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Comparison of several antiangiogenic regimens alone and with cytotoxic therapies in the Lewis lung carcinoma

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Abstract The efficacy of several potential antiangiogenic agents, TNP-470, minocycline, suramin, genistein, interferon δ4, 14(sulfated)-β-cyclodextrin and tetrahydrocortisol, alone and in combination with cytotoxic therapies was examined against primary and metastatic Lewis lung carcinoma. The antiangiogenic agents when administered as single agents or in two-agent combinations were only modestly active as antitumor agents. Three antiangiogenic agent combinations, TNP-470/minocycline, TNP-470/14(SO_4)βCD/THC and minocycline/14(SO_4)βCD/THC, produced significant increases in tumor growth delay and decreases in the number of lung metastases when administered along with cyclophosphamide compared with cyclophosphamide alone. Two antiangiogenic agent combinations, minocycline/interferon δ4 and minocycline/14(SO_4)βCD/THC, produced significant decreases in the number of lung metastases when administered alone with adriamycin compared with adriamycin alone. The antiangiogenic combinations of TNP-470/minocycline, TNP-470/suramin, TNP-470/genistein, TNP-470/interferon δ4 and TNP-470/14(SO_4)βCD/THC, resulted in increased tumor growth delays when administered along with CDDP, BCNU, fractionated radiation or 5-fluorouracil. There was not always a direct correlation between the antiangiogenic regimen that was most beneficial against the primary tumor as compared with disease metastatic to the lungs. These studies establish that a broad range of antiangiogenic therapies can interact in a positive manner with cytotoxic therapies.

Key words Antiangiogenic agents · Combination Therapy · TNP-470 · Suramin · Genistein · Minocycline

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Introduction

The growth of solid tumors requires the restructuring of normal tissue and the proliferation of normal cells of many types to support the tumor [16, 19, 26, 70]. The normal cells and tissue encompassed by the tumor is called the stroma. A tumor mass may consist of up to 90% stroma or as little as 10% stroma. Included within the stroma are blood vessels (endothelial cells, pericytes, mast cells), inflammatory cells (monocytes, neutrophils, macrophages), fibroblasts and connective tissue [16, 19, 26, 70]. These cells are normal because they do not metastasize, but, they are an integral part of malignant disease in that through signals produced by the malignant tumor cells they proliferate, invade surrounding normal tissue and allow nutrients to reach the malignant cells [16, 19, 26, 70]. Malignant disease therefore consists of the proliferation of normal and malignant cells. The corollary of this notion is that both normal and malignant cells within the tumor as well as the signaling pathways between the cells are valid targets for therapeutic attack.

The trigger for angiogenesis in tumors is still unknown. However, several polypeptides secreted by a variety of normal cells as well as several small molecules have been shown to be able to induce proliferation of endothelial cells and/or migration of endothelial cells leading to neovascularization [28–32, 44]. The angiogenic proteins identified thus far include: (1) basic and acidic fibroblast growth factors (b-FGF and a-FGF) [6, 38, 52, 53, 83, 88, 90, 108]; (2) vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) [14, 15, 69, 78, 84, 103]; (3) interleukin-8 (IL-8) [4, 5, 8, 54, 62, 76]; (4) platelet-derived-endothelial cell growth factor (PD-ECGF) [48]; (5) angiogenin [9]; (6) tumor necrosis factor-α (TNF-α) [75]; (7) transforming growth factor-α (TGF-α); and (8) transforming growth factor-β (TGF-β) [22, 91]. Among the angiogenic small molecules, 1-butyryl glycerol is secreted

by adipocytes that have differentiated from 3T3 fibroblasts [24]. The prostaglandins PGE_1 and PGE_2 [39], nicotinamide, adenosine, (12*R*)-hydroxyeicosatrienoic acid and okadaic acid have been reported to be highly angiogenic [25, 57, 63, 74].

