Combretastatin A4 Prodrug Study of Effect on the Growth and the Microvasculature of Colorectal Liver Metastases in a Murine Model

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ABSTRACT

Combretastatin A4P (CA4P) is a prodrug that, in active form, binds to tubulin microtubules of capillary endothelial cells. Studies to date indicate it has significant activity as a specific tumor vascular targeting agent. The goals were to assess the effects of CA4P on tumor growth and microvasculature of colorectal liver metastases in the mouse model, using stereological and histological methods to measure tumor growth, and vascular corrosion casting and laser doppler flowmetry to assess effect on the microvasculature. Continuous s.c. infusion of CA4P produced a major reduction in tumor growth. The percentage of the liver occupied by metastases decreased from 20.55 ± 13.3% in controls to 7.46 ± 5.99% in treated animals (P < 0.03). Ultrastructural study of tumor microvasculature after a single dose of CA4P revealed marked effects 1 h after treatment. There was loss of patent microvessels at the normal liver-tumor interface. Central microvascular density was reduced, with constriction and tapering of vessels. CA4P appeared to cause no damage to normal liver tissue or vasculature. Tumor blood flow decreased from 37.6 ± 13.9% in controls to 24.4 ± 6.1% in tumors >5 mm in diameter, 1 h after treatment with CA4P (P < 0.03). Quantitative histology of tissue at 6 and 24 h after CA4P treatment showed a significant increase in tumor necrosis (48.7 ± 21% and 55.5 ± 19% compared with controls, 20.6 ± 8%; P = 0.01). Continuous infusion with CA4P causes marked reduction in tumor volume. A single dose of CA4P causes major changes of the tumor microvasculature, reduction of tumor blood flow, and increase in tumor necrosis. CA4P has a potential role in the management of patients with liver metastases.

INTRODUCTION

In seeking drugs that can reduce tumor growth, recent attention has been directed to agents that affect the tumor microvasculature. These agents have been termed vascular targeting or antivascular agents. In contrast to antiangiogenic agents, which act by inhibiting proliferation of new tumor vasculature, vascular targeting agents specifically and rapidly act on preexisting tumor blood vessels and thus may prove useful in treatment of advanced cancers. CA4P is a vascular targeting agent that potently binds tubulin and appears to target tumor microvessels as a primary path to tumor destruction.

This agent is a member of a family of chemicals that have been extracted from the bark of the South African willow (1). It has been found to inhibit tumor growth at one-tenth of the maximum tolerated dose in mice (2). It has been shown to cause an increase in cellular necrosis in tumor models (3–6) and to delay tumor growth (3, 4, 7). Moreover, this drug has been thought to target tumor microvasculature from the finding of a rapid reduction in blood flow in a variety of tumor models (2, 7–9). Evidence of an effect of CA4P on the tumor microvasculature has not included ultrastructural studies. The rapid onset of blood flow disruption seen in vivo (2, 7–9) is thought to lead to increased tumor necrosis (5, 8, 9), possibly through damage to proliferating endothelial cells leading to inhibition of mitosis, and increased apoptosis in these cells (2, 3, 10, 11). The effectiveness of continuous dosing with CA4P on tumors at low doses as may be used in the clinical setting has not been studied. We hypothesize that CA4P inhibits metastatic tumor growth and that the mechanism by which this occurs is through a direct effect on the tumor microvasculature.

The broad aim of this study was to investigate the tumor growth inhibitory effect and antivascular effect of CA4P on a model of colorectal liver metastases in the mouse. Assessments include quantitative macroscopic and microscopic assessment of the effects of CA4P on tumor volume, tumor blood flow, and the ultrastructural changes in the tumor microvasculature.

MATERIALS AND METHODS

Animals. Male CBA mice (4–7 weeks of age) were purchased from Monash University Animal Services (Clayton, Australia) and maintained and used according to Monash University Animal Experimentation Ethics Committee guidelines.

