ACS chemical biology

Articles

pubs.acs.org/acschemicalbiology

Bypassing Fluoroquinolone Resistance with Quinazolinediones: Studies of Drug−Gyrase−DNA Complexes Having Implications for Drug Design

Karl Drlica,*[,†,⊥] Arkady Mustaev,[†] Tyrell R. Towle,[‡] Gan Luan,[†] Robert J. Kerns,*[,‡,⊥] and James M. Berger*[,§,∥,⊥]

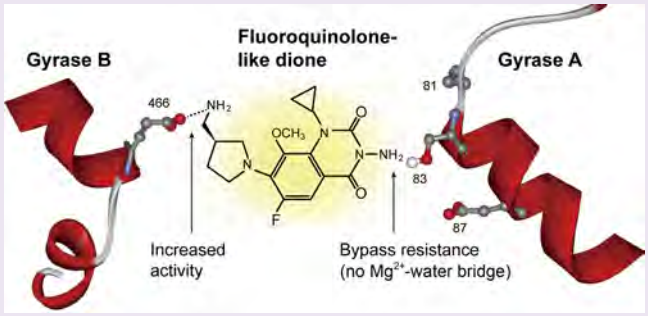
[†]Public Health Research Institute and Department of Microbiology & Molecular Genetics, New Jersey Medical School, Rutgers Biomedical and Health Sciences, 225 Warren Street, Newark, New Jersey 07103, United States

[‡]Division of Medicinal & Natural Products Chemistry, Department of Pharmaceutical Sciences & Experimental Therapeutics, University of Iowa College of Pharmacy, 115 South Grand Avenue, Iowa City, Iowa 52246, United States

[§]Molecular and Cell Biology Department, Quantitative Biosciences Institute, University of California, Stanley Hall, Berkeley, California 94720, United States

Supporting Information

ABSTRACT: Widespread fluoroquinolone resistance has drawn attention to quinazolinediones (diones), fluoroquinolone-like topoisomerase poisons that are unaffected by common quinolone-resistance mutations. To better understand differences between quinolones and diones, we examined their impact on the formation of cleaved complexes (drug−topoisomerase−DNA complexes in which the DNA moiety is broken) with gyrase, one of two bacterial targets of the drugs. Formation of cleaved complexes, measured by linearization of a circular DNA substrate, required lower concentrations of quinolone than dione. The reverse reaction, detected as resealing of DNA breaks in cleaved complexes, required higher temperatures and EDTA concentrations for quinolones than diones. The greater stability of quinolone-containing complexes was attributed to the unique ability of the quinolone C3/C4 keto acid to complex with magnesium and form a previously described drug−magnesium−water bridge with GyrA-Ser83 and GyrA-Asp87. A nearby substitution in GyrA (G81C) reduced activity differences between quinolone and dione, indicating that resistance due to this variation derives from perturbation of the magnesium−water bridge. To increase dione activity, we examined a relatively small, flexible C-7-3-(aminomethyl)pyrrolidinyl substituent, which is distal to the bridging C3/C4 keto acid substituent of quinolones. The 3-(aminomethyl)pyrrolidinyl group at position C-7 was capable of forming binding interactions with GyrB-Glu466, as indicated by inspection of crystal structures, computer-aided docking, and measurement of cleaved-complex formation with mutant and wild-type GyrB proteins. Thus, modification of dione C-7 substituents constitutes a strategy for obtaining compounds active against common quinolone-resistant mutants.



Fluoroquinolones are important antimicrobials that trap two bacterial topoisomerases, gyrase and topoisomerase IV, on DNA as complexes in which the DNA is broken. The complexes, commonly called cleaved complexes,[1,2] are the primary lesions responsible for quinolone activity.[3−5] The DNA ends in cleaved complexes are constrained by covalent linkage to the topoisomerase, which facilitates resealing of the DNA breaks after drug removal. Consequently, damage caused by the complexes, such as inhibition of DNA synthesis, is reversible when drug is removed.[6] Reversibility may be clinically important, since treatment of infections often involves fluctuating drug concentrations that may drop low enough to allow resealing of cleaved DNA. Resealing may reduce quinolone efficacy and increase pathogen survival. Pathogen survival can also arise from expansion of resistant subpopulations containing topoisomerase mutations that reduce drug binding and thereby cleaved complex formation. Thus, understanding how the structure of quinolone-class antimicrobials affects topoisomerase-mediated DNA cleavage and resealing is central to designing more effective drug candidates.

Cleaved complex formation can be assayed by incubating gyrase or topoisomerase IV with quinolone and circular DNA, followed by treatment with an ionic detergent, such as sodium dodecyl sulfate (SDS), to dissociate the complexes. Gel electrophoresis is then used to separate the DNA species; the appearance of linear DNA indicates complex formation.[7,8]

Received: August 8, 2014
Accepted: October 13, 2014

   dx.doi.org/10.1021/cb500629k | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

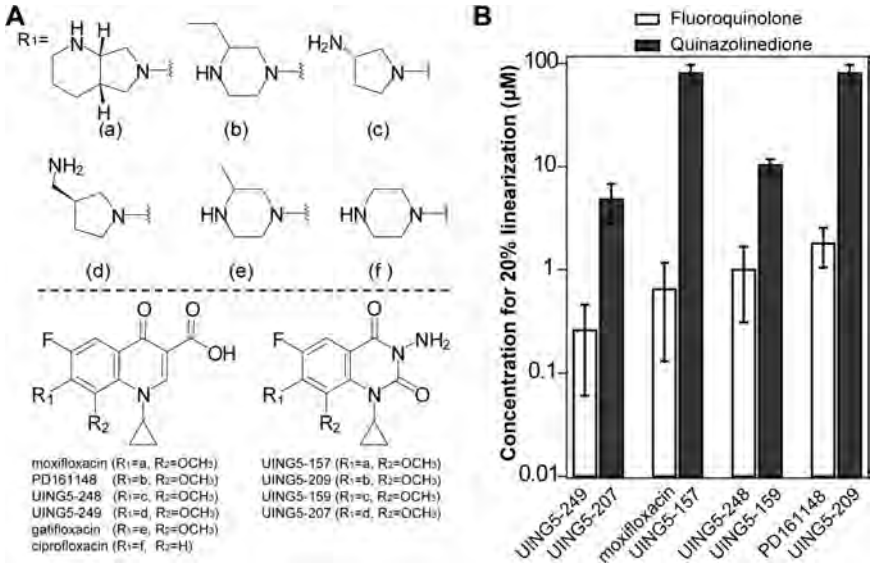


Figure 1. Structure and activity differences between quinolones and diones. (A) Compounds used in the present work. Structures of the C-7 substituents (R_1) are above the dashed line as a–f. Below the dashed line are fluoroquinolones (left) and quinazolinediones (right). The present report focuses on the 3-(aminomethyl)pyrrolidinyl C-7 group (structure d). (B) Formation of cleaved complexes occurs at lower concentrations of fluoroquinolone (empty bars) than quinazolinedione (filled bars). Cleaved complex formation was assayed as in Methods to determine the concentration of quinolone or dione required to linearize 20% of the plasmid DNA. Error bars represent standard deviation for four independent experiments.

Reversal of complex formation is observed as DNA resealing, i.e., the disappearance of linear DNA arising prior to dissociation of the complex with SDS. Reversal conditions, which involve destabilization of drug-bound enzyme–DNA complexes, include (i) dilution,[9] (ii) incubation at high temperature,[9] or (iii) treatment with ethylenediamine tetraacetic acid (EDTA).[7,10] EDTA chelates polyvalent metal ions, and its action can be explained by participation of Mg^{2+} in quinolone binding. Indeed, X-ray crystallographic studies of drug–enzyme–DNA complexes reveal that the C3/C4 keto acid moiety of fluoroquinolones coordinates a Mg^{2+} ion and forms a previously described drug–magnesium–water bridge to amino acid residues in helix-4 of either GyrA (gyrase) or ParC (topoisomerase IV).[11−14] Additional magnesium coordination occurs within the topoisomerase active site,[15] making it likely that multiple magnesium ions participate in cleaved complex formation and reversal.

