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ORIGINAL ARTICLE

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Sequence effect of irinotecan (CPT-11) and topoisomerase II inhibitors in vivo

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Abstract The DNA topoisomerases I and II are the target of several clinically important antineoplastic agents which produce DNA cleavage by stabilization of the covalent DNA-protein bond with resultant cell death after DNA synthesis is attempted. Depletion of the target topoisomerase and reciprocal changes in the other occur with drug treatment. *Purpose and methods*: To develop empiric treatment regimens of combinations and sequences of agents directed against topoisomerase I (irinotecan/CPT-11) and II (etoposide and doxorubicin), in vivo studies were performed in mice bearing the EMT-6 mammary tumor to assess efficacy, host tolerance and the resultant biochemical changes in topoisomerase mRNA and protein. *Results*: At 24 h after therapy, depletion of the target topoisomerase mRNA and protein with reciprocal increases in the alternate topoisomerase mRNA and, to a lesser extent, protein were noted. No therapeutic antagonism was found with any combination or sequence of agents, and therapeutic antagonism was noted with concurrent irinotecan/etoposide and sequential doxorubicin/irinotecan. Depletion of target topoisomerases by combined therapy beyond a threshold necessary for therapeutic efficacy produced no additional benefit. *Conclusions*: Antineoplastic therapy with combinations of topoisomerase I and II agents is feasible and may produce therapeutic synergy. The appropriate sequence may depend on the particular agents used. The rationale for such therapy, that topoisomerases I and II may have reciprocal and compensatory interactions, is supported by the biochemical data.

Key words Irinotecan · CPT-11 · Topoisomerase I · Topoisomerase II · Treatment sequence effects

Introduction

DNA topoisomerases are nuclear enzymes essential for DNA replication, RNA transcription, chromosomal condensation and mitotic chromatid separation [1–5]. The level of topoisomerase I is independent of cell cycle phases although cytotoxicity is manifest only in proliferating cells [5]. Topoisomerase II is cell cycle phase dependent, being absent in G_0/G_1 phase with an increase at the onset of S phase and during G_2 phase of the cell cycle [6]. Topoisomerases I and II are important targets for cancer chemotherapeutic agents [7]. The topoisomerase active agents currently in clinical use as antineoplastic agents stabilize a transient covalent enzyme-DNA complex, which produces DNA strand cleavage and apoptosis. Unlike the situation where elevated target enzymes confer resistance to an antineoplastic agent, as with thymidylate synthase and fluorouracil or dihyrofolate reductase and methotrexate, the enzyme is an essential component of the cytotoxicity of topoisomerase-active poisons. Camptothecin and the various camptothecin derivatives are topoisomerase I poisons which do not affect topoisomerase II [8]. Similarly, the topoisomerase II poisons such as etoposide and doxorubicin do not interact with topoisomerase I [9, 10].

Decreases in the level of either topoisomerase I or II confer resistance to the agents targeting the respective

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enzyme which may be a clinically relevant mechanism of resistance [11–13]. Cell lines, such as the P388 murine leukemia, may show a coordinate increase in the complementary topoisomerase when the target topoisomerase is decreased in the drug-resistant clones [11]. In vitro studies suggest a complex interaction between topoisomerase-acting drugs in that camptothecin, a topoisomerase I inhibitor, can negate the effect of a topoisomerase II-acting agent under certain conditions, such as concurrent rather than sequential administration [14, 15]. This might be due to the cell cycle arrest induced by camptothecin. The effects of topoisomerase schedule dependency may differ in vivo, where doses are lower and schedules different.

Given the dynamic interaction between topoisomerase I and topoisomerase II, the current study was undertaken to explore the in vivo therapeutic effect of sequence on the combination of topoisomerase I and II-acting drugs in a murine tumor model system. Doses were therapeutically relevant for antitumor activity, host tolerance, and ability to be used in combination therapy. To determine the biochemical factors responsible for the therapeutic results observed, changes in the RNA and protein levels of topoisomerase I and II were studied in murine EMT-6 mammary carcinoma cells produced by the topoisomerase I inhibitor CPT-11 (irinotecan) and the topoisomerase II inhibitors eptoposide and doxorubicin.

Materials and methods

Drugs

CPT-11 (irinotecan) was a gift from Dr. Mary Torkelson, Pharmacia and Upjohn, Kalamazoo, Mich. Etoposide (VP-16) and doxorubicin (adriamycin) were purchased from the Dana-Farber Cancer Institute pharmacy.

Tumor

The EMT-6 murine mammary carcinoma as an in vivo-in vitro tumor system. The EMT-6 tumor was carried in BALB/c mice (Taconic Farms, Germantown, N.Y.). For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted intramuscularly into the legs of BALB/c mice at 8 to 10 weeks of age [15, 16].

