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ORIGINAL ARTICLE

A hypotonic gel-forming eye drop provides enhanced intraocular delivery of a kinase inhibitor with melanin-binding properties for sustained protection of retinal ganglion cells

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Abstract

While eye drops are the most common ocular dosage form, eye drops for treating diseases of the posterior segment (retina, choroid, optic nerve) have yet to be developed. In glaucoma, eye drops are used extensively for delivering intraocular pressure (IOP)-lowering medications to the anterior segment. However, degeneration of retinal ganglion cells (RGCs) in the retina may progress despite significant IOP lowering, suggesting that a complementary neuroprotective therapy would improve glaucoma management. Here, we describe a hypotonic, thermosensitive gel-forming eye drop for effective delivery of sunitinib, a protein kinase inhibitor with activity against the neuroprotective targets dual leucine zipper kinase (DLK) and leucine zipper kinase (LZK), to enhance survival of RGCs after optic nerve injury. Further, binding of sunitinib to melanin in the pigmented cells in the choroid and retinal pigment epithelium (RPE) led to prolonged intraocular residence time, including therapeutically relevant concentrations in the non-pigmented retinal tissue where the RGCs reside. The combination of enhanced intraocular absorption provided by the gel-forming eye drop vehicle and the intrinsic melanin binding properties of sunitinib led to significant protection of RGCs with only once weekly eye drop dosing. For a chronic disease such as glaucoma, an effective once weekly eye drop for neuroprotection could result in greater patient adherence, and thus, greater disease management and improved patient quality of life.

Keywords Topical delivery · Thermosensitive · Melanin binding · Sustained delivery

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Introduction

The most common ocular dosage form is eye drops. Eye drops are used for a wide range of conditions affecting the front portion of the eye (anterior segment), such as for treatment of dry eye, conjunctivitis, corneal ulcers, and reduction of intraocular pressure (IOP) for glaucoma. However, the high frequency of required usage, often multiple times per day for IOP lowering drops, is a significant impediment to patient adherence and achieving appropriate clinical benefit [1]. Further, the utility of eye drops for treating conditions affecting the back of the eye (posterior segment), including the retina, choroid, and optic nerve, has been limited. Topically applied therapeutics encounter multiple layers of barriers to effective intraocular absorption, leading to very little reaching the posterior segment [2]. We recently described the development of a hypotonic, thermosensitive gel-forming eye drop that provided increased and sustained intraocular drug absorption without increased systemic drug exposure [3]. Upon application, the eye drop spread uniformly over the ocular surface prior to forming a thin, clear gel layer that was resistant to clearance from blinking [3]. Surprisingly, we found that this led to delivery of therapeutic levels of sunitinib malate, a broad-spectrum protein kinase inhibitor that is FDA approved for the treatment of a variety of cancers, in the posterior segment in pigs [3]. Presumably due to its ability to inhibit the vascular endothelial growth factor receptor (VEGFR) [4], we found that once daily dosing of sunitinib malate in the gel-forming eye drop (SunitiGel) in pigs provided protection against laser-induced choroidal neovascularization (CNV), whereas daily dosing of sunitinib malate in saline had no effect on CNV [3].

In glaucoma, axonal injury triggers the death of projection neurons called retinal ganglion cells (RGCs), a process that leads to vision loss. Dual leucine zipper kinase (DLK; MAP3K12) and leucine zipper kinase (LZK; MAP3K13) are key mediators of RGC injury and cell death [5–7], as well as signaling associated with axon degeneration and regeneration [7, 8]. Sunitinib was found to potently and in a dose-dependent manner promote the survival of human RGCs challenged by colchicine-induced axonal injury, presumably at least in part due to its ability to inhibit DLK and LZK [5]. Importantly, DLK/LZK inhibition has a neuroprotective effect that is independent of IOP lowering, and thus, is an attractive strategy for augmenting current approaches for glaucoma treatment. However, in contrast to the variety of IOP-lowering eye drops that act on anterior segment tissues, a DLK/LZK inhibitor must be delivered to the RGCs in the retina. Thus, we hypothesized that the unique ability of the SunitiGel eye drop formulation to deliver sunitinib malate to the posterior segment may also provide protection of RGCs from injury.

In our prior work, we observed that daily dosing of SunitiGel in pigs led to higher sunitinib concentrations in the choroid/retinal pigmented epithelium (RPE) than the retina [3]. One potential explanation is that sunitinib binds to melanin, which is produced in high amounts in the melanocytes in the choroid and in RPE cells [9, 10]. It has been described that for many drugs, melanin binding may lead to sequestration of drug, reducing therapeutic effect [11]. However, if the amount of unbound drug is high enough to maintain a therapeutic effect, melanin-bound drug may provide a local sustained release effect in the eye [12, 13]. In the context of glaucoma, drug bound to melanin in the choroid and RPE must also traverse to the other side of the retina to reach RGCs. If this is possible, SunitiGel eye drops may lead to prolonged protection. From a compliance perspective, reducing the required eye drop dosing frequency may lead to improved clinical outcomes [14]. Here, we describe protection of RGCs in an optic nerve crush model without signs of toxicity with once weekly eye drop dosing.

