Safety and Biodistribution Evaluation in CNGB3-Deficient Mice of rAAV2tYF-PR1.7-hCNGB3, a Recombinant AAV Vector for Treatment of Achromatopsia

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Applied Genetic Technologies Corporation (AGTC) is developing rAAV2tYF-PR1.7-hCNGB3, a recombinant adeno-associated virus (rAAV) vector expressing the human *CNGB3* gene, for treatment of achromatopsia, an inherited retinal disorder characterized by markedly reduced visual acuity, extreme light sensitivity, and absence of color discrimination. We report here results of a study evaluating safety and biodistribution of rAAV2tYF-PR1.7-hCNGB3 in CNGB3-deficient mice. Three groups of animals ($n = 35$ males and 35 females per group) received a subretinal injection in one eye of $1 \mu\text{l}$ containing either vehicle or rAAV2tYF-PR1.7-hCNGB3 at one of two dose concentrations (1×10^{12} or 4.2×10^{12} vg/ml) and were euthanized 4 or 13 weeks later. There were no test-article-related changes in clinical observations, body weights, food consumption, ocular examinations, clinical pathology parameters, organ weights, or macroscopic observations at necropsy. Cone-mediated electroretinography (ERG) responses were detected after vector administration in the treated eyes in 90% of animals in the higher dose group and 31% of animals in the lower dose group. Rod-mediated ERG responses were reduced in the treated eye for all groups, with the greatest reduction in males given the higher dose of vector, but returned to normal by the end of the study. Microscopic pathology results demonstrated minimal mononuclear cell infiltrates in the retina and vitreous of some animals at the interim euthanasia and in the vitreous of some animals at the terminal euthanasia. Serum anti-AAV antibodies developed in most vector-injected animals. No animals developed antibodies to hCNGB3. Biodistribution studies demonstrated high levels of vector DNA in vector-injected eyes but little or no vector DNA in nonocular tissue. These results support the use of rAAV2tYF-PR1.7-hCNGB3 in clinical studies in patients with achromatopsia caused by *CNGB3* mutations.

INTRODUCTION

ACHROMATOPSIA IS AN INHERITED retinal disorder characterized by markedly reduced visual acuity, nystagmus, photoaversion (intolerance of bright light), a small central scotoma, eccentric viewing, and complete or severe loss of color discrimination.¹ Unlike the common forms of inherited “color blindness,” in which there is difficulty distinguishing color differences but normal visual acuity, individuals with achromatopsia have markedly impaired visual function. Best visual acuity even under non-bright light conditions is usually about 20/200. Nystagmus develops during the first few weeks

after birth, and extreme light sensitivity with day-time blindness is a major feature of the disease. People with achromatopsia report that being color-blind is the least troublesome manifestation of this vision disorder.² Poor visual acuity (especially for distance) and hypersensitivity to bright light are much more disabling.

Approximately 50% of cases are caused by mutations in the cone photoreceptor-specific cyclic nucleotide-gated channel beta subunit (*CNGB3*) gene,³ 25% are caused by mutations in the cone-specific cyclic nucleotide gated channel alpha subunit (*CNGA3*) gene,⁴ and a small percentage are

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caused by mutations in other genes.^{5–9} Cone photoreceptor function is absent in patients with these mutations and in animal models with mutations in the homologous genes.^{10,11} Studies in the naturally occurring dog and knockout mouse models of achromatopsia caused by mutations in the *CNGB3* gene indicate that gene therapy using a recombinant adeno-associated virus (rAAV) vector expressing a normal human *CNGB3* gene (hCNGB3) can correct the defect caused by the abnormal CNGB3 protein and restore cone photoreceptor function.^{12,13}

AGTC is developing an rAAV vector expressing human CNGB3 (hCNGB3) as a potential product for treatment of achromatopsia caused by *CNGB3* mutations. The product, rAAV2tYF-PR1.7-hCNGB3, contains a cone-specific PR1.7 promoter,¹⁴ a codon-optimized human CNGB3 cDNA, an SV40 polyadenylation sequence, and is packed in an AAV2 capsid containing three tyrosine to phenylalanine (YF) mutations. As part of our efforts to develop this product, we conducted a toxicology and biodistribution study in CNGB3-deficient mice.