The search for antiangiogenic substances has primarily led to the discovery of proteins and small molecules that inhibit various steps in the breakdown of the basement membrane inhibitors of endothelial cell proliferation and/or endothelial cell migration [106]. These include naturally occurring proteins such as protamine; interferon-α; platelet factor 4; tissue inhibitors of metalloproteinases (TIMPs); peptides derived from cartilage, vitreous humor, smooth muscle, and aorta; as well as synthetic peptides such as synthetic laminin peptide (CDPG) YIGSR-NH_2 and somatostatin analogs such as somatoline [10, 21, 33, 41, 67, 85, 93, 115]. Antiangiogenic small molecules include naturally occurring heparins, a variety of steroids, several retinoids, warfarin and fumagillin, as well as synthetic agents such as sulfated chitin derivatives, sulfated cyclodextrins, SC44463, SC39026, derivatives of fumagillin and minocycline [33, 46, 47, 58, 59, 68, 73, 82, 87, 97]. Radiation also inhibits blood vessel growth [56, 80]. Many of these agents function by inhibiting enzymes that degrade the basement membrane. Therefore, in addition to being antiangiogenic, these treatments may function as antimetastatic agents by preventing invasion of tumor cells through the basement membrane [106, 109].

The angiostatic activity of several steroids was discovered some years ago; however, the mechanism by which these steroids inhibit vessel growth and/or produce regression of growing vessels is only now being elucidated [30, 46]. Tetrahydrocortisol is a steroid with pure antiangiogenic activity independent of either glucocorticoid or mineralocorticoid activity. Angiostatic steroids appear to induce basement membrane dissolution as part of their antiangiogenic action [46].

It has also been found that β-cyclodextrin tetradecasulfate in combination with hydrocortisone is 100 to 1000 times more effective than heparin in combination with hydrocortisone in inhibiting capillary formation in the chick embryo-chorioallantoic membrane assay and in preventing neovascularization induced by endotoxin in the rabbit cornea [33]. Recently, Thorpe et al. [102] reported a heparin–cortisol covalent conjugate which has greater biological activity than heparin plus the steroid.

Suramin, which like heparin is a polysulfonated molecule, interferes with binding of many growth factors, including b-FGF, to their receptors. Suramin is an inhibitor of angiogenesis, and a suppressor of endothelial cell growth and migration [20, 92, 94, 121]. Elegant studies by Takano et al. [95] have shown that suramin inhibits multiple control points of angiogenesis including those stimulated by b-FGF.

Several enzymes are involved in the degradation of the extracellular matrix during malignant tumor growth and invasion. Serine proteases and metalloproteases are the most prominent of these enzymes [2, 61, 106, 109]. Type IV collagen is the main component of the tight structure of the basement membrane. At least two specific type IV collagenases have been isolated and characterized [18, 81, 101]. Activity of type IV collagenase has been associated with the metastatic phenotype. Tetracyclines can inhibit tissue collagenase activity and tetracycline administration has been used in the treatment of periodontal disease, and gingival collagenolytic activity in diabetes, and to inhibit joint deterioration in patients with rheumatoid arthritis [36, 40]. This inhibitory activity has been associated with both type IV collagenase and interstitial collagenase [37]. Tamargo et al. [97] have reported that minocycline, a semisynthetic tetracycline with a relatively long circulating half-life, inhibits neovascularization in the rabbit cornea implanted with the VX2 carcinoma. Minocycline has been shown to be an active addition to anticancer treatment regimens [3, 98–100].

In 1987, Sidky and Borden reported that murine interferons inhibit tumor-induced and lymphocyte-induced angiogenesis [89]. The effect of the interferons is species specific, suggesting that, rather than acting at the endothelial cells, the interferons inhibit the signal inducing angiogenesis at the level of the tumor cells (human or murine). In the clinic, therapy with interferon alfa-2a improves Kaposi's sarcoma, the vascular tumor associated with human immunodeficiency virus (HIV) infection [65]. White and colleagues observed remarkable regression of pulmonary hemangiomatosis in a 7-year old patient after interferon alfa-2a therapy (115). Orchard et al. reported that two cases of hemangioma in infants regressed after the administration of interferon alfa-2a [77]. Interferon alfa-2a alone and in combination has been shown to be effective in several anticancer regimens [27, 45, 49, 105, 115, 116].

TNP-470 is a synthetic analog of the fungal secretion product called fumagillin [47, 99, 100]. Fumagillin has been shown to cause endothelial cells in culture to round-up and to inhibit angiogenesis in the chick embryo-chorioallantoic membrane assay [47] but is toxic to animals. Among synthetic derivatives of fumagillin, TNP-470 is the most potent angiogenesis inhibitor and is relatively nontoxic to animals. TNP-470 is a potent inhibitor of endothelial cell migration [12], endothelial cell proliferation and capillary tube formation [47, 58]. TNP-470 also inhibits angiogenesis as demonstrated in chick embryo-chorioallantoic membrane and the rabbit and rodent cornea [58]. TNP-470 also has been shown to inhibit the growth of primary and metastatic murine tumors as well as human tumor xenografts [11, 13, 50, 86, 96, 104, 117, 118, 120].

