Published in final edited form as:

Biochim Biophys Acta. 2016 March ; 1860(3): 569–575. doi:10.1016/j.bbagen.2015.12.019.

Fluoroquinolones stimulate the DNA cleavage activity of topoisomerase IV by promoting the binding of Mg²⁺ to the second metal binding site

Lisa M. Oppegard^{1,3}, Heidi A. Schwanz^{2,4}, Tyrell R. Towle^{2,5}, Robert J. Kerns², and Hiroshi Hiasa^{1,*}

Lisa M. Oppegard: brown235@umn.edu; Heidi A. Schwanz: heidiannschwanz@gmail.com; Tyrell R. Towle: tyrelltowler@gmail.com; Robert J. Kerns: robert-kerns@uiowa.edu; Hiroshi Hiasa: hiasa001@umn.edu

¹Department of Pharmacology, University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church Street SE, Minneapolis, Minnesota 55455, U.S.A

²Division of Medicinal and Natural Products Chemistry, Department of Pharmaceutical Sciences and Experimental Therapeutics, University of Iowa, 115 S Grand Ave., S321 Pharmacy Building, Iowa City, Iowa 52242, U.S.A

Abstract

Background—Fluoroquinolones target bacterial type IIA topoisomerases, DNA gyrase and topoisomerase IV (Topo IV). Fluoroquinolones trap a topoisomerase-DNA covalent complex as a topoisomerase-fluoroquinolone-DNA ternary complex and ternary complex formation is critical for their cytotoxicity. A divalent metal ion is required for type IIA topoisomerase-catalyzed strand breakage and religation reactions. Recent studies have suggested that type IIA topoisomerases use two metal ions, one structural and one catalytic, to carry out the strand breakage reaction.

Methods—We conducted a series of DNA cleavage assays to examine the effects of fluoroquinolones and quinazolinolones on Mg²⁺-, Mn²⁺-, or Ca²⁺-supported DNA cleavage activity of *Escherichia coli* Topo IV.

Results—In the absence of any drug, 20–30 mM Mg²⁺ was required for the maximum levels of the DNA cleavage activity of Topo IV, whereas approximately 1 mM of either Mn²⁺ or Ca²⁺ was sufficient to support the maximum levels of the DNA cleavage activity of Topo IV.

Fluoroquinolones promoted the Topo IV-catalyzed strand breakage reaction at low Mg²⁺ concentrations where Topo IV alone could not efficiently cleave DNA.

*Corresponding author: Hiroshi Hiasa, 6-120 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455, U.S.A., Tel. +1(612)626-3101. hiasa001@umn.edu.

³College of Undergraduate Health Sciences, Northwestern Health Sciences University, Bloomington, MN 55431, U.S.A.

⁴Boston Scientific, Maple Grove, MN 55311, U.S.A.

⁵BT Ingenuity, Mill Creek, WA 98082, U.S.A.

Competing interest

Authors declare that they have no competing interest.

Supporting data

Supplemental figures S1 and S2, and figure legends.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conclusions and General Significance—At low Mg^{2+} concentrations, fluoroquinolones may stimulate the Topo IV-catalyzed strand breakage reaction by promoting Mg^{2+} binding to metal binding site B through the structural distortion in DNA. As Mg^{2+} concentration increases, fluoroquinolones may inhibit the religation reaction by either stabilizing Mg^{2+} at site B or inhibition the binding of Mg^{2+} to site A. This study provides a molecular basis of how fluoroquinolones stimulate the Topo IV-catalyzed strand breakage reaction by modulating Mg^{2+} binding.

Keywords

divalent metal ion; DNA gyrase; fluoroquinolone; quinazolinodione; topoisomerase IV

1. Introduction

The bacterial type IIA topoisomerases, DNA gyrase and topoisomerase IV (Topo IV), are essential enzymes that play critical roles in many biological processes that involve DNA [1–5]. DNA gyrase and Topo IV consist of GyrA and GyrB subunits and ParC and ParE subunits, respectively. GyrA and ParC are DNA binding subunits, whereas GyrB and ParE subunits hydrolyze ATP. The active form of these enzymes is an A_2B_2 heterotetramer and both subunits are required to catalyze strand breakage and religation reactions. Type IIA topoisomerases catalyze the reaction by breaking both strands of a duplex DNA [referred to as the gate(G)-segment], passing another segment of DNA [referred to as the transfer(T)-segment] through the break (referred to as the DNA gate), and then religating the broken strands with ATP regulating the catalytic cycle [1–5].

Topoisomerases break and religate DNA strands by forming a covalent linkage between the enzyme and the DNA at the site of strand breakage [1–5]. The steady-state level of a topoisomerase-DNA covalent complex depends on the breakage-religation equilibrium, which is equivalent to the ratio of the strand breakage and religation rates. Some topoisomerase inhibitors, such as fluoroquinolones and etoposide, can increase the level of the topoisomerase-DNA covalent complex by stimulating the strand breakage reaction and/or inhibiting the religation reaction [6–11]. As a result, the topoisomerase-DNA covalent complex persists on the DNA as a topoisomerase-drug-DNA ternary complex. The formation of ternary complexes leads to the inhibition of DNA replication, the generation of double-strand breaks, and subsequent cell death. This class of topoisomerase inhibitors is often referred to as ‘topoisomerase poisons’ because of their unique mode of action [6–11]. It has been shown that fluoroquinolones poison *Escherichia coli* Topo IV by both stimulating the strand breakage reaction and inhibiting the religation reaction [12].

