OVERCOMING FLUOROQUINOLONE RESISTANCE: MECHANISTIC BASIS OF NON-QUINOLONE ANTIBACTERIALS TARGETING TYPE II TOPOISOMERASES

By

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DEDICATION

To all my family and church family who have supported and encouraged me over all my years in school.

To Him who I put all my trust. Commit your way to the Lord, trust also in Him and He shall bring it to pass. *Psalm 37:5*

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(-)SC	negatively supercoiled
(+)SC	positively supercoiled
A	alanine
ADP	adenosine diphosphate
Asp	aspartic acid
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
Cipro	ciprofloxacin
D	aspartate
DMSO	dimethyl sulfoxide
DS	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
F	phenylalanine
G	glycine
Glu	glutamic acid
Gyr	gyrase
H	histidine
L	leucine
Lin	linear
MDR-TB	multidrug-resistant tuberculosis

MGI	*Mycobacterium tuberculosis* gyrase inhibitor
Moxi	moxifloxacin
NBTI	novel bacterial topoisomerase inhibitor
Nick	nicked
Rel	relaxed
SDS	sodium dodecyl sulfate
Ser	serine
SS	single-stranded
TB	tuberculosis
V	valine
WT	wild-type
Y	tyrosine

CHAPTER I

INTRODUCTION

Topoisomerases

The topological state of DNA has a dramatic effect on nucleic acid processes in cells (Figure 1).[1-5] The genomes of most organisms are slightly negatively supercoiled (underwound), which enhances the opening of the double helix and facilitates replication and transcription.[1, 2, 6-9] In contrast, ahead of replication forks and transcription machinery, overwound DNA (positively supercoiled) accumulates and must be removed in order for replication to continue.[1, 2, 6-9] In addition, essential cellular processes such as recombination and replication introduce tangles and knots into the genome, impeding DNA tracking systems and preventing chromosomal segregation during cell division.[1, 2, 6-9] Replication leads to interlinking (catenation or tangling) of the sister chromatids, which must be resolved prior to mitosis.[1, 2 6-9] Because of the length of linear chromosomes in humans or the circular nature of chromosomes in bacteria, the topological strain cannot be resolved without help.[1, 2 1, 6-9] In order to relieve topological problems that arise in cells, all living organisms encode multiple enzymes to help regulate the topological state of their genome.[1, 6-9] Collectively, these enzymes are known as "topoisomerases," because they can interconvert DNA topoisomers. Topoisomerases function by creating transient breaks in the DNA backbone and are divided into two classes based on how many strands they cleave: type I enzymes create single-stranded breaks, and type II enzymes create double-stranded breaks (Figure 1).[1, 6-9] This dissertation will focus exclusively on type II enzymes.

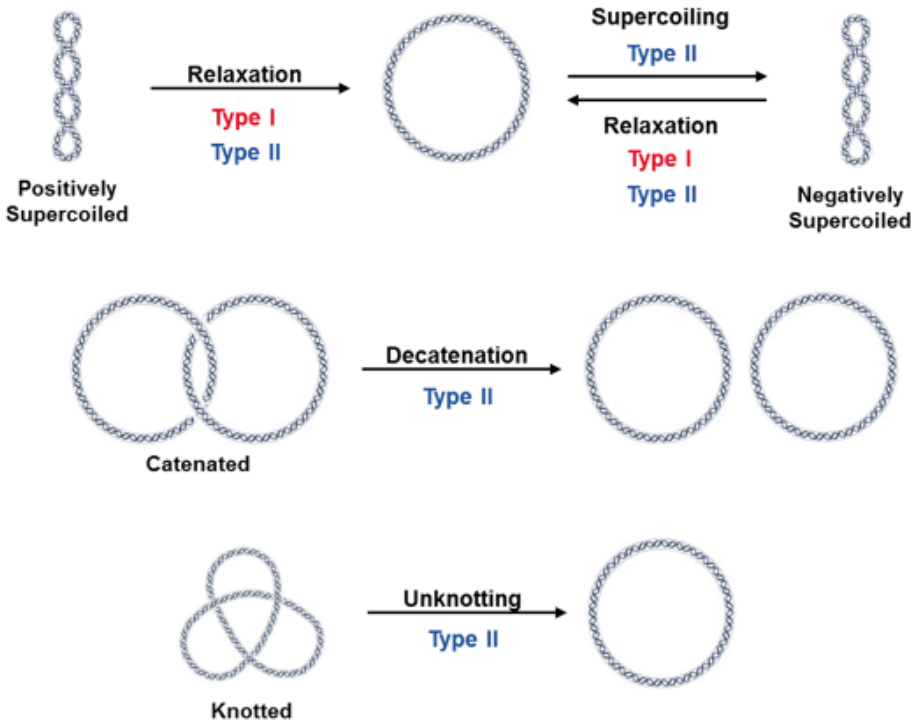


Figure 1. Actions of type I and type II topoisomerases. Type I topoisomerases create transient single-stranded DNA breaks allowing for the relaxation of both positively and negatively supercoiled DNA. Type II topoisomerases create transient double-stranded DNA breaks which allows them to relax positively and negatively supercoiled DNA and to untangle and unknot DNA molecules.

Bacterial type II DNA topoisomerases

The vast majority of bacteria encode two type II enzymes, gyrase and topoisomerase IV.[7, 10-12] These enzymes are essential for cell survival and both appear to be physiological targets for fluoroquinolones.[7, 11, 13, 14] In contrast, a handful of species encode only gyrase. This group includes a number of disease-causing organisms, including *Treponema pallidum* (syphilis),[15] *Helicobacter pylori* (stomach and intestinal ulcers),[16] *Campylobacter jejuni* (gastroenteritis),[17] *Mycobacterium leprae* (leprosy),[18] and *Mycobacterium tuberculosis* (tuberculosis).[19] In these species, gyrase takes on dual characteristics and can fulfill its own functions as well as those of topoisomerase IV.[20]

Gyrase was the first type II topoisomerase to be described in any species, originally reported in 1976.[21] It is an A_2B_2 heterotetramer in which the two subunit types are GyrA and GyrB.[7, 13, 22-25] The A subunits contain the active-site tyrosine residues that cleave the DNA (shown in blue in Figure 2). The B subunits form the N-terminal gate of the enzyme and contain the sites of ATP binding and hydrolysis (shown in green in Figure 2).[7, 13, 22-25]

The subunits of topoisomerase IV were first identified in Gram-negative species as being required for chromosome partitioning and were named ParC and ParE (blue and green in Fig. 2 respectively).[5, 7, 10, 24-28] Sequence analysis revealed that these proteins are homologous to GyrA and GyrB, respectively. In 1990, it was determined that the ParC/ParE complex was a heterotetramer that functioned as a distinct type II topoisomerase.[5, 7, 10, 24-28] The enzyme was subsequently named topoisomerase IV. Whereas the subunits of topoisomerase IV are denoted as ParC and ParE in Gram-negative species because of their historic roles in chromosome partitioning, they are called GrlA and GrlB, respectively (which comes from their initial name, gyrase-like proteins) in Gram-positive species.

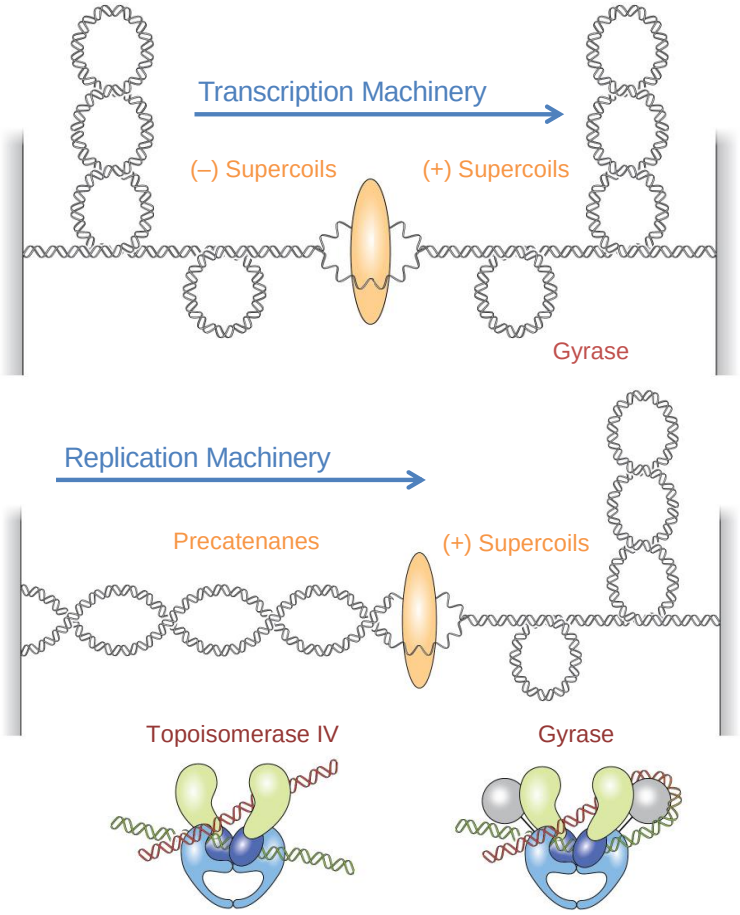


Figure 2. Cellular functions of bacterial type II topoisomerases. Topoisomerase IV (bottom left) uses a canonical double-stranded DNA passage mechanism. The enzyme can remove positive supercoils but acts primarily behind the replication fork (middle) to remove precatenanes and unlink daughter chromosomes. Gyrase (bottom right) uses a DNA-wrapping mechanism that is superimposed upon the double-stranded passage reaction. The enzyme removes positive DNA supercoils ahead of transcription (top) and replication (middle) complexes and maintains the negative superhelicity of the genome. Artwork by Ethan Tyler, NIH Medical Arts.

Gyrase and topoisomerase IV regulate DNA topology by using a double-stranded DNA passage mechanism, utilizing a catalytic cycle that is similar across all type II topoisomerases (Figure 3).[5, 7, 10, 24, 25, 29] A complete catalytic cycle requires a divalent metal ion (most often Mg^{2+}) and ATP. As a first step, gyrase and topoisomerase IV bind two segments of DNA, the "gate" or "G-segment" and the "transport" or "T-segment." Second, the gate segment is bent, and third, the enzymes use a two-metal-ion mechanism in concert with active site tyrosine residues to form an enzyme-bound double-stranded break in the G-segment.[5, 7, 10, 24, 25, 29] The scissile bonds on the two strands of the double helix are located across the major groove from one another. Cleavage results in 5'-overhanging termini with a four base cohesive stagger. In order to maintain genomic integrity during the DNA cleavage event, the enzymes form a covalent phosphotyrosine linkage between active site residues and the newly generated 5'-termini. This covalent enzyme-cleaved DNA complex is critical for the actions of fluoroquinolone antibacterials and is called the *cleavage complex.*[5, 7, 10, 24, 25, 29] After the cleavage event, the binding of two ATP molecules induces a conformational change that closes the N-terminal domain around the T-segment, which opens a gate in the G-segment and passes the T-segment through the gate. Rates of DNA strand passage are increased if one of the ATP molecules is cleaved. Next, the type II topoisomerase ligates the G-segment. Finally, the enzymes hydrolyze the second ATP molecule, which leads to the release of the T-segment and resets the enzyme for another round of catalysis.

Despite the sequence and structural similarities between gyrase and topoisomerase IV, differences in the C-terminal domains of GrlA/ParC and GyrA confer these enzymes with unique arrays of catalytic activities.[5, 7, 10, 24, 25, 29] The C-terminal domain of GrlA/ParC allows topoisomerase IV to interact with two distal DNA segments. Thus, the enzyme uses a "canonical" strand passage mechanism in which it captures existing intra- or intermolecular DNA crossovers

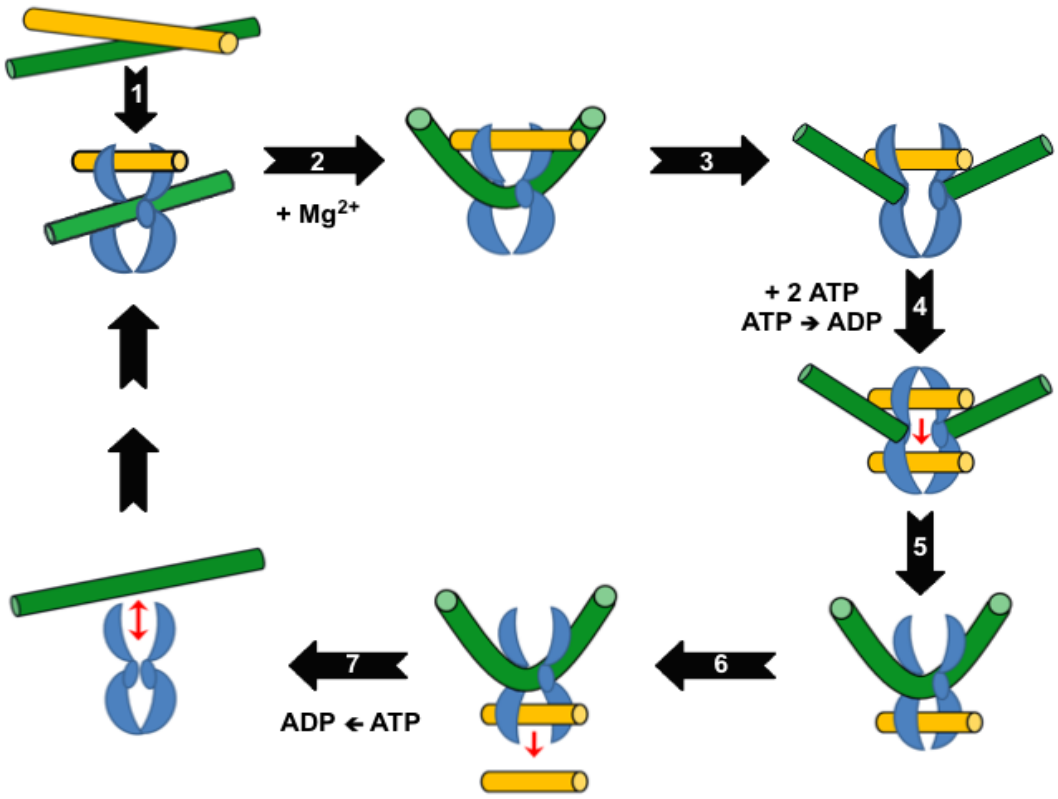


Figure 3. Catalytic cycle of type II topoisomerases. The enzyme is shown in blue, the DNA segment that is cleaved (gate segment, G-segment) is in green, and the DNA segment transported through the DNA break (transport segment, T-segment) is in yellow. The catalytic cycle has seven steps. The type II enzymes (1) bind to two DNA double helices; (2) bend the DNA gate segment; (3) generate a transient double-stranded break in the gate segment; (4) pass the transport segment through the DNA gate; (5) ligate the gate segment; (6) release the transport segment; and (7) release the gate segment and reset for another cycle.

(Figure 2, left).[5, 7, 10, 24, 25, 29] This allows the enzyme to relax (*i.e.*, remove) positive or negative DNA supercoils and to remove DNA tangles and knots in a highly efficient manner.[5, 7, 10, 24, 25, 29] Although topoisomerase IV is able to alleviate torsional stress ahead of DNA tracking systems and appears to play a role in regulating genomic superhelicity, its major function is to remove the precatenanes that form behind DNA replication forks (Figure 2, middle), separate daughter chromosomes following replication, and remove DNA knots that form during DNA recombination.[10, 30-35] To this point, if topoisomerase IV activity drops below threshold levels, cells die of mitotic failure (Figure 4).[5, 7, 10, 24, 25, 29]

In contrast to the canonical mechanism used by topoisomerase IV, gyrase uses a mechanism in which the C-terminal domain of the GyrA subunit (gray, Figure 2, right) wraps DNA, inducing a positive crossover between the G- and T-segments that mimics a positive supercoil.[10, 36-38] Because of this "wrapping" mechanism, the captured G- and T-segments are proximal to one another.[39] As a result, gyrase greatly favors the catalysis of intra- over intermolecular strand passage reactions. Consequently, the enzyme can efficiently alter superhelical density, but is very poor at removing tangles and knots.[39, 40] In addition, because gyrase always acts on the induced positive crossover, it works in a unidirectional manner;[10, 41] in the presence of ATP, the enzyme can remove positive, but not negative supercoils. Furthermore, gyrase is able to induce negative supercoils into relaxed DNA.[21, 42] The major cellular roles of gyrase stem from its DNA wrapping mechanism. Gyrase functions ahead of replication forks and transcription complexes to alleviate the torsional stress induced by DNA overwinding (Figure 2, middle and bottom).[24, 43] Furthermore, in conjunction with the ω protein, a type I topoisomerase, gyrase modulates the superhelicity of the bacterial chromosome, and allows the organism to maintain its genetic material

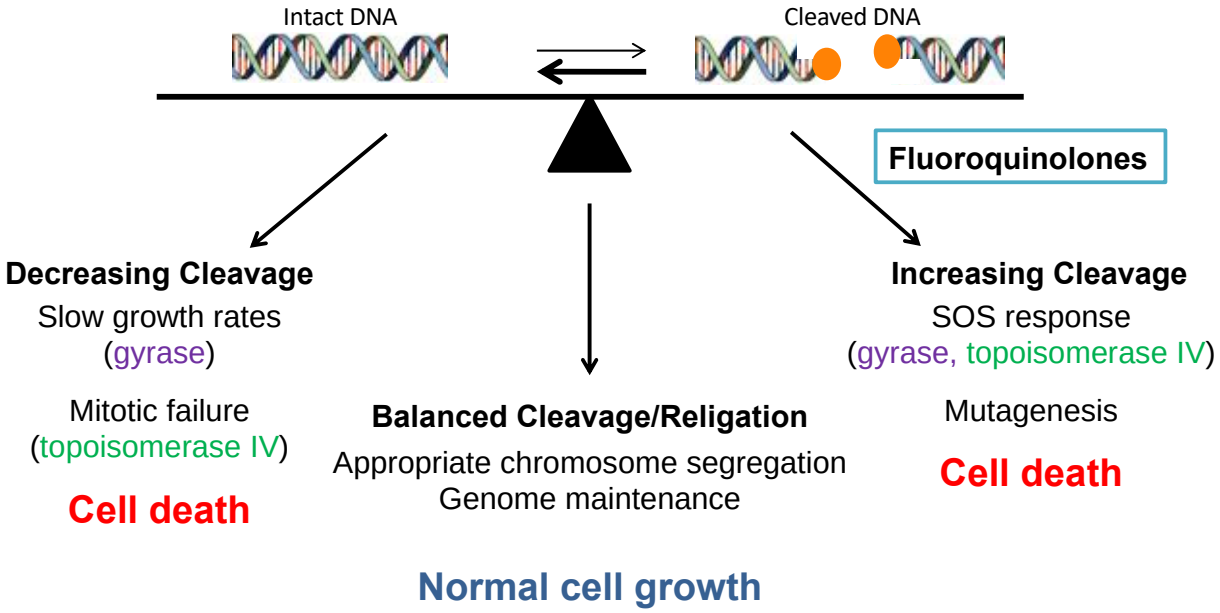


Figure 4. The critical balance of DNA cleavage and religation. The activity of bacterial type II topoisomerases must be controlled in the cell. When an appropriate level of cleavage complexes is maintained, topological problems are resolved and the cell can grow normally. If the levels of cleavage complexes decrease, slow growth rates and mitotic failure can cause cell death. Conversely, if the levels of cleavage complexes are too high, these breaks can block essential nucleic acid functions and induce the SOS response, generate mutations, and lead to cell death. Compounds (such as fluoroquinolones) that increase levels of gyrase or topoisomerase IV cleavage complexes act as topoisomerase "poisons" by converting the proteins to cellular toxins that have the potential to fragment the genome. These compounds are referred to as gyrase or topoisomerase IV "poisons," because they are said to poison these proteins, converting them to cellular toxins that have the potential to fragment the genome. Compounds also work on the other side of the balance and can inhibit overall catalytic leading to slow growth rates, mitotic failure, and cell death.

in an underwound state.[44] If gyrase activity in the cell drops, rates of replication/transcription are severely impacted (Figure 4).[5, 7, 10, 13, 25, 42] Furthermore, a number of pleiotropic effects on gene expression are observed due to changes in superhelicity of the bacterial chromosome.[45]

Although gyrase and topoisomerase IV are essential enzymes, they also pose a threat to the bacterial cell. Indeed, if a replication fork, transcription complex or DNA tracking system encounters and attempts to pass through a gyrase- or topoisomerase IV-mediated DNA cleavage complex, it can disrupt the complex and render the enzyme unable to ligate the DNA.[30] This event generates double-stranded DNA breaks that require recombination pathways to repair. Thus, these breaks block essential nucleic acid functions, induce the SOS response, generate mutations, and may lead to cell death.[5, 14, 46-49]

For the reasons described above, compounds that increase levels of gyrase or topoisomerase IV cleavage complexes have lethal actions against bacterial cells. These compounds are referred to as gyrase or topoisomerase IV "poisons,"[50] because they are said to poison these proteins, converting them to cellular toxins that have the potential to fragment the genome.[13, 14, 46, 47, 49] The term "poison" distinguishes these compounds from "catalytic inhibitors," which act primarily by robbing the cell of the catalytic functions of these enzymes (Figure 4).[29]

Fluoroquinolones

Fluoroquinolones are among the most efficacious and broad-spectrum oral antibacterials currently in clinical use.[14, 48, 51, 52] They are used as front-line treatments for a wide variety of infections caused by Gram-negative and Gram-positive bacteria.[53] Among the diseases treated with fluoroquinolones are urinary tract infections and pyelonephritis, sexually transmitted diseases, prostatitis, skin and soft tissue infections, chronic bronchitis, community-acquired and nosocomial pneumonia, and intra-abdominal and pelvic infections.[53] Fluoroquinolones are also the

first-line of prophylactic treatment for anthrax, the "biological agent most likely to be used" in a bioterrorist attack, according to the Centers for Disease Control and Prevention (CDC).[54] Furthermore, they are commonly used to treat tuberculosis in cases of resistance or patient intolerance to established regimens.[55]

Fluoroquinolones kill bacteria by increasing levels of double-stranded DNA breaks generated by gyrase and topoisomerase IV.[5, 7, 13, 14, 25] Unfortunately, fluoroquinolone usage is being threatened by an increasing prevalence of resistance, which extends to every bacterial infection treated by this drug class.[14, 51] The most common and clinically relevant form of resistance is target-mediated, which is caused by specific mutations in gyrase and topoisomerase IV.[56, 57] Therefore, it is critically important to understand how this drug class interacts with and alters the activity of its enzyme target to better guide drug development to overcome resistance.[25]

The history of the fluoroquinolones began in 1962, when Lesher *et al*. made the accidental discovery of nalidixic acid (Figure 5) as a by-product of the synthesis of the antimalarial compound chloroquine.[58] This first-generation quinolone displayed limited efficacy and was mainly used for the treatment of uncomplicated urinary tract infections caused by Gram-negative enteric bacteria.[59] In the 1980s, the second-generation of quinolones was established when norfloxacin (Figure 5) was synthesized.[49, 51, 59] This drug featured a fluorine at the C-6 position, making it the first true fluoroquinolone, and a cyclic diamine piperazine at the C-7 position. The fluorine at the C-6 position increased tissue penetration and has been included in every subsequent clinically relevant member of this drug class.[49, 51, 59, 60]

Even with improved tissue penetration, norfloxacin was still confined to the urinary tract and displayed low serum concentrations.[49, 51, 59, 60] However, its use was broadened to include sexually transmitted diseases.[49, 51, 59, 60]

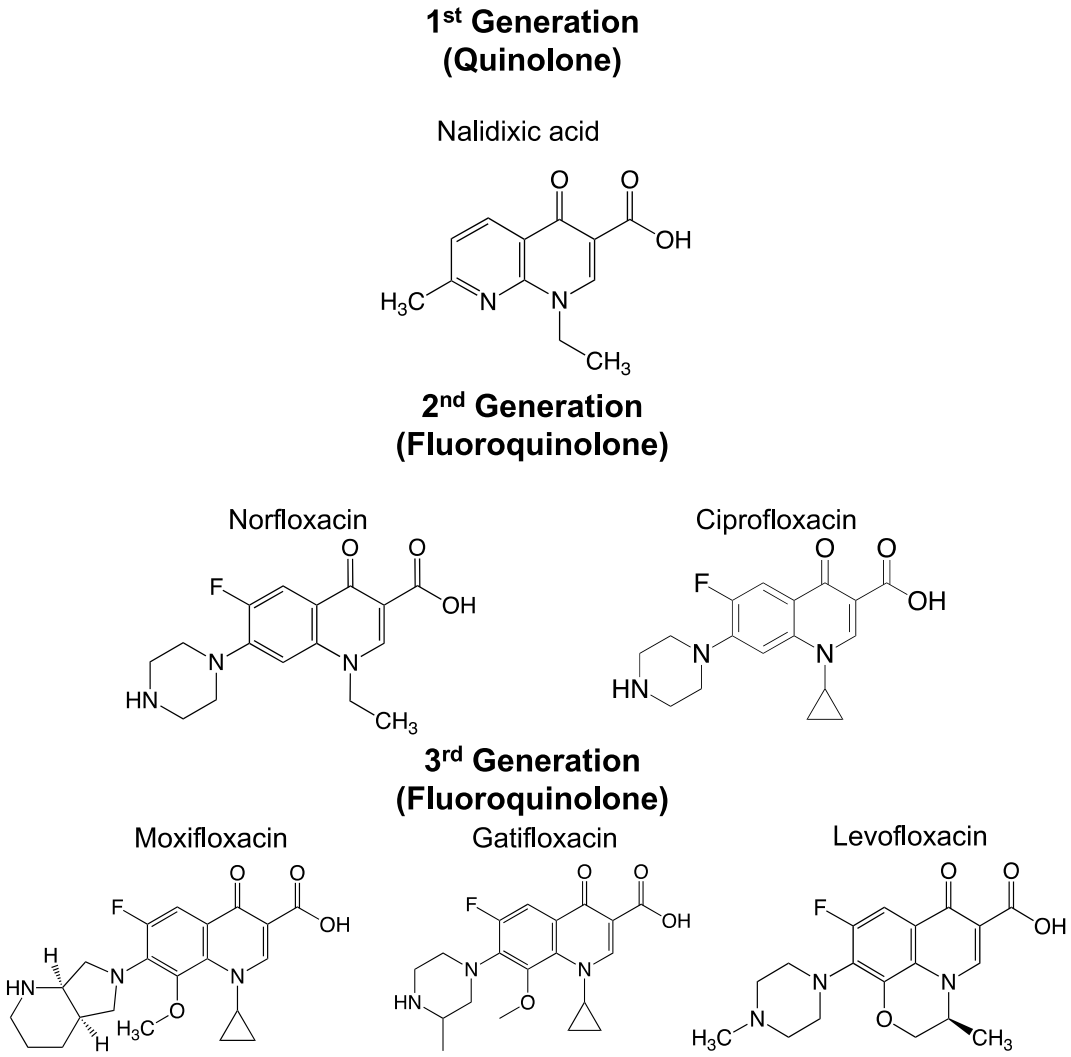


Figure 5. Fluoroquinolone structures. Nalidixic acid is a first-generation quinolone with limited efficacy for systemic infections and a narrow antibacterial spectrum. The second-generation fluoroquinolones (i.e. norfloxacin and ciprofloxacin) had improved efficacy, with ciprofloxacin being the most efficacious of the two. Ciprofloxacin had an improved antibacterial profile to include more Gram-positive bacteria and improved Gram-negative coverage. The third-generation fluoroquinolones (i.e. moxifloxacin, gatifloxacin, and levofloxacin) are the most efficacious and broad spectrum of the fluoroquinolones in clinical use today.

Ciprofloxacin (Figure 5) was the first fluoroquinolone to display efficacy toward both Gram-positive and Gram-negative bacterial species and was the first with sufficiently high tissue penetration and serum concentration to be used outside the urinary tract.[49, 51, 59] The clinical success of ciprofloxacin spawned the development of third-generation fluoroquinolones that include moxifloxacin, gatifloxacin, and levofloxacin (Figure 5).[49, 51, 59, 60] These drugs all exhibit improved half-lives compared to ciprofloxacin.[61] Moreover, they have extended the spectrum of fluoroquinolone activity to include a broader array of Gram-positive bacteria (including a number of respiratory infections), as well as atypical pathogens such as *Legionella pneumophila* and *Chlamydia pneumoniae*.[14, 48, 52, 53]

Fluoroquinolone mechanism

Fluoroquinolones are potent gyrase/topoisomerase IV poisons.[5, 13, 25, 57, 62-64] These drugs interact with both the protein and DNA within a cleavage complex and intercalate into the cleaved scissile bonds on the DNA backbone.[57, 64] Consequently, two fluoroquinolone molecules are required to stabilize double-stranded breaks induced by the type II bacterial enzymes (Figure 6). The presence of the intercalated fluoroquinolones likely produce some distortions within the enzyme active site; however, these drugs act primarily as "molecular doorstops" that form a physical barrier to DNA ligation.[57] Thus, the presence of fluoroquinolones inhibits gyrase- and topoisomerase IV-mediated DNA ligation. In addition, drugs that induce higher levels of enzyme-mediated DNA strand breaks appear to form more stable interactions within the cleavage complex and allow these complexes to persist for longer periods of time.[65, 66]

In addition to generating DNA strand breaks in the cell, fluoroquinolones also inhibit the overall catalytic strand passage activities of gyrase and topoisomerase IV.[5, 25, 48] As a result, there is debate as to whether the inhibition of strand passage contributes to drug efficacy in cells.

