Combretastatin A-4 Phosphate as a Tumor Vascular-Targeting Agent: Early Effects in Tumors and Normal Tissues

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ABSTRACT

The potential for tumor vascular-targeting by using the tubulin destabilizing agent disodium combretastatin A-4 3-O-phosphate (CA-4-P) was assessed in a rat system. This approach aims to shut down the established tumor vascularity, leading to the development of extensive tumor cell necrosis. The early vascular effects of CA-4-P were assessed in the s.c. implanted P22 carcinosarcoma and in a range of normal tissues. Blood flow was measured by the uptake of radiolabeled iodoantipyrine, and quantitative autoradiography was used to measure spatial heterogeneity of blood flow in tumor sections. CA-4-P (100 mg/kg i.p.) caused a significant increase in mean arterial blood pressure at 1 and 6 h after treatment and a very large decrease in tumor blood flow, which—by 6 h—was reduced approximately 100-fold. The spleen was the most affected normal tissue with a 7-fold reduction in blood flow at 6 h. Calculations of vascular resistance revealed some vascular changes in the heart and kidney for which there were no significant changes in blood flow. Quantitative autoradiography showed that CA-4-P increased the spatial heterogeneity in tumor blood flow. The drug affected peripheral tumor regions less than central regions. Administration of CA-4-P (30 mg/kg i.p.) in the presence of the nitric oxide synthase inhibitor, N\(^{-}\)-nitro-L-arginine methyl ester, potentiated the effect of CA-4-P in tumor tissue. The combination increased tumor vascular resistance 300-fold compared with less than 7-fold for any of the normal tissues. This shows that tissue production of nitric oxide protects against the damaging vascular effects of CA-4-P. Significant changes in tumor vascular resistance could also be obtained in isolated tumor perfusions using a cell-free perfusate, although the changes were much less than those observed in vivo. This shows that the action of CA-4-P includes mechanisms other than those involving red cell viscosity, intravascular coagulation, and neutrophil adhesion. The uptake of CA-4-P and combretastatin A-4 (CA-4) was more efficient in tumor than in skeletal muscle tissue and dephosphorylation of CA-4-P to CA-4 was faster in the former. These results are promising for the use of CA-4-P as a tumor vascular-targeting agent.

INTRODUCTION

The tumor vascularity is an attractive target for therapy because it is easily accessible by blood-borne anticancer agents, and most tumor cells rely on an intact vascular supply for their survival (1). Most of the research efforts in this area have focused on understanding the process of angiogenesis and identifying anti-angiogenic agents (2). Currently, there are several classes of anti-angiogenic agents in Phase II/III clinical trials for cancer, including inhibitors of matrix-metalloproteinases, agents that inhibit vascular endothelial growth factor or its receptor, and inhibitors of endothelial cell growth such as TNP-470 (3–5). Other agents that have shown promise in preclinical evaluation include the peptide inhibitors of endothelial cell proliferation, angiostatin (6) and endostatin (7).

In contrast to the anti-angiogenesis approach, anti-vascular approaches aim to cause a rapid and extensive shut-down of the established tumor vasculature, leading to secondary tumor cell death. Development of antibodies to specific epitopes on the tumor vasculature (8, 9) and vascular-targeted gene therapy (1) are two approaches that are receiving considerable attention. However, drug- and cytokine-based approaches to vascular targeting are also possible. Two main classes of drugs can induce vascular damage and subsequent hemorrhagic necrosis in tumors, namely the tubulin-binding agents (10, 11) and drugs related to flavone acetic acid (12–14). An example of the latter group, dimethylxanthene-acetic acid, is currently in Phase I clinical trials in the United Kingdom and New Zealand.

Tubulin-binding agents such as vincristine and vinblastine are potent anticancer drugs currently used in the clinic. These agents, at close to the maximum tolerated doses, can also induce extensive vascular damage in animal tumors (10, 11). Colchicine was the first tubulin-binding agent discovered to have antivascular effects, which resulted in hemorrhagic necrosis in human tumors (15). However, its toxicity prevented any further clinical evaluation. More potent vascular-damaging agents are currently being sought. Combretastatins are compounds that have been isolated from the South African tree Combretum caffrum. One of them, CA-4, has a high affinity for tubulin at or near the colchicine binding site, causing the destabilization of the tubulin polymers of the cytoskeleton (16, 17). We have shown that a more soluble derivative of CA-4, CA-4-P (18, 19), can cause vascular shut-down in the murine adenocarcinoma NT and the human breast carcinoma xenograft, MDA-MB-231 (20, 21). This compound is cleaved to the active CA-4 by endogenous nonspecific phosphatases. Most importantly, these effects can be achieved using relatively nontoxic doses (20). In vitro studies demonstrated a profound antiproliferative/cytotoxic effect against proliferating human umbilical vein endothelial cells but not against cells that were quiescent before and during drug exposure. Proliferating MDA-231 cells were much less affected than the human umbilical vein endothelial cells. Ex vivo perfusions showed that CA-4-P caused a significant increase in vascular resistance within minutes of drug exposure in the rat P22 carcinosarcoma but not in normal rat hind limb (20).

