Contribution of the 8-Methoxy Group to the Activity of Gatifloxacin against Type II Topoisomerases of *Streptococcus pneumoniae*

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The inhibitory activities (50% inhibitory concentrations [IC_{50}s]) of gatifloxacin and other quinolones against both DNA gyrase and topoisomerase IV of the wild-type *Streptococcus pneumoniae* IID553 were determined. The IC_{50}s of 10 compounds ranged from 4.28 to 582 µg/ml against DNA gyrase and from 1.90 to 35.2 µg/ml against topoisomerase IV. The inhibitory activity against DNA gyrase was more varied than that against topoisomerase IV among fluoroquinolones. The IC_{50}s for DNA gyrase of the 8-methoxy quinolones gatifloxacin and AM-1147 were approximately seven times lower than those of their 8-H counterparts AM-1121 and ciprofloxacin, whereas the IC_{50}s for topoisomerase IV were 1.5 times lower. Moreover, the IC_{50} ratios (IC_{50} for DNA gyrase/ IC_{50} for topoisomerase IV) of gatifloxacin, AM-1147, and moxifloxacin, which possess 8-methoxy groups, were almost the same. The 8-methoxy quinolones showed higher antibacterial activity and less mutant selectivity against IID553 than their 8-H counterparts. These results suggest that the 8-methoxy group enhances both target inhibition, especially for DNA gyrase, leading to potent antipneumococcal activity and dual inhibition against both DNA gyrase and topoisomerase IV in the bacterial cell.

Gatifloxacin shows potent activity against various respiratory pathogens, including *Streptococcus pneumoniae* (5, 11, 22), and is known to be a respiratory quinolone. Gatifloxacin is similar to ciprofloxacin in its chemical structure, except for substitutions of the methoxy group at position 8 (8-methoxy group) and the 3′-methyl group of the piperazinyl ring at position 7 (3′-methyl group) of the quinolone ring. In addition, gatifloxacin shows more potent activity and lower resistance selectivity against *S. pneumoniae* than ciprofloxacin (5, 7). Based on the antibacterial activity of gatifloxacin and its structurally related compounds as well as genetic analysis of quinolone-resistant mutants, we have hypothesized that the 8-methoxy group of gatifloxacin potentiates target inhibition, especially against DNA gyrase, and that 8-methoxy quinolones, including gatifloxacin, inhibit DNA gyrase at nearly the same level as topoisomerase IV inhibition in *S. pneumoniae* bacterial cells, leading to potent antibacterial activity and low mutant selectivity (7). Some investigators also have reported that introduction of the 8-methoxy group of quinolone ensures strong antibacterial activity, bactericidal activity, and/or prevention of the emergence of mutant strains in *Escherichia coli* (14, 26), *Staphylococcus aureus* (12, 13, 25), *S. pneumoniae* (7), and mycobacteria (2, 19, 24).

In this study, we cloned and expressed the *gyrA*, *gyrB*, *parC*, and *parE* genes of *S. pneumoniae* in *E. coli* and purified GyrA, GyrB, ParC, and ParE subunits to reconstitute DNA gyrase and topoisomerase IV in vitro. The inhibitory activities of quinolones, including gatifloxacin (8-methoxy, 7-piperazinyl-3′-methyl) and its structurally related compounds (AM-1121 [8-H, 7-piperazinyl-3′-methyl], AM-1147 [8-methoxy, 7-piperazinyl-3′-H], and ciprofloxacin [8-H, 7-piperazinyl-3′-H]), against the purified DNA gyrase and topoisomerase IV of *S. pneumoniae* were determined.

MATERIALS AND METHODS

Fluoroquinolones. Gatifloxacin and the other quinolones, except ciprofloxacin, were synthesized at Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan). Ciprofloxacin was purchased from Apin Chemicals Co. (Abingdon, United Kingdom). AM-1121 (8-H, 7-piperazinyl-3′-methyl) and AM-1147 (8-methoxy, 7-piperazinyl-3′-H) are analogues of gatifloxacin (8-methoxy, 7-piperazinyl-3′-methyl) (Table 1).

