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Combretastatin A-4, an Agent That Displays Potent and Selective Toxicity toward Tumor Vasculature¹

Graham G. Dark,² Sally A. Hill, Vivien E. Prise, Gillian M. Tozer, George R. Pettit, and Dai J. Chaplin

Tumour Microcirculation Group, Gray Laboratory Cancer Research Trust, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, United Kingdom [G. G. D., S. A. H., V. E. P., G. M. T., D. J. C.], and Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, Arizona 85287 [G. R. P.]

Abstract

Selective induction of vascular damage within tumors represents an emerging approach to cancer treatment. Histological studies have shown that several tubulin-binding agents can induce vascular damage within tumors but only at doses approximating the maximum tolerated dose, which has limited their clinical applicability. In this study, we show that the combretastatin A-4 prodrug induces vascular shutdown within tumors at doses less than one-tenth of the maximum tolerated dose. *In vitro* studies indicate that a short drug exposure results in profound long-term antiproliferative/cytotoxic effects against proliferating endothelial cells but not cells that are quiescent prior to and during drug exposure. Vascular shutdown, within experimental and human breast cancer models *in vivo* following systemic drug administration, was demonstrated with a reduction in functional vascular volume of 93% at 6 h following drug administration and persisted over the next 12 h, with corresponding histology consistent with hemorrhagic necrosis resulting from vascular damage. These actions against tumor vasculature and the broad therapeutic window demonstrate the clinical potential of these drugs and warrant further study to elucidate the mechanisms responsible for the antivasular effects of combretastatin A-4.

Introduction

Vasculature is critical to both the survival of a solid tumor mass and its continued growth. Tumor endothelium, therefore, represents a key target for the development of new approaches to cancer therapy. Most of the research efforts in this area have focused on the discovery of antiangiogenic agents (1). Two of the agents that have been shown to possess selective effects in preventing endothelial cell proliferation *in vitro* and tumor growth *in vivo* are fumagillol (2) and pentosan polysulfate (3). More recently, other agents such as linomide (4), thalidomide (5), and 2-methoxy-estradiol have been found to inhibit the process of angiogenesis (6). The increasing focus on antiangiogenic therapy has led to the rational search for more selective agents. Of particular interest are drugs that selectively inhibit the tyrosine kinase activity of Flk-1, the receptor for the angiogenic growth factor, vascular endothelial growth factor (7). However, in contrast to the antiproliferative effects of antiangiogenic therapy, antivasular approaches aim to cause a rapid and catastrophic shutdown in the vascular function of the tumor, leading to extensive secondary tumor cell death.

It has been established that several low molecular weight drugs can elicit irreversible vascular shutdown selectively within solid tumors. These drugs in general fall into two classes, the tubulin-binding agents

(8, 9) and drugs related to flavone acetic acid (10–13). Over the last decade, much effort at the preclinical level has focused on this latter group of compounds, which has led to the entry of dimethylxanthone-acetic acid into Phase I clinical trials in the United Kingdom and New Zealand. In contrast, the vascular-damaging effects of the tubulin-binding agents have received relatively little attention.

Colchicine was the first tubulin-binding agent noted to have some antivasular action, producing hemorrhagic necrosis in experimental tumours that resembled that produced by bacterial toxins (14). Furthermore, it was noted that the endothelial cells of growing capillaries appeared sensitive to its toxic actions (15). The therapeutic window was, however, very limited, and studies were curtailed due to toxicity. In recent years, a number of compounds have been isolated from the stem wood of the South African tree *Combretum caffrum*. These combretastatins show structural similarity to colchicine and possess a higher affinity for the colchicine binding site on tubulin than colchicine itself (16). We have reported previously that several novel tubulin-binding agents, including the combretastatins, could induce blood flow reduction in an experimental tumor system (8). This study was designed to assess, in more detail, the endothelial and antivasular effects of combretastatin A-4 and establish if it possessed a larger therapeutic window for its antivasular effects than agents studied previously.

Materials and Methods

Cell Culture. Our studies were performed with cells incubated under 1% O₂ (pO₂, 7.6 mmHg), 5% CO₂, balance N₂, because this may be more representative of the oxygenation within the tumor microenvironment. Indeed, studies have indicated that reduced oxygen tensions (pO₂ < 15 mmHg) are a common feature of human and experimental tumors (17). Furthermore, there is increasing evidence that even the endothelium in tumors is subjected to hypoxic or even anoxic conditions (18, 19). In addition, we performed some of our *in vitro* studies in the presence of tumor-conditioned medium, because endothelial cells in tumors are exposed directly to tumor-secreted factors that can modify their function (20).

