Published in final edited form as:

J Control Release. 2020 November 10; 327: 456–466. doi:10.1016/j.jconrel.2020.08.019.

Sunitinib malate-loaded biodegradable microspheres for the prevention of corneal neovascularization in rats

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Competing financial interests

Justin Hanes is a founder of Kala Pharmaceuticals, Inc. and GrayBug Vision, Inc. He owns company stock, which is subject to certain rules and restrictions under Johns Hopkins University policy. The terms of this arrangement are being managed by Johns Hopkins University in accordance with its conflict of interest policies.

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Abstract

Corneal neovascularization (NV) predisposes patients to compromised corneal transparency and visional acuity. Sunitinib malate (Sunb-malate) targeting against multiple receptor tyrosine kinases, exerts potent antiangiogenesis. However, the rapid clearance of Sunb-malate eye drops administered through topical instillation limits its therapeutic efficacy and poses a challenge for potential patient compliance. Sunb-malate, the water-soluble form of sunitinib, was shown to have higher intraocular penetration through transscleral diffusion following subconjunctival (SCT) injection in comparison to its sunitinib free base formulation. However, it is difficult to load highly water-soluble drugs and achieve sustained drug release. We developed Sunb-malate loaded poly(D,L-lactic-*co*-glycolic acid) (PLGA) microspheres (Sunb-malate MS) with a particle size of approximately 15 μm and a drug loading of 7 wt%. Sunb-malate MS sustained the drug release for 30 days under the *in vitro* infinite sink condition. Subconjunctival (SCT) injection of Sunb-malate MS provided a prolonged ocular drug retention and did not cause ocular toxicity at a dose of 150 μg of active agent. Sunb-malate MS following SCT injection more effectively suppressed the suture-induced corneal NV than either Sunb-malate free drug or the placebo MS. Local sustained release of Sunb-malate through the SCT injection of Sunb-malate MS mitigated the proliferation of vascular endothelial cells and the recruitment of mural cells into the cornea. Moreover, the gene upregulation of proangiogenic factors induced by the pathological process was greatly neutralized by SCT injection of Sunb-malate MS. Our findings provide a sustained release platform for local delivery of tyrosine kinase inhibitors to treat corneal NV.

Graphical Abstract

SCT injection of Sunb-malate MS

Sunb-malate MS reduced corneal neovascularization

Keywords

tyrosine kinase inhibitor; PLGA; controlled drug release; subconjunctival injection; trans-scleral delivery

1. Introduction

Corneal neovascularization (NV), or the ingrowth of new blood vessels into the typically avascular corneal stroma, is a pathological condition that can result from infection, chemical or traumatic injury, autoimmune disease, and cornea transplantation [1]. The avascularity and transparency of the cornea is key to its function as the anterior refractive surface of the eye [2], so corneal NV can compromise visual acuity [3, 4]. Aberrant blood vessel growth occurs with disruption of the homeostasis between angiogenic and antiangiogenic factors [3, 4], of which vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are primarily implicated [5, 6]. VEGF and its receptors (VEGFRs) are present in neovascularized corneas at higher concentrations than normal cornea [7–10]. However, the use of anti-VEGF agents alone, including monoclonal antibodies, ribonucleic aptamers, and VEGF trap has been demonstrated to have limited or partial effect in reducing pathological corneal NV in animal studies and clinical trials [5]. Additionally, sprouting endothelial cells secrete PDGF, which stimulates VEGF transcription via tyrosine kinase PDGF receptors

(PDGFRs) [11, 12]. Thus, inhibition of both VEGFRs and PDGFRs can significantly enhance the antiangiogenic efficacy [13].

