Combretastatin A-4 Phosphate Suppresses Development and Induces Regression of Choroidal Neovascularization

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PURPOSE. Combretastatin A-4 (CA-4) is a naturally occurring agent that binds tubulin and causes necrosis and shrinkage of tumors by damaging their blood vessels. In this study the effect of a CA-4 prodrug, combretastatin A-4-phosphate (CA-4-P), was tested in two models of ocular neovascularization.

METHODS. The effect of CA-4-P was quantitatively assessed in transgenic mice with overexpression of vascular endothelial growth factor in the retina (rh/VEGF mice) and mice with choroidal neovascularization (CNV) due to laser-induced rupture of Bruch’s membrane.

RESULTS. In rh/VEGF mice, daily intraperitoneal injections of 4.0 mg/kg CA-4-P starting at postnatal day (P)7, the time of onset of transgene expression, resulted in a significant reduction in the number of neovascular lesions and total area of neovascularization per retina at P21, compared with vehicle-injected mice. In mice with laser-induced rupture of Bruch’s membrane, daily intraperitoneal injections of 75 or 100 mg/kg CA-4-P resulted in a significant reduction in the area of CNV at rupture sites compared with vehicle-injected mice. In mice with established CNV, daily intraperitoneal injections of 100 mg/kg CA-4-P for 1 week resulted in a significant reduction in CNV area at rupture sites compared with the baseline area before treatment or the area of CNV in vehicle-treated mice.

CONCLUSIONS. These data indicate that CA-4-P suppresses the development of VEGF-induced neovascularization in the retina and both blocks development and promotes regression of CNV. Therefore, CA-4-P shows potential for both prevention and treatment of ocular neovascularization. (Invest Ophthalmol Vis Sci. 2003;44:3650–3655) DOI:10.1167/iovs.02-0985

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with different characteristics3,4 that impart selective toxicity to tumor vasculature.5 Combretastatin A-4-phosphate (CA-4-P) is a more soluble, inactive prodrug that is converted to CA-4 by endogenous nonspecific phosphatases.6 The selectivity of CA-4 for tumor vasculature allows for antitumor effects at doses of CA-4-P that are well tolerated.7 A single dose of 100 mg/kg is well tolerated in adult mice and has beneficial effects on several types of tumors.8,9,10 This finding led to a phase I study in patients with advanced cancer that showed a reasonable safety profile for 10-minute intravascular infusions of doses of 60 mg/m2 or less, administered every 3 weeks, and some preliminary evidence of possible efficacy, in that a patient with anaplastic thyroid cancer had a complete response.11 Therefore, CA-4-P shows considerable promise as a novel treatment for cancer.

Recently, it has been demonstrated that administration of CA-4-P results in microthrombus formation in new vessels surrounding a hyperplastic thyroid, indicating that its effects are not limited to tumor vasculature.12 In neonatal mice with oxygen-induced ischemic retinopathy, daily intraperitoneal injections of CA-4-P starting before the onset of neovascularization suppressed the development of retinal neovascularization.13 In this study, we sought to determine the effects of CA-4-P on subretinal and choroidal neovascularization.

MATERIALS AND METHODS

Treatment of Rhodopsin/VEGF Transgenic Mice

Mice were treated in accordance with the recommendations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Hemizygous rhodopsin/VEGF transgenic mice14,15 were given daily intraperitoneal injections of vehicle (n = 13 mice) or vehicle containing 2.2 (n = 5) or 4 mg/kg (n = 9) of CA-4-P (Oxigene, Inc., Boston, MA) between postnatal day (P)7 and P21. At P21, the mice were anesthetized and perfused with fluorescein-labeled dextran (2 × 108 molecular weight, Sigma-Aldrich, St. Louis, MO), and retinal flatmounts were prepared as previously described.15 Briefly, the eyes were removed and fixed for 1 hour in 10% phosphate-buffered formalin, and the cornea and lens were removed. The entire retina was carefully dissected from the eyecup, radically cut from the edge of the retina to the equator in all four quadrants, and flattened in aqueous mounting medium (Aquamount; BDH, Poole, UK) with photoreceptors facing upward. One retina of a mouse in the vehicle-treated group was severely damaged during dissection and could not be evaluated, leaving 25 retinas in the vehicle group for analysis. Flatmounts were examined by fluorescence microscopy (Axioskop; Zeiss, Thornwood, NY).

