Anti-Cancer Drugs 1996, 7, pp. 851–857

Camptothecin exhibits selective cytotoxicity towards human breast carcinoma as compared to normal bovine endothelial cells *in vitro*

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We have reported earlier that camptothecin (CPT) incorporated into multilamelar liposomes of appropriate lipid composition displayed effective anti-tumor activity with minimal host toxicity in a nude mouse model xenographed with the human breast carcinoma Clouser nut 1. To investigate this observation further, we have determined the differential effects of CPT on the Clouser tumor cells as well as normal vascular (BVEC) endothelial cells in culture. We report here that Clouser cells are ~200-fold more sensitive to CPT (IC_{50} = 4.0 nM) than the normal endothelial cells (IC_{50} ~ 1 μM) as assayed by MTT; however, CPT demonstrates a potent anti-proliferative activity on both cell lines at low drug concentrations as measured by [^{3}H]thymidine uptake. At higher concentrations (> 25.0 nM), however, the Clouser cells maintained a higher percentage of cells capable of incorporating [^{3}H]thymidine. No significant differences in the levels of topoisomerase I protein and *in vitro* enzymatic activity were seen; although, the Clouser cells showed a 2-fold greater incidence of cleavable complex formation by CPT *in vivo*. Based on the data presented here, we propose that the selective cytotoxic activity of CPT towards tumor cells may be a function of the tumor cells' reduced ability to prevent cleavable complex formation. We also propose that the antitumor effect of CPT may be enhanced *in vivo* by its anti-proliferative effect on vascular endothelial cells which are normally solicited to promote tumor growth.

Key words: Breast cancer, camptothecin, cleavable complexes, DNA topoisomerase I, endothelial cells.

Introduction

Previous work in our laboratory has shown that the plant alkaloid camptothecin (CPT), when incorporated into multilamelar liposomes of appropriate lipid composition, demonstrates excellent anti-tumor activity in a nude mouse model xenographed with the human Clouser nut 1 breast carcinoma.[1] Upon further investigation of this work we now show that CPT exhibits a selective cytotoxic action against the Clouser nut 1 human breast cell carcinoma *in vitro* and appears to only be cytostatic to normal venular endothelial cells (BVEC).

CPT is a lipophilic compound derived from the bark of a Chinese tree,[2] water soluble Na-CPT showed anti-tumor activity in clinical trials published in the early 1970s, but these trials were discontinued because of the toxic side effects.[3–5] It was not until the DNA relaxing protein topoisomerase I (topo I) was shown to be the cellular target of CPT,[6] and that this protein was over-expressed in a number of human tumors,[7] that further interest in CPT was renewed. CPT stabilizes the DNA topo I complex (cleavable complex) formation that occurs during DNA replication.[8,9] The observation that these complexes are rapidly reversed upon removal of CPT[10] and that CPT cytotoxicity is dependent upon DNA replication[11,12] has lead to the idea that collision of DNA replication forks with the cleavable complexes results in DNA strand breaks that are responsible for the cytotoxicity of CPT.[12] It has also been found that CPT has a pH labile lactone ring that is essential for its action against topo I.[13] The conversion of the lactone ring to the inactive carboxylate form at blood pH, therefore, may still be of great concern. Incorporation of the lipophilic parent compound into a suitable delivery system such as liposomes may offer a protective environment for the lactone ring and a greater efficacy in drug action.

In order for an antitumor agent to be effective *in vivo*, it must be able to elicit its cytotoxic effects on tumor cells with minimal toxicity on host cells. Previous work with melanoma and normal melanocytes by Pantazis *et al.*[19] has demonstrated that CPT may act with some selectivity towards tumor cells *in*

Supported by Public Health Service Grant CA67265 from the National Cancer Institute to SSD. This work is part of the doctoral thesis of MC.

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vivo and *in vitro*. Recent work from our laboratory has shown that CPT incorporated into liposomes of appropriate lipid composition is highly effective in the treatment of nude mice xenographed with the human Clouser nut 1 breast carcinoma cells with minimal toxic side effects.[1] To further determine the selective nature of CPT, here we characterize the differential activity of this compound on both Clouser breast carcinoma cells and normal venular endothelial cells *in vitro*. By determining the mechanisms involved in these two different cellular compartments, we may be able to further exploit the differences between normal and malignant tissues with respect to their response to drug action.

