxacin	1.0	1.0	0.25–2.0
	Sparfloxacin	0.25	0.5	0.125–0.5
	Ofloxacin	2.0	4.0	2.0–4.0
	Penicillin G	0.25	1.0	0.125–1.0
	Ceftriaxone	0.25	1.0	0.031–2.0
	Erythromycin	0.063	8.0	≤0.063–>16
	Clarithromycin	0.063	0.5	≤0.008–>16
	Tetracycline	0.25	>16	0.125–>16
	Vancomycin	0.25	0.5	0.063–0.5
S. pneumoniae, penicillin susceptible (8)	Trovafloxacin	0.125		0.063–0.125
	Ciprofloxacin	0.5		0.25–1.0
	Sparfloxacin	0.25		0.063–0.5
	Ofloxacin	2.0		2.0–4.0
	Penicillin G	0.015		≤0.008–0.063
	Ceftriaxone	0.015		≤0.008–0.25
	Erythromycin	0.03		0.015–8.0
	Clarithromycin	≤0.008		≤0.008–0.5
	Tetracycline	0.25		0.125–>16
	Vancomycin	0.25		0.063–0.5

Amplification and sequence of the QRDRs of *gyrA* and *grlA*. The changes in GrlA and GyrA associated with quinolone resistance in several representative first- and second-step mutants of 02J1016 and 02J1056 are summarized in Table 3. All first-step mutants examined by PCR had a substitution of Phe or Tyr at the serine at position 80 (Ser80) in the QRDR of GrlA (*S. aureus* coordinates [7]). This single-step mutation in 02J1016 led to mutants for which ciprofloxacin and trovafloxacin MICs were increased four- to eightfold. The MICs of ciprofloxacin increased to 4 to 8 μg/ml, while those of trovafloxacin were 0.5 μg/ml. This was also the case for strain 02J1056. No changes in the *gyrA* QRDR were observed in any of the first-step mutants of either strain.

All second-step mutants had changes in *gyrA*, in addition to changes in *grlA*. Mutations in both genes are associated with a significantly higher MIC of ciprofloxacin, generally in the range of 32 to 128 μg/ml. The MICs of trovafloxacin ranged from 4 to 16 μg/ml for the double mutants. The most common substitution in GyrA was a Tyr at Ser84 (*S. aureus* coordinates [18]), detected in 17 of 24 mutants examined by PCR (data for representative strains are presented in Table 3). A substitution of Phe for Ser84 was also found (3 of 24 mutants), and four mutants had a Lys substitution at Glu88. In strain 02J1056, the latter mutation seemed to confer the highest level of resistance to trovafloxacin (16 μg/ml), although this was not evident for strain 02J1016 (Table 3).

The MICs of the DNA gyrase B subunit inhibitor coumermycin A1 did not increase more than twofold for any of the mutants and decreased at least eightfold for mutant 02J1056-100-7. Susceptibility to tetracycline did not differ by more than twofold between the mutant and parent isolates.

Mouse lung infection studies with the parent and first-step mutant. Recent studies have indicated that under the standard conditions of the pneumococcal mouse lung infection model, quinolone-resistant mutants cannot be selected from strain 02J1016 during therapy with trovafloxacin or ciprofloxacin (8). Since mutants with resistance to trovafloxacin could not be selected in that model, we wished to determine the in vivo activity of trovafloxacin against one of our first-step *grlA* mutants compared with that observed against the original parent

TABLE 2. Selection of resistant *S. pneumoniae* with ciprofloxacin or trovafloxacin

