QUINONE METABOLITES OF ENVIRONMENTAL TOXINS POISON

TOPOISOMERASE IIα

By

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These five years of graduate school have come and passed. Sometimes it felt like the years were creeping along and other times I could not figure out where the time had gone. When I take a look back at myself, prior to this endeavor, I realize that I have grown, learned and matured. Although I have put forth a lot of effort in these years I know that I was not alone. I have had the fortune of knowing several people who have impacted my development as a person and as a scientist.

First I would like to thank my graduate school mentor Neil Osheroff. Neil was a great mentor that provided me with independence. He was never there looking over my shoulder to make sure I was doing what he wanted. He treated me like an adult from the onset and I appreciate that. Throughout my time at Vanderbilt Neil has continually challenged me to think about what my results really meant and to consider all possibilities. Without his guidance I would not have the knowledge base, experience and confidence to move forward with my career in science. I also would like to thank Neil for providing financial assistance during my graduate career.

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Larry Robertson and Dr. Gabriele Ludewig, he provided expertise and credibility in the field of PCB toxicity. Dr. Amy Ham supervised the mass spectrometry experiments performed and provided invaluable knowledge needed to interpret generated spectra. Dr. David Graves performed the previous NMR experiments that were the basis for my work with etoposide. Dr. Norma Dunlap and Dr. Paola Arimondo synthesized the podophyllotoxin derivatives used.

During my years working for Neil I have had the pleasure of working with friendly intelligent people. Jo Ann was my teacher when I first came into the lab. She demonstrated unparalleled patience in working with me. Jo Ann has also provided me with a resource for information regarding lab procedure. While my tenure in lab did not officially overlap with Dr. Amy Wilstermann we were bound by an unbreakable bond. I continued the work started by Amy, which entailed extracting NMR data from our collaborator. It took our collective efforts, but after five years of trying we finally secured the data and published the paper.

When I joined Neil's lab several students were there to show the ropes. Dr. Renier Velez-Cruz, my baymate, taught me infinite patience in the lab. Dr. Erin Baldwin, Dr. Jennifer Dickey, and Dr. A. Katie McClendon were the trio of women that were affectionately referred to as the Harem. We all had our fun, white water rafting, martini parties and the lot. R. Hunter Lindsey and I shared a similar laid-back smart-alec personality, it is unfortunate he only spent a year in the lab while I was there. To all of my former Osheroff labmates; best of luck in all of your ventures, although I do not think that you will need it. This leaves me with the younger members of the lab; Omari Bandele, Joseph Deweese, Steven Pitts and Amanda Gentry. Unfortunately most of you came to know me when I was in my embittered years of my graduate career. However, despite my lack of

spirit, it has been a pleasure to work with all of you. Steven, having you as my baymate for the past year has been a welcomed change of pace.

Although IGP groups tend to dissipate over time, I have felt that our incoming class was one of the closest classes to come through this program. We made it through our first year by indulging in Chili's happy hour and Thursdays at Bailey's. Even though we (Alex, Erin, Shannon, Mel, Nicole, Nikki, and Chris) were dubbed the "dirty people" we had the best times. All the others were just jealous. After my IGP year I joined the department of biochemistry with four of my IGP classmates; Cornelia Crooke, Heather Ball, Tiffany Ellis and Charles Mobley. It was a great comfort having you around during stressful times knowing that you going through the same things, especially pre-lims. It has been fun but our time here is ending. I know that you will be successful regardless of the path you choose. I will miss nerd night.

Outside of my IGP class I had the fortune of knowing several great people. Shannon and Carolene were my primary company after IGP and before pre-lims. Shannon and Carolene departed Nashville to pursue other interests much sooner than I, I miss the times we had together. Kim, Michelle, Mark and Lane are a group of great people that I also had the pleasure of knowing. Gatlinberg was a blast. Brian and Ron were two great guys that I came to know in my latter years in Nashville but whom have had many great times with.

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Finally, I want to thank my family. My mom and dad have been a great source of support and encouragement throughout my graduate career and my

life. My brother and sister have also provided me with an invaluable basis of support. Without my family this accomplishment would be significantly more difficult if not impossible.

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2'Cl-2,5pQ	2-(2-Chloro-phenyl)-[1,4]benzoquinone
3'Cl-2,5pQ	2-(3-chloro-phenyl)-[1,4]benzoquinone
3',5'Cl-2,5pQ	2-(3,5-dichloro-phenyl)-[1,4]benzoquinone
4'Cl-2,5pQ	2-(4-chloro-phenyl)-[1,4]benzoquinone
4'Cl-2,5HQ	4'-Chloro-biphenyl-2,5-diol
4'Cl-2,3oQ	3-(4-Chloro-phenyl)-[1,2]benzoquinone
4'Cl-3,4oQ	4-(4-chloro-phenyl)-[1,2]benzoquinone
amsacrine	4'-(9-acridinylamino)methanesulfon-m-anisidide
APAP	acetaminophen
ATP	adenosine triphosphate
Bcr	breakpoint cluster region
BQ	1,4-benzoquinone
CID	collision induced dissociation
CP-115,953	6,8-difluoro-7-(4'-hydroxyphenyl)-1-cyclopropyl-4-quinolone-3-carboxylic acid
DEPT	4'-demethylepipodophyllotoxin
DDEPT	4'-demethylepipodophyllotoxin A–ring diol
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPT	epipodophyllotoxin
etoposide	4'demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucoside)

hTop2αC170A	human topoisomerase IIα with a cys->ala mutation at residue 170
hTop2αC216A	human topoisomerase IIα with a cys->ala mutation at residue 216
hTop2αC300A	human topoisomerase IIα with a cys->ala mutation at residue 300
hTop2αC392A	human topoisomerase IIα with a cys->ala mutation at residue 392
hTop2αC405A	human topoisomerase IIα with a cys->ala mutation at residue 405
hTop2αC455A	human topoisomerase IIα with a cys->ala mutation at residue 455
hTop2αC392/405A	human topoisomerase IIα with a cys->ala mutations at residues 392 and 405
hTop2αC170/392/405A	human topoisomerase IIα with a cys->ala mutations at residues 170, 392 and 405
ICE bioassay	*in vivo* complex of the enzyme assay
kb	kilobases
kDa	kilodaltons
LC-MS/MS	liquid chromatography-mass spectrometry/mass spectrometry
MLL	mixed lineage leukemia
NAPQI	N-acetyl *p*-benzoquinone imine
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulfate
TCA	sodium tricloroacetate
Tris	tris-(hydroxymethyl)aminomethane

Z-VAD-fmk   Z-Val-Ala-DL-Asp-fluoromethylketone

CHAPTER I

INTRODUCTION

Malignant cells are frequently distinguished by rapid growth coupled with an impaired ability to activate cell cycle checkpoints and DNA repair pathways (*2, 3*). Consequently, DNA in cancerous tissues often sustains elevated rates of replication and transcription, despite a decreased competence to restore genomic integrity following damage. This dual property of high DNA metabolism and low genetic stability makes the double helix an attractive target for cancer chemotherapy. Indeed, several classes of widely utilized anticancer drugs act by damaging DNA, either directly or indirectly (*4*).

Multiple therapeutic strategies are used to damage DNA. Ultimately, these strategies block DNA replication or other essential nucleic acid processes, generate mutations, create DNA strand breaks, or induce gross chromosomal abnormalities. For example, methotrexate, which inhibits dihydrofolate reductase, decreases cellular thymine pools and promotes incorporation of deoxyuridine into chromosomes (*5*). The unnatural nucleotide AraC is incorporated into the genetic material and impairs replication (*6*). Mechlorethamine (nitrogen mustard) (*7*) and cisplatin (*8*) alkylate bases or crosslink the two strands of the double helix. Finally, bleomycin (*9*) or etoposide (*10*) generate DNA strand breaks by a chemical mechanism or by altering the activity of topoisomerases, respectively.

Some of the most effective anticancer drugs currently in clinical use damage DNA by targeting topoisomerases (*4*). These agents act in an insidious manner

and convert topoisomerases from essential enzymes to potent cellular toxins that fragment the genome to induce apoptosis (*11-17*). However, if the level of DNA strand breaks induced by topoisomerase II poisons is not lethal, repair of these breaks generate chromosomal aberrations that lead to specific types of leukemia (*11-17*). In addition to leukemias resulting from clinical topoisomerase II poisons. Epidemiological evidence has linked the exposure to environmental poisons and leukemia (bioflavanoids and benzene) (*18-23*). A common feature in these leukemias is the presence of translocations at chromosome band 11q23 (*13, 14, 24-26*).

Bioflavanoids directly interact with topoisomerase II to enhance double-stranded breaks in the genetic material (*27, 28*). Benzene, however, is believed to act through a quinone metabolite (benzoquinone) to enhance enzyme-mediated DNA cleavage (*29-32*). Similar to benzene, exposure to other environmental toxins or drugs that are metabolized to quinones demonstrate clastogenic activity in the liver (*33-36*). While the mechanism behind this damage is unknown, it is reminiscent of the actions of topoisomerase II-poisons.

Although type II topoisomerases have proven to be effective cancer chemotherapy targets and have been linked to the development of cancer, they regulate chromatin structure and are involved in virtually every process of DNA metabolism.

DNA Topology and Topoisomerases

DNA is essentially an extremely long double-stranded rope in which the two strands are interwound about one another (*37, 38*) (Figure 1). As a result, the

Labels in figure:
- Nuclear membrane
- Chromatin fiber
- Chromatin fiber
- Histone and nonhistone proteins
- Condensed fiber (30 nm diam.)
- Nucleosome (11 nm diam.)
- DNA (2 nm diam.)
- Nuclear pore
- Nuclear matrix fibers
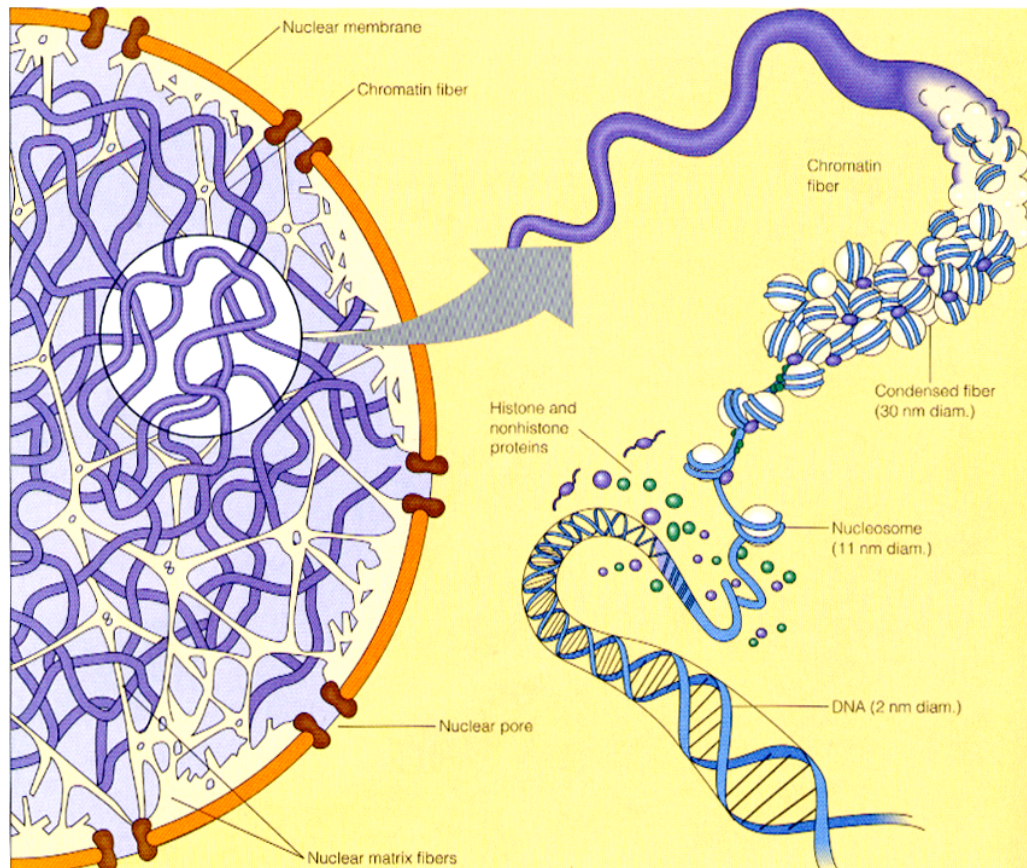
Figure 1. Model of DNA compaction inside of a cell nucleus. Stretched end to end, the DNA of a human cell measures approximately two meters in length and is condensed into a nucleus that is only five to ten microns in diameter. In addition to this compaction, DNA is composed of two anti-parallel inter-wound strands. Adapted from Ref. *(38)*.

topological properties of the genetic material profoundly influence virtually every major DNA process.

DNA is globally underwound (*i.e.*, negatively supercoiled) in all vertebrate species (*39-42*). This underwinding makes it easier to separate complementary DNA strands from one another and therefore greatly facilitates replication and transcription. Once the replication or transcription machinery begins to travel along the DNA template, however, deleterious effects of topology are manifested. Since helicases separate, but do not unwind the two strands of the double helix, fork progression results in acute overwinding (*i.e.*, positive supercoiling) of the DNA ahead of the tracking systems (*39, 41-43*). In contrast to underwinding, overwinding dramatically increases the difficulty of opening the double helix. Therefore, the accumulation of positive supercoils represents a formidable block to all DNA processes that require strand separation (*41, 43-46*).

The effects of DNA topology are further compounded by the extreme length of the double helix. The genetic material from a single human cell, which approaches two meters in length, exists in a nucleus that is only five to ten microns in diameter. Consequently, the double helix is subjected to the same forces and constraints as a room tightly packed from floor to ceiling with rope. Nuclear processes such as recombination and replication naturally generate knots and tangles in DNA, respectively (*47, 48*). If knots accumulate in the genome, DNA tracking systems are unable to separate the two strands of the double helix (*39, 42, 43, 47*). Moreover, if tangled (*i.e.*, catenated) daughter chromosomes are not separated prior to cell division, cells will die of mitotic failure (*16, 43, 49-51*).

The topological state of the double helix is regulated by ubiquitous enzymes known as topoisomerases, which act by creating transient breaks in the double helix (*15, 16, 41, 43, 49-54*). In order to maintain genomic integrity during this process, topoisomerases form covalent bonds between active site tyrosyl residues and the phosphate moieties of newly generated DNA termini (*15, 16, 41, 43, 49-54*). These covalent protein-cleaved DNA complexes are referred to as *cleavage complexes*. There are two classes of topoisomerases that are distinguished by the number of DNA strands that they cleave during their catalytic cycles.

Type I Topoisomerases

Type I topoisomerases act by generating a transient single-stranded break in the double helix followed by a single-stranded DNA passage event or controlled rotation about the break (*51, 54-56*) (Figure 2). As a result, these enzymes are able to alleviate torsional stress (*i.e.*, remove superhelical twists) in duplex DNA. Type I topoisomerases are involved in all DNA processes that involve tracking systems and play important roles in maintaining genomic integrity (*50, 54, 56, 57*).

Type II Topoisomerases

Mammalian type II topoisomerases are homodimeric enzymes that interconvert different topological forms of DNA by a double-stranded DNA passage mechanism (*43, 50, 51, 53, 57, 58*) (Figure 2). Briefly, these enzymes: 1) bind two separate segments of DNA, 2) create a double-stranded break in one of the segments, 3) translocate the second DNA segment through the cleaved nucleic acid "gate," 4) rejoin (*i.e.*, ligate) the cleaved DNA, and 5) release the translocated
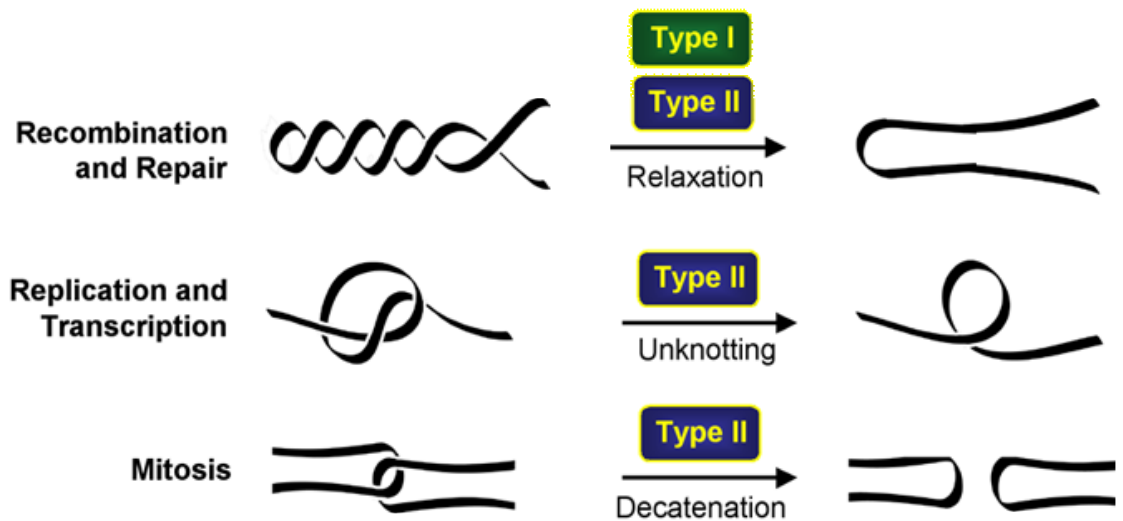
Figure 2. Topological problems associated with DNA metabolism. DNA is globally underwound in cells. During replication and transcription helicases are used to melt DNA ahead of tracking systems. Since helicases separate, but do not unwind the two strands of the double helix, fork progression results in acute overwinding (*i.e.*, positive supercoiling) of the DNA ahead of the tracking systems. This overwinding, if not resolved, can block the progression of the replication or transcriptional machinery. In addition to positive supercoiling, precatenanes form behind replication forks. If sister chromosomes are not decatedated, cells will die of mitotic failure. Finally, nuclear processes such as recombination and naturally generate knots and tangles in DNA, respectively. To resolve these topological problems associated with DNA, cells possess enzymes called topoisomerases.

segment through a gate generated at a C-terminal interface between the two protein subunits (*43, 50, 51, 53, 57-59*) (Figure 3). The two scissile bonds that are cut by type II topoisomerases are staggered and located across the major groove from one another (*43, 50, 51, 53, 57*). Thus, these enzymes generate cleaved DNA molecules that contain 4-base single-stranded cohesive ends at their 5′-termini (*60, 61*). During the cleavage event, all known type II topoisomerases covalently attach to the newly generated 5′-DNA termini.

Type II enzymes require two cofactors in order to carry out the catalytic double-stranded DNA passage reaction. First, they need a divalent cation for all steps beyond enzyme-DNA binding. Mg(II) appears to be used *in vivo* (*60-63*). Second, they use the energy of ATP to drive the overall DNA strand passage reaction (*64-67*).

As a result of their double-stranded DNA passage reaction, type II enzymes are able to remove superhelical twists from DNA and resolve knotted or tangled duplex molecules (*43, 50, 51, 53, 57*). They function in numerous DNA processes and are required for recombination, the separation of daughter chromosomes, and proper chromosome structure, condensation, and decondensation (*43, 50, 51, 53, 57*) (See figure 2).

Mammals express two distinct isoforms of the type II enzyme, topoisomerase IIα and topoisomerase IIβ (*51, 53, 68, 69*). These isoforms display a high degree (~70%) of amino acid sequence identity and similar enzymological characteristics, but differ in their protomer molecular masses (170 *vs.* 180 kDa, respectively) and are encoded by separate genes (*15, 16, 43, 51, 53, 68-73*).

Topoisomerase IIα and topoisomerase IIβ are both nuclear enzymes (mammals have no known mitochondrial type II enzymes), but have distinct
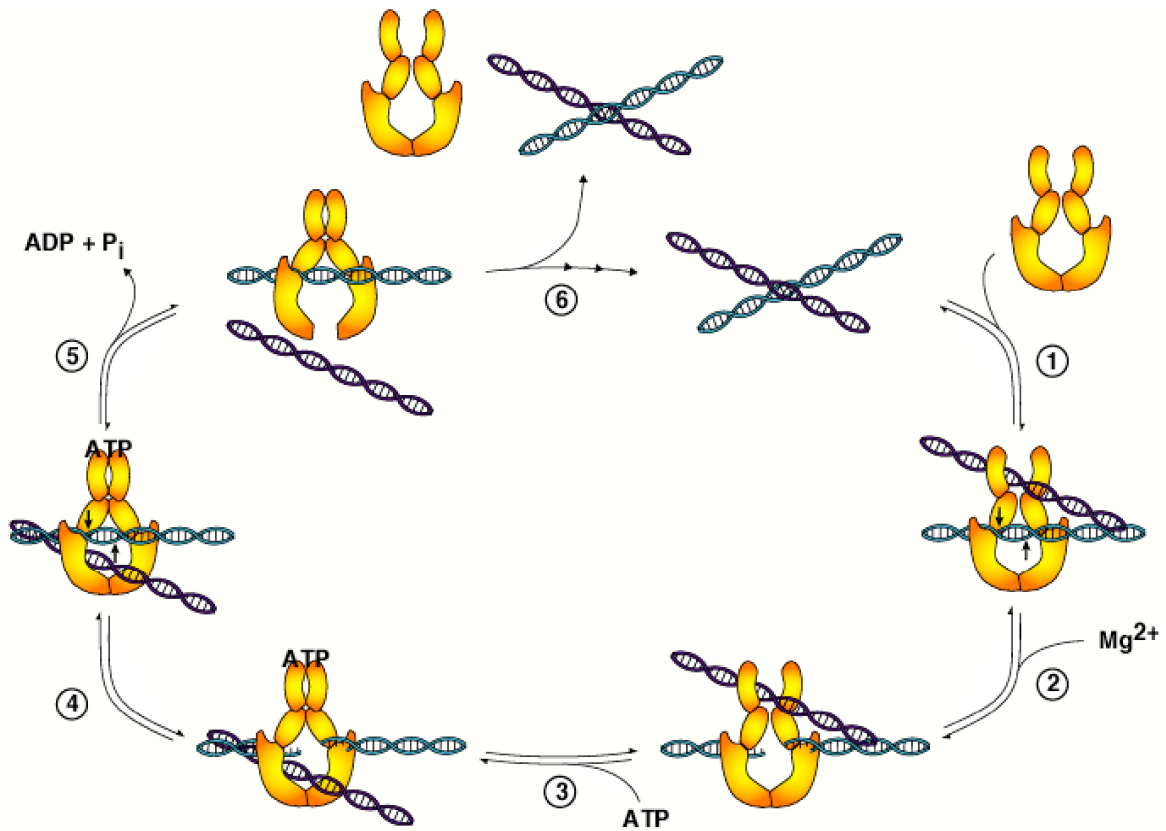
Figure 3. The catalytic cycle of type II topoisomerases. 1) Topoisomerase II binds to DNA, preferably at DNA crossovers and creates two staggered breaks in the sugar phosphate backbone of DNA to relax, untangle and decatenate DNA. 2) In order to prevent these scissile breaks in the DNA from becoming permanent double-stranded breaks, the enzyme covalently attaches to DNA at the active site tyrosine residue in the presence of a divalent cation. The covalent enzyme-DNA intermediate is known as the *cleavage complex*. 3) Once a cleavage complex is formed topoisomerase II binds ATP and undergoes a conformational change. This change in confirmation allows the enzyme to pass an intact strand of DNA through the cleaved DNA strand. 4) After topoisomerase II passes the intact DNA strand it religates the cleaved strand and 5) hydrolyzes ATP to complete the catalytic cycle and 6) releases the DNA substrate.

patterns of expression and cellular functions. Topoisomerase IIα is essential for the survival of actively growing cells and its concentration is upregulated dramatically during periods of proliferation (*74-76*). Furthermore, enzyme levels increase over the cell cycle and peak in G2/M (*76-78*). Topoisomerase IIα is found at replication forks and remains tightly associated with chromosomes during mitosis (*50, 79*). Thus, it is believed to be the isoform that functions in growth-dependent processes, such as DNA replication and chromosome segregation (*43, 50*).

In contrast to topoisomerase IIα, topoisomerase IIβ is dispensable at the cellular level. However, the β isoform is required for proper neural development (*80*). Expression of topoisomerase IIβ is independent of proliferative status and cell cycle, and the enzyme dissociates from chromosomes during mitosis (*50, 73, 76, 81*). Topoisomerase IIβ cannot compensate for the loss of topoisomerase IIα in mammalian cells, suggesting that these two isoforms do not play redundant roles in replicative processes (*73, 82, 83*). Although the physiological functions of topoisomerase IIβ have yet to be defined, recent evidence indicates roles in the transcription of hormonally- or developmentally-regulated genes (*84, 85*).

<u>Topoisomerase-targeted Anticancer Drugs</u>

As discussed above, all topoisomerases cleave DNA during their critical cellular functions. Although the strand breaks generated by these enzymes are transient in nature, they are potentially deleterious to the cell (Figure 4). When a nucleic acid tracking system, such as a replication or transcription complex, attempts to traverse a topoisomerase-DNA cleavage complex, it converts the
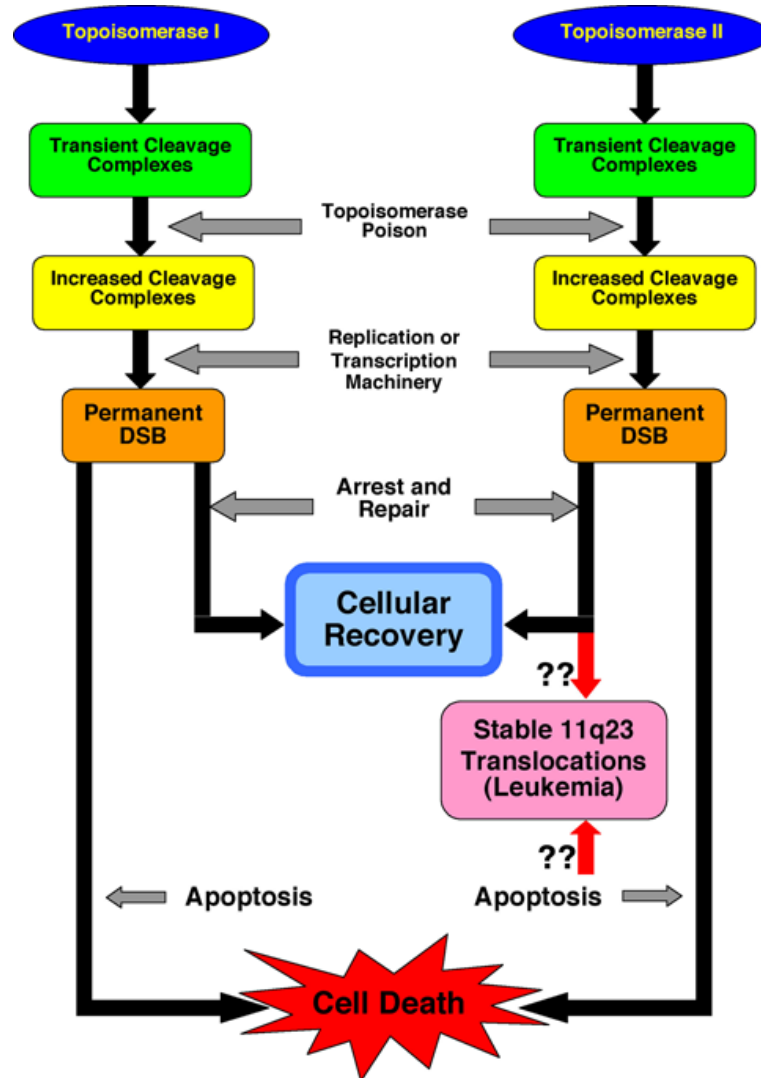
Figure 4. Topoisomerases as cellular toxins. Levels of transient DNA cleavage complexes generated by topoisomerase I or topoisomerase II are increased following exposure to a variety of topoisomerase poisons. These cleavage complexes are converted to permanent DNA strand breaks (DSB) as a result of collisions with replication or transcription complexes. If checkpoint and DNA repair pathways function properly and if levels of DSB do not overwhelm the cell, DNA damage is repaired, resulting in cellular recovery. If checkpoint or DNA repair pathways function are impaired or if levels of DSB overwhelm the cell, apoptotic pathways are initiated, resulting in cell death. Finally, in some cases, treatment of cells with topoisomerase II poisons induces the formation of stable leukemic translocations involving the *MLL* gene at chromosomal band 11q23. The source of the DNA breaks that initiate the translocations is controversial and is postulated to result from DNA cleavage mediated by topoisomerase II, apoptotic nucleases (coupled with abortive apoptosis), or both.

transient enzyme-DNA interaction to a permanent double-stranded break (*15, 41, 50, 53, 57*). The resulting strand breaks, as well as the inhibition of essential DNA processes, initiate recombination/repair pathways (*10, 86-94*). If the accumulation of breaks overwhelms the cell, they trigger apoptotic pathways (*11-17*). However, if the DNA strand breaks are not able to overwhelm the cell, surviving populations may contain chromosomal translocations or other aberrations (*11-17*).

Agents that increase levels of topoisomerase-DNA cleavage complexes are known as "topoisomerase poisons" because they convert these enzymes to potent cellular toxins (*10, 15, 16, 95, 96*). Topoisomerase poisons work by two mutually non-exclusive mechanisms. Some poisons act by inhibiting the ability of their topoisomerase target to ligate cleaved DNA intermediates (*10, 15, 16, 97*). Other poisons have little effect on the rate of enzyme-mediated ligation and are believed to act primarily by enhancing the forward rate of cleavage complex formation (*10, 15, 16, 98*). The exact mechanism by which this second group of drugs increases levels of DNA cleavage is unknown. They may specifically affect the forward rate of DNA scission. Alternatively, they may affect the enzyme-DNA binding equilibrium, as the level of topoisomerase-mediated DNA cleavage is proportional to the amount of enzyme bound. Clinically relevant topoisomerase-targeted agents appear to act primarily by inhibiting DNA ligation (*10, 15, 16, 97*).

A diverse group of natural and synthetic compounds increase the levels of topoisomerase-DNA cleavage complexes *in vitro* and in human cells. Several of these agents are in wide clinical use as anticancer therapeutics and represent

some of the most successful drugs currently used for the treatment of human malignancies.

Topoisomerase I-targeted Drugs

Topoisomerase I is the target for an emerging class of drugs based on camptothecin, a natural product derived from the bark of the Chinese yew tree, *Camptotheca acuminata* (*99*). These drugs represent some of the most active new agents in the clinic and show promise against malignancies that respond poorly to existing therapies, such as non-small cell lung cancer, metastatic ovarian cancer, and colorectal cancer (*4, 54, 56, 100*). Other drugs that target topoisomerase I, including the indolocarbazoles (*101*), the 5,11-diketoindenoisoquinolines (*102*), and the phenanthridines (*103*), currently are under clinical development.

Topoisomerase II-targeted Drugs

Topoisomerase II is the target for some of the most successful anticancer drugs currently used to treat human malignancies (*104*). It is estimated that one half of all chemotherapy regimens include these agents. Six topoisomerase II-targeted drugs are approved for use in the United States, with others being used worldwide (Figure 5) (*104*).

One of the first topoisomerase II-targeted agents to be developed was etoposide, which is derived from podophyllotoxin (*10, 105*). This natural product is found in *Podophyllum peltatum*, more commonly known as the May apple or mandrake plant (*10, 105*). Podophyllotoxin has been used as a folk remedy for over a thousand years (*10, 105*). It has well-established antimitotic properties,

Mitoxantrone

Amsacrine

R =

H₃C — = Etoposide

= Teniposide

R₁ = | **R₂ =** |
—CH₂OH | —OCH₃ | = Doxorubicin
—CH₃ | —OCH₃ | = Daunorubicin
—CH₂OH | —H | = Idarubicin
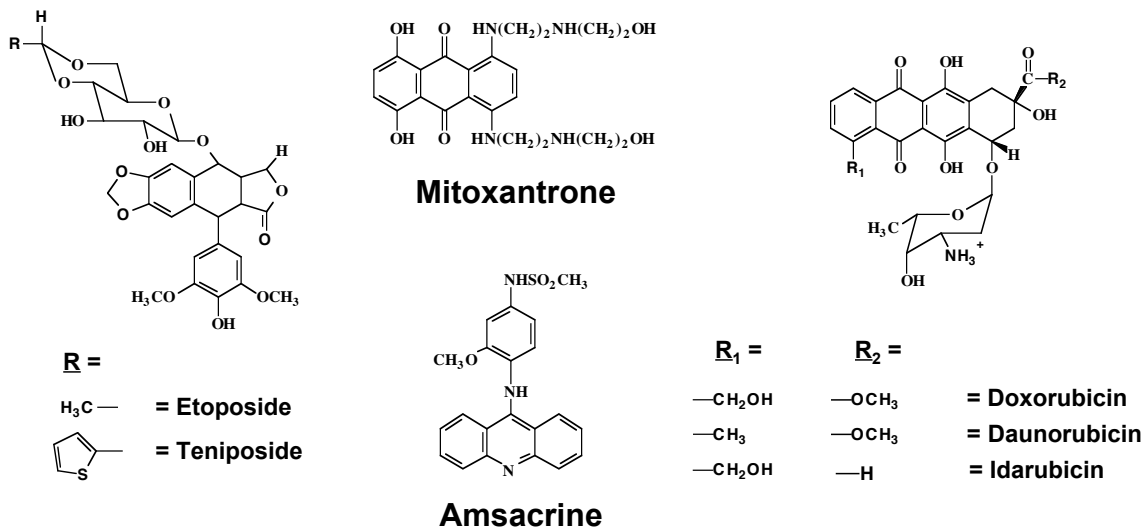
Figure 5. Structures of clinically used Topoisomerase II poisons.

which are related to its potent inhibition of tubulin polymerization (*10, 105*). Although clinical use of podophyllotoxin as an antineoplastic agent was prevented by high toxicity, two synthetic analogs, etoposide and teniposide, were developed (*105*). These analogs displayed increased antineoplastic activity and decreased toxicity compared to the parent compound. Etoposide was approved for clinical use against cancer in the mid-1980's (*10, 105*). Surprisingly (and in marked contrast to podophyllotoxin), etoposide displays no ability to inhibit tubulin polymerization. Rather, it kills cells by acting as a topoisomerase II poison (*106-108*).

Etoposide and other drugs, such as doxorubicin, are front-line therapy for a variety of systemic cancers and solid tumors, including leukemias, lymphomas, sarcomas, and breast, lung, and germline cancers. Every form of cancer that is considered to be curable by systemic chemotherapy utilizes drugs that target topoisomerase II in treatment regimens (*109*).

While topoisomerase II is believed to be <u>the</u> cytotoxic target of the drugs shown in Figure 5, the relative contributions of topoisomerase IIα and topoisomerase IIβ to the chemotherapeutic effects of these agents has yet to be resolved. Some drugs appear to favor one isoform or the other; however, no truly "isoform-specific" agents have been identified. The issue of isoform specificity has potential clinical ramifications. For example, since topoisomerase IIβ is present in all cell types, it may be responsible for mediating some of the toxic side effects of topoisomerase II poisons in non-malignant tissues. Alternatively, since topoisomerase IIα and topoisomerase IIβ are involved in different cellular processes, it may be that cleavage complexes formed with one or the other isoform are more likely to be converted to permanent DNA strand breaks.

Other Topoisomerase Poisons

In addition to the synthetically derived topoisomerase poisons that are used to treat cancer (and antibacterial quinolones that target the prokaryotic type II enzymes, DNA gyrase and topoisomerase IV), three other categories of topoisomerase poisons have been identified. These include natural products that are normal dietary components (bioflavonoids), toxic metabolites of drugs or industrial chemicals (quinones), and DNA-damaging agents (Figure 6). Thus far, compounds in the first two categories have been found to affect primarily the type II enzyme. However, DNA damaging agents increase cleavage mediated by both type I and II topoisomerases.

Bioflavonoids

The most prominent natural products with activity against mammalian topoisomerases are the bioflavonoids (*i.e.*, phytoestrogens). Bioflavonoids represent a diverse group of polyphenolic compounds that are components of many fruits, vegetables, and plant leaves (*110-113*). These compounds affect human cells through a variety of pathways; they are strong antioxidants, and efficient inhibitors of growth factor receptor tyrosine kinases (*110-113*). In addition, they are potent topoisomerase II poisons. It has been suggested that genistein (an isoflavone that is abundant in soy) has chemopreventative properties and that ingestion of this compound contributes to the low incidence of breast and colorectal cancers observed in the Pacific Rim (*110-113*). However,

Bioflavanoids

Quinone Metabolites
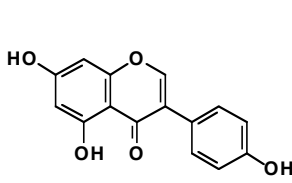
Genistein
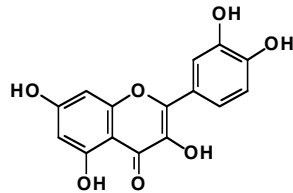
Quercetin
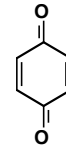
Benzoquinone
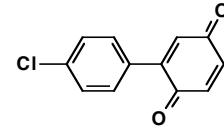
PCB Quinones
(4'Cl-2,5pQ)

DNA Damage
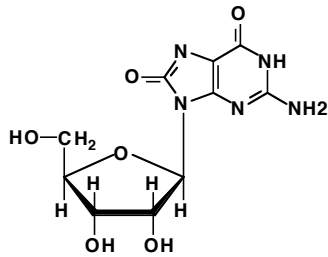
DNA Base Oxidation
(8-oxo-dG)
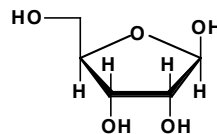
Abasic Site
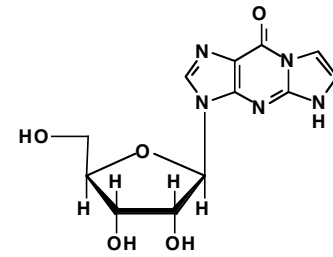
DNA Base Alkylation
(εdG)

Figure 6. Structures of environmental topoisomerase poisons. Structures are shown for selected bioflavonoids, quinones, and DNA lesions.

as discussed below, there also is evidence associating genistein consumption during pregnancy to the development of infant leukemias (*18, 20-23*).

Quinones

Quinones are highly reactive compounds that are often produced in the body as a result of detoxification or metabolism pathways. Quinones damage cells by generating oxygen radicals and by covalently modifying proteins and (to a lesser extent) nucleic acids (*32, 114-116*). Exposure to benzene [metabolized to benzoquinone (see Figure 8)], has been linked to the development of malignancies in rodents and the development of leukemia in humans (primarily acute myelogenous leukemia and acute non-lymphocytic leukemia) (*29, 31, 32*). Recent studies demonstrate that benzoquinone is a strong topoisomerase II-poison *in vitro* and in cultured human cells (*1, 117*). Benzoquinone requires adduction to the enzyme to inhibit function (*1, 117*). However, the mechanism by which quinone adduction enhances enzyme-mediate DNA cleavage is largely unknown. In addition to benzoquinone other quinones, including Menadione (the quinone known as vitamin K3), also display activity against topoisomerase II (*118*).

DNA Damage

Unlike the two categories of topoisomerase II poisons discussed above, DNA lesions are potent enhancers of DNA scission mediated by both the type I and II enzymes. Topoisomerase I is most sensitive to abasic sites, oxidative lesions, and alkylated bases (*90*). Topoisomerase II prefers lesions that distort the double helix

17

and is particularly sensitive to abasic sites and alkylated bases that contain exocyclic rings (*119-124*).

DNA damage increases cleavage at naturally occurring sites of topoisomerase I or topoisomerase II action. In all cases, lesions must be located proximal to the sites of cleavage in order to act as enzyme poisons. Whereas topoisomerase I is generally sensitive to lesions immediately upstream or downstream from the scissile bond (*90, 125*), topoisomerase II requires that damage be localized within the four-base stagger that separates the two scissile bonds on the opposite strands of the double helix (*119-124, 126*).

Although DNA damage increases levels of topoisomerase I and topoisomerase II cleavage complexes, the mechanism by which lesions alter the activity of these enzymes differs. DNA damage increases the concentration of topoisomerase I-DNA cleavage complexes primarily by inhibiting rates of enzyme-mediated DNA ligation (*90*). In contrast, damage has no obvious effects on rates of topoisomerase II-mediated DNA ligation and appears to act primarily by enhancing the forward rate of scission (*119, 121, 124, 126*).

The physiological benefits of DNA lesions as topoisomerase poisons, if any, are unclear. However, it is notable that topoisomerase I and topoisomerase II both appear to play roles in fragmenting genomic DNA during apoptosis (*125, 127-129*). It has been suggested that the apoptotic activities of topoisomerases are enhanced (or perhaps triggered) by DNA lesions that are generated following the release of oxygen radicals from permeable mitochondria in apoptotic cells (*125, 127-129*).

Topoisomerase II and Leukemia

In addition to its role as an essential cellular protein and target for anticancer drugs, clinical studies suggest that topoisomerase II initiates DNA strand breaks that can generate chromosomal translocations that trigger specific types of leukemia (see Figure 4). For example, ~2-3% of patients treated with regimens that include etoposide ultimately develop acute myelocytic leukemia (*14, 24-26*). Recently, correlations between the rising use of mitoxantrone to treat breast cancer and the development of secondary leukemias also have been noted (*130, 131*). The common feature in ~50% of these leukemias is the presence of translocations within an 8.3 kb breakpoint cluster region in the *MLL* (mixed lineage leukemia) gene at chromosome band 11q23 (*13, 14, 24-26*) (Figure 7).

