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Retina compatible interactions and effective modulation of blood ocular barrier P-gp activity by third-generation inhibitors improves ocular penetration of loperamide

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Abstract

Effective drug delivery to the deeper ocular tissues remains an unresolved conundrum mainly due to the expression of multidrug resistance (MDR) efflux proteins, beside tight junction proteins, in the blood ocular barriers (BOB). Hence, the purpose of the current research was to investigate the ability of the third-generation efflux protein inhibitors, elacridar (EQ) and tariquidar (TQ), to diminish P-glycoprotein (P-gp) mediated efflux transport of loperamide (LOP), a P-gp substrate, across the BOB in SD rats. Initially, western blot analysis confirmed the expression of P-gp in the iris-ciliary bodies (IC) and the retina-choroid (RC) in the wild type rats. Next, the ocular distribution of LOP, in the presence and absence of EQ/TQ (at two doses), was evaluated. The significantly higher aqueous humor (AH)/plasma (D_{AH}) and vitreous humor (VH)/plasma (D_{VH}) distribution ratios of LOP in the rats pretreated with EQ or TQ demonstrated effective inhibition of P-gp activity in the BOB. Interestingly, the modulation of P-gp activity by EQ/TQ was more pronounced at the lower dose. The normal functioning and architecture of the retina, as indicated by electroretinography (ERG) studies, confirmed the cytocompatibility of LOP and EQ/TQ interactions at the doses tested.

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Conflict of interest

The authors declare no conflicts of interest in this research.

Keywords

P-gp inhibitors; Electroretinography; Elacridar; Tariquidar; Blood aqueous barrier (BAB); Blood retinal barrier (BRB)

Introduction

Eye is a highly protected organ, by virtue of its complex anatomical and physiological microenvironments, making effective delivery of therapeutic cues into the deeper ocular compartments a challenging area in ophthalmic research.¹⁻³ The tight junction proteins and the multidrug resistance (MDR) efflux transporters play a major role in the functioning of the ocular barriers and may lead to sub-therapeutic levels in the ocular tissues.⁴⁻⁷ One of the most explored efflux transporter is P-glycoprotein (P-gp), an important efflux protein belonging to the ATP-binding cassette (ABC) transporter sub-family. P-gp is widely expressed in the plasma membranes of the brain, liver, lung, kidney, intestine and many other vital organs, protecting them from the chemical and biological toxic environments.⁸⁻¹⁰ Studies report that P-gp expression on the blood ocular barriers (BOB) shields the anterior and posterior segments of the ocular tissues from endobiotics and xenobiotics.^{11,12} Specifically, the ciliary epithelial cells in the blood-aqueous barriers (BAB) and the retinal endothelium and retinal pigmented epithelium (RPE) in the blood retinal barriers (BRB), express P-gp in both human and animal models.^{11,13} The strategic location and broad substrate specificity of P-gp accounts for a severe reduction in the penetration of numerous lipophilic molecules across the BOB and various other blood organ barriers.⁸⁻¹⁰

The feasibility of inhibiting P-gp and MDR proteins to enhance penetration of substrate molecules into the target organ has been demonstrated.^{14,15} Beside pharmacologically active, drugs like verapamil, quinidine, cyclosporine etc., emerged as the first-generation P-gp inhibitors, modulating efflux activity at the blood-organ interface.^{16,17} Nonetheless, the toxic and antagonistic effects associated with their pharmacological activity and their ability to modulate the kinetics of substrate drugs, limited their use as specific inhibitors.^{18,19} Interestingly, the second-generation P-gp inhibitors with diminished pharmacological activities were more potent and compatible in reducing the barrier characteristics.^{14,19,20} The third-generation P-gp inhibitors, like elacridar (EQ) and tariquidar (TQ), were developed with no pharmacological activity and with higher affinity to P-gp (at nanomolar concentrations).^{14,21,22} EQ and TQ alleviate breast cancer resistance protein (BCRP) mediated efflux transport of the substrate molecules.^{21,22} Both EQ and TQ have been extensively explored for modulating the P-gp transporter at the blood brain barrier (BBB) thereby, improving the blood to brain penetration of substrate molecules.^{8,21,22}

