Activity of Gemifloxacin against Quinolone-Resistant *Streptococcus pneumoniae* Strains In Vitro and in a Mouse Pneumonia Model

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Gemifloxacin is a novel fluoronaphthyridone quinolone with enhanced in vitro activity against *Streptococcus pneumoniae*. We investigated the activities of gemifloxacin and trovafloxacin, their abilities to select for resistance in vitro and in vivo, and their efficacies in a mouse model of acute pneumonia. Immunocompetent Swiss mice were infected with 10⁵ CFU of a virulent, encapsulated *S. pneumoniae* strain, P-4241, or its isogenic *parC*, *gyrA*, *parC gyrA*, and efflux mutant derivatives (serotype 3); and leukopenic mice were infected with 10⁷ CFU of two poorly virulent clinical strains (serotype 11A) carrying either a *parE* mutation or a *parC*, *gyrA*, and *parE* triple mutation. The drugs were administered six times every 12 h, starting at either 3 or 18 h postinfection. In vitro, gemifloxacin was the most potent agent against strains with and without acquired resistance to fluoroquinolones. While control mice died within 6 days, gemifloxacin at doses of 25 and 50 mg/kg of body weight was highly effective (survival rates, 90 to 100%) against the wild-type strain and against mutants harboring a single mutation, corresponding to area under the time-versus-serum concentration curve at 24 h (AUC₂₄)/MIC ratios of 56.5 to 113, and provided a 40% survival rate against a mutant with a double mutation (*parC* and *gyrA*). A total AUC₂₄/MIC ratio of 28.5 was associated with poor efficacy and the emergence of resistant mutants. Trovafloxacin was as effective as gemifloxacin against mutants with single mutations but did not provide any protection against the mutant with double mutations, despite treatment with a high dose of 200 mg/kg. Gemifloxacin preferentially selected for *parC* mutants both in vitro and in vivo.

Community-acquired pneumonia is the most common cause of death due to infection, with *Streptococcus pneumoniae* remaining the leading causative pathogen (27, 28, 33). The worldwide incidence of infections caused by pneumococci resistant to penicillin, macrolides, and other antimicrobials has increased at an alarming rate during the past two decades (3, 4). There is thus considerable interest in the use of alternative antimicrobials such as fluoroquinolones. Although fluoroquinolones are available, fluoroquinolones such as ciprofloxacin have limited effectiveness in this setting (7), and recently developed compounds appear to be more interesting for this purpose (5, 8, 35).

Several studies have shown that low-level quinolone resistance in pneumococci can result from mutations in the *parC* gene, which codes for topoisomerase IV (30). Increased levels of resistance occur following acquisition of additional mutations in *gyrA*, which encodes the A subunit of type II topoisomerase (DNA gyrase) (24). The impact of these two resistance mechanisms depends on the type of molecule considered (35). Moreover, recent studies have identified an efflux mechanism as a further cause of low-level resistance in pneumococci (11, 40). Combination of this efflux mechanism with mutations on the DNA gyrase (*gyrA*) or topoisomerase IV (*parC*) gene leads to higher levels of resistance.

Gemifloxacin (SB 265805) is a new fluoronaphthyridone quinolone with a 7-pyrrolidone substituent. This novel com-

pound has been shown to have excellent in vitro activity against both gram-negative and gram-positive pathogens (26, 37, 39) and against atypical pathogens such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila* (15, 16). Gemifloxacin is the most potent member of the quinolone class against isolates of *S. pneumoniae* with reduced susceptibilities to ciprofloxacin (13).

The aim of this study was to evaluate the efficacy of gemifloxacin in vitro and in a mouse model of acute pneumonia caused by an *S. pneumoniae* wild-type strain and by isogenic quinolone-resistant mutants in terms of both bactericidal activity and selection of resistance in comparison with the efficacy of trovafloxacin, a trifluoronaphthyridone quinolone which demonstrated a high degree of efficacy against *S. pneumoniae* both in vitro and in a mouse pneumonia model (8).

MATERIALS AND METHODS

Drugs. The antibiotics used in this study included the fluoroquinolones gemifloxacin (SmithKline Beecham Pharmaceuticals, Brentford, United Kingdom), trovafloxacin (Pfizer Laboratories, Groton, Conn.), ciprofloxacin and moxifloxacin (Bayer Laboratories, Sens, France), sparflaxacin (Rhône-Poulenc Rorer Laboratories, Nanterre, France), and norfloxacin (Merck Sharp & Dohme, Fontenay-sous-Bois, France). Ethidium bromide was purchased from Sigma (Saint Quentin Fallavier Cedex, France). The antibiotics were diluted in sterile water to the desired concentrations.

Bacterial strains. The strains used for the in vivo studies are shown in Table 1. *S. pneumoniae* P-4241 is a blood isolate (serotype 3) and an encapsulated strain which is virulent in a mouse model of acute pneumonia (100% lethal dose, 3.3 log₁₀ CFU/mouse). It is susceptible to both penicillin and quinolones (amoxicillin MIC, 0.03 µg/ml; ciprofloxacin MIC, 1 µg/ml). Isogenic *gyrA* and *parC* mutants were selected from parent strain P-4241 as reported previously (6). Strains 2500 and 2759 (serotype 11A) are poorly virulent clinical isolates carrying a single mutation in *parE* and mutations in *gyrA*, *parC*, and *parE*, respectively.

