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TNP-470/Minocycline/Cytotoxic Therapy: A Systems Approach to Cancer Therapy

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INTRODUCTION

AMONG THE various analogies that have been used to characterise the growth and invasiveness of solid malignant tumours, that of Dr Stephen Paget, known as the seed-and-soil hypothesis, has continued to provoke thought for over a 100 years [1, 2]. Paget, mainly through observations made by the study of autopsy data, realised that there existed predictable patterns of metastasis from specific tumours. Paget concluded that the soil of certain tissues must be more favourable to the growth of metastasis than that of other tissues [1, 2], and that the "remote organs cannot be altogether passive or indifferent" to the process of tumour growth. In other words, Paget recognised that the tissue in which malignant cells have implanted must be pro-actively involved in order for a tumour to grow. Although Paget's report applied specifically to metastatic disease, these same concepts apply to primary malignant disease—without the active involvement of normal cells in the vicinity of a malignant cell colony, a tumour will not grow. These normal cells are a major component of malignancy [3–10]. They are proliferating and they are invading. The corollary is that both the normal cells and the malignant cells involved in tumour growth as well as the chemical and mechanical signalling pathways that interconnect them are valid targets for therapeutic intervention. The recognised normal tissue compartment targets for therapeutic intervention are vascular components, extracellular matrix components, stromal and infiltrating cells. The ratio of these components can vary greatly so that some tumours appear to be masses of malignant cells, while in others, such as Hodgkin's disease, it is difficult to find the malignant cell. The integration of these concepts with classical cytotoxic anticancer therapies may be regarded as a systems approach to cancer treatment.

Tumours are dynamic, complex, living tissues undergoing the varied processes of tissue growth under the guidance of aberrant malignant cells. Cytotoxic anticancer therapies have focused solely on eradication of the malignant cell which is an absolute necessity in cancer therapy; however, even the most heroic therapeutic strategies rarely achieve cure of many tumour types. A broader look at the tumour reminds us that the growth processes of the tumour are normal processes, that the invasion processes of the tumour are normal processes, and that it is the inappropriate activation of these processes that comprises the morbidity of malignant disease. The tools are now at hand to make an important step forward in the therapeutic approach to solid tumours, that is, without losing sight of the importance of eradicating the malignant cell populations, to block normal processes critical to tumour maintenance and growth (and spread).

The question arises of how to integrate these new therapeutic agents into existing cancer treatment regimens which have been developed through great effort and ingenuity. These additional therapeutic agents are clearly directed toward new targets, that is, normal cells and extracellular enzymatic activities. Although these targets are critical to tumour growth, it is highly unlikely that agents directed toward these targets will lead to tumour cure. Therefore, the systems approach to therapy of choosing multiple targets to the goal would maintain cytotoxic therapy while incorporating new non-cytotoxic strategies. The current report will focus on our studies combining the administration of TNP-470, an agent directed toward proliferating endothelial cells, and minocycline, an agent directed toward extracellular matrix metalloproteinase activity, with standard cytotoxic anticancer therapies.

TNP-470 is a synthetic derivative of fumagillin, an antibiotic which has little antibacterial or antifungal activity but marked amoebicidal activity [11, 12]. It is a potent inhibitor of endothelial cell migration [13], endothelial cell proliferation [14] and capillary tube formation [15]. TNP-470 also inhibits angiogenesis as demonstrated in chick chorioallantoic membrane, the rabbit and rodent cornea [15]. TNP-470 has been shown to inhibit the growth of primary and metastatic murine tumours as well as human tumour xenografts [16–24]. Tetracycline antibiotics can inhibit tissue collagenase activity and tetracycline administration has been used in the treatment of periodontal disease [25], gingival collagenolytic activity in diabetes [25, 26], and to inhibit joint deterioration in patients with rheumatoid arthritis [27–29]. This inhibitory activity has been associated with both gelatinase (type IV collagenase) and interstitial collagenase [30]. Tamargo and associated [31] first reported that minocycline, a semisynthetic tetracycline with a relatively long

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circulating half-life, inhibited neovascularisation in the rabbit cornea implanted with the VX2 carcinoma.