The clinical potential of CA-4-P has been recognized by its recent entry into Phase I clinical trial in the United Kingdom. However, further information is required regarding its mechanism of action and vascular effects under in vivo conditions. Inhibition of NO synthesis has been found to enhance the tumor cytotoxicity induced by ischemia-reperfusion injury (22, 23) and photodynamic therapy, which are known to be vascular-damaging strategies. Paradoxically, administration of the vascular-damaging agent flavone acetic acid to tumor-bearing animals has been found to increase the circulating levels of nitrate (24) and to increase the activity of endothelial cell NOS in tumor tissue (25). This suggests that the production of NO may contribute to the cytotoxic action of this drug. However, although NO is known to be cytotoxic at high concentrations (26, 27), it is not known whether the net effect of increased NO production after FAA

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3The abbreviations used are: CA-4, combretastatin A-4; CA-4-P, disodium CA-4 3-O-phosphate; IAP, iodoantipyrine; MABP, mean arterial blood pressure; NO, nitric oxide; NOS, NO synthase; i-NAMe, N\(^{-}\)-nitro-L-arginine methyl ester.

4M. Korbelik, personal communication.
TUMOR VASCULAR TARGETING BY CA-4-P

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MATERIALS AND METHODS

Tumors. Early generation transplants of the P22 rat carcinosarcoma were used for these experiments (29). Tumors for blood flow experiments were grown in the left flank of 7–9-week-old male BD9 rats. One tumor per rat was implanted, and tumors were used for experimentation when the geometric mean tumor diameter reached 12–16 mm (approximately 2–3 weeks postimplantation). Mean tumor weight ± 1 SE for all of the tumors used was 1.33 ± 0.06 g. Tumors for isolated perfusions were grown in the right inguinal fat pad of 10–11-week-old male BD9 rats, such that their vascular supply developed solely from the superior epigastric vascular pedicle, to facilitate perfusion. This method has been described previously (20). Tumors were used 2–3 weeks postimplantation when they weighed 1.1 ± 0.2 g.

Blood Flow. CA-4-P (provided by Dr. G. R. Pettit, University of Arizona, Tempe, AZ) was administered i.p. at all of the doses, in a volume of 3 ml/kg, which was made up in 0.9% saline with a few drops of 5% Na2CO3. Animals were anesthetized approximately 30 min before the assay time for blood vessel catheterization. For testing the combination of CA-4-P and l-NAME, the latter was administered in the drinking water at a dose of 1.0 g/liter in 1% dextrose solution for 24 h before blood flow measurement. In addition, 1 mg/kg l-NAME was administered i.p. 15 min before and 15 min after CA-4-P administration. l-NAME for i.p. injection was made up in 0.9% saline in a volume of 1.7 ml/kg. Control animals were treated with the appropriate drug vehicle. In some rats, blood samples from the arterial catheter were withdrawn immediately before blood flow measurement and after heparinization for assay of combretastatin levels.

Tumor and normal tissue blood flow was measured using uptake of radio-labeled IAP. [14C]IAP (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) was used where spatial distribution of tumor blood flow was required. [125I]IAP (Institute of Cancer Research, Sutton, United Kingdom) was used in all of the other cases. Methods for using these tracers have been described previously (29). Briefly, animals were anesthetized with Hypnorm (fentanyl-fluanisone) and midazolam, and two tail veins and one tail artery were catheterized using polyethylene catheters (external diameter, 0.96 mm; internal diameter 0.58 mm). Animals were heparinized within minutes of blood flow measurement to avoid the interaction of heparin with any pro-coagulant effects of CA-4-P. Arterial blood pressure was monitored via the tail artery catheter up to the point of blood flow measurement.

