Combretastatin A-4 Prodrug in the Treatment of a Murine Model of Retinoblastoma

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Purpose. To evaluate the effect of subconjunctival injections of combretastatin A-4 phosphate (CA-4P) prodrug treatment on tumor vasculature and growth in an animal model of hereditary retinoblastoma.

Methods. Twenty-four, 12-week-old simian virus-40 T-antigen–positive mice received six subconjunctival CA-4P injections at doses of 0.5, 1.0, 1.5, and 2.0 mg delivered at 72-hour intervals to the right eye only. Six control animals received placebo treatment. All animals underwent serial ophthalmic evaluations and were euthanatized at 16 weeks of age, and eyes were obtained for histopathologic examination. Eyes were graded for presence or absence of tumor, delay of tumor growth, and intratumoral vascularity.

Results. The use of subconjunctivally injected CA-4P prodrug induced an extensive, dose-dependent decrease in microvessel density and led to significant tumor reduction in treated eyes compared with the placebo control ($P < 0.001$). No evidence of corneal, lenticular, choroidal, or retinal toxicity was observed by histopathologic evaluation.

Conclusions. Subconjunctival delivery of CA-4P is associated with extensive dose-dependent reduction in blood vessel count in this murine model of retinoblastoma. A combination treatment of retinoblastoma incorporating CA-4P may allow enhanced tumor reduction enabling a decrease in standard treatment doses of both chemotherapy and external beam radiotherapy. (*Invest Ophthalmol Vis Sci.* 2005;46:8–11) DOI: 10.1167/iovs.04-0751

Timely diagnosis and treatment of intraocular retinoblastoma have contributed to a marked improvement in patient survival.[1–3] Current treatment modalities, such as external beam radiotherapy and chemotherapy have focused on increased globe conservation.[4] Although efficacious, these treatment modalities have been associated with severe complications including an increased risk of secondary tumors in childhood survivors.[5–6] Due to existing concerns regarding significant morbidity and potential mortality associated with current therapies in the treatment of retinoblastoma, newer therapeutic modalities are being investigated.

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Vasculature is critical to the survival of a solid tumor mass, and angiogenesis has been found to be a prerequisite for continued tumor growth.[7–8] Angiogenesis involves the formation of new capillary blood vessels from preexisting vessels through a complex cascade of events.[8] Inhibition of one or more of these events is of potential therapeutic value for those pathologic conditions in which abnormal angiogenesis is a factor. Solid tumors exhibit the capacity to induce angiogenesis. Selective induction of vascular damage within tumors (i.e., selective damage of tumor-induced neovasculature) represents an emerging approach to cancer treatment. This has led to the development of many antiangiogenic agents for cancer therapy.[9–11] Clinical trials of vessel targeting therapy have yielded various results, with initial disappointing findings. However, current applications using targeted and adjuvant therapies have shown enhanced outcomes when applying these new treatment regimens.[9–10]

Retinoblastoma tumors are highly vascularized and dependent on their vascular supply. Retinoblastoma tumors form cuffs of viable cells that surround blood vessels and areas of necrosis are found removed from vessels.[12] This dependence on vascular supply makes retinoblastoma tumors optimal candidates for blood vessel–targeting therapy.

Combretastatin A-4 phosphate (CA-4P), a tubulin-binding agent originally isolated from the South African shrub *Combretum caffrum*, has emerged as a potent, selective vasculature-targeting drug.[13–15] This water-soluble phosphate prodrug is rapidly dephosphorylated in vivo to yield the parent drug, with a very short plasma half-life.[16]

Several studies have shown the efficacy of CA-4P in different tumor animal models such as murine colon adenocarcinoma, lung carcinomas, and mammary carcinoma, among others.[17–20] These studies have demonstrated that CA-4P induces tumor cell necrosis through a rapid, extensive, and long-term shutdown of vasculature within the different types of tumors without treatment-limiting systemic toxicity in the animals.[15,17–20] The precise mechanism of CA-4P's induction of vascular shutdown is not clear. It has been postulated that cytoskeletal changes in endothelial cells as a result of the tubulin-binding properties may contribute to endothelial conformational changes leading to increased tumor-vessel permeability and the disruption of blood flow.[21]

Phase I clinical trials to determine the pharmacokinetic, toxicity, and efficacy profiles of CA-4P as a single agent for the treatment of solid tumors have been completed in the United Kingdom and the United States.[22–24] Preliminary data indicate that CA-4P may be effective in targeting the vasculature of human tumors.