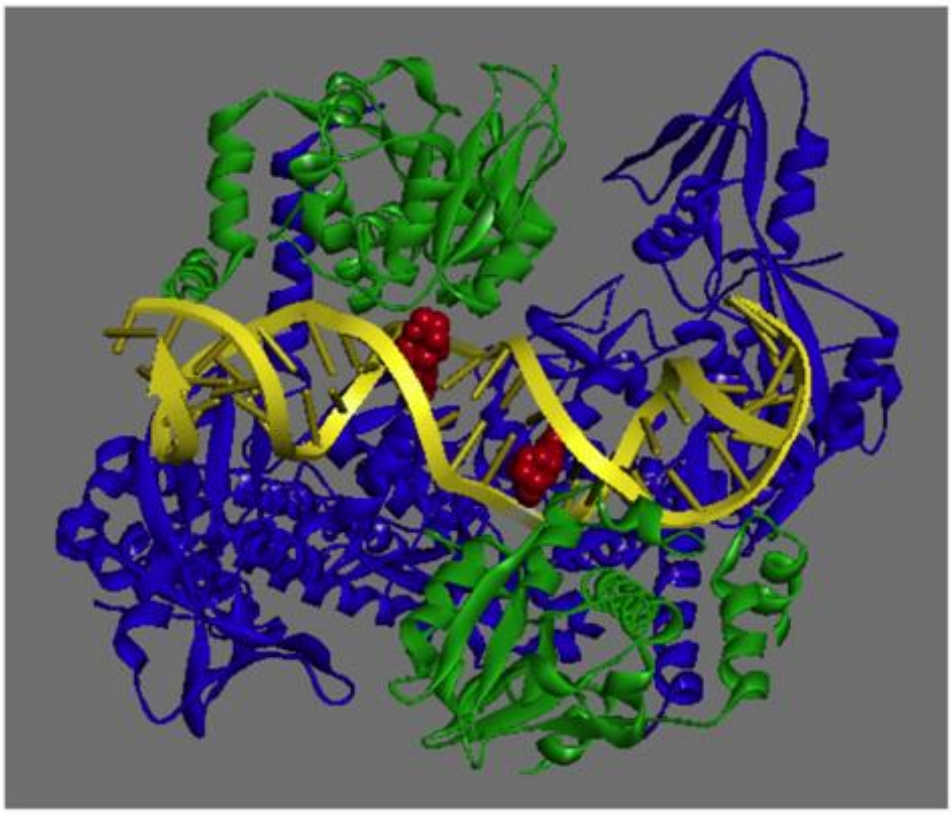


Figure 6. Crystal structure of moxifloxacin-stabilized topoisomerase IV-DNA cleavage complex in *Acinetobacter baumanii.* Two fluoroquinolone molecules are required to stabilize double-stranded breaks induced by the type II bacterial enzymes. The presence of the intercalated fluoroquinolone likely produces some distortions within the enzyme active site; however, these drugs act primarily as "molecular doorstops" that form a physical barrier to ligation and enhance stabilized DNA cleavage complexes. The catalytic core of the enzyme is shown; moxifloxacin in red; the topoisomerase IV A and B subunits are colored blue and green, respectively, and DNA is colored yellow. This structure is a top-view of the cleavage complex with two fluoroquinolone molecules intercalating four base pairs apart at the sites of DNA cleavage. (Adapted from Aldred et al., Ref. #25)

Although this issue has yet to be definitively decided, a recent study suggests that cell death results primarily from the enhancement of DNA cleavage.[67] In this study, the effects of ciprofloxacin on three different fluoroquinolone-resistant mutations of *Escherichia coli* topoisomerase IV that are associated with clinical resistance were examined *in vitro*. With all three enzymes, ciprofloxacin displayed virtually no ability to enhance DNA cleavage, but showed wild-type ability to inhibit DNA relaxation catalyzed by the type II enzymes. Therefore, it appears that the ability to induce DNA cleavage is the primary factor that determines fluoroquinolone-induced cytotoxicity.

Fluoroquinolone resistance

The World Health Organization (WHO) ranks fluoroquinolones as one of the five "highest priority" and "critically important" classes of antimicrobials.[68] However, due to their widespread use and over-use, resistance has been on the rise since the 1990s.[14, 25, 51] As an extreme example, the CDC has classified *Neisseria gonorrhoeae*, the causative agent of gonorrhea, as one of its top three "urgent level" drug-resistant threats to the United States.[69], primarily due to fluoroquinolone resistance. Along with the WHO, it has issued dire warnings that gonorrhea is on the precipice of joining HIV/AIDS and herpes as the third "incurable" sexually transmitted disease.[70]

Fluoroquinolones were used routinely to treat gonorrhea starting in 1993 and were used in more than 40% of the cases by the year 2003.[71-73] However, the use of fluoroquinolones as front-line therapy against this disease was discontinued in 2006 due to the high incidence of resistance; 22.4% of cases reported in the United States in 2015 were resistant to fluoroquinolones (this value rose to 32.1% among men who have sex with men).[71, 74, 75] In parts of Asia, fluoroquinolone resistance exceeds 80%.[75] Other infectious bacteria that need attention due to their high level of fluoroquinolone resistance include *Campylobacter* spp., *Salmonella* spp., and *Escherichia coli*.[76]

Thus far, three mechanisms of fluoroquinolone resistance have been described.[25, 47] The first is "target-mediated resistance," which results from specific mutations in gyrase or topoisomerase IV.[77-79] The second is "plasmid-mediated resistance," which is caused by the presence of extrachromosomal DNA fragments that encode three different classes of proteins.[25, 80, 81] Some plasmids encode acetylases, which modify and inactivate quinolones and other drugs. Others encode Qnr proteins, which the type II topoisomerases from binding to their DNA substrates or to fluoroquinolones. Still others encode efflux pumps, which decrease the fluoroquinolone concentration in cells. The third mechanism of fluoroquinolone resistance is "chromosome-mediated," in which the expression of efflux pumps is elevated or the expression of porins, which play a role in fluoroquinolone uptake, is downregulated.[25, 49, 80, 82]

Although the latter two mechanisms contribute significantly to fluoroquinolone resistance, the target-mediated mechanism is generally the first form of resistance that is observed in a species.[25, 83, 84] Furthermore, because target-mediated resistance represents the most common and clinically relevant form of resistance, the remainder of this chapter will focus on this mechanism.

Initial fluoroquinolone resistance is almost always associated with specific mutations in gyrase, topoisomerase IV, or both. For example, in a recent clinical study on drug resistance,[85] 97% of 60 fluoroquinolone-resistant isolates of *E. coli* carried mutations in gyrase, and 90% of these isolates also carried mutations in topoisomerase IV.

In general, the most commonly observed (up to ~90%) fluoroquinolone resistance mutation is in a highly conserved serine residue that was first described as Ser83 in the A subunit of *E. coli* gyrase.[86-90] This residue resides in helix-IV of GyrA. The majority of other resistance mutations usually map to a conserved glutamic/aspartic acid residue that is four amino acids downstream from the serine and also resides in helix-IV. Mutations at these positions often provide 10-fold or

higher reduction in susceptibility to clinically relevant fluoroquinolones. Corresponding mutations in *E. coli* topoisomerase IV also result in fluoroquinolone resistance *in vitro.*[87-90]

The prevalence of resistance mutations at the serine residue may reflect the fact that this residue is highly conserved but non-essential. To this point, the common mutations at this residue display no known phenotype, in cells or *in vitro*, with the exception of fluoroquinolone resistance. It is not clear why this residue is conserved; however, the presence of the serine appears to provide protection against nybomycin, a naturally occurring antibiotic.[91] Thus, it has been proposed there has been natural selection to maintain the serine in the bacterial genome. It is notable that mutations at the glutamic/aspartic residue generally decrease the overall catalytic activity of gyrase and topoisomerase IV.[79, 92] This may explain why a higher proportion of resistance mutations are observed at the serine residue.

To determine the contributions of gyrase and topoisomerase IV to fluoroquinolone resistance in cells, *E. coli* strains carrying these mutations in gyrase, topoisomerase IV, or both were analyzed for drug efficacy. Strains carrying mutant gyrase were ~10-fold less susceptible to fluoroquinolones. Although strains carrying mutant topoisomerase IV displayed little, if any resistance, those carrying mutations in both enzymes had ~100 fold decrease in susceptibility.[13, 14, 47, 48] This pattern of resistance strongly suggests that gyrase is the primary toxic target for fluoroquinolones in *E. coli* (Gram-negative), and topoisomerase IV is a secondary target for the drugs.

Since that initial set of experiments, the primary cellular target for fluoroquinolones in all other species has been identified by mutagenesis studies.[25, 93-98] The enzyme in which the first resistance mutations appear is believed to be the primary toxic target. Surprisingly, when these studies were carried out in *Streptococcus pneumoniae,* a Gram-positive species, the first mutations

appeared in topoisomerase IV.[99] Thus, it became dogma in the field that gyrase was the primary target for fluoroquinolones in Gram-negative species and topoisomerase IV was the primary target in Gram-positive species. While this axiom generally holds true, subsequent studies have found that there are often exceptions and that the target has to be determined on a species by species and drug by drug basis.[20, 65, 67, 92]

Role of the water-metal ion bridge in mediating fluoroquinolone resistance and gyrase/topoisomerase IV interactions

Although the association of the serine and acidic residues with fluoroquinolone resistance were established in the late 1980s,[100-103] the mechanism by which they lead to resistance was described only recently. Ultimately, the mechanistic basis for fluoroquinolone action and resistance turned out to be inextricably linked.[25, 65, 67, 104] Thus, these two important aspects of fluoroquinolone-enzyme interaction will be discussed together.

The initial insight into the roles of the serine and glutamic/aspartic acid residues of fluoroquinolone actions and resistance came from structural studies of cleavage complexes formed from topoisomerase IV or gyrase in the presence of fluoroquinolones.[57, 62-64] Although these studies all localized fluoroquinolones in the same binding pocket, which was proximal to the conserved amino acid residues, there was disagreement regarding drug orientation within the pocket. Furthermore, none of the studies found that the bound fluoroquinolone was close enough to either amino acid to form a direct interaction.

However, one of the structures (which examined the cleavage complex of *A. baumannii* topoisomerase IV formed in the presence of moxifloxacin) provided a potential mechanism by which mutations at the serine or glutamic/aspartic residue could lead to fluoroquinolone resistance.[57] It had long been known that the C3/C4 keto acid of fluoroquinolones chelate divalent

metal ions, but the physiological role of these bound metal ions, if any, were unknown. The structure of *A. baumannii* topoisomerase IV was the first to capture this fluoroquinolone-metal ion interaction within a cleavage complex. In this structure, the C3/C4 keto acid of moxifloxacin chelated a non-catalytic magnesium ion that appeared to be coordinated to four water molecules. Two of these water molecules were in sufficiently close proximity to Ser84 and Glu88 (equivalent to *E. coli* GyrA Ser83 and Glu87) to form hydrogen bonds. Thus, the authors suggested that this water-metal ion coordination might play a role in mediating interactions between fluoroquinolones and bacterial type II topoisomerases. A subsequent study that determined the structures of cleavage complexes formed with *M. tuberculosis* gyrase in the presence of moxifloxacin, 8-methyl-moxifloxacin, ciprofloxacin, levofloxacin, or gatifloxacin also observed the chelated metal ion, the associated water molecules, and the protein contacts.[66] A generalized diagram of the proposed water-metal ion "bridge" that facilitates fluoroquinolone interactions with the conserved serine and glutamic/aspartic residues is shown in Figure 7.[56, 67, 92]

The initial functional evidence for the existence and role for the water-metal ion bridge in mediating fluoroquinolone activity and resistance came from biochemical studies on *Bacillus anthracis* topoisomerase IV.[92] These studies utilized wild-type and drug-resistant enzymes that carried mutations in the serine (Ser81) and/or glutamic acid (Glu85) residues. The authors demonstrated that 1) the ability of fluoroquinolones to poison topoisomerase IV relied on the presence of a non-catalytic divalent metal ion; 2) mutations in either the serine or glutamic acid restricted the metal ions that could be used to support drug activity; 3) mutations in either amino acid decreased the affinity of the metal ion. Later studies extended these conclusions to topoisomerase IV from *E. coli* and gyrase from *B. anthracis* and *M. tuberculosis*.[25, 65, 67] Thus, it appears that the water-metal ion bridge is used to mediate fluoroquinolone-enzyme interactions in

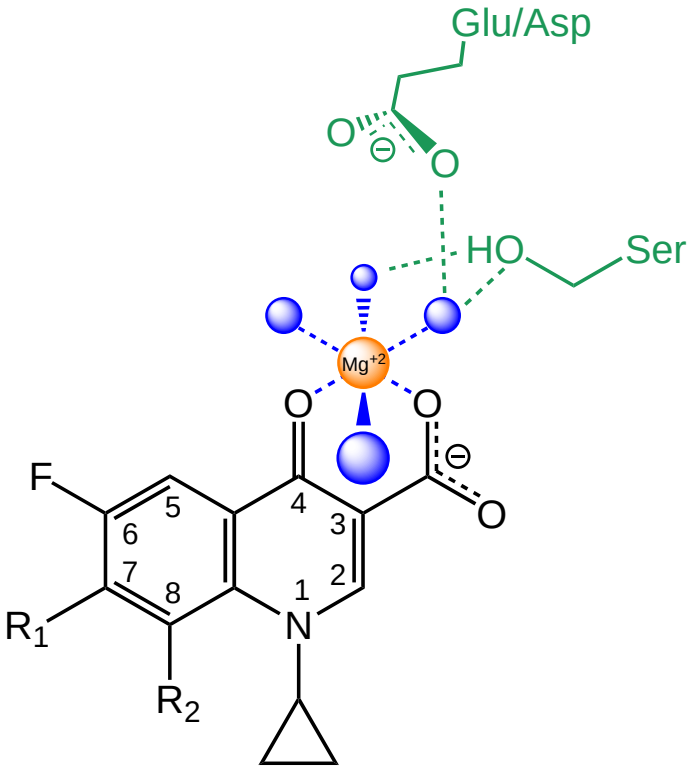


Figure 7. Water-metal ion bridge mediates critical interactions between fluoroquinolones and bacterial type II topoisomerases. A generic fluoroquinolone structure is depicted in black, water molecules are in blue, Mg2+ is in orange, and the coordinating serine and glutamic/aspartic acid residues are in green. Blue dashed lines indicate the interaction between the divalent metal ion, four water molecules and the C3/C4 keto acid of the fluoroquinolone. The green dashed lines represent hydrogen bonds between the serine or glutamic/aspartic acid side chain hydroxyl groups and the water molecules.

a variety of bacterial species. Furthermore, the loss of one or both of the amino acids that anchor the bridge is sufficient to disrupt these interactions and cause drug resistance.[56, 65, 67, 77]

Despite the importance and apparent "universality" of the water-metal ion bridge, it seems to be used differently by enzymes from different bacterial species. Whereas the bridge is critical for the binding of clinically relevant fluoroquinolones to *B. anthracis* gyrase and topoisomerase IV and *M. tuberculosis* gyrase, it is used primarily to align fluoroquinolones in the active site of *E. coli* topoisomerase IV.[25, 56, 65, 67, 92]

The divalent metal ion of the water-metal ion bridge interacts with fluoroquinolones through the C3/C4 keto acid of the drug scaffold.[56, 65, 67, 92] This may explain why clinically relevant fluoroquinolones can accommodate such a wide variety of substituents at the N1, C7, and C8 positions. Whereas substituents at the latter positions are unlikely to form critical gyrase or topoisomerase IV interactions, they may contribute minor or species-specific interactions. Furthermore, they may influence the pharmacokinetics of the drugs.

Finally, the water-metal ion bridge appears to be the feature of drug-enzyme interactions that allows discrimination between the bacterial and human type II topoisomerases. Indeed, the amino acids in human topoisomerase IIα that correspond to the serine and acidic residues of the bacterial helix-IV are methionine residues. This likely explains why clinically relevant fluoroquinolones display such poor activity against the human type II enzymes. If these methionine residues in topoisomerase IIα are converted to serine and glutamic acid residues the activity of ciprofloxacin and moxifloxacin against the human enzyme rises 4-5-fold.[92]

Overcoming target-mediated fluoroquinolone resistance

Currently, fluoroquinolones are the only antibacterials in clinical use that target gyrase or

topoisomerase IV.[13, 25, 57, 62-64] There are three approaches to overcoming antibacterial resistance: (1) discovering new targets in the bacterial cell, (2) developing fluoroquinolones that do not interact with type II topoisomerases via the water-metal ion bridge, or (3) finding new classes of compounds that target validated targets (i.e. bacterial type II topoisomerases), in which, the latter approach has resulted in several new classes, two of which are currently in clinical trials.

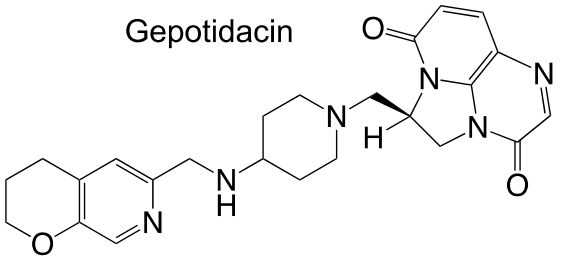
Novel Bacterial Topoisomerase Inhibitors (NBTIs)

NBTIs (Figure 8) were first reported by Coates *et al.* as a novel class of antibacterials[105] and subsequently were described as a class of compounds that inhibited the DNA supercoiling reaction of gyrase.[62, 106-110] It was not until 2007 that these compounds were found to have activity against bacterial type II topoisomerases.[111] Early studies demonstrated that at least some of the NBTIs are potent inhibitors of overall catalytic activity.[112] Later studies determined that some of these compounds could also poison the enzymes.[62]

Compared to fluoroquinolones, NBTIs are distinct in two major respects. First, structural studies demonstrate that only a single NBTI molecule interacts with the DNA in the active site of gyrase[62] It binds between the two scissile bonds and elongates the DNA in the active site of the enzyme. This is in contrast to the two fluoroquinolones (one at each cut scissile bond) that interact with DNA in the cleavage complex. Second, whereas fluoroquinolones stabilize double-stranded DNA breaks generated by gyrase or topoisomerase IV, NBTIs that act as gyrase/topoisomerase IV poisons induce only single-stranded DNA breaks.[62] Little else is known about how these compounds interact with gyrase or topoisomerase IV. NBTIs display high activity against bacterial cells that harbor fluoroquinolone-resistant mutations in gyrase and topoisomerase IV. However, no study examining purified fluoroquinolone-resistant mutant enzymes has been reported. One member of the NBTI family, gepotidacin, has completed phase II clinical trials for the treatment

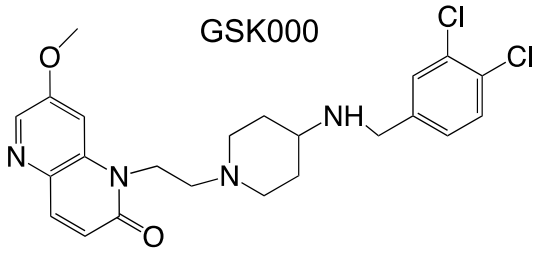
Novel Bacterial Topoisomerase Inhibitor (NBTI)

Gepotidacin



***Mycobacterium tuberculosis* Gyrase Inhibitor (MGI)**

GSK000



Spiropyrimidinetrione

Zoliflodacin

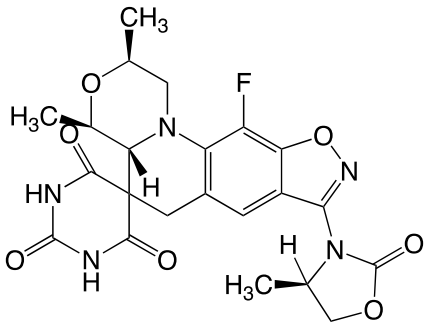


Figure 8. Novel gyrase/topisomerase IV-targeted compounds. NBTIs act as gyrase/topoisomerase IV poisons that induce only single-stranded DNA breaks, in contrast to fluoroquinolones, which induce double-stranded DNA breaks. Little else is known about how these compounds interact with gyrase or topoisomerase IV. Gepotidacin, is currently in Phase II clinical trials for the treatment of uncomplicated gonorrhea. MGIs were derived from NBTIs in an effort to optimize activity against *M. tuberculosis*. These compounds display high activity against wild-type and fluoroquinolone-resistant strains. The founding member of the spiropyrimidinetrione class of antibacterials is zoliflodacin (ETX0914/AZD0914). This drug maintains activity against multi-drug resistant *Pseudomonas aeruginosa* and is currently in Phase II clinical trials for the treatment of uncomplicated gonorrhea.

of skin/skin structure infections and uncomplicated urogenital gonorrhea[113] Gepotidacin will be discussed in greater detail in Chapter V.

Mycobacterium tuberculosis *gyrase inhibitors (MGIs)*

MGIs (Figure 8) were derived from NBTIs in an effort to optimize activity against *M. tuberculosis.*[114] These compounds display high activity against wild-type and fluoroquinolone-resistant strains. On the basis of mutagenesis studies, MGIs are believed to target gyrase, the only type II topoisomerase in *M. tuberculosis.* More in-depth biochemical studies with MGIs will be discussed in Chapter III.

Spiropyrimidinetriones

Spiropyrimidinetriones (Figure 8) are a novel class of gyrase/topoisomerase IV poisons. Similar to fluoroquinolones, these enzymes induce enzyme-mediated double-stranded DNA breaks.[115] The founding member of this class, zoliflodacin (ETX0914/AZD0914), maintains activity against multi-drug resistant *Pseudomonas aeruginosa*,[116] which contains fluoroquinolone-resistance mutations in the bridge-anchoring residues in both gyrase and topoisomerase IV. The drug is currently in Phase II clinical trials for the treatment of uncomplicated gonorrhea.[117]

Scope of the Dissertation

Fluoroquinolones are one of the most important and widely prescribed antibacterials in clinical use.[14, 48, 51, 52] However, their usefulness is being eroded by the rise of drug resistance. Of the mechanisms that decrease fluoroquinolone actions, those that result from mutations in gyrase and topoisomerase IV are the most common and detrimental.[56,57] Understanding how fluoroquinolones interact with their enzyme targets and how mutations alter these interactions has opened the door to new strategies for overcoming resistance. Among these are the development of

new drug classes that do not rely on the water-metal ion bridge for their actions. The goals of this dissertation are to investigate the mechanism of action of novel classes of gyrase/topoisomerase IV-targeted antibacterials and investigate their ability to overcome target-mediated fluoroquinolone resistance, and compare their action to those of fluoroquinolones.

Chapter I provides an introduction to topoisomerases, fluoroquinolones and resistance, and strategies to overcome resistance. Materials and methods used in this dissertation are described in Chapter II.

Chapter III describes the mechanism of action of a novel class of gyrase poisons: *M. tuberculosis* gyrase inhibitors (MGIs), a subclass of NBTIs, which were briefly discussed in the introduction. GSK000 and GSK325 demonstrate a different mechanism than fluoroquinolones, by stabilizing single-stranded DNA breaks as opposed to double-stranded DNA cleavage stabilized by the fluoroquinolones. In contrast to the NBTIs and the fluoroquinolones, the MGIs display specific activity against *M. tuberculosis* gyrase. A majority of the results in this chapter have been published.[118] Additional studies discussed in Chapter III examined the ability of the MGIs type of resistance (up-regulation of efflux pumps) in clinical isolates of *M. tuberculosis*.

Chapter IV examines the actions of the naphthyridone/aminopiperidine-based NBTIs, the parent class of the MGIs. GSK126 displays activity against a broad-spectrum of bacterial type II enzymes. This chapter focuses on its actions against *B. anthracis* gyrase as compared to *M. tuberculosis* gyrase. It will also describe the first examination of the activity of an NBTI against topoisomerase IV using *N. gonorrhoeae* (the enzyme of which GSK126 showed most activity) and *B. anthracis* topoisomerase IV (so we can examine a matched bacterial enzyme set) as model enzymes.

Chapter V describes the mechanistic and structural basis for the actions of gepotidacin, the most clinically relevant NBTI. Gepotidacin has successfully completed phase II clinical trials for the treatment of skin/skin structure infections (of which *Staphylococcus aureus* is a major contributor) and uncomplicated urogenital gonorrhea. Despite its success, little is known about its interactions with its target. This chapter will look into interactions of gepotidacin with *S. aureus* gyrase, which have been published.[119]

Conclusions and implications of the work presented in this dissertation are discussed in Chapter VI.

CHAPTER II

MATERIALS AND METHODS

DNA substrates

Negatively supercoiled pBR322 DNA was prepared from *E. coli* using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Positively supercoiled pBR322 DNA was prepared by treating negatively supercoiled molecules with recombinant *Archaeoglobus fulgidus* reverse gyrase as described previously.[120, 121] The number of positive supercoils induced by this process is comparable to the number of negative supercoils in the original pBR322 preparations.[120] In experiments that compared negatively and positively supercoiled DNA, the negatively supercoiled plasmid preparations were processed identically to the positively supercoiled molecules except that reaction mixtures did not contain reverse gyrase. Relaxed pBR322 plasmid DNA was generated by treating negatively supercoiled pBR322 with calf thymus topoisomerase I (Invitrogen) and purified as described previously.[122]

Type II topoisomerase enzymes

Bacterial enzymes

Wild-type *M. tuberculosis* gyrase subunits (GyrA and GyrB) and GyrA mutants ($GyrA^{A90V}$, $GyrA^{D94H}$, and $GyrA^{D94G}$) were expressed and purified as described previously.[65] *E. coli* topisomerase IV was provided by Keir C. Neuman (National Heart, Lung, and Blood Institute at National Institutes of Health). Wild-type *B. anthracis* gyrase and GyrA mutant ($GyrA^{S85L}$) was expressed and purified as described previously.[123] Wild-type and GrlA mutant ($GrlA^{S81F}$) *B. anthracis* topoisomerase IV was expressed and purified as described previously.[123] The wild-type *N. gonorrhoeae* gyrase, *N. gonorrhoeae* topisomerase IV and mutant ParC ($ParC^{D86N}$), *E. coli*

gyrase, and *S. aureus* gyrase were provided by Pan Chan (GlaxoSmithKline). The GyrB27-A56/Y123F fusion truncate of *S. aureus* gyrase (used for structural studies by Ben Bax) were expressed and purified as described previously.[62] Human topoisomerase IIα was expressed in yeast and purified as described by Kingma *et al*.[124]

Compounds

The MGIs GSK000 and GSK325 and the NBTI GSK126 were synthesized as described previously by Blanco *et al*.[114] In the paper by Blanco *et al.*, GSK126, GSK325, and GSK000 were identified as compounds 1, 2, and 3, respectively. Moxifloxacin was obtained from LKT Laboratories. Gepotidacin was provided by GlaxoSmithKline. Etoposide was purchased from Sigma-Aldrich. All the compounds were stored at 4 ºC as 20 mM stock solutions in 100% dimethylsulfoxide (DMSO).

Methods

Enzyme-mediated DNA cleavage

DNA cleavage reactions were based on the procedure of Aldred *et al*.[77] DNA cleavage reactions with *M. tuberculosis* gyrase contained 100 nM wild-type or mutant ($GyrA^{A90V}$, $GyrA^{D94H}$, and $GyrA^{D94G}$) gyrase (2:1 GyrA:GyrB ratio) and 10 nM positively or negatively supercoiled pBR322 in a total volume of 20 µL of 10 mM Tris-HCl (pH 7.5), 40 mM KCl, 6 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin (BSA, Sigma), and 10% glycerol. Unless stated otherwise, reactions were incubated at 37 ºC for 10 min. Enzyme-DNA cleavage complexes were trapped by adding 2 µL of 5% sodium dodecyl sulfate followed by 2 µL of 250 mM Na_2EDTA and 2 µL of 0.8 mg/mL Proteinase K (Sigma Aldrich). Reaction mixtures were incubated at 45 ºC for 30 min to digest the topoisomerases. Samples were mixed with 2 µL of loading buffer [60% sucrose, 10 mM Tris-HCl (pH 7.9), 0.5% bromophenol blue; and 0.5% xylene cyanol FF] and

were incubated at 45 ºC for 2 min before loading onto 1% agarose gels. Reaction products were subjected to electrophoresis in a buffer of 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA that contained 0.5 µg/mL ethidium bromide. DNA bands were visualized with medium-range ultraviolet light and quantified using an Alpha Innotech digital imaging system. DNA single- or double-stranded cleavage was monitored by the conversion of supercoiled plasmid to nicked or linear molecules, respectively and quantified in comparison to a control reaction in which an equal amount of DNA was linearized by digestion with EcoRI (New England BioLabs).

Reactions with *B. anthracis* gyrase contained 500 nM wild-type or $GyrA^{S85L}$ gyrase or at a 1:2 GyrA:GyrB ratio and 10 nM negatively supercoiled pBR322 in a total volume of 20 µL of 50 mM Tris-HCl (pH 7.5), 100 mM KGlu, 5 mM $MgCl_2$,1 mM dithiothreitol (DTT) and 50 µg/mL BSA. Reactions were incubated at 37 °C for 30 min, then stopped, digested, and analyzed as described above.

