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ORIGINAL ARTICLE

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Thrombospondin-1 plus irinotecan: a novel antiangiogenic-chemotherapeutic combination that inhibits the growth of advanced human colon tumor xenografts in mice

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Abstract Chemotherapy for the treatment of advanced or metastatic colon cancer, utilizing agents such as 5-fluorouracil (5-FU) and irinotecan (CPT-11), produce a 5-year survival of about 10%. Thus, the identification of new, effective, therapeutic regimens to treat this disease remains critically important. To this end, selected antiangiogenic agents, compounds that inhibit neovascularization, have been shown to produce a modest tumor growth-inhibitory effect with little systemic toxicity. Thus these agents are attractive candidates for use with conventional chemotherapeutic agents to treat this disease. To evaluate this approach, experiments were undertaken to assess the cytotoxic and antineoplastic activity of CPT-11 and the antiangiogenic agent thrombospondin-1 (TSP-1) in the HT-29 model of human colon cancer. These agents were chosen since CPT-11 is a camptothecin analogue efficacious in the treatment of colon cancer and TSP-1 is a human glycoprotein that possess antiangiogenic activity. As expected, in vitro studies revealed that a 5-day exposure to TSP-1 at concentrations up to 130 μg/ml was not cytotoxic alone and did not affect the cytotoxicity of CPT-11, or of its active metabolite SN38, in HT-29 cells. Similarly, in human umbilical vein endothelial cells, TSP-1 alone induced only a slight cell growth-inhibitory effect and did not significantly increase the cytotoxicity of either CPT-11 or SN38. The antineoplastic activities of TSP-1 and CPT-11 were assessed in athymic (nude) female mice bearing advanced subcutaneous xenografts of HT-29 cells. Mice received TSP-1 alone (5–40 mg/kg per day) intraperitoneally (i.p.), CPT-11 alone (100–300 mg/kg, i.p.), TSP-1 (10 mg/kg per day) plus CPT-11 (125 mg/kg), or TSP-1 (20 mg/kg per day) plus CPT-11 (150 mg/kg). TSP-1 was injected daily (Monday through Friday) for 4 weeks (20 injections in total) whereas CPT-11 was administered once weekly on days 0, 7, 14 and 21. By day 28, treatment with TSP-1 alone (5, 10 or 20 mg/kg per day) induced a dose-dependent inhibition of xenograft growth. Further, treatment with 10 or 20 mg/kg per day resulted in an average treated tumor size/control tumor size (T/C) on day 28 of 0.68 (range 0.64–0.71) or 0.58 (range 0.54–0.60), respectively. CPT-11 at all doses significantly inhibited tumor growth with an average T/C value of 0.21 (range 0.15–0.27). However, the 250 and 300 mg/kg regimens induced significant toxicity and mortality. When TSP-1 was combined with CPT-11, a significant ($P \leq 0.05$) inhibition of tumor growth also was observed (T/C ≤ 0.17, range 0.11–0.20). Importantly, this enhanced tumor growth inhibition was obtained without significant toxicity. The therapeutic implications of these findings are discussed.

Keywords Thombospondin-1 · Irinotecan · Colon tumor · Xenografts

Abbreviations *bFGF* Basic fibroblast growth factor · *CPT-11* Irinotecan · *5-FU* 5-Fluorouracil · *HUVEC* Human umbilical vein endothelial cells · *i.p.* Intraperitoneally · *SN38* 7-Ethyl-10-hydroxy camptothecin · *T/C* Treated tumor size/control tumor size · *TSP-1* Thrombospondin-1 · *VEGF* Vascular endothelial growth factor

Introduction

Colorectal cancer ranks third among all cancers in frequency and, in the United States alone, it is estimated that nearly 150,000 new cases were diagnosed in 2002. Over 55,000 Americans die each year from this disease [7, 41]. When diagnosed at an early stage, the 5-year survival rate for patients with colon cancer is 90%. However, fewer than 10% of patients with advanced or

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metastatic disease survive over this same period. Combination chemotherapy is the treatment of choice for advanced disease. Response rates of up to 50% have been observed in patients with metastatic colon cancer following treatment with selected combinations of 5-FU, CPT-11 and oxaliplatin [10, 15, 17, 39, 45]. Of these, the camptothecin analogue CPT-11 holds significant promise for continued clinical development. In vivo, CPT-11 is converted to the active metabolite SN38 that inhibits topoisomerase I, an enzyme that is required for the relaxation of DNA during replication, transcription, and repair [20, 36]. CPT-11 possesses activity against a variety of in vitro and in vivo tumor models, and in the clinical setting [27, 35].

