SU9518 Inhibits Proliferative Vitreoretinopathy in Fibroblast and Genetically Modified Müller Cell–Induced Rabbit Models

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PURPOSE. Proliferative vitreoretinopathy (PVR) is a complication of retinal detachment that can lead to surgical failure and vision loss. Previous studies suggest that a variety of retinal cells, including RPE and Müller glia, may be responsible. Platelet-derived growth factor receptor alpha (PDGFR α) has been strongly implicated in the pathogenesis, and found to be intrinsic to the development of PVR in rabbit models. We examine whether SU9518, a tyrosine kinase inhibitor with PDGFR α specificity, can inhibit the development of PVR in fibroblast and Müller cell rabbit models of PVR.

METHODS. SU9518 was injected in rabbit eyes along with fibroblasts, Müller cells (MIO-M1), or Müller cells transfected to increase their expression of PDGFR α (MIO-M1 α). Indirect ophthalmoscopy and histopathology were used to assess efficacy and toxicity.

RESULTS. SU9518 was an effective inhibitor of PVR in both fibroblast and Müller cell models of PVR. No toxic effects were identified by indirect ophthalmoscopy or histopathology.

CONCLUSIONS. SU9518 is an effective and safe inhibitor of PVR in rabbit models, and could potentially be used in humans for the treatment of this and other proliferative diseases of the retina involving fibrosis and gliosis. Further animal studies need to be performed to examine retinal toxicity and sustained delivery mechanisms. (*Invest Ophthalmol Vis Sci.* 2013;54:1392–1397) DOI:10.1167/iovs.12-10320

Proliferative vitreoretinopathy (PVR) occurs in 5% to 10% of rhegmatogenous retinal detachments.¹ It is a complex cellular process consisting of preretinal and subretinal membrane formation, intraretinal degeneration, gliosis, and contraction. As it is currently understood, the disease is characterized by migration and proliferation of RPE and glial cells along with synthesis of extracellular matrix (ECM) proteins, such as collagen or fibronectin, which organize into

retinal and vitreous membranes; and intraretinal glial cell proliferation, photoreceptor degeneration, and disorganization of retinal cell layers.^{2,3} In a way, PVR can be viewed as maladaptive and/or aberrant wound healing,⁴ the severity of which is often determined by clinical characteristics that include the size and location of the retinal tear, longevity of the detachment, and presence or absence of vitreous hemorrhage.

A debate remains regarding the extent of involvement of cells other than RPE, such as Müller glia, in the pathogenesis of PVR. Recent work demonstrating the reactivity of Müller glia during retinal detachment and other forms of retinal injury suggests that these cells, previously thought of as merely supportive and passive, may actually play a significant role in diseases involving retinal injury and degeneration, such as PVR. Although RPE cells have long been considered the principal mediators of this disease, Müller cell activation, migration, proliferation, and transformation have all been documented.^{5,6} Increased expression of glial fibrillary acidic protein (GFAP) and vimentin, indicative of increased reactivity, have been demonstrated in Müller glia in detached human retinas and experimental models of retinal detachment.^{7,8} Experimental detachment models have shown Müller cell proliferation, which peaks at 3 to 4 days after retinal detachment and continues at a slower rate for weeks to months,⁹ as well as migration of Müller cell processes and nuclei throughout the retinal layers and into the subretinal space.¹⁰ Certainly, the data support the need to explore more closely the capacity of these cells to actively participate in PVR pathogenesis.

Questions also exist as to what would be the ideal target of pharmacotherapies for the treatment and prevention of PVR. Although multiple cytokines and ligands have been implicated in the disease, platelet-derived growth factor (PDGF) and its receptor, PDGFR, have been shown in multiple studies to play a crucial role. PDGFR α , for example, is found extensively in preretinal membranes from PVR patients.^{11,12} Experimental models using mouse embryonic fibroblasts, as well as rabbit conjunctival fibroblasts, have helped distinguish the intrinsic role that PDGFR α , and not PDGFR β , plays in the pathogenesis of the disease.^{13,14} In fact, inhibition of the PDGFR α , either through its tyrosine kinase or the reactive oxygen species pathway, has been shown to be sufficient in these models to attenuate and/or inhibit the development of PVR.^{15,16}

The goal of this study was to determine whether SU9518 (3[5-(5-bromo-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrol-3-yl]propionic acid), a novel PDGFR α -specific tyrosine kinase inhibitor, can inhibit PVR at nontoxic doses in both Müller cell and fibroblast rabbit models of the disease.

