Mutant Prevention Concentration of Garenoxacin (BMS-284756) for Ciprofloxacin-Susceptible or -Resistant *Staphylococcus aureus*

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The new quinolone garenoxacin (BMS-284756), which lacks a C-6 fluorine, was examined for its ability to block the growth of *Staphylococcus aureus*. Measurement of the MIC and the mutant prevention concentration (MPC) revealed that garenoxacin was 20-fold more potent than ciprofloxacin for a variety of ciprofloxacin-susceptible isolates, some of which were resistant to methicillin. The MPC for 90% of the isolates (MPC$_{90}$) was below published serum drug concentrations achieved with recommended doses of garenoxacin. These in vitro observations suggest that garenoxacin has a low propensity for selective enrichment of fluoroquinolone-resistant mutants among ciprofloxacin-susceptible isolates of *S. aureus*. For ciprofloxacin-resistant isolates, the MIC at which 90% of the isolates tested were inhibited was below serum drug concentrations while the MPC$_{90}$ was not. Thus, for these strains, garenoxacin concentrations are expected to fall inside the mutant selection window (between the MIC and the MPC) for much of the treatment time. As a result, garenoxacin is expected to selectively enrich mutants with even lower susceptibility.

The fluoroquinolones are potent antibacterial agents that have been used successfully to control a variety of bacterial pathogens. Unfortunately, *Staphylococcus aureus* readily develops resistance to derivatives such as ciprofloxacin (1, 3, 18). In some surveys of hospital-acquired infection, more than 90% of the *S. aureus* isolates were found to be ciprofloxacin-resistant (1, 22). Nevertheless, many community-acquired infections due to *S. aureus* are still susceptible (8, 14, 17, 20), raising the possibility that new quinolones may be active enough to treat *S. aureus* infections successfully.

Fluoroquinolone activity can be measured in a variety of ways. For susceptible cells, activity is generally assessed in terms of the MIC, the concentration that prevents growth when 10^4 to 10^5 cells are applied to drug-containing agar. When large bacterial and patient populations are considered, activity can be assessed by the mutant prevention concentration (MPC) (5) to take into account subpopulations of resistant mutants that may be present prior to antimicrobial treatment. The MPC is defined as the MIC of the least susceptible single-step mutant (12, 24). For bacterial populations that already contain mutations that lower susceptibility, the MPC is equivalent to the MIC of the least-susceptible next single-step mutant. The MPC is approximated by the concentration that prevents growth when at least 10^{10} cells are applied to agar plates (when 10^9 to 10^{10} bacteria are treated with fluoroquinolone, the recovery of mutants is progressively reduced by increasing the drug concentration, as expected for concentrations that approach the MIC of the least susceptible mutant).

In principle, the interval between the lowest concentration that blocks wild-type growth and the MPC represents a concentration window in which resistant mutants are selected (26, 27). The properties of the selection window, when combined with drug pharmacokinetics for relevant tissues, provide a

framework that reflects the potential of a compound to selectively enrich resistant mutants.