The basis for the development of topoisomerase II-initiated leukemias has not been elucidated, but it appears to be related to the function of the protein product of the *MLL* gene. MLL is the human homolog of the *Drosophila* trithorax and yeast Set1 proteins, and is a histone methyltransferase that is involved in transcriptional regulation in hematopoietic cells (*26, 132-135*). Accumulating evidence suggests that the fusion of the MLL protein with other cellular partner proteins alters enzyme function and affects the differentiation of pluripotent hematopoietic stem cells or committed myeloid or lymphoid stem cells by deregulating the expression of *HOX* genes (*26, 132, 133, 135, 136*).

Infant acute lymphoblastic leukemias and some benzene-induced leukemias also display translocations involving chromosomal band 11q23 (*137*). Epidemiological and biochemical studies have established potential links between these malignancies and topoisomerase II. For example, the maternal consumption (during pregnancy) of foods that are high in genistein or other
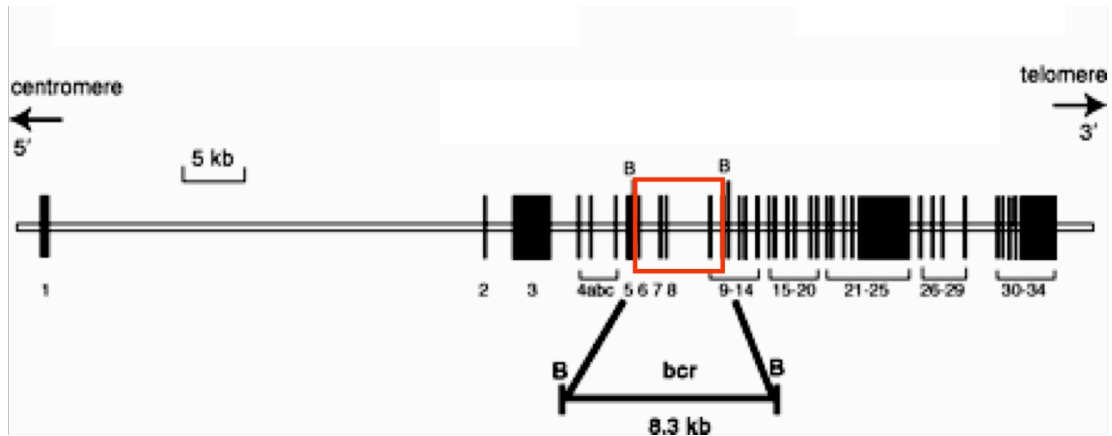
Figure 7. Diagram of the *MLL* gene locus and breakpoint cluster region. The *MLL* gene is located on chromosome band 11q23 and encodes a protein that is >400 kDa in size. Translocations and other aberrations involving this gene have been observed in patients with specific leukemia and have been linked to the activity of type II topoisomerases. The coding region contains an 8.3 kb breakpoint cluster region (bcr, boxed in red) flanked by BamHI sites (B).

naturally occurring topoisomerase II poisons increases the risk of developing infant acute lymphoblastic leukemias ~10–fold (*18, 20-23*). In addition, individuals with chronic exposure to benzene display an increasingly higher risk for leukemias with 11q23 chromosomal translocations if they are heterozygous or homozygous for the *C609T* polymorphism of the NAD(P)H:quinone oxidoreductase 1 (NQO1) (*138-142*). NQO1 is the enzyme that reduces benzoquinone to the less reactive hydroquinone (*138-140, 142*) (Figure 8). As discussed above, benzoquinone is a highly active topoisomerase II poison.

Although the involvement of topoisomerase II-mediated DNA cleavage in the development of leukemias with *MLL* translocations is widely accepted, the role of the enzyme-associated DNA strand breaks in triggering the chromosomal aberrations is controversial. Two hypotheses have been proposed (see Figure 4). The first postulates that the breaks induced by topoisomerase II play a direct role in the translocation process. In this case, the enzyme cleaves within the *MLL* gene and following processing and recombination/repair; the breaks are reattached to other sites in the genome (presumably that also were cleaved by the type II enzyme). Supporting this hypothesis, all *MLL* (and partner) chromosomal breakpoints identified in patient samples, including those with secondary and infant leukemias, are located in close proximity to *in vitro* sites of topoisomerase II-mediated DNA cleavage (*143-145*). Furthermore, leukemias with 11q23 chromosomal translocations are only observed in patients treated with topoisomerase II poisons, and are not seen following other anticancer therapies. This is despite the fact that radiation, DNA alkylation and crosslinking agents,
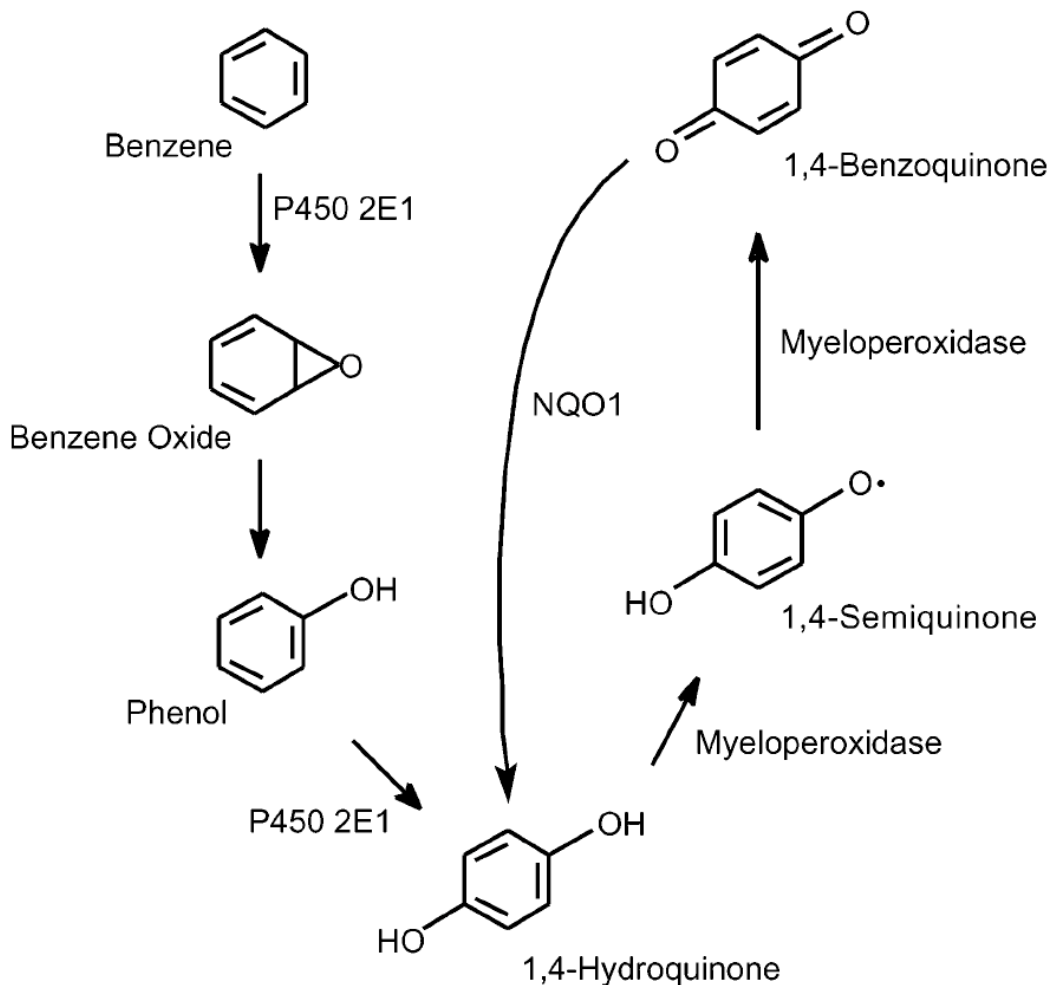
Figure 8. Metabolism of benzene to benzoquinone. Benzene is metabolized to benzene oxide in the liver by cytochrome P450 2E1 and converted to phenol by a nonenzymatic rearrangement. Phenol is converted to hydroquinone by a subsequent round of cytochrome P450 metabolism and non-enzymatic rearrangement. The now water soluble hydroquinone diffuses to the bone marrow and is metabolised, to benzoquinone by endogenous myeloperoxidase (*MPO*). Benzoquinone is converted back to hydroquinone by NAD(P)H:quinone oxidoreductase 1 (*NQO1*). Other metabolic and clearance pathways exist, but were omitted for simplicity. Adapted from (*1*).

and drugs such as bleomycin that chemically cleave DNA all generate chromosomal breaks and induce apoptotic pathways.

The second hypothesis states that the breaks induced by topoisomerase II play an indirect role in the translocation process. In this case, enzyme-mediated DNA cleavage induces apoptosis, which initiates chromosomal fragmentation (*146*). Occasionally (by processes that have yet to be described), apoptosis aborts and nuclease-generated breaks within the *MLL* gene are processed and reattached to other sites in the genome (*147*). Supporting this theory, a major site of apoptotic cleavage is located in the breakpoint cluster region of the *MLL* gene (*147-150*). Moreover, translocations involving 11q23 can be induced in cultured human cells by agents that trigger apoptosis, but do not target topoisomerase II (*147-150*). The discrepancy between patients and cellular studies regarding the requirement for topoisomerase II poisons to induce leukemic translocations is notable. The apoptotic model reconciles this discrepancy by further postulating that the inhibition of topoisomerase II function following treatment with poisons alters chromatin structure and that these alterations, coupled with nuclease action, are required for the translocation event in patients (*150*).

It is likely that the process that translates topoisomerase II-mediated DNA cleavage into 11q23 chromosomal translocations is highly complex and multifaceted. Both (or neither) of the above hypotheses may contribute to the process.

Scope of the Dissertation

Type II topoisomerases are necessary for proper chromosome structure and segregation (*43, 50, 53, 57, 58*). In addition to their essential functions, topoisomerase II has been a successful target for anticancer agents for almost four decades (*10, 104, 105, 151*). Topoisomerase II-targeted agents exploit the natural catalytic cycle of the enzyme to generate double-stranded DNA breaks (*10, 16, 95, 152-154*). These breaks in the genetic material, if present at high enough levels, induce programmed cell death pathways (*11-14, 16, 17*). However, insufficient generation of topoisomerase II-mediated DNA scission can lead to repair of the damage and can ultimately generate translocations, deletions and sister chromatid exchanges (*11-14, 16, 17*). Significant clinical evidence has linked topoisomerase II-targeted cancer chemotherapy regimens with the development of leukemias featuring 11q23 translocations (*13, 14, 24-26*). Exposure to benzene (which is metabolized to benzoquinone) also has been linked with the development of these leukemias (*139-142*).

Aside from the ability of benzoquinone to induce leukemia, other toxic quinone metabolites display genotoxic activity. However, this damage occurs primarily in the liver. Exposure to polychlorinated biphenyls (PCBs) and acetaminophen (metabolized to quinone and quinone imine compounds, respectively) induces chromosomal aberrations in this organ (*33-36*).

It is unclear how quinone and quinone imine metabolites induce DNA damage in the liver. However, the damage induced by these agents is consistent with the actions of topoisomerase II poisons. Furthermore, the exact role of the DNA breaks generated by exposure to clinical and environmental topoisomerase II poisons in the induction of leukemias is unclear. Therefore, the goals of this

dissertation are to 1) further characterize quinones as topoisomerase II poisons; 2) examine the molecular interactions between the anticancer agent etoposide and topoisomerase IIα; and 3) explore the ability of topoisomerase II-targeted agents to induce aberrations in the *MLL* gene in cultured human cells.

Chapter I of this dissertation reviews both type I and type II topoisomerases, as well as the role of each enzyme in treating and/or initiating human cancers. Chapter II describes the materials and methods utilized in the studies presented in Chapters III-VII.

Chapter III of this dissertation characterizes the reactive metabolite of acetaminophen, N-acetyl *p*-benzoquinone imine (NAPQI), as a topoisomerase II poison. Results indicate that NAPQI has the ability to induce double-stranded DNA breaks by inhibiting topoisomerase II-mediated ligation of cleaved nucleic acid molecules. Aside from enhancing enzyme-mediated DNA scission, NAPQI inhibits both the cleavage and relaxation activities of the enzyme when incubated in the absence of DNA. The amount of compound necessary to induce half the maximal inhibition of cleaved DNA substrate achieved was ~10–fold higher than that necessary to inhibit 50 percent of strand passage activity. This suggests a dual activity of this agent against the enzyme. Finally, the parent compound acetaminophen did not abrogate etoposide activity *in vitro* or in cultured human cells. The results of this study are published (*155*).

Chapter IV of this dissertation describes hydroquinone and quinone-based metabolites of PCBs as topoisomerase-poisons. All quinone-based PCB metabolites examined displayed significant activity as topoisomerase II poisons. These agents require covalent adduction to topoisomerase II and inhibited DNA ligation by the enzyme. The PCB hydroquinone, however, exhibited little activity

to enhance topoisomerase II-mediated double-stranded DNA breaks. Like NAPQI, these agents also inhibited the enzyme in the absence of DNA. A series of binding experiments suggest that these agents are capable of blocking the N-terminal gate of the protein by crosslinking the protomer subunits of topoisomerase II. This crosslinking activity appears to contribute to part of the activity of these agents against the enzyme. The results of this study are published (*156*).

Chapter V of this dissertation further explores the mechanism by which quinones act as topoisomerase II poisons. Quinones require adduction to function as topoisomerase II poisons. Therefore, sites of quinone adduction were mapped using mass spectrometry. Four cysteine residues were identified as sites of quinone adduction; cys170, cys392, cys405 and cys455. Mutations (C->A) were made at each position and the resulting enzymes were purified. Substitution of either cys 392 or cys405 reduced quinone sensitivity by ~50%. Simultaneous mutation of these residues did not confer further resistance to quinones. Topoisomerase II mutants carrying cys->ala mutations at residues 392 or 405 displayed faster rates of ligation in the presence of benzoquione. Furthermore, mutation of cys455 to ala generated a mutant enzyme that was 1.5– to 2–fold hypersensitive to quinones. This mutant displayed higher levels of clamp closed form of the enzyme in the presence of benzoquione. Taken together, these results suggest at least a dual activity of quinones against topoisomerase II. They adduct to cys 392 and cys405 to inhibit ligation and block the N-terminal protein gate increasing the concentration to DNA in the active sites of the enzyme. The results of this study are published or submitted (*157, 158*).

Chapter VI of this dissertation further examines the interactions between etoposide and topoisomerase II. Consistent with previous studies, the A–ring of the drug appears to be necessary for drug binding while the E–ring, appears to play a functional role in enhancing enzyme-mediated DNA scission. Addition of bulk at the 4' position of this ring significantly decreased binding of the resultant agent indicating that the E–ring rests in a confined pocket within the protein. Removal of the glycosidic moiety of etoposide has only minor effects on DNA cleavage that are not due to a decrease in drug enzyme-binding. Instead, results suggest that the glycosidic moiety of etoposide may interact with DNA in the enzyme-drug-DNA ternary complex. The results of this study have not yet been published.

Chapter VII of this dissertation studied the ability of two characterized topoisomerase II poisons to induce *MLL* aberrations in cultured human cells. Treatment of cells with either of these agents increase levels of topoisomerase II covalent DNA complexes and permanent double-stranded DNA breaks. Analysis of the *MLL* bcr revealed several aberrations of a 1.8 kb *XbaI* fragment that contains a translocation "hotspot" after exposure to etoposide or benzoquinone. Furthermore, inhibition of apoptosis decreased but did not eliminate these translocations. These data suggest that multiple pathways contribute to *MLL* gene aberrations that can lead to leukemias. Furthermore, this system can be used to analyze the role of type II topoisomerases in generating 11q23 translocations as well as the specific repair machinery involved. Finally, concluding remarks for this dissertation are found in Chapter VIII.

CHAPTER II

METHODS

<u>Materials</u>

Human topoisomerase IIα, top2αC170A, top2αC392A, top2αC405A, top2αC455A and top2αC392/405A were expressed in *Saccharomyces cerevisiae* (*159*) and purified as described previously (*160, 161*). Negatively supercoiled pBR322 DNA was prepared using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Human topoisomerase I was a gift from Dr. Mary Ann Bjornsti (St. Jude Children's Research Hospital). Mass spectrometry grade trypsin and chymotrypsin were obtained from Fisher and Princeton Separations, respectively. Restriction enzymes, phosphatase, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs. [γ-^{32}P]ATP and genistein were obtained from ICN, etoposide, NAPQI, acetaminophen, benzoquinone, hydroquinone and camptothecin were from Sigma, TOP-53 was a gift from Taiho Pharmaceuticals, amsacrine was a gift from Bristol-Myers Squibb, and CP-115,953 was a gift from Pfizer Global Research. 2-(2-Chloro-phenyl)-[1,4]benzoquinone (2'Cl-2,5pQ), 2-(3-chloro-phenyl)-[1,4]benzoquinone (3'Cl-2,5pQ), 2-(4-chloro-phenyl)-[1,4]benzoquinone (4'Cl-2,5pQ), 2-(3,5-dichloro-phenyl)-[1,4]benzoquinone (3',5'Cl-2,5pQ), 3-(4-Chloro-phenyl)-[1,2]benzoquinone (4'Cl-2,3oQ), 4-(4-chloro-phenyl)-[1,2]benzoquinone (4'Cl-3,4oQ), and 4'-Chloro-biphenyl-2,5-diol (4'Cl-2,5HQ) were the generous gifts of Dr. Hans J. Lehmler, University of Iowa. DEPT, EPT and DDEPT were the generous gifts of Dr. Norma Dunlap, Middle Tennessee State University.

Hydroxyphenyl-etoposide and Phenyl-etoposide were the generous gifts of David B. Berkowitz, University of Nebraska. Z-VAD-fmk was purchased from Axxora and was dissolved as a 15 mM stock in DMSO and stored at -70 °C. Etoposide, camptothecin, genistein, TOP-53, and amsacrine, were stored at 4 °C as 10 or 20 mM stock solutions in 100% DMSO. NAPQI and acetaminophen were prepared as 20 mM stock solutions in 100% DMSO and water, respectively, and used fresh for all experiments. The quinolone CP-115,953 was dissolved as 40 mM solution in 0.1 N NaOH, and stored at -20 °C. Immediately prior to use, the quinolone was diluted to 8 mM with 10 mM Tris, pH 7.9. All other chemicals were analytical reagent grade.

Procedures

Synthesis of PCB metabolites.

2'Cl-2,5pQ, 3'Cl-2,5pQ, 4'Cl-2,5pQ, 3',5'Cl-2,5pQ, 4'Cl-2,5HQ, 4'Cl-2,3oQ and 4'Cl-3,4oQ were synthesized by coupling of the appropriate chloroaniline with benzoquinone as described by Amaro *et al.* (*162*). 4'Cl-2,5HQ was generated by reducing 4'Cl-2,5pQ with sodium dithionite (*163*). 4'Cl-2,3oQ and 4'Cl-3,4oQ were generated by oxidizing the corresponding diol PCB derivatives with silver(I) oxide (*162, 164*). The [1,2]benzoquinones were used within 48 hours after their synthesis to minimize decomposition. In all cases, spectroscopic data were in agreement with proposed structures. Furthermore, the purity of all compounds was >98% as determined by gas chromatography ([1,4]benzoquinone) or gas chromatography-mass spectrometry (diols). The only

exception were the two [1,2]benzoquinones, which were characterized with ^{1}H nuclear magnetic resonance spectroscopy and used without further purification.

   Adduction of Topoisomerase IIα by Quinones.

   To determine sites of quinone adduction on human topoisomerase IIα, 135 nm enzyme was incubated in a total of 300 μL of cleavage buffer (10 mM Tris-HCl, pH 7.9, 100 mM KCl, 5 mM MgCl$_2$, 0.1 mM NaEDTA, and 2.5% glycerol) in the presence of 250 μM plumbagin for 10 min at 37 ˚C. Reactions were quenched with 20 μL of 45 mM DTT. Samples were applied to 30,000 NMWL filter columns (Millipore), washed with 10% methanol in 25 mM ammonium bicarbonate, and resuspended in 25 μL of 25 mM ammonium bicarbonate. DTT (2.5 μL of 45 mM) was added, and the protein was incubated for 15 min at 50 ˚C. Iodoacetamide (2.5 μL of 100 mM) was added and the sample was incubated at room temperature in the absence of light for 15 min. Additional DTT (2.5 μL of 45 mM) was added to quench the iodoacetamide, and the protein was incubated for 15 min at 50 ˚C. Samples were washed with 10% methanol in 25 mM ammonium bicarbonate and resuspended in 25 μL of 25 mM ammonium bicarbonate. Trypsin (0.1 μg) was added and samples were incubated for 16 h at 37 ˚C to digest the protein. Alternatively, chymotrypsin (0.1 μg) was added and samples were incubated for 6 h at room temperature. Peptides were eluted from the column, dried and reconstituted in 0.1% formic acid for mass spectral analysis.

   Residues in topoisomerase IIα that were adducted by quinone were identified by mass spectral analysis of the tryptic or chymotryptic peptides. The LC-MS/MS analyses were performed on a ThermoFinnigan LTQ linear ion trap mass spectrometer equipped with a ThermoFinnigan Surveyor LC pump and

autosampler, NanoSpray source (Thermo Electron), and Xcalibur 1.4 instrument control and data analysis software. HPLC separation of the tryptic peptides was achieved using a 100 μm x 11 cm C-18 capillary column (Monitor C18, 5 micron, 100 angstrom, Column Engineering) at a flow rate of 0.7 μL min^{-1}. Solvent A was H$_2$O with 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid. The gradient program was: 0–3 min, linear gradient from 0–5% B; 3–5 min, 5% B; 5–50 min, linear gradient to 50% B; 50–52 min, linear gradient to 80% B; 52–55 min, linear gradient to 90% B; 55–56 min, 90% B in solvent A. MS/MS scans were acquired using an isolation width of 2 m/z, an activation time of 30 ms, an activation Q of 0.250, and 30% normalized collision energy using 1 microscan and ion time of 100 for each MS/MS scan. The mass-spectrometer was tuned prior to analysis using the synthetic peptide TpepK (AVAGKAGAR). Some parameters may have varied slightly from experiment to experiment, but typically the tune parameters were as follows: spray voltage of 2.0 KV, capillary temperature of 160 °C, capillary voltage of 60 V, and tube lens of 130 V. Tandem MS analysis was performed using data-dependent scanning in which one full MS spectrum, using a full mass range of 400–2000 amu, was followed by 3 MS/MS spectra. Wild-type and modified peptides were identified using the SEQUEST algorithm (*165*), and the SEQUEST Browser software (Thermo Electron, San Jose, CA) using the human subset of the NCBI database. In addition, lists of theoretical or Sequest identified unmodified peptides were created and each peptide was run through P-Mod software to check for possible chemical modifications (*166*). The candidate modifications found by software were verified by visual inspection of corresponding spectra.

Generation of Mutant Human Topoisomerase IIα Proteins.

Mutations in the topoisomerase IIα PCR substrate were generated by cloning a *SalI-KpnI* fragment of YEpWob6 (*167*) that encoded the N-terminus of the human enzyme into pUC18. Site-directed mutagenesis was performed using the QuickChange II PCR system (Stratagene). The sequence of the forward and reverse primers used to generate the C170A mutation were GGAGCCAAATTG<u>GCT</u>AACATATTCAGTACCAAATTTACTGTGG and GAATATGTT<u>AGC</u>CAATTTGGCTCCATAGCCATTTCGACCACC, respectively. The sequence of the forward and reverse primers used to generate the C392A mutation were CTTTACAACCCAAGAGCTTTGGATCAACA<u>GCC</u>CAATTG-AGTG and GATAAATTTTTCACTCAATTG<u>GGC</u>TGTTGATCCAAAGCTCT-TGG, respectively. The sequence of the forward and reverse primers used to generate the C405A mutation were CAAAGCTGCCATTGGC<u>GCT</u>G-GTATTGTAGAAAGCATAC and CAGTTTAGTATGCTTTCTACAATACC<u>AGC</u>-GCCAATGGCAGC, respectively. The sequence of the forward and reverse primers used to generate the C455A mutation were CAGGGGGCCGAAACTCCACTGAG<u>GCT</u>ACGCTTATCC and CCCTCAGTCA-GGATAAGCGT<u>AGC</u>CTCAGTGGAGTTTCGGCCC, respectively. The mutagenized codons are underlined. To generate the C392/405A double mutation, sequential rounds of PCR were performed using the primers to generate the C392A and C405A mutations as above. Mutations were verified by sequencing and *SalI-KpnI* fragments were cloned back into YEpWob6. Mutant human topoisomerase IIα enzymes were purified as described above.

Plasmid DNA Cleavage.

DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff (*168*). Assay mixtures contained 135 nM topoisomerase IIα, and 10 nM negatively supercoiled pBR322 DNA in a total of 20 μL of cleavage buffer (10 mM Tris-HCl, pH 7.9, 100 mM KCl, 5 mM MgCl$_2$, 0.1 mM NaEDTA, and 2.5% glycerol) that contained 0 to 200 μM etoposide, DEPT, EPT, DDEPT, NAPQI, acetaminophen, PCB metabolite, plumbagin, or benzoquinone; or 50 μM genistein or amsacrine; or 5 μM CP-115,953. DNA cleavage was initiated by the addition of enzyme and mixtures were incubated for 6 min at 37 °C to establish DNA cleavage-religation equilibria. Enzyme-DNA cleavage intermediates were trapped by adding 2 μL of 5% SDS and 1 μL of 375 mM EDTA, pH 8.0. Proteinase K was added (2 μL of 0.8 mg/mL) and reactions were incubated for 30 min at 45 °C to digest the topoisomerase IIα. Samples were mixed with 2 μL of agarose loading buffer (60% sucrose in 10 mM Tris-HCl, pH 7.9, 0.5% bromophenol blue, and 0.5% xylene cyanol FF), heated for 15 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris-acetate, pH 8.3, 2 mM EDTA) that contained 0.5 μg/mL ethidium bromide. DNA cleavage was monitored by the conversion of negatively supercoiled plasmids to linear molecules. DNA bands were visualized by ultraviolet light and quantified using an Alpha Innotech digital imaging system.

In order to determine whether DNA cleavage by topoisomerase IIα was reversible, EDTA was added prior to treatment with SDS. To examine whether cleavage was protein-linked, proteinase K treatment was omitted. In reactions that tested whether PCB metabolite activity was affected by DTT, reactions

contained a final DTT concentration of 500 μM that was added either before or after cleavage complex formation and processed as above.

To analyze the effects of quinones on topoisomerase IIα in the absence of DNA, 100 μM etoposide, NAPQI, acetaminophen, PCB metabolite, or benzoquinone was incubated with the enzyme for 0 to 3 min at 37 °C in 15 μL of cleavage buffer. Cleavage reactions were initiated by adding negatively supercoiled pBR322 DNA in 5 μL of cleavage buffer. The concentrations of enzyme, DNA, and the agent in the final reaction mixtures were 135 nM, 10 nM, and 100 μM, respectively. Topoisomerase IIα DNA cleavage-religation equilibria were established and processed as described above.

Site-specific DNA Cleavage.

DNA sites cleaved by human topoisomerase IIα were determined by a modification of the procedure of O'Reilly and Kreuzer (*169*). 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. Reaction mixtures contained 0.35 nM DNA substrate and 60 nM topoisomerase IIα in 50 μL of cleavage buffer with 1 mM ATP. Assays were carried out in the absence of compound, or in the presence of 12.5 or 25 μM etoposide, 25 μM benzoquinone or PCB metabolite or 50 μM DEPT or 100 μM NAPQI or 250 μM EPT or DDEPT or 0–500 μM plumbagin. Reactions were initiated by the addition of the enzyme and were incubated for 10 min at 37 °C. Cleavage intermediates were trapped by adding 5 μL of 5% SDS followed by 5 μL of 250 mM NaEDTA, pH 8.0. Topoisomerase IIα was digested with proteinase K (5 μL of 0.8 mg/mL) for 30 min at 45 °C. Reaction products were precipitated twice in ethanol, dried, and

resuspended in 40% formamide, 8.4 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanole FF. Samples were subjected to electrophoresis in a 6% sequencing gel. The gel was then fixed in 10% methanol/10% acetic acid for 5 min, dried, and DNA cleavage products were analyzed on a Bio-Rad Molecular Imager FX.

DNA Religation.

DNA religation mediated by topoisomerase IIα was monitored according to the procedure of Byl *et al.* (*170*). Topoisomerase IIα DNA cleavage/religation equilibria were established as described above in the absence of compound, or in the presence of 100 μM etoposide, PCB metabolite, etoposide, benzoquinone, NAPQI, DEPT, EPT, or DDEPT. Religation was initiated by shifting reaction mixtures from 37 °C to 0 °C, and reactions were stopped at time points up to 40 s by the addition of 2 μL of 5% SDS followed by 1 μL of 375 mM NaEDTA, pH 8.0. Samples were processed and analyzed as described above for topoisomerase IIα plasmid DNA cleavage reactions.

DNA Ligation.

22 and 25-base oligonucleotides corresponding to residues 80–121 and 122–126 of pBR322 and its 47-mer complement were prepared on an Applied Biosystems DNA synthesizer. The sequences of the two top and single bottom strands were 5′-CCGTGTATGAAATCTAACAATG-3′ 5′-CGCTCATCGTCA-TCCTCGGCACCGT-3′ and 5′-ACGGTGCCGAGGATGACGATGAGCGCA-TTGTTAGATTTCATACACGG-3′, respectively. The 25-mer oligonucleotides were 5′-activated with *p*-nitrophenol according to Bromberg *et al*. (*171*). Single-

stranded 22-mer oligonucleotides were labeled on their 5′-termini with [^{32}P]phosphate and purified as described (*172*). Equimolar amounts of complementary oligonucleotides were annealed by incubating at 70 °C for 10 min and cooling to 25 °C.

DNA ligation reactions were carried out according to Bromberg *et al.* (*171*). Assays contained 200 nM wild-type human type IIα topoisomerase, 0–100 μM etoposide, DEPT, EPT, or DDEPT and 10 nM activated nicked oligonucleotide in a total of 20 μl of 10 mM Tris-HCl, pH 7.9, 135 mM KCl, 7.5 mM CaCl$_2$, 0.1 mM EDTA, and 2.5% glycerol. Reaction mixtures were incubated at 37 °C for 48 hours. Cleavage intermediates were trapped by adding 2 μL of 5% SDS followed by 1 μL of 375 mM NaEDTA, pH 8.0. Topoisomerase IIα was digested with proteinase K (2 μL of 0.8 mg/mL) for 30 min at 45 °C. Reaction products were treated as described above and subjected to electrophoresis in a 14% sequencing gel. The gel was then fixed in 10% methanol/10% acetic acid for 5 min, dried, and DNA cleavage products were analyzed on a Bio-Rad Molecular Imager FX.

DNA Binding.

The binding of topoisomerase IIα to linear DNA substrates was assessed using a nitrocellulose filter-binding assay. Either the linearized pBR322 DNA substrate described above or a ^{32}P-labeled double-stranded 50-mer oligonucleotide that contained a single topoisomerase II cleavage site (*173*) was employed. The oligonucleotide was prepared on an Applied Biosystems DNA synthesizer. The sequences of the top and bottom strands were 5′-TTGGTAT-CTGCGCTCTGCTGAAGCC↓AGTTACCTTCGGAAAAAGAGTTGGT-3′ and 5′-ACCAACTCTTTTTCCGAAGGT↓AACTGGCTTCAGCAGAGCGCAGATACCA

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A-3′, respectively. Arrows indicate cleavage sites by topoisomerase IIα The enzyme was incubated for 0–5 min at 37 °C in 15 μL of DNA binding buffer (10 mM Tris-HCl, pH 7.9, 30 mM KCl, 0.1 mM NaEDTA, and 2.5% glycerol) that contained no compound, PCB metabolite, or etoposide. Binding equilibria were initiated by addition of DNA in 5 μL of binding buffer and followed by incubation for 6 min at 37 °C. The final concentrations of topoisomerase IIα, DNA, and etoposide, PCB metabolite, or benzoquinone in the final reaction mixtures were 400 nM, 5 nM, and 100 μM, respectively. Nitrocellulose membranes (0.45 μm HA, Millipore) were prepared by incubation in DNA binding buffer for 10 min. Samples were applied to the membranes and filtered *in vacuo*. Membranes were washed 3 times with 1 mL of DNA binding buffer, dried, and submerged in 8 mL of scintillation fluid (Econo-Safe, Research Products International). Radioactivity remaining on the membranes was quantified using a Beckman LS 5000 TD Scintillation Counter.

Assays that monitored the binding of topoisomerase IIα to negatively supercoiled plasmid were carried out according to the procedure of Fortune *et al.* (*174*). Reaction mixtures contained enzyme and etoposide, PCB metabolite, or benzoquinone in 15 μL of binding buffer. Samples were incubated for 5 min at 37 °C. Binding was initiated by adding DNA in 5 μL of binding buffer and incubated at 37 °C for an additional 6 min. The final concentrations of topoisomerase IIα, DNA, and PCB metabolite in the final reaction mixtures were 0–400 nM, 5 nM, and 100 μM, respectively. 2 μL of 60% sucrose in 10 mM Tris-HCl, pH 7.9 was added to samples and were loaded without further processing onto a 1% agarose gel and subjected to electrophoresis in TBE buffer (100 mM

Tris-borate, pH 8.3, 2 mM EDTA). Gels were stained for 30 min with 0.5 μg/mL ethidium bromide and samples were analyzed as described above.

Protein Clamp Closing.

Filter binding assays were used to analyze the salt-stable closed-clamp of topoisomerase IIα (*175*). Briefly, 5 nM human topoisomerase IIα and 2 nM pBR322 were incubated for 5 min at 37 °C in a total of 90 μL of clamp closing buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, 8 mM MgCl$_2$). No compound, 100 μM PCB metabolite, benzoquinone or 250 μM plumbagin or a combination of 100 μM ICRF-193 + 2 mM ATP was added in a total of 10 μL of 10% DMSO. Mixtures were incubated for an additional 5 min at 37 °C.

In some experiments, 4'Cl-2,5pQ was incubated with topoisomerase IIα for 5 min in the absence of DNA. Following the addition of DNA, samples were incubated for an additional 5 min at 37 °C.

In all cases, binding mixtures were loaded onto glass fiber filters (Millipore) pre-incubated in clamp closing buffer, and filtered *in vacuo*. Filters were washed 3 times with clamp closing buffer (low salt wash), followed by 3 washes with clamp closing buffer that contained 1 M NaCl (high salt wash), followed by 3 washes with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.5% SDS. DNA was precipitated with isopropanol and loaded onto a 1% agarose gel in TAE buffer containing 0.5 μg/mL ethidium bromide. DNA was visualized as described above.

Protein Crosslinking.

The ability of quinones to crosslink the protomer subunits of human topoisomerase IIα was assessed using SDS polyacrylamide gels. Crosslinking mixtures contained 135 nM topoisomerase IIα in a total of 60 μL of cleavage buffer. The enzyme was incubated for 0–2 min at 37 °C in the presence of 100 μM 4′-Cl-2,5pQ or benzoquinone or for 6 min in the absence of compound or in the presence of 100 μM 4′-Cl-2,5pQ or 250 μM plumbagin. In dilution experiments, 80–800 nM topoisomerase IIα was incubated with 100 μM 4′-Cl-2,5pQ for 1 min. In all cases, reactions were quenched by the addition of 2 μL of 5 mM DTT and were incubated at 37 °C for an additional 2 min. Samples were precipitated with TCA and resuspended in 10 μL of H_2O. Laemmli buffer (10 μL, Bio-Rad) was added and samples were subject to electrophoresis in a 7.5% denaturing acrylamide gel (Bio-Rad) at 200 v for 1 h. The protein was visualized by coumassie staining.

Partial Proteolytic Mapping.

To identify peptides crosslinked by PCB metabolites, a modified Cleveland mapping protocol was utilized. Briefly, 3 μg of human topoisomerase IIα was treated with 100 μM 4′-Cl-2,5pQ for 0–2 min at 37 °C in 10 μL of cleavage buffer. Reactions were quenched with 1 μL of 5 mM DTT. Proteolysis was initiated by the addition of 0.4 ng of V8 protease (Pierce) and samples were incubated at 37 °C for 30 min. Protease reactions were quenched with 3 μL of 10% SDS followed by the addition of 15 μL of Laemmli buffer. Samples were subject to electrophoresis in a 4–20% denaturing acrylamide gel (Bio-Rad) at 200 v for 1.5 h. Protein was visualized by silver staining (Bio-Rad).

Topoisomerase II-Drug Binding.

Competition binding studies were performed using a nitrocellulose filter binding technique. Nitrocellulose membranes (0.45 μm HA; Millipore) were soaked in yeast binding buffer (10 mM sodium phosphate, pH 7.7, 250 mM KCl, 0.1 mM NaEDTA, and 5 mM $MgCl_2$) or DNA binding buffer for 10 min. Reaction mixtures contained 1.6 μM yeast topoisomerase II or human topoisomerase IIα and 20 μM [^{3}H]etoposide, as well as 0–100 μM etoposide, hydroxyphenyl-etoposide or phenyl-etoposide in a total of 60 μL of binding buffer. Samples were incubated for 6 min at 30 °C (yeast) or 37 °C (human) and applied to the nitrocellulose membranes *in vacuo*. Filters were immediately washed three times with 1 mL of ice-cold binding buffer, dried, and submerged in 8 mL of scintillation fluid (Econo-Safe; Research Products International). Radioactivity remaining on membranes was quantified using a Beckman LS 5000 TD scintillation counter. The amount of radioactive etoposide remaining on the filter in the absence of enzyme was subtracted prior to binding calculations.

DNA Cleavage Mediated by Topoisomerase IIα in CEM Cells.

Human CEM acute lymphoblastic leukemia cells (ATCC) were cultured under 5% CO_2 at 37 °C in growth media [RPMI 1640 medium (Cellgro by Mediatech, Inc.) containing 10% heat-inactivated fetal calf serum (Hyclone) and 2 mM glutamine (Cellgro by Mediatech, Inc.)]. The *In vivo* Complex of Enzyme (ICE) bioassay (*176, 177*) (as modified on the TopoGEN, Inc. website) was employed to determine the ability of PCB metabolites to induce topoisomerase IIα-mediated DNA breaks in CEM cells. Exponentially growing cultures were treated with 25 μM PCB metabolite for 6 h, 10 μM benzoquinone for 4 h or 10 μM

etoposide, DEPT, EPT, DDEPT or camptothecin for 2 h. Cells (~5 x 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 (~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 complexes formed between topoisomerase and DNA were detected using polyclonal antibodies directed against human topoisomerase IIα (Kiamaya Biochemical Co.) or human topoisomerase I (Biolegend) at a 1:1000 dilution.

H2AX Phosphoryolation Mediated by Topoisomerase II Poisons.

Human CEM acute lymphoblastic leukemia cells (ATCC) were cultured as described above. Cells (4 × 10^6) were seeded in a total of 10 mL of growth media. Phosphorylation of H2AX after exposure to topoisomerase II poisons was monitored by western blot. Cells were exposed to 25 μM etoposide for 2 h or 10 μM benzoquinone for 4 h. Cells were harvested and lysed with 50 μL 50 mM Tris-HCl, pH 7.9, 100 mM NaCl and 0.5% NP-40. A total of 35 μg of protein from cell lysates was loaded from each sample onto a 4-20% SDS-polyacrylamide gel (purchased from Sigma) and subjected to electrophoresis. Protein was then transferred to an activated PVDF membrane and γ-H2AX levels were determined by probing with a monoclonal anti-rabbit antibody directed to the H2AX phosphoprotein at a 1:2000 dilution.

Caspase-3 Activation

Human CEM acute lymphoblastic leukemia cells were cultured as described above. Cells (4×10^6) were seeded in a total of 10 mL of growth media. Cells were treated with 15 μM Z-VAD-fmk or DMSO vehicle 2 h prior to treatment with 25 μM etoposide for 2 h or 10 μM benzoquinone for 4 h, washed in 10 mL of growth media without compound. 2×10^6 cells were collected immediately following the drug washout and again 6 h later. Cells treated with Z-VAD-fmk prior to addition of topoisomerase II poison were maintained in the agent during the 6 h recovery period. Caspase-3 activation was determined by using the Apo-Alert Caspase-3 Colorimetric Assay kit (Clontech) as described by the manufacturer.

MLL Gene Aberrations Induced by Topoisomerase II Poisons.

Human CEM acute lymphoblastic leukemia cells were cultured as described above. Cells (8×10^6) were seeded in a total of 20 mL of growth media. Cells were exposed to 25 μM etoposide for 2 h or 10 μM benzoquinone for 4 h. Cells were washed in 10 mL of growth media without compound and were resuspended in 30 mL of growth media and 1×10^6 cells were collected every 24 hours for 5 days. An additional 1×10^6 cells cultured in the absence of either agent were also collected for comparison. In experiments that analyzed the role of apoptotic nucleases in generating *MLL* aberrations, cells were treated with 15 μM Z-VAD-fmk 2 h prior to the addition of topoisomerase II poison and throughout the recovery period. Cells (1×10^6) were harvested 3 days following exposure to either etoposide or benzoquinone.