Determination of MICs. 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. The MICs were determined by agar dilution methods with Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) containing 5% defibrinated horse blood. The MIC was defined as the lowest concentration of an antibacterial agent that inhibited visible growth after incubation for 20 h at 35°C.

Mutation frequencies. 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 containing 5% defibrinated horse blood with quinolones at one, two, four, and eight times the MICs. The selection plates were incubated at 35°C for 72 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).

Determination of MPCs. IID553 was incubated in Todd-Hewitt broth (Difco Laboratories) for 6 h. Cultures were centrifuged at $3,000 \times g$ for 15 min and resuspended in saline. Mutant strains were selected by plating the bacterial suspension (approximately 10^{10} CFU for each concentration of quinolones) on Mueller-Hinton agar containing 5% defibrinated horse blood with quinolones at 1, 2, 4, 8, and 16 times the MICs. The selection plates were incubated at 35°C for 72 h before being scored for the appearance of bacterial colonies. The mutant prevention concentration (MPC) was recorded as the lowest concentration of quinolone that allowed no growth of mutant strains.

Preparation of type II topoisomerases. The *gyrA*, *gyrB*, *parC*, and *parE* genes of *S. pneumoniae* were amplified from *S. pneumoniae* IID553 genomic DNA by PCR on a Hybaid thermal cycler with ELONGase Enzyme Mix (GIBCO BRL, Gaithersburg, Md.) and appropriate oligonucleotide primers (Table 2). The basic PCR parameters were 30 cycles of 95°C for 1 min, 55°C for 1 min, and 70°C for 2 min. The DNA fragments of *gyrA*, *parC*, and *parE* and pGEX-2T (Amersham Pharmacia Biotech) were digested with *Bam*HI, ligated, and transformed into *E. coli* DH5α (Toyobo, Tokyo, Japan). The DNA fragments of *gyrB* and

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TABLE 1. Chemical structures of gatifloxacin and its structurally related compounds ciprofloxacin, AM-1121, and AM-1147
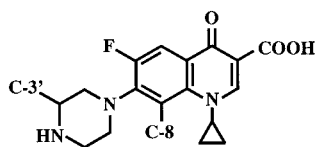
Quinolone	Substitution at:	
	C-8	C-3′
Gatifloxacin	OCH_3	CH_3
AM-1147	OCH_3	H
AM-1121	H	CH_3
Ciprofloxacin	H	H

pGEX-2T were digested with *Eco*RI and *Bam*HI, ligated, and transformed into *E. coli* DH5α. The nucleotide sequences of *gyrA*, *gyrB*, *parC*, and *parE* gene fragments that include regions corresponding to quinolone resistance-determining regions were identical to those of the wild-type strain of *S. pneumoniae* published previously in the National Center for Biotechnology Information database (NC 003098). The GyrA, GyrB, ParC, and ParE proteins of DNA gyrase and topoisomerase IV were purified separately as fusion proteins with glutathione *S*-transferase from overproducing strains of *E. coli*. Transformants were cultured in 2× YTG medium (tryptone, 16 g/liter; yeast extract, 10 g/liter; NaCl, 5 g/liter; glucose, 2% [pH 7.0]) containing 100 μg of ampicillin/ml at 37°C and induced at the mid-exponential phase by the addition of isopropyl-1-thio-β-D-(−)-galactopyranoside to a final concentration of 0.05 mM. After incubation for 16 to 17 h at 25°C, the cells were harvested and stored at −80°C until used. The cells were suspended in sonication buffer (50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol [DTT]) and sonicated on ice. Triton X-100 was added to a final concentration of 1%, and the suspension was centrifuged at 30,000 × *g* for 30 min The supernatant was loaded onto a glutathione-Sepharose 4B column previously equilibrated with sonication buffer containing 0.5% Triton X-100. The column was washed with 30 volumes of sonication buffer (with 0.5% Triton X-100) and 10 volumes of phosphate-buffered saline. The proteins were eluted with 1 volume of thrombin solution (80 U of thrombin/ml in phosphate-buffered saline). Concentration and buffer exchange were carried out with a Centriprep-10 (Millipore, Beverly, Mass.) with 50 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM EDTA, and 1 mM DTT, and the proteins were stored at −80°C.