Materials and methods

Chemicals and supplies

CPT (NSC 94600) was obtained from the Drug Development Branch, National Cancer Institute, NIH (Bethesda, MD) dissolved in dimethyl sulfoxide (DMSO) at 4 mM aliquots and stored at −70°C. Further dilutions were made in culture medium just prior to use. The final concentration of DMSO in culture did not exceed 0.1% (v/v) which is not toxic to cells. Cholesterol and thiazoyl blue (MTT) were purchased from Sigma (St Louis, MO). Dipalmitoylphosphatidylcholine (DPPC), phosphatidylinositol (PI) and sphingomyelin were obtained from Avanti Polar Lipids (Birmingham, AL). [Methyl-^{3}H]thymidine (6.7 Ci/mmol) was purchased from DuPont/NEN (Boston, MA). Monoclonal antibody C-21, which recognizes DNA topo I on immunoblots, was provided by Dr Y-C Cheng of Yale University (New Haven, CT). All other chemicals were reagent grade.

Cells and culture conditions

The Clouser nut 1 human breast carcinoma cells (provided by Dr BC Giovanella of the Stahelin Foundation for Cancer Research, Houston, TX) were grown in RPMI 1640 media (Gibco, Grand Island, NY) with 10% bovine calf serum (Hyclone, Logan, UT) in 5% CO_2 at 37°C. The bovine venular endothelial cell lines (provided by Dr S Wasi, Ontario, Canada) were grown in DMEM (Gibco) with high glucose, L-glutamine, 1 mM Na-pyruvate and 20% bovine calf serum (Hyclone) under 5% CO_2 at 37°C.

Antiproliferation assays

Antiproliferative activity of CPT was measured by the inhibition of [^{3}H]thymidine uptake into cellular DNA as described earlier.[28] Briefly, subconfluent monolayers were treated with various concentrations of CPT for 24 h, then rinsed with warm PBS and radiolabeled with 1.0 μCi/ml [^{3}H]thymidine for 4 h. The media was removed and the cells fixed with acetic acid:methanol (1:3 v/v). Cells were then rinsed twice with 80% methanol and finally digested for 1 h with 0.1 M NaOH and the radioactivity was measured in a TRI-CARB 2500TR liquid scintillation counter. Results were expressed as percent of untreated control and done in quadruplicate.

Liposome preparation

Multilamelar liposome-incorporated CPT (LCPT) was prepared as previously described.[1,29] The preparation used in the present study was comprised of DPPC:spingomyelin:CHOL:PI:CPT at a molar ratio of 2.4:6.6:1.0:0.05:1.0. The incorporation efficiency was 80% using an HPLC assay for measuring CPT concentration. The multilamelar vesicles were sterilized by passage through a 0.22 μm filter.

Cytotoxicity assay

The cytotoxicity of CPT and LCPT was evaluated by the MTT colorimetric method on quadruplicate samples following exposure to various concentrations of CPT, LCPT or control vehicle for 24 h, and incubation for two to three doubling times in drug-free medium as described previously.[30] The results are expressed as percent of control OD at 540 nm.

Isolation of nuclear extracts

The preparation of nuclear extracts containing topo I enzyme was accomplished according to a protocol supplied by TopoGEN (Columbus OH). Briefly, Cells from two 100 mm dishes were scraped in the media and centrifuged at 800 *g* for 3 min at 4°C. The cells were then resuspended in 5 ml of ice cold TEMP buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 4 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged again. The cell pellet was resuspended in 3 ml of ice cold TEMP, followed by 10 min incubation on ice, then dounced for six to eight strokes in a tight fitting homogenizer followed by

two centrifugation steps at 1500 *g* for 10 min in the cold to pellet the nuclei. The nuclear pellet was then resuspended in 1 ml of cold TEMP and centrifuged again. The pellet was then suspended in no more than four pellet volumes of cold TEP buffer (same as TEMP but lacking $MgCl_2$) and an equal volume of 1 M NaCl. The samples were then vortexed briefly and left on ice for 30 min followed by ultracentrifugation at 100 000 *g* for 1 h at 4°C. The supernatant was then collected and up to 20% glycerol was added followed by storage of the samples at −70°C.

Western blot analysis

Nuclear protein (20 μg) was separated by SDS PAGE using an 8% gel then electroblotted onto a nitrocellulose membrane. The membrane was then blocked in 5% milk for 1 h followed by 1 h incubation with the mouse monoclonal antibody C-21. The washed blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse antibody at room temperature. The membrane was then washed three times in PBS containing 0.1% (v/v) Tween 20. The membrane was then visualized using ECL chemiluminescence (Amersham, Arlington Heights, IL) according to manufactures instructions.

