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Suppression of gyrase-mediated resistance by C7 aryl fluoroquinolones

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ABSTRACT

Fluoroquinolones form drug-topoisomerase-DNA complexes that rapidly block transcription and replication. Crystallographic and biochemical studies show that quinolone binding involves a water/metal-ion bridge between the quinolone C3-C4 keto-acid and amino acids in helix-4 of the target proteins, GyrA (gyrase) and ParC (topoisomerase IV). A recent cross-linking study revealed a second drug-binding mode in which the other end of the quinolone, the C7 ring system, interacts with GyrA. We report that addition of a dinitrophenyl (DNP) moiety to the C7 end of ciprofloxacin (Cip-DNP) reduced protection due to resistance substitutions in *Escherichia coli* GyrA helix-4, consistent with the existence of a second drug-binding mode not evident in X-ray structures of drug-topoisomerase-DNA complexes. Several other C7 aryl fluoroquinolones behaved in a similar manner with particular GyrA mutants. Treatment of *E. coli* cultures with Cip-DNP selectively enriched an uncommon variant, GyrA-A119E, a change that may impede binding of the dinitrophenyl group at or near the GyrA-GyrA interface. Collectively the data support the existence of a secondary quinolone-binding mode in which the quinolone C7 ring system interacts with GyrA; the data also identify C7 aryl derivatives as a new way to obtain fluoroquinolones that overcome existing GyrA-mediated quinolone resistance.

INTRODUCTION

Fluoroquinolones are potent, broad-spectrum antimicrobials that trap DNA gyrase and DNA topoisomerase IV on DNA as drug–enzyme complexes (1,2). These complexes, which are called cleaved complexes because the DNA moiety is broken (3,4), block movement of RNA and DNA polymerases (5–7), thereby inhibiting bacterial replication (8,9) and transcription (10). At elevated concentrations, the quinolones also kill bacteria rapidly (11–13). As with other antimicrobials, clinical resistance to fluoroquinolones is seen among members of most pathogen species (14,15). Thus, we and others have sought derivatives that can bypass the effects of existing resistance mutations (16–20).

One approach for designing new derivatives is to understand in atomic detail how the quinolones interact with the target proteins. Towards that end X-ray crystallography has been performed with cleaved complexes (21–25). This work identifies a quinolone-binding site in which one end of the quinolone (the C7 ring system) is associated with the GyrB subunit of gyrase (ParE subunit of topoisomerase IV); the other end of the quinolone, the C3-C4 keto-acid moiety, lies near a magnesium ion and helix-4 of the GyrA subunit of gyrase (ParC subunit of topoisomerase IV). Subsequent biochemical studies support the idea that fluoroquinolones, through their C3-C4 keto-acid, form a stabilizing water-magnesium ion bridge with amino acids equivalent to *E. coli* GyrA-83 and GyrA-87 (18,26–28). The major resistance substitutions occur at these positions in GyrA and at equivalent positions in ParC (for examples in clinical isolates, see (29)). Moreover, formation of cleaved complexes with purified gyrase is sensitive to C7 ring changes in quinolone-class compounds (quinazolinediones) that can be countered by amino acid changes in GyrB (19). Thus,

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strong support exists for fluoroquinolone binding through the C3/C4-GyrA configuration observed by X-ray crystallography.

Evidence for an additional quinolone binding mode derives from data consistent with an inverted interaction between the quinolone and gyrase such that the C7 ring interacts with amino acids near GyrA helix-4 rather than with amino acids in GyrB. In an early example, the presence of an ethyl substituent on the distal end of a fluoroquinolone C7 piperazinyl ring raised minimal inhibitory concentration (MIC) for a GyrA substitution in *Mycobacterium smegmatis*. We speculated that steric hindrance occurred between the C7 moiety and GyrA helix-4 (30). More recently, we observed crosslinking between a C7 chloroacetyl derivative of ciprofloxacin (Cip-AcCl) and a Cys substitution in *E. coli* GyrA helix-4 (GyrA-G81C) (31). We also observed crosslinking between Cip-AcCl and GyrB-466C, which was expected from X-ray structures. Since GyrA-81 and GyrB-466 are far apart (17 Å), crosslinking of Cip-AcCl to both Cys substitutions indicates that fluoroquinolones bind to gyrase in at least two ways.

We reasoned that the secondary binding mode might allow particular substituents at the C7 end of quinolones to preferentially interact with GyrA, which would bypass the requirement for bridging between the other end of the quinolone, the C3/C4 moiety, and helix-4 of GyrA. Such a bypass would be seen as a reduction in protection afforded by the well-known resistance substitutions that map in GyrA helix-4 and participate in the water-magnesium ion bridge. We designed and synthesized a new set of quinolones that contained bulky, and in some cases charged, aryl substituents attached to the distal nitrogen of the C7 piperazinyl ring of ciprofloxacin, a clinically important fluoroquinolone. When we examined activity with a collection of quinolone-resistant gyrase mutants of *E. coli*, we found that addition of a dinitrophenyl group (DNP) lowered the extent to which many classic resistance mutations protect against fluoroquinolone-mediated poisoning of gyrase. These data provide functional relevance to a second mode for binding of quinolone to gyrase and thereby open a new avenue into understanding quinolone-gyrase biology. Moreover, the observations offer a novel platform for designing new quinolones that bypass existing fluoroquinolone resistance.

MATERIALS AND METHODS

Bacterial strains, growth conditions and susceptibility assays

Escherichia coli K-12 strains, listed in Table 1, were constructed by P1-mediated transduction (32). They were grown in LB liquid medium and on LB agar plates at 37°C (33). To reduce efflux, each strain was deficient in *tolC* through insertion of transposon Tn10. For determination of MIC, cells were grown to mid-exponential phase, diluted to 10^4–10^5 cfu in 1 ml aliquots containing quinolone varying in concentration by 2-fold increments and incubated overnight at 37°C. Growth was determined by visual inspection; the lowest quinolone concentration that blocked growth was taken as MIC.

Mutant prevention concentration (MPC) was measured as previously described (17,34). Briefly, a series of agar plates was prepared in which the concentration of quinolone varied over a broad range. *Escherichia coli* strain KD1397 (wild-type gyrase, *tolC*::Tn10), 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 frequency of resistance. The putative resistant colonies were then transferred to drug-free agar for a second round of growth, followed by transfer to agar containing antibacterial 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 (almost all presumptive mutants scored positive by this test). MPC was estimated as the lowest antibacterial concentration that restricted formation of all colonies when 10^{10} cfu were applied to agar plates.

Fluoroquinolones

Fluoroquinolone derivatives are listed in Table 2; structures and compound identifiers are shown in Figure 1. Norfloxacin and levofloxacin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA); moxifloxacin and ciprofloxacin were from Bayer Healthcare (West Haven, CT, USA). C7 aryl-modified quinolones were prepared using variations of known synthetic methods for similar compounds. Briefly, Cip-DNP, Cip-2NP, Cip-4NP, Cip-P, Cip-DFP, Cip-DAP, Cip-DMP, Cip-DOMeP and Cip-DCP were prepared by coupling the corresponding N-aryl piperazine to 7-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-quinolone-3-carboxylic acid. CipEt-DNP was similarly prepared using DNP-substituted ethylenediamine. Bay-DNP and Mox-DNP were prepared using the same quinolone core by first coupling with *cis*-octahydropyrrolo[3,4b]pyridine followed by arylation of the resulting C7 ring with 1-chloro-2,4-dinitrobenzene. Lev-DNP, Nor-DNP, Nap-DNP and CipOMe-DNP were prepared by coupling DNP-piperazine with the corresponding C7-Cl or C7-F quinolone core structure. Detailed procedures for construction and characterization of each derivative are provided in Supplementary Data.

