

Antiangiogenic Activity of Aganirsen in Nonhuman Primate and Rodent Models of Retinal Neovascular Disease after Topical Administration

Frank Cloutier,^{1,2} Matthew Lawrence,^{2,3} Robin Goody,³ Stéphanie Lamoureux,¹ Salman Al-Mahmood,⁴ Sylvie Colin,⁴ Antoine Ferry,⁴ Jean-Pascal Conduzorgues,⁵ Amel Hadri,⁵ Claus Cursiefen,⁶ Patricia Udaondo,⁷ Eric Viaud,⁴ Eric Thorin,^{4,8} and Sylvain Chemtob¹

PURPOSE. Aganirsen, an antisense oligonucleotide inhibiting insulin receptor substrate (IRS)-1 expression, has been shown to promote the regression of pathologic corneal neovascularization in patients. In this study, the authors aimed to demonstrate the antiangiogenic activity of aganirsen in animal models of retinal neovascularization.

METHODS. Eyedrops of aganirsen were applied daily in nonhuman primates after laser-induced choroidal neovascularization (CNV; model of wet age-related macular degeneration [AMD]) and in newborn rats after oxygen-induced retinopathy (OIR; model of ischemic retinopathy). Retinal aganirsen concentrations were assessed in rabbits and monkeys after topical delivery (21.5, 43, or 86 μ g). Clinical significance was further evaluated by determination of IRS-1 expression in monkey and human retinal biopsy specimens.

RESULTS. Topical corneal application of aganirsen attenuated neovascular lesion development dose dependently in African green monkeys. The incidence of high-grade CNV lesions (grade IV) decreased from 20.5% in vehicle-treated animals to 1.7% ($P < 0.05$) at the 86- μ g dose. Topical aganirsen inhibited retinal neovascularization after OIR in rats ($P < 0.05$); furthermore, a single intravitreal injection of aganirsen reduced OIR as

effectively as ranibizumab, and their effects were additive. Significantly, topical applications of aganirsen did not interfere with physiological retinal vessel development in newborn rats. Retinal delivery after topical administration was confirmed, and retinal expression of IRS-1 was demonstrated to be elevated in patients with subretinal neovascularization and AMD.

CONCLUSIONS. Topical application of aganirsen offers a safe and effective therapy for both choroidal and retinal neovascularization without preventing its normal vascularization. Together, these findings support the clinical testing of aganirsen for human retinal neovascular diseases. (*Invest Ophthalmol Vis Sci.* 2012;53:1195–1203) DOI:10.1167/iovs.11-9064

Retinal neovascular diseases such as retinopathy of prematurity, proliferative diabetic retinopathy, and exudative age-related macular degeneration (wet AMD) cause severe visual impairment in infants and adults.^{1,2} At present, these pathologic conditions are typically tackled with success in the clinical setting with vascular endothelial growth factor (VEGF) inhibitors³; however, this form of therapy requires intraocular injections at regular intervals with an associated risk for retinal detachment, hemorrhage, endophthalmitis,⁴ and, more rarely, systemic complications, the most important of which are deleterious cardiovascular effects associated with anti-VEGF inhibitor administration.^{5–8}

Ischemic retinopathies can also occur early in eye development, as is the case for retinopathy of prematurity, the major cause of childhood blindness in the industrialized world.⁹ The disorganized growth of retinal blood vessels is usually treated by laser photocoagulation. Although beneficial effects of anti-VEGF inhibitors have been reported for retinopathy of prematurity,⁷ these inhibitors can interfere with normal vascularization¹⁰ and its integrity,¹¹ not only of the retina⁷ but also of the brain.⁵ The developing retina and brain respond to regional hypoxia caused by the onset of neuronal activity by stimulating neovascularization (new vessel formation) through the secretion of VEGF (and other angiogenic factors) from various cell types.^{12–14} Accordingly, anti-VEGF inhibitors can potentially severely impair normal vascularization of neuroretinal tissue.

Acute and long-term complications of current anti-VEGF inhibitor therapy underscore the need for alternative and complementary therapeutic approaches.¹⁵ Hence, an “ideal” drug would interfere with and prevent pathologic retinal neovascularization, reduce vessel rarefaction, and allow normal vessel regrowth without affecting neuronal function and would be safely administered topically at a dosing interval that would encourage patient compliance. Here we used a primate choroidal neovascularization (CNV) and a rodent oxygen-induced retinopathy (OIR) model to compare the efficacy of aganirsen

From the ¹Department of Pediatrics, Ophthalmology, Pharmacology, Hôpital Ste-Justine, Université de Montréal, Montreal, Quebec, Canada; ²RxGen Inc., Hamden, Connecticut; ³Gene Signal Laboratories, Genopole Industries, Evry, France; ⁴CRID Pharma, Saint Gély du Fesc, France; ⁵Department of Ophthalmology, University of Cologne, Köln, Germany; ⁶Universidad Cardenal Herrera Oria, CEU, Nuevo Hospital Universitario y Politécnico La Fe, Valencia, Spain; and ⁸Department of Surgery, Montreal Heart Institute, Université de Montréal, Montreal, Quebec, Canada.

²Contributed equally to the work and therefore should be considered equivalent authors.

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Corresponding author: Eric Thorin, Department of Surgery, Montreal Heart Institute, 5000 Belanger Street, Montreal, Quebec H1T 1C8, Canada; eric.thorin@umontreal.ca.

to humanized anti-VEGF antibodies, which have been previously demonstrated to be biologically active in these preclinical models.^{16–18}

We and others have previously shown that aganirsen (also known as GS-101), an IRS-1 mRNA antisense, prevents injury-associated corneal neovascularization in rats¹⁹ and allows regression of proliferative corneal neovascularization in patients.²⁰ We further demonstrated that a single topical application of ³⁵S-aganirsen dissolved in saline could diffuse to the posterior chamber of the eye of a rabbit.¹⁹ Finally, we have previously investigated the safety, tolerability, and bioavailability of aganirsen eyedrops in healthy volunteers and showed that aganirsen is safe for human use.²¹ Taken together, these findings highlight the potential of aganirsen as an alternative treatment for abnormal neovascularization, potentially for multiple ocular indications.

Insulin receptor substrate (IRS)-1 has been reported to have an important role in retinal angiogenesis.²² Here, we investigate the efficacy of topical applications of aganirsen on choroidal neovascularization in a nonhuman primate model of wet AMD. We also tested the efficacy of aganirsen in a rat model of ischemic retinopathy (induced by supplemental oxygen exposure). Our findings uncover the first effective topical antiangiogenic agent in models of vasoproliferative AMD and in ischemic retinopathy, supporting its potential for future clinical applications.

METHODS

The procedures and protocols were approved by our institutional review board and were performed in accordance with our institutional guidelines and the Guide for the Care and Use of Laboratory Animals of France, Canada, and the United States. All experiments were performed in accordance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rabbits and rats were kept under standard conditions with free access to food and water (21°C; 12-hour light/12-hour dark cycle). African green monkeys were maintained at ambient temperature and lighting conditions (approximately 22°C–30°C; 12-hour light/12-hour dark cycle) at the primate facility at the St. Kitts Biomedical Research Foundation. Retina biopsy specimens from patients with proliferative vitreoretinopathy, subretinal neovascularization, and AMD were obtained from INSERM U450 (Paris, France) after approval of the institutional ethical review board in adherence to the Declaration of Helsinki (18th WMA General Assembly, Helsinki, Finland, June 1964).

