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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*

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Alternate mutations in the *grlA* and *gyrA* genes were observed through the first- to fourth-step mutants which were obtained from four *Staphylococcus aureus* strains by sequential selection with several fluoroquinolones. The increases in the MICs of gatifloxacin accompanying those mutational steps suggest that primary targets of gatifloxacin in the wild type and the first-, second-, and third-step mutants are wild-type topoisomerase IV (topo IV), wild-type DNA gyrase, singly mutated topo IV, and singly mutated DNA gyrase, respectively. Gatifloxacin had activity equal to that of tosufloxacin and activity more potent than those of norfloxacin, ofloxacin, ciprofloxacin, and sparfloxacin against the second-step mutants (*grlA gyrA*; gatifloxacin MIC range, 1.56 to 3.13 μg/ml) and had the most potent activity against the third-step mutants (*grlA gyrA grlA*; gatifloxacin MIC range, 1.56 to 6.25 μg/ml), suggesting that gatifloxacin possesses the most potent inhibitory activity against singly mutated topo IV and singly mutated DNA gyrase among the quinolones tested. Moreover, gatifloxacin selected resistant mutants from wild-type and the second-step mutants at a low frequency. Gatifloxacin possessed potent activity (MIC, 0.39 μg/ml) against the NorA-overproducing strain *S. aureus* NY12, the *norA* transformant, which was slightly lower than that against the parent strain SA113. The increases in the MICs of the quinolones tested against NY12 were negatively correlated with the hydrophobicity of the quinolones (correlation coefficient, −0.93; $P < 0.01$). Therefore, this slight decrease in the activity of gatifloxacin is attributable to its high hydrophobicity. Those properties of gatifloxacin likely explain its good activity against quinolone-resistant clinical isolates of *S. aureus* harboring the *grlA*, *gyrA*, and/or *norA* mutations.

Gatifloxacin (AM-1155, CG5501, BMS-206584) is a newly developed fluoroquinolone with broad-spectrum antibacterial activity (11, 12, 35, 36). The activity of this agent against quinolone-resistant *Staphylococcus aureus* is more potent than those of norfloxacin and ciprofloxacin (11, 35).

Quinolone resistance in *S. aureus* is known to be associated with altered expression or mutations of the genes *norA* (14, 22, 41), *gyrA* (3, 7, 13, 21, 25–27, 29, 32), *gyrB* (13), and *grlA* (4–6, 21, 27) (*flqA* [9, 33]).

norA encodes the quinolone-efflux protein NorA. Mutation in the presumptive *norA* promoter region has been shown to be associated with increased steady-state levels of the *norA* mRNA (14, 22). This observation is presumed to be due to the increased level of transcription of *norA*. Activation of *norA* transcription by insertion of a transposon into the promoter region has been suggested to be a quinolone resistance mechanism in clinical isolates of *S. aureus* (17). Recently, the existence of the *norA* induction system has also been suggested, and mutation of the induction system is thought to increase the level of *norA* gene transcription (15). These mutants seem to achieve resistance by increasing the level of efflux of fluoroquinolones from the cytoplasm of the cell.

The *gyrA* and *gyrB* genes encode the A and B subunits of DNA gyrase, respectively, and DNA gyrase is one of the target enzymes of fluoroquinolones. The *gyrA* or *gyrB* mutation was observed in the structural gene, and it is suggested that these mutations reduce the level of sensitivity of DNA gyrase to fluoroquinolones (8).

The *grlA* gene encodes the ParC subunit of topoisomerase IV (topo IV) (4). Topo IV is a type II topoisomerase with DNA decatenating activity (16) and is thought to be the primary target of several fluoroquinolones in wild-type quinolone-susceptible *S. aureus* strains (2, 4, 5, 21, 38). We have genetically determined the quinolone resistance mechanisms of the first- and second-step *S. aureus* mutants sequentially acquired by selection with norfloxacin and ofloxacin (6, 10). As was shown in previous studies (4, 5), the first- and second-step mutations occurred in the *grlA* and *gyrA* genes, respectively, which corresponded to the mutations in naturally occurring quinolone-resistant *S. aureus* strains. Recently, the occurrence of quinolone-resistant clinical isolates of *S. aureus* has been increasing, and *grlA* and/or *gyrA* mutations have been reported in such strains (4, 7, 10, 25–27, 29, 32). Therefore, fluoroquinolones with potent activity against *S. aureus* strains with altered DNA gyrase and topo IV as well as NorA overproduction are needed in the clinical setting. In addition, fluoroquinolones with a low frequency of selection for quinolone-resistant mutants are also needed.