For studies with cultured cells, fluoroquinolones and aryl-derivatives were dissolved in dimethyl sulfoxide to a final concentration of 10 mg/ml for testing. Dilutions were prepared with sterile distilled water, and solutions were kept at –20°C for several weeks during testing.

Cleaved-complex formation assays

The mutant *gyrA* G81C gene was constructed using the overlap extension PCR technique (35) and cloned into the pET-11c vector (36). The GyrA-81Cys protein was expressed in *E. coli* BL21(DE3)(36). Mutant and wild-type GyrA and wild-type GyrB proteins were purified as described (37,38). The wild-type ParC and ParE subunits of *E. coli* topoisomerase IV were expressed and purified separately, and active topoisomerase IV was reconstituted as described previously (39).

DNA cleavage reaction mixtures (25 μl) contained 50 mM Tris-HCl (pH 8.0 at 23°C), 10 mM $MgCl_2$, 50 mg/l bovine serum albumin, 1 mM ATP, 5 mg/l tRNA, 5 nM (or

Table 1. Bacterial strains used in the study

Strain number	Relevant genotype	Reference
KD1397	DM4100 *tolC6*::Tn10 *gyr*$^+$ (wild type)	(17)
KD2862	KD66 *tolC6*::Tn10 *gyrA* (S83L)	(17)
KD2864	KD1975 *tolC6*::Tn10 *gyrA* (A84P)	(17)
KD2866	KD1977 *tolC6*::Tn10 *gyrA* (D87Y)	(17)
KD2876	KD1909 *tolC6*::Tn10 *gyrA* (S83W)	(17)
KD2878	KD1911 *tolC6*::Tn10 *gyrA* (A67S)	(17)
KD2880	KD1913 *tolC6*::Tn10 *gyrA* (D87N)	(17)
KD2882	KD1915 *tolC6*::Tn10 *gyrA* (G81C)	(17)
KD2884	KD1917 *tolC6*::Tn10 *gyrA* (Q106H)	(17)
KD2886	KD1721 *tolC6*::Tn10 *gyrA* (A51V)	(17)
KD2932	KD1500 *tolC6*::Tn10 *gyrB* (D426N)	(17)
KD2934	KD1502 *tolC6*::Tn10 *gyrB* (K447E)	(17)
KD2955	KD1973 *tolC6*::Tn10 *gyrA* (D82A)	(17)
KD3253	KD1397 *tolC6*::Tn10 *gyrA* (A119E)	[a]This work

[a]A spontaneous mutant at 2 x MIC of Cip-DNP was obtained, and the gyrase mutation was moved into KD1397 by P1-mediated transduction by introducing nearby kanamycin-resistance marker.

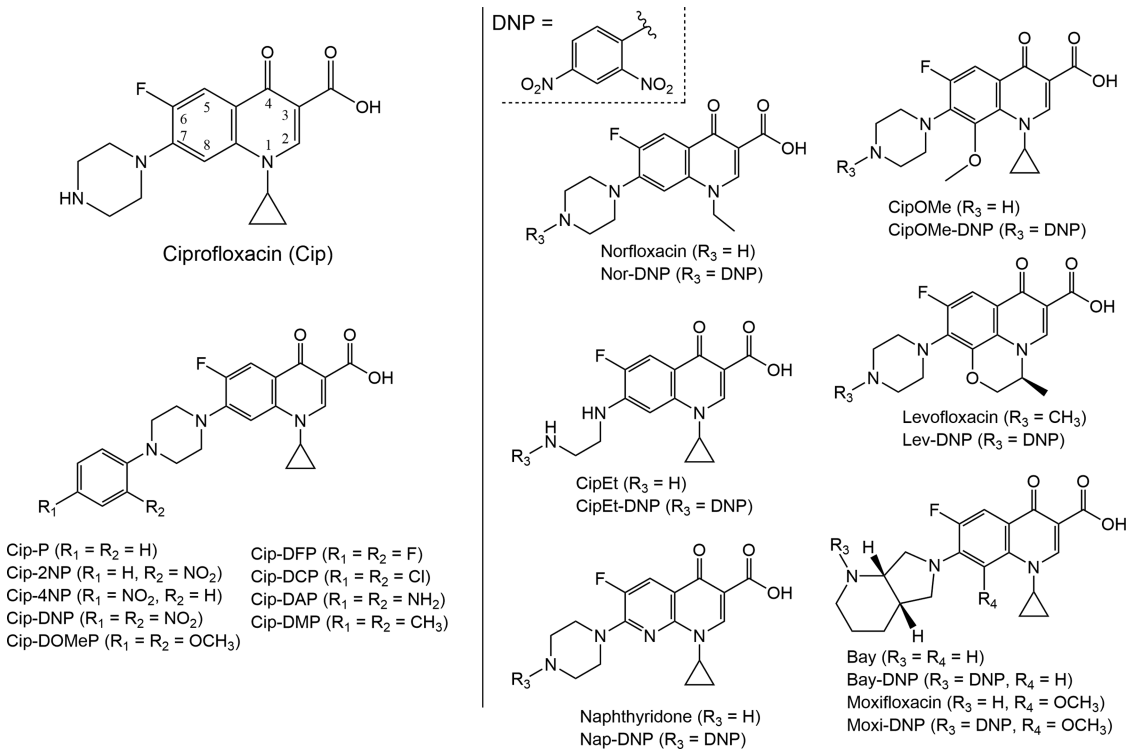

Figure 1. Structures of fluoroquinolones used in the study. Fluoroquinolones and related compounds are composed of two core rings with numbering of core positions shown for ciprofloxacin.

100 fmol) of plasmid pBR322 DNA, 7.5 nM (or 150 fmol) of wild-type gyrase, 25 nM (or 500 fmol) of GyrA-81C gyrase or 7.5 nM (or 150 fmol) of topoisomerase IV and the indicated concentrations of fluoroquinolone. Mixtures were incubated at 37°C for 15 min, and then sodium dodecyl sulfate was added to a concentration of 1% w/v followed by additional incubation at 37°C for 10 min. EDTA and proteinase K were then added to 50 mM and 100 mg/l, respectively, and incubation was continued for an additional 15 min at 37°C. The DNA products were purified by extraction with phenol/chloroform/isoamyl alcohol and then analysed by electrophoresis through vertical 1.2% w/v agarose gels at 2 V/cm for 15 h in TAE [50 mM Tris-HCl (pH 7.9 at 23°C), 40 mM sodium acetate and 1 mM EDTA] buffer that contained 0.5 mg/l ethidium bromide. After destaining in water, gels were photographed and quantified using an Eagle Eye II system (Agilent Technologies, Santa Clara, CA, USA).