IRS-1 mRNA Expression in Human Retina Biopsy Specimens

Three biopsy samples isolated from three patients with the same condition were pooled together to quantify the expression of IRS-1 transcripts. Total mRNAs were isolated using a mini-kit (RNeasy; Qiagen, Courtaboeuf, France). RNA yields and purity were assessed by spectrophotometric analysis. Real-time RT-PCR was performed as described previously.¹⁷ In brief, 15 ng total RNA was reverse-transcribed with oligo-dT primers and a cDNA synthesis kit (Transcriptor High Fidelity; Roche Applied Science, Meylan, France), and the synthesized cDNA was used immediately for real-time PCR amplification with the DNA-binding dye SYBR Green I for the detection of PCR products and the following primers: IRS-1 (sense, 5'-CTCAACTGGACATCACAGCAG-3'; antisense, 5'-AGGTCCTAGTTGTGAATCATGAAA-3'); GAPDH (sense, 5'-AGCTCACTGGCATGGCCCTTC-3'; antisense, 5'-GAGGTCCACCACCTGTGTC-3'). Real-time PCR reactions were carried out in a system equipped with continuous fluorescence detector (LightCycler 480; Roche Applied Science). Results were quantified using the equation: $\text{Copy}_{\text{TF}}/\text{Copy}_{\text{GAPDH}} = 2C(t)_{\text{GAPDH}} - C(t)_{\text{TF}}$. Results were expressed as the mean $\pm$ SEM of three experiments in which the levels of mRNA expression of IRS-1 in

the three types of subretinal membranes were normalized to the expression of GAPDH mRNA.

Laser-Induced CNV in Nonhuman Primates

The effect of topical aganirsen administration on laser-induced CNV was tested in 26 African green monkeys using laser parameters; the study design was similar to previously described methods.²³ Monkeys were anesthetized with ketamine (8 mg/kg) and xylazine (1.6 mg/kg) for all procedures. Topical twice-daily dosing with 54 μ L aganirsen or vehicle (ophthalmic emulsion composed of carbomer, caprylic/capric acid triglycerides, cetyl alcohol, glycerol and polyethylene glycol stearates, sodium hydroxide, and water) was initiated 2 days before laser photocoagulation. Topical dosing continued for 14 days after laser photocoagulation (16 days in total). Baseline examinations and ocular assessments were performed to confirm the health of the animals and the eyes. Monkeys were randomly assigned to 1 of 4 groups (six monkeys per experimental group, eight monkeys for the vehicle control group). On day 1, in both eyes of all 26 study monkeys, six laser spots were concentrically spaced approximately 1.5 disc diameters from the fovea at the anatomic periphery of the macula, within the temporal vascular arcades, as previously described.²³ Fundus photographs were taken immediately before and immediately after laser photocoagulation in each eye. Fundus images and angiograms were collected on the left eye (OS) and then the right eye (OD) on day 29 (week 4) after laser photocoagulation using a retinal camera (TRC-50X; Topcon, Tokyo, Japan) with digital imaging hardware (Canon 5D; Canon, Tokyo, Japan) and imaging software (New Vision Image Analysis System; Fundus Photo, St. Louis, MO). Fluorescein angiography in OS preceded angiography in OD by 5 to 24 hours to allow washout of the fluorescein between angiogram image series. Angiogram images were collected at the moment the intravenous fluorescein injection was completed and then 30 seconds and 3, 6, and 10 minutes later. Eyes were also examined by optical coherence tomography (OCT) (Heidelberg Instruments Spectralis OCT Plus system and Heidelberg Eye Explorer [Heyex] software v1.6.1.; Heidelberg Engineering, Vista, CA) on study day 29. Star-shaped OCT scans centered on each laser spot were performed consisting of 12 scans intersecting at 15° in a clock pattern with image averaging over 25 ART frames. At the end of the experiment, animals already under sedation with ketamine (10 mg/kg) were euthanized by intravenous sodium pentobarbital overdose, and eyes were collected.

Fluorescein Angiography Scoring

Graded scoring of angiograms was performed on a fluorescein angiogram series collected at week 4 after laser photocoagulation. At each lesion site, the extent of late-phase fluorescein leakage, an angiographic change associated with CNV, was rated as described previously²³ on a scale of I to IV. Scoring was conducted by two investigators who remained masked to treatment group. Eight eyes (of 52) exhibiting subretinal hemorrhage or scarring as a result of laser photocoagulation, which precluded accurate scoring, were excluded from subsequent analysis before unmasking.

ImageJ Analysis of Angiograms and OCT Images

Original week 4 angiogram jpg files were further assessed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>) applying previously described methods.²³ Quantitative CNV scoring was applied to acquired OCT images by definition of the principal axis of maximal CNV complex formation within each star-shaped scan at each laser lesion by a masked evaluator. The CNV complex area in the OCT image in that axis was then measured using the freehand tool within ImageJ to delineate the CNV complex boundary and to calculate maximum complex area in square micrometers (μm^2).

Rat OIR

An OIR rat model was used as previously described.²⁴ After hyperoxic exposure, animals were assigned at day 14 to topical and intravitreal treatment groups. In the first experimental group, animals were treated from day 14 to day 17 by two daily topical applications on one eye using the following: vehicle alone (10 mg ophthalmic emulsion, $n = 11$); vehicle with a scramble aganirsen oligonucleotide¹⁹ (4 mg/g, 80 μ g delivered daily, $n = 9$); or vehicle with aganirsen at the concentration of 0.25 (5 μ g delivered daily, $n = 12$), 0.5 (10 μ g delivered daily, $n = 11$), 1 (20 μ g delivered daily, $n = 10$), and 2 (40 μ g delivered daily, $n = 12$). In the second experimental group, animals were treated at postnatal day (P) 14 and P16 by intravitreal injection of 1 μ L in one eye of the following: vehicle alone (sterile NaCl 0.9%, $n = 8$); scramble aganirsen oligonucleotide (2 mg/mL, 2 μ g delivered, $n = 4$); or aganirsen at the concentration of 0.5 mg/mL (0.5 μ g delivered, $n = 7$), 1 (1 μ g delivered, $n = 8$), and 2 (2 μ g delivered, $n = 8$). In the third series of experiments, animals were treated at P14 and P16 by intravitreal injection of 1 μ L in one eye of ranibizumab (25 ng), aganirsen (0.5 μ g), or their combination. For intravitreal delivery, rats were anesthetized with halothane (~2.5%) and injected posterior to the limbus with a 10- μ L Hamilton syringe attached to a glass capillary of approximately 60 gauge. During the treatment period, rats were maintained in a cyclic lighting environment (80 lux; 12 hours dark/12 hours light). Finally, mothers of the litters were alternated between normoxic and hyperoxic conditions every 24 hours so that pulmonary complications known to arise in adult rats raised in a hyperoxic environment could be avoided. All pups were euthanized by decapitation under anesthesia (isoflurane 2%) at P18, at which time the eyes were enucleated, the anterior segments dissected, and the eyecups fixed overnight in 4% formalin. Doses detailed here are based on the estimated absorption of aganirsen in emulsion (~2%) and eye volumes (~25 μ L in third postnatal week vs. ~8 mL in adult).

Developmental Retinal Vascularization in Rats

The effect of aganirsen on the normal vascularization of the retina was tested in newborn rats, as previously described.²⁵ In this animal model, formation of the vascular plexus primarily occurs during the first postnatal week of life.²⁶ Under halothane anesthesia, 1 μ L in one eye of either vehicle alone (sterile NaCl 0.9%, $n = 6$) or vehicle containing aganirsen at a concentration of 0.5 mg/mL (0.5 μ g delivered, $n = 8$) was injected at P1 and P3. In another series of experiments, animals were treated from P1 to P4 with two daily topical applications on one eye of either vehicle alone (10 mg ophthalmic emulsion, $n = 8$) or vehicle containing aganirsen at a concentration of 1.2 mg/g (24 μ g delivered daily, $n = 7$). In all experiments, animals were euthanized by decapitation under anesthesia (isoflurane 2%) at P5, and the eyecups were fixed overnight.

