Anecortave Acetate as Single and Adjuvant Therapy in the Treatment of Retinal Tumors of $LH_{BETA}T_{AG}$ Mice

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Purpose. To evaluate the tumor control efficacy of the antiangiogenic agent anecortave acetate as single and combined therapy, in retinal tumor reduction using the $LH_{BETA}T_{AG}$ mouse model of retinoblastoma.

Methods. Group A: Ten-week-old, $LH_{BETA}T_{AG}$ mice received a single subconjunctival injection of anecortave acetate (1200, 600, 300, and 150 μg) delivered to right eyes only. Group B: Ten-week-old, $LH_{BETA}T_{AG}$ mice received a single subconjunctival injection of anecortave acetate (600, 300, and 150 μg) delivered to right eyes only, either during a cycle of carboplatin (six subconjunctival deliveries) or after the completed cycle. Carboplatin was delivered at the subtherapeutic concentration of 62.5 μg. All animals were euthanatized at 16 weeks of age, and the eyes were examined histopathologically.

Results. A statistically significant reduction in tumor burden was detected after a single periocular injection of anecortave acetate. The reduction of tumor burden followed a U-shaped dose–response curve. Tumor burden was significantly decreased when anecortave acetate and carboplatin were combined. However, varying doses and delivery schedule of these agents had significant impact on the effectiveness of the combined treatment. The most effective scheme was delivering a low dose (150–300 μg) of anecortave acetate after a complete cycle of carboplatin. Histopathological evaluation showed no signs of retinal toxicity to anecortave acetate delivery alone or in combination with carboplatin.

Conclusions. Anecortave acetate, as monotherapy or as adjuvant therapy, significantly controlled tumor burden in a murine model of retinoblastoma. Moreover, adjuvant therapy enabled the use of typically subtherapeutic carboplatin doses without decreasing efficacy of the therapy. (*Invest Ophthalmol Vis Sci.* 2006;47:1264–1268) DOI:10.1167/iovs.05-1194

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Supported by Alcon Laboratories, Inc., National Eye Institute Grant R01 EY013629 and Center Grant P30 EY014801; and an unrestricted grant to the University of Miami from Research to Prevent Blindness, Inc.

Submitted for publication September 7, 2005; revised October 19 and November 29, 2005; accepted January 19, 2006.

Disclosure: **M.-E. Jockovich**, Alcon Laboratories, Inc. (F); **T.G. Murray**, Alcon Laboratories, Inc. (F); **E. Escalona-Benz**, Alcon Laboratories, Inc. (F); **E. Hernandez**, Alcon Laboratories, Inc. (F); **W. Feuer**, Alcon Laboratories, Inc. (F)

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Retinoblastoma represents one of the most common malignant tumors of childhood, with an incidence of 1 in 15,000 live births.[1] Over the past century, significant advances in screening and treatment have led to virtually all children being cured of the primary eye cancer. Recently, clinical advances have focused on increasing tumor control and globe conservation with attendant preservation of sight.[2–6] Current available treatments include laser therapy, cryotherapy, external beam radiotherapy, charged-particle radiation, and systemic chemotherapy.[7,8] Serious concerns exist regarding the significant morbidity and potential mortality associated with current therapies in the treatment of retinoblastoma; therefore, newer therapeutic modalities are being investigated.[9,10]

Vasculature is critical to the survival of solid tumors, and angiogenesis has been found to be a prerequisite for continued tumor growth.[11,12] Angiogenesis involves the formation of new capillary blood vessels from preexisting vessels through a complex cascade of events.[12] The inhibition of one or more of these events is of potential therapeutic value for those pathologic conditions in which abnormal angiogenesis is a factor. This finding has led to the development of many antiangiogenic agents for cancer therapy.[13–15]

Retinoblastoma tumors are highly vascularized and depend on vascular supply for viability.[16] The capacity of these tumors to promote angiogenesis has been demonstrated.[17–19] Angiogenic factors, such as vascular endothelial growth factor (VEGF) and its receptors, Flt-1 and KDR, have been localized to areas of novel vasculature in human retinoblastoma tumors.[20–21] The angiogenic potential of retinoblastoma correlates with invasive growth and metastasis and is associated with poor prognosis.[22–24] The increased vascularity and propensity for stimulation of angiogenesis in retinoblastoma may make these tumors sensitive to vasculature targeting agents.