Cells were, therefore, preconditioned and maintained at 1% O₂. HUVECs³ used for studies had at least one passage at 1% before use. Tumor cells used for hypoxic conditions were continuously maintained at 1% O₂ and had at least five passages under hypoxia before use. By using carefully matched samples of tumor-stimulated and non-stimulated HUVECs, determination of the effects of tumor-secreted factors was possible.

HUVECs were isolated as primary explants, according to the method of Jaffe *et al.* (21). Cells from each cord were cultured separately on tissue culture grade plasticware coated with 0.2% gelatin and grown at 21% O₂ for their first passage. They were maintained in 10% HEPES buffered medium M199 (Sigma Chemical Co.), supplemented with 0.01% glutamine, 20% FCS, 20 µg/ml endothelial cell growth supplement (Advanced Protein Products) and 15 units/ml heparin (Sigma) and used at second passage. Human breast adenocarcinoma cells (MDA-MB-231) were purchased from the American Type Culture Collection and maintained in identical medium, but with the heparin

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² To whom requests for reprints should be addressed.

³ The abbreviations used are: HUVEC, human umbilical vein endothelial cell; MTD, maximum tolerated dose; TNF, tumor necrosis factor; FAA, flavone acetic acid.

and growth factors omitted and only 10% FCS, and were used from passages 5 to 12. Tumor-conditioned medium was collected 24 h after a medium change to a confluent tumor cell 150-cm² flask at 1% O₂, centrifuged at 1100 × g for 5 min to remove cellular debris, filter sterilized (0.2 μm), and supplemented with 10% FCS 20 μg/ml endothelial cell growth supplement (Advanced Protein Products) and 15 units/ml heparin (Sigma).

Preparation of Compounds. For the *in vitro* studies, combretastatin A-4 was dissolved in DMSO at a concentration of 5 mg/ml and then subsequently diluted in culture medium or tumor-conditioned medium. However, the limited solubility of combretastatin A-4 created several technical difficulties, which were overcome by the synthesis of a more soluble derivative, disodium combretastatin A-4 3-*O*-phosphate (prodrug; Ref. 22), which makes an ideal prodrug because the phosphate group can be cleaved by endogenous nonspecific phosphatases. Combretastatin A-4 prodrug was dissolved in PBS at 5 mg/ml and subsequently diluted in a similar manner to that for the parent compound. For the *in vivo* experiments, both drugs were injected i.p. Combretastatin A-4 was dissolved in DMSO and injected at 0.01 ml/5 g body weight. Combretastatin A-4 prodrug was dissolved in 0.9% saline and injected at 0.1 ml/10 g body weight. Tumor and hindlimb perfusion studies used a modified Krebs-Henseleit buffer, as described previously (23), containing 200 μM solution of either combretastatin A-4 or its phosphate prodrug.

Cell Viability Studies. Cell viability was determined by the use of a microculture neutral red technique. This assay provides a quantitative measure of the number of viable cells by determining the amount of neutral red taken up by lysozymes into viable cells in treated *versus* control (*i.e.*, untreated) cells. HUVEC were assessed by preconditioning at 1% O₂ for one passage and then initiating cultures in 96-well plates at 4.0×10^3 cells/well. Cells were allowed to attach overnight at 1% O₂ and 5% CO₂. Identical plates were then exposed to either fresh medium or tumor-conditioned medium for 22 h, followed by a 2-h exposure to the drug in medium/tumor-conditioned medium. The plates were washed twice with PBS and incubated at 37°C, 1% O₂, and 5% CO₂ for a period of 1 week or until the untreated control wells had become confluent. The surviving fraction was then assessed using the neutral red cytotoxicity assay (24), where the cells are incubated for 3 h in a solution of 39.6 μg/ml neutral red (Sigma). This was removed, and the cells were fixed with 100 μl of 0.5% formalin/1% calcium chloride and then solubilized with 100 μl of 1% acetic acid/50% ethanol at 37°C for 5 min with gentle agitation. The absorbance/well was measured at 540 nm by use of a Labsystems Multiskan MCC/340 Microplate reader. Each assay was performed at least three times, and a matrix of eight replicate wells per drug concentration was used. The same assay was used for the tumor cells (MDA-MB-231), except that they were preconditioned at 1% O₂ and 5% CO₂ for at least five passages and plated at 6×10^3 cells/well. To assess the effect against quiescent HUVECs, the cells were first grown in 25-cm² flasks at 1% O₂ and 5% CO₂ and once confluent, exposed to the drug for 2 h, washed twice in PBS, trypsinized, and plated onto 96-well plates at 4×10^3 cells/well, incubated for 1 week, and assayed using the neutral red assay. Results were plotted following statistical analysis using the mean of at least seven replicates and ±SE.

