

Antiangiogenic Effects of Camptothecin Analogues 9-Amino-20(S)-camptothecin, Topotecan, and CPT-11 Studied in the Mouse Cornea Model¹

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ABSTRACT

Angiogenesis has been correlated with increased invasion and metastases in a variety of human neoplasms. Inadequate inhibition of the growth of tumor microvessels by anticancer agents may result in treatment failure, rated clinically as progressive or stable disease. We have investigated the antiangiogenic properties of three camptothecin analogues, 9-amino-20(S)-camptothecin, topotecan, and camptosar (CPT-11), currently under investigation in clinical settings. Angiogenesis was induced by basic fibroblast growth factor in the cornea of inbred Swiss-Webster mice, with the aim of exploring the suppression of neovascularization by the analogues injected into the mice daily over a period of 6 days. The dose range chosen is known to inhibit, in the mouse model, the growth of various human tumor xenografts or murine tumors. The statistical analysis evaluated the association between the area of neoangiogenesis and the dose of the drugs tested and correlated the effects with observed drug toxicity. It was established that, as the drug doses increased, the area of neovascularization decreased, appearing to approximate a negative exponential curve. 9-Amino-20(S)-camptothecin at 6.89 and 8.26 $\mu\text{mol/kg}$ (2.5 and 3.0 mg/kg) and topotecan at 8.31 $\mu\text{mol/kg}$ (3.5 mg/kg), both drugs being delivered over a 6-day period, had statistically significant reduction (47.2–72.5%) of neoangiogenesis and acceptable toxicity. At higher doses of the two analogues, toxic body-weight losses and deaths were observed.

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CPT-11 showed statistically significant reduction of neoangiogenesis at a dose of 359 $\mu\text{mol/kg}$ (210 mg/kg) delivered over a 6-day course. Unlike camptothecin analogues, the nontoxic dose of vincristine did not induce a statistically significant inhibition of angiogenesis, and there was no dose-dependent escalation of antiangiogenic effects. The results indicate that camptothecins are most likely cytotoxic against two tumor compartments: in addition to tumor cells of epithelial origin, the drugs act against endothelial cells and prevent the growth of the tumor microvessels. We have hypothesized that treatment failure in some patients is due to incomplete or inadequate inhibition of the microvessel growth by camptothecins. Presumably, an intensive inhibition of the remaining tumor microvasculature in such patients could be achieved by combining a camptothecin with another antiangiogenic anticancer agent or with a highly selective angiogenic inhibitor exerting minimal dose-limiting toxicity. Such treatment by a camptothecin plus a less toxic inhibitor of angiogenesis can improve antitumor efficacy. To validate this concept, preclinical studies followed by clinical trials are planned.

INTRODUCTION

Camptothecin analogues 9-AC³ (NSC 603071), TTN (NSC 609699; 9-dimethylaminomethyl-10-hydroxycamptothecin), and CPT-11 {camptosar, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin} belong to a recently established class of anticancer agents (1–3). The camptothecins, compounds with a unique cytotoxic mechanism targeting the cellular enzyme DNA topoisomerase I, have been studied preclinically, and several are currently being studied clinically. 9-AC is currently in Phase I/II multi-institutional trials (3), with antitumor activity seen in patients with advanced epithelial ovarian, breast, small cell lung carcinoma, non-Hodgkin's lymphoma, and leukemia. The RR of ~30% was obtained in a Phase I trial among patients with advanced ovarian cancer failing cisplatin-based regimen and treated with a 21-day CI (4). The results remain to be confirmed in Phase II studies. TTN, another clinically researched camptothecin, was approved as second-line treatment of patients with ovarian cancer that is refractory to a cisplatin/taxol-based regimens (5, 6). The drug efficacy seems comparable to those of several new agents tested in recurrent ovarian cancer, such as gemcitabine, docetaxel, liposomal doxorubicin, or oral etoposide. Clinical research of CPT-11 shows broad antitumor activity under various schedules and dosages (7–9).

³ The abbreviations used are: 9-AC, 9-amino-20(S)-camptothecin; TTN, topotecan; RR, response rate; CI, continuous infusion; CR, complete response; PR, partial response; bFGF, basic fibroblast growth factor; QSAD, quantum satis ad dispensum.