Topoisomerase reactions. Both *S. pneumoniae* DNA gyrase and topoisomerase IV were reconstituted by incubation of each A and B subunit of the enzymes (GyrA-GyrB or ParC-ParE) on ice for at least 2 h. The topoisomerase activity was measured electrophoretically.

(i) Supercoiling activity of DNA gyrase. Reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 30 mM KCl, 4 mM ATP, 5 mM DTT, 2 mM spermidine HCl, 200 mM potassium glutamate, 50 μg of bovine serum albumin/ml, 40 μg of tRNA/ml, 1 U of reconstituted DNA gyrase, 50 ng of relaxed pBR322 DNA, and various concentrations of the quinolones tested were incubated at 37°C for 1 h. The maximum concentrations of quinolones in the reaction mixtures were as follows: AM-1121, ciprofloxacin, sparfloxacin, and norfloxacin, 1,000 μg/ml; levofloxacin, 600 μg/ml; gatifloxacin, AM-1147, moxifloxacin, and trovafloxacin, 200 μg/ml; and clinafloxacin, 50 μg/ml. Each quinolone was added to the reaction mixture after serial dilutions from maximum concentrations. The reaction was terminated by the addition of 1.4% sodium dodecyl sulfate and 90 μg of proteinase K/ml. After an additional 10 min of incubation at 37°C, one-fifth volume of a loading dye was added.

One unit of activity was defined as the amount of enzyme required to fully show supercoiling in the reaction under the above-mentioned conditions.

(ii) Decatenating activity of topoisomerase IV. The decatenating activity of topoisomerase IV, that is, the conversion of kinetoplast DNA (Topogene Inc., Columbus, Ohio) to the monomer, was measured as follows. Reaction mixtures (10 μl) containing 40 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 30 mM KCl, 2 mM ATP, 5 mM DTT, 2 mM spermidine HCl, 100 mM potassium glutamate, 50 μg of bovine serum albumin/ml, 1 U of reconstituted topoisomerase IV, 150 ng of kinetoplast DNA, and various concentrations of the quinolones tested were incubated at 37°C for 1 h. The maximum concentrations of quinolones in the reaction mixtures were as follows: Sparfloxacin and norfloxacin, 100 μg/ml;

gatifloxacin, AM-1147, AM-1121, ciprofloxacin, and levofloxacin, 50 μg/ml; and moxifloxacin, trovafloxacin, and clinafloxacin, 25 μg/ml. Each quinolone was added to the reaction mixture after serial dilutions from maximum concentrations. The reaction was terminated by the addition of 1.4% sodium dodecyl sulfate and 90 μg of proteinase K/ml. After an additional 10 min of incubation at 37°C, one-fifth volume of a loading dye was added.

One unit of activity was defined as the amount of enzyme required to fully show decatenation in the reaction under the above-mentioned conditions.

(iii) Inhibitory effects of quinolones. The reaction mixtures were electrophoresed on a 1% agarose gel. Quantification of DNA in agarose gels was carried out after ethidium bromide staining. The brightness of the bands corresponding to supercoiled pBR322 DNA and decatenated kinetoplast DNA were determined by densitometric analysis with a FMBIO II Multi View fluorescent image analyzer (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). The inhibitory effect of each quinolone against topoisomerases was assayed by determining the concentrations required to inhibit 50% of the enzyme reaction (IC_{50}s).

RESULTS

Inhibitory activities of fluoroquinolones against type II topoisomerases of *S. pneumoniae*. The IC_{50}s of 10 quinolones, including AM-1121 and AM-1147, against DNA gyrase and topoisomerase IV of *S. pneumoniae* are shown in Table 3. The IC_{50}s for DNA gyrase were more varied than those for topoisomerase IV among the quinolones. The IC_{50}s of the 10 compounds ranged from 4.28 to 582 μg/ml against DNA gyrase and from 1.90 to 35.2 μg/ml against topoisomerase IV. Also, the IC_{50} ratios (ratios of IC_{50}s for DNA gyrase to those for topoisomerase IV) of the 10 quinolones ranged from 2.25 to 20.1. This variation was caused by the variations in IC_{50}s for both type II topoisomerases, especially those for DNA gyrase.

