

Primary Targets of Fluoroquinolones in *Streptococcus pneumoniae*

HIDEYUKI FUKUDA^{1*} AND KEIICHI HIRAMATSU²

Central Research Laboratories, Kyorin Pharmaceutical Co., Ltd., 2399-1, Nogi, Shimotsuga, Tochigi 329-0114,¹ and
Department of Bacteriology, Juntendo University, 2-1-1, Hongo, Bunkyo, Tokyo 113-8421,² Japan

Received 6 July 1998/Returned for modification 24 August 1998/Accepted 25 November 1998

Mutants of wild-type *Streptococcus pneumoniae* IID553 with mutations in *parC* were obtained by selection with trovafloxacin, levofloxacin, norfloxacin, and ciprofloxacin. All of the *parC* mutants were cross-resistant to the selecting agents but were not resistant to gatifloxacin and sparfloxacin. On the other hand, *gyrA* mutants were isolated by selection with gatifloxacin and sparfloxacin. The *gyrA* mutants were cross-resistant to gatifloxacin and sparfloxacin but were not resistant to the other fluoroquinolones tested. These results suggest that in wild-type *S. pneumoniae* the primary target of trovafloxacin, levofloxacin, norfloxacin, and ciprofloxacin is topoisomerase IV, whereas the primary target of gatifloxacin and sparfloxacin is DNA gyrase.

Streptococcus pneumoniae is one of the most important pathogens and is responsible for community-acquired pneumonia, acute otitis media, and meningitis. Recently, the worldwide prevalence of penicillin-resistant *S. pneumoniae* has become a serious problem in clinical settings. In such situations, the use of classes of antibiotics other than β -lactam antibiotics, such as fluoroquinolones, has become necessary. Recently, antipneumococcal fluoroquinolones such as sparfloxacin, trovafloxacin, and gatifloxacin have been developed. The mechanisms of action of these fluoroquinolones against *S. pneumoniae* are under investigation (8, 10, 13, 14, 17).

DNA gyrase and topoisomerase IV (topo IV) serve as targets of the fluoroquinolones in bacteria. Topo IV and DNA gyrase are thought to be essential for DNA replication and the partition of replicated chromosomal DNA, respectively. Fluoroquinolones inhibit these enzyme reactions and then show antibacterial activity. DNA gyrase is known to be a primary target of fluoroquinolones in *Escherichia coli*. In contrast, topo IV seems to be a primary target of many fluoroquinolones in some gram-positive bacteria such as *Staphylococcus aureus* (4–7, 11, 18) and *S. pneumoniae* (8, 10, 13, 17). A recent study has indicated that the primary target of sparfloxacin, one of the antipneumococcal fluoroquinolones, seems to be a DNA gyrase in *S. pneumoniae* (14). These observations suggest that different fluoroquinolones can have different primary targets in *S. pneumoniae*. However, little information regarding the primary targets of the fluoroquinolones exists. In the study described here, we genetically determined the presumed primary targets of various fluoroquinolones, including those of the antipneumococcal fluoroquinolones sparfloxacin, gatifloxacin, and trovafloxacin, and studied the activities of these agents against target-altered mutant strains of *S. pneumoniae*.

Quinolone-resistant mutants of quinolone-susceptible wild-type *S. pneumoniae* IID553, which was obtained from the Japanese Society for Bacteriology, were isolated by selection. The mutants were selected on Mueller-Hinton agar plates with 5% defibrinated horse blood. Each plate contained 2 \times , 4 \times , 8 \times , 16 \times , and 32 \times the MICs of gatifloxacin, trovafloxacin, sparfloxacin, levofloxacin, ciprofloxacin, and norfloxacin, which

were synthesized at Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan. If no mutant was obtained by selection with these concentrations of the agents, the mutants were selected with 1 \times the MIC of the agents. The plates used for selection were incubated for at least 48 h before the plates were scored for the number of bacterial colonies.