Enzyme activity assay

Topo I activity was assayed using a kit supplied by TopoGEN and accomplished according to the manufactures instructions. Briefly, 1 μl of nuclear extract (of varying protein concentrations) was incubated for 30 min at 37°C with the supplied buffer (10 mM Tris–HCl, pH 7.9, 1 mM EDTA, 0.15 mM NaCl, 0.1% BSA, 0.1 mM spermidine and 5% glycerol) and supercoiled plasmid DNA (pHOT1). The reactions were then terminated by the addition of 5 μl stop buffer (5% sarkosyl, 0.125% bromophenol blue and 25% glycerol). The resulting supercoiled and relaxed topoisomers were separated on a 1% agarose gel at 70 mV for 3 h followed by staining with ethidium bromide. The gels were visualized and photographed under UV illumination using Polaroid 667 positive/negative film.

SDS–KCl co-precipitation

The SDS–KCl co-precipitation assay was performed according to Lui *et al.*[6] Cells were plated into six-well plates at a density of 1×10^5 cells/well and incubated overnight at 37°C. The cells were then labeled with 0.5 μCi/ml [^{3}H]thymidine for 24 h, washed and chased with unlabeled media for an additional 24 h. The cells were then treated with the appropriate doses for 1 h followed by washing three times with cold solution A (0.4 g glucose, 8 g NaCl, 0.35 g $NaHCO_3$ and 1 mM Na_2EDTA per liter, pH 7.4). The samples were then lysed in 1.2 ml of lysis buffer (1% SDS, 5 mM EDTA, 0.4 mg/ml salmon sperm DNA), pipetted five times through a 22-gauge needle and heated to 65°C for 15 min. A 50 μl aliquot of the lysate was taken to determine the activity of the whole sample. Approximately 250 μl of a 650 mM KCl solution was then added to 1 ml of the sample, vortexed for 5 s and then placed on ice for 10 min. The samples were then centrifuged for 10 min at 1000 *g*, resuspended in wash buffer (10 mM Tris HCl, 200 mM KCl, 1 mM Na_2EDTA, 0.01 mg/ml salmon sperm DNA) and heated to 60°C for 10 min. The samples were then placed on ice for 10 min followed by centrifugation and washing two times. The obtained pellet was dissolved in 500 μl of water at 65°C, placed in 10 ml scintillant and counted using a TRI-CARB 2500TR liquid scintillation counter. The data was expressed as fraction of total lysate, treated/control.

Results

Cytotoxic and cytostatic effects of CPT and LCPT

The cytotoxic effect of CPT on both Clouser and BVEC was determined using an MTT assay. Survival curves for the BVEC and Clouser cells following treatment with CPT and LCPT (1–1000 nM) for 24 h are shown in Figure 1. The Clouser cells showed a 200-fold greater sensitivity to CPT than the BVEC with IC_{50}'s of 4 and 1000 nM, respectively (Figure 1). However, the LCPT showed slightly less cytotoxic activity with IC_{50}'s of 10 nM for Clouser and greater than 1 μM for BVEC. In this study cytotoxicity assays measure the relative number of cells that survive treatment and therefore may not completely distinguish between cell death and growth inhibition. Because the BVEC numbers do not decrease below the original plating density prior to treatment, it is possible that CPT and LCPT are not killing the cells but only inhibiting their proliferation. The Clouser cells, however, show nearly total cell death at 100 nM treatment with either CPT or LCPT. The observed ability of CPT to inhibit the uptake of

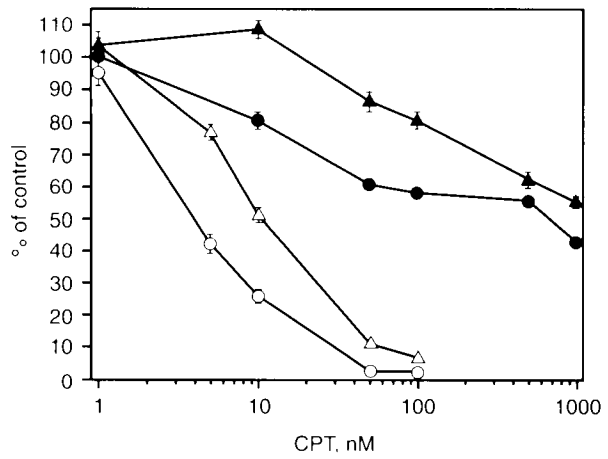

Figure 1. Dose response curves of Clouser nut 1 (open symbols)and BVEC (filled symbols) following 24 h treatment with either CPT (circles) or LCPT (triangles), as determined by thiazoyl blue assay. Points are means of quadruplicate determinations ± SE.