In the present work, we used a small but genetically diverse set of clinical isolates of *S. aureus*, as assessed by *spa* typing (23), to compare a new quinolone, garenoxacin (BMS-284756), with the older fluoroquinolone ciprofloxacin. Garenoxacin lacks the C-6 fluorine characteristic of fluoroquinolones, but it has a difluoro methoxyl moiety attached to the C-8 position. Earlier work showed that C-8 methoxyl and halogen moieties enhance the bacteriostatic and bactericidal activities of fluoroquinolones against *S. aureus*, particularly with resistant mutants (16, 28). Thus, we measured the MPC of garenoxacin to determine if this in vitro parameter would be below garenoxacin concentrations in serum for most of the dosing interval for ciprofloxacin-susceptible isolates. Since previous work (6, 9, 21, 25) had shown good activity as measured by standard MIC-based criteria and since guarded optimism had been expressed for using the compound against ciprofloxacin-resistant *S. aureus* (4, 6, 9, 25), the MPC was also measured for ciprofloxacin-resistant isolates. For ciprofloxacin-susceptible isolates, garenoxacin concentrations reported in the published literature were above the MPC determined in vitro; for ciprofloxacin-resistant isolates, they fell inside the mutant selection window.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Laboratory strain RN450 is a methicillin-susceptible, erythromycin-susceptible derivative of *S. aureus* strain 8325-4 (10). Strain 450 M is a methicillin-resistant transformant of RN450 (10) for which donor DNA was obtained from strain COL (2). Clinical isolates of *S. aureus*, obtained from a variety of sources, represented a wide variety of genotypes. Cells were cultured (19) with vigorous shaking at 37°C in CY medium (1% Casamino Acids, 1% yeast extract, 0.1 M NaCl, 0.5% glucose, and 0.05 M sodium glycerophosphate [pH 7.2]), and colonies were grown on GL agar (0.3% Casamino Acids, 0.3% yeast extract, 0.1 M NaCl, 0.2% sodium lactate, 0.1% glycerol, and 1.5% agar [pH 7.8]) incubated at 37°C.

To assay the selection of fluoroquinolone-resistant mutants with laboratory strains, *S. aureus* was grown to stationary phase, harvested by centrifugation, and resuspended in 25 to 50 volumes of fresh growth medium. Incubation was

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continued for 5 to 6 h, producing a culture containing 10^{10} to 10^{11} CFU/ml. Cells were diluted and applied at various volumes and concentrations to quinolone-containing agar at a maximum of 2×10^{10} CFU per 150-mm-diameter plate. Drug-containing plates were incubated at 37°C for 72 to 96 h to allow time for slow-growing colonies to be recovered. After colonies were counted, they were confirmed to be composed of resistant mutants by regrowth on agar containing quinolone at the concentration used to select the colonies.

Fluoroquinolones. Fluoroquinolones were obtained from Bristol-Myers Squibb (gatifloxacin and garenoxacin), Sigma (ciprofloxacin), and R. W. Johnson Pharmaceutical Research Institute (levofloxacin). Ciprofloxacin was dissolved in sterile water to give a final concentration of 10 mg/ml. Levofloxacin and gatifloxacin stock solutions were prepared similarly, except that about a 1/10 volume of 1 M NaOH was added to help dissolve both compounds. Garenoxacin was dissolved in 0.001 M acetic acid as a 5-mg/ml stock solution. Stock solutions were divided into 1-ml aliquots and stored at −80°C. Dilution series were prepared with autoclaved water. Solutions were occasionally stored at −20°C for several weeks.

Measurement of MIC and MPC. The MIC was determined by placing duplicate aliquots (10 or 20 μl) of serial dilutions of bacterial cultures onto GL agar plates containing various concentrations of quinolone. Colonies were counted after overnight incubation. For laboratory strains, preliminary determinations with twofold dilutions of the drug provided an approximate MIC. This measurement was followed by a second measurement, plus a replicate, that utilized linear drug concentration increments that did not exceed 50% per sequential increase. The MIC was defined as the minimal drug concentration that inhibited growth by 99% (MIC_{99}). For clinical isolates, twofold dilutions of the drug were used, and the MIC was taken as the lowest drug concentration that prevented the growth of 10^4 to 10^5 cells.

The MPC was defined as the lowest drug concentration that prevented recovery of colonies when more than 10^{10} cells, obtained as described above, were tested. For determination of the MPC, high-density cultures were prepared from overnight cultures grown in liquid medium followed by a 10-fold dilution and 4 h of incubation with shaking at 37°C. Cells were concentrated twofold by centrifugation. Preliminary determinations with twofold dilutions of the drug provided an approximate MPC. This measurement was followed by a second measurement, plus a replicate, that utilized linear drug concentration increments that did not exceed 50% per sequential increase.