Animals and Tumors. The experimental tumor systems used in this study were the murine adenocarcinoma NT (CaNT), the human breast carcinoma MDA-MB-231, and the rat carcinosarcoma P22. The murine and human tumors were initiated by injecting 0.05 ml of a crude tumor cell suspension (CaNT) or 5×10^5 tissue culture-maintained cells (MDA-MB-231) dorsally into 12–16-week-old CBA/Gy f TO or SCID mice, respectively. Animals were selected for drug treatment when their tumors reached 5–6 mm geometric mean diameter (150–300 mg). The rat tumor was grown as a tissue-isolated preparation in which the vascular supply is derived from a single artery and vein to facilitate *ex vivo* perfusion (see below). All animal studies were conducted in accordance with internal laboratory guidelines and the Animals (Scientific Procedures) Act 1986.

MTD. To guide the *in vivo* studies, experiments were performed to determine the MTD of combretastatin A-4 prodrug, following i.p. administration. The studies were based on guidelines published previously (25). Due to the limited drug availability and ethical considerations, the MTD was not accurately determined. However, our studies indicate that it is between 1000–1500 mg/kg in CBA/Gy f TO mice.

Isolated Tumor and Hindlimb Perfusion Studies. Early generations of the P22 transplanted rat carcinosarcoma were used for these experiments. Tissue-isolated tumors, the vascular supply of which was derived solely from

the superior epigastric vascular pedicle, were grown in the right inguinal fat pad of 10–11-week-old male BD9 rats. The method used was essentially as described previously (23), except that no attempt was made to physically enclose the growing tumor to prevent vessel ingrowth from surrounding normal tissues. Briefly, under Hypnorm and midazolam anesthesia, a small portion of the inguinal fat pad, immediately distal to the branching point of the superficial epigastric artery and vein, was surgically isolated from the bulk of the fat pad. A small (~1 mm³) piece of donor tumor was implanted into a pocket made in the fat, and the fat plus tumor was loosely sutured in position in the inguinal cleft to prevent twisting of the vascular pedicle while allowing movement of the growing tumor within the cleft. Tumors were used for experimentation when their vascular supply was seen to derive solely from the epigastric vascular pedicle (approximately 50% of preparations). Tumors were used after 2–3 weeks growth when they weighed 1–2 g.

The method for *ex vivo* perfusion of tumors has been described in detail elsewhere (26). Briefly, rats were anesthetized with Hypnorm and midazolam, and the femoral artery and vein were catheterized for connection to the perfusion apparatus. All branching vessels other than the tumor (superior epigastric) vessels were ligated or cauterized. A similar method was used for the hindlimb perfusions, except that the femoral vessels were catheterized proximal rather than distal to the superior epigastric branching vessels.

Tumors and hindlimbs were perfused with a cell-free modified Krebs-Henseleit buffer as described previously (23). After the start of the perfusions, rats were killed by i.v. administration of Euthatal (RMB Animal Health Ltd., Dagenham, United Kingdom), and tissues were left *in situ* for the duration of the experiment. Perfusate flow rate was maintained constant, and perfusion pressure was monitored continuously via a physiological pressure transducer connected to the afferent perfusion line, distal to a bubble trap. Vascular resistance was calculated from perfusion pressure ÷ perfusate flow rate and used as a measure of vascular tone. For tumor perfusions, the tumors were weighed at the end of the experiment, and specific vascular resistance was calculated in mmHg · (ml/g/min)⁻¹. For hindlimb perfusions, the exact mass of tissue perfused via the femoral vessels was unknown, and total vascular resistance was calculated in mmHg · (ml/min)⁻¹.