Drug and strain	Frequency of resistance selection with drug at the following concn (μg/ml)				
	4	2	1	0.5	0.25
Ciprofloxacin					
02J1054[a]	1×10^{-8}	1.5×10^{-7}	5.3×10^{-6}	$>1 \times 10^{-5}$[b]	$>1 \times 10^{-5}$
02J1056	$<1 \times 10^{-9}$	2.1×10^{-8}	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
02J1016	4.8×10^{-9}	9.1×10^{-8}	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
02J1010	$<7.9 \times 10^{-9}$	$<7.9 \times 10^{-9}$	9.2×10^{-8}	4.6×10^{-8}	$>1 \times 10^{-5}$
ATCC 6303	$<1.7 \times 10^{-9}$	5.3×10^{-8}	1.8×10^{-5}	$>1 \times 10^{-5}$	$>1 \times 10^{-5}$
Trovafloxacin					
02J1054[c]	$<8.0 \times 10^{-9}$	$<8.0 \times 10^{-9}$	$<8.0 \times 10^{-9}$	$<8.0 \times 10^{-9}$	3.6×10^{-7}
02J1056	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	8.9×10^{-9}	9.1×10^{-9}	3.2×10^{-8}
02J1016	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	1.1×10^{-8}
02J1010	$<7.9 \times 10^{-9}$	$<7.9 \times 10^{-9}$	$<7.9 \times 10^{-9}$	$<7.9 \times 10^{-9}$	2.3×10^{-8}
ATCC 6303	$<1.7 \times 10^{-9}$	$<1.7 \times 10^{-9}$	$<1.7 \times 10^{-9}$	3.5×10^{-9}	3.1×10^{-8}

[a] Ciprofloxacin MICs were 1 μg/ml for strains 02J1054, 02J1056, 02J1016, and ATCC 6303 and 0.25 μg/ml for strain 02J1010.
[b] Represents confluent plate growth of strain at or below the MIC for the strain.
[c] Trovafloxacin MICs were 0.063 μg/ml for strains 02J1054 and ATCC 6303. MICs were 0.125 μg/ml for strains 02J1056 and 02J1016 and 0.03 μg/ml for strain 02J1010.

strain. The data indicate that while the MIC of trovafloxacin increased fourfold for mutant 02J1016-30 compared with that for the parent (0.5 versus 0.125 μg/ml), the PD_{50} increased only 1.9-fold, with a value of 11.1 mg/kg of body weight (7.6 to 15.1 mg/kg) for the mutant, compared with 6.0 mg/kg (5.4 to 6.7 mg/kg) for the parent.

Ciprofloxacin was not efficacious against the parent in this model, even when it was dosed at 100 mg/kg (9), and it was therefore not tested against the mutant.

An experiment was carried out in order to correlate drug levels in blood and tissue with the efficacy observed in the lung model by using a trovafloxacin dose comparable to the PD_{50} obtained with mutant 02J1016-30. Mice infected with strain 02J1016-30 were given an oral dose of 12 mg of trovafloxacin per kg twice daily. After administration of the third dose, groups of mice were sacrificed over the ensuing 24 h, and the levels of trovafloxacin in serum and lung were determined. The data in Fig. 1 indicate that following the third dose of drug, peak drug levels in serum and lung were 5.1 μg/ml and 5.3 μg/g, respectively. Drug levels remained above the MIC for the first-step mutant (0.5 μg/ml) for more than 20 h.

DISCUSSION

While fluoroquinolones such as ciprofloxacin and ofloxacin have only moderate activity against *S. pneumoniae*, newer agents which have significantly greater in vitro potency against this important organism are in development. Trovafloxacin is a new naphthyridone agent of the fluoroquinolone class that possesses such potency advantages. In a recent review by Klugman and Gootz (16), the ranges in MIC_{90}s of trovafloxacin for *S. pneumoniae* in eleven studies were 0.06 to 0.25 μg/ml. The MIC_{90}s were similar for 1,285 penicillin-susceptible and 613 penicillin-resistant isolates. In a study by Visalli et al. (27), the bactericidal activity of trovafloxacin against six penicillin-susceptible and four penicillin-resistant pneumococci was 0 to 2 dilutions higher than the MIC. The highest MBC recorded in that study was 0.25 μg/ml. Trovafloxacin also showed enhanced bactericidal activity in an experimental pneumococcal meningitis model in rabbits compared with the activities of ciprofloxacin, ofloxacin, and temafloxacin (19).