Rat Retinal Flatmounts

After retinal fixation, flatmounts were prepared for staining with adenosine diphosphatase (ADPase). Specimens were photographed (40 $\times$, Axiophot microscope; Carl Zeiss Meditec GmbH, Oberkochen, Germany), and the retinal vascularization area and density were evaluated (ImagePro Plus 4.5; Media Cybernetics, Silver Spring, MD). For the OIR protocol, the severity of retinopathy was assessed using a retinal scoring system using the following criteria: blood vessel growth, blood vessel tufts, extraretinal neovascularization, central vasoconstriction, retinal hemorrhage, and blood vessel tortuosity.²⁷ Vascular tufts were evaluated on retinal flatmounts.

Rabbit Pharmacokinetic Analysis

We used male adult New Zealand albino rabbits ($n = 60$; age, 4 months; weight range, 2.14–3.34 kg; Bergerie de la combe aux loups, Boisset St. Priest, France). Each rabbit eye was subjected to topical application on the cornea of 50 μ L (~50 mg) ophthalmic emulsion used in the primate and rodent studies containing 1.72 mg/mL (1.72

mg/g) of aganirsen, giving a local delivery of 86 μ g active compound. Rabbits were then euthanized by intravenous injection of pentobarbital (100 mg/kg, auricular vein), and eyes were removed from their cavities and snap-frozen; iris, ciliary body, and retina were collected individually and stored at -75°C before analysis.

After the addition of 750 μ L NaCl 0.9% and tissue homogenization, supernatants were collected and transferred to a polypropylene vial before ionic chromatography analysis (DIONEX S.A., Voisins Le Bretonneux, France) coupled to UV detection (Waters W486; Waters SAS, Guyancourt, France). The UV scan (200–400 nm) of the solution was obtained, and the maximal absorption value was quantified using the formula: [aganirsen] ($\mu\text{g/mL}$) = $(A/\epsilon \times l) \times M \times \text{Fdil} \times 100$, where A = value of absorbance at maximum of absorption (Do), l = width of the tank (cm), ϵ = molar extinction coefficient (224,600 L/mol $\cdot$ cm), M = molecular mass of sodium aganirsen (8587.4152 g/mol), and Fdil = dilution factor of the solution. Data were then corrected for retinal weight and were reported in micrograms of aganirsen per gram of retina.

Retinal Concentration of Aganirsen in African Green Monkeys

Aganirsen concentrations were measured in the retinas of 12 African green monkeys at 90 minutes and at 8 hours after a single topical application of the emulsion of aganirsen on the cornea at a dose of 21.5, 43, and 86 μ g (four eyes per time point). At each time point, animals under sedation with ketamine (10 mg/kg) were euthanized by intravenous sodium pentobarbital overdose, and eyes were collected

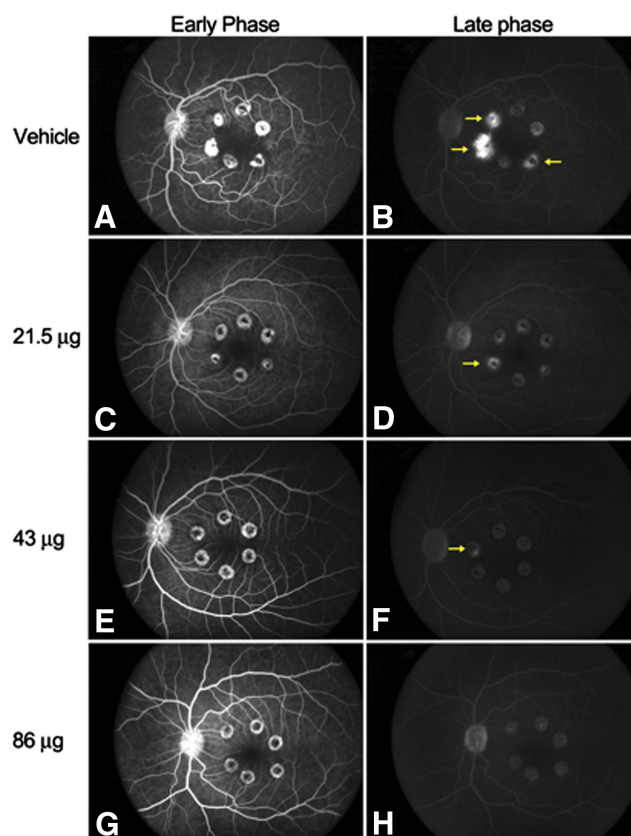


FIGURE 1. Fluorescein angiography of nonhuman primate eyes after laser photocoagulation and after topical administration of aganirsen or vehicle. Representative early (30-second) and late-phase (6-minute) fluorescein angiograms collected 4 weeks after laser photocoagulation from vehicle-treated animals (A, B) and from those receiving twice-daily topical doses of 21.5 μ g (C, D), 43 μ g (E, F), or 86 μ g (G, H) aganirsen from 2 days before laser photocoagulation to 14 days after it. Yellow arrows: grade IV CNV lesions.

and flash frozen. Frozen tissue was subdissected, retinal tissue was homogenized, and supernatant was collected for aganirsen determination.

IRS-1 Expression in the Retinas of African Green Monkeys

Because of the limited amount of tissue available, IRS-1 expression was measured in the retinas of African green monkeys at one time point only, 8 hours after a single topical application of the emulsion of aganirsen at the dose of 21.5, 43, and 86 μ g. IRS-1 was measured in homogenates by ELISA, as previously described.¹⁹

Statistical Analysis

Descriptive statistics were applied to data generated where appropriate. Graded (I-IV) scoring of lesions was analyzed using the Fisher's exact probability test, where incidence of grade IV, grade III, or both grades III and IV lesions was assigned "Yes" and any other grading was assigned "No." The incidence of grade I, grade II, or both grades I and

II lesions was evaluated in identical fashion. Validation of the laser-induced CNV model has demonstrated that, as with other CNV modeling studies in nonhuman primates,²⁸ clinically significant CNV is represented by grade III or grade IV lesion scores. Grade III and grade IV scores are represented by significant late leakage of fluorescein within, or extending beyond, the borders, respectively, of the laser-induced lesion. The effect of laser photocoagulation on the incidence of CNV was compared, in this manner, at day 29 between eyes receiving aganirsen and vehicle. ImageJ-based analysis of background-corrected mean fluorescein intensity and CNV complex area was analyzed using one-way ANOVA on Box-Cox-transformed values with post hoc analysis using Tukey-Kramer HSD. All statistics were performed using statistical analysis software (JMP; SAS Institute, Cary, NC). Rabbit data were analyzed using ANOVA, followed by a Tukey's multiple comparison test. Rodent data analysis was performed by ANOVA using the Dunnett posttest (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