Materials and methods

Material sources

Sunitinib malate was purchased from LC Laboratories. *N*-desethyl sunitinib and Sunitinib-d10 was purchased from Toronto Research Chemical (North York, ON, Canada). Balanced salt solution was purchased from Alcon Laboratory (Fort Worth, TX, USA). Melanin from *Sepia officinalis*, ferric ammonium citrate, saponin, bovine serum albumin (BSA), Tween 20, fetal bovine serum (FBS), Lutrol F127, trifluoroacetic acid, poly-D-lysine/laminin, and Triton X-100 were purchased from Sigma Aldrich (St. Louis, MO, USA). ARPE-19 (CRL-2302) and DMEM:F12 medium were purchased from the American Type Culture Collection (Manassas, VA, USA). DMEM with high glucose and pyruvate, Trypsin–EDTA (0.25%) with phenol red, rabbit anti-human TYRP1 antibody, Lab-Tek 4-well Permanox Chamber slides, Alexa Fluor 647 conjugated mouse anti-human ZO-1, Alexa Fluor 555 conjugated goat anti-rabbit IgG (H + L) secondary antibody, penicillin/streptomycin, Alexa Fluor 568 conjugated goat anti-rabbit IgG (H + L), 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI), 2-mercaptoethanol, Calcein AM, 384-well culture dishes, B-27 supplement, N-2 supplement, L-glutamine, PureLink RNA mini kits, SuperScript IV VILO Master Mix, TaqMan Fast Advanced Master Mix, Fluoromount-G, Image-iT™ Fixative Solution (4% formaldehyde, methanol-free) glyceraldehyde-3-phosphate dehydrogenase (Rn01775763_g1), γ-synuclein (Rn00581652_m1), and β-III tubulin (Rn01431594_m1) were ordered from Thermo Fisher Scientific (Waltham, MA, USA). Alexa Fluor 647 conjugated goat anti-mouse IgG H + L was purchased from Abcam (Cambridge, MA, USA). 1× phosphate-buffered saline (PBS), high-performance liquid chromatography (HPLC) grade acetonitrile, and water

were purchased from Fisher Scientific (Hampton, NH, USA). Endotoxin-Free Ultra-pure Water was purchased from EMD Millipore. Methylcellulose (400 cP) was purchased from Spectrum chemical (New Brunswick, NJ, USA). Hamilton 1700 Series gas tight syringes (25 µL, Model 1702 RN, 33 gauge and 30 gauge) were purchased from Hamilton Company (Reno, NV, USA). BD 1 mL TB syringe with 26G needles was purchased from BD (San Jose, CA, USA). Isoflurane was purchased from Baxter (Deerfield, IL, USA). Reverse-action forceps were purchased from World Precision Instruments (Sarasota, FL, USA). Rabbit anti γ-synuclein was purchased from GeneTex (Irvine, CA, USA). Mouse anti β-III tubulin was purchased from BioLegend (San Diego, CA, USA). Neomycin, polymyxin b, and bacitracin zinc ophthalmic ointment were purchased from Akorn (Lake Forest, IL, USA). CellTiter-Glo was purchased from Promega (Madison, WI, USA).

Induction of melanin expression in ARPE-19 cells

ARPE-19 cells were cultured with DMEM: F12 medium containing 10% FBS according to the vendor's protocol. Fully confluent APRE-19 cells were then cultured in the DMEM high glucose, pyruvate media with 250 µM of ferric ammonium citrate, and 1% FBS to induce melanogenesis [15]. The culture medium was changed twice per week without cell passage in T175 flasks. After 2 months, cells were detached from the flask using 0.25% trypsin for 10 min and centrifuged at 200 rcf for 3 min (Sorvall ST8R model, Thermo Fisher Scientific, Waltham, MA, USA). The intact cells were then resuspended in PBS, and 1 M cells per well were added to a 96-well plate to measure absorbance at 475 nm (BioTek Synergy SN236229 plate reader, Winooski, VT, USA) to evaluate melanin production. Absorbance at 475 nm was chosen based on performing a full-spectrum scan for the absorbance peak. For the immunohistochemical staining, the cells were cultured in Lab-Tek 4-well Permanox Chamber slides for confocal imaging. The control ARPE-19 cells were cultured in the chamber slide for 7 days. The induced ARPE-19 cells were cultured in the chamber slide for 2 months with the DMEM high glucose, pyruvate media and with 250 µM of ferric ammonium citrate and 1% FBS. The chamber slides were fixed in pure methanol at a room temperature for 5 min, and then washed in PBS for 5 min, three times. Cells were permeabilized with 0.5% saponin in PBS for 1 h followed by incubation for 1 h in blocking buffer containing 1% BSA, 2.2% glycine, and 0.1% Tween 20 in PBS. Cells were then incubated for 1 h with rabbit anti-human TYRP1 (1:200 dilution) and AF647 conjugated mouse anti-human ZO-1 primary antibody (1:50 dilution) in PBS containing 1% BSA and 0.1% Tween 20. Cells were then washed in PBS for 5 min, 3 times. Cells were then incubated with Alexa Fluor Plus 555 conjugated goat anti-rabbit IgG (H + L) (1:1000 dilution) in PBS containing 1% BSA and 0.1% Tween 20. After 1 h, cells were washed in PBS for 5 min, 3 times, followed by staining the cell nuclei with 4′,6-diamidino-2-phenylindole (DAPI,1:1000 dilution) in PBS for 10 min. Cells were then washed 3 times with PBS before taking images with a confocal LSM 710 microscope (Carl Zeiss, White Plains, NY, USA) at 40X magnification. Microscopic settings were adjusted to avoid introduction of any background fluorescence using non-melanin expressing control cells.

To characterize the contribution of melanin production to cell uptake and retention, 1 million each of ARPE-19 cells cultured for 2 months either according to the vendor's protocol or cultured to induce melanin production were collected and plated in 6-well plates. Sunitinib malate (25 µg/mL) in PBS was added to the cells for 6 h at 37 °C. The cells were then washed 3 times with PBS by centrifugation at 500 rcf for 5 min. After the last wash, the cells were incubated in acetonitrile at room temperature for 2 h to extract the drug. The samples were then centrifuged at 17,000 rcf at 37 °C for 30 min (accuSpin Micro 17R, Fisher Scientific, Hampton, NH, USA), and the supernatant was collected to measure the drug concentration by HPLC (Shimadzu LC-20) with a Luna® 5 µm C18(2) 100 Å, LC Column 250 × 4.6 mm (Phenomenex, Torrance, CA, USA). Acetonitrile and water were used as a mobile phase at the ratio of 55:45 with 0.1% trifluoroacetic acid. Chromatography was performed at 40 °C, and the UV spectrum was monitored at 470 nm, which was the wavelength at which peak absorption was observed with a full-spectrum scan.

