

# A Novel Eye Drop Formulation for Potential Treatment of Neovascular Age-Related Macular Degeneration

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**Purpose:** Drug delivery to posterior ocular tissues via topical eye drop administration is arduous due to the unique anatomy and physiology of the eye. Therefore, treatments for posterior eye disease have to be administered via intravitreal injection or systemic route, both of which have their drawbacks. Herein, the objective of this work was to demonstrate that a specially designed eye drop formulation could effectively deliver small-molecule vascular endothelial growth factor (VEGF) inhibitor to posterior ocular tissues for antiangiogenic therapy.

**Methods:** The unique eye drop formulation, termed ITRI AXN eye drops, was obtained from self-assembly of (2-hydroxypropyl)- $\beta$ -cyclodextrin with a VEGF tyrosine kinase inhibitor, a hydrophilic polymer, hypromellose, and a complex stabilizer, caffeine. In vivo ocular pharmacokinetics studies were performed with New Zealand White (NZW) rabbits and Non Human Primates (NHP). The antiangiogenesis effect was evaluated on the Long-Evans rat with laser-induced choroidal neovascularization and pigmented Dutch-Belted rabbits with VEGF-induced retinal neovascularization.

**Results:** The successful drug transport from ocular surface to posterior ocular cavity was indicated by a drug biodistribution pattern in pharmacokinetic studies. Excellent drug exposure in the choroid and retina with the concentrations of 900- and 750-fold greater than drug IC<sub>50</sub> 0.5 hours post the eye drop administration (drug level: 0.8%) was observed on the NHP study. The obtained formulation also demonstrated a comparable antiangiogenic outcome with the intravitreal injection of anti-VEGF antibody on rat and rabbit disease models.

**Conclusions:** Our eye drop formulation has demonstrated great promise in antiangiogenic therapy against retinal and choroidal neovascularization in animal models. The results suggest that the aim of this work can be successfully achieved by the novel eye drop formulation.

**Translational Relevance:** The preclinical results provide evidence that ITRI AXN eye drops could effectively deliver therapeutics to the choroid and retina for antiangiogenic therapy.

## Introduction

Neovascular age-related macular degeneration (nAMD), which manifested abnormal retinal and choroidal angiogenesis, has been recognized as a leading cause of blindness in developed countries.<sup>1</sup>

Age- and diabetes-mediated dysfunctions of endocrine system are considered as the major risk factors to induce nAMD.<sup>2</sup> Intravitreal injection (IVT) of anti-vascular endothelial growth factor (anti-VEGF) antibody has been served as the standard treatment against nAMD since 2006.<sup>3</sup> However, patient's fear of IVT, long-term periodic injection, and risk of IVT adverse

events such as bleeding, endophthalmitis, retinal detachment, and elevated intraocular pressure render the treatment imperfect and patient compliance is low.<sup>4,5</sup> Therefore, patients and doctors have yearned for a noninvasive treatment for nAMD.

Topical eye drops provide advantages over intravitreal injection due to its noninvasive, self-administering, and easy-to-use characteristics. However, the applications of eye drop products for nAMD treatment have been hindered due to the insufficient drug exposure at target sites (retina and choroid). This is why several new eye drop pipeline developments have been terminated in clinical stages.<sup>6,7</sup> The prerequisite of eye drop applications for nAMD treatment is effective drug delivery capable of overcoming the drug elimination via ocular clearance (including reflux tearing and aqueous humor outflow) and multiple ocular tissue barriers (such as tightly packed corneal epithelium, blood-aqueous barrier, and blood-retinal barrier).<sup>8</sup>

Several vascular endothelial growth factor (VEGF) tyrosine kinases inhibitors (TKIs) have been approved for cancer treatment due to their antiangiogenesis effect. One of them is axitinib, which was approved in 2012 by the United States Food and Drug Administration (FDA) for treatment of renal cell carcinoma. This drug is designed to be served as a high potent angiogenesis inhibitor by selectively blocking vascular endothelial growth factor receptors (VEGFR1, VEGFR2, and VEGFR3) and platelet-derived growth factor receptor (PDGFR).<sup>9</sup> Despite the success of axitinib and other VEGF TKIs in cancer treatments, the ophthalmic applications for nAMD are still impeded by its poor bioavailability in the posterior eye.<sup>10-12</sup>

Previously, we have developed a formulation with an enhanced ocular tissue-penetrating function and demonstrated superior bioavailability of a soft steroid drug, loteprednol etabonate, in anterior ocular tissues, compared to its commercial eye drop formulation.<sup>13</sup> In this work, the formulation was adapted and loaded with a previously FDA-approved VEGF tyrosine kinase inhibitor for developing an effective drug delivery system to treat choroidal and retinal angiogenesis. The eye drop formulation, termed as ITRI AXN eye drops, is a clear solution, the individual ingredient of which has been used in FDA-approved topical ophthalmic products. In vivo ocular pharmacokinetics were performed with New Zealand White (NZW) rabbits and Non Human Primates (NHP) to evaluate drug distribution after the eye drops administration. Ocular irritation and corrosion were conducted on New Zealand White rabbits according to the test guideline of OECD-405. The antiangiogenic effect of ITRI AXN eye drops was examined with the retinal neovascularization (RNV) model in rabbits and laser-induced

choroidal neovascularization (CNV) model in rats, respectively.

