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Killing of *Staphylococcus aureus* by C-8-Methoxy Fluoroquinolones

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C-8-methoxy fluoroquinolones were more lethal than C-8-bromine, C-8-ethoxy, and C-8-H derivatives for *Staphylococcus aureus*, especially when topoisomerase IV was resistant. The methoxy group also increased lethality against wild-type cells when protein synthesis was inhibited. These properties encourage refinement of C-8-methoxy fluoroquinolones to kill staphylococci.

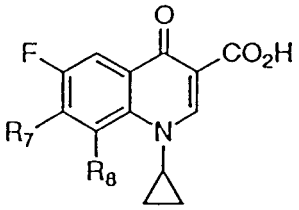
Fluoroquinolones are antibacterial agents that trap gyrase and topoisomerase IV on DNA (6). Current derivatives are only marginally effective against *Staphylococcus aureus*, largely because this bacterium readily acquires resistance mutations (6, 9, 11). In *S. aureus*, topoisomerase-based resistance occurs stepwise, with moderate levels arising from a single mutation in the primary target of the drug, topoisomerase IV, and higher levels from the accumulation of an additional mutation in gyrase (7, 8, 17). Since other forms of resistance are also rendering many therapeutic agents ineffective against *S. aureus*, we sought to improve fluoroquinolone action.

Several studies direct attention to substituents at the C-8 position of fluoroquinolones (R8 in Fig. 1). A computational approach indicated that C-8 fluorine or alkoxy groups, when coupled with an N-1 cyclopropyl moiety, would increase bacteriostatic activity (15). This was the case for 15 of 16 bacterial species subsequently tested (4). Another study showed that a C-8 chlorine increased activity against *Pseudomonas aeruginosa*, especially with strains containing a quinolone-resistant gyrase (14). The C-8 chlorine also increased activity against gyrase purified from the mutants. In a third example, a C-8 methoxy group increased lethality with *Escherichia coli*, particularly for a gyrase mutant (21). Fluoroquinolones with C-8 substitutents have also shown promise against gram-positive organisms (1, 3, 13), including fluoroquinolone-resistant clinical isolates of *S. aureus* (12). The present report compares C-8 substituents for enhancement of fluoroquinolone lethality with *S. aureus* carrying wild-type and characterized resistance alleles of *parC* (topoisomerase IV) and *gyrA* (gyrase).

S. aureus strains (7) included ciprofloxacin-resistant, first-step mutants 2-2 and 2-11, derived from RN4220, and resistant second-step mutants 2-32C128B and 11C128B, derived from 2-2 and 2-11, respectively. Ciprofloxacin and new fluoroquinolones PD161148, PD160793, PD135042, PD164488, and PD163449 (20) were prepared as 10-mg/ml solutions in 0.1 N NaOH. To measure fluoroquinolone lethality, cells were cultured with vigorous shaking at 37°C in CY medium (18), fluoroquinolone was added for 2 h during exponential growth, and the fraction of surviving cells was determined by plating cells on GL agar (18) lacking drug. Bacteriostatic activity was measured as ID_{50}, the fluoroquinolone concentration required to inhibit growth to half that observed in the absence of drug. For this measurement, overnight cultures were diluted 100-fold into CY medium containing various concentrations of fluoroquinolone; after overnight incubation at 37°C, untreated controls reached stationary phase while treated cultures grew to varying extents, as estimated by culture turbidity (A_{600}).

A C-8-methoxy fluoroquinolone (PD161148), which is effective against *E. coli* (21), was about fivefold more lethal than a C-8-H control (PD160793) against wild-type *S. aureus* when the concentration required to kill 99% of the cells (99% lethal dose [LD_{99}]) was measured (Fig. 2A). The difference between the compounds was about 10-fold for a *parC* mutant (strain 2-2) when LD_{50} was measured (the C-8-H compound was not effective enough for LD_{99} comparisons [Fig. 2B]) and about 7-fold for a *parC gyrA* double mutant (strain 2-32C1128B [Fig. 2C]). The reduced effect in the double mutant suggests that at least part of the C-8-methoxy effect is directed against gyrase.