Many synthetic and natural compounds with antiangiogenic properties have been identified and some have shown encouraging results in preclinical studies and clinical trials [13, 14, 24, 26, 33, 38]. Thrombospondin-1 (TSP-1), a 450-kDa human glycoprotein, is an antiangiogenic agent that regulates cell-cell and cell-substratum interactions. TSP-1 is synthesized by certain cells and platelets and, under defined conditions, can inhibit endothelial cell growth and adhesion on a fibronectin matrix [16, 21, 25]. In addition to these activities, the intact TSP-1 molecule, as well as specific peptide fragments, are able to induce apoptosis in endothelial cells [18, 25]. In preclinical studies TSP-1 alone has been shown to induce a modest tumor growth-inhibitory effect in mice bearing xenografts of human melanoma or squamous cell carcinoma [22, 40, 46]. These findings led us to hypothesize that antineoplastic regimens possessing significant activity could be developed by combining CPT-11 with the antiangiogenic agent TSP-1.

The present study was undertaken to assess the in vitro and in vivo tumor cell growth-inhibitory activity of TSP-1 plus CPT-11 in the clinically relevant HT-29 model of advanced human colon cancer. We now report that CPT-11 alone and SN38 alone inhibited both tumor cell and HUVEC growth. As a single agent, CPT-11 also effectively inhibited the growth of HT-29 xenografts in athymic (nude) mice, but this activity was limited by toxicity at higher doses. TSP-1 alone produced little cell growth inhibition in vitro and a modest xenograft inhibitory effect in vivo. Exposing HUVEC or HT-29 cells to either TSP-1 plus CPT-11 or TSP-1 plus SN38 did not induce an effect greater than that produced by either CPT-11 or SN38 alone. In contrast, combining TSP-1 with CPT-11 produced a dramatic and significant inhibition of xenograft growth without a concomitant increase in toxicity. The clinical implications of these findings are discussed and a preliminary report has appeared [1].

Materials and methods

Drugs

Purified recombinant human TSP-1 was generously provided by Protein Science Corporation (Meriden, Ct.) as a solution (4.6 mg/ml) in sterile saline. CPT-11 was purchased from Rhone-Poulenc Rorer (Milan, Italy), and SN38 was obtained from LKT Laboratories (St. Paul, Minn.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Immediately before use, TSP-1 and CPT-11 were diluted in saline to achieve the desired working concentrations.

Cell lines

The human colon adenocarcinoma cell line HT-29, obtained from the American Type Culture Collection (ATCC, Md.), was used in both in vitro and in vivo studies. Cells were cultured in sterile tissue culture flasks (Falcon Plastics, Oxnard, Calif.) as monolayers in RPMI 1640 medium (GIBCO, Life Technologies, N.Y.) supplemented with 10% fetal bovine serum (FBS, GIBCO) and were passed twice weekly. Cell cultures were maintained in a humidified incubator at 37°C in an atmosphere containing 5% CO_2. Under these conditions the doubling time was 18–24 h and cells in logarithmic growth were used in all studies. The ability of TSP-1, CTP-11, or CPT-11 plus TSP-1 to inhibit HUVEC (ATCC) proliferation was also evaluated. HUVEC were cultured in tissue culture flasks as monolayers in endothelial cell growth medium (ECGM, Clonetics, San Diego, Calif.) containing 2% FBS, 10 ng/ml of epidermal growth factor, 4 ng/ml of bFGF, and 5 ng/ml of VEGF. These cultures also were maintained in a humidified incubator at 37°C in an atmosphere containing 5% CO_2. The doubling time of HUVEC was 20–24 h.

Animals

Athymic (nude) female mice (Hsd, athymic nude mice nu/nu; Harlan, Indianapolis, Ind.) at 6–8 weeks of age were used to assess the antineoplastic activity of TSP-1 and CPT-11.

In vitro evaluation of antiproliferative activity

HT-29 cells or HUVEC (2×10^4) were added to each cell of a six-well cell culture plate (Costar, Corning, N.Y.) containing 4 ml of appropriate medium plus serum. Immediately thereafter, CPT-11, TSP-1, SN38, or their selected combinations were added to achieve concentrations of 0.25–2 μM for CPT-11, 0.1–10.0 nM for SN38, or 1–130 μg/ml for TSP-1. After 5 days, cells were trypsinized and cell numbers determined electronically (Coulter Model ZM; Coulter Electronics, Miami, Fl.). Cell growth inhibition was assessed as previously described [4, 8, 42]. Experiments were performed in duplicate and repeated at least three times.

