SYNTHETIC AND NATURAL PRODUCTS AS POISONS OF HUMAN TOPOISOMERASE II

By

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To my parents

Dennis and Beverly Ketron

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LIST OF ABBREVIATIONS

9AA	9-aminoacridine
ACR	acridine
Ala	alanine
bp	base-pair
Cys	cysteine
DTT	dithiothreitol
EC	epicatechin
ECG	epicatechin gallate
EDTA	ethylenediaminetetraacetic acid
EGC	epigallocatechin
EGCG	epigallocatechin gallate
EtBr	ethidium bromide
Etop	etoposide
kb	kilobase
kDa	kilodalton
MLL	mixed lineage leukemia gene
PCB	polychlorinated biphenyl
SC	supercoiled
SDS	sodium dodecyl sulfate
TIIα	human topoisomerase IIα
Topo	topoisomerase

CHAPTER I

INTRODUCTION

Topological relationships within the double helix (*i.e.*, DNA supercoiling, tangling, and knotting) significantly influence the processes by which the genetic information is passed from generation to generation, expressed, and recombined in all living systems (*1-3*). *In vivo*, the topological structure of DNA is regulated by ubiquitous enzymes called topoisomerases (*1, 2, 4-7*). These enzymes act by generating transient breaks in the backbone of the genetic material. Topoisomerases are separated into two major classes based on the number of DNA strands that they cleave: type I enzymes cut one strand of the double helix, while type II enzymes cut both (*1, 2, 4-7*). Among their many physiological functions, type II topoisomerases help to set global levels of DNA supercoiling, alleviate the torsional stress that accumulates in front of replication forks and transcription complexes, unlink daughter chromosomes that are generated during replication, and remove DNA knots that form during recombination events (*2, 4, 7-11*). However, because these enzymes generate double-stranded DNA strand breaks as requisite intermediates in their catalytic reactions, they also have the capacity to fragment the genome (*9, 10, 12, 13*). This potentially lethal feature of topoisomerase II can be amplified by the presence of compounds that stabilize enzyme-mediated DNA cleavage intermediates. Rather than depriving cells of the essential functions of topoisomerase II, these agents “poison” the enzymes

and convert them to potent cellular toxins (*4, 7, 10, 14, 15*). Thus, they are called topoisomerase II poisons to distinguish them from classic catalytic inhibitors. A number of laboratory-derived poisons of human topoisomerase II have been utilized as effective anticancer drugs (*4, 7, 10, 14, 15*). Additionally, several natural products recently have been identified as topoisomerase II poisons with chemotherapeutic and/or chemopreventive potential (*16-18*).

DNA Topology and Topoisomerases

DNA Topology

The genetic information of an organism is encoded in a linear array of DNA bases that is stored in the form of a double helix (*1-3*). Two critical features punctuate this elegant structure: base pairing and the intertwining of the two DNA strands. Both contribute to the physical integrity of the genome and provide the redundancy that is the underlying basis for DNA replication, recombination, and repair. In addition to the above, however, the interwound nature of the double helix imposes a number of topological constraints on the genetic material that affect all of its physiological functions (*1-3*).

Topology is a field of mathematics that is concerned with “relationships that are not altered by elastic deformation” (*1, 2*). How is this subject applied to DNA? As long as the ends of DNA are fixed in space and the double helix does not have free rotation, it can be considered to be a topologically closed system. Under these circumstances, the topological properties of DNA are defined as

those that cannot be altered without breaking one or both strands of the double helix (*1, 2*). In virtually every living system, chromosomes consist of extremely long DNA molecules that are circular or linear and are attached to membrane or protein supports. Thus, in general, this definition can be applied to all chromosomal DNA.

Topological relationships in DNA can be divided into two categories: relationships between the two strands of the double helix (*i.e.*, supercoiling) and relationships between different segments of duplex DNA (*i.e.*, tangling and knotting) (*1, 2*). Both affect DNA function in profound, but different, ways and are discussed below.

DNA Supercoiling

Double-stranded DNA that is free from torsional stress (*i.e.*, the classical Watson-Crick structure with ~10.4 base pairs per turn) is defined as “relaxed” (Figure 1; note that the DNA molecules in the figure are depicted as circular ribbon diagrams for simplicity. Similar topological structures exist in linear DNA molecules, as long as the ends of the molecule are fixed in space.) If torsional stress is applied by either under- or overwinding the DNA, molecules writhe about themselves to form superhelical twists (Figure 1) (*1, 2*). Hence, DNA that is under torsional stress is called “supercoiled” (SC). Underwound DNA molecules are defined as negatively supercoiled [(–)SC], and overwound molecules are defined as positively supercoiled [(+)SC].

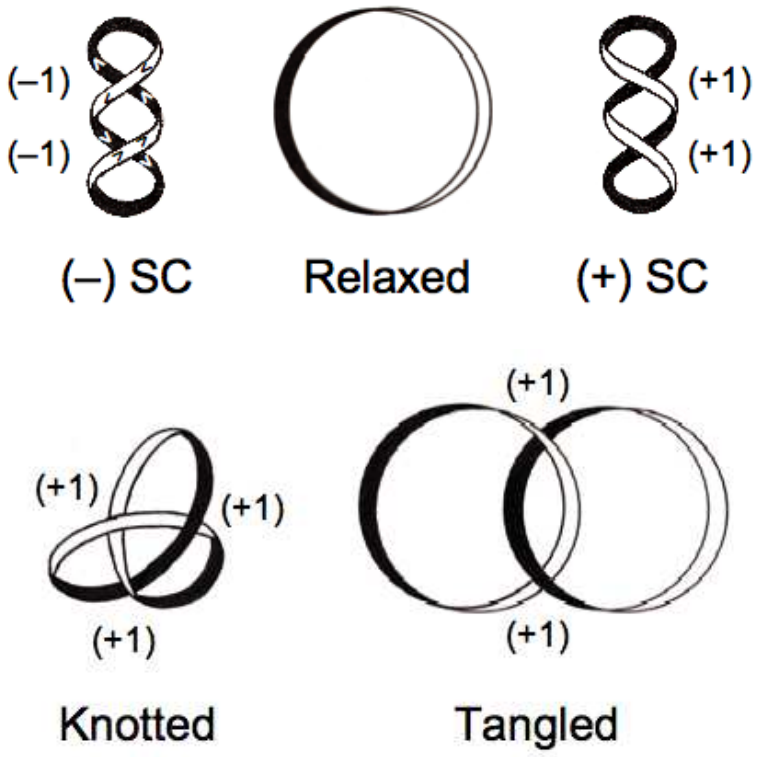


Figure 1. Topological relationships within DNA. Adapted from (*2*). DNA molecules are shown as circular ribbons for simplicity. Top: DNA with no torsional stress is referred to as "relaxed." Underwinding or overwinding DNA results in negative supercoils [(–)SC] or positive supercoils [(+)SC], respectively. The directionality of the DNA is shown by internal arrowheads in the (–)SC molecule. Supercoils are shown as writhes (DNA crossovers or nodes) for visual ease, but it should be noted that supercoils can be interconverted from writhes to twists. By convention, each writhe (denoted by the crossing of one DNA segment over another segment) is given an integral value of –1 or +1. Bottom: Intramolecular knots and intermolecular tangles also form in DNA.

Globally, chromosomal (and extrachromosomal) DNA in bacteria and eukaryotes is underwound ~6% (*2*). Because the two strands of the double helix must be separated in order for the genetic information to be replicated or expressed, under- and overwinding have important implications for DNA function (*1, 2*). Negative supercoiling introduces energy into the genetic material and facilitates strand separation. As a result, DNA underwinding generally increases rates of replication and transcription. In contrast, the movement of replication forks or transcription complexes (or other DNA tracking systems) through the double helix locally overwinds the DNA ahead of their actions (Figure 2). This positive supercoiling makes it more difficult to pull apart the double helix and impedes many essential cellular processes.

DNA Tangling and Knotting

The second aspect of DNA topology deals with relationships between separate segments of the double helix (Figure 1) (*1-3*). To this point, intermolecular tangles (also known as precatenanes) formed between daughter DNA molecules are produced during replication (Figure 2) and between sister chromatids during some recombination events. Furthermore, intramolecular knots are generated during other recombination events. Ultimately, because cells contain such a large amount of DNA (the DNA from the 46 chromosomes of a single human cell is ~2 meters in length and must be compacted into a nucleus that is ~5–10 μm in diameter), any process that involves movement of the genetic material is likely to produce DNA entanglements.

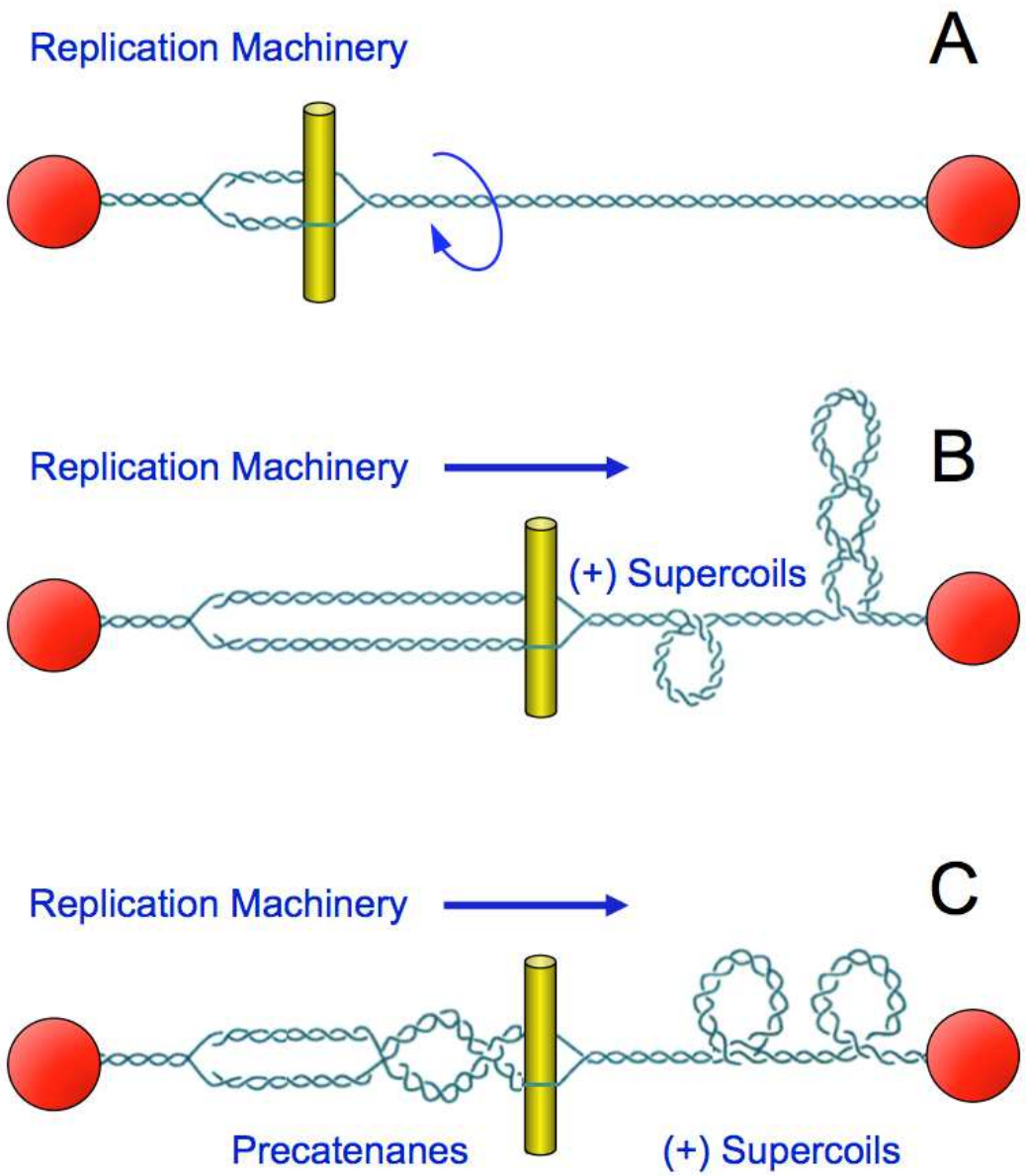


Figure 2. Generation of positive supercoils (+SC) ahead of and tangles behind DNA tracking systems. A. The ends of chromosomal DNA are anchored to membranes or the chromosome scaffold (represented by the red spheres) and are not free to rotate. Therefore, the linear movement of tracking systems (such as replication machinery represented by the yellow bars) through the double helix does not change the number of turns of the DNA. B. The above action compresses the turns into a shorter segment of the genetic material. Consequently, the double helix becomes increasingly overwound, generating positive (+) supercoils ahead of tracking systems. C. Some of the torsional stress induced by positive supercoiling slips behind the replication fork, generating DNA tangles (precatenanes).

Tangles and knots adversely affect a variety of essential nucleic acid processes. For example, DNA molecules that are tangled cannot be segregated during mitosis or meiosis. Moreover, the presence of knots makes it impossible to separate the two strands of the double helix. Consequently, DNA tangles and knots can be lethal to cells if they are not resolved.

DNA Topoisomerases

In order to regulate the superhelical density of DNA or resolve tangles and knots from the genetic material, the closed topological system has to be opened. This is accomplished by breaking the DNA backbone. However, the cell has to deal with these topological relationships in fundamentally different ways. Because the torsional stress associated with supercoiling can be modulated by either DNA rotation or strand passage, the number of supercoils can be altered by cleaving one or both strands of the double helix. In contrast, since the writhes associated with tangles and knots contain double-stranded DNA nodes, these topological structures can be removed only by creating double-stranded breaks in the DNA backbone.

Enzymes that regulate the topological structure of DNA are called topoisomerases (*1, 2, 4-7*). Because the ability to regulate DNA topology is essential for cell survival, these enzymes are encoded by all known species. Topoisomerases can be separated into two major classes, which are distinguished by the number of DNA strands that are cleaved and ligated by their respective enzymes (*1, 2, 4-7*). Type I topoisomerases act by cleaving one

strand of the double helix. Thus, they are able to regulate levels of DNA supercoiling. Type II topoisomerases act by cleaving both strands of the double helix. As a result, they can regulate the superhelical density of DNA and also can resolve tangles and knots in duplex DNA. (Note: type I topoisomerases can untangle and unknot DNA linkages if they are single-stranded in nature.) In order to maintain the integrity of the genome during the required DNA cleavage event, all topoisomerases form covalent bonds between active site tyrosyl residues and the DNA termini generated during the reaction. This covalent enzyme-cleaved DNA complex (known as the "cleavage complex") is a hallmark of topoisomerases.

Type I Topoisomerases

There are three classes of type I topoisomerases—type IA, IB, and IC—which are defined on the basis of homology and catalytic mechanism (*1, 2, 4-7, 19, 20*). With the exception of reverse gyrase, type I topoisomerases are denoted by "odd" numerals. Type IA enzymes are found in bacteria [topoisomerase I (also called ω protein), topoisomerase III, and reverse gyrase in thermophiles and hyperthermophiles], eukaryotes (topoisomerase III) and archaea (topoisomerase III and reverse gyrase). Type IB topoisomerases are found primarily in eukaryotes (nuclear topoisomerase I and mitochondrial Top1mt), but recently have been found in some bacterial species. Type IB enzymes also are encoded by poxviruses and mimiviruses. Finally, the type IC enzyme (topoisomerase V) is found exclusively in hyperthermophilic archaea.

Type II Topoisomerases

There are two classes of type II topoisomerases—type IIA and IIB—which are defined on the basis of homology (*2-7, 10, 11, 21, 22*). With the exception of gyrase, type II topoisomerases are denoted by “even” numerals. Most bacteria encode two type IIA enzymes (gyrase and topoisomerase IV). Eukaryotes, in contrast, encode only one type IIA enzyme, topoisomerase II. It should be noted, however, that vertebrate species express two closely related isoforms of the type IIA enzyme, topoisomerase IIα and topoisomerase IIβ. Archaea and plants encode the only known type IIB topoisomerase, topoisomerase VI. Because of the rarity of the type IIB class of topoisomerases, only type IIA topoisomerases will be considered here and “type II” will represent “type IIA” henceforth.

Type II topoisomerases regulate superhelical density and remove tangles and knots by the double-stranded DNA passage reaction depicted in Figure 3 (which uses eukaryotic topoisomerase II as a representative enzyme) (*2-5, 7, 10, 22, 23*). These enzymes require a divalent metal ion (Mg^{2+} appears to be the physiological ion) and ATP in order to carry out their complete catalytic cycle.

Type IIA enzymes bind two segments of DNA (Step 1). The first segment bound by the enzyme is the double helix that will be cleaved and is referred to as the “Gate-” or “G-segment.” The second segment is the double helix that will be transported through the transient DNA gate and is referred to as the “Transport-” or “T-segment.” DNA binding requires no cofactors. In the presence of the active site Mg^{2+} ions, type IIA topoisomerases sample the DNA for malleability (Step 2). Sequences that can be cleaved are bent to an angle of ~150º (depending on the

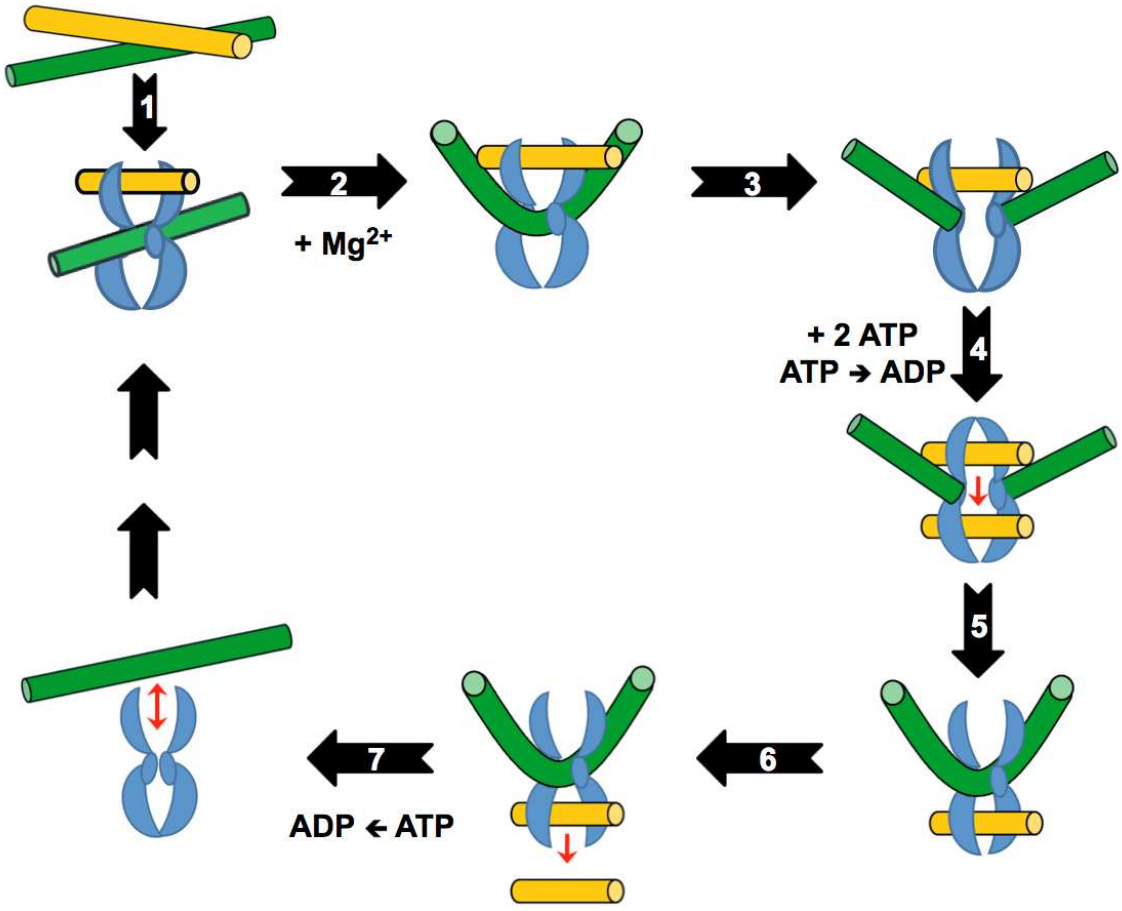


Figure 3. Catalytic cycle of type II topoisomerases. The homodimeric enzyme is shown in blue, the DNA double helix that is cleaved and acts as the DNA gate (G-segment) is shown in green, and the double helix that is transported through the DNA gate (T-segment) is shown in yellow. Details of the individual reaction steps are given in the text.

enzyme). Conversely, sequences that cannot be bent are not cleaved. A double-stranded break is generated in the G-segment (Step 3) using a noncanonical two-metal-ion mechanism. The type II enzymes contain two active site tyrosyl residues (located on different subunits), each of which makes a single-stranded DNA break. The scissile bonds on the two strands of the double helix are staggered, and cleavage generates 5'-termini with four-base single-stranded cohesive ends. During the scission event, type IIA topoisomerases covalently attach to the 5'-termini of the cleaved DNA. Two molecules of ATP are bound by the enzyme, which triggers the closing of the N-terminal protein gate, the opening of the DNA gate, and the translocation of the T-segment through the gate (Step 4). Although hydrolysis of the cofactor is not a prerequisite for DNA translocation, it appears that this step proceeds more rapidly if it is preceded by hydrolysis of one of the bound ATP molecules. The cleaved DNA is rejoined (Step 5), the T-segment is released through the C-terminal protein gate (Step 6), and, upon hydrolysis of the second ATP molecule, type IIA enzymes regain the ability to initiate a new round of catalysis (Step 7).

Topoisomerase II Function, Domain Organization and Isoforms

The eukaryotic type IIA enzyme, topoisomerase II, was discovered in 1980 (*2, 4, 5, 7, 10, 22, 23*). The enzyme (and collectively topoisomerase IIα and topoisomerase IIβ in vertebrates – see below) plays a number of essential roles in eukaryotic cells and participates in virtually every major process that involves movement or organization of the genetic material (*2, 4, 6, 7, 10, 11*). The enzyme

unlinks tangled daughter chromosomes following replication and resolves DNA knots that are formed during recombination. It also helps to alleviate the torsional stress that accumulates ahead of replication forks and transcription complexes. Topoisomerase II is required for proper chromosome condensation, cohesion, and segregation and appears to play roles in centromere function and chromatin remodeling. Furthermore, the enzyme is important for the maintenance of proper chromosome organization and structure and is the major non-histone protein of the mitotic chromosome scaffold and the interphase nuclear matrix.

In concert with histones, the enzyme also is responsible for maintaining the global underwinding of chromosomal DNA. Because nucleosomes wrap the genetic material in a left-handed superhelix (which underwinds the DNA), removal of the resulting compensatory positive supercoils by topoisomerase II leads to a net negative supercoiling of the eukaryotic genome (*1*). (Note: this activity is accomplished by gyrase in prokaryotic species, which has the unique ability among topoisomerases to introduce negative supercoils into relaxed DNA.)

Lower eukaryotic species such as yeast and fruit flies encode only a single type II topoisomerase (*i.e.*, topoisomerase II). However, as mentioned above, vertebrates express two isoforms, topoisomerase IIα and topoisomerase IIβ (*2, 4-7, 10, 11, 22*). These two proteins share extensive amino acid sequence identity (~70%) but are encoded by separate genes (located at chromosomal bands 17q21-22 and 3p24 in humans, respectively). Topoisomerase IIα and

topoisomerase IIβ also can be distinguished by their protomer molecular masses (~170 kDa and ~180 kDa, respectively).