RESEARCH DESIGN AND METHODS

Vector description, production, and characterization

The design of the rAAV2tYF-PR1.7-hCNGB3 vector is described in the companion article published in this issue of the journal.¹⁵ The vector was produced using a recombinant herpes simplex virus (rHSV) complementation system in suspension-cultured baby hamster kidney (sBHK) cells¹⁶ and characterized as described in the companion article published in this issue of the journal.¹⁵

Toxicology study design

The study was performed at Covance, the drug development business of Laboratory Corporation of America Holdings (LabCorp), and was conducted in compliance with Good Laboratory Practice for Nonclinical Laboratory Studies (GLP) requirements. The test system was homozygous CNGB3 knockout (KO) mice on a CB57BL/6 background originally obtained from Deltagen Inc. These CNGB3-deficient mice were produced at Taconic Laboratories by using sperm and oocytes collected from homozygous male and female CNGB3 KO mice.

The study was designed to evaluate toxicity, efficacy, and biodistribution of rAAV2tYF-PR1.7-hCNGB3 administered by subretinal injection in one eye of 1 μ l of vehicle (balanced salt solution with 0.014% Tween 20) or vector at a concentration of 1×10^{12} vg/ml (1×10^9 vg/eye) or 4.2×10^{12} vg/ml

(4.2×10^9 vg/eye) in a total of 210 CNGB3-deficient mice ($n = 35$ males and 35 females per group; Table 1). The other eye was untreated. Ten animals/sex/group were used for toxicology evaluation with ophthalmic examinations and pathological evaluations, 10 animals/sex/group used for biodistribution evaluation, and 15 animals/sex/group were used for efficacy evaluation.

Subretinal injections were performed in anesthetized animals as previously described¹⁷ with minor modifications. Briefly, under direct visualization of a surgical microscope, an incision was made in the cornea using a 30-gauge MVR blade, and a 33-gauge blunt-tipped needle was inserted through the corneal incision toward the back of the eye while avoiding the lens and penetrating the neuroretina to reach the subretinal space. One microliter of vector suspension with 0.1% fluorescein was slowly injected subretinally and the retina was visualized to confirm successful dosing. Residual dosing formulations were frozen for later testing by quantitative PCR (qPCR) to confirm the concentration of vector administered.

To obtain a sufficient number of successfully injected animals for evaluation (35/sex/group), a total of 60 male and 60 female animals per group received a subretinal injection in the right eye in subgroups of 24–36 animals on each of 8 days, with equal numbers of male and female animals in each of the three study groups each day. Study day 1 was the day of injection for each animal. The test and control articles contained 0.1% fluorescein to enable confirmation that the injection was delivered to the subretinal space. After ophthalmic examinations on study day 8, a sufficient number of successfully dosed animals to meet study design requirements were selected for inclusion in the study and the remaining animals not selected were euthanized and discarded without necropsy. Suc-

Table 1. Study design

Group	Number	Dosage level		
		Vector concentration, vg/ml	Injection volume, μ l	Total dose, vg
1	70 (35M/35F)	0 (control)	1	0
2	70 (35M/35F)	1×10^{12}	1	1×10^9
3	70 (35M/35F)	4.2×10^{12}	1	4.2×10^9

Animals were injected with test article or vehicle control in the right eye via subretinal injection on study day 1. The left eye was not treated. Within each group, 20 animals (10M/10F) were scheduled for toxicology evaluation, 20 animals (10M/10F) were scheduled for biodistribution evaluation, and 30 animals (15M/15F) were scheduled for efficacy evaluation. Half of the animals scheduled for toxicology and biodistribution evaluation were euthanized 4 weeks after injection, and the remaining animals were euthanized 3 months after injection.

cess of injection was based on delivery of dose to the subretinal space (evaluated at the time of dose administration), and level of procedure-related complications (animals with significant cataracts, hemorrhages, or other significant complications noted on study day 8 were removed from study).

To ameliorate postprocedure discomfort, all animals received buprenorphine, 2 mg/kg by subcutaneous injection on study day 1; topical atropine (1%) and neomycin/polymyxin B/0.1% dexamethasone applied to dosed eyes on study days 1, 2, 3, 5, and 7; and meloxicam administered via oral gavage, 2 mg/kg on study day 1 and 1 mg/kg on study days 2–7.

