COVALENT POISONS AND CATENATION: THE EFFECTS OF NATURAL PRODUCTS AND DNA TOPOLOGY ON REACTIONS CATALYZED BY TYPE II TOPOISOMERASES

By

Esha Deepak Dalvie

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 13, 2022

Nashville, Tennessee

Approved:

Neil Osheroff, Ph.D.

Frederick P. Guengerich, Ph.D.

James Dewar, Ph.D.

Emily Hodges, Ph.D.

Katherine Friedman, M.D.

.

To my Aai and Baba, who read to me from a biology book when I was two

and changed my whole life.

“It’s funny how day by day, nothing changes, but when you look back, everything is different.”

ACKNOWLEDGEMENTS

I would first like to thank my Ph.D. advisor, Dr. Neil Osheroff. Thank you for taking a chance on me, for supporting me, for encouraging me, for listening to me, and for being an amazing teacher and mentor over the past five years. You've made your lab a welcoming place and your belief in me has made me believe in myself. You have been so incredibly patient and immensely generous with your time, whether sending me draft edits at two in the morning or skyping me from New Zealand to help me write an abstract. From your excellent sense of humor and wonderful lessons and anecdotes I've learned both science and life skills, and they have made me a better scientist and a better person. I will take every lesson I have learned from this lab into my journey ahead. It has been an absolute honor to be a part of your lab and I have loved this time so much.

To my dissertation committee, Dr. Fred Guengerich, Dr. James Dewar, Dr. Emily Hodges, and Dr. Kathy Friedman: You all have been incredible teachers and your guidance and support has helped shape me into the scientist I have grown into. You held me to high standards, and though it was tough, I am so much better for it and I will always be grateful. Thank you for taking the time to assist me individually when I needed it and for being patient as I grew as a scientist. I have benefitted immensely from your expertise and wisdom.

To Jo Ann Byl: As I have conveyed many times before, you are the backbone of our lab. You work so hard and take the time to train us in safety, experiments, and becoming the best scientists we can be, while also checking in on how we are doing. I am so very fond of all our chats on every topic under the sun, and they never fail to make me smile.

To my former lab members Dr. Rachel Ashley, Dr. Lorena Infante Lara, Dr. Elizabeth Gibson, and Dr. Elirosa Minniti: Thank you for showing me the ropes and teaching me how to survive the

day-to-day of graduate school, but above all, thank you for being my friends. I will always cherish the fun adventures, get-togethers, and outings that very quickly made this lab feel like home.

To current lab members, Alexandria Oviatt, Jeffrey Jian, Jessica Collins, and Soziema Douda: You are all the siblings I never had! I have so much fun with you all, whether we're having game nights or just yelling jokes and teasing insults across our benches at each other. I have loved spending my days with you all. Thank you to Alexandria: I've been lucky enough to have been side by side with you on this journey since our first day of graduate school. You've become one of my closest, dearest friends and we've gone on so many adventures together, from hunting down the best chai in Nashville to running for trains in Italy. I hope we continue to have more fun times together. Thank you also to my bay-mate Jeff, who puts up with all my eccentric and capricious music tastes and has been a source of endless banter. I enjoy our crazy conversations, and you are the closest thing I have to a brother.

To all the students, both undergraduate and graduate, that I have trained, you have been my teachers as well. You all taught me how to best explain my endless diagrams and schematics and helped me understand my own work better. A special shoutout to Jordan Stacy, who helped me with my projects, provided me with much needed data, and did it all with a brilliant smile and a wonderful attitude. I'm glad you enjoyed the experience enough to come back to Vanderbilt as a graduate student!

Thank you to my undergraduate lab PIs, Dr. Larry Marnett at Vanderbilt and Dr. Dan Nomura at UC Berkeley. You were my first mentors, for inspiring my love of research, and for teaching me so much. I am exceedingly grateful for all your advice and guidance; it has been invaluable. Thank you to Dr. Will Beavers for being my introduction into lab work and for being patient with

me as I learned how to be a scientist. You continue to be a treasured mentor and friend to me even today, almost ten years later.

To my Aunt Brenda: Thank you for planting the seed of Vanderbilt into my head over a decade ago and opening your home and heart to me so many times. You provided so much kindness, encouragement, and boundless generosity, and you considered me a part of your family. You are the reason I came to Vanderbilt. I am so grateful you could see me start my science career, and I wish you had been able to see me graduate.

Thank you to all my dear friends that I have made over the years: Cailin, Florence, Dunya, Sarah, Matthew, Nicole, Natalie, Nikki, Melanie, Shannon, Meredith, and too many others to name here. You're all wonderful, lovely people and my life is so much better with you all in it. Thank you also for being patient when work made me too busy to hang out. I can't promise that will change, but I promise I really do love you all. Special thank you to Cailin for listening to me ramble on an almost-daily basis and not getting tired of me yet, and to Florence for celebrating my every accomplishment with such enthusiasm. I promise we'll live on the same coast again eventually. To my friends Margy, Steve, and Wendel: It has been so lovely to have you cheering me on through all these years. I am always buoyed by your kind words.

To my family: your support, even from so far away, has meant the world. To Kusum Maushi and Anand Kaka: you've been lifelong role models and I strive to be like you someday. To my Aji: no matter how many degrees I earn, I will never be as smart as you.

Thank you to my fur-sibling Toby for seeing me through from preschool to grad school and for being my constant companion over all those years. Thank you to Churro, who brings so much joy into every day with his boundless cheer, curiosity, and chaos. You've both taught me scheduling, discipline, and unconditional love.

To my Aai and Baba: You've been my cheerleaders my entire life, going through every high and low alongside me. You push me to be stronger and you make me better. You instilled in me a set of values and morals, curiosity, and a love of learning that has led me to pursue it my entire life. You are the reason I've made it this far and everything I am today is because of you.

Finally, I thank God for lifting me when I fall, holding my hand through both good and bad times, and protecting me through all of life's struggles. I owe this degree and my life to you.

TABLE OF CONTENTS

Page

DEDICATION iii

ACKNOWLEDGEMENTS iv

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xiii

Chapter

1. INTRODUCTION 1

DNA Topology 1
DNA Topoisomerases 4
Type II Topoisomerases 5
Enzyme Mechanics and Structure 6
Bacterial Type II Topoisomerases 11
Eukaryotic Type II Topoisomerases 13
Type II Topoisomerases as Therapeutic Targets 17
Antibacterial Drugs 22
Anticancer Drugs 24
Dietary and Medicinal Topoisomerase Poisons 25
The role of DNA Geometry on Type II Topoisomerase Activity 26
Scope of the Dissertation 30

2. METHODS 32

Enzymes 32
DNA Substrates 33
Drugs, Compounds, and Natural Products 35
DNA Cleavage 35
DNA Ligation 37
Persistence of Topoisomerase II-DNA Cleavage Complexes 37
Catenation 38
Cleavage of kDNA during Decatenation 39

3. 6,6'-DIHYDROXYTHIOBINUPHARADINE AS A POISON OF HUMAN TYPE II TOPOISOMERASES 41

Introduction....41
Results and Discussion....42
Conclusions....54

4. RECOGNITION OF DNA SUPERCOIL HANDEDNESS BY CATENATION CATALYZED BY TYPE II TOPOISOMERASES....57

Introduction....57
Results....58
Effects of DNA supercoil handedness on catenation catalyzed by human topoisomerase IIα and IIβ....58
Effects of DNA supercoil handedness on catenation catalyzed by bacterial topoisomerase IV....66
Effects of superhelical density on DNA catenation rates....68
Pathway to DNA catenation....70
Effects of catenation on DNA cleavage mediated by type II topoisomerases....80
Discussion....84

5. CONCLUSIONS AND IMPLICATIONS....88

Covalent Poisons and Natural Products....88
The Catenation/Decatenation Reaction and Recognition of DNA Supercoil Geometry....90

REFERENCES....95

LIST OF TABLES

Table Page

Chapter 1:

1.1. Properties of interfacial and covalent topoisomerase II poisons 21

Chapter 4:

4.1. $t_{1/2}$ and k values for Figure 4.1 and Figure 4.5.....61

LIST OF FIGURES

Figure Page

Chapter 1:

1.1. Topological relationships in DNA 2

1.2. The catalytic cycle of eukaryotic topoisomerase II 7

1.3. Domain structures of type II topoisomerases....10

1.4. The critical balance of DNA cleavage and religation....20

1.5. Structures of antibacterial and anticancer drugs 25

1.6. Geometry affects enzyme function during replication....27

Chapter 3:

3.1. Structure of 6,6'-dihydroxythiobinupharidine (DTBN) 43

3.2. DTBN increases levels of DNA double-stranded cleavage mediated by human type II topoisomerases....44

3.3. DNA cleavage induced by DTBN is mediated by human topoisomerase IIα 46

3.4. DTBN induces stable topoisomerase IIα-DNA cleavage complexes 47

3.5. The ability of DTBN to poison topoisomerase IIα is diminished by thiol containing compounds and reducing agents 50

3.6. DTBN inhibits the activity of human topoisomerase IIα when incubated with the enzyme prior to the addition of DNA....52

3.7. DTBN acts outside the active site of topoisomerase IIα....53

3.8. DTBN enhances DNA cleavage with a deletion construct of topoisomerase IIα that lacks the C-terminal domain. 55

3.9. DTBN does not compete with etoposide at the DNA cleavage-ligation active site of the enzyme 56

Chapter 4:

4.1. Human topoisomerase IIα shows supercoil preference during DNA catenation, while topoisomerase IIβ does not 60

4.2. Catenation of DNA by human topoisomerase IIα 62

4.3. Histone H1 shows similar affinity with different DNA topoisomers. 64

4.4. The condensing agent histone H1 is not required for supercoil handedness preference by topoisomerase IIα.....65

4.5. The C-terminal domain of human topoisomerase IIα is required for preferential catenation of negatively supercoiled DNA 67

4.6. Catenation of DNA by bacterial topoisomerase IV 69

4.7. 2D gel of DNA with varying levels of negative supercoiling.....71

4.8. 2D gel of DNA with varying levels of positive supercoiling 72

4.9. Effects of DNA with increasing levels of negative supercoiling on catenation 73

4.10. Effects of DNA with increasing levels of positive supercoiling on catenation 74

4.11. Catenation stopped with high heat.....76

4.12. Catenation stopped with SDS/EDTA versus high heat.....77

4.13. Production of catenation intermediates by human topoisomerase IIα.....78

4.14. Production of catenation intermediates by *B. anthracis* topoisomerase IV.....79

4.15. Catenation protects DNA from cleavage mediated by human topoisomerase IIα.....82

4.16. Amounts of linear kDNA do not increase after decatenation is complete.....83

4.17. Increases in DNA cleavage during decatenation reactions are not due to equilibration time 85

4.18. Catenation protects DNA from cleavage mediated by *E. coli* topoisomerase IV.....86

LIST OF ABBREVIATIONS

(-)SC	negatively supercoiled
(+)SC	positively supercoiled
ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	base pair
Cat	catenated
CI	confidence interval
DTBN	6,6'-dihydroxythiobinupharadine
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ETOP	etoposide
k	rate
kb	kilobase
kDNA	kinetoplast DNA
Lin	linear
Lk	linking number
Min	minicircle
Max	maxicircle
Nick	nicked
Rel	relaxed
SC	supercoiled

SDS	sodium dodecyl sulfate
Tw	twist
Wr	writhe
ΔLk	difference between the actual Lk and the Lk if the molecule were completely relaxed

CHAPTER ONE

INTRODUCTION

DNA is the most fundamental molecule in living systems. The sequence of base pairs within the double helix contains the information for every gene that encodes every vital process in a living cell. In humans, a diploid cell contains 6 billion bp of DNA, which is ~1.8 m in length. This must fit into a nucleus with diameter 10 μm and still be easily accessed and maintained (Kornberg and Baker 1992, Voet, Voet et al. 2002). As a result, DNA topology can become complicated.

DNA Topology

Topological properties of DNA are those that cannot be changed without breaking one or both strands of the double helix, assuming both ends are fixed in space (Bates and Maxwell 2005, Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Pommier, Sun et al. 2016, Ashley and Osheroff 2019). DNA Topology can be defined by mathematically by twist (Tw), writhe (Wr), and linking number (Lk). Twist refers to the number of double helical turns within a given segment of DNA. Positive twist is the right-handed twist on the Watson-Crick structure of DNA. Writhe is the number of times the double helix crosses itself when the DNA is projected in two dimensions. Crossovers are assigned a positive or negative value depending on the handedness: right-handed crosses are considered negative and left-handed crosses are considered positive (Figure 1.1). Linking number is the sum of twist and writhe and is invariant when the ends of DNA are fixed and the double helix is not broken. It is defined as follows:

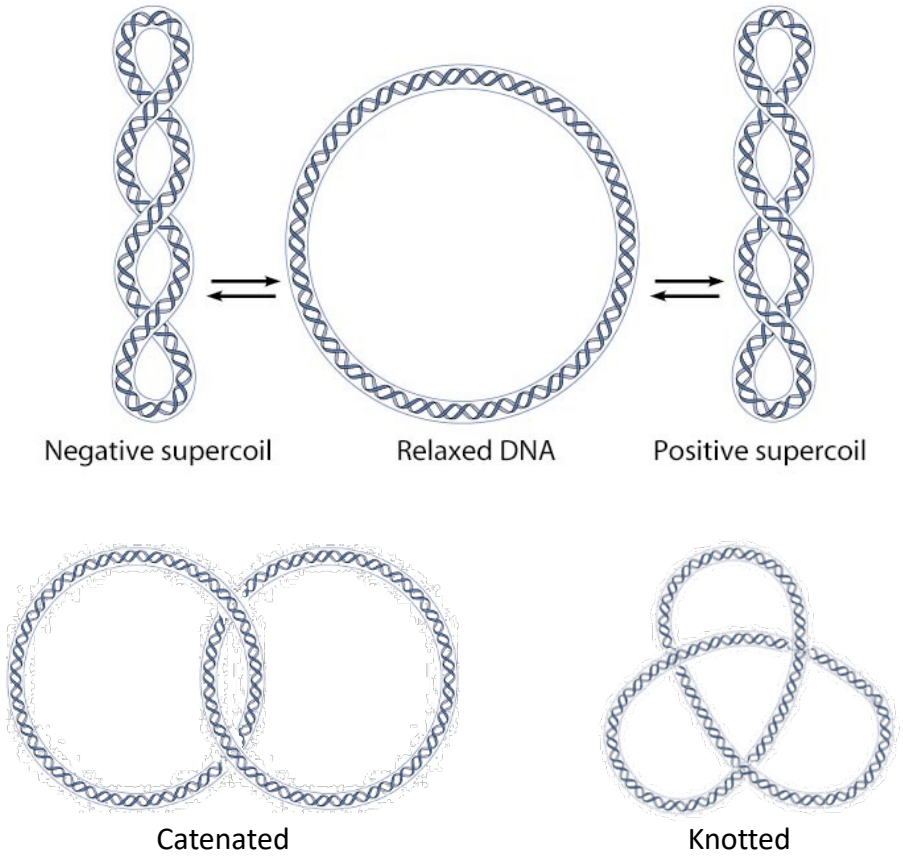

Figure 1.1: Topological relationships in DNA. Top: DNA that contains no torsional stress is considered "relaxed" (middle). Underwinding or overwinding the DNA results in negatively supercoiled (left) or positively supercoiled (right) DNA. These are depicted as Wr for visual clarity but Tw and Wr are interconvertible within these molecules. Bottom: Intermolecular catenanes (left) and intramolecular knots (right) can also form in DNA. In these cases, Tw and Wr are not interconvertible. Artwork by Dr. Rachel Ashley.

$$Lk = Tw + Wr$$

DNA is comprised of two interwound nucleic acid strands, and the genomes of most organisms are either very long, circular, or both (Wang 1996, Wang 2002, Bates and Maxwell 2005). These facts result in two distinct topological issues that all proliferating cells must cope with to survive.

The first issue is the torsional stress on the double helix: DNA in eubacteria and eukaryotes is globally ~6% underwound (Bates and Maxwell 2005, Deweese, Osheroff et al. 2008, Ashley and Osheroff 2019). Relaxed DNA is under no torsional stress and the double helix in this form would make one turn every 10.5 bp. Therefore, a relaxed DNA molecule with 1050 bp would have Lk = 100 (1050 ÷ 10.5 = 100). A right-handed plectonemically coiled double-helix will always have a positive Lk, as an Lk = 0 would mean that the DNA was completely melted with no crossings between the helix. DNA topology is often represented as the change in Lk, or ΔLk, which is defined as the difference between the actual Lk and the Lk if the DNA were completely relaxed. If $\Delta Lk \neq 0$, the DNA is under torsional stress. DNA under torsional stress is termed "supercoiled," as unconstrained over and underwound molecules writhe about themselves to form superhelical twists (one double helix wrapping around another) (Bates and Maxwell 2005, Deweese, Osheroff et al. 2008, Ashley and Osheroff 2019). Superhelical twists are a result of axial stress, which occurs when torsional stress can freely distribute across the molecule. Overwound DNA is referred to as positively supercoiled and has a positive ΔLk, while underwound DNA is referred to as negatively supercoiled and has a negative ΔLk (Bates and Maxwell 2005, Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Pommier, Sun et al. 2016, Ashley and Osheroff 2019).

Negative supercoiling adds energy into the genetic material and makes it easier to unwind during replication and transcription and facilitating both these processes (Liu and Wang 1987,

Wang 1996, Wang 2002, Schvartzman and Stasiak 2004). In contrast, DNA tracking systems such as replication forks and transcription machinery locally overwind the DNA ahead of these systems. This overwinding makes it harder to unwind the DNA and thereby can hinder many important cellular processes (Brill, DiNardo et al. 1987, Kim and Wang 1989, Wang 1996, Peter, Ullsperger et al. 1998, Wang 2002).

The second issue arises as a result of the long length of DNA (Bates and Maxwell 2005, Deweese, Osheroff et al. 2008, Ashley and Osheroff 2019). Nucleic acid knots (formed within a single DNA molecule) and tangles (between multiple DNA molecules) are commonly formed during processes such as DNA recombination and replication, respectively (Figure 1.1) (Wang 1996, Fortune and Osheroff 2000, Bates and Maxwell 2005, McClendon and Osheroff 2007, Deweese, Osheroff et al. 2008, Ashley and Osheroff 2019). DNA knots inhibit separation of the two strands of DNA and reduce the tensile strength of the genetic material (Liu, Deibler et al. 2009). DNA tangles, or catenanes, must be removed to allow the separation of sister chromatids during mitosis (Holm, Goto et al. 1985, Uemura, Ohkura et al. 1987, Baxter and Diffley 2008, Baxter, Sen et al. 2011, Bauer, Marie et al. 2012, Sen, Leonard et al. 2016). As a result, both topological structures can be lethal to cells if not removed.

DNA Topoisomerases

To maintain appropriate DNA supercoiling levels and remove knots and tangles, cells encode enzymes called topoisomerases (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Pommier, Sun et al. 2016, Austin, Lee et al. 2018, Ashley and Osheroff 2019). Topoisomerases modulate topological structures by creating transient breaks in the DNA backbone. There are two classes of

topoisomerases, defined by the number of DNA strands cleaved during reaction cycles. Type I topoisomerases create a transient single stranded break, or "nick," in the genetic material and facilitate controlled rotation about, or passage of another strand through, the nick (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Pommier, Sun et al. 2016, Austin, Lee et al. 2018, Ashley and Osheroff 2019) . Type II topoisomerases create a transient double stranded break through which they pass another intact double helix (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Pommier, Sun et al. 2016, Austin, Lee et al. 2018, Ashley and Osheroff 2019). To maintain genomic integrity during DNA cleavage, topoisomerases form covalent bonds between active site tyrosyl residues and newly generated DNA termini, forming complexes called "cleavage complexes." Type I topoisomerases can regulate DNA supercoiling and decatenation by creating transient single-stranded breaks followed by passage of the intact strand through the break, or by controlled rotation of the helix around the single-stranded break (Wang 1996, Champoux 2001, Leppard and Champoux 2005). Type II topoisomerases can resolve knots and tangles as well as removing torsional stress (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Pommier, Sun et al. 2016, Austin, Lee et al. 2018, Ashley and Osheroff 2019). This dissertation will focus on type II topoisomerases.

Type II Topoisomerases

There are two classes of type II topoisomerases: type IIA and type IIB, that are defined by homology. Bacteria encode two type IIA enzymes, DNA gyrase and topoisomerase IV. Eukaryotes, in contrast, encode only one type IIA enzyme, topoisomerase II (Austin and Marsh

1998, Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Pommier, Sun et al. 2016, Austin, Lee et al. 2018, Ashley and Osheroff 2019). Vertebrate species express two closely related isoforms of the enzyme, topoisomerase IIα and topoisomerase IIβ (Austin and Marsh 1998, Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Pommier, Sun et al. 2016, Austin, Lee et al. 2018, Ashley and Osheroff 2019). The only known type IIB topoisomerase, topoisomerase VI, exists in plants and archaea (Corbett, Schoeffler et al. 2005) and will not be further discussed in this dissertation.

Enzyme Mechanism and Structure

Type II topoisomerases regulate topology using a double-stranded DNA passage reaction, illustrated in Figure 1.2 using eukaryotic topoisomerase II as an example (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Vos, Tretter et al. 2011, Ashley and Osheroff 2019, Vann, Oviatt et al. 2021). Briefly, type II enzymes (in blue) (1) bind two DNA segments [the gate segment (in green), which will be cleaved by the enzyme, and the transport segment (in yellow), which will be passed through the open DNA gate]; (2) bend the gate segment; (3) create a double-stranded break in the gate segment; (4) translocate the transport segment through the cleaved double helix; (5) rejoin (i.e., ligate) the cleaved DNA; (6) release the transport segment through a gate in the protein; and (7) close the protein gate, regaining the ability to start a new round of catalysis. The scissile bonds on the two strands of the gate segment that are cut by type II topoisomerases are staggered. Type IIA enzymes generate cleaved DNA molecules that contain 4-base single-stranded cohesive ends at their 5′-termini.

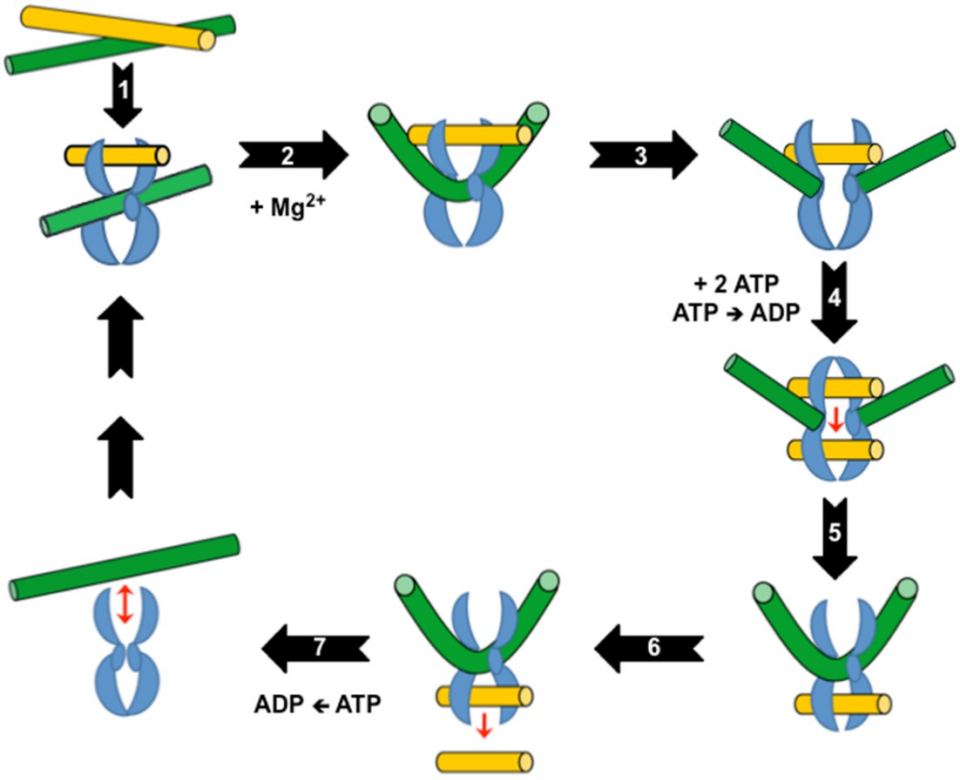

Figure 1.2: The catalytic cycle of eukaryotic topoisomerase II. The double-stranded DNA passage reaction of topoisomerase II can be separated into seven discrete steps. 1) Type II enzyme (blue) binding to two segments of DNA: the gate segment (green) and transport segment (yellow). 2) Bending of the gate segment, which requires the presence of Mg^{2+} (physiologically) or other divalent metal ions. These metal ions are required for all subsequent steps. 3) Double-stranded DNA cleavage of the gate segment (*i.e.*, formation of the cleavage complex). 4) Passage of the transport segment 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. 5) Ligation of the cleaved DNA gate segment. 6) Hydrolysis of the second ATP molecule, which allows release of the gate segment through a C-terminal gate in the protein and promotes 7) enzyme turnover and closing of the protein gate, which allows the enzyme to initiate a new round of catalysis. The protein is based on crystallographic studies of yeast topoisomerase II. Figure adapted from Ashley *et al*. (Ashley and Osheroff 2019).

Type II topoisomerases determine their sites of action by the ability to bend the gate segment (Lee, Dong et al. 2013, Jang, Son et al. 2019). This step is critical to the ability of these enzymes to recognize and cleave specific sites in the double helix. In addition, two cofactors are required for type II topoisomerases to carry out the catalytic double-stranded DNA passage reactions (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Ashley and Osheroff 2019). First, these enzymes require a divalent cation for all steps beyond enzyme-DNA binding. Magnesium(II) appears to be the divalent cation that the enzymes use in cells. Second, the energy of adenosine triphosphate (ATP) is used to drive the double-stranded DNA passage reaction. Type IIA topoisomerases do not require ATP for either DNA cleavage or ligation. However, the binding of this nucleoside triphosphate closes the top protein gate, which triggers DNA translocation, and its hydrolysis to adenosine diphosphate (ADP) and inorganic phosphate (Pi) is necessary for enzyme recycling. 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 (Lindsley and Wang 1993).

Type II topoisomerases share a number of common structural motifs that are shown in Figure 1.3 (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Ashley and Osheroff 2019, Dalvie and Osheroff 2021). 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) and acts as an A_2B_2 tetramer. GyrA contains the active site tyrosyl residue that forms the covalent bond with DNA during scission, and GyrB contains consensus sequences for the binding of ATP and the divalent metal ion. Bacterial topoisomerase IV is also an A_2B_2 tetramer comprised of two subunits (Figure 1.3) (Deweese,

Osheroff et al. 2008, Deweese and Osheroff 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Ashley and Osheroff 2019, Dalvie and Osheroff 2021). The A subunit contains the active site tyrosyl residue that forms the covalent bond with DNA during scission. The B subunit contains sequences for the binding of ATP and the divalent metal ion. In Gram-negative species, these subunits were first identified as proteins required for chromosomal partitioning. They 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.

A motif in the C-terminal domain of the GyrA subunit of gyrase forms a beta pinwheel (Corbett, Shultzaberger et al. 2004, Ashley, Dittmore et al. 2017). This 7-amino acid motif, termed the GyrA box, allows wrapping of the DNA to introduce negative supercoils and rapidly relax positive supercoils. It does this by constraining a local positive supercoil and inverting it to a negative supercoil upon T-segment transport (Corbett, Shultzaberger et al. 2004). The structure of the C-terminal domain of the ParC subunit of *Escherichia coli* topoisomerase IV contains a variable number of positively-charged "blades" that form a "broken pinwheel," which has the ability to bind but not wrap DNA (Corbett, Shultzaberger et al. 2004, Corbett, Schoeffler et al. 2005, Tretter, Lerman et al. 2010, Vos, Lee et al. 2013). The canonical GyrA box does not exist in the ParC C-terminal domain, but remnants of the motif are found in each of its three to eight blades (Tretter, Lerman et al. 2010, Vos, Lee et al. 2013, Ashley, Dittmore et al. 2017). While loss of the GyrA box abrogates the ability of gyrase to add negative supercoils into DNA, loss of the ParC C-terminal domain disrupts the ability of *E. coli* topoisomerase IV to distinguish between topologically distinct substrates (Corbett, Schoeffler et al. 2005, Vos, Lee et al. 2013, Ashley, Dittmore et al. 2017).