Eukaryotic type IIA topoisomerases are homologous to the bacterial type II enzymes (Figure 4) (*2, 4-6, 10, 11, 21, 24*). However, while bacterial type II topoisomerases consist of two copies of two separate proteins in an A_2B_2 heterotetramer (GyrA and GyrB for gyrase), eukaryotic topoisomerase II functions as a homodimer in which the two subunits have fused. On the basis of amino acid sequence comparisons with bacterial gyrase, each topoisomerase II protomer can be divided into three distinct domains. The N-terminal domain of the enzyme is homologous to GyrB and contains the binding site for ATP. The central domain is homologous to much of GyrA and contains the active site tyrosyl residue. The C-terminal domain of topoisomerase II, which occupies the same location on the protein as the C-terminal domain of GyrA, is highly variable. This region shares little to no sequence similarity to the equivalent region in DNA gyrase or topoisomerase IV and differs considerably between type II topoisomerases, even across eukaryotic species. The C-terminal domain of eukaryotic topoisomerase II contains nuclear localization sequences as well as amino acid residues that interact with cellular components or are phosphorylated *in vivo.*

It is not obvious why vertebrates encode two distinct topoisomerase II isoforms; differences between topoisomerase IIα and topoisomerase IIβ are subtle (*2, 4, 5, 7, 10, 11, 22*). The only major enzymatic characteristic that distinguishes the two isoforms from each other is the ability to recognize the

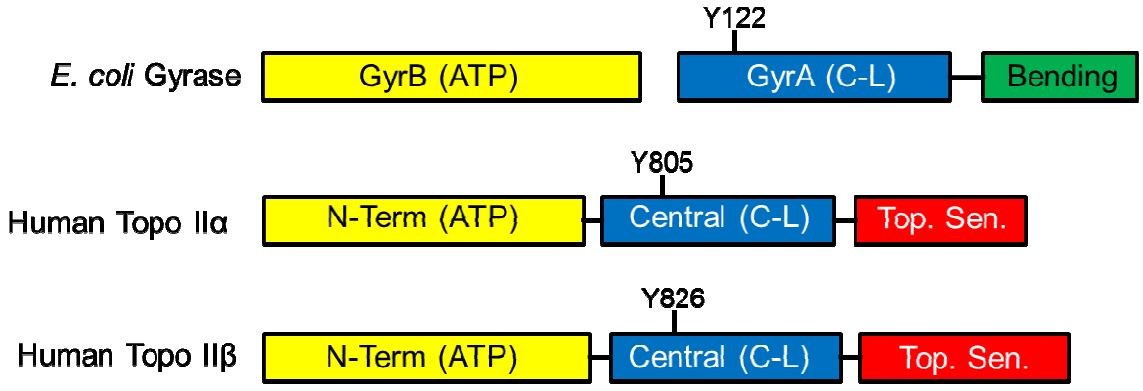


Figure 4. Domain structures of *E. coli* gyrase, human topoisomerase IIα and human topoisomerase IIβ. Regions of homology among the enzymes are indicated by colors. The N-terminal homology domains (yellow) contain the regions responsible for ATP binding and hydrolysis. The central homology domains (blue) contain the active site tyrosyl residue (Y122 for GyrA; Y805 and Y826 for the human isoforms α and β, respectively) that performs cleavage and ligation (C-L) and forms the covalent bond with DNA during scission. The variable C-terminal domains are shown in green or red. These domains are involved in DNA bending and topology sensing. Subunits and domains are drawn proportionally to their length. The active site tyrosyl residue is indicated for each enzyme.

handedness of DNA supercoils (*25*). While the α isoform removes positive DNA supercoils ~10–fold faster than it does negative, the β isoform removes both at similar rates. This topology sensing function of topoisomerase IIα is embodied in the C-terminal domain of the protein.

Topoisomerase IIα and topoisomerase IIβ have distinct patterns of expression and separate nuclear functions (*2, 4, 7, 10, 11*). Topoisomerase IIα is essential for the survival of proliferating cells and is regulated over cell and growth cycles. Enzyme levels increase throughout S-phase of the cell cycle and peak at the G_2/M boundary. Although topoisomerase IIα is nearly non-existent in quiescent or differentiated tissues, rapidly proliferating cells contain as many as ~500,000 copies of the enzyme. Topoisomerase IIα is associated with replication forks, and its ability to preferentially relax positive supercoils has led to speculation that it helps remove torsional stress ahead of the replication machinery. Furthermore, the enzyme remains tightly bound to chromosomes during mitosis. In light of the enzymological characteristics, regulation, and cell biology described above, it is believed that topoisomerase IIα is the isoform that functions in growth-related cellular processes.

Topoisomerase IIβ is dispensable at the cellular level, and its presence cannot compensate for the loss of topoisomerase IIα in mammalian cells (*2, 4, 5, 7, 10, 11, 26*). However, the β isoform is required for proper neural development in mice (*27*). In contrast to topoisomerase IIα, the concentration of topoisomerase IIβ is independent of the cell cycle, and high levels of this isoform are found in most cell types regardless of proliferation status (*26, 28, 29*). Topoisomerase IIβ

dissociates from chromosomes during mitosis. As with the ability to discern the handedness of DNA supercoils, the sequences that govern the association/dissociation of topoisomerase II with mitotic chromosomes reside in the C-terminal domain. Ultimately, the physiological functions of the β isoform have yet to be fully defined. However, recent evidence suggests that topoisomerase IIβ plays an important role in the transcription of hormonally- or developmentally-regulated genes (*30, 31*).

DNA Topoisomerase II as a Cellular Toxin

Topoisomerase II-DNA Cleavage Complexes

The covalent enzyme-DNA linkage formed during DNA scission (Figure 3, *Step 3*) plays two important roles in the topoisomerase II reaction mechanism (*3-5, 7, 10, 22, 24*). First, it conserves the bond energy of the sugar-phosphate DNA backbone. Second, because it does not allow the cleaved DNA chain to dissociate from the enzyme, the protein-DNA linkage maintains the integrity of the genetic material during the cleavage event. The covalent topoisomerase II-cleaved DNA reaction intermediate is referred to as the cleavage complex and is central to the catalytic cycle of the enzyme. The DNA cleavage/ligation equilibrium of the enzyme greatly favors ligation (*3-5, 7, 10, 22, 24*). Thus, topoisomerase II-DNA cleavage complexes normally are short-lived and are readily reversible. As described below, compounds that increase the longevity of cleavage complexes can have serious cellular consequences.

Topoisomerase II as a Cellular Toxin

Because topoisomerases generate DNA strand breaks as obligate reaction intermediates, they are intrinsically dangerous proteins (*2, 9, 10, 14, 15, 32, 33*). Thus, while necessary for cell viability, these enzymes also have the capacity to fragment the genome (Figure 5). As a result of this dual "Dr. Jekyll/Mr. Hyde" persona, cells maintain levels of cleavage complexes in a critical balance. If topoisomerase IIα cleavage drops below threshold levels, daughter chromosomes remain entangled following replication (*2, 4, 7, 8, 11*). Consequently, chromosomes cannot segregate properly, and cells die as a result of catastrophic mitotic failure (Figure 5).

Increased levels of topoisomerase IIα- or IIβ-DNA cleavage complexes also cause deleterious physiological effects, but for different reasons (Figure 5) (*2, 9, 10, 14, 15, 32, 33*). When replication forks, transcription complexes, or other DNA tracking proteins attempt to traverse covalently bound protein "roadblocks" in the genetic material, accumulated cleavage intermediates are converted to strand breaks that are no longer tethered by proteinaceous bridges. The ensuing damage induces recombination/repair pathways that can trigger mutations and other chromosomal aberrations. If the number of DNA breaks overwhelms the repair process, it can initiate cell death pathways (*9, 12, 34-36*). Conversely, if cells are not killed, DNA breaks can be converted to permanent chromosomal translocations that lead to specific forms of leukemia (*37, 38*).

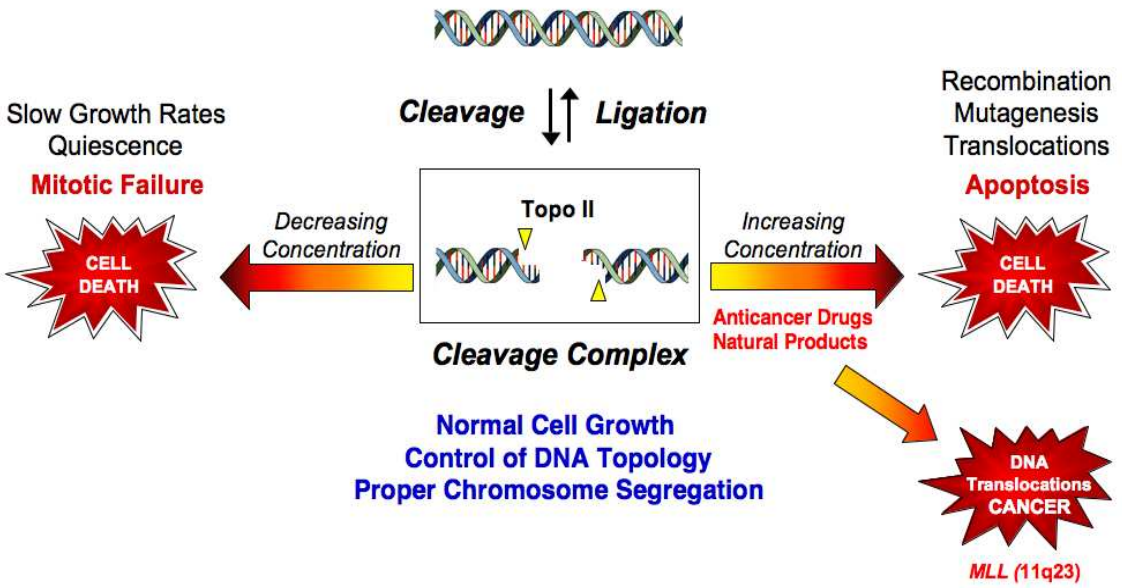


Figure 5. Topoisomerase II-DNA cleavage complex equilibrium. Adapted from (*10*). The formation of covalent DNA cleavage complexes is required for topoisomerases to perform their critical cellular functions. If the level of topoisomerase II-DNA cleavage complexes falls below threshold levels (left arrow), cells are unable to segregate their chromosomes and ultimately die of mitotic failure. If the level of cleavage complexes becomes too high (right arrow) the actions of DNA tracking systems can convert these transient complexes to permanent double-stranded breaks. The resulting DNA breaks, as well as the inhibition of essential DNA processes, initiate recombination/repair pathways and generate mutations, chromosome translocations, and other DNA aberrations. If the strand breaks overwhelm the cell, they can trigger apoptosis. This is the basis for the actions of several widely prescribed anticancer drugs that target topoisomerase II. However, if the increase in enzyme-mediated DNA strand breaks does not kill the cell, mutations or chromosomal aberrations may be present in surviving populations. In some cases, exposure to topoisomerase II-targeted agents has been associated with the formation of acute myeloid leukemias that involve the *MLL* (mixed lineage leukemia) gene at chromosome band 11q23 (lower right arrow).

Topoisomerase II Poisons

Compounds that alter topoisomerase II activity can be separated into two categories. Chemicals that decrease the overall activity of the enzyme are known as catalytic inhibitors (*39, 40*). Conversely, chemicals that increase levels of topoisomerase II-DNA cleavage complexes are said to “poison” the enzyme and convert it to a cellular toxin that initiates the mutagenic and lethal consequences described in Figure 5 (*2, 9, 10, 14, 15, 32, 33*). Because of their actions, these latter compounds are referred to as “topoisomerase II poisons” to distinguish them from catalytic inhibitors that do not increase the concentration of cleavage complexes (*2, 9, 10, 14, 15, 32, 33*). Although some topoisomerase poisons also inhibit overall activity, the “gain of function” induced by these compounds in the cell (*i.e.*, increased levels of cleavage complexes) is a dominant phenotype. Thus, they kill cells by a fundamentally different mechanism than that of most protein-targeted drugs (which act by robbing the cell of an essential function). As discussed below, a number of laboratory-synthesized and naturally occurring compounds act as topoisomerase II poisons, and thus display anticancer or chemopreventive properties.

Chemicals that function as topoisomerase II poisons act by two distinct mechanisms. Compounds utilizing the first mechanism are referred to as interfacial topoisomerase II poisons (*2, 9, 10, 14, 15, 32, 33*). These chemicals form non-covalent interactions with topoisomerase II at the protein-DNA interface in the vicinity of the active site tyrosine. They also interact with DNA within the ternary enzyme-DNA-poison complex and inhibit ligation by intercalating into the

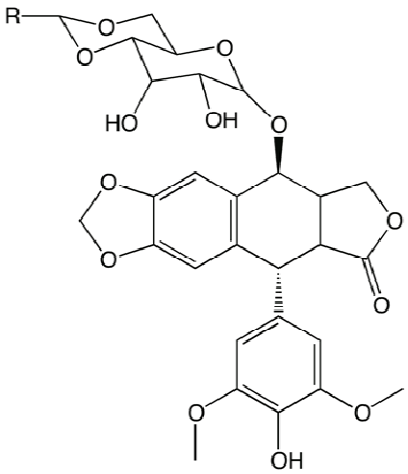
double helix at the cleaved scissile bond. Thus, they present a physical barrier to ligation and act as “molecular doorstops.” It is notable that the actions of interfacial topoisomerase II poisons are not affected by reducing agents, such as dithiothreitol, and that these compounds induce similar levels of enzyme-mediated DNA cleavage whether they are added to the binary topoisomerase II-DNA complex or are incubated with the enzyme prior to the addition of nucleic acid substrates.

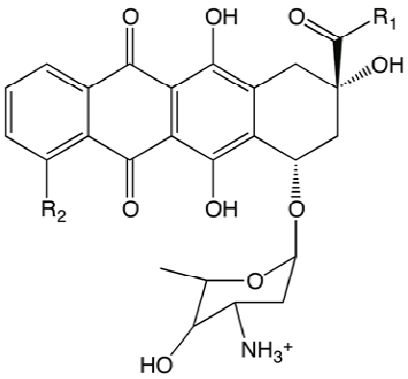
Unlike the interfacial poisons, compounds that use the second mechanism contain protein reactive groups. Because many of the original compounds that were examined underwent redox cycling (or oxidation/reduction reactions) as a prerequisite for activity, compounds that utilize this second mechanism were (and often still are) collectively referred to as “redox-dependent” topoisomerase II poisons (*10, 41-44*). (In light of the findings described in Chapter IV, the moniker “redox-dependent” has been found to be somewhat misleading, and I recommend that this class of compounds be referred to as “covalent” topoisomerase II poisons henceforth.) Most incorporate sulfhydryl-reactive groups such as quinones, isothiocyanates, or maleimides. In contrast to interfacial topoisomerase II poisons, covalent poisons adduct to the enzyme at amino acid residues outside of the active site. Moreover, their ability to poison topoisomerase II can be abrogated by reducing thiol nucleophiles. Finally, compounds within this second group enhance DNA cleavage when added to the protein-DNA complex, but display the distinguishing feature of inhibiting topoisomerase II activity when incubated with the enzyme prior to the addition of DNA.

There is evidence that some covalent topoisomerase II poisons function (at least in part) by crosslinking or closing the N-terminal protein gate of topoisomerase II (*42*). This could provide a mechanistic basis for stabilizing pre-existing cleavage complexes while excluding DNA binding to unoccupied enzymes. However, the precise details by which covalent topoisomerase II poisons increase levels of DNA cleavage complexes have yet to be determined.

Interfacial Topoisomerase II Poisons

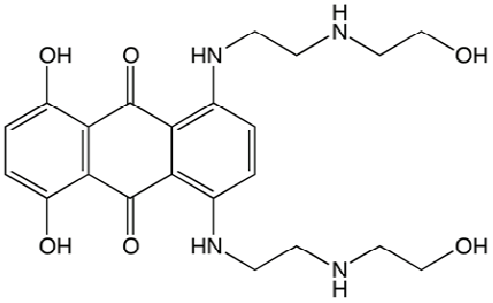
Some of the most important and widely prescribed anticancer drugs currently in clinical use are interfacial topoisomerase II poisons (Figure 6) (*2, 9, 10, 14, 15, 45*). One of the most important topoisomerase II-targeted anticancer drugs is etoposide, which is derived from podophyllotoxin (*46*). This natural product is produced by *Podophyllum peltatum,* more commonly known as the mayapple or American mandrake plant. Podophyllotoxin has been used as a folk remedy for over a thousand years and is an antimitotic drug that acts by preventing microtubule formation. The clinical use of this compound as an antineoplastic agent was prevented by high toxicity, but two synthetic analogs, etoposide and teniposide, displayed increased antineoplastic activity and decreased toxicity. Further analysis revealed that these drugs do not interact with microtubules; rather, they act as topoisomerase II poisons. Etoposide was approved for clinical use in the mid-1980s and for several years was the most widely prescribed anticancer drug in the world.



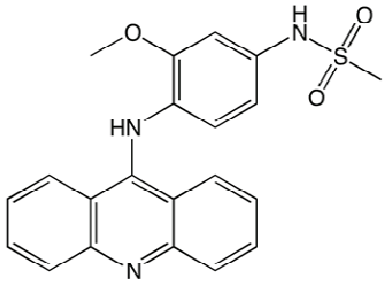


	R
Etoposide:	CH_3
Teniposide:	

	R_1	R_2
Doxorubicin:	CH_2OH	OCH_3
Daunorubicin:	CH_3	OCH_3
Idarubicin:	CH_2OH	H



Mitoxantrone



Amsacrine

Figure 6. Structures of selected interfacial topoisomerase II poisons. The demethyl-epipodophyllotoxins etoposide and teniposide, the anthracyclines doxorubicin, daunorubicin, and idarubicin, and the anthracenedione mitoxantrone are approved for clinical use in the United States. The acridine amsacrine is used in some salvage regimens for acute refractory myeloid leukemias.

Etoposide and drugs such as doxorubicin (and its derivatives) are front-line therapy for a variety of systemic cancers and solid tumors, including leukemias, lymphomas, sarcomas, lung cancers, and germline malignancies (*2, 9, 10, 14, 15*). Mitoxantrone is used to treat breast cancer, relapsed acute myeloid leukemia, and non-Hodgkins lymphoma. Amsacrine (which is discussed more extensively in Chapter III) also is used to treat relapsed acute myeloid leukemia. Ultimately, half of all anticancer regimens include topoisomerase II-targeted drugs.

Bioflavonoids are a diverse group of polyphenolic compounds that are constituents of many fruits, vegetables, legumes, and plant leaves (*47-52*). They are an integral component of the human diet and represent the most abundant natural source of antioxidants (*47-50, 52-54*).

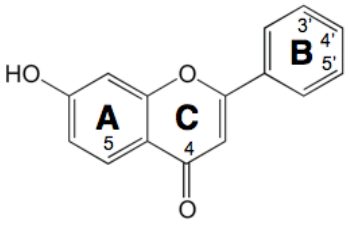
It is believed that the dietary intake of bioflavonoids provides a number of health benefits to adults (*47-52, 55, 56*). Epidemiological studies suggest that these compounds help protect against cancer, cardiovascular disease, osteoporosis, age-related diseases, and inflammation. The mechanistic basis for the physiological actions of bioflavonoids is not fully described, as they have a variety of effects on human cells. Beyond their antioxidant properties, many of these polyphenols are potent inhibitors of tyrosine kinases (*51, 57-62*), display anti-proliferative, pro-apoptotic, and genotoxic effects, and decrease the expression or function of several proteins that are involved in cell-cycle progression (*51, 52, 63-66*).

A variety of bioflavonoids (specifically flavones, isoflavones, and flavonols) have been examined for their abilities to enhance DNA cleavage mediated by human topoisomerase IIα and IIβ, and several were found to be potent topoisomerase II poisons *in vitro* and in cultured human cells (Figure 7) (*16, 17, 67, 68*). Among the bioflavonoids, genistein appears to have the highest activity against the human type II enzymes (*16, 17*). Many of the chemopreventive, cytotoxic, and genotoxic properties of flavones, isoflavones, and flavonols are consistent with their activities as topoisomerase II poisons. To this point, the sensitivity of cells to genistein has been correlated to the activity of the type II enzyme (*69, 70*).

With the exception of (–)-epigallocatechin gallate (EGCG; see below), flavones, isoflavones, and flavonols are interfacial topoisomerase II poisons and increase levels of cleavage complexes primarily by inhibiting enzyme-mediated DNA ligation (*16, 17*). In general, these compounds appear to be more efficacious against topoisomerase IIβ than the α isoform (*17*). Furthermore, cells that are depleted of topoisomerase IIβ are resistant to genistein (*70*). Therefore, it is believed that many of the cellular effects of flavones, isoflavones, and flavonols as topoisomerase II poisons are mediated primarily by the β isoform (*70*).

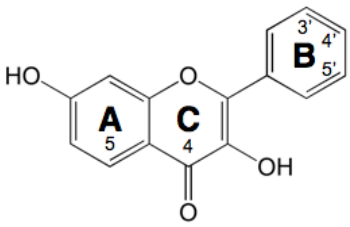
Covalent Topoisomerase II Poisons

Catechins represent another major and important class of bioflavonoids (*49, 50*). Green tea, which is one of the most commonly consumed beverages in the world, is a rich source of catechins and has been suggested to reduce the



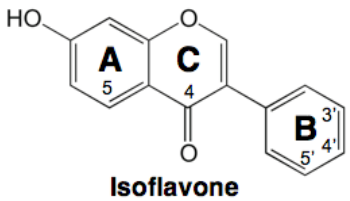
Flavone

Flavones	Position 5	3'	4'	5'	Topo II Activity
Luteolin	OH	OH	OH	H	++
Apigenin	OH	H	OH	H	+
Diosmetin	H	OH	OCH_3	H	+
Chrysin	OH	H	H	H	+/–



Flavonol

Flavonols	Position 5	3'	4'	5'	Topo II Activity
Myricetin	OH	OH	OH	OH	++
Quercetin	OH	OH	OH	H	++
Kaempferol	OH	H	OH	H	+
Fisetin	H	OH	OH	H	+
Isorhamnetin	OH	OCH_3	OH	H	–
Galangin	OH	H	H	H	–



Isoflavone

Isoflavones	Position 5	3'	4'	5'	Topo II Activity
Genistein	OH	H	OH	H	+++
Daidzein	H	H	OH	H	–
Biochanin A	OH	H	OCH_3	H	–

Figure 7. Structures of selected bioflavonoids. Adapted from (*17*). Flavones, flavonols, and isoflavones are shown, and the ability of each to enhance topoisomerase II-mediated DNA cleavage is indicated as >8-fold (+++), 6- to 8-fold (++), 3- to 6-fold (+), 2- to 3-fold (+/–), or <2-fold (–) over baseline.

incidence of breast, prostate, colorectal, and lung cancer in humans (*54, 71-73*). The most abundant catechins in green tea are EGCG and the related compounds (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), and (–)-epicatechin (EC) (Figure 8) (*54, 71-73*). Although EGCG and EGC are potent topoisomerase II poisons, neither ECG nor EC display any substantial activity against the human type II enzymes (*16, 74*). Thus, the ability of the catechins to poison topoisomerase II reflects the presence of three hydroxyl groups on the B-ring, with the D-ring having little relevance.

