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CHARACTERIZATION OF TOPOISOMERASE I AS A TARGET FOR CHEMOTHERAPY: EFFECT OF TOPOISOMERASE I POISONS AND DNA MODIFYING AGENTS ON ENZYME ACTIVITY.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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ABSTRACT

Topoisomerase I is a ubiquitous nuclear protein that alters DNA topology by breaking and then resealing single strands in DNA. Through this process, the enzyme plays an important role in DNA metabolic processes such as replication and transcription. Additionally, topoisomerase I is the cellular target for many anti-cancer agents that act by stabilizing a covalent intermediate formed between the enzyme and DNA thus converting topoisomerase I into a DNA damaging agent. Topotecan is a topoisomerase I poison that has shown promise in treating solid tumors. However, the drug is unstable under physiological conditions and converts to an inactive form within 30 minutes. Encapsulating topotecan in liposomes minimizes inactivation. The efficacy of topotecan in its free and liposome encapsulated forms was examined using a novel in vivo bioassay called ICE for In Vivo Complex of Enzyme. This bioassay uses antibodies to measure topoisomerase I-DNA covalent complexes formation in the presence and absence of poisons. Thus, the assay effectively measures topoisomerase I mediated DNA damage under various conditions. Liposome bound topotecan was found to be three to four fold more effective than free topotecan in inducing topoisomerase I mediated DNA damage. Enhanced efficacy of liposome bound topotecan is due to a combination of increased drug delivery to the cells as well as improved stability of
topotecan in the liposomal environment. Additionally, the ICE bioassay was clinically adapted to evaluate topotecan efficacy in patient samples. As part of a phase II clinical trial, topotecan induced topoisomerase I-DNA covalent complexes were analyzed in peripheral blood of patients undergoing treatment with the drug. Infusion of topotecan resulted in increased topoisomerase I mediated DNA damage in peripheral blood samples. Furthermore, a response to topotecan was also seen in tumor samples. Thus, the assay may be of predictive value in determining response to topoisomerase I poisons. Additional classes of agents that convert topoisomerase I into a DNA damaging agent were also identified. Through these studies it was determined that UV induced lesions in DNA stabilize covalent complexes formed between topoisomerase I and DNA. A similar topoisomerase I response was also seen in cisplatin and VM26 treated cells. These findings provide novel approaches to chemotherapeutic regimes which involve combinations of topoisomerase I poisons and lesion producing agents. Finally, the topoisomerase I response to lesions in DNA was not detected in some repair deficient cell lines implicating the enzyme in DNA repair processes.
To my grandfather, Shri N.B. Vasudevan
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LIST OF ABBREVIATIONS

Topo  Topoisomerase
CPT  Camptothecin
TPT  Topotecan
ICE  In Vivo Complex of Enzyme
DSPC  distearoylphosphatidylcholine
LIP-TPT  Liposome encapsulated topotecan
DPC  DNA Protein Crosslinks
SSB  Single strand breaks
DSB  Double strand breaks
UVB  Ultraviolet B
UVC  Ultraviolet C
CPD  Cyclobutane pyrimidine dimers
NER  Nucleotide Excision Repair
XPA  Xeroderma Pigmentosum complementation group A
XPD  Xeroderma Pigmentosum complementation group D
CHAPTER 1

INTRODUCTION

DNA topoisomerases are nuclear enzymes that alter the topology of DNA by catalyzing passage of DNA strands through one another (for reviews see, 1-9). DNA metabolic processes such as transcription and replication produce topological changes in DNA resulting in overwound, underwound and catenated DNA (10). These topological problems are resolved by topoisomerases. Thus, these enzymes play an important role in cellular transactions of DNA (1-9). Eukaryotic topoisomerases can be classified into two subfamilies - type I enzymes isomerize DNA by creating single strand breaks in DNA, whereas type II enzymes catalyze isomerization via transient double strand breaks in DNA (1-9).

TOPOISOMERASE I

Human topoisomerase I (topo I) is a 100 kDa monomeric protein (765 amino acids) encoded by a single copy gene located on chromosome 20q12-13.2 (11,12,13). Human topo I can be structurally divided into four domains: an N-terminal domain
which is dispensable for activity, the core domain, a linker region and the C-terminal domain which contains the active site tyrosine (14). The N-terminal and linker regions are not well conserved among organisms and sensitive to proteases. The N-terminal domain is believed to play a role in either modulating the activity of the catalytic domain or to be involved in protein-protein interactions. The linker region probably acts as a tether between the core region and C-terminal domain. The core region and C-terminal domain are fairly conserved and protease resistant. The core region probably plays a role in DNA topology recognition (14). The C-terminal domain contains the active site tyrosine (position 723) and is essential for catalytic activity (14). N-ethylmaleimide inhibition of topo I activity suggests the presence of an essential sulphhydryl group which may play a role in proton transfer during catalysis (15).

Mammalian topo I is expressed in all nucleated cells and its promoter region exhibits sequence features found in a number of housekeeping genes (such as high G+C content, high frequency of CpG islands, absence of a TATA and CCAAT box) (16,17). Several cellular transcription factors (such as SP1, OTF, CRE/ATF, AP1, NF-κB) have been implicated in regulating mammalian topo I transcription (16,18,19), but their roles have yet to be established. Topo I activity is also regulated by post-translational modifications, for example, phosphorylation by casein kinase II in vitro results in greater enzyme activity (20,21) while ADP-ribosylation inactivates topo I (22).
DNA PASSAGE MECHANISM OF TOPOISOMERASE I

The catalytic cycle of topo I involves the following steps: DNA binding, transient single strand DNA scission, passage of the intact strand through the break, resealing of the DNA followed by release of the enzyme (Fig. 1) (2). Both positive and negative supercoiled duplex DNAs are relaxed in this manner, altering the linking number of DNA in steps of one (23,25). Eukaryotic topo I is also capable of catenating double strand circles providing that one of the circles contains a pre-existing nick (24). The eukaryotic enzyme does not require divalent cations or a high energy cofactor for catalytic activity (25,26,27). Under optimal conditions, topo I acts via a processive mechanism wherein all supercoils are relaxed prior to enzyme/DNA dissociation (28,29). At high ionic strengths, catalysis occurs in a distributive fashion where only a few supercoils are relaxed prior to dissociation (30).

DNA binding

Eukaryotic topo I preferentially binds double strand DNA (dsDNA) (31) showing greater affinity for supercoiled DNA than relaxed DNA (32,33,35,36). This preference could be attributed to structural changes induced by DNA supercoiling, preferential binding of topo I to curved DNA, or to crossover points in DNA (36,37,38,39). Salt concentration effects on topo I-DNA binding indicate that electrostatic interactions play a role in substrate binding (40).
Figure 1: Mechanism of topoisomerase I action
Topo I binds to DNA (step 1) and cleaves one strand of DNA (step 2) with single strand break being stabilized by formation of a DNA-protein covalent complex. The intact strand is passed through the break (step 3) followed by resealing (step 4) and release (step 5). Through this mechanism topo I relaxes of supercoiled DNA in steps of one linking number.
Figure 1

1. Topo I binding
2. DNA Cleavage
3. Resealing
4. Release
5. Strand Passage
DNA Cleavage and Reunion.

Topo I binds both strands of DNA protecting a region of 15-19 base pairs in which the cleavage site is centrally located (43). Consensus sequence derivation for topo I binding reveals that the enzyme has a strong preference for the following hexadecameric motif (41,42)

![Cleavage Diagram]

Following DNA binding, topo I catalyzes single strand scission. Nucleotides 5' to the break site are important for cleavage (46), whereas bases 3' to the break site appear to be unimportant for enzyme cleavage (46). On the scissile strand, the first base 5' to the cleavage site (position -1) cannot be a purine (44,45) and positions -2 to -7 are essential for cleavage (43). However, in vitro and in vivo analysis of topo I cleavages reveal that not all sites are cleaved with equal efficiency, indicating that features other than the sequence of bases 5' to the break site are important for cleavage (6). Cleavage sites are likely to be determined by best steric fits between the active site and DNA strand (6).
The DNA cleavage and reunion reaction of topo I is governed by an equilibrium process (2). A simplified scheme for topo I-mediated DNA cleavage is as follows (2):

\[
E + S \rightleftharpoons E \cdot S \rightleftharpoons E \cdot S^* 
\]

In this scheme, E represents the enzyme, S is the DNA substrate and S* is the cleaved DNA. The product of the cleavage, or forward reaction (E•S*), is a covalent phosphotyrosine bridge formed between the enzyme and 3' phosphate of DNA leaving a free 5' OH (47,48,49,50). DNA cleavage occurs through transesterification of the phosphodiester bond by the active site tyrosine (46). A conformational change in the enzyme-DNA complex probably accompanies cleavage shielding the phosphotyrosyl intermediate from solvent (6). Thus, only the deoxyribosyl hydroxyl is available for reaction with the phosphotyrosyl intermediate during religation. Following passage of the intact strand through the break, the DNA is similarly resealed through transesterification in which the attacking nucleophile is the 5' hydroxyl group on the free end of the broken strand (46). Strand transfer shows no site specificity with respect to acceptor DNA, the only requirement being that acceptors must have a 5' OH terminus.
(6). HeLa topo I can transfer a single strand donor DNA to a range of acceptors including double strand nicked circles or linear double strand DNA with flush, 5' protruding or 5' recessed termini (6).

The cleavage-reunion equilibrium of topo I lies towards reunion with preference towards the sealed product (2). The phosphotyrosyl intermediate has a very short half-life and, importantly, cannot be detected under normal conditions (47,48,49,50), thus preventing inadvertent DNA damage during the catalytic cycle (the phosphotyrosyl intermediate is solvent labile).

Mechanistic Models for Enzyme Action

Two possible models exist for the mechanism of topo I action. During cleavage, the enzyme could covalently bind to one end of DNA leaving the non-covalently bound DNA end to rotate freely through the break prior to recapture by the enzyme thus removing superhelical turns (4). Alternately, the enzyme could bridge the single strand break, covalently bound to one end and non-covalently to the other end, while strand passage occurs through this bridge (4). However, the exact mode of action is still unknown.

BIOLOGICAL FUNCTION OF TOPOISOMERASE I

Many processes in DNA metabolism generate positive and negative supercoiling DNA which exert torsional strain on the helix (4,5,7,8,10). Excessive supercoiling is known to have strong effects on DNA structure and its interactions with other molecules.
Additionally, template topology has been shown to affect transcription (10). One major cellular function of topoisomerases is to prevent excess supercoiling of genomic DNA during various DNA metabolic processes.

Replification

During semi-conservative replication, as the parental strands are separated, positive and negative supercoiling occurs ahead of and behind the replication fork, respectively (51). This can distort the DNA and prevent the replication fork from proceeding along the helix (51). Thus, efficient movement of the replication fork requires a swiveling action to remove the positive supercoils ahead of the fork. Experiments with yeast suggest that the major replication swivel is provided by topo I, however, this function can be substituted by topo II (52,53). It has also been shown that the eukaryotic topo I is present in the vicinity of the SV40 replication fork suggesting a role for topo I in DNA replication (54).

Transcription

Wang and Liu have proposed a twin-supercoiled domain model of translocation wherein the transcription machinery translocates along the DNA duplex driving positive supercoiling ahead of and negative supercoiling behind RNA polymerase (55). Positive supercoils ahead of the transcription machinery may eventually lead to cessation of RNA polymerase movement (10). Topoisomerases are capable of removing such supercoils and, indeed, topo I has been shown to be associated with actively transcribed genes in
several eukaryotic systems by immunochemical and biochemical studies (56,57,58,59,55). Additionally, reduced RNA synthesis has been seen in many cases where topo I is inhibited (56,61,62). These observations are consistent with the notion that topo I is involved in transcription.

Other Processes

DNA supercoiling can result from protein translocation along the DNA helix. For example, it has been shown that type I and III restriction enzymes, helicases, and NTP-dependent enzymes that track along the DNA helix by looping or translocation generating supercoiled domains in DNA (63,64,64).

Genomic stability

It has been suggested that topoisomerases may play a role in suppressing mitotic recombination (4). In fact, yeast topoisomerase mutants have a much higher frequency of mitotic recombination (177). In these cells, low topoisomerase activity in heavily transcribed regions probably results in persisting supercoils in DNA which may stimulate recombination (4).

TOPOISOMERASE I-MEDIATED DNA DAMAGE

While topoisomerases are important for maintaining genomic stability, under certain conditions, they also cause genomic instability. Topoisomerases are targets of antibiotics and chemotherapeutic agents which act to stabilize covalent complexes formed between topoisomerases and DNA (7,8). Camptothecin (CPT), a topo I poison,
has been shown to inhibit religation during enzyme turnover, resulting in increased half-life of the covalent intermediate thus prolonging the nicked state of DNA (66,67). Stabilization of topo I-DNA covalent complexes could lead to double strand DNA breaks if topo I-mediated cleavage occurs in a single strand region of DNA, or opposing a nick (24). The presence of topo I-DNA covalent complexes could further produce irreversible double strand breaks and recombinogenic ends when a replication fork collides with the complex (66,69). Finally, stabilization of topo I-DNA covalent complexes may stimulate chromosomal deletions and rearrangements along with sister-chromatid exchange (70). Thus, topo I poisons effectively convert the enzyme into a DNA damaging agent.

It is also possible for topo I-DNA covalent complexes to become trapped in the absence of drugs under conditions where the enzyme or DNA is altered. Several yeast topo I mutants form DNA-enzyme covalent complexes which are more stable than wild type. This results in increased single and double strand breaks and increased illegitimate recombination frequency (71). Additionally, alterations in DNA, such as mismatched nucleotides, have also been shown to inhibit the religation step of topo I thus stabilizing enzyme-DNA covalent complexes (72). Hence, modifications of DNA by various agents may also induce topo I-mediated DNA damage.
SCOPE

Identification of topo I as the primary target of powerful anticancer drugs has generated much interest in the enzyme. *In vivo* assays that allow measurement of topo I-mediated DNA damage enable evaluation of the efficacy of topo I poisons. So far, DNA fragmentation assays have been used for measuring single strand and double strand breaks induced by poisons (73,74), however, these assays do not address the amount of DNA damage produced specifically by topo I. In developing novel assays for measuring topo I-mediated DNA damage *in vivo*, the unique nature of the topo I-DNA covalent intermediate can be exploited. The ICE bioassay (*in vivo* complex of enzyme) is an antibody based assay that allows specific measurement of topo I-DNA covalent complexes formed in intact cells. The number of topo I-DNA covalent complexes formed, in turn, reflects the amount of topo I-mediated DNA damage induced by various agents. Thus, the ICE bioassay allows for quantitative and specific measurement of the efficacy of agents that convert topo I into a DNA damaging agent.

This dissertation focuses on evaluating the role of topo I as a cellular target for chemotherapy. In these studies, the efficacy of the topo I poison, topotecan, has been evaluated in cultured cells using the ICE bioassay. Liposomal encapsulation of topotecan was used to improve the stability of the drug and the potency of the liposome encapsulated form of the drug was also examined. Given the importance of topo I in chemotherapy, the utility of the ICE bioassay in the clinical situation was examined.
Patients undergoing chemotherapy with topotecan were analyzed for topo I-mediated DNA damage to determine if the ICE bioassay would be a useful diagnostic tool to assess patient response to topotecan. Additionally, the effect of DNA modifications such as UV lesions and cisplatin crosslinks, on \textit{in vivo} topo I-DNA covalent complex formation was analyzed. Finally, the effect of UV-induced photoproducts on the catalytic activity of topo I was determined \textit{in vivo} and \textit{in vitro}. 
CHAPTER 2

ANALYSIS OF THE TOPOISOMERASE I POISON TOPOTECAN IN VITRO AND IN VIVO.

INTRODUCTION

Camptothecin (CPT), an active agent against murine leukemia, was isolated from the Chinese tree *Camptotheca acuminata* in 1966 (75). Initially, CPT was shown to cause alkali labile single strand breaks in DNA which rapidly resealed upon drug removal (76,78,81). Subsequent studies revealed that CPT induces DNA single strand breaks by stabilizing topo I-DNA covalent intermediates (66,67). In the absence of CPT, topo I turnover favors DNA religation with the covalent complex existing only transiently. In the presence of CPT, the religation step is slowed and, the equilibrium is shifted towards topo I-DNA covalent complex formation (66). This results in topo I-DNA adduct accumulation producing DNA single strand breaks (66,67,79). Thus, CPT acts as an antitumor agent by converting topo I into a DNA damaging agent. Removal of CPT results in the reversal of topo I-DNA covalent complexes shifting the equilibrium.
back towards religation (80). Topo I appears to be the sole target for CPT as yeast cells lacking functional topo I are resistant to the cytotoxic effects of the poison (81,82). Additionally, in mammalian cells a CPT resistant topo I has been isolated from CPT resistant cells (77,84).

The physical nature of CPT induced topo I-DNA covalent complexes is unknown. CPT has been shown to preferentially bind topo I-DNA adducts rather than topo I or DNA alone (81,85,86). CPT stabilizes topo I-DNA covalent complexes even if added after DNA cleavage has occurred. It has been proposed that CPT binds simultaneously to DNA and topo I after the covalent intermediate has formed resulting in a ternary complex that inhibits religation (81). CPT increases the intensity of the majority of basal topo I cleavages (i.e. those present in the absence of CPT) (42,44,87). Furthermore, in the presence of CPT, additional topo I cleavages are seen at sites undetectable in the absence of the poison (88). It is possible that CPT alters topo I cleavage by affecting the normal helix topology recognition.

Since topo I plays an important role in chemotherapeutic drug development, assays measuring the amount of topo I-mediated DNA damage provide important predictive information. While general DNA breaks can be determined by methods such as neutral and alkali elution (73,74), the ICE (in vivo Complex of Enzyme) bioassay is an antibody based assay that measures endogenous topo I-DNA covalent complexes. Covalent complexes reflect the amount of topo I-mediated DNA damage produced.
Additionally, while *in vitro* assays can be used to evaluate topo I poisons, these assays do not reflect the activity of the agents in the cellular environment. Complications such as biochemical modifications, degradation, drug uptake, and relocation to the sites of enzyme activity cannot be assessed by *in vitro* assays. The ICE bioassay determines the efficacy of topo I poisons within the context of the living cell, therefore, analogs and drug delivery variables can be tractably evaluated in any cell or tumor sample.

CPT was tested in clinical trials in the early 1970s, but was subsequently removed due to severe drug-related side effects (88,90,91). A variety of analogs of CPT have since been developed in an attempt to reduce side effects while retaining antitumor activity (92,93,99). CPT and its analogs are composed of a conjugated system of 5 rings (A-E) with an α-hydroxy lactone functionality in ring E, a pyridone moiety in ring D, and contains a chiral carbon at position 20 (Fig. 2A) (94). The binding site in the topo I-DNA covalent complex probably accommodates the entirety of the drug molecule. This is corroborated by the observation that the two distant rings (A and E) are critical for CPT activity (95). The lactone ring (E) is a critical structural feature for enzyme inhibition (95). Both substitutions of the hydroxyl group at position 20 and chemical modifications at position 21 inactivate the poison (95). Binding of CPT analogs to the topo I-DNA covalent complex is stereospecific as 20(R)-CPT is less active than 20(S)-CPT (95). Additionally, the lactone ring is
Figure 2: Structure of Camptothecin.

Panel A. Ring system of camptothecin.

Panel B. Hydrolysis of camptothecin. CPT lactone (left) exists in equilibrium with the open ring hydroxy acid (right) in a pH dependent manner.
Figure 2

A.

B.