To determine whether clinical regimens including TNP-470 and minocycline (Mino) have broad clinical applicability, studies were carried out in three solid tumour models: the Lewis lung carcinoma, the EMT-6 mammary carcinoma and the 9L gliosarcoma.

TNP-470, MINOCYCLINE AND RADIOTHERAPY

Although it may be ideal to initiate anti-angiogenic therapy prior to the onset of angiogenesis in a tumour macro-colony, it is unlikely that opportunity will arise in the clinic. However, to establish whether anti-angiogenic therapy could be compatible with cytotoxic therapies, administration of TNP-470 (subcutaneous, 30 mg/kg for mice and 25 mg/kg for rats, on alternative days, 8 injections) after and Mino (intraperitoneal, 10 mg/kg, daily, d4–18) was begun on day 4 post-tumour cell implantation in animals bearing the Lewis lung carcinoma, the EMT-6 mammary carcinoma or the 9L gliosarcoma when the tumours were a seed (2–4 mm^3). The administration of TNP-470 and Mino from day 4 to day 18 after tumour cell implantation resulted in small tumour growth delays in the Lewis lung carcinoma and the 9L gliosarcoma, but had no notable effect on the growth of the EMT-6 tumour (Table 1) [35–37, 40, 42, 49]. If radiation therapy alone, delivered locally to the tumour-bearing limb in fractions of 3 Gy daily for 5 days on days 7–11, was administered, each of the three tumours was moderately responsive (Table 1). When the animals were treated with TNP-470 and Mino prior to, during and after radiation therapy, there was a marked increase in the delay to tumour growth (Table 1). The increase in the delay to tumour growth was 3.5-fold, 2.2-fold and 1.6-fold for the Lewis lung carcinoma, the EMT-6 mammary carcinoma and the 9L gliosarcoma, respectively, compared to radiation therapy alone.

Although radiation is not often considered a molecular therapy, tissue response to radiation exposure is critically dependent upon the oxygen content of that tissue. Therefore, polarographic electrode measurements were used to determine the effect of administration of TNP-470/Mino on the oxygen content of each of the three tumours. The hypoxic fraction was defined as the percentage of the pO_2 readings < 5 mmHg. The Lewis lung carcinoma and the EMT-6 mammary carcinoma were very hypoxic under normal air conditions (Figure 1a,b). When animals bearing these tumours were treated with TNP-470/Mino for 5 days prior to the oxygen measurements, the hypoxic fractions of the tumours decreased from 92% to 75% for Lewis lung carcinoma and from 90% to 68% for EMT-6. The perflubron emulsion is an oxygen delivery agent which functions when a high oxygen content atmosphere is inspired [46, 47]. When the perflubron emulsion was administered to animals treated with TNP-470/Mino and the animals were allowed to breath carbogen (95% oxygen) during the pO_2 measurements, the hypoxic fractions in the Lewis lung carcinoma and the EMT-6 mammary were reduced to 45% and 37%, respectively. The hypoxic fraction of the 9L gliosarcoma growing subcutaneously in the hind-leg of the rat was 71% in air. When the animals were treated with TNP-470/Mino for 5 days prior to the pO_2 measurements, the hypoxic fraction decreased to 64%. With the addition of administration of the perflubron emulsion and carbogen breathing, treatment with TNP-470/Mino resulted in reduction of the hypoxic fraction to 34%.

Similar experiments were conducted using the human MCF-7 breast carcinoma cell line (Figure 1d). The administration of TNP-470 and Mino to female nude mice bearing human MCF-7 breast carcinoma xenografts resulted in a decrease of the hypoxic fraction of the tumour in air from 73% to 65%. Administration of perflubron emulsion and breathing carbogen together with TNP-470 and Mino further decreased the hypoxic fraction to 51%.

TNP-470, MINOCYCLINE AND CYTOTOXIC THERAPIES

Most anticancer chemotherapeutic agents are small molecules which diffuse from the vasculature through the cell layers into tumours. When animals bearing each of the three tumours were treated with the antitumour alkylating agent cyclophosphamide (150 mg/kg for for mice, 100 mg/kg for rats i.p. days 7, 9 and 11), the response of the tumour varied from 20.5 days growth delay in the Lewis lung carcinoma to 6.2 days growth delay in the EMT-6 mammary carcinoma, with 9.1 days growth delay in the 9L gliosarcoma (Table 2). The addition of the administration of TNP-470/Mino to treatment with cyclophosphamide markedly increased the growth delay produced by the drug by 2.2-fold, 2.2-fold and 1.6-fold in the Lewis lung tumours, the EMT-6 tumour and the 9L gliosarcoma, respectively. The delay in tumour growth for animals bearing the Lewis lung carcinoma was determined only for animals in which the tumours grew, with 40% of animals treated with TNP-470/Mino/cyclophosphamide cured.

