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Overcoming Target-Mediated Quinolone Resistance in Topoisomerase IV by Introducing Metal Ion-Independent Drug-Enzyme Interactions

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Abstract

Quinolones, which target gyrase and topoisomerase IV, are the most widely prescribed antibacterials worldwide. Unfortunately, their use is threatened by the increasing prevalence of target-mediated drug resistance. Greater than 90% of mutations that confer quinolone resistance act by disrupting enzyme-drug interactions coordinated by a critical water-metal ion bridge. Quinazolinediones are quinolone-like drugs, but lack the skeletal features necessary to support the bridge interaction. These compounds are of clinical interest, however, because they retain activity against the most common quinolone resistance mutations. We utilized a chemical biology approach to determine how quinazolinediones overcome quinolone resistance in *Bacillus anthracis* topoisomerase IV. Quinazolinediones that retain activity against quinolone-resistant topoisomerase IV do so primarily by establishing novel interactions through the C7 substituent, rather than the drug skeleton. Because some quinolones are highly active against human topoisomerase IIα, we also determined how clinically relevant quinolones discriminate between the bacterial and human enzymes. Clinically relevant quinolones display poor activity against topoisomerase IIα because the human enzyme cannot support drug interactions mediated by the water-metal ion bridge. However, the inclusion of substituents that allow quinazolinediones to overcome topoisomerase IV-mediated quinolone resistance can cause cross-reactivity against topoisomerase IIα. Therefore, a major challenge in designing drugs that overcome quinolone resistance lies in the ability to identify substituents that mediate strong interactions with the bacterial, but not the human, enzymes. Based on our understanding of quinolone-enzyme

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SUPPORTING INFORMATION

Supplementary Table S1 contains the full chemical, library, and abbreviated names for the compounds used in this study. Supplementary Methods, Supplementary Scheme S1, and Supplementary Figures S1–21 describe the synthesis and structural characterization of the quinolones and quinazolinediones generated for this study and the purification protocols for the enzymes. This material is available free of charge *via* the Internet at http://pubs.acs.org.

interactions, we have identified three compounds that display high activity against quinolone-resistant *B. anthracis* topoisomerase IV but low activity against human topoisomerase IIα.

Quinolones, including ciprofloxacin and moxifloxacin, are broad-spectrum antibacterial agents that are used to treat a wide variety of Gram-negative and Gram-positive infections.[1–6] Unfortunately, the clinical utility of quinolones is being threatened by the growing prevalence of resistance, which extends to nearly every bacterial infection that is treated with this drug class.[4,5] Quinolones kill bacteria by increasing levels of DNA strand breaks generated by gyrase and topoisomerase IV.[2,5,7–9] The most commonly seen resistance is target-mediated and is caused by specific point mutations in these two enzymes.[2,5–7,10–13]

Both gyrase and topoisomerase IV are essential to the bacterial cell. Gyrase alleviates positive DNA supercoils that accumulate during replication and regulates the superhelical density of the bacterial chromosome. Topoisomerase IV primarily unknots and untangles DNA and is required for chromosome segregation.[6,14,15] Both enzymes alter DNA topology by generating a double-stranded break in the genetic material and passing a separate double helix through the transient DNA gate.[6,14–17] Gyrase and topoisomerase IV are comprised of two protomer subunits (GyrA and GyrB in gyrase; GrlA and GrlB in Gram-positive topoisomerase IV) and have A_2B_2 quaternary structures. The A subunits contain the active site tyrosine residues involved in DNA cleavage and ligation, and the B subunits bind and hydrolyze ATP, which is required for overall catalytic activity.[6,8,14–16]

The most prevalent resistance-conferring mutations occur at a highly conserved serine residue in the A subunit of gyrase or topoisomerase IV[18] (originally described as Ser83 in *Escherichia coli* gyrase[19,20]). The second most prevalent mutations occur at a conserved acidic residue located four amino acids downstream (Glu87 in *E. coli* GyrA).[5,13,18,21] In general, alterations at these two amino acids are observed in >90% of quinolone-resistant clinical bacterial isolates.[13,21] Although the involvement of these amino acid residues in quinolone sensitivity has been known since the 1980s, the mechanistic basis by which their mutations cause drug resistance only recently has been described.[22,23]

Quinolones contain a C3/C4 keto acid that has long been known to chelate a divalent metal ion (Figure 1).[24–26] However, the role of this metal ion in drug function had not been demonstrated. A first indication for the importance of this chelated metal ion came from a recent structure of a ternary *Acinetobacter baumannii* topoisomerase IV-cleaved DNA-moxifloxacin complex (*i.e.*, cleavage complex).[27] Whereas previous structures had not captured the metal ion, the *A. baumannii* structure identified a chelated Mg^{2+} ion that appeared to be coordinated to four water molecules. Furthermore, two of these water molecules were situated close enough to Ser84 and Glu88 (equivalent to *E. coli* GyrA Ser83 and Glu87) to form hydrogen bonds. On the basis of this finding, as well as subsequent functional studies of quinolone (and related drugs) activity against wild-type and drug-resistant *Bacillus anthracis* topoisomerase IV, it was concluded that this water-metal ion interaction that “bridges” the drug to the enzyme plays a pivotal role in mediating quinolone activity (Figure 1).[23] The following model for quinolone action and the most common cause of resistance has emerged:

1. The water-metal ion bridge serves as the primary interaction between clinically relevant quinolones and bacterial type II topoisomerases.
2. The serine and acidic amino acid residues act as the anchor points that coordinate the bridge to the enzyme.

3. Mutations in the serine or acidic amino acid residues cause resistance by interfering with bridge-enzyme interactions. Thus, they decrease quinolone potency and the ability of these drugs to stabilize enzyme-DNA cleavage complexes.

An important ramification of the above model is that the most important interaction between clinically relevant quinolones and their bacterial enzyme targets is mediated through the drug skeleton (Figure 1). This may explain the tolerance for the structural diversity of substituents at the N1, C7, and C8 positions of this drug class.

Quinazolinediones (Figure 1) share structural homology with quinolones, but lack the C3/C4 keto acid that is required for metal ion chelation. Several studies have demonstrated that members of this drug class can overcome quinolone resistance caused by mutations in the serine or acidic amino acid residue,[22,23,28–32] suggesting that the quinazolinedione skeleton does not require the water-metal ion bridge to interact with bacterial type II topoisomerases. However, the quinazolinediones that maintain activity against resistant enzymes (and bacterial strains) feature a 3′-(aminomethyl)- or 3′-(aminoethyl)pyrrolidinyl substituent at the C7 position, which is not represented in any clinically used quinolone.[22,23,28–32] Therefore, it also is possible that quinazolinediones overcome resistance through additional drug-enzyme contacts mediated by the C7 substituent.

To resolve this issue, a series of quinazolinediones and quinolones that contained clinically utilized groups or a 3′-(aminomethyl)pyrrolidinyl group at the C7 position were tested for their activity against wild-type and quinolone-resistant GrlA^{S81F} or GrlA^{S81Y} *B. anthracis* topoisomerase IV. Results indicate that the quinazolinedione skeleton does not interact with the enzyme through metal ion-independent contacts. Rather, quinazolinediones are essentially quinolones that lack their most important interaction with the bacterial enzyme. Quinazolinediones that display high activity against wild-type and quinolone-resistant type II topoisomerases do so primarily by establishing novel interactions through the C7 (and other) ring substituents. Further studies establish that clinically relevant quinolones display poor activity against human topoisomerase II because the enzyme cannot support drug interactions mediated by the water-metal ion bridge. Unfortunately, the inclusion of C7 (and other) substituents that allow quinazolinediones to overcome quinolone resistance in bacterial type II enzymes can lead to cross-reactivity against human topoisomerase IIα. Therefore, a major challenge in designing quinolone-like drugs that overcome target-mediated resistance lies in the ability to identify substituents that mediate strong interactions with the bacterial, but not the human, type II enzymes. Three such compounds are identified in the present study.