Surprisingly (and in major contrast to the flavones, isoflavones, and flavonols), EGCG and EGC appear to be covalent (rather than interfacial) topoisomerase II poisons (*18, 74*). The mechanistic differences between bioflavonoid classes appear to be related to structural elements in the B- and C-rings (Figure 8) (*74*). First, while the C-4' hydroxyl of the B-ring is critical for bioflavonoids to act as interfacial topoisomerase II poisons (*16, 17, 67*), the inclusion of two additional B-ring hydroxyl groups increases redox activity (*75, 76*) and is required for compounds to act as covalent topoisomerase II poisons (*18, 74*). Second, the C-ring in flavones, isoflavones, and flavonols is aromatic, planar, and includes the C-4 keto group that allows the formation of the proposed pseudo ring with the C-5 hydroxyl (*77*). All of these elements are required for binding to human type II topoisomerases (*17, 74*). Because EGCG and EGC contain the catechin C-ring, they are unable to act as interfacial topoisomerase II poisons and function exclusively as covalent poisons. Moreover, ECG and EC lack the critical third hydroxyl group on their B-rings that would allow them to

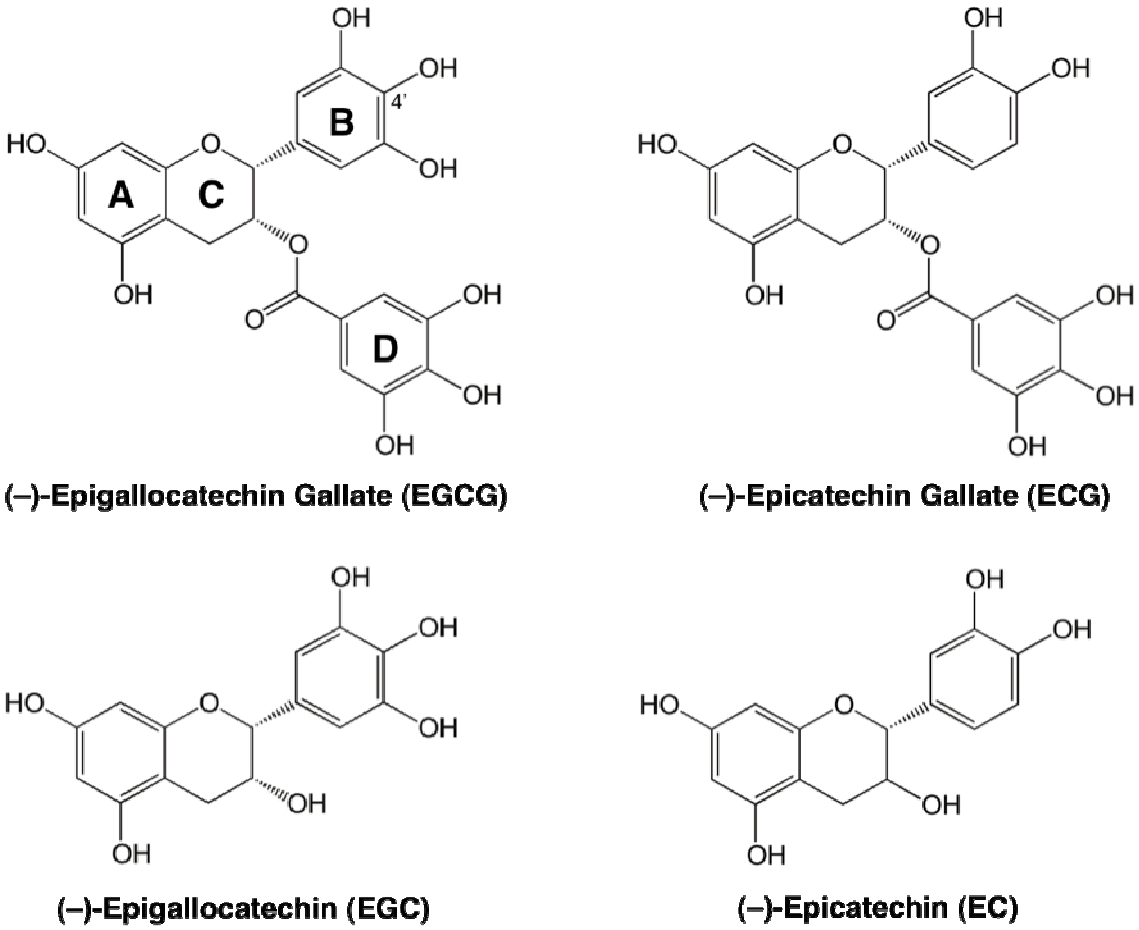


Figure 8. Structures of EGCG and related catechins.

function as covalent poisons. Therefore, they show virtually no activity against topoisomerase II. As predicted from the above, if three hydroxyl groups are included on the B-ring of a flavonol such as myricetin (see Figure 7), the compound acts as a dual function topoisomerase II poison and displays both interfacial and redox-dependent characteristics (*74*).

Dietary glucosinolates are found in cruciferous vegetables, including broccoli, cabbage, cauliflower, and kale (*78*). They are converted to bioactive isothiocyanates such as benzyl-isothiocyanate, phenethyl-isothiocyanate, and sulforaphane (Figure 9), upon hydrolysis by myrasinase (*79*). Many of these compounds inhibit cell proliferation, display chemopreventive properties, and inhibit tumor growth in xenograft models (*80-82*).

Isothiocyanates are topoisomerase II poisons *in vitro* and silencing topoisomerase IIα in cultured mouse embryonic fibroblasts decreases DNA damage induced by these compounds (*44*). As found for reactive quinone-based topoisomerase II poisons (*83*), isothiocyanates act as covalent poisons and modify several cysteine residues in human topoisomerase IIα (*44*). Consistent with a mechanism that requires cysteine modification, the ability of isothiocyanates to induce topoisomerase II-mediated DNA cleavage is abolished when compounds are co-incubated with excess glutathione (*44*).

Topoisomerase II-Associated Leukemias

Despite the importance of topoisomerase II as a target for anticancer drugs and chemopreventive agents, evidence suggests that DNA strand breaks

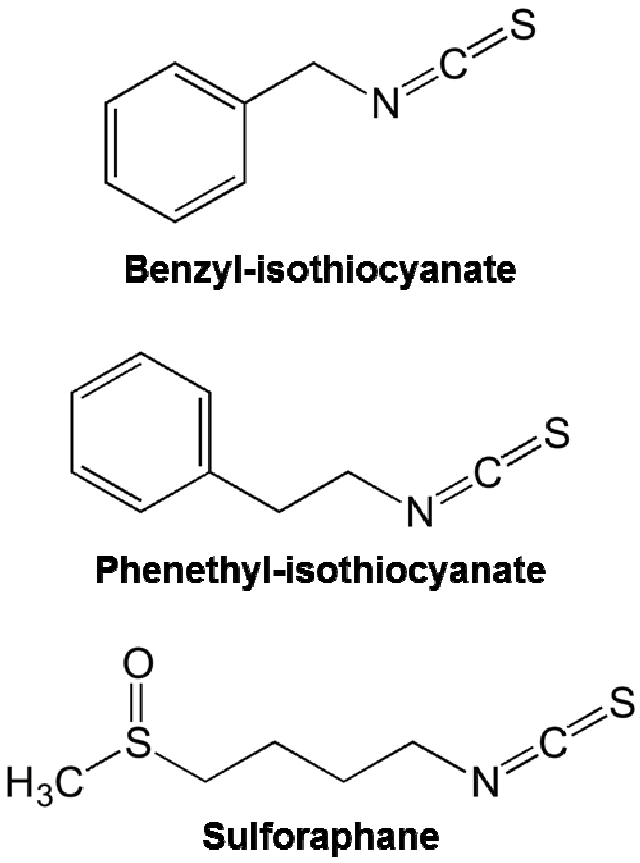


Figure 9. Structures of selected isothiocyanate-based topoisomerase II poisons.

generated by the enzyme can trigger chromosomal translocations associated with specific types of leukemia (Figure 5) (*9, 10, 37, 38, 84*). To this point, 2-3% of patients who receive regimens that include etoposide subsequently develop acute myeloid leukemias (AMLs) (*9, 10, 37, 38, 46, 84*). Most of these leukemias are characterized by translocations with breakpoints in the *MLL* (mixed lineage leukemia) gene at chromosomal band 11q23. The MLL protein is a histone methyltransferase that regulates (among other substrates) the *Hox* genes, which control proliferation in hematopoietic cells. Several breakpoints in *MLL* have been identified and are located in close proximity to topoisomerase II-DNA cleavage sites that are induced by etoposide (*85-89*).

In addition to treatment-related leukmias, ~80% of infants with AML or acute lymphoblastic leukemia (ALL) display translocations that involve the *MLL* gene (*9, 10, 37, 90*). The chromosomal translocations associated with these cancers have been observed *in utero*, indicating that infant leukemias are initiated during gestation. Epidemiological studies indicate that the risk of developing these infant leukemias increases >3-fold by the maternal consumption (during pregnancy) of foods that are rich in bioflavonoids and other naturally occurring topoisomerase II poisons (*91-93*). Consistent with this finding, treatment of cultured human cells with dietary bioflavonoids induces cleavage within the *MLL* gene (*90*). Compounds that display the highest activity in *in vitro* topoisomerase II-DNA cleavage assays show the greatest propensity to generate breaks in the *MLL* gene in cultured cells (*90*). Thus, the same topoisomerase II-active

phytochemicals that help to maintain health in human adults can have harmful effects on developing embryos.

Scope of the Dissertation

Despite the significance of topoisomerase II poisons for human health, details concerning the precise mechanism by which different classes of compounds poison the enzyme are not well understood. This dissertation seeks to further our understanding of the interaction between the type II enzyme and compounds that stimulate its activity, and to inform future studies relating to the rational design and discovery of new topoisomerase II poisons from artificial and natural sources.

An overview of DNA topology and DNA topoisomerases is provided in Chapter I. The materials and methods utilized in this dissertation are described in Chapter II.

Chapter III of this dissertation describes structure-activity relationship studies for the synthetic topoisomerase II poison amsacrine (*m*-AMSA) and investigates the role of drug-DNA interactions in the mechanism of action of this intercalative anticancer agent. This study grew out of initial efforts to explain the surprising difference in activity between the potent topoisomerase II poison *m*-AMSA and its closely related but inactive derivative *o*-AMSA. Ultimately, it was found that the head group of *m*-AMSA itself is a topoisomerase II poison. Furthermore, the role

of the intercalative acridine moiety seems to be to increase the local concentration of the drug at the active site of the enzyme.

Chapter IV of this dissertation examines the potential of the natural product curcumin—the active component of the spice turmeric, which has long been used in traditional Chinese and Ayurvedic medicine and is believed to have chemopreventive properties—to poison topoisomerase II under oxidizing conditions. The identification of quinone methide-containing metabolites of curcumin raised the question of whether some of the compound's activity might be effected through topoisomerase II. Although neither the parent compound nor the stable end product of oxidative metabolism display any activity towards the enzyme, intermediates along this pathway are shown to be potent covalent poisons of human topoisomerase II. Additionally, bioactive products of an alternate, degradative metabolic pathway of curcumin are found to possess no activity towards the type II enzyme. Finally, a complex formulation of turmeric is shown to have a stimulatory effect on human topoisomerase II-mediated DNA cleavage that reflects the activity of curcumin.

Concluding remarks and future directions, including preliminary data concerning several newly identified natural product topoisomerase II poisons, are found in Chapter V.

CHAPTER II

METHODS

Materials

Enzymes, Plasmid DNA, and Compounds

Human topoisomerase IIα, topoisomerase IIβ, and the mutant topoisomerase $IIα^{C392A/C405A}$ were expressed in *Saccharomyces cerevisiae* (*94*) and purified as described previously (*83, 95*). Human topoisomerase I was purchased from Topogen. Negatively supercoiled pBR322 DNA was prepared from *Escherichia coli* using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. [γ-^{32}P]ATP (~6000Ci/mmol) was obtained from Perkin-Elmer. *m*-AMSA and derivatives were synthesized as described previously (*96, 97*). Curcumin and 4',4"-dimethylcurcumin were synthesized as described previously (*98*). The bicyclopentadione oxidative product of curcumin was isolated from autoxidation reactions by high-performance liquid chromatography by Odaine N. Gordon. N-(4-amino-3-methoxyphenyl) methane-sulfonamide hydrochloride (*m*-AMSA head group), etoposide, vanillin, ferulic acid, feruloylmethane, thymoquinone, oleuropein and hydroxytyrosol were obtained from Sigma. The *m*-AMSA head group was stored at -20 °C as a 0.5 M stock solution in 100% DMSO. Potassium ferricyanide [$K_3Fe(CN)_6$] was obtained from Acros and was stored at -20 °C as a 50 mM stock solution in water. Turmeric was obtained from Spice Islands

Trading Company and was stored at -20 °C as a 37.5 mg/mL stock solution in 100% DMSO. All other drugs were stored at 4 °C as 20 mM stock solutions in 100% DMSO. All other chemicals were analytical reagent grade.

Procedures

Plasmid DNA Cleavage

DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff (*40*). Topoisomerase II DNA cleavage assays contained 220 nM human topoisomerase IIα, topoisomerase IIβ, or mutant topoisomerase $IIα^{C392A/C405A}$ and 10 nM negatively supercoiled pBR322 in a total of 20 µL of DNA cleavage buffer [10 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol]. DNA cleavage buffer contained ~2 µM residual dithiothreitol (DTT) that was carried over from the topoisomerase II storage buffer. Unless stated otherwise, reaction mixtures were incubated at 37 °C for 6 min, and enzyme-DNA cleavage complexes were trapped by the addition of 2 µL of 5% SDS followed by 2 µL of 250 mM EDTA (pH 8.0). Proteinase K (2 µL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. Samples were mixed with 2 µL of agarose gel loading buffer [60% sucrose in 10 mM Tris–HCl (pH 7.9), 0.5% bromophenol blue, and 0.5% xylene cyanol FF], heated at 45 °C for 5 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 µg/mL ethidium bromide. DNA bands were visualized with long-range ultraviolet

light and quantified using an Alpha Innotech digital imaging system. DNA cleavage was monitored by the conversion of supercoiled plasmid DNA to linear molecules.

Assays were carried out in the absence or presence of 0–50 µM *m*-AMSA or derivatives; 0-3.5 mM *m*-AMSA head group; 0-50 µM curcumin or derivatives (oxidation or degradation); 0-375 mg/mL turmeric solution; 0-50 µM thymoquinone; 0-200 µM etoposide; 0-200 µg/mL *Phillyrea latifolia* extract; 0-200 µM oleuropein; or 0-200 µM hydroxytyrosol. In some cases, assays were carried out in the presence of 0-50 µM $K_3Fe(CN)_6$; or in the presence of 0-3 mM DTT, which was added either before or after establishing topoisomerase II-mediated DNA cleavage complexes as indicated. Unless stated otherwise, curcumin (or a derivative) was always the last component added to corresponding reaction mixtures.

Molecular Modeling

The Calculate Energy Protocol within the Minimization Module of Discovery Studio 2.1 (Accelrys, Inc), was used for conformational space searching for *m*-AMSA and *o*-AMSA. Initially, *m*-AMSA and *o*-AMSA were input into Discovery Studio 2.1 using the Builder module. Atoms were assigned using the CHARMm forcefield. Geometries for each of the compounds were optimized using the minimization protocol within the simulation tool. Lowest energy structures for each of the compounds were derived using the conjugate gradient algorithm,

2000 steps, and a RMS gradient of 0.001. A dielectric of 1.0, nonbond list radius of 14.0, and spherical cutoff electrostatics were applied.

In the lowest energy structures, torsion angles 1 (rotation angle between C9 and the linking N) for *m*-AMSA and *o*-AMSA (-101.78^{o} and -102.82^{o}, respectively) were similar. The lowest energy torsion angles 2 (rotation angle between the linking N and C4') were -11.69^{o} for *m*-AMSA and -3.84º for *o*-AMSA. Using these rotation angles in the starting structures for *m*-AMSA and *o*-AMSA, changes to torsion 1 and torsion 2 were evaluated for their contributions to the overall potential energy of the drugs using the energy calculation module within the simulation protocol. Each of the torsion angles was modulated in ± 5º increments from its lowest energy value, and the energy was calculated for each torsion angle change. This method allowed the relative stability of the drug to be determined with respect to the lowest energy structure associated with each change in torsion angle.

DNA Cleavage Site Utilization

DNA cleavage sites were mapped using a modification (*99*) of the procedure of O'Reilly and Kreuzer (*100*). The pBR322 DNA substrate was linearized by treatment with *Hin*dIII. Terminal 5'-phosphates were removed by treatment with calf intestinal alkaline phosphatase and replaced with [^{32}P]phosphate using T4 polynucleotide kinase and [γ-^{32}P]ATP. The DNA was treated with *Eco*RI, and the 4332 bp singly-end-labeled fragment was purified from the small *Eco*RI-*Hin*dIII fragment by passage through a CHROMA SPIN+TE-100 column (Clontech).

Reaction mixtures contained 1 nM labeled pBR322 DNA substrate and 90 nM human topoisomerase IIα in 50 µL of DNA cleavage buffer supplemented with 1 mM ATP in the absence or presence of *m*-AMSA or derivatives. Reaction mixtures were incubated at 37 °C for 30 s, and enzyme-DNA cleavage complexes were trapped by the addition of 5 µL of 5% SDS followed by 3.75 µL of 250 mM EDTA (pH 8.0). Proteinase K (5 µL of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. DNA products were ethanol precipitated and resuspended in 5 µL of polyacrylamide gel loading buffer (40% formamide, 10 mM NaOH, 0.02% xylene cyanol FF, and 0.02% bromophenol blue). Samples were subjected to electrophoresis in denaturing 6% polyacrylamide sequencing gels. Gels were dried in vacuo, and DNA cleavage products were visualized with a Bio-Rad Molecular Imager FX.

DNA Intercalation

DNA intercalation was monitored as described previously (*40, 101*). When used as a substrate, relaxed plasmid DNA was generated by incubation with topoisomerase I (*40, 101*). Intercalation reaction mixtures contained 20 nM topoisomerase I, 5 nM relaxed or negatively supercoiled pBR322 DNA, and 0–150 µM *m*-AMSA or derivatives. Ethidium bromide (10 µM) and etoposide (100 µM) were included as positive and negative controls, respectively. Assays were carried out in a total of 20 µL of 50 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 50 mM KCl, 10 mM $MgCl_2$, and 0.5 mM DTT. Mixtures were incubated at 37 °C for 10

min, extracted with a phenol/chloroform/isoamyl alcohol mixture (25:24:1), and added to 3 µL of 0.77% SDS and 77 mM EDTA (pH 8.0). Samples were mixed with 2 µL of agarose gel loading buffer, heated at 45 °C for 5 min, and subjected to electrophoresis in a 1% agarose gel in 100 mM Tris-borate (pH 8.3) and 2 mM EDTA. Gels were stained with 1 µg/mL ethidium bromide, and DNA bands were visualized as described for plasmid DNA cleavage.

Competition with DNA Intercalators

A 50-bp oligonucleotide duplex was designed using a previously identified topoisomerase II cleavage site from pBR322 (*102*). Oligonucleotide sequences were generated using an Applied Biosystems DNA synthesizer. The 50-mer top and bottom sequences were 5'-TTGGTATCTGCGCTCTGCTGAAGCC↓AGTTACCTTCGGAAAAAGAGTTGGT-3' and 5'-ACCAACTCTTTTTCCGAAGGT↓AACTGGCTTCAGCAGAGCGCAGATACCAA-3', respectively (arrows denote cleavage sites). The bottom strand was labeled on the 5'-terminus with [γ-^{32}P]ATP using T4 polynucleotide kinase. Following labeling and gel purification, complementary oligonucleotides were annealed by incubation at 70 °C for 10 min and cooling to 25 °C.

DNA cleavage by human topoisomerase IIα was determined by a modification of the procedure of Fortune et al. (*102*). Reaction mixtures contained 220 nM human topoisomerase IIα and 100 nM double-stranded oligonucleotide in 10 µL of DNA cleavage buffer. Assays were carried out in the absence or

presence of 25 µM *m*-AMSA and 0–50 µM acridine, 9-aminoacridine, or ethidium bromide. Reactions were incubated for 10 min at 37 ℃. DNA cleavage products were trapped by the addition of 2 µL of 10% SDS, followed by 1 µL of 375 mM EDTA (pH 8.0). Samples were digested with proteinase K, and DNA products were ethanol precipitated and resuspended in 5 µL of polyacrylamide gel loading buffer. Samples were subjected to electrophoresis in denaturing 14% polyacrylamide sequencing gels. Gels were dried *in vacuo*, and DNA cleavage products were visualized as described above.

Cleavage of Phosphorothiolate Oligonucleotides

A duplex DNA oligonucleotide containing a single 3'-bridging phosphorothiolate linkage at the site of topoisomerase II-mediated cleavage was synthesized as described previously (*103*). DNA cleavage assays were carried out by a modification of the procedure of Fortune et al. (*40*). Oligonucleotide substrates were always 5’ end-labeled. All DNA cleavage reactions with human topoisomerase IIα contained 200 nM enzyme and 100 nM double-stranded oligonucleotide in a total of 20 µL of 10 mM Tris-HCl, pH 7.9, 135 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 2.5% glycerol. Reactions were initiated by the addition of enzyme and were incubated at 37 °C. Assays were carried out in the absence or presence of 3.5 mM *m*-AMSA head group. DNA cleavage products were trapped by the addition of 2 µL of 10% SDS followed by 2 µL of 250 mM EDTA, pH 8.0. Proteinase K (2 µL of 0.8 mg/mL) was added to digest the enzyme, and oligonucleotides were precipitated with ethanol. Cleavage products were

resolved by electrophoresis in a 14% denaturing polyacrylamide gel. To inhibit oxidation of cleaved oligonucleotides containing 3'-terminal –SH moieties and the formation of multimers in the gel, 100 mM DTT was added to the sample loading buffer. DNA cleavage products were visualized and quantified using a Bio-Rad Molecular Imager.

CHAPTER III

AMSACRINE AS A TOPOISOMERASE II POISON: IMPORTANCE OF DRUG-DNA INTERACTIONS

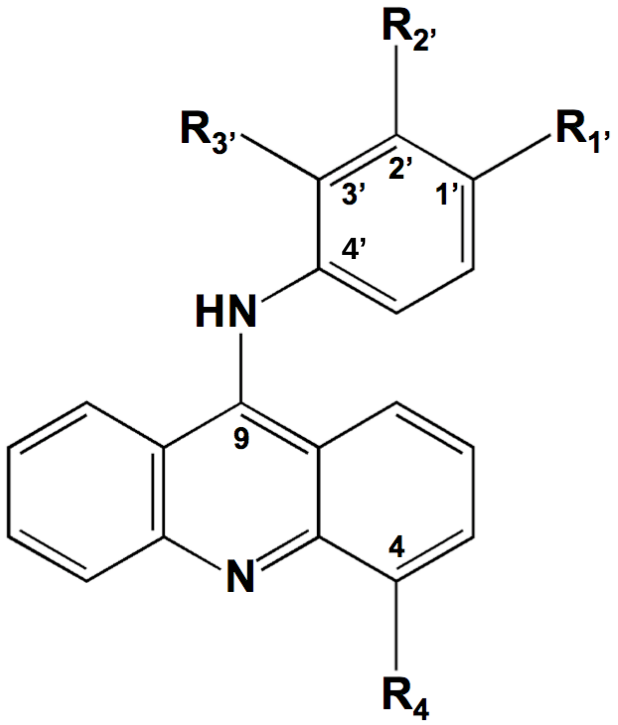
Introduction

Amsacrine (*m*-AMSA) is an acridine derivative with antineoplastic activity (*10, 15*). The drug is in multiple clinical trials for the treatment of hematological cancers in the United States (*104*) and is used to treat refractory acute lymphocytic and non-lymphocytic leukemias as well as Hodgkin's and non-Hodgkin's lymphomas in other countries (*10, 105-107*). *m*-AMSA kills cells by acting as a topoisomerase II poison and increases levels of covalent enzyme-cleaved DNA complexes primarily by decreasing rates of ligation (*9, 10, 14, 103, 108-111*). In vitro, *m*-AMSA displays similar activity toward the two isoforms of human topoisomerase II, α and β (*112, 113*). However, evidence suggests that the β isoform may be the more important target for the cytotoxic actions of the drug (*114-117*).

m-AMSA is a historically significant topoisomerase II-targeted anticancer drug. In a pioneering study published by Zwelling *et al*. in 1981, the authors proposed that *m*-AMSA targeted a topoisomerase based on the ability of the drug to induce protein-associated DNA strand breaks in treated human cells (*118*). Three years later, *m*-AMSA was the first drug demonstrated to poison mammalian topoisomerase II in vitro or in human cells (*108, 119*).