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3 The abbreviations used are: CA4P, combretastatin A4 prodrug; SEM, scanning electron microscopy.
Mouse Model of Colorectal Liver Metastases. Cells from a dimethyl hydrazine-induced primary colon cancer cell line were maintained in vivo in the flank of male CBA mice and passaged every 7–10 days. Briefly, this involved removing tumors at ~0.5 cm in diameter, mincing with fine scissors, and pushing the cells through a fine mesh with the use of a spatula and sterile saline. A 0.1-ml volume of this cell suspension was then injected s.c. into the flanks of a mouse. The mouse was monitored until the tumors were of sufficient size to be harvested for the intrasplenic injection model, which has been described and characterized previously (12).

Specifically, tumors were prepared as for passage and then 10 ml 0.1% trypsin at 37°C was added. After 10 min, 5–10 ml of DMEM containing 10% FCS were added, and the resulting suspension was centrifuged at 1–2000 rpm for 10 min. The pellet was resuspended in 0.02% EDTA in PBS and centrifuged as before. The pellet was then resuspended in 4°C Ringer’s solution containing 0.1% glucose. An aliquot was used to measure cell viability and concentration using trypan blue exclusion and a hemocytometer. A dilution of this suspension was made and divided into equal aliquots and each used for up to five to six animals. A final concentration of $1 \times 10^6$ cells/ml was injected. The cells were maintained on ice prior to injection. Mice were anesthetized using an i.p. injection of Ketamine/Xylazine mix (Ketamine 2% + Xylazine 0.1%) in normal saline at a dose of 0.05–0.1 ml/10 g of weight. The mouse was placed on its right side, and a 1.5-cm incision was made across the costal margin to expose the abdominal cavity above the spleen. The spleen was exteriorized along with the fascia and placed on chlorhexidine-soaked gauze. A 0.1-ml volume of cell suspension was slowly injected into the spleen using a 30-gauge needle over 20 s. Gauze was placed over the injection site while retracting the needle and the spleen was compressed for a 2-min period to allow the tumor cells to enter the portal system. A splenectomy was then performed using cautery, the skin was sutured, and the mouse was allowed to recover. This model produces multiple, distinct liver metastases by day 21 after induction.

Effect of CA4P on Tumor Growth. Doses of 1, 5, 10, 15, 25, and 50 mg/kg/day were studied. All study groups were accompanied by control groups. CA4P was delivered by constant s.c. infusion by the use of Alzet micro-osmotic pumps (No. 1002; Alzet Corp., Palo Alto, CA). An aqueous solution of CA4P was made up using PBS, and a 0.1-ml volume was inserted into each pump under sterile conditions. Each pump contained sufficient drug for 14 days of continuous dosing at the required concentration. Each mouse was anesthetized by i.p. injection of Ketamine/Xylazine mix, and the pumps were then inserted s.c. behind the head on day 10 after induction of liver metastasis, ensuring continuous dosing throughout the treatment period.

Stereological Assessment of Liver Metastases. Stereological assessment of liver metastases was performed on the continuous dose studies. At the end of the study period (day 21), the animals were killed, and the livers were removed and fixed in formalin for 1 week. The entire liver was cut into 1.5-mm slices using a multi-blade fractionator, and a subgroup of slices was used for measurement. Each slice was placed on a stereo microscope stage and imaged by a color video camera (JVC TK-870E) using a macro lens and visualized using a computer-based Image Analysis system (Chromatic; Leading Edge Pty Ltd). Images were captured digitally. An electronic cursor was used to delineate areas of tumor from normal liver.

The following measurements were calculated using stereological methods according to the Cavalieri principle (13): (a) total volume of liver (mm$^3$); (b) total volume of liver metastases (mm$^3$); (c) total volume of normal liver (mm$^3$); and (d) percentage of liver occupied by metastases.

Briefly, either every second or third slice was used for measurements, and the total volume of liver or metastases was calcu-
lated as follows: Volume_{total liver or tumor} = \sum \text{area}_{total liver or tumor} \times \text{thickness of tissue slice} \times 1/\text{fraction of total number of liver slices counted}. From this, the volume of normal liver was calculated by subtraction, and the percentage of liver occupied by metastases was calculated as total volume of metastases divided by the total volume of liver \times 100.

