

Activity of Gemifloxacin against Penicillin- and Ciprofloxacin-Resistant *Streptococcus pneumoniae* Displaying Topoisomerase- and Efflux-Mediated Resistance Mechanisms

VICTORIA J. HEATON,¹ COLIN E. GOLDSMITH,² JANE E. AMBLER,³ AND L. MARK FISHER^{1*}

Molecular Genetics Group, Department of Biochemistry, St. George's Hospital Medical School, University of London, London SW17 0RE,¹ Bacteriology Department, Belfast City Hospital Trust, Belfast BT9 7AB, Northern Ireland,² and SmithKline Beecham Pharmaceuticals, Harlow, Essex CM19 5AW,³ United Kingdom

Received 1 June 1999/Returned for modification 6 July 1999/Accepted 17 September 1999

Nine penicillin-resistant *Streptococcus pneumoniae* clinical isolates from Northern Ireland, resistant to ciprofloxacin (MICs, 2 to 64 µg/ml) through topoisomerase- and/or reserpine-sensitive efflux mechanisms, were highly susceptible to gemifloxacin (MICs, 0.03 to 0.12 µg/ml). Two strains (requiring a ciprofloxacin MIC of 64 µg/ml) carried known quinolone resistance mutations in *parC*, *parE*, and *gyrB*, resulting in S79F, D435V, and E474K changes, respectively. Thus, gemifloxacin is active against clinical strains exhibiting altered topoisomerase and efflux phenotypes.

The emergence and global spread of penicillin-resistant *Streptococcus pneumoniae* pose a major challenge to the effective control of pneumococcal disease (reviewed in references 1, 4, 8, 13, and 28). Over the last two decades, pneumococci with diminished penicillin susceptibility due to multiple alterations in penicillin binding proteins have been reported in New Guinea, South Africa, Spain, Hungary, France, and the United States, reaching a prevalence of 25 to 40% in many areas and up to 70% in Hungary. Penicillin resistance can occur independently or in association with reduced susceptibility to other antipneumococcal drugs, such as trimethoprim, tetracycline, chloramphenicol, erythromycin, and related antibiotics. Although the mechanisms of resistance to these antibiotics are thought to be distinct (e.g., resistance to erythromycin occurs through the modification of the ribosome or through altered macrolide efflux [16, 27]), diminished susceptibility to all these drugs is more common in penicillin-resistant than in penicillin-susceptible strains. Recent interest has therefore focused on agents that act by alternative mechanisms, notably the fluoroquinolones, such as ciprofloxacin, which kill bacteria by inhibiting DNA synthesis through interaction with either or both of the target enzymes DNA gyrase and topoisomerase IV (12, 14). Gyrase, an A₂B₂ tetramer encoded by the *gyrA* and *gyrB* genes, catalyzes ATP-dependent DNA supercoiling during DNA replication, whereas topoisomerase IV is made up of two C and two E subunits, specified by the *parC* and *parE* genes, and facilitates chromosome segregation (7, 29). Though the activity of ciprofloxacin against *S. pneumoniae* is borderline, the new fluoroquinolones that are coming into clinical use possess significantly greater antipneumococcal activity (25).

Gemifloxacin (SB265805, LB20304) is a highly potent fluoroquinolone which exhibits broad-spectrum activity with particular potency against penicillin-susceptible and -resistant strains of *S. pneumoniae* (5, 18). These features indicate that gemifloxacin could be useful in the treatment of community-acquired respiratory infections. However, one factor that could influence the utility of gemifloxacin and other new fluoro-

quinolones is the occurrence of quinolone-resistant pneumococcal strains selected by prior exposure to other quinolones. Such strains are currently uncommon but are being identified through surveillance (6). Resistance can arise either through altered efflux or through mutations in defined regions, termed the quinolone resistance-determining regions (QRDRs), of the topoisomerase IV and gyrase genes (7, 17, 19). Activity of gemifloxacin against clinical isolates with defined topoisomerase and altered efflux resistance mechanisms has not been reported thus far. To address this issue, we describe the first detailed characterization and drug responses of a panel of novel penicillin-resistant *S. pneumoniae* clinical strains from Northern Ireland (10), some of which display concomitant resistance to ciprofloxacin (11).