Lactone (Active) \[ \overset{\text{OH}^-}{\rightleftharpoons} \overset{\text{H}^+}{\text{Hydroxy (Inactive)}} \]
unstable at physiological pH, undergoing hydrolysis to the hydroxy acid which is pharmacologically inactive (Fig. 2B) (95,97). The lactone and hydroxy species are in equilibrium and at neutral pH 20% of the drug exists in the active lactone form (97).

Topotecan (TPT) is a water soluble derivative of CPT that shows promising anticancer activity and is currently being tested in phase II trials against a number of solid tumors (reviewed in 100,101). While numerous studies have been undertaken to investigate the pharmacological and cytotoxic properties of TPT, few have focused on examining the effects of TPT on topo I activity in vivo. Furthermore, improvement of the physiological stability of CPT analogs has not been addressed. In an attempt to increase drug stability in aqueous solutions, TPT was encapsulated in multilamellar, gel-phase vesicles composed of distearoylphosphatidylcholine (102). The vesicles can maintain a proton gradient across the lipid bilayer and can be formulated to preserve a stable internal aqueous compartment of pH 5 (102). Thus, TPT can be packaged within the aqueous portion of the lipid vesicle (LIP-TPT) and remain at a pH at which the lactone form predominates (102). Using the ICE bioassay the ability of TPT to induce topo I-DNA covalent complexes in vivo was tested. Additionally, the efficacy of LIP-TPT in vitro and in vivo was investigated and compared to free TPT.
MATERIALS AND METHODS

Reagents.

Adherent HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% defined, iron-supplemented bovine calf serum (Hyclone Laboratories, Logan, UT) and gentamycin sulfate (50 μg/ml). Human topo I was purified using the procedure of Muller and Trask (26) with modifications (103). One unit of topo I relaxes 250 ng of supercoiled DNA in 15 minutes at 37 °C. Plasmid pHOT1 contains a high affinity topo I cleavage site (104) cloned into pUC12. The Scl 70 antibody is a human polyclonal antibody to topo I and was isolated from scleroderma patients (105). Topotecan and liposomal topotecan were characterized and provided by Z. Mi and T. Burke (Ohio State University, College of Pharmacy). Multilamellar distearoylphosphatidylcholine vesicles containing TPT were prepared and characterized using the method described by Z. Mi and T. Burke (102). TPT concentrations were determined using HPLC methods (102). A typical liposome preparation has a drug/lipid ratio (mole/mole) of 1:20, and an encapsulation of approximately 40%.

Topoisomerase I activity assays.

Reactions containing 250 ng of negatively supercoiled plasmid DNA (pHOT1) and 20 nM (4 units) of human topo I in a final volume of 20 μl in 1 X TNE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl) were incubated for 30 minutes at 37 °C. Reactions were stopped by adding of 2 μl stop buffer (5% sarkosyl, 0.125%
bromophenol blue, 25% glycerol), followed by digestion with 50 µg proteinase K/ml for 30 minutes at 37 °C. An equal volume of chloroform:isoamyl alcohol (24:1) was added, the solution vortexed and briefly centrifuged (microfuge). The aqueous (blue) layer was loaded directly onto a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) containing 0.5 µg/ml ethidium bromide. Gels were run at 5V/cm for 30 minutes, destained with distilled water for 30 minutes, and photographed with a UV transilluminator. In some cases, the gels were photographed using Kodak TMAX 400 film and negatives scanned using a densitometer.

**Preparation of nuclear extracts for western blotting.**

HeLa cells (10^7 cells) were washed twice with cold TD (109 mM NaCl, 4.1 mM KCl, 0.56 mM Na_2PO_4, 20 mM Tris-HCl, pH 7.4) and scraped into two ml of TD. Cells were pelleted (3 minutes, 1000 x g) and resuspended in 2 ml of buffer A (100 mM NaCl, 50 mM KCl, 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM phenylmethysulfonylfluoride, 10% glycerol, 0.2% Nonidet P-40, 0.1% Triton X-100). Following a 10 minute incubation on ice, nuclei were centrifuged (10 minutes, 2000 x g) and lysed in 100 µl buffer A containing 1% SDS. The lysates were subjected to SDS-PAGE (7%), followed by electroblot transfer to nitrocellulose. Topo I was detected by probing the blot with the Scl 70 antibody using immunoblot techniques described below.
The ICE Bioassay.

Exponentially growing HeLa cells ($10^7$ cells per treatment) were incubated at 37°C with various inhibitors in serum free medium. At times specified in each experiment, medium was removed by aspiration and cells lysed by the direct addition of 1% sarkosyl (1-3 ml/dish, pre-equilibrated to 37°C). Lysates stored at -20 °C or assayed directly yielded identical results, therefore, some samples were stored frozen prior to processing. The lysates were overlaid onto CsCl density gradients containing four different density steps. To prepare the gradients, a stock solution of CsCl (density = 1.86 g/cc) was diluted in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to give four solutions of 1.37, 1.50, 1.72, 1.82 g/cc (solutions A-D, respectively). Solution D (2 ml) was placed in a SW41 polyallomer tube first, followed by 2 ml each of the successively dense CsCl solutions (C,B,A). The gradients were used directly without equilibration. The volume of the lysate was adjusted with lysis buffer (1% sarkosyl in TE) to 3 ml. The lysates were gently layered on top of the gradients and the tubes were centrifuged in a Beckman SW41 rotor at 31,000 RPM for 18-24 hours at 20 °C. Fractions (0.4 ml) were collected from the bottom of the gradients and DNA located in the gradient using fluorometry. Aliquots from each fraction (100 μl) were diluted with an equal volume of 25 mM sodium phosphate buffer (pH 6.5) and applied to Hybond C nitrocellulose membranes (Amersham) using a slot-blot vacuum manifold (Schleicher and Schuell). Nitrocellulose filters were analyzed by immunoblotting using the Scl70 antibody.
Immunoblotting analysis.

Nitrocellulose membranes were first equilibrated in 1 x TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween-20) for 15 minutes followed by incubation in TBST containing 5% non-fat dried milk (BLOTTO) for 2 hours. The filters were washed three times (10 minutes per wash) in TBST and incubated with the Scl 70 antibody (diluted 1:1000 in TBST) for 6 hours. The filters were washed three times (10 minutes per wash) with TBST followed by a 2 hour incubation in [125I]-Protein A (ICN) at 0.4 μCi/ml in TBST. The filters were washed three times (10 minutes per wash) with TBST and visualized by autoradiography. Autoradiograms were scanned using densitometry to quantify levels of topo I. The DNA concentration in each fraction was determined by fluorometry and topo I-DNA complexes were expressed by dividing the amount of topo I (arbitrary units derived from scanning) by the amount of DNA in each fraction.

Analysis of topotecan accumulation in HeLa cells.

Exponentially growing HeLa cells (10^7 per treatment) were incubated with TPT in 1 ml of serum free medium. Cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO_4, 1.4 mM KH_2PO_4) and scraped into 1 ml of PBS. The cells were then overlayed onto 750 μl of silicon oil followed by centrifugation at (5 minutes, 12,500 x g) room temperature. The silicon oil and medium were aspirated away and cells were lysed with 150 μl of acidified methanol (0.3N HCl in methanol). Samples
were then briefly centrifuged (1 minute to pellet cellular debris) and 50 μl aliquots of each sample were analyzed by HPLC to measure the amount of lactone drug as described previously (97,102).

RESULTS

Stabilization of topoisomerase I-DNA complexes by camptothecin and topotecan.

The ability of TPT to stabilize topo I-DNA complexes was first tested in vitro by topo I activity assays. Supercoiled plasmid DNA, containing a high affinity hexadecameric topo I recognition sequence (pHOT1) (104), was incubated in the presence and absence of topo I with various concentrations of TPT for 30 minutes. Reactions were terminated using a protein denaturant (sarkosyl) which traps the covalent intermediate formed between topo I and DNA. Reaction products were analyzed by agarose gel electrophoresis. However, since the intermediates are protein-bound, the samples were digested with proteinase K prior to gel analysis. In the case of plasmid DNA, the covalent intermediate consists of nicked or open circular DNA bound to topo I. In the absence of TPT, topo I completely relaxes supercoiled DNA (Fig. 3). Due to the rapid religation rate of the enzyme, low levels of the intermediate, open circular DNA is detected. TPT alone has no effect on supercoiled DNA, however, addition of TPT to reactions containing topo I and DNA, results in increased formation of open circular DNA in a dose dependent manner (Fig. 3). Therefore, TPT is effective in stabilizing topo I-DNA covalent complexes in vitro.
Figure 3: Topoisomerase I-DNA covalent complex stabilization by topotecan in vitro.

Supercoiled plasmid DNA was incubated with purified human topo I (20 nM) and increasing amounts of TPT at 37 °C for 30 minutes. Reaction products were analyzed by agarose gel electrophoresis in the absence of ethidium bromide. Gels were stained with ethidium bromide and photographed under ultraviolet light. Lane 1, supercoiled DNA marker; lane 2, DNA with 20 nM topo I; lane 3, DNA and TPT; Lane 4-8, DNA, topo I and increasing concentrations of TPT as indicated above the lanes.
Figure 3
Next, *in vivo* activity of CPT and TPT was tested using the ICE bioassay. In this assay, cells are treated with topo I poisons and covalent topo I-DNA intermediates are trapped within the cell using the ionic detergent, sarkosyl. Sarkosyl irreversibly arrests topo I-DNA complexes and dissociates non-covalent protein-DNA complexes (histones, transcription factors, etc.). Covalent complexes are resolved from free protein by cesium chloride gradient centrifugation which also serves to further disrupt electrostatic interactions between proteins and DNA. Since DNA and free protein band at very different densities (1.7 and 1.3 g/cc, respectively), crossover between free protein and DNA does not occur in these gradients. Topo I-DNA covalent complexes band at the density of DNA (1.7 g/cc), thus, topo I covalently bound to DNA is effectively separated from free topo I. Gradient fractions are then analyzed by immunoblotting techniques using antibodies against topo I to quantify topo I-DNA covalent complexes (Fig. 4). The amount of topo I-DNA covalent complexes formed can be represented as the amount of topo I (arbitrary value determined by densitometry) per µg of DNA. Measurement of topo I-DNA covalent complexes, in turn, reflects the amount of topo I-mediated DNA damage produced in cells.

Prior to analysis by the ICE bioassay, it was necessary to determine the specificity of the anti-topo I antibody. Crude nuclear extracts were prepared from HeLa cells and increasing amounts of extracts were submitted to SDS-PAGE followed by
Figure 4: Flow chart of the ICE bioassay.

Cells treated with topo I poisons to stabilize topo I-DNA covalent complexes formation are lysed using an ionic detergent (sarkosyl). Sarkosyl effectively dissociates electrostatic interactions between topo I and DNA while trapping covalent complexes. The lysates are loaded on cesium chloride step gradients to separate free topo I from DNA bound topo I. Fractions are collected from the gradients and DNA profiles determined by fluorometry. The fractions are also tested by immunoslot blots using an anti-topo I antibody. In the absence of drug, topo I is detected only in the free protein fraction. In the presence of a poison (CPT) topo I is also detected in the DNA peak, indicating the formation of topo I-DNA covalent complexes.
Topo I-DNA Non Covalent Interaction

Sarkosyl Lysis

DNA bound Topo I

Cesium Chloride Gradients

DNA Peak (DNA bound topo I)

Free Proteins (free topo I)

DNA Profile of Gradients

Fraction Number

No Drug

CPT

Immunoslot Blots

Figure 4
electrotransfer to nitrocellulose membranes. The nitrocellulose membranes were analyzed by standard Western blotting techniques using the Scl 70 antibody (a polyclonal anti-topo I antibody). Human placental topo I was used as a control in these blots. The Scl 70 antibody recognizes a single 100 kDa polypeptide in crude nuclear extracts and in purified topo I (Fig. 5) and was used for further studies.

The applicability of the ICE bioassay was first determined by testing the effects of the parental compound CPT on in vivo topo I-DNA covalent complex formation. HeLa cells were treated in the presence or absence of CPT (100 µM) for 30 minutes and analyzed by the ICE bioassay (Fig. 6). Topo I was detected as free protein (fraction 16-20) both in the presence and absence of CPT. The fractions at the top of the gradient (free proteins) contain largely cellular debris, membranes and protein aggregates. Consequently, the immunoblot signals from these crude fractions represent background binding of antibody or protein-A and do not represent specific topo I signals. Lysates from untreated cells have very low levels of topo I in the DNA peak (fractions 3-5) representing basal trapping of the enzyme on DNA. However, in the presence of CPT topo I is detected in the DNA peak indicating topo I-DNA covalent complex formation. Thus, efficient crosslinking of topo I on DNA requires the presence of CPT. These results demonstrate that the ICE bioassay can be used to evaluate topo I poison activity in the cellular context. Additionally, CPT-induced covalent complexes are specific to topo I as an antibody against topo II does not detect any protein in the DNA fractions (Fig. 6).
Figure 5: Western blot analysis of crude HeLa cell extracts probed with the Scl 70 antibody.

Nuclei isolated from HeLa cells were solubilized using SDS. Various amounts of extracts were subjected to SDS-PAGE followed by electrotransfer to nitrocellulose and analyzed by Western blotting. Lane 1, purified topo I probed with Scl 70; lanes 2-4, 25, 50 or 100 μg of extract probed with Scl 70, respectively.
Figure 5
Figure 6: Stabilization of topoisomerase I-DNA covalent complexes by camptothecin in vitro.

HeLa cells were subjected to analysis by the ICE bioassay as follows. Cells ($10^7$) were treated with 100 µM CPT, lysed with sarkosyl and loaded onto a cesium chloride gradient. Fractions from the gradient were analyzed for topo I by immunoblotting. DNA concentrations were measured by fluorometry. The graph shows the DNA profile from a representative gradient. Fractions were tested with the Scl 70 antibody to topo I as well as an anti-topo II antibody (slot blots are labeled accordingly).
Protein-DNA Complexes

Free Proteins

Topo I Antibody

No Drug

100 µM CPT

Topo II Antibody

No Drug

100 µM CPT

Figure 6
The ICE bioassay was also used to determine the efficacy of TPT. The yield of covalent complexes from each treatment was quantified by expressing total topo I relative to the total amount of DNA. The effect of topo I poisons can also be expressed in terms of the "ICE Index" which is the ratio of covalent complexes in drug versus no drug treatment. This index represents the fold increase in complex formation due to the presence of a test drug. At the same concentration of drug, TPT is two fold more effective in inducing topo I-mediated damage compared to the parental compound, CPT (Fig. 7). Additionally, TPT induces topo I-DNA covalent complexes in a dose dependent manner (Fig. 8).

The ICE Bioassay detects reversible topotecan induced topoisomerase I-DNA covalent complexes.

Drug induced topo I-DNA covalent complexes have been shown to dissociate upon drug removal or dilution in vitro (66,79). Enhanced formation of topo I-DNA covalent complexes in the presence of CPT is also reversed by the addition of high concentrations of NaCl (66,80). Evidence for reversibility in cells has also been detected by placing drug treated cells at a lower temperature (66,80). To determine whether the ICE bioassay can detect this reversibility, the following experiment was carried out. HeLa cells were treated with TPT (10 μM) for 30 minutes and either lysed directly in sarkosyl or incubated in drug free medium for an additional 30 minutes prior to lysis.
Figure 7: Comparison of camptothecin and topotecan activity in vivo.

HeLa cells ($10^7$) were treated with 100 μM TPT, 100 μM CPT, or no drug and analyzed by the ICE bioassay. The extent of complex formation was determined by dividing the total amount of topo I (from densitometry) by the total amount of DNA. The ICE index was determined by dividing poison-induced topo I-DNA covalent complexes by basal levels (i.e., in the absence of drug) of covalent complexes.
Figure 7

<table>
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<th>ICE Index (Fold Increase over no drug)</th>
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<td></td>
<td>1</td>
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<td>15</td>
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</table>
Figure 8: Relationship between the ICE index and topotecan concentration.

HeLa cells $\left(10^7\right)$ were treated with increasing amounts of TPT and analyzed by the ICE bioassay. The ICE index was determined by dividing drug-induced topo I-DNA complexes by basal level of covalent complexes.
ICE Index (fold increase over no drug)
(Fig. 9). When cells are treated with TPT and immediately lysed, topo I-DNA complexes are detected (lane 3), however, after a 30 minute drug-free chase, very low levels of topo I are detected in the DNA peak (87% of topo I-DNA covalent complexes are reversed). These results show that the ICE bioassay yields expected results based upon general biochemical behavior of topo I both in vitro and in vivo (66,80). Similarly, the ICE bioassay is also able to detect reversibility of topo I-DNA covalent complexes by high salt treatment. HeLa cells were exposed to TPT (10 μM) or no drug for 30 minutes followed by NaCl addition to a final concentration of 1M. Cells were lysed after 30 minutes with 1% sarkosyl and lysates were subjected to the ICE bioassay. As a control, HeLa cells treated with TPT or no drug were directly lysed with sarkosyl without high salt treatment (Fig. 10). The results show that high salt treatment also reverses TPT induced topo I-DNA adducts (87 % covalent complexes are reversed) consistent with in vitro studies. Thus, TPT induces reversible topo I-DNA covalent complexes similar to the parental compound CPT.

**Stability of topotecan at physiological pH.**

CPT and its analogs undergo hydrolysis at physiological pH and convert to an inactive open ring form (95,97). However, the rate of TPT hydrolysis has only been tested in physiological buffers and plasma (95,97). The rate of TPT hydrolysis in the
Figure 9: Reversal of topotecan-induced topoisomerase I-DNA complexes by drug removal.

HeLa cells were treated with 10 μM TPT (or no drug) for 30 minutes at 37 °C (two 100 mm petri dishes, 10^7 cells per dish). One of the pair was sarkosyl lysed while the other plate was incubated in drug free media for an additional 30 minutes followed by sarkosyl lysis (reversed). Lysates were analyzed by the ICE bioassay. Lane 1, no drug; lane 2, no drug treatment for 30 minutes followed by incubation in drug free medium for 30 minutes (mock treatment control); lane 3, treatment with 10 μM TPT for 30 minutes followed by direct lysis in sarkosyl; lane 4, treatment with 10 μM TPT for 30 minutes and in drug free media for 30 minutes prior to sarkosyl lysis.
**Figure 9**

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<table>
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(Fold Increase over No Drug)
Figure 10: Reversal of topotecan induced topoisomerase I-DNA complexes by high salt treatment.

HeLa cells were treated with 10 μM TPT (or no drug) for 30 minutes at 37 °C (two 100 mm petri dishes, 10^7 cells per dish). One of the pair was treated with NaCl to a final concentration of 1M and incubated for an additional 30 minutes followed by sarkosyl lysis (reversed). Lysates were analyzed by the ICE bioassay. Lane 1, no drug; lane 2, no drug treatment for 30 minutes followed by 1M NaCl treatment for 30 minutes (mock treatment control); lane 3, treatment with 10 μM TPT for 30 minutes followed by direct lysis in sarkosyl; lane 4, treatment with 10 μM TPT for 30 minutes followed by salt reversal.
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<tr>
<td>ICE Index</td>
<td>2.5</td>
<td>17</td>
<td>(87 % complex reversal)</td>
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</table>

(Fold Increase over no drug)

Figure 10

44
cellular environment was determined by incubating HeLa cells with TPT (10 μM) and measuring the amount of intracellular lactone (intact ring) TPT at various times post treatment by HPLC. The results show a time dependent decrease in lactone TPT (Fig. 11); within 30 minutes, 50% of TPT is converted to its inactive form.

