DNA LESIONS AS CELLULAR POISONS OF TOPOISOMERASE IIα

By

Renier Vélez-Cruz

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Approved:

Neil Osheroff, Ph.D.

Lawrence J. Marnett, Ph.D

Walter J. Chazin, Ph.D.

Michael P. Stone, Ph.D.

A mi familia, porque si no los tuviera a ellos, no tuviera nada…

To my family, because if I wouldn't have them, I'd have nothing…

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AML	acute myeloid leukemia
AP	apurinic/apyrimidinic
APE1	AP endonuclease 1
AraC	arabinose cytosine
ATP	Adenosine triphosphate
BER	base excision repair
bp	base pairs
CAA	2-chloroacetaldehyde
DMSO	dimethylsulfoxide
dRP	deoxyribose phosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
εdA	1,N^6-etheno-deoxyadenosine
εdC	3,N^4-etheno-deoxycytidine
εrC	3,N^4-etheno-2'-ribocytidine
εdG	1,N^2-etheno-deoxyguanosine
etoposide	4'-desmethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-b-D-glucopyranoside)
FRET	Fluorescence Resonance Energy Transfer
genistein	4',5,7-trihydroxyisoflavone
GyrA	gyrase A

GyrB	gyrase B
kDa	kilodaltons
M_1dG	pyrimido[1,2-*a*]purin-10(3H)-one-deoxyribose
MMS	Methyl methanesulfonate
MPG	Methyl *N*-purine DNA glycosylase
NMR	nuclear magnetic resonance
PdG	1,N^2-propano-deoxyguanosine
PI	Propidium Iodide
rC	2'-ribocytidine
SDS	sodium dodecyl sulfate
siRNA	Small Interfering RNA
THF	tetrahydrofuran
Tris	tris-(hydroxymethyl)aminomethane
UV	ultraviolet

CHAPTER I

INTRODUCTION

DNA Topology

Although the genetic information of an organism is encoded by the linear array of DNA bases that comprises its genome (1), the three-dimensional properties of the double helix dramatically affect how this information is expressed and passed from generation to generation (1). Some of the most important three-dimensional features of the genetic material are topological in nature (2).

The topological properties of DNA, including DNA under- and overwinding, knotting, and tangling, are defined as those that cannot be altered without breaking one or both strands of the double helix. Since DNA is comprised of two interwound nucleic acid strands and the genomes of all known organisms are very long or circular (or both), two distinct topological issues arise as a result of the structure of the genetic material (2-7). Proliferating cells must be able to cope with both of these in order to survive.

The first issue is related to the torsional stress on the double helix. DNA from all eukaryotes and eubacteria is globally underwound ~5-10% (2). DNA under torsional stress is termed supercoiled (underwound molecules are negatively supercoiled and overwound molecules are positively supercoiled), because underwound or overwound DNA writhes about itself to form superhelical twists. Negative supercoiling puts energy into the genetic material and makes it easier to separate the two strands of the double helix for replication and transcription (3,5,8). Thus, DNA underwinding dramatically

increases the rates of these two fundamental processes (9-14). In contrast, the movement of DNA tracking systems (such as replication forks and transcription complexes) through the double helix locally overwinds the DNA ahead of these complexes (9-14). Since overwinding makes it much harder to pull apart the double helix, this kind of torsional stress blocks many essential cellular processes, such as those mentioned above (9-14).

The second issue is related to the extreme length of genomic DNA. Nucleic acid knots (intramolecular) and tangles (intermolecular) are formed routinely during a variety of ongoing cellular processes including DNA recombination and replication (15-17). Both knots and tangles must be removed in order for daughter chromosomes to segregate properly during meiosis and mitosis (15-17). Failing to resolve these topological constrains results in mitotic catastrophe (18-20).

DNA Topoisomerases

Cells contain ubiquitous enzymes known as DNA topoisomerases that maintain the appropriate level of DNA supercoiling and remove knots and tangles from the genetic material (21-24). These enzymes modulate the topological structure of the genetic material by creating transient breaks in the backbone of the DNA. There are two types of DNA topoisomerases that can be distinguished by their reaction mechanisms. Type I enzymes create a transient single-stranded DNA break and catalyze the controlled rotation (or strand passage) of the other DNA strand through the nick (24-29). Type II enzymes create a transient double-stranded break in the genetic material, pass an intact double helix through the break, and religate the break (30-32). To maintain genomic integrity during their DNA cleavage events, topoisomerases form covalent linkages

between active site tyrosyl residues and the newly generated DNA termini. This covalent protein-cleaved DNA complex, known as the *cleavage complex*, is a hallmark of all topoisomerases irrespective of enzyme classification. Since type I topoisomerases create single-stranded breaks in the genetic material, they can regulate DNA supercoiling. However, since type II topoisomerases generate double-stranded breaks in the DNA, they can resolve knots and tangles in addition to removing torsional stress from the genetic material (33-37).

Although type I topoisomerases play an important role in cellular processes such as transcription and DNA replication, they are not essential (12,14,29,38). Type II topoisomerases are essential to all eukaryotic and prokaryotic organisms (33-37). They are highly conserved among species, and the eukaryotic enzymes appear to be direct descendents from ancestral bacterial proteins.

Eukaryotic Type II Topoisomerases

Domain Structures and Isoforms

The eukaryotic type II enzyme is known as topoisomerase II. It was discovered in 1980 and is a member of the type IIA homology subfamily (39-41) (Figure 1). Topoisomerase II can remove positive and negative superhelical twists from the double helix and can resolve DNA knots and tangles (33-37).

Type IIA Topoisomerases

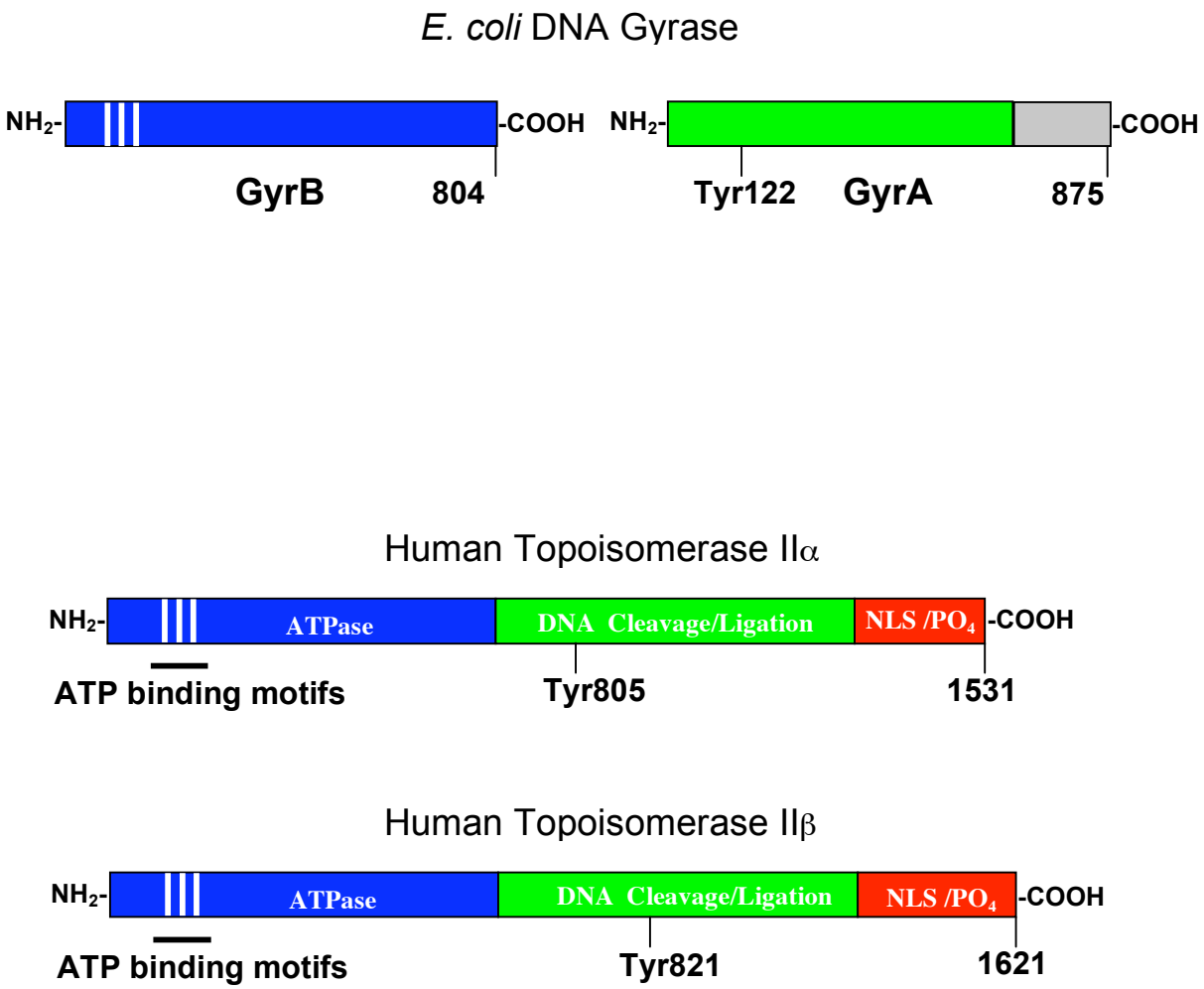


Figure 1. Domain structures of type IIA topoisomerases. Eukaryotic Type II topoisomerases are thought to be direct descendents from their bacterial counterpart, DNA gyrase. These enzymes belong to the type IIA homology subfamily of DNA topoisomerases. The eukaryotic enzymes can be separated into three different domains: an ATPase domain (blue), homologous to the GyrB domain in DNA gyrase; a DNA cleavage/ligation domain (green), homologous to GyrA domain in DNA gyrase, and a non-conserved C-terminal domain (red) that contains nuclear localization siganals (NLS) and phosphorylation sites (PO_4). Vertical lines (white) represent the conserved Walker A ATP binding domains. The active site tyrosine is labeled.

Eukaryotic type II topoisomerases are homodimeric enzymes with protomer molecular masses ranging from ~160-180 kDa (depending on the species) (42-45). On the basis of amino acid sequence comparisons with the bacterial type II enzyme, DNA gyrase, each enzyme monomer can be divided into three distinct domains (Figure 1). The N-terminal domain of the enzyme is homologous to the B subunit of DNA gyrase (GyrB) and contains consensus sequences for ATP binding and hydrolysis. The central domain is homologous to the A subunit of DNA gyrase (GyrA) and contains the active site tyrosyl residue that forms the covalent bond with DNA during the scission step. The C-terminal domain is not conserved and appears to have no corresponding region of homology with DNA gyrase. This variable region of the eukaryotic enzyme contains nuclear localization sequences as well as amino acid residues that are phosphorylated *in vivo*. The C-terminal is thought to be responsible for recognizing substrate topology (46,47).

Although some lower eukaryotic species, such as yeast and *Drosophila,* appear to have a single type II topoisomerase (*i.e.*, topoisomerase II), vertebrates contain two closely related isoforms, topoisomerase IIα and β (40,41,44,45,48-50). These two isoforms share extensive amino acid sequence identity (~70%), but are encoded by separate genes located at chromosomal bands 17q21-22 and 3p24, respectively. These isoforms can be distinguished by their protomer molecular masses (~170 kDa for α and ~180 kDa for β). Although both enzymes can complement yeast strains lacking topoisomerase II activity, topoisomerase IIα is essential for proliferating mammalian cells and its loss cannot be compensated by the β isoform (51-53).

The enzymological properties of both, human topoisomerase IIα and β, are virtually identical. The main difference found thus far between these isoforms is in their regulation

(54-57). Topoisomerase IIα is highly expressed in rapidly proliferating tissues and its expression is cell cycle-regulated, which peaks at the G_2/M boundary of the cell cycle (54). Based on its regulation, the α isoform is thought to be directly involved in processes such as DNA replication and chromosome segregation. Topoisomerase IIβ expression does not change throughout the cell cycle (57). The β isoform is thought to be responsible for ongoing nuclear processes. Topoisomerase IIβ appears to be dispensable at the cellular level, but is required for proper neural development in mice (44,45,50).

Enzyme Mechanism

Topoisomerase II interconverts different topological forms of DNA by its double-stranded DNA passage mechanism, as depicted in Figure 2. In order for topoisomerase II to maintain genomic integrity, each protomer forms a covalent linkage between the newly generated 5'- DNA terminal, and a tyrosine residue. This proteinaceous bridge, also known as the *cleavage complex*, is an enzyme-linked double-stranded DNA break and is a hallmark of all type II DNA topoisomerases.

The mechanism by which topoisomerase II interconverts different forms of DNA has been separated in a series of steps described below.

Step 1: Enzyme-DNA binding.

The first step in the catalytic cycle of topoisomerase II is the binding to its DNA substrate (58,59). This binding step seems to be governed by the topological nature of the nucleic acid rather than its sequence (58,59). Topoisomerase II can differentiate between positively and negatively supercoiled DNA substrates, and it preferentially relaxes

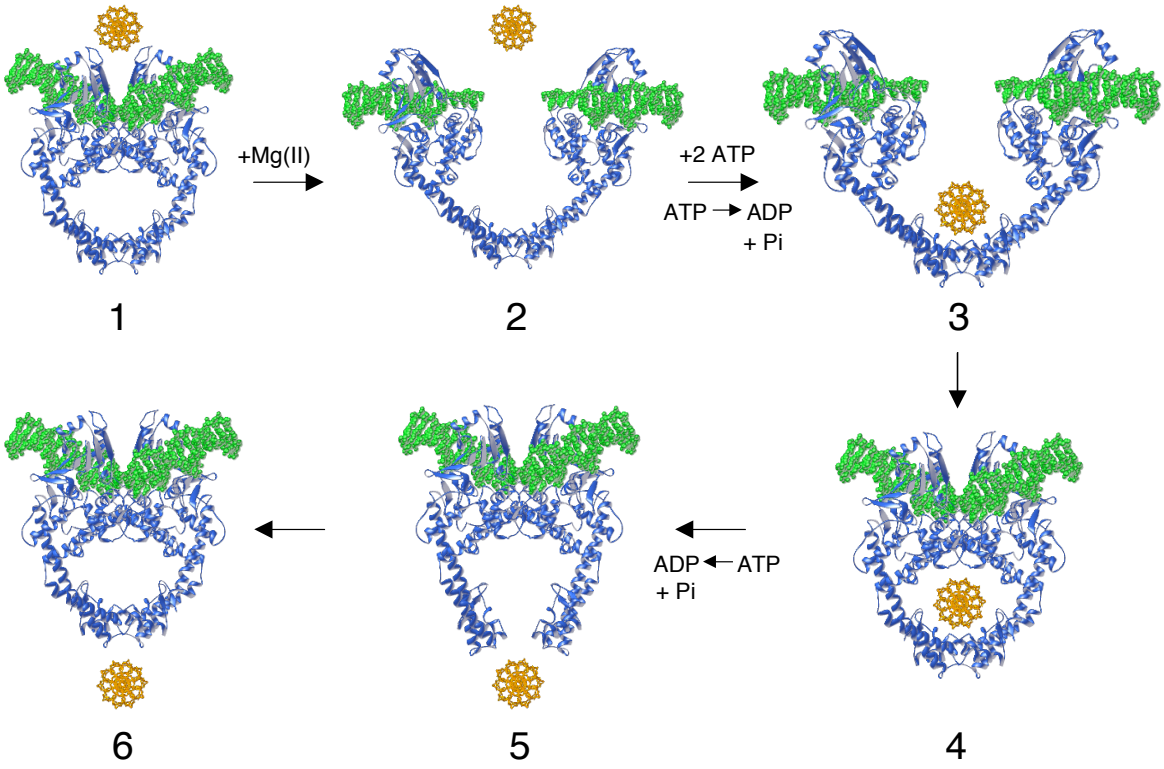


Figure 2. Topoisomerase II catalytic cycle. The complete double-stranded DNA passage reaction is shown as a series of six steps: 1) enzyme-DNA binding; 2) DNA cleavage (formation of *cleavage complex*); 3) double-stranded DNA passage; 4) DNA religation; 5) gate opening and release of the translocated DNA helix; 6) enzyme recycling. The protein (shown in blue) is based on the crystallographic structure of the catalytic core of yeast topoisomerase II. The two required co-factors ATP and Mg^{+2} are shown. Modeled DNA helices are shown in green (horizontal) and orange (coming out of the plane of the paper). Structures are courtesy of Dr. James M. Berger, University of California at Berkeley.

positively supercoiled DNA (46,47). This enzyme binds to DNA at preferred sequences known as *cleavage sites*, but there are not consensus sequences that can be used to predict cleavage sites (60).

Step 2: Generation of the pre-strand passage cleavage/religation equilibrium.

In the presence of a divalent cation, topoisomerase II establishes a DNA cleavage/religation equilibrium (61-65). Magnesium is thought to be the physiological cation, although some others such as Ca^{+2} and Mn^{+2} can substitute *in vitro*. This equilibrium is referred to as pre-strand passage to distinguish it from the cleavage/religation equilibrium that is established post- the strand-passage event. Under normal conditions this equilibrium lies towards the religated species of the reaction.

The DNA scission event results from a transesterification reaction, in which the enzyme generates a protein-linked double-stranded break in one of the DNA segments. During this reaction, two active-site tyrosyl residues (one per protomer) attack two phosphoryl groups on the two strands of the DNA backbone (61,64,66-73). As a result of this reaction, the enzyme becomes covalently attached to the 5'-termini of the cleaved DNA via phospho-tyrosyl bonds. These bonds are formed at the newly formed 5'-termini four bases apart and in opposite strands, yielding a DNA molecule with two 4-base 5'-overhangs. This covalent complex is known as the *cleavage complex*, and is of critical physiological importance.

The 3'-termini, although are not covalently attached to the enzyme, are held in place by non-covalent interactions with topoisomerase II (74). By remaining covalently attached to the DNA ends, this intermediate protects the genomic integrity of the cell.

Step 3: Double-stranded DNA passage.

Upon binding of two ATP molecules, the enzyme undergoes a conformational change that triggers the passage of a second double helix through the double-stranded DNA break previously generated (75,76,77,78-80). Concurrent with the structural change undergone by the enzyme, topoisomerase II becomes topologically linked to its nucleic acid substrate and acts as a "protein clamp" which cannot dissociate from circular molecules (78-80).

Step 4: Post strand-passage cleavage/religation equilibrium.

Following DNA translocation, a cleavage/religation equilibrium is re-established (65,77,81). Levels of DNA cleavage are generally higher in the presence of bound ATP.

Step 5: Gate opening and release of the translocated DNA helix.

Topoisomerase II hydrolyzes its high-energy co-factor (ATP), which triggers a conformational change on the enzyme that opens the protein clamp and confers the potential to release the topologically linked DNA molecule (77,79,82,83).

Step 6: Enzyme recycling.

Topoisomerase II returns to its original conformation and regains the ability to initiate a new round of catalysis (76,77).

Physiological Functions

Topoisomerase II plays a number of essential roles in eukaryotic cells and participates in virtually every major process that involves the genetic material. Topoisomerase IIα unlinks daughter chromosomes that are tangled following replication and resolves DNA knots that are formed during recombination and repair processes (34,35,37). It also helps to remove the positive DNA supercoils that are generated ahead

of replication forks and transcription complexes (84-86). Topoisomerase II is required for proper chromosome condensation, cohesion, and segregation, and appears to play roles in centromere function and chromatin remodeling (84,86). Finally, the type II enzyme is important for the maintenance of proper chromosome organization and structure, and is the major non-histone protein of the metaphase chromosome scaffold and the interphase nuclear matrix (87-91).

Assigning specific cellular functions to individual enzyme isoforms has been challenging. Primarily based on its regulation and abundance in proliferating tissues, topoisomerase IIα has been linked to the functions related to DNA replication and cell division (92). Topoisomerase II β is thought to be involved in ongoing nuclear functions not necessarily associated with cell growth (44,57,93-96).

Topoisomerase II: An Essential Enzyme and a Cellular Toxin

Topoisomerase IIα is essential to all cells. The activity, and thus the amount of topoisomerase II-DNA cleavage complexes in the cell must be tightly regulated. When topoisomerase II activity falls below a threshold level, cells display slower growth, defects in chromosome segregation, and can enter quiescence or cell death due to mitotic failure (Figure 3) (37,84,97,98). This effect can also be achieved by the use of catalytic inhibitors.

Even though topoisomerase II is essential, this enzyme generates a double-stranded DNA break during its catalytic cycle. This double-stranded DNA break poses a threat to the genomic integrity of the cell. The cellular amount of topoisomerase II cleavage complexes is normally low, and thus tolerated. Conditions that increase the concentration

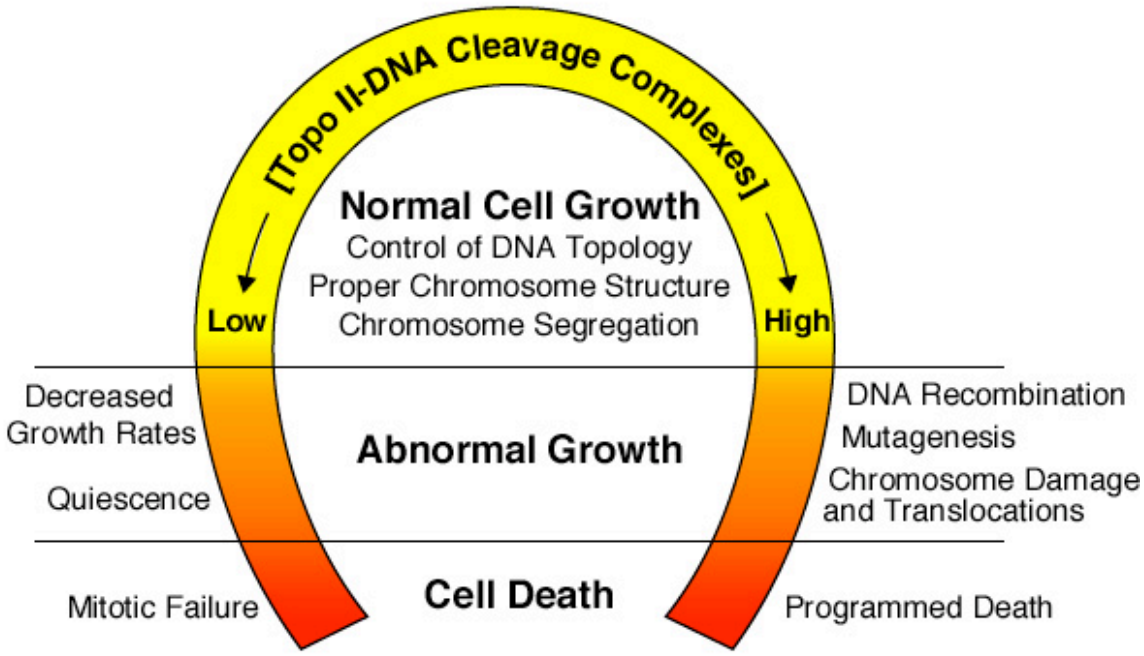


Figure 3. Topoisomerase II: an essential enzyme and a cellular toxin. Topoisomerase II activity is essential in all cells. Normal levels of topoisomerase II activity, and thus of topoisomerase II cleavage complexes, in the cell allow proper chromosomal segregation and function. Conditions or compounds that decrease the amount of topoisomerase II-DNA complexes (*i.e.,* catalytic inhibitors) lead to decreased growth rates culminating in quiescence and mitotic failure. Conversely, agents that increase in the levels of topoisomerase II cleavage complexes (*i.e.,* topoisomerase II poisons), induce mutagenic and recombinogenic events that can lead to cell death. Adapted from Fortune et al. (31).

or lifetime of these topoisomerase II-DNA cleavage complexes induce mutagenic and recombinogenic events that can lead to cell death (99-102) (Figure 3). Thus, although topoisomerase II is essential for cell survival, it also possesses the ability to fragment the genome.