RESULTS AND DISCUSSION

Contributions of the Quinazolinedione Skeleton *vs*. Substituents to Drug Activity Against Quinolone-Resistant Topoisomerase IV

To address the mechanism by which quinazolinediones overcome quinolone resistance, three series of quinazolinediones and quinolones that contained a piperazinyl, diazabicyclononyl, or 3′-(aminomethyl)pyrrolidinyl group at the C7 position (Figure 2) were tested for their activity against wild-type and quinolone-resistant GrlA^{S81F} or GrlA^{S81Y} *B. anthracis* topoisomerase IV. Each series also featured matched compounds that included a hydrogen, methyl, or methoxy group at the C8 position.

The first two series were based on the C7 substituents of ciprofloxacin (Figure 3) and moxifloxacin (Figure 4), respectively, which are in wide clinical use. As expected, all of the quinolones in both series (top panels) displayed high activity against wild-type *B. anthracis* topoisomerase IV but decreased activity against the resistant Ser81 mutant enzymes. In

contrast, the parallel quinazolinedione series (bottom panels) displayed poor activity against both the wild-type and mutant enzymes. These findings strongly suggest that (at least in compounds that incorporate these clinically relevant C7 substituents) the quinazolinedione skeleton does not form strong interactions with topoisomerase IV.

To test this conclusion, a series of quinazolinediones and quinolones that featured the 3′-(aminomethyl)pyrrolidinyl group at C7 were examined (Figure 5). Consistent with previous reports,[22,23,29,31,32] all of the quinazolinediones in this series (bottom panels) showed high activity against wild-type and mutant quinolone-resistant topoisomerase IV. Significantly, the parallel quinolone series (top panels) displayed similar results and maintained high activity against the resistant enzymes. Thus, in the presence of the C7 3′-(aminomethyl)pyrrolidinyl substituent, the drug skeleton (quinolone *vs.* quinazolinedione) makes little difference.

Taken together, the above results indicate that the quinazolinedione skeleton does not interact with the enzyme through metal ion-independent contacts. Rather, quinazolinediones are essentially quinolone derivatives that lack their most important interaction (mediated by the water-metal ion bridge) with bacterial type II topoisomerases. Furthermore, the ability of these compounds to act against wild-type gyrase and topoisomerase IV and to overcome the most common forms of quinolone resistance (caused by the lack of bridge function) results primarily from interactions formed by the 3′-(aminomethyl)pyrrolidinyl (and related) substituent at C7.

In addition to the C7 group, the substituent at C8 appears to make a minor contribution to the activity of both quinolones and quinazolinediones against the mutant enzymes (Figures 3–5). Generally, the inclusion of a methyl or methoxy group slightly enhanced activity (methyl ≥ methoxy > hydrogen) against the mutant enzymes. These substituents had little effect against wild-type topoisomerase IV.

Finally, the effects of the N3 amino group on quinazolinedione activity were examined (Figure 6). The loss of the group decreased the activities of compounds with a C7 piperazinyl or diazabicyclononyl group toward wild-type and mutant topoisomerase IV by ~50%. In contrast, in the presence of the C7 3′-(aminomethyl)pyrrolidinyl substituent, the loss of the N3 amino group had little effect on the activity of the quinazolinedione against topoisomerase IV. The differential effects of the N3 group may reflect the increased affinity of the 3′-(aminomethyl)pyrrolidinyl group for *B. anthracis* topoisomerase IV.

Interactions of Clinically Relevant Quinolones with Human Topoisomerase IIα

Clinically relevant quinolones, such as ciprofloxacin and moxifloxacin, display very little activity against human type II topoisomerases, even at concentrations well beyond therapeutic doses (Figures 7 and 8). However, the basis for this discrimination is not known. As discussed above, the primary interaction of clinically relevant quinolones with bacterial type II enzymes is mediated through the water-metal ion bridge.[23] Unlike gyrase and topoisomerase IV, human topoisomerase IIα and IIβ lack the serine and acidic amino acid residues that anchor the bridge. In the α isoform, for example, these residues are both methionine (Figure 7). Therefore, we propose that the loss of the bridge anchors in topoisomerase IIα and IIβ is the underlying basis for the discrimination of bacterial and human type II enzymes by clinically relevant quinolones.

To test this hypothesis, Met762 and Met766 in topoisomerase IIα were mutated to a serine and glutamic acid (hTop2A$^{M762S/M766E}$), respectively, which are the residues in *B. anthracis* topoisomerase IV that coordinate quinolones through the water-metal ion bridge (Figure 7). In contrast to results with the wild-type human enzyme, ciprofloxacin and

moxifloxacin both displayed concentration-dependent activity against hTop2A$^{M762S/M766E}$, increasing levels of DNA cleavage ≥4–fold over the range examined (Figure 7). Consistent with our results, an earlier study reported that converting amino acid residues in this region of topoisomerase IIα to those seen in *E. coli* gyrase (M762S/S763A/M766D) enhanced the ability of ciprofloxacin to inhibit DNA relaxation catalyzed by the triply mutated enzyme.[33] The fact that introducing bridge-coordinating amino acid residues sensitizes topoisomerase IIα to clinically relevant quinolones suggests that the wild-type human enzyme cannot support the water-metal ion bridge and (in some respects) is the equivalent of a quinolone-resistant topoisomerase IV.

As a control, the activities of cipro-dione and moxi-dione, which cannot support the water-metal ion bridge, against the wild-type and hTop2A$^{M762S/M766E}$ enzymes were assessed (Figure 7). These compounds contained the substituents found in ciprofloxacin and moxifloxacin, respectively, transferred to a quinazolinedione skeleton. Neither of the two compounds increased DNA cleavage mediated by either human enzyme. This finding further supports the conclusion that the enhanced activities of ciprofloxacin and moxifloxacin with hTop2A$^{M762S/M766E}$ are due to water-metal ion interactions coordinated by the quinolone skeleton, as opposed to interactions with the C7 or other substituents.

Taken together, the above results provide strong evidence that wild-type topoisomerase IIα cannot anchor the water-metal ion bridge, which explains why clinically relevant quinolones display little activity against the human enzymes.

Effects of the C7 3′-(Aminomethyl)pyrrolidinyl Substituent on the Activity of Compounds Against Human Topoisomerase II α

Quinolones that display high activity against eukaryotic type II topoisomerases have been reported.[34–37] For example, the ability of CP-115,955 to increase DNA cleavage mediated by human topoisomerase IIα (Figure 8, top left) is substantially greater than that of the widely-prescribed anticancer drug etoposide.[38–41] CP-115,955 differs from ciprofloxacin only by the substitution of a 4′-hydroxyphenyl ring for the piperazinyl ring at the C7 position. This finding strongly suggests that quinolone interactions with human type II topoisomerases can be driven by the C7 substituent.