The effect of TPT hydrolysis on topo I inhibition was tested in vitro by DNA relaxation assays. A time dependent study of TPT induced topo I-DNA adducts reveals that in the first 10 minutes, approximately 175 ng of nicked DNA was formed, however, by 30 minutes the yield of nicked DNA decreased to 75 ng, suggesting that the drug and/or enzyme is less active (Fig. 12A). To determine if the activity of the purified enzyme decays during the time course of the experiment, topo I was preincubated for 10 minutes or 60 minutes prior to addition of the DNA substrate. At lower concentrations of topo I there appears to be a reduction in enzyme activity as a result of the pre-incubation (Fig. 12B). However, at the highest concentration of topo I (the amount used in the above time course experiment, Fig 12A), the enzyme activity did not significantly decay during the course of the preincubation. Therefore, it is the activity of TPT, and not topo I, that is reduced over the course of the incubation.

Topoisomerase I-DNA complexes are stabilized by liposome encapsulated topotecan. In an attempt to increase the stability of TPT, gel phase lipid bilayers, composed of DSPC (102) that maintain a proton gradient across the bilayers were used to encapsulate TPT. Using this, TPT can be packaged in multilamellar DSPC liposomes with a reduced
Figure 11: Topotecan hydrolysis in cultured cells.

HeLa cells \((10^7)\) were treated with 10 \(\mu\)M TPT. At various times post treatment, cells were separated from extracellular drug and cell associated TPT was measured by HPLC as described in Materials and Methods. The amount of lactone or active form TPT was plotted as a function of time. The analysis was repeated in duplicate and each trial was plotted separately. ▲-Trial 1; ■-Trial 2.
Figure 11
Figure 12: Time course of topotecan induced topoisomerase I-DNA covalent complexes in vitro.

Panel A. TPT activity in vitro. Supercoiled plasmid DNA was incubated with 20 nM purified topo I (plus or minus 10 μM TPT as indicated) for different times. The DNA products were then visualized by ethidium bromide agarose gel electrophoresis. Lane 1, supercoiled DNA marker; lane 2, DNA and topo I; lanes 3-7, DNA, topo I and 10 μM TPT, reactions were stopped at the times indicated above the lane. In the presence of ethidium bromide relaxed DNA has higher mobility than supercoiled DNA.

Panel B. Effect of topo I preincubation on enzyme activity. Various concentrations of topo I were preincubated in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA at 37 °C for 10 minutes or 60 minutes followed by addition of the DNA substrate. Reactions were further incubated for 15 minutes at 37 °C, stopped by the addition of SDS and analyzed by agarose gel electrophoresis in the absence of ethidium bromide. In the absence of ethidium bromide, relaxed DNA has decreased mobility as compared to supercoiled DNA. Gels were then stained with ethidium bromide and photographed under UV light. Lanes 1 and 5, DNA alone; lanes 2 and 6, DNA and 20 nM topo I; lanes 3 and 7, DNA and 10 nM topo I; lanes 4 and 8, DNA and 5 nM topo I.
Figure 12
internal pH of 5 at which the drug is more stable (LIP-TPT). To determine if LIP-TPT can
access topo I-DNA complexes in vitro, topo I activity assays were performed with different
centrations of LIP-TPT (Fig. 13). The data show that DSPC liposomes alone do not
affect topo I, whereas LIP-TPT clearly increases the formation of nicked DNA. Evidently,
sufficient drug escapes the liposome to affect the topo I-DNA cleavage/religation equilibrium.
However, LIP-TPT is not as efficient as free TPT in trapping topo I-DNA covalent
complexes in vitro. Approximately 0.5 μM free TPT is required to completely convert
supercoiled DNA into nicked DNA (Fig 3) whereas 5 μM of LIP-TPT is required to see the
same effect (Fig 13).

Next, the ICE bioassay was used to compare free TPT and LIP-TPT induced topo I-
DNA covalent complexes in HeLa cells after a 2 hour exposure to each drug (Fig. 14). This
experiment was repeated four times with TPT and LIP-TPT and the results are summarized in
Fig. 14B. The data reveal that both forms of TPT induce topo I-DNA complexes in HeLa
cells, however, LIP-TPT was more efficient in stabilizing topo I-DNA complexes than free
TPT. The ICE index for free TPT was 3-4 fold compared to an ICE index for LIP-TPT of
10-14 fold. Therefore, LIP-TPT is 3-4 fold more efficient at causing topo I dependent DNA
damage than free TPT. Additionally, these experiments demonstrate the reproducibility of
the ICE bioassay. In HeLa cells, in the presence of 50 μM TPT, the 4 replicates gave an ICE
index of 4.12 ± 0.86 while in the presence of LIP-TPT, the ICE index is 12.76 ± 2.53.
Figure 13: Topoisomerase I cleavage in vitro using liposome bound topotecan.

Supercoiled plasmid DNA was incubated with human topo I (20 nM) with increasing amounts of LIP-TPT and incubated at 37 °C for 30 minutes. Reaction products were analyzed by agarose gel electrophoresis in the absence of ethidium bromide. Gels were stained with ethidium bromide and photographed under UV light. Lane 1, supercoiled DNA marker; lane 2, DNA and topo I; lanes 3, 4, DNA, topo I and drug free liposomes; lanes 5-9, DNA, topo I and increasing concentrations of LIP-TPT as indicated above the lanes.
Figure 13
Figure 14: Stabilization of topoisomerase I-DNA covalent complexes using free and liposome bound topotecan.

Panel A. HeLa cells (10^7) were treated with 50 μM TPT or 50 μM LIP-TPT for 2 hours. Cells were sarkosyl lysed and analyzed by the ICE bioassay.

Panel B. A graphical representation of the ICE bioassay data from Panel A. ICE bioassays were repeated in quadruplicate and the levels of complex formation were determined as described in “Materials and Methods”.

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Liposomes enhance topotecan stability.

The ability of liposomes to increase the stability of the biologically active form of the drug (102) may partially explain the enhanced activity of LIP-TPT. This effect is demonstrated *in vitro* when topo I activity assays are performed after pre-incubating LIP-TPT and free TPT for two hours (at physiological pH). Control reactions were set up with the same formulations of drug that were not pre-incubated (fresh TPT controls). Formation of nicked DNA was monitored by agarose gel electrophoresis. LIP-TPT showed similar activity whether added fresh or after pre-incubation (Fig. 15A). In contrast, pre-incubated free TPT was 2-4 fold less active relative to fresh TPT (Fig. 15B). It is apparent that the activity of free TPT is reduced by pre-incubation whereas LIP-TPT is relatively insensitive to pre-incubation.

Stability experiments with LIP-TPT vs. free TPT were also conducted in HeLa cells using the ICE bioassay. Free TPT or LIP-TPT was pre-incubated in culture medium (physiological pH) at 37 °C for two hours prior to exposure to HeLa cells for 30 minutes at 37 °C (Fig. 16). The ICE bioassay gave an ICE index of 10.0 ± 0.5 for fresh LIP-TPT and 8.5 ± 2.0 for pre-incubated LIP-TPT (20% reduction in drug activity). The ICE index for fresh TPT was 6.0 ± 0.2 and for pre-incubated TPT the value was 2.5 ± 0.5 (58% reduced); thus, TPT showed reduced efficacy relative to LIP-TPT as a result of pre-incubation. Interestingly, pre-incubated LIP-TPT still displayed higher activity than fresh TPT.
Figure 15: Comparison of the stability of free and liposome bound topotecan in vitro. 

Free and liposome encapsulated TPT was pre-incubated with DNA in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA at 37 °C for 2 hours. Topo I (20 nM) was added and the incubations continued for an additional 30 minutes. Reactions were terminated with sarkosyl and open circular DNA was measured by agarose gel electrophoresis. Parallel reactions were set up using fresh TPT as the free or liposome encapsulated forms as controls.

Panel A. Topo I inhibition using fresh and pre-incubated LIP-TPT.

Panel B. Topo I inhibition using fresh and pre-incubated TPT.
Figure 15

A

Liposome Bound Topotecan

% Open Circular DNA

Pre-incubated Drug
Fresh Drug

Topotecan Concentration (μM)

B

Free Topotecan

% Open Circular DNA

Pre-incubated Drug
Fresh Drug

Topotecan Concentration (μM)

Figure 15
Figure 16: Comparison of the stability of free and liposome bound topotecan *in vivo*.

Free and liposome bound TPT (50 µM) were pre-incubated in DMEM at 37 °C for 2 hours. HeLa cells (10^7 per treatment) were treated with fresh or pre-incubated drug (30 minutes at 37 °C) and topo I-DNA covalent complexes determined.

Panel A. ICE bioassay analysis of fresh and preincubated drug. Lane 1, no drug; lane 2, fresh LIP-TPT; lane 3, pre-incubated LIP-TPT; lane 4, fresh TPT; lane 5, pre-incubated TPT.

Panel B. Topo I-DNA complexes were quantified (by densitometry of the data in panel A, duplicate experiments) and the ICE index for each inhibitor was determined.
<table>
<thead>
<tr>
<th></th>
<th>No Drug</th>
<th>LIP-TPT</th>
<th>TPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preincubation</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Figure 16**

**A**
- Free Proteins
- Protein-DNA Complexes

**B**
- LIP-TPT
- LIP-TPT (Preincubated)
- TPT
- TPT (Preincubated)

ICE Index (Fold increase over No Drug)
Analysis of intracellular drug delivery.

Liposomes frequently increase the efficiency of intracellular drug delivery (102,107,108), therefore, the increased uptake and/or accumulation of TPT within the cell may explain the enhanced activity of LIP-TPT. To evaluate this, HeLa cells were treated with various concentrations of free TPT or LIP-TPT and intracellular TPT levels were measured after one hour by HPLC (Fig. 17). Liposome encapsulation clearly increases intracellular delivery of TPT. At 50 μM (the concentration used in the ICE bioassay), LIP-TPT resulted in approximately 7 fold more intracellular TPT compared to the free drug.

DISCUSSION

A quantitative bioassay (ICE) was developed to measure topo I-DNA covalent complexes in living cells. Since the ICE bioassay is antibody based it is highly specific for detecting topo I-DNA covalent complexes. Thus, the physiological activity of endogenous topo I can be examined directly with the ICE bioassay. Results are expressed as a function of DNA concentration which means that topo I complexes are being measured on a per cell basis. Topo I trapping in the presence and in the absence of poisons can be measured by the ICE bioassay. Basal trapping in the absence of poisons gives lower signals on immunoblots and is clearly less efficient as expected due to rapid religation by topo I. Topo I poisons, such as CPT and TPT inhibit religation and stabilize topo I-DNA covalent complexes resulting in increased topo I signals in the
Figure 17: Accumulation of cellular topotecan in HeLa cells.

HeLa cells (10^7) were treated with various concentrations of free TPT and LIP-TPT. Following a one hour incubation at 37 °C, cells were washed free of extracellular drug and cell-associated TPT was measured by HPLC methodologies as described in “Materials and Methods”.
Figure 17

TPT Accumulation (µM TPT/10^6 cells) vs. Input TPT (µM)

- Liposome TPT
- Free TPT

Input TPT (µM):
0 10 20 30 40 50 60

TPT Accumulation:
0.0 0.5 1.0 1.5 2.0 2.5 3.0
DNA fractions. The efficacy of these agents is represented by the ICE index which is the fold increase in drug induced covalent complexes over no drug. TPT shows a two fold enhancement in producing topo I mediated damage as compared to CPT. Additionally, TPT induced topo I DNA damage, as measured by the ICE bioassay, is dose dependent. The specificity of the ICE bioassay is further demonstrated by the observation that probing the DNA containing fractions from cells exposed to TPT gives a strong signal with the anti-topo I antibody but not with an anti-topo II antibody. Furthermore, conditions that strongly promote dissociation of topo I-DNA covalent complexes in vivo such as drug withdrawal and high salt are detectable by the ICE bioassay. Collectively, the foregoing points support the assertion that the ICE bioassay is a highly specific measure of DNA damage inflicted by topo I in the presence CPT and related agents.

There are several advantages of the ICE procedure for analyzing in vivo topo I-DNA covalent complexes. First, the method is simple and convenient, requiring only direct lysis of cells or tissues. Second because of its simplicity, direct lysis of actively metabolizing cells (under physiological conditions) can be accomplished. Cleavage/religation equilibria can be affected by different conditions, including raising or lower temperature (66,80), pH, ionic environment and drug depletion. To obtain a “freeze frame” of topo I cleavage/religation action, it is important that conditions remain constant up to the point of protein denaturation. The ICE bioassay requires no manipulation of cells prior to lysis. Third, the efficacy of a given topo I poison within
the context of the living cell is directly determined using the ICE method. Therefore, analogs and drug delivery variables can be tractably evaluated in any cell or tumor. Finally, the ICE assay can also be used to measure DNA damage induced by topo II specific poisons (108).

In this study, it was also shown that LIP-TPT is three to four fold more efficient at stabilizing topo I-DNA complexes in HeLa cells relative to free TPT. The mechanistic basis for enhancement appears to be related to the combined influence of an elevated stability of pharmacologically active drug, and increased intracellular delivery of TPT. CPT and related compounds have a binding preference for solid lipid bilayers and it is known that the biologically active lactone form is stabilized by lipid associations (97,102). The pre-incubation data reveal that LIP-TPT activity decayed by only 30% as compared to 60% of free TPT. This 2-fold difference is considerably less than the 7-fold increase in drug delivery detected with LIP-TPT. Therefore, it seems likely that the increased efficacy of LIP-TPT is best explained by greater intracellular drug delivery. Liposomes deliver their contents to the cell via specific mechanisms which are frequently more efficient than simple diffusion, hence, more drug is intracellularly delivered when TPT is liposome encapsulated. There are additional and related considerations to explain the enhanced activity of LIP-TPT such as interactions between TPT and proteins in cells. It has been previously reported that the lactone/hydroxy equilibrium of CPT is strongly affected by proteins that may interact preferentially with
one form or another (110). Thus, liposome bilayers may shield TPT from agents that preferentially bind the hydroxyl or inactive form. Additionally studies in tissue culture will be required to determine the precise mechanism, however, the data suggest that liposomes may be of utility for increasing the stability of active TPT under physiological conditions.

It is interesting that LIP-TPT seems to be active in vitro even though DSPC liposomes have been reported to be relatively impermeable to TPT (102). The fact that DSPC encapsulated drug clearly acts as an efficient poison with purified enzyme and DNA implies that sufficient drug is released in vitro. The underlying mechanism (simple release, slow diffusion or exchange, etc.) is not known but may be related to the ability of these drugs to interact with proteins in the micro-environment. As noted above, the lactone/hydroxy equilibrium is strongly affected by proteins that may preferentially interact with one form or another (110) and a similar mechanism may drive drug release. For example, direct binding of TPT to topo I or formation of a ternary binding complex between enzyme, DNA and drug may enhance release of drug. Figure 13 shows that, at the same concentration of drug, the activity of LIP-TPT in vitro is slightly lower than free TPT (at 0.5 μM, LIP-TPT shows about 60% of the activity of free TPT). These results support the notion that, while the liposome maintains TPT within the internal environment, it allows sufficient release to give biological activity (although reduced compared to the same concentration of free drug).
CONCLUSIONS

Through these studies, it has been shown that the ICE bioassay is a convenient and useful assay to measure DNA damage inflicted by topo I in the presence and absence of topo I poisons. TPT, a water soluble derivative of CPT, is effective in inducing reversible topo I-DNA covalent complexes in HeLa cells. Liposome encapsulation increases the efficiency of TPT in stabilizing topo I-DNA covalent complexes relative to free drug. Enhanced efficacy is largely due to increased intracellular delivery of TPT. Additionally, the liposome environment acts to increase TPT stability.
CHAPTER 3

ANALYSIS OF TOPOISOMERASE I-DNA COMPLEXES IN TOPOTECAN ADMINISTERED PATIENTS.

INTRODUCTION

The topo I inhibitor CPT, was evaluated in clinical studies in the late 1960s and early 1970s. Due to the inherent aqueous insolubility of CPT, the drug was modified and its water soluble sodium salt was used in clinical trials (reviewed in 101). Despite promising anti-tumor activity in phase I studies, the drug was not only ineffective but highly toxic in phase II trials of patients with gastrointestinal malignancies and melanomas (88,90,91). Myelosuppression, vomiting and diarrhea were often severe and, as a result, further clinical testing of CPT was ceased. Renewed interest in the study of camptothecins, 20 years later, has resulted due to a more complete understanding of their mechanism of action and the development of effective, water-soluble synthetic and semisynthetic derivatives of CPT (93). Currently, four analogs of CPT (TPT, irinotecan, 9-aminocamptothecin and GI 147211C) are undergoing clinical evaluation (Fig.18) (100,101).
The cellular effects of CPT include reversible chromosomal fragmentation, inhibition of DNA and RNA synthesis, and ultimately cell death (66,76,113). Topo I-mediated DNA damage induced by CPT and its analogs is different from other types of DNA damage because the covalent complexes reverse upon removal of the poison (76,113). The reversibility of this DNA damage suggests that CPT-induced cell death may result from the interaction of the covalent complex with DNA metabolic processes rather than inhibiting topo I relaxation activity (7). Inhibition of RNA synthesis is consistent with the observation that topo I associates with actively transcribing genes (56,57,58) and topo I-DNA covalent complexes occur preferentially within expressed genes (56,114,115,116). CPT-induced topo I-DNA covalent complexes appear to block elongation by impeding progression of RNA polymerase along the gene (116). Removal of CPT results in rapid and complete reversal of RNA inhibition probably due to dissociation of topo I-DNA covalent complexes (116). Similarly, inhibition of DNA synthesis appears to occur due to the accumulation of blocked replication forks caused by covalent complexes (78,117,118,119). However, unlike RNA synthesis, inhibition of DNA synthesis becomes partially irreversible following exposure to CPT (113). This is probably due to formation of topo I-DNA covalent complexes in the single strand regions of the replication fork, or collision of the advancing fork with a CPT-stabilized topo I-DNA adduct thereby resulting in irreversible double strand breaks (80). Stabilization of topo I-DNA covalent adducts also leads to sister chromatid exchange
Figure 18: Camptothecin and analogs undergoing clinical evaluation.

TPT = topotecan, 9-AC = 9-aminocamptothecin, CPT-11 = irinotecan.
**Figure 18**

<table>
<thead>
<tr>
<th>ANALOGS</th>
<th>( R_1 )</th>
<th>( R_2 )</th>
<th>( R_3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPT</td>
<td>( H )</td>
<td>( H )</td>
<td>( H )</td>
</tr>
<tr>
<td>TPT</td>
<td>( \text{OH} )</td>
<td>((\text{CH}_3)_2\text{NHCH}_2)</td>
<td>( H )</td>
</tr>
<tr>
<td>CPT-11</td>
<td>( \text{N-} )</td>
<td>( H )</td>
<td>( \text{C}_2\text{H}_5)</td>
</tr>
<tr>
<td>9-AC</td>
<td>( H )</td>
<td>( \text{NH}_2 )</td>
<td>( H )</td>
</tr>
</tbody>
</table>
and chromosomal aberrations (120). Therefore, CPT-induced cytotoxicity results from the interaction of topo I-DNA adducts with transcription and replication machinery producing DNA single strand and double strand breaks. Genomic fragmentation resulting from topo I-mediated DNA damage ultimately leads to G2 arrest and cell death (95,121).

TPT is a CPT derivative with higher aqueous solubility due to the presence of a charged dimethylaminomethyl group at position R2 (93). TPT possesses antitumor activity against a variety of transplantable mouse and human tumors (122,123,124). Based on preclinical screening results, phase I studies were performed with TPT (100,101). In all phase I studies, several anti-tumor responses were seen with myelosuppresion being the only dose limiting toxic side effect. Due to the promising results from phase I studies, phase II studies of TPT were undertaken for many tumor types. Studies are underway to identify the malignant diseases where TPT has greatest antitumor activity (100,101).