Cleavage complexes are particularly dangerous in the presence of DNA tracking enzymes such as polymerases or helicases that can attempt to traverse the covalently bound topoisomerase "roadblock" in the genetic material. Such a collision can result in conversion of transient enzyme-linked DNA breaks into permanent DNA breaks (Figure 4) (103). These permanent breaks in the genome trigger the generation of chromosomal insertions, deletions, translocations, and other aberrations, and when present in sufficient numbers, initiate a series of events that culminate in cell death (104-107).

Type II Topoisomerases as Therapeutic Targets

In addition to their diverse and critical physiological functions, the type II topoisomerases are targets for some of the most widely prescribed anticancer and antibacterial drugs in clinical use (108-110). In contrast to most enzyme-targeted drugs, these agents do not act by robbing cells of an essential enzyme activity. Rather, drugs that target type II topoisomerases kill cells by increasing the concentration of covalent enzyme-cleaved DNA complexes (*i.e.*, cleavage complexes) that are requisite intermediates formed during the double-stranded DNA passage reaction. Since these drugs that target type II topoisomerases convert these essential enzymes into potent cellular toxins that fragment the genome, they are referred to as topoisomerase II "poisons" to distinguish them from drugs that act as catalytic inhibitors.

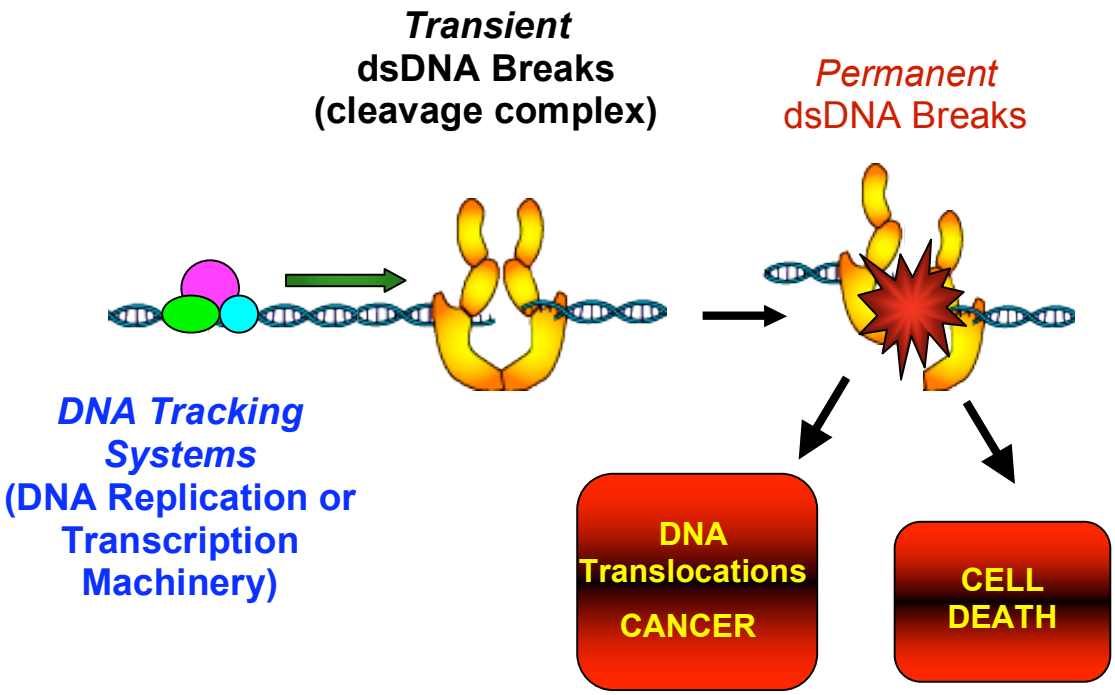


Figure 4. Mechanism of topoisomerase II poisons. DNA tracking systems such as DNA replication and transcription complexes have the potential of converting a transient enzyme-linked double-stranded DNA break into a permanent double stranded break. These breaks can induce mutagenic DNA repair events such as DNA translocations that can lead to cancer, or if overwhelm the cell can lead to cell death.

Exogenous Topoisomerase II Poisons

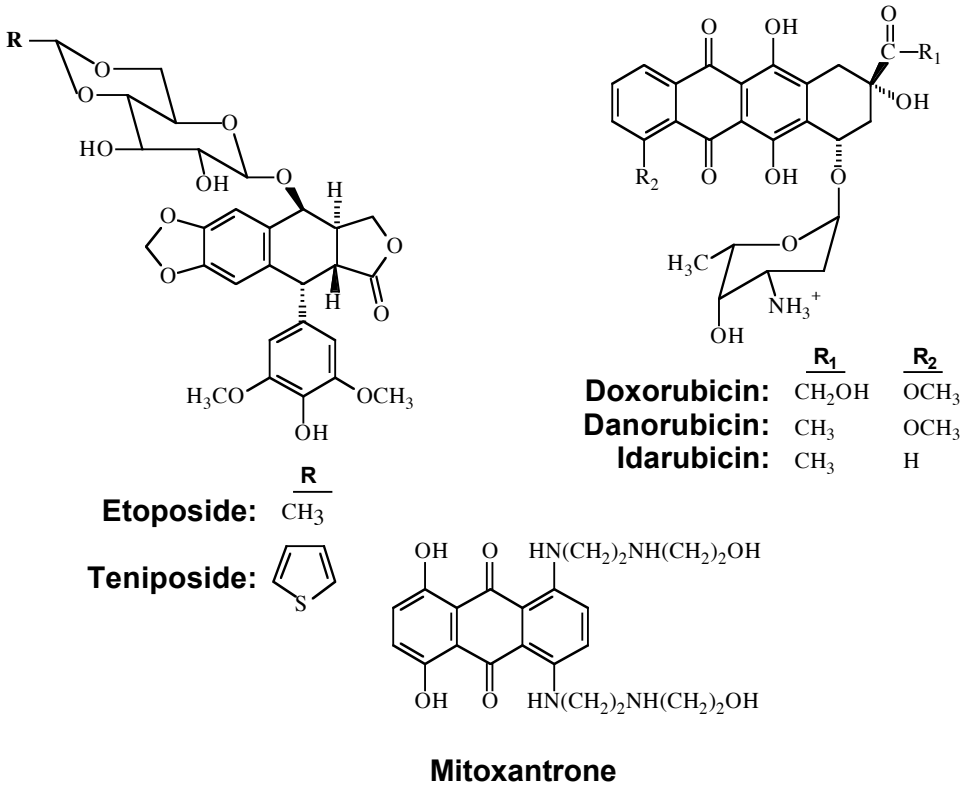
Currently, six topoisomerase II-targeted anticancer agents (Figure 5) are approved for use in the United States. Drugs such as etoposide and doxorubicin are front-line therapy for breast and lung cancers, as well as a variety of leukemias, lymphomas, and germ-line malignancies (108-110). Approximately one-half of all cancer chemotherapy regimens contain drugs targeted to topoisomerase II. Moreover, every form of cancer that can be cured by systemic chemotherapy is treated with these agents.

Due to the high abundance of topoisomerase IIα in rapidly proliferating cells, this isoform probably is the major important target of anticancer therapy. However, circumstantial evidence suggests that the β isoform also contributes to drug efficacy (111).

Topoisomerase II poisons increase the concentration of topoisomerase II-DNA cleavage complexes by two non-mutually exclusive mechanisms. Agents such as etoposide (Figure 5) inhibit the ability of topoisomerase II to religate the enzyme-mediated double-stranded DNA break (112,113). Other agents such as the quinolone CP-115-953 have very slight effect in enzyme-mediated religation, and thus are thought to accelerate the forward rate of cleavage complex formation (114).

In addition to these clinically used agents, a number of natural products and environmental pollutants have been shown to exert a poisoning ability against topoisomerase II. Genistein is a natural product prominent in soy that increases topoisomerase II-mediated DNA cleavage and its consumption has been linked to lower incidence of certain types of cancers in Asian populations (115,116).

Anticancer Drugs



Antibacterial Drugs

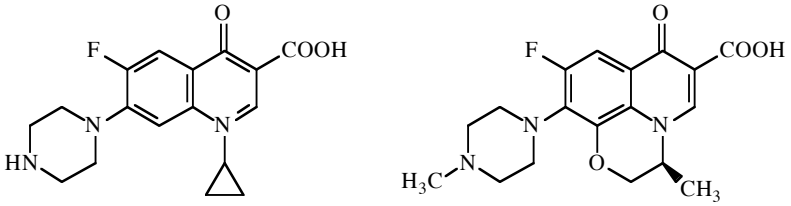


Figure 5. Structures of selected topoisomerase II poisons. Structures of selected anticancer drugs targeted to topoisomerase II and antibacterial drugs targeted to DNA gyrase and topoisomerase IV.

Topoisomerase II Poisons and Carcinogenesis

Notwithstanding the importance of topoisomerase II in cancer therapy, some circumstantial evidence suggests the participation of this enzyme in the generation of cancers (117-120). A small fraction of patients treated with topoisomerase II-targeted drugs develop Acute Myeloid Leukemias (AMLs) (117-120). Further characterization of a group of these patients revealed that the majority of these leukemias displayed a specific translocation in the chromosomal band 11q23 involving the *MLL* gene and in close proximity to known topoisomerase II cleavage sites (117,121,122).

Endogenous Topoisomerase II Poisons

The mechanism by which topoisomerase II poisons kill cells is rather unusual. The ability of poisons converting an essential enzyme into a potent *cellular toxin* with the capacity of fragmenting the genome suggest that these agents are taking advantage of pre-existing cellular pathways, and argues for the existence of endogenous topoisomerase II poisons. There are several lines of circumstantial evidence supporting this hypothesis. First, ~80% of infant leukemias display the same translocations in the *MLL* gene at the 11q23 chromosomal band as those found in etoposide-induced secondary leukemias. Second, there is strong correspondence between the breakpoints of the translocations found in infant leukemias and *in vitro* topoisomerase II-mediated DNA cleavage data. Since topoisomerase II poisons are believed to interact at the enzyme-DNA interface, it seems obvious to speculate that specific DNA lesions can function as endogenous topoisomerase II poisons.

DNA Lesions as Endogenous Topoisomerase II Poisons and the Positional Poison Model

Previous *in vitro* studies have shown that AP (AP, apurinic or apyrimidinic) sites, when located within the 4-base overhang of a topoisomerase II cleavage site, enhance the ability of topoisomerase II to cleave DNA as much as 16-fold (123-126). AP sites are much more potent topoisomerase II poisons than drugs. The DNA cleavage enhancement observed with a single AP site located within a topoisomerase II cleavage site can be compared to that of micromolar concentrations of etoposide.

In 1997 Kingma and Osheroff proposed the *positional poison model* (Figure 6) to explain how exogenous and endogenous topoisomerase II poisons (*i.e.,* drugs and DNA lesions, respectively) enhance the ability of topoisomerase II to cleave DNA (125). The positional poison model includes a number of features. First, consistent with a previous hypothesis on the actions of anticancer agents, it proposes that DNA lesions and drugs both enhance topoisomerase II-mediated DNA cleavage predominately by altering the structure of DNA duplex within the topoisomerase II cleavage site. For instance, some drugs that inhibit enzyme-mediated religation are thought to become physical blocks between the DNA termini and the enzyme, thus misaligning the termini and inhibiting the reaction (113,127). This second feature of the positional poison model is supported by the positional specificity of cleavage-enhancing DNA lesions, DNA binding, and drug cross-linking studies, all of which place exogenous topoisomerase II poisons on the DNA within the cleavage site (123,124,128-131).

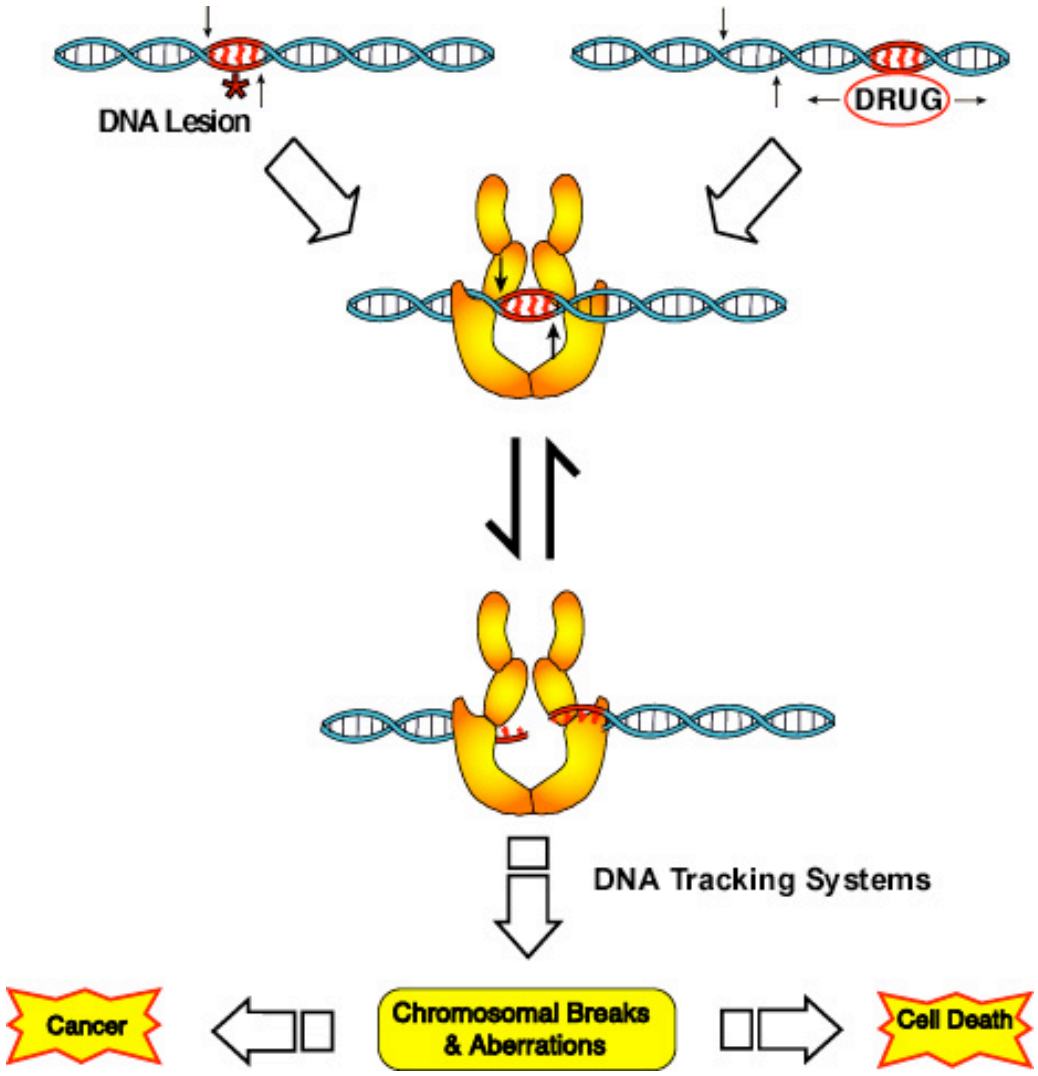


Figure 6. Positional poison model. This model proposes that drugs poison topoisomerase II by interacting at the enzyme-DNA interface and altering the DNA structure within the topoisomerase II cleavage site, thus mimicking the alterations induced by DNA lesions. Adapted from Kingma et al. (125)

Second, since most drugs interact with DNA in a relatively nonspecific fashion and are mobile, the model proposes that interactions with the enzyme are necessary to position the drug-induced alteration of DNA correctly within the cleavage site. This latter aspect of the model is supported by drug-enzyme binding and mutagenesis studies that provide strong evidence for interactions between topoisomerase II and anticancer agents (128). Since DNA lesions are immobile, interactions with the enzyme are not required to position the lesion correctly within the DNA. Therefore, while the efficacy of lesions is predetermined by their location within the cleavage site, the enzyme can control the specificity of the drugs.

Two other postulates are implied. One is that anticancer drugs enhance enzyme-mediated DNA cleavage because they induce structural alterations in DNA that mimic those of endogenous topoisomerase II poisons. The other is that anticancer agents ultimately kill cells by exploiting pre-existing processes that result from the interactions of topoisomerase II with DNA lesions.

Further studies in support of the positional poison model showed that sugar-ring modifications such as arabinose cytosine (AraC) also enhanced topoisomerase II-mediated DNA cleavage, though to a lesser extent than AP sites (132). More recent studies by Sabourin and Osheroff showed that small base-modifications such as methylation and oxidation of purines and pyrimidines were marginal topoisomerase II poisons (133). However, 1,N^6-etheno-deoxyadenosine (εdA), an endogenously-formed exocyclic DNA adduct present in human DNA, was a strong topoisomerase II poison with a potency similar to that of AP sites (133). These studies supports a correlation

between the distortion of the double helix induced by a DNA lesion and DNA cleavage enhancement mediated by topoisomerase II.

An NMR structure of an apurinic site within the sequence context of a topoisomerase II cleavage site revealed several structural features that could potentially be responsible for the poisoning effect of AP sites on topoisomerase II (134). First, loss of base-stacking at the lesion collapsed the major groove and shortened the distance between the two scissile phosphodiester bonds. Second, the apurinic lesion induced a kink that was located around the topoisomerase II cleavage site. Third, the base opposite the AP site became extrahelical and relocated to the minor groove. All of these structural alterations have the potential to influence interactions between topoisomerase II and its DNA substrate, and therefore can be responsible for the poisoning effect that DNA lesions exert on this enzyme.

Moreover, DNA lesions often induce kinks in the DNA and these bent substrates may be recognized or cleaved better by topoisomerase II (135-140). In addition to these structural features of the lesion-containing DNA, there are other potential traits of the damaged genetic material that could be responsible for the enhancement of topoisomerase II-mediated DNA cleavage. For instance, the flexibility and thermodynamic stability of the DNA helix is altered in duplexes containing DNA adducts (141-143).

DNA Repair and Endogenous Topoisomerase II Poisons

Endogenous DNA Damage

The cell is under constant attack from endogenous chemicals that can modify important cellular macromolecules. DNA is one of the most important macromolecules because it carries within itself the information that will be passed on from generation to generation. Among the most common and dangerous endogenous chemicals are alkylating oxidizing agents (144-150). Some of the modifications induced by these agents on DNA arrest critical cellular processes and are the underling cause of diseases such as cancer.

AP sites and εdA are the strongest topoisomerase II poisons from all the DNA lesions studied thus far (123-126,132,133). AP sites are the most abundant DNA lesions in the human genome (151-153). This lesion can be generated by spontaneous hydrolysis of the *N*-glycosydic bond or enzymatically, by the activity of DNA glycosylases (154-158). Recent studies in rodents estimate as many as 200,000 AP sites are generated per genome per day depending on the tissue (152).

The generation of AP sites is mutagenic, recombinogenic, and clastogenic in cells. An AP site is a non-informative residue in the DNA and blocks DNA polymerase elongation of replicative enzymes (159-162). Insertion of a residue opposite to an AP site is dependent on translesion synthesis polymerases, which are highly mutagenic (159-162). In the absence of translesion synthesis polymerases, a double-stranded DNA break is generated and recombination and repair pathways (which are also mutagenic) must be

used to bypass the lesion and/or repair the break (159-162). AP sites also arrest transcription polymerases (160,161).

Etheno adducts such as 3,N^4-etheno-deoxycytidine (εdC) and 1,N^2-etheno-deoxyguanosine (εdG) (in addition to εdA) are exocyclic DNA modifications that arise endogenously from lipid peroxidation byproducts (Figure 7) and can also be chemically induced (163-166). These adducts can be generated exogenously through vinyl chloride, which undergoes a P450-dependent conversion into 2-chloroethyleneoxide and react with DNA to produce these modifications. These lesions can also be induced in cells by treatment with bifunctional alkylating agents such as 2-chloroacetaldehyde (CAA) (163-166). Etheno adducts are mutagenic and carcinogenic and also interfere with cellular processes such as DNA replication and transcription (167). The abundance of etheno modifications under normal conditions is approximately a few hundred adducts per genome (153). This number is not nearly as high as the basal number of AP sites, however, these exocyclic modifications can be detected in human DNA at significant levels (153) and are believed to be significantly elevated during conditions of oxidative stress. Moreover, these lesions are converted to AP sites by DNA glycosylases and further processed by the base excision repair (BER) pathway.