Melanin binding assay

Melanin from *Sepia officinalis* (100 µg) was suspended in deionized water and centrifuged at 17,000 rcf for 30 min (accuSpin Micro 17R, Fisher Scientific, Hampton, NH, USA). The supernatant was discarded to remove small particles, and the pellet was resuspended by vortexing and washed in deionized water 3 more times. The melanin was then lyophilized for 7 days and stored in −20 °C freezer until use. Melanin, sunitinib malate, and *N*-desethyl sunitinib were suspended in PBS prior to mixing. The solutions were then mixed to obtain 400 µL of solution containing 100 µg melanin and sunitinib malate or *N*-desethyl sunitinib at concentrations ranging from 15.6–500 µg/mL. The solutions were then incubated on a 220-rpm orbital shaker (New Brunswick™ Innova® 2000, Eppendorf, Enfield, CT, USA), at 37 °C for 48 h in the dark, and then centrifuged at 17,000 rcf at 37 °C for 30 min. The supernatant was collected into new tubes and centrifuged at 17,000 rcf at 37 °C for 30 min to ensure the complete removal of melanin from the supernatant. The concentration of the sunitinib or *N*-desethyl sunitinib in the supernatant was quantified by HPLC as described above. The amount of bound drug was used to calculate the binding capacity (moles drug/mg melanin) and the dissociation constant (K_d) as previously described [16].

Eye drop formulation

SunitiGel was formulated to contain 12% (w/w) Pluronic F127 and 0.4% (w/v) sunitinib malate, as previously described [3]. F127 stock solution (20% w/w) was made by adding 2 g of F127 powder to 8 g of endotoxin-free ultrapure water and storing at 4 °C until fully dissolved. Sunitinib malate (1% w/v) was dissolved in water using an ultrasonic probe sonicator (Sonics, Vibra Cell VCX-750 with model CV334 probe, Newtown, CT, USA) by pulsing 1 s on/off at 40% amplitude for 5 min in a 4 °C water bath. The sunitinib malate solution was then mixed with the stock F127 solution at a 4:6 ratio (w/w) to make the final solution. To prepare the saline formulation, sunitinib malate (0.4% w/v) was dissolved in sterile normal saline using the same approach with the ultrasonic probe sonicator. As sunitinib malate is highly water soluble, the drug is fully dissolved when added to the Pluronic F127 solution (i.e., 100% drug loading efficiency). The material properties of the SunitiGel were described previously [3]. Of note, any properties of the gel could only be evaluated in vitro using higher concentration Pluronic F127 solutions above the critical gel concentration. The gelling temperature of a 16% (w/w) Pluronic F127 solution was found to be 29.0 ± 1.4 °C [3]. The dynamic viscosity at 37 °C at a low shear rate of 0.005 s^{-1} was 10,000 ± 335 Pa·s, which was ~ 25-fold higher than GenTeal lubricant eye gel [3]. However, at a high shear rate of 5000 s^{-1} to mimic blinking of the eyelids, the dynamic viscosity of the Pluronic F127 gel was reduced to 0.11 ± 0.0024 Pa·s, which was indistinguishable from GenTeal (0.13 ± 0.0097 Pa·s) [3].

Animal welfare statement

All experimental protocols were approved by the Johns Hopkins Animal Care and Use Committee. All animals were handled and treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for Use of Animals in Ophthalmic and Vision Research. A specific sex was not specified when ordering any species, so both male and female animals were used as provided. C57BL/6J and tyrosinase knockout mice on the C57BL/6J background (B6(Cg)-*Tyr*$^{c-2J}$/J) were purchased from The Jackson Laboratory. Brown Norway and Wistar rats were obtained from Charles River Laboratories. All animals were 6–8 weeks in age. Animals were anesthetized prior to euthanasia.

Pharmacokinetics

Animals were anesthetized using isoflurane (V3000PK, Coral Springs, FL) for 5 min prior to dosing to prevent any movement. Eye drops (5 µL) were delivered unilaterally prior to placing the animal back in the isoflurane chamber for additional 5 min. For the mouse studies, C57BL/6J and B6(Cg)-*Tyr*$^{c-2J}$/J mice (n = 7–8) were dosed once daily for 4 days, and the eyes were collected 24 h later for drug measurements. One outlier data point was removed from the C57BL/6J group after applying Grubbs test (see "Statistical methods"), resulting in n = 6 data points per group. For the rat studies, Brown Norway rats (n = 5—6) were dosed unilaterally three times, 1 week apart, and tissues were collected at 1, 4, and 7 days after the third dose for drug measurements. One outlier data point was removed from the groups collected at 1 and 7 days after the third dose after applying Grubbs test (see "Statistical methods"), resulting in n = 5 data points per group. Briefly, the eyes were enucleated and separated into posterior and anterior sections by cutting peripherally 1 mm behind the limbus area. The vitreous was carefully collected with a 1-mL tuberculin syringe with 26G needle attached. The retina and choroid/RPE layers were then separated. All specimens were stored in pre-weighed Eppendorf tubes at −80 °C until drug measurement.

Measurement of sunitinib in ocular tissues

Vitreous, retina, and choroid/RPE tissue samples were homogenized in 200–500 µL 1 × PBS using TissueLyser LT (Qiagen, Germantown, MD, USA) before extraction. Sunitinib was extracted from 50 µL of tissue homogenates with 150 µL of acetonitrile containing 2.5 ng/mL of the internal standard, sunitnib-d10. After centrifugation, the supernatant was then transferred to an autosampler vial for LC-MS/MS analysis. All ocular tissue samples were analyzed using a 1× PBS standard curve. Separation was achieved with a Waters Cortecs C18 (2.1 × 50 mm, 2.7 µm) column at room temperature using a gradient. Mobile phase A was water containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. The gradient started with mobile phase B held at 10% for 0.5 min and increased to 100% over 0.5 min; 100% mobile phase B was held for 1 min and then returned back to 10% mobile phase B and allowed to equilibrate for 1 min. Total run time was 3 min with a flow rate of 0.3 mL/min. The column effluent was monitored using a Sciex triple quadrupole™ 4500 mass-spectrometric detector (Sciex, Foster City, CA, USA) using electrospray ionization operating in positive mode. The spectrometer was programmed to monitor the following MRM transition 399.1 → 283.2 for sunitinib and 409.1 → 283.2 for the internal standard, sunitnib-d10. A calibration curve for sunitinib was computed using the area ratio peak of the analysis to the internal standard by using a quadratic equation with a $1/x^2$ weighting function over the range of 0.1–100 ng/mL for sunitinib with dilutions of up to 1:100 (v/v).