Drug-mediated magnesium coordination has been characterized by comparison of fluoroquinolones and quinazolinediones (diones), quinolone-like compounds (Figure 1A) that lack the carboxylate group needed for magnesium coordination with drug.[9,11,16−18] Diones are potentially important clinically because they exhibit striking activity against several types of fluoroquinolone-resistant mutant containing amino acid substitutions in gyrase and DNA topoisomerase IV.[17,19−21] For two of these substitution sites (equivalent to GyrA-83 and GyrA-87 in *Escherichia coli*), an absence of drug-mediated magnesium coordination has been implicated in the biochemical behavior of diones.[9,16,18] A third resistance site (GyrA-81) is not expected to directly participate in metal ion coordination, but a GyrA-G81C resistance substitution has been proposed to interfere with ion binding indirectly by sterically perturbing the geometry of the gyrase acidic group at a distance.[20] Such interference has not been examined biochemically. A consequence of the stabilizing magnesium bridge is that fluoroquinolones exhibit greater activity than diones with wild-type enzyme and cells.[18,19,21] Thus, a challenge is to find ways to stabilize dione-containing cleaved complexes, thereby increasing activity of an agent that may bypass existing resistance.

The present work began with a set of C-8-methoxy diones active against fluoroquinolone-resistant bacterial mutants.[19,20] To better understand differences between diones and quinolones, we examined their effects on gyrase-mediated DNA breakage and resealing using EDTA, thermal treatments, and a GyrA-G81C substitution. We then sought ways to both stabilize dione-containing cleaved complexes and increase dione activity. A 3-(aminomethyl)pyrrolidinyl substituent (hereafter called an aminomethyl pyrrolidine group) at position C-7 afforded increased activity for both dione and fluoroquinolone derivatives. Investigation of extant X-ray structures, along with docking studies, revealed a potential interaction between GyrB-Glu466 and the aminomethyl pyrrolidine group that might account for increased activity through a slightly altered binding orientation. In accord with this observation, a GyrB-E466C substitution preferentially decreased dione activity, supporting a stabilizing role for the GyrB interaction with the C-7 aminomethyl pyrrolidine group. Overall, the present work establishes the functional relevance of crystal structures of cleaved complexes, explains the action of the GyrA-G81C resistance substitution, addresses crossover activity of diones for human topoisomerase II, and provides a new approach for bypassing the protective activity of current quinolone-resistance mutations.

RESULTS AND DISCUSSION

Cleaved Complex Formation with Quinolone and Dione Pairs. Quinazolinediones and cognate fluoroquinolones having four different C-7 ring moieties (Figure 1A) were incubated at various concentrations with *E. coli* gyrase and plasmid DNA to form cleaved complexes that were assayed by the appearance of linear DNA (see Supporting Information

dx.doi.org/10.1021/cb500629k | ACS Chem. Biol. XXXX, XXX, XXX–XXX

Figure S1 for an example of electrophoretic separation of plasmid species). For each compound pair, the quinolone was 10−100 times more active than the dione, as judged by the concentration required to linearize ~20% of the DNA (Figure 1B). These results parallel earlier studies with cultured bacterial cells.[19,20] The UING5-249/UING5-207 pair was the most active; thus, subsequent work focused on these two compounds.

DNA Resealing Stimulated by EDTA Differs for Dione- and Quinolone-Containing Complexes. Previous studies have shown that type II DNA topoisomerases act through a two-metal mechanism[15,22,23] and that the metal chelator EDTA helps to stimulate resealing of broken DNA in cleaved complexes.[1,2,10,17,24] In an initial experiment, we formed cleaved complexes by incubating gyrase, plasmid DNA, and the fluoroquinolone UING5-249. Then we treated reaction mixtures with EDTA to reseal the DNA. Increasing EDTA concentration caused the recovery of linear DNA to drop, reach a broad minimum beginning at about 2 mM EDTA (the concentration of Mg^{2+} in the reaction mixture), and then increase (Figure 2A). Addition of Mg^{2+} after incubation with moderate concentrations of EDTA (2 mM) reversed the resealing stimulated by EDTA (Supporting Information Figure S2); incubation with Mg^{2+} after treatment with a high concentration of EDTA (50 mM) restored resealing (Supporting Information Figure S2). Thus, EDTA likely exerts its effects by sequestering Mg^{2+} ions away from the ternary drug−enzyme−DNA complex.

We reasoned that DNA resealing in complexes formed with diones might differ from that of quinolone-containing complexes because diones cannot participate in magnesium-mediated bridging of drug with GyrA. When we compared cognate dione (UING5-207) and quinolone (UING5-249) for EDTA-dependent effects on DNA resealing, the overall shapes of the curves were similar (Figure 2A). However, in the low EDTA concentration range, reversal of quinolone-mediated DNA cleavage occurred at a higher EDTA concentration than that observed for the dione. This reduced susceptibility of quinolone-containing complexes to EDTA-mediated DNA resealing is consistent with quinolones having an additional magnesium-dependent interaction that stabilizes their participation in cleaved complex formation.

The response to EDTA concentration may arise from low concentrations of EDTA promoting quinolone dissociation from the complex, thereby shifting gyrase from a DNA-breaking mode to a resealing one; if so, then high concentrations of EDTA would largely eliminate the remaining Mg^{2+} and thereby disrupt Mg^{2+}-dependent DNA resealing by gyrase. Nicked DNA was recovered at high EDTA concentration (Figure 2B), consistent with these conditions partially inhibiting gyrase-mediated resealing of DNA breaks. Partial resealing associated with high concentrations of EDTA (35 mM) has been observed previously with *Streptococcus pneumoniae* gyrase-containing complexes.[17] However, in that work, 35 mM EDTA failed to induce DNA resealing for an 8-methyl dione complexed with topoisomerase IV.[17] The difference at high EDTA concentration seen between the 8-methyl dione and the 8-methoxy derivative used here may derive from tighter binding of the methyl derivative, since formation of cleaved complexes with the methoxy compound requires higher concentrations than its cognate methyl analogue.[18,21]

Since high salt concentration can reverse DNA cleavage induced by some quinolones,[17] sodium ions present in EDTA solutions could, in principle, contribute to DNA resealing during EDTA treatment. However, we saw no stimulation of DNA resealing by sodium acetate (up to 250 mM, Supporting Information Figure S3). Likewise, 200 mM sodium acetate failed to reduce resealing temperature, in contrast to the effects of EDTA treatment (Supporting Information Figure S4). Thus, salt effects are probably not responsible for DNA resealing stimulated by low EDTA concentration.

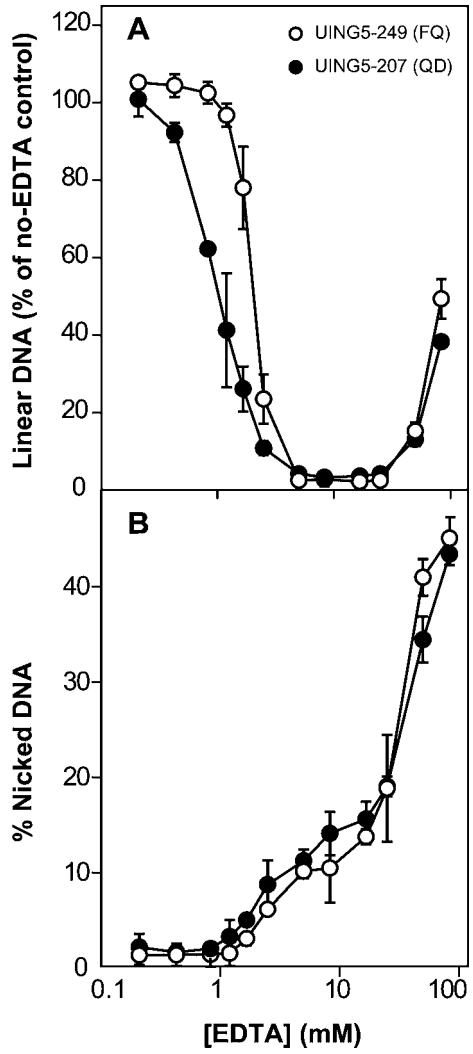


Figure 2. EDTA-mediated resealing of DNA in cleaved complexes formed with quinolone and dione. Incubations containing gyrase, DNA, and either fluoroquinolone (UING5-249 at 2 μM, empty circles) or dione (UING5-207 at 20 μM, filled circles) generated similar amounts of cleaved complexes for both compounds. Parallel reaction mixtures were incubated at the indicated concentration of EDTA for an additional 30 min before treatment with SDS and proteinase K. The fraction of total DNA in linear (A) or nicked (B) forms was determined by gel electrophoresis. Data were normalized to a zero-EDTA control, which was similar for quinolone and dione. Replicate experiments gave similar results; error bars indicate standard deviation.