A high soy food consumption has been hypothesized to be a factor in the lower incidence of certain human cancers in Asians than in others [7, 66]. Results from

animal experiments have suggested that the isoflavones in soybeans inhibit experimentally induced mammary tumors [7]. Genistein is a principal isoflavone in soybeans and is a protein tyrosine kinase inhibitor [17, 51, 55, 60, 64, 72, 79, 111, 112, 119, 122]. Genistein inhibits endothelial cell proliferation and in vitro angiogenesis at concentrations giving half-maximal inhibition of 5 and 150 μM, respectively [34].

The current studies were undertaken based on the hypothesis that the addition of effective antiangiogenic therapy to cytotoxic therapy would result in highly effective treatment regimens.

Material and methods

Drugs

Human interferon δ–4, a genetically engineered hybrid that consists of residues 5–62 of human interferon α2 and residues 64–166 of human interferon α1 that is active in murine systems was a gift from Dr. Paul Trotta (Schering Corporation, Bloomfield, N.J.). Suramin was a gift from Dr. Linda Paradiso (Parke-Davis Pharmaceutical Research, Warner-Lambert Co., Ann Arbor, Mich.). Genistein was purchased from ICN Biomedicals, (Aurora, Ohio). TNP-470 (AGM-1470) was a gift from Dr. K. Kitazawa (Takeda, Osaka, Japan). β-Cyclodextrin tetradecasulfate (14(SO_4)βCD) was prepared in our laboratory as previously described [98, 99]. 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 growth delay experiments

The Lewis lung tumor was carried in male C57BL mice (Taconic, Germantown, N.Y.). 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 at 8 to 10 weeks of age on day 0. Animals were randomized into treatment groups of six per condition on day 3.

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 eight injections, beginning on day 4, and/or were treated with minocycline (10 mg/kg), suramin (10 mg/kg) or genistein (100 mg/kg) i.p. daily for days 4 to 18 after tumor implantation and/or were injected i.v. with interferon δ4 (0.25×10^6 U) on days 4 through 8 and/or were implanted s.c. with 14-day mini-osmotic pumps (Alzet pumps, model 2002; Alza, Palo Alto, Calif.) containing 14 (SO_4) βCD (1 g/kg) and THC (125 mg/kg), a molar ratio of 1:1.

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) was 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 implantation. Radiation was delivered locally to the tumor-bearing limb as 3-Gy fractions daily on days 7 to 11. Adriamycin (1.75 mg/kg) or 5-fluorouracil α(30 mg/kg) were administered i.p. 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 six animals and the experiment was repeated three times. Days of tumor growth delay are the mean ± standard error for the treatment group compared with the control [98–100].

Lung metastases

The external lung metastases from animals treated as described above on day 20 after tumor implantation were counted manually and recorded as large if ≥ 3 mm in diameter. The data shown are the means from 6 to 12 pairs of lungs. Untreated control animals died from lung metastases in days 21 to 25. Parentheses in Tables 2 and 4 indicate the number of metastases and percentage of the total number of metastases that were recorded as large [98–100].

Results

The Lewis lung carcinoma growing in C57BL mice was chosen for these studies because this tumor is relatively resistant to many cancer therapies and because it metastasizes avidly to the lungs from subcutaneous implants. Because the Lewis lung carcinoma begins neoangiogenesis on about day 4 after tumor cell implantation [42, 43], that day was chosen as the beginning point for treatment with each of the potential antiangiogenic agents. Interferon δ4 was the most effective single agent amongst the six potential antiangiogenic agents tested, producing a tumor growth delay of 4 days (Table 1). TNP-470 and genistein also had some activity as single agents. Interestingly, the antitumor activity of these potential antiangiogenic agents did not increase when administered to the animals as two-drug combinations; however, the combinations of TNP-470/interferon δ4, minocycline/interferon, genistein/interferon δ4 and minocycline/genistein produced significant tumor growth delays compared with untreated control tumors.