Nucleotide sequence determination. The nucleotide sequences of the quinolone resistance-determining regions of *gyrA* and *parC* were determined after amplification of the respective DNA fragments from *S. aureus* chromosomal DNA templates by using PCR as previously described (14). Primers SA-parCseq (5′-ACG TCG TAT TTT ATA TGC AA-3′) and SA-gyrAfwd (5′-AGA TTA TGC GAT GAG TGT TAT CGT TGC-3′) were used for sequencing after PCR amplification of DNA fragments with primers SA-parCfwd (5′-TGA TGA GGA GGA AAT CTA GTG-3′), SA-parCrev (5′-GGA AAT CTT GAT GGC AAT AC-3′), SA-gyrAfwd (defined above), and SA-gyrArev (5′-TAG TCA TAC GCG CTT CAG TAT AAC GCA-3′). *spa* typing, which involves DNA sequence analysis of the variable number tandem repeats in the protein A (*spa*) gene, followed the protocol described previously by Shopsin et al. (23).

RESULTS

Bacteriostatic activities of fluoroquinolones against laboratory strains of *S. aureus*. To assess the ability of garenoxacin to block the growth of *S. aureus*, we measured the MIC_{99} and compared it to similar measurements for ciprofloxacin, levofloxacin, and gatifloxacin. Garenoxacin was 9- to 15-fold more active than ciprofloxacin and levofloxacin and 4-fold more active than gatifloxacin against a pair of laboratory strains (Table 1). Garenoxacin was also more active when the MPC was measured to assess the inhibition of mutant subpopulations (Table 1), but the differences were less than those measured with the MIC_{99}.

Since isolates of methicillin-resistant *S. aureus* (MRSA) frequently exhibit fluoroquinolone resistance (22), the possibility exists that enrichment of fluoroquinolone-resistant mutants is intrinsically elevated for MRSA strains. The data in Table 1 can be used to argue against this hypothesis: the MICs and the MPCs of several fluoroquinolones for strain 450 M, a *mecA+*

TABLE 1. Effect of *mecA+* on MICs and MPCs for *S. aureus*

Fluoroquinolone	MIC_{99} (μg/ml) for:		MPC (μg/ml) for:	
	Strain RN450	Strain 450 M (*mecA+*)	Strain RN450	Strain 450 M (*mecA+*)
Garenoxacin	0.02	0.01	0.3	0.2
Ciprofloxacin	0.3	0.14	4	3
Levofloxacin	0.18	0.12	2	2
Gatifloxacin	0.08	0.04	0.5	0.5

(methicillin-resistant) derivative of strain RN450, were not higher than those observed for strain RN450.

Recovery of fluoroquinolone-resistant mutants. Strains RN450 and 450 M were also used for the selection of resistant mutants on agar plates containing various concentrations of quinolone. As shown in Fig. 1, the presence of *mecA* reduced the MIC_{99} of garenoxacin by about 50% but in general the recoveries of mutants were similar for the two strains. The concentration at which no colony was recovered when 10^{10} cells were applied to agar plates was not higher for the *mecA+* strain. Similar results were obtained with ciprofloxacin and levofloxacin (data not shown). These data further demonstrate that *mecA* does not contribute directly to a high prevalence of ciprofloxacin resistance among MRSA isolates.

DNA was extracted from three resistant mutants of strain RN450 obtained from agar plates containing garenoxacin at concentrations near that represented by the inflection point in Fig. 1 (0.1 μg/ml). Nucleotide sequence analysis of the quinolone resistance-determining regions of *gyrA* and *parC* revealed that each mutant contained a Ser-to-Leu change at codon 84 in the GyrA protein (data not shown). No change was found in *parC*. These results support the conclusion that gyrase is the primary target of garenoxacin in *S. aureus* (4). In contrast to a previous study (4), we were able to obtain gyrase mutants in a single selection step.
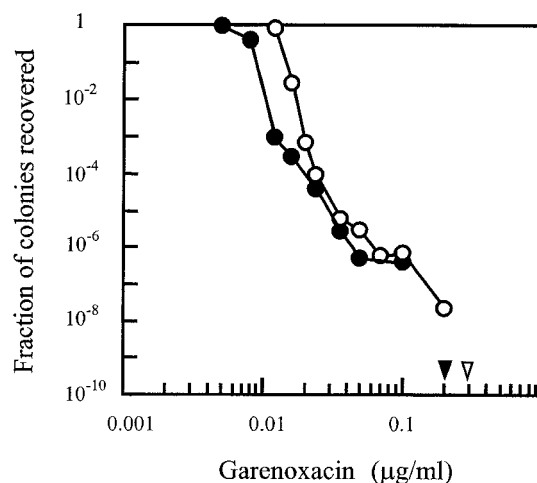