The frequencies of selection of mutant strains are presented in Table 1. Resistant mutants were obtained by selection with 16 \times the MIC or lower concentrations of sparfloxacin, 4 \times the MIC or lower concentrations of trovafloxacin, ciprofloxacin, and norfloxacin, and 2 \times the MIC of levofloxacin. No mutant strain was obtained by selection with 2 \times the MIC or higher concentrations of gatifloxacin, although a mutant could be obtained by selection with 1 \times the MIC of gatifloxacin.

In *S. pneumoniae*, the *gyrA* and *gyrB* genes encode the GyrA and GyrB subunits of DNA gyrase, respectively, and the *parC* and *parE* genes encode the ParC and ParE subunits of topo IV, respectively. To amplify those gene fragments including the quinolone resistance-determining region (QRDR) that correspond to the QRDRs of the *E. coli gyrA* and *gyrB* genes (19, 20), each pair of primers (sense and antisense primers) was synthesized as described by Pan et al. (13). The gene fragments were amplified by 25 PCR cycles with the genomic DNA of *S. pneumoniae* strains as templates. The PCR-amplified gene fragments were sequenced with 5'-biotinylated primers (5'-A AATCTGCTCGTATTACAGGGGATG-3'; nucleotide positions 187 to 211 of the *gyrA* gene [1]; 5'-CAGGGAACTAG CAGACTGTTCTTC-3', positions 1238 to 1262 of the *gyrB* gene [12]; 5'-GACAAGAGCTACCGTAAGTCGGCCAAG-3', positions 166 to 192 of the *parC* gene [12]; 5'-CAGCCCA ATCTAAGAATCCTGCTAAG-3', positions 1253 to 1278 of the *parE* gene [12]) by direct cycle sequencing (3).

Three mutants obtained by selection with each fluoroquinolone except gatifloxacin were randomly chosen and then analyzed. Only one mutant was obtained by selection with 1 \times the MIC of gatifloxacin, and it was also analyzed. Two types of mutants were obtained by selection with trovafloxacin; one of these formed smaller colonies than the parent and the other mutants. Therefore, these two types of trovafloxacin-resistant mutants were analyzed.

The MICs of the fluoroquinolones and the target-gene mutations for the mutant strains are presented in Table 2. Three types of mutants (types I to III) were obtained. Type I consisted of mutants selected with trovafloxacin, levofloxacin, cip-

* Corresponding author. Mailing address: Central Research Laboratories, Kyorin Pharmaceutical Co., Ltd., 2399-1, Mitarai, Nogi, Shimotsuga, Tochigi, 329-0114 Japan. Phone: 81-280-56-2201. Fax: 81-280-57-1293. E-mail: fvbb0984@mb.infoweb.ne.jp.

TABLE 1. Frequencies of appearance of mutant strains by selection with various quinolones

Selecting quinolone	Frequency at the following multiple of the MIC ^a :				
	2×	4×	8×	16×	32×
Gatifloxacin	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$
Sparfloxacin	$>2.2 \times 10^{-6}$	$>2.2 \times 10^{-6}$	$>2.2 \times 10^{-6}$	1.1×10^{-7}	$<1.1 \times 10^{-9}$
Trovaflaxacin	$>2.2 \times 10^{-6}$	6.2×10^{-8}	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$
Levofloxacin	1.5×10^{-7}	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$
Ciprofloxacin	$>2.2 \times 10^{-6}$	1.1×10^{-9}	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$
Norfloxacin	2.6×10^{-7}	2.2×10^{-9}	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$

^a The reproducibilities of the frequencies have been confirmed in repeated experiments.

rofloxacacin, and norfloxacin. This type of mutant possessed single-point mutations in the QRDR of the *parC* gene and was cross-resistant to the selecting agents but was not resistant to gatifloxacin and sparfloxacin. The decreases in the activities of trovafloxacin, norfloxacin, ciprofloxacin, and levofloxacin against these *parC* mutants were four-, four-, four-, and two-fold, respectively.

