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POTENTIATION OF CYTOTOXIC CANCER THERAPIES BY TNP-470 ALONE AND WITH OTHER ANTI-ANGIOGENIC AGENTS

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The ability of TNP-470, a synthetic analog of fumagillin which has been described as an anti-angiogenic agent, to potentiate cytotoxic cancer therapies was investigated *in vivo* in the murine FSaIIC fibrosarcoma and the Lewis lung carcinoma. TNP-470 was more toxic toward FSaIIC tumor cells from tumors treated *in vivo* than toward bone-marrow CFU-GM from the same animals. TNP-470 had a dose-modifying effect on the toxicity of cyclophosphamide toward FSaIIC tumor cells which amounted to an 8-fold increase in tumor-cell killing at a cyclophosphamide dose of 500 mg/kg. Treatment with TNP-470 and minocycline increased the permeability of the FSaII fibrosarcoma *in vivo* to the fluorescent dye Hoechst 33342 and increased the killing of both the bright and the dim tumor cells by cyclophosphamide. TNP-470, especially in combination with minocycline, formed a highly effective modulator combination for treatment of the Lewis lung carcinoma with cytotoxic cancer therapies against primary and metastatic disease. The combination of TNP-470/minocycline and cyclophosphamide led to 40 to 50% long-term survivors in Lewis-lung-carcinoma-bearing animals. Our results indicate that the use of anti-angiogenic modulators in cancer therapy is a very promising area for further study.

Solid tumor masses are composed of malignant cells and many normal cells which comprise the stroma of the tumor. Stroma includes connective tissue (fibroblasts), blood vessels (endothelial cells, pericytes) and inflammatory cells (mast cells, macrophages). In some tumors, the stroma can comprise up to 90% of the tumor mass (Connolly *et al.,* 1993). The malignant cells, through intercellular signaling, induce proliferation of the surrounding normal cells such as endothelial cells, a process which is necessary for tumor growth and perhaps tumor survival. The secretion by the malignant cells of intercellular signaling factors may alter the metabolic status not only of host normal cells in the vicinity of the tumor but also of responsive distal normal tissues. A novel approach to solid-tumor therapy, therefore, may include agents that inhibit proliferation of or that are cytotoxic to normal cells critical to tumor growth, in combination with agents directed toward eradication of malignant cells. Minocycline is a tetracycline and a collagenase inhibitor which has demonstrated anti-angiogenic activity (Alvarez Sotomayor *et al.,* 1992; Tamargo *et al.,* 1991; Teicher *et al.,* 1993). The characteristics of minocycline as a modulator of cytotoxic therapies in Lewis lung carcinoma have been described (Alvarez Sotomayor *et al.,* 1992; Teicher *et al.,* 1993).

TNP-470 is a synthetic analog of a fungal secretion product called fumagillin (Ingber *et al.,* 1990). Fumagillin was shown to cause endothelial cells in culture to round-up and to inhibit angiogenesis in the chick chorio-allantoic membrane assay (Ingber *et al.,* 1990). Fumagillin was toxic to animals. Therefore, synthetic derivatives of fumagillin were tested. TNP-470 was the most potent angiogenesis inhibitor of these derivatives that was non-toxic to animals. TNP-470 is a potent inhibitor of endothelial-cell migration (Brem *et al.,* 1991), endothelial-cell proliferation (Ingber *et al.,* 1990), and capillary tube formation (Kusaka *et al.,* 1991). TNP-470 also inhibits angiogenesis, as demonstrated in chick chorio-allantoic membrane, the rabbit and rodent cornea (Kusaka *et al.,* 1991). Subsequently, TNP-470 has been shown to inhibit proliferation of human tumor cells in culture and to slow or inhibit the growth of primary and metastatic murine tumors as well as human tumor xenografts (Brem and Folkman, 1993; Brem *et al.,* 1993; Kamei *et al.,* 1993; Takayamiya *et al.,* 1993; Yanase *et al.,* 1993).

Our study was based on the hypothesis that TNP-470 may be selectively cytotoxic toward endothelial cells within the tumor mass and that an agent with such selectivity would be a therapeutically useful addition to cytotoxic treatments directed toward tumor cells.

MATERIAL AND METHODS

Drugs

TNP-470 (AGM-1470) was a gift from Dr. K. Kitazawa (Takeda, Osaka, Japan), and 4-hydroperoxycyclophosphamide was a gift from Dr. J. Pohl (Asta, Frankfurt, Germany). β-cyclodextrin tetradecasulfate (14(SO_4)βCD) was prepared in our laboratory as described (Teicher *et al.,* 1992, 1993). Tetrahydrocortisol (THC), minocycline, melphalan (L-PAM), cyclophosphamide and *cis*-diamminedichloroplatinum(II) (CDDP) were purchased from Sigma (St. Louis, MO). Carmustine [BCNU; 1,3-bis(2-chloroethyl)-1-nitrosourea] was purchased from the Dana-Farber Cancer Institute pharmacy.

