

INTERACTIONS OF TYPE II TOPOISOMERASES WITH DIVALENT METAL IONS
AND THERAPEUTIC DRUGS

By

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
DEPT	4'-demethylepipodophyllotoxin
DMSO	dimethylsulfoxide
D-ring diol	11,13-O,O-4'-demethyl epipodophyllotoxin
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
etoposide	4'-demethylepipodophyllotoxin 9-(4,6-O-ethylidnine- β -D-glucoside)
ICE Bioassay	<i>in vivo</i> complex of the enzyme assay
kb	kilobases
kDa	kilodaltons
MLL	mixed lineage leukemia
NOE	nuclear Overhauser effect
retroDEPT	11-oxo,13-deoxo-4'-demethyl epipodophyllotoxin
retroetoposide	11-oxo,13-deoxo-etoposide
SDS	sodium dodecyl sulfate
STD	saturation transfer difference
tris	tris-(hydroxymethyl)aminomethane

CHAPTER I

INTRODUCTION

A month after the publication of their famous paper describing the structure of DNA, and a full 18 years before James Wang discovered the first topoisomerase, James Watson and Frances Crick published a second paper speculating on the implications of their discovery (1):

“Since the two chains in our model are intertwined, it is essential for them to untwist if they are to separate. As they make one complete turn around each other in 34 Å., there will be about 150 turns per million molecular weight, so that whatever the precise structure of the chromosome a considerable amount of uncoiling would be necessary. It is well known from microscopic observation that much coiling and uncoiling occurs during mitosis, and though this is on a much larger scale, it probably reflects similar processes on a molecular level. Although it is difficult at the moment to see how these processes occur without everything getting tangled, we do not feel that this objection will be insuperable.”

Watson and Crick realized that the cell would require a mechanism to overcome the inevitable topological issues that would arise due to the inherent properties of their newly discovered DNA structure.

DNA Topology

In almost all living organisms, DNA is globally underwound (2-5). This property decreases the amount of energy needed to break the hydrogen bonds between the bases of DNA and therefore facilitates strand separation (5-8). Separation of the two strands of the

double helix must occur for transcription and replication to be carried out by RNA and DNA polymerases.

As the two strands of DNA are unwound by a polymerase-associated helicase, compensatory over- and under-winding occurs. Over-winding ahead of, and under-winding behind, the replication or transcription machinery leads to positive and negative supercoiling, respectively (Figure 1) (9-12). Just as negative supercoiling facilitates normal DNA processes, positive supercoiling inhibits them. Because the ends of DNA are fixed in space (free rotation is hindered by DNA-associated proteins such as nucleosomes and membrane scaffolding, and bacterial DNA is circular), critical cellular processes such as replication and transcription would stall before completion without a mechanism to relieve the torsional stress (5,7,8,11). Furthermore, torsional stress ahead of DNA tracking systems is translated behind the fork and leads to the formation of precatenanes (Figure 1, bottom). Precatenanes link sister chromatids after replication and must be resolved in order for the chromosomes to be separated during mitosis (5,7,8,11).

The double helical nature of DNA is not the only topological challenge the genetic material presents to the cell. The human genome contains approximately 2 meters of DNA, which must be condensed into a nucleus roughly 5 microns in diameter (Figure 2) (11,13). Although the circular genome of *E. coli* is about 1000 times smaller than that of the human genome, considerable packing must also occur. The sheer length of DNA, along with its compaction into a relatively small area leads to problems such as knots (recombination) and tangles (replication), as well as catenanes formed during daughter chromosome condensation and segregation (10,11,13).

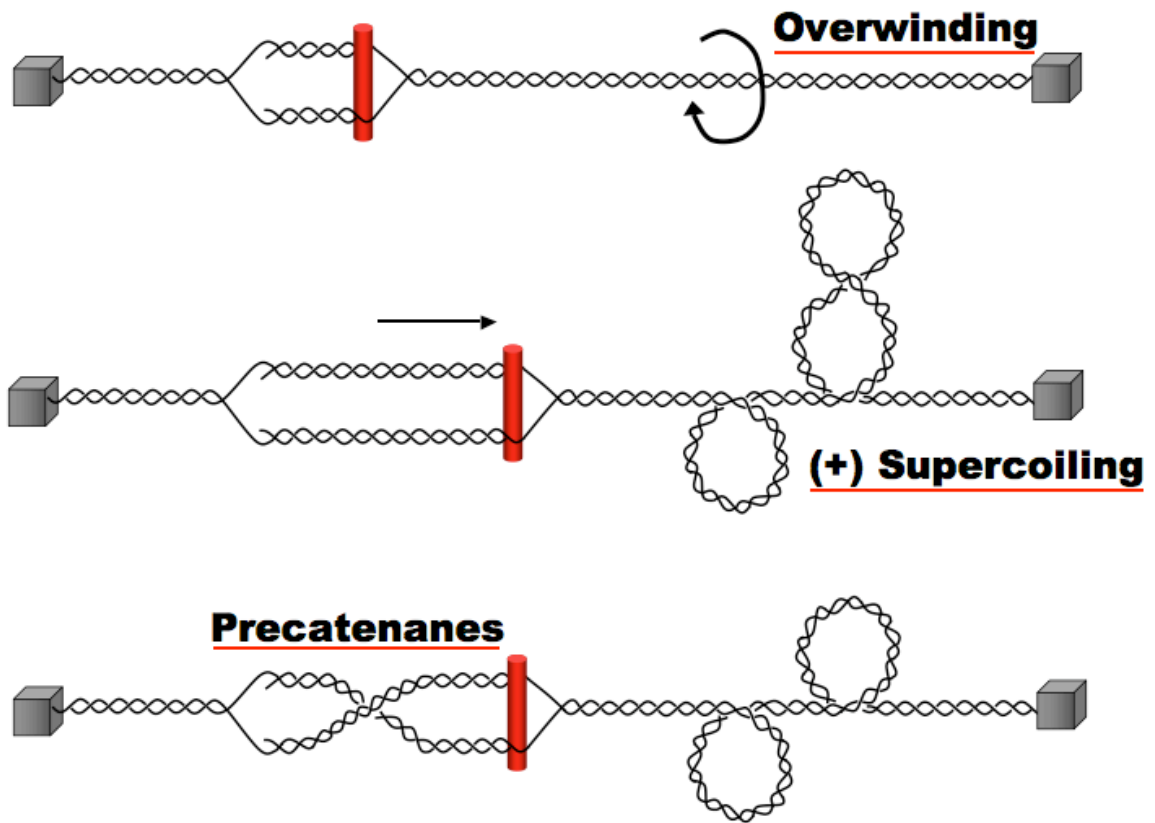


FIGURE 1. Topological challenges to the cell arise due to the DNA tracking systems during replication and transcription. As the replication/transcription machinery (red) traverses the double helix, overwinding occurs ahead of the fork because the ends of DNA are fixed to membranes or protein scaffolding (gray boxes). This torsional stress can be translated into positive supercoiling ahead of the fork and/or precatenanes behind.

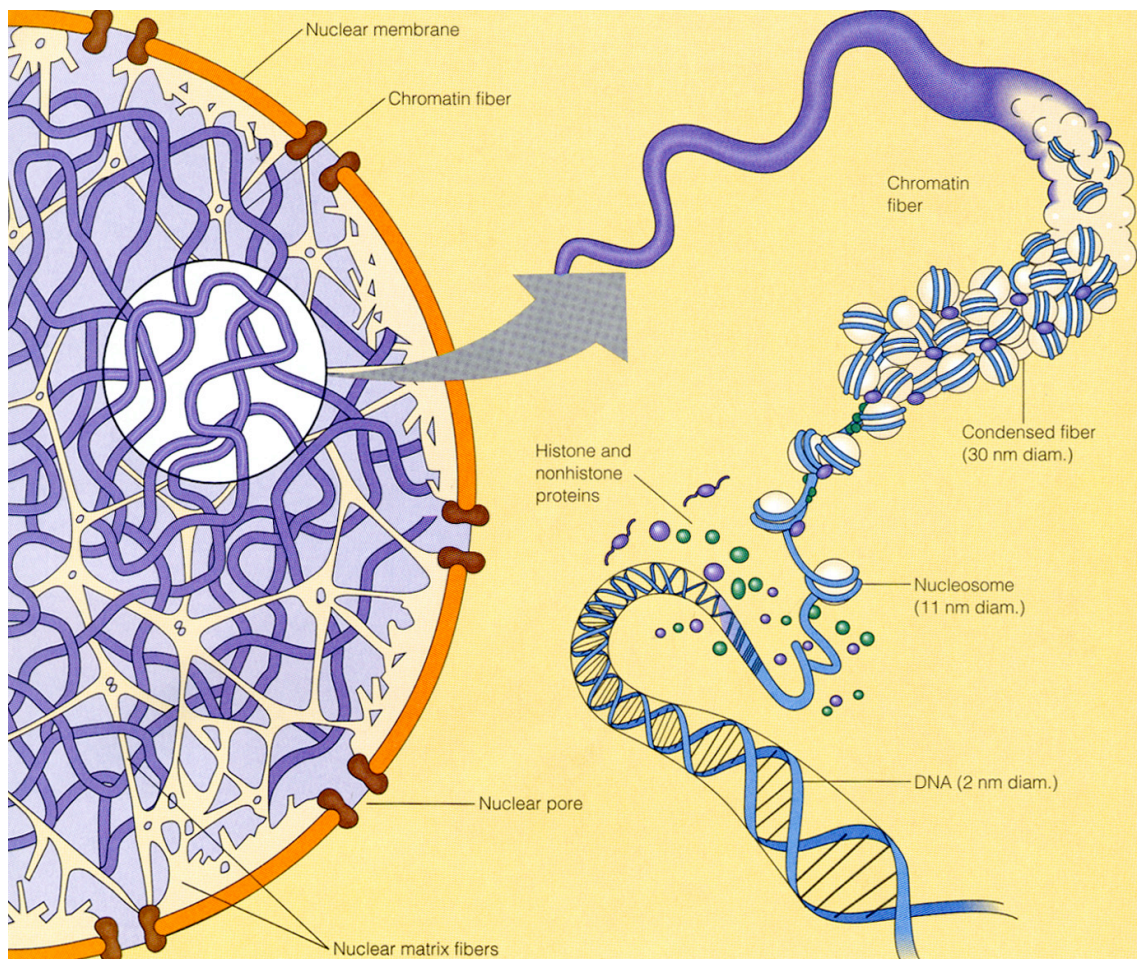


FIGURE 2. Approximately two meters of DNA must be condensed into nucleosomes and higher order structures in order to fit into the human nucleus, which is ~5 microns in diameter. This, along with the double-helical nature of DNA, would cause topological problems for the cell if not for topoisomerases. (From: Biochemistry (2nd Ed) C.K. Mathews & K.E. Van Holde. Benjamin/Cummings Publishing, Menlo Park, CA.)

The maintenance of proper DNA topology is controlled by a family of enzymes known as topoisomerases (11,13-17).

Topoisomerases

Topoisomerases are essential to all known living organisms. Topoisomerases generally have three fundamental roles in cells: 1) Maintenance of appropriate chromosome topology, 2) removal of positive supercoils, and 3) unlinking of sister chromatids during replication, strand separation and cell division (11,13-17). Topoisomerases are classified by the number of DNA strands that they cleave during their catalytic reactions. Type I topoisomerases create a single, transient break in the DNA and type II topoisomerases cleave both strands. All topoisomerases cleave DNA by the nucleophilic attack on a phosphate of the phosphodiester DNA backbone by a catalytic tyrosine on the enzyme (Figure 3) (11,13-17). Due to the vital role of topoisomerases in the survival of the cell, they are important drug targets for anticancer and antibacterial therapeutics (18-25).

Type I topoisomerases

There are two main subclasses of type I topoisomerases: type IA and type IB (11,13,17,26). Type IA topoisomerases alter topology by cleaving one strand of DNA through the creation of a 5' phosphotyrosine intermediate, passing the opposite strand through the break, and ligating the cleaved strand (11,13,17,26,27). They require a divalent metal ion but not ATP (reverse gyrase is the exception) (11,13,17,26,28).

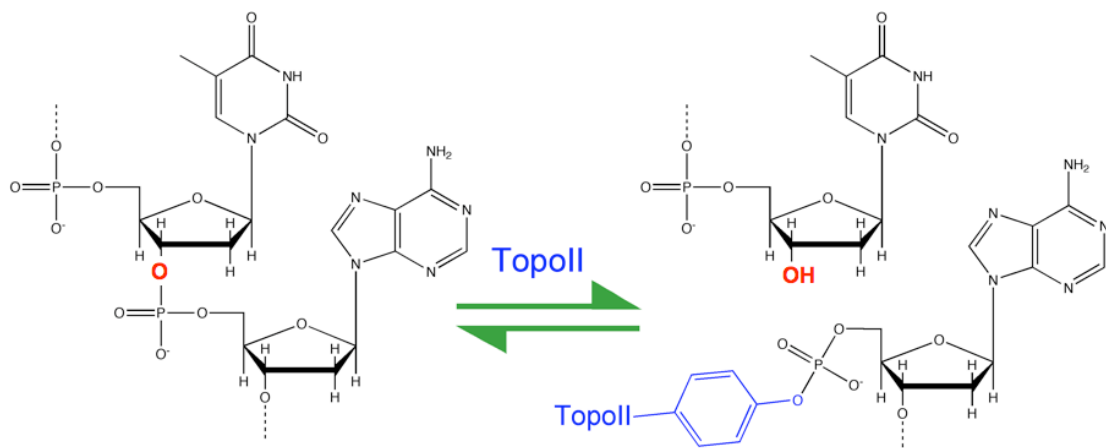


FIGURE 3. During DNA cleavage mediated by type II topoisomerases, the active site tyrosine of the enzyme becomes covalently bound to the 5'-terminal phosphate at the scissile bond of the DNA backbone, generating a free 3'-hydroxyl group (for simplicity, only the reaction at one strand of the double helix is shown). In the reverse of the cleavage reaction, the DNA backbone is ligated through nucleophilic attack of the enzyme-linked phosphate by the $-OH$ moiety.

Because Type IA topoisomerases act on DNA that contains single-stranded character, they are unable to relax positive supercoils.

Type IB topoisomerases create a transient single-stranded break in the DNA through a 3' phosphotyrosine covalent linkage (11,13,17,26). This leaves the 5' end of the DNA free to rotate around the break before the DNA is resealed. This class of enzymes does not require ATP or a divalent metal ion. In contrast to type IA, type IB topoisomerases can relax both positive and negative supercoils as a result of their controlled rotation mechanism (11,13,17,26).

The human type IB topoisomerase, topoisomerase I, is a target for several anticancer drugs (26,29-32). Drugs such as camptothecin, topotecan, and irinotecan inhibit the enzyme from ligating the cleaved strand of DNA (13,30,31,33).

Because they cut only one of the two strands of the double helix, type I topoisomerases can only relieve supercoiling and are not able to resolve knots or catenanes (Figure 4). These topological forms require type II topoisomerases.

Type II Topoisomerases

All living organisms encode at least one type II topoisomerase (13,17,34-37). This class of enzyme is able to relax (or in some cases induce) supercoils, untangle, and unknot DNA due to its ability to cleave both strands of the double helix (Figure 3). They are essential to cellular processes such as chromosome condensation, decondensation, and segregation (13,17,34-37).

Type II topoisomerases act by passing one segment of DNA through a separate cleaved segment of DNA (18,22,37-45). To begin their catalytic cycles (see Figure 5),

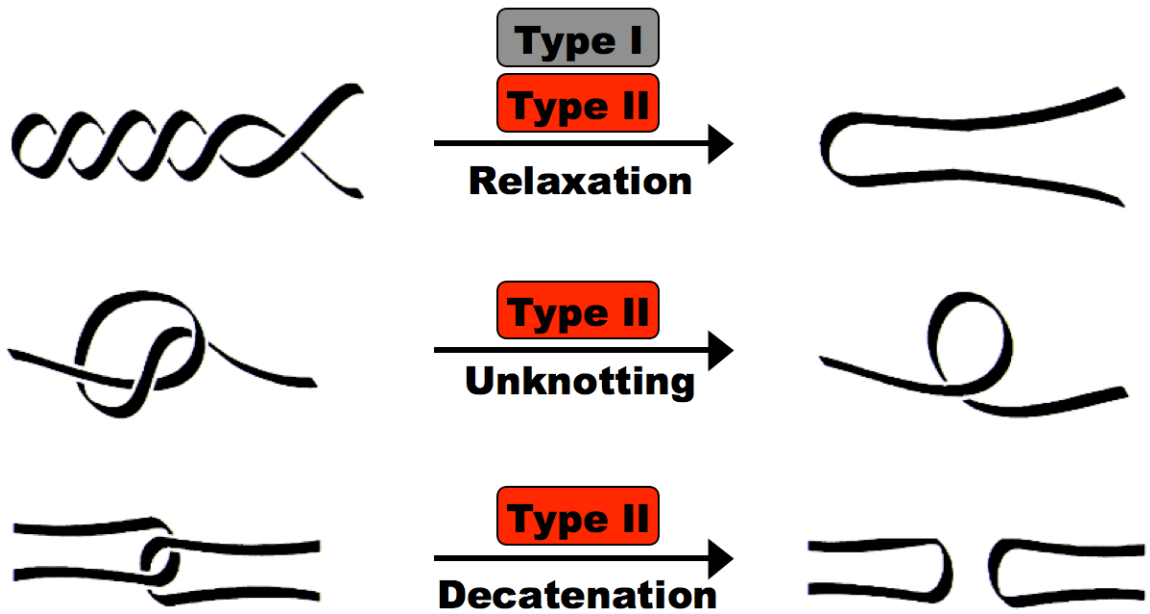


FIGURE 4. Topoisomerases resolve various topological problems that arise due to various cellular processes. Overwinding of DNA occurs ahead of replication and transcription machinery. Type I and type II topoisomerases are able to relax DNA, thereby eliminating the torsional stress and allowing these cellular processes to continue. Because type I topoisomerases only cleave one strand of the double helix, they are unable to deal with structures such as knots and catenanes. These topological configurations result during recombination and replication and can only be resolved by type II topoisomerases.

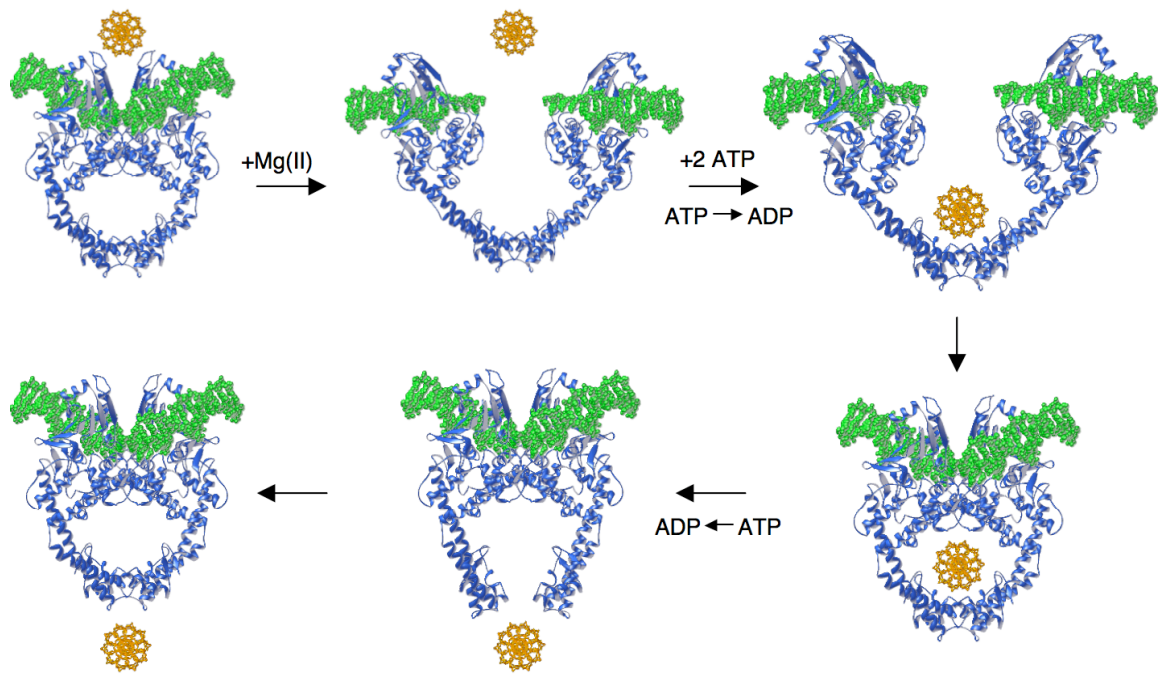


FIGURE 5. Type II topoisomerases (blue) preferably bind DNA at crossovers and, in the presence of a divalent cation, create a double strand break in one segment of the double helix (green). With the hydrolysis of an ATP molecule, the other segment of DNA (orange) is translocated through the break and the cleaved DNA is ligated. A second ATP molecule is hydrolyzed and the translocated segment is released and the enzyme can undergo another round of catalysis.

type II topoisomerases 1) bind two separate segments of DNA. 2) In the presence of divalent metal ions, the enzyme cleaves one of the segments (gate), creating a double-stranded break. This is accomplished through the nucleophilic attack by two active site tyrosyl residues on the scissile phosphate of the DNA backbone. This results in a 5'-phosphotyrosyl linkage on both strands of DNA, 4 bases apart from each other. The covalent enzyme-DNA intermediate is known as the "cleavage complex."