Combined inhibition of VEGFRs and PDGFRs can be achieved with tyrosine kinase inhibitors (TKIs). Topical administration of the TKIs sunitinib, pazopanib, sorafenib and axitinib was shown to have promising efficacy in treating corneal NV in both animal models and clinic trials [14–21]. However, topical eye drops have drawbacks, such as poor intraocular drug penetration and rapid clearance, which necessitate frequent, repetitive administration [22, 23]. For example, one clinical trial using pazopanib to treat corneal NV required eye drop application four times per day [19]. Frequent administration leads to poor patient compliance and compromised therapeutic efficacy [24]. An alternative option is subconjunctival (SCT) injection, which has been shown to provide high levels of water-soluble drugs to the anterior segment of the eyes of humans [25] and animals [26], mainly through the transscleral route that is more favored for water-soluble drugs [27, 28]. However, water-soluble drugs are typically cleared from the SCT injection site within a few hours [25, 26], leading to short-lived benefits. While it is challenging to formulate water-soluble drugs for sustained release [29], we hypothesized that continuous delivery of a TKI, such as sunitinib malate (Sunb-malate), following SCT injection may be effective in treating corneal NV. Here, we describe the development of biodegradable polymeric microspheres to provide sustained release of Sunb-malate (Sunb-malate MS) upon SCT injection, and demonstrate effective inhibition of corneal NV in a rat model *in vivo*.

2. Materials and methods

2.1 Materials

Poly(D, L-lactic-*co*-glycolic acid LA:GA 50:50, MW ~5.6 kDa, acid terminated) (PLGA) was purchased from Lakeshore Biomaterials (Evonik, Birmingham, AL). Sunitinib malate (Sunb-malate) and sunitinib free base (Sunb-base) were purchased from LC laboratories (Woburn, MA) (structures shown in Figure S1). Sunb-malate solution was prepared by dissolving Sunb-malate in phosphate buffered saline (PBS, pH 7.4) at a concentration of 0.5%. Polyvinyl alcohol (PVA) with MW ~25 kDa was purchased from Polysciences, Inc. (Warrington, PA). Other organic solvents were provided by Sigma-Aldrich (St. Louis, MO).

2.2 Animals

All rats were cared for and treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) concerning the use of animals in ophthalmic research. All experimental protocols were approved by the Johns Hopkins Animal Care and Use Committee. Male Sprague Dawley rats 6–8 weeks old were purchased from Harlan (Indianapolis, IN). The animals were anesthetized with intramuscular injection of a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg) during experimental procedures. Topical instillation of 0.5% proparacaine and 0.5% tropicamide were used for topical anesthesia and pupil dilation, respectively.

2.3. *Ex vivo* trans-scleral penetration of Sunb-malate and Sunb-base

The *ex vivo* trans-scleral transport assay was carried out using freshly excised sclera from intact healthy adult NZW rabbit eyeballs purchased through Pel-Freez Biologicals, LLC. The episcleral side was placed face down in the donor chamber of a 5 mL Franz diffusion cell (PermeGear, Inc., Hellertown, PA) as described previously [29]. The Sunb-base suspension was prepared by dispersing a Sunb-base DMSO solution (20 mg/mL) into PBS to achieve a concentration of 1 mg/mL. 0.3 mL of Sunb-base suspension (1 mg/mL in PBS) or Sunb-malate solution (1 mg/mL in PBS) were added to the donor chamber. PBS (pH 7.0) was placed in the receiver chamber, maintained at 37°C and stirred at 600 rpm. 0.4 mL of sample was taken from the receiver chamber at various time points and replaced with fresh PBS. The dissected rabbit sclera was observed with a Zeiss light microscope to ensure there were not observable macroscopic changes to the tissue integrity and continuity before and after the 6 h diffusion experiment. Drug levels were measured by HPLC on a Shimadzu Prominence LC system (Kyoto, Japan) equipped with a Pursuit 5 C18 column (Varian Inc., Lake Forest, CA). The mobile phase consisted of acetonitrile/water (60/40 v/v) containing 0.1% trifluoroacetic acid (flow rate = 1 mL/min), and column effluent was monitored by UV detection at 424 nm and 428 nm for Sunb-malate and Sunb-base, respectively.

The scleral permeability coefficient (P , cm/sec), which is a measure of the drug permeability that is independent of time and surface area, was estimated using the following equation [30]:

$$P = \frac{1}{C_d \times SA} \times \frac{\Delta Q}{\Delta t}$$

Where SA is the exposed surface area for transport within the Franz cell (0.2 cm²), Q is the cumulative amount of the drug transported across the sclera at any time t under sink conditions with intensive stirring, and C_d is the original drug concentration in the donor chamber. We assumed that we reached a steady state and C_d was approximately constant during the 6 h experiment. Data represent the mean of 3 independent scleral tissue samples.

