Sustained treatment of retinal vascular diseases with self-aggregating sunitinib microparticles

Hiroki Tsujinaka\textsuperscript{1,4}, Jie Fu\textsuperscript{1,4}, Jikui Shen\textsuperscript{1,4}, Yun Yu\textsuperscript{2,4}, Zibran Hafiz\textsuperscript{1}, Joshua Kays\textsuperscript{2}, David McKenzie\textsuperscript{2}, Delia Cardona\textsuperscript{2}, David Culp\textsuperscript{3}, Ward Peterson\textsuperscript{2}, Brian C. Gilger\textsuperscript{3}, Christopher S. Crean\textsuperscript{2}, Jin-Zhong Zhang\textsuperscript{2}, Yogita Kanan\textsuperscript{1}, Weiling Yu\textsuperscript{2}, Jeffrey L. Cleland\textsuperscript{2}, Ming Yang\textsuperscript{2}*, Justin Hanes\textsuperscript{1}* & Peter A. Campochiaro\textsuperscript{1}*

Neovascular age-related macular degeneration and diabetic retinopathy are prevalent causes of vision loss requiring frequent intravitreous injections of VEGF-neutralizing proteins, and under-treatment is common and problematic. Here we report incorporation of sunitinib, a tyrosine kinase inhibitor that blocks VEGF receptors, into a non-inflammatory biodegradable polymer to generate sunitinib microparticles specially formulated to self-aggregate into a depot. A single intravitreous injection of sunitinib microparticles potently suppresses choroidal neovascularization in mice for six months and in another model, blocks VEGF-induced leukostasis and retinal nonperfusion, which are associated with diabetic retinopathy progression. After intravitreous injection in rabbits, sunitinib microparticles self-aggregate into a depot that remains localized and maintains therapeutic levels of sunitinib in retinal pigmented epithelium/choroid and retina for more than six months. There is no intraocular inflammation or retinal toxicity. Intravitreous injection of sunitinib microparticles provides a promising approach to achieve sustained suppression of VEGF signaling and improve outcomes in patients with retinal vascular diseases.
age-related macular degeneration (AMD) is a highly prevalent cause of vision loss\(^1\). It is a neurodegenerative disease characterized by deposits called drusen, diffuse thickening of Bruch’s membrane beneath the retinal pigmented epithelium (RPE), and slow death of photoreceptors, RPE, and choriocapillaris beneath the macula resulting in gradual loss of central vision. A subgroup of AMD patients develop choroidal neovascularization (NV) in which new vessels originate in the choroid and extend under the RPE (type 1 choroidal NV) or through the RPE into the subretinal space (type 2), or new vessels originate from the deep capillary bed of the retina and grow through the outer nuclear layer (ONL) containing the nuclei of the photoreceptors into the subretinal space (type 3). Patients with any of these types of choroidal NV are said to have neovascular AMD (NVAMD) and often experience rapid reduction in vision due to leakage of plasma from NV causing fluid to collect under or within the macula, but the distinction is important because patients with type 3 choroidal NV carry a high risk of retinal atrophy, another source of visual loss\(^2\). The risk of developing NVAMD increases with advancing age and the severity of drusen and/or pigmentary changes in the macula; in the Age Related Eye Disease Study, the risk of developing NVAMD within 10 years was 48% for the oldest participants with the most extensive drusen and pigmentary changes\(^3\). Once NVAMD develops in one eye, there is a high risk for its development in the fellow eye. Studies in models of type 2 or type 3 choroidal NV like that seen in NVAMD implicated vascular endothelial growth factor (VEGF) as a critical stimulator\(^4\). Clinical trials showed that monthly intravitreous injections of the VEGF antagonist ranibizumab provided substantial improvement and stabilization of vision in patients with NVAMD\(^7,8\). However, in an extension study in which visits and injections were only required every 3 months, most of the visual gains obtained during 1 year of frequent injections were lost within a year\(^9\). A large multicenter clinical trial comparing monthly injections of ranibizumab or bevacizumab to injections only when intraretinal or subretinal fluid was present showed only a small decrease in overall number of injections in the latter groups over the course of 2 years and mean improvements in visual acuity were significantly better in patients receiving monthly injections\(^10\). Patients were returned to standard care and over the next 3 years the mean number of injections per year was reduced and the mean best-corrected visual acuity in each of the 4 groups declined to a level worse than baseline (loss of 3.3 letters from baseline)\(^11\). In contrast, a study in which patients with NVAMD were treated aggressively and given an average of 10.4 injections per year for 5 years, there was a large mean improvement of 14.1 letters\(^12\). In clinical practice, the mean number of intravitreous injections of a VEGF-neutralizing protein per year is substantially less than that in clinical trials and visual outcomes are much worse\(^13\). These data indicate that sustained suppression of VEGF provides the best visual outcomes in patients with NVAMD. Diabetic retinopathy and retinal vein occlusion are ischemic retinopathies that are prevalent causes of vision loss in working age individuals\(^14,15\). The most common cause of vision loss in each is macular edema due to leakage from retinal blood vessels\(^16–18\). Pilot trials demonstrated that intraocular injections of ranibizumab provided substantial reduction of intraretinal fluid in the macula and improvement in vision\(^19–21\). These findings were confirmed in phase 3 studies that led to FDA approval of ranibizumab for treatment of diabetic macular edema and retinal vein occlusion\(^22–24\). Follow-up studies showed that suppression of VEGF reduced progression of retinal vessel closure and caused improvement in perfusion in some patients with retinal vein occlusion or diabetic macular edema (DME)\(^25,26\), and improved background diabetic retinopathy in patients with DME\(^27\). These two effects of VEGF suppression are related because slowly progressive closure of retinal vessels is strongly associated with progression of background diabetic retinopathy\(^28\). Thus, increased VEGF in the retina drives disease progression of diabetic retinopathy and retinal vein occlusion and sustained suppression of VEGF can halt progression and cause improvement. We have developed an approach through which sustained suppression of VEGF signaling is achieved. We have incorporated sunitinib, a small molecule inhibitor of VEGF receptors and other tyrosine kinases that has been approved as an oral agent for treatment of renal cell carcinoma\(^29,30\), into microparticles (MPs) composed of blends of poly(lactic-co-glycolic acid) (PLGA) and PLGA conjugated to polyethylene glycol (PLGA-PEG). A different polymer MP formulation and manufacturing process is key to achieving three critical goals: (1) sustained release of effective levels of sunitinib for many months, (2) elimination of ocular inflammation typically observed following intravitreous injection of PLGA MPs, and (3) intraocular injection triggered self-aggregation of MPs into a depot near the site of injection that reduces dispersion of MPs into the visual axis. Here, we report the efficacy and pharmacokinetics of sunitinib MPs after intravitreous or subconjunctival injection in animal models of retinal and choroidal vascular diseases.

