Role of the Water–Metal Ion Bridge in Mediating Interactions between Quinolones and *Escherichia coli* Topoisomerase IV

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**ABSTRACT:** Although quinolones have been in clinical use for decades, the mechanism underlying drug activity and resistance has remained elusive. However, recent studies indicate that clinically relevant quinolones interact with *Bacillus anthracis* (Gram-positive) topoisomerase IV through a critical water–metal ion bridge and that the most common quinolone resistance mutations decrease drug activity by disrupting this bridge. As a first step toward determining whether the water–metal ion bridge is a general mechanism of quinolone–topoisomerase interaction, we characterized drug interactions with wild-type *Escherichia coli* (Gram-negative) topoisomerase IV and a series of ParC enzymes with mutations (S80L, S80I, S80F, and E84K) in the predicted bridge-anchoring residues. Results strongly suggest that the water–metal ion bridge is essential for quinolone activity against *E. coli* topoisomerase IV. Although the bridge represents a common and critical mechanism that underlies broad-spectrum quinolone function, it appears to play different roles in *B. anthracis* and *E. coli* topoisomerase IV. The water–metal ion bridge is the most important binding contact of clinically relevant quinolones with the Gram-positive enzyme. However, it primarily acts to properly align clinically relevant quinolones with *E. coli* topoisomerase IV. Finally, even though ciprofloxacin is unable to increase levels of DNA cleavage mediated by several of the Ser80 and Glu84 mutant *E. coli* enzymes, the drug still retains the ability to inhibit the overall catalytic activity of these topoisomerase IV proteins. Inhibition parallels drug binding, suggesting that the presence of the drug in the active site is sufficient to diminish DNA relaxation rates.

Ciprofloxacin and other quinolones kill bacteria by increasing levels of DNA strand breaks generated by type II topoisomerases.1−6 Nearly all bacteria encode two type II topoisomerases, gyrase and topoisomerase IV.2,6−14 Both enzymes are comprised of two protomer subunits and have an A2B2 quaternary structure.2,6,9,11,12,15 Gyrase consists of two GyrA subunits (that contain the active site tyrosine residues that mediate DNA cleavage and ligation) and two GyrB subunits (that bind ATP, which is required for overall catalytic activity). Topoisomerase IV consists of two ParC and two ParE subunits that are homologous to GyrA and GyrB, respectively.6−9,12 Gyrase and topoisomerase IV alter DNA topology by passing an intact double helix through a transient break that they generate in a separate segment of DNA.6−9,11−13,15 Both type II enzymes are essential for cell survival6−9,11−13 and are physiological targets for quinolone antibacterials.1,6,16−22

Although quinolones have been in clinical use against both Gram-positive and Gram-negative bacteria for several decades, the mechanism by which they interact with bacterial type II topoisomerases was determined only recently.23−29 Structural work with *Acinetobacter baumannii* topoisomerase IV suggests that the quinolone C3/C4 keto acid chelates a divalent metal ion, which interacts with the protein through water molecules that are coordinated by two highly conserved residues: a serine and an acidic amino acid located four positions downstream.26 Functional studies demonstrated that the proposed “water–metal ion bridge” exists and acts as the primary conduit by which clinically relevant quinolones interact with *Bacillus anthracis* (Gram-positive) topoisomerase IV (Figure 1).27−29 Partial disruption of the bridge, resulting from mutation of the serine or acidic residue, significantly decreased the potency (i.e., maximal drug–target affinity) of clinically relevant quinolones for the enzyme but had relatively little effect on drug efficacy (i.e., maximal drug-induced cleavage).27,28 Mutation of both amino acid residues, which completely disrupted bridge function, abrogated the

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ability of quinolones to increase topoisomerase IV-mediated DNA cleavage.

Quinolone resistance has risen steadily since the 1990s and is a threat to the continued clinical use of this drug class. Resistance is most often associated with specific mutations in topoisomerase IV and/or gyrase. Overwhelmingly, the most commonly mutated amino acid residues in Gram-positive and Gram-negative quinolone-resistant strains are the conserved serine (originally described as Ser83 in Escherichia coli GyrA) and acidic residue that anchor the water—metal ion bridge. The role of these residues in drug resistance underscores the importance of the bridge in mediating the clinical effects of quinolones.

Despite the broad-spectrum nature of quinolones, the ability (both potency and efficacy) of these drugs to enhance DNA cleavage mediated by topoisomerase IV and gyrase can differ substantially between species. Furthermore, while mutation of the serine and acidic residue is consistent across species, the substituted amino acids vary. For example, the most common resistance mutation is Ser → Phe in B. anthracis topoisomerase IV, but it is Ser → Ile in the E. coli enzyme.