## Materials and Methods

### Materials

(2-hydroxypropyl)- $\beta$ -cyclodextrin with an average molar substitution of hydroxypropyl group of 0.67 was purchased from Shandong Binzhou Zhiyuan Biotechnology Co., Ltd. (China). Hydroxypropyl methylcellulose (HPMC, Pharmacoat 2910) was supplied by Wei Ming Pharmaceutical Mfg. CO., Ltd. (Shin-Etsu, Japan). Caffeine was purchased from Siegfried PharmaChemikalien Minden GmbH (Germany). Glacial acetic acid (Pharma grade) and axitinib were purchased from Sigma-Aldrich (Germany) and Shilpa Medicare Limited (India), respectively. Distilledwater (UNISS, Taiwan) was used for the preparation of all solutions.

### Preparation of ITRI AXN Eye Drops

ITRI AXN eye drops were obtained from assembly of (2-hydroxypropyl)- $\beta$ -cyclodextrin with a tyrosine kinase inhibitor, axitinib, a hydrophilic polymer, hypromellose, and a complex stabilizer, caffeine. In brief, axitinib powder was first dissolved in glacial acetic acid, followed by mixing with an aqueous solution containing HPMC, caffeine, and HP $\beta$ CD with a certain ratio under sonication. The mixture was spray-dried and further desiccated under vacuum oven at 120°C for 24 hours to remove all of the solvents. To obtain the eye drops solution, the resulting powder was rehydrated with sterile water (water for injection), followed by filtration with a 0.2  $\mu$ m pore-sized filter. Drug content in the eye drop formulation was analyzed using HPLC. Sample osmolality was analyzed by Vapor Pressure Osmometer 5600, and the residual glacial acetic acid in the obtained formulation was determined by high performance liquid chromatography (HPLC).

### Animal Studies

All animal study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Care and Use Committee of National Defense Medical Center. A total of 42 male Long-Evans rats, weighing 200 to 250 g, were purchased from National Laboratory Animal Center, Taiwan. Adult male pigmented Dutch Belted (DB) and New Zealand

White (NZW) rabbits, weighing 2 to 2.5 kg, were purchased from Da-Zong pasture and HUEIJYUN breeding facility (Changhua City, Taiwan). Ten male cynomolgus monkeys, weighing 2.4 to 3.6 kg were used for nonhuman primate pharmacokinetic study, which is carried out by a contract research organization of Sundia MediTech Company, Ltd. The rats were housed in cages with stainless-steel mesh floors and allowed free access to food and water. The rabbits were housed in a rabbit laboratory of the animal center of National Chiao Tung University (NCTU). The housing conditions were kept under 12 hours light and 12 hours dark automatically, air conditioned  $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , and humidity-controlled at 40% to 70%. To ensure the health of the animals, clinical observation and recording were performed by veterinarians of NCTU and investigators of Industrial Technology Research Institute (ITRI) during the period of quarantine and experiment daily, respectively.

### Pharmacokinetic Study in NZW Rabbits

Rabbit eyes have been widely used for ophthalmic research due to their similar ocular size to human eyes. Six NZW rabbits were topically administered with 35  $\mu\text{L}$  of the eye drop formulation into both eyes by positive displacement pipette and gently restrained for 2 minutes after the dosing. At 0.5, 1.0, and 3.0 hours post dosing, two rabbits were euthanized at each time interval and ocular tissues including cornea, conjunctiva, aqueous humor, vitreous humor, choroid, and retina from both eyes of each rabbit were collected. Tissue homogenate containing drug were processed by deprotein method. The sample was oscillated for 5 minutes and then centrifuged at 12,000 rpm for 5 minutes. Ten microliter supernatant was analyzed by the liquid chromatography - tandem mass spectrometry (LC-MS/MS) system consisting of the Agilent HPLC 1200 with a Waters C18 column (XBirdge, C18, 3.5  $\mu\text{m}$ , 4.6 mm  $\times$  20 mm, Waters) using gradient elution and signal detection by the API 4000 mass spectrometer. A LC-MS/MS method has been developed for the quantitative determination of axitinib in NZW rabbit ocular tissue homogenate. The mass spectrometer was operated in the positive electrospray ionization (ESI) mode.

### Pharmacokinetic Study in Monkeys

NHPs were utilized for pharmacokinetic study due to their similarity to humans in ocular anatomy, especially the macula only existing in primates. The study was to investigate the ocular axitinib concentration of ITRI AXN eye drops following topical ocular administration in cynomolgus monkeys. Each animal

received a 30  $\mu\text{L}$  instillation of the ophthalmic solution (0.8%) into the conjunctival sac per eye. Both eyes were treated with the ocular formulation three times per day (dosing interval: 4 hours). Two animals per study group were euthanized at 1 hour, 6 hours, and 24 hours postdosing. The eyes were enucleated and dissected for collection of aqueous humor, choroid-RPE, cornea, iris-ciliary body, lens, retina, sclera, and vitreous humor. All solid ocular tissues needed to be rinsed three times, then stored at  $-70^{\circ}\text{C}$  until analysis. The tissue samples were quantitated by the LC-MS/MS.