**Results**

**Formulation and characterization of sunitinib MPs.** Our previous work showed that a dense PEG coating on polymer MPs is critical for minimizing the inflammation associated with intravitreous injection of MPs without a PEG coating\(^31\) therefore, sunitinib MPs were produced using a blend of PLGA-PEG and PLGA polymers to improve biocompatibility with ocular tissues. We next sought to improve the drug loading of sunitinib MPs to ensure that an adequate amount of sunitinib is delivered to provide therapeutic levels for several months by a single intravitreous injection with volume ≤50 µl. Because sunitinib has high solubility in acidic aqueous solutions (pH 1.2–6.8) and the solubility rapidly decreases at pH greater than 6.8, a relatively large volume of aqueous solution of pH 7.4 was used as the continuous phase, into which an organic solution containing dissolved sunitinib and polymers was emulsified to produce sunitinib MPs\(^32\). Due to the low aqueous solubility of sunitinib at pH 7.4, drug loss from the organic phase during MP production was minimized, thus resulting in high drug loading of over 10% (w/w) in MPs\(^33\). As shown in Fig. 1a, sunitinib was released from MPs in a sustained manner for over 2 months with minimal burst under sink conditions in vitro. The MPs designed for mouse experiments were smaller (13 ± 6 µm) than those designed for rabbit and minipig experiments (32 ± 9 µm), and they had a slightly faster release profile, but in the same range (Fig. 1b, closed squares). In contrast to sunitinib MPs, sunitinib drug suspension (Fig. 1b, open squares) was rapidly eluted and dissolved in release medium in vitro. The drug loading of the sunitinib MPs used in mice and rabbits was 10.6% and 12.0% (w/w), respectively.

Dispersion of MPs throughout the eye can block the visual axis and interfere with vision; therefore, MPs were modified to promote aggregation following intravitreous injection. As shown in Fig. 1c, following an injection of 50 µl sunitinib MP suspension and incubation at 37 °C for 2 h, a solid depot formed in a hyaluronate solution that could be grasped and isolated with forceps (Fig. 1c). One day after intravitreous injection of 50 µl of sunitinib MP suspension though a 27 g needle into the inferior vitreous of a rabbit, the aggregate of MPs could not be seen within the eye in primary position with a 30° fundus camera; however, with a contact lens on the eye which is grasped with a forceps and
achieved a much more sustained release over time. While MPs containing an equivalent amount of sunitinib (closed squares) was rapidly eluted and dissolved in release medium in vitro, a pair of forceps leads to the formation of a solid depot that can be isolated using a 50 µl sunitinib MP suspension in a hyaluronate solution at 37 °C.

**Sunitinib MPs suppress type 2 murine choroidal NV.** The murine model of laser-induced rupture of Bruch’s membrane results in choroidal NV that is similar to type 2 choroidal NV in patients with NVAMD, because the new vessels originate from the choroid and penetrate through Bruch’s membrane and the RPE into the subretinal space. Studies in this model helped to implicate VEGF as a critical stimulus for NVAMD and predicted the clinical benefits seen with aflibercept, a recombinant VEGF-neutralizing protein commonly used to treat patients with NVAMD. In order to assess the efficacy of sunitinib MPs over time, C57BL/6 mice were given an intravitreous injection of MPs containing 10 or 1 µg sunitinib in one eye and empty MPs in the fellow eye and then had laser-induced rupture of Bruch’s membrane at 3 locations in each eye at time points ranging from 1 to 24 weeks after injection. Compared with empty MP fellow eye controls, the mean area of choroidal NV at Bruch’s membrane ruptures sites was significantly less in eyes injected with MPs containing 10 µg sunitinib at each time point through week 24.