Type IIA topoisomerases require a divalent metal ion as a cofactor for their catalytic activities [1–5]. Mg^{2+} is the physiological divalent metal ion for type IIA topoisomerases, although these enzymes can use other divalent metal ions as a cofactor to catalyze certain reactions [13–16]. Biochemical studies on DNA gyrase [17], Topo IV [16] and human topoisomerase II [18–20], as well as a structural study of yeast topoisomerase II [21], support the notion that type IIA topoisomerases use a distinct variation of the classic ‘two-metal-ion mechanism’ [22, 23] to catalyze the strand breakage reaction. In the two-metal-ion mechanism for type IIA topoisomerases [16, 20, 21], Mg^{2+} at metal binding site A (Fig. 1) is

directly involved in the chemistry of the reaction by stabilizing the transition state. On the other hand, Mg^{2+} at metal binding site B (Fig. 1) plays a structural role by anchoring the -1 phosphate group. The strand breakage reaction is completed when two Mg^{2+} molecules simultaneously occupy both sites. A series of biochemical studies have shown that the binding affinity of Mg^{2+} to site A is higher than that of Mg^{2+} to site B [16, 17].

An alternative ‘moving metal ion mechanism’ [24] has been proposed based on the structural studies of drug-induced ternary complexes formed with DNA gyrase and Topo IV [25, 26]. In the moving metal ion mechanism, Mg^{2+} binds to same metal binding sites A and B, but only one of the two metal binding sites is occupied at any given time. A more recent study has demonstrated, however, that the binding of a fluoroquinolone (or a quinazolinodione) to the active site induces conformational changes in a type II topoisomerase that allow for occupancy of only one of the two metal binding sites [27]. Thus, the presence of only one metal ion at either site A or B in the drug-induced ternary complexes [24, 25] may be due to the formation of the ‘off-pathway’ endpoints that are trapped by the drugs [27].

Structural studies have shown that two molecules of either fluoroquinolone or quinazolinodione are intercalated between the $+1$ and -1 bases at the 4-base-pair staggered DNA breaks generated by DNA gyrase or Topo IV, and are anchored by drug-specific interactions with both the DNA binding subunit (GyrA or ParC) and the ATPase subunit (GyrB or ParE) of a topoisomerase [25, 26, 28]. The structure of the *Acinetobacter baumannii* Topo IV-moxifloxacin-DNA ternary complex has revealed the presence of a Mg^{2+} -water bridge between moxifloxacin and the conserved amino acid residues in the ParC subunit [26], suggesting that a Mg^{2+} molecule chelated by fluoroquinolone plays a direct role in the formation of topoisomerase-fluoroquinolone-DNA ternary complexes (Fig. S1). No such Mg^{2+} -water bridge has been found between a quinazolinodione and Topo IV in the ternary complex [25]. Both fluoroquinolones and quinazolinodiones cause similar structural distortions in DNA when they intercalate between the $+1$ and -1 bases at the sites of strand breakage [25, 26, 28].

We examined the effects of fluoroquinolones and quinazolinodiones on the DNA cleavage activity of *E. coli* Topo IV in the presence of Mg^{2+} , Mn^{2+} , or Ca^{2+} . Consistent with the report by Pitts *et al.* [16], approximately 1 mM of either Mn^{2+} or Ca^{2+} was sufficient to support the maximum levels of the DNA cleavage activity of Topo IV in the absence of any drug. In contrast, significantly higher concentrations (20–30 mM) of Mg^{2+} were required to support the maximum levels of the DNA cleavage activity of Topo IV, suggesting that the binding affinity of Mg^{2+} to metal binding site B is lower than that of either Mn^{2+} or Ca^{2+} , and that the binding of Mg^{2+} to metal binding site B is the rate-limiting step for the Topo IV-catalyzed strand breakage reaction. Fluoroquinolones and quinazolinodiones stimulated the Topo IV-catalyzed strand breakage reaction and the strongest enhancement of the DNA cleavage activity was detected in the presence of 1–2 mM Mg^{2+} . Thus, fluoroquinolones and quinazolinodiones stimulate the Topo IV-catalyzed strand breakage reaction by promoting Mg^{2+} binding to metal binding site B.

2. Materials and Methods

2-1. Biological and chemical reagents

The ParC and ParE subunits of *E. coli* Topo IV were expressed and purified, and the active enzyme was reconstituted as previously described [15, 29]. The plasmid pBR322 DNA and kinetoplast DNA were purchased from New England Biolabs (Ipswich, MA, U.S.A.) and TopoGEN (Port Orange, FL, U.S.A.), respectively.

The 8-methyl-quinazoline-2,4-dione (UIJR1-048) and the 8-methoxy-quinazoline-2,4-dione (UING5-207) were synthesized as previously described [30, 31]. Ciprofloxacin (CFX) and moxifloxacin were purchased from MP Biomedical Inc. (Irvine, CA, U.S.A.) and ChemPacific (Baltimore, MD, U.S.A.), respectively. Norfloxacin was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Other chemicals and reagents were purchased from Sigma-Aldrich, Thermo Fisher Scientific (Waltham, MA, U.S.A.), or Roche Applied Science (Indianapolis, IN, U.S.A.).

2-2. DNA cleavage assay

DNA cleavage reaction mixtures (20 μ l) contained 50 mM Tris-HCl (pH 8.0 at 23 °C), 10 mM dithiothreitol, 50 μ g/ml bovine serum albumin, 1 mM ATP, 5 μ g/ml tRNA, 100 fmol (as molecule) of plasmid pBR322 DNA, 200 fmol (as tetramer) of *E. coli* Topo IV and various concentrations of magnesium chloride, manganese chloride, or calcium chloride. When indicated, fluoroquinolones and quinazolinodiones were added to reaction mixtures at a concentration 3-fold higher than the CC₃ (defined as the concentration of drug needed to triple the amount of linear DNA generated above the background Topo IV level in the absence of drug [32]). Changes in the reaction conditions are indicated in the figure legends. Reaction mixtures were incubated for 15 min at 37 °C and then SDS was added to 1%, and the reaction mixtures were further incubated at 37 °C for 15 min. After addition of EDTA and proteinase K to a final concentration of 40 mM and 100 μ g/ml, respectively, the mixtures were incubated at 50 °C for 30 min. The DNA products were purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol) and then analyzed by electrophoresis through vertical 1.2% agarose gels at 2 V/cm for 12 hours in TAE buffer which contained 0.5 μ g/ml ethidium bromide. After destaining in water, gels were photographed and quantified using a Stratagene Eagle Eye II system (Agilent Technologies, Santa Clara, CA, U.S.A.).