Base Excision Repair

Oxidized and methylated bases, AP sites, and some exocyclic DNA adducts are repaired by the BER pathway (168-172). BER is a ubiquitous DNA repair pathway that removes bases with small modifications and repairs AP sites (173,174). Briefly, during BER a DNA adduct is: 1) recognized and removed, 2) the DNA backbone is incised

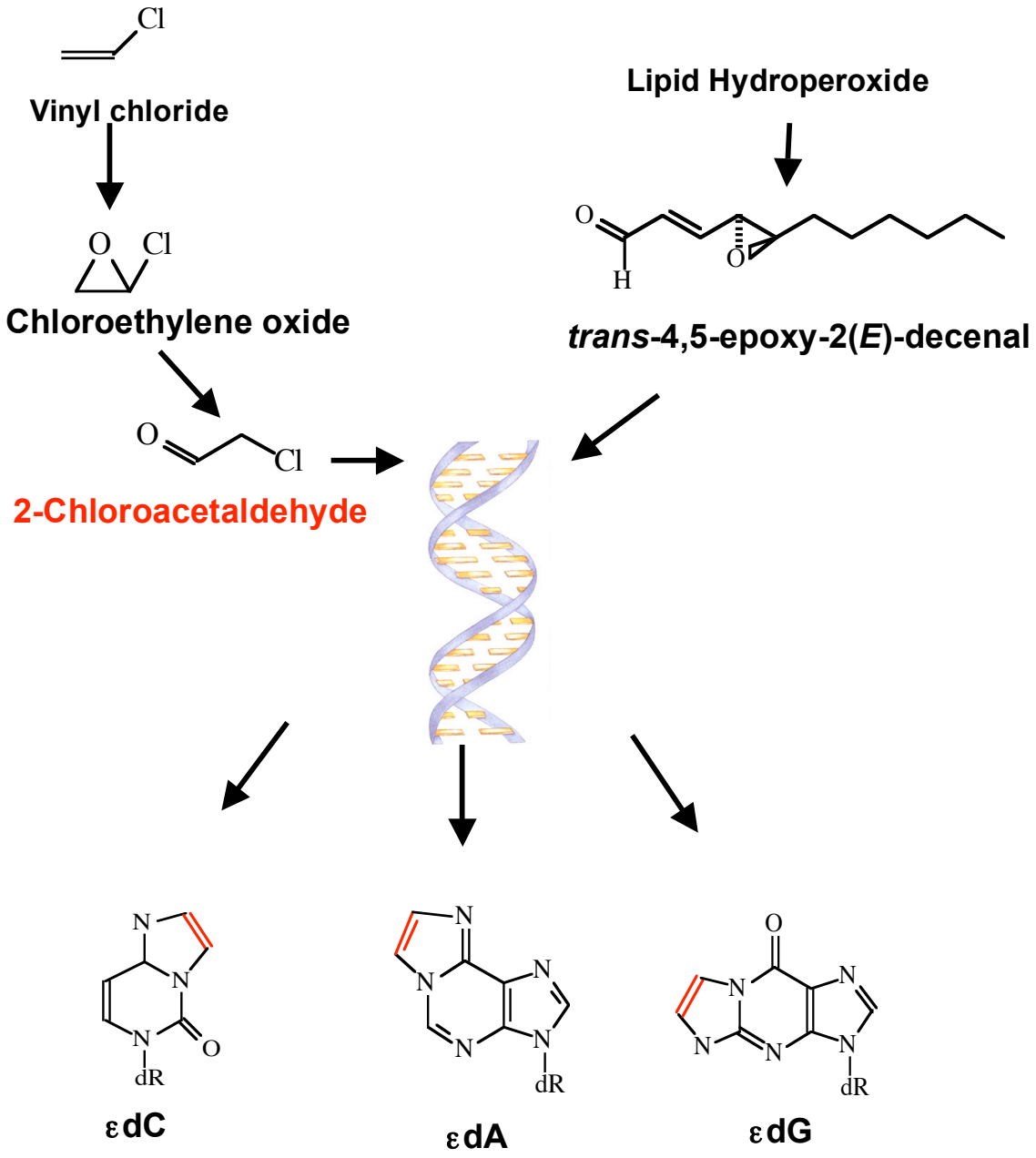


Figure 7. Generation of etheno adducts. Etheno adducts are among the most abundant DNA adducts found in the human genome (178-180). These adducts can arise endogenously from the reaction of lipid peroxidation bypoducts with DNA or exogenously by exposure to the carcinogen vinyl chloride and other organic compounds of industrial use.

at the resulting AP site, 3) the blocked terminal is processed, 4) the gap is filled, and 5) the strand is re-sealed.

There are two main BER sub-pathways: short-patch BER and long-patch BER (175-177). The main differences between them are the size of the gap and the enzymes involved. The first sub-pathway is characterized by the re-synthesis of a one-nucleotide gap by DNA polymerase β (pol β). The long-patch sub-pathway is characterized by the synthesis of a longer patch (8-12 nucleotides), and therefore the requirement of a replicative DNA polymerase (δ or ε), a flap endonuclease (FEN1), and the polymerase loading clamp, PCNA (176), (181-183). It is believed that long-patch repair is not as prominent as short-patch repair due to the limited expression of the additional factors required, which are cell cycle-dependent. It is estimated that more than 80% of BER occurs through the short-patch pathway. Repair of alkylation damage is thought be occur almost exclusively through the short-patch BER (175).

The short-patch BER pathway is also bifurcated. Which side of the fork is chosen depends on the DNA glycosylase utilized, and therefore on the type of DNA lesion to be repaired. Unlike simple glycosylases (such as *N*-methyl purine glycosylase, MPG, the glycosylase that recognizes alkylation damage), "complex" enzymes (such as OGG1, the glycosylase that recognizes oxidized bases) have an associated AP lyase activity (154,156,157,168,184). Consequently, after the enzyme has removed the base, it cleaves the DNA backbone on the 3'-end of the AP site. A blocked 3'-end results from this reaction. This 3'-blocked end must be processed by APE1, the major AP endonuclease in mammalian cells that possesses 3'-phosphodiesterase activity (185). Following the 3'-end

repair, pol β fills in the one-nucleotide gap and ligase I or ligase III/XRCC1 seals the nick on the DNA.

Base Excision Repair of Alkylation Damage

As described above, alkylation repair occurs almost exclusively through short-patch BER, and the DNA glycosylase involved is a "simple" glycosylase (155,177,186). The first step in BER is the excision of a base by a DNA glycosylase (Figure 8). This step grants specificity to the BER system, since glycosylases recognize specific substrates (although they cleave a variety of substrates with lower efficiency) (187,188). In humans, alkylation damage such as N^7-methylG and N^3-methylA, etheno adducts such as εdA and edG, normal purines, and hypoxanthine are recognized by *N*-methyl purine glycosylase (MPG), a "simple" glycosylase with no DNA lyase activity. This enzyme recognizes a DNA adduct and work by a "push and pull" mechanism in which they push the adducted base out of the DNA helix and pull it into their active site where hydrolysis of the *N*-glycosydic bond occurs (187). Simple glycosylases bind the product of their reactions (*i.e.*, an AP site) and thus are known to be product-inhibited. This particular characteristic of DNA glycosylases is believed to be advantageous to the cell because it protects the toxic BER intermediates from reacting with the cellular environment and enzymes not involved in repair processes (189-192).

The resulting AP site from a simple glycosylase (or generated by spontaneous hydrolysis) is further processed by an AP endonuclease. In humans, the major AP endonuclease is known as APE1 (193-195). Unlike $MPG^{-/-}$ mice, $APE1^{-/-}$ mice die during

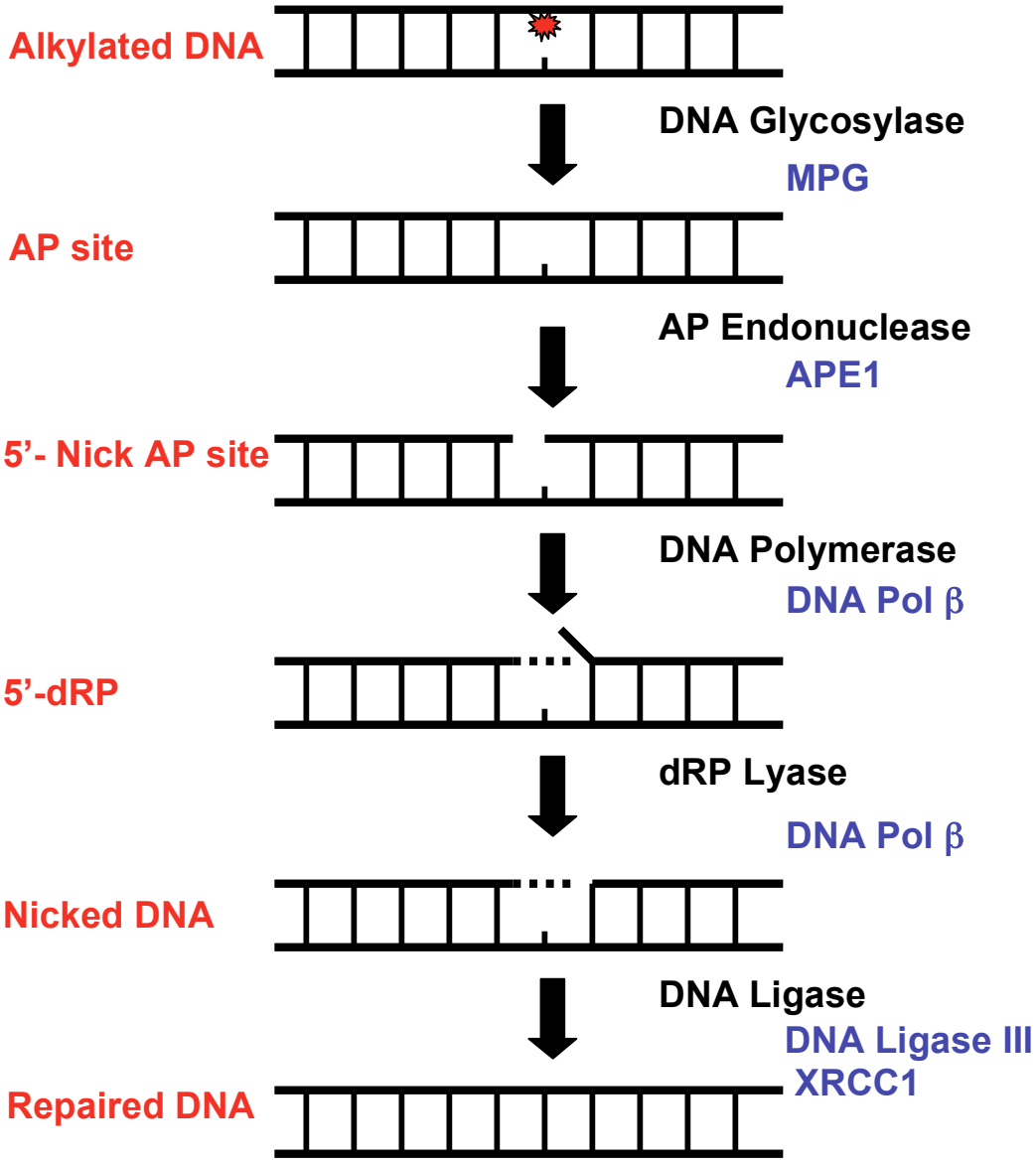


Figure 8. Simple Short-patch Base Excision Repair Pathway. AP sites and most of the alkylated DNA lesions are repaired through the short-patch "simple" BER pathway. This repair pathway is initiated by MPG, which generates an AP site. This AP site is further processed by APE1, which cleaves the phosphodiester bond 5' of the AP site, leaving a 3'-OH and a 5'-dRP flap. DNA polymerase β fills in the gap and its dRP lyase activity cleaves the 5'-dRP flap. Finally DNA ligase III/XRCC1 seals the nick to fully repair the DNA.

embryonic development. APE1 has an additional catalytic activity that is thought to be the reason for the embryonic lethality phenotype in these mice. This protein changes the oxidation-reduction state of some proteins and transcription factors (195). APE1 binds AP site-containing DNA and incise the duplex 5' to the AP site. This reaction results in a nick intermediate with a 5'-deoxyribose phosphate (dRP) flap, and a 3'-OH end. *In vitro* studies have shown that APE1 interacts with pol β and also enhances excision rates of some glycosylases (196). These data is in support of the model of "handing off" intermediates to protect them from the cellular environment (189,191).

The next step in the repair of alkylated damage is the synthesis step. DNA pol β is thought to be responsible for this activity in mammalian cells (177,197). This enzyme has two catalytic activities and both of them are important in BER. First, pol β synthesizes the one-nucleotide gap by its polymerization activity. Second, pol β excises the 5'-dRP flap with its dRP lyase activity. Although pol β is an essential enzyme, mouse pol $\beta^{-/-}$ fibroblast cell lines have been designed. Elegant studies have shown that the 5'-dRP lyase activity is the key activity for this repair pathway. Cells that lack this activity are hypersensitive to MMS and other alkylating agents, and accumulate a higher number of chromosomal aberrations upon MMS treatment (198).

Finally, the last step in BER is the sealing of the nick intermediate. This step is catalyzed *in vivo* by either ligase I or ligase III/XRCC1 (183,199).

Studies with synthetic oligonucleotides modeling BER intermediates demonstrated that these processed AP sites are strong topoisomerase IIα poisons (200). Moreover, some of these BER intermediates were shown to be suicide substrates for topoisomerase IIα. However, whether these substrates interact with the type II enzyme in a cellular

setting has yet to be demonstrated. Mounting structural and enzymological evidence suggest that each BER enzyme "hands off" the product of its reaction to the next enzyme in the pathway. The "baton" model proposes that the handing off the intermediates from one enzyme to the next provides the advantage of protecting toxic BER intermediates from the cellular milieu (188,191,194).

Scope of Dissertation

Although topoisomerase II is one of the most important targets for anti-cancer therapy, mounting circumstantial evidence links this enzyme with the generation of this disease. The unusual mechanism by which topoisomerase II poisons work suggest that these agents are taking advantage of pre-existing cellular pathways, and argues for the existence of endogenous topoisomerase II poisons. DNA lesions are very good candidates to be endogenous topoisomerase II poisons. Previous work has shown that AP sites and εdA are strong topoisomerase IIα poisons *in vitro*. The studies presented in this dissertation examine the interactions between topoisomerase IIα and DNA lesions, and presents evidence for the interaction between DNA lesions and topoisomerase IIα in a cellular system for the first time.

An overview of the enzymological and physiological functions of type II topoisomerases as well as a description of previous studies between this enzyme and some forms of DNA damage are presented in Chapter I. The methods utilized for the work presented in this dissertation is presented on Chapter II.

Chapter III further expands the spectrum of exocyclic DNA adducts that act as topoisomerase IIα *in vitro*, further characterizes the poisoning effect of DNA lesions on

topoisomerase IIα, and examines the potential interaction between etheno adducts and this enzyme in cultured cells. Studies indicate that AP sites and exocyclic DNA adducts are strong topoisomerase II poisons. The enhancement observed with sugar-ring modifications was marginal. Furthermore the increased levels of topoisomerase II-mediated DNA cleavage were not due to decreased rates of religation, nor to an increased affinity of topoisomerase IIα for adducted DNA, suggesting that these lesions poison topoisomerase II by accelerating the forward rate of DNA cleavage at the chemical scission step. Furthermore, fluorescence resonance energy transfer analysis showed that DNA bending of two representative DNA lesions correlated with topoisomerase IIα-mediated DNA cleavage enhancement. This finding supports the positional poison model. Finally, cells treated with CAA (generates etheno adducts) showed increased cellular levels of topoisomerase IIα-DNA breaks. This finding suggests that topoisomerase IIα interacts with exocyclic DNA lesions in cellular systems (201).

In addition to DNA lesions, BER intermediates have been shown to poison human topoisomerase IIα *in vitro* (200). In Chapter IV we examined whether topoisomerase II was able to interact with AP sites generated by BER in a physiological system. Cells treated with MMS (a methylating agent that generates lesions that are readily converted to AP sites by BER) showed an increase in the number of AP sites, which paralleled the increase of topoisomerase IIα-mediated DNA breaks in a dose-dependent fashion. Moreover, cells overexpressing MPG displayed higher baseline level of topoisomerase IIα-DNA breaks. Finally, cells with decreased levels of topoisomerase IIα displayed a slight resistance to MMS and a decreased amount of double stranded DNA breaks induced by this methylating agent. Taken together these data suggest that topoisomerase

IIα interacts with AP sites generated by BER in a physiological system and furthermore that topoisomerase IIα mediates some of the cytotoxic and genotoxic consequences of MMS.

Concluding remarks and future directions regarding the work presented in this dissertation are included in Chapter V.

CHAPTER II

METHODS

Materials

Human topoisomerase IIα was expressed and purified from *Saccharomyces cerevisiae* as described previously (126). Oligonucleotide substrates were prepared as described below. Negatively supercoiled DNA was prepared as previously described (47). T4 polynucleotide kinase was obtained from New England Biolabs Inc.; [γ-^{32}P]ATP (~5,000 Ci/mmol) was from ICN; dSpacer (tetrahydrofuran AP site analog), 3,N^4-etheno-2'-ribocytidine, and 2'-ribocytidine phosphoramidites were from Glen Research Corp., 3,N^4-etheno-deoxycytidine phosphoramidite was from Chem-Genes Inc. Etoposide was from Sigma and was stored at 25 °C as 20 mM stock solutions in 100% DMSO. The alkylating agents CAA [50% (v/v)], and methyl metanesulfonate (MMS) were also from Sigma and were stored at 25 °C as a 50 mM stock in water and DMSO, respectively. All other chemicals were analytical reagent grade.

Preparation of Topoisomerase IIα Substrates

DNA Adduct-containing Oligonucleotides

A 42-bp oligonucleotide corresponding to residues 1039-1081 of the *MLL* gene and its complementary strand were prepared on an Applied Biosystems DNA synthesizer. This substrate spans a previously mapped leukemic breakpoint at position 1067. The

sequences of the top and bottom strands were 5'-ATGATTGTACCACTGCAG↓TC-CAGCCTGGGTGACAAAGCAAAA-3' and 5'-TTTTGCTTTGTCACCCAGGC↓TG-GACTGCAGTGGTACAATCAT-3', respectively. This substrate contains a single strong cleavage site for topoisomerase II that has been well characterized (117,126). The points of topoisomerase II-mediated DNA scission are denoted by arrows.

Adducted DNA bases were inserted in the oligonucleotide at specific positions using phosphoramidite chemistry for the εdC (Chem Genes), εrC, rC, and THF (Glen Research). The M_1G, PdG, and εdG, lesioned oligos were synthesized as previously described (171,202-204). Wild-type strands oligonucleotides were labeled on the 5'-termini using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP, and gel-purified as described previously (200).

Methylated pBR322 plasmid

pBR322 (60 μg) was incubated at 37 °C for 1 h with increasing concentrations of MMS (0 to 5 μM) in 5 mM Tris-HCl (pH 8.0), 0.5 mM EDTA. Alkylated plasmid DNA was purified using Bio-Spin 6 chromatography columns (Bio-Rad) exchanging the buffer to fresh 5 mM Tris-HCl (pH 8.0), 0.5 mM EDTA. Buffer exchange was performed three times to ensure that the exclusion of MMS from the final DNA preparation. Under these conditions approximately 0-100 adducts were generated per plasmid.

FRET Analysis

Preparation of Fluorescently-labeled Oligonucleotides

A 16-mer oligonucleotide labeled on both ends with fluorophores and containing a centrally located topoisomerase II cleavage site was used for FRET studies. The sequences of the oligonucleotides synthesized for the top and bottom strands, respectively were: 5'-CTGCAG↓TCCAGCCTGG-3', and 5'-CCAGGC↓TGGACTGCAG-3'. Each top strand oligonucleotide contained either a normal base, or an AP site analog, or a 2'-OMeC base at the +2 position, and was labeled at its 5'- and 3'- end with Cyanine 3 and Fluorescein, respectively. Oligonucleotides were prepared using an Applied Biosystems DNA synthesizer, and purified by gel electrophoresis in the dark.

Double-stranded oligonucleotides were annealed after heating equimolar amounts of the top and bottom strands at 70 °C for 10 min and cooling to room temperature. Samples were stored at -20 °C in the dark prior to data collection.

Fluorescence Spectroscopy

Steady-state fluorescence measurements were acquired using a T-format spectrofluorometer (Photon Technologies). Absorbance measurements were acquired using the 8453 UV-Visible system (Hewlett-Packard). Measurements were done in quadruplicate on solutions with equivalent absorbance ($A_{433} < 0.05$) and corrected for background fluorescence and differences in excitation intensity. Measurements were acquired in 10 mM sodium phosphate buffer (pH 7.0). Quantum yields were determined from integrated fluorescence spectra (540 to 600 nm emission; excitation at 494 nm for

fluorescein and 546 nm for cyanine 3) taken from an equal absorbance of a fluorescein reference standard (Molecular Probes; quantum yield = 0.95).

Topoisomerase IIα Catalysis

Topoisomerase IIα-mediated Cleavage of Plasmid DNA

DNA cleavage reactions were performed as described by Fortune (205). Reactions contained 220 nM human topoisomerase IIα, and 10 nM negatively supercoiled pBR322 in cleavage buffer [10 mM Tris-HCl (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM NaEDTA, and 2.5% (v/v) glycerol]. Reactions were initiated by the addition of enzyme and incubated for 10 min at 37 °C to establish a DNA cleavage/religation equilibrium. Cleavage complexes were trapped by the addition of 2 μl of 5% (w/v) SDS and 1 μl of 375 mM NaEDTA (pH 8.0). Proteinase K was added (2 μl of 0.8 mg/ml) and reactions were incubated 30 min at 45 °C to digest topoisomerase IIα. Samples were mixed with 2 μl of 30% (w/v) sucrose, 0.5% (w/v) bromophebol blue, and 0.5% (w/v) xylene cyanol FF in 10 mM Tris-HCl (pH 7.9), heated for 5 min at 45 °C, and subjected to electrophoresis in a 1% (w/v) agarose gel in 40 mM Tris-acetate (pH 8.3), 2 mM EDTA, and 0.5 μg/ml ethidium bromide. Cleavage was monitored by the conversion of negatively supercoiled DNA to linear molecules. DNA bands were visualized by medium wavelength UV light, and quantified using an Alpha Innotech digital imaging system.

Site-specific DNA Cleavage Induced by DNA Lesions in Oligonucleotide Substrates

In all cases, double-stranded DNA substrates were generated by annealing equimolar amounts of complementary oligonucleotides at 70 °C for 10 min and cooling to 25 °C. DNA sites cleaved by human topoisomerase IIα in oligonucleotide substrates were determined as described previously (200). Reaction mixtures contained 220 nM human topoisomerase IIα and 100 nM double-stranded oligonucleotide in 20 μl of cleavage buffer. Reactions were started by the addition of the enzyme and mixtures were incubated at 37 °C for 10 min. DNA cleavage products were trapped by the addition of 2 μl of 10% (w/v) SDS followed by 1 μl of 375 mM NaEDTA (pH 8.0). Samples were digested with proteinase K (2 μl of a 0.8 mg/ml solution) for 30 min at 37 °C, precipitated twice in 100% (v/v) ethanol, rinsed once with 70% (v/v) ethanol, dried, and re-suspended in 40% (v/v) formamide, 8.4 mM EDTA, 0.02% (w/v) bromophenol blue, and 0.02% (w/v) xylene cyanole FF. DNA cleavage products were resolved by electrophoresis in 7 M urea, 14% polyacrylamide gels in 100 mM Tris-borate (pH 8.3), 2 mM NaEDTA, and were visualized and quantified on a Bio-Rad Molecular Imager FX system. Topoisomerase II-mediated DNA cleavage enhancement was calculated by dividing the percent DNA scission of the adducted oligonucleotide by the percent DNA scission of the corresponding wild-type oligonucleotide. The nomenclature of the positions on the top and bottom strands are assigned as follows: positions 5' to the cleavage site are named with negative numbers, whereas positions 3' to the cleavage site are named with positive numbers.

