Ocular Penetrance and Safety of the Dopaminergic Prodrug Etilevodopa

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Purpose: Animal models have demonstrated the role of dopamine in regulating axial elongation, the critical feature of myopia. Because frequent delivery of dopaminergic agents via peribulbar, intravitreal, or intraperitoneal injections is not clinically viable, we sought to evaluate ocular penetration and safety of the topically applied dopaminergic prodrug etilevodopa.

Methods: The ocular penetration of dopamine and dopaminergic prodrugs (levodopa and etilevodopa) were quantified using an enzyme-linked immunosorbent assay in enucleated porcine eyes after a single topical administration. The pharmacokinetic profile of the etilevodopa was then assessed in rats. A four-week once-daily application of etilevodopa as a topical eye drop was conducted to establish its safety profile.

Results: At 24 hours, the studied prodrugs showed increased dopaminergic derivatives in the vitreous of porcine eyes. Dopamine 0.5% (P = 0.0123) and etilevodopa 10% (p = 0.370) achieved significant vitreous concentrations. Etilevodopa 10% was able to enter the posterior segment of the eye after topical administration in rats with an intravitreal half-life of eight hours after single topical administration. Monthly application of topical etilevodopa showed no alterations in retinal ocular coherence tomography, electroretinography, caspase staining, or TUNEL staining.

Conclusions: At similar concentrations, no difference in ocular penetration of levodopa and etilevodopa was observed. However, etilevodopa was highly soluble and able to be applied at higher topical concentrations. Dopamine exhibited both high solubility and enhanced penetration into the vitreous as compared to other dopaminergic prodrugs.

Translational Relevance: These findings indicate the potential of topical etilevodopa and dopamine for further study as a therapeutic treatment for myopia.

Introduction

The prevalence of myopia (“nearsightedness”) is increasing at an alarming pace. In the past 50 years, myopia prevalence in the United States and Europe has doubled and in China has jumped from 20% to 90% of the population.¹ In the next 30 years, high myopia is expected to more than triple to near 10% of the global population.² Although there is substantive economic burden from refractive errors alone, high myopia carries significant visual morbidity including increasing risk of cataract, peripapillary deformation, optic neuropathy, retinal detachment, myopic degeneration, myopic foveoschisis, retinoschisis, macular holes, dome-shaped macula, and choroidal neovascularization—many of which cause irreversible vision loss.³

The critical feature of myopia is axial length elongation of the eye, which is responsible for both the refractive changes and pathologic consequences of myopia. Myopia is caused by excessive axial length elongation. Axial length homeostasis is a complex process that is incompletely understood. Currently evolving theories involve a visual stimuli trigger that is transduced from the retina to the sclera through chemical signals, eventually affecting scleral remodeling.⁴ Neurotransmitters, proteases, and growth factors have all
been implicated in this process. One such regulator is dopamine, a neurotransmitter released from amacrine and interplexiform cells of the retina, which has been shown to affect axial elongation.

While dopamine has not been clinically validated to date, pre-clinical data suggests that it is a critical regulator of axial elongation. Dopaminergic agents (i.e., dopamine, apomorphine, levodopa, ADTN, SKF-38393, quinpirole) appear highly effective at reducing axial length in animal models of myopia. Similarly, dopamine antagonists appear to halt this antimyopic effect (haloperidol, SCH23390, spiperone, sulpiridine, and 6-OHDA). Since initial studies with subconjunctival injections of apomorphine in chicks, dopaminergic agents have been shown to inhibit axial elongation in chicks, rabbits, guinea pigs, mice, tree shrews, and macaques. Dopaminergics are effective in inhibiting both form deprivation myopia, and lens-induced myopia, and have shown efficacy whether delivered systemically via the intraperitoneal route or locally via subconjunctival or intravitreal injection.