[^{3}H]thymidine was similar in both cell types showing a 50% inhibition at 5–8 nM, as indicated in Figure 2. Although the effect of CPT on [^{3}H]thymidine uptake was similar, additional differences were observed. At the lower doses (3–6 nM) [^{3}H]thymidine uptake was inhibited to a greater extent in the Clouser cells than in the BVEC. This was found to be reversed at higher doses (12–50 nM). This could be supportive of the idea that the CPT effect seen in the cytotoxicity assays on the BVEC could be due to an anti-proliferative activity rather than a cytotoxic effect.

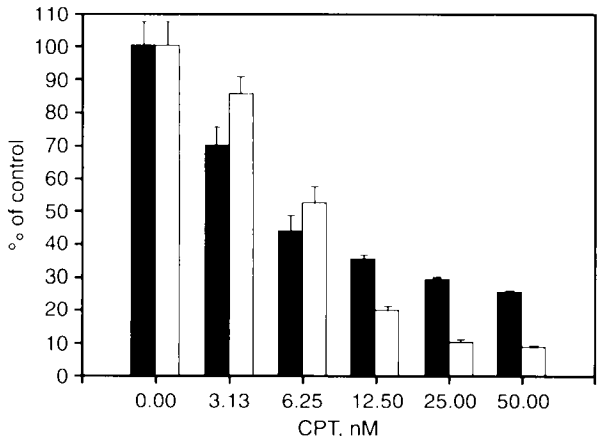

Figure 2. Inhibition of cell proliferation in Clouser nut 1 (filled columns) and BVEC (open columns) following CPT treatment. Cells (1×10^4) were seeded in 24-well plates and incubated in appropriate media containing serum in the absence or presence of CPT (3.13–50 nM) for 24 h. Incorporation of [^{3}H]thymidine was assayed as described in Materials and methods. Points are means of quadruplicate determinations ± SE.

Determination of topo I content and enzymatic activity

In order to determine if the differences in sensitivity between the Clouser and BVEC were due to increased topo I levels or increased topo I enzyme activity, both immunoblot analysis and plasmid relaxation assays were performed. Immunoblot analysis with a mouse monoclonal topo I antibody C-21 showed no detectable difference in the amount of topo I for each cell type as shown in Fig. 3. Pretreatment of cells with 50 nM CPT also had no effect on the relative levels of topo I. The level of topo I enzyme activity was also found to be similar between the two cell types as indicated in Figure 4, with nearly complete plasmid relaxation at 50 ng nuclear protein for each cell type. As with the case in the western analysis, pre-treatment with 50 nM CPT also had no effect on the topo I enzyme activity.

Quantitation of cleavable complex formation

Because there were no significant differences in the amount topo I protein or apparent enzymatic activity of topo I, the relative amount of cleavable complex formation in the two cell types was determined. Cleavable complex formation in the Clouser tumor cells was 2-fold greater than that observed in the BVEC, indicating an increase in DNA

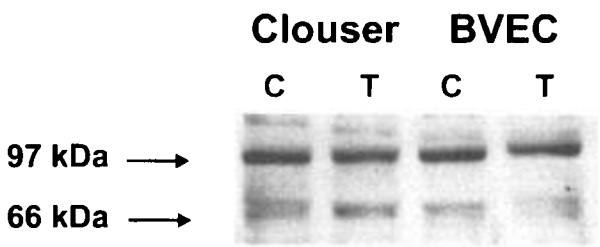

Figure 3. Topo I western blotting of nuclear extracts prepared from Clouser nut 1 and BVEC. Nuclear protein (20 μg) was subjected to 8% SDS PAGE, blotted onto nitrocellulose membranes and probed with a mouse monoclonal anti-topo I antibody as described in Materials and methods. Detection was by enhanced chemoluminescence. Lanes 1 and 2, Clouser and 50 nM CPT pretreated Clouser, respectively. Lanes 3 and 4, BVEC and 50 nM CPT pretreated BVEC, respectively.