2-3. Decatenation assay

Reaction mixtures (20 μ l) contained 50 mM Tris-HCl (pH 8.0 at 23 °C), 100 mM potassium glutamate, 10 mM dithiothreitol, 50 μ g/ml bovine serum albumin, 1 mM ATP, 180 fmol of kinetoplast DNA (as molecule), various concentrations of magnesium chloride, manganese chloride, or calcium chloride, and 50 fmol (as tetramer) of *E. coli* Topo IV. Reaction mixtures were incubated at 37 °C for 15 min and terminated by adding EDTA to 25 mM and further incubating at 37 °C for 5 min. The DNA products were analyzed by electrophoresis through vertical 1.2% agarose gels at 2 V/cm for 15 hours in TAE buffer. Gels were stained with ethidium bromide, and then photographed and quantified using a Stratagene Eagle Eye II system.

2-4. Molecular Modeling

The structure of the *A. baumannii* Topo IV-moxifloxacin-DNA ternary complex was obtained from PDB ID: 2XKK [26]. Four Mg^{2+} molecules are present in the PDB ID: 2XKK structure; two catalytic Mg^{2+} , one at each site A, and two Mg^{2+} chelated with moxifloxacin, one with each moxifloxacin. No Mg^{2+} was present in either of the two sites B in the PDB ID: 2XKK structure. Based on the positions of the two metal binding sites proposed by Schmidt *et al.* [21] and Pitts *et al.* [16], the site A and the site B, we manually modeled Mg^{2+} ions into each of the sites B of the PDB ID: 2XKK structure using SYBYL (Certara USA, Inc., Princeton, NJ, U.S.A.). The structure shown in Figure 1 thus has a Mg^{2+} ion present at each of the metal binding sites A and B. As indicated in the figure legend, the Mg^{2+} chelated by moxifloxacin is omitted for clarity. The amino acid numbering was changed to those in *E. coli* Topo IV based on the homologies between *A. baumannii* and *E. coli* Topo IVs.

3. Results and Discussion

3-1. A fluoroquinolone lowers Mg^{2+} concentrations required for Topo IV-catalyzed DNA cleavage

During our studies on the effects of fluoroquinolones and quinazolinediones on *E. coli* Topo IV [32], we noticed that 10 mM Mg^{2+} was required for efficient DNA cleavage by Topo IV, but fluoroquinolones could stimulate the DNA cleavage activity of Topo IV to similar levels in the presence of either 1 mM or 10 mM Mg^{2+} . These observations led us to examine the effects of fluoroquinolones on the concentrations of divalent metal ions required for optimal DNA cleavage activity of *E. coli* Topo IV. A DNA cleavage assay using a plasmid DNA as a substrate was first employed to measure the steady-state level of the DNA cleavage activity of Topo IV in the absence or presence of CFX. The concentrations of Topo IV and CFX were chosen to limit multiple cleavages of the plasmid DNA (data not shown), so that the steady-state level of the DNA cleavage activity of Topo IV could be measured as the amount of the full-length linear plasmid DNA generated after a 15-min incubation [32, 33].

The DNA cleavage assay was conducted at various Mg^{2+} concentrations, in the absence or presence of CFX (Fig. 2), and the DNA cleavage activity of Topo IV was measured by quantifying the amount of the full-length linear DNA (Fig. 3). In the absence of CFX, the DNA cleavage activity of Topo IV increased as the Mg^{2+} concentration increased and the maximum levels of DNA cleavage were detected when Mg^{2+} concentrations were 20–30 mM (Fig. 3A). The levels of DNA cleavage (8%–12%) we detected at 2.5–5 mM Mg^{2+} were lower than those of DNA cleavage (20%–25%) reported by Pitts *et al.* [16] although the level of DNA cleavage was measured as the amount of the full-length linear DNA in both studies. This difference may be due to a higher amount (1.75-times higher than the amount used in this study) of Topo IV used by Pitts *et al.* They might have detected even higher levels of DNA cleavage if they had increased the Mg^{2+} concentration beyond 10 mM. In fact, the level of DNA cleavage at 10 mM Mg^{2+} was higher than that of DNA cleavage at 5 mM Mg^{2+} in their study as well [16]. The increase in the amount of the nicked DNA appeared to coincide with that in the amount of the full-length linear DNA at various Mg^{2+}

concentrations (Fig. 2A). Thus, the Mg^{2+} concentration does not appear to affect coordination between two active sites of Topo IV.

CFX promoted the highest level of DNA cleavage at approximately 1–2 mM Mg^{2+} (Figure 3A). Additional assays were conducted to more closely measure the DNA cleavage activity of Topo IV at low (0–2 mM) Mg^{2+} concentrations (Fig. 3B). The level of CFX-induced, Topo IV-catalyzed DNA cleavage reached 50% of the maximal level and the maximum level when 0.75 mM and 1.25 mM Mg^{2+} were present, respectively. In contrast, in the absence of CFX, only the background level of Topo IV-catalyzed DNA cleavage was detected when up to 1 mM of Mg^{2+} was present. Thus, CFX promoted the Topo IV-catalyzed strand breakage reaction at low Mg^{2+} concentrations where Topo IV alone could not efficiently cleave DNA. Therefore, the presence of CFX significantly lowered (by more than 10-fold) the Mg^{2+} concentration required to achieve the maximum DNA cleavage activity of Topo IV. Although the addition of CFX significantly increased the amounts of the full-length linear DNA at 1–5 mM Mg^{2+} , the amounts of nicked DNA at 1–5 mM Mg^{2+} were essentially identical in the absence or presence of CFX (Figure 2), suggesting that CFX-induced strand breakage reaction by two active sites of Topo IV are coordinated more tightly than drug-independent strand breakage reaction by two active sites of Topo IV. It has been proposed that etoposide-induced structural distortions in DNA in a human topoisomerase II-etoposide-DNA ternary complex stabilize the binding of two etoposide molecules and thus couple strand breakage reaction by the two active sites of human topoisomerase II [34].