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TABLE 1. MICs and mutations in strains used for animal studies^a

Genotype [mutation(s)]	Strain	MIC ($\mu\text{g/ml}$)						
		GEM	TVA	MXF	SPX	CIP	NOR	EtBr
WT (<i>parC</i> , Ser79; <i>gyrA</i> , Ser81)	P-4241	0.03	0.125	0.125	0.5	1	8	4
<i>parC</i> (Ser79Tyr)	C42-R2	0.06	0.5	0.125	0.5	4	64	8
<i>gyrA</i> (Ser81Tyr)	Sp42-R1	0.06	0.125	0.25	1	1	8	4
<i>parC</i> (Ser79Tyr) <i>gyrA</i> (Ser81Tyr)	C42-Sp6	0.5	8	4	16	32	128	8
Efflux mutant (ND)	N42-6R1	0.06	0.18	ND	0.5	4	16	16
<i>parE</i> (Ile460Val + Asp435Asn)	2500	0.125	0.5	ND	0.5	16	>64	32
<i>parC</i> (Ser79Tyr) <i>gyrA</i> (Ser81Tyr) <i>parE</i> (Ile460Val)	2759	1	16.0	8	32	64	>64	32

^a Abbreviations: WT, wild type; GEM, gemifloxacin; TVA, trovafloxacin; MXF, moxifloxacin; SPX, sparfloxacin; CIP, ciprofloxacin; NOR, norfloxacin; EtBr, ethidium bromide; ND, not determined.

S. pneumoniae 1427 is a laboratory susceptible strain (gemifloxacin MIC, 0.06 $\mu\text{g/ml}$) that was used for in vitro selection of gemifloxacin-resistant mutants since it yielded more fluoroquinolone-resistant mutants than virulent strain P-4241 did.

MIC determinations. MICs were determined by the broth microdilution method in Columbia medium supplemented with 5% sheep blood (32).

Selection and analysis of first-step mutants resistant to gemifloxacin in vitro. Inocula of 10^9 to 10^{10} CFU of *S. pneumoniae* strain 1427 were plated on Columbia agar plates that were supplemented with 5% sheep blood and that contained gemifloxacin at final concentrations of one-half the MIC, the MIC, and two times the MIC. Emergence of resistant colonies was monitored for 48 h, and tests for the acquisition of mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* were performed. QRDRs were amplified by PCR with primer pair VGA3 (5'-CCGTCGCATTCTTACG) and VGA4 (5'-AGTTGCTCCATTAACCA) for *gyrA* and primer pair M0363 (5'-TGGGTGAAGCCGGTTCA) and M4271 (5'-TGCTGGCAAGACCGTG) for *parC* (34). PCR conditions were as follows: 1 cycle of 1 min at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 50°C, and 90 s at 72°C, with a final 5-min extension step at 72°C. PCR fragments were purified through spin columns (Qiagen, Hilden, Germany) and directly sequenced at the sequencing facility of the University of Geneva with the primers described above and an ABI A377 automatic sequencer (Applied Biosystems, Foster City, Calif.).

Analysis of strains recovered from mice. Bacteria were recovered from homogenized lungs of treated and untreated control animals. Lung homogenates (100 μl) were spread on Columbia agar plates supplemented with 5% sheep blood. Approximately 10 individual colonies were isolated at random after overnight culture at 37°C, and their resistance profiles were determined. Strains for which MICs were higher than the control value were further investigated. The QRDRs were amplified and sequenced as described above.

Infection of mice with *S. pneumoniae*. Animal studies were performed in accordance with prevailing regulations regarding the care and use of laboratory animals of the European Commission (17). Swiss mice (weight, 20 to 22 g; Ifa Credo, L'Arbresle, France) were infected by the intratracheal route with 40 μl of bacterial suspension at a dose of approximately 10^5 CFU of virulent *S. pneumoniae* per immunocompetent mouse and 10^7 CFU of a poorly virulent strain per leukopenic mouse. The animals were rendered leukopenic by intraperitoneal administration of 150 mg of cyclophosphamide/kg of body weight/day for 3 days, starting 4 days before infection. This treatment reduces leukocyte counts from about 7,000 to 1,000/ml of blood on the day of bacterial challenge and the neutrophil counts to about 100/ml (5). Both a high inoculum and leukopenia were required to induce pneumonia with poorly virulent strains.

Antibiotic treatment. Therapy was initiated 18 h after challenge with the wild-type virulent penicillin-susceptible strain (P-4241) and with the virulent quinolone-resistant mutants (*parC*, *gyrA*, efflux, and *parC gyrA* mutants) and 3 h after challenge with the poorly virulent *parE* and *parC gyrA parE* clinical strains. Early treatment initiation was required because the immunocompromised mice infected with the poorly virulent strains developed pneumonia shortly after they received a high bacterial inoculum. Immunocompetent mice infected with virulent strains developed pneumonia later, and thus, initiation of treatment was delayed. Gemifloxacin and trovafloxacin were given as six subcutaneous (s.c.) injections at 12-h intervals at doses of 6.25, 12.5, 25, and 50 mg/kg to mice challenged with the wild-type strain and the strains with single mutations. Trovafloxacin was given at doses of 100 and 200 mg/kg and gemifloxacin was given at doses of 50 and 75 mg/kg to mice challenged with the strain with the double mutation (*parC* and *gyrA*). Ciprofloxacin was injected at 100 and 250 mg/kg. Infected, untreated control mice received the same volume of isotonic saline. Each treatment group comprised between 12 and 16 animals. The observation

period was 10 days. Death rates were recorded daily, and cumulative survival rates were compared.