In this study, we compared the activity of gatifloxacin with those of various quinolones clinically used against the target-altered in vitro mutants of *S. aureus*, as well as a NorA-over-

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TABLE 1. Mutations in the QRDRs of *gyrA* and *grlA* genes accompanying sequential four-step mutations

Mutation (gene)	Strain				Reference
	MS5935	MS5952	MR5867	MR6009	
First step (*grlA*)	Ser-80(TCC)→Phe(TTC)	Ser-80(TCC)→Tyr(TAC)	Glu-84(GAA)→Lys(AAA)	Ser-80(TCC)→Tyr(TAC)	6
Second step (*gyrA*)	Ser-84(TCA)→Leu(TTA)	Ser-84(TCA)→Leu(TTA)	Ser-84(TCA)→Leu(TTA)	Glu-88(GAA)→Lys(AAA)	10
Third step (*grlA*)	Glu-84(GAA)→Lys(AAA)	Ala-116(GCA)→Val(GTA)	Ser-80(TCC)→Phe(TTC)	Glu-84(GAA)→Lys(AAA)	This study
Fourth step (*gyrA*)	Glu-88(GAA)→Val(GTA)	—[a]	Glu-88(GAA)→Lys(AAA)	Ser-84(TCA)→Leu(TTA)	This study

[a] —, the fourth-step mutant of MS5952 was not obtained from the third-step mutant.

producing laboratory strain, and determined the incidence of appearance of quinolone-resistant mutants.

MATERIALS AND METHODS

Quinolones. Ciprofloxacin, enoxacin, fleroxacin, lomefloxacin, norfloxacin, ofloxacin, pipemidic acid, sparfloxacin, and tosufloxacin were synthesized at Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan). Gatifloxacin (AM-1155, CG5501, BMS-206584) is a newly developed fluoroquinolone and was also synthesized at Kyorin Pharmaceutical Co., Ltd. Nalidixic acid was purchased from Sigma Chemical Co. (St. Louis, Mo.). The hydrophobicities of the quinolones were expressed as an apparent partition coefficient (log P) between chloroform and 0.1 M phosphate buffer (pH 7.4). The concentrations of the quinolones in the water phase were detected by high-pressure liquid chromatography.

Bacterial strains. Four clinical quinolone-susceptible *S. aureus* isolates, two of which were methicillin susceptible (strains MS5935 and MS5952) and the other two of which were methicillin resistant (strains MR5867 and MR6009), and their quinolone-resistant first- and second-step mutants selected sequentially with norfloxacin and ofloxacin have been described previously (10). The third- and fourth-step mutants were obtained from the second-step mutants by sequential selection with 4× the MICs of tosufloxacin and sparfloxacin, respectively. *S. aureus* SA113 and its *norA* transformant NY12 (NorA-overproducing strain) harboring plasmid pTUS20 with the *norA* gene have been described previously (34).

Antibacterial susceptibility. Antibacterial susceptibility testing was performed by an agar dilution method with Mueller-Hinton medium (Difco Laboratories, Detroit, Mich.). The MIC was defined as the lowest concentration of an antibacterial agent that inhibited the visible growth of the cells after 18 h of incubation at 37°C (11).

Statistical analysis. The MIC ratio was calculated as the ratio of the MIC for *norA* transformant NY12 to the MIC for parent strain SA113. Correlations between increments of the MICs for the *norA* transformant (log MIC ratio) and the hydrophobicities of the fluoroquinolones (log P) were tested by the linear regression test. A P value of less than 0.05 was considered statistically significant.