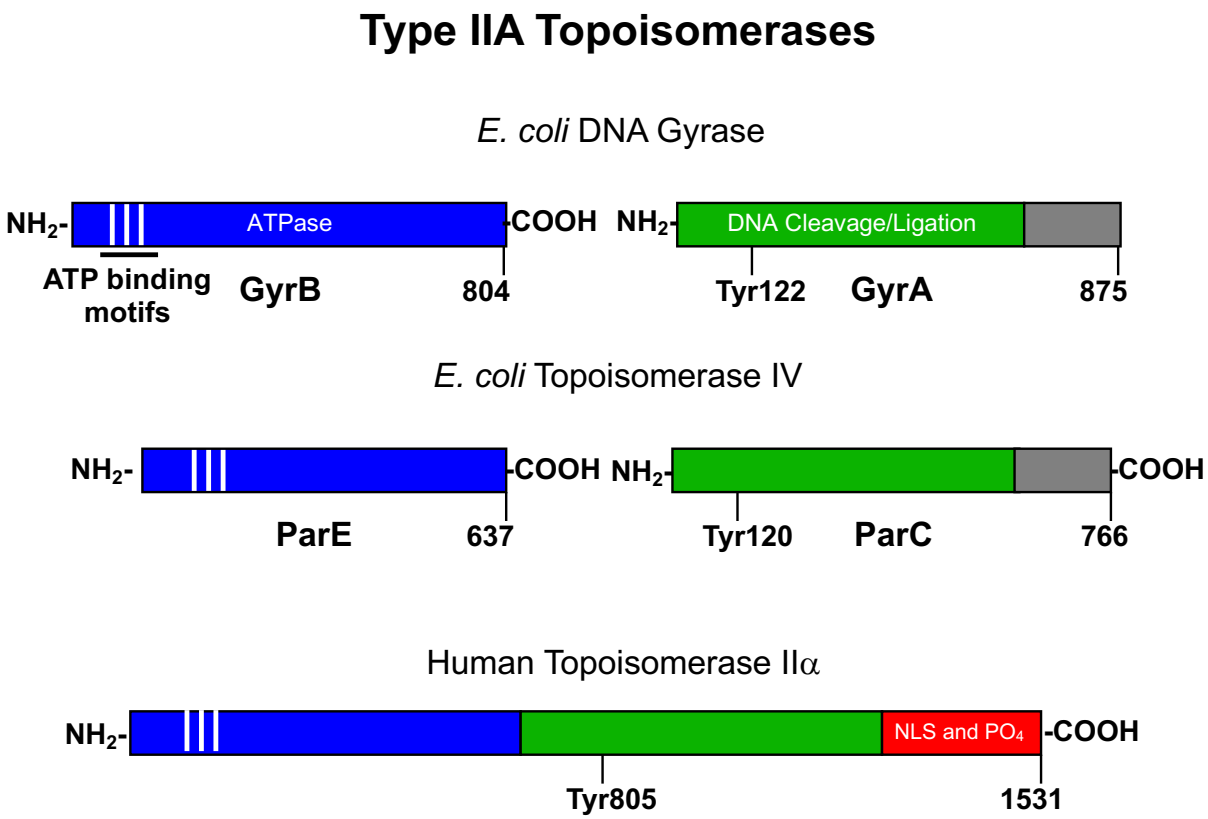

Figure 1.3: Domain structures of type II topoisomerases. The domain structures of three type IIA topoisomerases, bacterial (*Escherichia coli*) DNA gyrase and topoisomerase IV, and human topoisomerase II α are shown. Regions of homology among the enzymes are indicated by colors. The N-terminal (*i.e.*, GyrB) homology domains (blue) 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 N-terminal domain also contains the binding site for divalent metal ions. The central (*i.e.*, GyrA) homology domains (green) contain the active site tyrosyl residue that forms the covalent bond with DNA during scission. For human topoisomerase IIα, the variable C-terminal domain (red) contains nuclear localization sequences (NLS) and phosphorylation sites (PO_4). The active site tyrosine residue is indicated for each enzyme.

Eukaryotic type IIA topoisomerases are homologous to the bacterial enzymes (Figure 1.3) (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Ashley and Osheroff 2019, Dalvie and Osheroff 2021). However, the two subunits have fused into a single polypeptide with protomer molecular masses ranging from 160–180 kDa, depending on the species. Consequently, the eukaryotic enzymes function as homodimers. On the basis of amino acid sequence comparisons with bacterial gyrase, each topoisomerase II protomer can be divided into three distinct domains. The N-terminal domain of the enzyme is homologous to the B subunit of DNA gyrase (GyrB) and contains the binding site for ATP and the divalent metal ion. The central domain is homologous to the A subunit of DNA gyrase (GyrA) and contains the active site tyrosine. The C-terminal domain is variable and appears to have no corresponding region of homology in DNA gyrase. This region of the eukaryotic enzyme contains nuclear localization sequences as well as amino acid residues that are phosphorylated *in vivo*. An atomic structure of the C-terminal domain in eukaryotes has not been solved (McClendon, Gentry et al. 2008, Dalvie and Osheroff 2021).

Bacterial Type II Topoisomerases

DNA gyrase was the first type II topoisomerase to be discovered and was first reported in 1976 (Gellert, Mizuuchi et al. 1976, Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Ashley and Osheroff 2019). It is the only type II enzyme to retain its historical name. In contrast to other type II topoisomerases, DNA gyrase is the only enzyme that is capable of actively underwinding (i.e., negatively supercoiling) the double helix. It accomplishes underwinding by wrapping DNA around itself in a right-handed fashion (creating a positive supercoil) and carrying out its strand-passage

reaction in a unidirectional manner (thus converting a positive to a negative supercoil). As mentioned previously, this ability to wrap DNA is conferred by the beta pinwheel structure of the gyrase C-terminal domain (Corbett, Shultzaberger et al. 2004).

The ability of gyrase to wrap DNA during its strand passage reaction allows it to remove positive supercoils that accumulate in front of replication forks and transcription complexes even faster than it can introduce negative supercoils into relaxed DNA (Ashley, Dittmore et al. 2017). Thus, DNA gyrase plays a critical role in opening the double helix for these two physiological processes. In addition, DNA gyrase works in conjunction with the ω protein (a type I topoisomerase that removes negative supercoils from the double helix), to maintain the global balance of DNA supercoiling in bacterial cells (Mirkin, Zaitsev et al. 1984). Because of its DNA wrapping mechanism, DNA gyrase works primarily on DNA supercoiling; it is far less efficient at removing knots and tangles from the genome. In bacteria, these reactions are carried out primarily by the other type II topoisomerase, topoisomerase IV.

By the late 1980s, it had been known for several years that the ParC and ParE proteins were necessary for proper chromosome partitioning in bacteria. However, in 1990, it was discovered that these two subunits together constituted a second bacterial type II topoisomerase (Kato, Nishimura et al. 1990, Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Ashley and Osheroff 2019).

The catalytic properties of topoisomerase IV can be distinguished from those of DNA gyrase in two important ways (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Ashley and Osheroff 2019). First, although topoisomerase IV can remove positive and negative superhelical twists from DNA, it cannot actively underwind the double helix. Second, the ability of topoisomerase IV to

resolve DNA knots and tangles is considerably better than that of DNA gyrase. Because of these differences, the physiological roles of the two bacterial enzymes are distinct from one another. The primary cellular functions of topoisomerase IV are to unlink daughter chromosomes following DNA replication and to resolve DNA knots that are formed during recombination. Recently, it was found that topoisomerase IV removes positive supercoils from DNA more efficiently than it does negative supercoils (Vos, Lee et al. 2013, Ashley, Dittmore et al. 2017). Mutation and deletion studies suggest that this ability is due to specific sequences in the "blades" of the C-terminal domain and have led to speculation that the enzyme may also play a role ahead of DNA tracking systems to help alleviate the accumulation of torsional stress in the double helix (Vos, Lee et al. 2013, Ashley, Dittmore et al. 2017). However, the precise function of topoisomerase IV in this process has yet to be defined.

Eukaryotic Type II Topoisomerases

The eukaryotic type IIA enzyme, topoisomerase II, was discovered in 1980 (Liu, Liu et al. 1980, Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Ashley and Osheroff 2019). Topoisomerase II can remove positive or negative superhelical twists from the double helix and can resolve DNA knots and tangles. Eukaryotic species such as yeast and *Drosophila* encode only a single type II topoisomerase (i.e., topoisomerase II) (Goto and Wang 1982, Goto, Laipis et al. 1984, Goto and Wang 1984, Nolan, Lee et al. 1986). However, as discussed above, vertebrates contain two isoforms, topoisomerase IIα and topoisomerase IIβ (Austin and Marsh 1998, Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Gentry and Osheroff 2013, Austin, Lee et al. 2018, Ashley and Osheroff 2019).

These two isoforms share extensive amino acid sequence similarity (~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 of ~170 and ~180 kDa, respectively.

Topoisomerase II plays a number of essential roles in eukaryotic cells and participates in virtually every major process that involves the genetic material (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Pommier, Sun et al. 2016, Ashley and Osheroff 2019). It unlinks daughter chromosomes that are tangled during replication and resolves DNA knots that are formed during recombination. It also helps to remove the positive DNA supercoils that are generated ahead of replication forks and transcription complexes. 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.

It is not obvious why vertebrates encode two distinct isoforms of topoisomerase II (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Pommier, Sun et al. 2016, Ashley and Osheroff 2019). 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 from 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 (McClendon, Rodriguez et al. 2005). As with topoisomerase IV, this

ability of topoisomerase IIα is embodied in the C-terminal domain of the protein (McClendon, Gentry et al. 2008).

Topoisomerase IIα and topoisomerase IIβ have distinct patterns of expression and cellular functions (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Forterre and Gadelle 2009, Vos, Tretter et al. 2011, Chen, Chan et al. 2013, Pommier, Sun et al. 2016, Ashley and Osheroff 2019). Topoisomerase IIα is required for the survival of proliferating cells (Deweese and Osheroff 2009, Nitiss 2009, Chen, Chan et al. 2013, Ketron and Osheroff 2014, Pommier, Sun et al. 2016). Enzyme levels increase throughout S-phase of the cell cycle and peak at the G2/M boundary. Topoisomerase IIα is found almost exclusively in proliferating tissues, is associated with replication forks, and remains tightly bound to chromosomes during mitosis. Thus, it is believed to be the isoform that functions in growth-related processes, such as DNA replication and chromosome segregation. Topoisomerase IIα also appears to play roles in transcription.

Topoisomerase IIβ is non-essential at the cellular level but is believed to be required for proper neural development in mice (Yang, Li et al. 2000, Deweese and Osheroff 2009, Nitiss 2009, Chen, Chan et al. 2013, Ketron and Osheroff 2014, Austin, Lee et al. 2018). In contrast to the α isoform, the concentration of topoisomerase IIβ is independent of the cell cycle and this isoform is found at high concentrations in most cell types regardless of proliferation status. Topoisomerase IIβ dissociates from chromosomes during mitosis and cannot overcome the loss of topoisomerase IIα in mammalian cells. Although the physiological functions of the β isoform have yet to be fully defined, a number of studies indicate that it is important for the transcription of hormonally- or developmentally-regulated genes (Yang, Li et al. 2000, Deweese and Osheroff 2009, Nitiss 2009, Chen, Chan et al. 2013, Ketron and Osheroff 2014, Austin, Lee et al. 2018).

The C-terminal domain of topoisomerase IIα in vertebrates contains several sites for post-translational modification, including phosphorylation, SUMOlaytion, ubiquitination, and acetylation, as well as sites that allow for concentration of the enzyme at centromeres during mitosis (Linka, Porter et al. 2007, Antoniou-Kourounioti, Mimmack et al. 2019). Additionally, sites for SUMOylation in topoisomerase IIα in *Xenopus laevis* regulate the localization of binding proteins to the centromere during mitosis, and this may extend to the human enzyme as well (Ryu, Yoshida et al. 2015, Yoshida, Ting et al. 2016, Antoniou-Kourounioti, Mimmack et al. 2019, Dalvie and Osheroff 2021). In humans, sequences in the C-terminal domain of topoisomerase IIα direct localization to the nucleus and their absence in some drug-resistant cancer cell lines causes the enzyme to localize to the cytoplasm (Mirski, Gerlach et al. 1997). The C-terminal domain of human topoisomerase IIβ may perform a negative regulatory role that diminishes the binding of the enzyme with DNA due to an added sequence of amino acids that promotes protein-protein interactions over protein-DNA interactions (Gilroy and Austin 2011, Kozuki, Chikamori et al. 2017, Dougherty, Hawaz et al. 2021). The C-terminal domain of topoisomerase IIβ may also perform many of the same roles as that of topoisomerase IIα, but with reduced efficiency (Linka, Porter et al. 2007). The C-terminal domains of both human topoisomerase IIα and topoisomerase IIβ have several redundant roles with each other, such as supporting cell proliferation and regulating the decatenation checkpoint (Kozuki, Chikamori et al. 2017).

Human topoisomerase IIα relaxes positively supercoiled DNA faster than negatively supercoiled DNA, while human topoisomerase IIβ does not distinguish between positively and negatively supercoiled DNA during relaxation (McClendon, Rodriguez et al. 2005, McClendon, Dickey et al. 2006, McClendon, Gentry et al. 2008). Between topoisomerase IIα and topoisomerase IIβ, there is only ~31% amino acid sequence identity in the C-terminal domain as

compared to ~79% in their N-terminal and central domains, suggesting that the differences in recognition of supercoil handedness during relaxation may be due to the differences in amino acid sequence identity between the C-terminal domain of the isoforms (Austin, Sng et al. 1993, McClendon, Gentry et al. 2008). When the C-terminal domain was removed from topoisomerase IIα in the deletion construct hTIIαΔ1175 (Dickey and Osheroff 2005), topoisomerase IIα relaxed positively supercoiled and negatively supercoiled DNA at the same rate, suggesting that the C-terminal domain is required for recognition of supercoil handedness during relaxation (McClendon, Dickey et al. 2006, McClendon, Gentry et al. 2008). Additionally, when the C-terminal domain of topoisomerase IIα and topoisomerase IIβ were switched, topoisomerase IIβ was able to preferentially relax positively supercoiled DNA while topoisomerase IIα relaxed both positively and negatively supercoiled DNA at the same rate, further demonstrating that the ability to recognize supercoil handedness when relaxing DNA supercoils resides in the C-terminal domain (McClendon, Gentry et al. 2008). These results suggest that the catalytic core of topoisomerase IIα is indifferent to supercoil geometry during relaxation and does not differentiate between positive and negative supercoils, but that the C-terminal domain interacts uniquely with positively supercoiled DNA in a way that is able to facilitate relaxation. Therefore, when the C-terminal domain is removed, the enzyme relaxes positively and negatively supercoiled DNA at similar rates (McClendon, Gentry et al. 2008).

Type II Topoisomerases as Therapeutic Targets

Although type IIA topoisomerases carry out a number of critical physiological functions, they also generate double-stranded breaks in DNA as a requisite step in their reaction cycles (Figure 1.3) (Pendleton, Lindsey et al. 2014). Thus, they are fundamentally dangerous proteins

that have the potential to fragment the genome. (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Nitiss 2009, Aldred, Kerns et al. 2014, Ketron and Osheroff 2014, Pendleton, Lindsey et al. 2014, Pommier, Sun et al. 2016, Ashley and Osheroff 2019, Vann, Oviatt et al. 2021) Under normal conditions, covalent topoisomerase II-cleaved DNA complexes (*i.e.,* cleavage complexes) are tightly regulated, present at low steady-state levels, and short-lived. Consequently, their presence is tolerated in cells. However, conditions that significantly increase the concentration or lifetime of cleavage complexes trigger a cascade of mutagenic and potentially lethal events (Figure 1.4).

The potential lethality of cleavage complexes rises substantially when DNA tracking enzymes such as polymerases or helicases attempt to traverse the covalently bound topoisomerase "roadblock" in the genetic material (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Nitiss 2009, Aldred, Kerns et al. 2014, Ketron and Osheroff 2014, Pendleton, Lindsey et al. 2014, Pommier, Sun et al. 2016, Ashley and Osheroff 2019, Vann, Oviatt et al. 2021). Such an action disrupts cleavage complexes and prevents the bound enzyme from ligating the generated DNA breaks. As a result, transient cleavage complexes are converted to long-lived DNA breaks that may be resected by recombination repair pathways, triggering the generation of chromosomal insertions, deletions, translocations, and other aberrations. When these strand breaks are present in sufficient numbers, they initiate a series of events that culminates in cell death.

The inherent danger of cleavage complexes has made the type IIA topoisomerases targets for some of the most important antibacterial and anticancer drugs currently in clinical use (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Nitiss 2009, Aldred, Kerns et al. 2014, Ketron and Osheroff 2014, Pendleton, Lindsey et al. 2014, Pommier, Sun et al. 2016, Gibson, Ashley et al. 2018, Ashley and Osheroff 2019, Bax, Murshudov et al. 2019, Vann, Oviatt

et al. 2021). In contrast to most therapeutics, these agents do not act by robbing cells of an essential enzyme activity. Rather, drugs that target type II topoisomerases kill cells by dramatically increasing the concentration and stability of covalent enzyme-DNA cleavage complexes. Since the drugs that target type II 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 that act by robbing the cell of the catalytic functions of these enzymes.

There are two main types of topoisomerase poisons; Interfacial poisons act at the active site of the enzyme by intercalating into the cleaved scissile bond between the enzyme and DNA, and covalent poisons form covalent adducts with cysteine residues distal to the active site (Bandele and Osheroff 2008, Deweese and Osheroff 2009, Wu, Li et al. 2011, Pommier and Marchand 2012, Dalvie, Gopas et al. 2019, Vann, Oviatt et al. 2021). Although both enhance enzyme-mediated DNA cleavage, there are many properties that differentiate interfacial poisons from covalent poisons (Table 1.1).

Interfacial poisons such as etoposide act non-covalently at the cleavage/religation active site. They enhance DNA cleavage by inserting into the cleaved bond and physically block religation (Wu, Li et al. 2011, Pommier and Marchand 2012). In addition, interfacial poisons enhance DNA cleavage when incubated with the enzyme prior to the addition of DNA.

Covalent poisons such as benzoquinone adduct to the enzyme at a site distal to the active site (Wang, Mao et al. 2001, Lindsey, Bromberg et al. 2004, Deweese and Osheroff 2009, Ketron and Osheroff 2014). They enhance DNA cleavage by a mechanism believed to involve the ability to close the N-terminal protein gate (Wang, Mao et al. 2001, Bender, Lehmler et al. 2006, Bender and Osheroff 2007, Lindsey, Pendleton et al. 2014). As covalent poisons often form adducts with

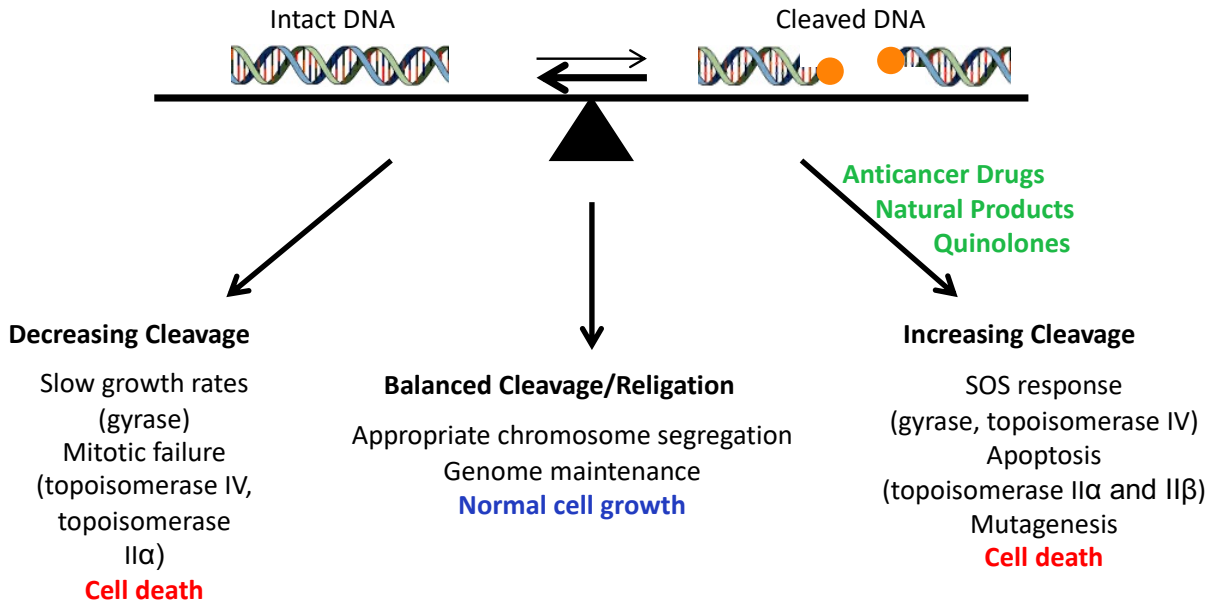

Figure 1.4: The critical balance of DNA cleavage and religation. The activity of type II topoisomerases must be tightly regulated in the cell. When cleavage/religation is balanced, the genome can be appropriately maintained and the cell can grow normally. If the level of cleavage/religation decrease, slow growth rates and mitotic failure can cause cell death. Conversely, if the levels of cleavage/relegation are too high, DNA damage can overwhelm the cell, leading to mutagenesis and cell death. Figure adapted from Pendleton *et al* (Pendleton, Lindsey et al. 2014).

Table 1.1: Properties of Interfacial and Covalent Topoisomerase II Poisons

Interfacial Poisons	Covalent Poisons
• Act non-covalently at the active site • Unaffected by reducing agents • Enhance enzyme-mediated DNA cleavage when added to the enzyme-DNA complex • Enhance DNA cleavage when incubated with the enzyme prior to the addition of DNA	• Covalently adduct the enzyme distal to the active site • Abrogated by reducing agents • Enhance enzyme-mediated DNA cleavage when added to the enzyme-DNA complex • Inhibit topoisomerase II when incubated with the enzyme prior to the addition of DNA

cysteine residues and require redox cycling, their effects can be abrogated by reducing agents (Wang, Mao et al. 2001, Lindsey, Bromberg et al. 2004, Deweese and Osheroff 2009, Ketron, Gordon et al. 2013). Furthermore, incubating a covalent poison with the enzyme prior to addition of DNA will inhibit DNA cleavage.

Antibacterial Drugs

DNA gyrase and topoisomerase IV are the targets for fluoroquinolones (Figure 1.5) (Aldred, Kerns et al. 2014, Gibson, Ashley et al. 2018, Bax, Murshudov et al. 2019). These drugs are the most active and broad spectrum oral antibacterial agents in clinical use and are among the most widely prescribed antibacterials worldwide. Drugs such as ciprofloxacin are prescribed routinely for a wide variety of Gram-negative bacterial infections, including gastrointestinal tract and bone and joint infections. Ciprofloxacin and related fluoroquinolones are also used to treat a number of sexually transmitted diseases as well as infection with anthrax. Newer fluoroquinolones, such as levofloxacin and moxifloxacin, display significant efficacy against Gram-positive bacterial infections, such as those found in the respiratory tract. They are also used for treating tuberculosis. Fluoroquinolones increase the level of cleavage complexes formed by DNA gyrase and topoisomerase IV. However, depending on the species of bacteria and the fluoroquinolone being used, either DNA gyrase or topoisomerase IV may be the more important drug target (Aldred, Kerns et al. 2014, Gibson, Ashley et al. 2018, Bax, Murshudov et al. 2019).

New classes of drugs that target bacterial type II topoisomerases are currently in development (Gibson, Ashley et al. 2018, Bax, Murshudov et al. 2019). The most advanced of these include Novel Bacterial Topoisomerase Inhibitors, or NBTIs, and spiropyrimidinetriones, or SPTs. As an example, gepotidacin, an NBTI, (Figure 1.5) is currently in phase three clinical trials,

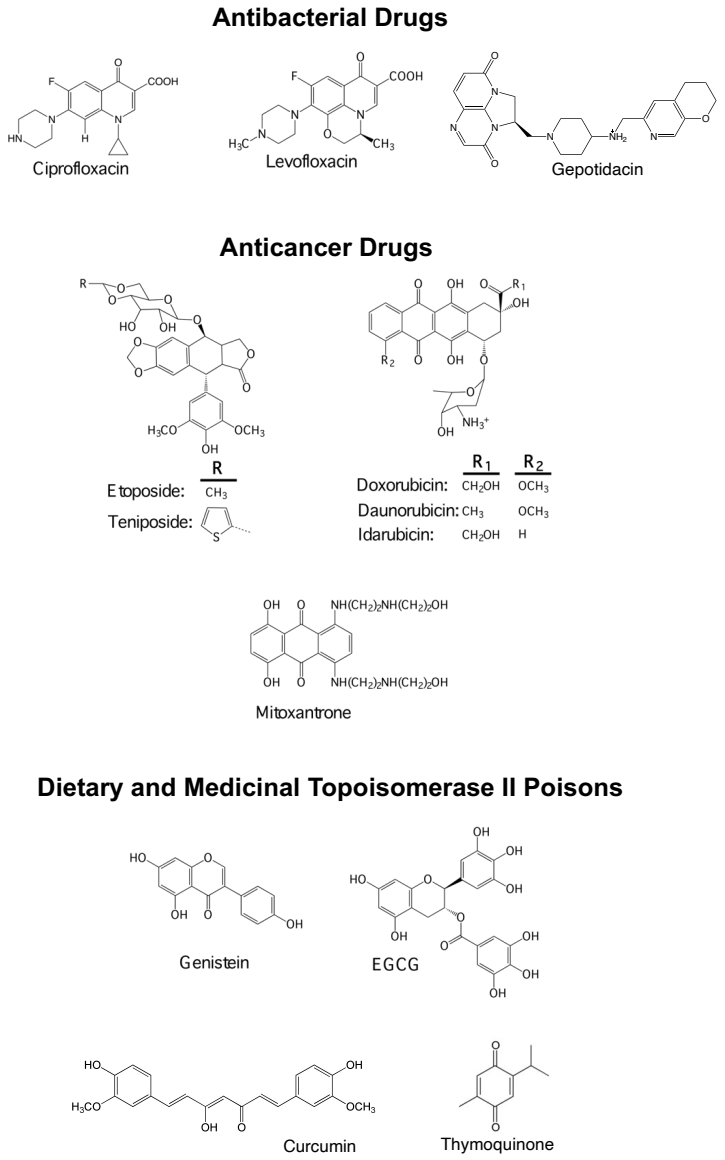

Figure 1.5: Structures of antibacterial and anticancer drugs. Structures of selected antibacterial drugs targeted to DNA gyrase and topoisomerase IV, anticancer drugs targeted to topoisomerase II, and dietary and medicinal topoisomerase II poisons.

as is zoliflodacin, an SPT (Gibson, Bax et al. 2019). In contrast to fluoroquinolones and SPTs, which generate enzyme-mediated double-stranded DNA breaks, NBTIs generate single-stranded breaks.

Anticancer Drugs

At the present time, six topoisomerase II-targeted anticancer agents (Figure 1.5) are approved for use in the United States (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Nitiss 2009, Aldred, Kerns et al. 2014, Ketron and Osheroff 2014, Pendleton, Lindsey et al. 2014, Pommier, Sun et al. 2016, Gibson, Ashley et al. 2018, Ashley and Osheroff 2019, Bax, Murshudov et al. 2019, Dalvie and Osheroff 2021). Additional drugs that target the type II enzyme are used worldwide. Drugs such as etoposide and doxorubicin are front-line therapy for breast and lung cancers, as well as a variety of leukemias, lymphomas, and germ-line malignancies. Mitoxantrone is gaining usage against breast cancers. 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.

Due to the high concentration of topoisomerase IIα and topoisomerase IIβ in rapidly proliferating cells, both isoforms probably play important roles in anticancer therapy. Unfortunately, because topoisomerase II-targeted drugs induce double- stranded breaks, their use is also associated with the generation of chromosomal translocations that result in secondary leukemias in a small proportion of patients (Felix 2001, Baldwin and Osheroff 2005, Joannides and Grimwade 2010, Joannides, Mays et al. 2011, Ezoe 2012, Rashidi and Fisher 2013, Pendleton, Lindsey et al. 2014). Circumstantial evidence suggests that the β isoform is primarily responsible

for this and other side effects of these important chemotherapeutic drugs (Smith, Cowell et al. 2014).