The second type of mutant (type II) comprised strains selected by gatifloxacin or sparfloxacin. These mutants possessed single-point mutations in the QRDR of the *gyrA* gene and were cross-resistant to the selecting agents but were not resistant to the other fluoroquinolones tested. The decreases in the activities of gatifloxacin and sparfloxacin against the *gyrA* mutants were two- and eightfold, respectively. This decrease in the activity of sparfloxacin against the *gyrA* mutants corresponds with those reported by Pan and Fisher (14).

The third type of mutant (type III) comprised those mutants selected with trovafloxacin. The mutants possessed no mutation in the QRDR of the target genes (*gyrA*, *gyrB*, *parC*, and *parE*). These mutants were resistant to trovafloxacin and sparfloxacin and formed colonies that were smaller than those of the parent and the other mutant strains. Some investigators have reported on quinolone-resistant mutant strains of *S. pneumoniae* that possess no mutation in the QRDRs of the *gyrA*, *gyrB*, *parC*, and/or *parE* genes, which suggests that these particular strains are permeability (efflux) mutants (2, 12, 13,

15, 16), one of which is cross-resistant to ethidium bromide (2). However, the type III mutants obtained in this study showed no change in susceptibility to ethidium bromide (data not shown). Further studies are necessary to analyze the mechanisms of quinolone resistance of the type III mutant strains.

Pan and Fisher (14) have reported that the mutants that possess the *gyrA* and *parC* mutations were obtained from wild-type *S. pneumoniae* by selection with sparfloxacin and ciprofloxacin, respectively (14). These *gyrA* and *parC* mutants were not resistant to ciprofloxacin and sparfloxacin, respectively (14). It was hypothesized from these results that the primary targets of sparfloxacin and ciprofloxacin are DNA gyrase and topo IV, respectively, in wild-type *S. pneumoniae*. Gootz et al. (8) have also reported that the *parC* mutants selected by ciprofloxacin are resistant not only to ciprofloxacin but also to trovafloxacin. These results suggest that the primary target of ciprofloxacin and trovafloxacin is likely topo IV. Regarding the target-altered resistant mutants, our results support the hypothesis and indicate that the primary targets of gatifloxacin and sparfloxacin are DNA gyrase; in addition, the primary target of trovafloxacin, levofloxacin, ciprofloxacin, and norfloxacin is topo IV in wild-type *S. pneumoniae*.

In *S. aureus*, sparfloxacin and gatifloxacin prevented the selection of quinolone-resistant *glaA* (*parC*) mutants (7, 18). The decreases in the activities of those fluoroquinolones against the *glaA* mutants were slight. These results suggest that

TABLE 2. Gene mutations in quinolone resistance-determining region and antibacterial activities of various quinolones against the resistant mutants^a

Strain	Selecting quinolone (concn [$\mu\text{g/ml}$]) ^b	No. of isolates	Mutation (codon) ^c		MIC ($\mu\text{g/ml}$)					
			<i>gyrA</i>	<i>parC</i>	Gat	Spa	Tro	Lev	Cip	Nor
Parent			None	None	0.39	0.39	0.10	0.78	0.78	6.25
Mutant										
Type I	Tro (0.20)	3	None	Ser79(TCT)→Tyr(TAT)	0.39	0.39	0.39	1.56	3.13	25
	Lev (3.13)	1	None	Asp83(GAT)→Asn(AAT)	0.39	0.39	0.39	1.56	3.13	25
	Lev (3.13)	2	None	Ser79(TCT)→Tyr(TAT)	0.39	0.39	0.39	1.56	3.13	25
	Nor (12.5 or 25)	3	None	Ser79(TCT)→Tyr(TAT)	0.39	0.39	0.39	1.56	3.13	25
	Cip (1.56)	1	None	Ser79(TCT)→Tyr(TAT)	0.39	0.39	0.39	1.56	3.13	25
	Cip (1.56)	2	None	Asp83(GAT)→Asn(AAT)	0.39	0.39	0.39	1.56	3.13	25
Type II	Spa (3.13 or 6.25)	3	Ser81(TCC)→Phe(TTC)	None	0.78	3.13	0.10	0.78	0.78	6.25
	Gat (0.39)	1	Ser81(TCC)→Tyr(TAC)	None	0.78	3.13	0.10	0.78	0.78	6.25
Type III	Tro (0.20 or 0.39)	3	None	None	0.39	0.78	0.39	0.78	0.78	6.25