**Subconjunctival sunitinib MPs suppress type 2 choroidal NV.** Subconjunctival injection is painless and allows injections of larger volumes. Mice had a 2 µl subconjunctival injection of MPs containing 20 or 2 µg sunitinib or empty MPs in one eye and no injection in the fellow eye. After 1 week, Bruch’s membrane was ruptured in 3 locations in each eye; the area of choroidal NV at Bruch’s membrane rupture sites was significantly less in eyes injected with MPs containing 1 µg sunitinib compared with fellow eye controls at 9 and 15 weeks after injection, but not 20 or 24 weeks after injection.

**Sunitinib MPs suppress murine type 3 choroidal NV.** Approximately, 30% of patients with NVAMD have type 3 choroidal NV (previously called retinal angiomatous proliferation) in which new vessels originate from the deep capillary bed of the retina, grow through the photoreceptor layer, and form networks of NV in the subretinal space. Transgenic mice in which the
rhodopsin promoter drives expression of VEGF in photoreceptors (rho/VEGF mice) provide a model of type 3 choroidal NV, which along with mice with type 2 choroidal NV was used to first demonstrate the efficacy of aflibercept\textsuperscript{6}. At postnatal day (P) 14, one eye of rho/VEGF mice was injected with MPs containing 10 µg sunitinib and the fellow eye was injected with an equivalent mass of empty MPs and, at weekly intervals, mice were euthanized and retinas were stained with *Griffonia simplicifolia* agglutinin (GSA) lectin and flat mounted with the photoreceptor side up showing dark green tufts of NV on the outer surface of the retina (subretinal space). Some of the tufts are surrounded by RPE (black pigment) and feeder vessels, portions of which are out-of-focus, because they extend from the deep capillary bed to the plane of focus, the outer surface of the retina. Compared with eyes injected with empty MPs, fellow eyes injected with MPs containing 10 µg sunitinib showed fewer tufts of subretinal NV by visual inspection and significantly less mean area of NV per retina at P21, P28, P35, and P42 (Fig. 4a). Eyes injected with 40 µg of aflibercept had fewer tufts of NV and significantly less mean area of NV per retina than control fellow eyes injected with PBS at P21, but not P28, P35, or P42 (Fig. 4b).

In clinical care, anti-VEGF agents are injected in eyes with established choroidal NV, and their major effect is to reduce leakage from the NV thereby reducing fluid in the retina, and...
improving vision. Fluorescein angiograms of rho/VEGF mice at P28, when there is extensive subretinal NV, does not have sufficient resolution to show individual buds of NV, but shows collections of extravascular fluid scattered throughout the retina, particularly in the posterior pole (Fig. 4c, top row). One week after intravitreous injection of MPs containing 10 µg sunitinib or an equivalent mass of empty MPs, sunitinib MP-injected eyes showed a marked reduction in extravascular fluorescein, while empty MP-injected eyes did not (Fig. 4c, bottom row). Fluorescein angiography provides qualitative assessment of leakage that is relevant to what is done in clinical practice, but it is not quantitative; however, measurement of serum albumin provides another assessment of retinal vascular leakage. There is no significant difference between fellow eyes for each group. *p < 0.05 for difference from all other groups by Kruskal Wallis test followed by Dunn’s test. Bar = 100 µm. Source data are provided as a Source Data file.

**Fig. 3 Subconjunctival injection of sunitinib MPs suppresses type 2 choroidal NV.** C57BL/6 mice received a subconjunctival injection of microparticles (MP) containing 20 or 2 µg of sunitinib (Suni) or empty MP in one eye and no injection in the fellow eye. At 1 week after injection, Bruch’s membrane was ruptured by laser photocoagulation at three locations in each eye. One week after laser, mice were euthanized and choroidal flat mounts were stained with FITC-labeled GSA lectin and the area of choroidal neovascularization (CNV) was measured at Bruch’s membrane rupture sites by an investigator masked with regard to treatment. The three values from each eye were averaged to give a single experimental value. The mean (±SEM) area of CNV was significantly less in eyes injected with MP containing 20 µg (a) or 2 µg (b) sunitinib vs. those injected with empty MP, but there was no significant difference between fellow eyes for each group. *p < 0.05 for difference from all other groups by Kruskal Wallis test followed by Dunn’s test.

**Sunitinib MPs reduce photoreceptor death in eyes with type 3 choroidal NV.** The incidence of macular atrophy is high in patients with type 3 choroidal NV treated with anti-VEGF agents. Measurement of ONL which contains the nuclei of photoreceptors provides an assessment of photoreceptor survival commonly used in models of inherited retinal degeneration. Rhodopsin kinase is an enzyme involved in phototransduction and its levels in retinal homogenates correlates with the number of healthy photoreceptors. In rho/VEGF mice with type 3 choroidal NV, photoreceptor cell death is accompanied by thinning of the ONL and reduction in retinal levels of rhodopsin kinase, which are substantially prevented by the potent antioxidant N-acetylcysteine, but are neither exacerbated nor improved by VEGF suppression. This indicates that oxidative damage is a major contributor to photoreceptor cell death that occurs in eyes with type 3 choroidal NV. At P14, rho/VEGF mice were given an intravitreous injection of MPs containing 10 µg sunitinib in one eye and empty microparticles in the fellow eye or 40 µg of aflibercept in one eye and PBS in the fellow eye. At P42, mean ONL thickness was significantly greater in sunitinib MP-injected eyes compared with empty MP-injected eyes at 3 of 6 measurement locations along the vertical meridian of the retina through the optic nerve, but there was no significant difference between aflibercept-injected and PBS-injected eyes (Fig. 5a, b). At P49,
immunoblots of retinal homogenates from sunitinib MP-injected eyes had significantly greater rhodopsin kinase/actin ratio compared with those from empty MP-injected eyes, but there was no difference between aflibercept- and PBS-injected eyes (Fig. 5c, d).