Bactericidal activity in vivo. The protocol used to study bactericidal activity was the same as that used for the mouse survival studies. The total CFU counts recovered from whole-lung homogenates were determined 6 h after the first treatment, which was initiated 18 h after bacterial challenge, and 13 h after the second, fourth, and sixth treatments at doses of 6.25, 12.5, 25, and 50 mg of gemifloxacin per kg. Three mice were used for each dose and time point. Mice were killed by intraperitoneal injection of sodium pentobarbital and were exsanguinated by cardiac puncture; blood was used for cultures. The lungs were removed and homogenized in 1 ml of normal saline. One hundred microliters of whole-lung homogenate or serial 10-fold dilutions of homogenates were plated on Columbia agar. Blood was cultured in brain heart infusion broth. After overnight culture, the colonies on the agar plates seeded with lung tissue samples were counted, and blood cultures were examined for turbidity. The results are expressed as the mean $\pm$ standard deviation $\log_{10}$ CFU per lung and as the number of positive or negative blood cultures for groups of three mice each.

Determination of gemifloxacin concentrations in serum and lung and pharmacokinetic (PK) analysis. Antibiotics were administered as a single s.c. dose of 25 mg of gemifloxacin or trovafloxacin per kg to both infected and uninfected mice. Infected mice were treated at 18 h postinfection. Serum and lung samples were collected from groups of six mice each at 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h after drug administration. All samples were frozen at -20°C and protected from light to avoid gemifloxacin degradation during analysis. Lung tissue samples were crushed in liquid nitrogen by magnetized crushing (Spex; Fisher Bioblock, Illkirch, France). Preparation of the calibration standard started with a standard stock solution. Approximately 14 mg of gemifloxacin (LB20304a) was placed into a labeled, volumetric flask and dissolved with methanol at a final concentration of 2.5 mg/ml. The final concentration for each solution was multiplied by 0.802, which converts the concentrations from the mesylate salt form to the free-base form (LB20304). The relevant stock solutions were diluted with methanol to give final spiking solutions of 1, 2.5, 4, 10, 25, 40, and 50 $\mu\text{g/ml}$. Aliquots (10 μl) of the spiking solutions were dispensed into mouse control serum (50 μl) and control lung tissue (50 mg) to give calibration standards equivalent to 0.2, 0.5, 0.8, 1.0, 2.0, 5.0, 8.0, and 10 $\mu\text{g/ml}$ of mouse serum and 0.02, 0.5, 0.8, 2.0, 5.0, 8.0, and 10 $\mu\text{g/g}$ of lung.

Serum samples (50 μl) or lung tissue samples (20 to 50 mg of lung tissue powder weighed exactly) were placed in labeled, silanized glass tubes; and 10% zinc sulfate (25 μl) was added, followed by the addition of 100 μl of methanol. The contents of the tubes were then mixed for approximately 30 s, and the tubes were centrifuged at $1,500 \times g$ for 20 min at 4°C . The supernatant (about 95 μl) was transferred into clean, tapered autosampler vials, and 20 μl of this was injected into a high-performance liquid chromatographic system. Determination of antibiotic concentrations was performed with an octadecyl silyl column (Novapak C₁₈; 4.6 by 150 mm; Waters, Milford, Mass.) coupled with spectrofluorometric detection at 280 and 415 nm for the excitation and the emission wavelengths, respectively. The mobile phase was a mixture of acetonitrile, sodium citrate buffer solution (pH 3.5), and water (22/15/63; vol/vol) with 0.1% trifluoroacetic acid adjusted to pH 4. The flow rate was 1.0 ml/min. The limit of quantitation was raised to 0.2 $\mu\text{g/ml}$, and the linearity was established over concentration ranges of 0.2 to 10.0 $\mu\text{g/ml}$ and 0.5 to 10.0 $\mu\text{g/g}$ for the serum and lung tissue samples, respectively. The coefficients of variation for the quality controls were less than 10% for the serum and lung tissue samples. The PK parameters for trovafloxacin were evaluated as described elsewhere (8).

PK analysis was based on a noncompartmental model (WinNonlin version 1.1; Pharsight, Mountain View, Calif.). The maximum concentrations in serum

TABLE 2. Analysis of first-step mutants generated upon exposure to gemifloxacin in vitro^a

Strain	MIC ($\mu\text{g/ml}$)				Sequence	
	GEM	MXF	EtBr	NOR	<i>gyrA</i>	<i>parC</i>
1427	0.06	0.5	8	8	WT (Ser81)	WT (Ser79)
Ge14-R1	0.125	0.5	8	32	ND	ND
Ge14-R2	0.125	0.5	8	32	WT	Ser79Phe
Ge14-R3	0.125	0.5	8	64	ND	Asp83Asn
Ge14-R4	0.125	0.5	8	32	ND	Asp83Asn

^a Abbreviations: GEM, gemifloxacin; MXF, moxifloxacin; EtBr, ethidium bromide; NOR, norfloxacin; WT, wild type; ND, not determined.

(C_{max}) were measured experimentally, while the area under the time-versus-serum concentration curve (AUC) from 0 to 24 h (AUC_{0-24}) and terminal half-life ($t_{1/2}$) were calculated by using WinNonlin software.