Table 1. Growth delay in three solid tumour models produced by fractionated radiation therapy alone or with administration of TNP-470 and minocycline

	Delay in tumour growth (days)*		
	TNP-470/Mino	X-rays	TNP-470/Mino/X-rays
Tumour			
Lewis lung carcinoma	1.8 ± 0.4	4.4 ± 0.3	15.3 ± 1.2
EMT-6 mammary carcinoma	0.7 ± 0.3	4.5 ± 0.8	9.8 ± 0.9
9L gliosarcoma	2.0 ± 0.4	5.4 ± 0.4	8.8 ± 0.7

* Tumour growth delay is the difference in days for treated tumours to reach 500 mm^3 compared with untreated control tumours. Untreated control tumours reach 500 mm^3 in approximately 14 days, 12 days and 19 days for the Lewis lung carcinoma, the EMT-6 mammary carcinoma and the 9L gliosarcoma, respectively. Mean ± SE of 15 animals. Minocycline (10 mg/kg) was administered i.p. daily on days 4–18. TNP-470 (30 mg/kg for mice and 25 mg/kg for rats) was administered s.c. on alternate days for eight injections, beginning on day 4. X-rays were delivered daily on days 7–11 locally to the tumour-bearing limb in fractions of 3 Gy. Each treatment group consisted of 5 animals for murine tumours and 4 for rats, and each experiment was repeated three times.