Anecortave acetate is an antiangiogenic agent that inhibits blood vessel growth in several preclinical models of angiogenesis, including rat mammary carcinoma, rabbit cornea, rat cornea, rat model of retinopathy of prematurity, and murine intraocular tumors.[25–30] Anecortave acetate is a cortisol derivative devoid of conventional glucocorticoid receptor–mediated activity (Clark AF et al., *IOVS* 1994;35:ARVO E-Abstract 1483).[31] As a result, it also does not demonstrate the significant deleterious ocular side effects associated with ocular glucocorticoid therapy (cataracts and elevation of intraocular pressure). Anecortave acetate has been shown to inhibit pathologic retinal angiogenesis, while not significantly affecting physiologic retinal microvasculature.[25] Furthermore, it has been shown to be safe in human clinical studies.[32–35] This drug may therefore hold therapeutic potential for several ocular conditions in which angiogenesis appears to play a critical pathophysiological role, including intraocular tumors.

The purpose of the present study was to evaluate the efficacy of anecortave acetate as a monotherapy and as an

Investigative Ophthalmology & Visual Science, April 2006, Vol. 47, No. 4

adjuvant therapy in controlling retinal tumor growth, using the $LH_{BETA}T_{AG}$ mouse model of retinoblastoma.

METHODS

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

The $LH_{BETA}T_{AG}$ transgenic mouse model used in the study has been characterized previously.[36–38] Briefly, a highly expressed transgene drives retinal tumor development by overexpression of the SV40 large T antigen. In transgenic animals bilateral, retinal tumors develop that resemble human retinoblastoma. At 10 weeks of age, tumors in this animal model are typically moderate in size (occupying approximately 20%–25% of the retinal area and 10%–25% of the ocular volume). At 16 weeks of age retinal tumors in these mice usually fill the orbit.

$LH_{BETA}T_{AG}$ mice, six animals per treatment group, were treated at 10 weeks of age. This number was determined with a power study performed on computer (Solo Power Analysis program; BMDP Statistical Software, Los Angeles, CA) based on pilot studies from our laboratory.

Subconjunctival Injections in Transgenic Mice

Only right eyes received subconjunctival injections, left eyes remained untreated and are used as the internal control.

$LH_{BETA}T_{AG}$ mice received a single subconjunctival injection of anecortave acetate (Alcon Pharmaceuticals, Fort Worth, TX) to the right eyes at doses of 1200, 600, 300, or 150 μg in a 20-μL volume. Anecortave acetate dilutions were performed in vehicle (provided by the manufacturer). Injections were delivered with a 33-gauge needle inserted into the superotemporal subconjunctival space.

For the combined treatment study anecortave acetate (600, 300, or 150 μg) was delivered after two (during the cycle) or six (completed cycle) carboplatin injections. Carboplatin was delivered at the subtherapeutic dose of 62.5 μg per injection,[39] delivered every 72 hours. Anecortave acetate was delivered 24 hours after the second or sixth carboplatin treatment.

Histopathological Study of Transgenic Mice

At 16 weeks of age, all animals were euthanatized with CO_2 fumes. Both eyes were enucleated and immediately immersion fixed in 10% formalin. The eyes were embedded in paraffin, sectioned serially in 5-μm sections, and processed for standard hematoxylin-eosin (H&E) analysis. Light microscopic examination was performed on all histopathologic sections in a masked fashion. Microscopic images of all hematoxylin and eosin (H&E)–stained sections (sixty 5.0-μm sections per eye) were obtained with a digital camera at a magnification of 40×. Tumor boundaries were traced and areas analyzed (Image Pro Express Software; Media Cybernetics, Silver Spring, MD) to determine the section with the largest tumor. The maximum tumor area was used in subsequent analyses.