Animals were observed daily for mortality and signs of illness. The body weight of each animal was obtained on the day of dosing, weekly thereafter, and on the day of euthanasia. Ophthalmic examinations were conducted by a board-certified veterinary ophthalmologist before dosing and on study day 8 for all dosed animals, and during study weeks 4 and 12 for animals scheduled for toxicity and efficacy evaluations. Both eyes were examined grossly, the adnexa and anterior portions of each eye were examined using a slit-lamp biomicroscope and the ocular fundus was examined using an indirect ophthalmoscope.

For animals scheduled for efficacy evaluations, electroretinography (ERG) testing included scotopic and photopic tests performed on each eye. Animals were dark-adapted for at least 2 hr before scotopic tests, and light-adapted for at least 10 min before photopic tests. Scotopic tests were done using single white flashes at intensities of –54, –48, –36, –30, –24, –16, –6, 0, 6, and 10 dB (range 0.00001 to 25 cd-s m⁻²). Photopic tests were done using single white flashes at intensities of –16, –6, 0, 6, and 10 dB, and flicker flashes at 0 dB, with an average of five trials (each trial encompassing a train of flashes) with interstimulus intervals of 0.2 sec (5 Hz), 0.1 sec (10 Hz), and 0.067 sec (15 Hz).

Half the animals in the biodistribution and toxicology arms of the study were euthanized 4 weeks after vector administration and the remaining animals were euthanized 3 months after vector administration. For animals in the efficacy evaluation subgroups, serum was collected at euthanasia for measurement of antibodies to AAV and CNGB3. For animals in the toxicology subgroups, blood was collected at euthanasia for hematology (hemoglobin, hematocrit, red cell count, red cell indices, reticulocyte count, platelet count, and white cell count with differential) and clinical chemistry (alanine

aminotransferase, blood urea nitrogen, total protein, albumin, and globulin). For animals in the biodistribution subgroups, blood for qPCR analysis was obtained on study days 3, 8 and at euthanasia.

For animals in the toxicology subgroups, at necropsy, a complete external and internal examination was performed, including body orifices and cranial, thoracic, and abdominal organs and tissues. Brain, heart, liver, gall bladder (drained), kidneys, spleen, thymus, lungs (with large bronchi), adrenals, ovaries, epididymides, and testes were weighed. Sections of the following tissues were stained with hematoxylin and eosin and examined histologically: injected eye, uninjected eye, adrenal glands, aorta, brain, cecum, colon, cervix, diaphragm, duodenum, epididymides, esophagus, femur with bone marrow (articular surface of the distal end), gall bladder (drained), Harderian glands, heart, ileum, jejunum, kidneys, lesions, liver, lung with large bronchi, lymph node (mesenteric and mandibular), mammary gland (females), optic nerves, ovaries, pancreas, parotid glands, pituitary gland, prostate, rectum, salivary gland (mandibular), sciatic nerve, seminal vesicle, skeletal muscle (quadriceps), skin/subcutis, spinal cord (cervical, thoracic and lumbar), spleen, sternum with bone marrow, stomach, testes, thymus, thyroid with parathyroid, urinary bladder, uterus, and vagina.

For animals in the biodistribution subgroups, samples of injected eye, uninjected eye, brain, liver, spleen, heart, lung, kidney, lymph nodes (mandibular), optic nerves, ovaries, and testes were obtained for qPCR analysis. Tissue samples collected for qPCR analysis were weighed in the prelabeled, preweighed sample collection tubes, placed in liquid nitrogen, and then stored in a freezer at –60°C to –80°C until shipped for analysis.

Detection of antibodies to AAV and CNGB3

Antibodies to the AAV2tYF capsid were measured using a neutralization assay as previously described.¹⁸ Antibodies to CNGB3 were determined by ELISA. Briefly, microtiter plates were coated with a truncated hCNGB3 protein consisting of N-terminal 215 amino acids generated from *Pichia pastoris* (Protein Potential) or mouse IgG (Sigma Aldrich) and incubated overnight. Plates were washed and blocked, and diluted mouse test serum or rabbit anti-hCNGB3 was added. The rabbit anti-hCNGB3 antibody was made for AGTC by 21st Century Biochemicals and used as a positive control in the assay because a mouse anti-CNGB3 antibody was not available. After sample incubation, a cocktail of horseradish peroxidase-conjugated

anti-mouse IgG and anti-rabbit IgG (Sigma Aldrich) was added to detect antibodies bound to hCNGB3. Tetramethyl benzidine substrate was then added and absorbance measured spectrophotometrically.