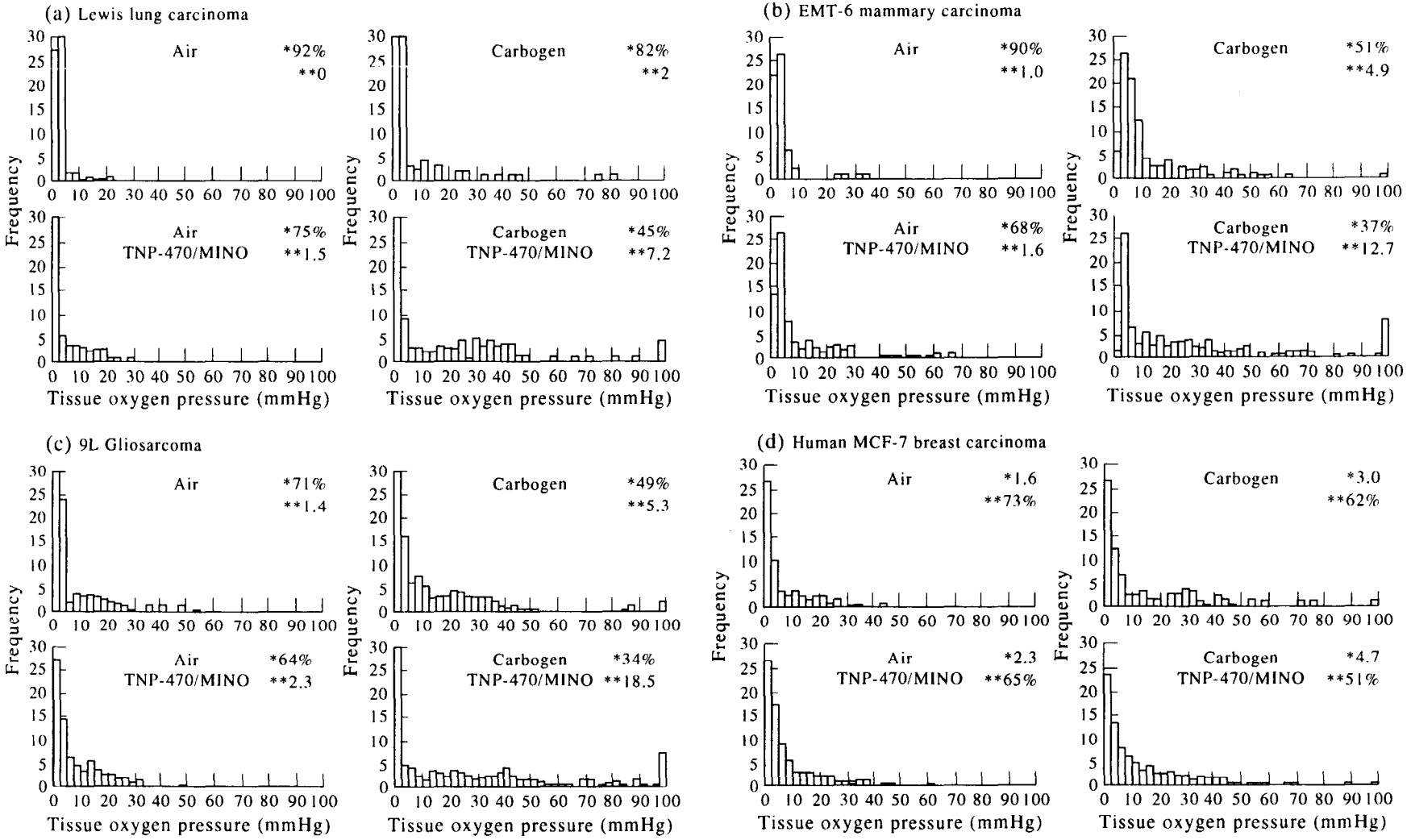

Figure 1. Histograms showing the oxygen profiles as well as the per cent of pO_2 readings* and median pO_2 values of the (a) murine Lewis lung carcinoma, (b) murine EMT-6 mammary carcinoma; (c) rat 9L gliosarcoma; and (d) human MCF-7 breast carcinoma measurements were made while the animals were breathing normal air and 15 min after intravenous administration of the oxygen delivery agent, perflubron emulsion (8 ml/kg), and initiation of carbogen (95% oxygen/5% carbon dioxide) breathing. Oxygen profiles are shown for tumours with no treatment or with TNP-470 (30 mg/kg for mice and 25 mg/kg for rats) administration subcutaneously on alternate days beginning on day 4 along with minocycline (10 mg/kg) administration intraperitoneally daily on days 4–8. Oxygen determinations were made on day 9 using an Eppendorf pO_2 histograph. Each profile represents 10 tumours with 50–60 measurements per tumour, therefore, n = 500–600 pO_2 measurements per histogram [47, 48].**

Table 2. Growth delay in three solid tumour models produced by chemotherapeutic agents alone or with administration of TNP-470 and minocycline

	Delay in tumour growth (days)*		
Tumour	TNP/Mino	CTX	TNP-470/Mino/CTX
Lewis lung carcinoma	1.8 + 0.4	20.5 + 1.7	44.8 + 2.8†
EMT-6 mammary carcinoma	0.7 ± 0.3	6.2 ± 0.5	13.8 ± 0.8
9L gliosarcoma	2.0 ± 0.4	9.1 ± 0.7	14.8 ± 1.1
		BCNU	TNP-470/Mino/BCNU
Lewis lung carcinoma		3.6 ± 0.4	14.6 ± 1.0
EMT-6 mammary carcinoma		5.1 ± 0.5	10.5 ± 1.3
9L gliosarcoma		5.3 ± 0.4	9.9 ± 0.8
		CDDP	TNP-470/Mino/CDDP
Lewis lung carcinoma		4.5 ± 0.3	10.9 ± 0.8
EMT-6 mammary carcinoma		7.5 ± 0.8	13.9 ± 1.1
9L gliosarcoma		9.4 ± 0.8	16.0 ± 1.4

* As described in footnote to Table 1. Cyclophosphamide (CTX) (150 mg/kg for mice and 100 mg/kg for rats) was administered i.p. on days 7, 9 and 11. BCNU (15 mg/kg) was administered i.p. on days 7, 9 and 11. CDDP (10 mg/kg for mice and 8 mg/kg for rats) was administered i.p. on day 7. † Delay in tumour growth in animals developing tumours. Forty per cent of treated animals with Lewis lung carcinoma were cured.