Detection of topoisomerase I and II transcripts

Topoisomerase I and II poly(A)$^+$ RNAs were detected by the method of reverse transcription-polymerase chain reaction (RT-PCR). The primers for the detection of topoisomerase II expression amplify a region adjacent to the ATPase domain (nucleotides 1394–1629), which is highly conserved in the mouse and Chinese Hamster. The sequences are 5′-CCAAACTTGATGATGCCA-3′ (forward) and 5′-CTGAAGACCCACAATCT-3′ (reverse). This set of primers recognizes only topoisomerase IIα, since no topoisomerase poly(A)$^+$ RNA is detectable in serum-starved EMT-6 unless it is transfected with topoisomerase IIα [17]. The primers for measuring topoisomerase I mRNA amplify a region around nucleotides 2300 to 2600. They are 5′-CTGGAGACCCACAATCT-3′ (forward) and 5′-CCAACCTTG ATGCCA-3′ (reverse). β-Actin was used as the control. Poly(A)$^+$ RNA was isolated from tumor tissues as previously described [15]. cDNA was synthesized from 1 μg of mRNA with the Superscript of cDNA Preamplification Kit (Gibco BRL, Grand Island, NY) in the presence of random hexamers. A 5-μl aliquot from 20 μl of the first strand cDNA preparation was added to the PCR reaction mix containing 1.0 *M* each of the forward and reverse primers (for either topoisomerase I or topoisomerase II), 200 *M* dNTPs, 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.3), 3.0 m*M* $MgCl_2$ and 2.5 units Ampli-Taq polymerase (Perkin-Elmer/Cetus) in a final volume of 50 l. The mixture was denatured at 94 °C, annealed at 52 °C and extended at 72 °C (1 min each) for 30 cycles followed by a 10-min extension at 72 °C on a DNA Thermal Cycler (Perkin-Elmer/Cetus, Foster City, Calif.). PCR products were analyzed on a 1.6% agarose gel with 0.5 g/ml ethidium bromide.

Western blot analysis of topoisomerase I and II proteins

Cellular extracts which contained the topoisomerase I and II proteins were prepared from frozen tissues as described previously with modifications [17]. Tumor specimens were homogenized using a tissue homogenizer (Tissue-Tearor, Fisher Scientific, Pittsburgh, Penn.) in a buffer containing 40 m*M* Na_2HPO_4, pH 7.8, 25 m*M* KCl, 1 *M* NaCl, 4 m*M* DTT, 5 m*M* EDTA, 5 m*M* EGTA, 10% glycerol, 0.5 μg/ml Pefabloc SC, Boehringer, Indiana Polis, Ind. 0.5 μg/ml leupepsin and 0.7 μg/ml pepstatin. Nuclear proteins were extracted by incubating the tissue homogenate on ice for 30 min, then polyethylene glycol (PEG) was added to a final concentration of 6%. The extract/PEG mix was further incubated on ice for 30 min followed by centrifugation at 27 000 *g* for 30 min at 40 °C. Protein concentrations were determined by Micro Protein BCA Kit (Pierce Rockford, Ill.). The extracted proteins (100 g/sample) were then analyzed by SDS-PAGE (7.5%), transferred to nitrocellulose filters and visualized by indirect immunodetection using human anti-topoisomerase I or rabbit anti-topoisomerase II antibodies (kindly donated by Dr. M.T. Muller, TopoGEN Columbus, Ohio), horseradish peroxidase (HRP) conjugates of goat antihuman or goat antirabbit antibodies (Pierce) and an ECL Kit (Amersham Arlington Heights, Ill.). Purified human topoisomerase I and II proteins (TopoGEN) were included as positive controls and for verification of the adequacy of storage and preparation. The signals of topoisomerase I and II proteins were quantified using the Bio-Max 1 D program (Kodak) and normalized by the laminin immunoreactivity detected as described above using rabbit antilaminin antibody (ICN Costa Mesa, Calif.). Anti-topoisomerase I antibody produced a single 100 kDa signal and the anti-topoisomerase II antibody was visualized only as the 170 kDa α form. The results are expressed as the average of two separate Western analyses.

Tumor excision assay

When the EMT-6 tumors were approximately 100 mm^3 in volume (8 days after tumor cell implantation), the animals were given intraperitoneal injections of various doses of CPT-11 (100, 200, 300 or 400 mg/kg), etoposide (10, 20, 30, 40 or 50 mg/kg) or doxorubicin (5, 10, 15 or 25 mg/kg). Mice were sacrificed 24 h after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage. The tumors were excised, and single cell suspensions were prepared as described previously [15, 16, 18]. The untreated tumor cell suspensions had a plating efficacy of 10–16%. The results are expressed as the surviving fraction ± SE of cells from treated groups compared with that of cells from untreated controls [15, 18].

Bone marrow toxicity

Bone marrow cells were taken from the same animals used for the tumor excision assay, and the assay for granulocyte/macrophage colony-forming units (GM-CFU) was carried out as described previously [15, 18]. Colonies of at least 50 cells were scored on a Acculite colony counter (Fisher, Springfield, N.J.). The results from three experiments, in which each group was measured in triplicate,

were averaged. The results are expressed as the surviving fraction ± SE of cells isolated from treated animals compared with that of cells isolated from untreated animals.

Tumor growth delay experiments

When the EMT-6 tumors were approximately 100 mm^3 in volume, treatment was initiated. Animals were treated by intraperitoneal injection with CPT-11 (20 mg/kg) once per day on days 7 through 11 or days 14 through 18 after tumor cell implantation, with etoposide (10 mg/kg) once per day on days 7 through 11 or days 14 through 18 after tumor cell implantation, or with doxorubicin (1.75 mg/kg) once per day on days 7 through 11 or days 14 through 18. Some treatment groups received CPT-11 (20 mg/kg) along with etoposide (10 mg/kg) or doxorubicin (1.75 mg/kg) daily on days 7 through 11 after tumor cell implantation, or received CPT-11 (20 mg/kg) on days 7 through 11 followed by etoposide (20 mg/kg) or doxorubicin (1.75 mg/kg) on days 14 through 18 after tumor cell implantation. Finally, some treatment groups received etoposide (10 mg/kg) or doxorubicin (1.75 mg/kg) on days 7 through 11 followed by CPT-11 (20 mg/kg) on days 14 through 18. The progress of each tumor was measured thrice weekly until it reached a volume of 500 mm^3. Tumor growth delay (TGD) was calculated as the time taken by each individual tumor to reach 500 mm^3 compared with the time in the untreated controls. Each treatment group had five animals, and the experiment was repeated three times. TGD times (days) are the means ± SE for the treatment group compared with those for the control group.