Statistical analysis. Survival curve data were analyzed by using the nonparametric Mantel-Cox log-rank test. *P* values of ≤ 0.05 were considered statistically significant.

RESULTS

Activity of gemifloxacin against *S. pneumoniae* strains with defined target mutations. The MICs for the selected strains are shown in Table 1. Gemifloxacin displayed excellent in vitro activity against the virulent penicillin-susceptible wild-type strain of *S. pneumoniae* (P-4241). Its activity was 4 times greater than those of trovafloxacin and moxifloxacin, 16 times greater than that of sparfloxacin, 32 times greater than that of ciprofloxacin, and 256 times greater than that of norfloxacin. The gemifloxacin and trovafloxacin MICs for the *parC* mutant, strain C42-R2 (Ser79Tyr), increased two- and fourfold, respectively. For *gyrA* mutant Sp42-R1 (Ser81Tyr), the gemifloxacin, moxifloxacin, and sparfloxacin MICs increased twofold, while the ciprofloxacin, norfloxacin, and trovafloxacin MICs remained unaffected. Mutant C42-Sp6, which had mutations in *parC* and *gyrA*, showed stronger increases in resistance, with the MICs of all quinolones tested being 16- to 64-fold higher than those observed for wild-type strain P-4241. Gemifloxacin has a lower MIC (0.5 $\mu\text{g/ml}$) than the other drugs for the mutant with the *gyrA* and *parC* double mutations. With the exception of the sparfloxacin MICs, the MICs of all quinolones increased two- to fourfold for efflux mutant N42-6R1. The gemifloxacin MICs for the clinical strain carrying a *parE* mutation (strain 2500) increased 2-fold, while the trovafloxacin and ciprofloxacin MICs increased 4- and 16-fold, respectively. The gemifloxacin MIC (1 $\mu\text{g/ml}$) for the clinical strain carrying a triple mutation (strain 2759) increased 32-fold, while the ciprofloxacin and trovafloxacin MICs increased 64- and 128-fold, respectively.

In vitro selection of *S. pneumoniae* strains resistant to gemifloxacin. Mutants were selected from strain 1427 on plates containing 0.125 μg of gemifloxacin per ml. After 48 h of incubation, resistant colonies were selected at a frequency of 3×10^{-9} . Four colonies from strain 1427 (Ge14-R1 to Ge14-R4) were tested for their resistance profiles and the acquisition of mutations in the QRDRs of *parC* and *gyrA* (Table 2). The MICs of gemifloxacin and norfloxacin were increased for all four colonies derived from strain 1427. Susceptibilities to ethidium bromide, a substrate of the PmrA efflux pump (19), remained unaffected. The *parC* QRDRs of three mutants were sequenced. Strain Ge14-R2 had a Ser-to-Phe substitution at

position 79, while mutants Ge14-R3 and Ge14-R4 had acquired an Asp-to-Asn substitution at position 83. Although gemifloxacin MICs were equally affected by mutations in *gyrA* and *parC* (Table 1), *parC* mutations were preferentially selected upon exposure to gemifloxacin (Table 2).

In vivo studies. (i) PK data. To establish the most appropriate antibiotic treatment, we first determined the gemifloxacin concentrations in serum and lung tissue. The total drug concentrations in serum and lung tissue measured after administration of a single s.c. injection of 25 mg of gemifloxacin per kg in uninfected animals (Fig. 1A) were similar to those in mice infected with virulent strain P-4241 (Fig. 1B). The AUCs and $t_{1/2}$ s were also not significantly affected by the infection status (Table 3), while for trovafloxacin the values of these PK parameters were two to three times higher than those in control animals. The C_{max} and AUCs of trovafloxacin were higher than those of gemifloxacin in both infected and uninfected animals, and the total AUC at 24 h (AUC_{24})/MIC ratios were also higher with trovafloxacin in infected animals, but gemi-

FIG. 1. Gemifloxacin concentrations in serum and lungs of uninfected (A) and infected (B) mice.

TABLE 3. PK and PK-pharmacodynamic parameters for serum and lung tissues of immunocompetent control and infected mice after administration of a single s.c. dose of gemifloxacin or trovafloxacin at 25 mg/kg

Mouse group, drug, and compartment	$C_{\max}$ ($\mu\text{g/ml}$ or $\mu\text{g/g}$)	$C_{\max}/\text{MIC}$ ratio	$C_{24\text{ h}}^a$ ($\mu\text{g/ml}$ or $\mu\text{g/g}$)	AUC_{0-24} ($\mu\text{g} \cdot \text{h/ml}$)	AUC/MIC ratio	$t_{1/2}$ (h)
Uninfected control						
Gemifloxacin						
Serum	2.4 ± 1.4^b			4.4 ± 1.4		1.0 ± 0.2
Lung	5.3 ± 1.3		1.72 ± 0.03	49.1 ± 9.7		20.7 ± 6.9
Trovafloxacin						
Serum	4.2^c		0.01	19		2.8
Lung	24.2		0.43	155		4.3
Mice infected with strain P-4241 ^d						
Gemifloxacin						
Serum	1.7 ± 1.4	56.7		3.4 ± 0.8	113	1.1 ± 0.2
Lung	4.7 ± 1.9	157	1.81 ± 0.31	43.8 ± 15.1	1,460	28.2 ± 19.0
Trovafloxacin						
Serum	5.7	45.6	0.5	49	392	7.4
Lung	21.2	170	3.7	250	2,000	11.6

^a $C_{24\text{ h}}$, total drug concentration at 24 h posttreatment.^b Values for gemifloxacin are means and standard deviations calculated for six serum and lung tissue samples taken 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h postdosing with gemifloxacin.^c Values for trovafloxacin are for six pooled samples of serum and lung tissue taken at the same times postdosing used for gemifloxacin (see footnote *b* above).^d The gemifloxacin MIC for strain P-4141 was 0.03 $\mu\text{g/ml}$; the trovafloxacin MIC was 0.125 $\mu\text{g/ml}$.

floxacin had longer $t_{1/2}$ s than trovafloxacin in the lungs of both the control and the infected animals (Table 3).