Amplification of *gyrA* and *grlA* gene fragments from *S. aureus* strains containing the sequence corresponding to the QRDR. The parts of the *gyrA* and *grlA* genes containing the sequence corresponding to the quinolone resistance-determining region (QRDR) of *gyrA* of *Escherichia coli* (40) were amplified by PCR. To amplify the *gyrA* gene fragment, two primers were designed on the basis of the sequence published by Margerrison et al. (19) and corresponded to nucleotide positions 2280 to 2320 (5′-TTGATGGCTGAATTACCTCAATC-3′) and positions 2733 to 2755 (5′-GACGGCTCTCTTTCATTACCATC-3′), respectively. The amplified fragment covered *gyrA* gene codon positions 1 to 157. For the amplification of the *grlA* fragment, two primers were designed on the basis of the sequence published by Ferrero et al. (4) and corresponded to nucleotide positions 2020 to 2043 (5′-TAGTGAGTGAAATAATTCAAGATT-3′) and positions 2400 to 2423 (5′-AACTCTTCAGCTAGTAAGCTTAAC-3′), respectively. The amplified fragment covered *grlA* gene codon positions 1 to 130. The gene fragments were amplified with genomic DNA of *S. aureus* strains as templates by 25 PCR cycles on a Perkin-Elmer thermal cycler with recombinant *Taq* DNA polymerase (Takara Shuzo Co., Ltd., Shiga, Japan). The genomic DNA of the *S. aureus* strains was extracted as described previously (31). The PCR conditions were 30 s at 94°C for denaturation, 30 s at 55°C for annealing, and 2 min at 72°C for primer extension. Amplification yielded 471- and 404-bp *gyrA* and *grlA* gene fragments, respectively, which were detected by agarose gel electrophoresis.

DNA sequencing. PCR-amplified *gyrA* and *grlA* gene fragments were sequenced with the 5′-biotinylated primers (5′-ATGAACAAGGTATGACACCG GAT-3′, corresponding to nucleotide positions 2443 to 2465 of the *gyrA* gene, and 5′-GCAATGTATTCAAGTGGTAATACAC-3′, corresponding to nucleotide positions 2173 to 2197 of the *grlA* gene) by direct cycle sequencing (1). The samples were subjected to electrophoresis in a 5% polyacrylamide gel containing 8 M urea at 45 W for 2.5 h. Thereafter, the DNA on the gel was transferred to a nylon membrane sheet (Boehringer Mannheim GmbH, Mannheim, Germany). The dried nylon membrane was then treated with a Phototope 6K detection kit (New England Biolabs Inc., Mass.), and the bands were visualized by exposing the membrane to Fuji RX X-ray film (Fuji Photo Film Co., Ltd., Kanagawa, Japan).

Calculation of frequency of emergence of resistant mutants selected by various quinolones. MS5952 and its first- and second-step mutants (10) were used as test strains. Quinolone-resistant mutants were selected on the plates containing gatifloxacin, norfloxacin, ofloxacin, ciprofloxacin, sparfloxacin, or tosufloxacin. The plates were incubated for 48 h before the plates were scored for the number of colonies that had grown. The incidence of emergence of resistant mutants was calculated as the ratio of the number of mutants to the number of inoculated bacteria (in CFU).

RESULTS

Isolation of third- and fourth-step quinolone-resistant mutants. The incidence of the third-step mutants from four sets of the second-step mutant ranged from 8.2×10^{-9} to $>2.1 \times 10^{-7}$ by selection with 4× the MIC of tosufloxacin. The fourth-step mutants were obtained from three sets of the third-step mutants except the third-step mutant of MS5952 by selection with 4× the MIC of sparfloxacin. The incidence of the fourth-step mutants ranged from 1.1×10^{-8} to 4.6×10^{-8}. The incidences were compatible with the incidences based on a single mutational event.

Mutation of the QRDR of the *grlA* and *gyrA* genes in the sequentially acquired quinolone-resistant mutants. Table 1 presents the mutations identified for the four isogenic sets of sequentially acquired mutants. The *grlA* and the additional *gyrA* gene mutations have been identified in the first- and second-step mutants, respectively (6, 10). The third-step mutations were again found in the *grlA* gene, indicating that the third-step mutants possessed single *gyrA* and double *grlA* mutations. All of the fourth-step mutants possessed an additional *gyrA* mutation. It was deduced that those mutations caused amino acid substitutions in the *grlA* and *gyrA* gene products (Table 1).

Antibacterial activity of gatifloxacin. Table 2 presents the antibacterial activities of the various quinolones against the sequentially acquired quinolone-resistant mutants. Generally, the activities of the quinolones decreased as the selection steps proceeded. The MIC ranges of gatifloxacin for wild-type strains and the first-, second-, third-, and fourth-step mutants were 0.05 to 0.10, 0.20, 1.56 to 3.13, 1.56 to 6.25, and 50 to 200 μg/ml, respectively. Gatifloxacin retained relatively potent activity against the wild-type strains as well as the mutants of up to the third step. Gatifloxacin displayed the most potent activity against the second- and third-step mutants except for the second-step mutant of strain MS5935, against which tosufloxacin and gatifloxacin were equally active. However, against the fourth-step mutants, gatifloxacin showed less activity. The observed increase in the MIC of gatifloxacin was 4 times or less for strains with the first- and third-step *grlA* mutations and 8 to 16 and 8 to 128 times for strains with the second- and fourth-step *gyrA* mutations, respectively. A similar profile of a decrease in activity was observed with sparfloxacin except for the much more drastic decreases in the activities of sparfloxacin for strains with the second-step *gyrA* mutation (32- to 256-fold increases in the MICs).