Chloramphenicol protects bacteria from the lethal action of the quinolones (5), presumably by blocking the induction of a protein responsible for releasing DNA breaks from quinolone-topoisomerase-DNA complexes (2). This protective effect occurred with wild-type *S. aureus* (compare Fig. 2A and D, filled



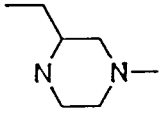
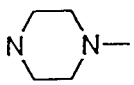
Compound	R7	R8
PD160793		-H
PD161148		-OMe
Ciprofloxacin		-H
PD135042		-OMe
PD164488		-OEt
PD163449		-Br

FIG. 1. Fluoroquinolone structures.

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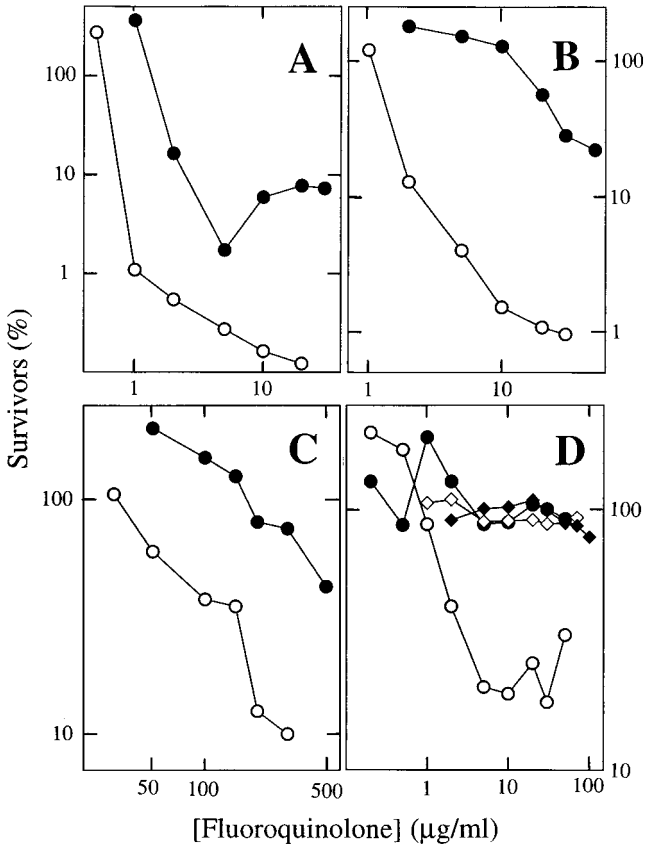

FIG. 2. Lethal action of fluoroquinolones. Aliquots from exponentially growing cultures of *S. aureus* were removed after incubation with the indicated concentrations of fluoroquinolones for 2 h, diluted, and plated on drug-free agar for determination of viable cells. (A) Strain RN4220 (wild type); (B) strain 2-2 (*parC* Cip^r); (C) strain 2-32C-128B (*parC* Cip^r *gyrA* Cip^r); (D) strain RN4220 (wild type; circles) or 2-2 (*parC* Cip^r; diamonds) treated with 20 μg of chloramphenicol per ml in addition to the indicated concentrations of fluoroquinolones. Drugs were PD161148 (C-8-methoxy; open symbols) and PD 160793 (C-8-hydrogen; filled symbols). Experiments were repeated three times with similar results.

circles). The effect was less complete for the C-8-methoxy compound PD161148 (Fig. 2D); thus, C-8-methoxy fluoroquinolones are more likely than C-8-H derivatives to kill nongrowing cells. A *parC* mutation blocked the chloramphenicol-insensitive activity of the C-8-methoxy compound (Fig. 2D, strain 2-2), indicating that fluoroquinolone action against its secondary target, gyrase, is sensitive to chloramphenicol. A comparable effect has been observed with *E. coli* (2, 16). There the secondary target is topoisomerase IV.

We next compared the effects of several C-8 substituents when added to ciprofloxacin. A methoxy group increased lethal activity (LD_{50}) against wild type and a *parC* mutant (strain 2-2) by factors of 4 and 30, respectively (compare circles in Fig. 3). A C-8-ethoxy compound behaved much like ciprofloxacin, and a C-8-bromine compound was intermediate (Fig. 3). A C-8-fluorine compound was previously shown to fall between C-8-methoxy and C-8-H compounds (12). Thus, the methoxy group is the most effective.

We postulated that the quinolones act in two steps (2). First, drug-topoisomerase-DNA complexes block DNA synthesis and eventually cell growth. This step is reversible. Lethal events arise subsequently when double-strand DNA breaks are released from the complexes. Thus, two compounds can have similar potencies for growth inhibition but different abilities to kill cells. Such a situation was seen with *E. coli* (21). With *S. aureus*, the C-8-methoxy substituent increased both bacteriostatic action (lowered ID_{50} [Table 1]) and bactericidal action (Fig. 2 and 3).