2.3 Preparation and characterization of Sunb-malate loaded PLGA microspheres

Sunb-malate loaded PLGA microspheres (Sunb-malate MS) were prepared in a room designated for sterile particle manufacturing using an emulsification method. The glassware, centrifuge tubes, and homogenizer tip used in the microparticle preparation were autoclaved before use. In brief, 50 mg Sunb-malate was dissolved in 0.625 mL dimethyl sulfoxide (DMSO) before mixing with 2.5 mL dichloromethane (DCM) solution containing 250 mg PLGA. The mixture was poured into 60 mL of sterile-filtered (Nalgene™ 0.2 µm PES filters) 1% PVA solution under homogenization at 5000 rpm using a L4RT High Shear mixer (Silverson, East Longmeadow, MA). The formed emulsion was added to an extra 100 mL 0.3% PVA solution under magnetic stirring at 700 rpm for 1 h. The suspension was placed in a vacuum chamber for another 3 h under stirring to remove residual solvent. The Sunb-malate MS were filtered through a 40 µm strainer, washed with DI water, and collected by centrifugation at 500× g for 10 min. The placebo microspheres (Placebo MS) were prepared with the same procedures without the addition of drug to the organic phase. The endotoxin

levels in particle solutions prepared for *in vivo* studies were confirmed to be lower than 0.5 EU/ml (the FDA limit for medical devices) using the ToxinSensor™ Chromogenic LAL endotoxin assay kit (GenScript, Piscataway, NJ) (not shown).

The microparticle size (volume mean $\pm$ SD) was measured on a Coulter Multisizer 4 (Beckman Coulter, Inc., Miami, FL). The ζ -potential was measured using a Zetasizer Nano ZS90 (Malvern, Southborough, MA) after being diluted in 10 mM NaCl solution (pH 7.4). The morphology of lyophilized Sunb-malate MS was studied by scanning electron microscopy (SEM) using a LEO/Zeiss Field-Emission scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany). The lyophilized MS was mounted onto a SEM stub with conductive double-sided carbon tape and then coated with gold by a sputter coater under vacuum. The SEM was operated with an accelerating voltage of 1kV and a working distance of 2.8 mm.

2.4 Drug loading and drug release *in vitro*

Lyophilized Sunb-malate MS powder was weighed and dissolved in DMSO. The solution was measured by UV-Vis at 441 nm on a BioTek Microplate Reader (Winooski, VT). The Sunb-malate concentration was calculated using a standard curve of Sunb-malate in DMSO. The drug loading (DL) and encapsulation efficiency (EE) were calculated as follows:

$$DL \text{ (\%)} = \frac{\text{amount of Sunb-malate in MS}}{\text{weight of MS}}$$

$$EE \text{ (\%)} = \frac{\text{actual drug loading}}{\text{theoretical drug loading}}$$

To study the *in vitro* drug release profile of Sunb-malate MS, 1 mL of Sunb-malate MS suspension in PBS (PH 7.4) was placed in a 1.5 mL siliconized Eppendorf tube. Tubes were placed on an orbital shaker in a 37°C incubator and shaken at 120 RPM. At predetermined time points, the suspension was centrifuged at 2000 $\times$ g for 5 min, and the supernatant was collected and replaced with 1 mL fresh PBS. The concentration of Sunb-malate in the collected supernatant was measured by UV-Vis and calculated using a standard curve for Sunb-malate in PBS.

2.5 Ocular retention of Sunb-malate *in vivo*

Thirty microliters of Sunb-malate MS or Sunb-malate solution (5 mg/mL Sunb-malate concentration) in PBS was administered to rats through SCT injection using a 27-gauge needle. At post-operation day (POD) 0, 1, 3, 7, 14 and 28, the whole eyeballs (n=4) were harvested. Sunb-malate exhibits autofluorescence, therefore we imaged the enucleated eyeballs containing the conjunctiva tissue with the Xenogen IVIS Spectrum optical imaging system (Caliper Life Sciences Inc., Hopkinton, MA) at the excitation and emission wavelength of 420 nm and 510 nm, respectively. The fluorescent images were analyzed using the Living Image 3.0 software (Caliper Lifesciences, Inc.), and the retention of sunitinib was quantified by comparing to the total fluorescence counts of the eye

immediately after SCT injection (0 d). Untreated rat eyes were used as the baseline to be subtracted at all samples.