The activities of the quinolones against *norA* transformant

TABLE 2. Antibacterial activities of various quinolones against sequentially acquired quinolone-resistant mutants

Strain and mutant[a]	MIC (μg/ml)					
	Gatifloxacin	Norfloxacin	Ofloxacin	Ciprofloxacin	Tosufloxacin	Sparfloxacin
MS5935	0.05	0.78	0.20	0.20	0.025	0.05
1st	0.20	12.5	0.78	1.56	0.20	0.10
2nd	3.13	100	12.5	12.5	3.13	25
3rd	6.25	200	100	100	25	50
4th	50	200	400	100	25	200
MS5952	0.10	0.78	0.20	0.39	0.05	0.10
1st	0.20	12.5	0.78	1.56	0.20	0.10
2nd	1.56	25	6.25	6.25	3.13	6.25
3rd	3.13	200	50	50	25	6.25
MR5867	0.05	0.78	0.39	0.20	0.025	0.05
1st	0.20	12.5	0.78	1.56	0.20	0.10
2nd	3.13	50	12.5	25	1.56	6.25
3rd	6.25	200	50	100	50	12.5
4th	200	200	400	100	50	200
MR6009	0.10	1.56	0.39	0.39	0.025	0.05
1st	0.20	12.5	0.78	1.56	0.20	0.10
2nd	1.56	50	12.5	12.5	3.13	3.13
3rd	1.56	200	25	100	12.5	6.25
4th	200	200	400	100	25	200

[a] Abbreviations: 1st, first-step mutant; 2nd, second-step mutant; 3rd, third-step mutant; 4th, fourth-step mutant. The fourth-step mutant of MS5952 was not obtained from the third-step mutant.

NY12 are presented in Table 3. The increase in the MICs of the quinolones for the *norA* transformant was negatively correlated with their hydrophobicity (correlation coefficient, -0.93; $P < 0.01$). Gatifloxacin had potent activity against *norA* transformant NY12 (MIC, 0.39 μg/ml). The MIC of gatifloxacin for NY12 was eight times higher than that for parent strain SA113. This increment was relatively lower than those for ciprofloxacin, enoxacin, lomefloxacin, norfloxacin, ofloxacin, pipemidic acid, and tosufloxacin; was equal to that for fleroxacin; and was higher than those for nalidixic acid and sparfloxacin.

Frequency of emergence of resistant mutants by selection with various quinolones. The frequencies of appearance of mutants from the wild-type strains and each step of the mutant strains by selection with various quinolones were determined. MS5952 and its first- and second-step mutants were used as the test strains for the selection. The results are presented in Table 4.

No resistant mutant was obtained from 2.0×10^9 CFU of the wild-type strain by selection with 4× the MIC or higher concentrations of sparfloxacin or gatifloxacin. From 2.6×10^9 CFU of the first-step mutants, resistant mutants were obtained by selection with 4× and 8× the MIC of gatifloxacin and even with 64× the MIC of sparfloxacin. In contrast, no mutant was obtained by selection with 4× the MIC or higher concentrations of norfloxacin or ciprofloxacin. No mutant was obtained from 5.3×10^9 CFU of the second-step mutants by selection with 4× the MIC or higher concentrations of sparfloxacin or gatifloxacin.

TABLE 3. Antibacterial activities of gatifloxacin and other quinolones against *norA* transformant NY12[a]

Quinolone	Hydrophobicity (log P)	MIC (μg/ml)		MIC ratio
		SA113 (parent)	NY12 (*norA* transformant)	
Nalidixic acid	+1.87	25	50	2
Sparfloxacin	+1.52	0.025	0.10	4
Tosufloxacin	+1.07	0.0125	0.20	16
Gatifloxacin	+0.69	0.05	0.39	8
Ofloxacin	+0.68	0.20	3.13	16
Fleroxacin	+0.66	0.39	3.13	8
Lomefloxacin	+0.09	0.39	6.25	16
Enoxacin	−0.03	0.78	25	32
Ciprofloxacin	−0.14	0.20	12.5	64
Norfloxacin	−0.59	0.78	50	64
Pipemidic acid	−0.85	12.5	800	64

[a] Log P is the apparent partition coefficient between chloroform and 0.1 M phosphate buffer (pH 7.4). The MIC ratio was calculated as the ratio of the MIC for *norA* transformant NY12 to the MIC for parent strain SA113. The increase in the MICs of the quinolones for the *norA* transformant (log MIC ratio) were negatively correlated with their hydrophobicity (log P) (correlation coefficient, -0.93; $P < 0.01$).