Molecular modelling

The structure of fluoroquinolone-topoisomerase cleaved complex was obtained from Protein Data Bank access number 2XKK (24) using the C8-H and C7 ring structure of ciprofloxacin rather than moxifloxacin. Placement of ciprofloxacin and its derivatives in subunit bridging and in-

Table 2. MIC of compounds with wild-type *E. coli*[a]

Compound name	Compound description	Designation	MIC (μM)	MIC relative to parental MIC
Ciprofloxacin	-	Cip	0.015	-
UIJR-002–7A	2-Nitrophenyl ciprofloxacin	Cip-2NP	0.035	2
UIJR-002–12A	4-Nitrophenyl ciprofloxacin	Cip-4NP	0.017	1
UIJR-002–10A	Phenyl ciprofloxacin	Cip-P	0.077	5
UIJR-002–9A	2,4-dinitrophenyl ciprofloxacin	Cip-DNP	0.031	2
UIJR-002–60-A	2,4-difluorophenyl-ciprofloxacin	Cip-DFP	1.24	82
UIJR-002–61-A	2,4-dimethylphenyl-ciprofloxacin	Cip-DMP	1.24	82
UIHS-IIa-67-A	2,4-diaminophenyl-ciprofloxacin	Cip-DAP	0.080	5
UIJR-002–65-A	2,4-dimethoxyphenyl-ciprofloxacin	Cip-DOMeP	1.18	78
UIBW1–066	2,4-dichlorophenyl ciprofloxacin	Cip-DCP	11.7	780
Moxifloxacin	-	Mox	0.025	-
UIHS-IIa-241	2,4-dinitrophenyl moxifloxacin	Mox-DNP	0.028	1
Norfloxacin	-	Nor	0.094	-
UIJR-002–16A	2,4-dinitrophenyl norfloxacin	Nor-DNP	0.41	4
Levofloxacin	-	Lev	0.022	-
UIJR-002–13A	2,4-dinitrophenyl levofloxacin	Lev-DNP	0.029	1
Bay y 3114	diazabicyclo-cipcore/des methoxy moxifloxacin	Bay	0.022	-
UIJR-002–69-A	2,4-dinitrophenyl-diazabicyclo-ciprofloxacin core	Bay-DNP	0.58	27
desethylene-ciprofloxacin[b]	-	CipEt	1.31	-
UIKLK-1–30-A	2,4-dinitrophenyl-desethylene-ciprofloxacin core	CipEt-DNP	1.33	1
UIHS-IIa-99	Naphthyridone [8N-Cip]	Nap	0.048	-
UIJR-002–17A	2,4-dinitrophenyl naphthyridone [8N-Cip]	Nap-DNP	0.125	3
UIHS-IIa-61–101	8-methoxy ciprofloxacin	CipOMe	0.011	-
UIJR-002–18A	2,4-dinitrophenyl 8-methoxy ciprofloxacin	CipOMe-DNP	0.059	5

[a]Strain KD1397.
[b]Purchased from Toronto Research Chemicals, Inc., 2 Brisbane Rd., North York, Ontario.

verted modes was performed manually on the 2XKK structure of topoisomerase IV-containing cleaved complexes using WebLab ViewerLight.

RESULTS

C7 aryl substituents of fluoroquinolones vary widely in effects on *E. coli* growth inhibition

To provide a baseline for comparison of compounds with gyrase mutants, we began by surveying activity with a gyr^+ culture of *E. coli*. A variety of compounds was synthesized in which aryl substituents were attached to the C7 ring system of ciprofloxacin and other commercially available fluoroquinolones (for structures see Figure 1). Most aryl substituents increased the MIC of wild-type cells (Table 2). An extreme example was seen with addition of dichlorophenyl to ciprofloxacin (Cip-DCP; MIC increased ∼800-fold). Difluorophenyl (Cip-DFP), dimethylphenyl (Cip-DMP), and dimethoxyphenyl (Cip-DOMeP) also increased MIC significantly (80-fold). A much smaller effect was seen with 2-nitrophenyl (Cip-2NP), 4-nitrophenyl (Cip-4NP), 2,4-diaminophenyl (Cip-DAP), 2,4-dinitrophenyl (Cip-DNP), and phenyl (Cip-P) derivatives that ranged from no effect to a 5-fold increase in MIC. Thus, our initial screen revealed a wide range of effects caused by extending ciprofloxacin via addition of an aryl moiety to the N4 position of the ciprofloxacin C7 piperazinyl ring. Some substituents, such as the dinitrophenyl group (DNP), had little effect on wild-type MIC and became the focus of the study.

Our initial survey included compounds that also differed at other positions in addition to the C7 aryl extensions. For example, the DNP modification had no effect on the MIC of moxifloxacin, an 8-methoxy compound that has a C7 diazobicyclo group. However, when the 8-methoxy group was absent, as in Bay y 3114, addition of the DNP moiety raised MIC by almost 30-fold (Bay y 3114 versus Bay-DNP) (Table 2). The opposite was observed with ciprofloxacin-DNP: the DNP moiety increased MIC by 5-fold when an 8-methoxy was added to ciprofloxacin (CipOMe versus CipOMe-DNP). Thus, the ability of a fluoroquinolone to block bacterial growth is affected by the C7 ring system (diazobicyclo for moxifloxacin or piperazinyl for ciprofloxacin) that separates the DNP group from the C8 moiety (H or methoxy) located on a quinolone core ring.

We also observed smaller long-distance effects. For example, addition of a DNP moiety to norfloxacin increased MIC by 4-fold, while the increase was only 2-fold when DNP was added to ciprofloxacin. Norfloxacin and ciprofloxacin differ only at the N1 position: norfloxacin has an ethyl moiety and ciprofloxacin a cyclopropyl. A previous energy minimization study indicated that moieties at positions 1 and 8 may restrict the rotation at C7 (40), a factor that could affect interactions between C7 aryl substituents and gyrase.

C7 piperazinyl dinitrophenyl group overcomes the protective effect of gyrase resistance mutations

We next examined the effect of the C7 aryl groups on growth of fluoroquinolone-resistant mutants. For these studies we measured MIC with a set of gyrase mutants (Table 1) that had the same genetic background, and then we normalized the result to MIC of the parental bacterial strain (such normalization reduces effects of factors such as drug uptake and efflux, which may differ among the test compounds). The resulting MIC ratio (mutant to wild type) reflects the ability of a particular amino acid substitution in gyrase

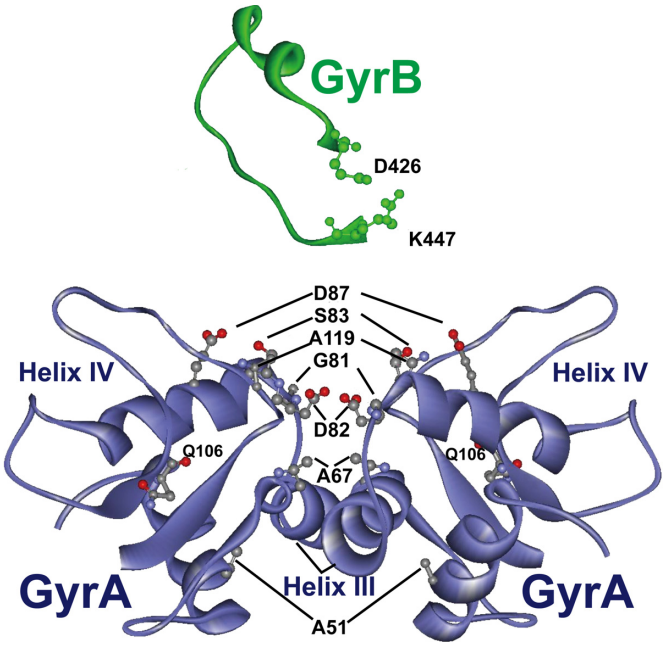

Figure 2. Location of resistance substitutions in gyrase. The figure shows the quinolone-binding region of GyrA adapted from the topoisomerase IV cleaved-complex structure of (24). The locations of resistance substitutions used in the present work are indicated using the *E. coli* GyrA numbering system.

to interfere with the inhibitory properties of a given fluoroquinolone at the level of cleaved-complex formation. A low ratio indicates that the test compound has strong antimutant activity. We emphasize that MIC and normalized MIC are used for different purposes: the former identifies compounds likely to have clinical utility and reveals clinical resistance, while the latter reflects drug-gyrase interactions within quinolone-gyrase-DNA complexes. The structure depicted in Figure 2 locates amino acid changes associated with reduced fluoroquinolone susceptibility ('resistance') that were used in the present work.