Inhibition of P-gp transporter in the BOB has been studied by several investigators.²³⁻²⁵ Previously, we reported the contribution of topically administered first-generation P-gp inhibitors in improving the mean residence time and reducing the clearance of systemically and intravitreally administered substrates in the vitreous humor of New Zealand albino rabbits, through inhibition of P-gp activity in the RPE.^{26,27} The interactions of first-generation inhibitors and substrate drugs with the other transporters expressed in the RPE,

beside P-gp, could have also contributed to the changes in the vitreal kinetics of substrate drugs.^{26,27} To rule out this, the impact of more specific third generation P-gp inhibitors (EQ/TQ) has not been examined.^{26,27} Moreover, the retinal vasculature in rabbits is insignificant, compared to humans and thus, it was difficult to draw conclusion about the role of the P-gp in the inner blood retinal barrier (retinal endothelium).²⁸ The effectiveness of the third-generation inhibitors (EQ and TQ) in regulating the P-gp efflux activity at the BOB in Sprague-Dawley (SD) rats, which closely mimics human choroid and retinal vasculature, has not been investigated till date.²⁹ Other studies have investigated the effect of the EQ or TQ on the BRB in humans, rabbits and *in vitro* cell models.²³⁻²⁵ Hence, in the current study, Sprague Dawley rats were utilized to investigate the impact of inhibition of P-gp activity, by specific third generation P-gp inhibitors, in the BAB and the BRB on the ocular penetration specific substrate drug (loperamide (LOP)) from systemic circulation. Moreover, the effect substrate-inhibitor interactions on retinal function has not yet been explored. In view of this, the current study aimed to examine the impact of third-generation P-gp inhibitors on the blood-ocular transport of LOP, a well-known P-gp substrate, in SD rats pretreated with EQ and/or TQ, for the first time. LOP has been investigated as a potent and specific substrate of P-gp transporter expressed in the BBB.⁸ Hence, LOP was selected as model substrate drug in this study. The expression of P-gp in the BAB and the BRB was confirmed by western blot analyses of the iris-ciliary bodies (IC) and retina choroid (RC) tissues. Electroretinography (ERG) studies were undertaken to evaluate the interaction of LOP-EQ or TQ with the retina.

Materials and Methods

Animals

All animal experiments were performed according to the approved protocols of the University of Mississippi Institutional Animal Care and Use committee (protocol# UM 15-003), the University of Tennessee Health Science Center Institutional Animal Care and Use committee and the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Male SD rats (6-8 weeks old), purchased from Horizon Discovery (Saint Louis, MO), were used. Animals were allowed free access to food and water.

Chemical Reagents

LOP and Glycofurool was procured from Sigma Aldrich (St. Louis, MO). EQ and TQ were acquired from Alfa Aesar (Haverhill, MA). Propylene glycol (1,2-Propanediol), Polyethylene glycol 400 (PEG-400), Polyethylene glycol 300 (PEG-300), Dimethyl Sulfoxide (DMSO) were purchased from Fisher Scientific (St. Louis, MO). All other high purity chemicals and HPLC grade solvents were also obtained from Fisher Scientific (St. Louis, MO).

P-gp protein expression by western blotting

P-gp expression was examined to confirm its presence in the BAB and BRB of the SD rats. The SD rats were euthanized by injecting an excess dose of ketamine and xylazine combination through the I.P. route (n=6). The eyes were enucleated immediately and the

ocular tissues (IC and RC) samples were collected. The tissues were harvested, and tissue lysates were prepared using 2X lysis buffer (Cell Signaling Technology, USA). Protein concentrations in the tissue lysates were estimated by Bradford assay (Bio-Rad USA). Equal amounts (20 µg) of proteins, from the various tissue lysates, were loaded into the wells of 10% SDS-PAGE mini-gels (Bio-Rad, USA), electrophoresed and the proteins were then transferred to nylon membranes (Cell Signaling Technology, USA) as per the western blot protocol. Membranes were then blocked in 5% non-fat dried milk in 1X TBST [20 mM Tris-HCl (pH 7.5), 137 mM NaCl and 0.1% (v / v) Tween 20] for 1h at room temperature. Blots were probed overnight with primary antibody MDR1/ABCB1 (Cat # 13978 Cell Signaling Technology) following the manufacturers protocol. The membranes were washed with 1X TBST and probed with anti-rabbit secondary antibody conjugated to HRP (Cat # 7074 Cell Signaling Technology) for 1 hr. For equal loading, samples were probed with beta actin antibody (Cell Signaling Technology, USA). The blots were developed with signal flame enhanced chemiluminescence (ECL) TM kit (Cell Signaling Technology, USA). The images were captured, processed and analyzed on ChemiDoC™ MP imaging system (Bio-Rad, USA). ImageJ, an image processing program developed at National Institutes of Health, was used to compute the area of the protein band in the blot. Further, relative P-gp protein intensity was calculated from the ratio of P-gp band area and the corresponding β-actin band area.