2.6 *In vivo* safety studies

To evaluate the ocular toxicity of Sunb-malate MS following SCT injection, 30 μ L Sunb-malate MS at concentrations of 5 and 0.5 mg/mL Sunb-malate in PBS were administered to both eyes of Sprague Dawley rats (n = 4 rats). The SCT injection of saline and placebo MS (2.5 mg particles per eye) were used as control. At POD 28, two rats were sacrificed to harvest the whole eyeballs with conjunctiva tissue for histological examination (n = 4 eyes). The injection site was marked with a 6-0 Nylon suture. The eyeballs were fixed in formalin, embedded in paraffin, sectioned along the anteroposterior axis (from cornea to optic nerve) to cut through the SCT injection site, and stained with the hematoxylin and eosin (H&E). The slides were observed and graded by a pathologist in a blinded manner.

2.7 The treatment of corneal NV

Corneal NV was induced by intrastromal suturing, as described previously [31, 32]. In brief, rats were anesthetized and their pupils were dilated prior to placing two intrastromal 10-0 nylon (Alcon Laboratories, Inc, Fort Worth, TX) suture stitches in the superior cornea under an operating microscope. The distance between the stitch and the limbus was approximately 2 mm, while there was a distance of 1 mm between the two stitches. After suturing, animals were immediately treated with a single SCT injection of 30 μ L of (1) PBS, (2) Placebo MS, (3) Sunb-malate MS (5 mg Sunb-malate/mL), and (4) Sunb-malate free drug solution (5 mg Sunb-malate/mL). Each group has n=6 rats (total 12 eyes). Erythromycin antibiotic ointment was applied to prevent potential infection and corneal drying. The rats were followed for 2 weeks.

2.8 Quantitative analysis of corneal NV

The corneas of all rats were examined by slit-lamp biomicroscope (SL120; Carl Zeiss AG, Oberkochen, Germany) and corneal photographs were taken with a digital camera by an ophthalmic photographer in a blinded manner. The area and length of vascularized cornea were quantified with Photoshop CS3.0 using the previously described method [31, 32]. An arc was drawn along the limbus, and the corneal NV area was calculated using the following equation:

$$\text{Corneal NV area} = \frac{\text{pixel of vascularized area}}{\text{pixel to occupy } 1 \text{ mm}^2 \text{ area}}$$

The vascularized area was evenly divided into six sections. The distance between vessel tips and the limbus at the five intersection points of the arc was measured. The five measured lengths were averaged to calculate the corneal NV length. These measurements were carried out in a blinded manner.

2.9 Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

At POD 7 and 14, three rats of each group were sacrificed and the corneas were collected. Because of the limited amount of cornea tissue from individual eyes, three corneas at the same condition were pooled together to get enough tissue for mRNA isolation and following measurement. Total mRNA was isolated with TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions, followed by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The mRNA expression levels of angiogenic and anti-angiogenic factors including VEGF, VEGFR1, VEGFR2, PDGF α , PDGF β , PDGFR α , PDGFR β , VE-cadherin, Ang1, MMP2, MMP9, bFGF and PECAM1, were quantified by RT-PCR with Fast SYBR® Green Master Mix using a 7100 Real Time PCR System (Applied Biosystems, CA). The primers are listed in Table S1. The mRNA expression levels were normalized to GAPDH. Each sample was repeated 3 times for the mRNA expression level quantification.

2.10 Immunostaining and confocal imaging

Eyes were enucleated and fixed with 4% paraformaldehyde (PFA) for 1 h at 4°C. Subsequently, the corneas were dissected, washed with PBS, cryoprotected in 15% sucrose PBS solution, and embedded in OCT compound. Serial corneal sections (30 μ m in thickness) were cut using a cryostat (Microm HM500 M), followed by immunostaining using the following antibodies: mouse platelet endothelial cell adhesion molecule-1 (PECAM-1, 1:500; Abcam), rabbit neural/glial antigen-2 (NG2, 1:500; Millipore), donkey anti-mouse Cy2 and donkey anti-rabbit Cy3 diluted in PBS containing 10% donkey serum and 0.1% Triton X-100. After overnight incubation at 4°C, the corneal sections were washed for three times in PBS and incubated with secondary antibody at room temperature for 2 h. The mounted corneal sections were imaged using Zeiss LSM 710 confocal microscopy (Carl Zeiss, Germany). To obtain the panoramic images of cornea, we serially aligned the corneal sections along the anteroposterior axis using Reconstruct 1.1.0 (J.C. Fiala, NIH) and performed a maximum-intensity projection.