Dietary and Medicinal Topoisomerase II Poisons

The most prevalent dietary topoisomerase II poisons are the bioflavonoids (e.g., phytoestrogens) (Figure 1.5) (Austin, Patel et al. 1992, Bandele and Osheroff 2007, Bandele, Clawson et al. 2008, Bandele and Osheroff 2008, Ketron and Osheroff 2014). These diverse polyphenolic compounds are components of many fruits, vegetables, legumes, and plant leaves. Bioflavonoids are an integral component of the human diet and represent the most abundant natural source of antioxidants. It is believed that the dietary intake of these compounds provides a number of health benefits, including protection against cancer, cardiovascular disease, osteoporosis, and inflammation. The mechanistic basis for the physiological actions of phytoestrogens is complex, and they have a variety of effects on human cells. However, several common bioflavonoids, including genistein and (–)-epigallocatechin gallate (EGCG), are potent topoisomerase II poisons (Bandele and Osheroff 2007, Bandele and Osheroff 2008, Azarova, Lin et al. 2010, Lopez-Lazaro, Calderon-Montano et al. 2011). Genistein is abundant in soy products and is believed to contribute to the lower incidence of specific types of cancers in the Pacific Rim. EGCG is the most abundant and biologically active polyphenol in green tea, and many of the therapeutic benefits of the beverage have been attributed to this compound. Beyond the bioflavonoids, curcumin is the flavor and olfactory component of turmeric, a widely used spice in Indian and Southeast Asian cooking, and displays antioxidant, anti-inflammatory, antibacterial, and chemopreventative properties (Lopez-Lazaro, Willmore et al. 2007, Ketron, Gordon et al. 2013). Finally, thymoquinone, which is the active component in black seed, is an ancient medicinal compound used to treat a variety of

diseases associated with inflammation. It also displays anticancer activity (Ashley and Osheroff 2014). The potential for a natural product to be a topoisomerase II poison will be further discussed in Chapter 3.

The role of DNA Geometry on Type II Topoisomerase Activity

As has been previously mentioned, DNA in eukaryotic and bacterial cells is globally underwound (i.e., negatively supercoiled) approximately 6% (Linka, Porter et al. 2007, Deweese and Osheroff 2009). Overwound (i.e., positively supercoiled) DNA that accumulates ahead of replication forks and transcription complexes results in torsional stress that must be alleviated for these processes to continue (Linka, Porter et al. 2007, Deweese and Osheroff 2009, Baxter, Sen et al. 2011). As this buildup of positive supercoils represents an acute temporal challenge for genomic activities, it is unsurprising that bacterial and human species encode at least one type II enzyme that preferentially relaxes overwound DNA.

Bacterial gyrase, which acts ahead of the replication machinery, has to deal with the accumulation of approximately 100 positive supercoils per second during replication and relaxes overwound DNA dramatically faster than it can generate negative supercoils in relaxed substrates (Figure 1.6) (Ashley, Blower et al. 2017, Ashley, Dittmore et al. 2017). Even though topoisomerase IV works primarily behind replication forks as a decatenase, it also has the capacity to preferentially remove positive over negative supercoils (Figure 1.6) (Ashley, Blower et al. 2017, Ashley, Dittmore et al. 2017).

In humans, topoisomerase IIα removes positive supercoils approximately 10-fold faster than it does negative supercoils (McClendon, Rodriguez et al. 2005, McClendon, Dickey et al. 2006, McClendon, Gentry et al. 2008). In contrast, topoisomerase IIβ, which does not appear to

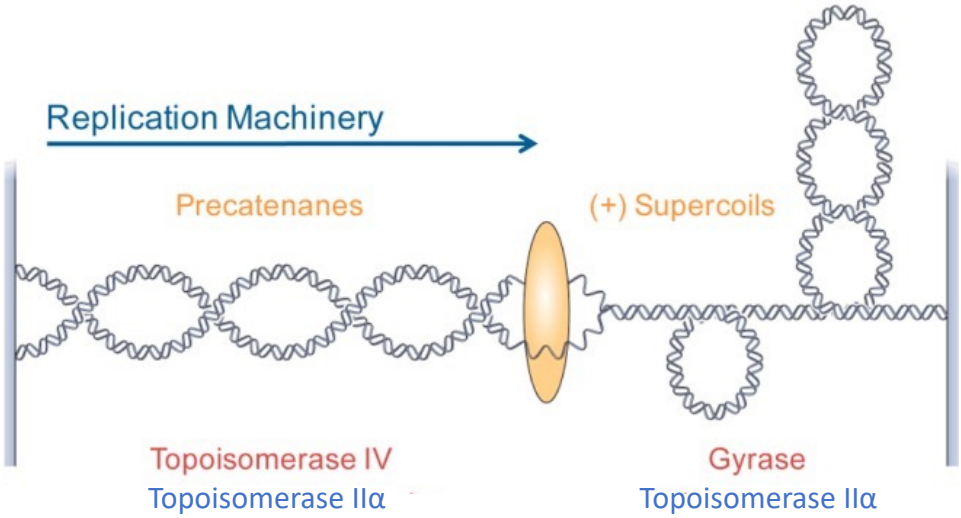

Figure 1.6: Geometry affects enzyme function during replication. In bacteria, topoisomerase IV acts behind the replication fork while gyrase acts ahead of the replication fork. In humans, topoisomerase IIα acts on both sides of the replication fork but prefers to act behind it. Figure adapted from Ashley *et. al* (Ashley, Dittmore et al. 2017).

play a role in replication, cannot distinguish between over and underwound DNA during the DNA relaxation reaction (McClendon, Rodriguez et al. 2005, McClendon, Dickey et al. 2006, McClendon, Gentry et al. 2008). As mentioned previously in this chapter, the ability of the α isoform to preferentially relax positive supercoils is associated with elements in the C-terminal domain of the enzyme, and when this domain was removed in a deletion construct, positively and negatively supercoiled DNA were relaxed at the same rate, suggesting that the C-terminal domain is required for recognition of supercoil handedness during relaxation (McClendon, Gentry et al. 2008). While there is evidence that topoisomerase IIα acts ahead of the replication fork, it is believed to primarily act behind it and it is not known where topoisomerase IIβ acts during replication (Figure 1.6) (McClendon, Rodriguez et al. 2005, Heintzman, Campos et al. 2019).

Type II topoisomerases can also recognize supercoil geometry during the DNA cleavage reaction. Bacterial gyrase maintains lower levels of cleavage complexes on positively supercoiled DNA, while topoisomerase IV shows no large difference in cleavage of positively versus negatively supercoiled DNA (Ashley, Blower et al. 2017, Ashley, Dittmore et al. 2017). This may reduce the effectiveness of gyrase as an antibacterial target, making it a safer enzyme, as it works ahead of the replication fork and therefore is less likely to cause permanent double stranded breaks (Ashley, Dittmore et al. 2017). Topoisomerase IV primarily works behind the replication fork as a decatenase, and although it maintains high levels of cleavage with positively supercoiled DNA, there are fewer chances for disruptions by tracking systems (Ashley, Dittmore et al. 2017).

Human topoisomerase IIα and IIβ both maintain ~3-4-fold lower levels of cleavage complexes on positively supercoiled DNA (McClendon and Osheroff 2006). As positively supercoiled DNA accumulates ahead of the replication fork, this supercoil handedness preference during cleavage helps prevent collisions between cleavage complexes and the advancing

machinery. Similarly, in the presence of non-intercalating drugs, topoisomerase IIα maintained lower levels of cleavage intermediates with positively supercoiled DNA. Topoisomerase IIβ did this as well, but in some instances, such as with genistein and TOP-53, the enzyme induced similar levels of cleavage between positively and negatively supercoiled DNA, suggesting that drug-induced stimulation of DNA cleavage by topoisomerase IIβ is more dangerous as it is more likely to cause permanent strand breaks (McClendon and Osheroff 2006). Intercalative drugs, however, saw greater levels of DNA cleavage of positively supercoiled DNA with both topoisomerase IIα and IIβ due to increased apparent positive supercoiling and preferential drug accumulation in negatively supercoiled substrates (McClendon and Osheroff 2006). This remained true even at high concentrations of drug, while negatively supercoiled DNA saw attenuated levels of DNA cleavage at those same high concentrations, suggesting that intercalative drugs maintain their effectiveness ahead of DNA tracking systems (McClendon and Osheroff 2006).

Cellular studies in bacteria and yeast have suggested that immediately prior to decatenation, DNA becomes positively supercoiled (Baxter, Sen et al. 2011, Zawadzki, Stracy et al. 2015). However, during this time, the DNA becomes associated with condensins and other mitotic factors that may influence the process. These studies suggest that type II topoisomerases may preferentially decatenate positively over negatively supercoiled DNA (Baxter, Sen et al. 2011, Zawadzki, Stracy et al. 2015). In support of this conclusion, single molecule experiments indicate that human topoisomerase IIα decatenates positively intertwined molecules approximately 2-fold faster than negatively supercoiled substrates (Seol, Gentry et al. 2013). The role of supercoil geometry in catenation/decatenation of DNA by type II topoisomerases will be further discussed in Chapter 4.

Scope of the Dissertation

The goals of this dissertation are to investigate the effects of a medicinal natural product on human type II topoisomerases, and to determine the effects of supercoiled DNA substrates on the catenation reaction of type II topoisomerases.

Chapter 1 introduces the concepts of DNA topology, type II topoisomerases, topoisomerase-targeting drugs, and how type II topoisomerases interact with DNA supercoil geometry. Parts of this chapter have been published (Dalvie and Osheroff 2021).

Chapter 2 describes the materials and methods used in this dissertation, parts of which have been published (Dalvie, Gopas et al. 2019).

Chapter 3 describes the effects of 6,6'-dihydroxythiobinupharadine (DTBN), a natural product from *Nuphar lutea*, the yellow water lily, on human type II topoisomerases. DTBN acts as a covalent poison that increases DNA cleavage mediated by topoisomerase IIα and IIβ. The results presented in this chapter have been published (Dalvie, Gopas et al. 2019).

Chapter 4 examines the recognition of DNA supercoil geometry by type II topoisomerases during the intermolecular catenation reaction. Previous studies have shown that type II topoisomerases recognize supercoil geometry during the intramolecular relaxation reaction. Results shown in this chapter demonstrate that human topoisomerase IIα and bacterial topoisomerase IV display supercoil handedness preference during catenation. However, while bacterial topoisomerase IV preferentially catenates positively supercoiled DNA over negatively supercoiled DNA, in parallel with the relaxation reaction, human topoisomerase IIα shows the opposite preference. Furthermore, this preference of human topoisomerase IIα is conveyed by the C-terminal domain. Finally, human topoisomerase IIα and *E. coli* topoisomerase IV both generate lesser amounts of DNA cleavage with catenated DNA than with decatenated DNA.

Chapter 5 discusses the conclusions and implications of the work presented in this dissertation.

CHAPTER TWO

METHODS AND MATERIALS

Enzymes

Recombinant human topoisomerase IIα, topoisomerase IIβ, and a deletion mutant containing residues 1–1175 of human topoisomerase IIα that lacked the C-terminal domain (Topo IIαΔCTD) were expressed in *Saccharomyces cerevisiae* and purified as described previously (Worland and Wang 1989, Kingma, Greider et al. 1997, Dickey and Osheroff 2005). Enzymes were untagged and purified by hydroxyapatite column and phosphocellulose column. The catalytic core of human topoisomerase IIα (Topo IIαcc; residues 431–1193) was a gift from J. E. Deweese and was expressed and purified as described previously (Wasserman, Austin et al. 1993, Biersack, Jensen et al. 1996, Oestergaard, Bjergbaek et al. 2004, Wendorff, Schmidt et al. 2012). Enzymes were stored at -80 °C as 1.5 mg/mL stocks in 50 mM Tris-HCl (pH 7.9), 0.1 mM NaEDTA, 750 mM KCl, and 40% (v/v) glycerol.

Bacillus anthracis topoisomerase IV subunits (GrlA and GrlB) and *Neisseria gonorrhea* topoisomerase IV subunits (ParC and ParE) were expressed in *E. coli* and purified using a C-terminal His-tag as described by Dong *et al* (Dong, McPherson et al. 2010). Untagged *E. coli* topoisomerase IV subunits (ParC and ParE) were expressed and purified using ion exchange chromatography as described by Peng and Marians (Peng and Marians 1993). Enzymes were stored at -80 °C in 40 mM HEPES-KOH (pH 7.6), 1 mM NaEDTA, 150 mM KGlu, and 40% (v/v) glycerol.

DNA Substrates

Negatively supercoiled pBR322 DNA was prepared from *E. coli* using a Plasmid Mega Kit (Qiagen) according to the manufacturer's protocol.

Relaxed pBR322 plasmid DNA was generated by treating negatively supercoiled pBR322 with calf thymus topoisomerase I (Invitrogen) (Aldred, Breland et al. 2014). Reaction mixtures contained 70 nM DNA and 60 units topoisomerase I in a total of 300 µL of 10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 175 mM KCl, 5 mM $MgCl_2$, and 2.5% (v/v) glycerol. Reactions were incubated at 37 °C for 30 min and halted by incubation at 75 °C for 10 min. Samples were extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and DNA was precipitated with cold ethanol. Relaxed plasmids were resuspended in 100 µL of 5 mM Tris-HCl (pH 7.4) containing 500 µM EDTA.

Positively supercoiled pBR322 was generated by treating negatively supercoiled DNA with recombinant *Archeoglobus fulgidus* reverse gyrase (Rodriguez 2002, McClendon, Rodriguez et al. 2005). Reaction mixtures contained 35 nM negatively supercoiled pBR322 DNA and 420 nM reverse gyrase in a total of 500 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 10 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 reaction mixtures 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 100 µL of 5 mM Tris-HCl (pH 7.4) containing 500 µM EDTA. The number of positive supercoils induced was similar to the number of negative supercoils in the original pBR322 preparations (McClendon, Rodriguez et al. 2005). As a control,

some experiments utilized negatively supercoiled plasmid preparations that were processed identically to the positively supercoiled DNA except that they did not contain reverse gyrase. Results were similar to those obtained with negatively supercoiled DNA prepared directly from *E. coli.*

Kinetoplast DNA (kDNA), a network of circular DNA characterized by catenated maxi-and mini-circles, was isolated from *Crithidia fasciculata* as described previously (Danzig 2005).

Substrates with varying levels of negative supercoiling were generated by treating relaxed pBR322 with calf thymus topoisomerase I in the presence of 0-0.35 mg/mL of chloroquine and purified as described previously (Aldred, Breland et al. 2014). Chloroquine was removed using phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. Substrates with varying levels of positive supercoiling were generated by treating relaxed DNA with 0-420 nM of recombinant *A. fulgidus* reverse gyrase and purified as described above. The supercoil states of the DNA were analyzed by two-dimensional gel electrophoresis as described in Gibson *et al* (Gibson, Oviatt et al. 2020). The first dimension was run for 2 h in a 1% agarose gel in 100 mM Tris-borate (pH 8.3) and 2 mM EDTA. The gel was then soaked in 100 mM Tris borate (pH 8.3), 2 mM EDTA containing 4.5 μg/mL chloroquine for 2 h with gentle shaking followed by electrophoresis in the orthogonal direction (90° clockwise) for 2 h in fresh 100 mM Tris borate (pH 8.3), 2 mM EDTA containing 4.5 μg/mL chloroquine. Gels were stained and DNA bands were visualized with ultraviolet light.

Drugs, Compounds, and Natural Products

6,6'-Dihydroxythiobinupharidine (DTBN; the active compound in *Nuphar lutea*, CAS Number 30343-70-5) and etoposide (CAS Number 33419-42-0) were purchased from Sigma-Aldrich.

A library of methanol/water extracts from 341 native Mediterranean plants (gathered primarily from the Tel Aviv University Botanical Garden or arid lands) was used for an initial screening to identify natural products that act as topoisomerase poisons (Sathiyamoorthy, Lugasi-Evgi et al. 1997, Sathiyamoorthy, Lugasi-Evgi et al. 1999, Ozer, Eisner et al. 2009). Extracts were prepared as described previously by Kaiser *et al.* (Kaiser, Yassin et al. 2007). Briefly, frozen plant material (1 g samples) was ground in a prechilled mortar containing liquid nitrogen. A methanol/water mixture [2 mL of a 50:50 (v/v) solution] was added, and slurries were mixed and kept on ice for 15 min. Mixtures were centrifuged at 10000 g for 5 min at room temperature using a Hermle Z160M microfuge. Supernatant liquids were removed and stored at −80 °C. The concentrations of extracts were determined gravimetrically. De-identified samples were dried *in vacuo*, numbered, and stored at -20 °C. Extracts were resuspended in deionized water at final concentrations of 2 mg/mL.

DNA Cleavage

DNA cleavage reactions were performed using the procedure of Fortune and Osheroff (Fortune and Osheroff 1998). Reaction mixtures contained 220 nM human topoisomerase IIα, 210 nM topoisomerase IIβ, 80 nM Topo IIαΔCTD, or 425 nM Topo IIαcc and 10 nM negatively supercoiled pBR322 in a total of 20 μL of DNA cleavage buffer [10 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM NaEDTA, and 2.5% (v/v) glycerol]. Assays were carried out in the

absence of compound or in the presence of 0-1000 μM DTBN. Unless stated otherwise, DTBN was added last to reaction mixtures. Samples were incubated at 37 °C for 6 min and enzyme-DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA, pH 8.0. Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. Samples were mixed with agarose gel loading buffer (2 μL of 60% sucrose, 10 mM Tris-HCl, pH 7.9, 0.5% bromophenol blue; and 0.5% xylene cyanol FF), heated at 45 °C for 5 min, and subjected to electrophoresis in 1% agarose gels in 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. Double-stranded DNA cleavage was monitored by the conversion of supercoiled plasmid DNA to linear molecules.

In reactions that determined whether DNA cleavage by human topoisomerase IIα was reversible, 2 μL of 250 mM EDTA was added to samples prior to treatment with SDS. In some experiments, DNA cleavage was performed in the presence of etoposide as a control.

To examine the effects of thiol-containing compounds and reducing agents on the actions of DTBN against topoisomerase IIα, 1 mM DTT, 1 mM glutathione, or 2.5 mM ascorbic acid were included in reaction mixtures.

To assess the effects of DTBN on human topoisomerase IIα prior to the addition of DNA, 293 nM enzyme was incubated in the presence of 667 μM DTBN (final concentration) for 0–2 min at 37 °C in 15 μL of DNA cleavage buffer. DNA cleavage was initiated by the addition of 10 nM negatively supercoiled pBR322 DNA (final concentration) to reaction mixtures (final volume of 20 μL), and samples were incubated at 37 °C for 6 min. Reactions were stopped, and samples were processed and analyzed as described above.

DNA Ligation

DNA ligation mediated by topoisomerase IIα was monitored according to the procedure of Byl *et al* (Byl, Fortune et al. 1999). Reaction mixtures contained 220 nM human topoisomerase IIα and 0 or 500 μM DTBN in a total of 20 μL of DNA cleavage buffer. In reactions that contained no compound, 5 mM $MgCl_2$ was replaced by 5 mM $CaCl_2$ to increase levels of baseline DNA scission (Osheroff and Zechiedrich 1987). DNA cleavage-ligation equilibria were established for 6 min at 37 °C as described above and ligation was initiated by shifting samples from 37 to 0 °C. Reactions were stopped at time points ranging from 0-30 s and analyzed as described above. DNA ligation was monitored by the loss of linear DNA.

Persistence of Topoisomerase II-DNA Cleavage Complexes

The stability of topoisomerase II-DNA cleavage complexes was determined using a modification of the procedure of Gentry *et al* (Gentry, Pitts et al. 2011). Initial reactions contained 440 nM topoisomerase IIα, 55 nM DNA, and 0 or 500 μM DTBN in a total of 20 μL of DNA cleavage buffer. Reactions were incubated for 10 min at 37 ºC to achieve DNA cleavage-ligation equilibrium and then diluted 20-fold with DNA cleavage buffer containing no divalent cation at 37 ºC. Samples (20 μL) were removed at times ranging from 0-240 min. DNA cleavage was stopped and samples were analyzed as described above. In assays that did not include DTBN, the 5 mM $MgCl_2$ in the DNA cleavage buffer was replaced with 5 mM $CaCl_2$ to increase baseline levels of DNA cleavage (Osheroff and Zechiedrich 1987). In all cases, levels of DNA cleavage were set to 100% at time zero, and the persistence of cleavage complexes was determined by the decay of linear reaction product over time.

Catenation

Catenation assays performed using human type II topoisomerases (topoisomerase IIα, topoisomerase IIβ, or Topo IIαΔCTD) contained 8.8 nM enzyme, 5 nM DNA (negatively supercoiled, positively supercoiled, or relaxed), 1 mM ATP, and 15 μg/mL of calf thymus histone H1 (Sigma-Aldrich) in 20 μL of 10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 175 mM KCl, 5 mM $MgCl_2$, and 2.5% (v/v) glycerol. Samples were incubated at 37 °C from 0-45 min and DNA catenation was stopped by the addition of 2 μL of 0.77% SDS and 77 mM EDTA (pH 8.0). Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. Samples were mixed with 2 μL of agarose gel loading buffer, heated at 45 °C for 5 min, and subjected to electrophoresis in a 1% agarose gel in 100 mM Tris-borate (pH 8.3) and 2 mM EDTA. Gels were stained in 1 μg/mL ethidium bromide and DNA bands were visualized with ultraviolet light and quantified using an Alpha Innotech digital imaging system. The rate of DNA catenation was monitored by the disappearance of monomer plasmid DNA. Rates were analyzed and measured using GraphPad Prism software.

Alternatively, catenation was catalyzed for 0-40 s in the presence of 220 nM human topoisomerase IIα or topoisomerase IIβ in buffers that lacked histone H1. Otherwise, reactions were carried out and analyzed as described above.

Catenation assays performed using bacterial topoisomerase IV contained 25 nM *B. anthracis* (1:2 GrlA:GrlB ratio), 10 nM *E. coli* (1:1 ParC:ParE ratio), or 10 nM *N. gonorrhea* (1:1 ParC:ParE ratio) enzyme, 5 nM DNA (negatively supercoiled, positively supercoiled, or relaxed), 1 mM ATP, and 15 μg/mL of calf thymus histone H1 in 20 μL of 40 mM HEPES (pH 7.6), 100 mM KGlu, 10 mM $Mg(OAc)_2$, and 25 mM NaCl. Samples were incubated at 37 °C for 0-45 min and DNA

catenation was stopped by the addition of 2 μL of 0.77% SDS and 77 mM EDTA (pH 8.0). Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. Samples were mixed with 2 μL of agarose gel loading buffer, heated at 45 °C for 5 min, and subjected to electrophoresis in a 1% agarose gel in 100 mM Tris borate (pH 8.3), 2 mM EDTA. DNA bands were visualized and analyzed as described above.

In experiments where catenation intermediates were tracked, reaction mixtures were as described above using 8.8 nM topoisomerase IIα or 25 nM *B. anthracis* topoisomerase IV. Samples were incubated at 25 °C for 0-45 min and catenation was stopped by incubation at 80 °C for 20 min. Reaction mixtures were cooled and the DNA was nicked by the addition of 10 U of nicking endonuclease Nt. BspQI (New England Biosciences) and incubating at 50 °C for 60 min. Reactions were stopped by the addition of 2 μL of 0.77% SDS and 77 mM EDTA (pH 8.0). Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzymes. Samples were mixed with 2 μL of agarose gel loading buffer, heated at 45 °C for 5 min, and subjected to electrophoresis in a 1% agarose gel in 100 mM Tris borate (pH 8.3) and 2 mM EDTA. DNA bands were visualized as described above.

Cleavage of kDNA During Decatenation

DNA decatenation assays with *E. coli* topoisomerase IV were based on previously published protocols (Anderson, Gootz et al. 1998, Aldred, McPherson et al. 2013). Assays contained 40 nM *E. coli* wild-type topoisomerase IV (1:1 ParC:ParE ratio) and 5 nM kDNA in 20 μL of 40 mM HEPES (pH 7.6), 100 mM KGlu, 10 mM $Mg(OAc)_2$, and 25 mM NaCl. Some reactions included 100 μM ciprofloxacin to enhance DNA cleavage. Reactions were incubated at 25 °C for 5 min to establish cleavage-ligation equilibrium. Decatenation was started with the addition of 50 μM ATP

and time points were taken over an interval from 0 to 2 min. Reaction mixtures were stopped by the addition of either 2 μL of 5% SDS and 2 μL 250 mM EDTA, pH 8.0, or 2 μL of a mixture of 0.77% SDS and 77.5 mM Na_2EDTA. Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. Samples were mixed with 2 μL of agarose gel loading buffer and incubated at 45 °C for 2 min before loading onto 1% agarose gels in 100 mM Tris-borate (pH 8.3), 2 mM EDTA, and 0.5 μg/mL ethidium bromide. DNA bands were visualized as described above.

DNA decatenation assays with human enzyme contained 110 nM topoisomerase IIα, 5 nM kDNA or relaxed DNA, and 50 nM ATP in 20 μL of 10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 175 mM KCl, 5 mM $MgCl_2$, and 2.5% glycerol. Some reactions contained 100 μM etoposide to enhance DNA cleavage. Reactions were incubated at 25 °C for 10 min to establish cleavage-ligation equilibrium. Decatenation was started with the addition of 50 μM ATP and time points were taken over an interval from either 0 to 2 min or 0 to 5 min. Reaction mixtures were stopped by the addition of either 2 μL of 5% SDS and 2 μL 250 mM EDTA (pH 8.0), or 2 μL of a mixture of 0.77% SDS and 77.5 mM Na_2EDTA. Proteinase K (2 μL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. Samples were mixed with 2 μL of agarose gel loading buffer and incubated at 45 °C for 2 min before loading onto 1% agarose gels in 100 mM Tris-borate (pH 8.3), 2 mM EDTA, and 0.5 μg/mL ethidium bromide. DNA bands were visualized as described above.

CHAPTER THREE

6,6'-DIHYDROXYTHIOBINUPHARADINE AS A POISON OF HUMAN TYPE II TOPOISOMERASES

Introduction

Beyond their critical physiological functions, topoisomerase IIα and IIβ are the targets for some of the most widely prescribed anticancer agents worldwide (Deweese and Osheroff 2009, Nitiss 2009, Pommier, Leo et al. 2010, Pommier 2013, Ketron and Osheroff 2014, Pendleton, Lindsey et al. 2014). As discussed in Chapter 1, topoisomerase II-targeted drugs act by stabilizing covalent enzyme-cleaved DNA complexes (cleavage complexes) that are requisite intermediates in the catalytic cycle of topoisomerase IIα and IIβ (Deweese and Osheroff 2009, Nitiss 2009, Pommier, Leo et al. 2010, Pommier 2013, Ketron and Osheroff 2014, Pendleton, Lindsey et al. 2014, Vann, Oviatt et al. 2021). Thus, these agents convert type II topoisomerases from essential enzymes to toxic proteins that fragment the genome. Drugs that act by this mechanism are referred to as topoisomerase II "poisons" to distinguish them from "catalytic inhibitors" that act by robbing the cell of the catalytic functions of these enzymes (Deweese and Osheroff 2009, Nitiss 2009, Pommier, Leo et al. 2010, Pommier 2013, Ketron and Osheroff 2014, Pendleton, Lindsey et al. 2014).

In addition to anticancer drugs, a number of phytochemicals with anticancer, chemopreventative, or other health-promoting properties act as topoisomerase II poisons (Ketron and Osheroff 2014). In an effort to identify additional natural products that act as poisons of human type II topoisomerases, we conducted a blind screen of a library of 341 Mediterranean plant

extracts. Species in the library were collected primarily from the Tel Aviv University Botanical Garden or arid lands. Previous work with this library determined that extracts from *Phillyrea latifolia L.* enhanced DNA cleavage mediated by human topoisomerase IIα, which led to the identification of hydroxytyrosol, oleuropein, and verbascoside as topoisomerase II poisons (Vann, Sedgeman et al. 2015).

Results and Discussion

In the present study, extracts from *Nuphar lutea*, the yellow water lily, were found to increase levels of DNA cleavage mediated by human topoisomerase IIα ~2.5-fold (not shown). This flower is found in ponds and marshes nearly worldwide (Ozer, Fishman et al. 2017). *N. lutea* extracts have been used for treatment of inflammation in the traditional medicine of Lebanon, Japan, and the Gitskan people of British Columbia, Canada (Johnson 2006, El Beyrouthy, Arnold et al. 2008, Nakae, Yokoi et al. 2012). They also have been reported to possess anti-leishmanial, anti-bacterial, and potentially anticancer properties (El-On, Ozer et al. 2009, El-On, Ozer et al. 2009, Ozer, El-On et al. 2010, Ozer, Levi et al. 2015, Ozer, Fishman et al. 2017, Levy, Chapple et al. 2019). 6,6'-Dihydroxythiobinupharidine (DTBN, Figure 3.1) is the active compound in *N. lutea* (Ozer, Fishman et al. 2017). Therefore, we determined the effects of DTBN on the DNA cleavage activities of human topoisomerase IIα and IIβ.