The differences in quinolone activity against bacterial type II enzymes raise the question of whether the water—metal ion bridge is utilized or functions in the same manner across species. Therefore, to address this issue, the interactions of ciprofloxacin and related drugs (quinazolinediones) with wild-type E. coli topoisomerase IV and a series of enzymes with mutations in the predicted bridge-anchoring residues (ParC Ser80 and Glu84) were analyzed. Results provide strong evidence that the water—metal ion bridge facilitates interaction between ciprofloxacin and the Gram-negative enzyme and is critical for quinolone activity. However, this interaction appears to play different roles in mediating quinolone activity in different species. Whereas the bridge facilitates the binding of clinically relevant quinolones to B. anthracis topoisomerase IV, it appears to properly align the drug in the active site of the E. coli enzyme to promote DNA cleavage.

**Experimental Procedures**

**Enzymes and Materials.** E. coli topoisomerase IV subunits (wild-type ParC, wild-type ParE, ParC Ser80L, ParC Ser80I, ParC Ser80F, and ParC E84K) were expressed and purified as described previously. The ParC mutants were prepared by site-directed mutagenesis using a QuickChange II XL site-directed mutagenesis kit (Agilent Technologies) and custom-synthesized primers containing the desired mutations. The entire coding region was sequenced (Macrogen USA) to ensure the presence of the desired mutation and the absence of unwanted alterations. In all assays, topoisomerase IV was used as a 1:1 ParC:ParE mixture.

Negatively supercoiled pBR322 plasmid DNA was prepared from E. coli using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Relaxed pBR322 plasmid DNA was generated by treatment with topoisomerase I for 30 min as described previously, followed by phenol/chloroform/isoamyl alcohol extraction, ethanol precipitation, and resuspension in 5 mM Tris·HCl (pH 8.5) and 500 μM ethylenediaminetetraacetic acid (EDTA). Histone H1 was obtained from Boehringer Mannheim.

Ciprofloxacin was obtained from LKT Laboratories, stored at −20 °C as a 40 mM stock solution in 0.1 N NaOH, and diluted 5-fold with 10 mM Tris·HCl (pH 7.9) immediately prior to use. 3-Amino-7-[(3S)-3-(aminomethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-8-methyl-2,4(1H,3H)-quinazolinedione (U1JR-1-048) was synthesized using established methods as previously reported.

For the sake of simplicity, this compound will be referred to as 8-methyl-3′-(AM)P-dione. 3-Amino-7-[(3S)-3-(aminomethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-2,4(1H,3H)-quinazolinedione (U1JR-2-245) and 7′-[(3S)-3-(aminomethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-8-methyl-2,4(1H,3H)-quinazolinedione (U1JR-2-97) were synthesized as previously reported. For the sake of simplicity, these drugs will be referred to as 8-H-3′-(AM)P-dione and 8-methyl-3′-(AM)P-non-amine-dione, respectively. The quinazolinediones were stored at 4 °C as 20 mM stock solutions in 100% DMSO.

All other chemicals were analytical reagent grade.

**DNA Relaxation.** DNA relaxation assays were based on the protocol of Fortune and Osheroff. Reaction mixtures (20 μL) contained 10 nM wild-type or mutant E. coli topoisomerase IV and 5 nM negatively supercoiled pBR322 in relaxation buffer [40 mM HEPES (pH 7.6), 100 mM potassium glutamate, 10 mM Mg(OAc)2, 50 mM NaCl, and 1 mM ATP] and were incubated at 37 °C. Relaxation was stopped at times ranging from 0 to 30 min by the addition of 3 μL of 0.77% sodium dodecyl sulfate (SDS) and 77.5 mM EDTA. Samples were mixed with 2 μL of agarse 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 100 mM Tris-borate (pH 8.3) and 2 mM EDTA. Gels were stained with 0.75 μg/mL ethidium bromide for 30 min. DNA bands were visualized with medium-range ultraviolet light and quantified using an Alpha Innotech digital imaging system. The percent relaxed DNA was determined by the loss of supercoiled DNA substrate.

Reactions that monitored the effects of ciprofloxacin on the relaxation activities of wild-type or mutant E. coli topoisomerase IV were incubated for 10 min (wild-type, ParC Ser80L, and ParC Ser80F), 20 min (ParC Ser80I), or 30 min (ParC E84K). The longer...
times utilized with the last two mutant enzymes reflect their slower basal rates of DNA relaxation. The amount of relaxed DNA observed in the absence of drug was set to 100% to facilitate direct comparisons between the effects of ciprofloxacin on the catalytic activities of the wild-type and mutant enzymes.