2.11 Statistical analysis

The data are presented as the average $\pm$ standard error of the mean (SEM). Two groups were compared using two-tailed Student's t-test and three or more groups were compared using one-way ANOVA with Bonferroni correction. Differences were considered to be statistically significant at a level of $p < 0.05$. Significance for multiple comparisons: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3. Results

3.1. Ex vivo transscleral drug transport

Transcleral penetration is the primary route for drug to enter the eye following SCT administration. Drugs delivered via eye drops are often pharmaceutical salts, such as Sunb-malate, whereas the lower solubility acid or base version, such as Sunb-base is typically more amenable to formulation for sustained release. Thus, we compared the transcleral permeation rates of Sunb-base and more water-soluble Sunb-malate in an *ex vivo* assay

using freshly excised rabbit sclera. We observed a 10-fold increase in transscleral permeation of Sunb-malate compared to Sunb-base at 6 h (Fig. 1A). The calculated apparent scleral permeability coefficient (P), which is independent of time and surface area, was 10-fold higher for Sunb-malate ($11.9 \pm 2.7 \times 10^{-6}$ cm/s) compared to Sunb-base ($1.24 \pm 0.07 \times 10^{-6}$ cm/s) (Fig. 1B).

3.2. Preparation and characterization of Sunb-malate MS

Sunb-malate has limited solubility in DCM, but can be dissolved in DMSO up to 100 mg/mL. The Sunb-malate DMSO solution and PLGA DCM solution were mixed at 1:4 ratio to form a homogeneous organic phase, which was used to prepare Sunb-malate MS using the emulsion solvent evaporation method. Because of the use of 20% of DMSO in the organic phase during the microsphere preparation, the Sunb-malate MS were porous (Figure 2A). Sunb-malate MS exhibited an average particle size of 12 ± 5 μ m (Table 1, Figure S2), and a high drug loading of 7 wt% of water soluble Sunb-malate in the hydrophobic MS (Table 1). The *in vitro* drug release studies showed that Sunb-malate was released steadily for up to 25 days without the obvious initial burst release phase (Figure 2B), that may be expected for release of a water-soluble drug from PLGA microparticles.

3.2. Ocular drug retention

IVIS imaging showed that autofluorescent Sunb-malate free drug solution was completely cleared within 1 day after SCT injection (Figure 3A). Widespread fluorescence signal on the whole eyeball was likely the result of leakage from the injection site in the conjunctiva tissue. In comparison, SCT injection of Sunb-malate MS demonstrated sustained fluorescence in the subconjunctival space over a period of 28 days (Figure 3B and 3C). There was a rapid ~40% loss of the fluorescence signal within the first day following SCT injection, which was likely a result of leakage from the injection site. Approximately 50% of the total Sunb-malate dose was retained at POD 14, and gradually diminished over POD 28 as the MS degraded and released Sunb-malate over time (Figure 3C).

3.3. Ocular safety of Sunb-malate MS

In order to determine the long-term ocular safety, we carried out the histological examination of the eyes 4 weeks after SCT injection of Placebo-MS and Sunb-malate MS. SCT injection of 2.5 mg Placebo-MS did not show any signs of obvious inflammation and foreign body responses in the conjunctiva tissue, and the cornea and limbus were healthy as well. Both the low dose and high dose of Sunb-malate MS (30 μ L of 0.5 mg and 5 mg Sunb-malate /ml) following SCT injection also did not induce obvious inflammation and foreign body responses in the conjunctiva tissue at the injection site, the limbus area and the cornea (Figure 4).