Table 1 Growth delay of the EMT-6 carcinoma produced by topoisomerase I and topoisomerase II inhibitors alone and in combination. Tumor growth delay is the difference in the time for treated tumors to reach 500 mm^3 compared with untreated control tumors. The data are presented as the means of 15 animals ± SEM. All of the drugs were administered by intraperitoneal injection once per day on the schedules shown

Treatment group	Tumor growth delay (days)
CPT-11 (20 mg/kg) days 7–11	3.5 ± 0.5
CPT-11 (20 mg/kg) days 14–18	3.2 ± 0.4
Etoposide (10 mg/kg) days 7–11	3.6 ± 0.4
Etoposide (10 mg/kg) days 14–18	2.9 ± 0.3
Doxorubicin (1.75 mg/kg) days 7–11	4.1 ± 0.3
Doxorubicin (1.75 mg/kg) days 14–18	3.3 ± 0.3
CPT-11/etoposide	8.6 ± 0.8*
CPT-11/doxorubicin	7.2 ± 0.7
CPT-11 → etoposide	8.1 ± 0.7
CPT-11 → doxorubicin	6.5 ± 0.6
Etoposide → CPT-11	7.4 ± 0.6
Doxorubicin → CPT-11	9.3 ± 0.9*

*Significantly greater than expected for additivity of the treatment, $P < 0.01$

Data analysis

Using the method of Deen and Williams [19], isobolograms were generated for the special case in which the dose of one agent is held constant. This method produces envelopes of additive effect for different levels of the variable agent. It is conceptually identical to generating a series of isobolograms and replotting the results at a constant dose of one agent on a log effect by the dose of the second-agent coordinate system.

Dose response curves for each agent alone were first generated. The envelopes of additivity shown were generated from a series of isoeffect curves derived from the complete dose response curves for each agent alone. Overall, combinations that produced the desired effect and that are within the envelope boundaries are considered additive. Those displaced to the left are supra-additive, while those displaced to the right are subadditive [20, 21]. This general approach can be extrapolated to the special case in which the level of an agent is held constant. Under these conditions, an isobologram can be derived that plots the expected effect for any level of the variable agent plus the constant agent combinations [22]. Experimentally, this approach is far simpler than classical isobologram methodology and readily facilitates determination of additive and nonadditive combinations [15].

Statistical comparisons for the TGD assays were carried out with the Dunn multiple comparisons test after a significant effect was found by analysis of variance [23, 24]. TGD that exceeded the limits of variability were reported as greater-than-additive if the TGD exceeded the upper boundary of the variance and less-than-additive if below the variance boundary. Each treatment group or treatment/sequence group reflects 15 animals, in three groups of 5. Numerical values for poly(A)$^+$ RNA and topoisomerase I and II protein represent the average of two experiments, normalized by densitometric scanning to the respective controls.

Results

CPT-11, etoposide, doxorubicin or combinations of CPT-11 and either etoposide or doxorubicin were used to assess the efficacy of each therapy and combinations of therapies on tumor cytotoxicity in the TGD assay. The TGD measures the therapeutic efficacy and overall host toxicity in the whole animal on a multiple dose schedule which is similar to the clinical schedule of each agent. The doses chosen were the maximal doses that produced no weight loss or obvious toxicity in the animals. Each drug was administered daily, whether used singly or in combination, by intraperitoneal injection for 5 days on either days 7 through 11 or days 14 through 18 (Table 1). CPT-11 (20 mg/kg) was administered alone, simultaneously with etoposide (10 mg/kg) or doxorubicin (1.75 mg/kg) or sequentially before or after etoposide or doxorubicin. Each of the drugs was an active antitumor agent against the EMT-6 tumor at the doses used. As expected, the TGD produced was greater when treatment was initiated on day 7 when the tumors were about 100 mm^3 in volume than when treatment was initiated on day 14 when the tumors were about 500 mm^3 in volume. Simultaneous administration of CPT-11 and etoposide resulted in a greater than additive TGD than simultaneous administration of CPT-11 and doxorubicin. When CPT-11 was administered first (days 7–11) followed by etoposide or doxorubicin (days 14–18), the TGD produced by the combination of CPT-11 followed by etoposide was greater than that produced by CPT-11 followed by doxorubicin. However, when etoposide or doxorubicin was administered first (days 7–11) followed by CPT-11 (days 14–18), the greatest TGD was obtained with the regimen of doxorubicin followed by CPT-11.

The tumor excision assay was used to assess the single dose cytotoxicity of each agent in vivo toward EMT-6 tumor cells and bone marrow CFU-GM in a dose-escalating manner. This is a measure of the relative cytotoxicity in an in vitro clonogenic assay after single

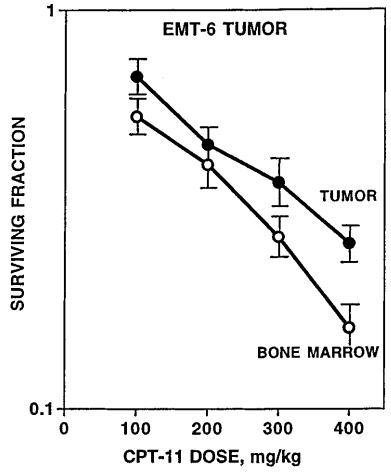


Fig. 1 Survival of EMT-6 tumor cells and of bone marrow CFU-GM from the same animals after treatment of the animals with various single doses of CPT-11. Points are the means of three experiments; bars are the SEM

dose in vivo therapy at doses not compatible with survival of the whole animal. CPT-11 killed increasing numbers of tumor cells with increasing dose in a log-linear manner (Fig. 1). CPT-11 was more cytotoxic toward the bone marrow CFU-GM than toward the EMT-6 tumor cells such that a dose of 200 mg/kg of CPT-11 killed 50% of the EMT-6 tumor cells but only 125 mg/kg of CPT-11 killed 50% of the bone marrow CFU-GM. The tumor excision assay was also used to assess the cytotoxicity of etoposide and doxorubicin in vivo toward EMT-6 tumor cells and bone marrow CFU-GM (Fig. 2). Both etoposide and doxorubicin were actually more cytotoxic toward EMT-6 tumor cells than toward bone marrow CFU-GM, in a similar dose-dependent manner.