Quantitation of vector in blood and tissues

DNA was extracted using DNeasy kits (Qiagen) and DNA concentration determined using PicoGreen (Molecular Probes, Inc.). A 0.2 μ g sample was subjected to TaqMan qPCR (Roche Molecular Systems, Inc.) analysis using the 7900HT Real-time PCR System (Life Technologies) in quadruplicate using primers and probe that target the hCNGB3 sequence in the vector or using primers and probe that target mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The cycle threshold (Ct) value was determined and compared with a standard curve based on serial dilutions of a plasmid containing known amounts of hCNGB3 or GAPDH sequences. All no-template control samples had Ct values >38, and each standard curve had a coefficient of variation <15%, R^2 values of >0.98, and a linear range of 10^1 to 10^7 copies per reaction. During assay validation the limit of detection (LOD) was determined to be 1 copy per sample and the lower limit of quantification (LLOQ) was determined to be 10 copies per sample (50 copies per μ g DNA) for both CNGB3 and GAPDH.

Statistical analyses

Analysis of variance (ANOVA) and pairwise comparisons were used to analyze body weight, body weight change, quantitative food consumption, continuous clinical pathology values, organ weight, organ:body weight percentage, and organ:brain weight percentage.

ERG data were analyzed by repeated-measure ANOVA for each sex, with *Time* as the within (repeated) variable and *Treatment* as the between variable. When ANOVA indicated that there was a significant overall *Group* $\times$ *Interval* interaction, Dunnett's test was used to evaluate the significance of *Control* versus *Treatment* mean comparisons. In addition, ERG traces were evaluated qualitatively for the presence of a cone signal. A cone signal was clearly present in wild-type mice to the brightest photopic single flash (10 dB photopic white single) and most clearly present to the flicker stimuli (5, 10, and 14.9 Hz). These photopic conditions were used to evaluate the treated groups and the untreated knockout mice, and to classify the cone signal as present or absent. The photopic ERG from the treated (right) eye was compared with the trace from the fellow (left) eye in order to determine whether a cone signal exceeded the (null) response of the un-

treated eye. A subjective rating was made as to whether the cone signal was present at levels similar to that seen in previous studies, partially present, or absent, using a 0–1 scale. In addition, a subjective assessment of rod function was also performed comparing the treated eye with the fellow eye. The subjective semiquantitative assessments were used primarily for verifying the results of the formal statistical analyses and to identify possible small subsets of animals that depart from the group trend that may not be captured by parametric statistics.

RESULTS

Characterization of rAAV2tYF-PR1.7-hCNGB3

Results of tests to characterize the drug substance and drug product used in this study are described in the companion article published in this issue of the journal.¹⁵ Analysis of the residual dosing formulation confirmed that the measured concentrations of the test article were consistent with the concentrations specified in the protocol.

Overall study results

There were no test-article-related changes in clinical observations, body weights, food consumption, ocular examinations, clinical pathology parameters, organ weights, or macroscopic observations at necropsy. There were seven unscheduled deaths or euthanasia in the study, all of which were considered procedure-related or incidental. One male from a toxicology subgroup in the lower dose vector group was euthanized in moribund condition on study day 23 because of procedure-related ocular inflammation. In the efficacy subgroups, one control male was found dead on study day 10 because of a rupture of the aortic root, one control female was euthanized in moribund condition on study day 43 because of ulceration of the skin, and four animals in the vector-treated groups died on study day 29 or 31 or while being handled for ERG testing. All animals in the biodistribution subgroups survived to their scheduled euthanasia.

No clear and consistent test-article-related effects were noted in any group. Ophthalmic examination findings during the study were comparable across groups, including those given vehicle control article, and were often attributable to the trans-corneal approach for subretinal injection, which typically causes minor intraocular trauma from the needle tip as it passes through the eye. Dosed right eyes had corneal scars at the injection site, incipient cataracts along the needle's path, and pigment alteration in the region of the fundus where the injection was given.

Figure 1. Rod-mediated electroretinography (ERG) responses in CNGB3-deficient mice injected with vehicle lower or higher dose of rAAV2tYF-PR1.7-hCNGB3 ($n = 13$ – 15 animals per sex per group at each time point). Scotopic ERG responses (means + standard error of mean) were recorded from males and females to representative flash strengths of $0.006 \text{ cd-s m}^{-2}$ (dim) and 2.5 cd-s m^{-2} (bright) and oscillatory potentials (OPs) at the three postdose intervals. Scotopic ERG b-wave and summed OP amplitude in high-dose males is depressed at week 5 and is largely recovered by week 13. Bars under asterisks indicate a significant overall $\text{Group} \times \text{Interval}$ interaction by ANOVA that did not reach significance for individual intervals in *post-hoc* tests.