As seen in Figure 3.2, DTBN enhanced DNA cleavage mediated by both isoforms but had a considerably larger effect on topoisomerase IIα. Whereas the compound increased levels of double-stranded DNA breaks generated by the α isoform ~8-fold (from ~2% to ~16% of the DNA substrate), it increased cleavage by the β isoform ~3-fold (from ~2% to ~6% of the DNA substrate).

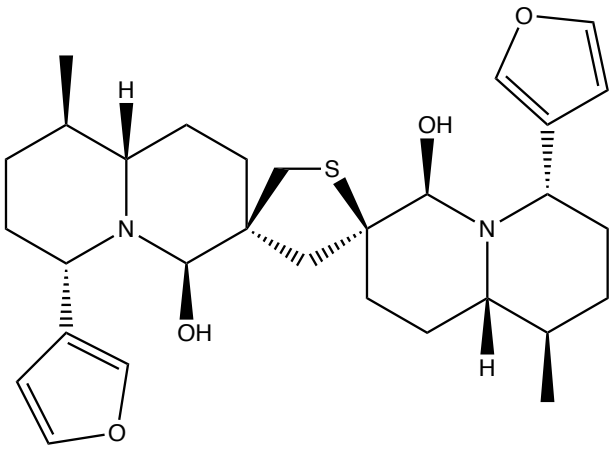

Figure 3.1: Structure of 6,6'-dihydroxythiobinupharidine (DTBN). DTBN is the active compound in *N. lutea*.

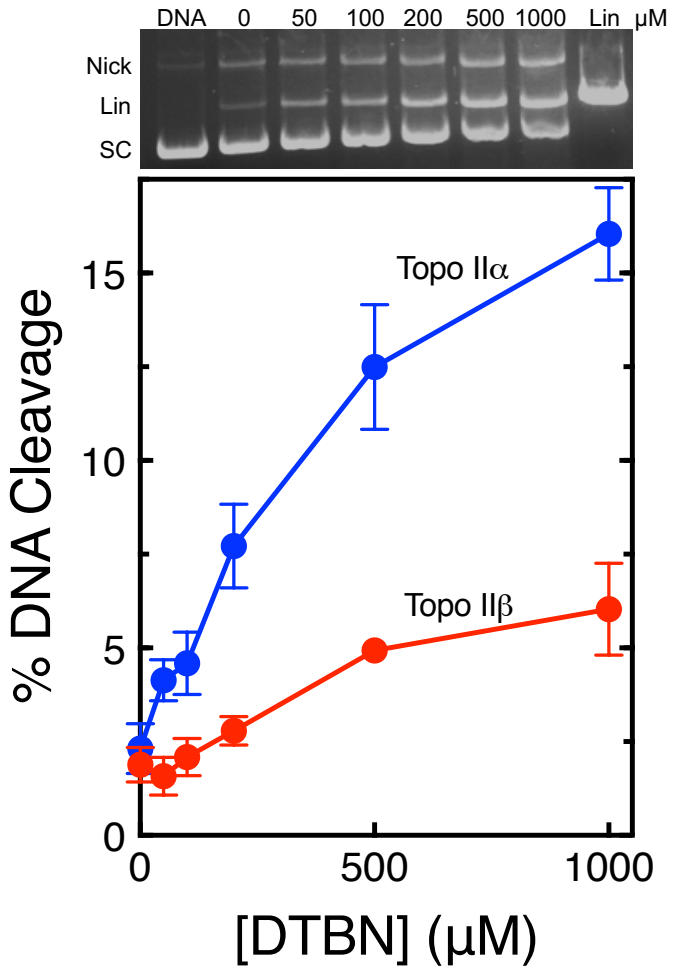

Figure 3.2: DTBN increases levels of DNA double-stranded cleavage mediated by human type II topoisomerases. The effects of DTBN on double-stranded DNA cleavage mediated by human topoisomerase IIα (Topo IIα, blue) and topoisomerase IIβ (Topo IIβ, red) are shown. DNA cleavage levels were normalized by comparison to linear standards, which were set to 100%. Error bars represent standard deviations of three independent experiments. A representative ethidium bromide-stained agarose gel containing an experiment with topoisomerase IIα is shown at the top. The positions of negatively supercoiled (SC), nicked (nick), and linear (lin) plasmid are shown.

Because of the enhanced activity of DTBN against topoisomerase IIα, this isoform was used for all the mechanistic studies that follow.

Prior to further analysis, two experiments were carried out to ensure that the DNA cleavage observed in the presence of DTBN was mediated by topoisomerase IIα (Figure 3.3). In the first, 500 μM DTBN was incubated with DNA in the absence of enzyme under the conditions of the DNA cleavage assay. No DNA cleavage was seen. In the second, topoisomerase IIα was included in assays, however, reactions were incubated with 25 mM EDTA prior to the addition of SDS (which traps the cleavage complex). The addition of this chelating agent removes the active site Mg^{2+} ions that are required for DNA scission (Osheroff 1987). Therefore, EDTA reverses DNA cleavage mediated by the type II enzyme (Osheroff 1987). This is seen in Figure 3.3; the addition of EDTA significantly diminished levels of double-stranded DNA scission. This reversal of cleavage is inconsistent with a non-enzymatic reaction and provides strong evidence that the DNA cleavage enhancement observed in the presence of DTBN is mediated by the human type II enzyme.

A previous study found that compounds that formed longer lasting cleavage complexes displayed greater cytotoxicity (Bandele and Osheroff 2008). Therefore, we determined the stability of cleavage complexes formed in the presence of DTBN (Figure 3.4). This was accomplished by establishing DNA cleavage-ligation equilibria and diluting samples 20-fold with buffer that included no divalent cation. Thus, once the DNA dissociates from topoisomerase IIα, it is unlikely that the enzyme will form new cleavage complexes. Whereas the $t_{1/2}$ of the cleavage complex was less than 30 s when formed in the absence of DTBN, it was stable over a 4 h time course in the presence of the compound.

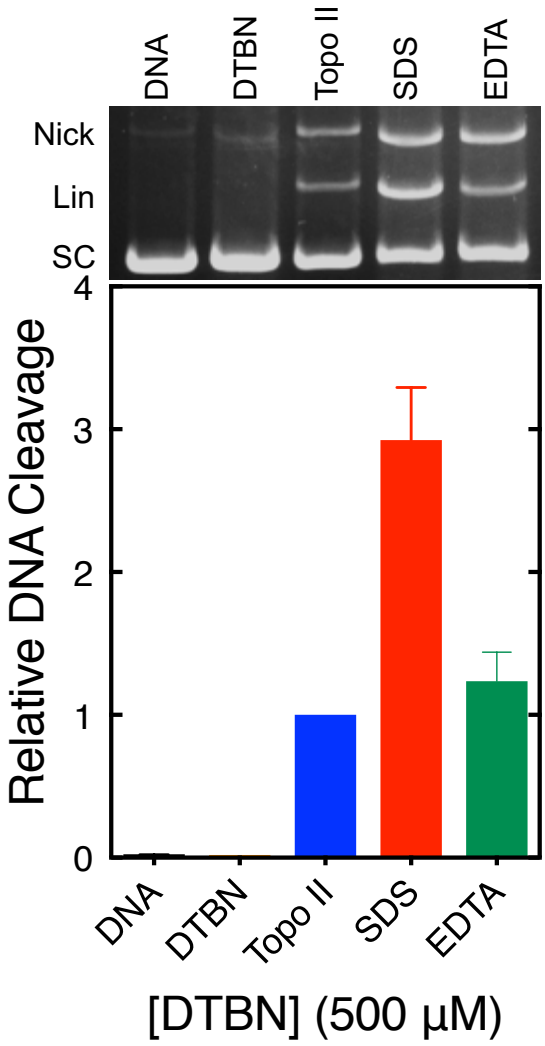

Figure 3.3: DNA cleavage induced by DTBN is mediated by human topoisomerase IIα. Assay mixtures contained DNA alone (DNA, black), DNA with DTBN in the absence of enzyme (DTBN, orange), topoisomerase IIα with DNA in the absence of DTBN (Topo II, blue), complete reactions stopped with SDS prior to the addition of EDTA (SDS, red), or complete reactions treated with EDTA prior to the addition of SDS (EDTA, green). Error bars represent the standard error of the mean of two independent experiments. A representative ethidium bromide-stained agarose gel is shown at the top and DNA positions are as indicated in Figure 3.2.

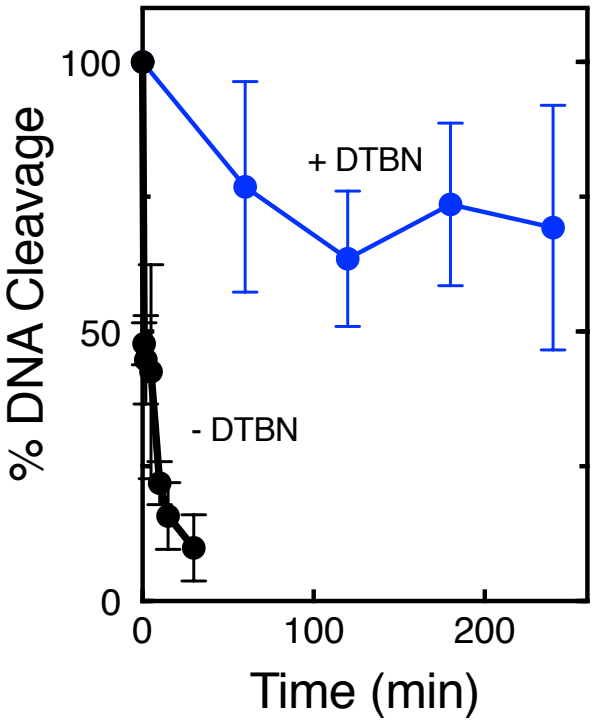

Figure 3.4: DTBN induces stable topoisomerase IIα-DNA cleavage complexes. Reactions were allowed to reach cleavage-ligation equilibrium, diluted with reaction buffer that lacked Mg^{2+}, and persistence was measured by assessing the loss of double-stranded breaks. DNA cleavage at time zero was set to 100%. Reactions included no compound (black) or 500 μM DTBN (blue). Error bars represent standard deviations of three independent experiments.

As discussed in Chapter 1, topoisomerase II poisons can be grouped into two different classes. Anticancer drugs such as etoposide interact noncovalently at the interface between the enzyme and its DNA substrate, contacting both (Pommier and Marchand 2012). Once DNA scission has taken place, the drug intercalates between the bases of the cleaved scissile bond, blocking ligation (Wu, Li et al. 2011, Pommier and Marchand 2012). Compounds that act in this non-covalent fashion are referred to as interfacial topoisomerase II poisons (Pommier and Marchand 2012).

In contrast, compounds such as benzoquinone interact covalently with topoisomerase II outside of the DNA cleavage active site (Lindsey, Bromberg et al. 2004, Deweese and Osheroff 2009, Ketron and Osheroff 2014). These compounds adduct cysteine (and potentially other) residues and are believed to enhance cleavage by stabilizing the dimerization of the N-terminal domain of the enzyme (Wang, Mao et al. 2001, Bender, Lehmler et al. 2006, Bender and Osheroff 2007, Lin, Zhou et al. 2011, Lindsey, Pendleton et al. 2014). Compounds that act by adducting the enzyme are referred to as covalent topoisomerase II poisons (Bender, Lehmler et al. 2006, Deweese and Osheroff 2009, Ketron and Osheroff 2014). In contrast to interfacial poisons, covalent poisons can have variable effects on enzyme-mediated DNA ligation. Whereas compounds such as benzoquinone strongly inhibit ligation, others such as hydroxytyrosol have relatively little effect (Lindsey, Bromberg et al. 2004, Vann, Sedgeman et al. 2015).

As a first step towards characterizing the mechanism by which DTBN enhances DNA cleavage, we examined the effects of the compound on the rate of DNA ligation mediated by human topoisomerase IIα. Unlike etoposide and other anticancer drugs, DTBN had very little effect on the rate of ligation ($t_{1/2}$ = 11 s versus 12.5 s in the presence or absence of DTBN, respectively). This finding suggests that the compound may act as a covalent poison of topoisomerase IIα.

To further elucidate the mechanistic basis for the actions of DTBN, we took advantage of several hallmark characteristics of covalent poisons that they do not share with interfacial poisons. First, because covalent poisons often adduct cysteine residues and require redox cycling, their activity against the type II enzyme can be diminished in the presence of thiol-containing compounds or reducing agents (Wang, Mao et al. 2001, Lindsey, Bromberg et al. 2004, Deweese and Osheroff 2009, Ketron, Gordon et al. 2013). Therefore, we examined the effects of these compounds on the activity of DTBN. It is possible that DTBN forms an iminium that may be reduced. As seen in Figure 3.5, the thiol reagents dithiothreitol and glutathione (1 mM) decreased the ability of DTBN to induce DNA cleavage by ~50%. In addition, the reducing agent ascorbic acid (2.5 mM) abrogated the effects of DTBN on DNA cleavage mediated by human topoisomerase IIα. These results are consistent with the covalent mechanism of poisoning.

Second, when covalent poisons are incubated with topoisomerase II prior to the addition of DNA, they inactivate the enzyme (Wang, Mao et al. 2001, Lindsey, Bromberg et al. 2004, Deweese and Osheroff 2009, Ketron, Gordon et al. 2013). This is likely due (in part) to the fact that they close the N-terminal protein gate which prevents plasmid DNA from entering the active site of the enzyme (Bender, Lehmler et al. 2006, Bender and Osheroff 2007, Mondrala and Eastmond 2010). There is also evidence that in the absence of DNA, covalent poisons may adduct a critical residue in the active site of the enzyme (Bender, Lehmler et al. 2006). When incubated with topoisomerase IIα prior to the addition of DNA, DTBN completely inactivated the enzyme within 120 s ($t_{1/2}$ = 30 s) (Figure 3.6). Once again, this finding indicates that DTBN acts as a covalent poison.

Third, because of their mechanism of action, covalent poisons require the presence of the N-terminal domain of topoisomerase II (Bender, Lehmler et al. 2006, Bender and Osheroff 2007, Mondrala and Eastmond 2010, Lindsey, Pendleton et al. 2014). Therefore, we examined the ability

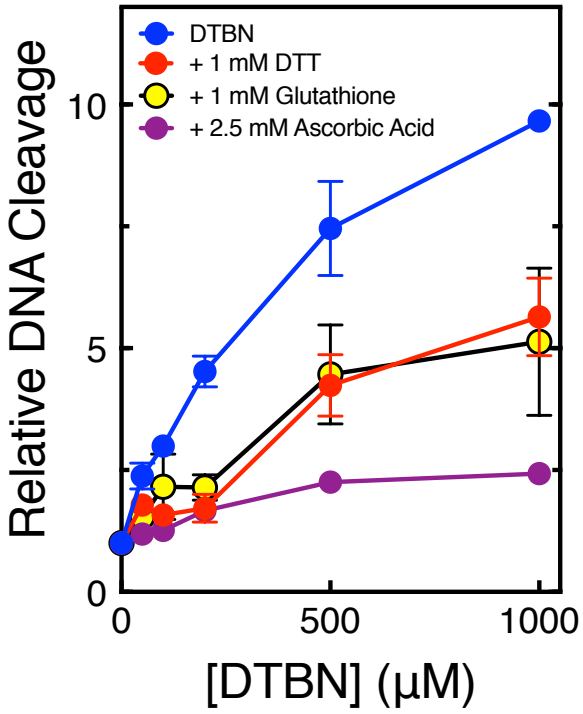

Figure 3.5: The ability of DTBN to poison topoisomerase IIα is diminished by thiol containing compounds and reducing agents. DNA cleavage reactions were carried out in the presence of 1 mM dithiothreitol (DTT) (red), 1 mM glutathione (yellow), or 2.5 mM ascorbic acid (purple). Error bars represent standard deviations of three independent experiments.

of DTBN to induce DNA cleavage with the catalytic core of human topoisomerase IIα. The catalytic core (residues 431 to 1193; see Chapter 1, Figure 1.3) includes the active site tyrosine residues that act as the nucleophiles for DNA scission as well as the TOPRIM domain that contains the residues necessary to bind the active site divalent metal ions (Lindsey, Pendleton et al. 2014). Although the catalytic core of the enzyme is competent to cleave and ligate DNA substrates, it lacks both the N-terminal and C-terminal domains of the enzyme and cannot carry out the DNA strand passage reaction (Lindsey, Pendleton et al. 2014). As seen in Figure 3.7, DTBN concentrations as high as 1 mM were unable to induce enzyme-mediated DNA cleavage with the catalytic core. This result contrasts with that with etoposide, which induced DNA cleavage at 50 and 100 μM.

To ensure that results with the catalytic core were due to the loss of the N-terminal domain, we examined the effects of DTBN on a topoisomerase IIα deletion construct that contained the N-terminal portion of the protein but lacked the C-terminal domain (Dickey and Osheroff 2005). The compound still retained the ability to induce cleavage with the C-terminal deletion construct (Figure 3.8). As a final control, we determined whether DTBN could interfere with the actions of etoposide in the catalytic core. Because etoposide is known to interact at the DNA cleavage active site of topoisomerase II (Deweese and Osheroff 2009, Wu, Li et al. 2011, Pommier and Marchand 2012, Ketron and Osheroff 2014) and covalent poisons act at residues outside the active site that impact the actions of the N-terminal domain, if DTBN is acting as a covalent poison, it should not be able to interfere with the actions of etoposide in the catalytic core of topoisomerase IIα (Lindsey, Pendleton et al. 2014). As seen in Figure 3.9, this was the case. DTBN had no effect on the ability of etoposide to induce enzyme-mediated DNA cleavage.

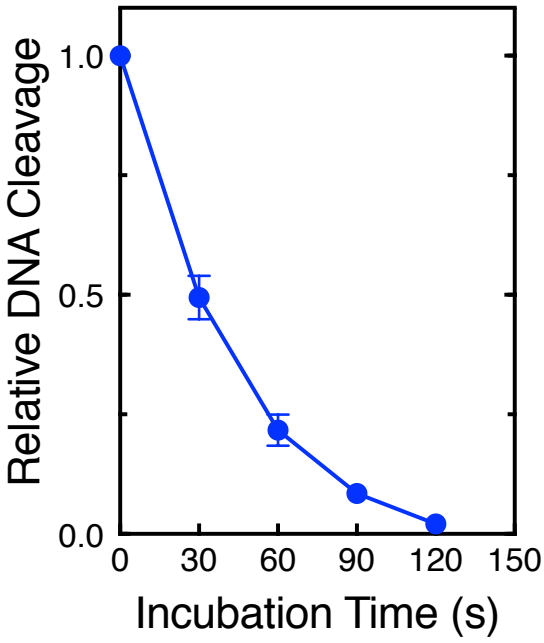

Figure 3.6: DTBN inhibits the activity of human topoisomerase IIα when incubated with the enzyme prior to the addition of DNA. Data represent incubation times for 500 μM DTBN and topoisomerase IIα prior to the addition of DNA. The effects on double-stranded breaks are shown. DNA cleavage levels were calculated relative to that at time zero which was set to 1.0. Error bars represent standard deviations of three independent experiments.

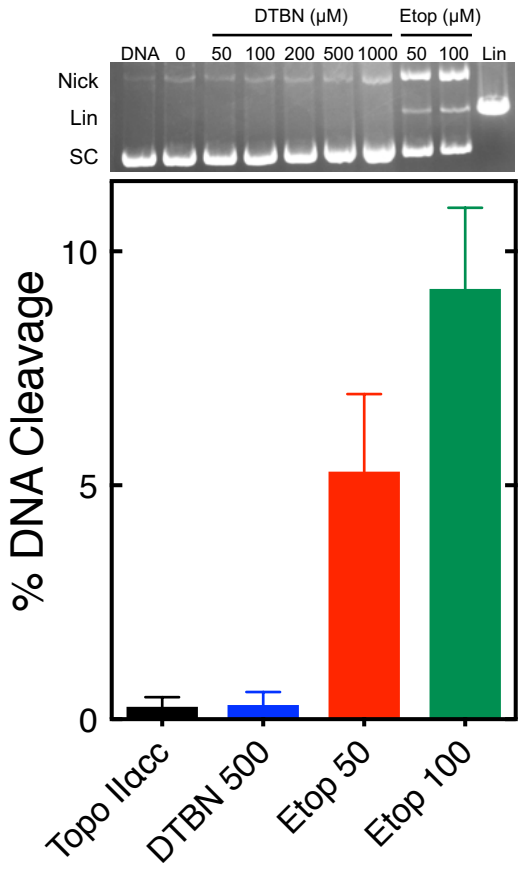

Figure 3.7: DTBN acts outside the active site of topoisomerase IIα. DNA cleavage assays utilized a human topoisomerase IIα deletion construct that was lacking both the N- and C-terminal domains (catalytic core; Topo IIαcc). The bar graph shows results for reactions that contained no compound (Topo IIαcc, black), 500 μM DTBN (DTBN 500, blue), or 50 or 100 μM etoposide (Etop 50, red; Etop 100, green). Error bars represent standard deviations of three independent experiments. A representative ethidium bromide-stained gel is shown at the top. The gel shows an assay with DNA alone (DNA), assays containing Topo IIαcc, and 0-1000 μM DTBN or 50 and 100 μM etoposide. A linear standard (Lin) is shown. DNA positions are as indicated in Figure 3.2.

Conclusions

Taken together, the findings described above provide strong evidence that DTBN is a covalent poison of human type II topoisomerases. They further suggest that at least some of the biological/medicinal effects of the compound may result from this activity. Finally, DTBN is unusual in that it has a greater effect on topoisomerase IIα than topoisomerase IIβ, which may mitigate some of the leukemogenic potential of the β isoform.

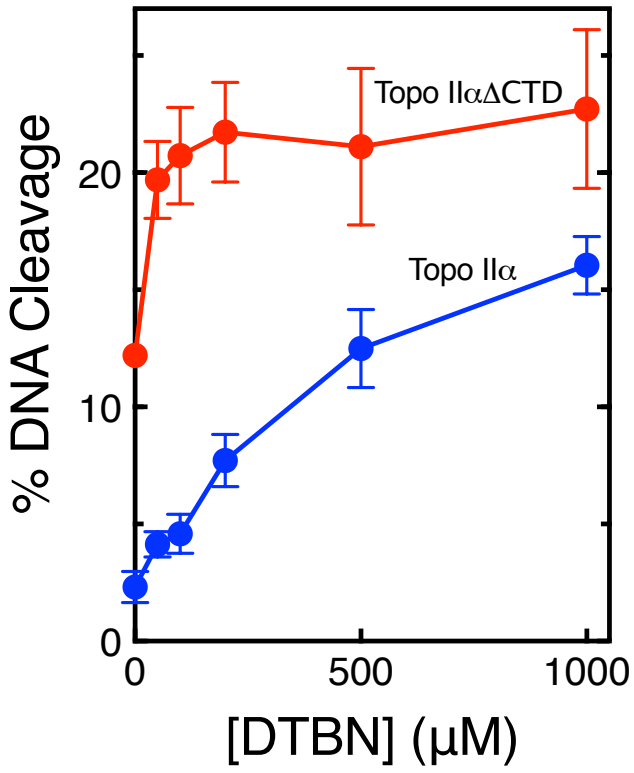

Figure 3.8: DTBN enhances DNA cleavage with a deletion construct of topoisomerase IIα that lacks the C-terminal domain. Assays utilized either the deletion construct (Topo IIαΔCTD, red) or full-length topoisomerase IIα (Topo IIα, blue). DNA cleavage levels were compared to linear standards, which were set to 100%. Error bars represent standard deviations of three independent experiments.

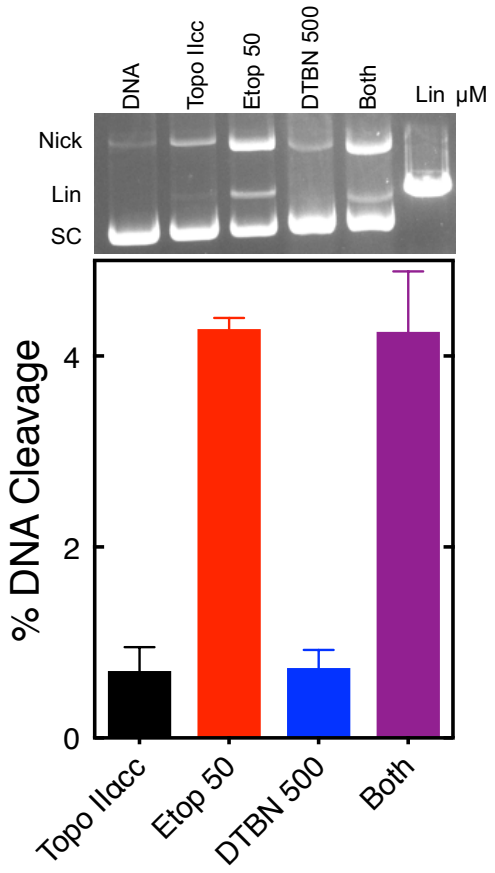

Figure 3.9: DTBN does not compete with etoposide at the DNA cleavage-ligation active site of the enzyme. DNA cleavage assays utilized the human topoisomerase IIα deletion construct that was lacking both the N- and C-terminal domains (Topo IIαcc). Assays were carried out in the absence of compound (Topo IIαcc, black), or in the presence of 50 µM etoposide (Etop 50, red), 500 µM DTBN (DTBN 500, blue), or both 50 µM etoposide and 500 µM DTBN simultaneously (Both, purple). DNA cleavage levels were compared to linear standards, which were set to 100%. Error bars represent standard deviations of three independent experiments. A representative ethidium bromide-stained gel is shown at the top. DNA positions are as indicated in Figure 3.2.

CHAPTER FOUR

RECOGNITION OF DNA SUPERCOIL HANDEDNESS DURING CATENATION CATALYZED BY TYPE II TOPOISOMERASES

Introduction

Previous studies suggest that catenation catalyzed by type II topoisomerases plays an important role in stabilizing interactions between sister chromatids as cells transition into mitosis (Nitiss 2009, Bauer, Marie et al. 2012, Sen, Leonard et al. 2016). Prior to the uncoupling of sister chromatids, eukaryotic topoisomerase II and bacterial topoisomerase IV appear to catalyze multiple cycles of catenation/decatenation, which switches to decatenation at the onset of anaphase (Sen, Leonard et al. 2016).

Immediately prior to decatenation of sister chromatids during mitosis in yeast and *E. coli*, the DNA becomes positively supercoiled. However, during the same time frame, it becomes associated with condensins and other mitotic factors that may influence the process (Baxter, Sen et al. 2011, Zawadzki, Stracy et al. 2015). These studies suggest that type II topoisomerases may preferentially decatenate positively over negatively supercoiled DNA (Baxter, Sen et al. 2011, Zawadzki, Stracy et al. 2015). In support of this conclusion, single molecule experiments indicate that human topoisomerase IIα decatenates positively intertwined molecules approximately 2-fold faster than negatively supercoiled substrates (Seol, Gentry et al. 2013).

As discussed in Chapter 1, DNA supercoil handedness has an important effect on type II topoisomerase activity. One important cellular function of type II topoisomerases is the

catenation/decatenation of DNA (Nitiss 2009, Bauer, Marie et al. 2012, Sen, Leonard et al. 2016). However, little is known about the influence of DNA supercoil handedness on this latter reaction.

Therefore, as a first step toward understanding the molecular conditions that influence the catenation/decatenation cycle, we investigated the effects of DNA topology on the ability of human topoisomerase IIα and IIβ and three species of bacterial topoisomerase IV to catenate DNA. As previously shown for relaxation reactions (McClendon, Rodriguez et al. 2005, Ashley, Dittmore et al. 2017), topoisomerase IIα and topoisomerase IV are able to distinguish supercoil geometry during the catenation reaction. However, the supercoil preference for topoisomerase IIα during the catenation process is opposite from that of relaxation.

Results

Effects of Supercoil Handedness on DNA Catenation Catalyzed by Human Topoisomerase IIα and IIβ

Previous studies demonstrated that human topoisomerase IIα can discern supercoil handedness during the intramolecular DNA relaxation reaction, preferentially removing positive over negative supercoils by an order of magnitude (McClendon, Rodriguez et al. 2005). In contrast, the β isoform was unable to distinguish supercoil geometry and relaxed positive and negative supercoils at similar rates (McClendon, Rodriguez et al. 2005). Therefore, to determine whether these relationships hold true for intermolecular double-stranded DNA strand passage events, the effects of supercoil handedness on the ability of topoisomerase IIα and β to catenate DNA substrates were examined.