At the appropriate time, blood flow was measured by flushing 0.3 MBq (8 μCi) of [125I]IAP or 0.6 MBq (15 μCi) of [14C]IAP into a tail vein catheter for 30 s, at a rate of 1.6 ml/min. During the 30-s period, free-flowing blood was collected at 1-s intervals into preweighed vials. At the end of the 30 s, the rat was killed by i.v. injection of Euthatal, and the tumor and various normal tissues were excised. Blood and tissue samples containing [125I]IAP were counted using a Wallac Autogamma well-counter. Blood samples containing [14C]IAP were counted using liquid scintillation on a Beckman LS1801 counter. Tumor samples containing [14C]IAP were rapidly frozen for cryostat sectioning. Tumor sections were exposed to autoradiographic film alongside [14C]-labeled methylmethacrylate microscales (Amersham Pharmacia Biotech) to determine tumor levels of [14C]IAP, as described previously (29).

Blood flow rates to tumor and normal tissues were calculated from tissue counts, the equilibrium partition coefficient for IAP in the different tissues, and the arterial input function derived from the arterial blood counts. This method is based on principles derived by Kety (30) and is described in detail elsewhere (31). Perfusion pressure for all of the tissues was assumed to change in direct proportion to MABP, and changes in tissue vascular resistance were calculated from MABP divided by blood flow rate.

Autoradiographic images were captured by camera and transformed pixel by pixel into blood-flow images, using the principles described above. Blood flow to necrotic regions of the tumor sections were excluded from further analysis, using in-house software developed using the Visilog Image Processing package (Noesis, Orsay, France). This involved overlaying images of histology onto the blood flow images and deleting pixels from the blood flow images that corresponded to delineated necrotic regions. The peripheral region of a section was defined as the rim of tissue equal to 10% of the section diameter. Sections cut from the center of tumors were used for these analyses. Sections cut through the whole tumor mass were used for all of the other analyses. The coefficient of variation of blood flow within a section was used as a measure of blood flow heterogeneity. The coefficient of variation was defined as the SD of the mean of all of the pixel blood flow values, expressed as a fraction of the mean value.

Isolated Perfusions. Tumors and hind limbs were isolated from the rat’s vascular supply and perfused ex vivo with a cell-free modified Krebs-Henseleit solution for 24 h before blood flow measurement. A significant difference between the 1- and 6-h time point is represented by * (P < 0.05) or ** (P < 0.01). A significant difference between the 1- and 6-h time point is represented by † (P < 0.05) or †† (P < 0.01).
buffer, as described previously (20, 31). The perfusate flow rate was main-
tained constant, and perfusion pressure was monitored continuously via a
physiological pressure transducer connected to the afferent perfusion line.
Changes in perfusion pressure were used as a measure of changes in vascular
resistance.

The effect of CA-4-P on vascular resistance in the tumor and hind limb
preparations was determined by adding CA-4-P (116 ± 14.6 μM) t o t h e
perfusate buffer such that the tissues were exposed to a constant level of
CA-4-P for the whole perfusion period. At the end of the perfusion period,
samples of perfusate, venous outflow, and tumor or skeletal muscle were taken
for determining the levels of CA-4-P and CA-4 as described below.

Measurement of CA-4-P and CA-4. Plasma levels of CA-4-P and CA-4
were determined in methanol extracts by reverse-phase high pressure liquid
chromatography, using an ion-pairing technique with fluorescence detection
(32). For alkaline phosphatase activity, tissues were homogenized in 4 volumes
PBS and 500-μl aliquots added to an equal volume of glycine buffer (pH 10.1).
These were incubated with CA-4-P at 37°C and, at intervals, 50-μl aliquots
were withdrawn and assayed as for plasma.

Statistics. All data were tested for normality of distribution and equal
variances between groups using a Shapiro-Wilk W test and an O’Brien’s F test,
respectively. In the majority of cases, these conditions were fulfilled and
differences between groups were tested for significance using either a Stu-
dent’s t test for unpaired data (comparison of two groups) or a standard
ANOVA followed by the Tukey-Kramer honest significance difference test
(comparison of more than two groups). The Wilcoxon rank sums test for
nonparametric data was used for groups that failed the test for normality, and
a Student’s t test for unequal variances was used where appropriate. JMP
Statistics for the Apple Macintosh was used for all of the analyses.