In vivo evaluation of antineoplastic activity

To evaluate drug toxicity and therapeutic effectiveness, mice received a subcutaneous injection of 5×10^6 HT-29 cells into the left axillary region. When the resulting xenografts were palpable (7–10 days), tumor weight was estimated by external caliper measurement [4, 9, 42]. Mice then were distributed by tumor weight into groups of five to eight animals. Immediately thereafter, mice were weighed and treatment with TSP-1, CPT-11, or their combination, was started. TSP-1 and CPT-11 were administered by i.p. bolus with the desired dose delivered in a volume of 0.1 ml/10 g body weight. TSP-1 doses evaluated were 5, 10, 20 and 40 mg/kg per day administered on five consecutive days (Monday through Friday) for 4 weeks. CPT-11 was administered at doses of 100, 125, 150, 200, 250 and 300 mg/kg once a week (Monday) for 4 weeks. In addition, two combinations of TSP-1 + CPT-11 were evaluated: TSP-1 (10 mg/kg per day) plus CPT-11 (125 mg/kg) and TSP-1 (20 mg/kg per day) plus CPT-11 (150 mg/kg), with each agent administered as described above. Experiments were repeated three times and results from individual

treatment regimens pooled. During treatment, animals were examined daily and mean tumor weight estimated twice weekly. Animal toxicity was assessed by determination of changes in body weight and mortality. All animal treatment protocols were approved prior to initiation by the Rhode Island Hospital Animal Care and Use Committee (RIH/IACUC).

Statistical analysis

Student's *t*-test was used to compare the tumor growth-inhibitory effects of the treatment regimens and P values ≤ 0.05 were considered significant [4, 9, 12, 42].

Results

In initial experiments, the ability of CPT-11, SN38 and TSP-1, alone or in selected combinations, to inhibit the growth of HT-29 human colon tumor cells or HUVEC was assessed. A 5-day exposure to CPT-11 inhibited the growth of HT-29 cells with an IC_{50} of about 1.1 μM while a similar exposure to SN38 inhibited HT-29 cell growth with an IC_{50} of about 6.5 nM. TSP-1 alone at concentrations ≤ 80 μg/ml, did not inhibit the proliferation of this tumor cell line. Increasing the concentration of TSP-1 to 130 μg/ml (the maximum concentration employed due to solubility limitations) inhibited tumor cell growth by 15%. To assess the antiproliferative interaction between CPT-11 and TSP-1, the effects of TSP-1 (10 to 130 μg/ml) on the growth-inhibitory activity of CPT-11 or SN38 in this tumor cell line were evaluated [6, 11]. These TSP-1 concentrations appeared not to affect the IC_{50} of either CPT-11 or SN38 in this human colon tumor cell line.

In parallel studies, a 5-day exposure to either CPT-11 or SN38 inhibited the growth of HUVEC with IC_{50} values of about 1.3 μM or about 3.5 nM, respectively. TSP-1 alone, at concentrations from 10 to 130 μg/ml inhibited cell growth by 14% to 45%. TSP-1 at concentrations ≤ 40 μg/ml appeared to slightly increase the growth-inhibitory activity of both CPT-11 and SN38 in HUVEC. However, at TSP-1 concentrations over 40 μg/ml this effect diminished and coexposure to TSP-1 did not alter the IC_{50} of either CPT-11 or SN38.

Next the antineoplastic activity of TSP-1 or CPT-11 in mice bearing advanced (about 50-mg) xenografts of HT-29 cells was assessed. After four cycles of TSP-1 at doses of 5, 10 and 20 mg/kg per day tumor size was reduced by 20%, 36% and 43% (P = NS), respectively, compared to controls (Fig. 1). Of interest, at 40 mg/kg per day TSP-1 resulted in a reduced antitumor effect compared to lower doses (Fig. 1). Treatment with TSP-1 alone was well tolerated and produced a maximal weight loss of 5%. Body weight returned to normal by day 28. No toxic deaths were observed (Table 1).