**DNA Catenation.** Catenation assays were based on the protocol of Fortune and Oshero\(^\text{27}\) as modified by Aldred et al.\(^\text{28}\) Reaction mixtures (20 μL) contained 20 nM wild-type or mutant *E. coli* topoisomerase IV and 5 nM relaxed pBR322 in relaxation buffer containing 25 mM NaCl (rather than 50 mM) supplemented with 5 μg/mL histone H1 and were incubated at 37 °C. Catenation was stopped at times ranging from 0 to 30 min by the addition of 2 μL of 250 mM EDTA (pH 8.0) followed by 2 μL of 1.25% SDS. Samples were mixed with 2 μL of agarose gel loading buffer, 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 EDTA containing 0.5 μg/mL ethidium bromide. DNA bands were visualized and quantified as described above. The percent catenated DNA was determined by the loss of relaxed monomers.

**DNA Cleavage.** DNA cleavage reactions were carried out using the procedure of Fortune and Oshero.\(^\text{27,46}\) Reaction mixtures contained 10 nM wild-type, ParC\(^\text{S80L}\), ParC\(^\text{S80I}\), or ParC\(^\text{S80F}\) or 25 nM ParC\(^\text{E84K}\) topoisomerase IV and 10 nM negatively supercoiled pBR322 in a total of 20 μL of cleavage buffer [40 mM Tris-HCl (pH 7.9), 2.5 mM MgCl\(_2\), 50 mM NaCl, and 2.5% (v/v) glycerol]. ParC\(^\text{E84K}\) was used at an increased concentration to account for its lower basal cleavage activities of the wild-type and Ser80 mutant enzymes. In some reactions, the concentration dependence of MgCl\(_2\) was examined or the divalent metal ion was replaced with CaCl\(_2\), ZnCl\(_2\), CdCl\(_2\), MnCl\(_2\), or NiCl\(_2\). 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 EDTA (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, 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 EDTA containing 0.5 μg/mL ethidium bromide. DNA bands were visualized and quantified as described above. DNA cleavage was monitored by the conversion of supercoiled plasmid to linear molecules.

Assays that monitored the DNA cleavage activities of wild-type and mutant *E. coli* topoisomerase IV in the absence of drugs substituted 2.5 mM CaCl\(_2\), for 2.5 mM MgCl\(_2\), in the cleavage buffer.

For assays that monitored competition between ciprofloxacin and 8-methyl-3′-(AM)P-dione, the level of DNA cleavage generated by the corresponding concentration of ciprofloxacin in the absence of the quinazolinedione was used as a baseline and was subtracted from the cleavage level seen in the presence of both compounds. Ciprofloxacin and 8-methyl-3′-(AM)P-dione were added simultaneously to reaction mixtures.

**DNA Religation.** DNA religation assays were carried out using the procedure of Robinson and Oshero.\(^\text{27,48}\) Reaction mixtures (20 μL) were identical to those described above for cleavage assays. Initial DNA cleavage/religation equilibria with wild-type or mutant ParC\(^\text{S80L}\). *E. coli* topoisomerase IV were established at 37 °C for 10 min. Religation was initiated by rapidly shifting the temperature from 37°C to 75°C. Reactions were stopped at times ranging from 0 to 135 s by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA (pH 8.0). Samples were digested with proteinase K and processed as above for DNA cleavage assays. Levels of DNA cleavage were set to 100% at time zero, and religation was determined by the loss of the linear reaction product over time.