3) In the presence of ATP, the enzyme passes the intact segment of DNA (transport) through the double strand break. The type II topoisomerase then 4) religates the break in the gate segment. This is accomplished by the nucleophilic attack of the tyrosyl-bound 5'-phosphate by the free 3'-OH of the DNA backbone. Finally, 5) upon the hydrolysis of ATP, the enzyme releases the transport segment and 6) closes the protein gate. The type II topoisomerase can then either dissociate from the DNA or go through another round of catalysis.

Type II topoisomerases act on the entire genome but it cleave DNA at preferred sites (46). Although general consensus sequences and DNA base preferences have been found, they are weak and there are many exceptions. It is not possible to use sequence information to consistently predict where type II topoisomerases will cleave DNA. It is therefore expected that the localized structure and properties of DNA such as flexibility have a great influence on topoisomerase II cleavage sites (47,48).

Topoisomerase Poisons

Type II topoisomerases are essential for the survival of proliferating cells but because their function requires the introduction of DNA double strand breaks, they also

have the capability of fragmenting and destabilizing the genome (11,13,18,22). Levels of cleavage complexes must be maintained within a critical range. If levels drop too low, chromosomes will not properly separate during mitosis and the cell could die due to mitotic failure (Figure 6, left) (11,13,18,22).

Cellular levels of cleavage complexes that are too high can also result in cell death. Helicases and polymerases associated with replication forks and transcription machinery can collide with these covalent topoisomerase II-DNA cleavage complexes (11,13,18,22,49). This causes the transient break to become permanent. The DNA breaks, as well as the error-prone products of recombination and repair, lead to mutations, deletions, and translocations. If the chromosomal damage is too great, programmed cell death pathways are triggered (Figure 6, right) (18,22,49-52).

There are two classes of compounds that directly effect cellular concentrations of topoisomerase cleavage complexes (11,13,18,22). The first class, catalytic inhibitors, decrease the concentration of cleavage complexes by blocking normal enzyme activity (11,13,18,22). The second, topoisomerase poisons, increase levels of the covalent topoisomerase-DNA intermediate (11,13,18,22). These compounds turn the enzyme into a cellular toxin, triggering double strand breaks in the genome, as described above. Poisons of type II topoisomerases include environmental toxins, dietary compounds, anticancer, and antibiotic drugs (11,13,18,22).

Bacterial Type II Topoisomerases

Virtually every eubacteria encodes two type II topoisomerases; gyrase and topoisomerase IV (17,19,24,37,53,54). These enzymes help to regulate the superhelical

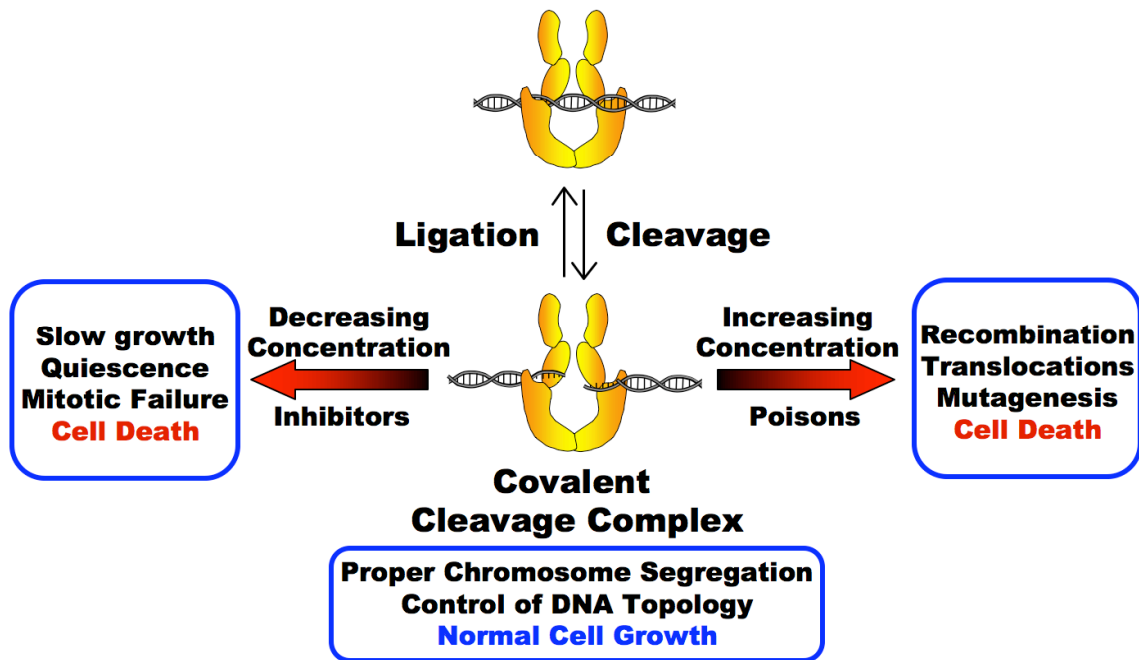


FIGURE 6. Type II topoisomerases are essential to all known living organisms. They carry out their necessary functions by the formation of covalent cleavage complexes. When the cellular concentration of these cleavage complexes becomes too low, such as in the presence of a catalytic inhibitor, proper control of DNA topology cannot be maintained and the cell can undergo slower growth, quiescence, mitotic failure, and even cell death. Conversely, when the concentration of cleavage complexes is too high, such as in the presence of a topoisomerase II poison, DNA strand breaks accumulate. DNA tracking systems such as helicases and polymerases can collide with the covalent topoisomerase-DNA complex, resulting in permanent double strand breaks. These breaks can lead to recombination, chromosomal translocations, and cell death.

density of the bacterial chromosome and remove knots and tangles from the double helix. While gyrase and topoisomerase IV both alter DNA topology by generating transient double-stranded breaks in the genetic material and passing an intact segment of DNA through the break, the two enzymes play different roles in the cell.

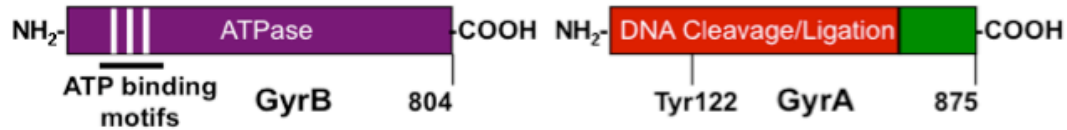
Gyrase is a heterotetramer containing two subunits of GyrA and two of GyrB (GyrA₂GyrB₂) (13,17,19,24,37,53,54). Gyrase has a three-domain structure consisting of the ATPase domain, the DNA cleavage/ligation domain, and the C-terminal domain (Figure 7). GyrA contains the active site tyrosine and C-terminal domain, while GyrB is responsible for ATP binding and hydrolysis (13,17,19,24,37,53,54).

Gyrase is the only known type II enzyme that is able to actively underwind the double helix (13,17,19,24,37,53,54). It is involved primarily in regulating the superhelical density of chromosomal DNA and alleviating torsional stress that accumulates ahead of DNA tracking systems. Generally, gyrase is more suited to deal with intramolecular DNA processes such as supercoiling. This is presumably due to the fact that it wraps DNA around itself, thereby bringing together two segments of the same localized section of the double helix (13,17,19,24,37,53,54).

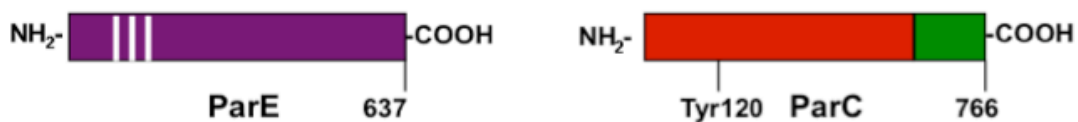
Topoisomerase IV shares the domain structure and ~40% sequence homology with gyrase. Like gyrase, it is a heterotetramer with two subunits of the DNA binding a cleavage domain (ParC) and two of the ATPase domain (ParE) (Figure 7) (13,17,19,24,37,53,54). Although it can relax positive and negative supercoils, topoisomerase IV does not wrap DNA and does not alleviate supercoiling nearly as well as gyrase (19,24,25,37,53-55). Topoisomerase IV, on the other hand, efficiently

Type IIA Topoisomerases

E. coli DNA Gyrase



E. coli Topoisomerase IV



Human Topoisomerase II α



Human Topoisomerase II β



FIGURE 7. Domain alignment of bacterial and human type II topoisomerases. *E. coli* gyrase and topoisomerase IV, along with human topoisomerase II α and β are shown. The colors represent regions of homology. The N-terminal domain (purple)(GyrB and ParE in *E. coli*) is responsible for ATP binding and hydrolysis. The three vertical white lines represent conserved “Bergerat fold” motifs. The central homology domain (red) contains the active site tyrosine that forms the covalent bond with the DNA backbone. The C-terminal domain (green) has the least sequence homology between species not closely related. In humans, this domain contains the nuclear localization sequence (NLS) and phosphorylation sites (PO₄)

decatenates DNA, and is the major enzyme that is responsible for unknotting and untangling the bacterial genome (19,24,25,37,53-55).

Antibacterial Drugs

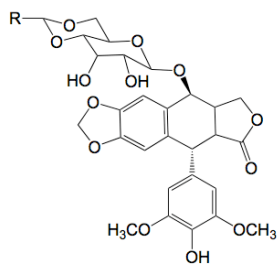
Beyond their critical physiological functions, gyrase and topoisomerase IV are targets for quinolone-based antibacterial drugs (13,19,20,23,24). Members of this drug class, such as levofloxacin and ciprofloxacin, are among the most active and broad-spectrum antibacterial agents currently in clinical use (Figure 8, right). Quinolones poisons cells by stabilizing covalent enzyme-cleaved DNA complexes, which are requisite intermediates in the DNA strand passage reactions of gyrase and topoisomerase IV (13,19,20,54,56-58). Cleavage complexes are converted to permanent strand breaks by collisions with helicases and other DNA tracking enzymes, triggering an SOS response in the bacterial cell (13,19,20,54,56-58).

Gyrase and topoisomerase IV both contribute to the cytotoxicity of quinolones (19,20,59-61). However, the enzyme primarily responsible for cell death appears to be species- and drug-dependent.

Human Type II Topoisomerases

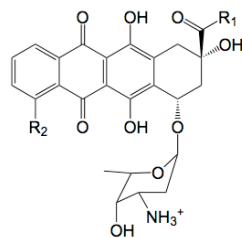
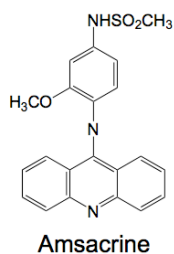
All eukaryotic type II enzymes are homodimeric and require ATP and divalent metal ions to perform their catalytic function (13,17). As seen in Figure 7, the eukaryotic type II topoisomerase retains the three-domain structure seen in the bacterial enzymes, although the domains are fused into one polypeptide. There is even a great deal of

Anti-Cancer Drugs



Etoposide: $\frac{\text{R}}{\text{CH}_3}$

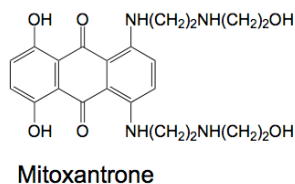
Teniposide:



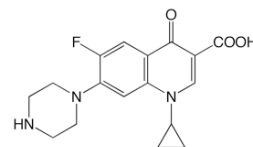
Doxorubicin: $\frac{\text{R}_1}{\text{CH}_2\text{OH}}$ $\frac{\text{R}_2}{\text{OCH}_3}$

Daunorubicin: CH_3 OCH_3

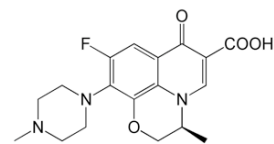
Idarubicin: CH_2OH H



Anti-Bacterial Drugs



Ciprofloxacin



Levofloxacin

FIGURE 8. Type II topoisomerase poisons. Left: Structures of clinically relevant chemotherapeutic drugs. Right: Examples of two anti-bacterial fluoroquinolones currently in clinical use.

sequence similarity between prokaryotic and eukaryotic excluding the C-terminus (13,17).

The genomes of lower eukaryotes such as yeast and *Drosophila* encode only one type II topoisomerase (34,62-64). In contrast, vertebrates, including humans, encode two closely related isoforms of topoisomerase II, topoisomerase II α and topoisomerase II β (11,17,36,37,65). The two isoforms have different molecular masses and are encoded by two separate genes (11,22,36,55,65-71). They display 70% amino acid sequence identity and share many enzymatic functions. The enzymes do, however, have different roles in the cell. The α isoform relaxes positive supercoils much faster than it does negative, but the β isoform does not discern the geometry of DNA supercoiling *in vitro* (72).

Topoisomerase II α and β also differ in their expression patterns. Topoisomerase II α is associated with replication forks and chromosomes during mitosis (16,73-76). Presumably due to its role removing tangles from DNA during replication and mitosis, the α isoform is essential for cell survival in proliferating cells. Its expression increases dramatically during periods of cellular growth (75,77-79). Expression of the α isoform is also regulated by the cell cycle, with levels peaking during G2/M (79-81).

In contrast to topoisomerase II α , topoisomerase II β is not required for cell survival (65,66). It has, however, been shown to be necessary for neuronal development in mice (76,82). Expression levels of topoisomerase II β are cell cycle independent and do not increase within highly proliferating cells and it is not found on chromosomes during mitosis (66,76,83). The β isoform cannot compensate for the loss of topoisomerase II α (66,75,84,85). This suggests that, while the isoforms are very similar in structure and

mechanism, their physiological roles are quite different. Recent evidence suggests that it may be involved in the transcription regulation of developmental genes (84,86).

Human Topoisomerase II poisons

Many naturally occurring compounds in the human diet poison topoisomerase II. These include bioflavonoids, which are found in fruits and vegetables (87-96). One such bioflavonoid, genistein, a prominent compound in soy, is a potent topoisomerase II poison and is believed to have chemopreventative properties in adults (88-91,94,95). (-)-epigallocatechin gallate (EGCG), a major component of green tea, also acts as a topoisomerase II poison (88,95,97). Environmental toxins such as quinones also poison the type II enzyme (98-106).

Several topoisomerase II poisons are in wide clinical use as effective chemotherapeutic drugs. These include amsacrine, daunorubicin, doxorubicin, idarubicin, mitoxantrone, teniposide, and etoposide (Figure 8, left) (21,107-110). They are effective in the treatment of a number of malignancies including small cell lung and germ-line cancers, sarcomas, as well as leukemias and lymphomas (21,107-110). These drugs inhibit religation of the cleaved DNA strand in the topoisomerase II cleavage complex, leading to double strand breaks, which can lead to the generation of chromosomal aberrations, destabilize the genome, and trigger cell death pathways (21).

Etoposide

Etoposide is a highly successful anticancer agent that has been used to treat a variety of blood-borne and solid human malignancies for nearly thirty years

(21,108,111,112). The drug is a derivative of podophyllotoxin, a naturally occurring folk remedy from mayapples that has been used for over a thousand years to treat various ailments (21,108). Podophyllotoxin blocks mitosis due to its ability to inhibit tubulin polymerization, but development as an anticancer drug was halted because of high toxicity (21,108). In the 1960's two podophyllotoxin derivatives, teniposide and etoposide, were developed in the hopes of overcoming this toxicity (21,108). Two decades later, the FDA approved the drugs for cancer chemotherapy treatments. While these drugs of reduced toxicity were more effective antineoplastic agents, the mechanism of action was unknown. In the mid-1980's researchers determined that topoisomerase II was the primary target of etoposide (21,108).

The individual contributions of topoisomerase α and β to the clinical efficacy of etoposide have yet to be determined. Etoposide has similar effects on levels of α - and β -mediated DNA cleavage *in vitro* and targets both isoforms in cultured human cells, suggesting that both enzymes have a role in the cellular efficacy of the drug (21,108,113). However, recent evidence suggests that topoisomerase II α may play a more prominent role in mediating the cytotoxic effects of topoisomerase II-targeted anticancer drugs (114-116). There is also evidence that topoisomerase II β is the isoform primarily responsible for off-site cardiotoxicity associated with topoisomerase II targeted chemotherapy (114-116). Coupled with the fact that the concentration of topoisomerase II α is generally higher in malignant as compared to corresponding normal tissues, most studies of etoposide action have focused on the α isoform (11,117-119).

Although etoposide has been an important chemotherapeutic drug during the last three decades, evidence suggests that its use also causes chromosomal translocations that

can lead to cancer formation (18,22,51,120-122). About 2-3% of patients treated with etoposide and other topoisomerase II-targeted regimens are eventually diagnosed with treatment-related leukemias (51,121). Most of these leukemias have translocations within a region of the mixed lineage leukemia (*MLL*) gene on chromosome 11 (51,121). The DNA breakpoints in the *MLL* gene and its translocation partner are typically near topoisomerase II cleavage sites.

Scope of the Dissertation

Type II topoisomerases are essential to all living organisms because of their roles in critical cellular processes such as replication, transcription, recombination, as well as chromosomal segregation, condensation, and decondensation (11,13-17). Type II topoisomerases also are important targets for anticancer and antibacterial drugs. These drugs use the catalytic activity of topoisomerase to create permanent double-stranded breaks in the genome, potentially leading to cell death (13,19,20,23,24,107-110).

Chapter I discusses DNA topology and bacterial and human topoisomerases, focusing on the type II enzyme. It includes an overview of the importance of these enzymes in the treatment of disease.

The materials and methods used in the research described in this dissertation are found in Chapter II.

Chapter III of this dissertation describes divalent metal ion usage in the bacterial topoisomerase IV cleavage reaction. It is demonstrated that DNA scission is enhanced when thiophilic metal ions are used with substrates that contain bridging sulfur atoms. This suggests that a metal ion is making a critical interaction with the 3'-bridging atom of the scissile phosphate of the DNA substrate. Furthermore, a series of experiments demonstrates that simultaneous binding of two metal ions is required for topoisomerase IV-mediated DNA cleavage.

Chapter IV explores interactions between the D-ring and glycosidic moieties of the anticancer drug etoposide with human topoisomerase II α . Previous experiments showed that these groups on etoposide do not interact with the enzyme in the binary protein-drug complex (138,139). Using a series of etoposide derivatives, it becomes clear

that the D-ring, and to a lesser extent, the glycosidic moiety, are important for drug activity. Changes in the D-ring also alter sites of enzyme-mediated DNA cleavage as well as decrease etoposide binding. These findings suggest that the D-ring of etoposide interacts with DNA in the topoisomerase II α cleavage complex.

Conclusions for the research described in this dissertation are summarized in Chapter V.

CHAPTER II

METHODS

Materials

E. coli topoisomerase IV (1:1 ratio of ParC and ParE subunits) was expressed in *E. coli* and purified as described by Peng and Mariani or by Corbett *et al* (123,124).. In the latter case, the cell pellet was resuspended in 20 mM HEPES (pH 7.5), 400 mM NaCl, 10% glycerol and 2 mM β -mercaptoethanol, lysed by sonication and centrifuged. The supernatant was loaded onto a HisTrap HP column (GE Healthcare) and the protein was eluted with a linear gradient of 0–200 mM imidazole. The protein containing fractions were pooled and desalted on a HiPrep desalting column (GE Healthcare). The His-tag was removed by overnight incubation with AcTEV at 4°C using an OD_{280nm} ratio 1:100 AcTEV:protein, and the cleaved protein was filtered through a HisTrap HP column. The protein was concentrated and further purified on a Superdex 200 10/300 GL column (GE Healthcare). The concentrations of ParC and ParE were quantified by UV absorption.

Human topoisomerase II α was expressed in *Saccharomyces cerevisiae* and purified as previously described (125,126). However, in the final step of the purification, topoisomerase II α was eluted from the phosphocellulose column (P81, Whatman) with buffer containing 10 mM sodium phosphate (pH 7.7), 750 mM KCl, 1 mM EDTA, 1 mM EGTA, and 0.5 mM dithiothreitol. For protein samples that were used in NMR experiments, the elution buffer was made up in D₂O (99.9%, Aldrich) instead of H₂O.

A 50-bp oligonucleotide duplex was designed using a previously identified topoisomerase II cleavage site from pBR322. This site corresponds to the sequence designated as site 2 by Fortune et al. (127). Wild-type oligonucleotide sequences were purchased from Sigma. The 50-mer top and bottom sequences were 5'-TTGGTATCTGCGCTCTGCTGAAGCC↓AGTTACCTTCGGAAAAAGAGTTGGT-3' and 5'-ACCAACTCTTTTTCCGAAGGT↓AACTGGCTTCAGCAGAGCGCAGATAACC AA-3', respectively (arrows denote sites of cleavage).

DNA containing a single 3'-bridging phosphorothiolate linkage at the scissile bond (-1/+1 position) was synthesized as described previously (128). Substrates containing a racemic phosphorothioate in place of a non-bridging oxygen at the scissile bond or at the -2/-1 position of the bottom strand were synthesized by Operon.

