MECHANISM OF QUINOLONE ACTION AND RESISTANCE IN BACTERIAL AND HUMAN TYPE II TOPOISOMERASES

By

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3’-(AM)P	3’-(aminomethyl)pyrrolidinyl
A	alanine
APP(NH)P	adenosine 5’-(β,γ-imido)triphosphate
Arg	arginine
ATP	adenosine triphosphate
bp	base pair
D	aspartic acid
DMSO	dimethylsulfoxide
DTT	dithiothreitol
E	glutamic acid
EDTA	ethylenediaminetetraacetic acid
F	phenylalanine
FQ	fluoroquinolone
Glu	glutamic acid
His	histidine
Ile	isoleucine
K	lysine
kDa	kilodalton
kDNA	kinetoplast DNA
L	leucine
Leu	leucine

Lys	lysine
M	methionine
Met	methionine
MWCO	molecular weight cut-off
NA	non-amino
Ni-NTA	nickel-nitrilotriacetic acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
R	arginine
RADAR	rapid approach to DNA adduct recovery
RT	room temperature
S	serine
SDS	sodium dodecyl sulfate
Ser	serine
TLC	thin layer chromatography
Tyr	tyrosine
V	valine
Val	valine
WT	wild-type
W	tryptophan
Y	tyrosine

CHAPTER I

INTRODUCTION

A wide variety of pharmaceuticals has been approved for use in the United States and throughout the world. These drugs are used to treat a number of maladies and afflictions, ranging from psychological disorders to cancer to bacterial infections. Some of these drugs are compounds that were found in nature and have a long history of use that pre-dates modern medicine. Many newer pharmaceuticals are either modified versions of compounds isolated from living organisms or were fully synthesized in the laboratory with the goal of identifying a compound with a specific bioactivity. Regardless of their origins, the mechanism(s) of action of many older drugs is not well understood. Quinolones are a class of synthetic antibacterials that fall into this category. Although their target enzymes – the bacterial type II topoisomerases, gyrase and topoisomerase IV – were discovered fourteen[1] and twenty-eight[2] years, respectively, after the first member of the class was introduced into the clinic,[3] much remains unknown about quinolone-topoisomerase interactions and the molecular mechanism(s) of quinolone action and resistance.

Quinolones

Nalidixic acid (which is actually a naphthyridine; Figure 1) is the founding member of the quinolone class of antibacterials and was one of the first completely synthetic antibacterials to be developed.[4] It was isolated as a byproduct of the synthesis

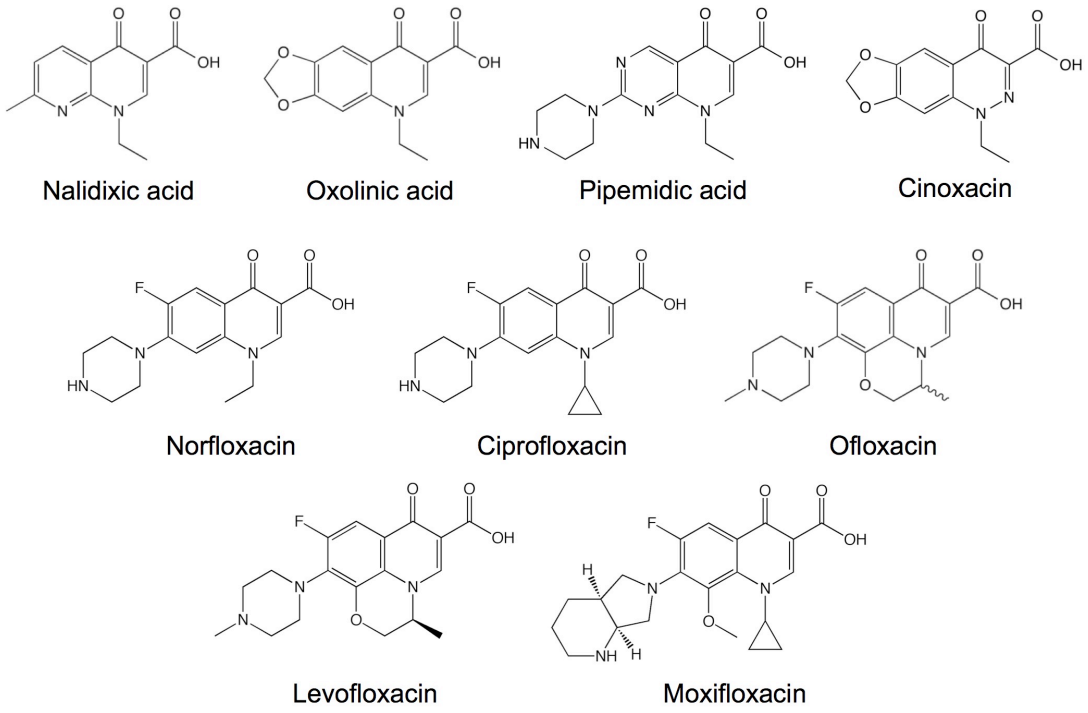


Figure 1. Quinolone structures. Nalidixic acid is the founding member of the quinolone class of antibacterials. Oxolinic acid, pipemidic acid, and cinoxacin are other first generation quinolones. Norfloxacin, ciprofloxacin, and ofloxacin are the most relevant second-generation quinolones. Levofloxacin (the levorotary isomer of ofloxacin) and moxifloxacin are newer generation quinolones.

of chloroquine (an antimalarial) in 1962 by George Lesher and colleagues.[5] Nalidixic acid was introduced into the clinic in the 1960s for the treatment of uncomplicated urinary tract infections caused by enteric bacteria.[3] By the 1970s, several additional first-generation quinolones, including oxolinic acid, pipemidic acid, and cinoxacin, had been synthesized and introduced into the clinic (Figure 1).[3,4,6-8]

In the early 1980s, a second generation of quinolones was developed.[3,4,6-8] These newer drugs spurred the transformation of the quinolones from a little-used drug class to the clinical mainstays that they are today. The second generation quinolones, highlighted by norfloxacin, ciprofloxacin, and ofloxacin, displayed considerably improved activity against gyrase, greater penetration into Gram-positive organisms, and enhanced pharmacokinetics and pharmacodynamics. The structural features that facilitated the improved activities were the introduction of a fluorine at the C6 position and a ring substituent (piperazine or methyl-piperazine) at C7 of the quinolone core (Figure 1).[3,4,6-8] Because of the inclusion of the fluorine, quinolones are often referred to as "fluoroquinolones."

Norfloxacin is considered to be the first broad-spectrum quinolone and was utilized to a far greater extent than nalidixic acid.[3,4,6-8] However, due to low serum levels and poor tissue penetration, norfloxacin was still restricted to the treatment of urinary tract infections and sexually transmitted diseases. Ciprofloxacin was the first quinolone that displayed significant activity outside of the urinary tract.[3,4,6-8] After more than twenty years in clinical use, ciprofloxacin remains one of the most commonly prescribed antibacterial drugs and is used to treat a variety of Gram-negative and, to a lesser extent, Gram-positive infections.[3,4,7,8]

The clinical success of ciprofloxacin spawned an array of newer generation quinolones that displayed even greater broad-spectrum activity, especially against Gram-positive respiratory tract infections. A number of these drugs have been approved for clinical use, with levofloxacin and moxifloxacin being two of the most important (Figure 1). The clinical success of levofloxacin can be partly attributed to its pharmacokinetics – treatment requires only a single pill per day.[7,9,10]

A number of diseases are currently treated with quinolones, including urinary tract infections and pyelonephritis, sexually transmitted diseases, prostatitis, skin and tissue infections, chronic bronchitis, community-acquired and nosocomial pneumonia, and intra-abdominal and pelvic infections.[11] Quinolones are also important in the treatment of multi-drug resistant tuberculosis, and moxifloxacin is in clinical trials as a front-line treatment for the disease.[12-16]

Bacterial Type II Topoisomerases

Nearly all species of bacteria encode two distinct (but homologous) type II topoisomerases, gyrase and topoisomerase IV.[17-23] Both of these enzymes are essential to the survival of bacterial cells. They play critical roles in most nucleic acid processes, help control levels of DNA under- and overwinding, and remove knots and tangles from the bacterial chromosome. Gyrase and topoisomerase IV modulate the topological state of DNA by passing an intact double helix through a transient double-stranded break that they generate in a separate segment of DNA.

The catalytic cycle of type II topoisomerases can be separated into six discrete steps (Figure 2). The enzyme begins by binding to DNA at helix-helix crossovers and

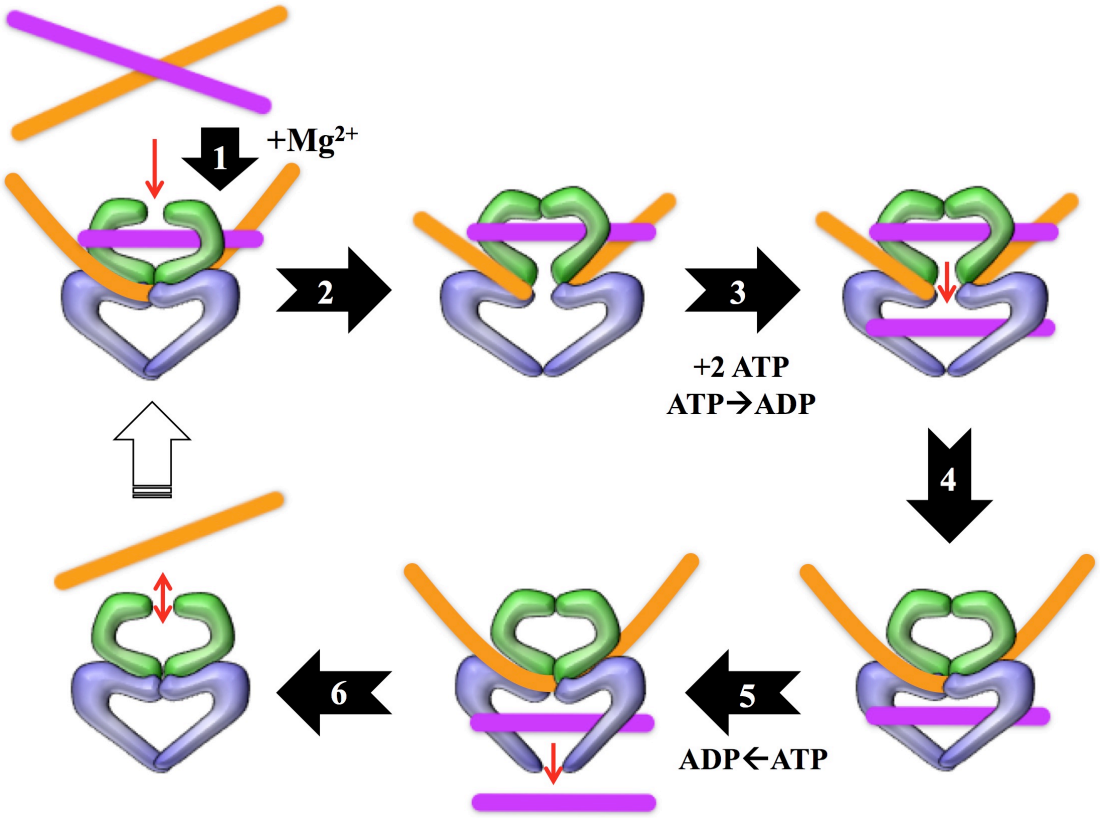


Figure 2. Catalytic cycle of type II topoisomerases. Step 1: The enzyme binds at helix-helix crossovers and bends the gate-, or G-, segment of DNA in the presence of divalent metal ions (the physiological ion is Mg^{2+}). Step 2: The enzyme cleaves and covalently attaches to the newly generated 5'-termini of the G-segment, generating the cleavage complex. Step 3: The enzyme hydrolyzes one molecule of ATP in order to pass the transfer-, or T-, segment of DNA through the cut it generated in the G-segment. Step 4: The enzyme religates the G-segment. Step 5: Upon hydrolysis of a second ATP molecule, the T-segment is released from the enzyme. Step 6: The enzyme releases the G-segment and resets for another round of catalysis.

sampling the genetic material for malleability. If the DNA is sufficiently flexible, the enzyme introduces an ~150° bend into the gate- (or G-) segment. The topoisomerase then cleaves and covalently attaches to the newly generated 5'-termini of the G-segment. Next, the transfer-segment (also called the T-segment) of DNA is passed through the double-stranded break in the G-segment. Following strand passage, the enzyme religates the DNA break that it created, releases both segments of DNA, and resets for another round of catalysis. Divalent metal ions (the physiological ion is Mg^{2+}) are required cofactors for DNA bending and the cleavage and religation reactions, and ATP binding and hydrolysis are required to drive progression through the catalytic cycle.[17-23]

The DNA cleavage and religation reactions, which constitute the core of topoisomerase function, utilize a non-canonical two-metal-ion mechanism.[24-26] Gyrase and topoisomerase IV generate staggered cuts in the DNA backbone that are 4-bp apart and on opposite strands (leaving a 5'-overhang). In order to maintain genomic integrity during this process, the enzymes form covalent bonds between active site tyrosine residues and the newly generated 5'-DNA termini.[17-19,22] These covalent enzyme-cleaved DNA complexes are known as "cleavage complexes" (Figure 2).

Both gyrase and topoisomerase IV are comprised of two distinct functional subunits and function as A_2B_2 heterotetramers (Figure 3).[17,19,22,23] The subunits in gyrase are GyrA and GyrB. In Gram-negative species, the genes encoding topoisomerase IV subunits were named as partitioning genes, while in Gram-positive species, they were named as gyrase-like genes.[17,19,27,28] Therefore, in topoisomerase IV, ParC (in Gram-negative species) and GrlA (in Gram-positive species) are equivalent to GyrA, and ParE and GrlB are equivalent to GyrB. The A subunit contains the active site tyrosine residue.

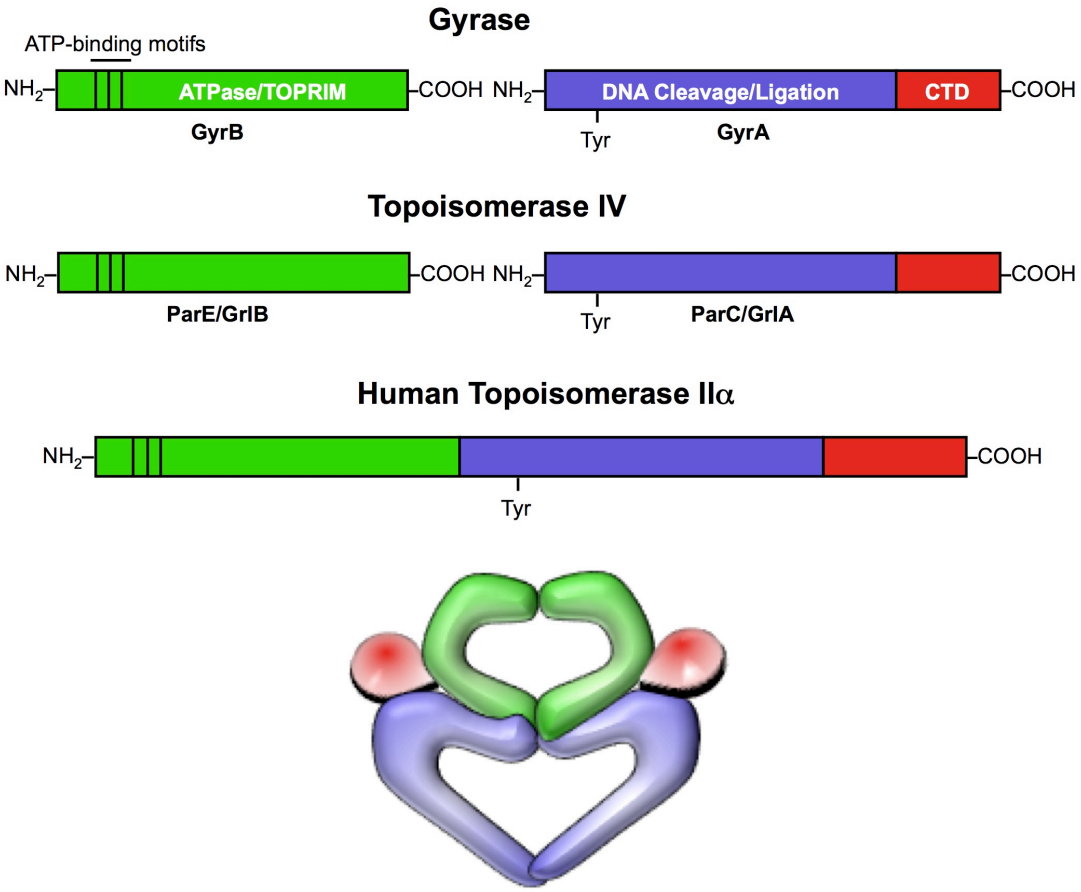


Figure 3. Domain structures of type II topoisomerases. Gyrase and topoisomerase IV are heterotetrameric enzymes consisting of two A subunits and two B subunits. The A subunits (blue and red; GyrA in gyrase and ParC/GrlA in Gram-negative/Gram-positive topoisomerase IV) contain the active site tyrosine residue that covalently attaches to the newly generated 5'-termini of DNA during the cleavage reaction. The C-terminal domains (CTD; red) of the A subunits are variable and allow gyrase to introduce negative supercoils into DNA. The B subunits (green; GyrB in gyrase and ParE/GrlB in Gram-negative/Gram-positive topoisomerase IV) contain the ATPase and TOPRIM domains, the latter of which binds the catalytic divalent metal ions essential for enzyme activity. Human topoisomerase IIα is homologous to the bacterial type II enzymes. However, during the course of evolution, the A and B subunits fused into a single polypeptide chain. Therefore, eukaryotic type II topoisomerases function as homodimers. A representation of the three-dimensional structure of type II topoisomerases is shown at the bottom.

The B subunit contains the ATPase domain as well as the TOPRIM domain, which binds the divalent metal ions involved in DNA cleavage and religation. Although there is strong sequence similarity between gyrase and topoisomerase IV, the C-termini of the A subunits are not well conserved. This portion of the protein is involved in topology recognition and allows gyrase, but not topoisomerase IV, to generate supercoils in DNA.[17,19,22,29-31]

Despite their mechanistic and structural similarities, gyrase and topoisomerase IV have separate physiological functions.[17,19,22,23] Gyrase is the only type II topoisomerase that can actively introduce negative supercoils into DNA. Consequently, the enzyme works in conjunction with the omega protein (a type I topoisomerase) to set the superhelical density of the bacterial chromosome. In addition, gyrase is primarily responsible for removing the torsional stress that accumulates in front of replication forks and transcription complexes.[17,19,22,23]

Topoisomerase IV appears to play a lesser role than gyrase in maintaining chromosomal superhelical density and alleviating torsional stress. Its major function is decatenating daughter chromosomes following replication and removing knots and tangles that accumulate in the bacterial chromosome as a result of fundamental cellular processes.[17,19,22,32,33]

Gyrase and Topoisomerase IV as Quinolone Targets

In order to carry out their critical physiological functions, gyrase and topoisomerase IV generate double-stranded breaks in the bacterial chromosome. Thus, while essential for cell survival, these enzymes have the potential to fragment the

genome.[17-19,22,23,34] Quinolones take advantage of this latter, and potentially lethal, characteristic and kill cells by increasing the concentration of enzyme-DNA cleavage complexes.[4,18,35-39] Thus, they are referred to as "topoisomerase poisons" because they convert gyrase and topoisomerase IV into cellular toxins.[35] In contrast, "catalytic inhibitors" block the overall catalytic functions of these enzymes without increasing levels of DNA strand breaks.

Quinolones bind non-covalently to gyrase and topoisomerase IV at the enzyme-DNA interface in the cleavage-religation active site.[40-43] Binding of the drugs in the cleavage complex is facilitated by interactions with both the protein and the DNA. Because quinolones insert into the DNA at both cleaved scissile bonds in an intercalative manner, they increase the steady-state concentration of cleavage complexes by acting as physical blocks to religation (Figure 4). Two drug molecules are required to increase levels of double-stranded DNA breaks because the scissile bonds on each strand are staggered.[4,18,36-39]

When replication forks, transcription complexes, or other DNA tracking systems collide with drug-stabilized gyrase- or topoisomerase IV-DNA cleavage complexes, these complexes are converted to permanent chromosomal breaks. The generation and accumulation of these DNA breaks trigger the SOS response and other DNA repair pathways. If the strand breaks overwhelm these processes, they can lead to cell death. This is the primary mechanism by which quinolones kill bacterial cells (Figure 5).[4,18,36-39]

Because quinolones stabilize cleavage complexes by inhibiting DNA religation, they also impair the overall catalytic functions of gyrase and topoisomerase IV. Thus, in addition to acting as poisons, quinolones can act as catalytic inhibitors. The

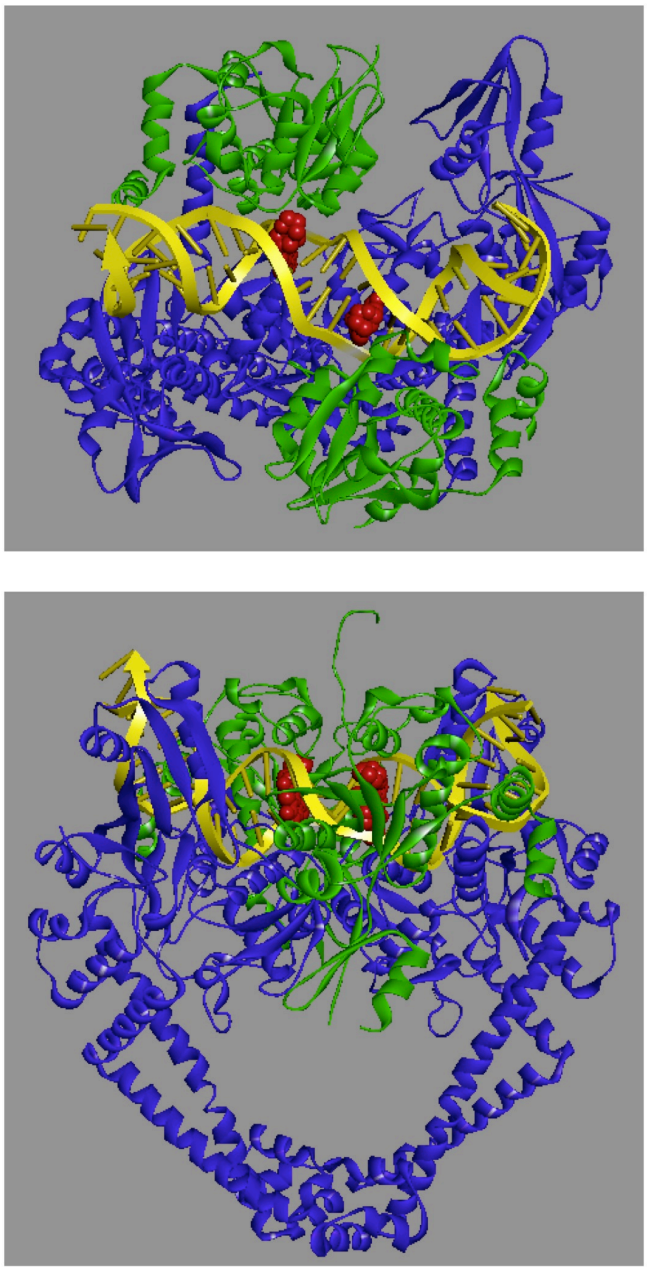


Figure 4. Crystal structure of a moxifloxacin-stabilized *Acinetobacter baumannii* topoisomerase IV-DNA cleavage complex. The catalytic core of the enzyme is shown. Moxifloxacin is shown in red, the topoisomerase IV A and B subunits are shown in blue and green, respectively, and DNA is shown in yellow. Top: A top view of the cleavage complex showing two quinolone molecules intercalating four base pairs apart at the sites of DNA cleavage. Bottom: A front view (rotated by 90° from the top view) of the cleavage complex. Protein Data Bank accession 2XKK was visualized using Discovery Studio 3.5 Visualizer (Accelrys Software Inc.). Adapted from Wohlkonig *et al*.[43]

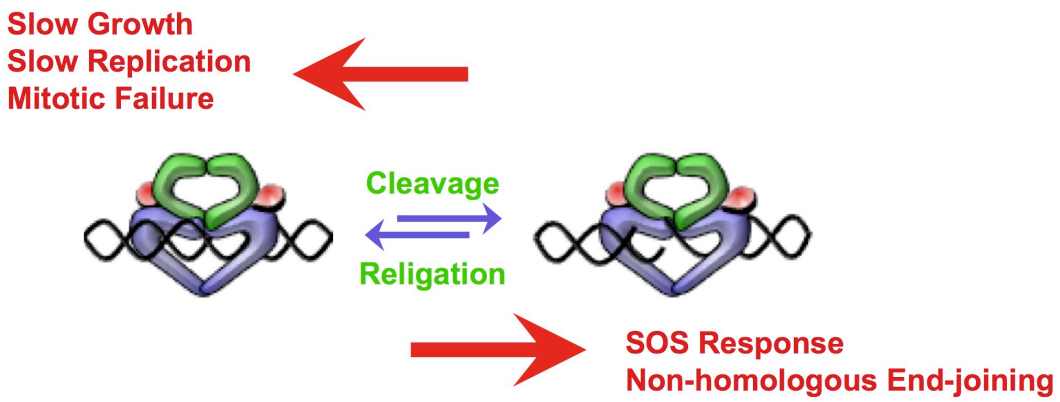


Figure 5. Bacterial type II topoisomerases are essential but potentially toxic enzymes. The balance between enzyme-mediated DNA cleavage and religation is critical for cell survival. If the level of gyrase-mediated DNA cleavage decreases, rates of DNA replication slow and impair cell growth (left). If the level of topoisomerase IV-mediated DNA cleavage decreases, cells are not able to untangle daughter chromosomes and ultimately die of mitotic failure (left). If the level of gyrase- or topoisomerase IV-mediated DNA cleavage becomes too high (right), the actions of DNA tracking systems can convert these transient complexes to permanent double-stranded breaks. The resulting DNA breaks initiate the SOS response and other DNA repair pathways and can lead to cell death.

accompanying loss of enzyme activity affects a number of nucleic acid processes and likely contributes to the overall toxicity of these drugs (Figure 5).[4,18,36-39]

Both gyrase and topoisomerase IV are physiologically relevant targets for quinolones. Gyrase was first identified as the cellular target of quinolones in 1977,[44,45] fifteen years after the introduction of these drugs into the clinic.[3] The later discovery (in 1990) of topoisomerase IV[2] raised the question of whether this enzyme is also a target of the quinolones. Based on an analysis of the minimum inhibitory concentration (MIC) of *Escherichia coli* strains carrying drug resistance mutations in gyrase, topoisomerase IV, or both enzymes, it was concluded that gyrase is the primary target for quinolones and that topoisomerase IV is a secondary drug target.[46] Consistent with this conclusion, quinolones are more potent against *E. coli* gyrase than topoisomerase IV[46] *in vitro* and induce higher levels of gyrase-DNA cleavage complexes in cells.[47]

Surprisingly, genetic studies in *Streptococcus pneumoniae* indicated that topoisomerase IV, rather than gyrase, was the primary cellular target for ciprofloxacin.[48] This led to the concept that gyrase is the primary target for quinolones in Gram-negative bacteria but that the opposite is true in Gram-positive species. However, subsequent studies (based on identifying which type II topoisomerase mutated first upon the treatment of bacterial cultures with quinolones) found that this paradigm does not hold in many cases. There are examples of Gram-positive bacteria in which gyrase is the primary target for quinolones. Furthermore, in a given bacterial species, different quinolones have been shown to have different primary targets.[49-51] Ultimately, the issue of quinolone targeting is still a matter of debate and the relative contributions of gyrase *vs*.

topoisomerase IV to quinolone action needs to be evaluated on a species-by-species and drug-by-drug basis.

Quinolone Resistance

Quinolone resistance has been steadily increasing since the 1990s.[11,52] This rise in resistance is threatening the clinical efficacy of the quinolones and has been observed in every bacterial species that is treated with this drug class.[8,11,39] A number of different quinolone resistance mechanisms have been reported and can be grouped into three categories: chromosome-mediated, plasmid-mediated, and target-mediated (Figure 6). The cellular alterations associated with each mechanism are not mutually exclusive and can accumulate to create strains that exhibit very high levels of quinolone resistance.

Chromosome-mediated Quinolone Resistance

The cellular concentration of quinolones is regulated by the opposing actions of diffusion-mediated drug uptake and pump-mediated efflux. In contrast to Gram-positive species, the outer membrane of Gram-negative bacteria poses an additional barrier that drugs must cross in order to enter the cell. Therefore, drug influx in Gram-negative species is facilitated by protein channels called porins. If the expression of porins is downregulated, it can lead to low-level resistance to quinolones (Figure 6).[4,52-55]

Enhanced expression of chromosome-encoded efflux pumps can also lead to quinolone resistance (Figure 6). Most commonly, the upregulation of these pumps is caused by mutations in regulatory proteins.[52,55,56] In general, changes in quinolone uptake and retention cause only low-level resistance and, in the absence of additional resistance

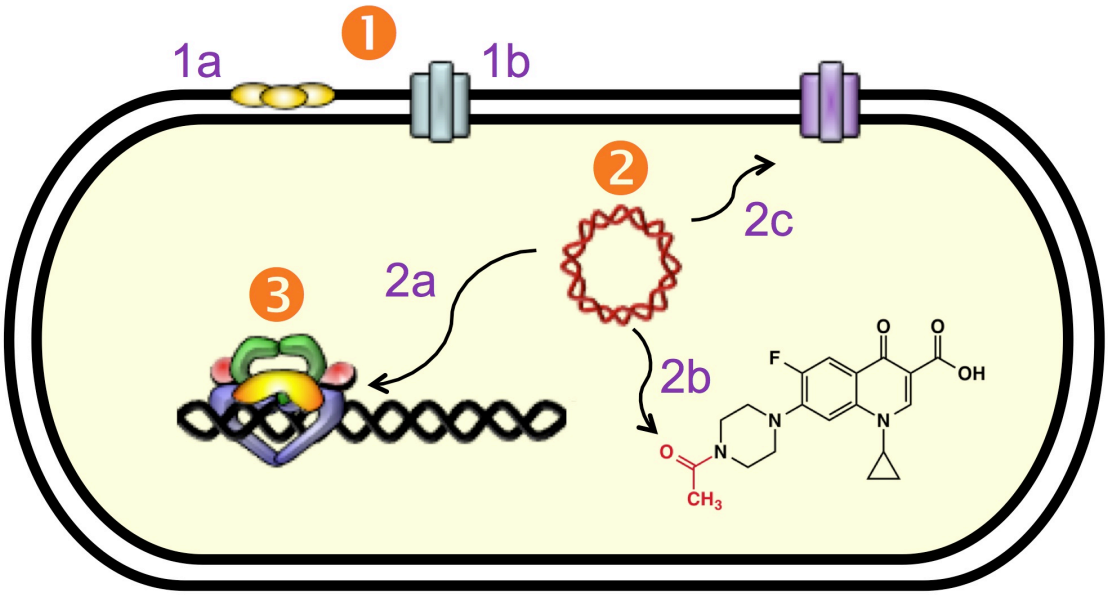


Figure 6. Mechanisms of quinolone resistance. 1. Chromosome-mediated resistance. 1a) Under-expression of porins in Gram-negative species decreases drug uptake. 1b) Over-expression of chromosome-encoded efflux pumps decreases drug retention in the cell. 2: Plasmid-mediated resistance. 2a) Qnr proteins (yellow) decrease topoisomerase-DNA binding and protect enzyme-DNA complexes from quinolones. 2b) Aac(6')-Ib-cr is an aminoglycoside acetyltransferase that acetylates the free nitrogen on the C7 ring of ciprofloxacin and norfloxacin, decreasing their effectiveness. 2c) Plasmid-encoded efflux pumps decrease the concentration of quinolones in the cell. 3: Target-mediated resistance. Mutations in gyrase and topoisomerase IV decrease quinolone-enzyme interactions.

mechanisms, do not appear to be a major clinical issue.[56] However, lowering the cellular concentration of quinolones creates a favorable background for other forms of resistance to develop and propagate.[57-59] In addition, these cellular changes can confer low levels of resistance to other antibacterial agents as these drugs also enter bacterial cells *via* diffusion and many efflux pumps are multi-drug and, thus, can eliminate a range of antibacterials from cells.[52,56]

Plasmid-mediated Quinolone Resistance

Recently, plasmids that carry quinolone resistance genes have been identified as an emerging clinical problem (Figure 6). Although the plasmid-encoded genes generally cause low-level (≤10-fold) resistance,[39,54,55,58-64] changes in quinolone sensitivity as high as ~250-fold has been reported.[39,54,55] Unlike chromosome-mediated resistance (and target-mediated resistance, discussed below), which is transmitted vertically from generation to generation, plasmid-mediated quinolone resistance can be transmitted horizontally (through bacterial conjugation) as well as vertically. Notably, plasmids that confer quinolone resistance typically carry additional genes that can cause resistance to other drug classes.[54,55,58,62]

Three families of genes are associated with plasmid-mediated quinolone resistance. The first are the Qnr genes, which encode small (~200 amino acid) pentapeptide repeat proteins.[54,55,65,66] Approximately 100 Qnr variants have been identified to date and have been classified into at least five distinct sub-families.[55,63,67,68] These proteins share homology with McbG and MfpA, which are DNA mimics.[54,55,65] The Qnr proteins appear to confer quinolone resistance by two different mechanisms.

Like McbG and MfpA, they decrease the binding of gyrase and topoisomerase IV to DNA. Thus, they protect cells from quinolones by lowering the number of available enzyme targets on the chromosome. They also bind to gyrase and topoisomerase IV and inhibit quinolones from entering cleavage complexes formed by the enzymes.[65,66,69,70]

The second plasmid-encoded protein associated with quinolone resistance is aac(6')-Ib-cr.[71,72] This protein is a variant of an aminoglycoside acetyltransferase that contains two specific point mutations, W102R and D179Y. This enzyme acetylates the unsubstituted nitrogen of the C7 piperazine ring of norfloxacin and ciprofloxacin, which decreases drug activity. Although both the wild-type and mutant aminoglycoside acetyltransferases are capable of acetylating other drugs, only the mutant enzyme is active against quinolones.[71,72]

The third group of plasmid-encoded quinolone resistance proteins is comprised of efflux pumps (that are distinct from those that are typically encoded on the bacterial chromosome). Thus far, three have been identified: OqxAB, QepA1, and QepA2.[55,73,74] Whereas the latter two proteins have been found in human bacterial infections, OqxAB is seen almost exclusively in animal infections.[55,75,76]

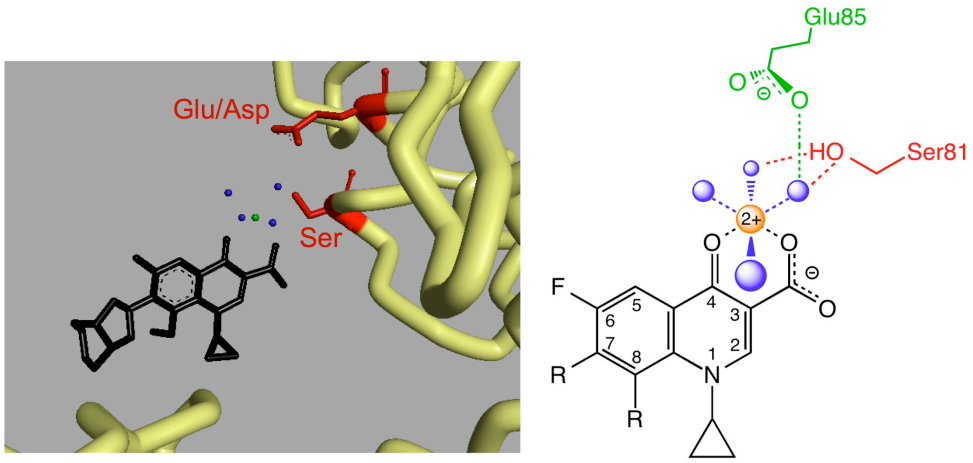
Target-mediated Quinolone Resistance

The most common form of quinolone resistance consists of point mutations in the target enzymes gyrase and topoisomerase IV (Figure 6). Generally, mutation of one type II enzyme confers ≤10-fold drug resistance. Selection for higher levels of resistance (~10- to 100-fold) usually yields strains with mutations in both enzymes.[18,36,37,39,49,77] Although mutations have been mapped throughout the A and B subunits of both gyrase and

topoisomerase IV in quinolone-resistant strains, the most commonly mutated amino acids are a specific, highly conserved serine residue (Ser83 by *E. coli* GyrA numbering) and an acidic residue that is located four positions downstream of the serine (Figure 7).[18,36,37,39,49,77-83] Typically, in both laboratory and clinical isolates, alterations at the serine comprise >90% of the mutant pool, with changes at the acidic residue comprising the bulk of the other mutations.[77,80-85]

The fact that specific mutations in gyrase and topoisomerase IV cause quinolone resistance strongly suggests that drug-protein interactions play an important role in stabilizing cleavage complexes. Thus, it has been assumed that these two amino acid residues play an integral role in mediating quinolone-enzyme interactions.