Gatifloxacin, moxifloxacin, and AM-1147, which possess an 8-methoxy group, have comparable inhibitory activities against type II topoisomerases, with IC_{50}s of 19.4 to 25.9 μg/ml for DNA gyrase and 4.05 to 7.27 μg/ml for topoisomerase IV. Moreover, the IC_{50}s of the 8-methoxy quinolones gatifloxacin and AM-1147 against DNA gyrase are approximately 7 times lower than those of their 8-H counterparts AM-1121 and ciprofloxacin, whereas the IC_{50}s of these 8-methoxy quinolones against topoisomerase IV are approximately 1.5 times lower. These results indicate that the 8-methoxy quinolones show

TABLE 2. DNA primers used for PCR amplification of DNA gyrase and topoisomerase IV of *S. pneumoniae*

Primer	Sequence[a]
gyrA	
Forward	5′-GGCAT<u>GGATCC</u>ATGCAGGATAAAAATTTAG-3′
Reverse	5′-GTAATATCA<u>GGATCC</u>CTGCTAGGATATTTGTC-3′
gyrB	
Forward	5′-GAAAAAGG<u>GGATCC</u>ATGACAGAAGAAATCAAAAATCTGC-3′
Reverse	5′-CTTGAA<u>GAATTC</u>GTAACGATTATATCATATTTTTTAGACC-3′
parC	
Forward	5′-GCTTTG<u>GGATCC</u>ATGTCTAACATTCAAAACATGTCCCTGG-3′
Reverse	5′-CTTC<u>GGATCC</u>CTTATTTATCTTCAGTAACTACTTCC-3′,
parE	
Forward	5′-GAATAGG<u>GGATCC</u>CTTGTGTCAAAAAAGGAAATC-3′
Reverse	5′-CACATAGG<u>GGATCC</u>ACATCCGACTCTAATTTCCAG-3′

[a] Restriction sites are underlined.

TABLE 3. Inhibitory activities for type II topoisomerases and antibacterial activities of gatifloxacin and the other quinolones

Quinolone	MIC (μg/ml) against IID553	IC$_{50}$ (μg/ml) against:		IC$_{50}$ ratio[a]	Target preference
		DNA gyrase	Topoisomerase IV		
Gatifloxacin	0.25	25.9	7.27	3.56	DNA gyrase
AM-1147	0.25	19.4	4.05	4.79	DNA gyrase
AM-1121	0.5	181	11.7	15.5	Topoisomerase IV
Ciprofloxacin	0.5	138	6.85	20.1	Topoisomerase IV
Clinafloxacin	0.063	4.28	1.90	2.25	DNA gyrase
Moxifloxacin	0.25	21.8	6.04	3.61	DNA gyrase
Sparfloxacin	0.25	145	8.28	17.5	DNA gyrase
Trovafloxacin	0.125	17.7	3.07	5.77	Topoisomerase IV
Levofloxacin	1	89.3	15.9	5.62	Topoisomerase IV
Norfloxacin	4	582	35.2	16.5	Topoisomerase IV

[a] IC$_{50}$ ratio, IC$_{50}$ against DNA gyrase/IC$_{50}$ against topoisomerase IV.

higher inhibitory activity against both target enzymes than their 8-H counterparts.

Resistance selection. The selectivities of mutant strains of fluoroquinolones were evaluated (Table 4). Sparfloxacin selected mutant strains at 16 times the MIC, and trovafloxacin and norfloxacin were selected at 4 times the MICs. AM-1121, ciprofloxacin, and levofloxacin also selected at two times the MICs. In contrast, gatifloxacin, AM-1147, clinafloxacin, and moxifloxacin selected no mutant strains at the MIC.

MPC. The MPCs of 10 quinolones were also determined (Table 4). The MPC is a novel concept for evaluation of the mutant selectivities of drugs. The MPCs of gatifloxacin, AM-1147, clinafloxacin, and moxifloxacin were same or lower than those of the other quinolones tested. The MPCs of gatifloxacin and AM-1147 were 1 μg/ml, which were lower than those of their 8-H counterparts, AM-1121 and ciprofloxacin.