Tumor excision assay

The FSaII fibrosarcoma adapted for growth in culture (FSaIIC) (Teicher *et al.,* 1987*a, b*) was carried in C3H/He male mice (Jackson Laboratories, Bar Harbor, ME). For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted intramuscularly into the legs of C3H/He male mice aged 8 to 10 weeks.

On day 7 after the tumor implant, TNP-470 (50, 70, 100, 150, 200, 300 mg/kg) was administered s.c. Cyclophosphamide (100, 300, 500 mg/kg i.p.) was administered alone on day 7 or, in combination with TNP-470 (3×30 mg/kg), on days 3, 5 and 7. On day 8 the mice were killed. The tumors were excised in sterile conditions in a laminar-flow hood and minced to a fine brei using 2 scalpels. Four tumors were pooled for each treatment group. Approximately 500 mg tumor brei was used to make each single-cell suspension. All reagents were sterilized with 0.22-μm Millipore filters and were added septically to the tumor cells. Each sample was washed in 20 ml α-MEM, after which the liquid was gently decanted and discarded. The samples were re-suspended in 450 units collagenase/ml (Sigma) and 0.1 mg DNase/ml (Sigma) and were incubated for 10 min at 37°C in a shaking water bath. The samples were re-suspended as above and incubated for another 15 min at 37°C after which the samples were filtered through 2 layers of sterile gauze. The samples were washed twice, then re-suspended in α-MEM supplements with 10% fetal bovine serum (FBS).

These single-cell suspensions were counted and plated in duplicate at 3 different cell concentrations for the colony-forming assay. No significant difference was observed in total

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cell yield from the pooled tumors in any treatment group. After 1 week, the plates were stained with crystal violet and colonies of >50 cells were counted. The untreated tumor-cell suspensions had a plating efficiency of 10 to 16%. The results are expressed as the surviving fraction (±SE) of cells from treated groups as compared with untreated controls (Teicher *et al.*, 1987*a, b*).

Bone-marrow toxicity

Bone marrow was taken from the same animals used for the tumor-excision assay. A pool of marrow from the femurs of 2 animals was obtained by gently flushing the marrow through a 23-gauge needle, and the CFU-GM assay was carried out as described (Teicher *et al.*, 1987*a,b*). CFU-GM were measured as follows: bone-marrow cells were suspended in supplemented McCoy's 5A medium containing 15% FBS, 0.3% agar (Difco, Detroit, MI), and 10% conditioned medium as a source of colony-stimulating activity. The colony-stimulating activity supplement was prepared by incubating L-929 mouse fibroblasts (2,500 cells/ml; Microbiological Associates, Bethesda, MD) with 30% FBS in McCoy's 5A medium for 7 days at 37°C in a humidified atmosphere containing 5% CO_2. The supernatant containing colony-stimulating activity was obtained by centrifugation of the medium at 10,000 *g* for 10 min at 4°C, followed by filtration in sterile conditions. The bone-marrow cell cultures were incubated for 7 days at 37°C and were fixed with 10% glutaraldehyde. Colonies of at least 50 cells were counted. The results of 3 experiments, in which determination for each group was made in duplicate at 3 cell concentrations, were averaged. The results are expressed as the surviving fraction from treated groups as compared with untreated controls.

Tumor subpopulation studies: tumor growth and Hoechst 33342 labeling

When the tumor volumes were approximately 100 mm^3 (about 1 week after tumor-cell implantation), animals were treated with cyclophosphamide (150 mg/kg, TNP-470 (3 × 30 mg/kg s.c. on days 3, 5 and 7) or minocycline (5 × 10 mg/kg i.p. on days 3 to 7) alone or in combination. Hoechst 33342 dye (2 mg/kg; Aldrich, Milwaukee, WI) dissolved in PBS was administered by tail-vein injection (0.25 ml) into tumor-bearing mice 24 hr after treatment. Tumor-cell suspensions were prepared by excising the tumor 20 min after i.v. administration of the dye (Teicher *et al.*, 1988, 1990) in sterile conditions, and single-cell suspensions of tumor cells were prepared for the colony-forming assay. To remove contaminating erythrocytes, 0.17 M NH_4Cl was added to the tumor-cell suspension for 3 min at room temperature just after filtering through gauze. The cells were then washed once with α-MEM supplemented with 10% FBS, filtered through a syringe fitted with a 40-μM nylon mesh filter to remove cell clumps, counted and centrifuged at 200 *g*. Cells were then re-suspended at a concentration of 2×10^6 cells/ml in complete medium.