Concurrent with clinical trials, adaptation of assays to quantify drug-induced topo I-DNA covalent complexes in patients can potentially serve as a method to individualize drug treatment. The ICE bioassay allows accurate measurement of the amount of topo I-mediated DNA damage produced in the presence of topo I specific poisons in cultured cells. Adapting the ICE bioassay to the clinical situation will provide a useful diagnostic tool for determining the efficacy of topo I poisons in cancer.
treatment. Additionally, screening patients prior to treatment may provide an avenue for candidate selection for treatment with topoisomerase drugs. The James Cancer Hospital at the Ohio State University was approved for phase II trials with TPT for non-Hodgkin’s lymphomas and melanomas. In collaboration with Dr. Eric Kraut at the James Cancer hospital, patients undergoing TPT treatment were analyzed for topo I-DNA covalent complexes using the ICE bioassay. In this study, peripheral blood from these patients was assayed at various times during and after TPT infusion for topo I mediated DNA damage. Additionally, tumor biopsy specimens from various sources were analyzed for TPT induced topo I-DNA covalent complex formation to examine tumor response to the drug.

MATERIALS AND METHODS

Reagents.

Topotecan was supplied by the Investigational Drug Branch of the National Cancer Institute.

Patients.

Four patients with non-Hodgkin’s lymphomas and nine patients with malignant melanomas entered into a phase II study of TPT due to refractory disease. Lymphoma patients were treated with 1.25 mg/m² and melanoma patients at 1.5 mg/m² TPT (178). TPT was administered by 30 minute intravenous infusions daily for a total of five days. Physical examination and complete blood counts were performed every three weeks.
Toxicity measurements and clinical assessments of disease were done at each visit and interim blood counts were done weekly. Objective tumor responses were made according to standard criteria.

**Pharmacokinetic sampling and analysis of plasma drug levels.**

On day four of chemotherapy, 2 ml blood samples were collected just prior to treatment (T0), at 5 minutes (T5), 25 minutes (T25) and 60 minutes (T60) after the start of the 30 minute infusion. To determine the fraction of the intact lactone form of TPT, 1.3 ml of each blood sample was centrifuged (2 minutes, 10,000 x g) and three separate 250 μl aliquots were added to 0.75 ml of cold DOSS solution (20 mM sodium dioctylsulfonosuccinate in methanol), vortexed for 15 seconds and centrifuged (2 minutes, 10,000 x g). The supernatants were decanted and combined into a single siliconized glass tube and promptly frozen in a dry ice/acetone bath. Prior to HPLC, 900 μl aliquots were mixed with 60 μl of an internal standard (16.7 μg/ml acridine in acetonitrile) and 400 μl of double distilled water. The tubes were vortexed, centrifuged briefly as above and the pH adjusted to 5.3 with either 3.5 M H₃PO₄ or 3.5 M KOH.

To determine the total TPT level, the remaining blood sample was centrifuged at 4 °C (5 minutes, 2000 x g) and the plasma transferred to a fresh tube and stored at -80 °C until assay. Prior to analysis, 300 μl of thawed plasma was mixed with 30 μl of 3.5 M H₃PO₄. The mixture was vortexed briefly and, after a 5 minute incubation at room temperature, 900 μl of cold DOSS, 60 μl of the acridine internal standard and 400 μl of
aqueous triethylamine (1:50 (v/v) triethylamine:water) was added and briefly vortexed. The mixture was centrifuged (6 minutes, 10,000g), 1.2 ml of supernatant added to a microcentrifuge tube, and the pH adjusted to 5.3. A standard curve was generated by adding 0, 5, 10, 20, 50, 100, 250 and 500 µl aliquots of 1 µg/ml stock solution of TPT (in acetonitrile) to siliconized glass tubes. The acetonitrile was evaporated to dryness under a flow of nitrogen gas at room temperature. Additionally, 1 ml of donor blank plasma was mixed into each tube.

Three aliquots (250 µl each) were analyzed separately for either total TPT or the intact lactone form of TPT by reverse phase HPLC. HPLC was performed using a Waters Nova-Pak C18 column (3.9 mm x 150 mm) with an acetonitrile/0.1M KH2PO4/aqueous triethylamine/DOSS eluent (275/250/2.1/2.223, v/v/v/w, respectively) at pH 6, at a flow rate of 1 ml/minute. Fluorescence of the lactone form of TPT and the internal standard (acridine) [excitation wavelength of 362 nm with a cutoff emission wavelength filter of 470 nm] was measured using a Spectaflow 980 programmable fluorescence detector. Using this technique, drug recovery from plasma was found to be 97-98%.
Sampling and analysis of topoisomerase I covalent complex formation in peripheral blood.

On the fourth day of infusion, peripheral blood was collected prior to infusion (T0), 5 minutes (T5) and 25 minutes (T25) after the start of infusion. Treatment was stopped after 30 minutes of infusion and 30 minutes later (T60) a final sample was taken. At each time point, 2 ml of blood was collected directly into tubes containing 0.1 ml of 20% sarkosyl to rapidly denature the cells and trap topo I-DNA complexes; blood samples were at body temperature at the time of lysis because there was no intervening handling of blood prior to lysis. Blood (2 ml) was also collected at each time point in heparin, incubated at 37 °C for 30 minutes in vitro and lysed with 0.1 ml of 20% sarkosyl. Blood lysates stored at -20 °C or processed directly yielded identical results; therefore, the samples were usually stored frozen prior to processing. The lysates were thawed and analyzed by the ICE bioassay as described in chapter 2.

Quantification of topoisomerase I-DNA complexes in peripheral blood.

Topo I signals on immunoblots were scanned by densitometry and compared to a standard curve generated from known concentrations of purified human topo I on the same immunoblot. DNA concentrations were determined using a DNA fluorometer (Hoeffer Scientific Instruments) and the amount of covalent complex expressed in terms of ng of topo I per µg DNA. Since the amount of DNA per cell is constant, expressing covalent complexes as ng enzyme per µg DNA corresponds to the absolute
number of topo I molecules bound per cell. Statistical analyses were done using the Wilcoxon Matched Pairs Signed Ranks Test. A p value of less than 0.10 indicates that the samples are significantly different, while a p value greater than 0.10 indicates that the samples are similar.

Preparation of cell suspensions from tumor explants.

Fresh tumor explants were obtained from patients through the Tissue Procurement facility at the James Cancer Hospital. The tumor samples were processed by the method of D’Ambrosio et al. (125) with modifications. The tumors were chopped into 1 mm³ pieces using two scalpels and transferred to a tube containing 20 ml of RPMI medium. Trypsin was added to a final concentration of 2.5 % and the tube was vigorously agitated at room temperature for 15 minutes. Trypsinization of the tumor specimen was stopped by adding fetal bovine serum (Hyclone Labs) to a final concentration of 10%. The cell suspension was filtered through a sieve (pore size = 40μm) to separate single cells from fibrous tissue and aggregates. The resulting cell suspension was collected and cells pelleted by a low speed centrifugation (10 minutes, 1000 x g). Cells were resuspended in 1 ml serum free RPMI medium and the number of cells was determined by hemocytometer counting. An aliquot of the cell suspension (50 μl) was diluted with 950 μl RPMI, and ethidium bromide and acridine orange dyes were added to a final concentration of 5 μg/ml and 1.5 μg/ml, respectively. Ethidium bromide stains dead cells while acridine orange stains live cells. The cells were visualized by
microscopy under ultraviolet light and the percent of living cells was determined. Cells (10⁶ per treatment) were incubated with 10 μM TPT or no drug for 30 minutes at 37 °C followed by ICE bioassay analysis.

Cytotoxicity assays.

The cytotoxic effects of TPT were determined by lactate dehydrogenase (LDH) release assays (Boehringer Mannheim Biochemicals). LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into the cell culture supernatant upon damage to the plasma membrane. Cells were plated at 2 x 10⁵ cells, 1 x 10⁵ and 5 x 10⁴ viable cells per well (9 total wells per cell concentration) in a 96 well microtiter plate in 200 μl of RPMI containing 10 % fetal bovine serum and incubated at 37 °C overnight. Prior to drug treatment, the medium was gently aspirated so that floating dead cells and debris were removed while the monolayer of living cells remained undisturbed. Cells were treated with (200 μl) of no drug, 10 μM TPT, or 1% Triton X-100 for 30 minutes at 37 °C. The Triton X-100 serves to determine the maximum amount of LDH released from cells. Following treatment, 100 μl of the medium was transferred to a fresh microtiter plate and an equal volume of LDH substrate was added. The reaction was incubated at room temperature for 30 minutes in the dark. The LDH activity is determined by an enzymatic assay: in the first step NAD⁺ is reduced to NADH/H⁺ by the LDH catalyzed oxidation of lactate to pyruvate. In the second step a tetrazolium salt INT (pale yellow) is reduced to formazan (red) by
transfer of $\text{H}/\text{H}^+$ from NADH/$\text{H}^+$. The amount of formazan dye produced is determined by measuring absorbance at 540 nm using an ELISA microtiter plate reader. The percent cytotoxicity was determined by the following equation:

$$\text{Cytotoxicity} = 100 \times \left( \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}} \right)$$

RESULTS

Correlation between the ICE bioassay and cellular toxicity of topotecan.

For the clinical trials, the absolute amount of topo I bound per $\mu$g DNA is used to measure topo I-DNA covalent complexes instead of the ICE index (fold increase over no drug). This allows for measurement of basal trapping of topo I on DNA in each patient as well as TPT induced covalent complexes. As a control, the absolute amount of topo I bound to DNA at various concentrations of TPT was first determined in HeLa cells (Table 1). In the absence of TPT, 0.6 ng of topo I were bound per $\mu$g of DNA and this increased to 6.4 ng of topo I in the presence of 25 $\mu$M TPT.

Prior to analyzing clinical specimens, it was necessary to determine if TPT-induced topo I-DNA covalent complexes correlated with the cell killing effects of the drug. To this end, HeLa cells were treated with various concentrations of TPT and analyzed either by the ICE bioassay or by cytotoxicity assays (measured by LDH
Table 1: Dose dependence of topoisomerase I-DNA covalent complexes.
HeLa cells ($10^5$) were treated with TPT for 30 minutes at 37 °C and analyzed by the ICE bioassay. Covalent complexes were determined as described in Materials and Methods.

<table>
<thead>
<tr>
<th>TPT Dose (μM)</th>
<th>Covalent Complexes (ng topo I per μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>0.5</td>
<td>2.1</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>5</td>
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<tr>
<td>10</td>
<td>5.7</td>
</tr>
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<td>25</td>
<td>6.4</td>
</tr>
</tbody>
</table>
release assays). Both topo I-DNA covalent complex formation and drug-induced cytotoxicity show a dose dependent response to TPT (Fig. 19A). Additionally, TPT-mediated topo I-DNA damage correlates with the cell killing effects of the drug (Fig. 19B).

**Formation of topoisomerase I-DNA covalent complexes in peripheral blood.**

An important requirement for the ICE bioassay is rapid and immediate lysis of cells to effect trapping of the topo I-DNA complexes. In the clinical situation, peripheral blood was chosen for ICE bioassay analyses, as blood can be quickly and easily collected into tubes containing sarkosyl to rapidly lyse the cells efficiently. The feasibility of measuring topo I-DNA covalent complexes in peripheral blood was first tested *in vitro*. Freshly drawn heparinized peripheral blood was incubated *in vitro* (in duplicate) with 10 μM TPT or no drug followed by ICE bioassay analysis (Fig. 20). In the absence of TPT, the duplicates gave an average of 5.1 ± 1.0 ng topo I per μg DNA; in the presence of TPT, duplicates averaged 18.1 ± 2.1 ng of topo I per μg DNA. Thus, the ICE bioassay can reproducibly detected TPT-induced topo I-DNA covalent complexes in peripheral blood.

Next, the ICE bioassay was used to measure topo I-DNA covalent complexes in patients undergoing treatment with TPT as they were receiving infusion of the drug. Patients receive a 30 minute infusion of TPT daily for a total of five days. On the fourth day, 2 ml of blood was collected in sarkosyl containing tubes prior to infusion (T0), 5
Figure 19: Comparison of topotecan induced topoisomerase I-DNA covalent complexes and topotecan cytotoxicity in HeLa cells.

Panel A. TPT induced topo I-DNA covalent complexes and drug cytotoxicity in HeLa cells. HeLa cells (10^6) were treated with 0, 0.5, 1, 5, 10, and 25 μM TPT for 30 minutes at 37 °C and analyzed by the ICE bioassay. In a parallel experiment, TPT cytotoxicity was measured at the same drug concentrations using LDH release assays (described in Materials and Methods).

Panel B. Relationship between TPT induced cytotoxicity and topo I-DNA covalent complexes in HeLa cells. Cytotoxicity and covalent complex formation data is from Panel A.
Cytotoxicity (% Cell Death)

Covalent Complexes (ng topo I per µg DNA)

Figure 19

B

Cytotoxicity (% Cell Death)

Covalent Complexes (ng topo I per µg DNA)

TPT Dose (µM)

Cytotoxicity (% Cell Death)

A
Figure 20: ICE bioassay in peripheral blood.

Fresh heparinized blood (2 ml per treatment) was collected from donors and either left untreated or drug treated (10 μM TPT) \textit{in vitro} for 30 minutes at 37 °C in duplicate (bars, range). The blood was sarkosyl lysed and subjected to the ICE bioassay protocol. Topo I-DNA covalent complexes were quantified as described in Materials and Methods.
Covalent Complexes (ng topo I/μg DNA)

Figure 20

- No Drug
- 10 μM TPT
and 25 minutes post infusion (T5 and T25) and 30 minutes after infusion was stopped (T60). An example of the *in vivo* ICE bioassay with a single patient is shown in Fig. 21. Prior to administration of TPT, low but detectable levels of topo I-DNA complexes were found (Fig. 21, T0, 3.6 ng topo I per µg DNA). Within 5 minutes post-infusion (T5), complex formation increased to 8.9 ng topo I per µg DNA (two fold increase). Reversal of TPT-induced topo I-DNA covalent complexes in peripheral blood was tested by collecting a parallel blood sample at 5 minutes in heparin followed by a 30 minute incubation *in vitro* at 37 °C prior to sarkosyl lysis. In this case, the value reverted back to 3.6 ng topo I per µg DNA. A significantly stronger signal was detected after 25 minutes (T25 = 26.7 ng topo I per µg DNA) which was also reversed after a 30 minutes chase *in vitro* to 5.5 ng topo I per µg DNA. Infusion of TPT was stopped at 30 minutes and a final blood sample was drawn 30 minutes later and lysed. This sample (T60) yielded 9.2 ng topo I per µg DNA showing that complex reversal proceeds to a greater extent *in vitro* (T25 reversal gave 5.5 ng topo I per µg DNA) than *in vivo*. The results from this single patient show that topo I-DNA covalent complexes can be detected in patient’s blood during TPT infusion therapy, complexes can be quantified and reverse upon drug withdrawal. Again, it is important to note that the free protein fractions largely contain cellular debris, membranes and protein aggregates. Thus, signals from the top of the gradient probably represent background binding of the antibody or protein-A and do not represent specific topo I signals.

85
Figure 21: Stabilization of topoisomerase I-DNA complexes in the peripheral blood of a Melanoma patient undergoing topotecan treatment.

The patient was treated with a 30 minute infusion of TPT each day for five consecutive days. On day four, 2 ml of peripheral blood was sampled at 0, 5, 25 and 60 minutes post infusion and directly lysed in sarkosyl to trap topo I-DNA complexes as described in "Methods". An additional 2 ml of blood was collected in heparin at each time point and incubated \textit{in vitro} for 30 minutes at 37 °C prior to lysis. Lysates were subjected to the ICE bioassay to measure of topo I/DNA adduct formation at each time point.

Panel A. Immunoblots of fractions from a melanoma patient undergoing TPT treatment. Measurements of topo I were taken from the fractions at the density of DNA (lower bracket).

Panel B. Histogram showing the number of topo I-DNA complexes for this patient at various times during TPT infusion.
Topo I-DNA covalent complexes was determined in 13 patients (4 lymphoma and 9 melanoma) undergoing TPT infusion therapy. Prior to TPT infusion, low but variable amounts of topo I (T0, mean = 3.9) were detected in the DNA fractions (Table 2) demonstrating that endogenous topo I can be trapped on DNA, although inefficiently, in the absence of the poison. Compared to baseline values, there was a clear increase in covalent complex formation at T5 and T25 (p = 0.0015 and p = 0.013, respectively). Complex formation increases with increasing exposure time to TPT; more complexes were detected at T25 than at T5 (T25 is greater than T5 with p = 0.019). The patient to patient variability (more than 10 fold in some cases) is greater than the variability attributed to experimental error within the assay itself (Fig. 20). Finally, the in vivo reversibility of TPT-mediated complexes can be seen in the T60 sample, where covalent complex formation does not significantly differ from the T0 basal level (p = 0.18). Additionally, in vitro reversal occurs more rapidly than in vivo reversal (T60 is greater than T25 reversed with p = 0.0469). In general, complex formation increases with increasing TPT exposure. Upon termination of infusion, complexes revert back to a lower basal level of formation.

**Relationship between topoisomerase I complex formation and clinical response.**

To determine if there is any relationship between the TPT plasma levels and topo I-DNA covalent complex formation, adduct formation in peripheral blood was compared with TPT (lactone) blood levels at T25 and T60 (see Table 3 for TPT levels). Given the
<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Patient Name</th>
<th>Cancer</th>
<th>Covalent Complexes (ng topo I per µg DNA)</th>
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<tbody>
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<td></td>
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<td></td>
<td>T0</td>
</tr>
<tr>
<td>1</td>
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<td>MI</td>
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</tr>
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<td>Melanoma</td>
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<td>Melanoma</td>
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</tr>
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<td>LA</td>
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<table>
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<th>Values</th>
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<td>Standard Deviation</td>
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Table 2: Topoisomerase I-DNA complex formation in patients undergoing topotecan treatment.
Table 3: Topotecan concentration in plasma from patients undergoing topotecan treatment.

*Pt. = patient
*Topotecan Concentrations were determined using standard HPLC techniques
*SD = Standard Deviation
*NA = Not Available
rather large biological variability and small sample size, no significant correlation can be established between the topo I-DNA covalent complexes and plasma level of TPT (Fig. 22). In contrast, under conditions where drug exposure can be controlled (in HeLa cells) increasing TPT concentration results in an increase in the ICE index (Fig. 19).

The amount of drug-stabilized topo I-DNA complexes may potentially parallel clinical response to TPT therapy. TPT-induced covalent complex formation at T25 was compared to toxicity, as measured by the percent change in neutrophils as well as the change in tumor size (Table 4). In this limited data set, no clear correlation was seen between covalent complexes and patient response to TPT.

Topoisomerase I-DNA covalent complex formation in tumor samples.

The applicability of the ICE bioassay in the clinical situation was further extended to tumor biopsy specimens from colon cancer, breast cancer, and melanoma patients. Single cell suspensions were prepared from fresh tumor biopsies as described in Materials and Methods and tested by the ICE bioassay for TPT activity on endogenous topo I (Table 5). Topo I-DNA covalent complexes were detected in all cases in the absence of TPT. There is a great deal of variability in basal levels of topo I trapping in these tumor samples (range 0.13-1.20 ng topo I/μg DNA). Addition of TPT results in an increase in topo I-DNA covalent complexes in all but one case. Again, there is a large
Figure 22: Comparison of topotecan plasma levels and topoisomerase I-DNA covalent complexes in peripheral blood samples.