CPT-11 alone was administered at doses ranging from 100 to 300 mg/kg and by day 21, a significant ($P \leq 0.05$) inhibition of tumor growth was apparent in all treated groups. Tumor growth inhibition on day 28 ranged from 67% to 89% (vs controls; Fig. 2). Tumor progression was seen in all treated mice within several days after treatment was discontinued. CPT-11 also induced dose-dependent toxicity in mice. The nadir of

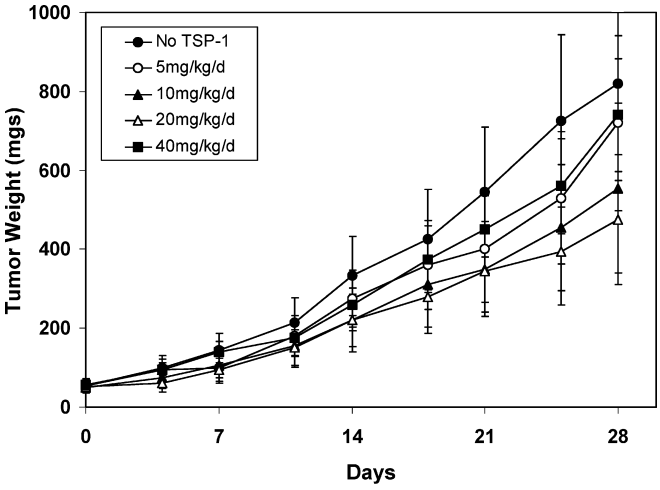

Fig. 1 The effect of various daily doses of TSP-1 on the growth of HT-29 xenografts in athymic (nude) female mice. Mice received a subcutaneous injection of 5×10^6 HT-29 cells into the left axillary region. When the resulting xenografts were palpable, tumor weight was estimated and mice were distributed by tumor weight into groups of five to eight animals. Immediately thereafter, mice were weighed and treatment with TSP-1 alone was started. TSP-1 was administered by i.p. bolus so that the desired dose was delivered in a volume of 0.1 ml/10 g body weight. TSP-1 doses evaluated were 5, 10, 20 and 40 mg/kg with doses administered daily on five consecutive days (Monday through Friday) for 4 weeks. Each experiment was repeated three times and each point represents the mean ± SD of 15–22 values

Table 1 TSP-1- and/or CPT-11-associated toxicity in athymic (nude) female mice. Groups of five to eight mice were treated with four weekly cycles of either TSP-1 alone (five injections per week), CPT-11 alone (one injection per week), or selected combinations of TSP-1 plus CPT-11. All injections were i.p., with doses administered in a volume of 0.1 ml/10 g body weight. TSP-1 doses ranged from 5 to 40 mg/kg per injection and CPT-11 doses ranged from 100–300 mg/kg per injection. During the injection regimen, and daily thereafter for a total of 35 days, mice were weighed and examined. Experiments were repeated a minimum of three times and mortality and weight loss data pooled

Agent	Dose (mg/kg)	n[a]	Weight loss (nadir %)	Mortality (% dead, day 28)
Vehicle control		22	0	0
TSP-1	5	15	−2	0
	10	22	−2	0
	20	22	0	0
	40	19	−5	0
CPT-11	100	15	−2	0
	125	22	−6	0
	150	22	−10	0
	200	17	−18	0
	250	16	−15	37
	300	16	−9	50
TSP-1 + CPT-11	10/125	22	−5	0
TSP-1 + CPT-11	20/150	18	−8	0