Rat optic nerve crush model

Rat eyes were topically anesthetized with 0.5% proparacaine hydrochloride prior to the procedure. The temporal

conjunctiva of the left eye was grasped with 0.12 mm toothed forceps and incised parallel to the limbus with sharp iris scissors. Dissection was performed using two pairs of curved blunt-tipped forceps, and the orbital fat and soft tissue were retracted to expose the orbital portion of the optic nerve. The optic nerve was crushed at a position 1.5–2 mm posterior to the globe using reverse-action forceps for 20 s. The orbital soft tissue was then repositioned over the nerve, and the conjunctiva was left to close by secondary intention. After the procedure, topical bacitracin-neomycin-polymyxin ophthalmic ointment was applied to both eyes to prevent infection. Two weeks later, the rats were sacrificed for further analysis.

RGC staining and counting

The eyes were then harvested with the optic nerve attached. The retinas were removed, incised for flat mounting, and post-fixed for 1 h in Image-iT™ Fixative Solution. The retinas were then washed with 0.5% Triton-100 in PBS for 30 min, followed by incubation for 3 days at 4 °C in a solution containing rabbit anti-gamma synuclein (1:250 dilution), mouse anti β-III tubulin (1:500 dilution), 1% Triton-100, and 1% bovine serum albumin in PBS. The retinas were then washed three times with 0.5% Triton-100 in PBS, and further incubated overnight at 4 °C in a solution containing goat anti-mouse IgG H&L Alexa Fluor 647 (1:1000), goat anti-rabbit IgG (H + L) Alexa Fluor 568 (1:1000), 1% Triton-100, and 1% bovine serum albumin in PBS. The retinas were again washed three times and incubated overnight in DAPI (1:1000 dilution) in PBS. The resulting retinal wholemount was then mounted on a slide using Fluoromount-G. Prepared retinas were imaged with a confocal LSM 710 microscope (Carl Zeiss, White Plains, NY, USA). For each retinal wholemount, 12 images were taken from the region 2–3 mm from the optic nerve (3 images per each of four retinal quadrants) at 40X magnification. Confocal images were viewed in ImageJ (National Institutes of Health) without modification of contrast or brightness. Retinal ganglion cells (RGCs) were identified by co-staining with DAPI and β-III tubulin. Investigators were masked as to the treatment when processing and imaging the tissues. RGCs were manually counted in a masked fashion by two independent observers. A third person was randomly assigned to count 9 out of the 12 images per retina flatmount to ensure consistency of counting results. If the count by the third observer was different than the average of the first two counters by more than ± 10%, the images were recounted by all three observers before being averaged. For each animal, the number of RGCs was normalized to the healthy contralateral eye and reported as a percentage.

qPCR assay

RNA was isolated from retina tissues using the Purelink RNA Mini Kit. cDNA was made using SuperScript IV VILO Master Mix. Quantitative real-time polymerase chain reaction (qPCR) was run on a QuantStudio 3 real-time PCR system (Waltham, MA, USA) using TaqMan Fast Advanced Master Mix. Each reaction was performed in triplicate for each primer with a no-template negative control. QuantSudio Design and Analysis Software were used to analyze the data using the $\Delta\Delta C_T$ method.

Topical dosing safety study

Histological evaluation was performed as previously described [3]. In brief, the Brown Norway rats were dosed unilaterally with SunitiGel once every 7 days for 21 days (total 4 topical doses) or with saline (untreated). One day later, the eyes were enucleated and placed in 4% paraformaldehyde before paraffin embedding, sectioning, and hematoxylin and eosin staining by the Johns Hopkins Reference Histology Laboratory. Histological sections were evaluated by a board-certified ophthalmic pathologist in a blinded manner for the signs of inflammation and ocular surface damage.

Statistical methods

Statistical analyses of two groups were conducted using two-tailed Student's *t* test. Differences were considered to be statistically significant at a level of $p < 0.05$. Statistical analysis was done using GraphPad Prism 8. Grubb's test (aka extreme studentized deviate method) was applied to remove outliers with significance level $\alpha = 0.05$.

Results

Melanin production increases sunitinib concentrations in cells in vitro and in ocular tissues in vivo

The binding of sunitinib and its primary active metabolite, *N*-desethyl sunitinib [17] to purified melanin was characterized in vitro by mixing varying amounts of drug with melanin and measuring the unbound amount of drug in the supernatant. This allowed for calculating the binding capacity and dissociation constant (K_d). As shown in Fig. 1a, the measured binding capacity of sunitinib and *N*-desethyl sunitinib (open circles) was much higher than that reported for other drugs in the literature (closed circles) [16, 18–21]. The dissociation constants were also lower than most drugs, indicating high binding strength (Fig. 1a). However, melanin is contained within melanosomes within the cell, so drug must also be taken up into cells to bind to melanin. Thus, we then cultured RPE cells to investigate drug uptake and binding to melanin within cells. When cultured in standard media, ARPE-19 cells