CA-4P has shown promising results in the inhibition of angiogenesis in a murine model of oxygen-induced proliferative retinopathy[25] and a murine model of laser-induced choroidal neovascularization.[26]

The capacity of retinoblastoma cells to promote angiogenesis has been demonstrated in various studies.[27–29] The purpose of the present study was to determine the safety and evaluate the effect of the CA-4P prodrug on the tumor vascu-

TABLE 1. Percentage of Vasculature Gradings in Each Eye by Extent of Vascular Reduction

CA-4P Dose (mg)	Grade 0: 0% Vascular Reduction	Grade 1: >0% Reduction	Grade 2: >25% Reduction	Grade 3: >75% Reduction
0.0	75.0 (16.7)	25.0 (16.7)	0.0 (0.0)	0.0 (0.0)
0.5	86.1 (34.0)	13.9 (34.0)	0.0 (0.0)	0.0 (0.0)
1.0	60.0 (54.8)	40.0 (54.8)	23.3 (43.5)	0.0 (0.0)
1.5	0.0 (0.0)	100.0 (0)	83.3 (19.2)	14.3 (31.1)
2.0	0.0 (0.0)	100.0 (0)	87.5 (25.0)	54.2 (45.9)
*P**	< 0.001	< 0.001	< 0.001	< 0.003

Data are the mean ± SD.
* Probability of linear trend.

lature and growth in a murine model of hereditary retinoblastoma.

METHODS

The study protocol was approved by the University of Miami, School of Medicine Animal Care and Use Review Board. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The transgenic mouse model used in the study has been characterized previously.[30–35] Briefly, a highly expressed transgene drives retinal tumor development by overexpression of the simian virus (SV)40 large T antigen (SV40 Tag). In transgenic animals, bilateral, heritable retinoblastoma develops that resembles human retinoblastoma. At 12 weeks of age, tumors in this animal model are typically moderate to large (occupying approximately 20%–25% of the retinal area and 10%–25% of the ocular volume; corresponding to Reese-Ellsworth groups III-IV).

Thirty SV40 Tag transgene-bearing mice, six animals per treatment group, were treated at 12 weeks of age. This number was determined with a power study on computer (Solo Power Analysis program; BMDP Statistical Software, Los Angeles, CA) based on pilot studies from our laboratory, in which multiple-dose, single-treatment, and multiple-treatment regimens were evaluated. These data suggest that six injections given at 72-hour intervals maximize the drug's ability to alter tumor vasculature and affect tumor burden.

Mice received C4-AP through subconjunctival injections. In the initial evaluation of focal therapy using this model system, we investigated intravitreal drug delivery. However, given the concern that intravitreal injection in the treatment of pediatric retinoblastoma might propagate the tumor, we evaluated subconjunctival delivery of the drug as a more clinically relevant treatment strategy.

Subconjunctival CA-4P Injections in Transgenic Mice

Twenty-four, 12-week-old SV40 Tag mice received subconjunctival CA-4P (OxiGene, Inc, Watertown, MA) injections in the right eye at doses of 0.5, 1.0, 1.5, and 2.0 mg every 72 hours for a total of six injections. CA-4P was dissolved in physiologic saline (balanced salt solution [BSS]; Alcon, Fort Worth, TX) at the appropriate concentrations. Injections were delivered with a 33-gauge needle inserted into the superotemporal subconjunctival space. A microvolume delivery pump was used to ensure accurate and reproducible delivery of a 20-μL volume. Six additional SV40 Tag mice received 20-μL subconjunctival injections of the saline to provide a placebo control.

Histopathological Study of Transgenic Mice

At 16 weeks of age, all animals were euthanatized with CO_2. Both eyes were enucleated and immediately immersion-fixed in 10% formalin. The eyes were embedded in paraffin, sectioned serially, and processed for standard hematoxylin-eosin (H&E) and periodic acid Schiff (PAS) staining. Light microscopic examination was performed on all histopathologic sections in a masked fashion.