Flow cytometry and sorting

The cells from tumors were analyzed and sorted using the Coulter Epics V instrument (Hialeah, FL). Hoechst 33342 dye fluorescence intensity was measured using an argon ion base with excitation at 350 to 360 nm (40 mW power) and emission was monitored with a 457-nm long-pass filter and a 530-nm short-pass filter. The fluorescence distribution was divided into 10 fractions based on Hoechst 33342 intensity. Two fractions of cells were collected, one containing the brightest 10% of cells and the other containing the dimmest 20% of cells. For any tumor-cell population, fluorescence intensity spans about 3 logs on a scale of 1 to 1000 cells were washed once with complete medium. After 1 week, colonies were stained with crystal violet and colonies of ≥50 cells were counted by eye. The plating efficiency for the unsorted population was 15.5 ± 2.7%. For the 10% brightest cells, the plating efficiency was 9.2 ± 1.6%; for the 20% dimmest cells, it was 5.5 ± 1.4%. The survival results are expressed as the surviving fraction ± SE of the treated bright and dim fractions compared with the bright and dim untreated controls respectively.

Tumor growth delay experiments

The Lewis lung tumor (Steel *et al.*, 1978) was carried in male C57BL mice (Taconic, Germantown, NY). For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted s.c. into the legs of male mice 8 to 10 weeks of age.

By day 4 after tumor-cell implantation, Lewis lung tumors had begun neovascularization. Animals bearing Lewis lung tumors were injected s.c. with TNP-470 (30 mg/kg) on alternate days for 8 injections, beginning on day 4, and/or were treated with minocycline (10 mg/kg) i.p. daily for days 4 to 18 after tumor implant and/or were implanted s.c. with 14-day mini-osmotic pumps (Alzet pumps, model 2002; Alza, Palo Alto, CA) containing 14 (SO_4)[67od] bCD (1 g/kg) and THC (125 mg/kg), a 1:1 molar ratio. When the Lewis lung tumors were approximately 100 mm^3 in volume, on day 7 after tumor cell implantation, cytotoxic therapy was initiated. CDDP (10 mg/kg) or melphalan (10 mg/kg) were administered i.p. on day 7. Cyclophosphamide (150 mg/kg) or BCNU (15 mg/kg) were administered i.p. on days 7, 9 and 11 after tumor implant. Radiation was delivered locally to the tumor-bearing limb as 3 Gray fractions daily on days 7 to 11.

The progress of each tumor was measured thrice weekly until it reached a volume of 500 mm^3. Tumor growth delay was calculated as the days taken by each individual tumor to reach 500 mm^3 as compared with the untreated controls. Each treatment group consisted of 6 animals, and the experiment was repeated 3 times. Days of tumor growth delay are the mean ± standard error for the treatment group compared with the control.

Lung metastases

The external lung metastases from animals treated as described above on day 20 after tumor implant were counted manually and scored as ≥3 mm in diameter. The data shown are the means from 6 to 12 pairs of lungs. Untreated control animals died from lung metastases on days 21 to 25. Parentheses on Tables II, IV and VI indicate the number of large (vascularized) metastases and percentage of the total number of metastases there were large (Teicher *et al.*, 1992, 1993).

RESULTS

TNP-470 was more toxic to FSaIIC tumor cells than to bone-marrow CFU-GM when the drug was administered systemically as single doses s.c. (Fig. 1). At doses of 70 mg/kg or more (up to 300 mg/kg), tumor-cell killing increased in a log-linear manner but with a very shallow slope. Fifty percent of FSaIIC tumor cells were killed by about 120 mg/kg of TNP-470. Only doses of TNP-470 of 150 mg/kg or more showed any toxicity toward bone-marrow CFU-GM. Cyclophosphamide killed increasing numbers of FSaIIC tumor cells and bone-marrow CFU-GM with increasing doses of the drug in a log-linear manner and with selectivity for tumor cells over bone-marrow CFU-GM. TNP-470 (3 × 30 mg/kg, s.c.) produced a surviving fraction of 0.70 in the FSaIIC tumor and was not cytotoxic to the bone-marrow CFU-GM. The combination of TNP-470 with cyclophosphamide resulted in dose modification of tumor-cell killing by the drug. At the standard dose range of cyclophosphamide, the combination produced about a 2-fold increase in tumor-cell killing as compared with the drug alone, while at the high-dose end of the survival curve the