The effectiveness against colorectal cancer was confirmed by clinical trials in three continents, and the drug was recently approved in the United States as second-line treatment of advanced colorectal adenocarcinoma (10). CPT-11 is a prodrug converted by decarboxylation into a biologically active form, SN-38 (7-ethyl-10-hydroxycamptothecin). The process, mediated by ubiquitous carboxylesterases, has been well established in the mouse experimental model (1). The conversion has variable interpatient efficacy and schedule dependency, with the conversion rate corresponding to ~3–15% (7, 8).

Stable disease, as compared with CRs or PRs or with no effects on measurable tumors, is seen in a relatively large number of patients treated with camptothecin analogues (3–7). “Stable disease” designates a treatment response that shows borderline changes in the volume of a solid tumor, short of PR (tumor shrinkage $\leq 50\%$ of the volume) but better than disease progression (growth expansion of the tumor volume by $\geq 25\%$). Such a response may indicate that the tissue cytokinetics of some tumors treated with camptothecins are in a steady state, characterized by the rate of cell division approximating the rate of cell death. Recent reports suggest that stable disease, without any detectable expansion in the tumor mass, can be caused by blocked angiogenesis exerted by antiangiogenic agents (reviewed in Ref. 11). Camptothecins may belong to such a group of agents, which show variable antiangiogenic effects depending on the type of the analogue and type of cancer, as well as on multiple factors including vascular architecture of tumors, as has been discussed by Holmgren *et al.* (12). Should the antiangiogenic effect be weak, however, the inhibition of angiogenesis is incomplete, and no response to treatment or stable disease results. Under the circumstances, the combination of an anticancer drug with an angiostatic agent that has no known toxicity or resistance may eradicate the tumor.

To explore this hypothesis, the model of angiogenesis in the mouse cornea (13, 14) has been used to investigate the potential antiangiogenic effects of camptothecins. The method quantitates bFGF-induced vascularization of the avascular corneal stroma. bFGF, a multifunctional single-chain peptide, is a potent stimulator of angiogenesis with complex effects on cell morphology, transformation, proliferation, and differentiation *in vitro* (12). A Hydron polymer pellet containing bFGF is implanted in the cornea, which induces an ordered series of biochemical changes required for angiogenesis. The induced angiogenesis is accompanied by minimal inflammation, and the method is highly reproducible (14), suitable for the exploration of drugs with potentially antiangiogenic effects (15, 16).

The method of bFGF-induced angiogenesis was used in this report with several research objectives: (a) to explore the potential antiangiogenic effects of 9-AC, TTN, and CPT-11 delivered systemically in daily i.m. injections over a period of 6 days; (b) to quantitate the effectiveness of normalized drug dosages of camptothecins and compare the results with another anticancer compound, vincristine; and (c) to correlate the antiangiogenic effects with drug toxicity.

MATERIALS AND METHODS

Mice. Inbred Swiss-Webster mice, 6–8 weeks old with the body weight of 22–33 g, were purchased from Taconic

Laboratories (Hudson Valley, NY) and maintained under veterinary supervision at the New York University Medical Center Berg Animal Facility, in accordance with the guidelines established by the NIH for the care of laboratory animals.

Drugs. One mg of 9-AC (IDEC Pharmaceuticals Corporation), formulated as a colloidal dispersion, was dissolved in 1 ml of special diluent (20% dextrose, USP; 0.9% NaCl, USP; sterile water for injection QSAD). Any additional dilution was done by adding saline (0.9% NaCl, USP; sterile water for injection QSAD). The drug was tested at levels ranging from 1.38 to 12.4 $\mu\text{mol/kg}$ (0.5–4.5 mg/kg) delivered over 6 days.

For the formulation of TTN (SmithKline Beecham Pharmaceuticals), 2.5 mg were dissolved in 1 ml of sterile water, with additional dilution in saline (0.9% NaCl, USP; sterile water for injection QSAD). Various dosages ranged from 3.56 to 17.8 $\mu\text{mol/kg}$ (1.5–7.5 mg/kg) per 6-day course.