Ciprofloxacin had roughly as much bacteriostatic activity as the other C-8-H compound (PD160793) with wild-type cells; however, it was less effective against resistant mutants (Table 1, rows 1 and 4). Ciprofloxacin lacks the ethyl moiety found on the piperizinyl ring of PD160793. But when a C-8-methoxy group was added to each, the ciprofloxacin derivative was two to three times more bacteriostatic (Table 1, rows 2 and 5) and twice as lethal, at least for wild-type cells (compare LD_{90}s in Fig. 2A and 3A). It may be useful to survey C-8-methoxy fluoroquinolones for additional structural improvements.

The striking effect of the C-8-methoxy group is especially evident when *S. aureus* carries a preexisting *parC* mutation or when *E. coli* carries a *gyrA* mutation (21). Fluoroquinolones that effectively kill first-step resistant mutants could greatly lower the ability of wild-type populations to acquire resistance if two mutations were required for resistance (for a discussion, see references 17, 19, and 21). Since the C-8-methoxy group also makes fluoroquinolones more effective against nongrowing cells (Fig. 2D), it should reduce the pool from which new

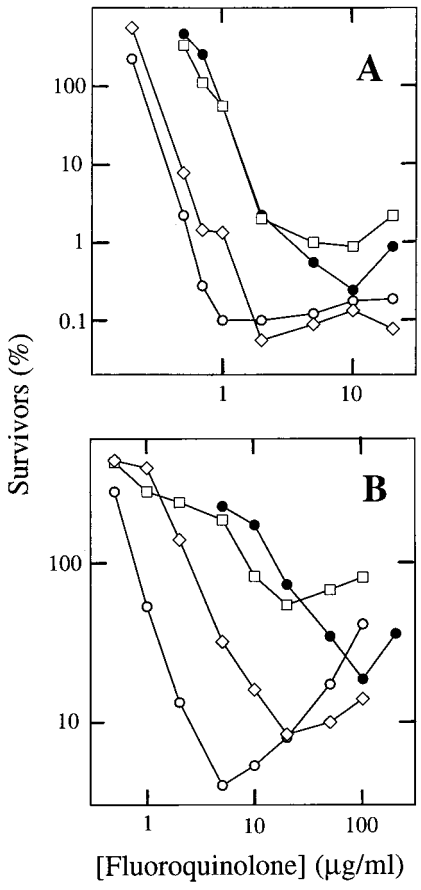

FIG. 3. Effects of C-8 variation on fluoroquinolone bactericidal activity. *S. aureus* wild type (A) (strain RN4220) and a *parC* mutant (B) (strain 2-2) were treated with the indicated concentrations of ciprofloxacin (C-8-hydrogen; filled circles), PD135042 (C-8-methoxy; open circles), PD164488 (C-8-ethoxy; squares), or PD163449 (C-8-bromine; diamonds) as described in the legend to Fig. 2.

TABLE 1. Bacteriostatic activities of fluoroquinolones against *S. aureus* mutants

Fluoroquinolone(s)	Bacteriostatic activity (ID_{50} [μg/ml])[a] for *S. aureus* strain:				
	RN4220 (wild type)	2-11 (*parC*) E84K[b]	2-2 (*parC*) S80Y	11C128B (*parC gyrA*) E84K S84L	2-32C128B (*parC gyrA*) S80Y S84L
PD160793 (C-8-H)	0.8	1.8	2.3	38	37
PD161148 (C-8-methoxy)	0.4	0.7	0.7	18	23
PD160793/PD161148	2.1	2.6	3.2	2.1	1.6
Ciprofloxacin	0.5	3.5	3.5	260	400
PD135042 (C-8-methoxy)	0.16	0.36	0.36	6.2	14
Ciprofloxacin/PD135042	3.1	9.7	9.7	42	30

[a] Determinations were made from three experiments that successively narrowed the range of fluoroquinolone concentrations required to determine ID_{50}.
[b] Mutational changes are indicated by the wild-type amino acid followed by the codon number and then the mutant amino acid. Abbreviations: E, glutamic acid; K, lysine; L, leucine; S, serine; Y, tyrosine.

mutants arise (10). Thus, C-8-methoxy fluoroquinolones are promising leads toward obtaining more-effective agents to control *S. aureus* infections.

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