Table 1 presents the antibiotic susceptibility profiles of 10 isolates, B1 through 33, collected from 1988 to 1995 by the Public Health Laboratory, Belfast City Hospital (unrelated susceptible strains 7785 and D5 are included for comparison). The strains belong to serotype 6B or 9V as determined by the standard Quellung method (sera from Statens Serum Institut, Copenhagen, Denmark). By pulsed-field gel electrophoresis (PFGE) of chromosomal *Sma*I DNA fragments according to the method of Lefevre et al. (15) (Pharmacia PFGE apparatus), strains B1, 27, 28, and 33 were indistinguishable from the Spanish/French 9V clone, whereas B5 and 24 were indistinguishable from the Spanish 6B clone, suggesting that these six strains may have originated from continental Europe. Strains B6, B10, and 4 each had a PFGE pattern that differed from each other and those of the other isolates. MICs were measured by twofold agar dilution (19). All 10 strains displayed intermediate- or high-level resistance to penicillin G (MIC ≥ 0.1 µg/ml), and 5 of 10 isolates were also highly resistant to erythromycin (MIC ≥ 16 µg/ml). With the exception of strain B10, for which the ciprofloxacin MIC of 1 µg/ml was in line with those for the susceptible strains 7785 and D5 (19), the other nine isolates were resistant to ciprofloxacin, requiring MICs of 2 to 64 µg/ml. Ofloxacin and sparfloxacin were effective against most strains. However, isolates 27 and 28 (which may be related) were highly resistant to ciprofloxacin and ofloxacin, requiring MICs of 64 and 16 µg/ml, respectively. Interestingly, all the isolates required gemifloxacin MICs in the range of 0.03 to 0.12 µg/ml (Table 1). Even strains 27 and 28,

* Corresponding author. Mailing address: Molecular Genetics Group, Department of Biochemistry, St. George's Hospital Medical School, University of London, London SW17 0RE, United Kingdom. Fax: 44 181 725 2992. E-mail: lfisher@sghms.ac.uk.

TABLE 1. Drug susceptibilities and topoisomerase QRDR statuses of *S. pneumoniae* clinical isolates from Northern Ireland^a

Strain	Serotype	PFGE pattern ^b	MIC ($\mu\text{g/ml}$) of:							Mutation(s) in the QRDR of ^c :			
			PEN ^d	ERY ^d	EBR	CIP (+ RES)	OFL	SPAR	GEMI	GyrA	ParC	GyrB	ParE
B1	9V	A	1	0.25	32–64	4 (1–2)	2	0.5	0.12	None	None	None	I460V
B5	6B	B	2	0.25	32–64	4 (0.5)	2	0.5	0.06	None	None	None	I460V
B6	9V	Unique	2	>16	32	4 (2)	2	0.5	0.06	None	None	None	I460V
B10	6B	Unique	1	0.25	8	1 (0.5)	1	0.5	0.03	None	None	None	None
4	9V	Unique	5	0.25	32	2 (0.5)	2	0.5	0.06	None	None	None	I460V
24	6B	B	1	>16	32	2–4 (1–2)	1	0.5	0.03	None	None	None	None
25	NT	ND	2	16	32	2 (0.5–1)	2	0.5	0.06	None	None	None	I460V
27	9V	A	1	>16	32–64	64 (8)	16	1	0.12	None	S79F	E474K	D435V, I460V
28	9V	A	1	>16	32–64	64 (8)	16	1	0.12	None	S79F	E474K	D435V, I460V
33	9V	A	1	0.25	32	2 (0.5)	2	0.5	0.06	None	None	None	I460V
7785	ND	ND	ND	ND	16	1–2 (ND)	2	0.5	0.06	None	None	None	None
D5	ND	ND	ND	ND	8–16	1 (ND)	ND	ND	ND	None	None	None	None

^a PEN, penicillin G; ERY, erythromycin; EBR, ethidium bromide; CIP, ciprofloxacin; RES, reserpine (at 7.5 $\mu\text{g/ml}$); OFL, ofloxacin; SPAR, sparfloxacin; GEMI, gemifloxacin; ND, not determined; NT, not typeable.