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METHODS

Major Reagents

Rabbit polyclonal antibodies against PDGFR α were purchased from Cell Signaling Technology (Beverly, MA), anti-GFAP was purchased from Zymed (San Francisco, CA), and tubulin and β -actin were purchased from Abcam (Cambridge, MA). Secondary antibodies (anti-rabbit and antimouse IgG) were purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA). SU9518 was obtained by material transfer agreement from Pfizer Co. (New York, NY). MIO-M1 human Müller cells were obtained by material transfer agreement from the Institute of Ophthalmology, University College London, from G. Astrid Limb, PhD, and Peng Tee Khaw, MD, PhD (patent application PCT/GB2004/005101).¹⁷ Primary rabbit conjunctival fibroblasts (RCFs) were obtained as previously described.¹³

Preparation of MIO-M1 α Cell Line and Western Blotting

The pLHDCX²-PDGFR α retrovirus was used to stably express the PDGFR α in immortalized MIO-M1 cells. Transfected cells were selected for resistance to histidinol toxicity.

Lysates of transfected MIO-M1 α cells were made for Western blot analysis to confirm increased expression of PDGFR α . Medium was removed and cells were collected using sterile cell scrapers in PBS. Lysates of these cells were created by incubation for 30 minutes at 4°C in radioimmunoprecipitation assay buffer (50 mM Tris HCl [pH 8.0], 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 2% protease inhibitor cocktail, 2% phosphatase inhibitor cocktail 1, 2% phosphatase inhibitor cocktail 2) (Sigma-Aldrich, St. Louis, MO), followed by 15 seconds of sonication and removal of cellular debris by centrifugation at 12,000 rpm for 12 minutes at 4°C.

Lysates of vitreous membranes of eyes injected with transfected Müller cells, with and without SU9518, were made for Western blot analysis to confirm the presence of MIO-M1 α cells. Rabbits were euthanized and the right eye enucleated after 4 weeks of observation post intravitreal cell injection as described below. The eyes were flash frozen at -80°C. The vitreous with membranous material of select rabbits was extracted while the eye was frozen after removal of all scleral, choroidal, and retinal tissue. This ensures complete retrieval of all vitreous material without loss of aqueous component or contamination by other ocular tissues and hematologic contamination. The vitreous specimen was centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and lysates were created using the procedure described above.

The protein content of the lysates was determined with the bicinchoninic acid protein assay (Thermo Fisher Scientific, Waltham, MA); 50 μ g of protein was resolved by 10% SDS-PAGE. The protein bands were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and the membrane was subjected to Western blot analysis using anti-PDGFR α or GFAP primary antibodies.

Band densities were quantified using ImageJ software (provided in the public domain by National Institutes of Health [NIH], <http://rsbweb.nih.gov/ij/>) and normalized to tubulin or β -actin expression.

Rabbit Model for PVR

PVR was induced in the right eyes of pigmented rabbits purchased from Covance (Denver, PA). Briefly, a gas vitrectomy was performed by injecting 0.1 mL of perfluoropropane (C₃F₈) (Alcon, Fort Worth, TX) into the vitreous cavity 4 mm posterior to the corneal limbus. Experimental rabbits were concomitantly injected with 300 μ g of SU9518 suspended in 0.1 mL of balanced salt solution (BSS); control rabbits were injected with 0.1 mL of BSS only. One week later, all rabbits received two injections: 0.1 mL of platelet-rich plasma (PRP); and 0.1 mL Dulbecco's modified Eagle's medium containing 2×10^5 RCF cells (11 eyes experimental, 8 eyes control), MIO-M1 cells (11 eyes experimental,