(ii) **Survival.** All untreated control mice died within 3 to 6 days after challenge with the different strains. Gemifloxacin was used to treat mice infected with virulent *S. pneumoniae* strain P-4241 and its fluoroquinolone-resistant derivatives (Table 4). Six injections of gemifloxacin at 50 and 25 mg/kg yielded rates of survival of 100 and 93%, respectively, among mice infected with wild-type strain P-4241, while 80% were protected with the 12.5-mg/kg dose. Even with the lowest dose of 6.25 mg/kg, a 40% survival rate was observed (Fig. 2A). Gemifloxacin at 50 and 25 mg/kg was also effective in mice infected with *parC* mutant C42-R2 (survival rates, 100 and 69%, respec-

tively) (Fig. 2B) and mice infected with *gyrA* mutant Sp42-R1 (survival rate, 100%) (Fig. 2C). Lower survival rates for the *parC* mutant than for the wild type were found at both 12.5 and 6.25 mg/kg (survival rates, 36 and 7%, respectively), while the survival rates for mice infected with the *gyrA* mutant and treated with the same doses remained relatively high (79 and 64%, respectively). All animals infected with efflux mutant N42-6R1 and *parE* mutant 2500 (Table 4) survived when they were treated with gemifloxacin at 25 and 50 mg/kg. Gemifloxacin remained effective at 50 and 75 mg/kg, with survival rates of 40 and 50%, respectively, when animals were infected with the mutant with the double mutation, C42-Sp6 (Fig. 2D), and was slightly effective at 50 mg/kg, with 25% survival among the

TABLE 4. Survival rates at 10 days postinfection in animals treated with gemifloxacin, trovafloxacin, or ciprofloxacin^a

Strain	% Survival								
	Gemifloxacin			Trovafloxacin				Ciprofloxacin	
	12.5 mg/kg	25 mg/kg	50 mg/kg	12.5 mg/kg	25 mg/kg	50 mg/kg	200 mg/kg	100 mg/kg	250 mg/kg
P-4241	80	93 ^b	100 ^c	91	100			50	77
<i>parC</i>	36 ^d	69 ^e	100 ^f	14	100	100		0	27
<i>gyrA</i>	79 ^g	100 ^g	100 ^g	46	92	100		0	80
<i>parC gyrA</i>	0	0	40 ^h			0	0		0
<i>parE</i>	92	100 ⁱ	100	55	56	90			
Efflux mutant	92 ^j	100 ^k	100 ^k	83	100	100		30	78

^a Treatments were initiated with gemifloxacin and trovafloxacin at 18 h postchallenge and with ciprofloxacin at 6 h postchallenge. Survival curves were compared by using the nonparametric Mantel-Cox log-rank test.^b Significant difference ($P < 0.05$) between gemifloxacin and ciprofloxacin at 100 mg/kg.^c Significant difference ($P < 0.01$) between gemifloxacin and ciprofloxacin at 100 mg/kg.^d Significant difference ($P < 0.001$) between gemifloxacin and ciprofloxacin at 100 mg/kg.^e Significant difference ($P < 0.01$) between gemifloxacin and ciprofloxacin at 250 mg/kg.^f Significant difference ($P < 0.0001$) between gemifloxacin and ciprofloxacin at 250 mg/kg.^g Significant difference ($P < 0.0001$) between gemifloxacin and ciprofloxacin at 100 mg/kg.^h Significant difference ($P < 0.0001$) between gemifloxacin and trovafloxacin at 200 mg/kg.ⁱ Significant difference ($P < 0.05$) between gemifloxacin and trovafloxacin at 25 mg/kg.^j Significant difference ($P < 0.01$) between gemifloxacin and ciprofloxacin at 100 mg/kg.^k Significant difference ($P < 0.001$) between gemifloxacin and ciprofloxacin at 100 mg/kg.

FIG. 2. Survival rates of gemifloxacin-treated mice challenged with either a virulent penicillin-susceptible pneumococcal strain or isogenic mutant derivatives. (A) P-4241 (wild type); (B) C42-R2 (*parC*); (C) Sp42-R1 (*gyrA*); (D) C42-Sp6 (*gyrA parC*).

mice infected with the mutant with a triple mutation (P-2759) (data not shown).

Trovafoxacin at doses of 50 and 25 mg/kg was as effective as gemifloxacin in mice infected with the wild type and mutants with single mutations (*gyrA*, *parC*, and efflux mutants), while gemifloxacin was significantly more effective than trovafoxacin at 25 mg/kg against the *parE* mutant ($P < 0.05$). Moreover, treatment regimens with very high doses of trovafoxacin (100 and 200 mg/kg) were totally ineffective (0% survival) against mutant C42Sp6 with double mutations in *parC* and *gyrA*. Gemifloxacin at 50 mg/kg was significantly more effective than trovafoxacin at 200 mg/kg ($P < 0.0001$) against the mutant with double mutations (Table 4).