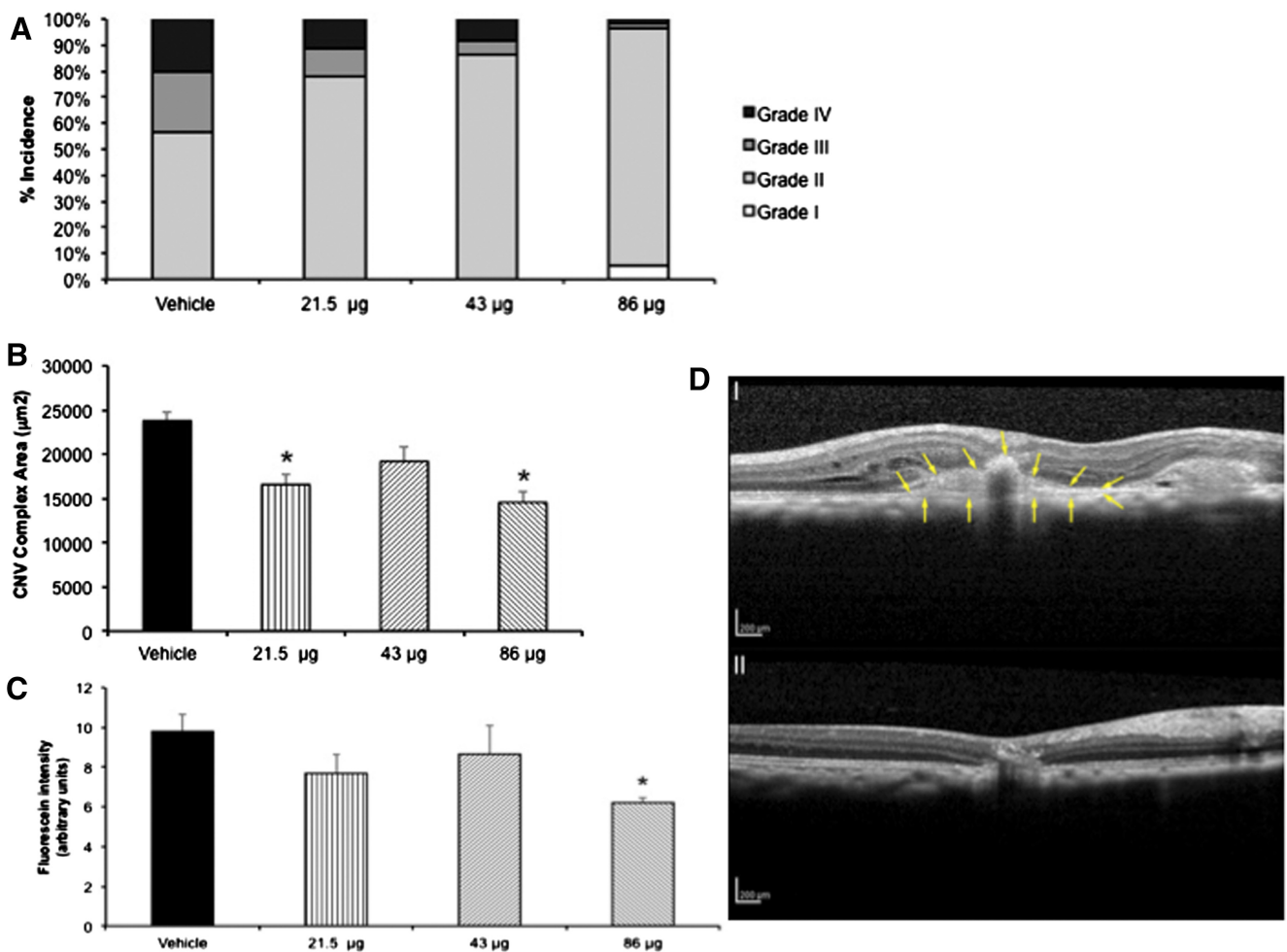


FIGURE 2. (A) Graded scoring of week 4 fluorescein angiography image series in African green monkeys after topical administration of aganirsen or vehicle formulations. Graph illustrates percentage incidence of each grade classification (36–78 lesions graded for each treatment group): I = no hyperfluorescence; II = hyperfluorescence without leakage and no significant residual staining in late-phase angiograms; III = hyperfluorescence early or mid-transit with late leakage and significant residual staining; IV = hyperfluorescence early or mid-transit with late leakage extending beyond the borders of the treated area. (B) Mean maximal CNV complex area at week 4 after laser treatment in aganirsen- and vehicle-treated eyes, as determined by ImageJ analysis of OCT images. Vertical bars indicate SEM (36–78 lesions graded for each treatment group). * $P < 0.0001$ compared with eyes receiving vehicle. Data presented represent back-transformed data after Box-Cox transformation of raw CNV complex area data for one-way ANOVA and post hoc evaluation. (C) Difference in late-phase (6-minute) mean relative fluorescein intensity week 4 after laser treatment in aganirsen- and vehicle-treated eyes, as determined by ImageJ analysis. Vertical bars: SEM (30–78 lesions graded for each treatment group). * $P = 0.0186$ versus vehicle-treated eyes. (D) Representative OCT images from eyes receiving aganirsen vehicle (I) and 86 μ g aganirsen (II) at 4 weeks after laser photocoagulation. Yellow arrows: CNV complex boundaries delineated during ImageJ analysis to calculate CNV complex area for each laser-induced lesion.

RESULTS

Expression of IRS-1 in Human Retina

The expression of IRS-1 mRNA transcript was low in retinal biopsy samples isolated from patients with proliferative vitreoretinopathy associated with glial or retinal pigment epithelium proliferation ($5.03 \pm 0.12 \times 10^{-2}$, $n = 3$), but was 12-fold higher ($P < 0.001$) in samples isolated from patients with subretinal neovascularization ($61.9 \pm 2.6 \times 10^{-2}$, $n = 3$) and 5-fold higher ($P < 0.001$) in samples collected from patients with age-related macular degeneration ($25.3 \pm 1.7 \times 10^{-2}$, $n = 3$).

Aganirsen Reduces Pathologic Neovascularization in a Primate Model

We evaluated the antiangiogenic efficacy of aganirsen in laser-induced CNV in African green monkeys. Topical administration of aganirsen at the highest dose (86 μ g) resulted in significant and near complete inhibition of CNV lesions (Fig. 1, representative angiograms) with the most pronounced neovascularization (grade IV) compared with vehicle-treated control eyes (Fig. 2A, $P = 0.000456$). The effect of aganirsen was dose dependent, but these changes were not significant at lower doses (43 μ g, $P = 0.08$; 21.5 μ g, $P = 0.09$). Our previous work in African green monkeys²³ and other nonhuman primate laser-induced CNV models²⁸ has demonstrated that clinically relevant CNV in nonhuman primates applies to grade III and grade IV lesions.^{23,28} The incidence of grade III lesions alone (Fig. 2A; 21.5 μ g, $P = 0.0418$; 43 μ g, $P = 0.0166$; 86 μ g, $P = 0.000132$) and the combined incidence of grade III and IV lesions after aganirsen (Fig. 2A; 21.5 μ g, $P = 0.0082$; 43 μ g, $P = 0.00133$; 86 μ g, $P < 0.0001$) was significantly lower than in vehicle-treated eyes at all doses.

Findings from densitometric assessment of fluorescein angiograms were consistent with graded scoring of CNV lesions, such that aganirsen (86 μ g) was again found to be effective compared with vehicle (Fig. 2C, $P = 0.0186$). OCT image analysis further revealed a reduction in CNV lesion size, as determined by assessment of maximal CNV complex area, in eyes receiving aganirsen compared with vehicle-treated controls (Figs. 2B, 2D; $P < 0.0001$). Otherwise OCT findings, as well as slit lamp examination and angiography, were all found to be normal after aganirsen.