DISCUSSION

On the basis of genetic analysis, it has been reported that the target preference in *S. pneumoniae* differs among the quinolones. Trovafloxacin, ciprofloxacin, AM-1121, levofloxacin, and norfloxacin prefer topoisomerase IV, whereas clinafloxacin, AM-1147, moxifloxacin, sparfloxacin, and gatifloxacin prefer DNA gyrase (5, 7, 15, 18). We have previously reported that

the target preferences of the quinolones in *S. aureus* can be anticipated based on determinations of the IC$_{50}$ ratios (21). Our data also generally show that the IC$_{50}$ ratios (IC$_{50}$ for DNA gyrase/IC$_{50}$ for topoisomerase IV) of quinolones preferring DNA gyrase are lower than those of quinolones preferring topoisomerase IV (Table 3). These results suggest that the IC$_{50}$ ratios are related to the target preference in *S. pneumoniae*, as they are in *S. aureus*.

It has been proposed that the MICs of quinolones are determined by the inhibitory activity against the preferential target (6, 9, 23). Gatifloxacin and AM-1147 showed twice the antibacterial activity of their 8-H counterparts, AM-1121 and ciprofloxacin, against wild-type strain IID553. It has been reported that the primary target of the 8-methoxy quinolones (AM-1147 and gatifloxacin) was DNA gyrase, although that of their 8-H counterparts (ciprofloxacin and AM-1121) was topoisomerase IV (7). Also, the IC$_{50}$s of the 8-methoxy quinolones gatifloxacin and AM-1147 against DNA gyrase are approximately 7 times lower than those of their 8-H counterparts AM-1121 and ciprofloxacin, whereas the IC$_{50}$s of these 8-methoxy quinolones against topoisomerase IV are approximately 1.5 times lower (Table 3). Furthermore, the MICs of the 8-methoxy quinolones for *gyrA* mutants were higher than those for the wild-type strain, although the MICs for *parC* mutants were almost the same as those for wild-type strain.

TABLE 4. Frequencies of appearance of mutant strains by selection with various quinolones

Quinolone	Frequency at the following multiple of the MIC[a]:						MPC (μg/ml)
	1×	2×	4×	8×	16×	32×	
Gatifloxacin	$<5.9 \times 10^{-10}$	$<5.9 \times 10^{-10}$	$<5.9 \times 10^{-10}$	$<5.9 \times 10^{-10}$	ND[c]	ND	1
AM-1147[b]	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	ND	1
AM-1121[b]	$>2.4 \times 10^{-6}$	$>2.4 \times 10^{-6}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	ND	8
Ciprofloxacin[b]	$>2.4 \times 10^{-6}$	$>2.4 \times 10^{-6}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	ND	8
Clinafloxacin	$<5.9 \times 10^{-10}$	$<5.9 \times 10^{-10}$	$<5.9 \times 10^{-10}$	$<5.9 \times 10^{-10}$	ND	ND	0.25
Moxifloxacin	$<5.9 \times 10^{-10}$	$<5.9 \times 10^{-10}$	$<5.9 \times 10^{-10}$	$<5.9 \times 10^{-10}$	ND	ND	1
Sparfloxacin[b]	ND	$>2.2 \times 10^{-6}$	$>2.2 \times 10^{-6}$	$>2.2 \times 10^{-6}$	1.1×10^{-7}	$<1.1 \times 10^{-9}$	4
Trovafloxacin[b]	ND	$>2.2 \times 10^{-6}$	6.2×10^{-8}	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	1
Levofloxacin[b]	ND	1.5×10^{-7}	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	4
Norfloxacin[b]	ND	2.6×10^{-7}	2.2×10^{-9}	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	$<1.1 \times 10^{-9}$	64

[a] The reproducibility of the frequencies has been confirmed in repeated experiments.
[b] Frequencies are from previous studies (5, 7).
[c] ND, not determined.

Hence, increases of the MICs of the 8-H counterparts for *parC* mutants were higher than those for *gyrA* mutants (7). These results suggest that the increase in the inhibitory activities of the 8-methoxy quinolones against DNA gyrase is one of the possible mechanisms underlying the potent antipneumococcal activity of these quinolones.