The interactions of CP-115,955 with topoisomerase IIα parallels those of compounds with the C7 3′-(aminomethyl)pyrrolidinyl substituent with quinolone-resistant *B. anthracis* topoisomerase IV (Figure 5). In the absence of the water-metal ion bridge necessary for interactions with clinically relevant quinolones, these compounds still display high activity against the human and bacterial type II enzymes, respectively. This raises the possibility that compounds containing the C7 3′-(aminomethyl)pyrrolidinyl substituent might display undesirable cross-reactivity against the human type II enzyme. With either a quinazolinedione or a quinolone that contained a C8 methyl group, compounds that included the C7 3′-(aminomethyl)pyrrolidinyl substituent displayed high activity against topoisomerase IIα (Figure 8, top left). This finding implies that the ability to design quinolones or related drugs that overcome drug resistance in bacterial gyrase or topoisomerase IV may be hampered by cross-reactivity toward human systems. Therefore, a major challenge in designing such drugs is the identification of substituents that support activity against bacterial, but not human, type II topoisomerases.

Defining Substituents on Quinolones and Quinazolinediones that Overcome Drug Resistance but Differentiate between *B. anthracis* Topoisomerase IV and Human Topoisomerase IIα

Although the C7 group appears to dominate interactions between quinolones or quinazolinediones and drug-resistant *B. anthracis* topoisomerase IV, the substituents at C8 (in both drug classes) and N3 (in quinazolinediones) appear to modulate drug activity to some extent (Figures 3–6).

With regard to quinolone interactions, human topoisomerase IIα is in many respects a "drug-resistant topoisomerase IV mutant" (see above). Therefore, in an effort to identify compounds with potent activity against wild-type and quinolone-resistant topoisomerase IV, but low activity against human type II enzymes, we examined the effects of C8 and N3 substituents on drug activity against topoisomerase IIα. All of the quinolones and quinazolinediones tested contained a C7 3′-(aminomethyl)pyrrolidinyl substituent and displayed high activity against wild-type and quinolone-resistant *B. anthracis* topoisomerase IV (Figures 5 and 6).

The nature of the groups at C8 and N3 had a larger influence on drug activity against topoisomerase IIα than they did against topoisomerase IV. The activities of compounds with C8 methoxy groups (data not shown) were somewhat lower than the corresponding compounds with C8 methyl groups (Figure 8, top left), although they still displayed reasonable activity against the human enzyme. In contrast, removal of the C8 or N3 substituent resulted in 3′-(aminomethyl)pyrrolidinyl-quinolones or -quinazolinediones that were able to differentiate between the bacterial and human enzymes. One quinolone and two quinazolinediones (Figure 8, bottom right) that displayed high activity against wild-type and mutant topoisomerase IV (Figures 5 and 6) showed virtually no activity against topoisomerase IIα at drug concentrations below 100 μM (Figure 8, top right). This concentration is well above the therapeutic window for clinically used quinolones.[42] Therefore, it is possible to design quinolones and related drugs that display high activity against the most common forms of quinolone-resistant topoisomerase IV but do not cross-react with human topoisomerase IIα.

The ability of quinolones (and related drugs) to differentiate between bacterial and human type II topoisomerases is critical to the clinical efficacy of this drug class. Clinically relevant quinolones discriminate between wild-type enzymes by utilizing a water-metal ion bridge in bacterial systems that cannot be used to support drug interactions in human systems. The key to the discrimination between drug-resistant topoisomerase IV and wild-type topoisomerase IIα relies on the relative dominance of the C7 group in mediating drug interactions. In the presence of a C7 3′-(aminomethyl)pyrrolidinyl substituent, the contributions of the C8 and N3 (in quinazolinediones) groups to drug-topoisomerase IV interactions are relatively unimportant. In contrast, these latter groups make a greater contribution to drug-topoisomerase IIα interactions. That being said, the C7 3′-(aminomethyl)pyrrolidinyl substituent still appears to be the most important of the three. To this point, quinolones or N3-amino-quinazolinediones that include the C7 groups of ciprofloxacin or moxifloxacin displayed low activity against the human type II enzyme, even in the presence of a methyl or methoxy group at C8 (data not shown).

A series of competition experiments was carried out to determine the mechanistic contributions of the C7 and C8 substituents and the quinolone *vs*. quinazolinedione skeleton to drug interactions with topoisomerase IIα. In these experiments, the ability of compounds (0–500 μM) to compete out DNA cleavage induced by 50 μM 8-methyl-3′-(AM)P-FQ was assessed (Figure 8, bottom left). Initial levels of DNA cleavage (*i.e.*, in the absence of competitor) were set to 1.00 to facilitate comparisons. The IC_{50} value for 8-H-3′-(AM)P-FQ

was ~50 μM, suggesting that the C8 group is important for drug efficacy but does not contribute significantly to binding. Presumably, interactions with the C8 methyl group help to properly position the drug in the cleavage complex. A similar result was seen with 8-H-3′-(AM)P-dione, suggesting that differences in levels of DNA cleavage induced by quinolones and quinazolinediones do not reflect changes in drug binding. In contrast, the IC_{50} value for ciprofloxacin [which differs from 8-H-3′-(AM)P-FQ only at the C7 position] was nearly 6-fold higher (~280 μM). This finding provides strong evidence that the C7 substituent plays an important role in driving binding interactions between drugs and the human enzyme.

Summary

The clinical utility of quinolones is being threatened by the increasing prevalence of target-mediated drug resistance. Greater than 90% of gyrase and topoisomerase IV mutations that confer quinolone resistance do so by disrupting enzyme-drug interactions coordinated by a critical water-metal ion bridge. We have utilized a novel chemical biology approach to determine how drugs overcome quinolone resistance in bacterial topoisomerase IV and how they interact with human topoisomerase IIα. As a result, we have identified a critical problem in designing quinolones (or related drugs) that overcome target-mediated quinolone resistance, namely cross-reactivity with the human type II enzyme. However, based on our understanding of quinolone-enzyme interactions, we have defined substituents that can overcome this issue. As a result, one quinolone and two quinazolinediones that display high activity against wild-type and quinolone-resistant *B. anthracis* topoisomerase IV but low activity against human topoisomerase IIα were identified. These compounds will be used as the starting point for further drug discovery aimed at overcoming the most common forms of quinolone resistance seen in bacterial infections.

METHODS

Materials and Enzymes

Ciprofloxacin was obtained from LKT Laboratories, and CP-115,955 was the generous gift of Thomas D. Gootz and Paul R. McGuirk (Pfizer Global Research). Both compounds were stored at −20 °C as 40 mM stock solutions in 0.1 N NaOH and diluted five-fold with 10 mM Tris–HCl (pH 7.9) immediately prior to use. Moxifloxacin was obtained from LKT Laboratories and was stored at 4 °C as a 20 mM stock solution in 100% DMSO. Etoposide was obtained from Sigma and stored at RT as a 20 mM stock solution in 100% DMSO. All other quinolones and quinazolinediones were synthesized and structurally characterized as described in the Supplementary Methods, Supplementary Scheme S1, and Supplementary Figures S1–20. 8-Methyl- and 8-methoxy-cipro were stored at −20 °C as 40 mM stock solutions in 0.1 N NaOH and diluted five-fold with 10 mM Tris–HCl (pH 7.9) immediately prior to use. All other quinolones and quinazolinediones were stored at 4 °C as 20 mM stock solutions in 100% DMSO. Supplementary Table S1 contains the full chemical, library, and abbreviated names for the compounds used in this study. Chemicals were analytical reagent grade.