**Sunitinib MPs reduce VEGF-induced leukostasis/ nonperfusion.** VEGF plays a critical role in the pathogenesis of diabetic retinopathy as well as NVAMD. Increased levels of VEGF in the retina cause leukostasis and closure of retinal vessels exacerbating retinal hypoxia, which is strongly associated with progression of diabetic retinopathy. Adult C57BL/6 mice were given an intravitreous injection of 200 ng of VEGF in each eye one week after injection of MP containing 10 µg sunitinib or 40 µg of aflibercept in one eye and an equivalent mass of empty MPs or PBS in the fellow eye. Twenty-four hour later, mice were perfused with PBS through the left ventricle to remove all non-adherent erythrocytes and leukocytes, and then perfused with conconavalin A to stain remaining adherent leukocytes. Compared with eyes injected with empty MPs, those injected with sunitinib MPs had a significant reduction in the mean number of adherent intravascular leukocytes (Fig. 6a). Similarly aflibercept-injected eyes had a significant reduction in the mean number of adherent intravascular leukocytes compared with PBS-injected eyes. This experiment was repeated, but the number of adherent leukocytes in vessels was visualized by immunohistochemical staining with anti-CD45 antibody and this also showed a significant reduction in leukostasis in eyes injected with sunitinib MPs compared with empty MPs (Fig. 6b).

The constant moderate expression of VEGF in rho/VEGF mice not only causes type 3 choroidal NV in the outer retina, but also causes gradual onset of leukostasis and retinal vessel closure in the inner retina thereby mimicking these aspects of diabetic retinopathy. Rho/VEGF mice (≥3 months old) had fluorescein angiography which showed areas of retinal nonperfusion and

![Fig. 4 Sunitinib MPs suppress murine type 3 choroidal NV substantially longer than aflibercept.](image-url)
then had intravitreous injection of MPs containing 10 µg sunitinib or an equivalent mass of empty MPs. Repeat fluorescein angiography 2 weeks after injection showed reperfusion of some previously nonperfused areas in sunitinib MP-injected retinas (Fig. 6c).

**Pharmacokinetics of sunitinib MPs in mice and rabbits.** As shown in Fig. 7a, at 30 days after an intravitreous injection of sunitinib MPs in C57BL/6 mice, 52% of the initial sunitinib MP dose still remained in the eye. In comparison, only 9% of the initial dose still remained in the eye 30 days after injection of an equivalent amount of sunitinib drug suspension. The data also correlate well with the in vitro drug release profiles in Fig. 1b. The results suggest that when not encapsulated in polymer MPs, sunitinib was rapidly cleared from the eye. In contrast, a much more sustained release of drug was achieved from sunitinib MPs. A single 50 µl intravitreous injection of MP suspension containing 1 mg sunitinib or a single 200 µl subconjunctival injection of MP suspension containing 2 mg sunitinib was administered to pigmented New Zealand rabbits and the rabbits were euthanized at selected time points to evaluate the levels of sunitinib in ocular tissues. Within 1 week after the intravitreous injection, the levels of sunitinib in the retina and RPE/choroid continued to increase and peaked at 3 months (Fig. 7b). Analysis for remaining sunitinib content in the retina and RPE/choroid continued to increase and peaked at 3 months. However, therapeutic levels of sunitinib were detected in the RPE/choroid for more than 6 months. The extended drug exposure is likely due to binding of sunitinib to melanin granules in the RPE/choroid, which served as a secondary depot for sunitinib.

The capacity of melanin to bind sunitinib was characterized in vitro by co-incubating sunitinib (100 ng to 2 mg) in 10 mg melanin (purified from sepsa officialis) in PBS and quantifying the amount of free sunitinib in solution after 1 h. Melanin-bound sunitinib was calculated by subtracting free sunitinib in solution from total sunitinib. As the sunitinib-to-melanin mass ratio increased from 0.001 to 20%, the percentage of melanin-bound
sunitinib decreased from 99.8 to ~80%. Thus, at a ratio of <10, 96–99.8% of the drug is bound to melanin and binding saturation occurs when this ratio is 10–20%. The combination of sunitinib release from MP s and subsequent release from the melanin secondary reservoir likely accounts for the sustained drug level seen in the retina and RPE/choroid for more than 6 months after a single intravitreous injection. Melanin content in pigmented rabbit choroid is in the range of 20–120 μg melanin per μg tissue, which is similar to that in humans.

A similar pharmacokinetic profile was obtained via subconjunctival injection. Within 1 week after administration of MPs containing 2 mg sunitinib, the levels of sunitinib in the retina and RPE/choroid were approximately 200-fold greater than the reported level required to inhibit VEGF receptors. The levels in the retina and RPE/choroid continued to increase through Day 28, the last time point in this pilot study (Fig. 7c).

