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Use of Gyrase Resistance Mutants To Guide Selection of 8-Methoxy-Quinazoline-2,4-Diones▽

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A series of 1-cyclopropyl-8-methoxy-quinazoline-2,4-diones was synthesized and evaluated for lowering the ratio of the antimicrobial MIC in gyrase resistance mutants to that in the *gyr*$^+$ (wild type) using isogenic strains of *Escherichia coli*. Dione features that lowered this ratio were a 3-amino group and C-7 ring structure (3-aminomethyl pyrrolidinyl < 3-aminopyrrolidinyl < diazobicyclo < 2-ethyl piperazinyl). The wild-type MIC was also lowered. With the most active derivative tested, many *gyrA* resistance mutant types were as susceptible as, or more susceptible than, wild-type cells. The most active 2,4-dione derivatives were also more active with two quinolone-resistant *gyrB* mutants than with wild-type cells. With respect to lethality, the most bacteriostatic 2,4-dione killed *E. coli* at a rate that was affected little by a *gyrA* resistance mutation, and it exhibited a rate of killing similar to its cognate fluoroquinolone at 10× the MIC. Population analysis with wild-type *E. coli* applied to agar showed that the mutant selection window for the most active 2,4-dione was narrower than that for the cognate fluoroquinolone or for ciprofloxacin. These data illustrate a new approach to guide early-stage antimicrobial selection. Use of antimutant activity (i.e., ratio of the antimicrobial MIC in a mutant strain to the antimicrobial MIC in a wild-type strain) as a structure-function selection criterion can be combined with traditional efforts aimed at lowering antimicrobial MICs against wild-type organisms to more effectively afford lead molecules with activity against both wild-type and mutant cells.

Fluoroquinolones are lethal antibacterial agents that are widely used for many bacterial infections; with some diseases, such as multidrug-resistant tuberculosis, they are sometimes considered to be agents of last resort. However, fluoroquinolone use is threatened by an increasing prevalence of resistance, now seen with almost every bacterial species treated. Even highly susceptible species, such as *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *Streptococcus agalactiae*, are exhibiting quinolone resistance (11, 21, 35, 36). A common strategy to bypass resistance is to seek new derivatives with increased ability to kill wild-type (susceptible) cells. Unfortunately, even highly lethal compounds can leave resistant mutants alive and able to amplify (13). As an alternative, we suggested that the choice of lead compounds in antibiotic discovery be guided toward those that have a very narrow mutant selection window, i.e., the MIC approximates the mutant prevention concentration (MPC), a measure of the mutant subpopulation MIC (5, 40, 41). With some gram-positive pathogens, particularly *Streptococcus pneumoniae*, this criterion has been approached using dual-targeted fluoroquinolones that have similar activities against both gyrase and DNA topoisomerase IV (8, 22–25, 30, 31). In this situation, the MIC of the less-susceptible target approximates the MPC, which creates a narrow window and restricts the recovery of resistant mutants in vitro. A more general approach is to seek lead compounds that block mutant as well as susceptible cell growth when acting against a single target. To our knowledge, such a strategy has not been incorporated into drug discovery programs.

Three significant features of quinolone class antimicrobials have been implicated in improving activity against resistant mutants and therefore provide a starting point for the design of new compounds with good antimutant activity. One is the 8-methoxy group, which in fluoroquinolones often lowers the ratio of the MIC in gyrase mutant strains (MIC_{mutant}) to the corresponding MIC in the wild type (MIC_{wt}) (4, 14, 42). Another feature is the structure of fluoroquinolone C-7-ring substituents, which influence the MPC (23, 28, 41). A third feature emerging from recent work of Ellsworth and coworkers is the conversion from a quinolone core structure to a quinazoline-2,4-dione structure (2). That change improves activity with gyrase mutants of *Escherichia coli*, *Staphylococcus aureus*, and *S. pneumoniae* (2, 6, 7). Although *E. coli* is not highly susceptible to 2,4-diones (2), susceptibility can be improved for in vitro studies with a *tolC* mutation. Moreover, many quinolone-resistant mutants are available (14, 37), which provides a way to broadly evaluate the ratio of the MIC_{mutant} to the MIC_{wt}.