Genes encoding His-tagged wild-type *B. anthracis* GrlA and GrlB and drug-resistant $GrlA^{S81F}$ and $GrlA^{S81Y}$ were expressed and purified as described previously.[43] Wild-type human topoisomerase IIα was expressed in *Saccharomyces cerevisiae*[44] and purified as described previously.[45] His-tagged mutant human topoisomerase IIα containing M762S/M766E ($hTop2A^{M762S/M766E}$) was generated by site-directed mutagenesis and expressed in *S. cerevisiae* as above. Supplementary Methods describe the construction and purification of the His-tagged topoisomerases. His-tagged wild-type topoisomerase IIα was prepared parallel to the mutant enzyme and displayed activities that were nearly identical to enzymes

prepared as described above by the protocol of Kingma *et al*.[45] (see Supplementary Figure S21).

Negatively supercoiled pBR322 plasmid DNA was prepared from *E. coli* using a Plasmid Mega Kit (Qiagen) as described by the manufacturer.

DNA Cleavage Mediated by Topoisomerase IV

DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff.[46] Reactions contained 75 nM wild-type or mutant topoisomerase IV and 10 nM negatively supercoiled pBR322 in a total of 20 μL of 40 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, and 2.5% (v/v) glycerol. Reaction mixtures were incubated at 37 °C for 10 min, and enzyme-DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM Na_2EDTA (pH 8.0). Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 45 min to digest the enzyme. Samples were mixed with 2 μL of agarose gel loading buffer [60% sucrose, 10 mM Tris–HCl (pH 7.9), 0.5% bromophenol blue, and 0.5% xylene cyanol FF], heated at 45 °C for 5 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris–acetate (pH 8.3) and 2 mM Na_2EDTA containing 0.5 μg/mL ethidium bromide. DNA bands were visualized with medium-range ultraviolet light and quantified using an Alpha Innotech digital imaging system. DNA cleavage was monitored by the conversion of supercoiled plasmid to linear molecules.

DNA Cleavage Mediated by Human Topoisomerase IIα

DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff[46]. Reactions that tested the effects of drugs on wild-type topoisomerase IIα contained 110 nM enzyme and 10 nM negatively supercoiled pBR322 in a total of 20 μL of 10 mM Tris–HCl (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 100 μM Na_2EDTA, 25 μM dithiothreitol, and 2.5% (v/v) glycerol. Reactions that compared the effects of drugs on wild-type and mutant ($hTop2A^{M762S/M766E}$) topoisomerase IIα contained 110 nM enzyme and 10 nM negatively supercoiled in a total of 20 μL of 10 mM Tris–HCl (pH 7.7), 5 mM $MgCl_2$, 140 mM KCl, 20 μM Na_2EDTA, 90 μM dithiothreitol, 7.5% (v/v) glycerol, and 1 mM ATP. The slight differences in buffer components (as compared to the above) reflect the fact that the mutant enzyme was more dilute than the wild-type enzyme preparation. These buffer differences had no effect on topoisomerase II activity. Reaction mixtures were incubated at 37 °C for 10 min and processed as described above for topoisomerase IV plasmid DNA cleavage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hooper DC. Clinical applications of quinolones. Biochim Biophys Acta. 1998; 1400:45–61. [PubMed: 9748496]
2. Hooper DC. Mechanisms of action of antimicrobials: focus on fluoroquinolones. Clin Infect Dis. 2001; 32(Suppl 1):S9–S15. [PubMed: 11249823]

3. Linder JA, Huang ES, Steinman MA, Gonzales R, Stafford RS. Fluoroquinolone prescribing in the United States: 1995 to 2002. Am J Med. 2005; 118:259–268. [PubMed: 15745724]
4. Andriole VT. The quinolones: past, present, and future. Clin Infect Dis. 2005; 41(Suppl 2):S113–119. [PubMed: 15942877]
5. Drlica K, Hiasa H, Kerns R, Malik M, Mustaev A, Zhao X. Quinolones: action and resistance updated. Curr Top Med Chem. 2009; 9:981–998. [PubMed: 19747119]
6. Pommier Y, Leo E, Zhang H, Marchand C. DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. Chem Biol. 2010; 17:421–433. [PubMed: 20534341]
7. Hooper DC. Mode of action of fluoroquinolones. Drugs. 1999; 58(Suppl 2):6–10. [PubMed: 10553698]
8. Anderson VE, Osheroff N. Type II topoisomerases as targets for quinolone antibacterials: turning Dr. Jekyll into Mr Hyde. Curr Pharm Des. 2001; 7:337–353. [PubMed: 11254893]
9. Drlica K, Malik M, Kerns RJ, Zhao X. Quinolone-mediated bacterial death. Antimicrob Agents Chemother. 2008; 52:385–392. [PubMed: 17724149]
10. Price LB, Vogler A, Pearson T, Busch JD, Schupp JM, Keim P. *In vitro* selection and characterization of *Bacillus anthracis* mutants with high-level resistance to ciprofloxacin. Antimicrob Agents Chemother. 2003; 47:2362–2365. [PubMed: 12821500]
11. Grohs P, Podglajen I, Gutmann L. Activities of different fluoroquinolones against *Bacillus anthracis* mutants selected *in vitro* and harboring topoisomerase mutations. Antimicrob Agents Chemother. 2004; 48:3024–3027. [PubMed: 15273116]
12. Bast DJ, Athamna A, Duncan CL, de Azavedo JC, Low DE, Rahav G, Farrell D, Rubinstein E. Type II topoisomerase mutations in *Bacillus anthracis* associated with high-level fluoroquinolone resistance. J Antimicrob Chemother. 2004; 54:90–94. [PubMed: 15190035]
13. Morgan-Linnell SK, Becnel Boyd L, Steffen D, Zechiedrich L. Mechanisms accounting for fluoroquinolone resistance in *Escherichia coli* clinical isolates. Antimicrob Agents Chemother. 2009; 53:235–241. [PubMed: 18838592]
14. Levine C, Hiasa H, Marians KJ. DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities. Biochim Biophys Acta. 1998; 1400:29–43. [PubMed: 9748489]
15. Champoux JJ. DNA topoisomerases: structure, function, and mechanism. Annu Rev Biochem. 2001; 70:369–413. [PubMed: 11395412]
16. Deweese JE, Osheroff MA, Osheroff N. DNA topology and topoisomerases: teaching a “knotty” subject. Biochem Molec Biol Educ. 2009; 37:2–10. [PubMed: 19225573]
17. Gentry, AC.; Osheroff, N. Encyclopedia of Biological Chemistry. Elsevier Inc; 2013. DNA topoisomerases: type II; p. 163-168.
18. Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol Mol Biol Rev. 1997; 61:377–392. [PubMed: 9293187]
19. Yoshida H, Kojima T, Yamagishi J, Nakamura S. Quinolone-resistant mutations of the gyrA gene of *Escherichia coli*. Mol Gen Genet. 1988; 211:1–7. [PubMed: 2830458]
20. Cullen ME, Wyke AW, Kuroda R, Fisher LM. Cloning and characterization of a DNA gyrase A gene from *Escherichia coli* that confers clinical resistance to 4-quinolones. Antimicrob Agents Chemother. 1989; 33:886–894. [PubMed: 2548439]
21. Li Z, Deguchi T, Yasuda M, Kawamura T, Kanematsu E, Nishino Y, Ishihara S, Kawada Y. Alteration in the GyrA subunit of DNA gyrase and the ParC subunit of DNA topoisomerase IV in quinolone-resistant clinical isolates of *Staphylococcus epidermidis*. Antimicrob Agents Chemother. 1998; 42:3293–3295. [PubMed: 9835531]
22. Aldred KJ, McPherson SA, Wang P, Kerns RJ, Graves DE, Turnbough CL Jr, Osheroff N. Drug interactions with *Bacillus anthracis* topoisomerase IV: biochemical basis for quinolone action and resistance. Biochemistry. 2012; 51:370–381. [PubMed: 22126453]
23. Aldred KJ, McPherson SA, Turnbough CL Jr, Kerns RJ, Osheroff N. Topoisomerase IV-quinolone interactions are mediated through a water-metal ion bridge: mechanistic basis of quinolone resistance. Nucleic Acids Res. 2013; 41:4628–4639. [PubMed: 23460203]
24. Marshall AJ, Piddock LJ. Interaction of divalent cations, quinolones and bacteria. J Antimicrob Chemother. 1994; 34:465–483. [PubMed: 7868402]