FIG. 1. Selection of resistant *S. aureus* mutants. Cells from *S. aureus* strain RN450 (open circles) or strain 450 M (filled circles) were applied to agar plates containing the indicated concentrations of garenoxacin as described in Materials and Methods. Concentrations at which no colony was recovered when 10^{10} cells were applied to agar are indicated by arrowheads.

TABLE 2. Activities of ciprofloxacin and garenoxacin against clinical isolates of *S. aureus*

Test compound	Ciprofloxacin phenotype of isolates	No. of isolates	No. of *spa* types	MIC (μg/ml)			MPC (μg/ml)			MPC/MIC ratio
				90%	Mode	Range	90%	Mode	Range	
Ciprofloxacin	Susceptible	20	15	0.6	0.4	0.2–0.6	8	3–4	2–12	13
Garenoxacin	Susceptible	20	15	0.03	0.015	0.005–0.03	0.4	0.3–0.4	0.07–0.8	13
	Resistant	22	10	3.2	0.8–1.2	0.04–4.8	>19.6	9.6–12.8	3.2–>28.8	>6

Effects of ciprofloxacin and garenoxacin on ciprofloxacin-susceptible clinical isolates. We examined two groups of bacterial isolates that were susceptible to ciprofloxacin. One comprised methicillin-susceptible *S. aureus* (MSSA) isolates collected from dialysis patients while the other comprised MRSA isolates from various sources. The isolates were genetically diverse, as indicated by DNA sequencing of the protein A gene repeats (*spa* typing). Little difference in the MICs was found for the two groups of strains (the MIC_{90} of garenoxacin was about 0.03 μg/ml, a value that is similar to that reported in other studies [15, 21]). However, the MPC was slightly higher for MRSA than for MSSA (the modal MPC of garenoxacin was 0.2 μg/ml for MSSA and between 0.3 and 0.4 μg/ml for MRSA). For susceptible clinical isolates, the MPC of ciprofloxacin for 90% of the isolates (MPC_{90}) was 8 μg/ml; the modal MPC and the MPC range were 3 to 4 and 2 to 12 μg/ml, respectively. These values are roughly 10- to 20-fold higher than those observed with garenoxacin. MPCs for isolates collected before quinolones were in widespread clinical use (isolates numbered 948 and 912) were the lowest (ciprofloxacin, 2 μg/ml; garenoxacin, 0.07 and 0.1 μg/ml, respectively), but the MICs were similar to those for the more recent isolates (ciprofloxacin, 0.3 and 0.4 μg/ml, respectively; garenoxacin, 0.015 μg/ml). These data are consistent with the extensive use of fluoroquinolones causing susceptibility as measured by the MPC to gradually decrease.

Effect of garenoxacin on ciprofloxcin-resistant clinical isolates. To assess the potency of garenoxacin against ciprofloxacin-resistant isolates, we measured the MICs and the MPCs for a diverse panel of isolates comprising 18 MRSA and 4 MSSA isolates. The MIC ranged between 0.04 and 4.8 μg/ml, the modal MIC was between 0.8 and 1.2 μg/ml, and the MIC_{90} was 3.2 μg/ml, similar to data in one report (15) and slightly higher than that in another (21). The range of MPCs was 3.2 to >29 μg/ml, the MPC_{90} was >19.6 μg/ml, and the modal MPC ranged between 9.6 and 12.8 μg/ml. These values, which are listed in Table 2, are 30- to 100-fold higher than those observed for ciprofloxacin-susceptible isolates.