In 12 animals, the concentration of aganirsen in the retina was measured after a single application of the emulsion of aganirsen at doses of 21.5, 43, and 86 μ g yielding a concentration of aganirsen of 0.28 ± 0.01 , 0.27 ± 0.03 , and 0.89 ± 0.11 μ M at 90 minutes and 0.45 ± 0.05 , 0.64 ± 0.13 , and 0.66 ± 0.09 μ M at 8 hours, respectively. The concentration of aganirsen in the retina was significantly greater at both 90 minutes ($P < 0.0001$) and 8 hours ($P < 0.009$) after administration of the highest dose of aganirsen compared with retina levels after administration of the lowest dose. In the same retinal samples after 8-hour exposure to aganirsen, the concentration of IRS-1 protein decreased from 31.8 ± 5.1 ng/g retina in samples exposed to the dose of 21.5 μ g aganirsen to 12.0 ± 2.1 ng/g retina ($P < 0.0039$) and 13.7 ± 4.7 ng/g retina ($P < 0.031$) in samples exposed to respective doses of 43 μ g and 86 μ g.

Distribution of Aganirsen in Rabbit Eyes

We evaluated the ocular distribution of aganirsen (1.72 mg/g) after topical application on rabbit eyes, a reliable model in which to study the kinetics of ophthalmic drugs.¹⁵ The average concentration of aganirsen peaked in the iris and ciliary body at 90 minutes (Fig. 3A). Similar findings were observed for the

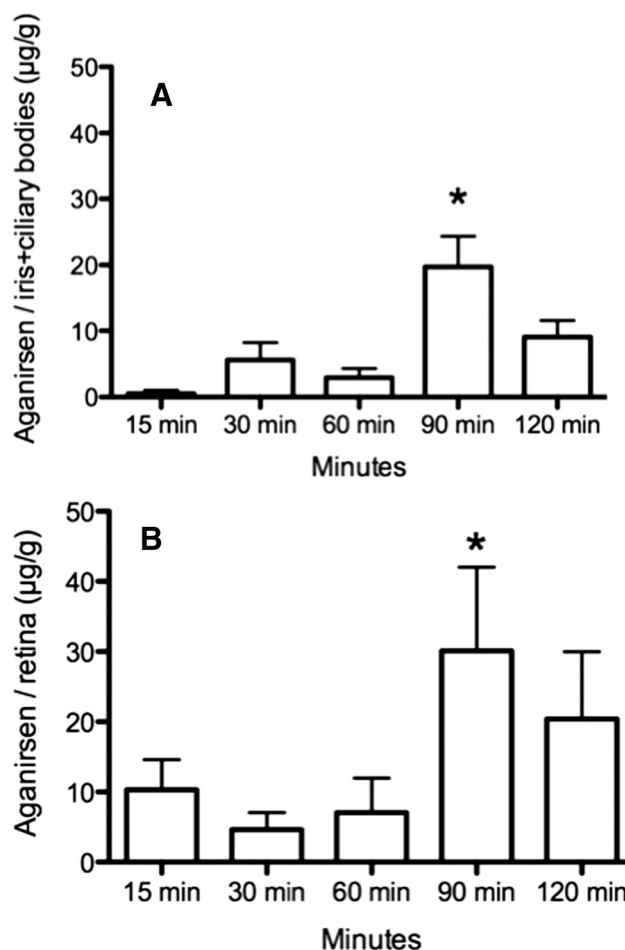


FIGURE 3. Concentrations of aganirsen in the iris and ciliary body (A) and retina (B) of rabbits ($n = 12$ per group) after a single 50 mg topical application of aganirsen (1.72 mg/g) on the cornea. Animals were euthanized at 15, 30, 60, 90, or 120 minutes after administration, and aganirsen was extracted from the tissues and quantified by UV light after ionic chromatographic separation. Results shown are mean $\pm$ SEM. * $P < 0.05$ compared with 30 and 60 minutes.

retina (Fig. 3B). Aganirsen was not detected in the untreated contralateral eye of rabbits ($n = 3$) unilaterally dosed. Importantly, the concentrations of aganirsen observed in the retina at 90 minutes were comparable to those (23 ± 11 μ g/g per retina) previously shown to prevent VEGF-induced capillary tubule-like formation of human endothelial cells.¹⁹

We estimated the percentage of aganirsen reaching the retina from the cornea: the average weight of the rabbit retina was 0.024 ± 0.012 g (mean $\pm$ SD). At the peak of the concentration in the retina (90 minutes), the amount of aganirsen was 30 ± 41 μ g/g retinal tissue (mean $\pm$ SD), equivalent to an average of 0.72 μ g aganirsen in the retina. The application of aganirsen (86 μ g) on the cornea led therefore to the delivery of 0.83% of the active compound.

Aganirsen Reduces Pathologic Neovascularization and Avascularity in a Rat Model

To further demonstrate that topical aganirsen reaches the retina in another model associated with aberrant neovascularization of the retina, this drug was tested in a rat model of OIR either by intraocular injection (Fig. 4A) or topical application (Fig. 4B). Topical application of aganirsen decreased preretinal neovascularization dose dependently to reach maximum effi-