When we examined ciprofloxacin for activity with mutant strains, we saw that GyrA substitutions known to confer clinical resistance had the highest values of mutant MIC normalized to wild-type MIC (Figure 3A), as seen in previous work (17). Addition of a DNP group to the C7 piperazinyl ring of ciprofloxacin produced a compound (Cip-DNP) that was surprisingly active with 10 GyrA and 2 GyrB variants (Figure 3B, the only exception was the GyrA Q106H variant [arrow labelled 106], which maps outside GyrA helix-4, the major quinolone-binding region). Related aryl compounds, such as a phenyl derivative or either 2- or 4-nitrophenyl derivatives of ciprofloxacin, behaved much like ciprofloxacin with the major resistance mutations (Figure 3C, D, and E). According to X-ray structures of cleaved complexes using other quinolones, a C7 aryl group is expected to interact with GyrB, not GyrA; thus, suppression of GyrA-mediated protection from Cip-DNP is not readily explained by existing X-ray structures.

C7 aryl substituents differ in activity with gyrase resistance mutants

Closer inspection and examination of additional compounds (Figures 3 and 4) identified a variety of differences among aryl derivatives. In the case of the 2,4-dinitrophenyl (Cip-DNP) and 2,4-difluorophenyl (Cip-DFP) derivatives of ciprofloxacin, we observed an overall increase in antimutant effect, while the activity of the unsubstituted phenyl derivative (Cip-P) and the mono-nitrophenyl compounds (Cip-2NP and Cip-4NP) depended on the particular resistance substitution. For example, only Cip-DNP and Cip-DFP exhibited improved antimutant activity with the major resistance determinants, but, relative to ciprofloxacin, many aryl groups increased activity with GyrA G81C (arrows labelled 81 in Figures 3 and 4). GyrA-81 is located on the GyrA–GyrA dimer interface where a GyrA-81-fluoroquinolone interaction might favour quinolone insertion into a secondary binding mode (see the Discussion).

Two other C7 phenyl derivatives also displayed selective antimutant activity. One, the 2,4-dichloro derivative (Cip-DCP), exhibited strong anti-mutant activity with the GyrA D82A strain (arrow labelled 82 in Figure 4F), but the compound had little activity with the other mutants (Figure 4F). This result appears to reflect a specific interaction between the chloro substituent and GyrA-82. Amino acid 82 is located at an end of GyrA helix-4; it does not participate in the water-Mg^{2+} bridge between fluoroquinolones and GyrA. The other example concerned the 2,4-dimethoxyphenyl derivative of ciprofloxacin (Cip-DOMeP). This compound showed exceptionally strong activity against the two GyrB mutants examined (unlabelled arrows, Figure 4E); however, little antimutant activity was observed with our panel of GyrA mutants. This result may be an example of an aryl derivative that interacts with gyrase largely in the primary binding mode revealed by X-ray crystallography but avoids the interference that these two GyrB substitutions normally exert on binding (compare with ciprofloxacin, Figure 3A). Thus, antimutant activity for some C7 derivatives is broad, while for others it is narrow.

We also replaced the phenyl group with a benzyl to insert a one-carbon spacer between the phenyl and the rings directly attached to fluoroquinolone position C7 (Supplementary Figure S1). With resistant mutants, compounds having the benzyl-containing C7 ring system exhibited slightly better activity than ciprofloxacin or moxifloxacin, but the benzyl derivatives (Cip-DNB and Mox-DNB) were far less active than Cip-DNP or Moxi-DNP (Supplementary Figure S2). By this criterion, extending the C7 aryl rings farther from the quinolone core does not improve antimutant activity.

Overall, aryl groups placed at the C7 end of fluoroquinolones have a variety of effects, many of which are not readily explained by quinolone binding at the site defined by X-ray crystallography.

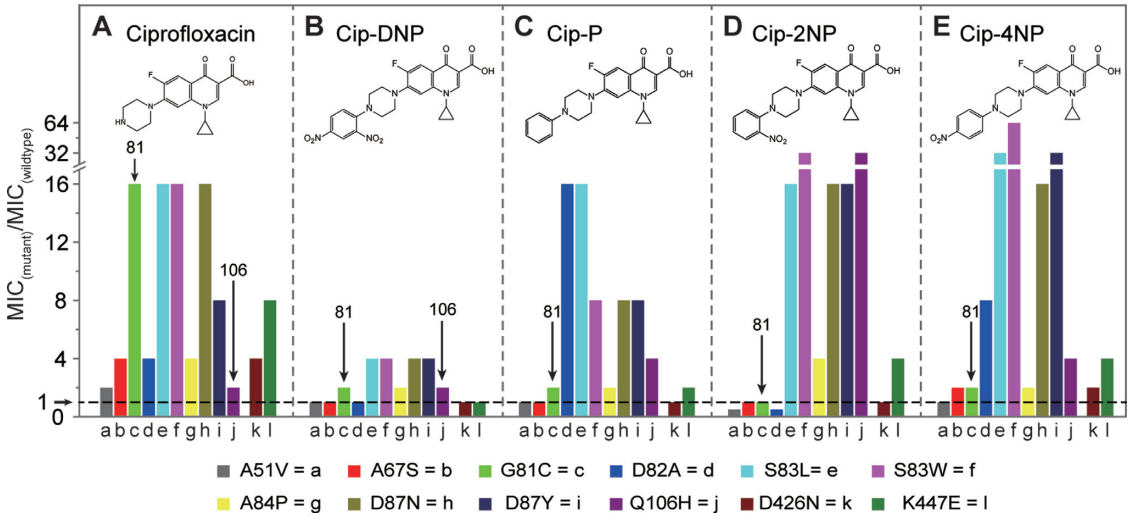

Figure 3. 2,4-dinitrophenyl derivative of ciprofloxacin has greater anti-mutant activity than related derivatives. The MIC_{mutant}/MIC_{wt} ratio was determined for the isogenic set of *gyrA* and *gyrB* mutants of *E. coli* listed in Table 1. A ratio of 1 is indicated by the arrow on the y axis and by the broken line. GyrA amino acid substitutions, arranged left to right, are A51V, A67S, G81C, D82A, S83L, S83W, A84P, D87N, D87Y and Q106H; at the right are GyrB variants D426N and K447E (amino acids 81 and 106 are indicated by labelled arrows; absolute MICs of compounds with the wild-type gyrase strain KD1397 are listed in Table 2). Test compounds are listed under each panel. Panel A: ciprofloxacin; panel B: 2,4-dinitrociprofloxcin (Cip-DNP); panel C, phenylciprofloxacin (Cip-P); panel D, 2-nitrociprofloxacin (Cip-2NP); panel E, 4-nitrociprofloxacin (Cip- 4NP). Similar results were obtained in replicate experiments.