Rod-mediated ERG responses

In all groups, including the control group, most of the right eyes given subretinal injections were noted to have mildly decreased scotopic ERG responses compared with the fellow left eye. This was most evident during study week 5. ANOVA also indicated that there was greater reduction of rod response in the right eyes of males given rAAV2tYF-PR1.7-hCNGB3 at higher dose ($4.2 \times 10^9 \text{ vg/eye}$) compared with the right eyes of males given control article (Fig. 1), suggesting that, in addition to an injection-related reduction, there was a transiently reduced rod-mediated ERG response resulting from subretinal injection of rAAV2tYF-

PR1.7-hCNGB3 at higher dose in male animals. By study week 13, however, all but one animal showed recovery of rod-mediated ERG responses in the vector-treated eye.

Cone-mediated ERG responses

Photopic ERG responses of eyes from each group are summarized in Table 2 and Fig. 2. None of the untreated eyes or vehicle-treated eyes had a cone-mediated ERG response. In the lower dose vector group, cone-mediated ERG responses were seen in the treated eye in 0 of 29 animals at study week 5, 5 of 28 animals at study week 9, and 8 of 27 animals at study week 13. In the higher dose vector group,

Table 2. Cone-mediated ERG responses after administration of rAAV2tYF-PR1.7-hCNGB3

Group	Sex	Treated eye			Untreated eye		
		Week 5	Week 9	Week 13	Week 5	Week 9	Week 13
Vehicle control	M	9.8 ± 5.7 (0/15)	10.3 ± 5.2 (0/15)	8.8 ± 5.5 (0/15)	9.6 ± 5.9 (0/15)	10.7 ± 5.1 (0/15)	9.1 ± 5.6 (0/15)
	F	13.5 ± 8.8 (0/15)	9.1 ± 5.2 (0/14)	7.4 ± 6.2 (0/14)	13.3 ± 6.6 (0/15)	9.2 ± 4.0 (0/14)	7.2 ± 7.1 (0/14)
$1 \times 10^9 \text{ vg/eye}$	M	11.8 ± 7.8 (0/14)	14.1 ± 4.8 (1/14)	13.2 ± 5.6 (2/13)	11.6 ± 8.1 (0/14)	9.3 ± 5.0 (0/14)	9.7 ± 3.6 (0/13)
	F	17.8 ± 7.2 (0/15)	20.3 ± 6.9 (4/14)	16.6 ± 8.29 (6/14)	12.4 ± 6.1 (0/15)	11.4 ± 5.6 (0/14)	7.8 ± 6.5 (0/14)
$4.2 \times 10^9 \text{ vg/eye}$	M	20.4 ± 10.9 (10/15)	41 ± 14.1 (13/15)	44.2 ± 18.2 (13/14)	8.9 ± 3.9 (0/15)	11 ± 4.8 (0/15)	11.1 ± 4.9 (0/14)
	F	30.8 ± 17.8 (13/15)	39.6 ± 23.1 (12/15)	42.8 ± 17.2 (13/15)	14.3 ± 12.0 (0/15)	14.2 ± 7.5 (0/15)	11.8 ± 11.7 (0/15)

Values are the mean $\pm$ SD of b-wave amplitudes (μV) recorded at 0 dB, 5 Hz single white flash for all animals in each group. The mean $\pm$ SD b-wave amplitude in normal C57BL/6 mice is 97.0 ± 42.3 . Values in parentheses are the number of eyes with cone-mediated electroretinography (ERG) response/total number of animals tested.

Figure 2. Cone-mediated ERG responses in CNGB3-deficient mice injected with vehicle lower or higher dose of rAAV2tYF-PR1.7-hCNGB3 ($n=13$ –15 animals per sex per group at each time point). Photopic ERG responses (means + standard error of mean) were recorded from males and females to 5, 10, and 15 Hz flicker rates at 2.5 cd-s m^{-2} at three postdose intervals. Photopic flicker amplitude from the treated eye of the high-dose group was significantly larger than that of controls at week 5 and continues to increase through weeks 9 and 13. Asterisks indicate significant difference from control group at each interval by ANOVA using Dunnett's test.

cone-mediated ERG responses were seen in 23 of 30 animals at study week 5, 25 of 30 animals at study week 9, and 26 of 29 animals at study week 13.