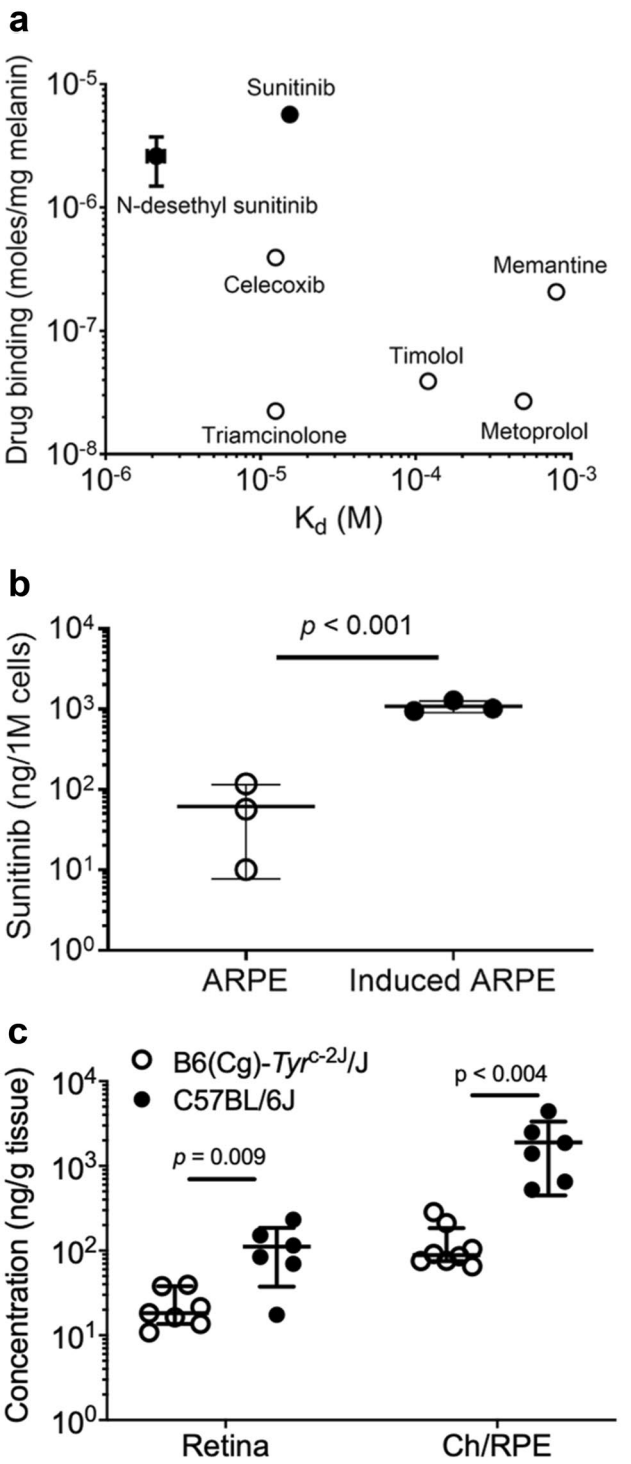

Fig. 1 **a** Sunitinib and *N*-desethyl sunitinib (closed circles) show high melanin binding, both in terms of capacity and binding strength (low dissociation constant, K_d), compared to values reported in the literature for other drugs (open circles [14, 16–19]). Measured values (closed circles) are shown as mean ± SD, $n = 3$. **b** ARPE-19 cells were cultured under normal conditions (ARPE) or under conditions that induce melanin production (induced ARPE) and incubated with sunitinib for 6 h. The cells were then collected and washed prior to extracting sunitinib. Data are shown as mean ± SD, $n = 3$. **c** C57BL/6J and tyrosinase knockout mice on the C57BL/6J background (B6(Cg)-*Tyr*$^{c-2J}$/J) were dosed unilaterally with SunitiGel once daily for 4 days. Retina and Ch/RPE were collected 24 h after the last dose ($n = 6$–8). Combined levels of sunitinib and *N*-desethyl sunitinib are shown as mean ± SD

do not produce much melanin. Melanin production can be stimulated by culturing in substrate-enriched media for an extended period of time [22]. Melanin production in ARPE-19 cells was confirmed by tyrosinase-related protein 1 (TYRP1) staining, brightfield microscopy, and light absorption (Supplementary Fig. S1). When non-induced (ARPE) and induced (induced ARPE) cells were incubated with sunitinib for 6 h, and the induced cells contained ~ 17-fold more sunitinib than the non-induced cells (Fig. 1b). We then wanted to make sure that drug bound to melanin in the choroidal melanocytes and RPE would be able to reach the cells in the retina, which is particularly important for the RGCs on the innermost side. To do this, we injected sunitinib in methylcellulose into the suprachoroidal space adjacent to the choroid [23]. Sunitinib is rapidly converted to *N*-desethyl sunitinib in vivo, which was previously shown to have similar activity to sunitinib in inhibiting VEGFR [24]. Here, we employed our RGC survival assay [6] and found that *N*-desethyl sunitinib also had similar activity to sunitinib in promoting primary mouse (Supplementary Fig. S2a) and human stem cell–derived (Supplementary Fig. S2b) RGC survival. Thus, for all drug measurements, combined concentrations of sunitinib and *N*-desethyl sunitinib are shown. As shown in Supplementary Fig. S3, combined drug levels in the Ch/RPE and retina of pigmented Brown Norway rats were significantly higher than of albino Wistar rats. To ensure that the increased drug levels in the retina of pigmented rats were not due to incomplete separation of the Ch/RPE, we measured the expression of retinal pigment epithelium-specific 65 kDa protein (RPE65) in separated retina and retina still attached to the Ch/RPE. The expression of RPE65 in the retina was < 3% of the retina/Ch/RPE, suggesting that the tissues were effectively separated (Supplementary Fig. S4). In the case of topically dosed sunitinib, drug must traverse various additional ocular tissues to reach the pigmented cells in the uveal tract and RPE. We also wanted to compare the effect of pigmentation on drug distribution in animals with the same genetic background. Thus, we dosed SunitiGel once daily for 4 days in pigmented mice (C57BL/6J) and isogenic albino tyrosinase knockout mice with the same genetic background (B6(Cg)-*Tyr*$^{c-2J}$/J) to characterize differences in ocular drug distribution.

As shown in Fig. 1c, the combined drug concentrations in the Ch/RPE and retina of pigmented mice were significantly higher (~ 15-fold and 5-fold, respectively) than the albino mice when collected 1 day after the last SunitiGel dose.