Recently, four crystal structures of quinolone-stabilized gyrase or topoisomerase IV cleavage complexes have been reported that shed light on quinolone-enzyme interactions.[40-43] Initial structures showed the quinolone bound to the enzyme near the serine and acidic resides, but the amino acids were not in close enough proximity to mediate drug binding.[40-42] However, a later structure captured a quinolone complex that contained a non-catalytic Mg^{2+} ion that was chelated by the C3/C4 keto acid of the drug (Figure 7).[43] The metal ion was coordinated by four water molecules, and two of these water molecules were shown to be stabilized by hydrogen bonds with the side chains of the highly conserved serine and acidic residues. Based on this structure and an earlier study that suggested a role for metal ions in quinolone action,[86] the authors suggested that this water-metal ion interaction "bridged" the drug to the enzyme and could account for why mutation of these residues results in quinolone resistance.[43]



Ab ParC	HPHGDSACYEAMVLM	93
Ab GyrA	HPHGDSAVYETIVRM	90
Ba GrlA	HPHGDSSVYEAMVRL	90
Ba GyrA	HPHGDSAVYETMVRM	94
Ec ParC	HPHGDSACYEAMVLM	89
Ec GyrA	HPHGDSAVYDTIVRM	92
Sa GrlA	HPHGDSSVYEAMVRL	89
Sa GyrA	HPHGDSSIYEAMVRM	93
Sp GrlA	HPHGDSSIYDAMVRM	88
Sp GyrA	HPHGDSSIYEAMVRM	90
hTIIα	YHHGEMSLMMTIINL	771
hTIIβ	YHHGEQALMMTIVNL	764

Figure 7. A water-metal ion bridge is proposed to facilitate quinolone-topoisomerase binding. Left: Crystal structure of a moxifloxacin-stabilized *A. baumannii* topoisomerase IV-DNA cleavage complex. Moxifloxacin is shown in black and the non-catalytic Mg^{2+} ion that is chelated by the C3/C4 keto acid of the quinolone and participates in the bridge interaction is shown in green. The four water molecules that fill out the coordination sphere of the Mg^{2+} ion are shown in blue. The backbone of selected portions of the protein amino acid chain is shown in yellow. The side chains of the serine and acidic residues that form hydrogen bonds with the water molecules in the water-metal ion bridge are shown in red. For clarity, DNA has been omitted from the picture. Protein Data Bank accession 2XKK was visualized using Discovery Studio 3.5 Visualizer (Accelrys Software Inc.). Adapted from Wohlkonig *et al.*[43] Right: Simplified diagram of the water-metal ion bridge. A generic quinolone is depicted in black, the non-catalytic Mg^{2+} is orange, water molecules are blue, and the coordinating serine and acidic residues (*B. anthracis* topoisomerase IV numbering) are red and green, respectively. Blue dashed lines indicate the octahedral coordination sphere of the divalent metal ion. The red or green dashed lines represent hydrogen bonds between the serine side chain hydroxyl group or the acidic residue side chain carboxyl group and the water molecules. Bottom: Sequence alignment of the A subunits showing the serine and acidic residues (red) that coordinate the water-metal ion bridge. Sequences of *A. baumannii* (*Ab*), *Bacillus anthracis* (*Ba*), *E. coli* (*Ec*), *Staphylococcus aureus* (*Sa*), and *S. pneumoniae* (*Sp*) gyrase (GyrA) and topoisomerase IV (ParC/GrlA) are shown. The homologous regions of human topoisomerase IIα (hTIIα) and IIβ (hTIIβ), which lack the residues necessary to coordinate the water-metal ion bridge interaction, are shown for comparison.

Quinolones and Human Type II Topoisomerases

Like bacteria, humans also express two type II topoisomerases, topoisomerase IIα and topoisomerase IIβ.[19,22,23,28,34] Both the α and β isoforms share significant amino acid sequence similarity with the bacterial enzymes. However, during the course of evolution, the genes encoding the A and B subunits fused, resulting in a single polypeptide chain (Figure 3). Consequently, the human type II enzymes function as homodimers.[19,22,28]

Topoisomerase IIα is essential to human cells, and its level of expression oscillates with the cell cycle.[22,34] Topoisomerase IIβ is essential during neuronal development, but appears to be dispensable once this development is complete. Unlike the α isoform, topoisomerase IIβ is expressed at a constant level throughout the cell cycle.[22,34] Both enzymes function through the same catalytic cycle described above for gyrase and topoisomerase IV (Figure 2) and maintain the genome by removing supercoils from the DNA, resolving knots and tangles that are introduced into the genome during normal cellular processes, and unlinking daughter chromosomes.[22,34]

The human type II topoisomerases are targeted by various anticancer chemotherapeutics, including etoposide, doxorubicin, and amsacrine (Figure 8). Similar to the quinolones, these drugs act as poisons, stabilizing cleavage complexes and inducing cell death by causing an accumulation of chromosome breaks that overwhelm DNA repair processes.[22,34] Although the clinically relevant quinolones do not display any appreciable activity against human type II topoisomerases, some experimental quinolones, including CP-115,953 and CP-115,955 (Pfizer), have high activity against the human (and bacterial) enzymes (Figure 8).[87-90] The basis for the discrimination between

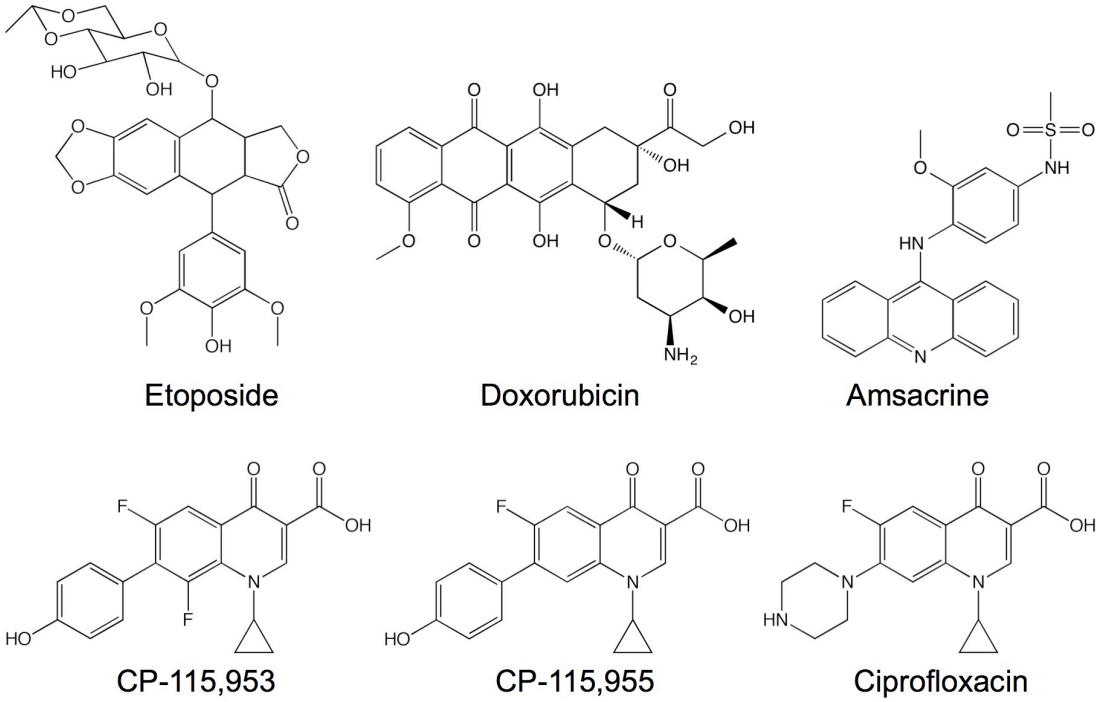


Figure 8. Structures of drugs that target human type II topoisomerases. Etoposide, doxorubicin, and amsacrine are important anticancer drugs. CP-115,953 and CP-115,955 are experimental quinolones that have high activity against both human and bacterial type II topoisomerases. Ciprofloxacin (a clinically relevant quinolone that is not active against human type II enzymes) is shown for comparison.

human and bacterial topoisomerases by the clinically relevant quinolones and the dual targeting of the enzymes by some experimental quinolones is not known.

Scope of the Dissertation

Due to the clinical significance of the quinolone class of antibacterials and the increasing prevalence of resistance that extends to every species of bacteria treated with these drugs, it is important to define and understand the molecular mechanisms of quinolone action and resistance. Therefore, the goals of this dissertation are to: further define quinolone-topoisomerase interactions in order to shed on light how quinolones stabilize cleavage complexes and inhibit religation; determine how mutations in gyrase and topoisomerase IV cause quinolone resistance; establish a basis for the discrimination between bacterial and human type II enzymes by clinically relevant quinolones and for the dual targeting of these enzymes by some experimental quinolones; and outline the roles of quinolone substituents so that this knowledge may used in designing new quinolones or quinolone-like drugs that overcome resistance without cross-reacting with the human system or that specifically target the human enzymes for potential use as anticancer chemotherapeutics.

An introduction to bacterial and human type II topoisomerases and the quinolone antibacterials is described in Chapter I. The materials and methods used in this dissertation are described in Chapter II.

Chapter III describes the characterization of the enzymatic activities of wild-type and quinolone-resistant mutant topoisomerase IV enzymes from *Bacillus anthracis*. Portions of these findings have been published[91,92] and demonstrate that mutations that

cause quinolone resistance do not alter the cleavage activities of the enzymes. Therefore, these mutations cause resistance by altering drug-enzyme interactions.

Chapter IV describes the effects of quinolones and a quinazolinedione on the enzymatic activities of wild-type and quinolone-resistant mutant topoisomerase IV enzymes from *B. anthracis*. Portions of these findings have been published[91,92] and demonstrate that quinolone resistance mutations decrease quinolone potency by reducing drug-enzyme interaction. In addition, these mutations also interfere with the ability of quinolones, but not quinazolinediones, to inhibit enzyme-mediated DNA religation and to form stable cleavage complexes.

Chapter V describes functional biochemical evidence supporting the water-metal ion bridge interaction between quinolones and bacterial type II topoisomerases that was identified in a crystal structure.[43] A portion of these findings have been published[91,92] and demonstrate that the water-metal ion bridge is the major point of interaction between the quinolones and topoisomerase IV. In addition, they show that the conserved serine and acidic residues act as the protein anchors of the bridge and explain how mutations at these residues cause quinolone resistance, namely by disrupting the water-metal ion bridge. Finally, these findings suggest that quinazolinediones interact with topoisomerases independently of a metal-ion bridge interaction and overcome quinolone resistance by establishing other contacts with the enzymes.

Chapter VI describes the characterization of wild-type and two quinolone-resistant mutant topoisomerase IV enzymes from *E. coli*. Results indicate that the mutations cause quinolone resistance by decreasing drug efficacy and, to a lesser extent, drug potency. In addition, the ability of a quinolone, but not a quinazolinedione, to inhibit

enzyme-mediated DNA religation and form stable cleavage complexes is reduced. Moreover, it appears that quinolone interactions with *E. coli* topoisomerase IV are mediated through the water-metal ion bridge.

Chapter VII describes the evaluation of the structure-activity relationships of three series of quinolones, quinazolinediones, and non-amino-quinazolinediones that are based on ciprofloxacin, moxifloxacin, and an experimental quinazolinedione containing a C7 3'-(aminomethyl)pyrrolindinyl group. The majority of this work has been published[93] and demonstrates that, as a whole, quinazolinediones are poorer drugs than their quinolone counterparts because they are essentially quinolones that lack their most important interaction (*i.e.*, the water-metal ion bridge) with the enzymes. Therefore, quinazoliendiones that overcome quinolone resistance caused by mutations in gyrase and topoisomerase IV do so by forming additional interactions with the enzymes through their C7 (and other) substituents. Furthermore, clinically relevant quinolones do not cross-react with the human type II topoisomerases because these enzymes cannot support the water-metal ion bridge interaction. For this reason, the C7 groups that facilitate interactions with the bacterial type II enzymes can also form interactions with the human enzymes, resulting in compounds with undesirable cross-reactivity. However, the C8 and N3 substituents modulate activity against human type II enzymes to a much greater extent than they do with the bacterial enzymes. As a result, three compounds have been identified that maintain high activity against quinolone-resistant mutant *B. anthracis* topoisomerase IV but do not show cross-reactivity with human topoisomerase IIα.

Chapter VIII describes the evaluation of the structure-activity relationships of a series of quinolones, quinazolinediones, and non-amino-quinazolinediones based on CP-

115,955, an experimental quinolone with high activity against both human and bacterial type II topoisomerases. Results indicate that CP-115,955-based compounds have high activity against human topoisomerase IIα because the enzyme has a very specific interaction with the C7 4'-hydroxyphenyl moiety of the drug. The relative activities of the compounds *in vitro* are also reflected in the intracellular context. Thus, it may be possible to develop certain quinolones as anticancer chemotherapeutics. The C7 4'-hydroxyphenyl group does not appear to facilitate strong interactions with *B. anthracis* topoisomerase IV, as these compounds (like those based on the clinically relevant quinolones ciprofloxacin and moxifloxacin) do not have high activity against quinolone-resistant mutant topoisomerase IV enzymes. This observation demonstrates that there are differences between the human and bacterial enzymes that can be distinguished by the C7 group and provides hope that a moiety can be identified that selectively targets the wild-type and quinolone-resistant mutant bacterial enzymes.

Concluding remarks on the research presented in this dissertation are provided in Chapter IX.

CHAPTER II

METHODS

Materials

Enzymes

B. anthracis topoisomerase IV enzymes were purified using the procedure of Dong *et al*.[94] with modifications. Genes encoding wild-type *B. anthracis* GrlA and GrlB were individually PCR-amplified from *B. anthracis* Sterne 34F2 chromosomal DNA and cloned into the pET15b (Novagen) expression vector, which provided an N-terminus 6×His tag. Mutant quinolone-resistant $GrlA^{S81F}$, $GrlA^{S81Y}$, $GrlA^{E85K}$, $GrlA^{E85A}$, $GrlA^{S81F/E85K}$, $GrlA^{S81F/E85A}$, and $GrlA^{V96A}$ were generated by QuikChange (Stratagene) site-directed mutagenesis of the wild-type expression vector. Recombinant subunits were individually expressed in an *E. coli* BL21(DE3) ΔslyD strain. Cells were lysed by resuspension in CelLytic B (Sigma) containing protease inhibitors (Roche Complete Protease Inhibitor Cocktail, EDTA-free) and one passage through a French press at 20,000 psi. The cell lysate was centrifuged at 20,000 × *g* for 30 min at 4°C to remove debris. The cleared lysate was incubated with 2 mL Ni-NTA agarose beads (Qiagen) for 1 h at 4°C with end-over-end rotation. Following batch binding, the beads were pelleted by centrifugation at 100 × *g* at 4°C and were then washed with a buffer containing 1 M NaCl, 60 mM imidazole, and 20 mM Tris-HCl (pH 7.9) for 30 min at 4°C with end-over-end rotation. Next, the beads underwent a series of four 1-min washes with a buffer

containing 500 mM NaCl, 60 mM imidazole, and 20 mM Tris-HCl (pH 7.9). Finally, the beads were loaded into a column, and the proteins were eluted at 4°C with 12 mL of a buffer containing 500 mM NaCl, 1 M imidazole, and 20 mM Tris-HCl (pH 7.9). Twelve 1-mL fractions were collected, and fractions 2-11 were combined, injected into a 20kDa MWCO Slide-a-Lyzer dialysis cassette (Thermo), and dialyzed against 200 mM NaCl and 50 mM Tris-HCl (pH 7.5) at 4°C for 4 hours. The proteins were then dialyzed overnight at 4°C into 200 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 20% glycerol and were subsequently stored at -80°C. Protein concentrations were determined by A_{280} measurements, and the quality and purity of the subunits was determined by Coomassie staining following separation on a 7.5% SDS-PAGE gel. In all assays, topoisomerase IV was used as a 1:1 mixture of GrlA:GrlB.

E. coli topoisomerase IV enzymes (wild-type ParC, wild-type ParE, $ParC^{S80L}$, and $ParC^{E84K}$) were expressed and purified as previously described.[95,96]

Human topoisomerase IIα (wild-type and mutant) was expressed in *Saccharomyces cerevisiae* as previously described.[97] The wild-type enzyme was expressed from YEpWob6 and purified as previously described.[98] A modified YEpWob6 vector containing the wild-type human topoisomerase IIα gene and a 6×His tag at the C-terminus[99] was obtained from Dr. Joseph Deweese (Lipscomb University). Mutations in the His-tagged gene were generated by QuikChange Lightning (Agilent) site-directed mutagenesis using the primer pair 5'-GTCTTCTTATCATCATGGTGAGTCGTCACTA ATGGAGACCATTATCAATTTGGC and 5'-GCCAAATTGATAATGGTCTCCATTA GTGACGACTCACCATGATGATAAGAAGAC (mutated positions are underlined). The mutated gene encoding the M762S and M766E mutations was sequenced for

accuracy using the primer 5'-GGTTTGAAACCAGGTCAGAG. Yeast cells containing human topoisomerase IIα$^{M762S/M766E}$ were lysed in Y-PER Plus (Thermo) containing 1 mM β-mercaptoethanol and protease inhibitors as described by the manufacturer. Unless otherwise noted, the following purification steps were carried out at 4°C. After addition of 500 mM NaCl, the lysate was centrifuged for 10 min at 19000 × *g*. The cleared lysate was subjected to batch binding to 2 mL Ni-NTA agarose beads (Qiagen) for 30 min. The beads were then washed for 30 min in wash buffer 1 [1 M NaCl, 30 mM imidazole, 20 mM Tris-HCl (pH 7.9), 1 mM β-mercaptoethanol, and protease inhibitors] followed by four 1-min washes at RT in wash buffer 2 (wash buffer 1 containing 500 mM NaCl rather than 1 M NaCl). Mutant human topoisomerase IIα was eluted with 12 mL of elution buffer [500 mM NaCl, 1 M imidazole, 20 mM Tris-HCl (pH 7.9), 1 mM β-mercaptoethanol, and protease inhibitors], collecting 1-mL fractions. Fractions 2-11 were injected into a 20kDa MWCO Slide-a-Lyzer dialysis cassette (Thermo) and dialyzed for 4 hours into dialysis buffer 1 [750 mM KCl, 50 mM Tris-HCl (pH 7.7), 100 μM EDTA, and 500 μM DTT] and for 16 hours into dialysis buffer 2 (dialysis buffer 1 containing 40% glycerol). The purified protein was stored at -80°C. Protein concentration was determined by Bradford analysis, and the quality and purity of the enzyme was determined by Coomassie staining following separation on a 7.5% SDS-PAGE gel. 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*.[98] (Figure 9).

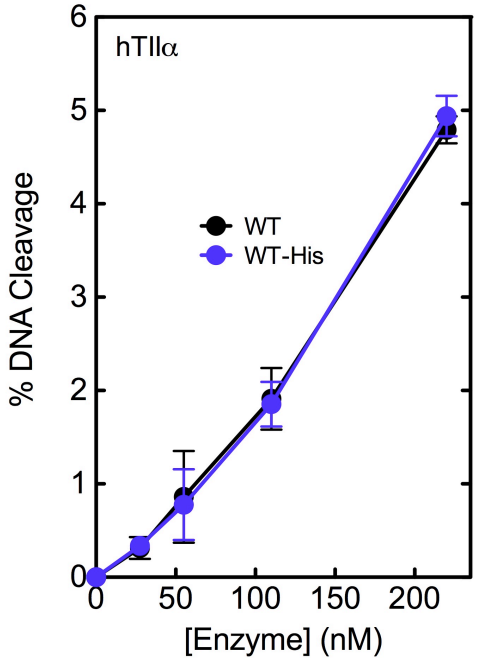


Figure 9. DNA cleavage activities of His-tagged (WT-His) and untagged (WT) wild-type human topoisomerase IIα. Cleavage levels were assessed in the absence of drugs with 5 mM Ca^{2+}, rather than Mg^{2+}, as the divalent metal ion. Error bars represent the standard deviation of three independent experiments.

DNA Substrates

Negatively supercoiled pBR322 plasmid DNA was prepared from *E. coli* using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Kinetoplast DNA (kDNA) was isolated from *Crithidia fasciculata* as previously described.[100] Relaxed pBR322 plasmid DNA was generated by treatment with topoisomerase I for 30 min as previously described,[101] followed by phenol-chloroform-isoamyl alcohol extraction, ethanol precipitation, and resuspension in 5 mM Tris-HCl (pH 8.5) and 500 μM EDTA. Linear radiolabeled pBR322 was prepared as follows. Plasmid pBR322 DNA was linearized by treatment with *Hin*dIII. Terminal 5'-phosphates were removed by treatment with calf intestinal alkaline phosphatase and replaced with [^{32}P]phosphate using T4 polynucleotide kinase and [γ-^{32}P]ATP. The DNA was treated with *Eco*RI, and the 4,332 bp singly-end-labeled fragment was purified from the small *Eco*RI-*Hin*dIII fragment by passage through a CHROMA SPIN+TE-100 column (Clontech).

Quinolones and Quinazolinediones

Ciprofloxacin was obtained from LKT Laboratories, stored at -20°C as a 40 mM stock solution 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 levofloxacin, sparfloxacin, and norfloxacin were obtained from Sigma-Aldrich. All other quinolones and quinazolinediones were synthesized using established methods as previously described.[102-107] A list of these compounds can be found in Table 1. 8-Methyl-cipro, 8-methoxy-cipro, CP-115,955, 8-methyl-955, and 8-methoxy-955 were stored at -20°C as 40 mM stock solutions in 0.1 N NaOH and diluted five-fold with 10 mM

Table 1. Full chemical, library, and abbreviated names of compounds used in this work.

Name	Library name	Chemical name
Cipro-dione	UIHS-IIa-249	3-amino-7-(1-piperazinyl)-1-cyclopropyl-6-fluoro-2,4(1*H*,3*H*)-quinazolinedione
8-Methyl-cipro	UILI-2-89	1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid
8-Methyl-cipro-dione	UILI-2-87	3-amino-7-(1-piperazinyl)-1-cyclopropyl-6-fluoro-8-methyl-2,4(1*H*,3*H*)-quinazolinedione
8-Methyl-cipro-NA-dione	UILI-2-75	7-(1-piperazinyl)-1-cyclopropyl-6-fluoro-8-methyl-2,4(1*H*,3*H*)-quinazolinedione
8-Methoxy-cipro	UIHS-IIa-101	1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid
8-Methoxy-cipro-dione	UIHS-IIa-253	3-amino-7-(1-piperazinyl)-1-cyclopropyl-6-fluoro-8-methoxy-2,4(1*H*,3*H*)-quinazolinedione
Moxi-dione	UIHS-IIa-251	3-amino-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-8-methoxy-2,4(1*H*,3*H*)-quinazolinedione
8-Methyl-moxi	UIHS-IIa-45	1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-b]pyridin-6-yl]-4-oxo-3-quinolinecarboxylic acid
8-Methyl-moxi-dione	UILI-2-81	3-amino-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-8-methyl-2,4(1*H*,3*H*)-quinazolinedione
8-Methyl-moxi-NA-dione	UILI-2-83	7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-8-methyl-2,4(1*H*,3*H*)-quinazolinedione
8-H-moxi	UIHS-IIa-239	1-Cyclopropyl-6-fluoro-1,4-dihydro-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-b]pyridin-6-yl]-4-oxo-3-quinolinecarboxylic acid
8-H-moxi-dione	UIHS-IIa-247	3-amino-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-2,4(1*H*,3*H*)-quinazolinedione
8-Methyl-3'-(AM)P-FQ	UIHS-I-303	1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-[(3*S*)-3-(aminomethyl)-1-pyrrolidinyl]-4-oxo-3-quinolinecarboxylic acid
8-Methyl-3'-(AM)P-dione	UIJR-1-048	3-amino-7-[(3*S*)-3-(aminomethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-8-methyl-2,4(1*H*,3*H*)-quinazolinedione
8-Methyl-3'-(AM)P-NA-dione	UILI-2-97	7-[(3*S*)-3-(aminomethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-8-methyl-2,4(1*H*,3*H*)-quinazolinedione
8-Methoxy-3'-(AM)P-FQ	UING-5-249	1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[(3*S*)-3-(aminomethyl)-1-pyrrolidinyl]-4-oxo-3-quinolinecarboxylic acid

Table 1. (*Continued*)

Name	Library name	Chemical name
8-Methoxy-3'-(AM)P-dione	UING-5-207	3-amino-7-[(3*S*)-3-(aminomethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-8-methoxy-2,4(1*H*,3*H*)-quinazolinedione
8-H-3'-(AM)P-FQ	UIHS-IIIa-35	1-Cyclopropyl-6-fluoro-1,4-dihydro-7-[(3*S*)-3-(aminomethyl)-1-pyrrolidinyl]-4-oxo-3-quinolinecarboxylic acid
8-H-3'-(AM)P-dione	UIHS-IIa-245	3-amino-7-[(3*S*)-3-(aminomethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-2,4(1*H*,3*H*)-quinazolinedione
CP-115,953	CP-115,953	1-Cyclopropyl-6,8-difluoro-1,4-dihydro-7-(4-hydroxyphenyl)-4-oxo-3-quinolinecarboxylic acid
CP-115,955	CP-115,955	1-Cyclopropyl-6-fluoro-1,4-dihydro-7-(4-hydroxyphenyl)-4-oxo-3-quinolinecarboxylic acid
955-dione	UILI-2-61	3-amino-7-(4-hydroxyphenyl)-1-cyclopropyl-6-fluoro-2,4(1*H*,3*H*)-quinazolinedione
955-NA-dione	UILI-2-63	7-(4-hydroxyphenyl)-1-cyclopropyl-6-fluoro-2,4(1*H*,3*H*)-quinazolinedione
8-Methyl-955	UIHS-IIIa-23	1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(4-hydroxyphenyl)-4-oxo-3-quinolinecarboxylic acid
8-Methyl-955-dione	UILI-2-297	3-amino-7-(4-hydroxyphenyl)-1-cyclopropyl-6-fluoro-8-methyl-2,4(1*H*,3*H*)-quinazolinedione
8-Methyl-955-NA-dione	UILI-2-257	7-(4-hydroxyphenyl)-1-cyclopropyl-6-fluoro-8-methyl-2,4(1*H*,3*H*)-quinazolinedione
8-Methoxy-955	UIHS-IIIa-27	1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(4-hydroxyphenyl)-4-oxo-3-quinolinecarboxylic acid
8-Methoxy-955-dione	UILI-3-11	3-amino-7-(4-hydroxyphenyl)-1-cyclopropyl-6-fluoro-8-methoxy-2,4(1*H*,3*H*)-quinazolinedione
8-Methoxy-955-NA-dione	UILI-2-289	7-(4-hydroxyphenyl)-1-cyclopropyl-6-fluoro-8-methoxy-2,4(1*H*,3*H*)-quinazolinedione

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 DMSO.

Other Materials

[γ-^{32}P]ATP (~6000 Ci/mmol) and [γ-^{32}P]ATP (~3000 Ci/mmol) were obtained from PerkinElmer. Histone H1 was obtained from Boehringer Mannheim. Plastic-backed 20×20 cm polyethyleneimine-impregnated cellulose F thin layer chromatography (TLC) plates were obtained from EMD Chemicals. Rabbit anti-human topoisomerase IIα antibody was obtained from Abcam. Donkey anti-rabbit antibody (IRDye 680 LT) for use with the Li-Cor Odyssey system was obtained from Li-Cor. Nitrocellulose membrane was acquired from PerkinElmer.

Bacillus anthracis Topoisomerase IV Assays

DNA Relaxation

DNA relaxation assays were based on the protocol of Fortune and Osheroff.[108] Reaction mixtures (20 μL) contained 18.75 nM wild-type or mutant 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 0-30 min by the addition of 3 μL of stop solution [0.77% SDS and 77.5 mM EDTA]. 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 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 AlphaInnotech digital imaging system. The percent relaxed DNA was determined by the loss of supercoiled DNA substrate.

Kinetoplast DNA Decatenation

Decatenation assays were carried out by the procedure of Anderson *et al.*[109] Reaction mixtures (20 μL) contained 18.75 nM wild-type or mutant topoisomerase IV and 0.3 μg kinetoplast DNA (kDNA) in relaxation buffer and were incubated at 37°C. Decatenation was stopped at times ranging from 0-30 min by the addition of 3 μL of stop solution. 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 100 mM Tris-borate (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 decatenated DNA was determined by the appearance of monomeric circular DNA molecules.

Plasmid DNA Catenation

Catenation assays were based on the protocol of Fortune and Osheroff.[110] Reactions (20 μL) contained 50 nM wild-type or mutant topoisomerase IV and 5 nM relaxed pBR322 in relaxation buffer containing 25 mM NaCl (rather than 50 mM) and supplemented with 5 μg/mL histone H1 and were incubated at 37°C. Catenation was stopped at times ranging from 0-30 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 100 mM Tris-borate (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 or the appearance of catenated products retained in the wells (both yielded similar results).

Non-turnover catenation reactions contained 200 nM wild-type or mutant topoisomerase IV and substituted 1 mM APP(NH)P for ATP. Prior to electrophoresis, reactions were treated with proteinase K (2 μL of a 0.8 mg/mL solution) at 45°C for 45 min to digest the enzyme.

ATP Hydrolysis

ATP hydrolysis assays were carried out as described by Kingma *et al.*[111] Reactions (20 μL) contained 100 nM wild-type or mutant topoisomerase IV and 85 nM negatively supercoiled pBR322 in relaxation buffer supplemented with 150 nM [γ-^{32}P]ATP and were incubated at 37°C. Reactions carried out in the absence of DNA contained 500 nM wild-type or mutant topoisomerase IV. Reactions were terminated at 0-14 min by spotting 2 μL on a TLC plate. Standards were spotted at the top of each plate and consisted of 1, 2, and 3 μL of a 50-fold diluted reaction. Plates were developed in 400 mM NH_4HCO_3. Following air-drying, plates were covered in plastic wrap and exposed for 30 min to a K screen. The K screen was visualized and hydrolyzed phosphate was quantified using a Bio-Rad Molecular Imager FX.

Plasmid DNA Cleavage

DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff.[108] Reactions contained 75 nM wild-type or mutant topoisomerase IV and 10 nM negatively supercoiled pBR322 in a total of 20 μL of cleavage buffer [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 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 topoisomerase IV in the absence of drugs substituted 10 mM $CaCl_2$ for 10 mM $MgCl_2$ in the cleavage buffer. Ca^{2+} supports higher levels of topoisomerase IV-mediated DNA cleavage in the absence of drugs, thereby facilitating quantification of basal enzyme activity. It should be noted that Ca^{2+} does not significantly alter the reversibility or the stability of cleavage complexes as compared to Mg^{2+}.

Assays that assessed the DNA cleavage activities of the wild-type and mutant enzymes in the presence of drugs contained 0-500 μM of the indicated compound. For assays that monitored competition between two drugs, the compounds were added simultaneously to reaction mixtures, and the final concentration of the compounds is

indicated. In these competition assays, the level of cleavage seen with the corresponding concentration of the competing drug in the absence of the drug held at a constant concentration across the assay was used as a baseline and was subtracted from the cleavage level seen in the presence of both compounds. 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 $NiCl_2$. The final concentration of these alternative metal ions in the reactions is indicated in the relevant figures.

DNA Cleavage Site Utilization

DNA cleavage sites were mapped using a modification[112] of the procedure of O'Reilly and Kreuzer.[113] Reaction mixtures contained 50 nM wild-type or mutant topoisomerase IV and 1 nM radiolabeled pBR322 DNA substrate in 50 μL of DNA cleavage buffer in the absence or presence of compounds. Reaction mixtures were incubated at 37°C for 10 min, and enzyme-DNA cleavage complexes were trapped by the addition of 5 μL of 5% SDS followed by 3 μL of 250 mM EDTA (pH 8.0). Proteinase K (5 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45°C for 45 min to digest the enzyme. DNA products were precipitated with ethanol and resuspended in 5 μL of polyacrylamide gel loading buffer [10% agarose gel loading buffer, 80% formamide, 100 mM Tris-borate (pH 8.3), and 2 mM EDTA]. Samples were subjected to electrophoresis in denaturing 6% polyacrylamide sequencing gels. Gels were dried *in vacuo*, and DNA cleavage products were visualized with a Bio-Rad Molecular Imager FX.

DNA Religation

DNA religation assays were carried out using the procedure of Robinson and Osheroff.[114] Reactions (20 μL) contained 75 nM wild-type or mutant topoisomerase IV and 10 nM negatively supercoiled pBR322 in cleavage buffer containing 5 mM $MgCl_2$ (rather than 10 mM). Reactions carried out in the presence of drug contained the indicated compound at the concentration listed. Reactions carried out in the absence of drug substituted 1 mM $CaCl_2$ for 5 mM $MgCl_2$. Initial DNA cleavage/religation equilibria were established at 37°C for 10 min. Religation was initiated by rapidly shifting the reaction temperature from 37 to 75°C. The shift to high temperature allows enzyme-mediated religation but prevents new rounds of DNA cleavage from occurring. Thus, it results in a unidirectional sealing of the cleaved DNA. Reactions were stopped at times ranging from 0-135 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 described above for plasmid cleavage assays. Levels of DNA cleavage were set to 100% at time zero, and religation was determined by the loss of linear cleavage 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.*[115] Initial reaction mixtures contained 375 nM wild-type or mutant topoisomerase IV, 50 nM DNA, and 20 μM (for the wild-type enzyme) or 200 μM (for the mutant enzymes) ciprofloxacin or 20 μM 8-methyl-3'-(AM)P-dione in a total of 20 μL of DNA cleavage buffer. Reactions 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 0-300 min, and DNA cleavage was stopped with 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 plasmid cleavage assays. Levels of DNA cleavage were set to 100% at time zero, and the persistence of cleavage complexes was determined by the decay of the linear reaction product over time.

Escherichia coli Topoisomerase IV Assays

Assays utilizing *E. coli* topoisomerase IV were carried out as described above for *B. anthracis* topoisomerase IV with the following modifications. All assays utilizing wild-type or $ParC^{S80L}$ topoisomerase IV contained 10 nM enzyme. All assays utilizing $ParC^{E84K}$ topoisomerase IV contained 25 nM enzyme. Cleavage buffer contained 2.5 mM $MgCl_2$ (rather than 10 mM). In some reactions, 2.5 mM $CaCl_2$ was substituted for the $MgCl_2$. Religation assays carried out in the absence of drugs did not substitute $CaCl_2$ for $MgCl_2$. The persistence of cleavage complexes was measured from 0-30 min, and initial reactions contained 50 nM wild-type or $ParC^{S80L}$ topoisomerase IV.

Human Topoisomerase IIα Assays

Plasmid DNA Cleavage

DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff.[108] 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 EDTA, 25 μM DTT, and 2.5% (v/v) glycerol. Reactions that compared the effects of drugs on wild-type and mutant M762S/M766E topoisomerase IIα contained 110 nM enzyme and 10 nM negatively supercoiled pBR322 plasmid DNA in a total of 20 μL of 10 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 140 mM KCl, 20 μM EDTA, 90 μM DTT, 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 IIα activity. Reaction mixtures were incubated at 37°C for 10 min and processed as described above for topoisomerase IV plasmid DNA cleavage.