DISCUSSION

The increasing prevalence of antimicrobial resistance makes it important that new agents be considered for their propensity to selectively enrich resistant mutants. It has been suggested that mutant enrichment occurs when the concentration of the antimicrobial agent is high enough to block the growth of susceptible cells (above the MIC) but not that of resistant mutants (below the MPC) (26, 27). Compounds that can be dosed so that relevant concentrations at the site of infection exceed the MPC are expected to enrich fewer mutants than

those whose concentrations do not exceed the MPC, provided that mutation frequencies are similar (26, 27). For example, ciprofloxacin, a compound that readily selects resistant *S. aureus* mutants (1, 3), has an in vitro MPC_{90} of about 8 μg/ml when the value is determined for a small but genetically diverse set of ciprofloxacin-susceptible *S. aureus* (Table 2) isolates. Pharmacokinetic measurements reported in the published literature indicate that ciprofloxacin concentrations in serum do not exceed the MPC (Fig. 2A). The MPC_{90} of garenoxacin was lower (0.4 μg/ml). Pharmacokinetic measurements with garenoxacin indicate that human serum drug concentrations will exceed this value for the entire dosing period (24 h) at the recommended 400-mg once-daily dosage (Fig. 2B), even if 75% of the agent is considered unavailable due to protein binding (11). We emphasize that the MPC has not been measured in vivo; determining whether concentrations that exceed the MPC measured in vitro are sufficient to block the development of resistance requires clinical studies.

Another strategy for restricting the development of resistance is to find compounds that have very narrow mutant selection windows ($MPC/MIC_{99} = 1$). With *S. aureus*, fluoroquinolone structure affects this ratio (27). In the present work, the ratios of the MPC of ciprofloxacin to the MIC_{99} of ciprofloxacin for laboratory strains RN450 and 450 M were 13 and 21, respectively. The ratios of the MPC of garenoxacin to the MIC_{99} of garenoxacin were similar (15 and 20 for RN450 and 450 M, respectively). Average values for clinical isolates, which were expected to be lower due to the use of the standard MIC rather than the MIC_{99}, were about 13 for both ciprofloxacin and garenoxacin (Table 2). Thus, the mutant selection window for garenoxacin, which is about twice that of gatifloxacin (Table 1), is not unusually narrow.

Many *S. aureus* isolates that exhibit resistance to methicillin are also ciprofloxacin resistant. Since selection of resistance by garenoxacin produced similar results with $mecA^+$ and *mecA* mutant laboratory strains (Fig. 1) and with MRSA and MSSA clinical isolates (MPCs were similar [data not shown]), it is likely that factors other than *mecA* are involved in the fluoroquinolone resistance of MRSA. The association between MRSA and ciprofloxacin resistance may stem from the more frequent exposure of MRSA to fluoroquinolones and to the dissemination of ciprofloxacin-resistant strains in institutional settings.