Genomic DNA from treated and untreated cells was purified using a DNA Wizard Genomic DNA Isolation kit (Promega). DNA (5 μg) was digested with 40 units of *XbaI* (New England Biolands) in 200 μL of buffer supplied by the manufacturer. DNA was ethanol precipitated and ligated with 2000 units of T4 DNA ligase (New England Biolabs) in 300 μL of buffer supplied by the manufacturer to form circular DNA PCR substrates. DNA was ethanol precipitated and resuspended in 75 μL of 10 mM Tris-HCl, pH 7.7. Samples were further purified by passing the circularized DNA through a TE-100 column (Clontech) to remove excess ATP.

A modified procedure described by Libura *et al.* was used to analyze the *MLL* bcr (*178*). Two rounds of PCR were performed to amplify the 1.8 kb *XbaI* region of the *MLL* bcr. The forward primers for the first and second round of PCR were 5′- TCTACAAGTGCCAGGGGTCT-3′ and 5′-AATAGCATGCTGCCT-GCACTGCACTCCTAA-3′, respectively. The reverse primers for the first and second round of PCR were 5′- CCCGACGTGGATTTTCTTTA-3′ and 5′-GATCG-TAGGATATGTCCCTTATAAATGACAAACTACTGCTTCC-3′, respectively. PCR reactions were carried out using a Long Template PCR kit (Roche Applied Biosciences). PCR mixtures for the first round of PCR contained 200 ng of circularized DNA (described above), with 125 ng of the appropriate forward and reverse primer, 2.5 μL of 10 mM dNTP (Sigma), 3.75 units of long template PCR polymerase mix in a total of 50 μL of the supplied "buffer 3". 2 μL of the first round reaction mix was used in lieu of 200 ng of circularized DNA in the second round reaction mix. For the first set of PCR the temperature pattern was as follows: 35 cycles at 94°C for 30 s, 54°C for 45 s, followed by 68°C extension period for 3 min. The extension period was extended by 2 min after the first 10

cycles and each 10 cycles thereafter. The second set of PCR the temperature pattern was as follows: 35 cycles at 94°C for 30 s, 56°C for 45 s, followed by 68°C extension period for 3 min. The extension period was extended by 2 min after the first 10 cycles and each 10 cycles thereafter. In both sets of PCR, the final length of the extension period for the final 5 cycles was 7 min.

Following PCR, 2 μL of agarose loading buffer was added to samples and amplified circularized DNA fragments were subject to gel eletrophorese in 1% agarose in TAE buffer containing 0.5 μg/mL ethidium bromide. DNA bands were visualized by ultraviolet light using an Alpha Innotech digital imaging system.

CHAPTER III

N-ACETYL-*p*-BENZOQUINONE IMINE, THE TOXIC METABOLITE OF
ACETAMINOPHEN, IS A TOPOISOMERASE II POISON

Introduction

Acetaminophen is the most widely used analgesic in the United States and
the world and is contained in more than one hundred prescription and non-
prescription products (*179, 180*). Unfortunately, the drug is also the second
leading cause of toxic drug overdoses in the United States (*181, 182*) and
accounts for ~50% of hospital admissions for poisoning in the United Kingdom
(*181, 182*). Over 56,000 cases of acetaminophen poisoning were reported in the
United States in the year 2000, resulting in 100 deaths (*181, 182*).

Acetaminophen is metabolized in the liver (*183*). Normally, the drug is
cleared from the body by sulfation or glucuronidation followed by renal
excretion (*179, 184-187*). However, if these two processes become overwhelmed
or if the cytochrome P450 system has been induced by prior insult to the liver
(such as alcohol ingestion), acetaminophen is converted to NAPQI by CYP2E1
and to a lesser extent by CYP1A2 and CYP3A4 (*179, 184-190*) (Figure 9). NAPQI
is cleared primarily by conjugation to glutathione (*179, 184-187*).

All of the harmful effects of acetaminophen have been attributed to the
formation of NAPQI (*34, 179, 184-187*). This reactive metabolite is toxic to cells
and induces apoptosis and necrosis in cultured cells and *in vivo* (*34, 191*). In
addition, the compound is genotoxic in humans and genotoxic and carcinogenic
in animals (*33, 34*). It generates DNA strand breaks, gaps, and other
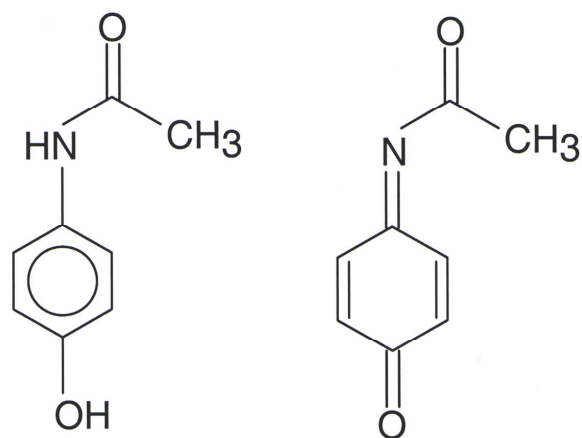
Figure 9. Stuctures of acetaminophen and NAPQI.

chromosomal aberrations, and induces sister chromatid exchange (*33, 34*). The mechanistic basis for the actions of NAPQI is not fully understood. Since the compound can alkylate and oxidize proteins, it was initially believed that NAPQI toxicity was due to a direct inactivation of cellular enzymes (*34, 184, 192*). More recent studies, however, suggest that toxicity is also facilitated by the depletion of cellular glutathione (*34, 184, 193*). This depletion opens the cell to a variety of reactive chemicals (including oxygen radicals), which inactivate enzymes and damage the genetic material (*34, 193*). Finally, since depletion of glutathione allows the accumulation of NAPQI in cells, it is once again believed that (at least) some of the cytotoxic/genotoxic effects of NAPQI may be caused by the direct alkylation and/or oxidation of cellular proteins by the compound (*34*).

The specific cellular proteins that are modified by NAPQI following acetaminophen metabolism are not well characterized. However, the genotoxic effects of NAPQI are consistent with the actions of topoisomerase II-targeted drugs. Since the genotoxic events triggered by NAPQI resemble those of topoisomerase II poisons, the effects of the compound on human topoisomerase IIα were examined.

Results

NAPQI Increases DNA Cleavage Mediated by Human Topoisomerase IIα.

The plasma concentration of acetaminophen in humans following a standard drug dose ranges from 50–100 *μM* (*34, 179, 184-187, 194*). The average plasma concentration in patients who suffer accidental acetaminophen overdose

is ~250 μM drug, while that in patients who intentionally overdose is ~950 μM drug (*181*). With these values in mind, the effects of 0–200 μM acetaminophen and NAPQI on DNA cleavage mediated by human topoisomerase IIα were determined (Figure 10).

NAPQI increased levels of topoisomerase II-mediated double-stranded DNA breaks in a concentration-dependent manner. The highest relative amount of DNA scission was observed at 100 μM NAPQI and was >5–fold higher than the no drug control. This cleavage enhancement is as compared to the widely used anticancer drug, etoposide, which stimulated enzyme-mediated DNA cleavage ~8–fold at a similar concentration. In marked contrast, acetaminophen had a marginal effect on the DNA cleavage activity of human topoisomerase IIα. These results indicate that metabolism of acetaminophen to NAPQI converts the parent compound to a strong topoisomerase II poison.

Several controls were carried out to ensure that the DNA cleavage seen in Figure 10 was mediated by topoisomerase IIα (Figure 11). First, no linear DNA was generated by NAPQI in the absence of enzyme. Second, scission was reversed when EDTA was added to the reaction prior to denaturation of topoisomerase IIα with SDS. This reversibility is consistent with an enzyme-mediated reaction. Third, the electrophoretic mobility of the cleaved DNA (*i.e.*, linear band) was dramatically retarded in the absence of proteinase K treatment, indicating that all of the cleaved plasmid molecules were covalently attached to topoisomerase IIα. These findings provide strong evidence that the double-stranded DNA breaks observed in the presence of NAPQI are generated by human topoisomerase IIα.
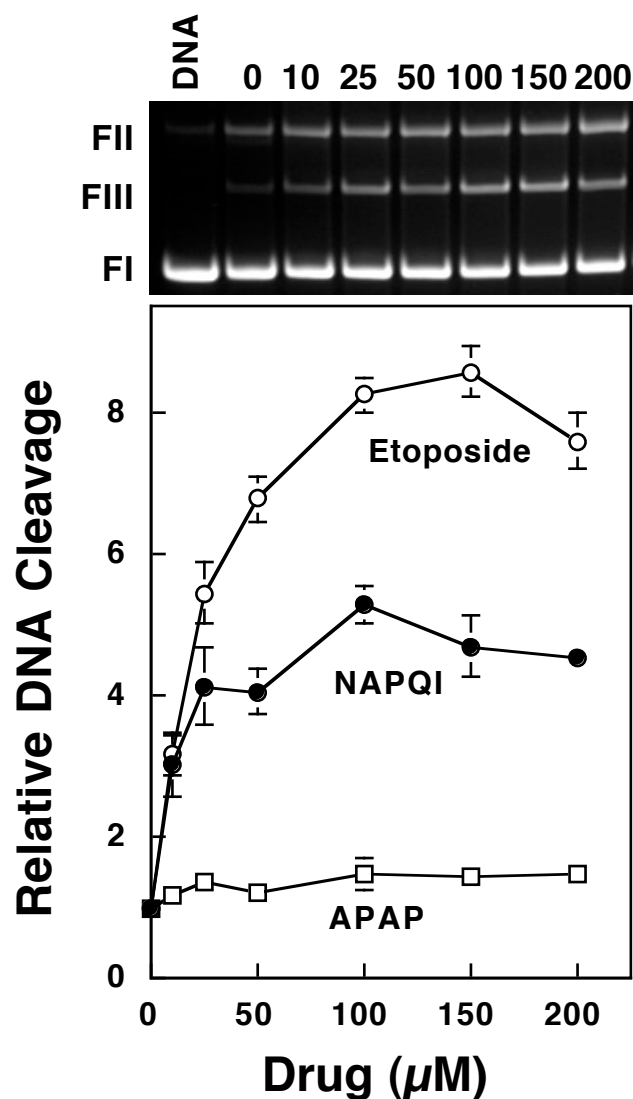
Figure 10. NAPQI stimulates DNA cleavage mediated by human topoisomerase IIα. An ethidium bromide-stained agarose gel of DNA cleavage reactions carried out in the presence of 0-200 μM NAPQI is shown at top. A lane containing DNA in the absence of enzyme (DNA) is shown as a control. The mobilities of negatively supercoiled DNA (form I, FI), nicked circular plasmid (form II, FII), and linear molecules (form III, FIII) are indicated. Levels of DNA cleavage were quantified and expressed as a fold enhancement over reactions that were carried out in the absence of drug. Assays mixtures contained NAPQI (closed circles), etoposide (open circles) or acetaminophen (APAP, open squares). Error bars represent the standard deviation of three independent experiments.
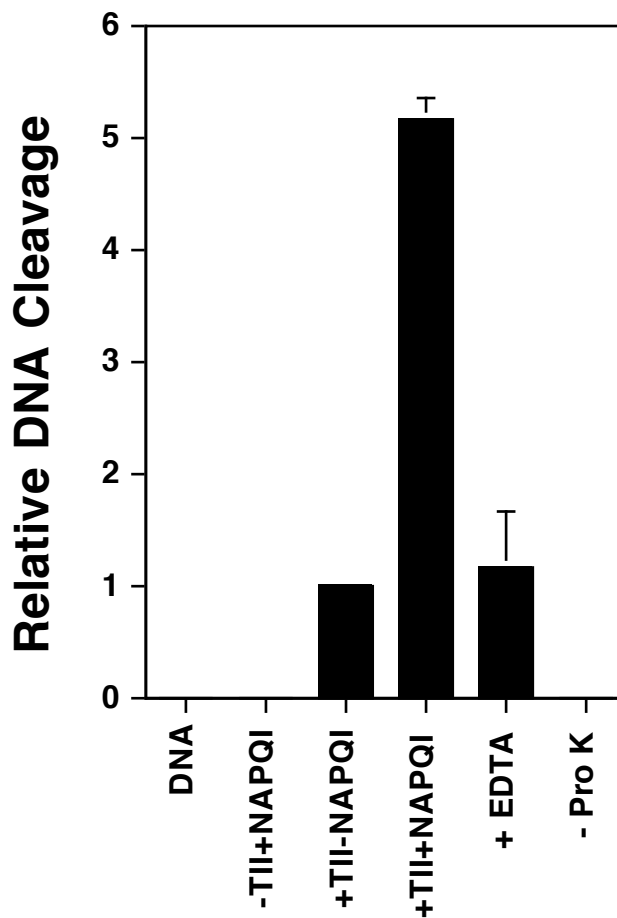
Figure 11. DNA cleavage in the presence of NAPQI is mediated by topoisomerase IIα. Levels of DNA cleavage were quantified and expressed as a fold enhancement over reactions that were carried out in the absence of drug. Data for DNA alone (DNA) or with NAPQI (NAPQI) in the absence of enzyme are shown. DNA cleavage mediated by human topoisomerase IIa in the absence (TII–NAPQI) or presence (TII+NAPQI) of 100 μM NAPQI was examined. To determine whether the DNA cleavage observed in the presence of NAPQI was protein-linked, proteinase K treatment was omitted (-Pro K). Reversibility of reactions containing NAPQI was examined by adding EDTA prior to SDS treatment (+EDTA). Error bars represent the standard deviation of three independent experiments.

To further examine the effects of NAPQI on DNA scission mediated by the human type II enzyme, singly end-labeled linear pBR322 plasmid was used as a substrate. This linear DNA allows cleavage to be monitored at the site-specific level. As seen in Figure 12, NAPQI increased DNA scission at every site cleaved by the enzyme in the absence of drug. It also, induced cleavage at a number of additional DNA sites. Levels of cleavage induced by 100 μM NAPQI were similar to those observed in reactions that contained 25 μM etoposide. This result is consistent with assays that employed negatively supercoiled plasmid substrate (see Figure 10). While the DNA sites cleaved by topoisomerase IIα in the presence of the two drugs were similar, site utilization differed. A number of sequences appeared to be preferentially cleaved when NAPQI was present in reactions.

Recent reports indicate that agents that are reactive toward protein sulhydryl groups increase levels of DNA breaks generated by human topoisomerase IIα when they are incubated with the enzyme in the presence of DNA (*118, 195*). A common characteristic of these "alkylating topoisomerase II poisons" is that they rapidly inactivate topoisomerase IIα when they are incubated with the enzyme in the absence of its DNA substrate (*118*). Since NAPQI is reactive toward protein sulhydryl groups and alkylates a number of proteins (*34, 192*), similar incubation studies were performed (Figure 13). NAPQI rapidly inactivated human topoisomerase IIα in the absence of DNA. Within one min, the enzyme lost ~90% of its DNA cleavage activity. These results suggest that the effects of NAPQI on the human enzyme may be due (at least in part) to a covalent modification of the protein.
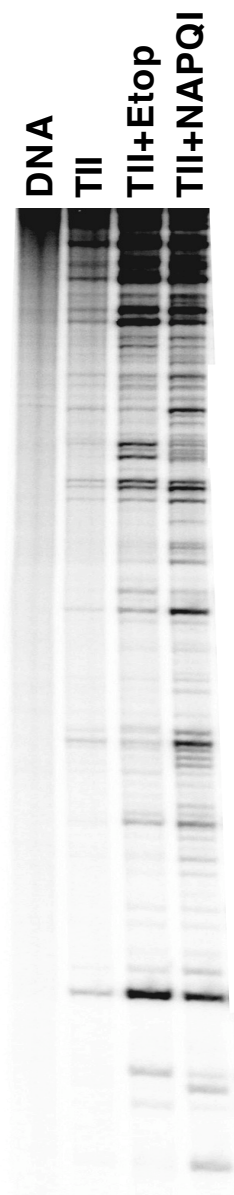
Figure 12. DNA cleavage site utilization of human topoisomerase IIα in the presence of NAPQI. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions contained no drug (TII), 25 μM etoposide (TII+Etop), or 100 μM NAPQI (TII+NAPQI). A DNA control is shown in the far left lane (DNA). Data are representative of three independent assays.
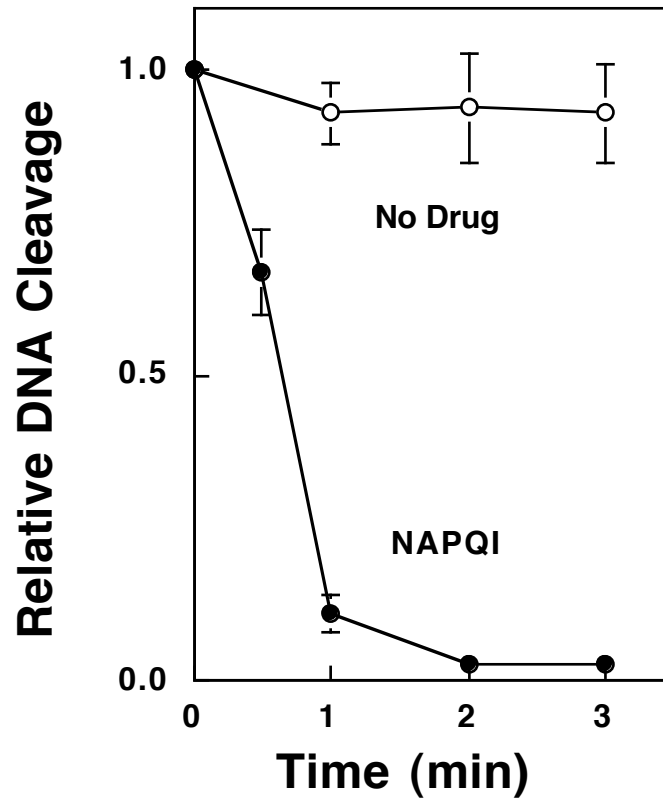
Figure 13. NAPQI rapidly inactivates human topoisomerase IIα in the absence of DNA. Reaction mixtures were incubated in the absence (open circles) or presence of 100 μM NAPQI (closed circles) for 0 to 3 min prior to the addition of DNA. Levels of DNA cleavage in reaction mixtures that were not pre-incubated (*i.e.*, time zero) were set to 100%. Error bars represent the standard deviation of three independent experiments.

NAPQI Inhibits DNA Religation Mediated by Human Topoisomerase IIα.

Topoisomerase II poisons increase levels of enzyme-mediated DNA breaks by two non-mutually exclusive mechanisms. Whereas etoposide appears to act primarily by inhibiting the ability of topoisomerase II to religate DNA breaks, other poisons such as quinolones appear to act primarily by enhancing the forward rate of DNA scission (*15, 16, 97, 98, 109, 196*).

To determine the mechanistic basis for the actions of NAPQI against human topoisomerase IIα, the effects of the compound on enzyme-mediated religation were examined (Figure 14). The rate of religation in the presence of NAPQI was several–fold slower than observed in the absence of drug and approached levels of inhibition seen with etoposide (not shown). While the enzyme religated <15% of the cleaved DNA in 30 s in reactions that contained 25 μM NAPQI, the $t_{1/2}$ value for religation in the absence of drug was ~10 s. On the basis of these values, it is proposed that NAPQI increases levels of topoisomerase II-mediated DNA breaks primarily by inhibiting the ability of the enzyme to religate DNA.

NAPQI Inhibits DNA Strand Passage Catalyzed by Human Topoisomerase IIα.

A number of well characterized topoisomerase II poisons inhibit the overall catalytic activity of the enzyme (*197-201*). However, in many cases, the ability to increase DNA cleavage and inhibit overall catalytic activity require significantly different drug concentrations. This finding implies that different drug binding sites on topoisomerase II may be responsible for the two events. Therefore, to determine the relationship between cleavage enhancement and catalytic inhibition for NAPQI, the effects of the drug on topoisomerase II-catalyzed DNA relaxation were examined.
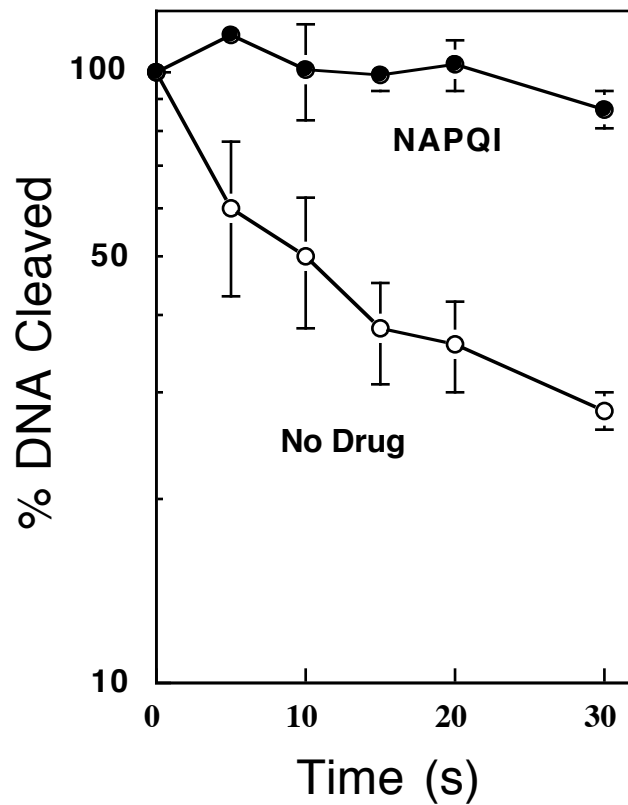
Figure 14. DNA religation mediated by human topoisomerase IIα is inhibited by NAPQI. DNA religation mediated by topoisomerase IIα was examined in the absence of drug (open circles), or in the presence of 25 μM NAPQI (closed circles). Samples were incubated at 37 ˚C to establish DNA cleavage/religation equilibria and were then shifted to 0 ˚C to initiate religation. The amount of DNA cleavage observed at equilibrium for each drug was set to 100% at time zero. DNA ligation was quantified by the loss of linear cleaved molecules. Points represent the average of three independent experiments.

NAPQI was a strong inhibitor of DNA relaxation catalyzed by human topoisomerase IIα. When catalytic levels of the enzyme (~5 nM) were employed in assays, 50% inhibition was observed at nearly stoichiometric levels of NAPQI (~5 nM) (not shown). In order to compare results of catalytic and DNA cleavage assays directly, relaxation was carried out using the same concentration of topoisomerase IIα that was utilized for DNA scission (~135 nM). Short time courses (30 s) and low ATP concentrations (250 μM) were employed in these latter experiments so that inhibition could be monitored accurately. As seen in Figure 15, 50% inhibition of DNA relaxation at high topoisomerase IIα concentrations was observed at ~1.5 μM NAPQI. This drug concentration is as compared to the ~15 μM NAPQI that was required to enhance DNA cleavage to 50% of its maximal value. These results suggest that the inhibition of overall catalytic activity and the stimulation of DNA cleavage by NAPQI may be due to separate events.

NAPQI is a Topoisomerase II Poison in Cultured Human Cells.

The ICE bioassay was employed to determine whether NAPQI affects levels of DNA cleavage mediated by topoisomerase IIα in cultured human CEM cells. In this assay, cells are lysed with an ionic detergent, and proteins that are covalently attached to genomic DNA are separated from free proteins by sedimentation through a CsCl cushion. The pelleted DNA from cultures treated with no drug, 50 or 100 μM NAPQI, or 10 μM etoposide (for comparison) was blotted and probed with a polyclonal antibody specific for human topoisomerase IIα. As seen in Figure 16, levels of topoisomerase IIα that were covalently
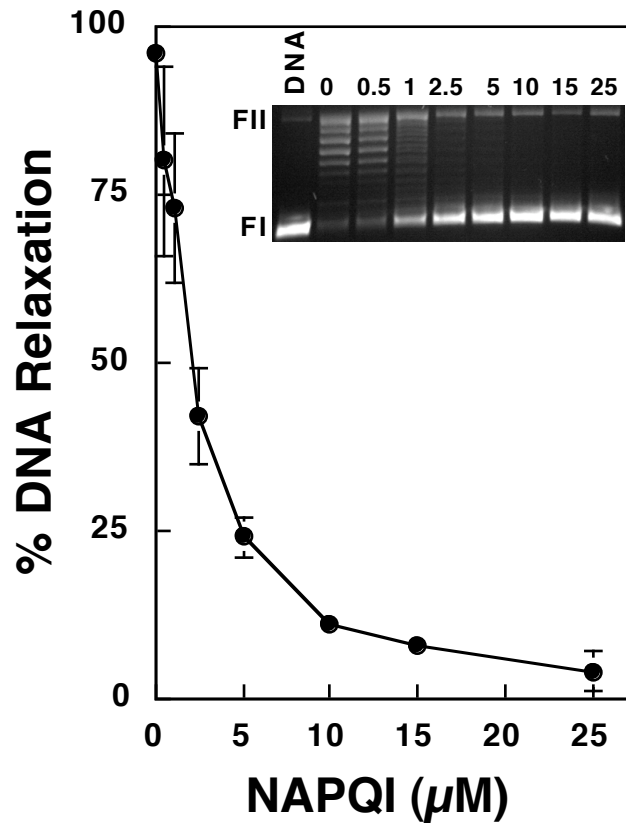
Figure 15. NAPQI inhibits the overall catalytic activity of topoisomerase IIα. DNA relaxation reactions catalyzed by topoisomerase IIα were carried out for 30 s in the presence of 0-25 μM NAPQI. DNA relaxation was quantified by the disappearance of negatively supercoiled DNA substrate. Error bars represent the standard deviation of three independent experiments. The inset shows an ethidium bromide-stained agarose gel of DNA relaxation reactions carried out in the presence of 0-25 μM NAPQI. A lane containing DNA in the absence of enzyme (DNA) is shown as a control. The mobilities of negatively supercoiled DNA (form I, FI) and nicked circular plasmid (form II, FII) are indicated.

Etoposide

No Drug

50 μM NAPQI

100 μM NAPQI
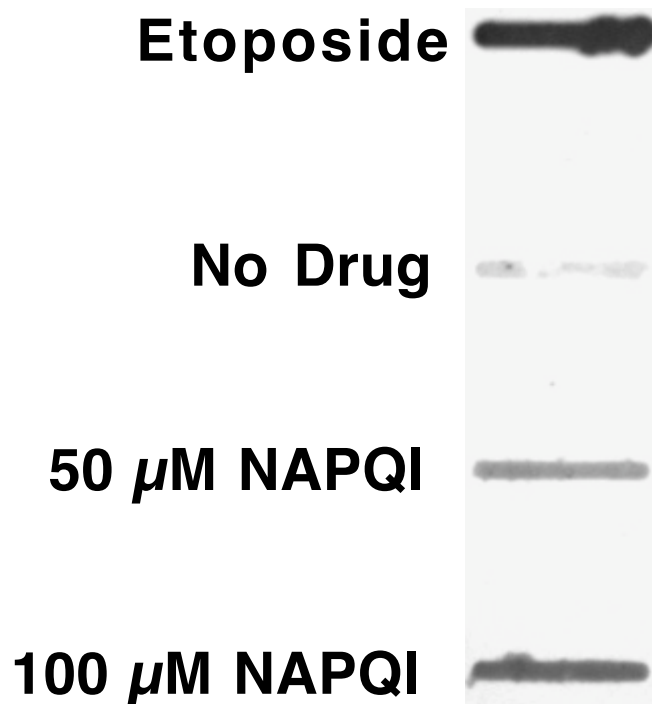
Figure 16. NAPQI enhances human topoisomerase IIα-mediated DNA cleavage in treated human CEM cells. The ICE bioassay was used to monitor the level of cleavage complexes in cells treated with NAPQI. DNA (10 μg) from cultures treated with no drug, 50 or 100 μM NAPQI, or 10 μM etoposide was blotted onto a nitrocellulose membrane. Blots were probed with a polyclonal antibody directed against human topoisomerase IIα. Results are representative of two independent experiments.

attached to DNA following treatment with NAPQI increased several–fold over the drug-free control.

It should be noted that some cleavage-independent topoisomerase IIα-DNA crosslinking was observed *in vitro* in DNA cleavage assays that contained NAPQI. Therefore, it is possible that a portion of the covalent topoisomerase IIα-DNA complex observed in NAPQI-treated CEM cells actually reflects protein-DNA crosslinking rather than enzyme-mediated DNA scission. However, the topoisomerase IIα:DNA base pair ratio (~1:325) used in the *in vitro* cleavage assays was >20 times higher than estimated (~1:7500) for CEM cells (*202*). When *in vitro* experiments were carried out using topoisomerase IIα:DNA base pair ratios that approximated the cellular condition, no significant protein:DNA crosslinking was observed (not shown). Therefore, it is likely that NAPQI acts as a topoisomerase II poison in cultured human cells.

Acetaminophen Does Not Compete with Etoposide for Human Topoisomerase IIα.

Acetaminophen does not significantly increase enzyme-mediated DNA cleavage *in vitro* (see Figure 10). However, it still is possible that the drug interacts with topoisomerase IIα at the same site as anticancer agents such as etoposide, but does not induce DNA cleavage. Such is the case for the antibacterial quinolone ciprofloxacin. This drug targets prokaryotic type II topoisomerases and has little effect on DNA scission mediated by the eukaryotic enzyme (*198, 203, 204*). However, ciprofloxacin competes with etoposide and several other anticancer drugs, blocking their ability to increase DNA cleavage by topoisomerase II (*204*).

Considering the wide use of products that contain acetaminophen, it is important to know whether this drug alters the efficacy of topoisomerase II-targeted chemotherapeutic agents. Therefore, two experiments were carried out to determine the effects of acetaminophen on the actions of etoposide against human topoisomerase IIα. First, an *in vitro* competition assay was performed. In this assay, etoposide (25 μM) was mixed with acetaminophen (0–500 μM) and then added to a topoisomerase IIα-DNA complex. Even at a concentration of 500 μM acetaminophen (which is 3–5 times higher than the upper range of the normal non-toxic dose found in blood), no decrease in etoposide-induced DNA cleavage was observed (Figure 17). Therefore, if acetaminophen interacts with topoisomerase IIα at all, it probably does not interact with the binding site utilized by anticancer drugs such as etoposide.

Second, ICE bioassays were performed to examine the effects of acetaminophen on covalent topoisomerase IIα-DNA complexes in human CEM cells. As seen in Figure 17, no increase in complex formation was observed in the presence of 150 μM acetaminophen (the upper limit for non-toxic drug doses). Furthermore, pre-incubation of cells with 150 μM acetaminophen had no effect on levels of topoisomerase IIα-DNA complexes that were induced by 10 μM etoposide. Thus, it appears that normal therapeutic doses of acetaminophen do not significantly interfere with the actions of etoposide on topoisomerase IIα in treated cells.
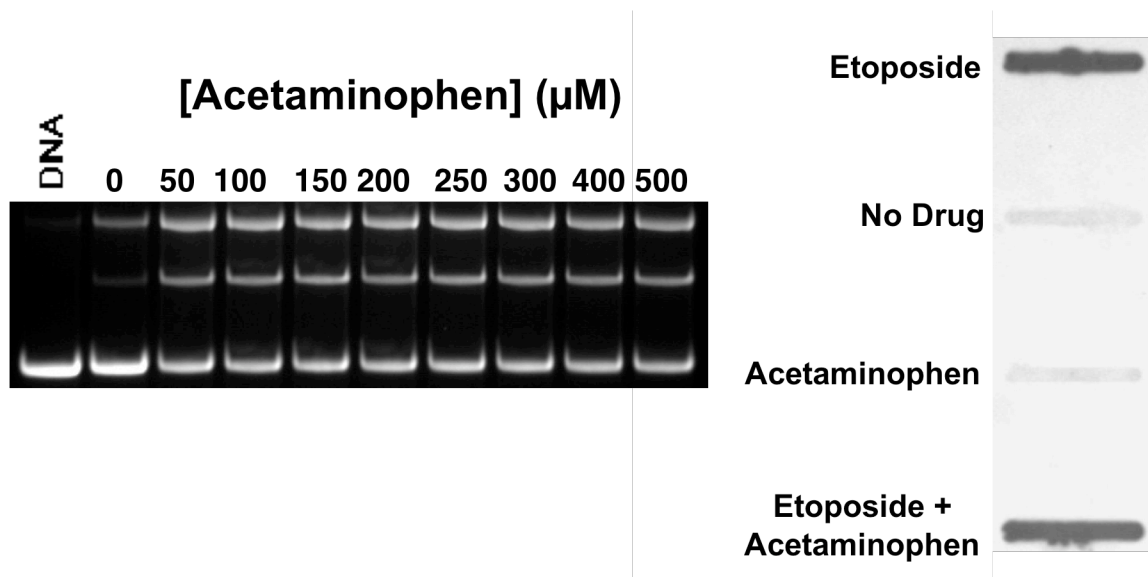
Figure 17. Acetaminophen does not interfere with the ability of etoposide to increase DNA cleavage mediated by human topoisomerase IIα *in vitro* and cultured human cells. Left: An ethidium bromide-stained agarose gel of DNA cleavage reactions carried out in the presence of 25 μM etoposide and 0-500 μM acetaminophen is shown. Reactions containing DNA in the absence (DNA) or presence of topoisomerase IIα (Topo II), but in the absence of drugs are shown as controls. Levels of DNA cleavage were quantified and expressed as a fold enhancement over reactions that contained no acetaminophen. Error bars represent the standard deviation of three independent experiments. Right: The ICE bioassay was used to monitor the level of cleavage complexes in cells. DNA (10 μg) from cultures treated with no drug, 150 μM acetaminophen, 10 μM etoposide or 150 μM acetaminophen plus 10 μM etoposide was blotted onto a nitrocellulose membrane. Blots were probed with a polyclonal antibody directed against human topoisomerase IIα. Results are typical of two independent experiments.

Discussion

Although acetaminophen is the most widely used analgesic in the world, it causes hepatotoxicity if ingested in doses that are beyond its normal therapeutic window (*34, 179-182, 184-187*). Acetaminophen-induced liver damage also can occur if the organ has been compromised by previous insults (*185-187, 189, 190*). All of the harmful effects of acetaminophen have been attributed to its reactive metabolite, NAPQI (*185-187, 194*). This compound induces DNA strand breaks and other genotoxic events in a variety of treated cells and also promotes apoptosis and necrosis (*33, 34, 191*). Results of the present study indicate that NAPQI is a potent poison of human topoisomerase IIα and induces enzyme-mediated double-stranded DNA breaks approximately half as well as etoposide, a widely prescribed anticancer drug.

Relationships between the enhanced topoisomerase IIα-mediated DNA cleavage and the cytotoxic/genotoxic effects of NAPQI are not known. However, exposure of cells to topoisomerase II poisons causes DNA strand breaks and many of the other cytotoxic/genotoxic events seen following NAPQI treatment (*11-17, 109*). While NAPQI reacts with a number of proteins *in vivo* and induces oxidative damage in cells (*34*), the present findings are consistent with a role for topoisomerase IIα in mediating the toxicity of this acetaminophen metabolite.

It is presumed that NAPQI increases levels of cellular DNA cleavage by human topoisomerase IIα by a mechanism that involves the direct interaction of the compound with the enzyme-DNA complex. However, NAPQI-treatment also generates DNA adducts in cells by inducing oxidative stress (*34*). Many of these adducts enhance topoisomerase II-mediated DNA cleavage, or are converted to

abasic sites, which are strong topoisomerase II poisons (*119, 120, 122, 161, 205-208*). Therefore, it is possible that some of the increase in enzyme-mediated DNA scission that is observed following cellular exposure to NAPQI results from an interaction of topoisomerase IIα with drug-induced DNA damage.

As determined by experiments carried out *in vitro* or in cultured human cells, acetaminophen does not significantly raise levels of DNA cleavage generated by topoisomerase IIα. Furthermore, it does not antagonize the poisoning effects of etoposide. Therefore, normal doses of acetaminophen should neither cause topoisomerase II-mediated DNA damage, nor decrease the efficacy of topoisomerase II-based cancer chemotherapy.

Structural changes between acetaminophen and NAPQI (*i.e.*, the loss of two hydrogen molecules; see Figure 9) are relatively minor. The primary difference between the two compounds is related to their chemical reactivity. While hydroquinones (*i.e.*, acetaminophen) are relatively inert toward proteins, quinones (*i.e.*, NAPQI) are very reactive toward protein sulfhydryl groups and other residues (*34*). Recent work suggests that thiolation agents enhance topoisomerase II-mediated DNA scission (*118, 195*). Therefore, it is possible that NAPQI alkylation of topoisomerase IIα plays a role in stabilizing DNA cleavage complexes.

In summary, exposure to NAPQI increases levels of DNA cleavage mediated by topoisomerase IIα *in vitro* and in cultured cells. These findings suggest that at least some of the cytotoxic/genotoxic effects caused by acetaminophen overdose may be mediated by the actions of NAPQI as a topoisomerase II poison.

CHAPTER IV

POLYCHLORINATED BIPHENYL QUINONE METABOLITES POISON
HUMAN TOPOISOMERASE IIα: ALTERING ENZYME FUNCTION BY
BLOCKING THE N-TERMINAL PROTEIN GATE

Introduction

Polychlorinated biphenyls (PCBs) represent a class of compounds with two aromatic six-member rings that contain from one to ten chlorine atoms (*209, 210*). These chemicals were employed in a variety of large-scale industrial applications from the 1930s to the 1970s (*209-211*). For example, PCBs were used as organic diluents, lubricants, and cooling fluids, and were commonly utilized in the production of adhesives, paper, pesticides, and flame retardants. One of the properties that made PCBs so attractive for industrial purposes was their high stability and low biodegradability (*209, 211*). As a result, however, these compounds accumulated in the environment. Due to human health concerns, most countries banned the production and use of PCBs in the 1970s (*209, 211*). Three decades later, these compounds remain persistent environmental contaminants (*209, 211-213*).

Exposure to PCBs is associated with a broad spectrum of human health problems, including neurotoxicity, hepatotoxicity, hypothyroidism, and immunodeficiency (*209, 211, 214, 215*). PCBs are complete carcinogens in rodents and display genotoxic activity *in vitro* and *in vivo* (*35, 36*). For example, in laboratory studies, these compounds induce chromosomal aberrations and sister chromatid exchanges in cultured human lymphocytes (*216*). Similar chromosomal abnormalities have been found in the peripheral lympocytes of

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workers who were occupationally exposed to PCBs, with longer exposures correlating with greater DNA damage (*217, 218*). Finally, PCBs cause liver cancer in mice and rats and are listed by the Environmental Protection Agency as probable human carcinogens (*209, 215, 219*).

The underlying basis for the chromosomal damage induced by PCBs is not understood. However, it is believed that these compounds do not trigger DNA damage directly. Rather, they act through a series of phenolic and quinone-based metabolites (*162, 220-223*). It has been proposed that the generation of reactive oxygen species by redox cycling, the depletion of glutathione by adduction, or the generation of DNA lesions by oxidation may play a role in chromosome damage (*162, 220-223*). In addition, PCB quinone metabolites form protein adducts through cysteine residues in treated cells (*223*). The specific cellular proteins that are modified by these compounds are not well characterized. However, the genotoxic effects of PCBs are consistent with the actions of topoisomerase II poisons and a previous study found that these compounds inhibited enzyme-catalyzed DNA decatenation (*223*). Since the genotoxic events triggered by PCBs resemble those of topoisomerase II poisons, the effects of a variety of quinone and hydroquinone PCB metabolites on human topoisomerase IIα were examined.

PCB Metabolites Enhance DNA Cleavage Mediated by Human Topoisomerase IIα.

The PCB metabolites used in the present study are shown in Figure 18. The compounds were either mono- or di-chlorinated, and included a series of *p*-quinones and *o*-quinones, as well as a *p*-hydroquinone.

All of the quinone-based PCB metabolites increased DNA cleavage mediated by topoisomerase IIα greater than 4–fold (Figure 19). As seen in the left panel, 4'Cl-2,5pQ was equipotent to etoposide and increased cleavage ~8–fold. On the basis of titrations from 0–200 μM metabolites (not shown), levels of cleavage enhancement plateaued at ~100 μM for all of the quinones examined. In contrast to 4'Cl-2,5pQ, which was the most active quinone metabolite, the corresponding hydroquinone conger, 4'Cl-2,5HQ, displayed very little activity against topoisomerase IIα. No significant increase in DNA scission was observed up to 200 μM 4'Cl-2,5HQ (Figure 19, left panel). These results strongly suggest that PCB quinones are potent topoisomerase II poisons in vitro, but that PCB hydroquinones are far less active against the enzyme.