DISCUSSION

We identified mutations in the specific regions of *grlA* or *gyrA* genes in quinolone-resistant mutants of *S. aureus*, suggesting that either topo IV or DNA gyrase serves as a primary target of quinolones, as described previously (2, 4, 5, 21, 38). In wild-type quinolone-susceptible *S. aureus*, topo IV seems to be a primary target of several quinolones such as norfloxacin (2, 38), ciprofloxacin (2, 4, 5, 21, 38), and sparfloxacin (2, 38).

In *Streptococcus pneumoniae*, the primary target of several fluoroquinolones also seems to be topo IV (20, 23, 30). Pan and Fisher recently obtained resistant mutants of *S. pneumoniae* by selection with sparfloxacin (24). Those mutants possessed no *parC* mutation but possessed a *gyrA* mutation and displayed no altered susceptibility to ciprofloxacin but a lower level of susceptibility to sparfloxacin compared with that of their parent strains. On the other hand, the *parC* mutant, which was selected by ciprofloxacin, showed no altered susceptibility to sparfloxacin but had a lower level of susceptibility to ciprofloxacin (24). These results suggest that the primary targets of

TABLE 4. Frequency of appearance of quinolone-resistant strains from the wild type and each mutant strain of MS5952 by selection with various quinolones

Strain	Quinolone	Frequency at the following multiple of the MIC[a]:				
		4×	8×	16×	32×	64×
Wild	Gatifloxacin	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
	Norfloxacin	3.3×10^{-8}	1.0×10^{-8}	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
	Ofloxacin	1.5×10^{-8}	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
	Ciprofloxacin	3.9×10^{-8}	1.0×10^{-9}	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
	Tosufloxacin	4.8×10^{-8}	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
	Sparfloxacin	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
First-step mutant	Gatifloxacin	8.5×10^{-9}	1.9×10^{-9}	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$
	Norfloxacin	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$
	Ofloxacin	2.3×10^{-9}	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$
	Ciprofloxacin	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$
	Tosufloxacin	8.0×10^{-8}	1.9×10^{-8}	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$
	Sparfloxacin	$>3.8 \times 10^{-7}$	1.7×10^{-8}	5.4×10^{-9}	3.5×10^{-9}	2.7×10^{-9}
Second-step mutant	Gatifloxacin	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$
	Norfloxacin	9.3×10^{-8}	1.5×10^{-9}	$<1.9 \times 10^{-10}$	ND[b]	ND
	Ofloxacin	5.9×10^{-8}	1.3×10^{-9}	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$
	Ciprofloxacin	1.9×10^{-7}	3.8×10^{-8}	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$	ND
	Tosufloxacin	$>1.9 \times 10^{-7}$	3.7×10^{-8}	$<1.9 \times 10^{-10}$	ND	ND
	Sparfloxacin	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$

[a] The reproducibilities of the frequencies have been confirmed in repeat experiments.
[b] ND, not determined because those quinolones did not dissolve in the medium.

sparfloxacin and ciprofloxacin are DNA gyrase and topo IV in *S. pneumoniae*, respectively (24).

Topo IV and DNA gyrase are essential for bacterial cell growth. The bacteria cannot remain alive if either of the enzyme reactions is inhibited by fluoroquinolones. It is proposed that the susceptibility of *S. aureus* to quinolones is primarily determined by which one of the two target enzymes, topo IV or DNA gyrase, is more sensitive to quinolones (38). A similar hypothesis has been proposed in the case of *E. coli*, in wild-type strains for which the primary target of quinolones seems to be DNA gyrase (18).