A variety of divalent cations, such as Co^{2+}, Ni^{2+}, Pb^{2+}, and Mn^{2+}, can satisfy the requirement for a noncatalytic metal ion in formation of cleaved complexes with both quinolone and dione.[9] However, several metal ions, including Ca^{2+}, Cd^{2+}, Zn^{2+}, and Ba^{2+}, also preferentially form complexes with dione-type compounds.[9] Although the basis for these metal ion differences

dx.doi.org/10.1021/cb500629k | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

is not known, the data fit with the idea that in cleaved complexes the metal dependencies of quinolones and diones differ.

Temperature-Promoted DNA Resealing Differs between Dione- and Quinolone-Containing Complexes. In addition to the effects on EDTA-induced resealing, we reasoned that an additional stabilizing magnesium interaction might also increase the reversal temperature of complexes formed with fluoroquinolones relative to that with cognate diones. When cleaved complexes, formed by incubation of drug, gyrase, and plasmid DNA, were subjected to 5 min incubations at various temperatures, the production of linear DNA showed a distinct decrease over the same temperature range at which supercoiled DNA increased (Supporting Information Figure S5; the fraction of nicked DNA grew slightly as temperature increased). For comparison of dione and quinolone, we determined the temperature at which the proportion of linear DNA had decreased by 50%: dione-containing complexes exhibited overall lower DNA resealing temperatures than did complexes formed with cognate quinolones (Figure 3A and Supporting Information Table S1). In general, compounds having a greater stimulatory effect on DNA cleavage tended to exhibit a higher DNA resealing temperature (Figure 3B). The lower thermal stability of cleaved complexes formed with diones was observed over a broad concentration range in which raising dione concentration raised the resealing temperature (Supporting Information Figure S6). These data suggest that DNA resealing is facilitated by dissociation of quinolone/dione from the complexes. Overall, the results of thermal resealing experiments are consistent with a role for drug−enzyme stability in favoring cleaved complex formation, a result that accords with the observed stabilizing effects of water−magnesium ion bridging between fluoroquinolones and GyrA/ParC.

In previous work, a thermal resealing difference was not observed during comparison of an 8-methyl dione with several commercial quinolones.[16] In those experiments, complexes were formed with wild-type topoisomerase IV followed by incubation for various times at only a single temperature, 75 °C. The present work suggests that 75 °C is likely to be above the transition temperature, which could make that particular test insensitive to compound structure.

Dione-Containing Complexes Are Less Sensitive than Quinolone-Containing Complexes to a GyrA-G81C Substitution. X-ray analysis of crystal structures for cleaved complexes suggests that drug binding occurs close enough to GyrA-Gly81 for a Cys substitution to sterically perturb quinolone-mediated magnesium bridging.[11,20] Thus, cleaved complexes formed with GyrA-G81C gyrase and quinolones were expected to have properties similar to those of complexes formed with diones. Indeed, when dione UING5-207 and its cognate quinolone (UING5-249) were compared for complex formation with wild-type gyrase, 10- to 20-times more dione was required to generate the same amount of linear DNA (Figure 4Ai). By contrast, the difference was only 2-fold when GyrA-G81C was used (Figure 4Aii). Similarly, examination of DNA resealing by GyrA-G81C gyrase at various EDTA concentrations revealed no difference between dione- and fluoroquinolone-containing complexes at low EDTA concentration (Figure 4B) unlike the result obtained with wild-type gyrase, Figure 2A). The GyrA-G81C substitution also lowered DNA resealing temperature more for the fluoroquinolone than for the dione, bringing them to similar levels (Figure 4C).

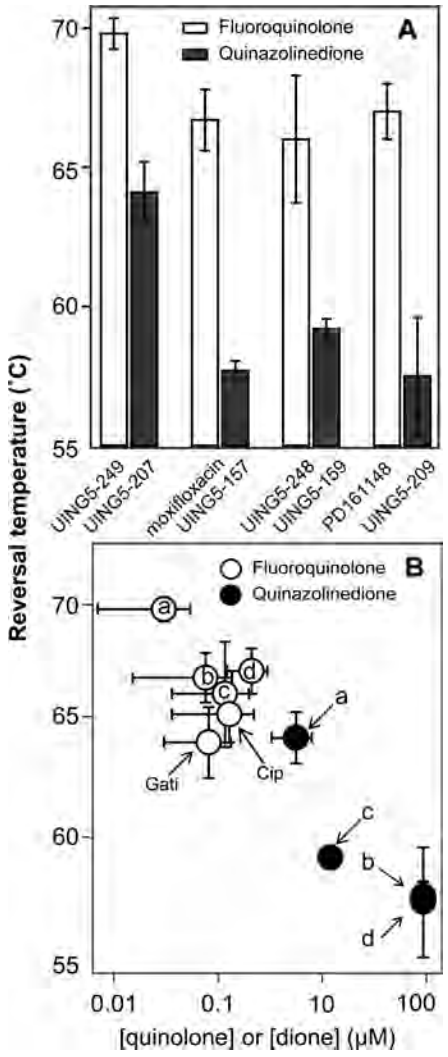


Figure 3. Thermal reversal of cleaved complexes formed with quinolones and diones. (A) Comparison of quinolones and cognate diones. Cleaved complexes were formed as described in Methods and then incubated at various temperatures to determine the temperature causing 50% resealing of DNA. (B) Relationship between drug concentration required for complex formation and thermal resealing of DNA. Concentrations of fluoroquinolone (empty circles) or quinazolinedione (filled circles) required to trap 20% of the plasmid DNA in a linear form were determined as in Figure 1B, and temperatures at which 50% of the linear DNA was resealed were determined. Letters indicate paired fluoroquinolones and diones (a = UING5-249/207; b = moxifloxacin/UING5-157; c = UING5-248/159; d = PD161148/UING5-209); Cip, ciprofloxacin; Gati, gatifloxacin. Error bars indicate standard deviation of the mean.

Overall, these results are consistent with the GyrA-G81C substitution exerting a destabilizing effect on the fluoroquinolone-mediated magnesium linkage that is absent in dione-containing complexes. Thus, the GyrA-G81C substitution joins substitutions at GyrA-83 and GyrA-87 in acting by impeding the formation of the quinolone−magnesium−water−enzyme bridge.[9,16]

Although substitution at GyrA-81 can be strongly protective with cultured cells[6,19,20] and with purified gyrase (Figure 4A), it is seldom associated with clinical resistance,[25] perhaps due to loss of mutant fitness in human hosts. Interestingly, at high EDTA concentrations, GyrA-G81C gyrase exhibited a reduced ability to reseal dione-containing complexes (Figure 4B).

