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Hydrophilicity of Quinolones Is Not an Exclusive Factor for Decreased Activity in Efflux-Mediated Resistant Mutants of *Staphylococcus aureus*

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The elevated expression of the *norA* gene is responsible for efflux-mediated resistance to quinolones in *Staphylococcus aureus* (E. Y. W. Ng, M. Trucksis, and D. C. Hooper, Antimicrob. Agents Chemother. 38:1345–1355, 1994). For *S. aureus* transformed with a plasmid containing the cloned *norA* gene, SA113(pTUS20) (H. Yoshida, M. Bogaki, S. Nakamura, K. Ubukata, and M. Konno, J. Bacteriol. 172:6942–6949, 1990), and an overexpressed mutant, SA-1199B (G. W. Kaatz, S. M. Seo, and C. A. Ruble, J. Infect. Dis. 163:1080–1086, 1991), the MICs of norfloxacin increased 16 and 64 times compared with its MICs for the recipient and wild-type strains, SA113 and SA-1199, respectively. MICs of CS-940, however, increased only two and eight times, even though these two fluoroquinolones are similarly hydrophilic (apparent log*P*s of approximately −1). No good correlation was found, among 15 developed and developing quinolones, between the increment ratio in MICs and hydrophobicity ($r = 0.61$). Analysis of the quantitative structure-activity relationship among 40 fluoroquinolones revealed that the MIC increment ratio was significantly correlated with the bulkiness of the C-7 substituent and bulkiness and hydrophobicity of the C-8 substituent of fluoroquinolones ($r = 0.87$) and not with its molecular hydrophobicity ($r = 0.47$). Cellular accumulation of norfloxacin in SA-1199B was significantly lower than that in SA-1199, and it was increased by addition of carbonyl cyanide *m*-chlorophenyl hydrazone. On the other hand, accumulations of CS-940 in these strains were nearly identical, and they were not affected by addition of the protonophore.

Fluoroquinolone antimicrobial agents are potent against gram-positive and -negative bacteria, *Mycoplasma* species, and *Chlamydia* species, and they are widely used clinically (15). Their primary target is considered to be DNA gyrase, and recently topoisomerase IV has also been considered to be involved as a target and may be the dominant target in *Staphylococcus aureus* (10). Both enzymes are essential type II topoisomerases, and they alter the linking number of a double-stranded DNA molecule in steps of two. They are holoenzymes, consisting of two A subunits and two B subunits. The *gyrA* and *gyrB* genes encode A and B subunits for DNA gyrase, and the *grlA* (*parC*) and *grlB* (*parE*) genes encode those for topoisomerase IV, respectively (1, 14, 22, 29, 38).

As fluoroquinolones have been used increasingly, some bacteria, especially methicillin-resistant *S. aureus* and *Pseudomonas aeruginosa*, have developed resistance to them (3, 31, 32). In *S. aureus*, four quinolone resistance mechanisms have been reported: (i) point mutation in the *gyrA* and *gyrB* genes is related to quinolone resistance (9, 12, 16); (ii) a membrane protein, NorA, contributes to active efflux-mediated resistance; (iii) *cfxB-ofxC* (*flqA*) confers quinolone resistance in *S. aureus*, although its mechanism is still unknown (35); and (iv) point mutations in the *grlA* gene are also responsible for quinolone resistance (10).

The *norA* gene was first cloned from a quinolone-resistant clinical isolate (36). Many authors (20, 25, 27, 39) insist that this gene confers higher-level resistance to hydrophilic quinolones, such as norfloxacin and enoxacin, than to hydrophobic quinolones. This resistance is caused by an energy-dependent, carrier-mediated efflux of drugs from cells, and cellular accumulation of norfloxacin was found to be increased by treatment with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a protonophore. Kaatz et al. (20) cloned and determined the nucleotide sequence of the *norA1199* gene, which is considered to be nearly identical to the *norA* gene, with 91% homology (this gene is designated *norA* hereafter in this paper). This gene's product is predicted to contain 12 membrane-spanning domains and to show significant homology with other transport proteins (e.g., Bmr and Tet species). Thus, NorA is classified as a member of the major facilitator superfamily among transport proteins (21, 30). Although a point mutation in the *norA* gene was first considered to be responsible for the expression of resistance to quinolones (27), some authors later revealed an increased transcriptional level of the *norA* gene (20, 25).

In a previous study (34), we found that approximately 12% of strains without the GyrA mutation were resistant to norfloxacin and enoxacin but not to CS-940 and AM-1155, even though the four fluoroquinolones show similar hydrophilicities. We considered the possibility that efflux-mediated resistance is responsible for at least part of the resistance in strains with no GyrA mutation. We investigated the relationship between hydrophobicity of quinolones and the increase in their MICs, mainly for a *norA* mutant, compared with those for the parent strain. We found that the decrease in activity was significantly correlated with other factors, the bulkiness of the C-7 substituent and bulkiness and hydrophobicity of the C-8 substituent of quinolones, and not with molecular hydrophobicity.