Since 4'Cl-2,5pQ was the most active metabolite, it was used as the "model" quinone for control experiments designed to determine whether the enhanced DNA cleavage induced by PCB metabolites was mediated by topoisomerase IIα (Figure 20). First, no linear DNA was observed in reactions that contained 100 μM 4'Cl-2,5pQ, but lacked enzyme. Second, the electrophoretic mobility of the cleaved DNA (*i.e.*, the linear band) was dramatically reduced in the absence of proteinase K treatment, indicating that all of the cleaved plasmid molecules were covalently attached to topoisomerase IIα. Third, scission was reversed when
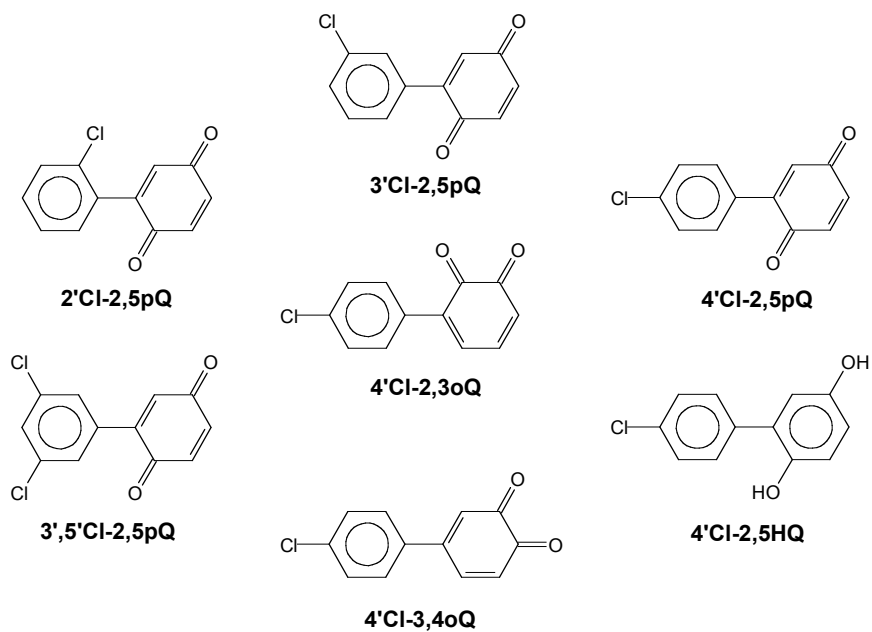
Figure 18. Structures of PCB metabolites.
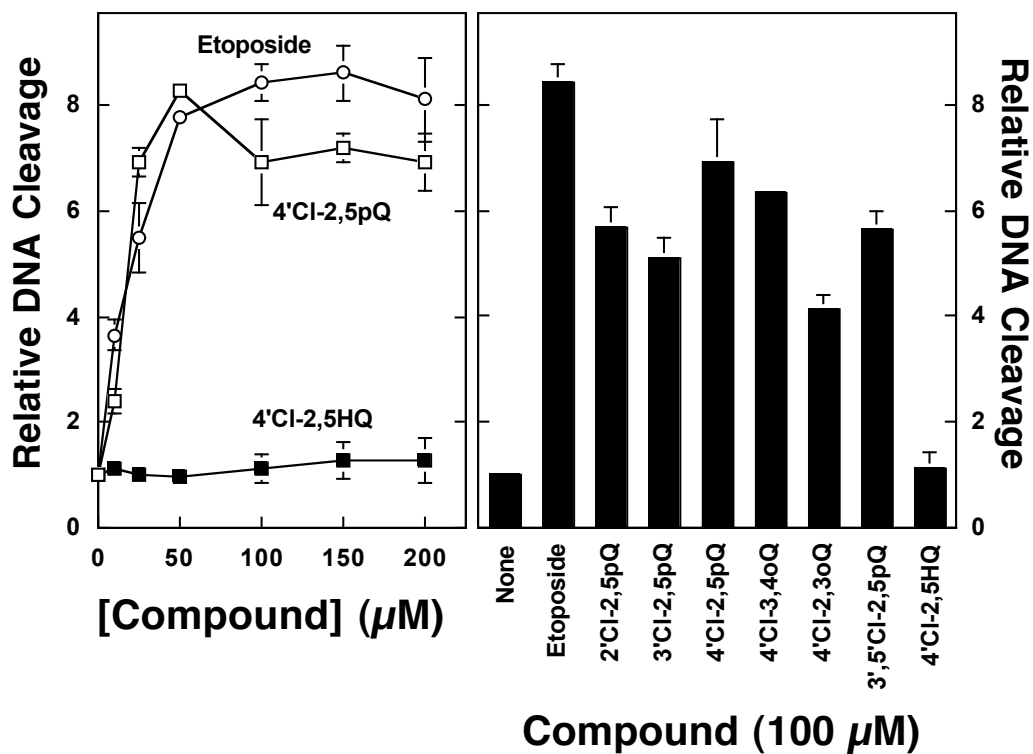
Figure 19. PCB metabolites stimulate DNA cleavage mediated by human topoisomerase IIα. Levels of DNA cleavage were quantified and expressed as a fold enhancement over reactions that were carried out in the absence of metabolites. Cleavage titrations are shown in the left panel. Reaction mixtures contained 0–200 μM 4'Cl-2,5pQ (open squares), or 4'Cl-2,5HQ (closed squares). A parallel titration with etoposide (open circles) is shown as a control. Relative DNA cleavage enhancement induced by 100 μM PCB metabolites is shown in the right panel. Error bars represent the standard deviation of at least three independent experiments.
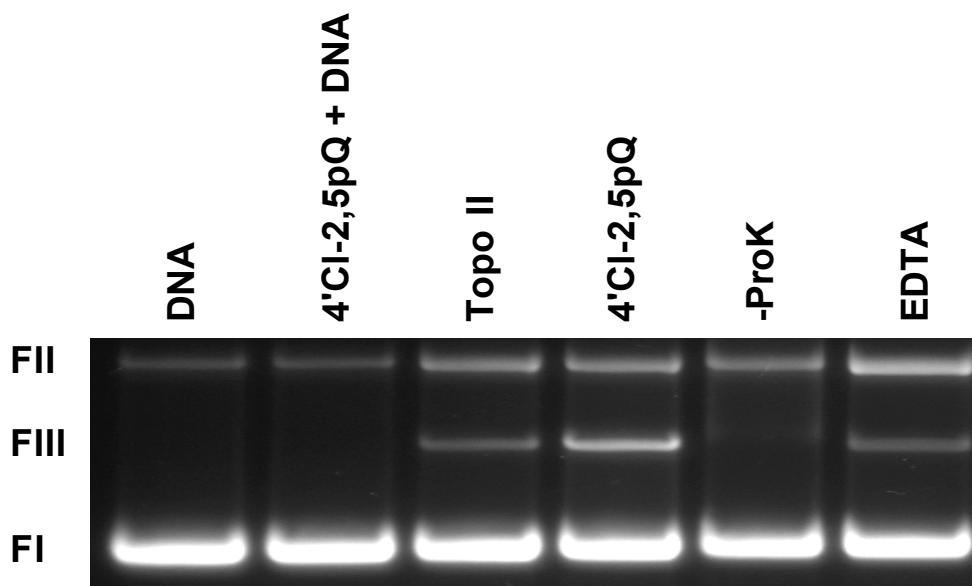
Figure 20. DNA cleavage induced by PCB metabolites is mediated by topoisomerase IIα. An ethidium bromide-stained agarose gel of DNA cleavage reactions is shown. DNA controls lacking enzyme were in the absence (DNA) or presence of 4′Cl-2,5pQ (4′Cl-2,5pQ + DNA). DNA cleavage mediated by human topoisomerase IIα in the absence (Topo II) or presence (4′Cl-2,5pQ) of 100 μM 4′Cl-2,5pQ was examined. To determine whether the DNA cleavage observed in the presence of 4′Cl-2,5pQ was protein-linked, proteinase K treatment was omitted (-Pro K). Reversibility of reactions containing 4′Cl-2,5pQ was examined by adding EDTA prior to SDS treatment (EDTA). The electrophoretic mobilities of negatively supercoiled DNA (form I, FI), nicked circular plasmid (form II, FII), and linear molecules (form III, FIII) are indicated. Data are representative of two independent assays.

EDTA was added to reaction mixtures before cleavage complexes were trapped by the addition of SDS. This reversibility is inconsistent with a non-enzymatic reaction. Taken together, the above findings provide strong evidence that PCB quinones increase DNA cleavage through an enzyme-mediated reaction.

Previous studies suggest that quinones alter the activity of topoisomerase IIα by covalently modifying the enzyme (*1, 118, 223*). This is the primary reason why quinones are believed to be more active toward the enzyme than hydroquinones (*117, 223*). If protein adduction plays an important role in the actions of PCB metabolites, once a quinone has modified topoisomerase IIα, it should not be able to dissociate from the enzyme. Therefore, order-of-addition experiments were carried out to address the effects of the reducing agent DTT on the actions of 4'Cl-2,5pQ (Figure 21). As expected, no enhancement of enzyme-mediated DNA scission was observed when 100 μM quinone was incubated with 500 μM DTT prior to its addition to the topoisomerase IIα-DNA complex. Conversely, once a cleavage complex was established in the presence of 4'Cl-2,5pQ, DTT had no effect on the enhancement of DNA scission. In control experiments, DTT did not significantly alter levels of DNA cleavage mediated by topoisomerase IIα in the absence of the quinone (Figure 21) or in the presence of the anticancer drug etoposide (which does not form a covalent protein adduct, not shown) (*1, 118, 223*). These findings indicate that PCB metabolites increase DNA strand breaks generated by topoisomerase IIα by forming covalent adducts with the enzyme.

Quinones can undergo enzyme dependent redox cycling to generate free radicals that damage nucleic acids (*114, 222*). In addition, some biologically relevant quinones can form adducts with DNA (*30, 114, 220, 224*). Since many

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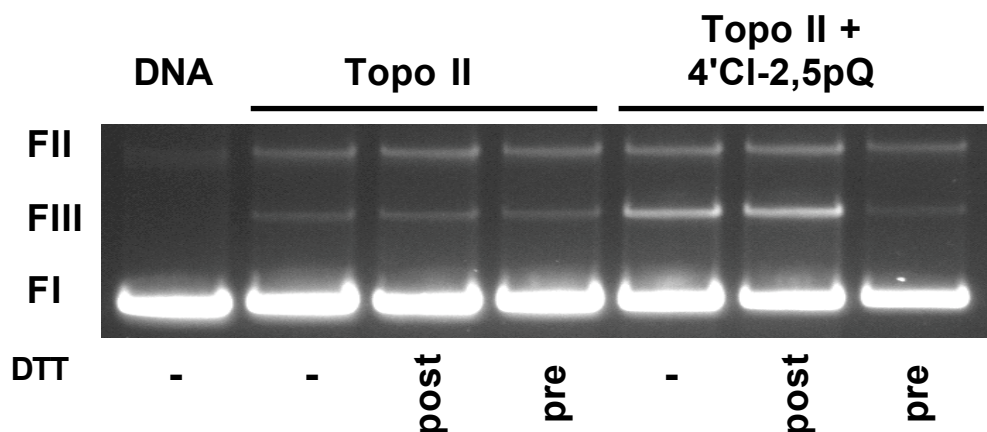
Figure 21. Effects of reducing agents on the ability of 4'Cl-2,5pQ to enhance DNA cleavage mediated by human topoisomerase IIα. A representative ethidium bromide-stained agarose gel is shown. The DNA substrate is shown as a control in lane 1. Reactions were carried out in the absence (lanes 2-4) or presence of 100 μM 4'Cl-2,5pQ (lanes 5-7). Reactions contained no reducing agent (-, lanes 1, 2 and 5), 500 μM DTT that was added to reaction mixtures after topoisomerase II-DNA cleavage complexes were established (post, lanes 3 and 6), or 500 μM DTT that was incubated with 4'Cl-2,5pQ prior to the addition of the quinone to cleavage reactions (pre, lanes 4 and 7). The mobilities of supercoiled (FI), nicked circular (FII), and linear (FIII) DNA molecules are as in Figure 20. Results are representative of four independent experiments.

DNA lesions have been shown to poison human topoisomerase IIα (*121, 122, 124, 126, 161, 207*), it is possible that the enhancement of DNA scission by the PCB metabolites is due to a modification of the plasmid substrate. To address this issue, 100 μM 4'Cl-2,5pQ was incubated with negatively supercoiled pBR322 for 6 min, but removed by gel filtration prior to the addition of the enzyme. No enhancement of DNA cleavage was observed under these conditions (data not shown). Thus, modification of DNA does not appear to contribute to the effects of PCB metabolites on topoisomerase IIα.

To determine the effects of PCB metabolites on the DNA cleavage site specificity of topoisomerase IIα, a singly end-labeled fragment of pBR322 was used as the substrate. This 4330 bp linear DNA allows cleavage to be monitored at specific sites. Four of the quinone metabolites were used for cleavage mapping, including both mono- and di-chlorinated *p*-quinones. All of them increased topoisomerase IIα-mediated DNA scission at a number of distinct sequences (Figure 22). As determined by visual inspection, the quinones displayed DNA cleavage site specificities that were identical to one another, but differed significantly from that of etoposide. Furthermore, strong sites of cleavage enhancement appeared to correspond to sites cut by topoisomerase IIα in the absence of poisons. This finding suggests that PCB quinones may be acting distal to the active site of the enzyme, since drugs that act at the scissile bonds generally alter cleavage site utilization by topoisomerase II (*225*). Consistent with the global DNA cleavage data, 4'Cl-2,5HQ had little effect on enzyme-mediated DNA scission and yielded a cleavage pattern that was similar to that of the topoisomerase IIα control (Figure 22).
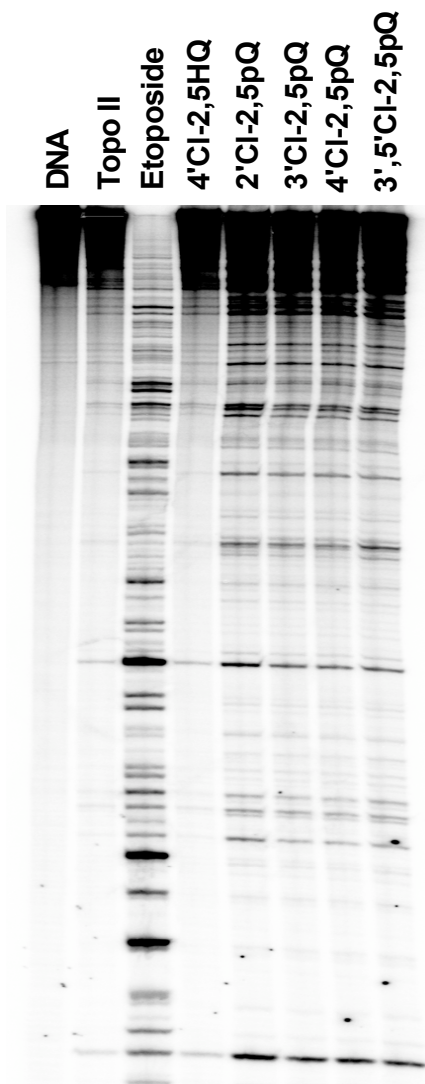
Figure 22. DNA cleavage site utilization by human topoisomerase IIα in the presence of PCB metabolites. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions contained no compound (Topo II), 12.5 μM etoposide, or 25 μM of the indicated PCB. A DNA control is shown in the far left lane. Data are representative of at least three independent experiments.

PCB Quinones Inhibit DNA Religation Mediated by Human Topoisomerase IIα.

Topoisomerase II poisons increase levels of enzyme-mediated DNA breaks by two non-mutually exclusive mechanisms (*10, 15, 16, 109*). Drugs such as etoposide act primarily by inhibiting the ability of topoisomerase IIα to religate DNA breaks (*97, 226*). In contrast, drugs such as quinolones have little effect on strand rejoining and appear to act primarily by increasing the forward rate of DNA scission (*98, 227*).

To determine the mechanism by which PCB metabolites increase levels of topoisomerase II-generated DNA strand breaks, their effects on rates of religation mediated by human topoisomerase IIα were assessed (Figure 23). All of the PCB quinones examined strongly inhibited the ability of the enzyme to reseal cleaved DNA molecules, suggesting that this inhibition is the primary mechanism by which these compounds increase levels of topoisomerase II-DNA cleavage complexes. Considerably less inhibition was seen for 4'Cl-2,5HQ.

Exposure of Topoisomerase IIα to PCB Quinones in the Absence of DNA Inhibits Enzyme Activity.

Although quinones act as topoisomerase II poisons when incubated with enzyme-DNA complexes, several studies indicate that they are potent inhibitors of enzyme activity when incubated with human topoisomerase IIα prior to the addition of DNA (*1, 118, 155*). It is not known whether these two properties of quinones share a common basis for action.

To determine whether PCB metabolites also display inhibitory properties, these compounds were incubated with topoisomerase IIα in the absence of DNA and their effects on enzyme-mediated DNA cleavage were characterized. As seen

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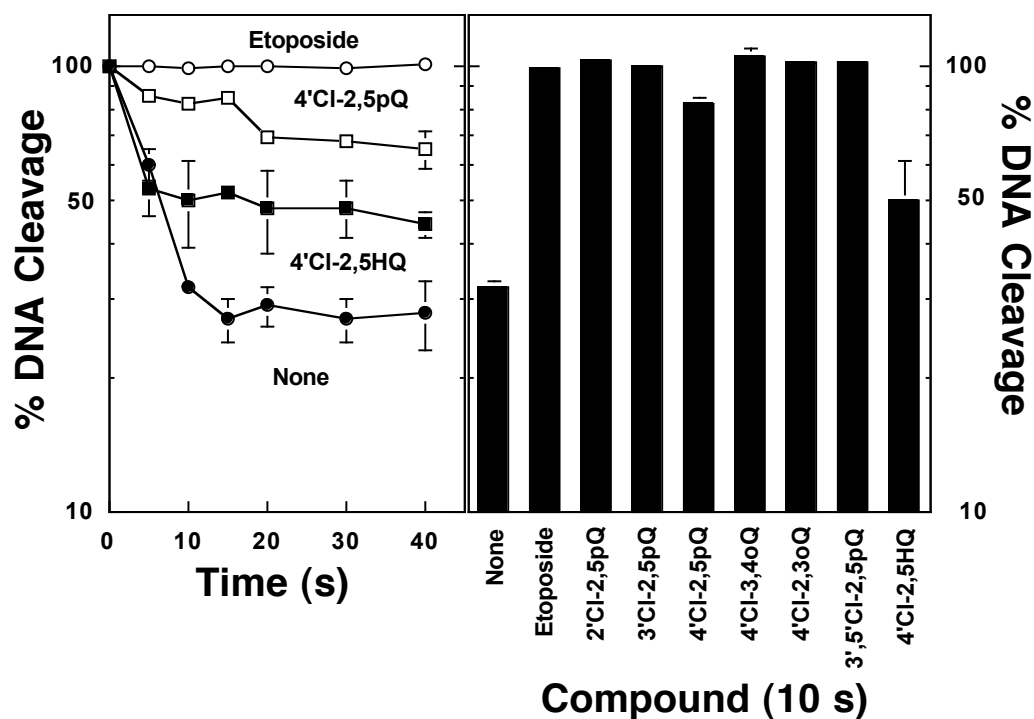
Figure 23. DNA religation mediated by human topoisomerase IIα is inhibited by PCB metabolites. Left panel: DNA religation was examined in the absence of compound (none, closed circles), or in the presence of 100 μM etoposide (open circles), 100 μM 4'Cl-2,5pQ (open squares) or 100 μM 4'Cl-2,5HQ (closed squares). Right panel: Representative religation data for PCB metabolites at 10 s. In all cases, samples were incubated at 37 ˚C to establish DNA cleavage/religation equilibria and were then shifted to 0 ˚C to initiate religation. Levels of DNA cleavage observed at equilibrium were set to 100% at time zero. DNA religation was quantified by the loss of linear cleaved molecules. Error bars represent the standard deviation of at least three independent experiments.of at least three independent experiments.

in Figure 24, exposure of topoisomerase IIα to PCB quinones prior to the addition of plasmid rapidly decreased the ability of the enzyme to cleave DNA. Inhibition by all of the quinones (at 100 μM) was nearly complete within 30 s. In contrast, no inhibition was observed following a 3 min exposure to etoposide. While incubation with 4'Cl-2,5HQ decreased DNA cleavage, the rate of inhibition was substantially slower than observed with the corresponding quinone. Taken together, these results indicate that PCB metabolites, like other quinone-based compounds (*1, 118, 223*) and ICRF-193 (which acts by trapping the N-terminal protein clamp of topoisomerase II) (*228*), are mixed function inhibitors of topoisomerase IIα. When incubated with the enzyme-DNA complex, they act as topoisomerase II poisons; when incubated with the enzyme in the absence of a nucleic acid substrate, they block topoisomerase II function.

Exposure of Topoisomerase IIα to PCB Quinones Prior to the Addition of DNA Inhibits the Binding of the Enzyme to Circular Plasmid Substrates.

As a first step toward defining the mechanism by which incubation of PCB quinones with topoisomerase IIα in the absence of DNA impairs enzyme activity, the effects of these compounds on enzyme-DNA binding was assessed. Initial experiments utilized negatively supercoiled circular plasmids as substrates. Exposure of topoisomerase IIα to several *p*-quinones (at 100 μM) sharply decreased the formation of non-covalent enzyme-DNA complexes (Figure 25, left top panel and bottom summary panel). In contrast, equivalent concentrations of etoposide or 4'Cl-2,5HQ had no significant affect on binding.

Additional experiments examined the effects of PCB metabolites on the binding of topoisomerase IIα to linear plasmids. Similar patterns were observed
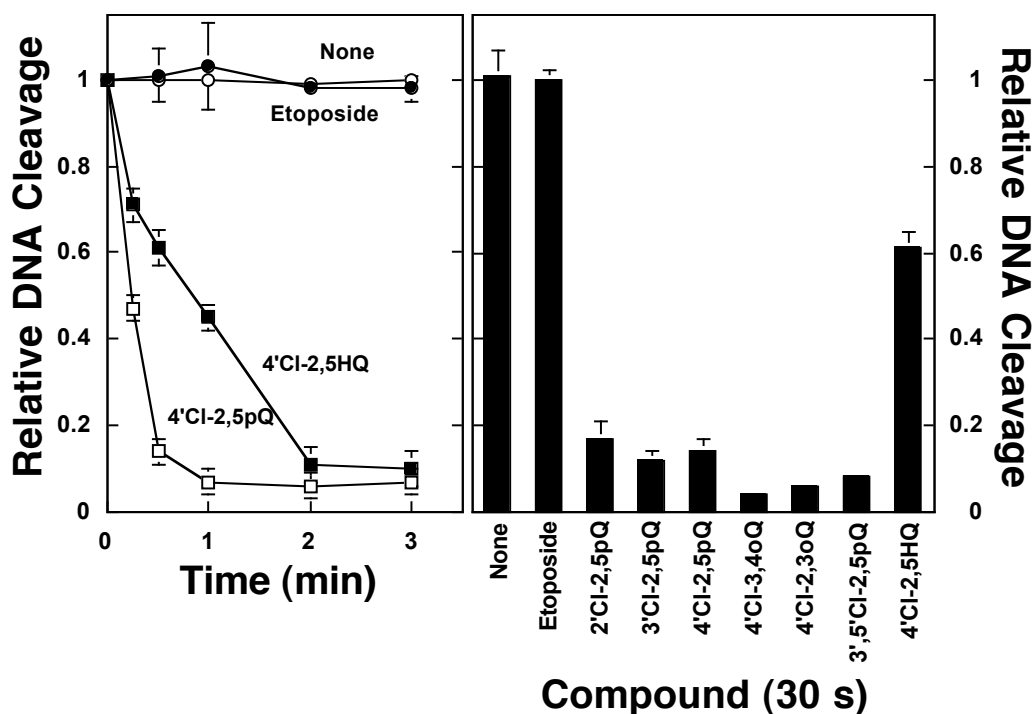
Figure 24. PCB metabolites rapidly inactivate human topoisomerase IIα in the absence of DNA. PCB metabolites were incubated with the enzyme prior to the addition of DNA and the effects of these compounds on enzyme activity were assessed by DNA cleavage assays. Left panel: Time course for the incubation of topoisomerase IIα with no compound (closed circles), 100 μM etoposide (open circles), 100 μM 4'Cl-2,5pQ (open squares) or 100 μM 4'Cl-2,5HQ (closed squares). Right panel: Representative values for PCB metabolites at 30 s. Levels of DNA cleavage for reactions in which metabolites and DNA were added simultaneously (*i.e.*, time zero) were set to 1.0. Error bars represent the standard deviation of at least three independent experiments.
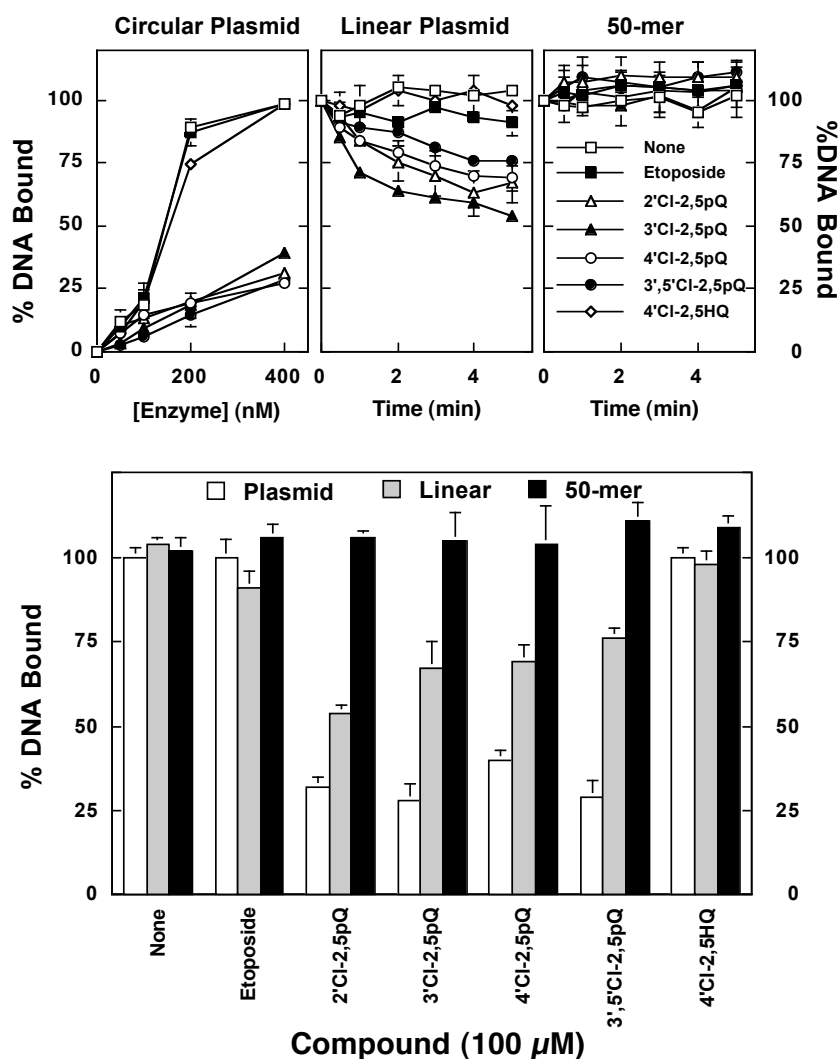
Figure 25. Substrate-dependent effects of PCB metabolites on topoisomerase IIα-DNA binding. Assays employed a negatively supercoiled circular plasmid (top left panel), a linear plasmid (top middle panel), or a duplex 50-mer oligonucleotide (top right panel) as the substrate. For experiments that used the circular plasmid, 0-400 nM enzyme was incubated with no compound (none), 100 μM etoposide, or 100 μM PCB metabolite for 5 min prior to the addition of DNA. For assays that used linear substrates (linear plasmid or the 50-mer), 400 nM enzyme was incubated with no compound, 100 μM etoposide, or 100 μM PCB metabolite for 0–5 min prior to the addition of DNA. The bottom summary panel is a representative graph including all three substrates in assays carried out under identical reaction conditions (exposure of 400 nM enzyme to compounds for 5 min prior to the addition of DNA). Error bars represent the standard deviation of at least three independent experiments

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with the linear substrate; however, the decrease in binding was less dramatic (Figure 25, center top panel and bottom summary panel). In general, the inhibition observed with linear DNA was ~1/2 that seen with circular substrates.

The linear plasmid substrates were >4,300 bp in length. To further investigate the differences seen between linear and circular substrates, the effects of PCB metabolites on the ability of topoisomerase IIα to bind short DNA fragments was examined. The substrate used for these experiments was a duplex 50-mer that contained a DNA cleavage site for the human enzyme (*173*). As seen in Figure 25 (right top panel and bottom summary panel), exposure of topoisomerase IIα to PCB quinones prior to the addition of DNA had no substantial effect on the ability of the enzyme to bind the oligonucleotide.

In summary, PCB quinones sharply impair the binding of topoisomerase IIα to circular DNA substrates, display a smaller effect on the binding to linear molecules, and have no effect on the binding to oligonucleotides. These findings are not consistent with the idea that quinones adduct an amino acid residue in the active site of topoisomerase IIα. Rather, as discussed below, they suggest that the compounds may alter enzyme function by affecting the N-terminal gate of topoisomerase II.

4′Cl-2,5pQ Blocks the N-Terminal Protein Gate of Human Topoisomerase IIα.

During the catalytic cycle of type II topoisomerases, the "T-helix" (*i.e.*, the DNA helix that is "transported" through the transient double-stranded break that the enzyme generates in a separate helix) enters the protein through a gate that is comprised of the N-terminal domains of the two protomer subunits of the enzyme (*15, 16, 57, 58, 109*). Upon ATP binding, topoisomerase II undergoes a

conformational change that closes the N-terminal gate and induces passage of the T-helix through the DNA break (*15, 16, 57-59, 65, 109*). Formation of this N-terminal clamp does not allow the T-helix to exit the protein through the original gate (*15, 16, 57-59, 65, 109, 229, 230*). Thus, it promotes a unidirectional strand passage event (*59*). In addition, clamp closure does not allow a second T-helix to enter the enzyme through the N-terminal gate until the strand passage event is completed and the first T-helix has exited topoisomerase II through a C-terminal gate (*15, 16, 57-59, 109, 229, 230*).

Closure of the N-terminal gate in the presence of DNA results in the formation of a protein clamp that topologically encircles the DNA, forming a non-covalent, but salt-stable complex with circular nucleic acids (*65, 229*). If the gate is closed prior to the addition of DNA, circular nucleic acids are prevented from entering the active site of topoisomerase II (*229*). Linear DNA molecules, however, are still able to access the active site by feeding directly through the central annulet of the enzyme (*65, 229*). As discussed above, treatment of topoisomerase IIα with PCB quinones prior to the addition of DNA inhibits the binding of circular plasmid, but has a distinctly lesser effect with linear plasmid or oligonucleotide substrates. Since this finding is consistent with an effect on the N-terminal clamp, the ability of 4′Cl-2,5pQ and 4′Cl-2,5HQ to induce clamp closure was examined.

In order to assess clamp closing, topoisomerase IIα was incubated with negatively supercoiled plasmid and the mixture was applied to a glass fiber filter. In the absence of a stable protein clamp, circular DNA (which does not bind to glass fibers) passes through the filter in the flow through, low salt (LS), or high salt (HS) washes. Alternatively, DNA trapped by stable protein clamps is

eluted from the filter only after denaturation of the enzyme with SDS (Figure 26, inset).

Previously, stable clamps have been observed for wild-type human topoisomerase IIα only in the presence of a non-hydrolyzable ATP analog or in the presence of ICRF-193 and ATP (*65, 229, 230*). Similar results were seen in the present study (Figure 26, left panel). Less than 3% of the circular plasmid substrate formed a salt-stable complex with the enzyme in the absence of an ATP analog, while ~10% did so in the presence of 100 μM ICRF-193 and ATP. Remarkably, when 100 μM 4′Cl-2,5pQ was incubated with a mixture of topoisomerase IIα and DNA, even in absence of ATP, ~20% of the initial plasmid substrate formed a salt-stable non-covalent complex with the enzyme (left panel). In a set of parallel experiments, ~8% of the plasmid formed a salt-stable complex following incubation with 100 μM 4′Cl-2,5HQ. Finally, incubation of topoisomerase IIα with 100 μM 4′Cl-2,5pQ prior to the addition of DNA reduced the formation of salt-stable complex by nearly 10–fold (right panel). Taken together, the above results strongly suggest that exposure of topoisomerase IIα to PCB quinones blocks the N-terminal protein gate even in the absence of a high-energy cofactor.

4′Cl-2,5pQ Crosslinks the Subunits of Topoisomerase IIα.

Data shown in Figure 21 indicate that PCB quinones alter the activity of topoisomerase IIα by covalently adducting the enzyme. Adduction results in the formation of a covalent bond between the sulfhydryl (or other amino acid group such as an amine) and one of the hydrogenated carbons on the quinone ring (*231, 232*).
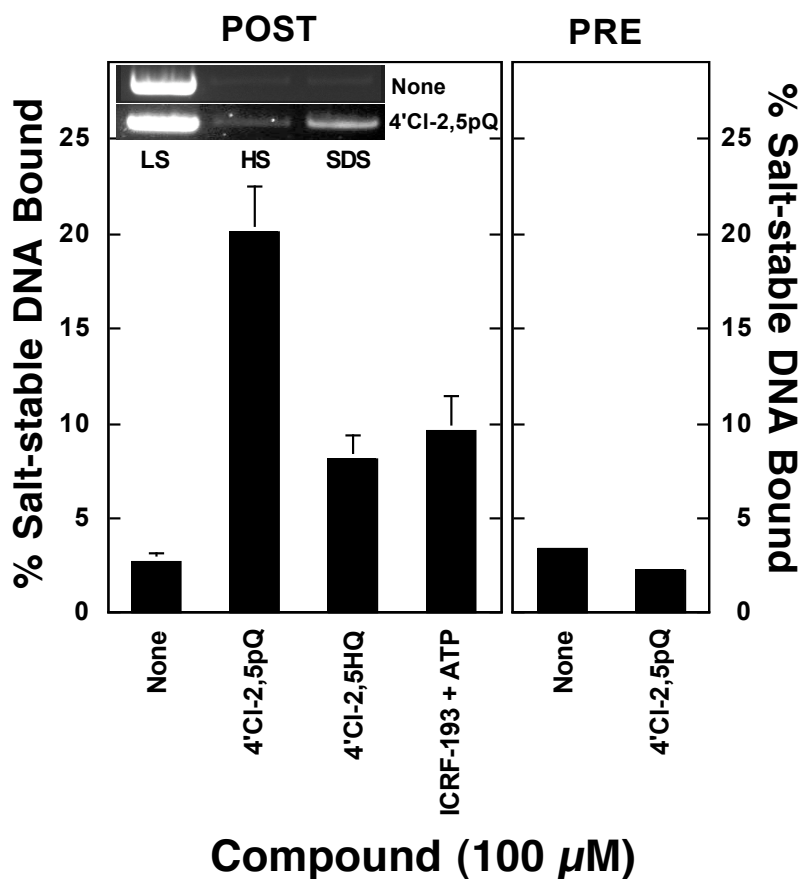
Figure 26. 4′Cl-2,5pQ blocks the N-terminal gate of human topoisomerase IIα. Filter binding assays were used to analyze the salt-stable closed-clamp of topoisomerase II. Reactions contained no compound (None), 100 μM 4′Cl-2,5pQ or 100 μM 4′Cl-2,5HQ in the absence of ATP, or 100 μM ICRF-193 + 2 mM ATP. DNA eluted from filters after sequential low salt (LS), high salt (HS), or SDS (SDS) washes was subjected to electrophoresis in an agarose gel. Salt-stable non-covalent enzyme-DNA complexes were quantified by the amount of plasmid that did not elute until the SDS wash relative to the total plasmid eluted in all three washes. Left panel: Compounds were incubated with topoisomerase IIα after the addition of plasmid (POST). Right panel: 4′Cl-2,5pQ was incubated with enzyme prior to the addition of plasmid (PRE). Error bars represent the standard deviation of four independent experiments. A representative ethidium bromide-stained agarose gel is shown in the inset in the left panel.

Quinone compounds are capable of crosslinking proteins in the cell (*231, 233, 234*). Previous studies demonstrate that benzoquinone (and derivatives), as well as PCB quinones, are capable of adducting to more than one sulfhydryl group (*223, 231*). To this point, a single molecule of 2′Cl-2,5pQ has been shown to adduct to three separate glutathione molecules, one at each of the compounds electrophilic sites (*223*) (see Figure 28E). Thus, the PCB quinones shown in Figure 1 are able to act as protein crosslinking agents.

Therefore, three possible mechanisms are consistent with the actions of PCB quinones (Figure 27). First, covalent modification of topoisomerase IIα by quinones may induce the N-terminal gate to close in a manner that is similar to that observed in the presence of ATP (model A). While this model is formally possible, it should be noted that clamp closure in the absence of a nucleotide cofactor has not been reported (*65, 229, 230*). Second, quinones may adduct residues above the position where the T-segment normally rests prior to strand passage and sterically block entrance or exit of the helix (model B). However, since there are no reported structures of topoisomerase IIα with an intact, but open, N-terminal gate, it is not known whether PCB metabolites or other quinones would have enough bulk to act in this fashion. Third, PCB quinones may crosslink the two protomers of topoisomerase IIα, thereby blocking the N-terminal gate of the protein (model C).

To help distinguish between these three models, the ability of 4′Cl-2,5pQ to crosslink the two subunits of human topoisomerase IIα was assessed (Figure 28A and B). Following incubation with the PCB quinone, a distinct new protein band appeared with an apparent molecular mass of ~330 kDa. This mass is approximately twice that of the protomer molecular mass of topoisomerase IIα
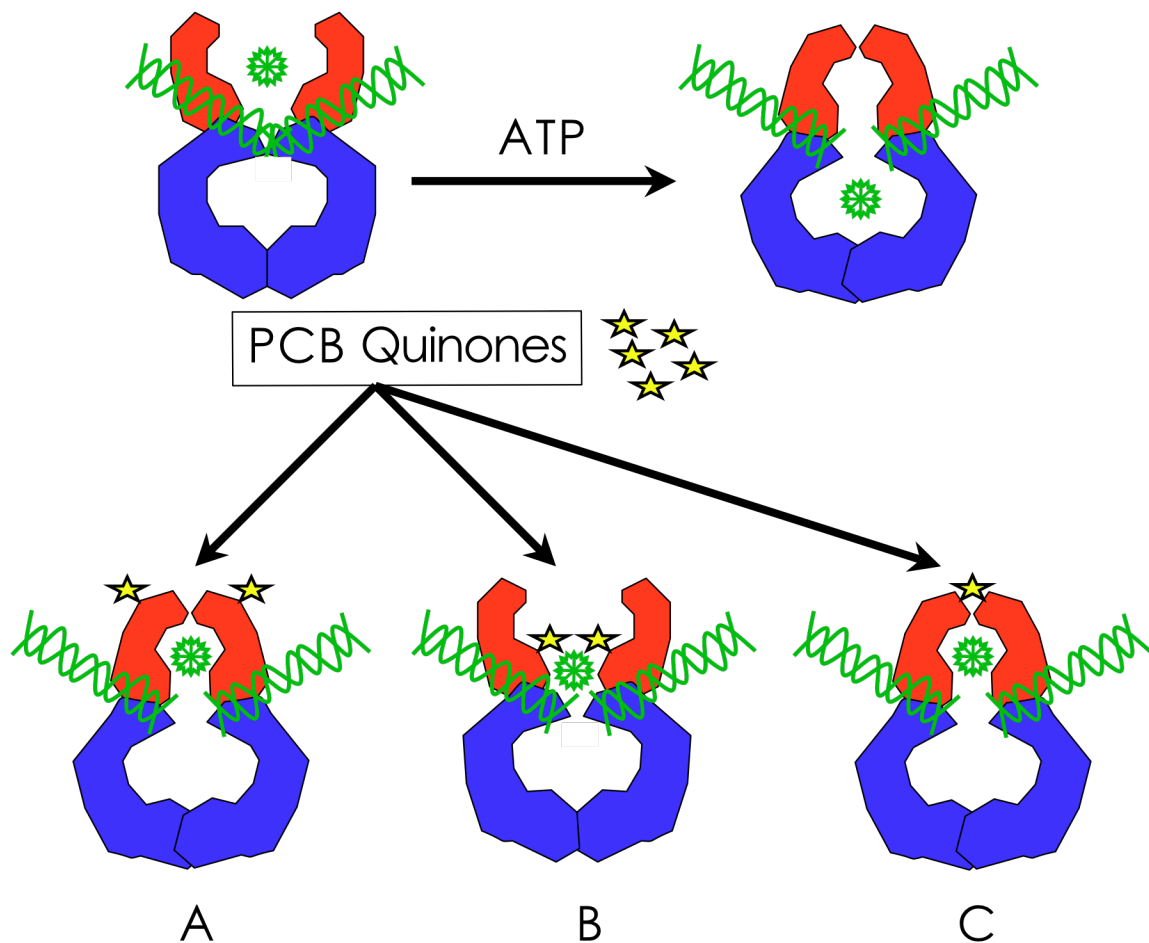
Figure 27. Models for how PCB Quinones block the N-terminal gate of human topoisomerase IIα. (A) PCB quinones induce natural clamp closure of the enzyme as seen with ATP (above). (B) PCB quinones adduct to positions above the T-segment that sterically block entrance or exit of the helix. (C) PCB quinones crosslink the two protomers of human topoisomerase IIα.
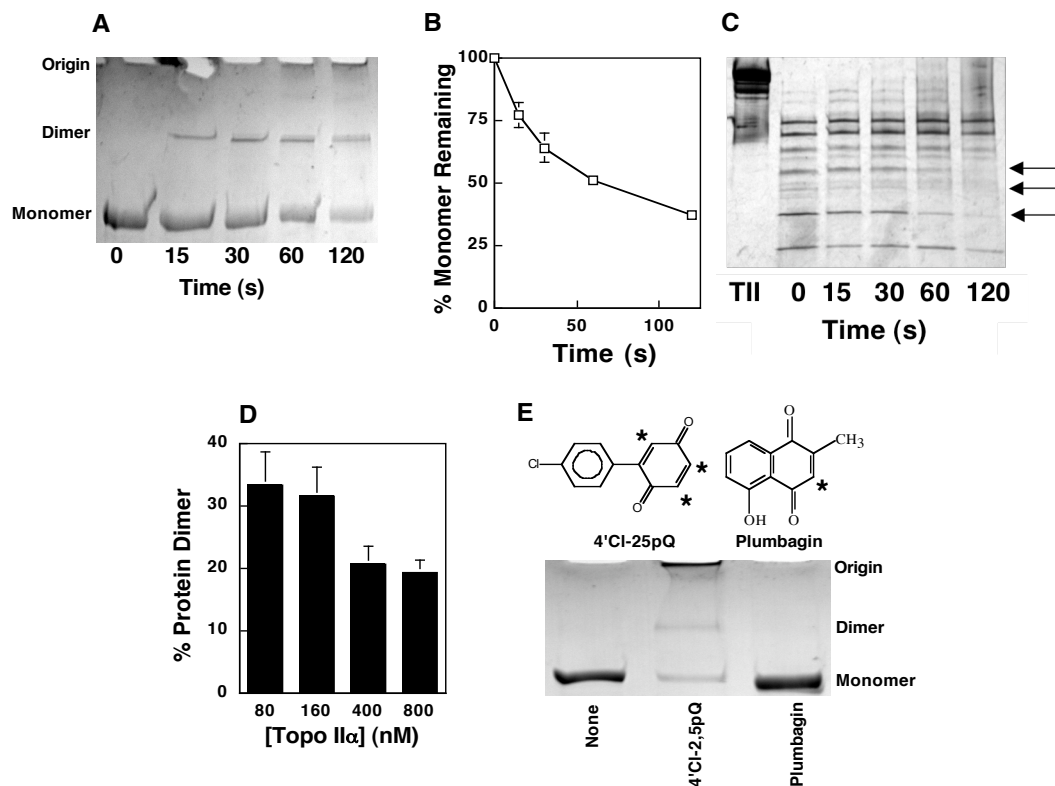
Figure 28. 4′Cl-2,5pQ crosslinks the protomer subunits of human topoisomerase IIα. Panel A shows a coumassie-stained denaturing polyacrylamide gel in which 135 nM topoisomerase IIα was treated 100 μM 4′Cl-2,5pQ for 0–2 min. The mobility of a topoisomerase IIα subunit monomer and dimer is indicated as well as the origin of the gel. The gel is representative of two independent experiments. Panel B quantifies the loss of the monomeric topoisomerase IIα subunit over time as a result of crosslinking by 4′Cl-2,5pQ. Data were derived from the gel shown in Panel A and a parallel experiment. Error bars represent the standard error of the mean for these two experiments. Panel C displays a partial proteolytic map of topoisomerase IIα following exposure to 100 μM 4′Cl-2,5pQ over time. A silver-stained polyacrylamide gel that is representative of four independent experiments is shown. Arrows indicate specific polypeptide bands that diminished following exposure to the PCB quinone. Panel D quantifies the formation of SDS-stable dimers when 80–800 nM topoisomerase IIα was treated with 100 μM 4′Cl-2,5pQ for 1 min. Error bars represent the standard deviation of four independent experiments. Panel E shows a coumassie-stained denaturing polyacrylamide gel in which 135 nM topoisomerase IIα was treated with no compound (None), 100 μM 4′Cl-2,5pQ, or 250 μM plumbagin for 6 min. Structures of both compounds are shown with sites of potential protein adduction marked by asterisks. The gel is representative of two independent experiments.