### Bioanalysis of Ocular and Plasma Samples

The organic solvent used to extract the samples was 100% acetonitrile with internal standards (IS) for axitinib in all matrices. Propranolol was used as IS for quantification. The 60  $\mu\text{L}$  vitreous humor, lens, retina, choroid-RPE, and sclera samples were mixed with 180  $\mu\text{L}$  ACN containing IS in each EP tube. The 20  $\mu\text{L}$  aqueous humor, cornea, and iris-ciliary body samples were mixed with 60  $\mu\text{L}$  ACN containing IS in each EP tube. All mixtures were vortexed for 10 minutes and centrifuged for 10 minutes (13,000 rpm,  $4^{\circ}\text{C}$ ). Then, supernatant was transferred to a 96-well plate with twofold water and shaken for 10 minutes. Finally, 4  $\mu\text{L}$  supernatant was injected into the LC-MS/MS system for analysis.

The LC-MS/MS system used in the studies consists of an AB Sciex QTRAP 5500 (Applied Biosystems, Foster City, CA), a high-performance liquid chromatography (HPLC) system (Shimadzu, Columbia, MD) and an autosampler (Shimadzu). The HPLC were performed on XSelect HSS T3 C18 Column (2.5  $\mu\text{m}$ , 2.1 mm  $\times$  50 mm; Waters Corp., Milford, MA) at flow rate of 0.8 ml/min and ACQUITY UPLC HSS T3 C18 Column (1.8  $\mu\text{m}$ , 2.1 mm  $\times$  50 mm; Waters Corp.) at flow rate of 0.5 ml/min for various monkey samples. The mobile phase composition and gradient status were listed in Table 1. The phase A mixed the methanol, acetonitrile, formic acid, 1M ammonium acetate, and water at 100:100:4:8:3800 ratio to obtain phase A containing 2 mM ammonium acetate and 0.1% formic acid in water with 5% ACN:MeOH = 1:1. In addition, the phase B mixed the methanol, acetonitrile, formic acid, and 1M ammonium acetate at 2000:2000:4:8 ratio to obtain phase B containing 2 mM ammonium acetate and 0.1% formic acid in ACN:MeOH = 1:1. Working standards at concentrations in the 0.1 to 50 ng/mL range were prepared for axitinib. The original data for this study was collected and calculated by AB Sciex mass spectrometer software analyst 1.6.1. Data statistics and calculations were performed on Microsoft Office Excel.

**Table 1.** Mobile Phase Composition and Gradient Status in HPLC Analysis

Mobile Phase	Time (min)	Phase A (%)	Phase B (%)
		2 mM NH <sub>4</sub> AC&0.1%FA in 5% (ACN:MeOH = 1:1) in Water	2 mM NH <sub>4</sub> AC&0.1%FA in (ACN:MeOH = 1:1)
Gradient elution	0.10	80	20
	1.40	40	60
	1.50	5	95
	2.40	5	95
	2.50	80	20
	3.00	80	20

### In Vivo Ocular Irritation Test

In vivo irritation of the proposed formulation was evaluated according to OECD guideline for the testing of chemicals (OCDE 405). The eye drop formulation was instilled into both eyes of each rabbit ( $N = 3$ ). Each eye received 35  $\mu$ L formulation solution three times per day (dosing interval: 3 hours). The ophthalmic irritation signs were evaluated at predosing, 1 hour after each dose and 24 hours after the initial dose by clinical observation and slit-lamp microscopy.

### VEGF-Induced RNV Model on Rabbits

To mimic pigmented human eyes in the event that the drug binds to melanin, the pigmented DB rabbits were utilized for nAMD model establishment and evaluation of therapeutic efficacy. Pigmented rabbits were sedated with 0.1 mg/kg atropine (Astar) subcutaneous injection. A combination of 10 mg/kg tiletamine-zolazepam mixed agent (Zoletil50) and 6 mg/kg xylazine (Rompun) were injected intramuscularly for anesthesia. The animal eyes were topically anesthetized with 1 to 2 drops of 0.5% Alcaine (Alcon) and induced mydriasis by instillation of a mydriatic (Mydrin-P). Antibiotic ointment was administered to the lower lid of the eyes. After the previous procedures, 1  $\mu$ g recombinant human VEGF 165 (Peprotech) was injected into the vitreous by using a 29-gauge needle to establish the retinal neovascularization model.<sup>14</sup>

### Pharmacodynamic Evaluation of the Obtained Eye Drop

After the VEGF injection, the animals were randomly divided into three groups ( $N = 3$ ): VEGF group received one dose of intravitreal phosphate buffered saline as the control group. Avastin group received one dose of intravitreal bevacizumab (Avastin). The eye drop group was topically treated with 35  $\mu$ L of the ITRI AXN eye drops (drug content: 0.8% (w/v)) four times a day during the study period.

Fundus fluorescence angiography (FA) examinations were conducted using Heidelberg Spectralis HRA (Heidelberg Engineering) to evaluate the pharmacodynamics. Baseline examination was performed before the VEGF injection, and follow-up examinations were performed at day 2 and day 4 after the VEGF injection. After the anesthetized procedure as previous described, the fluorescein was administered into the marginal ear vein. Photographs of the fundus were taken within 10 minutes after the fluorescein injection. Leakage area was quantitatively measured by fluorescein angiography image analysis based on brightness using open-source software (ImageJ) to assessment the pharmacodynamics.<sup>15</sup>

### Laser-Induced CNV Model on Rats

To establish a rat CNV lesion model, adult male Long-Evans rats were anesthetized by ketamine (35 mg/kg, intramuscular injection) and xylazine (5 mg/kg, intramuscular injection). Then, all animals were administered 2.5% phenylephrine HCL and 1% atropine sulfate to dilate the pupils. Six argon laser spots (150 mW, 100 ms, 100  $\mu$ m; MC-500 Multicolor laser, Nidek) were applied to each fundus around the optic disc.