**Effect of CA4P on the Microvasculature of Colorectal Liver Metastases.** Tumor-bearing mice were dosed with a single i. p. injection of CA4P at 100 mg/kg on day 21 after induction. Groups of mice were randomized to be killed at 1, 6, or 24 h after CA4P treatment. The livers were then either subjected to microvascular corrosion casting, laser doppler flowmetry, or quantitative histological evaluation.

**Microvascular Corrosion Casting.** The technique has been described in detail previously (14). Briefly, mice were anesthetized, and an incision was made to expose the heart and thoracic aorta. The aorta was cannulated, and the vascular system was flushed with warm saline containing heparin (10 IU/ml), papaverine (12 mg/ml), and 6% polyvinyl pyrollidone (PVP40). The right atrium was punctured, and when the effluent was clear, 5 ml of an acrylic resin of Mercox CL-2B (Okenshoji Co., Tokyo, Japan), methyl methacrylate (Sigma Chemical Co., St. Louis, MO), and catalyst MA was infused at a pressure of 160 mm Hg to allow microvascular filling. The resin was allowed to polymerize overnight at room temperature, and then the liver was carefully excised and placed in 20% KOH at 37°C to allow tissue digestion. The KOH was changed after 2–5 days, and the resin casts were washed in distilled water. The casts were then frozen in water and while frozen, cut into segments using a high-speed cutting tool (Dremel; Moto-Tool, Elk Grove Village, WI). This produced an undamaged cut surface. Suitable casts were mounted on aluminum stubs with electrodag 415 (Acheson Colloids, Port Huron, MI) to allow electrical contact, gold coated with a Baltec SCD005 sputtercoater, and viewed on a scanning electron microscope (Hitachi SEM). Scanning electron micrograph digital images for all tumors visible in each specimen were captured using Spectrum Imaging software at \times40, \times80, and \times150 magnification.

**Laser Doppler Flowmetry.** Laser doppler flowmetry was performed on a selection of tumors visible on the upper surface of the liver on day 21 after tumor induction. Blood flow measurements were taken with a LaserFlo Blood Per-
fusion Monitor (Model TSI 403A, St. Paul, MN). This technique has been described previously (12). Briefly, the mouse was anesthetized and placed on a heating pad, and the liver was exposed by a midline abdominal incision. Care was taken not to touch the surface of the liver. An aqueous transmission gel was applied to the surface of the laser doppler probe. The laser probe was attached to a counter weighted floating arm, and the tip was placed on the surface of the liver or tumor. This allowed the probe to lightly rest on the tissue surface, as well as to always maintain contact with the surface of the liver with the movement due to breathing. After a 10-min period, readings became stable.

A series of 8–10 sequential blood flow readings (at 1-min intervals) were taken from normal liver and then from adjacent tumor. Tumor diameters were also noted. Tumor blood flow was expressed as the percentage of blood flow relative to normal liver for each animal. The laser probe measured blood flow up to a depth of $\approx 1$ mm.

Quantitative Histological Assessment. Liver slices were processed using a standard paraffin processing protocol. Sections (3 $\mu$m) were cut and stained with H&E. Histological assessment of tumors was performed with the use of a drawing tube attachment (BH2-DA) on an Olympus light microscope (BHT, Olympus, Japan). Areas of necrosis within tumors were drawn and subsequently digitized using a computer-based digitizing tablet attached to an Image measuring program (Measure; Capricorn Graphics, Melbourne, Australia). The area of tumor necrosis was expressed as a percentage of total tumor volume.

Data Analysis. Data were represented as mean $\pm$ SE unless otherwise stated. Statistical analysis was performed using either $t$ test for (un)equal variance or $Z$ test for means where appropriate. $P \leq 0.05$ was considered significant.