[a]Total number of animals treated at that dose

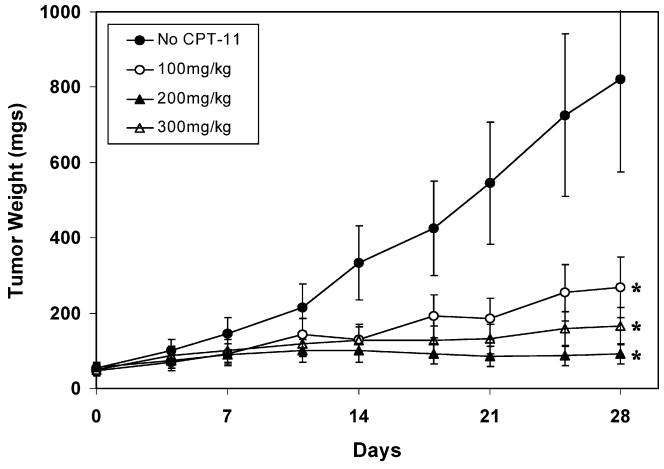

Fig. 2 The effect of various doses of CPT-11 on the growth of HT-29 xenografts in athymic (nude) female mice. Mice received a subcutaneous injection of 5×10^6 HT-29 cells into the left axillary region. When the resulting xenografts were palpable, tumor weight was estimated and mice were distributed by tumor weight into groups of five to eight animals. Immediately thereafter, mice were weighed and treatment with CPT-11 alone was started. CPT-11 was administered by i.p. bolus so that the desired dose was delivered in a volume of 0.1 ml/10 g body weight. Doses evaluated were 100, 125, 150, 200, 250 and 300 mg/kg with doses administered once weekly for 4 weeks. Each experiment was repeated three times and each point represents the mean ± SD of 15–22 values. $*P < 0.05$ vs control (no CPT-11), day 21 through day 28 for the 100 mg/kg dose group, day 14 through day 28 for the 200 mg/kg dose group, day 18 through day 28 for the 300 mg/kg dose group. For clarity, curves representing the xenograft growth-inhibitory effect produced by doses of 125, 150 and 250 mg/kg are not included

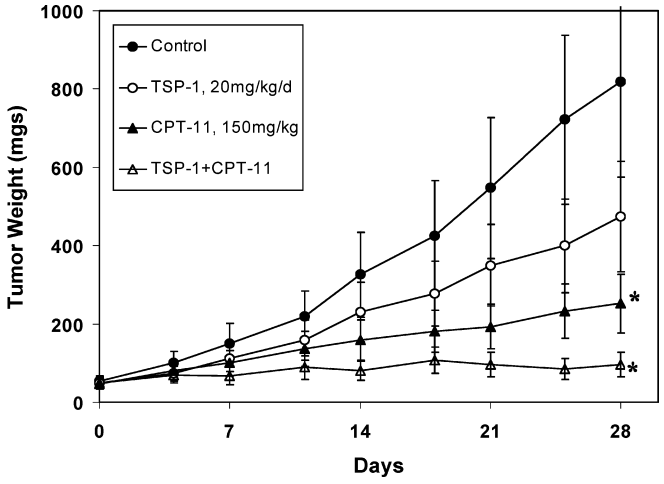

Fig. 3 The effect CPT-11 alone (150 mg/kg), TSP-1 alone (20 mg/kg), or their combination, on the growth of HT-29 xenografts in athymic (nude) female mice. Mice received a subcutaneous injection of 5×10^6 HT-29 cells into the left axillary region. When the resulting xenografts were palpable, tumor weight was estimated and mice were distributed by tumor weight into groups of five to eight animals. Immediately thereafter, mice were weighed and treatment with CPT-11 alone, TSP-1 alone, or their combination, was started. CPT-11 and TSP-1 were administered by i.p. bolus so that the desired dose was delivered in a volume of 0.1 ml/10 g body weight. The TSP-1 dose evaluated was 20 mg/kg administered daily on five consecutive days (Monday through Friday) for 4 weeks. CPT-11 was administered at a dose of 150 mg/kg once weekly for 4 weeks. Each experiment was repeated three times and each point represents the mean ± SD of 18–22 values. $*P \leq 0.05$ vs control, day 25 through day 28 for the CPT-11 alone group, day 14 through day 28 for CPT-11 plus TSP-1 group

body weight loss (Table 1) was observed in the week following the first injection and the mean body weight of mice treated with doses of < 250 mg/kg had returned to pretreatment values by day 28. Mice treated with doses of 250 or 300 mg/kg never completely recovered from CPT-11-associated toxicity and these dosage regimens produced 37% and 50% mortality, respectively (Table 1).

The antineoplastic activity of two combinations of CPT and TSP-1 was next evaluated. Specifically, TSP-1 (10 mg/kg per day) plus CPT-11 (125 mg/kg) and TSP-1 (20 mg/kg per day) plus CPT-11 (150 mg/kg). Both combinations were well tolerated with no toxic deaths. Nadir body weight losses of 5% and 8%, respectively, were observed (Table 1). Importantly, in spite of this low systemic toxicity, a significant tumor growth inhibition was observed with both combinations (Fig. 3). TSP-1 (10 mg/kg per day) plus CPT-11 (125 mg/kg) and TSP-1 (20 mg/kg per day) plus CPT-11 (150 mg/kg) were nearly equally effective and inhibited tumor growth by 84% and 89%, respectively ($P \leq 0.05$ by day 14). In addition, xenograft growth inhibition was improved compared to regimens employing the same dose regimen of CPT-11 alone and approximated the tumor growth inhibition observed in mice treated with CPT-11 alone at doses of 250 and 300 mg/kg.