[γ -³²P]ATP (~5000 Ci/mmol) was obtained from PerkinElmer NEN. Single-stranded oligonucleotides were labeled on their 5'-termini using T4 polynucleotide kinase (New England Biolabs). Following labeling and gel purification, complementary oligonucleotides were annealed by incubation at 70 °C for 10 min and cooling to 25 °C.

Etoposide was purchased from Sigma. Retroetoposide, DEPT, retroDEPT, and the D-ring diol were synthesized as described previously (129). All drugs were stored at 4 °C as 20 mM stock solutions in 100% DMSO. Drugs used for NMR experiments were stored in 100% d-DMSO.

Procedures

Topoisomerase IV-mediated cleavage of negatively supercoiled plasmid substrates

DNA cleavage assays were carried out using the procedure of Fortune and Osheroff (130). Unless otherwise stated, assay mixtures contained 35 nM topoisomerase IV (1:1 ratio of ParC and ParE subunits) and 10 nM negatively supercoiled pBR322 DNA in a total of 20 μ L of 40 mM Tris-HCl (pH 7.9), 2.5 mM MgCl₂, MnCl₂, or CaCl₂ and 2.5% glycerol. DNA cleavage mixtures were incubated for 10 min at 37 °C, and enzyme-DNA cleavage intermediates were trapped by adding 2 μ L of 5% SDS and 1 μ L of 375 mM EDTA (pH 8.0). Proteinase K was added (2 μ L of a 0.8 mg/mL solution), and reaction mixtures were incubated for 30 min at 45 °C to digest the topoisomerase IV. Samples were mixed with 2 μ L of 60% sucrose in 10 mM Tris-HCl (pH 7.9), 0.5% bromophenol blue and 0.5% xylene cyanol FF, heated for 2 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate, (pH 8.3) and 2 mM EDTA containing 0.5 μ g/mL ethidium bromide. DNA cleavage was monitored by the conversion of negatively supercoiled plasmid DNA to linear molecules. DNA bands were visualized by medium wavelength ultraviolet light and quantified using an Alpha Innotech digital imaging system.

Topoisomerase II α -mediated cleavage of negatively supercoiled plasmid substrates

DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff (130). Unless stated otherwise, assay mixtures contained 150 nM topoisomerase II α and 10 nM negatively supercoiled pBR322 DNA in a total of 20 μ L of topoisomerase II α cleavage buffer [10 mM Tris-HCl (pH 7.9), 100 mM KCl, 5 mM MgCl₂, 0.1 mM

NaEDTA, and 2.5% glycerol] that contained 0 to 200 μ M etoposide, retroetoposide, DEPT, retroDEPT, or D-ring diol. DNA cleavage was initiated by the addition of enzyme and mixtures were incubated for 6 min at 37 °C to establish DNA cleavage-ligation equilibria. Samples were processed as above with the topoisomerase IV plasmid cleavage reactions.

Topoisomerase IV-mediated cleavage of oligonucleotide substrates

DNA cleavage assays were carried out by a modification of the procedure of Dewese et al. (130). Oligonucleotide substrates were always 5'-end-labeled. Unless otherwise stated, cleavage reactions contained 70 nM topoisomerase IV (1:1 ratio of ParC and ParE subunits) and 100 nM double-stranded oligonucleotide in a total of 10 μ L of 40 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, MnCl₂ or CaCl₂, and 2.5% glycerol. In some cases, the concentration of divalent cation was varied or combinations of the cations were used. Reactions were initiated by the addition of enzyme and were incubated for up to 10 min at 37 °C. DNA cleavage products were trapped by the addition of 2 μ L of 10% SDS. DNA products were precipitated in 100% ethanol, washed with 70% ethanol, dried and resuspended in 5 μ L of sample loading buffer (40% formamide, 10 mM NaOH, 0.02% xylene cyanol FF and 0.02% bromophenol blue). Cleavage products were resolved by electrophoresis in 14% denaturing polyacrylamide gels. To inhibit oxidation of cleaved oligonucleotides containing 3'-terminal -SH moieties and the formation of multimers in the gel, 100 mM DTT was added to the sample loading buffer (). DNA cleavage products were visualized and quantified using a Bio-Rad Molecular Imager.

Reversal of Topoisomerase IV cleavage complexes of oligonucleotide substrates

Assays were performed in the preceding section with the following changes: Either the cleavage reaction was terminated by the addition of SDS after 5 min or 2 μ L of 5 M NaCl was added to the cleavage reaction after 5 min and the solution was incubated for 5 min more before the addition of SDS. High salt conditions allow for ligation, but will not support DNA cleavage.

Topoisomerase II α -mediated cleavage of oligonucleotide substrates

Assays were performed as above with topoisomerase IV with the following differences. A 47-bp oligonucleotide corresponding to residues 80–126 of pBR322 and its complement were employed to compare the DNA cleavage site specificity of etoposide to that of retroetoposide (131). Oligonucleotide substrates were purchased from Sigma. The sequences of the top and bottom strands were 5'-CCGTGTATGAAATCTAACAAT**X**↓CGCTCATCGTCATCCTC-GGCACCGT-3' and 5'-ACGGTGCCGAGGATGACGATG↓AGCG**Z**ATTGTTAGATTTCA-TACACGG-3', respectively. This substrate contains a single strong cleavage site for topoisomerase II that has been well characterized (132-134). Arrows denote points of scission. Oligonucleotides were prepared that contained a G (found in the wild-type sequence), C, A, or T at the -1 position on the top strand (denoted by the bold X). Complementary bottom strand oligonucleotides contained a C, G, T, or A, respectively, at the position denoted by the bold Z.

Reaction mixtures contained 220 nM human topoisomerase II α and 100 nM double-stranded oligonucleotide in 20 μ L of topoisomerase II α cleavage buffer

containing 0–500 μM etoposide or retroetoposide. Reactions were incubated for 10 min at 37 °C.

Topoisomerase IV-mediated cleavage of linear plasmid substrates

DNA cleavage sites were mapped using a modification of the procedure of O'Reilly and Kreuzer (135). A linear 4330 bp fragment (HindIII/EcoRI) of pBR322 plasmid DNA singly labeled with ^{32}P on the 5'-terminus of the HindIII site was used as the cleavage substrate. The plasmid was linearized by treatment with HindIII. Terminal 5'-phosphates were removed by treatment with calf intestinal alkaline phosphatase and replaced with [^{32}P]phosphate using T4 polynucleotide kinase and [$\gamma\text{-}^{32}\text{P}$]ATP. The DNA was treated with EcoRI, and the 4330 bp singly end-labeled fragment was purified from the small EcoRI-HindIII fragment by passage through a CHROMA SPIN+TE-100 column (Clontech).

Reaction mixtures contained 1.4 nM labeled linear DNA and 35 nM topoisomerase IV (1:1 ratio of ParC and ParE subunits) in 50 μL of 40 mM Tris-HCl (pH 7.9), 0–2.5 mM MgCl_2 , MnCl_2 or CaCl_2 and 2.5% glycerol. Reactions were initiated by the addition of enzyme and were incubated for 10 min at 37 °C. Cleavage intermediates were trapped by adding 5 μL of 5% SDS followed by 3 μL of 250 mM EDTA (pH 8.0). Topoisomerase IV was digested with proteinase K (5 μL of a 0.8 mg/mL solution) for 30 min at 45 °C. DNA products were precipitated twice in 100% ethanol, washed in 70% ethanol, dried and resuspended in 5 μL of sample loading buffer. Samples were subjected to electrophoresis in 6% denaturing polyacrylamide gels. DNA cleavage products were visualized and quantified as above.

Topoisomerase II α -mediated cleavage of linear plasmid substrates

Assays were performed as above with topoisomerase IV with the following differences. Reaction mixtures contained 0.7 nM labeled pBR322 DNA and 90 nM human topoisomerase II α in 20 μ L of DNA cleavage buffer supplemented with 0.5 mM ATP in the absence of drug or in the presence of 10 μ M etoposide, 25 μ M DEPT, or 250 μ M retroetoposide, retroDEPT, or D-ring diol. Reaction mixtures were incubated for 6 min.

Drug-induced DNA cleavage mediated by topoisomerase II α in cultured human CEM cells

The In vivo Complex of Enzyme (ICE) bioassay (136,137) (as modified on the TopoGEN, Inc. website) was employed to determine the ability of 25 μ M etoposide, retroetoposide, DEPT, retroDEPT, or D-ring diol to induce topoisomerase II α -mediated DNA breaks in CEM cells. Human CEM acute lymphoblastic leukemia cells (ATCC) were cultured under 5% CO₂ at 37 °C in RPMI 1640 medium (Cellgro by Mediatech, Inc.) containing 10% heat-inactivated fetal calf serum (Hyclone) and 2 mM glutamine (Cellgro by Mediatech, Inc.).

Exponentially growing cultures were treated with DMSO or drugs for 1 h. Cells ($\sim 5 \times 10^6$) were harvested by centrifugation and lysed by the immediate addition of 3 mL of 1% sarkosyl. Following gentle dounce homogenization, cell lysates were layered onto a 2 mL cushion of CsCl (1.5 g/mL) and centrifuged in a Beckman NVT 90 rotor at 80,000 rpm ($\sim 500,000$ g) for 5.5 h at 20 °C. DNA pellets were isolated, resuspended in 5

mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and blotted onto nitrocellulose membranes using a Schleicher and Schuell slot blot apparatus. Covalent cleavage complexes formed between topoisomerase II α and chromosomal DNA were detected using a polyclonal antibody directed against human topoisomerase II α (Kiamaya Biochemical Co.) at a 1:1000 dilution.

STD ¹H NMR spectroscopy

NMR spectra were generated using conditions similar to those described previously (138,139). All NMR experiments were performed at 283 K using a Bruker Avance 700 MHz spectrometer equipped with a 5 mm cryoprobe with z gradients. NMR buffers contained 10 mM sodium phosphate (pH 7.7), 250 mM KCl, 0.1 mM Na₂EDTA, and 5 mM MgCl₂. Samples (400 μ L) contained 5 μ M human topoisomerase II α and 500 μ M etoposide, retroetoposide, DEPT, retroDEPT, or D-ring diol and were maintained at 4 °C until data were collected. STD ¹H NMR experiments employed a pulse scheme similar to that reported by Mayer and Meyer (140). A 2 s saturation pulse was used for the saturation, with on- and off-resonance irradiation frequencies of 0.5 and -71 ppm, respectively. The water signal was suppressed using excitation sculpting with gradients. For each experiment (on- and off-resonance irradiation), 256 scans were collected with a 2 s recycle time. Difference spectra were prepared by subtracting the on-resonance spectrum from the off-resonance spectrum. Signals resulting in the difference spectrum represent the NOE difference signals generated by the transfer of irradiation energy from the enzyme to the bound ligand. Ligand protons in close spatial proximity with the enzyme displayed larger NOE signals. Mapping of the NOE signals with their proton

assignments on the ligand revealed the ligand-binding epitope to human topoisomerase

II α . Spectra were processed using Bruker Topspin software.

CHAPTER III

USE OF DIVALENT METAL IONS IN THE DNA CLEAVAGE REACTION OF TOPOISOMERASE IV

Introduction

As discussed in Chapter I, virtually every eubacteria encodes two type II topoisomerases, gyrase and topoisomerase IV (17,19,24,37,53,54). Gyrase is primarily involved in primarily in regulating the superhelical density of chromosomal DNA and alleviating torsional stress (17,19,24,37,53,54). Topoisomerase IV efficiently decatenates DNA, and is the major enzyme that is responsible for unknotting and untangling the bacterial genome. In addition to their critical physiological functions, both enzymes contribute to the cytotoxicity of the quinolone antibacterial drugs (17,19,24,37,53,54).

Despite the importance of gyrase and topoisomerase IV in the regulation of DNA topology in bacteria and the treatment of infectious diseases, many aspects of the DNA cleavage reaction are poorly understood. To this point, an area of emerging interest is the use of divalent metal ions in catalyzing the DNA scission event (141,142).

Based on the ability of mutant gyrase proteins to utilize metal ions, Noble and Maxwell (143) proposed that the bacterial enzyme utilizes a two-metal-ion phosphotransferase/hydrolase mechanism similar to that of DNA primases and polymerases (14,144). In this mechanism, one divalent cation activates a catalytic water or ribose hydroxyl for nucleophilic attack, the other coordinates a leaving group, and both stabilize the pentavalent transition state. While the mutagenesis approach employed in this study provided evidence for the use of two divalent metal ions, it was not able to

provide information on the specific roles of the metal ions in the DNA cleavage/ligation reaction of gyrase.

A greater understanding of metal ion usage by type II topoisomerases was provided by enzymological and mutagenesis studies with human topoisomerase II α and topoisomerase II β (142,145-149). These studies indicated that the human type II enzymes also employ a two-metal-ion mechanism. Furthermore, they suggested that one of the metal ions has an important interaction with the bridging oxygen of the scissile bond that greatly stimulates DNA cleavage. An interaction with the non-bridging oxygen of the scissile bond also was noted, but the ability of this interaction to stimulate DNA cleavage varied between the two human isoforms (142,145-149).

A recent crystal structure of the covalent yeast topoisomerase II-cleaved DNA complex contained both metal ions and led to the proposal that eukaryotic topoisomerase II employs a novel variation of the canonical two-metal-ion mechanism for DNA cleavage (Figure 9) (150). In this model, one metal ion (metal ion A) plays a direct role in the transition chemistry, most likely by promoting the leaving of the ribose 3'-OH. The other metal ion (metal ion B) appears to play a structural role in anchoring the DNA during cleavage.

Structures for non-covalent and covalent complexes formed between bacterial type II topoisomerases and DNA have been reported recently (151-154). In contrast to the work with yeast topoisomerase II, some of these structures contained divalent metal ions at site A or B, but none contained ions at both sites. These results led to an alternative proposal for the use of divalent cations by bacterial type II enzymes, suggesting that they use a "moving metal ion mechanism" in which only one of the two metal ion sites is filled at

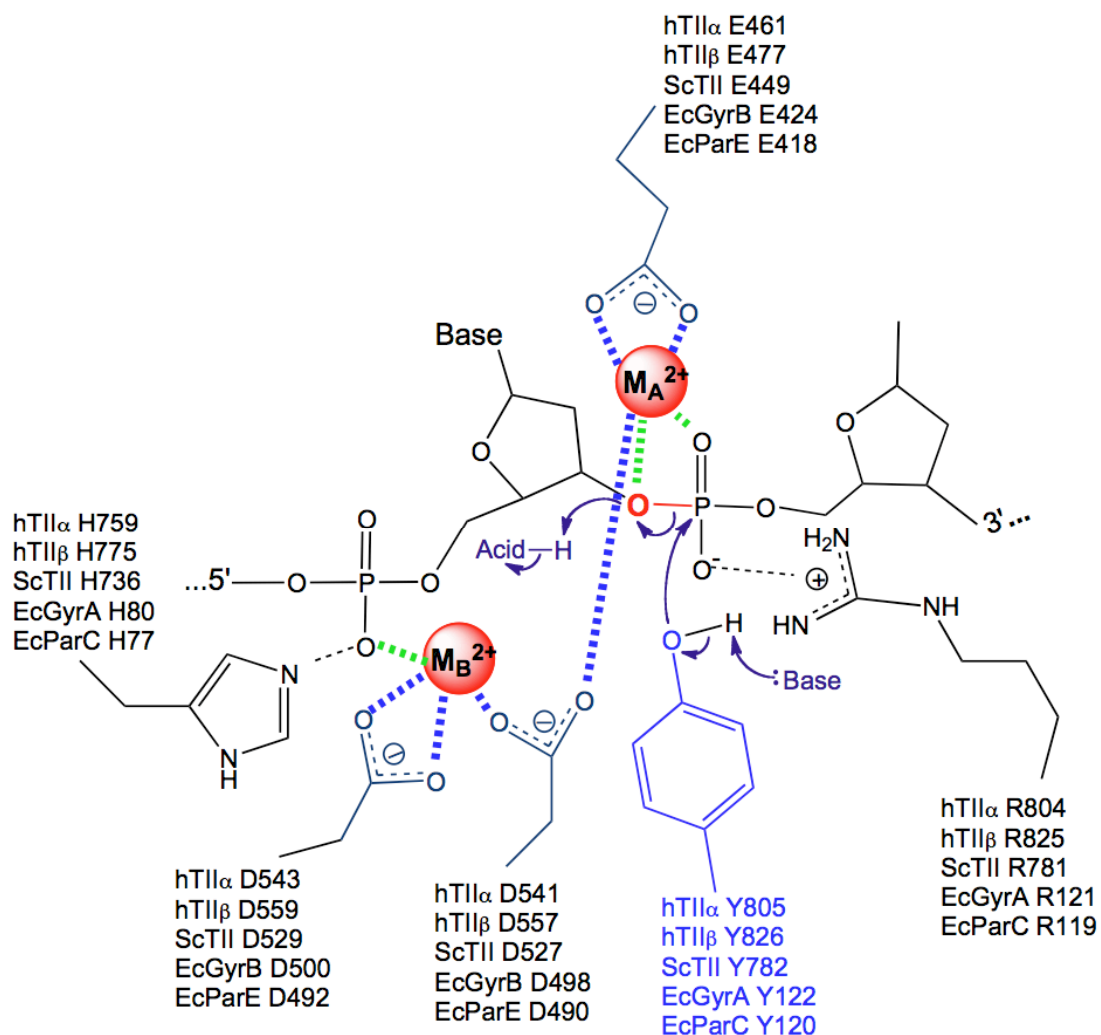


FIGURE 9. Proposed two-metal-ion mechanism for DNA cleavage and ligation mediated by type II topoisomerases. A novel variant of the canonical two-metal-ion mechanism employed by primases and polymerases is shown. Amino acids in the active site that are postulated to function in catalysis by human topoisomerase II α and topoisomerase II β , and by *Saccharomyces cerevisiae* topoisomerase II are indicated. The corresponding amino acids in the ParE and ParC subunits of *E. coli* topoisomerase IV are shown in italics. Metal ions (M_A^{2+} and M_B^{2+}) are highlighted in red. Interactions between metal ions and nucleic acid and acidic amino acid residues are denoted in green and blue, respectively. In the proposed model, M_A^{2+} makes critical contacts with the 3'-bridging atom (red) and a non-bridging atom of the scissile phosphate and plays a role in the transition chemistry and in stabilizing the leaving 3'-terminal oxygen. M_B^{2+} contacts the non-bridging oxygen of the phosphate that connects the -1 and -2 bases upstream from the scissile bond and plays a structural role in anchoring the DNA during scission. Figure is adapted from Schmidt, *et al.* (150).

any given time (153-156).

In order to resolve this fundamental issue regarding the use of divalent metal ions by the bacterial type II enzymes, we carried out enzymological studies with *E. coli* topoisomerase IV. These studies utilized a series of divalent metal ions with varying thiophilicities in conjunction with DNA cleavage substrates that replaced specific bridging and non-bridging oxygen molecules with sulfur. Results strongly suggest that topoisomerase IV mediates DNA scission *via* a two-metal-ion mechanism and that one of the required divalent cations makes critical interactions with the bridging atom of the scissile bond during the cleavage event. Thus, it is proposed that the bacterial and eukaryotic type II topoisomerases utilize a common metal ion-dependent mechanism for cleaving DNA.

