ROLE OF DNA INTERACTIONS IN MODULATING THE ACTIVITY OF HUMAN TOPOISOMERASES AND ANTICANCER DRUGS

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

May, 2011

Nashville, Tennessee

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ACKNOWLEDGEMENTS

I would like to thank Dr. Neil Osheroff for giving me the opportunity to do my graduate research in his laboratory, for guiding me along this winding road to a completed dissertation and for providing opportunities to expand my scientific horizons. I would also like to thank my committee, Drs. Michael Stone, Martin Egli, Zu-Wen Sun, and Daniel Kaplan for all of their support, encouragement, constructive critiques and ideas that added to my graduate education. Finally, I would be remiss in not thanking Marlene Jayne. Marlene, thank you for all of the conversations and encouragement and keeping me on track and on deadline.

The projects that I worked on during my graduate career would not have been possible without the invaluable contributions of our collaborators. To the Denmark group- Anni Andersen, Birgitta Knudsen, Marie Brinch, Sissel Juul, and Simon Benson- for providing human topoisomerase I enzyme and topoisomerase II C-terminal deletion mutants, and for many fruitful discussions that added to the quality of this work and several manuscripts. To Dr. Chapin Rodriguez for providing the construct for *Archaeoglobus fulgidus* reverse gyrase, enabling the generation of the positively supercoiled substrates used in this work. To Dr. Christian Bailly at Pierre Fabre for providing F14512, without which Chapter V of this dissertation would not exist. To Drs. David Graves and Michael Jablonsky at UAB for their assistance with NMR experiments. And finally, to Drs. Keir Neuman and Yeonee Seol at the NIH for patiently teaching me single molecule techniques and allowing me to work with you in Bethesda for several weeks. I regret that no data from these trips made the dissertation cut, but I

take with me a greatly expanded knowledge of experimental techniques and some very fond and fun memories.

I would not have survived graduate school without the technical and, most importantly, emotional support of Jo Ann Byl. You saved my sanity on more than one occasion and were always there with helpful suggestions, a listening ear, or a shoulder. Jo Ann, there aren't enough words to tell you how much I appreciate you.

I would not be where I am today without the assistance and encouragement of previous members of the Osheroff lab. The friendliness and enthusiasm of Drs. Katie McClendon, Ryan Bender, Omari Bandele and Joseph Deweese convinced me to join this group. Katie provided the start of my project and invaluable training in topoisomerase research techniques. I'd also like to give a special shout out to Joe. We shared a bay for many years. Your engrained calmness, untiring work ethic, and endless well of encouragement convinced me to keep showing up and fighting through whatever problems arose.

I came into the lab with Steven Pitts. Steve, I don't even know what to say. Your dry wit was a constant source of amusement. To Adam Ketron and Katie Aldred, I will miss our lunchtime Ken Kens. Katie- you have been a breath of fresh air this last year. Thanks for listening. And finally to our undergraduate student, Anne Timmel, thank you for providing assistance above and beyond your job description and for introducing me to the many rules of shotgun. I will never again hear that word without thinking about pirates.

To Leah Potter, Mariam Eapen, Michelle Shepard, Jessica Hickey, Amy Dickey and the rest of my community group- thank you for providing a shoulder, an ear, an

escape, friendship, reality, a reminder of the truth, a cup of coffee or even the random take out lasagna on my door step. No matter where life leads us, I will never forget these years living life together in Nashville.

To Pamela Monahan and Jennifer Luedtke Kennedy, I can't imagine my life without you in it. Though we are spread out geographically, we embarked on this journey together. From all of the times at UTK to the phone calls and emails and trips to Knoxville and Illinois and here and the many weddings we attended during graduate school, thank you for not giving up on me and for tracking me down and staying in touch even when I neglect to return my phone calls for days on end. Never change.

And finally, I have to thank my family. You were my biggest support and biggest distraction. You reminded me that there was life outside of graduate school. To my brother, Andrew, I have so enjoyed living close to you these past few years and getting to know my nieces, Callia and Sadie. I will miss most being able to just drop by and be Mani for a few hours. To my parents, Camille and Enoch Gentry, I wouldn't have made it without you. Thanks for always listening, encouraging me to finish what I begin, and being willing to take a drive down I-40 whenever I needed some face to face support. I am blessed beyond words to have you on my side.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
amsa	amsacrine
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATP	adenosine triphosphate
bp	base-pair
CTD	C-terminal domain
DEPT	Demethyl epipodophyllotoxin
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
etop	etoposide
Gal	galactose
Gyr A	gyrase A
Gyr B	gyrase B
H	histidine
K	lysine
kDa	kilodalton
MLL	mixed lineage leukemia
NLS	nuclear localization sequence
PML	promyelocytic leukemia

R	arginine
RARA	retinoic acid receptor α
SC	supercoil
SDS	sodium dodecyl sulfate
σ	specific linking difference
TTP	thymidine triphosphate
WRN	Werner syndrome gene
Y	tyrosine

CHAPTER I

INTRODUCTION

The human body is composed of 10 trillion cells, each of which contains 2 meters of DNA compacted into a nucleus that is ~10 μm in diameter. Added together, our bodies contain over 12 billion miles of DNA, which must be maintained and replicated faithfully to ensure necessary cellular functions and to prevent a variety of nefarious illnesses, including cancer. The unique complimentary nature of DNA provides a redundant code that allows the genome to be replicated and repaired in a manner that promotes genomic stability (1-3). However, due to the length, double helical structure, and packaging of the DNA molecule into chromosomes, the cell must overcome significant topological problems (including over and underwinding, knotting and tangling) that arise as a result of normal cellular processes such as replication, transcription and recombination (4-8). These topological problems are resolved by DNA topoisomerases.

DNA Topology

Topological properties of DNA are those that cannot be altered without breaking one or both strands of the double helix (4). Because DNA is comprised of two interwound nucleic acid strands, and the genomes of all known organisms are very long or circular (or both), two distinct topological issues arise (4,6-8). Proliferating cells must be able to cope with both of these topological issues in order to survive.

The first issue is related to the torsional stress on the double helix. DNA from all species of eubacteria and eukaryotes is globally underwound ~6% (4,9). DNA under torsional stress is termed supercoiled, because underwound (or overwound) molecules writhe about themselves to form superhelical twists (4,9,10). Underwound and overwound molecules are called negatively and positively supercoiled, respectively (Figure 1). Negative supercoiling puts energy into the genetic material and makes it easier to separate the two strands of the double helix for replication and transcription (4,9,10). Thus, DNA underwinding increases the rates of these two fundamental processes. In contrast, the movement of DNA tracking systems (such as replication forks and transcription complexes) through the double helix locally overwinds the DNA ahead of their actions. Since overwinding makes it harder to pull apart the double helix, it blocks many essential cellular processes (4,11,12) (Figure 2).

The second issue is related to the extreme length of genomic DNA. Nucleic acid knots (intramolecular) and tangles (intermolecular) are formed routinely during a variety of ongoing cellular processes including DNA recombination and replication (Figure 1). DNA knots impair the ability to separate the two strands of the double helix and reduce the tensile strength of the genetic material. DNA tangles must be resolved in order for daughter chromosomes to segregate properly during meiosis and mitosis (4,6-9,11). Consequently, these two topological structures can be lethal to cells if they are not resolved.

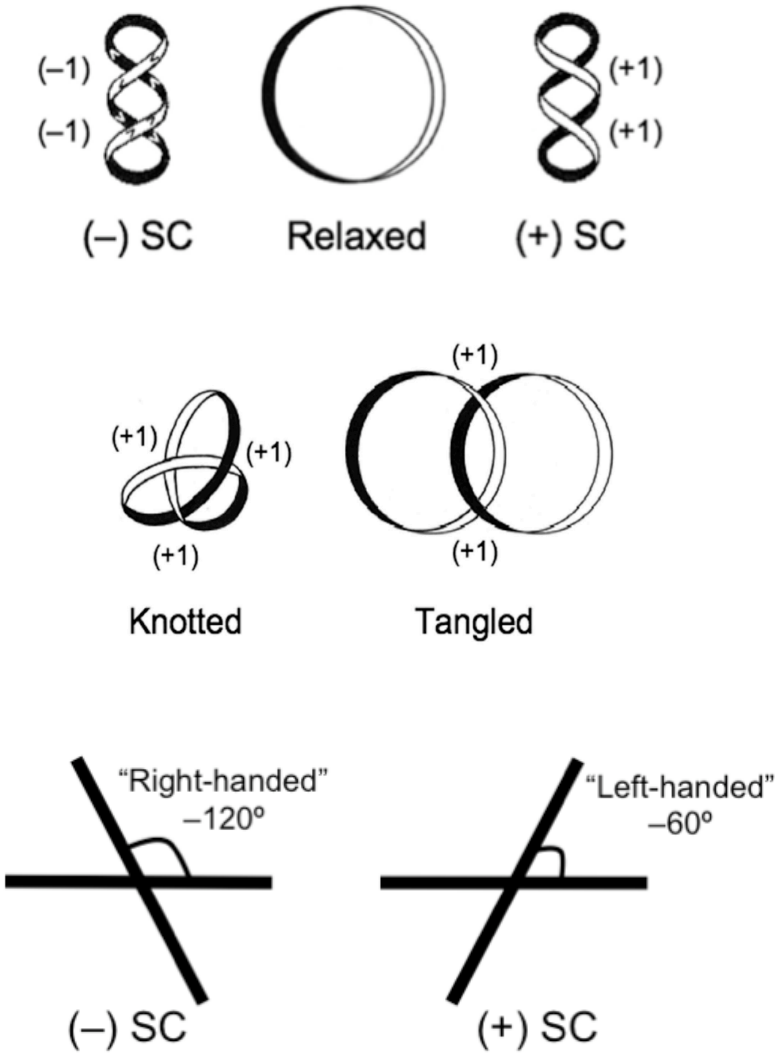


Figure 1. Topological relationships within DNA. DNA molecules are shown as circular ribbons for simplicity. Top: Relaxed DNA is not under torsional stress. DNA underwinding and overwinding results in negative [(–) SC] or positive supercoiling [(+) SC], respectively. The directionality of the DNA is shown by arrowheads. Supercoils are shown as writhes. By convention, each writhe is given an integral value of -1 or +1. Middle: Intramolecular knots and intermolecular tangles can also form in DNA. Bottom: Negative and positive supercoils are denoted as "right-handed" and "left-handed", respectively. Adapted from (9).

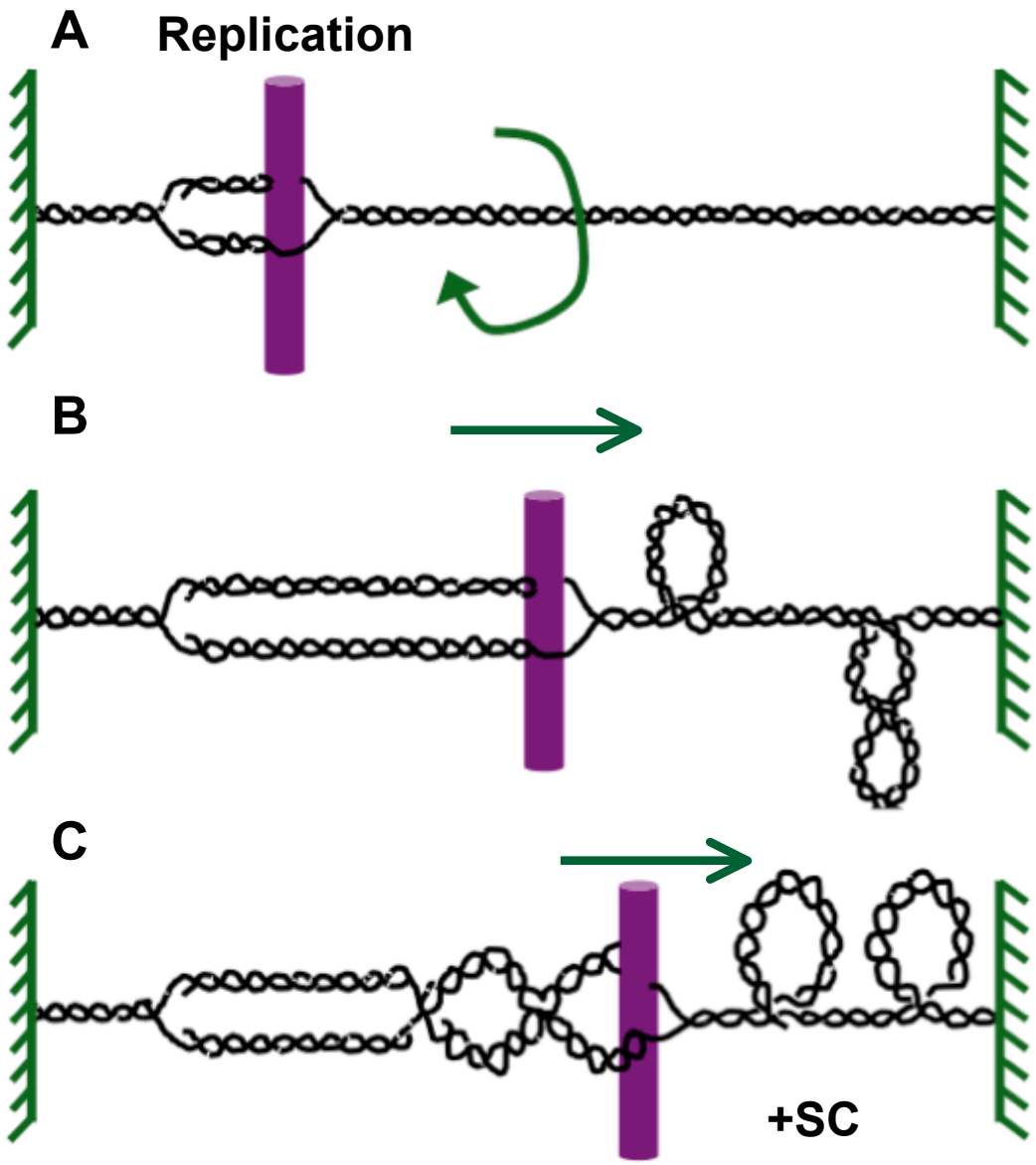


Figure 2. Topological changes associated with nuclear processes. A, DNA ends are tethered to membranes or the chromosome scaffold (represented by green lines) and are unable to rotate. Therefore, separation of the double helix and movement of DNA tracking systems (such as replication machinery represented by purple rods) through the immobilized DNA segment induces acute overwinding (i.e, positive supercoiling) (B). C, Compensatory underwinding (negative supercoiling) behind the replication machinery allows some of the torsional stress in the pre-replicated DNA to be translated to the newly replicated daughter molecules. Adapted from (15).

DNA Topoisomerases

In order to maintain the appropriate level of DNA supercoiling and remove knots and tangles from the genetic material, cells encode enzymes known as topoisomerases (5,6,13-15). Topoisomerases modulate these topological structures by creating transient breaks in the DNA backbone. There are two classes of topoisomerases that can be distinguished by the number of DNA strands that they cleave during their catalytic cycles. Type I topoisomerases alter DNA topology by creating a transient single-stranded break in the genetic material and facilitating controlled rotation of the double helix about (or passage of the opposite strand through) the nick (16-19). Type II topoisomerases act by generating a transient double-stranded break, through which they pass a separate intact double helix. To maintain genomic integrity during DNA cleavage, topoisomerases form covalent links between active site tyrosyl residues and the newly generated DNA termini. These covalent protein-cleaved DNA complexes, known as "cleavage complexes," are hallmarks of all topoisomerases irrespective of enzyme classification (5,6,14,16-20). Because type I topoisomerases create single-stranded breaks in the genetic material, they can regulate levels of DNA supercoiling. However, because type II topoisomerases generate double-stranded breaks in the DNA backbone, they can resolve knots and tangles in addition to removing torsional stress from the genetic material (Figure 3). Both type I and type II topoisomerases perform critical cellular functions that allow the progression of virtually all events in the cell that involve the genetic material (13,15-17,19).

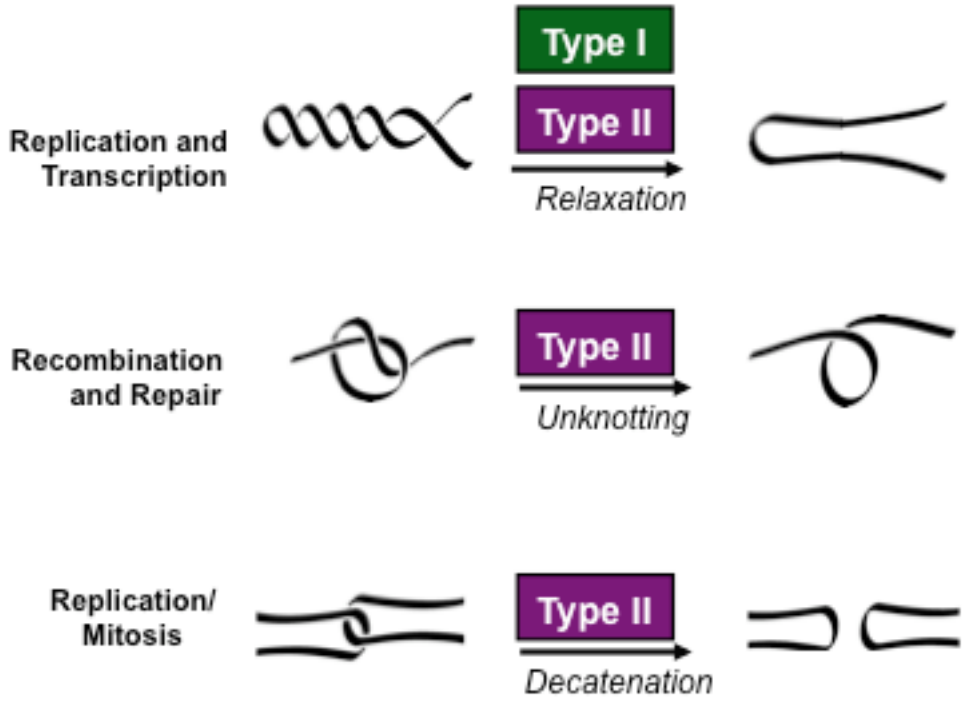


Figure 3. Topological structures resolved by DNA topoisomerases. DNA supercoils that result from replication and transcription are relaxed by both topoisomerase I and topoisomerase II. Due to its double stranded cleavage/strand passage mechanism, type II topoisomerases resolve knots and tangles in the genetic material that result from recombination and repair processes. Type II topoisomerases also decatenate interlocked sister chromatids at mitosis.

Type I Topoisomerases

With one exception, type I topoisomerases are denoted by "odd" numerals. Historically, type I topoisomerases have been divided into two subfamilies: type IA and type IB (6,16,19,21,22). There are several key differences between the two subfamilies. First, type IA topoisomerases covalently attach to the 5'-terminus of DNA during the cleavage reaction and resolve topological issues *via* a single-strand passage mechanism (16,19,21-24). In contrast, type IB enzymes covalently attach to the 3'-terminus of the cleaved DNA strand and act *via* a controlled rotation mechanism (16-18,25,26). Second, type IA enzymes require single-stranded DNA as a substrate and thus are capable of relaxing only negatively supercoiled substrates (21,22,27). Type IB enzymes do not require single-stranded substrates and can relax both negatively and positively supercoiled molecules (16,17,25-27). Third, while neither subfamily (with the exception noted below) requires a high–energy cofactor for enzymatic activity, type IA members do require a divalent cation for catalysis, while type IB members do not. However, catalysis by type IB topoisomerases is stimulated by divalent cations (16,18). Fourth, to date there are no known drugs that target topoisomerases in the IA subfamily. In contrast, several classes of molecules that have chemotherapeutic potential target type IB topoisomerases, specifically the eukaryotic enzymes (18,27,28). The clinical importance of these compounds and their actions against human topoisomerase I will be discussed later.

Prokaryotes encode several type I enzymes, including topoisomerase I and topoisomerase III, which belong to the type IA subfamily (16,21,22). Additionally, many hyperthermophilic prokaryotes encode reverse gyrase. This enzyme, which introduces positive supercoils into the genomic material, is the only type I topoisomerase that

requires ATP for catalysis (16,19,21,22). *Archaeoglobus fulgidus* reverse gyrase (29,30) was used to generate the positively supercoiled substrates employed in this dissertation.

Humans encode four type I topoisomerases: nuclear topoisomerase I, mitochondrial topoisomerase I (mt topoisomerase I), and topoisomerase IIIα and IIIβ (Table I). Nuclear and mitochondrial topoisomerase I are type IB enzymes while both topoisomerase III isoforms belong to the type IA subfamily (6,15-17). Nuclear topoisomerase I and topoisomerase IIIα are required for proper development (31,32). Less is known about mt topoisomerase I and topoisomerase IIIβ, though mice that lack topoisomerase IIIβ display infertility, aneuploidy and reduced lifespan (33). Because the activity of human nuclear topoisomerase I is a focus of work presented in this dissertation, this enzyme will be discussed further below.

Human Topoisomerase I Catalytic Cycle

Human topoisomerase I, a 91kD monomeric enzyme, carries out its catalytic function in five discrete steps (16-18) (Figure 4):

Step 1: Noncovalent binding of the enzyme to a DNA substrate. Topoisomerase I binds preferentially at nodes of DNA crossovers and thus prefers supercoiled substrates of either handedness (34).

Step 2: Generation of a single stranded break in one strand of the double helix and covalent attachment of the enzyme to the 3'-terminus of the cleaved DNA strand, creating the "cleavage complex". Human topoisomerase I shows some substrate preference for DNA surrounding the site of cleavage [5'–(A/T)(G/C)(A/T)T–3'] with the enzyme forming a covalent attachment to the 3' thymine (17).

Step 3: Relief of torsional stress in the DNA by a 'controlled rotation' mechanism in which DNA relaxation proceeds by rotation of the free 5' hydroxyl DNA end around the intact DNA strand. This relaxation is 'controlled' due to steric interactions by the surrounding topoisomerase I enzyme that restrict free movement (17,25,26).

Step 4: Religation of the cleaved DNA strand in a reaction that is the reverse of the cleavage reaction. This action regenerates an intact DNA substrate and the active site tyrosine of topoisomerase I. The catalytic mechanism of topoisomerase I allows it to relax DNA supercoils in steps of *n*, meaning that more than one supercoil can be relaxed before DNA religation takes place (16,17,25,26).

Step 5: Dissociation of the enzyme from the DNA substrate or initiation of a new round of catalysis.

Human Topoisomerase I Domain Structure

Human topoisomerase I consists of four domains defined by crystal structure and limited proteolysis studies (16-19,21,35) (Figure 4). The N-terminal domain (amino acids 1-214) is a poorly conserved region that contains multiple nuclear localization signals and interaction sites for several proteins including p53, nucleolin, and WRN helicase (36-38). This region of the protein is dispensable for activity *in vitro*. The core domain (amino acids 215-635) contains all of the residues needed for enzyme catalysis with the exception of the catalytic tyrosine. The core domain also provides structures for DNA substrate recognition and binding, and can be further divided into three subdomains (core subdomains I-III) based on structural studies. The linker domain (amino acids 636-712), which connects the core subdomain III to the C-terminal domain, is protease

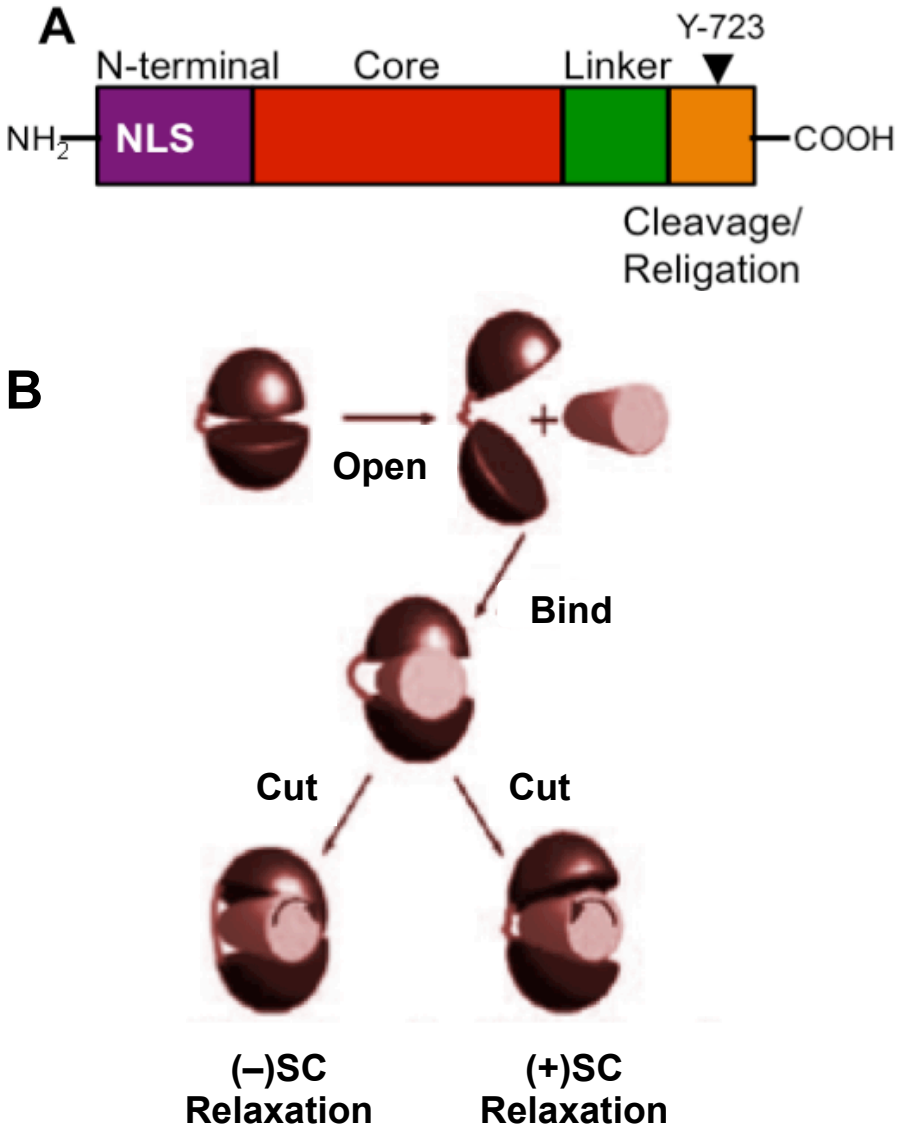


Figure 4. Domain structure and enzyme mechanism of human topoisomerase I. A, Domain organization of human topoisomerase I: N-terminal domain (purple), core domain (red), linker (green) and C-terminal domain (orange) are shown. The N-terminal domain contains nuclear localization sequences (NLS) and sites for protein interactions. The conserved core contains residues required for catalysis and substrate recognition. The C-terminal domain contains the active site tyrosine. B, Schematic of topoisomerase I mechanism [adapted from (26)]. The lobed structure formed by topoisomerase I is shown as semi-circles connected by a putative hinge. Negative [(–)SC] and positive [(+)SC] supercoils are relaxed by controlled rotation in a clockwise or counter-clockwise direction, respectively.

sensitive and dispensable for catalytic activity. The C-terminal domain (amino acids 713-765) contains the catalytic tyrosine. The core and C-terminal domain together are sufficient to reconstitute relaxation activity *in vitro* (16,17,19).

Structurally, topoisomerase I forms a bilobed clamp around the DNA duplex (16,17,19,21,26). The upper lobe, or cap, is made up of core subdomains I and II. The lower lobe is made up of core subdomain III and the C-terminal domain. A putative hinge region is located at the top of core subdomain III and is thought to control the opening and closing of topoisomerase I around DNA. Parts of the N-terminal domain are hypothesized to interact with DNA and the putative hinge. Steric interactions between DNA and portions of topoisomerase I require the bound DNA substrate to rotate in different directions in order to relax negative or positive DNA supercoils (16,17,25,26). The tight clamping of the two lobes of topoisomerase I around the DNA duplex creates a central cavity at the base of which the catalytic tyrosine is embedded (17).

The active site of human topoisomerase I contains a pentad of catalytic amino acids, R488, K532, R590, H632, and Y723 (the catalytic tyrosine), that are highly conserved among members of the type IB subfamily. The first four amino acid residues help position the scissile phosphate for nucleophilic attack by Y723, which generates the phosphodiester linkage between the enzyme and the 3' phosphate of the DNA backbone (16,17,19). This covalent linkage preserves the integrity of the genetic material during enzyme catalysis.

Cellular Roles of Human Topoisomerase I

Human topoisomerase I is constitutively expressed throughout the cell cycle. Topoisomerase I is thought to play a key role in relaxing the positive supercoils that are generated ahead of replication and transcription machinery so that these processes can be carried to completion (11,15-17). To this point, topoisomerase I is enriched in the nucleolus, where 40-60% of cellular transcription occurs on rDNA (17). Apart from its cleavage-ligation topoisomerase function, the enzyme also is implicated in transcriptional co-activation, seemingly facilitating the formation of the transcription activation complex TFIID–TFIIA on promoters (39). In addition to regulating topological issues associated with replication and transcription processes, topoisomerase I also may play roles in chromosome condensation (40), chromatin remodeling (41), DNA repair (37,38), and RNA splicing (42,43).