Whereas some topoisomerase II-targeted drugs, such as etoposide, have little if any interaction with DNA in the absence of enzyme (*46, 120, 121*), *m*-AMSA was designed to be a DNA binding agent (*96, 97*). To this point, *m*-AMSA is one of the most widely studied intercalative topoisomerase II poisons (*122, 123*). The drug is comprised of an acridine moiety coupled to a 4'-amino-methanesulfon-*m*-anisidide head group. It has long been known that moving the anisidide methoxy group from the *meta* (3') to the *ortho* (2') position (see Figure 10) attenuates drug activity against mammalian topoisomerase II, despite the fact that the resulting *o*-AMSA is a stronger intercalator than *m*-AMSA (*108, 118, 124-126*).

The relative activity of *m*-AMSA *vs. o*-AMSA against topoisomerase II indicates that DNA binding cannot be the sole determinant of drug function. Moreover, it brings into question the precise role of DNA intercalation in the action of *m*-AMSA and the contributions of head group substituents to topoisomerase II poisoning. Therefore, to more fully analyze structure-function relationships and the role of DNA binding in the action of *m*-AMSA, a series of derivatives was analyzed. Results indicate that much of the activity and specificity of *m*-AMSA as a topoisomerase II poison is embodied in the head group. DNA intercalation also is important for optimal drug function, being used primarily to increase the affinity of *m*-AMSA for the topoisomerase II-DNA cleavage complex.



Name	$R_{1'}$	$R_{2'}$	$R_{3'}$	R_4
m-AMSA	$-NHSO_2CH_3$	-H	$-OCH_3$	-H
AMSA	$-NHSO_2CH_3$	-H	-H	-H
o-AMSA	$-NHSO_2CH_3$	$-OCH_3$	-H	-H
1'-OH 3'-OCH_3	-OH	-H	$-OCH_3$	-H
1'-OH	-OH	-H	-H	-H
1'-OH 2'-OCH_3	-OH	$-OCH_3$	-H	-H
3'-OCH_3	-H	-H	$-OCH_3$	-H
N-Phenyl	-H	-H	-H	-H
2'-OCH_3	-H	$-OCH_3$	-H	-H
4-methyl-*m*-AMSA	$-NHSO_2CH_3$	-H	$-OCH_3$	$-CH_3$

Figure 10. Structure of *m*-AMSA and derivatives.

Results and Discussion

Contributions of m-*AMSA Head Group Substituents to Drug-Induced DNA Cleavage by Human Type II Topoisomerases*

It has long been known that the activity of *o*-AMSA as a topoisomerase II poison is dramatically lower than that of *m*-AMSA (see Figure 10 for drug structures) (*108, 118, 124-126*). As seen in Figure 11, *m*-AMSA enhanced DNA cleavage mediated by human topoisomerase IIα or topoisomerase IIβ ~7– to 8–fold as compared to no drug reactions, whereas *o*-AMSA displayed almost no ability to poison either enzyme. This difference is despite the fact that the only change between *m*- and *o*-AMSA is the position of the methoxy group (3' *vs.* 2', respectively). The molecular basis underlying this difference in drug activity has not been delineated and several questions have yet to be addressed. For example, does the low activity of *o*-AMSA reflect the loss of a critical interaction between the 3'-methoxy of *m*-AMSA and topoisomerase II or DNA, or does the 2'-methoxy of *o*-AMSA sterically hinder interactions of the drug in the enzyme-DNA complex (or a combination of both)?

Therefore, the ability of AMSA (a derivative of *m*-AMSA that is lacking the methoxy substituent) to stimulate topoisomerase II-mediated DNA cleavage was determined. If the activity of AMSA were similar to that of *m*-AMSA, it would suggest that the methoxy group does not enhance drug activity when in the 3'-position, but rather inhibits activity when in the 2'-position. Alternatively, if the activity of AMSA were similar to that of *o*-AMSA, it would imply that the 3'-methoxy is critical for topoisomerase II poisoning. Results are shown in panels A

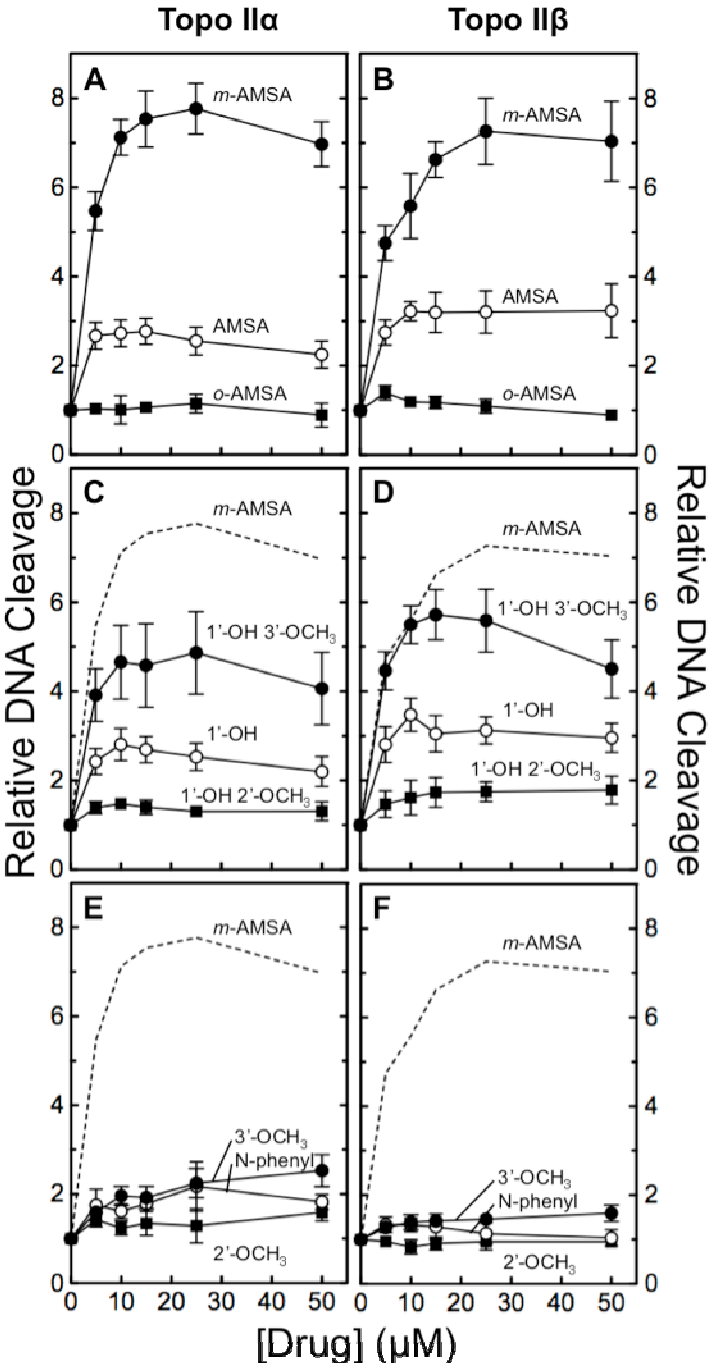


Figure 11. Enhancement of topoisomerase II-mediated DNA cleavage by *m*-AMSA and derivatives. The effects of *m*-AMSA (closed circles), AMSA (open circles), and *o*-AMSA (squares) (panels A and B), 1'-OH 3'-OCH_3 (closed circles), 1'-OH (open circles) and 1'-OH 2'-OCH_3 (squares) (panels C and D), and 3'-OCH_3 (closed circles), N-phenyl (open circles), and 2'-OCH_3 (squares) (panels E and F) on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα (panels A, C, and E) and topoisomerase IIβ (panels B, D, and F) were determined. Error bars represent the standard deviation of three independent experiments. Data for *m*-AMSA are included as dashed lines in panels C-F for comparison.

and B of Figure 11. The activity of AMSA was intermediate to those of *m*-AMSA and *o*-AMSA, increasing levels of DNA cleavage ~3–fold. This finding indicates that the presence of the 3'-methoxy positively affects drug function and is necessary for optimal activity, while the presence of the 2'-methoxy impairs drug interactions.

To further explore the role of the methoxy group in drug function, the activities of compounds that contained a 3'-, 2'-, or no methoxy were compared in two additional series: one that replaced the 1'-methanesulfonamide with a 1'-hydroxy moiety (Figure 11, panels C and D) and another that lacked a 1'-substituent (panels E and F). Results were similar to those described above for the 1'-methanesulfonamide series. Compounds with a 3'-methoxy always induced the highest levels of DNA cleavage, compounds with a 2'-methoxy had little effect on enzyme activity, and compounds lacking the methoxy were intermediate.

Although the above relationships regarding the methoxy group were consistent across the three series, the nature of the 1'-substituent had a profound effect on drug activity. Comparing compounds with a 3'-methoxy, the activity of that with a 1'-methanesulfonamide was greater than that with a 1'-hydroxy, which was much greater than that with no substituent at the 1' position. Thus, it appears that the ability of the 1'-substituent to form hydrogen bonds (or other interactions) is important for drug activity against human type II topoisomerases.

The above results indicate that the 3'-methoxy and 1'-methanesulfonamide positively impact the ability of *m*-AMSA to poison topoisomerase II, while the 2'-

methoxy of *o*-AMSA impairs this process. However, they do not provide an understanding of the underlying mechanism by which these substituents affect drug activity. Therefore, modeling studies were carried out with *m*-AMSA and *o*-AMSA to address this issue (Figure 12).

As determined by energy minimization calculations, the orientation of the head group in *m*-AMSA appears to be much more constrained than it is in *o*-AMSA (Figure 12). The lowest potential energy for *m*-AMSA (8.85 kcal/mol) was observed when torsion angle 1 (rotation angle between C9 and the linking N, see Figure 1) was set to -101.78^{o}. Torsion angle 1 could be changed by -15^{o} to +30^{o} (a total of 45^{o}) without significant changes in energy (range of 12.63 at -15^{o} to 12.1 kcal/mol at +30^{o}). Rotations past -15^{o} and +30^{o} resulted in a marked increase in the potential energy of the structure. Similar energy profiles were observed for changes to torsion angle 2 (rotation angle between the linking N and C4'). The lowest energy (8.85 kcal/mol) was observed at an angle of 11.69^{o}. Incremental changes to torsion angle 2 followed by energy calculations revealed a significant increase in the potential energy of the molecule if torsion angle 2 was rotated more than -25^{o} and +60^{o} from the starting -11.69^{o}.

In contrast to *m*-AMSA, *o*-AMSA appears to have a much broader low energy conformational space. Starting with the lowest energy conformation (-102.82^{o}), torsion angle 1 could be changed by -40^{o} to +45^{o} (a total of 85^{o}) with relatively small fluctuations in the potential energy of the molecule (range of 14.4 kcal/mol at -40^{o} to 14.8 kcal/mol at +45^{o}). The lowest energy for torsion angle 2

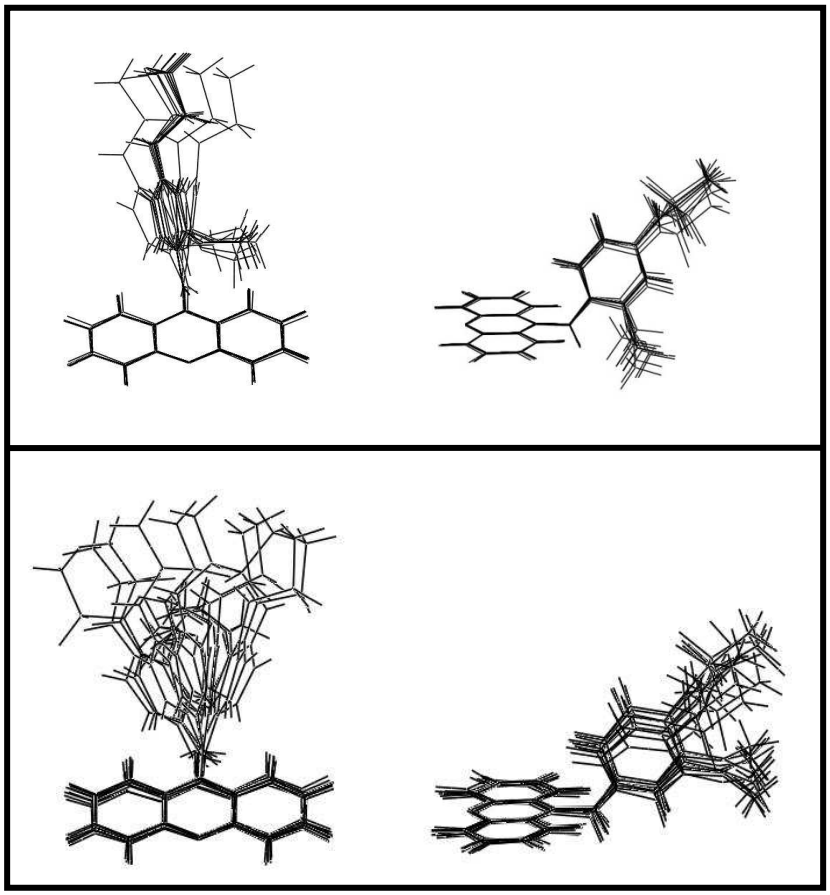


Figure 12. Energy minimization models of *m*-AMSA and *o*-AMSA. Low-energy structures (within ± 5 kcal/mol of the optimized structures for *m*-AMSA (top) and *o*-AMSA (bottom) are shown. Front and side views are shown at left and right, respectively. Courtesy of Dr. David E. Graves, University of Alabama at Birmingham.

was observed at -3.84º and the angle could be rotated with near full rotational freedom.

On the basis of these modeling studies, the following is proposed: the 3'-methoxy enhances drug activity by restricting the head group to a narrow range of favorable conformations. Conversely, when the 3'-methoxy is missing in AMSA or moved in *o*-AMSA, drug activity drops because the head group is no longer constrained to a favored orientation. Finally, the activity of *o*-AMSA is even lower than that of AMSA because, in addition to the unrestricted head group, the presence of the 2'-methoxy may impose steric constraints that further inhibit interactions of the 1'-substituent or other portions of the head group with the protein or DNA.

To determine whether changes in the above substituents affect the specificity of the drug class, sites of DNA cleaved by human topoisomerase IIα in the presence of *m*-AMSA and several derivatives were determined (Figure 13). Similar cleavage maps were observed for *m*-AMSA, AMSA (which lacks the methoxy group), and 1'-OH 3'-OCH_3 (which contains a hydroxy in place of the 1'-methanesulfonamide group of the parent drug). However, minor differences with regard to site specificity and utilization were observed. This result suggests that portions of the *m*-AMSA head group may have interactions with DNA as well as the protein in the ternary enzyme-drug-DNA complex.

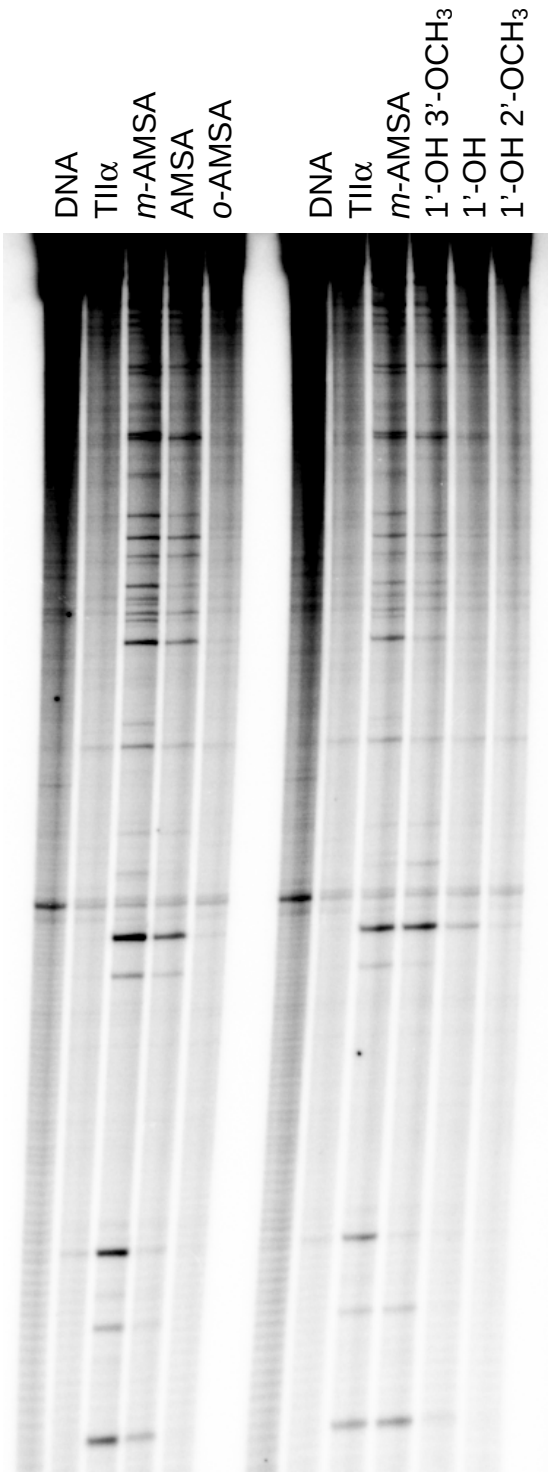


Figure 13. DNA cleavage site specificity and utilization by human topoisomerase IIα in the presence of *m*-AMSA and derivatives. A singly end-labeled linear 4332 bp fragment of pBR322 was used as the cleavage substrate. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions were carried out in the absence of drug (TIIα), or in the presence of 10 μM *m*-AMSA; 25 μM AMSA, 1'-OH 3'-OCH_3, or 1'-OH; or 100 μM *o*-AMSA or 1'-OH 2'-OCH_3. DNA standards (DNA) also are shown. Results are representative of three independent experiments.

DNA Intercalation

m-AMSA was originally designed as a DNA-binding drug (*96, 97*). However, there is no clear correlation between the strength of DNA binding (as determined by intercalation) and drug activity against topoisomerase II. As discussed earlier, *o*-AMSA, which intercalates more strongly than *m*-AMSA, displays little ability to poison the type II enzyme (*108, 118, 124-126*). Therefore, to more fully explore relationships between DNA binding and topoisomerase II poisoning, the ability of the compounds described in Figure 1 to intercalate was determined.

The DNA intercalation assay is based on the fact that intercalative agents induce constrained negative supercoils and compensatory unconstrained positive superhelical twists in covalently closed circular DNA (Figure 14, top). Therefore, as the concentration of an intercalative compound increases, a plasmid that is negatively supercoiled or relaxed (i.e., contains an equilibrium distribution of topoisomers whose mean number of superhelical twists is zero) appears to become positively supercoiled. Treatment of an intercalated plasmid with topoisomerase I removes the unconstrained positive DNA supercoils. Subsequent extraction of the compound allows the local drug-induced unwinding to redistribute in a global manner and manifest itself as a net negative supercoiling of the plasmid (Figure 14, bottom). Thus, in the presence of an intercalative agent, topoisomerase treatment converts plasmids (through a completely relaxed intermediate population) to a distribution of negatively supercoiled molecules. Representative intercalation assay gels for *m*-AMSA and all derivatives (as well as ethidium bromide) are shown in Figure 15.

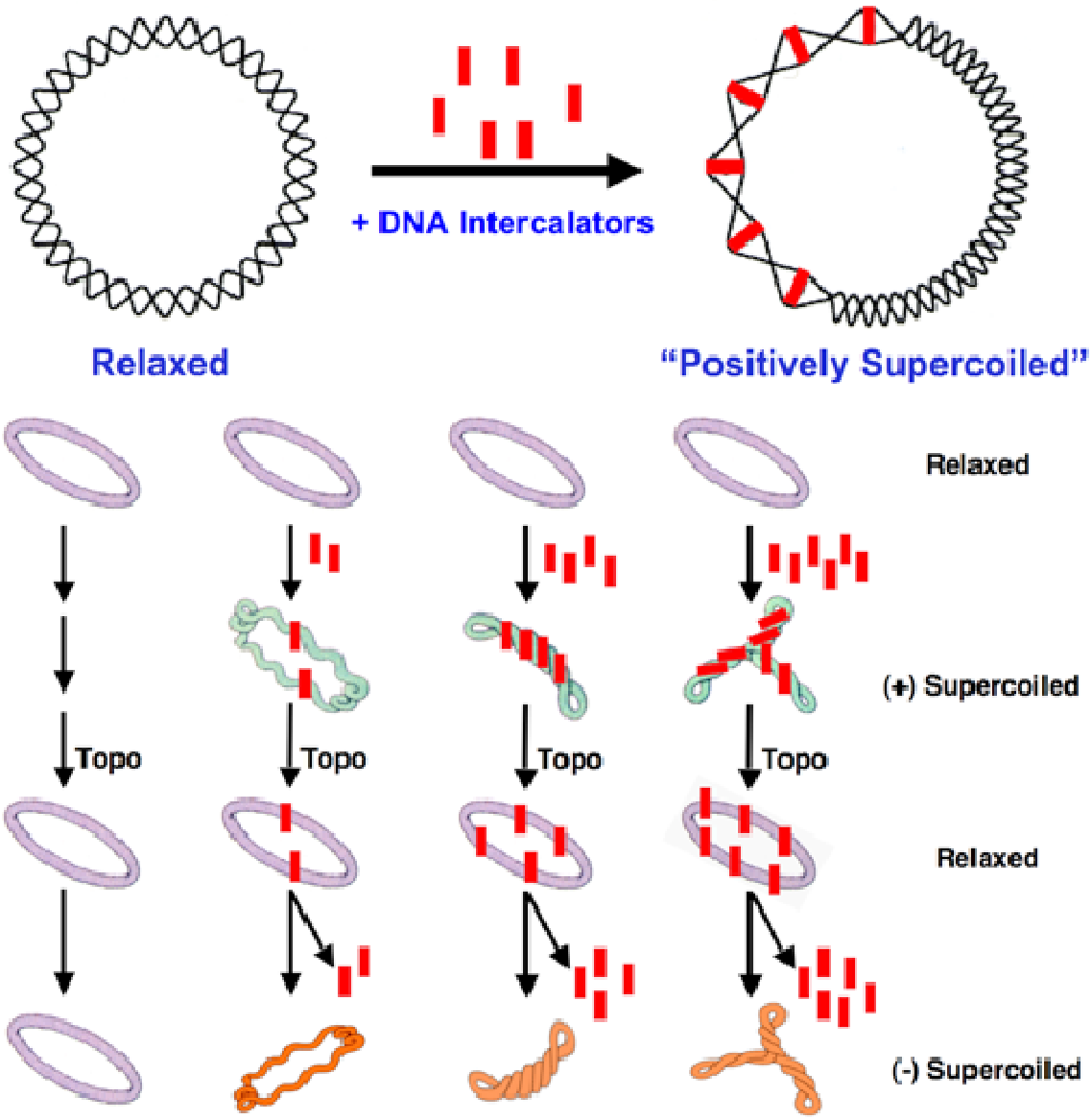


Figure 14: Schematic of the DNA intercalation assay.