It has been demonstrated that catalytically-inactive mutant DNA gyrase and Topo IV can form ternary complexes with a fluoroquinolone and DNA [35, 36] and that ternary complex formation with a catalytically-inactive mutant Topo IV induces local structural distortions in DNA [37]. These results suggest that fluoroquinolones are capable of binding to Topo IV on DNA prior to the strand breakage reaction and inducing structural distortions in DNA. We propose that at low Mg^{2+} concentrations where Topo IV alone cannot efficiently catalyze the strand breakage reaction, CFX binds to Topo IV on DNA and induces structural distortions in DNA, which influence metal binding sites to promote the binding of Mg^{2+} to metal binding site B, which in turn stimulates the strand breakage reaction.

We did detect low levels of both the decatenation activity (Fig. 5A) and the DNA cleavage activity (Fig. 3B) of Topo IV at 1 mM Mg^{2+} . Thus, we cannot preclude the possibility that CFX binds to Topo IV after the strand breakage reaction even at low Mg^{2+} concentrations. The binding of CFX induces conformational changes that could block the binding of Mg^{2+} to metal binding site A [28], which in turn would inhibit the religation reaction. It is also possible that the intercalation of CFX between the bases at the –1 and +1 positions [25, 26] could spatially block the religation reaction.

At Mg^{2+} concentrations high enough to efficiently support the strand breakage reaction, CFX still stimulated the DNA cleavage activity of Topo IV (Fig. 3A). This is likely caused by inhibiting the religation reaction as a result of binding of CFX to Topo IV after the strand breakage reaction. It has been recently reported that both CFX and 8-methyl-quinazoline-2,4-dione inhibit the religation reaction catalyzed by *E. coli* Topo IV in the

presence of 2.5 mM Mg^{2+} [38]. The inhibition of the religation reaction can be caused by stabilization of Mg^{2+} at metal binding site B, preventing Mg^{2+} from binding to metal binding site A [28], and/or spatially interfering the DNA end-joining step [25, 26].

3-2. Mn^{2+} and Ca^{2+} can support the DNA cleavage activity of Topo IV

Since we found that CFX greatly altered the concentrations of Mg^{2+} required for the DNA cleavage activity of Topo IV, we next examined if CFX also affects the concentrations of other divalent metal ions required to support the DNA cleavage activity of Topo IV. DNA cleavage assays were performed in the presence of various concentrations of either Mn^{2+} or Ca^{2+} (Figs. 4 and S2). In the absence of CFX, Mn^{2+} supported reduced levels of the Topo IV-catalyzed strand breakage reaction. The highest level of DNA cleavage was detected in the presence of 1–2 mM Mn^{2+} and the level of DNA cleavage reduced as the Mn^{2+} concentration further increased. No cleavage was detected when 30 mM or higher concentrations of Mn^{2+} were present. These results are consistent with previous observations [16, 39]. It has been proposed that DNA gyrase forms an inactive conformation in the presence of high concentrations of Mn^{2+} [39]. This may also be the case with Topo IV. The addition of CFX stimulated Mn^{2+} -supported DNA cleavage activity of Topo IV but did not alter the optimal Mn^{2+} concentrations for the DNA cleavage activity of Topo IV (Fig. 4).

Ca^{2+} induced greatly elevated levels of Topo IV-catalyzed DNA cleavage in the absence of CFX (Figs. 4 and S2). The optimal Ca^{2+} concentration to support the DNA cleavage activity of Topo IV was 1–2 mM and the level of DNA cleavage gradually decreased as the Ca^{2+} concentration further increased. The addition of CFX did not significantly affect the levels of Ca^{2+} -supported DNA cleavage activity of Topo IV and did not alter the optimal Ca^{2+} concentrations for the DNA cleavage activity of Topo IV (Fig. 4). It is likely that Ca^{2+} alone could promote the maximum level of the Topo IV-catalyzed strand breakage reaction and the addition of CFX would, therefore, not increase the level of DNA cleavage. Alternatively, CFX may not bind to a Topo IV-DNA complex when Ca^{2+} is used as a cofactor.

3-3. Topo IV cannot complete the catalytic cycle using Ca^{2+} as a cofactor

We also tested if all three divalent metal ions can support the catalytic activity of Topo IV. As expected [15], Topo IV could catalyze both the decatenation reaction (Fig. 5A) and the relaxation reaction (data not shown) using Mg^{2+} as a cofactor. Mn^{2+} was also able to support the catalytic activity of Topo IV (Fig. 5B), although only in a more narrow concentration range (1–2.5 mM) than Mg^{2+} . The Mn^{2+} concentrations that support the Topo IV-catalyzed DNA cleavage (Fig. 4) also supported the catalytic activity of Topo IV (Fig. 5B). These results demonstrate that Mn^{2+} can function as a cofactor to support the complete catalytic cycle, including the ATP hydrolysis, of Topo IV.

In contrast, we found that Topo IV could not catalyze either the decatenation reaction (Fig. 5C) or the relaxation reaction (data not shown) when Ca^{2+} was used as the cofactor. Thus, although *E. coli* Topo IV efficiently binds and cleaves DNA in the presence of Ca^{2+} (Fig. 4), it cannot complete the catalytic cycle using Ca^{2+} as the cofactor (Fig. 5C). It has been proposed that the two ATP molecules bound by a type IIA topoisomerase are hydrolyzed

asynchronously and that the strand passage step is tightly coupled with the release of Pi from the first ATP hydrolysis [40, 41]. Thus, it is possible that *E. coli* Topo IV cannot carry out the strand passage step because Ca^{2+} is the wrong metal ion to support its ATPase activity. It is interesting to note that *E. coli* gyrase [17, 41], but not *Mycobacterium tuberculosis* gyrase [42, 43], can efficiently catalyze the supercoiling reaction using Ca^{2+} as a cofactor. Furthermore, human topoisomerase II [44] can also complete the catalytic cycle using Ca^{2+} as a cofactor, whereas *Drosophila melanogaster* topoisomerase II failed to do so [13]. These differences may be due to the inability of certain enzymes to use Ca^{2+} as a cofactor for ATP binding and/or ATP hydrolysis. Because divalent metal ions have multiple roles in the catalytic cycle of type IIA topoisomerases, further studies are necessary to determine why only certain type IIA topoisomerases can use Ca^{2+} as a cofactor to complete the catalytic cycle.