Lactone or active TPT levels from patients was compared with drug-induced topo I-DNA covalent complexes. Data for TPT and levels and complex formation was obtained from Table 3 and 2 respectively.

Panel A. Data from samples at T25.

Panel B. Data from samples at T60.
<table>
<thead>
<tr>
<th>Patient Name</th>
<th>Toxicity (measured as % Change in Neutrophils)</th>
<th>% Change in Tumor Size</th>
<th>Cov. Comp</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Initial Count</td>
<td>Final Count</td>
<td>% Change</td>
</tr>
<tr>
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</tr>
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<td>HA</td>
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</tr>
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<td>WA</td>
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</tr>
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<td>SC</td>
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<td>HR</td>
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<td>3640</td>
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</tr>
<tr>
<td>JO</td>
<td>4093</td>
<td>1276</td>
<td>69</td>
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</table>

Table 4: Comparison of topoisomerase I-DNA covalent complexes, % toxicity and tumor size in patient samples.
variation in topo I-mediated DNA damage induced by TPT in these tumor samples. These results indicate that, similar to patient response, individual tumor samples also show large variations in TPT-induced topo I-DNA covalent complexes.

The cell killing effects of TPT on the tumor cells was also tested by cytotoxicity assays and compared to TPT induced topo I-DNA covalent complexes (Table 5). Again with this limited data set no correlation was seen between TPT induced cell killing and TPT induced topo I-DNA covalent complexes (Fig. 23). Thus, the ICE bioassay is effective in measuring TPT induced covalent complexes in tumor cells. However it remains to be seen if topo I mediated DNA damage induced by TPT correlates to the cell killing effects of the drug.

DISCUSSION

The ICE bioassay can effectively measure TPT induced topo I-DNA covalent complexes in peripheral blood and biopsy tumor samples. In peripheral blood, TPT addition elevated complex formation in all cases tested; increasing exposure to drug results in a corresponding increase in complex formation. The amount of topo I-DNA covalent complexes at T0 and T25 (Table 2) were statistically different (p = 0.0015). There was significant variation in TPT stimulated complex formation between patients. For example, at T25, the range was from 7.7 to 86.7 ng per μg DNA (12 fold); the T5
<table>
<thead>
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<th>Cytotoxicity (% Cell Death)</th>
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<tr>
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</tr>
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</tr>
<tr>
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<td>Colon</td>
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<th>Values</th>
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<th>Cytotoxicity (% Cell Death)</th>
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<tr>
<td>Standard Deviation</td>
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</table>

Table 5: Topoisomerase I-DNA covalent complexes and cytotoxicity in tumor samples.
Figure 23: Relationship between topotecan induced topoisomerase I-DNA covalent complexes and topotecan cytotoxicity in tumor samples.

Cytotoxicity and topo I-DNA covalent complex formation data was obtained from Table 5.
Figure 23

TPT induced cytotoxicity
(% Cell Death)

TPT induced covalent complexes
(ng topo I per μg DNA)
range varied over 30 fold. The patient to patient variability cannot be explained by experimental errors in the ICE bioassay itself because replicates from the in vitro experiment agree within 10-20%.

Among the explanations for the large patient to patient variability, one obvious possibility is the level of TPT in plasma in patients. Indeed, the total amount of TPT in plasma at T25 ranged from 25.5 to 114.1 ng TPT/ml. However, the sample size was too small to allow comparison of plasma TPT with appropriate levels of statistical significance and power. Additionally, the active form of TPT has an α-hydroxylactone ring moiety which can be hydrolyzed to produce an inactive hydroxy acid. At physiological pH, the lactone form of the drug is rapidly hydrolyzed to the hydroxy acid (as seen in Chapter 2, Fig 11). It is possible that the interpatient variability is due to the conversion of TPT to the inactive open ring form. Analysis of the intact form of TPT in plasma (Table 3) showed that patients had a mean of 76% intact drug (standard deviation of 16%) at the time of ICE bioassay sampling. It is unlikely that large scale inactivation of TPT contributes to the significant degree of variation seen with the ICE bioassay. Variations in total levels of topo I in peripheral blood cells in different patients may be responsible for the variability; however, topo I is abundant in resting lymphocytes and behaves like a constitutively expressed protein (179,180). Although topo I expression is not linked to the cell cycle, CPT cytotoxicity has been shown to be greater in S phase cells (86,181,182). Therefore, the variability in patient samples may be linked to the cell
cycle status of the peripheral blood cells. Finally, the immune status of patients could be an important variable to consider since T and B cell populations may be differentially active between patients. TPT induces higher levels of topo I-DNA covalent complexes in peripheral blood lymphocytes undergoing mitogenic stimulation compared to resting lymphocytes (M Muller, unpublished data).

The amount of drug-stabilized topo I-DNA complexes may potentially parallel clinical response to TPT therapy; however, in the limited data set, no clear correlation is seen between topo I-DNA complexes and tumor size or toxicity as measured by changes in neutrophils (Table 4). It is difficult to draw firm conclusions from this small sample size, thus additional data is required.

It is interesting to note that the mean basal trapping of topo I on DNA in peripheral blood is about 6 fold higher than in HeLa cells. The peripheral blood samples used in these studies have approximately one million cells. One million HeLa cells have been estimated to have 100 ng of topo I. Assuming a similar abundance of topo I in nucleated cells in peripheral blood, in the absence of TPT, between 1 to 13% of the total cellular topo I is trapped per µg DNA. After 25 minutes of TPT treatment, this value ranges from about 8 to 87%. In comparison, one million HeLa cells have 0.6% total topo I bound to DNA in the absence of TPT and 6% bound in the presence of the drug. In vitro studies with peripheral blood (Fig. 20) also show elevated levels of basal topo I trapping. It
is possible that the presence of blood components in these samples either increases the basal activity of topo I on DNA or increases the background signals in the immunoblots.

One important advantage of the ICE bioassay is its direct applicability to biopsy tumor samples. Similar to peripheral blood, a great deal of variation is seen in topo I-DNA covalent complexes in the presence and absence of TPT. However, the basal trapping of topo I on DNA in tumor samples is lower than in peripheral blood and more comparable to HeLa cells. The increase in topo I-DNA covalent complexes upon the addition of TPT in each case ranges from 2 to 10 fold. These results illustrate the ICE bioassay’s diagnostic power in evaluating tumor response to topo I poisons. However, due to the lack of correlation between topo I response and cytotoxic effects of TPT, it remains to be determined if the number of topo I-DNA covalent complexes in tumor cells reflects TPT efficacy. Additionally, matched tumor and peripheral blood samples need to be compared to ascertain if peripheral blood cells will be a surrogate indicator of tumor response.

CONCLUSIONS

The ICE bioassay can be employed to measure of topo I-mediated DNA damage induced by TPT in peripheral blood. Complex formation in peripheral blood of patients undergoing treatment with TPT follows the same trends as in HeLa cells. However, there is significant interpatient variability in TPT-induced covalent complexes. With the
limited data set, a clear correlation between adduct formation and cytotoxicity in patients remains elusive. The ICE bioassay can be further employed to analyze cell suspensions prepared from tumor samples. Thus, it has been possible to adapt the ICE bioassay to the clinical situation and monitor topo I mediated DNA damage in patient samples.
CHAPTER 4

THE EFFECT OF DNA MODIFICATIONS ON IN VIVO TOPOISOMERASE I-
DNA COVALENT COMPLEX FORMATION

INTRODUCTION

The emerging role of topo I in cancer chemotherapy has lead to the search for additional classes of agents that target the enzyme. Distortions in DNA structure have been shown to affect the activity of several DNA processing enzymes, such as restriction endonucleases (126). Since topoisomerases play in a role in DNA metabolic processes, DNA distortions may affect the activity of these enzymes. UV photoproducts are often used as a model system for exploring the effects of DNA modifications on cellular processes (127). Exposure to the UVB region (290-320 nm) of sunlight results primarily in the formation of cyclobutane dimers and pyrimidine (6-4) pyrimidinome photoproducts (127). A minor class of photoproducts, DNA/protein crosslinks (DPCs) are also produced and these are always accompanied by the formation of single strand breaks (SSBs) (129,133). However, unlike the major photoproducts which are removed
through the action of DNA repair systems (128), DPC and SSB levels increases with
time post irradiation (129,130,131,132,133). The isolation of a mutant cell line derived
from ICR 2A frog cells, which is hypersensitive to UVB and deficient in DPC/SSB
formation reveals that DPCs and SSBs play an important biological role (131). In
addition, a study of cell lines obtained from systemic lupus erythematosus (SLE) patients
showed that cell lines exhibiting hypersensitivity to UVB also displayed a deficiency in
the formation of DPC (132). In contrast, the SLE cell lines which exhibited normal
levels of survival following exposure to simulated sunlight also showed a normal DPC
response (132). These results are consistent with the conclusion that DPCs, which form
following UV irradiation, may represent some form of cellular response to DNA
damage. The effect of UV photoproducts on topo I activity is particularly interesting
since topo I forms covalent bonds with DNA accompanied by DNA single strand breaks
during catalysis. Thus, it is possible that topo I is involved in these UVB induced DPCs.

In collaboration with Dr. Barry Rosenstein (Mount Sinai Medical Center), the
effect of UV photoproduts on topo I activity was examined in vivo. The ICE bioassay
was used to measure topo I-DNA covalent complex formation in intact cells following
UVB irradiation. The time and dose dependence of these UVB induced topo I-DNA
covalent complexes was tested. Additionally, DPC deficient SLE cells were analyzed
for the UV-topo I response.
The effect of three other DNA modifying agents on topo I-DNA covalent complex formation was also studied. First, cisplatin was used to introduce intra- and inter-strand DNA crosslinks. Cisplatin kinks the major groove of DNA by about 50° to 60° (134). Pyrimidine dimers, on the other hand, kink DNA by about 27° (135). The effect of cisplatin induced DNA distortions on topo I activity in vivo was tested using the ICE bioassay. Second, VM26 was used to induce topo II-DNA covalent complexes in vivo (152). VM26 is specific to topo II and has no effect on topo I activity when tested in vitro with purified topo I and DNA (152). The effect of topo II induced lesions on topo I-DNA covalent complexes was determined in vivo using the ICE bioassay. Finally, cells were treated with ionizing radiation which induces single and double strand breaks in DNA thus resulting in gaps, breaks and exchanges at the chromosomal level (127,136). Ionizing radiation has been shown to potentiate cell killing induced by topo I poisons (153,154), however the mechanism of this synergy is unknown. Therefore, it was of interest to study the effect of ionizing radiation on topo I-DNA covalent complex formation in HeLa cells.

MATERIALS AND METHODS

Reagents.

Cells were cultured in 75 cm² flasks (Coming) in CO₂ independent medium (GIBCO) supplemented with 10% defined, iron-supplemented bovine calf serum (GIBCO), penicillin (100 units/ml) and streptomycin (100 µg/ml).
UVB and UVC Irradiation.

HeLa cells (one T-75 flask per treatment, $5 \times 10^6$ cells per flask) were washed twice with 1 X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$) and overlaid with 15 ml of PBS. The cells (placed on ice) were then exposed to UVB produced by two Westinghouse FS40 SunLamps (4.5 J/m$^2$/sec), filtered through the polystyrene flasks tops to remove all wavelengths shorter than approximately 290 nm (137). Spectroscopic examination of the flask material was performed to ensure that the transmission characteristics did not significantly change between flasks. Under these conditions, most of the DNA damage was produced by the 290-310 nm wavelength region (137). In addition, cells were grown in 100 mm dishes, washed with PBS and irradiated in the absence of the dish lid covers to UVC produced by four GE G15T8 germicidal lamps (0.35 J/m$^2$/second). The fluence rates were measured using an IL 1700 radiometer. Following irradiation, medium was replaced and cells were incubated at 37 °C. At various times post UV treatment, cells were harvested using the ICE bioassay.

Cisplatin and VM26 treatment.

HeLa cells (1 flask per treatment) were treated with 50 μM VM26 or 15 μM cisplatin (provided by A. Johnson, Department of Pharmacology, Ohio State University). At various times post treatment, drug containing medium was removed and the cells lysed with sarkosyl (final concentration 1%). Topo I-DNA covalent complex formation was measured by the ICE bioassay.
Ionizing Radiation.

HeLa cells (one T-75 flask per treatment, $5 \times 10^6$ cells per flask) were placed on ice and irradiated with X-ray generator at a dose of 1000 cGy. The cells were returned to the incubator and harvested at various times post irradiation by the ICE bioassay.

Preparation of crude nuclear extracts for topoisomerase I activity assays.

HeLa cells were washed twice with PBS (5 ml per wash) and scraped into 5 ml of PBS. The cells were pelleted by centrifugation (10 minutes, 1000 x g) and resuspended in a hypotonic buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4 mM MgCl$_2$). The cells were disrupted by dounce homogenization (pestle B, 10 strokes) and nuclei pelleted by centrifugation (10 minutes, 2000 x g). The nuclei were resuspended in a high salt buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4 mM MgCl$_2$, 0.8M NaCl, 20 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride) and placed on ice for 30 minutes. Finally, the debris was pelleted by a high speed centrifugation (1 hour, 100,000 x g). The supernatants were used for topo I activity assays. Prior to testing, the protein concentrations were determined using the BioRad Protein Assay Reagent (BioRad Labs).

Topoisomerase I activity assays.

Reactions containing 250 ng of negatively supercoiled plasmid DNA (pHOT1) and 75 ng of crude extracts in a final volume of 20 μl in 1 X TNE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl) were incubated for 30 minutes at 37°C. Reactions were stopped by the adding 2 μl stop buffer (5% sarkosyl, 0.125%
bromophenol blue, 25% glycerol). An equal volume of chloroform:isoamyl alcohol (24:1) was added, the solution was vortexed and briefly centrifuged (microfuge). The aqueous (blue) layer was directly loaded onto a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) containing 0.5 µg/ml ethidium bromide. Gels were run at 5 V/cm for 30 minutes, destained with distilled water for 30 minutes, and photographed with a UV transilluminator. In some cases, the gels were photographed using Kodak TMAX 400 film and negatives scanned using a densitometer.

**Preparation of nuclear extracts for western blotting.**

HeLa cells (10^7 cells) were washed twice with cold TD (109 mM NaCl, 4.1 mM KCl, 0.56 mM Na₂PO₄, 20 mM Tris-HCl, pH 7.4) and scraped into 2 ml of TD. Cells were pelleted (3 minutes, 1000 X g) and resuspended in 2 ml of buffer A (100 mM NaCl, 50 mM KCl, 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, 10% glycerol, 0.2% Nonidet P-40, 0.1% Triton X-100). Following a 10 minute incubation on ice, nuclei were centrifuged (10 minutes, 2000 X g) and lysed in 100 µl buffer A containing 1% SDS. The lysates were analyzed by SDS-PAGE (7%), followed by electroblot transfer to nitrocellulose. Topo I was detected by probing the blot with the Scl 70 antibody.
RESULTS

Analysis of topoisomerase I-DNA covalent complexes in UVB irradiated cells.

To evaluate the role of topo I in UVB induced DPCs, HeLa cells were treated with UVB at a fluence of 10 kJ/m² and analyzed at various times post irradiation using the ICE bioassay. HeLa cells not exposed to UVB show relatively low levels of topo I-DNA covalent complexes (Fig. 24A, Lane 2). In the control experiment, unirradiated HeLa cells treated with 10 μM CPT for 30 minutes at 37 °C showed formation of topo I-DNA covalent complexes (Fig. 24A, Lane 1). Cells irradiated with UVB also showed formation of topo I-DNA covalent complexes even in the absence of CPT. The topo I UVB response was detected within minutes after exposure to 10 kJ/m² UV and complexes continued to increase up to 5 hours post irradiation (Fig. 24B). To determine if UVB induced topo I-DNA covalent complexes result from general UV crosslinking of proteins to DNA, the same samples were tested for topo II-DNA covalent complex formation. Topo II-DNA adducts were not detected at any time following UVB treatment, indicating that covalent complex formation in these studies is specific to topo I (Fig. 24B). However, topo II-DNA covalent complexes can be seen in HeLa cells treated with etoposide (a topo II poison), demonstrating the ability of the anti-topo II antibody to specifically detect topo II-DNA adducts (Fig 24A, Lane 4).

To examine whether the topo I-UVB response represents irreversible trapping of the enzyme on DNA as a result of UV crosslinking, the reversibility of the covalent complexes was tested with high salt treatment. As described in Chapter 2, addition of
Figure 24: Effect of UVB irradiation on topoisomerase I-DNA covalent complex formation.

HeLa cells (10^7 cells per treatment) were subjected to various treatments and were harvested by the ICE bioassay.

Panel A shows the control experiments using topoisomerase inhibitors. For the topo I control, HeLa cells were treated with 10 μM camptothecin (CPT) or no drug for 30 minutes at 37 °C followed by ICE bioassay. Similarly, for the topo II control, HeLa cells were treated with 50 μM Etoposide (Etop.) or with no drug and the blot was probed with an antibody to topo II.

Panel B shows topo I and II response to UV irradiation. HeLa cells were treated with 10 kJ/m^2 UVB irradiation and incubated at 37 °C for various times post irradiation. Cells were analyzed by the ICE bioassay using both anti-topo I and anti-topo II antibodies. The DNA containing fractions from both blots are shown.
Figure 24
NaCl prior to detergent lysis releases the enzyme from DNA by reversing topo I-DNA covalent complexes (66). To verify that UVB induced topo I-DNA covalent complexes exhibit the same characteristics, HeLa cells were exposed to UVB and at various times post irradiation, treated with NaCl (final concentration, 1M) 30 minutes prior to analysis by the ICE bioassay. As a control, CPT treated HeLa cells were subjected to high salt reversal or left untreated prior to ICE bioassay analysis (Fig. 25). High salt treatment results in a 87% reduction in camptothecin induced topo I-DNA covalent complexes. In UVB treated cells, there is a 89% reduction in topo I-DNA complexes as a result of the high salt treatment. UVB induced topo I-DNA covalent complexes respond to the high salt treatment in a manner similar to CPT induced complexes. Thus, UVB induced topo I-DNA covalent complexes represent the catalytic covalent intermediate and do not represent irreversible trapping of topo I on DNA.

To determine the time course of the UVB-topo I response, HeLa cells were treated with three doses of UVB and topo I-DNA covalent complexes quantified at various times post irradiation (Fig 26). The results show that topo I-DNA covalent complexes can be detected within 12 minutes post UVB (1.5-3 fold increase over no incubation) and the topo I-UVB response continued to increase up to 5 hours post treatment (3-8 fold over no incubation).
Figure 25: Reversal of UVB induced topoisomerase I-DNA covalent complexes.

HeLa cells (2 plates per treatment, 10^7 cells per plate) were treated with UVB at a fluence of 10 kJ/m² or with 10 μM camptothecin (CPT) for 30 minutes at 37 °C. One plate from each pair was then treated with NaCl to a final concentration of 1M and incubated for an additional 30 minutes followed by sarkosyl lysis to reverse complexes. The other plate from each pair was directly lysed with sarkosyl. Lysates were analyzed by the ICE bioassay and topo I-DNA covalent complexes quantified.
Covalent Complexes (ng topo I/µg DNA)

- 10 µM CPT (- Salt)
- 10 µM CPT (+Salt) 87 % Reversal
- 10 kJ/m² UVB (- Salt)
- 10 kJ/m² UVB (+ Salt) 89 % Reversal

Figure 25
Figure 26: Time dependent analysis of UVB induced topoisomerase I-DNA covalent complexes.