Statistical Analyses

Tumor size response to anecortave acetate dose was investigated with analysis of variance. Orthogonal polynomial decomposition was used to test for linear and quadratic trend effects of dose. The influence of delivery of anecortave acetate relative to carboplatin delivery was assessed by fitting tumor size as the dependent variable in a regression model with three independent variables: anecortave acetate dose, anecortave acetate delivery time relative to carboplatin delivery, and dose delivery time interaction. Similar results were obtained when models were constructed in which globe size was included as an independent variable and in which the ratio of tumor size to globe size was the dependent variable. The influence of combining both therapeutic agents, relative to untreated control subjects or to the single anecortave acetate treatment, was performed by using a least-significant-difference post hoc comparison after one-way ANOVA on tumor size square root transformed to effect homogeneity of variance.

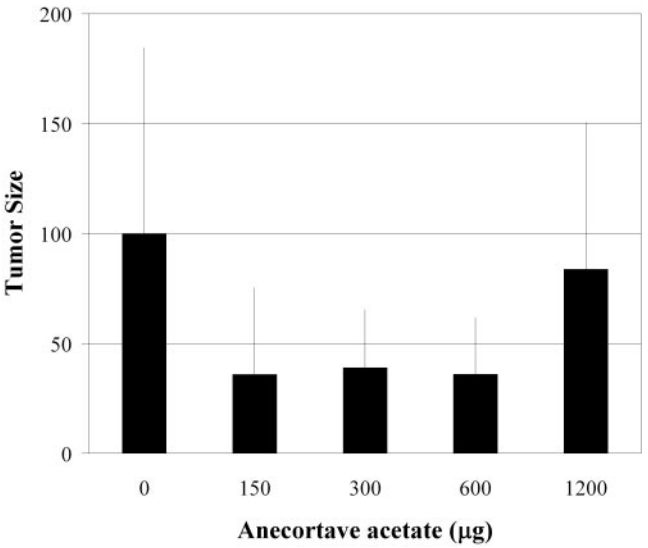

FIGURE 1. Anecortave acetate, single therapy. A statistically significant reduction in tumor burden ($P = 0.012$) was measured after a single treatment of anecortave acetate. Tumor areas were normalized to the mean of the untreated controls. Error bars, SD.

RESULTS

A significant ($P = 0.012$, quadratic dose–response) reduction in tumor size was seen at the lower doses (150–600 μg) of anecortave acetate tested (Fig. 1). Whereas the highest dose (1200 μg) did not significantly reduce tumor size. Variability in tumor size was not significantly related to anecortave acetate dose ($P = 0.12$, Levine test). The dose–response curve followed a U-shaped, biphasic trend. Although, tumor size in treated eyes was markedly reduced compared with that in fellow eyes and untreated control eyes, complete control of tumor burden did not occur at the tested doses. All eyes harbored tumors in fellow, untreated eyes.

We evaluated the hypothesis that adjuvant therapy with anecortave acetate could reduce the necessary dose of the toxic chemotherapeutic drug carboplatin needed to treat retinoblastoma successfully. In this study, various doses of anecortave acetate were combined with a constant subtherapeutic dose of carboplatin. Previous studies have established the tumor control dose $(TCD)_{50}$ of carboplatin at six of 138.5 μg injections.[39] Thus, a dose known to provide minimal tumor control (62.5 μg) was tested. To asses the effect of delivery schedule in the combined treatment, two delivery schemes were tested. Anecortave acetate was delivered during the six injection carboplatin treatment (after two carboplatin deliveries) or after the carboplatin series was completed.

Complete tumor control was observed in two of the six eyes that received 150 μg anecortave acetate after six carboplatin deliveries. Statistical analysis found tumor size to be significantly related to anecortave acetate dose ($P = 0.043$; Figs. 1, 2) and time of anecortave acetate delivery relative to carboplatin ($P = 0.027$; Fig. 2). A statistical interaction between these two variables was found ($P = 0.063$). Further analyses of tumor size were performed, including control animals receiving no treatment and animals treated with anecortave acetate at doses of 150 and 300 μg alone or after the complete carboplatin cycle. This analysis demonstrated that anecortave acetate reduced tumor sizes compared with control