**Persistence of Topoisomerase IV–DNA Cleavage Complexes.** The persistence of topoisomerase IV–DNA cleavage complexes established in the presence of drugs was determined using the procedure of Gentry et al.\(^\text{27,49}\). Initial reaction mixtures contained 50 nM wild-type or mutant ParC\(^\text{S80L}\). *E. coli* topoisomerase IV, 50 nM DNA, and 20 μM ciprofloxacin or 8-methyl-3′-(AM)P-dione in a total of 20 μL of DNA cleavage buffer. Reaction mixtures were incubated at 37 °C for 10 min and then diluted 20-fold with DNA cleavage buffer warmed to 37°C. Samples (20 μL) were removed at times ranging from 0 to 30 min, and DNA cleavage was monitored by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA (pH 8.0). Samples were digested with proteinase K and processed as described above for DNA cleavage assays. Levels of DNA cleavage were set to 100% at time zero, and the persistence of cleavage complexes was determined by the loss of the linear reaction product over time.
Enzymatic Activities of Wild-Type and Mutant Quinolone-Resistant *E. coli* Topoisomerase IV. All of the functional evidence for the existence and role of the water—metal ion bridge in mediating quinolone activity comes from studies of *B. anthracis* (a Gram-positive species) topoisomerase IV.\(^{27,28}\) As a first step toward determining whether the water—metal ion bridge is a general mechanism of quinolone—topoisomerase interaction, we characterized the interactions of drugs with wild-type, ParC\(^{S80L}\), ParC\(^{S80I}\), ParC\(^{S80F}\), and ParC\(^{E84K}\) topoisomerase IV from *E. coli* (a Gram-negative species). All of these mutations occur at the amino acid residues predicted (by sequence alignments) to anchor the water—metal ion bridge in this species. The S80L, S80I, and E84K mutants have been identified in clinical isolates of quinolone-resistant *E. coli*.\(^{38–41}\) The S80L and E84K mutants are resistant to quinolones in *vitro*.\(^{16,30,31}\) However, the purified ParC\(^{S80I}\) enzyme (which contains the most common Ser80 mutation found in clinical isolates\(^{38–41}\)) has not yet been characterized. The S80F mutation parallels the most common quinolone resistance mutation seen in *B. anthracis*.\(^{33–37}\) and provides for direct comparisons with the previously characterized Gram-positive enzyme.\(^{27,28}\)

Quinolones require the activity of type II topoisomerases in order to kill cells.\(^1–6\) Consequently, topoisomerase IV mutations could cause quinolone resistance either by altering drug—enzyme interactions or by diminishing enzyme activity. Therefore, wild-type, ParC\(^{S80L}\), ParC\(^{S80I}\), ParC\(^{S80F}\), and ParC\(^{E84K}\) *E. coli* topoisomerase IV were examined for their abilities to relax, catenate, and cleave DNA in the absence of drugs (Figure 2).

ParC\(^{S80L}\) and ParC\(^{E84K}\) topoisomerase IV relaxed negatively supercoiled plasmid DNA and catenated relaxed plasmids at a rate that was nearly identical to that of the wild-type enzyme. Unexpectedly, ParC\(^{S80F}\) relaxed negatively supercoiled plasmid DNA ∼3-fold slower than did the wild-type enzyme. This mutant also catenated relaxed plasmids slower than did the wild-type enzyme. ParC\(^{E84K}\) relaxed and catenated DNA at a rate that was even slower than those of the wild-type enzyme and the Ser80 mutants. This mutation, as well as the equivalent mutation (E85K) in *B. anthracis* topoisomerase IV,\(^{28}\) has been shown to display decreased rates of DNA relaxation and decatenation.\(^{50}\) In the case of the *B. anthracis* topoisomerase IV E85K mutant enzyme, the decreased catalytic activity was due to a defect in the DNA strand passage event, which takes place following DNA cleavage.\(^{28}\)

The wild-type and Ser80 mutant *E. coli* enzymes all displayed similar abilities to cleave DNA, suggesting that quinolone resistance conferred by these mutations results from altered drug—enzyme interactions rather than decreased enzyme activity. In contrast to results with the *B. anthracis* E85K mutant enzyme,\(^{28}\) *E. coli* ParC\(^{E84K}\) topoisomerase IV displayed a DNA cleavage ability that was approximately half that of the wild-type enzyme. Therefore, this topoisomerase IV mutation may cause quinolone resistance in *E. coli* cells by affecting the activity of the enzyme in addition to (or instead of) altering drug activity.

**Effects of Ciprofloxacin and 8-Methyl-3′-(AM)P-dione on Wild-Type and Quinolone-Resistant *E. coli* Topoisomerase IV.** To determine how the ParC Ser80 and Glu84 mutations cause quinolone resistance, the ability of ciprofloxacin to enhance DNA cleavage mediated by the wild-type and mutant *E. coli* enzymes was assessed (Figure 3, left). The quinolone displayed little ability to increase the level of DNA cleavage mediated by ParC\(^{S80L}\), ParC\(^{S80I}\), ParC\(^{S80F}\), and ParC\(^{E84K}\) as compared to that of the wild-type enzyme. In contrast to results with the equivalent *B. anthracis* enzymes,\(^{27,28}\) the mutant *E. coli* topoisomerase IV enzymes displayed little to no increase in ciprofloxacin-enhanced DNA cleavage at high drug concentrations (Figure 3, right).

