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Contributions of the 8-Methoxy Group of Gatifloxacin to Resistance Selectivity, Target Preference, and Antibacterial Activity against *Streptococcus pneumoniae*

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Gatifloxacin (8-methoxy, 7-piperazinyl-3′-methyl) at the MIC selected mutant strains that possessed *gyrA* mutations at a low frequency (3.7×10^{-9}) from wild-type strain *Streptococcus pneumoniae* IID553. AM-1147 (8-methoxy, 7-piperazinyl-3′-*H*) at the MIC or higher concentrations selected no mutant strains. On the other hand, the respective 8-H counterparts of these two compounds, AM-1121 (8-*H*, 7-piperazinyl-3′-methyl) and ciprofloxacin (8-*H*, 7-piperazinyl-3′-*H*), at one and two times the MIC selected mutant strains that possessed *parC* mutations at a high frequency (>2.4×10^{-6}). The MIC of AM-1147 increased for the *gyrA* mutant strains but not for the *parC* mutant strains compared with that for the wild-type strain. These results suggest that fluoroquinolones that harbor 8-methoxy groups select mutant strains less frequently and prefer DNA gyrase, as distinct from their 8-H counterparts. The in vitro activities of gatifloxacin and AM-1147 are twofold higher against the wild-type strain, eight- and twofold higher against the first-step *parC* and *gyrA* mutant strains, respectively, and two- to eightfold higher against the second-step *gyrA* and *parC* double mutant strains than those of their 8-H counterparts. These results indicate that the 8-methoxy group contributes to enhancement of antibacterial activity against target-altered mutant strains as well as the wild-type strain. It is hypothesized that the 8-methoxy group of gatifloxacin increases the level of target inhibition, especially against DNA gyrase, so that it is nearly the same as that for topoisomerase IV inhibition in the bacterial cell, leading to potent antibacterial activity and a low level of resistance selectivity.

Streptococcus pneumoniae is one of the most important pathogens and is responsible for community-acquired pneumonia, acute otitis media, and meningitis. Recently, the worldwide prevalence of penicillin-resistant *S. pneumoniae* has become a serious problem in clinical settings. Therefore, antibiotics that possess potent activity against penicillin-resistant as well as penicillin-susceptible *S. pneumoniae* are urgently needed.

Some of the recently developed fluoroquinolones have improved activities against respiratory pathogens, including *S. pneumoniae*, and are expected to be useful as chemotherapeutic agents for the treatment of patients infected with such pathogens (1, 6, 11, 12, 20, 21, 33, 34). Recent clinical assessments of the susceptibility of *S. pneumoniae* to antibacterial agents have indicated that most clinical isolates continue to retain their quinolone susceptibility (9, 15, 31). Nevertheless, an outbreak of quinolone-resistant *S. pneumoniae* has recently been reported (K. Weiss, C. Restieri, M. Laverdiere, R. J. Davidson, A. McGeer, J. De Azavedo, and D. E. Low, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., Abstr. 824, p. 110, 1999). In conjunction with the increasing clinical use of fluoroquinolone for the treatment of respiratory infections, the increasing prevalence of quinolone resistance is anticipated in *S. pneumoniae*, as recently occurred in methicillin-resistant *Staphylococcus aureus*. Therefore, it is important to try to prevent the acquisition of quinolone resistance in *S. pneumoniae*.

In number of studies on the in vitro selection of quinolone-resistant strains of *S. pneumoniae*, investigators have reported observing different frequencies of resistance selectivity among fluoroquinolones (4, 8). It has been suggested that gatifloxacin and clinafloxacin possess potent antipneumococcal activities and select mutant strains less frequently than other fluoroquinolones because of their inhibition of DNA gyrase and topoisomerase IV (TopoIV), which occur at nearly the same levels in bacterial cells (dual-targeting property) (8, 23). On the other hand, it has been reported that the ease of resistance selectivity in *S. pneumoniae* correlated with the susceptibilities of the agents to the bacterial NorA-type efflux system (4).