Type II Topoisomerases

Type II topoisomerases are essential to all prokaryotic and eukaryotic organisms. They are highly conserved among species, and the eukaryotic enzymes appear to be direct descendents of ancestral bacterial proteins (6,14,16,19,22,24). There are two classes of type II topoisomerases, type IIA and type IIB, which are defined on the basis of homology. With one exception, type II topoisomerases are denoted by "even" numerals. Bacteria encode two type IIA enzymes, DNA gyrase and topoisomerase IV (6,14,16,19,22,24). Eukaryotes, in contrast, encode only one type IIA enzyme, topoisomerase II (44,45). It should be noted, however, that vertebrate species express two closely related isoforms of the enzyme, topoisomerase IIα and topoisomerase IIβ (13-

16,46,47). Archaea and plants express the only known type IIB topoisomerase, topoisomerase VI. More in depth studies of the type IIB topoisomerase can be found in recent reviews (16,24,48). Because type IIA topoisomerases are considered in work in this dissertation, these enzymes will be discussed further below.

Topoisomerase II Enzyme Mechanism

Type II topoisomerases interconvert different topological forms of DNA by the double-stranded DNA passage reaction depicted in Figure 5, which uses eukaryotic topoisomerase II as a representative enzyme. Note that the eukaryotic enzyme functions as a homodimer while the prokaryotic enzymes work as heterotetramers. Type II topoisomerases carry out their catalytic function in six discrete steps (5,6,14-16,19,20):

Step 1: Binding of the enzyme to two DNA segments, the gate segment and the transfer segment. Type II topoisomerases bind preferentially to DNA crossovers (34).

Step 2: Creation of a double-stranded break in the gate segment of DNA, generating the cleavage complex. The scissile bonds on the two strands of the double helix cut by topoisomerase II are staggered, and the enzyme generates cleaved DNA molecules that contain 4-base single-stranded cohesive ends at their 5'-termini (20,49-51).

Step 3: Translocation of the transfer segment of DNA through the cleaved gate double helix.

Step 4: Rejoining (*i.e.*, religation) of the cleaved DNA, establishing a post-strand passage cleavage/religation equilibrium.

Step 5: Release of the translocated DNA helix through the opening of the C-terminal gate of the type II enzyme.

Step 6: Closing of the protein gate, returning the enzyme to its original conformation. The type II enzyme can now either dissociate or start a new round of catalysis.

Type II enzymes require two cofactors in order to carry out their catalytic double-stranded DNA passage reactions. First, they need a divalent cation for all steps beyond enzyme-DNA binding (52-55). While magnesium(II) appears to be the divalent cation that the enzymes use *in vivo,* other divalent metal ions such as calcium and manganese are sufficient for enzyme cleavage *in vitro* (52-54). Second, they use the energy of adenosine triphosphate (ATP) to drive the overall DNA strand passage reaction (56-60). Type IIA topoisomerases do not require ATP for DNA cleavage or ligation. However, the binding of this nucleoside triphosphate triggers DNA translocation (Figure 5, step 3), and its hydrolysis to adenosine diphosphate (ADP) and inorganic phosphate (Pi) is necessary for enzyme recycling (Figure 5, step 5). Normally, type II enzymes bind two molecules of ATP. Although hydrolysis of the cofactor is not a prerequisite for the strand passage event, it appears that this step proceeds more rapidly if it is preceded by hydrolysis of one of the bound ATP molecules (56-60).

Topoisomerase II Domain Structures

Type II topoisomerases share a number of common structural motifs that are shown in Figure 6. The founding type II enzyme, bacterial DNA gyrase, is comprised of two distinct subunits, GyrA and GyrB (molecular mass ≈ 96 kDa and 88 kDa, respectively),

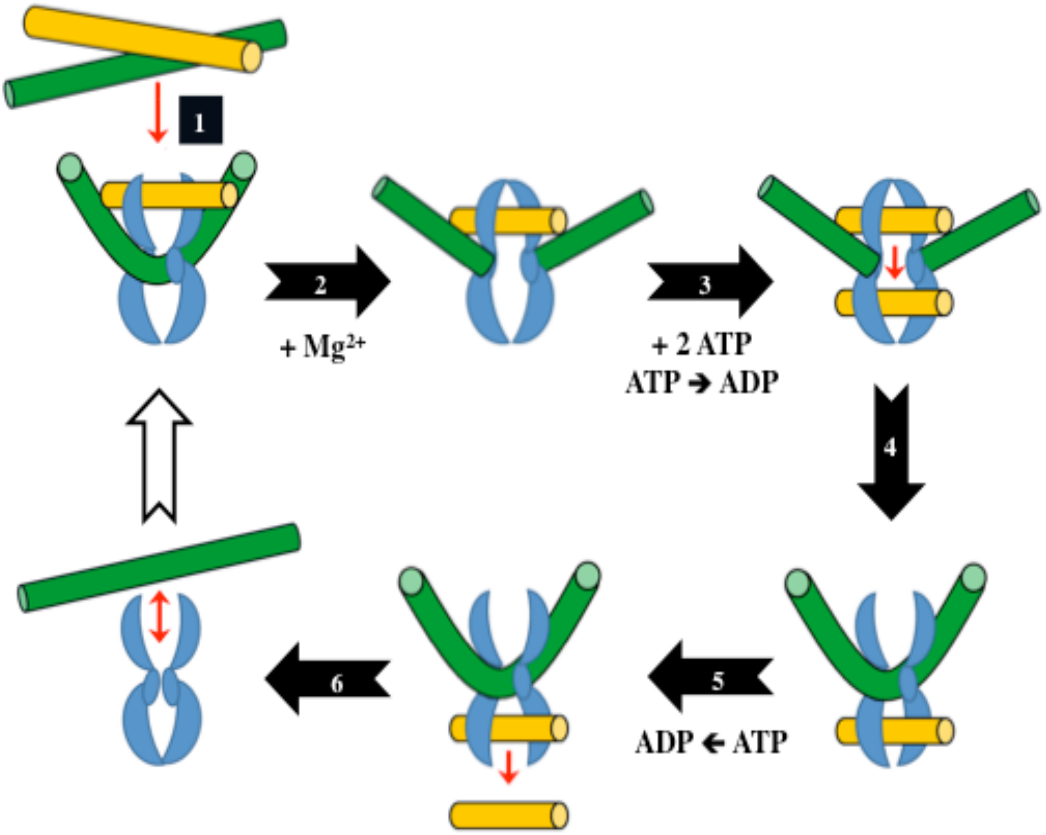


Figure 5. The double-stranded DNA passage reaction of topoisomerase II can be separated into six discrete steps. 1) DNA binding to regions of helix-helix juxtaposition. 2) Double-stranded DNA cleavage (*i.e.*, formation of the cleavage complex), which requires the presence of Mg^{2+} (physiologically) or other divalent metal ions. 3) Double-stranded DNA passage through the DNA gate generated by cleavage. This reaction requires the binding of 2 ATP molecules, and strand passage proceeds more rapidly if one of the two ATP molecules is hydrolyzed. 4) Ligation of the cleaved DNA. 5) Hydrolysis of the second ATP molecule, which allows release of the DNA through a C-terminal gate in the protein and promotes 6) enzyme turnover, which allows the enzyme to initiate a new round of catalysis. The protein (shown in blue) is based on crystallographic studies of yeast topoisomerase II. Modeled DNA helices are shown in green (horizontal) and yellow (coming out of the plane of the paper). Adapted from (20).

and acts as an A_2B_2 tetramer (14,16,19,61-63). GyrA contains the active site tyrosyl residue that forms the covalent bond with DNA during scission, and GyrB contains consensus sequences for ATP binding. Bacterial topoisomerase IV is also an A_2B_2 tetramer comprised of two subunits. In Gram-negative species, these subunits were first identified as proteins required for chromosomal partitioning, and are designated ParC (molecular mass ≈ 88 kDa) and ParE (molecular mass ≈ 70 kDa), which are homologous to the A and B subunits of DNA gyrase. In Gram-positive species, the subunits of topoisomerase IV are designated as the gyrase-like proteins GrlA and GrlB, respectively (14,16,19,61-63).

Eukaryotic type IIA topoisomerases are homologous to the bacterial enzymes (6,14,16,19,22,24). However, the two subunits have fused into a single polypeptide with protomer molecular masses ranging from ~160-180 kDa (depending on the species) (Figure 6). On the basis of amino acid sequence comparisons with bacterial gyrase, each topoisomerase II protomer can be divided into three distinct domains (6,14,16,19). The N-terminal domain of the enzyme is homologous to the B subunit of DNA gyrase (GyrB) and contains the binding site for ATP. The central domain is homologous to the A subunit of DNA gyrase (GyrA) and contains the active site tyrosyl residue. The C-terminal domain of the eukaryotic enzyme contains nuclear localization sequences as well as amino acid residues that are phosphorylated *in vivo* (64-70). As will be discussed in Chapter III, the C-terminal domains of both the prokaryotic and eukaryotic type II enzymes also are important for enzyme recognition of DNA topology. However, because work in this dissertation focuses on human topoisomerases (Table I), only the eukaryotic type II enzyme will be discussed in more detail below.

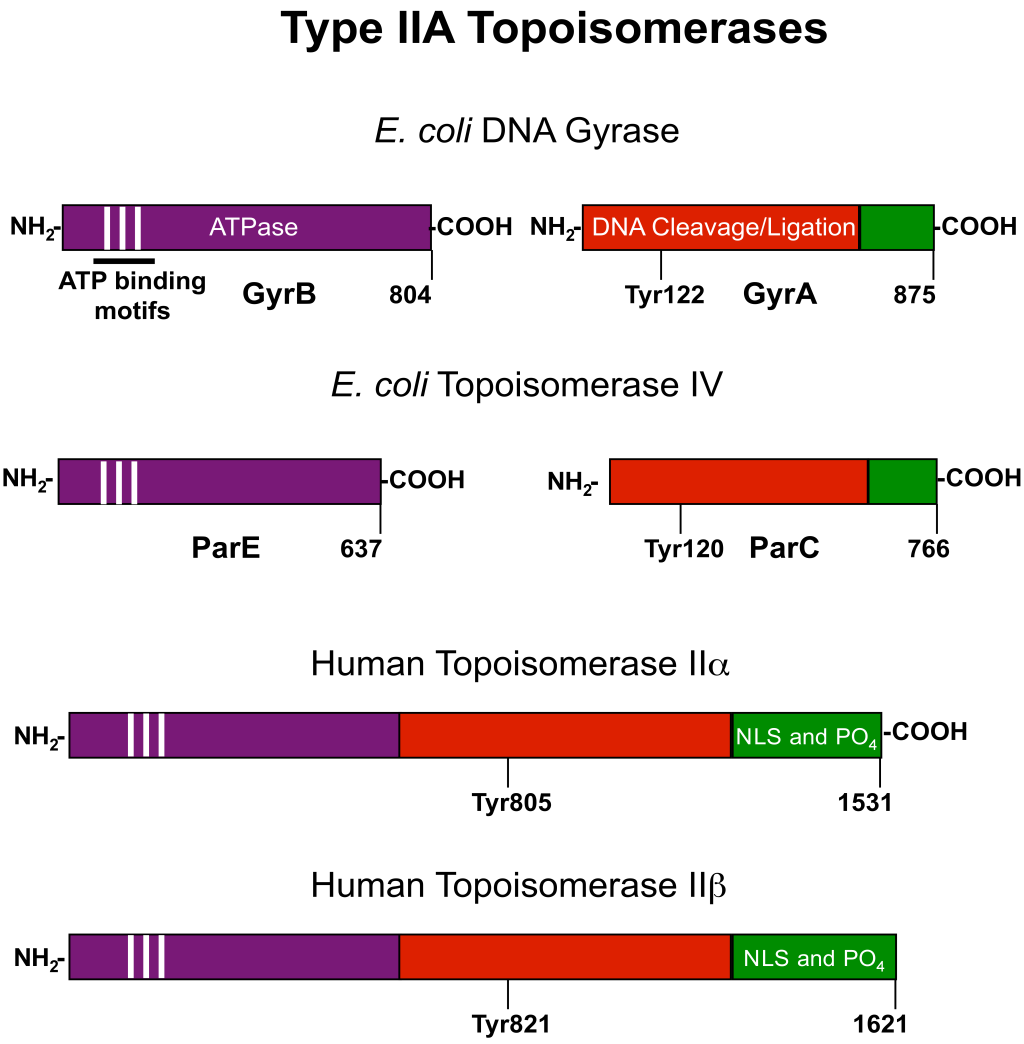


Figure 6. Domain structures of type II topoisomerases. The domain structures of four type IIA topoisomerases, bacterial (*Escherichia coli*) DNA gyrase and topoisomerase IV, and human topoisomerase IIα and IIβ are shown. Regions of homology among the enzymes are indicated by colors. The N-terminal (*i.e.*, GyrB) homology domains (purple) contain the regions responsible for ATP binding and hydrolysis. The vertical white stripes represent the three conserved motifs of the "Bergerat fold" that define the ATP-binding domain. The central (*i.e.*, GyrA) homology domains (red) contain the active site tyrosyl residue that forms the covalent bond with DNA during scission. For human topoisomerase IIα and IIβ the variable C-terminal domain (green) contains nuclear localization sequences (NLS) and phosphorylation sites (PO_4).

Table 1: Human DNA topoisomerases

Topoisomerase	**Family**	**Linkage**	**Cofactor Requirement**		**Subunit Structure**
			Metal Ion	ATP	
Nuclear topoisomerase I	IB	3’	-	-	monomer
mt topoisomerase I	IB	3’	-	-	monomer
Topoisomerase IIα	IIA	5’	+	+	homodimer
Topoisomerase IIβ	IIA	5’	+	+	homodimer
Topoisomerase IIIα	IA	5’	+	-	monomer
Topoisomerase IIIβ	IA	5’	+	-	monomer

Eukaryotic Type II Topoisomerases

The eukaryotic type II enzyme, topoisomerase II, was discovered in 1980 (71). Eukaryotic species such as yeast and *Drosophila* encode only a single type II topoisomerase (*i.e.*, topoisomerase II) (44,45,72-74). However, as discussed above, vertebrates contain two isoforms, topoisomerase IIα and topoisomerase IIβ. These two isoforms share extensive amino acid sequence identity (~70%) but are encoded by separate genes (located at chromosomal bands 17q21-22 and 3p24 in humans, respectively). Topoisomerase IIα and topoisomerase IIβ also can be distinguished by their protomer molecular masses (~170 kDa and ~180 kDa, respectively) (14,75-78).

Cellular Roles of Eukaryotic Topoisomerase II

Topoisomerase II plays a number of essential roles in eukaryotic cells and participates in virtually every major process that involves the genetic material (13-16,19,20). The enzyme unlinks daughter chromosomes that are tangled following replication and resolves DNA knots that are formed during recombination (Figure 3). It also helps to remove the positive DNA supercoils that are generated ahead of replication forks and transcription complexes (20,79,80). Topoisomerase II is required for proper chromosome condensation, cohesion, and segregation, and appears to play roles in centromere function and chromatin remodeling. Finally, topoisomerase II is important for the maintenance of proper chromosome organization and structure, and is the major non-histone protein of the mitotic chromosome scaffold and the interphase nuclear matrix (14,15,81-83).

It is not obvious why vertebrate species encode two distinct topoisomerase II isoforms. Enzymological differences between topoisomerase IIα and topoisomerase IIβ are subtle, and relationships between these isoforms are not well defined. The only major enzymological characteristic that distinguishes topoisomerase IIα and topoisomerase IIβ is the fact that the α isoform removes positive DNA supercoils ~10–fold faster than it does negative, while the β isoform removes both at similar rates (79). The structural features of topoisomerase IIα that promote this enzymological difference will be discussed in Chapter III.

Topoisomerase IIα and topoisomerase IIβ have distinct patterns of expression and separate cellular functions. Topoisomerase IIα is essential for the survival of proliferating cells and is regulated over both cell and growth cycles (84-87). Enzyme levels increase throughout S-phase of the cell cycle and peak at the G_2/M boundary (86,88,89). Topoisomerase IIα is found almost exclusively in proliferating tissues, is associated with replication forks, and remains tightly bound to chromosomes during mitosis (13,87,90-92). Thus, it is believed to be the isoform that functions in growth-related processes, such as DNA replication and chromosome segregation.

Topoisomerase IIβ is dispensable at the cellular level but is required for proper neural development in mice (46,93,94). In contrast to the α isoform, the concentration of topoisomerase IIβ is independent of the cell cycle, and this isoform is found in most cell types regardless of proliferation status. Topoisomerase IIβ dissociates from chromosomes during mitosis and cannot compensate for the loss of topoisomerase IIα in mammalian cells (46,92,93). Although the physiological functions of the β isoform have yet to be

fully defined, recent evidence indicates involvement in the transcription of hormonally- or developmentally-regulated genes (95,96).

Topoisomerases as Cellular Toxins

The concentrations of topoisomerase-DNA cleavage complexes are tightly regulated in cells (Figure 7). These complexes normally have a short lifespan and are readily reversible. While topoisomerase activity is necessary for cellular survival, too few or too many topoisomerase cleavage complexes can have a detrimental effect on the cell. If concentrations of topoisomerase II-DNA cleavage complexes drop below threshold amounts, topological problems that arise during replicative processes are not resolved, and daughter chromosomes remain entangled, resulting in catastrophic mitotic failure. If concentrations of topoisomerase I- or topoisomerase II-DNA cleavage complexes are too high, several negative outcomes are possible, including mutagenic translocations or cell death due to genome fragmenting (13-15,20,27,80,97-100).

In addition to their varied and critical physiological functions, topoisomerases are targets for some of the most active antibacterial (targeting bacterial type II topoisomerases) and anticancer drugs (targeting human topoisomerase I and both isoforms of human topoisomerase II) in clinical use. These agents do not act by robbing cells of essential topoisomerase activity. Rather, drugs that target topoisomerases kill cells by dramatically increasing the concentration of covalent enzyme-DNA cleavage complexes (20,27,28,80,101-104).

The potential lethality of cleavage complexes rises substantially when DNA tracking enzymes such as polymerases or helicases attempt to traverse the covalently

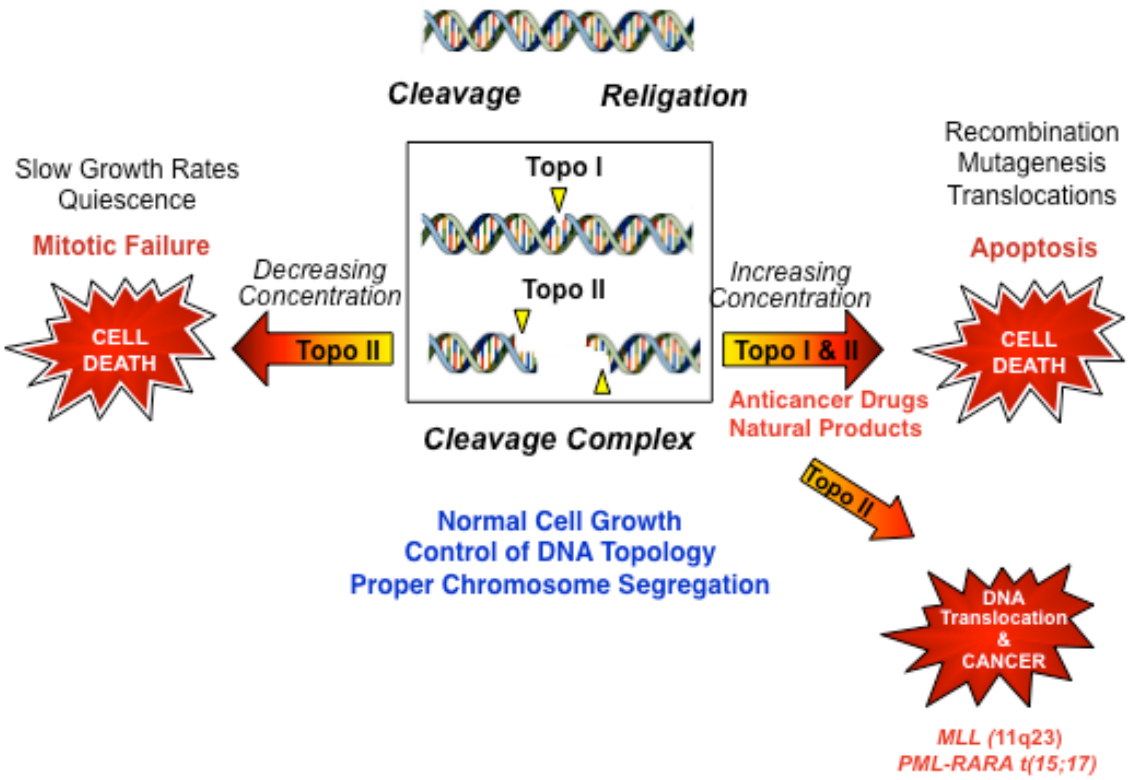
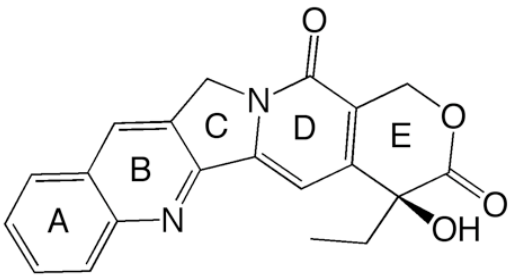


Figure 7. Effects of topoisomerase-cleavage complexes in the cell. Topoisomerase I and II cleavage complexes are transient in nature, and their cellular concentration is tightly regulated. If levels of topoisomerase II cleavage complexes fall too low, cells are unable to undergo chromosome segregation, resulting in slow growth rates and mitotic failure. If levels of either topoisomerase I or topoisomerase II cleavage complexes are too high, they can be converted to permanent strand breaks which can lead to chromosome breakage and translocations. Both of these pathways can culminate in cell death. In a small percentage of patients treated with topoisomerase II poisons, DNA translocations at the MLL and PML-RARA chromosomal loci result in therapy-related leukemias. Adapted from (20).

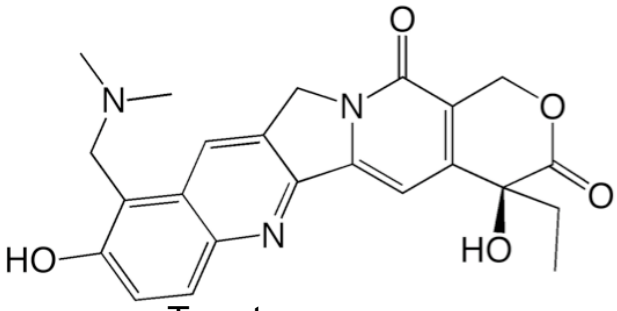
bound topoisomerase "roadblock" in the genetic material. Such an action disrupts cleavage complexes and converts transient enzyme-mediated DNA breaks to permanent DNA breaks (20,80,98,99). These permanent breaks in the genome trigger the generation of chromosomal insertions, deletions, translocations, and other aberrations, and when present in sufficient numbers, initiate a series of events that culminates in cell death (20,80,98-100,105-107). Because the drugs that target topoisomerases convert these essential enzymes to potent cellular toxins that fragment the genome, they are referred to as topoisomerase "poisons" to distinguish them from drugs that act as catalytic inhibitors.

Human Topoisomerase I in Chemotherapy

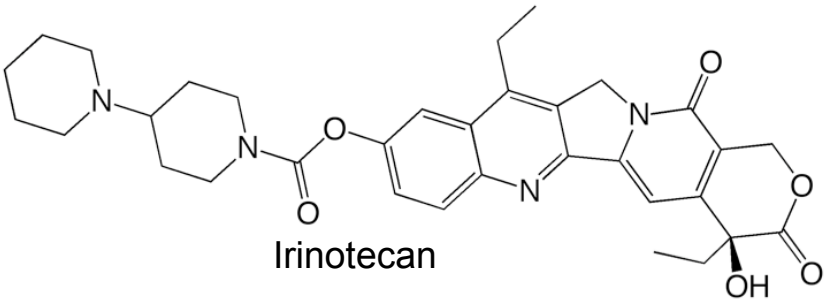
Human topoisomerase I is the target of an emerging class of drugs based on the parent compound camptothecin (27,28,108) (Figure 8). Camptothecin was first discovered in 1966 during a screening for natural products with anticancer activity and was originally isolated from the bark of the Chinese Yew tree *Camptotheca acuminata* (109). Camptothecin acts by inhibiting the DNA religation reaction of topoisomerase I, trapping covalent topoisomerase I-nicked DNA complexes on the genetic material. Two derivatives of camptothecin, topotecan (a water soluble formulation) and irinotecan (a prodrug that is activated *in vivo*) are approved worldwide for the treatment of colorectal, gynecological, and other cancers. A third derivative, belotecan is approved for use in South Korea. These topoisomerase I-targeted anti-cancer agents are of special importance because they are active against a spectrum of cancers that have few other treatment options (27,28,108).



Camptothecin



Topotecan



Irinotecan

Figure 8. Topoisomerase I-targeted anticancer drugs. Camptothecin is a naturally occurring compound originally isolated from the bark of *Camptotheca acuminata*. Topotecan and Irinotecan are clinically prescribed derivatives that are used to treat a wide spectrum of cancers that have few other treatment options.

Several lines of evidence demonstrate that the anticancer properties of camptothecin result from its actions on topoisomerase I. First, yeast that lack topoisomerase I are immune to the effects of camptothecin (110,111). Second, experiments to select for camptothecin resistance yield specific causal point mutation in topoisomerase I (112). Third, plants that produce camptothecin contain point mutations in their topoisomerase I enzymes that render them resistant to the effects of the drug (27,112,113).

Though effective, these camptothecin-based drugs are not without limitations. At physiological pH, the opening of the lactone E-ring of the compound readily inactivates camptothecin (27,28,112). Additionally, camptothecin binds serum albumin and is thus less accessible for cellular uptake (114). Topotecan and irinotecan are more stable than camptothecin and display reduced albumin binding. Other mechanisms that contribute to camptothecin resistance include cellular exclusion by drug efflux pumps, reduced topoisomerase I protein levels, and topoisomerase I mutations that decrease the sensitivity of the enzyme for these compounds (27,104,115-119). Thus, the development of new drugs that target topoisomerase I is of great clinical interest, and several new families of compounds that poison topoisomerase I currently are under investigation (27,28,118,120,121). The effects of DNA topology on the actions of anti-cancer drugs that affect topoisomerase I and implications for new classes of drugs that target the enzyme will be discussed in Chapter IV.

Human Topoisomerase II in Chemotherapy

At the present time, six topoisomerase II-targeted anticancer agents are approved for use in the United States (Figure 9). Additional drugs that target the type II enzyme are

used worldwide. Drugs such as etoposide and doxorubicin (and their derivatives) are front-line therapy for breast and lung cancers, as well as a variety of leukemias, lymphomas, and germ-line malignancies (20,80,122-125). Mitoxantrone is used to treat breast cancer as well as remitting cases of multiple sclerosis, an autoimmune disease (126,127). Both mitoxantrone and amsacrine are used to treat relapsed acute myeloid leukemia (128). Approximately one-half of all cancer chemotherapy regimens contain drugs targeted to topoisomerase II. Moreover, every form of cancer that can be cured by systemic chemotherapy is treated with these agents (97,122,125,128).

The topoisomerase II-targeted anti-cancer agents currently in clinical use act by interfering with the ability of the enzyme to religate the cleaved DNA strands (80,97,125). Doxorubicin, mitoxantrone, and amsacrine have additional effects due to their ability to intercalate into the double helix (80,129-132). The affects that intercalative compounds have on the activity DNA topoisomerases will be explored in Chapter IV.

Topoisomerases were first suggested to be the target for certain anticancer drugs in the late 1970s when the laboratory of Dr. Kurt Kohn observed that protein-associated DNA breaks formed in cells treated with intercalative compounds (133,134). In the early 1980s, the laboratory of Dr. Leroy Liu demonstrated that etoposide and related compounds specifically increased levels of DNA cleavage mediated by mammalian topoisomerase II *in vitro* and *in vivo* (129,135,136). In the late 1980s, the laboratory of Dr. Neil Osheroff showed that these compounds interfered specifically with the cleavage-ligation reaction of the type II enzyme (137). Clinical development of new compounds that target the human enzyme is still robust today.