Two cytotoxic anticancer drugs, cyclophosphamide and adriamycin, were selected for the initial comparison of the potential of the various antiangiogenic treatments within a therapeutic regimen. Cyclophosphamide, a bifunctional antitumor alkylating agent, was very effective as a single agent against the Lewis lung carcinoma producing a growth delay of 19 days (Table 1). Four of the single antiangiogenic agents, minocycline, suramin, interferon δ4 and 14(SO_4)βCD/THC, were effective in producing increased tumor growth delay in combination with cyclophosphamide. Six two-agent combinations, TNP-470/minocyline, TNP-470/genistein, TNP-470/interferon δ4, TNP-470/14(SO_4)βCD/THC, minocycline/interferon δ4 and minocycline/14(SO_4)βCD/THC, were effective in increasing the tumor growth delay produced by cyclophosphamide. Adriamycin, a DNA intercalator and topoisomerase II inhibitor, administered as a single agent daily for 5 days produced a tumor growth delay of 5 days in the Lewis lung carcinoma (Table 1). Two of the single antiangiogenic agents, minocycline and 14(SO_4)βCD/THC, were effective in producing

Table 1 Growth delay of the Lewis lung tumor produced by potential antiangiogenic agents alone or along with a standard regimen of cyclophosphamide or adriamycin. Tumor growth delay is the difference in days for treated tumors to reach 500 mm^3 compared with untreated control tumors. Untreated control tumors reached 500 mm^3 in about 14 days (values are means ± SE of 15 animals). Cyclophosphamide (150 mg/kg) was administered by intraperitoneal injection on days 7, 9 and 11 after tumor cell implantation. Adriamycin (1.75 mg/kg) was administered by intraperitoneal injection daily on days 7 through 11. Minocycline (10 mg/kg), suramin (20 mg/kg) and genistein (100 mg/kg) were administered intraperitoneally daily on days 4 to 18. TNP-470 (30 mg/kg) was administered subcutaneously on alternate days for eight injections, beginning on day 4. Interferon δ4 (0.25×10^6 U) was administered intravenously daily on days 4 through 8. 14(SO_4)β-cyclodextrin (1 g/kg) along with tetrahydrocortisol (125 mg/kg) were administered over days 4 through 18 by subcutaneous continuous infusion from a mini-osmotic pump.

Treatment group	Tumor growth delay (days) Alone	+ Cyclophosphamide	+ Adriamycin
–	–	19.3 ± 1.7	5.3 ± 0.4
TNP-470 (30 mg/kg × 8)	2.1 ± 0.4*	25.3 ± 2.2*	5.6 ± 0.6
Minocycline (10 mg/kg × 14)	1.2 ± 0.4	29.1 ± 1.8*	7.4 ± 0.6
Suramin (20 mg/kg × 14)	1.4 ± 0.3	31.5 ± 2.7*	6.6 ± 0.7
Genistein (100 mg/kg × 14)	2.4 ± 0.4*	23.5 ± 1.8	6.0 ± 0.6
Interferon δ4 (0.25×10^6 U × 5)	4.0 ± 0.4*	27.7 ± 2.3*	5.1 ± 0.5
14(SO_4)βCD (1 g/kg over 14 days)			
Tetrahydrocortisol (125 mg/kg over 14 days)	0.6 ± 0.3	33.0 ± 2.8**	7.2 ± 0.6
TNP-470/Minocycline	1.8 ± 0.4	44.5 ± 3.3**	15.3 ± 1.3**
TNP-470/suramin	0.4 ± 0.3	25.7 ± 2.5*	6.6 ± 0.7
TNP-470/genistein	1.9 ± 0.3	36.1 ± 2.8**	9.8 ± 1.1**
TNP-470/interferon δ4	2.5 ± 0.4*	37.7 ± 3.0**	9.9 ± 1.1**
TNP-470/14(SO_4)βCD/THC	1.5 ± 0.3	49.2 ± 3.6**	12.3 ± 1.5**
Minocycline/suramin	0.5 ± 0.3	21.2 ± 1.8	6.6 ± 0.5
Minocycline/genistein	2.4 ± 0.4*	21.3 ± 1.9	9.1 ± 0.9*
Minocycline/interferon δ4	2.6 ± 0.4*	31.5 ± 2.9**	7.4 ± 0.6
Minocycline/14(SO_4)βCD/THC	1.2 ± 0.3	48.8 ± 3.5**	10.6 ± 1.1**
Suramin/genistein	1.2 ± 0.3	19.6 ± 1.8	7.4 ± 0.5
Suramin/interferon δ4	1.3 ± 0.4	18.6 ± 1.9	7.8 ± 0.7
Genistein/interferon δ4	3.2 ± 0.4*	24.1 ± 2.1	6.7 ± 0.6

