

Advances in Brief

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

Graham G. Dark, 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 produg 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 anti-proliferative/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 antivascular effects of combretastatin A-4.

Introduction

Vascularity 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, antivascular 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 dimethylxanthenone-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 antivascular 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 antivascular effects of combretastatin A-4 and establish if it possessed a larger therapeutic window for its antivascular 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.
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/g body weight. Combretastatin A-4 prodrug was dissolved in 0.9% saline and injected at 0.1 ml/g body weight. Tumour 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. HUVECs were assessed by preconditioning at 1% O₂ for one passage and then initiating cultures in 96-well plates at 4.0 × 10³ 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 × 10³ 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 × 10³ 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 × 10⁴ 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 tumor 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).

MDT. 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 i.p. in male BD9 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 Hypnorn 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 Hypnorn 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. Perfusion 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/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 of 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
EFFECT OF COMBRETASTATIN A-4 ON TUMOR VASCULATURE

**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 × 10⁵) were exposed to the drug for 2 h at 1% O₂ and 5% CO₂ 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 (34). 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.

**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 (b) prodrug. 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.

**EFFECT OF COMBRETASTATIN A-4 ON TUMOR VASCULATURE**

EX 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. 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 × 10⁵) were exposed to the drug for 2 h at 1% O₂ and 5% CO₂ 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 (34). 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.

**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 (b) prodrug. 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.

---

1831
Effects are produced when administered at doses less than 10% of the tumor vascular function. In contrast to agents tested previously, these reflect the extensive loss of viable cells in the treated tumor. There is a 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.

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. The mechanism for this effect is unknown. Fig. 2c shows a similar plot for the normal rat hindlimb before and during infusion of 200 μM 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.

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 dimethylxanthenone-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.
levels (10). Thus, because the mechanism of vascular effects of tubulin-binding agents appears to be different from that of the flavonoids, 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.

References

6. Fotsis, T., Zhang, Y., Pepper, M. S., Adlercreutz, H., Montesano, R., Nawroth, P. P.,
EFFECT OF COMBRETASTATIN A-4 ON TUMOR VASCULARITY


Downloaded from cancerres.aacrjournals.org on January 5, 2018. © 1997 American Association for Cancer Research.
Combretastatin A-4, an Agent That Displays Potent and Selective Toxicity toward Tumor Vasculature

Graham G. Dark, Sally A. Hill, Vivien E. Prise, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/57/10/1829

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/57/10/1829. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.