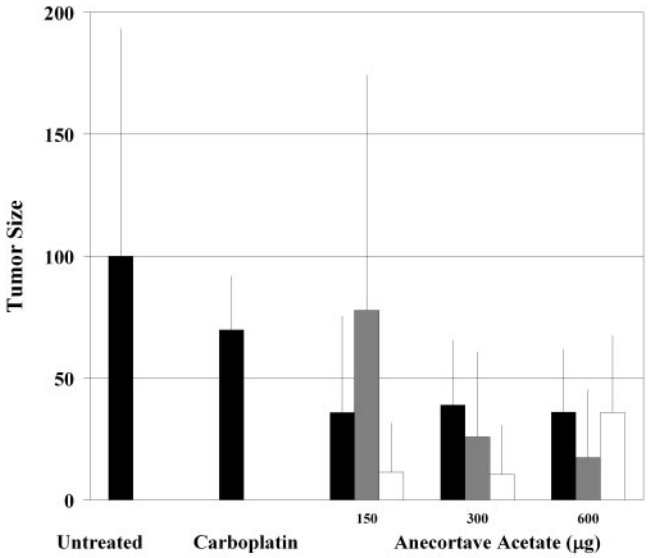

FIGURE 2. Anecortave acetate and carboplatin combined therapy. Tumor response after combined therapy with anecortave acetate delivered after two (▨) or six (□) carboplatin injections. Untreated, carboplatin only (62.5 μg), and the anecortave acetate control (■) are shown for comparison. Tumor size was significantly related to anecortave acetate dose ($P = 0.043$) and time of anecortave acetate delivery relative to carboplatin ($P = 0.027$). A statistical interaction between these two variables was found ($P = 0.063$). Tumor areas were normalized to the mean of the untreated control. Error bars, SD.

tumors ($P = 0.039$) and that anecortave acetate delivery after a complete carboplatin cycle further reduced tumor sizes compared with anecortave acetate treatment alone ($P = 0.008$).

Histopathology showed no evidence of corneal, lenticular, choroidal, or retinal toxicity after anecortave acetate delivery as monotherapy, or combined with subtherapeutic carboplatin (Fig. 3). The uninvolved retina in the treated eyes showed normal morphology in all experimental eyes. No evidence of systemic toxicity was observed.

DISCUSSION

Inhibition of angiogenesis has been proposed as a therapeutic strategy for solid tumors[13–15] including pediatric malignancies.[40] Antiangiogenic therapy is emerging as a possible treatment option for retinoblastoma, given the tumor's dependence on vascular supply and its potential to promote angiogenesis, particularly in cases of advanced disease.

In the $LH_{BETA}T_{AG}$ model of retinoblastoma, two different vessel-targeting agents were effective treatments for this malignancy. Previously, we have reported that the first vessel-targeting agent, combretastatin A4, a tubulin-binding agent that disrupts blood flow through immature vasculature, effectively reduced retinal tumor burden in $LH_{BETA}T_{AG}$ mice.[41] In this study, we present data on the second vasculature targeting agent, the antiangiogenic anecortave acetate, that suggest that inhibition of novel blood vessel formation is also effective in retinal tumor reduction in this model. Although a statistically significant reduction in tumor size was measured after a single treatment with either vessel-targeting agent, complete control of tumor burden is not achieved. These data suggest that the most effective clinical application of vessel-targeting agents in the treatment of solid tumors is obtained in combination with existing anticancer treatment modalities, including chemotherapy.

A challenge of using antiangiogenic agents as therapeutics is dose optimization. Many antiangiogenic agents display a nonconventional (U-shaped, biphasic) dose response.[42–44] This dose–response curve was also seen when anecortave acetate is used in the treatment of retinal tumor burden within $LH_{BETA}T_{AG}$ mice (this study) and in the treatment of subfoveal lesions in age-related macular degeneration.[34,35] This atypical dose–response was also evident in the combined treatment, where optimal delivery schedule was dose specific. Explanations of this atypical dose–response have included receptor desensitization,[45] problems with drug penetration at higher doses or dose-dependent differences in biological response.[46] However, the mechanism leading to this dose–response curve is still to be elucidated. This fact must be considered in the design of human anticancer trials.