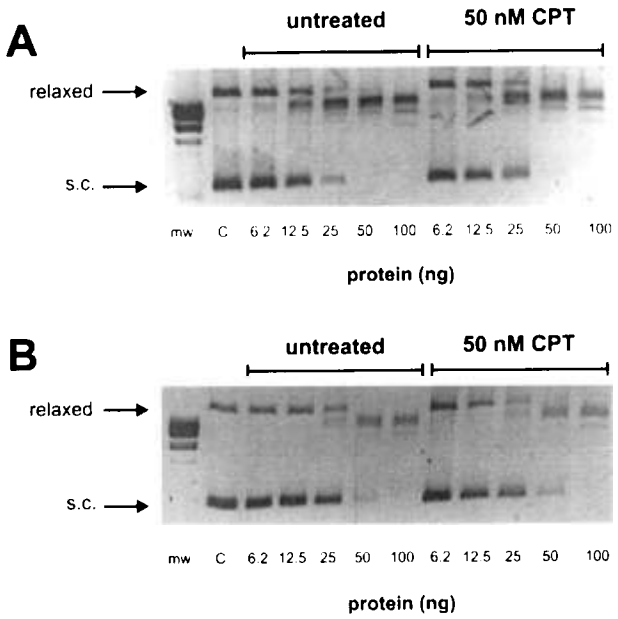

Figure 4. Measurement of topo I catalytic activity in nuclear extracts of Clouser (A) and BVEC (B) cells. The indicated amounts of nuclear protein from both untreated and 50 nM CPT pretreated cells were analyzed for DNA relaxing activity as described in Materials and methods. Increased topo I activity is seen as a loss of the supercoiled substrate DNA (s.c.) to the relaxed DNA with increasing amounts of nuclear extract.

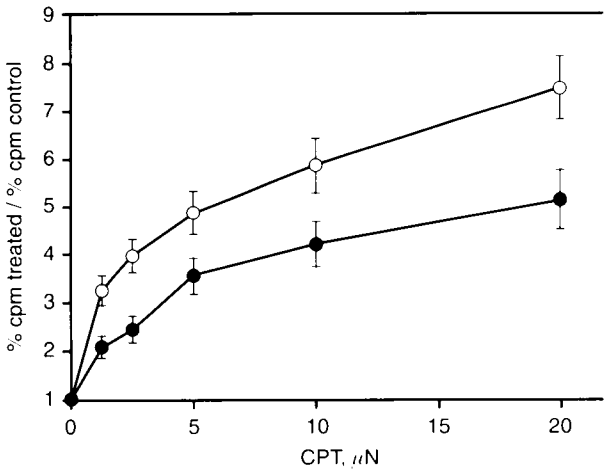

Figure 5. Cleavable complex formation by CPT in Clouser (open circles) and BVEC (filled circles) cells. Cells with [^{3}H]thymidine labeled DNA were treated with various doses of CPT for 1 h followed by precipitation of cleavable complexes as described in Materials and methods. Data are presented as percent counts precipitated in treated samples divided by percent counts precipitated in control samples, where a higher number indicates greater cleavable complex formation. Points are means of quadruplicate determinations ± SE.

damage induced by CPT (Figure 5). The difference in cleavable complex formation between cell types was similar at all of the drug concentrations used (1.25–20 μM). This data provides a good correlation with the cytotoxicity data and a potential mechanism for the selectivity of CPT observed.

Discussion

Previous work in our laboratory has shown that CPT can be effectively incorporated into liposomes and that this delivery system was highly effective in the treatment of a nude mouse model xenographed with the human breast carcinoma Clouser nut 1.[1] Because the success of the CPT treatment in the previous work was seen without any obvious toxic side effects, we further investigated the apparent selective action of CPT by characterizing its effect on both the malignant cells (Clouser) and non-malignant cells (BVEC) *in vitro*. We show in this work that the Clouser cells are much more susceptible to the cytotoxic activity of CPT than the BVEC; however, CPT elicits a very potent anti-proliferative activity on both the Clouser and BVEC cell types at non-cytotoxic levels. This difference in sensitivity could not be explained by topo I protein levels or *in vitro* enzymatic activity, which was the same in both cell types. It was found, however, that the Clouser cells developed a greater number of cleavable complexes compared to the BVEC. This suggests that the normal cells are capable of eliciting some compensatory action that provides a level of protection against the action of CPT. Indeed some evidence exists to show that normal cells are capable of cellular G_2 arrest upon treatment with higher doses of CPT.[21] Malignant cells, however, showed an arrest in S phase. The G_2 pause in the cell cycle may allow for repair of damaged DNA thus preventing the cytotoxicity of CPT.