CPT-11 (Pharmacia and Upjohn) was formulated in sterile water QSAD at the concentration of 20 mg per 1 ml. Heating to 90°C, sonication and vortexing were required to dissolve the drug, and additional dilution was achieved with saline or 5% galactose in sterile water for injections QSAD. Increased temperature during drug formulation was without detectable effects on CPT-11 integrity because this was determined by HPLC analysis.⁴ Additional dilution with D5W adjusted the concentration to desired dose levels ranging from 51.3 to 359.0 $\mu\text{mol/kg}$ (30–210 mg) delivered over 6 days.

Vincristine sulfate (Vincasar PFS injections, Pharmacia Inc., Columbus, OH), diluted by sterile water at concentrations resulting in dosages of 1.08–4.88 $\mu\text{mol/kg}$ (1.0–4.5 mg/kg), which were delivered over 6 days. The drug was tested as a control with unproved antiangiogenic activity (15).

Experimental Procedure. Corneal neovascularization was induced in Swiss-Webster mice using a well established procedure (13, 14). Hydron polymer-coated pellets of sucrose aluminum sulfate, containing 100 ng of bFGF each, were placed into corneal pockets prepared by an incision placed 1 mm toward corneal center away from the limbic vessel. In each experiment, mice were randomized into five to six groups, with six to seven mice per group.

Mice received daily i.m. injections of placebo or various concentrations of 9-AC, TTN, or CPT-11. Each course of placebo or drug administration consisted of six injections of 0.1 ml delivered on consecutive days into alternating thighs. Using an identical schedule, we administered vincristine i.p. in some experiments. The body weight of individual mice was taken on day 0 prior to commencing the experiment and again on day 6 when drug administration was completed. The experimental protocol was approved by the Institutional Animal Care and Use Committee.

Evaluation of Responses. On day 6, each cornea of anesthetized mice was evaluated by the same observer using slit-lamp biomicroscopy, and pictures of the cornea were taken in two projections (Fig. 1). The observer was blinded as to the experimental drug and dosage used in the group under scrutiny. With the mouse eye proptosed by a jeweler’s forceps, the

⁴ M. Potmesil, unpublished observations.