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(170 kDa), suggesting that two subunits of the protein were crosslinked in the presence of 4'Cl-2,5pQ. Partial proteolytic mapping experiments demonstrated that specific topoisomerase IIα peptides were crosslinked by the quinone (Figure 28C). These results favor model C.

In addition to the dimer band, an apparent high molecular weight protein species was observed at the origin at incubation times of 1 min or longer (Figure 28A). After a 6 min exposure to 100 μM 4'Cl-2,5pQ, most of the enzyme remained at the origin (Figure 28E). It is notable that this protein species was soluble and did not precipitate upon centrifugation. Consequently, the protein at the origin does not appear to be an insoluble aggregate and is believed to represent a high molecular species that is too large to effectively enter the gel. Thus, it appears that the quinone is able to establish crosslinks between different topoisomerase IIα homodimers (*i.e.*, intermolecular crosslinks).

If model C is correct, however, 4'Cl-2,5pQ must be able to crosslink the two promoter subunits of an individual enzyme homodimer (*i.e.*, intramolecular crosslink). Therefore, dilution experiments (from 800 down to 80 nM topoisomerase IIα) were carried out to determine whether the 330 kDa band resulted from an inter- or intramolecular crosslinking event. If the 330 kDa band arose from crosslinking of two separate topoisomerase IIα molecules, it should decrease proportionally as the concentration of the protein decreases. Conversely, if the band arose from crosslinking two protomers of an individual topoisomerase IIα homodimer, its intensity should be independent of protein concentration. As seen in Figure 28D, the 330 kDa band did not decrease at lower protein concentrations. This finding indicates that the dimer band most likely is formed by an intramolecular crosslinking event. Based on these data, we propose

that PCB quinones block the N-terminal gate of topoisomerase IIα, at least in part, by crosslinking the two protomer subunits of the enzyme.

As a control, the effects of plumbagin on topoisomerase IIα were examined (Figure 28E). Although similar in size to the PCB metabolites, this compound has a quinone ring that contains only a single reactive site (marked by the asterisk in the figure). By comparison, 4'Cl-2,5pQ has three potential sites of adduction (also marked by asterisks). Even though plumbagin covalently adducts to cysteine residues on human topoisomerase IIα (determined by mass spectrometry), it displayed very little ability to enhance enzyme-mediated DNA cleavage (*118*) or to inhibit enzyme activity in the absence of nucleic acids (data not shown). Following a 6 min incubation with 250 μM plumbagin, no protein dimers or high molecular weight species were observed. Hence, as expected for a quinone that has only a single site of adduction, plumbagin displays no ability to induce protein-protein crosslinks. These findings are not consistent with models A or B, which require only a single site of adduction per quinone molecule. Rather, they support the importance of protomer-protomer crosslinks, as postulated in model C.

PCB Metabolites Increase Levels of DNA Cleavage Mediated by Topoisomerase IIα in Cultured Human Cells.

Since PCB metabolites act as topoisomerase II poisons in vitro, the ICE bioassay was employed to determine whether these compounds increase DNA cleavage mediated by topoisomerase IIα in human cells. In this assay, cultured CEM leukemia cells were lysed with an ionic detergent, and proteins that were covalently attached to genomic DNA were separated from free proteins by

sedimentation through a CsCl cushion. The pelleted DNA from cultures treated with no compound or 25 μM PCB metabolites for 6 h was blotted and probed with a polyclonal antibody directed against human topoisomerase IIα. Results for cells treated with 10 μM etoposide for 2 h are shown for comparison. In all cases, less than 10% cell death was observed over the course of experiment. As seen in Figure 29, levels of topoisomerase IIα that were covalently attached to DNA increased 3– to 4–fold following treatment of cells with PCB metabolites (as compared to ~7–fold with etoposide).

The covalent topoisomerase II-DNA complexes monitored in the ICE bioassay are believed to represent cleavage complexes. However, since some quinones can induce protein-DNA crosslinks, it is possible that a portion of the observed complexes may actually reflect crosslinks rather than cleavage. We do not believe that this is the case for the following reasons. First, no protein-DNA crosslinking was observed in experiments with purified topoisomerase IIα, even under conditions that employed enzyme:base pair ratios (~1:325) that were >20 times higher than those estimated (~1:7500) for CEM cells (*202*) and quinone concentrations (100 μM) that were 4 times higher than those used in the cellular studies (see Figure 20). Second, treatment of CEM cells with 25 μM 4'Cl-2,5pQ did not increase levels of covalent topoisomerase I-DNA complexes (Figure 29). It is notable that 4'Cl-2,5pQ does not stimulate topoisomerase I-mediated DNA cleavage in vitro (data not shown). Since the type I enzyme is intimately associated with chromosomal DNA, this latter finding argues against 4'Cl-2,5pQ acting as a general protein-DNA crosslinking reagent. On the basis of these two control experiments, we conclude that PCB metabolites are topoisomerase II poisons in cultured human cells.
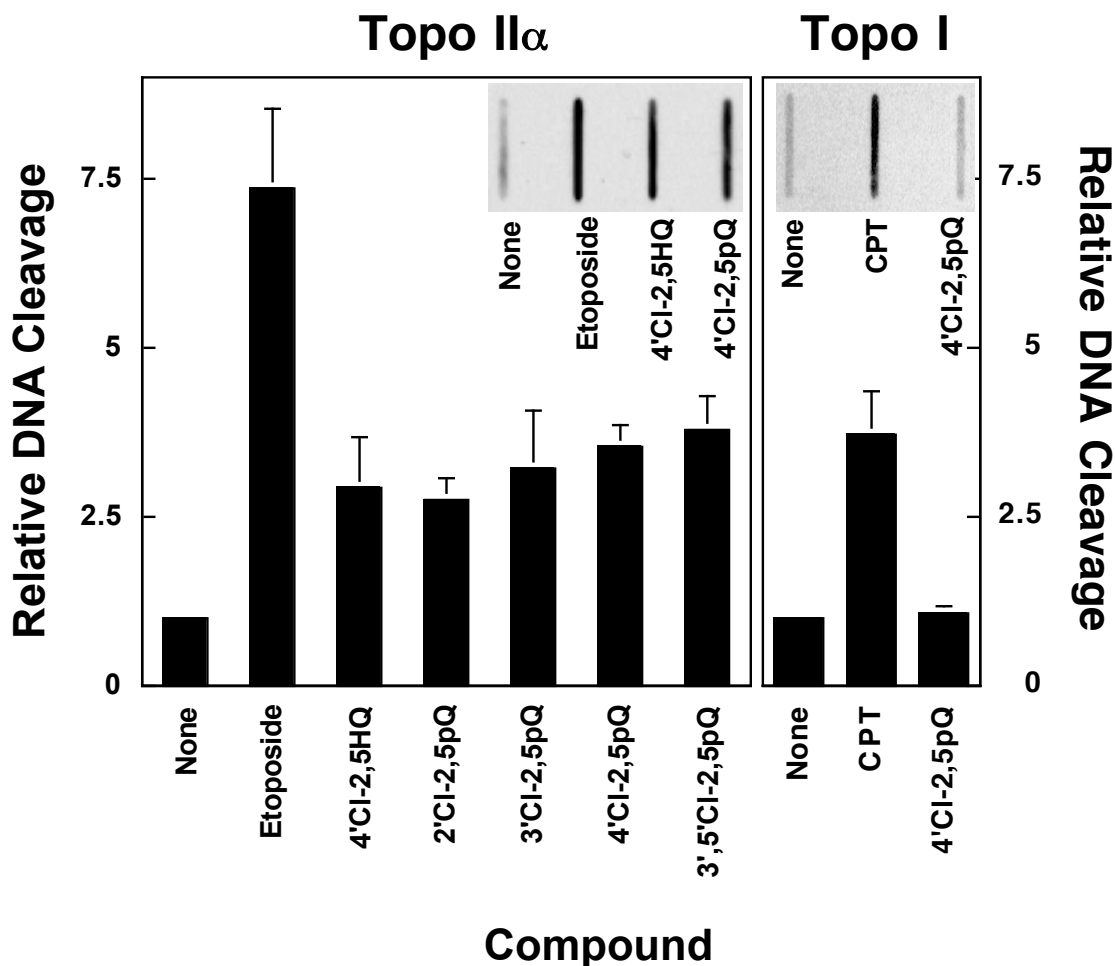
Figure 29. PCB metabolites increase topoisomerase IIα-mediated DNA cleavage in human CEM cells. The ICE bioassay was used to monitor the level of cleavage complexes in cells treated with PCB metabolites. DNA (10 μg) from cultures treated with no compound (None), 25 μM of the indicated PCB metabolite for 6 h, or 10 μM etoposide or 10 μM camptothecin (CPT) for 2 h was blotted onto a nitrocellulose membrane. Blots were probed with a polyclonal antibody directed against human topoisomerase IIα (left) or human topoisomerase I (right). Relative DNA cleavage was calculated relative to levels of cleavage complexes observed in untreated cells. Error bars represent the standard deviation of three independent experiments. Representative immunoblots are shown in the insets.

Although 4'Cl-2,5HQ displayed minimal cleavage enhancing activity in vitro, it induced topoisomerase II-mediated DNA scission in CEM cells. However, since these leukemic cells display peroxidase activity (*235*), it is likely that the hydroquinone was activated to 4'Cl-2,5pQ within the cell.

Discussion

Exposure to PCBs induces a variety of chromosomal aberrations in humans and treated human cells (*209, 211*), but the underlying basis for this genomic damage has yet to be defined. Multiple pathways are likely to be involved, such as oxidative damage due to redox cycling, glutathione depletion, or adduction of protein thiols (*162, 220-223*).

Since the genotoxic effects of PCBs are consistent with the actions of topoisomerase II poisons and quinones have been shown to enhance DNA scission mediated by the type II enzyme (*1, 118, 155*), we examined the effects of several PCB metabolites on the activity of human topoisomerase IIα. Results indicate that a variety of PCB quinones, including mono- and di-chlorinated compounds and both *p*- and *o*-quinones, are potent topoisomerase IIα poisons in vitro and in cultured human cells. Furthermore, as demonstrated previously with other bioreactive quinones (*1, 118, 155*), incubation of topoisomerase IIα with PCB metabolites in the absence of DNA leads to a rapid loss of enzyme activity.

While quinones appear to act by covalently adducting topoisomerase IIα (*1, 118, 155*), the mechanism by which these compounds alter enzyme activity is not understood. Moreover, it is not known whether the same series of events is

responsible for both the enhancement of DNA cleavage and the inhibition of enzyme activity. Results with PCB metabolites suggest that these two opposing actions of quinones may be linked, at least in part, to an effect on the N-terminal gate of topoisomerase IIα. Based on the differential ability of the quinone-treated enzyme to bind circular and linear forms of DNA, and the generation of salt-stable non-covalent complexes between topoisomerase IIα and circular plasmids in the presence of 4'Cl-2,5pQ, it appears that PCB metabolites block the N-terminal gate of the protein.

The effects of 4'Cl-2,5pQ on topoisomerase IIα are in many respects similar to those described for ICRF-193 and related bisdioxopiperazines. These compounds alter enzyme function by stabilizing the closed-clamp form of topoisomerase II (*230, 236, 237*). If the clamp is closed in the absence of a nucleic acid substrate, DNA cleavage is inhibited because DNA is unable to enter the active site of the enzyme (*230*). Conversely, if the clamp closed after DNA binding, levels of cleavage rise modestly (2– to 3–fold) (*238*). This latter effect is presumably due to the increased concentration of non-covalent enzyme-DNA complexes. In this regard, cleavage enhancement by 4'Cl-2,5pQ (~8–fold) was considerably higher than induced by ICRF-193. However, the PCB quinone also was more efficient at trapping a salt-stable complex between topoisomerase IIα and supercoiled plasmid.

The above similarities notwithstanding, the mechanism by which 4'Cl-2,5pQ traps the salt-stable complex is very different than seen with ICRF-193. While bisdioxopiperazines act in a strictly non-covalent manner (*230*), the quinones appear to require covalent adduction to the protein (*1, 118, 155*). Furthermore, bisdioxopiperazines stabilize the closed N-terminal protein clamp by inhibiting

ATP hydrolysis (*228*). In contrast, PCB quinones induce a salt-stable enzyme-DNA complex even in the absence of a nucleotide triphosphate cofactor and have only a modest effect on ATP hydrolysis (data not shown). Based on protein crosslinking experiments, we propose that PCB quinones block the N-terminal gate of topoisomerase IIα by crosslinking the two protomer subunits of the enzyme (Figure 27, model C). If this hypothesis is correct, reactive amino acids (most likely N-terminal residues) on the two protomers must be in close enough proximity in the absence of ATP (<8 Å) to bond to the same quinone molecule. Since the N-terminal gate of topoisomerase IIα has to be at least 20 Å across to accommodate the T-segment, this proposal implies that the N-terminal domain of topoisomerase IIα must be very flexible and dynamic in the absence of ATP.

Two complicating points should be noted. First, while exposure to 4'Cl-2,5pQ resulted in the preferential loss of individual peptides, no specific higher molecular mass peptide species were identified (Figure 28C). Rather, a high molecular mass smear was observed. This finding suggests that quinone crosslinking of the topoisomerase IIα protomers may involve multiple sites of adduction rather than unique amino acid residues. Second, under conditions that paralleled those employed for DNA cleavage experiments, high molecular mass topoisomerase IIα multimers were observed following exposure to PCB quinones (Figure 28A and E). This indicates that intermolecular protein-protein crosslinking also is taking place. It is not known how (or if) these intermolecular events contribute to either the enhancement of enzyme-mediated DNA cleavage or the inhibition of enzyme activity by quinones.

Finally, although the evidence indicates that blocking the N-terminal protein gate plays an important role in the actions of quinones against topoisomerase IIα,

additional possibilities cannot be excluded. To this point, quinone-treated enzyme binds oligonucleotide substrates, but the adducted protein is not capable of cleaving the bound 50-mers (data not shown). Furthermore, levels of clamp closing and protein crosslinking are not as great as levels of enzyme inhibition under similar conditions. Thus, in the absence of structural data, alternative mechanisms must still be considered.

In conclusion, PCB metabolites are reactive chemicals that are health hazards to humans and are probable carcinogens. The toxic and mutagenic effects of these compounds are complex and most likely driven by a variety of pathways. Although the ultimate relationship of our findings to the health effects of PCBs is not known, the present work suggests that topoisomerase IIα may play a role in mediating at least some of the chromosomal damage induced by these environmental contaminants.

CHAPTER V

QUINONE-INDUCED ENHANCEMENT OF DNA CLEAVAGE BY HUMAN
TOPOISOMERASE IIα: DUAL ACTIVITY OF QUINONES AGAINST THE
ENZYME

Introduction

Topoisomerase II poisons play important roles in both the treatment and the development of human cancers. Approximately one-half of all cancer chemotherapy regimens contain drugs that target the type II enzyme. However, a small number of patients who receive therapy that includes topoisomerase II-targeted drugs develop secondary leukemias. These drug-related leukemias commonly feature aberrations (deletions, duplications, and translocations) in the mixed-lineage leukemia (*MLL*) gene at chromosomal band 11q23 (*13, 14, 24, 25, 239-242*). In addition, bioflavonoids, which are beneficial for adult health, are believed to increase the risk of infant leukemias that include *MLL* rearrangements when ingested during pregnancy (*18, 20-23*).

Recently, a number of toxic quinone-based metabolites of drugs or environmental pollutants have been shown to be potent human topoisomerase II poisons (*118*). These include *N*-acetyl-*p*-benzoquinone imine (derived from acetaminophen) (*155*), benzoquinone (derived from benzene) (*1, 117*), as well as several PCB metabolites (*156*). Exposure to these compounds causes DNA strand breaks and other chromosomal aberrations, and has been linked to a variety of human health problems, including cancer (*19, 35, 243-245*).

The quinone-based topoisomerase II poisons differ from "classic" topoisomerase II poisons (such as etoposide and bioflavonoids) in two ways. First, quinones are mixed-function inhibitors of the type II enzyme: they increase DNA scission when added to a topoisomerase II-DNA complex, but inhibit scission when incubated with the enzyme prior to the addition of nucleic acids (*1, 117, 155, 156, 223, 246-249*). Second, in contrast to compounds like etoposide (which interact with topoisomerase II in a non-covalent manner), quinones act by covalently attaching to the protein (*1, 117, 118, 155, 156, 223*).

It is not known how this covalent modification increases levels of cleavage complexes and it is possible that quinones affect enzyme activity by more than one process. Religation experiments suggest that these compounds function at least in part by impairing the ability of topoisomerase II to rejoin cleaved DNA termini (*1, 117, 155, 156*). In addition, quinones block the N-terminal gate of the protein, thereby increasing the concentration of DNA associated with topoisomerase II by trapping the substrate in a non-covalent complex (*156*). Molecular sizing experiments suggest that quinones block the N-terminal gate by crosslinking the two protomer subunits of topoisomerase IIα (*156*).

Because of the human health concerns of quinone-based topoisomerase II poisons and their unusual mode of action against the type II enzyme, sites of quinone adduction on human topoisomerase IIα were mapped by mass spectrometry.

Identification of Cysteine Residues in Human Topoisomerase IIα that are Adducted by Quinones.

In an effort to further dissect the mechanistic basis for the actions of quinones as topoisomerase II poisons, amino acid residues in human topoisomerase IIα that are covalently modified by these compounds were identified by mass spectrometry. Initial attempts utilized benzoquinone as the modification agent. Unfortunately, no individual amino acid residues were identified in these experiments. This is most likely due to the ability of benzoquinone to crosslink multiple residues in the protein (*223*).

To overcome this technical difficulty, plumbagin, which has only a single reactive site, was employed (Figure 30). Plumbagin is a topoisomerase II poison, however, it is at least 10–fold less potent than benzoquinone (Figure 30). In addition, the compound does not effectively block the N-terminal gate of the enzyme (Figure 30) and does not crosslink the protomer subunits (*156*). These data support the hypothesis that multiple processes contribute to the increase in cleavage complexes by quinone-based topoisomerase II poisons.

As determined by LC-MS/MS analysis of tryptic and chymotryptic peptides derived from human topoisomerase IIα, four cysteine residues were adducted by plumbagin, cys170, cys392, cys405, and cys455. Representative
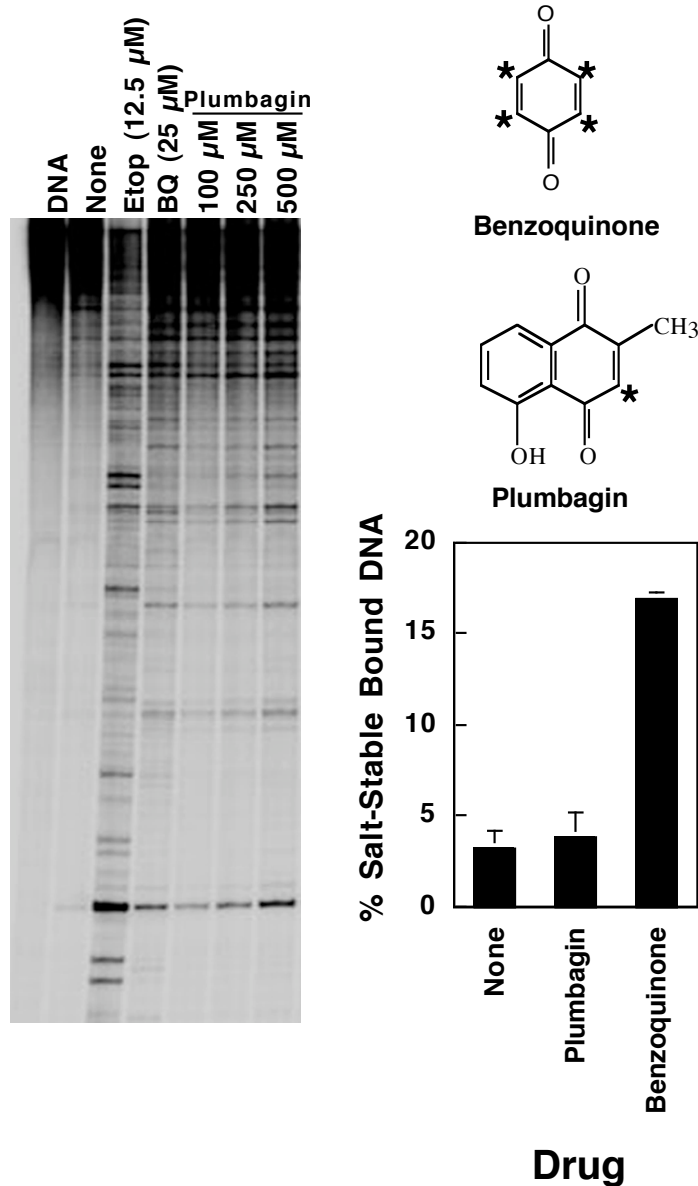
Figure 30. DNA cleavage enhancement by human topoisomerase IIα in the presence of plumbagin. Upper Right: The structures of plumbagin and benzoquinone are shown. Reactive sites on the compounds are indicated by stars. Left: An autoradiogram of a polyacrylamide gel of DNA cleavage reactions is shown. Assays contained no compound (None), 12.5 μM etoposide, or 25 μM benzoquinone or 100–500 μM plumbagin. A DNA control is shown in the far left lane. Data are representative of two independent experiments. Lower Right: Results of topoisomerase II protein clamp closing assays are shown. The bar graph represents levels of salt-stable bound DNA formed when topoisomerase II-DNA complexes were treated with no drug (None), 250 μM plumbagin or 100 μM benzoquinone. Error bars represent the standard deviation of four independent experiments.

spectra are shown in Figure 31. Modeling studies based on the structure of yeast topoisomerase II place cys170, cys392 cys405 in the N-terminal domain and cys455 in the B′-subdomain of the catalytic core of the enzyme (*58, 250*) (Figure 32). As determined in a previous study, all of these residues exist in the protein as free sulfhydryls (*i.e.*, not part of a disulfide bridge) with the exception of cys455, which is free only10–15% of the time (*251*).

DNA Cleavage Mediated by N-terminal Mutant Human Topoisomerase IIα Enzymes.

In order to assess the contributions of the above four cysteine residues to the actions of quinones as topoisomerase II poisons, each of the residues was individually mutated to an alanine residue and the resulting mutant enzymes (top2αC170A, top2αC392A, top2αC405A, and top2αC455A, respectively) were purified and characterized. With the exception of top2αC455A, all of the mutant enzymes displayed wild-type DNA cleavage activity in the absence of topoisomerase II poisons (Figure 33A). Top2αC455A exhibited a cleavage activity that was ~50% higher than wild-type (see Figure 39). Since the basal properties of this mutant enzyme were altered, it will be discussed later in this chapter.

To further analyze the properties of top2αC170A, top2αC392A, and top2αC405A, the sensitivity of these mutant enzymes to topoisomerase II poisons was determined. Etoposide, a *classic* topoisomerase II poison, as well as benzoquinone and the PCB metabolite 4′Cl-2,5pQ, two *quinone-based* topoisomerase II poisons, were utilized for these experiments. As shown in Figure 33B, all displayed wild-type sensitivity to the anticancer drug etoposide. In contrast, two of the mutant enzymes, top2αC392A and top2αC405A, were less
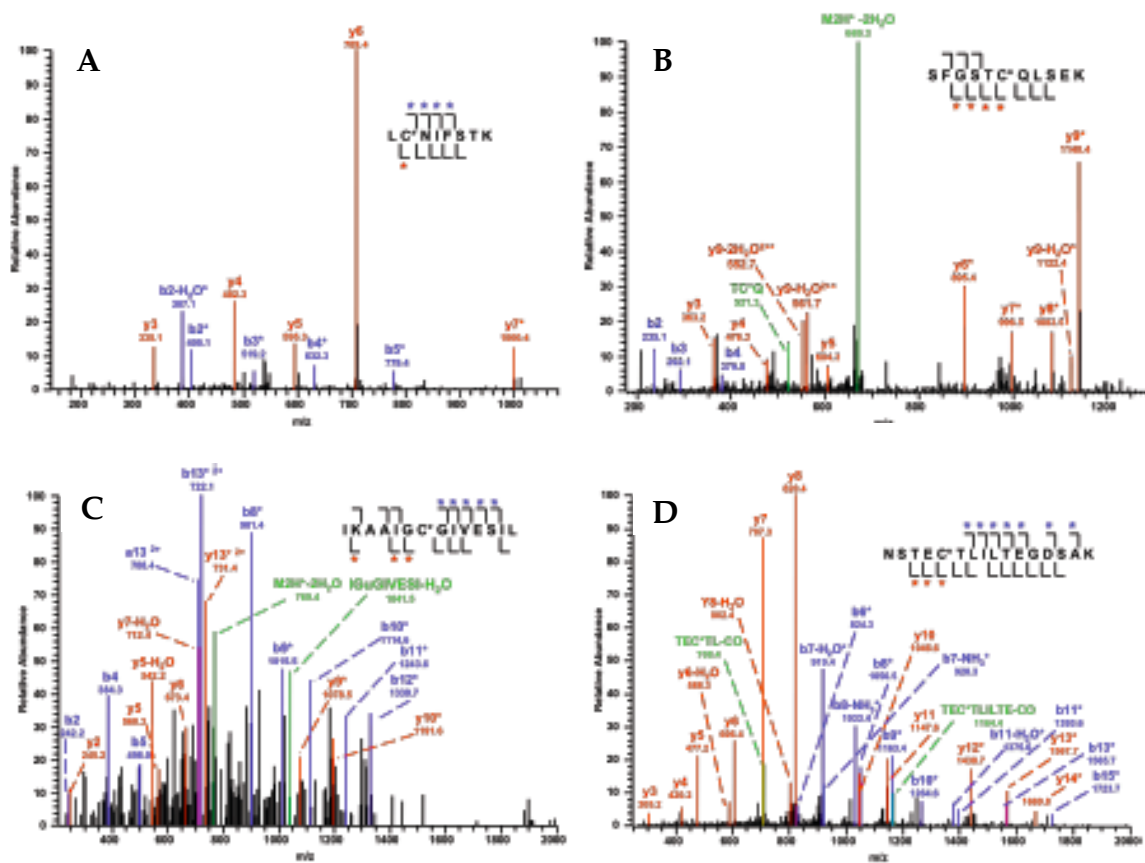
Figure 31. Plumbagin adducts to cysteine residues of human topoisomerase IIα. Low-energy CID spectra of peptides that contain the adducted a) cys170, b) cys392, c) cys405 or d) cys455 residues are shown. Fragment ions are labeled according to the accepted nomenclature. b-ions are coded blue, y-ions are coded red, and precursor ions are coded green. Asterisks denote b- or y-ions that contain the adducted cysteine residue. The insets show the sequence of the adducted peptides; the asterisk denotes the adducted cysteine residue and the black lines denote the identified cleavages.
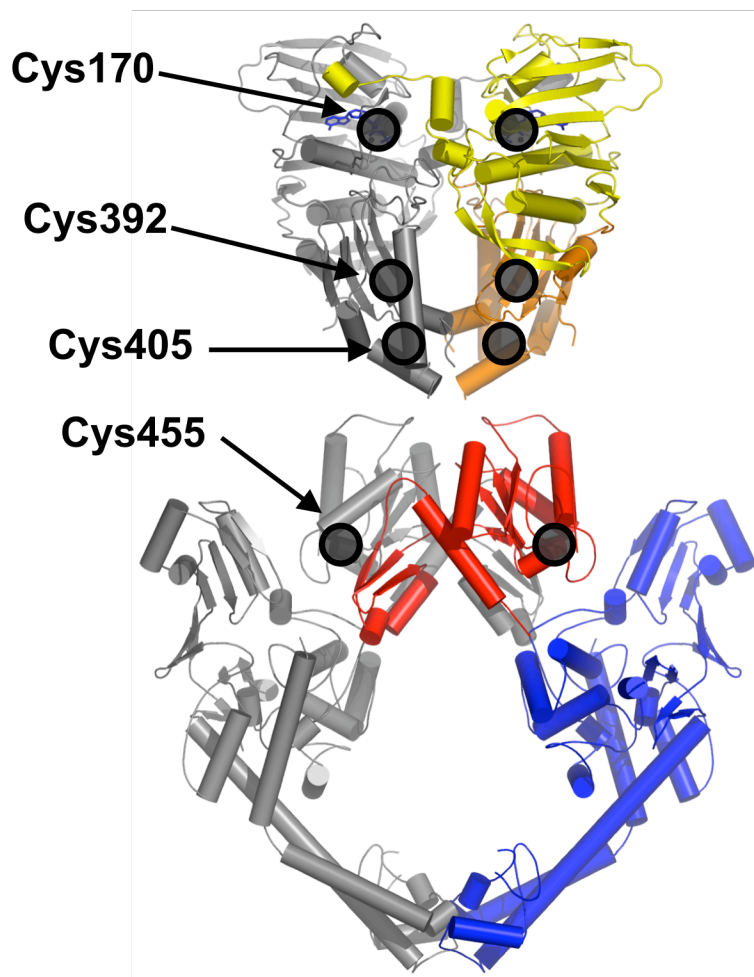
Figure 32. Sites of adduction by plumbagin in human topoisomerase IIα.
Residues adducted by quinones were identified by mass spectrometry. A
composite of the crystal structures of the yeast catalytic core and N-terminal
domain is shown and the locations of the homologous cysteine residues
adducted in human topoisomerase IIa are indicated by shaded circles. These
residues were mutated to alanines using mutagenesis PCR to evaluate their
role in quinone action. Adapted from Refs. (*58, 250*).
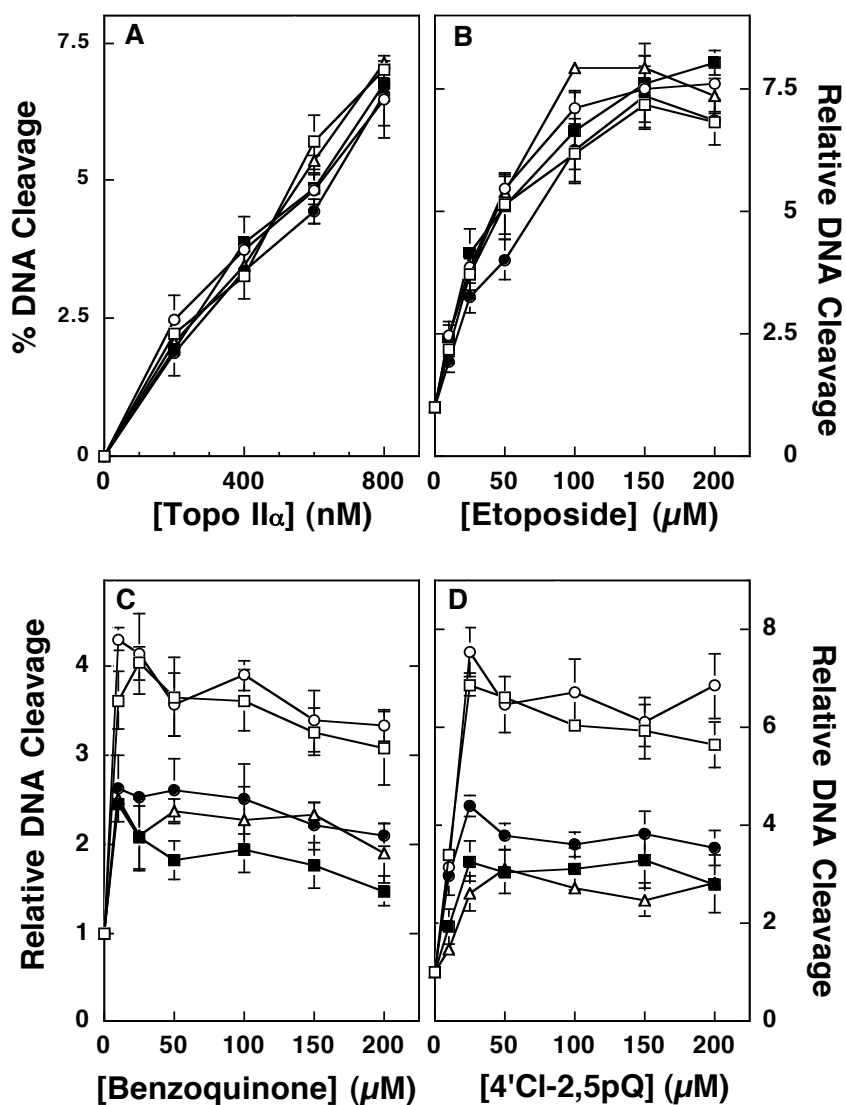
Figure 33. DNA cleavage activity of N-terminal mutant human topoisomerase IIα enzymes. Panel A: Cleavage activity was assessed using 0–800 nM topoisomerase IIα in the absence of quinones. Assay mixtures contained wild-type enzyme (WT, open squares), top2αC170A (C170A, open circles), top2αC392A (C392A, filled squares), top2αC405A (C405A, filled circles), or top2αC392/405A (C392/405A, open triangles). Panel B: DNA cleavage reactions were carried out in the presence of 0–200 µM etoposide. Panel C: DNA cleavage reactions were carried out in the presence of 0–200 µM benzoquinone. Panel D: DNA cleavage reactions were carried out in the presence of 0–200 µM 4'Cl-2,5pQ. Error bars represent the standard deviation of at least three independent experiments.

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responsive to benzoquinone (Figure 33C) and 4′Cl-2,5pQ (Figure 33D) than was wild-type topoisomerase IIα. The decreased sensitivity did not appear to be due to a reduced affinity for quinone, because at saturating levels of benzoquinone or 4′Cl-2,5pQ, levels of DNA cleaved by the mutant enzymes were only one-half of those observed with the parental enzyme.

The finding that mutation of cys392 or cys405 causes partial resistance toward quinones can be explained by two possibilities. For example, one residue may be involved in the inhibition of DNA religation while the other may be involved in blocking the N-terminal gate. If this were the case, an enzyme that carries alanine residues at both positions (top2αC392/405A) should display enhanced resistance over either singly-mutated enzyme.

Alternatively, adduction of these residues by quinones may enhance topoisomerase IIα-mediated DNA cleavage by the same mechanism (religation or the N-terminal gate). However, it is necessary to modify both residues in order to observe an effect. If this were the case, the doubly-mutated enzyme should display a sensitivity to quinones that is similar to that of either singly-mutated enzyme.

To address this mechanistic issue, top2αC392/405A was generated and isolated. Like top2αC392A and top2αC405A, the doubly-mutated enzyme exhibited wild-type DNA cleavage activity in the absence (Figure 33A) or presence (Figure 33B) of etoposide, but displayed ~one-half of the sensitivity to either benzoquinone (Figure 33C) or 4′Cl-2,5pQ (Figure 33D). These findings suggest that quinone adduction of cys392 and cys405 enhances topoisomerase IIα-mediated DNA cleavage as part of the same process.

Finally, two additional cysteine residues, cys216 and cys300, have been shown to exist in the protein as free sulfhydryls (*251*). Even though they were not identified as sites of quinone adduction in the present work, top2αC216A and top2αC300A were generated and analyzed. Neither enzyme displayed any resistance to benzoquinone or 4'Cl-2,5pQ (not shown).

Effects of Benzoquinone on DNA Religation Mediated by N-terminal Mutant Human Topoisomerase IIα Enzymes.

To further examine the role of cys392 and cys405 in facilitating the actions of quinones against human topoisomerase IIα, the abilities of wild-type enzyme, top2αC170A top2αC392A, top2αC405A, and top2αC392/405A to religate cleaved DNA in the absence or presence of benzoquinone were determined (Figure 34). In the absence of the quinone, all of the enzymes religated DNA to a similar extent (~50% ligation was observed in 10 s). However, in the presence of benzoquinone, top2αC392A, top2αC405A, and top2αC392/405A exhibited faster rates of religation (~40% ligation in 10 s) as compared to either wild-type topoisomerase IIα or top2αC170A (~20% ligation in 10 s). These findings support the conclusions of the preceding section and strongly suggest that adduction of cys392 and cys405 by quinones increases levels of DNA cleavage complexes by inhibiting the ability of the enzyme to religate cleaved nucleic acid substrates.

Ability of Benzoquinone to Block the N-terminal Gate of N-terminal Mutant Human Topoisomerase IIα Enzymes.