Assays that monitored competition between two drugs were carried out as described above for topoisomerase IV.

Persistence of Topoisomerase IIα-DNA Cleavage Complexes

Persistence of topoisomerase IIα cleavage complexes was assessed as described above for topoisomerase IV. Initial reactions contained 550 nM enzyme and 20 μM CP-115,955 or 200 μM 955-dione. Following a 25-fold dilution, the persistence of cleavage complexes was measured from 0-1 min.

Intracellular Cleavage Complex Formation

Levels of topoisomerase IIα cleavage complexes formed in human CEM cells upon drug treatment were determined using the rapid approach to DNA adduct recovery (RADAR) assay[116] with modifications. Cells were grown in RPMI 1640 + L-glutamine

culture medium supplemented with 10% bovine growth serum. 600,000 cells were seeded at a density of 150,000 cells/mL in 6-well cell culture plates. Following a 24 h incubation at 37°C, cells were treated with drugs for 1 h at 37°C. Cells were harvested by centrifugation (500 × *g* for 5 min at RT) and lysed with 4 M guanidine thiocyanate, 20 mM Tris-HCl, 20 mM EDTA, 2% Triton X-100, 1% Sarkosyl, 1% DTT, and 100 mM NaOAc, at pH 6.5 (personal communication with Kostantin Kiianitsa, University of Washington). Lysates were sheared with four passages through a 21-gauge needle and ethanol precipitated as described[116] for at least 20 min. Precipitated DNA was pelleted by centrifugation at 18,000 × *g* for 20 min at 4°C. Pellets were washed and resuspended in 8 mM NaOH as described.[116] DNA solutions were subjected to at least eight shearing passes through a 21-gauge needle. Levels of DNA-topoisomerase IIα cleavage complexes were determined by immunoblotting for topoisomerase IIα on 0.5 μg genomic DNA. Immunoblots were visualized and quantified using the Li-Cor Odyssey system.

CHAPTER III

ENZYMATIC ACTIVITIES OF WILD-TYPE AND MUTANT QUINOLONE-RESISTANT TOPOISOMERASE IV FROM *BACILLUS ANTHRACIS*

Introduction

Quinolones kill bacterial cells by increasing levels of double-stranded DNA breaks generated by the bacterial type II topoisomerases gyrase and topoisomerase IV.[4,18,35-39] However, the molecular mechanism by which quinolones interact with and stabilize cleavage complexes is not well understood. A recent structural study has suggested that quinolones interact with the bacterial type II enzymes through a water-metal ion interaction that "bridges" the drug to the protein through a serine and an acidic residue.[43] These two residues are also the most commonly mutated amino acids in quinolone-resistant bacterial strains.[18,36,37,39,49,77-83]

To further our understanding of quinolone-topoisomerase interactions and how specific mutations in the enzyme result in quinolone resistance, it is necessary to first characterize the enzymatic activities of wild-type and mutant quinolone-resistant topoisomerase IV in the absence of drugs. Topoisomerase mutations could cause quinolone resistance by altering drug-enzyme interactions, which could decrease drug efficacy and/or drug potency. Alternatively, mutations could affect the enzyme directly by decreasing its concentration in the cell, its stability, and/or its level of activity. While mutations that affect drug-enzyme interactions could be overcome by designing new drugs, resistance that is caused by the latter effects on the enzyme cannot.

Topoisomerase IV from *B. anthracis* was used as the model to characterize the basal (*i.e*. in the absence of drugs) activity levels of wild-type and quinolone-resistant mutant enzymes. The mutant topoisomerases that were evaluated include $GrlA^{S81F}$, $GrlA^{S81Y}$, $GrlA^{E85K}$, and $GrlA^{V96A}$. The Ser81 and Glu85 mutant enzymes contain alterations at the amino acid residues suggested to participate in the water-metal ion bridge interaction (Figure 7) identified in the crystallographic study[43] discussed earlier. These two residues are highly conserved across bacterial species and are the residues most commonly mutated in quinolone-resistant strains.[18,36,37,39,49,77-83] The Val96 mutant enzyme contains an alteration at an amino acid located on the same α helix (specifically, helix α4) as Ser81 and Glu85, but it is not in close enough proximity to the drug binding pocket to participate in the water-metal ion bridge interaction that is proposed to facilitate quinolone-topoisomerase binding. Val96 mutations are observed much less frequently than Ser81 or Glu85 mutations in drug-resistant clinical and laboratory strains.[18,36,37,39,49,77-85] Characterization of the enzymatic activities of these enzymes will provide information regarding the nature of the most commonly observed quinolone resistance mutations.

Results and Discussion

Characterization of the Catalytic Activities of Wild-type and Quinolone-Resistant Mutant Topoisomerase IV

The cellular functions of topoisomerase IV include the removal of DNA supercoils and the unlinking of daughter chromosomes. These catalytic activities were assessed *in vitro* by evaluating the ability of the enzyme to relax negatively supercoiled

plasmid DNA and decatenate the mini- and maxi-circles that make up kinetoplast DNA (kDNA).

GrlA^{S81F} and GrlA^{S81Y}, as well as GrlA^{V96A}, topoisomerase IV relax DNA at approximately the same rate as the wild-type enzyme (Figure 10). The Ser81 mutants can also decatenate kDNA at a rate that is identical to that of wild-type topoisomerase IV (Figure 10). Therefore, these mutations of Ser81 and Val96 do not affect the catalytic activity of the enzyme. In contrast, GrlA^{E85K} displays a significantly decreased rate of DNA relaxation as compared to the wild-type enzyme. A similar decrease in catalytic activity has been reported for the equivalent Glu→Lys mutation in *E. coli* topoisomerase IV.[117] This finding raised the possibility that GrlA^{E85K} topoisomerase IV displays quinolone resistance because it has low overall activity. Therefore, the Glu85 mutant enzyme was further evaluated to shed light on the underlying basis of its decreased catalytic rate.

First, the ability of GrlA^{E85K} topoisomerase IV to hydrolyze ATP was characterized. In the absence of DNA, the basal rates of ATP hydrolysis catalyzed by the mutant and wild-type enzymes were indistinguishable (Figure 11). In the presence of DNA, the hydrolysis rate of the mutant enzyme was slightly slower than that of wild-type (Figure 11), but was not sufficiently impaired to account for the large difference in the rate of DNA relaxation discussed above. Thus, the impaired overall activity of GrlA^{E85K} does not appear to reflect an altered interaction with the ATP cofactor.

Second, the ability of GrlA^{E85K} topoisomerase IV to carry out catalytic and non-turnover DNA catenation was assessed. As expected based on the DNA relaxation results, the mutant enzyme catalyzed DNA catenation (in the presence of ATP) at a rate

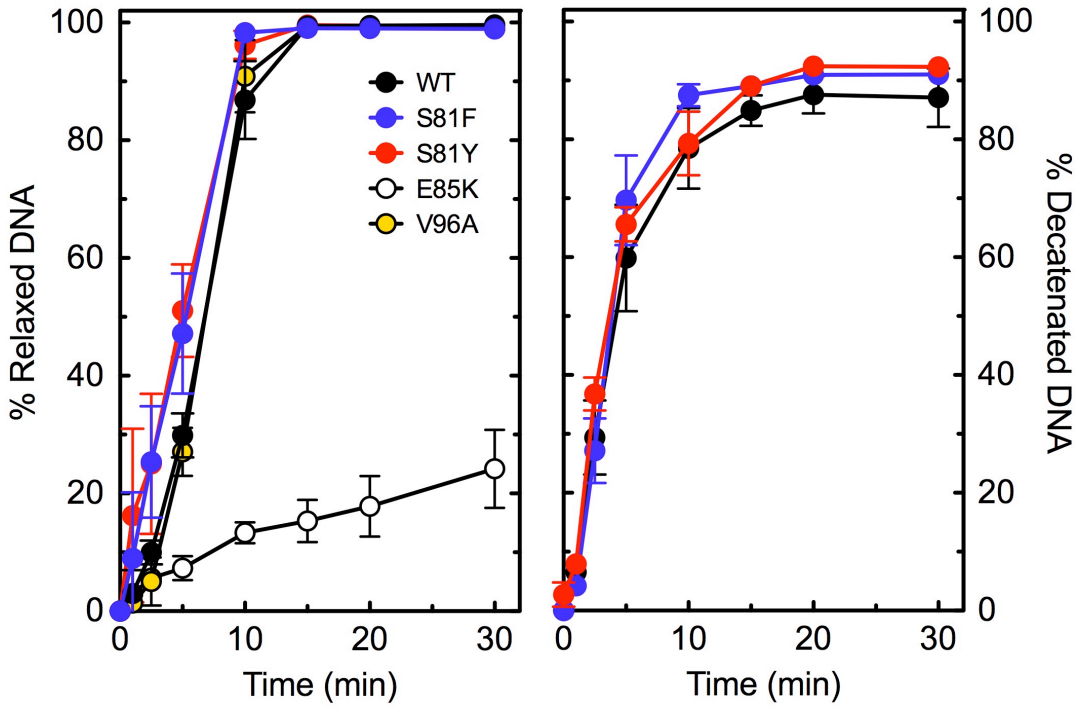


Figure 10. Catalytic activities of wild-type and quinolone-resistant mutant *B. anthracis* topoisomerase IV enzymes. The ability of wild-type, GrlA^{S81F}, GrlA^{S81Y}, GrlA^{E85K}, and GrlA^{V96A} topoisomerase IV to relax negatively supercoiled pBR322 plasmid DNA is shown on the left. The ability of wild-type, GrlA^{S81F}, and GrlA^{S81Y} topoisomerase IV to decatenate kinetoplast DNA is shown on the right. Error bars represent the standard deviation of at least three independent experiments.

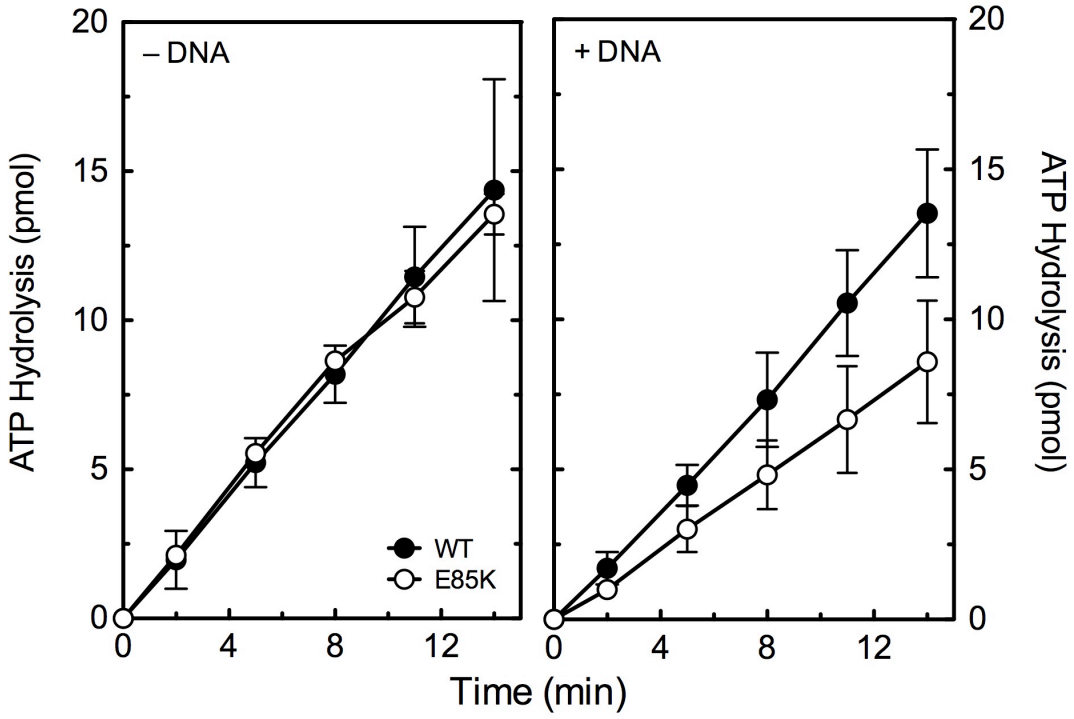


Figure 11. ATP hydrolysis catalyzed by wild-type and GrlA^{E85K} topoisomerase IV from *B. anthracis*. The abilities of the enzymes to hydrolyze ATP in the absence (left) or presence (right) of negatively supercoiled pBR322 plasmid DNA are shown. Error bars represent the standard deviation of at least three independent experiments.

that was much slower than that of the wild-type enzyme (Figure 12). Non-turnover reactions utilized APP(NH)P, a non-hydrolyzable ATP analog that allows each topoisomerase IV enzyme to carry out only one DNA strand passage event. In contrast to wild-type topoisomerase IV, GrlA^{E85K} displayed very little ability to catalyze DNA strand passage under non-turnover conditions (Figure 12). Therefore, the decreased catalytic rate of the mutant enzyme likely occurs at a step that precedes enzyme turnover.

Characterization of the DNA Cleavage Activities of Wild-type and Quinolone-Resistant Mutant Topoisomerase IV

Quinolones kill bacterial cells primarily by increasing levels of DNA cleavage mediated by gyrase and topoisomerase IV.[4,18,35-39] Because the cleavage step occurs early in the catalytic cycle of the enzymes and does not depend on ATP,[17-23] mutations can have a different effect on enzyme-mediated DNA cleavage than they do on catalytic processes, such as relaxation or decatenation. Therefore, even if a mutant enzyme has altered catalytic activity, it may retain a wild-type level of cleavage activity, which is the function that is important for quinolone action. Thus, the ability of wild-type, GrlA^{S81F}, GrlA^{S81Y}, GrlA^{E85K}, and GrlA^{V96A} topoisomerase IV to cleave DNA was examined.

Although the DNA cleavage reaction is ATP independent, a divalent metal ion is required for the enzyme to cut the double-helix.[17-26] The cleavage reaction of *B. anthracis* topoisomerase IV could be supported by Mg^{2+}, Ca^{2+}, and Mn^{2+} (Figure 13, inset). As observed for other prokaryotic and eukaryotic type II enzymes,[26,118-120] high levels of DNA scission were seen in reactions that contained Ca^{2+}. A number of other divalent metal ions (Ba^{2+}, Sr^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Pb^{2+}) and even a few trivalent metal

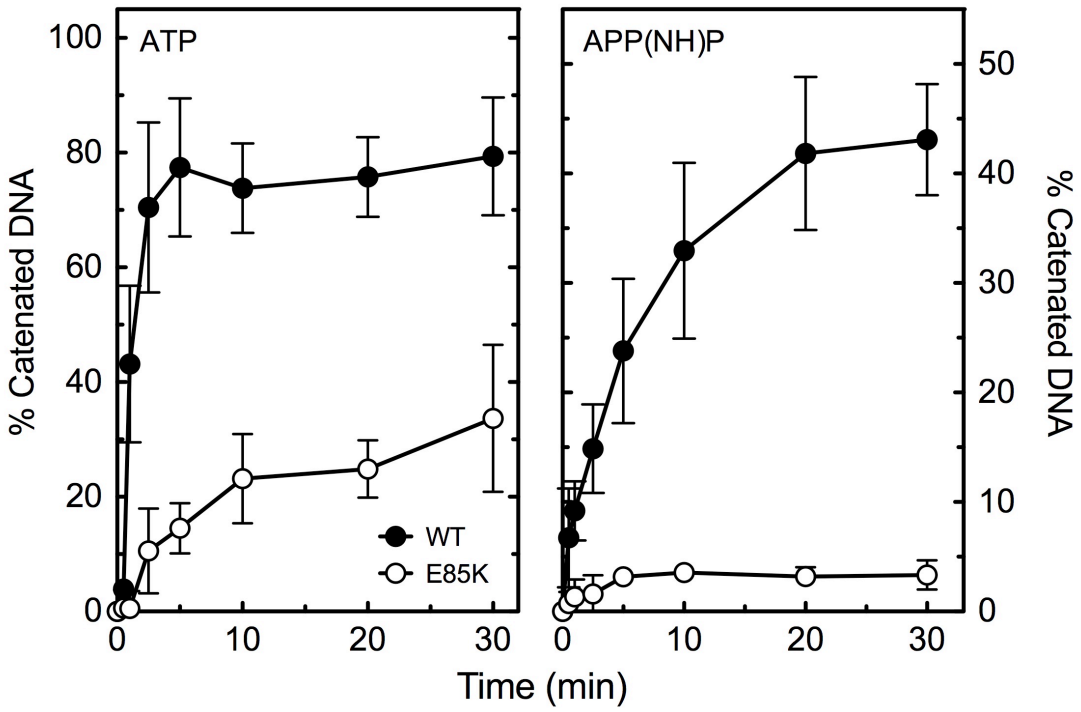


Figure 12. Catalytic and non-turnover catenation mediated by wild-type and GrlA^{E85K} topoisomerase IV from *B. anthracis*. The ability of the enzymes to catalyze catenation of relaxed pBR322 plasmid DNA is shown on the left. The ability of the enzymes to carry out strand passage is shown on the right. These "non-turnover" assays utilized the non-hydrolyzable ATP analog APP(NH)P and relaxed pBR322 plasmid DNA to observe a single DNA strand passage (*i.e.*, catenation) event. Error bars represent the standard deviation of at least three independent experiments.

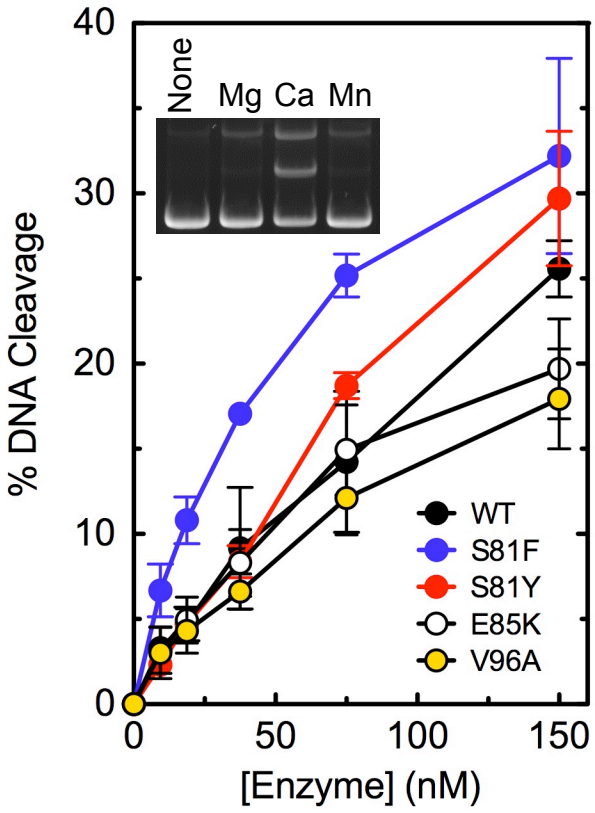


Figure 13. DNA cleavage activities of wild-type, GrlA^{S81F}, GrlA^{S81Y}, GrlA^{E85K}, and GrlA^{V96A} topoisomerase IV from *B. anthracis*. The ability of the enzymes to cleave negatively supercoiled pBR322 plasmid DNA in the absence of drugs is shown. Assays were carried out in the presence of 10 mM $CaCl_2$. Error bars represent the standard deviation of at least three independent experiments. The inset shows an agarose gel of a typical DNA cleavage assay mediated by wild-type topoisomerase IV in the absence of divalent metal ion (None) or in the presence of 10 mM Mg^{2+}, Ca^{2+}, or Mn^{2+}.

ions (Al^{3+}, Eu^{3+}, and Tb^{3+}) could also support varying levels of enzyme-mediated DNA cleavage (not shown).

Because Ca^{2+} supported much higher (and, thus, more readily quantifiable) levels of DNA cleavage than did Mg^{2+}, it was used in assays that assessed the ability of the enzymes to cleave DNA in the absence of drugs. As seen with DNA relaxation and decatenation, $GrlA^{S81F}$, $GrlA^{S81Y}$, and $GrlA^{V96A}$ topoisomerase IV retained wild-type levels of activity (Figure 13). In contrast to results seen with the catalytic assays, the $GrlA^{E85K}$ mutant enzyme also cleaved plasmid DNA with an activity that was similar to that of wild-type topoisomerase IV (Figure 13). This latter finding suggests that the steps of the catalytic cycle of $GrlA^{E85K}$ that precede the strand passage event [enzyme-DNA binding, DNA bending, and DNA cleavage[17-19,22,27,28,34,121]] are relatively unaffected in the mutant enzyme and implies that the Glu85→Lys mutation decreases the overall catalytic activity of *B. anthracis* topoisomerase IV by impairing the DNA strand passage event. Furthermore, it provides strong evidence that quinolone resistance in $GrlA^{E85K}$, as well as $GrlA^{S81F}$, $GrlA^{S81Y}$, and $GrlA^{V96A}$, topoisomerase IV is not caused by a general loss of activity and likely reflects altered drug-enzyme interactions.

Summary of Conclusions

Bacillus anthracis $GrlA^{S81F}$, $GrlA^{S81Y}$, and $GrlA^{V96A}$ mutant topoisomerase IV retain wild-type levels of both catalytic and cleavage activities. However, the $GrlA^{E85K}$ enzyme displays a significantly impaired catalytic ability, while at the same time retaining wild-type levels of cleavage activity. In addition, this enzyme hydrolyzes ATP at a similar rate to wild-type topoisomerase IV, both in the absence and presence of

DNA. Finally, in non-turnover catenation reactions, the GrlA^{E85K} mutant shows very little ability to link plasmid DNA molecules. Taken together, these results indicate that the Glu→Lys mutation likely decreases the catalytic activity of the enzyme by impairing strand passage. Moreover, all of these mutations appear to cause quinolone resistance by altering drug-enzyme interactions.

CHAPTER IV

EFFECTS OF QUINOLONES AND A QUINAZOLINEDIONE ON WILD-TYPE AND MUTANT QUINOLONE-RESISTANT TOPOISOMERASE IV FROM *BACILLUS ANTHRACIS*

Introduction

The most clinically significant form of quinolone resistance is caused by point mutations in the topoisomerase enzymes targeted by these drugs.[18,36,37,39,49,77] A specific serine and acidic residue, located four positions downstream of the serine, are the residues that are most frequently mutated in resistant clinical and laboratory strains.[18,36,37,39,49,77-83] (In *B. anthracis* topoisomerase IV, these residues are Ser81 and Glu85.) Both of these amino acids are highly conserved across bacterial species. Although the involvement of these amino acid residues in quinolone resistance has been known for well over a decade, the mechanistic basis by which their alteration leads to resistance remains an enigma.

A recent crystallographic study suggested that quinolones bind to type II topoisomerases through a metal ion chelated by the C3/C4 keto acid moiety of the drug.[43] In this structure, the metal ion was stabilized by four water molecules, two of which were coordinated by the serine and acidic residues. The authors suggested that this interaction was important for quinolone-enzyme binding and that mutation of either the serine or acidic residue could cause quinolone resistance by disrupting this interaction.[43] As a first step toward testing this idea and shedding light on the molecular mechanism of quinolone action and resistance, the effects of quinolones on wild-type, $GrlA^{S81F}$, $GrlA^{S81Y}$,

GrlA^{V96A}, and GrlA^{E85K} topoisomerase IV were evaluated. For comparison, the activity of a quinazolinedione against these enzymes was also examined. Quinazolinediones have been to shown to overcome quinolone resistance caused by topoisomerase IV and gyrase mutations.[91-93,105,107,122-124] These compounds are structurally similar to quinolones, but they lack the C3/C4 keto acid necessary to chelate metal ions. Thus, they presumably maintain activity against mutant enzymes by functioning independently of bridging water-metal ion interactions.

Results and Discussion

Effects of Quinolones and a Quinazolinedione on DNA Cleavage Mediated by Wild-Type and Mutant Quinolone-Resistant Topoisomerase IV

Quinolones kill bacteria by increasing the number of topoisomerase-DNA cleavage complexes in the cell. These normally transient breaks are converted to permanent strand breaks that can overwhelm DNA repair processes and ultimately lead to cell death.[4,18,35-39] To begin exploring how quinolones interact with topoisomerases, a number of clinically relevant quinolones[39] – ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin, and sparfloxacin – were tested for their ability to enhance DNA cleavage mediated by wild-type topoisomerase IV (Figure 14). Ciprofloxacin was the most potent quinolone and, together with levofloxacin, was the most efficacious. Because these two drugs are also the most frequently prescribed members of the class, they were then tested with GrlA^{S81F} and GrlA^{S81Y} topoisomerase IV.

As predicted from quinolone resistance studies with *B. anthracis* cultures[77,84,85] and previous studies with gyrase and topoisomerase IV from other bacterial species that

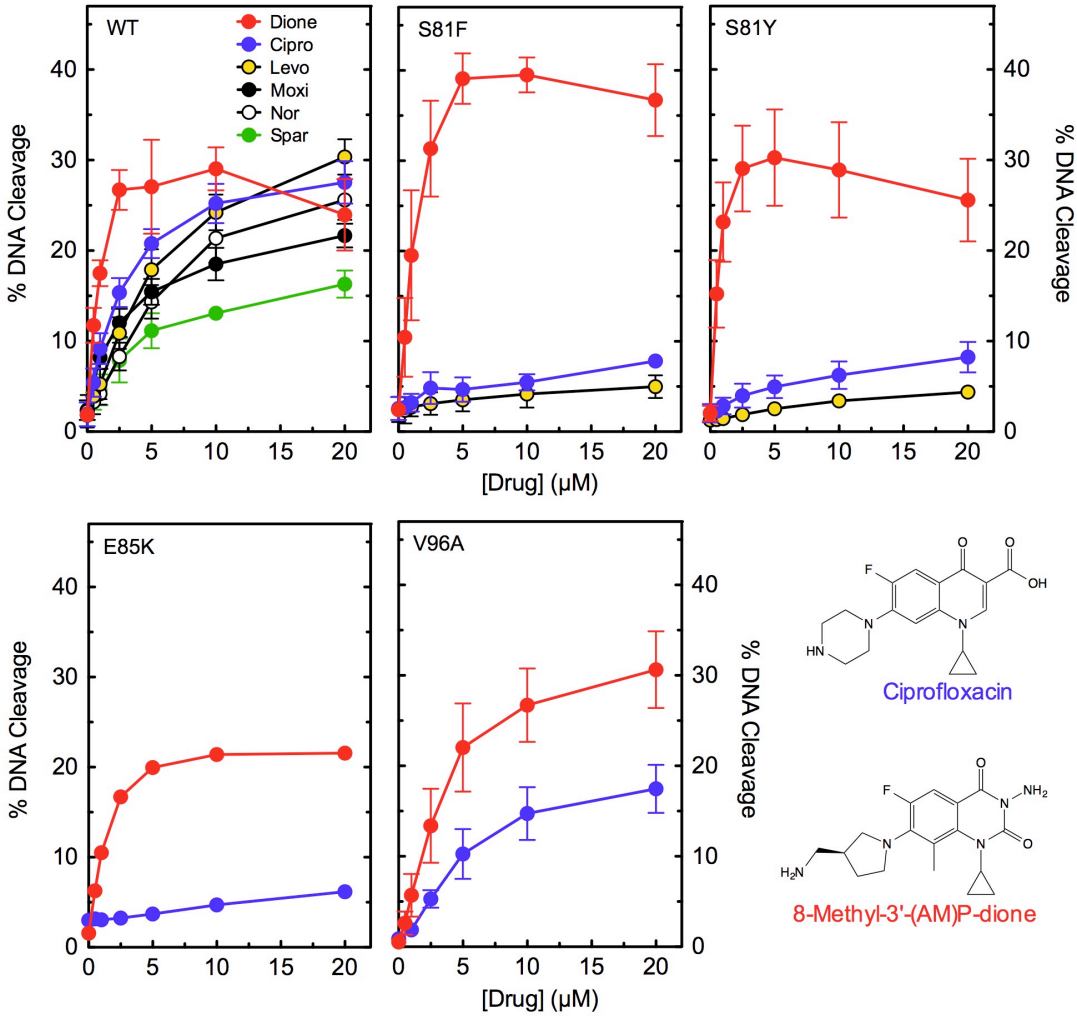


Figure 14. Effects of quinolones and 8-methyl-3'-(AM)P-dione on the DNA cleavage activities of wild-type, GrlA^{S81F}, GrlA^{S81Y}, GrlA^{E85K}, and GrlA^{V96A} topoisomerase IV from *B. anthracis*. Results with ciprofloxacin and 8-methyl-3'-(AM)P-dione are shown for all enzymes. Results with levofloxacin are shown for wild-type, GrlA^{S81F}, and GrlA^{S81Y}. Results with norfloxacin, moxifloxacin, and sparfloxacin are shown for wild-type. The structures of ciprofloxacin and 8-methyl-3'-(AM)P-dione are also shown. Error bars represent the standard deviation of at least three independent experiments.

carry mutations at this serine residue,[78,119,123-125] ciprofloxacin and levofloxacin displayed decreased activity against GrlA^{S81F} and GrlA^{S81Y} topoisomerase IV (Figure 14). The quinolone concentration required to triple levels of enzyme-mediated DNA cleavage (CC_3) rose ~27- to 42-fold with GrlA^{S81F} topoisomerase IV (as compared to the wild-type enzyme) and ~10- to 17-fold with GrlA^{S81Y} topoisomerase IV (Table 2).

Because ciprofloxacin displayed slightly higher activity than levofloxacin against both serine mutants, it was also tested with GrlA^{E85K} and GrlA^{V96A} topoisomerase IV. The GrlA^{E85K} mutant enzyme displayed significant resistance to ciprofloxacin (Figure 14). The CC_3 (~43 μM) rose ~80-fold as compared to wild-type (~0.5 μM) (Table 2). The GrlA^{V96A} mutant enzyme showed a much lower level of resistance to ciprofloxacin than did the other mutant enzymes (Figure 14). The CC_3 was ~2.5-fold higher against this mutant enzyme than against the wild-type enzyme (Table 2). Because the V96A mutation is located away from the proposed water-metal ion interaction discussed above, it is not surprising that this mutant enzyme retains more sensitivity to the quinolone than do the topoisomerases possessing mutations in the amino acids suggested to facilitate quinolone-enzyme interactions through this mechanism.

To determine the effects of the mutations on the efficacy of quinolone action, maximal levels of DNA cleavage were obtained by extending the drug concentration range (Figure 15). Levels of DNA cleavage observed with the mutant enzymes at high concentrations of ciprofloxacin approached those seen at lower drug concentrations with wild-type topoisomerase IV. Similar results were observed for levofloxacin and moxifloxacin (Figure 15). Drug efficacy (*i.e.*, the maximal level of DNA cleavage) ranged from 0.61 to 1.2 for the various mutant topoisomerase IV enzymes as compared to

Table 2. Potency[a] and efficacy values of ciprofloxacin, levofloxacin, moxifloxacin, and 8-methyl-3'-(AM)P-dione against wild-type, GrlA^{S81F}, GrlA^{S81Y}, GrlA^{E85K}, and GrlA^{V96A} topoisomerase IV from *B. anthracis*.

	Cipro		**Levo**		**Moxi**		**Dione**	
Enzyme	CC_3, µM	Max. clvg., %	CC_3, µM	Max. clvg., %	CC_3, µM	Max. clvg., %	CC_3, µM	Max. clvg., %
WT	0.53	30.0	1.45	30.4	—	23.5	0.20	29.0
S81F	22.3 (42.0)[b]	26.9 (0.90)	38.8 (26.7)	29.3 (0.96)	—	25.0 (1.07)	0.30 (1.5)	39.5 (1.36)
S81Y	9.10 (17.2)	23.4 (0.78)	15.0 (10.3)	23.5 (0.77)	—	22.4 (0.95)	0.18 (0.90)	30.3 (1.04)
E85K	42.8 (80.8)	18.3 (0.61)	—	26.6 (0.87)	—	28.0 (1.20)	0.35 (1.75)	21.6 (0.74)
V96A	1.30 (2.45)	20.0 (0.67)	—	—	—	—	0.25 (1.25)	35.0 (1.20)

[a]CC_3, the concentration of drug required to triple the percent cleavage observed in the absence of drug, is used as an indicator of potency.

Values in parentheses are a relative comparison of mutant values to wild-type values, as calculated by dividing the mutant value by the wild-type value.

— Indicates value was not determined.

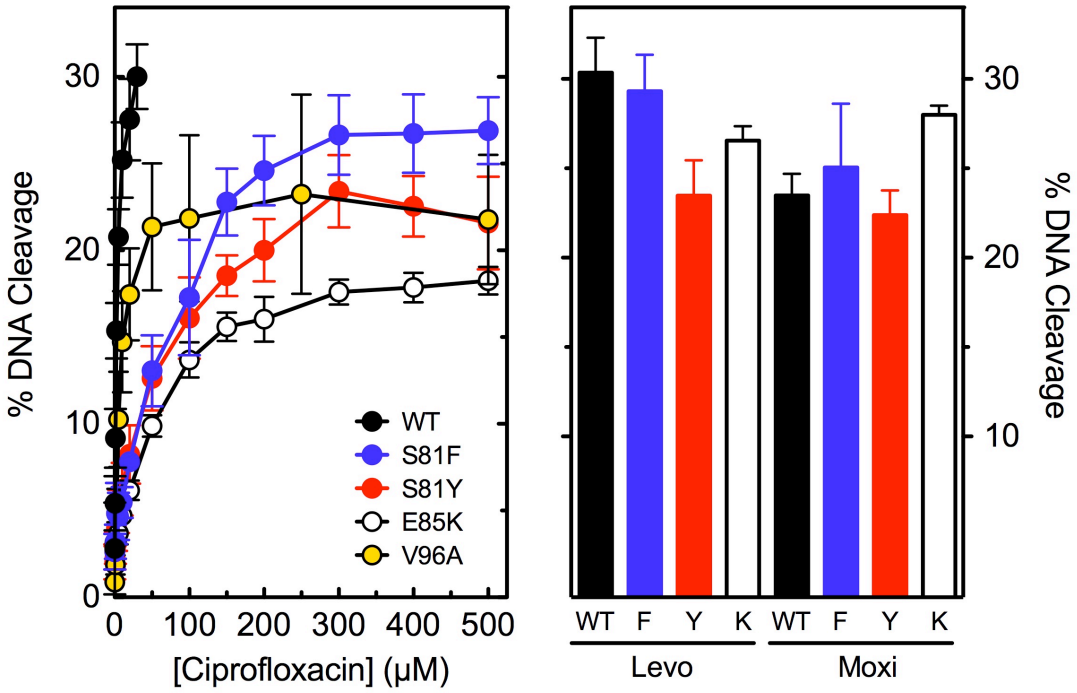


Figure 15. Maximal levels of quinolone-induced DNA cleavage mediated by wild-type and quinolone-resistant mutant *B. anthracis* topoisomerase IV enzymes. A titration is shown for ciprofloxacin with wild-type, GrlA^{S81F}, GrlA^{S81Y}, GrlA^{E85K}, and GrlA^{V96A} (left). Maximal levels of DNA cleavage induced by wild-type, GrlA^{S81F}, GrlA^{S81Y}, and GrlA^{E85K} in the presence of levofloxacin and moxifloxacin are shown at the right. Levofloxacin and moxifloxacin concentrations with the wild-type enzyme were 20 μM and with the mutant enzymes were 500 μM. Error bars represent the standard deviation of at least three independent experiments.

the wild-type enzyme (Table 2). These results indicate that quinolone resistance caused by the S81F, S81Y, E85K, and V96A mutations primarily reflects a decrease in drug potency (*i.e.*, affinity for the enzymes) rather than efficacy. Thus, it should be possible to overcome resistance if quinolones or quinolone-like drugs with higher affinities for the most common mutant type II enzymes can be developed.