11 eyes control), or MIO-M1 α (10 eyes experimental, 11 eyes control). Although 11 rabbits were chosen for each experimental and control group, eyes with complications that could confound the results and interfere with accurate observation, such as vitreous hemorrhage and cataract formation, were eliminated from the study. The extent of retinal detachment was evaluated by indirect ophthalmoscopy with a handheld +30-diopter fundus lens at days 2, 4, and 7, and weekly thereafter for a total of 4 weeks. Extent of PVR was graded according to the Fastenberg classification from grades 0 through 5.¹⁸ All ophthalmic examinations and grading were performed by one observer (GV). Fundus images were obtained using the Heine Omega 2C Indirect Ophthalmoscope (Heine USA Ltd., Dover, NH). On day 28 post cell injection, the animals were euthanized and the eyes were enucleated. All surgeries were performed under aseptic conditions and pursuant to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols for the use of animals were approved by the Schepens Eye Research Institute and University of Massachusetts Medical School Animal Care and Use Committees. Mann-Whitney test for nonparametric data ($P < 0.05$) was used for statistical analysis.

Animal Dosing and Sample Preparation for Pharmacokinetic Studies

Seven pigmented rabbits were injected in the right eye with 300 μ g of SU-9518 1 week after gas vitrectomy as described above. One rabbit was injected with gas only and used for control. One rabbit was euthanized and enucleated at each time point on days 3, 5, 7, 14, 21, 28, and 35. The eyes were immediately flash frozen at -80°C. The vitreous was extracted while the eyes were frozen to ensure complete retrieval of the vitreous material without other ocular tissue or hematologic contamination, with complete removal of the sclera, choroid, and retina. The vitreous samples were centrifuged for 10 minutes at 10,000 rpm to remove all vitreous collagenous and cellular components and the supernatant removed and retained. To 200 μ L of vitreous was added 10 ng of sunitinib as an internal standard. Samples were mixed and protein was precipitated with 600 μ L of acetonitrile by centrifugation. The resulting supernatant was removed, dried under nitrogen, and reconstituted in 20 μ L of 60% acetonitrile with 0.1% (vol/vol) formic acid. The calibrants were prepared by spiking 200 μ L of control vitreous with 10 ng of sunitinib and variable amounts of SU-9518 to cover a concentration range of 3 to 300 ng/mL. Calibrants were then processed as described above for the samples.

Liquid Chromatography–Mass Spectroscopy

Liquid chromatography–mass spectroscopy (LC-MS)/MS was performed on an Aquity UPLC (Waters Co., Milford, MA) interfaced to a Quattro Premier XE (Waters Co.) triple-quadrupole mass spectrometer.

Using an injection volume of 1 μ L and a flow rate of 35 μ L per minute, compounds were separated using a Zorbax 300SB (Agilent Technologies, Inc., Santa Clara, CA) 3.5- μ m C18 column (150 $\times$ 0.5 mm) using gradient elution with a mobile phase consisting of water with 0.1% (vol/vol) formic acid and acetonitrile containing 0.1% formic acid. The gradient conditions were as follows: 0 to 1 minute, 60% acetonitrile containing 0.1% formic acid; 5.0 minutes, 95% acetonitrile containing 0.1% formic acid; 6.0 minutes, 95% acetonitrile containing 0.1% formic acid; 6.1 minutes, 60% acetonitrile containing 0.1% formic acid; 10 minutes, stop. The mass spectrometer (Quattro Premier XE; Waters Co.) was operated in positive ion electrospray mode using a voltage of 4.4 kV and a source temperature of 150°C. The mass spectrometer (Quattro Premier XE; Waters Co.) was operated at unit mass resolution for Q1 and decreased resolution for Q3 in the multiple reaction monitoring (MRM) mode, with a dwell time of 50 ms. The MRM transitions for SU-9815 were 389.0 $\rightarrow$ 329 and 392 $\rightarrow$ 331, whereas sunitinib used 399 $\rightarrow$ 283 and 399 $\rightarrow$ 326 and had optimized collision energy values of 23, 23, 31, and 31 eV. Data were analyzed by Quanlynx (version 4.1; Waters Co.) by fitting the sample data to the calibration

FIGURE 4. SU9518 inhibits PVR in rabbits injected with MIO-M1 α cells transfected to increase expression of PDGFR α . Mann-Whitney analysis showed a statistically significant difference ($P < 0.05$) at day 14 and day 28 between both groups.