RESULTS

A CA-4-P dose of 100 mg/kg i.p. was chosen for study because, in
mice, this dose was relatively nontoxic and induced extensive hem-
orrhagic necrosis and vascular shut-down in several tumor types (20,
21). Fig. 1 shows the effect of CA-4-P (100 mg/kg i.p.) on MABP and
heart rate. MABP was significantly raised by about 30% at 1 h after
treatment but, although still significantly raised, was returning toward
the baseline by 6 h after treatment. The heart rate was significantly
reduced at 1 h after treatment, probably reflecting a reflex bradycardia
in response to the raised blood pressure. However, at 6 h, it was
significantly increased by about 25% above control levels. Plasma
levels of CA-4-P were 120.9 ± 4.2 μM at 1 h posttreatment and
15.7 ± 1.0 μM at 6 h posttreatment. The corresponding values for
CA-4 were 15.7 ± 1.0 μM and 0.43 ± 0.04 μM at the 1- and 6-h time
points, respectively.

Fig. 2 shows the effect of CA-4-P (100 mg/kg i.p.) on blood flow
to tumor and normal tissues, 1 and 6 h after treatment. Blood flow was
significantly reduced at both time points in the tumor, the skin
overlying the tumor, the contralateral skin, the skeletal muscle, and the spleen. Blood flow was also significantly reduced in the brain but only at 6 h. Blood flow was not significantly reduced from control levels in the heart and kidney at either time point. Blood flow was increased in the small intestine at 1 h but not at 6 h. Blood flow reduction was more profound at 6 h than at 1 h for tumor, skin, spleen, and brain. The most striking characteristic of this data is that the blood flow reduction to the tumor was much greater than in any of the normal tissues. At 6 h, tumor blood flow was reduced approximately 100-fold compared with approximately 7-fold for the spleen, which was the most sensitive normal tissue.

Fig. 3 shows the corresponding changes in vascular resistance in tumor and normal tissues after the administration of CA-4-P (100 mg/kg i.p.) and using MABP as a relative measure of perfusion pressure in all of the cases. Because MABP was increased at both time points, the changes in vascular resistance were even more striking than the blood flow changes shown in Fig. 2. Again, the increase in vascular resistance was much greater for the tumor than for the normal tissues. Note the scale used in Fig. 3 for the tumor panel compared with the scales used for the normal tissue panels. These calculations indicate that there were significant increases in vascular resistance in the heart and kidney, despite the absence of significant blood flow changes. This indicates that the increase in MABP was sufficient to maintain blood flow to these tissues despite an increased resistance to flow. In the small intestine, vascular resistance at 1 h and 6 h after CA-4-P treatment was not significantly different from the control level, which suggests that the observed increase in blood flow at 1 h (Fig. 2) was due entirely to the increase in MABP and not to a decrease in the resistance to flow.

The effect of CA-4-P (100 mg/kg i.p.) on the spatial distribution of tumor blood flow in 20-μm-thick tumor cryostat sections is shown in Fig. 4. These computed images illustrate the spatial heterogeneity of tumor blood over a sampling period of 30 s. Some of the very low flow regions in the untreated tumors relate to necrotic regions, which account for 11 ± 6% of the tumor volume. The lower panel of tumor images graphically illustrates the profound reduction in tumor blood flow attained at 1 h after treatment with CA-4-P. On the scale used, blood flow is only apparent at the tumor periphery, and this is much reduced compared with untreated tumors.

Quantitative analysis of the spatial distribution of tumor blood flow was carried out on images such as those shown in Fig. 4. These results are shown in Fig. 5. The coefficient of variation for blood flow increased approximately 4-fold with CA-4-P, in the sections taken as a whole and in peripheral and central regions of the sections (Fig. 5a). This indicates that the heterogeneity of blood flow was significantly increased by CA-4-P in all of the regions. Fig. 5b shows that the blood
flow to the periphery of untreated tumors is approximately twice as high as the blood flow to the center. After treatment with CA-4-P, this differential increases, with blood flow to the periphery being approximately 3–4 times as high as to the center. This confirms the impression of the maintenance of a residual flow in the periphery of tumors given in the images shown in Fig. 4.