RESULTS

Stereological Assessment of Tumor Growth. The maximal effect of CA4P on stereological assessment was seen with a dose of 15 mg/kg/day. The percentage of liver occupied by metastases was $20.6 \pm 13.3\%$ in the control group and $7.5 \pm 6.0\%$ in treated animals ($P = 0.03$; Fig. 1). The total volume of metastases was reduced by CA4P treatment from $543 \pm 448$ mm$^3$ to $163 \pm 138$ mm$^3$ ($P < 0.05$). There was not a consistent effect by the lower doses of 10, 5, and 1 mg/kg/day. A constant s.c. dose of 25 and 50 mg/kg/day CA4P exhibited systemic toxicity as well as local skin ulceration around the area contain-
ing the osmotic pump. In this model, a dose of 50 mg/kg/day was fatal to mice within 3–6 days of dosing.

**Effect of CA4P on Tumor Microvasculature.** The microvascular architecture of normal mouse liver and of this model of liver metastases has been described in detail previously (12). Liver metastases receive a direct vascular supply from the adjacent hepatic sinusoids, which become more compressed with tumor growth (Fig. 2A). Small metastases 0.5–1 mm in diameter have an abnormal tumor microvasculature containing a complex of large-diameter dilated and tortuous vessels centrally (Fig. 2b).

The effect of a single dose of CA4P (100 mg/kg i.p.) on the microvasculature of normal liver tissue was investigated. Study by SEM did not show any architectural distortion or change in integrity of the sinusoids in normal liver.

CA4P treatment produced a marked effect on the microvasculature of liver metastases. The effect was most pronounced at 1 and 6 h after treatment. Some effects remained at 24 h after treatment. The tumor microvasculature showed the following particular changes:

(a) There was a marked reduction in the density of patent tumor vessels at the tumor-host interface. This resulted in the formation of a halo zone at the margin of the tumor, with only central vessels showing filling of resin (Fig. 3).

(b) By 6 h, central dilated vessels had become flattened and constricted with multiple spindly branches undergoing smooth tapering toward areas of complete vascular occlusion (Fig. 4).

(c) Some tumors showed areas of extravasation of the casting material with the formation of plaque-like structures and nodular thickening (Fig. 4).

(d) There was a size relationship with tumors <1.0 mm in diameter frequently showing no specific change after CA4P. Larger tumors manifest more pronounced effects from CA4P treatment, particularly at the tumor-host interface.

(e) In untreated tumors, normal liver sinusoids were obscured by the tumor vessels at the interface (Fig. 5a). However, after CA4P treatment, the absence of tumor microvessels in this zone enabled viewing of normal liver sinusoids (Fig. 5b).

(f) CA4P treatment had no identifiable effect on the normal liver microvasculature.

These particular changes were present in most tumors, although the response within tumor regions varied.

**Laser Doppler Flowmetry Studies.** Blood flow was measured in blood flow units in both liver and tumor tissue. The blood flow readings in both the liver and tumor remained stable during the period of measurement (Fig. 6). CA4P treatment produced a significant reduction in relative tumor blood flow. The mean flow rate through liver metastases was found to be 37.6 ± 14% of the flow in the adjacent normal liver. In tumors >5 mm in diameter, CA4P resulted in a mean flow rate that was 24.4 ± 6% of normal liver flow rate at 1 h after treatment, which was significantly different from untreated control (P < 0.03; Fig. 7). There was no significant decrease in relative blood flow at 6 and 24 h after treatment.

**Quantitative Histological Assessment.** CA4P increased tumor necrosis in a time-dependent manner. The percentage of liver volume showing necrosis increased from 21.6 ± 3.1% at 1 h to 48.7 ± 21% at 6 h and 55.5 ± 19% at 24 h after treatment, respectively (P = 0.01 for 6 and 24 h; Fig. 8). Untreated tumors showed a mean percentage necrosis of 20.6 ± 8%. The areas of necrosis showed nuclear and cellular fragmentation accompanied by an amorphous eosinophilic exudate. In some areas, cells had pyknotic nuclei and indistinct cytoplasm. Viable tumor cells were seen on the edges of the necrotic areas (Fig. 9). Frequently, areas showed complete cellular degeneration with only a rim of viable tumor cells remaining. Viable cells were seen arranged concentrically around patent tumor blood vessels. In comparison, tumor microvessels that appeared to show blockage were...
surrounded by necrotic tumor cells (Fig. 10). A heterogeneous response of growth and death within tumors was evident with areas showing frequent mitotic cells and apoptotic cells.