DNA cleavage reactions with *B. anthracis* topoisomerase IV contained 100 nM enzyme (1:2 GrlA:GrlB ratio) and 10 nM or negatively supercoiled pBR322 in a total volume of 20 µL of Bacterial Topoisomerase Reaction Buffer [40 mM Tris-HCl (pH 7.9), 50 mM NaCl, and 12.5% glycerol] containing 10 mM $MgCl_2$. Reactions were incubated at 37 °C for 10 min, then stopped, digested, and analyzed as described above.

DNA cleavage reactions with *E. coli* topoisomerase IV contained 10 nM enzyme (1:1 ParC:ParE ratio) and 10 nM negatively supercoiled pBR322 in 20 µL Bacterial Topoisomerase Reaction Buffer containing 5 mM $MgCl_2$. Reactions were incubated at 37 °C for 10 min, then stopped, digested, and analyzed as described above.

DNA cleavage reactions with *E. coli* gyrase contained 50 nM enzyme (1:1 GyrA:GyrB ratio) and 10 nM negatively supercoiled pBR322 in 20 µL Bacterial Topoisomerase Reaction

Buffer containing 10 mM $MgCl_2$. Reactions were incubated at 37 °C for 10 min, then stopped, digested, and analyzed as described above.

DNA cleavage reactions with *N. gonorrhoeae* gyrase and topoisomerase IV contained 10 nM enzyme (1:1 ParC:ParE ratio) and 10 nM negatively supercoiled pBR322 in 20 µL Bacterial Topoisomerase Reaction Buffer containing 5 mM $MgCl_2$. Reactions were incubated at 37 °C for 10 min, then stopped, digested, and analyzed as described above.

DNA cleavage reactions with *S. aureus* gyrase contained 75 nM enzyme (1:1 GyrA:GyrB ratio) and 10 nM positively or negatively supercoiled pBR322 in 20 µL of 50 mM Tris-HCl (pH 7.5), 100 mM KGlu, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 50 µg/mL BSA. Reactions were incubated at 37 °C for 30 min, then stopped, digested, and analyzed as described above.

In some cases with the bacterial enzymes, 1.5 mM ATP was included in reaction mixtures or the $MgCl_2$ in the cleavage buffer was replaced with 5 mM $CaCl_2$, except with *M. tuberculosis*, which was replaced with 6 mM $CaCl_2$.

DNA cleavage reactions with human topoisomerase IIα were performed as described previously.[125] Reaction mixtures contained 150 nM topoisomerase IIα and 10 nM negatively supercoiled pBR322 DNA in 20 µL of 10 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol. Reaction mixtures were incubated for 6 min at 37 °C then stopped, digested, and analyzed as described above.

In each of the reactions, the enzymes were incubated in the presence or absence of the compounds listed above.

DNA Ligation

DNA ligation assays were carried out in the absence or presence of GSK000 or moxifloxacin following the procedure of Robinson and Osheroff.[126] Reaction mixtures (20 µL)

contained 100 nM wild-type *M. tuberculosis* gyrase and 10 nM negatively supercoiled pBR322 in 10 mM Tris-HCl (pH 7.5), 40 mM KCl, 6 mM $MgCl_2$, 0.1 mg/mL BSA, and 10% glycerol. In experiments carried out in the absence of drug, $MgCl_2$ in the cleavage buffer was replaced with 6 mM $CaCl_2$ to increase baseline levels of DNA cleavage. DNA cleavage–ligation equilibria were established at 37 °C for 10 min. Ligation was initiated by shifting the samples from 37 °C to 75 °C. Reactions were stopped at times ranging from 0 to 210 s and were digested, processed, and visualized as described above. Levels of MGI-induced single-stranded and moxifloxacin induced double-stranded DNA cleavage were set to 1 at 0 s, and ligation was assessed by the loss of nicked or linear reaction product, respectively, over time.

Molecular modeling

The structure of GSK000 in a ternary complex with *M. tuberculosis* gyrase was modeled by Ben Bax using Coot,[127] MOE,[128] and Maestro (Schrödinger Release 2017-2: Maestro, Schrödinger, LLC, New York, NY, 2017). Drug placement was based on the crystal structure of the NBTI GSK299423 in a ternary complex with *S. aureus* gyrase [Protein Data Bank (PDB) code 2XCS] and the crystal structure of a cleavage complex of *M. tuberculosis* gyrase with moxifloxacin (PDB code 5BTA).

Stability of gyrase-DNA cleavage complexes

The persistence of gyrase-DNA cleavage complexes was determined using the procedure of Aldred *et al.*[77] For *M. tuberculosis* gyrase, assays were carried out in the absence or presence of GSK000 or moxifloxacin. Initial reaction mixtures contained 500 nM gyrase, 50 nM negatively supercoiled pBR322, and 10 μM GSK000 or 50 μM moxifloxacin in cleavage buffer a total volume of 20 μL. In experiments carried out in the absence of drug, the $MgCl_2$ in the cleavage buffer was replaced with 6 mM $CaCl_2$ to increase baseline levels of DNA cleavage. Reaction mixtures were

incubated at 37 ºC for 10 min to allow cleavage complexes to form, and were then diluted 20–fold with 37 ºC 1X reaction buffer without divalent metal ion. Samples (20 µL) were removed at times ranging from 0–240 min. DNA cleavage was stopped and samples were digested, processed, and visualized as described above. Levels of MGI-induced single-stranded and moxifloxacin-induced double-stranded DNA cleavage were set to 1 at time zero, and the stability of cleavage complexes was determined by the loss of single- or double-stranded DNA cleavage, respectively, over time.

For persistence assays with *S. aureus* gyrase, reactions were carried out in the absence or presence of gepotidacin or moxifloxacin and reaction mixtures contained 375 nM gyrase, 50 nM negatively supercoiled pBR322, and 5 µM gepotidacin or 25 µM moxifloxacin in 20 µL of 50 mM Tris-HCl (pH 7.5), 100 mM KGlu, 5 mM $MgCl_2$, 1 mM DTT, and 50 µg/mL BSA. In experiments carried out in the absence of drug, the $MgCl_2$ in the cleavage buffer was replaced with 5 mM $CaCl_2$ to increase baseline levels of DNA cleavage.[65, 129] Reaction mixtures were incubated at 37 ºC for 30 min to allow cleavage complexes to form, and were then diluted 20–fold with 37 ºC 1X cleavage buffer that lacked divalent metal ion. Samples (20 µL) were removed at times ranging from 0–240 min. DNA cleavage was stopped and samples were processed, visualized, and quantified as described above. Levels of gepotidacin-induced single-stranded or moxifloxacin-induced double-stranded DNA cleavage were set to 100% at time zero, as was enzyme-mediated DNA cleavage in the absence of drug. The stability of cleavage complexes was determined by the loss of single- or double-stranded DNA cleavage, respectively, over time.

DNA supercoiling and relaxation

DNA supercoiling and relaxation assays were based on previously published protocols.[65, 77] In each of the reactions, the enzymes were incubated in the presence or absence of the MGIs, NBTIs, or moxifloxacin.

M. tuberculosis gyrase supercoiling and relaxation assays contained 25 nM gyrase, 5 nM relaxed or positively supercoiled pBR322, 1 mM DTT and 1.5 mM ATP in 20 µL of 10 mM Tris-HCl (pH 7.5), 40 mM KCl, 6 mM $MgCl_2$, 0.1 mg/mL BSA, and 10 % glycerol. Reactions were incubated at 37 °C for 30 min (DNA supercoiling assays) or 1 min (DNA relaxation assays). The chosen assay lengths represent the minimum time required to completely supercoil or relax the DNA in the absence of drug. Reaction mixtures were stopped by the addition of 3 µL of stop solution [0.77% SDS and 77.5 mM EDTA]. Samples were mixed with 2 µL of loading buffer and were incubated at 45 ºC for 2 min before being subjected to electrophoresis on 1% agarose gels in 100 mM Tris-borate (pH 8.3) and 2 mM EDTA. Gels were stained with 1 µg/mL ethidium bromide for 30 min and DNA bands were visualized with medium-range ultraviolet light and quantified using an Alpha Innotech digital imaging system.

B. anthracis topoisomerase IV wild-type and mutant ($GrlA^{S81F}$) DNA relaxation assays contained 50 nM topoisomerase IV, 5 nM supercoiled pBR322, and 1.5 mM ATP in 20 µL of 40 mM HEPES (pH 7.6), 100 mM KGlu, 10 mM $Mg(OAc)_2$, and 50 mM NaCl. Reactions were incubated for 25 min at 37 °C, stopped, processed, and analyzed as described above.

For wild-type and mutant ($ParC^{D86N}$) *N. gonorrhoeae* topoisomerase IV, DNA relaxation assays contained 10 nM topoisomerase IV, 5 nM relaxed pBR322, and 1 mM ATP in 20 µL of 40 mM HEPES (pH 7.6), 100 mM KGlu, 10 mM $Mg(OAc)_2$, and 50 mM NaCl. Reactions were incubated for 25 min at 37 °C, stopped, processed, and analyzed as described above.

For assays with wild-type *B. anthracis* gyrase or mutant $GyrA^{S85L}$, (1000 nM enzyme at a 1:1 GyrA:GyrB ratio) were incubated for 5 min at 37 °C in 100 mM Tris-HCl (pH 7.5), 350 mM KGlu, and 100 µg/mL BSA, then diluted two-fold with a mixture containing DNA, Mg^{2+}, and ATP for a final reaction volume of 20 µL. The final concentrations of reactants were 500 nM gyrase, 5

nM relaxed DNA, and 1.5 mM ATP in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 175 mM KGlu, and 50 μg/mL BSA. Reactions were incubated at 37 °C for 30 min, stopped, processed, and analyzed as described above.

S. aureus gyrase supercoiling/relaxation assays contained 20 nM gyrase, 5 nM relaxed or positively supercoiled pBR322, 1.5 mM ATP, 1 mM DTT in 20 μL of 50 mM Tris-HCl (pH 7.7), 20 mM KCl, 300 mM KGlu, 5 mM $MgCl_2$, and 0.05 mg/mL bovine serum albumin. Reactions were incubated at 37 °C for 25 min (DNA supercoiling assays) or 0.5 min (DNA relaxation assays). Reactions were stopped, processed, and analyzed as described above.

Crystallization of gepotidacin in complex with S. aureus *gyrase*

Crystallization studies were completed by our collaborator Ben Bax. Crystals of gepotidacin with the *S. aureus* GyrB27-A56/Y123F fusion truncate and 20-12p-8 duplex DNA were grown by microbatch under oil and frozen as described previously (Table 1).[62, 130] The 20-12p-8 is a 20mer DNA duplex made by annealing complementary 8mers and 12mers such that the four base-pair overhang from the 12mers is complementary (Watson strand: 5'-AGCCGTAG-3' + 5'-GTACCTACGGCT-3'; Crick strand: 5'-AGCCGTAG-3' + 5'-GTACCTACGGCT-3').[62] The 12mer contains a 5' phosphate moiety, equivalent to the scissile phosphate, but not covalently linked to the 3' OH of the preceding nucleotide or Tyr123 of GyrA. The symmetric doubly nicked DNA was used because it has been optimized to provide diffractable crystals with NBTIs.[62, 130] Furthermore, in crystal structures of NBTIs formed with asymmetric DNA substrates, the DNA has static disorder around the same axis of the complex.[130-132]

Data to 2.31Å were collected on a single frozen crystal of gepotidacin with the *S. aureus* GyrB27-A56/Y123F fusion truncate and 20-12p-8 duplex DNA on beamline ID23-2 at the ESRF on a Mar 225 CCD.[62] Data were processed and merged with HKL and SCALEPACK[133], the

Data collection	
Beamline	ESRF ID23-2
Space group	$P6_1$
Cell dimensions a,b,c (Å); α, β, γ (°)	92.73, 92.73, 408.78; 90.0, 90.0, 120.0
Wavelength (Å)	0.8726
Resolution range (Å)	40-2.31 (2.35-2.31)
No. of unique reflections	85905 (4236)
Multiplicity	5.5 (5.6)
Completeness (%)	99.7 (99.9)
R_{merge} (%)	9.4 (48.0)
I/σI	19.2 (3.7)
Refinement	
Resolution (Å)	2.31 (2.37-2.31)
No. reflections (work/free)	82431 /3426
R_{work}/ R_{free} (%)	16.7/20.5 (22.3/28.3)
No. Atoms	12711
Protein	10926
DNA	850
Ligand/ion	130
Water	805
B-factors	
Protein	35.6
DNA	36.8
Ligand/ion	44.9*
Water	40.3
R.m.s deviations	
Bond lengths (Å)	0.007
Bond angles (º)	1.46

*Includes 9 glycerols; average B-factor for gepotidacin is 37.3.

Table 1. Crystallographic parameters for the structure of the interaction between gepotidacin and the *S. aureus*-gyrase DNA cleavage complex.

structure was solved by rigid body refinement from other structures in the same cell (PDB codes: 2xcs, 5iwi),[62] and refined with refmac[134] and phenix.refine.[135] The restraint dictionary for gepotidacin was made with Acedrg.[136] See Table 1 for crystallographic details.

The deposited crystallographic coordinates, which represent the millions of complexes in the crystal, contain two crystallographic copies of the compound in the unit cell, related by C2 symmetry. In any one complex there will only be one compound bound, sitting on the molecular 2-fold, and single biological complexes with one compound can be readily derived from the crystal structures. Note these complexes use a nomenclature which is non-conventional by PDB standards, because the structures are of a fusion protein in which residues from the C-terminal region of *S. aureus* GyrB (411-644) have been fused to amino-acids from the N-terminus of GyrA (2-491). In our standard 'BA-x' nomenlature the residues in the first GyrBA fusion have CHAINID B if they are from GyrB and CHAINID A, if they are from GyrA. This nomenclature is extended to inhibitors which are given CHAINID I – for inhibitor. NBTIs are given the residue number 2 (*i.e.*, I2), for the 'second' inhibitor site on the twofold axis (the first site which stabilizes cleavage complexes is the cleavage site, occupied by inhibitors such as fluoroquinolones). In structural figures that were created using Pymol[137], carbon atoms in the DNA are green, those in the first $GyrBA^{core}$ fusion truncate subunit are cyan in GyrA and magenta in GyrB, and those in the second subunit are grey or black. Carbon atoms in NBTIs are yellow or orange, and oxygen, nitrogen, and sulfur atoms are red, blue, and yellow, respectively. Water molecules are shown in red.

Determination of minimum inhibitory concentrations (MICs)

MIC values for GSK000, GSK325, and GSK126 against cultured *E. coli* (7623) cells were determined as described in Blanco, *et al.* [114] MIC values determined with clinical *M. tuberculosis isolates* with GSK000 and GSK325 were determined as described previously by Palomino, *et al*.[138]

DNA cleavage site utilization

DNA cleavage sites with *M. tuberculosis* gyrase in the presence and absence of the MGIs and moxifloxacin were mapped using a modification[139] of the procedure of O'Reilly and Kreuzer.[140] The pBR322 DNA substrate was linearized by treatment with *Hin*dIII. Terminal 5′-phosphates were removed by treatment with calf intestinal alkaline phosphatase and were replaced with [^{32}P]phosphate using T4 polynucleotide kinase and [γ-^{32}P]ATP. The DNA was then treated with *Eco*RI, and the 4332-base pair singly end-labeled fragment was purified from the small *Eco*RI–*Hin*dIII fragment by passing it through a CHROMA SPIN+TE-100 column (Clontech). Reaction mixtures contained 500 nM wild-type *M. tuberculosis* gyrase and 5 nM radiolabeled pBR322 DNA substrate in 50 μL of DNA cleavage buffer with 6 mM $CaCl_2$ in the absence or presence of MGIs or moxifloxacin. Reaction mixtures were incubated at 37 °C for 10 min, and enzyme-DNA cleavage complexes were trapped by the addition of 5 μL of 10% 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 30 min to digest the enzyme. Alternatively, cleavage complexes were enriched by K^+-SDS precipitation of gyrase, prior to Proteinase K treatment.[141] This was accomplished by adding 25 μL of 2.5 M KCl and incubating at -20 °C for 10 min. Mixtures were centrifuged at 18,000 x g for 10 min. Pellets were resuspended in 50 μL of 10 mM Tris-borate (pH 7.9) and 1 mM EDTA and were then treated with Proteinase K as described above. In both cases, DNA products were precipitated with ethanol and resuspended in 6 μL of 40% formamide, 10 mM NaOH, 0.02% bromophenol blue, and 0.02% xylene cyanol FF. Samples were heated at 75 °C for 2 min and were subjected to electrophoresis in a denaturing 6% polyacrylamide sequencing gel in 100 mM Tris-borate (pH 8.3) and 2 mM EDTA. Gels were dried *in vacuo*, and DNA cleavage products were visualized with a Bio-Rad Molecular Imager FX.

CHAPTER III

MECHANISM OF ACTION OF *MYCOBACTERIUM TUBERCULOSIS* GYRASE INHIBITORS (MGIS): A NOVEL CLASS OF GYRASE POISONS

Introduction

Tuberculosis is a lung infection caused by the bacterium *M. tuberculosis*, an aerobic bacillus that stains neither Gram-positive nor Gram-negative.[142] It is one of the leading causes of mortality worldwide and recently surpassed HIV/AIDS as the deadliest disease caused by a single infectious agent.[143] In 2017, there were an estimated 10 million new cases of tuberculosis reported world-wide and 1.6 million people died from the disease. Of these reported cases, an estimated 558,000 were diagnosed with rifampin-resistant disease and of those cases 82% had multi-drug resistant tuberculosis.[144]

The standard treatment regimen for tuberculosis includes rifampin, isoniazid, pyrazinamide, and ethambutol.[142, 145] However, fourth-generation fluoroquinolone antibacterials, such as moxifloxacin (Figure 9) and levofloxacin, are critical drugs for treating patients who have multidrug-resistant tuberculosis or are intolerant of first-line therapies.[55] Unfortunately, fluoroquinolone resistance is on the rise and is starting to impact the treatment of tuberculosis.[143]

As discussed in Chapter I, most bacteria encode two type II topoisomerases, gyrase and topoisomerase IV.[4, 5, 10, 13, 25, 146, 147] However, *M. tuberculosis* is unusual in that it encodes only gyrase, which carries out the cellular functions of both type II enzymes.[148-150] Thus, it is an ideal antibacterial target for disrupting *M. tuberculosis* DNA replication and transcription.

The lack of available drugs and the rising incidence of drug resistance and intolerance point to a need for the development of new antitubercular agents.[143] Three approaches have been used

to address this issue: the discovery of new antibacterial targets, alterations of fluoroquinolones that do not interact via the water-metal ion bridge, and the development of new drugs that act through validated targets, but do not succumb to current resistance patterns, as mentioned in the introduction. Using this latter approach, a new class of naphthyridone/aminopiperidine-based drugs that target bacterial type II topoisomerases has been described. These drugs are known as "novel bacterial topoisomerase inhibitors" (NBTIs),[62] one of which (GSK126)[114] is shown in Figure 9. NBTIs differ from fluoroquinolones in three important respects. First, some members of this drug family do not enhance enzyme-mediated DNA cleavage (and therefore are not classified as gyrase "poisons") and act strictly as catalytic inhibitors.[62, 107, 108, 110, 147] Second, those NBTIs that do enhance DNA cleavage appear to stabilize primarily single-stranded (as opposed to double-stranded) DNA breaks generated by bacterial type II topoisomerases.[107, 110, 147] Consistent with this observation, the crystal structure of a *Staphylococcus aureus* gyrase-DNA cleavage complex formed in the presence of the NBTI GSK299423 contains only one drug molecule (centrally located between the two scissile bonds), as compared to two (one at each scissile bond) for fluoroquinolones.[62, 65] Third, NBTIs retain activity against cells that express clinically relevant mutations in gyrase or topoisomerase IV that are associated with fluoroquinolone resistance.[62, 107, 108, 110] In addition, while an S83L mutation in *E. coli* GyrA increased the IC_{50} of ciprofloxacin from 0.35 μM to 15 μM, the activity of the NBTI GSK299423 was not altered by this mutation ($IC_{50} \approx$ 0.10 μM).[62]

Unfortunately, little else has been published regarding the actions of NBTIs against bacterial type II topoisomerases. NBTIs display relatively poor activity against *M. tuberculosis* gyrase.[114] However, to develop NBTI-like drugs that act against tuberculosis, Blanco *et al.* used a high-throughput screen to identify a subclass of naphthyridone/aminopiperidine-containing

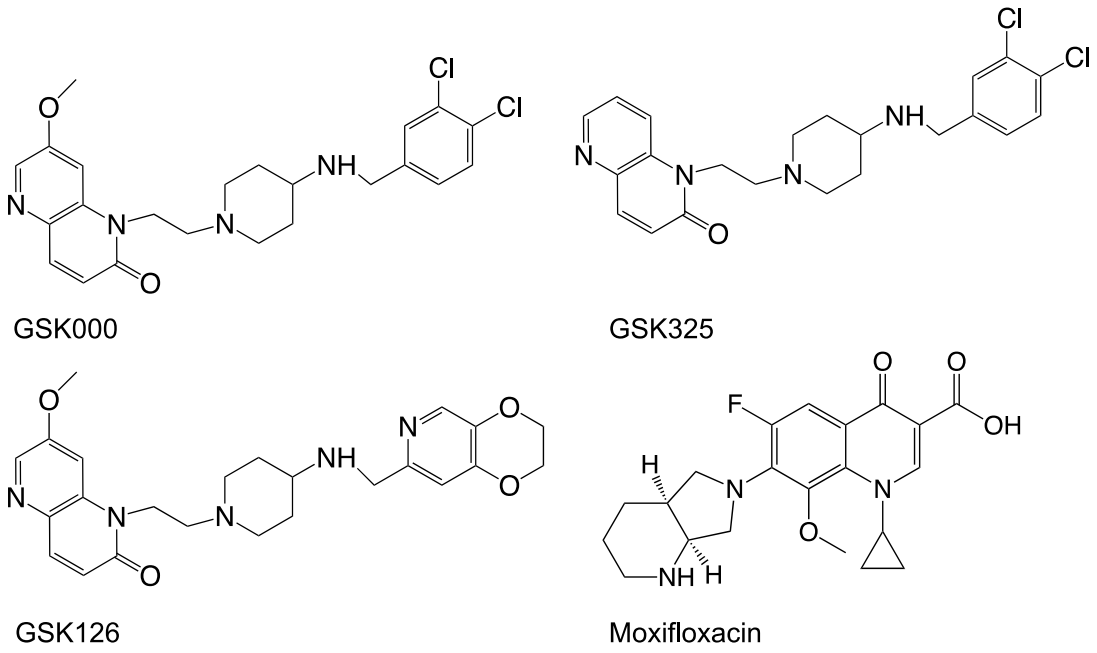


Figure 9. Structures of selected compounds that alter the activity of gyrase. GSK000 and GSK325 are *Mycobacterium tuberculosis* Gyrase Inhibitors (MGIs); GSK 126 is a NBTI; and moxifloxacin is a fluoroquinolone antibacterial.

compounds that displayed activity against *M. tuberculosis* cells in culture and the disease in mouse models.[114] Due to structural and activity differences compared to NBTIs, compounds in this class are known as "*Mycobacterium tuberculosis* gyrase inhibitors" (MGIs). The MGIs are represented by GSK000 and GSK325 in Figure 9. On the basis of genetic/mutagenesis studies in *M. tuberculosis* cells, the authors suggested that gyrase was the primary physiological target of MGIs.[114] However, DNA cleavage studies with purified *M. tuberculosis* gyrase had yet to be reported for any MGI.

Given the potential clinical impact of MGIs for the treatment of tuberculosis, it is critical to understand how they interact with and affect the activity of their target. Therefore, the mechanism of action of MGIs was characterized against purified *M. tuberculosis* gyrase. GSK000 and GSK325 were potent enhancers of gyrase-mediated DNA cleavage. In contrast to fluoroquinolones, the MGIs induced only single-stranded DNA breaks and suppressed the ability of gyrase to generate double-stranded breaks. Furthermore, they maintained activity against gyrase enzymes that harbored the three most common fluoroquinolone resistance mutations in tuberculosis and displayed no activity against human topoisomerase IIα. These findings provide critical mechanistic insight into the actions of MGIs against their cellular target and establish a framework for understanding their actions against tuberculosis.

Results and Discussion

MGIs induce gyrase-mediated single-stranded DNA breaks

Despite the fact that MGIs appear to target gyrase in *M. tuberculosis* cells,[114] their effects on the DNA cleavage activity of the enzyme had yet to be characterized *in vitro*. Therefore, the effects of GSK000 and GSK325 on the DNA cleavage activity of purified *M. tuberculosis* gyrase were determined and compared to those of the NBTI GSK126. As seen in Figure 10, all three of

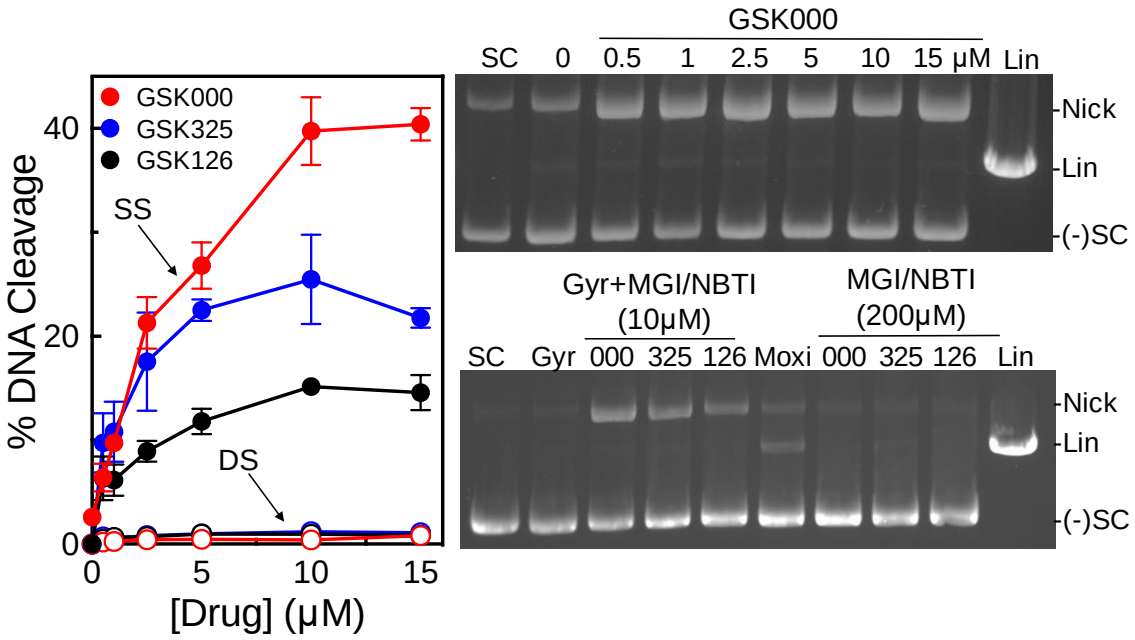


Figure 10. MGIs induce single-stranded DNA breaks mediated by *M. tuberculosis* gyrase. The left panel shows the quantification of single-stranded (SS, closed circles) and double-stranded (DS, open circles) DNA breaks induced by GSK000 (red), GSK325 (blue), or GSK126 (black) in the presence of *M. tuberculosis* gyrase. Error bars represent the standard deviation (SD) of four independent experiments. The top right gel shows DNA cleavage products produced by gyrase that was incubated with increasing concentrations of GSK000. The bottom right gel shows DNA products following cleavage reactions containing 10 μM GSK000 (000), GSK325 (325), or GSK126 (126), or 20 μM moxifloxacin (Moxi) in the presence of gyrase or 200 μM GSK000, GSK325, or GSK126 in the absence of enzyme. Negatively supercoiled (SC) and linear (Lin) DNA controls are shown along with a reaction that contained gyrase, but no drug (Gyr). The mobilities of negatively supercoiled DNA [(-)SC], nicked circular DNA (Nick), and linear DNA (Lin) are indicated. Gels are representative of at least four independent experiments.

the compounds increased levels of single-stranded, but not double-stranded DNA breaks. This is in contrast to the effects of moxifloxacin on *M. tuberculosis* gyrase, which induces primarily double-stranded breaks (Figure 10, lower right gel). GSK000 was the most efficacious compound and increased levels of single-stranded DNA breaks ~21-fold (from 2% cleavage at baseline to 42% maximal cleavage in the presence of the compound) as compared to GSK325 (~12.5-fold enhancement, 25% maximal cleavage) and GSK126 (~7.5-fold enhancement, 15% maximal cleavage) (Figure 10, left panel). These data are consistent with the previous cellular studies, which reported that GSK000 was more cytotoxic than GSK325 and that the NBTI GSK126 had little effect on the growth of *M. tuberculosis* cells.[114]

As a control, all three compounds were incubated with a negatively supercoiled plasmid in the absence of gyrase. Even at a concentration of 200 μM (a concentration 20-fold higher than needed to induce maximal DNA scission in the presence of enzyme), no enhancement of double-stranded or single-stranded breaks was observed (Figure 10, lower right gel). Therefore, the DNA breaks observed in Figure 10 do not appear to be due to a chemical reaction between the MGIs/NBTI and DNA.