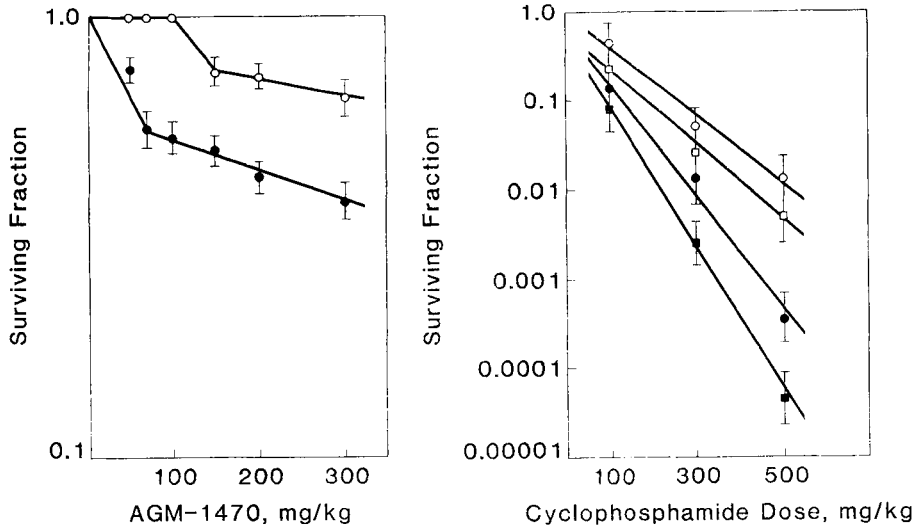

FIGURE 1 – Survival of FSaIIC tumor cells from tumors (●) and bone-marrow CFU-GM (○) from the same animals after treatment with single doses of TNP-470 (50, 70, 100, 150, 200 or 300 mg/kg, s.c.) on day 7 or with single doses of cyclophosphamide (100, 300 or 500 mg/kg i.p.) on day 7 alone (tumor, ●; bone-marrow CFU-GM, ○) or in combination with TNP-470 (30 mg/kg, s.c.) on days 3, 5 and 7 (tumor, ■; bone-marrow CFU-GM, □). Data are means of 3 independent experiments ± SEM.

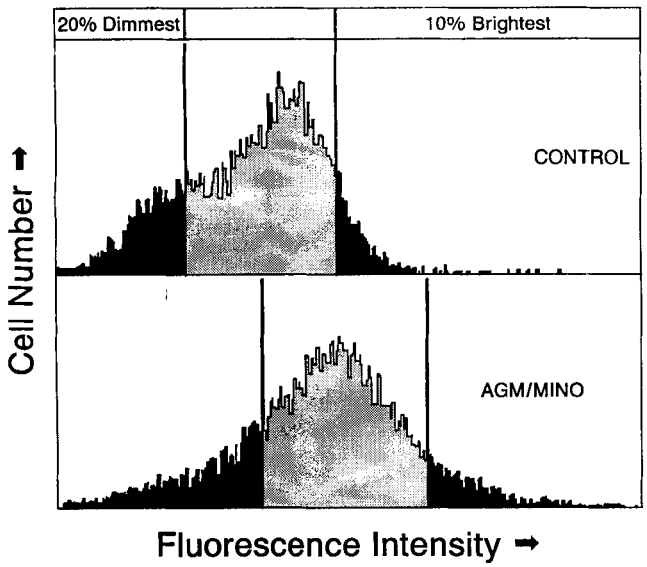

FIGURE 2 – Fluorescence distribution in FSaIIC tumor cells after i.v. injection of tumor-bearing animals with Hoechst 33342 (2 mg/kg). Data are for an untreated control tumor and a tumor treated with TNP-470 (3 × 30 mg/kg, s.c.) and minocycline (5 × 10 mg/kg, i.p.).

combination produced about an 8-fold increase in tumor-cell killing compared with the drug alone. Although the increase in the killing of bone-marrow CFU-GM was not significant, the trend showed about a 2-fold increased killing of bone-marrow CFU-GM by the combination over the dosage range of cyclophosphamide studied.