Some quinazolinediones, including 8-methyl-3′-(AM)P-dione, have been shown to maintain activity against quinolone-resistant type II enzymes and function in a manner that is independent of the water—metal ion bridge.\(^{27–29,45–52}\) Therefore, this drug was tested against wild-type and mutant ParC\(^{S80L}\), ParC\(^{S80I}\), ParC\(^{S80F}\), and ParC\(^{E84K}\) topoisomerase IV. All of the enzymes maintained high sensitivity to 8-methyl-3′-(AM)P-dione (Figure 3, middle). Thus, as reported previously for *B. anthracis* topoisomerase IV,\(^{27,28}\) mutations in amino acid residues predicted to anchor the water—metal ion bridge caused resistance that was quinolone-specific.

Next, experiments were carried out to determine whether the quinolone and quinazolinedione increase the concentration of

![Figure 3](https://dx.doi.org/10.1021/bi500682e)
E. coli topoisomerase IV cleavage complexes by inhibiting enzyme-mediated DNA religation (Figure 4). Because ciprofloxacin was not able to enhance DNA cleavage mediated by ParC<sup>S80I</sup>, ParC<sup>S80F</sup> or ParC<sup>E84K</sup> topoisomerase IV, only the wild-type and ParC<sup>S80L</sup> enzymes were examined. Although less dramatic than results with the Gram-positive B. anthracis<sup>27,28</sup> and Staphylococcus aureus<sup>6,57</sup> topoisomerase IV enzymes, ciprofloxacin and 8-methyl-3′-(AM)P-dione both slowed the DNA religation rate of the wild-type enzyme ~4–5-fold. A similar effect of ciprofloxacin on the religation rate of the wide-type enzyme has been reported.<sup>58</sup> As expected, the ParC<sup>S80L</sup> resistance mutation impairs quinoline, but not quinazolinone-dione, function. Similar results were reported for B. anthracis enzymes containing mutations at the equivalent serine residue.<sup>27</sup>

To further characterize the effects of drugs on DNA cleavage mediated by E. coli topoisomerase IV, the persistence of cleavage complexes was determined (Figure 5). Once again, the only mutant enzyme examined was ParC<sup>S80L</sup> topoisomerase IV. DNA cleavage complexes formed with wild-type and mutant E. coli topoisomerase IV were significantly less stable than those formed with the equivalent B. anthracis enzymes.<sup>27,28</sup> The t<sub>1/2</sub> values for the decay of wild-type E. coli topoisomerase IV cleavage complexes were ~4.4 and ~5.3 min in the presence of ciprofloxacin and 8-methyl-3′-(AM)P-dione, respectively. The t<sub>1/2</sub> values of wild-type B. anthracis cleavage complexes in the presence of these drugs were ~90 and 280 min, respectively.<sup>27</sup> The t<sub>1/2</sub> values of cleavage complexes formed with ParC<sup>S80L</sup> topoisomerase IV in the presence of both ciprofloxacin and 8-methyl-3′-(AM)P-dione decreased severalfold as compared to that of the wild-type enzyme (Figure 5, inset). However, a much larger reduction (~22-fold vs ~6-fold) was seen with the quinolone. In contrast, with B. anthracis topoisomerase IV, mutations at the equivalent serine decreased the stability of quinolone-induced cleavage complexes but had little effect on the stability of those formed in the presence of the quinazolinone-dione.<sup>27</sup>

**Correlation between Quinolone Affinity and the Inhibition of DNA Relaxation Catalyzed by Wild-Type and Mutant E. coli Enzymes.** Because ciprofloxacin displayed little ability to enhance DNA cleavage mediated by ParC<sup>S80L</sup>, ParC<sup>S80F</sup>, ParC<sup>S80I</sup>, and ParC<sup>E84K</sup> topoisomerase IV even up to 500 μM drug (see Figure 3), a competition experiment was carried out to determine whether quinoline resistance was due to a lack of drug interaction. In this experiment, the ability of ciprofloxacin (0–200 μM) to compete out DNA cleavage induced by 10 μM 8-methyl-3′-(AM)P-dione was determined (Figure 6, left). With ParC<sup>S80L</sup> and ParC<sup>S80F</sup>, ciprofloxacin competed well against the quinazolinone-dione, decreasing the level of cleavage by 50% at ~12 and ~17 μM quinolone, respectively. Because these IC<sub>50</sub> values are similar to the concentration of quinazolinone-dione used in the competition assay, it appears that the decreased activity of ciprofloxacin toward these two E. coli mutant enzymes does not result from a loss of drug affinity. This finding is in contrast to previous results with B. anthracis S81F topoisomerase IV. With the B. anthracis enzyme, a 7.5-fold molar excess of ciprofloxacin reduced the activity of 8-methyl-3′-(AM)P-dione by <40%, indicating a marked decrease in quinolone binding.<sup>27</sup>