As discussed above, quinolone resistance is most often associated with mutations at the serine and acidic residues. Although the underlying basis for quinolone resistance generated by these mutations is unknown, it has been suggested that it is related to the ability of quinolones to bind Mg^{2+} ions. Quinolones require divalent metal ions to unwind DNA,[123,126,127] and it has long been assumed that Mg^{2+} is required to coordinate the interactions of quinolones in the enzyme-DNA complex.[39,86,128] Based on the ability of ciprofloxacin to alter conformational equlibria in *E. coli* GyrA (but not $GyrA^{S83W}$) in the presence of Mg^{2+} ions, Sissi *et al*.[86] suggested that the serine was involved in mediating quinolone-Mg^{2+}-protein interactions. This conclusion was supported by the crystallographic study that showed a bridging interaction between the quinolone and enzyme (mediated by a serine and acidic residue) that was facilitated by a Mg^{2+} ion chelated by the quinolone keto acid.[43]

If the above hypothesis is correct, then quinolone-like drugs that do not require the water-Mg^{2+} bridge might be relatively unaffected by mutations at these residues. Quinazolinediones are structurally similar to quinolones, but they lack the C3/C4 keto acid that is necessary to bind metal ions (Figure 14). Two quinazolinediones are known to display similar or better activity than quinolones against a variety of wild-type and quinolone-resistant Gram-positive and Gram-negative bacterial species *in vitro*, in

culture, and in a murine acute lethal Gram-positive infection model.[105,122-124,129] Therefore, a quinazolinedione was tested for activity against wild-type and quinolone-resistant mutant *B. anthracis* topoisomerase IV.

8-Methyl-3'-(AM)P-dione induced DNA cleavage mediated by wild-type topoisomerase IV to a similar extent as did the quinolones (Figure 14 and Table 2). However, this drug was ~2.5 times more potent (as reflected by the CC_3 value) than ciprofloxacin. In contrast to results with the quinolones, $GrlA^{S81F}$, $GrlA^{S81Y}$, $GrlA^{E85K}$, and $GrlA^{V96A}$ topoisomerase IV displayed little to no resistance to the quinazolinedione (Figure 14 and Table 2). With $GrlA^{S81F}$ topoisomerase IV, the CC_3 concentration for the quinazolinedione rose only 1.5-fold, and the level of maximal DNA cleavage was actually 36% higher than observed with the wild-type enzyme. With $GrlA^{S81Y}$ topoisomerase IV, both potency and efficacy were slightly better than those seen with the wild-type enzyme. With $GrlA^{E85K}$ topoisomerase IV, the CC_3 for the quinazolinedione rose only 1.75-fold compared to wild-type (0.2 *vs*. 0.35 μM), and drug efficacy with the mutant enzyme was ~75% of that seen with wild-type topoisomerase IV (~22% *vs*. 29% maximal DNA cleavage). Finally, with $GrlA^{V96A}$ topoisomerase IV, the CC_3 rose only 1.25-fold, and drug efficacy was 20% higher than that seen with the wild-type enzyme.

The striking sensitivity of the quinolone-resistant topoisomerase IV enzymes to 8-methyl-3'-(AM)P-dione is consistent with the hypothesis that the proposed water-Mg^{2+} bridge interaction that coordinates the bacterial type II enzyme with quinolones is a major determinant for drug action. Although structurally similar to quinolones, quinazolinediones appear to interact with type II topoisomerases independently of the

bridge interaction and represent quinolone-like drugs that retain high activity against the most common quinolone resistance mutations.

Biochemical Basis for Quinolone Action and Resistance in Wild-type and Mutant Quinolone-Resistant Topoisomerase IV

Several experiments were carried out to further characterize the biochemical mechanism of quinolone resistance in *B. anthracis* topoisomerase IV and the differences between quinolones and quinazolinediones. First, sites of DNA cleaved by wild-type, $GrlA^{S81F}$, and $GrlA^{S81Y}$ topoisomerase IV were examined in the presence of ciprofloxacin, levofloxacin, CP-115,953, and 8-methyl-3'-(AM)P-dione (Figure 16). Some differences in site utilization were seen, and overall levels of quinolone-induced DNA cleavage mediated by the mutant enzymes dropped. However, no major differences in cleavage site specificity were observed for any drug-enzyme combination. Although some DNA cleavage bands became very faint in the presence of quinolones and the resistant enzymes, these bands were visible at higher drug concentrations (not shown).

These findings indicate that the resistance of $GrlA^{S81F}$ and $GrlA^{S81Y}$ topoisomerase IV to quinolones and the ability of 8-methyl-3'-(AM)P-dione to overcome the resistance are not related to the selection of DNA cleavage sites by *B. anthracis* topoisomerase IV. They further suggest that drug-DNA interactions formed in the presence of the mutant enzymes are essentially those formed in the presence of wild-type topoisomerase IV (albeit weaker with quinolones) and that the quinazolinedione-cleavage complex interactions are similar to those established by quinolones.

The increased quinolone CC_3 values for $GrlA^{S81F}$, $GrlA^{S81Y}$, $GrlA^{E85K}$, and $GrlA^{V96A}$ topoisomerase IV suggest that quinolones bind less tightly to the mutant

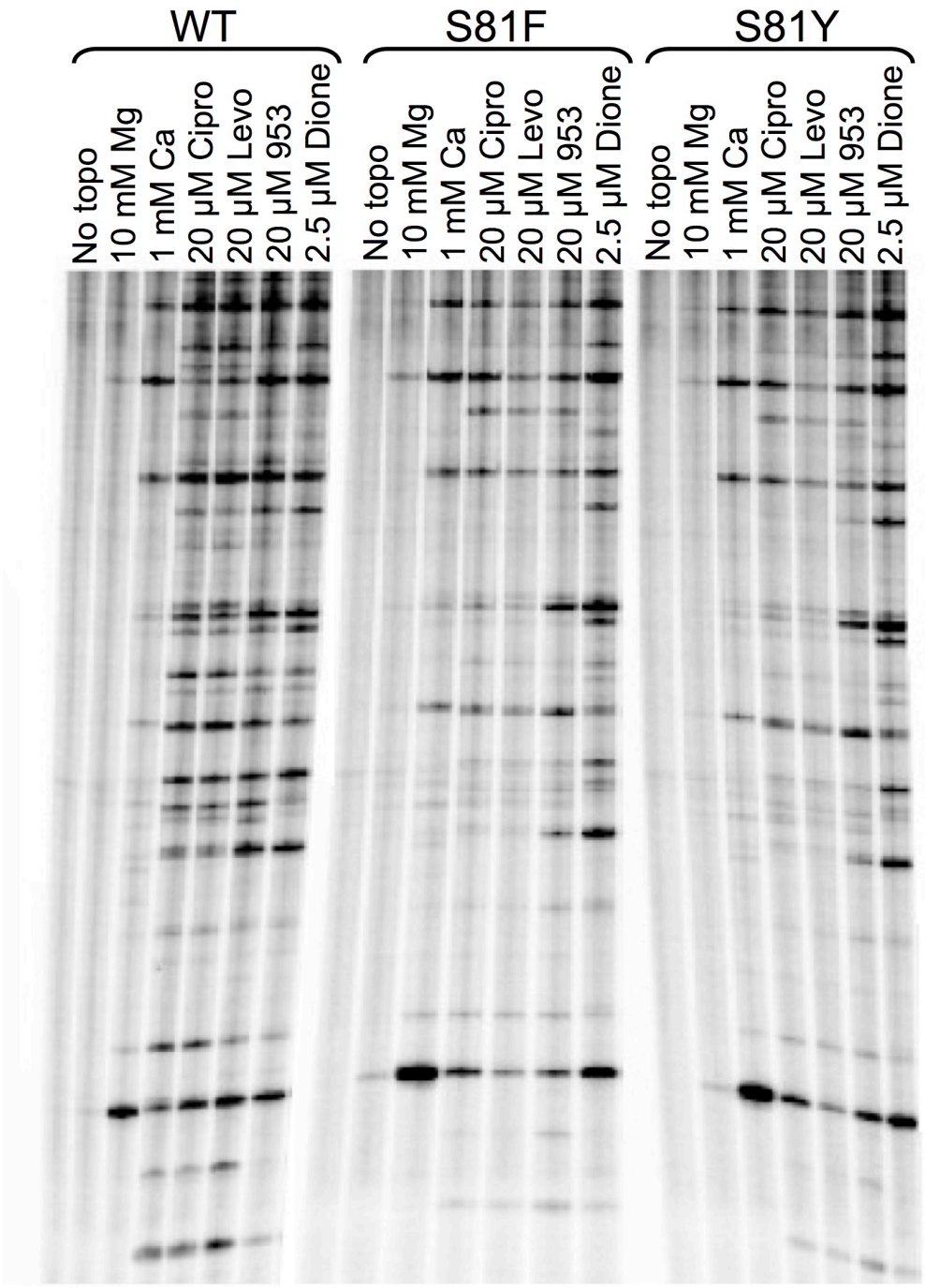


Figure 16. Effects of quinolones and 8-methyl-3'-(AM)P-dione on DNA cleavage site selection by wild-type, $GrlA^{S81F}$, and $GrlA^{S81Y}$ *B. anthracis* topoisomerase IV. An autoradiogram of a polyacrylamide gel identifying DNA sites cleaved by the enzymes is shown. Reactions contained no enzyme (No topo), or topoisomerase IV in the presence of the indicated concentrations of Mg^{2+} or Ca^{2+}, or ciprofloxacin, levofloxacin, CP-115,953, or 8-methyl-3'-(AM)P-dione. Mg^{2+} (10 mM) was used in all drug-containing reactions. The autoradiogram is representative of at least three independent experiments.

enzymes than to wild-type topoisomerase IV. This finding is consistent with previous binding and kinetic studies with serine mutants of *E. coli* gyrase and *Staphylococcus aureus* topoisomerase IV.[119,124,125,130] In contrast, the high affinity of 8-methyl-3'-(AM)P-dione for the mutant enzymes appears to be maintained. Therefore, the basis underlying these differences was explored by examining the ability of ciprofloxacin to compete with the quinazolinedione.

Competition assays took advantage of the fact that lower levels of DNA cleavage were observed in the presence of the quinolone. Therefore, the relative contributions of ciprofloxacin and 8-methyl-3'-(AM)P-dione to DNA cleavage were determined by comparing levels of scission generated in the presence of both drugs to the cleavage observed in the presence of either drug alone. Even at quinolone concentrations that were 7.5 times higher than that of the quinazolinedione, ciprofloxacin contributed less than half of the drug-induced DNA cleavage (Figure 17). This finding suggests that ciprofloxacin is unable to compete effectively with 8-methyl-3'-(AM)P-dione for binding to $GrlA^{S81F}$ and $GrlA^{S81Y}$ topoisomerase IV-DNA complexes and provides further evidence that the mutation at Ser81 of *B. anthracis* topoisomerase IV has a greater impact on the affinity of the quinolone than the quinazolinedione for the enzymes.

Next, the ability of drugs to inhibit topoisomerase IV-mediated DNA religation was assessed. Although quinolones impair DNA religation mediated by type II topoisomerases, their effects range from modest (<2-fold) to strong (~10-fold), depending on the enzyme species and drug employed.[87,109,125,131] In the presence of wild-type *B. anthracis* topoisomerase IV, quinolones and the quinazolinedione had a similar moderate effect on enzyme-mediated DNA religation, increasing $t_{1/2}$ values by ~3-fold (as

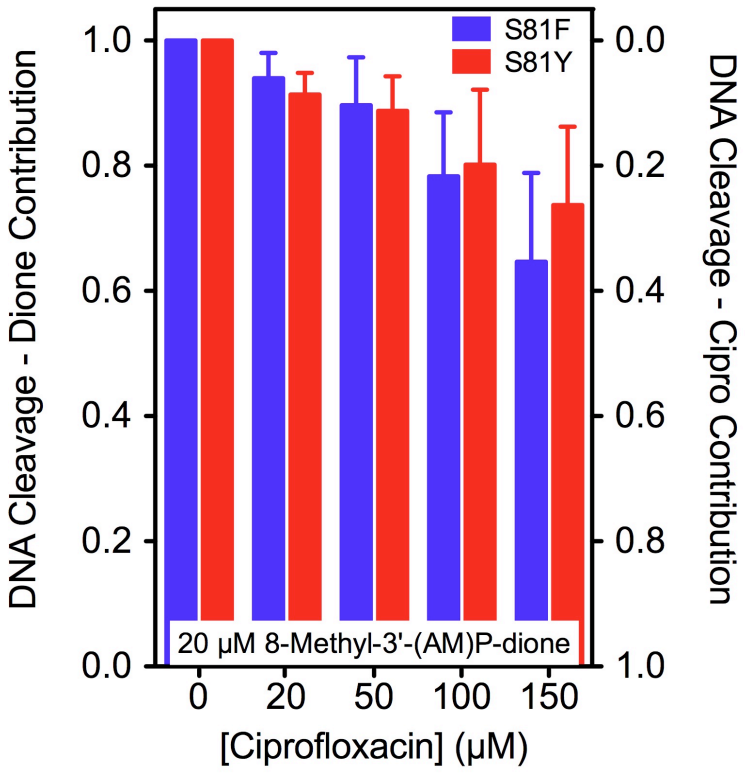


Figure 17. Competition between ciprofloxacin and 8-methyl-3'-(AM)P-dione for *B. anthracis* topoisomerase IV. The ability of 0-150 µM ciprofloxacin to compete with 20 µM 8-methyl-3'-(AM)P-dione for GrlA^{S81F} and GrlA^{S81Y} *B. anthracis* topoisomerase IV was determined using DNA cleavage assays. Both drugs were added to reaction mixtures simultaneously. The relative contribution of the quinazolinedione (left axis) to the total level of DNA cleavage was calculated as follows: (% DNA cleavage with both drugs − % DNA cleavage with ciprofloxacin only) ÷ (% DNA cleavage with quinazolinedione only − % DNA cleavage with ciprofloxacin only). The relative contribution of ciprofloxacin to the total level of DNA cleavage (1 minus the above equation) can be read from the right axis. Error bars represent the standard deviation of at least three independent experiments.

compared to reactions carried out in the absence of drug; Figure 18). However, the two drug classes had markedly different effects on religation mediated by the quinolone-resistant Ser81 and Glu85 mutant topoisomerase IV enzymes. Whereas 8-methyl-3'-(AM)P-dione maintained (or even increased) its ability to inhibit religation, very little inhibition was seen in the presence of quinolones (Figure 18). Notably, the quinazolinedione was used at 20 μM with the wild-type and mutant enzymes, while the quinolones were ten times more concentrated when used with the mutant enzymes than when used with wild-type (200 μM vs. 20 μM). These results imply that the Ser81 and Glu85 resistance mutations impair quinolone function as well as binding. In contrast to Ser81 and Glu85 mutations, the V96A mutation had little effect on the ability of either the quinolone (50 μM) or quinazolinedione (20 μM) to inhibit religation (Figure 18).

Finally, the effects of resistance mutations on the persistence of drug-induced cleavage complexes were determined. This was accomplished by establishing DNA cleavage-religation equilibria with wild-type or mutant topoisomerase IV in the presence of ciprofloxacin (20 μM with wild-type; 50 μM with $GrlA^{V96A}$; 200 μM with Ser81 and Glu85 mutants) or 8-methyl-3'-(AM)P-dione (20 μM), diluting reaction mixtures 20-fold, and monitoring the decay of cleavage complexes over time (Figure 19). The $t_{1/2}$ for DNA cleavage induced by ciprofloxacin dropped ~20-fold with the Ser81 and Glu85 mutant enzymes, while the drop seen with 8-methyl-3'-(AM)P-dione was (at most) ~30%. With the V96A mutant enzyme, the $t_{1/2}$ for DNA cleavage induced by ciprofloxacin dropped only ~5-fold, but, with the quinazolinedione, it dropped ~40%. These findings indicate that quinolone resistance correlates with a decreased stability of the topoisomerase IV-drug-DNA ternary complex, while the high sensitivity of the mutant quinolone-resistant

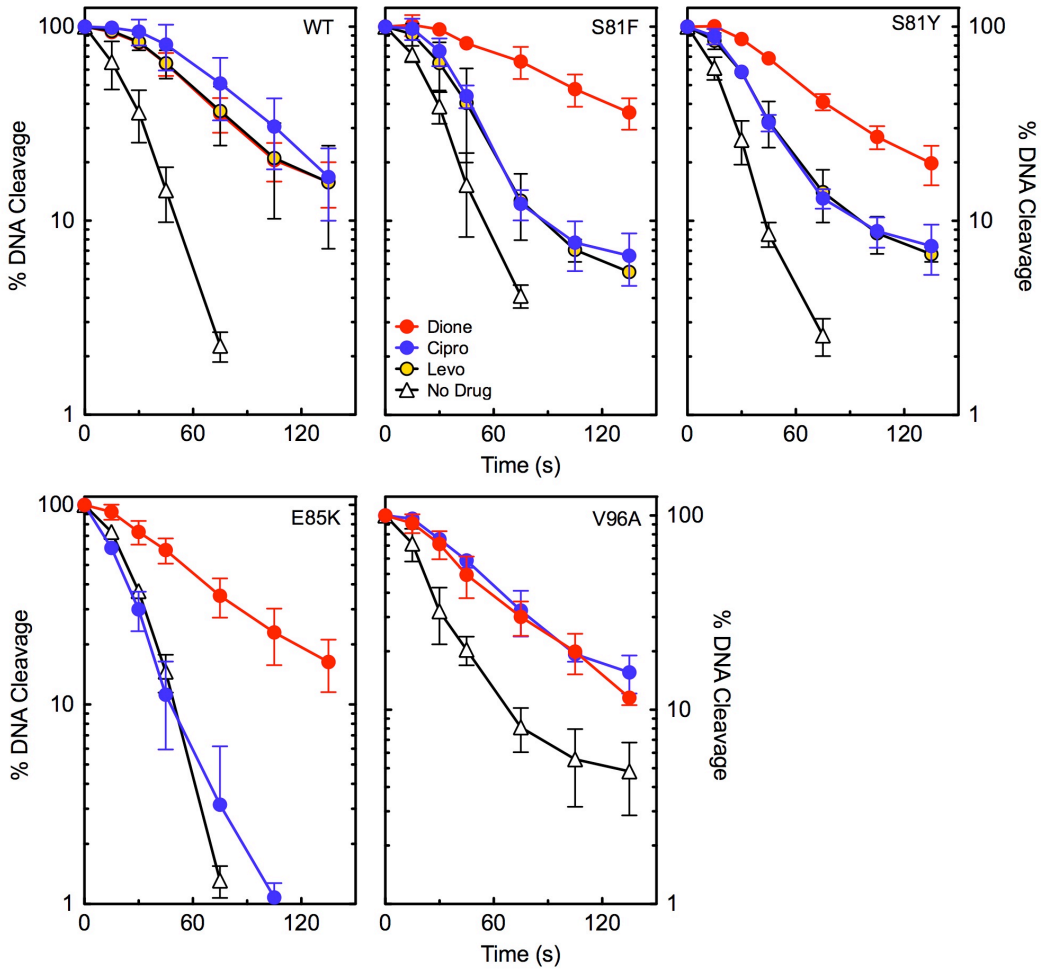


Figure 18. Effects of quinolones and 8-methyl-3'-(AM)P-dione on the DNA religation activities of wild-type, GrlA^{S81F}, GrlA^{S81Y}, GrlA^{E85K}, and GrlA^{V96A} topoisomerase IV from *B. anthracis*. Results for assays carried out in the absence of drugs or in the presence of ciprofloxacin or 8-methyl-3'-(AM)P-dione are shown for all enzymes. Results with levofloxacin are also shown for wild-type, GrlA^{S81F}, and GrlA^{S81Y}. Religation was assessed by monitoring the loss of double-stranded DNA breaks (linear product) over time. Cleavage at time zero was set to 100%. Quinolone concentrations were 20 µM in assays that examined wild-type topoisomerase IV and were increased to 50 µM in assays that examined GrlA^{V96A} and 200 µM in assays that examined the Ser81 and Glu85 mutant enzymes. The concentration of 8-methyl-3'-(AM)P-dione was 20 µM in all assays. Reactions carried out in the absence of drugs replaced 5 mM Mg^{2+} with 1 mM Ca^{2+} in order to achieve readily quantifiable levels of DNA cleavage. Error bars represent the standard deviation of at least three independent experiments.

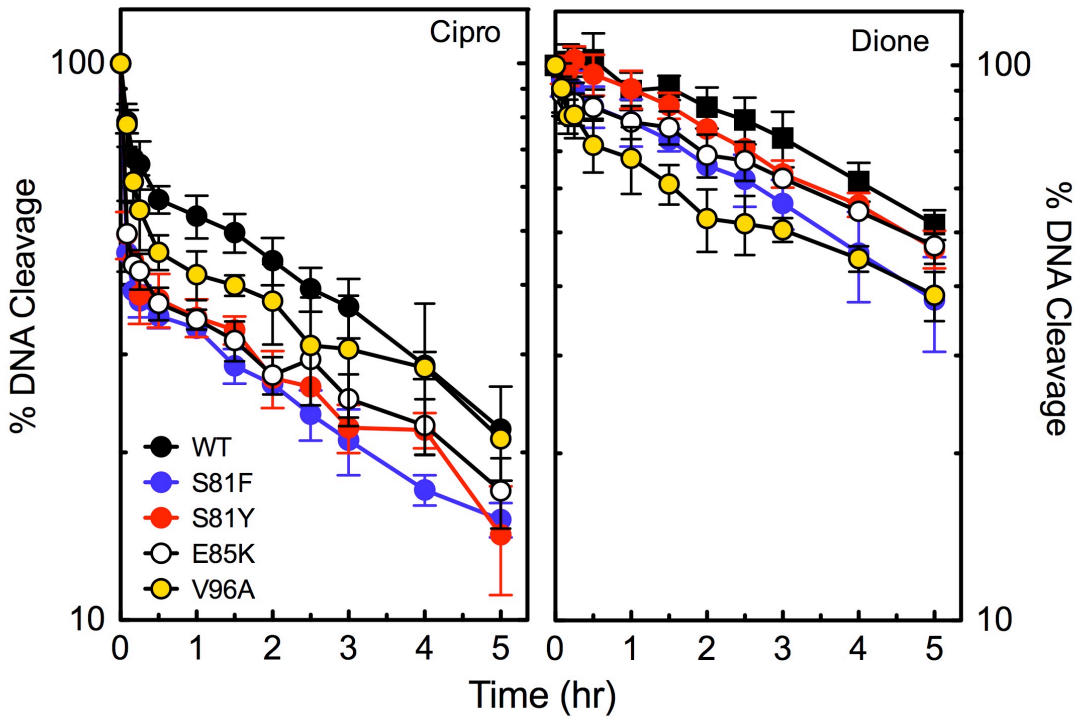


Figure 19. Effects of ciprofloxacin and 8-methyl-3'-(AM)P-dione on the persistence of ternary enzyme-drug-DNA cleavage complexes formed with wild-type, GrlA^{S81F}, GrlA^{S81Y}, GrlA^{E85K}, and GrlA^{V96A} topoisomerase IV from *B. anthracis*. Initial DNA cleavage-religation reactions were allowed to come to equilibrium and were then diluted 20-fold with DNA cleavage buffer. The persistence of cleavage complexes was assessed by monitoring the loss of double-stranded DNA breaks (linear product) over time. Cleavage at time zero was set to 100%. The concentration of ciprofloxacin (left panel) was 20 μM in assays that examined wild-type topoisomerase IV and was increased to 50 μM in assays that examined GrlA^{V96A} and 200 μM in assays that examined the Ser81 and Glu85 mutant enzymes. The concentration of 8-methyl-3-(AM)P-dione was 20 μM in all assays. Error bars represent the standard deviation of at least three independent experiments.

topoisomerase IV enzymes to the quinazolinedione correlates with the maintenance of a stable ternary complex.

Summary of Conclusions

The GrlA^{S81F}, GrlA^{S81Y}, GrlA^{E85K}, and GrlA^{V96A} mutations in topoisomerase IV cause quinolone resistance by decreasing drug potency (*i.e*. drug-enzyme affinity). These mutations also impair the ability of quinolones to inhibit enzyme-mediated DNA religation and decrease the stability of ternary enzyme-drug-DNA cleavage complexes. 8-Methyl-3'-(AM)P-dione is a quinolone-like drug that retains high activity against the quinolone-resistant mutant enzymes. Wild-type topoisomerase IV is also very sensitive to this compound, and its potency and efficacy against the wild-type enzyme actually exceed that of the quinolones. Presumbly, due to the lack of a C3/C4 keto acid group, this quinazolinedione interacts with the enzymes independently of the proposed water-metal ion bridge interaction, thus explaining how the drug maintains activity against quinolone-resistant mutant topoisomerase IV enzymes. Unlike the quinolones, the quinazolinedione inhibits religation mediated by the mutant type II enzymes, interacts more tightly with these enzymes than does ciprofloxacin, and forms very stable cleavage complexes. Finally, topoisomerase IV cleaves DNA at the same set of sites (although at different relative frequencies) in the presence of quinolones and quinazolinediones, indicating that both drug classes interact with the enzyme-DNA cleavage complex in a similar manner.

CHAPTER V

FUNCTIONAL EVIDENCE FOR A WATER-METAL ION BRIDGE MEDIATING THE MAJOR INTERACTION BETWEEN QUINOLONES AND *BACILLUS ANTHRACIS* TOPOISOMERASE IV

Introduction

Quinolones have long been known to chelate a variety of divalent metal ions,[132-134] which have been hypothesized to enhance a number of drug functions.[39,86,128,134,135] For example, quinolones bind DNA and alter the conformation of *E. coli* GyrA but only in the presence of divalent metal ions.[86,127,135] A recent crystallographic study[43] suggested that quinolones interact with bacterial type II topoisomerases through a bridging, non-catalytic Mg^{2+} ion that is in turn stabilized by four water molecules that are coordinated to the side chains of a serine and an acidic amino acid residue through hydrogen bonds (Figure 7). This serine and acidic amino acid are the two residues that are most commonly mutated in quinolone-resistant bacterial strains.[18,36,37,39,49,77-83] Typically, in both laboratory and clinical isolates, alterations at the serine residue comprise >90% of the mutant pool, with changes at the acidic residue comprising the bulk of the other mutations.

Quinazolinediones lack the C3/C4 keto acid necessary to chelate metal ions. Presumably, they overcome quinolone resistance caused by serine or acidic residue mutations by interacting with the enzyme independently of metal ions. This observation is consistent with the water-metal ion bridge interaction hypothesis. However, the existence of the water-metal ion bridge and its role in mediating quinolone-topoisomerase

interactions has not been directly tested or functionally corroborated. A potential confounding issue is that type II topoisomerases require two divalent metal ions per active site to support DNA cleavage.[24-26,42,120,136,137] One of these "catalytic" metal ions binds with high affinity to site A and mediates the chemistry of DNA scission and religation. The other binds with lower affinity to site B and appears to play a structural role aligning the double helix during these processes. In addition, *B. anthracis* topoisomerase IV is a promiscuous enzyme (as discussed in Chapter III) that can utilize a variety of metal ions in the DNA cleavage reaction. Thus, it was difficult to separate the requirement of a non-catalytic Mg^{2+} (or other divalent) ion for quinolone action from the requirement for catalytic ions.

Three approaches were used to test the validity of the water-metal ion bridge hypothesis. Experiments were carried out to determine whether quinolone action requires divalent metal ions, whether the serine and acidic residues anchor the proposed water-metal ion bridge interaction, and how mutations in these two amino acids could affect the metal ion requirement. The quinazolinedione was tested alongside the quinolones because it maintains activity against quinolone-resistant mutant topoisomerase IV and appears to be a metal-ion-independent drug.

Results and Discussion

Metal Ion Requirements for Quinolone-induced DNA Cleavage

In order to establish whether quinolone function requires non-catalytic metal ions, it was necessary to segregate the functions of the catalytic and non-catalytic metal ions.

Therefore, a variety of divalent (and trivalent) cations were screened for the ability to support DNA cleavage mediated by wild-type *B. anthracis* topoisomerase IV in the absence of drugs or in the presence of ciprofloxacin or 8-methyl-3'-(AM)P-dione. Each metal ion was tested over a range of 50 μM to 10 mM and optimized for activity with the wild-type enzyme. Mg^{2+}, Mn^{2+}, Co^{2+}, Sr^{2+}, Ni^{2+}, Pb^{2+}, Al^{3+}, Tb^{3+}, and Eu^{3+} supported topoisomerase IV-mediated DNA cleavage under all three conditions (not shown). However, four divalent metal ions – Ca^{2+}, Cd^{2+}, Zn^{2+}, and Ba^{2+} – supported DNA cleavage differentially. Although they supported basal enzyme activity and high levels of DNA cleavage in the presence of 8-methyl-3'-(AM)P-dione, each showed little or no ability to support DNA cleavage enhancement by ciprofloxacin (Figure 20; results with Ba^{2+} are not shown). These results strongly suggest that quinolones, but not quinazolinediones, require a non-catalytic divalent metal ion in order to induce DNA cleavage.

Ca^{2+}, Cd^{2+}, Zn^{2+}, and Ba^{2+} have all been shown to complex with quinolones.[133,134,138-141] Therefore, when chelated by ciprofloxacin, these ions likely cannot support the proper coordination geometry to form a functional water-metal ion bridge. However, an alternative interpretation is plausible. Sub-millimolar concentrations of metal ions were used in experiments because higher concentrations of Cd^{2+} and Zn^{2+} impaired enzyme activity and higher concentrations of Ca^{2+} and Ba^{2+} significantly raised basal levels of DNA cleavage. Thus, it is possible that they did not support ciprofloxacin-induced DNA cleavage because the drug sequesters the divalent cations and prevents their use in the active site of the enzyme. Two controls indicate that this is not the case (Figure 20). First, no enhancement of DNA cleavage was observed at the lowest

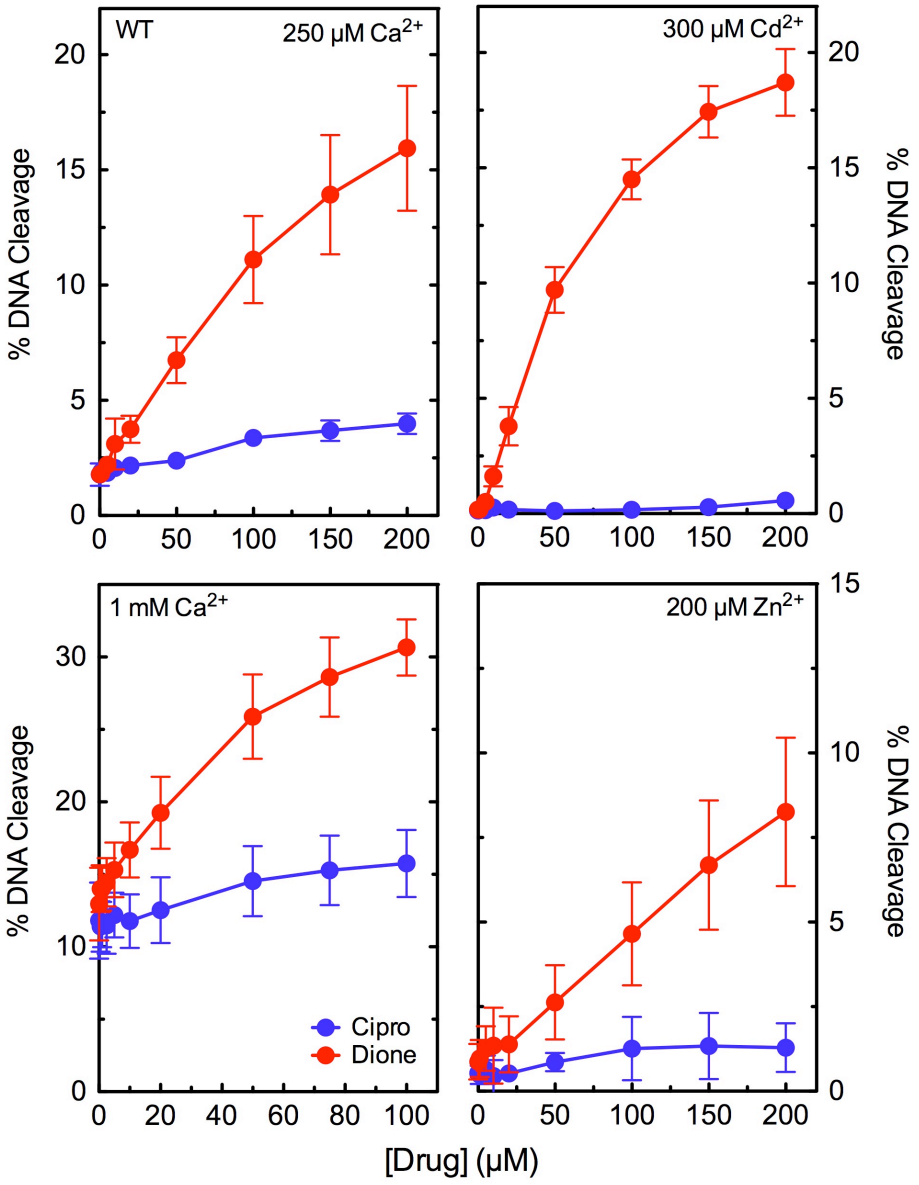


Figure 20. Effects of alternative metal ions on drug-induced DNA cleavage mediated by wild-type *B. anthracis* topoisomerase IV. Assays were carried out with ciprofloxacin or 8-methyl-3'-(AM)P-dione. The indicated divalent metal ions were substituted for the Mg^{2+} used in standard assays. Cd^{2+} (300 μM; top right) and Zn^{2+} (200 μM; bottom right) were utilized at the concentration that gave maximal enzyme-mediated DNA cleavage activity. Ca^{2+} was utilized at 250 μM (top left) or 1 mM (bottom left). Error bars represent the standard deviation of at least three independent experiments.

concentrations of ciprofloxacin (<50 μM) at which concentrations of metal ions would be more than sufficient to support the DNA cleavage reaction of topoisomerase IV. In contrast, significant levels of DNA cleavage were seen in the same concentration range of 8-methyl-3'-(AM)P-dione. Second, experiments were repeated at 1 mM Ca^{2+}. Even though drug effects were tempered by the high levels of basal (*i.e.*, no drug) topoisomerase IV-mediated DNA cleavage, it is obvious that the quinazolinedione, but not the quinolone, further increases DNA scission.

Roles of GrlA Ser81 and GrlA Glu85 in the Water-Metal Ion Bridge Interaction

The proposed water-metal ion bridge appears to be anchored to the topoisomerase through a serine and an acidic amino acid. These highly conserved residues, located in the A subunit of the enzyme, correspond to Ser81 and Glu85 in *B. anthracis* topoisomerase IV GrlA. If these residues play a role in the water-metal ion bridge interaction, their mutation may impact the variety of metal ions that can form a functional bridge and support quinolone activity. Therefore, experiments were carried out to identify metal ions that could distinguish between wild-type and mutant topoisomerase IV.