Isobologram methodology was used to analyze simultaneous single dose combinations of CPT-11 and etoposide or doxorubicin for additivity/synergy in cytotoxicity in vivo toward EMT-6 tumor cells and bone marrow CFU-GM. When CPT-11 (400 mg/kg) was administered simultaneously with etoposide (50 mg/kg)

Fig. 2 Survival of EMT-6 tumor cells and of bone marrow CFU-GM from the same animals after treatment of the animals with various single doses of etoposide or doxorubicin. Points are the means of three experiments; bars are the SEM

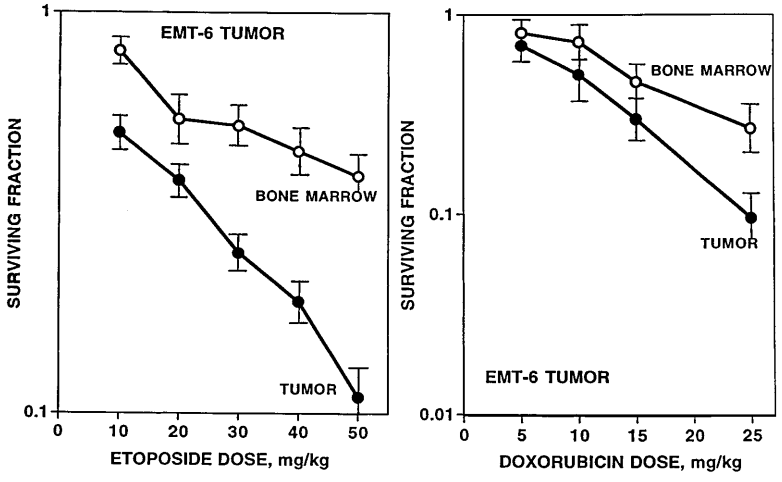
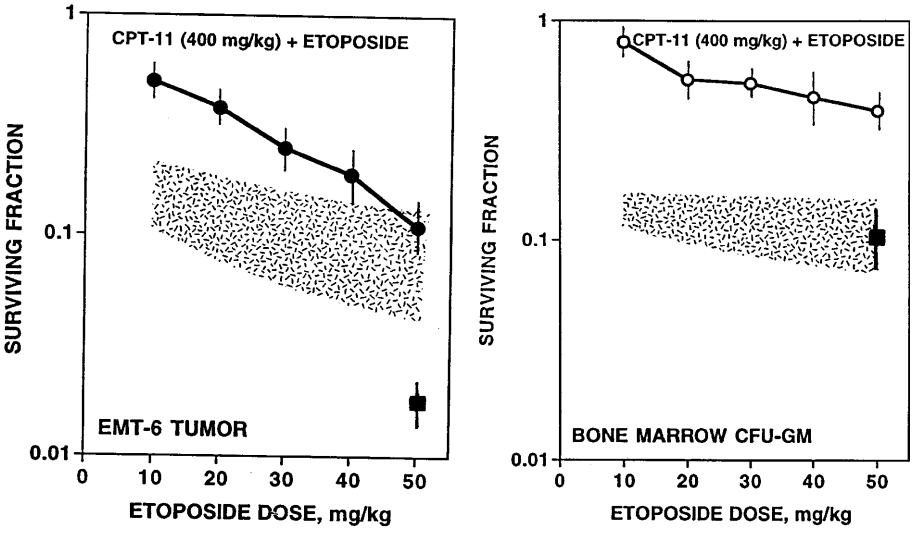


Fig. 3 Survival of EMT-6 tumor cells and bone marrow CFU-GM after treatment of tumor-bearing animals with combinations including CPT-11 (400 mg/kg) and etoposide. The shaded area is the envelope of additivity determined by isobologram analysis. Points are the means of three experiments; bars are the SEM



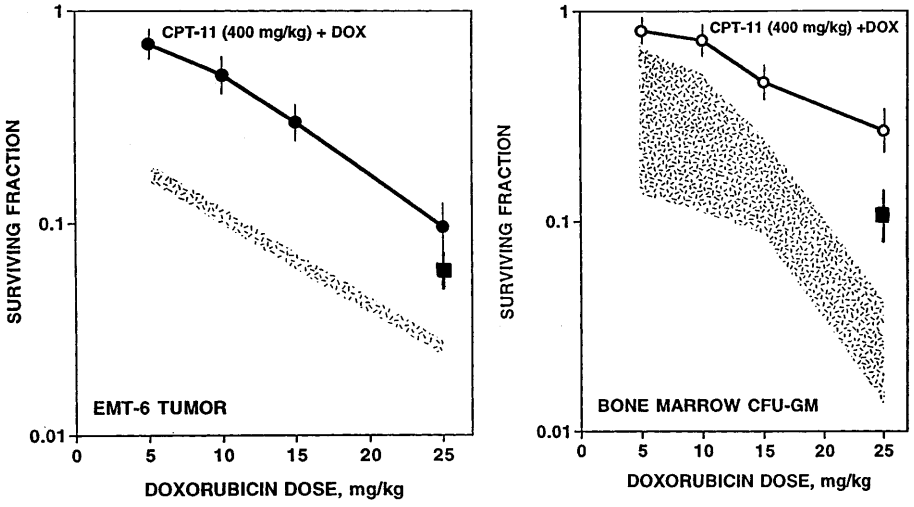


Fig. 4 Survival of EMT-6 tumor cells and bone marrow CFU-GM after treatment of tumor-bearing animals with combinations including CPT-11 (400 mg/kg) and doxorubicin. The shaded area is the envelope of additivity determined by isobologram analysis. Points are the means of three experiments; bars are the SEM

(Fig. 3), combination treatment regimens resulted in greater than additive tumor cell killing for the two drugs.