Comparison with ciprofloxacin showed that only 50% of mice infected with the wild type strain P-4241 were protected by this quinolone, despite treatment with a high dose of 100 mg/kg and early treatment at 6 h after challenge. A high dose of 250 mg/kg yielded about 80% survival in animals infected

with the wild-type strain, the *gyrA* mutant, and the efflux mutant and only 27% survival in animals infected with the *parC* mutant, while none of the mice survived when they were challenged with C42Sp6, which carried the double mutation *gyrA* and *parC*. None of the animals survived when the first treatment was delayed until 18 h postinfection. Gemifloxacin was significantly more effective than ciprofloxacin against all study strains (Table 4).

(iii) Bacterial clearance. Bacterial growth in the lungs of mice infected with strain P-4241 was observed from 22 h postinfection ($5.2 \log_{10}$ CFU/ml) until death at 67 h postinfection ($8 \log_{10}$ CFU/ml). Blood cultures were always positive for the untreated controls. The lungs and blood of mice infected with wild-type strain P-4241 were completely cleared after two injections of gemifloxacin at 50 or 25 mg/kg and four injections at 12.5 mg/kg. At the lowest dose, 6.25 mg/kg, bacterial regrowth in both the lungs and blood was observed 13 h after six treatments (Table 5). In animals infected with the *gyrA* or *parC*

TABLE 6. Analysis of wild-type- and mutant-derived strains isolated from untreated controls and gemifloxacin-treated mice^a

Strain	MIC (µg/ml)					Sequence	
	NOR	CIP	GEM	SFX	EtBr	<i>gyrA</i>	<i>parC</i>
4241	8	1	0.03	0.5	4	Ser81	Ser79, Arg95
C1, C2	8	1/2	0.03	0.5	4	ND	ND
T1-1, T1-2	64	8	0.06	1	4/8	ND	Ser79Tyr + Arg95Cys
C42-R2	64	4	0.06	2	8	Ser81	Ser79Tyr
(<i>parC</i>)							
C1, C2	64	8	0.06	2	8	ND	ND
T1, T2	64	8	0.06	2	8	ND	ND
Sp42-R1	16	ND	0.06	2	ND	Ser81Tyr	ND
(<i>gyrA</i>)							
C1, C2	16	ND	0.06	2	ND	Ser81Tyr	ND
T1 to T3	128	ND	0.5	8	ND	Ser81Tyr	Ser79Tyr + Arg95Cys
T4 to T6	32	ND	0.25	4	ND	Ser81Tyr	Arg95Cys
C42-Sp6	128	64	0.5	32	ND	Ser81Tyr	Ser79Tyr
(<i>gyrA parC</i>)							
C1, C2	128	64	0.5	32	ND	ND	ND
T1 to T4	128	64	0.5	32/64	ND	ND	ND
N42-6R1	16-32	4	0.06	1	16	ND	ND
C1, C2	16	4	0.06	1	16	ND	ND
T1, T2	16/64	4/8	0.06	1	16	ND	Ser79Tyr + Arg95Cys

^a The mice were treated with gemifloxacin at 6.25 mg/kg. Abbreviations: C, control mice; T, treated mice; NOR, norfloxacin; CIP, ciprofloxacin; GEM, gemifloxacin; SFX, sparfloxacin; EtBr, ethidium bromide; ND, not determined.

mutant, the lungs and blood were completely cleared after two injections of gemifloxacin at 50 and 25 mg/kg but not after two injections at 12.5 mg/kg. Bacterial clearance from the lungs and blood was also incomplete when animals infected with the *gyrA parC* (Table 5) or *gyrA parC parE* mutants were treated with 50 mg of gemifloxacin per kg. Bacterial clearance correlated with survival.

(iv) In vivo emergence of resistance. Emergence of resistance was evaluated in infected animals, which were either untreated or treated with gemifloxacin (Table 6).

In mice infected with the wild-type strain and treated with gemifloxacin at 6.25 mg/kg, resistance emerged in two clones of one isolate (clones T1-1 and T1-2), with each clone carrying two *parC* mutations: the frequent Ser79Tyr substitution and the rare Arg95Cys substitution. The MICs for these clones were higher than those for isolates from mice infected with the wild type or the untreated controls. This is in agreement with the results of the in vitro study, in which *parC* mutants were selected after exposure to gemifloxacin.

No significant changes in MICs were observed for strains isolated from mice infected with *parC* mutant C42-R2 when the mice were either treated or not treated with gemifloxacin. The resistance phenotype was thus conserved in all clones analyzed, suggesting that the *parC* mutation is stably maintained in vivo, even in the absence of antibiotic pressure in the case of the untreated control mice.