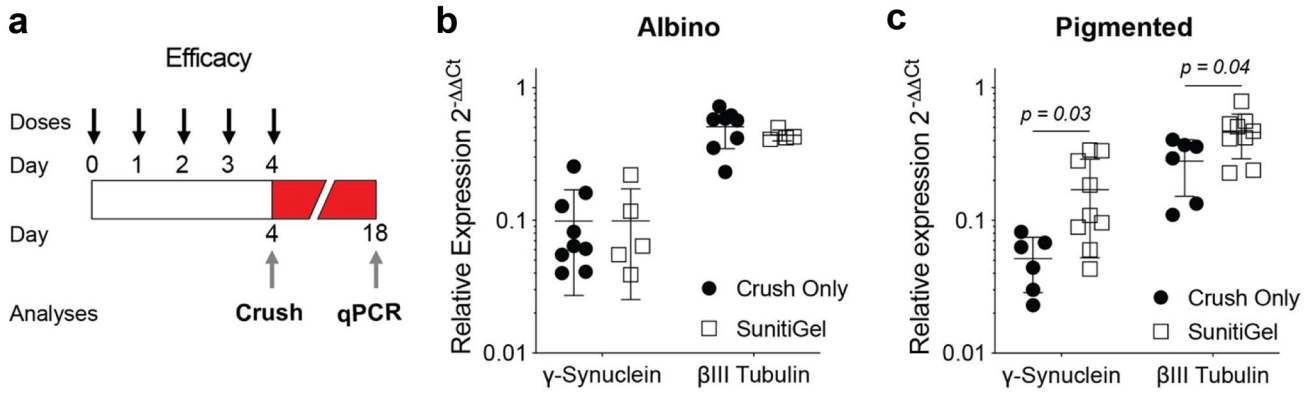

Fig. 2 **a** Schematic showing once daily treatment with SunitiGel for a total of 5 topical doses (arrows) prior to optic nerve crush (day 4). Tissues were collected 14 days later (day 18) to perform qPCR to assess expression of γ-synuclein and βIII-tubulin. **b** No differences in gene expression were observed in albino Wistar rats ($n = 4$–9) whether treatment was (SunitiGel) or was not (crush only) given prior to the optic nerve crush. **c** Increased gene expression was observed in pigmented Brown Norway rats ($n = 6$–9) that received SunitiGel treatment prior to optic nerve crush. Data are shown as mean ± SD

SunitiGel provides sustained protection of RGCs in an optic nerve crush model

To determine whether SunitiGel would provide protection of RGCs in vivo, once daily dosing for a total of 5 doses was performed prior to the optic nerve crush procedure in rats. Two weeks later, expression of key genes known to be downregulated in injured RGCs (γ-synuclein and βIII-tubulin [25–27]) was characterized (Fig. 2a). In albino Wistar rats, treatment with SunitiGel had no effect on gene expression compared to no treatment prior to crush (crush only) (Fig. 2b). In contrast, in pigmented Brown

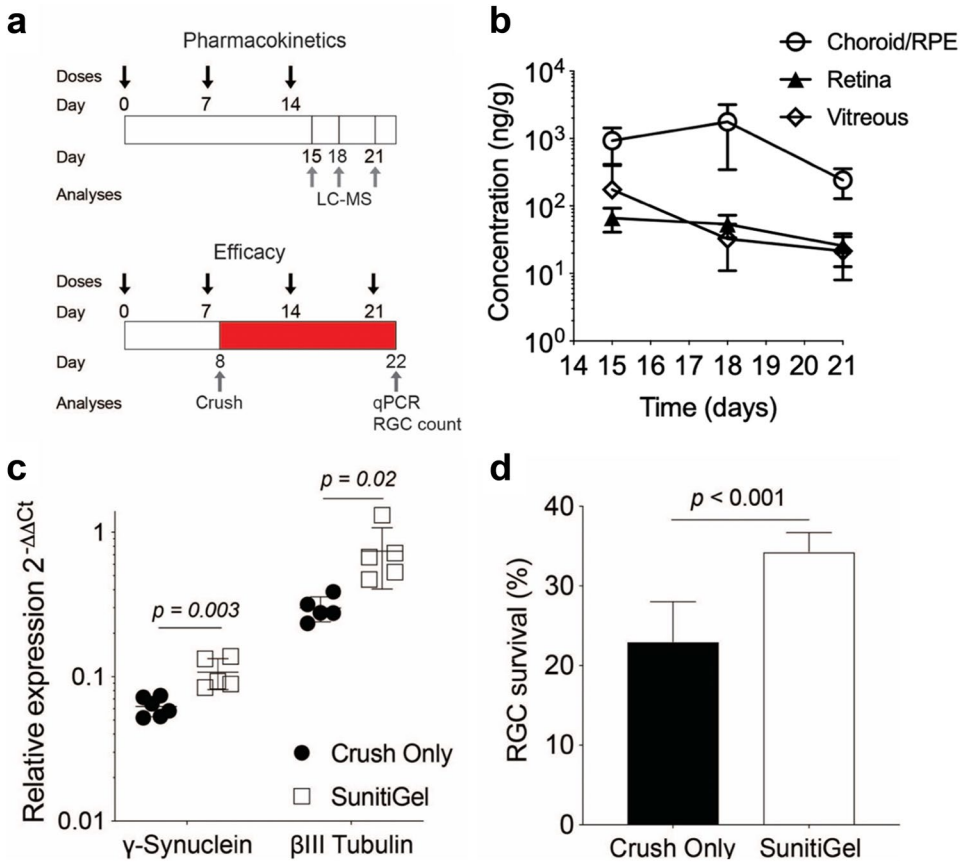

Fig. 3 **a** Schematics showing the timeline for pharmacokinetic and efficacy studies in pigmented Brown Norway rats with once weekly dosing (arrows) of SunitiGel. A total of three doses were given in the pharmacokinetic study, and tissues were collected at 1, 4, and 7 days after the last dose. For efficacy experiments, optic nerve crush occurred 1 day after the second dose, and tissues were collected for qPCR and RGC counting 1 day after the fourth dose. **b** Combined levels of sunitinib and *N*-desethyl sunitinib in the retina, Ch/RPE, and vitreous ($n = 5$–6) over time after the third weekly dose. Data are shown as mean ± SD. **c** Increased gene expression level of γ-synuclein and βIII-tubulin was observed in rats ($n = 4$—5) receiving SunitiGel treatment compared to crush only. Data are shown as mean ± SD. **d** RGC counting in whole retinal flat mounts ($n = 11$–12) confirms increased survival with SunitiGel treatment. Data are shown as mean ± SD

Norway rats, the SunitiGel treatment prior to optic nerve crush led to a significant increase in both γ-synuclein (~ 3.3-fold) and βIII-tubulin (~ 1.7-fold) expression (Fig. 2c).