Given DNA with superhelical twists, type II topoisomerases generally carry out an intramolecular double-stranded DNA passage reaction, relaxing plasmids as opposed to catenating

them (Osheroff 1998, Champoux 2001, Fortune and Osheroff 2001). This is likely due to the repulsion of plasmids from one another because of the strong negative charge of the sugar-phosphate backbone. However, if the negative charges on the DNA are neutralized by the presence of a polycation, such as histone H1, type II topoisomerases switch from catalyzing intramolecular DNA relaxation to intermolecular catenation (Krasnow and Cozzarelli 1982). Under these conditions, no relaxation of the DNA is observed (see the gel in Figure 4.2). Therefore, unless stated otherwise, histone H1 was included as the "DNA condensing agent" in all assays discussed below.

In parallel to relaxation, topoisomerase IIα was able to distinguish supercoil handedness during catenation whereas topoisomerase IIβ catenated negatively supercoiled, positively supercoiled, and relaxed DNA at similar rates ($t_{1/2} \approx 6$ min, 4 min, and 10 min, respectively, $k \approx$ 0.11, 0.19, and 0.07 %/min, respectively) (Figure 4.1, Table 4.1). However, in contrast to the intramolecular reaction, the α isoform displayed a different substrate preference during intermolecular DNA strand passage. Indeed, topoisomerase IIα catenated negatively supercoiled DNA ($t_{1/2} \approx 7.5$ min, $k \approx 0.09$ %/min) nearly 5 times faster than positively supercoiled substrates ($t_{1/2} \approx 37$ min, $k \approx 0.02$ %/min) (Table 4.1). Furthermore, positively supercoiled DNA did not become fully catenated even after 120 min.

There are two potential caveats to the results shown in Figure 4.1: first, even though the presence of histone H1 in reactions neutralizes the charge in DNA and shifts topoisomerase II-catalyzed reactions from relaxation to catenation, there is still a chance that relaxation is taking place prior to catenation. If this were the case, the interconversion of substrates from supercoiled to relaxed could alter the kinetics of catenation. However, this does not appear to be the case as no

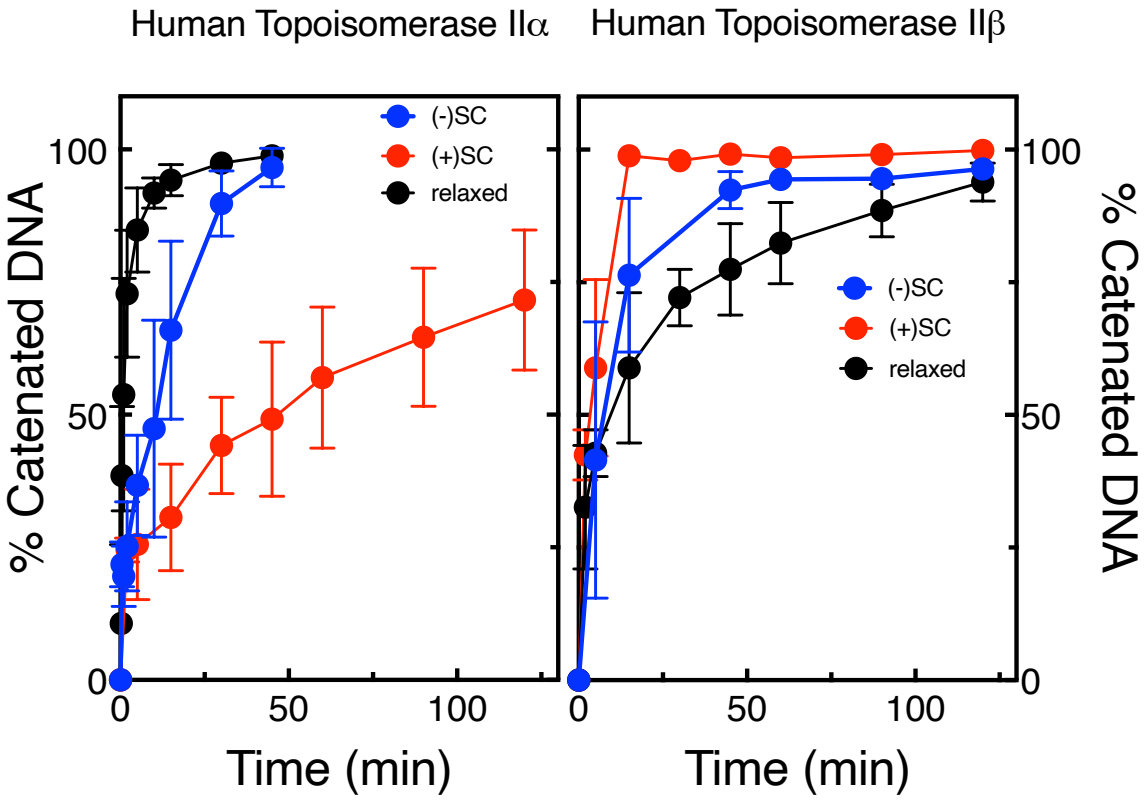

Figure 4.1: Human topoisomerase IIα shows supercoil preference during DNA catenation, while topoisomerase IIβ does not. Catenation of negatively supercoiled [(-)SC], positively supercoiled [(+)SC], and relaxed DNA by human topoisomerase IIα *(left panel)* and human topoisomerase IIβ *(right panel)*. Topoisomerase IIα displays a preference for catenating relaxed DNA and (-)SC DNA over (+)SC DNA. Topoisomerase IIβ displays little discrimination between the three DNA substrates. Error bars represent the standard deviations of at least three independent experiments.

Table 4.1: $t_{1/2}$ and k values for Figure 4.1 and Figure 4.5

	Topoisomerase IIα			Topoisomerase IIβ			Topoisomerase IIαΔCTD		
	$t_{1/2}$ (min)*	Rate (k) (%/min)	95% CI of k†	$t_{1/2}$ (min)*	Rate (k) (%/min)	95% CI of k†	$t_{1/2}$ (min)*	Rate (k) (%/min)	95% CI of k†
(-)SC	7.5	0.092	0.054 to 0.15	6.2	0.11	0.076 to 0.16	43	0.016	0.013 to 0.02
(+)SC	37	0.019	0.014 to 0.024	3.6	0.19	0.11 to 0.32	25	0.027	0.019 to 0.037
Rel	0.81	0.85	0.59 to 1.18	9.6	0.072	0.036 to 0.17	51	0.014	0.009 to 0.018

*$t_{1/2}$ = 0.693/k

†CI: Confidence Intervals

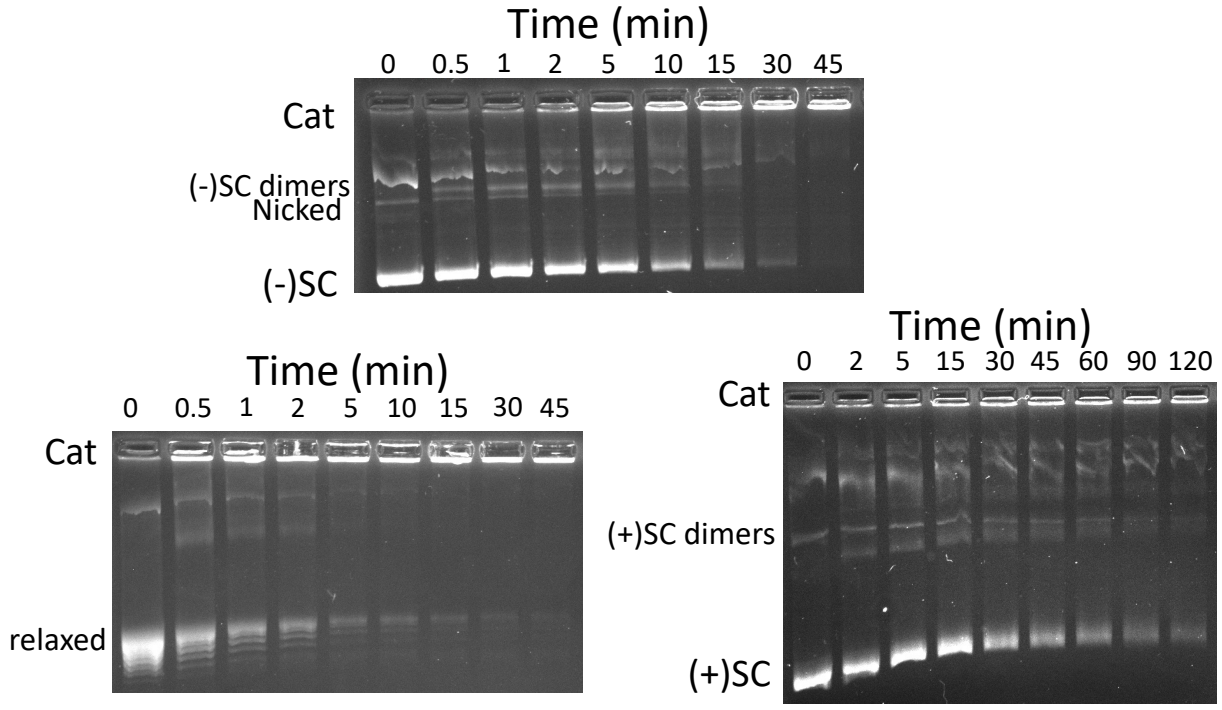

Figure 4.2: Catenation of DNA by human topoisomerase IIα. Catenation of negatively supercoiled [(-)SC] *(top),* relaxed *(bottom left)* and positively supercoiled [(+)SC] *(bottom right)* DNA by human topoisomerase IIα shows monomeric substrate disappearing as catenated product (Cat) appears as time progresses with no relaxed monomeric DNA intermediates.

relaxed monomer intermediates were observed during the course of catenation reactions (Figure 4.2).

Second, although there is no evidence in the literature that the topological state of DNA influences its interactions with histones, it is possible that histone H1 displays different affinities for positively supercoiled, negatively supercoiled, and relaxed DNA. As changes in histone affinity could affect rates of catenation, two experiments were carried out to address this issue: in the first, electrophoretic mobility shift assays were used to assess the affinity of histone H1 for positively and negatively supercoiled DNA. The observed shifts for the different DNA species appeared to be similar (Figure 4.3). In the second experiment, catalytic levels of topoisomerase IIα and IIβ (8.8 nM) were replaced with high concentrations of enzyme (440 nM), and histone H1 was omitted from the catenation assays. It has been shown previously that condensing agents such as histones are not necessary for catenation at high concentrations of topoisomerase II (Osheroff 1986). Presumably, the high levels of the enzyme are sufficient to neutralize charge of the DNA to allow the intramolecular double-stranded DNA passage reaction. Although the rates of catenation were considerably faster at high enzyme concentrations, topoisomerase IIα was still able to preferentially catenate negatively ($t_{1/2} \approx 4$ s) over positively supercoiled DNA ($t_{1/2} \approx 12$ s) (Figure 4.4). Thus, it appears that the ability of topoisomerase IIα to distinguish supercoil handedness during catenation is not related to potential differences in the interactions of histone H1 with positively supercoiled, negatively supercoiled, and relaxed DNA.

The ability of topoisomerase IIα to recognize supercoil handedness during the intramolecular DNA relaxation reaction resides in the C-terminal domain of the enzyme (McClendon, Gentry et al. 2008). When this portion of the enzyme is deleted, topoisomerase IIα no longer preferentially relaxes positive DNA supercoils, and the rate of relaxation of the positively supercoiled substrate

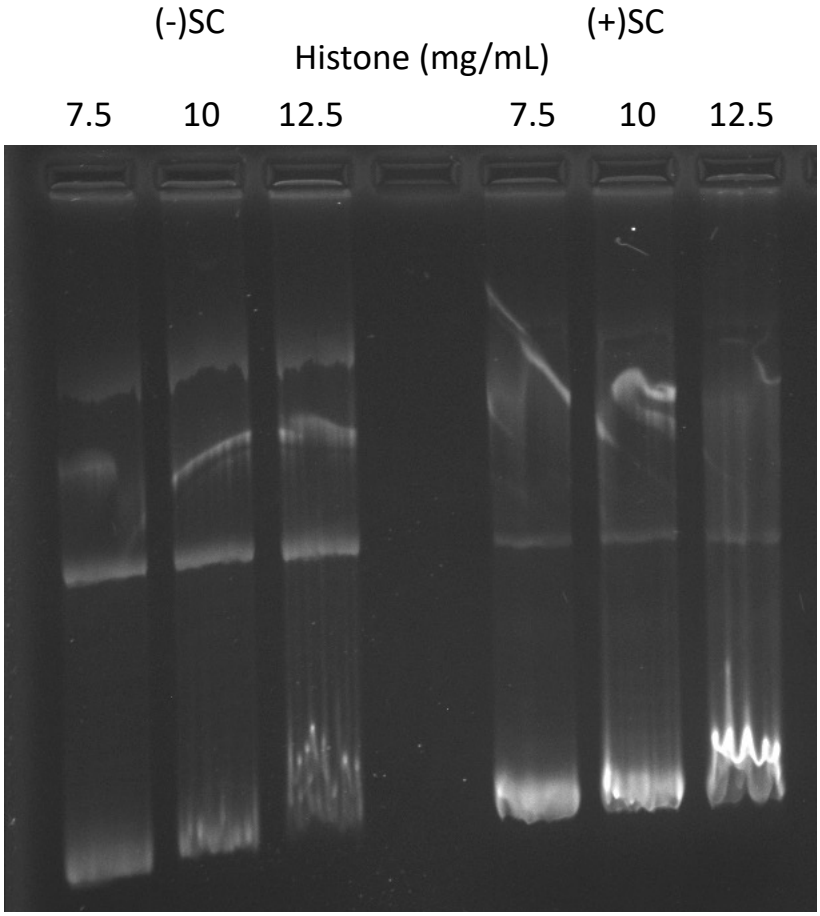

Figure 4.3: Histone H1 shows similar affinity with different DNA topoisomers. Electrophoretic mobility shift assays were performed with negatively supercoiled [(-)SC] and positively supercoiled [(+)SC] DNA incubated with varying amounts of histone H1. The observed shifts are similar suggesting that (-)SC and (+)SC DNA have similar affinities to histone H1.

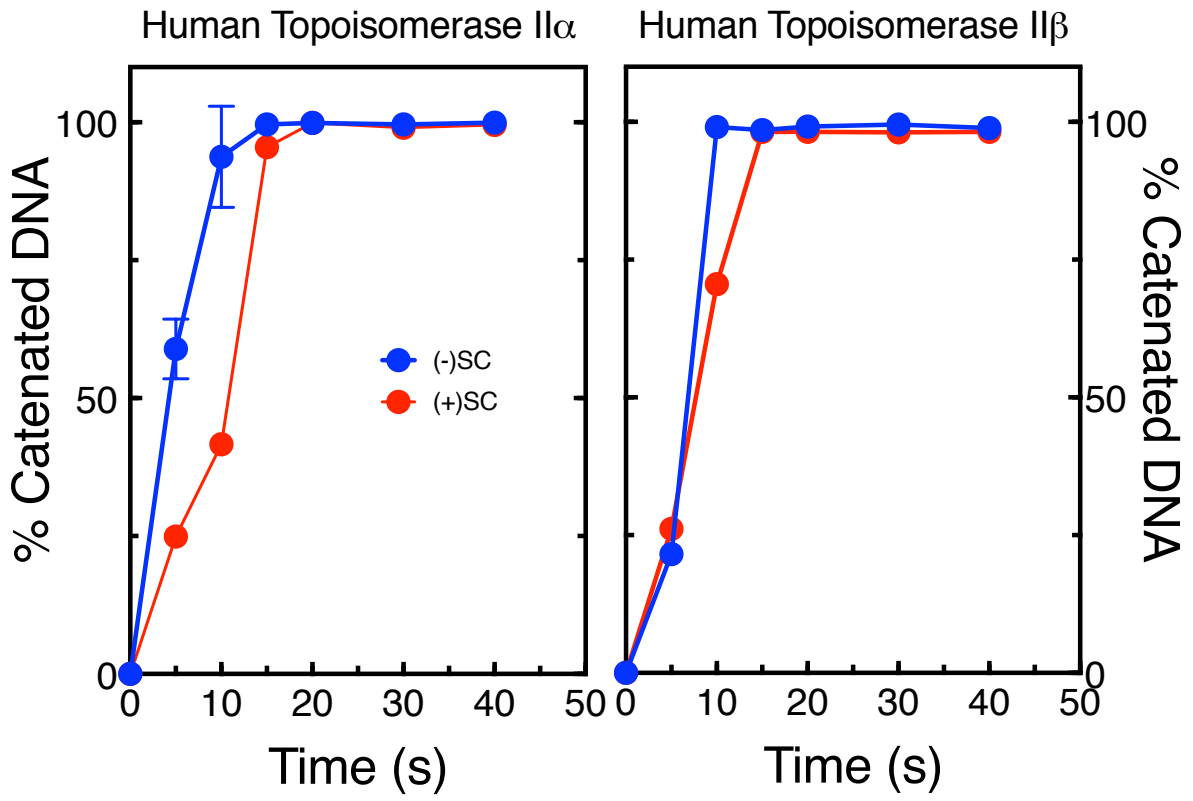

Figure 4.4: The condensing agent histone H1 is not required for supercoil handedness preference by topoisomerase IIα. *Left panel:* Catenation of negatively supercoiled [(-)SC] and positively supercoiled [(+)SC] DNA with high concentrations of topoisomerase IIα was observed in the absence of histone H1. The enzyme showed preference for catenation of negatively supercoiled DNA. *Right panel:* Catenation of (-)SC and (+)SC DNA with high concentrations of topoisomerase IIβ was observed in the absence of histone H1. The enzyme showed little discrimination between the two substrates. Error bars represent the standard deviations of at least three independent experiments.

falls to those observed with negatively supercoiled substrates. To determine whether the ability to distinguish supercoil handedness during catenation also resides in the C-terminal domain of topoisomerase IIα, DNA catenation reactions were carried out using a deletion construct that terminated at residue 1175 and lacked this domain (Topo IIαΔCTD). Similar to results seen with DNA relaxation, Topo IIαΔCTD no longer preferentially catenated negatively supercoiled and relaxed substrates (Figure 4.5). In fact, rates of catenation for these DNA topoisomers were similar and dropped below those observed with positively supercoiled DNA (Table 4.1). Therefore, the C-terminal domain of topoisomerase IIα appears to play a critical role in allowing the enzyme to distinguish DNA substrates during both intra- and intermolecular double-stranded DNA passage reactions.

Effects of Supercoil Handedness on DNA Catenation Catalyzed by Bacterial Topoisomerase IV

As discussed above, previous studies indicate that bacterial topoisomerase IV and gyrase preferentially remove positive supercoils in relaxation assays (Ashley, Blower et al. 2017, Ashley, Dittmore et al. 2017). However, because topoisomerase IV is the bacterial type II topoisomerase that is primarily responsible for catenating/decatenating DNA, we examined the effects of DNA topology on the ability of topoisomerase IV to catenate plasmid substrates. Topoisomerase IV from three species, Gram-negative *E. coli* and *N. gonorrhea*, and Gram-positive *B. anthracis*, were used for these experiments. The three enzymes displayed a strong preference for catenating positively supercoiled over negatively supercoiled substrates (Figure 4.6). In all cases, rates of catenation with positively supercoiled DNA were at least an order of magnitude faster than those observed with negatively supercoiled plasmid. Unlike human topoisomerase IIα, which displayed the opposite preference for supercoil handedness during catenation as opposed to DNA relaxation

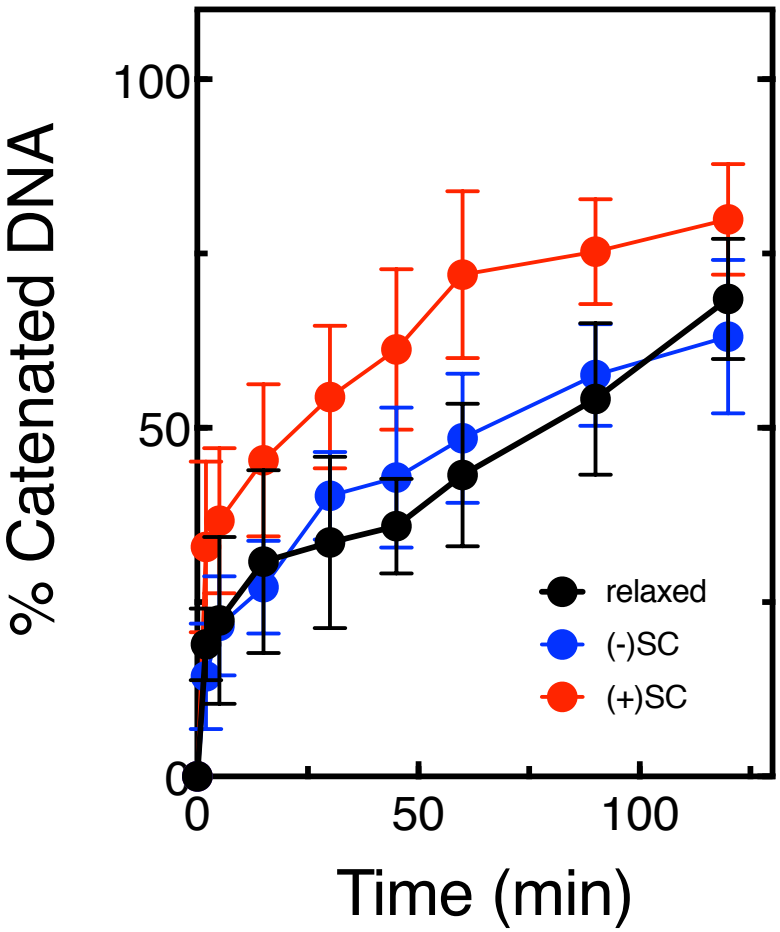

Figure 4.5: The C-terminal domain of human topoisomerase IIα is required for preferential catenation of negatively supercoiled DNA. Catenation of negatively supercoiled [(-)SC], positively supercoiled [(+)SC], and relaxed DNA by a deletion construct of human topoisomerase IIα enzyme that lacked the C-terminal domain is shown. The enzyme construct lacked the ability to preferentially catenate (-)SC DNA. Error bars represent the standard deviations of at least three independent experiments.

assays, the preference of bacterial topoisomerase IV for positive supercoils during intermolecular catenation aligns with its preference for intramolecular relaxation reactions. This finding suggests that human topoisomerase IIα and bacterial topoisomerase IV may regulate catenation/decatenation reactions differently.

A curious difference between the three bacterial enzymes was observed regarding their catenation of relaxed substrates. Catenation rates for relaxed plasmids were slower than those for negatively supercoiled DNA with *E. coli* topoisomerase IV, faster than negatively supercoiled substrates with the enzyme *N. gonorrhoeae* topoisomerase IV, and similar to positively supercoiled DNA with *B. anthracis*. At the present time, the underlying basis for these differences is not known.

Effects of Superhelical Density on DNA Catenation Rates

Given the differences between rates of DNA catenation for relaxed, negatively, and positively supercoiled substrates, we examined the influence of superhelical density on the reaction. Two potential outcomes can be envisioned. First, there could be an approximately "linear" relationship between superhelical density and catenation rates. In this case, increases in levels of supercoiling (either positive or negative) would lead to a proportional change in reaction rates. Second, there could be a sharp transition between levels of supercoiling and catenation rates. In this case, a distinct switch in reaction rates would be observed at a specific superhelical density.

To distinguish between these two possibilities, DNA species with a specific range of DNA supercoils were generated. Samples with differing degrees of negative supercoils were prepared by treating relaxed DNA with topoisomerase I in the presence of varying amounts of the intercalator chloroquine (Figure 4.7). Conversely, species with differing degrees of positive

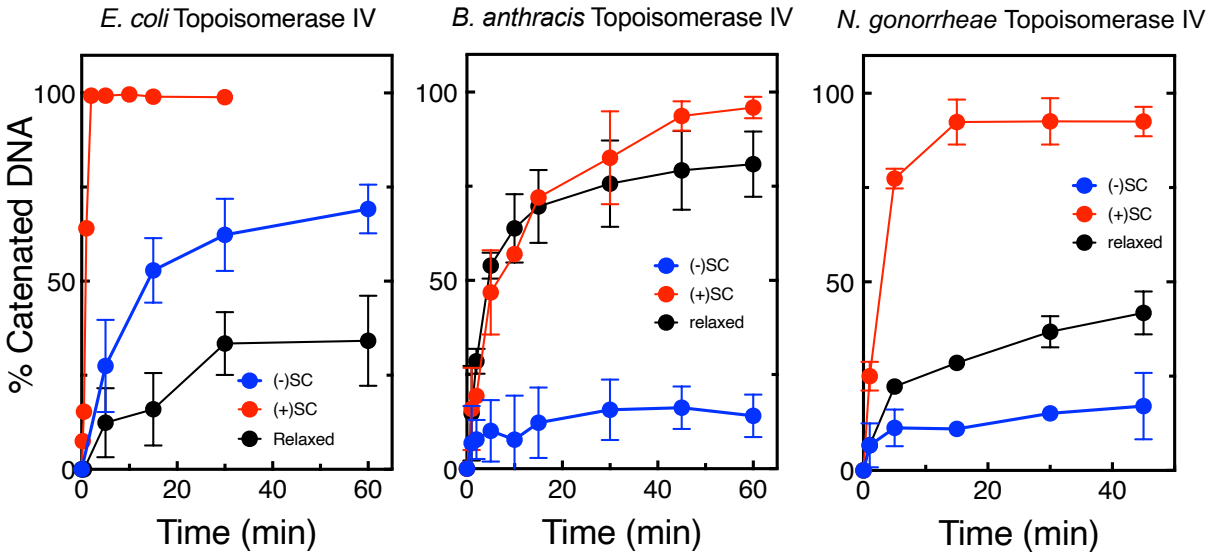

Figure 4.6: Catenation of DNA by bacterial topoisomerase IV. Negatively supercoiled [(-)SC], positively supercoiled [(+)SC], and relaxed DNA were catenated by *E. coli* topoisomerase IV, *B. anthracis* topoisomerase IV, or *N. gonorrhoeae* topoisomerase IV. All three enzymes catenated (+)SC DNA faster than (-)SC DNA. Error bars represent the standard deviations of at least three independent experiments.

supercoils were created by treating DNA with varying amounts of reverse gyrase (Figure 4.8). These DNA species were evaluated for superhelical density using a 2D gel electrophoresis system (Figure 4.7) (Gibson, Oviatt et al. 2020).

Initial experiments were carried out with *B. anthracis* topoisomerase IV because of the large difference between rates of catenation observed between relaxed and negatively supercoiled DNA. As seen in Figure 4.9, rates of catenation decrease sharply between 0.15 and 0.25 mg/mL chloroquine. These values correspond to substrates containing ~3-8 negative supercoils (median ≈ 5 supercoils) and ~5-11 negative supercoils (median ≈ 8). Thus, the transition for catenation rates between relaxed and negatively supercoiled substrates occurs relatively abruptly with substrates containing 5 or fewer supercoils acting like relaxed plasmids while those containing ~8 and above acting like negatively supercoiled substrates. Figure 4.10 shows catenation over the transition between relaxed and positively supercoiled DNA. As shown in Figure 4.6, *B. anthracis* topoisomerase IV catenates relaxed and positively supercoiled DNA at similar rates. Therefore, as expected, substrates with varying degrees of positive supercoiling show similar amounts of catenation.