Assessment of Perfused Vascular Volume. Estimates of functional vascular volume in the murine breast carcinoma NT were obtained by use of the DNA-binding fluorescent dye Hoechst 33342 (27). Following the i.v. injection of Hoechst 33342 (10 mg/kg) into untreated and drug treated mice (100 mg/kg combretastatin A-4 prodrug), the tumors were excised at 1 min postinjection, frozen, sectioned, and visualized under UV excitation. Comparative sections were taken from untreated control mice and animals that received 100 mg/kg combretastatin A-4 prodrug 1, 6, or 18 h previously. Vascular volumes were determined using a random point scoring system based on that described by Smith *et al.* (27) and Chalkley (28), and results are expressed as a percentage of the vascular volume obtained in tumors from untreated animals.

Histological Sections. Histological sections were taken from the murine carcinoma NT and human MDA-MB-231 tumors from untreated control animals and at 24 h after i.p. injection of a single dose of 100 mg/kg combretastatin A-4 prodrug.

Results

Cell Viability Studies. The results indicate that combretastatin A-4 has a concentration-dependent action on both the tumor cells and the HUVECs (Fig. 1a). However, due to the limited solubility of combretastatin A-4, the experiments were repeated with the more soluble prodrug. The cytotoxicity profile for the prodrug indicated no loss of activity compared to the parent drug (Fig. 1b). However, it was clear that there is greater toxicity toward the endothelial cells compared to the tumor cells for both the parent compound and the prodrug, with the suggestion of further enhancement of toxicity against HUVECs in the presence of tumor-secreted factors (Fig. 1c). Further studies examining the effects of the prodrug on nonproliferating endothelium indicated that this selective toxicity is primarily directed toward proliferating endothelium (Fig. 1c).

Isolated Tumor and Hindlimb Perfusion Studies. The time course and tumor selectivity of the vascular effects of 200 μM combretastatin A-4 and 200 μM of its prodrug were evaluated using an *ex*