**Ocular tolerability and safety of sunitinib MPs in minipigs.** Following 2 repeat intravitreous injections 20 weeks apart of 0.25, 0.5, or 1 mg/eye, sunitinib MPs were well-tolerated during a 40-week observation period with regard to all endpoint assessments included in a repeat-dose ocular toxicity study in minipigs. The no-observed-adverse-effect-level is above the highest dose tested in this study. There were no sunitinib MP-associated ocular findings with exception of a transient, expected minor, focal yellow discoloration to vitreous humor and lens observed in some animals. This discoloration resolved over time and was considered secondary to the presence of sunitinib. It was not considered adverse. Sporadic presence of pigment on anterior lens capsule during pretest and dosing period in the left (control PBS-injected) or the right (sunitinib MP-injected) eyes. This was considered incidental as it appeared sporadic and also during pretest and in the left control eyes.

Group mean average values for IOP readings in males were considered within normal limits at all time points with exception of Group 1 in both eyes at the Day 198 time point and Group 3 in the left eye at the Day 43 and Day 142 time points. These findings were considered incidental and not associated with test article administration since they affected control eyes. Group mean average values for IOP readings in females were considered within normal limits at all time points.

Electroretinography (ERG) assessments were also conducted in this study on all animals pretest and Weeks 19, 30, and 39 using the Roland Consult Retiport Gamma ERG system. Amplitude and latency values were measured from tracings. Each ERG assessment consisted of a series of stimuli with flash intensity of 0.0025, 0.25, and 2.5 cd.s/m² for scotopic phase assessments following dark adaptation, and with flash intensity...
Days 7 and 28 (region RPE-choroid and retina in one eye of each animal were collected administered to both eyes of pigmented New Zealand rabbits. Central given a 1 µg intravitreous injection of either MPs containing 1 mg sunitinib in rabbits, the drug levels were injected and maintained for at least 6 months. The ocular toxicity of sunitinib MPs was evaluated by measuring drug levels in plasma and ocular tissues. Sunitinib levels in plasma and ocular tissues were evaluated by LC-MS at each time point. Sunitinib levels in plasma and ocular tissues were evaluated by a validated bar) in both eyes. Immediately after injection or at 30 days after injection. Six months after a 50 µl intravitreous injection of MPs containing 10 µg sunitinib had persistent peak activity 6 months after injection, far longer than an intravitreous injection of 40 µg of aflibercept. While intravitreous injection is the projected route of administration, subconjunctival injection of MPs, a less invasive approach, may be an option because it provides local suppression of type 2 choroidal NV in the injected eye without an identifiable systemic effect in the fellow eye. In rho/VEGF mice, intravitreous injection of MPs containing 10 µg sunitinib provides sustained inhibition of subretinal NV for at least 4 weeks, the longest time point tested, while an intravitreous injection of 40 µg of aflibercept inhibited subretinal NV for 1 week. Much of the clinical benefit of anti-VEGF agents comes from rapid reduction of leakage from established subretinal NV and sunitinib MPs provided dramatic reduction in leakage within 1 week of injection similar to that seen with intravitreous injection of 40 µg of aflibercept. The minipig eye is closer in size to a human eye and may be more predictive for the duration of action. An intravitreous injection of sunitinib MPs resulted in a consolidated depot in the inferior choroid and >50-fold higher than the Ki in retina.

In this study, we have incorporated sunitinib, a small molecule tyrosine kinase inhibitor that blocks signaling through VEGF receptors, into biodegradable polymer MPs that after intravitreous injection provide dose-dependent, long duration suppression of type 2 choroidal NV. A single dose of MPs containing 10 µg sunitinib had persistent peak activity 6 months after injection, far longer than an intravitreous injection of 40 µg of aflibercept. While intravitreous injection is the projected route of administration, subconjunctival injection of MPs, a less invasive approach, may be an option because it provides local suppression of type 2 choroidal NV in the injected eye without an identifiable systemic effect in the fellow eye. In rho/VEGF mice, intravitreous injection of MPs containing 10 µg sunitinib provides sustained inhibition of subretinal NV for at least 4 weeks, the longest time point tested, while an intravitreous injection of 40 µg of aflibercept inhibited subretinal NV for 1 week. Much of the clinical benefit of anti-VEGF agents comes from rapid reduction of leakage from established subretinal NV and sunitinib MPs provided dramatic reduction in leakage within 1 week of injection similar to that seen with intravitreous injection of 40 µg of aflibercept. The minipig eye is closer in size to a human eye and may be more predictive for the duration of action. An intravitreous injection of sunitinib MPs resulted in a consolidated depot in the inferior vitreous base well outside the visual axis. The size of the depot gradually decreased over time and it was barely visible by Month 5 when a second injection of MPs was given. There was no identifiable ocular toxicity at Month 6, 1 month after the second injection. Six months after a 50 µl intravitreous injection of MPs containing 1 mg of sunitinib in rabbits, the drug levels were >1000-fold higher than the Ki for VEGFR2 and VEGFR1 in RPE/choroid and >50-fold higher than the Ki in retina.

To achieve these impressive results, a number of challenges were overcome. The three key challenges for ocular administration of MPs were inflammation from the PLGA MPs, duration of action (6 months or longer), and dispersion of MPs after injection. The pro-inflammatory effects of PLGA MPs were eliminated by the use of a hydrophilic coating (PEG) that forms on the surface of MPs when PLGA-PEG is used in the
formulation. The duration of action over 6 months was accomplished by the combination of prolonged release of sunitinib from the MPs and the binding of sunitinib to melanin granules in the RPE/choroid, which served as a secondary depot of sunitinib. Finally, surface treatment facilitated self-aggregation of MPs and the formation of a solid depot upon injection into the vitreous, thus mitigating the risk of interfering with vision by dispersed MPs.