Gatifloxacin harbors a characteristic methoxy group at the 8 position of the quinolone ring. The contributions of the methoxy groups of certain fluoroquinolones, including gatifloxacin, to antibacterial activity and/or resistance selectivity have been investigated in some bacteria. The methoxy group has been shown to correlate with the prevention of emergence of the mutant strains and/or potent in vitro activity against *Escherichia coli* (17, 38), *S. aureus* (13, 14, 39), and mycobacteria (5, 29, 37).

In the study described in this report, we investigated the contribution of the 8-methoxy group of gatifloxacin to resistance selectivity, target preference, and the antibacterial activity against *S. pneumoniae*.

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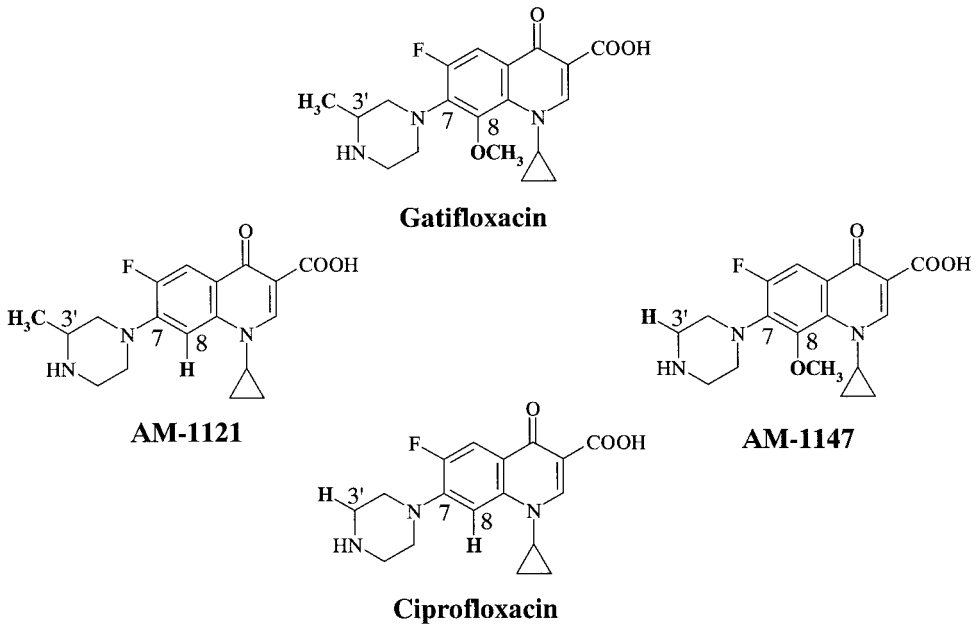

FIG. 1. Chemical structures of gatifloxacin (8-methoxy, 7-piperazinyl-3′-methyl) and its structurally related compounds ciprofloxacin (8-*H*, 7-piperazinyl-3′-H), AM-1121 (8-*H*, 7-piperazinyl-3′-methyl), and AM-1147 (8-methoxy, 7-piperazinyl-3′-*H*).

MATERIALS AND METHODS

Fluoroquinolones. Figure 1 shows the fluoroquinolones used in the present study. Gatifloxacin (8-methoxy, 7-piperazinyl-3′-methyl) and the structurally related compounds ciprofloxacin (8-*H*, 7-piperazinyl-3′-*H*), AM-1121 (8-*H*, 7-piperazinyl-3′-methyl), and AM-1147 (8-methoxy, 7-piperazinyl-3′-*H*) were synthesized at Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan. These fluoroquinolones were used for the selection of the mutant strains and susceptibility testing.