Gatifloxacin had a lower level of activity against the first-step *grlA* (topo IV) mutants than against the wild-type strains. This indicated that the topo IV mutations caused an apparent decrease in bacterial susceptibility to gatifloxacin. Therefore, topo IV seems to be a primary target of gatifloxacin in wild-type strains. Gatifloxacin had a lower level of activity against the second-step *gyrA* mutants than against the first-step mutants. This indicates that an alteration in DNA gyrase caused the decrease in susceptibility to gatifloxacin in the second-step mutants. Therefore, DNA gyrase seems to be a primary target of gatifloxacin in the first-step *grlA* mutants. Along the same line of reasoning, singly mutated topo IV and singly mutated DNA gyrase seem to be primary targets of gatifloxacin in the second- and third-step mutants, respectively. The primary target in the fourth-step mutant could not be determined because fifth-step mutants were not obtained, nor were the target gene mutations and quinolone susceptibilities of the fifth-step mutants determined.

Gatifloxacin had activity almost equal to that of tosufloxacin and activity more potent than those of norfloxacin, ofloxacin, ciprofloxacin, and sparfloxacin against the second-step mutants (harboring singly mutated *grlA* and singly mutated *gyrA* genes) and had the most potent activity against the third-step mutants (harboring doubly mutated *grlA* and singly mutated *gyrA* genes). These data suggest that gatifloxacin possesses more potent inhibitory activity against the presumptive primary targets in the second- and third-step mutants, singly mutated topo IV and singly mutated DNA gyrase, than the other fluoroquinolones tested.

It has been observed that the MIC of sparfloxacin for a *grlA* mutant was almost equal to that for its parent wild-type strain and that no mutant was obtainable from the wild-type strain (38). This observation was interpreted as indicating that the inhibitory activity of sparfloxacin against wild-type DNA gyrase is almost equal to that against wild-type topo IV (39). The decreases in the activities of gatifloxacin accompanying the first-step *grlA* mutations were only fourfold or less. This may be because the sensitivity of the wild-type DNA gyrase to gatifloxacin is close to that of wild-type topo IV. Also, the activity of gatifloxacin against the second-step mutants was almost equal to that against the respective third-step mutants, and no mutant was obtained from the second-step mutant by selection with 4× the MIC or higher concentrations of gatifloxacin. These results suggest that both the singly mutated DNA gyrase and the singly mutated topo IV in the second-step mutants possess nearly the same sensitivities to gatifloxacin.

On the other hand, the increment in the MIC of gatifloxacin accompanying the second-step *gyrA* mutation was 8- to 16-fold. Also, the second-step mutants were obtained from the first-step mutant of MS5952 by selection with 4× and 8× the MIC of gatifloxacin. The increment of the MIC accompanying the second-step mutation is considered to correlate with the decrease in topo IV sensitivity caused by the first-step *grlA* mutation. The increment in the MIC of sparfloxacin accompanying the second-step mutation was 32- to 256-fold, and among the quinolones tested, the incidence of selection of second-step mutants was the highest for sparfloxacin. Therefore, sparfloxacin may be more vulnerable than gatifloxacin to the alteration in topo IV at the enzyme level. This correlates with the fact that gatifloxacin, when compared to sparfloxacin, showed the same or a lower level of activity against the wild-type and the first-step mutants but a higher level of activity against the second- and third-step mutants.

No mutant was obtained from the first-step mutant of MS5952 by selection with 4× the MIC or a higher concentra-

tion of norfloxacin or ciprofloxacin. The increases in the MICs of these agents accompanying the second-step mutation in MS5952 were slight (two to four times). This indicates that norfloxacin and ciprofloxacin inhibit singly mutated topo IV and the wild-type DNA gyrase in the first-step mutant with almost the same potency.

Gatifloxacin also showed potent activity against the *norA* transformant, which was slightly lower than that against the parent strain SA113. It is reported that the hydrophobicity of quinolones is not an exclusive factor responsible for decreased activity against efflux-mediated quinolone-resistant *S. aureus* (28). However, other investigators suggested that the hydrophobicity of quinolones seems to be one of the factors influencing the excretion capability of the NorA pump (37, 41). Our data also indicate that the increases in the MICs of the quinolones for the *norA* transformant are negatively correlated with the hydrophobicity of the quinolones (correlation coefficient, -0.93; $P < 0.01$). Therefore, the lower level of decrease in the activity of gatifloxacin against the *norA* transformant seems to be attributable to its high hydrophobicity.

The MIC of gatifloxacin at which 90% of recent quinolone-resistant clinical isolates of *S. aureus* in Japan are inhibited was reported to be from 6.25 to 12.5 μg/ml (11, 35); these MICs were equal to or slightly higher than the MIC of gatifloxacin for the third-step mutants. We isolated the fourth-step mutants, which were much less susceptible to all fluoroquinolones including gatifloxacin (MIC, 50 to 200 μg/ml). Such strains might be anticipated to occur in clinical situations.