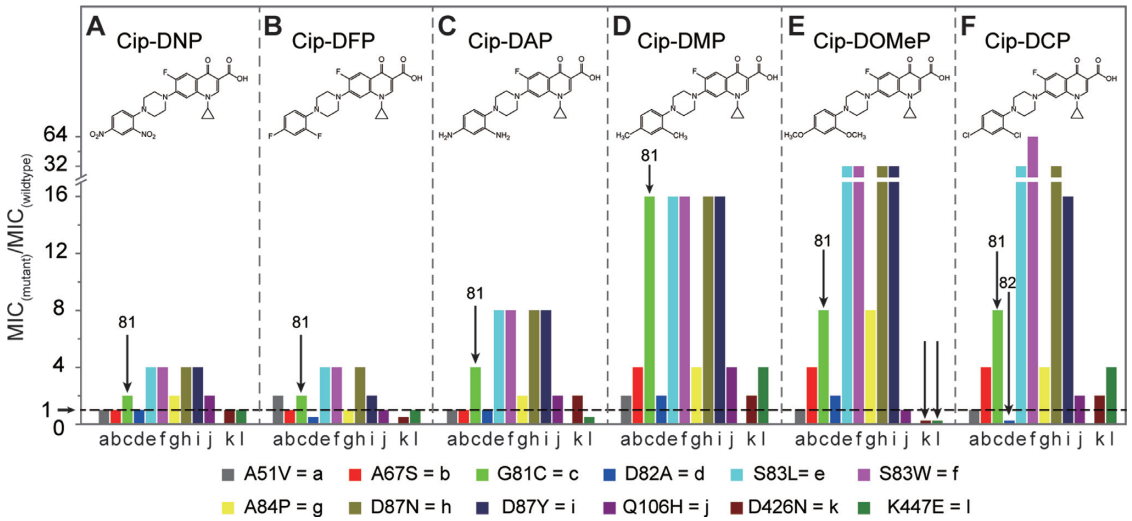

Figure 4. Anti-mutant activity of 2,4-nitrophenyl ciprofloxacin relative to other 7-aryl derivatives. MIC for C7-aryl derivatives of ciprofloxacin with mutant and wild-type *E. coli* was determined and displayed as described in Figure 3. Panel A: 2,4-dinitrophenyl (Cip-DNP); panel B: 2,4-difluorophenyl (Cip-DFP); panel C: 2,4-diamino (Cip-DAP); panel D: 2,4-dimethyl (Cip-DMP); panel E: 2,4-dimethoxy (Cip-DOMeP); panel F: 2,4-dichloro (Cip-DCP). Amino acids 81 and 82 are indicated by labelled arrows; unlabelled arrows indicate GyrB substitutions. Similar results were obtained in a replicate experiment.

Activity of a C7 dinitrophenyl substituent with gyrase resistance mutants is influenced by other fluoroquinolone substituents

To determine whether the effects of the dinitrophenyl (DNP) moiety are influenced by other fluoroquinolone substituents, we performed several additional comparisons. In one, we found that attaching the DNP group to norfloxacin *reduced* the overall ability of the compound to block growth of the major GyrA mutants (Figure 5D). This DNP effect on norfloxacin is opposite to that seen with ciprofloxacin in which the DNP moiety *increased* antimutant activity. The difference must arise from the replacement of the N1 cyclopropyl group of ciprofloxacin with an N1 ethyl, since, as pointed out above, that is the only difference between norfloxacin and ciprofloxacin. In another comparison, replacing the piperazinyl ring with a desethylene group (Cip-Et) generated a more flexible C7 system. The resulting DNP derivative (Cip-Et-DNP) was less active than Cip-DNP against our panel of GyrA mutants (Figure 5E). Thus, multiple aspects of quinolone structure influence C7 aryl effects.

Other fluoroquinolone substituents also allowed the C7 DNP moiety to reduce the protective effects of particular GyrA substitutions. For example, placing a dinitrophenyl