Pathway to DNA Catenation

Although high molecular weight catenanes (i.e., those that are too large to enter the gel) likely have very complex and diverse structures, two general pathways can be envisioned by which type II topoisomerases can mediate their formation. In the first, dimeric structures are formed and act as a nucleation point for the further incorporation of monomeric plasmids. In this case, dimers and potentially higher order structures would appear as steady-state intermediates in the generation of complexes that no longer enter the agarose gel. In the second pathway, the enzyme might display

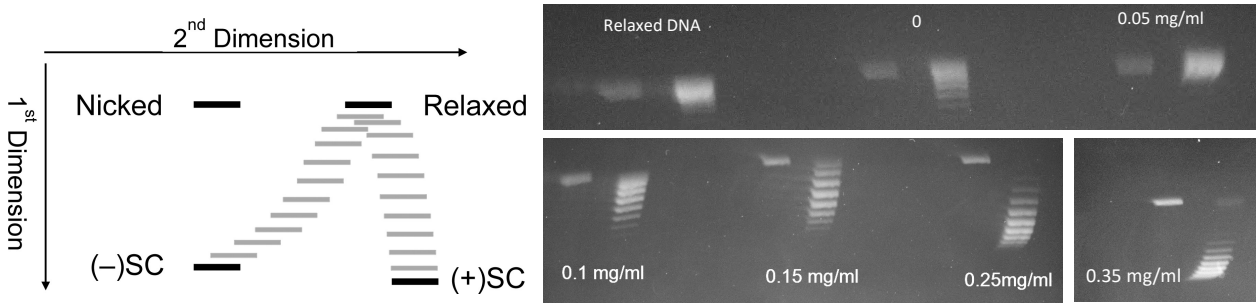

Figure 4.7: 2D gel of DNA with varying levels of negative supercoiling. Left: Schematic depicting the migration of topoisomers during 2D gel electrophoresis. The positions of nicked, relaxed, negatively supercoiled [(−)SC], and positively supercoiled [(+)SC] DNA are shown as black bands. Gray bands represent DNA topoisomers of intermediate supercoiling. Partially negatively supercoiled molecules migrate as the arc between relaxed and (−)SC DNA and partially positively supercoiled molecules migrate as the arc between (+)SC and relaxed DNA. Figure adapted from Gibson and Oviatt *et. al* (Gibson, Oviatt et al. 2020). Right: 2D gel of DNA with varying levels of negative supercoiling**.** Increasing amounts of chloroquine was added to relaxed DNA and treated with topoisomerase I. The chloroquine was then removed to create products with different amounts of negative supercoiling.

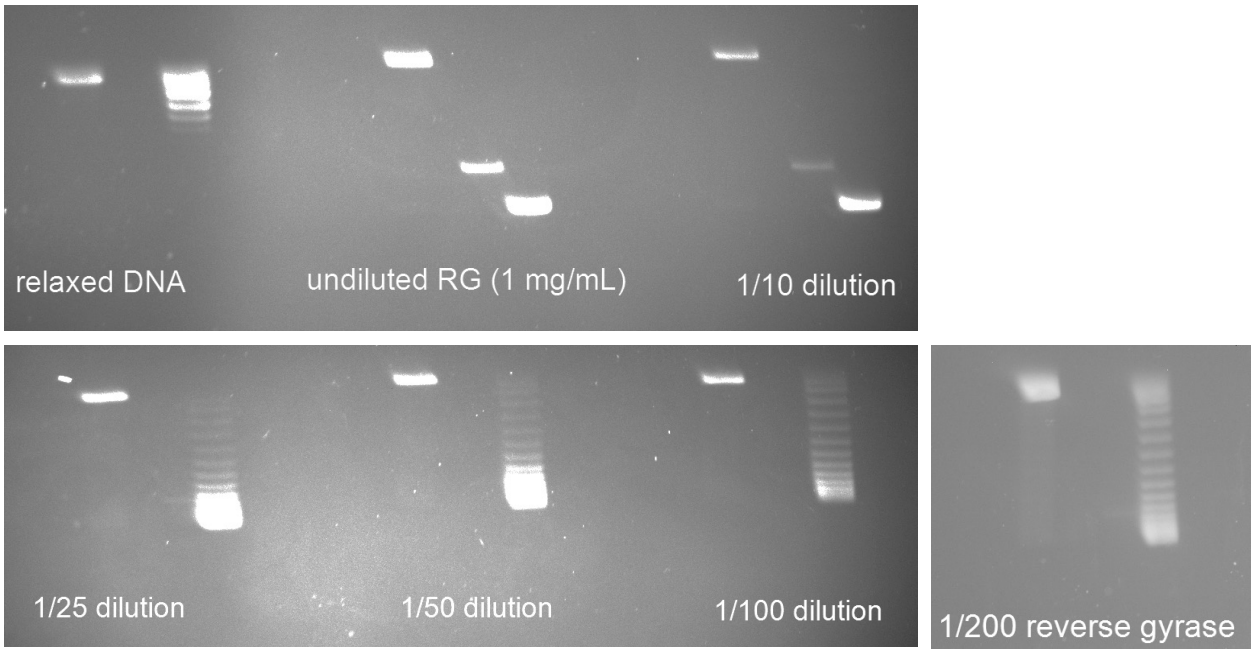

Figure 4.8: 2D gel of DNA with varying levels of positive supercoiling. A titration of reverse gyrase was used on relaxed DNA and the DNA was purified to create products with increasing amounts of positive supercoiling.

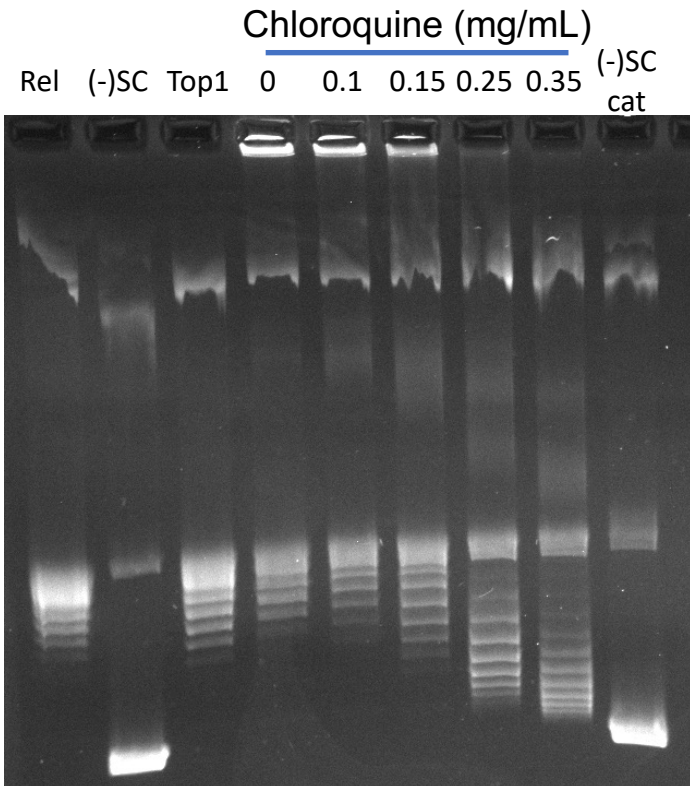

Figure 4.9: Effects of DNA with increasing levels of negative supercoiling on catenation. DNA substrates with increasing amounts of negative supercoiling were created using a titration of chloroquine and topoisomerase I. When negatively supercoiled DNA species were catenated using *B. anthracis* topoisomerase IV for 10 minutes, catenation decreased after a ~5-8 negative supercoils were added. Sample labeled Top1 was an enzyme control for topoisomerase I that did not contain any chloroquine or topoisomerase IV.

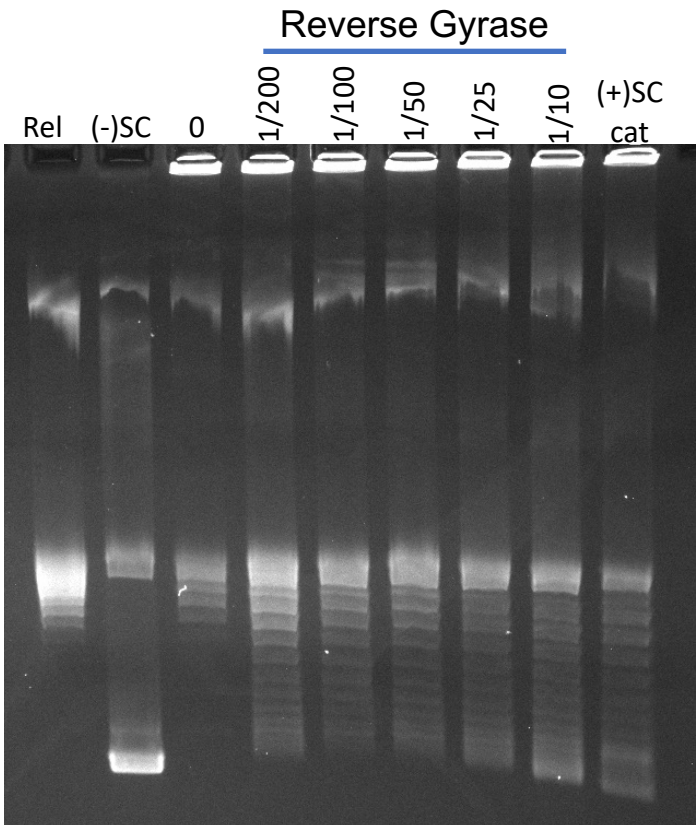

Figure 4.10: Effects of DNA with increasing levels of positive supercoiling on catenation. DNA with increasing amounts of positive supercoiling was created using a titration of reverse gyrase. As relaxed DNA and positively supercoiled DNA has similar rates of catenation by *B. anthracis*, the amount of catenation was unchanged as positive supercoils were added.

a preference for monomeric plasmids, converting them all to dimers before higher-order structures are observed. The following set of experiments was carried out to examine the pathway by which type II topoisomerases catenate DNA and to determine if plasmids with different supercoil handedness follow the same pathway.

DNA catenation assays utilizing human topoisomerase IIα and *B. anthracis* topoisomerase IV were performed in the presence of histone H1 as described in Figure 4.2 and Figure 4.6, respectively, with the exception that reaction mixtures were incubated at 25 ºC (as opposed to 37 ºC) to slow catenation rates. In addition, samples were treated with the nicking endonuclease Nt.BspQI after catenation was terminated but before gel electrophoresis to eliminate potential supercoiling differences between products and simplify the kinetic analysis. To maintain endonuclease activity, catenation reactions were terminated by incubation at 80 ºC rather than with the addition of SDS/EDTA mixtures. Catenation was effectively terminated by this heat protocol (Figure 4.11, Figure 4.12).

Results with topoisomerase IIα are shown in Figure 4.13. Starting with negatively supercoiled monomeric plasmids, steady-state intermediates presumed to be dimers and trimers are visible over the disappearance of monomeric substrates. Similar results were seen when positively supercoiled substrates were used, albeit at slower reaction rates. These results suggest that topoisomerase IIα catenates DNA via the second pathway in which monomers are linked to higher-order structures. Furthermore, this pathway is unaffected by supercoil handedness.

To determine if these relationships also held for a bacterial type II topoisomerase, parallel experiments were carried out using *B. anthracis* topoisomerase IV (Figure 4.14). Similar conclusions appear to hold for the bacterial enzyme, although positively supercoiled substrates were catenated much more rapidly than negatively supercoiled plasmids.

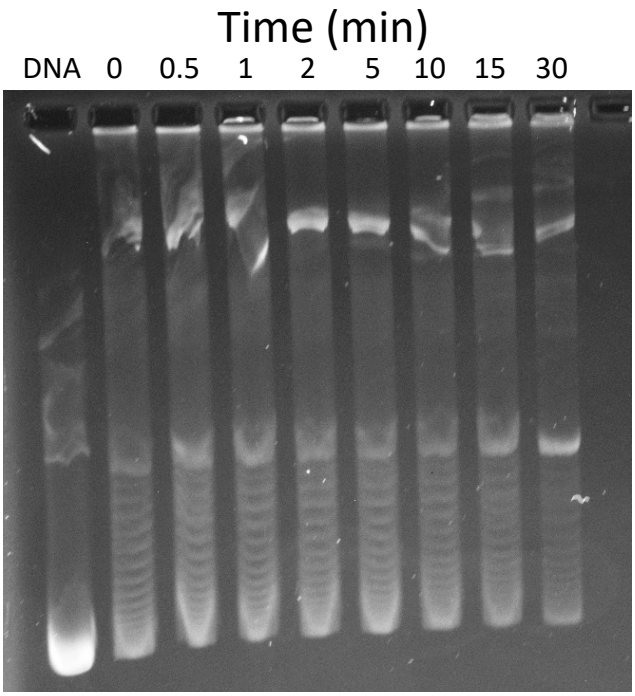

Figure 4.11: Catenation stopped with high heat. Catenation of (-)SC DNA with human topoisomerase IIα was incubated at 25 °C for 5 min and then transferred to 80 °C and observed over time. Nicked DNA increased over time at high heat but amounts of catenation did not increase indicating that the catenation reaction was effectively stopped by incubation at the higher temperature.

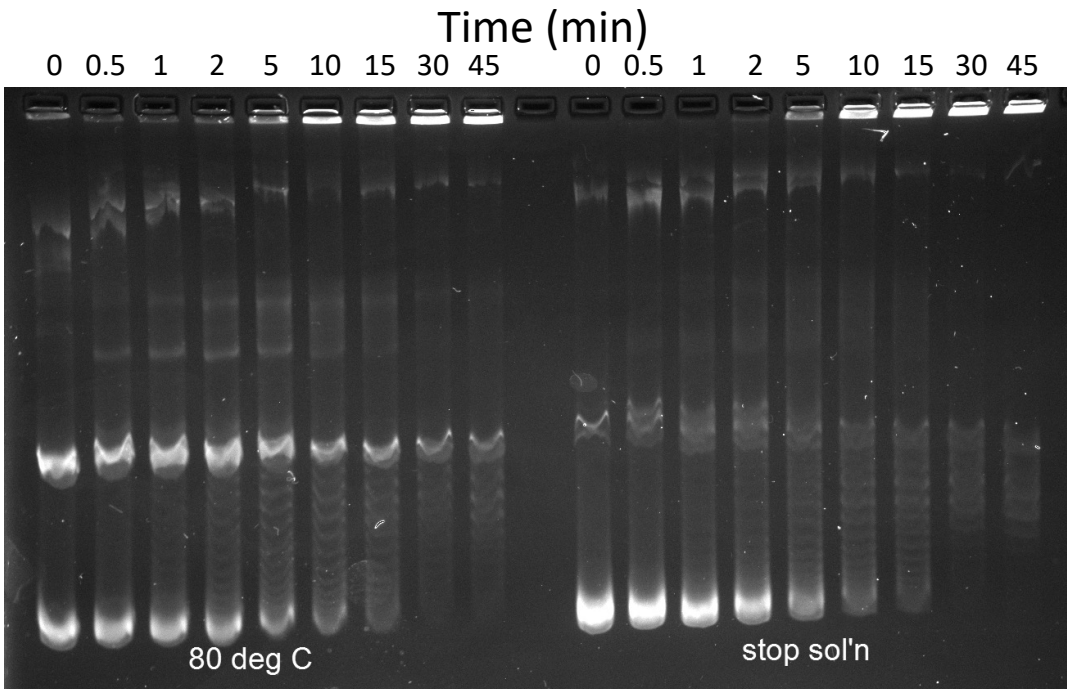

Figure 4.12: Catenation stopped with SDS/EDTA versus high heat. Catenation of (-)SC DNA with human topoisomerase IIα was stopped with either high heat at 80 °C for 20 minutes (left) or with SDS/EDTA (stop solution) (right). Catenation stopped with high heat shows increased nicked DNA.

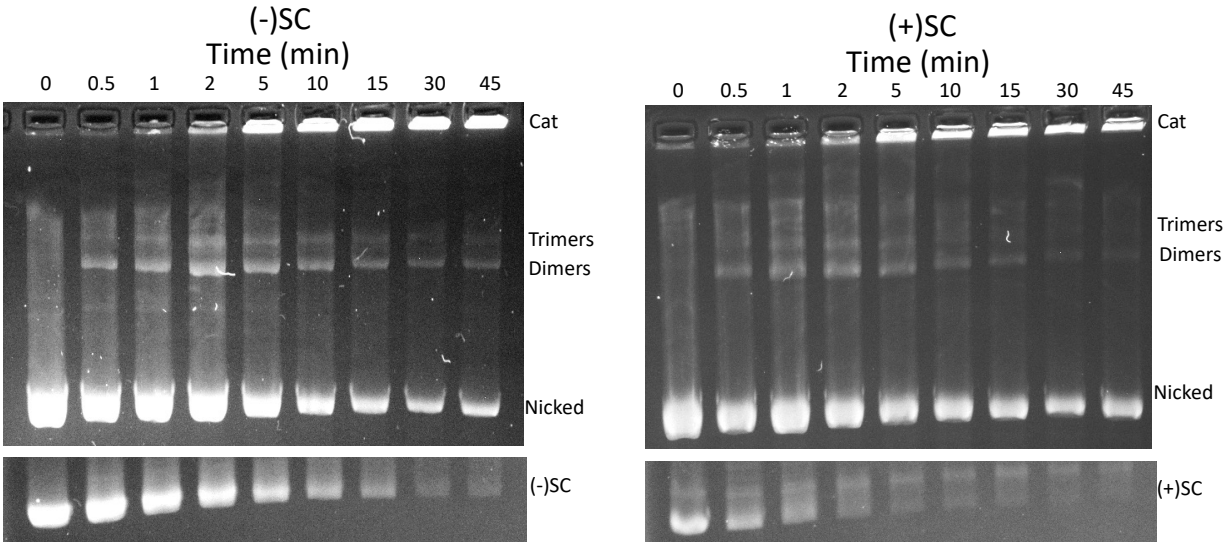

Figure 4.13: Production of catenation intermediates by human topoisomerase IIα. Catenation of negatively supercoiled DNA [(-)SC] (*top left*) and positively supercoiled DNA [(+)SC] (*top right*) by human topoisomerase IIα was carried out at 25 °C, terminated at 80 °C, and nicked by nicking endonuclease Nt.BspQI. Monomeric unnicked substrate is displayed for comparison (*bottom*). As reaction proceeds, steady state intermediates are visible over the disappearance of monomeric substrates.

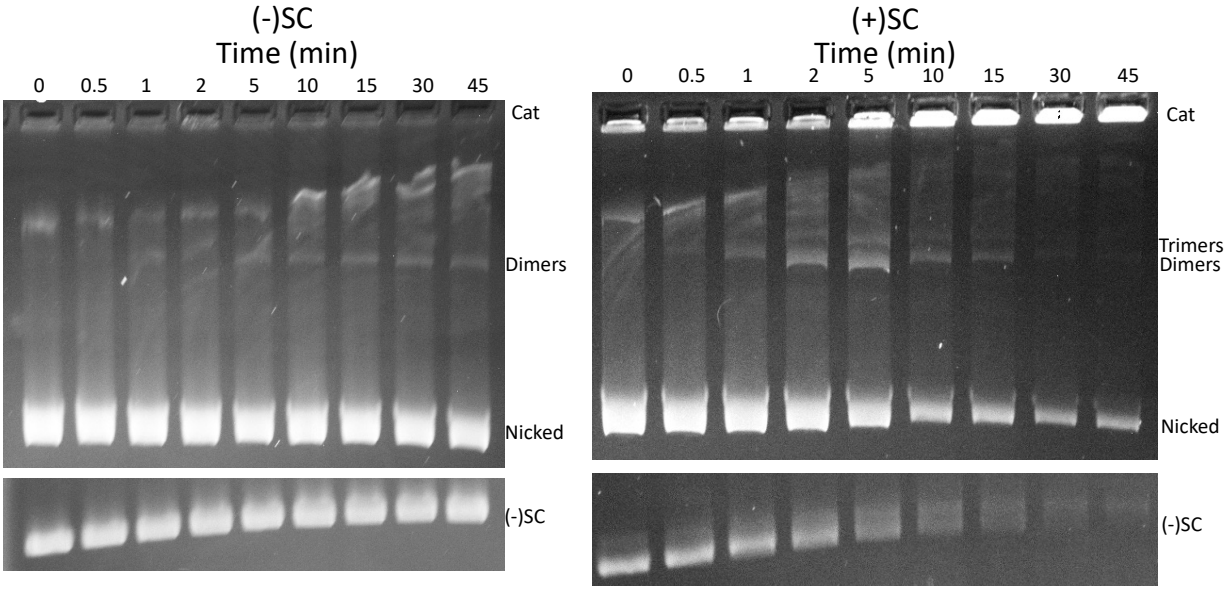

Figure 4.14: Production of catenation intermediates by *B. anthracis* topoisomerase IV. Catenation of negatively supercoiled DNA [(-)SC] (*top left*) and positively supercoiled DNA [(+)SC] (*top right*) by *B. anthracis* topoisomerase IV was carried out at 25 °C, terminated at 80 °C, and nicked by nicking endonuclease Nt.BspQI. Monomeric unnicked substrate is displayed for comparison (*bottom*). As reaction proceeds, steady state intermediates are visible over the disappearance of monomeric substrates.

Effects of Catenation on DNA Cleavage Mediated by Type II Topoisomerases

Type II topoisomerases generate transient double-stranded breaks as requisite intermediates in their double-stranded DNA passage reactions (Pendleton, Lindsey et al. 2014). To maintain genomic integrity while the DNA is cleaved, these enzymes become linked to the DNA through covalent bonds made between the newly generated 5′-terminal DNA phosphates and the 4′-OH substituent of active site tyrosine residues (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Nitiss 2009, Aldred, Kerns et al. 2014, Ketron and Osheroff 2014, Pendleton, Lindsey et al. 2014, Pommier, Sun et al. 2016, Ashley and Osheroff 2019, Vann, Oviatt et al. 2021). These covalent enzyme-DNA complexes are known as cleavage complexes. If ligation of the double helix in cleavage complexes is impeded, they become extremely dangerous to the cell (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Nitiss 2009, Aldred, Kerns et al. 2014, Ketron and Osheroff 2014, Pendleton, Lindsey et al. 2014, Pommier, Sun et al. 2016, Ashley and Osheroff 2019, Vann, Oviatt et al. 2021). Consequently, a number of compounds that stabilize cleavage complexes are used as front-line anticancer and antibacterial drugs that convert type II topoisomerases into lethal enzymes through generation of double-stranded breaks and fragmentation of the genome (Deweese, Osheroff et al. 2008, Deweese and Osheroff 2009, Nitiss 2009, Aldred, Kerns et al. 2014, Ketron and Osheroff 2014, Pendleton, Lindsey et al. 2014, Pommier, Sun et al. 2016, Gibson, Ashley et al. 2018, Ashley and Osheroff 2019, Bax, Murshudov et al. 2019, Vann, Oviatt et al. 2021). Catenated sister chromatids might be particularly susceptible to the dangers of cleavage complexes as the forces induced by the mitotic spindle have the potential to pull apart these structures, generating double-stranded breaks.

To address the issues outlined above, the effects of catenation on DNA cleavage mediated by type II topoisomerases were examined. Kinetoplast DNA (kDNA), a highly catenated network of

mitochondrial DNA maxi- and minicircles (38 kb and 2.5 kb, respectively) from the unicellular eukaryote *C. fasciculata*, was used for this study (Lukeš, Guilbride et al. 2002). kDNA is generally nicked, and consequently is incapable of maintaining supercoils (Rauch, Perez-Morga et al. 1993, Chen, Rauch et al. 1995, Lukeš, Guilbride et al. 2002). DNA cleavage was monitored over the course of a decatenation assay carried out by human topoisomerase IIα (Figure 4.15). Cleavage complexes were trapped by terminating reactions with SDS, which rapidly denatures the type II enzyme before it can ligate the cleaved DNA (Bandele and Osheroff 2009). Levels of DNA cleavage in reaction mixtures were increased by the addition of etoposide, an anticancer drug that stabilizes cleavage complexes. Under the conditions employed, decatenation was completed in ~120 s. Over the course of the decatenation assay, levels of enzyme-mediated DNA cleavage increased ~8-fold (Figure 4.15). This finding suggests that DNA that exists in a highly catenated network is less likely to contain DNA strand breaks generated by topoisomerase IIα than is DNA in monomeric substrates.

A caveat to the above conclusion is that the enhancement of DNA cleavage represents the time it takes to form an enzyme-DNA-drug-ATP complex rather than a conversion from catenated to monomeric DNA. Three controls suggest this is not the case. First, the enzyme-DNA-drug complex was formed and allowed to reach DNA cleavage-ligation equilibrium prior to the initiation of decatenation by the addition of ATP. Second, reactions were followed for 300 s, well beyond the time required for complete decatenation (Figure 4.16), and no additional increase in DNA cleavage was observed once decatenation was complete. Third, a parallel reaction was carried out using relaxed monomeric plasmid substrate to monitor the time to DNA cleavage-ligation equilibrium in the presence of ATP. The relaxed monomeric substrate was used because it did not change topological state over the course of the reaction. As seen in Figure 4.17,

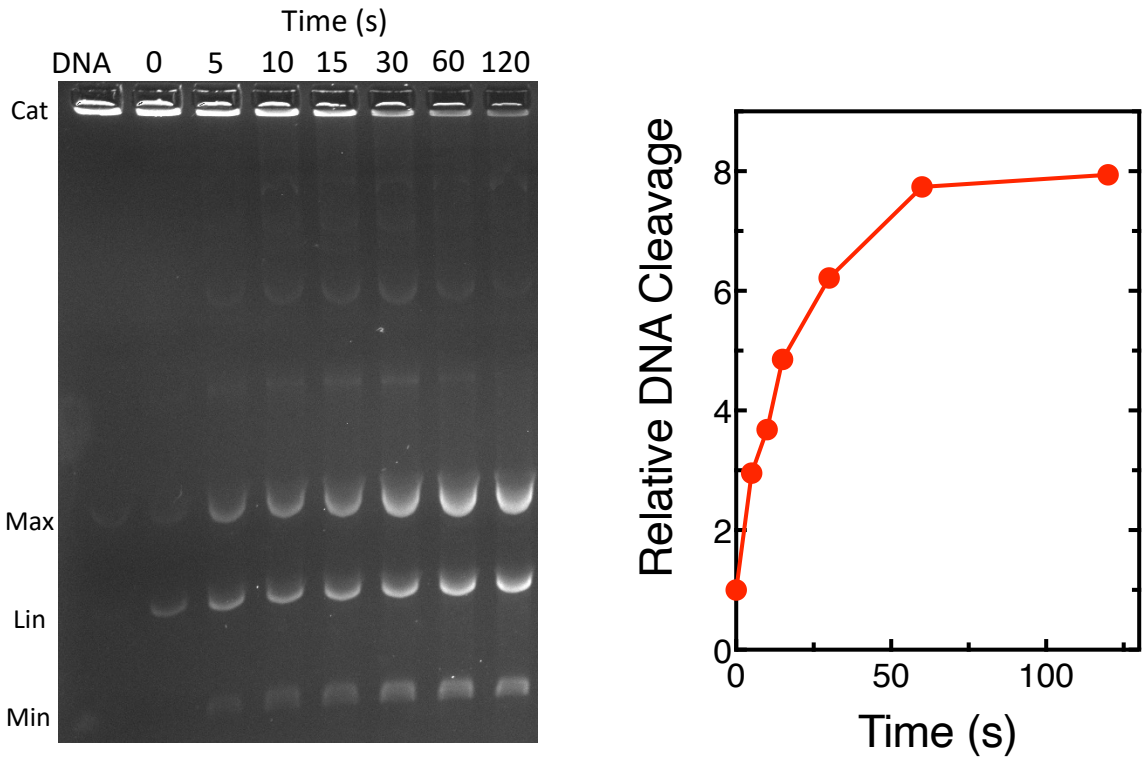

Figure 4.15: Catenation protects DNA from cleavage mediated by human topoisomerase IIα. kDNA was allowed to reach cleavage-ligation equilibrium and then decatenated over time with human topoisomerase IIα in the presence of etoposide. Reactions were stopped with 5% SDS to trap cleavage complexes. As kDNA (cat) decatenated into minicircles (min) and maxicircles (max), the amount of linear DNA (lin) increased suggesting that catenated kDNA maintains a lower level of cleavage complexes with catenated kDNA than with monomeric substrates. A representative graph is shown at right.