DNA Religation Mediated by Topoisomerase IIα

DNA religation assays were carried out by a modification of the procedure of Osheroff and Zechiedrich (65). DNA cleavage/religation equilibria were established in cleavage buffer as described in the preceding section with the exception that the 5 mM $MgCl_2$ in the reaction buffer was replaced by 5 mM $CaCl_2$. Topoisomerase IIα-DNA cleavage complexes were trapped by the addition of NaEDTA (6 mM final concentration). NaCl was added (500 mM final concentration) to prevent re-cleavage. Religation was initiated by the addition of $MgCl_2$ (0.1 mM final concentration) and terminated at times up to 60 s by the addition of 2 μl of 10% (w/v) SDS. Samples were analyzed as described above. The apparent first order rate of DNA religation was determined by quantifying the loss of the cleaved DNA product.

Topoisomerase IIα-DNA Binding Affinity

The effects of lesions on the affinity of human topoisomerase IIα for DNA were monitored by a competitive nitrocellulose filter-binding assay. Nitrocellulose filters (0.45 μm, Millipore) were equilibrated in binding buffer [10 mM Tris-HCl (pH 7.9), 40 mM KCl, 0.1 mM NaEDTA, and 2.5% (v/v) glycerol]. Assays were performed in the absence of a divalent cation to avoid topoisomerase IIα-mediated DNA cleavage. Binding was initiated by the addition of 220 nM human topoisomerase IIα to a mixture that contained 50 nM [^{32}P]-labeled wild-type oligonucleotide and 0 to 200 nM of cold competitor oligonucleotide in binding buffer (20 μl total volume). Binding mixtures were incubated at 37 °C for 10 min, transferred to nitrocellulose filters, and washed three times with binding buffer. Filters were placed in 10 mL of Econo-Safe scintillation fluid (Research

Product International) and the amount of [^{32}P]-labeled wild-type oligonucleotide that remained bound to the filter was quantified using a Beckman LS 5000TD scillination counter.

Experiments Performed in Cultured Human Cancer Cell Lines

Cell Culture

Human CEM (leukemic cell line), MDA-MB-231, and MCF-7 (both breast cancer cell lines) cells were obtained from ATCC. Cells were cultured under 5% CO_2 at 37 °C in RPMI 1640 medium (Cellgro by Mediatech, Inc.), containing 10% heat-inactivated bovine growth serum (Hyclone) and 2 mM glutamine (Cellgro by Mediatech, Inc.).

DNA Cleavage Mediated by Topoisomerase IIα in Cultured Human Cells

The In vivo Complex of Enzyme (ICE) bioassay (as modified on the TopoGEN, Inc. website) was employed to determine the effects of CAA and MMS on topoisomerase IIα-associated DNA breaks in treated cells. Exponentially growing cultures were treated with these alkylating agents for 4 h. MDA-MB-231 cells (~5×10^6) were incubated with trypsin prior to centrifugation at 1,500 rpm for 5 min. Following centrifugation, cells were lysed by the immediate addition of 3 ml of 1% (w/v) sarkosyl in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. Following gentle douncing, lysates were layered onto a 2 ml cushion of CsCl (1.5 g/ml) and centrifuged at 80,000 rpm for 5.5 h at 20 °C. DNA pellets were isolated, re-suspended 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 IIα and DNA were detected using a polyclonal antibody directed against human topoisomerase IIα (Kiamaya Biochemical Co.) at a 1:2,000 dilution. The secondary antibody used was anti-rabbit IgG conjugated to horseradish peroxidase. The amount of cleavage complexes was visualized using an ECL kit (Amersham) and quantified using an Alpha Innotech Digital Imager system. Relative DNA cleavage was calculated by dividing the intensity of the band of generated during each treatment by that of the non-treated sample.

Quantification of AP Sites in Genomic DNA

MDA-MB-231 cells were treated with increasing concentrations of MMS for 4 h. Cells were trypsinized and harvested as described above. Genomic DNA was extracted using the GETpure DNA isolation kit (Dojindo) following the manufacturer's directions. The proteinase K incubation that accompanied the extraction was performed at room temperature for 30 min to avoid further damage of the genomic DNA. The amount of AP sites was determined using the aldehyde reactive probe (Dojindo). This probe reacts with the aldehyde form of AP sites (151). By treating the DNA with an excess of the probe, AP sites are converted to biotin-tagged AP sites, which can be detected colorimetrically by peroxidase-conjugated avidin. The number of AP sites was calculated by constructing a standard curve with standards from Dojindo. Samples were read in a microplate reader using 450 nm wavelength. Relative levels of AP sites were calculated by dividing the AP sites in each sample by the amount determined for the non-treated sample.

Cellular Cytotoxicity

Cellular cytotoxicity was assessed by determining the fraction of metabolically active cells following treatment with MMS. MDA-MB-231 cells were plated in 96-well plates at 1×10^5 cells/ml for 24 h to allow cells to attach. After attachment, cells were exposed to increasing concentrations of MMS for up to 2 h, incubated with fresh media, and allowed to grow for an additional 36 h. WST-8 (Dojindo) (10 μl) was added to each well. This reagent is reduced by mitochondrial dehydrogenases in metabolically active cells rendering a yellow product (formazan). The amount of formazan dye generated by the activity of dehydrogenases in cells can be measured spectrophotometrically (450 nm) and is directly proportional to the number of living cells in each well. The 96-well plates were read in a microplate reader at 450 nm and cell viability was determined as compared to the viability of the non-treated wells.

Protein Immunoblot Analysis

MDA-MB-231 cells were trypsinized and harvested as described above. Cells were re-suspended in 50 mM Tris pH 7.9, 150 mM NaCl, 0.5% (v/v) NP-40, and protease inhibitor cocktail (Roche), and incubated on ice for 30 min. The protein concentration these whole cell lysates was determined by Bradford assays. Lysates were diluted 1:1 in Laemmli buffer, boiled for 15 min and equal amounts of protein were subjected to electrophoresis on denaturing polyacrylamide gels for 1 h at 200 V (35 mA). Proteins were blotted onto PDVF membranes (Hy-bond) in 25 mM Tris (pH 8.3), 192 mM glycine for 1h at 100 V (300 mA) at 4 °C. After blotting, membranes were blocked with 5% (w/v) non-fat dried milk in 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% (v/v) Tween-

20 for 20 min. Membranes were probed with the appropriate antibodies overnight at 4 °C (monoclonal α-human topoisomerase IIα and α-human topoisomerase IIβ antibodies were a generous gift from Dr. Akihiko Kikuchi from the University of Nagoya, Japan; monoclonal α-histone phopho-H2AX (Ser 139) and polyclonal α-PARP-1 antibodies were obtained from Upstate Cell Signaling; monoclonal α-MPG was a generous gift from Dr. Rabindra Roy, University of Texas Medical Branch, Galveston, TX). After washing the primary antibody, membranes were incubated with the secondary antibody (α-rabbit or α-mouse) α-IgG conjugated to horseradish peroxidase (Amersham). In some cases, membranes were stripped by incubation in 0.2 M NaOH for 15 min at room temperature. After stripping, the membranes were blocked and probed as described above. The amount of protein was visualized using an ECL kit.

Small Interfering RNA (siRNA) Transfection

MDA-MB-231 cells were seeded in 6-well plates the day before transfection at a cell density of 5×10^4 cells/ml. siRNA Smart Pools against human topoisomerase IIα and a scrambled control sequence pool were obtained from Dharmacon Inc. RNA duplexes were stored at –20 °C as 20 μM stocks. Oligofectamine™ (Invitrogen) was used as a transfecting reagent and transfections were performed as described in the manufacturer's instructions. Oligofectamine™ solution was made by mixing 4 μl of Oligofectamine™ with 12 μl of serum-free medium, and incubating at room temperature for 15 min. Cells were rinsed twice with serum-free medium. An additional 800 μl of serum-free medium and 200 μl of transfection solution [5 μl of siRNA, 15 μl of Oligofectamine™ solution and 180 μl of serum-free medium (Opti-Mem I, Gibco)] were added to each well. After 4

h, 1 ml of RPMI 1640 medium with 20% (v/v) fetal bovine serum was added to each well and cells were allowed to grow for 72 to 96 h. The final concentration of siRNA in the culture medium was 50 nM. Cells were treated with MMS as described above or harvested for immunoblot analysis, cytotoxicity, sister chromatid exchange (SCE), or cell cycle analysis by flow cytometry.

Cell Cycle Analysis by Flow Cytometry

MDA-MB-231 cells were transfected with siRNA (topoisomerase IIα or scrambled sequence control pools) and cultured as described in the preceding section. Cells were prepared and analyzed by flow cytometry using propidium iodide staining. Briefly, ~1 × 10^6 cells were trypsinized, harvested by centrifugation at 1,500 rpm for 2 min, washed with fresh media, re-suspended in 70% (v/v) ethanol, and stored 4 °C overnight. Cells were washed in PBS and incubated at 37 °C for 15 min in PBS containing propidium iodide (25 μg/ml) and RNase A (50 μg/ml). Samples were filtered through a 40-μm (pore size) mesh (Small Parts, Inc., Miami Lakes, FL) and subjected to flow cytometry on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Ten thousand events were analyzed for each sample, and DNA histograms indicating the fraction of cells with DNA content characteristic of G_1, S, and G_2 were generated by ModFit LT (Verity Software House, Topsham, ME). The fraction of apoptotic cells was determined by measuring the cells with sub-G_1 DNA content.

CHAPTER III

EXOCYCLIC DNA LESIONS STIMULATE DNA CLEAVAGE MEDIATED BY HUMAN TOPOISOMERASE IIα IN VITRO AND IN CULTURED CELLS

Introduction

The human genome is under constant attack by a plethora of endogenous and environmental chemicals that damage DNA. Alkylating agents are among the most common of these DNA-reactive chemicals, and can be either monofunctional or bifunctional in nature (145,148). The former generate singly-modified bases (*i.e.*, methylated, ethylated, etc.), while the latter often produce exocyclic DNA adducts. Alkylated bases can be highly mutagenic and carcinogenic (206).

The interaction of repair proteins, DNA polymerases, and RNA polymerases with adducted bases has been documented (161,167,207-209). However, relatively little is known about how damaged bases affect the activities of other nuclear proteins. In this regard, DNA topoisomerases are among the few enzymes that have been investigated. When AP sites or other specific lesions are located in proximity to a topoisomerase I (210-215) or topoisomerase II (123-126,132,133,216) cleavage site, they often have a dramatic effect on DNA scission mediated by these two enzymes.

Previous studies demonstrated that AP sites and DNA adducts are often potent poisons of topoisomerase I (210-215) or II (123-126,132,133,216) *in vitro*. Lesions act in a position-specific manner. The scissile bonds cleaved by topoisomerase II on the two strands of the double helix are staggered by four bases. When AP sites are located within this four-base stagger, they stimulate topoisomerase II-mediated DNA cleavage as much

as 20–fold (125). In contrast, when they are located immediately outside of the scissile bonds, they often inhibit DNA cleavage (125).

In addition, there appears to be a correlation between the ability of a DNA adduct to distort the double helix and its potential to act as a topoisomerase II poison (133). AP sites and εdA, both of which induce kinks in DNA (133), increase enzyme-mediated DNA cleavage ~10– to 20–fold. In contrast, adducts such as 8-oxo-deoxyguanosine, O^6-methyl-deoxyguanosine, 8-oxo-deoxyadenosine, and N^6-methyl-deoxyadenosine, which induce little distortion in DNA, have relatively small (if any) effects on DNA cleavage (133).

Although some DNA damaging agents induce topoisomerase I-mediated DNA cleavage in cultured cells (214,217), DNA lesions have not as yet been shown to act as topoisomerase II poisons in human cells. Therefore, the present study investigated the effects of alkylated bases on DNA scission mediated by human topoisomerase IIα. Results indicate that a variety of exocyclic base adducts, including εdC, εdG, M_1dG, and PdG (see Figure 9) enhance DNA cleavage by the type II enzyme ~5– to 17–fold. In addition, CAA (a reactive product of vinyl chloride metabolism), which is carcinogenic and mutagenic, induces sister chromatid exchanges, and induces etheno base adducts in DNA, (163,165), is a potent topoisomerase IIα poison in cultured human cells.

Results

The presence of base adducts in the genetic material can lead to mutations because replicative and repair DNA polymerases often insert wrong nucleotides opposite of adducted bases (206,218,219). In addition, lesions can lead to chromosomal aberrations

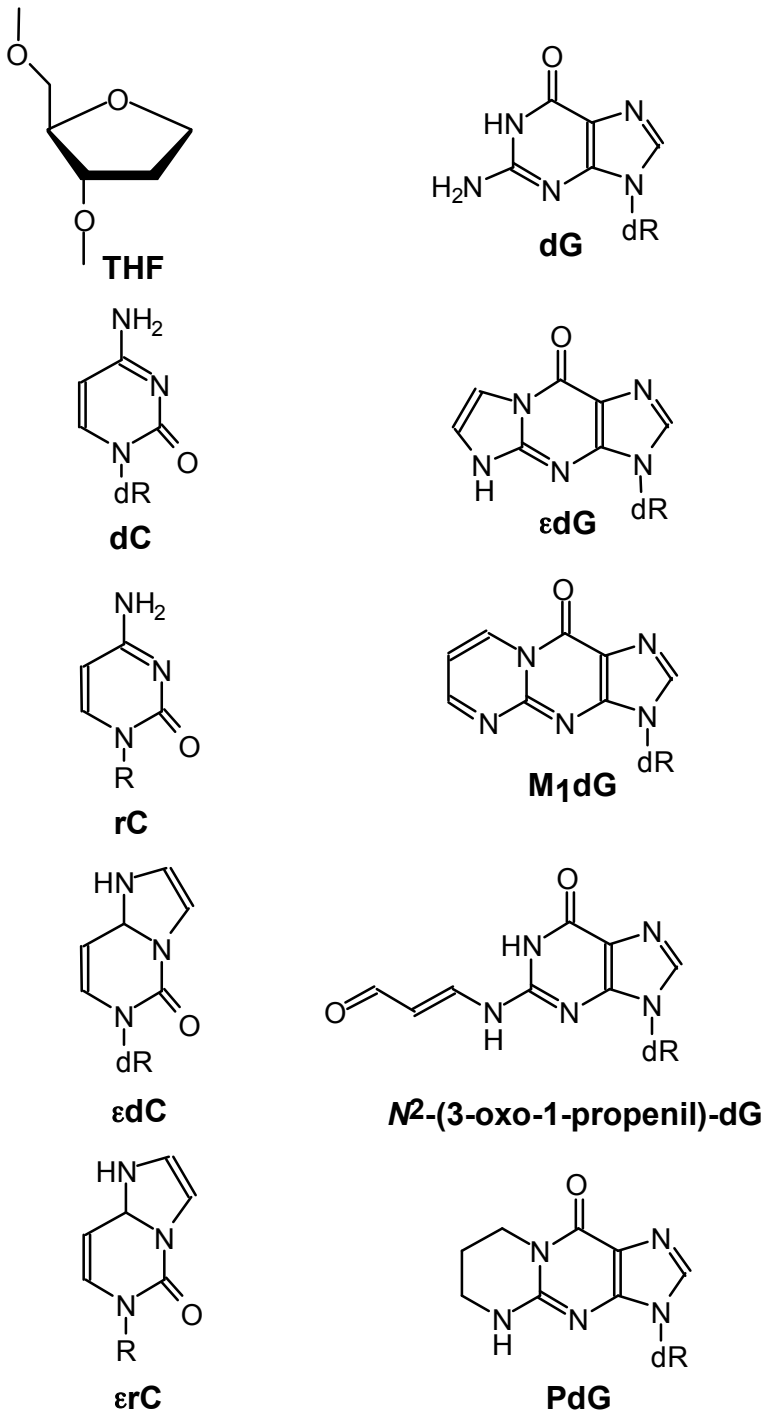


Figure 9. Structures of DNA lesions. The structures of the different DNA lesions studied are shown. THF is used as an analog of an AP site. Etheno adducts are produced endogenously by lipid peroxidation byproducts (178,179) and exogenously by exposure to vinyl chloride (224). M_1dG is produced endogenously by malondialdehyde and base propenals (ring-closed and ring-opened structures) (146,225) and PdG is an unnatural adduct used commonly as a M_1dG analog (136,226,227).

such as insertions, deletions, rearrangements, and translocations (219-221). Many of these latter effects are attributed to recombination pathways that are triggered when lesions stall DNA replication forks (160,161,167,208,222). However, similar chromosomal aberrations are observed following treatment of cells with topoisomerase II poisons (30-32,101,102,223).

Effects of Exocyclic DNA Adducts on DNA Cleavage Mediated by Human Topoisomerase IIα.

In order to further explore the potential of DNA lesions to act as topoisomerase II poisons, the effects of a number of exocyclic base adducts (Figure 9) on DNA cleavage mediated by human topoisomerase IIα were determined. The oligonucleotide substrate used for these studies contains a single well-characterized cleavage site for human topoisomerase IIα (126). The sequence is derived from the breakpoint cluster region of the *MLL* oncogene at chromosomal band 11q23 and contains a leukemic chromosomal translocation breakpoint identified in a patient that had been treated with etoposide (117). Since εdA lesions are strong topoisomerase II poisons and stimulate DNA cleavage ~8-10–fold (133), initial studies focused on εdC and εdG.

Etheno adducts are generated in the cell by two major pathways. First, they are formed by exposure of DNA to *trans-4*,5-epoxy-2(*E*)-decenal, which is a byproduct of endogenous lipid peroxidation (179). Second, they are formed by environmental exposure to a known carcinogen, vinyl chloride, and related compounds such as urethane (224). As seen in Figure 10, εdC adducts were strong position-specific poisons of topoisomerase IIα. When located between the scissile bonds at the +2 and +3 positions, respectively, these adducts stimulated DNA cleavage mediated by human topoisomerase IIα ~6– and

12–fold. Although the +2 etheno adduct did not have as pronounced an effect on DNA cleavage as did an apyrimidinic site at the +2 position, cleavage stimulation by the +3 εdC was greater (12–fold). Moreover, as reported for AP sites, εdC adducts that were located immediately outside of the scissile bonds (at the -3 or +6 positions) inhibited enzyme-mediated DNA scission (Figure 10).

Sugar-ring modifications are modest topoisomerase II poisons (132,216,228). For example, inclusion of ribonucleotides between the scissile bonds stimulates DNA cleavage up to ~2–fold (see inset, Figure 10). To determine whether the effects of base- and sugar-ring modifications on topoisomerase II are synergistic, the εdC adducts at the +2 and +3 positions were replaced with εrC. Little additional cleavage enhancement was observed at the +2 position, and a modest increase was seen at the +3 position. Therefore, the effects of the two modifications, while potentially additive, do not appear to be synergistic.

The effects of εdG on DNA cleavage mediated by human topoisomerase IIα also were examined (Figure 11). εdG was a moderate to strong topoisomerase II poison and stimulated DNA cleavage ~6– and 5–fold when substituted at the +2 and +3 positions, respectively. Similar to results with εdC, the presence of an εdG adduct at the +2 position had a smaller effect on DNA cleavage than the corresponding AP (apurinic) site, but had a larger effect than the AP site when present at the +3 position. Once again, the presence of an adduct located immediately outside of the scissile bonds (at the -3 position inhibited DNA scission by the enzyme (Figure 11).

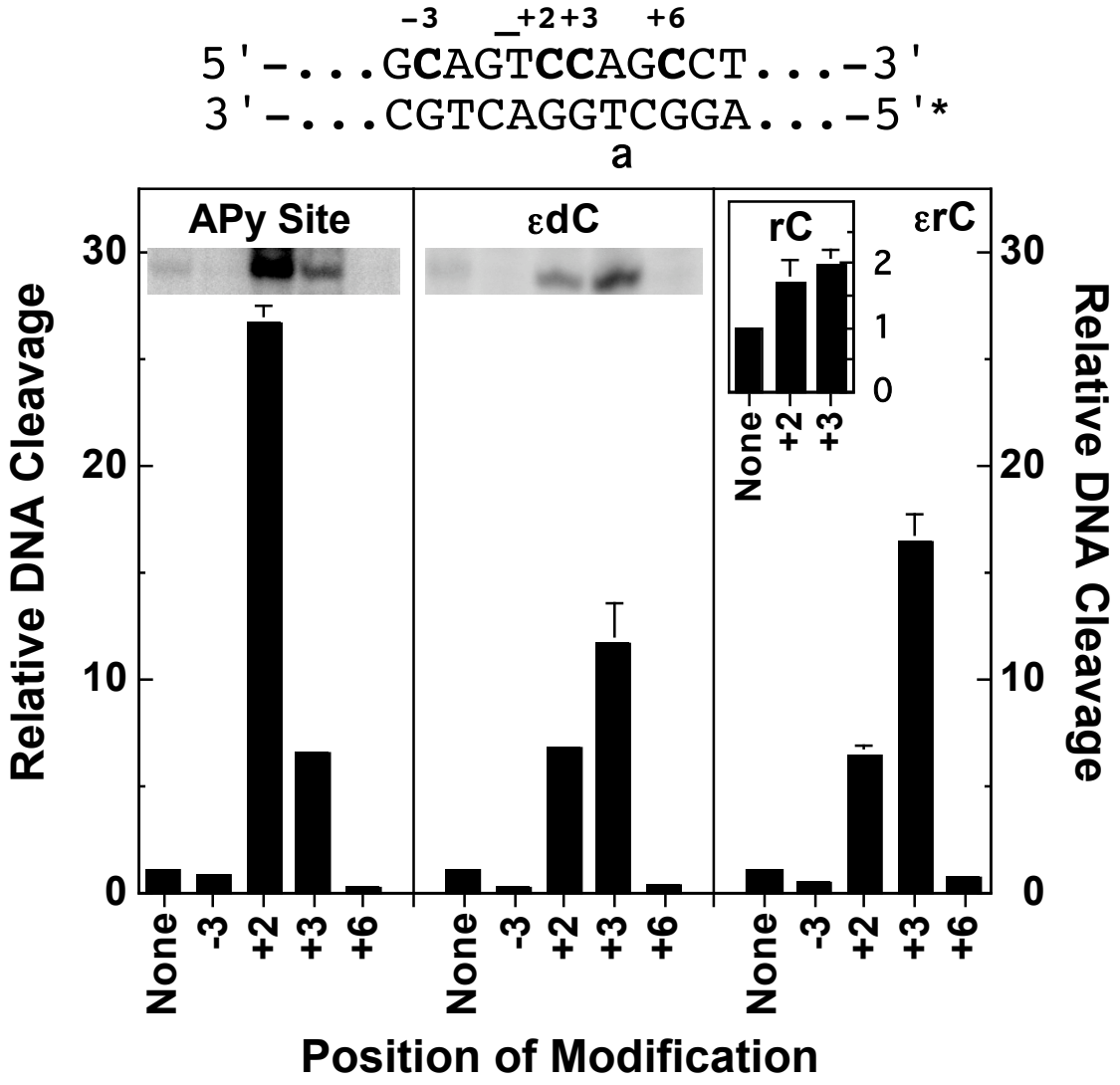


Figure 10. εdC and εrC adducts are position-specific topoisomerase IIα poisons. The central sequence of the 42-mer oligonucleotide substrate used to monitor cleavage of the bottom strand is shown. Bold-face indicates the modified positions. The asterisk denotes the position of the 5'-radiolabel and the arrows indicate the points of topoisomerase II-mediated DNA cleavage. The effects of εdC or εrC adducts, as well as the corresponding apyrimidinic (APy) sites on DNA cleavage mediated by human topoisomerase IIα are shown. The positions of adducts relative to the scissile bonds are given. The scissile bonds are between the –1 and +1 bases. The effects of ribonucleotides (rC) are shown for comparison in the inset. Relative DNA cleavage was calculated by normalizing levels of scission of the unmodified oligonucleotide (None) to 1. Error bars represent the standard deviations of three independent experiments. Representative autoradiograms showing the DNA cleavage product generated in the presence APy and εdC lesions are included above the corresponding bar.