^a The mutants were selected on the plates containing the selecting agents after at least a 48 h of incubation at 37°C. On the other hand, the MICs were defined as the lowest concentrations at which no visible growth was observed after incubation at 37°C for 18 to 20 h. None of the strains possessed mutations in the QRDR of *gyrB* and *parE* genes. Abbreviations: Gat, gatifloxacin; Spa, sparfloxacin; Tro, trovafloxacin; Lev, levofloxacin; Nor, norfloxacin; Cip, ciprofloxacin.

^b The concentrations of the agents used for selection.

^c The codon positions of *gyrA* and *parC* are designated according to the numbering of Balas et al. (1) and Pan and Fisher (12), respectively.

agents such as sparfloxacin and gatifloxacin inhibit both DNA gyrase and topo IV at nearly the same levels (7). In this study, we showed that gatifloxacin selected mutants of *S. pneumoniae* at a lower frequency. Also, gatifloxacin had a slight decrease in activity (twofold) against the *gyrA* mutants. These observations might further suggest that gatifloxacin inhibits both wild-type DNA gyrase and topo IV at nearly the same levels in *S. pneumoniae*. Recently, Zhao et al. (21) have reported that fluoroquinolones with a substituted methoxy moiety at the C-8 position of the quinoline ring select quinolone-resistant mutants less frequently. Gatifloxacin also possesses a methoxy moiety at the C-8 position (9), which may explain why it prevents selection of the resistant mutants.

Since penicillin-resistant *S. pneumoniae* is prevalent worldwide in clinical settings, the use of classes of antibiotics other than β -lactam antibiotics, such as antipneumococcal fluoroquinolones and especially those that select for resistant mutants less frequently, will be necessary. Our observations, together with additional studies on the target enzymes, should provide us with the necessary information to develop potent antipneumococcal fluoroquinolones that prevent the selection of resistant strains of *S. pneumoniae*.