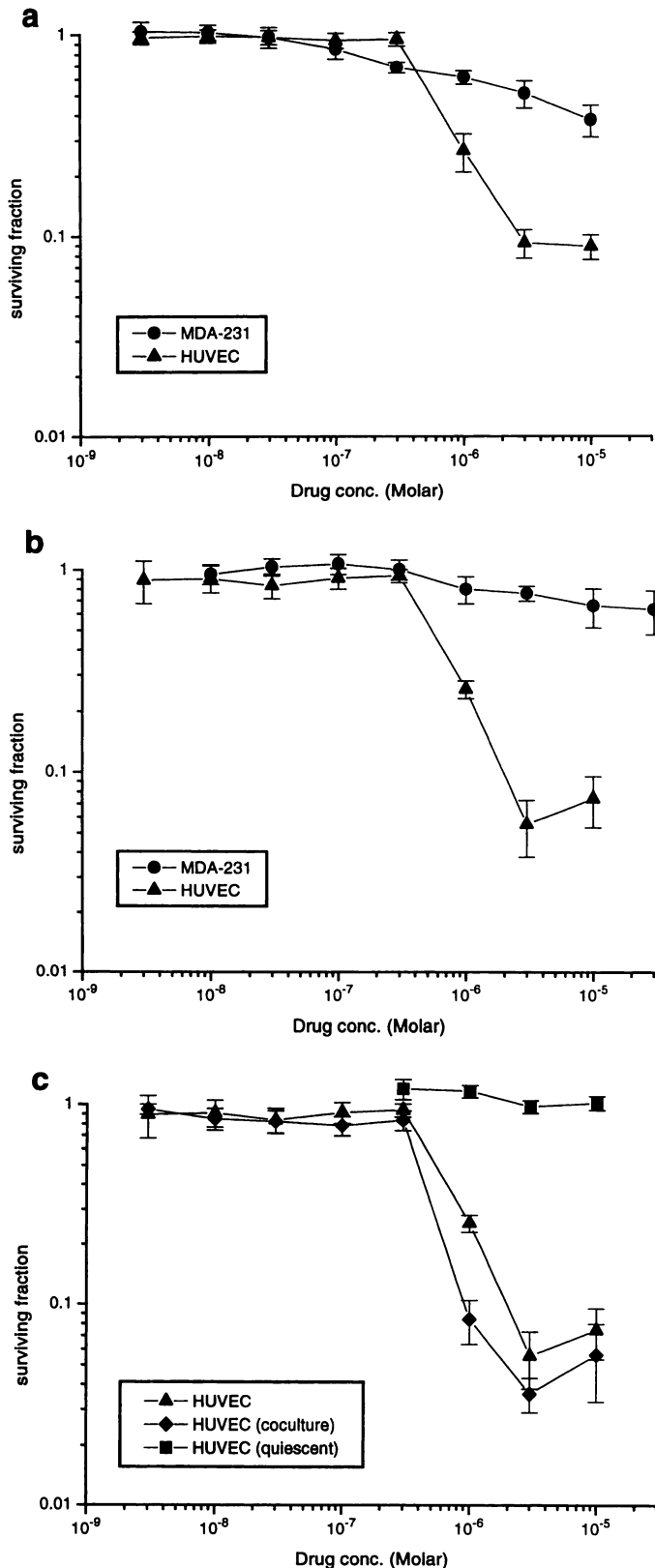


Fig. 1. The cytotoxicity profiles of combretastatin A-4 (a) and the derived prodrug (b and c) against HUVECs (▲) and MDA-MB-231 breast adenocarcinoma cells (●). The cells (1×10^5) were exposed to the drug for 2 h at 1% O_2 and 5% CO_2 in a tumor microenvironment model system and subsequently maintained under the same conditions. The relative number of cells at 6 days after exposure was determined using the neutral red cytotoxicity assay (24). The effect of coculture and exposure to tumor-secreted factors for 24 h prior and during drug exposure can be seen to reduce the surviving fraction (c, ◆). If endothelial cells are quiescent (i.e., nonproliferating) for the period of drug exposure, no significant drug cytotoxicity is observed (c, ■), even at the higher concentrations. Bars, SE.

vivo perfusion system. This dose represents the peak plasma level of the prodrug following i.p. administration of 100 mg/kg in mice. Fig. 2 shows the effect of combretastatin A-4 (Fig. 2a) and its prodrug (Fig. 2b) on vascular resistance of the rat P22 tumor perfused *ex vivo*. In both cases, vascular resistance increased by a factor of at least 3 within 20 min of the onset of drug infusion. The prodrug, but not the parent drug, also induced vasomotion in the tumor, as shown in Fig.