HeLa cells ($10^7$ cells per treatment) were treated with UVB irradiation at a fluence of 7.5, 10, and 20 kJ/m$^2$ and analyzed at various times using the ICE bioassay. The yield of topo I-DNA covalent complexes was determined and plotted as a function of time. The analysis was repeated in duplicate and each trial plotted separately. (Trial 1, O; Trial 2, □)

Panel A: 7.5 kJ/m$^2$.
Panel B: 10 kJ/m$^2$
Panel C: 20 kJ/m$^2$
The UV fluences used in the above experiments represent relatively high doses and it is possible that the UVB-topo I response detected is not physiologically relevant but simply a result of UV induced cell death. To address this point, the UVB-topo I response at lower doses of UV irradiation was examined. HeLa cells were treated with UVB fluences ranging from 0.3 kJ/m\(^2\) to 10 kJ/m\(^2\) and tested at 5 hours post irradiation for the topo I-UVB response (Fig. 27). The results show that the topo I-UVB response increases with increasing dose and can be detected at fluences as low as 0.3 kJ/m\(^2\).

To test if UVB alters topo I levels, Western Blot analysis was performed with UVB treated HeLa cells at various times post-irradiation. The analysis revealed that after 1.5 hours post irradiation, total topo I polypeptide levels increase by about 2 fold (Fig. 28). Therefore, an increase in total protein levels cannot fully account for the enhanced topo I-DNA covalent complex formation detected following UV irradiation. The UV-topo I response probably results from increased topo I activity or redistribution of topo I on genomic DNA.

To determine if the catalytic activity of topo I increases due to UVB irradiation, extracts prepared from UVB treated HeLa cells were tested for topo I relaxation activity (Fig. 29). Extracts were incubated with supercoiled plasmid DNA (pHOT1) and the formation of relaxed DNA products was monitored by agarose gel electrophoresis. The time required for complete relaxation of supercoiled DNA was
Figure 27: Dose dependence of topo I-DNA covalent complexes.

HeLa cells ($10^7$ cells per treatment) were treated with UVB fluences of 0, 0.3, 1, 3, 7.5, and 10 kJ/m$^2$ for 5 hours followed by ICE bioassay analysis. Topo I-DNA covalent complexes were quantified and plotted as a function of dose.
Figure 28: Topoisomerase I polypeptide levels in UVB treated HeLa cells.

HeLa cells were treated with a UVB fluence of 10 kJ/m². At various times post UV treatment, cells were harvested by direct addition of 1% sarkosyl. Equal amounts of total protein (50 μg) from each lysate were subjected to 7.5% SDS-PAGE. Proteins on the gel were transferred to nitrocellulose and analyzed by standard Western blotting techniques using the ScI70 antibody to topo I.
Figure 28
Figure 29: Topoisomerase I relaxation activity in UVB irradiated cells.

HeLa cells were treated with UVB at a fluence of 10 kJ/m² and extracts prepared at various times post irradiation. Equal amounts of extracts (75 ng) were incubated with supercoiled plasmid DNA (pHOT1) at 37 °C and reactions were stopped after 1, 5, 15, 30, 45, and 60 minutes by the addition of SDS (final concentration 1%). The reactions were analyzed by agarose gel electrophoresis in the absence of ethidium bromide. Gels were stained with ethidium bromide and visualized under UV light. The amount of time required to obtain complete relaxation was determined and indicated in the table below.
Figure 29
determined for all times post irradiation. The results show that topo I catalytic activity increases by about two fold immediately after UVB irradiation but remains constant at all other times post UVB irradiation (Fig. 29).

The direct involvement of topo I in UVB induced DPCs was tested by examining SLE cell lines deficient in DPC formation for the UVB-topo I response. Three SLE cell lines were examined for topo I-DNA covalent complex formation using the ICE bioassay and compared to a wild type diploid fibroblast cell line (Fig 30). Both the SLE3 and SLE4 cell lines show low levels of UV induced topo I-DNA covalent complex which do not increase with time post irradiation. SLE5 cells appear to show the normal UVB-topo I response at early times, but after 0.2 hours covalent complexes start to decrease. Thus, the DPC and SSB deficient SLE cell lines are also deficient in the UVB topo I response indicating that topo I is largely responsible for UV induced DPCs and SSBs.

UVB induced topoisomerase I-DNA covalent complexes in G1 arrested and exponentially growing cells.

The CPT induced topo I response has been shown to be cell cycle dependent. CPT mediated topo I damage is greater in S phase cells than in G1 arrested cells (86,155,156). To determine if UVB induced topo I-DNA covalent complexes are also cell cycle dependent, the UVB-topo I response was compared in G1 arrested and exponentially growing cells. Cells treated with CPT alone were used as controls (Fig. 31, inset). Both G1 and exponentially growing cells display a large increase in topo I
Figure 30: UVB induced topoisomerase I-DNA covalent complexes in SLE cell lines.

Normal diploid fibroblasts and three cell lines (10^7 cells per treatment) derived from systemic lupus erythmatosus patients were treated with 10 kJ/m² UVB irradiation and analyzed at various times post irradiation. (Normal, •; SLE3, ■; SLE 4, ▲; and SLE 5, ▼).
Figure 30
Figure 31: Topoisomerase I-DNA covalent complex formation in G1 and exponentially growing cells.

GM4390 cells (a diploid human fibroblast cell line) were arrested in G1 by growing cells in medium containing 0.5% serum. Exponentially growing cells were prepared by seeding cells 48 hours prior to treatment. The cultures ($10^7$ cells per treatment) were treated with a UVB fluence of 10 kJ/m$^2$ and analyzed by the ICE bioassay at various times post-UVB treatment for topo I-DNA covalent complexes. The control experiment was performed on the same cells that were not UVB irradiated but were treated with 10 μM camptothecin for 30 minutes at 37 °C prior to lysis (inset).
Covalent Complexes (ng topo I/μg DNA)

Figure 31

Time Post Treatment (hours)

0 1 2 3 4 5 6

0 0.4 0.8 1.2 1.6 2.0

Covalent Complexes (ng topo I/μg DNA)

Exponential

G1

The image contains a graph plotting the formation of covalent complexes over time post treatment, with specific details indicating exponential and G1 phases.
adduct formation following UVB-irradiation. There appears to be very little difference between covalent complex formation in G1 arrested and S phase cells, in fact, overall levels of complex formation is somewhat higher in G1 arrested cells (Fig. 31). On the other hand, S phase cells show a two fold increase in CPT induced topo I-DNA covalent complexes as compared to G1 arrested cells. The results demonstrate that the topo I-UVB response is S-phase independent, as would be expected for a process that is linked to DNA damage response, whereas CPT induced topo I-DNA covalent complexes are enhanced in exponentially growing cells (Fig. 31).

Effect of other DNA modifications on topoisomerase I-DNA covalent complex formation.

HeLa cells were exposed to 0.1 kJ/m² of 254 nm UV (UVC) which induces approximately the same levels and types of DNA damage produced by 10 kJ/m² of UVB (185). It was found that the levels of topo I-DNA adduct formation in UVC treated cells was slightly lower than UVB irradiated cells (less than two fold) but the kinetics of the UVC-topo I response were similar to the UVB response (Fig 32). This result supports the notion that the topo I-DNA complexes form in response to the creation of UV induced lesions.

To determine if a topo I response can also be produced by other DNA modifying agents, cells were treated with cisplatin and analyzed by the ICE bioassay at various times post treatment. Similar to the UVB-topo I response, cisplatin also induces topo I-DNA covalent complexes (Fig. 33). The levels and kinetics of the cisplatin induced topo I-DNA adducts are very similar to the UVB response at 20 kJ/m².
Figure 32: Kinetic analysis of UVC induced topoisomerase I-DNA covalent complexes.
HeLa cells ($10^7$ cells per treatment) were treated with UVC at a fluence of 100 J/m$^2$ and analyzed at various times post irradiation using the ICE bioassay. The number of topo I-DNA covalent complexes formed was determined and compared to UVB (10 kJ/m$^2$) induced covalent complexes. (UVC, ■; UVB, ●)
Figure 32

Covalent Complexes (ng topo I/μg DNA)

Time Post Treatment (hours)

- UVB
- UVC

Figure 32
Figure 33: Topoisomerase I-DNA covalent complexes in cisplatin treated cells. HeLa cells ($10^7$ cells per treatment) were treated (in duplicate) with 15 μM cisplatin and analyzed at various times post treatment for topo I-DNA covalent complex formation.
Figure 33
Next, cells were treated with VM26 to create topo II lesions on DNA and the effects of these lesions on topo I-DNA covalent complex formation were determined. VM26 also induces topo I-DNA covalent complexes in a time dependent manner and the levels are similar to that seen with UVB irradiation at 20 kJ/m² (Fig. 34). VM26 induced topo II-DNA covalent complexes reverse upon drug withdrawal in a manner similar to CPT induced topo I-DNA covalent complexes. To determine if removing the topo II lesions reverses topo I-DNA covalent complexes, cells were treated with VM26 for one hour followed by a drug free incubation. Withdrawal of VM26 reverses topo II-DNA covalent complexes thus removing topo II lesions (Fig. 35A). Topo I-DNA covalent complex formation was also monitored at various times post VM26 removal. Topo I-DNA covalent complexes are detected after one hour of VM26 treatment but these complexes are reversed to basal levels within 2 hours of VM26 removal (Fig. 35B).

Finally, cells were treated with ionizing radiation, which produces a variety of lesions on DNA, the most predominant being single and double strand breaks. Irradiated cells were tested at various times post treatment for topo I-DNA covalent complexes by the ICE bioassay. In contrast to UVB topo I response, ionizing radiation produces very low levels of topo I-DNA covalent complexes which do not increase with time (Fig. 36).
Figure 34: Topoisomerase I-DNA covalent complexes in VM26 treated cells. 
HeLa cells (10^7 cells per treatment) were treated with 50 μM VM26 and analyzed at various times post treatment for topo I-DNA covalent complex formation. The 0 hour time point represents cells that were treated with VM26 and then immediately lysed with sarkosyl. In the absence of any treatment (mock) covalent complex formation is 0.4±0.2 ng topo I per μg DNA. The analysis was repeated in duplicate.
Figure 34
Figure 35: Reversal of VM26 induced topoisomerase I-DNA covalent complexes. HeLa cells (10^7 cells per treatment) were treated with 50 μM VM26 for 1 hour followed by incubation in drug free medium. Topo II- and topo I-DNA covalent complexes were monitored during the drug treatment as well as during the drug free chase.

Panel A. Topo II-DNA covalent complex formation.

Panel B. Topo I-DNA covalent complex formation.
Figure 35

A. Topo II-DNA complexes

B. Topo I-DNA complexes

Remove VM26
Figure 36: Topoisomerase I-DNA covalent complex formation in cells treated with ionizing radiation.

HeLa cells ($10^7$ cells per treatment) were treated with ionizing radiation with 1000 cGy and analyzed at various times post treatment for the formation of topo I-DNA covalent complexes.
Figure 36

Ionizing Radiation

Covalent Complexes (ng topo I/μg DNA)

Time Post Treatment (hours)

10 kJ/m²
DISCUSSION

Exposure of human cells to UVB radiation induces the formation of topo I-DNA covalent complexes without the need for CPT to stabilize the complexes. The kinetics of topo I-DNA complex formation were similar to DPC/SSB formation in HeLa cells (129). A comparison of the levels of UVB induced DPCs and topo I-DNA covalent complexes reveals that at 5 hours post UVB treatment approximately 10 enzyme molecules were bound per megabase of DNA as compared to about 2-4 DPCs or SSB per megabase (133). This suggests some association between topo I and UVB-induced DPC, however, an accurate comparison cannot be made due to the relative sensitivity of each method. These data are consistent with the idea that the appearance of DPCs and SSBs in UVB irradiated cells results from increased topo I-DNA adducts following UV irradiation. Further evidence of topo I involvement in UVB-induced DPCs comes from the SLE cell lines which are deficient in DPC formation following UV irradiation as well as in the UVB-topo I response. Finally, DPCs and SSBs are also detected in CPT treated cells (73), indicating that the UV response agrees with topo I mechanism of action.

The UV response detected using the ICE bioassay is not due to irreversible trapping or photochemical crosslinking of topo I on DNA. When topo I-DNA covalent complexes are arrested on DNA as part of the normal catalytic cycle of topo I (using detergents to trap the intermediates), complexes are readily reversed by either drug
removal or treatment with high salt. Treating cells with high salt reverses the UV
induced complexes to the same extent as seen with CPT dependent complexes (Fig. 25).
Photochemically trapped protein/DNA adducts would not be salt labile. A more likely
interpretation is that UV induced DNA lesions alter the catalytic activity of endogenous
topo I on genomic DNA. Optimal detection of covalent complexes requires doses in
excess of 7.5 kJ/m². However, these findings are not likely to be attributed to simple cell
death induced by high UVB doses. The topo I response was detected within minutes
after UVB exposure, well before the onset of cytotoxicity and/or apoptosis.
Furthermore, covalent complex formation displays a linear response with dose and can
be detected at lower UV doses which result in relatively little cell killing (Fig. 27).

The topo I response to lesions in DNA is not unique to UV-induced
photoproducts. Topo I-DNA covalent complexes can be detected in cells treated with
other agents that cause conformational changes in DNA. DNA crosslinking using
cisplatin, as well as topo II lesions on DNA, also induces an in vivo topo I response.
Additionally, removal of topo II lesions reverses topo I-DNA covalent complex
formation indicating that these complexes are formed in response to lesions on DNA. In
contrast, no topo I response is seen in cells treated with ionizing radiation which
primarily induces single and double strand breaks in DNA. Thus, it is apparent that
different types of DNA modifications invoke different responses from topo I. There
could be several reasons why ionizing radiation does not induce topo I-DNA covalent
complex formation. Double and single strand breaks in DNA may not distort the DNA sufficiently for topo I recognition and activity, whereas the crosslinking agents may alter the local conformation of the helix, similar to UV photoproducts, thus recruiting topo I to these sites. Alternately, the lack of response in cells treated with ionizing radiation may result from different repair pathways. Double and single strand breaks induced by ionizing radiation are repaired in cells by a different mechanism (largely DNA ligation) as compared to UV photoproducts, crosslinking agents such as cisplatin, and bulky adducts (nucleotide excision repair). Finally, topo I catalytic activity was shown to be down regulated in some tumor cells by posttranslational modifications in cells treated with ionizing radiation (143). This might explain the low levels of topo I-DNA covalent complex formation seen in HeLa cells.

The exact mechanism by which DNA distortions affect topo I activity on genomic DNA is unknown. In UV treated cells, topo I polypeptide levels as well as catalytic activity show a slight increase following UV irradiation, however, this increase is not sufficient to explain the in vivo UV-topo I response. Numerous studies have shown that steric factors influence the interaction of the topo I with DNA (48,138,139). Topo I has been shown to preferentially bind supercoiled DNA over relaxed DNA (14). Alterations of the DNA topological state by UV dimers (140) or crosslinking agents could increase the affinity of topo I for DNA. As a result, there may be more topo I cleavages on modified DNA leading to an increased abundance of
topo I-DNA covalent complexes. Conformational changes induced by dimers may not only be localized to the site of the altered pyrimidines but may be propagated into neighboring sequences (140). Therefore, additional topo I sites may be produced in DNA in the presence of lesions. Also, changes in the structural context of DNA may result in optimal conformation of topo I sites that are normally not accessible to the enzyme producing more topo I cleavages (140).

Alternately, lesion induced topo I-DNA covalent complexes may arise due to inhibition of the topo I religation step (140). The presence of a dimer at a topo I cleavage site may result in misalignment of the two ends of DNA at the site of the nick preventing religation. Furthermore, the presence of dimers at sites removed from the topo I cleavage site may alter the local topology at a cleavage site preventing religation by the enzyme. It is also possible that the presence of lesions in the passing strand at the time of strand passage may slow down diffusion through the protein-DNA bridge which may be detected as an increase in topo I-DNA covalent complexes (140).

Finally, given the possible biological role of DPC in the cellular response to UV irradiation (133,131,132), the formation of topo I-DNA covalent complexes following UV irradiation may be related to repair processes such as nucleotide excision repair (NER) (127). Although there is no direct proof that topo I is a repair factor, this enzyme has been implicated in DNA repair processes (142,143,72,144,145). There is strong evidence that several of the factors involved in transcription also play roles in repair.
Topo I localizes to sites of active transcription (56,57,149) and, thus, may also play an additional role in repair processes. In UV-irradiated cells, lesions are thought to be detected by repair complexes scanning the DNA for conformational abnormalities, including those produced by cyclobutane pyrimidine dimers and 6-4 photoproducts (150). It is possible that topo I senses these helical distortions given the recent report showing that topo I can recognize DNA mismatches in vitro (72). Additionally, the repair process itself is likely to produce topological alterations in DNA that could involve topo I (151). Nucleosome rearrangements, which occur following UV irradiation (151) to allow access of the repair machinery to lesions, may require topo I for topology adjustments.

CONCLUSIONS

Topo I is a key protein involved in the formation of UV-induced DPCs. Cells treated with UV irradiation, cisplatin and VM26 show a time dependent increase in topo I-DNA covalent complex formation. However, ionizing radiation has no effect on topo I adduct formation. UVB induced topo I-DNA covalent complexes are reversible, similar to CPT induced covalent complexes. The data indicate that topo I may play a role in the cellular response to DNA lesions. Covalent complex formation may be a result of increased affinity of topo I for lesion containing DNA, altered topo I cleavage/religation equilibrium or topo I involvement in DNA repair.
CHAPTER 5
THE ROLE OF TOPOISOMERASE I IN THE CELLULAR RESPONSE TO DNA DAMAGE.

INTRODUCTION

In the previous chapter it was shown that UV irradiation induces the formation of salt labile topo I-DNA covalent complexes in vivo, however, the mechanism of this response is unknown. Among the possibilities for increased topo I-DNA adduct formation could be enhanced topo I binding to lesion containing DNA. Topo I has been shown to have an increased affinity for DNA that is torsionally stressed (39,38). Indeed, topo I shows preferential binding to supercoiled DNA (39). The torsional strain in supercoiled DNA could alter the pitch of the helix or produce unique structures in DNA associated with supercoiling, either of which could be recognized by topo I (39). Additionally, supercoiling produces more nodes or crossover points in the DNA to which topo I binds with higher affinity (39,38). Topo I has also been shown to have a preference for curved DNA (157,37,34). UV lesions introduce significant deformation
of the DNA double helix producing kinks in DNA (127,158). Thus, if topo I binds conformationally altered DNA with greater affinity, there may be increased topo I cleavages on lesion containing DNA and correspondingly increased covalent complex formation.

DNA lesions may also affect topo I activity by altering the cleavage/religation equilibrium of the enzyme, leading to increased stabilization of topo I-DNA covalent complexes. It has been demonstrated that topo I can be covalently trapped on DNA containing mismatches in vitro (72), even in the absence of poisons. The presence of a mismatched base adjacent to the topo I cleavage site inhibits religation by topo I. However, the effect of mismatches removed from topo I cleavage sites was not tested. More recently, it was demonstrated that the presence of UV lesions on DNA inhibits topo I relaxation activity in vitro (140). Reduced enzymatic activity correlates with increased topo I-DNA covalent complex formation due to alterations in the cleavage/religation equilibrium of topo I (140).