Toxicology results

There were no test-article-related macroscopic observations at necropsy. The only test-article-related microscopic findings were minimal mononuclear cell infiltrates in the retina at the interim euthanasia and minimal mononuclear cell infiltrates in the vitreous at the interim and terminal euthanasia in some animals in the higher dose group and one animal in the lower dose group (Table 3).

Antibodies to AAV2tYF capsids and CNGB3 protein

Sera collected 3 months after injection of test article or control were analyzed for antibodies to AAV2tYF and CNGB3. Anti-AAV2tYF neutralizing antibody titers were <5 in all vehicle-injected animals. Antibody titers increased in 7 of 14 animals in the lower vector dose group and 13 of 14 animals in the higher vector dose group (Fig. 3). No animals developed antibodies to CNGB3.

Biodistribution results

At the interim euthanasia, vector DNA was detected in the injected eye in 8 of 10 animals in the

lower dose group and 9 of 10 animals in the higher dose group. However, for two animals it appears that the left and right eye were mislabeled, because high amounts of vector DNA were detected in the uninjected eye, whereas no vector DNA was detected in the injected eye (Table 4). For injected eyes in which vector DNA was detected, levels at the interim euthanasia ranged from 331,125 to 3,472,195 vg/ μg DNA (geometric mean 1,547,039) in the lower dose group and from 200 to 11,337,148

Table 3. Histological findings after administration of rAAV2tYF-PR1.7-hCNGB3

Dose level (vg/eye)	Males			Females		
	0	1×10^9	4.2×10^9	0	1×10^9	4.2×10^9
Interim euthanasia						
Number examined	5	4	5	4	5	5
Infiltrate, mononuclear cell, retina						
Minimal	0	0	4	0	1	2
Infiltrate, mononuclear cell, vitreous						
Minimal	0	0	3	0	0	2
Terminal euthanasia						
Number examined	5	5	5	5	5	5
Infiltrate, mononuclear cell, vitreous						
Minimal	0	0	3	0	0	2

Results are shown for treated eyes. There were no abnormalities seen in untreated eyes.

Figure 3. Serum anti-AAV2tYF neutralizing antibody titers in CNGB3-deficient mice injected with vehicle lower or higher dose of rAAV2tYF-PR1.7-hCNGB3 ($n=7$ males and 7 females per group).

vg/ μ g DNA (geometric mean 1,484,424) in the higher dose group. Levels at the terminal euthanasia ranged from 248 to 1,998,690 vg/ μ g DNA (geometric mean 13,501) in the lower dose group and from 2,097 to 2,086,672 vg/ μ g DNA (geometric mean 19,443) in the higher dose group. In addition to the two animals in which the left and right eye appeared to be mislabeled, low levels of vector DNA

were also detected in four other uninjected eyes, including one uninjected eye in the vehicle control group (Table 4).

Vector DNA was detected in the blood at study day 3 in 6 of 32 animals in the lower vector dose group (range 224 to 2,266 vg/ μ g DNA, geometric mean 616) and 12 of 32 animals in the higher vector dose group (range 150 to 14,956 vg/ μ g DNA, geometric mean 1,045). Vector DNA was detected in the blood at study day 8 in 2 of 28 animals in the lower vector dose group (range 145 to 738 vg/ μ g DNA, geometric mean 327) and 6 of 32 animals in the higher vector dose group (range 345 to 3,642 vg/ μ g DNA, geometric mean 628). Vector DNA was detected in the blood at the terminal euthanasia in 1 of 10 animals in the lower vector dose group (73 vg/ μ g DNA). Vector DNA was not detected in blood from any vehicle control animal or any animal at the interim euthanasia.