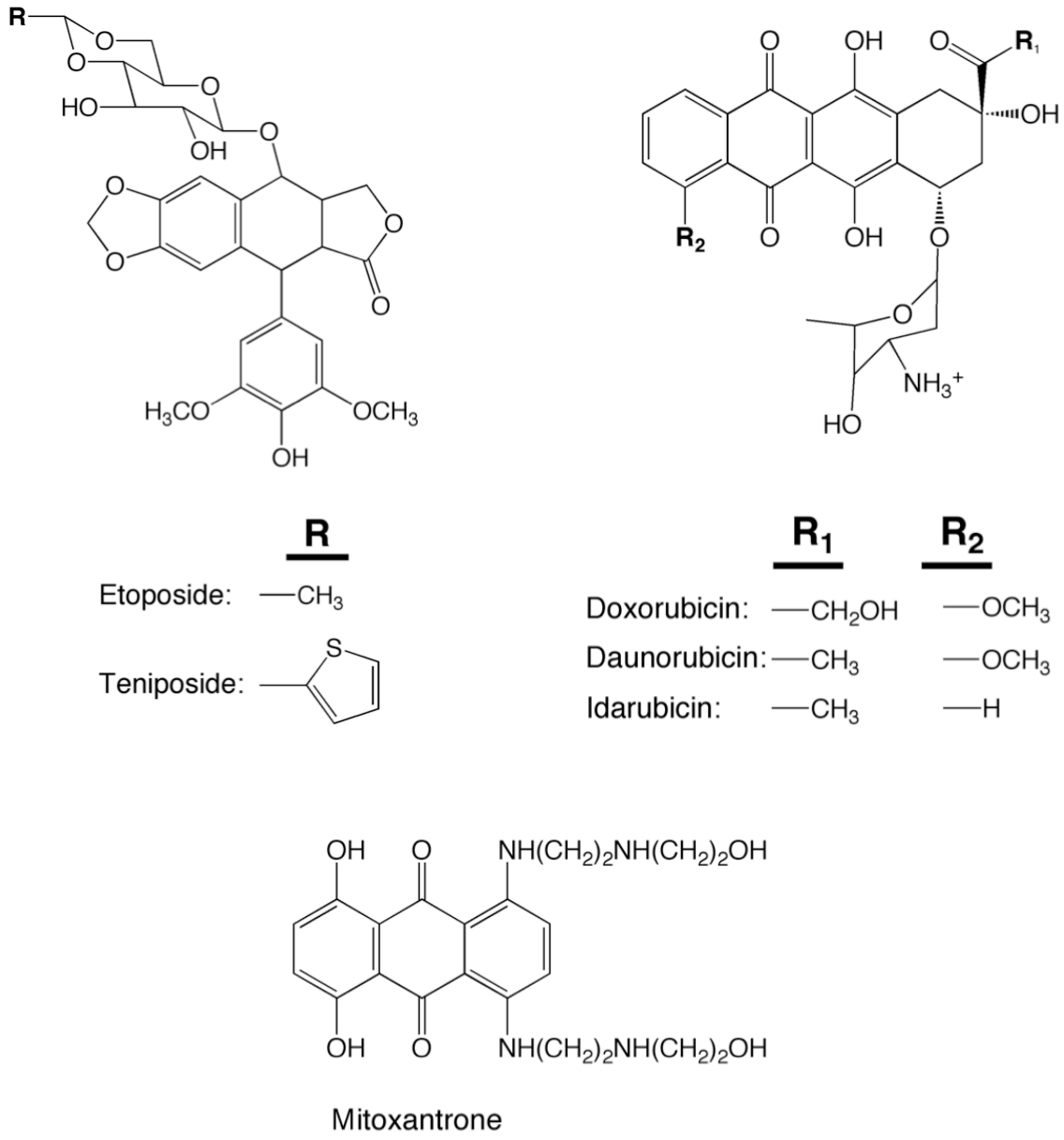


Figure 9. Topoisomerase II-targeted anticancer drugs. Clinically approved topoisomerase II-targeted agents are shown. These compounds are termed poisons because they act by increasing cellular levels of covalent topoisomerase II-DNA cleavage complexes.

Due to the high concentration of topoisomerase IIα in rapidly proliferating cells, this isoform probably is the more important target of anticancer therapy. However, circumstantial evidence suggests that the β isoform also contributes to drug efficacy (20,80,97). Treatment of patients with drugs that target topoisomerase II, while potentially curative, comes with a cost. Topoisomerase II poisons can cause numerous side effects, including myelosuppression and gastrointestinal and cardiac toxicities. Additionally, ~2-3% of patients treated with topoisomerase II-targeted agents develop specific treatment-related acute myeloid leukemias (AML) that include chromosomal translocations in the *MLL* (mixed lineage leukemia) breakpoint region at chromosomal band 11q23 (107,138,139). Occurrences of acute promyelocytic leukemias with t(15;17) chromosomal translocations after treatment with topoisomerase II poisons also have been reported (140,141).

Several studies suggest that topoisomerase IIβ may play a more important role than topoisomerase IIα in mediating off-target toxicities and generating leukemic chromosomal translocations (142-144). Currently, there is no method available for specifically targeting topoisomerase II poisons to cancer cells. To this point, interactions of etoposide-based compounds with DNA and topoisomerase II, and implications for more selective means of targeting these compounds to cancer cells will be discussed in Chapter V.

Scope of the Dissertation

Topoisomerases play critical roles in regulating the topological state of the genetic material. Topoisomerase I has long been assumed to act ahead of replication forks to relieve the positive torsional stress that accumulates during this process. Recent studies provide evidence that topoisomerase IIα preferentially relaxes positively supercoiled DNA and also may be acting to relieve positive superhelical stress associated with replication (15,16,80). However, very few studies have examined these enzymes on their purported cellular substrates. In addition to their cellular functions, topoisomerases are the targets for some of the most widely prescribed anticancer drugs currently in clinical use (20,27,28,97). These drugs act by stabilizing covalent topoisomerase-DNA cleavage complexes. It is the accumulation of these cleavage complexes ahead of DNA tracking systems that ultimately leads to cell death (20,27,80). Therefore, the goal of this dissertation is to more fully characterize the effects that DNA interactions have on the activities of topoisomerase I and topoisomerase II and drugs that target these enzymes.

Chapter I of this dissertation reviews DNA topology and type I and type II topoisomerases. Additionally, Chapter I discusses the role of human topoisomerase I and topoisomerase II as cellular toxins and chemotherapeutic targets. Chapter II describes the materials and methods utilized in the work presented in Chapters III-V.

Chapter III of this dissertation describes the structural features of human topoisomerases that are required for the recognition of DNA topology. Previous studies have shown that topoisomerase IIα, but not topoisomerase IIβ preferentially relaxes positively supercoiled DNA (79). Recent work shows that the C-terminal domain of the enzyme is required for this activity. Therefore, studies were undertaken to determine

which portions of the C-terminal domain are required for the recognition of DNA supercoil handedness for relaxation. My contributions to this project, which are presented in Chapter III, demonstrate that almost the entire C-terminal domain of topoisomerase IIα is required for the preferential relaxation of positive supercoils. Furthermore, removal of even the endmost 30 amino acids abolished this ability. These results have been published (145).

If topoisomerase I is acting ahead of replication forks, its preferred substrate should be positively supercoiled DNA. Therefore, the relaxation and cleavage activity of human topoisomerase I were characterized on positively supercoiled substrates. Results presented in Chapter III show that topoisomerase I preferentially relaxes and cleaves positively supercoiled substrates. It is notable that the work on DNA relaxation was a collaborative project and that only my contributions are shown here. A more in-depth characterization of this property of topoisomerase I has been published in Frohlich *et al.* (146). I spearheaded the characterization of topoisomerase I cleavage on positively supercoiled DNA, and these results have been published in Gentry *et al.* (147).

Chapter IV of this dissertation examines the effects that DNA supercoil geometry has on topoisomerase-targeted anticancer agents. Results show that topoisomerase I maintains higher concentrations of cleavage complexes on overwound substrates in the presence of both camptothecin-based and non-camptothecin based anti-cancer drugs. This is thought to be due to increased baseline levels of cleavage on positively supercoiled substrates rather than an altered drug interaction in the enzyme-DNA complex. Additionally, intercalative compounds that are not traditional topoisomerase I poisons

enhance the ability of the enzyme to cleave negatively supercoiled substrates *in vitro* and in a human cancer cell line. These results have been published in Gentry *et al*. (147).

Results presented in Chapters III and IV show that the interaction of drugs with DNA can have a profound effect on the activity of both topoisomerase I and topoisomerase II. Chapter V of this dissertation examines the activity of F14512, a novel etoposide derivative with strong DNA interactions, against type II topoisomerases. F14512 contains a spermine moiety in place of the C4 gycosidic group of etoposide (148,149). This compound was developed to increase drug uptake *via* the polyamine transport system. However, the addition of the spermine moiety to the etoposide core resulted in a compound that is a DNA binder (148). Results show that F14512 is a potent poison of topoisomerase IIα and topoisomerase IIβ but does not change the fundamental mechanism of etoposide action (*i.e.*, inhibition of DNA ligation). Rather, the linkage between the spermine and the drug core appears to enhance the stability of etoposide in the ternary enzyme-drug-DNA complex. Results from this work currently are under review in Gentry *et al*. Chapter VI contains conclusions that can be drawn from my work on topoisomerases.

CHAPTER II

METHODS

Materials

Human topoisomerase IIα and topoisomerase IIβ were expressed in *Saccharomyces cerevisiae* and purified as described previously (150). A set of mutant human topoisomerase IIα enzymes that were partially deleted in their CTD or that replaced amino acids starting with residue 1501 with the corresponding portion of human topoisomerase IIβ were constructed, expressed in *S. cerevisiae* JEL-1*Δtop1* under the control of the yeast Gal promoter, and purified as described previously (151). Human topoisomerase I was expressed in *S. cerevisiae* top1 null strain RS190 (a gift from R. Sternglanz, State University of New York at Stony Brook) and purified as described previously (152). *Archaeoglobus fulgidus* reverse gyrase was expressed in *Escherichia coli* C41(DE3) and purified according to Rodriguez (29). Negatively supercoiled pBR322 DNA was prepared from *E. coli* DH5α using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Restriction enzymes, klenow exo$^-$, calf phosphatase, and T4 polynucleotide kinase were from New England Biolabs. [α-^{32}P]dATP (6000 Ci/mmol), [α-^{32}P]TTP (3000 Ci/mmol) and [γ-32P]ATP (~5000Ci/mmol) were obtained from NEN. Camptothecin, ethidium bromide, 9-aminoacridine, etoposide, and ciprofloxacin were from Sigma. Topotecan was from Alexis Biochemicals. Amsacrine was a gift from Dr. David Graves (University of Alabama at Birmingham). NSC706744 and NSC725776 were gifts from Dr. Yves Pommier (NCI). Tas-103 and TOP-53 were gifts from Taiho

Pharmaceuticals. F14512 (patent WO 2005/100363) was synthesized as described previously (148,149) and was a gift from Dr. Christian Bailly (Pierre Fabre). 4′-Demethyl epipodophyllotoxin (DEPT) was the gift of Dr. Norma Dunlap (Middle Tennessee State University). Camptothecin and topotecan were stored at -20 ºC as 10 mM stock solutions in 100% DMSO and water, respectively. Amsacrine, 9-aminoacridine, etoposide, TOP-53 and F14512 were stored at 4 ºC as 20 mM stock solutions in 100% DMSO. TAS-103 and ethidium bromide were stored at 4 ºC as 10 mM and 2.5 mM stock solutions in water, respectively. Ciprofloxacin was stored at -20 °C as a 40 mM stock solution in 0.1N NaOH. All other chemicals were analytical reagent grade.

Procedures

Preparation of Positively Supercoiled DNA

Positively supercoiled pBR322 DNA was prepared by incubating negatively supercoiled plasmids with *Archaeoglobus fulgidus* reverse gyrase using a protocol modified from McClendon *et al*. (79). Reaction mixtures contained 18 nM negatively supercoiled pBR322 DNA and 250 nM reverse gyrase in a total of 250 µL of 50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM $MgCl_2$, and 1 mM ATP. Reactions were incubated at 95 °C for 5 min, halted by the addition of 13 µL of 375 mM EDTA, and cooled on ice. Proteinase K was added (10 µl of 4 mg/ml), and the reactions were incubated at 45 °C for 30 min to digest the enzyme. Samples were extracted with phenol: chloroform: isoamyl alcohol (25:24:1), and positively supercoiled DNA was precipitated with cold ethanol. Plasmids were resuspended in 25 µL of 5 mM Tris-HCl, pH 7.4,

containing 500 μM EDTA. To remove residual ATP, reactions were further purified through Bio-Spin 6 nucleic acid purification columns (BioRad) according to the manufacturer's protocol. To make certain that differences between negatively and positively supercoiled substrates were not influenced by the temperature or other conditions used in the preparation protocol, negatively supercoiled plasmids were treated in a parallel fashion, except that reverse gyrase was omitted from reaction mixtures.

The average number of superhelical twists present in DNA substrates and the resulting σ values were determined by electrophoretic band counting relative to relaxed molecules. Typical of plasmids isolated from *E. coli*, negatively supercoiled plasmids contained ~15–17 negative superhelical twists per molecule ($\sigma \approx -0.035$ to -0.039). Positively supercoiled plasmids contained ~15–17 positive superhelical twists per molecule ($\sigma \approx +0.035$ to $+0.039$). Thus, the supercoiled substrates employed in this body of work contained equivalent numbers of superhelical twists but were of opposite handedness. To ensure that equal amounts of plasmid were used in all experiments, the DNA concentration was assessed by spectrophotometric analysis and confirmed by ethidium bromide staining of linearized plasmid substrates.

Plasmid DNA Relaxation

Topoisomerase I. Topoisomerase I plasmid DNA relaxation reactions were based on the protocol of Frohlich *et al*. (146). Reaction mixtures contained 3.75 nM topoisomerase I, and 5 nM negatively or positively supercoiled DNA in a total of 20 μL of topoisomerase I relaxation buffer (50 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 50 mM KCl, 10 mM $MgCl_2$, and 0.5 mM DTT). Mixtures were incubated at 37 ºC for 0-10 min

and stopped by the addition of 3 μL of 0.5% SDS and 77 mM EDTA. Samples were mixed with agarose gel loading buffer (60% sucrose in 10 mM Tris-HCl, pH 7.9) and subjected to electrophoresis in 1% agarose gels in 100 mM Tris-borate, pH 8.3 and 2 mM EDTA. Following electrophoresis, gels were stained for 30 min with 0.5 μg/mL ethidium bromide. DNA bands were visualized by UV light and were quantified using an Alpha Innotech digital imaging system (San Leandro, CA). DNA relaxation was monitored by measuring loss of the initial supercoiled substrate.

Topoisomerase II. Topoisomerase II DNA relaxation assays were based on the protocol of McClendon *et al*. (79). Reaction mixtures contained 1-2 nM wild-type or mutant human topoisomerase IIα enzymes, 1 mM ATP, and 5 nM negatively or positively supercoiled pBR322 DNA in a total of 20 μL of topoisomerase II relaxation buffer (10 mM Tris-HCl, pH 7.9, 175 mM KCl, 5 mM $MgCl_2$, 0.1 mM NaEDTA, and 2.5% glycerol). Mixtures were incubated at 37 ºC for the time indicated and stopped by the addition of 3 μL of 0.5% SDS and 77 mM EDTA. Samples were mixed with agarose gel loading buffer, and subjected to electrophoresis in 1 % agarose gels in 100 mM Tris-borate, pH 8.3 and 2 mM EDTA. Gels were stained for 30 min with 0.5 μg/mL ethidium bromide. DNA bands were visualized and quantified as above. DNA relaxation was monitored both by the formation of the fully relaxed product and by the loss of the initial supercoiled substrate.

Plasmid DNA Cleavage

Topoisomerase I. Unless indicated otherwise, topoisomerase I DNA cleavage reactions were carried out by incubating 5 nM positively or negatively supercoiled

pBR322 plasmid DNA with 11 nM human topoisomerase I in 20 μL of topoisomerase I cleavage buffer (10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 2.5% glycerol). Except where noted, reactions were incubated for 2 min at 37 ºC, and enzyme-DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS followed by 1 μL of 375 mM EDTA, pH 8.0. Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and protein samples were incubated for 30 min at 45 ºC to digest the enzyme. Samples were mixed with 2 μL of agarose gel loading buffer, heated for 5 min at 45 ºC, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate, pH 8.3, and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. DNA bands were visualized with ultraviolet light and quantified using an Alpha Innotech digital imaging system. DNA cleavage was monitored by the conversion of covalently closed circular supercoiled plasmid DNA to nicked molecules.

The effects of anticancer drugs on topoisomerase I-mediated cleavage of positively or negatively supercoiled plasmid DNA were assessed in the presence of 0–10 μM camptothecin, topotecan, NSC 706744 or NSC725776, or 0–100 μM rebeccamycin. The effects of intercalators were assessed in the presence of 0–20 μM ethidium bromide, 0–20 μM TAS-103, 0–100 μM 9-aminoacridine, or 0–400 μM amsacrine.

Topoisomerase II. Topoisomerase II cleavage reactions were carried out using the procedure of Fortune and Osheroff (153). Topoisomerase II DNA cleavage assays contained 110 nM human topoisomerase IIα or 220 nM human topoisomerase IIβ and 10 nM negatively or positively supercoiled pBR322 in a total of 20 μL of topoisomerase II cleavage buffer (10 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol). Reactions were incubated for 6 min at 37 ºC, and processed as

in the above section. DNA cleavage was monitored by the conversion of covalently closed circular supercoiled plasmid DNA to nicked or linear molecules. DNA cleavage reactions were performed in the absence of ATP so that the topological state of the DNA would not change during the course of the reaction. It should be noted that the nucleotide cofactor does not influence the mechanism of topoisomerase II-mediated DNA scission.

Topoisomerase II assays were carried out in the absence of compound or in the presence of 0-100 μM etoposide, DEPT, TOP-53, or F14512. Some reactions also contained 0-1000 μM ciprofloxacin or 0–500 μM spermine.

Site-specific Cleavage of Circular DNA

Site-specific DNA cleavage reactions contained 7 nM negatively or positively supercoiled pBR322 DNA and 14 nM human topoisomerase I (in the absence or presence of 5 μM topotecan) in a total of 160 μL of topoisomerase I cleavage buffer. Samples were incubated for 2 min at 37 ºC, and enzyme-DNA cleavage complexes were trapped by the addition of 16 μL of 1% SDS followed by 8 μL 375 mM EDTA. Proteinase K (3 μL of a 4 mg/mL solution) was added, and mixtures were incubated for 30 min at 45 ºC to digest the topoisomerase I. Reaction products were purified by passage through Qiaquick spin columns (Qiagen) as described by the manufacturer. Plasmids were linearized by treatment with *Eco*RI and labeled with Klenow (exo-) in the presence of [α-^{32}P]dATP and [α-^{32}P]TTP. Samples were treated with *Hin*dIII, and the singly end-labeled 4,330 bp DNA fragment was purified by passage through a CHROMA SPIN + TE-10 column (Clontech). Reaction products were normalized for radioactivity. Equivalent counts were mixed with 5 μL of polyacrylamide gel loading buffer (80% formamide, 10

mM sodium hydroxide, 1 mM sodium EDTA, 0.1 % xylene cyanol, and 0.1% bromophenol blue), and subjected to electrophoresis in 6% polyacrylamide sequencing gels. Gels were dried *in vacuo*, and DNA cleavage products were visualized with a Bio-Rad Molecular Imager FX.

Site-specific Cleavage of Linear DNA

Topoisomerase I. To determine specific sites of topoisomerase I cleavage on a linear substrate, BR322 was linearized, labeled, and the 4,330 bp *Eco*RI-*Hin*dIII fragment was isolated as described in the preceding section. DNA cleavage reactions (20 μL) contained 4.4 nM labeled linear pBR322 DNA and 20 nM human topoisomerase I, in the absence of compounds or in the presence of topoisomerase I-targeted anticancer drugs (10 μM camptothecin or topotecan) or DNA intercalators (20 μM ethidium bromide, 20 μM TAS-103, 100 μM 9-aminoacridine, or 200 μM amsacrine). Reactions were incubated for 2 min at 37 ºC and enzyme-DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS followed by 1 μL of 375 mM EDTA, pH 8.0. Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and protein samples were incubated for 30 min at 45 ºC to digest the topoisomerase I. Reaction products were ethanol precipitated and resuspended in 6 μL of polyacrylamide gel loading buffer. Samples were subjected to polyacrylamide gel electrophoresis and analyzed as described in the preceding section.

Topoisomerase II. Linear DNA cleavage sites utilized by topoisomerase II were mapped using a modification (154) of the procedure of O'Reilly and Kreuzer (155). The pBR322 DNA substrate was linearized by treatment with *Hin*dIII. Terminal 5'-phosphate was removed by treatment with calf intestinal alkaline phosphatase and replaced with

[^{32}P]phosphate using T4 polynucleotide kinase and [γ-32P]ATP. The DNA was treated with *Eco*RI, and the 4332 bp singly end labeled fragment was purified from the small *Eco*RI-*Hin*dIII fragment by passage through a CHROMA SPIN+TE-100 column (Clontech). Reaction mixtures contained 0.7 nM labeled pBR322 DNA substrate and 110 nM human topoisomerase IIα or topoisomerase IIβ in 20 μL of DNA cleavage buffer supplemented with 0.5 mM ATP in the absence or presence of 1–5 μM F14512, 5–10 μM TOP–53, or 5–10 μM etoposide. Reaction mixtures were incubated at 37 °C for 6 min and processed and imaged as in the proceeding section.

Ligation of Cleaved DNA

Topoisomerase I. Topoisomerase I DNA cleavage-ligation equilibria were established for 2 min at 37 ºC in topoisomerase I cleavage buffer that contained 10 μM camptothecin or 5 μM topotecan, as described in the section on topoisomerase I-mediated cleavage of plasmid DNA. Ligation was initiated by the addition of NaCl to a final concentration of 300 mM and terminated from 5 to 45 s by the addition of 2 μL 5% SDS. Samples were processed and analyzed as described for plasmid DNA cleavage. The percent DNA cleavage at time 0 was set to 100%, and ligation was monitored by quantifying the loss of nicked DNA over time.

Topoisomerase II. DNA ligation mediated by topoisomerase IIα was monitored according to the procedure of Byl *et al*. (156). DNA cleavage-ligation equilibria were established for 6 min at 37 °C in the absence or presence of 10 μM F14512, 50 μM TOP53, or 50 μM etoposide. Ligation was initiated by shifting samples from 37 to 0 °C. Reactions were stopped at time points ranging from 0-30 s by the addition of 2 μL of 5%

SDS followed by 2 μL of 250 mM EDTA, pH 8.0. Samples were processed and analyzed as described under plasmid DNA cleavage. Linear DNA cleavage product at time zero was set to 100% and DNA ligation was monitored by the loss of linear DNA.

DNA Intercalation

Intercalation reaction mixtures contained 20 nM topoisomerase I and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of topoisomerase I relaxation buffer. These experiments also contained 0–10 μM ethidium bromide or TAS–103, 0–50 μM 9-aminoacridine, 0-100 μM amsacrine, or 0-100 μM F14512. Reactions containing 20 μM ethidium bromide or 100 μM etoposide were included as positive and negative controls, respectively. Mixtures were incubated at 37 °C for 10 min, extracted with a phenol/chloroform/isoamyl alcohol mixture (25:24:1), and added to 3 μL of 0.77% SDS and 77 mM EDTA (pH 8.0). Samples were mixed with 2 μL of agarose gel loading buffer, heated at 45 °C for 5 min, and subject to electrophoresis in a 1% agarose gel in 100 mM Tris-borate (pH 8.3) and 2 mM EDTA. Gels were stained with 1 μg/mL ethidium bromide, and DNA bands were visualized as described for plasmid DNA relaxation.

The DNA intercalation assay is based on the fact that intercalative agents induce constrained negative supercoils and compensatory unconstrained positive superhelical twists in covalently closed circular DNA (157,158). Therefore, as the concentration of an intercalative compound increases, a plasmid that is negatively supercoiled or relaxed (*i.e.*, contains no superhelical twists) appears to become positively supercoiled. Treatment of an intercalated plasmid with topoisomerase I or topoisomerase II removes the

unconstrained positive DNA superhelical twists. Subsequent extraction of the compound allows the local drug-induced unwinding to redistribute in a global manner and manifest itself as a net negative supercoiling of the plasmid. Thus, in the presence of an intercalative agent, topoisomerase treatment converts relaxed plasmids to negatively supercoiled molecules.

Formation of Topoisomerase I-DNA Cleavage Complexes in Cultured Human Cells

Human CEM leukemia cells were cultured under 5% CO_2 at 37 °C in RPMI 1640 medium (Cellgro by Mediatech, Inc) containing 10% heat-inactivated fetal calf serum (Hyclone). The *in vivo* complex of enzyme (ICE) bioassay (as modified on the TopoGen, Inc., website) was utilized to determine levels of topoisomerase I-DNA cleavage complexes formed in the presence of anticancer drugs and/or intercalative compounds. Exponentially growing cultures were treated in absence of compound or presence of 10 μM ethidium bromide, 5 μM topotecan, or 10 μM ethidium bromide + 5 μM topotecan for 1 h. Cells (~5 x 10^6) were harvested by centrifugation and lysed by the immediate addition of 3 mL of 1% sarkosyl. Following gentle homogenization in a Dounce homogenizer, cell lysates were layered onto a 2 mL cushion of CsCl (1.5 g/mL) and centrifuged at 45,000 rpm for 15 hr at 20 °C. DNA pellets were isolated, resuspended in 5 mM Tris-HCl, pH 8.0, and 0.5 mM EDTA, normalized for the amount of DNA present, and blotted onto nitrocellulose membranes using a Schleicher and Schuell slot blot apparatus. Covalent complexes formed between human topoisomerase I and DNA were detected using a polyclonal antibody directed against human topoisomerase I (Topogen) at a 1:3000 dilution.

STD [^{1}H] NMR Spectroscopy

NMR spectra were generated using conditions similar to those described previously (159,160). All NMR experiments were performed at 283 K using a Bruker Avance 700 MHz spectrometer equipped with a 5 mm cryoprobe with z gradients. NMR buffers contained 10 mM sodium phosphate (pH 7.7), 250 mM KCl, 0.1 mM Na_2EDTA, and 5 mM $MgCl_2$. Samples (400 μL) contained 5 μM human topoisomerase IIα and 500 μM F14512 and were maintained at 4 °C until data were collected. STD [^{1}H]-NMR experiments employed a pulse scheme similar to that reported by Mayer and Meyer (161). A 2 s saturation pulse was used for the saturation, with on- and off-resonance irradiation frequencies of 0.5 and 17 ppm, respectively. The water signal was suppressed using excitation sculpting with gradients. For each experiment (on- and off-resonance irradiation), 256 scans were collected with a 2 s recycle time. Difference spectra were prepared by subtracting the on-resonance spectrum from the off-resonance spectrum. Signals resulting in the difference spectrum represent the nuclear Overhauser enhancement (NOE) difference signals generated by the transfer of irradiation energy from the enzyme to the bound ligand. Ligand protons in close spatial proximity with the enzyme displayed larger NOE signals. Mapping of the NOE signals with their proton assignments on the ligand revealed the ligand-binding epitope to human topoisomerase IIα. Spectra were processed using Bruker Topspin software.

Persistence of DNA Cleavage Complexes

The persistence of topoisomerase II-DNA cleavage complexes was determined using a modification of the procedure of Bandele *et al*. (162). Initial reactions contained 50 nM

DNA, 550 nM topoisomerase IIα and 100 μM etoposide or 10 μM F14512 in a total of 20 μL of topoisomerase II cleavage buffer. Reactions were incubate at 37 ºC for 6 minutes then diluted 20 fold with 1x DNA cleavage buffer while remaining at 37 ºC. 20 μL of reaction mix was removed and stopped with 2 μL 5% SDS at the indicated times. DNA cleavage at time 0 was set to 100 % and persistence of cleavage complexes determined as the decay of linear product over time.

CHAPTER III

STRUCTURAL FEATURES OF TOPOISOMERASES REQUIRED FOR THE RECOGNITION OF DNA TOPOLOGY

Introduction

Historically, models have placed topoisomerase I ahead of nucleic acid tracking systems, acting to relieve positive torsional stress generated during replication and transcription. In contrast, models placed topoisomerase II behind the fork, acting to resolve DNA knots and tangles between newly replicated sister chromatids (11,14-17). Recently, however, studies have generated data that strongly suggests a role for human topoisomerase IIα ahead of replication forks (15,16,80). These studies also began to describe structural features of this enzyme that are important for its activity on positively supercoiled substrates. For example, it has been shown that topoisomerase II can compensate for the loss of topoisomerase I in *S. cerevisiae*, but the loss of both enzymes abruptly halts DNA synthesis (163,164). This finding indicates that the type II enzyme can assume the role of topoisomerase I, relaxing positive DNA supercoils ahead of the replication machinery. Additionally, topoisomerase IIα is associated with DNA replication forks in human cells (165). Finally, both isoforms of human topoisomerase II have been shown to recognize the geometry of DNA supercoils for cleavage (157). However, only topoisomerase IIα recognizes supercoil handedness during relaxation, removing positive supercoils >10 times faster than negative supercoils (79). A recent study has shown that the ability to preferentially relax positively supercoiled DNA is dependent on the C-terminal domain (CTD) of topoisomerase IIα. Results presented in this chapter indicate that almost the entire CTD is required for this preferential relaxation.