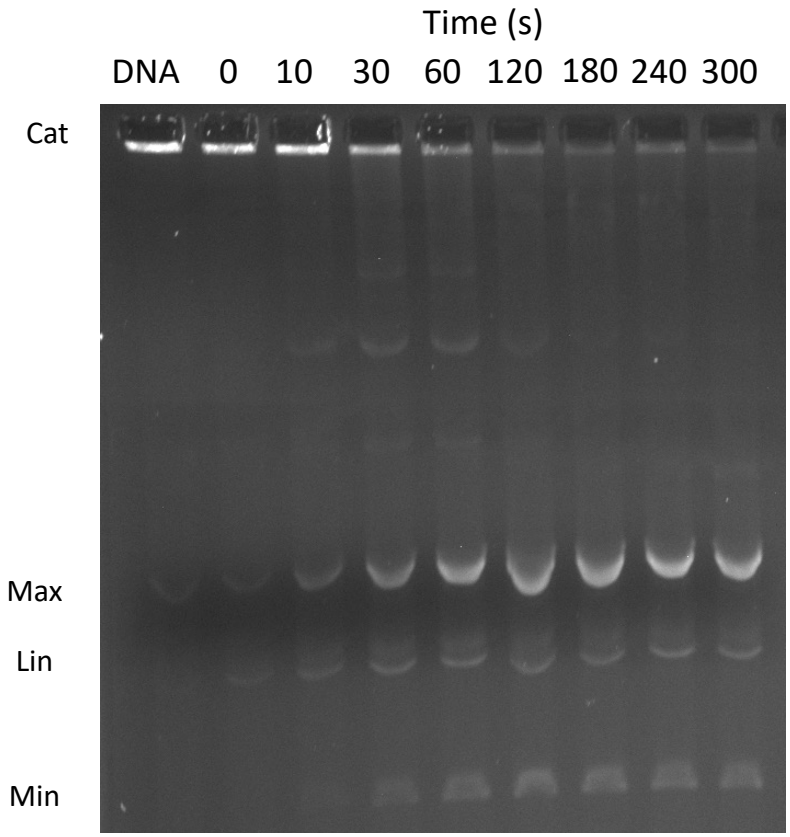

Figure 4.16: Amounts of linear kDNA do not increase after decatenation is complete. kDNA was allowed to reach cleavage-ligation equilibrium and then decatenated over 300 s with human topoisomerase IIα. Reactions were stopped with 5% SDS to trap cleavage complexes. kDNA (Cat) became completely decatenated by 120 s, after which point the amount of linear DNA (Lin) stopped increasing and remained constant.

cleavage-ligation equilibrium was reached within 10 s and no further increase in DNA cleavage was observed over the course of the 300 s assay. Taken together, these controls support the conclusion that catenation protects DNA from cleavage mediated by topoisomerase IIα.

To determine whether a similar conclusion can be drawn with bacterial type II topoisomerases, a parallel decatenation/DNA cleavage experiment was carried out using *E. coli* topoisomerase IV. This enzyme was chosen because it maintains high levels of cleavage complexes (Aldred, Breland et al. 2014, Ashley, Dittmore et al. 2017). Consequently, the experiment shown in Figure 4.18 was carried out in the absence of cleavage-enhancing antibacterial drugs. As seen with the human enzyme, DNA cleavage increased over the course of the decatenation assay. Although this increase was not as pronounced as seen with the human enzyme (cleavage rose a little over 2-fold over the course of the assay), it suggests that catenation also protects DNA from cleavage mediated by the bacterial type II topoisomerase.

Discussion

Although the genomes of organisms ranging from bacteria to humans are globally underwound (negatively supercoiled) (Linka, Porter et al. 2007, Deweese and Osheroff 2009), regions that are upstream from replication forks, transcription complexes, and other DNA tracking machinery can be severely overwound (positively supercoiled) (Linka, Porter et al. 2007, Deweese and Osheroff 2009, Baxter, Sen et al. 2011). These changes in supercoil handedness can have profound effects on nucleic acid processes.

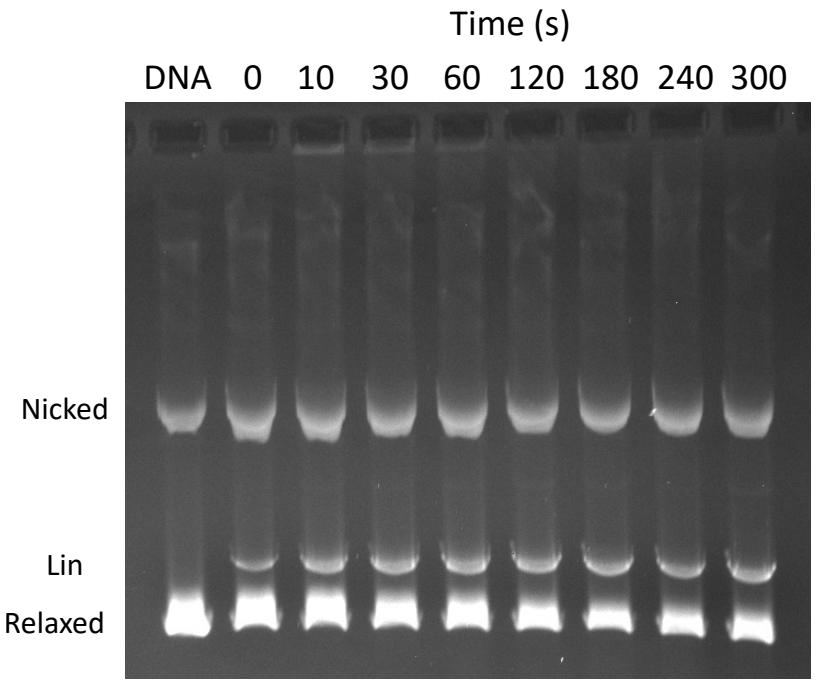

Figure 4.17: Increases in DNA cleavage during decatenation reactions are not due to equilibration time. Relaxed monomeric DNA was allowed to reach cleavage-ligation equilibrium and then decatenated over 300 s with human topoisomerase IIα. Reactions were stopped with 5% SDS to trap cleavage complexes. Cleavage-ligation equilibrium was reached within 10 s and no further increases in linear DNA (Lin) was seen, suggesting that increases seen with kDNA are not due to equilibration time.

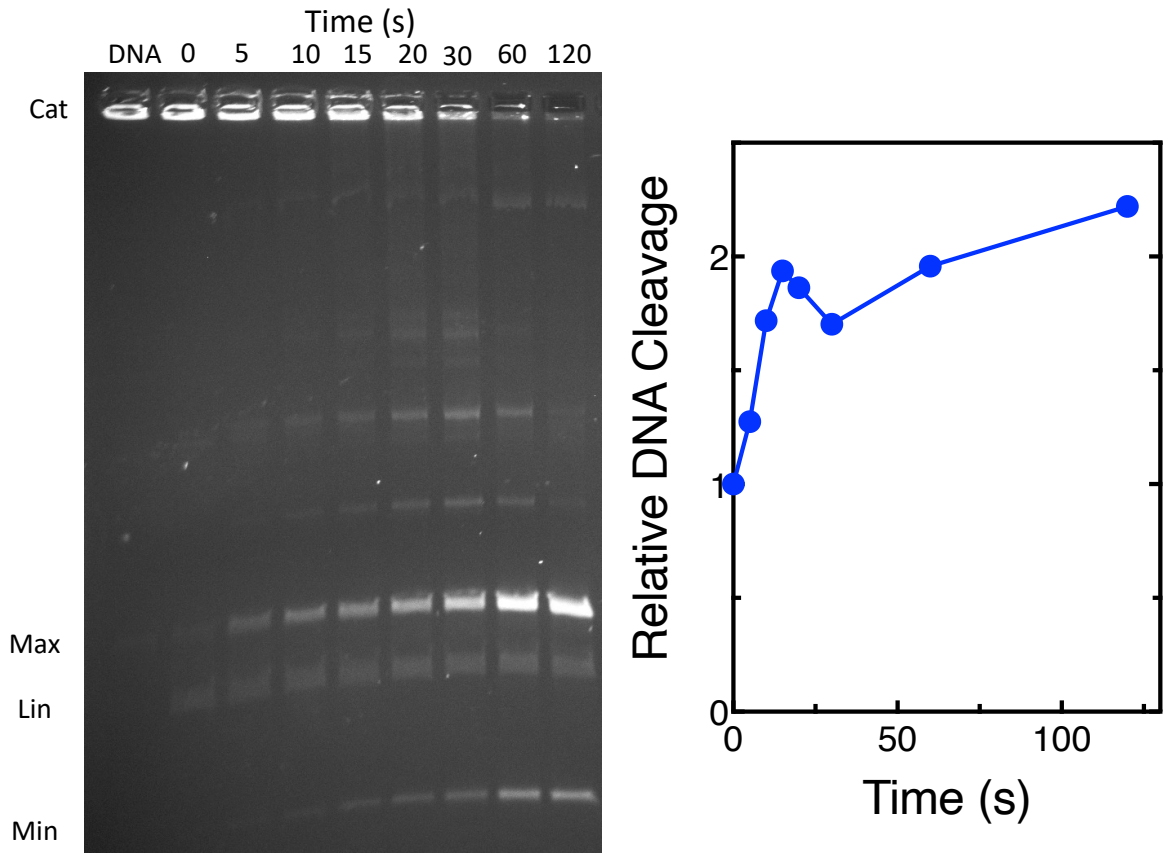

Figure 4.18: Catenation protects DNA from cleavage mediated by *E. coli* topoisomerase IV. kDNA was allowed to reach cleavage-ligation equilibrium and then decatenated over time with *E. coli* topoisomerase IV. Reactions were stopped with 5% SDS to trap cleavage complexes. As kDNA (Cat) decatenated into minicircles (Min) and maxicircles (Max), the amount of linear DNA (Lin) increased suggesting that catenated kDNA holds lower levels of cleavage complexes than decatenated kDNA. A representative graph is shown at right.

The generation of intertwined (i.e., catenated) DNA molecules represents one such nucleic acid process (Nitiss 2009, Bauer, Marie et al. 2012, Sen, Leonard et al. 2016). The presence of DNA catenanes is precarious. While their formation stabilizes interactions between daughter chromosomes, a lack of resolution can have serious consequences for genomic stability (Nitiss 2009, Bauer, Marie et al. 2012, Sen, Leonard et al. 2016). Therefore, the current work was carried out to determine what the effects of supercoil handedness are on the ability of human and bacterial type II topoisomerases to catenate DNA.

In parallel to DNA relaxation reactions, topoisomerase IIα was able to distinguish between different supercoiled states of DNA whereas topoisomerase IIβ was not. Surprisingly, the α isoform catenated relaxed and underwound molecules faster than it did overwound substrates, a supercoil preference that is opposite from what was found for DNA relaxation. The reason for this opposite preference for supercoil handedness is not known, however it may have evolved to decrease the possibility of intertangling DNA molecules ahead of approaching replication complexes. In contrast, topoisomerase IV from three bacterial species preferentially catenated positively supercoiled DNA. However, as this enzyme acts primarily behind replication forks, this preference may not be deleterious to cellular processes. Furthermore, as DNA precatenanes appear to be positively supercoiled (Postow, Crisona et al. 2001), this behavior may help keep daughter chromosomes together until separation in anaphase.

Finally, both topoisomerase IIα and topoisomerase IV, the type II enzymes that are involved in chromosome segregation, maintain lower levels of cleavage complexes in catenated as opposed to monomeric DNA. This allows these enzymes to perform their cellular functions in a safer manner as catenated daughter chromosomes may be subject to stress generated by the mitotic spindle that could lead to irreversible DNA cleavage.

CHAPTER 5

CONCLUSIONS AND IMPLICATIONS

Covalent Poisons and Natural Products

Natural products are some of the most productive sources of compounds that have been used for drug development (Newman and Cragg 2012, Cragg and Newman 2013). Natural products and their derivatives have been used as the basis for almost half of the anticancer drugs used worldwide, and many of the topoisomerase II poisons derived from natural products show chemotherapeutic or chemopreventative properties (Austin, Patel et al. 1992, Champoux 2001, Bandele and Osheroff 2007, Lopez-Lazaro, Willmore et al. 2007, Bandele, Clawson et al. 2008, Bandele and Osheroff 2008, Deweese and Osheroff 2009, Nitiss 2009, Lopez-Lazaro, Willmore et al. 2010, Pommier, Leo et al. 2010, Lin, Zhou et al. 2011, Vos, Tretter et al. 2011, Ketron, Gordon et al. 2013, Timmel, Byl et al. 2013, Vann, Oviatt et al. 2021). Drugs such as etoposide and doxorubicin are used to treat a variety of hematological and solid tumors and mitoxantrone is used in the treatment of breast cancer and autoimmune diseases such as multiple sclerosis (Hasan, Mays et al. 2008, Pendleton, Lindsey et al. 2014). In addition to anticancer drugs, a number of phytochemicals with anticancer, chemopreventative, or other health-promoting properties act as topoisomerase II poisons (Ketron and Osheroff 2014). Among these compounds are bioflavonoids (Austin, Patel et al. 1992, Bandele and Osheroff 2007, Bandele, Clawson et al. 2008), catechins (Bandele and Osheroff 2008, Lopez-Lazaro, Calderon-Montano et al. 2011), curcumin (Lopez-Lazaro, Willmore et al. 2007, Ketron, Gordon et al. 2013), isothiocyanates (Lin, Zhou et al. 2011),

and antioxidants such as hydroxytyrosol, oleuropein, and verbascoside (Vann, Sedgeman et al. 2015).

Chapter 3 discusses the effects of 6,6'-dihydroxythiobinupharidine (DTBN), a natural product from *Nuphar lutea*, the yellow water lily, on human type II topoisomerases. DTBN enhanced DNA cleavage mediated by both topoisomerase IIα and IIβ, a property required of a topoisomerase poison. Unusually, it showed greater effect on topoisomerase IIα, which may mitigate the leukemogenic potential of the β isoform. Further results suggested that DTBN may be a covalent poison of topoisomerase II. Cleavage complexes induced by DTBN were seen to be stable for over 4 h, and incubation with the enzyme prior to the addition of DNA caused inhibition of DNA cleavage. Covalent poisons adduct cysteine residues and require redox cycling, and as a result their actions can be diminished by the use of a reducing agent or thiol containing compound. In the presence of the thiol compounds dithiothreitol and glutathione and the reducing agent ascorbic acid, the activity of DTBN on topoisomerase IIα decreased 50%, an effect that is consistent with covalent poisoning. It is possible that DTBN may form an iminium that is affected by reducing agents and thiol-containing compounds.

One of the major differences between covalent poisons and interfacial poisons is the location at which the compound acts. Interfacial poisons such as etoposide act at the enzyme active site, whereas covalent poisons act at a site distal to the active site, usually at the N-terminal domain (Wang, Mao et al. 2001, Bender, Lehmler et al. 2006, Bender and Osheroff 2007, Lin, Zhou et al. 2011, Pommier and Marchand 2012, Lindsey, Pendleton et al. 2014). DTBN, consistent with the properties of a covalent poison, was unable to induce DNA cleavage in the absence of the N-terminal domain.

All these results taken together suggest that DTBN is a covalent poison of human type II topoisomerases. *N. lutea* extracts have previously been used as a treatment for inflammation in the traditional medicine of Lebanon, Japan, and the Gitskan people of British Columbia, Canada. They have also been reported to have antibacterial, antileishmanial, and potential anticancer properties (El-On, Ozer et al. 2009, El-On, Ozer et al. 2009, Ozer, El-On et al. 2010, Ozer, Levi et al. 2015, Ozer, Fishman et al. 2017, Levy, Chapple et al. 2019). As DTBN is the active compound in *N. lutea*, it is likely that some of these properties and medicinal uses come from interactions with topoisomerases.

Further studies with DTBN could include addition of the compound to cells to determine whether it still displays effects of a topoisomerase poison when not in a purified system. The compound may have to be modified to allow entry into cells, as it has a molecular weight of over 500 g/mol. As mentioned earlier, many topoisomerase II poisons that are used as anticancer drugs were initially developed from natural products. If DTBN shows effectiveness as a topoisomerase poison in cells, it could be investigated further for potential as a therapeutic.

A caveat to studying DTBN would be the requirement of a very high dose. Results shown in Chapter 3 suggest that 1 mM DTBN induces ~16% cleavage of the DNA substrate. The high amount of the compound required to produce this DNA cleavage effect would mean that a concentration would need to be found for use in cells that is high enough to produce the desired outcome without causing an undue level of off-target effects on the cell.

The Catenation/Decatenation Reaction and Recognition of DNA Supercoil Geometry

As mentioned in Chapter 1, the genomes of eukaryotes and bacteria are globally underwound (negatively supercoiled) (Linka, Porter et al. 2007, Deweese and Osheroff 2009). Despite this,

regions that are upstream from replication forks, transcription complexes, and other DNA tracking machinery can be severely overwound (positively supercoiled) (Linka, Porter et al. 2007, Deweese and Osheroff 2009, Baxter, Sen et al. 2011). These changes in supercoil handedness can have profound effects on nucleic acid processes.

Catenation of DNA is one such nucleic acid process. While catenane formation stabilizes interactions between daughter chromosomes, catenanes can pose a danger as well, as a lack of resolution can have serious consequences for genomic stability (Nitiss 2009, Bauer, Marie et al. 2012, Sen, Leonard et al. 2016). Therefore, experiments discussed in Chapter 4 investigate the effects of DNA supercoil handedness on the intramolecular catenation reaction catalyzed by type II topoisomerases.

As mentioned earlier in this dissertation, several type II topoisomerases recognize supercoil handedness of DNA during enzymatic function. Notably, topoisomerase IIα and topoisomerase IV both show preference for positively supercoiled DNA over negatively supercoiled DNA during the intermolecular relaxation reaction (McClendon, Rodriguez et al. 2005) (Ashley, Dittmore et al. 2017).

Consistent with these previous findings, human topoisomerase IIα and bacterial topoisomerase IV both showed preference of supercoil handedness during catenation. However, topoisomerase IIα preferentially catenated negatively supercoiled DNA, which was the opposite preference to what had been seen with relaxation. The reason for this opposite preference for supercoil handedness is not known but may have evolved to decrease the possibility of intertangling DNA molecules ahead of approaching replication complexes. In contrast, topoisomerase IV from three bacterial species preferentially catenated positively supercoiled DNA, although, as this enzyme acts primarily behind replication forks, this preference may not be dangerous to cellular processes.

Furthermore, as DNA precatenanes appear to be positively supercoiled (Postow, Crisona et al. 2001), this may help keep daughter chromosomes together until separation during anaphase.

Studies performed in bacterial and yeast cells indicate that DNA becomes positively supercoiled prior to decatenation in the presence of condensins and other mitotic factors (Baxter, Sen et al. 2011, Zawadzki, Stracy et al. 2015). As bacterial topoisomerase IV also preferentially relaxes and preferentially catenates positively supercoiled DNA over negatively supercoiled DNA, one can speculate that topoisomerase IV may show a general preference for positively supercoiled DNA during double-stranded DNA strand passage events. If this is the case, it is possible that topoisomerase IV acts by a different mechanism than human topoisomerase IIα, as the human enzyme shows different supercoil handedness preference between the relaxation and catenation reactions.

For future studies, it may be interesting to collaborate with a lab that does 3D modeling to determine how catenation with different supercoil handedness may vary between the substrate species. It would be interesting to model in particular how the different type II enzymes bind and interact differently with varying substrates during catenation and may help elucidate the differences between catenation by topoisomerase IIα and by topoisomerase IV.

Further investigation would also involve the study of the decatenation reaction to determine if the patterns seen with catenation by type II topoisomerases are also true of the reverse reaction. This would be studied by observing how catenated substrates of different supercoiling state decatenate into monomeric product. If topoisomerase IIα preferentially catenates negatively supercoiled DNA over positively supercoiled DNA, it may follow that the enzyme may preferentially decatenate positively supercoiled DNA over negatively supercoiled DNA. In yeast cells, the positive supercoiling of DNA drives decatenation by topoisomerase II prior to mitosis

(Baxter, Sen et al. 2011). It remains to be seen if this is an intrinsic property of the enzyme that continues in a purified system devoid of mitotic factors.

Results discussed in Chapter 4 also showed that human topoisomerase IIα and *E. coli* topoisomerase IV maintain lower levels of cleavage complexes in catenated DNA than in decatenated DNA. The kDNA substrate used to test the cleavage of catenated versus decatenated DNA is characterized by being a highly catenated network of DNA circles (Lukeš, Guilbride et al. 2002). It can be hypothesized that catenated daughter chromosomes may be subject to stress generated by the mitotic spindle which could lead to irreversible DNA cleavage, and therefore lower levels of DNA cleavage on catenated DNA may allow for an evolutionarily safer enzyme.

As it is possible that kDNA is too highly tangled for the enzyme to properly cleave the DNA, it would be beneficial to test the cleavage of lower order catenanes, such as dimers, as compared to monomeric substrate and thereby determine if the results are consistent with those observed with kDNA.

kDNA is also highly nicked and therefore cannot hold supercoils (Rauch, Perez-Morga et al. 1993, Lukeš, Guilbride et al. 2002). It would be interesting to observe whether cleavage of catenated DNA changes with the addition of supercoiling. A collaboration was established with Dr. Tony Maxwell of the John Innis Centre to obtain a substrate that could be used to form dimers and could also be supercoiled, but unfortunately supply chain issues prevented me from obtaining and using it. The system involved an engineered plasmid that contained two resolvase sites that would allow for the creation of a singly-linked catenane (i.e., a dimer) that was small enough to fit into an agarose gel and could be supercoiled (Waraich, Jain et al. 2020). This substrate would still be a fascinating utility for future experiments to observe cleavage of dimers versus monomers.

Catenation of DNA occurs at an important point in the cell cycle: catenation is used to stabilize interactions between daughter chromosomes, ensuring that separation does not occur too early as the cell prepares for mitosis (Nitiss 2009, Bauer, Marie et al. 2012, Sen, Leonard et al. 2016). However, as disruption of DNA replication and the mitotic process can have grave consequences on a cell, many factors must be considered, including the location of replication and mitotic machinery relative to the activity of topoisomerases. As discussed above and in Chapter 4, human topoisomerase IIα, for example, may show preference for catenation of negatively supercoiled DNA and for cleavage of decatenated DNA as an evolutionary response to protect the DNA from risk of breakage. Therefore, the study of the effects of supercoil handedness on catenation by type II topoisomerases allows for greater insight into a delicately balanced process and could be used to help tailor future therapeutics to destabilize a cancer cell during a critical moment in the cell cycle.

References

Aldred, K. J., E. J. Breland, V. Vlckova, M. P. Strub, K. C. Neuman, R. J. Kerns and N. Osheroff (2014). "Role of the water-metal ion bridge in mediating interactions between quinolones and Escherichia coli topoisomerase IV." Biochemistry **53**(34): 5558-5567.

Aldred, K. J., R. J. Kerns and N. Osheroff (2014). "Mechanism of quinolone action and resistance." Biochemistry **53**(10): 1565-1574.

Aldred, K. J., S. A. McPherson, C. L. Turnbough, Jr., R. J. Kerns and N. Osheroff (2013). "Topoisomerase IV-quinolone interactions are mediated through a water-metal ion bridge: mechanistic basis of quinolone resistance." Nucleic Acids Res **41**(8): 4628-4639.

Anderson, V. E., T. D. Gootz and N. Osheroff (1998). "Topoisomerase IV catalysis and the mechanism of quinolone action." J Biol Chem **273**(28): 17879-17885.

Antoniou-Kourounioti, M., M. L. Mimmack, A. C. G. Porter and C. J. Farr (2019). "The Impact of the C-Terminal Region on the Interaction of Topoisomerase II Alpha with Mitotic Chromatin." Int J Mol Sci **20**(5).

Ashley, R. E., T. R. Blower, J. M. Berger and N. Osheroff (2017). "Recognition of DNA Supercoil Geometry by Mycobacterium tuberculosis Gyrase." Biochemistry **56**(40): 5440-5448.

Ashley, R. E., A. Dittmore, S. A. McPherson, C. L. Turnbough, Jr., K. C. Neuman and N. Osheroff (2017). "Activities of gyrase and topoisomerase IV on positively supercoiled DNA." Nucleic Acids Res **45**(16): 9611-9624.

Ashley, R. E. and N. Osheroff (2014). "Natural products as topoisomerase II poisons: Effects of thymoquinone on DNA cleavage mediated by human topoisomerase IIα." Chem. Res. Toxicol. **27**(5): 787-793.

Ashley, R. E. and N. Osheroff (2019). Regulation of DNA Topology by Topoisomerases: Mathematics at the Molecular Level, Cham, Springer International Publishing.

Austin, C. A., K. C. Lee, R. L. Swan, M. M. Khazeem, C. M. Manville, P. Cridland, A. Treumann, A. Porter, N. J. Morris and I. G. Cowell (2018). "TOP2B: The First Thirty Years." International Journal of Molecular Sciences **19**(9): 2765.

Austin, C. A. and K. L. Marsh (1998). "Eukaryotic DNA topoisomerase IIβ." Bioessays **20**: 215-226.

Austin, C. A., S. Patel, K. Ono, H. Nakane and L. M. Fisher (1992). "Site-specific DNA cleavage by mammalian DNA topoisomerase II induced by novel flavone and catechin derivatives." Biochem J **282 (Pt 3)**: 883-889.

Austin, C. A., J. H. Sng, S. Patel and L. M. Fisher (1993). "Novel HeLa topoisomerase II is the II beta isoform: complete coding sequence and homology with other type II topoisomerases." Biochim Biophys Acta **1172**(3): 283-291.

Azarova, A. M., R. K. Lin, Y. C. Tsai, L. F. Liu, C. P. Lin and Y. L. Lyu (2010). "Genistein induces topoisomerase IIβ- and proteasome-mediated DNA sequence rearrangements: Implications in infant leukemia." Biochem. Biophys. Res. Commun. **399**(1): 66-71.

Baldwin, E. L. and N. Osheroff (2005). "Etoposide, topoisomerase II and cancer." Curr. Med. Chem. Anticancer Agents **5**(4): 363-372.

Bandele, O. J., S. J. Clawson and N. Osheroff (2008). "Dietary polyphenols as topoisomerase II poisons: B ring and C ring substituents determine the mechanism of enzyme-mediated DNA cleavage enhancement." Chem. Res. Toxicol. **21**(6): 1253-1260.

Bandele, O. J. and N. Osheroff (2007). "Bioflavonoids as poisons of human topoisomerase IIα and IIβ." Biochemistry **46**(20): 6097-6108.

Bandele, O. J. and N. Osheroff (2008). "The efficacy of topoisomerase II-targeted anticancer agents reflects the persistence of drug-induced cleavage complexes in cells." Biochemistry **47**(45): 11900-11908.

Bandele, O. J. and N. Osheroff (2008). "(-)-Epigallocatechin gallate, a major constituent of green tea, poisons human type II topoisomerases." Chem. Res. Toxicol. **21**(4): 936-943.

Bandele, O. J. and N. Osheroff (2009). "Cleavage of plasmid DNA by eukaryotic topoisomerase II." Methods Mol Biol **582**: 39-47.

Bates, A. D. and A. Maxwell (2005). DNA Topology, Oxford University Press.

Bauer, D. L. V., R. Marie, K. H. Rasmussen, A. Kristensen and K. U. Mir (2012). "DNA catenation maintains structure of human metaphase chromosomes." Nucleic Acids Research **40**(22): 11428-11434.

Bax, B. D., G. Murshudov, A. Maxwell and T. Germe (2019). "DNA Topoisomerase Inhibitors: Trapping a DNA-Cleaving Machine in Motion." J Mol Biol **431**(18): 3427-3449.

Baxter, J. and J. F. Diffley (2008). "Topoisomerase II inactivation prevents the completion of DNA replication in budding yeast." Mol Cell **30**(6): 790-802.

Baxter, J., N. Sen, V. L. Martinez, M. E. De Carandini, J. B. Schvartzman, J. F. Diffley and L. Aragon (2011). "Positive supercoiling of mitotic DNA drives decatenation by topoisomerase II in eukaryotes." Science **331**(6022): 1328-1332.

Bender, R. P., H. J. Lehmler, L. W. Robertson, G. Ludewig and N. Osheroff (2006). "Polychlorinated biphenyl quinone metabolites poison human topoisomerase IIα: Altering enzyme function by blocking the N-terminal protein gate." Biochemistry **45**(33): 10140-10152.

Bender, R. P. and N. Osheroff (2007). "Mutation of cysteine residue 455 to alanine in human topoisomerase IIα confers hypersensitivity to quinones: Enhancing DNA scission by closing the N-terminal protein gate." Chem. Res. Toxicol. **20**(6): 975-981.

Biersack, H., S. Jensen, I. Gromova, I. S. Nielsen, O. Westergaard and A. H. Andersen (1996). "Active heterodimers are formed from human DNA topoisomerase IIα and IIβ isoforms." Proc. Natl. Acad. Sci. USA **93**(16): 8288-8293.

Brill, S. J., S. DiNardo, K. Voelkel-Meiman and R. Sternglanz (1987). "Need for DNA topoisomerase activity as a swivel for DNA replication for transcription of ribosomal RNA." Nature **326**(6111): 414-416.

Byl, J. A., J. M. Fortune, D. A. Burden, J. L. Nitiss, T. Utsugi, Y. Yamada and N. Osheroff (1999). "DNA topoisomerases as targets for the anticancer drug TAS-103: Primary cellular target and DNA cleavage enhancement." Biochemistry **38**(47): 15573-15579.

Champoux, J. J. (2001). "DNA topoisomerases: Structure, function, and mechanism." Ann. Rev. Biochem. **70**: 369-413.