Quantitation of VEGF-Induced Neovascularization

Retinal flatmounts were examined by fluorescence microscopy at 400× magnification, which provides a narrow depth of field, so that when focusing on NV on the outer edge of the retina the remainder of the retinal vessels are out of focus, allowing easy delineation of the NV.15 The outer edge of the retina, which corresponds to the subreti-
nal space in vivo, is easily identified and therefore there is standard-
ization of focal plane from slide to slide. Images were digitized with a
three-color charge-coupled device (CCD) video camera (IK-TU40A;
Toshiba, Tokyo, Japan) and a frame grabber. Image analysis software
(Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was set to
recognize fluoroescently stained neovascularization and used to deline-
ate each of the lesions throughout the entire retina and calculate the
number of lesions per retina, the area of each lesion, and the total area
of neovascularization per retina.

Preventive Treatment of Laser-Induced CNV

Laser photocoagulation-induced rupture of Bruch’s membrane was used
to generate CNV. Briefly, 4 to 5-week-old female C57BL/6j mice
were anesthetized with ketamine hydrochloride (100 mg/kg body
weight), and the pupils were dilated with 1% tropicamide. Three burns
(group 1; mice were treated with daily intraperitoneal injections of vehicle
obtaining experimental CNV,16 and therefore only burns in which a
indicates rupture of Bruch’s membrane, is an important factor in
the retina. Production of a bubble at the time of laser burn, which
were performed in the 9, 12, and 5 o’clock positions of the posterior pole of
duration, 120 mW) were delivered to each retina with the slit lamp
delivery system of a photocoagulator (OcuLight GL; Iridex, Mountain
View, CA) and a handheld coverslip used as a contact lens. Burns were
indicated by the absence of a vaporization bubble, resulted in the
absence of a vaporization bubble, resulted in the following number of rupture sites for analysis in each group: group 1, 19; group 2, 21; group 3, 22; group 4, 5; and group 6, 37.

Elimination of burns that had not ruptured Bruch’s membrane, as
indicated by the absence of a vaporization bubble, resulted in the following number of rupture sites for analysis in each group: group 1, 185; group 2, 41; group 3, 51; group 4, 58; group 5, 17; and group 6, 92.

Treatment of Established CNV

Adult female C57BL/6 mice (n = 25) had laser treatment of three
locations in each eye, as described earlier. Only burns in which a
bubble was produced were included in the study. After laser burn, mice
were treated with daily intraperitoneal injections of vehicle (group 1; n = 41) or vehicle containing 10 (group 2; n = 10), 20
(group 3; n = 11), 50 (group 4; n = 11), 75 (group 5; n = 4), or 100
(group 6; n = 19) mg/kg CA-4-P. After 2 weeks, mice were perfused
with fluorescein-labeled dextran, and choroidal flatmounts were pre-
pared as described for retinal flatmounts, except that the eyeball rather
than the retina was cut with radial cuts and mounted. In each group,
some eyes were unusable due to traumatic injuries from fighting
among the mice or damage incurred during enucleation or dissection,
resulting in the following number of eyes in each group: group 1, 76;
group 2, 19; group 3, 21; group 4, 22; group 5, 8; and group 6, 37.

Measurement of the Area of CNV

The area of CNV lesions was measured in choroidal flatmounts.17
Flatmounts were examined by fluorescein microscopy, and images
were digitized using a three-color CCD video camera and a frame
grabber. Image-analysis software (Image-Pro Plus; Media Cybernetics)
was used to measure the total area of hyperfluorescence associated
with each burn, corresponding to the total fibrovascular scar.

Statistical Analysis

In mice with laser-induced rupture of Bruch’s membrane, CNV areas
were analyzed with a linear mixed model.18 This model is analogous to
analysis of variance (ANOVA), but allows analysis of all CNV area
measurements from each mouse, rather than average CNV area per
mouse, by accounting for correlation between measurements from
the same mouse. The advantage of this model over ANOVA is that it
accounts for differing precision in mouse-specific average measure-
ments arising from a varying number of observations among mice. A
log transformation was used on the area measurements before analysis
so that they better met the normal distribution assumption of the
analytic model. Probabilities for comparison of treatments were ad-
justed for multiple comparisons by the Dunnett method. P ≤ 0.05 was
considered statistically significant.

In rho/VEGF transgenic mice, the data for the number of neovas-
cular lesions per retina, the average size of neovascular lesions, and the
total area of neovascularization per retina were analyzed separately
with a linear mixed model, as described earlier. A log transformation
was applied to the number of lesions and the total area measurements
before analysis, so that they better met the model assumption of
normally distributed data. Probabilities for the comparison of treat-
ments were adjusted for multiple comparisons using the Dunnett
method. P ≤ 0.05 was considered statistically significant.