In this study, the IC_{50}s of sparfloxacin for both enzymes were similar to those of ciprofloxacin, although sparfloxacin had more potent antibacterial activity than ciprofloxacin against IID553. Furthermore, the IC_{50} ratio of sparfloxacin was 17.5, which was similar to those of norfloxacin and ciprofloxacin. However, sparfloxacin seems to prefer DNA gyrase, as this agent selected *gyrA* mutant strains with high frequency and showed no decrease in antibacterial activity against *parC* mutant strains compared with the wild-type strain (5). There are some discrepancies between inhibitory activity against target enzymes and either antipneumococcal activity or target preference. Other investigators have already observed these discrepancies in sparfloxacin (10, 16, 17); however, possible causes of these discrepancies remain unclear.

Gatifloxacin, AM-1147, moxifloxacin, and clinafloxacin showed less mutant selectivity than the other quinolones (Table 4). It has been reported that some fluoroquinolones selected the mutant strains of *S. pneumoniae* and *S. aureus* with low frequency, as these agents seem to inhibit both DNA gyrase and topoisomerase IV at nearly the same level in bacterial cells (dual-targeting property) (5, 7, 8, 15). As such, these four quinolones seem to possess the dual-targeting property, even though the target preference of these quinolones seems to be DNA gyrase (5, 15, 18). Furthermore, gatifloxacin and AM-1147 selected no mutant strains at the MIC or higher concentrations, whereas their 8-H counterparts, AM-1121 and ciprofloxacin, selected the resistant mutants at two times the MIC. These results suggest that the 8-methoxy group of quinolones contributes to enhancement of the inhibition of DNA gyrase, leading to the dual-targeting property of the agents in bacterial cells.

Recently, in order to evaluate drug activity, the novel concept designated MPC has been proposed. The MPC has been defined as the minimal drug concentration that allows no mutant recovery when approximately 10^{10} cells are applied to drug-containing agar (3). The MPCs of gatifloxacin, AM-1147, clinafloxacin, and moxifloxacin were the same or lower than those of the other quinolones tested (Table 4). These results suggest that gatifloxacin, AM-1147, clinafloxacin, and moxifloxacin are expected to restrict the selection of resistant mutants in a clinical setting. Dong et al. reported that the 8-methoxy quinolones possessed lower MPCs against mycobacteria (3, 4) and *S. aureus* (3). Blondeau et al. (1) determined the MPCs against clinical isolates of *S. pneumoniae* and also suggested that gatifloxacin and moxifloxacin restricted the selection of resistant mutants. Our results for the MPCs agree with those studies and suggest that both the dual-targeting property and potent antipneumococcal activity of 8-methoxy quinolones contribute to low MPCs.

The IC_{50} ratios of gatifloxacin, AM-1147, clinafloxacin, and moxifloxacin were found to be 3.56, 4.79, 2.25, and 3.61, respectively. We have previously hypothesized that the IC_{50} ratio is important for predicting target preference, including the dual-targeting property, in bacterial cells (21). Similar findings

were observed in whole-cell assays with gyrase mutant and topoisomerase IV mutant of *S. aureus*. Roychoudhury et al. (20) have shown that the cell growth inhibition of quinolones against gyrase mutant was correlated to the IC_{50} for topoisomerase IV of the wild-type strain and have suggested that the dual-targeting property could be anticipated by the ratio of the cell growth inhibition against each mutant. In the present study, gatifloxacin, AM-1147, clinafloxacin, and moxifloxacin showed almost the same IC_{50} ratios, suggesting that this range of IC_{50} ratios might represent the dual-targeting property of quinolones in *S. pneumoniae* bacterial cells.

In these enzyme studies, we have clarified that the 8-methoxy group of gatifloxacin and AM-1147 contributes to an enhancement of both target inhibition, especially for DNA gyrase, leading to potent antipneumococcal activity and the dual-targeting property of 8-methoxy quinolones in *S. pneumoniae* bacterial cells. Further studies on the contributions of the other substituents at position 8 of the quinolone ring to target inhibition will give us useful information for the discovery of favorable quinolones against *S. pneumoniae*.

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