Efficacy in the pigmented rats suggested that the presence of melanin in the eye was important for delivering therapeutic concentrations of sunitinib to the RGCs, and the timeline of 2 weeks after the crush without additional treatment suggests a sustained delivery effect facilitated by melanin binding. Thus, we next tested a treatment schedule of once weekly SunitiGel dosing, where both intraocular drug levels and efficacy in pigmented rats were assessed (Fig. 3a). For drug measurements, ocular tissues were collected at 1, 4, and 7 days after the third weekly dose. The combined drug concentration remained relatively high in the Ch/RPE (242.2 ng/g) and retina (25.6 ng/g) for up to 7 days after the last dose (Fig. 3b). We then performed the optic nerve crush 1 day after a second weekly dose, with two additional weekly doses prior to collecting tissues to assess gene expression (Fig. 3a). The weekly SunitiGel treatment provided significantly increased gene expression after optic nerve crush (Fig. 3c). In contrast, weekly treatment of sunitinib in saline as a vehicle (Sunit) had no effect on gene expression levels compared to no treatment (crush only) (Supplementary Fig. 5), confirming that achieving increased intraocular sunitinib absorption was required to take advantage of the melanin binding effect. To confirm that the increase in gene expression with SunitiGel was due to increased RGC survival, we then repeated the SunitiGel treatment study with whole retinal flatmounts to stain and count RGCs. As shown in Fig. 3d, the once weekly SunitiGel treatment led to a significant increase in RGC survival (34%) compared to crush only (23%).

Once weekly dosing of SunitiGel has no discernible toxicity to the cornea or retina in healthy pigmented rats

We previously observed that twice daily treatment with the gel-forming vehicle for 5 weeks in rabbits was safe and well-tolerated [3]. And while it was previously shown that binding of drugs to melanin was not inherently toxic [28], we wanted to determine whether accumulation of sunitinib in pigmented cells would cause ocular toxicity. Pigmented rats were treated once weekly for a total of four doses of SunitiGel. One day later, ocular tissues were obtained for histological staining. The cornea and retina of rats treated with SunitiGel were indistinguishable from that of untreated rats (Fig. 4).

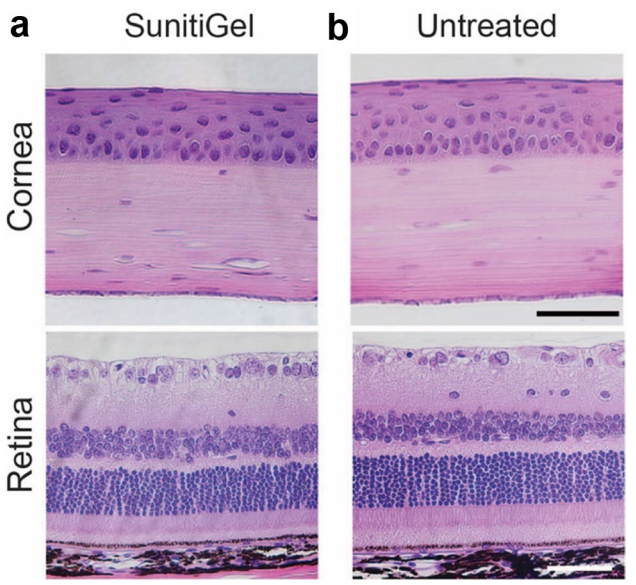

Fig. 4 Brown Norway rats were dosed unilaterally with **a** SunitiGel once every 7 days for 21 days (total 4 topical doses) or **b** untreated. One day later, the eyes ($n = 3$) were enucleated for sectioning and staining. Hematoxylin and eosin stained sections of the cornea (top) and retina (bottom) were indistinguishable between groups. Scale bar = 50 µm

Discussion

While eye drops are noninvasive and relatively simple for patients to use, there is room for improvement in their design and performance. This is particularly true for therapeutic applications that require delivery to the posterior segment, which is typically achieved through intraocular injections. Efforts toward developing eye drops for delivering drugs to the posterior segment have historically been unsuccessful, even when dosing 4–6 times per day (ClinicalTrials.gov Identifier: NCT00509548, NCT02355028) [2, 29–31]. Indeed, we previously found that daily dosing of sunitinib malate in saline was ineffective in preventing laser-induced CNV in both mice and pigs, whereas daily dosing of SunitiGel reduced CNV spot size by about half in pigs [3]. Similarly here, treatment with sunitinib malate in the form of SunitiGel was effective in protecting RGCs in the rat optic nerve crush model, whereas dosing sunitinib malate in saline was not. The gel-forming eye drop vehicle is formulated to be hypotonic and below the polymer critical gel concentration, which prevents premature gelation at high room temperature, and allows for spreading over the ocular surface prior to gelation [3]. The thin, uniform, clear gel layer resists clearance from blinking and holds drug against the surface of the eye for longer periods, increasing intraocular absorption [3]. As we demonstrate here, using the hypotonic

gel-forming vehicle to deliver a drug with high melanin binding capacity leads to accumulation of drug and prolonged therapeutic drug levels in the eye, which with once weekly dosing provided protection of RGCs in a rat optic nerve crush model. Whereas many eye drops used for IOP lowering in glaucoma treatment require multiple drops per day, leading to adherence problems [32, 33], such a dose-sparing approach for neuroprotection could be convenient for patients and improve glaucoma management [34]. Patient compliance to treatment regimens is particularly important for chronic diseases like glaucoma, which can often require treatment for decades [35, 36].