Results and Discussion

DNA cleavage mediated by E. coli topoisomerase IV is promoted by an interaction between a divalent metal ion and the bridging atom of the scissile phosphate

E. coli topoisomerase IV, like other type II topoisomerases, requires a divalent metal ion to support DNA cleavage and overall catalytic activity (157). Although the physiological metal ion appears to be magnesium, the bacterial enzyme can utilize a variety of divalent cations for the DNA cleavage reaction *in vitro* (157,158). Figure 10 shows the effects of metal ion concentration on topoisomerase IV-mediated DNA cleavage (*left panel*) as well as time courses for cleavage at constant metal ion concentrations (*right panel*). When negatively supercoiled plasmid was used as the substrate, Ca^{2+} supported the highest levels of DNA scission over a broad range of metal ion concentrations (*left panel*). Mg^{2+} and Mn^{2+} both supported DNA cleavage to a lesser

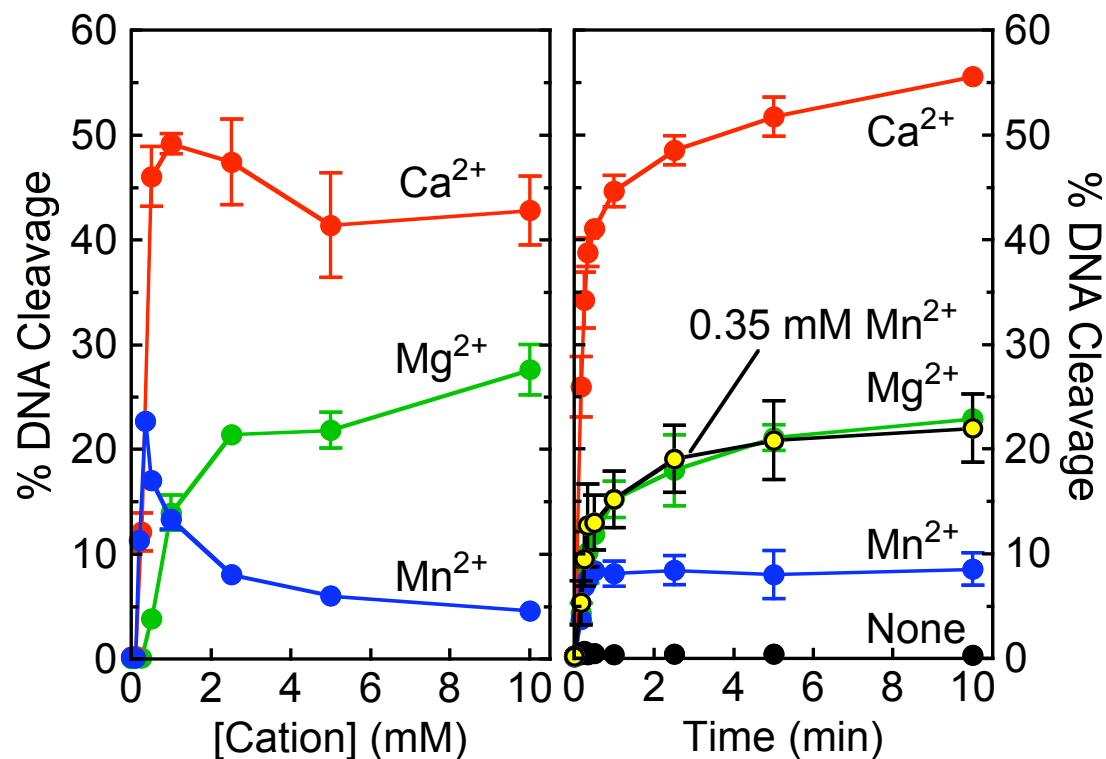


FIGURE 10. Cleavage of pBR322 plasmid by *E. coli* topoisomerase IV in the presence of different divalent metal ions. The effects of cation concentration on DNA cleavage (10 min reactions) are shown in the *left panel*. Mg²⁺ (green), Mn²⁺ (blue), or Ca²⁺ (red) were titrated from 0.1 to 10 mM. Time courses for DNA cleavage in the presence of 2.5 mM divalent metal ions are shown in the *right panel* (Mg²⁺, green; Mn²⁺, blue; Ca²⁺, red). Time courses in the absence of metal ion (open squares) and at the optimal concentration for Mn²⁺ (0.35 mM Mn²⁺, yellow) also are shown. Error bars represent the standard deviation of three independent experiments.

extent (2– to 4–fold lower than Ca^{2+}), but the optimal concentration of Mn^{2+} (~0.35 mM) was nearly an order of magnitude lower than that of Mg^{2+} (~2.5 mM).

Biochemical and structural studies with human and yeast type II topoisomerases suggest that active site metal ions in the eukaryotic enzyme interact with DNA at the bridging oxygen of the scissile bond (-1/+1 position), and non-bridging oxygen atoms at the scissile bond and the -2/-1 position (see Figure 9) (142,147-150). While the interactions at the scissile bond are believed to help mediate the chemistry of DNA cleavage, the interaction with the -2/-1 non-bridging oxygen is thought to play a structural role in the reaction (150). In order to characterize the role of the metal ion in the DNA cleavage reaction of topoisomerase IV, a series of oligonucleotide substrates was employed that substituted sulfur for the bridging (*i.e.*, phosphorothiolate) or non-bridging (*i.e.*, phosphorothioate) oxygen atoms described above (Figure 11).

Wild-type oligonucleotides or substrates with substituted non-bridging atoms, establish rapid DNA cleavage-ligation equilibria. Treatment of these cleavage complexes with high salt allows for DNA ligation but not cleavage because dissociation occurs once the enzyme is no longer covalently bound to the DNA (Figure 12, left). In contrast, virtually no ligation of the phosphorothiolate substrate was observed following the addition of high salt to the topoisomerase IV-DNA cleavage complexes (Figure 12, middle/right). This is because the 3'-terminal -SH moiety that is generated following cleavage of the phosphorothiolate is a poor nucleophile for phosphorous (159,160).

Metal ion-DNA interactions were determined by comparing the ability of topoisomerase IV to cleave substrates containing oxygen or sulfur atoms in the presence of metal ions of varying “hardness” (*i.e.*, thiophilicity) (159,160). Experiments took

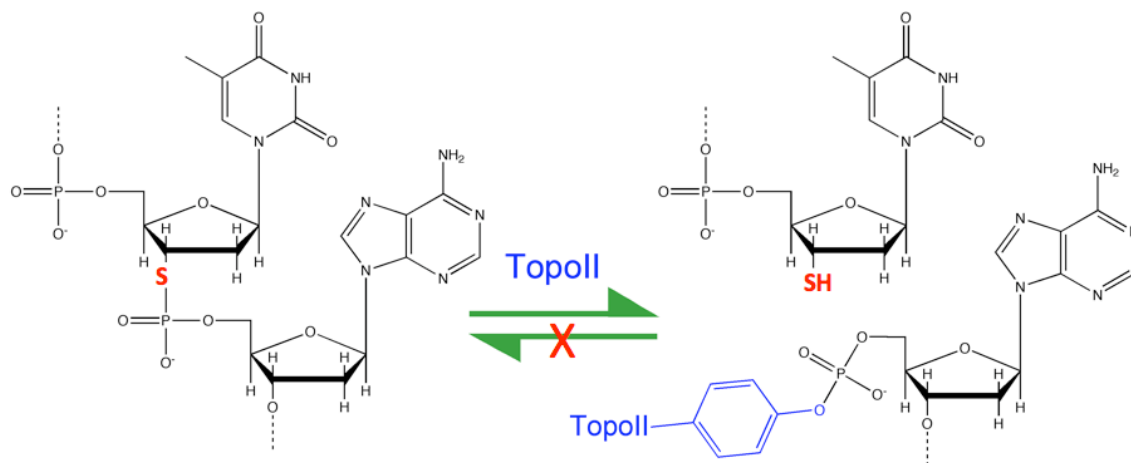


FIGURE 11. A phosphorothiolate substrate, with a sulfur replacing the 3' bridging oxygen, is shown. The topoisomerase-mediated cleavage reaction proceeds just as with the wild-type substrate. The active site tyrosine of the enzyme covalently binds to the 5' phosphate, but in this case, a 3'-terminal -SH group is generated. This sulfhydryl group is a weak nucleophile and does not support ligation of the DNA. This allows for the study of cleavage in the absence of the possibly confounding ligation reaction.

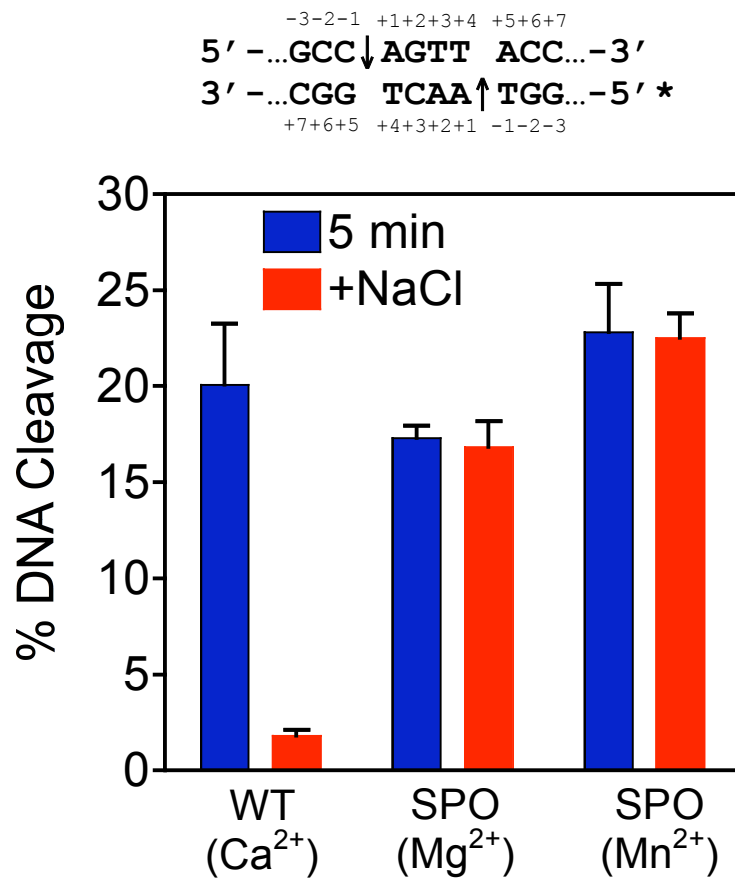


FIGURE 12. Topoisomerase IV-mediated cleavage of a 50-mer oligonucleotide with a 3'-bridging phosphorothiolate at the scissile bond is non-reversible. The central portion of the DNA cleavage substrate is depicted above the graphs. The asterisk denotes the 5'-end-labeled strand and the scissile bonds are indicated by arrows. All modifications are on the bottom strand. The topoisomerase IV cleavage reaction of a wild-type oligonucleotide was terminated by SDS after 5 min (WT, blue bar). When NaCl, instead of SDS, was added to the cleavage reactions after 5 min, cleavage of the WT substrate was reversed (WT, red bar). When the same was done with the phosphorothiolate substrates, DNA cleavage was irreversible. The divalent cations used in these experiments were chosen for their ability to support high levels of topoisomerase IV-mediated DNA cleavage.

advantage of the fact that “soft” metal ions such as Mn^{2+} (which are larger and more polarizable) often prefer sulfur over oxygen as an inner-sphere ligand, whereas “hard” metals such as Mg^{2+} and Ca^{2+} (which have a smaller ionic radius and are non-polarizable) usually coordinate more readily with oxygen (161-167).

Results with the wild-type oligonucleotide (Figure 13, left) were similar to those obtained with plasmid substrate. Once again, Ca^{2+} supported (by far) the highest levels of topoisomerase IV-mediated DNA cleavage. The only difference between results with the wild-type oligonucleotide and plasmid substrates was the fact that Mn^{2+} consistently supported higher levels of cleavage with the oligonucleotide than did Mg^{2+} .

A substrate containing a 3'-bridging sulfur was employed to determine whether metal ion interactions with the bridging/leaving group facilitate catalysis. If this were the case, relative levels (or rates) of scission with substrates that contain a 3'-bridging phosphorothiolate should increase in the presence of soft metals and decrease in reactions that contain hard metals.

As seen in Figure 13 (right), metal ions produced a dramatically different cleavage pattern with the 3'-bridging phosphorothiolate as compared to the wild-type substrate. Cleavage rates were significantly higher and the levels of topoisomerase IV-mediated DNA scission were nearly 10-fold higher with the sulfur-containing substrate in the presence of the soft metal Mn^{2+} . Conversely, the hard metal Ca^{2+} , which supported the highest levels of cleavage with the wild-type substrate, generated the slowest rates of cleavage and the lowest levels of scission with the sulfur-containing oligonucleotide. Despite the fact that cleavage complexes accumulate over time with the 3'-bridging phosphorothiolate, levels of cleavage seen with Ca^{2+} fell more than 10-fold as compared

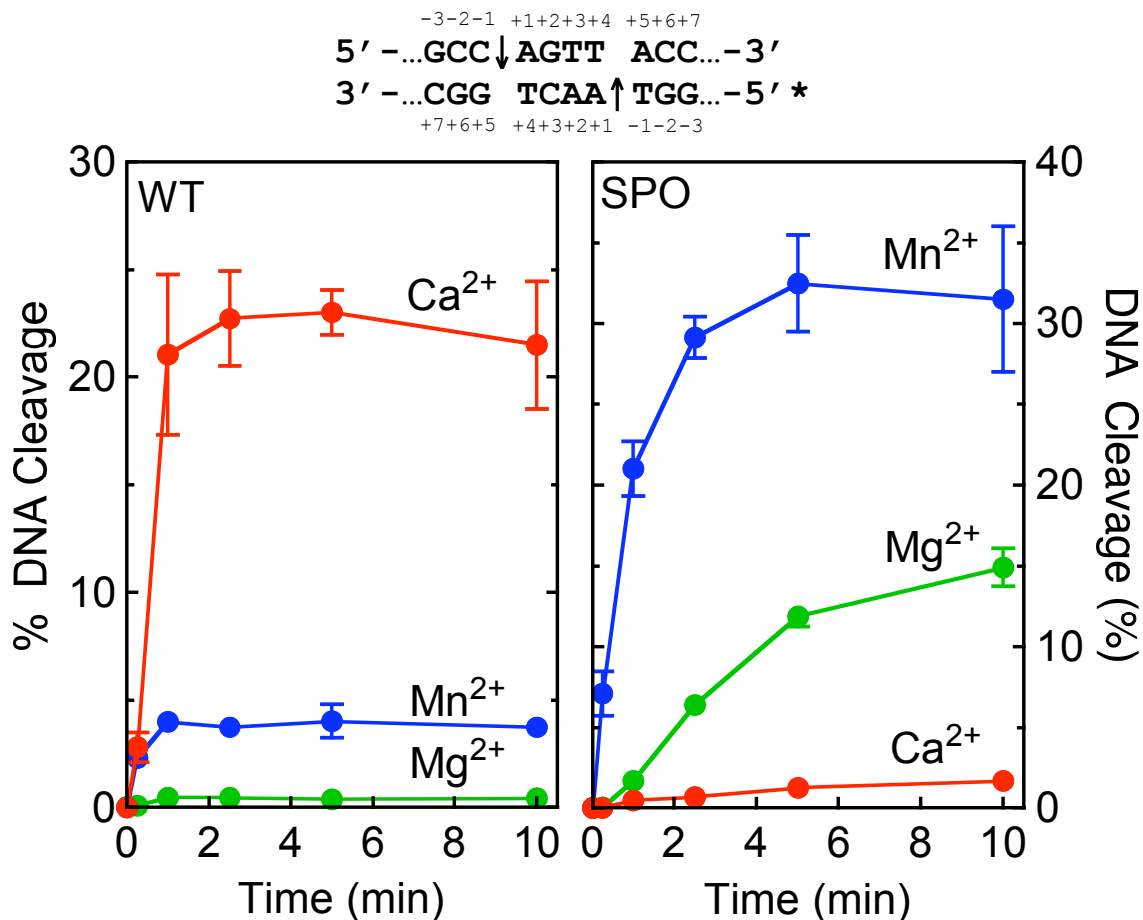


FIGURE 13. Cleavage of oligonucleotide substrates by topoisomerase IV in the presence of different divalent metal ions. The central portion of the DNA cleavage substrate is depicted above the graphs. The asterisk denotes the 5'-end-labeled strand and the scissile bonds are indicated by arrows. All modifications are on the bottom strand. Results for the wild-type oligonucleotide (WT, left), and substrates containing a 3'-bridging phosphorothiolate at the scissile (-1/+1) bond (SPO, right) are shown. DNA cleavage was carried out in the presence of 10 mM Mg²⁺ (green), Mn²⁺ (blue), or Ca²⁺ (red). Error bars represent the standard deviation of three independent experiments.

to wild-type reactions. The precipitous drop in Ca^{2+} -supported scission of the sulfur-containing substrate, coupled with the increased scission observed with Mn^{2+} , demonstrates that there is an important interaction between the metal ion and the 3'-bridging atom of the scissile bond in the active site of *E. coli* topoisomerase IV that promotes the DNA cleavage event. Similar conclusions have been drawn for human topoisomerase II α and topoisomerase II β (147,148).

Divalent metal ion interactions with non-bridging phosphate atoms

Interactions between metal ion A and the non-bridging oxygen atom of the scissile phosphate have been observed in the crystal structure of the covalent yeast topoisomerase II-cleaved DNA complex (150). While similar interactions in the active site of human topoisomerase II α and topoisomerase II β have been proposed on the basis of kinetic experiments, their importance in the DNA cleavage reaction of these enzymes differs considerably (142,147,148). The relative ability of topoisomerase II α to utilize different divalent metal ions with substrates that contain a racemic phosphorothioate at the non-bridging position of the scissile phosphate (-1/+1) was similar to that observed with the wild-type substrate (148). In contrast, the interaction between the metal ion and the non-bridging oxygen appears to play a more substantial role in the DNA scission reaction of topoisomerase II β (147). The relative ability of Ca^{2+} to support cleavage of the racemic non-bridging phosphorothioate decreased and fell below that of Mg^{2+} (147).

Given these differences, we wanted to determine whether the use of metal ions by topoisomerase IV was more similar to that of topoisomerase II α or topoisomerase II β . Therefore, we examined the ability of different metal ions to support topoisomerase IV-

mediated cleavage of the non-bridging phosphorothioate (-1/+1) substrate. As seen in Figure 14 (left), the relative use of metal ions approximated that seen with the wild-type substrate. Once again, the order of metal ion utilization was $\text{Ca}^{2+} > \text{Mn}^{2+} > \text{Mg}^{2+}$. On the basis of these data, *E. coli* topoisomerase IV appears to utilize metal ion A (at least with respect to interactions at the non-bridging oxygen of the scissile phosphate) in a manner similar to that of human topoisomerase II α .

As discussed above, the crystal structure of the covalent yeast topoisomerase II-DNA cleavage complex revealed a novel interaction between metal ion B and the non-bridging oxygen of the phosphate at the -2/-1 position (150). It is believed that this contact plays a structural role in anchoring the double helix during the cleavage/ligation reaction. Therefore, the effects of this interaction on rates of DNA cleavage were determined. Once again, the use of metal ions was similar to those seen with the wild-type substrate (Figure 14, right). These findings cannot rule out an interaction between metal ion A or B and the -2/-1 non-bridging oxygen atoms. However, if these interactions exist in the active site of topoisomerase IV, their effects on the overall rate of DNA cleavage appear to be equivocal.

A two-metal-ion mechanism for DNA cleavage mediated by topoisomerase IV

Biochemical, kinetic, and mutagenesis studies with human topoisomerase II α and topoisomerase II β indicate that both DNA cleavage/ligation active sites contain two divalent metal ions (145-149). Furthermore, the eukaryotic enzymes cleave DNA using a two-metal-ion mechanism in which metal ion site A and site B must both be occupied (142,147-149). This model for eukaryotic topoisomerase II is strongly supported by a

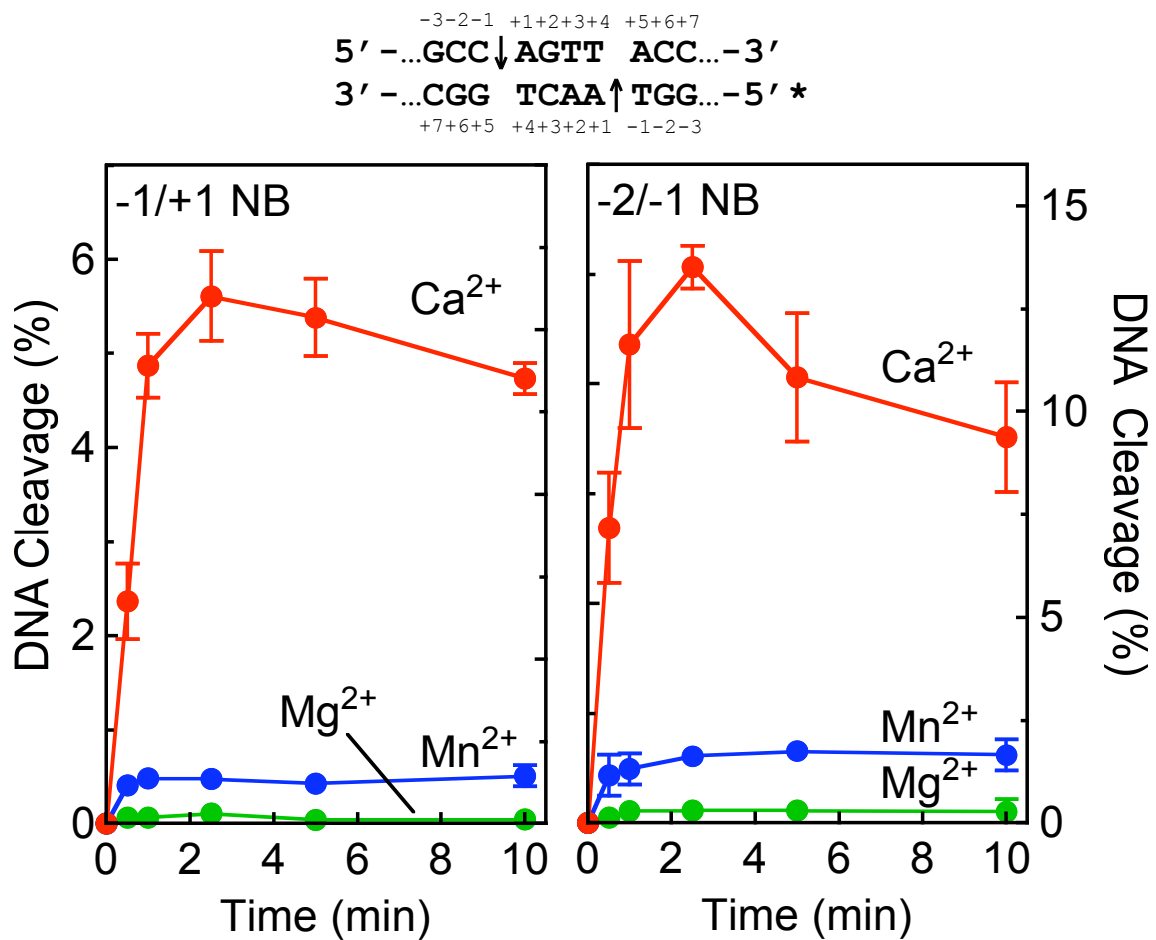


FIGURE 14. Cleavage of oligonucleotide substrates by topoisomerase IV in the presence of different divalent metal ions. The central portion of the DNA cleavage substrate is depicted above the graphs. The asterisk denotes the 5'-end-labeled strand and the scissile bonds are indicated by arrows. All modifications are on the bottom strand. Results for the non-bridging phosphorothioate at the scissile bond (-1/+1 NB, left), and a non-bridging phosphorothioate at the -2/-1 position (-2/-1 NB, right) are shown. DNA cleavage was carried out in the presence of 10 mM Mg²⁺ (green), Mn²⁺ (blue), or Ca²⁺ (red). Error bars represent the standard deviation of three independent experiments.

recent structural study of the covalent yeast topoisomerase II-cleaved DNA complex (150). In this structure, both metal ion sites on each enzyme protomer are filled simultaneously.