In contrast to the results described above, the ability of ciprofloxacin to compete with the quinazolinone-dione was significantly impaired with ParC<sup>S80L</sup> and ParC<sup>E84K</sup> (Figure 6, left). IC<sub>50</sub> values with these mutant enzymes were ~65 and ~143 μM quinolone, respectively. These results indicate that
the S80L and E84K mutations have a significant effect on the affinity of ciprofloxacin for the *E. coli* topoisomerase IV—DNA complex.

Although quinolones are believed to kill bacterial cells primarily by increasing levels of DNA strand breaks, they also can impair cellular functions by inhibiting type II topoisomerases from carrying out their critical physiological functions. This catalytic inhibition may contribute to their toxicity toward bacterial cells.\(^1\)–\(^6\) Therefore, the effects of ciprofloxacin on DNA relaxation mediated by ParCS\(^{80L}\), ParCS\(^{80I}\), ParCS\(^{80F}\), and ParCE\(^{84K}\) topoisomerase IV were compared to those on DNA relaxation mediated by the wild-type enzyme (Figure 6, right).

Even though ciprofloxacin did not enhance DNA cleavage mediated by ParCS\(^{80L}\) and ParCS\(^{80F}\) topoisomerase IV (see Figure 3), the drug inhibited DNA relaxation catalyzed by the mutant enzymes with an IC\(_{50}\) value (\(\sim 5 \mu M\)) that was the same as that observed with wild-type *E. coli* topoisomerase IV. The level of inhibition was correspondingly reduced with ParCS\(^{80L}\) and ParCE\(^{84K}\) topoisomerase IV, which displayed lower affinities for ciprofloxacin (as determined by the competition assays described above). A similar effect of ciprofloxacin on DNA relaxation catalyzed by the mutant S80L and E84K enzymes has been reported.\(^{50}\) These results strongly suggest that the ability of quinolones to inhibit the overall catalytic activity of topoisomerase IV reflects drug binding in the DNA cleavage/religation active site of the enzyme. However, inhibition of DNA relaxation appears to be unrelated to the ability of quinolones to enhance enzyme-mediated DNA cleavage.

**Role of the Water—Metal Ion Bridge in Facilitating Quinolone Activity against *E. coli* Topoisomerase IV.** To determine whether quinolone interactions with *E. coli* topoisomerase IV are mediated by the water—metal ion bridge,\(^{26–28}\) three experiments were carried out to analyze the metal ion requirements of ciprofloxacin-induced and 8-methyl-3′-[(AM)P]-dione-induced DNA cleavage.

In the first experiment, we examined the metal ion requirement for quinolone-induced DNA cleavage with wild-type *E. coli* topoisomerase IV. Thus, a variety of divalent and trivalent metal ions that support enzyme function were tested for their ability to support ciprofloxacin activity. Three metal ions (Ca\(^{2+}\), Zn\(^{2+}\), and Cd\(^{2+}\)) that supported enzyme-mediated DNA cleavage but did not support quinolone activity against the enzyme were identified (Figure 7). As a control, Ca\(^{2+}\), Zn\(^{2+}\), and Cd\(^{2+}\) were tested with 8-methyl-3′-[(AM)P]-dione, a metal ion-independent drug.\(^{26–28}\) In contrast to ciprofloxacin, the quinazolinedione maintained activity against the enzyme, regardless of the metal ion utilized. These findings suggest that clinically relevant quinolones such as ciprofloxacin require divalent metal ions to induce DNA cleavage mediated by *E. coli* topoisomerase IV.