Melanin is a macromolecule localized within melanosomes in cells in the iris, ciliary body, choroid, and RPE [37, 38]. The primary function of melanin in the eye is to absorb incoming light to protect ocular tissues and improve image quality [10, 39]. Ocular melanin content appears to vary more as a function of eye color in the uveal melanocytes than in the pigmented epithelia of the iris, retina, and ciliary body [40]. It has been known for decades that some drugs can bind to melanin in the eye, which can affect their biodistribution and activity [41–43]. However, it is difficult to predict whether binding to melanin will be beneficial or detrimental to the intended pharmacologic effect. In the case of the mydriatic drug atropine, binding to the pigment in the iris was described to significantly prolong pupil dilation [12, 44]. Alternatively, in the case of brimonidine, high binding to the pigmented iris-ciliary body resulted in a larger IOP-lowering response at early times, but not a sustained IOP-lowering effect [45, 46]. Many other ocular hypotensive drugs, including timolol, pilocarpine, and epinephrine, have been described to be less effective in more highly pigmented eyes because of the reduction in freely available drug [47, 48]. It was described that binding of celecoxib to melanin in the choroid/RPE reduced delivery to the retina and vitreous via transscleral penetration in pigmented rats [16]. Intravitreal injection of polymeric microparticles that released sunitinib for about 2 months in vitro prevented neovascularization in multiple mouse models and led to persistent drug levels in pigmented rabbits for at least 6 months due to melanin binding [49]. However, drug binding in the choroid/RPE does not guarantee that drug would also be able to effectively reach the RGCs on the other side of the retina to provide protection against injury. Here, we demonstrated that daily topical dosing of SunitiGel not only provided increased intraocular penetration of sunitinib malate compared to a standard liquid saline vehicle, but binding to melanin in the eye provided prolonged therapeutic protection of RGCs with even once weekly dosing of SunitiGel.

Medication adherence is an essential issue for the success of the treatment of chronic diseases, and it has been shown that adherence improves when the required frequency of taking medication decreases [50–53]. For example, medication adherence and persistence were increased with a once weekly treatment compared to once daily treatment of osteoporosis and type 2 diabetes [54–57]. Moreover, patients taking a once weekly medication for type 2 diabetes reported increased quality of life and treatment satisfaction compared with patients taking once daily medication [58, 59]. Depending on the type, IOP-lowering eye drops must be used 1–3 times per day, where adherence has been reported as low as 39% [1]. For this reason, numerous strategies have already been developed for increasing adherence to glaucoma treatments. A comprehensive smartphone-based and tablet-based glaucoma application has been developed that includes eye drop reminders, a visual field tutorial, medical and ocular data storage, and educational videos [60]. Such apps could be useful for reminding patients to also use a weekly eye drop for neuroprotection in addition to their daily IOP-lowering drops.

The studies described here are not without limitations. First, while we have previously demonstrated that the SunitiGel can provide therapeutically relevant delivery of sunitinib to the posterior segment in both rabbits and pigs with daily dosing, it would be valuable to confirm that prolonged drug levels allow for once weekly dosing in these larger species as well. Among other species-related differences in ocular structure, intraocular distribution of melanin has also been shown to be species dependent [9]. The safety data shown here is also promising, though longer-term chronic dosing in rabbits would be a valuable next step for evaluating intraocular safety of drug accumulation in melanin-producing cells. It was previously described that drug binding to melanin is not predictive of ocular toxicity [28]. It is also encouraging that no safety concerns were observed in a Phase 1/2a clinical trial of an intravitreal sustained delivery system for sunitinib malate [61]. From a disease perspective, it would also be worthwhile to confirm RGC protection with once weekly dosing in a glaucoma model involving IOP elevation, which was previously demonstrated with a sustained delivery system delivering the protein kinase inhibitor tozasertib, which like sunitinib also has inhibitory activity against DLK/LZK [6]. Further, prior reports described some regional variability in melanin production in some species [9], and it would be valuable to determine whether this leads to spatial variation in protection of RGCs by melanin-binding drugs like sunitinib.

The approach described here has high potential for clinical translation. Oral sunitinib malate (Sutent®) is already FDA-approved for treatment of some cancers, and importantly, a formulation that provides sustained intraocular delivery of sunitinib is in phase 2 clinical trials for neovascular age-related macular degeneration [62] and macular edema (ClinicalTrials.gov Identifier: NCT04085341).

Further, Pluronic F127 (also known as Poloxamer 407), a key component of SunitiGel, is already a component of several approved eye drop products, though typically at concentrations less than 2% (w/w). Safety studies will likely be required with higher polymer concentrations dosed as an eye drop. However, the history of use of these components in the eye is likely to be beneficial when planning the scope of Investigational New Drug (IND)-enabling studies as a next step in development. A safe and efficacious once weekly eye drop for neuroprotection in glaucoma would likely have a significant impact in helping to maintain the vision of patients with glaucoma.

Conclusions

Here, we described a translational approach for improved topical ocular delivery of a small-molecule protein kinase inhibitor for protection of retinal ganglion cells (RGCs). Enhanced delivery to the posterior segment provided by the gel-forming eye drop vehicle allowed for sunitinib binding to melanin in the pigmented choroid/retinal pigment epithelial cells, which provided sustained drug levels in the non-pigmented retina. Together, this allowed for increased survival of RGCs after optic nerve crush with only once weekly eye drop dosing. Importantly, once weekly dosing in healthy rats showed no signs of corneal or retinal toxicity. A once weekly neuroprotective eye drop treatment has the potential to improve the management of glaucoma.

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Author contribution Conceptualization: L.M.E, J.H., D.J.Z., Y.C.K. Data collection and analysis: H.T.H., M.D.S., C.A.B., H.H., N.M.A., A.H., K.T.L., R.T.C., H.K., M.B.A., U.R., P.K., C.E., I.P. Writing and editing: L.M.E., Y.C.K., H.T.H., N.M.A., I.P., D.J.Z. All the authors read and approved of the final version of the manuscript.

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Availability of data and materials The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate All institutional and national guidelines for the care and use of laboratory animals were followed.

Consent for publication All authors give consent for publication.

Competing interests Y.C.K., L.M.E., H.T.H., and J.H. are inventors on patents/patent applications related to this technology. J.H. and L.M.E. are co-founders of a start-up company planning to develop the eye drop technology for clinical use. DJZ and CAB are inventors on patents related to sunitinib and neuroprotection. Some of these patents have been licensed to Graybug Vision and to Oriole Therapeutics. J.H. is a co-founder and equity owner of Graybug Vision, and both J.H. and Johns Hopkins own company stock. DJZ is a co-founder and equity owner in Oriole Therapeutics. These arrangements have been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies. All other authors declare that they have no competing interests.

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