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TABLE 1. Log*P*s of 15 quinolone antimicrobial agents and their activities for *S. aureus* SA-1199, SA-1199B, SA113, and SA113(pTUS20) and *E. coli* HB101 and HB101(pTUS829)

Compound	MIC (μg/ml)[a] for:						$\log P_{app}$				cLog*P*
	SA-1199	SA-1199B	SA113	SA113 (pTUS20)	HB101	HB101 (pTUS829)	Observed[b]	In reference[c]:			
								7	13	39	
Piromidic acid	3.13	12.5	3.13	12.5	6.25	6.25	0.93		1.07		1.14
Oxolinic acid	0.78	1.56	1.56	3.13	0.20	0.20	0.61	−0.13	0.35	−0.07	0.97
Nalidixic acid	25	100	25	50	1.56	1.56	0.43	−0.27	0.59	0.53	0.91
Sparfloxacin	0.10	0.20	0.05	0.20	0.0015	0.0015	0.01			−0.04	1.54
Tosufloxacin	0.10	0.78	0.05	0.39	0.006	0.012	−0.33				2.30
Ofloxacin	0.20	1.56	0.20	3.13	0.025	0.05	−0.41	−0.71	−0.48	−0.96	2.17
Fleroxacin	0.39	3.13	0.39	3.13	0.05	0.10	−0.61	−0.85	−1.10		2.37
AM-1155	0.10	0.39	0.10	0.78	0.0015	0.012	−0.85				1.93
CS-940	0.10	0.20	0.05	0.39	0.0015	0.0015	−0.90				2.23
Ciprofloxacin	0.39	6.25	0.39	6.25	0.003	0.10	−1.09	−0.82	−1.70	−1.22	1.46
Norfloxacin	0.78	50	0.78	25	0.05	0.78	−1.14	−0.79	−2.00	−1.15	1.64
Enoxacin	1.56	25	0.78	25	0.10	0.39	−1.24		−2.15	−1.22	0.77
Lomefloxacin	0.78	6.25	0.78	12.5	0.10	0.20	−1.28				2.31
Cinoxacin	100	400	100	200	1.56	1.56	−1.88		−1.52		0.26
Pipemidic acid	25	1,600	25	800	0.78	12.5	−1.96	−0.82	−1.52		−0.18

[a] Each value is the median of more than three independent determinations.
[b] *n*-Octanol–Britton Robinson buffer (pH 7.0).
[c] *n*-Octanol–0.1 M phosphate buffer (pH 7.2).

MATERIALS AND METHODS

Bacterial strains and drugs. *S. aureus* SA-1199 (wild type) and SA-1199B (*norA*) were kindly provided by G. W. Kaatz, Wayne State University (19). *S. aureus* SA113, *Escherichia coli* HB101, and the recipient strains transformed with plasmids containing the cloned *norA* gene, SA113(pTUS20) and HB101(pTUS829) (20), were kindly provided by H. Yoshida, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. A standard strain, *S. aureus* FDA 209P JC, was also used. Nalidixic acid, piromidic acid, pipemidic acid, oxolinic acid, cinoxacin, and CCCP were obtained commercially from Sigma Chemical Co. (St. Louis, Mo.). All fluoroquinolones used were synthesized by Ube Industries, Ltd., Ube, Japan.

Susceptibility test. MICs were determined by the agar dilution method (17) in Mueller-Hinton II agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.), using approximately 5×10^4 CFU. The MICs were read after 20 h of incubation at 37°C. Quinolones listed in Table 1 were first dissolved in only enough 1 N NaOH (less than 1/10 volume) to make 10× solutions in water. When 1 volume of drug dilution series and 9 volumes of the medium were mixed, the pH of each mixture at 45°C was neutral, ranging from 6.8 to 7.3.

Partition coefficient of quinolones. Log*P*s in *n*-octanol–Britton Robinson buffer (pH 7.0) were determined according to Organization for Economic Cooperation and Development guidelines (28). Quinolones were dissolved in 44 or 50 ml of the mixture (1:1 or 1:10 in volume) to prepare 50 μg/ml. The solution was shaken at room temperature for 5 min and then at 25°C for 30 min; then it was separated into a water phase and an organic phase by centrifugation. Portions of each phase were subjected to reversed-phase high-pressure liquid chromatography on an Inertosil ODS-2 column (15 cm by 4.6-mm inside diameter; GL Sciences, Ltd., Tokyo, Japan) in an acetonitrile–methanol–10 mM phosphate buffer (pH 3.0) solvent system. Determination of quinolone concentrations was carried out in quadruplicate, and the apparent log*P* (ratio of saturated concentration in the organic phase to that in water phase in logarithm [$\log P_{app}$]) was calculated from the average concentrations.