25. Turel I. The interactions of metal ions with quinolone antibacterial agents. Coord Chem Rev. 2002; 232:27–47.
26. Serafin A, Sta3czak A. The complexes of metal ions with fluoroquinolones. Russ J Coord Chem. 2009; 35:81–95.
27. Wohlkonig A, Chan PF, Fosberry AP, Homes P, Huang J, Kranz M, Leydon VR, Miles TJ, Pearson ND, Perera RL, Shillings AJ, Gwynn MN, Bax BD. Structural basis of quinolone inhibition of type IIA topoisomerases and target-mediated resistance. Nat Struct Mol Biol. 2010; 17:1152–1153. [PubMed: 20802486]
28. Huband MD, Cohen MA, Zurack M, Hanna DL, Skerlos LA, Sulavik MC, Gibson GW, Gage JW, Ellsworth E, Stier MA, Gracheck SJ. *In vitro* and *in vivo* activities of PD 0305970 and PD 0326448, new bacterial gyrase/topoisomerase inhibitors with potent antibacterial activities versus multidrug-resistant Gram-positive and fastidious organism groups. Antimicrob Agents Chemother. 2007; 51:1191–1201. [PubMed: 17261623]
29. German N, Malik M, Rosen JD, Drlica K, Kerns RJ. Use of gyrase resistance mutants to guide selection of 8-methoxy-quinazoline-2,4-diones. Antimicrob Agents Chemother. 2008; 52:3915–3921. [PubMed: 18765690]
30. Pan XS, Gould KA, Fisher LM. Probing the differential interactions of quinazolinedione PD 0305970 and quinolones with gyrase and topoisomerase IV. Antimicrob Agents Chemother. 2009; 53:3822–3831. [PubMed: 19564360]
31. Oppegard LM, Streck KR, Rosen JD, Schwanz HA, Drlica K, Kerns RJ, Hiasa H. Comparison of *in vitro* activities of fluoroquinolone-like 2,4- and 1,3-diones. Antimicrob Agents Chemother. 2010; 54:3011–3014. [PubMed: 20404126]
32. Malik M, Marks KR, Mustaev A, Zhao X, Chavda K, Kerns RJ, Drlica K. Fluoroquinolone and quinazolinedione activities against wild-type and gyrase mutant strains of *Mycobacterium smegmatis*. Antimicrob Agents Chemother. 2011; 55:2335–2343. [PubMed: 21383100]
33. Hammonds TR, Foster SR, Maxwell A. Increased sensitivity to quinolone antibacterials can be engineered in human topoisomerase IIα by selective mutagenesis. J Mol Biol. 2000; 300:481–491. [PubMed: 10884345]
34. Robinson MJ, Martin BA, Gootz TD, McGuirk PR, Moynihan M, Sutcliffe JA, Osheroff N. Effects of quinolone derivatives on eukaryotic topoisomerase II. A novel mechanism for enhancement of enzyme-mediated DNA cleavage. J Biol Chem. 1991; 266:14585–14592. [PubMed: 1650363]
35. Robinson MJ, Martin BA, Gootz TD, McGuirk PR, Osheroff N. Effects of novel fluoroquinolones on the catalytic activities of eukaryotic topoisomerase II: Influence of the C-8 fluorine group. Antimicrob Agents Chemother. 1992; 36:751–756. [PubMed: 1323952]
36. Elsea SH, Osheroff N, Nitiss JL. Cytotoxicity of quinolones toward eukaryotic cells. Identification of topoisomerase II as the primary cellular target for the quinolone CP-115,953 in yeast. J Biol Chem. 1992; 267:13150–13153. [PubMed: 1320012]
37. Elsea SH, McGuirk PR, Gootz TD, Moynihan M, Osheroff N. Drug features that contribute to the activity of quinolones against mammalian topoisomerase II and cultured cells: correlation between enhancement of enzyme-mediated DNA cleavage in vitro and cytotoxic potential. Antimicrob Agents Chemother. 1993; 37:2179–2186. [PubMed: 8257142]
38. Hande KR. Etoposide: four decades of development of a topoisomerase II inhibitor. Eur J Cancer. 1998; 34:1514–1521. [PubMed: 9893622]
39. Hande KR. Clinical applications of anticancer drugs targeted to topoisomerase II. Biochim Biophys Acta. 1998; 1400:173–184. [PubMed: 9748560]
40. Holden JA. DNA topoisomerases as anticancer drug targets: from the laboratory to the clinic. Curr Med Chem Anticancer Agents. 2001; 1:1–25. [PubMed: 12678768]
41. Baldwin EL, Osheroff N. Etoposide, topoisomerase II and cancer. Curr Med Chem Anticancer Agents. 2005; 5:363–372. [PubMed: 16101488]
42. Kim, M-K.; Nightingale, CH. Pharmacokinetics and pharmacodynamics of the fluoroquinolones. In: Andriole, VT., editor. The Quinolones. 3. Academic Press; San Diego: 2000. p. 169-202.
43. Dong S, McPherson SA, Wang Y, Li M, Wang P, Turnbough CL Jr, Pritchard DG. Characterization of the enzymes encoded by the anthrose biosynthetic operon of *Bacillus anthracis*. J Bacteriol. 2010; 192:5053–5062. [PubMed: 20675481]

44. Worland ST, Wang JC. Inducible overexpression, purification, and active site mapping of DNA topoisomerase II from the yeast *Saccharomyces cerevisiae*. J Biol Chem. 1989; 264:4412–4416. [PubMed: 2538443]

45. Kingma PS, Greider CA, Osheroff N. Spontaneous DNA lesions poison human topoisomerase IIα and stimulate cleavage proximal to leukemic 11q23 chromosomal breakpoints. Biochemistry. 1997; 36:5934–5939. [PubMed: 9166762]

46. Fortune JM, Osheroff N. Merbarone inhibits the catalytic activity of human topoisomerase IIα by blocking DNA cleavage. J Biol Chem. 1998; 273:17643–17650. [PubMed: 9651360]