As discussed above, quinones that are strong topoisomerase II poisons contain multiple electrophilic carbons (*i.e.*, sites of adduction) and block the N-
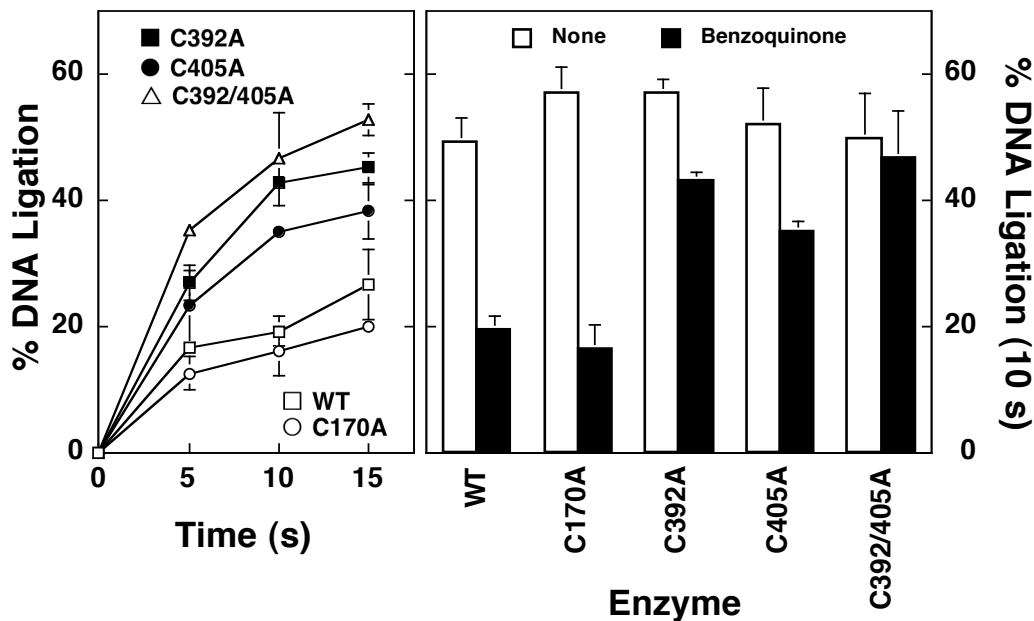
Figure 34. Quinone-resistant mutant human topoisomerase IIα enzymes display higher rates of DNA religation in the presence of benzoquinone. Left Panel: DNA religation reactions were carried out in the presence of 100 μM benzoquinone. Assay mixtures contained wild-type enzyme (WT, open squares), top2αC170A (C170A, open circles), top2αC392A (C392A, filled squares), top2αC405A (C405A, filled circles), or top2αC392/405A (C392/405A, open triangles). Samples were incubated at 37 ˚C to establish DNA cleavage/religation equilibria. Reactions were shifted to 0 ˚C to initiate religation. DNA religation was quantified by the loss of linear cleaved molecules. Right Panel: Representative DNA religation data at 10 s is shown. DNA cleavage/religation equilibria were established in the presence (filled bars) or absence (None, open bars) of benzoquinone. Error bars represent the standard deviation of at least three independent experiments.

terminal gate of human topoisomerase IIα by crosslinking the two protomers of the enzyme (*156, 223*). This action has no direct effect on the DNA cleavage-religation equilibrium of the enzyme. However, since it traps the non-covalent protein-DNA complex, it increases levels of scission by raising the concentration of DNA in the active site of topoisomerase II. Therefore, to determine whether any of the cysteine residues identified by mass spectrometry as sites of quinone adduction in topoisomerase IIα are involved in blocking the N-terminal gate of the enzyme, DNA binding, protein clamp closing, and protomer crosslinking experiments were performed.

Initial studies examined the ability of quinone-treated enzymes to bind circular and linear forms of DNA. As seen in Figure 35, treatment of wild-type topoisomerase IIα with benzoquinone prior to the addition of DNA differentially affects the ability of the enzyme to bind these DNA substrates. By blocking the N-terminal gate of topoisomerase IIα, quinones impair the ability of circular DNA to diffuse into the active site in the central annulus of the protein. This action decreased levels of circular DNA binding to ~30% of the levels observed in the absence of quinone. In contrast, linear forms of DNA, especially short oligonucleotides (double-stranded 50-mer), are able to enter the "closed-clamp" form of the enzyme and bind to the active site (~80% and 100% binding, respectively, as compared to parallel "no quinone" controls). DNA binding results for quinone-treated top2αC170A, top2αC392A, top2αC405A, and top2αC392/405A were nearly identical to those observed for the wild-type enzyme (Figure 35).

The second set of experiments utilized a "clamp closing" assay. In these experiments, the enzyme was incubated with circular DNA substrates prior to
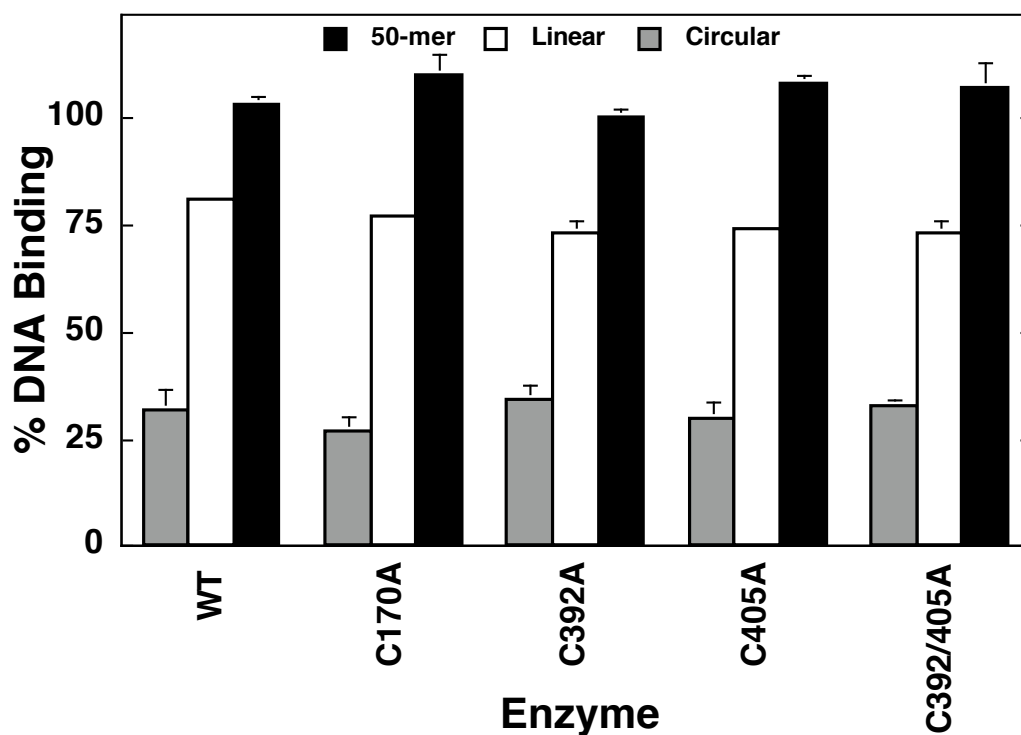
Figure 35. Substrate-dependent effects of benzoquinone on N-terminal mutant topoisomerase IIα-DNA binding. Assays employed a negatively supercoiled circular plasmid (shaded bars), a linear plasmid (open bars), or a duplex 50-mer oligonucleotide (filled bars) as the substrate. Enzymes were incubated with 100 μM benzoquinone for 5 min prior to the addition of DNA. DNA binding in the absence of benzoquinone was set to 100%. Error bars represent the standard deviation of at least three independent experiments.

the addition of benzoquinone. Blocking the N-terminal gate traps the circular substrate in the central annulus of topoisomerase IIα, generating a non-covalent protein-DNA complex that is stable in 1 M NaCl (*175*). Benzoquinone treatment increased the concentration of salt-stable protein-DNA complexes ~4–fold (from ~4.5% to ~17% of the total DNA) when wild-type topoisomerase IIα was employed (Figure 36). Once again, similar results were observed for all of mutant enzymes. Levels of salt-stable complexes formed with top2αC392A, top2αC405A, and top2αC392/405A were slightly lower (~13–14% of the total DNA) than seen with wild-type enzyme in the presence of benzoquinone. However, levels of salt-stable protein complexes formed with these enzymes also were lower (~3.2–3.6% of the total DNA) in the absence of quinone (Figure 36).

The third set of experiments examined the ability of benzoquinone to crosslink the two protomer subunits of human topoisomerase IIα. Exposure of the enzyme to quinones efficiently crosslinks the two protomer subunits of the protein homodimer. This event is monitored by gel electrophoresis under denaturing conditions and is characterized by the loss of the ~170 kDa protomer and the subsequent generation of a new protein species with an apparent molecular mass of ~330 kDa. As seen in Figure 37, treatment of wild-type topoisomerase IIα as well as the mutant enzymes with benzoquinone resulted in a rapid loss of protein monomer. The half-life for protomer crosslinking (~15 s) was similar for all of the enzymes examined. To address the possibility that all three cysteine residues (cys170, cys392, and cys405) are involved in crosslinking with only one being required at any given time, a triple mutant of topoisomerase IIα (top2αC170/392/405A) was generated. The half-life for protomer crosslinking

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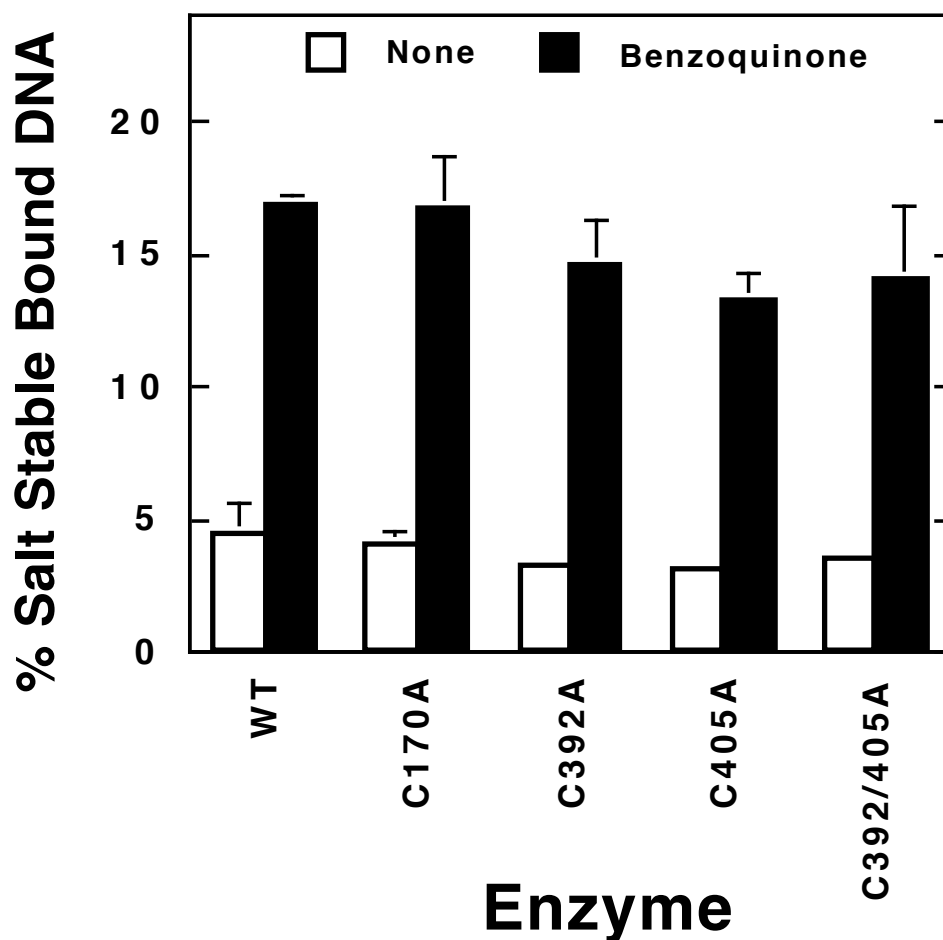
Figure 36. Benzoquinone blocks the N-terminal gate of N-terminal mutant human topoisomerase IIα enzymes. Filter binding assays were used to analyze the salt-stable closed-clamp of topoisomerase II. Enzyme-DNA complexes were established and further incubated in the absence (None, open bars) or presence (filled bars) of 100 μM benzoquinone. Samples were applied to glass fiber filters, DNA was eluted by sequential washes in low salt, high salt, and SDS, and eluted samples were subjected to electrophoresis in an agarose gel. Salt-stable non-covalent enzyme-DNA complexes were quantified by the amount of plasmid that did not elute until the SDS wash relative to the total plasmid eluted in all three washes. Error bars represent the standard deviation of at least three independent experiments.
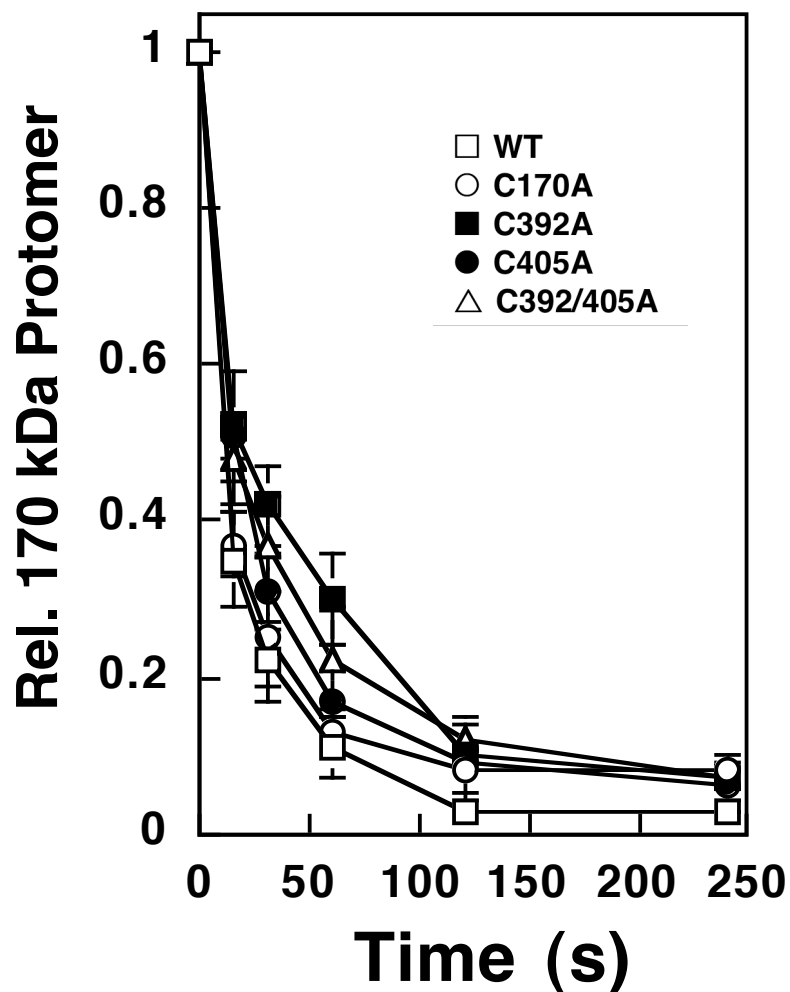
Figure 37. Benzoquinone crosslinks the protomer subunits of N-terminal mutant human topoisomerase IIα enzymes. Assay mixtures contained 135 nM wild-type enzyme (WT, open squares), top2αC170A (C170A, open circles), top2αC392A (C392A, filled squares), top2αC405A (C405A, filled circles), or top2αC392/405A (C392/405A, open triangles) that was treated with 100 μM benzoquinone for 0–4 min. Samples were subject to electrophoresis in a polyacrylamide gel and protein was visualized by coomassie staining. The level of 170 kDa enzyme protomer that was present in the absence of benzoquinone was set to 1. Error bars represent the standard deviation of at least three independent experiments.

of this enzyme was similar to that of the double top2αC392/405A mutant (data not shown).

Taken together, the experiments described above provide strong evidence that cys170, cys392, and cys405 are not required for quinones to block the N-terminal of topoisomerase IIα or crosslink the two protomer subunits of the protein. Thus, the adduction of cys392 and cys405 by benzoquinone appears to enhance DNA scission mediated by topoisomerase IIα primarily by inhibiting the ability of the enzyme to religate cleaved nucleic acid molecules.

Human Topoisomerase IIα Carrying a Cys455–>Ala Mutation (top2αC455A) is Hypersensitive to Quinones.

The previously characterized Cys–>Ala mutants (top2αC392A, top2αC405A, or top2αC392/405A) displayed wild-type DNA cleavage activity in the absence of topoisomerase II poisons. In contrast, top2αC455A exhibited a cleavage activity with negatively supercoiled plasmid DNA that was ~50% higher than that of the wild-type enzyme (Figure 38). Enhanced DNA cleavage was not due to an increase in DNA binding by the mutant enzyme. In fact, top2αC455A displayed a lower binding affinity for negatively supercoiled DNA than did wild-type topoisomerase IIα. In addition, as determined by nitrocellulose filter binding experiments the mutant and wild-type enzymes bound similar levels of linear plasmid DNA or oligonucleotide (data not shown).

To further analyze the properties of top2αC455A, its sensitivity toward topoisomerase II poisons was compared to that of wild-type human topoisomerase IIα. It should be noted that the data shown in Figure 39 is plotted as relative DNA cleavage. Thus, the data account for the enhanced scission
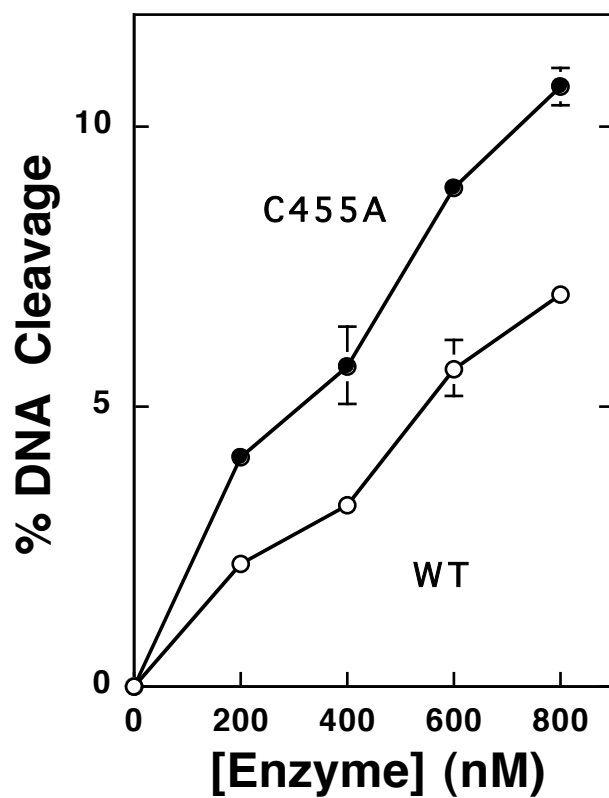
Figure 38. DNA cleavage activity of wild-type human topoisomerase IIα and top2αC455A. Cleavage activity was assessed using 0–800 nM enzyme. Assay mixtures contained wild-type enzyme (WT, open circles) or top2αC455A (C455A, closed circles). Error bars represent the standard deviation of at least three independent experiments.
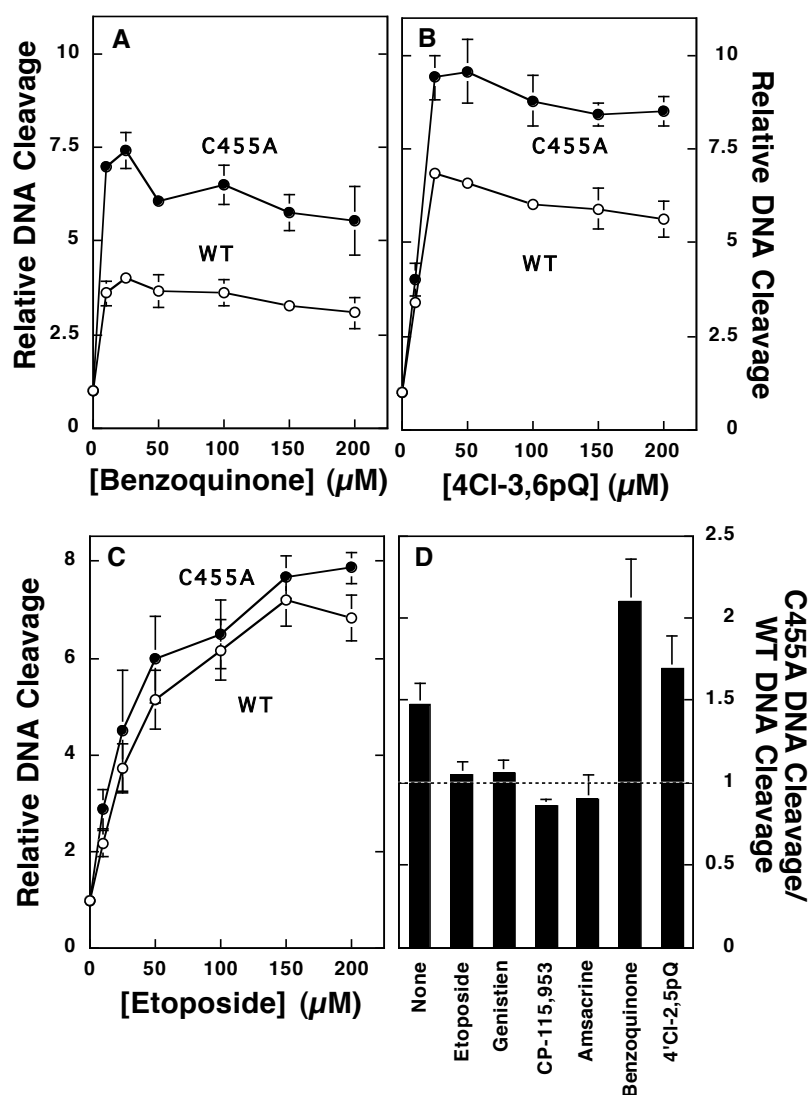
Figure 39. Effects of quinones and traditional topoisomerase II poisons on the DNA cleavage activity of top2αC455A. Assay mixtures contained wild-type topoisomerase IIα (WT, open circles) or top2αC455A (C455A, closed circles). Relative levels of DNA cleavage are shown. For both the wild-type and mutant enzymes, the level of DNA cleavage in the absence of topoisomerase II poisons was set to 1.0. Panel A: DNA cleavage was assessed in the presence of 0–200 μM benzoquinone. Panel B: DNA cleavage was assessed in the presence of 0–200 μM 4'Cl-2,5pQ. Panel C: DNA cleavage was assessed in the presence of 0–200 μM etoposide. Panel D: DNA cleavage was assessed in the presence of 50 μM etoposide, genistein, amsacrine, benzoquinone, or 4'Cl-2,5pQ, or 5 μM CP-115,953. Values represent the ratio of DNA cleavage generated by top2αC455A divided by that generated by the wild-type enzyme. The dotted line represents equal sensitivities for the indicated compound by both enzymes. Values below the line indicate resistance, while those above the line indicate hypersensitivity. Error bars represent the standard deviation of at least three independent experiments.

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activity of top2αC455A by setting the initial level of DNA cleavage in the absence of topoisomerase II poisons to 1.0 for both the mutant and wild-type enzymes.

As seen in Figure 39A, B, and D, the mutant enzyme was hypersensitive (~1.5– to 2–fold) to two quinone-based topoisomerase II poisons, benzoquinone and the PCB quinone 4'Cl-2,5pQ. The enhanced sensitivity did not appear to result from an increased affinity for the quinones, because at saturating (*i.e.*, plateau) concentrations of benzoquinone or 4'Cl-2,5pQ, levels of DNA cleaved by the mutant enzyme were always higher than those observed for the wild-type enzyme.

To determine whether the enhanced susceptibility of top2αC455A was unique to quinones, the sensitivity of the enzyme toward a series of traditional (*i.e.*, non-covalent) topoisomerase II poisons was assessed. Drugs from four different classes were employed for these experiments, including etoposide (a demethylepipodophyllotoxin), genistein (an isoflavone), CP-115,953 (a quinolone), and amsacrine (an anilinoacridine). As seen in Figure 39C and D, the sensitivity of the mutant enzyme toward these traditional topoisomerase II poisons was similar to that of wild-type topoisomerase IIα. Therefore, the hypersensitivity of top2αC455A appears to be specific to quinone-based topoisomerase II poisons.

Basis for the Quinone Hypersensitivity of top2αC455A.

As discussed above, quinones have two effects on human topoisomerase IIα that may contribute to their actions as topoisomerase II poisons: they inhibit the ability of the enzyme to ligate cleaved DNA and they block the N-terminal gate of the protein. As a first step toward determining the mechanistic basis for the

quinone hypersensitivity of top2αC455A, the ability of the enzyme to ligate DNA was characterized. In the absence of quinones, the mutant enzyme ligated DNA ~1/3 slower than did wild-type topoisomerase IIα (Figure 40). This decreased ligation rate probably accounts (at least in part) for the higher levels of DNA cleavage generated by top2αC455A in the absence of topoisomerase II poisons.

Addition of benzoquinone to reaction mixtures decreased the ability of top2αC455A to ligate DNA (Figure 40). Levels of ligation mediated by top2αC455A dropped ~2.8–fold (from 33.1% to 11.9%) in the presence of the quinone. This drop was similar to the 2.5–fold decrease observed for wild-type topoisomerase IIα (from 49.0% to 19.3%). Therefore, the heightened sensitivity of top2αC455A to quinones does not appear to be related to effects on DNA ligation.

To further explore the basis for quinone hypersensitivity, the ability of benzoquinone to block the N-terminal protein gate of top2αC455A was characterized (*175*) (Figure 41). In the absence of quinone, top2αC455A and wild-type topoisomerase IIα trapped similar levels of DNA in a salt-stable non-covalent complex (4.5% vs. 5.6%, respectively). However, in the presence of benzoquinone, a dramatic difference was observed. Whereas the level of DNA trapped by the wild-type enzyme rose to 16.8%, that trapped by top2αC455A was ~3 times higher (47.1%). These data strongly suggest that the enhanced ability of quinones to block the N-terminal gate of top2αC455A contributes to the hypersensitivity of the enzyme toward these topoisomerase II poisons. Furthermore, this activity may contribute to enzyme-mediated DNA breaks.

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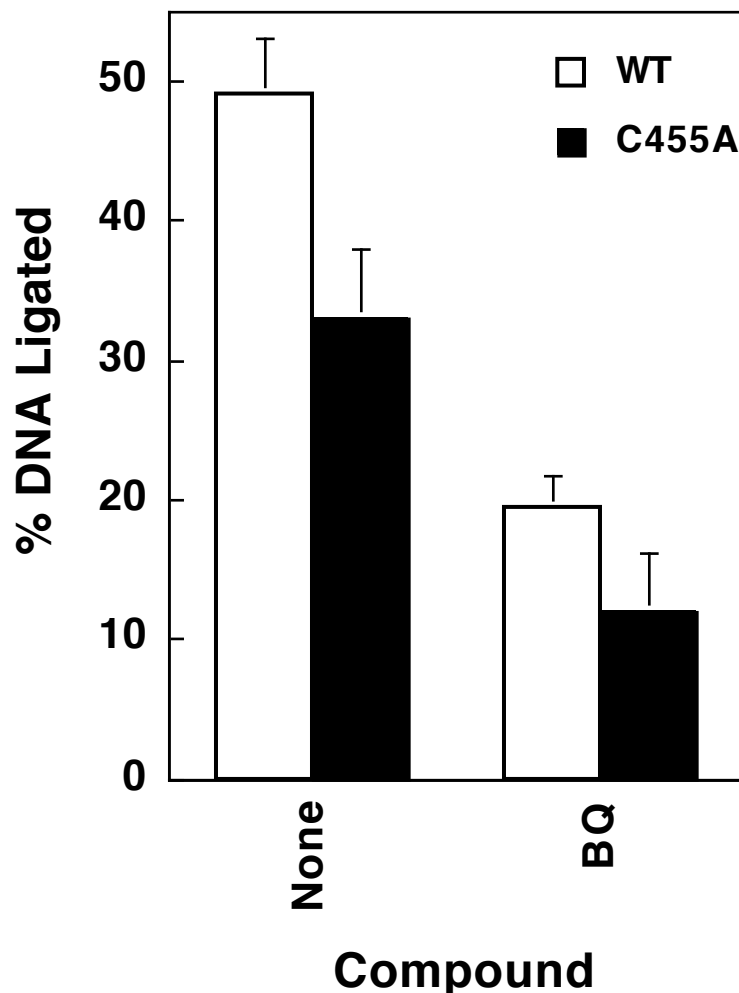
Figure 40. DNA ligation mediated by wild-type human topoisomerase IIα and top2αC455A in the absence and presence of benzoquinone. Samples contained wild-type topoisomerase IIα (WT, open bars) or top2αC455A (C455A, closed bars) and were incubated at 37 ˚C to establish DNA cleavage/ligation equilibria. Reactions were shifted to 0 ˚C for 10 s, and DNA ligation was quantified by the loss of linear cleaved molecules. DNA cleavage/ligation equilibria were established in the absence (None) or presence (BQ) of 100 μM benzoquinone. Error bars represent the standard deviation of at least three independent experiments.
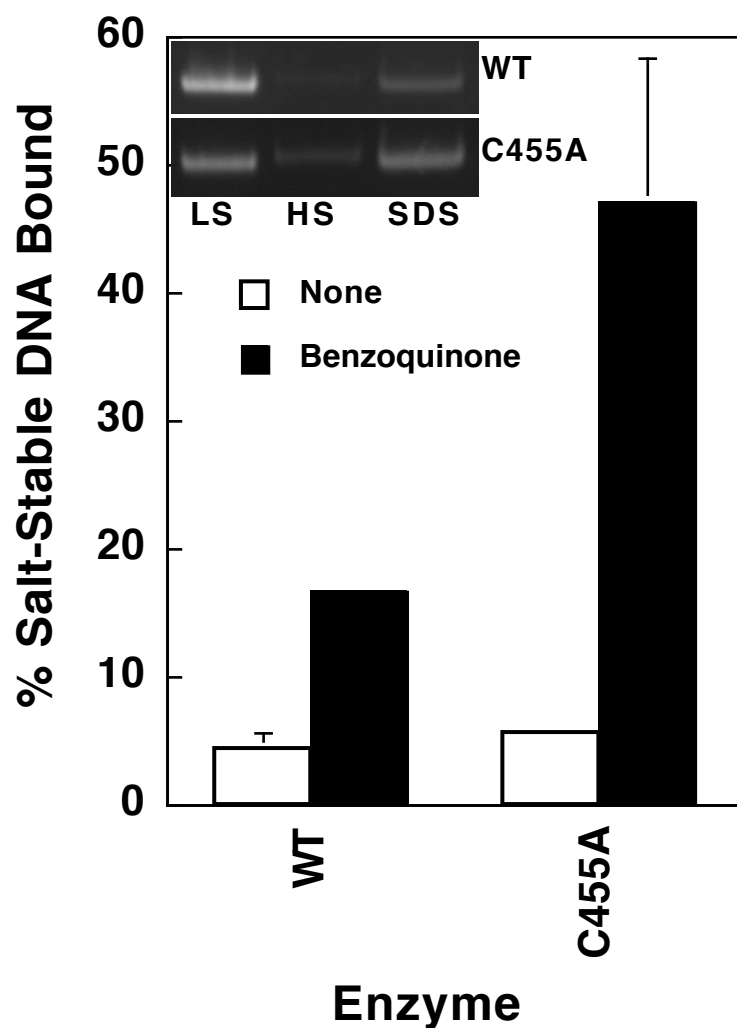
Figure 41. Ability of quinones to close the N-terminal gate of wild-type human topoisomerase IIα (WT) and top2αC455A (C455A). Filter binding assays were used to analyze the salt-stable closed-clamp form of topoisomerase II. Enzyme-DNA complexes incubated in the absence (None, open bars) or presence (filled bars) of 100 μM benzoquinone. Samples were applied to glass fiber filters, eluted by sequential washes in low salt (LS), high salt (HS), and SDS, and eluted DNA was subjected to electrophoresis in an agarose gel. Representative gels are shown in the inset. Salt-stable non-covalent enzyme-DNA complexes were monitored by quantifying the amount of plasmid in the SDS wash relative to the total plasmid eluted in all three washes. Error bars represent the standard deviation of at least three independent experiments.

A variety of drug-related and environmental quinone-based metabolites are potent topoisomerase II poisons. These compounds act by adducting the protein, and previous studies suggest that they increase levels of enzyme-DNA cleavage complexes by at least two potentially independent mechanisms (*1, 117*). Quinones act directly on the DNA cleavage-religation equilibrium of topoisomerase II by inhibiting rates of religation. Alternatively, they block the N-terminal gate of the protein and thereby raise levels of cleavage complexes by increasing the local concentration of DNA in the active site of the enzyme. It is not known whether these two effects result from quinone adduction to the same amino acid residue(s) in topoisomerase IIα or whether they are mediated by modification of separate residues.

Although anticancer drugs and other classic topoisomerase II poisons function at the active site of the enzyme (*10, 15, 16, 95, 96, 252*), sites at which quinone-based poisons adduct the protein are not known. Therefore, the present study identified amino acid residues that are modified by quinones and determined their role in the actions of these compounds as topoisomerase II poisons. Four cysteine residues were identified by mass spectrometry as sites of quinone adduction: cys170, cys392, cys405, and cys455. Mutations (cys–>ala) were individually generated at each of these positions. Mutations at cys392 or cys405 resulted in enzymes with reduced sensitivity to benzoquinone or 4'Cl-2,5pQ. Top2αC392A, top2αC405A, and the double mutant top2αC392/405A all displayed ~50% resistance to these compounds. Furthermore, top2αC392/405A (the only mutant enzyme tested) was ~2– to 4–fold resistant to plumbagin (data

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not shown). Results of DNA religation and binding studies, together with protein clamp closing and crosslinking experiments, strongly suggest that adduction of cys392 and cys405 increases levels of topoisomerase IIα-mediated DNA cleavage primarily by inhibiting the ability of the enzyme to reseal nucleic acid breaks.

Studies with top2αC392A, top2αC405A, and the double mutant top2αC392/405A indicate that cys392 and cys405 both are required to observe inhibition of DNA religation by quinones. Given the close proximity of these two residues (Figure 32), an intriguing possibility is that quinones such as benzoquinone act by forming an intra-protomer crosslink between cys392 and cys405. This could help explain why mono-adducting quinones, such as plumbagin, are weak topoisomerase II poisons. Future structural studies will be required to explore this issue.

Cys455 also was identified as a site of quinone adduction by mass spectrometry. The mutant enzyme was ~1.5– to 2–fold hypersensitive to benzoquinone and 4'Cl-2,5pQ, but displayed wild-type sensitivity to traditional topoisomerase II poisons. The ability of benzoquinone to inhibit DNA ligation mediated by top2αC455A was similar to that of wild-type topoisomerase IIα. However, the quinone induced ~3 times more clamp closure with the mutant enzyme when compared to wild-type topoisomerase IIα. These findings strongly support the hypothesis that quinones increase levels of topoisomerase II-associated DNA strand breaks, at least in part, by blocking the N-terminal gate of the enzyme.

Since Cys455 is located in the in the catalytic core of topoisomerase IIα as opposed to the N-terminal domain (*58, 250*), it is unlikely that the residue plays a direct role in clamp closure. Why then does the Cys455–>Ala mutation enhance

the effects of quinones on the N-terminal gate of topoisomerase II? At least three possibilities exist. First, Cys455 may simply represent a "sink" for quinone adduction. This would diminish levels of compound available to modify amino acid residues involved in clamp closure. Second, adduction of Cys455 may attenuate the ability of quinones to block the N-terminal gate of topoisomerase II. Therefore, removal of the residue would enhance the actions of quinones on clamp closure. Third, the existence of Cys455 as part of a disulfide bridge may impede closure of the N-terminal gate. To this point, the majority of Cys455 exists in a disulfide bridge; at any given time, only 10 to 15% of the residue exists as a free sulfhydryl (*251*). Thus, quinone adduction of Cys455 would enhance the ability of other quinone molecules to close the N-terminal gate by preventing the disulfide bridge from reforming. Mutation of Cys455–>Ala would prevent the formation of the disulfide bridge and enhance clamp closing by mimicking the effects of quinone adduction.

The first possibility appears to be unlikely. If the sole role of Cys455 in quinone action was to draw these compounds away from other residues, levels of DNA cleavage mediated by wild-type topoisomerase IIα would eventually approach those seen with top2αC455A as quinone concentrations reached saturation. As seen in Figure 2, this was not the case. At the present time, it is not possible to distinguish between the second and third possibilities (or an even more complicated scenario). Further mechanistic and structural studies most likely will be required to address this complex issue.

In conclusion, quinones are topoisomerase II poisons that act by adducting the enzyme. Results of the present study indicate that adduction of cys392 and cys405 is important for the actions of quinones and increases levels of

enzyme-DNA cleavage complexes primarily by inhibiting DNA religation (Figure 34). Furthermore, evidence suggests that quinones also contribute to topoisomerase II-mediated DNA scission by blocking the N-terminal protein gate. As discussed in previous chapters, this latter effect may inhibit enzyme activity in the absence of DNA. Taken together, these data support the hypothesis that quinones enhance DNA scission by the enzyme by at least two independent mechanisms (Figure 42).

Crosslink the N-terminus In the absence of DNA and inhibit the enzyme

Crosslink the N-terminus in the presence of DNA and increase non-covalent protein-DNA complexes - may contribute to cleavage enhancement

Adduct (crosslink?) C392 and C405 to stabilize cleavage complexes
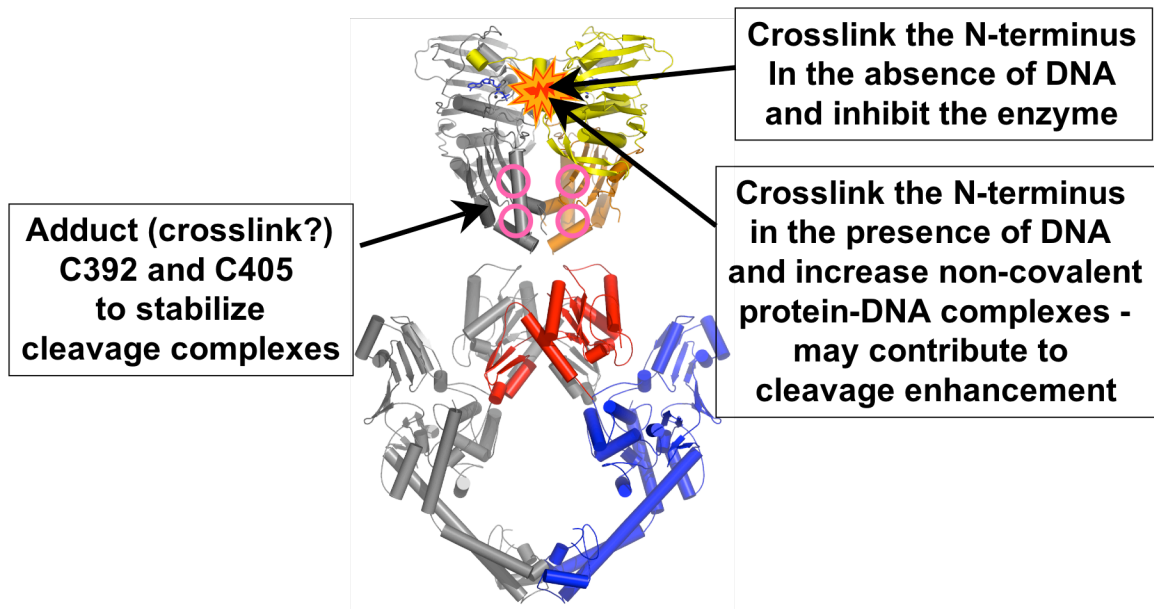
Figure 42. Summary of activities of quinones against topoisomerase IIα. Quinones adduct to residues cys392 and cys405 to inhibit ligation of the cleaved DNA substrate. Quinones also have the ability to close the N-terminal protein gate. In the absence of DNA this activity inhibits enzyme activity. Conversely, in the presence of DNA, this acitivity enhances DNA scission by trapping DNA in the active site of the enzyme. Adapted from Refs. (*58, 250*).

CHAPTER VI

TOPOISOMERASE II-DRUG INTERACTION DOMAINS: IDENTIFICATION OF SUBSTITUENTS ON ETOPOSIDE THAT INTERACT WITH THE ENZYME

Introduction

Podophyllotoxins, derived from the Mayapple or Mandrake root, have been used to treat various ailments for over a millennium (*253*). Although podophyllotoxins were used in the treatment of many diseases, the toxicity of these agents is a limiting factor. This toxicity prompted the development of etoposide among other podophyllotoxin derivatives (*105*).

Etoposide is one of the most successful chemotherapeutic agents used for the treatment of human cancers (*10, 104, 105, 151*). The drug currently is in its third decade of clinical use and is front line therapy for a variety of malignancies, including leukemias, lymphomas, and several solid tumors (*10, 104, 105, 151*). Until the development of paclitaxel and related compounds, etoposide was the most widely prescribed anticancer drug in the world.

The cellular target of etoposide is topoisomerase II (*10, 104-106, 151*). The drug acts by inhbiting ligation of topoisomerase II-mediated double-stranded breaks (*97, 196*). Even though the cellular target and mechanism of action of etoposide have been known for over 20 years, the interacting domains of the drug with its protein target are largely unknown.

Several lines of evidence indicate that interactions between topoisomerase II and etoposide, as opposed to drug-DNA interactions, are critical for drug activity and mediate the entry of etoposide into the ternary enzyme-drug-DNA

complex. First, mutation of specific residues in topoisomerase II dramatically affects the ability of etoposide to increase levels of enzyme-DNA cleavage complexes (*10, 15, 109, 160, 254-258*). Second, etoposide binds weakly (if at all) to DNA in the absence of topoisomerase II (*259*). Third, the drug binds to yeast topoisomerase II and human topoisomerase IIα in the absence of nucleic acids and a mutant yeast enzyme (yTop2H1011Y) that is resistant to etoposide displays a reduced binding affinity for the agent (*260, 261*). Fourth, etoposide displays a similar kinetic affinity for topoisomerase II-DNA cleavage complexes formed at sites with markedly different levels of scission enhancement (*262*). Finally, DNA breaks accumulate more rapidly when etoposide is incubated with topoisomerase II prior to the addition of DNA (as compared to the opposite order of addition) (*262*).