Unlike anticholinergic agents currently in use, dopaminergic agents have limited effects on accommodation and, without crossover antimuscarinic activity, have no effect on pupillary dilation. In animal testing, dopaminergics are more effective at preventing myopia. Whereas even low-dose atropine 0.01% has shown potential toxicity on electroretinography (ERG), dopaminergics have not exhibited any toxicity. On the basis of these data, dopaminergic compounds appear to inhibit axial elongation and therefore may be candidates for the treatment of myopia.

A major factor limiting clinical development of dopaminergic compounds for the treatment of myopia has been the method of delivery. Most dopaminergic compounds are either poorly soluble or have limited intraocular penetration when applied topically. Myopia progression for juvenile-onset myopia primarily occurs between the ages of 6 and 21 years of age. Frequent delivery of dopaminergic agents via peribulbar, intravitreal, or intraperitoneal injections is not clinically viable. It is therefore necessary to develop topical dopaminergic agents that have high ocular penetration allowing for topical delivery in children with progressive myopia. A potential solution is the use of dopaminergic prodrugs. Levodopa, a dopamine prodrug, has demonstrated efficacy at slowing axially elongation in the chick after topical administration. However, it is significantly less effective when delivered topically rather than intravitreally. Enhancing both lipophilicity and solubility with esterification is expected to further enhance corneal penetration. Such an approach has the potential to achieve a higher intravitreal dopamine concentration after topical administration. Etilevodopa is an ester prodrug of levodopa and is hypothesized to result in improved intracocular penetration after topical administration. This study sought to assess the ex vivo ocular penetration and in vivo safety of etilevodopa.

### Methods

#### Ocular Penetration of Dopamine and Dopaminergic Derivatives Ex Vivo

To assess the vitreous penetration, dopamine (dopamine hydrochloride; Sigma-Aldrich, St. Louis, MO, USA), and etilevodopa (BOC Sciences, Shirley, NY, USA) were freshly dissolved in phosphate buffered saline solution (PBS, pH 7.4; Aniara, West Chester, OH, USA) at the concentration of 0.5%, whereas levodopa (Sigma-Aldrich) was freshly dissolved in distilled water at 0.5%. Thirty microliters of each solution was placed onto the cornea of enucleated porcine eyes (Animal Technologies, Tyler, TX, USA; at least six porcine eyes were studied for each treatment), which were no more than 30 hours since enucleation and were maintained on ice throughout experimentation. PBS was used as a vehicle control. The vitreous humor sample was collected after 24 hours for quantitative measurement of dopaminergic derivative level using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturers’ protocol (dopamine ELISA kit; Immuno-Biological Laboratories, Inc., Minneapolis, MN, USA). The penetration to both anterior chamber and vitreous of etilevodopa at 10% (this concentration represented the maximum solubility of etilevodopa) was further studied at 0, 0.5, 1, 6, 12, 18, 24, 48, and 72 hours to establish a time-penetration curve (at least three porcine eyes were studied at each time point).

#### Animals

The use of rats followed the animal study guidelines of the Association of Research in Vision and Ophthalmology and was approved by the Administrative Panel on Laboratory Animal Care (APLAC) at Stanford University. Both male and female Long Evans rats (six weeks of age; body weight 150 to 200 g; Charles River, Wilmington, MA, USA) were housed at constant temperatures, with a 12-hour light/dark cycle and food and water available as desired.
Pharmacokinetics of Topical Etilevodopa in Long Evans Rat Eyes

To assess the short-term pharmacokinetic effect, 5 μL of etilevodopa 10% was instilled onto a rat eye. At one, two, four, eight, and 24 hours, rats were sacrificed (at least three rats were studied at each time point). Serum was obtained by allowing the blood sample to clot at room temperature for 0.5 to 1 hour, followed by centrifuging at 13,000 rpm for 10 minutes (MiniSpin Eppendorf; Thermo Fisher Scientific, Waltham, MA, USA). Serum was then frozen at −80°C until tested. Eyes were enucleated and collected for quantitative measurement of dopaminergic derivative levels. Concentrations of dopaminergic derivative in both whole eye and vitreous/retina were measured for pharmacokinetic evaluation. Briefly, tissue samples were incubated with 150 μL lysis buffer (CelLytic MT Cell Lysis Reagent; Sigma-Aldrich) for 0.5 to 1 hour, followed by centrifuging at 13,000 rpm for 10 minutes, and the supernatant was collected. All samples were immediately frozen at −80°C until tested. The level of dopaminergic derivative was measured in both serum and eye using a dopamine ELISA kit (Immuno-Biological Laboratories, Inc.) according to the manufacturer’s instruction.