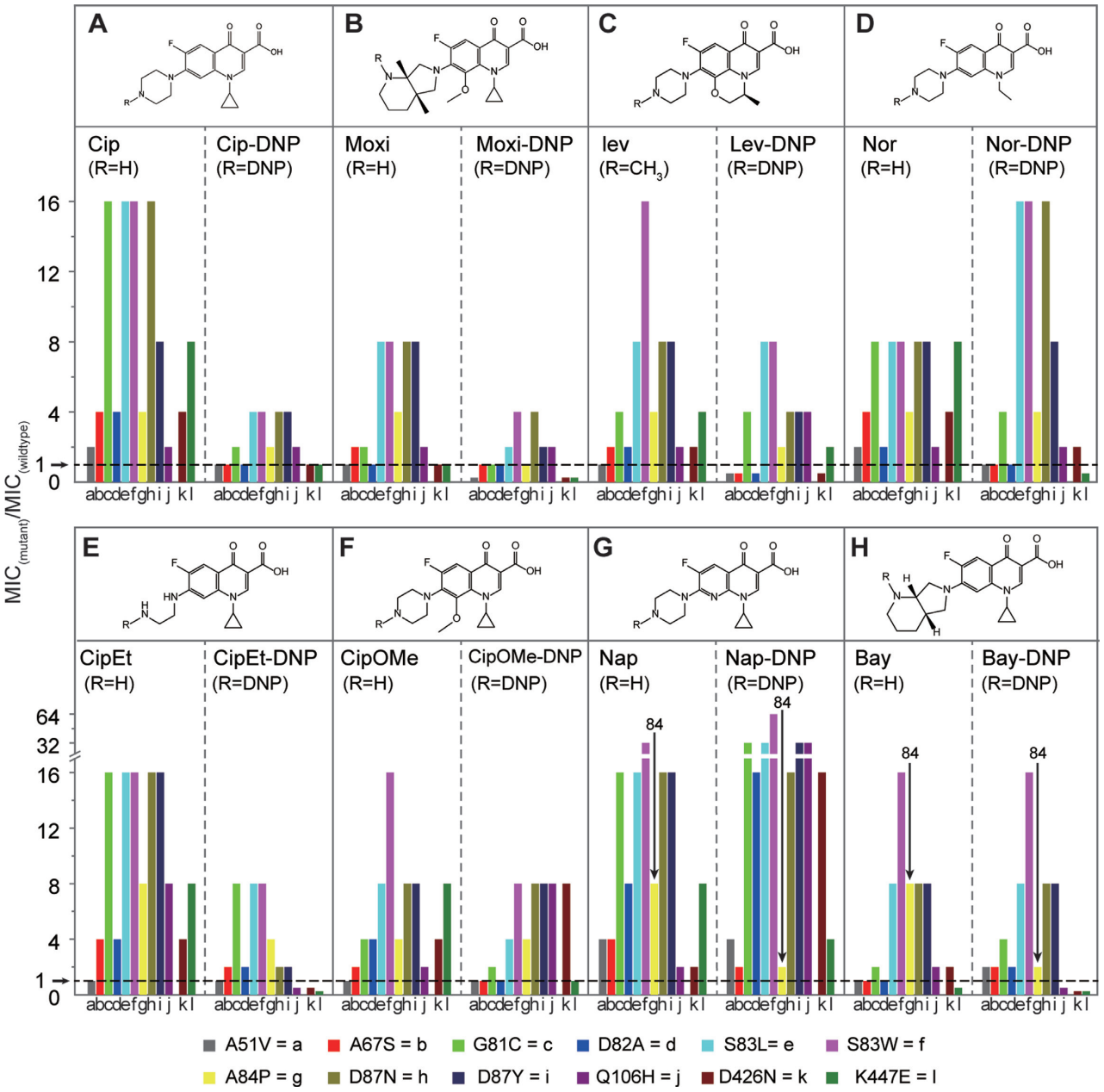

Figure 5. Anti-mutant activity due to 2,4-dinitrophenyl moiety depends on core ring substituents. MIC for C7-aryl derivatives of ciprofloxacin and related compounds with mutant and wild-type *E. coli* was determined and displayed as described in Figure 3. Panel A: ciprofloxacin and ciprofloxacin-DNP (Cip-DNP); panel B: moxifloxacin and moxifloxacin-DNP (Mox-DNP); panel C: levofloxacin and levofloxacin-DNP (Lev-DNP); panel D: norfloxacin and norfloxacin-DNP (Nor-DNP); panel E: desethylene ciprofloxacin (CipEt) and desethylene-ciprofloxacin-DNP (CipEt-DNP); panel F: 8-methoxy ciprofloxacin (CipOMe) and its DNP derivative; panel G: naphthyridone (Nap) and naphthyridone-DNP (Nap-DNP); panel H, Bay y 3114 and Bay-DNP. Amino acid 84 is indicated by labelled arrow. Similar results were obtained in a replicate experiment.

moiety on a naphthyridone derivative of ciprofloxacin (N at position 8 rather than C) generally decreased activity with GyrA resistance substitutions (Nap versus Nap-DNP, Figure 5G). However, with the minor resistance substitution GyrA A84P, the DNP group increased activity (arrows labelled 84 in Figure 5G). Particularly good activity with GyrA A84P was also seen when a DNP group was added to the C8-H derivative of moxifloxacin (Bay-DNP, arrows labelled 84 in Figure 5H); with most resistance substitutions, Bay-DNP was similar to its parent compound Bay y 3114. Other examples of minor resistant mutants are listed in Supplementary Table S2. It appears that quinolone position 8, in addition to effects of position 1 described above, exerts important effects on C7 ring activity. Previous energy minimization calculations place the N1-cyclopropyl and C8-methoxy moieties in a *trans* configuration outside the plane of the core quinolone rings such that the two substituents restrict C7 ring rotation (40). Thus, long-distance effects between C7 ring substituents and moieties at positions 1 and

8 are expected, but interactions between the C7 moiety and any particular amino acid cannot be predicted.

In summary, C7-aryl quinolones can overcome the protective effects of some GyrA resistance substitutions in ways that depend on the structure of the aryl and other quinolone substituents (summarized according to mutants in Supplementary Figure S3). These relationships are not readily explained by X-ray structures of cleaved complexes in which C7 moieties are far from the GyrA residues involved in resistance.

Effect of C7 dinitrophenyl moiety on selection of resistant mutants

To gain insight into binding of aryl quinolones to gyrase, we examined resistant mutants selected by Cip-DNP, the derivative showing the most antimutant activity. Nucleotide sequence analysis of DNA extracted from mutants selected on agar containing 1- to 2-times MIC Cip-DNP revealed that a surprising fraction of the mutants (7/29) contained an Ala to Glu substitution at GyrA position 119 (Supplementary Table S3). We confirmed that the A119E substitution was responsible for decreased activity by transferring the mutant *gyrA* gene to an isogenic *E. coli* background (KD1397) using P1-mediated transduction. A 16-fold increase in MIC was observed for Cip-DNP with the GyrA A119E variant (KD3254) relative to wild type, while an increase of only 4-fold was observed for ciprofloxacin (Supplementary Table S4). This recovery of GyrA rather than ParC variants as first-step mutants indicated that gyrase, not topoisomerase IV, is the site of the secondary binding seen with Cip-DNP.

The ability of GyrA A119E to preferentially protect *E. coli* from Cip-DNP is not predicted by X-ray structures of cleaved complexes; therefore, it may help define a second mode of quinolone binding. GyrA-119 is located near, but not in, helix-4 (see Figure 2 for location). Recovery of this unusual substitution is most easily explained by the C7 region of Cip-DNP locating near GyrA rather than GyrB. However, most of the mutants selected by Cip-DNP contained GyrA alterations that are commonly found (substitutions were observed at GyrA positions 81, 83 and 87, Supplementary Table S3). Our current interpretation is that an equilibrium condition exists between two quinolone-binding modes. We note that with the low drug concentrations used in our mutant selection procedure, commercially available quinolones tend to select non-gyrase mutants (41–43), a trend seen in the present work with ciprofloxacin (Supplementary Table S3). Non-*gyrA* mutants appear to be less readily selected by the DNP-containing derivative for reasons that are not understood.

Selection of resistance can also be examined on a population basis by determining concentrations that restrict the selective enrichment of mutant subpopulations present in large bacterial cultures. Compounds can be compared for their ability to block mutant growth by determining the concentration required to prevent colony formation when more than 10^{10} cells are plated, a value called the MPC (44,45). To measure MPC, *E. coli* cultures were grown to stationary phase, applied to agar plates containing various concentrations of ciprofloxacin or C7 derivatives of ciprofloxacin, and the fraction of input cells forming colonies was determined. The ratio of MPC to MIC approximates the concentration range over which mutant enrichment occurs: compounds that are more restrictive for mutant growth will exhibit a lower ratio. The MPC/MIC ratio was lower for Cip-DNP and the difluorophenyl derivative of ciprofloxacin (Cip-DFP) than for ciprofloxacin (Table 3), as expected from anti-mutant activity (Figure 4). The dimethoxyphenyl derivative of ciprofloxacin (Cip-DOMeP), which had weaker antimutant activity than ciprofloxacin (Figures 3 and 4), showed a higher ratio of MPC/MIC (Table 3). Thus, population analysis confirms that addition of a dinitrophenyl moiety to the C7 end of ciprofloxacin restricts the selective enrichment of resistant mutants.

Effect of C7 aryl group on the inhibition of the activities of purified topoisomerases

We have initiated studies to determine how well biochemical measurements of cleaved-complex formation recapitulate the inhibition of culture growth (MIC) when a putative secondary binding mode/site is considered. For this comparison, we examined ciprofloxacin and Cip-DNP for cleaved-complex formation using wild-type gyrase and a GyrA G81C mutant form of gyrase. Reaction mixtures containing gyrase, supercoiled plasmid DNA and fluoroquinolones at various concentrations were incubated and subsequently treated with sodium dodecyl sulfate to disrupt cleaved complexes that had formed. Then plasmid DNA was examined by gel electrophoresis to assess the percent having a linear form as an indication of cleaved-complex formation. As expected, mutant gyrase was less susceptible than wild-type gyrase to ciprofloxacin (33-fold decrease, column 2 in Table 4). With Cip-DNP, the loss of biochemical susceptibility due to the G81C substitution was 10-fold less (3.4-fold decrease, column 6 in Table 4). With cultured cells, Cip-DNP lowered the protective effect of the G81C substitution by 8-fold relative to ciprofloxacin (columns 4 and 8, Table 4). Thus, a similar difference between ciprofloxacin and Cip-DNP was observed for cleaved-complex formation and MIC (columns 9 and 10, Table 4).