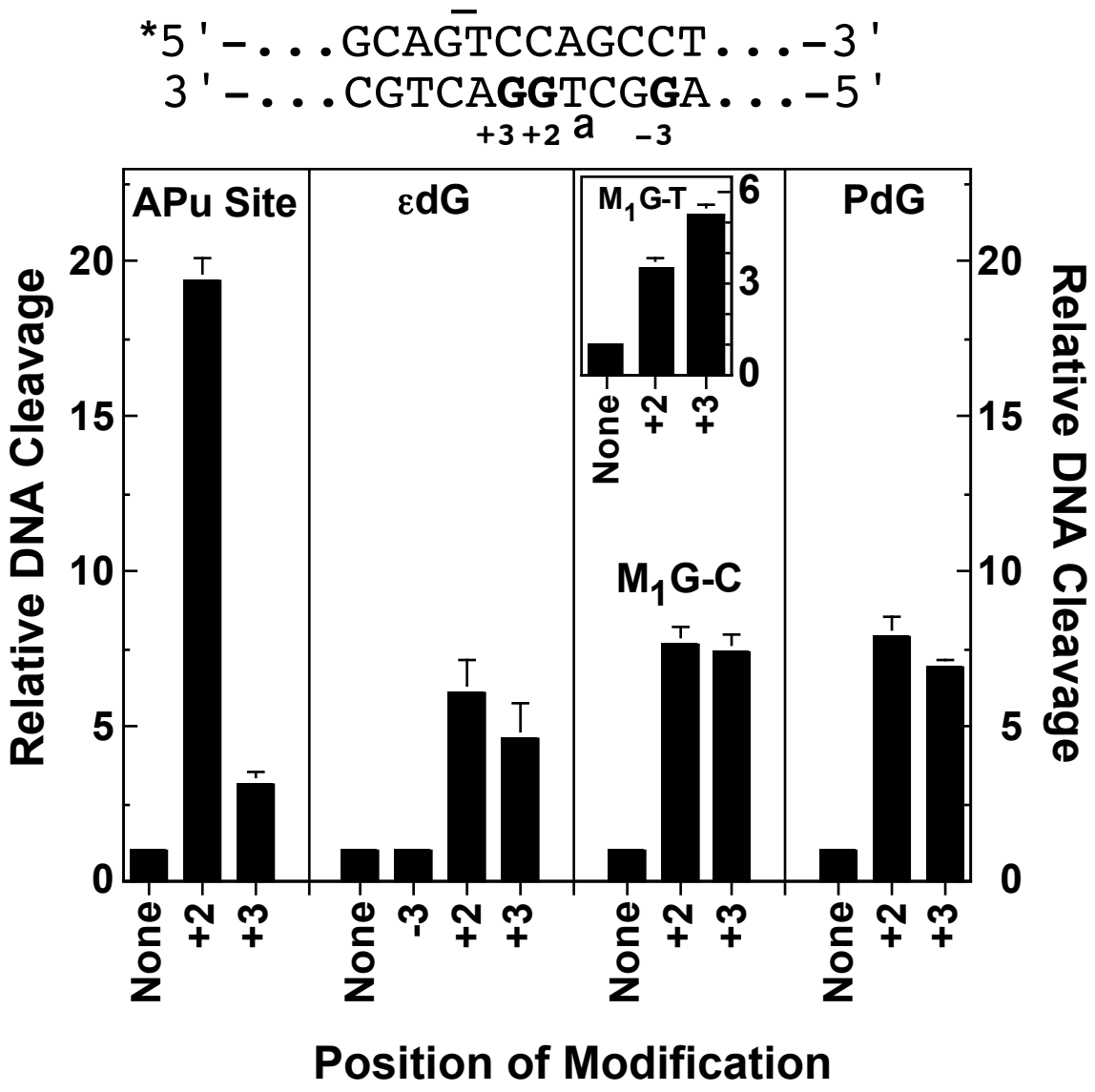


Figure 11. εdG, M_1dG, and PdG are position-specific human topoisomerase IIα poisons. The central sequence of the 42-mer oligonucleotide substrate used to monitor cleavage of the top strand is shown as in Figure 2. Bold-face indicates the modified positions. The effects of εdG, M_1dG, or PdG adducts, as well as the corresponding apurinic site (APu) on DNA cleavage mediated by topoisomerase IIα are shown. Results for M_1dG opposite to thymine are shown for comparison in the inset. Relative DNA cleavage was calculated by normalizing levels of scission of the unmodified oligonucleotide (None) to 1. Error bars represent the standard deviations of three independent experiments.

Since the etheno adducts all stimulated DNA scission mediated by human topoisomerase IIα, the effects of the structurally related exocyclic lesion, M_1dG (see Figure 11), on DNA cleavage were examined. M_1dG is a naturally occurring exocyclic adduct detected in human DNA (225,229-231). It is formed by reaction of DNA with malondialdehyde, a byproduct of thromboxane biosynthesis and a product of oxidative degradation of polyunsaturated lipids, and other base propenals (146).

M_1dG stimulated DNA cleavage ~7– to 8–fold, which was slightly higher than values observed for εdG (Figure 11). There is a caveat to these results, however. In some sequences, when M_1dG is located directly across from a cytosine, the exocyclic ring opens (see Figure 9) (140,232). In contrast to M_1dG, the ring-opened form (*i.e.*, N^2-(3-oxo-1-propenyl)-deoxyguanosine) induces minimal distortion into the DNA backbone (140). To further explore this issue, the effects of M_1dG on topoisomerase II-mediated DNA cleavage were examined when the bases across from these adducts were changed to thymine residues (Figure 11, inset). M_1dG has been observed in a ring-closed structure when situated across from a thymine (232). Results were similar to those obtained with cytosine-containing sequences. Two possible conclusions can be drawn from these findings. Either M_1dG is a strong topoisomerase II poison in both the ring-opened and ring-closed forms, or the adduct remains in the ring-closed form in both of the oligonucleotide substrates.

Due to the uncertainty regarding the exocyclic structure of M_1dG, the effects of PdG on topoisomerase II-mediated DNA cleavage were characterized. Although PdG is not a naturally occurring DNA adduct, it has been used extensively as a stable model for the ring-closed form of M_1dG (136,226,227). It is structurally similar to M_1dG, except

that the exocyclic ring is aliphatic rather than aromatic (see Figure 9). In addition, the exocyclic ring of PdG never opens (136,226,227). Results for PdG are shown in Figure 11. DNA cleavage enhancement was identical to that observed for M_1dG. On the basis of these data, we suggest that M_1dG exists in a closed-ring form in the DNA sequence used for the present study. Together with the results of the etheno base adducts, these findings indicate that a variety of exocyclic base adducts are position-specific poisons of human topoisomerase IIα.

Effects of Exocyclic Base Adducts on DNA Religation Mediated by Human Topoisomerase IIα.

Topoisomerase II poisons can increase levels of enzyme-DNA cleavage complexes by opposite mechanisms that are not mutually exclusive. Some anticancer drugs, such as etoposide, strongly inhibit the ability of topoisomerase II to ligate cleaved DNA molecules (30-32,113). In contrast, other drugs such as the quinolone CP-115,953 have little effect on rates of ligation and presumably raise the concentration of cleavage complexes by increasing the overall rate of DNA cleavage (30-32,114). This latter rate reflects both the binding constant of topoisomerase II for its nucleic acid substrate (*i.e.*, the formation of the noncovalent enzyme-DNA complex) as well as the forward rate of the chemical DNA scission event.

Previous studies indicate that a variety of DNA lesions act on topoisomerase II by the latter mechanism (123-126,132,133,233). To determine if this was the case for the exocyclic base adducts used in the present work, the effects of εdC, εrC, εdG, M_1dG, and PdG on the ability of human

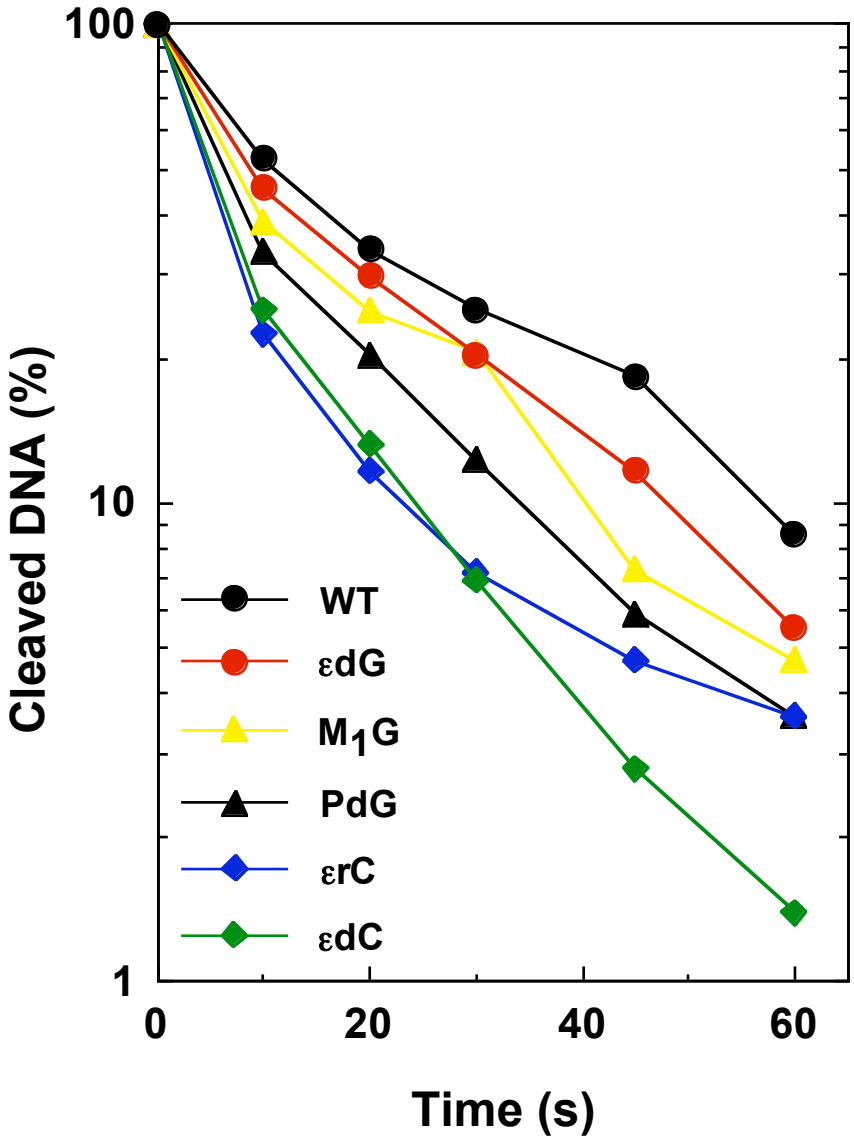


Figure 12. Alkylated DNA bases do not inhibit DNA religation mediated by human topoisomerase IIα. The effects of DNA adducts at the +3 position of the top strand on DNA religation mediated by topoisomerase IIα are shown. Oligonucleotide substrates are as in Figures 2 and 3. DNA religation was monitored on the strand opposite the lesions. The amount of DNA cleavage observed at equilibrium for each substrate was set to 100% at time zero. DNA religation was quantified by the loss of cleaved molecules. Data represent the average of three independent experiments.

Table 1. Apparent first-order rates of religation of damage-containing oligonucleotides by human topoisomerase IIα[a].

DNA Lesion	Rate (s^{-1})
Wild-type	0.055
εdG	0.077
M_1dG	0.099
PdG	0.115
εdC	0.125
εrC	0.138

[a]DNA lesions were located at the +3 position as shown in Figures 10 and 11.

topoisomerase IIα to religate DNA was determined. Results for lesions located at the +3 position are shown in Figure 12 and Table 1. The apparent first order rate of religation for the unmodified sequence was 0.055 s^{-1}. In all cases, religation rates were higher for oligonucleotide substrates that contained adducts. Similar results were obtained when adducts were located at the +2 position (not shown). The fastest rate obtained (0.138 s^{-1} for εrC) was ~2.5–fold higher than seen with the parental unmodified substrate. These results support previous findings and indicate that exocyclic base adducts do not increase levels of topoisomerase II-DNA cleavage complexes by inhibiting enzyme-mediated DNA ligation.

Effects of Exocyclic Base Adducts on Topoisomerase IIα-DNA Binding

The above results suggest that exocyclic base adducts raise levels of enzyme-DNA cleavage complexes by increasing the overall rate of cleavage complex formation (which reflects both the enzyme–DNA binding constant and the actual rate of DNA scission). At the present time, it is not possible to determine directly the rate of the DNA scission event. Therefore, in order to further clarify the mechanistic basis for the increased cleavage of damaged DNA, the effects of lesions on the ability of human topoisomerase IIα to bind its nucleic acid substrate were determined.

A competitive nitrocellulose DNA filter binding assay was employed. The human enzyme was incubated with a mixture of radiolabeled unmodified oligonucleotide and a non-labeled competitor oligonucleotide that contained no damage, an AP site, or εdC, εdG, M_1dG, PdG, or rC at the +2 position. Selected competition curves are shown in Figure 13, and IC_{50} values for all of the oligonucleotides are given in Table 2. IC_{50}

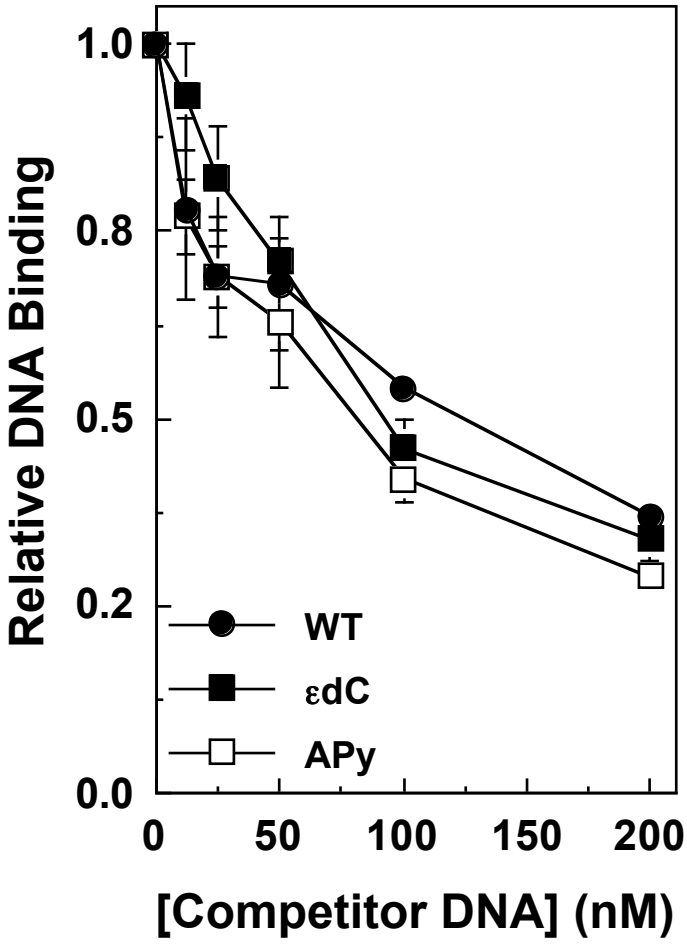


Figure 13. The presence of exocyclic DNA adducts does not affect the binding affinity of human topoisomerase IIα for DNA. Results from a competitive nitrocellulose filter-binding assay are shown. Oligonucleotide substrates are as in Figures 10 and 11. [^{32}P]-labeled unmodified oligonucleotide (50 nM) was incubated with topoisomerase IIα and increasing concentrations of unmodified (WT) oligonucleotide or substrates containing an apyrimidinic site (APy) or an εdC adduct at the +2 position of the top strand. Error bars represent the standard deviations of three independent experiments.

Table 2. Topoisomerase IIα-DNA nitrocellulose filter-binding competition assays [a].

DNA Lesion	IC_{50} (nM) ± SD
Wild type	115 ± 13
Apy	78 ± 9
Apu	104 ± 13
εdC	100 ± 7
εdG	96 ± 4
M_1dG	103 ± 15
PdG	114 ± 16
rC	92 ± 13

[a]DNA lesions were located at the +2 position as shown in Figures 10 and 11. IC_{50} values represent the concentration of damage-containing oligonucleotides required to decrease the binding of radio-labeled unmodified oligonucleotide (50 nM) to human topoisomerase IIα (220 nM) by 50%. Values are the averages of three independent experiments with their respective standard deviations.

values for the undamaged and damaged substrates were similar and ranged from 78–115 nM. These results indicate that the increased levels of topoisomerase II-mediated DNA cleavage observed with substrates that contain exocyclic base adducts (at least at the +2 position) are not due to an increased binding affinity of the human enzyme for damaged DNA. Together with the present and previous (123-126,132,133,233) religation data, these findings suggest that the forward rate of the DNA scission event catalyzed by topoisomerase IIα is accelerated when damaged bases are located between the scissile bonds.

Topoisomerase IIα-mediated DNA Cleavage Enhancement Correlates With DNA Bending of Lesion-containing Oligonucleotides

Why exogenous poisons enhance the DNA cleavage activity of topoisomerase II has been a long-standing question. Osheroff and Kingma proposed nearly a decade ago the positional poison model (Figure 6) attempting to explain why exogenous and endogenous poisons of topoisomerase II alter the DNA cleavage activity of this enzyme (125). This model suggests a correlation between topoisomerase II-mediated DNA cleavage enhancement and the distortion induced by the specific DNA lesions within the cleavage site.

An NMR structure of an apurinic site within a topoisomerase IIα cleavage site sequence context revealed, among other features, a kink in the DNA duplex (134). In an attempt to further define the structural features that govern enzyme-mediated DNA cleavage enhancement, a fluorescence resonance energy transfer (FRET) system was designed to examine a potential correlation between this catalytic activity and DNA bending of lesion-containing duplexes.

The system consisted of a doubly-labeled wild-type oligonucleotide and a complementary strand containing a DNA lesion (Figure 14). The top strand is labeled on its 5'-end with fluorescein (fluorescence donor) and on its 3'-end with cyanine 3 (fluorescence acceptor).

DNA bending can be estimated by measuring the FRET efficiency from fluorescein to cyanine 3. The sample is irradiated at the fluorescein absorbance peak (494 nm wavelength). Fluorescein will absorb the photons and emit photons with lower energy (526 nm). This latter emission is absorbed by the fluorescence acceptor (cyanine 3) and released at an even lower energy (563 nm). Thus, by irradiating the sample at 494 nm, and measuring the fluorescence emission of cyanine 3 at 563 nm we can calculate the energy transfer efficiency, and therefore the distance between the fluorophores. As described on Figure 14, a straight DNA molecule would display a lower FRET efficiency than a bent molecule, since the fluorophores would be farther away in the former (234).

Two model lesions were chosen for this study: an AP site and a 2'-methoxyribo cytosine lesion, in addition to the wild-type sequence. AP sites are strong topoisomerase II poisons *in vitro* (123-126,133,201). On the other hand, small modifications on the DNA such as sugar-ring modifications have been shown to have a marginal effect in topoisomerase II-mediated DNA cleavage (Figure 10) (132,201).

As seen on Figure 14 the sugar-ring modification had a marginal effect in enzyme-mediated DNA cleavage enhancement, when compared to that of an AP site. This pattern of topoisomerase IIα -mediated DNA cleavage is paralleled by the efficiency of energy transfer from the donor-acceptor pair in our system (Figure 15).