Finally, UV topo I response may result from topo I involvement in repair processes. UV induced lesions invoke nucleotide excision repair (NER) (127) for removal of photoproducts. Through the isolation of cell lines derived from three repair defective human hereditary diseases, xeroderma pigmentosum (XP), Cockayne’s syndrome and a photosensitive form of tirchothiodystrophy, the steps and several of the human genes involved in NER have been identified (127). The first step in NER is
damage recognition by the XPA gene (159), followed by incision of the damaged strand on opposite sides of the lesion by the XPG and XPF nucleases (160,161,162,163). Excision of the lesion is facilitated by unwinding of the helix by the XPB and XPD proteins (164). Finally, a new DNA segment is synthesized, using the undamaged strand as a template, and ligated to the parental strand (128,166). Topo I may be recruited by NER to adjust the topology of DNA as repair proceeds. DNA unwinding at repair sites to allow repair enzymes to excise and refill the region of DNA containing the lesion could result in some supercoiling on either side of the repair patch similar to transcriptionally induced supercoiling. As a result, topo I might be recruited to these sites to relax DNA supercoiling. Additionally, overall genomic repair requires nucleosome repositioning to provide the repair machinery lesion site access (151). This repositioning can result in topological changes in DNA which may require the action of topoisomerases. Finally, for a number of DNA lesions, it has been demonstrated that active genes are repaired more efficiently than inactive genes (167,168,169). In fact, there is a strong relationship between transcription and DNA repair (170). Transcribed regions of genomic DNA are believed to be under torsional stress (167,168,169) and topo I has been shown to be associated with genes undergoing active transcription and may also play a role in repair (56,57,149). Thus, nucleotide excision repair may influence topo I relocalization to repair sites resulting in increased topo I activity and an abundance of covalent complexes.
The goals of this work were 1) to determine the affinity of topo I for UV-modified DNA \textit{in vitro}, 2) to investigate the effect of cyclobutane pyrimidine dimers on topo I activity \textit{in vitro} 3) to determine topo I cleavage sites on UV-damaged DNA \textit{in vitro}, and 4) to analyse repair deficient cell lines for UV induced topo I-DNA covalent complexes.

**MATERIALS AND METHODS**

**Topoisomerase I activity assays.**

Reactions containing 250 ng of negatively supercoiled plasmid DNA (pHOT1) and human topo I in a final volume of 20 μl in 1X TGS buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 5% glycerol, 0.1% bovine serum albumin, 0.1 mM spermidine) were incubated for 30 minutes at 37 °C. Reactions were stopped by adding 5 μl of stop buffer (5% sarkosyl, 0.125% bromophenol blue, 25% glycerol). An equal volume of chloroform:isoamyl alcohol (24:1) was added, the solution vortexed and briefly centrifuged (microfuge). The aqueous (blue) layer was directly loaded onto a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) containing 0.5 μg/ml ethidium bromide. Gels were run at 5 V/cm for 2 hours, destained with distilled water for 30 minutes, and photographed with a UV transilluminator using a photodocumentation unit. The image files were analyzed using an image quantification program (ImageQuant, Molecular Dynamics) to quantify the reaction products. To
detect topo I-DNA covalent complexes, reactions were stopped by adding of 5 μl of stop buffer and treated with 50 μg proteinase K/ml for 30 minutes at 37 °C prior to CIA extraction and gel analysis.

**Preparation of End-labeled DNA fragments.**

Plasmid pHOT1 was linearized with EcoRI and 3’ end-labeled with the Klenow fragment of E. coli in the presence of [³²P]-αdATP. The linear fragment was purified using Sephadex G-50 spin columns to separate the end labeled DNA from free nucleotides. The double end-labeled linear plasmid was used for SDS-K⁺ precipitation assays. For mobility shift assays and topo I cleavage assays, a 500 bp fragment (uniquely 3’ end-labeled) was generated by subjecting linear pHOT1 3’ end-labeled at the Æ ‘ coRI site to a second digestion with SspI. Uniquely end-labeled fragments were separated from the parental fragment on preparative polyacrylamide gels followed by electroelution and ethanol precipitation to purify the fragments.

**Preparation of UV Irradiated DNA.**

UV irradiated DNA was prepared by exposure to 254 nm UV light from a germicidal lamp at a fluence rate of 2 J m⁻² s⁻¹. The samples were kept on ice to avoid heating and evaporation. The doses employed in the study ranged from 0 to 2000 J/m². The number of cyclobutane pyrimidine dimers (CPD) produced at each dose is as follows: 50 J/m², 1 CPD/1600 bp; 100 J/m², 1 CPD/800 bp; 200 J/m², 1 CPD/400 bp; 400 J/m², 1 CPD/200 bp; 800 J/m², 1 CPD/100 bp; 1200 J/m², 1 CPD/70 bp; 2000 J/m², 1 CPD/40 bp.
Mobility Shift Assays.

Approximately 20,000 CPM of the uniquely 3' end-labeled 500 bp EcoRI-SspI fragment was incubated with purified human topo I at 37 °C for 30 minutes in 1 X TGS in 20 µl reactions. The reactions were adjusted to 10% glycerol and loaded on a 4% native polyacrylamide gel in 0.25 X TBE (22.5 mM Tris-borate, 0.6 mM EDTA). The gels were run at 100 V for 6 hours, dried and analyzed by autoradiography. The gels were also quantified used a phosphoimager (Molecular Dynamics).

SDS-K Precipitation Assays.

Topo I was incubated with linear 3' end-labeled pHOTI in 20 µl reactions in 1 X TGS buffer at 37 °C for 30 min. Reactions were terminated with the addition of SDS to a final concentration of 1%, followed by the addition of 250 µl buffer A1 (10 mM Tris-HCl, pH 8.0, 20 µg/ml bovine serum albumin, 20 µg/ml calf thymus DNA and 1% SDS). The mixture was vortexed and further incubated at 37 °C for 5 minutes followed by the addition of 25 µl of 2.5 M KCl. After vortexing a second time, the mixtures were incubated on ice for 30 minutes to allow for precipitation. The precipitates were collected by filtering through glass fiber discs (GF/C) pre-wetted with buffer B (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM KCl). The precipitates were loaded onto filters in a Millipore sample manifold (model 1225) with the vacuum off. The reaction tubes were rinsed 3-4 times with cold buffer B and the rinses added to the filter cup. A vacuum was then applied and the filters were washed 4 times with 5 ml of cold buffer B.
per wash. The filters were washed once with 10 ml of 95% ethanol (-20 °C) and dried under an infrared lamp. The amount of radioactivity on the filters was determined by liquid scintillation counting.

Topoisomerase I Cleavage Assays.

The 500 bp EcoRI-SspI 3’ end-labeled fragment was incubated with topo I (40 nM) in 20 μl reactions containing 10 mM Tris-HCl (pH 8.0), 60 mM NaCl, 3 mM CaCl₂, 0.1M sucrose, 1 mM dithiothreitol, 100 μg/ml BSA, 7% DMSO and 5% glycerol at 37 °C for 30 minutes. Cleavages were trapped by adding of SDS and EDTA to final concentrations of 1% and 10 mM respectively, followed by a 10 minute incubation at 45 °C. Following the addition of NaCl to 0.8 M, the samples were precipitated with ethanol and digested with 3 ml of 250 μg/ml proteinase K in 0.5% SDS at 37 °C for 30 minutes. An equal volume of loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 5 mM EDTA, pH 8.0 in deionized formamide) was added and samples were adjusted with loading buffer to contain the same number of CPM/μl, heated to 95 °C for 5 minutes and loaded on denaturing polyacrylamide sequencing gels (8%).

RESULTS

Affinity of topoisomerase I for UV photoproducts.

Mobility shift assays provide a useful tool for examining interactions between proteins and DNA. To test the affinity of topo I for UV treated DNA, the 500 bp 3’ end-labeled EcoRI-SspI fragment was irradiated with UVC at 200 J/m², incubated with various
amounts of topo I and analyzed by mobility shift assays. Since UVC induces nearly similar levels (within a factor of two) of topo I-DNA covalent complexes as UVB, UV lesions were induced by irradiation at 254 nm (UVC) in all the experiments described in this chapter as it is readily available from a germicidal lamp while UVB requires a sunlamp. The UVC dose used in this experiment corresponds to a UVB dose of 20 kJ/m². Topo I appears to bind the UV irradiated probe with the same affinity as unirradiated probe at all concentrations of topo I tested in mobility shift assays (Fig. 37). Addition of the anti-topo I antibody to the reaction results in a supershift of the complex with both probes, indicating that these complexes are specific to topo I. At higher concentrations of topo I, low molecular weight complexes are also detected. However, these are probably due to minor degradation products of full length topo I as these complexes are also supershifted by the anti-topo I antibody. It is possible that due to the small size of the probe, topo I may not be encountering many lesions on the DNA at this dose. At 200 J/m², approximately one cyclobutane pyrimidine dimer (CPD) is produced per 400 bp, thus, enzyme binding to DNA may remain unaffected by the UV treatment. However, increasing the doses of UV irradiation up to 2000 J/m² (producing 10 lesions per 400 bp) also has no effect on topo I binding to DNA (Fig. 38). In fact, there appears to be a slight decrease in topo I-DNA complexes at the highest UV dose. Again, at all doses tested, the topo I-DNA complexes are supershifted when the anti-topo I antibody is
Figure 37: DNA binding activity of topoisomerase I.
A 500 bp 3' end labeled EcoRI-SspI fragment from pHOT1 was irradiated with UVC at a fluence of 200 J/m² and incubated with various amounts of topo I at 37 °C for 30 minutes (Lanes 1-6). Reactions were stopped by the addition of glycerol to 10% and loaded on a 6% native polyacrylamide gel. Gels were dried and visualized by autoradiography. As a control, unirradiated probe was subjected to the same treatment (Lanes 7-12). Lane 1 and 7, probe alone; lane 2 and 8, probe with 10 nM topo I; lane 3 and 9, probe with 20 nM topo I; lane 4 and 10, probe with 40 nM topo I; lane 5 and 11, probe with 80 nM topo I; lane 6 and 12, probe with 20 nM topo I and 1 µl of the anti-topo I antibody.
Figure 37
Figure 38: UV dose dependent binding of topoisomerase I to irradiated DNA. A 500 bp 3' end labeled EcoRI-SspI fragment from pHOT1 was irradiated with UVC at various doses and incubated with 10 nM of topo I at 37 °C for 30 minutes or with 10 nM of topo I and 1 μl of the anti-topo I antibody. Reactions were stopped by the addition of glycerol to 10% and loaded on a 6% native polyacrylamide. Gels were dried and visualized by autoradiography. The doses used are indicated on the gel.

AB - probe + topo I + anti-topo I antibody; TI - probe + topo I; I - free probe; II - topo I-DNA complexes; III - antibody supershifted complexes.
Figure 38
added to the reaction. To further evaluate the binding affinity of topo I to UV lesion containing DNA, competition assays were performed. In this case, the UV treated probe (2000 J/m²) was competed either with cold unirradiated competitor (EcoRI-SspI fragment, 0 J/m²) or with cold UV irradiated competitor (EcoRI-SspI fragment, 2000 J/m²). Both UV irradiated and unirradiated fragments compete with topo I binding to UV irradiated probe to the same extent (Fig. 39). Thus, there appears to be no difference in the total binding of topo I to DNA in the presence or absence of lesions, however, since the probes used in these experiments are small this may not accurately reflect the situation in vivo.

Since mobility shift assays primarily measures non-covalent binding of topo I to DNA, the covalent binding of topo I to lesion containing DNA was tested by SDS-K⁺ precipitation assays (171). In these assays, linear 3’ end-labeled pHOT1 (3 kb) was UV irradiated at 0, 500 and 2000 J/m² and incubated with various amounts of topo I. Topo I-DNA covalent complexes were trapped by the addition of SDS. KCl was added to the reaction which results in the formation of a SDS-K⁺ precipitate. Since SDS binds proteins, SDS-K⁺ will also effectively precipitate proteins. On the other hand, SDS does not bind DNA, so only DNA that is covalently bound to protein will co-precipitate with SDS-K⁺ while free nucleic acids will remain in the supernatant. Additionally, electrostatic interactions between topo I and DNA will be disrupted by SDS, thus, the assay specifically measures covalent complex formation. The precipitate is collected on
Figure 39: Analysis of topoisomerase I binding affinity to UV treated probe.
Mobility shift assays were performed with 10 nM topo I and the 3' end-labeled EcoRI-SspI probe in the presence of increasing amounts of unirradiated cold competitor (EcoRI-SspI fragment) or UV treated cold competitor (EcoRI-SspI fragment). The dried gels were scanned using a phosphoimager to quantify the amount of topo I-DNA complexes formed. Topo I-DNA complexes in the absence of any competitor was set to 100 %.
Figure 39

% Complex Formation vs Competitor DNA (ng)

UV Treated Probe

Unirradiated Competitor

Irradiated Competitor

Figure 39
glass fiber filters and the amount of DNA trapped in covalent complexes is measured by scintillation counting. The results show that topo I-DNA covalent complex formation increases in a dose dependent manner at all concentrations of topo I tested (Fig. 40). With 20 nM of topo I, UV-induced lesions increase the abundance of topo I-DNA covalent complexes by about 30%.

**Effect of UV photoproducts on topoisomerase I catalytic activity.**

The results from the SDS-K⁺ assays indicate that the presence of UV lesions produces an increase in the amount of topo I-DNA covalent complexes *in vitro*. However, this UV induced enhancement could be a result of increased topo I binding to DNA or from an alteration in the cleavage/religation equilibrium of the enzyme. Thus, it was necessary to determine the effect of UV photoproducts on the ability of human topo I to relax supercoiled DNA. Supercoiled plasmid DNA (pHOT1, described in Chapter 2) was treated with increasing UVC doses from 0 to 2000 J/m² and incubated with various amounts of purified human topo I. The reactions were allowed to proceed for 30 minutes and relaxed DNA was separated from the supercoiled DNA by agarose gel electrophoresis. The reaction products were quantified by densitometric scanning of the gel photographs and plotted as a function of topo I (Fig. 41). In the control experiment, the effect of CPT on topo I relaxation activity was measured (Fig. 42). CPT clearly inhibits topo I relaxation activity at all concentrations of topo I tested. Likewise, the presence of UV lesions also appears to inhibit the relaxation activity of topo I as
Figure 40: UV dose dependent of covalent binding of topoisomerase I to DNA in vitro.

A 3' end-labeled linear probe was generated by from pHOT1, UV irradiated and incubated with various amounts of topo I for 30 minutes at 37 °C. The reactions were stopped by adding SDS and covalent complexes precipitated as described in Materials and Methods. The amount of covalent complexes was quantified by determining the percent of DNA bound to the filters.
Figure 40

% DNA Bound

Topo I (nM)

2000 J/m²

500 J/m²

0 J/m²
Figure 41: Topoisomerase I relaxation assays in the presence of UV lesions.
Plasmid pHOT1 was UV irradiated at various doses and incubated with 0, 1.25, 2.5, 5, 10 and 20 nM of topo I for 30 minutes at 37 °C. The reaction products were visualized by agarose gel electrophoresis. The gels were also quantified by densitometry as described in Materials and Methods and the amount of relaxed DNA produced was determined as a function of total DNA present. Lanes 1-6: no UV; Lanes 7-12: 400 J/m²; Lanes 13-18: 800 J/m²; Lanes 19-24: 400 J/m².
Figure 41
Figure 42: Topoisomerase I relaxation assays in the presence of camptothecin
Plasmid pHOT1 was incubated with 0, 1.25, 2.5, 5, 10 and 20 nM of topo I and various
amounts of CPT for 30 minutes at 37 °C. The reaction products were visualized by
agarose gel electrophoresis. The gels were also quantified by densitometry as described
in Materials and Methods and the amount of relaxed DNA produced was determined as a
function of total DNA present. Lanes 1-6: no drug DNA; Lanes 7-12: 5 μM CPT; Lanes
13-18: 10 μM CPT; Lanes 19-24: 50 μM CPT.
Figure 42

Lane 1  2  3  4  5  6  7  8  9  10  11  12

Lane 13  14  15  16  17  18  19  20  21  22  23  24

% Relaxed DNA

Topo I (nM)
evidenced by a reduction in the amount of relaxed DNA when comparing unirradiated plasmid with irradiated DNA (treated at 2000 J/m²). To further illustrate this point, the amount of relaxed DNA produced by 5 nM of topo I is plotted as a function of UV fluence or CPT concentration (Fig. 43). In the absence of CPT, topo I (5 nM) relaxes approximately 35% of the DNA while in the presence of 50 μM CPT only 12% of the DNA is relaxed representing a 3 fold reduction in enzyme activity. Similarly, topo I produces 33% relaxed product with unirradiated DNA while DNA treated with a fluence of 2000 J/m² reduces topo I relaxation activity to 8%. These results indicate that there is a dose dependent reduction in topo I relaxation activity in the presence of UV lesions similar to the effect seen with CPT. Thus, UV lesions appear to alter the cleavage/religation equilibrium by stabilizing topo I-DNA covalent complexes and inhibiting DNA religation. However, at higher concentrations of topo I, the UV induced inhibition is diminished; 20 nM topo I produces approximately 100% relaxed molecules in the absence of UV lesions and this is reduced to 75% in the presence of lesions. In contrast, CPT induced topo I inhibition is still seen at the higher concentration of topo I. With 20 nM topo I, 80% of the DNA is relaxed in the absence of CPT and 20% relaxed in the presence of CPT.

To further confirm that the UV dependent inhibition of topo I relaxation activity occurs through topo I-DNA covalent complex stabilization, the amount of the intermediate open circular DNA produced was determined. For this purpose the above
Figure 43: Dependence of topoisomerase I relaxation activity on UV dose or camptothecin concentration.
Topo I (5 nM) was incubated with plasmid pHOT1 for 30 minutes at 37 °C and reaction products were analyzed by agarose gel electrophoresis. The amount of relaxed DNA produced was determined as described and plotted as a function of CPT concentration (Panel A) or UV dose (Panel B).
Figure 43

A

% Relaxed DNA

CPT Concentration (μM)

B

% Relaxed DNA

UV Dose (J/m²)
relaxation assays were repeated, however, to visualize protein bound open circular DNA, the samples were treated with proteinase K prior to gel electrophoresis. The results show that there is a UV dose dependent increase in the formation of open circular DNA (Fig. 44). At the highest UV dose, there is a three fold increase in topo I-DNA covalent complexes (55% open circular DNA) as compared to untreated DNA (18%). This is consistent with the three fold decrease in topo I relaxation activity.

Nucleotide Localization of topoisomerase I-mediated DNA breaks stimulated by UV photoproducts.

Covalent complexes represent an intermediate in the topo I catalytic cycle which consist of DNA with a single strand nick at the site where the enzyme is covalently bound to DNA. In general, topo I mediated cleavages are nonrandomly introduced in the DNA helix. Cleavage sites are characterized by weak sequence specificity (44,172) as well as the conformation of the helix (i.e., bends or curves) (157,37,34,173). UV lesions introduce a small but significant deformation in the DNA helix which could affect the topo I cleavage reaction. Since UV lesions stabilize topo I-DNA covalent complexes the influence of UV photoproducts on the cleavage properties of human topo I was characterized by mapping break sites. This was compared to the specificity of sites cleaved by topo I in the presence of CPT.
Figure 44: Dose dependence of topoisomerase I-DNA covalent complex formation *in vitro*.

DNA relaxation assays were performed using unirradiated or UV irradiated plasmid pHOT1 and 20 nM of topo I, and covalent complexes trapped by the addition of SDS. Reaction products were analyzed by agarose gel electrophoresis following treatment of samples with proteinase K to facilitate detection of the intermediate open circular DNA. The gels were quantified by densitometry and the amount of open circular DNA produced determined as a function of total DNA.
Figure 44

Graph showing the percentage of open circular DNA against UV dose (J/m²) for Topo I (units) conditions.