Chen, J., C. A. Rauch, J. H. White, P. T. Englund and N. R. Cozzarelli (1995). "The topology of the kinetoplast DNA network." Cell **80**(1): 61-69.

Chen, S. H., N.-L. Chan and T.-S. Hsieh (2013). "New mechanistic and functional insights into DNA topoisomerases." Ann. Rev. Biochem. **82**(1): 139-170.

Corbett, K. D., A. J. Schoeffler, N. D. Thomsen and J. M. Berger (2005). "The structural basis for substrate specificity in DNA topoisomerase IV." J Mol Biol **351**(3): 545-561.

Corbett, K. D., R. K. Shultzaberger and J. M. Berger (2004). "The C-terminal domain of DNA gyrase A adopts a DNA-bending beta-pinwheel fold." Proc Natl Acad Sci U S A **101**(19): 7293-7298.

Cragg, G. M. and D. J. Newman (2013). "Natural products: a continuing source of novel drug leads." Biochim Biophys Acta **1830**(6): 3670-3695.

Dalvie, E. D., J. Gopas, A. Golan-Goldhirsh and N. Osheroff (2019). "6,6'-Dihydroxythiobinupharidine as a poison of human type II topoisomerases." Bioorg Med Chem Lett **29**(15): 1881-1885.

Dalvie, E. D. and N. Osheroff (2021). DNA Recognition/Processing | DNA Topoisomerases: Type II☆. Encyclopedia of Biological Chemistry III (Third Edition). J. Jez. Oxford, Elsevier**:** 479-486.

Danzig, R. (2005). Proliferation of biological weapons into terrorist hands. The Challenge of Proliferation: A Report from the Aspen Strategy Group. K. M. Campbell. Washington, D.C, The Aspen Institute**:** 65–84.

Deweese, J. E., M. A. Osheroff and N. Osheroff (2008). "DNA Topology and Topoisomerases: Teaching a "Knotty" Subject." Biochem Mol Biol Educ **37**(1): 2-10.

Deweese, J. E. and N. Osheroff (2009). "The DNA cleavage reaction of topoisomerase II: Wolf in sheep's clothing." Nucleic Acids Res. **37**(3): 738-748.

Dickey, J. S. and N. Osheroff (2005). "Impact of the C-terminal domain of topoisomerase IIα on the DNA cleavage activity of the human enzyme." Biochemistry **44**(34): 11546-11154.

Dong, S., S. A. McPherson, Y. Wang, M. Li, P. Wang, C. L. Turnbough, Jr. and D. G. Pritchard (2010). "Characterization of the enzymes encoded by the anthrose biosynthetic operon of Bacillus anthracis." J Bacteriol **192**(19): 5053-5062.

Dougherty, A. C., M. G. Hawaz, K. G. Hoang, J. Trac, J. M. Keck, C. Ayes and J. E. Deweese (2021). "Exploration of the Role of the C-Terminal Domain of Human DNA Topoisomerase IIα in Catalytic Activity." ACS Omega **6**(40): 25892-25903.

El Beyrouthy, M., N. Arnold, A. Delelis-Dusollier and F. Dupont (2008). "Plants used as remedies antirheumatic and antineuralgic in the traditional medicine of Lebanon." J. Ethnopharm. **120**(3): 315-334.

El-On, J., L. Ozer, J. Gopas, R. Sneir, H. Enav, N. Luft, G. Davidov and A. Golan-Goldhirsh (2009). "Antileishmanial activity in Israeli plants." Ann Trop Med Parasitol **103**(4): 297-306.

El-On, J., L. Ozer, J. Gopas, R. Sneir and A. Golan-Goldhirsh (2009). "Nuphar lutea: in vitro anti-leishmanial activity against Leishmania major promastigotes and amastigotes." Phytomedicine **16**(8): 788-792.

Ezoe, S. (2012). "Secondary leukemia associated with the anti-cancer agent, etoposide, a topoisomerase II inhibitor." Int. J. Environ. Res. Public Health **9**(7): 2444-2453.

Felix, C. A. (2001). "Leukemias related to treatment with DNA topoisomerase II inhibitors." Med. Pediatr. Oncol. **36**(5): 525-535.

Forterre, P. and D. Gadelle (2009). "Phylogenomics of DNA topoisomerases: their origin and putative roles in the emergence of modern organisms." Nucleic Acids Research **37**(3): 679-692.

Fortune, J. M. and N. Osheroff (1998). "Merbarone inhibits the catalytic activity of human topoisomerase IIα by blocking DNA cleavage." J. Biol. Chem. **273**(28): 17643-17650.

Fortune, J. M. and N. Osheroff (2000). "Topoisomerase II as a target for anticancer drugs: When enzymes stop being nice." Prog. Nucleic Acid Res. Mol .Biol. **64**: 221-253.

Fortune, J. M. and N. Osheroff (2001). "Topoisomerase II-catalyzed relaxation and catenation of plasmid DNA." Methods Mol Biol **95**: 275-281.

Gellert, M., K. Mizuuchi, M. H. O'Dea and H. A. Nash (1976). "DNA gyrase: an enzyme that introduces superhelical turns into DNA." Proceedings of the National Academy of Sciences **73**(11): 3872-3876.

Gentry, A. C. and N. Osheroff (2013). DNA topoisomerases: Type II. Encyc. Biol. Chem. a. L. M. D. Lennarz W.J. Waltham, MA, Academic Press. **2:** 163-168.

Gentry, A. C., S. L. Pitts, M. J. Jablonsky, C. Bailly, D. E. Graves and N. Osheroff (2011). "Interactions between the etoposide derivative F14512 and human type II topoisomerases: Implications for the C4 spermine moiety in promoting enzyme-mediated DNA cleavage." Biochemistry **50**(15): 3240-3249.

Gibson, E. G., R. E. Ashley, R. J. Kerns and N. Osheroff (2018). Bacterial Type II Topoisomerases and Target-Mediated Drug Resistance. Antimicrobial Resistance in the 21st Century. I. W. Fong, D. Shlaes and K. Drlica. Cham, Springer International Publishing**:** 507-529.

Gibson, E. G., B. Bax, P. F. Chan and N. Osheroff (2019). "Mechanistic and Structural Basis for the Actions of the Antibacterial Gepotidacin against Staphylococcus aureus Gyrase." ACS Infect Dis **5**(4): 570-581.

Gibson, E. G., A. A. Oviatt and N. Osheroff (2020). "Two-Dimensional Gel Electrophoresis to Resolve DNA Topoisomers." Methods Mol Biol **2119**: 15-24.

Gilroy, K. L. and C. A. Austin (2011). "The Impact of the C-Terminal Domain on the Interaction of Human DNA Topoisomerase II α and β with DNA." PLOS ONE **6**(2): e14693.

Goto, T., P. Laipis and J. C. Wang (1984). "The purification and characterization of DNA topoisomerases I and II of the yeast Saccharomyces cerevisiae." J. Biol. Chem. **259**(16): 10422-10429.

Goto, T. and J. C. Wang (1982). "Yeast DNA topoisomerase II. An ATP-dependent type II topoisomerase that catalyzes the catenation, decatenation, unknotting, and relaxation of double-stranded DNA rings." J. Biol. Chem. **257**(10): 5866-58672.

Goto, T. and J. C. Wang (1984). "Yeast DNA topoisomerase II is encoded by a single-copy, essential gene." Cell **36**(4): 1073-1080.

Hasan, S. K., A. N. Mays, T. Ottone, A. Ledda, G. La Nasa, C. Cattaneo, E. Borlenghi, L. Melillo, E. Montefusco, J. Cervera, C. Stephen, G. Satchi, A. Lennard, M. Libura, J. A. Byl, N. Osheroff, S. Amadori, C. A. Felix, M. T. Voso, W. R. Sperr, J. Esteve, M. A. Sanz, D. Grimwade and F. Lo-Coco (2008). "Molecular analysis of t(15;17) genomic breakpoints in secondary acute promyelocytic leukemia arising after treatment of multiple sclerosis." Blood **112**(8): 3383-3390.

Heintzman, D. R., L. V. Campos, J. A. W. Byl, N. Osheroff and J. M. Dewar (2019). "Topoisomerase II Is Crucial for Fork Convergence during Vertebrate Replication Termination." Cell Rep **29**(2): 422-436 e425.

Holm, C., T. Goto, J. C. Wang and D. Botstein (1985). "DNA topoisomerase II is required at the time of mitosis in yeast." Cell **41**(2): 553-563.

Jang, Y., H. Son, S. W. Lee, W. Hwang, S. R. Jung, J. A. W. Byl, N. Osheroff and S. Lee (2019). "Selection of DNA Cleavage Sites by Topoisomerase II Results from Enzyme-Induced Flexibility of DNA." Cell Chem Biol **26**(4): 502-511 e503.

Joannides, M. and D. Grimwade (2010). "Molecular biology of therapy-related leukaemias." Clin. Transl. Oncol. **12**(1): 8-14.

Joannides, M., A. N. Mays, A. R. Mistry, S. K. Hasan, A. Reiter, J. L. Wiemels, C. A. Felix, F. L. Coco, N. Osheroff and E. Solomon (2011). "Molecular pathogenesis of secondary acute promyelocytic leukemia." Mediterranean journal of hematology and infectious diseases **3**(1).

Johnson, L. M. (2006). "Gitksan medicinal plants--cultural choice and efficacy." J Ethnobiol Ethnomed **2**: 29.

Kaiser, J., M. Yassin, S. Prakash, N. Safi, M. Agami, S. Lauw, E. Ostrozhenkova, A. Bacher, F. Rohdich, W. Eisenreich, J. Safi and A. Golan-Goldhirsh (2007). "Anti-malarial drug targets: screening for inhibitors of 2C-methyl-D-erythritol 4-phosphate synthase (IspC protein) in Mediterranean plants." Phytomedicine **14**(4): 242-249.

Kato, J., Y. Nishimura, R. Imamura, H. Niki, S. Hiraga and H. Suzuki (1990). "New topoisomerase essential for chromosome segregation in E. coli." Cell **63**(2): 393-404.

Ketron, A. C., O. N. Gordon, C. Schneider and N. Osheroff (2013). "Oxidative metabolites of curcumin poison human type II topoisomerases." Biochemistry **52**(1): 221-227.

Ketron, A. C. and N. Osheroff (2014). "Phytochemicals as anticancer and chemopreventive topoisomerase II poisons." Phytochem. Rev. **13**(1): 19-35.

Kim, R. A. and J. C. Wang (1989). "Function of DNA topoisomerases as replication swivels in Saccharomyces cerevisiae." J Mol Biol **208**(2): 257-267.

Kingma, P. S., C. A. Greider and N. Osheroff (1997). "Spontaneous DNA lesions poison human topoisomerase IIα and stimulate cleavage proximal to leukemic 11q23 chromosomal breakpoints." Biochemistry **36**(20): 5934-5939.

Kornberg, A. and T. A. Baker (1992). DNA replication, Wh Freeman New York.

Kozuki, T., K. Chikamori, M. D. Surleac, M. A. Micluta, A. J. Petrescu, E. J. Norris, P. Elson, G. A. Hoeltge, D. R. Grabowski, A. C. G. Porter, R. N. Ganapathi and M. K. Ganapathi (2017). "Roles of the C-terminal domains of topoisomerase IIα and topoisomerase IIβ in regulation of the decatenation checkpoint." Nucleic acids research **45**(10): 5995-6010.

Krasnow, M. A. and N. R. Cozzarelli (1982). "Catenation of DNA rings by topoisomerases. Mechanism of control by spermidine." J Biol Chem **257**(5): 2687-2693.

Lee, I., K. C. Dong and J. M. Berger (2013). "The role of DNA bending in type IIA topoisomerase function." Nucleic acids research **41**(10): 5444-5456.

Leppard, J. B. and J. J. Champoux (2005). "Human DNA topoisomerase I: Relaxation, roles, and damage control." Chromosoma **114**(2): 75-85.

Levy, D. H., I. L. C. Chapple, L. Shapira, A. Golan-Goldhirsh, J. Gopas and D. Polak (2019). "Nupharidine enhances Aggregatibacter actinomycetemcomitans clearance by priming neutrophils and augmenting their effector functions." J Clin Periodontol **46**(1): 62-71.

Lin, R. K., N. Zhou, Y. L. Lyu, Y. C. Tsai, C. H. Lu, J. Kerrigan, Y. T. Chen, Z. Guan, T. S. Hsieh and L. F. Liu (2011). "Dietary isothiocyanate-induced apoptosis via thiol modification of DNA topoisomerase IIalpha." J. Biol. Chem. **286**(38): 33591-33600.

Lindsey, R. H., Jr., K. D. Bromberg, C. A. Felix and N. Osheroff (2004). "1,4-Benzoquinone is a topoisomerase II poison." Biochemistry **43**(23): 7563-7574.

Lindsey, R. H., M. Pendleton, R. E. Ashley, S. L. Mercer, J. E. Deweese and N. Osheroff (2014). "Catalytic core of human topoisomerase IIα: Insights into enzyme–DNA interactions and drug mechanism." Biochemistry **53**(41): 6595-6602.

Lindsley, J. E. and J. C. Wang (1993). "On the coupling between ATP usage and DNA transport by yeast DNA topoisomerase II." J. Biol. Chem. **268**(11): 8096-8104.

Linka, R. M., A. C. Porter, A. Volkov, C. Mielke, F. Boege and M. O. Christensen (2007). "C-terminal regions of topoisomerase IIα and IIβ determine isoform-specific functioning of the enzymes *in vivo*." Nucleic Acids Res. **35**(11): 3810-3822.

Liu, L. F., C. C. Liu and B. M. Alberts (1980). "Type II DNA topoisomerases: Enzymes that can unknot a topologically knotted DNA molecule *via* a reversible double-strand break." Cell **19**(3): 697-707.

Liu, L. F. and J. C. Wang (1987). "Supercoiling of the DNA template during transcription." Proc. Natl. Acad. Sci. USA **84**(20): 7024-7027.

Liu, Z., R. W. Deibler, H. S. Chan and L. Zechiedrich (2009). "The why and how of DNA unlinking." Nucleic Acids Res. **37**(3): 661-671.

Lopez-Lazaro, M., J. M. Calderon-Montano, E. Burgos-Moron and C. A. Austin (2011). "Green tea constituents (-)-epigallocatechin-3-gallate (EGCG) and gallic acid induce topoisomerase I- and topoisomerase II-DNA complexes in cells mediated by pyrogallol-induced hydrogen peroxide." Mutagenesis **26**(4): 489-498.

Lopez-Lazaro, M., E. Willmore and C. A. Austin (2010). "The dietary flavonoids myricetin and fisetin act as dual inhibitors of DNA topoisomerases I and II in cells." Mutat. Res. **696**(1): 41-47.

Lopez-Lazaro, M., E. Willmore, A. Jobson, K. L. Gilroy, H. Curtis, K. Padget and C. A. Austin (2007). "Curcumin induces high levels of topoisomerase I- and II-DNA complexes in K562 leukemia cells." J Nat Prod **70**(12): 1884-1888.

Lukeš, J., D. L. Guilbride, J. Votýpka, A. Zíková, R. Benne and P. T. Englund (2002). "Kinetoplast DNA Network: Evolution of an Improbable Structure." Eukaryotic Cell **1**(4): 495-502.

McClendon, A. K., J. S. Dickey and N. Osheroff (2006). "Ability of viral topoisomerase II to discern the handedness of supercoiled DNA: bimodal recognition of DNA geometry by type II enzymes." Biochemistry **45**(38): 11674-11680.

McClendon, A. K., A. C. Gentry, J. S. Dickey, M. Brinch, S. Bendsen, A. H. Andersen and N. Osheroff (2008). "Bimodal recognition of DNA geometry by human topoisomerase II alpha: preferential relaxation of positively supercoiled DNA requires elements in the C-terminal domain." Biochemistry **47**(50): 13169-13178.

McClendon, A. K. and N. Osheroff (2006). "The geometry of DNA supercoils modulates topoisomerase-mediated DNA cleavage and enzyme response to anticancer drugs." Biochemistry **45**(9): 3040-3050.

McClendon, A. K. and N. Osheroff (2007). "DNA topoisomerase II, genotoxicity, and cancer." Mutat. Res. **623**(1-2): 83-97.

McClendon, A. K., A. C. Rodriguez and N. Osheroff (2005). "Human topoisomerase IIα rapidly relaxes positively supercoiled DNA: Implications for enzyme action ahead of replication forks." J. Biol. Chem. **280**(47): 39337-39345.

Mirkin, S. M., E. N. Zaitsev, I. G. Panyutin and V. I. Lyamichev (1984). "Native supercoiling of DNA: The effects of DNA gyrase and ω protein in E. coli." Molecular and General Genetics MGG **196**(3): 508-512.

Mirski, S. E., J. H. Gerlach, H. J. Cummings, R. Zirngibl, P. A. Greer and S. P. Cole (1997). "Bipartite nuclear localization signals in the C terminus of human topoisomerase II alpha." Exp Cell Res **237**(2): 452-455.

Mondrala, S. and D. A. Eastmond (2010). "Topoisomerase II inhibition by the bioactivated benzene metabolite hydroquinone involves multiple mechanisms." Chem. Biol. Interact. **184**(1-2): 259-268.

Nakae, H., A. Yokoi, H. Kodama and A. Horikawa (2012). "Comparison of the Effects on Rib Fracture between the Traditional Japanese Medicine Jidabokuippo and Nonsteroidal Anti-Inflammatory Drugs: A Randomized Controlled Trial." Evid Based Complement Alternat Med **2012**: 837958.

Newman, D. J. and G. M. Cragg (2012). "Natural products as sources of new drugs over the 30 years from 1981 to 2010." J Nat Prod **75**(3): 311-335.

Nitiss, J. L. (2009). "DNA topoisomerase II and its growing repertoire of biological functions." Nat. Rev. Cancer **9**(5): 327-337.

Nitiss, J. L. (2009). "Targeting DNA topoisomerase II in cancer chemotherapy." Nat. Rev. Cancer **9**(5): 338-350.

Nolan, J. M., M. P. Lee, E. Wyckoff and T. S. Hsieh (1986). "Isolation and characterization of the gene encoding Drosophila DNA topoisomerase II." Proc. Natl. Acad. Sci. USA **83**(11): 3664-3668.

Oestergaard, V. H., L. Bjergbaek, C. Skouboe, L. Giangiacomo, B. R. Knudsen and A. H. Andersen (2004). "The transducer domain is important for clamp operation in human DNA topoisomerase IIα." J. Biol. Chem. **279**(3): 1684-1691.

Osheroff, N. (1986). "Eukaryotic topoisomerase II. Characterization of enzyme turnover." J Biol Chem **261**(21): 9944-9950.

Osheroff, N. (1987). "Role of the divalent cation in topoisomerase II mediated reactions." Biochemistry **26**(20): 6402-6406.

Osheroff, N. (1998). "DNA topoisomerases." Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression **1400**(1): 1-2.

Osheroff, N. and E. L. Zechiedrich (1987). "Calcium-promoted DNA cleavage by eukaryotic topoisomerase II: trapping the covalent enzyme-DNA complex in an active form." Biochemistry **26**(14): 4303-4309.

Ozer, J., N. Eisner, E. Ostrozhenkova, A. Bacher, W. Eisenreich, D. Benharroch, A. Golan-Goldhirsh and J. Gopas (2009). "Nuphar lutea thioalkaloids inhibit the nuclear factor kappaB pathway, potentiate apoptosis and are synergistic with cisplatin and etoposide." Cancer Biol. Ther. **8**(19): 1860-1868.

Ozer, J., D. Fishman, B. Eilam, A. Golan-Goldhirsh and J. Gopas (2017). "Anti-Metastatic Effect of Semi-Purified Nuphar Lutea Leaf Extracts." J Cancer **8**(8): 1433-1440.

Ozer, J., T. Levi, A. Golan-Goldhirsh and J. Gopas (2015). "Anti-inflammatory effect of a Nuphar lutea partially purified leaf extract in murine models of septic shock." J Ethnopharmacol **161**: 86-91.

Ozer, L., J. El-On, A. Golan-Goldhirsh and J. Gopas (2010). "Leishmania major: anti-leishmanial activity of Nuphar lutea extract mediated by the activation of transcription factor NF-kappaB." Exp Parasitol **126**(4): 510-516.

Pendleton, M., R. H. Lindsey, Jr., C. A. Felix, D. Grimwade and N. Osheroff (2014). "Topoisomerase II and leukemia." Ann. N.Y. Acad. Sci. **1310**(1): 98-110.

Peng, H. and K. J. Marians (1993). "Escherichia coli topoisomerase IV. Purification, characterization, subunit structure, and subunit interactions." J Biol Chem **268**(32): 24481-24490.

Peter, B. J., C. Ullsperger, H. Hiasa, K. J. Marians and N. R. Cozzarelli (1998). "The structure of supercoiled intermediates in DNA replication." Cell **94**(6): 819-827.

Pommier, Y. (2013). "Drugging topoisomerases: Lessons and challenges." ACS Chem. Biol. **8**(1): 82-95.

Pommier, Y., E. Leo, H. Zhang and C. Marchand (2010). "DNA topoisomerases and their poisoning by anticancer and antibacterial drugs." Chem. Biol. **17**(5): 421-433.

Pommier, Y. and C. Marchand (2012). "Interfacial inhibitors: Targeting macromolecular complexes." Nat. Rev. Drug. Discov. **11**(1): 25-36.

Pommier, Y., Y. Sun, S.-y. N. Huang and J. L. Nitiss (2016). "Roles of eukaryotic topoisomerases in transcription, replication and genomic stability." Nat. Rev. Mol. Cell Biol. **17**(11): 703-721.

Postow, L., N. J. Crisona, B. J. Peter, C. D. Hardy and N. R. Cozzarelli (2001). "Topological challenges to DNA replication: Conformations at the fork." Proceedings of the National Academy of Sciences **98**(15): 8219-8226.

Rashidi, A. and S. I. Fisher (2013). "Therapy-related acute promyelocytic leukemia: a systematic review." Med. Oncol. **30**(3): 625.

Rauch, C. A., D. Perez-Morga, N. R. Cozzarelli and P. T. Englund (1993). "The absence of supercoiling in kinetoplast DNA minicircles." Embo j **12**(2): 403-411.

Rodriguez, A. C. (2002). "Studies of a positive supercoiling machine. Nucleotide hydrolysis and a multifunctional "latch" in the mechanism of reverse gyrase." J Biol Chem **277**(33): 29865-29873.

Ryu, H., M. M. Yoshida, V. Sridharan, A. Kumagai, W. G. Dunphy, M. Dasso and Y. Azuma (2015). "SUMOylation of the C-terminal domain of DNA topoisomerase IIalpha regulates the centromeric localization of Claspin." Cell Cycle **14**(17): 2777-2784.

Sathiyamoorthy, P., H. Lugasi-Evgi, P. Schlesinger, I. Kedar, J. Gopas, Y. Pollack and A. Golan-Goldhirsh (1999). "Screening for Cytotoxic and Antimalarial Activities in Desert Plants of the Negev and Bedouin Market Plant Products." Pharm. Biol. **37**(3): 188-195.

Sathiyamoorthy, P., H. Lugasi-Evgi, P. Van-Damme, A. Abu-Rabia, J. Gopas and A. Golan-Goldhirsh (1997). "Larvicidal Activity in Desert Plants of the Negev and Bedouin Market Plant Products." Internat. J. Pharmacog. **35**(4): 265-273.

Schvartzman, J. B. and A. Stasiak (2004). "A topological view of the replicon." EMBO Rep. **5**(3): 256-261.

Sen, N., J. Leonard, R. Torres, J. Garcia-Luis, G. Palou-Marin and L. Aragon (2016). "Physical Proximity of Sister Chromatids Promotes Top2-Dependent Intertwining." Mol Cell **64**(1): 134-147.

Seol, Y., A. C. Gentry, N. Osheroff and K. C. Neuman (2013). "Chiral discrimination and writhe-dependent relaxation mechanism of human topoisomerase IIalpha." J Biol Chem **288**(19): 13695-13703.

Smith, K. A., I. G. Cowell, Y. Zhang, Z. Sondka and C. A. Austin (2014). "The role of topoisomerase II beta on breakage and proximity of RUNX1 to partner alleles RUNX1T1 and EVI1." Genes Chromosomes Cancer **53**(2): 117-128.

Timmel, M. A., J. A. Byl and N. Osheroff (2013). "Epimerization of green tea catechins during brewing does not affect the ability to poison human type II topoisomerases." Chem. Res. Toxicol. **26**(4): 622-628.

Tretter, E. M., J. C. Lerman and J. M. Berger (2010). "A naturally chimeric type IIA topoisomerase in Aquifex aeolicus highlights an evolutionary path for the emergence of functional paralogs." Proc Natl Acad Sci U S A **107**(51): 22055-22059.

Uemura, T., H. Ohkura, Y. Adachi, K. Morino, K. Shiozaki and M. Yanagida (1987). "DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in S. pombe." Cell **50**(6): 917-925.

Vann, K. R., A. A. Oviatt and N. Osheroff (2021). "Topoisomerase II Poisons: Converting Essential Enzymes into Molecular Scissors." Biochemistry **60**(21): 1630-1641.

Vann, K. R., C. A. Sedgeman, J. Gopas, A. Golan-Goldhirsh and N. Osheroff (2015). "Effects of olive metabolites on DNA cleavage mediated by human type II topoisomerases." Biochemistry **54**(29): 4531-4541.

Voet, D., J. G. Voet and C. W. Pratt (2002). Fundamentals of biochemistry, Wiley New York.

Vos, S. M., I. Lee and J. M. Berger (2013). "Distinct regions of the Escherichia coli ParC C-terminal domain are required for substrate discrimination by topoisomerase IV." J Mol Biol **425**(17): 3029-3045.

Vos, S. M., E. M. Tretter, B. H. Schmidt and J. M. Berger (2011). "All tangled up: How cells direct, manage and exploit topoisomerase function." Nat. Rev. Mol. Cell. Biol. **12**(12): 827-841.

Wang, H., Y. Mao, A. Y. Chen, N. Zhou, E. J. LaVoie and L. F. Liu (2001). "Stimulation of topoisomerase II-mediated DNA damage *via* a mechanism involving protein thiolation." Biochemistry **40**(11): 3316-3323.

Wang, J. C. (1996). "DNA topoisomerases." Annu. Rev. Biochem. **65**: 635-692.

Wang, J. C. (2002). "Cellular roles of DNA topoisomerases: A molecular perspective." Nat. Rev. Mol. Cell. Biol. **3**(6): 430-440.

Waraich, N. F., S. Jain, S. D. Colloms, W. M. Stark, N. P. Burton and A. Maxwell (2020). "A novel decatenation assay for DNA topoisomerases using a singly-linked catenated substrate." Biotechniques **69**(5): 356-362.

Wasserman, R. A., C. A. Austin, L. M. Fisher and J. C. Wang (1993). "Use of yeast in the study of anticancer drugs targeting DNA topoisomerases: Expression of a functional recombinant human DNA topoisomerase IIα in yeast." Cancer Res. **53**(15): 3591-3596.

Wendorff, T. J., B. H. Schmidt, P. Heslop, C. A. Austin and J. M. Berger (2012). "The structure of DNA-bound human topoisomerase IIα: Conformational mechanisms for coordinating inter-subunit interactions with DNA cleavage." J. Mol. Biol. **424**(3-4): 109-124.

Worland, S. T. and J. C. Wang (1989). "Inducible overexpression, purification, and active site mapping of DNA topoisomerase II from the yeast *Saccharomyces cerevisiae*." J. Biol. Chem. **264**(8): 4412-4416.

Wu, C. C., T. K. Li, L. Farh, L. Y. Lin, T. S. Lin, Y. J. Yu, T. J. Yen, C. W. Chiang and N. L. Chan (2011). "Structural basis of type II topoisomerase inhibition by the anticancer drug etoposide." Science **333**(6041): 459-462.

Yang, X., W. Li, E. D. Prescott, S. J. Burden and J. C. Wang (2000). "DNA topoisomerase IIβ and neural development." Science **287**: 131-134.

Yoshida, M. M., L. Ting, S. P. Gygi and Y. Azuma (2016). "SUMOylation of DNA topoisomerase IIalpha regulates histone H3 kinase Haspin and H3 phosphorylation in mitosis." J Cell Biol **213**(6): 665-678.

Zawadzki, P., M. Stracy, K. Ginda, K. Zawadzka, C. Lesterlin, A. N. Kapanidis and D. J. Sherratt (2015). "The Localization and Action of Topoisomerase IV in Escherichia coli Chromosome Segregation Is Coordinated by the SMC Complex, MukBEF." Cell Rep **13**(11): 2587-2596.