In the present study we synthesized a series of 1-cyclopropyl-8-methoxy-quinazoline-2,4-diones and determined the ratio of the MIC_{mutant} to the MIC_{wt} (*gyr*$^+$) with a set of 12 isogenic *E. coli* strains, each containing a *gyrA* or *gyrB* quinolone resistance mutation. By varying dione structure at the N-3 and C-7 positions, we were able to identify derivatives that brought the ratio of MIC_{mutant} to MIC_{wt} close to unity. The MIC_{wt} was also reduced; moreover, resistant mutants were selected over a much narrower concentration range for the most active 8-methoxy-2,4-dione tested than for a cognate fluoroquinolone or for ciprofloxacin, a fluoroquinolone commonly used to treat gram-negative infections. These experiments illustrate an an-

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TABLE 1. Bacterial strains

Strain	Relevant genotype[a]	Source or reference
KD1397	DM4100 *tolC6*::Tn*10 gyr*$^+$ (wild type)	29; this study
KD2862	KD66 *tolC6*::Tn*10 gyrA*(*S83L*)	26; this study
KD2864	KD1975 *tolC6*::Tn*10 gyrA*(*A84P*)	14; this study
KD2866	KD1977 *tolC6*::Tn*10 gyrA*(*D87Y*)	14; this study
KD2876	KD1909 *tolC6*::Tn*10 gyrA*(*S83W*)	14; this study
KD2878	KD1911 *tolC6*::Tn*10 gyrA*(*A67S*)	14; this study
KD2880	KD1913 *tolC6*::Tn*10 gyrA*(*D87N*)	14; this study
KD2882	KD1915 *tolC6*::Tn*10 gyrA*(*G81C*)	14; this study
KD2884	KD1917 *tolC6*::Tn*10 gyrA*(*Q106H*)	14; this study
KD2886	KD1721 *tolC6*::Tn*10 gyrA*(*A51V*)	14; this study
KD2932	KD1500 *tolC6*::Tn*10 gyrB*(*D426N*)	20; this study
KD2934	KD1502 *tolC6*::Tn*10 gyrB*(*K447E*)	20; this study
KD2955	KD1973 *tolC6*::Tn*10 gyrA*(*D82A*)	14; this study

[a] All strains in the present study contained a *tolC* deficiency to lower the antimicrobial MIC.

timutant approach for guiding the development of new antimicrobial agents.

MATERIALS AND METHODS

Fluoroquinolones and 8-methoxy-quinazoline-2,4-diones. PD161148 was a generous gift from John Domagala, Parke-Davis Division of Pfizer Pharmaceutical Co. Moxifloxacin and ciprofloxacin were obtained from Bayer AG. FQ-c (UING5-248) and FQ-d (UING5-249) were synthesized by substituting (*S*)-3-aminopyrrolidine (Alfa Aesar, Ward Hill, MA) and (*R*)-3-*N*-Boc-aminomethyl pyrrolidine (Astatech, Inc., Bristol, PA) into the C-7 position of 1-cyclopropyl-6,7-difluoro-8-methoxy-4-oxo-3-quinolinecarboxylic acid (3B Scientific Corp., Libertyville, IL) using standard methods for coupling and Boc deprotection (3, 27). The C-7 variants of 1-cyclopropyl-6-fluoro-8-methoxy-1H-quinazoline-2,4-dione (NH dione) and 3-amino-1-cyclopropyl-6-fluoro-8-methoxy-1H-quinazoline-2,4-dione (N-NH_2 dione) were synthesized by substituting (*S*)-3-aminopyrrolidine, (*R*)-3-*N*-Boc-aminomethyl pyrrolidine, (*S*,*S*)-*cis*-octahydropyrrolo[3,4-b]pyridine (3B Pharmachem International Co., Wuhan, People's Republic of China) or 2-ethylpiperazine (Atlantic SciTech Group, Bristol, PA) into the C-7 position of 3-H- or 3-amino-1-cyclopropyl-6,7-difluoro-8-methoxy-1H-quinazoline-2,4-dione. De novo synthesis of the requisite quinazoline-2,4-dione intermediates and introduction of the C-7 groups was performed with minor modifications to previously described methods (1, 10, 32, 33). The structure of each compound was characterized by nuclear magnetic resonance and high-resolution mass spectroscopy. All compounds were dissolved to 10 mg/ml in either dimethyl sulfoxide (diones) or 0.1 N NaOH (quinolones) prior to use.

^{1}H nuclear magnetic resonance (300 MHz, dimethyl sulfoxide-d_6) assignments for trifluoroacetic acid salt forms of the 2,4-diones are summarized below.

Dione-a (UING5-48). 1-Cyclopropyl-7-(3-ethylpiperazin-1-yl)-6-fluoro-8-methoxy-1H-quinazoline-2,4-dione, δ = 11.41 (s, 1H), 9.10 (m, 1H), 8.83 (m, 1H), 7.42 (d, J = 12 Hz, 1H), 3.68 (s, 3H), 3.57 to 3.37 (m − overlap with H_2O, 3H), 3.21 to 3.14 (m, 5H), 1.62 (m, 2H), 1.00 to 0.94 (m, 5H), 0.57 (m, 2H).