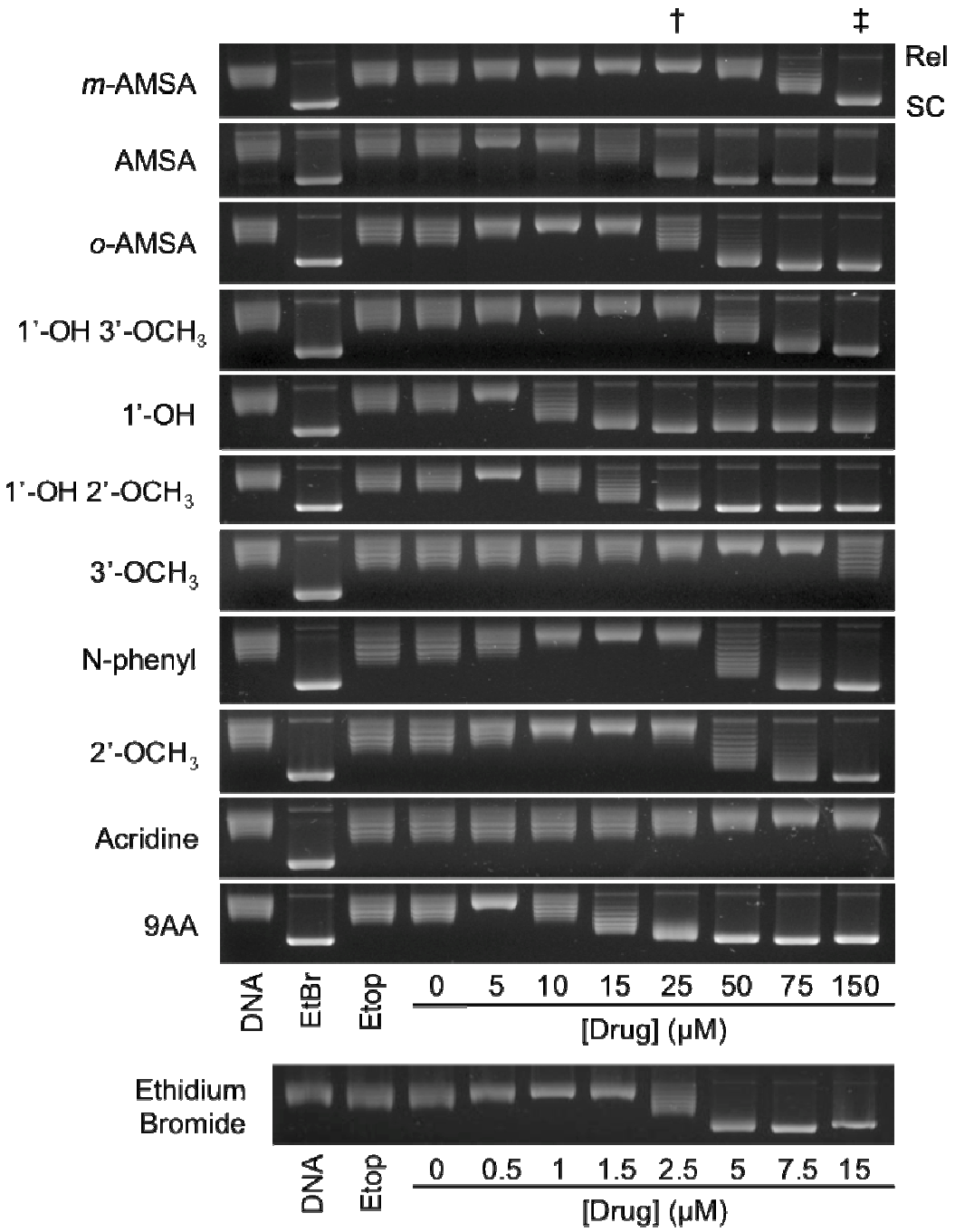


Figure 15. DNA intercalation by *m*-AMSA and derivatives. The abilities of 0–150 μM *m*-AMSA, AMSA, *o*-AMSA, 1'-OH 3'-OCH_3, 1'-OH, 1'-OH 2'-OCH_3, 3-OCH_3, N-Phenyl, 2'-OCH_3, 9-aminoacridine (9AA), acridine, and ethidium bromide to intercalate into DNA were determined using a topoisomerase I-based supercoiling assay. Representative ethidium bromide (EtBr)-stained agarose gels are shown. The effects of 10 μM EtBr and 100 μM etoposide (Etop) are included as positive and negative controls, respectively. Relaxed DNA standards (DNA) also are shown. Results are representative of three independent experiments. As described and recorded in Table 1, concentrations of intercalators required to yield "fully relaxed" (Rel) and "fully supercoiled" (SC) plasmid are used for comparative purposes. Lanes that include these concentrations for *m*-AMSA are indicated by a dagger (†) and double dagger (‡), respectively.

In order to compare the relative abilities of compounds to intercalate, two data points were employed: the concentration of drug that converts the population of plasmids to the most relaxed form (*i.e.*, the highest band seen on the gels in Figure 15) following treatment with topoisomerase I and the concentration of drug that converts the initial population to the fully supercoiled form (*i.e.*, the lowest band seen on the gels in Figure 15). These values for *m*-AMSA and all derivatives are listed in Table 1.

The chemical nature of the 1'-substituent had a consistent effect on drug intercalation into DNA, with the strength of intercalation being: 1'-hydroxy series > 1'-methanesulfonamide series > no 1'-substituent series. The presence and position of the methoxy group also affected drug intercalation in a consistent manner, with the strength of intercalation being: no methoxy > 2'-methoxy > 3'-methoxy. Despite these findings, as originally observed for *m*-AMSA and *o*-AMSA (*108, 118, 124-126*), there appears to be little correlation between the strength of DNA binding and enhancement of topoisomerase II-mediated DNA cleavage. For example, while members of the 1'-hydroxy series are stronger intercalators than corresponding members of the 1'-methanesulfonamide series, they are weaker topoisomerase II poisons.

One caveat regarding the above observations should be noted: all of the *m*-AMSA derivatives that were examined contained altered substituents on the head group. Since portions of the head group are likely to interact with topoisomerase II or the scissile bond in the cleavage complex, it is possible that the same alterations that strengthen DNA intercalation also interfere with these

Table 1. DNA intercalation by *m*-AMSA and derivatives[a]

Compound	Fully Relaxed	Fully Supercoiled
	Concentration (μM)	Concentration (μM)
m-AMSA	25	150
AMSA	5-10	50
o-AMSA	10	75
1'-OH 3'-OCH_3	15	150
1'-OH	5	25
1'-OH 2'-OCH_3	5	25-50
3'-OCH_3	50-75	>150
N-Phenyl	15-25	75
2'-OCH_3	15	150
9-aminoacridine	5	50
4-methyl-*m*-AMSA	15	75
Acridine	75	>>150
Ethidium Bromide	1	5-7.5

[a]The concentrations of compounds required to convert the plasmid substrate to a "fully relaxed" or "fully supercoiled" population was assessed by the topoisomerase I DNA supercoiling assay described in Figure 15.

critical interactions (or vice versa). This could explain the lack of correlation between drug activity and DNA intercalation. Therefore, to address the issue of DNA binding without changing substituents on the head group, the ability of 4-methyl-*m*-AMSA to intercalate DNA and poison topoisomerase IIα was evaluated. The methyl substituent in this compound is on the acridine ring. A previous crosslinking study using a photoactivated *m*-AMSA analog (*127*) demonstrated that the acridine moiety interacts with DNA in the ternary topoisomerase II-drug-DNA complex (*128*).

Intercalation studies (Figure 16 inset and Table 1) indicate that 4-methyl-*m*-AMSA binds DNA with an affinity that is approximately twice that of *m*-AMSA. Similarly, the potency of 4-methyl-*m*-AMSA (as determined by a topoisomerase IIα-DNA cleavage assay), was ~2–fold higher than that of *m*-AMSA. Despite the change in potency, the methylated and unmethylated compounds displayed comparable efficacies (maximal levels of DNA cleavage).

The correlation between DNA binding and cleavage observed for 4-methyl-*m*-AMSA suggests that intercalation plays an important role in drug action by increasing the affinity of *m*-AMSA for the ternary complex. If this suggestion is correct, inhibiting the ability of *m*-AMSA to intercalate should attenuate drug action. To test this prediction, competition experiments were carried out in the presence of acridine (a weak intercalator), 9-aminoacridine (a strong intercalator), and ethidium bromide (a stronger intercalator) (see Table 1 and Figure 15 for intercalation data).

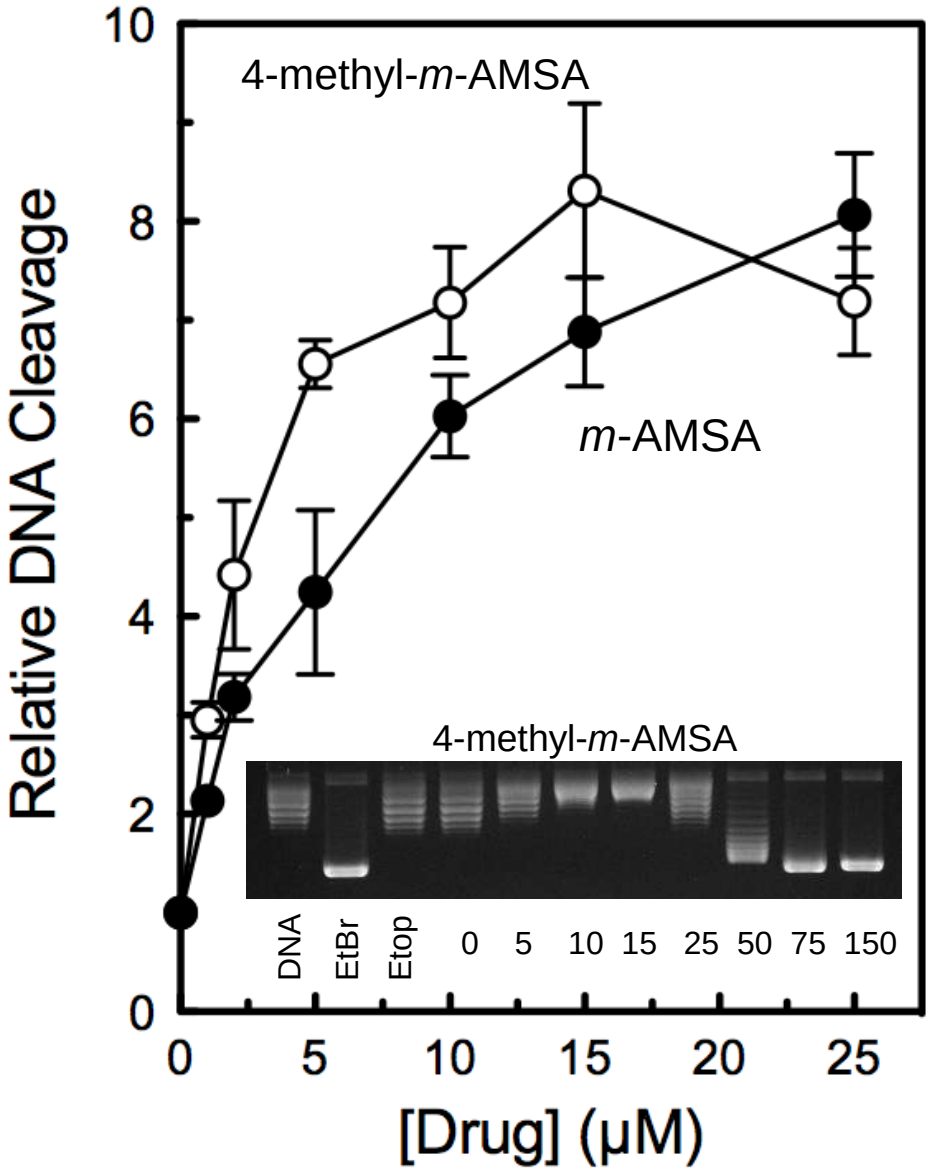


Figure 16. Enhancement of topoisomerase IIα-mediated DNA cleavage by 4-methyl-*m*-AMSA. The effects of 4-methyl-*m*-AMSA (open circles) on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα are compared to those of *m*-AMSA (closed circles). Error bars represent the standard deviation of three independent experiments. The inset shows a representative topoisomerase I-based intercalation assay for 4-methyl-*m*-AMSA.

As seen in Figure 17, acridine, 9-aminoacridine, and ethidium bromide inhibited topoisomerase IIα-mediated DNA cleavage induced by 25 μM *m*-AMSA to an extent proportional to their DNA binding strengths. Acridine showed little inhibition of DNA cleavage at 50 μM. In contrast, 9-aminoacridine and ethidium bromide inhibited *m*-AMSA-induced DNA cleavage by 50% at ~21 and ~7 μM, respectively.

It is notable that intercalators can decrease topoisomerase II-mediated cleavage by interfering with DNA binding or by altering the apparent topology of DNA (making the substrate appear to be positively supercoiled) in a closed topological system (*113*). To minimize the effects of intercalators on baseline levels of DNA cleavage mediated by human topoisomerase IIα, an oligonucleotide system was utilized for the competition experiments. Indeed, at 50 μM ethidium bromide, the highest concentration of the strongest intercalator employed (Table 1), baseline levels of enzyme-mediated DNA cleavage dropped only 16% (Figure 17, inset).

Taken together, the above findings support the conclusion that DNA intercalation plays an important role in the actions of *m*-AMSA as a topoisomerase II poison.

Activity of the m-*AMSA Head Group*

A number of topoisomerase II-targeted drugs contain a multi-ring system attached to a head group (*10, 15*). For example, the anticancer drug etoposide is comprised of a glycosylated polycyclic core (albeit non-intercalative) that is linked

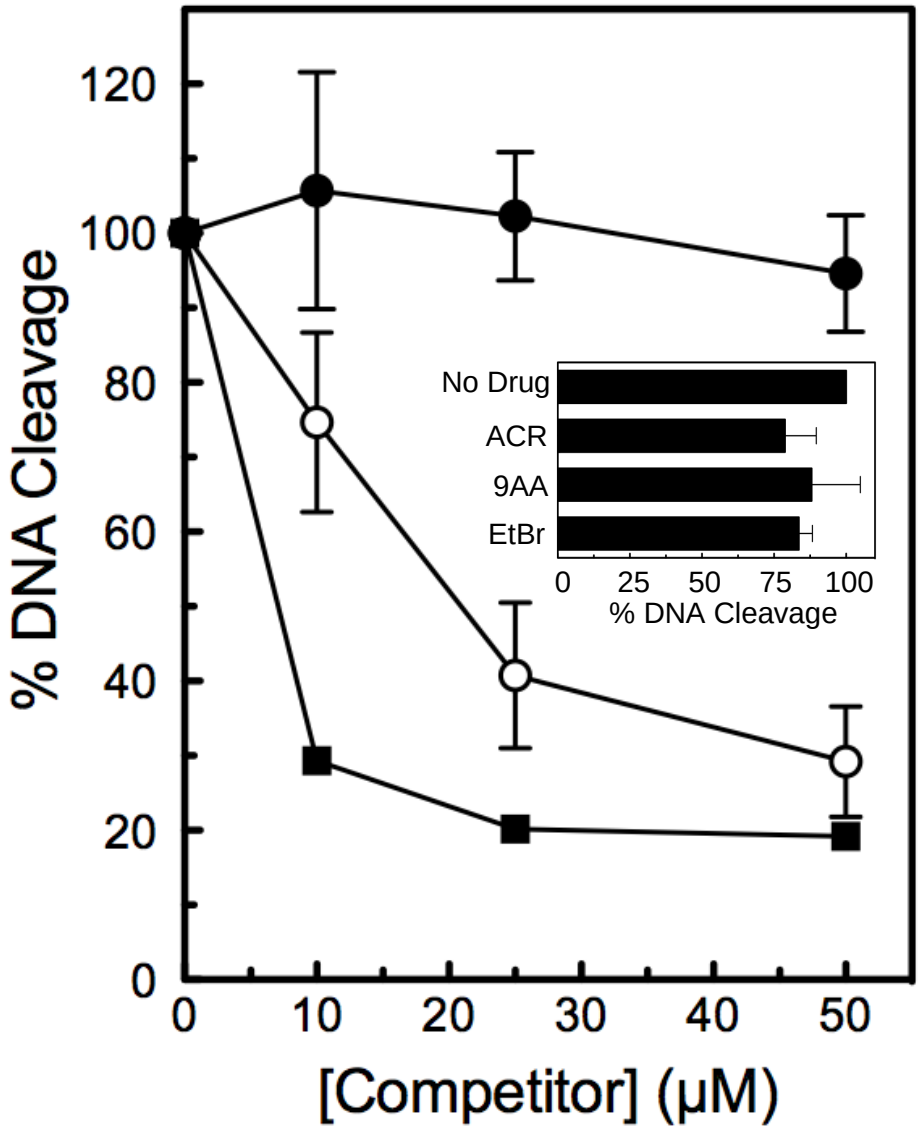


Figure 17. Inhibition of *m*-AMSA-induced topoisomerase IIα-mediated DNA cleavage by intercalators. The abilities of 0–50 μM acridine (ACR, closed circles), 9-aminoacridine (9AA, open circles), and ethidium bromide (EtBr, squares) to inhibit DNA cleavage induced by 25 μM *m*-AMSA were determined. A singly end-labeled 50-mer oligonucleotide substrate was used as the cleavage substrate. The level of DNA cleavage in the presence of 25 μM *m*-AMSA and the absence of competitor was set to 100%. The inset shows the effects of 50 μM ACR, 9AA, and EtBr on DNA cleavage mediated by human topoisomerase IIα in the absence of *m*-AMSA. The baseline level of DNA cleavage in the absence of intercalators was set to 100%. Error bars represent the standard deviation of three independent experiments for both the figure and the inset.

to a 3',5'-dimethoxy-4'-hydroxyphenyl head group (E-ring) (*10, 15, 46*). A recent structure of a covalent human topoisomerase IIβ-DNA cleavage complex formed in the presence of etoposide indicates that the polycyclic drug core interacts primarily with DNA, while the head group is positioned at the interface between the enzyme and the cleaved scissile bond and interacts with both the protein and DNA (*129*). Saturation transfer difference NMR studies of the etoposide-topoisomerase IIα binary complex coupled with activity studies also suggest strong interactions between portions of the polycyclic core/glycosyl group and DNA, and between the head group and the enzyme (*130-132*).

Consistent with the studies on etoposide, the findings described above for *m*-AMSA imply that the acridine core and head group of the drug may play different and complementary functions in stabilizing the topoisomerase II-DNA complex. It is proposed that the acridine portion of *m*-AMSA is largely responsible for DNA binding, while the head group interacts with the enzyme and the scissile bond in the ternary complex.

In order to address this hypothesis, the ability of the isolated *m*-AMSA head group to enhance DNA cleavage mediated by human topoisomerase IIα was assessed (unfortunately, the *o*-AMSA head group was unavailable for testing). As seen in Figure 18 (left panel), the detached head group stimulated DNA scission ~8– to 9–fold, which is comparable to levels observed with ~25 μM *m*-AMSA. In contrast to the parent drug, however, ~3.5 mM head group was required to induce this level of cleavage. Thus, the efficacy of the isolated head

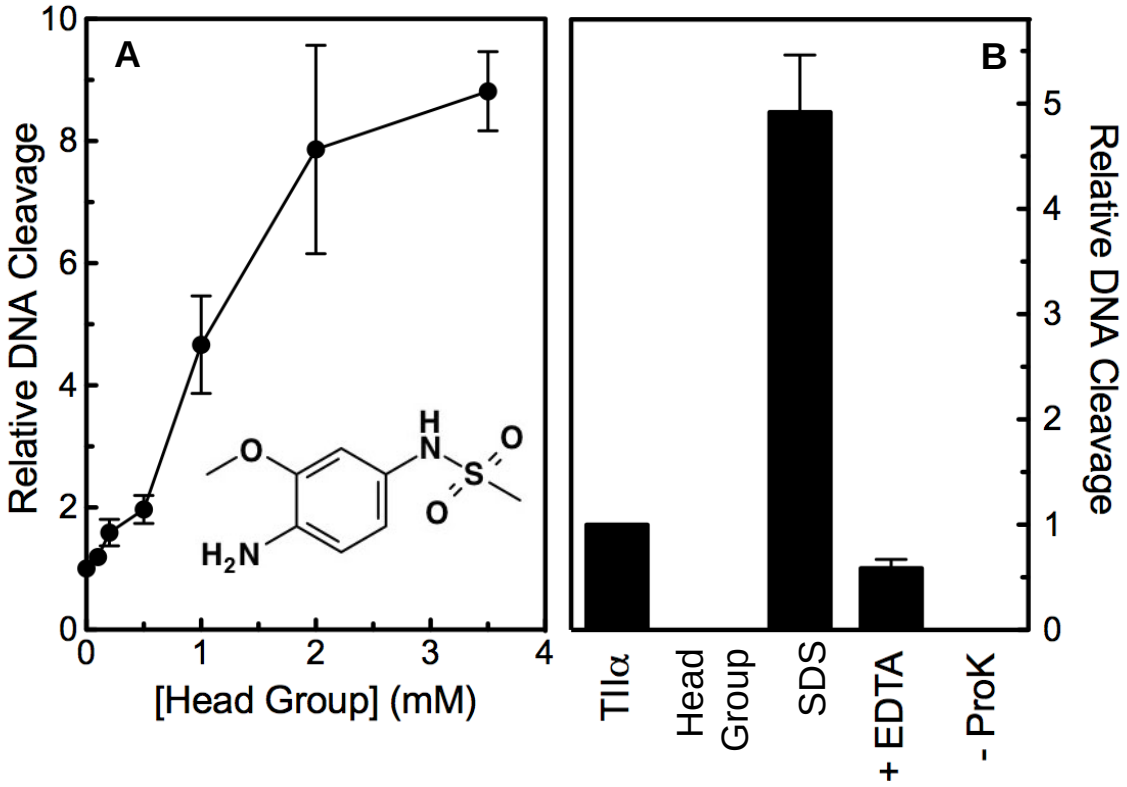


Figure 18. Enhancement of topoisomerase IIα-mediated DNA cleavage by the detached *m*-AMSA head group. Panel A shows the effects of the isolated *m*-AMSA head group (structure shown as inset) on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα. determined. Panel B shows a series of control experiments that confirm that DNA cleavage induced by the isolated head group is mediated by human topoisomerase IIα. Reactions contained DNA and enzyme in the absence of *m*-AMSA head group (TIIα), DNA and 3 mM head group in the absence of enzyme, or complete reaction mixtures treated with SDS prior to adding EDTA (SDS). The reversibility of DNA cleavage induced by 3 mM head group was determined by incubating reactions with EDTA prior to trapping cleavage complexes with SDS (EDTA). To determine whether DNA cleavage induced by 3 mM head group was protein-linked, proteinase K treatment was omitted (-ProK). Error bars for both panels represent standard deviations for three independent experiments.

group is similar to that of the parent compound, but the potency is >2 orders of magnitude lower.

To ensure that the detached head group was inducing DNA cleavage through an effect on topoisomerase IIα, several control experiments were carried out (Figure 18, right panel). No DNA cleavage was observed in the absence of enzyme. In addition, DNA scission was reversed by the addition of EDTA, which chelates the essential divalent cation (*133*), and no free linear DNA was seen in reactions that were not treated with proteinase K to digest the topoisomerase II. Thus, the isolated head group induces DNA cleavage through its effects on the type II enzyme.

As discussed in Chapter I, traditional topoisomerase II poisons stabilize enzyme-mediated DNA cleavage by blocking religation of the cleaved nucleic acid substrate (as opposed to increasing the forward rate of cleavage). To determine which of these two mechanisms is utilized by the isolated *m*-AMSA head group, cleavage of a phosphorothiolate oligonucleotide substrate was monitored over time. In this modified nucleic acid, the oxygen of the 3'-bridging phosphate at the scissile bond has been replaced with a sulfur atom. The resulting substrate is readily cleaved by topoisomerase II, but the free 3'-sulfhydryl group does not facilitate subsequent DNA religation. Thus, treatment of phosphorothiolate substrates with topoisomerase II results in an accumulation of DNA cleavage products with time, and traditional topoisomerase II poisons have little to no effect on this accumulation. Only compounds that stimulate the forward

rate of cleavage have a positive effect on cleavage of the phosphorothiolate substrate.

As seen in Figure 19, the isolated *m*-AMSA head group had no significant effect on the rate of topoisomerase II-mediated cleavage of the phosphorothiolate oligonucleotide substrate. Similar results were observed previously for the parent compound (*103*). These findings indicate that the isolated *m*-AMSA head group does not stimulate the forward rate of enzyme-mediated DNA cleavage, and by inference, that the compound inhibits DNA religation much like the parent compound *m*-AMSA.

Studies described above predict that the intercalation of *m*-AMSA is mediated by the acridine portion of the drug. If this is the case, the isolated head group would not be expected to intercalate. Indeed, even at concentrations as high as 4 mM, no intercalation by the head group was observed (Figure 20).