To assess the long-term pharmacokinetic effect, 5 μL of etilevodopa 10% was administered once daily to a rat eye. At 14 and 28 days, rats were sacrificed, and serum was obtained for measurement of dopaminergic derivative levels (four rats were studied at each time point).

Clinical Examination, Optical Coherence Tomography (OCT) Imaging and ERG Measurement

Etilevodopa 10% 5 μL was administered as an eye drop once daily to one eye of each rat for 28 consecutive days, and the contralateral eye received PBS as a control. To assess the tolerability after administering etilevodopa over time, clinical examination was conducted at 14 and 28 days during treatment. To assess retinal structure and function, spectral-domain OCT (Spectralis HRA + OCT instrument; Heidelberg Engineering, Heidelberg, Germany) and full-field ERG (D300; Diagnosys LLC, Lowell, MA, USA) were also performed at 14 and 28 days. Briefly, rats were first anesthetized with intraperitoneal injection of a mixture of ketamine hydrochloride (75 mg/kg; Hospira, Inc., Lake Forest, IL, USA) and xylazine (5 mg/kg; Bedford Laboratories, Bedford, OH, USA). The corneas were topically anesthetized with tetracaine 0.5% (Alcon Laboratories, Inc. Fort Worth, TX, USA), and the pupils were dilated with tropicamide 1% (Akorn, Inc., Lake Forest, IL, USA) and phenylephrine hydrochloride 2.5% (Akorn, Inc.).

For clinical examination, 1% methylcellulose and a plastic coverslip were applied to the cornea to enhance visualization. A Zeiss OPMI MDO S5 Microscope (Prescott’s, Inc., Monument, CO, USA) with an Excelis camera (ACCU-SCOPE, Inc., Commack, NY, USA) was used to digitally record ocular photos.

For OCT, 1% methylcellulose and a coverslip were placed on the anesthetized rat cornea. A commercially available 78-D double aspheric fundus lens (Volk Optical, Inc., Mentor, OH, USA) was mounted in front of the OCT and images were taken with proprietary software (Eye Explorer, version 3.2.1.0; Heidelberg Engineering). A raster scan of 19 equally spaced horizontal B-scans centered on the optic nerve was captured.

For full-field ERG, rats were dark-adapted overnight. A gold wire loop was placed on the cornea of both eyes, a reference electrode was placed subcutaneously on the nose, and a ground electrode was placed in the tail. ERG responses were recorded from both eyes simultaneously. The flash intensity was set at six increasing intensities of 0.0001, 0.001, 0.01, 0.1, 1, and 3 cd · s/m². ERG recordings were averaged over 10 presentations of a single one-millisecond flash with a 10-second interstimulus interval. The A-wave amplitude was measured from the baseline to trough, while the B-wave was measured from the trough to peak.

Histology and Immunohistochemistry

To assess for inflammation and apoptosis, 5 μL of etilevodopa 10% was administered as a once-daily eye drop to one eye of each rat, and the contralateral eye received PBS as a control. Rat eyes were enucleated at 14 and 28 days after treatment and fixed in 4% paraformaldehyde (VWR, Visalia, CA, USA) overnight at 4°C, dehydrated in graded series of sucrose and alcohol, frozen, and cut into 12 μm sections onto glass slides.

For inflammation, frozen sections were first fixed in 10% formalin (Sigma-Aldrich) for 20 minutes and washed in water, followed by staining the nuclei with hematoxylin for three minutes and washing in water again. The slides were then dipped in lithium carbonate (bluing agent) for 45 seconds, washed in water, immersed in 95% ethanol (2-3 dips), and stained with eosin for another three minutes. After dehydrating in 95% ethanol for one minute and then in 100% ethanol for two minutes, the slides were mounted with Cytoseal
60 and imaged using Nikon Eclipse E800 microscope (Nikon Corp., Tokyo, Japan).