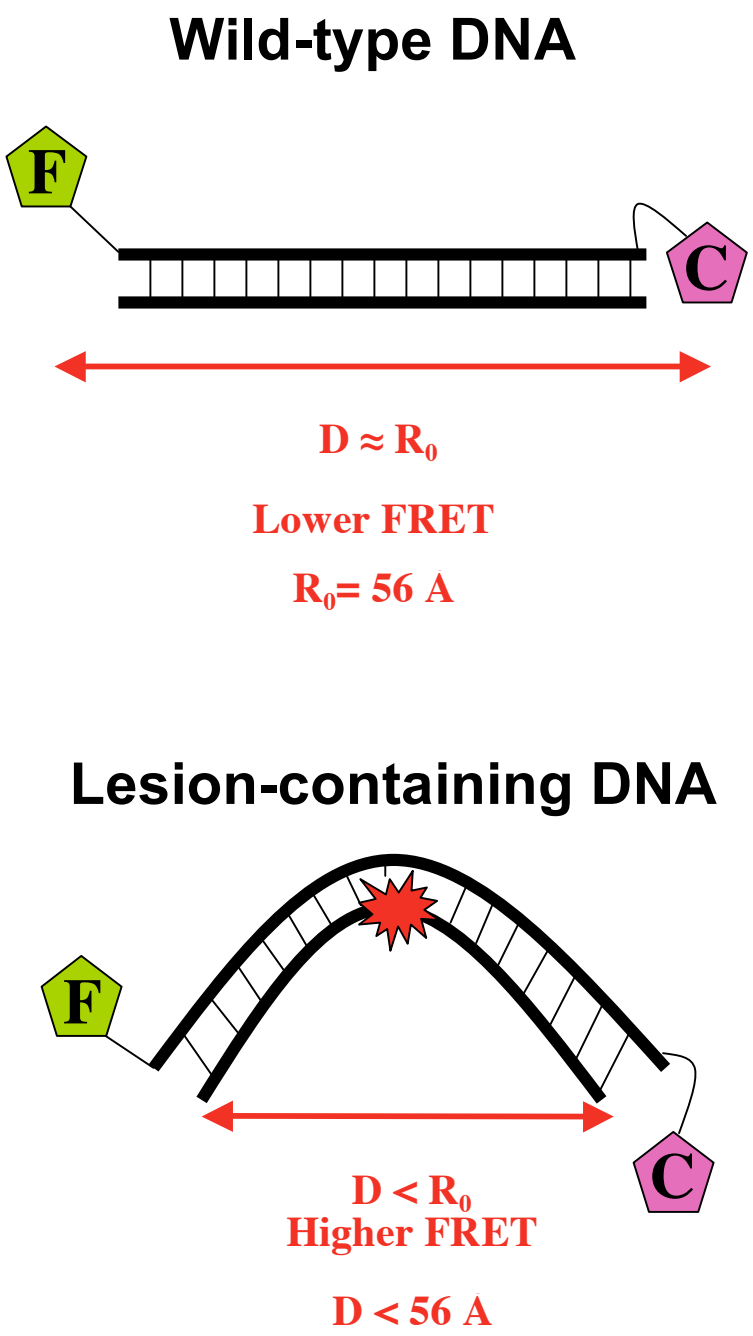


Figure 14. FRET system to measure DNA bending of adducted DNA oligonuclotides. A double-stranded 16-mer oligonucleotide was designes. The top strand was doubly-labeled with two fluorophores (F, Fluorescein, and C, Cyanine 3). Flourescein is the energy donor and cyanine 3 is the energy acceptor. The sample is irradiated at 494 nm (exitation maxima of Fluorescein), and the fluorescence emission of cyanine 3 was detected. Since cyanine 3 is not excited at 494 nm, only the fluorescence transfer from fluorescein will be detected.

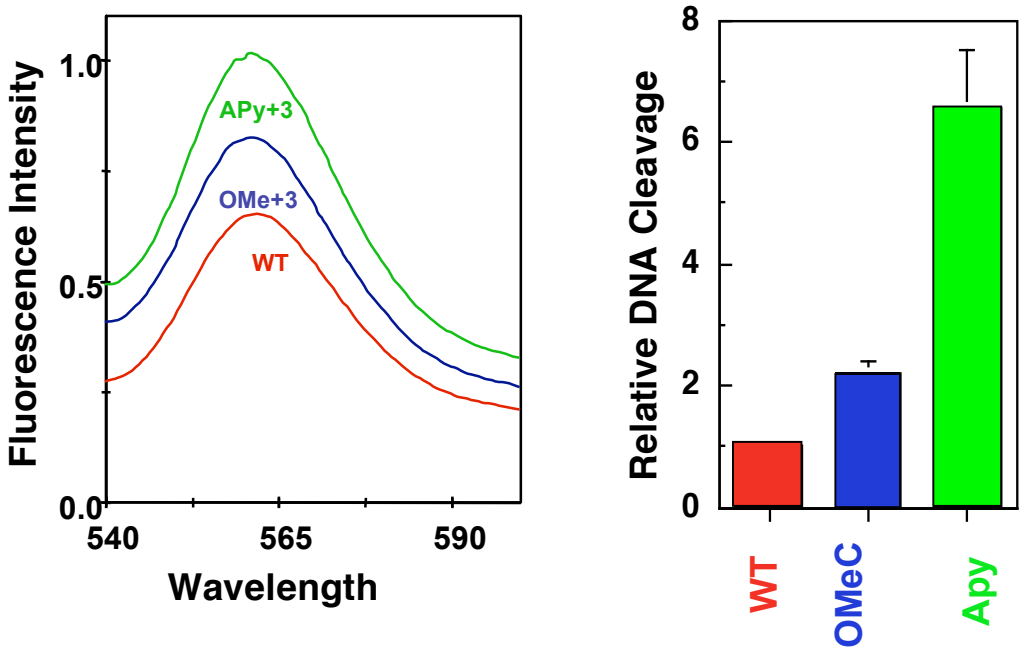


Figure 15. FRET analysis shows a correlation between DNA bending of adducted DNA oligonucleotides and topoisomerase II-mediated DNA cleavage enhancement. Left: Fluorescence emission of cyanine 3 as a result of energy transfer was monitored for the wild-type (WT), 2'-methoxyC (OMeC), and apyrimidinic site (APy). Right: topoisomerase IIα-mediated DNA cleavage enhancement for the same oligonucleotides.

Enhancement of DNA Cleavage Mediated by Topoisomerase IIα in Cultured Human Cells Treated with CAA

The cellular consequences of any given DNA lesion are determined by the proteins that first interact with it (207). For example, if a glycosylase encounters an alkylated base, it is likely that the DNA damage will be converted into an AP site and channeled into the BER pathway (209). However, if a DNA polymerase attempts to traverse that same lesion, the damage may trigger recombination pathways or be fixed in the genome as a permanent mutation (161,167,208,219,222).

Previous studies indicate that some DNA lesions increase levels of topoisomerase I-DNA cleavage complexes in cultured cells (214,217). This finding implies that if topoisomerase I encounters a DNA adduct, the original damage can lead to the generation of protein-linked single-stranded DNA breaks.

Thus, the actions of topoisomerases have the potential to dramatically alter the genotoxicity of specific forms of DNA damage.

All of the *in vitro* data suggest that type II topoisomerases, like their type I counterparts, should interact with DNA lesions in the cell. However, physiological encounters between topoisomerase II and DNA damage have yet to be demonstrated. Therefore, the effects of CAA on levels of DNA cleavage mediated by topoisomerase IIα were determined in cultured human cells. CAA is one of the reactive products of vinyl chloride metabolism and induces etheno-base adducts, such as εdG, εdC, and εdA, in treated cells (163,164,235).

As determined using the ICE bioassay (Figure 16), treatment of human CEM leukemia cells with 125 or 250 μM CAA increased levels of topoisomerase IIα-mediated DNA cleavage ~3.3– or 3.9–fold, respectively. Although substantial, this increase is less

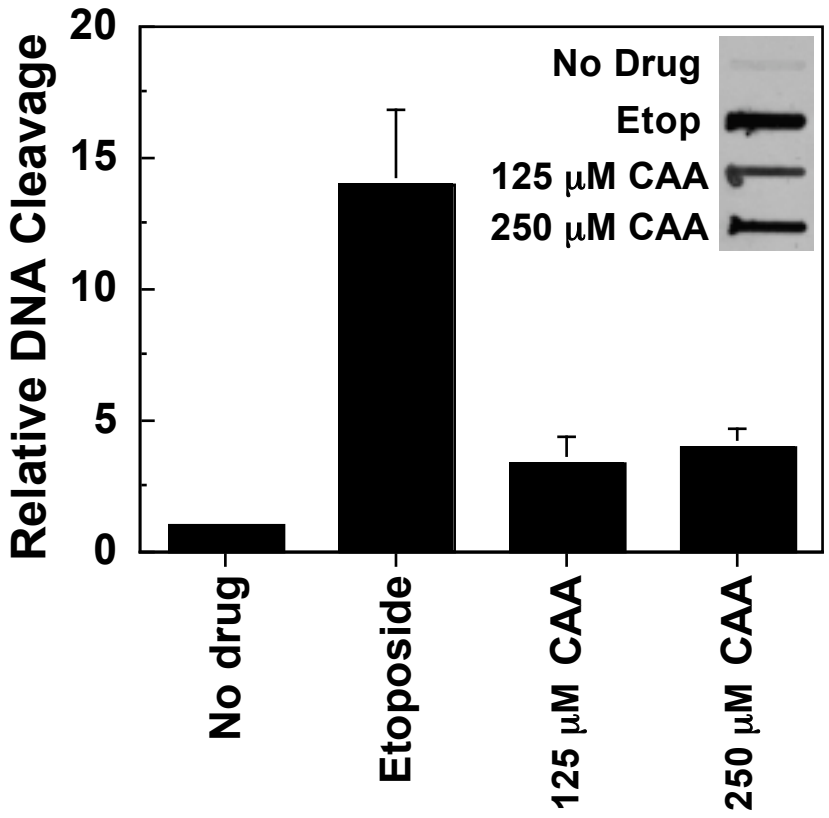


Figure 16. CAA induces topoisomerase IIα-mediated DNA cleavage in cultured human CEM cells. The ICE bioassay was used to monitor levels of enzyme-DNA cleavage complexes. Cells were exposed to 50 μM etoposide (Etop) for 1h, or to 125 or 250 μM CAA (CAA) for 4h. Cells were lysed and pelleted through a CsCl cushion to separate topoisomerase IIα-DNA cleavage complexes from free enzyme. Pellets were slot-blotted and probed for human topoisomerase IIα. A typical slot-blot is shown in the inset. Relative DNA cleavage was calculated by normalizing levels of scission in untreated cultures (No Drug) to 1. Error bars represent the standard deviations of four independent experiments.

than observed following treatment of cells with 50 μM etoposide. However, it should be noted that etoposide increases levels of topoisomerase II-linked DNA breaks through a direct interaction with the enzyme. In contrast, CAA acts through the generation of DNA lesions. While the cellular concentration of base adducts induced by the DNA damaging agent is not known, it is highly likely that it is significantly lower than the initial concentration (125 or 250 μM) of CAA used to treat cells.

Increases in DNA scission mediated by human topoisomerase IIα also were observed when MDA-MB-231 or MCF-7 breast cancer cells were treated with CAA (not shown). Levels of cleavage rose ~1.8– and 2.5-fold, or ~2.3– and 4.8–fold when these two cell lines were treated with 125 or 250 μM CAA, respectively.

Since CAA is a bifunctional alkylating agent, it is possible that the increase in covalent topoisomerase IIα-DNA complexes seen in Figure 16 actually represents enzyme-DNA crosslinks rather than enzyme-mediated scission. However, we do not believe that this is the case. The concentrations of CAA used for these studies, 125 and 250 μM, are ~3 orders of magnitude lower than that normally used to induce protein-DNA crosslinks with more reactive agents such as formaldehyde. Moreover, a previous study demonstrated that treatment of human Burkitt's lymphoma cells with 750 μM CAA did not significantly increase levels of protein-DNA crosslinks (236).

To further address this issue, three control experiments were carried out (not shown). First, no protein-DNA crosslinks were observed when topoisomerase IIα was incubated with plasmid molecules and 250 μM CAA. Second, no p53-DNA crosslinks were observed in CEM cells following exposure to 250 μM CAA. Finally, treatment of cultured human cells with methyl methanesulfonate, a monofunctional alkylating agent

that cannot generate protein-DNA crosslinks, increased levels of covalent topoisomerase IIα-DNA complexes (unpublished results).

Taken together, the data presented above provide strong evidence that topoisomerase IIα interacts with DNA damage in cultured human cells and that this interaction increases levels of DNA cleavage mediated by the enzyme.

Discussion

The presence of base adducts in the genetic material can lead to mutations because replicative and repair DNA polymerases often insert wrong nucleotides opposite of adducted bases (206,218,219). In addition, lesions can lead to chromosomal aberrations such as insertions, deletions, rearrangements, and translocations (219-221). Many of these latter effects are attributed to recombination pathways that are triggered when lesions stall DNA replication forks (167,208,222). However, similar chromosomal aberrations are observed following treatment of cells with topoisomerase II poisons (32,101,102,223).

Topoisomerase II is an essential enzyme that removes knots and tangles from the genetic material (30,237). However, because the enzyme generates a protein-linked double-stranded DNA break as a requisite step in its catalytic cycle, topoisomerase II also has the potential to fragment the genome every time it functions (30,40,41,237,238). As a result of this latter property of topoisomerase II, this enzyme plays a central role in cancer. A number of drugs that act as topoisomerase II poisons are used as front-line chemotherapeutic agents for the treatment of human malignancies. Conversely, evidence indicates that under some circumstances, exposure to topoisomerase II poisons (drugs,

natural products, or environmental pollutants) can lead to the generation of specific leukemias.

In addition to chemicals that increase levels of topoisomerase II-mediated DNA cleavage, several DNA lesions have been found to poison the type II enzyme *in vitro* (123-126,132). For example, when located within the 4-base stagger that separates the scissile bonds of a topoisomerase II DNA cleavage site, AP sites increase enzyme-mediated scission as much as 10– to 20–fold (123-126,133). Previous studies from this laboratory suggest that the ability to distort the double helix contributes to the potential of a lesion to act as a topoisomerase II poison (125,133). Therefore, to further characterize the range of DNA lesions that alter enzyme function, the effects of bulky exocyclic adducts on the DNA cleavage activity of human topoisomerase IIα were assessed.

εdC, εdG, M_1dG, and PdG all were moderate to strong position-specific topoisomerase IIα poisons. When located in the +2 or +3 positions between the scissile bonds they increased levels of DNA cleavage ~5– to 17–fold. Enhanced cleavage did not appear to result from an increased affinity of topoisomerase IIα for the adducted DNA or a decreased rate of enzyme-mediated religation. Therefore, it is concluded that these lesions acted as topoisomerase II poisons by accelerating the forward rate of enzyme-mediated DNA scission. This conclusion is consistent with prior studies on DNA lesions (123-126,132,133) and evidence that suggests that the recognition of DNA cleavage sites by topoisomerase II is governed by chemical steps rather than by the enzyme-DNA binding step (113,127,239).

The mechanistic basis for the enhancement of topoisomerase II-mediated DNA scission by DNA adducts is not understood. However, it is believed that they act by

distorting the double helix (32,114,125,133). When this distortion is located between the two scissile bonds of a topoisomerase II DNA cleavage site, it facilitates interactions within the active site of the enzyme and accelerates rates of scission. However, when it is located immediately outside of the scissile bonds, the DNA distortion presumably alters the alignment of the cleavage sequence within the active site and decreases scission. It is notable that εdC, εdG, M_1dG, and PdG introduce kinks into DNA (139,140,227,232). All of these adducts project their exocyclic ring into the major groove of the double helix and disrupt base pairing (139,140,227,232). Beyond these general features, it is difficult to compare the precise alterations in DNA structure that are induced by εdC, εdG, M_1dG, and PdG, as the structural data for each was generated within difference DNA sequence contexts.

It has been known for nearly a decade that some DNA lesions (primarily AP sites) could increase topoisomerase II-mediated DNA cleavage *in vitro* (123-126,132,133). However, it had never been demonstrated that lesions act as topoisomerase II poisons in living systems. Results of the present study indicate that levels of DNA cleavage mediated by topoisomerase IIα rise substantially when cultured human cells are exposed to CAA, a chemical reagent that induces the formation of etheno base adducts *in vivo* (163-165). On the basis of this finding, we believe that DNA lesions are capable of acting as physiological poisons of topoisomerase II.

The first step of BER, the pathway that repairs etheno adducts, is the conversion of alkylated bases to AP sites by a DNA glycosylase (240,241). Since AP sites are strong topoisomerase II poisons, it is not known whether increased DNA cleavage by topoisomerase IIα ultimately results from an interaction with the etheno adducts or rather

with the resulting AP sites, or a combination of both. It has been proposed that proteins in the BER pathway act in a cooperative fashion, with each handing the processed repair intermediate to the subsequent protein in the pathway (188). Although a variety of BER intermediates are topoisomerase II poisons *in vitro* (200), it has yet to be determined whether the enzyme can successfully compete with APE1 or DNA polymerase β for access to AP sites generated by this repair pathway *in vivo*. This important issue remains the subject of future investigation.

Results of the present study indicate that DNA lesions have the potential to act as topoisomerase II poisons in cultured human cells. The physiological ramifications of this finding are not known. Formation of a topoisomerase II-DNA cleavage complex proximal to the site of a DNA adduct provides no obvious advantage for the repair of that adduct. If anything, generation of a protein-associated double-stranded break adjacent to a DNA lesion would diminish the chances of successful repair and greatly increase the likelihood of mutagenesis or chromosomal rearrangements. In this regard, topoisomerase II-mediated DNA cleavage has been implicated in initiating specific types of leukemia (242-244). These leukemias generally display chromosomal translocations with breakpoints that originate within the *MLL* gene at chromosomal band 11q23 (117,242-247). They are observed in cancer patients treated with drug regimens that include topoisomerase II poisons (118,119,245-247). They also are observed in infant leukemias and are associated with the ingestion of naturally occurring topoisomerase II poisons (117,242-247). Thus, interactions of topoisomerase II with DNA lesions may be deleterious in nature and may simply be the price that the cell has to pay to have an enzyme that can pass one DNA double helix through another.

Conversely, if the genetic material of a cell sustains sufficiently high levels of damage, the resulting increase in topoisomerase II-mediated DNA cleavage may help trigger apoptotic pathways (32,248,249) that remove the damaged cell from the population. In this case, interactions of topoisomerase II with DNA lesions could play a positive role in the survival of an organism. Whether the cellular recognition of DNA damage by topoisomerase II ultimately is demonstrated to have a negative or a positive physiological effect (or both), it is clear that this interaction provides an additional layer of complexity that the cell has to cope with in order to maintain the integrity of its genome.

CHAPTER IV

TOPOISOMERASE IIα INTERACTS WITH BASE EXCISION REPAIR INTERMEDIATES AND GENERATES DOUBLE-STRANDED DNA BREAKS IN CULTURED HUMAN CELLS

Introduction

Human cells are under constant assault by a wide variety of environmental and endogenous chemicals. These agents produce a diverse array of damage to cellular macromolecules, including nucleic acids (145,148,149). Since DNA carries the genetic information, damage to this macromolecule is particularly dangerous. For example, chemicals that cause base modifications or loss can generate mutations due to misincorporation by replicative or repair DNA polymerases (250,251). Agents that cause DNA strand breaks or produce lesions that block essential nuclear functions trigger mutagenic recombination events or cell death pathways (252,253).

In order to cope with DNA damage, cells have evolved multiple repair systems with overlapping specificities (209,254). Small (*i.e.*, non-bulky) base alterations, including many alkylated and oxidized lesions, as well as AP sites, generally are repaired by the BER pathway (155,158,186). Two pathways are used in human cells: short-patch BER, in which only the damaged base is excised, or long-patch BER, in which 2-13 residues are excised. Short-patch BER is employed most often (175,177,182).

Short-patch BER is initiated by the recognition and subsequent excision of the damaged base by a specific DNA glycosylase (Figure 8) (154,209,255). This reaction converts the original lesion to an AP site, which ultimately is removed and replaced with

the appropriate base by the combined actions of APE1 and DNA pol β. Finally, the DNA strand is resealed by DNA ligase I or the ligase III/XRCC1 complex (175,177,182).

It is notable that short-patch BER is itself a bifurcated pathway. The fork that is chosen is determined by the glycosylase that removes the damaged base (184,256). If the glycosylase is monofunctional (*i.e.*, contains no associated lyase activity), cleavage by APE1 generates a 5'-dRP flap that is removed by pol β after it synthesizes the missing base (183). If the glycosylase is bifunctional (*i.e.*, contains associated lyase activity), the the glycosylase removes the base and cleaves 3' of the AP site generating a 3′-unsaturated aldehydic (α,β-4-hydroxy-2-pentenal) end (3′-dRP) that is repaired by APE1 (257). The one-nucleotide gap that is filled in by pol β. Alkylated bases are most often removed by simple DNA glycosylases and repaired by the pathway depicted on Figure 8 (155,177).

In addition to DNA glycosylases, many alkylated lesions are recognized by topoisomerase II (133,201). Previous studies indicate that AP sites and many exocyclic DNA lesions are topoisomerase II potent poisons *in vitro* (133,201). Furthermore, when cultured human cells are treated with the alkylating agent CAA, which generates a variety of etheno-adducts in mammalian systems, levels of DNA cleavage mediated by topoisomerase IIα increase ~4–fold (201). This latter finding suggests that the human enzyme is able to recognize exocyclic DNA adducts in a physiological setting. However, it is not yet known whether topoisomerase IIα interacts with other forms of DNA damage in the cell.

AP sites are the most abundant form of DNA damage *in vivo* (152,153). It is estimated that 10,000 of these lesions are formed by spontaneous hydrolysis per genome

per day in living systems ranging from *Escherichia coli* to humans (258,259). Far greater numbers are generated by the actions of DNA glycosylases during the first step of BER (154). AP sites and model BER intermediates (that contain processed AP sites) also are very strong topoisomerase II poisons *in vitro* (200). Taken together, these findings raise the possibility that topoisomerase IIα interacts with AP sites in human cells. To address this issue, AP sites were generated in MDA-MB-231 breast cancer cells by treatment with MMS or by imbalancing BER by the transient overexpression of MPG. Results indicate that AP sites, including those generated during BER, are cellular poisons of topoisomerase IIα. Moreover, the type II enzyme appears to mediate a portion of the clastogenic and cytotoxic effects induced by MMS.

Results

Treatment of human cells with the alkylating agent CAA increases levels of DNA cleavage mediated by topoisomerase IIα (Figure 16) (201). This finding suggests that the human enzyme recognizes exocyclic DNA lesions in a cellular system. However, the etheno-adducts generated by CAA can be converted to AP sites by BER, and AP sites are strong topoisomerase II poisons *in vitro* (123-125,133,172,201). Thus, it is possible that interactions between topoisomerase IIα and BER intermediates contribute to (or are the underlying cause of) the increase in enzyme-mediated DNA cleavage.

Although AP sites are the most prevalent form of DNA damage in the cell, the vast majority of these lesions (which are generated by BER) are believed to be associated with repair enzymes that protect them from other proteins (188,194). As a result, it is not clear whether topoisomerase II is able to interact with AP sites in chromosomal DNA.