Antiangiogenic and cytotoxic chemotherapy potentially yield maximum effects when combined, because different compartments of the tumor are targeted: cancer cells and endothelial cells.[47] Targeting vasculature, however, may compromise delivery of chemotherapy to the tumor and antagonize the effect of the combined therapy.[48] The efficacy of the combined treatment modality thus depends on appropriate vasculature targeting relative to chemotherapy. As seen in the present study, delivery of the lowest dose of anecortave acetate during carboplatin treatment led to antagonism between the two therapies. Conversely, an additive effect was detected when vessels were targeted after the complete cycle of chemotherapy. These results can be interpreted to mean that vessel targeting during chemotherapy compromises further delivery of carboplatin. Vessel targeting after chemotherapy may trap carboplatin within tumors yielding better tumor control.

Focally delivered carboplatin has been advocated due to existing concerns regarding significant morbidity and potential mortality caused by drug-related toxicity. Focal delivery has the benefits associated with chemoreductive treatment and conceivably spares children the associated toxicities and mutagenic potential of systemic delivery of chemotherapy.[10] We have recently shown that focal delivery of carboplatin effectively distributes more drug to the ocular tissues than does systemic delivery.[49] However, clinical trials to assess the efficacy of subconjunctival carboplatin suggest that although efficacious, toxicities such as optic atrophy[50] and ocular motility

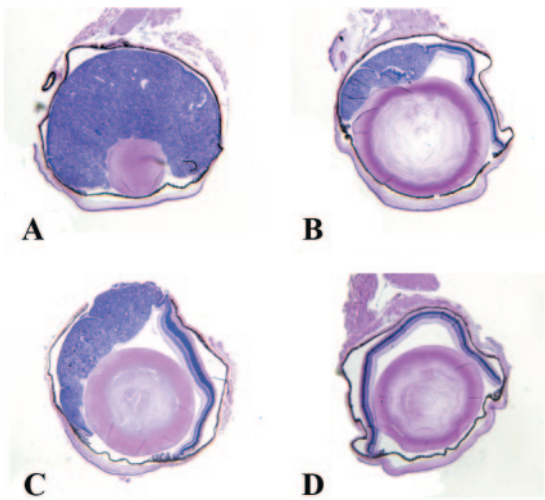

FIGURE 3. Histopathology sections of enucleated globes. Representative globe sections of 16-week-old $LH_{BETA}T_{AG}$ mice: (**A**) untreated; (**B**) 150 μg anecortave acetate; (**C**) 150 μg anecortave acetate after two carboplatin injections; (**D**) 150 μg anecortave acetate after six carboplatin injections. H&E; magnification, ×40.

changes due to soft tissue alterations[51] are observed. Results from the present study suggest that carboplatin doses can be markedly reduced when combined with vessel-targeting adjuvant therapy, without compromising the efficacy of the drug. This combined treatment modality may provide an excellent therapeutic option for children with advanced disease.

In summary, therapy using vasculature targeting agents in combination with subconjunctival chemotherapy may represent a novel option in the treatment of pediatric retinoblastoma. The potential of combination treatment incorporating anecortave acetate or other vessel-targeting agents may allow enhanced tumor reduction enabling a decrease in standard treatment doses for chemotherapy. However, dosage regimens must be optimized to prevent antagonizing effects.

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E R R A T U M

Erratum in: "Noninvasive Measurement of Rodent Intraocular Pressure with a Rebound Tonometer" by Wang et al. (*Invest Ophthalmol Vis Sci.* 2005;46:4617–4621).

The fourth sentence of the last paragraph of the Introduction should read, "Prototypes of this instrument were shown to produce meaningful data in human subjects, rats, and mice.[12,13]"

The following reference should be included in the citations at the end of this sentence. The reference was inadvertently omitted by the authors. Danias J, Kontiola AI, Filippopoulos T, Mittag T. Method for the noninvasive measurement of intraocular pressure in mice. *Invest Ophthalmol Vis Sci.* 2003;44:1138–1141.