Assessment of Tumor Vasculature. Blood vessel detection was performed by using a technique previously described.[36] Microscopic images were obtained of all PAS-stained tumor sections (twenty-one 5.0-μm sections per eye). Sections were viewed with a microscope (Axioplan Axiophot; Carl Zeiss Meditec, Dublin, CA) and images captured with a digital camera (Axiocam with Axiovision; Carl Zeiss Meditec). Vessel quantitation was performed on digitized microscopic images (Axiovision software; Carl Zeiss Meditec) and processed with the digital imaging software (AxioVision; Carl Zeiss Meditec). Tumor vasculature was graded with a vasculature-grading scale ranging from 0 to 3 as follows: 3, tumors showing vascular reduction ≥75% ($0–2.36 \times 10^{-5}$ vessels/μm^2); 2, tumors demonstrating vascular reduction <5% but >25% (2.36×10^{-5} to 9.7×10^{-5} vessels/μm^2); 1, tumors showing vascular reduction of ≤25% (9.7×10^{-5} to 1.5×10^{-4} vessels/μm^2); and 0, tumors with no evidence of vascular reduction (2.14×10^{-4} vessels/μm^2). Analysis of vasculature grading was performed by two separate investigators (EE-B, BH) masked to treatment dose. This grading scale was validated by evaluation of inter- and intra-reader reliability, establishing a reliability index of >98%.

Assessment of Tumor Burden. Tumor area was measured as a function of the globe area. Microscopic images of all H&E-stained sections (sixty 5.0-μm sections per eye) were obtained with a digital camera at a magnification of 40×. Tumor and globe boundaries were traced using a digitizing tablet (Intuos; Wacom Technology Co., Vancouver, WA). For each eye an image-analysis application (Image Pro Express Software; Media Cybernetics, Silver Spring, MD) was used to calculate the percentage of tumor occupying the globe area.

Statistical Analyses

The effect of dose on tumor vasculature was assessed with analysis of variance with orthogonal polynomial decomposition to check for linear trends. The percentage of locations in each scale category were determined for each eye and then means and standard deviations of these percentages were calculated for all the eyes in the study.

Tumor areas were analyzed with analysis of variance. Orthogonal polynomial decomposition was used to test for a linear trend with doses. Statistical analysis was performed on the count data, which did not violate the assumption of homoscedasticity. Results were displayed as the percentage of tumor areas in the control eyes.

RESULTS

Subconjunctival injections of CA-4P induced a dose-dependent reduction in vessel density in the transgenic retinoblastoma mouse tumors (Table 1). At the lowest dose studied, 0.5 mg of CA-4P, on average, >13.9% of the treated tumors showed vascular reduction. After treatment with a dose of 1.0 mg CA-4P, >40.0% of the tumors showed some vascular reduction. After treatment with the higher doses of 1.5 and 2.0 mg CA-4P, all tumors showed a decrease in vessel density with respect to the untreated control. A dose of 2.0 mg CA-4P resulted in more than a 75.0% reduction of vessel density in more than half of the treated tumor-bearing eyes.

Histopathologic examination at age 16 weeks of all treated eyes ($n = 24$) and placebo control eyes ($n = 6$) suggested that the use of subconjunctival injections of CA-4P induced tumor reduction in a dose-dependent manner in the transgenic retinoblastoma mice ($P = 0.002$; Figs. 1, 2). Eyes receiving 2.0, 1.5, and 1.0 mg of CA-4P demonstrated greater tumor reduction at 16 weeks of age after treatment. Complete tumor control was not detected with any of the doses used. The dose–response approached a plateau at >1.0 mg CA-4P.

No evidence of corneal, lenticular, choroidal, or retinal toxicity was observed by histopathologic evaluation. No evidence of systemic toxicity was observed.

Discussion

The use of subconjunctival CA-4P prodrug in the murine retinoblastoma tumors induced an extensive, dose-dependent reduction of vessel density and significant tumor reduction in treated eyes compared with placebo-treated control eyes.

Several investigators have assessed vascular shutdown in other nonocular solid tumors after administration of CA-4P and have demonstrated extensive cell loss persisting up to 24 hours after treatment.[14–15,17–18,37–38] In these studies, although vascular cytotoxic effects and intratumoral necrosis were evident, this was not translated into any significant effect on tumor growth. It has been hypothesized that this continued tumor growth is attributable to an actively proliferating rim of viable cancer cells at the periphery of tumors that are supplied by normal peripheral vessels, which are not affected by CA-4P.[14–15,17] In our study, the retinoblastoma tumors had a relatively longer drug exposure because of the multiple-dose schedules, which translated into a significant tumor reduction. However, this reduction in tumor did not result in total tumor eradication, even at the highest tested doses. Boehle et al.[19] have also documented delayed tumor growth in their in vitro and in vivo experiments of human non–small-cell lung cancer in a murine xenotransplantation model, with the use of CA-4P.