After CNV induction, the rats were randomly divided into six groups ( $N = 6$ ): Vehicle group topically received the solution of drug-free formulation three times daily as the control group. Eylea group received one dose of intravitreal aflibercept. The eye drop groups were topically treated with the eye drop formulations (20  $\mu$ L/dose) with different drug levels from 0.2% to 1.2% (w/v) three times a day for 14 days.

### Fundus Photography and Fluorescein Angiography

The cornea was anesthetized with topical Alcaine (0.5%) and the pupils were dilated with atropine (1%) and phenylephrine (10%). Methylcellulose eye gel was applied to prevent the dehydration of the cornea during

imaging. The retinal fundi of the rat were viewed and photographed with a Micron IV fundus camera (Phoenix Research Labs, Inc., Pleasanton, CA). All images were collected by using the specialty Micron IV software (StreamPix; NorPix, Inc., Montreal, Quebec, Canada). Following a subcutaneous injection of 0.1 mL 2.0% fluorescein sodium solution (Fluorescite 10%; Alcon), fluorescein angiographs were viewed and captured with a green filter attached to the Micron III fundus camera at 5 minutes post injection. Images were exported as tagged image files (.tif) and fluorescence intensity was quantified by using a custom macro in ImageJ v1.47.

### Optical Coherence Tomography Acquisition and Analysis

A Phoenix Micron IV retinal microscope with image-guided OCT was used for imaging. OCT was performed using an SD-OCT, which provides a longitudinal resolution of 1.8  $\mu\text{m}$  and a transverse resolution of 3  $\mu\text{m}$  with a 3.2 mm field of view and 1.2 mm imaging depth at the retina. After general anesthesia, the rats were placed on the imaging platform, and the head was positioned at an angle to allow the penetration of light vertical to the cornea from the temporal side. The retinal structure was obtained by linear scanning around the lesion site. At least three clear captures were obtained for each eye. The center of the lesion was defined as the midline passing through the area of RPE-Bruch's membrane rupture. OCT was only utilized for monitoring the CNV model establishment, especially for the bubble formation in Bruch's membrane. The rat eye with the lesions containing no bubble or bubble with hemorrhage was excluded from this study.<sup>16</sup>

### Statistics

For single pairwise comparisons, Student's *t*-test was applied. In all tests, *P* values < 0.05 were considered to be statistically significant. Data were presented as arithmetic mean  $\pm$  standard error of mean (SEM).

## Results and Discussion

### Characterization of ITRI AXN Eye Drops

Topical administration of ITRI AXN eye drops was designed to serve as an alternative treatment of nADM, to replace the current invasive intravitreal injection of an anti-VEGF antibody. Though the aqueous solubility of axitinib is rather low, approximately 0.2  $\mu\text{g}/\text{mL}$ ,<sup>17</sup> the prepared formulation was a clear solution with a

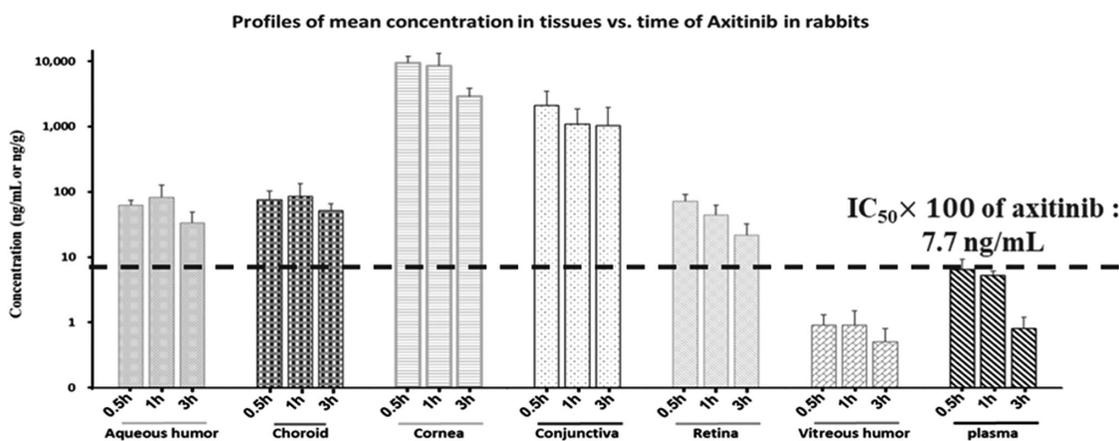
concentration up to 8 mg/ml, which was made possible by forming the highly water-soluble supramolecular complexes between drug molecules and cyclodextrins, assisted by the selectively chosen water-soluble polymer, HPMC, and caffeine, all of which form ternary complexes and stabilization of HP $\beta$ CD/drug association, respectively.<sup>13,18</sup> The rationale for why the caffeine could serve as a complexation stabilizer is possibly attributed to the sealing effect of caffeine to fill up the residual space of HP $\beta$ CD after drug encapsulation within the cavity of HP $\beta$ CD. Similar phenomena have also been observed in the other eye drop formulation as shown in our previous work.<sup>13</sup> The axitinib content in the eye drop formulation was 8.494  $\pm$  132.1% (w/v%) according to HPLC analysis. The pH, osmolality, and viscosity of the eye drop formulation were measured to be 6.75, 580  $\pm$  2.89 mOsm/kg and 9.26 cP, respectively, all of which falls into acceptable criteria of ophthalmic eye drops. The eye drops are chemically and physically stable at 25°C/45% relative humidity for at least 6 months and at 40°C/25% relative humidity for at least 3 months, without any significant variation in product appearance and drug content (variation less than 10%). Detailed stability data was described in Table 2. The level of residual acetic acid in each sample is lower than 400 ppm, which is below the limit (5000 ppm) according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline Q3C-R7. The resulting product also met the acceptance criteria in sterility test according to guidance described in United States Pharmacopeia Chapter <71>. These results suggest that ITRI AXN eye drops exhibit appropriate features for eye drop applications.