Fig. 6 shows the effect of combining CA-4-P (30 mg/kg i.p.) with the NOS inhibitor, l-NAME, on vascular resistance in the tumor and normal tissues. In this experiment, both CA-4-P alone and l-NAME alone increased MABP by around 22%, which is somewhat less than the increase observed for 100 mg/kg CA-4-P shown in Fig. 1. The rats drank very similar amounts of water, such that the total dose of l-NAME received in the drinking water was 99.7 ± 9.2 mg/kg for the rats given l-NAME alone and 98.9 ± 4.3 mg/kg for the rats given the combination of l-NAME and CA-4-P. The combination of l-NAME and CA-4-P increased blood pressure by 53%, which suggests an additive effect of the two drugs (results not shown). Fig. 6 shows that l-NAME increased tumor vascular resistance by a factor of around 5, which is similar to the increase observed previously for Nω-nitro-l-arginine (l-NNA) at early times after bolus i.v. administration (33). CA-4-P increased tumor vascular resistance by a factor of 17, which is very similar to the higher dose of 100 mg/kg shown in Fig. 3. A comparison of Fig. 3 and Fig. 6 shows that the vascular resistance increases that are observed in the normal tissues with CA-4-P are also very similar for the two doses of CA-4-P and are much smaller than that observed in the tumor. l-NAME tended to increase vascular resistance in the normal tissues although the effects are much smaller than that observed in the tumor and did not reach statistical significance for any of the tissues except for the skin overlying the tumor and kidney. Combining l-NAME with CA-4-P caused a very large increase in tumor vascular resistance to around 300 times that of control tumors. This effect was more than additive (Fig. 6). The combination also tended to increase vascular resistance in the normal tissues with a significantly higher vascular resistance for the combination than for CA-4-P alone in the skin, brain, heart, kidney, and small intestine. However, the combined effect was much smaller than that observed in the tumor and may have been no more than additive.

Table 1 compares the tissue levels of CA-4-P and CA-4 relative to the perfusate levels at the end of the perfusion in the isolated perfused tumors and the hind limbs shown in Fig. 7. The concentrations of CA-4-P and CA-4 in the tumor perfusate were 120.9 ± 3.8 μM and 6.5 ± 3.1 μM, respectively, which closely matched the plasma levels in the rats treated with 100 mg/kg CA-4-P at 1 h after treatment. The total level of combretastatin (CA-4-P plus CA-4) in the tumor tissue at the end of perfusion, was significantly higher than that in the skeletal muscle of the hind limbs (Table 1). This reflects favorable conditions within the tumors for extravasation of the compound(s)
and/or transport of the compound(s) within the extravascular space. The level of CA-4 was nearly 8 times higher in the tumor tissue than in the perfusate (P < 0.01), whereas the level of CA-4 in skeletal muscle was not significantly different from that in the perfusate. This suggests that dephosphorylation of CA-4-P to CA-4 was much more efficient in the tumor than in the hind limb preparation. This hypothesis was supported by data in Fig. 8, which show rapid dephosphorylation of CA-4-P in tumor tissue but not in skeletal muscle tissue. In the tumor homogenate, a concentration of CA-4-P in excess of 400 μM was completely dephosphorylated to CA-4 within 1 h of exposure time. However, in the skeletal muscle homogenate, very little dephosphorylation was achieved within a 2-h exposure, and over 6 h of exposure was required for complete conversion to CA-4.

**DISCUSSION**

Results in Figs. 1, 2, and 3 show that the vascular-damaging effects of CA-4-P were larger in tumor tissue than in any of the normal tissues investigated. Although significant increases in vascular resistance were calculated for all of the normal tissues at one or both of the time points studied, which suggests some vascular damage, blood flow was maintained at or above control levels in the heart, kidney, and small intestine. This compares with a 100-fold reduction in blood flow to the tumor. These results suggest that CA-4-P at 100 mg/kg causes vasoconstriction and/or an increase in blood viscosity in the normal tissues, which—in the heart, kidney, and small intestine—is balanced or exceeded by an increase in tissue perfusion pressure, thus preventing any decrease in blood flow. This conclusion is based on the assumption that the tissue perfusion pressure changes in direct proportion to changes in MABP, which seems reasonable.

The observed increase in MABP may have been due entirely to an increase in total peripheral resistance, suggested by a 3-fold decrease in blood flow to skeletal muscle. The slight recovery of MABP toward control levels at 6 h after treatment may have been due to a decrease in cardiac output, suggested by the small decrease in blood flow to the myocardium between 1 and 6 h. These results are encouraging for obtaining a therapeutic benefit for CA-4-P. It will be important to determine whether reducing the dose of CA-4-P can achieve significant reductions in tumor blood flow with minimum effect in normal tissues. CA-4-P (100 mg/kg) is approximately one-tenth of the maximum-tolerated dose in mice (20). However, significant decreases in blood flow to some of the normal tissues and an indication of a fall in cardiac output suggest that the maximum-tolerated dose in rats is significantly lower than this. Data for 30 mg/kg (Fig. 6) show a very similar effect to 100 mg/kg at 1 h after treatment. However, there may be differences at later time points, which are currently under investigation.