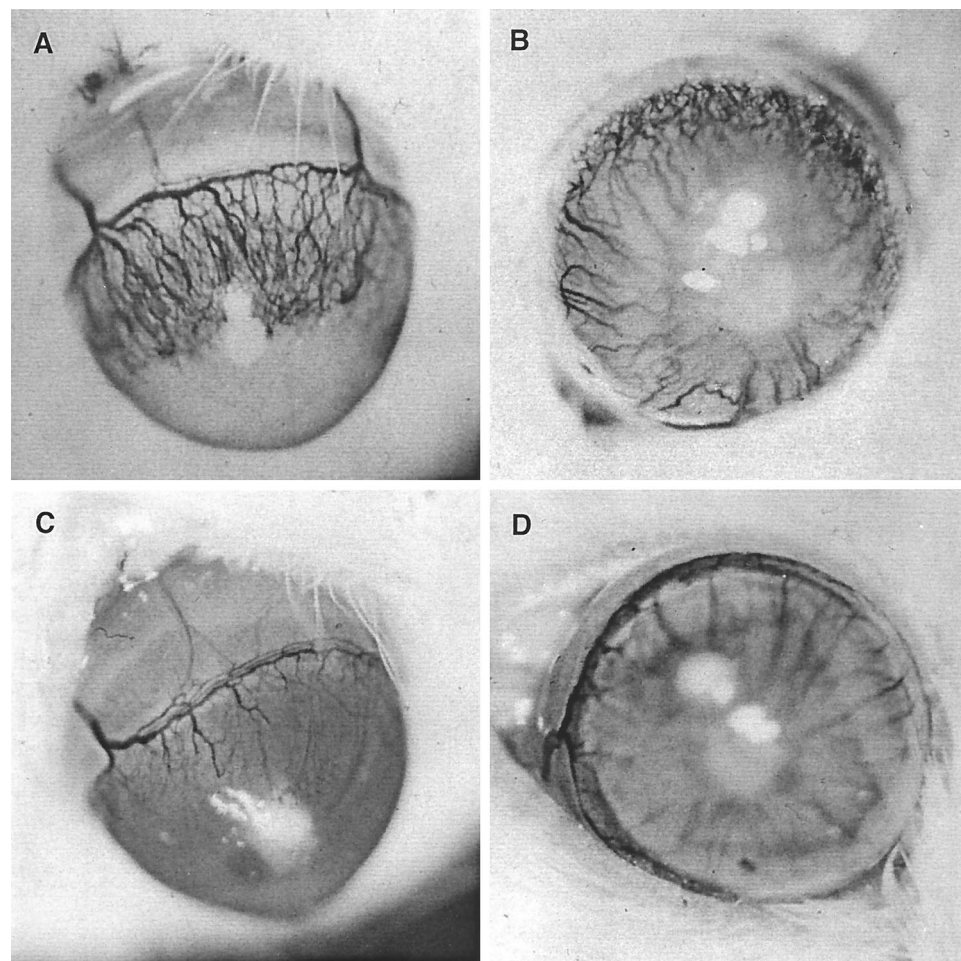


Fig. 1 Photographs of bFGF-induced neovascularization observed in mouse corneas. Lateral (A and C) and front (B and D) views of the corneas: A and B, a control mouse treated with placebo; C and D, mouse treated with 9-AC (9.64 $\mu\text{mol/kg}$ per course). Details of the experiments are described in "Materials and Methods."

maximal vessel length between the limbic vessel and bFGF pellet was measured. Using an *en face* photograph, we evaluated the maximal area of intense neovascularization in clock hours. The angiogenesis was quantitated by calculating the vascular area in mm^2 , using a modified formula for a half-ellipse:

$$\text{Vascular area} = \text{Vessel length (in mm)} \\ \times \text{clock hours} \times 3.14 \times 0.2$$

This formula provides the most accurate approximation of the area of neovascularization between the limbic vessel and the pellet (14). Reduction of neovascularization was calculated for both corneas of individual mice as the decrease in percentage relative to the mean vascular area for placebo-treated control mice.

Statistical Consideration. When multiple comparisons were performed in this study, the Tukey-Kramer procedure⁵ was used to reduce the probability of a type 1 (α) error, caused by evaluating the data more than once. In a similar manner, when

multiple comparisons were evaluated using experimentally derived means compared to a control mean, the procedure of Dunnett (17) was used. When multiple comparisons were not applied, Student's *t* test was performed to detect a difference in means at a conventional level of $P < 0.05$.

RESULTS

Antiangiogenesis of 9-AC. A total of 15 groups of six to seven mice each were injected in three independent experiments over a 6-day period with either placebo or various doses of the drug. There was a dose-dependent reduction in neovascularization ranging from 12.4% for the dose of 1.38 $\mu\text{mol/kg}$ to 92.2% for 12.4 $\mu\text{mol/kg}$ (Fig. 2 and Table 1), with statistically highly significant inhibition of angiogenesis seen for doses ≥ 6.89 $\mu\text{mol/kg}$. Irreversible toxicity, defined as a $>20\%$ reduction in body weight during the time of treatment (18–20)⁴ or deaths of experimental animals were seen in some groups. Significant weight loss of 26% was observed in one of three groups of mice treated with 6.89 $\mu\text{mol/kg}$, and at the two highest doses of 9.64 and 12.4 $\mu\text{mol/kg}$ (Table 1). The two highest doses also caused toxic deaths in a total of 5 of 26 mice.

Antiangiogenesis of TTN. There were two independent experiments and a total of 10 groups with seven mice each

⁵ J. W. Tukey, The problem of multiple comparisons, unpublished manuscript, 1953.