For apoptosis, sections were stained with cleaved caspase-3 (Cell Signaling, Beverly, MA, USA). Briefly, sections were first washed with washing buffer (0.1% Triton X-100 in PBS) three times for five minutes each, blocked with 5% bovine serum albumin (Fraction V; Sigma-Aldrich) in washing buffer for one hour, followed by incubating in primary antibody-cleaved caspase-3 (1:200 in washing buffer) for two hours. After washing three times for five minutes each, sections were incubated in secondary antibody (AlexaFluor 488 donkey anti-rabbit IgG; 1:400; Invitrogen, Waltham, MA, USA) in washing buffer for one hour, followed by washing another three times and mounting with 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting media (Vectashield; Vector Laboratories, Burlingame, CA, USA). Slides were imaged using Leica DMI8 microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was also performed to further detect apoptosis using TUNEL label mix kit (Sigma-Aldrich). TUNEL reaction mixture was prepared using the nucleotide-labeling mix combined with the TUNEL enzyme (Sigma-Aldrich), and the assay was performed according to the manufacturer’s instructions.

Statistical Analysis

Statistical analyses were performed using Prism 9 (Graphpad Software, San Diego, CA, USA). All results are expressed as mean ± SEM and the differences among groups were assessed with statistical tests noted in the figure legends. A P value < 0.05 was considered to be statistically significant.

Results

Ocular Penetration of Dopamine and Dopaminergic Derivatives Ex Vivo

The vitreous penetration of topical dopamine and dopaminergic drugs was assessed using enucleated porcine eyes. As shown in Figure 1, all studied drugs, that is, dopamine, levodopa and etilevodopa, at 0.5% were able to penetrate the eye and reach the vitreous (Fig. 1) at 24 hours after treatment, with a concentration of 375.1 (P = 0.0123), 11.9 (P = 0.0028), and 9.7 ng/mL (P = 0.0047), respectively. Etilevodopa achieved significant vitreous concentrations when applied as a 10% solution (36.2 ng/mL, P = 0.0370). The time-penetration profile of etilevodopa at 10% was further studied over a 72-hour period (Fig. 2). After a single topical administration into a porcine eye, etilevodopa could continuously diffuse into the anterior chamber (Fig. 2A) and vitreous (Fig. 2B), reaching a peak concentration of 87.3 and 87.7 ng/mL, respectively at 72 hours. As a reference, in porcine eyes receiving PBS vehicle control, dopaminergic derivatives in the anterior chamber were 0.86 ng/mL and in the vitreous 3.3 ng/mL.

In Vivo Pharmacokinetics of Etilevodopa

The in vivo 24-hour pharmacokinetic profile following a single topical treatment of etilevodopa 10% is shown in Figure 3A and the Table. A peak concentration of 7797.4 and 118.9 ng/mL were achieved in the whole eye and retina/vitreous at one hour after the treatment, respectively. This concentration declined to 62.1 ng/mL for the whole eye and 39.9 ng/mL for the retina/vitreous at eight hours after the treatment and maintained this level until 24 hours. In serum, a maximum concentration of 5.7 ng/mL was also achieved one hour after drug treatment, and the concentration fell to 0.48 ng/mL 24 hours after treatment. For the whole eye, etilevodopa has an area under the curve (AUC) of 4298.5 ng/mL, a half -life (T1/2) of 1.4 hours, and a mean resident time of 1.4 hours, whereas in the retina/vitreous, it has an AUC of...
Figure 2. Ex vivo pharmacokinetic profile of anterior chamber (A) and vitreous (B) penetration of etilevodopa 10% over 72 hours. Etillevodopa continuously diffused into the anterior chamber and vitreous of porcine eye from the ocular surface over a 72-hour period after a single administration of topical etilevodopa 10%. The results are graphed as a log-linear plot. All values represent the mean ± SEM, and at least three porcine eyes were studied for each time point.