dx.doi.org/10.1021/cb500629k | ACS Chem. Biol. XXXX, XXX, XXX−XXX

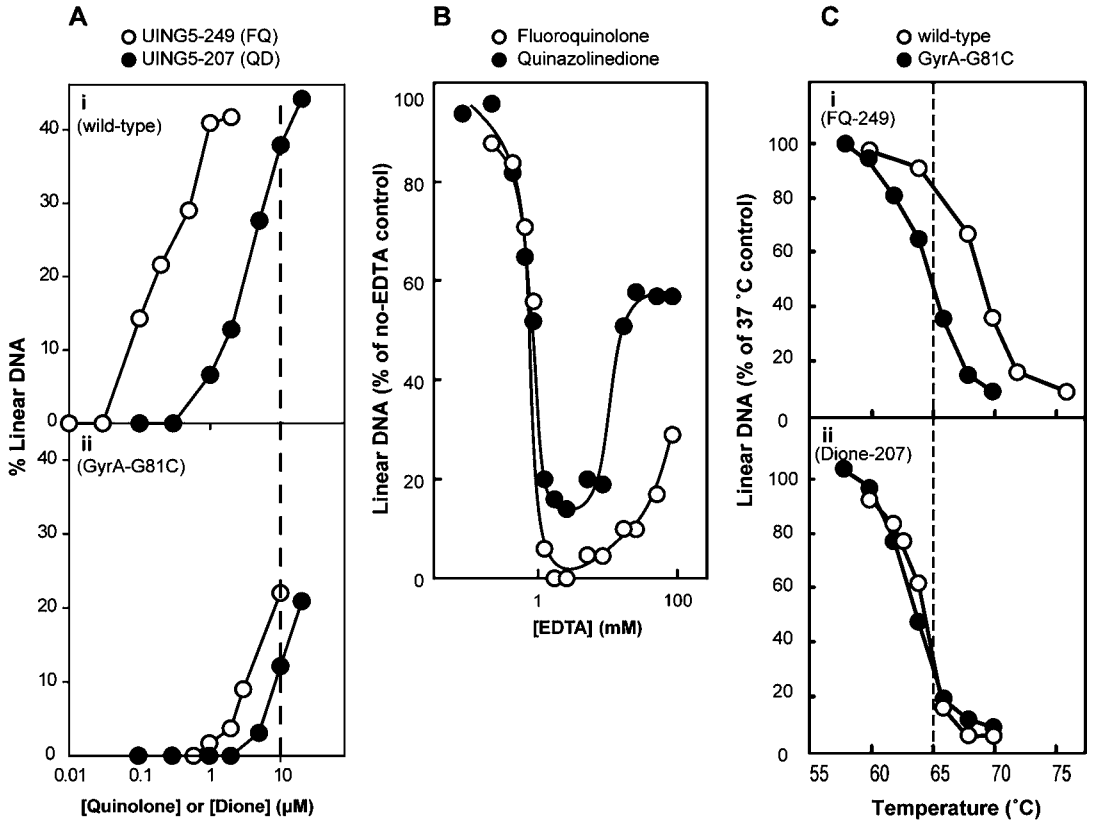


Figure 4. Effect of GyrA-G81C gyrase on dione and quinolone activity. (A) Compound concentration effects on complex formation: (i) wild-type gyrase and (ii) GyrA-G81C gyrase. Symbols: empty circles, fluoroquinolone UING5-249; filled circles, dione UING5-207. Similar results were obtained with four replicate experiments. (B) DNA resealing stimulated by EDTA. Reaction mixtures containing cleaved complex were treated with the indicated concentrations of EDTA and analyzed as in Figure 2. Linear DNA was quantified and expressed as a percent of the zero-EDTA control. Symbols: filled circles, dione UING5-207 at 80 μM; empty circles, fluoroquinolone UING5-249 at 40 μM. Similar results were obtained with three independent replicate experiments. (C) DNA resealing stimulated by high temperature. Reaction mixtures containing cleaved complexes were treated and analyzed as in Figure 3. (i) Complexes formed with fluoroquinolone UING5-249; (ii) complexes formed with dione UING5-207. Symbols: empty symbols, wild-type gyrase; filled symbols, GyrA81C gyrase. Similar results were obtained with four replicate experiments.

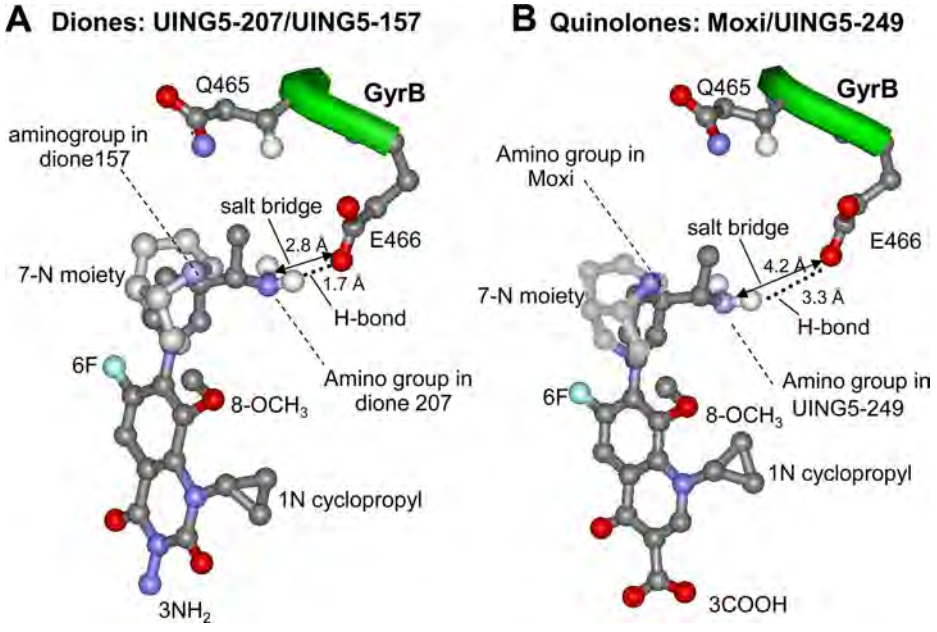


Figure 5. Location of C-7 ring substitutents relative to amino acid 466 of GyrB in cleaved complexes. A potential salt bridge and hydrogen bond between the C-7 ring systems and GyrB-466E are shown. (A) Dione binding. The C-7 ring system of UING5-157 is shown in light gray, whereas that of UING5-207 is in dark gray. (B) Quinolone binding. The C-7 ring system of moxifloxacin is shown in light gray, whereas that of UING5-249 is in dark gray.

dx.doi.org/10.1021/cb500629k | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