About 20% of patients with NVAMD treated with anti-VEGF agents develop patches of macular atrophy due to photoreceptor cell death over the course of 2 years. A major risk factor for the development of atrophy is the presence of type 3 choroidal NV. In rho/VEGF mice with type 3 choroidal NV, photoreceptor cell death resulting in retinal atrophy is due to oxidative damage and while aflibercept does not increase photoreceptor death and loss of function, it does not significantly reduce photoreceptors death or maintain rhodopsin kinase, a critical component of the visual transduction cascade that is a marker for photoreceptor survival and function. In contrast, sunitinib MP promoted photoreceptors survival as demonstrated by significantly greater outer nuclear thickness and higher rhodopsin kinase level in rho/VEGF mice treated with sunitinib MP. This suggests that sunitinib MPs may reduce atrophy in patients with type 3 choroidal NV. This could provide an important advantage over currently available anti-VEGF agents. While the mechanism by which sunitinib promotes photoreceptor survival in rho/VEGF mice is uncertain, one possibility is inhibition of the dual leucine zipper kinase, an activity of sunitinib that has been demonstrated to promote survival of injured ganglion cells.

In addition to the major benefits in NVAMD, anti-VEGF agents provide benefits in the ischemic retinopathies, diabetic retinopathy and retinal vein occlusions. Anti-VEGF agents reduce excessive vascular leakage, reduce macular thickening, and improve vision in patients with diabetic macular edema or macular edema due to retinal vein occlusion. The main impediment to obtaining these outstanding outcomes in clinical practice is the need for repeated intravitreal injections, a problem that could be overcome by the long duration of action of sunitinib MPs. Anti-VEGF agents have also been demonstrated to reduce retinal non-perfusion in retinal vein occlusion and diabetic retinopathy, which drives disease progression in both and. As a result, injections of anti-VEGF agents cause regression of diabetic retinopathy and the retinopathy associated with retinal vein occlusions.

Sunitinib MPs significantly reduced VEGF-induced leukocytic plugging and retinal nonperfusion. Since activation of VEGFR1 on leukocytes promotes leukocyte recruitment which contributes to leukocyte plugging, sunitinib MP, which blocks both VEGFR2 and VEGFR1 and have a long duration of action, may be ideal for treatment of diabetic retinopathy in the absence of macular edema.

In summary, we have demonstrated that a single intravitreal injection of sunitinib MPs provides prolonged suppression of type 2 and type 3 choroidal NV in mice and therapeutic levels in RPE/choroid and retina of rabbits for at least 6 months. The prolonged suppression of VEGF signaling provided by sunitinib MPs has a high chance of improving the treatment of patients with NVAMD, diabetic retinopathy, and retinal vein occlusion.

**Methods**

**Preparation of drug-loaded microparticles.** Polymer MPs loaded with sunitinib were prepared using a single emulsion solvent evaporation method. Briefly, 565.6 mg of PLGA 7525 4A and PLGA-PEG5k (Evonik Corporation, Piscataway, NJ) were dissolved in 4 ml dichloromethane (DCM) and mixed with a solution of 90 mg sunitinib malate (TAPI, Parsippany, NJ) in 2 ml DMSO. The mixture was homogenized in 200 ml of an aqueous solution of 1% polyvinyl alcohol (25 kDa, Polysciences, Warrington, PA) in 1× PBS using a laboratory mixer (LSM-A, Silverson Machines, East Longmeadow, MA) for 1 min. For MPs tested in mouse models, a 1.5” workhead was used and for those tested in rabbits, a 0.75” workhead was used. The MPs were hardened by stirring at room temperature for 2 h to allow DCM to evaporate and then collected by sedimentation, washed thrice with cell culture grade water (HyClone, Fisher, Pittsburgh, PA) and lyophilized. The dry powder of sunitinib MPs was suspended in a cold solution containing 70% ethanol and 75 mM NaOH and stirred for 3 min, collected, washed and lyophilized again. For animal studies, the lyophilized MPs were suspended in a 0.1% sodium hyaluronate solution at desired concentration. A pulled glass micropipette or a 27-G needle was used for injections in mice and rabbits, respectively.

**Drug release from microparticles.** Sunitinib MPs were suspended in 4 ml PBS (pH 7.4) containing 1% polysorbate 20 at 2.5 mg/ml and incubated at 37 °C on a rotating platform (150 rpm). At selected time points, 3 ml of the release medium was collected and replaced with 3 ml fresh release medium. Sunitinib content in release medium was measured by UV absorbance at 440 nm.