**Figure 6.** Competition between ciprofloxacin and 8-methyl-3′-[(AM)P]-dione and the effects of ciprofloxacin on DNA relaxation. The ability of 0–200 \(\mu M\) ciprofloxacin to compete with 10 \(\mu M\) 8-methyl-3′-[(AM)P]-dione for ParCS\(^{80L}\) (S80L, blue), ParCS\(^{80I}\) (S80I, red), ParCS\(^{80F}\) (S80F, green), and ParCE\(^{84K}\) (E84K, yellow) *E. coli* topoisomerase IV was determined using DNA cleavage assays (left). Both drugs were added to reaction mixtures simultaneously. For ParCS\(^{80I}\), the level of cleavage seen in the presence of ciprofloxacin alone was used as a baseline and subtracted from the total observed in the presence of both drugs. The level of DNA cleavage observed in the presence of only the quinazolinedione was set to 1.0 to facilitate direct comparisons. The effects of ciprofloxacin on the relaxation activities of wild-type (WT, black), ParCS\(^{80L}\) (S80L, blue), ParCS\(^{80I}\) (S80I, red), ParCS\(^{80F}\) (S80F, green), and ParCE\(^{84K}\) (E84K, yellow) topoisomerase IV are shown at the right. Wild-type, ParCS\(^{80L}\), and ParCS\(^{80F}\) relaxation reactions were stopped at 10 min, ParCS\(^{80I}\) reactions at 20 min, and ParCE\(^{84K}\) reactions at 30 min to account for the different baseline DNA relaxation rates of the different enzymes. The amount of relaxed DNA observed in the absence of drug (‘0’ point) was set to 100% to facilitate direct comparisons. Error bars represent the standard deviation of three or more independent experiments.

**Figure 7.** Effects of alternative metal ions on drug-induced DNA cleavage mediated by wild-type *E. coli* topoisomerase IV. Assays were carried out with ciprofloxacin (Cipro, blue) or 8-methyl-3′-[(AM)P]-dione (Dione, red). The indicated divalent metal ions were substituted for the Mg\(^{2+}\) used in standard assays. The concentrations of Cd\(^{2+}\) (600 \(\mu M\), right) and Zn\(^{2+}\) (400 \(\mu M\), middle) that were utilized gave maximal enzyme-mediated DNA cleavage activity. Ca\(^{2+}\) was utilized at a concentration of 400 \(\mu M\) to minimize the basal level of cleavage seen in the absence of drug (left). Error bars represent the standard deviation of three or more independent experiments.

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In the second experiment, we determined whether a mutation of ParC Ser80 of \(E. coli\) topoisomerase IV altered metal ion utilization by ciprofloxacin. Only metal ions that could be used at millimolar concentrations were assessed to ensure that catalytic and noncatalytic sites were saturated. We found that \(Ni^{2+}\) could support low-level ciprofloxacin activity against the wild-type enzyme but not against the ParC\(^{S80L}\) mutant (Figure 8). A similar result was seen with \(Mn^{2+}\) (not shown). As described above, 8-methyl-3′-[(AM)]P-dione was examined as a control. \(Ni^{2+}\) and \(Mn^{2+}\) supported the activity of the quinazolinedione against both enzymes (Figure 8 and not shown). These results indicate that a mutation at Ser80 restricts the variety of metal ions that ciprofloxacin can utilize to enhance DNA cleavage, suggesting that the residue plays a role in anchoring the water–metal ion bridge that coordinates the quinolone to \(E. coli\) topoisomerase IV.

In the third experiment, Mg\(^{2+}\) titrations were carried out to determine whether mutation of a bridge-anchoring residue alters the affinity of the coordinating metal ion. DNA cleavage induced by the quinolone and quinazolinedione displayed a similar requirement for Mg\(^{2+}\) when wild-type topoisomerase IV was used (Figure 9). In contrast, the Mg\(^{2+}\) requirements for DNA cleavage mediated by ParC\(^{S80L}\) were different with the two drugs. While the metal ion utilization for 8-methyl-3′-[(AM)]P-dione closely resembled that seen with the wild-type enzyme, ciprofloxacin required higher levels of Mg\(^{2+}\) to support DNA cleavage (Figure 9). The requirement for higher Mg\(^{2+}\) concentrations to support quinolone- but not quinazolinedione-induced DNA cleavage by the mutant enzyme provides further evidence that interactions between quinolones (but not quinazolinediones) and \(E. coli\) topoisomerase IV are coordinated by a metal ion.

Taken together, the results described above provide strong evidence for the existence and use of the water–metal ion bridge to mediate interactions between ciprofloxacin and \(E. coli\) topoisomerase IV. They also suggest that Ser80 plays a critical role in anchoring the bridge that coordinates the quinolone to the Gram-negative enzyme and that quinolone resistance that accompanies mutation of this residue is caused by a disruption or alteration of the bridge. Similar conclusions have been reported for \(B. anthracis\) topoisomerase IV.\(^{27,28}\)

**DISCUSSION**

Although quinolones have been in clinical use for decades,\(^{59}\) the mechanism underlying drug activity and resistance remained elusive. However, a recent structural study with \(A. baumannii\)\(^{26}\) topoisomerase IV and functional studies with \(B. anthracis\) topoisomerase IV\(^{27,28}\) provide strong evidence that clinically relevant quinolones interact through a critical water–metal ion bridge and that the most common quinolone resistance mutations decrease drug activity by disrupting this bridge. To determine whether the water–metal ion bridge also is utilized by a Gram-negative species, we examined interactions between quinolones and quinazolinediones...
between quinolones (and related drugs) and wild-type and mutant E. coli topoisomerase IV. All of the mutations examined were in the serine and glutamic acid residues predicted to anchor the bridge to the enzyme. Results indicate that the water–metal ion bridge is essential for the actions of quinolones against E. coli topoisomerase IV. Thus, the bridge represents a common and critical mechanism that underlies quinolone function with both a Gram-positive species and a Gram-negative species.