It is likely that the topological state of DNA is regulated during the critical replication and transcription processes by both topoisomerase I and topoisomerase II. The relative contribution of the individual enzymes to these processes is an area of active investigation. However, in order to provide a complete picture of what is occurring *in vivo*, we must have an understanding of the catalytic activity of these enzymes.

If topoisomerase I is acting ahead of replication forks to relieve accumulating positive supercoils, then it stands to reason that this enzyme would recognize and preferentially act on a positively supercoiled substrate. However, until recently, most studies have examined enzyme-mediated cleavage using negatively supercoiled DNA or linear substrates. Therefore, the ability of human topoisomerase I to relax and cleave positively supercoiled DNA was examined. Results indicate that topoisomerase I is able to distinguish between the handedness of DNA supercoils during both catalytic processes. Furthermore, the enzyme is shown to both preferentially relax and cleave overwound as compared to underwound substrates.

Results and Discussion

Structural Features of Human Topoisomerase IIα Required for Topology Recognition

Topoisomerase IIα and IIβ possess a high degree of amino acid sequence identity in their N-terminal ATPase and central DNA cleavage/ligation domains (~79% identity). However, the two enzymes diverge considerably in their CTDs (~31% identity) (14,16,45,166-168). To date, the only enzymological difference found between the two isoforms is the ability of topoisomerase IIα, but not topoisomerase IIβ, to preferentially

remove positive superhelical twists. Conversely, both enzymes maintain lower levels of DNA cleavage intermediates with positively supercoiled substrates (79,157). These findings suggest that type II topoisomerases discern DNA supercoil geometry in a bimodal fashion, using separate portions of the protein to distinguish nucleic acid handedness during different enzymatic processes.

Because topoisomerase IIα and IIβ differ most significantly in their CTDs, it was proposed that this portion of topoisomerase IIα is responsible for the ability of the enzyme to preferentially relax positively supercoiled DNA. Indeed, removal of the CTD of the α isoform rendered the enzyme unable to preferentially relax positively supercoiled substrates. Furthermore, when the CTD of topoisomerase IIβ was replaced with that of the α isoform, the resulting enzyme preferentially relaxed positively supercoiled substrates. In contrast, a chimeric topoisomerase IIα that carried the CTD of the β isoform lost its ability to recognize the geometry of DNA supercoils during relaxation (145). These findings demonstrate that the ability to preferentially relax positive DNA supercoils resides in the CTD of topoisomerase IIα. Experiments presented below were undertaken to determine which portions of the CTD of human topoisomerase IIα confer this ability.

Elements in the CTD of Human Topoisomerase IIα that Confer the Ability to Recognize Supercoil Geometry During DNA Relaxation

There currently is no information available for the three-dimensional structure of the CTD of human topoisomerase IIα. However, crystal structures for the CTDs of the two bacterial type II enzymes, DNA gyrase (GyrA) and topoisomerase IV (ParC), have been solved (169-172). The CTD of GyrA adopts a unique fold, called a β-pinwheel, which

forms a closed circular structure made up of six "blades" (170,172). The outer rim of each blade contains a cluster of positively charged amino acids that are believed to interact with DNA (170,172). The CTD of ParC adopts a "broken β-propeller" structure that is similar to the β-pinwheel of GyrA, but lacks ring closure (169,171). Depending on the bacterial species, the CTD of ParC is predicted to contain as few as three and as many as eight blades. Once again, the outer rims of the blades contain positively charged amino acids. The CTDs of GyrA and ParC both have been shown to bind and bend DNA (170).

Extensive efforts were made by previous laboratory members to model the CTD of human topoisomerase IIα based on the structures of the bacterial type II enzymes. Unfortunately, homology between the CTDs of the different topoisomerase II species was too low to generate a predicted structure with confidence. However, amino acid sequence alignments comparing the CTDs of topoisomerase IIα with those of GyrA and ParC revealed similarities. Like the bacterial enzymes, human topoisomerase IIα contains a series of positively charged amino acid clusters that could potentially interact with DNA. Ten such clusters were identified and are indicated with red bars in Figure 10.

As a first attempt to narrow down the region(s) of the topoisomerase IIα CTD that is responsible for the preferential relaxation of positively supercoiled DNA, a series of nested C-terminally deleted enzymes was generated. This series contained full-length topoisomerase IIα, a mutant truncated human enzyme that included all of the predicted clusters of positive amino acids (truncated following amino acid 1521, hTop2αΔ1521), and enzymes that lacked one (hTop2αΔ1501), two (hTop2αΔ1476), or six clusters

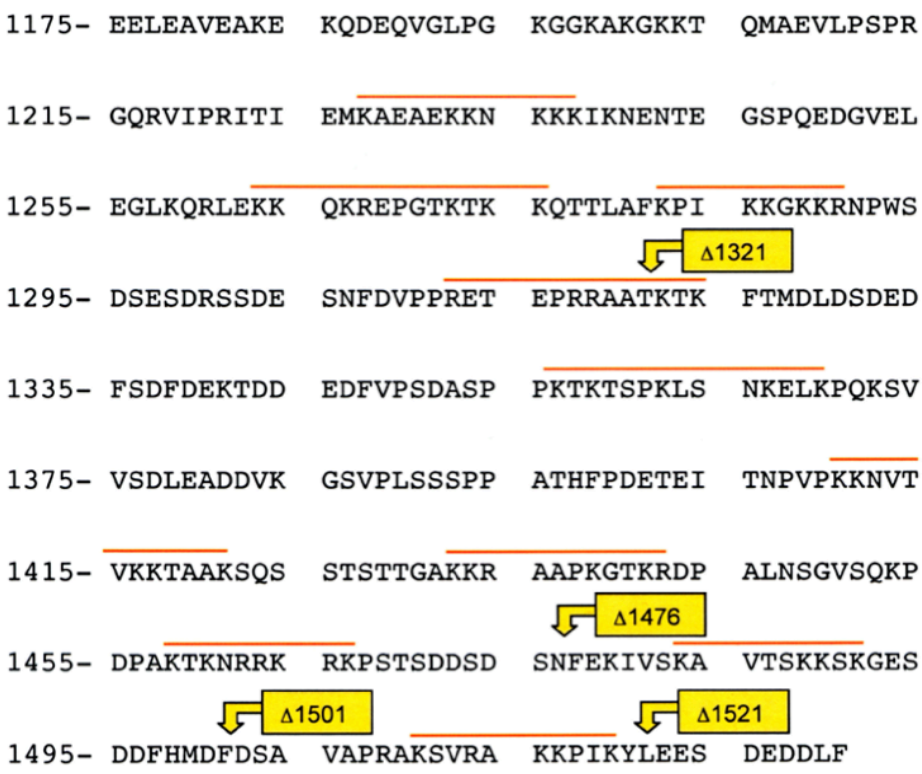


Figure 10. Sequence of the CTD of human topoisomerase IIα. Positively charged amino acid clusters corresponding to those of bacterial DNA gyrase and topoisomerase IV are demarcated by red bars. Points of C-terminal truncations in topoisomerase IIα mutant enzymes are indicated.

(hTop2αΔ1321), respectively. Time courses for the relaxation of negatively and positively supercoiled plasmids by wild–type topoisomerase IIα, these truncated enzymes, and hTop2αΔ1175 are shown in Figure 11.

The recognition of DNA geometry by hTop2αΔ1521 during relaxation was similar to that of the full-length enzyme. However, removal of even a single cluster of positively charged amino acids from the CTD abolished the ability of topoisomerase IIα to discern the handedness of its DNA substrate. Comparable rates of relaxation of negatively and positively supercoiled plasmids were observed for hTop2αΔ1501, hTop2αΔ1476, and hTop2αΔ1321. These findings imply a role for the positively charged amino acid clusters of the CTD of topoisomerase IIα in the recognition of DNA geometry during strand passage. They also suggest that the removal of even the most C-terminal cluster of positively charged residues has the potential to impact the ability of topoisomerase IIα to preferentially remove positive superhelical twists.

To further investigate the role of the C-terminal cluster of positive amino acids, a chimeric enzyme was constructed (hTop2αΔ1501/β) in which residues 1502-1531 of human topoisomerase IIα were replaced with the corresponding C-terminal region of topoisomerase IIβ (residues 1585-1621). As seen in Figure 12, the chimeric enzyme did not maintain the ability to discern the handedness of DNA supercoils during relaxation. These data show that the loss of preferential relaxation of positively supercoiled DNA with the removal of even one positively charged amino acid cluster is not due to a general loss of CTD size. Instead, this data provides additional evidence that the most C-terminal cluster of positive amino acids in topoisomerase IIα is required to preferentially relax positively supercoiled DNA substrates.

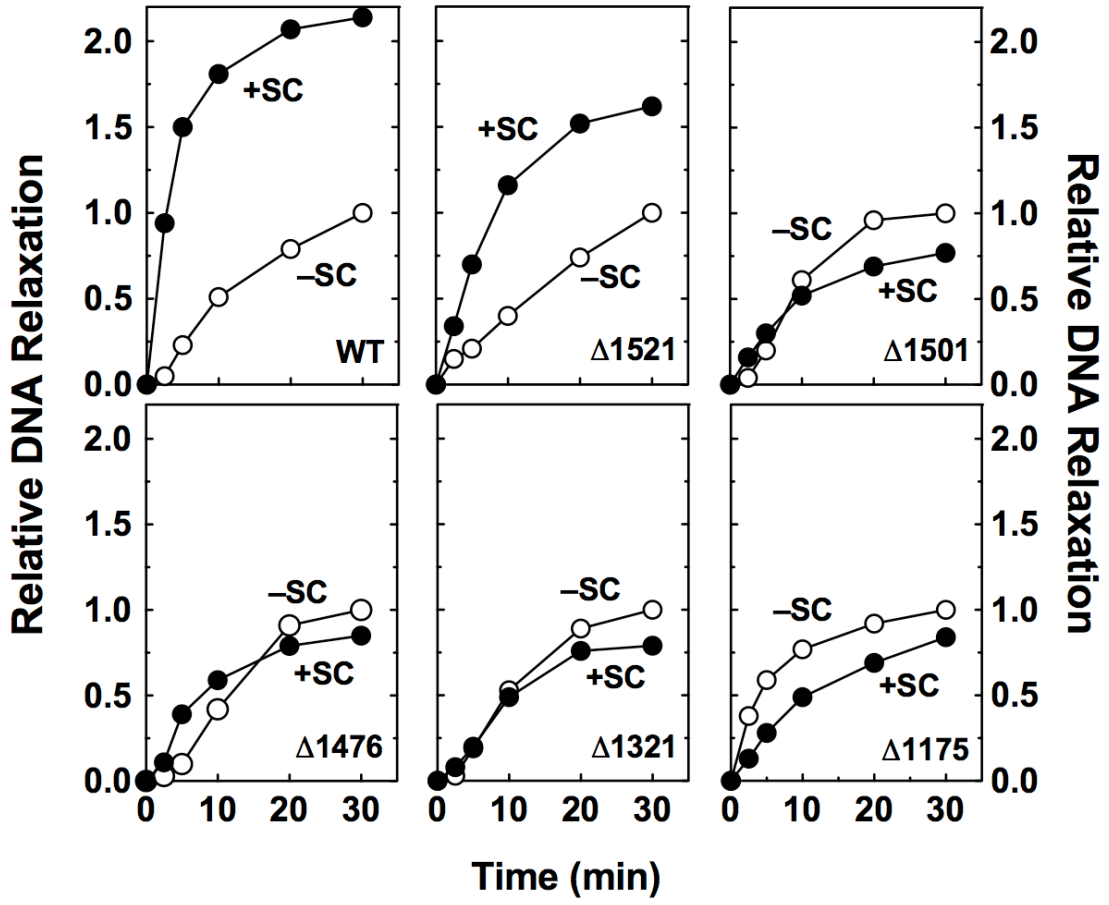


Figure 11. Time courses for relaxation of negatively and positively supercoiled substrates by C-terminally truncated topoisomerase IIα mutant enzymes. The ability of hTop2αΔ1521, hTop2αΔ1501, hTop2αΔ1476, and hTop2αΔ1321 (lacking zero, one, two and six clusters of positive charges, respectively) to relax negatively [(-)SC, open circles] and positively supercoiled substrates [(+)SC, closed circles] is shown. Results with full-length human topoisomerase IIα and hTop2αΔ1175 also are included. To aid comparisons, levels of DNA relaxation for negatively supercoiled substrates were normalized to 1.0 at 30 min.

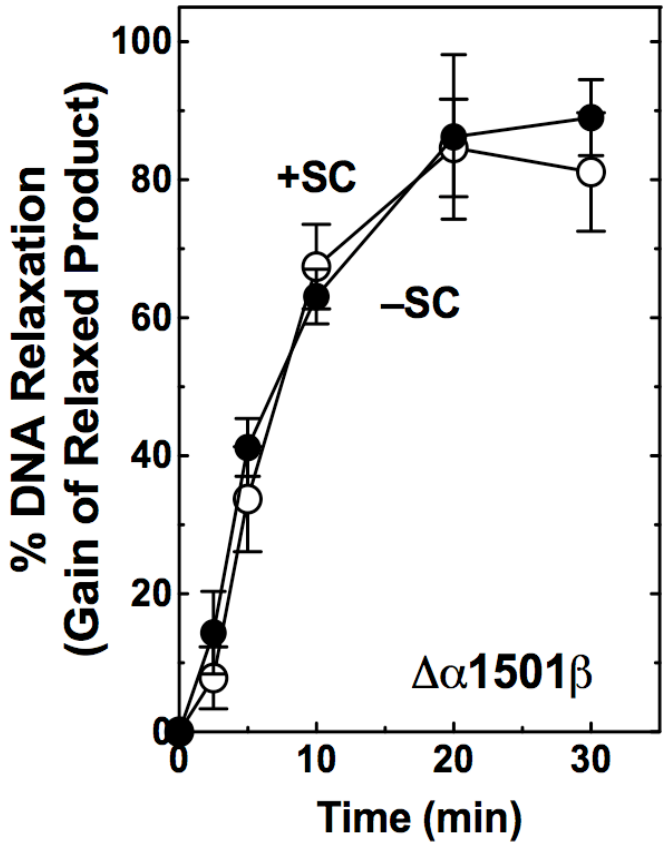


Figure 12. Time course for relaxation of negatively and positively supercoiled substrates by hTop2αΔ1501/β. hTop2αΔ1501/β is a chimeric enzyme in which residues 1502-1531 of human topoisomerase IIα were replaced with the corresponding C-terminal region of topoisomerase IIβ (residues 1585-1621)]. Ability of the chimeric enzyme to relax negatively [(-)SC, open circles] and positively supercoiled substrates [(+)SC, closed circles] is shown. Error bars represent the standard deviation of three independent assays.

It is notable that the overall rate of relaxation of positively supercoiled DNA dropped (~5– to 6–fold) as the length of the mutant enzymes decreased. This suggests that the presence of the CTD enhances the DNA relaxation activity of topoisomerase IIα with positively supercoiled substrates. In some cases (hTop2αΔ1501, hTop2αΔ1476, and hTop2αΔ1321), however, mutant enzymes also displayed a modest (~2–fold) increase in the rate of relaxation of negatively supercoiled plasmids or the decatenation of kDNA (data not shown). Whether this apparent increase in activity reflects a negative regulatory function of the CTD in the relaxation of negatively supercoiled substrates or a slight variation in the level of active enzyme in the topoisomerase IIα preparations is not known at the present time.

The Ability of Human Topoisomerase I to Distinguish the Handedness of DNA Supercoils

As discussed above, topoisomerase I is thought to act ahead of replication forks and transcription complexes to relax positively supercoiled substrates. While computer simulations have examined the relaxation mechanism of human topoisomerase I on under- and overwound substrates (26), few studies have examined the catalytic activity of the enzyme on its purported physiological substrate. Thus *in vitro* studies were performed to characterize the ability of this critical cellular enzyme to relax and cleave positively supercoiled DNA.

Topoisomerase I Preferentially Relaxes Positively Supercoiled Substrates

Similar to topoisomerase IIα, topoisomerase I was found to relax positively supercoiled DNA more rapidly than negatively supercoiled DNA (Figure 13). This result

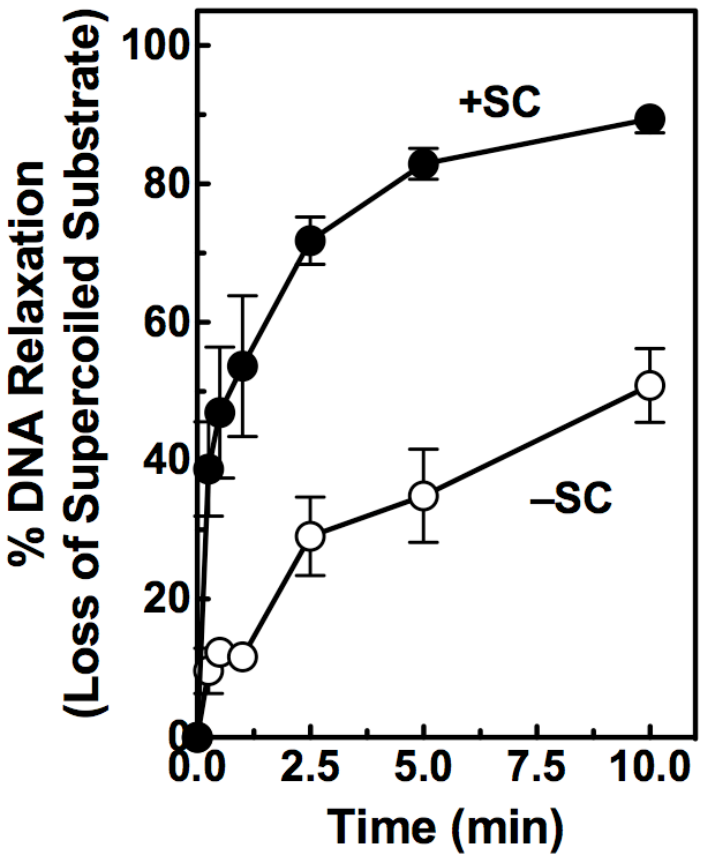


Figure 13. Time course for relaxation of negatively and positively supercoiled substrates by human topoisomerase I. Ability of the enzyme to relax negatively [(-)SC, open circles] and positively supercoiled substrates [(+)SC, closed circles] is shown. Supercoil relaxation is quantified by the loss of the initial supercoiled substrate. Error bars represent the standard deviation of three independent assays.

supports the current model of topoisomerase I cellular action and is not unexpected of an enzyme that is proposed to act ahead of replication and transcription machinery. In collaboration with Birgitta Knudsen's group in Aarhus Denmark, this preferential relaxation of positively supercoiled DNA by human topoisomerase I was examined in more detail (146). Consistent with the results shown in Figure 13, human topoisomerase I was found to relax positively supercoiled substrates 20-50 times more rapidly than negatively supercoiled substrates under processive conditions (*i.e.,* low salt) and 3-10 times faster under distributive conditions (*i.e.* high salt). Additionally, this effect could not be attributed to preferential binding of the enzyme to positively supercoiled substrates, as no significant difference was observed in experiments that measured the ability of negatively and positively supercoiled DNA substrates to compete with a radiolabeled linear substrate for binding to an active site topoisomerase I mutant.

Topoisomerase I carries out supercoil relaxation by a controlled rotation mechanism. Recent single molecule studies suggest that the enzyme relaxes supercoiled DNA in a stepwise torque-dependent manner, in which relaxation occurs by rotation of the free 5'-hydroxyl DNA end around the intact DNA strand (25). A molecular dynamics simulation study of topoisomerase I by Sari and Andricioaei proposed that the mechanism of controlled rotation differs depending on the handedness of the DNA supercoils in the substrate, in part because the direction of rotation of the DNA within the enzyme core differs for negatively and positively supercoiled substrates (see Chapter I, Figure 4) (26). They suggest that relaxation of negatively supercoiled DNA involves stretching of the hinge region of the protein with the assistance of residues in the N-terminal domain,

while relaxation of positively supercoiled DNA involves a separation of the lips of the enzyme (26).

Previous studies have shown the importance of the N-terminal domain of topoisomerase I in controlling DNA relaxation of negatively supercoiled DNA (173). While the relaxation of negatively supercoiled DNA by wild-type human topoisomerase I is inhibited by the anti-cancer compound camptothecin, relaxation of similar substrates by topoisomerase I enzymes containing N-terminal truncations was unaffected by the drug.

Interestingly, in the collaborative study, topoisomerase I enzymes that have N-terminal truncations exhibited 40– to 60– fold inhibition of positively supercoiled DNA relaxation in the presence of camptothecin (146). These results provide strong evidence that the mechanism of controlled rotation relaxation of supercoiled DNA by human topoisomerase I does in fact differ depending on the handedness of the supercoiled substrate and that regions of the N-terminal domain are important in the control of removal of negative supercoils by the enzyme.

Topoisomerase I Preferentially Cleaves Positively Supercoiled DNA

As discussed above, topoisomerase cleavage complexes formed ahead of DNA tracking systems (*i.e.*, on positively supercoiled portions of the genome) are those most likely to be converted to permanent strand breaks (11,15,27,28,174-176). Given the importance of the topoisomerase I-DNA cleavage reaction to the physiological and pharmacological functions of the enzyme, the ability of human topoisomerase I to cleave positively supercoiled substrates was examined.

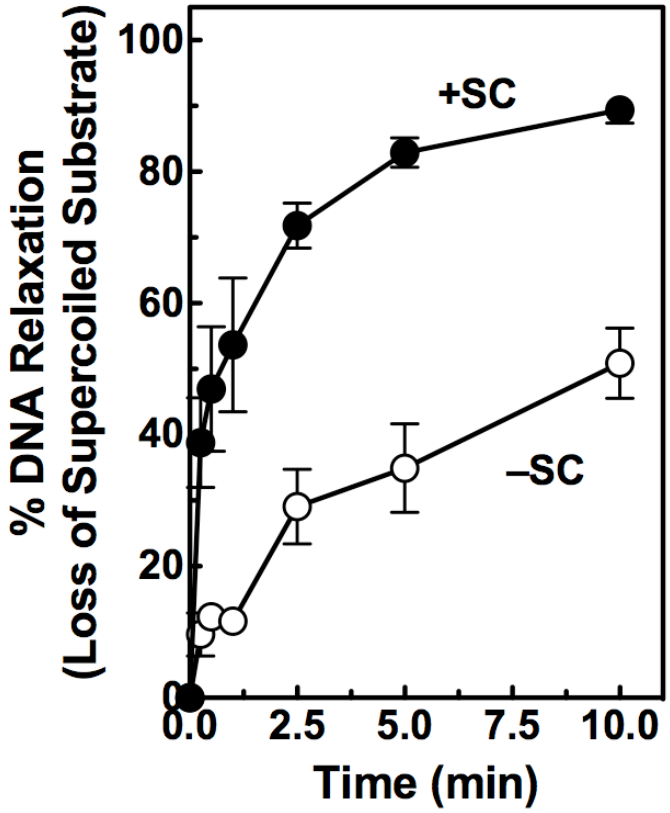


Figure 14. Topoisomerase I maintains higher levels of cleavage complexes with positively supercoiled DNA. The ability of increasing concentrations of human topoisomerase I to cleave positively [(+)SC, closed circles] and negatively [(–)SC, open circles] supercoiled pBR322 plasmid DNA is shown. Error bars represent the standard deviation of at least three independent experiments.

As a first step in characterizing the cleavage activity of human topoisomerase I, enzyme-mediated DNA cleavage of overwound and underwound DNA was assessed over a broad enzyme concentration range (~3–45 nM). As seen in Figure 14, topoisomerase I demonstrated the ability to discern the geometry of DNA over the entire concentration range, maintaining a concentration of cleavage complexes that was ~3 times greater with substrates that contained positive as compared to negative supercoils.

Conclusions

Topoisomerase IIα preferentially removes positive superhelical twists, and the ability of the enzyme to distinguish DNA supercoil geometry during relaxation is embedded in the CTD of the enzyme. Though no structural data is available for the CTD, sequence alignment with bacterial type II enzymes reveals 10 clusters of positively charged amino acids that are similar to those that fold into DNA-interacting blades in gyrase and topoisomerase IV. Results show that removal of even one of these positively charged clusters attenuates the ability of topoisomerase IIα to preferentially relax positively supercoiled substrates.

Experiments described in this chapter also show that human topoisomerase I relaxes positively supercoiled substrates more rapidly than negatively supercoiled substrates. However, this enzyme also maintains ~3– to 4– fold higher concentrations of cleavage complexes on positively supercoiled DNA. Taken together, these findings strongly suggest that human topoisomerase I is an enzyme that is designed to act primarily on overwound substrates.

However, because topoisomerase I maintains higher concentrations of cleavage complexes on positively supercoiled DNA, this is an inherently dangerous enzyme to have ahead of replication forks, as stabilization of topoisomerase I-cleavage complexes on this substrate are more likely to result in collisions with DNA tracking systems that convert transient cleavage complexes to permanent strand breaks. Therefore, the effects of DNA supercoil handedness on the actions of topoisomerase I-targeted anti-cancer drugs will be explored in the following chapter.

CHAPTER IV

THE GEOMETRY OF DNA SUPERCOILS AFFECTS THE ACTIVITY OF TOPOISOMERASE–TARGETED AGENTS

Introduction

Beyond their important cellular functions, human topoisomerase I and topoisomerase II are targets for a number of highly effective anticancer agents that act at the enzyme-DNA interface, stabilizing topoisomerase-DNA cleavage complexes (20,27,28,80,108,123,167). The accumulation of these cleavage complexes ahead of DNA tracking systems is believed to kill cells by several different mechanisms. First, the presence of cleavage complexes or accumulating positive supercoils ahead of the replication or transcription machinery impairs these essential cellular functions. Second, the presence of blocked replication forks induces replication re-start pathways that generate DNA strand breaks. Third, collisions between DNA tracking systems and covalent topoisomerase roadblocks convert transient cleavage complexes to permanent DNA strand breaks (20,27,28,80,108,123,167,176).

As discussed in Chapter III, type II topoisomerases maintain lower levels (~2– to 4–fold) of cleavage complexes with positively supercoiled as opposed to negatively supercoiled DNA (79,145,157). While this feature makes the enzyme safer to function ahead of replication and transcription complexes, it may make topoisomerase II less sensitive to the actions of anticancer drugs. In contrast, human topoisomerase I maintains higher levels of cleavage complexes with positively supercoiled substrates (146,157). This property makes the type I enzyme a potentially better target for therapeutic agents.

However, it also suggests that topoisomerase I is intrinsically more dangerous to the cell than the type II enzyme.

Because of the fundamental role that topoisomerase I plays in a number of critical nuclear processes and in the treatment of human malignancies, it is important to more fully characterize the actions of anticancer drugs against topoisomerase I on a positively supercoiled substrate. Thus, results in this chapter will show that topoisomerase I maintains higher levels of cleavage complexes with positively as opposed to negatively supercoiled substrates in the presence of several classes of topoisomerase I poisons. Furthermore, this effect correlates with a decreased rate of ligation with overwound DNA. Finally, the effects of intercalative compounds on the actions of both topoisomerase I and topoisomerase II on under- and overwound DNA will be discussed. Results show that intercalating agents that make covalently closed DNA appear to be positively supercoiled enhance topoisomerase I-mediated DNA cleavage *in vitro* and in cultured human cells.

Results and Discussion

Effect of DNA Supercoil Geometry on Cleavage Mediated by Human Topoisomerase I in the Presence of Camptothecin-based Anticancer Drugs

Camptothecin-based compounds have been in clinical use for over a decade (27,28). Topotecan is approved to treat ovarian, cervical and small cell lung cancers that are refractory to other courses of treatment. Irinotecan, a prodrug converted to its active metabolite *in vivo* by carboxylesterase, is used to treat colorectal cancers. Belotecan is used to treat ovarian cancer. A third camptothecin derivative, gimatecan, is in clinical

trials and shows some activity in treating gliomas (27,28,108). However, the activity of these compounds has not been examined in depth on their proposed primary physiological substrate. Therefore, as a first step in characterizing the actions of topoisomerase I poisons in the context of positively supercoiled DNA, the ability of camptothecin and topotecan to poison human topoisomerase I was determined on underwound and overwound substrates.

As seen in Figure 15, higher levels of cleavage were observed with camptothecin and topotecan and positively supercoiled DNA. In both cases, ~3–fold more drug-induced scission was observed with positively supercoiled (as compared to negatively supercoiled) substrates over the range of camptothecin and topotecan concentrations examined. Because this level of enhancement is similar to that seen in the absence of topoisomerase I poisons, it is proposed that increased drug efficacy on overwound DNA is due primarily to an increase in baseline levels of cleavage rather than an altered drug interaction in the enzyme-DNA complex.