### Pharmacokinetic Studies in Rabbits and NHP

The ocular pharmacokinetics of topical administration of ITRI AXN eye drops were assessed. The exposure criteria was set 100-fold above the half maximal inhibitory concentration (IC<sub>50</sub>) of axitinib (100-fold of axitinib IC<sub>50</sub>: 7.7 ng/mL) to overcome the possible tissue and plasma protein binding (99%).<sup>17</sup> As shown in Figure 1, biodistribution pattern of axitinib given as a single dose of the eye drop formulation to NZW rabbit eyes demonstrated that the drug exposure at the retina and choroid was 935- and 980-fold greater than IC<sub>50</sub> of drug. In comparison, topical administration of 2% axitinib suspension on rabbit eyes resulted in drug exposure on the retina less than the quantification limit of the current analytic method (0.5 ng/mL). These results demonstrate efficient drug delivery to posterior ocular tissues of our formulation.

**Table 2.** Stability Studies of the Eye Drop Formulation at Various Storage Temperatures and Humidity

Sampling Interval	Storage Conditions	
	25°C/40%RH Drug Content Variation	40°C/25%RH Drug Content Variation
Day 0	106%	103%
1 month	105%	105%
2 months	109%	107%
3 months	106%	104%
4 months	108%	–
5 months	109%	–
6 months	108%	–



**Figure 1.** Biodistribution of axitinib in NZW rabbits after ocular administration of ITRI AXN eye drops.

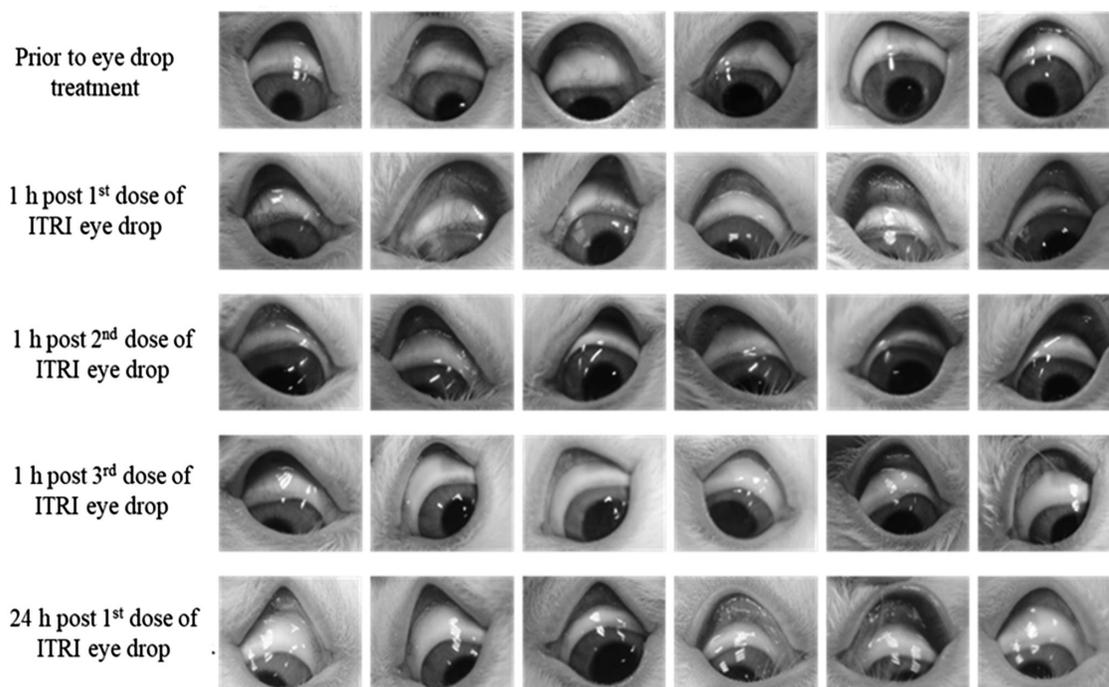
Cynomolgus monkeys were also utilized for the ocular pharmacokinetic study because only primates have a macula. Following topical ocular administration of ITRI AXN eye drops to the monkey eyes three times daily, the mean concentrations of axitinib in aqueous humor, choroid-RPE, cornea, iris-ciliary

body, lens, retina, sclera, and vitreous humor at each time interval were shown in Table 3. The accuracy of quality control samples was between 87.1% ~120%. The results showed that the drug level in the retina and choroid remains high and above preset therapeutic concentrations over the time period of 24 hours,