Isolates recovered before quinolone use was widespread exhibited susceptibilities (as measured by the MICs) that were similar to those observed for other ciprofloxacin-susceptible isolates. However, the MPCs were lower. This result is consistent with the recent accumulation of mutations that have little effect on the growth of the bulk population in the presence of
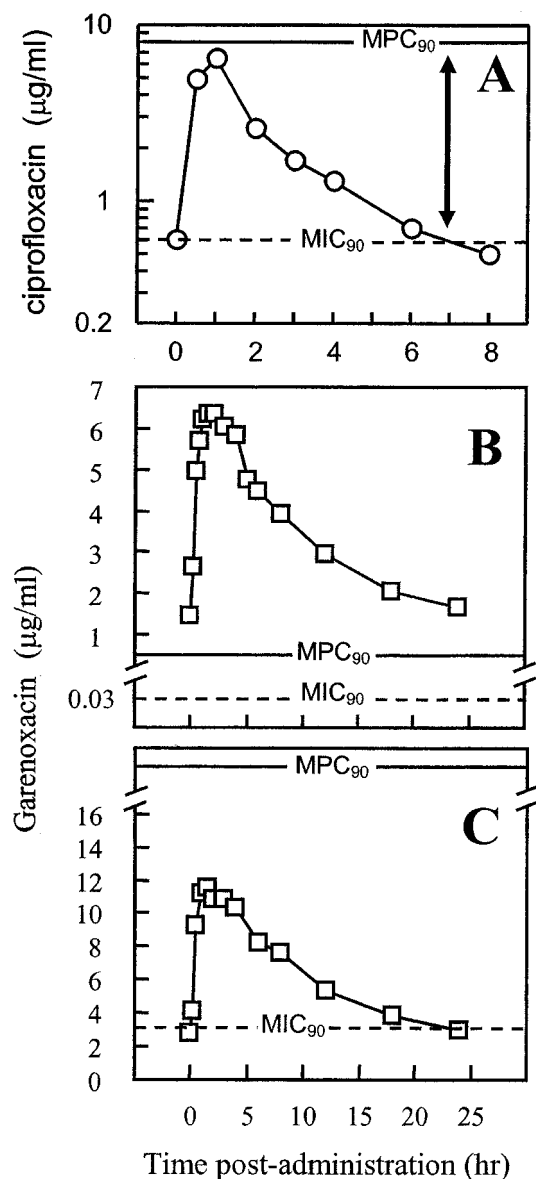
FIG. 2. Quinolone pharmacodynamics with *S. aureus*. (A) Cipro-
floxacin and susceptible *S. aureus*. The MIC_{90} and the MPC_{90} were
obtained from the data in Table 2; pharmacokinetic data were deter-
mined for 400-mg doses of ciprofloxacin administered intravenously
three times per day (13). (B) Garenoxacin and susceptible *S. aureus*.
The MIC_{90} and the MPC_{90} were obtained from the data in Table 2;
pharmacokinetic data were determined for 400-mg doses of garenoxa-
cin administered orally once daily (7). (C) Garenoxacin and ciprofloxa-
cin-resistant *S. aureus*. The MIC_{90} and the MPC_{90} were obtained from
the data in Table 2; pharmacokinetic data were determined for 600-mg
doses of garenoxacin administered orally once daily (P. Hale, personal
communication).

quinolone but increase the contribution of resistant subpopu-
lations to growth at high drug concentrations. A similar phe-
nomenon has been seen with *Escherichia coli* (29). In that case,
a *parC* (DNA topoisomerase IV) mutation had no effect on the
MIC but dramatically increased the recovery of *gyrA* (gyrase)
mutants.

For the isolates that were already resistant to ciprofloxacin,
the MIC_{90} of garenoxacin was 3.2 µg/ml, roughly eight times
higher than the MPC_{90} for the susceptible isolates. This ob-
servation suggests that multiple mutations were present in
some of the resistant strains, as has been documented by other
studies (15, 21). Since the MIC for resistant isolates is below
achievable serum drug concentrations (Fig. 2C), it is conceiv-
able that treatment of ciprofloxacin-resistant *S. aureus* with
garenoxacin might sometimes cure infection. However, the
MPC_{90} of the isolates already resistant to ciprofloxacin was
>19.6 µg/ml, which is well above the serum drug levels
achieved with garenoxacin, even if daily doses are raised to 600
mg (Fig. 2C). Indeed, the MPC-based pharmacodynamics of
garenoxacin and the mutants (Fig. 2C) are similar to those of
ciprofloxacin and fully susceptible isolates (Fig. 2A). Since
ciprofloxacin selected resistant mutants rapidly (1, 3), we pre-
dict that additional mutations will be fixed in *S. aureus* if
garenoxacin is used against ciprofloxacin-resistant strains.
Those mutations would then preclude the use of garenoxacin
in combination therapy.

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