Mechanistic Basis for Increased Topoisomerase I-mediated DNA Cleavage of Positively Supercoiled Substrates

Although topoisomerase I preferentially relaxes and cleaves overwound molecules, it binds positively and negatively supercoiled DNA with similar affinities (146). Therefore, the enhanced cleavage of overwound molecules must result from a different aspect of the enzyme-DNA interaction. One possibility is that topoisomerase I cleaves a broader selection of sites in overwound DNA. Therefore, sites of enzyme-mediated scission were mapped in positively and negatively supercoiled substrates. Mapping in the absence of drugs is difficult due to the low level of cleavage. Consequently, topotecan was included

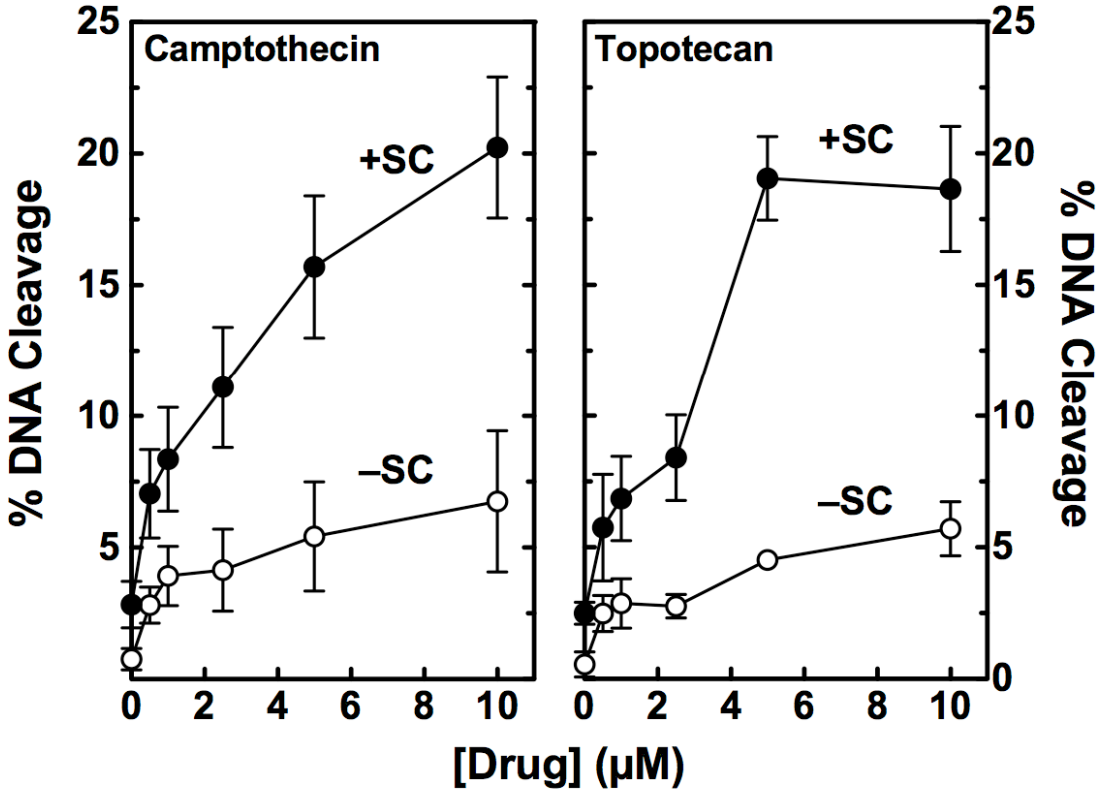


Figure 15. Effects of DNA supercoil handedness on topoisomerase I-mediated DNA cleavage in the presence of anticancer drugs. The ability of topoisomerase I to cleave positively [(+)SC, closed circles] and negatively [(–)SC, open circles] supercoiled pBR322 plasmid DNA in the presence of 0–10 μM camptothecin (left panel) or topotecan (right panel) is shown. Error bars represent the standard deviation of four independent experiments.

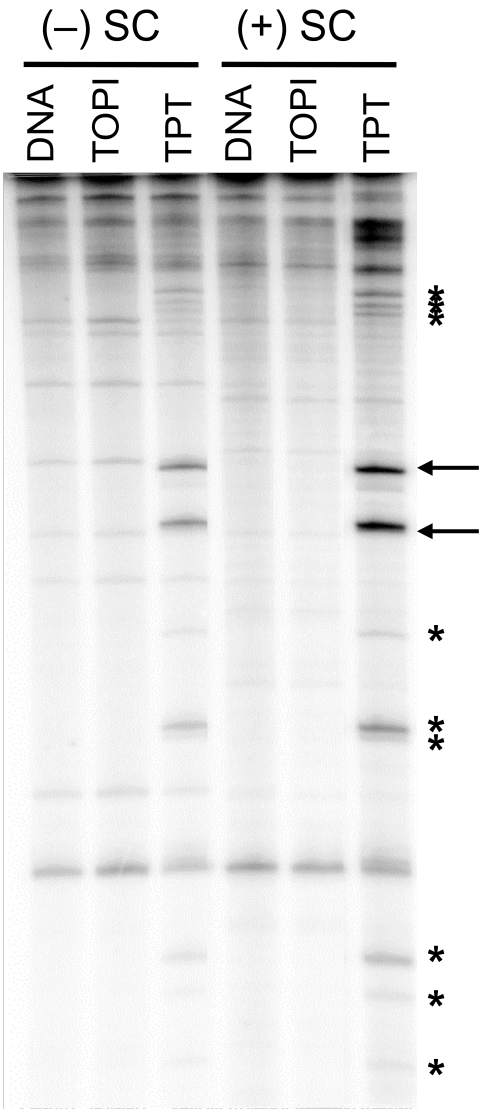


Figure 16. Effects of DNA supercoil handedness on sites of topoisomerase I-mediated DNA cleavage. DNA sites cleaved by topoisomerase I were mapped in negatively [(–)SC] and positively [(+)SC] supercoiled pBR322 plasmid DNA in the absence (TOP1) or presence (TPT) of 5 μM topotecan. Untreated DNA is shown as a control (DNA). Major and minor sites of cleavage are denoted by arrows and asterisks, respectively.

in experiments to increase the overall level of scission. Four to five major and several minor sites of cleavage were observed in the presence of the anticancer drug (Figure 16). In general, corresponding sites were observed in both substrates, but levels of scission were higher when positively supercoiled plasmid was used. Thus, differences in site specificity probably are not the major cause for the enhanced cleavage with overwound substrates.

A second possibility is that topoisomerase I maintains higher concentrations of cleavage complexes with overwound molecules because the enzyme ligates these substrates more slowly. In the absence of drugs, rates of enzyme-mediated ligation are too quick to monitor at the bench. Therefore, camptothecin or topotecan (both of which reduce the rate of ligation) were included in assays. In the presence of either drug, topoisomerase I ligated positively supercoiled plasmids more slowly than it did negatively supercoiled DNA (Figure 17). Relative rates of ligation (based on calculated apparent first order constants) for positively vs. negatively supercoiled DNA were ~2.6– and 7.0–fold slower in reactions that contained camptothecin and topotecan, respectively. On the basis of this finding, it is proposed that topoisomerase I maintains higher levels of cleavage complexes with overwound substrates (at least in part) because it ligates them more slowly than it does underwound DNA.

Effects of DNA Supercoil Geometry on Cleavage Mediated by Human Topoisomerase I in the Presence of Non-camptothecin Poisons

Camptothecin-based compounds have several limitations, including severe side effects and spontaneous inactivation at physiological pH by opening of the E-ring of the compounds (27,28,112). Because of these limitations and because drugs with common

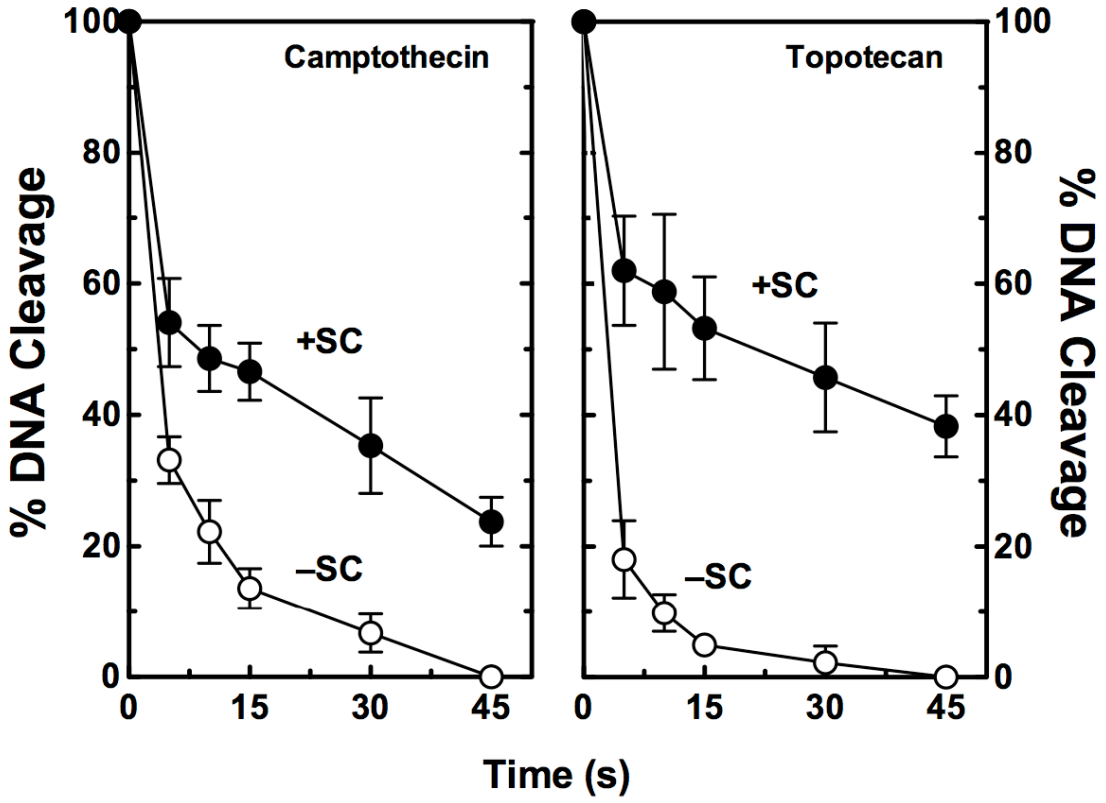


Figure 17. Effects of DNA supercoil handedness on topoisomerase I-mediated DNA ligation in the presence of anticancer drugs. The ability of topoisomerase I to ligate positively [(+)SC, closed circles] and negatively [(–)SC, open circles] supercoiled pBR322 plasmid DNA was monitored in the presence of 10 μM camptothecin (left panel) or 5 μM topotecan (right panel). DNA ligation was initiated by the addition of 300 mM NaCl. Levels of cleavage at time zero were set to 100%. Error bars represent the standard deviation of three independent experiments.

primary cellular targets often display different clinical activities, several classes of novel non-camptothecin topoisomerase I poisons currently are being developed (27,28,108) (Figure 18). Three classes of compounds thus far have clinical potential. The indolocarbazoles are based on naturally occurring alkaloids and have undergone early phase clinical trials (27,120). The phenanthridines are expected to enter clinical trials in the near future (27). Finally, several idenoisoquinolines, a class of drugs discovered as novel topoisomerase I inhibitors by the Developmental Therapeutics Program at the National Cancer Institute, have been selected for clinical development (27,177,178).

To examine the activity of non-camptothecin based topoisomerase I poisons on positively supercoiled DNA, three compounds were utilized. The first, rebeccamycin, is an indolocarbazole antitumor antibiotic (120). This drug has been shown to poison topoisomerase I *in vitro* and in cells. Moreover, yeast that display reduced sensitivity to camptothecin maintain sensitivity to rebeccamycin (179). As seen in Figure 19, though not as potent a poison as camptothecin or topotecan, rebeccamycin induced 3– to 4–fold higher concentrations of cleavage complexes with overwound substrates.

Two idenoisoquinolines, NSC706744 and NSC725776, also were examined. This class of topoisomerase I poisons display several characteristics that make them favorable candidates for clinical development. First, they do not display the chemical instability seen with camptothecin-based compounds. Second, they display different site selectivity than camptothecins. Finally, they are not substrates of ABC membrane transporters, which suggests that the idenoisoquinolines may not display drug resistance in the same instances as camptothecins (28). As seen in Figure 20, both idenoisoquinoline compounds displayed activity similar to that of topotecan, with NSC706744 being a more

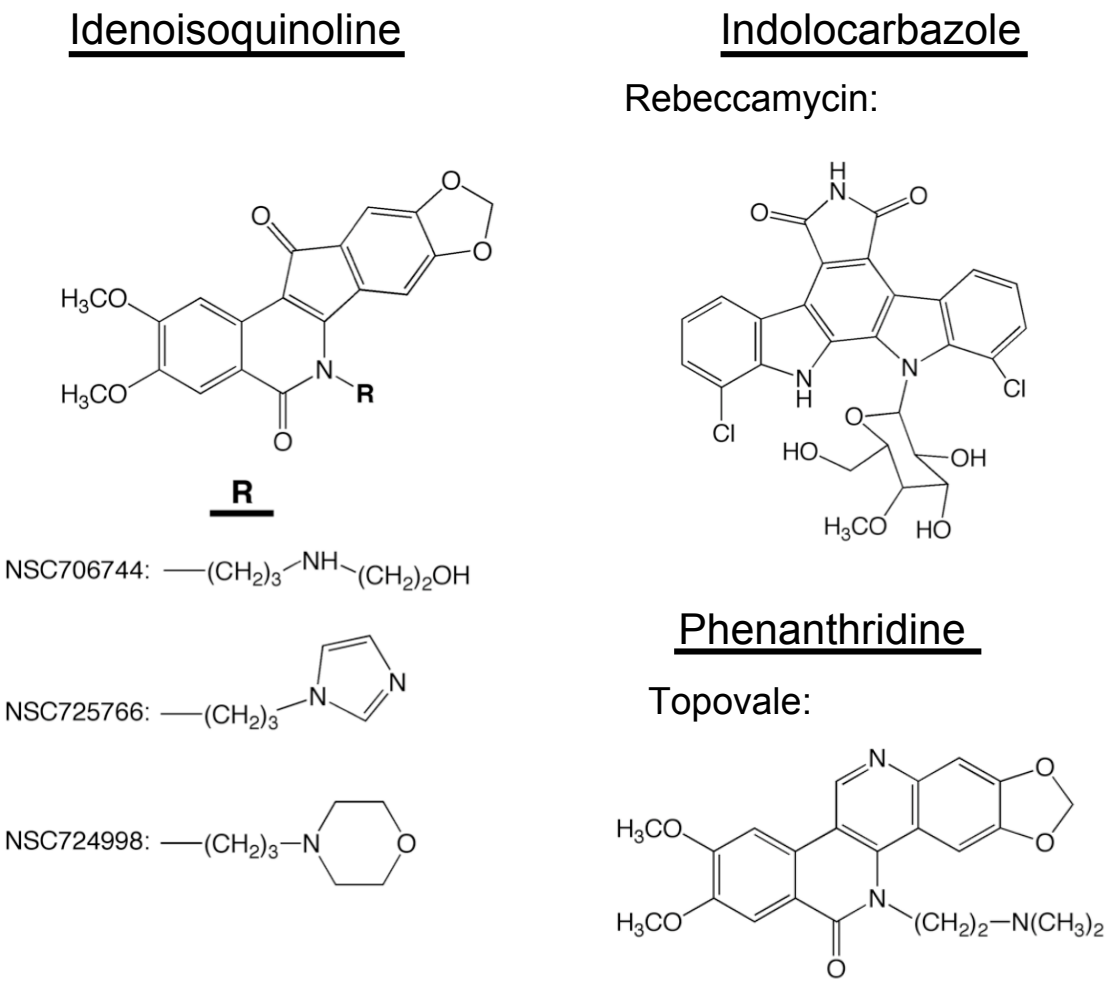


Figure 18. Non-camptothecin–based topoisomerase I targeted agents. Representatives of three classes of drug compounds that target topoisomerase I and have clinical potential are shown.

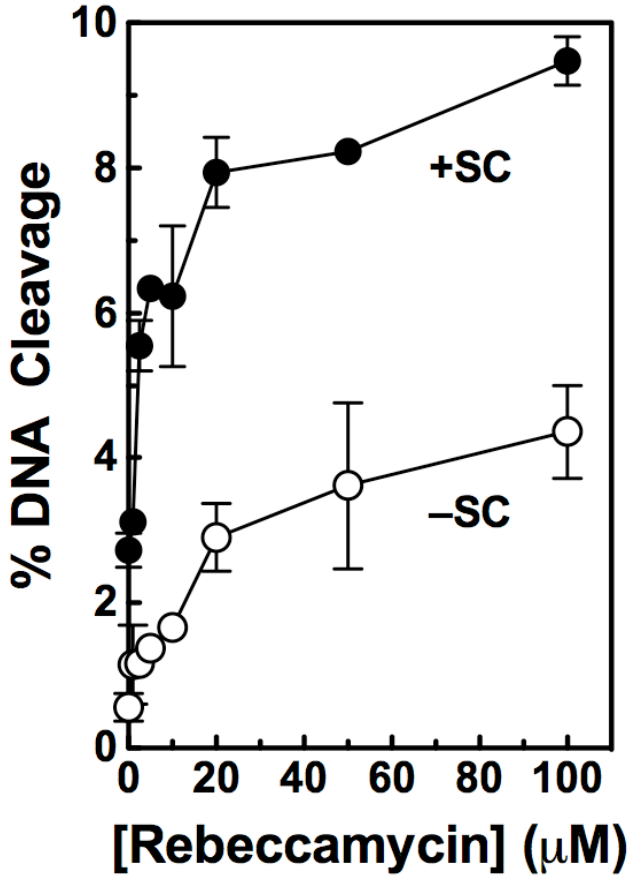


Figure 19. Effects of DNA supercoil handedness on topoisomerase I-mediated DNA cleavage in the presence of the indolocarbazole rebeccamycin. The ability of topoisomerase I to cleave positively [(+)SC, closed circles] and negatively [(–)SC, open circles] supercoiled pBR322 plasmid DNA in the presence of 0–100 μM rebeccamycin is shown. Error bars represent the standard deviation of three independent experiments.

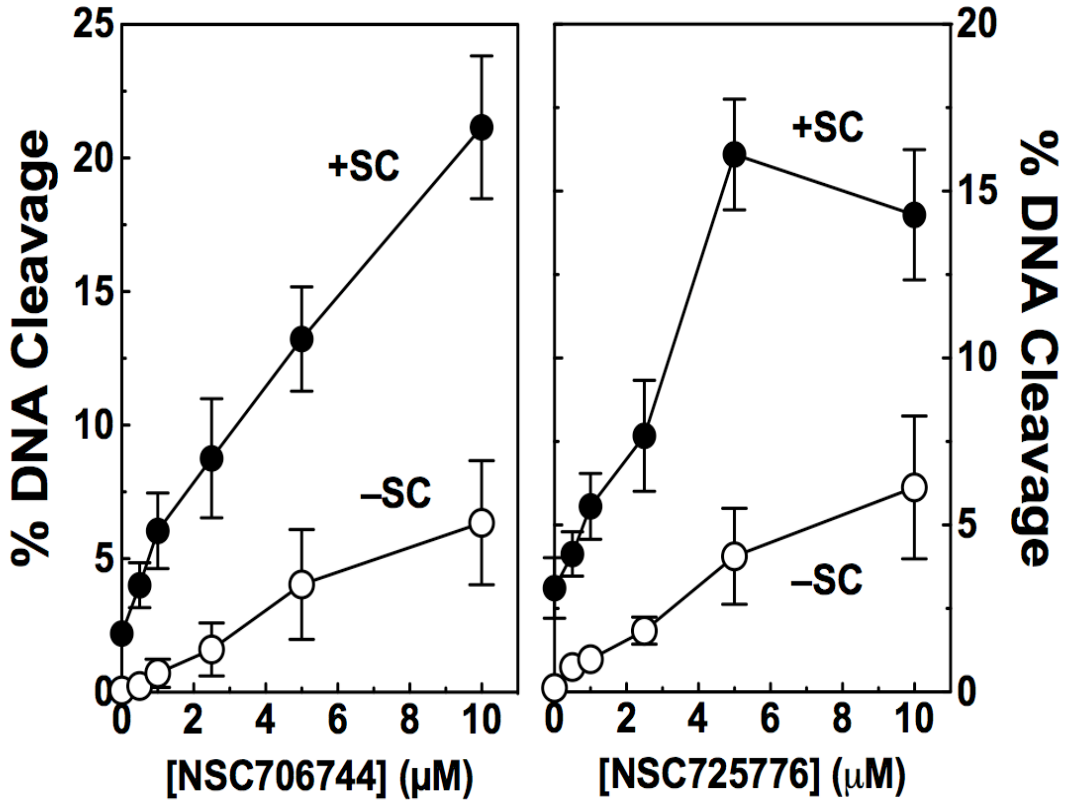


Figure 20. Effects of DNA supercoil handedness on topoisomerase I-mediated DNA cleavage in the presence of idenoisoquinolines. The ability of topoisomerase I to cleave positively [(+)SC, closed circles] and negatively [(–)SC, open circles] supercoiled pBR322 plasmid DNA in the presence of 0–10 μM NSC706744 (left panel) and NSC725776 (right panel) is shown. Error bars represent the standard deviation of at least three independent experiments.

potent poison of topoisomerase I than NSC725776. The drugs also maintained 3– to 4–fold higher levels of topoisomerase I-cleavage complexes on positively supercoiled substrates. As with the camptothecins, it is proposed that increased drug efficacy on overwound DNA in the presence of rebeccamycin and the idenoisoquinoline compounds is due primarily to an increase in baseline levels of cleavage rather than an altered drug interaction in the enzyme-DNA complex.

Effects of DNA Intercalators on Underwound and Overwound DNA

In contrast to topoisomerase I, topoisomerase II maintains lower concentrations of cleavage complexes with positively supercoiled substrates both in the absence of drugs and in the presence of classic non-intercalative poisons such as etoposide. However, this attribute does not hold in the presence of compounds that intercalate into DNA (157). The binding of intercalators to DNA locally opens (*i.e.*, underwinds) the double helix (157,180,181). In a covalently closed plasmid, this local underwinding is balanced by a compensatory global overwinding of the unconstrained (*i.e.*, unbound) DNA. Thus, even though the overall topological state of the plasmid has not changed, the presence of intercalative agents make the DNA available to the enzyme appear to be positively supercoiled (Figure 21).

Ethidium bromide, a strong DNA intercalator that does not enhance topoisomerase II-mediated cleavage, inhibits the ability of the type II enzyme to cleave negatively supercoiled DNA ~6–fold and positively supercoiled DNA ~2–fold. Interestingly, concentrations of cleavage complexes on negatively and positively supercoiled substrates are virtually identical at concentrations of ethidium bromide at which maximum DNA

intercalation has occurred (157). However, intercalative topoisomerase II poisons have a markedly different effect on enzyme-mediated DNA cleavage. In the presence of intercalative poisons topoisomerase II-mediated cleavage of negatively supercoiled substrates increases at low drug concentrations then drops as full drug intercalation is reached. In contrast, cleavage of positively supercoiled substrates increases with drug concentration and plateaus at concentrations of full drug intercalation (157).

The above results are thought to result from two effects on DNA. First, as drug intercalates into the DNA, the overall topological state of the substrate appears to be more positively supercoiled. The drop in cleavage levels seen with intercalative compounds on negatively supercoiled substrates can be explained by the lower baseline levels of DNA scission that topoisomerase II maintains on overwound substrates (79,157). Additionally, drug accumulation in the double helix could be inhibiting enzyme binding and catalysis. This does not, however, explain the higher levels of scission generated in the presences of intercalative poisons on overwound substrates. Positively supercoiled plasmids are under positive torsional stress even in the absence of intercalator. Thus, it is likely that overwound molecules cannot bind as many intercalative compounds as underwound DNA. Therefore, topoisomerase II activity on positively supercoiled substrates is less likely to be inhibited by the accumulation of intercalative compounds in the genetic material.

To test the theory that overwound molecules bind lower levels of intercalative compounds compared to underwound molecules, the effects of ethidium bromide binding into positively and negatively supercoiled DNA were determined. Briefly, increasing concentrations of under- and overwound plasmids were subjected to electrophoresis on

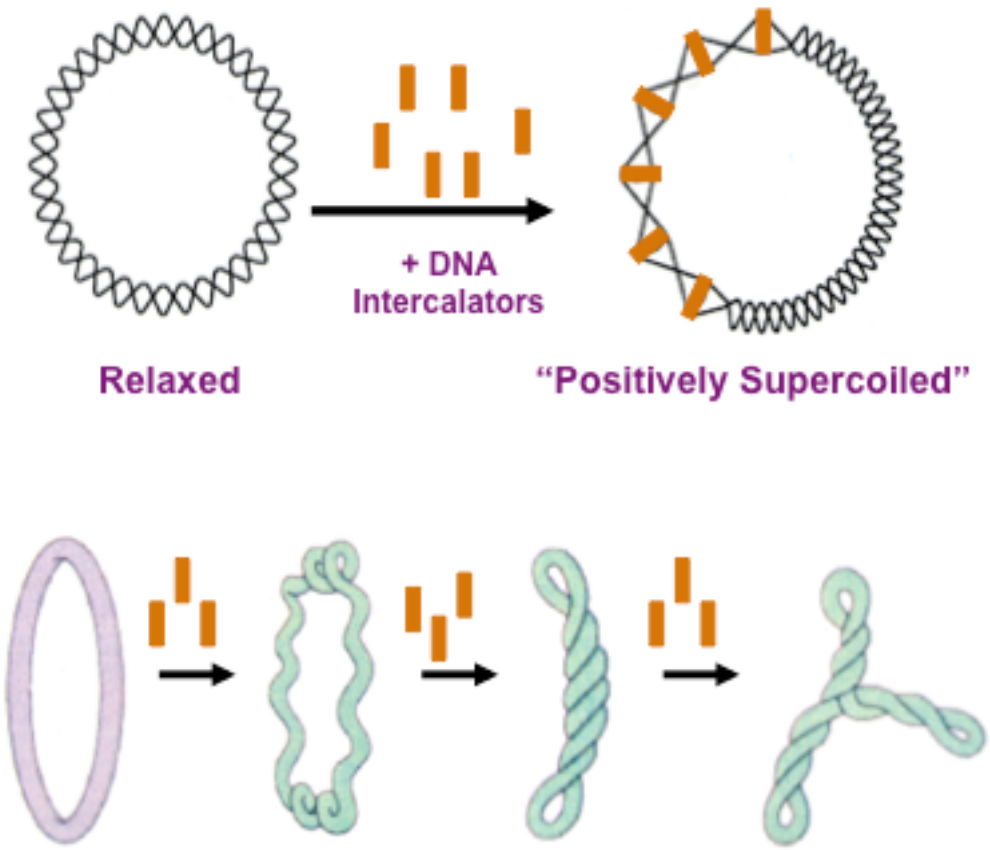


Figure 21. Intercalative compounds effect the appearance of covalently closed DNA. The binding of intercalators to DNA locally opens (*i.e.*, underwinds) the double helix. In a covalently closed plasmid, this local underwinding is balanced by a compensatory global overwinding of the unconstrained (*i.e.*, unbound) DNA.