3-4. The highest enhancement of Topo IV-catalyzed DNA cleavage by CFX is detected at 1 mM Mg^{2+}

To directly compare the effects of CFX on the DNA cleavage activity of Topo IV supported by the three divalent metal ions at various concentrations, we expressed the effect of CFX on Topo IV-catalyzed DNA cleavage as a fold-enhancement of the DNA cleavage activity over drug-independent DNA cleavage activity under the same reaction conditions (Fig. 6). A very strong effect (greater than a 20-fold enhancement) of CFX on the DNA cleavage activity of Topo IV was detected in the presence of 1 mM Mg^{2+} . At higher Mg^{2+} concentrations (5–50 mM), the effect of CFX was modest (a 5-fold enhancement or less) and relatively constant. As described above, we propose that the strong effects of CFX on the DNA cleavage activity of Topo IV at low Mg^{2+} concentrations are caused by an increase in the binding affinity of Mg^{2+} to metal binding site B promoting the strand breakage reaction, whereas the modest effects of CFX at high Mg^{2+} concentrations is caused by stabilization of cleaved DNA by Mg^{2+} at site B inhibiting the religation reaction.

The effect of CFX on the DNA cleavage activity of Topo IV was low (a 3-fold enhancement or less) and relatively constant when Mn^{2+} (0.5–30 mM) was used as a cofactor (Fig. 6). Because CFX did not stimulate Topo IV-catalyzed DNA cleavage in the presence of Ca^{2+} (0.5–50 mM), no significant enhancement was detected in the presence of various concentrations of Ca^{2+} (Fig. 6).

3–5. Quinazolidinediones can also lower the Mg^{2+} concentration required for Topo IV-catalyzed DNA cleavage

A Mg^{2+} -water bridge between the fluoroquinolone and Topo IV (Fig. S1) contributes to formation of the Topo IV-fluoroquinolone-DNA ternary complex [24]. Because fluoroquinolones inherently chelate Mg^{2+} and this Mg^{2+} is brought into the ternary complex, an important consideration is whether fluoroquinolones might increase the local Mg^{2+} concentration at fluoroquinolone-induced ternary complexes and, thus, promote binding of Mg^{2+} to metal binding site B, which would lead to the stimulation of the Topo IV-catalyzed strand breakage reaction. Quinazolidinediones, which are structurally similar to fluoroquinolones (Fig. S1), do not chelate Mg^{2+} and thus quinazolidinediones do not bring Mg^{2+} into ternary complexes. There is no Mg^{2+} -water bridge between 8-methyl-

quinazoline-2,4-dione and Topo IV in a Topo IV-(8-methyl-quinazoline-2,4-dione)-DNA ternary complex [25]. Therefore, if fluoroquinolone-mediated stimulation of the Topo IV-catalyzed strand breakage reaction at low Mg^{2+} concentrations is caused by the chelation of Mg^{2+} by the fluoroquinolone, quinazolinodiones should not display this same effect on the strand breakage reaction. To test this possibility, we measured the DNA cleavage activity of Topo IV at various Mg^{2+} concentrations in the presence of 8-methyl-quinazoline-2,4-dione (Fig. 7), 8-methoxy-quinazoline-2,4-dione (data not shown), moxifloxacin (Fig. 7), and norfloxacin (data not shown). Quinazolinodiones stimulated the DNA cleavage activity of Topo IV in a manner similar to fluoroquinolones (Fig. 7). These results demonstrate that quinazolinodiones, like fluoroquinolones, stimulate the Topo IV-catalyzed strand breakage reaction at low Mg^{2+} concentrations, suggesting that the chelation of Mg^{2+} by fluoroquinolones does not contribute to the stimulation of the Topo IV-catalyzed strand breakage reaction at lower Mg^{2+} concentrations.

Structural studies of Topo IV-drug-DNA ternary complexes formed with either a fluoroquinolone or quinazolinodione have revealed a striking similarity between fluoroquinolones and quinazolinodiones in their modes of binding in ternary complexes. Both compounds, despite their differences [32, 45], intercalate between the -1 and +1 bases at the active sites and cause the bound DNA to be distorted [24, 25, 27]. Biochemical studies have shown that fluoroquinolones can form ternary complexes with Topo IV and DNA prior to the strand breakage reaction and induce structural distortions in DNA [35, 37]. Thus, we propose that at low Mg^{2+} concentrations where Topo IV alone cannot efficiently catalyze the strand breakage reaction, both fluoroquinolones and quinazolinodiones might influence metal binding sites through the distortion of the DNA structure to increase the affinity of Mg^{2+} to metal binding site B and promote the Topo IV-catalyzed strand breakage reaction. As discussed above, we cannot rule out the possibility that the binding of CFX to Topo IV after the strand breakage reaction at low Mg^{2+} concentrations leads to the inhibition of the religation reaction [25, 26, 28]. However, Lee *et al.* [46] reported that Mg^{2+} -mediated DNA bending by human topoisomerase II is critical for the regulation of cleavage of the two DNA strands by human topoisomerase II. Thus, Mg^{2+} and the local distortion of DNA appear to play critical roles in both coordinating the strand breakage reaction by the two active sites of type IIA topoisomerases and modulating the effect of topoisomerase poisons on type IIA topoisomerases.