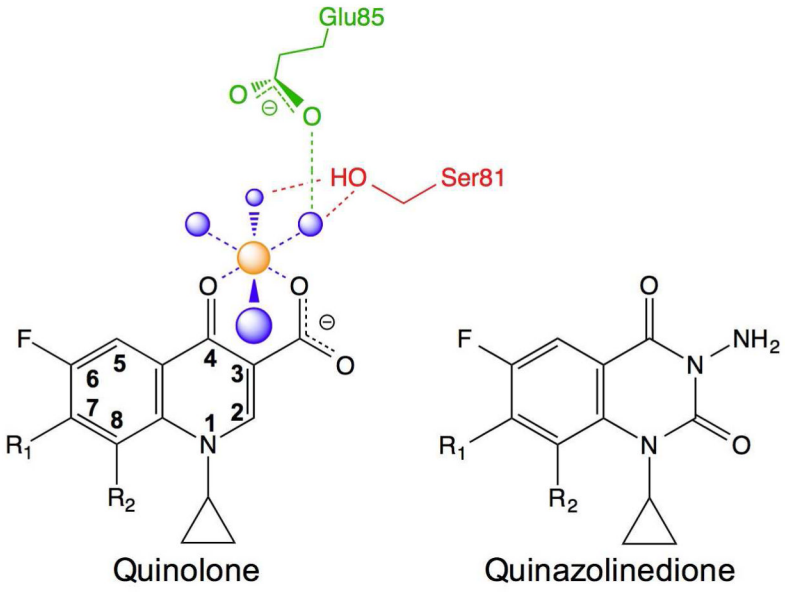
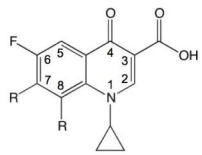
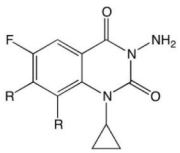
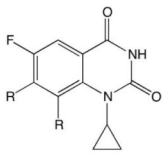


Figure 1.

Diagram of the water-metal ion bridge interaction. The water-metal ion bridge is the major point of interaction between clinically relevant quinolones and bacterial type II topoisomerases.[23] A generic quinolone structure is depicted in black, water molecules are in blue, Mg^{2+} is in orange, and the coordinating serine and glutamic acid residues (Ser81 and Glu85 in *B. anthracis* topoisomerase IV) are in red and green, respectively. Blue dashed lines indicate the octahedral coordination sphere of the divalent metal ion interacting with four water molecules and the C3/C4 keto acid of the quinolone. The red dashed lines represent hydrogen bonds between the serine side chain hydroxyl group and two of the water molecules. The green dashed line represents a hydrogen bond between the glutamic acid side chain carboxyl group and one of the water molecules. Adapted from Wohlkonig *et al*.[27] and Aldred *et al*.[23] A generic quinazolinedione structure is shown for comparison.

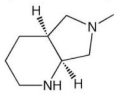
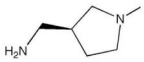
Position	C7	C8		
		Fluoroquinolone	Quinazolinedione	"Non-amino" quinazolinedione
	Piperazinyl (cipro)	-H	-H	
		$-CH_3$	$-CH_3$	$-CH_3$
		$-OCH_3$	$-OCH_3$	
	Diazabicyclononyl (moxi)	-H	-H	
		$-CH_3$	$-CH_3$	$-CH_3$
		$-OCH_3$	$-OCH_3$	
	3′-(aminomethyl)pyrrolidine [3′-(AM)P]	-H	-H	
		$-CH_3$	$-CH_3$	$-CH_3$
		$-OCH_3$	$-OCH_3$	

Figure 2.

Structures of matched quinolones and quinazolinediones utilized in this study. Supplementary Table S1 contains the full chemical, library, and abbreviated names for the compounds used in this study.

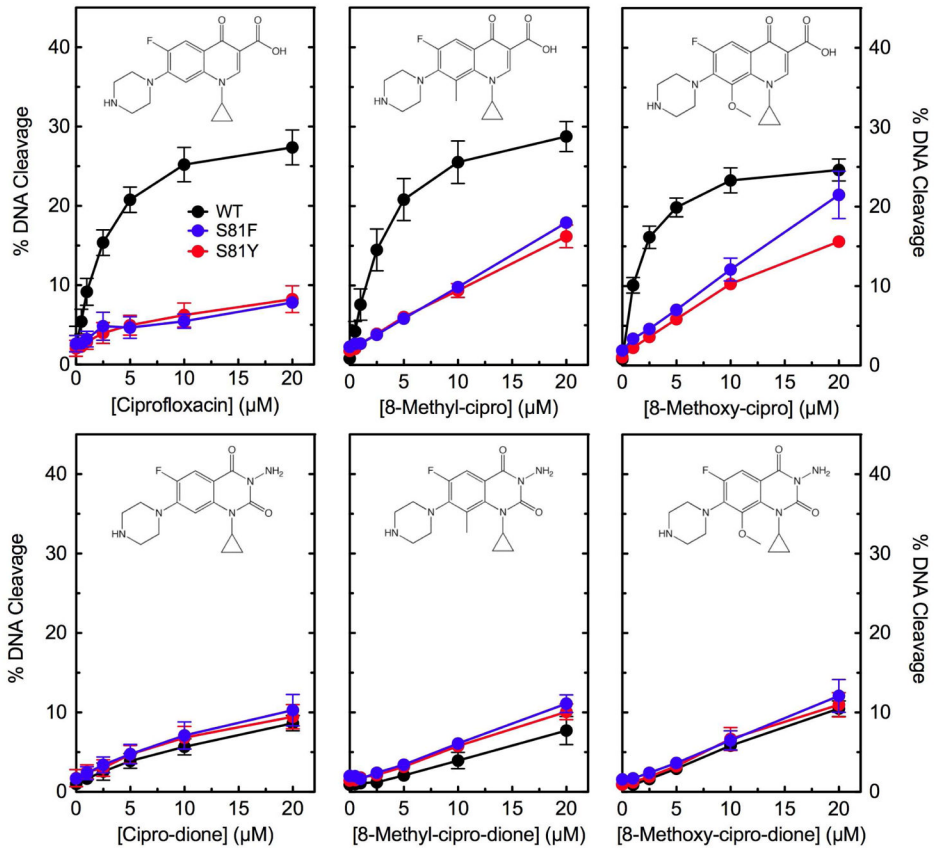


Figure 3.

Effects of a series of quinolones and quinazolinediones based on the C7 substituent of ciprofloxacin on the DNA cleavage activity of *B. anthracis* topoisomerase IV. The ability of wild-type (WT, black circles), GrlA^{S81F} (S81F, blue circles), and GrlA^{S81Y} (S81Y, red circles) topoisomerase IV to cleave negatively supercoiled pBR322 DNA in the presence of quinolones (top panels) or quinazolinediones (bottom panels) containing a C7 piperazinyl group and a C8 hydrogen (left panels), methyl (middle panels), or methoxy (right panels) group is shown. Drug structures are shown in the corresponding panels. Error bars represent the standard deviation of three or more independent experiments.

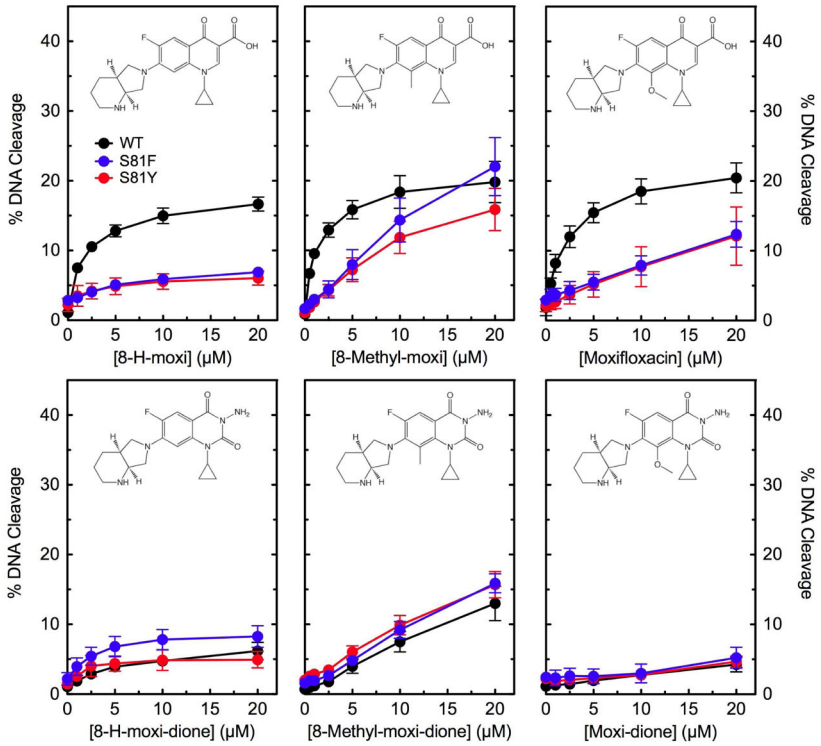


Figure 4.