Little or no vector DNA was detected in nonocular tissue (Fig. 4). The most common nonocular tissues in which DNA was detected were the optic nerves and spleen. At both the interim and terminal euthanasia, vector DNA was detected in left or right optic nerve from 9 of 10 animals in the lower dose group and 10 of 10 animals in the higher dose group. At the interim euthanasia vector, DNA was detected in the spleen from 2 of 10 animals in the

Table 4. Vector DNA in uninjected and vector-injected eyes

Group	Males						Females			
Interim euthanasia										
Vehicle control										
Animal number	A15148	A15149	A15150	A15151	A15154	A15328	A15329	A15332	A15333	A15334
Uninjected eye	—	—	—	—	—	—	276	—	—	—
Injected eye	—	—	—	—	—	—	—	—	—	—
1 × 10 ⁹ vg/eye										
Animal number	A15207	A15208	A15209	A15210	A15211	A15387	A15388	A15389	A15390	A15392
Uninjected eye	—	3,002,795	—	—	—	—	—	—	—	1,744
Injected eye	331,125	—	3,472,195	—	1,091,418	1,570,374	2,387,928	2,703,449	1,944,275	1,326,547
4.2 × 10 ⁹ vg/eye										
Animal number	A15267	A15268	A15269	A15271	A15273	A15447	A15448	A15449	A15451	A15452
Uninjected eye	—	1,332,518	256	—	—	—	53	—	—	—
Injected eye	6,870,181	—	6,585,354	5,689,621	11,337,148	2,508,298	2,225,189	1,795,587	5,982,799	200
Terminal euthanasia										
Vehicle control										
Animal number	A15162	A15163	A15165	A15166	A15169	A15342	A15344	A15345	A15346	A15348
Uninjected eye	—	—	—	—	—	—	—	—	—	—
Injected eye	—	—	—	—	—	—	—	—	—	—
1 × 10 ⁹ vg/eye										
Animal number	A15223	A15224	A15226	A15228	A15229	A15402	A15403	A15404	A15405	A15409
Uninjected eye	—	—	—	—	—	—	—	757	—	—
Injected eye	—	—	6,111	—	339,992	—	436	1,998,690	—	248
4.2 × 10 ⁹ vg/eye										
Animal number	A15282	A15283	A15285	A15287	A15289	A15462	A15464	A15467	A15468	A15469
Uninjected eye	—	—	—	—	—	—	—	—	—	—
Injected eye	724,310	210,323	159	—	—	2,086,672	36,472	—	—	2,097

Data expressed as vector genome copies (vg) per μ g DNA; dash (—), less than lower limit of quantification (50 vg/ μ g of total DNA).

Figure 4. Biodistribution of rAAV2tYF-PR1.7-hCNGB3 in nonocular tissue in CNGB3-deficient mice injected with vehicle lower or higher dose of rAAV2tYF-PR1.7-hCNGB3 ($n=5$ males and 5 females per group). Values are the geometric mean concentration of vector DNA per μg host DNA determined by qPCR in tissues obtained at week 5 (interim euthanasia) or month 3 (terminal euthanasia). Error bars indicate the antilog of the mean + SD of the $\log_{10}$ transformed data used to calculate the geometric mean. The horizontal dashed line at 50 copies per μg DNA indicates the lower limit of quantification (LLOQ). Samples with results $<\text{LLOQ}$ were assigned a value of 10 to calculate the geometric mean.

lower dose group and 3 of 10 animals in the higher dose group. Vector DNA was detected in no more than one sample from any other tissue at either euthanasia time point. A low level of vector DNA was detected in the ovary of one animal at the interim euthanasia (100 $\text{vg}/\mu\text{g}$ DNA) and from the testis of one animal at the terminal euthanasia (374 $\text{vg}/\mu\text{g}$ DNA).

DISCUSSION

Results of this study showed that subretinal administration of rAAV2tYF-PR1.7-hCNGB3 at either of two dose levels in CNGB3-deficient mice was well tolerated with no test-article-related clinical, clinical pathology, or gross pathology findings. Ophthalmic examination findings during the dosing phase were attributed to local trauma caused by the subretinal injection procedure. Histological exami-

nation of eyes postmortem demonstrated minimal mononuclear cell infiltrates in the eyes of some of the animals in the higher vector dose group.