4. Conclusion

Significantly higher concentrations of Mg^{2+} than either Mn^{2+} or Ca^{2+} were required for the Topo IV-catalyzed strand breakage reaction, suggesting that Mg^{2+} has a weak affinity to metal binding site B. The binding of either a fluoroquinolone or quinazolinodione greatly stimulated the DNA cleavage activity of Topo IV at low Mg^{2+} concentrations, where Topo IV alone cannot efficiently catalyze the strand breakage reaction. Fluoroquinolones are capable of forming ternary complexes with Topo IV and DNA prior to the strand breakage reaction and inducing local structural distortions in DNA. Based on these results, we propose that at low Mg^{2+} concentrations where Topo IV binds to DNA but cannot catalyze the strand breakage reaction, fluoroquinolones (and quinazolinodiones) bind to Topo IV, and influence metal binding sites through the distortion of the DNA structure to increase the

affinity of Mg^{2+} to metal binding site B. This leads to the stimulation of the Topo IV-catalyzed strand breakage reaction. At high Mg^{2+} concentrations, fluoroquinolones (and quinazolinodiones) inhibit the religation reaction. Thus, providing a molecular basis of how fluoroquinolones either stimulate the strand breakage reaction or inhibit the religation reaction through effects on Mg^{2+} binding. Thus, this study provides evidences for a molecular basis of how fluoroquinolones either stimulate the strand breakage reaction or inhibit the religation reaction by modulating the binding of Mg^{2+} to metal binding sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by National Institutes of Health grant R01 AI087671 to RJK and HH, a National Institutes of Health Predoctoral Traineeship in Biotechnology (T32GM008365) to HAS, Predoctoral Fellowships from the American Foundation for Pharmaceutical Education to HAS and TRT, and an American Chemical Society Division of Medicinal Chemistry Predoctoral Fellowship sponsored by Richard B. Silverman to TRT.

Abbreviations

CFX	ciprofloxacin
Topo	topoisomerase

References

1. Champoux JJ. DNA topoisomerases: structure, function, and mechanism. *Annu Rev Biochem.* 2001; 70:369–413. [PubMed: 11395412]
2. Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol.* 2002; 3:430–440. [PubMed: 12042765]
3. Nitiss JL. DNA topoisomerase II and its growing repertoire of biological functions. *Nat Rev Cancer.* 2009; 9:327–337. [PubMed: 19377505]
4. Vos SM, Tretter EM, Schmidt BH, Berger JM. All tangled up: how cells direct, manage and exploit topoisomerase function. *Nat Rev Mol Cell Biol.* 2011; 12:827–841. [PubMed: 22108601]
5. Bates AD, Berger JM, Maxwell A. The ancestral role of ATP hydrolysis in type II topoisomerases: prevention of DNA double-strand breaks. *Nucleic Acids Res.* 2011; 39:6327–6339. [PubMed: 21525132]
6. Heddle JG, Barnard FM, Wentzell LM, Maxwell A. The interaction of drugs with DNA gyrase: a model for the molecular basis of quinolone action. *Nucleosides Nucleotides Nucleic Acids.* 2000; 19:1249–1264. [PubMed: 11097055]
7. 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]
8. Li TK, Liu LF. Tumor cell death induced by topoisomerase-targeting drugs. *Annu Rev Pharmacol Toxicol.* 2001; 41:53–77. [PubMed: 11264450]
9. Drlica K, Malik M. Fluorouinolones: action and resistance. *Curr Top Med Chem.* 2003; 3:249–282. [PubMed: 12570763]
10. Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer.* 2009; 9:338–350. [PubMed: 19377506]
11. 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]

12. Anderson VE, Gootz TD, Osheroff N. Topoisomerase IV catalysis and the mechanism of quinolone action. *J Biol Chem.* 1998; 273:17879–85. [PubMed: 9651393]
13. Osheroff N, Zechiedrich EL. Calcium-promoted DNA cleavage by eukaryotic topoisomerase II: Trapping the covalent enzyme-DNA complex in an active form. *Biochemistry.* 1987; 26:4303–4309. [PubMed: 2822084]
14. Barnard FM, Maxwell A. Interaction between DNA gyrase and quinolones: Effects of alanine mutations at GyrA subunit residues Ser(83) and Asp(87). *Antimicrob Agents Chemother.* 2001; 45:1994–2000. [PubMed: 11408214]
15. Hiasa H. The Glu-84 of the ParC subunit plays critical roles in both topoisomerase IV-quinolone and topoisomerase IV-DNA interactions. *Biochemistry.* 2002; 41:11779–11785. [PubMed: 12269820]
16. Pitts SL, Liou GF, Mitchenall LA, Burgin AB, Maxwell A, Neuman KC, Osheroff N. Use of divalent metal ions in the DNA cleavage reaction of topoisomerase IV. *Nucleic Acids Res.* 2011; 39:4808–4817. [PubMed: 21300644]
17. Noble CG, Maxwell A. The role of GyrB in the DNA cleavage-religation reaction of DNA gyrase: a proposed two metal-ion mechanism. *J Mol Biol.* 2002; 318:361–371. [PubMed: 12051843]
18. West KL, Meczes EL, Thorn R, Turnbull RM, Marshall R, Austin CA. Mutagenesis of E477 or K505 in the B' domain of human topoisomerase II β increases the requirement for magnesium ions during strand passage. *Biochemistry.* 2000; 39:1223–1233. [PubMed: 10684600]
19. Leontiou C, Lakey JH, Lightowlers R, Turnbull RM, Austin CA. Mutation P732L in human DNA topoisomerase II β abolishes DNA cleavage in the presence of calcium and confers drug resistance. *Mol Pharmacol.* 2006; 69:130–139. [PubMed: 16239602]
20. Deweese JE, Burgin JE, Osheroff N. Human topoisomerase II α uses a two-metal-ion mechanism for DNA cleavage. *Nucleic Acids Res.* 2008; 36:4883–4893. [PubMed: 18653531]
21. Schmidt BH, Burgin AB, Deweese JE, Osheroff N, Berger JM. A novel and unified two-metal mechanism for DNA cleavage by type II and IA topoisomerases. *Nature.* 2010; 465:641–644. [PubMed: 20485342]
22. Wang J, Yu P, Lin TC, Konigsberg WH, Steitz TA. Crystal structures of an NH₂-terminal fragment of T4 DNA polymerase and its complexes with single-stranded DNA and with divalent metal ions. *Biochemistry.* 1996; 35:8110–8119. [PubMed: 8679562]
23. Nowotny M, Yang W. Stepwise analyses of metal ions in RNase H catalysis from substrate destabilization to product release. *EMBO J.* 2006; 25:1924–1933. [PubMed: 16601679]
24. Oezguen N, Schein CH, Peddi SR, Power TD, Izumi T, Braun W. A “moving metal mechanism” for substrate cleavage by the DNA repair endonuclease APE-1. *Proteins.* 2007; 68:313–323. [PubMed: 17427952]
25. Laponogov I, Pan XS, Veselkov DA, McAuley DA, Fisher LM, Sanderson MR. Structural basis of gate-DNA breakage and resealing by type II topoisomerases. *PLoS One.* 2010; 5:e11338. [PubMed: 20596531]
26. 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]
27. Wendorff TJ, Schmidt BH, Heslop P, Austin CA, Berger JM. The structure of DNA-bound human topoisomerase II alpha: conformational mechanisms for coordinating inter-subunit interactions with DNA cleavage. *J Mol Biol.* 2012; 424:109–124. [PubMed: 22841979]
28. Laponogov I, Sohi MK, Veselkov DA, Pan XS, Sawhney R, Thompson AW, McAuley KE, Fisher LM, Sanderson MR. Structural insight into the quinolone-DNA cleavage complex of type IIA topoisomerases. *Nat Struct Mol Biol.* 2009; 16:667–669. [PubMed: 19448616]
29. Oppegard LM, Hamann BL, Streck KR, Ellis KC, Fiedler HP, Khodursky AB, Hiasa H. *In vivo* and *in vitro* patterns of the activity of simocyclinone D8, an angucyclinone antibiotic from *Streptomyces antibioticus*. *Antimicrob Agents Chemother.* 2009; 53:2110–2119. [PubMed: 19273673]
30. Ellsworth, EL.; Showalter, HDH. Antibacterial agents. US patent. 0114458 A1. 2003.