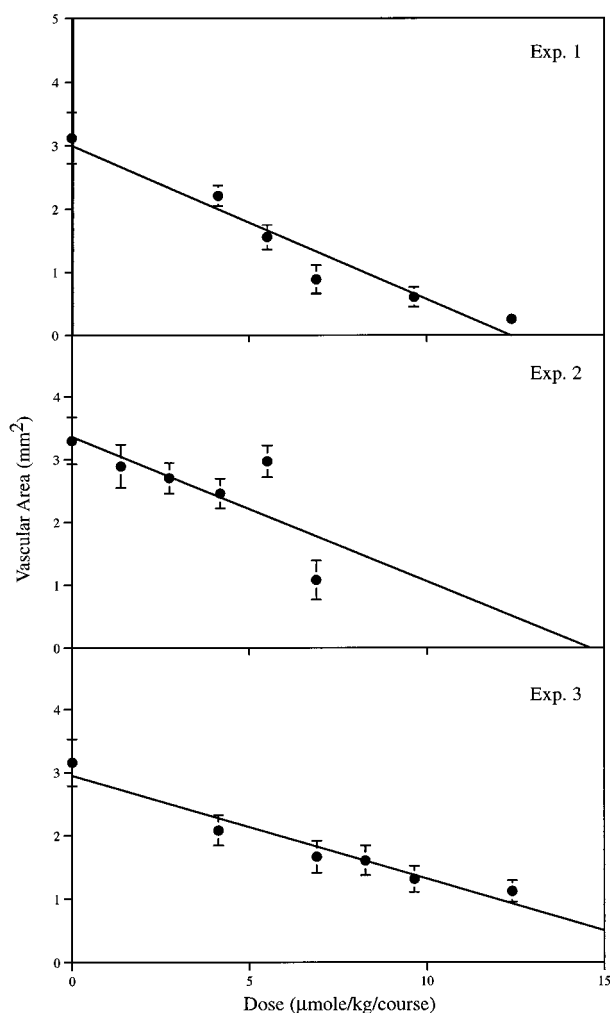


Fig. 2 Exps. 1–3, the effect of various doses of 9-AC on the corneal vascular area, used as a correlate of neoangiogenesis and its inhibition. Data points, means; bars, SE; lines, regression analyses.

injected with placebo or various drug concentrations. The antiangiogenic effects were dose dependent: the area of neovascularization was reduced by 18.6% at the 5.93 $\mu\text{mol/kg}$ dose, and the reduction escalated to 78.8% for the dose of 15.4 $\mu\text{mol/kg}$ (Fig. 3 and Table 1). Toxicity was detected at dose levels of $\geq 10.7 \mu\text{mol/kg}$, with a body weight decrease of $>20\%$ in most of the groups and death of one of seven mice at 17.8 $\mu\text{mol/kg}$.

Antiangiogenesis of CPT-11. This drug was tested in two independent experiments using seven groups of six to seven mice each. Statistically significant reduction of corneal neovascularization to 45.3% was observed at the dose of 359.0 $\mu\text{mol/kg}$ (Fig. 3 and Table 1). There was no drop in the body weight during the time of the experiment, and there were no toxic deaths.

Antiangiogenesis of Vincristine. Vincristine was applied at four dose levels. The nontoxic dose of 1.08 $\mu\text{mol/kg}$ over 6 days (1 mg/kg) did not cause statistically significant reduction of neovascularization. Although higher doses of 2.71 and 4.88 $\mu\text{mol/kg}$ over 6 days (2.5 and 4.5 mg/kg) induced

statistically significant reduction of neovascularization by 49.9 and 51.0%, these drug concentrations were toxic and caused deaths in 7 of 21 mice.