increase in the pathogenicity of the MIO-M1 α cell line versus naïve MIO-M1 cells, which is comparable to that of RCFs. This is consistent with the observed increased PDGFR α expression in MIO-M1 α cells, which is comparable to PDGFR α expression in RCFs.¹⁹

To answer the question of whether SU9518 is having a toxic versus inhibitory effect on the injected cells, Western blot analysis was performed of lysates from the vitreous membranes of eyes injected with MIO-M1 α , with and without SU9518. The result, using a human-specific primary antibody, showed the presence of GFAP-positive cells, regardless of stage of PVR (Fig. 6). As expected, the membranes from untreated eyes with more severe PVR had higher levels of GFAP when compared with those from SU9518-treated eyes with less severe PVR. In the absence of treatment with SU9518 and in the presence of a ligand-rich environment with PRP, one would expect the injected cells to proliferate; however, the presence of human GFAP in all selected specimens at significant levels is consistent with the presence of viable MIO-M1 α cells even in the presence of SU9518. This suggests that SU9518 acts in this

FIGURE 5. SU9518 does not alter the behavior of nontransfected MIO-M1 cells, which are very limited in their capacity to induce PVR. Mann-Whitney analysis showed no statistically significant difference at any of the time points between experimental and control eyes.

FIGURE 6. Western blot of vitreous membrane lysates of eyes injected with MIO-M1 α cells shows preservation of GFAP expression in both SU9518-treated (D, E) and -untreated (A–C) eyes. As expected, GFAP expression is highest in eyes with more severe PVR (A–C). Relative band densities controlled for β -actin expression are shown.

model through inhibition of proliferation, rather than a toxic effect on the injected cells.

Toxicity

Eyes injected with SU9518 free-base alone showed no signs of retinal toxicity by indirect ophthalmoscopy, with preservation of the medullary rays. Light microscopy of retinal sections at the medullary rays showed complete preservation of all retinal layers with intact nerve fiber layer and photoreceptor outer segments (Fig. 7).

Pharmacokinetics

Pharmacokinetics of 300 μ g of SU9518 free-base injected in the rabbit vitreous followed a single compartment, first-order elimination model (Fig. 8), with a half-life of 4.15 days calculated as $0.693/K$, where K is 0.17. Concentrations in ng/mL and nMol are listed in the Table. By day 14, SU9518 reached an average plateau concentration of 41.26 ng/mL or 106.04 nMol (molecular weight = 389 g/mole and 1 ng/mL = 2.57 nMol), which is sustained through the course of the last 3 weeks of the study. This is well above the documented 50% inhibitory concentration of 53 nMol for SU9518 inhibition of PDGF-induced BrdU incorporation in mouse fibroblasts.²⁰

DISCUSSION

Retinal gliosis and fibrous proliferation is an important component of not only PVR, but other conditions such as proliferative diabetic retinopathy (PDR), retinopathy of prematurity (ROP), and disciform scar formation in exudative age-related macular degeneration (ARMD). It presents an often insurmountable challenge, limiting visual recovery and/or

FIGURE 7. Toluidine blue O staining of the rabbit retina 35 days postinjection of 300 μ g of SU9518 free-base shows intact nerve fiber layer and photoreceptor outer segments with no disorganization of the retinal layers, and no evidence of autolysis or processing artifacts at $\times 25$ (a) and $\times 40$ (b) magnification.

FIGURE 8. Aqueous concentration of SU9518, 300µg, in the rabbit vitreous follows a one-phase decay model. A stable therapeutic concentration is achieved by day 14 and sustained over the course of 3 weeks.

leading to visual loss. Inhibition of fibrosis in intraocular diseases, therefore, remains a challenge and a therapeutic priority.