We began our investigation with a susceptibility study of penicillin-susceptible and -resistant isolates of *S. pneumoniae*. The data in Table 1 are consistent with those from studies reported by others, indicating that the MIC_{90} of trovafloxacin is 4-, 8-, and 32-fold lower than the MIC_{90}s of sparfloxacin, ciprofloxacin, and ofloxacin, respectively. The data for the 55 pneumococcal isolates were also stratified for penicillin-resistant, -intermediate, and -susceptible isolates. While a large proportion of our strains had reduced susceptibilities to beta-lactams and macrolides, trovafloxacin generated the same MIC_{50}s and MIC_{90}s for all three groups. For the isolates in this collection least susceptible to trovafloxacin, the MICs were 0.25 μg/ml.

In order to compare the frequency of emergence of resistance in vitro, we chose four clinical strains (two macrolide- and penicillin G-susceptible strains and two macrolide- and penicillin G-resistant strains) and one American Type Culture Collection control strain for the selection studies. Initial experiments involved the testing of ciprofloxacin and trovafloxacin each at the clinically relevant concentrations of 0.25 to 4 μg/ml. This comparison revealed that at concentrations ≥0.5 μg/ml, resistance selection with trovafloxacin occurred at a relatively low frequency ($\leq 9.1 \times 10^{-9}$ per cell plated). At ciprofloxacin concentrations of 2 to 4 μg/ml (at least twofold greater than the MIC), the frequencies of mutation to resistance tended to be higher ($<1.0 \times 10^{-9}$ to 1.5×10^{-7}). We were not able to obtain mutants resistant to either drug at concentrations ≥10 times the MIC (data not shown). These data agree with those from a previous study (3), which found that in vitro resistance selection with *S. aureus* across a similar concentration range occurred at a lower frequency with trovafloxacin than with ciprofloxacin. The frequency of resistance selection in our study was influenced by the drug concentrations chosen. At comparable factors above the MIC of each drug, the frequency of mutation appeared to be similar for both fluoroquinolones. The greater potency of trovafloxacin accounts for the lower frequency of resistance selection observed at the clinically relevant concentrations of 1 to 4 μg/ml.

Since under these selection conditions, first-step mutants were obtained at a higher frequency with ciprofloxacin, we performed further experiments with strains 02J1016 and 02J1056 on medium containing four times the MIC of the drug. All of the first-step mutants that we studied were generally fourfold less susceptible to ciprofloxacin and trovafloxacin than the parent, while susceptibility to the gyrase B subunit inhibitor coumermycin A1 and to tetracycline did not change

TABLE 3. Characteristics of first- and second-step mutants of *S. pneumoniae*

Group and strain	MIC (μg/ml)				Amino acid change	
	Ciprofloxacin	Trovafloxacin	Coumermycin	Tetracycline	GyrA	GrlA
Parent						
02J1016	1	0.125	0.125	0.125		
02J1056	1	0.125	0.125	0.25		
First-step mutant[a]						
1016-27	8	0.5	0.125	0.125		Ser80 to Phe[b]
1016-36	4	0.5	0.125	0.125		Ser80 to Tyr
1056-13	8	0.5	0.25	0.25		Ser80 to Phe
1056-100	8	0.5	0.063	0.25		Ser80 to Phe
Second-step mutant[c]						
1016-27-11	128	4	0.25	0.125	Ser84 to Tyr[d]	Ser80 to Phe
1016-27-23	64	16	0.125	0.125	Ser84 to Phe	Ser80 to Phe
1016-36-1	64	16	0.031	0.063	Ser84 to Tyr	Ser80 to Tyr
1016-36-24	64	8	0.125	0.125	Ser84 to Phe	Ser80 to Tyr
1016-36-43	128	16	0.031	0.063	Glu88 to Lys[e]	Ser80 to Tyr
1016-36-70	64	16	0.125	0.063	Glu88 to Lys	Ser80 to Tyr
1056-13-2	256	8	0.125	0.25	Ser84 to Tyr	Ser80 to Phe
1056-13-18	128	16	0.125	0.25	Glu88 to Lys	Ser80 to Phe
1056-13-22	64	8	0.125	0.25	Ser84 to Tyr	Ser80 to Phe
1056-100-4	32	4	0.25	0.25	Ser84 to Tyr	Ser80 to Phe
1056-100-7	64	16	≤0.015	0.25	Glu88 to Lys	Ser80 to Phe
1056-100-78	32	4	0.25	0.25	Ser84 to Tyr	Ser80 to Phe