**Table 3.** Ocular Biodistribution of Axitinib in Monkeys After Topical Administration of ITRI AXN Eye Drops

Tissue/Time Point	1 Hour Mean ± SD	6 Hours Mean ± SD	24 Hours Mean ± SD
Aqueous humor (ng/mL)	3.9 ± 3.2	4.6 ± 1.3	1.4 ± 0.9
Choroid-RPE (ng/g)	71.0 ± 32.5	61.7 ± 38.8	64.7 ± 32.3
Cornea (ng/g)	2757.0 ± 1022.0	2787.0 ± 2013.3	806.3 ± 519.9
Iris-ciliary body (ng/g)	80.9 ± 31.9	320.7 ± 164.8	644.1 ± 310.4
Lens (ng/g)	32.5 ± 11.3	12.0 ± 6.5	12.6 ± 6.2
Retina (ng/g)	55.5 ± 66.9	22.4 ± 15.6	5.9 ± 2.0
Sclera (ng/g)	669.5 ± 703.1	501.5 ± 377.7	444.3 ± 105.8
Vitreous humor (ng/mL)	8.7 ± 17.0	1.2 ± 1.5	0.4 ± 0.3
Plasma (ng/mL)	27.3 ± 8.5	23.1 ± 23.1	4.6 ± 4.9

The 6<sup>th</sup> hour sampling was 2 hours after the second dose and the 24<sup>th</sup> hour sampling was 24 hours after the first dose.



**Figure 2.** Representative pictures of the in vivo rabbit ocular irritation test. The NZW rabbit treated with 30  $\mu$ L of ITRI AXN eye drops three times daily.

except for the 24 hour time point of retina, which was 5.9 ng/g, lower than our preset therapeutic concentration criteria, which was 100 ng/g. Drug retention in the choroid and iris-ciliary body was observed, which is likely attributed to the melanin binding of axitinib in pigmented monkeys.<sup>19,20</sup> The drug reaching the retina or other low melanin tissues (lens, sclera, aqueous humor, vitreous humor, etc.) was eliminated faster, compared to the choroid and iris-ciliary body.

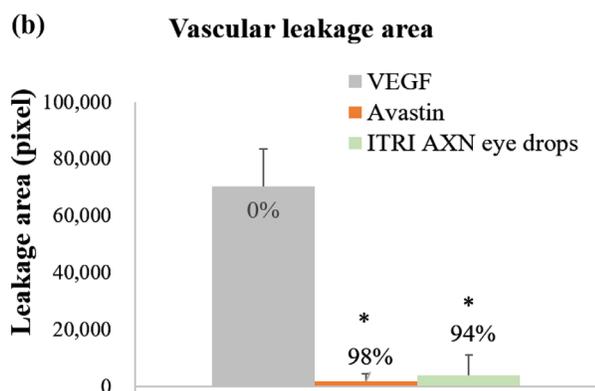
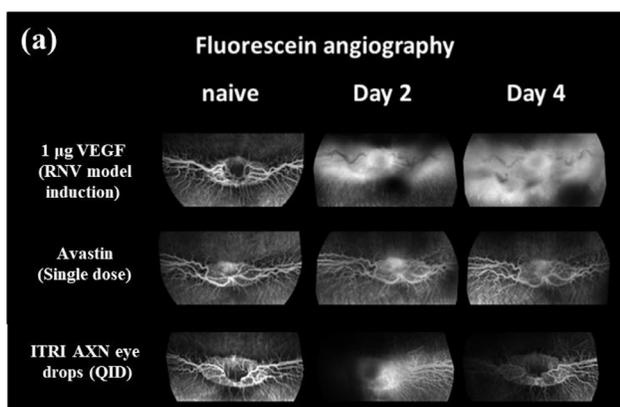
### Ocular Irritation Study

The safety of ITRI AXN eye drops in terms of the acute ocular irritation and corrosion was evaluated on the rabbits which received the eye drop three times daily. The assessment was performed in accordance with the test guideline of OECD-405. As shown in Figure 2, all rabbits receiving ITRI AXN eye drops at preset time intervals demonstrated a rather benign effect on rabbit eyes without any ocular irritation and corrosion, as evidenced by a lack of apparent side effects on corneal structure and integrity (average total score 0). These data strongly suggest the nonirritant property of the obtained formulation.