* $P < 0.01$; ** $P < 0.005$ compared with untreated control or compared with cyclophosphamide or adriamycin

increased tumor growth delay in combination with adriamycin. Six two-agent combinations, TNP-470/minocyline, TNP-470/genistein, TNP-470/interferon δ4, TNP-470/14(SO_4)βCD/THC, minocycline/genistein and minocycline/14(SO_4)βCD/THC, were effective in increasing the tumor growth delay produced by adriamycin. In general, combination regimens including suramin were less effective therapeutically.

None of the potential antiangiogenic agents, single or in two-agent combinations, reduced the number of lung metastases on day 20 in animals implanted subcutaneously with the Lewis lung tumor (Table 2). However, four of the potential antiangiogenic therapies, genistein, interferon δ4, TNP-470/interferon S4 and minocycline/interferon δ4, significantly decreased the percentage of large lung metastases (≥ 3 mm in diameter) potentially vascularized on day 20. Cyclophosphamide treatment decreased the number and percentage of large lung metastases on day 20 from that in untreated controls. Four of the potential antiangiogenic agents, TNP-470, minocycline, suramin and 14(SO_4)βCD/THC, administered along with cyclophosphamide further decreased the number and percentage of large lung metastases from treatment with cyclophosphamide. Four of the potential antiangiogenic combinations, TNP-470/minocyline, TNP-470/suramin, TNP-470/14(SO_4)βCD/THC and minocycline/14(SO_4)βCD/THC, produced significant decreases in the number of lung metastases when administered along with cyclophosphamide compared with cyclophosphamide alone. Treatment of the Lewis lung tumor-bearing animals with adriamycin as a single agent did not decrease the number of lung metastases on day 20 but did decrease the percentage of lung metastases large enough to be vascularized on day 20 compared with untreated control animals (Table 2). Only four potential antiangiogenic treatments, 14(SO_4)βCD/THC, TNP-470/interferon δ4, minocycline/interferon δ4 and minocycline/14(SO_4)βCD/THC, were effective in decreasing the number of lung metastases when administered along with adriamycin compared with adriamycin alone.

Several potential antiangiogenic treatments including TNP-470 were studied in therapeutic regimens with other cytotoxic anticancer treatments (Tables 3 and 4). CDDP, an antitumor alkylating agent, produced a tumor growth delay of 4.5 days when administered as a single agent to animals bearing the Lewis lung carcinoma. Treatment with TNP-470/minocycline or TNP-470/14(SO_4)βCD/THC along with CDDP more than

Table 2 Number of lung metastases on day 20 from subcutaneous Lewis lung tumors produced by potential antiangiogenic agents alone or along with a standard regimen of cyclophosphamide or adriamycin

Treatment group	Mean number of lung metastases (% large)		
	Alone	+ Cyclophosphamide	+ Adriamycin
–	21 (63)	12 (40)	18 (39)
TNP-470 (30 mg/kg × 8)	21 (51)	6 (30)	15 (48)
Minocycline (10 mg/kg × 14)	20 (50)	6 (33)	15 (57)
Suramin (20 mg/kg × 14)	19 (47)	5 (33)	15 (38)
Genistein (100 mg/kg × 14)	22 (41)	10 (29)	15 (40)
Interferon δ4 (0.25×10^6 U × 5)	21 (40)	12 (38)	19 (45)
$14(SO_4)$βCD (1 g/kg over 14 days)			
Tetrahydrocortisol (125 mg/kg over 14 days)	20 (60)	6 (32)	7 (35)
TNP-470/minocycline	18 (54)	2 (25)	15 (45)
TNP-470/suramin	23 (43)	7 (27)	14 (45)
TNP-470/genistein	21 (60)	11.5 (33)	22 (49)
TNP-470/interferon δ4	22 (35)	10 (29)	16 (36)
TNP-470/$14(SO_4)$βCD/THC	18 (50)	2 (50)	15 (40)
Minocycline/suramin	21 (47)	9 (23)	16 (52)
Minocycline/genistein	19 (53)	12 (35)	22 (32)
Minocycline/interferon δ4	21 (37)	11 (34)	13 (48)
Minocycline/$14(SO_4)$βCD/THC	17 (46)	0.5 (20)	7 (57)
Suramin/genistein	21 (54)	12 (40)	15 (40)
Suramin/interferon δ4	20 (50)	12 (50)	17 (41)
Genistein/interferon δ4	20 (50)	9 (26)	18 (39)