Bacterial strains. Quinolone-susceptible, wild-type *S. pneumoniae* IID553 (a type strain collected at the Institute of Medical Science, University of Tokyo) was provided through the Japanese Society for Bacteriology. Overnight cultures of IID553 on Mueller-Hinton agar plates containing 5% defibrinated horse blood were suspended in saline. The mutant strains were selected by plating the bacterial suspension (approximately 10^9 CFU) on Mueller-Hinton agar plates containing 5% defibrinated horse blood with gatifloxacin, ciprofloxacin, AM-1121, and AM-1147 at 1, 2, 4, 8, and 16 times the MICs. The selection plates were incubated aerobically at 37°C for at least 48 h before being scored for the number of bacterial colonies. The incidence of the appearance of resistant strains was calculated as the ratio of the number of colonies that emerged to the number of bacteria inoculated (in CFU).

Four types of first-step mutant strains possessing a deduced alteration in either the GyrA subunit (S81F or S81Y) or the ParC subunit (S79Y or D83N) have been obtained from wild-type strain IID553 by selection with various fluoroquinolones (8). The second-step mutant strains have continuously been selected from the first-step *gyrA* and *parC* mutant strains (9). The results of the second-step mutation showed that all of the second-step mutant strains possessed deduced alterations in both ParC and GyrA subunits, as described in Table 3. In order to investigate the antibacterial activities of gatifloxacin and its related compounds, these first- and second-step mutant strains were used for MIC determinations.

Mutations of QRDRs of the *parC, parE, gyrA*, and *gyrB* genes. To amplify gene fragments, including the quinolone resistance-determining region (QRDRs) of the *gyrA, gyrB, parC*, and *parE* genes, which correspond to the QRDRs of the *E. coli gyrA* and *gyrB* genes (35, 36), each pair of primers, the sequences of which were the same as those reported by Pan et al. (24), was synthesized. The gene fragments were amplified, using the genomic DNAs of *S. pneumoniae* 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 PCR conditions were as follows: 30 s at 94°C for denaturation, 30 s at 55°C for annealing, and 2 min at 72°C for primer extension. The PCR-amplified gene fragments were sequenced with 5′-biotinylated primers (5′-AAATCTGCTCGTATTACAGGG GATG-3′, nucleotide positions 187 to 211 of the *gyrA* gene [3]; 5′-CAGGGAA ACTAGCAGACTGTTCTTC-3′, nucleotide positions 1238 to 1262 of the *gyrB* gene [25]; 5′-GACAAGAGCTACCGTAAGTCGGCCAAG-3′, nucleotide positions 166 to 192 of the *parC* gene [25]; 5′-CAGCCCAATCTAAGAATCCTG CTAAG-3′, nucleotide positions 1253 to 1278 of the *parE* gene [25]) by direct cycle sequencing. 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 by use of a Phototope 6K detection kit (New England Biolabs Inc., Mass.), and the bands were visualized by exposing the membrane to X-ray film.

MIC determination. The MICs of each fluoroquinolone for the resistant strains were determined. The MIC was defined as the lowest concentration of an antibacterial agent that inhibited visible growth of the cells on Mueller-Hinton agar plates with 5% defibrinated horse blood after 18 to 20 h of incubation at 37°C (12).

RESULTS AND DISCUSSION

Resistance selection. Gatifloxacin at the MIC selected mutant strains at a low frequency (3.7×10^{-9}). AM-1147 at the MIC or higher concentrations selected no mutant strains. On the other hand, ciprofloxacin and AM-1121 at one and two times their MICs selected mutant strains at high frequencies ($>2.4 \times 10^{-6}$) (Table 1). These results indicate that the compounds harboring an 8-methoxy group selected mutant strains less frequently than their 8-H counterparts did. For some fluoroquinolones, the presence of an 8-methoxy group has been shown to correlate with the prevention of the emergence of mutant strains of *E. coli* (38), mycobacteria (5), and *S. aureus* (13). The potent bactericidal activities of the agents have been interpreted as factors that influence resistance selectivity in these bacteria. On the other hand, it has been reported that some fluoroquinolones selected mutant strains of *S. pneumoniae* or *S. aureus* less frequently, since those agents seemed to inhibit DNA gyrase and TopoIV at nearly the same levels in bacterial cells (dual-targeting property) (8, 23). Therefore, the