### Antiangiogenesis Effect on Animal Disease Model

#### Retinal Neovascularization Model

The antiangiogenic effects of ITRI AXN eye drops on retinal neovascularization model of rabbits were evaluated. VEGF protein was given intravitreally at day 0 to induce retinal vascular leakage to simulate exudative neovascular age-related macular degeneration (nAMD). The fluorescein, a fluorescent tracer, was intravenously administered for observation of blood leakage by fundus fluorescence angiography. As shown in Figure 3a, significant blood leakage at either optic disc or medullary wings was observed 2 days post VEGF injection, suggesting the successful production of a disease model. This phenomenon persisted at least for 2 days (from day 2 to day 4). The blood leakage in the optic disc of the Avastin (anti-VEGF antibody)-treated group was dramatically reduced at day 2 and day 4, resulting from the neutralization reaction of the anti-VEGF antibody against VEGF. The substantially reduced intraocular VEGF level is inadequate to generate angiogenesis and alter vascular permeability in retina. Similar blood leakage inhibition was observed in the group topically receiving the ITRI AXN eye drops with a 94% reduction of vascular leakage area

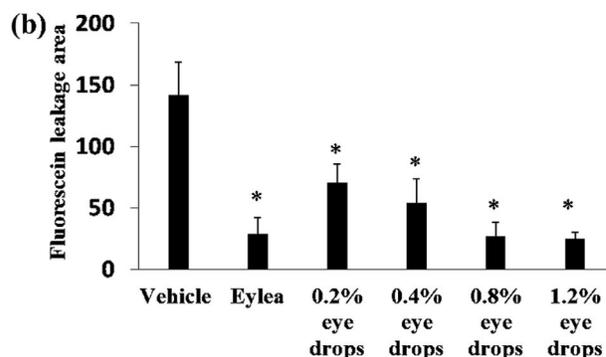
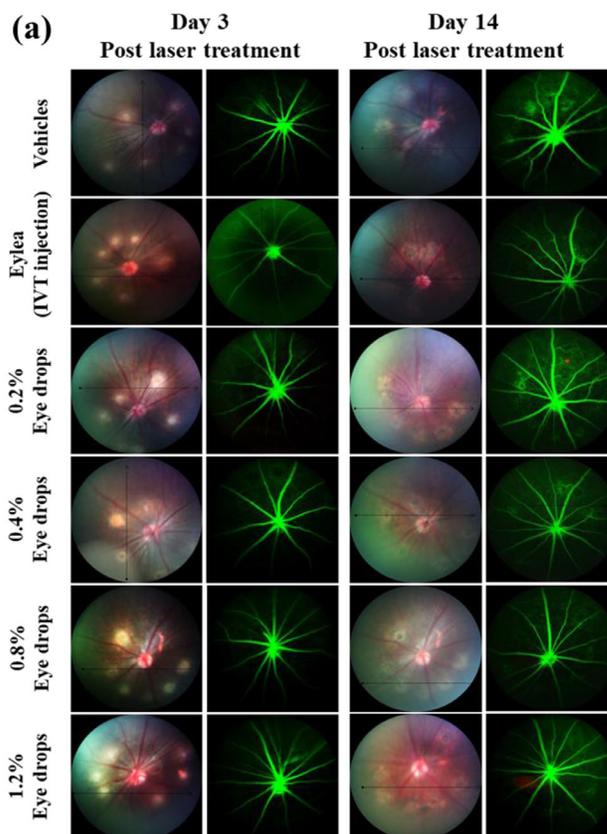


**Figure 3.** (a) Fluorescein angiography prior to induction of retinal neovascularization model and posttherapeutic treatment with either intravitreal injection of Avastin or topical administration of ITRI AXN eye drops on DB rabbits at day 2 and day 4. (b) Quantification of vascular leakage area from the intravenously injected fluorescein signals in fluorescein angiography of the rabbits treated with either Avastin or ITRI AXN eye drops at day 4. Symbols and error bars are mean  $\pm$  SD \* $P$  < 0.05, compared to VEGF group.

as compared to 98% reduction of the Avastin group shown in Figure 3b. The results obviously demonstrated the superior therapeutic potential of the eye drop approach for nAMD treatment.

### Choroid Neovascularization Model

The laser-induced CNV model, the most commonly used and mainstay model for nAMD therapeutic evaluation, was utilized to evaluate the dose effects. Six laser spots were applied in the rat’s eyes. Laser injury on Bruch’s membrane located between the retinal pigment epithelium and the capillary lamina of the choroid induces angiogenesis capable of modeling the hallmark pathology of neovascular AMD.<sup>21</sup> To determine dose-dependent therapeutic outcome, the eye drop formulations with the drug levels of 0.2% to 1.2% (w/v) were topically administered to the rats four times per day for 14 days. Rats intravitreally injected with Eylea, the first-line available anti-VEGF antibody drug for nAMD treatment, were served as the positive control. The



**Figure 4.** (a) Fundus photography and fluorescein angiography of the rats treated with either intravitreal injection of Eylea or topical administration of the eye drop formulations with the drug level in a range of 0 (vehicle) to 1.2% (w/v) at day 3 and day 14 post CNV induction. (b) Quantification of vascular leakage area from the intravenously injected fluorescein signals in fluorescein angiography of the rats treated with either Eylea or the eye drop formulations at day 14. Symbols and error bars are mean  $\pm$  SD \* $P$  < 0.05, compared to vehicle group.

angiogenesis progression in each group was monitored at day 3 and day 14 post laser-induced CNV induction. Data revealed that six CNV lesions could be observed in each group without significant hemorrhage at day 3 (Fig. 4a), suggesting the successful establishment of a CNV model without blood vessel damage directly induced by the laser. A great reduction of

blood leakage was observed at day 14 in the group intravitreally injected with Eylea, while the fluorescein signal of the group topically receiving the vehicle remained high. This indicates the pronounced antiangiogenic effect of anti-VEGF antibodies. Similar to that, the eye drops-treated groups all demonstrated significant retardation of blood leakage ( $P < 0.05$ ) in comparison with the vehicle group (Fig. 4b). Moreover, the higher the drug level in the eye drop formulation, the better the antiangiogenic effect until therapeutic plateau was reached for the 0.8% and 1.2% eye drops, as evidenced by the comparable outcome between 0.8% and 1.2% eye drops. There is no statistically significant difference between the groups of Eylea and 0.8% eye drops. These results clearly demonstrated the great potential of the eye drop approach for replacement of anti-VEGF injections in nAMD treatment.