Hoechst 33342 is a DNA-binding fluorescent dye which diffuses passively from the vasculature through cell layers (Teicher *et al.*, 1988, 1990). Figure 2 shows typical fluorescence distributions for FSaIIC tumor cells after tumor-bearing animals were injected i.v. with a tracer amount of the dye. The control tumor shows a fluorescence distribution typical of untreated or treated tumors (Teicher *et al.*, 1990). When tumors were treated with TNP-470 and minocycline (Alvarez Sotomayor *et al.*, 1992; Tamargo *et al.*, 1991; Teicher *et al.*, 1993), there was a marked shift toward greater brightness of the entire tumor-cell population, so that the 10% brightest and the 20% dimmest cell sub-populations were composed of cells containing much more dye than the same sub-populations in the control tumor. As described (Teicher *et al.*, 1990), cyclophosphamide was about 6-fold more toxic toward bright cells than toward dim cells (Fig. 3). Treatment of the animals with TNP-470 (3 × 30 mg/kg) or minocycline (5 × 10 mg/kg) along with cyclophosphamide resulted in a 2.5- to 3-fold increase in the killing of the bright and the dim sub-populations compared with cyclophosphamide alone. Treatment with the combination of TNP-470 and minocycline along with cyclophosphamide was most effective, resulting in a 3.6-fold increase in killing of bright cells and a 6.8-fold increase in the killing of dim cells compared with cyclophosphamide alone.

The Lewis lung carcinoma growing in C57BL mice was chosen for tumor-growth-delay studies because this tumor is relatively resistant to many cancer therapies and because it metastasizes avidly to the lungs from s.c. implants. In order to study tumor growth delay, each of the cytotoxic therapies was administered at full standard dose and schedule. TNP-470, administered s.c. on alternate days beginning on day 4 and continuing until day 18, was a moderately effective modulator of the cytotoxic therapies (Table I). TNP-470 as a single modulator was most effective with melphalan, BCNU and radiation, increasing the tumor growth delay produced by these treatments 1.8- to 2.4-fold. TNP-470 along with minocycline (10 mg/kg) administered i.p. daily on days 4 to 18 comprised a highly effective modulator combination. The increases in tumor growth delay produced by the modulator combination TNP-470/minocycline along with the cytotoxic therapies ranged from 2- to 4-fold. In the treatment group receiving TNP-470/minocycline and cyclophosphamide, approximately 40% of the animals were long-term (> 120 days) survivors. Each of the cytotoxic therapies (including radiation delivered locally to the tumor-bearing limb) produced a reduction in the number of lung metastases found on day 20

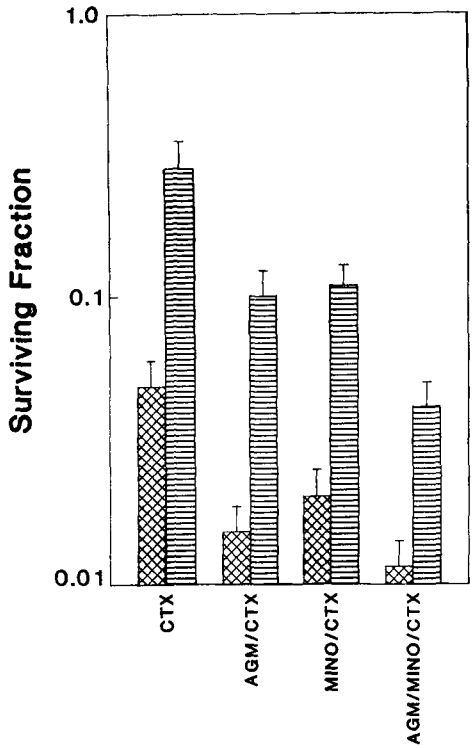

FIGURE 3 – Survival of sub-populations based on Hoechst 33342 fluorescence intensity of FSaIIC cells from animals bearing FSaIIC tumors treated with a single dose of cyclophosphamide (150 mg/kg, i.p.) on day 7 alone or in combination with TNP-470 (3 × 30 mg/kg, s.c.) on days 3, 5 and 7 and/or minocycline (5 × 10 mg/kg, i.p.) on days 3 to 7. The bars show the survival of bright cells (▩) and dim cells (▤). Data are means of 3 independent experiments ± SEM.

(Table II). None of the modulators altered the number of lung metastases or the percentage of large (vascularized) lung metastases on day 20, nor was the number of lung metastases different from those obtained with the cytotoxic therapies, except in the case of cyclophosphamide: many animals treated with this drug and modulator combination had very few metastases on day 20, most of them very small.