^b A, identical to that of the Spanish/French 9V clone; B, same as that of the Spanish 6B clone.

^c With the exception of 7785 and D5, all strains harbored a silent mutation (GTT→GTC) at codon 88 of the *gyrA* sequence. Mutations (by reference to the 7785 DNA sequence) were as follows: ParC, S79F (TCT→TTT); GyrB, E474K (GAA→AAA); and ParE, I460V (ATC→GTC) and D435V (GAC→GTC).

^d Data from the National Public Health Laboratory, Colindale, London, United Kingdom.

which were highly resistant to ciprofloxacin, required gemifloxacin MICs of only 0.12 $\mu\text{g/ml}$, the lowest for the quinolones tested.

To clarify the molecular basis underlying the enhanced activity of gemifloxacin over that of ciprofloxacin and other quinolones, it was important to determine the resistance mechanisms operating in these strains. Recent studies have suggested that altered efflux is commonly involved in the ciprofloxacin resistance of *S. pneumoniae* clinical isolates (2, 3, 9). Reversal of resistance to ciprofloxacin by the efflux pump inhibitor reserpine and an increased MIC of the pump substrate ethidium bromide are both routinely taken as evidence indicating an efflux phenotype (2, 3). We found that reserpine at 7.5 $\mu\text{g/ml}$ lowered the ciprofloxacin MICs for all the clinical isolates we tested two- to eightfold (Table 1) but by itself had no effect on bacterial growth (not shown). Moreover, compared to ciprofloxacin-susceptible strains B10, D5, and 7785, the same strains required two- to eightfold increases in the ethidium bromide MIC. These data are consistent with the operation of an efflux system which appears relatively inefficient in extruding sparfloxacin and gemifloxacin in many of these strains.

PCR was used to amplify the *gyrA*, *gyrB*, *parC*, and *parE* QRDRs of clinical isolates, with chromosomal DNA as a template and with the primer pairs shown in Table 2. Conditions for PCR and for asymmetric PCR (to provide single-stranded DNA) prior to DNA sequence analysis were as described previously (19, 21, 22). The *gyrA* sequences were identical among the strains, and to that of 7785 and D5, except for a silent mutation at codon 88 (Table 1) (19, 23). Similarly, the *parC* and *gyrB* sequences were identical to that of strain 7785, except for isolates 27 and 28, in which the sequences encoded changes of S79F in ParC and E474K in GyrB (Table 1). In addition, the sequences in strains 27 and 28 encoded a D435V ParE change. Finally, except for those in isolates B10 and 24, the *parE* QRDR specified an I460V alteration (whose presence did not correlate with ciprofloxacin MICs). No other mutations were detected in the QRDRs. The *parC* mutation converting S79 (the equivalent residue to the quinolone resistance hot spot S83 in *Escherichia coli* GyrA [30]) to Phe is known to confer ciprofloxacin resistance on *S. pneumoniae* (26). Similarly, the *parE* mutation changes D435 to Val in a highly conserved EGDSA motif at the position equivalent to D426 in *E. coli* GyrB (31), whose mutation to Asn is known to confer quino-

lone resistance (24). Finally, the *gyrB* mutation resulting in a E474K alteration at the protein level downstream of the PLRGK motif has been reported previously for a first-step *S. pneumoniae* mutant selected with clinafloxacin (22). Thus, it is likely that all three topoisomerase mutations, along with altered efflux, contribute to the high-level ciprofloxacin resistance of strains 27 and 28.