Lane 1  2  3  4  5  6  7  8  9  10  11

Signs indicating the presence or absence of Topo I (units) at different UV doses.
The 500 bp 3' end-labeled EcoRI-SspI probe was UV irradiated and incubated with topo I for 30 minutes at 37 °C. Reactions were stopped with the addition of SDS, treated with proteinase K and reaction products analyzed on denaturing polyacrylamide sequencing gels. Topo I cleavage sites in DNA containing UV lesions were compared to topo I cleavage sites in the presence of CPT. Additionally, UV irradiated fragments were also treated with T4 endonuclease V, which specifically cleaves DNA at the site of CPDs, to determine the relationship between topo I sites and UV dimers. In the absence of CPT and UV lesions, 5 topo I break sites are detected, however, these bands are faint, consistent with the rapid religation of topo I during its normal catalytic cycle (Fig. 45, lane 6). In the presence of CPT, topo I cleavages are seen at the same 5 sites but the abundance of breaks is much greater. Additionally, numerous other sites are also cleaved by topo I in the presence of CPT. Topo I cleavages on UV treated probes occur at the same sites detected with untreated probe. UV treatment results in a dose dependent increase in the abundance of topo I breaks at three of the sites (site 1, 3 and 5). Analysis of the relative distribution of UV induced CPDs reveals that all three topo I cleavage sites occur in close proximity to CPD clusters in lesion containing DNA. Cleavage at site 2 is unaffected by the presence of UV lesions and is also not located near any CPD sites. Cleavage site 4 lies very close to CPD sites but the number of topo I breaks does not show a dose dependent increase. It is possible that the CPD at this site affects topo I cleavage by producing new topo I sites rather than increasing the cleavage intensity. Indeed, two novel topo I breaks sites are detected in UV treated probes - one above site 4 (UV1) and one above site 5 (UV2).
Figure 45: Localization of topoisomerase I mediated cleavage sites in UV lesion containing DNA.

The 3' end-labeled EcoRI-SspI probe (500 bp) was UV irradiated with various doses and incubated with 40 nM of topo I for 30 minutes at 37 °C. The reactions were terminated by adding SDS to trap covalent complexes to allow mapping of break sites. Reaction products were analyzed on a 8% polyacrylamide sequencing gel and visualized by autoradiography. Lane 1: untreated probe alone; Lane 2: UV treated probe (2000 J/m^2) alone; Lane 3-5: endonuclease V and DNA treated at 0, 400 and 2000 J/m^2, respectively; Lanes 6-10: topo I and DNA treated at 0, 400, 800, 1200 and 2000 J/m^2; Lane 11 and 12: untreated probe with topo I and 10 and 50 μM CPT respectively.

TI: topo I cleavage sites.

UV: UV induced topo I cleavage site.
Topoisomerase I-DNA covalent complexes in repair deficient cell lines.

The above experiments indicate the UV irradiation alters the topo I cleavage/religation equilibrium causing an increase in topo I-DNA covalent complex formation. However, this increase in covalent complex formation is approximately 2 to 3 fold over untreated samples at the highest dose tested. Since there is approximately a 7 to 8 fold increase in topo I-DNA covalent complexes in vivo, it is possible that there are other factors that induce the in vivo UV topo I response.

To determine if topo I plays a role in DNA repair processes resulting in the in vivo stimulation of topo I-DNA covalent complexes, cells lines that are deficient in NER were examined for the UV-topo I response. These cell lines are derived from xeroderma pigmentosum (XP) patients; so far 7 different complementation groups have been identified which are defective to various extents in nucleotide excision repair pathway. Cell lines derived from the complementation group A and D were UV irradiated and analyzed for topo I-DNA covalent complexes (Fig. 46). As a control, wild type diploid fibroblasts were also examined for the UV-topo I response. XPA cells are deficient in a protein that may participate in some aspect of lesion recognition during NER (159). UV irradiated XPA cells show lower levels of topo I-DNA covalent complex formation as compared to wild type cells. However, the kinetics of the UVB-topo I response in XPA cells appear to be similar to normal cells. XPD cells, are deficient in a protein which has an ATP-dependent helicase activity (164,163). XPD cells, on the other hand, are
Figure 46: UVB induced topoisomerase I-DNA covalent complexes in repair deficient cells.
Cell lines derived from xeroderma pigmentosum complementation group A and D were treated with UVB at a fluence of 10 kJ/m² and analyzed at various times post treatment for the formation of topo I-DNA covalent complexes using the ICE bioassay. Normal diploid fibroblasts were used as a control. Normal, ⬤; XPA, ■; XPD, ▲.
Figure 46
deficient in the topo I-UVB response showing baseline levels of topo I-DNA covalent complex formation at all times post irradiation (Fig. 46). Western blot analysis reveals that both cell lines contain nearly identical amounts of topo I compared to normal cells (Fig. 47), thus, these results are not due to differences in protein levels. Additionally, XPA and XPD cells were also analyzed for CPT response and it was found that CPT induces the same levels of topo I-DNA covalent complex formation in these cells as normal cells (Fig. 48). Thus, XPA and XPD cells contain topo I that is functionally capable of forming covalent complexes with DNA.

**Effect of combining camptothecin and DNA modifications on topoisomerase I-DNA covalent complex formation.**

Since DNA modifications induce the formation of topo I-DNA covalent complexes, novel combinatorial approaches to tumor cell killing can be explored. To test the effect of addition of topo I poisons on lesion induced topo I-DNA covalent complex formation, HeLa cells were treated with UV irradiation to induce lesions in DNA followed by addition of CPT (10 μM final concentration) 30 minutes prior to lysis. The effect of CPT addition was determined at 0.5 and 5 hours post UV irradiation using the ICE bioassay (Fig. 49). The results show that CPT enhances the UV-topo I response in an additive manner.
Figure 47: Western blot analysis of repair proficient and deficient cell lines. Normal, XPA and XPD cells were prepared for western blot analysis by lysing cells with 1% sarkosyl.

Equal amounts of total protein from each lysate were subjected to 7.5% SDS-PAGE. Proteins on the gel were transferred to nitrocellulose and analyzed by standard Western blotting techniques using the Scl70 antibody to topo I. Lane 1: normal cells; Lane 2 and 3: duplicate samples from XPA cells; Lane 4 and 5: duplicate samples from XPD cells; Lane 6: extracts from HeLa cells.
Figure 47
Figure 48: Camptothecin induced topoisomerase I-DNA covalent complexes in repair deficient cells.

XPA and XPD cell lines were treated with 10 μM CPT or no drug for 30 minutes at 37°C followed by sarkosyl lysis and ICE bioassay analysis. As a control, diploid fibroblast cells were subjected to the same treatment.
Figure 48

Covalent Complexes (ng topo I/μg DNA)

- Normal
- XPA
- XPD

CPT
Figure 49: Effect of camptothecin treatment on UV induced topoisomerase I-DNA covalent complexes.

HeLa cells were treated with a UVB dose of 10 kJ/m² and either treated immediately with 10 μM CPT for 30 minutes or incubated for 4.5 hours followed by a 30 minute CPT treatment. Cells were lysed by the addition of sarkosyl and lysates analyzed by the ICE bioassay. As a control, unirradiated cells were treated with 10 μM CPT for 30 minutes followed by ICE bioassay analysis.
Figure 49
Next, the effect of CPT on cisplatin treated cells was determined at two different concentrations of cisplatin. HeLa cells were treated with cisplatin and incubated for 5 hours. CPT (10 μM) was added to the cells 30 minutes prior to harvesting by the ICE bioassay (Fig. 50). The results show that at both concentrations of cisplatin used, CPT enhances formation of topo I-DNA covalent complexes.

DISCUSSION

The consequences of UV lesions, as well as DNA repair mechanisms, on the activity of human topo I was investigated to determine the basis of the in vivo UV-topo I response. One of the factors that influence the formation of UV induced topo I-DNA covalent complexes is the effect of UV lesions on topo I turnover. The products of UV irradiation, CPD and (6-4) photoproducts, produce significant local distortions in the helix which can interfere with the proper catalytic activity of topo I. Indeed, the presence of UV photoproducts inhibits DNA relaxation activity of topo I by approximately 30 % when close to 10 lesions were introduced per 400 bp of DNA. Decreased relaxation activity correlates with increased stabilization of topo I-DNA covalent complexes indicating that the religation step in the topo I catalytic cycle is inhibited. Topo I-DNA covalent complex formation increases linearly with UV dose similar to the effects seen with CPT. This UV dependent enhancement of topo I mediated breaks appears to be due to a reduction in the closure rate of broken DNA. Thus, the in vivo UV-topo I response can be partially explained by the inhibition of relaxation activity.
Figure 50: Effect of camptothecin treatment on cisplatin induced topoisomerase I-DNA covalent complexes.

HeLa cells were treated with cisplatin doses of 15 or 60 μM, incubated for 4.5 hours followed by treatment with 10 μM CPT for 30 minutes. Cells were lysed by the addition of sarkosyl and lysates analyzed by the ICE bioassay. As a control, untreated cells were exposed to 10 μM CPT for 30 minutes followed by ICE bioassay analysis.
Figure 50

Covalent Complexes (ng topo/µg DNA)

- +
No Cisplatin

- +
15 µM Cisplatin

- +
60 µM Cisplatin

CPT
Localization of the breakage sites at the nucleotide level revealed that UV lesions cause increase breaks at the same sites seen on untreated DNA. Interestingly, these topo I sites are present in close proximity to CPD clusters in the DNA. The presence of these CPDs may alter the local DNA conformation at the break sites causing misalignment of the two ends of the break site leading to inhibition of religation. However, the UV irradiated probes contain a heterogeneous population of molecules with different lesion sites and frequency. Therefore, it is difficult to assess which lesions surrounding the topo I cleavage site are effective in stabilizing the breaks. No clear novel topo I sites are detected in the presence of UV lesions, however, since the probe used in these experiments is small, it possible that effects of UV lesions at sites removed from the photoproducts are not being detected. It is possible that as UV dose increases topo I stabilized cleavages at additional sites but each of them are formed with a very low frequency. Indeed, there two faint novel cleavage sites are detected only in the presence of UV lesions (Fig. 45). Additionally, other new sites may not be detected as discreet bands but as an increase in the overall background.

The inhibition of topo I relaxation activity by UV lesions does not sufficiently explain the in vivo response of topo I to UV irradiation. Topo I-DNA covalent complexes increase by as much as 7 fold in UV treated cells while only a 3 fold reduction in relaxation activity is seen in vitro. It is possible that topo I may be a component of DNA repair processes which leads to targeting of topo I to its sites of
action on lesion containing chromatin. The results from the repair deficient cell lines implicate a coupling of topo I activity with DNA repair. XPD cells were found to be defective in the UV-topo I response as measured by the ICE bioassay. The XPD gene encodes an ATP dependent helicase (164,163) that presumably assists in unwinding the helix at the repair site. This unwinding could result in supercoiling in close proximity to the lesion site and topo I may be driven to these repair sites because of its inherent affinity for topologically altered DNA, although topo I/helicase protein-protein interactions cannot be ruled out. In the absence of an active helicase there will be lower levels of DNA unwinding resulting in fewer sites with localized supercoiling leading to less topo I recruitment under these conditions (in XPD cells). Additionally, the XPD gene is also involved in transcription and is a component of the TFIH complex. Since topo I is closely associated with active transcription, the lack of response in XPD cells may suggest an involvement of topo I in transcription coupled repair. XPA cells also show lower levels of topo I DNA complex formation although the kinetics of complex formation seem to be similar to the wild type response. It is possible that there are other proteins involved in lesion recognition, thus the absence of this gene may not severely affect topo I recruitment to repair sites.

Another interesting aspect of the UV-topo I response is the speculation that topoisomerases are responsible for sister chromatid exchange following lesion formation (174,175,138). Indeed illegitimate recombination has been observed in cells treated with
CPT (176). It is possible that DNA distortions in the vicinity of unrepaired lesions may alter the function of these enzymes such that rejoining could occur incorrectly. The finding that UV induced lesions stabilize topo I-DNA covalent complexes supports the idea that this crosslinking may be responsible for chromosomal rearrangements following UV irradiation.

CONCLUSIONS

The rate of plasmid relaxation by topo I is reduced in the presence of UV photoproducts. This is a result of increased stabilization of topo I-DNA covalent complexes. Thus, the in vivo response to UV photoproducts can partially be explained by the alteration in the topo I cleavage/religation equilibrium. UV lesions do not appear to produce major novel cleavages by topo I - minor sites are detected at higher doses of UV treatment. Examination of repair deficient cell lines also implicates topo I in DNA repair processes as XPD cells do not show a UV induced topo I response. Thus, the in vivo topo I response following UV irradiation appears to be a combination of altered cleavage/religation equilibrium and topo I activity in repair. Finally, topo I mediated DNA damage induced by UV photoproducts and cisplatin are enhanced by the addition of CPT.
DNA topoisomerases are unique enzymes that alter the topological state of DNA without changing its chemical structure. These enzymes have been found to participate in nearly all aspects of nucleic acid metabolism and are important for the maintenance of chromosome and nuclear structure. The establishment of topoisomerases as targets of many anticancer agents has expanded the study of these enzymes into the areas of pharmacology and clinical medicine.

This thesis has focused on topo I and its role in cancer chemotherapy. The formation of topo I-DNA covalent complexes is the key step in effective tumor cell killing. The persistence of these covalent complexes results in topo I-mediated DNA damage that ultimately leads to cell death. These endogenous topo I-DNA covalent complexes can be directly and specifically measured by the ICE bioassay, thus enabling evaluation of topo I-mediated DNA damage under various conditions. The assay can
be used to monitor the effects of poisons that stabilize topo I-DNA covalent complexes in the cellular environment. From this work several important issues regarding the role of topo I in cancer therapy have emerged.

First, the new topo I poison TPT was found to be effective in stabilizing topo I-DNA covalent complexes. While the drug has shown promise in clinical trials, the interactions between TPT and topo I have remained untested. Furthermore, complex formation shows a strong correlation with the cell killing effects of TPT, indicating that the assay accurately reflects the efficacy of the drug.

Second, the issue of TPT instability at physiological pH was addressed by encapsulating the drug in liposomes. Liposomal encapsulation vastly improved the efficacy of TPT both by increasing drug delivery to cells and enhancing TPT stability. Thus, liposome delivery systems may be of potential utility for introducing TPT into cancer patients. However, additional studies are required to determine the feasibility of liposome encapsulated TPT in the clinical situation. It is important to note that liposome encapsulation does not necessarily improve the efficacy of all drugs. For example, encapsulation of SN38 (another analog of CPT) in DSPC liposomes does not appear to increase the activity of this drug in the cellular environment (data not shown). In this case, it may be necessary to use different formulations of liposome to enhance SN38 efficacy. Thus, prior to clinical evaluation, it is important to test all drugs in cultured cells where the concentration ranges and different manipulations of the drug (such as liposome encapsulation) can be evaluated.
Third, the assay can be adapted to the clinical situation to measure the efficacy of topo I poisons in patient samples. Preclinical and clinical trials of drugs should be accompanied by specific assays that investigate the relationship between chemotherapeutic agents and their targets. The ICE bioassay has enabled us to evaluate topo I-mediated DNA damage in patients undergoing treatment with TPT. The results are very promising as, in all cases tested, TPT was able to induce topo I-DNA covalent complexes in a time dependent fashion. Both peripheral blood samples and tumor cells showed an increase in endogenous topo I-DNA covalent complex formation upon treatment with TPT. While these studies have revealed no correlation between patient response and topo I-DNA covalent complex formation, the sample size tested was very small. Continued testing of patients undergoing treatment with TPT may reveal patterns between topo I-DNA covalent complex formation and patient response. Additionally, testing patients undergoing treatment with other topo I poisons should also be carried out to determine if the assay applicable in all situations. Preliminary data from our lab indicates that topo I-DNA covalent complexes can be detected in peripheral blood of patients undergoing treatment with 9-AC (an analog of CPT). Thus, the ICE bioassay could be an important diagnostic tool for individualizing treatment with topo I poisons. Fourth, other classes of agents that induce topo I mediated DNA damage have been identified. The formation of topo I-DNA covalent complexes in cells treated with cisplatin and VM26 provides a novel approach for topo I directed cancer chemotherapy. A similar topo I response is also seen in UV treated
cells. Regardless of the mechanism by which these covalent complexes are formed, the resultant increase in topo I activity on chromatin can be exploited to induce topo I-mediated DNA damage. Finally, these studies have provided great potential for rational designs of combinatorial drug regimes involving topo I poisons and lesion inducing agents. DNA modifying agents can be used in combination with topo I poisons to improve treatment against cancer. DNA modifications can be used to increase localization of topo I on chromatin, followed by treatment with CPT analogs to stabilize topo I-DNA covalent complexes. Indeed, the combination of cisplatin and CPT, as well as UV and CPT, enhances the formation of topo I-DNA covalent complexes producing greater topo I mediated DNA damage and cell killing.

Since the ICE bioassay provides a tractable end point, this assay is of great utility in the investigation of topo I as a target for chemotherapy. The assay is very versatile - it can be used in cultured cells where conditions can be controlled as well as in patient samples. A variety of agents can be tested for their ability to induce topo I mediated DNA damage. It is clear that these studies need not be restricted to topo I poisons as other agents are also capable of stabilizing topo I-DNA covalent complexes. The ICE bioassay can also be used to compare various combinations of DNA modifying agents and topo I poisons to evaluate the amount of topo I mediate damage that is produced. This in turn, can be translated to the clinical situation for developing combinatorial approaches to cancer therapy.
Interestingly, the UV topo I response also implicates the enzyme in DNA repair processes. When tested \textit{in vitro}, UV lesions alter topo I catalytic turnover by stabilizing topo I-DNA covalent complexes. However, this effect is not sufficient to explain the \textit{in vivo} UV topo I response. The most striking result is seen with the repair deficient cell lines which are also deficient in UV induced topo I-DNA covalent complexes. If lesions were influencing topo I by altering enzyme turnover or increasing the affinity of topo I binding to DNA, one would not expect to see the lack of UV topo I response in these cell lines. Particularly interesting is the XPD cell lines which are completely devoid of the UV topo I response. One possible explanation for the XPD data is that topo I interacts directly with the XPD helicase and is thus recruited to repair sites. Since the XPD helicase is also a transcription factor, there may be some involvement of topo I in transcription coupled repair. Another possibility is that topo I is attracted to the supercoiling induced by the helicase at repair patches thus increasing topo I activity at these sites. The exact mechanism by which the XPD protein affects topo I activity during repair needs to be determined. An important question to ask is whether transfecting the XPD gene into the XPD deficient cell line restores the UV-topo I response. These studies are currently underway in our lab.

Recent studies from our lab have also revealed a lack of UV-topo I response in a p53 mutant cell line. An increase in p53 levels has been detected in cells following DNA damage (183) and p53 has been shown to bind lesions (184). Additionally, it has been demonstrated that p53 interacts with topo I \textit{in vivo}. Thus, p53 may also be
involved in the recruitment of topo I to repair sites. Collectively, these data provide some evidence for topo I involvement in DNA repair. DNA repair, like all other processes involving nucleic acid metabolism will cause topological alterations in DNA which could recruit topo I activity. However, the role of topo I in the cellular response to lesions has yet to be elucidated.

The work presented in this thesis reveals that topo I is becoming an increasingly attractive candidate as an enzyme that is recruited in cellular responses to DNA lesions as well offering itself as a important chemotherapeutic target. By the very nature of the reaction topo I catalyzes, the enzyme will continue to be implicated in processes where DNA conformation is altered, including DNA repair. Future work promises to identify novel combinatorial approaches to anti-tumor therapy involving topo I poisons. Other agents that can be used in combination with topo I poisons will be revealed through systematic assessment of topo I-DNA covalent complex formation in the presence of both compounds via the ICE bioassay.
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