Metal ions that displayed optimal activity in the millimolar range were utilized for these studies to ensure that both the catalytic and non-catalytic sites were saturated over the drug range tested. Although Mg^{2+} (as well as Co^{2+}; not shown) supported quinolone-induced DNA cleavage mediated by wild-type, $GrlA^{S81F}$, and $GrlA^{E85K}$ topoisomerase IV, Mn^{2+} and Ni^{2+} could only do so with the wild-type enzyme (Figure 21). In contrast, all four of these metal ions supported quinazolinedione-induced DNA cleavage with wild-type and mutant topoisomerase IV (Figure 21, results with Co^{2+} are not shown). These

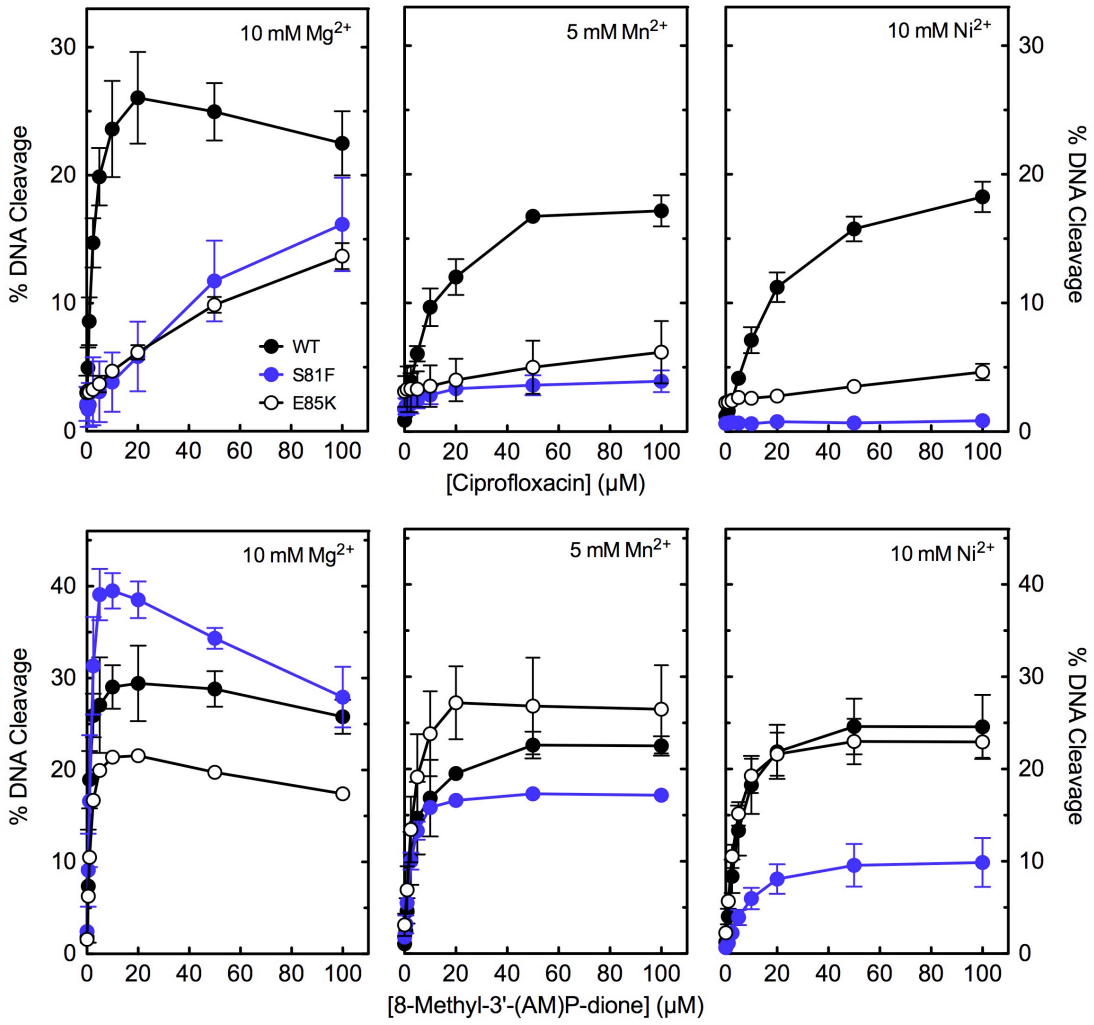


Figure 21. Effects of alternative metal ions on drug-induced DNA cleavage mediated by wild-type, GrlA^{S81F}, and GrlA^{E85K} topoisomerase IV from *B. anthracis*. Results are shown for cleavage mediated by the enzymes in the presence of ciprofloxacin (top) or 8-methyl-3'-(AM)P-dione (bottom) and Mg^{2+} (left), Mn^{2+} (middle), or Ni^{2+} (right). Metal ions were utilized at the concentration that yielded maximal enzyme activity (10 mM for Mg^{2+} and Ni^{2+}, 5 mM for Mn^{2+}). Error bars represent the standard deviation of at least three independent experiments.

findings support the hypothesis that Ser81 and Glu85 play a role in quinolone-enzyme interaction. In addition, they suggest that mutation of either of the two amino acids that are proposed to anchor the bridge (and hence the quinolone) to the enzyme restricts the variety of divalent cations that can be used to form a functional water-metal ion bridge.

The serine and acidic residues are proposed to hydrogen bond with and stabilize the water molecules that fill the coordination sphere of the Mg^{2+} ion that is chelated by the quinolone keto acid. Therefore, mutation of these residues may decrease the ability of Mg^{2+} to bind and form the water-metal ion bridge. To determine whether the Ser→Phe/Tyr and Glu→Lys mutations alter the affinity of metal ions in the proposed bridge, the Mg^{2+} dependence of quinolone- and quinazolinedione-induced topoisomerase IV-mediated DNA cleavage was analyzed.

The quinolone and quinazolinedione displayed similar requirements for Mg^{2+} when wild-type topoisomerase IV was employed; half-maximal and maximal DNA cleavage were observed at ~0.35 mM and ~1 mM Mg^{2+}, respectively (Figure 22). In contrast, the two drugs displayed markedly different Mg^{2+} requirements for DNA cleavage mediated by $GrlA^{S81F}$, $GrlA^{S81Y}$, and $GrlA^{E85K}$. While the metal ion utilization for 8-methyl-3'-(AM)P-dione with the mutant enzymes closely resembled that seen with the wild-type enzyme, ciprofloxacin required higher levels of Mg^{2+} to support DNA cleavage (Figure 22). In the presence of the Ser→Phe mutation, levels of Mg^{2+} required to generate half-maximal and maximal DNA cleavage increased to ~0.75 mM and ~2.5 mM Mg^{2+}, respectively, in the presence of the quinolone. The Ser→Tyr mutation is a more conservative mutation than Ser→Phe because the side chains of both serine and tyrosine contain a terminal –OH group. However, levels of Mg^{2+} required by $GrlA^{S81Y}$ to

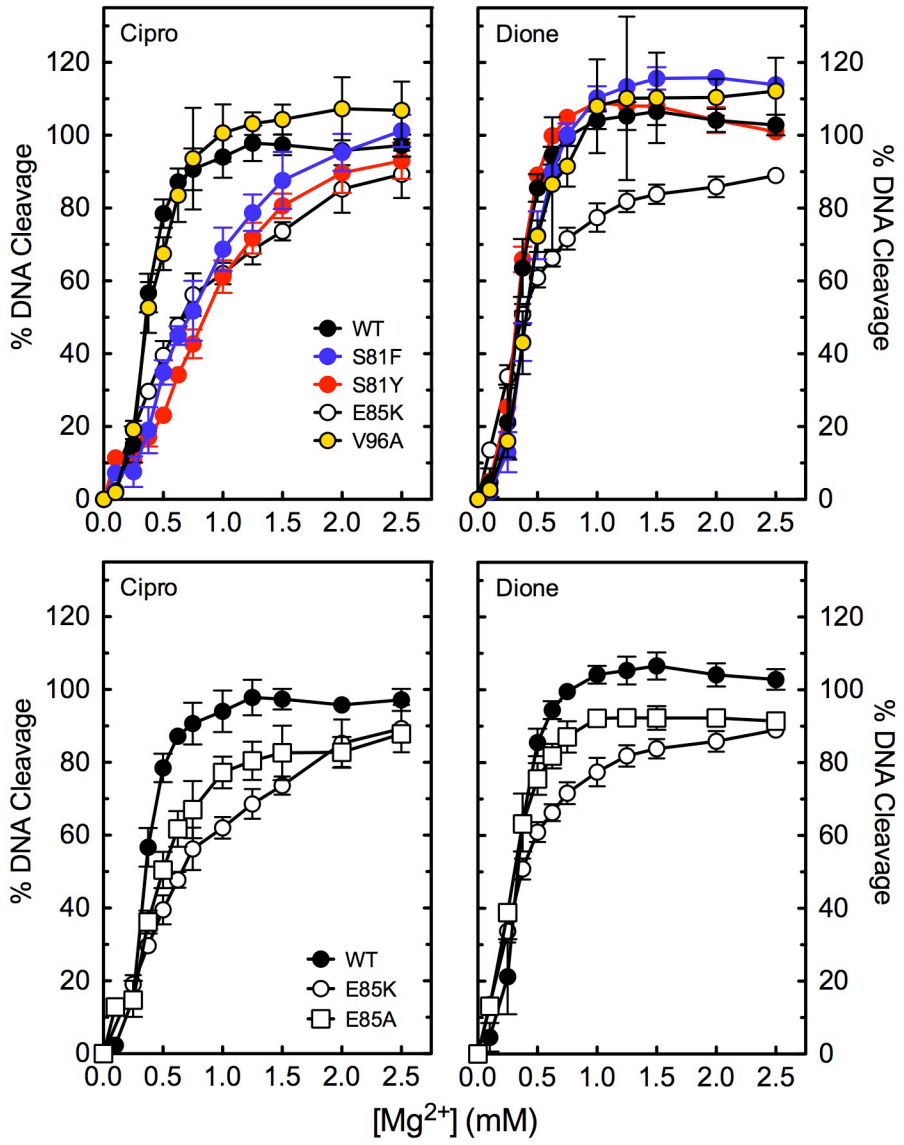


Figure 22. Effects of Mg^{2+} on DNA cleavage mediated by wild-type and quinolone-resistant mutant topoisomerase IV from *B. anthracis*. Results are shown for 50 µM ciprofloxacin (left) and 10 µM 8-methyl-3'-(AM)P-dione (right) with the wild-type, $GrlA^{S81F}$, $GrlA^{S81Y}$, $GrlA^{E85K}$, and $GrlA^{V96A}$ enzymes in the top panels. Results for $GrlA^{E85A}$ (with wild-type and $GrlA^{E85K}$ for comparison) are shown in the bottom panels. DNA cleavage for each drug-enzyme pair was normalized to 100% at 10 mM Mg^{2+} to facilitate direct comparisons. Error bars represent the standard deviation of at least three independent experiments.

generate half-maximal and maximal quinolone-induced DNA cleavage (~0.85 mM and ~3.0 mM Mg^{2+}) were actually slightly higher than those required by $GrlA^{S81F}$. Finally, in the presence of the Glu→Lys mutation, these values were ~0.66 mM and ~3.0 mM Mg^{2+} when the quinolone was utilized. The requirement for higher Mg^{2+} concentrations to support quinolone- but not quinazolinedione-induced DNA cleavage by the mutant enzymes provides further evidence for the water-metal ion bridge that is proposed to coordinate quinolone-topoisomerase IV binding as well as the roles of Ser81 and Glu85 in anchoring the bridge to the enzyme.

The K_D values for Mg^{2+} binding to metal ion sites A and B are ~0.1 mM and ~1 mM, respectively, for *E. coli* topoisomerase IV.[26] Because half-maximal DNA cleavage was observed at ~0.35 mM Mg^{2+} for both the quinolone and the quinazolinedione with wild-type *B. anthracis* topoisomerase IV, it is likely that this value reflects the affinity of metal ion B rather than the metal ion used to mediate quinolone-protein binding. This supposition implies that the affinity of the metal ion involved in the protein-water-Mg^{2+}-quinolone interaction is higher than that of active site metal ion B, and that the Mg^{2+}-quinolone interaction only becomes limiting in the presence of mutations (such as those at Ser81 and Glu85) that disrupt the coordination of the water-Mg^{2+} bridge. Consequently, it is probable that the GrlA S81→F, S81→Y, and E85→K mutations in *B. anthracis* topoisomerase IV actually lower the affinity of the quinolone-bridging Mg^{2+} by more than the approximately two-fold effect seen in Figure 22.

The mutation of Glu85→Lys introduces a positively charged amino acid side chain in the place of a negatively charged one. Especially in the context of the water-metal ion bridge where this side chain participates in hydrogen bonding with water

molecules and is in close proximity to a positively charged metal ion, the effects on metal ion utilization seen with the GrlA^{E85K} mutant topoisomerase IV may be the result of the nature of the mutation rather than the simple loss of the bridge anchor. For example, the positive charge on the lysine could alter quinolone binding by repelling the divalent metal ion that is chelated by the quinolone. To test for this possibility, the properties of GrlA^{E85A} topoisomerase IV were examined. This enzyme displayed cleavage activities in the absence of drug that were similar to those of GrlA^{E85K} (Figure 23). Furthermore, the effects of drugs on GrlA^{E85A} topoisomerase IV paralleled those seen with the Glu→Lys mutant enzyme (Figure 24). Ciprofloxacin was able to poison GrlA^{E85A} topoisomerase IV at high drug concentrations, while the mutation did not have any significant effect on the ability of the quinazolinedione to enhance topoisomerase IV-mediated DNA cleavage. Finally, as seen with the Glu→Lys mutation, GrlA^{E85A} required an increased concentration of Mg^{2+} to support maximal levels of DNA cleavage in the presence of ciprofloxacin, but not in the presence of 8-methyl-3'-(AM)P-dione (Figure 22).

The GrlA^{V96A} mutation in topoisomerase IV causes resistance to ciprofloxacin, albeit at a lower level than do the mutations at Ser81 and Glu85, and 8-methyl-3'-(AM)P-dione maintains high activity against this mutant enzyme. Based on crystallographic studies,[43] Val96 does not appear to participate in the water-metal ion bridge interaction. Therefore, this mutation should not affect the concentration of Mg^{2+} required to achieve maximal levels of quinolone-induced DNA cleavage. As expected, GrlA^{V96A} topoisomerase IV required wild-type concentrations of Mg^{2+} to achieve maximal levels of DNA cleavage in the presence of both the quinolone and the quinazolinedione (Figure 22). This finding provides further evidence that the serine and acidic residues act as the

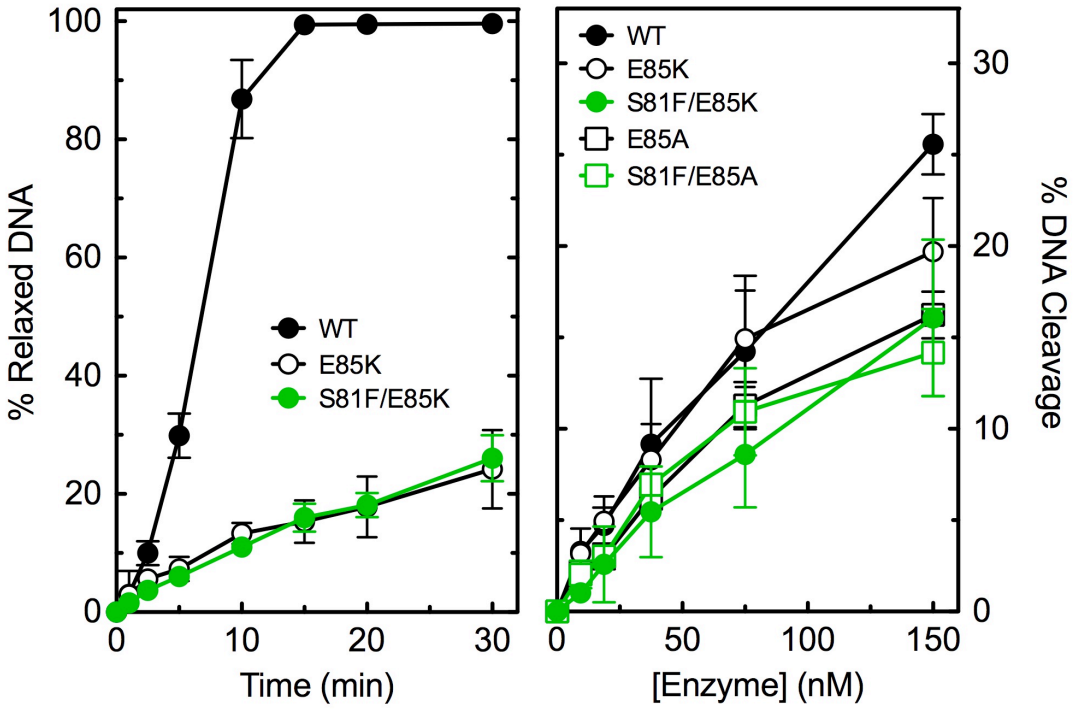


Figure 23. DNA relaxation and cleavage activities of *B. anthracis* GrlA$^{S81F/E85K}$ topoisomerase IV. The catalytic activity of the doubly-mutated enzyme was determined by assessing its ability to relax negatively supercoiled pBR322 plasmid DNA (left). The ability of the enzyme to cleave negatively supercoiled plasmid DNA in the absence of drugs is shown on the right. Cleavage assays utilized 10 mM Ca^{2+} as the divalent metal ion. The abilities of GrlA^{E85A} and GrlA$^{S81F/E85A}$ to cleave DNA in the absence of drugs are also shown. Results with wild-type and GrlA^{E85K} are shown for comparison. Error bars represent the standard deviation of at least three independent experiments.

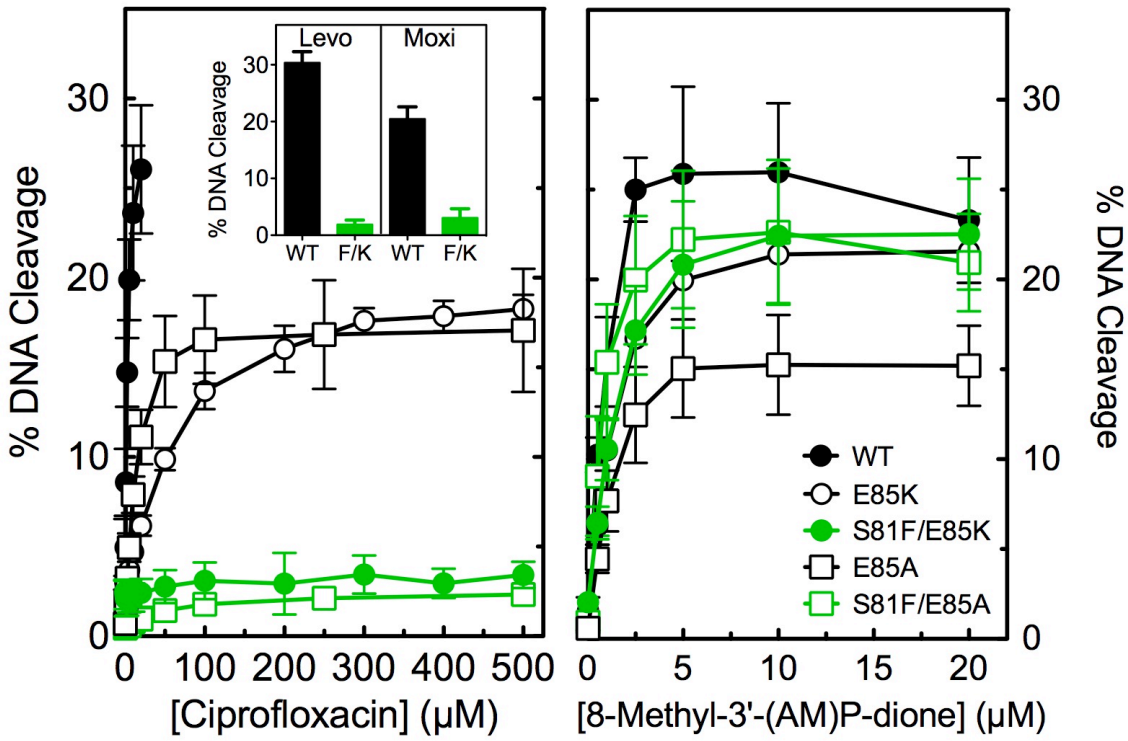


Figure 24. Effects of ciprofloxacin and 8-methyl-3'-(AM)P-dione on the DNA cleavage activities of GrlA^{E85A}, GrlA$^{S81F/E85K}$, and GrlA$^{S81F/E85A}$ *B. anthracis* topoisomerase IV. DNA cleavage mediated by the wild-type and GrlA^{E85K} enzymes is shown for comparison. Titrations to 500 μM ciprofloxacin (left) and 20 μM 8-methyl-3'-(AM)P-dione (right) are shown. The inset shows the maximum level of levofloxacin- and moxifloxacin-induced DNA cleavage generated by GrlA$^{S81F/E85K}$ topoisomerase IV at 500 μM drug. The maximum level of cleavage induced by these drugs (at 20 μM) with wild-type is shown for comparison. Error bars represent the standard deviation of at least three independent experiments.

protein anchors for the water-metal ion bridge. In addition, it suggests that target-mediated quinolone resistance can be caused by mechanisms other than disruption of the water-metal ion bridge interaction. Because Val96 is located on the same α helix as Ser81 and Glu85, one possibility is that it causes quinolone resistance by slightly repositioning the quinolone and the intact water-metal ion bridge so that the drug loses some ability to stabilize cleavage complexes.

The above findings strongly suggest that the GrlA S81→F, S81→Y, and E85→K mutations in *B. anthracis* topoisomerase IV disrupt the water-Mg^{2+} bridge that mediates quinolone-protein binding in the cleavage complex. This conclusion supports the hypothesis that quinolone resistance results from the disruption of this critical quinolone-protein interaction[43] and also provides an explanation for the lack of quinazolinedione resistance seen with these mutant enzymes.

The increased Mg^{2+} concentration required to support ciprofloxacin-induced DNA cleavage by $GrlA^{S81F}$, $GrlA^{S81Y}$, and $GrlA^{E85K}$ topoisomerase IV suggests that the mutant proteins can still bind the quinolone-bridging Mg^{2+} but do so with diminished affinity. Furthermore, because DNA cleavage assays utilize 10 mM Mg^{2+}, which is significantly above the saturating metal ion concentration seen for these mutant enzymes (~3 mM), the decreased potency of quinolones against the resistant topoisomerases cannot be attributed solely to decreased Mg^{2+} binding. Thus, it is likely that quinolones still interact with $GrlA^{S81F}$, $GrlA^{S81Y}$, and $GrlA^{E85K}$ topoisomerase IV through a water-Mg^{2+} bridge. However, because serine and glutamic acid residues appear to coordinate two of the four water molecules that contact the Mg^{2+} ion, the water-metal ion bridge formed with the mutant enzymes is likely altered and less stable. As a result, quinolones display a

decreased potency for DNA cleavage, exhibit a reduced ability to inhibit DNA religation, and form less stable cleavage complexes with the mutant enzymes. In contrast, because quinazolinediones presumably do not require the water-Mg^{2+} bridge to mediate their interactions with bacterial type II topoisomerases, mutation of the serine and glutamic acid residues has little effect on the ability of 8-methyl-3'-(AM)P-dione to inhibit enzyme-mediated DNA religation and form stable ternary enzyme-drug-DNA complexes. Taken together, these results support the conclusion that Ser81 and Glu85 play important roles in mediating quinolone-enzyme interactions by anchoring the water-metal ion bridge.

Simultaneous Mutation of the Amino Acid Residues that Anchor the Water-Metal Ion Bridge Interaction

Ser81 or Glu85 mutant topoisomerase IV enzymes appear to interact with quinolones through a partially functional (or altered) water-metal ion bridge. This could account for the observation that these mutations cause quinolone resistance, but high concentrations of quinolone can induce wild-type levels of DNA cleavage mediated by the mutant enzymes. Therefore, to further explore the significance of the water-metal ion bridge in quinolone-topoisomerase interaction, $GrlA^{S81F/E85K}$ topoisomerase IV was characterized. This enzyme contains mutations at both of the residues that anchor the water-metal ion bridge to the protein.

The double mutant displayed catalytic and DNA cleavage activities that were similar to those of $GrlA^{E85K}$ topoisomerase IV (Figure 23). However, unlike results with the individual $GrlA^{S81F}$ and $GrlA^{E85K}$ mutants, ciprofloxacin and other clinically relevant quinolones displayed virtually no ability to enhance DNA cleavage mediated by

GrlA$^{S81F/E85K}$ topoisomerase IV, even at high drug concentrations (Figure 24). In contrast, 8-methyl-3'-(AM)P-dione induced near wild-type levels of DNA cleavage with the double mutant (Figure 24).

The decreased potency of quinolones with the individual GrlA^{S81F} and GrlA^{E85K} mutants (Figure 14) strongly suggests that impaired bridge function decreases the ability of quinolones to bind in the cleavage complex. Because quinolones induced virtually no DNA cleavage enhancement with GrlA$^{S81F/E85K}$ topoisomerase IV, it was impossible to determine quinolone potency with the double mutant. Therefore, a competition experiment was used to assess the effects of the double mutation on quinolone affinity (Figure 25). Because structural studies indicate that the interaction domains of quinolones and quinazolinediones in the cleavage complex overlap almost completely,[40-43] the ability of ciprofloxacin (0-500 μM) to compete out DNA cleavage mediated by GrlA$^{S81F/E85K}$ topoisomerase IV in the presence of 5 μM 8-methyl-3'-(AM)P-dione was determined. Even at a 100-fold molar excess of quinolone over quinazolinedione, little competition was observed (DNA cleavage dropped by ~20%). This result indicates that in the absence of the water-metal ion bridge, the relative affinity of ciprofloxacin [compared to 8-methyl-3'-(AM)P-dione] for the topoisomerase IV-DNA complex decreases >100-fold.

The above results strongly suggest that the proposed water-metal ion bridge represents the major conduit by which ciprofloxacin and other clinically relevant quinolones interact with topoisomerase IV in the DNA cleavage complex. However, as discussed above, it is possible that the insertion of the positively charged lysine residue in place of Glu85 disrupts enzyme-quinolone interactions by a mechanism that reflects more than the simple loss of the glutamic acid anchor. Although this was not the case in the

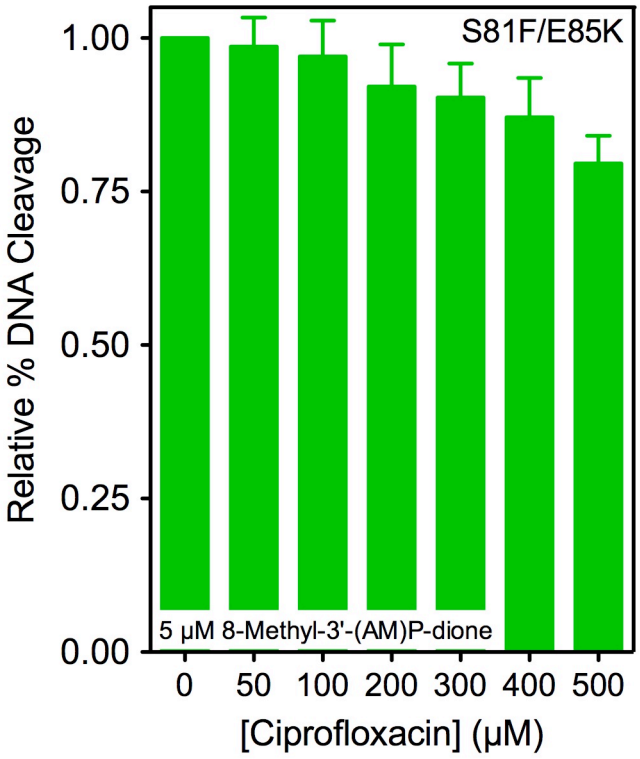


Figure 25. Ability of ciprofloxacin to compete out DNA cleavage induced by 8-methyl-3'-(AM)P-dione with $GrlA^{S81F/E85K}$ topoisomerase IV from *B. anthracis*. The quinazolinedione was utilized at a concentration of 5 µM. The quinolone and quinazolinedione were added to reactions simultaneously. Error bars represent the standard deviation of at least three independent experiments.

mutant topoisomerase IV enzyme containing a mutation only at the Glu85 residue, it is possible that the formation of a partially functional water-metal ion bridge facilitated by Ser81 obscured some effects of this Glu→Lys mutation. Therefore, GrlA$^{S81F/E85A}$ topoisomerase IV was also examined. This mutant enzyme displayed DNA cleavage activities in the absence of drug that were similar to its Glu85→Lys counterpart (Figure 23). In addition, ciprofloxacin (even at high concentrations) was still unable to enhance DNA cleavage mediated by this double mutant, and the activity of the quinazolinedione was unaffected.

These results provide further functional evidence supporting the water-metal ion bridge model for quinolone-topoisomerase IV interactions. GrlA$^{S81F/E85K}$ cannot support the water-metal ion bridge interaction, even in a partially functional form. Thus, the double mutation abrogates the activity of ciprofloxcin against the enzyme, but has little effect on the activity of a quinazolinedione that interacts with the protein independently of the bridge. An important ramification of the water-metal ion bridge model is that the most important interaction between clinically relevant quinolones and their bacterial enzyme targets is mediated by the drug core (Figure 7). This may explain the tolerance for the structural diversity of substituents at the N1, C7, and C8 positions of the quinolone drug class.

Summary of Conclusions

The water-metal ion bridge is the primary interaction between clinically relevant quinolones and bacterial type II topoisomerases. The highly conserved serine and acidic amino acid residues (Ser81 and Glu85 in *B. anthracis* GrlA) act as the anchor points for

coordination of the bridge to the enzyme. Mutation of either of these anchoring residues interferes with the formation of a fully functional bridge and results in a quinolone-resistant topoisomerase IV enzyme. Simultaneous mutation of both the serine and acidic residue completely abolishes the function of the water-metal ion bridge, which results in an enzyme that is fully resistant to quinolone activity, even at high drug concentrations. The significance of the water-metal ion bridge interaction (which is facilitated by the quinolone core rather than substituents) to quinolone activity may explain how these drugs can tolerate structurally diverse substituents at the N1, C7, and C8 positions. Although the bridge plays a pivotal role in mediating quinolone activity, mutation of amino acid residues that do not participate in this water-metal ion interaction can still cause some level of quinolone resistance. Thus, target-mediated quinolone resistance can occur by a mechanism other than disruption of the bridge.

CHAPTER VI

QUINOLONE INTERACTIONS WITH *ESCHERICHIA COLI* TOPOISOMERASE IV

Introduction

The primary interaction between clinically relevant quinolones and *B. anthracis* (Gram-positive) topoisomerase IV is mediated through a water-metal ion bridge. The serine and acidic residues that anchor the bridge to the enzyme are highly conserved across the bacterial kingdom, suggesting that the water-metal ion bridge may be the primary interaction between quinolones and all bacterial type II topoisomerases. As a step toward establishing the bridge as a universal mechanism of quinolone-topoisomerase interaction, *E. coli* (Gram-negative) topoisomerase IV was examined.

Experiments utilized wild-type and quinolone-resistant $ParC^{S80L}$ and $ParC^{E84K}$ topoisomerase IV and evaluated the effects of quinolones and a quinazolinedione on their activities. The metal ion dependence of quinolone-induced DNA cleavage was also examined. Ser80 and Glu84 are equivalent to Ser81 and Glu85 of *B. anthracis* GrlA and are, thus, the amino acid residues that would anchor the water-metal ion bridge in this enzyme. Although topoisomerase IV appears to be the secondary target for quinolones in *E. coli*,[46] the $ParC^{S80L}$ and $ParC^{E84K}$ mutations are commonly observed in quinolone-resistant clinical isolates.[80-83] The ParC Ser80→Ile mutation is more common in the clinic than the Ser80→Leu mutation,[80-83] but $ParC^{S80L}$ was chosen for these studies due to it more often being utilized in the laboratory in previous studies on quinolone resistance in this species.[46,117,142-144]

Results and Discussion

Enzymatic Activities of Wild-type and Quinolone-Resistant ParC^{S80L} and ParC^{E84K} Topoisomerase IV

As discussed in Chapter III, mutations in topoisomerase IV can cause quinolone resistance either by disrupting quinolone-enzyme interactions and causing a decrease in quinolone efficacy and/or potency, or by directly affect the enzyme, such as by causing it be unstable or less active. Therefore, the relaxation and cleavage activities of the wild-type and quinolone-resistant ParC^{S80L} and ParC^{E84K} enzymes were first evaluated in the absence of drugs.

As expected, ParC^{S80L} topoisomerase IV relaxed negatively supercoiled plasmid DNA at a rate that was nearly identical to that of the wild-type enzyme (Figure 26). ParC^{E84K} was previously shown to have a significantly decreased rate of DNA relaxation,[117] very similar to that of GrlA^{E85K} from *B. anthracis* topoisomerase IV. Presumably, this mutation has the same effect on *E. coli* topoisomerase IV as it does on the *B. anthracis* enzyme and, thus, causes a defect in strand passage. For this reason, both mutant *E. coli* enzymes were examined for their ability to mediate DNA cleavage.

Wild-type and ParC^{S80L} *E. coli* topoisomerase IV displayed similar abilities to cleave DNA in the absence of drugs (Figure 27). Unexpectedly, the Glu84→Lys mutant enzyme displayed a DNA cleavage ability that was approximately half that of the wild-type enzyme. Therefore, unlike in the *B. anthracis* enzyme, this mutation may cause quinolone resistance by affecting the activity of the enzyme, rather than (or in addition to) decreasing drug potency and/or efficacy.

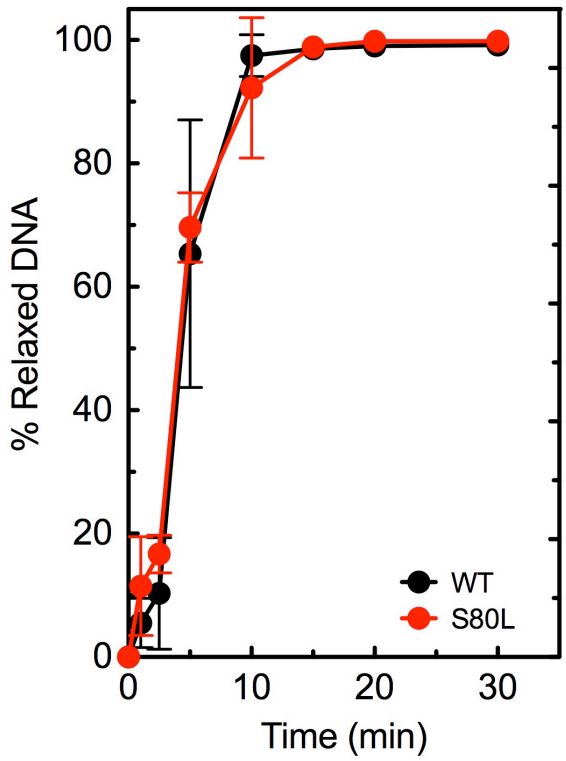


Figure 26. Catalytic activities of wild-type and ParC^{S80L} topoisomerase IV from *E. coli*. The ability of the enzymes to relax negatively supercoiled pBR322 plasmid DNA is shown. Error bars represent the standard deviation of at least three independent experiments.

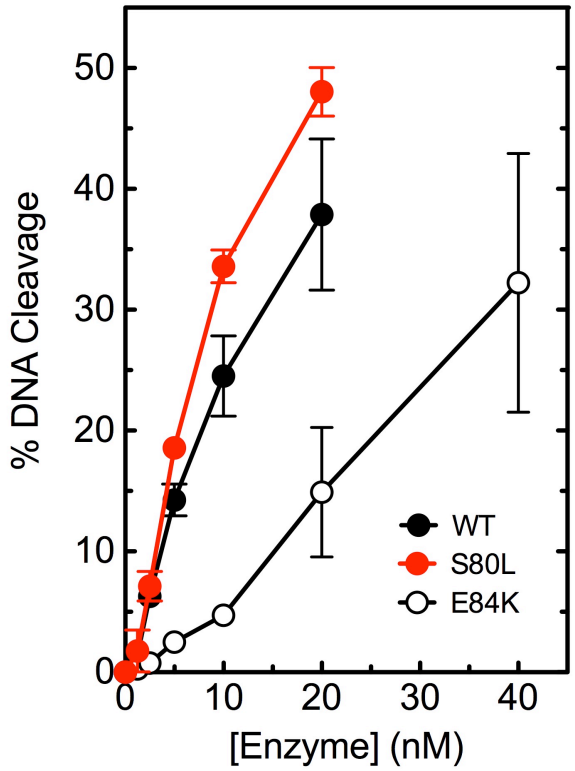


Figure 27. DNA cleavage activities of wild-type, $ParC^{S80L}$, and $ParC^{E84K}$ topoisomerase IV from *E. coli*. The ability of the enzymes to cleave negatively supercoiled pBR322 plasmid DNA in the absence of drugs is shown. Assays were carried out in the presence of 2.5 mM $CaCl_2$. Error bars represent the standard deviation of at least three independent experiments.

As observed with *B. anthracis* topoisomerase IV, a number of different metal ions can support the cleavage activity of the wild-type *E. coli* enzyme (not shown). Therefore, this enzyme also appears to have a promiscuous catalytic metal ion usage profile.