TABLE 1. Frequencies of appearance of mutant strains by selection with gatifloxacin and its related compounds

Selecting quinolone	Substitutions at the following position[a]:		Frequency at the following multiple of the MIC[b]:				
	8	3′	1	2	4	8	16
Gatifloxacin	OCH_3	CH_3	3.7×10^{-9}	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$
AM-1121	H	CH_3	$>2.4 \times 10^{-6}$	$>2.4 \times 10^{-6}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$
AM-1147	OCH_3	H	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$
Ciprofloxacin	H	H	$>2.4 \times 10^{-6}$	$>2.4 \times 10^{-6}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$

[a] 8, position C-8 of the quinolone ring; 3′, position 3′ of the C-7 piperazinyl moiety.
[b] The reproducibility of the frequency was confirmed by repeated experiments.

dual-targeting property of the agents that harbor an 8-methoxy group might be one of the causes of the low frequency of resistance selectivity in *S. pneumoniae*.

It has been reported that the ease of resistance selectivity in *S. pneumoniae* correlates with the susceptibilities of the agents to the NorA-type efflux system (4). We have tested the effects of reserpine (10 μg/ml), which is an inhibitor of the NorA-type streptococcal PmrA efflux system (10), on the activities of gatifloxacin and the related compounds. No effects of reserpine on the MICs of these quinolones for wild-type and mutant strains were observed (H. Fukuda, unpublished data). These results suggest that the activities of these compounds are little influenced by the intrinsic PmrA and/or other reserpine-sensitive efflux systems.

As described previously (18), to investigate the differences in the magnitude of the effects of reserpine between the compounds in detail, studies of the activities against *S. pneumoniae* strains that possess an activated reserpine-sensitive efflux system will be necessary. Unfortunately, we have no efflux system-activated *S. pneumoniae* strains. However, we have an *S. aureus norA* strain and have determined the staphylococcal NorA efflux system susceptibilities of some fluoroquinolones, including gatifloxacin and ciprofloxacin, as the ratio of the MIC for the *S. aureus norA* strain to the MIC for its parent strain (MIC ratio) (7). On the other hand, we have also investigated resistance selectivity in *S. pneumoniae* (8). The NorA efflux system-susceptible quinolones norfloxacin and ciprofloxacin selected mutant strains of *S. pneumoniae* at a high frequency. However, the NorA efflux system-resistant quinolone sparfloxacin selected mutant strains of *S. pneumoniae* at a high frequency. We have also investigated the antibacterial activities of AM-1121 and AM-1147 against the *S. aureus norA* strain and its parent strain. The NorA efflux system susceptibilities of AM-1147 and AM-1121 were almost the same as that of ciprofloxacin (MIC ratios, 32 to 64). It is not clear, on the basis of these results, whether the ease of resistance selectivity in *S. pneumoniae* correlates with the susceptibilities of the agents to the NorA-type efflux system.

Target preference. We and other workers have already obtained first-step mutant strains of *S. pneumoniae* by selection with various fluoroquinolones (8, 11, 16, 19, 23, 24, 26, 28, 30). Trovafloxacin, levofloxacin, ciprofloxacin, and norfloxacin select *parC* mutant strains, whereas gatifloxacin, sparfloxacin, clinafloxacin, and moxifloxacin select *gyrA* mutant strains. These genetic studies suggest that the former and the latter groups of fluoroquinolones show preferences for TopoIV and DNA gyrase, respectively. On the other hand, studies with purified pneumococcal DNA gyrase and TopoIV have shown that TopoIV is more sensitive to sparfloxacin and clinafloxacin according to a simple comparison of the 50% inhibitory concentrations (IC_{50}s) (22, 27). The IC_{50}s of these quinolones for the target enzymes were determined with different in vitro assay systems, the conditions of which were also different from the conditions in bacterial cells. Therefore, target preference cannot necessarily be determined by simple comparison of the IC_{50}s in vitro. Further studies on the correlation between the IC_{50} ratio (IC_{50} for TopoIV/IC_{50} for DNA gyrase) and target preference in bacterial cells will be needed.