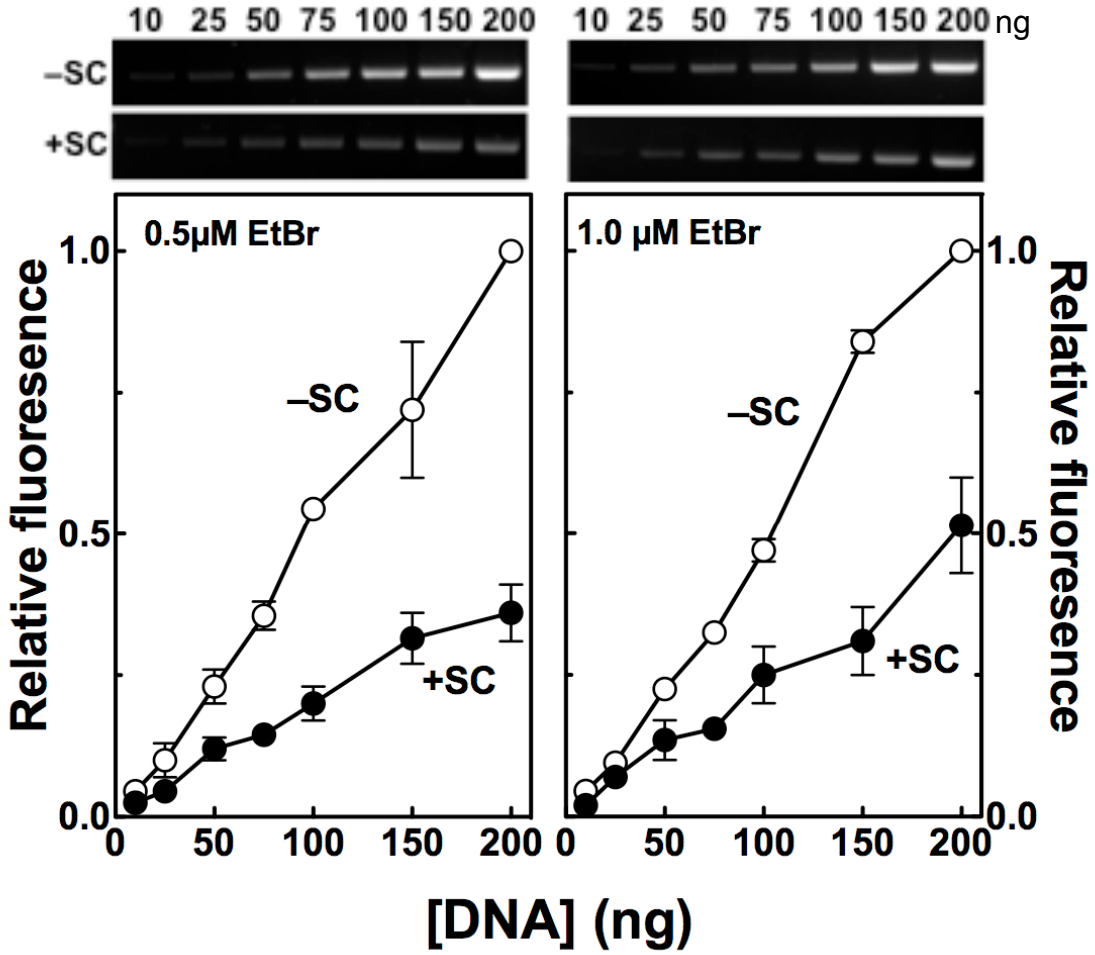


Figure 22. Overwound plasmids bind less ethidium bromide than underwound. The ability of 10-200 ng of negatively [(–)SC, open circles] or positively [(+)SC, closed circles] supercoiled plasmids to bind 0.5 μM or 1 μM ethidium bromide was determined. Concentration of plasmid DNA was confirmed by spectrophotometric analysis. Top, representative agarose gels. Bottom, quantification of three independent assays. To aid comparisons, levels of fluorescence were normalized to that seen with 200 ng (–)SC DNA.

agarose gels containing increasing concentrations of ethidium bromide. As can be seen in Figure 22, negatively supercoiled plasmids exhibited higher amounts of fluorescence than did positively supercoiled. Indeed, overwound molecules displayed 2– to 3–fold lower relative fluorescence than did underwound substrates. There is no evidence that the handedness of DNA supercoils affects the intrinsic fluorescence properties of ethidium bromide. Therefore, this set of experiments serves as proof of principle that differences in levels of fluorescence between the two substrates can best be interpreted as a decreased binding of ethidium bromide in positively supercoiled plasmids. A decreased binding of intercalative compounds to positively supercoiled DNA could expand the effective concentration range over which intercalative topoisomerase II poisons enhance cleavage complex formation.

DNA Intercalators as Topological Poisons of Topoisomerase I

Because intercalators make DNA available to topoisomerases appear to be positively supercoiled, and topoisomerase I prefers a positively supercoiled substrate, it was hypothesized that an intercalative topoisomerase I poison might have enhanced activity compared to a non-intercalative poison. However, to date there are no known compounds that both intercalate into DNA and poison human topoisomerase I in a practical concentration range. Therefore, the effects of several general DNA intercalators on topoisomerase I cleavage were determined. Four different intercalators, ethidium bromide, 9-aminoacridine, TAS-103, and amsacrine, were employed. Significant intercalation was observed over the concentration ranges employed (see Figure 23, insets).

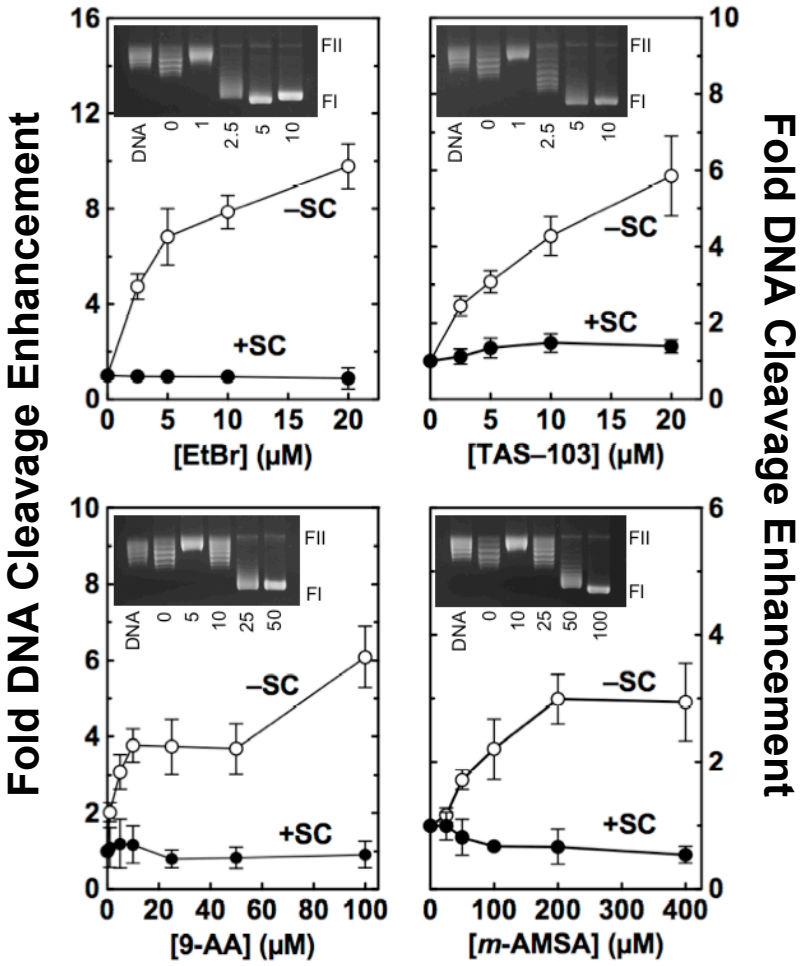


Figure 23. Effects of DNA intercalators on topoisomerase I-mediated cleavage of plasmid DNA. Cleavage of positively [(+)SC, closed circles] and negatively [(–)SC, open circles] supercoiled pBR322 plasmid DNA was monitored in the presence of ethidium bromide (EtBr), TAS-103, 9-aminoacridine (9-AA), or amsacrine (*m*-AMSA). Data were plotted as relative (*i.e.*, fold) DNA cleavage enhancement for simplicity. Fold DNA cleavage enhancement was calculated by normalizing levels of scission in the absence of intercalator to a relative value of 1.0. Error bars represent the standard deviation of at least three independent experiments. Insets show representative gels of DNA intercalation assays using relaxed plasmids in the absence of enzyme (DNA) or in the presence of the indicated concentration (μM) of compound (see Experimental Procedures for the interpretation of intercalation assays). The positions of supercoiled (FI) and nicked circular (FII) molecules are indicated.

As seen in Figure 23, all of the intercalators examined enhanced topoisomerase I-mediated DNA cleavage when added to negatively supercoiled plasmids. Ethidium bromide was the most potent and efficacious of the compounds tested. Approximately 10–fold enhancement of DNA cleavage was observed at 20 μM ethidium bromide. The other three compounds enhanced cleavage ~3– to 6–fold.

Two possible conclusions can be drawn from the above results. The intercalators may be "topological poisons" of topoisomerase I, enhancing DNA scission by making the negatively supercoiled substrate appear to be positively supercoiled. Alternatively, they may be previously undescribed "interfacial" topoisomerase I poisons (*i.e.*, poisons such as camptothecin that function at the enzyme-DNA interface) (27). Three experiments were carried out to distinguish between these possibilities. In the first, the effects of intercalators on topoisomerase I-mediated DNA cleavage were determined using positively supercoiled substrates. Since these plasmids are already overwound, the addition of intercalative compounds should have very little effect on the apparent topology of the DNA. In all cases, virtually no enhancement of cleavage was observed when intercalators were present in assays that examined overwound substrates (Figure 23).

In the second experiment, levels and sites of topoisomerase I-mediated cleavage in the presence of ethidium bromide, 9-aminoacridine, TAS-103, or amsacrine were monitored using radioactively end-labeled linear DNA (Figure 24). Since linear DNA is a topologically open system, the opening of the double helix by intercalators does not result in the accumulation of positive superhelical twists. Intercalator concentrations used in this assay corresponded to those that generated maximal cleavage with negatively

supercoiled substrates (see Figure 23). No enhancement of cleavage was seen with any of the compounds. This is in marked contrast to camptothecin or topotecan, both of which greatly increased levels of scission.

In the third experiment, the effects of ethidium bromide on topoisomerase I-mediated DNA cleavage were assessed in the presence of camptothecin or topotecan (Figure 25). Once again, the intercalator increased scission only when negatively supercoiled plasmid was used. These results suggest that ethidium bromide affects topoisomerase I cleavage by a mechanism that is distinct from that of interfacial poisons such as camptothecin and topotecan.

Taken together, these findings provide strong evidence that the intercalative compounds examined have no intrinsic activity against topoisomerase I and are not classical interfacial poisons. Since intercalators only affected enzyme-mediated DNA cleavage when underwound covalently closed substrates were employed, it is concluded that these compounds are topological poisons of topoisomerase I and enhance enzyme-mediated scission by altering the apparent superhelical state of the double helix.

Effects of Intercalators on Topoisomerase I-mediated DNA Cleavage in Cultured Human Cells

Since the genetic material in human cells is globally underwound, DNA intercalators might influence the ability of topoisomerase I to cleave the double helix *in vivo*. To assess this possibility, the effects of ethidium bromide on topoisomerase I-mediated DNA scission were determined in human CEM cells (Figure 26). Levels of topoisomerase I-DNA cleavage complexes rose ~2.5–fold when cells were treated with 10 μM ethidium bromide. Similar to the *in vitro* results seen in Figure 27, the intercalator also enhanced

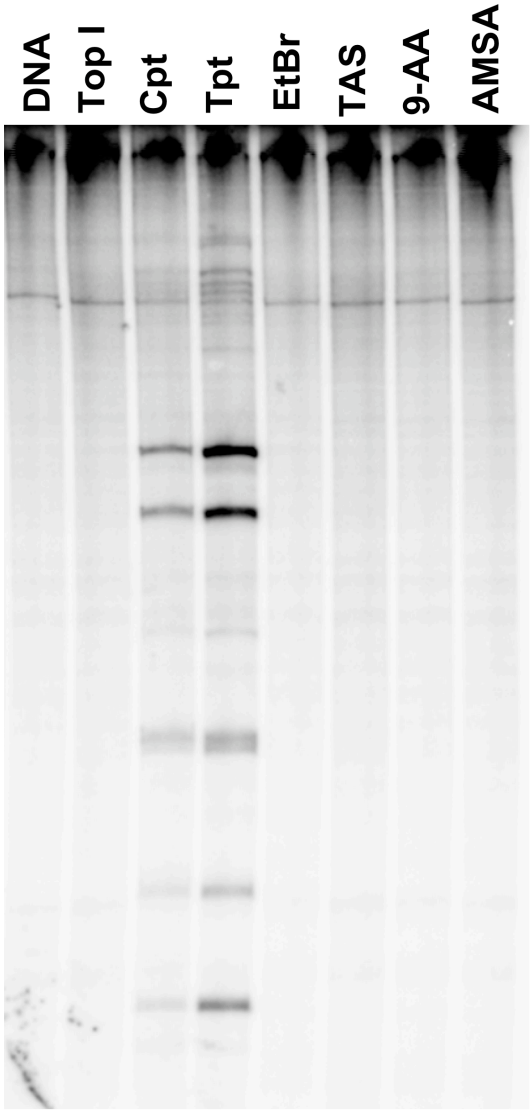


Figure 24. Effects of anticancer drugs and DNA intercalators on topoisomerase I-mediated cleavage of linear DNA. The ability of anticancer drugs [5 μM camptothecin (Cpt) or 10 μM topotecan (Tpt)] and DNA intercalating agents [20 μM ethidium bromide (EtBr), 20 μM TAS-103 (TAS), 100 μM 9-aminoacridine (9-AA), or 200 μM amsacrine (AMSA)] to enhance cleavage of a 3'-end labeled DNA substrate was determined. The autoradiograph is representative of three independent experiments.

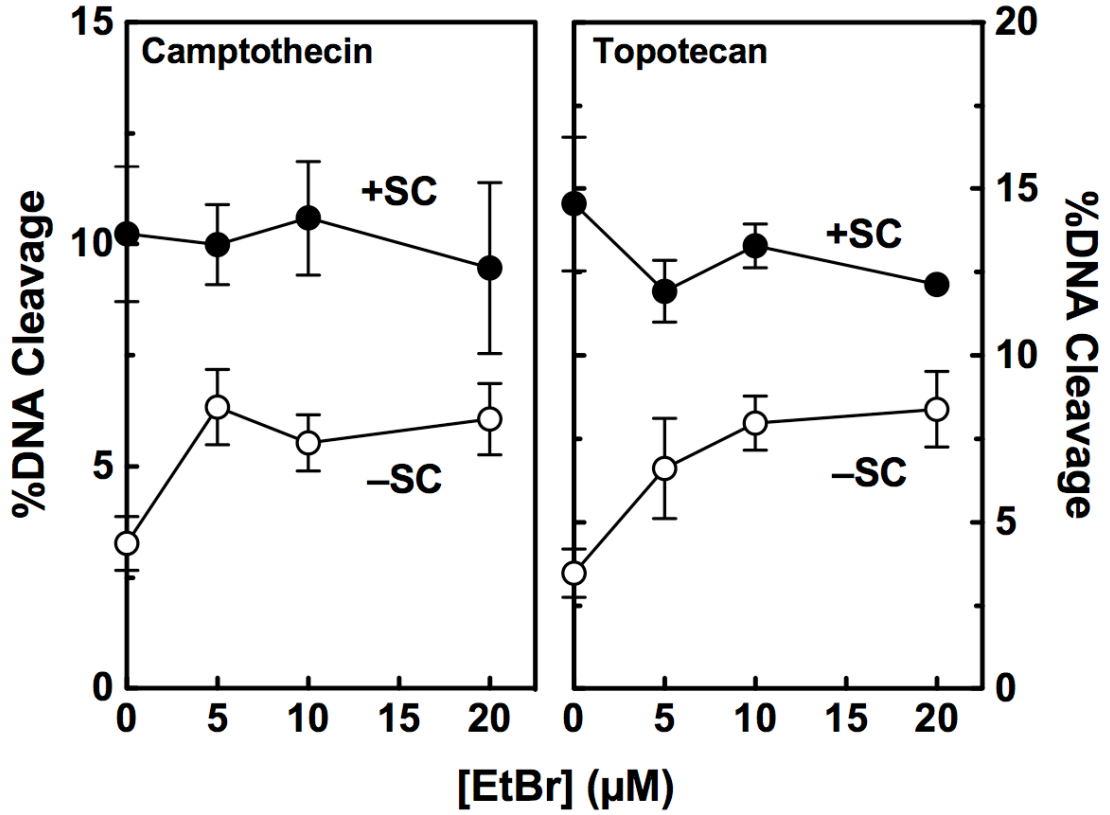


Figure 25. Effects of DNA intercalators on topoisomerase I-mediated cleavage of plasmid DNA in the presence of anticancer drugs. Cleavage of positively [(+)SC, closed circles] and negatively [(–)SC, open circles] supercoiled pBR322 plasmid DNA was monitored in the presence of 0–20 μM ethidium bromide and either 2.5 μM camptothecin (left panel) or 5 μM topotecan (right panel). Error bars represent the standard deviation of at least three independent experiments.

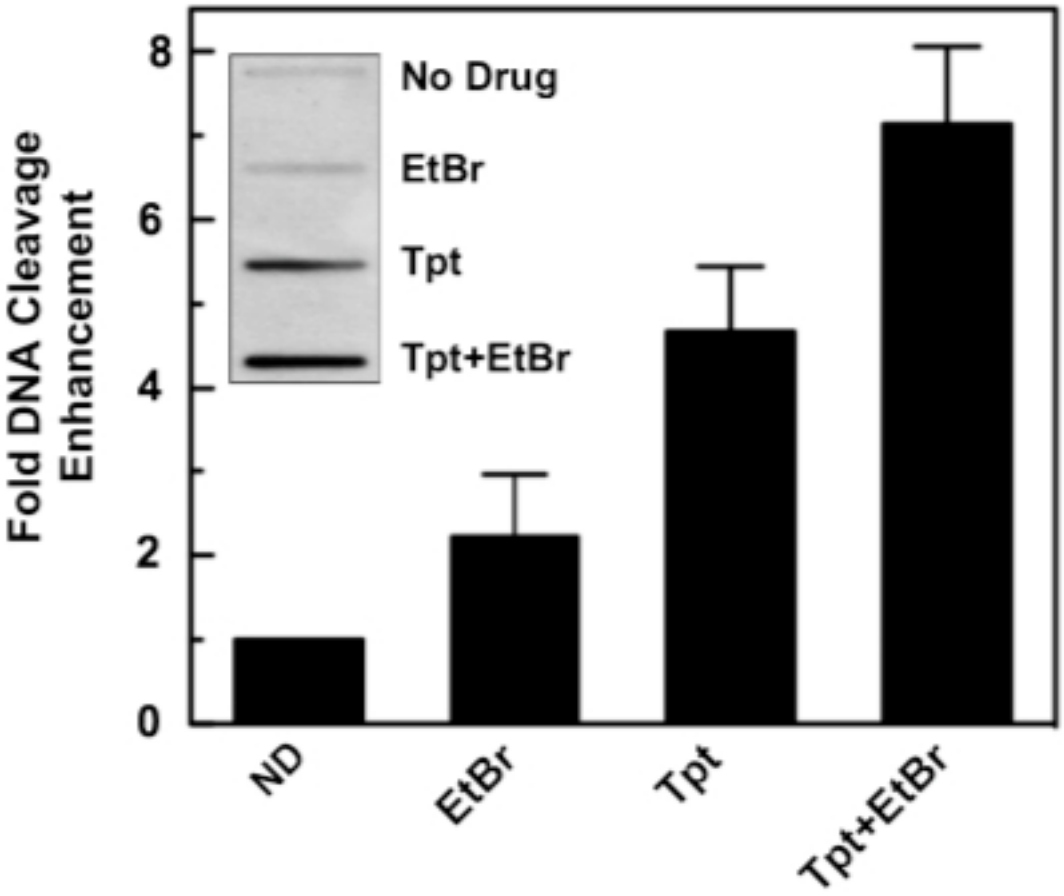


Figure 26. Effects of ethidium bromide on topoisomerase I-mediated DNA cleavage in cultured human CEM cells. Cells were treated for 1 h with no drug (ND), 10 μM ethidium bromide (EtBr), 5 μM topotecan (Tpt) or both ethidium bromide and topotecan (Tpt+EtBr). Topoisomerase I-DNA complexes were monitored using the ICE bioassay (see inset for a representative blot). Levels of topoisomerase I-mediated DNA cleavage in the absence of drug were set to a relative value of 1. Error bars represent the standard deviation of at least three independent experiments.

scission in cells that were treated with 5 μM topotecan. These results indicate that ethidium bromide can poison topoisomerase I in human cells.

Conclusions

Results in this chapter show that topoisomerase I maintains higher levels of cleavage complexes (~3– to 4–fold) with overwound substrates in the presence of camptothecins and other topoisomerase I poisons. The handedness of DNA supercoils does not alter the cleavage sites utilized in the presence of camptothecin-based compounds. Rather, the enhanced cleavage of overwound DNA correlates with decreased rates of enzyme-mediated DNA ligation on this substrate. Because topoisomerase I maintains higher baseline levels of cleavage with positively supercoiled DNA (see Chapter III), it is proposed that increased drug efficacy on overwound DNA seen in the presence of nonintercalative topoisomerase I poisons examined is due primarily to an increase in baseline levels of cleavage rather than an altered drug interaction in the enzyme-DNA complex.

DNA intercalators that have little or no intrinsic effect on topoisomerase I function enhanced the ability of the enzyme to cleave covalently closed negatively supercoiled substrates. Therefore, it is suggested that these compounds act by altering the perceived topological state of the double helix, making underwound DNA appear to be overwound. Due to the novel mechanism of action of intercalators on the type I enzyme, it is proposed that these compounds be referred to as topological poisons of topoisomerase I. It is notable that two of the intercalators examined are known topoisomerase II poisons (129,156). Therefore, while the primary cellular affects of these drugs likely results from

their poisoning of topoisomerase II, indirect poisoning of topoisomerase I due to changes in the topological state of DNA might also contribute to drug efficacy.

CHAPTER V

INTERACTIONS BETWEEN THE ETOPOSIDE DERIVATIVE F14512 AND HUMAN TYPE II TOPOISOMERASES: IMPLICATIONS FOR THE C4 SPERMINE MOIETY IN PROMOTING ENZYME-MEDIATED DNA CLEAVAGE

Introduction

Results presented in the previous chapters indicate that drugs that change the topological state of DNA can profoundly effect the responses of type I and type II topoisomerases. This chapter explores the effects that altering a drug to increase its ability to interact with DNA has on the ability of the compound to poison type II topoisomerases.

As mentioned in Chapter I, etoposide is a widely prescribed anticancer agent that is used as front line therapy to treat a variety of human malignancies (122-125). The drug is currently in its fourth decade of clinical use and kills cells by stabilizing covalent topoisomerase II-cleaved DNA complexes (20,80,97,104,122-125). Although regimens that include etoposide generate successful outcomes against a range of cancers, the cytotoxic nature of the drug induces significant side effects, including cardiotoxicity, myelosuppression, and gastrointestinal toxicity (122,123,182,183). Furthermore, ~2-3% of patients that are treated with etoposide go on to develop specific secondary leukemias that involve the mixed lineage leukemia (*MLL*) gene at chromosomal band 11q23 (107,125,138,139). Since the toxic side effects of etoposide are predominantly mechanism-based (*i.e.*, result from the actions of the drug against topoisomerase II), they are difficult to eliminate. Etoposide and other anticancer drugs affect both isoforms of human topoisomerase II, topoisomerase IIα and topoisomerase IIβ (20,80,97,104,157).

Evidence suggests that topoisomerase IIβ may play a more important role than topoisomerase IIα in mediating off-target toxicities and generating leukemic chromosomal translocations (142-144).

One way to decrease the off-target toxicity of etoposide and related drugs is to increase their specificity for malignant tissues. To this point, the polyamine transport system is more active in proliferating cells than resting cells, and the uptake of polyamines by some cancer cells is even greater (184-187). Therefore, in an effort to enhance the uptake of etoposide by cancer cells, F14512 was developed (Figure 27) (148,149). This derivative replaces the carbohydrate moiety at the C4 position of etoposide, which does not interact with topoisomerase II in the binary enzyme-drug complex (159,160), with the polyamine spermine. The spermine moiety is conjugated to the etoposide core through a glycyl spacer and was selected for optimal activity among a large series of conjugates (148,149).

F14512 is taken up by mammalian cells through the polyamine transport system and, compared to etoposide, displays increased potency against most cancer cell lines (148). Furthermore, F14512 is more active than the parent compound against a mouse MX-1 human breast tumor xenograft model and displays a higher therapeutic index and lower systemic toxicity (148). The drug also displays high activity against a variety of other xenograph models, including solid tumors (188) and leukemia (189), and currently is in phase I clinical trials for the treatment of acute myeloid leukemia.

Although the spermine moiety originally was attached to F14512 in order to change the cellular uptake properties of etoposide, the inclusion of the polyamine had two additional effects (148). First, it altered the DNA binding properties of the compound.

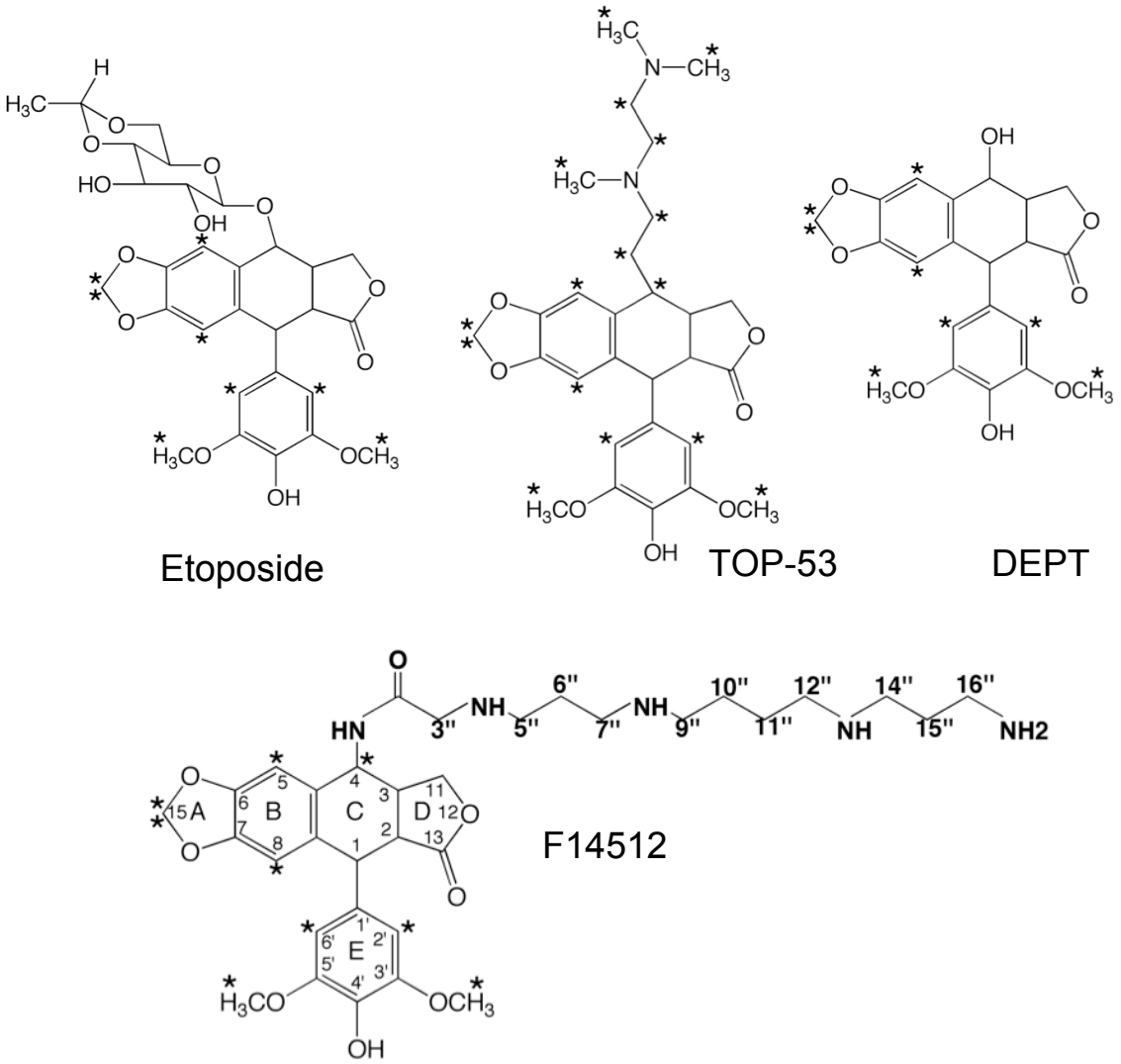


Figure 27. Structure of etoposide and derivatives. The spermine moiety at the C4 position of F14512 is indicated by the box. Asterisks mark protons that interact with topoisomerase II in the binary enzyme-drug complex as determined by STD [^{1}H]-NMR spectroscopy (26, 27) (see Figure 28 and Table 2).

While etoposide displays little interaction with DNA (in the absence of topoisomerase II) (190,191), F14512 is a DNA binder, most likely interacting with the double helix through the minor groove (148). Second, as determined by preliminary *in vitro* studies, F14512 appears to be a more efficacious topoisomerase II poison than etoposide (148).

Given the high activity of F14512 in preclinical cancer models, it is important to understand how the drug interacts with potential cellular targets. Therefore, the effects of the C4 spermine moiety on drug function against human type II topoisomerases were analyzed. Results indicate that the C4 polyamine increases the potency and efficacy of the drug against both isoforms of human topoisomerase II, but does not change the fundamental mechanism of etoposide action (*i.e.*, inhibition of DNA ligation). Rather, the linkage between the spermine and the drug core appears to enhance the stability of etoposide in the ternary enzyme-drug-DNA complex.