**DISCUSSION**

The use of vascular targeting agents is an exciting new treatment option currently undergoing investigation. CA4P is a tubulin binding agent that has shown promising antivascular effects in animal models. Past studies using angiogenesis inhibitors that reduce the formation of new blood vessels have been shown to delay tumor growth (15–17). However, unlike vascular targeting agents such as CA4P, they do not act on preexisting tumor vasculature. In this study, we have established the effects of CA4P on the mouse model of colorectal liver metastases.

Our study of tumor microvascular corrosion casting showed direct morphological evidence of the effects of CA4P
on the microvasculature of the tumor. A significant decrease in
density of patent vessels is seen as early as 1 h after treatment
and up to 24 h after one dose of CA4P. The most prominent
effect is on the tumor vessels at the interface with normal liver.
Loss of these vessels creates a zone of relative avascularity
around the tumor. Large tumors seem to be affected more than
small tumors. Small tumors have their own tumor neovascula-
ture but also receive oxygenation and nutrients through direct
diffusion from the adjacent normal liver sinusoidal system. This
may explain why they are less susceptible to the effects of
CA4P. Another group studying rat rhabdomyosarcomas has also
found that CA4P has a greater effect on large tumors than small
tumors (18).

One feature of the effect of CA4P on tumor microvascu-
lature is the heterogeneity of the response within tumors. Some
areas of tumor show more complete filling, with constricted
blood vessels, whereas others show a total absence of blood
supply. This heterogeneous response to a single dose of CA4P
has been noted previously (8). In support of this focal micro-
vascular effect, a single 100 mg/kg dose of CA4P produces a
significant reduction in tumor blood flow and increased tumor
necrosis, with destruction of a large part of the tumor mass. This

Fig. 8 Effect of CA4P (100 mg/kg) on percentage of tumor
necrosis by quantitative histology. Values represent means;
bars, SE. *, P = 0.01
damage to the microvasculature and subsequent reduction in tumor blood flow precedes the tumor cell necrosis. The pattern of tumor cell necrosis suggests cell death from nutrient and oxygen deprivation, induced by vascular deficiency (19). A viable rim of tumor cells was seen mainly at the tumor-host interface and surrounding patent blood vessels. Viable tumor cells at the periphery of tumors may be receiving oxygenation and nutrients by diffusion from the adjacent normal liver sinusoids, as in small tumors. Continuous low-dose (15 mg/kg/day s.c.) treatment with CA4P significantly inhibited growth of colorectal liver metastases in this model, when treatment commenced on day 10 after tumor induction. This effect has not been demonstrated previously. Past studies have shown a delay in tumor growth only with intermittent high-dose treatment (4).

CA4P binds to tubulin microtubules and disrupts the intracellular network of proliferating endothelial cells (3). Microtubules are directly involved with cell functions such as mitosis and maintenance of cell shape (20). The primary effect of CA4P appears to be damage to the tumor microvascular endothelial cells. This may result in a change of shape (11) in these cells, leading to a reduction or cessation of blood flow within the tumor. Vascular occlusion has been shown to produce marked tumor cell death (21), attributable to the resultant ischemia and nutrient deficiency. Apoptosis has also been shown to be induced in proliferating endothelial cells in vitro (10) when incubated with CA4P for 24 h. However, in our study, the occurrence of vascular changes within 1 h and cellular necrosis at 6 h suggest that apoptosis of endothelial cells is not the primary mode of action.

We did not find any evidence to suggest that single-dose or continuous low-dose CA4P damages normal liver vasculature or hepatocytes in this study, although systemic toxicity was noted with the continuous high-dosage regimen of 25 and 50 mg/kg/day. High doses of CA4P have been associated with increased mean arterial blood pressure and heart rate (8); therefore, the finding of the lowest effective dose would significantly decrease the risk of any side effects for use in human disease.

In conclusion, CA4P is an effective vascular targeting agent that produces rapid, significant tumor microvascular changes and tumor necrosis. The agent has potential for the control of metastatic disease of the liver. This application would be helped by further study to identify optimal dosage and sequence of administration.

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