Mutations in topo IV and/or DNA gyrase have been reported in clinical isolates of quinolone-resistant *S. aureus* (4, 6, 7, 10, 25–27, 29, 32). Gatifloxacin had activity more potent than those of norfloxacin, ciprofloxacin, ofloxacin, tosufloxacin, and sparfloxacin against many clinical isolates of *S. aureus* harboring *gyrA* mutations (29). The presumptive activation of *norA* transcription has also been reported to be one of the quinolone resistance mechanisms in clinical isolates of *S. aureus* (17). In this study, we showed that gatifloxacin has relatively potent activity against some target-altered as well as NorA-overproducing laboratory strains and prevents the selection of quinolone-resistant strains with the first- and third-step *grlA* mutations. Those properties of gatifloxacin likely explain its good activity against quinolone-resistant clinical isolates of *S. aureus* harboring the *grlA*, *gyrA*, and/or *norA* mutations as well as quinolone-susceptible strains.

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REFERENCES

1. **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.
2. **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.
3. **Fasching, C. E., F. C. Tenover, T. G. Slama, L. M. Fisher, S. Sreedharan, M. Oram, K. Willard, L. M. Sinn, D. N. Gerding, and L. R. Peterson.** 1991. *gyrA* mutations in ciprofloxacin-resistant, methicillin-resistant *Staphylococcus aureus* from Indiana, Minnesota, and Tennessee. J. Infect. Dis. **164:**976–979.
4. **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.
5. **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.
6. **Fukuda, H., S. Hori, and K. Hiramatsu.** 1996. The *grlA* mutation in norfloxacin-resistant first-step mutants and clinical isolates of *Staphylococcus aureus*. J. Infect. Chemother. **2:**98–101.
7. **Goswitz, J. J., K. E. Willard, C. E. Fasching, and L. R. Peterson.** 1992. Detection of *gyrA* gene mutations associated with ciprofloxacin resistance in methicillin-resistant *Staphylococcus aureus*: analysis by polymerase chain reaction and automated directed DNA sequencing. Antimicrob. Agents Chemother. **36:**1166–1169.
8. **Hooper, D. C., and J. S. Wolfson.** 1993. Mechanisms of bacterial resistance to quinolones, p. 97–118. *In* D. C. Hooper and J. S. Wolfson (ed.), Quinolone antimicrobial agents, 2nd ed. American Society for Microbiology, Washington, D.C.
9. **Hooper, D. C.** 1995. Quinolone mode of action. Drugs **49:**10–15.
10. **Hori, S., Y. Ohshita, Y. Utsui, and K. Hiramatsu.** 1993. Sequential acquisition of norfloxacin and ofloxacin resistance by methicillin-resistant and -susceptible *Staphylococcus aureus*. Antimicrob. Agents Chemother. **37:**2278–2284.
11. **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.
12. **Hosaka, M., S. Kinoshita, A. Toyama, M. Otsuki, and T. Nishino.** 1995. Antibacterial properties of AM-1155, a new 8-methoxy quinolone. J. Antimicrob. Chemother. **36:**293–301.
13. **Ito, H., H. Yoshida, M. Bogaki-Shonai, T. Niga, H. Hattori, and S. Nakamura.** 1994. Quinolone resistance mutations in the DNA gyrase *gyrA* and *gyrB* gene of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **38:**2014–2023.
14. **Kaatz, G. W., S. M. Seo, and C. A. Ruble.** 1993. Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **37:**1086–1094.
15. **Kaatz, G. W., and S. M. Seo.** 1995. Inducible NorA-mediated multidrug resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **39:**2650–2655.
16. **Kato, J., Y. Nishimura, R. Imamura, H. Niki, S. Hiraga, and H. Suzuki.** 1990. New topoisomerase essential for chromosome segregation in *E. coli*. Cell **63:**393–404.
17. **Kojima, T., J. Yamagishi, Y. Oyamada, H. Yoshida, H. Hattori, M. Inoue, and S. Nakamura.** 1995. Analysis of quinolone resistance genes in a clinical isolate of quinolone-resistant MRSA. Drugs **49**(Suppl. 2)**:**182–184.
18. **Kumagai, Y., J. Kato, K. Hoshino, T. Akasaka, K. Sato, and H. Ikeda.** 1996. Quinolone-resistant mutants of *Escherichia coli* DNA topoisomerase IV *parC* gene. Antimicrob. Agents Chemother. **40:**710–714.
19. **Margerrison, E. E. C., R. Hopewell, and L. M. Fisher.** 1992. Nucleotide sequence of the *Staphylococcus aureus gyrB-gyrA* locus encoding the DNA gyrase A and B proteins. J. Bacteriol. **174:**1596–1603.
20. **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.
21. **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.
22. **Ng, E. Y. W., M. Trucksis, and D. C. Hooper.** 1994. Quinolone resistance mediated by *norA*: physiologic characterization and relationship to *flqB*, a quinolone resistance locus on the *Staphylococcus aureus* chromosome. Antimicrob. Agents Chemother. **38:**1345–1355.
23. **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.
24. **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.
25. **Sreedharan, S., M. Oram, B. Jensen, L. R. Peterson, and L. M. Fisher.** 1990. DNA gyrase *gyrA* mutations in ciprofloxacin resistant strains of *Staphylococcus aureus*: close similarity with quinolone resistance mutations in *Escherichia coli*. J. Bacteriol. **172:**7260–7262.
26. **Sreedharan, S., L. R. Peterson, and L. M. Fisher.** 1991. Ciprofloxacin resistance in coagulase-positive and -negative staphylococci: role of mutations at serine 84 in DNA gyrase A protein of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **35:**2151–2154.
27. **Takahata, M., M. Yonezawa, S. Kurose, N. Futakuchi, N. Matsubara, Y. Watanabe, and H. Narita.** 1996. Mutations in the *gyrA* and *grlA* genes of quinolone-resistant clinical isolates of methicillin-resistant *Staphylococcus aureus*. J. Antimicrob. Chemother. **38:**543–546.
28. **Takenouchi, T., F. Tabata, Y. Iwata, H. Hanzawa, M. Sugawara, and S. Ohya.** 1996. Hydrophilicity of quinolones is not an exclusive factor for decreased activity in efflux-mediated resistant mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**1835–1842.
29. **Takenouchi, T., C. Ishii, M. Sugawara, Y. Tokue, and S. Ohya.** 1995. Inci-