Results and Discussion

Interactions between F14512 and Human Topoisomerase IIα

Previous studies indicate that interactions between topoisomerase II and etoposide are critical for drug activity and mediate the entry of etoposide into the ternary enzyme-drug-DNA complex (159,160,192,193). Furthermore, drug contacts in the binary topoisomerase II-drug complex, including the C15 geminal protons of the A–ring, the C5 and C8 protons of the B–ring, and the C2' and C6' protons and the 3'– and 5'–methoxyl protons of the pendant E–ring (as defined by STD [^{1}H]-NMR spectroscopy, Table 2) have predictive value for the actions of etoposide within the ternary drug-enzyme-DNA

complex (159,160). Indeed, alterations of A-ring and E-ring substituents dramatically decrease the efficacy of etoposide against topoisomerase II. In contrast, removal of the C-4 glycosidic group (which generates DEPT, Figure 27) has relatively little effect on the ability of the drug to poison topoisomerase II (160).

F14512 shares an identical core (rings A–E) with etoposide but contains a spermine group in place of the glycosidic moiety at the C4 position (Figure 27). Therefore, as a first step in characterizing the activity of the compound against human type II topoisomerases, STD [^{1}H]-NMR spectroscopy was employed to define the substituents on F14512 that contact topoisomerase IIα in the binary enzyme-drug complex. With the exception of one additional contact with the enzyme (the C4 proton of the C-ring) the substituents on the drug core that contacted topoisomerase IIα in the binary complex were identical to those previously described for etoposide (Table 2 and Figure 28) (159,160). It is notable that no significant nuclear Overhauser enhancement (NOE) signals were observed for any of the protons of the spermine moiety of F14512. This result suggests that there is limited interaction, if any, between this portion of F14512 and the enzyme.

Although the C4 substituents of etoposide and F14512 do not interact with topoisomerase IIα, it is possible to substitute this position with a protein-binding group. To this point, TOP-53 is a derivative of etoposide that contains a C4 aminoalkyl side chain (Figure 27) (194). In contrast to results with etoposide or F14512, every proton associated with this side chain of TOP-53 contacts topoisomerase IIα in the binary complex (159). Furthermore, the presence of the aminoalkyl group increases the binding

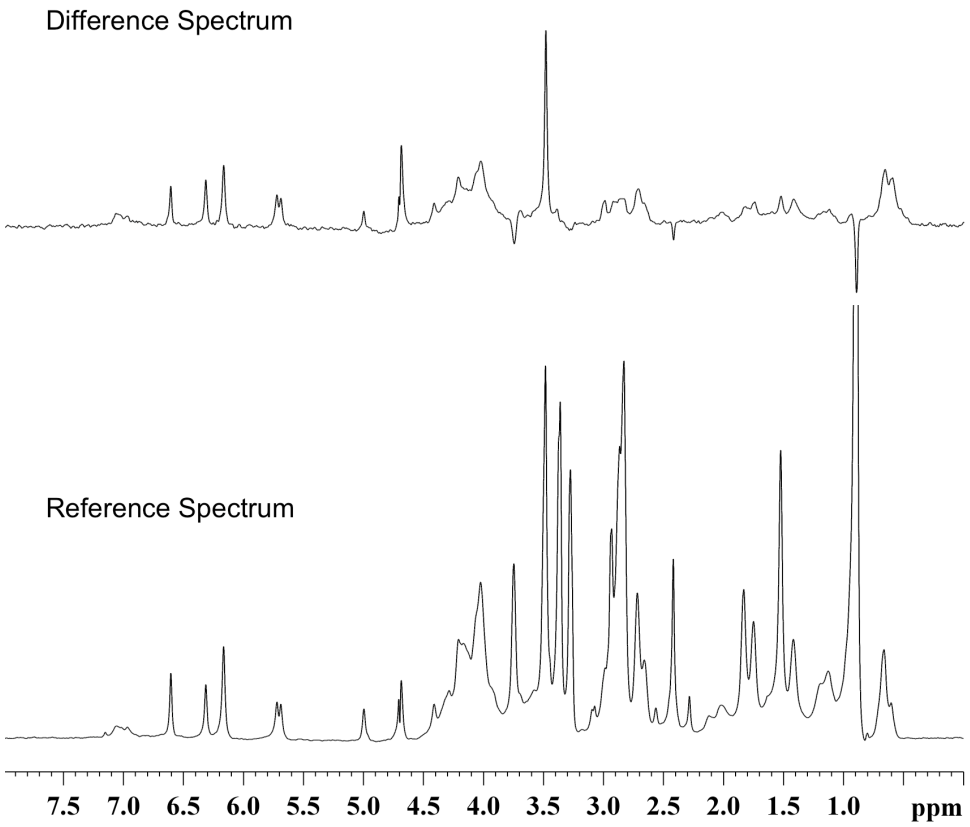


Figure 28. Interaction of F14512 with human topoisomerase IIα as determined by STD [^{1}H]-NMR spectroscopy. Difference (top) and reference (off-resonance, bottom) spectra are shown. Spectra are representative of at least two independent experiments.

Table 2: Drug Substituents that Interact with Human Topoisomerase IIα in the Binary Enzyme-Drug Complex as Determined by STD [^{1}H]-NMR Spectroscopy[a]

Etoposide		F14512	
Substituent Proton	Resonance	Substituent Proton	Resonance
1	4.39	1	4.52
2	3.33	2	3.18
3	2.83	3	2.99
4	4.82	**4**	**5.00**
5	**6.70**	**5**	**6.61**
8	**6.32**	**8**	**6.32**
11R, 11S	4.15, 4.20	11R, 11S	3.90, 4.30
15R, 15S	**5.70**	**15R, 15S**	**5.70**
2', 6'	**6.18**	**2', 6'**	**6.17**
3', 5'-OCH$_3$	**3.49**	**3', 5'-OCH$_3$**	**3.49**
1"	4.43	3"	3.78
2"	3.07	5"	2.97
3"	3.33	6"	2.05
4"	3.17	7"	3.04
5"	3.24	9", 12"	2.90
6", 6"	3.42, 4.01	10", 11"	1.72
7"	4.70	14", 16"	3.07
-CH$_3$	1.10	15"	1.97

[a]Resonances that display nuclear Overhauser effects in STD [^{1}H]-NMR spectroscopy experiments along with the substituent protons that they represent are indicated in bold.

affinity of TOP-53 for topoisomerase II and significantly enhances the potency and efficacy of the drug against the type II enzyme (159,195).

In contrast to the C4 aminoalkyl side chain of TOP-53, the spermine moiety of F14512 interacts with DNA and converts the drug to a DNA binder (148). Taken together, these results suggest that the C4 substituents of compounds in the etoposide family have the potential to influence drug activity by two different mechanisms: enhanced binding to the enzyme or enhanced binding to DNA. Consequently, the influence of the C4 spermine on F14512 activity was investigated more fully.

F14512 Poisons Human Type II Topoisomerases

Preliminary results using human topoisomerase IIα suggest that F14512 is a more efficacious poison than etoposide (148). Therefore, to examine the activity of F14512 in greater detail, the effects of the drug on DNA cleavage mediated by human type II topoisomerases were compared to those of etoposide and TOP-53. F14512 was several–fold more potent and more efficacious than either drug against both topoisomerase IIα (Figure 29) and topoisomerase IIβ (Figure 30). In all cases, F14512 generated the highest levels of double-stranded, single-stranded, and total topoisomerase II-mediated DNA strand breaks (Figure 31). These results demonstrate that the inclusion of the DNA-binding C4 spermine in F14512 has a marked effect on drug activity. Furthermore, this effect is even greater than that generated by the enzyme-binding aminoalkyl group in TOP-53.

It is notable that F14512 is more active against topoisomerase IIα than topoisomerase IIβ. Comparable or higher levels of DNA scission were observed with the

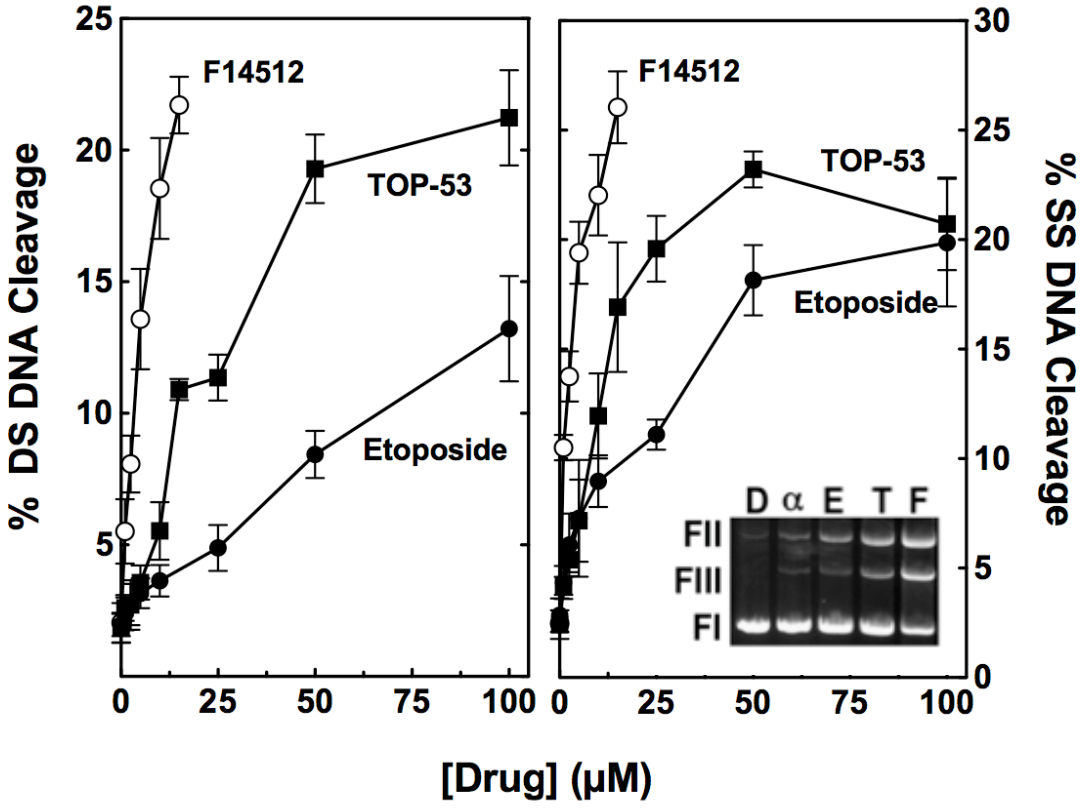


Figure 29. F14512 is a potent topoisomerase IIα poison. The effects of etoposide (closed circles), TOP-53 (closed squares), and F14512 (open circles) on the cleavage of negatively supercoiled pBR322 plasmid DNA by human topoisomerase IIα were determined. The percentage of double-stranded (left panel) and single-stranded (right panel) cleavage is shown. Error bars represent the standard deviation of 3 independent experiments. The inset in the right panel shows an agarose gel of topoisomerase IIα-mediated cleavage of negatively supercoiled pBR322 plasmid DNA in the absence of drug (α) or in the presence of 15 μM etoposide (E), TOP-53 (T), or F14512 (F). A DNA standard (D) also is shown. The positions of supercoiled (form I, FI), nicked circular (form II, FII), and linear (form III, FIII) molecules are indicated.

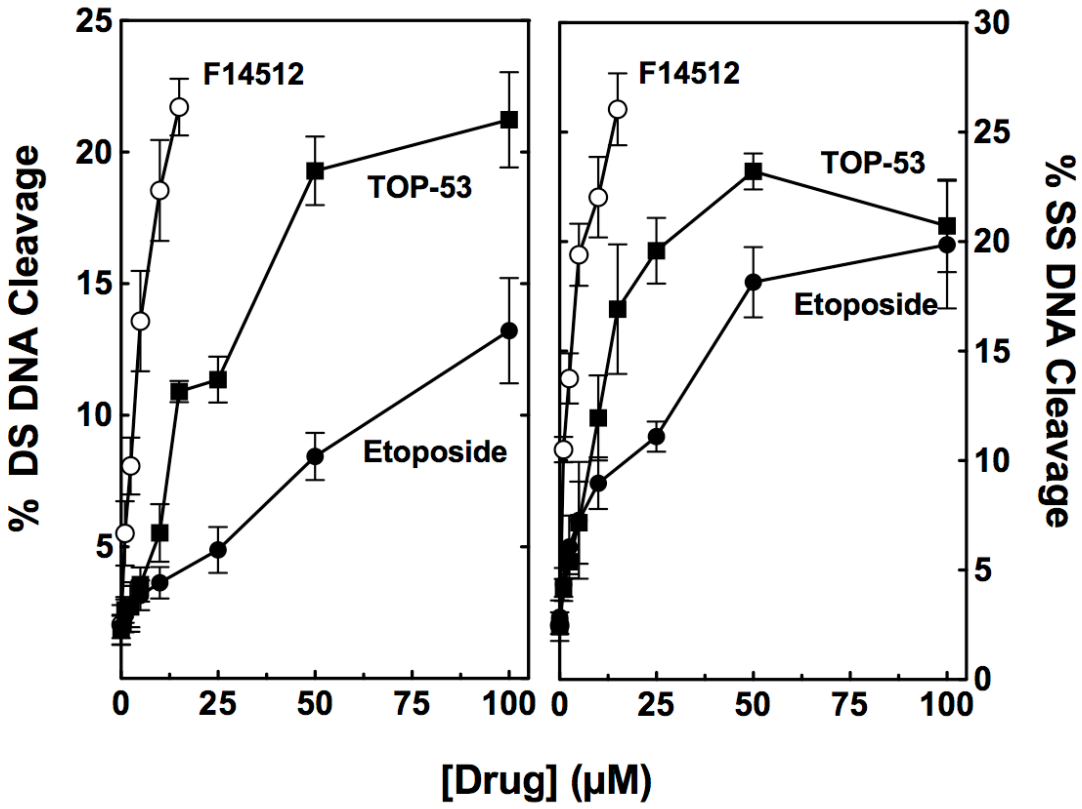


Figure 30. F14512 is a potent topoisomerase IIβ poison. The effects of etoposide (closed circles), TOP-53 (closed squares), and F14512 (open circles) on the cleavage of negatively supercoiled pBR322 plasmid DNA by human topoisomerase IIβ were determined. The percentage of double-stranded (left panel) and single-stranded (right panel) cleavage is shown. Error bars represent the standard deviation of 3 independent experiments.

α isoform, despite the fact that the enzyme concentration employed was only one half that used in topoisomerase IIβ assays. Furthermore, at concentrations of F14512 that exceeded 15 μM, multiple cleavage events per plasmid were observed with topoisomerase IIα, such that it became impossible to accurately quantify levels of double-stranded DNA breaks (Figure 29). It has been suggested the α isoform of human topoisomerase II may be a more important therapeutic target for topoisomerase II-directed anticancer agents than topoisomerase IIβ, while the latter isoform contributes more to the off-target toxicities of these drugs (142-144). Consequently, the higher activity that F14512 displays against topoisomerase IIα may provide therapeutic advantages.

Eukaryotic type II topoisomerases are homodimeric proteins (50,196,197). Each protomer active site of the enzyme cuts one strand of the DNA to generate a double-stranded break (51). The two enzyme protomers are not fully coordinated and, as a result, also create single-stranded breaks (198-200). The double-stranded:single-stranded cleavage ratio differs between drug classes, with etoposide yielding a relatively low ratio (~0.5:1 at 10 μM drug) with topoisomerase IIα (Figure 31, *inset*) (198). Of the three drugs, F14512 induced the highest ratio of double-stranded:single-stranded breaks (~1:1 at 10 μM drug with topoisomerase IIα) (Figure 31, *inset*).

Type II topoisomerases require ATP binding and hydrolysis for overall catalytic activity (56-58). Numerous studies have demonstrated that etoposide displays maximal activity only in the presence of the high-energy cofactor (201), although the mechanistic basis for this property is not understood. As seen in Figure 32, levels of topoisomerase IIα-mediated DNA cleavage induced by etoposide and TOP-53 drop ~3– and ~2–fold,

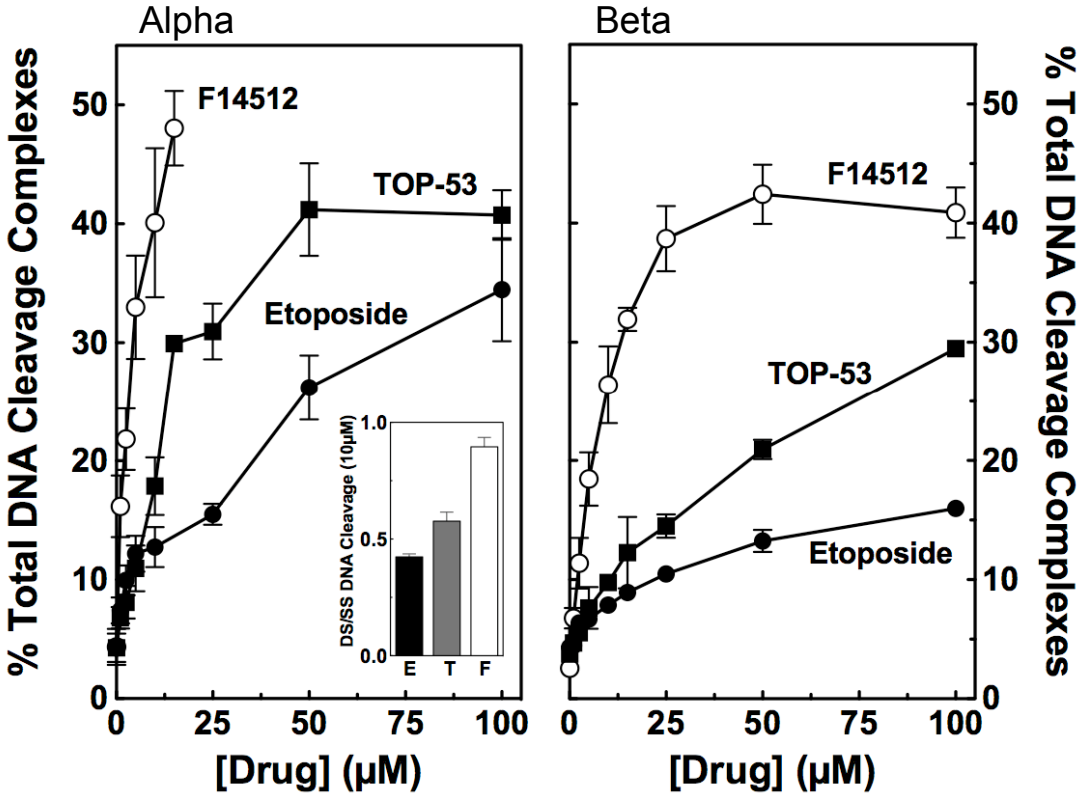


Figure 31. F14512 is a potent topoisomerase II poison. The effects of etoposide (closed circles), TOP-53 (closed squares), and F14512 (open circles) on total DNA cleavage mediated by human type II topoisomerases were determined. The percentage of total DNA cleavage by topoisomerase IIα (left panel) and IIβ (right panel) is shown. Error bars represent the standard deviation of 3 independent experiments. The inset in the left panel shows the ratio of double-stranded:single-stranded (DS/SS) breaks generated by topoisomerase IIα in the presence of 10 μM etoposide) (E), TOP-53 (T), or F14512 (F).

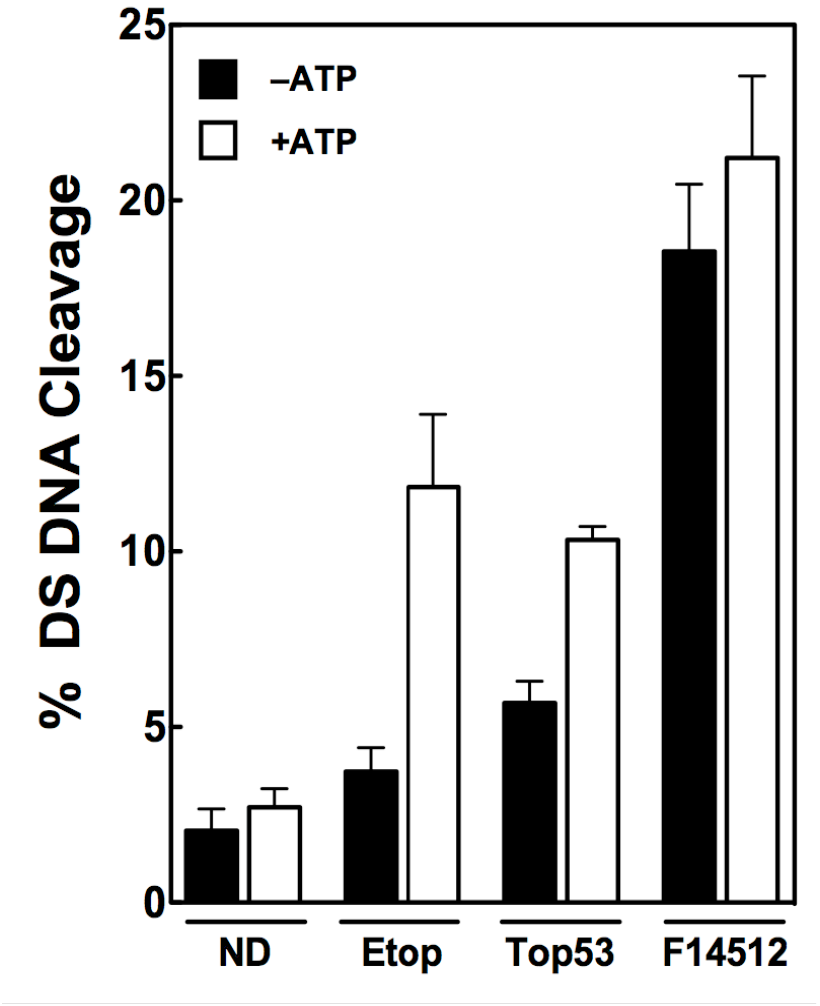


Figure 32. Effects of ATP on drug-stimulated DNA cleavage mediated by human topoisomerase IIα. Assays were carried out in the absence (closed bars) or presence (open bars) of 0.5 mM ATP. Reactions contained no drug (ND) or 10 μM etoposide, TOP-53, or F14512. Error bars represent the standard deviation of 3 independent experiments.

respectively, in the absence of ATP. In contrast, F14512 was far better at maintaining activity in the absence of ATP, with cleavage levels declining only 15% in assays that lacked the cofactor.

Because the C4 spermine moiety of F14512 interacts with DNA, it is possible that it alters the cleavage site specificity of topoisomerase IIα and topoisomerase IIβ. In order to assess this issue, the DNA sites that are cleaved by the human enzymes in the presence of etoposide, TOP-53, and F14512 were determined (Figure 33). A similar (but not identical) array of sites was cleaved with all three drugs. This finding suggests that the addition of the spermine moiety to the etoposide core does not promote site-specific DNA interactions and is consistent with the general DNA properties of the polyamine.

Etoposide increases levels of covalent topoisomerase II-DNA cleavage complexes by inhibiting the ability of the enzyme to ligate DNA breaks (137,202). Therefore, a ligation assay was employed to determine whether the inclusion of the spermine moiety alters the mechanistic basis for drug action. As seen in Figure 34, etoposide, TOP-53, and F14512 are all potent inhibitors of DNA ligation mediated by human topoisomerase IIα. Given the strong inhibition, it is not possible to determine whether the higher levels of DNA cleavage generated by F14512 reflect an enhanced inhibition of the ligation reaction.

F14512–DNA Interactions are Non-intercalative

It has long been known that etoposide binds weakly if at all to DNA in the absence of topoisomerase II (190,191). In contrast, a previous study that assessed changes in DNA melting temperatures, as well as the ultraviolet and circular dichroism spectra of

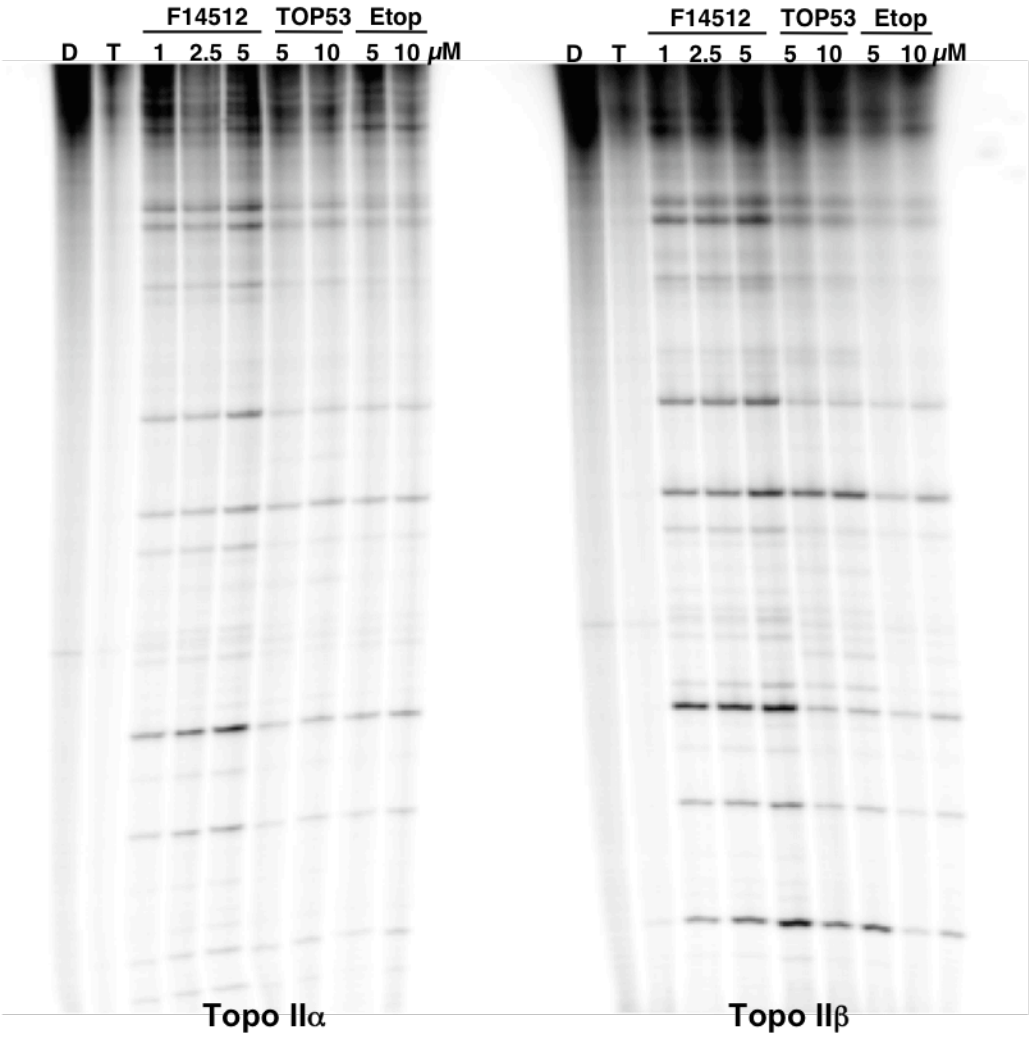


Figure 33. Effects of etoposide derivatives on sites of DNA cleavage mediated by human topoisomerase IIα (left) and topoisomerase IIβ (right). An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions were carried out in the absence of drug (TII), or in the presence of the indicated concentrations of F14512, TOP-53, or etoposide. A DNA standard (DNA) also is shown. Results are representative of 3 independent experiments.

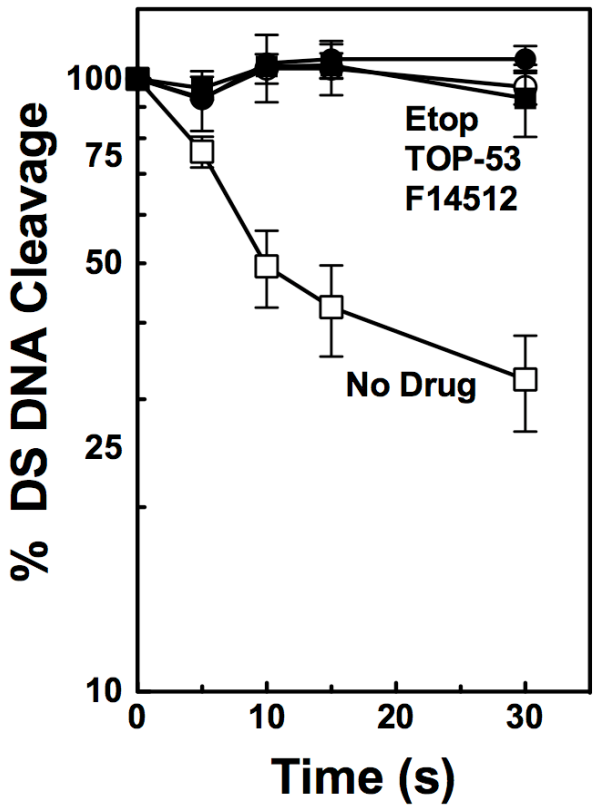


Figure 34. Effects of etoposide derivatives on DNA ligation mediated by human topoisomerase IIα. Reactions were carried out in the absence of drug (open squares) or in the presence of 50 μM etoposide (closed circles), 50 μM TOP-53 (closed squares), or 10 μM F14512 (open circles). Ligation is expressed as the percent loss of linear DNA, which was set to 100% at time zero. Error bars represent the standard deviation of 3 independent experiments.