[a] First-step mutants were selected on agar plates containing 4 μg of ciprofloxacin per ml.
[b] Ser80 according to *S. aureus* coordinates for *grlA* (7).
[c] Second-step mutants were selected on agar plates containing 32 μg of ciprofloxacin per ml.
[d] Ser84 according to the *S. aureus* coordinates for *gyrA* (18).
[e] Glu88 according to the *S. aureus* coordinates for *gyrA* (18).

for most mutants (Table 3). For the first-step mutants, ciprofloxacin MICs were 4 to 8 μg/ml, while trovafloxacin MICs were 0.25 or 0.5 μg/ml.

Two first-step mutants from each pneumococcal strain were used in a second step of selection by plating cells on medium containing 32 μg of ciprofloxacin per ml (four times the MIC). Second-step mutants occurred at a frequency similar to that observed with the first-step mutants ($\sim 10^{-9}$). For the second-step mutants, the MICs of ciprofloxacin were high (32 to 256 μg/ml), while the MICs of trovafloxacin were 4 to 16 μg/ml.

Since stepwise selection to ciprofloxacin resistance in *S. aureus* has been shown to involve separate sequential mutations in *grlA* and *gyrA*, we used gene amplification to study the QRDRs of both genes in our mutants. Two pairs of oligonucleotide primers derived from *S. pneumoniae*, coding for 254- and 285-bp fragments from *grlA* and *gyrA*, respectively, were used. The results of the amplification and sequence analysis indicate that, as in the case with *S. aureus*, substitution at Ser80 of GrlA was associated with the first step of resistance. Among the mutants listed in Table 3, all had substitutions of Phe or Tyr at Ser80. No changes in *gyrA* were detected in these mutants. Substitution at Ser80 was also observed by Ferrero et al. (6) in their first-step mutants in *S. aureus*. In addition, those investigators found a Glu84 to Lys or Leu substitution in GrlA. They also found no change in GyrA in first-step mutants. The MIC of ciprofloxacin for their mutants was 8 μg/ml, which is comparable to the MIC range of 4 to 8 μg/ml that we observed for our first-step pneumococcal mutants.

Gene amplification of the same QRDRs of the second-step mutants in our study revealed additional changes in *gyrA*. Of the mutants characterized by PCR, 20 of 24 had a Tyr or Phe at Ser84 substitution and 4 had a Glu88 to Lys substitution. While Ferrero et al. (6, 7) noted all of these changes in GyrA mutants of *S. aureus*, they also found decreased levels of fluoroquinolone accumulation in some of their second-step mutants. Preliminary work with our mutants failed to detect such changes in permeability. It is interesting that for some of the double mutants in Table 3 containing identical changes in GyrA and GrlA the MICs of ciprofloxacin or trovafloxacin were different (e.g., strains 02J1056-13-2 and 02J1056-100-78). While each level of resistance was selected in a single step, it cannot be ruled out that additional, as yet uncharacterized mutations exist in some of these strains.