When animals bearing each of the three tumours were treated with the nitrosourea BCNU (15 mg/kg, i.p., days 7, 9 and 11), tumour growth delay between 3.6 and 5.3 days was observed (Table 2). Administration of TNP-470 and Mino together with BCNU resulted in marked increases in growth delay of 4.1-fold, 2.1-fold and 1.9-fold in animals bearing the Lewis lung, the EMT-6 and the 9L tumour, respectively. Treatment with cisplatin (CDDP 10 mg/kg for mice, 8 mg/kg for rats, i.p., day 7), produced a growth delay of between 4.5 days and 9.4 days, and co-administration of TNP-470 and Mino resulted in a marked increase in growth delay of 2.4-fold, 1.9-fold and 1.7-fold in animals bearing the Lewis lung, the EMT-6 and the 9L tumour respectively (Table 2).

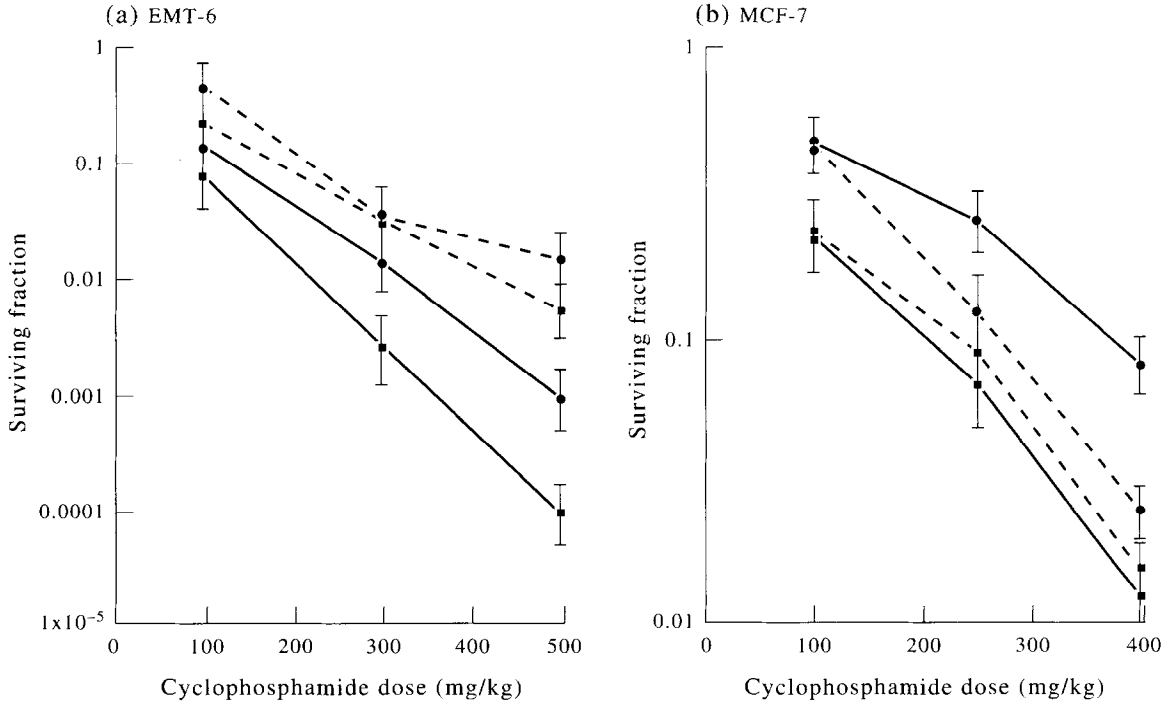

Figure 2. Survival of (a) EMT-6 or (b) MCF-7 tumour cells (——) or bone marrow CFU-GM (- - - -) after treatment of tumour bearing animals with single doses of cyclophosphamide alone (●) (on day 8) or in combination with TNP-40 (30 mg/kg, s.c. on days 4, 6 and 8) and minocycline (10 mg/kg, i.p.) on days 4–8 (■). Data are the means of three independent experiments; bars are S.E.M. Animals were sacrificed 24 h after completion of treatment. Four tumours from two animals were pooled for each treatment group, and 500 mg of tumour were used to make each single cell suspension. Bone marrow from the femurs of the same animals was used to determine the survival of the CFU-GM. The cell suspensions were plated in duplicate at three different concentrations of the colony-forming assay. The results are expressed as the surviving fraction of the cells from treated groups as compared with untreated controls [35, 38, 39]