The same isobologram analysis of simultaneous treatment regimens was used to assess the single dose additivity/synergy of CPT-11 (400 mg/kg) and doxorubicin (25 mg/kg). Simultaneous administration of CPT-11 and doxorubicin resulted in less-than-additive killing of EMT-6 tumor cells in vivo (Fig. 4). The EMT-6 tumor cell killing observed was similar to that obtained with doxorubicin alone. A similar pattern pertained in the killing of the bone marrow CFU-GM by combination of CPT-11 and doxorubicin.

To better understand the biochemical events associated with combined topoisomerase I and II directed drug therapy poly(A)$^+$ RNA and topoisomerase protein were isolated from the tumors 24 h after the last day of single or combination therapy in the TGD assay (Fig. 5, Table 2). Using a semiquantitative RT-PCR technique, the topoisomerase I-active agent CPT-11 alone increased the expression of topoisomerase II poly(A)$^+$ RNA and decreased the expression of topoisomerase I poly(A)$^+$ RNA. The administration of etoposide or doxorubicin alone increased the expression of topoisomerase I poly(A)$^+$ RNA and decreased the levels of topoisomerase II poly(A)$^+$ RNA. Concurrent CPT-11 and etoposide or doxorubicin administration decreased the levels of both topoisomerase I and II mRNA. Sequential administration most reflected the effect of the last agent administered, although the sequence of doxorubicin followed by CPT-11 produced no significant change. There was a reciprocal interaction between topoisomerase II and topoisomerase I poly(A)$^+$ RNA expression with a decrease in RNA expression in the target topoisomerase and a rise in the other.

The levels of the topoisomerase I and the topoisomerase II protein in the EMT-6 tumors were also affected by treatment of the animals with CPT-11, etoposide and doxorubicin alone and in combination (Fig. 5, Table 2). CPT-11 alone had no significant effect on topoisomerase I or II protein levels at 24 h. Etoposide and doxorubicin individually decreased topoisomerase II levels but had little or no effect on topoisomerase I levels at 24 h. Simultaneous treatment decreased both the levels of topoisomerase I and II; the combination of doxorubicin and CPT-11 produced greater decreases than the combination of etoposide and CPT-11 but produced less therapeutic benefit (Table 1). Sequential treatment of the animals with CPT-11 followed by etoposide or doxorubicin resulted in increased levels of the topoisomerase I enzyme, which would be expected from the earlier increase in poly(A)$^+$ RNA resulting from etoposide or doxorubicin treatment. The topoisomerase II enzyme levels were decreased by sequential CPT-11 followed by doxorubicin but not by CPT-11 followed by etoposide. The sequential combination containing doxorubicin, as with the simultaneous treatment, resulted in a greater decrease in topoisomerase II but produced less therapeutic benefit than sequential CPT-11/etoposide.

Sequential treatment with either of the two topoisomerase II agents first followed by CPT-11 produced different patterns of topoisomerase protein expression than seen with the agents individually or with the sequence of CPT-11 administered first. Etoposide preceding CPT-11 produced the largest decrease in topoisomerase II levels observed with any etoposide administration, but no change in topoisomerase I levels. Doxorubicin followed by CPT-11, produced no change in topoisomerase II levels (as was seen with CPT-11 alone at these doses) but markedly reduced the topoisomerase I levels. Unlike the case where a greater decrease in topoisomerase II levels at 24 h was associated with less tumor cytotoxicity, lower levels of topoisomerase I were associated with a greater therapeutic effect. The results are summarized in Table 2.

Discussion

Topoisomerase I and II are the targets of several highly effective antineoplastic agents. Resistance to these