Effects of Ciprofloxacin and 8-Methyl-3'-(AM)P-dione on Wild-type and Quinolone-Resistant ParC^{S80L} and ParC^{E84K} Topoisomerase IV

To determine how the ParC Ser80→Leu and Glu84→Lys mutations cause quinolone resistance, the ability of ciprofloxacin to enhance DNA cleavage mediated by wild-type and mutant *E. coli* topoisomerase IV was assessed. As expected, ciprofloxacin displayed little ability to enhance DNA cleavage mediated by ParC^{S80L} and ParC^{E84K} as compared to the wild-type enzyme (Figure 28). However, in contrast to results with the equivalent *B. anthracis* enzymes, the *E. coli* mutant enzymes displayed little to no increase in ciprofloxacin-enhanced DNA cleavage, even at high drug concentrations (Figure 28). Based on these results, it appears that ParC^{S80L} causes quinolone resistance in *E. coli* topoisomerase IV by decreasing both the potency and efficacy of the drug, while ParC^{E84K} appears to cause resistance primarily through an efficacy effect. Although the ParC^{S80L} and ParC^{E84K} mutations seem to have somewhat different effects on quinolone activity than do the equivalent mutations in *B. anthracis* topisomerase IV, these mutant enzymes, as well as the wild-type enzyme, maintained sensitivity to 8-methyl-3'-(AM)P-dione (Figure 28).

Because ciprofloxacin displayed no ability to enhance DNA cleavage mediated by ParC^{E84K} topoisomerase IV, a competition experiment was carried out to determine whether the quinolone maintained any ability to bind to the mutant enzyme. In this

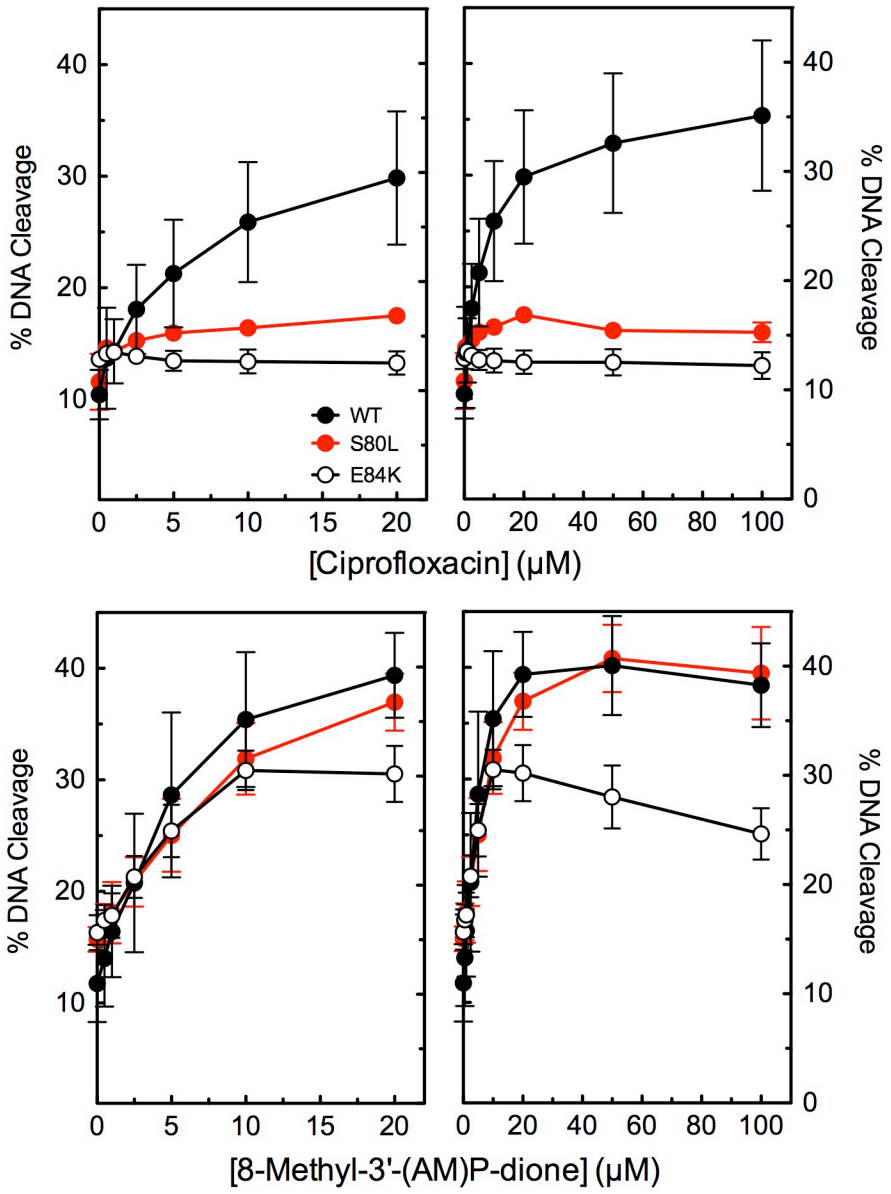


Figure 28. Effects of ciprofloxacin and 8-methyl-3'-(AM)P-dione on the DNA cleavage activities of wild-type, ParC^{S80L}, and ParC^{E84K} topoisomerase IV from *E. coli*. Cleavage induced by clinically relevant concentrations of the quinolone (top) and quinazolinedione (bottom) are shown on the left. Maximal levels of cleavage induced by high concentrations of the drugs are shown on the right. Error bars represent the standard deviation of at least three independent experiments. Note that the ParC^{E84K} enzyme was used at a 2.5-fold higher concentration than the wild-type and ParC^{S80L} enzymes to account for its decreased basal enzyme activity seen in Figure 27. This allows direct comparisons between the three enzymes to be made.

experiment, the ability of ciprofloxacin (0-500 μM) to compete out cleavage induced by 10 μM 8-methyl-3'-(AM)P-dione was determined. Even at a ten-fold excess of quinolone over quinazolinedione, approximately half of the quinazolinedione-induced cleavage complexes remained (Figure 29). Therefore, the Glu84→Lys mutation not only prevents quinolones from enhancing enzyme-mediated DNA cleavage, but it also decreases their ability to interact with the enzyme.

Next, experiments were carried out to determine whether quinolones and the quinazolinedione increase the concentration of *E. coli* topoisomerase IV cleavage complexes by inhibiting enzyme-mediated DNA religation. Because ciprofloxacin was not able to enhance $ParC^{E84K}$ topoisomerase IV DNA cleavage, only the wild-type and $ParC^{S80L}$ enzymes were tested. Although less dramatic than results with *B. anthracis* topoisomerase IV, ciprofloxacin did slow the DNA religation rate of the wild-type enzyme as compared to the rate seen in the absence of drug (Figure 30). As expected, the rate of DNA religation mediated by $ParC^{S80L}$ in the presence of ciprofloxacin was significantly decreased and was nearly indistinguishable from the rate observed in the absence of drug (Figure 30). In contrast, the quinazolinedione slowed the rate of DNA religation mediated by both wild-type and $ParC^{S80L}$ *E. coli* topoisomerase IV (Figure 30). These results imply that the resistance mutation impairs quinolone, but not quinazolinedione, function.

Finally, the persistence of cleavage complexes formed by wild-type and $ParC^{S80L}$ topoisomerase IV in the presence of drugs was determined. Overall, the *E. coli* topoisomerase IV cleavage complexes (Figure 31) appear to be less stable than their *B. anthracis* topoisomerase IV counterparts (Figure 19). The $t_{1/2}$ of wild-type *E. coli*

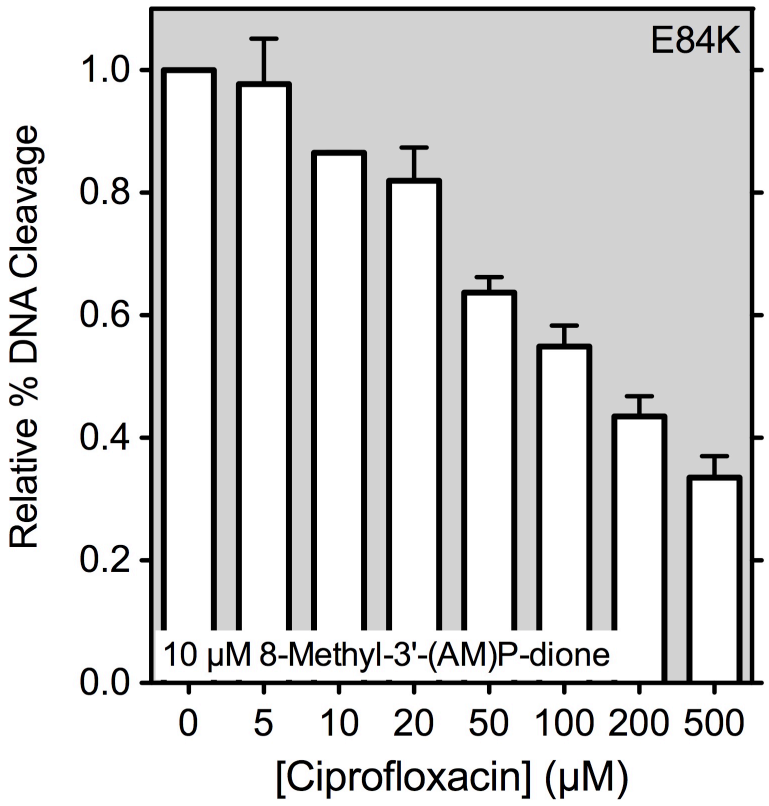


Figure 29. Ability of ciprofloxacin to compete out DNA cleavage induced by 8-methyl-3'-(AM)P-dione with $ParC^{E84K}$ topoisomerase IV from *E. coli*. The quinazolinedione was utilized at a concentration of 10 μM. The quinolone and quinazolinedione were added to reactions simultaneously. Error bars represent the standard deviation of at least three independent experiments.

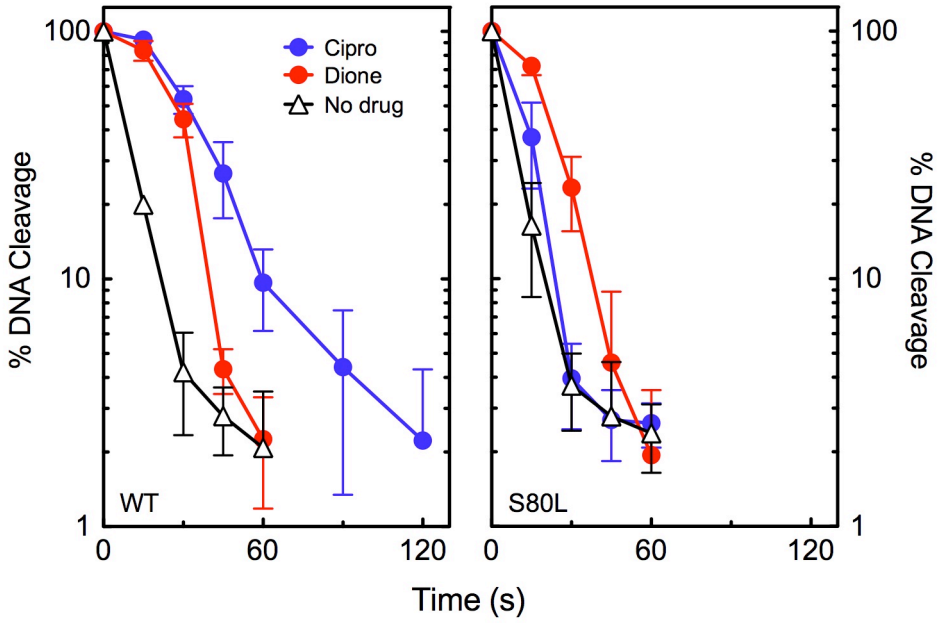


Figure 30. Effects of ciprofloxacin and 8-methyl-3'-(AM)P-dione on the DNA religation activities of wild-type and ParC^{S80L} topoisomerase IV from *E. coli*. Results for assays carried out in the absence of drugs are also shown. Religation was assessed by monitoring the loss of double-stranded DNA breaks (linear product) over time. Cleavage at time zero was set to 100%. Drugs were used at 20 μM in all assays. Error bars represent the standard deviation of at least three independent experiments.

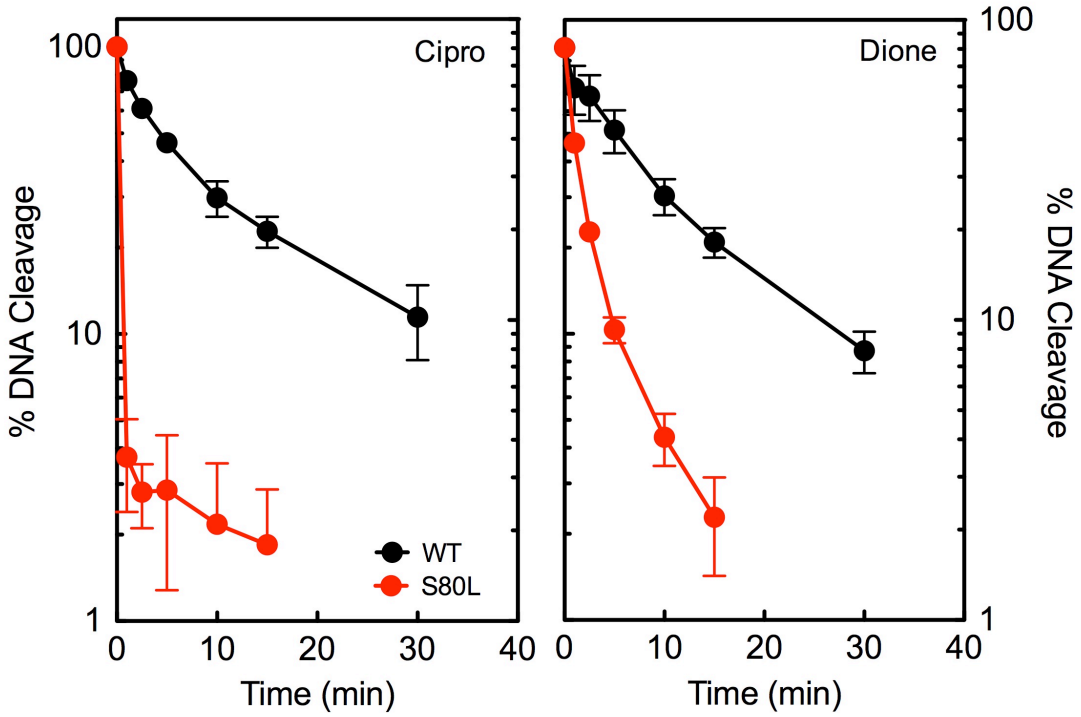


Figure 31. Effects of ciprofloxacin and 8-methyl-3'-(AM)P-dione on the persistence of ternary enzyme-drug-DNA cleavage complexes formed with wild-type and ParC^{S80L} topoisomerase IV from *E. coli*. Initial DNA cleavage-religation reactions were allowed to come to equilibrium and were then diluted 20-fold with DNA cleavage buffer. The persistence of cleavage complexes was assessed by monitoring the loss of double-stranded DNA breaks (linear product) over time. Cleavage at time zero was set to 100%. Drugs were used at 20 μM in all assays. Error bars represent the standard deviation of at least three independent experiments.

topoisomerase IV cleavage complexes formed in the presence of ciprofloxacin was very similar to that of complexes formed in the presence of 8-methyl-3'-(AM)P-dione (Figure 31). As expected, the persistence of $ParC^{S80L}$ cleavage complexes formed in the presence of ciprofloxacin was significantly decreased as compared to wild-type (Figure 31). However, the mutant quinazolinedione-induced cleavage complexes were also much less stable than the equivalent wild-type cleavage complexes (Figure 31). Despite this decreased stability, the $ParC^{S80L}$ quinazolinedione-induced cleavage complexes were still more stable than those formed in the presence of the quinolone. Therefore, quinolone resistance caused by the Ser80→Leu mutation correlates with a decreased stability of the topoisomerase IV-drug-DNA ternary complex. In addition, even though 8-methyl-3'-(AM)P-dione can induce high levels of $ParC^{S80L}$ cleavage complexes, they are less stable than those formed by the wild-type enzyme in the presence of the quinazolinedione.

Metal Ion Requirements for Quinolone-induced DNA Cleavage Mediated by Wild-type and $ParC^{S80L}$ Topoisomerase IV

To determine whether quinolone-enzyme interactions in *E. coli* topoisomerase IV are mediated by the water-metal ion bridge, a Mg^{2+} titration was carried out. The quinolone and quinazolinedione displayed similar requirements for Mg^{2+} when wild-type topoisomerase IV was employed; half-maximal and maximal DNA cleavage were observed at ~0.42 mM and ~1 mM Mg^{2+}, respectively (Figure 32). In contrast, the two drugs displayed markedly different Mg^{2+} requirements for DNA cleavage mediated by $ParC^{S80L}$. 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 32). Levels of Mg^{2+} required to generate half-maximal

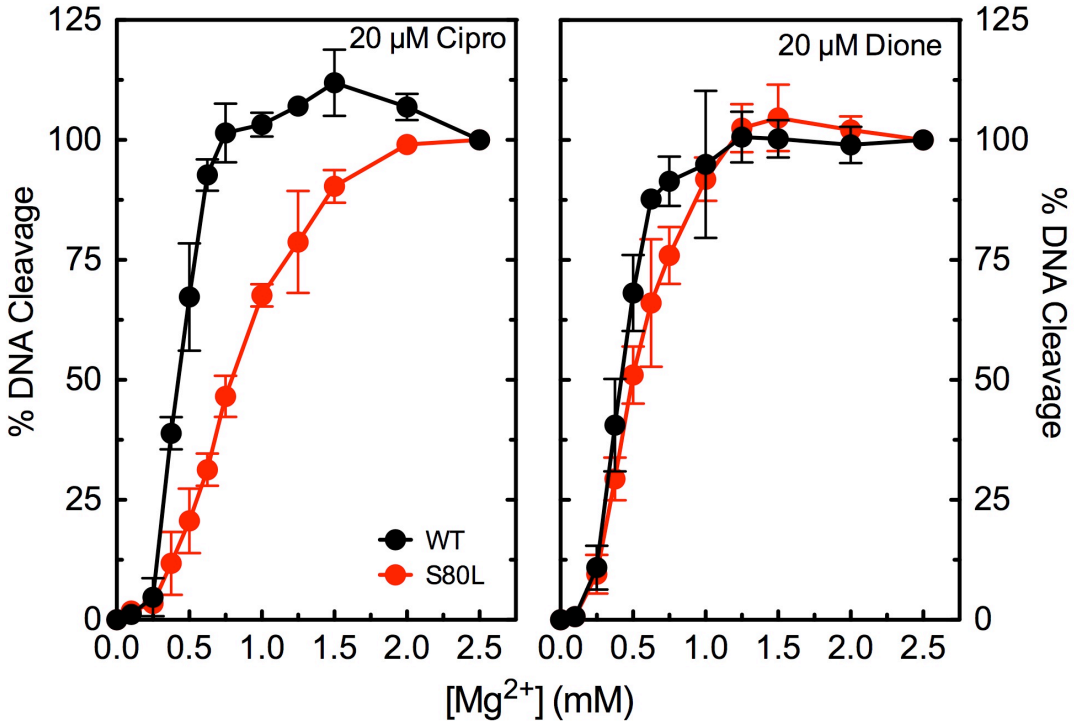


Figure 32. Effects of Mg^{2+} on DNA cleavage mediated by wild-type and $ParC^{S80L}$ topoisomerase IV from *E. coli*. Results are shown for 20 μM ciprofloxacin (left) and 20 μM 8-methyl-3'-(AM)P-dione (right). DNA cleavage for each drug-enzyme pair was normalized to 100% at 2.5 mM Mg^{2+} to facilitate direct comparisons. Error bars represent the standard deviation of at least three independent experiments.

and maximal DNA cleavage rose to ~0.79 mM and ~2 mM Mg^{2+}, respectively, in the presence of the quinolone. The requirement for higher Mg^{2+} concentrations to support quinolone- but not quinazolinedione-induced DNA cleavage by the mutant enzyme suggests that interactions between quinolones and *E. coli* topoisomerase IV are mediated by the water-metal ion bridge and that Ser80 plays a role in anchoring the bridge to the enzyme.

Summary of Conclusions

$ParC^{S80L}$ and $ParC^{E84K}$ topoisomerase IV from *E. coli* are resistant to quinolones but, like the wild-type enzyme, are sensitive to 8-methyl-3'-(AM)P-dione. Both of these mutations have larger effects on quinolone efficacy than do the equivalent mutations in *B. anthracis* topoisomerase IV, and ciprofloxacin actually displayed no ability to enhance DNA cleavage mediated by $ParC^{E84K}$. As seen with the *B. anthracis* Ser81 mutants, the ParC Ser80→Leu mutation decreases the ability of ciprofloxacin to inhibit enzyme-mediated DNA religation and also decreases the stability of quinolone-induced cleavage complexes. Moreover, it appears that the water-metal ion bridge is functional and a major point of interaction with quinolones in *E. coli* topoisomerase IV. Because the serine and acidic residues that anchor the bridge are highly conserved throughout the bacterial kingdom and the bridge interaction is functional in both a Gram-positive (*B. anthracis*) and Gram-negative (*E. coli*) topoisomerase IV, it appears that the water-metal ion bridge is not species-specific and is the major mechanism by which quinolones interact with all bacterial type II topoisomerases.

CHAPTER VII

OVERCOMING TARGET-MEDIATED QUINOLONE RESISTANCE

Introduction

Structural studies indicate that quinolones and quinazolinediones interact with topoisomerase IV in the same drug-binding pocket.[41] Several functional studies have demonstrated that members of the quinazolinedione drug class can overcome quinolone resistance caused by mutations in the serine or acidic amino acid residue,[105,107,123,124,129] suggesting that the quinazolinedione core does not require the water-metal ion bridge to interact with bacterial type II topoisomerases. Consistent with this idea, the C2 carbonyl of quinazolinediones appears to form a hydrogen bond with a conserved arginine residue (Arg119 in *B. anthracis* GrlA).[41,107] However, this interaction is expected to be weaker than the water-metal ion bridge interaction utilized by quinolones. Although quinazolinediones have been shown to maintain activity against resistant enzymes (and bacterial strains), they feature a 3'-(aminomethyl)pyrrolidinyl or 3'-(aminoethyl)pyrrolidinyl group at the C7 position (Figure 33), which is not represented in any clinically used quinolone.[105,107,123,124,129] Therefore, it is possible that quinazolinediones overcome resistance through the introduction of additional drug-enzyme contacts mediated by the C7 substituent, rather than through interactions mediated by the drug core.

Although it appears that the quinazolinediones are a drug class that could be devloped to combat quinolone-resistant infections, there have been no studies examining

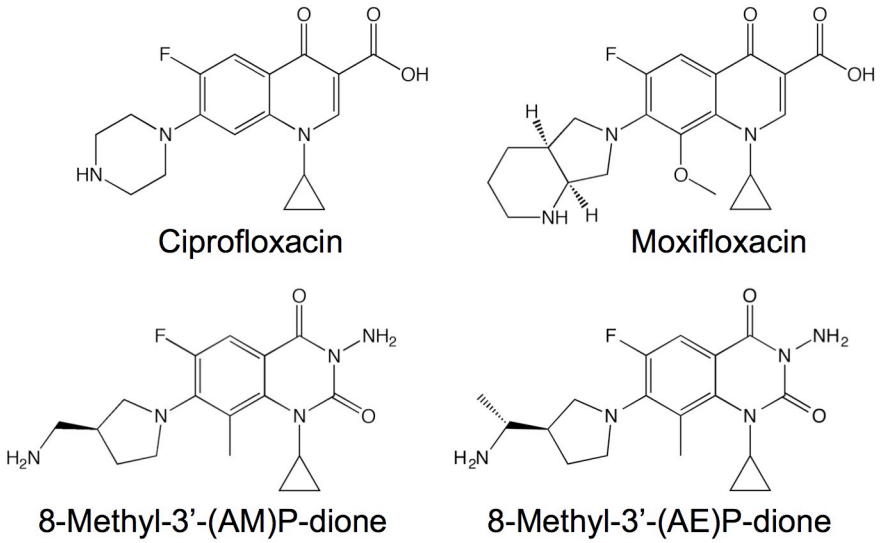


Figure 33. Structures of quinolones and quinazolinediones. Ciprofloxacin and moxifloxacin are clinically relevant quinolones. Ciprofloxacin contains a piperazinyl group at the C7 position. Moxifloxacin contains a diazabicyclononyl group at the C7 position. 8-Methyl-3'-(AM)P-dione and 8-methyl-3'-(AE)P-dione are quinazolinediones that have been shown to overcome quinolone resistance caused by topoisomerase mutations. 8-Methyl-3'-(AM)P-dione contains a 3'-(aminomethyl)pyrrolidinyl group at the C7 position. 8-Methyl-3'-(AE)P-dione contains a 3'-(aminoethyl)pyrrolidinyl group at the C7 position. The two different C7 groups of these quinazolinediones are not represented in any clinically relevant quinolone.

the effects of these compounds on human type II topoisomerases. Some quinolones have long been known to have high activity against the human enzymes,[87-90] so it is possible that some quinazolinediones may also have undesirable cross-reactivity.

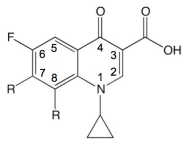
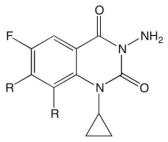
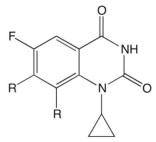
To resolve these issues and provide further understanding of target-mediated quinolone resistance, a series of quinazolinediones and quinolones that contain clinically utilized groups or a 3'-(aminomethyl)pyrrolidinyl group at the C7 position were tested for activity against wild-type and quinolone-resistant $GrlA^{S81F}$, $GrlA^{S81Y}$, and $GrlA^{E85K}$ *B. anthracis* topoisomerase IV. These drugs were also tested with human topoisomerase IIα to determine how clinically relevant quinolones differentiate between the human and bacterial type II enzymes and to determine potential causes of cross-reactivity with the human enzymes.

Results and Discussion

Contributions of the Quinazolinedione Core 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 34) were tested for activity against wild-type and quinolone-resistant $GrlA^{S81F}$, $GrlA^{S81Y}$, and $GrlA^{E85K}$ 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 35) and moxifloxacin (Figure 36), respectively, which are in wide clinical use. As expected,

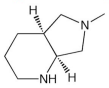
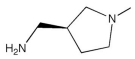
Position	C7	Fluoroquinolone C8	Quinazolinedione C8	"Non-amino" quinazolinedione C8
Piperazinyl (cipro)		-H	-H	
		$-CH_3$ $-OCH_3$	$-CH_3$ $-OCH_3$	$-CH_3$
Diazabicyclononyl (moxi)		-H	-H	
		$-CH_3$ $-OCH_3$	$-CH_3$ $-OCH_3$	$-CH_3$
3'-(Aminomethyl)pyrrolidinyl [3'-(AM)P]		-H $-CH_3$ $-OCH_3$	-H $-CH_3$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

Figure 34. Structures of matched quinolones and quinazolinediones used in Chapter VII. These compounds are based on the clinically relevant quinolones ciprofloxacin and moxifloxacin and the quinazolinedione 8-methyl-3'-(AM)P-dione that overcomes resistance caused by topoisomerase mutations. "Non-amino" quinazolinediones are identical to quinazolinediones except that they lack the N3 amino group. Table 1 contains the full chemical, library, and abbreviated names for these compounds.

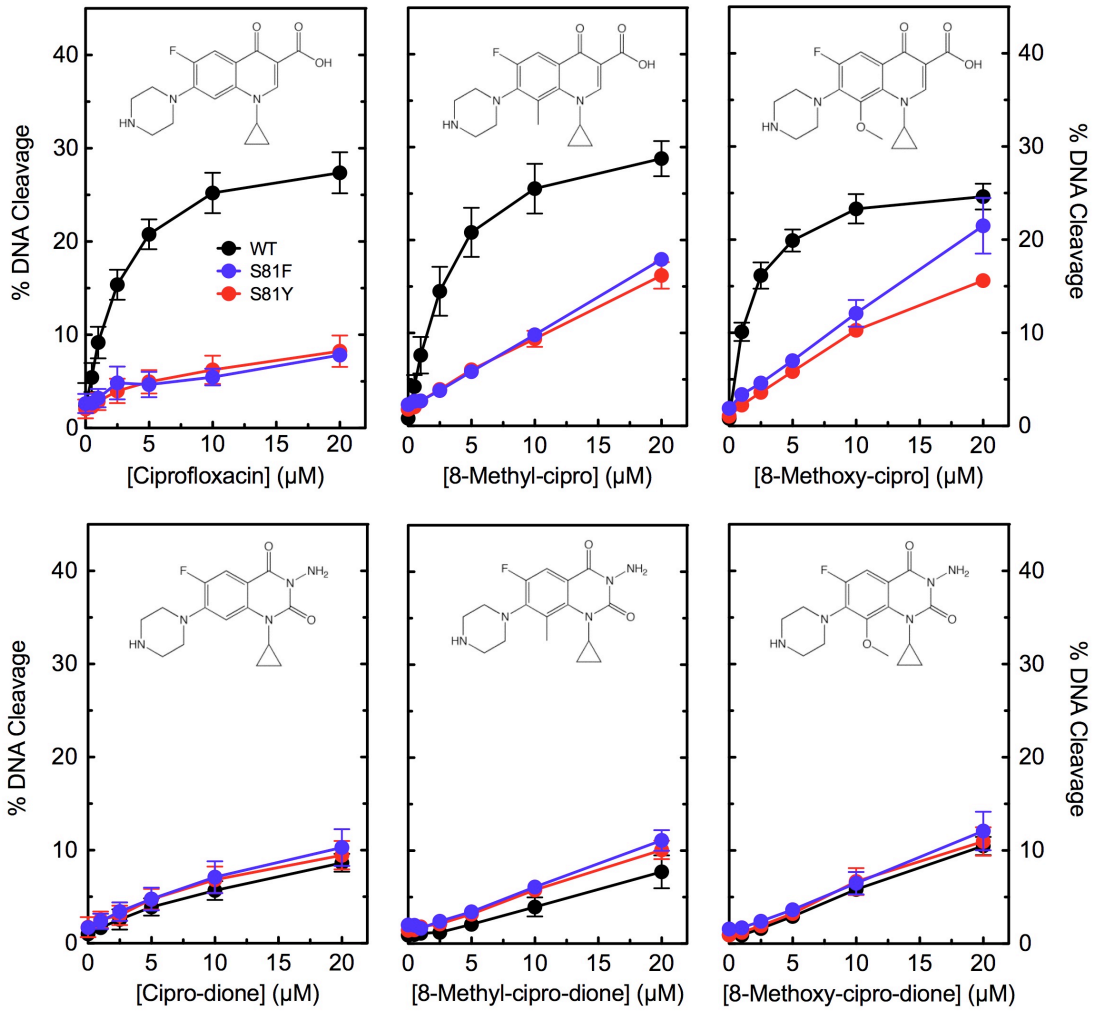


Figure 35. Effects of a ciprofloxacin-based series of quinolones and quinazolinediones on the DNA cleavage activity of *B. anthracis* topoisomerase IV. The ability of wild-type, GrlA^{S81F}, and GrlA^{S81Y} topoisomerase IV to cleave negatively supercoiled pBR322 DNA in the presence of quinolones (top) or quinazolinediones (bottom) containing a C7 piperazinyl group and a C8 hydrogen (left), methyl (middle), or methoxy (right) group is shown. Drug structures are shown in the corresponding panels. Error bars represent the standard deviation of at least three independent experiments.

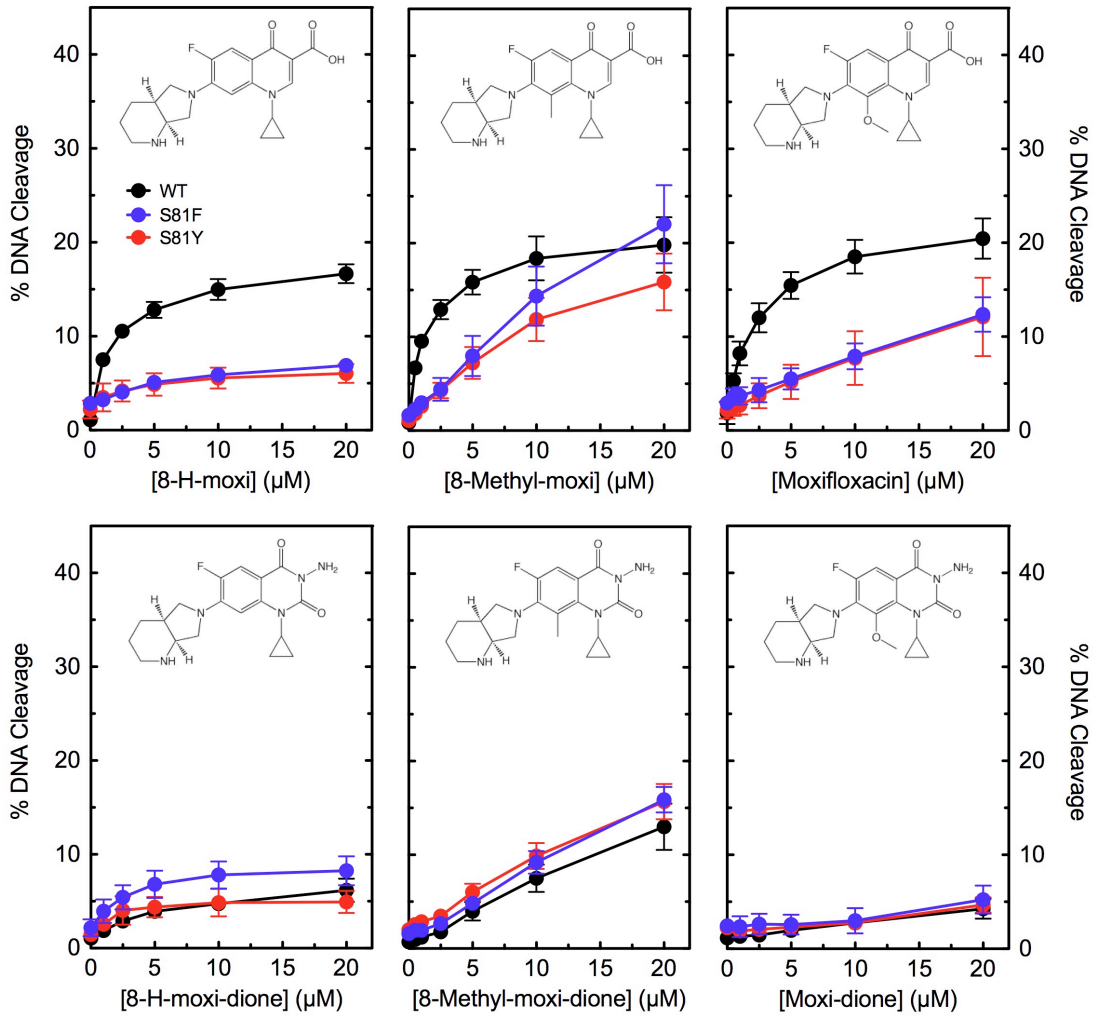


Figure 36. Effects of a moxifloxacin-based series of quinolones and quinazolinediones on the DNA cleavage activity of *B. anthracis* topoisomerase IV. The ability of wild-type, GrlA^{S81F}, and GrlA^{S81Y} topoisomerase IV to cleave negatively supercoiled pBR322 DNA in the presence of quinolones (top) or quinazolinediones (bottom) containing a C7 diazabicyclononyl group and a C8 hydrogen (left), methyl (middle), or methoxy (right) group is shown. Drug structures are shown in the corresponding panels. Error bars represent the standard deviation of at least three independent experiments.

all of the quinolones in both series displayed high activity against wild-type *B. anthracis* topoisomerase IV but decreased activity against the resistant Ser81 and Glu85 mutant enzymes. In contrast, the parallel quinazolinedione series 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 core does not form strong interactions with topoisomerase IV.