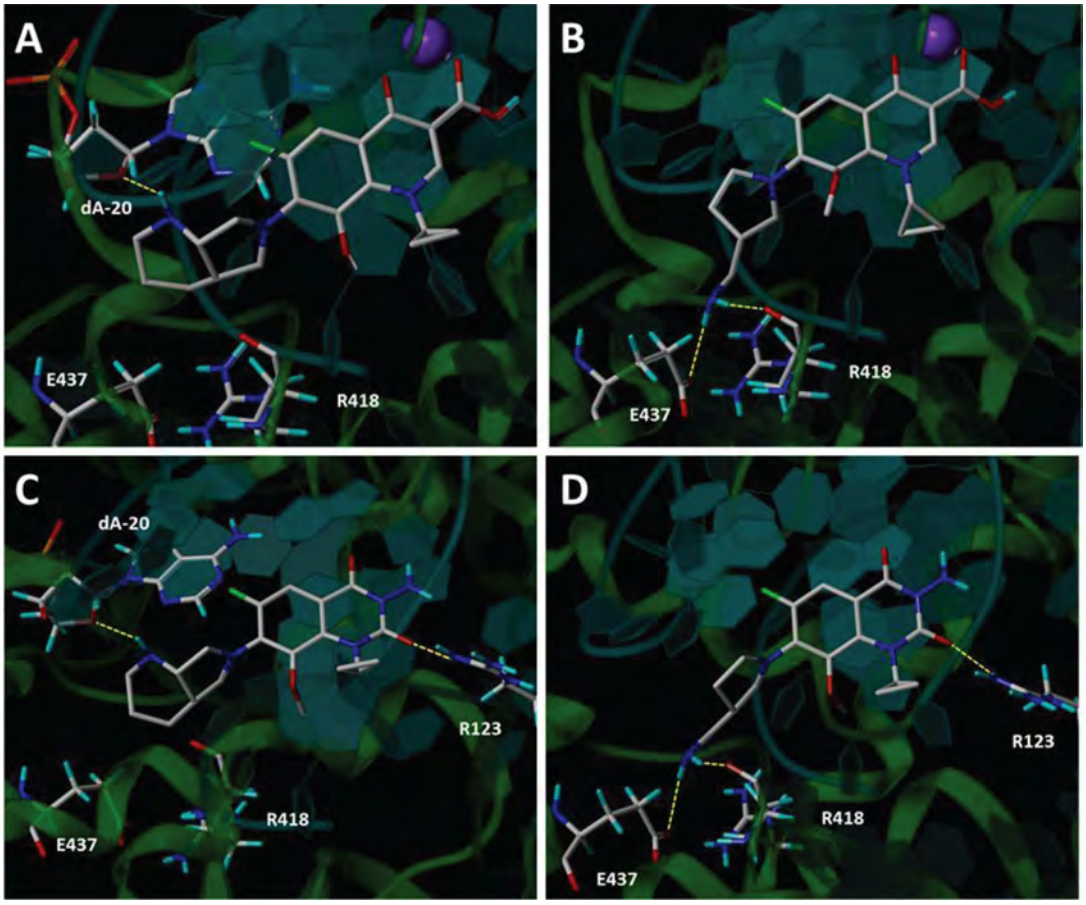


Figure 6. Docking of quinolones and diones with wild-type enzyme. Representative cleaved complex interactions were taken from the top 20 binding poses obtained from docking moxifloxacin (A), UING5-249 (B), UING5-157 (C), and UING5-207 (D) into the 2XKK (wild-type) crystal structure. Hydrogen bonds are shown as dashed yellow lines. The top scoring and most consistent pose for moxifloxacin and UING5-157 shows a hydrogen bond to the deoxyribose oxygen of dA-20; top pose of UING5-249 and UING5-207 shows hydrogen bonds from the C-7 aminomethyl pyrrolidine primary amine to E437 and R418. Both diones were anchored to ParC-Arg123 through a hydrogen bond with their C-2 carbonyl group.

Although additional work is required to understand this phenomenon, it is likely that GyrA-G81C gyrase may still allow tighter binding of the quinolone than the dione (Figure 4Aii).

C-7 Ring Substituents Interact with GyrB. Comparison of diones having various C-7 rings identified UING5-207 as the most active at forming cleaved complexes (Figure 1B). This result is consistent with UING5-207 being more active than other 8-methoxy diones, as determined with *Bacillus anthracis* topoisomerase IV.[18] To better understand this elevated activity, we examined published X-ray structures derived from compounds similar to UING5-249 and UING5-207 in complex with type-II topoisomerases and DNA.[11−14] When we substituted dione UING5-207 and UING5-249 for moxifloxacin in X-ray structure PDB 2XKK (moxifloxacin complexed with *Acinetobacter baumannii* topoisomerase IV; a comparable gyrase structure is not presently available), we noticed during manual docking that the primary amine of the C-7 aminomethyl pyrrolidine group is in a position where it could potentially form a salt bridge/hydrogen bond with Glu466 of GyrB (Figure 5A,B). Manually rotating the C-7 group of moxifloxacin to make this same binding contact revealed that the secondary amine of the moxifloxacin C-7 group was unlikely to extend far enough to form a similar binding interaction with GyrB Glu466. Thus, the C-7 aminomethyl pyrrolidine substituent appeared to afford an additional binding contact with GyrB.

We next turned to computer-aided docking and fitting. The 20 best binding poses for each fluoroquinolone and dione were obtained by first docking with the 2XKK topoisomerase IV crystal structure of cleaved complexes formed with moxifloxacin. The poses were then overlaid and examined for differences in the relative orientations of functional groups and for binding contacts with DNA and protein (see Supporting Information Figures S7, S8, and Table S2 for the 20 docked poses). Docking of moxifloxacin into the fluoroquinolone-binding site of the 2XKK crystal structure revealed that the C-7 group preferentially adopts a conformation that creates a hydrogen bond to the deoxyribose oxygen of dA-20 (see Figure 6A for top-scoring binding pose). In contrast, many of the top binding poses for the UING5-249 fluoroquinolone docked into this site exhibited a 180° rotation of the C-7 aminomethyl pyrrolidine group such that the primary amine of this moiety could form a hydrogen bond with both ParE-Glu437 (corresponding to *E. coli* GyrB-466) and the backbone carbonyl of nearby ParE-Arg418 (Figure 6B). Indeed, docked poses for UING5-249 revealed that the C-7 aminomethyl pyrrolidine group is capable of simultaneously forming hydrogen bonds with multiple residues. However, in the rigid C-7 ring system of moxifloxacin, the amine group is directly linked to the pyrrolidine and cannot reach ParE-Glu437. A

dx.doi.org/10.1021/cb500629k | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

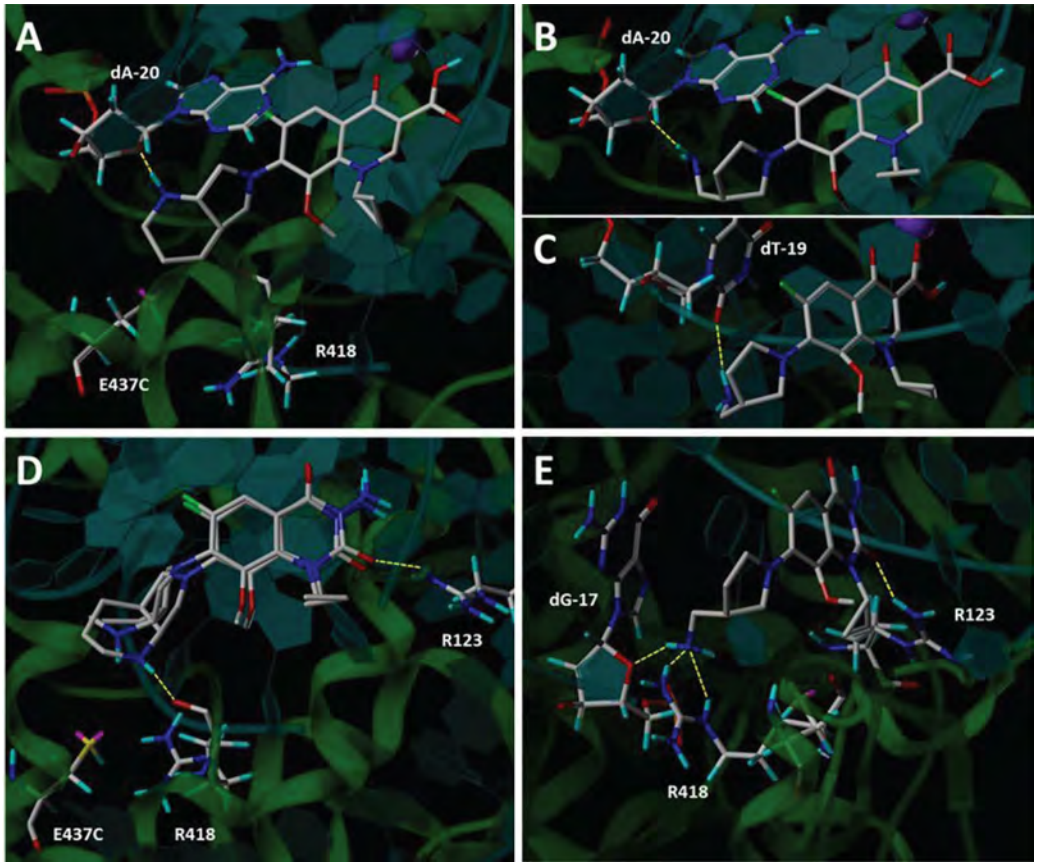


Figure 7. Docking of quinolones and diones with ParE/GyrB mutant enzyme. Representative ternary complex interactions were taken from the top 20 binding poses obtained from docking moxifloxacin (A), UING5-249 (B, C), UING5-157 (D), and UING5-207 (E) into the 2XKK crystal structure modified from ParE-Glu437 to Cys437. Hydrogen bonds are shown as dashed yellow lines. The top pose for moxifloxacin (A) showed formation of the same hydrogen bond to dA-20 as that found with the wild-type structure. However, when UING5-249 was docked into the E437C mutant structure (B, C), the top scoring poses no longer showed hydrogen bonds to the 437 position or to R418. Instead, the C-7 amine rotated about 180° to form hydrogen-bond interactions with DNA (dA-20 or dT-19). In the case of docking UING5-157 (D) into the mutant enzyme, the top two poses (shown) either form a hydrogen bond to R418 or form no electrostatic/hydrogen-bond interaction. When UING5-207 (E) was docked, its top pose displayed hydrogen bonds from the C-7 amine to both the R418 side chain and to the deoxyribose oxygen of dG-17.

similar difference between binding contacts of the C-7 groups was seen with the cognate diones, UING5-157 and UING5-207 (Figure 6C,D).