3.4. Efficacy of Sunb-malate MS on corneal NV

All animals were examined by slit-lamp biomicroscopy at POD 5, 7 and 14 to evaluate the corneal NV under the treatment of Sunb-malate MS, Sunb-malate free drug and Placebo-MS. Radially-oriented new blood vessels invaded into the cornea from the limbus toward the suture stitches by POD 5 and further grew to reach the stitches by POD 14 for Placebo-MS

treated rats (Figure 5A). SCT injection of Sunb-malate solution did not show improvement over the Placebo-MS in inhibiting the ingrowth of corneal NV. There were no statistically significant differences in terms of corneal NV length (Figure 5B) and area (Figure 5C) between Sunb-malate solution and Placebo-MS treated groups, except for a small decrease in corneal NV area at POD 5 with Sunb-malate injection compared to Placebo-MS. In contrast, the Sunb-malate MS significantly suppressed the sprouting of new blood vessels at POD 5 and further slowed the ingrowth of corneal NV over time up to POD 14 (Figure 5B,C). The histopathological analysis further demonstrated that the ingrowth of new blood vessels, corneal inflammation, and corneal edema induced by the intrastromal suturing extensively existed in the Sunb-malate and Placebo-MS treated corneas, and were greatly suppressed at both POD 7 and 14 by the SCT injection of Sunb-malate MS (Figure 6).

3.5 Sunb-malate MS downregulated the mRNA expression levels of angiogenic effectors

The mRNA expression of angiogenic factors, endothelial cell markers and matrix metalloproteases was determined by RT-PCR at both POD 7 and POD 14. The quantitative analysis at POD 7 showed that the expression of angiogenesis-associated genes in the cornea, including VEGF, VEGFR1, PDGFb, PDGFRs, VE-cadherin, bFGF, MMPs and Ang1, was significantly decreased by SCT injection of Sunb-malate MS as compared to Placebo-MS and Sunb-malate free drug, although there was no statistically significant difference in the mRNA levels of VEGFR2 and PDGFa between Sunb-malate MS and Sunb-malate free drug treatment groups (Figure 7). We obtained a similar result at POD 14 (Figure S3), suggesting a continuous suppression of angiogenesis-associated gene expression with SCT injection of Sunb-malate MS.

3.6 Sunb-malate MS suppressed the mural cell recruitment

To investigate the effect of SCT injection of Sunb-malate MS on the ingrowth of vascular endothelial cells and the subsequent recruitment of vascular mural cells including pericytes around capillaries and smooth muscle cells around larger vessels, we collected the corneas following SCT injection of Sunb-malate MS for immunohistochemical analysis (Figure 8). The panoramic images of cornea showed that the growth of corneal vasculature was conspicuously suppressed by SCT injection of Sunb-malate MS when compared with SCT injection of Placebo-MS (Figure 8). Interestingly, the recruitment of NG2-positive mural cells was more mitigated than the PECAM-positive endothelial cells by SCT injection of Sunb-malate MS (Figure 8).

4. Discussion

Corneal NV represents an important cause of compromised corneal transparency and decreased visual acuity. Previous studies have demonstrated that VEGF is essential for induction of corneal NV in both human corneas and animal models. Therefore, current antiangiogenic treatment targeting against VEGF and VEGF-associated molecules offers a promising way to mitigate corneal NV [6, 33–36]. The blockade of VEGF signaling pathway by topical administration of bevacizumab reduced the corneal NV but was not sufficient for a profound inhibition [37]. Disruption of both VEGF and PDGF signaling with Sunb-malate or a combination of Sunb-malate and bevacizumab is more effective than

blocking VEGF alone in multiple angiogenesis models [37, 38]. Unfortunately, poor corneal penetration and the rapid drug clearance from the ocular surface greatly compromise the therapeutic efficacy of topical eye drops to treat ocular diseases. Thus, topical drugs have to be administered frequently, which can lead to poor patient compliance and compromised therapeutic efficacy [39, 40]. Here, we encapsulated Sunb-malate into biodegradable PLGA MS and provided sustained inhibition of the suture-induced corneal NV in rats.