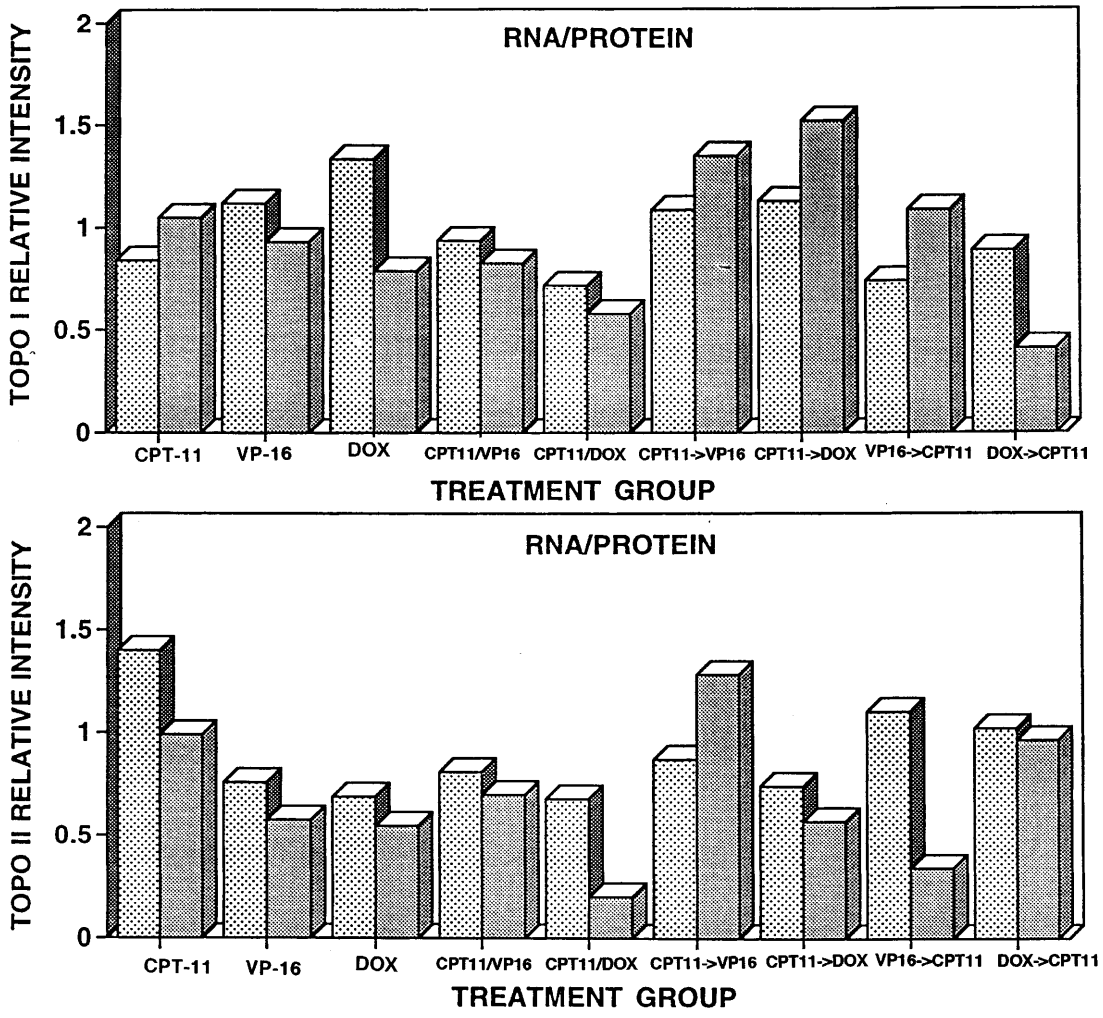


Fig. 5 Relative intensity of topoisomerase I and topoisomerase II bands derived from semiquantitative RT-PCR of 1 μg poly(A)$^+$ RNA (*dotted bars*) prepared from EMT-6 tumors after treatment of the tumor-bearing animals. Primers were to total topoisomerase I and total topoisomerase II. Relative intensity of the topoisomerase I and topoisomerase II bands derived from Western blots (*filled bars*) of total protein from EMT-6 tumors after treatment of the tumor-bearing animals. The antibodies to topoisomerase I and topoisomerase II were gifts from Dr. Mark Muller (TopoGEN)

agents is multifactorial. Increased efflux via membrane proteins like P-glycoprotein/*mdr* or *mrp* may prevent the accumulation of cytotoxic levels of all the available topoisomerase II agents but are unlikely to contribute significantly to resistance to the camptothecin derivatives except perhaps topotecan [25]. Genomic mutations which alter the enzyme amino acid sequence may reduce the drug-induced stability imparted to the DNA-topoisomerase covalent complex and confer high level resistance in vitro, but a similar role clinically is unproven [26, 27]. Several experimental cell lines and some clinical data implicate decreased levels of either topoisomerase I or II, respectively [11–13, 28]. Since all the currently useful clinical topoisomerase I and II directed drugs poison cells by trapping the topoisomerase enzyme in a deadly embrace with DNA, decreased protein levels would confer resistance. Concomitant increased levels or function in the other topoisomerase have been observed in these situations, suggesting that combined topoisomerase I and II directed therapy may be a rational therapeutic goal, although cell culture studies have shown that the simultaneous exposure of cells to a topoisomerase I inhibitor and a topoisomerase II inhibitor can result in antagonism (less-than-additive cytotoxicity) of the two drugs [14, 15]. Despite these experimental observations, combined topoisomerase I and II therapy in the clinic has produced either less than expected benefit or excessive toxicity [29, 30].

With this background, an in vivo study of the therapeutic efficacy of several possible combinations and schedules of clinically available topoisomerase-directed agents was performed along with biochemical assays of topoisomerase I and II levels as a means to better understand the therapeutic interaction of topoisomerase I and II and antineoplastic drugs directed at the topoisomerases. In the tumor excision combination studies, simultaneous administration of CPT-11 and etoposide resulted in greater-than-additive tumor cytotoxicity and

Table 2 Topoisomerase I and II levels (0 = 0 ± 19% change; +/− = 20–39% change; ++/−− = 40–59% change; +++/−−− = 60–79% change; ++++/−−−− = >80% change)

	CPT-11	Etoposide	Doxorubicin	CPT-11/ etoposide	CPT-11/ doxorubicin	CPT-11 → etoposide	CPT-11 → doxorubicin	Etoposide → CPT-11	Doxorubicin → CPT-11
Topoisomerase I (poly(A)$^+$ RNA)	−(0.2)	0	+(0.3)	0	−(0.3)	0	0	−(0.3)	0
Topoisomerase I	0	0	−(0.2)	−(0.2)	−−(0.4)	++(0.4)	++(0.5)	0	−−−(0.6)
Topoisomerase II (poly(A)$^+$ RNA)	++(0.4)	0	−(0.2)	−(0.2)	−(0.3)	0	−(0.2)	0	0
Topoisomerase II	0	−−(0.4)	−−(0.4)	−−(0.3)	−−−−(0.8)	+(0.2)	−−(0.4)	−−(0.7)	0
Tumor growth delay	3.5	3.6	4.1	8.6*	7.2	8.1	6.5	7.4	9.3*

* $P < 0.01$

simultaneous administration of CPT-11 and etoposide resulted in greater-than-additive tumor cytotoxicity and simultaneous administration of CPT-11 and doxorubicin resulted in additive tumor cytotoxicity. In the TGD studies, where the individual drug doses were divided over several days, greater-than-additive antitumor effects were demonstrated for combinations of CPT-11 and both doxorubicin and etoposide, although the optimal sequence differed for each combination. Simultaneous administration of CPT-11 and etoposide again produced greater-than-additive cytotoxicity where simultaneous CPT-11 and doxorubicin were again additive. In the sequential administration studies, the sequence of CPT-11 followed by either etoposide or doxorubicin produced additive antitumor cytotoxicity. The opposite sequence, doxorubicin followed by CPT-11, produced therapeutic synergy whereas etoposide followed by CPT-11 was additive.