In addition to the preferred binding contacts between the primary amine of the aminomethyl pyrrolidine group with ParE-Glu437 (*E. coli* GyrB-Glu466), the dione UING5-207 also adopted several poses in which the primary amine of the aminomethyl pyrrolidine formed single hydrogen-bonding interactions with ParE-Glu437 and the backbone carbonyl of ParE-Arg418 (Figure 6D). An interaction of the moxifloxacin C-7 group with ParE-Arg418 was also observed, but only in a few of the lower-scoring poses (Supporting Information Table S2). Thus, the fluoroquinolone and dione derivatives containing a C-7 aminomethyl pyrrolidine appear to be capable of forming binding interactions with GyrB residues, whereas the C-7 group of moxifloxacin and the cognate dione, UING5-157, preferentially interact with dA-20. As expected from crystal structures, docking the fluoroquinolone required the C-3/C-4 ketoacid−magnesium interaction to anchor the fluoroquinolones to helix-4. To observe dione docking, the compounds needed to be anchored through their C-2 carbonyl group to ParC-Arg123, as seen in the PDB 3LTN structure of *S. pneumoniae* topoisomerase IV bound to dione and DNA.[11]

We next performed docking with the GyrB-E466C substitution by introducing the equivalent change (ParE-E437C) into the 2XKK crystal structure of cleaved complexes formed with *A. baumannii* topoisomerase IV. With moxifloxacin, the top 20 binding poses were similar to the top 20 binding poses generated using wild-type enzyme (Figure 7A; see Supporting Information Figures S7 and S8 for visualization of all 20 binding poses), as expected from moxifloxacin lacking a contact with GyrB-Glu466. Thus, the ParE-E437C (GyrB-E466C) substitution appears to have little effect on the preferred interaction of the C-7 group of moxifloxacin. In contrast, the top 20 binding poses of the fluoroquinolone UING5-249 with the ParE-E437C variant showed greater variation for C-7 group orientation and binding contacts: the primary amine of the C-7 aminomethyl pyrrolidine group formed hydrogen bonds to dA-20, to dT-19, to the backbone carbonyl of ParE-Arg418, or simply failed to form any polar or electrostatic interaction (two DNA contacts are exemplified in Figures 7B,C). Thus, the Cys substitution severely disrupts interaction with GyrB-466. Similar differences were seen with the cognate diones (Figure 7D,E), indicating that the C-7 aminomethyl pyrrolidine group might interact with wild-type GyrB and thus provide a stabilizing effect with GyrB that is not seen with the C-7 diazabicylco compounds (i.e., moxifloxacin

dx.doi.org/10.1021/cb500629k | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

and UING5-157). Finally, a consistent observation throughout comparison of the top binding poses was that, as compared to the fluoroquinolone, the positioning and orientation of the dione core structures were highly conserved in all binding poses due to the restriction imposed by the hydrogen bond between the dione C-2 carbonyl group and Arg123 of ParC (see overlapping structures in Supporting Information Figures S7 and S8).

To provide an experimental test for an interaction between the C-7 aminomethyl pyrrolidine group and GyrB-Glu466, we compared cleaved complex-forming activity of diones UING5-207 and UING5-157 using a GyrB-E466C mutant gyrase available from other work.[6] With wild-type gyrase, UING5-207 was about 10-fold more active than the moxifloxacin-like dione UING5-157 (Figure 8A,B). Replacement of the anionic glutamate with the shorter, uncharged cysteine side chain reduced the activity more for UING5-207 than for UING5-157, presumably due to a loss of C-7 ring interactions between UING5-207 and GyrB-Glu466 (the core portions of diones position similarly in mutant and wild-type proteins, Supporting Information Figures S7 and S8). The differences shown in Figure 8A,B,E are consistent with the GyrB-E466C substitution removing a preferred binding contact for the C-7 aminomethyl pyrrolidine group of dione UING5-207. The unique binding interactions between the aminomethyl pyrrolidine and GyrB thus appears to improve the ability of diones with this substituent to poison gyrases bearing helix-4 substitutions compared to derivatives having other C-7 groups that have been examined to date.

The fluoroquinolone comparison (UING5-249 vs moxifloxacin) with GyrB E466C was more complex because the substitution could perturb both GyrB-C-7 ring interactions and formation of the magnesium−water bridge at the other end of the fluoroquinolone. When the top 20 poses for each docking run were considered as a whole (Supporting Information Figures S7 and S8), both moxifloxacin and the UING5-249 fluoroquinolone docked in a generally less-ordered fashion to GyrB-E466C gyrase than to the wild-type enzyme, and the docking orientation around the magnesium ion was less ordered with mutant gyrase. In addition, UING5-249 was unable to form a hydrogen bond to GyrB-466 when it was cysteine. These observations suggest that both fluoroquinolones would exhibit lower activity with GyrB-E466C gyrase and that the reduction for UING5-249 would be slightly greater. Such was the case when cleaved complex-forming activity was measured (Figure 8C−E). Efforts are now being made to obtain crystal structures of cleaved complexes formed with gyrase and either UING5-249 or UING5-207 to confirm and extend conclusions derived from docking and DNA cleavage studies. Dione-resistant mutants are also being examined to locate GyrB substitutions and determimne their impact on drug susceptibility.

Crossover Activity with Human Topoisomerase II. For reasons that were previously unclear, the C-7 aminomethyl pyrrolidine group confers crossover activity for fluoroquinolones and diones with human topoisomerase II.[18] When we compared spatial orientation of amino acid residues in the cleaved complex region of the crystal structures for *A. baumannii* topoisomerase IV (2XKK)[14] with that of human topoisomerase IIβ (3QX3),[26] we found that human topoisomerase II has glutamic acid (E522) and arginine (R503) residues in its TOPRIM domain that are conserved with respect to ParE-Glu437 and ParE-Arg418 in topoisomerase IV.

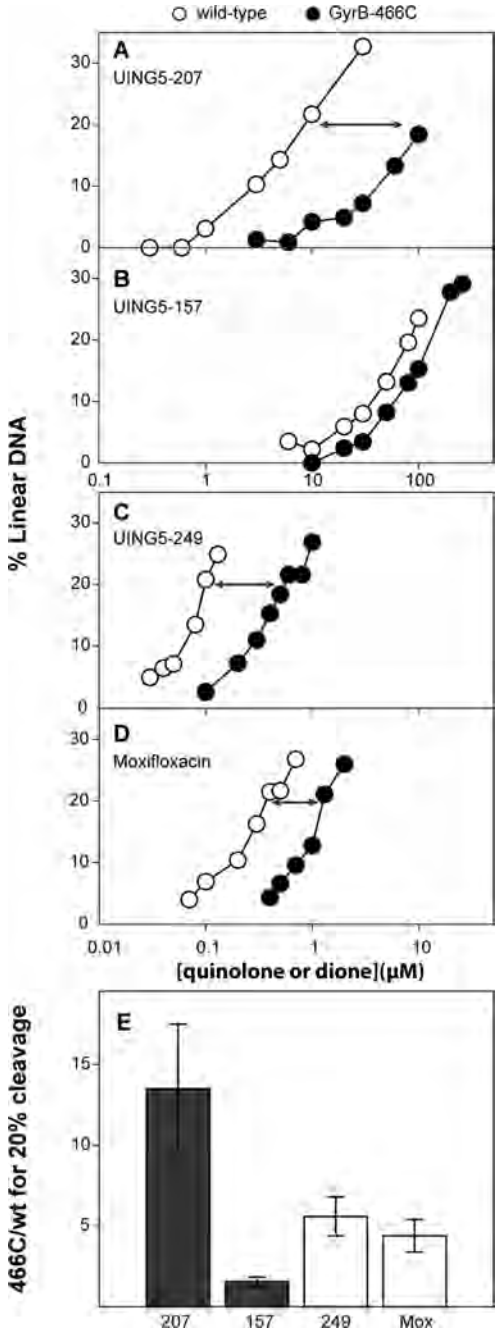


Figure 8. C-7 ring substituent affects GyrB-466C-mediated reduction in complex-forming activity. Cleaved complexes were formed at the indicated concentrations of fluoroquinolone or dione. Percent linear DNA was determined by gel electrophoresis: (A) dione UING5-207, (B) dione UING5-157, (C) fluoroquinolone UING5-249, (D) moxifloxacin. Symbols: empty circles, wild-type gyrase; filled circles, GyrB-466C gyrase. (E) Comparison of cleavage activities. Compound concentration required to achieve 20% linear DNA for GyrB-466C gyrase was divided by that value for wild-type gyrase, determined for each test compound (average values from multiple experiments; error bars represent standard deviation). Arrows indicate 20% linear DNA.