Dione-b (UING5-47). 1-Cyclopropyl-6-fluoro-8-methoxy-7-[(*4aS*,*7aS*)-octahydro-6H-pyrrolo[3,4-b]pyridin-6-yl]-1H-quinazoline-2,4-dione, δ = 11.20 (s, 1H), 9.43 (br, 1H), 8.60 (br s, 1H), 7.31 (d, J = 13Hz, 1H), 4.00 (m, 1H), 3.86 to 3.13 (m, 9H), 2.96 (m, 1H), 2.62 (br, 1H), 1.78 to 1.70 (m, 4H), 1.00 (m, 1H), 0.88 (m, 1H), 0.61 (m, 1H), 0.49 (m, 1H).

Dione-c (UING5-63). 7-[(*S*)-3-Aminopyrrolidin-1-yl]-1-cyclopropyl-6-fluoro-8-methoxy-1H-quinazoline-2,4-dione, δ = 11.25 (s, 1H), 8.16 (br, 3H), 7.32 (d, J = 13Hz, 1H), 3.88 to 3.53 (m, 5H), 3.50 (s, 3H), 3.17 (m, 1H), 2.25 (m, 1H), 2.00 (m, 1H), 0.96–0.90 (m, 2H), 0.58 to 0.55 (m, 2H).

Dione-d (UING5-200). 7-[(*S*)-3-Aminomethylpyrrolidin-1-yl]-1-cyclopropyl-6-fluoro-8-methoxy-1H-quinazoline-2,4-dione, δ = 11.18 (s, 1H), 8.10 (br, 3H), 7.26 (d, J = 12 Hz, 1H), 3.71 to 3.45 (m–overlap with H_2O, 8H), 3.13 (br, 1H), 2.89 (br, 2H), 2.05 (br, 1H), 1.70 (br, 1H), 0.91 (br, 2H), 0.53 (br, 2H).

NH_2-dione-a (UING5-209). 3-Amino-1-cyclopropyl-7-(3-ethylpiperazin-1-yl)-6-fluoro-8-methoxy-1H-quinazoline-2,4-dione, δ = 9.13 (m, 1H), 8.86 (m, 1H), 7.48 (d, J = 12 Hz, 1H), 4.49 (br–exchange with H_2O, 2H), 3.73 to 3.14 (m, 11H), 1.63 (m, 2H), 1.00 to 0.94 (m, 5H), 0.60 (m, 2H).

NH_2-dione-b (UING5-157). 3-Amino-1-cyclopropyl-6-fluoro-8-methoxy-7-[(*4aS*,*7aS*)-octahydro-6H-pyrrolo[3,4-b]pyridin-6-yl]-1H-quinazoline-2,4-dione, δ = 9.10 (m, 1H), 8.47 (m, 1H), 7.37 (d, J = 12 Hz, 1H), 6.54 (br, 2H), 4.04 (m, 1H), 3.87 to 2.90 (m, 9H), 2.61 (br, 1H), 1.86 to 1.65 (m, 4H), 1.05 (m, 1H), 0.89 (m, 1H), 0.65 (m, 1H), 0.50 (m, 1H).

NH_2-dione-c (UING5-159). 3-Amino-7-[(*S*)-3-aminopyrrolidin-1-yl]-1-cyclopropyl-6-fluoro-8-methoxy-1H-quinazoline-2,4-dione, δ = 8.17 (br, 3H), 7.37 (d, J = 13 Hz, 1H), 4.50 (br–exchange with H_2O, 2H), 3.89 to 3.54 (m, 5H), 3.51 (s, 3H), 3.29 (m, 1H), 2.25 (m, 1H), 1.99 (m, 1H), 0.99 to 0.93 (m, 2H), 0.63–0.57 (m, 2H).

NH_2-dione-d (UING5-207). 3-Amino-7-[(*S*)-3-aminomethylpyrrolidin-1-yl]-1-cyclopropyl-6-fluoro-8-methoxy-1H-quinazoline-2,4-dione, δ = 8.21 (br, 3H), 7.31 (m, 1H), 4.50 (br–exchange with H_2O, 2H), 3.67 to 3.24 (m, 9H), 2.86 (br, 2H), 2.06 (br, 1H), 1.69 (br, 1H), 0.91 (br, 2H), 0.54 (br, 2H).

Bacterial strains, growth conditions, and susceptibility determinations. *E. coli* K-12 strains, listed in Table 1, were constructed by P1-mediated transduction (34); they were grown in LB liquid medium and on LB agar plates at 37°C (18). To reduce efflux, each strain was deficient in *tolC* though insertion of transposon Tn*10*. For determination of MIC, cells were grown to mid-exponential phase, diluted to 10^{4-5} CFU/ml in tubes containing quinolone or dione, and incubated overnight at 37°C. Growth was determined by visual inspection, with the lowest quinolone or dione concentration that blocked growth being taken as MIC; the MIC_{99} was the antimicrobial concentration in agar that blocked colony formation by 99%. Lethal activity was assessed by incubation of exponentially growing liquid bacterial cultures with either dione or quinolone for the times and concentrations indicated in the figure legends, followed by dilution and enumeration of the CFU by plating on drug-free agar. Bacterial survival was expressed as a fraction of the CFU present at the time of drug addition.