Concentrations of the four microspecies in solution. Fluoroquinolone molecules contain two proton-binding sites, and they exist in four microscopic protonation forms in solution. Microconstants and macroconstants were determined by nuclear magnetic resonance (NMR) spectroscopy. Each sample was dissolved in 500 μl of D_2O, and the pH was adjusted with NaOD or DCl. The ^1H NMR spectra were obtained at various pH values at 30°C on a JEOL GX-270 NMR spectrometer. The fraction of protonation of each functional moiety at a given pH was calculated from the chemical shift values of the proton near the functional group, 2H for the carboxyl group, 2′H and 3′H of the 7-piperazine ring for the amino group of norfloxacin, and 7′-methyl for the amino group of CS-940. Microconstants and macroconstants were then obtained from these data by the nonlinear least-squares method as described by Takács-Novák et al. (33), with minor modifications. Concentrations of the four microspecies at an arbitrary pH were calculated by using microconstants according to the definition equations.

Accumulation of fluoroquinolones in bacterial cells. Accumulation of norfloxacin and CS-940 was determined by the modified method of Chapman and Georgopapadakou (7) and Mortimer and Piddock (24). Cells were grown in antibiotic medium 3 (Penassay broth; Difco Laboratories, Detroit, Mich.) to an optical density at 660 nm of 1 and were harvested by centrifugation at 4,000 × *g* at 4°C for 15 min. The cells were washed once with 50 mM potassium phosphate buffer (pH 7.0) and were resuspended in the same buffer at 40 mg (wet cell weight)/ml. After preincubation of the suspension at 37°C for 10 min, fluoroquinolone was added to the suspension to achieve a final concentration of 10 μg/ml, and the suspension was further incubated at 37°C with shaking. CCCP, at a final concentration of 2 μg/ml, was added to the suspension 10 min after the drug addition. Four 0.5-ml aliquots were removed at fixed times ranging from 15 s to 28 min after the addition of fluoroquinolone. One of the four samples was used to determine the optical density at 660 nm. For the remaining three samples, the cells were immediately harvested, washed once with the ice-cold phosphate buffer, suspended in 1 ml of 0.1 M glycine-HCl (pH 3.0), and kept at room temperature overnight to extract fluoroquinolones. The suspension was centrifuged, and the supernatant was diluted appropriately with 0.1 M glycine-HCl. The amount of cell-associated fluoroquinolone was determined on a Hitachi model 650-60 fluorescence spectrophotometer. The excitation and emission wavelengths used were, respectively, 277 and 443 nm for norfloxacin and 304 and 451 nm for CS-940. Samples without fluoroquinolones were processed in the same manner as backgrounds. It took approximately 2 min from sampling to resuspension in drug-free phosphate buffer. The cell dry weight-versus-log of the optical density at 660 nm standard curve was constructed and used for adjustment.

Structure-activity relationship. Quantitative structure-activity relationship analysis was carried out using the programs QSAR+ (Molecular Simulations, Inc., San Diego, Calif.) and HANSCH3X (Pomona College, Pomona, Calif.). Log*P* was predicted with the program cLogP (Daylight Chemical Information Systems, Inc., Irvine, Calif.). This program calculates log*P*, assuming that a molecule is in the nonprotonated, neutral form.

PCR and direct sequencing. Three pairs of 20- or 21-mer primers were synthesized to amplify the regions encompassing the quinolone resistance-determining region of the respective *gyrA*, *gyrB*, and *grlA* genes, according to the sequences reported previously (5, 10, 16). They were 5′-AATGAACAAGGTATGACACC and 5′-TACGCGCTTCAGTATAACGC to amplify codons 54 through 127 of the *gyrA* gene, 5′-GTAACACGTCGTAAATCAGCG and 5′-CATCCACATCG GCATCAGTC to amplify codons 402 through 513 of the *gyrB* gene, and 5′-A GTCGGTGATGTTATTGGTC and 5′-AGCTTCAGTGTAACGCATTG to amplify codons 67 through 122 of the *grlA* gene. PCR and direct sequencing were carried out as previously described (34).

RESULTS

Hydrophobicity of quinolones, their activities, and structure-activity relationship. Table 1 shows $\log P_{app}$s of 15 quinolone antimicrobial agents and their activities for three pairs of wild-type and derivative resistant strains tested. Among them, the relationship for the pair SA-1199 and SA-1199B was selected and shown in Fig. 1. $\log P_{app}$s obtained in this study were in good agreement with those of other investigators (7, 13, 39). A higher $\log P_{app}$ indicates a more hydrophobic com-