Table 3 Growth delay of Lewis lung tumors produced by potential antiangiogenic agents along with various cytotoxic anticancer therapies. Tumor growth delay is the difference in days for treated tumors to reach 500 mm^3 compared with untreated control tumors. Untreated control tumors reached 500 mm^3 in about 14 days. (values are means ± SE of 15 animals). The antiangiogenic agents were administered as described for Table 1. CDDP (10 mg/kg) was administered by intraperitoneal injection on day 7. BCNU (15 mg/kg) was administered by intraperitoneal injection on days 7, 9 and 11. 5-Fluorouracil (30 mg/kg) was administered by intraperitoneal injection daily on days 7 through 11. X-rays were delivered daily on days 7 to 11 locally to the tumor-bearing limb.

Treatment group	Tumor growth delay (days)					
	Alone	TNP-470/ Minocycline	TNP-470/ suramin	TNP-470/ genistein	TNP-470/ interferon δ4	TNP-470/14 (SO_4)βCD/THC
CDDP (10 mg/kg)	4.5 ± 0.3	10.9 ± 0.8**	8.6 ± 1.0*	7.7 ± 0.6*	8.5 ± 0.7*	10.6 ± 0.7**
BCNU (15 mg/kg × 3)	3.6 ± 0.4	14.6 ± 1.2**	7.0 ± 0.8*	8.0 ± 0.8*	10.3 ± 1.0**	10.6 ± 1.1**
X-rays (3 Gy × 5)	4.4 ± 0.3	15.3 ± 1.3**	5.5 ± 0.5	9.3 ± 0.9*	9.9 ± 1.0**	10.3 ± 0.9**
5-Fluorouracil (30 mg/kg × 5)	3.6 ± 0.3	8.4 ± 0.9*	3.8 ± 0.4	6.6 ± 0.7	4.9 ± 0.5	8.5 ± 0.6*

* $P < 0.01$; ** $P < 0.005$ compared with the cytotoxic therapy alone

doubled the tumor growth delay produced by the drug. BCNU, a nitrosourea which undergoes a complex chemical breakdown resulting in DNA alkylation and strand-breaking, produced 3.6 days of tumor growth delay of the Lewis lung carcinoma. The most effective antiangiogenic treatments in combination with BCNU were TNP-470/minocyline, TNP-470/interferon δ4 and TNP-470/$14(SO_4)$βCD/THC resulting in a three- to four-fold increase in the tumor growth delay produced. Fractionated radiation therapy is cytotoxic primarily by production of DNA strand-breaks via oxygen radicals. Five fractions of 3 Gy produced a tumor growth delay of 4.4 days. The antiangiogenic treatments, TNP-470/minocyline, TNP-470/interferon δ4 and TNP-470/$14(SO_4)$βCD/THC were most effective in increasing the tumor growth delay produced by fractionated radiation therapy. 5-Fluorouracil, an antitumor antimetabolite, produced a growth delay of 3.6 days in the Lewis lung carcinoma. Administration of TNP-470/minocycline or TNP-470/$14(SO_4)$βCD/THC along with 5-fluorouracil resulted in a greater than two-fold increase in the tumor growth delay produced by 5-fluorouracil. None of these antiangiogenic treatment combinations decreased the number of lung metastases on day 20 when administered along with the cytotoxic therapies compared with the cytotoxic therapies alone (Table 4).