Blood flow to the tumor was significantly lower at 6 h after treatment than at 1 h. A similar time course was observed for the skin, spleen, brain and heart. In vitro studies have shown that CA-4-P induces endothelial shape changes with a consequent increase in permeability of an endothelial cell monolayer to macromolecules. It is conceivable that an increase in vascular permeability to macromolecules is the primary event in vivo and that subsequent events develop relatively slowly to account for the observed time course. These events may include an increase in interstitial fluid pressure, an increase in blood viscosity, procoagulative effects, vascular collapse, and the induction of cytokines. The reason for the tumor selectivity of CA-4-P is not clear from these results but may relate to differences in proliferation rate of endothelial cells in tumors and normal tissues, as suggested previously (20), or in the rate of dephosphorylation of CA-4-P to its active form (see below).

The increase in tumor blood flow heterogeneity after CA-4-P indicates that there is a wide variation in response to the drug in different tumor microregions. This has been observed in real-time, using intravital microscopy of the P22 tumor growing in a transparent window chamber. The heterogeneity may also reflect temporal fluctuations in blood flow that are asynchronous between different tumor regions. However, the results with the isolated perfused tumors (Fig. 7) suggest that any temporal fluctuations in blood flow actually occur throughout the whole tumor mass. The ratio of blood flow in the periphery of tumors/blood flow in the center doubled after CA-4-P treatment. This has also been observed using intravital microscopy of the P22 tumor and is consistent with the observation, in mouse tumors and human tumor xenografts, that a viable rim of tumor cells survives to repopulate the tumor after CA-4-P treatment (20). This is the most likely explanation for the rather moderate tumor regrowth delay observed for CA-4-P administered as a single agent (21). The remaining viable tumor rim represents a potential target for conventional cytotoxic treatments, and the combination of CA-4-P with cisplatinum or fractionated radiation treatment has been found to significantly enhance the effect of either treatment given alone (21). A dose of 100 mg/kg CA-4-P was found to cause irreversible vascular changes in the adenocarcinoma NT (CaNT) tumor in mice up to 24 h after treatment (21). However, any recovery in tumor blood flow would also limit the cytotoxic effect of CA-4-P, and this should be investigated in other tumor models over a wide dose range.

The reason for the reduced effect of CA-4-P on blood vessels in the

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5 G. Dark and S. Galbraith, personal communication.
6 Unpublished data.
tumor periphery is unclear at present. It may be that there are more incorporated normal vessels in the periphery whose response to CA-4-P is different from that of central vessels. Intravital microscopy has shown that peripheral tumor tissue retains some blood flow after CA-4-P treatment but becomes hemorrhagic with dilated blood vessels. This compares with central regions in which there is a major loss of the visible vasculature, and the tissue appears pale.6 This suggests that vascular permeability changes may be more profound in the periphery, where most of the extravasation of macromolecules occurs under unperturbed conditions (34). However, any resulting increase in interstitial fluid pressure may have a more catastrophic effect on tumor blood flow in the center, where the unperturbed pressure is high (35). These possibilities remain to be tested.

The combination of L-NAME and 30 mg/kg CA-4-P significantly enhanced the vascular effect of either agent alone in the tumor and in most of the normal tissues with the exception of the kidney and small intestine, in which the effect of the combination was very similar to that of L-NAME alone. However, the striking effect was the very large increase in vascular resistance observed in the tumor compared with any of the normal tissues, such that coadministration of L-NAME increased the normal:tumor blood flow ratio achieved with CA-4-P treatment alone. These results show that tissue production of NO protects against the damaging effects of CA-4-P. However, in common with many other tumor types, the P22 tumor produces high levels of NO (Refs. 33, 36 and Footnote7) and is much more sensitive to CA-4-P than any of the normal tissues studied. Therefore, although differences in NO production between tumor and normal tissues could explain the increased normal:

tumor blood flow ratio achieved by adding a NOS inhibitor, they cannot explain the innate selectivity of CA-4-P for tumor tissue.

NO production is important for the maintenance of blood flow to the P22 tumor (33), and it is possible that NO, via its vasodilatory properties, maintains some blood flow even in the face of endothelial damage induced by CA-4-P. In the presence of L-NAME, this residual flow would be lost with the consequences that we observed. The combined effect of L-NAME and CA-4-P on tumor blood flow is also reminiscent of the increased tumor cytotoxicity observed when NOS inhibition is combined with ischemia-reperfusion injury (22, 23) and photodynamic therapy.4 These strategies are both vascular-damaging via the induction of oxidative stress. It may be that vascular damage induced by CA-4-P involves the production of damaging oxygen species that, in the presence of NO, are redirected along a less damaging pathway than occurs in the presence of a NOS-inhibiting analogue of L-arginine, such as L-NAME. This would be more apparent in the high NO-producing P22 tumor than in the normal tissues.