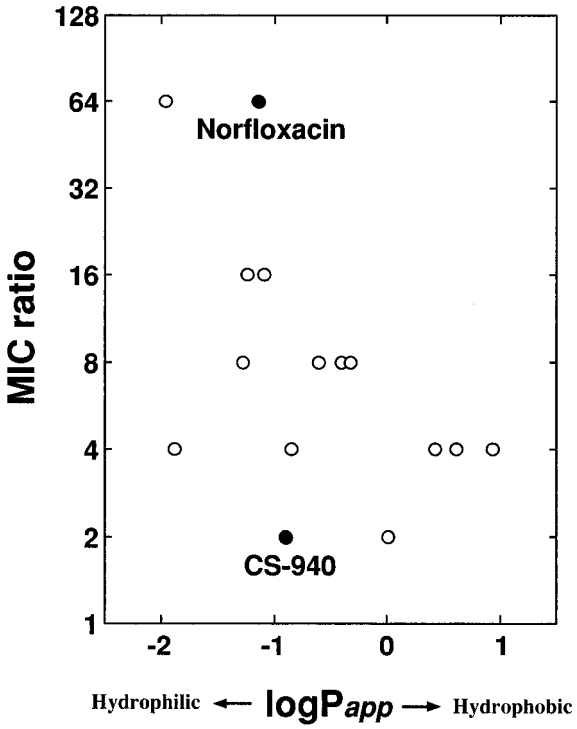


FIG. 1. Relationship between the hydrophobicity of a quinolone and an increase in its MIC by the *norA* mutation. The hydrophobicity is expressed as the logP_{app}. The MIC ratio is a quotient of the MIC of a given quinolone for SA-1199B divided by its MIC for SA-1199.

pound. MICs of all quinolones for SA-1199B (*norA*) were higher than MICs for SA-1199 (wild type), and the magnitude of increment varied among quinolones. The magnitude of the MIC increase was only 2 times for CS-940 as well as for sparfloxacin and oxolinic acid; it was 16 times for enoxacin and ciprofloxacin and as much as 64 times for norfloxacin and pipemidic acid. The following correlation equation was obtained between the increment ratio (R) of the MIC for SA-1199B to the MIC for SA-1199 and a hydrophobic parameter, logP_{app}: $-\log_2 R = 1.02\ (\pm 0.80) \times \log P_{app} - 2.27\ (\pm 0.87)$ ($r = 0.61$; SD = 1.32), where r is a correlation coefficient, SD is the standard deviation, and the 95% confidence limits are shown in parentheses. The two parameters were not well correlated. Similar tendencies were observed between SA113 and SA113(pTUS20) and between HB101 and HB101(pTUS829). The magnitude of the MIC increment for *E. coli* was smaller than that for *S. aureus*. Since the extent of the increment among quinolones was largest for the pair SA-1199 and SA-1199B, this pair was used in subsequent experiments. It is known that the calculated log$P$, cLog$P$, deviates significantly from the logP_{app} according to the number of ionizing groups, such as amines and carboxylic acids, in the molecules. When the compounds that do not have an alkylamine group in the C-7 substituent (piromidic acid, oxolinic acid, and nalidixic acid) and one that has an extra amine at the C-5 position (sparfloxacin) were excluded, cLog$P$ was reasonably correlated with logP_{app} as shown in the following equation: $\log P_{app} = 0.51\ (\pm 0.23) \times \text{cLog}P - 1.86\ (\pm 0.40)$ ($r = 0.86$; SD = 0.28). Therefore, cLog$P$ was used in the further analysis.

Table 2 shows chemical structures of 40 amphoteric fluoroquinolones containing the quinoline nucleus, their MICs for SA-1199 and SA-1199B, and seven chemical parameters predicted by a computer. As described in the previous section, the correlation equation between the MIC increment ratio of these compounds and cLog$P$ was found: $-\log_2 R = 2.83\ (\pm 1.99) \times \text{cLog}P - 0.45\ (\pm 0.37) \times \text{cLog}P^2 - 6.19\ (\pm 2.52)$ ($r = 0.47$; SD = 1.32; optimum cLog$P$ = 3.17 [2.64 to 6.19]). This formula would be interpreted as showing that the correlation coefficient was not significant and the SD was relatively large. Taking every chemical parameter as an independent variable, a multiplex correlation equation was found (among the parameters used, MR_1, MR_7, and MR_8 are molar refractivities for the N-1, C-7, and C-8 substituents, respectively, and they indicate the bulkiness of each substituent; L_1, a sterimol parameter, indicates the length for the N-1 substituent; π_7 and π_8 are the hydrophobic substituent constants for the C-7 and C-8 substituents, respectively): $-\log_2 R = 5.09\ (\pm 2.56) \times MR_7 - 0.41\ (\pm 0.25) \times MR_7^2 + 2.11\ (\pm 0.84) \times MR_8 + 3.77\ (\pm 1.66) \times \pi_8 - 19.78\ (\pm 6.23)$ ($r = 0.87$; SD = 0.77; optimum MR_7 = 6.14 [5.66 to 7.90]). This quadrinomial equation indicated a reasonable correlation between R; MR_7, MR_8, and π_8, and a smaller SD. From signs of the first, third, and fourth terms, it was indicated that the compound containing the bulkier substituents at the C-7 and C-8 positions and more hydrophobic substituent at the C-8 position showed a smaller MIC increment ratio.