Figure 3. In vivo pharmacokinetic effect of 24 hours (A) and four weeks (B) treatment of etilevodopa. (A) Concentration of dopaminergic derivatives at 24 hours was quantified in whole Long Evans rat eye, retina/vitreous and serum after a single treatment of etilevodopa 10% as eye drop. The concentrations declined over 24 hours. The results are graphed as a log-linear plot. All values represent the mean ± SEM, n ≥ 3. (B) Concentration of dopaminergic derivatives in the rats serum at 14 and 28 days after once-daily topical administration of etilevodopa 10%. All values represent the mean ± SEM, and four rats were studied at each time point.

Table. Noncompartmental Analysis of Dopaminergic Derivative Concentrations in the Whole Rat Eye, Retina/Vitreous, and Blood After a Single Topical Treatment of Etillevodopa 10% In Vivo

<table>
<thead>
<tr>
<th>Group</th>
<th>AUC_{0→∞} (ng/mL)</th>
<th>T_{1/2} (h)</th>
<th>Mean Resident Time (h)</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole eye</td>
<td>4298.5</td>
<td>1.4</td>
<td>1.4</td>
<td>0.95</td>
</tr>
<tr>
<td>Retina/Vitreous</td>
<td>817.5</td>
<td>8.0</td>
<td>11.7</td>
<td>0.56</td>
</tr>
<tr>
<td>Blood</td>
<td>11.2</td>
<td>3.4</td>
<td>4.6</td>
<td>0.95</td>
</tr>
</tbody>
</table>

AUC, area under the curve; T_{1/2}, half-life.

817.5 ng/mL, a T_{1/2} of 8.0 hours and a mean resident time of 11.7 hours. In the blood, it has an AUC of 11.2 ng/mL, a T_{1/2} of 3.4 hours and a mean resident time of 4.6 hours. Of note, the dopaminergic derivative concentration of retina/vitreous and serum in untreated Long Evans rats were found to be 20.16 and 0.10 ng/mL, respectively.

Figure 3B shows the dopaminergic derivatives concentration in the rat serum at 14 and 28 days after once-daily etilevodopa administration. A concentration of 0.47 and 0.25 ng/mL was achieved at 14 and 28 days, respectively, which was comparable to serum concentration at 24 hours after a single treatment of etilevodopa (i.e., 0.48 ng/mL).
Clinical, Structural, and Functional Assessments

After once-daily treatment of etilevodopa 10% eye drop, the overall retina morphology and blood vessels remained unchanged (Fig. 4A) at 14 and 28 days. The cornea and lens remained clear throughout the duration of the study. No retinal detachment, edema or inflammation was observed in either the PBS control or the treatment groups. Figure 4B compares the OCT images of the retina of rats receiving PBS control or etilevodopa 10% treatment. OCT did not demonstrate any qualitative structural changes in the retina at 14 and 28 days. Retinal function after the daily treatment of etilevodopa was measured using ERG recordings of the rod, cone, and mixed response. Figure 4C

Figure 4. Clinical, structural and functional assessment of Long Evans rat eyes after topical administration of etilevodopa. Etilevodopa 10% 5 μL was administered as an eye drop to one eye of each rat once daily, while the contralateral eye received PBS as control. Representative photos of a rat eye (A) at days 14 and 28 show no evidence of inflammatory response, corneal opacity, fundus abnormalities, or conjunctival redness. Ocular coherence tomography (OCT) images of the retina (B) demonstrate no qualitative changes in retinal structure at 14 or 28 days. (C) Comparison of electroretinography (ERG) waveform of etilevodopa with PBS control treated eyes at 14 and 28 days. No statistically significant changes in the A-wave amplitude (D) or B-wave amplitude (E) were observed at studied time points. All values represent the mean ± SEM and 4 rats were studied at each time point. Statistical comparisons were performed with two-way ANOVA followed by Sidak analysis.
shows the ERG waveforms of rat eyes treated with PBS control and etilevodopa at 14 and 28 days. The range of fluctuation of the ERG amplitudes of etilevodopa treated eyes was similar to control eyes. The comparison of both of A- and B-wave amplitude between the treated and control eyes are shown in Figures 4D and 4E, and there was no statistically significant difference in A- or B-wave amplitudes at either 14 or 28 days.