Two different approaches were employed to address this issue. In the first, human cells were treated with MMS. This alkylating agent creates methylated bases that are readily converted to AP sites by BER (162,174). In the second, AP sites were generated by overexpressing MPG, a glycosylase that initiates BER (168).

Treatment of Human Cells with MMS Generates AP Sites and Increases DNA Cleavage Mediated by Topoisomerase IIα

MMS is a monofunctional alkylating agent that induces the formation of several different methylated bases (168,174). The most common adducts produced are N^7-methylG (~89%), N^3-methylA (~9%), and O^6-methylG (<0.3%). The two major adducts generated by this alkylating agent are repaired by BER (155).

Levels of DNA cleavage mediated by topoisomerase IIα in cultured cells were determined using the ICE bioassay, which monitors the amount of covalent enzyme-DNA complexes that is formed during the scission reaction. (Figure 17, left panel, *inset*). Treatment of MDA-MB-231 cells with MMS (0–1 mM) increased levels of DNA cleavage by topoisomerase IIα >4–fold in a dose-dependent manner (Figure 17, left panel). This finding demonstrates that exposure to MMS generates topoisomerase II poisons in cultured human cells.

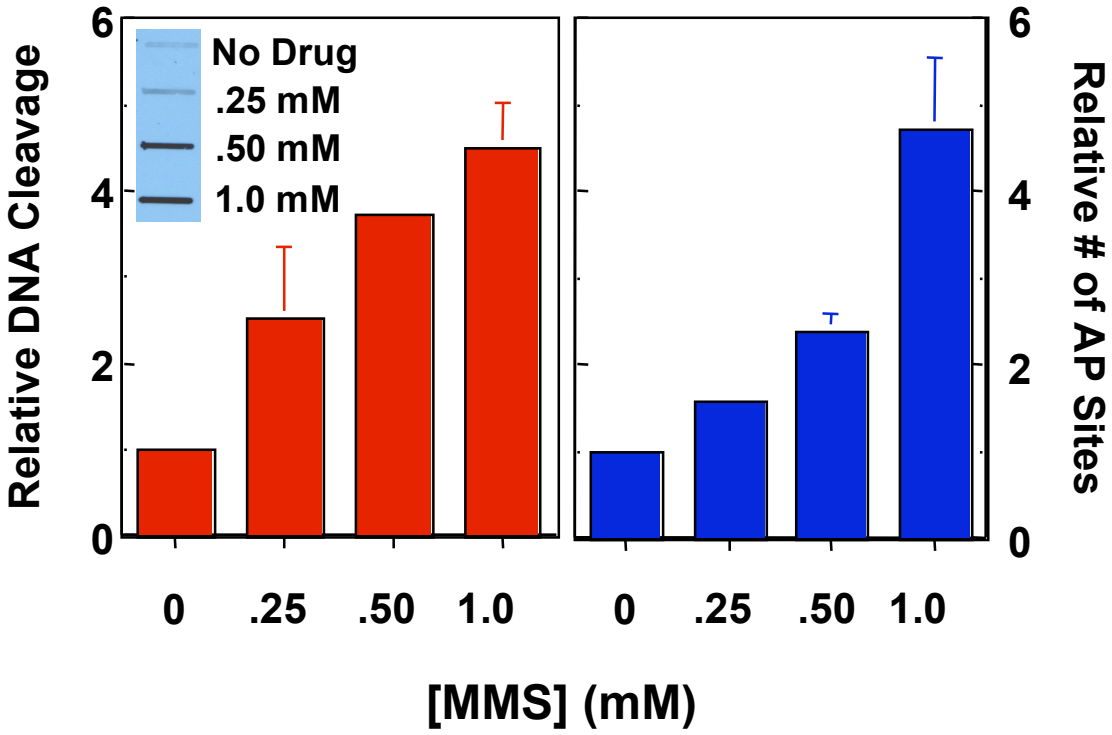


Figure 17. Treatment of cells with MMS increased the levels of topoisomerase IIα DNA cleavage complexes and the number of AP sies. MDA-MB-231 cells were treated with increasing concentrations of MMS for 4h and the amount of topoisomerase IIα cleavage complexes (*left*) and AP sites (*right*) were determined. The relative DNA cleavage was determined by using the ICE Bioassay and the relative number of AP sites was determined by using the aldehyde reactive probe. The *inset* in left panel is a representative blot from the ICE bioassay. Values represent the average of three independent experiments and error bars represent the standard deviation.

There are four possible explanations for this result. First, MMS itself may be a topoisomerase II poison. This is not the case, as direct incubation of topoisomerase IIα with the alkylating agent did not increase DNA cleavage (data not shown).

Second, under some conditions, topoisomerase IIα excises chromosomal loops as part of the apoptotic response. It is not known what triggers this enzyme-mediated DNA event, but it appears to occur late in apoptosis (260). To address this possibility, the proteolytic cleavage of poly(ADP-ribose) polymerase-1 (PARP-1) was monitored during MMS treatment. PARP-1 is cleaved at a specific site by caspase-3 as one of the earliest steps in apoptosis (261). Consequently, this event is a sensitive monitor of initiation of cell death pathways. Under the conditions and time frame (4 h) used for our experiments, no cleavage of PARP-1 was observed when MDA-MB-231 cells were treated with MMS concentrations as high as 2 mM (see Figure 22). Therefore, the increase in DNA cleavage by topoisomerase IIα does not appear to result from initiation of an apoptotic pathway.

Third, the increase in covalent topoisomerase IIα DNA cleavage complexes in MMS-treated cells may result from the generation of AP sites, which are strong topoisomerase II poisons (123-125,133,201). MMS incubation has been shown to induce AP sites in chromosomal DNA in a variety of systems, ranging from bacteria to humans (161,174,262,263). As seen in Figure 17 (right panel), under the conditions employed, MMS treatment of MDA-MB-231 cells increased levels of AP sites nearly 5–fold. This increase is similar to that observed for DNA cleavage by topoisomerase IIα. This finding suggests that the increase in cellular AP sites contributes to the stimulation of DNA scission by the type II enzyme in MMS-treated cells.

Fourth, topoisomerase IIα may recognize and cleave MMS-generated methylated base adducts prior to their conversion to AP sites by BER. To this point, previous studies have examined the effects of methylated bases on human topoisomerase IIα, including O^6-methylG, N^6-methylA, and 5-methylC, and all of them were poor topoisomerase II poisons (133). In most cases, DNA cleavage either decreased in the presence of lesions or increased less than 2–fold. Unfortunately, phosphoramidites are not available for the two major products of MMS-treatment, N^7-methylG or N^3-methylA. Therefore, to determine whether methylated bases generated by MMS stimulate topoisomerase II-mediated DNA cleavage, pBR322 was treated with this alkylating agent up to a concentration that was in 500-fold molar excess over that of the plasmid (Figure 18). The products of MMS treatment were marginal topoisomerase II poisons. Levels of DNA cleavage by human topoisomerase IIα rose no more than 1.2–fold at low MMS:pBR322 ratios (<20:1) and decreased below baseline levels at ratios in excess of 100:1. These data indicate that the methylated bases produced by MMS do not poison topoisomerase IIα.

Taken together, the above findings strongly suggest that the increase in DNA cleavage by topoisomerase IIα observed in MMS-treated MDA-MB-231 cells does not result from an interaction between the enzyme and MMS or from an interaction between the enzyme and the methylated bases. Rather, it is caused by an interaction between the enzyme and the AP sites that are generated during the repair of the methylated DNA.

Overexpression of MPG Increases DNA Cleavage Mediated by Topoisomerase IIα

As a second approach to determining physiological interactions between topoisomerase IIα and AP sites, MPG was overexpressed in MDA-MB-231 cells by

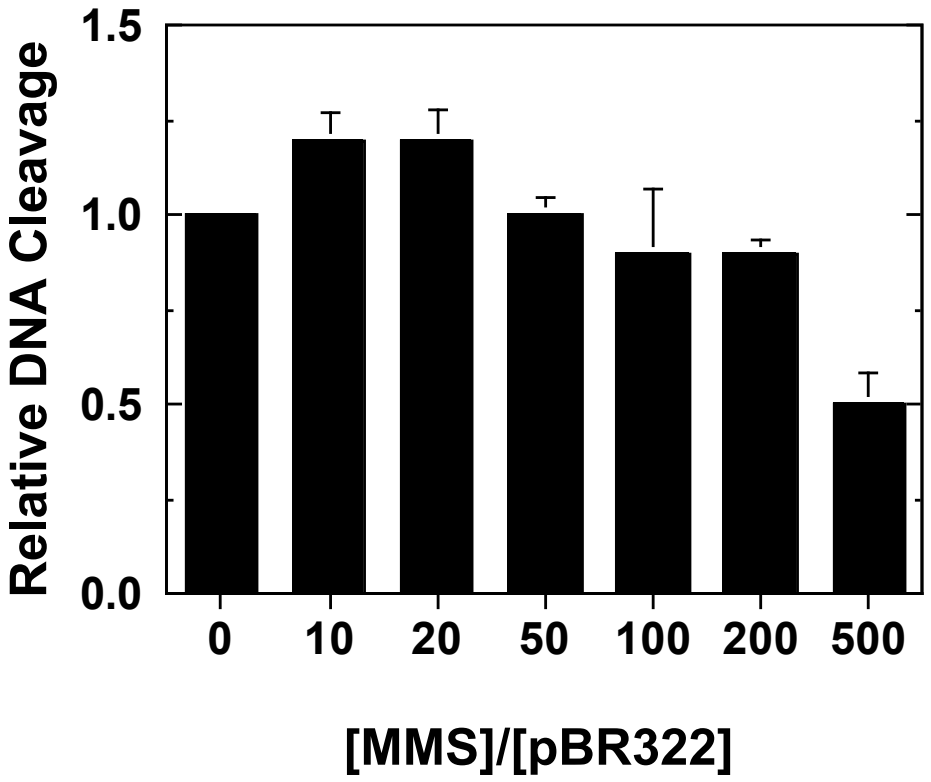


Figure 18. Methylated bases do not poison human topoisomerase IIα *in vitro*. pBR322 was treated with increasing concentrations of MMS for 1 h to generate methylated bases. MMS-treated DNA had a marginal effect in cleavage enhancement at lower concentrations of MMS and an inhibitory effect at higher concentrations.

transient transfection of an adenoviral vector that encoded the HA-tagged MPG gene under the cytomegalovirus early promoter (264,265). Human MPG and related glycosylases such as yeast Mag1 are the enzymes that remove N^7-methylG and N^3-methylA from DNA and initiate BER (168,266). They also remove unmodified purines from the genome. Overexpression of these glycosylases increases the mutagenicity, clastogenicity, and cytotoxicity, of cells following treatment with MMS or other methylating agents (263-265). In budding yeast the enhanced effects of these agents are abrogated by the coordinate overexpression of AP endonuclease (263,267,268). Consequently, it has been concluded that overexpression of MPG or Mag1 increases numbers of AP sites in the cell.

Overexpressing MPG to generate AP sites has two advantages over the initial MMS studies used to evaluate cellular interactions between these lesions and topoisomerase IIα. Since MPG removes unmodified purines from DNA, there is no need to generate a "parent" adduct that is repaired by BER. In addition, it ensures that all of the AP sites that result from overexpression are created by the BER pathway.

As seen in Figure 19 (*inset*), following transfection, levels of MPG were dramatically higher than seen in cells that were transfected with the empty vector. Furthermore, consistent with earlier studies on MPG, overexpression of this enzyme in MDA-MB-231 cells increased the cytotoxicity of MMS ~3– to 4–fold (Figure 19) (264). This demonstrates that the virally-expressed glycosylases is active. Finally, overexpression of MPG increased levels of DNA cleavage mediated by topoisomerase IIα nearly 4–fold, even in the absence of external DNA damaging agents (Figure 20). It should be noted that higher levels of DNA cleavage complexes were induced in the

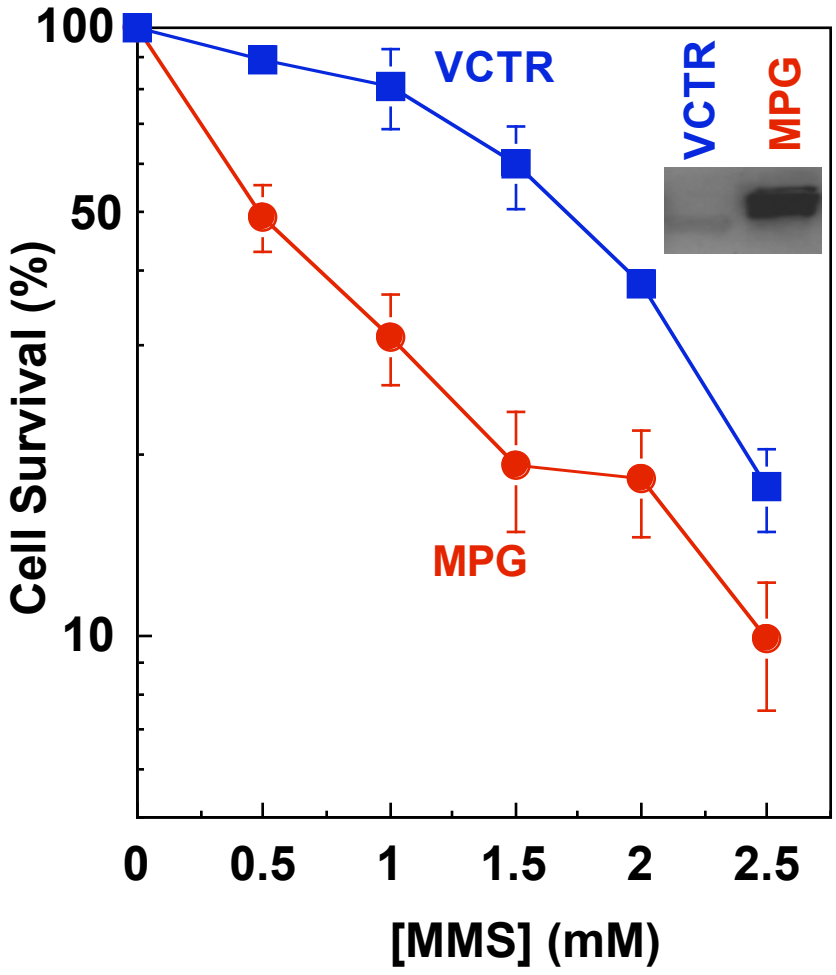


Figure 19. Cells overexpressing MPG are hypersensitive to MMS. MDA-MB-231 cells were infected with an adenoviral vector either empty (VCTR) or containing human MPG (MPG). Twenty-four hours after infection cells were treated with increasing concentrations of MMS for 1 h. Cell viability was assessed 36 h after exposure to MMS as described in the methods section. The values represent averages of three independent experiments and the error bars represent the standard deviations.

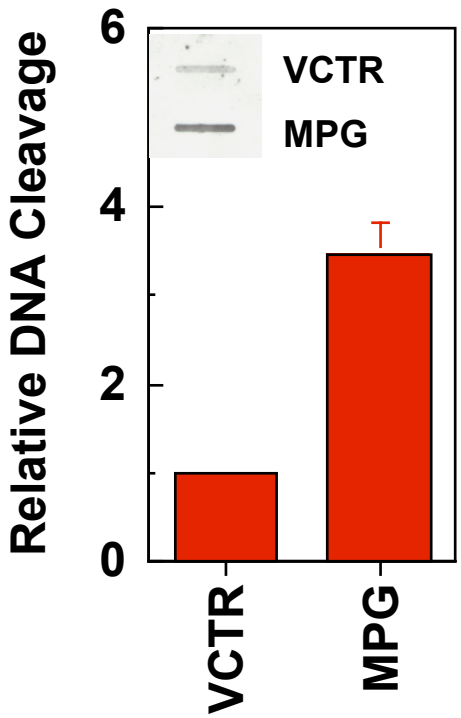


Figure 20. Overexpression of MPG increases the basal level of human topoisomerase IIα-DNA cleavage complexes in cultured cells. MDA-MB-321 cells were infected with an adenoviral vector that overexpresses MPG (MPG) or an empty vector (VCTR). Cells were subjected to the ICE Bioassay 24 h post-transfection. The inset is a representative blot. Values represent the averages of three independent experiments with their respective standard deviations.

presence of 100 μM MMS (data not shown). However, quantification was felt to be unreliable due to difficulties in obtaining DNA pellets from MMS-treated cells that overexpressed MPG.

These results provide further evidence that topoisomerase IIα recognizes and cleaves AP sites in a cellular setting. In addition, they strongly suggest that the α-isoform of the type II enzyme is able to interact with AP lesions that are generated during the process of BER.

Topoisomerase IIα Mediates a Fraction of the Clastogenic Effects of MMS

In additional to the mutagenic effects of MMS, treatment of cells with this alkylating agent also generates permanent double-stranded breaks in the genome (264,269-271). The appearance of these strand breaks has been attributed primarily to the induction of recombination pathways triggered by replication fork arrest. The attempted repair of closely juxtaposed AP sites on opposite strands of the double helix also has been implicated. However, since MMS treatment increases cellular levels of topoisomerase IIα-DNA cleavage complexes, it is possible that at least some of the clastogenic effects of MMS are mediated by topoisomerase IIα.

Therefore, as a first step towards determining the potential role of topoisomerase IIα in mediating the double-stranded DNA breaks induced by MMS, cellular levels of the enzyme were decreased by transient transfection with a siRNA pool specific for the α-isoform. Seventy-two hours after transfection with the siRNA, topoisomerase IIα could no longer be detected by immunoblot analysis (Figure 21, *inset*). As a control, levels

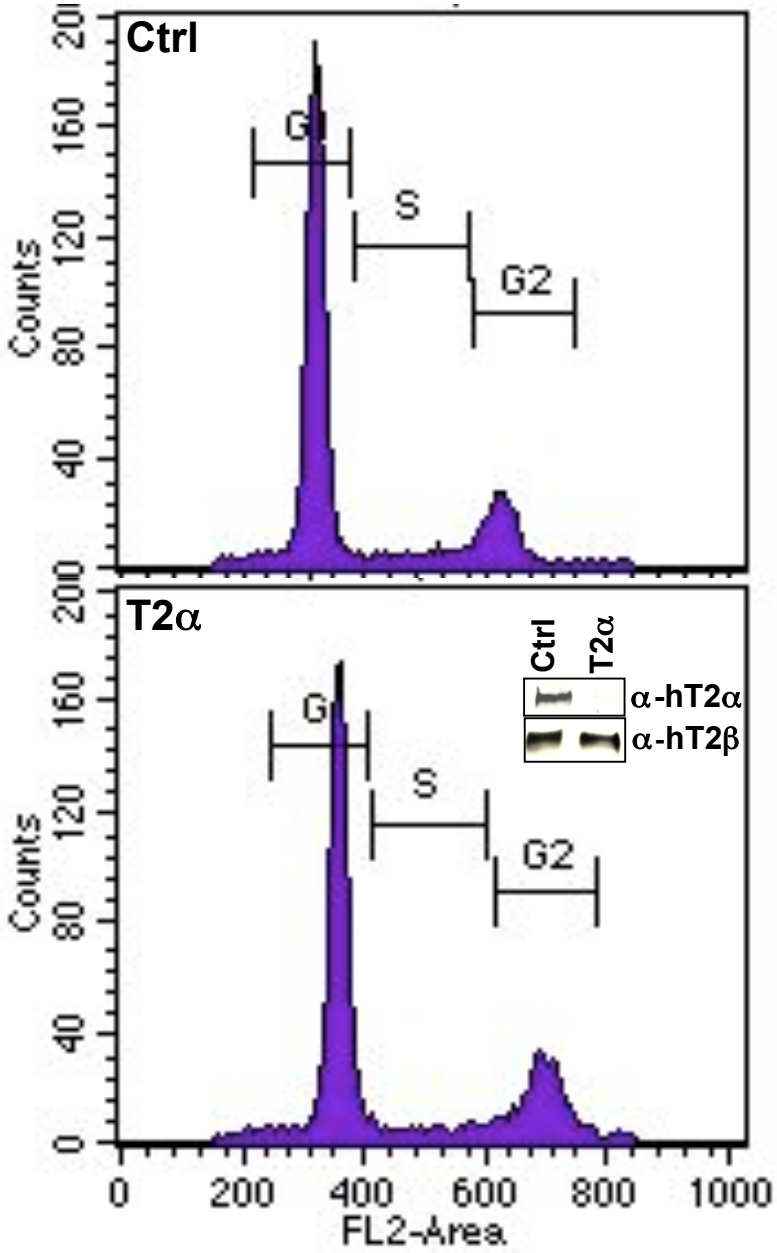


Figure 21. Depletion of human topoisomerase IIα does not cause a gross change in the cell cycle. MDA-MB-321 cells were transfected with siRNA pools against a scrambled control (Ctrl) or human topoisomerase IIα (T2α). Ninety-six hour after transfection cells were harvested, fixed, and the DNA content was determined by PI staining. The levels of topoisomerase IIα were undetectable by immunoblot after 96 h, the levels of topoisomerase IIβ were monitored as a control.

of topoisomerase IIβ were monitored during the course of siRNA treatment. No decrease in the β-isoform was observed (Figure 21, *inset*).

It is likely that transfected MDA-MB-231 cells still contain low levels of topoisomerase IIα, because cultures continued to grow (albeit somewhat more slowly) and maintained a wild-type morphology. DNA content analysis by flow cytometry indicates that topoisomerase IIα-depleted cultures traverse the cell cycle with a modest accumulation of cells in G_2/M (22% as compared to 16% in control cultures) (Figure 21).

Double-stranded DNA breaks in MDA-MB-231 cells were monitored by quantifying the phosphorylated form of histone H2AX (γH2AX). This histone variant is phosphorylated on Ser139 in response to double-stranded breaks and is considered to be dose-responsive barometer for this form of DNA damage (272-275). Following treatment with 2 mM MMS, levels of γH2AX in topoisomerase IIα-depleted MDA-MB-231 cells were ~40% lower than those in cells treated with a non-specific siRNA pool (Figure 22). The phosphorylation of H2AX did not result from the induction of apoptosis, as no cleavage of PARP-1 was observed under the experimental conditions (Figure 22). These data suggest that topoisomerase IIα plays a role in creating a portion of the double-stranded DNA breaks that are generated by MMS. It is notable that levels of γH2AX were lower in topoisomerase IIα-depleted cells even in the absence of external DNA damaging agents (Figure 22). This result implies that a substantial number of the permanent double-stranded DNA breaks present in cultured human cells are generated by the actions of this essential enzyme.