Population analysis profiles. Population analysis was performed as previously described (43). Briefly, a series of agar plates was prepared in which the concentration of NH_2-dione-d, the cognate fluoroquinolone (FQ-d), or ciprofloxacin varied over a broad range. Wild-type *E. coli* (*gyr*$^+$, strain KD1397), grown to stationary phase in LB liquid medium, was applied to each plate in amounts that allowed a small number of colonies to form. These putative mutants were counted to obtain a preliminary score, and the colonies were transferred to drug-free agar for a second round of growth, followed by transfer to agar containing drug at the same concentration used initially for selection. Strains that showed growth of separated, individual colonies on drug-containing plates after transfer were scored as resistant mutants.

RESULTS

Effect of fluoroquinolone and quinazoline-2,4-dione structure on the ratio of MIC with a quinolone-resistant mutant (i.e., the MIC_{mutant}) to MIC with the wild type (*gyr*$^+$) (MIC_{wt}). MIC was determined for the 8-methoxy-quinazoline-2,4-diones and corresponding fluoroquinolones shown in Fig. 1 using wild-type *E. coli* and 12 isogenic quinolone-resistant mutants (listed in Table 1; in the present study the wild-type strain carried a deficiency of *tolC* to reduce dione efflux). When the

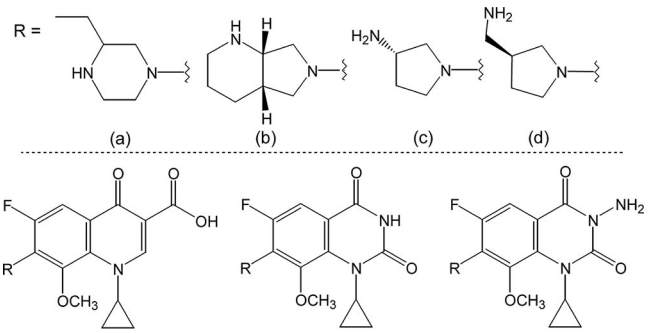

FIG. 1. Structures of 8-methoxy-quinazoline-2,4-diones and corresponding 8-methoxy fluoroquinolones.

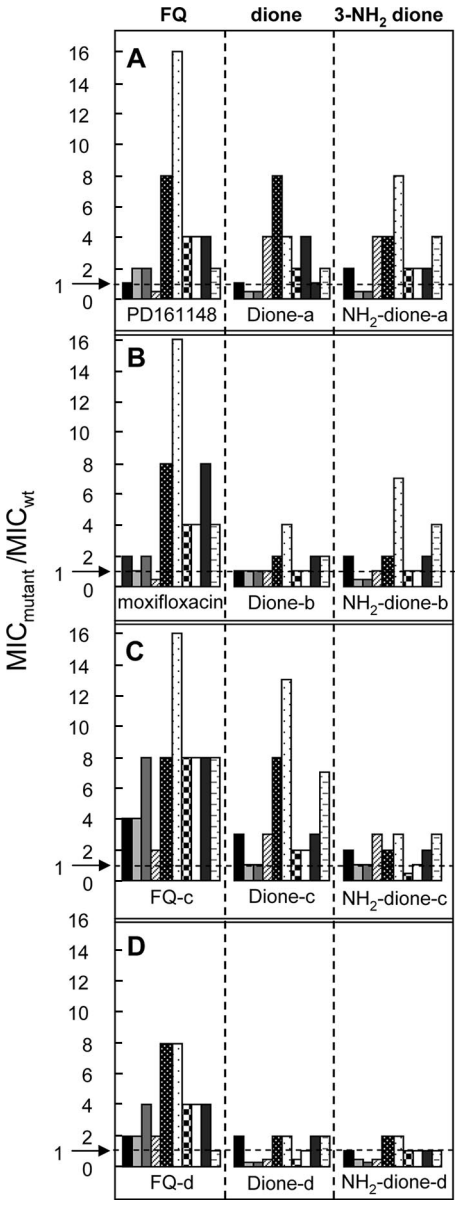

FIG. 2. Bacteriostatic activity of 8-methoxy-quinazoline-2,4-diones and corresponding 8-methoxy fluoroquinolones with wild-type and quinolone-resistant *gyrA* mutants of *E. coli*. Isogenic, *tolC*-deficient strains of *E. coli* containing alleles of *gyrA* were incubated overnight in LB broth containing the indicated quinazoline-2,4-dione or its cognate fluoroquinolone to determine MIC. The MIC_{mutant}/MIC_{wt} (strain KD1397) ratio was determined for each mutant. A ratio of 1 is indicated by an arrow and broken line. The GyrA amino acid substitutions, arranged left to right, were A51V, A67S, G81C, D82A, S83L, S83W, A84P, D87Y, D87N, and A106H. The MICs for wild-type cells were 0.0078, 0.031, 0.016, and 0.004 μg of moxifloxacin, PD161448, FQ-c, and FQ-d/ml, respectively. Panels are arranged according to C-7 ring structure: A, 2-ethyl piperazinyl; B, diazobicyclo; C, (*S*)-3-aminopyrrolidinyl; D, (*S*)-3-aminomethyl pyrrolidinyl. Similar results were obtained in a replicate experiment.