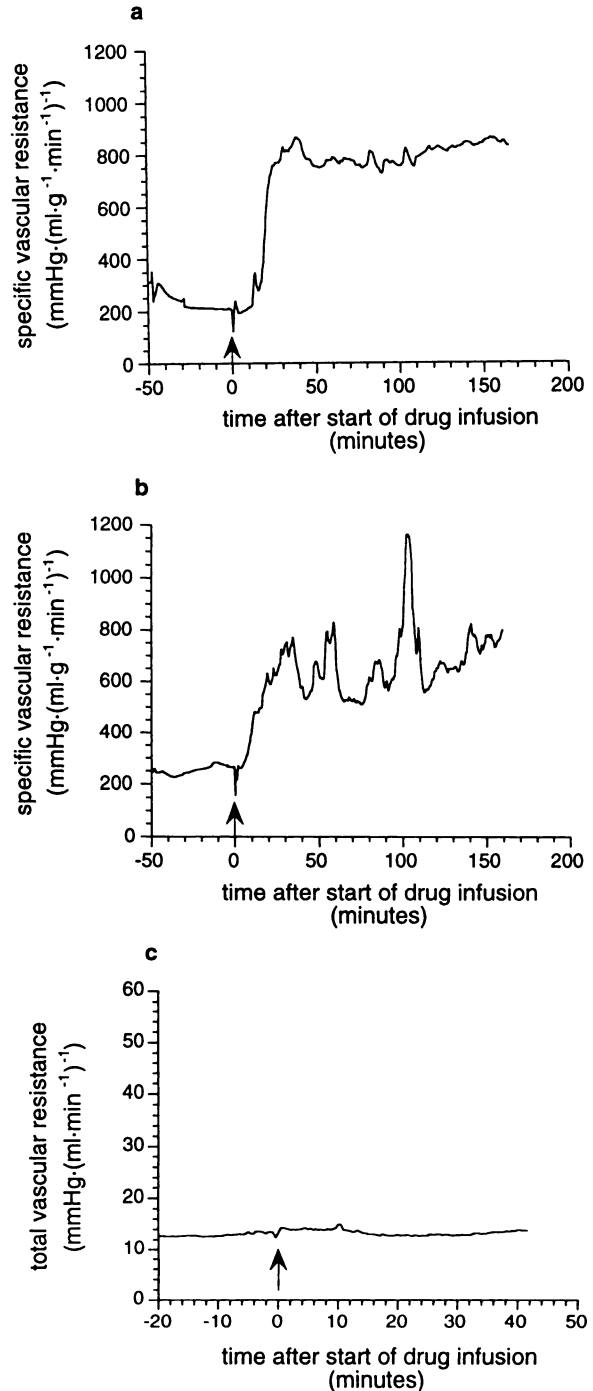


Fig. 2. Specific vascular resistance of the P22 rat tumor perfused *ex vivo* before and during constant infusion of 200 μM combretastatin A-4 (a) and 200 μM combretastatin A-4 prodrug (b). Specific vascular resistance is expressed per gram tumor, and each trace represents perfusion of a single tumor. Arrow, the start of drug infusion at time zero. c, total vascular resistance of the normal rat hindlimb, before and during constant infusion of 200 μM combretastatin A-4 prodrug. The mass of tissue perfused was unknown, and the trace represents perfusion of a single hindlimb preparation. Arrow, the start of drug infusion at time zero.

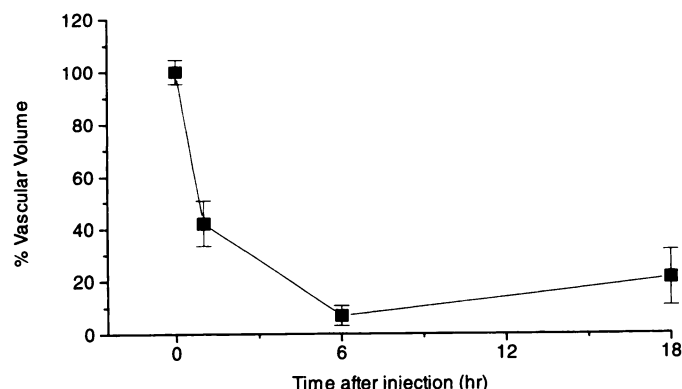


Fig. 3. Functional vascular volume in the carcinoma NT tumor as a function of time following the administration of combretastatin A-4 prodrug. Data are expressed as a percentage of the perfusion measured in untreated tumors; bars, the mean $\pm$ SE of five tumors.

2b by the oscillations in vascular resistance during drug infusion. The mechanism for this effect is unknown. Fig. 2c shows a similar plot for the normal rat hindlimb before and during infusion of 200 μ M combretastatin A-4 prodrug. In contrast to the tumor, the prodrug had no effect on vascular resistance in the hindlimb, demonstrating selectivity for tumor vasculature.

Tumor Perfusion Studies following Systemic Drug Administration. Quantitative analysis of the tumor sections indicated a 93% reduction in functioning vascular volume at 6 h following treatment (Fig. 3), and little recovery was apparent by 18 h.

Histological Studies. Our *in vivo* work has indicated a MTD of combretastatin A-4 prodrug in excess of 1000 mg/kg. The effects of a single dose of 100 mg/kg combretastatin A-4 prodrug on histological structure of both the carcinoma NT tumor and the human MDA-MB-231 tumor at 24 h postinjection compared with untreated controls can be seen in Fig. 4. Sections from untreated and treated tumors were stained simultaneously, and the apparent reduction in stain intensity reflects the extensive loss of viable cells in the treated tumor. There is marked hemorrhagic necrosis consistent with widespread vascular shutdown, and only a small rim of viable tumor remains, equivalent to less than 5% of the tumor mass.

Discussion

As with all approaches to cancer therapy, vascular targeting is only realistic if significant selectivity between tumor and normal tissue response can be achieved. The potential of antibody and gene therapy-directed methods, coupled with the fact that the target cell is next to the blood stream, has led to much focus being given to such approaches (29–31). In contrast, relatively little effect has been afforded to drug discovery in this area, and promising agents may not have been identified during drug screening programs, due to focus on direct antitumor action. The agents that have been reported to elicit marked vascular shutdown within tumors include lipopolysaccharide, TNF, FAA, colchicine, and vinblastine, but they only elicit antivascular effects at doses approaching the MTD and with evidence of significant morbidity (9, 11–13, 15, 32). There is, therefore, a need to identify new agents with improved selectivity that is manifest within a large therapeutic window. Our present studies demonstrate the selective action of combretastatin A-4, and its more soluble prodrug, against proliferating endothelium *in vitro*, as well as *in vivo* effects on tumor vascular function. In contrast to agents tested previously, these effects are produced when administered at doses less than 10% of the MTD and without detectable morbidity.