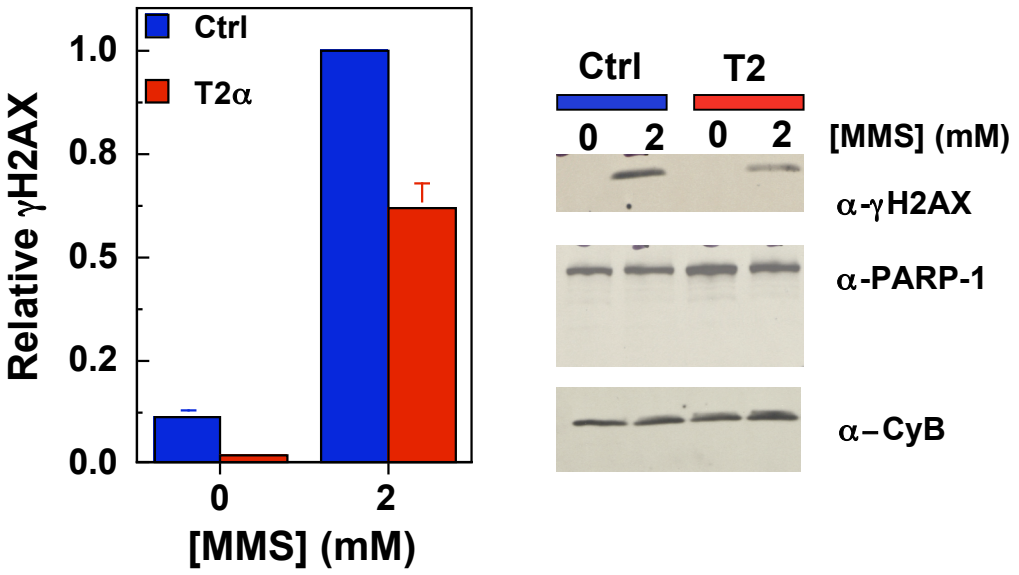


Figure 22. Depletion of Human topoisomerase IIα decreases the double-stranded DNA breaks induced by MMS. MDA-MB-321 cells were transfected with siRNA pools against a scrambled control (Ctrl) or human topoisomerase IIα (T2α). Ninety-six hour after transfection cells were treated with the indicated concentrations of MMS for 4 h and protein immuno analysis was performed for histone γ-H2AX to monitor double-stranded DNA breaks, PARP-1 to monitor apoptosis, and cyclophilin B was monitored as a loading control. Quantification of the histone γ-H2AX is shown on the left. The values represent the averages for three independent experiments with their respective standard deviations.

Consistent with the above findings, topoisomerase IIα-depleted MDA-MB-231 cells display a slight (~30%) resistance to MMS (Figure 23). Thus, at least some of the cytotoxic effects of this DNA methylating agent appear to result from the interaction between the type II enzyme and the AP sites that are formed as a consequence of repair pathways.

Discussion

A number of DNA lesions, including exocyclic base adducts, are strong topoisomerase II poisons *in vitro* (123,124,133). A recent study demonstrated that treatment of human cells with CAA, a bifunctional alkylating agent that generates etheno-adducts, increases physiological levels of topoisomerase IIα-associated DNA breaks (201). This finding indicates that DNA adducts also act as cellular poisons of the human enzyme.

AP sites are the most common DNA lesions formed *in vivo* and are a common mutagenic intermediate for many forms of damage (209,255,276). Despite the fact that these lesions induce high levels of topoisomerase II-mediated DNA cleavage in purified systems, it is not known whether the enzyme interacts with AP sites in a cellular system. Two complementary approaches were used to address this issue. The first utilized MMS, a monofunctional alkylating agent that generates methylated bases that are not topoisomerase II poisons, but are converted to AP sites in cells by BER. Treatment of MDA-MB-231cells with MMS resulted in a dose-dependent increase in DNA cleavage mediated by topoisomerase IIα and a corresponding increase in AP sites (Figure 17). The

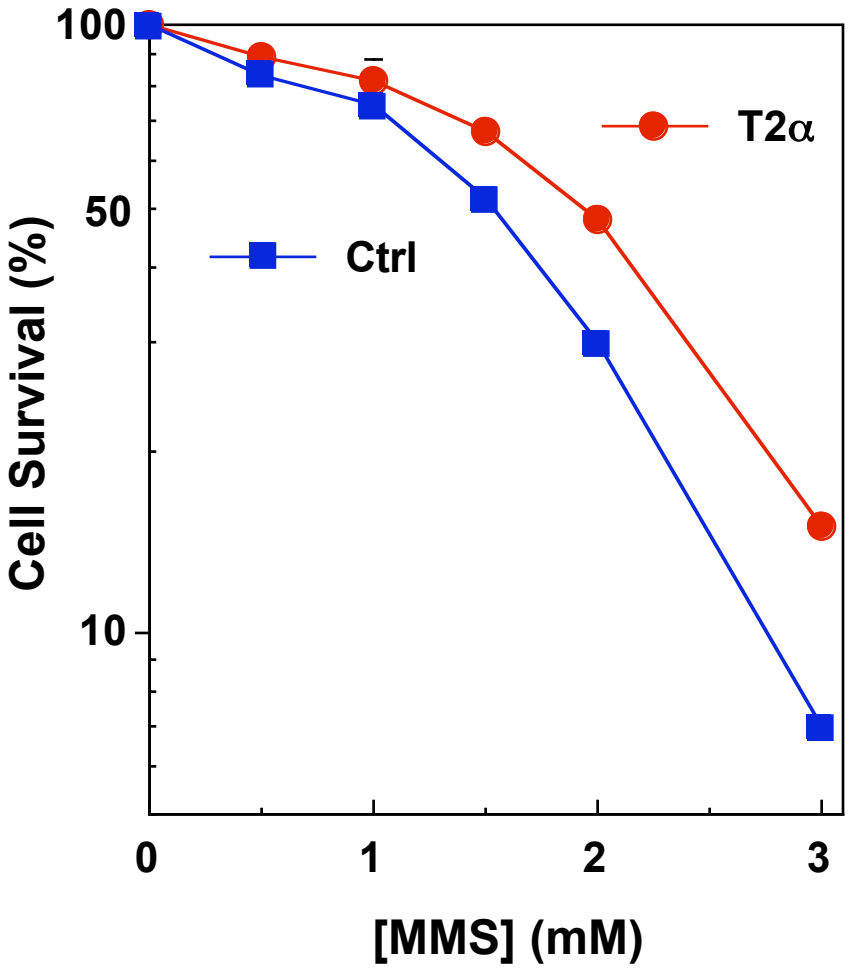


Figure 23. Cells depleted of human topoisomerase IIα are slightly resistant to MMS. MDA-MB-231 cells were transfected with siRNA pools against a scrambled control (Ctrl) or human topoisomerase IIα (T2α). Ninety-six hour after transfection cells were plated and treated with increased concentrations of MMS for 1 h. Cell viability was assessed 36 h after exposure to MMS as described in the methods section. The values represent averages of three independent experiments and the error bars represent the standard deviations.

second approach imbalanced BER by overexpressing MPG, which generates AP sites by removing unmodified purine bases. Once again, a substantial increase in DNA cleavage mediated by topoisomerase IIα was observed (Figure 20). Taken together, these results provide strong evidence that AP sites generated by BER act as topoisomerase IIα poisons in human cells.

During the process of BER, small DNA lesions are converted to AP sites and strand breaks. As a result, repair intermediates generated by BER enzymes are in many cases more dangerous to the cell than the initial lesion. On the basis of enzymological and structural data, a "baton" model has been proposed for short-patch BER (188,194). In this model, each enzyme remains associated with the product of its reaction and subsequently "hands off" the repair intermediate to the following enzyme in the pathway. As a result, BER enzymes are believed to sequester repair intermediates, thus protecting them from potentially harmful interactions with non-repair proteins.

Results of the present study indicate that topoisomerase IIα interacts with AP sites generated by BER in human cells. This finding implies that the type II enzyme is able to disrupt the flow of repair intermediates from one BER enzyme to the next, and "intercept" the DNA baton. It is not known at which step(s) topoisomerase IIα interrupts the BER pathway. Intact AP sites, such as those generated by glycosylases are strong topoisomerase II poisons *in vitro* (125,200). Furthermore, the concentration of topoisomerase IIα in proliferating human cells and the affinity of the enzyme for DNA are in the same range as those of APE1 (194,277,278). However, it is notable that BER intermediates containing nicked AP sites or deoxyribosephosphate flaps, such as those generated by APE1 or DNA polymerase β also are poisons or suicide substrates for

human topoisomerase IIα (200). Therefore, further enzymological studies will be required to determine the specific step(s) of BER at which topoisomerase IIα can intercept the repair intermediate.

High concentrations of MMS (mM range) are believed to kill mammalian cells primarily by the generation of double-stranded DNA breaks (264,279). However, the path(s) that leads from AP sites to these double-stranded breaks has not yet been fully described. Topoisomerase IIα-depleted cells display lower levels of double-stranded breaks and a slight resistance to the methylating agent. Thus, the type II enzyme appears to mediate at least a portion of the clastogenic and cytotoxic effects of MMS.

Because it is imperative for living systems to protect the integrity of their genetic material, cells have evolved multiple and redundant DNA repair pathways (209,254). As a result, the first protein that recognizes and associates with damaged DNA may determine the pathway by which the lesion is repaired (207). Recent evidence suggests that interactions between DNA damage and non-repair proteins also can affect the fate of the lesion (207). For example, if a DNA polymerase or topoisomerase encounters an adduct before the arrival of a repair protein, recombination pathways may be required to restore the DNA. Beyond the ability of topoisomerase IIα to compete for lesions with repair proteins, the present study indicates that the enzyme also is able to disrupt BER and "hijack" processed lesions during the repair process. This finding suggests that repair circuits can be rewired mid-pathway, and that the intended and ultimate destinations of a DNA adduct may be more dynamic than originally believed.

CHAPTER V

CONCLUSIONS

Although topoisomerase II is an essential enzyme, it threatens the genomic integrity of the cell every time it carries out its strand-passage reaction. During this required catalytic step, the enzyme generates a protein-linked double-stranded break. In the presence of DNA tracking systems, this transient break can become a permanent double-stranded DNA break and trigger mutagenic repair pathways or apoptosis. Under normal conditions, the concentration of this intermediate is very low, and thus tolerated by the cell. In the presence of exogenous topoisomerase II poisons, the concentration of this intermediate is increased above physiological levels and this essential enzyme is transformed into a lethal cellular toxin (30,280,281).

The unusual mechanism by which topoisomerase II poisons work argues for the existence of endogenous poisons of this enzyme. These endogenous poisons may be missing link between topoisomerase II and some specific infant leukemias (118-120,246). The work presented in the preceding chapters further expands the spectrum of DNA lesions that act as topoisomerase IIα poisons *in vitro*, provides a better understanding of the mechanism by which these lesions poison the enzyme, and provides, for the first time, evidence supporting a cellular interaction between the enzyme and different forms of DNA damage.

Conclusions and Perspectives Regarding the Interaction Between Topoisomerase IIα, AP Sites. and Exocyclic DNA Adducts *in vitro* and in Cultured Cells

Since AP sites and εdA lesions poison the type II enzyme *in vitro*, it was of interest to expand the spectrum of DNA lesions that could poison the enzyme, and characterize the mechanism by which they increased enzyme-mediated DNA cleavage enhancement (201).

Exocyclic DNA adducts, such as those induced by the environmental exposure to vinyl chloride, (Figure 7) have long been known to be carcinogenic and mutagenic (165,167,172,179,206,218,219,221,224,282-284). Moreover, these family of etheno adducts are known to interfere with critical cellular processes such as DNA replication and transcription (167,172), and induce chromosomal aberrations in cultured cells and animal models (218,219,221,224,283,285-287). These adducts also arise from endogenous sources by the reaction of lipid peroxidation byproducts with DNA (179,288,289). Similarly, malondialdehyde, a lipid metabolism byproduct, produces the adduct know as M_1dG (144,146,148-150). Studies on M_1dG reveal that this adduct is also mutagenic in bacteria and interferes with cellular processes (208,290).

In Chapter III the interaction between topoisomerase IIα and these adducts was examined (201). These studies show that when located within a topoisomerase II cleavage site these adducts enhance the ability of this enzyme to cleave DNA (Figure 10 and 11). Furthermore, the mechanism by which these lesions enhanced the ability of topoisomerase IIα to cleave DNA did not involve an inhibition of the DNA religation (Figure 12 and Table 1), or an enhanced affinity of the enzyme for these lesions (Figure 13 and Table 2). A correlation between topoisomerase II-mediated DNA cleavage enhancement and the extent to which DNA lesions induced a kink in the duplex was

observed (at least with two model lesions) (Figure 15). Finally, treatment of cultured cells with the alkylating agent CAA that is known to induce the formation of etheno adducts in cells, increased the levels of topoisomerase IIα-DNA cleavage complexes (Figure 16). This finding suggests that topoisomerase IIα is able to interact with exocyclic DNA adducts in cells.

Etheno adducts are recognized by DNA glycosylases and repaired by the BER pathway in cells (171,240,241). The cellular interaction observed in Figure 16 may be between topoisomerase IIα and the alkylated base or between the enzyme and the repair intermediates, which have been shown to be potent poisons of topoisomerase IIα *in vitro* (200). Moreover, the cellular consequences of this interaction are unknown. There is no obvious advantage to the cell in allowing this interaction to take place. In fact, it has been postulated that the BER intermediates in the cell are at most times associated with BER enzymes, and thus protected from other proteins (188,194).

However, the interaction between topoisomerase IIα and DNA lesions could be an alternate pathway employed when the BER system is impaired or saturated, thus re-directing this type of damage to be repaired through recombinatorial repair pathways. This "detour" of the standard repair pathway may be deleterious to the cell, since BER is a highly proficient low-error pathway, whereas recombinatorial repair is error-prone (291-297).

Despite the importance of topoisomerase II-targeted drugs in anticancer treatment, these pharmacological regimens have been linked to the generation of this disease (118,120,245). The induction of several forms of cancer has also been linked to excessive amounts of DNA damage and elevated levels of mutagenic and carcinogenic DNA

lesions. It would be of interest to determine whether the interaction between topoisomerase IIα and these forms of DNA damage would be linked to the generation of specific types of cancer.

Conclusions and Perspectives Regarding the Interaction of Topoisomerase IIα with BER Intermediates and the Generation of Double-stranded Breaks in Cultured Cells

Although much enzymological data suggested that topoisomerase II could interact with some forms of DNA damage in the cell, there was no evidence of such interaction until the studies described in Chapter III (201). This finding raised questions regarding the consequences of this interaction. The studies described in Chapter IV are our first attempt to address the potential consequences of the cellular interactions between topoisomerase IIα and AP sites generated by the BER pathway (298).

Many forms of DNA damage have been shown to enhance the ability of topoisomerase IIα to cleave DNA (123,126,132,133,201). While exocyclic DNA adducts such as etheno bases and M_1dG are strong topoisomerase II poisons, methylated bases and sugar-ring modifications have a marginal effect in enzyme-mediated DNA cleavage. MMS is a monofunctional alkylating agent that generates methylated bases, which themselves do not poison topoisomerase IIα. The lesions generated by MMS are readily repaired by the BER pathway, and thus converted to AP sites by the actions of DNA glycosylases (154,156-158,186,299).

In the studies described in Chapter IV, treatment of cells with MMS produced an increase in the levels of topoisomerase IIα-DNA cleavage complexes in a dose-dependent manner (Figure 17). Consistent with the fact that methylated bases are not topoisomerase II poisons, a concomitant increase in AP sites was observed upon MMS treatment.

Furthermore, overexpression of MPG increased the basal levels of topoisomerase IIα-DNA cleavage complexes, thus confirming the interaction between the type II enzyme and AP sites generated by the BER pathway (Figure 20). Finally, cells with undetectable amounts of topoisomerase IIα displayed a reduced amount of double-stranded DNA breaks induced by MMS, and were slightly resistant to MMS as compared to wild-type cells (Figure 22 and 23). These studies confirm the physiological interaction between topoisomerase IIα and DNA damage. Moreover, these studies demonstrate that topoisomerase IIα plays a role in the clastogenicity and cytotoxicity of MMS.

The mechanism by which MMS generates double-stranded DNA breaks is still unclear. Many or the reports ascribe the origin of these breaks to DNA replication-fork arrest (147,270,300). However, these breaks also have been observed in cells that are not actively replicating and roughly 50% of the breaks do not colocalize with the replication factor PCNA in replicating cells (271). Although further studies need to be done, it is likely that topoisomerase IIα may be responsible for a fraction of the MMS-induced double-stranded DNA breaks that are replication-independent (Figure 24).

The BER pathway is thought of as vital, since it removes most of the endogenously formed DNA lesions in the genome (155,158,299). In addition, knockout mice for some of the enzymes (such as the APE1 and pol β) involved in this pathway display an embryonic lethal phenotype (175,177,301-304). Originally, it was thought that the redox activity of APE1 was responsible for the embryonic lethality; however, recent evidence demonstrates that it is actually the AP endonuclease activity what is required for development of the organism (195). This finding highlights the importance of the BER

pathway and the heavy load of AP sites generated endogenously under normal conditions.

Considering the heavy load of AP sites generated in tissues and the limited amounts of repair proteins, it is likely that topoisomerase IIα interacts with these forms of DNA damage. Specific overexpression and downregulation of BER components yields an "imbalance" in this pathway. Cells with imbalanced BER display many of the phenotypical characteristics as those treated with topoisomerase II poisons. Cells treated with topoisomerase II poisons display increased mutagenesis, double-stranded DNA breaks, sister chromatid exchanges and recombination, and chromosomal breakage (264,265,269,305,306). Now that we know that topoisomerase II can interact with DNA damage in a cellular setting, it would be of interest to investigate which (if any) effects of DNA damage are mediated by this non-repair enzyme.

It is notable that the interaction between a base modification and topoisomerase IIα may convert a potentially mutagenic lesion into a permanent double-stranded DNA break, which is a strong inducer of apoptosis (105). If we consider that topoisomerase IIα would only interact with BER intermediates when this system is saturated or impaired, the interaction between the intermediate and this enzyme can function as an apoptotic inducer. This pathway would place topoisomerase IIα as a cellular barometer for DNA damage, thus ascribing a new physiological function to this enzyme. Further experiments will be required to address this hypothesis.

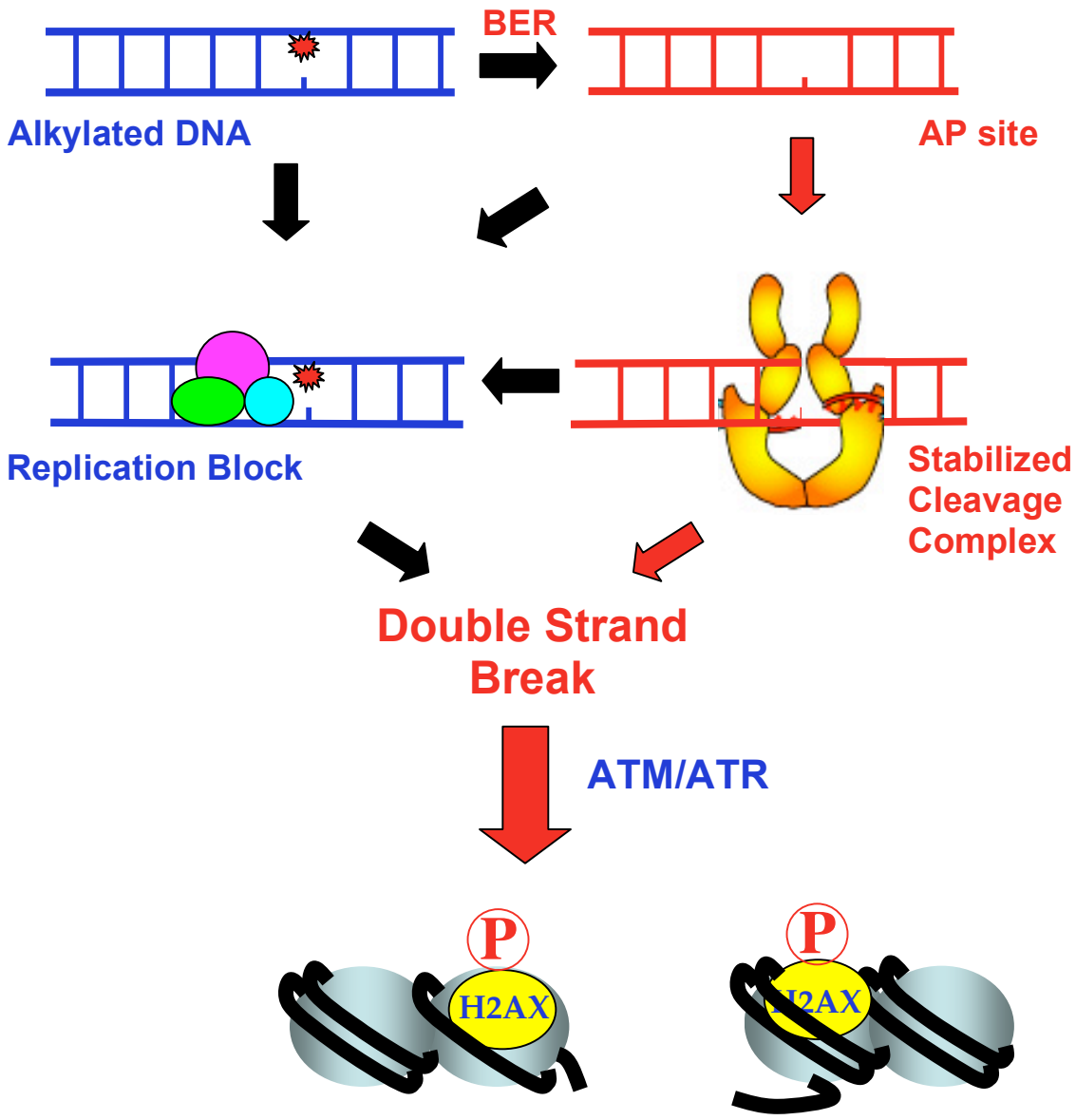


Figure 24. Topoisomerase IIα mediates some of the genotoxic effects of alkylation damage. Alkylated bases such as etheno adducts and methylated bases are normally repaired through BER. When left unrepaired, these adducts can stall DNA replication and thus induce double-stranded DNA breaks. Approximately 50% of the breaks observed upon MMS treatment are replication-independent. These replication-independent breaks could be mediated by topoisomerase IIα.

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