There was no relationship between the molecular hydrophobicity, cLog$P$, and substituent hydrophobicity at the C-8 position, π_8.

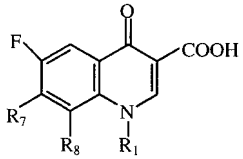
Microconstants and concentration of microspecies. From pH titration using ^{1}H NMR, microconstants between zwitterionic and negatively charged microspecies and between uncharged and negative microspecies were, respectively, 7.44 ± 0.31 and 8.51 ± 0.05 for norfloxacin and 7.93 ± 0.21 and 9.04 ± 0.01 for CS-940. Macroconstants were then calculated from these values; they were 8.57 and 6.35 for norfloxacin and 9.09 and 6.04 for CS-940, respectively. Further, respective calculated concentrations of negative, uncharged, zwitterionic, and positively charged microspecies at pH 7.0 were, respectively, 2, 6, 70, and 18% for norfloxacin and 1, 6, 80, and 10% for CS-940.

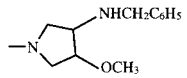
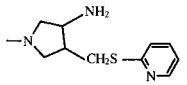
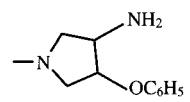
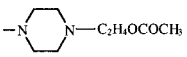
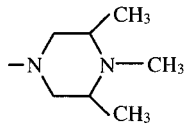
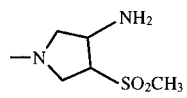
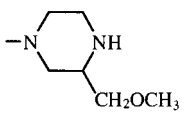
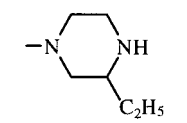
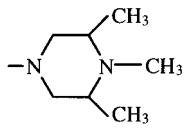
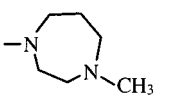
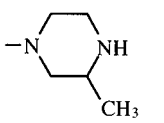
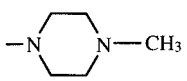
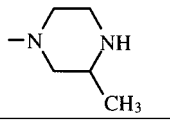
Accumulation of norfloxacin and CS-940 in cells. Figure 2 shows a relationship between the outer concentrations of norfloxacin and CS-940 and their cellular accumulations in a standard strain, *S. aureus* FDA 209P JC. There was a linear correlation between the outer concentration of each fluoroquinolone and its accumulation, at least up to the outer concentration of 800 μg/ml, at 10 min after drug addition.

Figure 3 shows cellular accumulations of norfloxacin and CS-940 in SA-1199 and SA-1199B and the effect of CCCP on the accumulations. In SA-1199, the accumulation of norfloxacin reached near equilibrium at 5 min; thereafter it remained at almost the same level up to 30 min, and the addition of CCCP did not affect the accumulation. In SA-1199B, the accumulation of norfloxacin was significantly lower than that observed in SA-1199 ($P < 0.001$ at 5, 10, 20, and 30 min; Student's t test), and the accumulation rose from 164 to 187% by the addition of CCCP to levels similar to those observed in SA-1199. On the other hand, the accumulations of CS-940 were similar in the two strains. It had already reached equilibrium at 2 min, the levels were higher than the accumulation of norfloxacin in SA-1199B, and the addition of CCCP did not affect the accumulation.

Point mutations in the *gyrA*, *gyrB*, and *grlA* genes. Because SA-1199B was isolated from an experimental rabbit endocarditis model that involved repetitive ciprofloxacin administrations (18), association of another resistance mechanism(s) should be excluded. Direct sequencing of the PCR products

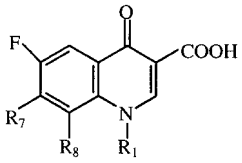
TABLE 2. Chemical structures, antibacterial activities, and chemical parameters of 40 fluoroquinolones



Compound	Substituent			MIC (μg/ml)[a] for:		Parameter[b]						cLog*P*
	R_1	R_7	R_8	SA-1199	SA-1199B	MR_1	MR_7	MR_8	L_1	π_7	π_8	
1			—OCH_3	0.20	0.39	2.28	7.04	1.65	4.14	1.81	−0.08	3.55
2			—F	0.10	0.20	2.28	7.01	1.04	4.14	0.61	0.14	2.55
3			—OCH_3	0.10	0.20	2.28	6.11	1.65	4.14	1.22	−0.08	2.96
4			—OCH_3	0.20	0.39	2.28	5.49	1.65	4.14	0.85	−0.08	2.59
5	—C_2H_4F		—OCH_3	1.56	3.13	1.97	4.84	1.65	4.70[c]	1.57	−0.08	3.20
6			—F	1.56	3.13	2.28	4.78	1.04	4.14	−1.06	0.14	0.88
7			—$OCHF_2$	0.10	0.20	2.28	4.52	1.68	4.14	−0.30	0.22	1.73
8			—$OCHF_2$	0.39	0.78	2.28	4.37	1.68	4.14	0.72	0.22	2.76
9			—$OCHF_2$	0.10	0.20	2.28	4.37	1.68	4.14	0.71	0.22	2.75
10			—OCH_3	0.20	0.39	2.28	4.37	1.65	4.14	0.62	−0.08	2.36
11			—OCF_3	0.78	1.56	2.28	3.91	1.69	4.14	0.19	0.48	2.49
12			—$OCHF_2$	0.20	0.39	2.28	3.91	1.68	4.14	0.53	0.22	2.56
CS-940			—$OCHF_2$	0.10	0.20	2.28	3.91	1.68	4.14	0.19	0.22	2.23