Discussion

Excitement regarding the potential of antiangiogenic therapy was widely generated by the report of White

Table 4 Number of lung metastases on day 20 from subcutaneous Lewis lung tumors produced by potential antiangiogenic agents along with various cytotoxic anticancer therapies

Treatment group	Mean number of lung metastases (% large) TNP-470 Alone	TNP-470/ Minocycline	TNP-470/ suramin	TNP-470/ genistein	TNP-470/ interferon δ	TNP-470/14 (SO_4)βCD/THC
CDDP (10 mg/kg)	15 (34)	18 (45)	14 (38)	14 (37)	15 (39)	18 (50)
BCNU (15 mg/kg × 3)	18 (41)	13 (38)	16 (30)	16 (40)	17 (28)	14 (43)
X-rays (3 Gy × 5)	18 (40)	12 (42)	16 (30)	15 (30)	15 (34)	11 (36)
5-Fluorouracil (30 mg/kg × 5)	22 (38)	21 (36)	24 (41)	21 (37)	20 (33)	15 (45)

et al. [115] who used interferon α to treat a pulmonary hemangiomatosis. This treatment not only prevented further progress of the disease but resulted in regression of the excess capillaries in the patient's lungs. Since that time, many clinical studies have appeared correlating angiogenesis-related properties such as serum or urine levels of b-FGF and microvessel density by immunohistochemical staining with prognosis for disease-free survival [1, 23, 35, 71, 107, 113, 114]. Of these, the most notable was a report by Weidner et al. [114] of a prospective, blinded study of 165 consecutive patients with primary invasive breast carcinoma in which the microvessels were highlighted by immunocytochemical staining to detect factor VIII-related antigen. Weidner et al. [114] concluded that microvessel density in the area of the most intense neovascularization in invasive breast carcinoma is an independent and highly significant prognostic indicator for overall and relapse-free survival in patients with early-stage breast carcinoma.

The molecules described herein as antiangiogenic agents represent a wide variety of molecular structures with a wide variety of biological effects and targets. Most often these agents have been generally classified as antiangiogenic by their effects in an in vitro bioassay system. The diversity in this group of molecules gives strength to the potential of this approach in therapeutic applications. The biological and biochemical pathways involved in angiogenesis are numerous and redundant. It is likely that there are many angiogenic factors and many pathways by which to promote tumor invasion. Therefore, it is likely that blockade of more than one of the pathways related to angiogenesis and/or invasion will be necessary to impact on the natural progress of a malignant disease. The incorporation of antiangiogenic agents into therapeutic regimens represents an important challenge.

The successful treatment of cancer requires the eradication of all malignant cells and, therefore, treatment with cytotoxic therapies. The compatibility of antiangiogenic therapy with cytotoxic therapeutic treatments is not obvious. If the angiogenic and invasive characteristics of a tumor can be identified, that is expression of angiogenic factors and expression of extracellular proteases, and the molecular targets of antiangiogenic agents elucidated, then tumor-specific antiangiogenic therapeutic regimens could be developed. Northern blot analysis has shown that the Lewis lung carcinoma expresses high levels of b-FGF and low levels of VEGF (b-FGF : VEGF; 10 : 1). In this study the most generally efficacious antiangiogenic treatments were: (1) TNP-470/minocycline, (2) TNP-470/interferon δ4, (3) TNP-470/14(SO_4)βCD/THC and (4) minocycline/14(SO_4)βCD/THC. Although the mechanism(s) of action of these agents have not been fully elucidated, it is most probable that each of these has a different molecular target. There was no clear benefit achieved by using antiangiogenic agent combinations alone compared with the single agents. Suramin, in particular, was more effective as a single agent than when administered with other potential antiangiogenic agents. For the most part, those treatment combinations that were most effective against the primary subcutaneous tumors were also most effective in reducing the number of lung metastases on day 20.

The most effective cytotoxic therapy used in this study was cyclophosphamide. By increasing the tumor growth delay produced by cyclophosphamide in this tumor by 2- to 2.5-fold using the antiangiogenic treatments, TNP-470/minocyline, TNP-470/14(SO_4)βCD/THC or minocycline/14(SO_4)βCD/THC, 40–50% cures have been achieved [100]. Increases in tumor growth delay of this same order of magnitude were also achieved with these and other antiangiogenic treatments as shown on Table 3. However, cures were not seen, because the cytotoxic therapies were not as effective as cyclophosphamide. Perhaps the important factor in translating these findings to clinical studies is that the addition of antiangiogenic agents to treatment is most likely to make very good therapeutic regimens better.

The focus of this study was on the therapeutic application of antiangiogenic agents to the treatment of solid tumors. The results in this in vivo model of an established solid tumor were very positive and provide direction for future clinical trials including these drugs. Two general conclusions may be drawn. First, combinations of antiangiogenic agents evoke a greater effect on tumor response to therapy than does treatment with single agents of this type. Second, treatment with antiangiogenic agents can interact in a positive way with a variety of cytotoxic therapies.

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