Discussion

TSP-1 is a natural angiogenesis inhibitor, similar to the better-known proteins angiostatin and endostatin. The latter two molecules have been shown to inhibit the growth of model tumors in vivo, supporting the notion that tumor angiogenesis is an explorable target for cancer therapy [2, 34]. Although the antiangiogenic activity of TSP-1 has been confirmed [40, 46], other studies have shown that TSP-1, under certain conditions, can enhance tumor cell invasion by supporting the formation of microemboli between platelets and tumor cells [43]. However, the plasma concentration of TSP-1 in cancer patients can be tenfold above baseline without accompanying hematological or treatment side effects and without correlation to an aggressive tumor phenotype or a compromised treatment outcome [31, 44]. Thus, exact relationships between plasma TSP-1 concentrations and tumor growth have yet to be defined.

The results of the present study demonstrated that TSP-1 doses of 10 and 20 mg/kg per day produced a modest inhibition of tumor growth without remarkable toxicity in mice. Using the same model we assessed the effect of CPT-11 alone or combined with TSP-1. A dramatic antitumor effect was observed in all CPT-11-treated groups, but significant toxicity was observed at doses ≥250 mg/kg. It is important that a significantly

enhanced antitumor effect was observed after combining CPT-11 with TSP-1 as compared to the same doses of these agents alone, without enhanced toxicity. Consequently, TSP-1 increased the therapeutic index of this clinically important camptothecin analogue. These findings were predicted by our in vitro experiments. Over the range of concentrations evaluated in both HUVEC and HT-29 cells, TSP-1 neither produced a significant cytotoxic effect nor significantly affected the IC_{50} of either CPT-11 or SN38. This suggests that the enhanced antitumor effect seen in vivo resulted from a direct cytotoxic effect of CPT-11 on tumor cells combined with an alternative tumor growth-inhibition mechanism induced by exposure to TSP-1, presumably inhibition of tumor angiogenesis. These results support findings of others showing that an antitumor effect greater than that produced with single agents can be obtained by combining a chemotherapeutic agent with an angiogenesis inhibitor [19, 22].

Still to be determined is the exact mechanism responsible for the reduced tumor growth inhibition observed using the 40 mg/kg per day TSP-1 dosing regimen. Peptide fragments of TSP-1 have been found to exert various effects on cell growth and proliferation. It is intriguing to speculate that at higher concentrations, an activity associated with a TSP-1 fragment, and not apparent at lower doses, could be prevalent [37]. This could also explain the observation that, at TSP-1 concentrations below 40 μg/ml, a positive interaction between CPT-11 and TSP-1 was observed in HUVEC. Alternatively, the reduced tumor growth-inhibitory activity of higher doses of TSP-1 may reflect enhanced tumor growth secondary to TSP-1-induced interactions between tumor cells and platelets, as suggested by Tuszynski et al. [43]. Finally, the reduced activity of higher TSP-1 doses may reflect an effect similar to that recently described for the suppressor of cytokine signaling (SOCS) family of proteins. These proteins are considered negative regulators of cytokine signal transduction [32] and are expressed when cells are exposed to very high concentrations of cytokine [28, 30]. The lack of tumor growth inhibition following treatment with the 40 mg/kg per day regimen could reflect a similar inhibitory mechanism induced by exposure to high concentrations of TSP-1. In any case, while the mechanisms remain unclear, our findings reveal that this endogenous human protein, detected in the circulation at concentrations up to 600 ng/ml [3, 44], possesses antineoplastic activity in this model of chemoresistant human colon cancer.

Studies are planned to evaluate TSP-1 in alternative models of human breast, renal, and bladder cancers. The rationale for these studies derives from the observation that, in patients with these tumors, a poor prognostic factor is tumor vascularization supported by bFGF [5, 23, 29]. Since the effect of bFGF is inhibited by TSP-1, tumor growth inhibition by TSP-1 alone in these models could be more pronounced than that observed in the HT-29 model. Independent of future findings, our present study revealed that combinations of TSP-1 and CPT-11 possess exploitable antineoplastic activity in a model of human colon cancer. Reflecting the fact that CPT-11 is now considered a front-line therapy for advanced or metastatic colon cancer, clinical translation and evaluation of these findings is warranted.

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