Effects of a series of quinolones and quinazolinediones based on the C7 substituent of moxifloxacin on the DNA cleavage activity of *B. anthracis* topoisomerase IV. The ability of wild-type (WT, black circles), $GrlA^{S81F}$ (S81F, blue circles), and $GrlA^{S81Y}$ (S81Y, red circles) topoisomerase IV to cleave negatively supercoiled pBR322 DNA in the presence of quinolones (top panels) or quinazolinediones (bottom panels) containing a C7 diazabicyclononyl group and a C8 hydrogen (left panels), methyl (middle panels), or methoxy (right panels) group is shown. Drug structures are shown in the corresponding panels. Error bars represent the standard deviation of three or more independent experiments.

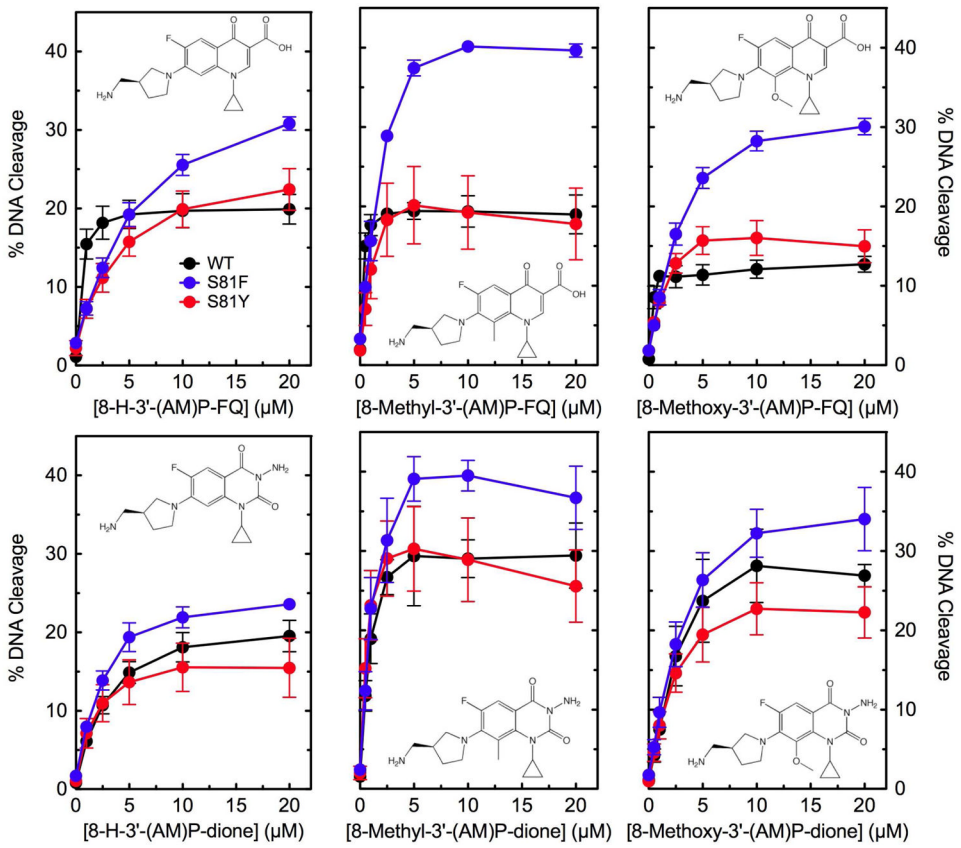


Figure 5.

Effects of a series of quinolones and quinazolinediones based on the C7 substituent of 8-Methyl-3′-(AM)P-dione on the DNA cleavage activity of *B. anthracis* topoisomerase IV. The ability of wild-type (WT, black circles), GrlA^{S81F} (S81F, blue circles), and GrlA^{S81Y} (S81Y, red circles) topoisomerase IV to cleave negatively supercoiled pBR322 DNA in the presence of quinolones (top panels) or quinazolinediones (bottom panels) containing a C7 3′-(aminomethyl)pyrrolidinyl [3′-(AM)P] group and a C8 hydrogen (left panels), methyl (middle panels), or methoxy (right panels) group is shown. Drug structures are shown in the corresponding panels. Error bars represent the standard deviation of three or more independent experiments.

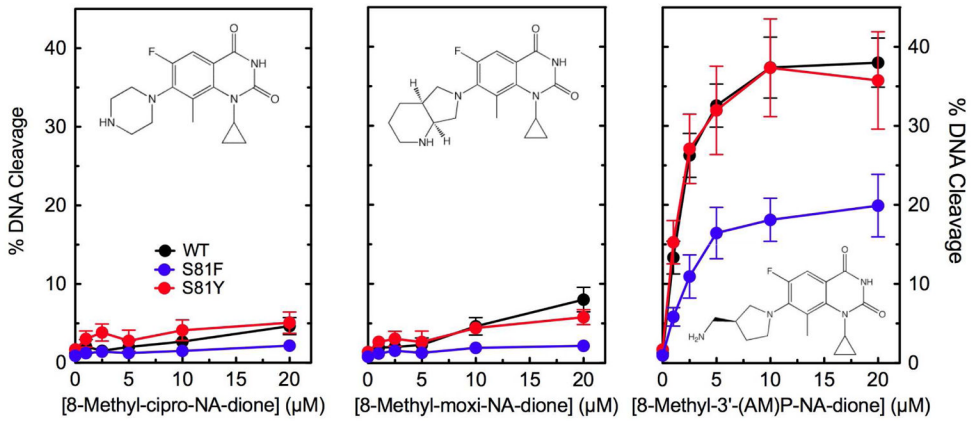


Figure 6.

Effects of quinazolinediones lacking the N3 amino group on the DNA cleavage activity of *B. anthracis* topoisomerase IV. The ability of wild-type (WT, black circles), GrlA^{S81F} (S81F, blue circles), and GrlA^{S81Y} (S81Y, red circles) topoisomerase IV to cleave negatively supercoiled pBR322 DNA in the presence of "non-amino" quinazolinediones containing a C8 methyl group and a C7 piperazinyl (left panel), diazabicyclononyl (middle panel), or 3′-(aminomethyl)pyrrolidinyl group is shown. Drug structures are shown in the corresponding panels. Error bars represent the standard deviation of three or more independent experiments.