The efficacy of the modulator combination of TNP-470/minocycline against primary Lewis lung tumors is compared with that of other potential anti-angiogenic modulator combinations in Table III. The most effective combination with CDDP was 14 (SO_4)βCD/THC/minocycline, while the other cytotoxic therapies using the 3 modulator combinations were approximately equally effective, resulting in 40 to 50% long-term survivors. None of the modulator combinations alone was effective against metastatic disease, although in each case the percent of large metastases on day 20 was reduced (Table IV). There was a trend toward the combination of 14(SO_4)βCD/THC/minocycline being the most effective modulation of cytotoxic therapies against metastatic disease.

In the data presented in Tables I to IV, modulator treatment was begun on day 4, when the primary tumor was a seed initiating angiogenesis, and continued through day 18 when the primary tumor had "fully matured" (Brem and Folkman, 1993; Brem *et al.*, 1993). To determine the efficacy of the modulator combination against establishing disease, TNP-470 and minocycline were administered on different schedules, while the cytotoxic treatments remained as previously described (Table V). The modulator schedule on days 4 to 11

TABLE I – GROWTH DELAY OF THE LEWIS LUNG TUMOR PRODUCED BY VARIOUS ANTI-CANCER TREATMENTS ALONE OR IN COMBINATION WITH POTENTIAL ANTI-ANGIOGENIC MODULATORS

Treatment group	Tumor growth delay, days[1]			
	Alone	+ minocycline[2]	+ TNP-470	+ TNP-470/mino
—	—	1.2 ± 0.4	2.1 ± 0.4	1.8 ± 0.4
CDDP (10 mg/kg)	4.5 ± 0.3	5.0 ± 0.3	6.0 ± 0.5	10.9 ± 0.8
Cyclophosphamide (3 × 150 mg/kg)	21.5 ± 1.7	32.4 ± 1.8	25.3 ± 2.2	44.8 ± 2.8[3]
Melphalan (10 mg/kg)	2.7 ± 0.3	4.3 ± 0.3	6.0 ± 0.5	8.5 ± 0.6
BCNU (3 × 15 mg/kg)	3.6 ± 0.4	5.2 ± 0.4	6.3 ± 0.5	14.6 ± 1.0
X-rays (5 × 3 Gray)	4.4 ± 0.3	7.8 ± 0.6	10.6 ± 1.1	15.3 ± 1.2

[1]Tumor growth delay is the difference in days for treated tumors to reach 500 mm^3 compared with untreated control tumors. Untreated control tumors reach 500 mm^3 in about 14 days. Mean ± SE of 15 animals.–[2]Minocycline (10 mg/kg) was administered i.p. daily on days 4 to 18. TNP-470 (30 mg/kg) was administered s.c. on alternate days for 8 injections, beginning on day 4. CDDP and melphalan were administered i.p. on day 7. Cyclophosphamide and BCNU were administered i.p. on days 7, 9 and 11. X-rays were delivered daily on days 7 to 11 locally to the tumor-bearing limb.–[3]Long-term survivors 5/12 (> 120 days).

TABLE II – NUMBER OF LUNG METASTASES ON DAY 20 FROM S.C. LEWIS LUNG TUMORS AFTER VARIOUS ANTI-CANCER THERAPIES ALONE OR IN COMBINATION WITH POTENTIAL ANTI-ANGIOGENIC MODULATORS

Treatment group	Mean number of lung metastases (% large)			
	Alone	+ minocycline	+ TNP-470	+ TNP-470/mino
—	20 (62)	20 (50)	21 (51)	18 (54)
CDDP (10 mg/kg)	13 (58)	11 (48)	14.5 (34)	14 (50)
Cyclophosphamide (3 × 150 mg/kg)	12 (40)	6 (33)	6 (30)	2 (25)
Melphalan (10 mg/kg)	13 (48)	11 (50)	15 (47)	15 (45)
BCNU (3 × 15 mg/kg)	16 (53)	15 (38)	15.5 (45)	13 (38)
X-rays (5 × 3 Gray)	15 (40)	13 (30)	10 (40)	12 (42)

began when the tumors were seeds and extended through the cytotoxic treatments and on days 7 to 11 it allowed modulator administration during the same period as the cytotoxic therapies, while on days 7 to 18 it allowed initiation of modulator administration at the same time as initiation of cytotoxic therapy and extended the modulators for 1 week after completion of the cytotoxic therapies. The most effective therapies were those begun on day 4, when the tumor burden was smallest. However, the subsequent treatment schedules resulted in enhanced tumor growth delays compared with the cytotoxic therapies alone. The modulator schedules including cyclophosphamide and beginning on day 4 resulted in 1.7- and 2.1-fold increased tumor growth delay, while those beginning on day 7 resulted in 1.3- and 1.5-fold increased tumor growth delay as compared with cyclophosphamide treatment alone. BCNU on days 7, 9 and 11, along with the modulator schedules beginning on day 4, produced 3.3- and 4-fold increases in tumor growth delay, while those beginning on day 7 produced a 2.9-fold increase in tumor growth delay. Finally, the schedules beginning on day 4 resulted in 2.4- and 3.5-fold increases and those beginning on day 7 in a 1.5-fold increase in tumor growth delay. Varying the modulator administration schedules did not appear to affect response of the metastatic disease to the therapies (Table VI). Only in the case of cyclophosphamide