RESULTS

Effect of CA-4-P on the Development of Subretinal Neovascularization in Rho/VEGF Transgenic Mice

Litters of hemizygous rho/VEGF transgenic mice were divided
into three groups and between P7 and P21 were given daily
intraperitoneal injections of vehicle, or vehicle containing 2.2
or 4 mg/kg CA-4-P. At P21, mice treated with vehicle (Fig. 1A)
or 2.2 mg/kg CA-4-P (Fig. 1B) showed numerous neovascular
lesions (arrows). In contrast, fewer neovascular lesions were
present in mice treated with 4.0 mg/kg CA-4-P (Fig. 1C, ar-
rows). Image analysis confirmed that there were significantly
fewer neovascular lesions and that each had a significantly
smaller area than those in the retinas of vehicle-treated mice
(Fig. 1D). There was no difference in number or area of lesions
between vehicle-treated mice and mice treated with 2.2 mg/kg
CA-4-P. The total area of neovascularization per retina was
0.01415 ± 0.00432 mm² in mice treated with 4 mg/kg CA-4-P,
which was significantly less than that in mice treated with
vehicle (0.06112 ± 0.03436 mm², P = 0.0033). The total area
of neovascularization per retina was 0.04671 ± 0.01059 mm²
in mice treated with 2.2 mg/kg CA-4-P, which was not sig-
ificantly different from that in vehicle-treated mice (P = 1.00).

Effect of CA-4-P on the Development of CNV at Sites of Rupture of Bruch’s Membrane

Adult mice tolerate much higher levels of CA-4-P than neonatal
mice, which experience high mortality at doses above 5 mg/
kg.13 Because previous studies had demonstrated that single
doses of 100 mg/kg are well-tolerated in adult mice,5,8–10 we
selected this for our highest dose in adults. Six of 25 mice
were treated with 100 mg/kg per day for 2 weeks died near the end
of the treatment period, suggesting that this is at or near the
maximum tolerated dose for a 2-week treatment period. There
were no deaths in mice treated with 100 mg/kg per day for 1
week (see companion study described later), nor in mice
treated with 75 mg/kg per day or lower doses for 2 weeks.

Treatment of adult C57BL/6 mice with 10, 20, or 50 mg/kg
CA-4-P for 2 weeks after laser-induced rupture of Bruch’s mem-
bane resulted in CNV that had no significant difference in area
compared with CNV in mice treated with vehicle (Fig. 2). Mice
were treated with 75 mg/kg CA-4-P had a moderate and statistically
significant decrease in the area of CNV (Figs. 2E, 2G). Mice

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treated with 100 mg/kg CA-4-P had a large, statistically significant decrease in CNV lesion size (P < 0.0001; Figs. 2F, 2G).

**Effect of CA-4-P on Established CNV**

To determine whether CA-4-P has any effect on already established CNV, mice were not treated for 1 week after laser-induced rupture of Bruch’s membrane, and the baseline amount of CNV was measured (Fig. 3A). The remainder of the mice were treated with vehicle or vehicle containing 100 mg/kg CA-4-P, and after an additional week the amount of CNV at Bruch’s membrane rupture sites was measured. There was no significant difference in size between baseline CNV lesions (Fig. 3A) and those in mice treated for the subsequent week with vehicle (Figs. 3B, 3D). However, mice treated for the subsequent week with 100 mg/kg CA-4-P had significantly smaller CNV lesions (Fig. 3C) than those at baseline (Fig. 3A) or those in vehicle-treated mice (Figs. 3B, 3D). This indicates that treatment with CA-4-P for 1 week resulted in partial involution of CNV.

**DISCUSSION**

In this study, we have demonstrated that a tubulin-binding agent, CA-4-P suppresses the development of subretinal neovascularization in rdhopsin/VEGF transgenic mice and suppresses the development of CNV at Bruch’s membrane rupture sites. This suggests that when administered before onset of an angiogenic stimulus, CA-4-P can prevent these two forms of ocular neovascularization. A recent study has shown that CA-4-P can also prevent ischemia-induced retinal neovascularization in mice. Therefore, CA-4-P joins a growing list of drugs that have potential as prophylactic agents for retinal and/or choroidal neovascularization. However, CA-4-P also caused partial regression of established CNV, a finding that sets it apart from other drugs. To our knowledge, intraocular gene transfer of pigment epithelium-derived factor (PEDF) is the only other gene-therapy–based or drug treatment that has been shown conclusively to cause partial regression of CNV, and therefore CA-4-P is in select company. Therefore, similar to PEDF gene transfer, treatment with CA-4-P has potential for treatment of established CNV.