## Discussion

VEGF is the key mediator of angiogenesis in nAMD, which leads to abnormal blood vessels that leak fluid or blood into the macula. In this study, we formulated an eye drop with VEGF inhibitor, termed ITRI AXN eye drops, and the pharmacokinetic and pharmacodynamic results showed that sufficient drug reached the choroid and retina leading to similar treatment efficacy as intravitreal injected VEGF antibody. This may be clinical translational relevance in ophthalmology and medicine.

The superior delivery efficiency was made possible by combining caffeine and HP $\beta$ CD, which form stable supermolecular complex with the poorly water-soluble axitinib, leading to a clear aqueous solution in high-level axitinib content. The drug aqueous solubility was substantially enhanced from ca 0.2  $\mu\text{g}/\text{mL}$  to at least 12,000  $\mu\text{g}/\text{mL}$ . The pharmacokinetic studies of rabbits and NHP showed that the drug exposure at the retina and choroid was 700- to 900-fold greater than  $\text{IC}_{50}$  of drug after topical administration of the eye drops. It should be noted that the other eye drop formulations have also shown promise in ocular drug delivery in preclinical studies but failure in clinical trials due to lack of efficacy.<sup>6,7,22,23</sup> The clinical trial failures were possibly attributed to the insufficient drug exposure at target tissues, arising from the significant difference between drug tissue binding in animals and humans. Horita et al. have found species differences in ocular pharmacokinetics and pharmacological activity of regorafenib and pazopanib eye drops among rats, rabbits, and monkeys.<sup>24</sup> The drug tissue binding in different species was a major factor affecting the

unbound drug fraction in target tissues to exert therapeutic effect. Jousseaume et al. developed a regorafenib eye drop formulation for nAMD treatment but terminated the study in phase IIa.<sup>6</sup> They analyzed the failure and proposed that a threshold of the unbound drug level in target tissues for exerting therapeutic efficacy should be set so that drug tissue binding issue can be overcome. To this end, the drug exposure threshold of 100-fold drug  $\text{IC}_{50}$  (axitinib  $\text{IC}_{50}$ : 7.7  $\text{ng}/\text{mL}$ ) to overcome the possible tissue and plasma protein binding (99%) has been set as a major criterion in this work for developing our eye drop formulation. Consistent with the pharmacokinetic results shown above, the effective antiangiogenic effect on target tissues by using ITRI AXN eye drops were demonstrated in rabbits and monkeys.

To evaluate therapeutic potential of the eye drop approach, the preclinical animal models of retinal and choroidal neovascularization, which have been considered as major features of nAMD, were employed in this work. It should be noted that melanin is an important pigment widely existing in human ocular tissues, such as the iris, ciliary body, choroid, and retinal pigment epithelium.<sup>25</sup> The presence of melanin could affect the drug distribution in ocular tissues after topical drug administration.<sup>26–28</sup> To this end, the pigmented animals, DB rabbits and Long-Evans rats, were utilized for nAMD model establishment. VEGF was intravitreally injected into the rabbit eyes for induction of the retinal neovascularization model. After being treated with the eye drop formulation, a comparable antiangiogenic outcome to the group injected intraocularly with anti-VEGF antibody to neutralize VEGF was observed. This demonstrates the great antiangiogenic potential of the eye drop approach for nAMD treatment. Antiangiogenesis study on the other nAMD model of choroid neovascularization, which is the mainstay model for nAMD therapeutic evaluation, was also performed. Again, the eye drop formulations showed a significant antiangiogenic effect, even in those low-dose groups (0.2% and 0.4% eye drops). The decrease of blood vessel leakage in the choroid was associated with elevated drug levels in eye drop formulations. Maximum therapeutic effect was observed in the group of 0.8% and 1.2% eye drops. There is no statistically significant difference between the groups of Eylea and 0.8% eye drops. These results signified that the eye drop approach can be utilized for replacement of anti-VEGF injections in nAMD treatment.

Ocular irritation assessment was also evaluated on rabbits. After receiving the eye drop formulation three times daily, the score for ocular lesions is zero, reflecting that the rabbit eyes remained rather benign without occurrence of acute irritation and corrosion.

In conclusion, ITRI AXN eye drops demonstrated a great potential in drug delivery to posterior ocular cavity with a rather high-level drug exposure at the retina and choroid. By virtue of the sufficient therapeutic level in target sites of nAMD, the obtained eye drop formulation shows a great promise in antiangiogenic outcome against the retinal and choroidal neovascularization model in vivo while eye irritation remains rather benign. These results suggested that the goal of this study can be successfully achieved by our formulation. We believe further clinical studies are justified.

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