We also performed cleaved-complex formation assays with *E. coli* topoisomerase IV by methods described in (46). For ciprofloxacin, topoisomerase IV activity was 150-fold lower than with wild-type gyrase and 4-fold lower than observed with the GyrA G81C mutant gyrase (column 1, Table 4). These data are consistent with topoisomerase IV being a secondary target for ciprofloxacin in *E. coli*, as previously reported (for example, (47)). For Cip-DNP, the difference was even greater: topoisomerase IV activity was 370-fold lower than with wild-type gyrase and 100-fold lower than with GyrA G81C gyrase (column 5, Table 4). These data support the conclusion that a first-step Cip-DNP-resistant mutant is unlikely to be due to an amino acid substitution in topoisomerase IV.

DISCUSSION

Two general findings emerged from the work described above. First, addition of a dinitrophenyl (DNP) group to the

Table 3. MPC of ciprofloxacin derivatives

Compound name	Designation	MPC (μM)	$MIC_{(99)}$ (μM)[a]	$MPC/MIC_{(99)}$[a]
ciprofloxacin	Cip	0.36	0.018	20
UIJR-002–9A	Cip-DNP	0.51	0.034	15
UIJR-002–60-A	Cip-DFP	3.96	0.4	10
UIJR-002–61-A	Cip-DMP	19.9	1.0	20
UIJR-002–65-A	Cip-DOMeP	60.2	1.5	40

[a]$MIC_{(99)}$ was the antimicrobial concentration in agar that blocked colony formation by 99%.

Table 4. DNA cleavage by purified gyrase and topoisomerase IV

Enzyme	Ciprofloxacin (Cip)[a] [col. 1-4]				Cip-DNP[a] [col. 5-8]				Cip/Cip-DNP	
	DNA cleavage (CC_3)[b]	CC_3 relative to wt	MIC[c]	MIC relative to wt	DNA cleavage (CC_3)[b]	CC_3 relative to wt	MIC[c]	MIC relative to wt	CC_3 relative to wt	MIC relative to wt
	1	2	3	4	5	6	7	8	9	10
Gyrase (wt)	0.097 ± 0.003	1	0.015	1	1.2 ± 0.04	1	0.031	1	1	1
Gyrase (G81C)	3.2 ± 0.7	33	0.240	16	4.1 ± 1.2	3.4	0.062	2	9.7	8
Topoisomerase IV (wt)	14.2 ± 3.3	NA[d]	NA[d]	NA[d]	450 ± 50	NA[d]	NA[d]	NA[d]	NA[d]	NA[d]

[a]All concentrations expressed as μM.
[b]CC_3 represents cleaved-complex formation measured as concentration of fluoroquinolone required to triple the amount of linear plasmid DNA detected following sodium dodecyl sulfate treatment after incubation of gyrase, fluoroquinolone and DNA (46).
[c]MIC data taken from Table 1.
[d]Not applicable because no topoisomerase IV mutant was examined.

C7 piperazinyl ring of ciprofloxacin (Cip-DNP) increases bacteriostatic activity with GyrA resistance mutants commonly found in clinical practice. This observation reveals a new avenue for designing fluoroquinolones to overcome existing resistance. Second, the effect of the C7 DNP group with GyrA mutants fits with the idea that quinolones have at least two modes of binding to gyrase (31). As pointed out in the Introduction, X-ray structures and biochemical experiments show that (i) the C7 ring system interacts with regions of GyrB/ParE and (ii) the C3/C4 keto-acid moiety interacts with helix-4 of GyrA/ParC, the latter through a water-metal ion bridge (Figure 6A and B show renderings of ciprofloxacin and Cip-DNP guided by the X-ray structure of protein data base entry 2XKK (24)). Resistance substitutions in GyrA helix-4 interfere with formation of the water-metal bridge and therefore fluoroquinolone binding (18,19,31). However, the structure derived from X-ray crystallography cannot explain many observations with C7 aryl compounds. For example, the protective effect of GyrA resistance substitutions depends on the type and distribution of substituents attached to the phenyl moiety (Figures 3 and 4), the specific moiety located between the phenyl group and carbon-7 of the quinolone core (piperazinyl, desethylene, or diazobicyclo, Figure 4), and even a one-carbon linker between the phenyl and the piperazinyl rings (phenyl versus benzyl moieties, Supplementary Figure S2). Thus, the data in the present report force us to consider alternate configurations.

One alternative, based on crosslinking between GyrA G81C and a C7 chloroacetyl derivative of ciprofloxacin (31), is a structure in which the drug is inverted by 180° within the binding site observed by X-ray crystallography. In Figure 6C we show a speculative model anchored by the C7 end of ciprofloxacin located near GyrA G81C, as indicated by crosslinking (31). This model postulates insertion of the C7 dinitrophenyl substituent between active center residues GyrA Arg-121 and GyrA Tyr-122, perhaps as π–π and *p*–π stacking interactions (similar interactions are described in (48)). Such an insertion explains selective enrichment of the uncommon GyrA A119E resistance variant by Cip-DNP: this amino acid substitution would interfere with insertion of the DNP group by forming a salt bridge between the Glu-119 side chain and Arg-121 (Figure 6C). The inverted model for quinolone binding removes the C3/C4 keto-acid of the drug from the vicinity of GyrA helix-4, explaining the reduction of protection due to resistance substitutions at GyrA positions 83 and 87 (Figure 3): if no keto-acid-water-magnesium-GyrA bridge forms, substitutions at these two positions may have little effect. Likewise, removal of the C7 end of Cip-DNP from GyrB would explain the absence of protection from GyrB resistance alleles (Figure 3). Indeed, in the inverted mode (Figure 6C), the DNP moiety is located far (15–20 Å) from the region of GyrB where fluoroquinolone C7 moieties are found in X-ray structures of cleaved complexes. Thus, we now have an imprecise, but testable model for a secondary binding mode.

Another alternative is an inverted orientation as in Figure 6C but with the fluoroquinolone rotated 180° around its vertical axis to give the structure shown in Figure 6D. This mode, also anchored by the proximity of GyrA G81C to the C7 end of ciprofloxacin, posits that the DNP moiety can stack into the interface between the GyrA subunits such that it has a *p*–π interaction with R121, but it does not insert between R121 and Y122. In this rotated, inverted configuration, the protective effect of the A119E substitution is also explained by formation of a salt bridge. Placement of the C7 phenyl moiety near GyrA D82A may explain the strong effect of the dichlorophenyl group on this substitu-