The results described here indicate that gemifloxacin is highly potent against multidrug-resistant *S. pneumoniae* clinical isolates whose ciprofloxacin resistance accrues from altered topoisomerase targets and/or putative efflux mechanisms. Activity against strains 27 and 28 was particularly impressive, requiring a gemifloxacin MIC of 0.12 $\mu\text{g/ml}$, which is 500-fold lower than that of ciprofloxacin. The presence of a *parC* mutation in strains 27 and 28 affecting S79 in ParC is consistent

TABLE 2. Oligonucleotides used to amplify or sequence QRDRs of *S. pneumoniae* *gyrA* and topoisomerase IV genes

Oligonucleotide (gene)	Use(s) ^a	Sequence (nucleotide position, 5'→3') ^b
VGA9 (<i>gyrA</i>)	P	AAGTGAAGGCAAGGGCG (184 to 167) ^c
VGA4 (<i>gyrA</i>)	P, As	ACCAGTTGCTCCATTAAC (513 to 496)
VGA3 (<i>gyrA</i>)	S	ACCGTCGCATTCTTTACG (128 to 145)
M0362 (<i>parC</i>)	P	ATGTGAATGACTATGTC (–377 to –361)
M5884 (<i>parC</i>)	P	ATACGAAGAGCATCACGG (893 to 876)
M4721 (<i>parC</i>)	As	TGCTGGCAAGACCGTTGG (471 to 454)
M0363 (<i>parC</i>)	S	TGGGTTGAAGCCGGTTCA (105 to 122)
M4025 (<i>gyrB</i>)	P	TTCTCCGATTTCCTCATG (1096 to 1113)
M4026 (<i>gyrB</i>)	P, As	AGAAGGGTACGAATGTGG (1563 to 1546)
VGBQ (<i>gyrB</i>)	S	AACCTTCCAGGGAACTAGC (1231 to 1250)
XS01 (<i>parE</i>)	P	TGAAGCGATTGAGTTCCA (651 to 668)
M0361 (<i>parE</i>)	P	ATCCGACTCTAATTTCCA (–376 to –393) ^d
S6399 (<i>parE</i>)	As	TCTGCTCCAACCCGCA (1469 to 1452)
S6398 (<i>parE</i>)	S	AAGGCGCGTGATGAGAGCC (1180 to 1198)

^a Oligonucleotides were used for PCR amplification (P) and asymmetric PCR (As) and as primers for DNA sequence analysis (S).

^b Based on sequences in references 20 and 23. Oligonucleotides VGA9, VGA4, M5884, M4721, M4026, M0361, and S6399 are based on noncoding strand nucleotide sequences.

^c Nucleotide positions refer to the *ldh* gene upstream of *gyrA* (23).

^d Nucleotide positions in the *parC* gene downstream of *parE* (20).

with selection by ciprofloxacin, which targets topoisomerase IV (17, 19, 24). However, association of the *parC* change with the particular *parE* and *gyrB* mutations identified here (Table 1) has not been reported previously. Ciprofloxacin normally selects *parC* (or *parE*) changes and then *gyrA* mutations (19). Conceivably, the relatively small effects of the topoisomerase mutations on gemifloxacin MICs for strains 27 and 28 (compared to those on the ciprofloxacin MIC) could arise from gemifloxacin targeting GyrA. These aspects are presently under investigation. Irrespective of these various considerations, the data presented here suggest that gemifloxacin may be useful in the treatment of both ciprofloxacin-resistant and multi-drug-resistant *S. pneumoniae* infections.

We thank Xiao-Su Pan for help and advice.

This work was supported by a project grant from SmithKline Beecham.