L-arginine analogues are undergoing clinical trial for use in the prevention of endotoxic shock (37). Their role in potentiating CA-4-P treatment requires further evaluation. In particular, it is possible that, because NO is a key modulator of neutrophil adhesion, L-NAME administration enhances neutrophil adhesion during CA-4-P treatment. The associated oxidative burst could account for the increased damage associated with the combined administration of CA-4-P and a NOS inhibitor.

Results for isolated perfusions of tumor and normal rat hind limbs confirm our previous preliminary data (20) and show that there was a significant increase in vascular resistance in tumors but not hind limbs with constant infusion of CA-4-P. These results confirm the differential observed in the whole-animal studies for the vascular effects of tumor periphery is unclear at present. It may be that there are more incorporated normal vessels in the periphery whose response to CA-4-P is different from that of central vessels. Intravital microscopy has shown that peripheral tumor tissue retains some blood flow after CA-4-P treatment but becomes hemorrhagic with dilated blood vessels. This compares with central regions in which there is a major loss of the visible vasculature, and the tissue appears pale.6 This suggests that vascular permeability changes may be more profound in the periphery, where most of the extravasation of macromolecules occurs under unperturbed conditions (34). However, any resulting increase in interstitial fluid pressure may have a more catastrophic effect on tumor blood flow in the center, where the unperturbed pressure is high (35). These possibilities remain to be tested.

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L-arginine analogues are undergoing clinical trial for use in the prevention of endotoxic shock (37). Their role in potentiating CA-4-P treatment requires further evaluation. In particular, it is possible that, because NO is a key modulator of neutrophil adhesion, L-NAME administration enhances neutrophil adhesion during CA-4-P treatment. The associated oxidative burst could account for the increased damage associated with the combined administration of CA-4-P and a NOS inhibitor.

Results for isolated perfusions of tumor and normal rat hind limbs confirm our previous preliminary data (20) and show that there was a significant increase in vascular resistance in tumors but not hind limbs with constant infusion of CA-4-P. These results confirm the differential observed in the whole-animal studies for the vascular effects of

Fig. 6. The effect of combining CA-4-P (30 mg/kg) with the NOS inhibitor L-NAME (approximately 100 mg/kg over a 24-h period). Tissues were assayed at 1 h after treatment with CA-4-P. Values are means ± 1 SE for control (n = 4, clear columns); L-NAME alone (n = 4, hatched columns); CA-4-P alone (n = 5, black columns); and L-NAME + CA-4-P (n = 5, broken hatched columns). A significant difference from the control value is represented by * (P < 0.05) or ** (P < 0.01). A significant difference between the combination treatment and CA-4-P alone is represented by † (P < 0.05) or †† (P < 0.01).
CA-4-P in tumor versus normal tissue. In addition, they show that tumor vascular effects can occur in the absence of RBCs, platelets, clotting factors, and neutrophils, such that the abnormalities in hemodynamics, intravascular coagulation, and neutrophil adhesion cannot fully account for the vascular effects of CA-4-P. It is possible that the rapid increase in tumor vascular resistance in the isolated perfusions was due to rapid changes in endothelial cell shape or endothelial cell detachment that could occlude flow directly or increase vascular permeability to albumin in the perfusate, causing increased interstitial perfusion pressure, as described above. In addition, CA-4-P may cause a direct vasoconstriction. The concentration of CA-4-P used in the isolated perfusions was similar to the plasma levels observed in the whole-animal studies at 1 h after treatment, which indicates that the tissues in the isolated perfusions were exposed to levels of CA-4-P that were similar to those in the tissues in the whole-animal studies. Despite this, comparison of Fig. 3 and 7 show that the increase in tumor vascular resistance was much greater in the whole-animal studies than in the isolated perfusions. This suggests that coagulation and other mechanisms relating to blood components, such as neutrophil adhesion, are likely to be involved in the mechanism of action of CA-4-P in vivo. It is also possible that the systemic effects of CA-4-P in the whole-animal studies result in increased circulating levels of vasoactive hormones, which contribute to the overall effects of the drug. Fluctuations in tumor vascular resistance, which occurred on a time-scale of several minutes, were a characteristic of the isolated tumor perfusions. Large fluctuations do not normally occur under unperturbed conditions but have been observed, for instance, during the administration of the vasoconstrictor, phenylephrine (38). Similar effects have been noted for isolated perfusions of arteries supplying the P22 tumor (39), which suggests that vasomotion of arterial blood vessels may be responsible for our results. Vasomotion is a feature of normal arteries and some microvessels, although it tends to occur at a higher frequency than that observed in the present study. At present, the mechanisms controlling vasomotion are not fully understood, and it has been suggested that, in isolated perfused arteries, it is not strictly controlled. Rather, it may be an example of temporal chaos (40). Experiments are in progress to determine whether CA-4-P induces similar fluctuations in vascular resistance under in vivo conditions.