A number of experiments were carried out to further describe the enhancement of single-stranded DNA cleavage by MGIs. Because GSK000 was the most efficacious compound, it was used as the focus for these studies. First, when DNA cleavage reactions were stopped by the addition of EDTA (which reverses gyrase-mediated cleavage by chelating the active-site divalent metal ions) or NaCl (which reverses cleavage by disrupting enzyme-DNA binding) prior to the addition of SDS (which traps DNA cleavage complexes by denaturing the enzyme), levels of single-stranded breaks generated in the presence of GSK000 dropped precipitously (Figure 11).

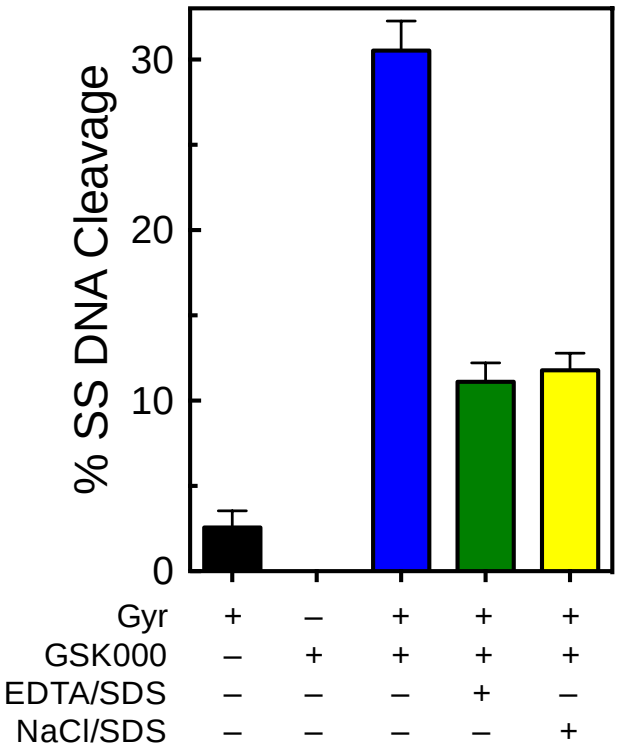


Figure 11. DNA cleavage induced by GSK000 is mediated by *M. tuberculosis* gyrase. The bar graph shows results from reactions that contained negatively supercoiled DNA in the presence of *M. tuberculosis* gyrase (Gyr, black bar), GSK000 (200 µM) in the absence of gyrase (GSK000), or complete reaction mixtures containing 10 µM GSK000 and gyrase that were stopped with SDS prior to the addition of EDTA (blue bar), and reactions that were treated with EDTA (green bar) or NaCl (yellow bar) prior to SDS. Error bars represent the SD of at least 4 independent experiments.

This finding confirms that DNA cleavage enhancement induced by the MGI was mediated by gyrase.

Second, some topoisomerase II poisons, such as the anti-cancer drug etoposide, generate primarily single-stranded breaks at low drug concentrations, but induce high levels of double-stranded breaks at high concentrations.[151, 152] Presumably, this reflects the difficulty of a second drug molecule entering the DNA-cleavage complex. Therefore, to determine whether MGIs can also be pushed to induce double-stranded DNA breaks at high concentrations, a 60-min time course for gyrase-mediated DNA cleavage was carried out at 10 and 200 μM GSK000 (Figure 12, left panel). Similar results were observed under both conditions. Even at 200 μM drug over a time course (60 min) that was 6 times longer than used for standard DNA cleavage assays, no generation of double-stranded breaks was observed. Thus, it appears that GSK000 induces only gyrase-mediated single-stranded cleavage.

Third, even though gyrase does not require ATP to mediate either DNA cleavage or ligation, it needs the high-energy co-factor to carry out its complete DNA strand passage reaction.[13, 25] The DNA cleavage reactions shown in Figures 10-12 were carried out in the absence of ATP. Thus, to determine whether the high-energy co-factor influences the ability of MGIs to induce single- vs. double-stranded DNA breaks, a titration of GSK000 (0-15 μM) was carried out in the absence or presence of 1 mM ATP. As seen in Figure 12 (right panel), ATP had no effect on the levels of single-stranded DNA cleavage or the ability of gyrase to induce double-stranded breaks.

Taken together, the above findings provide strong evidence that MGIs induce only single-stranded DNA breaks mediated by *M. tuberculosis* gyrase.

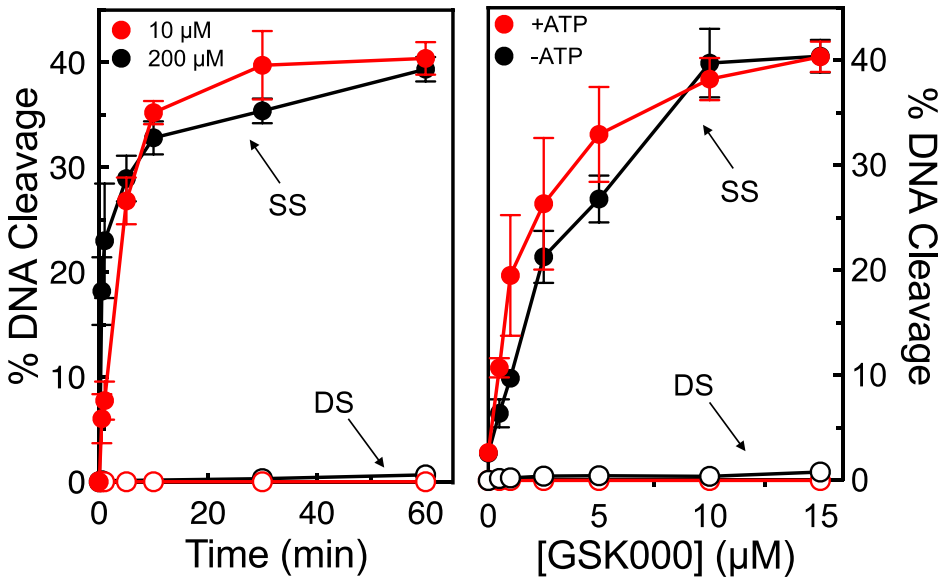


Figure 12. GSK000 enhances only single-stranded DNA breaks mediated by *M. tuberculosis* gyrase. The panel on the left shows the enhancement of gyrase-mediated single-stranded (SS, closed circles) or double-stranded (DS, open circles) DNA breaks generated by gyrase over time in reactions that contained 10 µM (red) or 200 µM (black) GSK000. The right panel shows the effects of GSK000 on gyrase-mediated DNA cleavage in the presence (red) or absence (black) of ATP (1 mM). Error bars represent the SD of at least 3 independent experiments.

GSK000 acts by stabilizing cleavage complexes formed by M. tuberculosis gyrase

Fluoroquinolone antibacterials increase levels of enzyme-mediated DNA strand breaks by stabilizing the covalent enzyme-DNA complexes that are formed upon scission of the genetic material.[13, 14, 25, 47, 65, 147, 153-155] These drugs do so by intercalating into the DNA at each of the cleaved scissile bonds, blocking the ability of gyrase to rejoin the newly generated DNA ends.[25, 62-64, 66, 147] However, only a single NBTI molecule (which is structurally related to the MGIs) appears to bind between the scissile bonds, rather than intercalating within them and stretches the DNA.[62, 114] Consequently, it is not obvious how MGIs increase levels of gyrase-mediated DNA strand breaks. To this point, DNA lesions increase topoisomerase II-mediated DNA cleavage by altering the structure of the genetic material within the cleavage site, allowing the enzyme to bend[156] and cleave DNA faster.[157, 158] Therefore, two approaches were utilized to determine whether GSK000 alters the stability of cleavage complexes formed by *M. tuberculosis* gyrase. In both cases, the rate of loss of MGI-induced single-stranded DNA breaks was compared to the rate of loss of double-stranded breaks induced by the fluoroquinolone moxifloxacin.

As a first approach, I monitored the effects of GSK000 on the rate of enzyme-mediated DNA ligation, which was determined by shifting cleavage complexes from 37 ºC to 75 ºC (a temperature that allows ligation, but not cleavage of the DNA). As seen in Figure 13 (left panel), the rate of gyrase-mediated DNA ligation in the presence of 10 µM GSK000 was ~3 times slower than observed in the absence of drug. This diminution in the rate of ligation is similar to that seen in the presence 50 µM moxifloxacin and suggests that GSK000 stabilizes cleavage complexes to a similar extent as the fluoroquinolone.

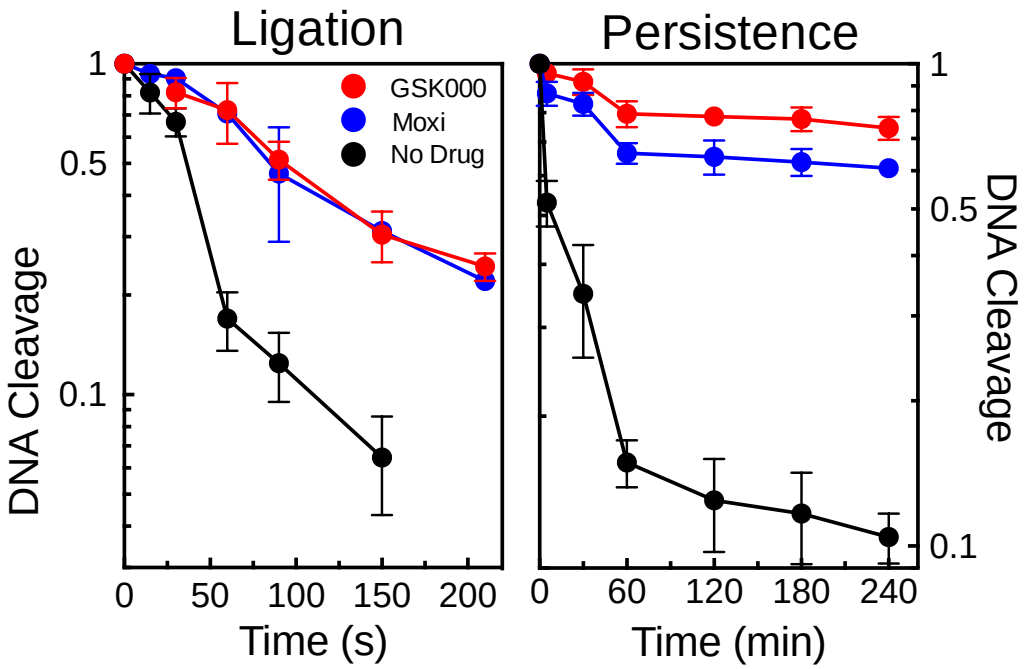


Figure 13. Effects of GSK000 and moxifloxacin on ligation and persistence of cleavage complexes mediated by *M. tuberculosis* gyrase. The rate of gyrase-mediated DNA ligation (left) and the stability of ternary gyrase–drug–DNA cleavage complexes (right) were monitored by the loss of single-stranded DNA breaks in the presence of 10 μM GSK000 (red) or the loss of double-stranded DNA cleavage in the absence of drug (black) or in the presence of 50 μM moxifloxacin (blue). Levels of DNA cleavage at time 0 (42% single-stranded breaks in the presence of GSK000 and 36% double-stranded breaks in the presence of moxifloxacin) were set to 1 to allow direct comparison. Error bars represent the SD of at least 3 independent experiments.

For the second approach, I monitored the effects of GSK000 on the persistence (stability) of cleavage complexes following a 20-fold dilution of reaction mixtures. This assay reflects the rate at which the drug dissociates from the ternary complex, given that enzyme-DNA-drug ternary complexes are much less likely to form when they are diluted. As seen in Figure 13 (right panel), GSK000 increased the lifetime of cleavage complexes to an extent even greater than that seen with moxifloxacin. Thus, it appears that GSK000 increases the level of gyrase-mediated single-stranded DNA breaks primarily by increasing the stability of cleavage complexes.

GSK000 and moxifloxacin induce gyrase-mediated DNA cleavage at a different array of sites

Sites of gyrase-mediated DNA scission were mapped in the presence of GSK000 or moxifloxacin to determine whether MGIs and fluoroquinolones display the same cleavage specificity (Figure 14). Linear end-labeled pBR322 was used for this experiment. Although overlap between the cleavage patterns generated by the MGI and the fluoroquinolone was observed (sites of similar cleavage are indicated by blue arrows), a number of unique sites or sites where utilization differed between the two drugs were observed. Representative sites that were cleaved more frequently in the presence of the MGI (red arrows) or the fluoroquinolone (green arrows) are indicated. These differences likely reflect the difference in the way MGIs and fluoroquinolones interact with *M. tuberculosis* gyrase. Similar DNA cleavage results were obtained when cleavage complexes were enriched by K^+-SDS precipitation of the gyrase prior to treatment with Proteinase K and electrophoresis (Figure 14). This provides further evidence that the cleavage observed was mediated by gyrase.

GSK000 suppresses double-stranded DNA breaks generated by M. tuberculosis *gyrase*

Previous studies have demonstrated that topoisomerase II poisons like etoposide are able to act independently at each scissile bond.[151] Consequently, drug action on the Watson strand often

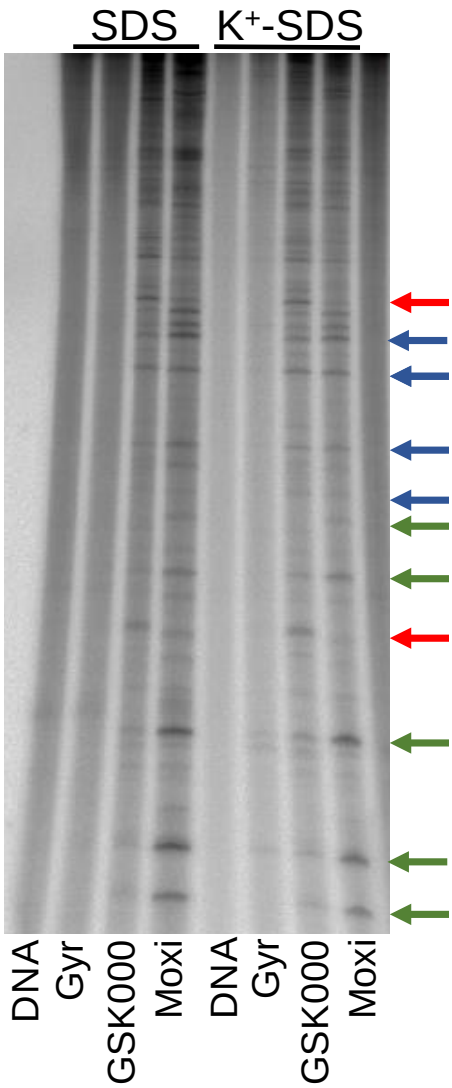


Figure 14. Effects of GSK000 and moxifloxacin on the sites of DNA cleavage generated by *M. tuberculosis* gyrase. An autoradiogram of a polyacrylamide gel is shown. Reaction mixtures contained DNA with no enzyme (DNA), enzyme in the absence of drug (Gyr), or enzyme in the presence of 100 µM GSK000 (GSK000) or 100 µM moxifloxacin (Moxi). The left- and right-hand sides of the gel show reactions processed without or following K^+-SDS precipitation of DNA cleavage complexes. Red arrows indicate representative strong sites where GSK000 induced greater levels of DNA cleavage than did moxifloxacin, green arrows indicate representative strong sites where moxifloxacin induced greater levels of cleavage than did GSK000, and blue arrows indicate representative sites where GSK000 and moxifloxacin induced similar levels of cleavage. The autoradiogram is representative of at least 4 independent experiments.

has relatively little effect on levels of cleavage or rates of ligation on the Crick strand.[151] Because MGIs/NBTIs do not appear to interact with the individual scissile bonds on the Watson and Crick strands, it is not obvious whether their presence coordinately affects cleavage on both strands. Thus, these compounds may enhance cleavage on only one strand of the double helix without affecting the other. Alternatively, the propensity of MGIs to induce single-stranded breaks suggests that they may suppress the ability of the enzyme to cut both strands of the double-helix.

The low level of double-stranded DNA breaks induced by *M. tuberculosis* gyrase in the absence of drugs makes it difficult to differentiate between these two possibilities. Therefore, $MgCl_2$ in DNA cleavage reactions was replaced with $CaCl_2$. This latter divalent metal ion can replace Mg^{2+} in the active site of type II topoisomerases.[129] Although most properties of the DNA cleavage and ligation reactions remain unchanged, considerably higher levels of enzyme-mediated double-stranded breaks are generated in the presence of Ca^{2+}.[65, 129] In the case of *M. tuberculosis* gyrase, background levels of enzyme-mediated double-stranded DNA cleavage increased to ~10% (from 2% in Mg^{2+}).

As seen in Figure 15, GSK000 induced high levels of single-stranded DNA breaks in the presence of Ca^{2+}. However, this enhancement of single-stranded DNA cleavage was accompanied by a decrease in double-stranded DNA-cleavage (a representative gel is shown at the top, with quantification shown in the bottom panel). This finding strongly suggests that the induction of cleavage by one MGI molecule actually suppresses cleavage of the second strand. The molecular basis for this property is not known. However, it likely results from a distortion of the DNA in the active site of the enzyme, similar to what is generated in the presence of NBTIs.[62]

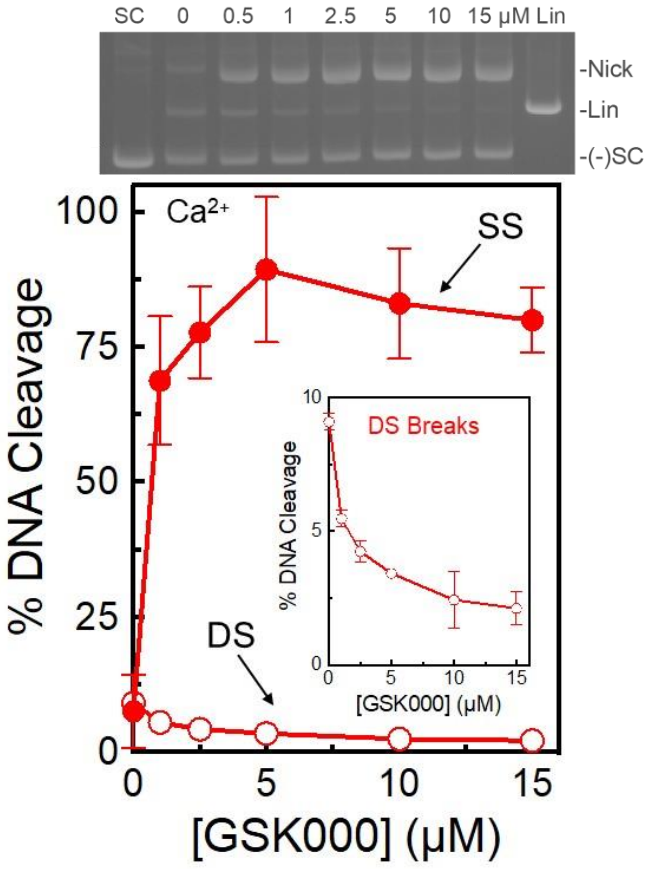


Figure 15. GSK000 suppresses double-stranded DNA breaks generated by *M. tuberculosis* gyrase. The gel (top) shows DNA cleavage products following incubation of gyrase with increasing concentrations of GSK000 in the presence of Ca^{2+}. Negatively supercoiled (SC) and linear controls (Lin) are shown. The gel is representative of at least 4 independent experiments. The graph quantifies the effects of GSK000 on *M. tuberculosis* gyrase-mediated single-stranded (SS, closed circles) and double-stranded (DS, open circles) DNA cleavage. Error bars represent the SD of at least 4 independent experiments.

The actions of GSK000 and moxifloxacin on the induction of cleavage by M. tuberculosis *gyrase are mutually exclusive*

Because of the mechanistic and structural differences between the MGIs and fluoroquinolones, it is not known whether they can simultaneously affect DNA cleavage activity of a single gyrase enzyme. As a first step toward addressing this issue, modeling studies were carried out by Ben Bax, a collaborator, to address whether it is theoretically possible to form ternary gyrase-DNA-drug structures that include both GSK000 and moxifloxacin (Figure 16). On the basis of these studies, it does not seem possible for both drugs to interact simultaneously in the same ternary complex. NBTIs are believed to trap the enzyme in the CRsym conformation, in which only one compound can be accommodated within the DNA. In the model of the GSK000 complex (Figure 16B), which is in the CRsym conformation, the DNA is well ordered and there is no room at either cleavage site to accommodate an additional compound.[159] The moxifloxacin structure (Figure 16A) is in an intermediate conformation in which the subunits have moved slightly apart allowing a second compound to be accommodated in the DNA.[159] Note that the superimposition of the GSK000 and moxifloxacin structures (Figure 16C) requires that basepairs overlap with the compounds.

To confirm the conclusions of the modeling studies, a competition study was utilized to determine whether GSK000 and moxifloxacin are capable of acting within the same ternary complex. In the absence of the MGI, moxifloxacin readily induced enzyme-mediated double-stranded DNA breaks (~36% in the presence of 100 μM drug), but also induced ~12% single-stranded breaks (peaking at 25 μM) (Figure 17, left panel). In the competition study, *M. tuberculosis* gyrase was saturated with 10 μM GSK000 (yielding the typical ~40% single-stranded DNA breaks) (Figure 17, right panel) followed by incubation with 0-200 μM moxifloxacin.

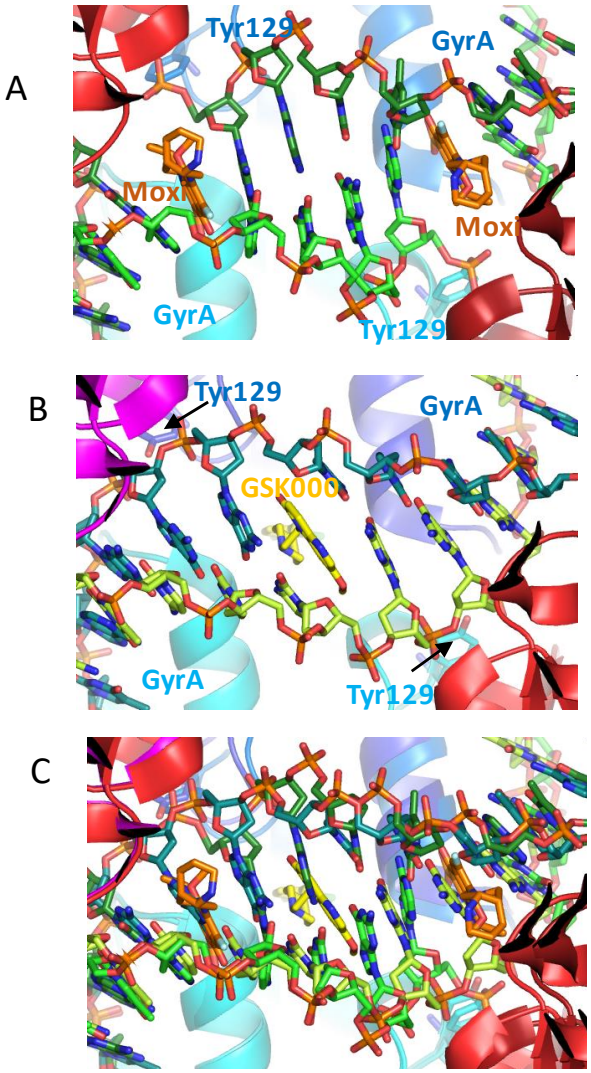


Figure 16. M**odeling studies suggest that moxifloxacin and GSK000 cannot interact simultaneously in the same drug-enzyme-DNA ternary complex**. **A.** View down the two-fold axis of a crystal structure of a ternary complex formed with *M. tuberculosis* gyrase, DNA, and moxifloxacin.[66] Gyrase subunits are shown in cartoon representation, in blue/cyan (GyrA) or red/dark red (GyrB). The catalytic tyrosine (Tyr 129) which has cleaved the DNA is shown in stick representation. Moxifloxacin (orange carbons) and DNA (green carbons) are also shown in stick representation. **B.** Model of a complex of GSK000 (yellow carbons) with *M. tuberculosis* gyrase and uncleaved DNA that was based on the crystal structure of GSK299423 with *Staphylococcus aureus* gyrase.[62] **C.** Superimposition of A and B require that base-pairs overlap with the compounds.

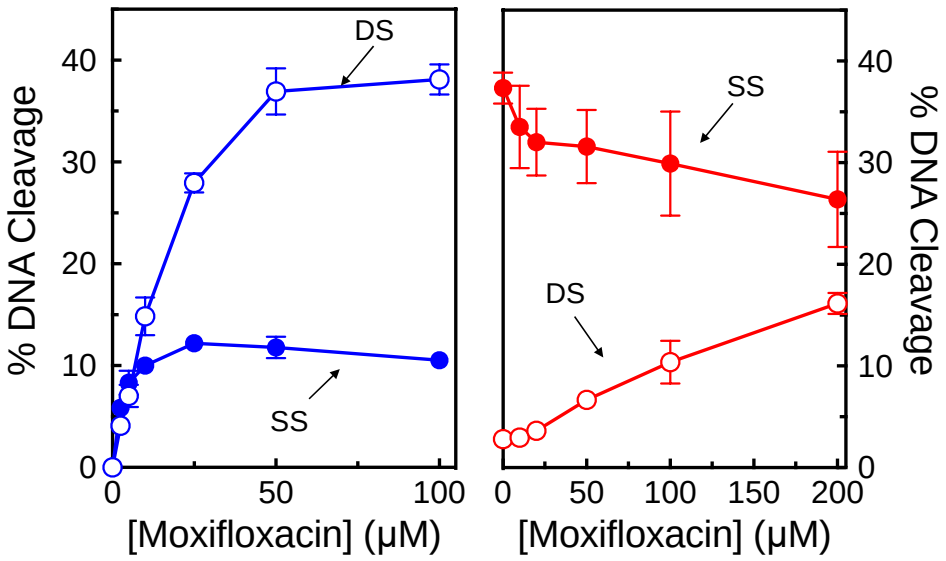


Figure 17. The actions of GSK000 and moxifloxacin on gyrase-mediated DNA cleavage are mutually exclusive. In the left panel, enhancement of *M. tuberculosis* gyrase-mediated single-stranded (SS, closed circles) and double-stranded (DS, open circles) DNA cleavage is shown in the presence of moxifloxacin alone (blue). In the right panel, gyrase was saturated with 10 μM GSK000 followed by a subsequent titration of 0-200 μM moxifloxacin. Error bars represent the SD of at least 3 independent experiments.

Changes in the level of double- and single-stranded breaks were monitored (Figure 17, right panel) to determine if there was competition or additivity between the two compounds. As the concentration of moxifloxacin increased, levels of double-stranded breaks rose, albeit to a lesser extent than seen in the absence of the MGI (similar changes in DNA cleavage were observed if gyrase was incubated with moxifloxacin prior to the addition of GSK000). Because GSK000 does not generate double-stranded DNA breaks, this increase demonstrates that the fluoroquinolone was able to stabilize cleavage complexes in reaction mixtures that also contain the MGI. However, it does not differentiate whether the GSK000 in reaction mixtures was replaced by moxifloxacin or was still situated within the ternary complex.

To distinguish between these latter possibilities, I monitored the single-stranded breaks that are generated in the presence of both drugs (Figure 17, right panel). In contrast to double-stranded breaks, single-stranded breaks decreased in the presence of moxifloxacin. This finding suggests that moxifloxacin acts only when it can displace GSK000 from the complex. Had both drugs been present in the ternary complex, levels of single-stranded breaks would have been expected to rise, because both drugs induce enzyme-mediated single-stranded DNA cleavage. Taken together, these results strongly suggest that the actions of MGIs and fluoroquinolones utilize mutually exclusive mechanisms to induce *M. tuberculosis* gyrase-mediated DNA cleavage.

GSK000 induces lower levels of gyrase-mediated DNA cleavage on positively supercoiled DNA

In the cell, gyrase removes the positive supercoils that accumulate ahead of replication forks and transcription complexes.[10, 24, 43] As these DNA tracking systems encounter the acting gyrase, transient cleavage complexes can be converted to non-ligatable DNA strand breaks that induce the SOS DNA damage response pathway.[160] When this pathway is overwhelmed, the DNA breaks can lead to cell death. Because drug-stabilized cleavage complexes formed ahead of moving

forks and transcription complexes are most likely to be converted to non-ligatable strand breaks, cleavage complexes stabilized on positively supercoiled DNA are the most dangerous for the cell.[29, 161-163] A recent study by Ashley *et al.* demonstrated that *M. tuberculosis* gyrase maintains 2-3-fold lower levels of cleavage complexes on positively compared to negatively supercoiled DNA in the absence or presence of fluoroquinolones.[20] This attribute makes gyrase a safer enzyme (for cells) to work ahead of DNA tracking systems, but may also affect the cytotoxicity of fluoroquinolones.

Because MGIs/NBTIs interact differently with DNA within the cleavage complex than do fluoroquinolones, I wanted to determine whether GSK000 differentially affected DNA scission mediated by *M. tuberculosis* gyrase on negatively and positively supercoiled substrates. As seen in Figure 18, gyrase induced ~3-4-fold lower levels of single-stranded breaks with positively supercoiled DNA. Thus, the effects of DNA supercoil geometry on the actions of MGIs and fluoroquinolones appear to be similar.