ratio of MIC_{mutant} to MIC_{wt} with *gyrA* mutants was compared for PD161148 and two diones with the same C-7 ring structure (dione-a and NH_2-dione-a), the two diones exhibited lower ratios with most mutants (Fig. 2A). Lower MIC_{mutant}/MIC_{wt} ratios were also observed with dione derivatives having the same C-7 group as moxifloxacin (dione-b and NH_2-dione-b, Fig. 2B), and subsequently the (*S*)-3-aminopyrrolidine C-7 moiety (dione-c and NH_2-dione-c, Fig. 2C). Changing the 3-H-dione core C-7-ring structure to (*S*)-3-aminomethyl pyrrolidine (dione-d) further lowered the MIC_{mutant}/MIC_{wt} ratio (Fig. 2D); this C-7 group also gave some reduction in the ratios against *gyrA* mutants for the corresponding fluoroquinolone, FQ-d (Fig. 2D). Introduction of the (*S*)-3-aminomethyl pyrrolidine group at C-7 of the 3-amino-8-methoxy-dione core provided NH_2-dione-d. This 3-amino dione displayed the lowest MIC_{mutant}/MIC_{wt} ratio against *gyrA* mutants, which was at or near 1 (Fig. 2D).

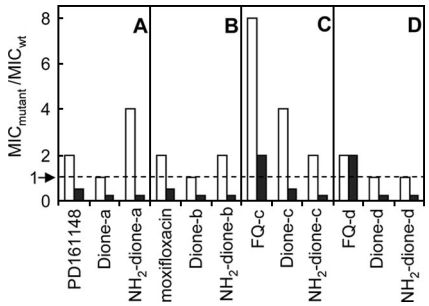

FIG. 3. Bacteriostatic activity of 8-methoxy-quinazoline-2,4-diones and corresponding 8-methoxy fluoroquinolones with wild-type and *gyrB* mutants of *E. coli*. The MIC_{mutant}/MIC_{wt} ratio was determined for the two *gyrB* mutants as in Fig. 2 (strain KD2932 [D426N], □; strain KD2934 [K447E], ■). The compounds tested are indicated in the figure. A ratio of 1 is indicated by an arrow and broken line. Similar results were obtained in a replicate experiment.

Low MIC_{mutant}/MIC_{wt} ratios were also observed with diones against *gyrB* mutants (Fig. 3). with the GyrB K447E variant, MIC_{mutant}/MIC_{wt} ratios for the four fluoroquinolones revealed that two parent quinolones were more active (ratio < 1) against the *gyrB* mutant (PD161148 and moxifloxacin) and two were less active (ratio > 1, FQ-c and FQ-d). In contrast, each of the 2,4-dione derivatives afforded MIC_{mutant}/MIC_{wt} ratio below 1. With the GyrB D426N variant, the MIC_{mutant}/MIC_{wt} ratios for the four fluoroquinolones ranged from 2 to 8, while the ratios for 2,4-diones ranged from 1 to 4. However, for the diones that were optimal against *gyrA* mutants, dione-d and NH_2-dione-d, MIC_{mutant}/MIC_{wt} ratios against both *gyrB* mutants were similarly at or below 1 (Fig. 3D). Thus, preparation and evaluation of even a small series of 2,4-diones revealed an ability to identify diones that eliminate the capability of known gyrase mutations to raise MIC of *E. coli*.

Effect of quinazoline-2,4-dione structure on absolute MIC. *E. coli* is not very susceptible to quinazoline-2,4-diones (2). The MICs for dione derivatives with C-7 groups as found in PD161148 and moxifloxacin were ≥10 μg/ml (Fig. 4). Changing the C-7 ring to (*S*)-3-aminopyrrolidine or (*S*)-3-aminomethyl pyrrolidine lowered the dione MIC. The greatest lowering of the MIC was observed with the introduction of these C-7 groups into diones bearing a 3-amine group (NH_2-dione-c and NH_2-dione-d). In this situation wild-type MIC was improved by ~20-fold (Fig. 4). Thus, changes that lowered the ratio of mutant to wild-type MIC also lowered the absolute MIC.