We therefore investigated the mutations of the QRDRs in the *gyrA* and *parC* genes of the first-step mutant strains selected with gatifloxacin and its related compounds. Gatifloxacin and ciprofloxacin naturally selected the *gyrA* and the *parC* mutant strains, respectively (Table 2). AM-1121 selected the *parC* mutant strains (Table 2). These results suggest that gatifloxacin prefers DNA gyrase and that AM-1121 and ciprofloxacin prefer TopoIV in the wild-type strain.

TABLE 2. Mutations in QRDRs of the *gyrA* and *parC* genes of representative mutant strains

Selecting quinolone	Strain	Mutation[a]	
		gyrA	*parC*
Gatifloxacin	GF9821	S81(TCC)→Y(TAC)	None
	GF9822	S81(TCC)→F(TTC)	None
AM-1121	219841	None	D83(GAT)→N(AAT)
	219842	None	S79(TCT)→Y(TAT)
Ciprofloxacin	CF9841	None	S79(TCT)→Y(TAT)
	CF9842	None	D83(GAT)→N(AAT)

[a] No mutant was obtained by selection with AM-1147 at the MIC or higher concentrations. The codon positions of *gyrA* and *parC* are according to the numbering of Balas et al. (3) and Pan and Fisher (25), respectively.

TABLE 3. Activities of gatifloxacin and its related compounds against target-altered mutant strains

Strain[a] (amino acid substitutions)	MIC (μg/ml)			
	Gatifloxacin	AM-1121	AM-1147	Ciprofloxacin
Wild-type strain IID553 (ParC, none; GyrA, none)	0.39	0.78	0.39	0.78
First-step mutant strains				
NF9884 (ParC, S79Y; GyrA, none)	0.39	3.13	0.39	3.13
LF9853 (ParC, D83N; GyrA, none)	0.39	3.13	0.39	3.13
SF9863 (ParC, none; GyrA, S81F)	0.78	1.56	0.78	1.56
GF9821 (ParC, none; GyrA, S81Y)	0.78	1.56	0.78	1.56
Second-step mutant strains				
NG9951 (ParC, S79Y; GyrA, S81F)	6.25	25	6.25	25
NG9952 (ParC, S79Y; GyrA, E85K)	6.25	25	12.5	25
ST9941 (ParC, S79F; GyrA, S81F)	6.25	25	6.25	25
SN9981 (ParC, D83Y; GyrA, S81F)	3.13	25	3.13	12.5

[a] NF9884, LF9853, SF9863, and the second-step mutant strains were obtained in previous experiments (8, 9).

No mutant strains were obtained by selection with the MIC or higher concentrations of AM-1147 (Table 1). However, the MIC of AM-1147 increased for the first-step *gyrA* mutant strains but not for the first-step *parC* mutant strains compared with that for the wild-type strain (Table 3). These results suggest that AM-1147 prefers DNA gyrase. In *S. aureus*, we reported that the 8-methoxy groups of gatifloxacin and AM-1147 contribute to enhancement of DNA gyrase inhibition rather than TopoIV inhibition (M. Takei, H. Fukuda, Y. Oomori, and M. Hosaka, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 758, p. 80, 2000). Moreover, Pestova et al. (28) suggest that the 8-methoxy quinolone moxifloxacin prefer DNA gyrase in *S. pneumoniae*. Therefore, the 8-methoxy groups of the fluoroquinolones might contribute to the target preference for DNA gyrase by enhancing DNA gyrase inhibition in the wild-type *S. pneumoniae* strain. Some quinolones with 8-halogen groups, such as clinafloxacin (8-chlorine) and sparfloxacin (8-fluorine), also prefer DNA gyrase (23, 26). Therefore, substituents other than the methoxy group at the C-8 position might be correlated with the target preference.