REFERENCES

- Balas, D., E. Fernández-Moreira, and A. G. de la Campa. 1998. Molecular characterization of the gene encoding the DNA gyrase A subunit of *Streptococcus pneumoniae*. *J. Bacteriol.* **180**:2854–2861.
- Baranova, N. N., and A. A. Neyfakh. 1997. Apparent involvement of a multidrug transporter in the fluoroquinolone resistance of *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **41**:1396–1398.
- Beck, S., T. O’Keeffe, J. M. Coull, and H. Köster. 1989. Chemiluminescent detection of DNA: application for DNA sequencing and hybridization. *Nucleic Acids Res.* **17**:5115–5123.
- Blanche, F., B. Cameron, F.-X. Bernard, L. Maton, B. Manse, L. Ferrero, N. Ratet, C. Lecoq, A. Goniot, D. Bisch, and J. Crouzet. 1996. Differential behaviors of *Staphylococcus aureus* and *Escherichia coli* type II DNA topoisomerases. *Antimicrob. Agents Chemother.* **40**:2714–2720.
- Ferrero, L., B. Cameron, B. Manse, D. Lagneaux, J. Crouzet, A. Famechon, and F. Blanche. 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target of fluoroquinolones. *Mol. Microbiol.* **13**:641–653.
- Ferrero, L., B. Cameron, and J. Crouzet. 1995. Analysis of *gyrA* and *grlA* mutations in stepwise-selected ciprofloxacin-resistant mutants of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **39**:1554–1558.
- Fukuda, H., S. Hori, and K. Hiramatsu. 1998. Antibacterial activity of gatifloxacin (AM-1155, CG5501, BMS-206584), a newly developed fluoroquinolone, against sequentially acquired quinolone-resistant mutants and the *norA* transformant of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **42**:1917–1922.
- Gootz, T. D., R. Zaniewski, S. Haskell, B. Schmieder, J. Tankovic, D. Girard, P. Courvalin, and R. J. Polzer. 1996. Activity of the new fluoroquinolone trovafloxacin (CP-99,219) against DNA gyrase and topoisomerase IV mutants of *S. pneumoniae* selected in vitro. *Antimicrob. Agents Chemother.* **40**:2691–2697.
- Hosaka, M., T. Yasue, H. Fukuda, H. Tomizawa, H. Aoyama, and K. Hirai. 1992. In vitro and in vivo antibacterial activities of AM-1155, a new 6-fluoro-8-methoxy quinolone. *Antimicrob. Agents Chemother.* **36**:2108–2117.
- Muñoz, R., and A. G. De La Campa. 1996. ParC subunit of topoisomerase IV of *Streptococcus pneumoniae* is a primary target of fluoroquinolones and cooperates with DNA gyrase A subunit in forming resistance phenotype. *Antimicrob. Agents Chemother.* **40**:2252–2257.
- Ng, E. Y., M. Trucksis, and D. C. Hooper. 1996. Quinolone resistance mutations in topoisomerase IV: relationship to the *flqA* locus and genetic evidence that topoisomerase IV is the primary target and DNA gyrase is the secondary target of fluoroquinolones in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **40**:1881–1888.
- Pan, X. S., and L. M. Fisher. 1996. Cloning and characterization of the *parC* and *parE* genes of *Streptococcus pneumoniae* encoding DNA topoisomerase IV: role in fluoroquinolone resistance. *J. Bacteriol.* **178**:4060–4069.
- Pan, X. S., J. Ambler, S. Mehtar, and L. M. Fisher. 1996. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **40**:2321–2326.
- Pan, X. S., and L. M. Fisher. 1997. Targeting of DNA gyrase in *Streptococcus pneumoniae* by sparfloxacin: selective targeting of gyrase or topoisomerase IV by quinolones. *Antimicrob. Agents Chemother.* **41**:471–474.
- Perichon, B., J. Tankovic, and P. Courvalin. 1997. Characterization of a mutation in the *parE* gene that confers fluoroquinolone resistance in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **41**:1166–1167.
- Piddock, L. J. V., Y. F. Jin, and M. J. Everett. 1997. Non-*gyrA*-mediated ciprofloxacin resistance in laboratory mutants of *Streptococcus pneumoniae*. *J. Antimicrob. Chemother.* **39**:609–615.
- Tankovic, J., B. Perichon, J. Duval, and P. Courvalin. 1996. Contribution of mutations in *gyrA* and *parC* genes to fluoroquinolone resistance of mutants of *Streptococcus pneumoniae* obtained in vivo and in vitro. *Antimicrob. Agents Chemother.* **40**:2505–2510.
- Yamagishi, J., T. Kojima, Y. Oyamada, K. Fujimoto, H. Hattori, S. Nakamura, and M. Inoue. 1996. Alterations in DNA topoisomerase IV *grlA* gene responsible for quinolone resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **40**:1157–1163.
- Yoshida, H., M. Bogaki, M. Nakamura, and S. Nakamura. 1990. Quinolone resistance determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. *Antimicrob. Agents Chemother.* **34**:1271–1272.
- Yoshida, H., M. Bogaki, M. Nakamura, L. M. Yamanaka, and S. Nakamura. 1991. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. *Antimicrob. Agents Chemother.* **35**:1647–1650.
- Zhao, X., C. Xu, J. Domagala, and K. Drlica. 1997. DNA topoisomerase targets of fluoroquinolones: a strategy for avoiding bacterial resistance. *Proc. Natl. Acad. Sci. USA* **94**:13991–13996.