The neutral red assay used in the *in vitro* experiments cannot

distinguish between cytotoxic and antiproliferative effects of the test compounds. However, if the reduction in cell number is the result of a prolonged antiproliferative rather than cytotoxic effect, it would have to be maintained over a 7-day period following a short drug exposure of 2 h to explain the result. Both drugs produced a concentration-dependent effect with reduction in endothelial and tumor cell numbers, with the tumor cells inherently more resistant than endothelial cells. From previous experiments, we have determined that the proliferation rates of the endothelial and tumor cells under investigation are similar, when under the conditions used in these studies. This suggests that the difference in sensitivity between these cell types is intrinsic rather than related to differences in proliferation rate. Moreover, if the proliferation rate is reduced, due to the endothelial cells becoming quiescent prior to and during drug exposure, they become particularly resistant to the effects of these agents over the dose range investigated. Why proliferating endothelial cells show such sensitivity to the *in vitro* effects of combretastatin A-4 or its prodrug is not yet known. The antimitotic effect of preventing spindle formation would affect the tumor and endothelial cells in a similar manner because of their similar proliferation rates. However, our studies have also shown similar effects with other tubulin-binding agents, which indicate that endothelial cells may be predisposed to such agents.

The cytoskeleton mediates a number of important endothelial cellular functions other than the mitotic spindle and chromosome segregation, including intracellular organization, cell morphology, cilia motility, angiogenesis, and the intracellular transport of molecules from the site of synthesis to the cell surface via microtubule motor proteins (33). Antiangiogenic activity both *in vitro* and *in vivo* has been reported for 2-methoxy-estradiol, which also binds to tubulin, indicating that our *in vitro* results may reflect antiangiogenic activity in addition to cytotoxic effects. Further work is now ongoing to establish which, if any, of these processes represent the selective target for the drug effects on proliferating endothelium.

Although the *in vitro* results indicate potential antiangiogenic and/or vascular toxicity of combretastatin A-4, it is likely that the mechanism responsible for the *in vitro* effect cannot solely explain the *in vivo* effects observed. The studies in the isolated tumor system indicate that vascular shutdown is seen within 20 min of the start of drug infusion. Because the perfusate does not contain platelets or clotting factors, intravascular coagulation is not involved with the effects seen in this model. It is possible that the observed increase in vascular resistance is induced by rapid changes in endothelial cell shape or endothelial cell detachment, which could occlude flow. Whatever the mechanism involved, these studies indicate that the combretastatin A-4 prodrug has a direct and selective effect on tumor vasculature.

The *in vivo* blood flow and histological studies with two experimental breast tumor models confirm the selective effects of combretastatin A-4 prodrug on tumor vasculature when administered systemically to tumor-bearing mice. At 100 mg/kg, rapid and prolonged blood flow shutdown is evident, and both human and murine tumor models show extensive necrosis within 24 h. Additional studies have indicated similar effects at doses between 25 and 1500 mg/kg, with morbidity only detected when approaching the MTD, thus indicating a broad therapeutic window for this compound. However, previous work with FAA, another agent that mediates its antitumor effect via vascular damage, has shown that the extent of such effects is tumor line dependent (34). It is of interest to note that the vascular effects of FAA and dimethylxanthene-acetic acid are mediated in large part through TNF release (35). However, combretastatin A-4 prodrug, like vinblastine, does not invoke any detectable increase in serum TNF