31. German N, Malik M, Rosen J, Drlica K, Kerns RJ. Use of gyrase resistance mutants to guide selection of 8-methoxyquinazoline-2,4-diones. *Antimicrob Agents Chemother.* 2008; 52:3915–3921. [PubMed: 18765690]
32. 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]
33. Pfeiffer ES, Hiasa H. Determination of the primary target of a quinolone drug and the effect of quinolone resistance-conferring mutations by measuring quinolone sensitivity based on its mode of action. *Antimicrob Agents Chemother.* 2007; 51:3410–3412. [PubMed: 17606687]
34. Wu CC, Li TK, Farh L, Lin LY, Lin TS, Yu YJ, Yen TJ, Chiang CW, Chan NL. Structural basis of type II topoisomerase inhibition by the anticancer drug etoposide. *Science.* 2011; 333:459–462. [PubMed: 21778401]
35. Hiasa H, Yousef DO, Marians KJ. DNA strand cleavage is required for replication fork arrest by a frozen topoisomerase-quinolone-DNA ternary complex. *J Biol Chem.* 1996; 271:26424–26429. [PubMed: 8824300]
36. Critchlow SE, Maxwell A. DNA cleavage is not required for the binding of quinolone drugs to the DNA gyrase-DNA complex. *Biochemistry.* 1996; 35:7387–93. [PubMed: 8652515]
37. Marians KJ, Hiasa H. Mechanism of quinolone action. A drug-induced structural perturbation of the DNA precedes strand cleavage by topoisomerase IV. *J Biol Chem.* 1997; 272:9401–9409. [PubMed: 9083078]
38. Aldred KJ, Breland EJ, Vlková V, Strub MP, Neuman KC, Kerns RJ, Osheroff N. Role of the water-metal ion bridge in mediating interactions between quinolones and *Escherichia coli* topoisomerase IV. *Biochemistry.* 2014; 53:5558–67. [PubMed: 25115926]
39. Sissi C, Chemello A, Vazquez E, Mitchenall LA, Maxwell A, Palumbo M. DNA gyrase requires DNA for effective two-site coordination of divalent metal ions: further insight into the mechanism of enzyme action. *Biochemistry.* 2008; 47:8538–8545. [PubMed: 18642932]
40. Harkins TT, Lewis TJ, Lindsley JE. Pre-steady-state analysis of ATP hydrolysis by *Saccharomyces cerevisiae* DNA Topoisomerase II. 2 Kinetic mechanism for the sequential hydrolysis of two ATP. *Biochemistry.* 1998; 37:7299–7312. [PubMed: 9585544]
41. Baird CL, Harkins TT, Morris SK, Lindsley JE. Topoisomerase II drives DNA transport by hydrolyzing one ATP. *Proc Natl Acad Sci USA.* 1999; 96:13685–13690. [PubMed: 10570133]
42. Karkare S, Yousafzai F, Mitchenall LA, Maxwell A. The role of Ca^{2+} in the activity of *Mycobacterium tuberculosis* DNA gyrase. *Nucleic Acids Res.* 2012; 40:9774–9787. [PubMed: 22844097]
43. Aubry A, Fisher LM, Jarlier V, Cambau E. First functional characterization of a singly expressed bacterial type II topoisomerase: the enzyme from *Mycobacterium tuberculosis*. *Biochem Biophys Res Commun.* 2006; 348:158–165. [PubMed: 16876125]
44. Gilroy KL, Austin CA. The impact of the C-terminal domain on the interaction of human DNA topoisomerase II α and β with DNA. *PLoS One.* 2011; 6:e14693. [PubMed: 21358820]
45. Pan XS, Gould KA, Fisher LM. Probing the differential interactions of quinazolinone PD 0305970 and quinolones with gyrase and topoisomerase IV. *Antimicrob Agents Chemother.* 2009; 53:3822–3831. [PubMed: 19564360]
46. Lee S, Jung SR, Heo K, Byl JA, Deweese JE, Osheroff N, Hohng S. DNA cleavage and opening reactions of human topoisomerase II α are regulated via Mg^{2+} -mediated dynamic bending of gate-DNA. *Proc Natl Acad Sci USA.* 2010; 109:2925–2930. [PubMed: 22323612]
47. Yoshida H, Bogaki M, Nakamura M, Yamanaka LM, Nakamura S. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. *Antimicrob Agents Chemother.* 1991; 35:1647–1650. [PubMed: 1656869]