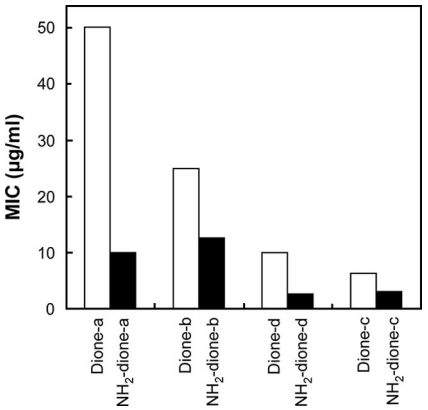

FIG. 4. Bacteriostatic activity of 8-methoxy-quinazoline-2,4-diones. The MIC was determined with wild-type *E. coli* for each of the compounds indicated in the figure. Bars: □, 3-H diones; ■, 3-NH_2 diones. Similar results were obtained in a replicate experiment.

Lethal activity of diones. The effect of 3-amino-8-methoxy-quinazoline-2,4-dione structure on lethal activity was examined initially by comparison with the cognate 8-methoxy fluoroquinolone using wild-type *E. coli*. When NH_2-dione-a and PD161148 were compared for the rate of rapid killing at 10× the MIC, the fluoroquinolone was about two times faster (Fig. 5A). When survival was measured at a variety of concentrations during an incubation of 2 h, NH_2-dione-b was 10-fold less lethal at a high concentration (Fig. 5B). NH_2-dione-d, the dione exhibiting the lowest MIC and MIC ratio, was more lethal than NH_2-dione-a (Fig. 5C and D). Since the compounds differ only in C-7 ring structure, that moiety contributes to dione lethality. Lethal activity for NH_2-dione-d, relative to its cognate fluoroquinolone (FQ-d), was similar (Fig. 5E) or slightly higher (Fig. 5F). We also compared the lethal activity of NH_2 dione-d with dione-d. The two exhibited equal rates of killing at 10× the MIC; NH_2-dione-d was more lethal at lower dione concentrations (data not shown). Thus, the 8-methoxy quinazoline-2,4-diones kill *E. coli* rapidly.

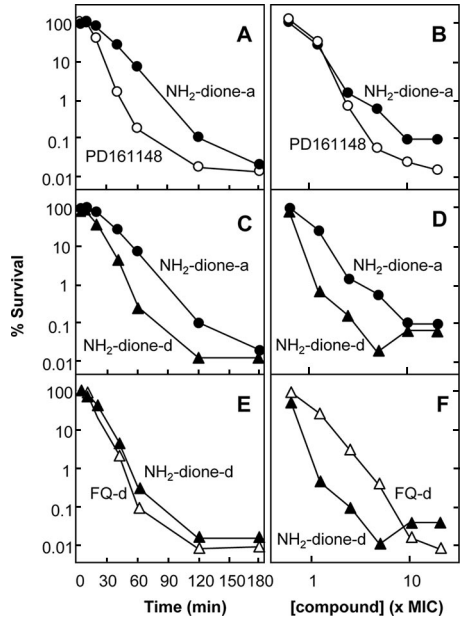

FIG. 5. Bactericidal activity of 8-methoxy-quinazoline-2,4-diones with wild-type *E. coli*. Exponentially growing *E. coli* (strain KD1397) was incubated with fluoroquinolone or quinazoline-2,4-dione for the indicated times at 10× the MIC (A, C, and E) or for 2 h at the indicated concentrations as a function of MIC (B, D, and F), and viable cell numbers were determined by plating on drug-free agar. Panels A and B show findings for PD161148 (○) and NH_2-dione-a (●). Panels C and D show findings for NH_2-dione-a (●) and NH_2-dione-d (▲). Panels E and F show findings for FQ-d (△) and NH_2-dione-d (▲). Similar results were obtained in a replicate experiment.

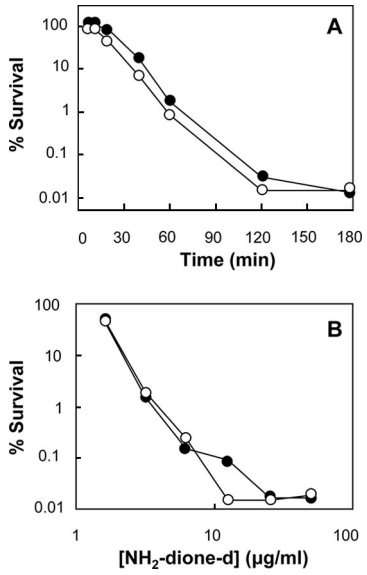

FIG. 6. Bactericidal activity of 8-methoxy-quinazoline-2,4-diones with wild-type *E. coli* and a *gyrA* resistant mutant. (A) Rate of killing. Exponentially growing cultures of wild-type *E. coli* (strain KD1397, ○) or a GyrA D87Y variant (strain KD2866, ●) were incubated for the indicated times with NH_2-dione-d at 10× the MIC (25 μg/ml for both strains). Aliquots were removed, diluted, and plated on drug-free LB agar for determination of viable cells. (B) Effect of quinazoline-2,4-dione concentration. Exponentially growing cultures of wild-type *E. coli* (○) or a GyrA D87Y variant (●) were incubated with the indicated concentrations of NH_2-dione-d for 2 h. Similar results were obtained in a replicate experiment.