Hundreds of etoposide derivatives have been analyzed in an effort to establish structure-activity relationships within this drug class (*263-272*). Despite the importance of protein-drug interactions, none of these studies have identified any of the substituents on etoposide that interact with topoisomerase II. However, a recent study from our laboratory utilized saturation transfer difference [^{1}H]-nuclear magnetic resonance (STD [^{1}H]-NMR) spectroscopy (*273-277*) and protein-drug competition binding assays to define the groups on the drug that associate with the type II enzyme. As shown in Figure 43, substituents on the A–, B–, and E–rings of etoposide interact with yeast topoisomerase II and human topoisomerase IIα. More specifically, NOE signals were observed for the geminal protons of the A–ring (6.0 ppm), the H5 and H8 protons of the B–ring (7.0 and 6.6 ppm, respectively), as well as the H2′ and H6′ protons (6.4 ppm) and the 3′– and 5′–methoxyl protons of the pendent E–ring (3.8 ppm). In contrast, no
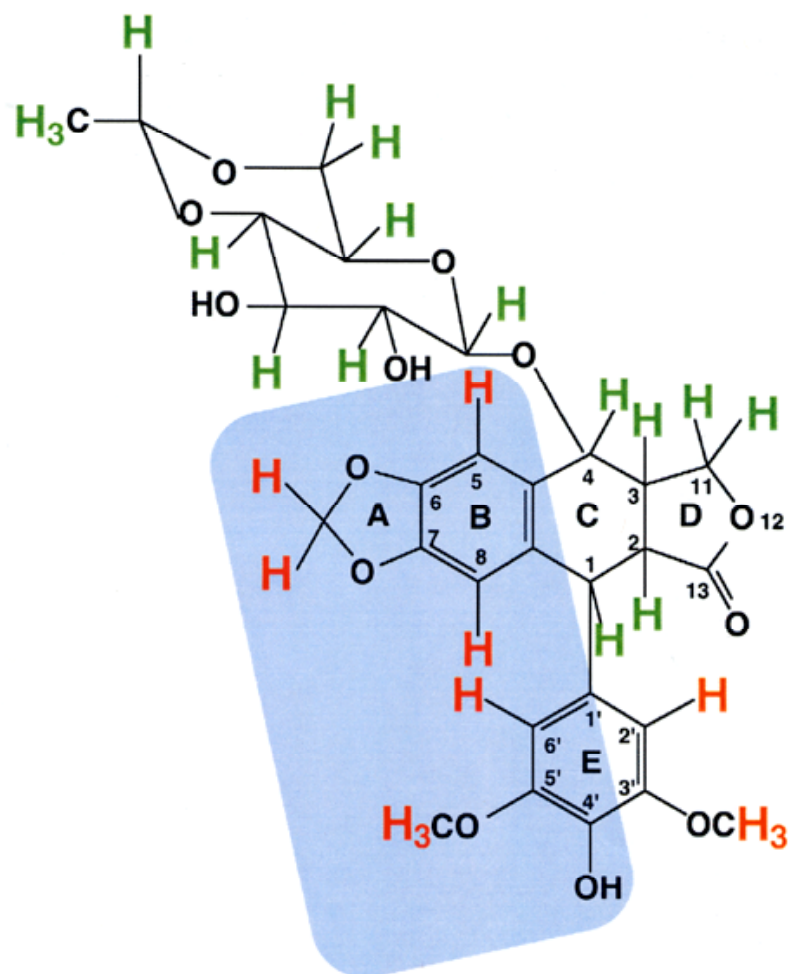
Figure 43. Summary of etoposide substituents that interact with type II topoisomerases. Protons that interact with the enzyme are shown in red, those that do not are shown in green. Hydroxyl protons that were obscured by the water peak and could not be visualized are indicated in black. The shaded region on etoposide, including portions of the A–, B– and E–rings, is proposed to interact with topoisomerase II in the binary drug-enzyme complex. Adapted from Ref. *(278)*.

significant NOE signals arising from the C–ring, the D–ring, or the C4 glycosidic moiety were observed, suggesting that there is limited or no contact between these portions of etoposide and the enzyme in the binary complex (*278*).

In order to further characterize the contributions of the A– and E– rings and glycosidic moiety of etoposide to drug function several podophyllotoxin derivatives were employed. Results indicate that the geminal protons of the A–ring of etoposide bind to topoisomerase II and are necessary for drug-protein interactions. In addition, the glycosidic moiety has minor contributions *in vitro* and is dispensable for efficient drug activity. The E–ring appears to play a functional role, but additional bulk at the 4' position hinders drug-enzyme binding.

<u>Results</u>

Previously, the interacting hydrogens of etoposide with the yeast type II enzyme were mapped using STD-NMR (*278*). geminal protons of the A–ring, the H5 and H8 protons of the B–ring, as well as the H2' and H6' protons and the 3'– and 5'–methoxyl protons of the pendent E–ring. To verify the ability of the STD-NMR technique to predict interacting drug domains, a variety of podophyllotoxin derivatives were tested for their ability to poison topoisomerase II (See Figures 44 and 46).

E–ring Substituents Mediate Drug Activity.

A previous study in our lab examined the ability two etoposide derivatives, hydroxyphenyl-etoposide and phenyl-etoposide, to act as topoisomerase II poisons (*278*). Removal of the 3'- and 5'-methoxyl groups from the E–ring of

etoposide (hydroxyphenyl-etoposide; Figure 44) decreased drug activity ~60% (*278*). Further removal of the 4'-hydroxyl group (phenyl-etoposide; see Figure 44), decreased drug activity to nearly baseline (*278*). However, despite the loss of these critical substituents, the pendant E–rings of the agents still maintain a close association with topoisomerase II in the binary complex (*278*). The results of this study suggests that while the 3'– and 5'–methoxyl groups and the 4'-hydroxyl of the E–ring are important for drug function, are not required for drug binding.

Therefore, the current study employed nitrocellulose filter binding competition assays to further define the contribution of these groups to drug binding. As seen in Figure 45, the ability of hydroxyphenyl-etoposide and phenyl-etoposide to compete with [^{3}H]etoposide for binding to yeast topoisomerase II was similar to that of etoposide. On the basis of these results, we conclude that the 3'– and 5'– methoxyl groups and the 4'–hydroxyl of the etoposide E–ring play critical functional roles for drug action, but do not mediate binding of the drug to the type II enzyme.

The Glycosydic Moiety of Etoposide Does not Contribute to Drug-induced DNA Cleavage Mediated by Human Topoisomerase IIα.

It has long been known that the glycosidic moiety of etoposide plays important physiological roles (*10, 104, 105, 151*). The presence of this group keeps etoposide from interacting with tubulin. Beyond these physiological functions, it is not clear whether the glycosidic moiety of etoposide plays any direct role in enhancing topoisomerase II-mediated DNA cleavage. To this point, no contacts
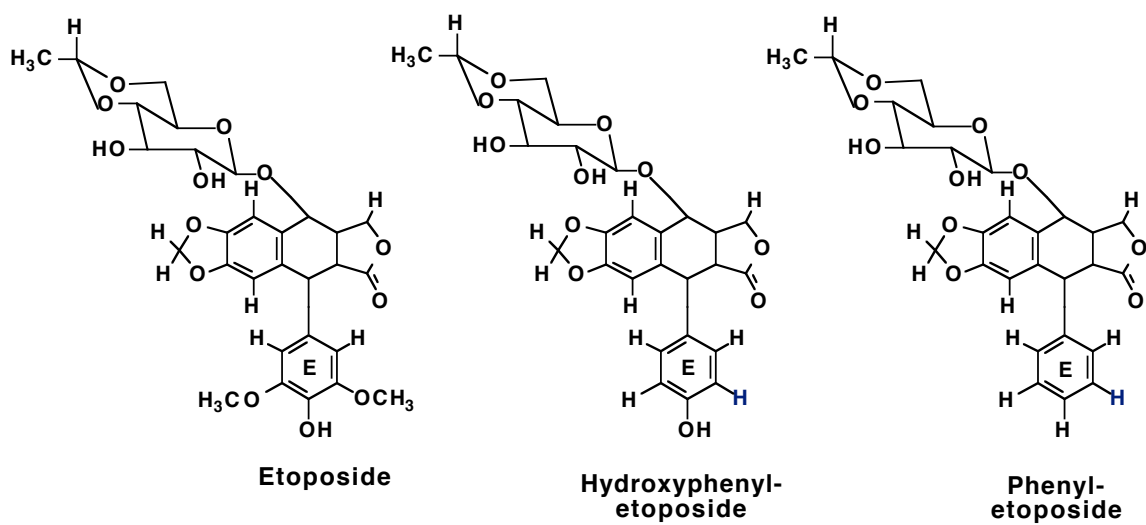
Figure 44. Structure of Etoposide and E–ring etoposide derivatives.
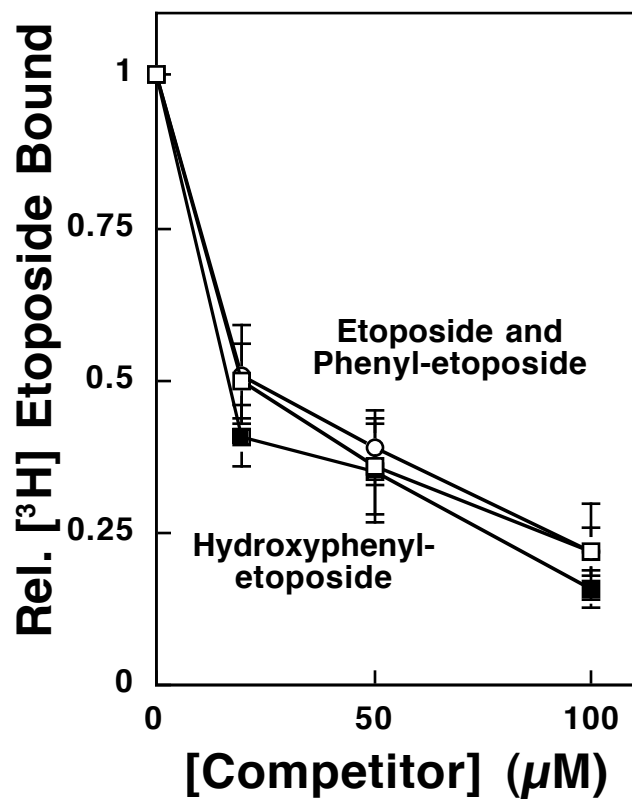
Figure 45. Binding of etoposide and derivatives to yeast topoisomerase II.
Reaction mixtures contained 20 μM [^{3}H]etoposide and 0–100 μM etoposide
(open squares), hydroxyphenyl-etoposide (closed squares), or phenyl-
etoposide (open circles). Levels of [^{3}H]etoposide binding to yeast
topoisomerase II observed in the absence of competitor drug were set to 1.
Error bars represent the standard deviation of three independent experiments.

were observed between topoisomerase II and any glycosidic protons (*278*). However, the hydroxyls of this moiety were unable to be monitored. It is notable that substitution of the glycosidic moiety with a flexible and charged amino-alkyl side chain, as in TOP-53, significantly enhanced drug-enzyme binding and drug activity against topoisomerase II (*278*). Furthermore, contacts were observed between the enzyme and every observable proton of this side chain in the binary complex.

In order to determine the functional relevance of the sugar moiety of etoposide, the activity of 4′-Demthylepipodophyllotoxin (DEPT) was characterized. As seen in figure 46, DEPT has all of the same components of etoposide but lacks the sugar moiety linked to the C-ring. The ability of 4′-DEPT to induce topoisomerase IIα-mediated DNA cleavage was assessed using DNA cleavage assays. As seen in Figure 47, left panel, 4′-DEPT also increased levels of enzyme-mediated double stranded DNA breaks similar, although slightly lower, to those induced by etoposide. Consistent with previous NMR results, it appears that the sugar moiety so not significantly contribute to the activity of etoposide.

Intact *A– and E–ring Moieties are Necessary to Efficiently Induce Topoisomerase IIα-Mediated DNA Cleavage.*

A previous study indicated that the A-ring was important for drug binding to topoisomerase II and the E–ring was involved in drug function (*278*). Thus, two additional podophyllotoxin derivatives were utilized in this study (see Figure 46). Epipodophyllotoxin (EPT) has a 4′-methoxy group present on the E– ring of the molecule to examine the sensitivity of drug function to additional
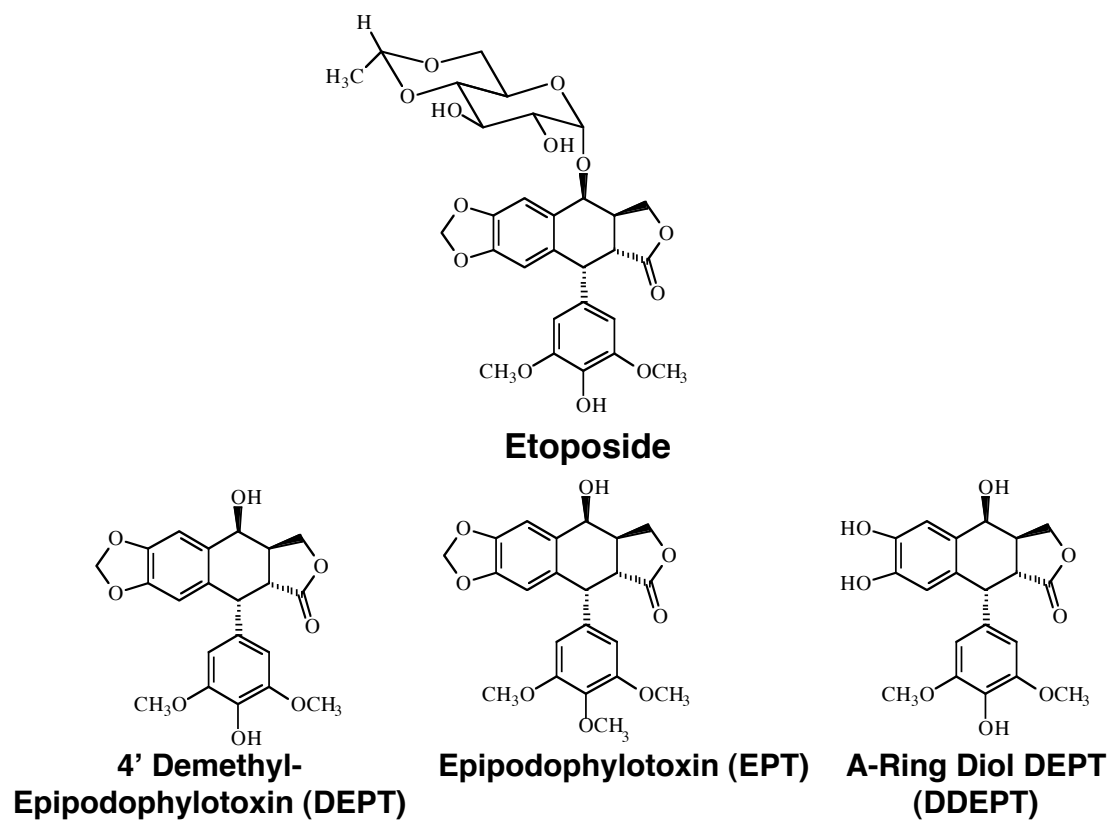
Figure 46. Structure of etoposide and podophyllotoxin derivatives.
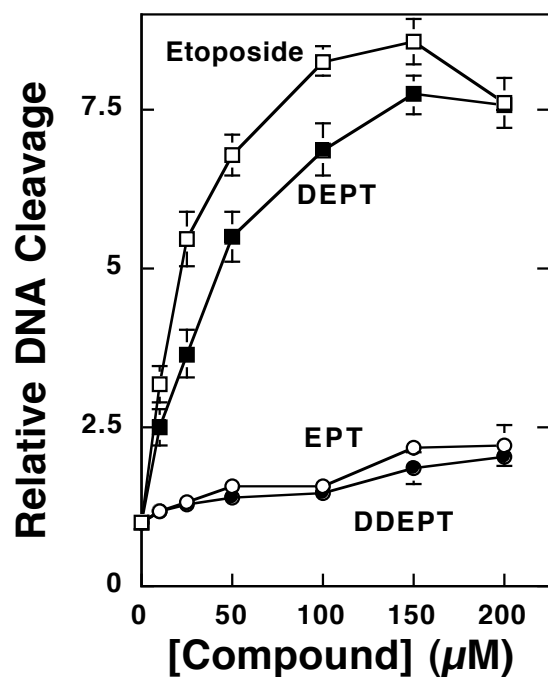
Figure 47. Effect of podophyllotoxin derivatives on DNA cleavage mediated by human topoisomerase IIα. Levels of DNA cleavage were quantified and expressed as a fold enhancement over reactions that were carried out in the absence of podophyllotoxin derivatives. Reaction mixtures contained 0–200 μM etoposide (open squares), DEPT (closed squares), EPT (open circles), or DDEPT (closed circles). Right Error bars represent the standard deviation of at least three independent experiments.

bulk at the 4′ position. A–ring 4′-Demethylepipodophyllotoxin diol, (DDEPT) contained an A–ring that was opened to remove the interacting geminal protons.

As a first step to determine the effects of the above modifications, the ability EPT and DDEPT to induce topoisomerase II-mediated DNA cleavage was assessed. Neither EPT nor DDEPT were able to efficiently stimulate single- or double-stranded DNA breaks mediated by the enzyme (figure 47). These data suggest that the geminal protons of the A–ring are essential for etoposide function and the addition of bulk at the 4′ position greatly decreases drug efficiency.

The A– and E–Rings of Etoposide are Important in Inhibiting DNA Ligation Mediated by Human Topoisomerase IIα.

It has been established that the primary mode of action of etoposide is the inhibition of topoisomerase II-induced ligation of the cleaved DNA substrate (*10, 97, 196*). Therefore, the ability of the podophyllotoxin derivatives to inhibit DNA ligation was assessed. Since there was a difference observed in the ability of etoposide and the derivatives to induce single- and double-stranded DNA breaks (Figure 47), enzyme-mediated ligation of both types of breaks was monitored.

Ligation of double-stranded DNA breaks was monitored using a plasmid-based system. Consistent with previous DNA cleavage experiments, etoposide and DEPT inhibited similar amounts of topoisomerase II-mediated ligation (Figure 48). EPT and DDEPT were unable to inhibit DNA ligation of cleaved nucleic acids.
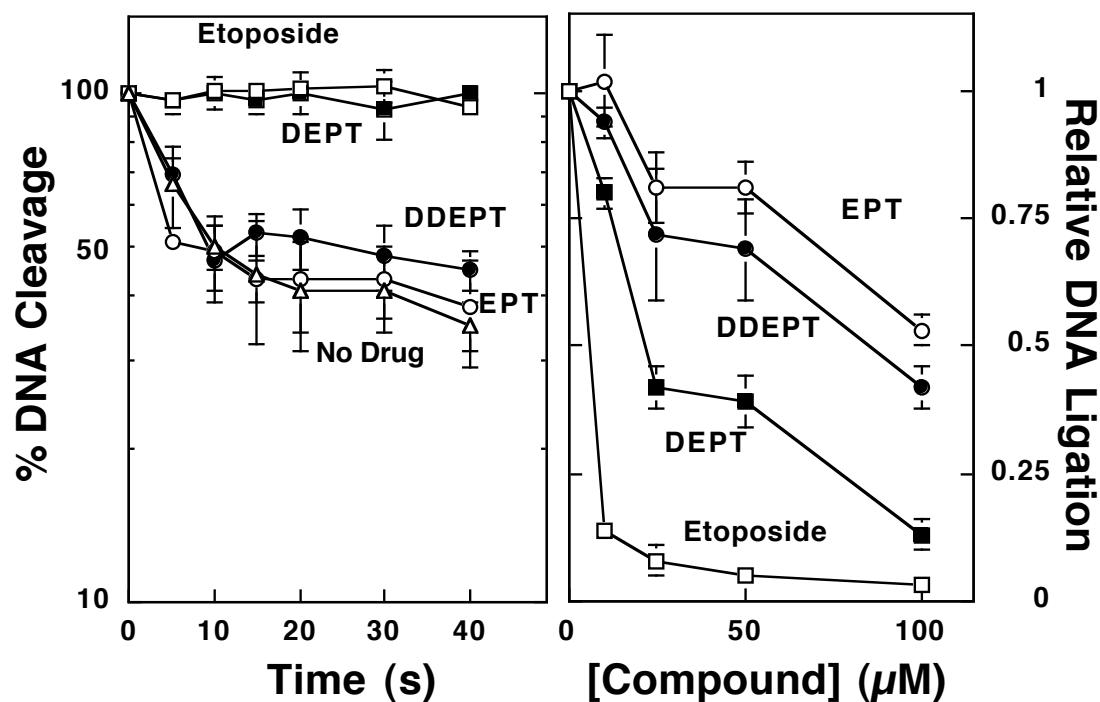
Figure 48. DNA ligation mediated by human topoisomerase IIα in the presence of podophyllotoxin derivatives. Left panel: DNA ligation of plasmid was examined in the absence of compound (No drug, open triangles), or in the presence of 100 μM etoposide (open squares), DEPT (closed squares), EPT (open circles), or DDEPT (closed circles). DNA religation was quantified by the loss of linear cleaved molecules. Right panel: DNA ligation of a nicked oligonucleotide substrate was examined in the presence of 0-100 μM etoposide (open squares), DEPT (closed squares), EPT (open circles), or DDEPT (closed circles). DNA religation was quantified by the loss of nicked DNA substrates. Error bars represent the standard deviation of at least three independent experiments.

In order to assess the ability of the podophyllotoxin derivatives to inhibit enzyme-mediated DNA ligation, a nicked oligonucleotide was utilized (*171*). Again, consistent with cleavage data, 4'-DEPT displayed the ability to inhibit topoisomerase II-mediated ligation but to a lesser extent than etoposide (Figure 48). In contrast, both EPT and DDEPT displayed a limited ability to inhibit ligation of a DNA nick despite being unable to induce double-stranded breaks by topoisomerase II.

Specific Alterations of the A– and E–rings of Etoposide Impair Drug Binding to Human Topoisomerase IIα.

In previous NMR experiments, protons of the A– B– and E– rings of etoposide were in intimate association with the enzyme (*278*). However, removal of essential components of the E–ring impaired drug function without disrupting binding to topoisomerase II (*278*) (See Figure 45). In order to assess whether components of the A–ring are necessary for drug binding and the effect of additional bulk at the 4' position of the E–ring on drug-enzyme interactions, the ability of the podophyllotoxin derivatives bind to human topoisomerase IIα was assessed.

As seen in figure 49, removal of the sugar moiety of etoposide did not affect drug binding of the resulting compound (DEPT). However, addition of bulk on the E–ring (EPT) greatly reduced the ability of the agent to bind to its enzyme target. Furthermore, opening of the A–ring of etoposide also greatly inhibited drug binding. These data suggest that the A–ring of etoposide makes necessary interactions with topoisomerase II, while the E–ring of the drug potentially rests in a space-restricted pocket within the enzyme. The data also indicate that the
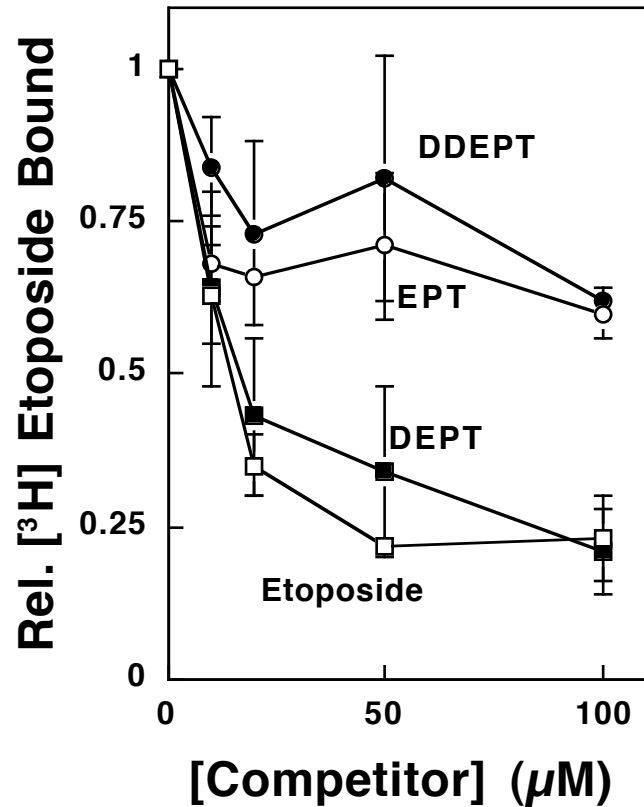
Figure 49. Binding of etoposide and podophyllotoxin derivatives to human topoisomerase IIα. Competition nitrocellulose filter binding assays were utilized. Reaction mixtures contained 20 μM [³H]etoposide and 0–100 μM etoposide (open squares), DEPT (closed squares), EPT (open circles) or DDEPT (closed circles). Levels of [³H]etoposide binding to human topoisomerase IIα observed in the absence of competitor drug were set to 1. Error bars represent the standard deviation of three independent experiments.

minor contributions of the glycosidic moiety to cleavage do not involve drug binding to topoisomerase IIα.

Loss of the Glycosidic Moiety Slightly Alters Cleavage Site Utilization of Topoisomerase IIα.

One property of etoposide is that this agent possesses cleavage-site sequence specificity when incubated in the presence of topoisomerase IIα and a DNA substrate (*106, 225*). This indicates that some portion of the molecule interacts with DNA in the ternary complex. The loss of the sugar moiety at the C4 position of etoposide slightly impairs the ability of the resulting compound to induce cleavage by topoisomerase II, but does not alter the ability of the agent to bind to the enzyme. This data confirms previous NMR results that the glycosidic moiety does not interact with the enzyme in the binary complex. However, it is possible that the A– and E–ring substituents as well as the glycosidic moiety influence cleavage site utilization by the enzyme. Therefore, to determine whether the glycosidic moiety of etoposide influences cleavage site utilization of topoisomerase II, cleavage assays utilizing a ^{32}P singly-end labeled DNA substrate were performed.

Since Etoposide induced far more single strand breaks than any of the podophyllotoxin derivatives, a smaller concentration of etoposide was used in this assay. Shown in Figure 50, there were modest differences in site utilization between etoposide and the podophyllotoxin derivatives. Based on visual inspection, DEPT enhanced cleavage mainly at sites preferred by etoposide, however, some minor differences in site utilization were observed. The results suggest that the sugar moiety of etoposide may play a small role in influencing
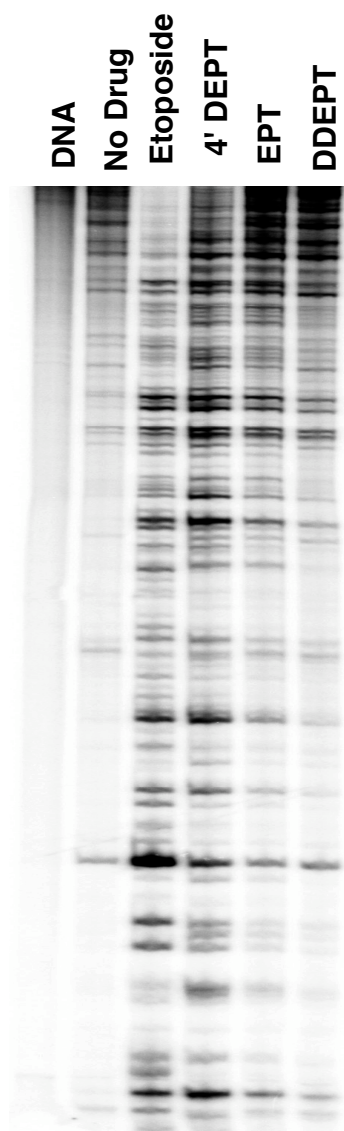
Figure 50. DNA cleavage site utilization by human topoisomerase IIα in the presence of podophyllotoxin derivatives. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions contained no compound (Topo II), 25 μM etoposide, 50 μM DEPT, or 250 μM EPT or DDEPT. A DNA control is shown in the far left lane. Data are representative of at least three independent experiments.

the site utilization of topoisomerase IIα possibly by altering the binding geometry of the drug-enzyme complex.

Cleavage Mediated by Topoisomerase IIα in Cultured Human Cells in the Presence of Podophyllotoxin Derivatives.

It has long been known that the glycosidic moiety of etoposide plays important physiological roles (*10, 104, 105, 151*). The presence of this group keeps etoposide from interacting with tubulin (*10, 104, 105, 151*). Beyond these physiological functions, it is not clear whether the glycosidic moiety of etoposide plays any direct role in enhancing topoisomerase II-mediated DNA cleavage. Therefore, the ability of podophyllotoxins lacking the glycosidic moiety of etoposide to raise cellular levels of topoisomerase II cleavage complexes was determined. Consistent with cleavage data, DEPT increased the amount of topoisomerase II covalently bound to DNA while EPT and DDEPT were ineffective (Figure 51). The results indicate that the loss of the glycosidic moiety does not significantly alter their inherent ability of the podophyllotoxin derivatives to induce topoisomerase II-mediated breaks in the genetic material.

Discussion

Although etoposide is one of the most widely prescribed drugs used for the treatment of human cancers (*10, 104, 105, 151*), the specific ring substituents that mediate its interactions with topoisomerase II have been difficult to define. It has long been known that the glycosidic moiety of etoposide plays important physiological roles, including inhibiting the drug from interacting with tubulin. The recent use of STD [^{1}H]-NMR has enabled drug-enzyme interactions to be

No Drug

Etoposide (10 *μ*M)

DEPT (10 *μ*M)

EPT (50 *μ*M)

DDEPT (50 *μ*M)
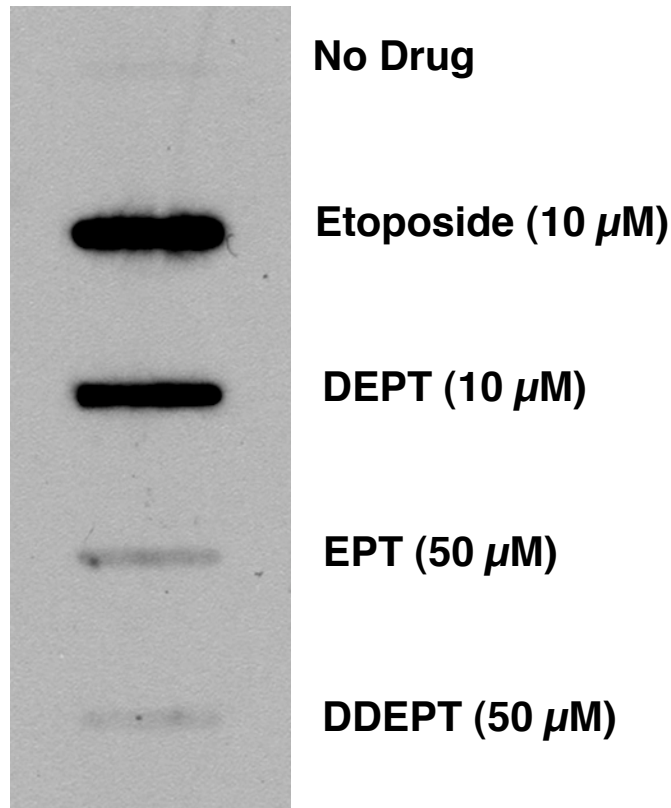
Figure 51. DEPT enhances human topoisomerase IIα-mediated DNA cleavage in treated human CEM cells. The ICE bioassay was used to monitor the level of cleavage complexes in cells treated with podophyllotoxin derivatives. DNA (10 *μ*g) from cultures treated with no drug, 10 *μ*M etoposide or DEPT or 50 *μ*M EPT or DDEPT was blotted onto a nitrocellulose membrane. Blots were probed with a polyclonal antibody directed against human topoisomerase IIα. Results are representative of three independent experiments.

characterized at the proton level (*278*). Results indicate that protons on the A–ring, B–ring, and pendent E–ring are in close contact with yeast topoisomerase II and human topoisomerase IIα in the binary enzyme-ligand complex. These findings are summarized in Figure 43, which highlights hydrogens that interact with topoisomerase II in red and those for which no interactions were observed in green.

Although no interactions were observed between topoisomerase II and the protons of the etoposide glycosidic moiety, it is possible that this moiety may influence the cleavage activity of the enzyme. In fact, removal of the sugar group had modest effects on topoisomerase II-induced DNA cleavage activity induced by the agent. DEPT and etoposide only differed in their ability to induce single-stranded nucleic acid breaks, with etoposide inducing far more of these breaks. This may be due to a decreased ability of DEPT to inhibit ligation of nicked DNA substrates. In topoisomerase II-binding assays, removal of the sugar group did not alter the ability of etoposide to bind to the enzyme. However, interactions of DEPT with the topoisomerase II-DNA-drug ternary complex, in comparison to etoposide, were altered as determined by changes in DNA cleavage site utilization.

Based on previous NMR data, further alterations were made to DEPT to assess the functional importance of the A– and E–rings. Removal of the interacting geminal protons of the A–ring greatly reduced the ability of the resulting agent (DDEPT) to enhance DNA cleavage mediated by topoisomerase II (see Figure 47). Loss of E–ring substituents did not alter drug affinity for the enzyme but greatly impaired the ability of the compound to increase DNA breaks generated by topoisomerase II (see Figure 45) (*278*). Additionally,

addition of bulk at the 4' position of the E–ring also greatly dimished the activity of the agent (EPT, see Figure 47). Lack of activity by either of these agents was due to a reduced affinity for the enzyme (see Figure 49).

Removal of the glycosidic moiety of etoposide did not greatly alter the ability of the drug (DEPT) to induce double-stranded DNA breaks (see Figure 47, left panel). Thus, the role of this moiety appears to primarily be physiological and not necessary for activity. Several lines of evidence suggest that the glycosidic moiety prevents etoposide-tubulin binding allowing the drug to be available to target type II topoisomerases in the cell (*10, 104, 105, 151*). As determined by cellular experiments, removal of the glycosidic moiety of etoposide did not alter the activity of DEPT in cellular systems (see Figure 51). Therefore, it appears that the presence of this group reduces the inhibition of tubulin polymerization instead of increasing molecules of etoposide available to target topoisomerase II.

In conclusion, consistent with previous NMR results, the A–ring is necessary for drug binding. Although the E–ring substituents have been attributed to drug function, the presence of additional bulk at the 4' position of the E–ring greatly diminished drug binding. This finding indicates that the E–ring of etoposide rests in a pocket too small to accommodate additional bulk. Furthermore, the glycosidic moiety plays a limited role in enhancing topoisomerase II-mediated breaks in the genetic material by interacting with the DNA in the ternary complex (Figure 52). However, further structural studies will be necessary to address with possibility.

Finally, previous NMR results determined that substituents of the A– and E–ring, the A–ring are necessary for drug binding. Consistent with this data,

disruption of substituents necessary for binding abrogates drug activity. Thus, the STD-NMR has the ability analyze the molecular interactions of drugs with their protein targets. This finding indicates that the E–ring of etoposide rests in a pocket too small to accommodate additional bulk. Furthermore, the glycosidic moiety plays a limited role in enhancing topoisomerase II-mediated breaks in the genetic material by interacting with the DNA in the ternary complex (Figure 52). However, further structural studies will be necessary to address with possibility.
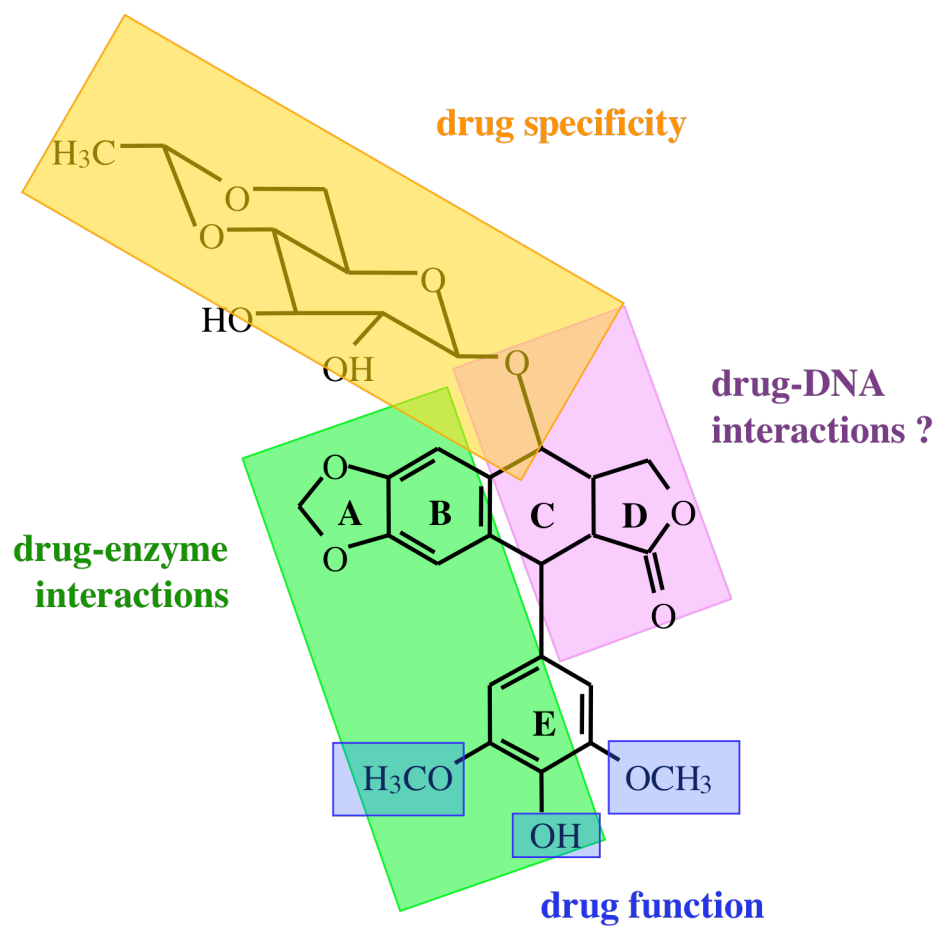
Figure 52. Summary of functional domains of etoposide.

CHAPTER VII

GENERATION OF ABERRATIONS IN THE *MLL* GENE RESULTING FROM
CELLULAR EXPOSURE TO TOPOISOMERASE II POISONS

Introduction

Type II topoisomerases are essential enzymes that are necessary for proper

chromosome maintenance (*15, 41, 43, 50, 51, 53, 57*). These enzymes also are the

targets of a variety of successful anticancer chemotherapy agents (*10, 104, 105,

151*). In addition to its role as an essential cellular protein and target for

anticancer drugs, mounting evidence suggests topoisomerase II initiates

chromosomal translocations that lead to specific types of leukemia (see Figure 4).

Approximately 2-3% of patients treated with regimens that include etoposide

ultimately develop acute myelocytic leukemia (*14, 24-26*). Approximately one-

half of these leukemias posses translocations within an 8.3 kb breakpoint cluster

region in the *MLL* (mixed lineage leukemia) gene at chromosome band 11q23 (*13,

14, 24-26*). These leukemias with *MLL* translocations are seen following exposure

to other anticancer drugs.

In addition these leukemias induced by topoisomerase II-targeted drugs,

exposure to environmental topoisomerase II poisons has been associated with

the development of leukemias that display translocations involving

chromosomal band 11q23 (*137*). Consumption of foods that are high in

bioflavanoids (such as genistein, see Figure 6) by pregnant mothers during

gestation increases the risk of developing specific infant leukemias by an order of

magnitude (*18, 20-23*). Furthermore, individuals with chronic exposure to

benzene display a higher risk for leukemias with 11q23 chromosomal translocations. Benzene is not believed to induce DNA strand breaks directly, instead the compound induces DNA breaks through a series of phenolic and quinone-based metabolites. Additionally, individuals that are heterozygous or homozygous for the *C609T* polymorphism of the NAD(P)H:quinone oxidoreductase 1 (NQO1, the enzyme that reduces benzoquinone to the less reactive hydroquinone, Figure 8) (*138-142*). As described above, benzoquinone is a highly active topoisomerase II poison.

The involvement of topoisomerase II-mediated DNA cleavage in the initiation of leukemias with *MLL* translocations is widely accepted. However, the specific role of the enzyme-associated DNA strand breaks in triggering the chromosomal aberrations is controversial. Two potential mechanisms have been suggested (see Figure 4). The first postulates that the breaks induced by topoisomerase II play a direct role in the translocation process. In this case, processing of enzyme-generated breaks in the *MLL* gene by cellular repair proteins would generate the 11q23 aberration. Supporting this hypothesis, all *MLL* (and partner) chromosomal breakpoints identified in patient samples, including those with secondary and infant leukemias, are located in close proximity to, and in some cases precisely at, *in vitro* sites of topoisomerase II-mediated DNA cleavage (*143-145*). Furthermore, leukemias with 11q23 chromosomal translocations are only observed in patients treated with topoisomerase II poisons (*14, 24, 279, 280*), and are not seen following other anticancer therapies. This is despite the use of other cancer chemotherapeutic agents generate chromosomal breaks and induce apoptotic pathways.