DISCUSSION

In this introductory study, we have attempted to evaluate the potential contribution of camptothecin analogues to the suppression of the angiogenic processes. The dose range of camptothecin analogues used in the study included drug concentrations that, given weekly, have antitumor activity in the mouse model. The pure, optically active 20(S) form of 9-AC, given i.m. at 1.0–2.5 mg/kg/week (2.75–6.89 $\mu\text{mol/kg}$ over 4–6 weeks) induced CR or PR of various human xenografts carried by immunodeficient “nude” mice (18, 19). TTN was injected on various schedules into thymectomized CBA/CAJ mice bearing xenografts of tumor lines derived from childhood rhabdomyosarcoma. In most of the lines, CR and PR were achieved with dosages of 1.5 and 2.0 mg/kg (3.56 and 4.75 μmol) over 5 days, given in repeated courses (20). CPT-11 was used against the L1210 cell line, inoculated into CD2F₁ mice, at total doses of 100 and 200 mg/kg (171.1 and 342.2 $\mu\text{mol/kg}$, respectively) delivered over a span of 9 days. The treatment reached the T/Cs [T/C (%) = median survival days of drug-treated/median survival days of untreated controls \times 100] of 288 and 500% (21). Similarly, CPT-11 at a dose of 50–200 mg/kg (85.5–342.2 $\mu\text{mol/kg}$) per week for 2 weeks induced 12–100% of CR or PR in most xenograft lines of human tumors implanted into thymectomized CBA/CAJ mice (22). Statistical evaluation of dose-response effects shows that, as the dose of injected camptothecins increased, the area of corneal neovascularization decreased, appearing to approximate a negative exponential curve (Figs. 2–4). Using Dunnett’s method of statistical evaluation of the differences between corneal areas of neovascularization in placebo- and drug-treated mice, we found significant differences for intermediate- to high-treatment dosages. The experiments show statistically significant inhibitory effects of 9-AC, TTN, and CPT-11 on the proliferation of the cellular components of capillaries and on the formation of the microvasculature (Table 1). Such effects are consistent with tumor-growth inhibition caused by drug interference with tumor microvessels.

In the mouse, camptothecin analogues apparently differ in the dynamics of toxicities that develop during the treatment. It was shown for 9-AC and TTN that the treated mice recover from a body weight loss of $<20\%$. Higher weight losses result in early deaths, usually during the first week of treatment (18–20).⁴ Both analogues, 9-AC at 6.89–8.26 $\mu\text{mol/kg}$ per course and TTN at 8.31 $\mu\text{mol/kg}$, effectively inhibited angiogenesis at a dose range that has induced acceptable toxicity. Unlike 9-AC or TTN, even the highest doses of CPT-11 applied over 6 days did not cause any significant body weight loss or toxic deaths in our experiments (Table 1). This is consistent with observations in other preclinical studies showing that, except for the dose of 200 mg/kg per week (342.2 $\mu\text{mol/kg}$) that caused 4% of toxic deaths, CPT-11 has little toxicity in terms of early body-weight loss or deaths (21, 22).

The effects of various anticancer drugs on angiogenesis may differ, depending on the method chosen for the screening

Table 1 Antiangiogenic activity of various drugs, significance of differences, and toxicity

Drug	Dose/course ($\mu\text{mol/kg}$)	% of inhibition of angiogenesis ^a	Significance of differences ^b (<i>P</i>)	Weight change (%) ^c	No. of deaths/total no. per group	
9-AC	1.38	12.4	NS	+9.1	0/7	
	2.75	18.2	NS	+2.1	0/7	
	4.13	30.9	0.075	-0.6	0/6	
		25.5	NS	0.0	0/7	
		34.2	0.039	-5.8	0/7	
	5.51	48.8	0.013	-13.0	0/7	
		10.0	NS	-8.5	0/7	
		72.5	<0.001	-26.3	0/6	
	6.89	47.2	0.001	-16.0	0/7	
		67.2	<0.001	-18.9	0/7	
		49.4	0.001	-18.4	0/7	
	8.26	81.3	<0.001	-27.3	0/6	
		58.5	<0.001	-21.3	2/7	
		92.2	<0.001	-40.0	2/6	
	12.4	66.5	<0.001	-20.6	1/7	
39.7		0.013	-7.8	0/7		
18.6		NS	+4.7	0/7		
TTN	3.56	38.4	0.02	-9.8	0/7	
		52.4	<0.001	-12.5	0/7	
	5.93	40.0	0.015	-17.8	0/7	
		42.9	0.009	-26.4	0/7	
	8.31	48.6	0.001	-21.8	0/7	
		78.8	<0.001	-24.5	0/7	
	10.7	61.6	<0.001	-12.5	0/7	
		64.0	<0.001	-22.5	1/7	
	CPT-11	17.8	14.8	NS	+1.7	0/6
			4.5	NS	+1.5	0/7
51.3		11.4	NS	-0.6	0/6	
		8.5	NS	+6.5	0/7	
103.0		46.5	NS	-3.0	0/6	
		25.1	NS	+6.5	0/7	
205.0		42.5	NS	+9.2	0/7	
		45.3	0.027	+12.1	0/7	
Vincristine		1.08	43.5	NS	-8.2	0/7
		2.71	49.9	0.022	-6.1	2/7
	3.79	53.1	NS	-30.0	4/7	
	4.88	51.0	0.014	-11.6	1/7	

^a Inhibition of angiogenesis in cornea of drug-treated mice, relative to angiogenesis of control placebo-treated mice, equal to 100%.