Noble and Maxwell found that mutant gyrase proteins with substitutions at residues believed to complex with metal ions displayed greater DNA cleavage in the presence of Mg^{2+} and Ca^{2+} than did either divalent cation alone (143). Based on these findings, they proposed that the prokaryotic type II enzyme utilized a two-metal-ion mechanism.

This conclusion was challenged by a recent crystallographic study that examined non-covalent complexes between *Staphylococcus aureus* gyrase, DNA, and inhibitors (153). Although structures were generated that contained a metal ion at site A or B, none were observed that included metal ions at both sites. Based on these findings with gyrase, the authors proposed that type II topoisomerases utilize a moving metal ion mechanism in which only one of the two metal ion sites in each protomer is filled at any given time (153,155,156). In this model, metal ion binding to site B promotes DNA binding, albeit in a conformation that does not support efficient DNA cleavage. Once this conformation has been achieved, the first metal ion dissociates and is replaced by a second metal ion bound to site A, which promotes efficient DNA cleavage.

Recently, two structures of the covalent *Streptococcus pneumoniae* topoisomerase IV-cleaved DNA complex were reported. One of the structures contained no metal ions (151), while the other contained a single Mg^{2+} (consistent with metal ion A) at each active site (154). Although the authors “[could not] exclude the participation of a more weakly

bound Mg^{2+} ,” they also suggested the possibility that topoisomerase IV might utilize a single dynamic metal ion for DNA cleavage (154).

An important issue should be noted regarding the proposed moving (or dynamic) metal ion mechanism: the affinity of metal ion B, which would be the first to bind the enzyme in this model, is ~10-fold lower than that of metal ion A, which is the cation that participates directly in the chemistry of the cleavage reaction (147-149).

Two independent approaches were used to determine whether bacterial type II topoisomerases utilize a two-metal-ion or a moving metal ion mechanism for DNA cleavage. In the first, the concentration dependence of divalent metal ions for DNA cleavage mediated by *E. coli* topoisomerase IV was assessed. Initial studies utilized end-labeled linear pBR322 as the cleavage substrate. Several striking features were seen (Figure 15). The most obvious is that no DNA scission was observed at low concentrations of Mg^{2+} , Mn^{2+} , or Ca^{2+} . For each of the three metal ions, cleavage products were generated only after a threshold concentration had been reached (~0.05-0.25 mM, depending on the metal ion), and began to accumulate coordinately for all observable DNA bands. Furthermore, when quantified, the metal ion dependence of DNA cleavage appeared to be sigmoidal in shape (Figure 16).

These data strongly suggest that each enzyme protomer contains two distinct sites for metal ion binding in the topoisomerase IV-DNA complex, and that both are critical for cleavage of each strand of the double helix. One metal ion binds to the complex with higher affinity (metal ion A) and saturates the site at levels that reflect the threshold concentration at which cleavage appears. The second (metal ion B) also is essential for DNA cleavage and associates with the topoisomerase IV-DNA complex with apparent

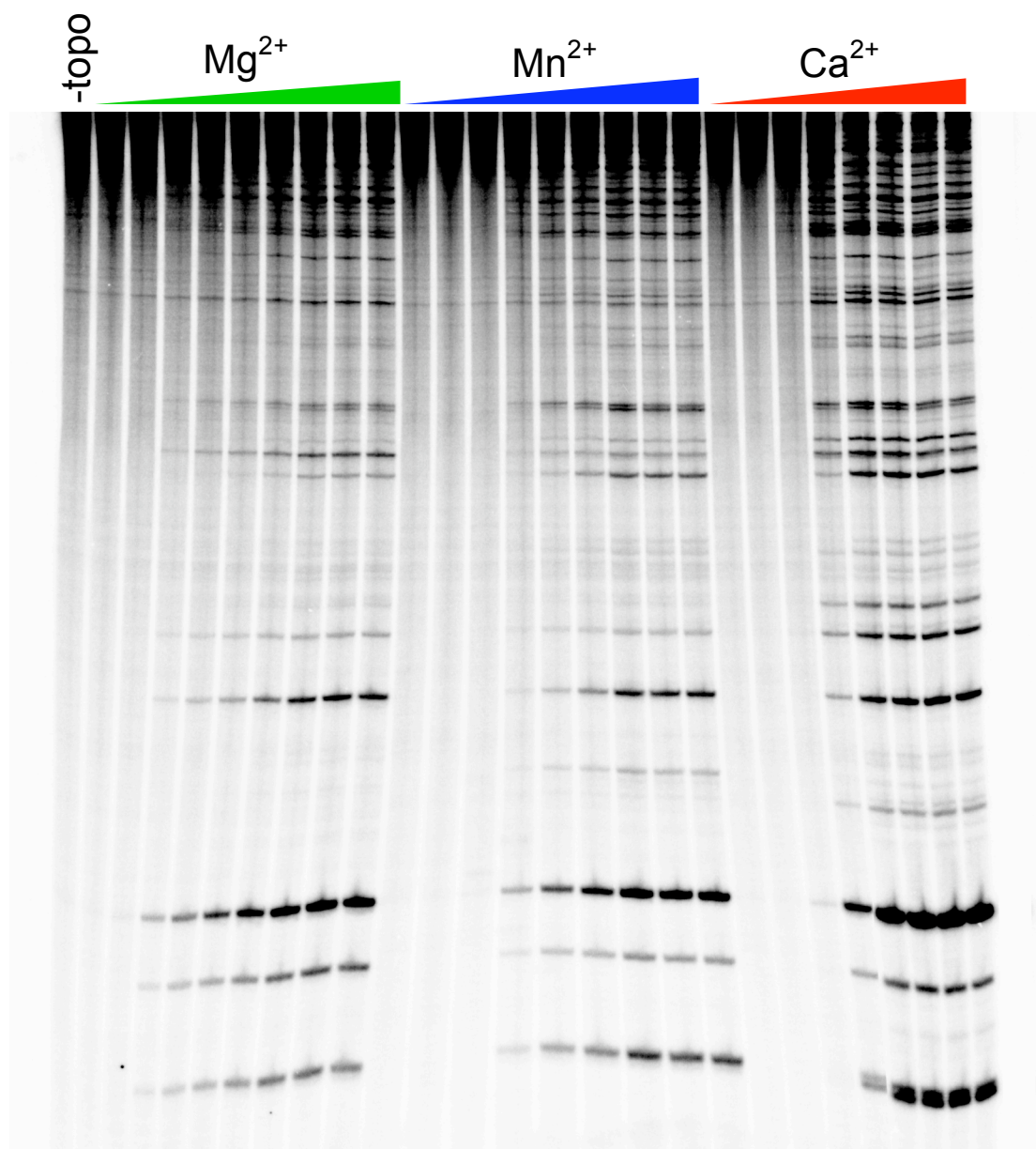


FIGURE 15. Metal ion concentration dependence for topoisomerase IV-mediated DNA cleavage of linearized pBR322 plasmid. A 4330 bp fragment of pBR322 was singly end-labeled and employed as the cleavage substrate. Reaction products were resolved by acrylamide gel electrophoresis. DNA cleavage in the presence of varying concentrations of Mg^{2+} , Mn^{2+} , or Ca^{2+} (left, center, or right, respectively) is shown. The gel is representative of three independent experiments.

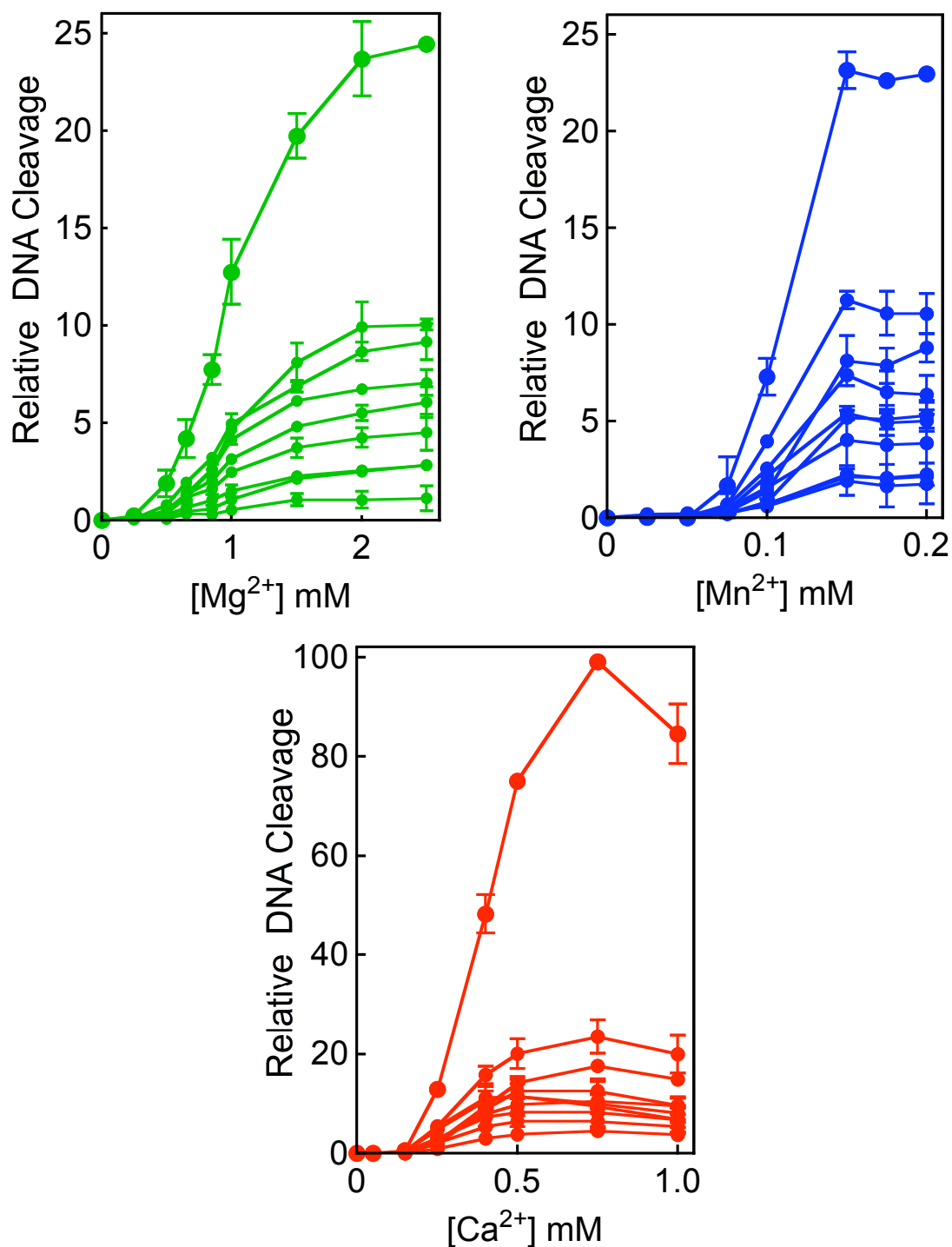


FIGURE 16. Metal ion concentration dependence for topoisomerase IV-mediated DNA cleavage of linearized pBR322 plasmid. Data from Figure 15 and additional experiments were quantified. Results for Mg²⁺ (top left), Mn²⁺ (top right), and Ca²⁺ (bottom) are shown. All values were quantified relative to the most abundant cleavage product (generated at 0.75 mM Ca²⁺), which was set to 100. Error bars represent the standard deviation of three independent experiments.

K_D values of ~1.0, 0.12, and 0.40 mM, respectively for Mg^{2+} , Mn^{2+} , and Ca^{2+} . It is not obvious how the concentration dependence of DNA cleavage seen in Figures 15 and 16 can be interpreted by a model in which both metal ion-binding sites are not filled simultaneously.

Results similar to those seen with the plasmid substrate also were observed using the oligonucleotide cleavage substrate described earlier. A biphasic concentration dependence for Ca^{2+} -supported cleavage of the wild-type substrate and for Mn^{2+} -supported cleavage of the phosphorothiolate substrate are shown (Figure 17). Once again, these findings suggest a model that requires two simultaneously bound metal ions to support DNA scission mediated by *E. coli* topoisomerase IV.

As a second approach to determine the number of metal ions required to support the DNA cleavage reaction of bacterial type II topoisomerases, metal ion mixing experiments were performed (143,147,148). Initial studies utilized the wild-type oligonucleotide substrate and mixtures of Mg^{2+} and Ca^{2+} . This metal ion pair was used because Mg^{2+} supports DNA cleavage of the wild-type substrate poorly, even at high concentrations, while Ca^{2+} efficiently supports high levels of scission. The first time courses for DNA cleavage were generated in the presence of either 10 mM Mg^{2+} , a saturating level, or 1 mM Ca^{2+} , a concentration that saturates the site for metal ion A without appreciably filling the site for metal ion B (see Figure 17). As seen in Figure 18, similar low levels of cleavage were generated when either one of the metal ions was included in assays. However, when both metal ions were present in reactions, rates of cleavage increased several-fold above levels predicted from the simple arithmetic sum of scission observed in the presence of the individual metal ions. Furthermore, a large

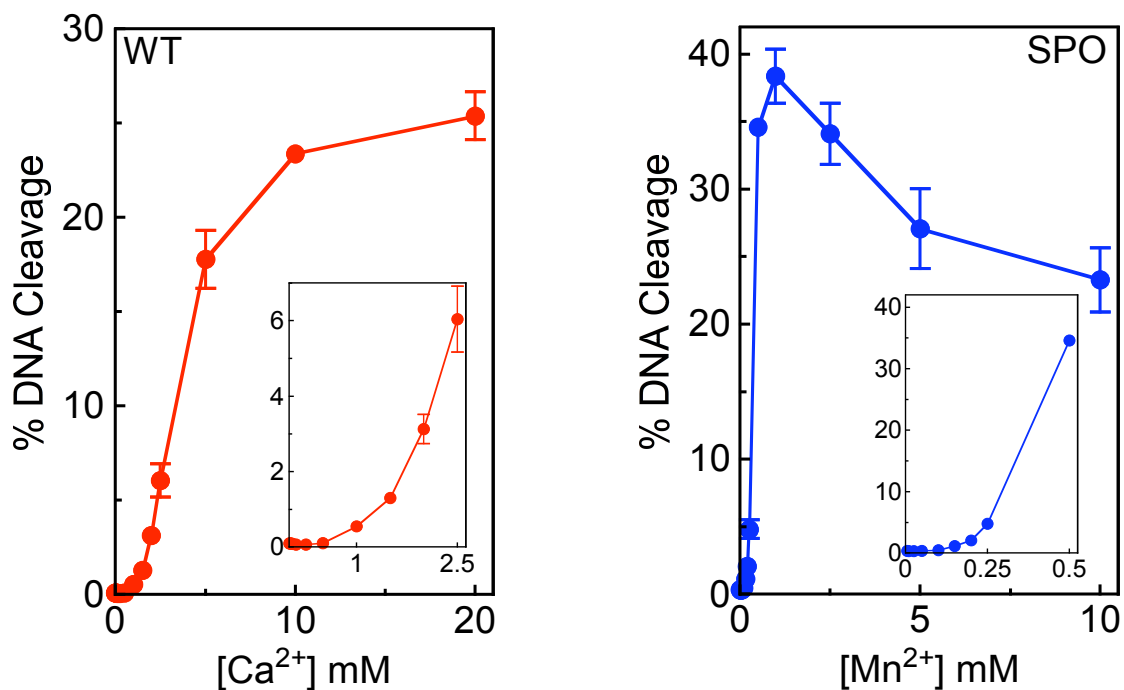


FIGURE 17. Metal ion concentration dependence for DNA cleavage by topoisomerase IV with wild-type (WT) and phosphorothiolate (SPO) oligonucleotide substrates. Left, cleavage of a wild-type oligonucleotide in the presence of 5 μ M to 20 mM Ca^{2+} . The inset shows concentrations up to 2.5 mM Ca^{2+} . Right, cleavage of an oligonucleotide containing a 3'-bridging phosphorothiolate in the presence of 5 μ M to 10 mM Mn^{2+} . The inset shows concentrations up to 0.5 mM Mn^{2+} . All DNA cleavage reactions were incubated for 10 min. Error bars represent the standard deviation of three independent experiments.

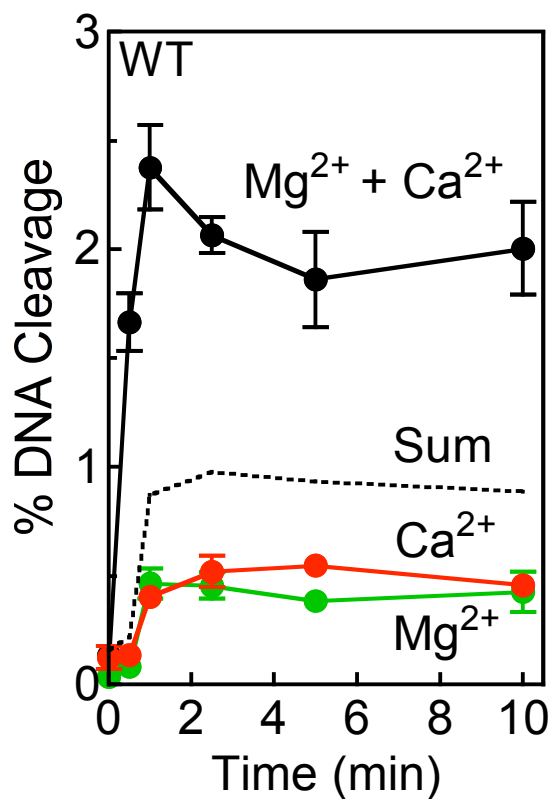


FIGURE 18. Cleavage of the wild-type (WT) oligonucleotide substrate in the simultaneous presence of two different divalent metal ions. Time courses were carried out in the presence of 10 mM Mg²⁺ alone (green), 1 mM Ca²⁺ alone (red), or a mixture of 10 mM Mg²⁺ and 1 mM Ca²⁺ (black). The arithmetic sum of the cleavage with Mg²⁺ or Ca²⁺ alone is represented by the dotted line. Error bars represent the standard deviation of three independent experiments.

enhancement in the rate and level of DNA scission (compared to calculated sums) was observed when 10 mM Mg^{2+} was combined with a range of Ca^{2+} concentrations (0.1–1 mM) that partially filled or saturated the high affinity metal ion site (Figure 19).

Even more dramatic results were observed when the oligonucleotide that contained a bridging phosphorothiolate was employed as the substrate. These experiments employed Ca^{2+} , which supports only low levels of cleavage of the sulfur-containing substrate, and Mn^{2+} , which supports the highest levels of topoisomerase IV-mediated scission of this substrate. The first experiment used 10 mM Ca^{2+} and 100 μM Mn^{2+} , which (as above) represents the concentration that fills only metal ion site A (see Figure 17). As seen in Figure 20, the initial rate of cleavage was ~200-fold faster and the final level of cleavage increased ~20-fold in reactions that contained both metal ions as compared to the arithmetic sum of cleavage observed in reactions that contained only one of the two individual metal ions. Once again, similar results were observed in reactions that combined 10 mM Ca^{2+} and 1–100 μM Mn^{2+} (Figure 21).

In the experiments discussed above, rates and levels of DNA cleavage were greatly enhanced by mixing low concentrations of a metal ion that effectively fills site A with saturating levels (*i.e.*, high enough to fill site B) of a second metal ion that cannot act effectively at site A. The most straightforward interpretation of the above findings is that the first (*i.e.*, low concentration) metal ion acts primarily at site A and the second (*i.e.*, saturating) metal ion acts primarily at site B. Taken together, these results argue for the simultaneous use of two metal ions during the DNA cleavage reaction mediated by bacterial topoisomerase IV and provide strong evidence for a two-metal-ion mechanism for DNA cleavage.