Although the amino acid sequences adjacent to the glutamate residue (DEVLASQEV and KQIMENAEI in *A. baumannii* topoisomerase IV and human topoisomerase II, respectively) are not highly conserved, spatial positioning of the glutamate residue relative to DNA and drug binding is highly conserved (3D conservation). Moreover, the nearby arginine-containing

dx.doi.org/10.1021/cb500629k | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

sequences are highly conserved (PIRGKILN vs PLRGKILN). These similarities suggest that anchoring of the drug to the cleavage site through the conserved binding interaction between the C-7 aminomethyl pyrrolidine group and the TOPRIM domain glutamic acid and/or arginine (as observed with topoisomerase IV, Figure 6B,D) contributes to the poisoning activity with helix-4 variants of *B. anthracis* topoisomerase IV and wild-type human topoisomerase II. Indeed, the binding contacts formed by the C-7 aminomethyl pyrrolidine group that impart activity against helix-4 variants of bacterial type-II topoisomerases are likely to be the same features and contacts that impart C-7 aminomethyl pyrrolidine fluoroquinolone and dione activity against human topoisomerase II.

Concluding Remarks. The traditional response to antibiotic resistance has been the creation of new compounds to which bacteria are not yet resistant. The present work focused on just such a strategy for quinolone-like quinazolinediones. Judicious choice of C-7 ring substituents, and likely many other substituents yet to be explored, can provide the stabilization needed to generate quinolone-class derivatives that have activity against fluoroquinolone-resistant type II topoisomerases. Diones bypass much of the protective effect of common GyrA-mediated resistance mutations because they do not form the drug−magnesium−water−GyrA bridge characteristic of fluoroquinolones. We found that dione activity can be increased through a specific C-7 ring interaction with *E. coli* GyrB-466. Since the most active compounds with purified *E. coli* gyrase were generally the most active with cultured cells (Supporting Information Table S1), biochemical studies of the type described are likely to be useful for finding more effective agents.

In the course of the present work, the Osheroff and Kerns laboratories found that quinolone/dione C-7 substituents that facilitate action with bacterial topoisomerase helix-4 variants also facilitate action with human topoisomerase II,[18] a result explained by the findings reported here. Although such activity may cause topoisomerase II-based toxicity in mammalian hosts, other aspects of dione/quinolone structure, such as substituents at the C-8 position, can be altered to discriminate activity between human and bacterial enzymes.[18] We expect the experimental and computational modeling approach presented above to provide a platform for designing new quinolone-class antibacterials having both elevated activity with wild-type and fluoroquinolone-resistant bacteria, along with reduced activity against human topoisomerase II.

METHODS

GyrA and GyrB subunits of *E. coli* gyrase, including those with GyrA-G81C and GyrB-E466C substitutions, were expressed and purified separately as described previously[6,27] and in Supporting Information. Moxifloxacin and ciprofloxacin were obtained from Bayer Healthcare; gatifloxacin was from Bristol-Meyers-Squibb; PD161148 was from Parke-Davis Division of Pfizer Corp. Other fluoroquinolones and quinazolinediones were synthesized as described previously.[19] Formation and detection of cleaved complexes was as described previously[6] and in Supporting Information. In all cases, a quinolone or dione was used to form cleaved complexes.

Resealing of plasmid DNA was accomplished in two ways. In one, reaction mixtures, prepared and incubated to obtain cleaved complexes, were incubated in EDTA at 37 °C for 30 min before SDS (1% w/v) and proteinase K (100 μg mL^{-1}) were added for an additional 15 min incubation. In the other, aliquots of reaction mixtures, incubated as described for complex formation, were incubated at various temperatures for 5 min followed by incubation in 1% SDS and 100 μg mL^{-1} proteinase K for another 15 min at 37 °C. In both reaction schemes, EDTA concentration was brought to 50 mM prior to applying reaction mixtures to agarose gels. Plasmid species were separated by gel electrophoresis and quantified.

For modeling studies, an initial manual analysis and manipulation of fluoroquinolone-gyrase cleaved complexes was performed using structures obtained from PBD accession numbers 2XKK for *A. baumannii* topoisomerase IV[14] and 3LTN for *S. pneumoniae* topoisomerase IV[11] using WebLab ViewerLight. For computer-aided docking, SYBYL-X 1.3 was used to prepare the 2XKK crystal structure for ligand docking.[28] Standard Tripos procedures were followed for SYBYL-X 1.3 (Certara, L.P.). Briefly, metal bonds were removed, atom types in the ligands were fixed, missing side chains were added, protein termini were charged, hydrogen atoms were added, and a staged minimization of the entire complex was performed. Details are provided in the Supporting Information. The quinazoline-2,4-dione ligands were docked in two steps. In the first step, dione UING5-157, the cognate of moxifloxacin, was docked according to the procedure in the Supporting Information except that the magnesium ion of the magnesium−water bridge was removed prior to docking. After docking, the top 20 binding poses were examined, and the highest scoring pose that showed a hydrogen-bond interaction between the dione C-2 carbonyl and ParC-Arg123 was used as the template for subsequent docking of UING5-157 and UING5-207. The parameters of the second docking run were identical to the first except that deviation from the ParC-Arg123 hydrogen-bond-forming binding pose template was required to exceed 5 pK_d/Å^2 to be scored. This very small penalty had the effect of fixing the dione core in the position revealed by the 3LTN crystal structure[11] while allowing the C-7 substituents to sample various binding interactions with GyrB and DNA. Binding poses were examined and visualized using the SYBYL-X 1.3 software.

ASSOCIATED CONTENT

Supporting Information

Comparison of fluoroquinlones and cognate quinazolinediones for properties of cleaved complexes and growth inhibition, tabulation of hydrogen bonds formed with the terminal amine of each C-7 group, separation of plasmid DNA species by gel electrophoresis, biphasic effect of EDTA on resealing of DNA in fluoroquinolone-containing cleaved complexes, effect of sodium acetate concentration on DNA linearization in drug−gyrase−DNA complexes, effect of sodium acetate on the thermal resealing of cleaved complexes, temperature-mediated changes in relative abundance of plasmid DNA species in cleaved complexes, increased temperature-induced DNA resealing due to increased inhibitor concentration, docking of quinolones and diones with wild-type enzyme, docking of quinolones and diones with ParE-E437C (GyrB-E466C) mutant enzyme, relative activities of gyrases, and detailed descriptions of methods. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*(K.D.) E-mail: drlicaka@njms.rutgers.edu.
*(R.J.K.) E-mail: robert-kerns@uiowa.edu.
*(J.M.B.) E-mail: jberge29@jhmi.edu.

Present Address

‖Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, United States.

Author Contributions

[⊥]Drs. Drlica, Kerns, and Berger contributed equally to experimental design, obtaining funding, and manuscript preparation.