Highlights

- Fluoroquinolones promote the binding of Mg^{2+} to site B to stimulate strand breakage
- Fluoroquinolones may inhibit religation by stabilizing Mg^{2+} binding at site B
- Quinazoliniones act by the same mechanisms as fluoroquinolones
- Topoisomerase IV cannot complete the catalytic reaction using Ca^{2+} as a cofactor

Figure 1. The positions of two Mg^{2+} molecules at the catalytic sites in the Topo IV-fluoroquinolone-DNA ternary complex

The structure of the *A. baumannii* Topo IV-moxifloxacin-DNA ternary complex was obtained from PDB ID: 2XKK [26]. Four Mg^{2+} ions are shown; one at each site A and one at each site B. Based on the positions of two metal binding sites proposed by Schmidt *et al.* [21] and Pitts *et al.* [16], the B site Mg^{2+} ions were manually modeled into the structure. The four Mg^{2+} molecules shown in the catalytic sites of Topo IV are two molecules each at metal binding sites A (purple spheres) and sites B (yellow spheres). The amino acid numbering in *A. baumannii* Topo IV was changed to that in *E. coli* Topo IV. Two moxifloxacin molecules are depicted in space-filling representation and the backbone of DNA is shown in green. For clarity, Topo IV is omitted except for the active site tyrosine (phosphotyrosine) of *E. coli* ParC, two conserved aspartic acid residues, Asp-490 and Asp-492, of *E. coli* ParE that directly interact with the Mg^{2+} ions at site B, and the Asp-420 residue in the conserved EGDSA motif in the TOPRIM domain of *E. coli* ParE, where both fluoroquinolone- and quinazolinodione-resistant mutations have been mapped [45, 47]. The Mg^{2+} molecules that bridge between moxifloxacin and the ParC subunit are also omitted for clarity.

Figure 2 (single column)

Figure 2. Effect of Mg^{2+} concentration on Topo IV-catalyzed DNA cleavage in the absence or presence of CFX

Representative results of the DNA cleavage assay are shown. DNA cleavage reactions contained 100 fmol (as a molecule) of plasmid pBR322 DNA, 200 fmol (as a tetramer) of *E. coli* Topo IV and the indicated concentrations of magnesium chloride (Mg), and were performed in the absence (Panel A) or presence of 45 μ M CFX (Panel B).

Figure 3. CFX lowers the optimal Mg^{2+} concentration for Topo IV-catalyzed DNA cleavage
 The DNA cleavage assay was performed in the presence of various concentrations of Mg^{2+} as described in Materials and Methods, and the level of Topo IV-catalyzed DNA cleavage was measured as the amount of linear DNA. One set of assays was carried out in the presence of a wide range of Mg^{2+} concentrations (A), the other in a narrow range of lower Mg^{2+} concentrations (B). Each assay was repeated three times. The average values are plotted as a function of Mg^{2+} concentration and error bars indicate standard deviations. For both panels: $\circ$, in the absence of CFX; $\bullet$, in the presence of 45 μ M CFX.

Figure 4. CFX does not alter the optimal concentration of either Mn²⁺ or Ca²⁺ for Topo IV-catalyzed DNA cleavage

The DNA cleavage assay was performed in the presence of various concentrations of either Mn²⁺ ($\triangle$, in the absence of CFX; $\blacktriangle$, in the presence of 45 μ M CFX) or Ca²⁺ ($\square$, in the absence of CFX; $\blacksquare$, in the presence of 45 μ M CFX) as described in Materials and Methods, and the level of Topo IV-catalyzed DNA cleavage was measured as the amount of linear DNA. Each assay was repeated three times. The average values are plotted as a function of either Mn²⁺ or Ca²⁺ concentration and error bars indicate standard deviations.

Figure 5. Mg²⁺ and Mn²⁺, but not Ca²⁺, can support the decatenation activity of Topo IV
The decatenation assay was performed in the presence of the various concentrations of either magnesium chloride (Mg²⁺, Panel A), manganese chloride (Mn²⁺, Panel B), or calcium chloride (Ca²⁺, Panel C) as described in Materials and Methods. In panel C, the control lane shows the DNA products from a decatenation reaction in the presence of 10 mM magnesium chloride.

Figure 6. The strongest enhancement of Topo IV-catalyzed DNA cleavage by CFX is detected at approximately 1 mM Mg²⁺

The ratio of Topo IV-catalyzed DNA cleavage in the presence of 45 μ M CFX to that of Topo IV-catalyzed DNA cleavage in the absence of CFX was plotted as a function of Mg²⁺ (●), Mn²⁺ (▲), or Ca²⁺ (■) concentration, using the results shown in Figs. 3 and 4.

Figure 7. Both fluoroquinolones and quinazolinones can lower the optimal Mg²⁺ concentration required for Topo IV-catalyzed DNA

The DNA cleavage assay was performed in the presence of 45 μ M CFX (●), 30 μ M moxifloxacin (●), 10 μ M 8-methyl-quinazoline-2,4-dione (UIJR1-048, ■), or in the absence of any drug (○) as described in Materials and Methods. Each assay was repeated three times. The average values are plotted as a function of Mg²⁺ concentration and error bars indicate standard deviations. The results for CFX shown in Fig. 3 are shown again in this figure for comparison.