Inflammation and Apoptosis

Histological observations including hematoxylin and eosin staining and immunohistochemistry assay (cleaved caspase-3 and TUNEL staining) were performed to further study the intraocular compatibility of etilevodopa in rat eyes. Hematoxylin and eosin staining (Fig. 5) shows that the retinal layer was intact at 14 and 28 days in both PBS control and etilevodopa treated rat eyes, with no infiltration of inflammatory cells, fibrosis or morphological changes. Next, apoptotic biomarkers, cleaved caspase-3 and TUNEL were labeled separately to further assess the effect of etilevodopa (Figs. 6 and 7).31,32 Figure 6 demonstrates the expression of cleaved caspase-3+ DAPI+ cells predominately located in inner nuclear layer in both PBS control and treatment groups at studied time points. There was no TUNEL+ DAPI+ cells observed in both PBS control and etilevodopa-treated groups at 14 and 28 days (Fig. 7). Of note, sections treated with DNase I to induce DNA stand breaks before TUNEL labeling procedure (according to manufacturer’s instruction, Sigma-Aldrich) were studied as positive control (Supplementary Fig. S1).

Discussion

Animal models of myopia have consistently demonstrated a regulatory role of dopamine in the development of myopia. This occurs via dopaminergic amacrine cells that signal to the retinal pigment epithelium and choroid, leading ultimately to scleral remodeling and retinal neurogenesis. Given the potential role of dopamine in regulating axial elongation and the feasibility of topical administration for long-term treatment, dopaminergic agents have been explored as topical therapeutics. Unfortunately, dopaminergic agents often have poor solubility and ocular penetration limiting their usefulness as topical agents. The goal of this investigation was to determine whether improved ocular penetration would occur with dopaminergic prodrugs.

We assessed ocular penetration of dopamine and dopaminergic derivatives, including levodopa and etilevodopa. Levodopa is a dopamine precursor that is converted into dopamine via dopamine decarboxylase. It has recently been shown to be effective at slowing myopia progression in chicks after intravitreal administration and, to a lesser extent, after topical administration.25,29 Esterification has been an effective means of enhancing ocular penetration through improved solubility and penetration through the corneal stroma. Accordingly, dopaminergic ester prodrug, etilevodopa,
was synthesized and ocular penetration was compared to levodopa. Etilevodopa is an ester prodrug of levodopa, and levodopa has demonstrated efficacy in preventing myopia progression in animal models when administered intravitreally, and to a lesser extent topically. It was therefore expected that etilevodopa would exhibit enhanced solubility and ocular penetration, making it a superior therapeutic myopia candidate.

We initially assessed ocular penetration in vitreous in enucleated porcine eyes (<30 hours old). Porcine eyes served as our ex vivo model as the globe size, cornea thickness, ratio of length of the cornea to eye-globe diameter, and histological structure of porcine eyes mimic the human eye. For direct comparison, all drugs were freshly prepared at 0.5% (the maximum concentration achievable with levodopa). An ELISA was used to quantify the presence of dopaminergic derivatives in the anterior chamber and vitreous. In vehicle-treated eyes, there was a higher quantity of dopaminergic derivatives in the vitreous compared to the anterior chamber. This is consistent with dopamine’s role as a retinal neurotransmitter. Because dopamine diffuses from the retina, through the vitreous, and into the anterior chamber, vitreous concentrations are expected to be higher than the anterior chamber. Prior studies in humans have found a 3.5-fold higher concentration of vitreous dopamine (0.7 ng/mL) as compared to anterior chamber dopamine (0.2 ng/mL). This study found a similar ratio of dopaminergic derivatives in control-treated eyes—0.86 ng/mL in the anterior chamber versus 3.3 ng/mL in the vitreous. The higher levels of background dopaminergic-