Both the head group and the intercalative acridine moiety are required for the high potency of *m*-AMSA as a topoisomerase II poison. Because intercalation alters the structure of DNA, acridine may increase the potency of *m*-AMSA by a specific or a general mechanism. In the first case, intercalation by the acridine moiety of an individual *m*-AMSA molecule increases the affinity of the attached head group for the enzyme and the scissile bond. In the second case, intercalation by *m*-AMSA molecules outside of the topoisomerase II active site alters the local DNA structure in a manner that increases the affinity of a different *m*-AMSA head group for the scissile bond. To distinguish between these two possibilities, the importance of the linkage between the acridine moiety and the

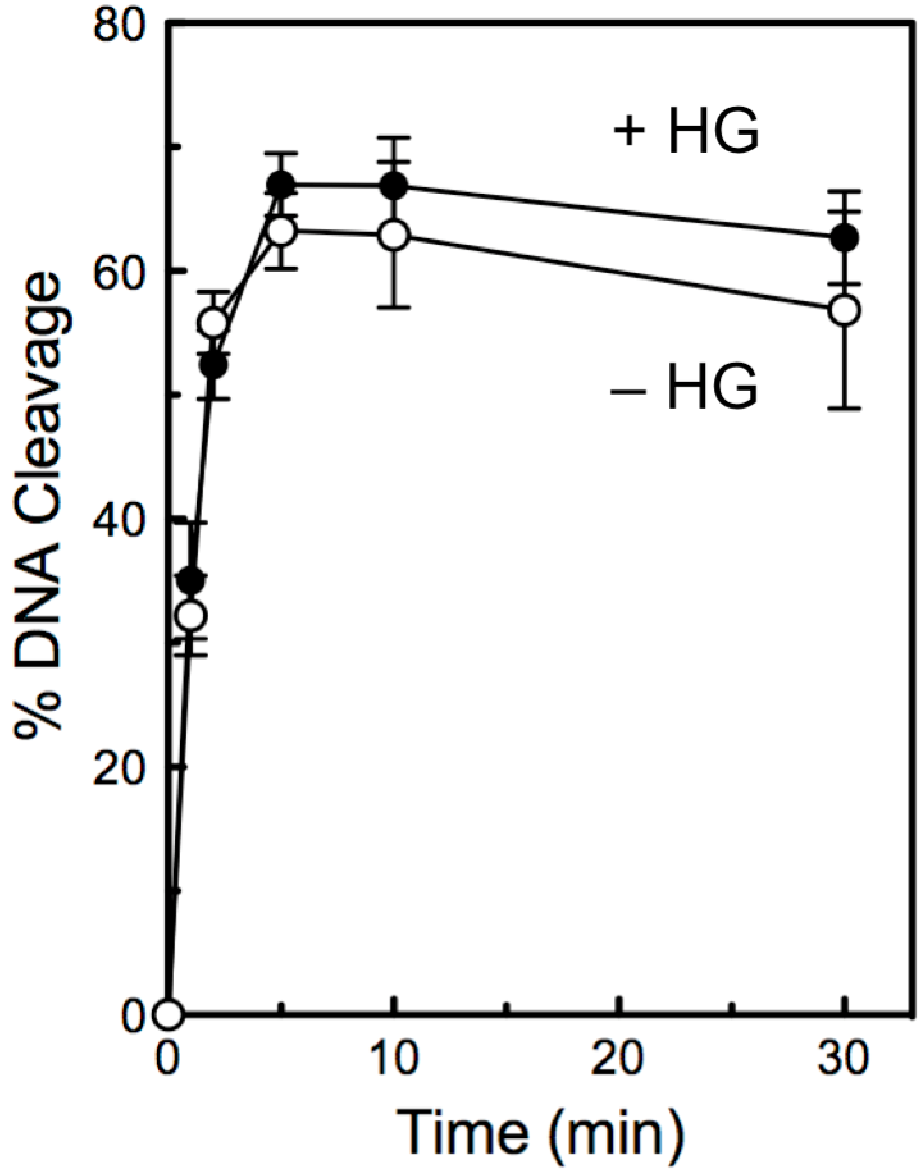


Figure 19. Effect of *m*-AMSA head group on the forward rate of topoisomerase IIα-mediated DNA scission. Time courses for cleavage of the bottom strand of the oligonucleotide containing a phosphorothiolate S-P linkage at the scissile bond of the bottom strand in the absence (– HG, open circles) or presence (+ HG, closed circles) of 3.5 mM *m*-AMSA head group. Error bars represent the standard deviation of three independent experiments.

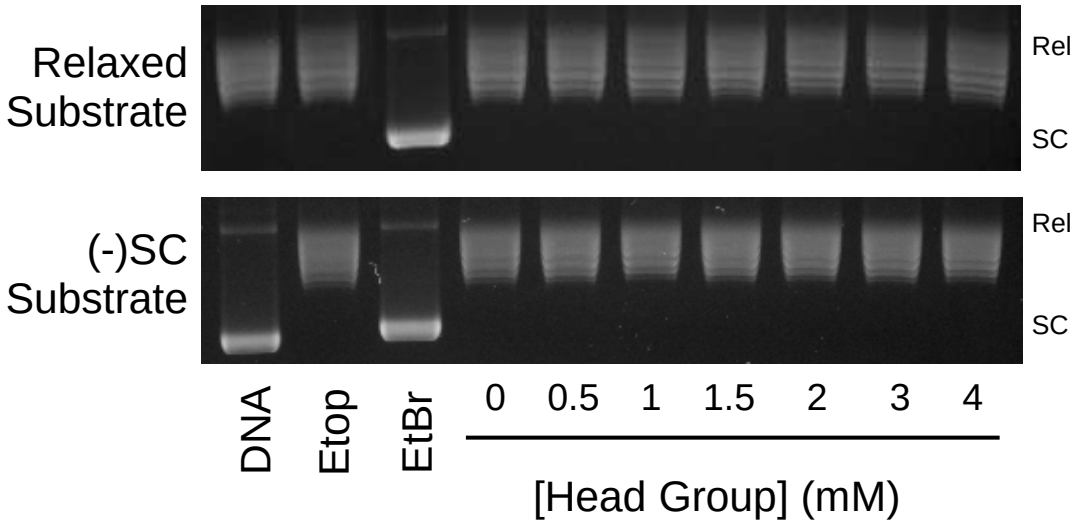


Figure 20. The isolated *m*-AMSA head group does not intercalate in DNA. Representative topoisomerase I-based DNA intercalation assay gels are shown for the isolated *m*-AMSA head group. Assays starting with either relaxed or negatively supercoiled DNA plasmid substrates are included. Etoposide (Etop, 100 µM) and ethidium bromide (EtBr, 10 µM) are included as positive and negative controls, respectively. DNA standards (DNA) also are shown. Results are representative of three independent experiments.

head group for drug function was characterized. As seen in Figure 21 the activity of a 1:1 mixture of detached head group:acridine was lower than that of the head group alone, and the potency of the mixture was considerably lower than that of *m*-AMSA (in which the head group and acridine moiety are linked). This finding provides strong evidence that the linkage between the acridine moiety and the head group is critical for the potent activity of *m*-AMSA.

Because the *m*-AMSA head group has *para* amino substituents, it has the potential to undergo redox cycling with concomitant amino-imino transformation. Previous studies indicate that quinone-based compounds can poison topoisomerase II by a mechanism that differs from that of interfacial poisons such as *m*-AMSA (*18, 42, 134, 135*). This alternate mechanism involves covalent attachment of the drug to the enzyme (*10, 18, 42, 83, 135*). It is not known whether imino-based compounds can also act as covalent poisons of topoisomerase II. Therefore, to determine if a proportion of the activity of the *m*-AMSA head group against human topoisomerase IIα reflects a covalent mechanism, the reducing agent DTT was added to DNA cleavage reactions. DTT blocks redox cycling, and reduces quinones to the corresponding hydroquinones and imines to the corresponding amines. Thus, it abrogates the effects of covalent poisons on topoisomerase II.

No significant decrease in the activity of *m*-AMSA was seen in the presence of DTT, but incubation of the head group with the reducing agent did lower drug activity (Figure 22). However, because the head group retained the

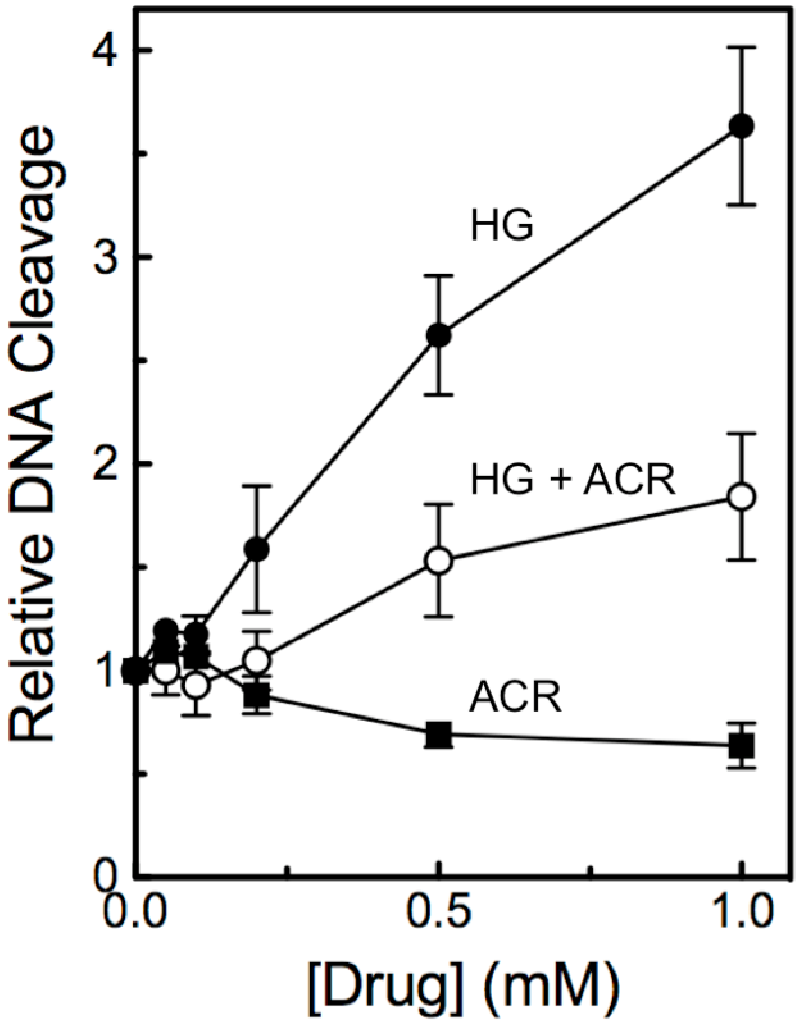


Figure 21. Covalent linkage of the head group to the acridine moiety is necessary for the high potency of *m*-AMSA as a topoisomerase II poison. The ability of the detached head group (HG, closed circles) or a 1:1 mixture of head group + acridine (HG + ACR, open circles) to stimulate topoisomerase IIα-mediated DNA cleavage is shown. A control experiment assessing the effects of acridine alone on the DNA cleavage activity of topoisomerase IIα (ACR, squares) also is shown. Error bars represent the standard deviation of three independent experiments.

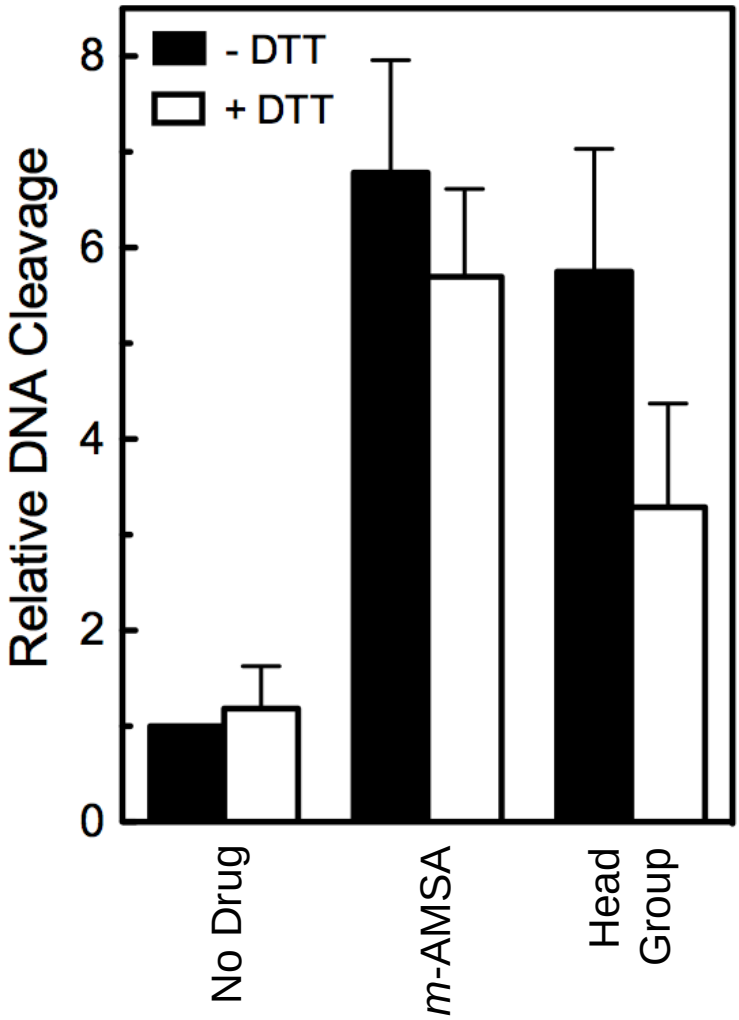


Figure 22. Partial redox-dependence of the isolated *m*-AMSA head group as a topoisomerase II poison. The effects of 25 μM *m*-AMSA or 3 mM *m*-AMSA head group on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα in the absence (closed bars) or presence (open bars) of 3 mM dithiothreitol (DTT) are shown. A control experiment carried out in the absence of drugs also is shown (No Drug). DNA cleavage levels are relative to those induced by the enzyme in the absence of drug or DTT. Error bars represent the standard deviation of three independent experiments.

majority of its activity in the presence of DTT, it is concluded that at least part of its activity toward topoisomerase IIα reflects a traditional, redox-independent mechanism like that of the parent drug.

To further explore the properties of the head group, sites of topoisomerase IIα-mediated DNA cleavage induced by the compound were determined. Sites cleaved in the presence of the head group represented a subset of those induced by *m*-AMSA (Figure 23). Therefore, the head group appears to be responsible for much of the specificity of the drug.

Conclusions

Although *m*-AMSA was the first compound shown to poison eukaryotic topoisomerase II, the specific functions of the individual components of the drug are still undefined. Taken together, the findings of the present study suggest that the activity and specificity of *m*-AMSA reside largely in the head group. Both the 3'-methoxy and 1'-methanesulfonamide substituents contribute positively to drug efficacy. Finally, the linkage between the head group and the intercalative acridine moiety provides a strong DNA anchor for the drug and, consequently, dramatically increases the affinity of *m*-AMSA for the topoisomerase II-DNA cleavage complex.

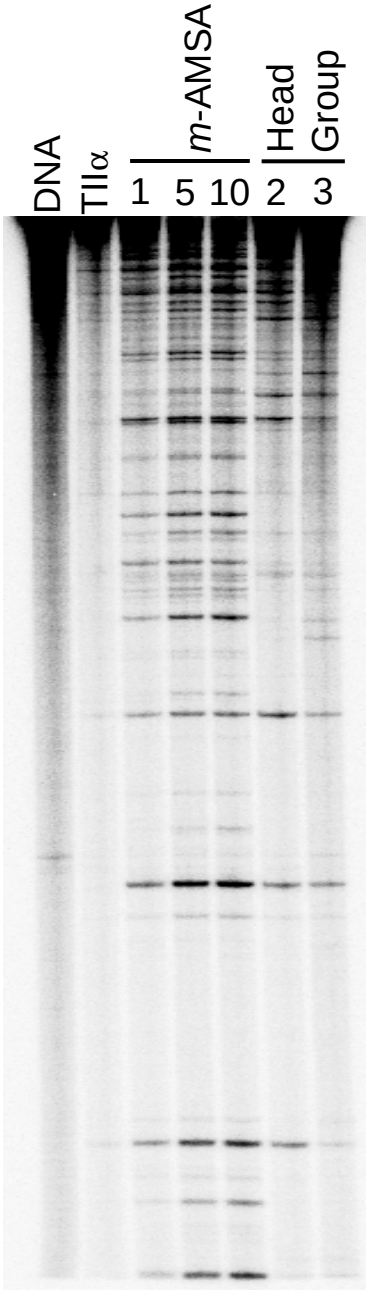


Figure 23. DNA cleavage site specificity and utilization by human topoisomerase IIα in the presence of the *m*-AMSA head group. A singly end-labeled linear 4332 bp fragment of pBR322 was used as the cleavage substrate. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions were carried out in the absence of drug (TIIα), or in the presence of 1, 5, or 10 µM *m*-AMSA or 2 or 3 mM head group. A DNA standard (DNA) also is shown. Results are representative of three independent experiments.

CHAPTER IV

OXIDATIVE METABOLITES OF CURCUMIN POISON HUMAN TYPE II TOPOISOMERASES

Introduction

Turmeric is a common spice that is used in curries and a variety of other Asian cuisines (*136-138*). It is isolated from the rhizomes of *Curcuma longa*, which is an herbaceous perennial of the ginger family (*138*).

Curcumin (Figure 24) is the principal flavor and color component of turmeric. Beyond its culinary uses, curcumin is believed to positively impact human health and commonly is used in traditional Chinese herbal medicine and Ayurvedic medicine (*137, 138*). The compound has antioxidant, anti-inflammatory, and antibacterial activities (*139, 140*). Furthermore, it appears to have chemopreventive properties against a variety of human malignancies and currently is in clinical trials as an anticancer agent (*139-144*).

Curcumin has poor oral bioavailability and is unstable under physiological conditions (*98, 145-148*). Thus, it has been suggested that curcumin metabolites mediate at least some of the biological effects of the parent compound (*149-152*). Several metabolic pathways have been proposed. Following oral administration, curcumin often is conjugated to form glucuronides or sulfates. Alternatively, if administered intraperitoneally, it can undergo reductive reactions to form a variety of hydrogenated curcuminoids. However, most studies have concluded

that neither the conjugated nor the reduced products of curcumin are biologically active (*152*).

Curcumin also can undergo spontaneous autoxidation in aqueous solutions at physiological pH (Figure 24) (*98*). This reaction gives rise to novel products that have potential for biological activity. Autoxidation of curcumin is stimulated by peroxidases and oxidizing agents and is initiated by hydrogen abstraction from one of the two phenolic hydroxyl moieties (*98*). Following this abstraction, the reaction is proposed to proceed through several unstable and reactive intermediates, including an electrophilic quinone methide radical (*98*). The final product of curcumin autoxidation is a dioxygenated bicyclopentadione (*98*).

In addition to oxidation, the heptadienone chain of curcumin can be fragmented into vanillin, ferulic acid, feruloylmethane, and related compounds (see Figure 31) (*152*). These fragmentation products have been shown to display antioxidant and anti-inflammatory properties (*152*). However, at physiological pH, degradation of the curcumin heptadienone chain appears to be a minor reaction (*153*).

As discussed in Chapter I, several naturally occurring polyphenols that can form quinones display activity against human type II topoisomerases (*10*). Many of these, including bioflavonoids such as myricetin (which is common in grapes, berries, and other fruits and vegetables) and catechols such as EGCG (which is the active polyphenol in green tea) (*16-18*) are believed to have chemopreventive properties (*54, 72, 73*). All of these compounds increase levels of DNA cleavage mediated by the type II enzymes (*16-18*).

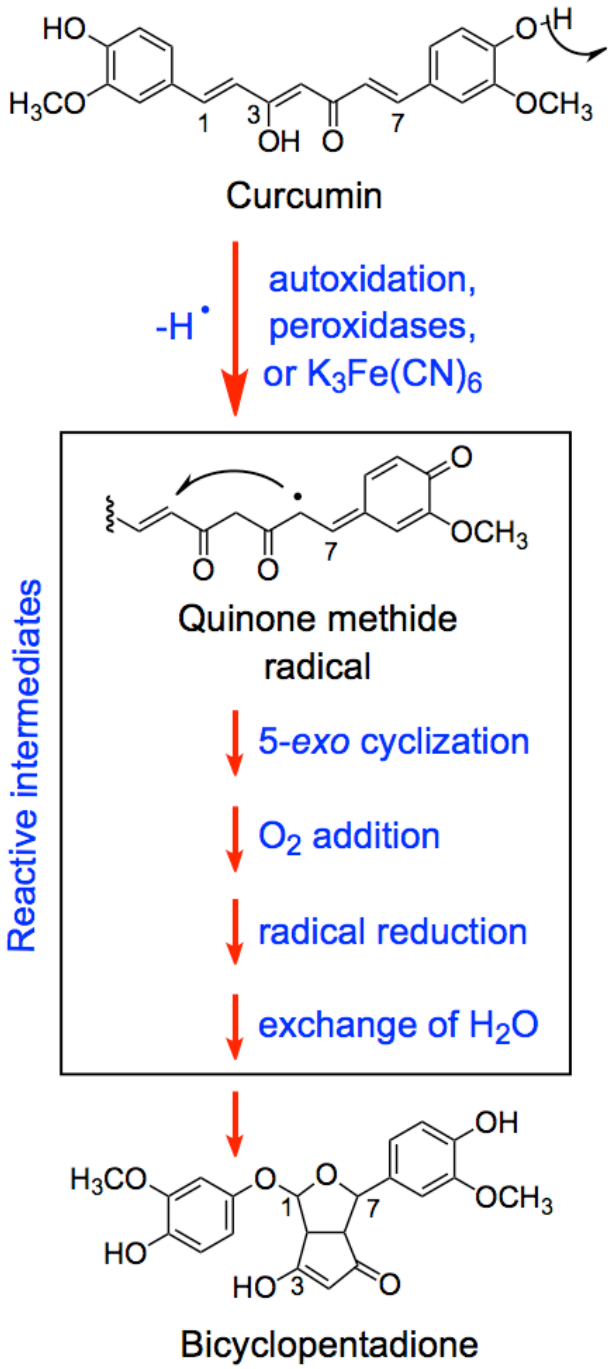


Figure 24. Oxidative transformation of curcumin. Adapted from Griesser *et al*. (*98*)

Treatment of human cells with curcumin induces DNA cleavage complexes formed by topoisomerase IIα and IIβ (*154*). Cleavage complex formation is prevented by the addition of an antioxidant, suggesting the importance of oxidative pathways in curcumin activity against the type II enzymes (*154*).

An earlier study found that curcumin could induce topoisomerase II-mediated DNA cleavage *in vitro* (*155*). However, because curcumin can undergo oxidation and degradation in aqueous solution, it is not clear whether the parent compound or metabolites (or both) are the topoisomerase II-reactive species. Therefore, the ability of curcumin to poison human type II topoisomerases under conditions in which the compound remains stable or undergoes oxidation was tested. Additionally, the activities of vanillin, ferulic acid, and feruloylmethane toward the human enzymes were examined. Results indicate that oxidative metabolites of curcumin poison human topoisomerase IIα and IIβ. In contrast, neither the parent compound nor its fragmentation products displayed significant activity toward the human enzymes.

Results and Discussion

Oxidative Metabolites of Curcumin Enhance DNA Cleavage Mediated by Human Type II Topoisomerases

Although curcumin increases levels of DNA cleavage mediated by topoisomerase IIα and IIβ in cultured human cells (*154*), the ability of the compound to affect enzyme activity in purified systems has not been well characterized. Therefore, the effects of the phytochemical on the human type II

enzymes were determined. As seen in Figure 25, curcumin displayed no activity toward either topoisomerase IIα and IIβ. However, in the presence of an oxidizing agent, such as potassium ferricyanide [$K_3Fe(CN)_6$], curcumin became a potent topoisomerase II poison. Between 4– and 5–fold DNA cleavage enhancement was observed with human topoisomerase IIα and IIβ, respectively. The activation of curcumin required stoichiometric concentrations of $K_3Fe(CN)_6$, and the oxidant had no effect on topoisomerase II-mediated DNA cleavage in the absence of the phytochemical (Figure 26, left).