REFERENCES

- Baquero, F., J. A. García-Rodríguez, J. García de Lomas, L. Aguilar, and The Spanish Surveillance Group for Respiratory Pathogens. 1999. Antimicrobial resistance of 1,113 *Streptococcus pneumoniae* isolates from patients with respiratory tract infections in Spain: results of a 1-year (1996–1997) multicenter surveillance study. *Antimicrob. Agents Chemother.* **43**:357–359.
- 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.
- Brenwald, N. P., M. J. Gill, and R. Wise. 1998. Prevalence of a putative efflux mechanism among fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **42**:2032–2035.
- Campbell, G. D., Jr., and R. Silberman. 1998. Drug-resistant *Streptococcus pneumoniae*. *Clin. Infect. Dis.* **26**:1188–1195.
- Cormican, M. G., and R. N. Jones. 1997. Antimicrobial activity and spectrum of LB20304, a novel fluoronaphthyridone. *Antimicrob. Agents Chemother.* **41**:204–211.
- Doern, G. V., M. A. Pfaller, M. E. Erwin, A. B. Brueggemann, and R. N. Jones. 1998. The prevalence of fluoroquinolone resistance among clinically significant respiratory tract isolates of *Streptococcus pneumoniae* in the United States and Canada—1997 results from the SENTRY antimicrobial surveillance program. *Diagn. Microbiol. Infect. Dis.* **32**:313–316.
- Drlica, K., and X. Zhao. 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol. Mol. Biol. Rev.* **61**:377–392.
- Felmingham, D., and J. Washington. 1999. Trends in the antimicrobial susceptibility of bacterial respiratory tract pathogens—findings of the Alexander Project 1992–1996. *J. Chemother.* **11**:5–21.
- Gill, M. J., N. P. Brenwald, and R. Wise. 1999. Identification of an efflux pump gene, *pmrA*, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **43**:187–189.
- Goldsmith, C. E., J. E. Moore, and P. G. Murphy. 1997. Pneumococcal resistance in the UK. *J. Antimicrob. Chemother.* **40**:11–18.
- Goldsmith, C. E., J. E. Moore, P. G. Murphy, and J. E. Ambler. 1998. Increased incidence of ciprofloxacin resistance in penicillin-resistant pneumococci in Northern Ireland. *J. Antimicrob. Chemother.* **41**:420–421.
- Hoshino, K., A. Kitamura, I. Morrissey, K. Sato, J.-I. Kato, and H. Ikeda. 1994. Comparison of inhibition of *Escherichia coli* topoisomerase IV by quinolones with DNA gyrase inhibition. *Antimicrob. Agents Chemother.* **38**:2623–2627.
- Johnson, A. P. 1998. Antibiotic resistance among clinically important gram-positive bacteria in the UK. *J. Hosp. Infect.* **40**:17–26.
- Khodursky, A. B., E. L. Zechiedrich, and N. R. Cozzarelli. 1995. Topoisomerase IV is a target of quinolones in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **92**:11801–11805.
- Lefevre, J. C., G. Faucon, A. M. Sicard, and A. M. Gase. 1993. DNA fingerprinting of *Streptococcus pneumoniae* strains by pulsed-field gel electrophoresis. *J. Clin. Microbiol.* **31**:2724–2728.
- McCracken, G. H., Jr. 1997. Microbiologic activity of the newer macrolide antibiotics. *Pediatr. Infect. Dis. J.* **16**:432–437.
- Muñoz, R., and A. G. De La Campa. 1996. ParC subunit of DNA 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.
- Oh, J.-I., K.-S. Paek, M.-J. Ahn, M.-Y. Kim, C. Y. Hong, I.-C. Kim, and J.-H. Kwak. 1996. In vitro and in vivo evaluations of LB20304, a new fluoronaphthyridone. *Antimicrob. Agents Chemother.* **40**:1564–1568.
- 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. 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., 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.
- Pan, X.-S., and L. M. Fisher. 1998. DNA gyrase and topoisomerase IV are dual targets of clinafloxacin action in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **42**:2810–2816.
- Pan, X.-S., and L. M. Fisher. 1999. *Streptococcus pneumoniae* DNA gyrase and topoisomerase IV: overexpression, purification, and differential inhibition by fluoroquinolones. *Antimicrob. Agents Chemother.* **43**:1129–1136.
- 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. 1994. New quinolones and gram-positive bacteria. *Antimicrob. Agents Chemother.* **38**:163–169.
- 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.
- Thornberry, C., P. Ogilvie, J. Kahn, and Y. Mauriz. 1997. Surveillance of antimicrobial resistance in *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in the United States in the 1996–1997 respiratory season. *Diagn. Microbiol. Infect. Dis.* **29**:249–257.
- Tomasz, A. 1997. Antibiotic resistance in *Streptococcus pneumoniae*. *Clin. Infect. Dis.* **24**:S85–S88.
- Wang, J. C. 1996. DNA topoisomerases. *Annu. Rev. Biochem.* **65**:635–692.
- 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.