In order for drug treatment to be effective in any malignant disease, the agents used must demonstrate a certain level of selectivity. The greater the selectivity towards the tumor tissue, the greater the therapeutic index for the anti-neoplastic drugs. Because anti-cancer drugs currently being used in the clinic lack this selectivity, treatment of malignancies has been relatively ineffective. In the case of CPT, however, a clear selectivity is seen in the malignant Clouser cells as compared to the normal BVEC cells with respect to the growth inhibition assays. The [^{3}H]thymidine uptake data showed that CPT can inhibit DNA synthesis in both cell types at

very low concentrations. When the concentration is increased, CPT becomes cytotoxic to the Clouser cells; however, the relative number of BVEC never drops below the number observed before treatment. This would indicate that CPT may only be cytostatic to the BVEC cells rather than cytotoxic. Because tumor growth and development is dependent upon normal endothelial growth to provide a vasculature system, an agent that can both kill tumor cells as well as cause a non-cytotoxic inhibition of endothelial cell growth may prove to be very effective in the treatment of human malignancies.

Previous work has shown that CPT analogs have good antitumor activity against some gastrointestinal cancers[5] as well as colon carcinomas.[20,21] It was also observed that topo I was over-expressed in many advanced malignancies as compared to normal tissues,[7-22] suggesting a possible explanation of the observed selectivity of CPT. However, in cells tested here there were no significant differences in the amount of topo I protein. It was also shown that treatment of these cells with CPT did not significantly affect topo I levels. Since topo I is an enzyme and the activity of this enzyme is required for the cytotoxic activity of CPT, the amount of enzyme activity would be of more importance here than the protein levels. It is possible that post-translational modifications of this enzyme could alter its activity *in vitro*. Previous work by Pommier *et al.*[23] showed phosphorylation of topo I to be required for the enzyme activity and the cleavable complex formation. The relative *in vitro* enzyme activity of the nuclear extracts from Clouser and BVEC cells, however, was not different. This suggests that no modifications have occurred that alter the overall relaxing activity of the enzyme. Pre-treatment with CPT also failed to initiate any changes in the activity of topo I, removing the possibility that the topo I in the BVEC was being inactivated. Despite the relatively equal levels of topo I protein and enzyme activity, the levels of cleavable complex formation was significantly different for the two cell types. Recent work by our laboratory has shown that nuclear extracts obtained from BVEC and BLEC (normal bovine lymphatic endothelial cells) are less susceptible to CPT induced stabilization of the cleavable complex *in vitro*[24] when compared to several malignant breast cell lines.

Correlation between cleavable complex formation and cytotoxicity has been demonstrated for several tumor cell types.[25] A mechanistic explanation for the differences in the cleavable complexes, however, has yet to be produced. Although the cleavable complex formation data correlate with the cytotoxicity data, a 2-fold increase in Clouser cells may not be sufficient to account for the drastic differences in cytotoxicity. Since CPT is cytotoxic to Clouser cells and appears to only be cytostatic to BVEC, it would appear that differences in the ability of these cells to regulate their cell cycle may play a key role in their response to CPT. It has been shown that CPT-induced DNA damage can up-regulate the tumor suppressor protein p53 in wild-type p53 competent cells.[26] The p53 status of the Clouser cells has not been determined; however, p53 mutations are very common in almost all types of tumors.[27] The p53 protein in the BVEC would be normal and assumed to function in a normal cell cycle checkpoint pathway. It is possible that mutations in p53 or other cell cycle regulatory proteins may play a significant role in the cells response to CPT treatment. Further genetic analysis of the Clouser tumor cells and BVEC will be necessary to determine additional mechanisms responsible for the sensitivity of both cell types to CPT.

In summary, our data showed CPT has selective cytotoxic activity towards the Clouser nut 1 human breast carcinoma as compared to normal bovine venular endothelial cells *in vitro*. This selectivity could not be explained by the levels of topo I protein or enzyme activity which were not different in the two cell types; however, the cytotoxicity data does correlate well with the cleavable complex formation which showed a 2-fold greater incidence in the Clouser cells. Our data also suggest that CPT is cytostatic to both Clouser and BVEC at concentrations far below the IC_{50} for the BVEC.

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(Received 13 August 1996; accepted 5 September 1996)