Effects of GSK000 on the catalytic activities of M. tuberculosis *gyrase*

Some NBTIs appear to act by inhibiting the catalytic activities of bacterial type II topoisomerases rather than by poisoning these enzymes.[62, 107, 108, 110, 147] In addition, the MGIs have been shown to inhibit the DNA supercoiling reaction catalyzed by *M. tuberculosis* gyrase.[114] Therefore, the effects of GSK000 on the catalytic activities of *M. tuberculosis* gyrase were assessed and compared to those of moxifloxacin. As seen in Figure 19 (top left and right panels), the IC_{50} values (~0.5 vs. 1 μM, respectively) for the inhibition of gyrase-catalyzed supercoiling of relaxed DNA by GSK000 and moxifloxacin were similar. This is in contrast to their abilities to kill *M. tuberculosis* cells[114] or enhance DNA

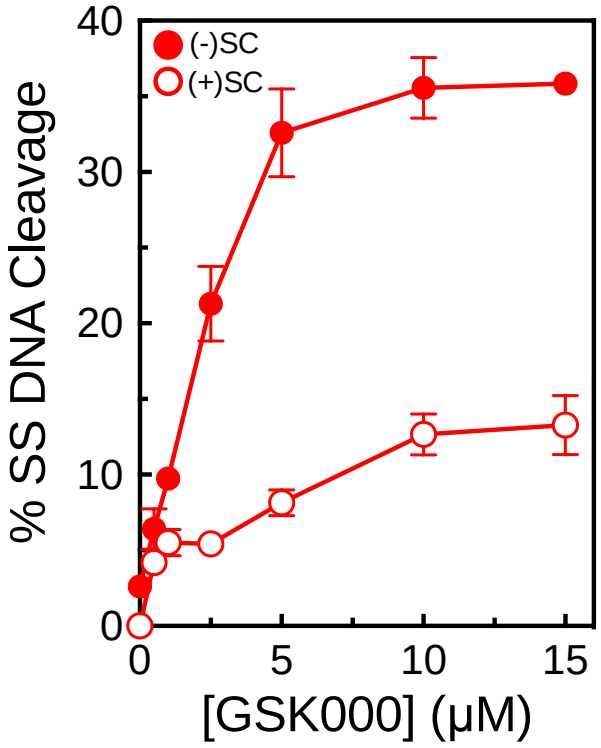


Figure 18. GSK000 maintains lower levels of gyrase-mediated single-stranded DNA breaks on positively supercoiled DNA. The effects of GSK000 on the enhancement of gyrase-mediated single-stranded cleavage of negatively (closed circles) and positively (open circles) supercoiled DNA is shown. Error bars represent the SD of at least 3 independent experiments.

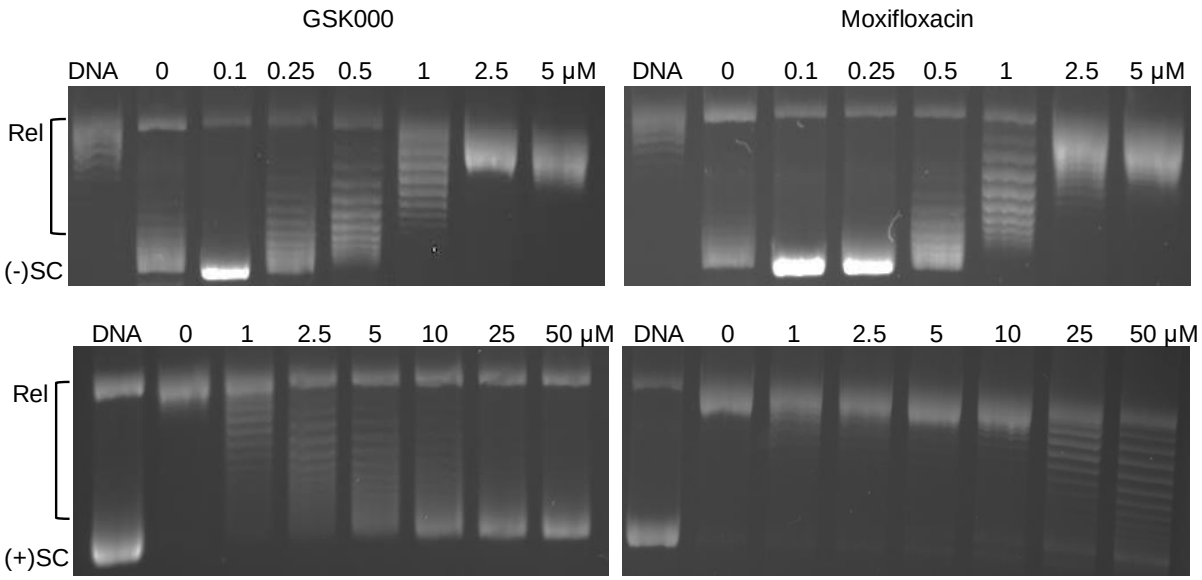


Figure 19. Inhibition of gyrase catalyzed reactions by GSK000 and moxifloxacin. The effects of GSK000 (left panels) and moxifloxacin (right panels) on the supercoiling of relaxed DNA (top panels) and the relaxation of positively supercoiled DNA (bottom panels) are shown. The positions of relaxed (Rel), negatively supercoiled [(-)SC], and positively supercoiled [(+)SC] DNA are indicated. Gels are representative of at least four independent

cleavage mediated by *M. tuberculosis* gyrase (see Figures 10 and 17). In these latter two cases, GSK000 is ≥10-fold more potent than moxifloxacin. These data strongly suggest that the mechanism of cell killing by these two drugs is not directly associated with their ability to inhibit DNA supercoiling catalyzed by *M. tuberculosis* gyrase.

In contrast to drug effects on DNA supercoiling, GSK000 was a more potent inhibitor of the gyrase-catalyzed relaxation of positively supercoiled DNA than was moxifloxacin (Figure 19, bottom left and right panels). The IC_{50} of the MGI (~2.5 to 5 μM) was at least an order of magnitude lower than that seen with moxifloxacin. The removal of positive DNA supercoils generated by replication forks represents a critical step in the elongation of DNA replication. Therefore, these results cannot rule out a role for the inhibition of gyrase-catalyzed removal of positive supercoils (in addition to their effects on gyrase-mediated DNA cleavage) in cell death induced by MGIs.

GSK000 preferentially acts against M. tuberculosis *gyrase*

MGIs were originally selected for their ability to kill *M. tuberculosis* cells.[114] However, it is not known whether the activity of MGIs toward this species reflects a broadened spectrum of drug activity or an increased specificity for *M. tuberculosis.* Therefore, the ability of GSK000 to induce DNA cleavage mediated by gyrase and topoisomerase IV from Gram-negative *E. coli* and *N. gonorrhoeae,* and Gram-positive *B. anthracis* was assessed (Figure 20). GSK000 displayed much lower activity against enzymes from these other species than it did for *M. tuberculosis* gyrase. Therefore, at least in regard to the interaction of MGIs with their enzyme target, it appears that the structural differences between MGIs and NBTIs increase the specificity of these drugs for *M. tuberculosis* gyrase rather than broadening their spectrum of action.

In order to extend this conclusion from biochemical studies to the organismal level, it will be necessary to carry out extensive microbiological studies on a variety of bacterial species.

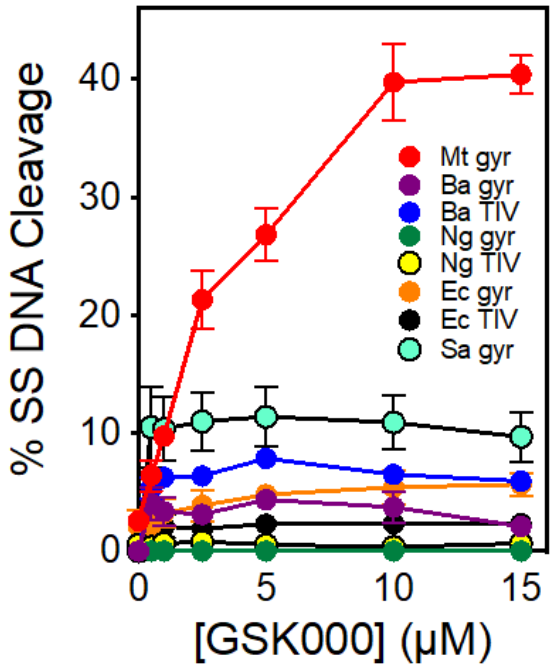


Figure 20. GSK000 acts preferentially against *M. tuberculosis* gyrase. The effects of GSK000 on single-stranded DNA cleavage mediated by *M. tuberculosis* gyrase (Mt gyr, red), *B. anthracis* gyrase (Ba gyr, purple) and topoisomerase IV (Ba TIV, blue), *N. gonorrhoeae* gyrase (Ng gyr, green) and topoisomerase IV (Ng TIV, yellow), *E. coli* gyrase (Ec gyr, orange), topoisomerase IV (Ec TIV, black), and *Staphylococcus aureus* gyrase (Sa gyr, aqua) are shown. Error bars represent the SD of at least 3 independent experiments.

However, a comparison carried out by a collaborator, Monica Cacho, of the activity of GSK000, GSK325, and GSK126 toward cultured *M. tuberculosis* (H37Rv) and *E. coli* (7623) cells is consistent with an enhanced specificity of MGIs toward *M. tuberculosis* gyrase.

Whereas, the reported minimum inhibitory concentration (MIC) values of GSK000 and GSK325 for *M. tuberculosis* were <0.01 μM and 0.08 μM, respectively, the MIC for the NBTI GSK126 was considerably higher (0.5 μM).[114] In contrast, for *E. coli*, the MIC of GSK126 (0.26 μM) was substantially lower than those of the two MGIs GSK000 and GSK325 (2.2 μM and 2.3 μM, respectively). It is also notable that the MICs for GSK000 and GSK325 against *E. coli* are considerably higher than those reported with *M. tuberculosis*.

If the biochemical and microbiological data described above are representative of other species, an enhanced specificity of MGIs could have advantages for its clinical use. Tuberculosis is sometimes misdiagnosed as pneumonia, which is treated with fluoroquinolones.[164, 165] Unfortunately, this initial treatment is associated with the development of fluoroquinolone-resistant *M. tuberculosis*.[165-168] If MGIs display specificity for *M. tuberculosis,* they would not be used to treat a misdiagnosed patient, potentially leading to fewer cases of resistance. In addition, anti-tuberculosis regimens are normally prescribed for several months.[55, 145] Thus, patients taking MGIs are potentially less likely to incur the adverse drug events associated with the long-term use of a broad-spectrum antibacterial in the regimen.[142]

MGIs share two properties with fluoroquinolones that may ultimately affect their clinical development. First, the MGIs inhibition of the hERG potassium channel may need to be decreased.[114] Second, the reported frequency of spontaneous resistance mutations for GSK126 (no data is available for the MGIs) is higher than that of moxifloxacin.[114] However, this value is similar to or lower than other drugs routinely used to treat tuberculosis.

MGIs maintain activity against M. tuberculosis *gyrase enzymes carrying the most common mutations associated with fluoroquinolone resistance*

In a previous study, GSK000 and GSK325 maintained cytotoxic activity against *M. tuberculosis* cells that carried gyrase mutations $GyrA^{A90V}$ or $GyrA^{D94G}$, both of which elicit fluoroquinolone resistance.[114] However, the effects of these two MGIs on the DNA cleavage activity of *M. tuberculosis* gyrase enzymes that harbor these fluoroquinolone resistance mutations are unknown. Therefore, the effects of GSK000 and GSK325, as well as GSK126, on gyrase enzymes containing the $GyrA^{A90V}$ or $GyrA^{D94G}$ mutations or a $GyrA^{D94H}$ mutation were determined. These three gyrase alterations represent the most common mutations associated with fluoroquinolone resistance in tuberculosis.[154]

As seen in Figure 21, GSK000 and GSK325 retained the ability to induce the mutant gyrase enzymes to generate single-stranded DNA breaks. GSK000 retained full activity against all three mutant enzymes (left panel). Although the activity of GSK325 (right panel) was slightly lower when incubated with $GyrA^{D94H}$, the compound maintained full activity against $GyrA^{A90V}$ and $GyrA^{D94G}$. These results demonstrate that MGIs are able to overcome the most common causes of target-mediated fluoroquinolone resistance in tuberculosis.

I took advantage of these findings with the fluoroquinolone-resistant mutant enzymes to reexamine the moxifloxacin-GSK000 competition studies (Figure 17). In contrast to results with the wild-type enzyme, moxifloxacin was unable to compete with the actions of GSK000 against $GyrA^{D94G}$ (Figure 22). Even at 200 μM moxifloxacin, virtually no decrease in single-stranded breaks or increase in double-stranded breaks was observed. This finding confirms that the competition between moxifloxacin and GSK000 seen in Figure 17 was due to the replacement of the MGI by the fluoroquinolone in the active site of gyrase.

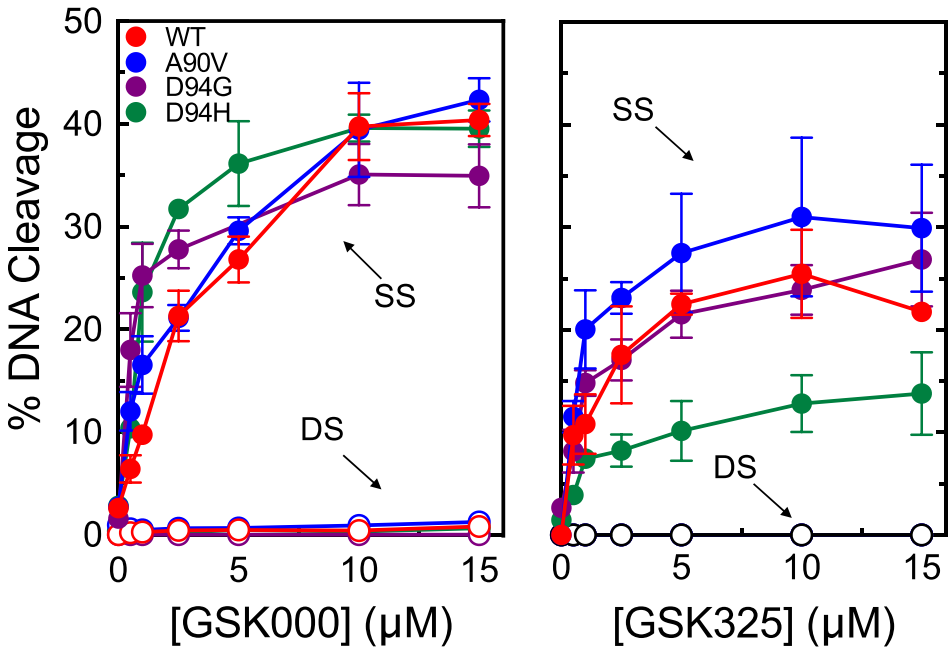


Figure 21. MGIs maintain activity against *M. tuberculosis* gyrase containing the most common mutations associated with clinical fluoroquinolone resistance. Effects of the MGIs GSK000 (left panel) an d GSK325 (right panel) on wild-type (red) *M. tuberculosis* gyrase and gyrase containing the fluoroquinolone resistance mutations at $GyrA^{A90V}$ (blue), $GyrA^{D94G}$ (purple), or $GyrA^{D94H}$ (green) are shown. Single-stranded (SS) and double-stranded (DS) DNA breaks are denoted by closed and open circles respectively. Error bars represent the SD of at least 3 independent experiments.

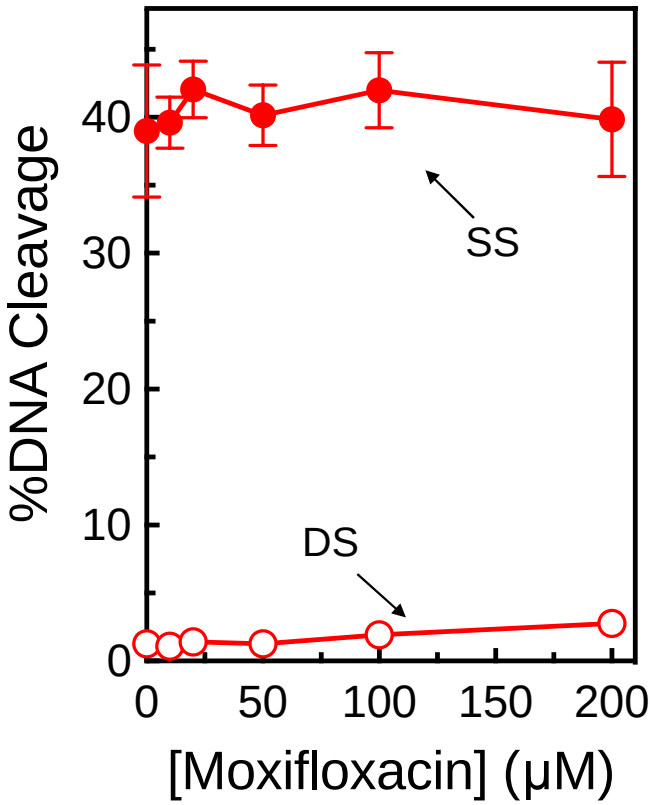


Figure 22. The actions of GSK000 and moxifloxacin on DNA cleavage mediated by GyrA^{D94G}. The mutant fluoroquinolone-resistant gyrase was saturated with 10 μM GSK000 followed by a subsequent titration of 0-200 μM moxifloxacin. Error bars represent the SD of at least 3 independent experiments.

MGIs do not induce DNA cleavage mediated by human topoisomerase IIα

Bacterial and human type II topoisomerases share a significant level of amino acid identity.[4] Although clinically relevant fluoroquinolone antibacterials display low activity against the human type II enzymes, some members of this drug class cross over into mammalian systems and are potent poisons of human type II topoisomerases.[13, 25, 77, 104, 169] Because poisoning the human enzyme precludes the clinical use of these fluoroquinolones as antibacterial agents, it is important to assess the activity of gyrase-targeted drugs against the human enzymes.

The ability of GSK000 and GSK325, as well as the NBTI GSK126, to induce DNA strand breaks mediated by topoisomerase IIα is shown in Figure 23. None of the drugs displayed any effect on DNA cleavage mediated by the human enzyme. This result is in marked contrast to the high levels of single- and double-stranded breaks induced by etoposide, a clinically important anti-cancer drug.[151, 170]

MGIs retain wild-type activity with fluoroquinolone-resistant clinical isolates

Results from Blanco, *et al*. and my enzymological studies demonstrate that the MGIs can overcome the most prevalent gyrase mutations seen in the clinic ($GyrA^{A90V}$, $GyrA^{D94G}$, or $GyrA^{D94H}$).[114, 118] Even though target-mediated resistance with fluoroquinolones is the most common and usually the first type of resistance observed, resistance can also be caused due to an upregulation of efflux pumps.[25] These mutations result in lower drug concentrations in the *M. tuberculosis* cell, dampening the fluoroquinolones cytotoxic activity. I determined the MIC for the MGIs against four patient isolates that either contained no or a low percentage of a mutation in gyrase, the wild-type H37Rv strain, and two patient isolates that contained the $GyrA^{A90V}$ or the $GyrA^{D94G}$ mutation as a control (Table 2). These mutations were tested by the Sterling group with the addition of efflux pump inhibitors and based on these results the resistance detected suggests

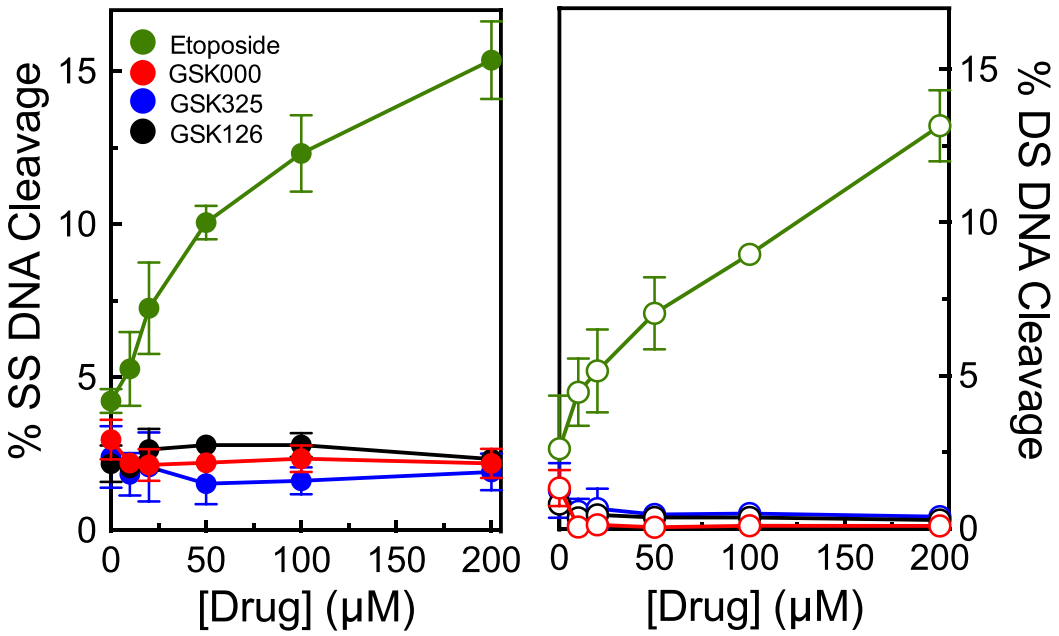


Figure 23. MGIs/NBTI do not enhance DNA cleavage mediated by human topoisomerase IIα. The left and right panels show the effects of the MGIs GSK000 (red) and GSK325 (blue) and the NBTI GSK126 (black) on single-stranded (closed circles) and double-stranded (open circles) DNA cleavage mediated by the human enzyme. The effects of etoposide (green), a widely prescribed anti-cancer drug, on topoisomerase IIα are shown as a positive control. Error bars represent the SD of at least 3 independent experiments.

Isolate	Mutation (GyrA)	Frequency
H37Rv	---	---
1711	A90V	99.9%
853	D94G	99.8%
31	A90V	8%
584	D94G	2.6%
1359	D94Y	2.9%
1299	D94N	2.5%

	H37Rv	1711	853	31	584	1359	1299
Ofloxacin	1	8	16	4	4	8	8
Moxifloxacin	0.5	4	8	4	4	8	8
Ofloxacin + Reserpine	NT	8	16	2	0.5	1	2
Ofloxacin + Verapamil	NT	8	16	1	0.5	1	2
GSK000	<0.125	0.5	1	1	1	0.5	2
GSK325	0.125	0.25	0.5	2	2	1	4

Table 2. Table of mutation frequency (top) and MIC values (bottom) for patient isolates of *M. tuberculosis*. The mutation frequency denoted is from whole genome sequencing. MIC data was determined using the resazurin microtiter assay and carried out in triplicate. All MIC values are in µg/mL and NT denotes not tested.

that an upregulation of efflux pumps is preventing a high enough cellular concentration of fluoroquinolone to kill the cells (Table 2). Against all four isolates GSK000 and GSK325 were able to maintain activity and have MIC values that were equal to or less than those seen with the fluoroquinolones plus the efflux pump inhibitor (Table 2). The results seen with the control strains were similar to the results from Blanco *et al*.[114] Not only can the MGIs overcome target-mediated resistance, but these results suggest they can also overcome resistance due to efflux upregulation.

Conclusions

Tuberculosis is one of the leading causes of morbidity worldwide and drug resistance and intolerance are prevalent. Consequently, there is a desperate need for the development of new anti-tubercular drugs that overcome resistance and/or are better tolerated by patients. Although there is a continual search for new drug targets, another approach is to develop novel compounds with high activity against validated targets, but that still retain activity in the face of current resistance profiles.

MGIs were selected for activity against *M. tuberculosis*, and, based on genetic studies and their structural similarities to NBTIs, they were believed to target gyrase.[114] However, little was known about their interactions with the *M. tuberculosis* type II enzyme. Therefore, the activity of two MGIs was characterized against *M. tuberculosis* gyrase. Results indicate that MGIs are potent gyrase poisons. These compounds induce only single-stranded enzyme-mediated DNA breaks and suppress the ability of gyrase to cut both strands of the double helix. MGIs appear to be selective for *M. tuberculosis* gyrase over other species, retain activity against the most common mutations that lead to fluoroquinolone resistance, display no activity against human topoisomerase IIα, and retain activity against clinical isolates that are fluoroquinolone-resistant due to altered efflux pump activity. These findings suggest that MGIs have potential as anti-tubercular drugs, especially in

the case of fluoroquinolone-resistant disease. The mechanistic and cellular studies described above provide a basis for future structure-activity studies directed toward improving drug activity, while addressing physiological toxicities and mutation rates.

CHAPTER IV

ACTIONS OF A NAPTHYRIDONE/AMINOPIPERIDINE-BASED ANTIBACTERIAL TARGETING BACTERIAL TYPE II TOPOISOMERASES

Introduction

Although most NBTIs display poor activity against *M. tuberculosis*, as described in chapter III, GSK126 (napthyridone/aminopiperidine-based NBTI) was identified in a screen as having moderate activity against the bacterium (Figure 9).[114] In the previous chapter, I characterized the mechanistic basis for the actions of MGIs against *M. tuberculosis* gyrase. However, relatively little is known about the actions of the parent class of NBTIs against gyrase. Furthermore, there have been no reports describing the activity of NBTIs against any species of topoisomerase IV.

In order to address these important issues, I analyzed the actions of GSK126 against a variety of bacterial type II topoisomerases. These studies will provide direct comparisons with the MGIs. Results indicate that GSK126 has a broader spectrum of activity against gyrase and topoisomerase IV than the MGIs, but displays similar mechanistic characteristics. Like the MGIs, the NBTI induced only single-stranded breaks, and suppressed the ability of the enzymes to generate double-stranded breaks. Furthermore, it maintained activity against a variety of fluoroquinolone resistant mutant enzymes. Finally, GSK126 utilizes a similar mechanism of action against both gyrase and topoisomerase IV.

Results and Discussion

MGIs are currently the most well-characterized members of the napthyridone/aminopiperidine-based NBTI-like drugs. However, their selection for activity for *M. tuberculosis* resulted in a group of compounds with narrow specificity. As seen in Figure 24 (left),

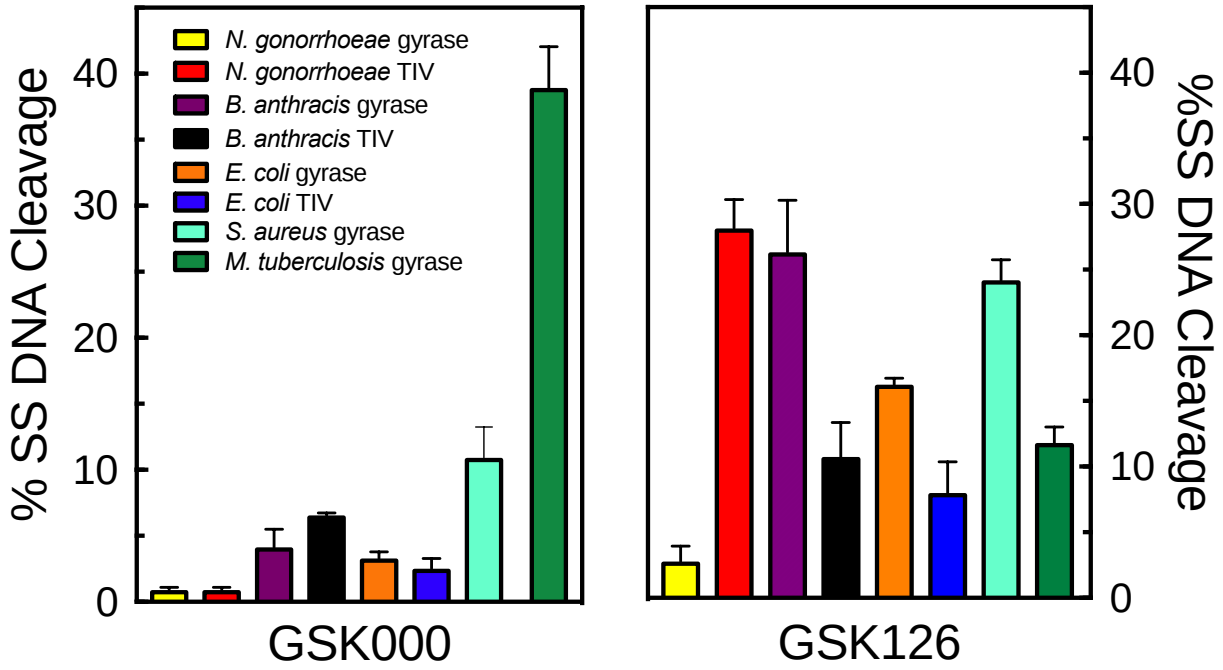


Figure 24. GSK126 displays activity against both Gram-positive and Gram-negative type II topoisomerases enhancing only single-stranded (SS) DNA breaks. In contrast to the NBTI, GSK000 shows specificity towards *M. tuberculosis* gyrase. The peak cleavage mediated by each bacterial type II topoisomerase is displayed.

the most active MGI, GSK000, induces high levels of single-stranded DNA breaks with only *M. tuberculosis* gyrase. In contrast, the activity of the compound against *N. gonorrhoeae*, *B. anthracis*, *E. coli* gyrase and topoisomerase IV, and *Staphylococcus aureus* gyrase ranged from virtually none to weak. This narrow specificity range raises questions regarding the spectrum of action of the parent NBTI series and whether the mechanism of the napthyridone/aminopiperidine-based compounds are translatable across species.

Therefore, the ability of GSK126 to induce enzyme-mediated single-stranded breaks was examined for each of the type II enzymes discussed above. In contrast to the MGI, the NBTI displayed a much broader specificity (Figure 24, right). The compound showed high activity against *B. anthracis* gyrase and *N. gonorrhoeae* topoisomerase IV and at least moderate activity against every other enzyme with the exception of *N. gonorrhoeae* gyrase. In order to assess the basis for the actions of GSK126 against bacterial type II topoisomerases, I decided to examine the activity of the compound against three separate species of gyrase and topoisomerase IV. I chose to use *B. anthracis* gyrase and *N. gonorrhoeae* topoisomerase IV due to the high efficacy of GSK126, *M. tuberculosis* gyrase to afford direct comparisons to MGIs, and *B. anthracis* topoisomerase IV to have a matched set of enzymes from a gram-positive species.