**Mouse model of laser-induced choroidal NV.** Wild-type female 4-week-old C57BL/6N mice were used for the experiments (Charles River, Wilmington, MA). Mice were treated in accordance with the Association for Research in Vision and Ophthalmology Guidelines on the care and use of animals in research. All protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Laser photocoagulation-induced rupture of Bruch’s membrane was used to generate choroidal NV. Briefly, 4–5-week-old C57BL/6 mice were anesthetized, pupils were dilated with 1% tropicamide (Alcon Labs, Inc., Fort Worth, TX), and one eye was given an intravitreal injection of MPs containing 10 or 1 µg sunitinib in one eye and an equivalent mass of empty MPs in the fellow eye, or they were given an injection of 40 µg of aflibercept in one eye and PBS in the fellow eye. At various time points after injection ranging from 1 to 24 weeks after injection, the mice had rupture of Bruch’s membrane by laser photocoagulation at 9, 12, and 3 o’clock positions of the posterior pole in each eye with 352 nm diode laser photocoagulation (75 µm spot size, 0.1 s duration, 120 mW) using the slit lamp delivery system of an OcuLight GL Photocoagulator (Iridex, Mountain View, CA, USA) and a hand-held cover slide as a contact lens. One week after rupture of Bruch’s membrane, mice were euthanized, eyes were removed, anterior segments, retinas, and vitreous was removed, and eye cups were stained with fluorescein isothiocyanate (FITC)-conjugated GSA (Vector Laboratories, Burlingame, CA), and flat mounted. Flat mounts were examined by fluorescence microscopy and the area of each choroidal NV was measured by image analysis with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) by an observer masked with respect to experimental groups. The three areas obtained in each eye were averaged to give a single experimental value.

In some experiments, C57BL/6 mice were given a subconjunctival injection of MPs containing 20 or 2 µg sunitinib or an equivalent mass of empty MPs in one eye and no injection in the fellow eye. At 1 week after injection, Bruch’s membrane was ruptured by laser photocoagulation at 3 locations in each eye and after 1 week the area of choroidal NV at Bruch’s membrane sites was measured.

**Transgenic mice with VEGF expression in photoceptors.** Rho/VEGF transgenic mice, in which the rhodopsin promoter drives expression of VEGF in photoreceptors have increased levels of VEGF starting at P7 and develop multiple areas of subretinal NV by P21. At P14, rho/VEGF mice of either sex, were given an intravitreal injection of MPs containing 10 µg sunitinib in one eye and an equivalent mass of empty MPs in the other eye or 40 µg of aflibercept in one eye and PBS in the other eye. At P21, P28, P35, or P42, mice were euthanized and retinas were stained with FITC-labeled GSA lectin and flat mounted with photoceptors facing up. Retinal flat mounts were examined by fluorescence microscopy and the area of subretinal NV was measured by image analysis by a masked observer.

**Fluorescein angiography.** Rho/VEGF mice were anesthetized, pupils were dilated with 1% tropicamide, and fundus photographs were obtained with a Micron III Retinal Imaging Microscope (Phoenix Research Laboratories Inc., Pleasanton, CA) before and at several time points after intraperitoneal injection of 50 µl of 25% fluorescein (AK-Fluor; Lake Forest, IL).
plate was read at 450 and 570 nm with SpectraMax Plus (Molecular Devices, San Jose, CA).

Measurement of retinal vascular leakage with Evans Blue dye. C57BL/6N mice, 5–7 weeks old of either sex, were given an intravitreal injection of MP containing 10 µg sunitinib in one eye and an equivalent mass of empty MPs in the fellow eye followed 1 week later by an intravitreal injection of 100 ng of VEGF in each eye. After 12 h, mice were given an intraperitoneal injection of 300 µl of Evans Blue solution (20 mg/ml; Sigma) and after 1 h they were anesthetized, the chest was opened to expose the heart, the right atrium was cut for drainage, a 27-G cannula was inserted into the left ventricle, and the vasculature was flushed with PBS for 3 min. Retinas were dissected, weighed, and incubated in 150 µl formamide at 70 °C for 18 h. Retina-formamide extracts were centrifuged at 16,000 g, 60 µl of supernatant was added to a 96-well plate and optical density was measured at 620 nm on a plate reader. A standard curve was used to determine the amount of Evans Blue per mg retina.

Pharmacokinetic studies in mice and rabbits. Twenty male or female 5–7-week-old C57BL/6N mice were anesthetized and the pupils were dilated as previously described. Each mouse was given bilateral intravitreal injections of either MPs containing 1 µg sunitinib or an equivalent dose of sunitinib drug suspension. Immediately after injection or at 30 days after injection, 5 mice from each condition were euthanized and the whole globes were collected and transferred to a bioanalytical facility for measurement of drug levels by liquid chromatography–mass spectrometry (LC–MS). The amount of drug remaining in the eye at 30 days was compared to and presented as a percentage of the initial dose injected in the eye.

Safety studies in minipigs. Ocular tolerability and safety was tested in 15 male and 15 female 6-month-old Yucatan minipigs in an independent laboratory using Good Laboratory Practice (Charles River Laboratories, Mattawan, MI). The minipigs were treated ethically in accordance with the Association for Research in Vision and Ophthalmology Guidelines on the care and use of animals in research and protocols were approved by the Power of Research. Care and use committee. A 20 µl suspension of MPs containing 1 mg sunitinib malate was administered to both eyes through a 27 G needle. A 500 µl blood sample was collected from the rabbit’s ear vein into K₂ EDTA tubes, the plasma separated by centrifugation and frozen at ≤70 °C. Eyes were enucleated immediately after sacrifice. Only one eye was collected for pharmacokinetic ocular tissue analysis. The following ocular tissues were collected at 24 h, Days 3 and 10, and Months 1–6, 8, and 11: central region RPE-chorioid, retina, and vitreous humor. Tissue was harvested into precooled tubes. Ocular tissue and plasma was stored frozen (at or below ≤70 °C) in Eppendorf tubes after collection and transferred to the bioanalytical facility at designated intervals for analysis.