Figure 6. Cleaved caspase-3 immunostaining analysis of rat eyes at 14 and 28 days after topical treatment of 5 μL etilevodopa 10% or PBS once daily. (A) Cleaved caspase-3+ DAPI+ immunostaining was primarily observed in the inner nuclear layer of both PBS control group eyes and treatment group eyes at day 14 and 28 days. Scale bar: 50 μm. (B) Bar graph shows no statistically significant change in the number of cleaved caspase-3+ DAPI+ cells between control and treatment groups at studied time points. All values represent the mean ± SEM. Statistical comparisons were performed with two-way ANOVA followed by Sidak analysis.
gic derivatives stem from quantification of not just dopamine but other dopaminergic derivatives (like 3,4-Dihydroxyphenylacetic acid (DOPAC)) and possible increased dopamine release from the retina after enucleation of porcine eyes. At 24 hours, all studied drugs at 0.5% showed increased dopaminergic derivatives into the vitreous (Fig. 1). We found that dopamine at 0.5% had the highest level of ocular penetration. Despite etilevodopa being an ester prodrug, it did not exhibit enhanced corneal penetration as compared to levodopa at a given concentration. However, because of its enhanced solubility, etilevodopa can readily achieve higher concentrations (10% etilevodopa vs. 0.5% levodopa) in standard physiological buffers facilitating clinical translation use. Interestingly, dopamine 0.5% exhibited the highest degree of ocular penetration, with a 10-fold higher intravitreal concentration as compared to etilevodopa 10% after topical administration in porcine eyes. There are several physiological and anatomical factors that can affect drug penetration through the cornea. The cornea consists of a lipophilic epithelial layer on the outside, a hydrophilic stromal layer in the middle and a much less lipophilic endothelial layer on the inside. The lipophilic layer can hinder the penetration of hydrophilic drugs, whereas the hydrophilic layer prevents the penetration of lipophilic drugs. Thus, drug molecules need to be amphiphilic in order to pass through the cornea. Through this mechanism, ester prodrugs have consistently demonstrated enhanced corneal penetration and explain the improved anterior chamber and vitreous concentrations that were observed. However, unlike other ester prodrugs, we were unable to demonstrate improved corneal penetration after topical administration of etilevodopa as compared to levodopa. One possible explanation is that etilevodopa is rapidly hydrolyzed on the ocular surface before passing through the cornea. Another possibility is that etilevodopa and levodopa are primarily passing through the conjunctiva/sclera and not the cornea, making esterification less useful. Additional research is needed to further elucidate these differences.

Similar to the ex vivo results, etilevodopa was able to enter the posterior segment in vivo (Fig. 3). After a single topical administration, a peak concentration of 7797.4, 118.9 and 5.7 ng/mL of etilevodopa was achieved in the whole rat eye, retina/vitreous and serum, respectively, at one hour (Fig. 3), and it reduced to 68.4, 30.3 and 0.48 ng/mL, respectively, at 24 hours. Increased dopamine levels are expected to increase dopamine receptor activity, which has been demonstrated to alter axial elongation. Although direct measurements of myopic eye growth after topical treatment of etilevodopa were not performed in this study, previous studies have shown that topical levodopa treatment slowed ocular growth and inhibited form deprivation myopia development in a dose-dependent manner in chicks. Our work is the first to show ocular penetration with topical etilevodopa treatment in rats, and the finding supports the topical application of dopaminergic compounds as a potentially viable treatment approach for myopia. Additionally, based on our ex vivo data, etilevodopa 10% exhibited a higher vitreous penetration than levodopa 0.5% (etilevodopa: 36.2 ng/mL, levodopa: 11.9 ng/mL, $P = 0.0079$). As a reference, Thomson et al. demonstrated an EC$_{50}$ for...
a reduction of axial elongation with topical levodopa of 0.05%. They further showed a stronger effect size with an intravitreal administration of levodopa (EC50 of 0.0008 mM in a 10 μL dose). Using a chick vitreous volume of 200 μL, this EC50 is an intravitreal concentration of approximately 7.9 ng/mL. All tested compounds achieved intravitreal concentrations above this EC50 in our ex vivo model. However, the ex vivo porcine model significantly understimates losses because of tear film turnover and conjunctival blood flow. Therefore, in an in vivo model, only dopamine 0.5% and to a lesser extent etilevodopa 10% would be expected to exceed the intravitreal EC50 concentration required to prevent axial elongation.