In mice infected with *gyrA* mutant Sp42-R1, the MIC profiles for all strains isolated from untreated control mice remained unchanged. However, six of six colonies isolated from gemi-

TABLE 5. Time course of bacterial clearance from lungs and blood of mice infected with the P-4241 wild-type strain or with its mutant C42Sp6 with a double mutation (*gyrA* and *parC*) and treated with gemifloxacin

Time postdosing ^b	Time p.i. ^c (h)	Bacterial count (CFU/ml) ^a after treatment with gemifloxacin at the indicated doses (mg/kg)									
		P-4241					C42Sp6 (<i>gyrA, parC</i>)				
		None	6.25	12.5	25	50	None	6.25	12.5	25	50
6 h after one Tt	24 h	5.2 ± 1.9 (3/3)	3.2 ± 0.3 (3/3)	2.8 ± 0.1 (3/3)	2.2 ± 0.9 (3/3)	3.4 ± 0.3 (3/3)	5.4 ± 1.7 (3/3)	4.2 ± 1.6 (3/3)	3.6 ± 0.7 (2/2)	3.8 ± 0.8 (3/3)	3.0 ± 0.7 (3/3)
13 h after two Tt	41 h	6.2 ± 0.4 (3/3)	2.5 ± 0.4 (2/3)	1.6 ± 0.5 (3/3)	1.0 ± 0.2 (0/3)	<1 ^d (0/3)	5.9 ± 0.9 (3/3)	6.5 ± 0.3 (3/3)	4.7 ± 0.8 (3/3)	3.5 ± 0.3 (3/3)	3.6 ± 0.2 (3/3)
13 h after four Tt	67 h	>8.0 ^e	1.7 ± 0.8 (0/3)	<1 (0/3)	<1 (0/3)	<1 (0/3)	>8.0 ^e	6.5 ± 0.3 (3/3)	4.7 ± 0.8 (3/3)	5.0 ± 0.4 (3/3)	2.0 ± 1.5 (1/3)
13 h after six Tt	91 h		4.3 ± 2.0 (2/3)	<1 (0/3)	<1 (0/3)	<1 (1/3)		— ^e	6.5 (1/1)	2.5 ± 1.3 (3/3)	3.0 ± 0.04 (2/2)

^a Bacterial counts in lung were expressed as log₁₀ CFU per milliliter of whole-lung homogenate; the values are means ± standard deviations. Values in parentheses are the number of animals with positive blood cultures/total number of animals.

^b Tt, treatment (the first treatment was given 18 h after bacterial challenge).

^c p.i., postinfection.

^d Less than the limit of detection (1 log₁₀ CFU/ml) in all three animals.

^e All mice died.

floxacin-treated animals displayed newly acquired mutations in *parC*. Three clones (clones T4 to T6) had a single *parC* mutation (Arg95Cys), while three other clones (clones T1 to T3) had two mutations in *parC* (Ser79Tyr and Arg95Cys). These results show that a *gyrA* mutation facilitates the selection of subsequent mutations in *parC*. With the exception of clone T4, the MICs for the clones containing the two *parC* mutations were higher than those for the clones carrying only one mutation.

No significant changes in MICs were observed for isolates from untreated and gemifloxacin-treated animals infected with the mutant with the double mutation, C42-Sp6. The QRDRs of these strains were therefore not sequenced.

The norfloxacin and ciprofloxacin MICs were increased for one of three colonies isolated from gemifloxacin-treated animals infected with efflux mutant N42-6R1. This clone (clone T2) had acquired a new *parC* mutation.

DISCUSSION

In our study, gemifloxacin displayed the lowest MIC for a virulent penicillin-susceptible wild-type strain of *S. pneumoniae* (P-4241), followed by the MICs of trovafloxacin, moxifloxacin, sparfloxacin, ciprofloxacin, and norfloxacin. Additionally, gemifloxacin had the lowest MICs for quinolone-resistant pneumococci, irrespective of the quinolone resistance mechanism. This was the case for quinolone-resistant mutants harboring a single mutation in *gyrA* (Ser81Tyr), *parC* (Ser79Tyr), and *parE* (Ile460Val or Asp435Asn) as well as for an efflux mutant. Gemifloxacin also demonstrated the lowest MICs for strains harboring double mutations (*parC* and *gyrA*) or triple mutations (*parC*, *gyrA*, and *parE*). Our data are in accordance with those of several other investigators (10, 12, 14, 22, 25, 29, 36, 38), which indicate that the rank order of the in vitro potencies of quinolones against *S. pneumoniae* is as follows: gemifloxacin $\geq$ garenoxacin, clinafloxacin $>$ moxifloxacin $>$ gatifloxacin, trovafloxacin, and grepafloxacin $>$ sparfloxacin $>$ levofloxacin $>$ ciprofloxacin. Gemifloxacin was more effective than these quinolones against laboratory strains carrying mutations in *parC* (Ser79Tyr, Ser79Phe, Asp83Asn, or Ala189Val); *gyrA* (Glu87Lys, Ser81Phe, or Ser83Tyr); *gyrB* (Glu474Lys); *parE* (Asp435Val or Ile460Val); the efflux mechanism; *parC* and *gyrA*; and *parC*, *gyrA*, and *parE*. In addition, gemifloxacin demonstrated a higher level of activity than moxifloxacin, gatifloxacin, ciprofloxacin, and levofloxacin against penicillin-susceptible and -resistant *S. pneumoniae* strains (38).