dence of various *gyrA* mutants in 451 *Staphylococcus aureus* strains isolated in Japan and their susceptibilities to 10 fluoroquinolones. Antimicrob. Agents Chemother. **39:**1414–1418.
30. **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.
31. **Tokue, Y., S. Shoji, K. Satoh, A. Watanabe, and M. Motomiya.** 1992. Comparison of a polymerase chain reaction assay and a conventional microbiologic method for detection of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **36:**6–9.
32. **Tokue, Y., K. Sugano, D. Saito, T. Noda, H. Ohkura, Y. Shimosato, and T. Sekiya.** 1994. Detection of novel mutations in the *gyrA* gene of *Staphylococcus aureus* by nonradioisotopic single-strand conformation polymorphism analysis and direct DNA sequencing. Antimicrob. Agents Chemother. **38:** 428–431.
33. **Trucksis, M., J. S. Wolfson, and D. C. Hooper.** 1991. A novel locus conferring fluoroquinolone resistance in *Staphylococcus aureus*. J. Bacteriol. **173:** 5854–5860.
34. **Ubukata, K., N. Itoh-Yamashita, and M. Konno.** 1989. Cloning and expression of the *norA* gene for fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **33:**1535–1539.
35. **Wakabayashi, E., and S. Mitsuhashi.** 1994. In vitro antibacterial activity of AM-1155, a novel 6-fluoro-8-methoxy quinolone. Antimicrob. Agents Chemother. **38:**594–601.
36. **Wise, R., N. P. Brenwald, J. M. Andrews, and F. Boswell.** 1997. The activity of the methylpiperazinyl fluoroquinolone CG5501: a comparison with other fluoroquinolones. J. Antimicrob. Chemother. **39:**447–452.
37. **Yamada, H., S. Kurose-Hamada, Y. Fukuda, J. Mitsuyama, M. Takahata, S. Minami, Y. Watanabe, and H. Narita.** 1997. Quinolone susceptibility of *norA*-disrupted *Staphylococcus aureus*. Antimicrob. Agents Chemother. **41:** 2308–2309.
38. **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.
39. **Yamagishi, J.** 1997. Personal communication.
40. **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.
41. **Yoshida, H., M. Bogaki, S. Nakamura, K. Ubukata, and M. Konno.** 1990. Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J. Bacteriol. **172:**6942–6949.