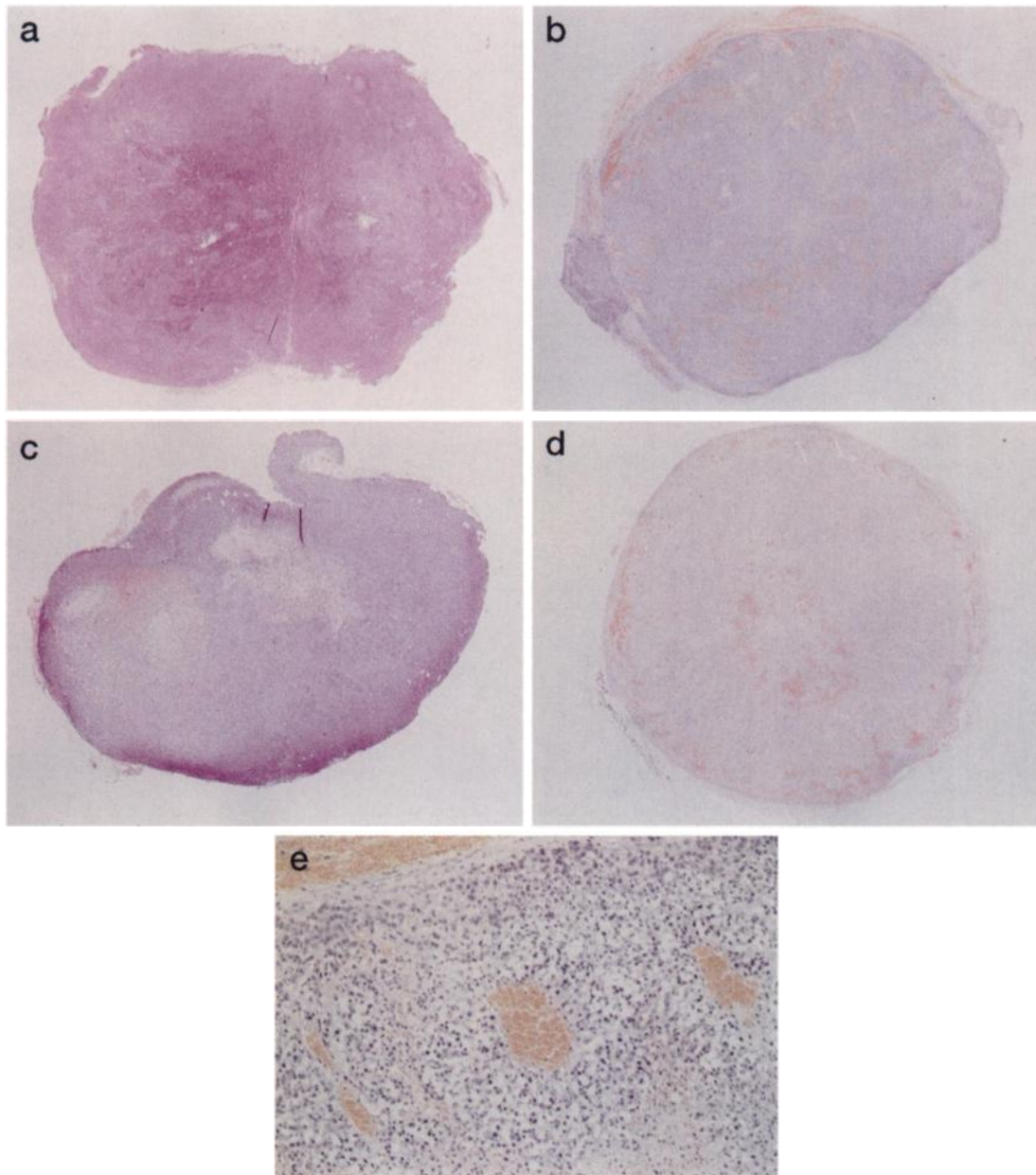


Fig. 4. Histological sections from two breast tumor models. The specimens are untreated control (a, carcinoma NT; c, MDA-MB-231) and 24 h postinjection of a single i.p. dose of 100 mg/kg combretastatin A-4 prodrug (b, carcinoma NT; d, MDA-MB-231). $\times 2$. The high power image (e, $\times 40$) demonstrates the intravascular coagulation that occurs following treatment with combretastatin A-4 prodrug (100 mg/kg) to the carcinoma NT tumor.

levels (10).⁴ Thus, because the mechanism of vascular effects of tubulin-binding agents appears to be different from that of the flavanoids, a different spectrum of tumor sensitivity might be expected.

The present studies show that combretastatin A-4 prodrug can induce potent and selective antivascular effects against tumor-associated endothelium, but significantly, this antimitotic compound produces these effects at concentrations less than one-tenth of the MTD and shows a wide therapeutic window. Additional studies have indicated that the limiting toxicity is platelet depletion. This is to be expected, because the budding of platelets from megakaryocytes is a critical tubulin-driven process within the vascular compartment (36). The apparent selectivity of the effects seen now warrant much more detailed study of the cellular and molecular mechanisms involved in addition to investigation of the therapeutic potential of the drug in single and multiple dose schedules, both alone or in combination with

other chemotherapeutic agents. In summary, the current studies provide further evidence for the concept of vascular-directed cancer therapy and moreover, emphasize that selectivity can be achieved using drug-based approaches.

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