Cone-mediated ERG function was at least partially restored in 90% of animals in the higher dose group (4.2×10^{12} vg/ml), and 31% of animals in the lower dose group (1×10^{12} vg/ml). Cone-mediated ERG responses were detected starting at study week 5 in the higher dose group and at a lower incidence starting at study week 9 in the lower dose group. However, because of species differences in the efficiency of AAV vectors packaged in capsids of different serotypes to enter cells, and of promoters to drive transgene expression after vector entry into cells, caution must be exercised when using efficacy results from studies in mice to assist in defining a minimally effective dose in humans. For example, in CNGB3-deficient mice, vectors containing a chimeric cone transducin promoter, which is highly efficient in driving transgene expression in murine cones, were more efficient in restoring cone ERG function than were vectors containing the PR1.7 promoter, but the chimeric cone transducin promoter was unable to drive transgene expression in nonhuman primate cones.¹⁴ The rAAV2tYF-PR1.7-hCNGB3 vector includes the 1.7 kb L-opsin promoter because it directs strong transgene expression in all three types of primate cone photoreceptors after subretinal injection.¹⁴ In that study, a vector expressing green fluorescent protein (GFP) driven by the PR1.7 promoter and packaged in AAV2tYF capsids achieved high-level transgene expression in most primate cone photoreceptors when given by subretinal injection at a dose of 2×10^{11} vg/ml and in all primate cone photoreceptors when given at a dose of 5×10^{11} vg/ml . When defining the appropriate dose levels for use in human clinical trials, it seems reasonable to use results from studies in nonhuman primates in addition to results in a murine disease model.

Scotopic, rod-mediated ERG responses were statistically significantly reduced during study week 5 in the injected eye in the higher vector dose group compared with animals given vehicle, but this finding was not considered clinically significant and returned to normal by the end of the study. The vector concentrations used in this study, which were chosen based on results of preliminary studies that demonstrated rescue of cone function in CNGB3-deficient mice, are consistent with the concentrations to be administered in a planned phase 1/2 clinical trial.¹⁹

Antibodies to AAV at titers ranging from 1:40 to 1:2560 developed in 50% of animals in the lower vector dose group and $>90\%$ of animal in the higher

vector dose group (Fig. 3). These antibody titers were similar to those seen in a study in which rAAV2tYF-CB-hRS1, an AAV vector expressing retinoschisin (RS1), was administered by intravitreal injection at dose levels of 1×10^9 or 4×10^9 vg per eye in RS1-deficient mice on a C57BL/6 background.²⁰ In contrast, a study by Li et al. reported that injection of an rAAV2-CB-GFP vector at dose levels of 2×10^9 vg per eye in C57BL/6 mice resulted in high levels of neutralizing antibodies when the vector was administered by intravitreal injection but neutralizing antibodies did not develop when the vector was administered by subretinal injection.²¹ The reasons for these discordant results are not apparent. Antibody titers at 3 months after subretinal injection of rAAV2tYF-PR1.7-hCNGB3 in CNGB3-deficient mice given $1 \mu\text{l}$ at 4.2×10^{12} vg/ml (Fig. 3) were also similar to titers in nonhuman primates given the same vector at $300 \mu\text{l}$ at 4×10^{12} vg/ml, although the range of titers in the primates (1:160 to 1:320) was narrower than that in mice.¹⁵

Results of biodistribution testing demonstrated high levels of vector DNA in eyes injected with vector (Table 4) and little or no vector DNA in other tissues (Fig. 4). At the interim euthanasia, the geometric mean concentration of vector DNA in vector-injected eyes was approximately 1,500,000 vg per μg DNA at both dose levels, which is much higher than the 1,810 to 6,579 vg per μg DNA seen in eyes injected with rAAV2tYF-CB-hRS1 at a dose of 4×10^9 vg per eye in a previous study.²⁰ This is consistent with more efficient transduction of retinal cells after subretinal injection than after intravitreal injection.

In summary, subretinal injection of rAAV2tYF-PR1.7-hCNGB3 in CNGB3-deficient mice was as-

sociated with no clinically important toxicology findings, rescue of cone-mediated ERG responses in vector-treated eyes, and vector DNA detection limited primarily to vector-injected eyes. These results support the use of rAAV2tYF-PR1.7-hCNGB3 in clinical studies in patients with achromatopsia caused by CNGB3 mutations. A phase 1/2 clinical trial evaluating rAAV2tYF-PR1.7-hCNGB3 is scheduled to begin in 2016.¹⁹

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AUTHOR DISCLOSURE

G.Y., S.M., P.M.R., D.R.K., and J.D.C. are employees and shareholders of AGTC and have a conflict of interest to the extent that this work potentially increases their financial interests. W.W.H. and the University of Florida have a financial interest in the use of AAV therapies and are shareholders of AGTC. Covance is the drug development business of Laboratory Corporation of America Holdings (LabCorp). Content was developed by a scientist (L.M.S.) who at the time was affiliated with the LabCorp Clinical Trials or Tandem Labs brands, now part of Covance. None of the other authors has a competing financial interest.

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