Antibacterial activity. We previously obtained second-step mutant strains from first-step *gyrA* and *parC* mutant strains with gatifloxacin, trovafloxacin, levofloxacin, ciprofloxacin, and sparfloxacin (9). All of the fluoroquinolones selected the *gyrA* and the *parC* mutant strains from the first-step *parC* and *gyrA* mutants, respectively. The results of studies of the second-step mutations showed that all of the second-step mutant strains possessed both *parC* and *gyrA* mutations (9).

To investigate the contribution of the 8-methoxy group to the antibacterial activity, we studied the activities of gatifloxacin and its related compounds against various types of the target-altered first- and second-step mutant strains as well as the wild-type strain.

Against the wild-type strain, the activities of the 8-methoxy quinolones gatifloxacin and AM-1147 were two-fold higher than those of their respective 8-H counterparts, AM-1121 and ciprofloxacin (Table 3). Moreover, the activities of these 8-methoxy quinolones were eight-and twofold higher against the first-step *parC* and *gyrA* mutant strains, respectively, and two- to eightfold higher against the second-step *gyrA* and *parC* mutant strains with double mutations (Table 3). These results indicate that the 8-methoxy group contributes to enhancement of antibacterial activity against target-altered mutant strains as well as the wild-type strain.

The results of studies of strains with second-step mutations suggest that the fluoroquinolones prefer DNA gyrase and TopoIV in the first-step *parC* and *gyrA* mutant strains of *S. pneumoniae*, respectively (9). On the basis of the assumed target preferences of the fluoroquinolones, 8-methoxy quinolones seem to prefer DNA gyrase in the wild-type strains and the *parC* mutant strains, and their inhibition of DNA gyrase might be greatly correlated with their activities against the wild-type and *parC* mutant strains, whereas 8-methoxy quinolones seem to prefer TopoIV in the *gyrA* mutant strains, and their inhibition of TopoIV might be greatly correlated with their activities against the *gyrA* mutant strains.

The MICs of the 8-H quinolones ciprofloxacin and AM-1121 increased not only for the first-step *parC* mutant strains but also for the *gyrA* mutant strains compared with those for the wild-type strain, although the increase in the MIC for the *gyrA* mutant strains was less than that for the *parC* mutant strains (Table 3). This slight crossover effect has previously been reported in *S. pneumoniae* (32) and *S. aureus* (13). These results suggest that the preferential target (TopoIV) inhibition of 8-H quinolones is correlated with the activity against the wild-type strain and also that the secondary target (DNA gyrase) inhibition might be slightly involved in the activity.

Concluding comments. It is hypothesized that the 8-methoxy group of gatifloxacin increases the level of target inhibition, especially against DNA gyrase, so that it is nearly the same as that for TopoIV inhibition in the bacterial cell, leading to potent antibacterial activity and a low level of resistance selectivity in *S. pneumoniae*, although further enzyme analysis will be necessary to validate this hypothesis.

Alovero et al. (2) suggested that some C-7 substituents of fluoroquinolones affect not only antibacterial activity but also target preference in *S. pneumoniae*. However, the piperazinyl 3′-methyl group of gatifloxacin at the C-7 position did not contribute to enhancement of the antibacterial activities or the target preferences of the compounds used in the present study. Further study of the contribution of C-8 and C-7 substituents to target preference, resistance selectivity, and antibacterial

activity will provide important information for the development of quinolones that possess potent antibacterial activity and low levels of resistance selectivity.

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