Dopamine is a major neurotransmitter in the vertebrate retina and plays a significant role in regulation of center-surround antagonism. It has both a synaptic and paracrine effect. Given its role in retinal signal processing, concern for retinal toxicity has been raised. Controversy still remains as to potential toxicity of levodopa due to increased oxidative stress on striatal neurons in Parkinson’s disease. Although in vitro studies have demonstrated toxic effects, most in vivo studies have failed to replicate such toxicity. In our study, four-week application of etilevodopa as a topical eye drop showed no evidence of direct toxicity. Structural testing with OCT did not demonstrate any change in retina morphology at 14 and 28 days, whereas functional testing with ERG failed to identify reductions in A- or B-wave amplitude (Fig. 4). Histology and immunohistochemistry demonstrated that etilevodopa did not change retinal architecture or induce any inflammation or cell death (apoptosis) compared to PBS control (Figs. 5–7). Together, these initial safety studies suggest that topical etilevodopa may have a safety profile that supports exploration as a long-term therapeutic treatment for myopia.

Delivery of dopaminergic agents via peribulbar, intravitreal, or intraperitoneal injections through the wall of the eye can lead to several complications such as ocular irritation, inflammation, conjunctivitis, internal ocular bleeding, and formation of cataracts, especially with repeated injections. These complications are not associated with topical eye drops. It is known that topical drug treatment has potential off-target effects due to systemic distribution. However, in this study, the serum concentration at 14 and 28 days after once-daily etilevodopa treatment were comparable to the concentration after a single treatment at 24 hours (Fig. 3), suggesting that the systemic distribution for etilevodopa is limited, and it does not accumulate in the body after repeated daily treatment, which may minimize any nonocular off-target side effects. Further supporting these findings, we observed no obvious changes in rat behavior during the 28-day study, including activity levels, aggression, feeding pattern, and weight gain. However, additional testing is required to confirm the lack of effect on behavioral changes.

Despite our positive results, there were a number of limitations that should be addressed. Anatomic differences between rat and human eye can impair translation of pharmacokinetic studies. Furthermore, we used a dopamine ELISA that has high cross-reactivity with dopaminergic derivatives. Although the ELISA detected levodopa and etilevodopa with similar sensitivity, it had slightly better sensitivity for dopamine. This may have resulted in an underestimation of the total quantity of levodopa and etilevodopa in the vitreous. Although these safety studies showed no evidence of toxicity, our study was only conducted for 28 days, which may be too short to identify long-term effects of etilevodopa administration. Future long-term safety studies will be needed. There is currently no clinical data available on the use of topical etilevodopa in the ophthalmic setting. It is currently unknown whether topical etilevodopa administration will result in significant increases in systemic dopaminergic activity. Although we did not observe frank behavioral changes in our preclinical study, increases in systemic dopamine (and its derivatives) were identified after topical administration, raising potential long-term safety issues.

**Conclusion**

We have successfully demonstrated that topical administration of etilevodopa can penetrate the eye and enter the posterior segment. Topical etilevodopa did not exhibit enhanced ocular penetration as compared to levodopa but, because of its enhanced solubility, was able to be applied in a more concentrated form and thus result in greater intravitreal concentrations. Surprisingly, dopamine exhibited the greatest ocular penetration after topical administration. Considering the feasibility of using topical administration, these findings highlight the potential of dopamine and etilevodopa for further study as a therapeutic treatment for myopia.

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