Funding

This work was supported by the National Institutes of Health [R01AI073491 to K.D., R01AI87671 to R.J.K., and R01CA077373 to J.M.B.]. Funding for open access charge: National Institutes of Health.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank members of the Berger laboratory, especially K. Jude, for technical advice, M. Malik for preparing figures, and the following for many experimental suggestions and critical comments on the manuscript: M. Gennaro, K. Jude, M. Malik, R. Pine, and X. Zhao. K.D. was a Visiting Scholar, University of California, Berkeley, CA.

■ REFERENCES

(1) Osheroff, N., and Zechiedrich, E. (1987) Calcium-promoted DNA cleavage by eukaryotic topoisomerase II: trapping the covalent enzyme-DNA complex in an active form. *Biochemistry 26*, 4303−4309.

(2) Howard, M. T., Neece, S. H., Matson, S. W., and Kreuzer, K. N. (1994) Disruption of a topoisomerase−DNA cleavage complex by a DNA helicase. *Proc. Natl. Acad. Sci. U.S.A. 91*, 12031−12035.

(3) Snyder, M., and Drlica, K. (1979) DNA gyrase on the bacterial chromosome: DNA cleavage induced by oxolinic acid. *J. Mol. Biol. 131*, 287−302.

(4) Drlica, K., Malik, M., Kerns, R. J., and Zhao, X. (2008) Quinolone-mediated bacterial death. *Antimicrob. Agents Chemother. 52*, 385−392.

(5) Kreuzer, K. N., and Cozzarelli, N. R. (1979) *Escherichia coli* mutants thermosensitive for deoxyribonucleic acid gyrase subunit A: effects on deoxyribonucleic acid replication, transcription, and bacteriophage growth. *J. Bacteriol. 140*, 424−435.

(6) Mustaev, A., Malik, M., Zhao, X., Kurepina, N., Luan, G., Oppegard, L., Hiasa, H., Marks, K., Kerns, R., Berger, J., and Drlica, K. (2014) Fluoroquinolone−gyrase−DNA complexes: two modes of drug binding. *J. Biol. Chem. 289*, 12300−12312.

(7) Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T., and Tomizawa, J.-L. (1977) Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. *Proc. Natl. Acad. Sci. U.S.A. 74*, 4772−4776.

(8) Sugino, A., Peebles, C., Kruezer, K., and Cozzarelli, N. (1977) Mechanism of action of nalidixic acid: purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. *Proc. Natl. Acad. Sci. U.S.A. 74*, 4767−4771.

(9) Aldred, K., McPherson, S., Turnbough, C., Kerns, R., and Osheroff, N. (2013) Topoisomerase IV−quinolone interactions are mediated through a water-metal ion bridge: mechanistic basis of quinolone resistance. *Nucleic Acids Res. 41*, 4628−4639.

(10) Pan, X., Dias, M., Palumbo, M., and Fisher, L. (2008) Clerocidin selectively modifies the gyrase-DNA gate to induce irreversible and reversible DNA damage. *Nucleic Acids Res. 36*, 5516−5529.

(11) Laponogov, I., Pan, X., Veselkov, D., McAuley, K., Fisher, L., and Sanderson, M. (2010) Structural basis of gate-DNA breakage and resealing by type II topoisomerases. *PLoS One 5*, e11338.

(12) Laponogov, I., Sohi, M., Veselkov, D., Pan, X., Sawhney, R., Thompson, A., McAuley, K., Fisher, L., and Sanderson, M. (2009) Structural insight into the quinolone−DNA cleavage complex of type IIA topoisomerases. *Nat. Struct. Mol. Biol. 16*, 667−669.

(13) Bax, B., Chan, P., Eggleston, D., Fosberry, A., Gentry, D., Gorrec, F., Giordano, I., Hann, M., Hennessy, A., Hibbs, M., Huang, J., Jones, E., Jones, J., Brown, K., Lewis, C., May, E., Saunders, M., Singh, O., Spitzfaden, C., Shen, C., Shillings, A., Theobald, A., Wohlkonig, A., Pearson, N., and Gwynn, M. (2010) Type IIA topoisomerase inhibition by a new class of antibacterial agents. *Nature 466*, 935−940.

(14) Wohlkonig, A., Chan, P., Fosberry, A., Homes, P., Huang, J., Kranz, M., Leydon, V., Miles, T., Pearson, N., Perera, R., Shillings, A., Gwynn, M., and Bax, B. (2010) Structural basis of quinolone inhibition of type IIA topoisomerases and target-mediated resistance. *Nat. Struct. Mol. Biol. 17*, 1152−1153.

(15) Deweese, J., and Osheroff, N. (2010) The use of divalent metal ions by type II topoisomerases. *Metallomics 2*, 450−459.

(16) Aldred, K., McPherson, S., Wang, P., Kerns, R., Graves, D., Turnbough, C., and Osheroff, N. (2012) Drug interactions with *Bacillus anthracis* topoisomerase IV: biochemical basis for quinolone action and resistance. *Biochemistry 51*, 370−381.

(17) Pan, X., Gould, K., and Fisher, L. M. (2009) Probing the differential interactions of quinazolinedione PD 0305970 and quinolones with gyrase and topoisomerase IV. *Antimicrob. Agents Chemother. 53*, 3822−3831.

(18) Aldred, K., Schwanz, H., Li, G., McPherson, S., CL Turnbough, J., Kerns, R., and Osheroff, N. (2013) Overcoming target-mediated quinolone resistance in topoisomerase IV by introducing metal ion-independent drug-enzyme interactions: implications for drug design. *ACS Chem. Biol. 8*, 2660−2668.

(19) German, N., Malik, M., Rosen, J., Drlica, K., and Kerns, R. (2008) Use of gyrase resistance mutants to guide selection of 8-methoxy-quinazoline-2,4-diones. *Antimicrob. Agents Chemother. 52*, 3915−3921.

(20) Malik, M., Marks, K., Mustaev, A., Zhao, X., Chavda, K., Kerns, R., and Drlica, K. (2011) Fluoroquinolone and quinazolinedione activities against wild-type and gyrase mutant strains of *Mycobacterium smegmatis*. *Antimicrob. Agents Chemother. 55*, 2335−2343.

(21) Oppegard, L. M., Streck, K., Rosen, J., Shwanz, H. A., Drlica, K., Kerns, R. J., and Hiasa, H. (2010) Comparison of *in vitro* activities of fluoroquinolone-like 2,4- and 1.3-diones. *Antimicrob. Agents Chemother. 54*, 3011−3014.

(22) Noble, C., and Maxwell, A. (2002) The role of GyrB in the DNA cleavage−religation reaction of DNA gyrase: a proposed two metal-ion mechanism. *J. Mol. Biol. 318*, 361−371.

(23) Pitts, S., Liou, G., Mitchenall, L., Burgin, A., Maxwell, A., Neuman, K., and Osheroff, N. (2011) Use of divalent metal ions in the DNA cleavage reaction of topoisomerase IV. *Nucleic Acids Res. 39*, 4808−4817.

(24) Sander, M., and Hsieh, T. (1983) Double strand DNA cleavage by type II DNA topoisomerase from *Drosophila melanogaster*. *J. Biol. Chem. 258*, 8421−8428.

(25) Maruri, F., Sterling, T., Kaiga, A., Blackman, A., vanderHeijden, Y., Mayer, C., Cambau, E., and Aubry, A. (2012) A systematic review of gyrase mutations associated with fluoroquinolone-resistant *Mycobacterium tuberculosis* and a proposed gyrase numbering system. *J. Antimicrob. Chemother. 67*, 819−831.

(26) Wu, C., Li, T., Farh, L., Lin, L., Lin, T., Yu, Y., Yen, T., Chiang, C., and Chan, N. (2011) Structural basis of type II topoisomerase inhibition by the anticancer drug etoposide. *Science 333*, 459−462.

(27) Schoeffler, A., May, A., and Berger, J. (2010) A domain insertion in *Escherichia coli* GyrB adopts a novel fold that plays a critical role in gyrase function. *Nucleic Acids Res. 38*, 7830−7844.

(28) Jain, A. (2003) Surflex: fully automatic flexible molecular docking using a molecular similarity-based search engine. *J. Med. Chem. 46*, 499−511.