Curcumin undergoes rapid oxidation in aqueous solutions (Figure 26, top right) (*98*). However, the compound was much more stable in the buffer used for topoisomerase II-mediated DNA cleavage assays, and little oxidation was observed over the 6-min course of the reaction (Figure 26, bottom right). The increase in curcumin stability was due largely to the $MgCl_2$ that was included in the assay buffer (data not shown). Complete oxidation of curcumin was observed in assay mixtures shortly after addition of $K_3Fe(CN)_6$ (Figure 26, bottom right).

Several control reactions with human topoisomerase IIα were performed to ensure that the DNA cleavage enhancement observed with oxidized curcumin was mediated by the type II enzyme (Figure 27). No DNA scission was seen in the presence of curcumin and $K_3Fe(CN)_6$ when the type II enzyme was left out of reactions. Furthermore, topoisomerase IIα-mediated DNA cleavage induced by oxidized curcumin was reversed when the active site Mg^{2+} ions were chelated with EDTA prior to trapping cleavage complexes with SDS. This reversibility is not consistent with an enzyme-independent reaction. Finally, cleaved plasmid

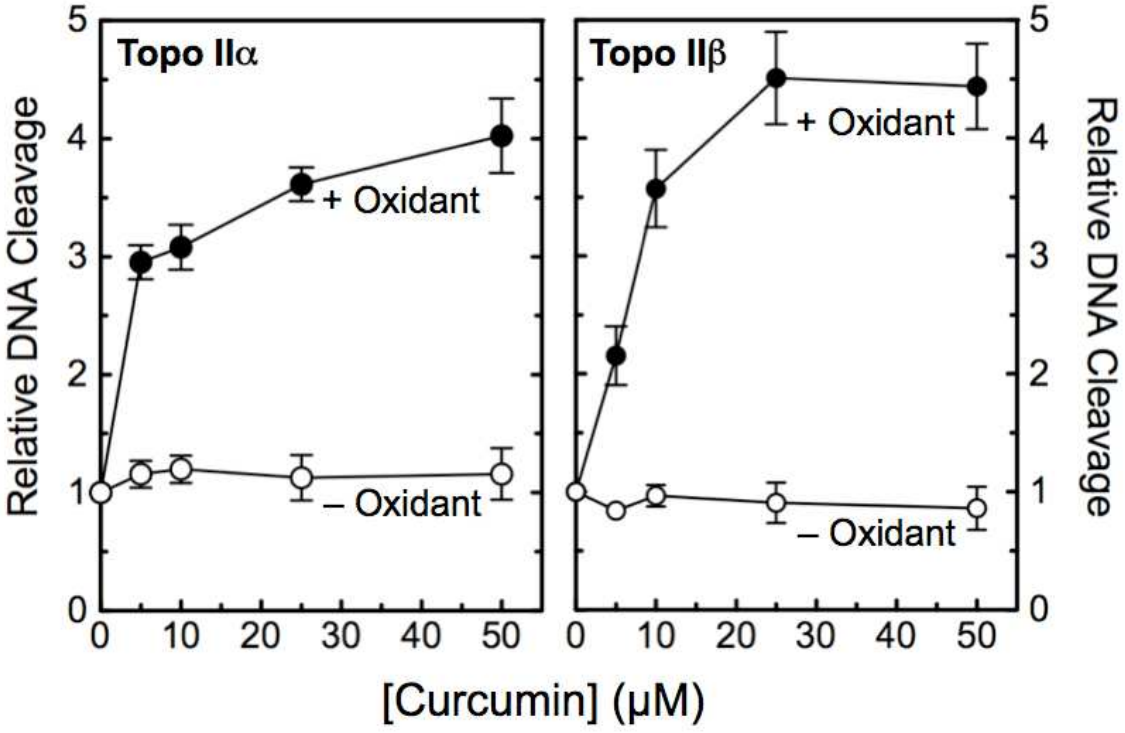


Figure 25. Enhancement of topoisomerase II-mediated DNA cleavage by curcumin in the presence of oxidant. The effects of curcumin on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα (left) and topoisomerase IIβ (right) were determined in the absence (open circles, – Oxidant) or presence (closed circles, + Oxidant) of 50 μM $K_3Fe(CN)_6$. Error bars represent standard deviations for three independent experiments.

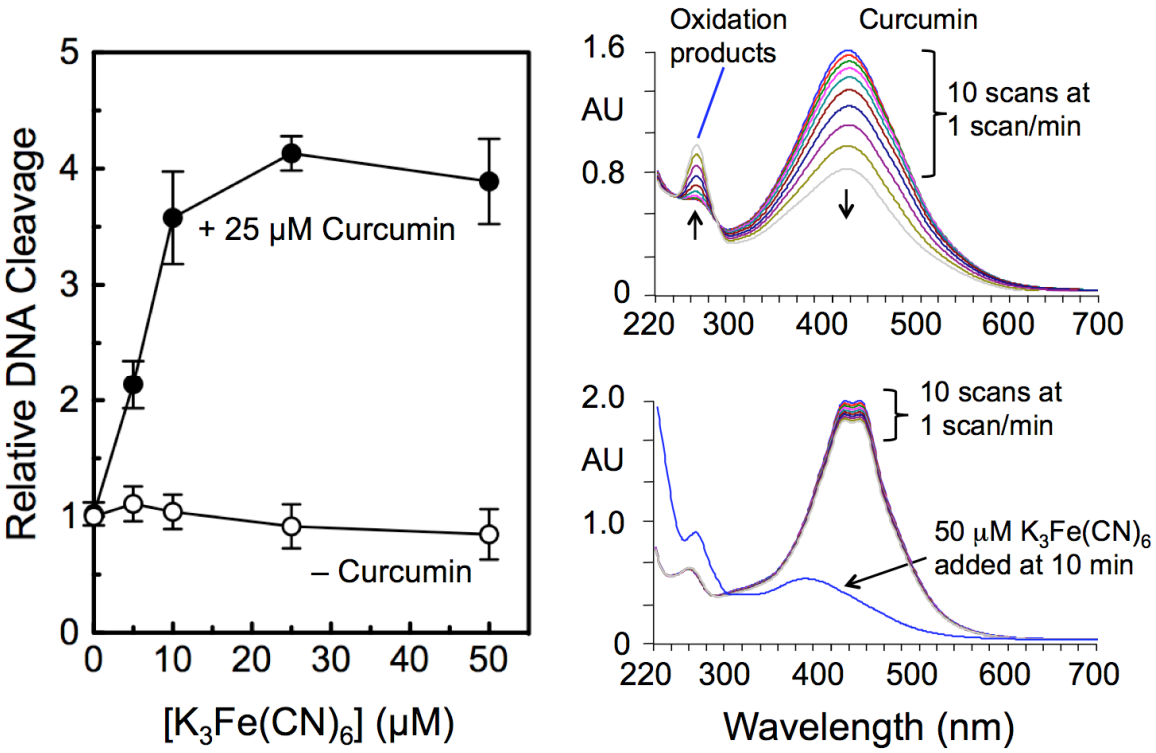


Figure 26. Effects of $K_3Fe(CN)_6$ on curcumin oxidation and the DNA cleavage activity of human topoisomerase IIα. Left: The effects of $K_3Fe(CN)_6$ on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα were determined in the absence (open circles) or presence (closed circles) of 25 µM curcumin. Error bars represent standard deviations for three independent experiments. Right: Ultraviolet/visible spectroscopic analysis of the loss of curcumin (maximum wavelength at 430 nm) and appearance of oxidized products (peak at 263 nm) in Tris-HCl buffer (pH 7.9) (top) and in topoisomerase II assay buffer (pH 7.9) (bottom). Scans were obtained at a frequency of one per min. $K_3Fe(CN)_6$ was added to the reaction in topoisomerase II assay buffer at 10 min. Spectra courtesy of Odaine N. Gordon, Vanderbilt University.

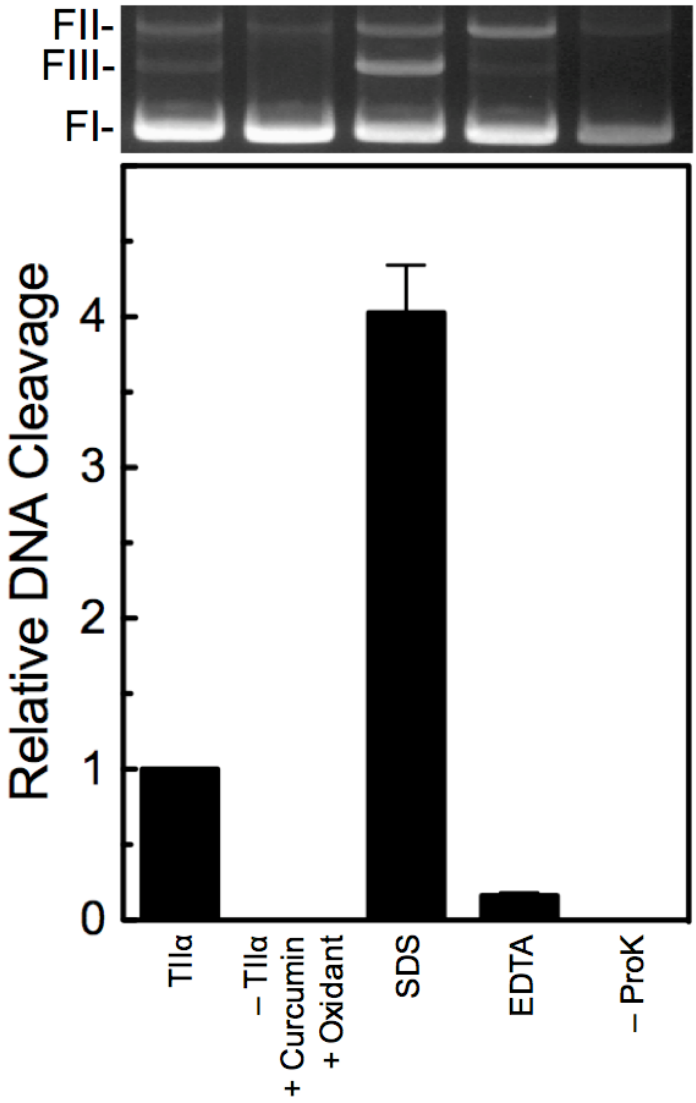


Figure 27. DNA cleavage induced by oxidized curcumin is reversible and protein-linked. Assay mixtures contained enzyme in the absence of curcumin or oxidant (TIIα), 50 µM curcumin and 50 µM $K_3Fe(CN)_6$ in the absence of enzyme [-TIIα□+ Curcumin + Oxidant], or complete reactions treated with SDS prior to adding EDTA (SDS). To determine whether DNA cleavage induced by oxidized curcumin was reversible, reactions were incubated with EDTA prior to trapping cleavage complexes with SDS (EDTA). To determine whether DNA cleavage induced by oxidized curcumin was protein-linked, proteinase K treatment was omitted (-ProK). Error bars represent standard deviations for three independent experiments. A representative agarose gel is shown at the top. DNA lanes correspond to the bars shown in the graph. The positions of negatively supercoiled (form I, FI), nicked (form II, FII), and linear (form III, FIII) plasmid DNA are indicated.

products were covalently linked to topoisomerase II. In the absence of proteinase K, the linear DNA band disappeared and was replaced by a band that remained at the origin of the gel (not shown). These results demonstrate that the DNA scission observed in the presence of curcumin and $K_3Fe(CN)_6$ is mediated by the type II enzyme.

The findings described above provide strong evidence that oxidized metabolites of curcumin, rather than the parent compound, are responsible for the enhancement of topoisomerase II-mediated DNA cleavage. As further evidence supporting this conclusion, the ability of 4',4"-dimethylcurcumin to poison human topoisomerase IIα was determined. Since the methyl groups protect the 4'- and 4"-hydroxyl moieties from hydrogen abstraction, the compound undergoes oxidation rates that are >1000–fold slower than that of curcumin (*98*). As seen in Figure 28, no enhancement of topoisomerase II-mediated DNA cleavage was observed in the absence or presence of $K_3Fe(CN)_6$.

Upon treatment with an oxidizing agent, curcumin is rapidly converted to a stable bicyclopentadione (Figures 24 and 26). En route to this ultimate oxidation product, the parent compound moves through a series of reactive quinone methide intermediates (Figure 24) (*98*). As discussed below, a number of quinone-based compounds have been shown to poison type II topoisomerases (*41, 42, 134, 135*). Therefore, two experiments were carried out to determine whether the quinone methide intermediates (as opposed to the final bicyclopentadione) are the more likely compounds that poison topoisomerase II in the presence of an oxidant. In the first experiment, curcumin was incubated

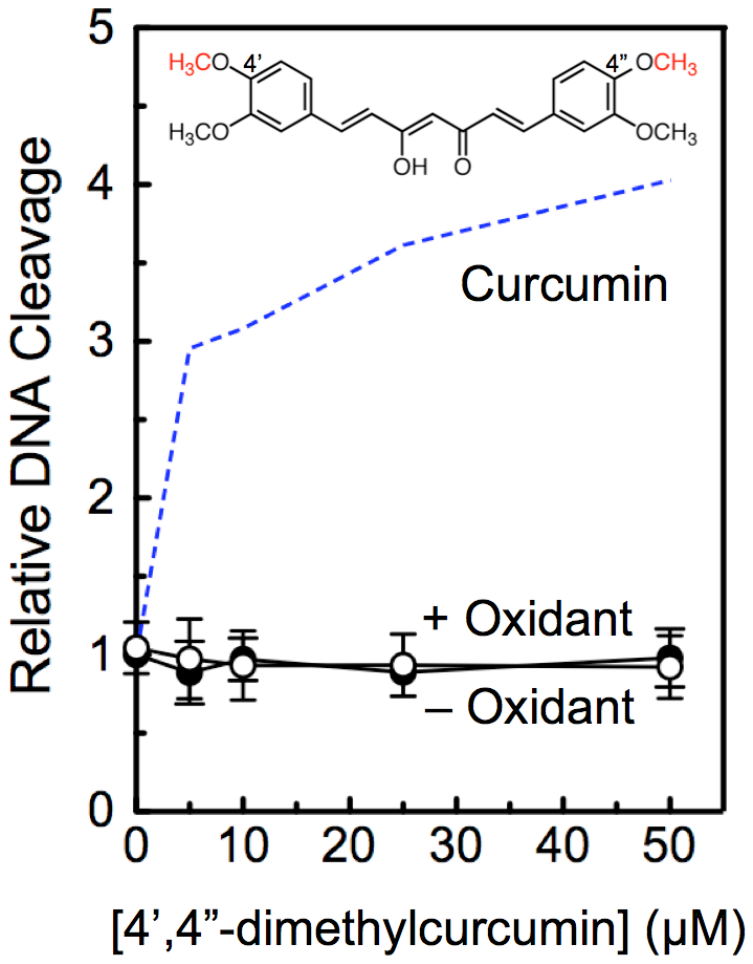


Figure 28. Effects of 4',4"-dimethylcurcumin on topoisomerase II-mediated DNA cleavage. The effects of 4',4"-dimethylcurcumin (structure at top, 4'- and 4"-methyl groups highlighted in red) in the absence (open circles, – Oxidant) or presence (closed circles, + Oxidant) of 50 μM $K_3Fe(CN)_6$ on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα were determined. Data for oxidized curcumin (dashed line from Figure 2) are included for comparison. Error bars represent standard deviations for three independent experiments.

with $K_3Fe(CN)_6$ for 10 min prior to its addition to a topoisomerase IIα-DNA cleavage reaction. Under these conditions, the majority of the parent phytochemical was converted to the bicyclopentadione (Figure 26, bottom right). As seen in Figure 29 (inset), no enhancement of DNA scission was observed. In the second experiment, purified bicyclopentadione was added to DNA cleavage assays in the absence or presence of $K_3Fe(CN)_6$ (Figure 29). Virtually no enhancement of topoisomerase IIα-mediated DNA cleavage was observed under either condition. Thus, it appears that the quinone methide metabolites of curcumin are the chemical species that poison topoisomerase II.

Oxidative Metabolites of Curcumin Are Covalent Topoisomerase II Poisons

To reemphasize a point discussed in Chapter I, compounds that poison type II topoisomerases can be categorized into two broad classes: interfacial *vs.* covalent (formerly "redox-dependent"). Members of the first group interact with topoisomerase II at the protein-DNA interface (in the vicinity of the active site tyrosine) in a non-covalent manner (*10, 14, 156*). Interfacial topoisomerase II poisons include several anticancer drugs (*e.g.*, etoposide) and dietary bioflavonoids (*e.g.*, genistein) and display a number of characteristic properties (*10, 18, 41-43, 74, 134*). Primarily, because their actions against topoisomerase II do not depend on redox chemistry, they are unaffected by the presence of reducing agents. Furthermore, these compounds induce similar levels of enzyme-mediated DNA scission whether they are added directly to the binary

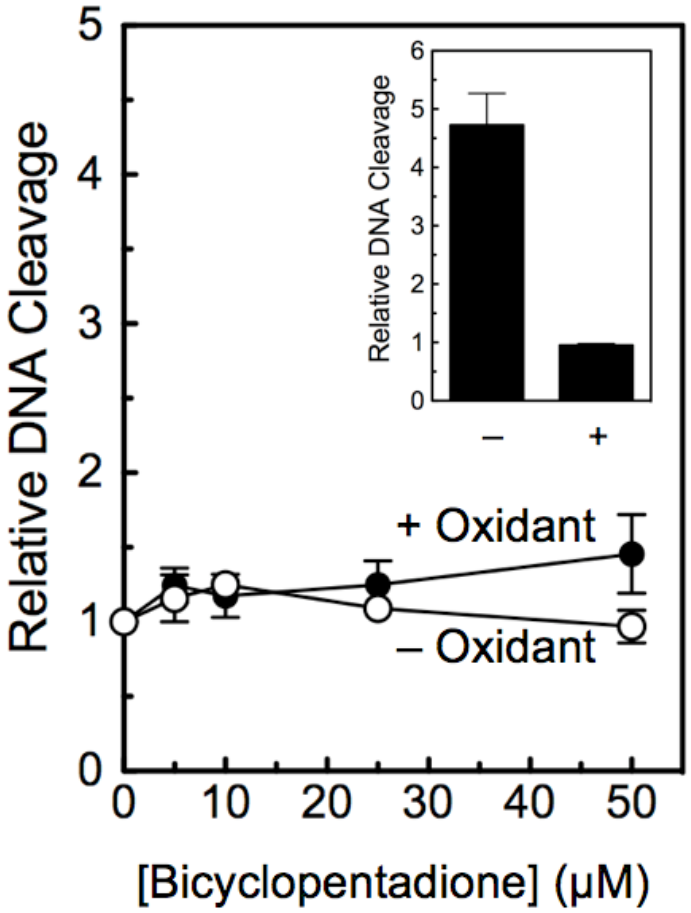


Figure 29. Effects of bicyclopentadione on topoisomerase II-mediated DNA cleavage. The effects of bicyclopentadione (structure shown in Figure 1) in the absence (open circles, – Oxidant) or presence (closed circles, + Oxidant) of 50 μM $K_3Fe(CN)_6$ on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα were determined. Inset: curcumin was incubated in the absence (-) or presence (+) of $K_3Fe(CN)_6$ for 10 min before addition to topoisomerase IIα-DNA cleavage assay mixtures that contained 50 μM $K_3Fe(CN)_6$. Error bars represent standard deviations for three independent experiments.

topoisomerase II-DNA complex or are incubated with the enzyme prior to the addition of the nucleic acid substrate (*10, 41*).

Topoisomerase II poisons in the second class form covalent adducts with the enzyme at amino acid residues distal to the active site (*83, 157*). The best-characterized members of this group are quinones, such as 1,4-benzoquinone and polychlorinated biphenyl (PCB) metabolites (*41, 42, 135*). In contrast to interfacial topoisomerase II poisons, the ability of covalent poisons to form topoisomerase II adducts (and, consequently, increase enzyme-mediated DNA cleavage) requires them to be in an oxidized form. Thus, DNA cleavage enhancement is blocked by the presence of reducing agents (*10, 18, 41-43, 134*). However, once final protein adducts are formed, their oxidation state appears to be irrelevant. As a result, if reducing agents are added to assay mixtures after DNA cleavage-ligation equilibria have been established in the presence of a quinone-based poison, they are unable to reverse the cleavage enhancement (*10, 41, 42, 134*). Finally, while covalent poisons enhance topoisomerase II-mediated DNA cleavage when added to the enzyme-DNA complex, they inactivate topoisomerase II when incubated with the protein prior to the addition of DNA (*10, 41, 42, 134*).

Curcumin requires an oxidant in order for it to increase topoisomerase II-mediated DNA cleavage. Furthermore, many of the proposed metabolites in the oxidation pathway of the compound contain quinones (*98*). Thus, it seems likely that the active metabolites of curcumin function as covalent (as opposed to

interfacial) topoisomerase II poisons. Four approaches were utilized to address this hypothesis.

In the first, a 5–fold molar excess of DTT over curcumin and $K_3Fe(CN)_6$ was added to assay mixtures prior to the start of reactions. As expected (considering that curcumin requires oxidation for activation), no enhancement of topoisomerase IIα-mediated DNA cleavage was observed (Figure 30, left).

In the second, DTT was added to reaction mixtures after cleavage complexes had been established. As seen in Figure 30 (left), levels of DNA scission remained high. As discussed above, this finding suggests that the oxidized metabolites of curcumin form covalent topoisomerase II adducts.

In the third, curcumin was incubated with human topoisomerase IIα in the presence of $K_3Fe(CN)_6$ prior to the addition of DNA (Figure 30, middle). As predicted for covalent poisons, enzyme activity fell to nearly zero with a $t_{1/2}$ of 0.9 min. In contrast, the activity of topoisomerase IIα was considerably more stable when the enzyme was incubated with either curcumin ($t_{1/2}$ = 10.4 min) or $K_3Fe(CN)_6$ ($t_{1/2}$ = >10 min) alone prior to the addition of DNA (not shown).

In the fourth, the ability of oxidized curcumin to stimulate DNA cleavage mediated by topoisomerase $IIα^{C392A/C405A}$ was determined. A previous study established that quinones can adduct human topoisomerase IIα at Cys392 and Cys405 and that topoisomerase $IIα^{C392A/C405A}$ is partially (~2–fold) resistant to covalent poisons, such as benzoquinone and PCB quinones, but not to interfacial poisons (*83*). As seen in Figure 30 (right), the ability of oxidized curcumin to

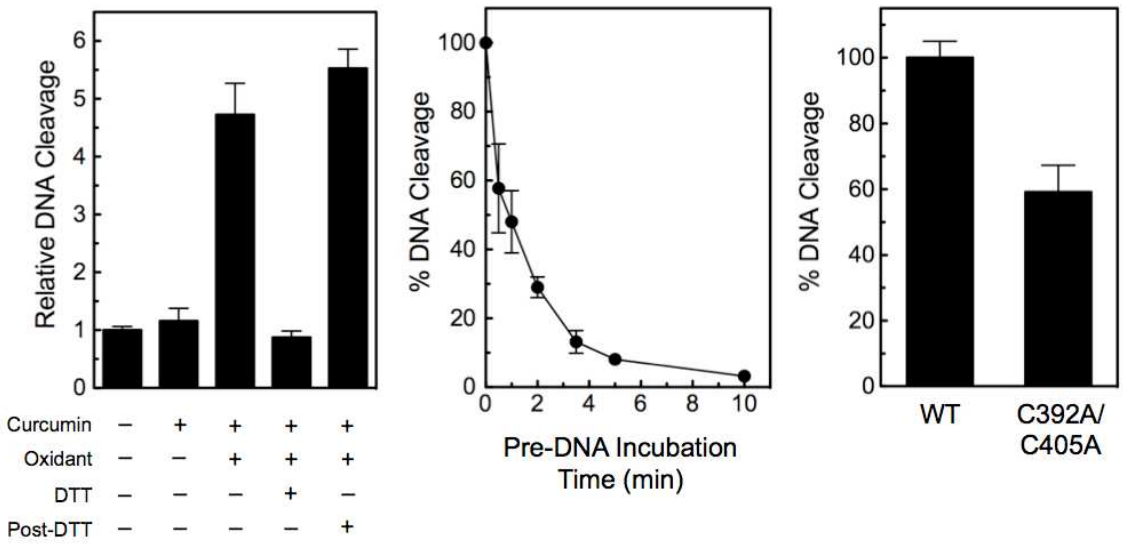


Figure 30. Oxidized curcumin intermediates act as covalent topoisomerase II poisons. Left: The effects of DTT on the enhancement of topoisomerase IIα-mediated DNA cleavage by oxidized curcumin intermediates were determined. Reaction mixtures contained DNA and human topoisomerase IIα in the absence or presence of 50 μM curcumin, 50 μM $K_3Fe(CN)_6$, or 250 μM DTT. In some reactions, DTT was added after the establishment of enzyme-DNA cleavage complexes (Post-DTT). Middle: The effects of oxidized curcumin intermediates on topoisomerase IIα activity when compounds were incubated with the enzyme prior to the addition of DNA. Human topoisomerase IIα was incubated with a combination of 50 μM curcumin and 50 μM $K_3Fe(CN)_6$ for 0-10 min prior to the addition of DNA to initiate 6 min cleavage reactions. Right: The effects of oxidized curcumin intermediates on the enhancement of DNA cleavage by human wild-type topoisomerase IIα (WT) and mutant quinone-resistant topoisomerase $IIα^{C392A/C405A}$ (C392A/C405A) were determined. Error bars for all three panels represent standard deviations for three independent experiments.

increase DNA cleavage mediated by topoisomerase IIα$^{C392A/C405A}$ was ~60% that of wild-type enzyme.