We next examined the ability of the most bacteriostatic dione, NH_2-dione-d, to kill a GyrA D87Y variant (the MIC of this variant equaled wild-type MIC, which allowed a direct comparison of the two strains). As shown in Fig. 6A, NH_2-dione-d killed mutant and wild-type cells at an equal rate at a concentration of 10× the MIC. Lethal action of NH_2-dione-d at various concentrations was also similar against both wild-type and *gyrA* resistant strains (Fig. 6B). Thus, the presence of a *gyrA* resistance mutation does not affect the ability of this dione to block growth or kill *E. coli*.

Selection of resistant mutants. To examine the ability of the most active 2,4-dione to restrict the selection of resistant mutants, population analysis was performed with wild-type *E. coli* (strain KD1397). Cells grown to stationary phase were applied in various numbers to agar plates containing various concentrations of either the NH_2-dione-d or fluoroquinolone, and colonies were obtained after incubation at 37°C. To estimate the fraction of input cells that formed resistant colonies, colonies were counted after incubation for 2 days, transferred to

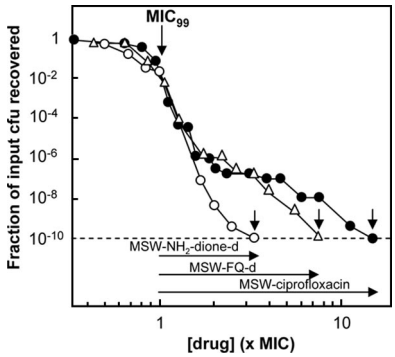

FIG. 7. Effect of dione and fluoroquinolone concentration on the recovery of resistant mutants. *E. coli* strain KD1397 (*gyr*$^+$) was applied to agar plates containing the indicated concentrations of NH_2-dione-d (○), FQ-d (△), or ciprofloxacin (●) expressed as a function of the MIC_{99} (0.7, 0.004, and 0.006 μg/ml, respectively). After incubation, the fraction of input CFU recovered as colonies that regrew on the selecting drug concentration was determined. Mutant selection windows (MSW) are indicated at the bottom of the figure; unlabeled arrows indicate MPCs.

drug-free agar, and then retested for growth on NH_2-dione-d or fluoroquinolones at the concentration initially used for mutant selection. The fraction that tested positive by retest was used to correct the initial colony counts. The point at which a population analysis profile intersects the dashed line in Fig. 7 represents the MPC as a multiple of MIC_{99} (unlabeled arrows). Mutants were selected over a narrower concentration range for NH_2-dione-d than for its cognate fluoroquinolone (FQ-d) or for ciprofloxacin (Fig. 7). Thus, compounds with a low ratio of MIC_{mutant} to MIC_{wt} also have a low ratio of MPC to MIC.

DISCUSSION

According to the mutant selection window hypothesis, three ways exist to restrict the emergence of resistance: (i) maintain the drug concentration above the window, (ii) use combination therapy involving two or more agents of different classes, and (iii) eliminate the window (MIC = MPC). The third idea has been explored using "dual-targeting" fluoroquinolones (reference 23 and references therein) and by eliminating one of the two targets to broaden the selection window (12). In the present study, we synthesized a series of quinolone-like molecules and approximated MPC by measuring MIC with a collection of isogenic quinolone-resistant gyrase mutants. Synthesis and evaluation of 8-methoxy-quinazoline-2,4-dione derivatives and cognate fluoroquinolones (structures in Fig. 1) identified analogs for which the ratio of MIC_{mutant} to MIC_{wt} approached unity. This antimutant strategy contrasts with the traditional quinolone-discovery approach in which lowering the MIC with various wild-type organisms is the goal (MIC-based examples for quinazoline-2,4-diones have been reported recently) (7, 32).

For the antimutant strategy described here, initial lead compounds need have only modest wild-type MICs in order to identify the structural features of subsequent compounds that contribute to lowering the MIC_{mutant}/MIC_{wt} ratio. Ultimately, structure-activity relationships from both traditional MIC-lowering studies and antimutant studies, as described above, are expected to provide combined structural elements that lead to new compounds that are highly active against both wild-type and mutant cells. Issues of bioavailability and toxicity remain to be considered.