Continued on following page

TABLE 2—*Continued*



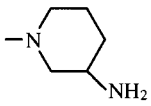
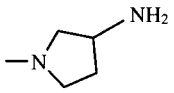
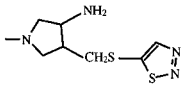
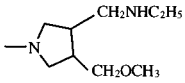
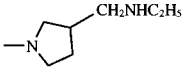
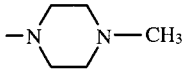
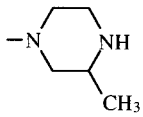
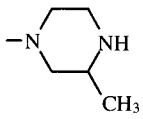
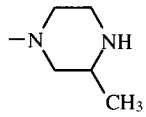
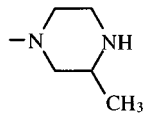
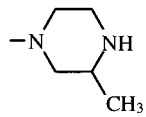
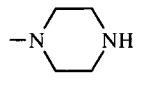
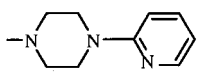
Compound	Substituent			MIC (μg/ml)[a] for:		Parameter[b]						cLog*P*
	R_1	R_7	R_8	SA-1199	SA-1199B	MR_1	MR_7	MR_8	L_1	π_7	π_8	
13			—$OCHF_2$	0.10	0.20	2.28	3.91	1.68	4.14	0.13	0.22	2.16
14			—OCF_3	0.39	0.78	2.28	3.44	1.69	4.14	−0.43	0.48	1.87
15			—F	0.20	0.78	2.28	6.61	1.04	4.14	0.04	0.14	1.98
16			—OCH_3	0.10	0.39	2.28	5.92	1.65	4.14	−0.59	−0.08	1.14
17	—C_2H_5		—F	0.20	0.78	1.96	4.84	1.04	4.11	0.30	0.14	2.42
18	—$NHCH_3$		—$OCHF_2$	1.56	6.25	1.86	3.91	1.68	3.53[c]	0.53	0.22	3.30
19	—$C_6H_3F_2$		—$OCHF_2$	1.56	6.25	3.57	3.91	1.68	6.87[c]	0.19	0.22	4.04
20	—C_6H_5		—$OCHF_2$	12.5	50	3.54	3.91	1.68	6.28	0.19	0.22	3.75
21	—$CHCH_2$		—$OCHF_2$	3.13	12.5	2.01	3.91	1.68	4.29[c]	0.19	0.22	2.84
22	—$CH(CH_3)_2$		—$OCHF_2$	6.25	25	2.42	3.91	1.68	4.11	0.19	0.22	2.71
AM-1155			—OCH_3	0.10	0.39	2.28	3.91	1.65	4.14	0.19	−0.08	1.93
23	—CH_3		—OC_2H_5	12.5	50	1.49	3.44	2.11	3.00	−0.33	0.45	1.59
24	—C_6H_4F		—H	0.10	0.78	3.56	5.74	1.03	6.87[c]	1.50	0.00	4.95

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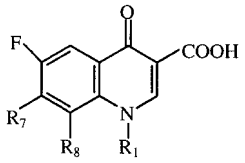
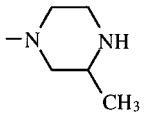
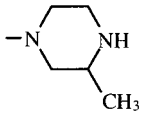
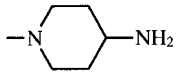
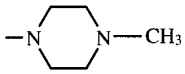
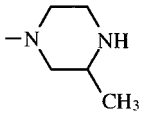
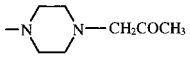
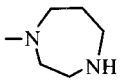
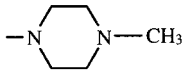
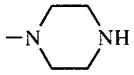
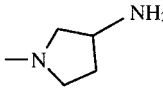
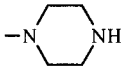
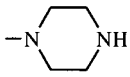
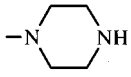
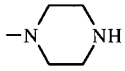
TABLE 2—*Continued*