Despite the importance of the bridge, it appears to play different roles in mediating quinolone activity against B. anthracis and E. coli topoisomerase IV. The water–metal ion bridge forms the primary binding contact of clinically relevant quinolones with the Gram-positive enzyme.27,28 Partial disruption of the bridge dramatically decreases the level of quinolone binding, but near wild-type levels of DNA cleavage can be generated at high quinolone concentrations. In contrast, partial disruption of the bridge in E. coli topoisomerase IV abrogates drug enhancement of DNA cleavage but, in some cases, has little to no effect on quinolone affinity. This finding implies that the water–metal ion bridge plays a crucial role in orienting clinically relevant quinolones in the cleavage complex, allowing them to optimally stimulate DNA scission. However, other binding contacts with the enzyme or DNA substrate can mediate drug binding in a manner that is not optimal for inducing DNA cleavage.

Even though ciprofloxacin is unable to increase levels of DNA cleavage mediated by several of the Ser80 and Glu84 mutant E. coli enzymes, the drug still retains the ability to inhibit the overall catalytic activity of these topoisomerase IV proteins. Inhibition parallels drug affinity, suggesting that the presence of the drug in the active site is sufficient to diminish the rate of DNA relaxation. These findings further indicate that the ability of quinolones to inhibit topoisomerase IV catalysis is not caused by decreased rates of DNA religation. Quinolones can kill bacteria by stimulating gyrase- and topoisomerase IV-mediated DNA cleavage or by robbing cells of the critical catalytic functions of these enzymes. The fact that clinically observed resistance mutations in Ser80 can abolish drug-induced DNA cleavage without affecting the ability of quinolones to inhibit catalytic activity implies that cleavage enhancement is the dominant, lethal effect of the drug.

Finally, if quinolone-like drugs that overcome the most common forms of resistance are to be developed, they must display activity against a broad range of gyrase and topoisomerase IV species. Two quinazolinedione derivatives that retain activity against quinolone-resistant B. anthracis topoisomerase IV but not human topoisomerase IIa have been described.29 These same drugs also retained activity against wild-type and quinolone-resistant E. coli topoisomerase IV. Thus, it may be possible to develop novel broad-spectrum drugs that are capable of quelling the rising tide of quinolone resistance.

Figure 10. Effects of N3 and C8 substituents on DNA cleavage mediated by wild-type and quinolone-resistant E. coli topoisomerase IV. 8-H-3′-(AM)P-dione and 8-methyl-3′-(AM)P-non-amino-dione (middle and right, respectively) were tested for the ability to enhance DNA cleavage mediated by the wild-type (WT, black) and quinolone-resistant ParC80L (S80L, blue) and ParC84K (E84K, yellow) enzymes. The activity of 8-methyl-3′-(AM)P-dione (left) against these enzymes is shown for comparison. The structures of the compounds are shown in their respective panels. Error bars represent the standard deviation of three or more independent experiments.

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3′-(AM)P(3′-(aminomethyl)pyrrolidinyl)-8-methyl-3′-(AM)P-dione, 3′-amino-7′-[(3S)-3′-(aminomethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-8-methyl-2,4′(1H,3H)-quinazolinedione; 8-H-3′-(AM)P-dione, 3′-amino-7′-[(3S)-3′-(aminomethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-2,4′(1H,3H)-quinazolinedione; 8-methyl-3′-(AM)P-3′-non-amino-dione, 7′-[(3S)-3′-(aminomethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-8-methyl-2,4′(1H,3H)-quinazolinedione.

**ABBREVIATIONS**

Biochemistry

Inc., Amsterdam.

Topoisomerase: The enzyme from *Staphylococcus aureus*: Different patterns of quinolone-induced (2000) Selective targeting of topoisomerase IV and DNA gyrase in *Mycobacterium tuberculosis*; 68, 385–392.

Quinolone-mediated bacterial death. *Antimicrob. Agents Chemother.* 52, 385–392.

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