For the subconjunctival injection study, a 200 µl suspension of MPs containing 2 mg sunitinib was administered to both eyes through a 27 G needle. Only one eye was collected for bioanalytical tissue analysis. Central region RPE-chorioid and retina were collected Days 7 and 28. Sunitinib levels in plasma and ocular tissues were evaluated by a validated extraction and the LC–MS method.

| Table 1 Ocular tolerability and safety in Yucatan minipigs. |
|----------------|----------------|--------------------|-----------------|----------------|----------------|
| **Group**      | **Test material** | **Sunitinib malate (mg/eye)** | **Control** | **Injection volume (µl)** | **Males** | **Females** |
| 1              | Sunitinib MP    | 0.25                | PBS           | 12              | 3              | 3 |
| 2              | Sunitinib MP    | 0.5                 | PBS           | 12              | 3              | 3 |
| 3              | Sunitinib MP    | 0.5                 | PBS           | 24              | 3              | 3 |
| 4              | Sunitinib MP    | 1.0                 | PBS           | 24              | 3              | 3 |

A suspension (12–24 µl) of sunitinib microparticles (MPs) containing 0.25, 0.5, or 1 mg of sunitinib was injected into one eye and the fellow eye was injected with phosphate-buffered saline (PBS). Injections were repeated on Day 149. Slit lamp examination, indirect ophthalmoscopy, and measurement of IOP were performed by a veterinary ophthalmologist. No statistically significant differences were observed. ERGs were performed prior to the initiation of the study and in Weeks 19, 30, and 39. At the end of each 40-week observation period, the minipigs were euthanized and the eyes were collected.

Leukostasis was also assessed by immunohistochemical staining to identify CD45-positive cells in retinal vessels. C57BL/6 mice of either sex at 5–7 weeks were given an intravitreal injection of MP containing 10 µg sunitinib in one eye and an equivalent mass of empty MPs in the fellow eye followed 1 week later by an intravitreal injection of 100 ng of VEGF in each eye. After 12 h, mice were euthanized, eyes were removed and fixed in 2% PFA at 4 °C. After washing, retinas were dissected and examined by fluorescence microscopy. The number of intravascular CD45-positive cells in retinal vessels was measured by an investigator masked with respect to treatment group.

For the subconjunctival injection study, a 200 µl suspension of MPs containing 2 mg sunitinib was administered to both eyes through a 27 G needle. Only one eye was collected for bioanalytical tissue analysis. Central region RPE-chorioid and retina were collected Days 7 and 28. Sunitinib levels in plasma and ocular tissues were evaluated by a validated extraction and the LC–MS method.

Statistical analyses. No sample size calculations were done in this study. Sample sizes were estimated based upon previous similar experiments done in the past. We controlled for covariates by injecting the same animal in one eye with control and the fellow eye with the drug. Results are presented as the mean ± standard error of the mean. A Shapiro–Wilk normality test was conducted for each experiment, showing that the data did not follow a normal distribution. Therefore, statistical comparisons between two groups were done with a two-sided Mann–Whitney test.
and comparisons between more than two groups were done by Kruskal–Wallis test followed by Dunn’s test using GraphPad Prism software (GraphPad Software, La Jolla, CA).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All data generated during this study are included in this published article (and its Supplementary Information files). The supplementary source data file contains the source data underlying Figs. 2a–d, 3a, b, 4a–e, 5b–d, 6a, b, and 7a–c.

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**Author contributions**

H.T. performed the experiments, collected and analyzed the data, prepared figures, edited and approved the paper. J.F. designed the microparticles, synthesized the microparticles, performed the experiments, collected and analyzed the data, edited and approved the paper. J.S. performed the experiments, collected the data, analyzed the data, edited and approved the paper. Y.Y. improved and synthesized the microparticles, performed the experiments, collected and analyzed the data, edited and approved the paper. Z.H. assisted in performance of the experiments and collected the data. J.K. improved and synthesized the microparticles, performed the experiments, collected and analyzed the data, edited and approved the paper. D.M. synthesized the microparticles, performed the experiments, collected and analyzed the data, edited and approved the paper. D.Ca. synthesized the microparticles, performed the experiments, collected and analyzed the data, edited and approved the paper. B.C.G. performed the experiments, collected and analyzed the data, edited and approved the paper. Y.K. analyzed the data, edited and approved the paper. J.L.C. provided resources, designed the experiments, edited and approved the paper. M.Y. improved and synthesized the microparticles, performed the experiments, collected and supervised the experiments, analyzed the data, wrote first draft of the paper, edited and approved the paper. P.A.C. designed and supervised the experiments, analyzed the data, wrote first draft of the paper, edited and approved the paper.

**Competing interests**

J.H., J.F. and P.A.C. are founders of Graybug Vision which has a commercial interest in sunitinib microparticles, and they have equity in Graybug and receive remuneration for consultation. Johns Hopkins University has patents on which J.H. and J.F. are authors that are licensed to Graybug Vision. All of these relationships are being managed by the Johns Hopkins University conflict of interest committee. Y.Y., D.M., D.Ca., W.Y. and M.Y. are employees of Graybug Vision. J.K., W.P., J.Z.Z. and J.L.C. are former employees of Graybug Vision. The remaining authors declare no competing interests.

**Additional information**

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**Correspondence** and requests for materials should be addressed to M.Y., J.H. or P.A.C.

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