Taken together, these findings are consistent with the hypothesis that the oxidized intermediates of curcumin can be classified as covalent topoisomerase II poisons.

Degradation Products of Curcumin Do Not Poison Topoisomerase II

Under the conditions of the DNA cleavage assays, no significant degradation of curcumin to vanillin, ferulic acid, or feruloylmethane was observed (not shown). However, because some of these compounds display biological activity (*152*), the effects of vanillin, ferulic acid, and feruloylmethane on topoisomerase IIα-mediated DNA cleavage were determined. As seen in Figure 31, none of the compounds increased levels of DNA scission in the absence or presence of $K_3Fe(CN)_6$. Therefore, curcumin fragmentation products do not appear to be interfacial or covalent topoisomerase II poisons.

Oxidized Turmeric Is a Topoisomerase II Poison

Since curcumin generally is ingested in the form of turmeric (*137, 138*), the powdered spice was dissolved and assessed for its ability to stimulate DNA cleavage mediated by human topoisomerase IIα (Figure 32). In the absence of an oxidant, the spice had no significant effect on enzyme-mediated DNA scission. However, when $K_3Fe(CN)_6$ was included in reactions, turmeric stimulated DNA cleavage >4–fold at concentrations of 200-400 µg/mL. As

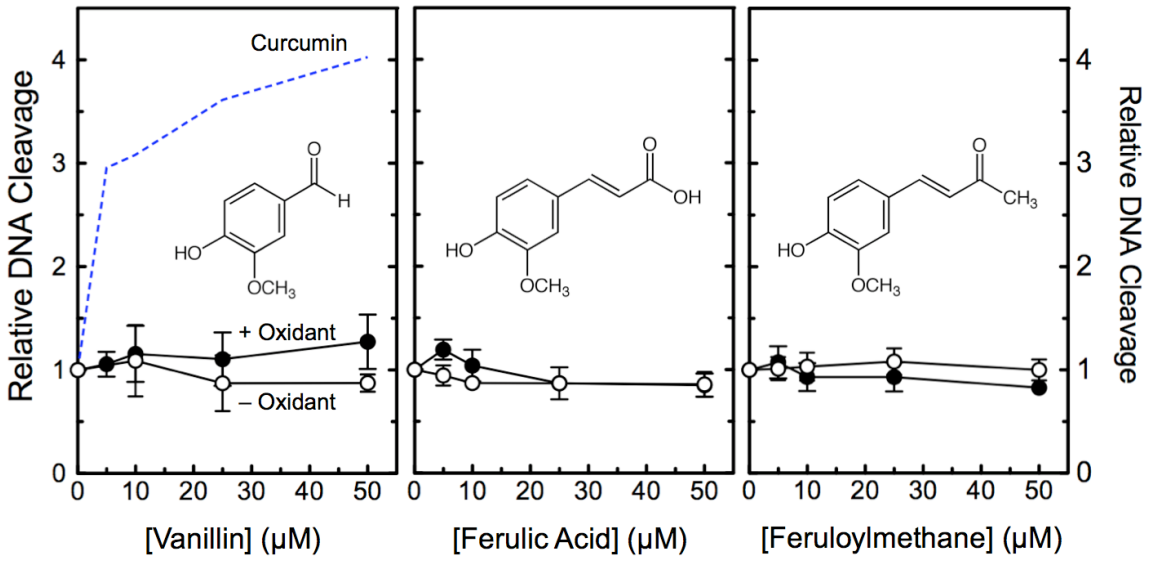


Figure 31. Effects of curcumin degradation products on topoisomerase II-mediated DNA cleavage. The effects of vanillin (left), ferulic acid (middle), or feruloylmethane (right) on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα were determined in the absence (open circles, – Oxidant) or presence (closed circles, + Oxidant) of 50 μM $K_3Fe(CN)_6$. Data for curcumin (dashed line from Figure 2) are included in the left panel for comparison. Error bars represent standard deviations for three independent experiments.

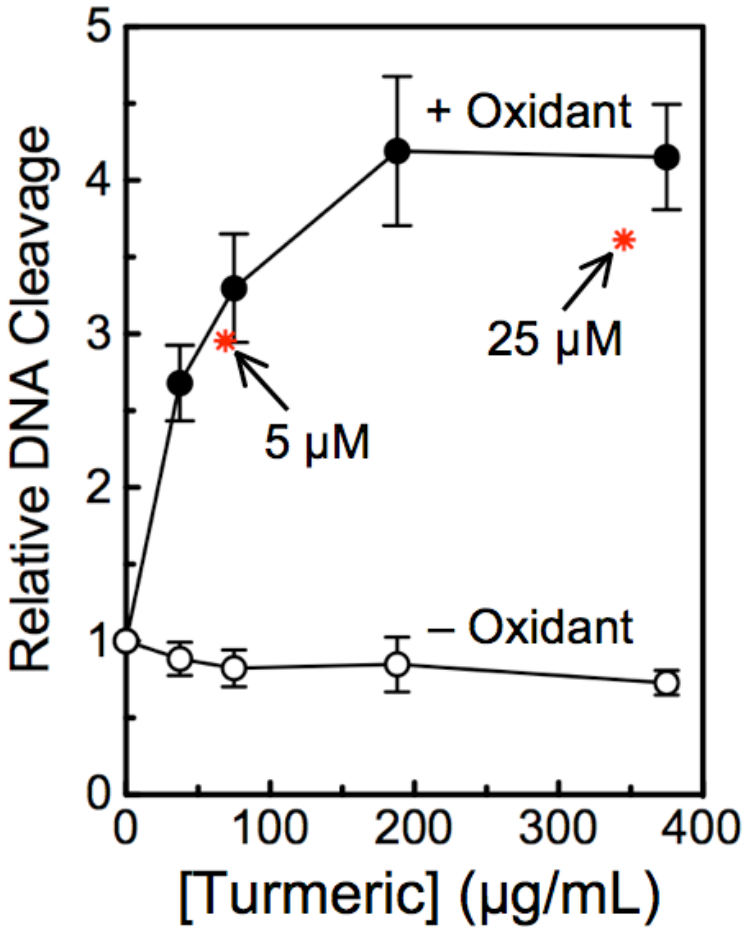


Figure 32. Effects of turmeric on topoisomerase II-mediated DNA cleavage. The effects of turmeric on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα were determined in the absence (open circles, – Oxidant) or presence (closed circles, + Oxidant) of 50 µM $K_3Fe(CN)_6$. The turmeric stock solution was determined to contain ~2.7% curcumin. On the basis of this concentration, DNA cleavage results for 5 and 25 µM oxidized curcumin intermediates (asterisks) are overlaid at the associated turmeric concentrations for comparison. Error bars represent standard deviations for three independent experiments.

determined by high-performance liquid chromatography, the turmeric solution contained ~2.7% curcumin. On the basis of this value, DNA cleavage data for 5 and 25 μM curcumin [+$K_3Fe(CN)_6$] were overlaid for comparison (asterisks). As seen in Figure 32, there is a strong correlation between the activities of turmeric and curcumin. Therefore, even within the more complex spice formulation, oxidized curcumin appears to function as a topoisomerase II poison.

Conclusions

Oxidized metabolites of curcumin, even in a solution of turmeric, are covalent poisons of human type II topoisomerases. In contrast, degradation products of the parent compound do not affect topoisomerase II-mediated DNA cleavage.

Curcumin displays a number of medically relevant biological properties, including antioxidant, anti-inflammatory, antibacterial, and chemopreventive activities (*139-141, 144*). The compound also is in cancer chemotherapy trials (*142, 143*). A number of chemopreventive natural products (including genistein and EGCG) and several highly successful anticancer drugs (including etoposide and doxorubicin) are potent topoisomerase II poisons (*4, 7, 10, 14-18, 74*). Coupled with the findings that 1) curcumin enhances DNA cleavage mediated by topoisomerase IIα and IIβ in cultured human cells and 2) cleavage enhancement is abrogated by antioxidant treatment (*154*), the above findings suggest that at least some of the anticancer activities of curcumin may be mediated by the effects of its oxidized metabolites on the type II topoisomerases.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The Role of Drug-DNA Interactions in the Activity of *m*-AMSA

Amsacrine (*m*-AMSA) is an anticancer agent that displays activity against refractory acute leukemias as well as Hodgkin's and non-Hodgkin's lymphomas. The drug consists of an intercalative acridine moiety coupled to a 4'-amino-methanesulfon-*m*-anisidide head group. Although *m*-AMSA was designed as a DNA binding agent, the ability to intercalate does not appear to be the sole determinant of drug activity. Therefore, to more fully analyze structure-function relationships and the role of DNA binding in the action of *m*-AMSA, several series of derivatives were analyzed for the ability to enhance DNA cleavage mediated by human topoisomerase IIα and topoisomerase IIβ and to intercalate DNA. Results suggest that much of the activity and specificity of *m*-AMSA as a topoisomerase II poison is embodied in the head group, while DNA intercalation is used primarily to increase the affinity of *m*-AMSA for the topoisomerase II-DNA cleavage complex.

As a result of these findings, it is postulated that new derivatives of *m*-AMSA may be synthesized by transplanting the head group onto different intercalative moieties. In essence, each one of these could act as a unique "vessel" for bringing the enzyme-active head group to the enzyme-DNA interface. For example, efforts are underway to synthesize a hybrid of *m*-AMSA and ethidium bromide, the latter of which is a much stronger DNA intercalator than

acridine (Figure 33). This compound may provide a scaffold for developing more potent poisons of topoisomerase II with varying pharmacokinetics.

Curcumin as a Topoisomerase II-Targeted Anticancer Agent

The polyphenol curcumin is the principal flavor and color component of the spice turmeric. Beyond its culinary uses, curcumin is believed to positively impact human health and displays antioxidant, anti-inflammatory, antibacterial, and chemopreventive properties. It also is in clinical trials as an anticancer agent. In aqueous solution at physiological pH, curcumin undergoes spontaneous autoxidation that is enhanced by oxidizing agents. The reaction proceeds through a series of quinone methide and other reactive intermediates to form a final dioxygenated bicyclopentadione product. Results suggest that intermediates in the curcumin oxidation pathway are covalent topoisomerase II poisons while the parent compound and the stable final product have no effect on enzyme activity. Degradation products of curcumin (vanillin, ferulic acid, and feruloylmethane) are not topoisomerase II poisons. Finally, even in the complex turmeric formulation, oxidized curcumin intermediates appear to function as topoisomerase II poisons.

Further work is needed to identify the specific oxidative metabolite(s) of curcumin that is(are) responsible for poisoning topoisomerase II. A number of curcumin derivatives are being synthesized that will help to further characterize the oxidation pathway of curcumin.

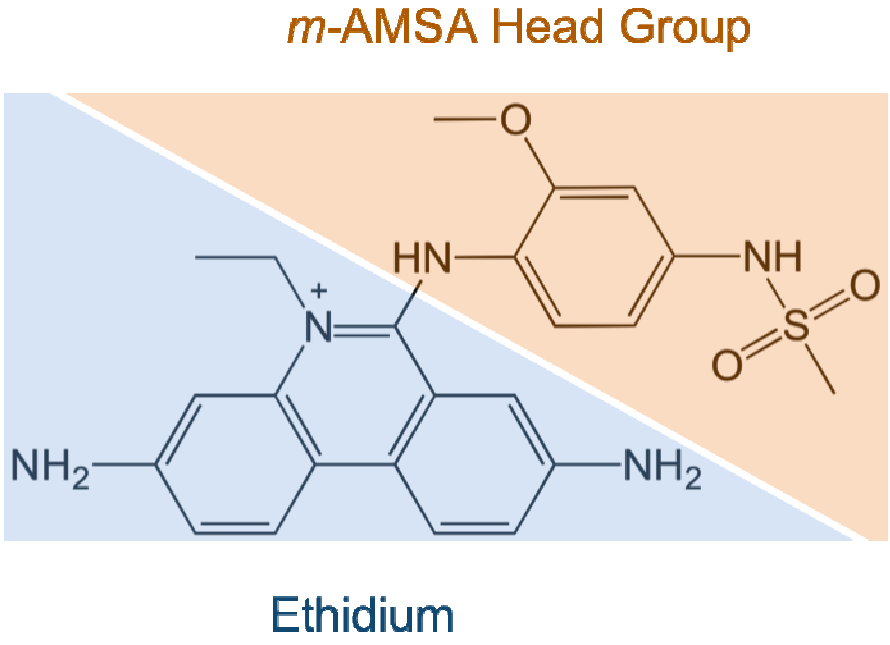


Figure 33: Speculative hybrid of ethidium bromide and *m*-AMSA. The intercalative ethidium moiety is shaded in blue; the head group of *m*-AMSA is shaded in orange.

Natural Products as a Source For Novel Topoisomerase II Poison Discovery

Because of the work on curcumin presented in Chapter IV, other bioactive constituents of traditional Chinese medical herbs were considered as potential topoisomerase II poisons. Thymoquinone—an active component of the spice black cumin, or *Nigella sativa* seeds—has been studied for its anti-inflammatory and anticancer activities (*158, 159*).

As seen in Figure 34, thymoquinone is indeed a potent covalent topoisomerase II poison, as evidenced by its stimulation of enzyme-mediated DNA cleavage ~5–fold at a concentration of 50 µM and its sensitivity to the presence of the reducing agent DTT. Further structure-activity studies are underway to elucidate the effects of the alkyl side chains on drug activity.

Another result of studies carried out on natural products is a collaboration that has developed between Dr. Osheroff's laboratory and that of Dr. Avi Golan of Ben-Gurion University of the Negev in Israel. A number of desert plant extracts are being screened for topoisomerase II poison activity, and at least one significant hit has been identified (Figure 35). A solution of *Phillyrea latifolia* extract enhanced DNA cleavage mediated by the enzyme ~7.5–fold at a concentration of 200 µg/mL. Two of the extract's most abundant, active components, oleuropein and hydroxytyrosol (*160*)—(both of which are also quite abundant in olives) (*161, 162*), also had significant positive effects (~10– and ~12.5–fold, respectively) on topoisomerase II-mediated DNA cleavage, each at a concentration of 200 µM. These levels of activity are comparable to that of the well-established anticancer drug etoposide at the same concentration.

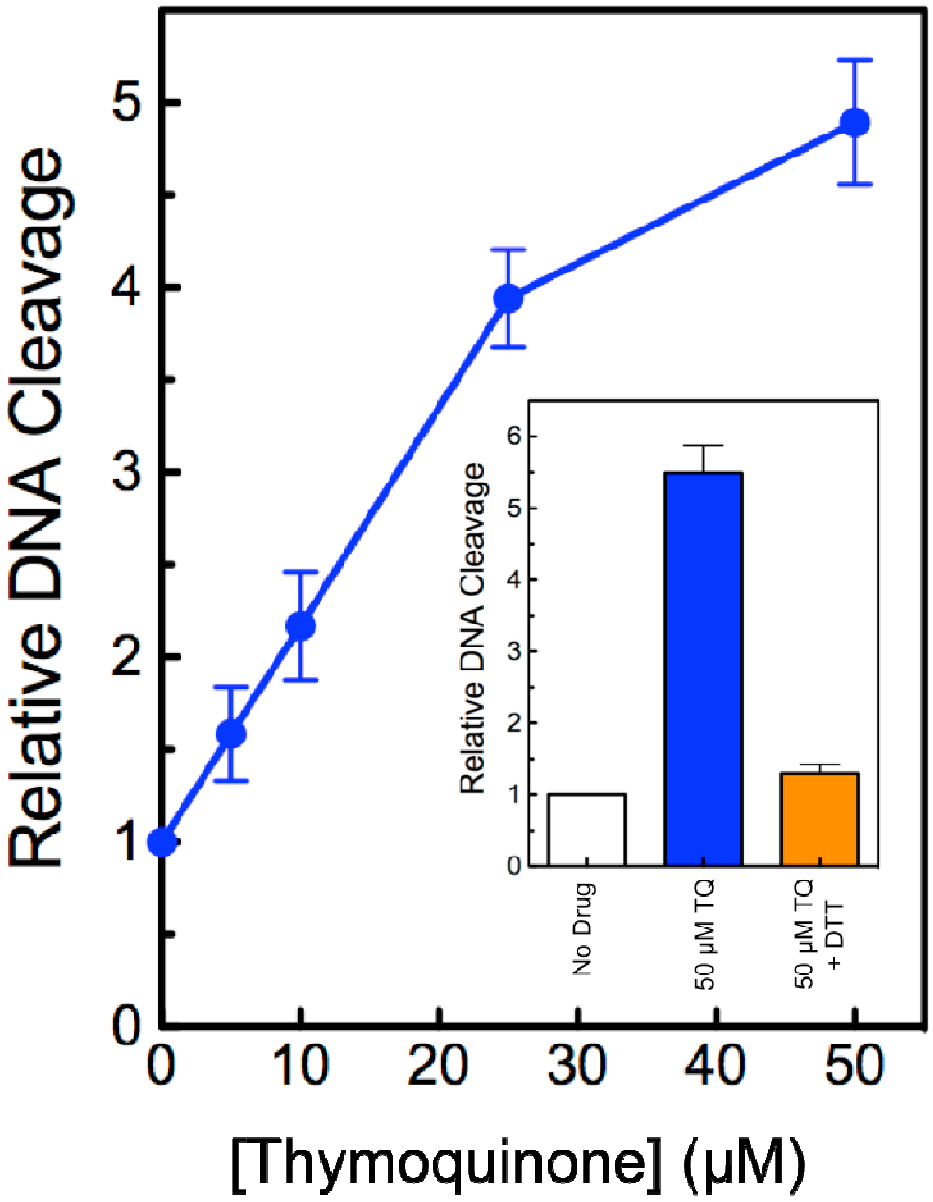


Figure 34: Enhancement of topoisomerase IIα-mediated DNA cleavage by thymoquinone. The effects of thymoquinone on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα were determined. The inset shows the effect of DTT on DNA cleavage stimulated by thymoquinone. Error bars represent the standard deviation of three independent experiments. Courtesy of Rachel Hoffmann, Vanderbilt University.

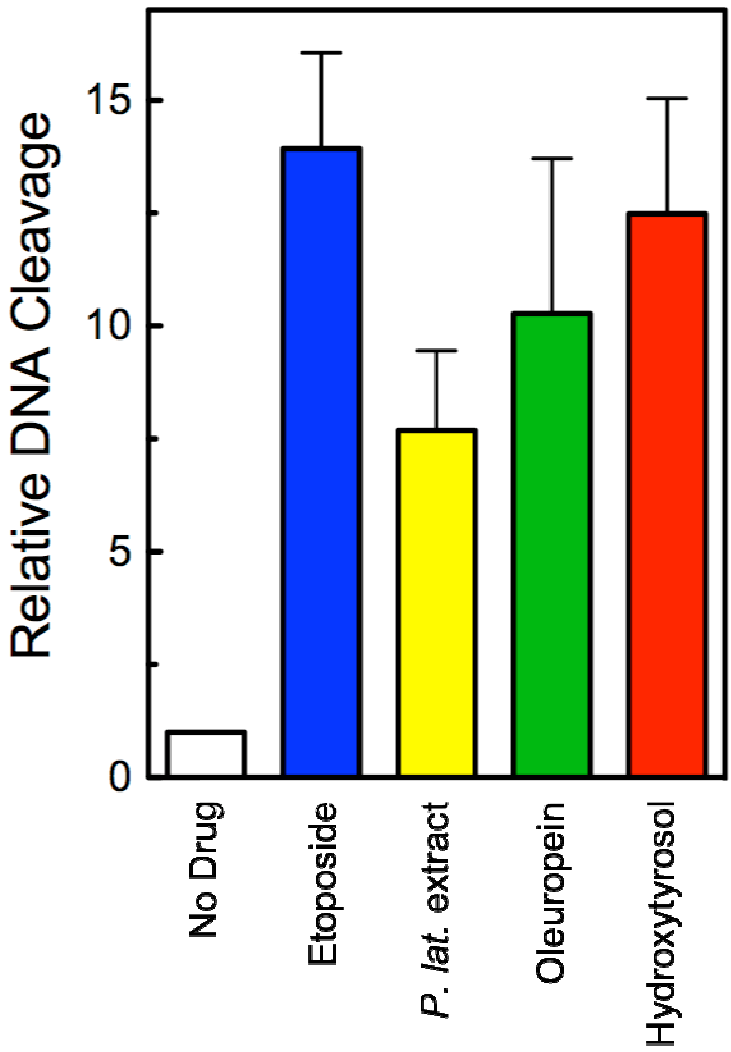


Figure 35: Enhancement of topoisomerase IIα-mediated DNA cleavage by an extract solution of *Phillyrea latifolia* (*P. lat.*) and two of its most abundant constituent phytochemicals. The effects of 200 µg/mL *P. lat.* extract (yellow bar), 200 µM oleuropein (green bar), and 200 µM hydroxytyrosol (red bar) on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase IIα are compared to those of 200 µM etoposide (blue bar). Error bars represent the standard deviation of three independent experiments. Courtesy of Carl Sedgeman, Vanderbilt University.

Oleuropein and hydroxytyrosol will be further characterized as topoisomerase II poisons, and the search for more novel poisons of the enzyme will continue as more plant extracts are screened.

Clearly, natural products are a rich source of topoisomerase II poisons, and future discoveries of novel enzyme-targeted compounds will be impeded primarily by the sheer diversity of plants to screen. Traditional herbal medicine systems (Chinese, Ayurvedic, etc.) represent an excellent entry point to this search because practitioners of these institutions have collected a wealth of knowledge over the centuries concerning specific plants and their associated remedies. Until recently, this treasury of information has been largely inaccessible to Western medical science due to the scarcity of organized records. However, a contemporary study by Ehrman et al. (*163*) has taken an important step towards making this exciting frontier more tractable by analyzing the distribution patterns of 8411 compounds from 240 Chinese herbs. Highly distinctive patterns emerged linking specific herbs and spices and their uses in traditional Chinese medicine with the classes of phytochemicals they contain. As a whole, the study suggests that it may be worthwhile to consider links between ethnopharmacological and molecular data when probing these largely as-yet untapped resources of potential new therapeutic agents. I believe these associations will prove invaluable in the search for novel topoisomerase II-targeted drugs.

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