TABLE III – GROWTH DELAY OF THE LEWIS LUNG TUMOR PRODUCED BY VARIOUS ANTI-CANCER TREATMENTS ALONE OR IN COMBINATION WITH POTENTIAL ANTI-ANGIOGENIC MODULATORS

Treatment group	Tumor growth delay, days[1]			
	Alone	+ 14(SO_4)βCD/ THC/mino	+14(SO_4)βCD/ THC/TNP-470	+ mino/ TNP-470
—	—	1.2 ± 0.4	1.5 ± 0.3	1.8 ± 0.4
CDDP (10 mg/kg)	4.5 ± 0.3	26.2 ± 2.5	10.6 ± 0.7	10.9 ± 0.8
Cyclophos-phamide (3 × 150 mg/kg)	21.5 ± 1.7	48.8 ± 3.3 (5/12)[2]	49.2 ± 3.4 (6/12)[3]	44.8 ± 2.8 (5/12)[3]
Melphalan (10 mg/kg)	2.7 ± 0.3	10.5 ± 0.9	12.2 ± 1.4	8.5 ± 0.6
BCNU (3 × 15 mg/kg)	3.6 ± 0.4	9.8 ± 0.8	10.6 ± 1.1	14.6 ± 1.0
X-rays (5 × 3 Gray)	4.4 ± 0.3	12.6 ± 1.2	10.3 ± 0.9	15.3 ± 1.2

[1]See Table I for definition.–[2]Lived a normal lifespan (~2 years).–[3]Long-term survivors (> 180 days).

TABLE IV – NUMBER OF LUNG METASTASES ON DAY 20 FROM S.C. LEWIS LUNG TUMORS AFTER VARIOUS ANTI-CANCER THERAPIES ALONE OR IN COMBINATION WITH POTENTIAL ANTI-ANGIOGENIC MODULATORS

Treatment group	Mean number of lung metastases (% large)			
	Alone	+ 14(SO_4)βCD/ THC/mino	+ 14 (SO_4)βCD/ THC/TNP-470	+ mino/ TNP-470
—	20 (62)	17 (46)	18 (50)	18 (54)
CDDP (10 mg/kg)	13 (58)	8 (42)	15 (40)	14 (50)
Cyclophos-phamide (3 × 150 mg/kg)	12 (40)	1 (0)	2 (50)	2 (25)
Melphalan (10 mg/kg)	13 (48)	7 (50)	15 (40)	15 (45)
BCNU (3 × 15 mg/kg)	16 (53)	14 (45)	14 (43)	13 (58)
X-rays (5 × 3 Gray)	15 (40)	9 (43)	11 (36)	12 (42)

was it evident that beginning modulator administration on day 7 led to decreased efficacy of the therapy against metastatic disease.

DISCUSSION

The various methods used in the search for anti-cancer drugs include (1) measuring cytotoxicity toward mammalian tumor cells in culture; (2) determining anti-tumor activity in murine transplantable tumor models; and (3) searching for inhibitors of presumed critical biochemical processes involved in tumor-cell proliferation. All these methods have focused on the malignant cell. Several laboratories, notably that of Dr. J. Folkman (Folkman, 1987, 1989; Folkman and Ingber, 1987; Folkman and Klagsbrun, 1987; Folkman *et al.*, 1983, 1989; Ingber *et al.*, 1990), recognizing that normal cell proliferation is necessary for solid-tumor growth, have used a proliferating-normal-cell system, the chick chorio-allantoic membrane, as a screen for agents which may be selectively cytotoxic toward endothelial cells. Several unique classes of molecules have demonstrated significant activity in the chick chorio-allantoic membrane "screen" as well as in other pre-clinical assays focusing on endothelial-cell proliferation. Our study discovered molecules from 4 different structural classes (a cyclodextrin, a steroid, a tetracycline and a di-epoxide fumagillin antibiotic) through this process. All the cytotoxic therapies described here are presumably DNA-targeted, producing either cross-links or strand-breaks in cellular DNA. These cytotoxic therapies are most effective against proliferating cells and are active in the chorio-allantoic membrane assay (Steiner, 1992). Conversely, among the anti-angiogenic modulators, TNP-470 and, to a much lesser degree, minocycline have demonstrated cytotoxicity toward tumor cells (Alvarez Sotomayor *et al.*, 1992; Tamargo *et al.*, 1991; Teicher *et al.*, 1993). Whether the anti-angiogenic agents are selectively cytotoxic toward endothelial cells in tumors remains an open question. The mechanism(s) of action of the anti-angiogenic modulators remain to be elucidated; however, cellular DNA is presumably not their target.