Fibroblasts have traditionally been used in animal models of PVR due to the reliability and predictability of the pathology they induce.^{13,21} Fibrosis in the retina, however, is the result of proliferation by other cell types, including RPE and glial cells. Models using RPE cells, even when transfected to increase expression of PDGFR α at very high levels, are less reliable and have a longer, more protracted course.²² We developed a novel model of PVR using transfected Müller cells because of their ability to mimic fibroblasts in the rabbit model. When transfected to increase expression of PDGFR α , which is ubiquitously absent in unstimulated cells grown in vitro, Müller cells mimic fibroblasts both in the severity of the pathology as well as the timeline. Work in our laboratory has demonstrated increased expression of PDGFR α in Müller and RPE cells when placed in co-culture in vitro, suggesting a synergistic effect in the context of retinal injury.¹⁹ The importance that PDGFR α plays in Müller cell behavior is highlighted in our study by (1) the significant increase in fibroblastic pathogenicity in these cells with small, less than 4-fold increases in PDGFR α expression; and (2) the difference in efficacy of SU9518 in the MIO-M1 versus MIO-M1 α models. It is possible that during certain pathologic states, such as PVR, Müller cells increase their expression of PDGFR α as they migrate and proliferate, thereby altering their behavior and phenotype. Although ideally one would observe this in vivo, this would be particularly difficult given the observed ability of Müller cells to transform and lose identifiable cell markers.⁶

There is a difference in efficacy of inhibition of PVR in RCF versus MIO-M1 α -injected eyes. Although not statistically significant, the observed difference in inhibition might be the result of the increased plasticity of MIO-M1 cells. MIO-M1 cells have the capacity to exhibit stem cell-like behavior, with increased expression of multiple growth factor receptors (Velez G, et al. *IOVS* 2009; 50: ARVO E-Abstract 1295). Although PDGFR α has been shown to be intrinsic in the development of severe PVR, other growth factor receptors have also been shown to play a role, such as those for TGF- β and FGF. It is possible that, despite successful inhibition of PDGFR α by SU9518, upregulation of other growth factors allows these cells to bypass, to a certain extent, PDGFR α inhibition.

TABLE. Average Concentrations in ng/mL and nMol for All Samples Tested Are Shown

Sample	ng/mL (SD)	nMol
Day 3	135.3 (1.2)	347.8
Day 5	90.5 (15.4)	232.5
Day 7	111.9 (4.6)	287.6
Day 14	42.8 (6.0)	110.0
Day 21	39 (10.9)	100.1
Day 28	56.3 (5.2)	144.7
Day 35	39.1 (1.0)	100.5

Tyrosine kinase inhibitors are small-molecule compounds that interfere with receptor function. Although many have the capacity to inhibit multiple receptor kinases, there is a wide range of variability in terms of efficacy and receptor specificity. SU9518 is a novel synthetic indolinone and multikinase inhibitor with specificity against the cellular PDGFR kinase; and potentially inhibits PDGFR-induced cell proliferation. Cell-based assays show inhibition of PDGFR-induced phosphorylation occurs within 5 minutes after drug exposure and persists for more than 6 hours after drug removal.²³ In a mouse model of pulmonary fibrosis, SU9518 was found to be an effective inhibitor of fibrosis through PDGFR α inhibition.²⁰ In vitro studies have shown that SU9518 can potentially inhibit irradiated fibroblast and endothelial cell proliferation.²⁴ In a rat balloon arterial injury-induced stenosis model, both oral and subcutaneous administration of SU9518 was found to reduce intimal thickening of the carotid artery.²³ In our study, SU9518 demonstrates effective inhibition of PVR when using both fibroblasts and Müller cells in a rabbit model.

The mechanistic and pharmacologic properties of this compound make it well suited for the potential treatment of intraocular disease. SU9518 is extremely insoluble in pH-neutral aqueous environments, such as the vitreous. Because of this, pretreatment with SU9518 prior to cell injection allowed for improved bioavailability of the agent once cells were injected, intrinsically acting as a sustained release depot. This same property also makes this drug an ideal agent for sustained release delivery approaches, such as biodegradable and nonbiodegradable intraocular and extraocular implants. It is possible to envision this agent being used as a pretreatment in patients with high-risk characteristics (large retinal tear, vitreous hemorrhage) for the development of PVR; or in all patients undergoing retinal detachment surgery for the prevention of secondary PVR. It could also be used as an adjunct in the management of other intraocular diseases, such as PDR, ARMD, and ROP, in which slowly progressive retinal gliosis, fibrosis, and membrane contraction play an important role. Further studies in animal models of these diseases are warranted.

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