DNA, concluded that F14512 is a DNA binding drug (148). While changes in circular dichroism suggest that F14512 binds to the double helix through the minor groove, the study did not determine the DNA binding mode of the drug (148).

A previous study found that attachment of spermine to anthracene (which is a poor DNA intercalator) generated an intercalative drug (203). Therefore, two experiments were carried out to determine whether F14512 intercalates into DNA. The first experiment utilized a topoisomerase I-DNA relaxation assay to monitor intercalation. As seen in Figure 35 (*top*), F14512 displayed no ability to intercalate over the concentration range that promoted topoisomerase II-mediated DNA cleavage (0–100 μM).

The second experiment takes advantage of the finding that topoisomerase IIα maintains 2– to 3–fold higher levels of DNA cleavage complexes with negatively supercoiled DNA as compared to positively supercoiled molecules (79,145). While this relationship is maintained over a broad concentration range in the presence of non-intercalative topoisomerase II poisons, as described in Chapter IV, it does not hold when intercalative drugs are utilized (145). In this latter case, cleavage levels of negatively supercoiled plasmids relative to positively supercoiled substrates drop as the concentration of intercalator increases. Over the concentration range of F14512 examined, the percent cleavage of negatively supercoiled plasmids remained 2– to 3–fold higher than seen with the corresponding positively supercoiled substrate (Figure 35, *bottom*). These findings provide strong evidence that F14512 does not utilize an intercalative mode to bind DNA.

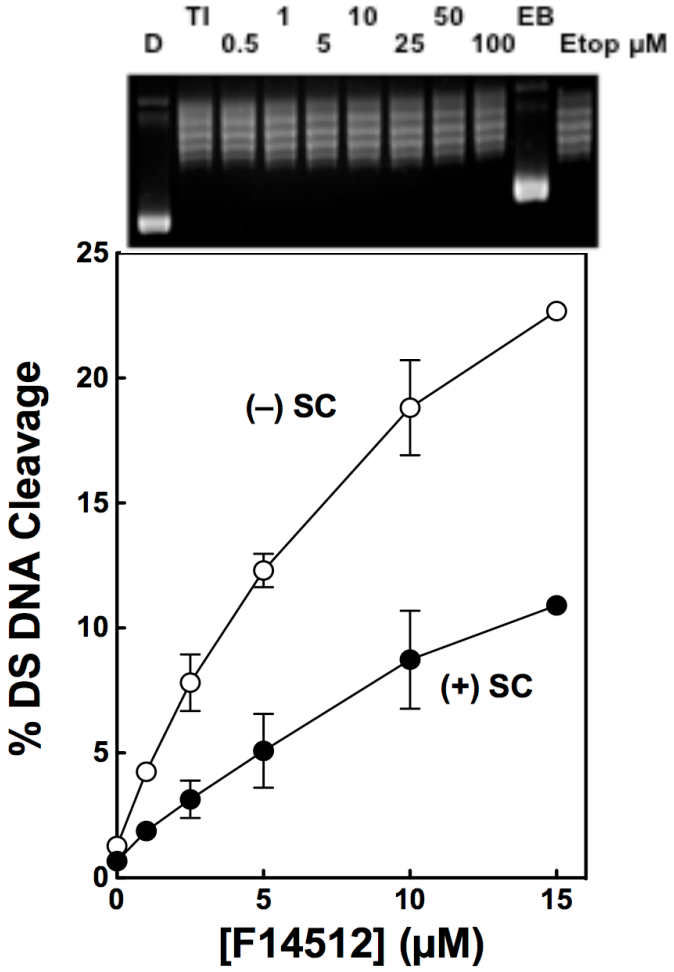


Figure 35. F14512 is not a DNA intercalator. The ability of F14512 to intercalate into negatively supercoiled pBR322 plasmid DNA was determined using a topoisomerase I relaxation assay (top). An agarose gel is shown. DNA relaxation was carried out in the absence of drugs (TI), or in the presence of 0–100 µM F14512, 20 µM ethidium bromide (EB), or 20 µM etoposide (Etop). A DNA standard (D) also is shown. The gel is representative of three independent experiments. The bottom panel shows the effects of F14512 on the ability of human topoisomerase IIα to cleave negatively (open circles) and positively (closed circles) supercoiled pBR322 plasmid molecules. Error bars represent the standard deviation of 3 independent experiments.

Enhanced Stability of the Ternary Topoisomerase IIα-F14512-DNA Complex

F14512 displays similar contacts as etoposide with human topoisomerase IIα in the binary enzyme-drug complex (see Table 2 and Figure 28), but has additional interactions with the double helix in the binary DNA-drug complex (148). Since F14512 is a more potent topoisomerase II poison than etoposide, we wanted to see if these DNA interactions contribute to an enhanced stability of the spermine-linked drug in the ternary topoisomerase IIα-drug-DNA complex. Two approaches were used to address this issue. In the first, the persistence of DNA cleavage complexes established in the presence of F14512 or etoposide was determined (162). This was accomplished by establishing DNA cleavage-ligation equilibria in the presence of 10 μM F14512 or 100 μM etoposide, diluting reaction mixtures 20–fold, and monitoring the decay of cleavage complexes over time. As seen in Figure 36, cleavage complexes formed in the presence of F14512 persisted 5– to 10–fold longer than equivalent complexes induced by etoposide. This suggests that F14512 forms a more stable ternary complex than etoposide.

In the second approach, the ability of the quinolone ciprofloxacin to compete with F14512 or etoposide for binding in the ternary complex was determined. Ciprofloxacin is an antibacterial topoisomerase II poison (101,204) that displays little ability to stimulate DNA cleavage mediated by the eukaryotic type II enzyme (205). However, the quinolone interacts with eukaryotic topoisomerase II and shares a binding site with anticancer drugs (205). Thus, it can inhibit the ability of these drugs to enhance DNA scission (205).

As seen in Figure 37, ciprofloxacin competed with both F14512 and etoposide, but the ciprofloxacin:F14512 ratio required to decrease DNA cleavage was 5– to 10–fold higher than seen with etoposide. Taken together, these experiments indicate that ternary

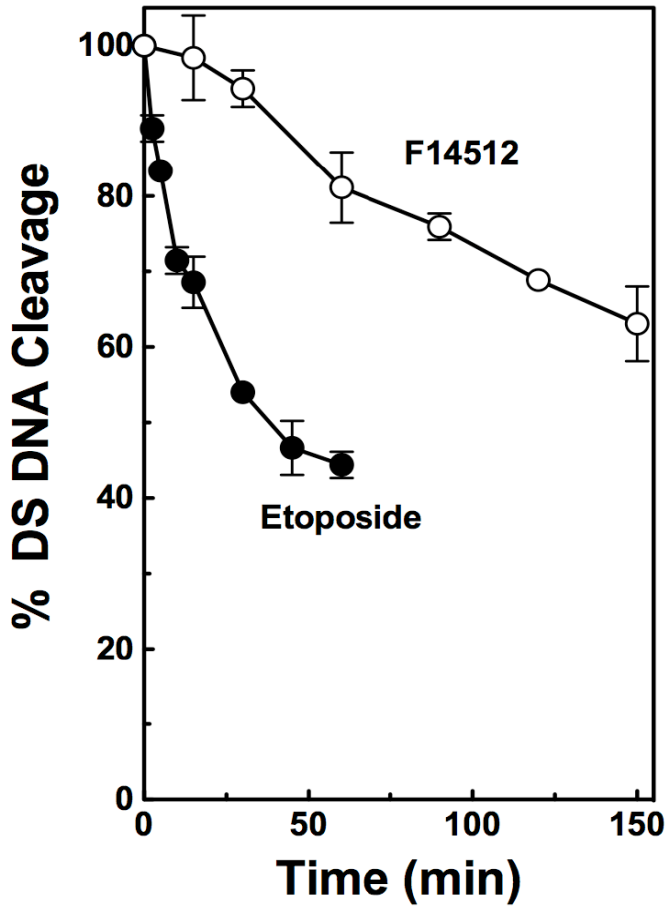


Figure 36. Effects of etoposide derivatives on the persistence of ternary topoisomerase IIα-drug-DNA cleavage complexes. Assays were carried out in the presence of etoposide (100 μM) or F14512 (10 μM) and negatively supercoiled pBR322 plasmid DNA. After initial reaction mixtures attained DNA cleavage-ligation equilibria, they were diluted 20–fold with DNA cleavage buffer. The persistence of cleavage complexes was assessed by monitoring the loss of double-stranded DNA breaks (linear product) over time. Cleavage at time zero was set to 100%. Error bars represent the standard error of 2 independent assays.

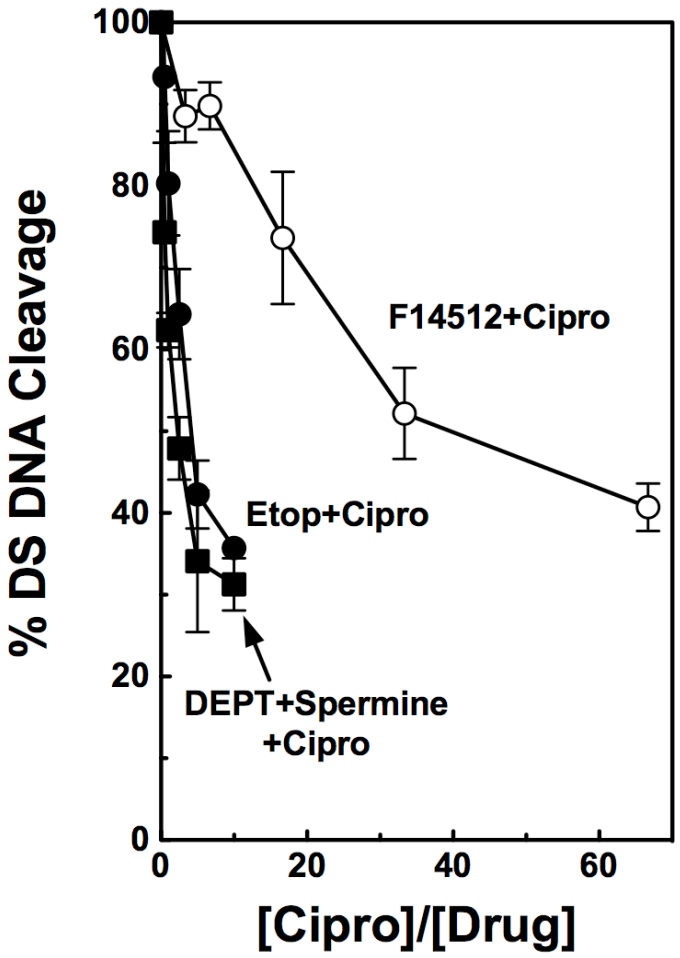


Figure 37. F14512 binds more tightly than etoposide in the ternary topoisomerase IIα-drug-DNA complex. A ciprofloxacin competition assay was employed. DNA cleavage complexes were established in the presence of etoposide (Etop), F14512, or a 1:1 mixture of DEPT + spermine and 0–1000 μM ciprofloxacin (Cipro). Levels of DNA cleavage generated by human topoisomerase IIα in the presence of ciprofloxacin alone were subtracted from those seen in the presence of etoposide derivatives + ciprofloxacin. Cleavage in the absence of ciprofloxacin was set to 100%, and results are presented as a ratio of ciprofloxacin concentration to etoposide derivative. Error bars represent the standard deviation of 3 independent experiments.

enzyme-drug-DNA complexes formed in the presence of F14512 are more stable than those formed with etoposide. This enhanced stability provides a probable basis for the greater potency and efficacy of F14512 compared to etoposide.

Importance of the Spermine-Drug Linkage to the Enhanced Activity of F14512 Against Topoisomerase IIα

Experiments in the previous section suggest that the spermine-mediated binding of F14512 to DNA is responsible for the enhanced activity of the drug. However, spermine-DNA interactions also neutralize nucleic acid charge and allow condensation of the double helix (206-208). Furthermore, polyamines such as spermine and spermidine can alter interactions between type II topoisomerases and DNA and are used routinely as DNA condensation agents to convert intramolecular to intermolecular DNA strand passage reactions (*i.e.*, DNA relaxation to catenation) (209).

Therefore, to determine whether the spermine moiety alters F14512 activity by a specific effect on drug interactions in the ternary complex or by a general effect on DNA charge/structure, the importance of the spermine-drug linkage to the activity of F14512 was examined. As a first step, the stability of the ternary enzyme-drug-DNA complex was assessed when it was formed in the simultaneous presence of the unlinked etoposide core (*i.e.*, DEPT, see Figure 27) and spermine. As assessed by the ciprofloxacin competition assay (Figure 37), the stability of a 1:1 DEPT:spermine mixture was similar to that of etoposide and was ~10–fold lower than that of F14512. This finding indicates that the drug-spermine linkage is critical for the enhanced stability of F14512 in the ternary complex.

The effects of the drug-spermine linkage on the activity of F14512 also were determined. As seen in Figure 38, spermine (up to 100 μM) had little influence on levels of DNA cleavage mediated by human topoisomerase IIα (210). Moreover, DNA cleavage enhancement observed in the presence of a 1:1 DEPT:spermine mixture was comparable to that of the drug core in the absence of the polyamine. A similar result was seen comparing a 1:1 etoposide:spermine mixture to the activity of etoposide alone. Taken together, these results provide strong evidence that, in the concentration ranges examined, spermine does not enhance topoisomerase II-mediated DNA cleavage by a general effect on DNA charge or structure. Furthermore, the drug-spermine linkage is critical for the high activity of F14512.

Conclusions

F14512 is a novel etoposide derivative that contains a spermine group in place of the C4 glycosidic moiety (148). The linkage between the drug core and spermine converts etoposide to a DNA binder (148). The presence of the spermine also enhances the selectivity of the drug in cancers that over-express an active polyamine transport system (148,188,189). Results shown here indicate that F14512 is a more potent and efficacious topoisomerase II poison than etoposide, and this enhanced activity correlates with an increased stability of the ternary topoisomerase II-drug-DNA complex. The covalent linkage between the etoposide core and spermine is critical for the enhanced activity of F14512. Additionally, the drug is more potent and efficacious than TOP-53, an etoposide derivative that contains a C4 aminoalkyl group that strengthens drug-enzyme binding. These findings highlight the utility of a C4 DNA binding group and provide a rational

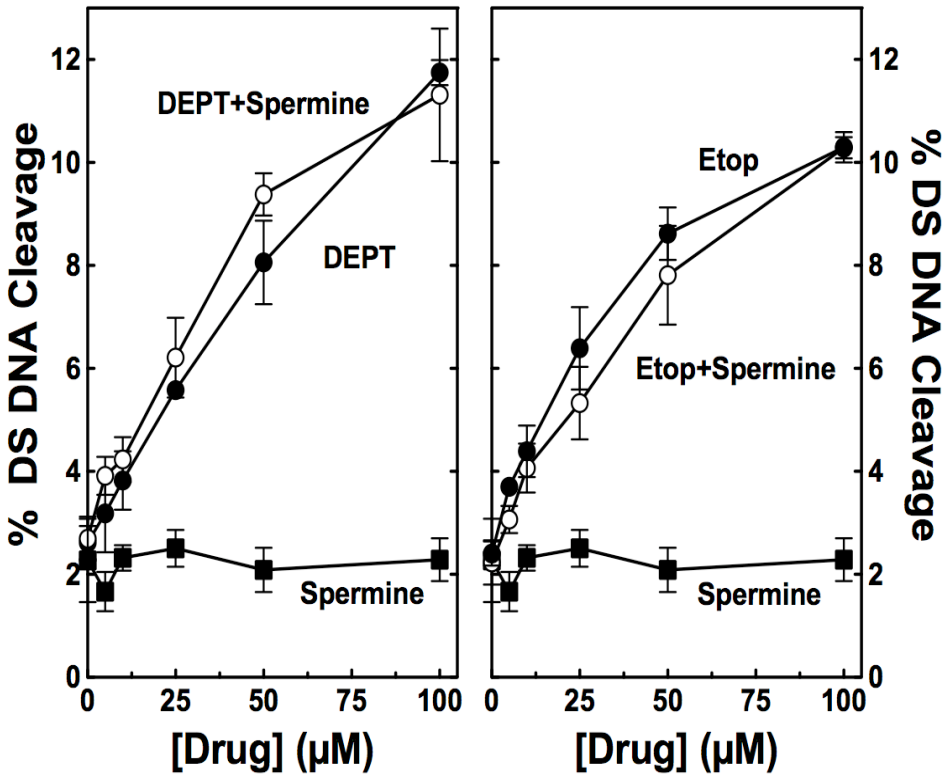


Figure 38. The covalent linkage of the C4 spermine moiety to the etoposide core is necessary for the enhanced DNA cleavage seen in the presence of F14512. The ability of DEPT or a 1:1 mixture of DEPT + spermine (left panel), and etoposide (Etop) or a 1:1 mixture of etoposide + spermine (right panel) to enhance topoisomerase IIα-mediated DNA cleavage is shown. Control experiments assessing the effects of spermine alone on the DNA cleavage activity of topoisomerase IIα are shown in both panels. Error bars represent the standard deviation of 3 independent experiments.

basis for the development of novel and more active etoposide-based topoisomerase II poisons. Finally, the dual function of the C4 spermine moiety (*i.e.*, enhanced cellular uptake in cancers with active polyamine transport and greater activity against topoisomerase II) supports the ongoing clinical development of F14512.

CHAPTER IV

CONCLUSIONS

The three-dimensional properties of DNA dramatically affect how the cell accesses and reproduces its genetic information. Some of the most important three-dimensional relationships in DNA are topological in nature, including underwinding and overwinding, knotting, and tangling (3,4,8-11). These topological problems are resolved by the concerted actions of DNA topoisomerases (5,6,13,15,16). In addition to their critical cellular functions, topoisomerases are the targets for some of the most widely prescribed anticancer drugs currently in clinical use (20,27,28,80,104,125). The work presented in this dissertation provides a more complete understanding of the interplay between DNA interactions, human topoisomerase activity, and anticancer drugs that act at the enzyme-DNA interface.

Structural Features of Topoisomerases Required for the Recognition of DNA Topology

During replication, unwinding of the double helix and synthesis of new complementary DNA strands results in the accumulation of underwound DNA behind the replication fork and overwound DNA ahead of the fork (4,9,11). A similar redistribution of DNA supercoils arises during transcription. Chapter III of this dissertation describes the regions of topoisomerase IIα that are necessary for recognition of DNA topology ahead of replication forks (*i.e.* overwound DNA). This chapter also provides strong

evidence that topoisomerase I is an enzyme that is designed to act primarily on overwound DNA.

Portions of Topoisomerase II Required for Preferential Relaxation of Positively Supercoiled DNA

Topoisomerase IIα recognizes supercoil handedness during relaxation, and removes positive supercoils >10 times faster than negative supercoils (79). Experiments presented in Chapter III show that almost the entire CTD of topoisomerase IIα is required for this activity. Although no structural data of the CTD of this enzyme is available, this protein domain contains clusters of positively charged amino acids that could interact with DNA in a manner similar to that seen with prokaryotic type II enzymes. The CTDs of gyrase and topoisomerase IV fold into pinwheel structures wherein the clusters of positively charged residues form blades that are capable of binding and bending DNA. Attempts to express the CTD of topoisomerase IIα apart from the N-terminal and core of the enzyme were unsuccessful, likely do to the predicted unstructured nature of this domain apart from the rest of the enzyme (James Berger, personal communication).

To date, only structures of the N-terminal and core domains of eukaryotic topoisomerase II are available. Once more structural data become available, it will be interesting to see if the CTD of topoisomerase IIα resembles that of the prokaryotic enzymes. However, to narrow down the region(s) of the topoisomerase IIα CTD that is responsible for the preferential relaxation of positively supercoiled DNA, a series of nested C-terminally deleted enzymes that removed clusters of positively charged amino acids was generated. Removing even one blade of positively charged residues from the CTD of topoisomerase IIα attenuated the ability of the enzyme to preferentially relax

overwound DNA. Replacing these last 30 amino acid residues with the corresponding region of topoisomerase IIβ did not restore this activity.

Unlike topoisomerase IIα, topoisomerase IIβ does not distinguish supercoil geometry for relaxation. However, replacing the CTD of the β isoform with that of the α isoform does confer the ability to distinguish supercoil geometry for relaxation (145). It would be interesting to replace just the final cluster of positively charged residues of topoisomerase IIβ with those of the α isoform. While results presented in this dissertation show that the last 30 residues of topoisomerase IIα are necessary for preferential relaxation of positively supercoiled DNA, swapping these residues of topoisomerase IIα into the CTD of topoisomerase IIβ would provide information on whether this cluster alone was sufficient.

Topoisomerase I Preferentially Relaxes and Cleaves Overwound DNA

If topoisomerase I acts ahead of replication forks to relieve accumulating positive supercoils, then it stands to reason that this enzyme would recognize and preferentially act on an overwound DNA substrate. Results presented in Chapter III show that topoisomerase I preferentially relaxes and cleaves positively supercoiled substrates.

Previous studies have shown that truncation of the N-terminal domain of topoisomerase I does not effect the ability of the enzyme to relax supercoiled substrates. However, while relaxation of negatively supercoiled DNA by full-length topoisomerase I is inhibited by the anti-cancer compound camptothecin, relaxation of similar substrates by topoisomerase I enzymes containing N-terminal truncations was unaffected by the drug (173). Results generated during the collaborative study of topoisomerase I relaxation

mentioned in this dissertation showed that relaxation of positively supercoiled substrates by N-terminally truncated topoisomerase I enzymes was inhibited in the presence of camptothecin. These results suggest that the N-terminal portion of the enzyme, while not strictly necessary for enzymatic function, plays a role in the removal of negative supercoils. Whether N-terminal deletions of topoisomerase I differentially affect the ability of the enzyme to cleave negatively or positively supercoiled substrates in the presence of anticancer drugs is still unknown.

The increased concentration of cleavage complexes generated with positively supercoiled substrates appears to correlate with decreased rates of enzyme-mediated DNA ligation (see Chapter IV). This seems counterintuitive given the fact that topoisomerase I also preferentially relaxes positive supercoils (189). However, since the enzyme removes multiple superhelical twists per event, ligation may not be the limiting step of the relaxation reaction. Alternatively, if the enzyme is less likely to ligate cleaved DNA, it may actually remove superhelical twists more rapidly.

The Geometry of DNA Supercoils Affects the Activity of Topoisomerase-targeted Agents

Beyond their important cellular functions, human topoisomerase I and topoisomerase II are targets for a number of highly effective anticancer agents that act at the enzyme-DNA interface and stabilize topoisomerase-DNA cleavage complexes (20,27,28,80,108,123,167). Type II topoisomerases maintain lower levels (~2– to 4–fold) of cleavage complexes with positively supercoiled as opposed to negatively supercoiled DNA (79,145,157). While this feature makes topoisomerase II safer to function ahead of replication and transcription complexes, it may make the enzyme less sensitive to the

actions of anticancer drugs. In contrast, as shown in Chapter III, human topoisomerase I maintains higher levels of cleavage complexes (~3–to 4–fold) with positively supercoiled substrates. This property makes topoisomerase I a potentially better target for therapeutic agents and suggests that the enzyme is intrinsically more dangerous to the cell than topoisomerase II. Studies in Chapter IV examine how DNA topology alters the actions of anticancer drugs that target human topoisomerases.

Results show that topoisomerase I maintains higher concentrations of cleavage complexes on overwound substrates in the presence of both camptothecin-based and non-camptothecin based anticancer drugs. Similar cleavage sites were utilized on both substrates. Enhanced topoisomerase I-mediated cleavage of positively supercoiled substrates in the presence of these poisons is thought to be due to increased baseline levels of cleavage on positively supercoiled substrates rather than an altered drug interaction in the enzyme-DNA complex.

Unexpected results were generated when intercalative compounds were included in topoisomerase I reactions. Intercalation of a compound into a covalently closed plasmid causes local unwinding of the double helix at the site of intercalation and compensatory positive supercoiling at distal sites on the plasmid. As topoisomerase I prefers to cleave positively supercoiled substrates, it was hypothesized that an intercalative topoisomerase I poison might have enhanced activity compared to a non-intercalative poison. Because there are as yet no known intercalative topoisomerase I poisons, experiments utilized intercalative compounds that do not poison topoisomerase I in the classic interfacial manner.

No enhancement of topoisomerase I-mediated cleavage enhancement of positively supercoiled or linear DNA was seen in the presence of intercalative compounds. In contrast, cleavage enhancement was seen when a negatively supercoiled substrate was employed. At least some of the enhanced topoisomerase I-mediated cleavage of underwound DNA seen in the presence of intercalative compound can be ascribed to the higher baseline levels of cleavage that topoisomerase I maintains on overwound substrates. However, cleavage enhancement of negatively supercoiled substrates in the presence of intercalators exceeded total cleavage levels maintained on positively supercoiled substrates. Thus, it is proposed that these compounds are topological poisons of topoisomerase I and enhance enzyme-mediated scission by altering the apparent superhelical state of the double helix.

In light of the *in vitro* results, and because the genetic material in human cells is globally underwound, it was hypothesized that DNA intercalators might also influence the ability of topoisomerase I to cleave the double helix *in vivo*. Indeed, results in Chapter IV show that enhanced trapping of topoisomerase I cleavage complexes was seen in a human cancer cell line that was treated with ethidium bromide. Treatment of cells with both ethidium bromide and topotecan lead to a higher enhancement of cellular topoisomerase I cleavage complex trapping than either drug alone. These results indicate that intercalative compounds may be capable of poisoning topoisomerase I *in vivo*. Two of the intercalative compounds used in the *in vitro* studies are known topoisomerase II poisons (129,156). Therefore, it would be interesting to examine whether or not intercalative topoisomerase II poisons also indirectly affect topoisomerase I activity *in*

vivo. The cellular results also suggest that an intercalative topoisomerase I poison might trap more topoisomerase I cleavage complexes *in vivo* than a non-intercalative poison.

The Effects of Enhanced Drug-DNA Interaction on DNA Cleavage Mediated by Topoisomerase II

Chapter V examines the effects that attaching a DNA interacting group at the C4 position of the core of the classic topoisomerase II poison etoposide has on the ability of the compound to poison type II topoisomerases. F14512 is an etoposide derivative that contains a spermine moiety in place of the C4 glycosidic moiety. F14512 was originally developed to increase the cellular uptake of etoposide by targeting the drug to the polyamine transport system which is upregulated in some cancer cells (148). However, the addition of the spermine group had an added effect on the compound in that it turned the drug into a DNA binder (148). Experiments in Chapter V show that F14512 is a stronger poison of both isoforms of topoisomerase II than is etoposide. Furthermore, unlike etoposide, F14512 maintains robust activity in the absence of ATP. The enhanced activity of F14512 correlates with an increased stability of the ternary topoisomerase II-drug-DNA complex, and experiments show that the spermine-drug core linkage is critical for this attribute.

The ability of F14512 to poison topoisomerase II also was compared to that of TOP-53. TOP-53 is an etoposide derivative that contains an aminoalkyl group at the C4 position. As determined by STD [^{1}H]-NMR spectroscopy, every proton in the C4 group of TOP-53 displays interactions with topoisomerase IIα in the absence of DNA; however, this drug is not known to interact with DNA in the absence of enzyme. Because no interactions were seen between topoisomerase II and the protons in the C4 moiety of

F14512, these drugs allow a comparison of similar etoposide-based compounds that have enhanced interactions with the enzyme (TOP-53) or DNA (F14512). F14512, the drug that interacted more strongly with DNA, was a more potent topoisomerase II poison *in vitro* than the drug (TOP-53) with enhanced enzyme interactions. Whether these results are unique to these two drugs or predictive for other compounds that poison topoisomerase II remains to be seen.

Drug resistance is a common roadblock to the successful treatment of human ailments. Several mechanisms contribute to resistance, including drug efflux, target protein down-regulation, and development of point mutations in target proteins that render the drug ineffective (27,112,116,211-214). One can speculate that increased cellular uptake *via* the polyamine system and tighter DNA interactions could help to counterbalance some drug efflux issues. Additionally, increased interactions with DNA and tighter binding in the enzyme-drug-DNA complex could help to overcome some instances of resistance due to protein point mutations. While topoisomerase point mutations are not a major cause of resistance in cancer therapy, antibacterial resistance due to type II topoisomerase point mutations is prevalent (215-217). Therefore, it would be interesting to see if attaching a spermine group to a known quinolone antibacterial topoisomerase II poison enhances the ability of the drug to overcome resistance mutations.

Ultimately, these findings demonstrate the utility of a C4 DNA binding group and provide a rational basis for the development of novel and more active etoposide-based topoisomerase II poisons.

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