Several investigators have suggested that the most likely clinical application of vascular targeting agents in the treatment of solid tumors is in combination with existing anticancer treatment modalities, including chemotherapy, other antiangiogenic drugs, hyperthermia, and/or radiotherapy. Several preclinical studies have demonstrated the potential of CA-4P to enhance the antitumor activity of conventional cancer therapies.[17,38–42]

We believe it is unlikely that CA-4P can eradicate retinoblastoma tumors as a single agent, but this agent may ultimately have an important application as an adjuvant to current regimens such as chemotherapy, external beam radiotherapy, and local laser ablative therapy.

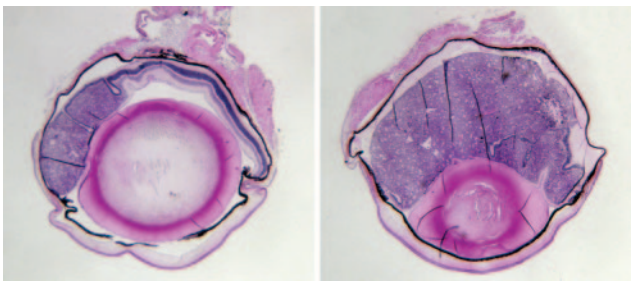

Figure 1. Histopathology sections of enucleated globes. Representative eye treated with 1.5 mg CA-4P, demonstrating reduction in tumor size (*left*). Control eye with large intraocular tumor (*right*). Stain, H&E. Magnification, ×40.

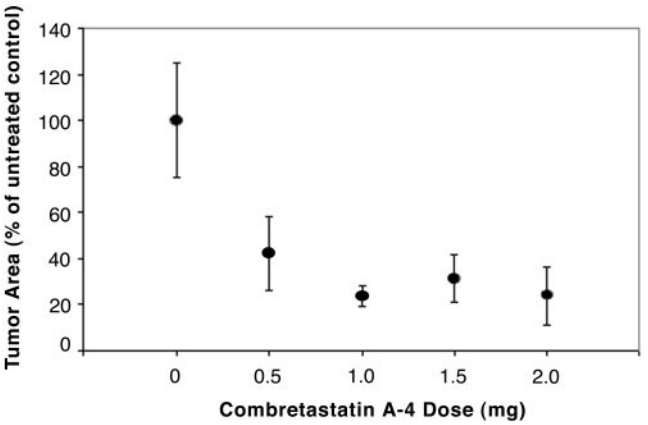

Figure 2. Percentage reduction in tumor areas in treated compared with control eyes. CA-4P induced tumor reduction in a dose-dependent manner ($P = 0.002$).

In vivo experiments have indicated a maximum tolerated dose (MTD) of CA-4P in excess of 1000 mg/kg.[15,43] These studies have also demonstrated that this agent induces vascular shutdown within tumors at doses less than one tenth of the MTD. Furthermore, in vitro experiments indicate that a short drug exposure results in profound long-term cytotoxic effects against proliferating endothelial cells but not cells that are quiescent before and during drug exposure.[15] In our study, no signs of ocular toxicity were found by histopathologic examination in any of the treated eyes. Furthermore, animals did not display evidence of systemic toxicity. However, this may not exclude the possibility of unrecognized toxicity. The doses used were well below those demonstrated to be toxic and below those doses associated with extensive hemorrhagic necrosis in several other tumor types.[14–15,17,37] These findings underscore the potential clinical importance of CA-4P in the treatment of retinoblastoma due to its significant selectivity and broad therapeutic window.

In summary, subconjunctival delivery of CA-4P is associated with extensive dose-dependent vascular closure in this murine model of retinoblastoma. The potential of combination treatment of retinoblastoma incorporating CA-4P may allow enhanced tumor reduction, enabling a decrease in standard treatment doses of both chemotherapy and external beam radiotherapy. Future studies evaluating combined treatment modalities are currently under way.

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