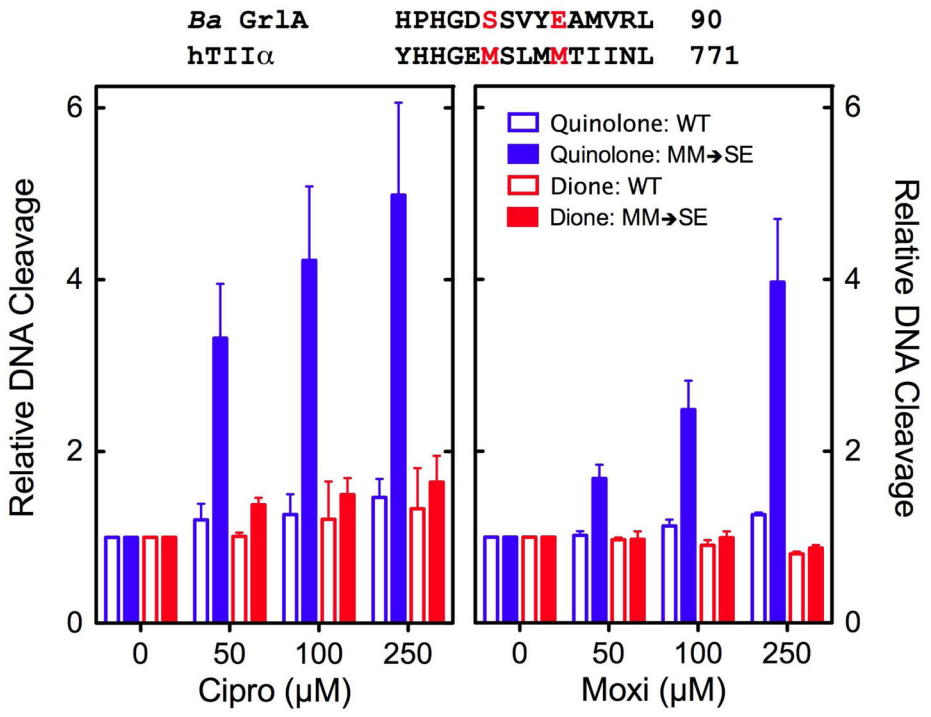


Figure 7.

Effects of ciprofloxacin, moxifloxacin, and their matched quinazolinediones on the DNA cleavage activities of wild-type and mutant (hTop2A$^{M762S/M766E}$) human topoisomerase IIα. A sequence alignment of wild-type *B. anthracis* GrlA (*Ba* GrlA) and human topoisomerase IIα (hTIIα) is shown at the top. The methionine residues in the human enzyme that correspond to the serine and glutamic acid residues (red) that coordinate the water-metal ion bridge in *B. anthracis* topoisomerase IV were mutated to those seen in the bacterial enzyme (hTop2A$^{M762S/M766E}$, MM→SE). The effects of ciprofloxacin (blue) and cipro-dione (red; left panel) and moxifloxacin (blue) and moxi-dione (red; right panel) on the DNA cleavage activity of wild-type (open bars) and mutant (solid bars) human topoisomerase IIα are shown. Error bars represent the standard deviation of three or more independent experiments.

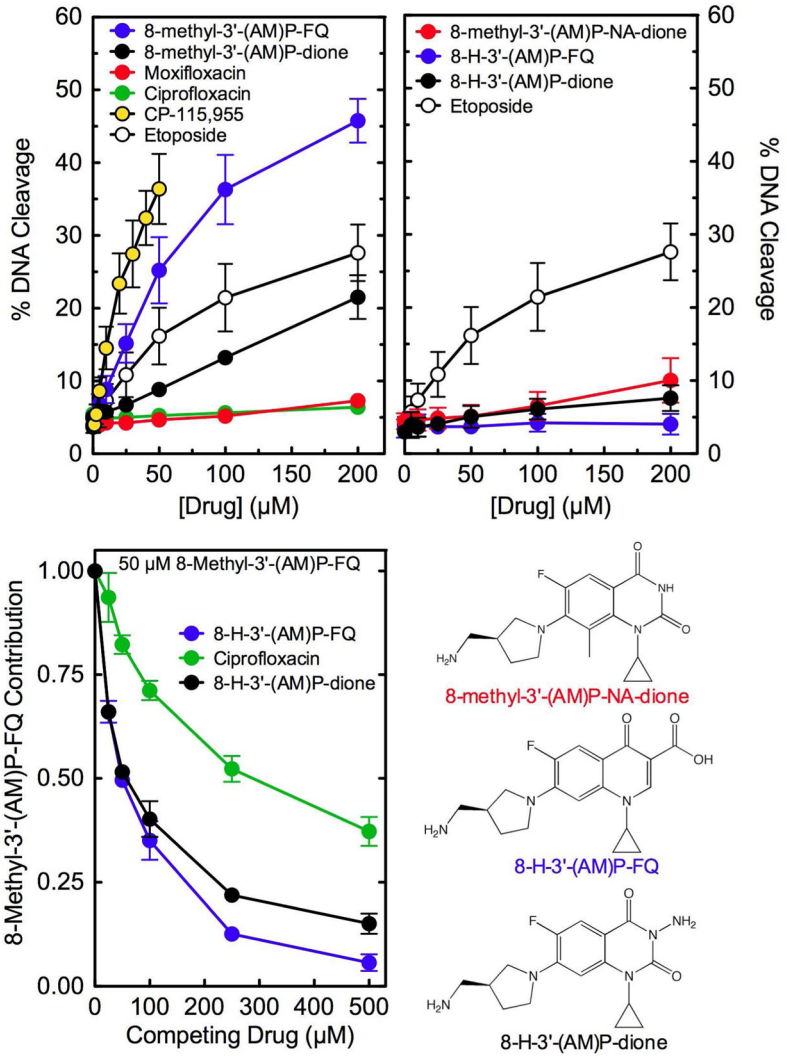


Figure 8.

Effects of a series of quinolones and quinazolinediones based on the C7 substituent of 8-Methyl-3′-(AM)P-dione on the DNA cleavage activity of human topoisomerase IIα. Top left panel: The ability of the human type II enzyme to cleave negatively supercoiled pBR322 DNA in the presence of a quinolone (FQ; blue circles) or a quinazolinedione (dione; black circles) containing a C7 3′-(aminomethyl)pyrrolidinyl [3′-(AM)P] group and a C8 methyl group is shown. Results for moxifloxacin (red circles), ciprofloxacin (green circles), CP-115,955 (yellow circles), and etoposide (open circles) are shown for comparison. Top right panel: The ability of the human type II enzyme to cleave negatively supercoiled pBR322 DNA in the presence of a quinolone (blue circles) or quinazolinedione (black circles) containing a C7 3′-(aminomethyl)pyrrolidinyl group and a hydrogen at C8 or a "non-amino" quinazolinedione (NA-dione; red circles) containing a C7 3′-(aminomethyl)pyrrolidinyl group and a C8 methyl group. Bottom left panel: The ability of 8-H-3′-(AM)P-FQ (blue circles), ciprofloxacin (green circles), or 8-H-3′-(AM)P-dione (black circles) to compete out DNA cleavage induced by 8-methyl-3′-(AM)P-FQ (50 μM) with human topoisomerase IIα. 8-Methyl-3′-(AM)P-FQ and the competing drug were added to reactions simultaneously. Initial levels of DNA cleavage (*i.e.*, in the absence of competitor) were set to 1.00 to facilitate comparisons. Error bars represent the standard

deviation of three or more independent experiments. The structures of a quinolone and two quinazolinediones that overcome quinolone resistance in the bacterial enzyme but do not have cross-reactivity with the human enzyme are shown on the bottom right.