To test this conclusion, a series of quinazolinediones and quinolones that feature the 3'-(aminomethyl)pyrrolidinyl group at C7 were examined (Figure 37). Consistent with previous reports,[105,107,124] all of the quinazolinediones in this series showed high activity against wild-type and mutant quinolone-resistant topoisomerase IV. Significantly, the parallel quinolone series displayed similar results and maintained high activity against the resistant enzymes. Thus, in the presence of the C7 3'-(aminomethyl)pyrrolidinyl substituent, the drug core (quinolone *vs.* quinazolinedione) makes little difference.

Taken together, these results indicate that the quinazolinedione core 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 the 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.

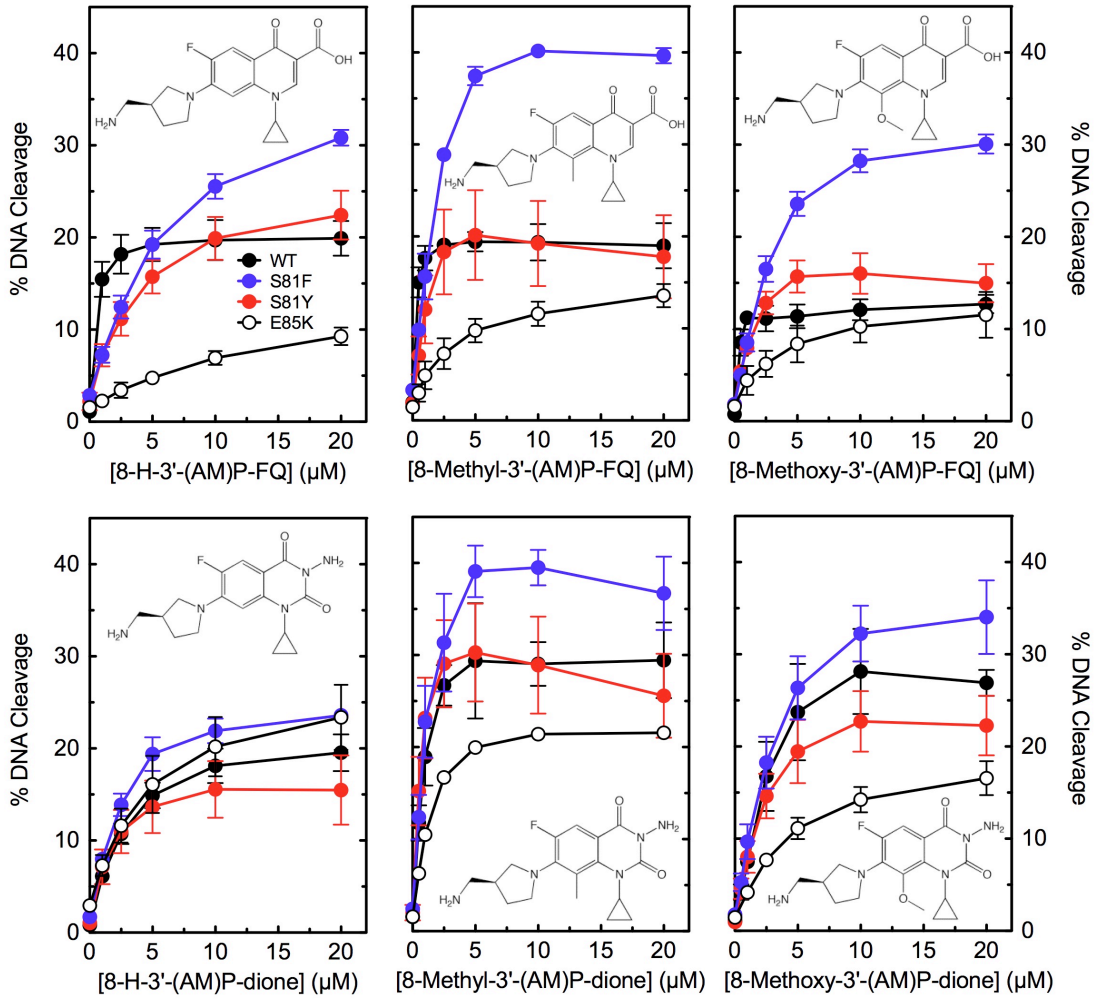


Figure 37. Effects of an 8-methyl-3'-(AM)P-dione-based series of quinolones and quinazolinediones on the DNA cleavage activity of *B. anthracis* topoisomerase IV. The ability of wild-type, GrlA^{S81F}, GrlA^{S81Y}, and GrlA^{E85K} topoisomerase IV to cleave negatively supercoiled pBR322 DNA in the presence of quinolones (top) or quinazolinediones (bottom) containing a C7 3'-(aminomethyl)pyrrolidinyl group and a C8 hydrogen (left), methyl (middle), or methoxy (right) group is shown. Drug structures are shown in the corresponding panels. Error bars represent the standard deviation of at least three independent experiments.

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 35-37). Generally, the inclusion of a methyl or methoxy group slightly enhances 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 38). 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. These 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 (Figure 39). However, the basis for this discrimination is not known. As discussed above, the primary interaction between clinically relevant quinolones and bacterial type II enzymes is mediated through the water-metal ion bridge. 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, these residues are both methionine (Figure 39), and, in the β isoform, they are a glutamine and a

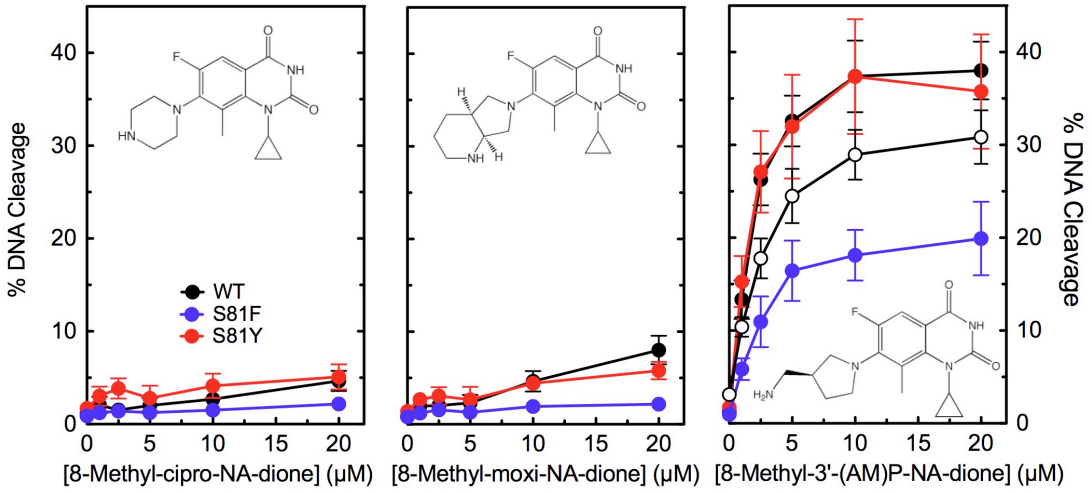


Figure 38. Effects of quinazolinediones lacking the N3 amino group on the DNA cleavage activity of *B. anthracis* topoisomerase IV. The ability of wild-type, GrlA^{S81F}, and GrlA^{S81Y} topoisomerase IV to cleave negatively supercoiled pBR322 DNA in the presence of "non-amino" quinazolinediones containing a C8 methyl group and a C7 piperazinyl (left), diazabicyclononyl (middle), or 3'-(aminomethyl)pyrrolidinyl (right) group is shown. Results with 8-methyl-3'-(AM)P-NA-dione and GrlA^{E85K} topoisomerase IV are also shown. Drug structures are shown in the corresponding panels. Error bars represent the standard deviation of at least three independent experiments.

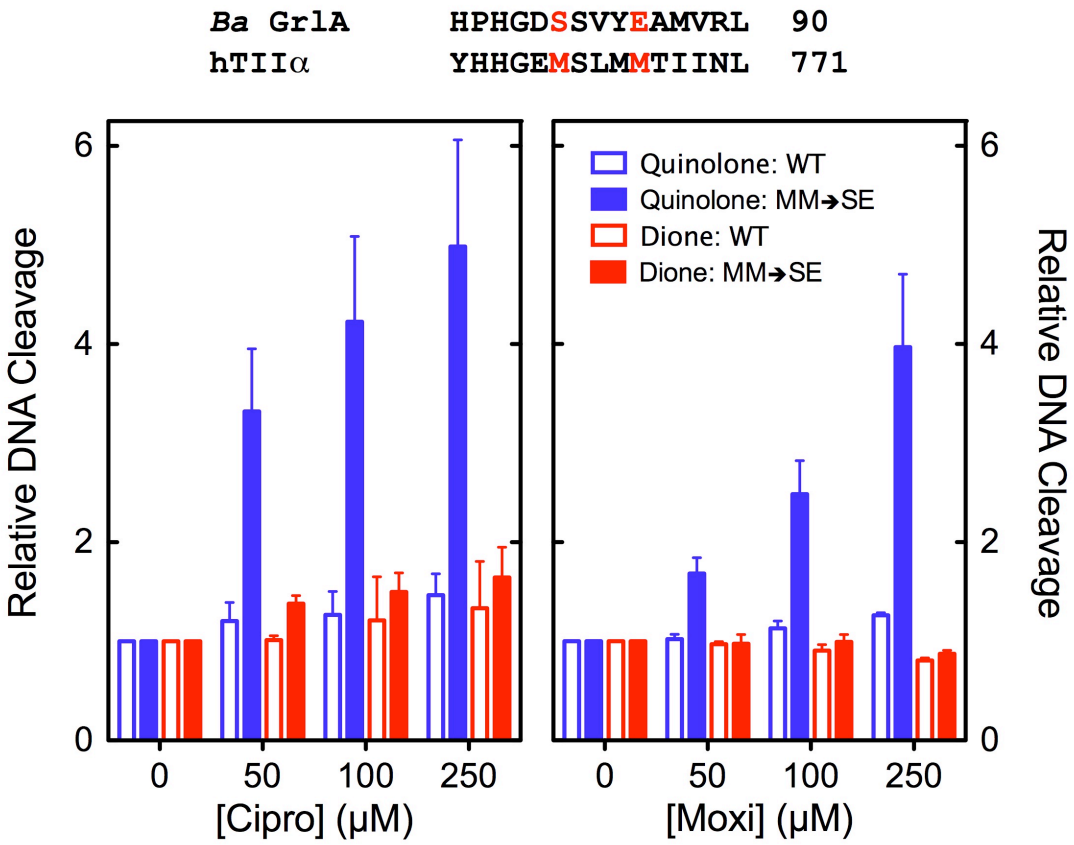


Figure 39. Effects of ciprofloxacin, moxifloxacin, and their matched quinazolinediones on the DNA cleavage activities of wild-type and mutant (hTop2A$^{M762S/M766E}$) human topoisomerase IIα. A sequence alignment of wild-type *B. anthracis* GrlA and human topoisomerase IIα is shown at the top. The methionine 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. The effects of ciprofloxacin and cipro-dione (left) and moxifloxacin and moxi-dione (right) on the DNA cleavage activities of wild-type and mutant human topoisomerase IIα are shown. Error bars represent the standard deviation of at least three independent experiments.

methionine. Therefore, the loss of the bridge anchors in topoisomerase IIα and IIβ could account for the discrimination between 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 39). In contrast to results with the wild-type human enzyme, both ciprofloxacin and moxifloxacin displayed concentration-dependent activity against hTop2A$^{M762S/M766E}$, increasing levels of DNA cleavage ≥4-fold over the range examined (Figure 39). Consistent with these 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.[145] 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 39). These compounds are identical to ciprofloxacin and moxifloxacin, except that they utilize the quinazolinedione core rather than the quinolone core. Neither of the two compounds increased DNA cleavage mediated by the wild-type or mutant 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 core, 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 IIα

Quinolones that display high activity against eukaryotic type II topoisomerases have been reported.[87-90] To this point, CP-115,955 and CP-115,953 increase DNA cleavage mediated by human topoisomerase IIα (Figure 40) to a greater extent than does the widely prescribed anticancer drug etoposide.[146-149] 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 parallel those of compounds with the C7 3'-(aminomethyl)pyrrolidinyl substituent with quinolone-resistant *B. anthracis* topoisomerase IV. 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 finding raises the possibility that compounds containing the C7 3'-(aminomethyl)pyrrolidinyl substituent might display undesirable cross-reactivity against human type II topoisomerases. With either a quinazolinedione or a quinolone that contained a C8 methyl group, compounds that included the C7

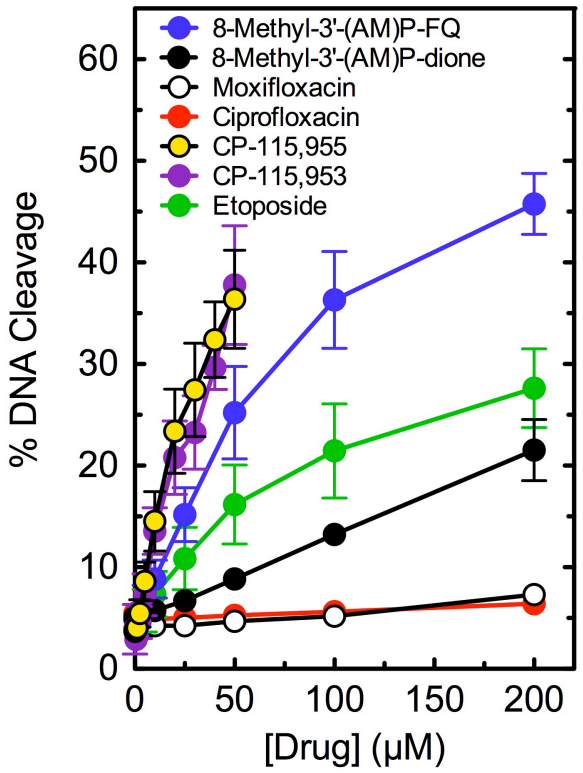


Figure 40. Effects of 8-methyl-3'-(AM)P-dione and 8-methyl-3'-(AM)P-FQ on the DNA cleavage activity of human topoisomerase IIα. These compounds contain a 3'-(aminomethyl)pyrrolidinyl group at the C7 position and a methyl group at the C8 position. Results for moxifloxacin, ciprofloxacin, CP-115,955, CP-115,953, and etoposide are shown for comparison. Error bars represent the standard deviation of at least three independent experiments.

3'-(aminomethyl)pyrrolidinyl substituent displayed high activity against topoisomerase IIα (Figure 40). This finding implies that the ability to design quinolones or related drugs that overcome resistance in bacterial gyrase or topoisomerase IV may be hampered by cross-reactivity with the human enzymes. 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 35-38).

With regard to quinolone interactions, human topoisomerase IIα largely mimics a drug-resistant topoisomerase IV mutant. 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) the effects of C8 and N3 substituents on drug activity against topoisomerase IIα were examined. 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 37 and 38).

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 were somewhat lower than the corresponding compounds with C8 methyl groups (Figure 41), 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 42) that displayed high activity against wild-type and mutant topoisomerase IV (Figures 37 and 38) showed virtually no activity against topoisomerase IIα at drug concentrations below 100 μM (Figure 42). This concentration is well above the therapeutic window for clinically used quinolones.[150] 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 supported 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 groups to drug-topoisomerase IV interactions are relatively unimportant. In contrast, these groups appear to be a determining factor in whether a compound has activity against human topoisomerase IIα. That being said, the C7 3'-(aminomethyl)pyrrolidinyl substituent still seems to be the most important of the

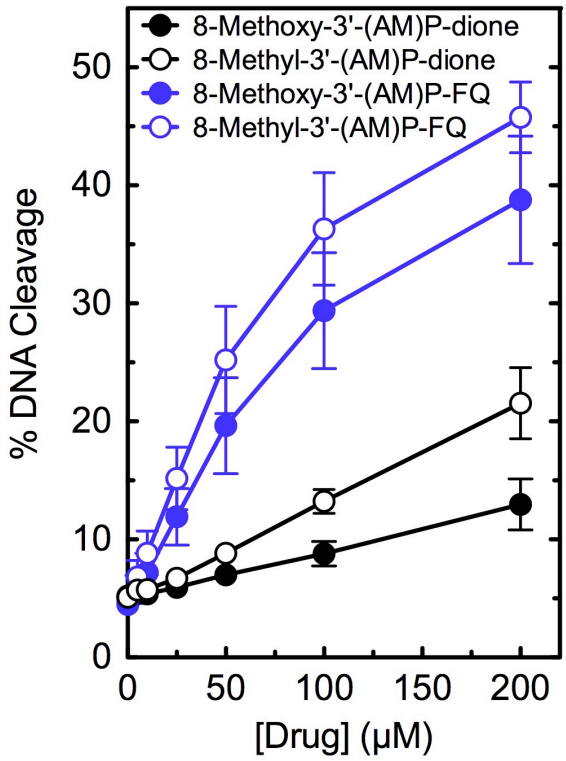


Figure 41. Effects of 8-methoxy-3'-(AM)P-dione and 8-methoxy-3'-(AM)P-FQ on the DNA cleavage activity of human topoisomerase IIα. These compounds contain a 3'-(aminomethyl)pyrrolidinyl group at the C7 position and a methoxy group at the C8 position. Results for the C8 methyl versions of these compounds are shown for comparison. Error bars represent the standard deviation of at least three independent experiments.

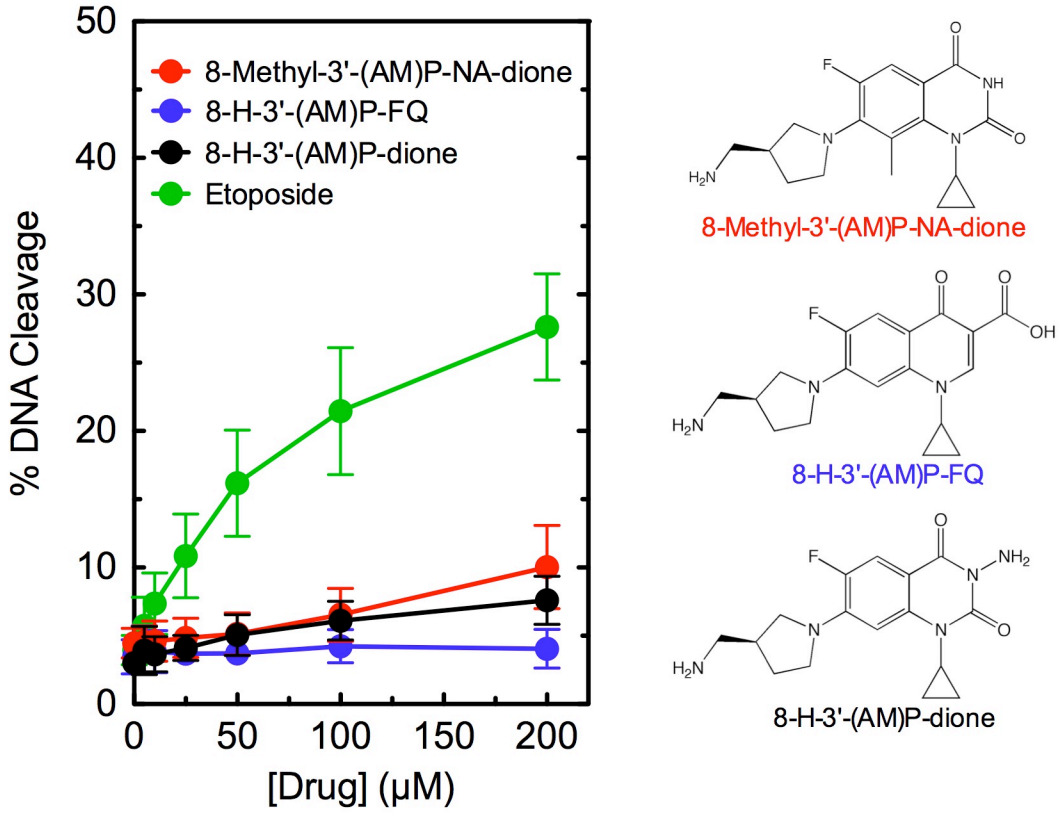


Figure 42. Effects of 8-methyl-3'-(AM)P-dione-based compounds on the cleavage activity of human topoisomerase IIα. The ability of the human type II enzyme to cleave negatively supercoiled pBR322 DNA in the presence of a quinolone and a quinazolinedione containing a C7 3'-(aminomethyl)pyrrolidinyl group and a hydrogen at C8 and a "non-amino" quinazolinedione containing a C7 3'-(aminomethyl)pyrrolidinyl group and a C8 methyl group are shown. The structures of these compounds (which overcome quinolone resistance in mutant *B. anthracis* topoisomerase IV) are at the right. Results with etoposide are shown for comparison. Error bars represent the standard deviation of at least three independent experiments.

three. To this point, quinolones or N3-amino-quinazolinediones that include the C7 groups of ciprofloxacin or moxifloxacin display low activity against the human type II enzyme, even in the presence of a methyl or methoxy group at C8 (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 core to drug interactions with topoisomerase IIα. In these experiments, the ability of compounds to compete out DNA cleavage induced by 50 μM 8-methyl-3'-(AM)P-FQ was assessed (Figure 43). The IC_{50} 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} 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 of Conclusions

Because the quinazolinedione core does not appear to mediate any strong interactions with the bacterial type II topoisomerases, quinazolinediones are essentially quinolones that lack their most important interaction with the bacterial enzymes. Thus, quinazolinediones that display high activity against wild-type and quinolone-resistant

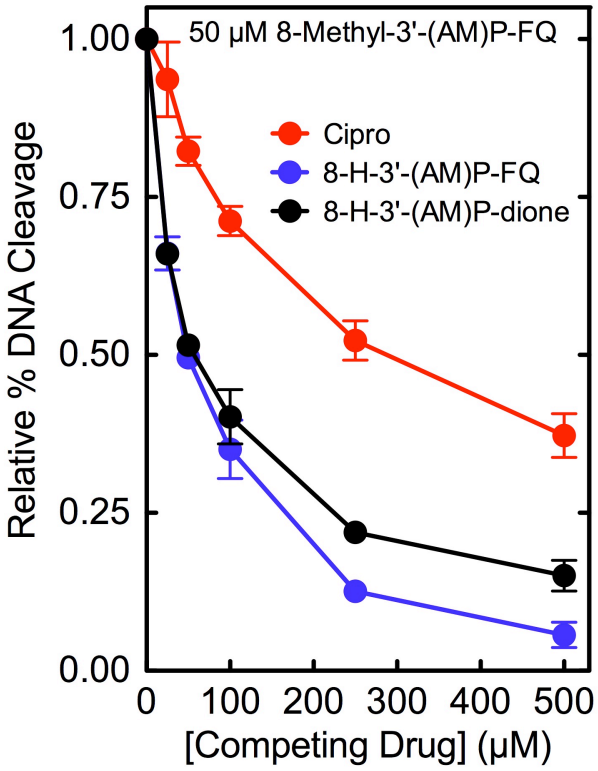


Figure 43. Ability of ciprofloxacin, 8-H-3'-(AM)P-FQ, and 8-H-3'-(AM)P-dione to compete out DNA cleavage induced by 50 μM 8-methyl-3'-(AM)P-FQ with human topoisomerase IIα. 8-Methyl-3'-(AM)P-FQ and the competing drug were added to reactions simultaneously. Initial levels of DNA cleavage (*i.e.,* in the absence of competitor) were set to 1.00 to facilitate comparisons. Error bars represent the standard deviation of at least three independent experiments.

type II topoisomerases do so primarily by establishing novel interactions with the enzymes through the C7 substituent. The N3 and C8 substituents make minor contributions to the interactions of these drugs with the bacterial enzymes. Clinically relevant quinolones display poor activity against human topoisomerase IIα 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 with human topoisomerase IIα (Figure 44). 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 – 8-H-3'-(AM)P-FQ, 8-H-3'-(AM)P-dione, and 8-methyl-3'-(AM)P-NA-dione – have been identified. Levofloxacin (which contains a C7 group similar to that of ciprofloxacin but has a tricyclic drug core) shows very little ability to compete out DNA cleavage induced by 8-methyl-3'-(AM)P-FQ with human topoisomerase IIα (not shown). Therefore, it may also be possible to use a tricyclic quinolone core (like that of levofloxacin) to selectively target the bacterial type II topoisomerases. For example, substituting a 3'-(aminomethyl)pyrrolidinyl group for the N-methylpiperazinyl C7 group on levofloxacin may result in a quinolone that has high activity against quinolone-resistant bacterial enzymes (due to drug-enzyme interactions mediated by the C7 group) but no activity against the human enzyme (due to selectivity provided by the cyclization between the C8 and N1 substituents).

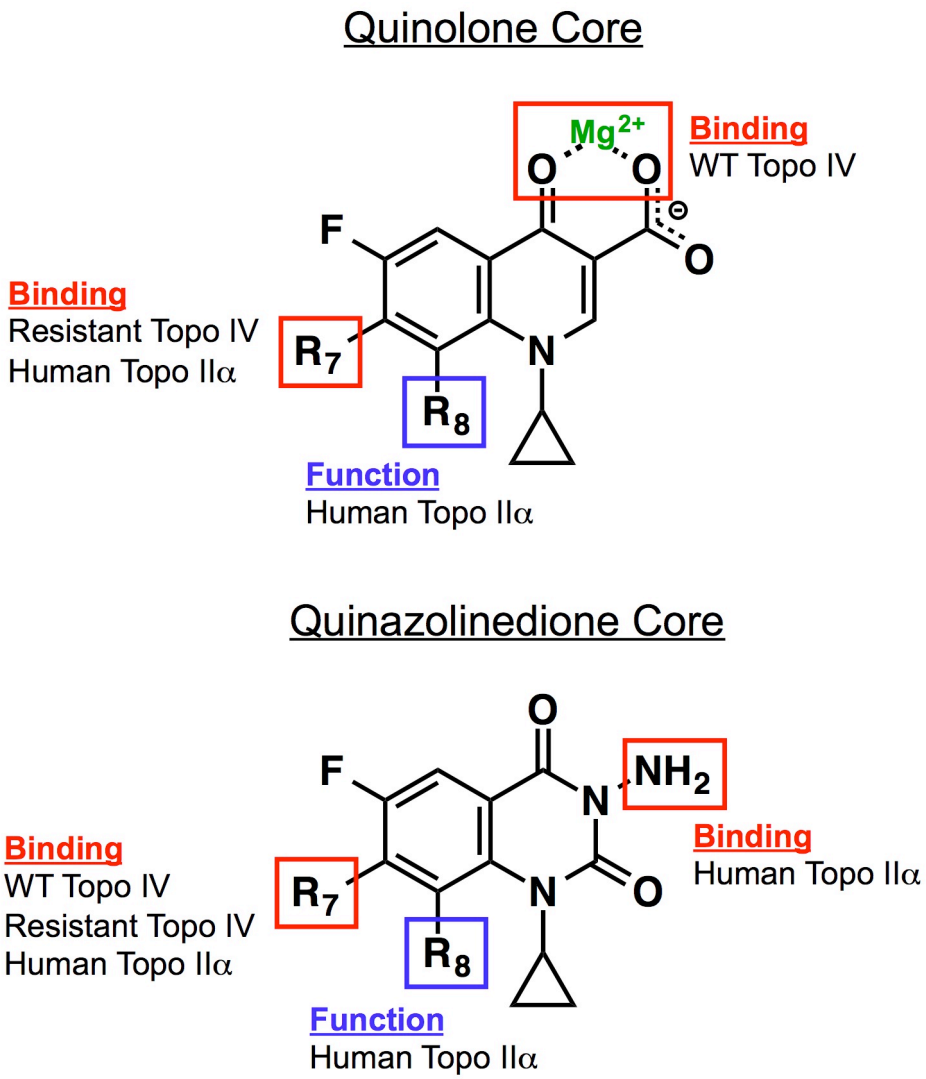


Figure 44. Roles of substituents and core elements of quinolones and quinazolinediones in mediating drug activity against bacterial and human type II topoisomerases. Quinolones: The binding of clinically relevant quinolones to topoisomerase IV is mediated primarily through the water-metal ion bridge. The binding of quinolones that overcome resistance is mediated primarily by the C7 substituent. Quinolone binding to human topoisomerase IIα is also mediated by the C7 substituent. The C8 group affects the ability of quinolones to act against the human type II enzyme but is not required for drug binding. Quinazolinediones: Interactions between quinazolinediones and topoisomerase IV (wild-type and quinolone-resistant) are mediated through the C7 substituent. The effects of the C7 and C8 substituents on quinazolinedione activity against topoisomerase IIα are the same as described for the quinolones. The N3 amino group plays a role in the binding of quinazolinediones to the human enzyme.

CHAPTER VIII

QUINOLONES WITH POTENTIAL AS ANTICANCER CHEMOTHERAPEUTICS

Introduction

Human type II topoisomerases are targeted by various anticancer agents, including etoposide, doxorubicin, mitoxantrone, and amsacrine.[22,34] These drugs function similarly to the quinolones in that they enhance topoisomerase-mediated DNA cleavage and ultimately kill cells by increasing the concentration of double-stranded DNA breaks to the point that they overwhelm repair processes and trigger apoptosis.[22,34]

Some quinolones, including CP-115,953 and CP-115,955, have high activity against both human and bacterial type II topoisomerases.[87-90] However, little is known regarding the similarities and differences between their interactions with the human and bacterial enzymes. To explore how these drugs interact with type II topoisomerases, a series of CP-115,955-based compounds was tested with wild-type and quinolone-sensitive M762S/M766E human topoisomerase IIα and wild-type and quinolone-resistant $GrlA^{S81F}$ and $GrlA^{S81Y}$ *B. anthracis* topoisomerase IV. Compounds that were examined included quinolones, quinazolinediones, and non-amino-quinazolinediones containing a 4'-hydroxyphenyl substituent at the C7 position and a C8 hydrogen, methyl, or methoxy group. A subset of these compounds was also tested in human CEM cells to determine whether the activities observed *in vitro* were paralleled in the context of the cell.

Results and Discussion

Effects of CP,115-955-based Compounds on DNA Cleavage Mediated by Wild-type and Quinolone-Resistant GrlA^{S81F} and GrlA^{S81Y} Topoisomerase IV from B. anthracis

CP-115,955 is an experimental quinolone that differs from ciprofloxacin only by the inclusion of a 4'-hydroxyphenyl group in place of the piperazinyl moiety at the C7 position. As discussed in Chapter VII, this drug (and CP-115,953, which differs from CP-115,955 only by the inclusion of a fluorine at the C8 position) has high activity against both bacterial and human type II topoisomerases. Therefore, much like the 3'-(aminomethyl)pyrrolidinyl group, it may have cross-reactivity with the human system due to the C7 4'-hydroxyphenyl mediating strong interactions with both enzymes. To test this hypothesis, the effects of a series of quinolones, quinazolinediones, and non-amino-quinazolinediones on DNA cleavage mediated by wild-type and quinolone-resistant topoisomerase IV were examined.

As expected, all three quinolones showed high activity against wild-type *B. anthracis* topoisomerase IV (Figure 45). However, the GrlA^{S81F} and GrlA^{S81Y} mutant enzymes were resistant to these compounds (Figure 45). This finding suggests that CP-115,955 (and related quinolones) interacts with bacterial type II topoisomerases through the water-metal ion bridge and that the C7 4'-hydroxyphenyl group, like the C7 groups of the clinically relevant quinolones, mediates little interaction with the bacterial enzymes.

As observed with the quinazolinediones containing a clinically relevant C7 group (discussed in Chapter VII), CP-115,955-based quinazolinediones showed decreased activity against both wild-type and mutant topoisomerase IV (Figure 45). In addition, the removal of the N3 amino group decreased the activity of these quinazolinediones by

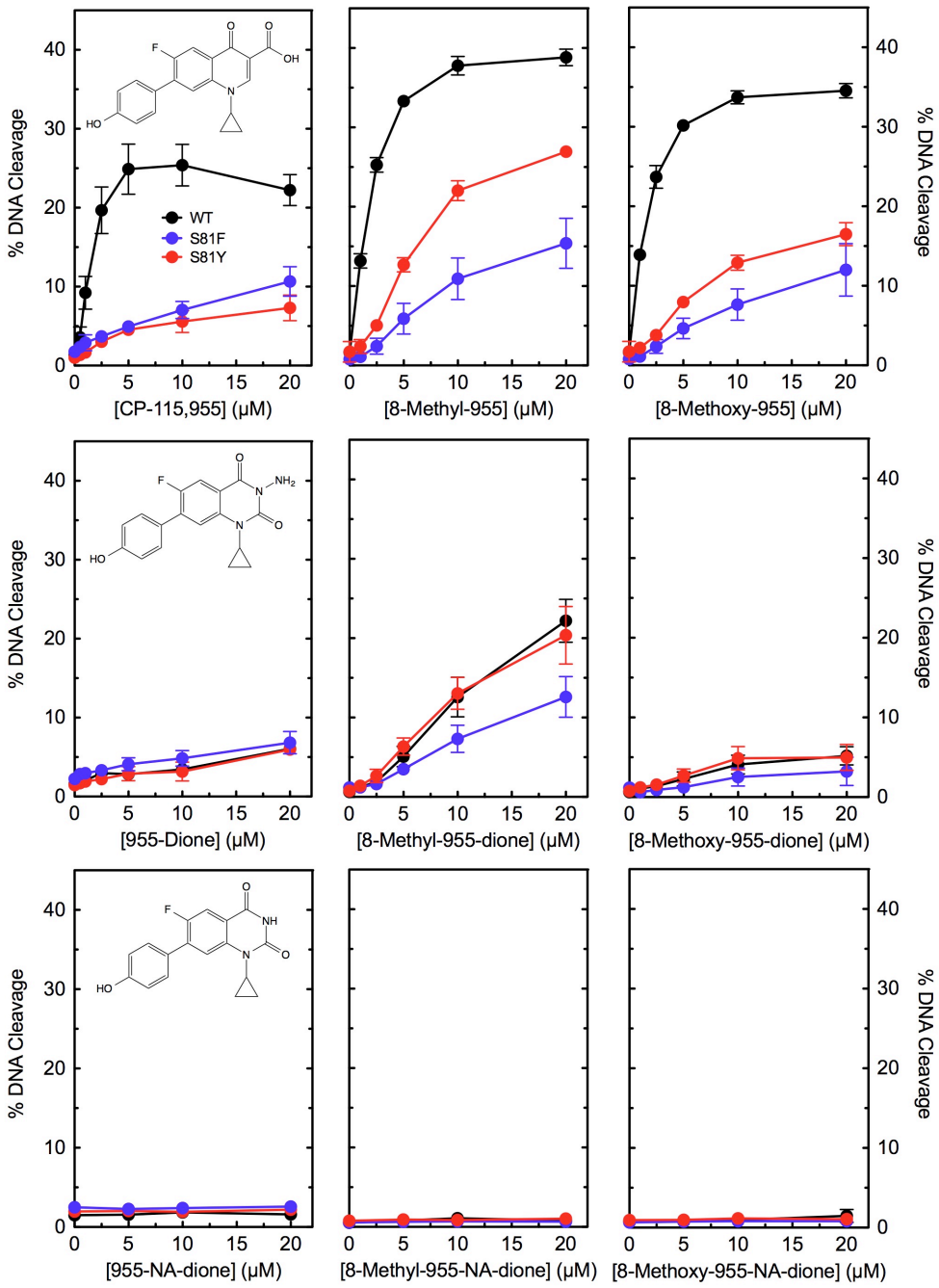


Figure 45. Effects of a CP-115,955-based series of quinolones, quinazolinediones, and non-amino-quinazolinediones on the DNA cleavage activity of *B. anthracis* topoisomerase IV. The ability of wild-type, GrlA^{S81F}, and GrlA^{S81Y} topoisomerase IV to cleave negatively supercoiled pBR322 DNA in the presence of quinolones (top), quinazolinediones (middle), or non-amino-quinazolinediones (bottom) containing a C7 4'-hydroxyphenyl group and a C8 hydrogen (left), methyl (middle), or methoxy (right) group is shown. Representative drug structures are shown in the left panels. Error bars represent the standard deviation of at least three independent experiments.

≥50% (Figure 45). Finally, the inclusion of a C8 methyl or methoxy group somewhat enhanced the activity of the compounds against the bacterial enzymes (Figure 45).