Studies of topoisomerase I and II poly(A)$^+$ RNA using a semiquantitative RT-PCR technique and protein levels occurring after treatment were undertaken to provide a biochemical correlate for the empiric therapeutic observations. When either topoisomerase I or II was the target of a particular drug, there was a marked rise in the poly(A)$^+$ RNA message for the other topoisomerase. This observation suggests that counter-regulatory pathways may exist as a response to either the catalytic unavailability of a particular topoisomerase or to the cleavable complex formed with DNA and the resultant DNA damage mediated by the target topoisomerase. Less consistent data were seen in the protein levels. The time-points 24 h after treatment may have been too early to allow for protein synthesis from the increased mRNA, perhaps due to the antiproliferative effect of the drugs themselves or there may be a different (i.e. post-transcriptional) regulatory mechanism for the enzymes. Additional time-points and studies of RNA and protein turnover will be needed to clarify this point.

It is unlikely that the topoisomerases were still complexed to DNA since the reversibility of the DNA-topoisomerase complexes is rapid (95% at 1 h) and the immunoblots were performed 24 h after the last dose of a topoisomerase-active agent [31]. Since a PEG extraction of protein was used, DNA or DNA complexes were removed and were not visible on the immunoblots. Proteolytic loss of topoisomerase II protein after the induction of DNA strand breaks occurs and post-translational depletion of topoisomerase I has been recently reported [31, 32]. The recovery of topoisomerase I levels after CPT-11 appears more rapid than the recovery of topoisomerase II after etoposide or especially doxorubicin administration. The increase in topoisomerase I protein levels after the sequential treatments directed against topoisomerase I (CPT-11) followed by topoisomerase II (etoposide or doxorubicin) was unexpected, since topoisomerase I levels are considered to be constant and have not been previously noted to be elevated after a single treatment (course) with topoisomerase II-directed agents.

While topoisomerase I-active agents are not yet used clinically against breast cancer, CPT-11/irinotecan is very active against murine mammary carcinomas and human breast cancer cell lines [33, 34] and topotecan has activity in clinical breast cancer [35]. Although activity may be greater against other cancer cell types, non-myelosuppressive toxicity (principally gastrointestinal) occurs at or slightly above the maximum tolerated dose for both CPT-11/irinotecan and topotecan. Dose escalation of topoisomerase I-directed agents is therefore unlikely to be practical. Changes in schedule to divide the dose further (prolonged infusions or oral formulations) is one approach to allow dose escalation to further enhance the therapeutic efficacy of the topoisomerase I agents. The observation reported here, that topoisomerase I- and topoisomerase II-directed agents can increase the target protein for the other class of agents, may allow a second approach to enhancing the cytotoxic efficacy. The particular sequence of agents may be important and not empirically obvious. Our observations suggest that combinations and sequences of drug administrations can produce greater than additive tumor cytotoxicity without additive host toxicity, that sequences other than a topoisomerase I agent followed by a topoisomerase II agent may be better, and that doxorubicin should be considered in these combinations.

Overall these observations suggest that therapeutic strategies directed at combined topoisomerase I and II targets have potential clinical benefit if the therapeutic ratio can be maintained or improved. These findings support the observation that the topoisomerase enzymes must be present and interact with the drug and DNA to form the cytotoxic species. Excessively high levels of topoisomerase-directed agents, particularly topoisomerase I-directed agents, may produce only increased toxicity without enhanced therapeutic benefit. Continued clinical investigation of topoisomerase I inhibitor/topoisomerase II inhibitor combination regimens is warranted, with attention to the importance of schedule and recognition of the potential uniqueness of differing combinations.