The activity of GSK126 against gyrase

The effects of GSK126 on gyrase-mediated DNA cleavage by the enzymes from *B. anthracis* and *M. tuberculosis* are shown in Figure 25. Although the NBTI was an order of magnitude more potent against *B. anthracis* gyrase, GSK126 induced only single-stranded DNA breaks with both enzymes. To further assess the ability of GSK126 to induce single- vs. double-stranded breaks, DNA cleavage reactions with *B. anthracis* and *M. tuberculosis* gyrase were carried out at NBTI concentrations as high as 200 μM and at reaction times up to six times longer than required to

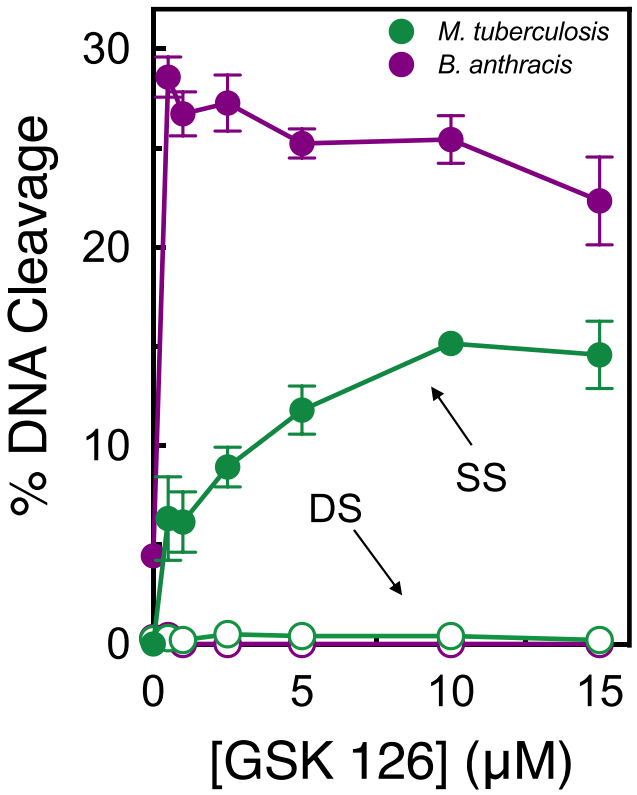


Figure 25. GSK126 enhances only single-stranded DNA breaks mediated by *B. anthracis* gyrase (purple) and *M. tuberculosis* gyrase (green). The graph shows the enhancement of *B. anthracis* and *M. tuberculosis* gyrase-mediated single-stranded (SS, closed circles) or double-stranded (DS, open circles) DNA cleavage. No enhancement of DS DNA breaks was observed with any of the bacterial type II enzymes tested.

achieve DNA cleavage-ligation equilibrium. Under all conditions examined, only single-stranded breaks were observed (Figure 26). Furthermore, no double-stranded breaks were observed in the presence of ATP, which is required for overall catalytic activity (Figure 27).

The double-stranded cleavage of DNA by type II topoisomerases is carried out by two coordinated single-stranded cleavage events.[151] Thus, the single-stranded DNA breaks that are generated in the presence of GSK126 can reflect two different mechanisms. The compound in any given cleavage complex induces cleavage only at one of the scissile bonds. Alternatively, after cleavage at one scissile bond, the presence of GSK126 alters the structure of the enzyme-DNA complex such that the second DNA strand cannot be cut. To distinguish between these two possibilities, the $MgCl_2$ in DNA cleavage assays was replaced with $CaCl_2$. This latter divalent metal ion raises baseline levels of DNA cleavage, so that double-stranded breaks can be observed even in the absence of drugs (compare levels of double-stranded DNA breaks in the absence of GSK126 in Figure 25 with Figure 28). As seen in Figure 28, the rise in single-stranded DNA breaks mediated by *B. anthracis* and *M. tuberculosis* gyrase at increasing concentrations of GSK126 was accompanied by a coordinate decrease in double-stranded breaks. This finding suggests that cleavage of one scissile bond in the presence of GSK126 suppresses cleavage at the opposite scissile bond.

Taken together, the above data provide strong evidence that the napthyridone/aminopiperidine-based NBTIs, like the napthyridone/aminopiperidine-based MGIs (GSK000 and GSK325) induce only gyrase-mediated single-stranded DNA breaks. Although NBTIs and fluoroquinolones both bind in the active site of gyrase in the cleavage complex, they do not appear to interact with the same residues of the enzyme. Consequently, cells that harbor mutations in the two residues that lead to fluoroquinolone resistance appear to retain sensitivity to

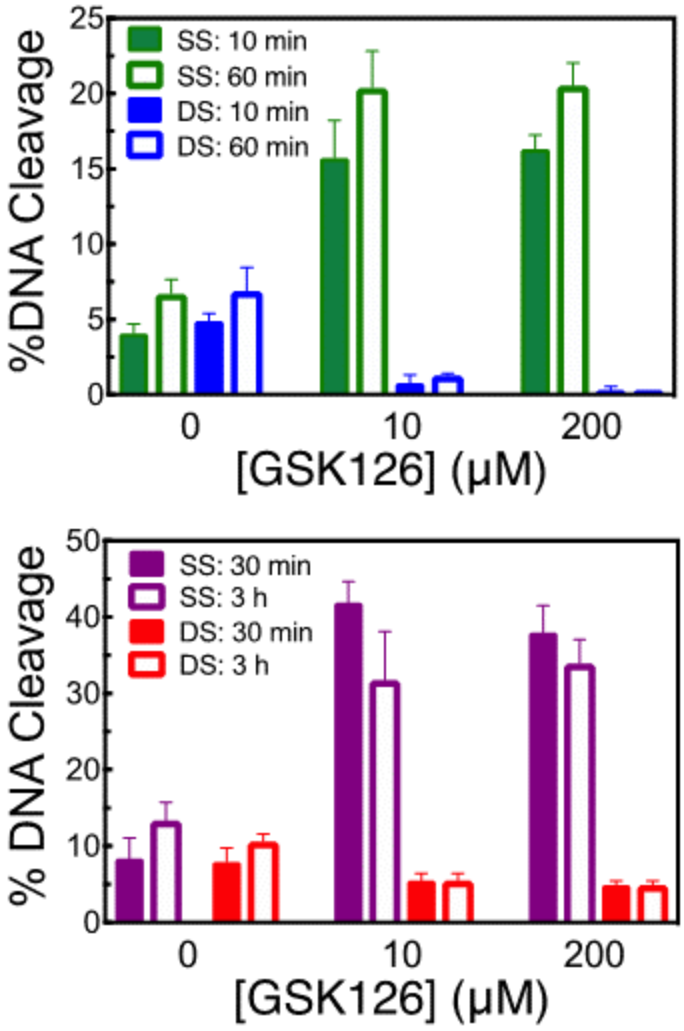


Figure 26. GSK126 enhances only single-stranded DNA breaks mediated by *M. tuberculosis* (top) and *B. anthracis* (bottom) gyrase. The top panel shows the enhancement of *M. tuberculosis* gyrase-mediated single-stranded (green bar) or double-stranded (blue bar) DNA breaks generated by gyrase at 10 min (closed) or 60 min (open) in the absence or presence of 10 μM or 200 μM GSK126. The bottom panel shows the enhancement of *B. anthracis* gyrase-mediated single- (purple bar) or double-stranded (red bar) DNA breaks at 30 min (closed) or 3 h (open) in the absence or presence of 10 μM or 200 μM GSK126. Error bars represent the SD of at least 3 independent experiments.

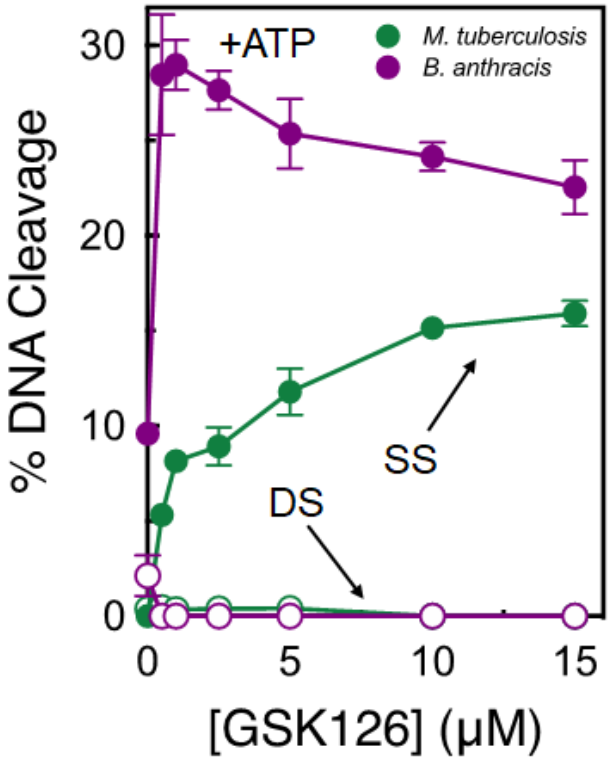


Figure 27. GSK126 enhances only single-stranded DNA breaks mediated by *B. anthracis* (purple) and *M. tuberculosis* (green) gyrase. The graph shows the effects of GSK126 on the enhancement of gyrase-mediated single-stranded (SS, closed circles) or double-stranded (DS, open circles) in the presence of ATP (1.5 mM). Refer to Figure 25 for comparison of reactions without ATP. Error bars represent the SD of at least 3 independent experiments.

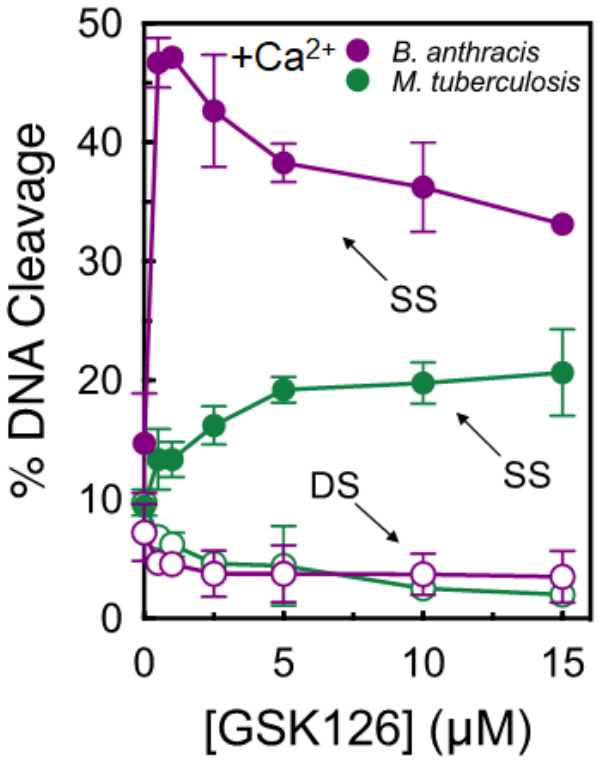


Figure 28. GSK126 induces single-stranded (SS) DNA breaks while suppressing double-stranded (DS) DNA breaks mediated by *B. anthracis* and *M. tuberculosis* gyrase. The graph quantifies the effects of GSK126 on *B. anthracis* and *M. tuberculosis* gyrase-mediated single-stranded (SS, closed circles) and double-stranded (DS, open circles) DNA cleavage. Error bars represent the SD of at least 3 independent experiments.

NBTIs. This relationship holds true at the enzyme level for MGIs, but with the exception of data shown in Chapter III, very little is known about how the presence of fluoroquinolone resistance mutations in gyrase affect the ability of NBTIs to induce enzyme-mediated DNA cleavage. As shown in Figure 29 (left), GSK126 maintained activity against *M. tuberculosis* gyrase that contained the most common fluoroquinolone-resistant mutations. If anything, GSK126 displayed higher activity against $GyrA^{A90V}$ and $GyrA^{D94G}$. A similar trend is seen for the ability of GSK126 to induce cleavage with *B. anthracis* gyrase that harbors the $GyrA^{S85L}$ fluoroquinolone-resistance mutation (Figure 29, right).

The activity of GSK126 against topoisomerase IV

Depending upon the bacterial species and the specific drug, the primary cellular target of fluoroquinolones may be gyrase, topoisomerase IV, or both enzymes. However, less is known about the targeting of NBTIs. As of yet, the role of topoisomerase IV in the activity of NBTIs has not been described. Furthermore, no previous reports have described the effects of NBTIs or related compounds on the activity of topoisomerase IV. Therefore, the effects of GSK126 on DNA cleavage mediated by *N. gonorrhoeae* and *B. anthracis* topoisomerase IV were characterized.

Results with topoisomerase IV paralleled those described above with gyrase. GSK126 only increased enzyme-mediated single-stranded breaks, over a titration range (Figure 30), at high NBTI concentrations, and at longer reaction times (Figure 31) or in the presence of ATP (Figure 32). Because topoisomerase IV generally displays higher levels of baseline (i.e., in the absence of drug) DNA cleavage, the decrease in double-stranded breaks at increasing concentration of GSK126 is apparent, even in reactions containing $MgCl_2$. This drop becomes even more evident in reactions that contained $CaCl_2$ (Figure 33). For example, with *N. gonorrhoeae* topoisomerase IV the increase in single-stranded DNA breaks from 24 to 62% induced by the addition of 1 µM

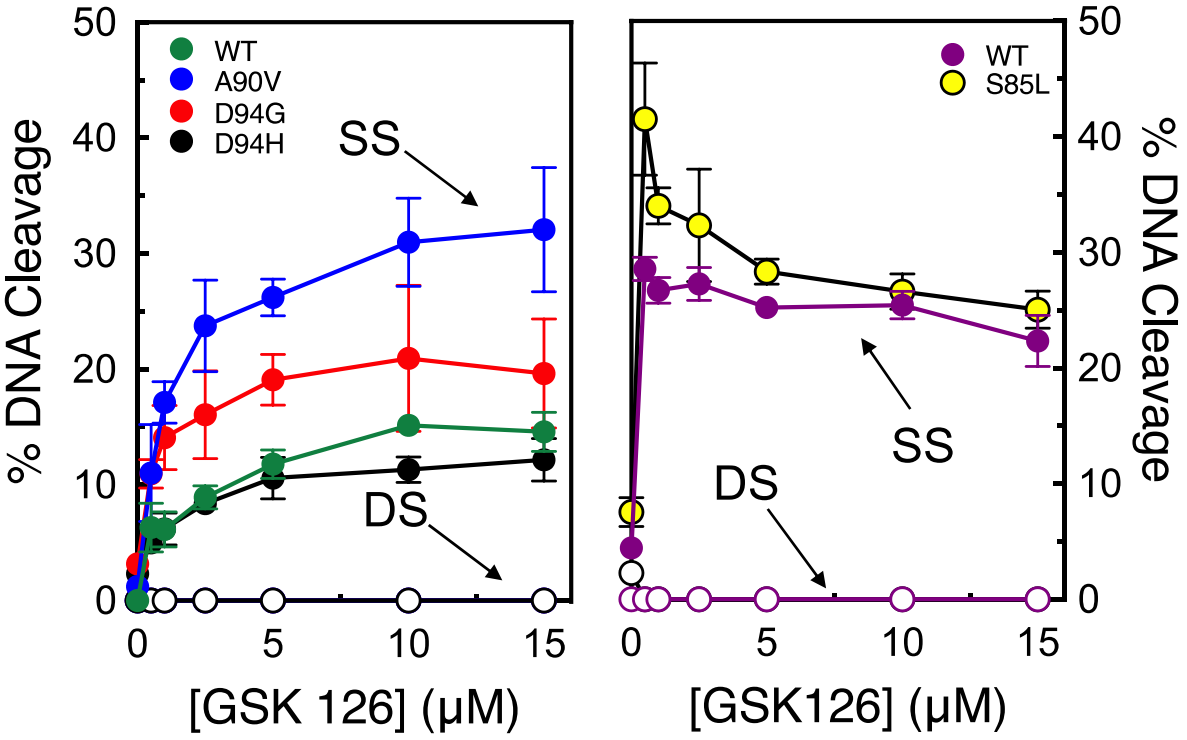


Figure 29. GSK126 maintains activity against *M. tuberculosis* gyrase and *B. anthracis* gyrase mutations associated with clinical fluoroquinolone resistance. In the left panel, the effects of GSK 126 on wild-type (green) *M. tuberculosis* gyrase and gyrase containing the fluoroquinolone resistance mutations at $GyrA^{A90V}$ (blue), $GyrA^{D94G}$ (red), or $GyrA^{D94H}$ (black) are shown. In the right panel, the effects of GSK126 on wild-type (purple) and $GyrA^{S85L}$ (yellow) are shown Single-stranded (SS) and double-stranded (DS) DNA breaks are denoted by closed and open circles respectively. Error bars represent the SD of at least 3 independent experiments.

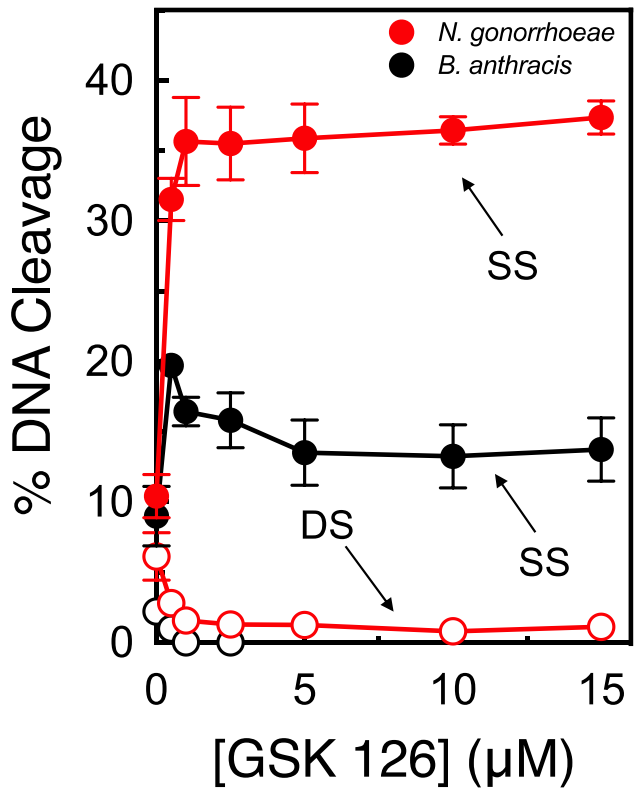


Figure 30. GSK126 enhances only single-stranded DNA breaks mediated by *N. gonorrhoeae* (red) and *B. anthracis* (black) topoisomerase IV. The graph shows the enhancement of *N. gonorrhoeae* and *B. anthracis* topoisomerase-mediated single-stranded (SS, closed circles) or double-stranded (DS, open circles) DNA cleavage. No enhancement of DS DNA breaks was observed with any of the bacterial type II enzymes tested.

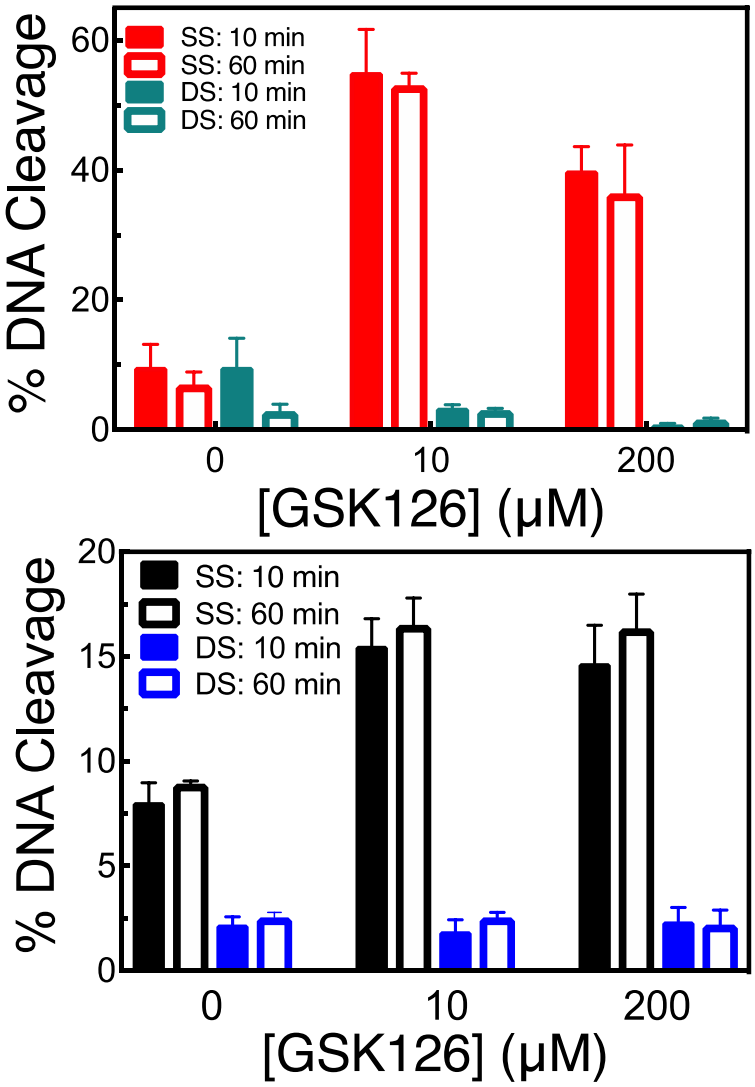


Figure 31. GSK126 enhances only single-stranded DNA breaks mediated by *N. gonorrhoeae* (top) and *B. anthracis* (bottom) topoisomerase IV. The top panel shows the enhancement of *N. gonorrhoeae* topoisomerase IV-mediated single-stranded (red bar) or double-stranded (teal bar) DNA breaks generated by gyrase at 10 min (closed) or 60 min (open) in the absence or presence of 10 μM or 200 μM GSK126. The bottom panel shows the enhancement of *B. anthracis* topoisomerase IV-mediated single- (black bar) or double-stranded (blue bar) DNA breaks at 10 min (closed) or 60 min (open) in the absence or presence of 10 μM or 200 μM GSK126. Error bars represent the SD of at least 3 independent experiments.

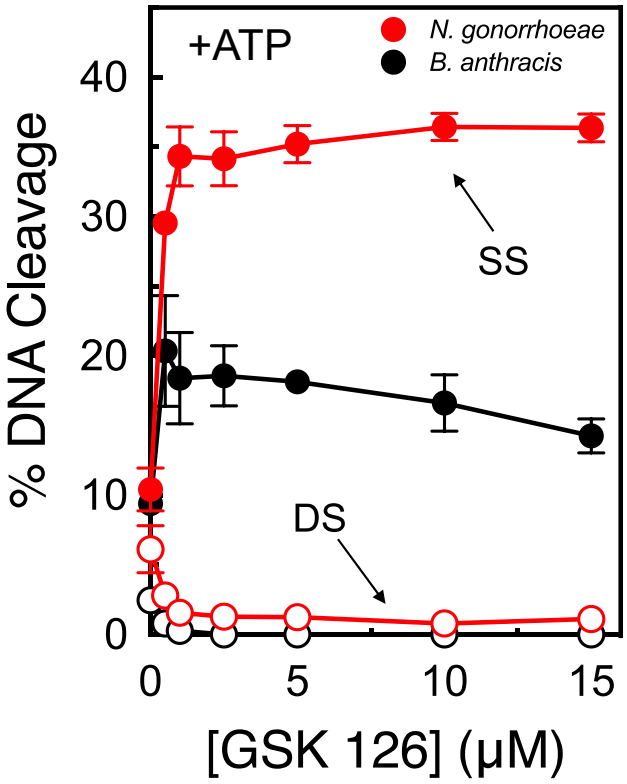


Figure 32. GSK126 enhances only single-stranded DNA breaks mediated by *N. gonorrhoeae* (red) and *B. anthracis* (black) topoisomerase IV. The graph shows the effects of GSK126 on the enhancement of topoisomerase IV-mediated single-stranded (SS, closed circles) or double-stranded (DS, open circles) DNA breaks in the presence of ATP (1.5 mM). Refer to Figure 30 for comparison of reactions without ATP. Error bars represent the SD of at least 3 independent experiments.

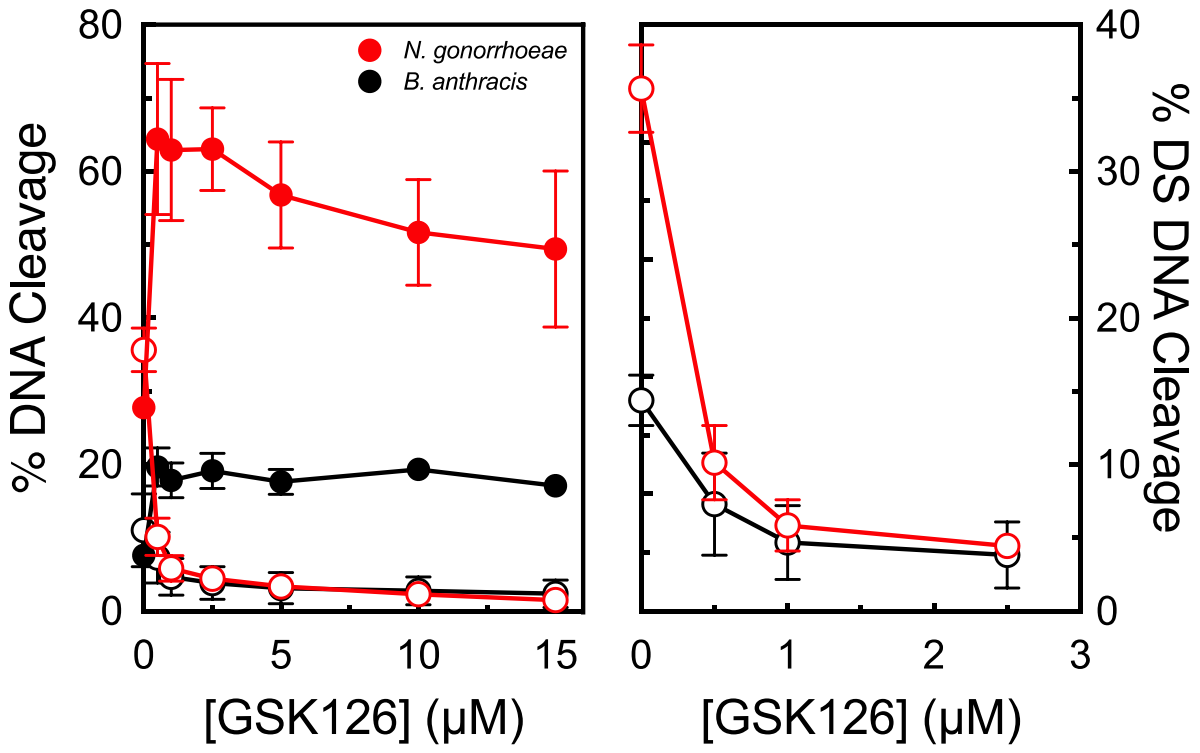


Figure 33. GSK126 induces single-stranded (SS) DNA breaks while suppressing double-stranded (DS) DNA breaks mediated by *N. gonorrhoeae* (red) and *B. anthracis* (black) topoisomerase IV. In the left panel, the graph quantifies the effects of GSK126 on *N. gonorrhoeae* and *B. anthracis* topoisomerase IV-mediated single-stranded (SS, closed circles) and double-stranded (DS, open circles) DNA cleavage. The right panel is zoomed in on the double-stranded brakes to show the steep decrease in double-stranded cleavage. Error bars represent the SD of at least 3 independent experiments.

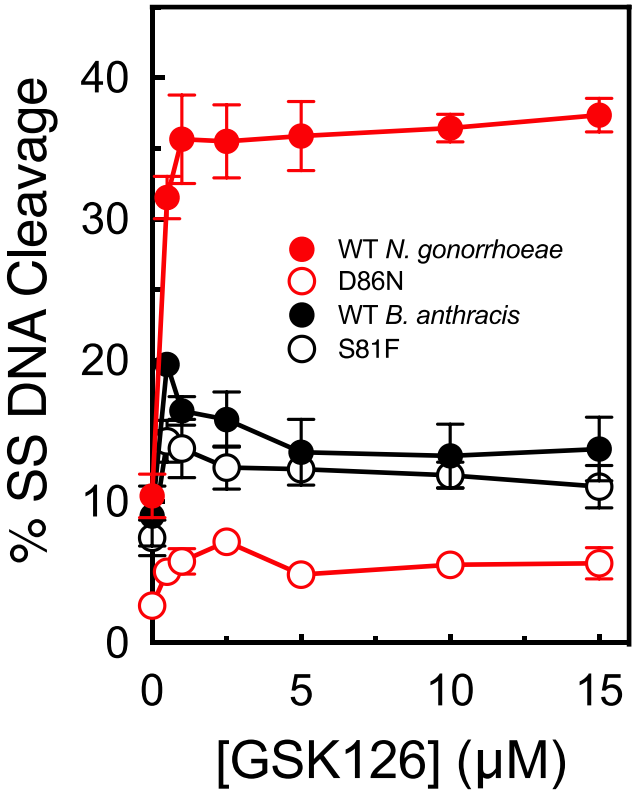


Figure 34. GSK126 retains activity with *B. anthracis* topoisomerase IV (black) fluoroquinolone resistance mutation, but has little effect on *N. gonorrhoeae* topoisomerase IV (red) NBTI-resistant mutant enzyme. Enhancement of single-stranded DNA breaks are shown for wild-type (closed circles) and mutant (open circles) topoisomerase IV enzymes. Errors represent the SD of at least 3 independent experiments.