^b Significance calculated using Dunnett's method (see "Materials and Methods"). NS, not significant with $P \geq 0.05$.

^c The difference (in %) between the average body weight on day 0 and day 6 of a treatment course.

(15, 16). A method identical with ours has identified vincristine, at the dose level of 0.2 mg/kg/day (0.22 $\mu\text{mol/kg}$) given over 5 days, as a drug without any effects on angiogenesis (15). In our study, ~5–22-fold higher dose of vincristine showed neoangiogenic suppression of 51% on average. Such dose escalation did not increase significantly antiangiogenesis but resulted in severe toxicity (Table 1).

The precise mechanism of antiangiogenesis exerted by camptothecins remains unclear. The growth and renewal of tumor vasculature is a dynamic process, presumably ongoing in the periphery of tumor nodules. This may generate a well-oxygenated region with mitotic activity and tumor growth. It could be suggested that cytotoxic interaction between a camptothecin and DNA topoisomerase I complex targeting normal proliferating endothelial cells in the capillaries serves as the mechanism limiting the growth of microvessels. Thus, it appears that camptothecin cytotoxicity is targeted not only against the cells of a solid tumor, but also against its endothelial cells, which comprise the tumor capillary network. Vascular renewal and growth inhibition by camptothecins, combined with cyto-

toxic effects against tumor cells, results in growth inhibition and shrinkage of the tumor. Most likely, if the drug delivery is interrupted, the tumor vasculature and tumor mass start to regrow. Daily application of camptothecins, as compared to doses separated by several days, has been used in our experiments. Such frequent or continuous treatment schedule has therapeutic advantages that were observed in preclinical (reviewed in Refs. 1 and 3), as well as in clinical studies (4, 23).

The study of antiangiogenesis, as it relates to various anticancer chemotherapeutic agents, may have its implication in the treatment of aggressive tumors that have shown increased microvascularization and enhanced capacity to metastasize (11, 12). We have hypothesized that incomplete or inadequate inhibition of the microvessel growth by camptothecins in some patients may result in a failing response, rated in clinical evaluation as progressive or stable disease. Presumably, more intensive inhibition of the remaining microvasculature could be achieved in these patients by combining a camptothecin with another antiangiogenic anticancer agent such as Taxol (15) or other compounds, preferably without overlapping dose-limiting

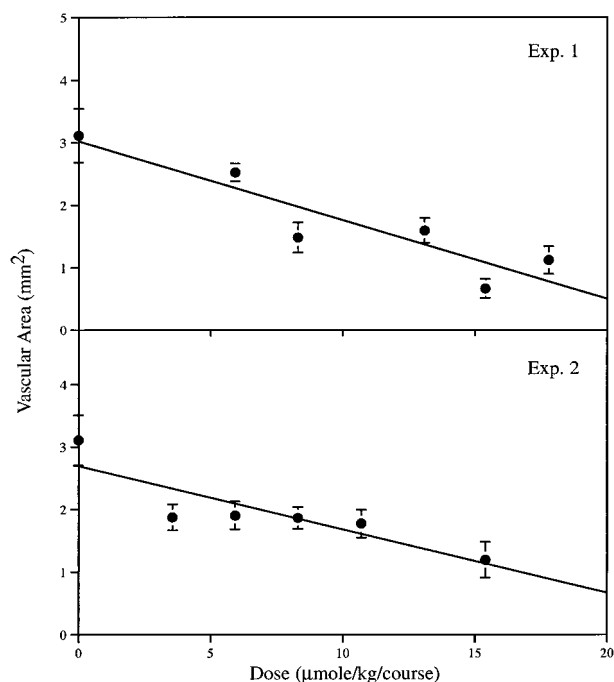


Fig. 3 Exps. 1–2, the effect of various doses of TTN on the corneal vascular area, used as a correlate of neoangiogenesis and its inhibition. Data points, means; bars, SE; lines, regression analyses.