Compound	Substituent			MIC (μg/ml)[a] for:		Parameter[b]						cLog*P*
	R_1	R_7	R_8	SA-1199	SA-1199B	MR_1	MR_7	MR_8	L_1	π_7	π_8	
25	—C_6H_4F		—$OCHF_2$	3.13	25	3.56	3.91	1.68	6.87[c]	0.19	0.22	3.89
26	—C_2H_4F		—$OCHF_2$	6.25	50	1.97	3.91	1.68	4.70[c]	0.19	0.22	2.12
27			—OCH_3	0.39	3.13	2.28	3.91	1.65	4.14	−0.44	−0.08	1.30
Fleroxacin	—C_2H_4F		—F	0.39	3.13	1.97	3.91	1.04	4.70[c]	0.53	0.14	2.37
Lomefloxacin	—C_2H_5		—F	0.78	6.25	1.96	3.91	1.04	4.11	0.19	0.14	2.31
28	—C_2H_5		—H	0.78	12.5	1.96	4.87	1.03	4.11	0.39	0.00	2.35
29	—CH_3		—OCH_3	1.56	25	1.49	3.91	1.65	3.00	−0.23	−0.08	1.15
30	—CH_2OCH_3		—H	3.13	50	2.11	3.91	1.03	4.91	0.53	0.00	1.85
31	—$NHCH_3$		—OCH_3	3.13	50	1.86	3.44	1.65	3.53[c]	−0.33	−0.08	2.14
32	—C_6H_4F		—OCH_3	0.78	12.5	3.56	3.44	1.65	6.87[c]	−0.43	−0.08	2.98
Ciprofloxacin			—H	0.39	6.25	2.28	3.44	1.03	4.14	−0.33	0.00	1.46
33	—CH_2OCH_3		—H	12.5	400	2.11	3.44	1.03	4.91	−0.33	0.00	1.00
34	—$C_2H_4OCH_3$		—H	12.5	800	2.58	3.44	1.03	6.04[c]	−0.33	0.00	0.90
Norfloxacin	—C_2H_5		—H	0.78	50	1.96	3.44	1.03	4.11	−0.33	0.00	1.64

[a] Each value is the median of more than three independent determinations.

[b] MR_1, MR_7, and MR_8, molar refractivities for the N-1, C-7, and C-8 substituents, respectively; L_1, sterimol parameter for N-1 substituent; π_7 and π_8, the hydrophobic substituent constants for the C-7 and C-8 substituents, respectively.

[c] Estimated value based on those compiled in reference 37.

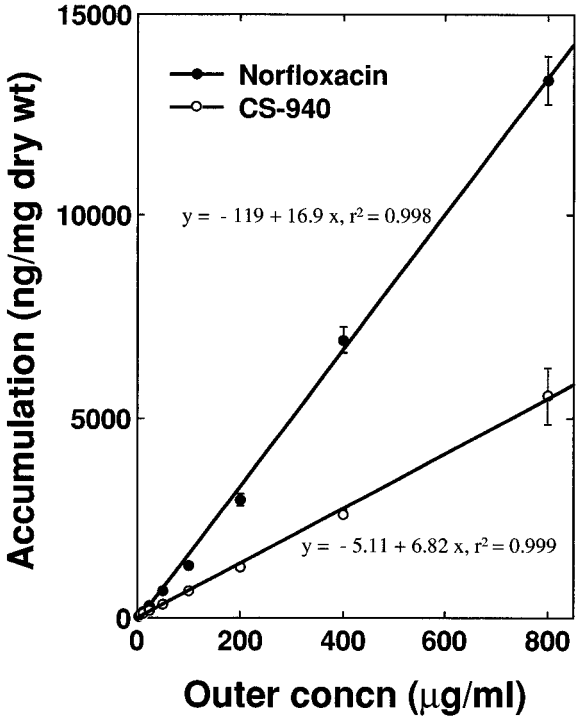


FIG. 2. Cellular accumulation of norfloxacin and CS-940 in *S. aureus* FDA 209P JC. Ten minutes after the fluoroquinolone addition, samples were removed, and the concentration of each fluoroquinolone extracted from cells was determined by a fluorescence spectrophotometer. Each value represents the mean ± SD from three triplicate experiments.