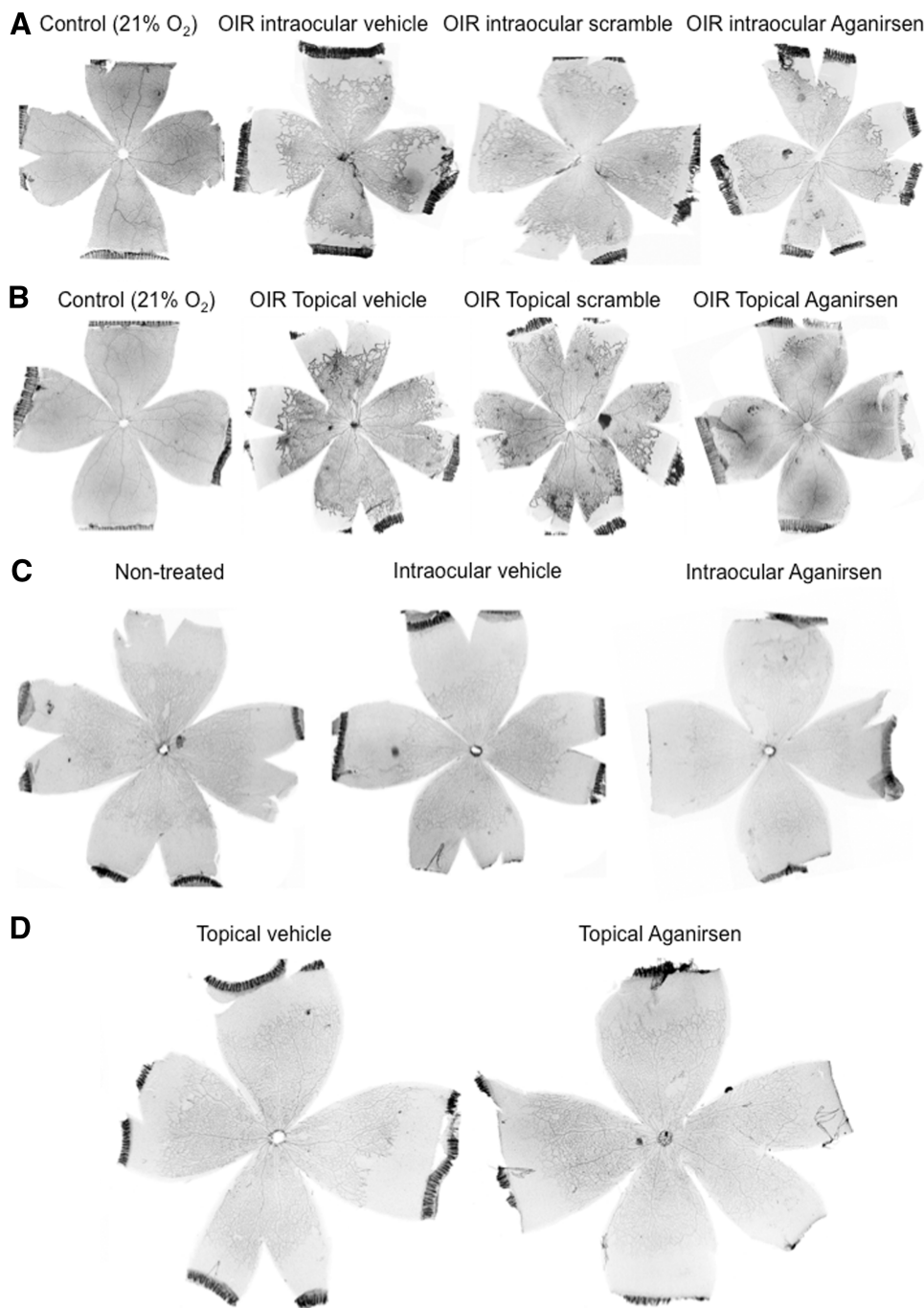


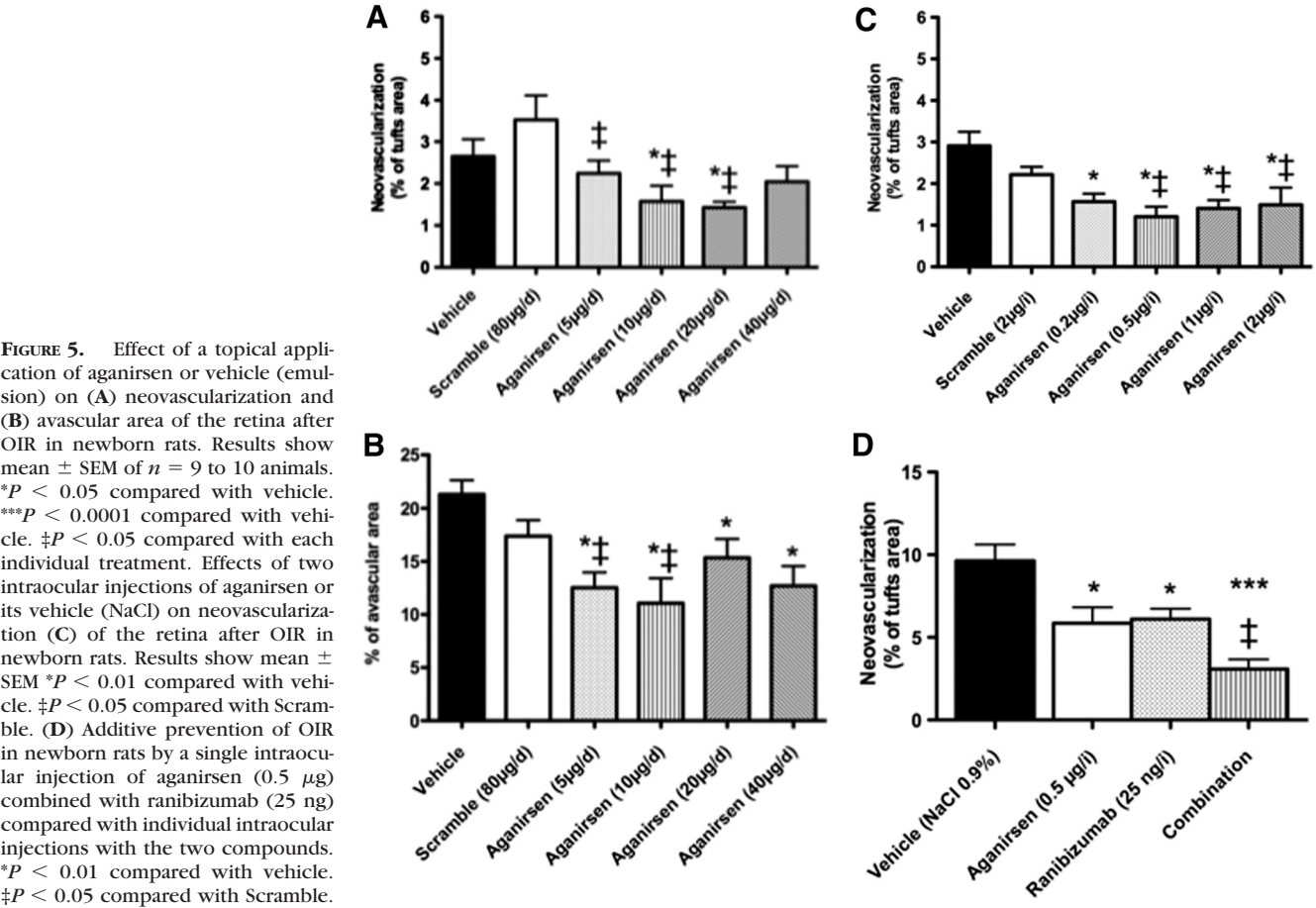
FIGURE 4. Representative retina flat-mounts showing (A) neovascularization of the retina after OIR in newborn rats treated by intraocular injection of aganirsen (0.5 μ g/injection), (B) neovascularization of the retina after OIR in newborn rats treated by daily topical applications of aganirsen (10 μ g/application), normal vascularization and its density in the retina during normal development in newborn rats treated or not treated by (C) intraocular injections or (D) topical applications of aganirsen (doses in Table 1).

cacy of $\sim 50\%$ at 20 μ g (Fig. 5A); at higher doses, efficacy was diminished. Aganirsen did not aggravate the degree of avascularity in OIR; in fact, aganirsen reduced the avascular retinal area (Fig. 5B). Consistent with these observations, aganirsen (topical or intraocular; Fig. 4C) did not interfere with normal vascular development (Table 1). Importantly, topical aganirsen was as effective as intravitreal treatment in reducing preretinal neovascular tufts, consistent with robust intraocular penetration of aganirsen (Fig. 5C). Finally, we compared the effects of aganirsen with those of ranibizumab and found both drugs to be equivalently effective and to have additive efficacy (Fig. 5D).

DISCUSSION

AMD and ischemic retinopathies are major causes of blindness. Both conditions are associated with neovascularization, which

contributes to abnormally leaky vessels that can breach the inner limiting membrane of the retina and invade the vitreous, resulting in subretinal and vitreal hemorrhage, retinal detachment, and eventually blindness. The only effective drugs approved to target neovascularization in these ocular disorders are anti-VEGF compounds. Recently, however, we reported that aganirsen, an antisense oligonucleotide that inhibits the expression of IRS-1, has antiangiogenic activity after administration on the rat cornea^{19,29} and has no side effects after corneal topical application in human healthy volunteers.²⁰ In the present study, we provide the first evidence that IRS-1 is overexpressed in the retinas of patients with subretinal neovascularization and AMD, two conditions associated with pathologic neovascularization, compared with retinal samples isolated from patients with proliferative vitreoretinopathy, a con-



dition associated with glial or retinal pigment epithelial proliferation without neovascularization. Moreover, we show that topically (on the cornea) applied aganirsen reaches the posterior ocular chamber and prevents CNV in nonhuman primates and preretinal neovascularization in a model of ischemic retinopathy without adversely affecting normal revascularization and developmental angiogenesis. Collectively, these data support the importance of IRS-1 as a key component of abnormal neovascular proliferation responses in the retina.