GSK126 is accompanied by a decrease in double-stranded breaks from 37 to 4%. Thus, it is clear that the strong enhancement of topoisomerase IV-mediated single-stranded breaks by GSK126 is accompanied by the suppression of cleavage at the second scissile bond on the opposite strand.

I examined two separate drug-resistance mutations in topoisomerase IV. GSK126 displayed wild-type activity against *B. anthracis* topoisomerase IV $GrlA^{S81F}$, which is a fluoroquinolone resistance mutant. In contrast, the ability of GSK126 to induce DNA cleavage with *N. gonorrhoeae* $ParC^{D86N}$, which has been identified in NBTI-resistant cell lines, was significantly impaired (Figure 34).

Conclusions

Based on these studies, the NBTIs have clinical promise as a new drug class. However, there is paucity of published data describing the interactions of these compounds with their type II topoisomerases targets. Results of the present work indicate that the NBTI GSK126 enhances single-stranded DNA cleavage mediated by gyrase and topoisomerase IV from a variety of gram-positive and gram-negative bacterial species and can overcome some of the most common fluoroquinolone resistance mutations found in *M. tuberculosis* gyrase and *B. anthracis* gyrase and topoisomerase IV. Continued studies with the NBTIs and their enzyme targets will allow for the potential development of a set of consistent "rules" that govern the actions of NBTIs against enzymes and bacterial cells, which in turn may hasten the development of this potentially important class of compounds.

CHAPTER V

MECHANISTIC AND STRUCTURAL BASIS FOR THE ACTIONS OF THE ANTIBACTERIAL GEPOTIDACIN AGAINST *STAPHYLOCOCCUS AUREUS* GYRASE

Introduction

In Chapter IV, I compared the effects of GSK126 the parent NBTI to the MGI subclass of compounds and explored its activity against several bacterial type II topoisomerases. However, the most clinically advanced NBTI is gepotidacin (Figure 35).[171-173] This first-in-class triazaacenaphthylene-based compound is one of a very few antibacterials currently in active development that acts by a novel mechanism.[174] Gepotidacin has successfully completed phase II trials for the treatment of acute bacterial skin/skin structure infections (including those caused by *S. aureus*) and for the treatment of uncomplicated urogenital gonorrhea (caused by *N. gonorrhoeae*) with no significant adverse events.[171-173] It also displays robust *in vitro* activity against a range of bacterial species, including fluoroquinolone-resistant strains.[175]

Despite the clinical promise of gepotidacin, nothing has been reported for this compound regarding its interactions with any bacterial type II topoisomerase. Due to the clinical focus of gepotidacin towards skin/skin structure infections, I characterized the actions of gepotidacin against *S. aureus* gyrase. The compound was a potent inhibitor of gyrase catalytic activity. Furthermore, it induced high levels of gyrase-mediated single-stranded DNA breaks; no double-stranded breaks were observed even at high gepotidacin concentrations or extended reaction times. Finally, as determined by a 2.31Å resolution crystal structure of gepotidacin with a *S. aureus* GyrB27-A56/Y123F fusion truncate and DNA by our collaborator Ben Bax, a single molecule of the compound was situated in the midway between the two DNA scissile bonds cleaved by the

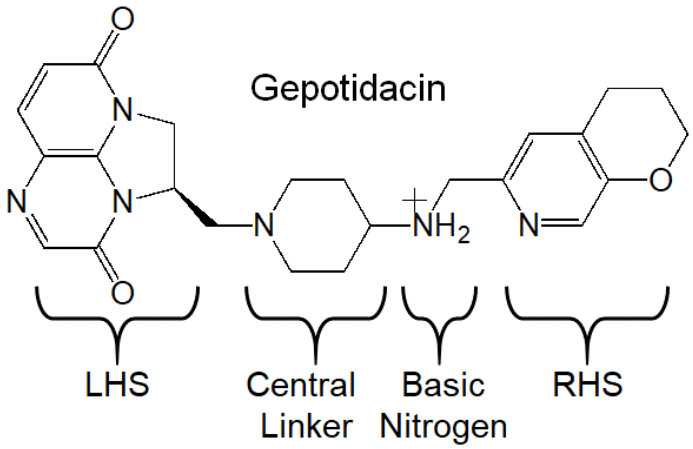


Figure 35. Structure of gepotidacin. Gepotidacin is composed of a triazaacenaphthylene on the left-hand side (LHS), a central linker region, a basic nitrogen, and a pyranopyridine on the right-hand side (RHS).

enzyme. The left-hand side (triazaacenaphthylene) of gepotidacin sat in the DNA on the twofold axis of the complex, midway between the two DNA cleavage sites, and the right-hand side (pyranopyridine) sat in a pocket on the twofold axis between the two GyrA subunits. This work provides important mechanistic insight into how gepotidacin acts against one of its most clinically relevant bacterial targets.

Results and Discussion

Inhibition of gyrase catalytic activity by gepotidacin.

The NBTI GSK126 displayed broad-spectrum enzyme activity. As a comparison, I looked at the effect gepotidacin had on enzyme-mediated DNA cleavage activity on a range of bacterial type II enzymes. Similar to GSK126, gepotidacin displayed broad-spectrum activity in enhancing enzyme-mediated DNA cleavage (data not shown). However, I focused my studies on *S. aureus* gyrase because of the high clinical relevance and the high activity.

As a prelude to determining how gepotidacin affects the inhibition of enzyme catalytic activity, *S. aureus* gyrase activity was assessed in the absence of gepotidacin. Starting with positively supercoiled DNA, I followed the time course for the enzyme to remove the positive supercoils and then convert the relaxed DNA to a negatively supercoiled plasmid. As reported previously for gyrase from *B. anthracis, E. coli*, and *M. tuberculosis*,[20, 176] the *S. aureus* enzyme removed positive supercoils much more rapidly than it introduced negative supercoils into relaxed DNA (Figure 36). Whereas all of the positive supercoils were gone within 60 s, it took at least 20 min to convert the plasmid into fully negatively supercoiled DNA. This >20-fold time difference between the two reactions likely reflects the acute temporal requirement to rapidly remove positive supercoils that would accumulate in front of the replication fork as compared to the maintenance of steady state levels of negative DNA supercoiling.[20, 176]

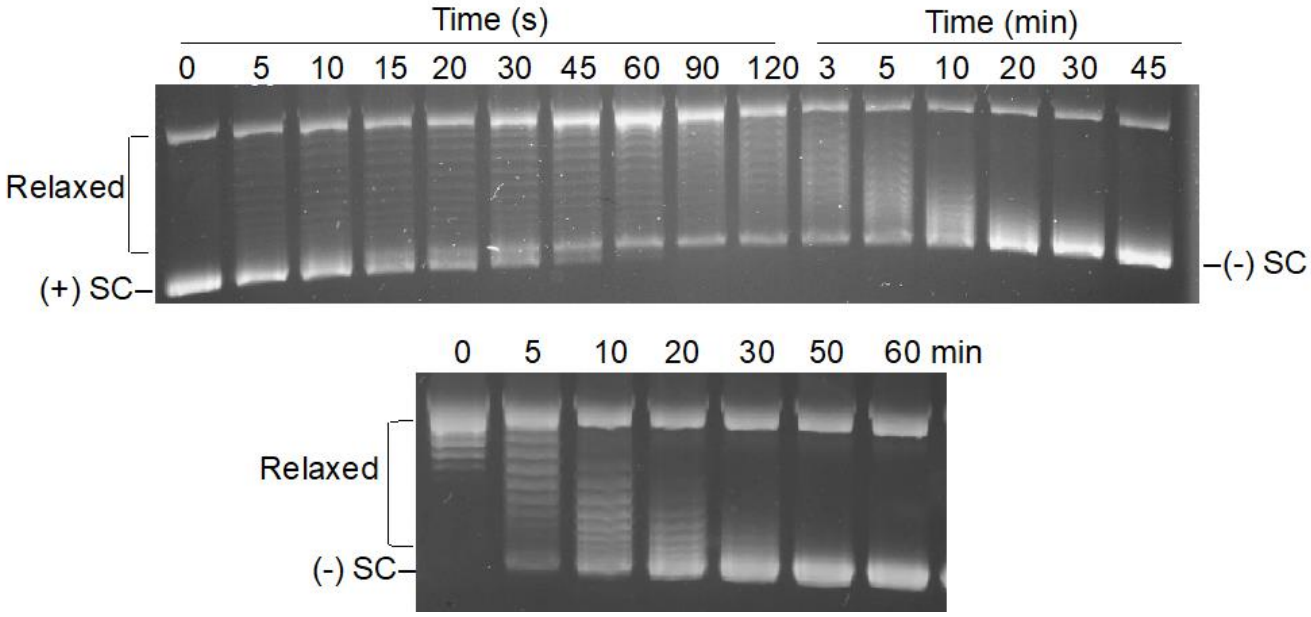


Figure 36. *S. aureus* gyrase removes positive supercoils more rapidly than it introduces negative supercoils into relaxed DNA. Time courses are shown for the relaxation of positively supercoiled plasmid followed by the introduction of negative supercoils (top) and the negative supercoiling of relaxed plasmid (bottom). The positions of positively supercoiled [(+)SC], relaxed, and negatively supercoiled [(−)SC] DNA are indicated on the gels. The gel images are representative of at least three independent experiments.

As seen in Figure 37, gepotidacin was a potent inhibitor of gyrase activity. The IC_{50} for inhibition of DNA supercoiling (top left panel) was ~0.047 μM and that for the relaxation of positive DNA supercoils (top right panel) was ~0.6 μM. In contrast, the IC_{50} values for the inhibition of supercoiling and relaxation by moxifloxacin, a clinically relevant fluoroquinolone were ~11.5 μM (bottom left panel) and ~73 μM (bottom right panel), respectively. Thus, gepotidacin is considerably more potent (~240- and 120-fold, respectively) than moxifloxacin in its ability to inhibit the two critical catalytic functions of *S. aureus* gyrase.

Enhancement of gyrase-mediated DNA cleavage by gepotidacin

Because some NBTIs induce DNA scission by bacterial type II topoisomerases, (also see data for GSK126 in the previous chapter),[10, 29, 62, 106, 110, 118] I examined the effects of gepotidacin on the ability of *S. aureus* gyrase to cleave DNA (Figure 38). Like GSK126, all of the breaks created in the presence of gepotidacin were single-stranded. Furthermore, when gyrase was left out of reaction mixtures, no DNA cleavage was observed even at 200 μM gepotidacin and incubation times (3 h) that were 6 times longer than used normally.

Initial experiments to further characterize the induction of gyrase-mediated DNA cleavage by gepotidacin utilized negatively supercoiled plasmid (Figure 39, left panel, blue). Gepotidacin strongly enhanced gyrase-mediated DNA cleavage in the nanomolar range [EC_{50} (concentration required to induce 50% maximal DNA cleavage) ≈ 130 nM] and generated single-stranded DNA breaks in more than 30% of the initial substrate at low micromolar concentrations. Moreover, gepotidacin was considerably more potent than moxifloxacin, which required micromolar levels to induce substantial levels of double-stranded DNA cleavage (EC_{50} ≈ 2 μM) (Figure 39, right panel, black).

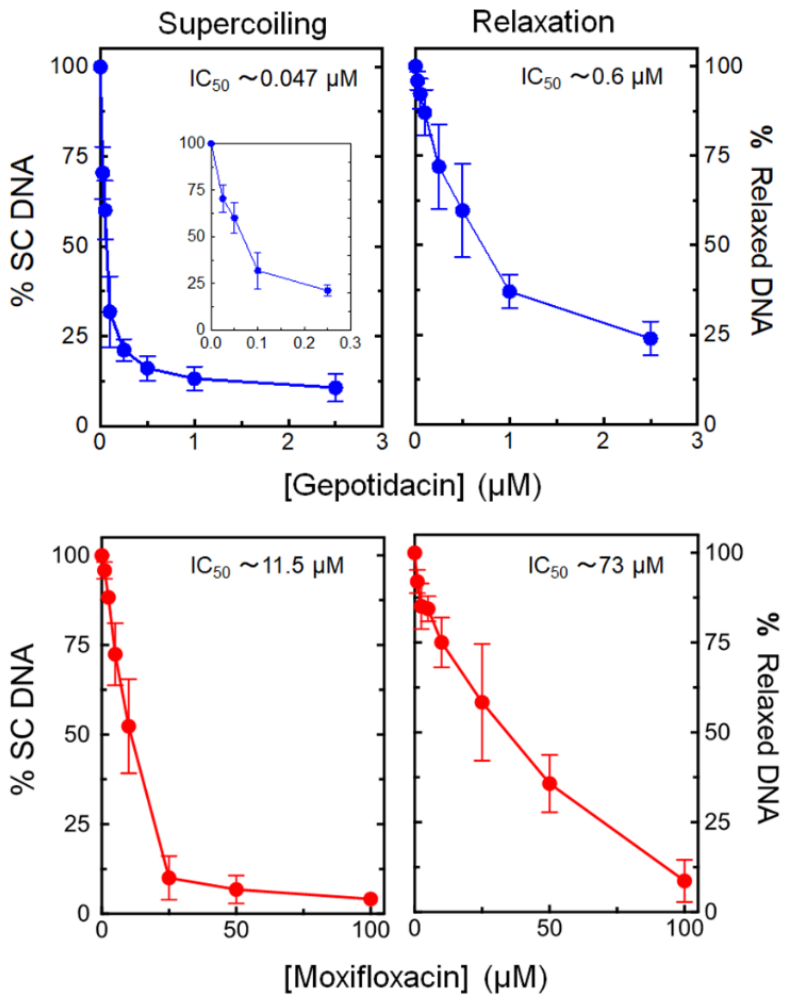


Figure 37. Gepotidacin and moxifloxacin inhibit DNA supercoiling and relaxation reactions catalyzed by *S. aureus* gyrase. The effects of gepotidacin (blue, top panels) and moxifloxacin (red, bottom panels) on the supercoiling of relaxed DNA (left panels) and the relaxation of positively supercoiled DNA (right panels) are shown. Error bars represent the standard deviation (SD) of at least three independent experiments.

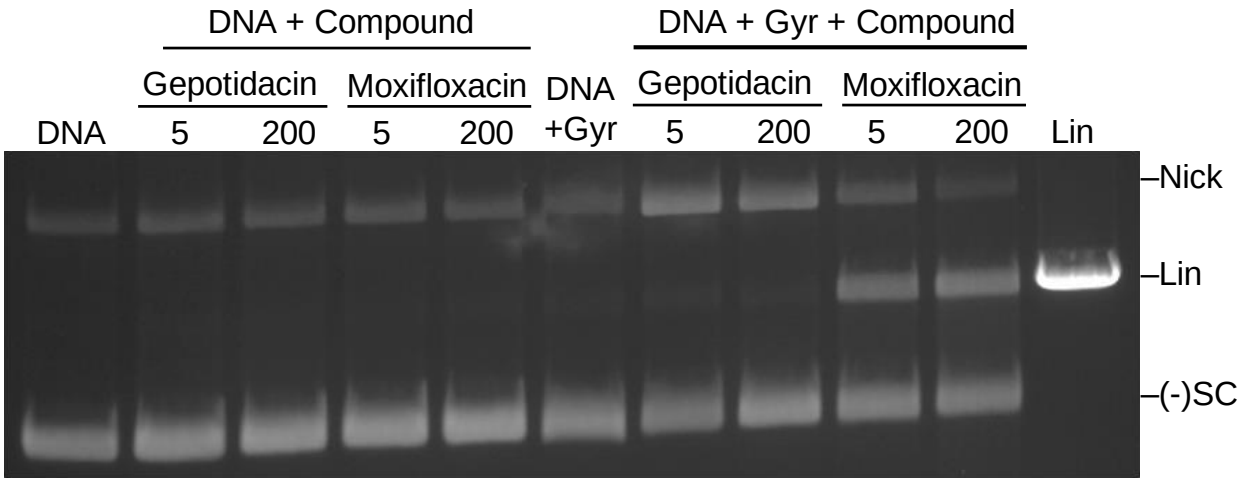


Figure 38. Gepotidacin induces single-stranded DNA breaks in the presence of gyrase. The gel shows DNA products following cleavage reactions containing 5 or 200 μM gepotidacin or moxifloxacin in the absence or presence of *S. aureus* gyrase. The positions of negatively supercoiled [(−)SC], nicked (Nick), and linear (Lin) DNA are indicated on the gels. The gel images are representative of at least three independent experiments.

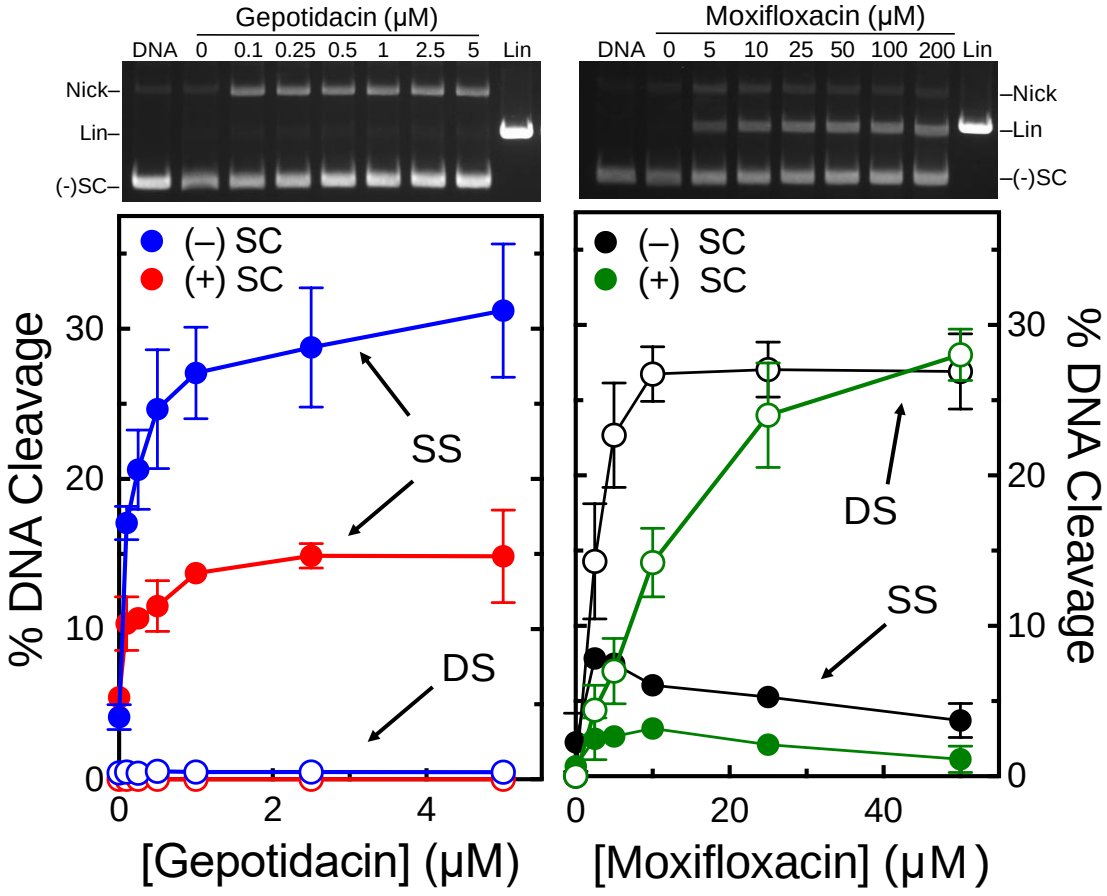


Figure 39: Gepotidacin is a potent enhancer of gyrase-mediated single-stranded DNA cleavage. The left panel shows the effects of gepotidacin on gyrase-mediated single- (SS, closed circles) and double-stranded (DS, open circles) DNA cleavage of negatively (blue) and positively (red) supercoiled DNA. The right panel shows the effects of moxifloxacin on gyrase-mediated single- and double-stranded DNA cleavage of negatively (black) and positively (green) supercoiled DNA. Error bars represent the SD of at least three independent experiments. The gels shown at the top are representative cleavage assays with negatively supercoiled DNA. The mobilities of negatively supercoiled DNA [(−)SC], nicked circular DNA (Nick), and linear DNA (Lin) are indicated on the gels.

Because drug-stabilized cleavage complexes formed on positively supercoiled DNA ahead of replication forks and transcription complexes are most likely to be converted into permanent strand breaks,[10, 29, 106] I also examined the effects of gepotidacin on gyrase-mediated cleavage of positively supercoiled DNA (Figure 39, left panel, red). As reported for other species of gyrase with fluoroquinolones and MGIs,[20, 118, 153, 176] gepotidacin induced ~2–fold lower levels of single-stranded breaks in the presence of the *S. aureus* enzyme and positively supercoiled as compared to negatively supercoiled plasmid. In addition, the potency of gepotidacin with positively supercoiled DNA was slightly higher than that seen with negatively supercoiled substrate ($EC_{50} \approx$ 180 nM). As with negatively supercoiled plasmid, no double-stranded breaks were observed.

In contrast to the results with gepotidacin, the efficacy of moxifloxacin-induced double-stranded breaks in positively supercoiled plasmid (Figure 39, right panel, green) was similar to that seen with negatively supercoiled DNA. However, the potency of the drug fell ~8–fold with positively supercoiled substrates ($EC_{50} \approx$ 17 or 2 μM with positively or negatively supercoiled DNA, respectively). As above, gepotidacin was considerably (~10– to 100–fold) more potent against *S. aureus* gyrase than moxifloxacin.

To determine whether gepotidacin displays the ability to induce gyrase-mediated double-stranded breaks at high concentrations, DNA cleavage assays were carried out in the presence of 200 μM compound, which is ~40 times the concentration needed to induce maximal levels of single-stranded DNA breaks (Figure 40). The DNA cleavage profile for 200 μM gepotidacin was identical to that observed for 5 μM compound, even over a time course that was 6 times longer than used under standard conditions (Figure 40). As seen at lower drug concentrations, only single-stranded breaks were observed.

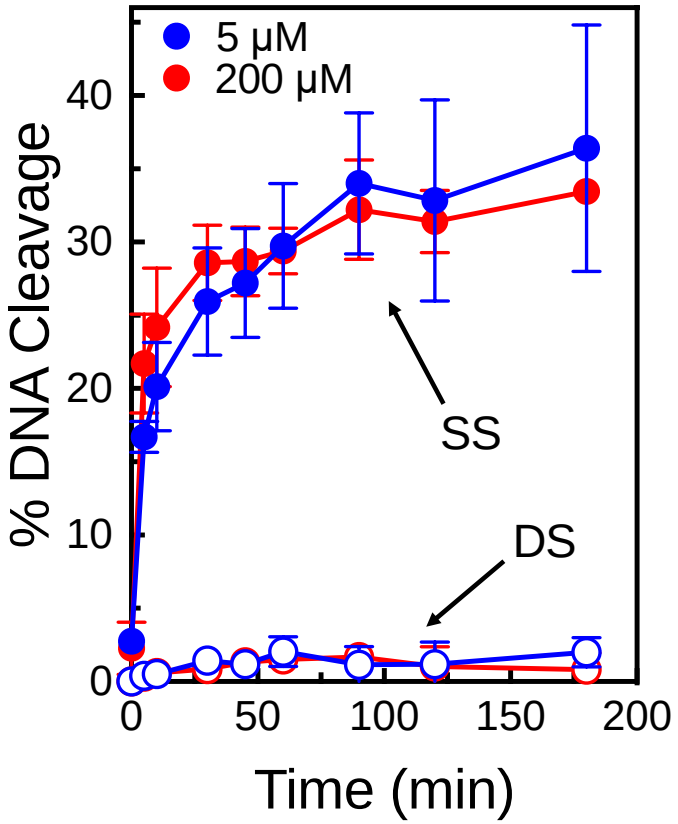


Figure 40. Gepotidacin stabilizes only single-stranded DNA breaks mediated by *S. aureus* gyrase. The enhancement of single-stranded (SS, closed circles) and double-stranded (DS, open circles) over time in the presence of 5 µM (blue) and 200 µM (red) gepotidacin are shown. Error bars represent the SD of at least three independent experiments.

As mentioned previously for other type II enzymes, *S. aureus* gyrase does not require ATP in order to cleave DNA, the high energy cofactor is necessary for DNA strand passage and enzyme turnover.[13, 177] The DNA cleavage assays shown in earlier figures did not include ATP in reaction mixtures. Therefore, the effects of gepotidacin on gyrase-mediated DNA cleavage were carried out in the presence of 1.5 mM ATP to determine whether the high-energy cofactor effects the ability of the compound to generate single- vs. double-stranded DNA breaks (Figure 41). Whereas the compound is more potent in the presence of ATP ($EC_{50} \approx 40$ nM as compared to $EC_{50} \approx 130$ nM in the absence of ATP), no double-stranded DNA cleavage was observed.

Taken together the above data lead to the conclusion that gepotidacin induces only single-stranded DNA breaks mediated by *S. aureus* gyrase.

Gepotidacin induces stable gyrase-DNA cleavage complexes

In general, the ability of topoisomerase-targeted compounds to kill cells correlates with the stability of cleavage complexes formed in their presence.[178] Therefore, I examined the stability of *S. aureus* gyrase-DNA cleavage complexes that were generated in gepotidacin-containing reactions. This was accomplished by monitoring the decay in DNA scission following a 20-fold dilution of reaction mixtures into a buffer that lacked the divalent metal ion required for cleavage. This assay reflects the rate at which the ternary complex dissociates, given that these complexes are unlikely to re-form in a diluted reaction mixture that lacks substantial levels of divalent metal ion. As seen in Figure 42, gyrase-DNA cleavage complexes formed in the presence of gepotidacin were highly stable and displayed little dissociation even 4 h after dilution. The stability of these single-stranded DNA cleavage complexes appeared to be similar to or marginally greater than that of the doubly cleaved counterparts generated in the presence of moxifloxacin. In contrast, in the absence of the NBTI or fluoroquinolone the lifetime of single- or double-stranded

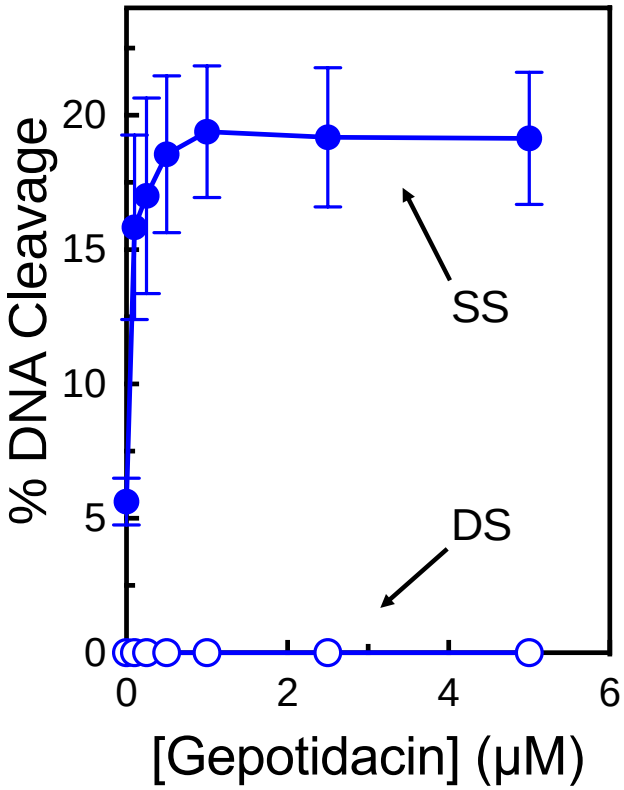


Figure 41. Gepotidacin enhances only single-stranded DNA breaks mediated by *S. aureus* gyrase in the presence of ATP. The enhancement of gyrase-mediated single-stranded (SS, closed circles) or double-stranded (DS, open circles) DNA breaks generated by gyrase in the presence of 1.5 mM ATP is shown. Error bars represent the SD of at least three independent experiments.

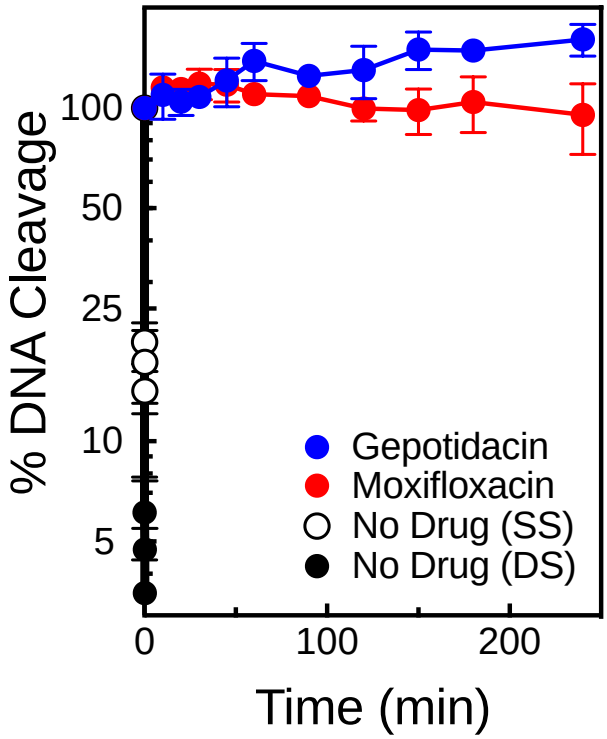


Figure 42. **Gepotidacin induces stable DNA cleavage complexes formed by *S. aureus* gyrase**. The persistence of ternary gyrase–drug–DNA cleavage complexes was monitored by the loss of single-stranded DNA breaks in the presence of 5 µM gepotidacin (blue) or double-stranded DNA cleavage in the presence of 25 µM moxifloxacin (red), or the loss of single- (open circle, white) or double-stranded DNA cleavage in the absence of drug (closed circle, black). Levels of DNA cleavage were set to 100% at time zero to allow for direct comparisons. Error bars represent the SD of at least three independent experiments.