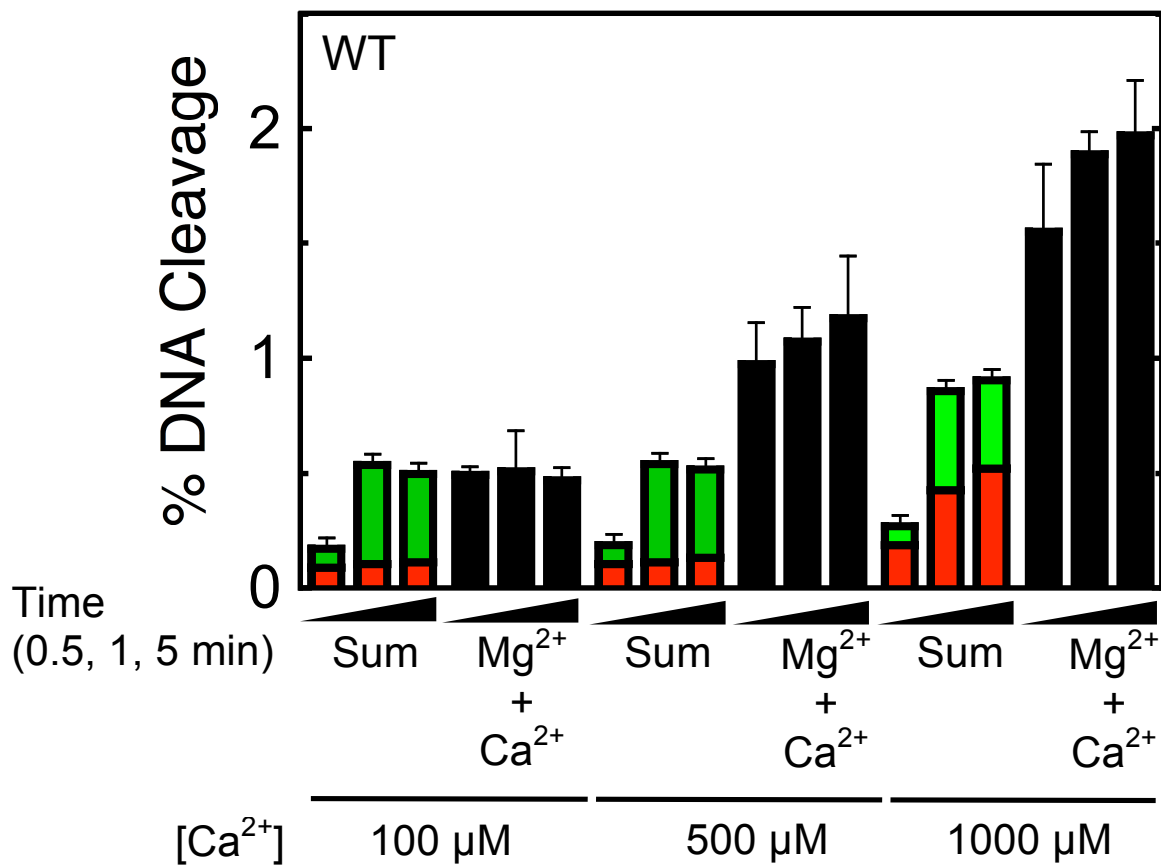


FIGURE 19. Topoisomerase IV-mediated cleavage of the wild-type (WT) oligonucleotide in the presence of different divalent metal ions. Cleavage reactions were carried out for 0.5, 1, or 5 min in the presence of 10 mM Mg²⁺ alone (green bars), 100-1000 μM Ca²⁺ alone (red bars), or a mixture of 10 mM Mg²⁺ and 100-1000 μM Ca²⁺ (black bars). The calculated sum of cleavage in the presence of Mg²⁺ or Ca²⁺ alone is shown (stacked green and red bars). Error bars represent the standard deviation of three independent experiments.

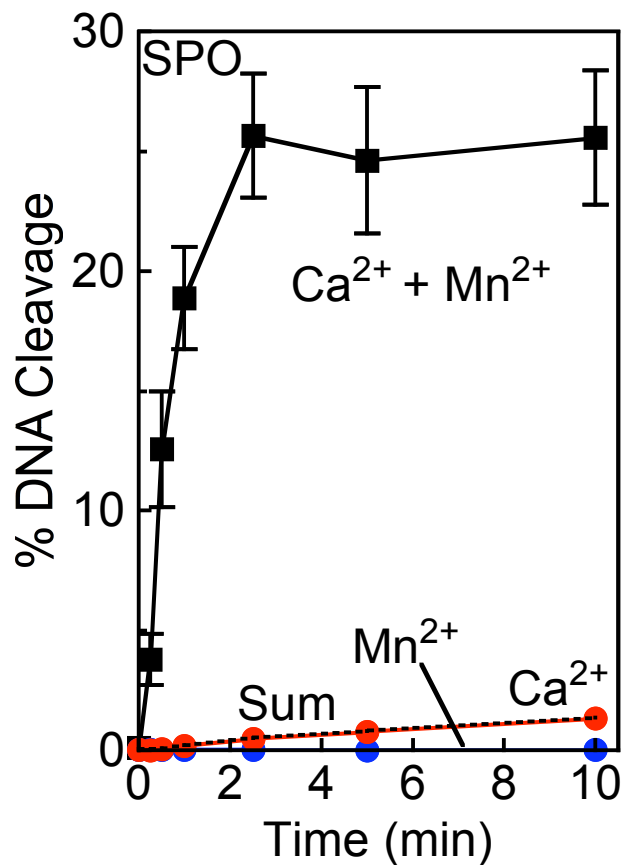


FIGURE 20. Cleavage of the 3'-bridging phosphorothiolate (SPO) oligonucleotide substrate in the simultaneous presence of two different divalent metal ions. Time courses were carried out in the presence of 10 mM Ca²⁺ alone (red), 100 μM Mn²⁺ alone (blue), or a mixture of 10 mM Ca²⁺ and 100 μM Mn²⁺ (black). The arithmetic sum of the cleavage with Ca²⁺ or Mn²⁺ alone is represented by the dotted line. Error bars represent the standard deviation of three independent experiments.

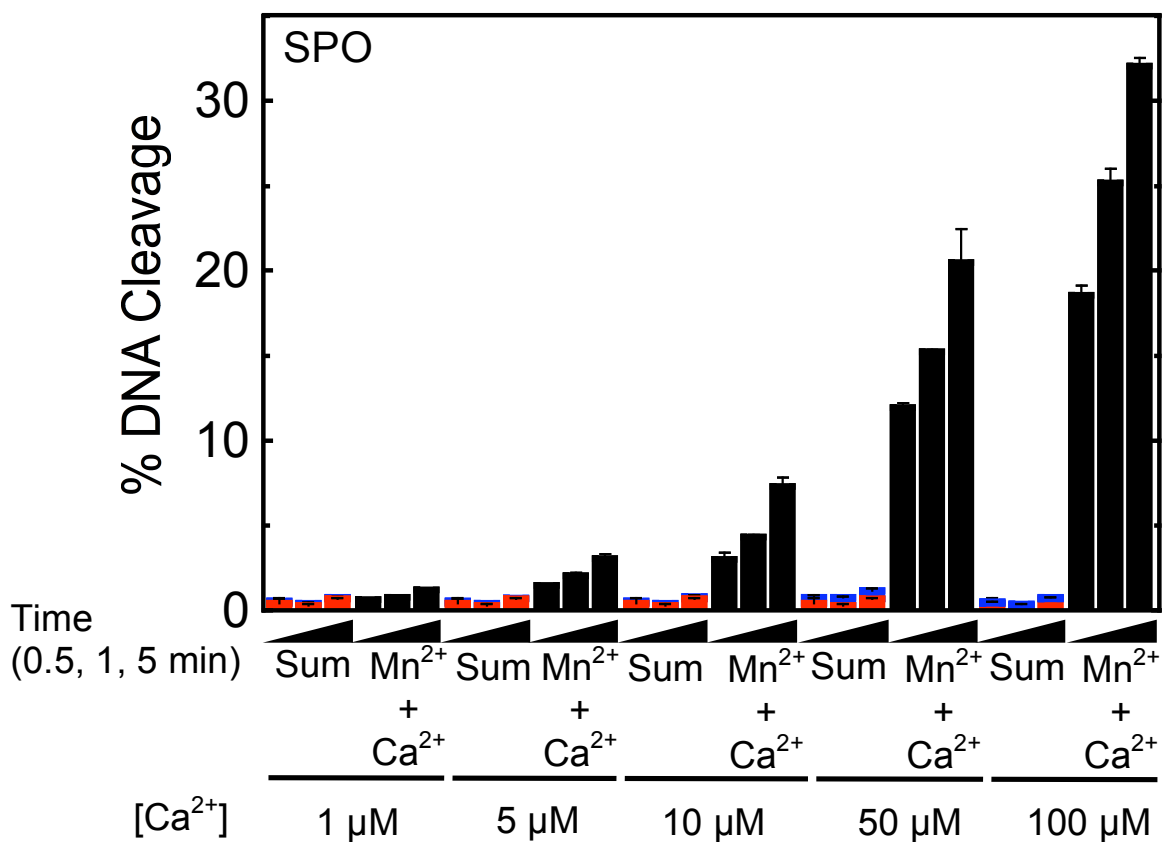


FIGURE 21. Topoisomerase IV-mediated cleavage of the 3'-bridging phosphorothiolate (SPO) oligonucleotide in the presence of different divalent metal ions. Cleavage reactions were carried out for 0.5, 1, or 5 min in the presence of 10 mM Ca^{2+} alone (red), 1-100 μM Mn^{2+} alone (blue), or a mixture of 10 mM Ca^{2+} and 1-100 μM Mn^{2+} (black bars). The calculated sum of cleavage in the presence of Mg^{2+} or Ca^{2+} alone is shown (stacked red and blue bars). Error bars represent the standard deviation of three independent experiments.

Although the importance of metal ion binding to sites A and B is clear, specific relationships between the two sites have yet to be delineated. An intriguing question is whether the metal ion in site B on one ParC subunit might work in concert with the metal ion in site A on the other ParC subunit to promote DNA scission (153). Indeed, there is precedent for trans cooperation between residues on opposite protomers of yeast topoisomerase II in the catalysis of DNA cleavage (168,169). At the present time, we cannot distinguish between a model in which site A and B cooperate in cis or trans.

Although type II topoisomerases require divalent metal ions in order to cleave the double helix, the role of these cations in promoting DNA scission by the bacterial type II enzymes has been controversial. Based on the findings discussed above, we propose that topoisomerase IV cleaves DNA using a two-metal-ion mechanism similar to that shown in Figure 9. In this model, metal ion A makes a critical interaction with the 3'-bridging atom of the scissile phosphate and facilitates DNA cleavage mediated by the bacterial type II enzyme.

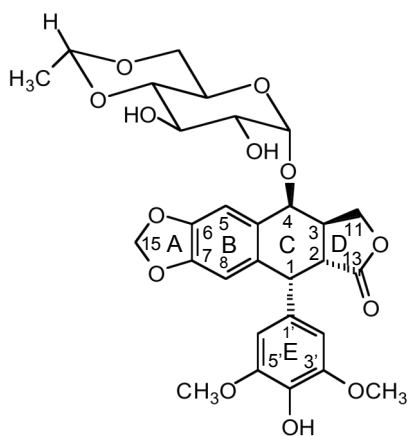
CHAPTER IV

CONTRIBUTIONS OF THE D-RING TO THE ACTIVITY OF ETOPOSIDE AGAINST HUMAN TOPOISOMERASE II α : POTENTIAL INTERACTIONS WITH DNA IN THE TERNARY ENZYME-DRUG-DNA COMPLEX

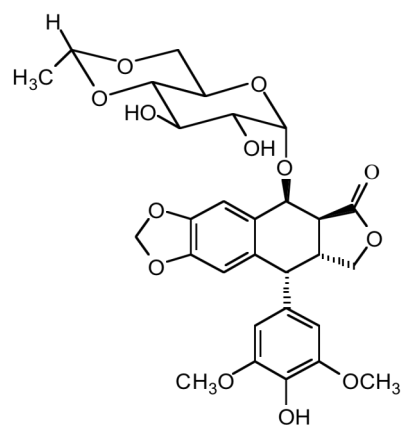
Introduction

Etoposide is a highly successful anticancer agent that has been used to treat a variety of blood-borne and solid human malignancies for nearly thirty years (21,108,111,112). Etoposide kills cells by stabilizing covalent topoisomerase II-cleaved DNA complexes that are formed during the DNA strand passage reaction of the enzyme (22,170-172). These transient cleavage complexes are converted to permanent DNA strand breaks by collisions with DNA tracking systems, which generate chromosomal aberrations, destabilize the genome, and trigger cell death pathways. Recent evidence suggests that topoisomerase II α may play a more prominent role in mediating the cytotoxic effects of topoisomerase II-targeted anticancer drugs.

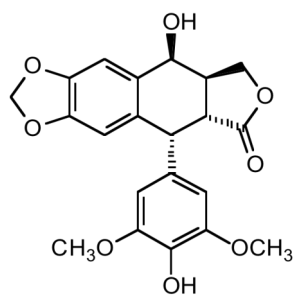
Previous studies indicate that interactions between topoisomerase II and etoposide are critical for drug activity and mediate the entry of etoposide into the ternary enzyme-drug-DNA complex (119,138,139,173,174). Therefore, substituents on etoposide that contact topoisomerase II in the binary enzyme-drug complex have been identified by STD ^1H NMR spectroscopy (138,139). These substituents include the C15 geminal protons of the A-ring, the C5 and C8 protons of the B-ring, the C2' and C6' protons of the pendant E-ring, and the 3'- and 5'-methoxyl protons of the E-ring (Figure 22). In contrast, no protein contacts have been observed for the C1 and C4 protons of the C-ring, the C2 and C3 protons that bridge the C- and D-rings, the C11 protons of the D-ring, and



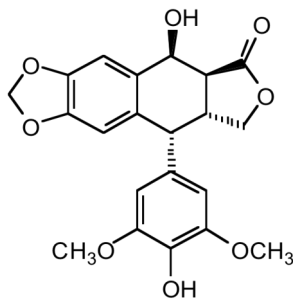
Etoposide



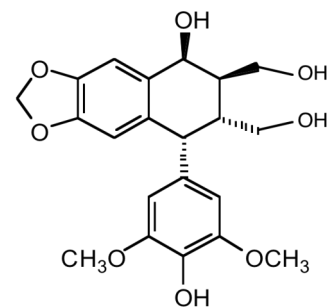
Retroetoposide



Demethylepipodophyllotoxin
(DEPT)



Retrodemethylepipodophyllotoxin
(retroDEPT)



D-Ring Diol

FIGURE 22: Structures of etoposide, retroetoposide, DEPT, retroDEPT and the D-ring diol.

any protons on the C4 glycosidic moiety. An identical set of drug contacts were seen in a binary complex between topoisomerase II α and DEPT, an etoposide derivative that lacks the C4 glycosidic group (Figure 22).

Protein-drug contacts in the binary complex have predictive value for the actions of etoposide within the ternary topoisomerase II-drug-DNA complex (138,139). Opening of the A-ring or altering the 3', 4', or 5' substituents of the E-ring dramatically decreases the efficacy of etoposide, but does not affect the specificity of DNA cleavage (138,139). Thus, it was concluded that etoposide interacts with topoisomerase II through the A-, B-, and E-rings. Conversely, the loss of the C4 glycosidic group in DEPT has relatively little effect on the ability of the drug to enhance topoisomerase II-mediated DNA scission, but does induce subtle changes in cleavage specificity and site utilization (139). Consequently, it has been suggested that the C4 glycoside, along with the D-ring, may mediate interactions with DNA in the cleavage complex (139).

Therefore, to explore potential interactions between the D-ring and DNA during topoisomerase II-mediated DNA cleavage, a series of etoposide derivatives, including retroetoposide, retroDEPT, and the D-ring diol were characterized. Results indicate that D-ring alterations diminish the ability of etoposide to enhance DNA cleavage mediated by human topoisomerase II α in vitro and in cultured human cells. They also decrease etoposide binding in the ternary enzyme-drug-DNA complex and alter sites of enzyme-mediated DNA cleavage. Based on these findings, it is concluded that the D-ring of etoposide has important interactions with DNA in the topoisomerase II cleavage complex.

Results and Discussion

Interactions between topoisomerase II and substituents on the A-ring, E-ring, and C4 position of etoposide in the binary enzyme-drug complex (identified by STD ^1H NMR spectroscopy) predict the ability of etoposide to stabilize the covalent topoisomerase II-DNA cleavage complex (138,139). However, retroetoposide and retroDEPT display reduced cytotoxicity (~8- and 32-fold, respectively, as compared to etoposide) against murine leukemia L1210 (129) despite the fact that NMR studies indicate that none of the protons on the D-ring of etoposide (*i.e.*, the C2, C3 and C11 protons) contact human topoisomerase II α in the binary complex (138,139). Although differences in drug cytotoxicity may reflect physiological attributes such as uptake or efflux, etc., this finding suggests that alterations in the D-ring of etoposide may affect drug activity towards topoisomerase II α in the ternary enzyme-drug-DNA complex.

Activity of etoposide D-ring derivatives against human topoisomerase II α

To address the role of the D-ring in etoposide function, the effects of retroetoposide, DEPT, retroDEPT, and the D-ring diol (see Figure 22 for structures) on DNA cleavage mediated by human topoisomerase II α were compared to that of etoposide (Figure 23). As reported previously (139), the activity of DEPT was ~80-90% that of the parent compound. In contrast, the ability of retroetoposide, retroDEPT, and the D-ring diol to stimulate enzyme-mediated DNA cleavage was reduced substantially. Levels of DNA scission observed in the presence of retroetoposide were ~2- to 3-fold lower than seen with etoposide. The activity of retroDEPT was similarly reduced as compared to DEPT and was lower than that of retroetoposide. Thus, the effects of the two changes in

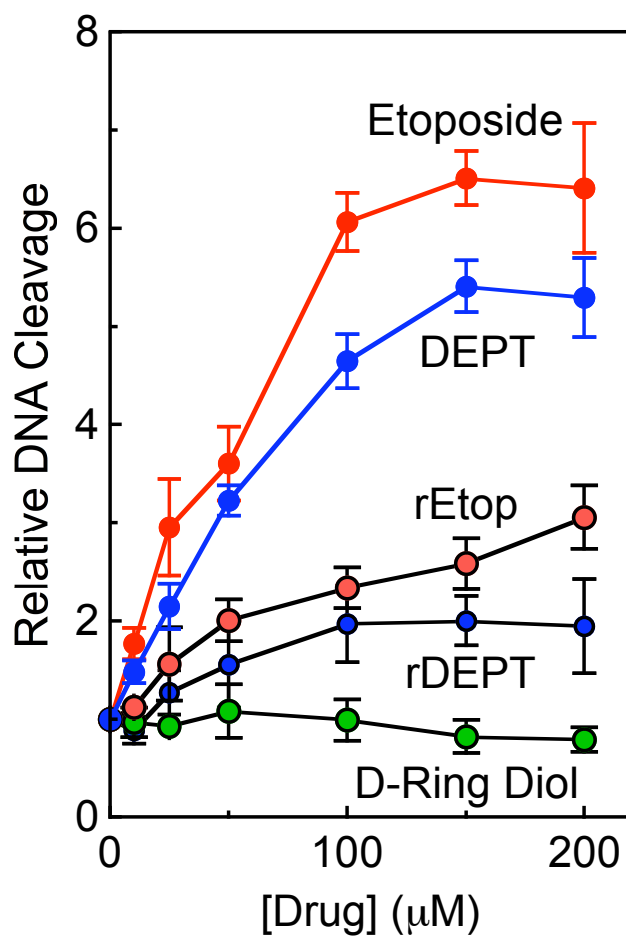


FIGURE 23: Effects of etoposide derivatives on DNA cleavage mediated by human topoisomerase $\text{II}\alpha$. Levels of double-stranded DNA cleavage were expressed as a fold-enhancement over reactions that were carried out in the absence of drug. Assay mixtures contained 0–200 μM etoposide (red), DEPT (blue), retroetoposide (rEtop, outlined red), retroDEPT (rDEPT, outlined blue), or D-ring diol (outlined green). Error bars represent the standard deviation of three independent experiments.

etoposide to generate retroDEPT (*i.e.*, the loss of the C4 glycoside and the transposition of the C13 ketone to the C11 position) appear to be additive. Finally, the D-ring diol displayed no ability to stimulate DNA cleavage mediated by topoisomerase II α .

The above results suggest that the decreased cytotoxicity of retroetoposide and retroDEPT are due, at least in part, to a decreased activity against topoisomerase II. Together with the findings for the D-ring diol, DNA cleavage results provide strong evidence that substituents on the D-ring of etoposide can profoundly affect the ability of the drug to act as a topoisomerase II poison.

It has been suggested that the diminished cellular activity of retroetoposide results from a deleterious interaction between the C4 glycoside and the C11 carbonyl group (129). However, since the decreased activity of retroDEPT compared to DEPT was similar to that for retroetoposide compared to etoposide, this explanation seems unlikely.