There are many differences between neovascularization in tumors and CNV, and some agents such as interferon-α, which have been shown to inhibit some types of tumor angiogenesis, do not inhibit CNV. Therefore, it is hazardous to predict the effect of drugs on ocular neovascularization based on effects on tumor neovascularization. However, now that it has been demonstrated that CA-4-P causes regression of CNV, it is reasonable to consider findings in tumor models to formulate possible mechanisms by which CA-4-P may have this effect.

Within 2 hours of a single intraperitoneal dose of 150 mg/kg CA-4 or 100 mg/kg CA-4-P, signs of hemorrhagic necrosis occur and vascular resistance; vasoconstriction, which is partially ameliorated by nitric oxide (NO) and exacerbated by NO synthase (NOS) inhibitors; increased vascular permeability; platelet thrombi; and vascular shutdown. In cultured endothelial cells, CA-4-P causes shape changes and apoptosis of proliferating cells, but not of quiescent cells. It appears that the cytoskeleton of newly formed cells is sensitive to CA-4-P, whereas the cytoskeleton of mature cells is not. This appears to underlie the preferential sensitivity of endothelial cells in tumor vessels, which unlike those in normal vessels, become thrombogenic, resulting in hemorrhagic necrosis of tumors. The present study suggests that this differential sensitivity also applies to adult mice with CNV.
We did not address toxicity of CA-4-P in our study, but our data suggest that a dose of 100 mg/kg per day is near the maximum tolerated dose in adult mice when given for 2 weeks, but is well tolerated for 1 week. Increasing the number or frequency of doses seems to increase toxicity. Doses as low as 25 mg/kg per day given every 12 hours cause severe damage to liver vasculature and death within 5 days. A phase 1 trial in patients with advanced cancer demonstrated that an intravenous dose of 60 mg/m² or less every 5 weeks is safe and well tolerated, with some preliminary evidence of efficacy. Clinical trials for ocular neovascularization in adult humans could be designed to take advantage of safety data for CA-4-P that is...
being generated in oncology trials. Neonatal mice are particularly sensitive to the effects of CA-4-P. Mice survive a dose of 4 mg/kg between P7 and P21, which significantly suppresses VEGF-induced neovascularization in the retina, but doses above 5 mg/kg result in a high rate of mortality. We postulate that cytoskeletal maturation occurs more slowly and to a lesser extent in neonates, making pathologic neovascularization exquisitely sensitive to CA-4-P, but other vessels throughout the body are only slightly less sensitive. This suggests that CA-4-P is not appropriate for treatment of retinopathy of prematurity or any other neovascular diseases in infants.

Although it is not certain that the mechanism by which CA-4-P causes partial regression of CNV is the same as the mechanism by which it affects tumor vessels, it is certainly a reasonable hypothesis and deserves investigation in future studies. It is also important to determine whether in treatment of CNV there is synergism between CA-4-P and hyperthermia, as is the case with treatment of tumors. Similarly, it is important to investigate potential synergism between CA-4-P and photodynamic therapy. While gaining this information, it would be reasonable to consider a clinical trial based on safety data obtained in a phase I study in patients with advanced cancer.

**References**


**FIGURE 3.** CA-4-P caused regression of established CNV. Adult C57BL/6 mice had rupture of Bruch’s membrane at three locations in each eye by laser photocoagulation. After 1 week, some mice were perfused with fluorescein-labeled dextran, and choroidal flatmounts were examined by fluorescence microscopy to establish the baseline amount of CNV (A). The remainder of the mice were given daily intraperitoneal injections of vehicle (B) or vehicle containing 100 mg/kg CA-4-P (C). Measurement of CNV area by image analysis showed that mice treated with CA-4-P had significantly less CNV than mice treated with vehicle (D). The CNV area in CA-4-P-treated mice was also significantly less than that at baseline before treatment (D) indicating that CA-4-P caused regression of the CNV. There was no statistically significant difference between the baseline area of CNV and the area in mice treated with vehicle. The number of rupture sites evaluated in each group was: 1 week baseline, 42; 2 weeks vehicle-treated, 31; and 2 weeks CA-4-P-treated, 38. *P* = 0.0003 by linear mixed model for comparison versus vehicle at 2 weeks; †*P* = 0.0002 by linear mixed model for comparison with baseline CNV area at 1 week. Probabilities are adjusted for multiple comparisons by the Dunnett method. Bar = 100 μm