DNA cleavage complexes formed by *S. aureus* gyrase following dilution was <10 s.

Gepotidacin suppresses gyrase-mediated double-stranded DNA cleavage

Cleavage of the two strands of the double helix by type II topoisomerases are coordinated but individual events (i.e., cleavage at one scissile bond does not necessarily affect cleavage at the other).[151] Thus, the single-stranded DNA cleavage that occurs in the presence of gepotidacin can reflect two different mechanisms. Either the compound induces cleavage at only one of the two scissile bonds in any cleavage complex or the cleavage of one scissile bond in the presence of gepotidacin alters the enzyme-DNA complex such that the second DNA strand cannot be cut. The latter mechanism was found to be the case for MGIs.[118]

Unfortunately, the low baseline level of double-stranded DNA cleavage mediated by *S. aureus* gyrase makes it difficult to distinguish between these two possibilities. To overcome this difficulty, I substituted the $MgCl_2$ in DNA cleavage assays with $CaCl_2$ (Figure 43). Although the fundamental properties of DNA cleavage and ligation are not altered by this substitution,[65, 129] baseline levels of enzyme-mediated double-stranded DNA cleavage in the presence of Ca^{2+} (~15%) are substantially higher as compared to those observed in Mg^{2+}-containing reactions (<1%). As seen in Figure 43, the rise in single-stranded DNA cleavage induced by increasing concentrations of gepotidacin was accompanied by a coordinate decrease in levels of double-stranded DNA cleavage. This result provides strong evidence that cleavage of one scissile bond in the presence of gepotidacin suppresses the ability of *S. aureus* gyrase to cleave the scissile bond on the opposite strand.

Gepotidacin can displace moxifloxacin from the active site of S. aureus *gyrase*

Previous structural studies with NBTIs indicate that they bind to the DNA cleavage/ligation site of bacterial type II topoisomerases.[62, 131, 132] Although their site of interaction within the

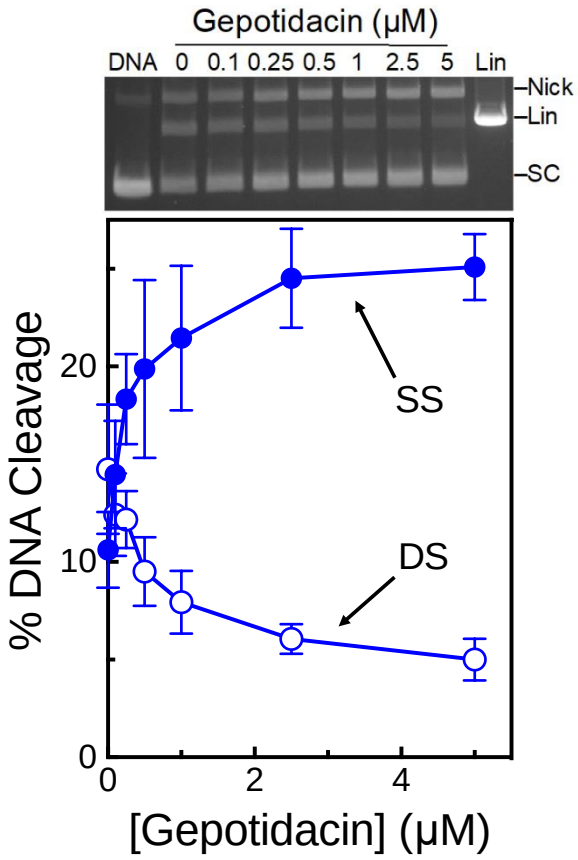


Figure 43. Gepotidacin suppresses double-stranded DNA breaks generated by *S. aureus* gyrase. The effects of gepotidacin on *S. aureus* gyrase-mediated single-stranded (SS, closed circles) and double-stranded (DS, open circles) DNA cleavage are shown. Reactions were carried out in the presence of Ca^{2+} rather than Mg^{2+} to increase levels of baseline DNA cleavage. Error bars represent the SD of at least three independent experiments. The gel shown at the top is representative of at least three independent experiments. The mobilities of negatively supercoiled DNA [SC], nicked DNA (Nick), and linear DNA (Lin) are indicated on the gels.

enzyme-DNA complex is not identical to those of fluoroquinolones, as discussed in chapter III, a modeling study by Ben Bax examined the MGI GSK000 and moxifloxacin suggested that the two compounds could not coexist in the active site of *M. tuberculosis* gyrase.[118] Therefore, a cleavage competition assay was carried out to determine whether gepotidacin and moxifloxacin can occupy the same cleavage complex established with *S. aureus* gyrase. In this assay, cleavage complexes were formed in the presence of a mixture of 25 μM moxifloxacin and increasing concentrations of gepotidacin (0-100 μM). Competition was monitored by the loss of double-stranded DNA breaks, which could have been induced only by moxifloxacin. As seen in Figure 44, levels of double-stranded breaks dropped ~95% in the presence of gepotidacin, which indicates that the binding of the NBTI and moxifloxacin in the active site of the *S. aureus* gyrase-DNA cleavage complex are mutually exclusive.

Structure of the S. aureus-*DNA-gepotidacin ternary complex*

Given the results of the competition studies and the fact that previous NBTIs have been localized to the DNA cleavage/ligation active site of bacterial type II topoisomerases, Ben Bax, a collaborator, further examined gepotidacin interactions within the enzyme-DNA complex. To this end, he determined a 2.31Å resolution crystal structure of gepotidacin with the *S. aureus* GyrB27-A56/Y123F fusion truncate and nicked DNA (Figure 45). In contrast to fluoroquinolones, only a single molecule of gepotidacin binds in the active site of the *S. aureus* gyrase-DNA cleavage complex. Briefly, gepotidacin interacts with GyrA Asp83, but not with GyrA Ser84 or Glu88, the two amino acid residues that anchor the water-metal ion bridge that facilitates protein interactions with fluoroquinolones and most commonly mutated in fluoroquinolone-resistant isolates.[57, 106, 177]

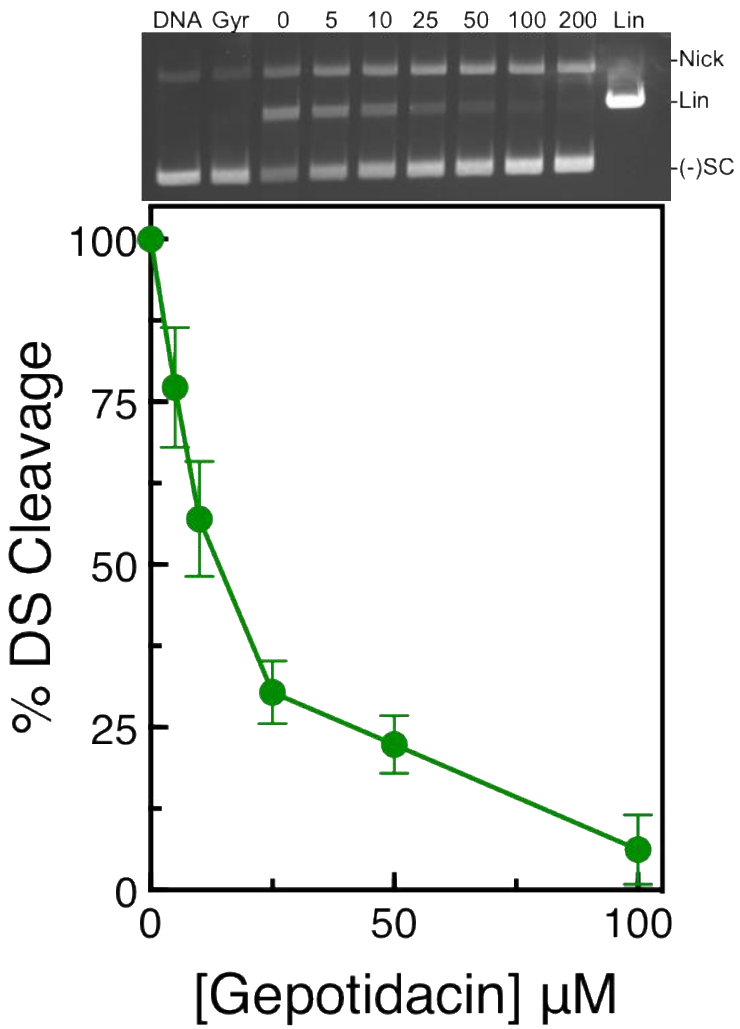


Figure 44. The actions of gepotidacin and moxifloxacin on *S. aureus* gyrase-mediated DNA cleavage are mutually exclusive. A DNA cleavage/ligation equilibrium was formed in the presence of a saturating concentration of moxifloxacin (25 µM) plus 0-100 µM gepotidacin. Competition was monitored by the loss of moxifloxacin-induced double-stranded DNA breaks. Error bars represent the SD of at least 3 independent experiments.

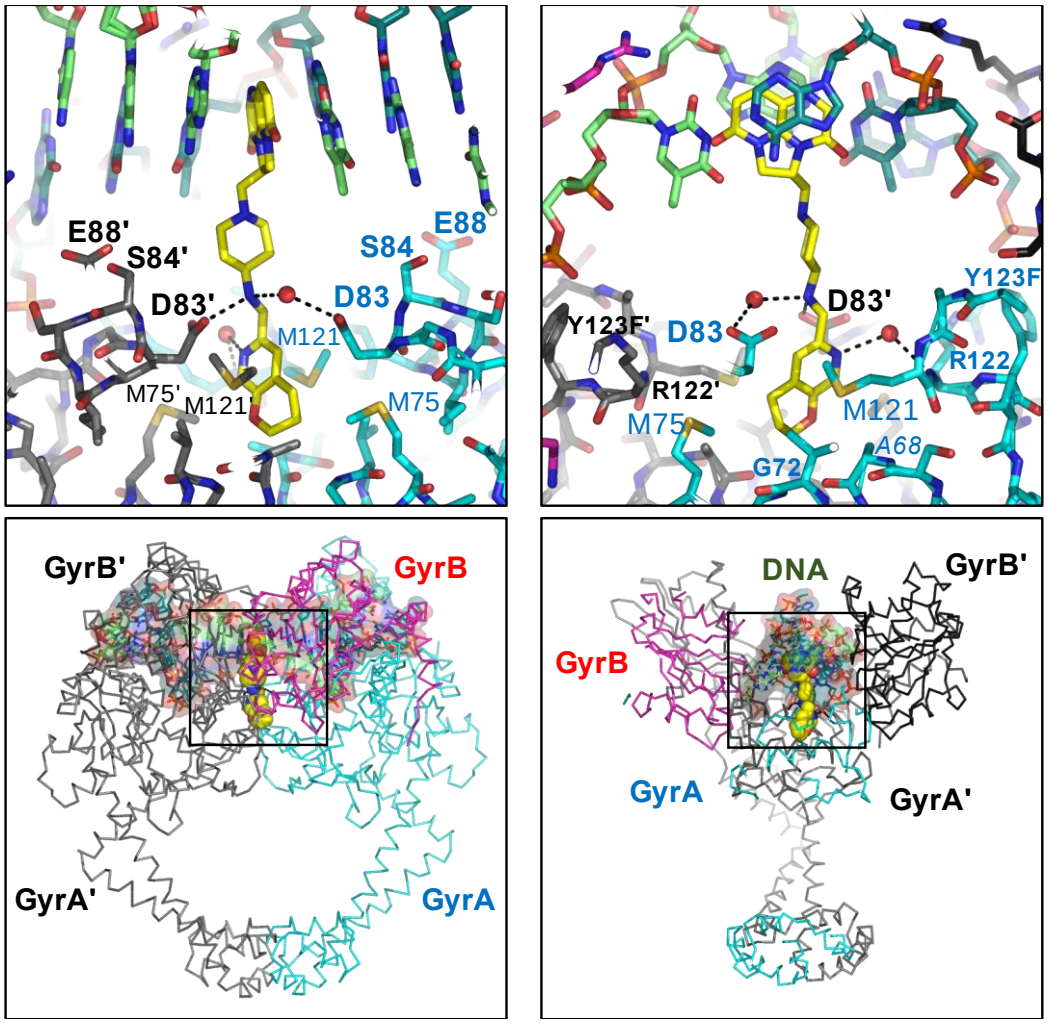


Figure 45. Views of a gepotidacin complex formed with *S. aureus* gyrase and DNA at a resolution of 2.31Å. The top left panel shows gepotidacin binding on the 2-fold axis of the complex midway between the two DNA cleavage sites; the top right panel is an approximately orthogonal (90º) view of the same structure. The bottom left and right panels show the same views as the corresponding top panels, but zoomed out to show the subunits of gyrase. In these panels, gepotidacin is shown in spheres, DNA with semi-transparent surface, and proteins as ribbons. In all panels, carbon atoms in the DNA are green, those in the first GyrBA core fusion truncate subunit are cyan/blue in GyrA and magenta in GyrB, and those in the second subunit are grey or black. Carbon atoms in gepotidacin are yellow, and oxygen, nitrogen, and sulfur atoms are red, blue, and yellow, respectively. Water molecules are shown as small red spheres.

Consistent with the structure, mutation of Asp83 in *S. aureus* or its equivalent residue, ParC Asp86 in *N. gonorrhoeae* are associated with elevated minimum inhibitory concentrations for gepotidacin.[171, 173] The basic nitrogen in gepotidacin interacts with GyrA' Asp83' directly and with Asp83 of the second gyrase subunit via a water molecule. The nitrogen in the RHS of gepotidacin interacts with a water molecule hydrogen bonded to the main-chain NH of Arg122 from GyrA (Figure 46). The left-hand side (triazaacenaphthylene) of gepotidacin sits in the DNA on the same axis of the complex, midway between the two DNA cleavage sites, and the right-hand side (pyranopyridine) sits in a pocket on the same axis between the two GyrA subunits (Figure 45).

Conclusions

Due to the rise in antibacterial drug resistance, there is a critical need for the development of new agents that retain activity against resistant infections. One approach, which led to the development of the NBTIs, is to identify compounds that work against clinically relevant validated targets, such as the bacterial type II topoisomerases, gyrase and topoisomerase IV, and retain activity against fluoroquinolone-resistant enzymes. Although gepotidacin and other NBTIs bind in the same DNA cleavage/ligation active site of gyrase as fluoroquinolones, they display distinct interactions. Whereas, fluoroquinolones interact with *S. aureus* gyrase through water-mediated contacts with Ser84 and Asp88 in the GyrA subunit, the basic nitrogen of gepotidacin interacts directly with GyrA Asp83 from one subunit, and indirectly via a water with Asp83 from the second GyrA subunit. Hence, NBTIs and related compounds are able to retain activity against the most common gyrase mutations that are resistant to clinically relevant fluoroquinolones.

Gepotidacin is a first-in-class triazaacenaphthylene NBTI. Despite its success in clinical trials against skin/skin structure infections (such as those caused by *S. aureus*) and uncomplicated urogenital gonorrhea, nothing had been reported about its interactions with its bacterial type II

topoisomerase targets. Therefore, the current work characterized the activity of the compound against *S. aureus* gyrase. Gepotidacin was a potent inhibitor of gyrase activity and enhanced enzyme-mediated DNA cleavage. In contrast to fluoroquinolones, the compound enhanced only single-stranded DNA breaks. Further studies will be required to determine how gepotidacin blocks the ability of gyrase to cut the second strand of the double helix. However, it is likely that this property is due (at least in part) to the ability of the compound to distort the double helix following cleavage of the first strand. Finally, it will be interesting to determine whether it is the stimulation of enzyme-mediated DNA cleavage or the inhibition of the type II topoisomerases that ultimately leads to cell death following treatment with gepotidacin.

CHAPTER VI

CONCLUSIONS AND IMPLICATIONS

Antibacterial drug resistance is on the rise. According to a recent review, by 2050, 10 million deaths per year will result from antimicrobial resistant infections, which will surpass the amount of deaths per year caused by all types of cancer.[179] Many of the bacteria discussed in this dissertation are among the CDC's urgent (*N. gonorrhoeae*) or serious (*M. tuberculosis* and *S. aureus*) threat list due to their antimicrobial resistance.[69] Fluoroquinolone antibacterials are among the WHO's critically important list of antimicrobials due to their use for the treatment of a variety of Gram-positive and Gram-negative bacterial infections that affect millions of people worldwide.[180] However, due to overuse and misuse, the clinical use of fluoroquinolones is becoming limited.[25, 47, 69, 155] As mentioned in Chapter I, there are three strategies to combat fluoroquinolone resistance : (1) Find new antibacterial targets; (2) create fluoroquinolones that do not interact via the water-metal ion bridge; and/or (3) find new classes of compounds that target bacterial type II topoisomerases.

In this dissertation, I have characterized four novel bacterial topoisomerase-targeted drugs from two different classes: the MGIs (GSK000 and GSK325) and the NBTIs (GSK126 and gepotidacin). It is critically important to understand how these compounds interact with their target and assess their ability to overcome fluoroquinolone-resistant bacterial type II topoisomerases. Understanding their mechanism and how they potentially interact with their target can aid in the design of better compounds and help with resistance predictions.

M. tuberculosis, the causative bacterium of TB is one of the leading causes of morbidity and mortality worldwide.[76, 143, 144] The incidence of drug resistance and intolerance are prevalent

and has become an international issue. The WHO has a global "End TB strategy" to decrease the number of new cases by 80% and decrease the number of deaths by 90% by 2030.[181] The urgent need for new treatments for this wide-spread disease led to a UN General Assembly High-Level Meeting with the WHO this past year to try to gain some impetus in the WHO's strategic plan.[181] The worldwide impact denotes that there is a desperate need for the development of new anti-tubercular drugs.

The work in Chapter III explored the mechanism of a novel group of compounds, the MGIs. These compounds are a subclass of the napthyridone/aminopiperidine-based NBTIs that resulted from a screen of NBTIs that displayed activity against *M. tuberculosis*. However, little was known regarding the interaction of these compounds with the bacterial type II enzyme. Therefore, I examined the effects of two MGIs, GSK000 and GSK325, on *M. tuberculosis* gyrase. MGIs greatly enhanced DNA cleavage mediated by the bacterial enzyme. In contrast to fluoroquinolones, MGIs induced only single-stranded DNA breaks under a variety of conditions. MGIs work by stabilizing covalent gyrase-cleaved DNA complexes and appear to suppress the ability of the enzyme to induce double-stranded breaks. The drugs displayed little activity against type II topoisomerases from several other bacterial species, suggesting that these drugs display at least some specificity for *M. tuberculosis* gyrase. Furthermore, MGIs maintain activity against *M. tuberuclosis* gyrase enzymes that contained the three most common fluoroquinolone resistance mutations seen in the clinic, but displayed no activity against human topoisomerase IIα. Futhermore, GSK000 and GSK325 were able to overcome fluoroquinolone-resistance suspected to be caused by an upregulation of efflux pumps in clinical *M. tuberculosis* isolates. These findings suggest that MGIs have potential as anti-tubercular drugs, especially in the case of fluoroquinolone-resistant disease. However, based on the studies from Blanco, *et al* GSK000 and GSK325 interact with the human

ether-à-go-go-related gene (hERG) channels with an IC_{50} of <2 μM.[114] The acceptable limit for FDA drug approval is >30 μM.[182] Inhibition of these channels can lead to QT-interval prolongation in the heart, arrythmias, and potentially sudden cardiac death.[182] GSK126 had an IC_{50} >50 μM, so the minor structural changes resulting in specific *M. tuberculosis* gyrase activity also led to an unacceptable inhibition of the hERG channels.[114] More development needs to be focused on generating derivatives of GSK000 to identify compounds that retain their specificity for TB but do not inhibit the hERG channels.

One of the problems with fluoroquinolones today is the widespread use, which increases the likelihood of a patient already being exposed to them and their infection already having some level of fluoroquinolone resistance. If a drug is developed that has specificity for TB, the drug can be limited to those patients that have the disease. These drugs will have less widespread use, decreasing the overall resistance from overuse and misuse. The MGIs would be an ideal new drug class to help millions worldwide if a few alterations can be made to decrease the interaction with hERG and maintain its efficacy and specificity towards TB.

Another approach to combatting resistance is finding drug classes that synergize with one another and/or shorten the treatment time. The current regimen to treat TB includes rifampin, isoniazid, pyrazinamide, and ethambutol.[145] To be effective, this four-drug regimen is prescribed for four months, then reduced to rifampin and isoniazid for an additional two months.[145] If any resistance is detected, a combination of antibacterial drugs is often prescribed for up to two years. This can be very costly for the patient and cumbersome because of all the side effects. To further the work to find new classes to treat tuberculosis, there needs to be an effort to finding drug combinations that synergize to lower the concentrations of drug to be taken to lower the side effect profile and also hopefully shorten treatment time.

After exploring the properties of MGIs and how they interact with their enzyme target, I characterized GSK126, which is the parent compound of the MGI subclass. Note the small structural differences between the NBTI, GSK126, and those of the most efficacious MGI, GSK126 (Figure 9). The left-hand side of both compounds is identical; however, the right-hand side was altered from a bicyclic ring structure with two oxygens, to a single aromatic ring with two chlorines. First, I examined the ability of GSK126 to enhance DNA-cleavage with a broad range of bacterial type II topoisomerases. Like the MGIs, GSK126 enhanced only single-stranded DNA breaks; however, in contrast to GSK000, GSK126 displayed a broad range of activity against both Gram-positive and Gram-negative bacterial type II enzymes.

There is a paucity of published data describing the interactions of the NBTI class of compounds with their type II topoisomerase targets, so there is not a defined set of rules for how they interact across species or with gyrase vs. topoisomerase IV. Based on the initial survey of cleavage activity, I furthered my studies with three separate species of gyrase and topoisomerase IV (*M. tuberculosis*, *N. gonorrhoeae*, and *B. anthracis*). I chose to use *B. anthracis* gyrase and *N. gonorrhoeae* topoisomerase IV due to the high efficacy of GSK126, *M. tuberculosis* gyrase to afford direct comparisons to MGIs, and *B. anthracis* topoisomerase IV to have a matched set of enzymes from a Gram-positive species.

Results of the present work indicate that the NBTI GSK126 enhances single-stranded DNA cleavage mediated by gyrase and topoisomerase IV from the selected species and that it also is a potent inhibitor of overall catalytic activity by these enzymes. Another important property to note is that GSK126 had a high IC_{50} for the hERG channels, which is in steep contrast to the MGIs and other NBTIs that are in the literature.[114, 183] Because there are such few structural differences between the NBTI and MGI, the substituents on the right-hand side of the molecule seem to make

the difference in the toxicity profile. Further explorations should be made to determine which chemical entities contributed to both the tuberculosis specificity and the hERG inhibition.

Potency, efficacy, and toxicity aside, these findings make it likely that NBTIs display similar mechanisms of action against most bacterial type II topoisomerases as compared with the MGIs. This result will allow for the potential development of a set of consistent "rules" that govern the actions of NBTIs against enzymes and bacterial cells, which in turn may hasten the development of this potentially important class of compounds.

In terms of NBTI development, the most clinically advanced NBTI is gepotidacin, a triazaacenaphthylene-based compound. As discussed in chapter V, this compound has successfully completed phase II trials for the treatment of acute bacterial skin/skin structure infections and for the treatment of uncomplicated urogenital gonorrhea. It also displays robust *in vitro* activity against a range of bacterial species, including fluoroquinolone-resistant strains. However, despite this clinical promise, little was known about its interactions with its antibacterial targets. I first did a survey of the ability of gepotidacin to enhance DNA cleavage with bacterial type II topoisomerases from *S. aureus*, *M. tuberculosis*, *E. coli*, *B. anthracis*, and *N. gonorrhoeae*. Like GSK126 and the MGIs, gepotidacin enhanced only single-stranded DNA breaks, but the compound displayed broad spectrum enzyme activity similar to that of GSK126. Unfortunately, gepotidacin showed no substantial activity towards *M. tuberculosis* gyrase.

Due to the clinical importance of novel treatments for the treatment of *S. aureus*, I characterized the mechanism of action of gepotidacin against *S. aureus* gyrase. Gepotidacin was a potent inhibitor of gyrase-catalyzed supercoiling of relaxed DNA and relaxation of positively supercoiled substrates. The compound induced high levels of stable gyrase-mediated single-stranded breaks, with no enhancement of double-stranded DNA breaks at high concentration, long

cleavage times, or in the presence of ATP. Moreover, gepotidacin suppressed the formation of double-stranded breaks. *In vitro* competition suggests that gyrase binding by gepotidacin and fluoroquinolones are mutually exclusive. Finally, Ben Bax determined a crystal structure of gepotidacin with the *S. aureus* GyrB27-A56/Y123F fusion truncate and DNA, which shows the compound sits on the twofold axis between the DNA cleavage complex and in the twofold axis of the GyrA subunits. My work provides novel mechanistic insight into the actions of gepotidacin against an important clinical antibacterial target. To determine the exact mechanism, further studies will be needed to look at the ability of gepotidacin to the suppress double-stranded DNA breaks. Based on the structure, it is likely to be due in part to the ability of the compound to distort the double helix following cleavage of the first strand.

As seen in previous studies with fluoroquinolones and NBTIs, the compounds do not seem to have a set of "rules" of how they interact with their antibacterial target with regards to species or gyrase or topoisomerase IV. Future work will include a mechanistic study of how gepotidacin interacts with the type II enzymes from the Gram-negative *N. gonorrhoeae* to determine if gepotidacin acts in a similar manner as it does with the Gram-positive *S. aureus.* Furthermore, having access to clinical resistance data from patient isolates from the clinical trials, it is feasible to determine whether the stimulation of enzyme-mediated DNA cleavage or the inhibition of the type II enzymes leads to cell death following treatment with gepotidacin.

Although bacterial type II topoisomerases are just two of the limited number of antibacterial targets in cells, they are important targets. To be able to get ahead of the rise in resistance, the full mechanism of antibacterials, especially new classes being investigated, need to be explored. Knowing the mechanism and where the compounds interact with their target can help predict resistance and potentially prevent or overcome it. Also, finding what targets the

antibacterials work best against and limiting the use and overuse of these drugs can help greatly with this epidemic of antimicrobial resistant infections. If the trend of antimicrobial drug discovery stays as it is, there will be as many as ~10 million deaths per year due to antimicrobial resistant infections and a total GDP loss of ~100 trillion dollars.[179] My mechanistic studies of the MGIs (GSK000 and GSK325) and the NBTIs (GSK 126 and gepotidacin) are just a stepping stone toward understanding how these compounds work and kill cells.

Bacterial type II topoisomerases are well-validated antibacterial targets; however, the usability of fluoroquinolones in the clinic is on the decline. These new drug classes (NBTIs and MGIs) that are able to interact with their target outside of the water-metal ion bridge demonstrates that bacterial topoisomerases can still be explored as an antibacterial target. Also, the NBTIs and MGIs induce only-single stranded breaks, which might be easier to repair and have lower cytotoxic activity than double-stranded breaks. However, gepotidacin has successfully completed phase II clinical trials for both skin/skin structure infections and uncomplicated urogenital gonorrhea, indicating this compound has cytotoxic activity. It is not known whether the generation of single- vs. double-stranded DNA breaks are more toxic to bacterial cells. Therefore, studies need to be undertaken to further investigate the impact of single- vs. double-stranded DNA breaks on toxicity in *N. gonorrhoeae* and *S. aureus* bacterial cultures.

Finally, there is a general trend regarding the targeting of fluoroquinolones to the two bacterial type II topoisomerases. Topoisomerase IV is generally the primary target of fluoroquinolones in Gram-positive species, while gyrase is generally the primary target in Gram-negative species. The primary cellular target for NBTIs has not yet been established. To determine the fate of this class of compounds, cellular studies with the NBTIs should be carried out to determine the primary target of these compounds (if there is one) and if the resistance mutations

introduced by these compounds into gyrase and/or topoisomerase IV affect the expression levels of the enzymes or simply affects the interaction of the compound with its target. Furthermore, to determine the future usability of the NBTIs, the rates of spontaneous mutation development needs to be determined. Furthermore, these novel compounds can also be used as tools to shed light on why the serine and acidic residues that the fluoroquinolones interact with via the water-metal ion bridge are conserved across species. Characterizing the key differences between these two classes and carrying out an in-depth evaluation of the structure-activity relationships can impact the entire drug development approach of type II topoisomerase-targeted antibacterials. To prevent a post-antibiotic era, as so aptly quoted by Sun Tzu in the *Art of War*, "If you know the enemy and know yourself, you need not fear the results of a hundred battles." If you know the enzyme, and you know the antibacterial structure-activity relationships and resistance patterns, you need not fear a hundred infections.

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