Etoposide increases levels of cleavage complexes by inhibiting the ability of topoisomerase II to ligate DNA (175,176). Therefore, the effects of the D-ring derivatives on DNA ligation mediated by topoisomerase II α were determined. As seen in Figure 24 (left panel), all of the compounds displayed a reduced activity compared to etoposide, with the order being the same as seen in DNA cleavage assays (etoposide > DEPT > retroetoposide > retroDEPT >> D-ring diol). Moreover, there was a strong correlation between the ability of the drugs to inhibit ligation and increase levels of DNA cleavage (Figure 25). These results indicate that the derivatives increase levels of topoisomerase II-mediated strand breaks by the same mechanism as that of the parent compound (*i.e.*, inhibition of DNA ligation).

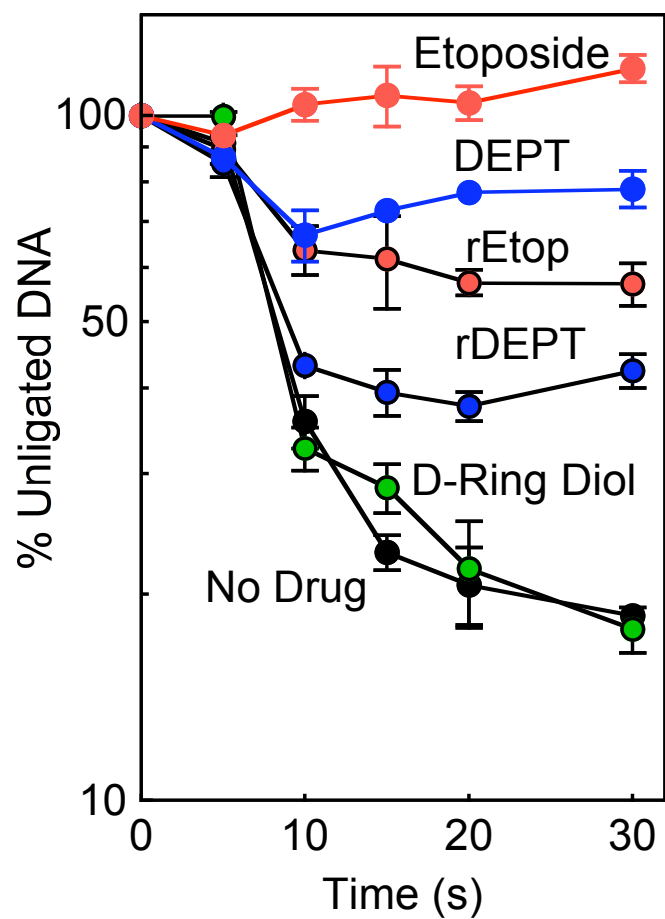


FIGURE 24: DNA ligation was examined in the absence of compound (No Drug, black) or in the presence of 100 μ M etoposide (red), DEPT (blue), retroetoposide (rEtop, outlined red), retroDEPT (rDEPT, outlined blue), or D-ring diol (outlined green). Error bars represent the standard deviation of three independent experiments.

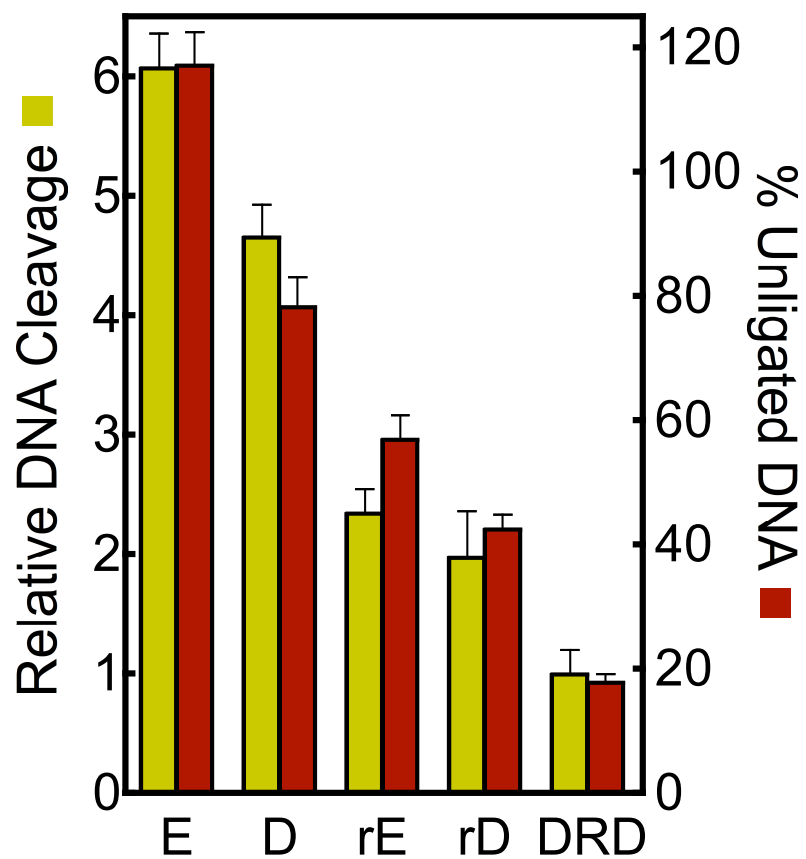


FIGURE 25: Comparison of DNA cleavage (dark yellow, 100 μ M drug) and ligation (dark red, 30 s) mediated by human topoisomerase II α in the presence of etoposide (E), DEPT (D), retroetoposide (rE), retroDEPT (rD), or D-ring diol (DRD). Error bars represent the standard deviation of three independent experiments.

As discussed above, retroetoposide and retroDEPT are cytotoxic to leukemia cells, but are less potent than etoposide (129). Therefore, to determine whether the reduced cytotoxicity of D-ring derivatives correlates with a decreased activity against topoisomerase II α , we assessed the ability of DEPT, retroetoposide, retroDEPT, and the D-ring diol to induce DNA cleavage by the type II enzyme in cultured human CEM leukemia cells. As seen in Figure 26), drug activity correlated with results of the in vitro DNA cleavage assays: DEPT, retroetoposide, and retroDEPT (in that order) induced reduced cellular levels of cleavage complexes compared to etoposide, and the D-ring diol displayed no activity. These findings strongly suggest that the cytotoxicity of retroetoposide and retroDEPT reflects drug activity against topoisomerase II α .

Interactions of etoposide D-ring derivatives with human topoisomerase II α

Although the C2, C3, and C11 protons associated with the D-ring of etoposide do not appear to interact with topoisomerase II α in the binary complex (138,139), alterations in the D-ring profoundly affect the ability of the drug to poison topoisomerase II α in vitro and in cultured human cells (Figures 23 and 26). This dichotomy suggests one of two things: either alterations in the D-ring affect contacts between etoposide and the enzyme in the binary complex, or this portion of etoposide has critical interactions in the ternary complex that are not reflected in the absence of DNA. To address these possibilities, interactions between topoisomerase II α and retroetoposide, retroDEPT, and the D-ring diol were identified by STD ^1H NMR spectroscopy.

Off-resonance and difference spectra for samples containing topoisomerase II α and retroetoposide, retroDEPT, and the D-ring diol are shown in Figure 27. Resonances

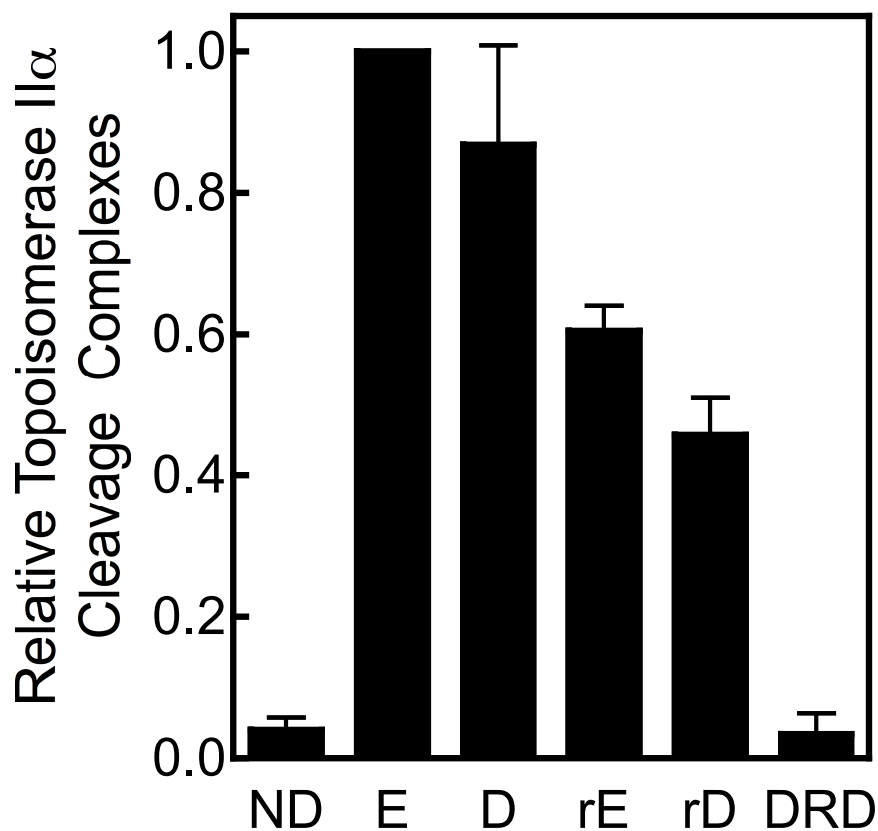


FIGURE 26: Levels of topoisomerase II α -DNA cleavage complexes formed in human CEM leukemia cells that were treated with etoposide derivatives. DNA samples (10 μ g) from cultures treated with no drug (ND) or 25 μ M etoposide (E), DEPT (D), retrotoposide (rE), retroDEPT (rD), or D-ring diol (DRD) for 1 h were blotted onto a nitrocellulose membrane and probed with a polyclonal antibody directed against human topoisomerase II α . Error bars represent the standard deviation of three independent experiments. A representative blot is shown above.

for all visible protons for these drugs, as well as etoposide and DEPT, are given in Table 1.

With the exception of one additional contact with the enzyme (the C4 proton of the C-ring), the substituents on retroetoposide and retroDEPT that interact with human topoisomerase II α in the binary complex were the same as those previously described for etoposide and DEPT (138,139). However, the nuclear Overhauser enhancement (NOE) signals seen for the E-ring protons of both drugs were substantially broader than found with etoposide in the binary complex. This finding suggests that movement of the C13 carbonyl group to the C11 position may alter the conformation around the E-ring of etoposide and affect interactions between this portion of the drug and the enzyme.

In contrast to retroetoposide and retroDEPT, the substituents on the D-ring diol that contact topoisomerase II α in the binary complex were identical to those of etoposide and no broadening of the E-ring proton resonances were observed. This result indicates that the loss of the D-ring does not alter drug interactions in the binary complex and implies that the lack of DNA cleavage enhancement induced by this derivative results from a specific difference in drug interactions within the ternary complex. Therefore, a competition assay was carried out to address this possibility. As seen in Figure 28, the D-ring diol was unable to displace 50 μ M etoposide from the ternary topoisomerase II α -DNA cleavage complex, even at concentrations that were 10-fold higher than that of the parent compound. Thus, despite the fact that the D-ring diol and etoposide display similar contacts with topoisomerase II α in the binary complex, their interactions in the ternary complex (at least with regard to strength) appear to differ. Because the difference between the binary and ternary complex is the presence of DNA, this finding suggests

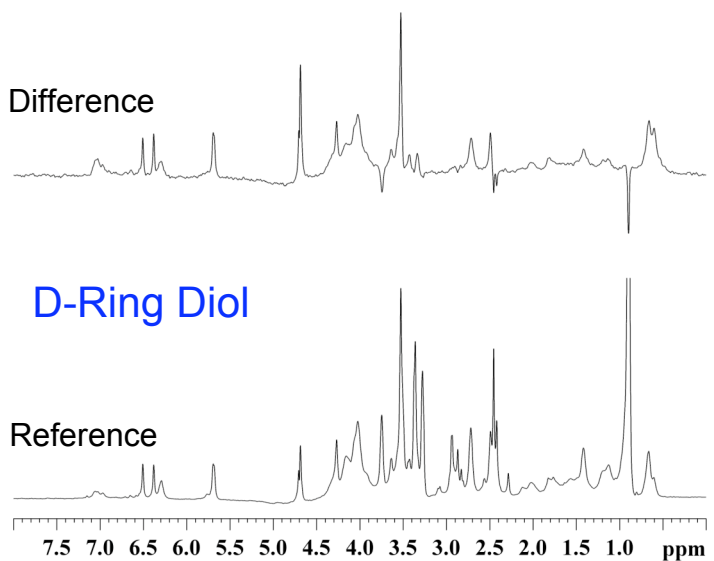
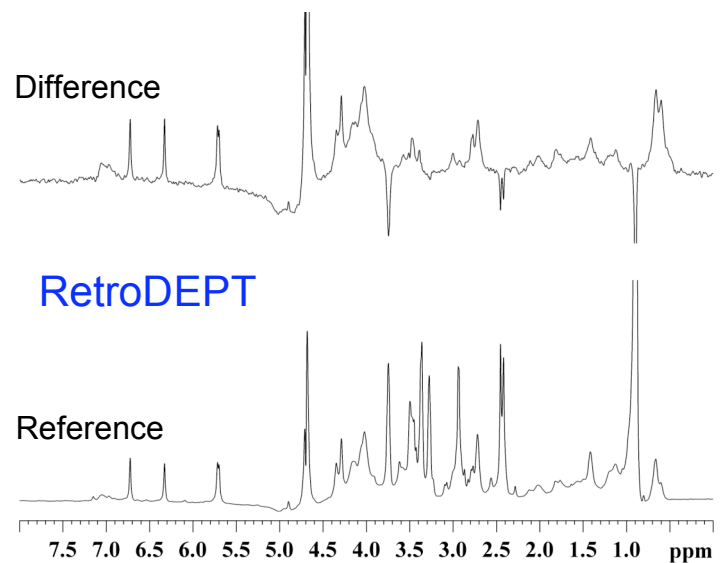
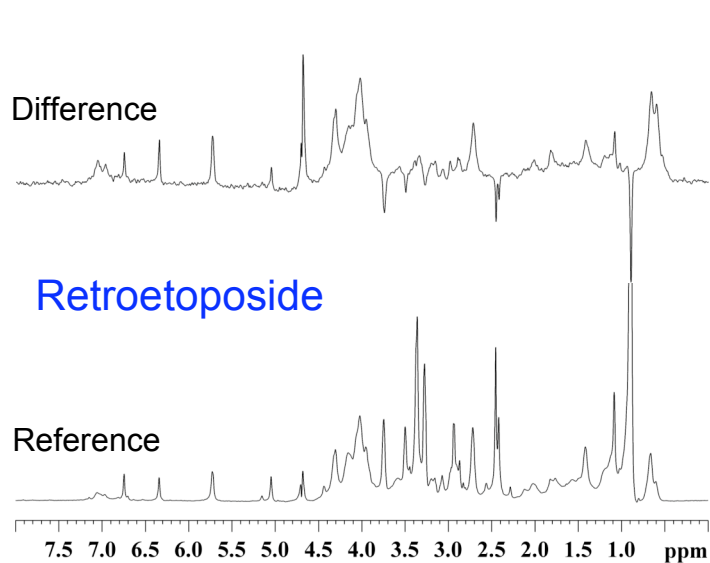


Figure 27: Interaction of retroetoposide (left top), retroDEPT (right top), and D-ring diol (bottom) with human topoisomerase II α as determined by STD ^1H NMR spectroscopy. Difference and reference (off resonance) spectra are shown for each drug. Spectra are representative of at least two independent experiments.

Table 1. Drug Substituents That Interact with Human Topoisomerase II α in the Binary Enzyme–Drug Complex As Determined by STD ^1H NMR Spectroscopy^a

etoposide		retroetoposide		DEPT		retroDEPT		D-ring diol	
substituent	resonance	substituent	resonance	substituent	resonance	substituent	resonance	substituent	resonance
1	4.39	1	4.31	1	4.38	1	4.26	1	4.28
2	3.33	2	3.21	2	3.10	2	3.21	2	2.50
3	2.83	3	2.88	3	2.81	3	2.76	3	2.5
4	4.82	4	5.07	4	4.77	4	4.84	4	4.59
5	6.70	5	6.75	5	6.70	5	6.70	5	6.53
8	6.32	8	6.33	8	6.28	8	6.30	8	6.32
11R, 11S	4.15, 4.20	13R, 13S	3.34, 4.34	11R, 11S	4.09, 4.25	13R, 13S	3.45, 4.32		
15R, 15S	5.70	15R, 15S	5.73	15R, 15S	5.67	15R, 15S	5.68	15R, 15S	5.66
2', 6'	6.18	2', 6'	6.15^b	2', 6'	6.11	2', 6'	6.07^b	2', 6'	6.26
3'-OCH₃, 5'-OCH₃	3.49	3'-OCH₃, 5'-OCH₃	3.49^b	3'-OCH₃, 5'-OCH₃	3.45	3'-OCH₃, 5'-OCH₃	3.59^b	3'-OCH₃, 5'-OCH₃	3.54
1''	4.43	3''	4.44					11-CH ₂	3.44, 3.65
2''	3.07	5''	3.00					13-CH ₂	3.35, 3.53
3''	3.33	6''	3.37						
4''	3.17	7''	3.17						
5''	3.24	9'', 12''	3.08						
6'', 6''	3.42, 4.01	10'', 11''	3.40, 3.97						
7''	4.70	14'', 16''	4.64						
-CH ₃	1.10	15''	1.10						

^a Resonances that display nuclear Overhauser effects in STD ^1H NMR spectroscopy experiments along with the substituent protons that they represent are indicated in bold and are underlined. ^b Resonances were substantially broadened.

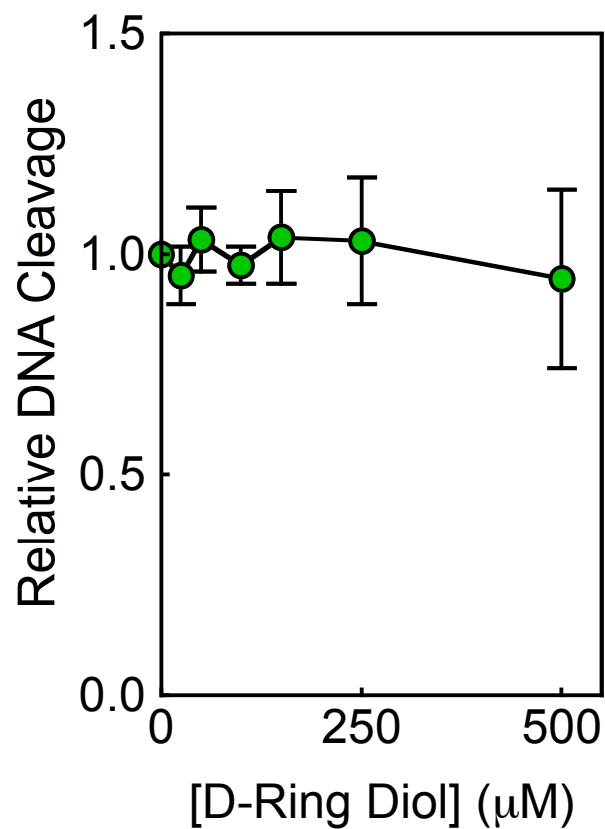


FIGURE 28: Competition between the D-ring diol and etoposide in the ternary complex. Topoisomerase II α -mediated DNA cleavage in the presence of 50 μ M etoposide and increasing concentrations (up to 500 μ M) of D-ring diol is shown as fold-enhancement over etoposide alone. Error bars represent the standard deviation of three independent experiments.

that the D-ring may have important interactions with the double helix in the covalent topoisomerase II α -drug-DNA cleavage complex.

Effects of D-ring substitutions on the specificity of topoisomerase II α -mediated DNA cleavage

A previous study demonstrated that removal of the C4 glycoside alters the specificity of etoposide-induced DNA cleavage by human topoisomerase II α (also shown in Figure 29) (139). Based on this finding, it was proposed that the sugar moiety of etoposide interacts with DNA in the cleavage complex. Because the etoposide D-ring does not appear to contact topoisomerase II α in the binary complex, but modification of this group significantly diminishes drug activity, sites of cleavage were mapped to determine if the D-ring also has the potential to interact with the double helix in the cleavage complex.