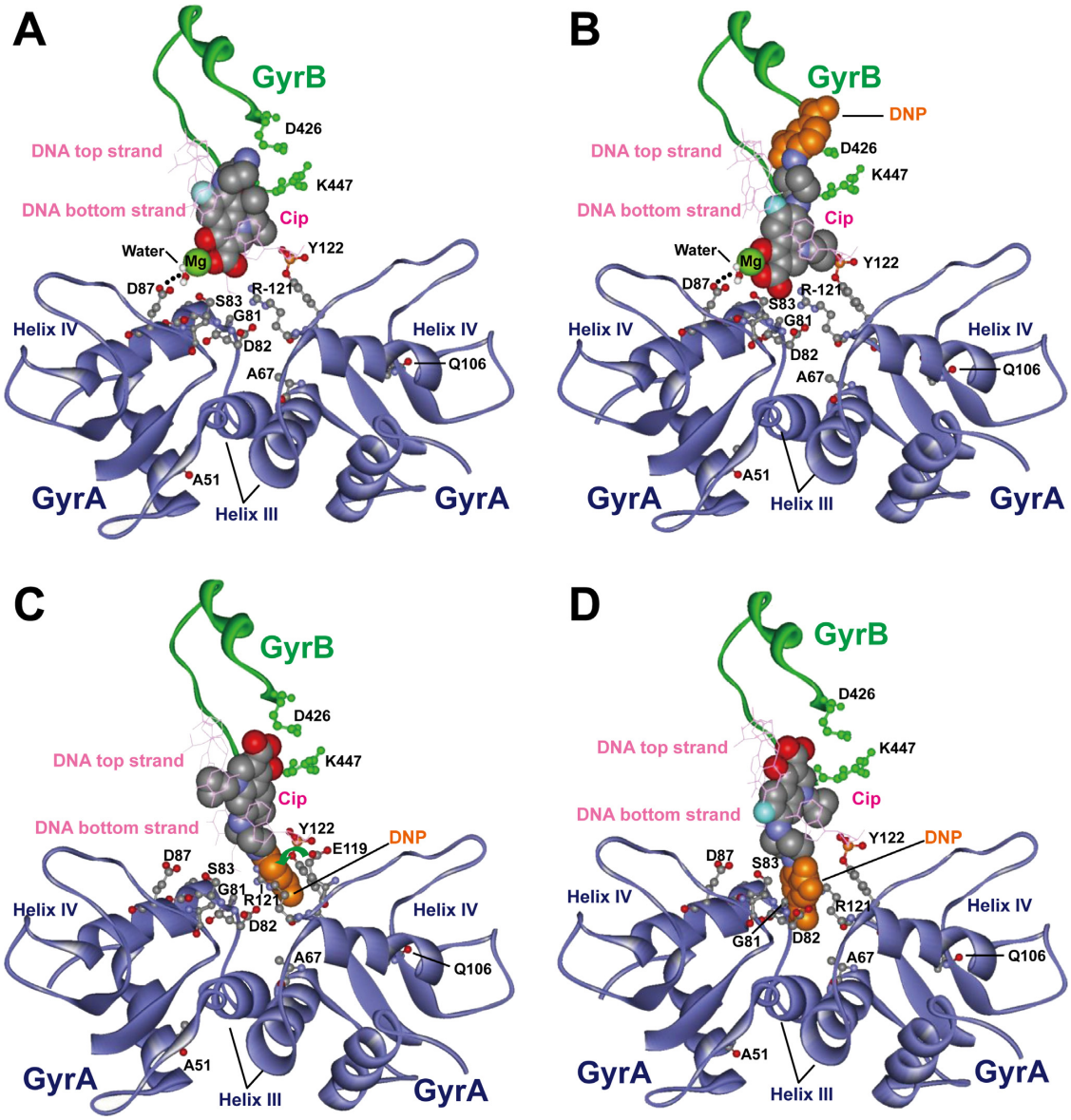

Figure 6. Models for binding of Cip-DNP to GyrA in cleaved complexes. (**A**) Ciprofloxacin in cleaved complex. (**B**) Modelled primary binding mode for Cip-DNP derived from X-ray structure of fluoroquinolone complexed to DNA and topoisomerase IV (24). The DNP moiety is shown in orange. (**C**) Inverted binding mode. Cip-DNP is shown extending to position 119. (**D**) Inverted binding mode rotated 180° around the vertical axis.

tion (Figure 4F). For the two inverted orientations we use the term alternate binding mode rather than alternate binding site, since the binding site is the same as revealed by X-ray crystallography. A separate binding site (1), which spans the GyrA-GyrA dimer interface, has not been ruled out or distinguished experimentally from the inverted modes (see Supplementary Figure S4).

Although substitutions at GyrA position 119 are uncommon, two (V and E) have been observed previously in *Salmonella enterica* Typhimurium (49) and *Mycoplasma hominis* (50). The less protective, A119V, elicits no change in MIC with *M. hominis* (50), and with purified *E. coli* gyrase it confers no protection from ciprofloxacin (51). Thus, it is not surprising that with *E. coli* we selected only the A119E allele. However, the A119V substitution is highly protective (25-fold) with the plant *Arabidopsis thaliana* (51), thereby pointing to a difference between *E. coli* and chloroplast gyrases that may be structurally interesting.

We have initiated comparative studies with ciprofloxacin and Cip-DNP using biochemical assays for gyrase containing the GyrA G81C resistance substitution. When the protective effect of the substitution was assessed by normalizing mutant to wild-type activity (formation of cleaved complexes), Cip-DNP exhibited roughly 10-times more activity than ciprofloxacin; MIC measurements showed a similar (8-fold) activity enhancement when normalized to wild-type levels (Table 4). These data indicate that the C7 moiety interacts in a positive way with GyrA G81C gyrase. A more comprehensive biochemical characterization of the C7 aryl compounds and mutant enzymes is in progress.

A striking feature of the data presented above is the different effects that the C7-DNP substituent can have on structurally similar fluoroquinolones for a given mutant. For example, the DNP derivatives of ciprofloxacin and its naphthyridone cognate behave very differently with gyrase mutants, suggesting that they may favour different binding modes. Having the primary (C3/C4-GyrA) and secondary (C7-GyrA) binding modes in equilibrium would explain why Cip-DNP selected resistance substitutions at both GyrA-119 and positions that participate in the water-magnesium ion bridge (GyrA-83 and GyrA-87). According to this view, fluoroquinolones could shift from one binding mode to another as part of cleaved-complex formation and/or as a function of drug concentration. Indeed, a binding mode shift has been suggested to explain an anomalous stabilization of cleaved complexes occurring during thermal reversal (31). Since quinolones can bind gyrase in the absence of DNA cleavage (7,52), it is possible that an initial binding occurring in one mode shifts to another mode following DNA cleavage.

The potential existence of a distinct, secondary binding site (Supplementary Figure S4) raises the possibility that at high drug concentration this site and the primary binding site can both be filled to create a four-drug complex. Such an event could explain why binding of radioactive enoxacin to gyrase-DNA complexes occurs in a two-step manner as concentration is increased (53). If a four-drug complex is a precursor to lethal events, which are currently unexplained by X-ray structures of cleaved complexes, we could begin to understand why rapid, quinolone-mediated killing requires higher concentrations than blocking growth.

In conclusion, the present work provides a functional framework for biochemical and structural studies. For example, we identified C7-dinitrophenyl derivatives as starting points for examining the secondary binding mode/site. Our suggestion that drug binding to the two modes is governed by equilibrium considerations may initially require crosslinking (31) to capture quinolone in the secondary binding mode. The present work also proposes C7 aryl fluoroquinolones as a way to bypass existing fluoroquinolone resistance. Other approaches include quinazolinediones (17,54), novel bacterial topoisomerase inhibitors (23,55,56) and spiropyrimidinetriones (57,58). The quinazolinediones do not form the C3/C4 interaction with GyrA helix-4 observed with fluoroquinolones (18), and as a consequence they tend to have lower bacteriostatic activity (17,19,59). Unlike the quinazolinediones, the C7 aryl fluoroquinolones can exhibit little loss of bacteriostatic activity (Table 2). Indeed, some DNP derivatives show a lower absolute MIC against some resistant mutants than does their parental compound. It will now be interesting to synthesize quinazolinediones containing a C7 aryl substituent to determine whether two moieties that contribute to bypass of resistance have additive effects.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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