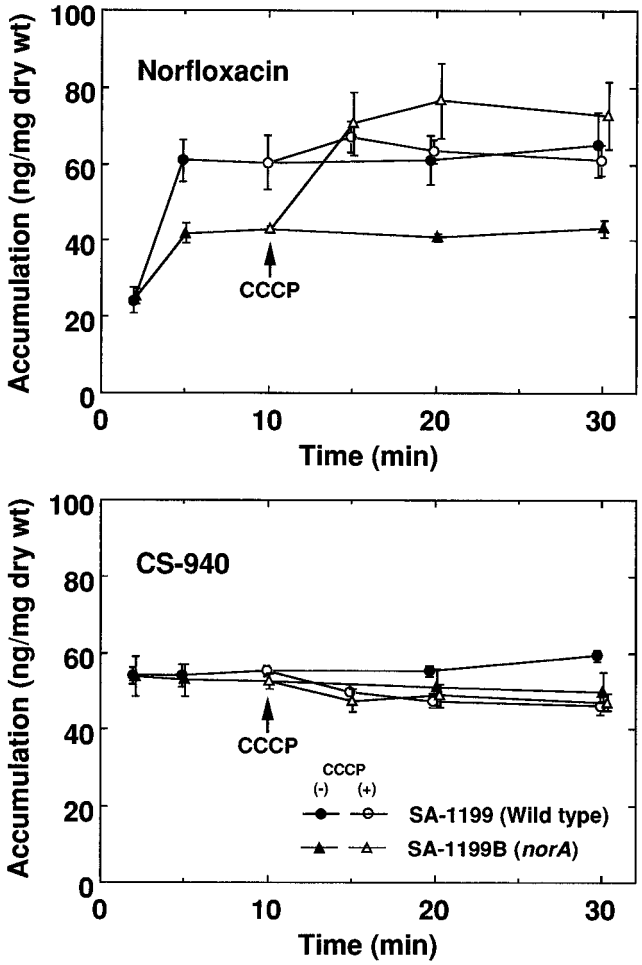


FIG. 3. Cellular accumulation of norfloxacin and CS-940 in *S. aureus* SA-1199 and SA-1199B. Fluoroquinolone was added to cell suspensions at a final concentration of 10 μg/ml. After 10 min, CCCP was added to the suspensions at a final concentration of 2 μg/ml. Portions were removed at the given times, and the concentration of each fluoroquinolone extracted from cells was determined with a fluorescence spectrophotometer. Each value represents the mean ± SD from three triplicate experiments.

revealed that SA-1199 and SA-1199B possess only silent differences at codon 110 (Phe, TTT→TTC) in the *gyrA* gene and at codons 466 (Lys, AAA→AAG) and 485 (Phe, TTT→TTC) in the *gyrB* gene. No point mutation was observed at codons 80 and 84 in the *grlA* gene.

DISCUSSION

From the discovery of *norA* resistance, it has been believed that activity of hydrophilic quinolones is decreased to a larger extent than that of hydrophobic quinolones, on the basis of findings obtained from a comparison among a small number of quinolones. From a comparison of MICs of a series of fluoroquinolone congeners for SA-1199 and SA-1199B, the decrease in antibacterial activity by *norA* mutation was poorly correlated with the hydrophobicity of a quinolone molecule and was rather well correlated with its bulkiness at the C-7 substituent and bulkiness and hydrophobicity at the C-8 substituent. Therefore, we suggest that bulkiness at the C-7 substituent and bulkiness and hydrophobicity at the C-8 substituent would be a factor influencing the decrease in activity of quinolones in efflux-mediated resistant mutants of *S. aureus.*

Whole-cell accumulation in a standard *S. aureus* strain was proportional to outer concentrations of norfloxacin and CS-940 at 10 min after drug additions, when equilibrium had already been attained across the cytoplasmic membrane. Uptake of quinolones was considered to be not by way of any specific mechanism but by simple diffusion, as others have claimed (6, 19, 20, 23). The method used in this study is thought to be sufficient to determine the steady-state concentration and the effect of CCCP, because accumulation of norfloxacin in cells of SA-1199B was significantly increased by addition of CCCP, as was observed in another method using a radioisotope and dinitrophenol (19). Cellular accumulation of CS-940 in SA-1199B was almost identical to that in SA-1199, and it was not affected by addition of CCCP. The affinities of quinolones to NorA presumably correlate with the extent to which *norA* causes resistance to these compounds. Therefore, CS-940 was considered to be a poor substrate for the transport protein and to be hardly expelled from cells, therefore leading to the small magnitude of the MIC increase of CS-940.

Furet et al. (11) proposed a model of the role of the four microspecies of amphoteric fluoroquinolones. According to the model, zwitterionic and uncharged species are associated with transport across the cytoplasmic membrane of bacterial cells. At pH 7.0, used in this study, concentrations of uncharged species of both CS-940 and norfloxacin were calculated to be equal. However, the concentration of dipolar species of CS-940 was higher than that of norfloxacin by 10%. This would explain why accumulation of CS-940 reached equilibrium faster than norfloxacin did. The equilibrium concentration of CS-940 in SA-1199 was nearly equal to that of norfloxacin, since equilibrium is reached when the cytoplasmic

concentration of the uncharged form becomes equal to that of the form in the outer fluid (26). Some authors (4, 8, 23) have claimed that the hydrophobicity of a quinolone is positively correlated with its uptake in this organism; however, others (2) observed no direct relationship between the two factors.

SA-1199B is derived from a genetically undefined clinical strain, SA-1199. No point mutation resulting in a significant amino acid substitution was found in quinolone resistance-determining regions of the *gyrA*, *gyrB*, and *grlA* genes in SA-1199 and SA-1199B by direct sequencing of the PCR products. Genetic analysis of the *flqA* mutation is virtually impossible, and this mutation might correspond to the *grlA* mutation (10, 14). Moreover, we reconfirmed a higher transcriptional level of *norA* in SA-1199B than in SA-1199 (data not shown), as described by Kaatz et al. (20), indicating that the resistance mechanism expressed in SA-1199B is solely drug efflux mediated.

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