The second possibility is that the breaks induced by topoisomerase II play an indirect role in the translocation process. In this case the role of topoisomerase II-mediated DNA cleavage would trigger apoptosis, which generates chromosomal breaks in the *MLL* gene by activated apoptotic nucleases (*146*). Experimental evidence suggests that apoptosis induced by topoisomerase II poisons sometimes aborts (by processes that have yet to be described). Nuclease-generated breaks within the *MLL* gene are repaired (*147*), sometimes resulting in the formation of chromosomal translocations. Sequencing studies suggest that a major site of apoptotic cleavage is located in the bcr of the *MLL* gene (*147-150*). Moreover, translocations involving 11q23 can be induced in cultured human cells by agents that trigger apoptosis, but do not target topoisomerase II (*147-150*). To reconcile the patient data with the apoptotic model, it is proposed that the inhibition of topoisomerase II alters chromatin structure. This alteration, coupled with nuclease action, is required for the translocation event in patients (*150*).

It is likely that the process that translates topoisomerase II-mediated DNA cleavage into 11q23 chromosomal translocations is highly complex and multifaceted. Both (or neither) of the above hypotheses may contribute to the process. Therefore, the ability of topoisomerase II poisons to induce *MLL* aberrations in various cellular backgrounds was examined.

Results

The human *MLL* gene is located at chromosome band 11q23. The protein product of this gene is an important regulator of the HOX genes and is necessary for proper hemopoetic development (*26, 132-135*). A common feature (~50%) of

topoisomerase II-induced leukemias is aberrations in the *MLL* gene at chromosomal band 11q23 (*13, 14, 24-26*). This chromosomal break typically occurs in the intronic region upstream of the ninth exon within the bcr (*26*) (Figure 53).

The basis for the development of topoisomerase II-initiated leukemias has not been elucidated, but it appears to be related to disrupting the natural function of the *MLL* gene. MLL is the human homolog of the *Drosophila* trithorax and yeast Set1 proteins, and is a histone methyltransferase that is involved in transcriptional regulation in hematopoietic cells (*26, 132-135*). Thus, translocations in this region alter the differentiation of pluripotent hematopoietic stem cells or committed myeloid or lymphoid stem cells.

Etoposide and Benzoquinone Increase Levels of Topoisomerase IIα-DNA Complexes and Double-Stranded DNA Breaks.

It has long been known that topoisomerase II poisons increase levels of cleavage complexes in cells. As shown in Figure 54, treatment of cultured human CEM cells with either etoposide or benzoquinone enhances levels of topoisomerase II that is covalently attached to DNA.

The variant of histone H2A, H2AX, is rapidly phosphorylated on ser139 following the generation of double-stranded DNA breaks (*281, 282*). Using this protein as a marker, the ability of agents to induce DNA breaks can be examined by observing levels of the H2AX phosphoprotein (γ-H2AX). As determined by monitoring γ-H2AX, treatment of cells with etoposide or benzoquinone induces permanent double-stranded nucleic acid breaks in cells (Figure 54).
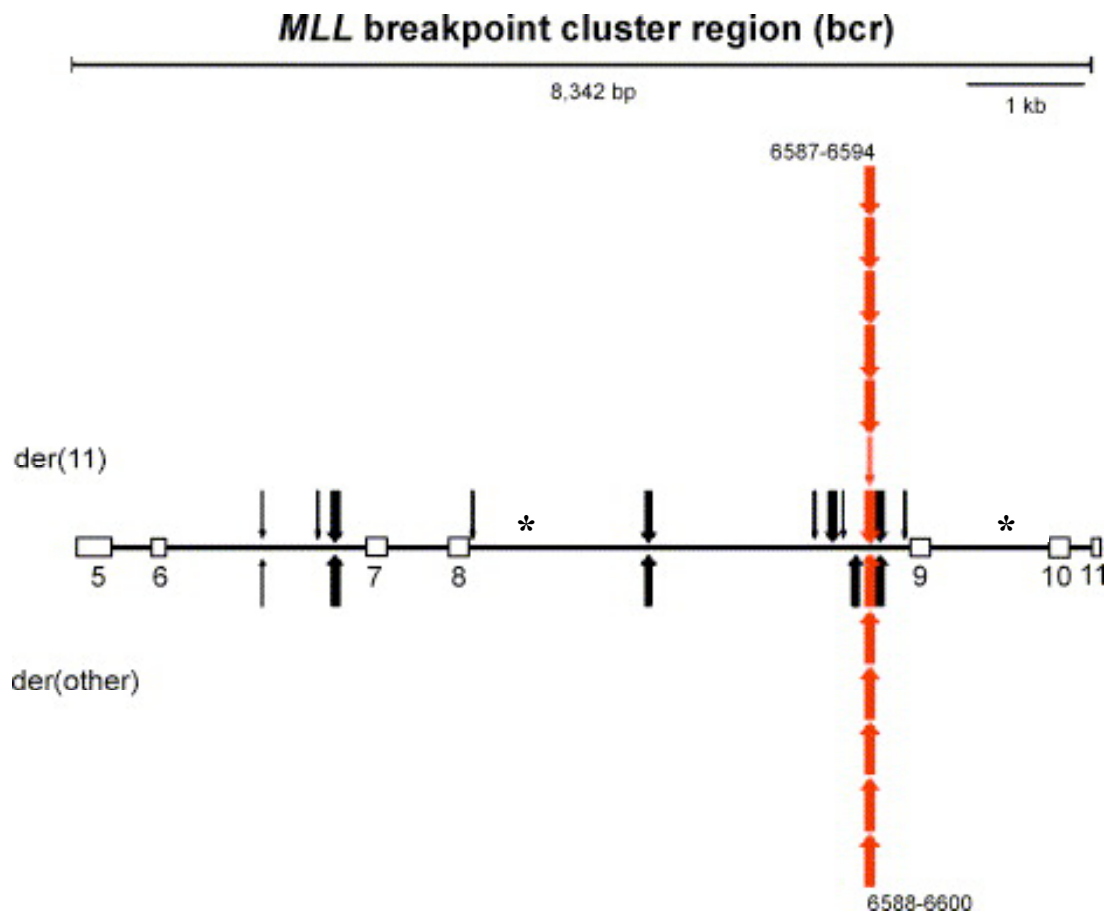
Figure 53. Diagram of the *MLL* break point cluster region (bcr) of chromosomal band 11q23. The *MLL* bcr is an 8.3 kb *BamHI* fragment composed of exonic and intronic sequences spanning exon 5 through exon 11. Arrows indicate clinically observed *MLL* translocation sites found in patients with leukemia. The red arrows indicate the translocation "hotspot" of this gene locus. Asterisks indicate approximate sites of *XbaI* cleavage. Adapted from Ref. *(26)*.

Generation of MLL Aberrations in Cultured Human Cells Following Treatment with Topoisomerase II-targeted Agents.

A 8.3kb region of the open reading frame of the gene is where most of the clinically significant translocations in MLL are observed (*26*). This region spans the 5th through 11th exons and is termed the breakpoint cluster region (bcr) (See Figures 7 and 53). Since the vast majority of clinically observed breaks in the bcr occur in a region upstream of exon 9, a PCR based system was developed to analyze this clinically significant region of the *MLL* gene (*178*).

Flanking the translocation "hotspot" 3' of exon 9 are *XbaI* sites with 1.8 kb of DNA separating the two sites (see Figure 53) (*178*). Therefore, this region was analyzed in the current study. Human CEM leukemia cells were treated with etoposide (25 *μ*M for 2 h) or benzoquinone (10 *μ*M for 4 h). After removal of the agents, genomic DNA was isolated from treated cells over the course of a 5 day recovery period in the absence of agent (Figure 55). For comparison, DNA from untreated CEM cells was also isolated. DNA was digested with *XbaI* and fragments were circularized. The 1.8 kb *MLL* fragment was amplified by sequential rounds of PCR were performed using primers specific for this region. By circularizing the genomic DNA, amplification of the bcr region is possible regardless of the presence of an unknown translocation partner.

After exposure to etoposide or benzoquinone, the analyzed region of the *MLL* bcr displayed several differences in distance between the two *XbaI* sites. Most of the fragments amplified were smaller in size (~500 to 1700 bases) than that of the wild-type (1800 bases) (Figure 56). Smaller fragments sizes are indicative of deletions, but could also be a result of a translocation that shortens
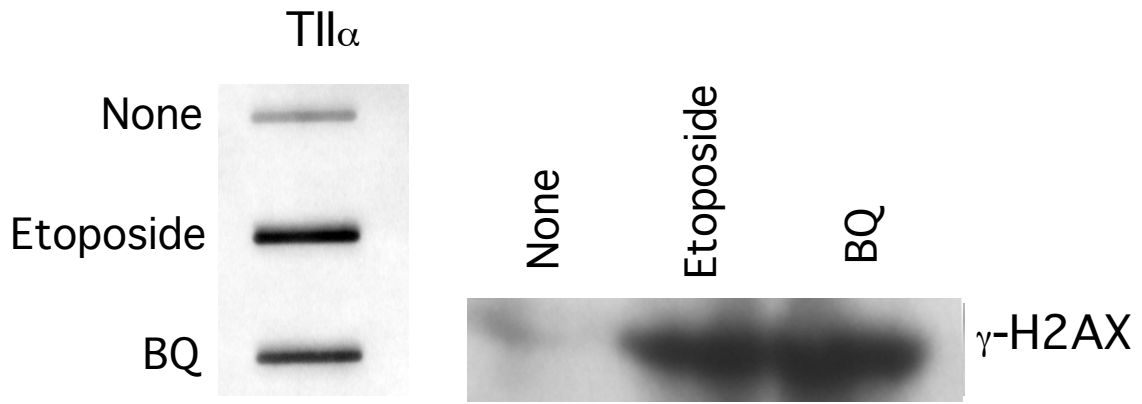
Figure 54. Etoposide and benzoquinone increase covalent topoisomerase II-DNA complexes and permanent double stranded breaks in cells. Cultured CEM leukemia cells were treated with no compound (none), etoposide (25 μM for 2hr) or benzoquinone (10 μM for 4 h) Left: DNA (10 μg) from cells was blotted onto nitrocellulose filters. Levels of topoisomerase IIα cleavage complexes were determined by use of a monoclonal antibody directed toward topoisomerase IIα. Right: Protein (35 μg) from cell lysates was subject to SDS-PAGE and transferred to activated PVDF membrane. Levels of γ-H2AX were determined by use of a monoclonal antibody directed toward the H2AX phosphoprotein. Data are representative of two independent experiments.
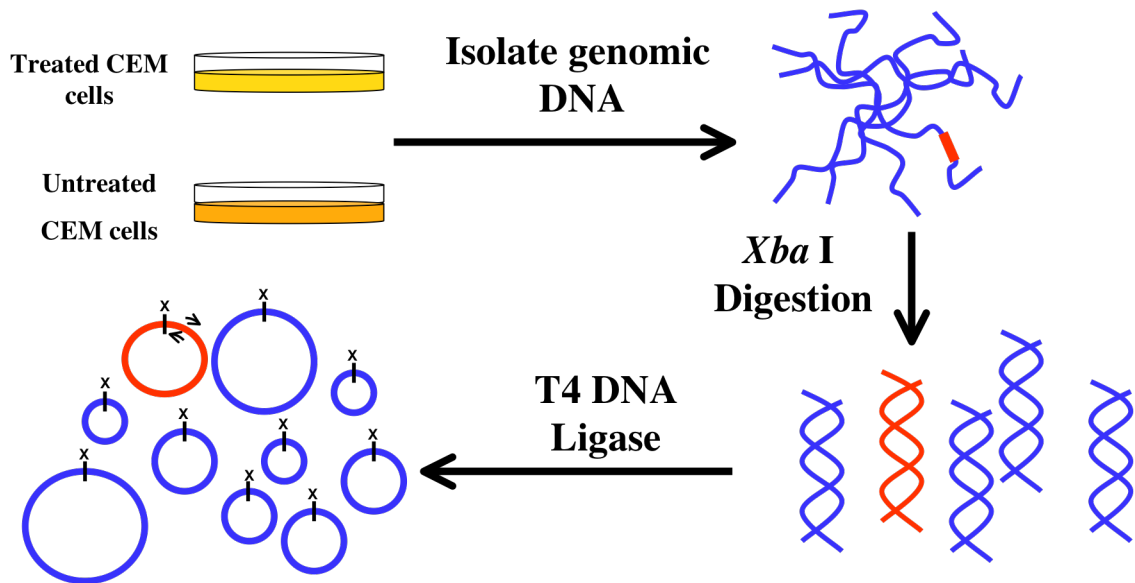
Figure 55. Method for generating substrates for PCR of the *MLL* bcr. DNA from treated cells was isolated, digested with *XbaI*, and subjected to ligation to circularize the DNA. The 1.8 kb *XbaI* region of the *MLL* gene is indicated in red. Primers were designed to bind to the known *MLL* sequence to avoid problems with amplifying the sequence of an unknown translocation partner. The "x" indicates newly generated *XbaI* sites in the circular PCR substrates.
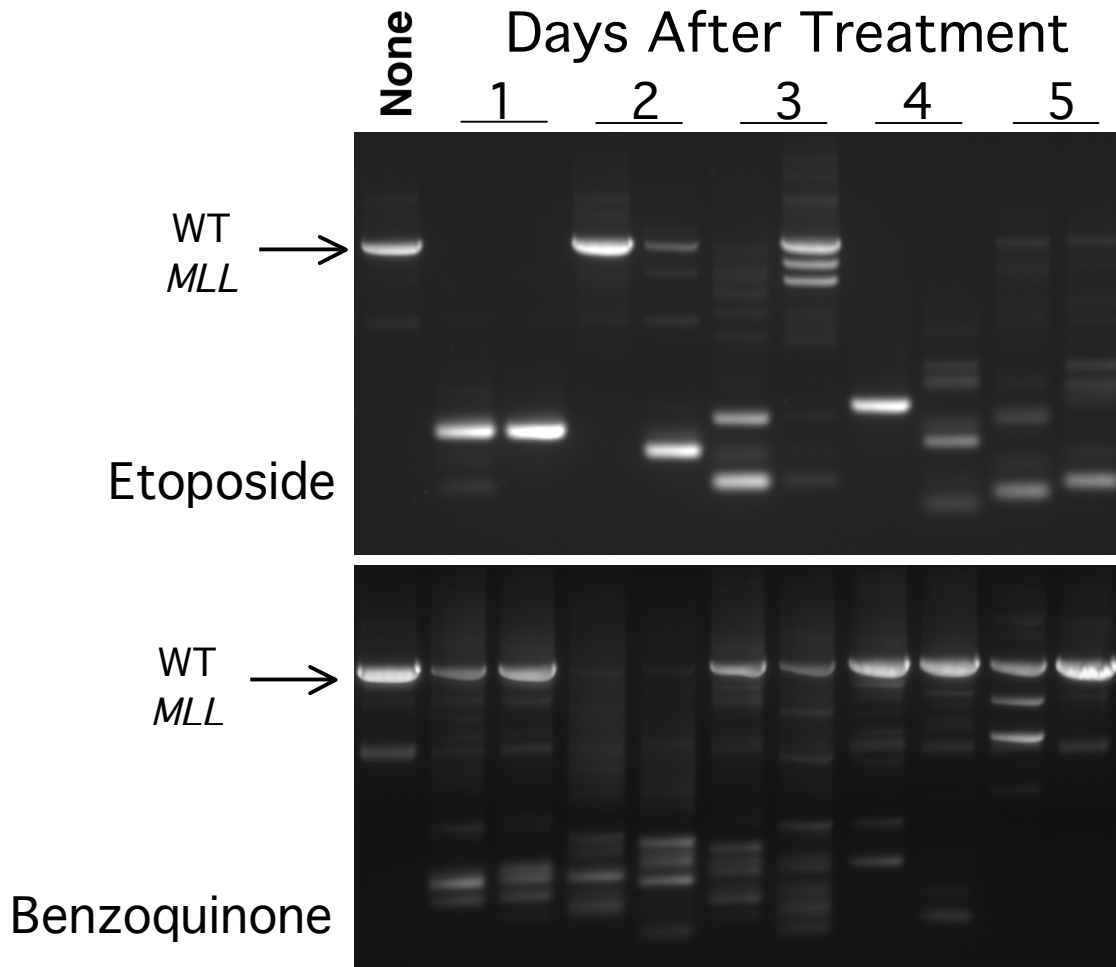
Figure 56. Etoposide and benzoquinone induce aberrations in a 1.8 kb *XbaI* fragment of the *MLL* bcr. Cultured CEM leukemia cells were treated etoposide (25 μM for 2hr, top gel) or benzoquinone (10 μM for 4 h, bottom gel). Circular DNA substrates were prepared as described previously. Cells (1×10^6) were harvested every 24 h following treatment for a total of 5 days. Cells incubated in the absence of agent are shown for comparison on the far left (none). The 1.8 kb wild-type fragment is indicated. Data are representative of two independent experiments.

the length between the *XbaI* cleavage sites. Senquencing studies will be required to determine the nature of these aberrations.

It is interesting to note that over time, cells treated with benzoquinone appear to recover most of the wild-type 1.8 kb *XbaI* fragment of the *MLL* bcr (Figure 56). This recovery is not observed for cells treated with etoposide. This difference in recovery may be the result of a difference in the amount of damage induced by each agent or by the activation of alternate repair pathways.

Multiple Pathways Contribute to Alterations in the MLL bcr.

As previously mentioned, the precise mechanism that generates 11q23 alterations following exposure to topoisomerase II poisons is unknown. Although these agents induce double-stranded breaks in the genetic material, it has also been suggested that the activation of apoptotic nucleases generates strand breaks that ultimately lead to *MLL* aberrations. Therefore, the ability of topoisomerase II-mediated breaks to induce changes in the 1.8 kb *XbaI* fragment of the *MLL* bcr was examined in the presence and absence of activated apoptotic cascades.

During activation of the apoptotic pathway, proteases called caspases are activated in a cascade-like fashion by cleaving at specific sites in the primary sequence of their substrates. During mitochondrial apoptosis caspase-9 is activated by the apopotosome, which in turn activates caspase-3 (*283*). Activated caspase-3 cleaves and inactivates proteins necessary for genomic maintenance as well as suppressors of apoptotic nucleases (Figure 57) (*284*). These proteins include DNA-pk (necessary for DNA repair) and the inhibitor of caspase activated DNAse (inhibits DNA cleavage by caspase activated DNAse). The

153
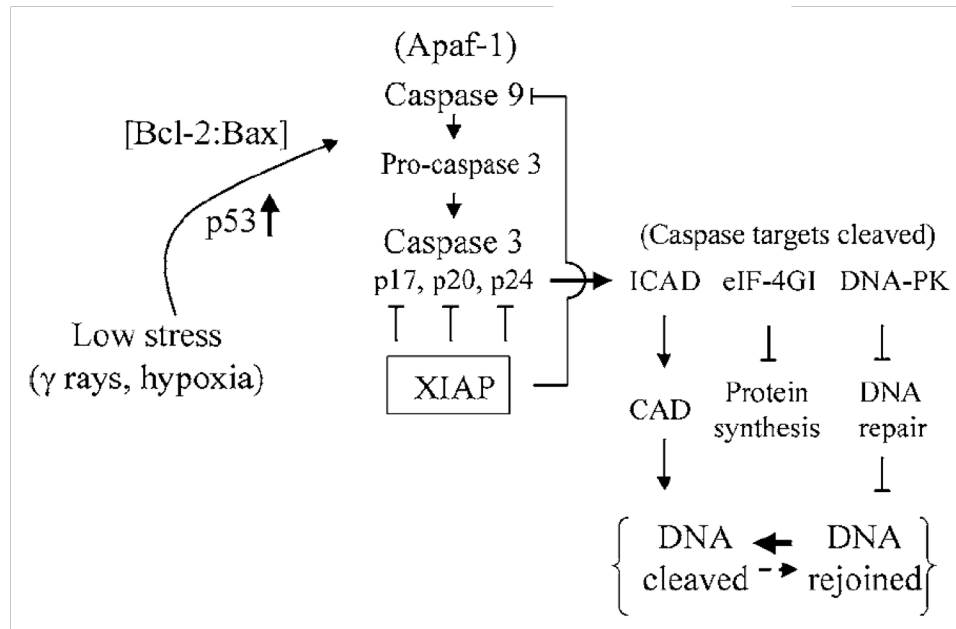
Figure 57. Initiation of apoptosis in human cells. Conditions that generate substantial DNA damage or cellular hypoxia cause the release of cytochrome c from the mitochondria. Cytochrome c activates caspase-9 which, in turn, activates caspase-3 (from pro-caspase-3). Upon activation, caspase-3 cleaves and inactivates a variety of cellular proteins including inhibitor of caspase activated DNase (ICAD) and DNA-PK. Inactivation of these proteins allows for chromatin fragmentation as well as the inhibition of protein sythesis. Several cellular proteins that inhibit apoptotic signaling also are shown (Bcl-2:Bax and XIAP). Adapted from Ref. *(148)*.

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inactivation of these protein, among others, promotes typical DNA fragmentation during apoptosis.

The protein-based inhibitor Z-VAD-fmk, upon cleavage by caspases, inhibits further activity by these enzymes effectively preventing apoptosis (*285*). Shown in Figure 58, treatment of cells with Z-VAD-fmk prior to the addition of etoposide or benzoquinone efficiently inhibited the activity of caspase-3, indicating efficient inhibition of the apoptotic cascade.

Analysis of cells treated with both Z-VAD-fmk and either etoposide or benzoquinone displayed lower levels of aberrations in the 1.8 kb *XbaI* fragment of the *MLL* bcr (Figure 59). However, inhibition of apoptosis did not eliminate the generation of alterations. These data suggest that the apoptotic machinery is involved in producing aberrations in the *MLL* gene. However, the presence of *MLL* alterations in the absence of activated caspases suggests that topoisomerase II-mediated DNA cleavage also plays a direct role in the generation of *MLL* aberrations. It should be noted that the analysis of cells treated with etoposide was obscured by the high level of alterations observed. Sequencing experiments will require fewer, more defined, genetic changes.

<div align="center">Discussion</div>

Exposure to clinical and environmental topoisomerase II poisons has been linked to the development of leukemias harboring translocations at chromosomal band 11q23 (*14, 24-26, 137, 286*). The role of topoisomerase II-mediated DNA breaks in generating 11q23 aberrations is unclear. Current evidence suggests that at least two pathways contribute to the formation of these translocations.
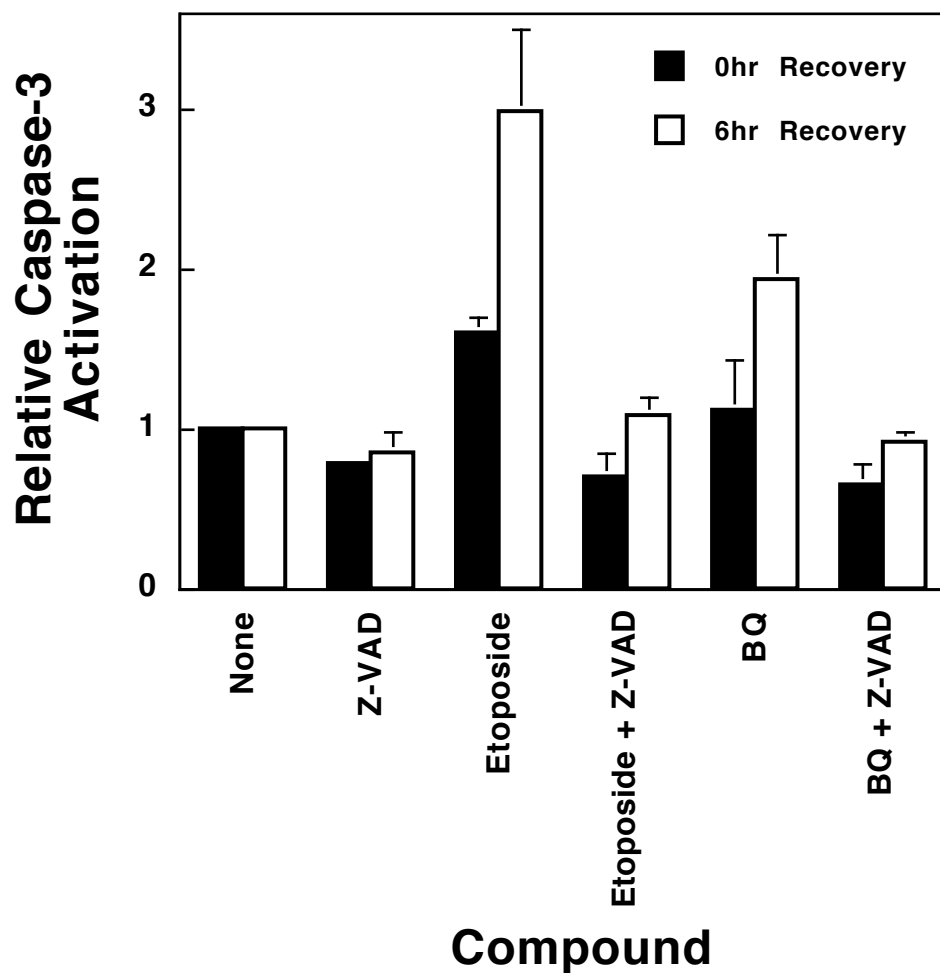
Figure 58. Z-VAD-fmk inhibits apoptosis induced by etoposide or benzoquinone. Cultured CEM leukemia cells were treated with 15 μM Z-VAD-fmk or DMSO vehicle 2hr prior to the addition of etoposide (25 μM for 2hr) or benzoquinone (10 μM for 4 hr). Cells were harvested following treatment (0 hr, filled bars) or allowed to recover in the absence of drug for 6 hr (open bars). Caspase-3 activation was expressed as a fold enhancement over cells cultured in the absence of compound. Error bars represent the standard error of the mean for two independent experiments.
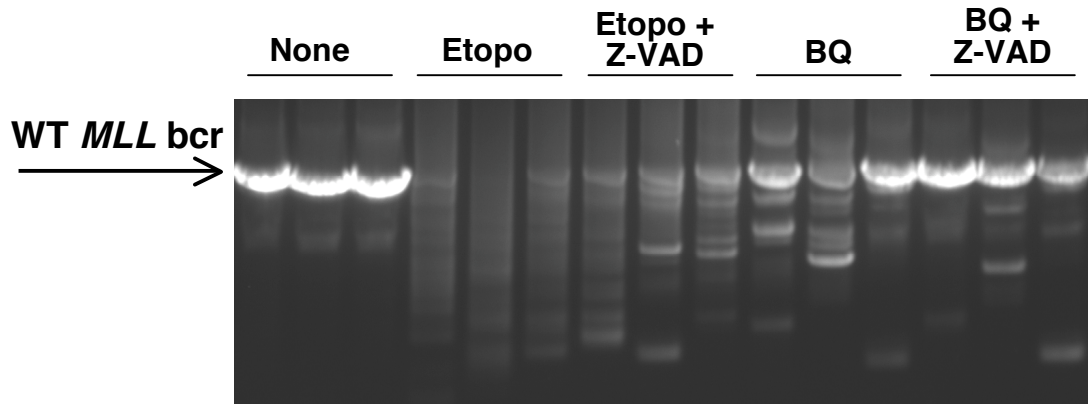
Figure 59. Inhibition of apoptosis does not block topoisomerase II-mediated *MLL* aberrations. Cultured CEM leukemia cells were treated etoposide (25 μM for 2hr, top gel) or benzoquinone (10 μM for 4 hr, bottom gel) in the presence or absence of 15 μM Z-VAD-fmk. Circular DNA substrates were prepared as described previously. Cells (1×10^6) were harvested 3 days following treatment with topoisomerase II poison. Cells treated with Z-VAD-fmk prior to the addition of poison were maintained in 15 μM of the agent throughout the recovery period. Cells incubated in the absence of agent are shown for comparison on the far left (none). The 1.8 kb wild-type fragment is indicated.

Due to the discrepancy in the mechanism behind the formation of 11q23 translocations, a cell based system was utilized to examine the roles of type II topoisomerases and apoptotic nucleases in generating these breaks in the presence of topoisomerase II poisons (*178*). A large number of clinically significant translocations occur at a specific site upstream of exon 9 (*26*). Thus a 1.8 kb *XbaI* fragment encompassing this site was analyzed.

Etoposide and benzoquinone increase cellular concentrations of topoisomerase II cleavage complexes as verified by the ICE bioassay (see Figure 54). Additionally, both compounds raised levels of permanent double-stranded breaks in CEM cells determined by examining levels of H2AX phosphorylation. Following a short treatment of CEM cells with etoposide or benzoquinone, significant alterations were observed in the monitored region of the *MLL* bcr over a 5 day recovery period (see Figure 56). After treatment with benzoquinone, as opposed to etoposide, cells more efficiently and accurately repaired the *MLL* bcr. This suggests either the two agents induced differential levels of enzyme-induced cleavage or alternate repair pathways were activated.

The contributions of DNA strand breaks produced by topoisomerase II and apoptotic nucleases in generating *MLL* gene aberrations is unclear. Therefore, the ability of etoposide and benzoquinone to induce these alterations in the absence of apoptotic nuclease activation was determined. Treatment of cells with Z-VAD-fmk prior to the administration of either compound reduced levels of caspase-3 activation to those observed in the absence of a topoisomerase II poison (see Figure 58). In the absence of apoptotic nuclease activation, the amount of aberrations in the 1.8 kb *XbaI* fragment of the *MLL* bcr were dimished but not absent (see Figure 59). These data indicate that the apoptotic machinery,

in this cell-based system, is involved in the generation of *MLL* alterations. However, the inability of Z-VAD-fmk to fully diminish these aberrations indicates that DNA damage generated by topoisomerase II activity directly contributes to the formation of alterations in chromosomal band 11q23.

CHAPTER VIII

CONCLUSIONS

Topoisomerase II is an essential enzyme that regulates DNA topology in cells (*43, 50, 51, 53, 57, 58*). This enzyme has been a very successful target for the chemotherapeutic treatment of human cancers. For example, etoposide has been in clinical use for almost 4 decades and was the most widely described cancer chemotherapy agents used until the development of taxols (*10, 104, 105, 151*). This agent kills cells by increasing levels of II topoisomerase II-mediated double-stranded breaks (*15, 41, 43, 50, 51, 53, 57, 97, 196*). Despite the wide clinical use of etoposide, the functional portions of the molecule that have yet to be fully determined.

Despite the effectiveness of topoisomerase II-targeted agents in the clinic, treatment with these agents has been linked to the development of specific type of leukemias, displaying 11q23 translocations (*14, 24-26*). Furthermore, leukemias with 11q23 aberrations have also been observed following the exposure to benzene (*30, 32, 287-289*). Benzene itself is not believed to generate the DNA breaks leading to these translocations. Instead evidence suggests that a variety of phenolic and quinone metabolites, most notably benzoquione, cause this damage in the bone marrow (*30, 32, 289*). Recently benzoquinone was shown to act as a strong topoisomerase II poison *in vitro* and in cultured human cells (*1*).

Aside from benzoquinone activity in the bone marrow, several other quinone and quinone imine metabolites of drugs and environmental toxins demonstrate teratogenic activity in the liver (*33-36*). The basis for this activity is

not known. However, the DNA damage induced by these agents is suggestive of the actions of topoisomerase II poisons. Furthermore, the mechanism behind leukemias that arise following exposure to clinical and environmental toxins is unclear. These areas of discrepancy were the basis of this dissertation.

Overall Conclusions

A number of conclusions can be drawn from the results presented in this dissertation. First, both NAPQI and a variety of PCB quinones are topoisomerase II poisons *in vitro* and in cultured human cells (see Figures 10-12, 16, 19, 22, and 29). However, when incubate with topoisomerase II in the absence DNA, of these agents rapidly inhibited the ability of the enzyme to cleave and relax negatively supercoiled DNA plasmids (see Figures 13, 15, and 24). Also, PCB quinones appear to require adduction to the enzyme to function (see Figure 21). Evidence suggests that these agents have multiple activities against the enzyme.

Second, due to the ability of quinones to adduct to proteins, sites of adduction on topoisomerase IIα were determined (see Figures 31 and 32). Of the four sites identified (cysteines 170, 392, 405 and 455), mutation of cys392 and/or cys405 to alanine reduced the sensitivities of the resulting mutant enzymes to quinones by ~50% (see Figures 33C and D). As a mechanism of quinine resistance, these enzymes display rates of DNA ligation that are ~2–fold faster than that of the wild-type enzyme in the presence of benzoquinone (see Figure 34). Alternatively, a cys455->ala mutation of topoisomerase IIα confers hypersensitivity to quinones (see Figures 39A, B, and D). This mutant enzyme was ~3–fold more sensitive to N-terminal clamp closure, when compared with the wild-type enzyme, in the presence of benzquione (see Figure 41). Studies

with these cys->ala topoisomerase IIα mutants demonstrate that quinones enhance enzyme-induced DNA cleavage by at least two independent mechanisms; adduction to cysteine residues 392 and 405 to inhibit topoisomerase II-mediated DNA ligation and closure of the N-terminal protein gate that traps DNA in the active site of the enzyme (see Figure 42).

Third, consistent with previous NMR experiments, disruption of the A– or E–rings of etoposide significantly alter drug activity (see Figures 46-50). The data also indicate that the glycosidic moiety, believed to primarily play physiological roles, plays minor roles in enhancing topoisomerase II-mediated DNA scission (see Figures 46-51). Furthermore, removal of the glycosidic moiety of etoposide did not alter the ability of the resulting compound to induce topoisomerase II-mediated DNA breaks in cells relative to *in vitro* experiments (see Figure 51).

Finally, both etoposide and benzoquinone induce aberrations in the *MLL* bcr in cultured human cells. A 1.8 kb *XbaI* fragment of the *MLL* bcr, containing the observed clinical translocation hotspot (*26*), was analyzed using a ligase mediated PCR technique (*178*). Following treatment of human CEM cells with etoposide or benzoquinone, significant aberrations were observed in this region (see Figure 56). Furthermore, cells treated with benzoquione appeared to recover the wild-type 1.8 kb fragment over time. Cells treated with etoposide did not display a similar ability to recover. Treatment of cells with Z-VAD (and inhibitor of apoptosis), aberrations in the 1.8 kb *XbaI* fragment of the *MLL* bcr were reduced but still present (see Figure 59). These data indicate that apoptotic nucleases play a limited role in generating aberrations in the *MLL* gene locus. Furthermore, these data suggest that DNA breaks mediated by topoisomerase II directly contribute to alterations in the *MLL* bcr.

The results described in this dissertation lead to further knowledge of the mechanism of topoisomerase II poisons as chemotherapeutic and carcinogenic agents. Previous results indicate that rodent exposure to either acetaminophen or PCBs generates chromosomal aberrations including sister chromatid exchanges, translocations and deletions as well as cancers of the liver (*33-36*). This work establishes a causal link between metabolites of acetaminophen (NAPQI) and PCBs (PCB quinones) and topoisomerase II in the generation of chromosomal aberrations. NAPQI and PCB quinones are topoisomerase II poisons. Furthermore, acetaminophen, the parent compound of NAPQI, did not antagonize etoposide function in purified systems or in cultured human cells (see Figure 17). This indicates that the co-administration of acetaminophen with etoposide will not reduce the clinical efficiency of the topoisomerase II poison.

Prior to this study the mechanism of quinone action was largely unknown. This work established a basic mechanism for quinone action against the type II enzyme. Mass spectrometry and mutation studies indicate that quinones enhance topoisomerase II-mediated DNA cleavage by at least two independent activities. First, quinone adduction to cysteine residues 392 and 405 inhibits ligation of cleaved DNA molecules by the enzyme. Second, quinones close the N-terminal gate of topoisomerase II by crosslinking the two protomer subunits together.

The work described in chapter VI of study described the activity of several podophyllotoxin derivatives. It is important to note that these derivatives were designed based on data obtained in a previous experiment (*278*). These results verify the saturation transfer difference NMR technique as a sufficient

determinant of interacting portions drugs with their enzyme target. Finally, these studies suggest that modifications of etoposide designed to increase efficiency, availability or reduce toxic side effects should focus on the glycosidic moiety at the C4 position.

The work in chapter VII describes a cell-based system used to analyze the ability to topoisomerase II poisons to induce aberrations in the *MLL* gene at chromosomal band 11q23. Results suggest that multiple pathways are responsible for the induction of *MLL* translocations. Furthermore, this system provides a powerful tool to further analyze the relative contributions of several cellular activities (*i.e.* topoisomerases, apoptotic nucleases, repair machinery, etc.) in the generation of 11q23 translocations that lead to human leukemias.

One simplified model that this work suggests is that quinones adduct to topoisomerase II to induce DNA strand breaks that lead to the development of leukemias. If this were the case then increasing the relative amount of cellular sulfhydryls available (free cysteine and glutathione) may decrease the amount of quinone available to act as a topoisomerase II poison. In laboratory animals, ingestion of whey protein increase levels of glutathione in the liver (*290-292*). Furthermore, ingestion of whey protein decreased oxidative damaged to the liver and kidneys following a burn injury. (*292*). Thus, individuals who have been exposed to quinone precursors can ingest increased amounts of whey protein. This would increase cellular levels of free cysteine and glutathione available to neutralize quinones and thus limit their mutagenic activities.

Future Directions

As described in chapter V, several residues on topoisomerase IIα were identified as sites of quinone adduction. Mutation of these sites revealed several mechanistic properties of quinones as topoisomerase II poisons. One point of interest of the lab would be to determine whether the activity of these agents against topoisomerase IIβ is similar, at the molecular level, to that of topoisomerase IIα. Furthermore, the yeast enzyme, which is partially resistant to stimulation of DNA cleavage by these agents (*118*) could be used to further define the role of blocking the N-terminal gate of the protein. This enzyme is lacking a cysteine residue that is homologous to the cys405 residue in human topoisomerase IIα. Thus, determining the ability of quinones to inactivate and crosslink the two protomer subunits and close the N-terminal clamp of the yeast enzyme could further establish a role for the crosslinking activity of quinones in inactivating topoisomerase II as well as enhancing enzyme-mediated DNA cleavage.

Mutation of residue cys455 to ala generated a mutant enzyme that displayed elevated DNA cleavage activity and was hypersensitive to quinones. Cys455 is unique among other cysteine residues identified as sites of quinone adduction as it exists, at least some of the time, in a disulfide bond with cysteine residue 427 (*251*). Current studies are underway to examine the importance of this disulfide to quinone activity. Two possibilities exist; 1) the lack of the ability of residue 455 to form a disulfide bond with residue 427 enhances the ability of quinones to block the N-terminal gate of the protein 2) quinone adduction to residue inhibits the ability of quinones to close the N-terminal clamp. To delineate between these

two possibilites a top2αC427A will be generated and tested for sensitivity to quinones.

Chapter VI further examined the interactions between etoposide and topoisomerase IIα. Results from this study demonstrate that the A–ring of the molecule is necessary for drug binding and that drug activity is sensitive to alterations in the E–ring. Previous NMR experiments examined the interacting protons of etoposide with yeast topoisomerase II and human topoisomerase IIα in the absence of DNA. However, topoisomerase II-targeted agents appear to have a base preference at sites of drug induced cleavage (*106, 225*). This indicates that some portion of the molecule probably interacts with DNA in the drug-enzyme-nucleic acid ternary complex. Therefore, it is of the interest of the lab to examine whether topoisomerase II poisons have the ability to interact with DNA in the ternary complex. Data indicate that protons of the D–ring and glycosidic moiety do not interact with the enzyme, however, this does not exclude them from interacting with DNA in the cleavage complex. Future STD-NMR studies analyzing the enzyme-drug-DNA complex are planned to determine whether portions of the molecule interact with DNA in the ternary complex. Furthermore, current studies are focusing on characterizing the activity of etoposide and podophyllotoxins with D–ring modifications to determine the functional importance of this moiety.

STD-NMR is an effective technique to dissect the molecular interactions between drugs and their targets. Therefore, future STD-NMR studies will examine the interacting groups a variety of topoisomerase II-targeted agents. This is further facilitate the development of topoisomerase II poisons for anticancer treatments.

The final data chapter in this dissertation described a PCR-based system to analyze *MLL* aberrations following treatment of cultured cells with topoisomerase II poisons. This system is a powerful to analyze the factors that contribute to translocations at chromosomal band 11q23. The types of aberrations (deletions or translocations) that are induced by topoisomerase II and apoptotic nucleases can be addressed by sequencing observed aberrations following treatment of cells with topoisomerase II poisons. To accomplish this goal, several experiments would need to be carried in a variety of cellular backgrounds (*i.e.,* in the absence of type II topoisomerases or apoptotic nucleases). Therefore, siRNA knockdowns of both topoisomerase IIα and topoisomerase IIβ are currently being optimized.

Initial results using the PCR-based system indicate that treatment of cells with benzoquinone induces a repair pattern that is different than that of cells treated with etoposide. Therefore, it is of interest to determine if the repair machinery involved in repair of benzoquinone enhanced topoisomerase II-mediated double-stranded DNA breaks is different from the machinery that repairs DNA breaks induced in the presence of etoposide. To accomplish this aim, levels of *MLL* aberrations induced by etoposide will have to be reduced to levels observed in the presence of benzoquinone. If etoposide-induced damage appears to have a different repair pattern when compared to cells treated with benzoquinone, knockdowns of necessary repair proteins can be accomplished using siRNA. This will allow the contributions of specific DNA repair pathways in the repair of topoisomerase II-mediated DNA cleavage in the presence of various agents that target the enzyme to be determined.

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