A single topical application of aganirsen as an ophthalmic emulsion led to its dose-dependent accumulation in the retina within 90 minutes both in rabbits and in monkeys. Findings are consistent with our previous observations that, on a single topical application, aganirsen saturates the conjunctival epithelium and is taken up by the cornea and the sclera,¹⁹ followed by its distribution to the posterior segment of the eye. Importantly, all doses of aganirsen led to a sustained concentration of

the compound in the monkey retina up to 8 hours. A greater concentration of aganirsen ($2.81 \pm 1.34 \mu\text{M}$, $n = 12$; derived from Fig. 3B) was obtained in the rabbit retina 90 minutes after topical application of an 86- μg dose; the concentration tended to decline slightly at 120 minutes ($1.29 \pm 0.41 \mu\text{M}$, $n = 12$). Together these data demonstrate that aganirsen is readily available to the retina after topical application. In addition, the concentration achieved in the retina was within the range of the effective concentration of aganirsen shown to prevent VEGF-induced capillary tubulelike formation of human endothelial cells.¹⁹ Accordingly, intravitreal (intraocular) injections of aganirsen reduced neovascularization by ~50% in the rat OIR model at the optimal dose tested, confirming that aganirsen is a potent antiangiogenic agent. Data are in line with our previous findings revealing that aganirsen reduced injury-induced corneal neovascularization by ~35%,¹⁹ an efficacy rate comparable to that of topical bevacizumab (Avastin; Genentech, South San Francisco,

TABLE 1. Effects of Topical Applications (24 µg/day) and a Single Intravitreal Injection (0.5 µg/injection) of Aganirsen on Normal Vascularization and Its Density in the Retina during Development in Newborn Rats

Application	Vehicle		Aganirsen	
	Vascular Area	Density	Vascular Area	Density
Topical	65.2 ± 2.4	33.5 ± 1.0	64.3 ± 2.0	36.2 ± 1.3
n	8	8	7	7
Intraocular	53.6 ± 1.8	39.9 ± 1.7	51.9 ± 2.8	34.2 ± 2.5
n	6	7	8	8

Data are percentages expressed as mean ± SEM. n, number of animals.

CA), which reduces chemically induced corneal neovascularization in rats³⁰ and mice³¹ by 30%.

An important feature of our findings is that topical application of aganirsen effectively reduces retinal neovascularization by ~50% at the optimal dose tested, indicating that aganirsen penetrates to the posterior chamber of the eye at the required concentration. This is clinically relevant because it could eliminate the need for monthly intravitreal injections of antiangiogenic agents such as glucocorticoids and anti-VEGF,^{2,15} avoiding associated side effects⁴ and providing an alternative to more destructive laser photocoagulation, a continued mainstay of treatment for macular edema.² Intraocular administration of antiplacental growth factor, which binds to VEGF Flt1 receptors, and anti-VEGF reduces retinal neovascularization in a mouse model by 30% to 70%.⁶ Although anti-VEGF inhibitors such as bevacizumab, ranibizumab, and pegaptanib sodium fail to inhibit laser-induced retinal/choroidal neovascularization in rats, possibly because of late intervention (3 weeks after injury),³² selective monoclonal anti-VEGF neutralizing antibodies demonstrated antiangiogenic activity on the rat cornea^{18,33} and choroid¹⁷ and prevented the normal vascularization of the retina.¹⁴ The finding that aganirsen effectively inhibited neovascularization in our rat model of OIR with an efficacy comparable to that of anti-VEGF inhibitors and without effects on developmental vascularization supports the clinical evaluation of aganirsen as an alternative therapy for ischemic retinopathies.

Topical administration of aganirsen resulted in significant and near-complete inhibition of the highest grade CNV lesion formation in a nonhuman primate model of AMD at the dose of 86 μ g, which was the middle range of topical doses previously explored safely in humans.²¹ At 21.5 μ g and 43 μ g, a modest but nonsignificant reduction in the incidence of grade IV CNV lesions was observed, as was a dose-dependent reduction in IRS-1 protein expression, suggestive of a nonsaturated pharmacologic distribution at the posterior pole over the dose range explored. We have previously shown that intravitreal administration of bevacizumab can inhibit grade IV lesions in this primate model to an extent comparable to that seen with aganirsen.²³ Although we did not directly compare the effects of topical aganirsen with the two standards of care (bevacizumab and ranibizumab), based on previous data from the African green monkey model²³ and other nonhuman primate models,²⁸ successful inhibition of grade IV lesions is notable; along these lines, the addition of aganirsen to ranibizumab halved the extent of neovascularization compared with aganirsen or ranibizumab treatment alone in the OIR model.

Anti-VEGF therapy is associated with undesired structural and functional impairments. Anti-permeability properties of anti-VEGF therapy may excessively alter the choroidal fenestration essential for normal choroid function.³⁴ Bevacizumab can also inhibit normal vascular development^{12–14} and lead to mitochondrial disruption of the inner segments of photoreceptors, resulting in increased proapoptotic protein expression.³⁵ These effects have not been observed with aganirsen; consistent with this claim, retinal vascular development was found to be normal after aganirsen administration in the rat, as were other ocular examination findings in the monkey.

Aganirsen not only reduces aberrant neovascularization, it attenuates avascularity and does not interfere with normal vascular developmental, a profile optimal for an antiangiogenic compound. Because VEGF partakes in normal vascular development, this beneficial profile of aganirsen is not shared with anti-VEGF molecules. Hence, aganirsen seems to inhibit angiogenesis, in part independently of inhibiting VEGF expression. Interestingly, IRS-1 deficiency in the mouse does not affect the normal growth of vessels, but it does reduce pathologic neovascularization.²² Nonetheless, it is unlikely that aganirsen is

specific to endothelial cells, though no adverse effects have been evidenced during preclinical and clinical development of the product.^{19,21} The structure of aganirsen makes it indeed an amphipathic compound (Supplementary Material, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9064/-/DCSupplemental>), allowing for its penetration through the lipophilic corneal and conjunctival epithelium (and cells in general) and through the highly hydrated corneal stroma and sclera (extracellular matrix in general). We speculate, therefore, that IRS-1 exerts a distinct role in normal versus pathologic neovascularization based on the anti-inflammatory effect of aganirsen, as previously shown in cultured endothelial cells and in rats.^{19,29} In the latter studies, we showed that aganirsen (GS-101) reduced interleukin-1 (a potent proinflammatory factor) production both in vitro and in vivo and reduced the expression of VEGF. These effects were not associated with a change in ERK1/2 activity but were associated with a reduction in Akt activity.¹⁹ Importantly, as shown in the monkey retina, complete inhibition of IRS-1 expression is not required to confer antiangiogenic efficacy, further supporting the safety of aganirsen use. Taken together, the efficacy of aganirsen for aberrant neovascularization, its safety, and its ocular distribution on topical application suggest a promising therapeutic profile for further testing in the clinical setting for conditions involving potent increases in IRS-1 expression, such as in retinopathies associated with pathologic neovascularization.