TABLE V – GROWTH DELAY OF LEWIS LUNG TUMOR PRODUCED BY VARIOUS ANTI-CANCER TREATMENTS ALONE OR IN COMBINATION WITH TNP-470 AND MINOCYCLINE ADMINISTERED ON VARIOUS SCHEDULES

Treatment group	Tumor growth delay, days[1] TNP-470 + minocycline				
	Alone	Days 4–11	Days 7–11	Days 7–18	Days 4–18
—	—	1.4 ± 0.3	0.6 ± 0.3	0.9 ± 0.3	1.8 ± 0.4
Cyclophos-phamide (3 × 150 mg/kg)	21.5 ± 1.7	37.1 ± 2.7	28.8 ± 2.4	32.1 ± 2.9	44.8 ± 2.8
BCNU (3 × 15 mg/kg)	3.6 ± 0.4	11.8 ± 1.4	10.4 ± 1.7	10.6 ± 1.5	14.6 ± 1.6
X-rays (5 × 3 Gray)	4.4 ± 0.3	10.4 ± 1.6	6.6 ± 1.2	6.7 ± 1.0	15.3 ± 1.7

[1]See Table I for definition.

TABLE VI – NUMBER OF LUNG METASTASES ON DAY 20 FROM S.C. LEWIS LUNG TUMORS AFTER VARIOUS ANTI-CANCER THERAPIES ALONE OR IN COMBINATION WITH TNP-470 AND MINOCYCLINE ADMINISTERED ON VARIOUS SCHEDULES

Treatment group	Mean number of lung metastases (% large) TNP-470 + minocycline				
	Alone	Days 4–11	Days 7–11	Days 7–18	Days 4–18
—	20 (62)	17 (57)	20 (52)	20 (48)	18 (54)
Cyclophosphamide (3 × 150 mg/kg)	12 (40)	2 (25)	4 (18)	4 (28)	2 (25)
BCNU (3 × 15 mg/kg)	16 (53)	17 (41)	12 (42)	14 (25)	15 (45)
X-rays (5 × 3 Gray)	15 (40)	15 (38)	17 (34)	15 (38)	12 (42)

To determine whether TNP-470 and the anti-tumor alkylating agents or radiation could interact positively in the killing of tumor cells, we examined these combinations in cell culture. Increased cell killing was observed in some cases, especially with TNP-470 and 4-hydroperoxycyclophosphamide or radiation (hypoxic cells at low pH). *In vivo,* however, we observed positive modulation with each of the 4 anti-tumor alkylating agents and radiation, though the greatest enhancements in tumor growth delay were with cyclophosphamide and radiation therapy, as the cell culture studies may have indicated.

The distribution of Hoechst 33342 into tumors grown during treatment with TNP-470 and minocycline was increased as compared with untreated control tumors, indicating that the treated tumors were more easily penetrated by this lipophilic dye. The vasculature forms the first barrier to penetration of molecules into tumors. Although the modulator treatments we administered did not completely inhibit angiogenesis in these

tumors, the vasculature of the treated tumors may be impaired. Overall, therefore, it is probable that the main targets for the anti-angiogenic modulators are extracellular matrix processes and/or tumor endothelial cells, and that inhibition and/or impairment of these non-malignant functions may improve therapeutic responses when used in combination with cytotoxic therapies.

The goal of the addition of modulators to therapeutic combinations is to take a good therapy and, without additional toxicity, "push" it to cure. Cyclophosphamide is a good drug against the Lewis lung carcinoma, although the doses and schedules used in our study produced no long-term survivors with cyclophosphamide. Adding anti-angiogenic modulators to the treatment with cyclophosphamide produced a cure rate of 40 to 50%, with the primary and the metastatic disease eradicated. Cures were achieved only when the modulator treatments extended from day 4 to day 18 after tumor implantation.

We report activity of TNP-470 (alone or in combination) together with anti-tumor alkylating agents in 2 different murine tumors via 2 endpoints. Our results indicate that the use of anti-angiogenic modulators in cancer therapy is very promising and that further investigation of this approach is warranted.

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