References

1. Wang J (1991) DNA topoisomerases: why so many? J Biol Chem 266: 6659–6662
2. Holm CT, Gato T, Wang JC, Botstein D (1985) DNA topoisomerase II is required at the time of mitosis in yeast. Cell 41: 553
3. Gasser S, Laroche T, Falquet J, Tour EBDL, Laemmli U (1986) Metaphase chromosome structure: involvement of topoisomerase II. J Mol Biol 188: 613–629
4. Adachi Y, Luke M, Laemmli UK (1991) Chromosome assembly in vitro: topoisomerase II is required for condensation. Cell 64: 137–148
5. Champoux J (1990) Mechanistic aspects of type-I topoisomerases. In: Wang J, Cozarelli N (eds) DNA topology and its biological effects. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1990, pp 217–242
6. Heck M, Earnshaw W (1986) Topoisomerase II: a specific marker for cell proliferation. J Cell Biol 103: 2569–2581
7. Caron P, Wang J (1993) DNA topoisomerases as a target of therapeutics: a structural overview, In: Andoh T, Ikeda H, Oguro M (eds) Molecular biology of DNA topoisomerases and its application to chemotherapy. CRC, Boca Raton, 1993, pp 1–18
8. Hsiang Y, Hertzberg R, Hecht S, Liu L (1985) Camptothecin induces protein linked DNA breaks via mammalian DNA topoisomerase II. J Biol Chem 260: 14873–14878
9. Kohn K, Pommier Y, Kerrigan D, Markovits J, Covey J (1987) Topoisomerase II as a target of anticancer drug action in mammalian cells. NCI Monogr 4: 61–71
10. Pommier Y, Kohn K (1989) Topoisomerase II inhibition by antitumor intercalators and demethyepipodophyllotoxins. In: Glazer R (ed) Developments in cancer chemotherapy. CRC, Boca Raton, 1989, pp 175–196
11. Tan K, Mattern M, Eng W-K, McCabe F, Johnson R (1989) Non-productive rearrangement of DNA topoisomerase I and II genes: correlation with resistance to topoisomerase inhibitors. J Natl Cancer Inst 81: 1732–1735
12. Sugimoto J, Tsukahara S, Oh-hara T, Isoes T, Tsuiuo T (1990) Decreased expression of DNA topoisomerase I in camptothecin-resistant tumor cell lines as determined by monoclonal antibody. Cancer Res 50: 6925–6930
13. Friche E, Danks M, Schmidt C, Beck W (1991) Decreased DNA topoisomerase II in daunorubicin-resistant Ehrlich ascites tumor cells. Cancer Res 51: 1129–1136
14. Kaufman S (1991) Antagonism between camptothecin and topoisomerase II directed chemotherapy agents in a human leukemia cell line. Cancer Res 51: 4213–4218
15. Teicher BA, Holden SA, Eder JP, Herman TS, Antman KH, Frei E III (1990) Preclinical studies relating to the use of thiotepa in the high-dose setting alone and in combination. Semin Oncol 17: 18–32
16. Teicher BA, Holden SA, Jacobs JL (1987) Approaches to defining the mechanism of enhancement by Fluosol-DA 20% with carbogen of melphalan antitumor activity. Cancer Res 47: 513–518
17. Eder J, Chan V-W, Ng S-W, Rizvi N, Zacharoulis S, Teicher B, Schippner L (1995) DNA topoisomerase II alpha expression is associated with alkylating agent resistance. Cancer Res 55: 6109–6116
18. Teicher BA, Rose CM (1984) Perfluorochemical emulsions can increase tumor radiosensitivity. Science 223: 934–936
19. Deen DF, Williams ME (1979) Isobologram analysis of X-ray-BCNU interactions in vitro. Radiat Res 79: 483–491
20. Steel GG, Peckham MJ (1979) Exploitable mechanisms in combined radiotherapy-chemotherapy: the concept of additivity. Oncol Biol Phys 15: 85–91
21. Berenbaum MC (1977) Synergy, additivism and antagonism in immunosuppression. A critical review. Clin Exp Immunol 28: 1–18
22. Dewey WC, Stone LE, Miller HH, Giblak RE (1971) Radiosensitization with 5-bromodeoxyuridine of Chinese hamster cells X-irradiated during different phases of the cell cycle. Radiat Res 47: 672–688
23. Teicher BA, Holden SA, Ara G, Liu J-TC, Robinson MF, Flodgren P, Dupuis N, Northey D (1993) Cyclooxygenase inhibitors: in vitro and in vivo effects on antitumor alkylating agents in the EMT-6 murine mammary carcinoma. Int J Oncol 2: 145–153
24. Teicher BA, Korbut TT, Menon K, Holden SA, Ara G (1994) Cyclooxygenase and lipoxygenase inhibitors as modulators of cancer therapies. Cancer Chemother Pharmacol 33: 515–522
25. Hendricks C, Rowinsky E, Grochow L, Donehower R, Kaufman S (1992) Effect of P-glycoprotein expression on the accumulation and cytotoxicity of topotecan (SKF 104864), a new camptothecin analogue. Cancer Res 52: 2268–2278
26. Chan V, Ng S-W, Eder J, Schnipper L (1993) Molecular cloning and identification of a point mutation in the topoisomerase IIc DNA from an etoposide resistant Chinese hamster ovary cell line. J Biol Chem 268: 2160–2165

27. Andoh T, Ishii K, Suzuki Y, Kusunoki Y, Takemoto Y, Okada K (1987) Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. Proc Natl Acad Sci USA 84: 5565–5569
28. Doyle L (1994) Topoisomerase expression in cancer cell lines and clinical samples. Cancer Chemother Pharmacol [Suppl] 34: S32–40
29. Ando M, Eguchi K, Shinkai T (1996) Phase I study of sequentially administered CPT-11 and VP-16 for metastatic non-small cell lung cancer. Proc Am Soc Clin Oncol 15: 480
30. Goto K, Nishiwaki Y, Saijo T (1995) A phase II study of irinotecan (CPT-11) and etoposide (VP-16) for metastatic non-small cell lung cancer (NSCLC): Japanese Clinical Oncology Group (JCOG) trial. Proc Am Soc Clin Oncolol 14: 362
31. Beidler A, Cheng Y-C (1995) Camptothecin induction of a time- and concentration-dependent decrease of topoisomerase I and its implication in camptothecin activity. Mol Pharmacol 47: 907–914
32. Fu Q, Grill S, Chen H, Cheng Y-C (1997) The role of proteases in camptothecin-induced topoisomerase I downregulation (abstract). Proc Am Assoc Cancer Res 38: 15
33. Bissery M, Vrignaud P, Lavelle F, Chabot G (1996) Experimental antitumor activity and pharmacokinetics of the camptothecin analog irinotecan (CPT-11) in mice. Anticancer Drugs 7: 437–460
34. Kawato Y, Furuta T, Aonuma M (1991) Antitumor activity of a camptothecin derivative, CPT-11, against human tumor xenografts in nude mice. Cancer Chemother Pharmacol 28: 192–198
35. O'Reilly S, Kennedy M, Rowinsky E, Donehower R (1995) Vinorelbine and the topoisomerase I inhibitors: current and potential roles in breast cancer chemotherapy. Breast Cancer Res Treat 33: 1–17