Taken together, these results indicate that CP-115,955-based quinolones and quinazolinediones interact with topoisomerase IV in much the same way that quinolones and quinazolinediones containing clinically relevant C7 groups [rather than a C7 3'-(aminomethyl)pyrrolidinyl group] interact with the bacterial enzyme. The primary interaction between the quinolones and the bacterial type II enzymes is the water-metal ion bridge, the quinazolinediones are essentially quinolones that lack their most important interaction with the enzymes, and the C8 and N3 substituents display some ability modulate the activities of the compounds.

Effects of CP-115,955-based Compounds on Wild-type and Quinolone-sensitive M762S/M766E Human Topoisomerase IIα

To explore the interactions between CP-115,955-based compounds and human type II topoisomerases, the drugs were first tested for their ability to enhance DNA cleavage mediated by wild-type topoisomerase IIα. As expected, all three quinolones displayed high activity against the enzyme (Figure 46). This activity actually exceeded that of the anticancer drug etoposide. As observed with 3'-(aminomethyl)pyrrolidinyl-containing compounds, the activities of CP-115,955-based quinazolinediones against topoisomerase IIα were reduced as compared to their quinolone counterparts (Figure 46). In addition, removal of the N3 amino group further decreased the activities of the quinazolinediones (Figure 46).

Combined with observations from Chapter VII, these results indicate that quinolones tend to have higher activity against all type II topoisomerases than do

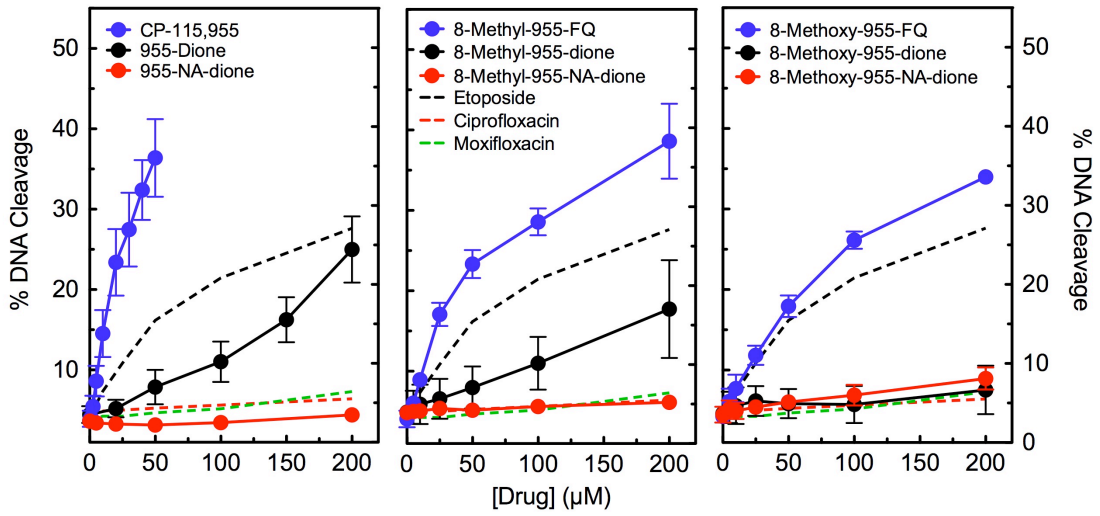


Figure 46. Effects of a CP-115,955-based series of quinolones, quinazolinediones, and non-amino-quinazolinediones on the DNA cleavage activity of human topoisomerase IIα. The ability of the enzyme to cleave negatively supercoiled pBR322 DNA in the presence of quinolones, quinazolinediones, or non-amino-quinazolinediones containing a C7 4'-hydroxyphenyl group and a C8 hydrogen (left), methyl (middle), or methoxy (right) group is shown. Results with etoposide, ciprofloxacin, and moxifloxacin are shown for comparison. Error bars represent the standard deviation of at least three independent experiments.

quinazolinediones. The basis for this difference of activity in the bacterial enzymes can be attributed to the loss of the water-metal ion bridge interaction upon changing from a quinolone core to a quinazolinedione core. However, because wild-type human type II topoisomerases cannot mediate the bridge interaction, the basis for this difference in activity in the human system is not clear. Presumably, either a portion of the quinolone core mediates a weak, non-bridge interaction with the enzyme or a portion of the quinazolinedione core sterically clashes with the enzyme.

In contrast to results with the 3'-(aminomethyl)pyrrolidinyl series of quinolones and quinazolinediones, the inclusion of a C8 group in the CP-115,955 series decreased, rather than increased, the activities of the compounds against human topoisomerase IIα (Figure 46). These results suggest that a very specific interaction occurs between the C7 4'-hydroxyphenyl group and the enzyme and the presence of a C8 group can alter this interaction. The C8 group could alter C7-enzyme interactions by changing the electronics and/or planarity of the compounds. It is also possible that the 4'-hydroxyphenyl group slips into a tight pocket in the enzyme, thus causing steric crowding between the C8 methyl or methoxy group and the protein.

Next, competition experiments were carried out to determine why some compounds that contained a C7 4'-hydroxyphenyl group did not have activity against human topoisomerase IIα. Thus, the ability of ciprofloxacin, 8-methoxy-955-dione, and 955-NA-dione to compete out cleavage induced by 20 μM CP-115,955 was assessed.

Ciprofloxacin displayed very little ability to compete with CP-115,955 for binding to the human enzyme (Figure 47). This indicates that there is a strong interaction

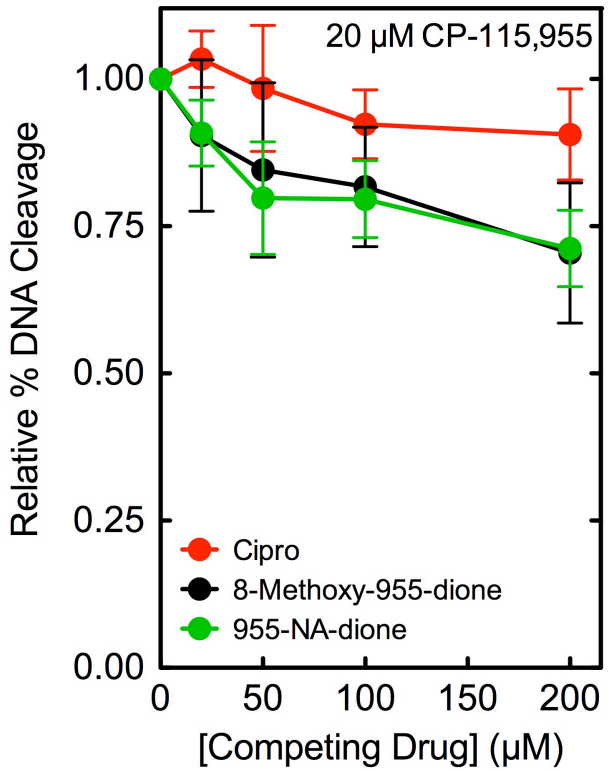


Figure 47. Ability of ciprofloxacin, 8-methoxy-955-dione, and 955-NA-dione to compete out DNA cleavage induced by 20 μM CP-115,955 with human topoisomerase IIα. CP-115,955 and the competing drug were added to reactions simultaneously. Initial levels of DNA cleavage (*i.e.,* in the absence of competitor) were set to 1.00 to facilitate comparisons. Error bars represent the standard deviation of at least three independent experiments.

between topoisomerase IIα and the C7 4'-hydroxyphenyl group of CP-115,955 that cannot be facilitated by the C7 piperazinyl moiety of ciprofloxacin.

8-Methoxy-955-dione and 955-NA-dione displayed similar and weak abilities to compete with CP-115,955 (Figure 47). Results with 8-methoxy-955-dione suggest that the inclusion of a C8 substituent decreases the binding of the drugs to the enzyme, rather than positioning the drugs in a way that allows them to bind but not enhance cleavage. This is opposite of the effect seen with the C7 3'-(aminomethyl)pyrrolidinyl series (see Chapter VII), in which quinolones and quinazolinediones that did not enhance topoisomerase IIα-mediated DNA cleavage could bind to the enzyme equally as well as those in the series that did enhance cleavage. Results with 955-NA-dione suggest that the N3 amino group of the quinazolinedione core mediates some interaction with the human type II enzyme. This parallels the effects of the removal of the N3 substituent in the 3'-(aminomethyl)pyrrolidinyl series. Thus, the N3 amino group plays the same role in quinazolinedione-enzyme interactions regardless of the structure of the C7 substituent.

Mutation of Met762 and Met766 of human topoisomerase IIα to the residues seen in *B. anthracis* topoisomerase IV that coordinate the water-metal ion bridge sensitizes the human enzyme to clinically relevant quinolones (Figure 39). CP-115,955 interacts with bacterial type II topoisomerases through the bridge interaction, while the C7 group facilitates its activity against wild-type topoisomerase IIα. To determine whether the introduction of a second strong interaction between CP-115,955 and the human enzyme would increase the activity of the compound, its ability to enhance DNA cleavage mediated by hTop2A$^{M762S/M766E}$ was assessed. The levels of cleavage induced by CP-115,955 in the presence of wild-type and mutant topoisomerase IIα were

indistinguishable (Figure 48). Similar results were observed with wild-type topoisomerase IV and the quinolones and quinazolinediones containing a C7 3'-(aminomethyl)pyrrolidinyl group (Figure 37).

Although CP-115,955 did not induce higher levels of DNA cleavage mediated by hTop2A$^{M762S/M766E}$ than by wild-type topoisomerase IIα, the cleavage complexes established by the mutant enzyme may be more stable than those established with the wild-type enzyme due to the additional point of drug-enzyme interaction. Therefore, the persistence of cleavage complexes established by the mutant and wild-type enzymes in the presence of 20 μM CP-115,955 and 200 μM 955-dione was determined. With wild-type topoisomerase IIα, drug-induced cleavage complexes were ~2.5-fold more stable than those formed in the absence of drugs (Figure 49). In addition, cleavage complexes formed by the wild-type enzyme in the presence of CP-115,955 were more stable than those formed in the presence of 955-dione, as the quinazolinedione was utilized at a ten-fold higher concentration than the quinolone (Figure 49). Furthermore, hTop2A$^{M762S/M766E}$ cleavage complexes formed in the presence of CP-115,955 were ~3-fold more stable than those formed by the wild-type enzyme in the presence of this drug, while there was no difference in the persistence of cleavage complexes formed by the two enzymes in the presence of 955-dione (Figure 49).

These results indicate that quinolone-induced cleavage complexes are more stable than quinazolinedione-induced cleavage complexes. Moreover, although the introduction of a second strong interaction between the enzyme and CP-115,955 (mediated by the M762S and M766E mutations) did not result in higher levels of cleavage complexes being formed, those that were established were more stable.

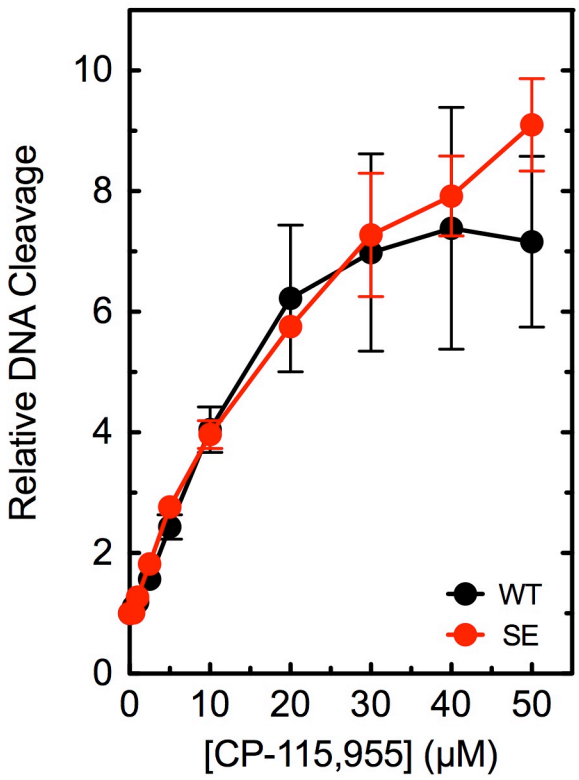


Figure 48. DNA cleavage induced by CP-115,955 with wild-type and M762S/M766E mutant human topoisomerase IIα. The mutant enzyme can support the water-metal ion bridge interaction, while the wild-type enzyme cannot. Error bars represent the standard deviation of at least three independent experiments.

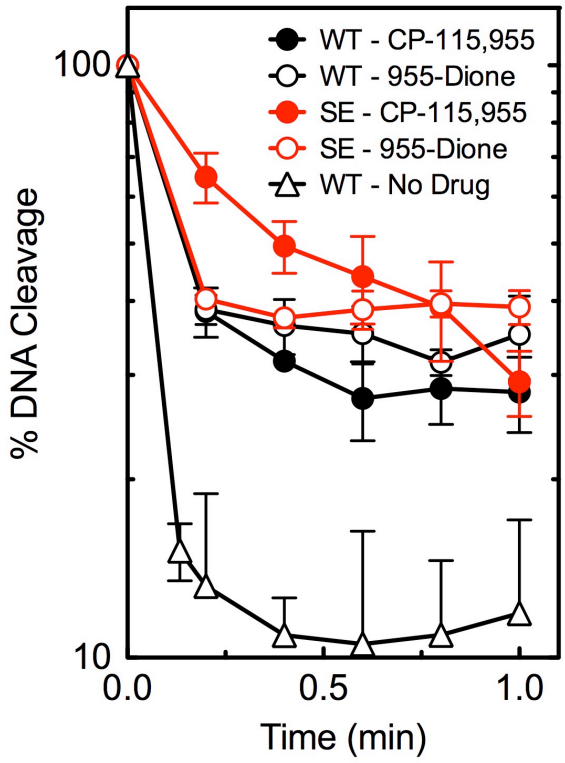


Figure 49. Effects of CP-115,955 and 955-dione on the persistence of ternary enzyme-drug-DNA cleavage complexes formed with wild-type and M762S/M766E mutant human topoisomerase IIα. The stability of wild-type cleavage complexes formed in the absence of drugs is also shown. The mutant enzyme can support the water-metal ion bridge interaction, while the wild-type enzyme cannot. Initial DNA cleavage-religation reactions were allowed to come to equilibrium and were then diluted 25-fold with DNA cleavage buffer. The persistence of cleavage complexes was assessed by monitoring the loss of double-stranded DNA breaks (linear product) over time. Cleavage at time zero was set to 100%. CP-115,955 was utilized at a concentration of 20 μM, and 955-dione was used at a concentration of 200 μM. Error bars represent the standard deviation of at least three independent experiments.

Effects of CP-115,955-based Compounds on Levels of Topoisomerase IIα-DNA Cleavage Complexes Formed in Human CEM Cells

In order to determine whether CP-115,955-based compounds can induce the formation of cleavage complexes in human cells, CP-115,955, 955-dione, and 955-NA-dione were tested in CEM cells using the RADAR assay. In this assay, cells are treated with the compounds for 1 h and intracellular cleavage complexes are trapped by rapid lysis. The DNA can then be isolated by ethanol precipitation, and the amount of topoisomerase IIα that is covalently attached to DNA can be measured by immunoblotting. Etoposide was also tested as a positive control.

CP-115,955 and 955-dione both increased levels of topoisomerase IIα cleavage complexes formed in cells, while 955-NA-dione showed no ability to do so (Figure 50). The relative activities of these compounds in cells (CP-115,955 > 955-dione > 955-NA-dione) reflected their abilities to induce enzyme-mediated DNA cleavage in *in vitro* cleavage assays. However, unlike extracellular observations, the activity of CP-115,955 was significantly less than that of etoposide when tested in the cellular context. This may be due to the fact that the CP-115,955-based compounds were not fully soluble in the cell culture media. However, it is also possible that the cellular uptake and retention of etoposide exceeds that of the CP-115,955-based compounds. Additionally, in *in vitro* cleavage assays, etoposide induces higher levels of topoisomerase IIα-mediated single-stranded DNA breaks than does CP-115,955. Because single- *vs.* double-stranded DNA breaks are not distinguished in the RADAR assay, this could also account for the difference between the two drugs in the cellular context.

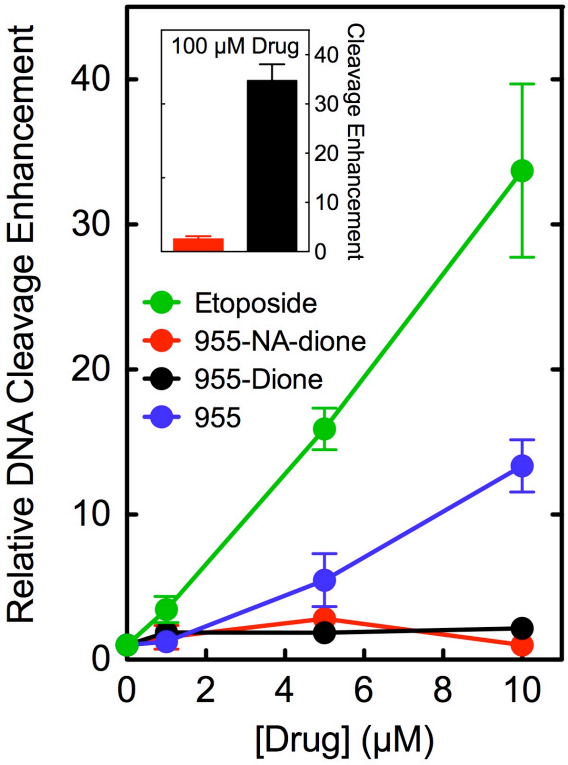


Figure 50. Levels of drug-induced DNA cleavage mediated by topoisomerase IIα in CEM cells. The ability of CP-115,955, 955-dione, and 955-NA-dione to induce DNA cleavage by topoisomerase IIα was determined using the RADAR assay following a 1 hr treatment. Levels of etoposide-induced DNA cleavage are shown for comparison. The inset shows the ability of 100 μM 955-dione and 955-NA-dione to induce intracellular DNA cleavage. Error bars represent the standard error of the mean for two independent experiments.

Summary of Conclusions

CP-115,955 and its derivative quinolones interact with bacterial type II topoisomerases through the water-metal ion bridge, just as clinically relevant quinolones do. In contrast, these drugs interact with human topoisomerase IIα through their C7 4'-hydroxyphenyl moiety. Therefore, this C7 group can distinguish between the bacterial and human type II topoisomerases and demonstrates that there are differences between the bacterial and human enzymes that could potentially be taken advantage of in order to develop quinolones that overcome target-mediated quinolone resistance without having cross-reactivity with the human system. In addition, quinolones tend to have higher activity than quinazolinediones against both bacterial and human type II topoisomerases, and the N3 amino group of the quinazolinediones modulates the activities of these drugs against both enzymes. In contrast to results with the 3'-(aminomethyl)pyrrolidinyl series of compounds, the addition of a C8 methyl or methoxy group decreases the activities of CP-115,955-based drugs against human topoisomerase IIα, primarily by decreasing drug-enzyme interaction. The introduction of a second strong interaction between the quinolones and the human enzyme by the M762S and M766E mutations does not result in an increase in the level of cleavage induced by the drugs (as compared to the wild-type enzyme), but it does result in hTop2A$^{M762S/M766E}$ cleavage complexes being more stable than their wild-type counterparts. Finally, CP-115,955 and 955-dione can induce the formation of topoisomerase IIα cleavage complexes in CEM cells, and their relative activities are reflective of those observed in extracellular cleavage assays. Therefore, it may be possible to develop some quinolones for use as anticancer drugs and optimize their efficacy using *in vitro* cleavage and structure-activity relationship studies.

CHAPTER IX

CONCLUSIONS

Over a period of a few decades, quinolones have transformed from a small and unimportant class of drugs used primarily to treat urinary tract infections to one of the most frequently prescribed classes of antibacterials in the world.[3,4,151] Today, they are used to treat a wide variety of Gram-negative and Gram-positive bacterial infections. Unfortunately, quinolone usage is threatened by the rising occurrence of resistance, which has been observed in every species that is treated by this drug class.[8,11,39]

Although there are various mechanisms of quinolone resistance, the most common and most clinically significant form is target-mediated resistance, which arises as a result of point mutations in the gyrase and topoisomerase IV enzymes that are targeted by these drugs.[18,36,37,39,49,77] The most frequent quinolone resistance mutations occur at a specific, highly conserved serine residue and an acidic residue located four positions downstream.[18,36,37,39,49,77-83] The involvement of these residues in the development of resistance to quinolones has been known for many years, but the specific mechanism by which they reduce drug action was not known. The work described in this dissertation provides mechanistic details on the interactions between quinolones and topoisomerase IV and how enzyme mutations cause resistance to the drugs. Furthermore, it details the basis for discrimination between bacterial and human type II topoisomerases by the clinically relevant quinolones and outlines the roles of various drug substituents in

mediating interaction with and activity against the enzymes. Taken together, this information suggests that a "mechanistic" approach to drug discovery holds potential.

Quinolone Interactions with Type II Topoisomerases

Interactions of Quinolones with B. anthracis *Topoisomerase IV*

Mutations at Ser81 and Glu85 in the A subunit of *B. anthracis* topoisomerase IV are the most common alterations associated with quinolone resistance.[18,36,37,39,49,77-83] As described in Chapter III, the GrlA^{E85K} mutation (but not mutations at Ser81) decreases the catalytic DNA relaxation and DNA catenation activities of the enzyme by interfering with strand passage. This may explain why alteration of the serine is significantly more common than alteration of the acidic residue. However, the observation that mutation of Ser→Phe or Tyr does not adversely affect enzymatic activities in the absence of drug raises an interesting question: why is an amino acid residue in gyrase and topoisomerase IV that has no apparent function other than to provide sensitivity to a class of synthetic antibacterials so highly conserved across the bacterial kingdom? An intriguing possibility comes from a study on nybomycin, an antibiotic produced by *Streptomyces* spp. This compound displays little activity against *S. aureus* strains that express wild-type gyrase. However, nybomycin is active against strains that express a Ser→Leu quinolone-resistant GyrA.[152] Thus, the conserved serine residue may actually represent a "resistance mutation" that provides protection against naturally occurring antibiotics. Studies on the effects of nybomycin on wild-type and quinolone-resistant mutant topoisomerase IV from *B. anthracis* could shed light on the evolutionary significance of Ser81.

As described in Chapter IV, quinolones increase the level of double-stranded DNA breaks generated by topoisomerase IV by stabilizing covalent enzyme-cleaved DNA complexes and inhibiting enzyme-mediated DNA religation. The $GrlA^{S81F}$, $GrlA^{S81Y}$, $GrlA^{E85K}$, and $GrlA^{V96A}$ mutations decrease quinolone potency, such that appreciable levels of enzyme-mediated DNA cleavage can only be achieved at high drug concentrations. Thus, it may be possible to overcome quinolone resistance by designing a drug that has stronger and/or more interactions with the enzyme. In addition, these mutations decrease the stability of quinolone-induced cleavage complexes and decrease the ability of quinolones to inhibit enzyme-mediated DNA religation. It appears that quinolone potency correlates with cleavage complex stability and the ability of the drug to inhibit religation. Therefore, a drug that interacts more strongly with the mutant enzymes will likely also regain the ability to form stable cleavage complexes and inhibit DNA religation.

Quinazolinediones have previously been shown to overcome resistance caused by mutations at the serine or acidic residues.[91-93,105,107,122-124] Presumably, these compounds do not depend on these residues for interaction with the enzyme. Accordingly, 8-methyl-3'-(AM)P-dione maintained the ability to enhance enzyme-mediated DNA cleavage at low drug concentrations, even in the presence of the mutations that caused resistance to the quinolones. In addition, this compound was able to form stable cleavage complexes and inhibit DNA religation in the presence of the enzymes that were tested. Therefore, the quinazolinedione drug class could potentially be developed for use in the clinic to combat quinolone-resistant bacterial infections.

A recent crystallographic study suggested that the main interaction between quinolones and bacterial type II topoisomerases is mediated through a bridging divalent metal ion.[43] Chapter V describes the functional evidence supporting the existence of this "water-metal ion bridge" and its importance in quinolone-topoisomerase interactions.

In the presence of alternative metal ions, a quinazolinedione, but not a quinolone, could induce DNA cleavage mediated by wild-type *B. anthracis* topoisomerase IV. This shows that quinolone function requires the presence of a divalent metal ion. Mutation of Ser81 or Glu85 restricted the variety of metal ions that could support quinolone (but not quinazolinedione) activity, indicating that these residues play important roles in mediating quinolone-enzyme interaction. Finally, higher levels of Mg^{2+} were required to support maximal quinolone-induced (but not quinazolinedione-induced) DNA cleavage in the presence of $GrlA^{S81F}$, $GrlA^{S81Y}$, and $GrlA^{E85K}$ topoisomerase IV than in the presence of the wild-type enzyme. In contrast, $GrlA^{V96A}$ topoisomerase IV required wild-type levels of Mg^{2+} to achieve maximal DNA cleavage in the presence of both the quinolone and the quinazolinedione. These results demonstrate that mutation of the amino acid residues that anchor the bridge decreases the affinity of Mg^{2+} for the interaction. $GrlA^{S81F/E85K}$ topoisomerase IV was fully resistant to ciprofloxacin, but sensitive to 8-methyl-3'-(AM)P-dione, and the quinolone could not compete with the quinazolinedione for interaction with the enzyme. Thus, quinolones interact with bacterial type II topoisomerases primarily through a single, strong interaction facilitated by the conserved serine and acidic residues.

Taken together, these findings support the existence of the water-metal ion bridge, indicate that it is the primary interaction between the drug and the enzyme, and identify

the serine and acidic residues as the protein anchors of the bridge. The significance of the water-metal ion bridge interaction, which is facilitated by the quinolone core, to drug activity may explain how these drugs can tolerate structural diversity of the N1, C7, and C8 substituents. Moreover, these results provide evidence that the most common forms of target-mediated resistance (*i.e.*, mutations of the serine and acidic residue) cause quinolone resistance through a common mechanism – the disruption of the water-metal ion bridge. Therefore, at least in theory, it should be possible to overcome most instances of target-mediated resistance by designing a quinolone or quinolone-like drug that no longer depends on the water-metal ion bridge for its primary interaction with gyrase or topoisomerase IV.

Interactions of Quinolones with E. coli Topoisomerase IV

Chapter VI describes the characterization of wild-type, ParC^{S80L}, and ParC^{E84K} topoisomerase IV from *E. coli*. Similar to results with Ser81 mutants from *B. anthracis*, the ParC^{S80L} mutation does not adversely affect the catalytic or cleavage activities of the enzyme. The ParC^{E84K} mutation has previously been shown to have a decreased catalytic ability.[117] Presumably, this mutant enzyme (like the GrlA^{E85K} mutant from *B. anthracis*) has a defect in strand passage. Unexpectedly, ParC^{E84K} showed a decreased ability (~50% that of wild-type) to cleave DNA in the absence of drugs. Thus, this may partially account for the level of quinolone resistance conferred by the Glu84→Lys mutation.

The Ser80→Leu and Glu84→Lys mutations caused quinolone resistance by decreasing not only drug potency but also drug efficacy. Although the resistance profiles varied somewhat from those seen with *B. anthracis* topoisomerase IV, the mutations still

had little effect on the activity of 8-methyl-3'-(AM)P-dione, suggesting that a drug that overcomes resistance in one species will also maintain activity against other quinolone-resistant bacterial strains.

The $ParC^{E84K}$ mutation completely abolished ciprofloxacin activity against the enzyme by significantly decreasing the ability of the drug to interact with the mutant protein. Topoisomerase IV has been shown to be the secondary cellular target for quinolones in *E. coli*.[46] Thus, the enzyme may have some intrinisic level of resistance that makes it a poorer target than gyrase in the bacterial cell. This could account for the Ser80 and Glu84 mutations having a more devastating effect on quinolone sensitivity *in vitro* than the equivalent mutations do on the sensitivity of *B. anthracis* topoisomerase IV to quinolones *in vitro*.

As observed with *B. anthracis* topoisomerase IV, $ParC^{S80L}$ required higher concentrations of Mg^{2+} than did the wild-type enzyme to achieve maximal levels of ciprofloxacin-induced, but not quinazolinedione-induced, DNA cleavage. These results indicate that the water-metal ion bridge is the major point of interaction between quinolones and *E. coli* topoisomerase IV. Thus, the bridge interaction could potentially be a universal mechanism for the binding of quinolones with bacterial type II topoisomerases. This suggests that a quinolone or quinolone-like drug that can overcome these mutations and maintain activity against quinolone-resistant strains will not be species-specific and could be useful in treating a wide range of bacterial infections.

Interactions of Quinolones with Human Topoisomerase IIα

As described in Chapter VII, human type II topoisomerases lack the amino acid residues necessary to coordinate the water-metal ion bridge. Thus, clinically relevant quinolones do not have activity against these enzymes because they lack the ability to form necessary binding interactions.

As described in Chapter VIII, some experimental quinolones have very high activity against human type II topoisomerases that actually exceeds that of the anticancer drug etoposide. CP-115,955 and related compounds, which contain a C7 4'-hydroxyphenyl group, display high activity against the human enzyme because the C7 group facilitates a specific interaction between the drug and the protein. The addition of a substituent at C8 and/or changing from a quinolone core to a quinazolinedione core decreased the activity of the compounds against this enzyme by decreasing their ability to bind with the enzyme. The presence of a C8 substituent could have this effect by altering the planarity and/or electronics of the drug. It is also possible that there are spatial constraints in the region of the enzyme where the C7 group interacts so that the enzyme cannot physically facilitate the presence of a group at the C8 position. There are two possible explanations for quinolones having higher activity than their quinazolinedione counterparts. The quinolone core could mediate a weak, non-bridge interaction between the drug and the enzyme, or a portion of the quinazolinedione core could sterically clash with the enzyme due to spatial constraints.

Although CP-115,955 did not induce higher than wild-type levels of DNA cleavage mediated by hTop2A$^{M762S/M766E}$, the cleavage complexes formed by the mutant enzyme were more stable than those formed by wild-type topoisomerase IIα in the

presence of this drug. Therefore, it appears that multiple strong drug-enzyme interactions do not enhance the induction of DNA cleavage, but they do prolong the half-life of the DNA breaks that are generated. Presumably, drugs that induce cleavage complexes with longer half-lives would be more cytotoxic due to the increased likelihood of the complexes being transformed into permanent DNA strand breaks.

The abilities of CP-115,955, 955-dione, and 955-NA-dione to induce DNA cleavage in cultured human cells reflected the relative activities of the drugs against the human type II enzyme *in vitro*. Thus, it may possible to develop certain quinolones for use as anticancer chemotherapeutics. In addition, it appears that optimization of the substituents for interaction with and activity against the human enzymes can be based on *in vitro* cleavage assays.

Overcoming Quinolone Resistance

Mutations in gyrase and topoisomerase IV are the most common cause of high-level quinolone resistance.[18,36,37,39,49,77] Therefore, the development of novel quinolones or quinolone-like drugs that retain activity against these mutated enzymes has the potential to greatly extend the clinical efficacy of the quinolone drug class. The initial hurdle to the development of drugs against resistant gyrase and topoisomerase IV was the concept that it would be impractical to design quinolones against every common mutation. This concept was based on the assumption that different mutations caused resistance by different mechanisms. However, as discussed in Chapters V and VI, the vast majority of clinically relevant mutations cause quinolone resistance through a common mechanism, namely by disrupting the water-metal ion bridge.

As described in Chapter VII, some quinazolinediones maintain activity against quinolone-resistant topoisomerase IV enzymes. However, their activity is due to the structure of their C7 groups, not the drug core interacting with the protein independently of the water-metal ion bridge. Although the quinazolinediones, as a whole, may be a weaker drug class than the quinolones, they could be a useful platform in the laboratory for substituent optimization. If a quinazolinedione is identified that has high activity against the bacterial enzymes, its substituents could be transferred to the quinolone core to generate a drug with multiple strong protein interactions. Such a drug could be more difficult for a bacterium to evolve resistance against because the organism would likely require at least two simultaneous mutations (rather than sequential accumulation) to survive and generate an infection with a clinically significant level of resistance.

Human type II topoisomerases are not sensitive to the clinically relevant quinolones because these enzymes cannot facilitate the water-metal ion bridge interaction. However, they are sensitive to some experimental quinolones, including CP-115,955, due to interactions formed between the C7 4'-hydroxyphenyl group and the enzyme. Therefore, in the context of quinolone interactions, the human enzymes are similar to mutant quinolone-resistant topoisomerase IV enzymes. For this reason, the C7 group of quinolones and quinazolinediones can mediate interactions with both quinolone-resistant topoisomerase IV and wild-type human topoisomerase IIα and cause undesirable cross-reactivity. Thus, it may be difficult to identify a C7 moiety that selectively targets the resistant bacterial enzymes. Notably, however, the C8 and N3 substituents can modulate activity of the drugs against the human enzyme while having little effect on activity against the bacterial enzymes. Therefore, a key to designing a clinically relevant

drug that overcomes quinolone resistance may be identifying C8 substituents that can be tolerated by the bacterial enzymes but not the human enzymes. (Figure 44 outlines the roles of quinolone and quinazolinedione substituents in mediating drug binding and function against bacterial and human type II topoisomerases.) Three compounds – 8-H-3'-(AMP)-FQ, 8-H-3'-(AM)P-dione, and 8-methyl-3'-(AM)P-NA-dione – have been identified that overcome quinolone resistance in *B. anthracis* topoisomerase IV without having cross-reactivity with human topoisomerase IIα. However, these compounds still bind to the human enzyme as well as the compounds that did enhance enzyme-mediated DNA cleavage. Thus, they could have undesirable effects on the activity of the human type II topoisomerases in the cellular context. If this is the case, these compounds can be used as the starting point for further drug discovery aimed at overcoming the most common forms of quinolone resistance seen in bacterial infections. For example, the use of a tricyclic drug core, like that of levofloxacin, may provide selectivity for the bacterial enzymes. Therefore, the combination of a tricyclic core with the 3'-(aminomethyl)pyrrolidinyl C7 group may generate a drug that overcomes quinolone resistance without binding to the human enzyme.

As discussed in Chapter VIII, the C7 4'-hydroxyphenyl group of CP-115,955 (and derivative quinolones and quinazolinediones) facilitates strong interactions with human topoisomerase IIα but not with wild-type or quinolone-resistant mutant *B. anthracis* topoisomerase IV. These findings demonstrate that there are differences between the human and bacterial enzymes that can be sensed by the C7 substituent of the drugs. Thus, it should be possible to identify C7 (or other) substituents that can selectively target the

bacterial type II topoisomerases and maintain activity against quinolone-resistant mutant enzymes. Overall, a mechanistic approach to drug development appears valid.

Future Directions

After parallel studies with *B. anthracis* gyrase have been completed, the effects of quinolones and quinazolinediones on intracellular levels of type II topoisomerase-mediated DNA cleavage can be assessed. Some compounds tested in Chapter VII not only induced high levels of double-stranded DNA breaks, but also induced high levels single-stranded DNA breaks (not shown). It is expected that single-stranded DNA breaks can contribute to the induction of DNA damage response pathways and aid in fragmentation of the genome. Thus, studies with bacterial cultures can also be used to determine the relative importance of double-stranded *vs*. single-stranded DNA breaks in cell kill and whether the *in vitro* ability of drugs to induce double- and/or single-stranded DNA cleavage correlates with their cellular toxicity. In addition, the ability of quinolones to kill bacterial cells under altered metal ion conditions could provide insight into quinolone function in the human body and how patient physiology could impact and alter drug efficacy from person to person. Cellular studies can also inform on whether/how mutations in the type II enzymes affect their expression levels and cellular concentrations and which enzyme (gyrase or topoisomerase IV) is the primary target of various quinolones. These studies may also shed light on why the serine and acidic residues that anchor the water-metal ion bridge are so highly conserved across bacterial species. Based on results in Chapter III, the acidic residue is important for catalytic activity. The serine residue, on the other hand, does not appear to be important for the cleavage or catalytic

abilities of the enzyme, but may be important for resistance to natural antibiotics (such as nybomycin). However, these mutations may have effects on the enzyme or bacterial cell growth that are not apparent in the *in vitro* assays used in this work.

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