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References

1. Brown G, Brown MM. Let us wake the nation on the treatment for age-related macular degeneration. *Curr Opin Ophthalmol*. 2010; 21(3):169–171.
2. Kollias AN, Ulbig MW. Diabetic retinopathy: early diagnosis and effective treatment. *Dtsch Arztebl Int*. 2010;107(5):75–83.
3. Martin DF, Maguire MG, Ying GS, Grunwald JE, Fine SL, Jaffe GJ. Ranibizumab and bevacizumab for neovascular age-related macular degeneration. *N Engl J Med*. 2011;364(20):1897–1908.
4. Sampat KM, Garg SJ. Complications of intravitreal injections. *Curr Opin Ophthalmol*. 2010;21(3):178–183.
5. Saint-Geniez M, Maharaj AS, Walshe TE, et al. Endogenous VEGF is required for visual function: evidence for a survival role on Muller cells and photoreceptors. *PLoS One*. 2008;3(11):e3554.
6. Van de Veire S, Stalmans I, Heindryckx F, et al. Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease. *Cell*. 2010;141(1):178–190.
7. Micieli JA, Surkont M, Smith AF. A systematic analysis of the off-label use of bevacizumab for severe retinopathy of prematurity. *Am J Ophthalmol*. 2009;148(4):536–543, e2.
8. Ueta T, Yanagi Y, Tamaki Y, Yamaguchi T. Cerebrovascular accidents in ranibizumab. *Ophthalmology*. 2009;116(2):362.
9. Chen ML, Guo L, Smith LE, Dammann CE, Dammann O. High or low oxygen saturation and severe retinopathy of prematurity: a meta-analysis. *Pediatrics*. 2010;125(6):e1483–e1492.
10. Ruiz de Almodovar C, Lambrechts D, Mazzone M, Carmeliet P. Role and therapeutic potential of VEGF in the nervous system. *Physiol Rev*. 2009;89(2):607–648.
11. Saint-Geniez M, Kurihara T, Sekiyama E, Maldonado AE, D'Amore PA. An essential role for RPE-derived soluble VEGF in the maintenance of the choriocapillaris. *Proc Natl Acad Sci U S A*. 2009; 106(44):18751–18756.
12. Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed

- retinal vessels and has implications for retinopathy of prematurity. *Nat Med*. 1995;1(10):1024-1028.
13. Stone J, Itin A, Alon T, et al. Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *J Neurosci*. 1995;15(7 pt 1):4738-4747.
 14. Ozaki H, Seo MS, Ozaki K, et al. Blockade of vascular endothelial cell growth factor receptor signaling is sufficient to completely prevent retinal neovascularization. *Am J Pathol*. 2000;156(2):697-707.
 15. Edelhauser HF, Boatright JH, Nickerson JM. Drug delivery to posterior intraocular tissues: Third Annual ARVO/Pfizer Ophthalmics Research Institute Conference. *Invest Ophthalmol Vis Sci*. 2008;49(11):4712-4720.
 16. Akkoyun I, Karabay G, Haberal N, et al. Structural consequences after intravitreal bevacizumab injection without increasing apoptotic cell death in a retinopathy of prematurity mouse model. *Acta Ophthalmol*. 2010 Jul 30 [Epub ahead of print].
 17. Hua J, Spee C, Kase S, et al. Recombinant human VEGF165b inhibits experimental choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2010;51(8):4282-4288.
 18. Sener E, Yuksel N, Yildiz DK, et al. The impact of subconjunctivally injected EGF and VEGF inhibitors on experimental corneal neovascularization in rat model. *Curr Eye Res*. 2011;36(11):1005-1013.
 19. Al-Mahmood S, Colin S, Farhat N, Thorin E, Steverlynck C, Chemtob S. Potent in vivo antiangiogenic effects of GS-101 (5'-TATCCG-GAGGGCTCGCCATGCTGCT-3'), an antisense oligonucleotide preventing the expression of insulin receptor substrate-1. *J Pharmacol Exp Ther*. 2009;329(2):496-504.
 20. Cursiefen C, Bock F, Horn FK, et al. GS-101 antisense oligonucleotide eye drops inhibit corneal neovascularization: interim results of a randomized phase II trial. *Ophthalmology*. 2009;116(9):1630-1637.
 21. Kain H, Goldblum D, Geudelin B, Thorin E, Beglinger C. Tolerability and safety of GS-101 eye drops, an antisense oligonucleotide to insulin receptor substrate-1: a 'first in man' phase I investigation. *Br J Clin Pharmacol*. 2009;68(2):169-173.
 22. Jiang ZY, He Z, King BL, et al. Characterization of multiple signaling pathways of insulin in the regulation of vascular endothelial growth factor expression in vascular cells and angiogenesis. *J Biol Chem*. 2003;278(34):31964-31971.
 23. Goody RJ, Hu W, Shafiee A, et al. Optimization of laser-induced choroidal neovascularization in African green monkeys. *Exp Eye Res*. 2011;92(6):464-472.
 24. Dorfman A, Dembinska O, Chemtob S, Lachapelle P. Early manifestations of postnatal hyperoxia on the retinal structure and function of the neonatal rat. *Invest Ophthalmol Vis Sci*. 2008;49(1):458-466.
 25. Sapieha P, Sirinyan M, Hamel D, et al. The succinate receptor GPR91 in neurons has a major role in retinal angiogenesis. *Nat Med*. 2008;14(10):1067-1076.
 26. Sennlaub F, Valamanesh F, Vazquez-Tello A, et al. Cyclooxygenase-2 in human and experimental ischemic proliferative retinopathy. *Circulation*. 2003;108(2):198-204.
 27. Higgins RD, Yu K, Sanders RJ, Nandgaonkar BN, Rotschild T, Rifkin DB. Diltiazem reduces retinal neovascularization in a mouse model of oxygen induced retinopathy. *Curr Eye Res*. 1999;18(1):20-27.
 28. Lichtlen P, Lam TT, Nork TM, Streit T, Urech DM. Relative contribution of VEGF and TNF-alpha in the cynomolgus laser-induced CNV model: comparing the efficacy of bevacizumab, adalimumab, and ESBA105. *Invest Ophthalmol Vis Sci*. 2010;51(9):4738-4745.
 29. Andrieu-Soler C, Berdugo M, Doat M, Courtois Y, BenEzra D, Behar-Cohen F. Downregulation of IRS-1 expression causes inhibition of corneal angiogenesis. *Invest Ophthalmol Vis Sci*. 2005;46(11):4072-4078.
 30. Manzano RP, Peyman GA, Khan P, et al. Inhibition of experimental corneal neovascularisation by bevacizumab (Avastin). *Br J Ophthalmol*. 2007;91(6):804-807.
 31. Bock F, Onderka J, Dietrich T, et al. Bevacizumab as a potent inhibitor of inflammatory corneal angiogenesis and lymphangiogenesis. *Invest Ophthalmol Vis Sci*. 2007;48(6):2545-2552.
 32. Lu F, Adelman RA. Are intravitreal bevacizumab and ranibizumab effective in a rat model of choroidal neovascularization? *Graefes Arch Clin Exp Ophthalmol*. 2009;247(2):171-177.
 33. Amano S, Rohan R, Kuroki M, Tolentino M, Adamis AP. Requirement for vascular endothelial growth factor in wound- and inflammation-related corneal neovascularization. *Invest Ophthalmol Vis Sci*. 1998;39(1):18-22.
 34. Ameri H, Chader GJ, Kim JG, Sadda SR, Rao NA, Humayun MS. The effects of intravitreal bevacizumab on retinal neovascular membrane and normal capillaries in rabbits. *Invest Ophthalmol Vis Sci*. 2007;48(12):5708-5715.
 35. Inan UU, Avci B, Kusbeci T, Kaderli B, Avci R, Temel SG. Preclinical safety evaluation of intravitreal injection of full-length humanized vascular endothelial growth factor antibody in rabbit eyes. *Invest Ophthalmol Vis Sci*. 2007;48(4):1773-1781.