toxicities. TNP-470, a synthetic fumagillin analogue with a wide spectrum of activity against various tumor types, selectively inhibits proliferation and migration of endothelial cells, which are the source of various growth factors with angiogenic properties. It was shown in Phase I and II trials that TNP-470 requires a prolonged administration of nontoxic concentrations inhibiting rather than eradicating treated tumors (24–27). This invites the option to combine TNP-470 or related drugs with another agent targeted at tumor cells. Preclinical and clinical studies combining TNP-470 with taxol have already been initiated (24, 26). In a similar way, camptothecins can be combined with TNP-470, BB-94, or BB2516 (marimastat). The latter two agents, investigated in Phase I/II clinical trials, are inhibitors of matrix metalloproteinases, which are responsible for proteolytic degradation of the extracellular matrix. Degradation of the extracellular matrix is a key feature of the angiogenic process, and by blocking such an essential step, the metalloproteinase inhibitors prevent tumor growth and metastatic dissemination (28, 29). To bring the antiangiogenic property of camptothecins to full potential, prolonged low-dose application can be the schedule of choice. Still another avenue of exploration is the combination of camptothecins with angiostatin, endostatin, or vasculostatin, compounds that belong to the emerging class of endogenous inhibitors of angiogenesis (27, 30). Treatments that involve the combination of a camptothecin with a nontoxic inhibitor of angiogenesis may attack two different targets involved in tumor proliferation, namely, epithelial tumor cells and normal endothelial cells and may result in improved antitumor activity. To validate this concept, however, carefully controlled preclinical studies followed by clinical trials will have to estab-

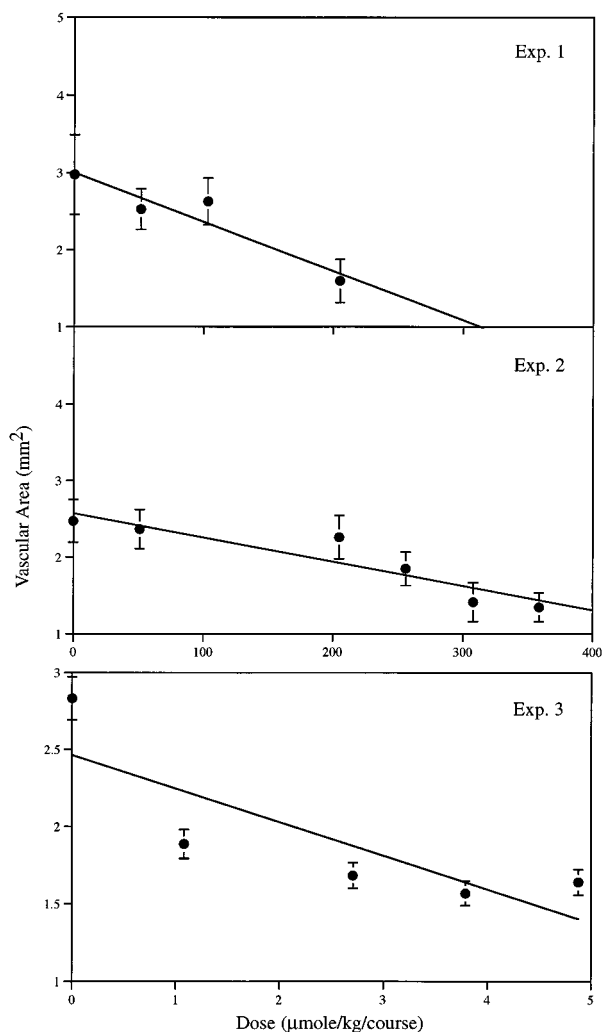


Fig. 4 The effect of various doses of CPT-11 (Exps. 1–2) and vincristine (Exp. 3) on the corneal vascular area, used as a correlate of neoangiogenesis and its inhibition. Data points, means; bars, SE; lines, regression analyses.

lish appropriate drug dosages and an effective treatment schedule.

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