A comparison was made between the effect of TNP-470 and Mino administration on the killing of tumour cells and the killing of bone marrow CFU-GM, as a representative sensitive normal tissue. Animals bearing the EMT-6 tumour or the MCF-7 breast carcinoma xenograft were treated with cyclophosphamide (100, 300, 500 mg/kg, i.p., day 8) alone or with TNP-470 (30 mg/kg, s.c., days 4, 6 and 8) and Mino (10 mg/kg, i.p., days 4–8). The EMT-6 tumour was more sensitive to cyclophosphamide than the MCF-7 tumour such that a cyclophosphamide dose of 400 mg/kg killed approximately 2.5 logs of EMT-6 cells and approximately 1.3 logs of MCF-7 cells (Figure 2). Administration of TNP-470 and Mino to animals bearing the EMT-6 tumour resulted in increased tumour cell killing, which increased with higher cyclophosphamide doses. At a cyclophosphamide dose of 500 mg/kg, there was a 9-fold increase in EMT-6 tumour cell killing in the animals receiving TNP-470 and Mino compared with those receiving cyclophosphamide only. There was also an increase in MCF-7 tumour cell killing in animals treated with TNP-470 and Mino and cyclophosphamide, and at a cyclophosphamide dose of 400 mg/kg, there was a 7-fold increase in MCF-7 tumour cell killing in animals also treated with TNP-470 and Mino compared with those receiving cyclophosphamide only. Bone marrow sensitivity to cyclophosphamide was not drastically altered by the addition of TNP-470 and Mino (Figure 2).

CONCLUSION

The vasculature forms the first barrier to penetration of molecules into tumours. Although the anti-angiogenic agent treatments administered in this study did not completely inhibit angiogenesis in these tumours, the vasculature present in the treated tumours may be impaired compared to control tumours. It may be that under anti-angiogenic treatment conditions, tumours develop a blood flow system resembling sinusoids of the liver or spleen where the endothelial lining is discontinuous and the basement membrane is incomplete [50, 51]. Each of the three animal tumours described were able to grow at normal or near normal rates during the 2 weeks of anti-angiogenic therapy. The hypothesis is that the main targets for the anti-angiogenic agents are extracellular matrix processes and/or tumour endothelial cells, and that inhibition and/or impairment of these non-malignant functions can improve therapeutic responses when agents directed toward these targets are used in combination with cytotoxic therapies.

The incorporation of anti-angiogenic agents and/or anti-metastatic 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 anti-angiogenic therapy and/or anti-invasion agents with cytotoxic chemotherapeutic agents is not obvious [52]. While it could be possible that with anti-angiogenic therapy a less vascular tumour would develop thus producing a tissue mass where the delivery of large and small molecules to the malignant cells would be more difficult, the result of anti-angiogenic therapy in the three animal tumours studied appeared to be a tissue mass where delivery of small molecules occurred more readily [35–37, 40, 42, 49]. Anti-angiogenic therapy increased the response of three different animal tumours to both radiation therapy and chemotherapy. This result demonstrates that this therapeutic strategy may be broadly applicable in the clinic and may be broadly compatible with cytotoxic therapies.

It is likely that there will be heterogeneity in the responsiveness of tumours to anti-angiogenic therapy. Within the small sample size of three animal tumours in this study, the responsiveness of the Lewis lung carcinoma was most increased when TNP-470 and Mino was administered along with the cytotoxic therapies. Only when a very good cytotoxic therapy (cyclophosphamide) was combined with TNP-470 and Mino administration as a treatment regimen for the Lewis lung tumour were cures obtained. The human MCF-7 breast carcinoma xenograft was as responsive as the murine EMT-6 mammary carcinoma to treatment with TNP-470 and Mino both in increased tumour oxygenation and in increased tumour cell killing by cyclophosphamide. Although the vasculature of the MCF-7 xenografts is murine, this finding provides an indication that administration of TNP-470 and Mino in a treatment regimen along with chemotherapy and/or radiation therapy could improve treatment outcome in patients.

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