SCT injection of water-soluble drugs was found to provide improved retention and increased drug levels in the aqueous compared to eye drops because of the depot effects from subconjunctival space [25, 41]. However, drugs can be cleared quickly following the SCT administration, typically within a few hours [25, 26]. Transscleral diffusion is believed to be one important route for intraocular drug penetration following SCT injection [25, 42], and water-soluble small molecule drugs showed improved transscleral drug penetration because of the much higher concentration gradients compared to hydrophobic drugs [29, 43, 44]. Water soluble dexamethasone sodium phosphate exhibited greater than 10-fold increase in transscleral drug penetration than hydrophobic dexamethasone *ex vivo* [29], leading to improved therapeutic efficacy for treating experimental autoimmune uveitis [29], corneal neovascularization [32] and corneal transplantation [26]. Sunb-malate can be dissolved in PBS at concentrations of at least 25 mg/mL, and we almost observed no drug retention in the eye within 24 h following the SCT injection of Sunb-malate free drug solution to rats. In our previous study, only 0.4% of original dose of water-soluble dexamethasone sodium phosphate remained at the injection site 2 h after SCT injection in rats [26]. Through the encapsulation of water-soluble Sunb-malate into biodegradable PLGA MS, we achieved a significantly longer retention of Sunb-malate up to 28 days with 50% drug retention at POD 14. Particles can be well retained in the injection site following the initial injection and particle leakage [26, 45]. We previously demonstrated that fluorescent non-degradable particles (100–500 nm) were well-retained in the conjunctiva tissue for at least 60 days after subconjunctival injection [26]. Enhanced retention of particles following SCT injection and sustained drug release contributed to prolonged drug retention in the injection site. The efficacy of SCT injection of Sunb-malate MS in inhibiting corneal NV was dependent on two key factors: (1) successful encapsulation of water-soluble Sunb-malate into biodegradable polymeric MS and (2) efficient intraocular penetration of water-soluble Sunb-malate released in the subconjunctival space. Indeed, we observed no effect on corneal NV in the suture-induced corneal NV model when we administered PLGA microspheres loaded with hydrophobic Sunb-base (data not shown). The glass transition temperature (T_g) of PLGA decreases upon exposure to water [46], leading to self-aggregation into a localized depot after intraocular injection [47–49]. The PLGA MS depot in the conjunctiva tissue will decrease in size as PLGA is degraded and gradually cleared by hydrolysis to produce lactic and glycolic acids, which will be eventually eliminated via the Krebs cycle by conversion to carbon dioxide and water [32].

Angiogenesis involves a complicated pathological process including the formation of endothelial tubes, the recruitment of mural cells and the upregulation of pro-angiogenic factors. Upregulation of VEGF promotes endothelial cell proliferation, migration and capillary tube formation [50] and the activation of PDGF signaling facilitates vascular mural cell recruitment to the endothelial tube [51, 52]. Sunb-malate selectively suppressed the

phosphorylation and activation of both VEGFR2 and PDGFR β [53]. We observed that blocking VEGF and PDGF signaling pathway with Sunb-malate MS inhibited the invasion of PECAM1-positive endothelial cells and, more strikingly, the recruitment of NG2-positive mural cells. We hypothesize that the continuous interference of multiple receptor tyrosine kinases with the sustained release of Sunb-malate from Sunb-malate MS causes the deficient endothelial cell-mural cell interactions and a significant loss of mural cells. Our studies contribute to the understanding of a potential cellular mechanism underlying the inhibition of corneal NV.

Furthermore, the upregulation of endothelial cell markers (VE-cadherin and PECAM1), metalloproteinases (MMP2 and MMP9), and proangiogenic factors and their receptors (VEGF, PDGFs, bFGF, Ang1, VEGFRs and PDGFRs) was largely abolished by SCT injection of Sunb-malate MS in the rats. MMPs participate in the degradation of extracellular matrix and the remodeling of vascular basement membrane, which are required for angiogenesis [54]. VEGF, PDGFs, bFGF and Ang1 were involved in regulating angiogenesis by binding and activating the corresponding receptor tyrosine kinases on the cell surface [55]. Here, sustained suppression of many pro-angiogenic factors and angiogenesis-associated proteases was achieved with Sunb-malate MS in the suture-induced animal model, demonstrating long-lasting anti-angiogenic activities at the molecular level.

5. Conclusion

Biodegradable PLGA microspheres successfully encapsulated high content Sunb-malate, and provided sustained release of water-soluble Sunb-malate and prolonged drug retention in the subconjunctival space for 4 weeks. SCT injection of Sunb-malate MS significantly inhibited corneal NV in the suture-induced corneal NV rat model, evidenced by significantly lower corneal NV area and vessel length, decreased mRNA expression of many pro-angiogenic factors and angiogenesis-associated proteases, and suppressed the mural cell recruitment. Sustained release of Sunb-malate by SCT injection of Sunb-malate MS could provide improved efficacy, good safety, and potential patient compliance. SCT injection of long-lasting Sunb-malate MS presents a potential therapeutic strategy for treating corneal NV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work has been supported by the Raymond Kwok Family Research Fund, the Andreas Dracopoulos Research fund, the George and Lavinia Blick Research Fund, the Eye Bank Association of America/Richard Lindstrom Research Grant 2013, Ralph E. Powe Junior Faculty Enhancement Award, the National Institutes of Health (R01EY027827, P30-EY001765, UG3DA048768), the FDA (HHSF223201810114C), and an unrestricted departmental grant from Research to Prevent Blindness (RPB).