In a recent study the QRDRs of *gyrA* and *grlA* from clinical

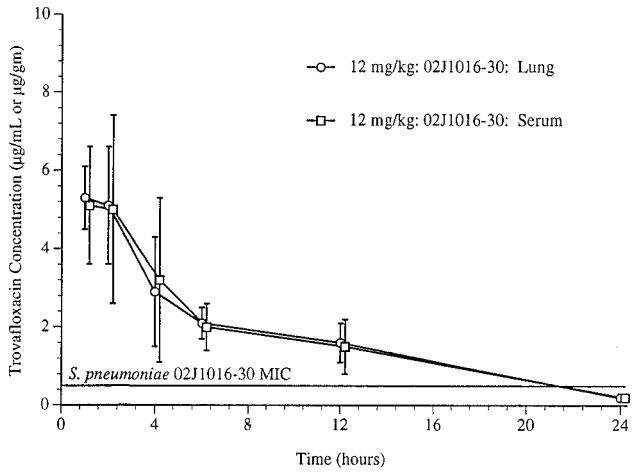

FIG. 1. Levels of trovafloxacin in serum and lungs of mice infected with *S. pneumoniae* 02J1016-30.

S. pneumoniae isolates were amplified and sequenced, revealing that Asp84 to His and Ser80 to Phe changes in GrlA were responsible for ciprofloxacin resistance (24). Changes were not observed in either *gyrA* or *gyrB*. Similar mutations were also observed in two mutants selected in the laboratory. However, for three laboratory mutants selected with ciprofloxacin, the MICs of that drug were 8 to 16 μg/ml, and no detectable changes in *gyrA*, *gyrB*, or *grlA* were found. Also, one mutant for which the MIC of ciprofloxacin was 64 μg/ml had a Ser84 to Phe substitution in GyrA, yet it had no change in the QRDR of GrlA.

Results from our study and from those of Tankovic et al. (24) suggest that fluoroquinolone resistance in *S. pneumoniae* can be associated with several mutations, some of which are yet to be identified. In their study, four of eight mutants examined lacked a mutation in *parC*, while all of the first-step mutants tested in our study had a mutation in this gene. Our study indicates that during sequential mutation, high-level resistance in *S. pneumoniae* occurs in two steps: first, through a change in GrlA and then through a second step involving a change in GyrA. This is similar to the results observed with *S. aureus* and supports the notion that topoisomerase IV is a primary lethal site for fluoroquinolones in these organisms. This role for topoisomerase IV in the gram-positive bacteria examined to date is in direct contrast to the results found with *Escherichia coli* (11, 13, 21).

New fluoroquinolones such as trovafloxacin appear to retain relatively potent activity against first-step pneumococcal mutants with changes in topoisomerase IV. Trovafloxacin was efficacious against a lethal lung infection in mice caused by such a mutant. While the MIC of trovafloxacin for mutant 02J1016-30 increased fourfold (0.5 μg/ml), the PD_{50} increased only 1.9-fold compared with that for the parent. Under the dosing regimen used, drug levels in the blood and lung tissue of infected mice exceeded the MIC for the first-step mutant for more than 20 h. The efficacy of trovafloxacin against wild-type and GrlA mutants of *S. pneumoniae* likely results from its greater intrinsic potency against this species and its higher levels in blood and tissue compared with those of some other fluoroquinolones. In studies with healthy humans, a single 200-mg oral dose of trovafloxacin produced mean peak levels in blood of 2.2 μg/ml 6 h after dosing and a mean concentration of 6.1 μg/g in cells obtained from bronchoalveolar lavage fluid (17). At 18 to 24 h after dosing, mean levels in serum and cells from bronchoalveolar lavage fluid were 1.1 μg/ml and 5.2 μg/g, respectively. These human pharmacokinetic data and the in vitro selection to resistance data obtained in the current study suggest that trovafloxacin should be highly efficacious against infections caused by wild-type pneumococci. Selection to resistance should be an infrequent event, and trovafloxacin may still demonstrate efficacy against first-step mutants with changes in topoisomerase IV. It is hoped that the use of older, less potent fluoroquinolones for respiratory tract indications will be restricted in an attempt to prevent the in vivo selection of strains with high-level resistance caused by the accumulation of multiple mutations. Such careful use will preserve the efficacies of newer agents such as trovafloxacin with an expanded spectrum of activity compared with those of currently marketed fluoroquinolones. Such therapeutic options will be important for *S. pneumoniae* isolates which, in recent years, have shown a steady decrease in susceptibility to many other classes of antimicrobial agents.

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