Our results demonstrate that quinazoline-2,4-diones can be modified to drastically reduce the protective effects of quinolone-resistant mutations in *gyrA* and *gyrB* of *E. coli* (Fig. 2 and 3). Since specific structural features of quinolones contribute to rapid lethality (9, 15, 17), it was important to test 2,4-diones for that activity. The most bacteriostatic dione in the present study, NH_2-dione-d, exhibited rapid lethality that was similar to fluoroquinolones when normalized to MIC to correct for drug uptake and/or efflux (Fig. 5); moreover, lethal activity was not affected by a *gyrA* mutation (Fig. 6). We conclude that the 2,4-diones retain the lethal activity typical of fluoroquinolones.

Two structural features of the 8-methoxy-quinazoline-2,4-diones studied here were important for antimutant activity: the C-7 ring structure and the amine at position 3. The 3-amino group increased antimutant activity to where *gyrA* resistance alleles had almost no effect. In some cases, mutants were more susceptible than wild-type cells, particularly with the nalidixic acid-resistant GyrB variants (hypersusceptibility has been previously observed with the GyrB Lys-447 to Glu variant) (38). A role for the structure of the C-7 ring was not surprising, since that has been observed with fluoroquinolones (28, 38, 41). In the present study the 3-aminomethyl pyrrolidine C-7 ring conferred more activity to diones than the piperazinyl ring of PD161148 or the diazobicyclo ring system of moxifloxacin. Several other highly active quinolone derivatives (e.g., clinafloxacin and sitafloxacin) also contain a substituted pyrrolidine at C-7 (23). Detailed explanations for these and other dione features await a structural model for fluoroquinolone-gyrase-DNA binding.

We used 8-methoxy substituted 2,4-diones because 8-methoxy fluoroquinolones, such as moxifloxacin, have been ascribed superior antimutant activity for the fluoroquinolones (4, 42). The 8-methoxy group on fluoroquinolones also contributes to the rapid killing of cells in the absence or presence of ongoing protein biosynthesis (16). However, previous MIC-based investigations, primarily against wild-type and MDR gram-positive organisms, demonstrated that 8-methoxy-quinazoline-2,4-diones typically have higher MICs than the corresponding 8-methyl derivatives (6, 7, 32). That result contrasts with 8-methoxy and 8-methyl fluoroquinolone derivatives (otherwise structurally identical) having similar MICs (19, 39). Thus, while C-7 variants of 8-methyl-quinazoline-2,4-diones are expected to possess lower MICs than corresponding 8-methoxy derivatives against wild-type strains, the 8-methoxy-quinazoline-2,4-diones were anticipated to be better lead compounds for structural modification to lower the MIC_{mutant}/MIC_{wt} ratio and to achieve rapid killing of mutant cells. The present study identified 8-methoxy-2,4-diones with excellent antimutant activity, lethality against quinolone-resistant mutants, and a narrow mutant selection window. Thus, inherent MIC must not be a limiting factor in choosing lead compounds for antimutant SAR studies. Studies are in progress to determine whether analogous 8-methyl diones exhibit antimutant and rapid killing activities similar to the 8-methoxy diones. A positive finding would demonstrate the convergence of MIC-

based studies and antimutant-based studies to afford new lead compounds against wild-type and mutant cells; a negative finding would demonstrate divergence of MIC-based SAR and antimutant-based SAR, at least within this initial, small set of compounds.

One limitation of this study is that a single strain and derivate mutants of one species were used. Nevertheless, the principles of the antimutant approach should apply to many other species once isogenic batteries of resistant mutants are available. Indeed, preliminary work indicates that the 2,4-diones behave in a similar way with *E. coli* and *Mycobacterium smegmatis* (M. Malik et al., unpublished observations).

The data described above support the mutant selection window hypothesis: as predicted by the window hypothesis, a compound (NH_2-dione-d) with a low MIC_{mutant}/MIC_{wt} ratio will select resistant mutants from a wild-type culture over a narrower drug concentration range than a compound (FQ-d) exhibiting a higher ratio (Fig. 7; a vertical response is expected to be optimal). The population heterogeneity observed for ciprofloxacin in Fig. 7 was expected, since in similar experiments a variety of different fluoroquinolone-resistant mutants were recovered (43). Analysis of dione-resistant mutants, which has been performed with *S. pneumoniae* (7), reveals alterations in *gyrB* and *parE*. Studies are in progress to characterize dione-resistant *E. coli* mutants in order to contrast such mutants with the *gyrB* mutants examined above that have an MIC_{mutant}/MIC_{wt} ratio at or below 1. New mutants will then be added to the panel of test strains to further refine the compound selection process.

In conclusion, use of the antimutant approach should produce new lead structures that have a very narrow mutant selection window (41); this should help restrict the amplification of resistant mutants (40). Such compounds should allow less enrichment of resistant mutants than agents that kill only susceptible cells. Both features should help restrict the emergence of resistance in ways not considered by criteria used in traditional approaches.

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