Previous reports (31) showed that exposure to subinhibitory concentrations of gemifloxacin in vitro selected mutants with single modifications in *parC* (S79Phe, Asp83Asn, or Lys137Asn), *gyrA* (Ser81Phe, Ser81Tyr, Glu85Ala, or Glu85Lys), or *parE* (Arg447Ser or Ile460Val). Boos et al. (9) also described multistep resistance selection in *S. pneumoniae* with subinhibitory concentrations of gemifloxacin and five other quinolones. Derived clones had single mutations in *gyrA* or *parC* alone or double mutations in *gyrA* and *parC* with the classical alterations in *ParC* (Ser79Phe or Tyr and Asp83Tyr) and *GyrA* (Ser81Phe or Tyr). Heaton et al. (23) also showed that gemifloxacin targets both enzymes. Morrissey and George (29) reported that gemifloxacin has a higher affinity for topoisomerase IV (*ParC*, *ParE*) than for gyrase (*GyrA*, *GyrB*).

In our in vitro experiments, we found that gemifloxacin preferentially selects for mutations in the *parC* gene (Ser79Phe or Asp83Asn). This is in agreement with our in vivo results, in which *parC* mutants selected after treatment with gemifloxacin carried the frequent Ser79Tyr substitution and the rare Arg95Cys substitution. Surprisingly, with the wild-type strain, the *gyrA* mutant, and the efflux mutant, gemifloxacin treatment yielded strains that carried two mutations in the *parC* gene. This could mean that at least two cycles of selection occurred in the animal during repeated gemifloxacin administration. Indeed, the mutant prevention concentration of gemifloxacin has been determined in vitro by Hansen et al. (21) and was shown to be equal to 1 μ g/ml, which is close to the maximal concentrations in serum of 1.7 and 2.4 μ g/ml determined in our study, which might explain the possible selection of mutants in vivo with the lowest gemifloxacin dose of 6.25 mg/kg. Of note, in vitro studies suggested that mutations in *gyrA* or *parC*, or even both genes, generated only a minor biological cost for *S. pneumoniae*, since the growth rates of the mutants were comparable to those of the wild type (20), an observation confirming the maintenance of these mutations in strains isolated from untreated control animals.

Our in vivo results showed that gemifloxacin is as effective as trovafloxacin against the wild-type strain. This efficacy was not adversely affected by quinolone resistance due to a single mutation (*parC*, *gyrA*, *parE*, or an efflux mutation). However, the main differences in efficacy were observed against strains carrying multiple target mutations. Even at the very high dose of 200 mg/kg, trovafloxacin was completely ineffective against the mutant with the double mutation, whereas gemifloxacin provided 50% survival against this mutant at only 75 mg/kg.

The in vivo efficacy of gemifloxacin was mainly due to its high in vitro potency against wild-type and fluoroquinolone-resistant *S. pneumoniae* strains. However, other factors, and particularly PK-pharmacodynamic parameters, may explain its in vivo efficacy. For fluoroquinolones, data suggest that a total AUC₂₄/MIC ratio of 100 to 125 correlates with optimal clinical and microbiological outcomes in seriously ill patients infected with gram-negative enteric pathogens or *Pseudomonas aeruginosa* (18). Ambrose et al. (2) observed a relationship between a free drug AUC₂₄/MIC ratio >33.7 and the microbiological response in patients with community-acquired respiratory tract infections involving *S. pneumoniae* and treated with levofloxacin or gatifloxacin. From these results, the total drug AUC₂₄/MIC ratios for the two drugs could be extrapolated to 42 and 44, respectively, since in humans the levels of serum protein binding are equal to 24 and 20% for levofloxacin and gatifloxacin, respectively.

Our data suggest that with the 25-mg/kg dose, the total AUC₂₄/MIC ratio of 113 against P-4241 was associated with a survival rate of 93% and an AUC₂₄/MIC ratio of 56.5 against *parC* and *gyrA* mutants was associated with survival rates of 69 and 100%, respectively. Conversely, with the 6.25-mg/kg dose, the total AUC₂₄/MIC ratio was equal to 28.5 and was associated with poor efficacy and the emergence of resistant mutants.

However, even though the gemifloxacin MICs were similar for the *parC* and *gyrA* mutants, gemifloxacin at 25 mg/kg showed decreased efficacy against the *parC* mutant but not against the *gyrA* mutant. This suggests that *ParC* is the more important target for gemifloxacin, which could explain the

preferential selection of *parC* mutants during gemifloxacin therapy.

Allen et al. (1) studied the PKs and tolerability of gemifloxacin after administration of single oral doses to healthy volunteers. They found that the mean $C_{\max}$ s increased with increasing dose: the mean $\pm$ standard deviation $C_{\max}$ s were 1.48 ± 0.39 and 3.86 ± 1.09 $\mu\text{g/ml}$ following the administration of 320- and 600-mg doses, respectively, which are expected to be effective. These concentrations are similar to that obtained with a dose of 25 mg/kg in mouse serum, with $C_{\max}$ equal to 2.4 ± 1.4 $\mu\text{g/ml}$ in noninfected animals. The serum AUC in our mouse model was equal to 4.4 ± 1.4 $\mu\text{g} \cdot \text{h/ml}$ for the control animals, which is near that obtained in healthy volunteers after treatment with 160 mg (5.48 ± 1.24 $\mu\text{g} \cdot \text{h/ml}$).

In conclusion, gemifloxacin was effective in an experimental model of pneumococcal pneumonia induced by both quinolone-susceptible and quinolone-resistant strains of *S. pneumoniae*. However, the total AUC₂₄/MIC ratio must be >28.5 , as this ratio was found to be associated with poor efficacy and the emergence of resistant mutants. Moreover, at lower doses, gemifloxacin preferentially selects for *parC* mutants.

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