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Figure 1.

Ex vivo trans-scleral diffusion of Sunb-malate solution (1 mg/mL in PBS) and Sunb-base suspension (1 mg/mL in PBS) with rabbit sclera. (A) Cumulative drug transport (μ g) and (B) apparent permeability coefficients (P) for Sunb-malate and Sunb-base. Mean $\pm$ SEM (standard error of mean); Student's t-test, *** $p < .001$; $n=3$ sclera tissues per time point.

Figure 2. (A) Scanning electron microscopy (SEM) image and (B) *in vitro* drug release profile of Sunb-malate MS. Mean $\pm$ SD (n = 3 repeat release samples).

Figure 3.

The representative IVIS fluorescence images of rat whole eyeballs enucleated at different time points after SCT injection of 30 μ L of (A) Sunb-malate (5 mg/mL in PBS) and (B) Sunb-malate MS (5 mg/mL Sunb-malate). (C) The ocular retention curve of Sunb-malate free drug and Sunb-malate MS following SCT injection in the rat eyes. Mean $\pm$ SEM, n=4 eyes per time point.

Figure 4.

The representative HE images of the eyes with SCT injection of 30 μ L of Placebo-MS (2.5 mg) and Sunb-malate MS (5 and 0.5 mg/mL Sunb-malate) at POD 28. (A) the limbus area containing both conjunctiva and cornea (magnification 100x), (B) the conjunctiva of the injection site (magnification 400x), and (C) the cornea (magnification 400x).

Figure 5.

(A) The representative slit lamp examination photos and the quantitative analysis of (B) corneal neovascularization area and (C) vessel length of the corneas with intrastromal suturing following the SCT injection of 30 μ L of Sunb-malate MS (5 mg/mL Sunb-malate), Sunb-malate solution (5 mg/mL Sunb-malate) and Placebo MS (2.5 mg) at POD 5, 7, and 14. Mean $\pm$ SEM; n=6 corneas per time point; Student's t-test, * p < 0.05; ** p < 0.01; *** p < 0.001.

Figure 6.

Representative histological images of rat corneas with intrastromal suturing following SCT injection of 30 μ L of (A) Placebo-MS (2.5 mg), (B) Sunb-malate (5 mg/mL Sunb-malate), and (C) Sunb-malate MS (5 mg/mL Sunb-malate) at POD 7 and POD14. (D) (A) Histology images of a healthy rat cornea. Magnification 200x.

Figure 7.

RT-PCR analysis revealed the strong suppression of the mRNA expression levels of angiogenic effectors by SCT injection of 30 μ L of Sunb-malate MS (5 mg/mL Sunb-malate) compared to Sunb-malate free drug (5 mg/mL Sunb-malate) and Placebo MS (2.5 mg) at POD 7. Mean $\pm$ SEM; n=3 repeats from the pooled 3 corneas of the same condition; Student's t-test, * p < 0.05; ** p < 0.01; *** p < 0.001.

Figure 8.

SCT injection of Sunb-malate affects corneal vascular growth and morphology. (A) The representative photo of the whole cornea tissues with intrastromal suturing following the SCT injection of 30 μ L of Placebo-MS (2.5 mg) and Sunb-malate MS (5 mg/mL Sunb-malate) at POD 14. (B) Vascular endothelial cells and (C) vascular mural cells were immunostained with anti-PECAM-1 (green) and anti-NG2 antibodies (red), respectively. (D) The merged confocal images of the immunostained cornea tissues. Suturing positions are marked with an asterisk. Scale bar: 400 μ m.

Table 1.

Physiochemical characteristics of PLGA microspheres

	Size (μm)	Surface charge (mV)	Drug loading
Sunb-malate MS	12 ± 5	-1.1 ± 0.2	7%
Placebo-MS	14 ± 9	-0.7 ± 0.2	N/A

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