As seen in Figure 29, there were substantial differences in the cleavage specificity/site utilization of topoisomerase II α in the presence of etoposide *vs.* retroetoposide. Similar differences were seen when comparing cleavage maps generated in the presence of DEPT *vs.* retroDEPT. In addition, opening of the D-ring (D-ring diol) abolished all sites of drug-induced DNA cleavage. These results indicate that the specificity of etoposide-induced DNA cleavage is governed, at least in part, by the D-ring. Furthermore, results are consistent with the hypothesis that the D-ring contacts DNA in the topoisomerase II α cleavage complex. Because movement of the C13 carbonyl to the C11 position appears to affect the conformation of the etoposide E-ring, an alternative hypothesis is that D-ring alterations affect cleavage specificity by indirectly

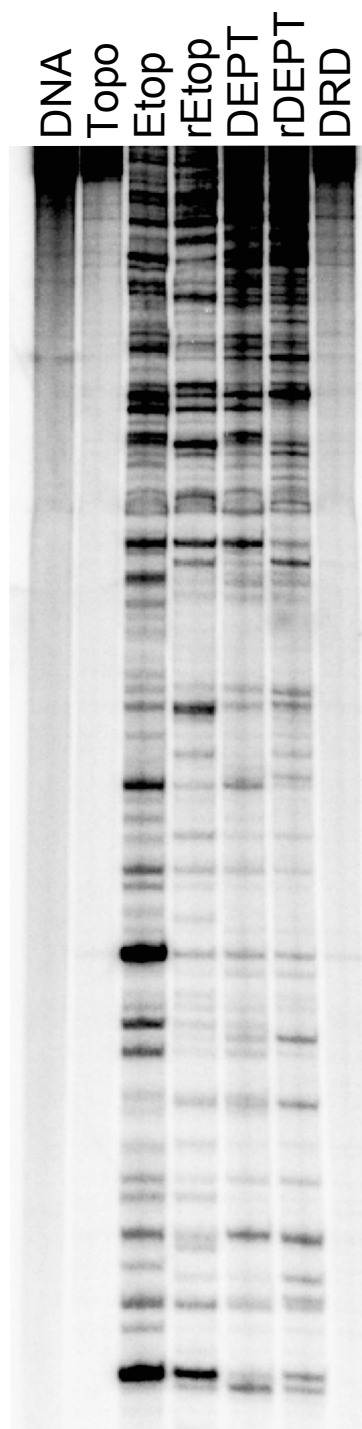


FIGURE 29: DNA cleavage site specificity and utilization by human topoisomerase II α in the presence of etoposide derivatives. A singly end-labeled linear 4332 bp fragment of pBR322 was used as the cleavage substrate. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions contained topoisomerase II α with no drug (Topo), 10 μ M etoposide (Etop), 250 μ M retroetoposide (rEtop), 25 μ M DEPT (DEPT), 250 μ M retroDEPT (rDEPT), or 250 μ M D-ring diol (DRD). A DNA control (DNA) also is shown. Data are representative of four independent experiments.

changing protein-drug contacts. However, two findings argue against this latter interpretation. First, the D-ring diol displays no ability to induce topoisomerase II α -mediated DNA cleavage, despite the fact that the protein-drug contacts in the binary complex are the same as those seen with etoposide. Second, a previous study found that derivatization of the etoposide E-ring significantly diminishes levels of drug-induced DNA cleavage, but has no effect on the site specificity of human topoisomerase II α (138).

To further examine the effects of D-ring derivatization on the cleavage specificity of the type II enzyme, the ability of etoposide and retroetoposide to induce topoisomerase II α -mediated DNA cleavage of an oligonucleotide substrate was compared. Etoposide displays specificity for the -1 base relative to the scissile bond and generally prefers to cleave substrates with a C at this position (46,177). With the oligonucleotide used for cleavage experiments, etoposide preferentially induced cleavage when the substrate contained a -1 C or A (Figure 30). Scission levels dropped ~2-fold when a T or G was present at this position.

Compared to etoposide, retroetoposide displays a decreased specificity for a -1 C or A (Figure 30). In fact, levels of topoisomerase II α -mediated DNA scission with the -1 G substrate were comparable to those seen in the presence of C or A. This finding confirms that changes in the D-ring of etoposide alter the DNA cleavage specificity of the drug.

Although etoposide is in its fourth decade of clinical use, relationships between drug activity and interactions within the binary topoisomerase II-drug complex and the ternary topoisomerase II-drug-DNA complex have been addressed only recently. Based

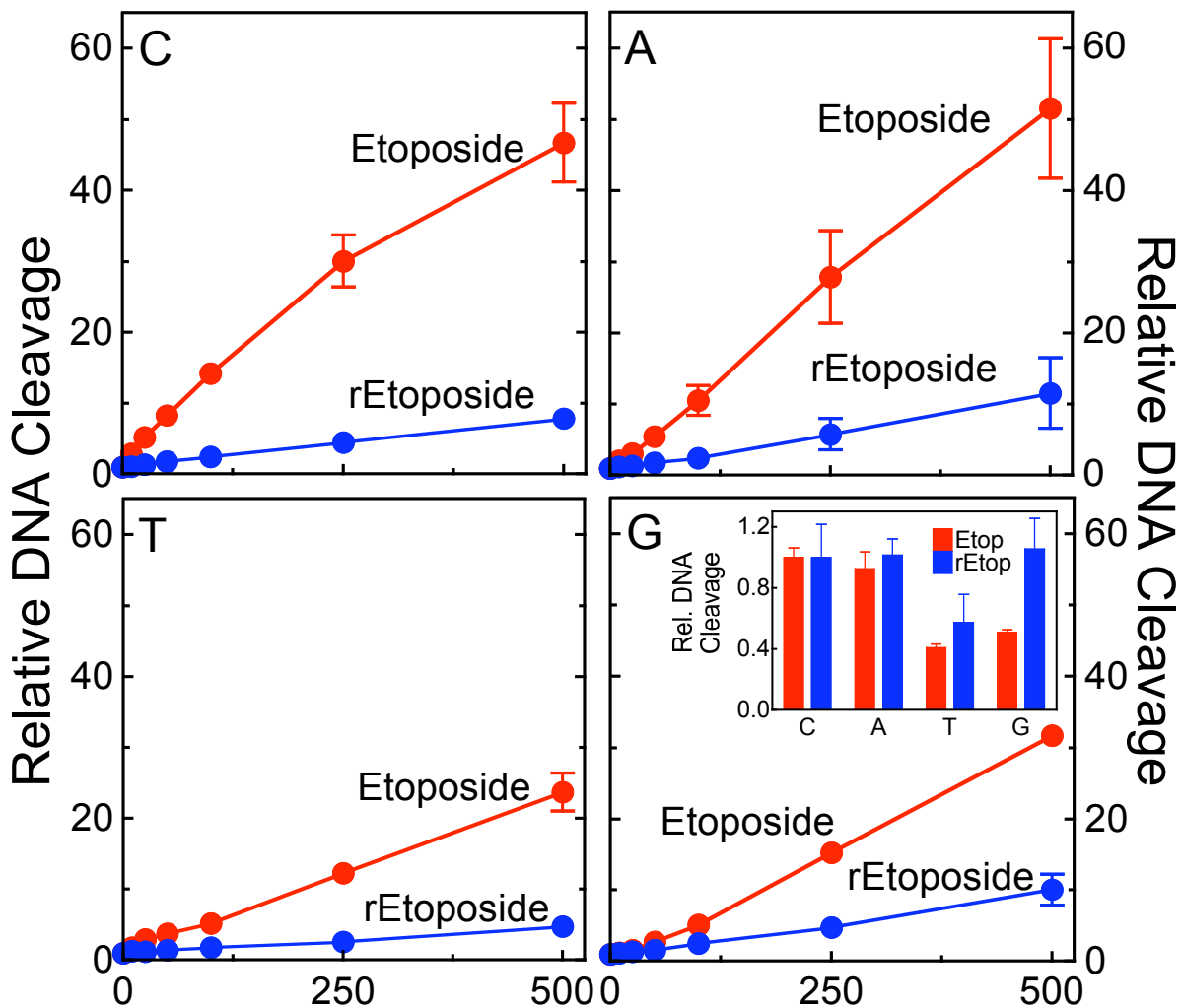


FIGURE 30: Oligonucleotide sequence specificity of etoposide (red) versus retroetoposide (blue). Drug titrations were performed using an oligonucleotide substrate that contained a strong cleavage site for human topoisomerase II α . The oligonucleotide was synthesized with either a C, A, T, or G at the -1 position relative to the scissile bond. The inset in the lower right panel compares DNA cleavage levels induced by 250 μ M etoposide (red bars) or retroetoposide (blue bars) relative to cleavage of the oligonucleotide containing a C at the -1 position, which was set at 1 for both drugs. Error bars represent the standard deviation of three independent experiments.

on the results of STD ^1H NMR spectroscopy and enzyme-drug binding in the binary complex, as well as drug competition and DNA cleavage in the ternary complex, a model has emerged (Figure 31). In this model, the binding of etoposide to human topoisomerase II α is driven by interactions with the A-ring, B-ring, and potentially by stacking interactions with the E-ring (138,139). The E-ring methoxyl groups and the 4'-OH moiety are important for drug function, but do not contribute substantially to enzyme-drug binding or DNA cleavage specificity (138,139).

Neither the C4 glycoside nor the D-ring of etoposide contact the enzyme in the binary complex (138,139). While removal of the sugar has little effect on drug activity, it subtly alters the specificity of DNA cleavage. In contrast, D-ring modifications profoundly affect the ability of etoposide to induce DNA scission and alter the cleavage specificity of topoisomerase II α . Taken together, we propose that interactions between etoposide and DNA in the ternary complex are driven primarily by the D-ring, with additional contributions from the C4 sugar. This hypothesis is supported by recent studies on F14512, an etoposide derivative that replaces the C4 glycoside with a spermine moiety (178-180). The presence of the spermine enhances DNA interactions and increases the potency of the drug against human type II topoisomerases (Figure 32) (178-180). Thus, by targeting the C4 moiety and the D-ring, it may be possible to develop novel etoposide derivatives with increased activity and/or altered cleavage specificity.

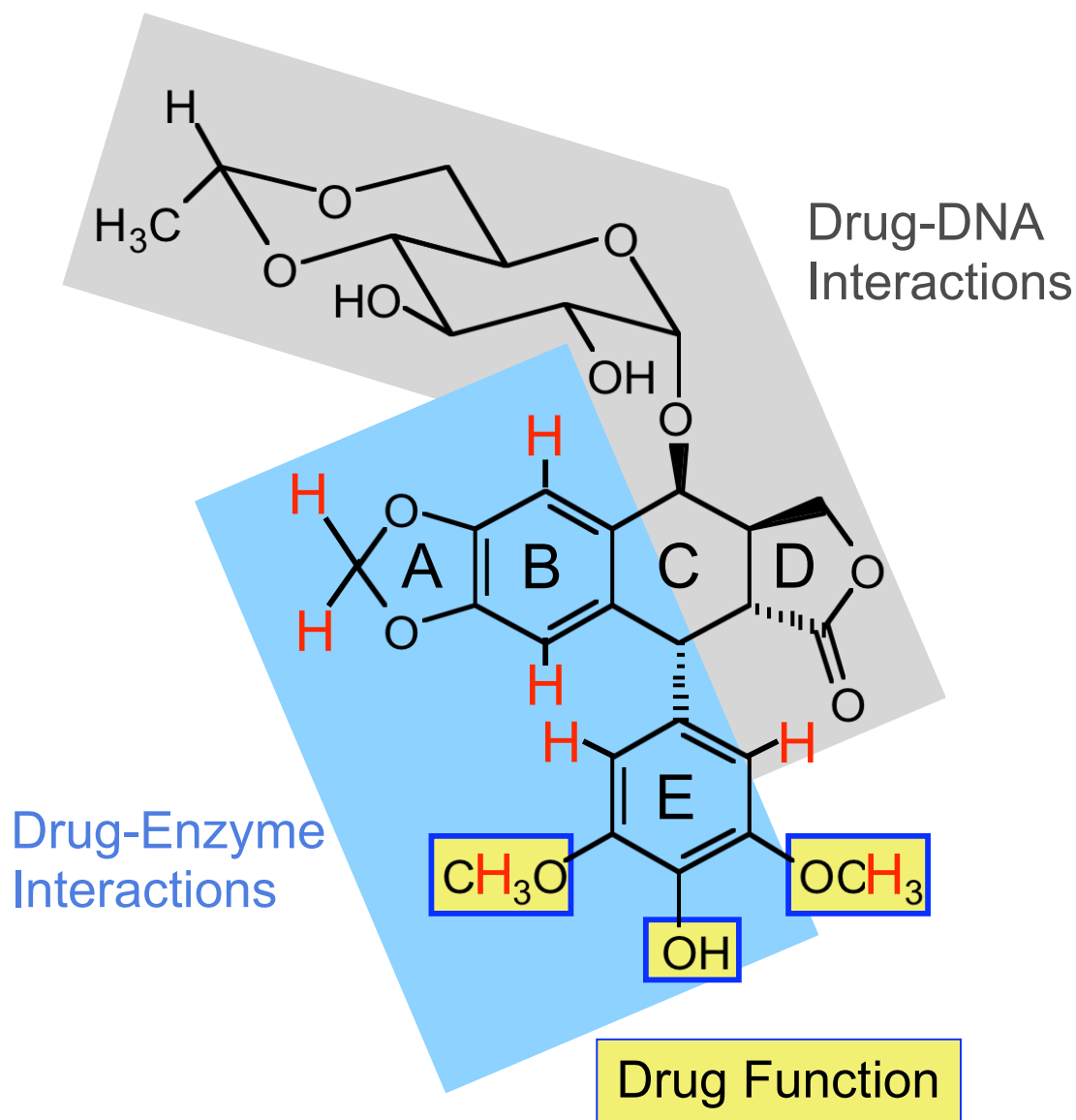


FIGURE 31: Summary of etoposide substituents that interact with human topoisomerase II α . Protons that interact with the enzyme (as determined by STD ^1H NMR spectroscopy) are shown in red. Interactions between hydroxyl protons and the enzyme were obscured by the water peak and could not be visualized. The blue region on etoposide, including portions of the A-, B- and E-rings, is proposed to interact with topoisomerase II α in the binary drug-enzyme complex. E-ring substituents highlighted with yellow boxes are important for drug function and interact with the enzyme, but do not appear to contribute significantly to binding. We propose that interactions between etoposide and DNA in the ternary complex (shaded in gray) are driven primarily by the D-ring, with additional contributions from the C4 sugar.

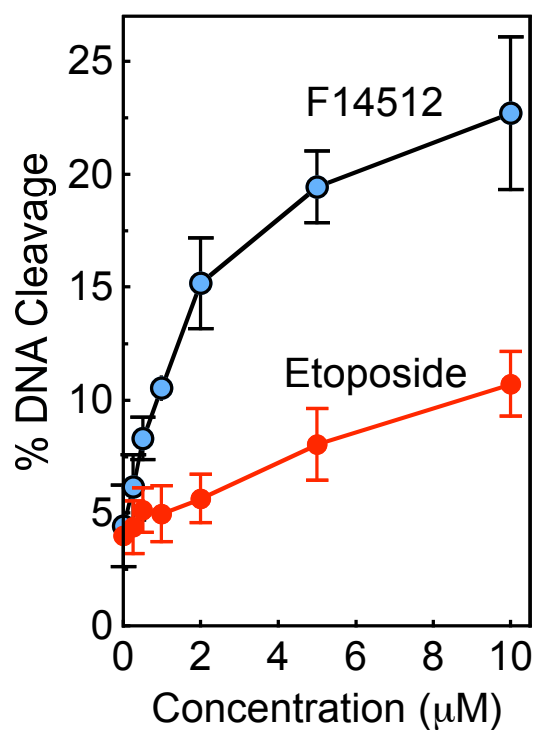
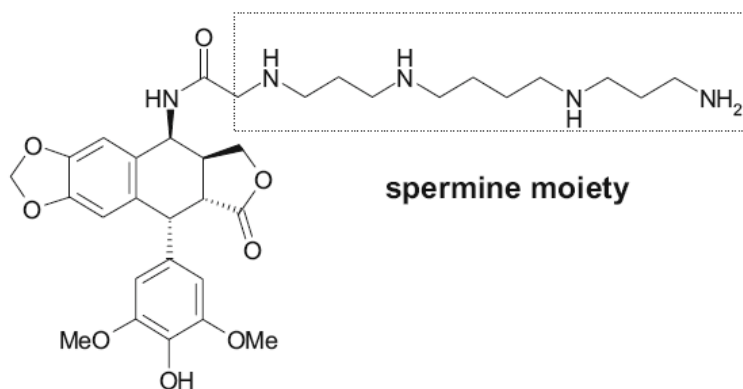


FIGURE 32: Effects of etoposide (red) and the etoposide derivative, F14512 (blue) on DNA cleavage mediated by human topoisomerase II α . Percent double-stranded DNA cleavage is shown. Assay mixtures contained 0–10 μ M drug and 72 nM topoisomerase II α . The structure of F14512 is shown above. Error bars represent the standard deviation of three independent experiments.

CHAPTER V

CONCLUSIONS

Topoisomerases are enzymes that regulate DNA topology and are essential to all known living organisms. Cellular processes such as replication, transcription, recombination, as well as chromosome condensation, decondensation, and segregation require topoisomerases. Type II topoisomerases are targets for antibacterial and anticancer therapeutic drugs.

Metal Ion Usage by Topoisomerase IV

Despite the importance of gyrase and topoisomerase IV in the regulation of DNA topology in bacteria and the treatment of infectious diseases, many aspects of the DNA cleavage reaction are poorly understood. To this point, an area of emerging interest is the use of divalent metal ions in catalyzing the DNA scission event.

The role of metal ions in the cleavage reaction mediated by bacterial type II enzymes has been controversial. Early evidence with gyrase indicated a two-metal-ion mechanism for topoisomerase type II DNA cleavage. This was subsequently supported by studies with the human enzyme. Recent crystallographic studies with prokaryotic type II enzymes have suggested that both metal ion sites might not be filled simultaneously. These authors proposed that type II topoisomerases might utilize a moving metal ion mechanism in which only one of the two metal ion sites in each protomer is filled at any given time.

To resolve this critical issue, the DNA cleavage reaction of *Escherichia coli* topoisomerase IV was characterized. We utilized a series of divalent metal ions with varying thiophilicities in conjunction with oligonucleotides that replaced bridging and non-bridging oxygen atoms at (and near) the scissile bond with sulfur atoms. DNA scission was enhanced when thiophilic metal ions were used with substrates that contained bridging sulfur atoms. This suggests that a metal ion is making a critical interaction with the 3'-bridging atom of the scissile phosphate and facilitates DNA scission by the bacterial type II enzyme. This evidence addresses metal ion interactions within the cleavage complex but not the number of metal ions.

The metal-ion dependence of topoisomerase IV-mediated DNA cleavage is sigmoidal in nature. This suggests that one metal ion binds to and saturates the higher affinity metal binding site followed by a second metal ion binding at the lower affinity site. Metal ion mixing experiments further supported the simultaneous two-metal-ion model.

It is clear that biochemical studies are needed to fully understand the role of metal ions in type II topoisomerases. These enzymes are dynamic and difficult to crystallize. The addition of DNA, divalent cations, and drugs makes structural studies all the more difficult. While there is general agreement between biochemical and structural data on the positions of the metal ion binding sites, there are questions about the temporal occupation of these sites.

Ultimately, structures of type II topoisomerase-DNA complexes have been generated with no metal ions, one metal ion (in one of two positions), or two metal ions. Because structural studies only show a static enzyme in one particular conformation, in one

specific set of conditions, it has been difficult to generate a coherent model from crystallographic studies alone.

Understanding the interactions between DNA, enzyme, and metal ions in the active site of topoisomerases will lead to a greater ability to design drugs that exploit the dynamics of the cleavage complex. Compounds could be designed or altered to specifically bind DNA in the cleavage complex or take advantage of a particular cleavage complex intermediate. Type II topoisomerases have several moving parts and added variables such as DNA and metal ions impart many spatial and temporal opportunities to take advantage of the enzyme in the search for treatments and cures for diseases.

Interactions of Etoposide with DNA in the Topoisomerase II α Cleavage Complex

Etoposide is has been used to treat various human malignancies for nearly thirty years. The drug kills cells by stabilizing covalent topoisomerase II-cleaved DNA complexes that are then converted to permanent DNA strand breaks by collisions with DNA tracking systems. These strand breaks generate chromosomal aberrations and also trigger apoptosis.

Previous studies identified groups on etoposide that interact with topoisomerase II α . The D-ring and C4 glycosidic moiety of etoposide did not interact with the enzyme in the binary enzyme-drug complex. To explore potential interactions between the D-ring and DNA during topoisomerase II-mediated DNA cleavage, a series of etoposide derivatives, including retroetoposide, retroDEPT, and the D-ring diol were characterized.

D-ring alterations diminish the ability of etoposide to enhance DNA cleavage mediated by human topoisomerase II α in vitro and in cultured human cells. This shows

that, while the D-ring does not appear to be interacting with topoisomerase II α in the binary complex, it is important for drug activity. Changes in the D-ring also decrease etoposide binding in the ternary enzyme-drug-DNA complex and alter sites of enzyme-mediated DNA cleavage. Based on these findings, it is concluded that the D-ring of etoposide has important interactions with DNA in the topoisomerase II cleavage complex.

To date, there are no topoisomerase II crystal structures that contain etoposide or any other anticancer drug. It is therefore not precisely known how etoposide interacts with the topoisomerase II cleavage complex. Understanding the orientation, position, and important substituents of etoposide in the cleavage complex could lead to the design of drugs with greater efficacy and possibly more specificity. Recent studies that increase drug-DNA interactions by replacing the C-4 glycoside of etoposide with a spermine moiety show that greater understanding of the importance and interactions of etoposide substituents can lead to more efficacious drugs.

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