Finally, we found that the uptake of combretastatin into tumor tissue and the dephosphorylation of CA-4-P to its active form, CA-4, were much higher in tumor tissue than in skeletal muscle. The active form of combretastatin, CA-4, binds to plasma proteins, and this seems to reduce its activity (18). Therefore, there may be an advantage for using CA-4-P rather than CA-4 beyond its increased solubility. The polar CA-4-P will not readily cross intact cell membranes but may have easier access through the relatively "leaky" tumor vasculature. The high concentration of total drug found in the P22 tumor may also be driven by the efficient dephosphorylation of CA-4-P to CA-4 found in tumors compared with skeletal muscle. The level of alkaline phosphatase in a tumor homogenate was found to be significantly higher than that in a homogenate of skeletal muscle. Alkaline phosphatase is found at high levels in endothelial cells and in the underlying adventitia of larger vessels and is commonly used as a vascular marker in normal tissues (41). The relatively high level of alkaline phosphatase activity in the P22 tumor was, therefore, most likely derived from the tumor vasculature. The plasma half-life of CA-4-P is short (15 min in female CBA mice), which suggests that the phosphatase activity in the vasculature of different tissues may influence the amount of active drug taken up into endothelial cells. Paradoxically, levels of alkaline phosphatase in tumor blood vessels tend to be rather low compared with those in normal tissues, such as the heart and brain (42). This is believed to account partially for the reduced tumor effect of the radioprotector, WR-2721, which requires dephosphorylation to WR-1065 for its activity (43). However, most rodent and xenografted human

Table 1 Levels of CA-4-P and CA-4 in tumor and skeletal muscle of the hind limb relative to levels in the perfusate of ex vivo perfusions

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CA-4-P (fraction of perfusate level)</th>
<th>CA-4 (fraction of perfusate level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>0.72 ± 0.05**</td>
<td>7.77 ± 2.67*</td>
</tr>
<tr>
<td>Hind limb</td>
<td>0.22 ± 0.06</td>
<td>1.09 ± 0.09</td>
</tr>
</tbody>
</table>

CA-4-P and CA-4 are expressed as mean ± SE. Tissues were derived from a single preparation (4) for each tissue. Significant differences between tumor and hind limb are indicated by * (P < 0.05) and ** (P < 0.01).
tumors tested in our laboratory respond to CA-4-P at relatively nontoxic doses,8 which suggests that phosphatase activity in the vasculature is not the primary determinant of the differential in effect of CA-4-P between tumor and normal tissues. Despite this inference, the difference in response between the P22 tumor and the normal hind limb, in our studies, does correlate with phosphatase activity. Currently, we do not know how phosphatase activity in the P22 tumor compares with that in other tumors. A study of the relationship between phosphatase activity and responsiveness to CA-4-P in a range of tumor types would provide insight into the mechanism of action of this drug.

In summary, the present study shows that the effect of CA-4-P on tissue blood flow and vascular resistance is much greater in the P22 tumor than in a range of normal tissues. The spatial distribution of tumor blood flow after CA-4-P administration explains our previous finding that a peripheral rim of tumor cells survives CA-4-P treatment to repopulate the tumor. NO protects against the vascular effects of CA-4-P, and the combination of CA-4-P and a NO inhibitor increases the differential between CA-4-P-induced vascular damage in tumor and normal tissues. An increase in tumor vascular resistance is achieved even in the absence of RBCs, platelets, clotting factors, and neutrophils, although the effect is not as large as that observed in vivo. This study confirms the therapeutic potential of CA-4-P as a tumor vascular targeting agent and begins to unravel the mechanism of action of the drug under in vivo conditions.

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Combretastatin A-4 Phosphate as a Tumor Vascular-Targeting Agent: Early Effects in Tumors and Normal Tissues

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