Preparation and evaluation of loperamide, elacridar and tariquidar solutions

LOP intravenous solution (1 mg/mL) was prepared by dissolving an appropriate quantity in a mixture of propylene glycol and saline (1:1 v/v). EQ solution (2.5 mg/mL), for intravenous administration, was obtained by dissolving an accurately weighed amount of EQ in DMSO (5 % v/v) and then the final volume was made up with glycofurol (5% v/v), PEG 300 (20% v/v), ethanol (30% v/v) and saline (40 % v/v). A clear solution of 2.5 mg/mL of TQ was prepared by dissolving an accurately weighed quantity of TQ in DMSO (5% v/v), glycofurol (10% v/v) PEG 400 (30% v/v), propylene glycol (25% v/v), and the required volume adjusted by the addition of saline. The LOP, EQ and TQ solutions were tested for pH, assay, precipitation in phosphate buffered saline (pH 7.4), stability at room temperature and for loss during filtration across 0.22µm nylon filter (Millipore, USA).

Quantification of EQ and TQ

High performance liquid chromatography-ultraviolet spectrometry (HPLC-UV) method was adopted to quantify the EQ content in all the samples.³⁰ A mixture of acetonitrile (ACN) and 0.05 M ammonium acetate (45:55 % v/v) mobile phase, pH adjusted to 5.0 with orthophosphoric acid, was pumped through a Luna® C₁₈(2) 100 Å column (Phenomenex, 4.6 mm x 250 mm; 5µ particle size), at a flow rate of 1 mL/min, by Waters 600 controller pump equipped HPLC system. With the aid of Waters 717 plus Autosampler, 25 µL of sample was injected into the mobile phase and the column eluent was monitored at λ_{max} (detection wavelength) of 254 nm using Waters 2487 dual λ absorbance detector and the response peaks were integrated with the help of an Agilent 3395 integrator.

Similarly, TQ was quantified under similar chromatographic conditions but with a different mobile phase comprising of a mixture of acetonitrile (ACN) and 0.2 M ammonium acetate

(40:60 % v/v) mobile phase with pH adjusted to 4.5 with glacial acetic acid. The eluents were tested at λ_{max} (detection wavelength) of 222 nm.³¹

Ocular distribution studies

The ability of EQ and TQ to modify the P-gp activity in the BOB to improve the blood-ocular penetration of LOP was estimated through ocular distribution studies in SD rats. The rats were placed in 5 treatment groups (n=6), so that each study group receive only one treatment, and received LOP (0.5 mg/kg); LOP (0.5 mg/kg) + EQ (1 mg/kg); LOP (0.5 mg/kg) + EQ (2.5 mg/kg); LOP (0.5 mg/kg) + TQ (1 mg/kg); and LOP (0.5 mg/kg) + TQ (2.5 mg/kg), through the tail vein. Animals receiving both substrate and inhibitor, were injected with P-gp inhibitor (EQ or TQ) 30 min prior to the LOP (0.5 mg/kg) administration. At the end of 120 min post LOP dosing, the rats were anesthetized by an intraperitoneal (I.P.) administration of a combination of ketamine (35 mg/kg) and xylazine (3.5 mg/kg) and the blood was collected via cardiac puncture. The animals were then euthanized with an excess dose of ketamine and xylazine combination injection through the I.P. route. Immediately after euthanization, the eyes were enucleated to separate the aqueous humor (AH) and vitreous humor (VH). The AH and VH collected from both eyes in each rat was pooled. The plasma was separated from the blood samples after centrifugation at 10,000 rpm for 5 min. The plasma, AH and VH samples were kept at -80 °C until further analysis were performed.

Sample preparation

To 80 μL plasma, 20 μL AH and 40 μL VH tissue samples, 10 μL , 2.5 μL , and 5 μL , respectively, of 1 $\mu\text{g/mL}$ ketoconazole (internal standard (IS)) solution was added. Then ice-cold acetonitrile (2 parts) was introduced into the samples (1 part) to extract drug and internal standard from the biological matrices with complete precipitation of proteins. Furthermore, the samples were vortexed and then centrifuged at 13000 rpm for 15 min and the clear supernatant solution was collected. The eluents in the supernatant solution (2 μL) were quantified using the method described in the earlier section. The extraction efficiency (EE), limit of detection (LOD) and linearity of LOP in plasma (10-160 ng/mL), AH (0.5-40 ng/mL) and VH (0.5-20 ng/mL) was assessed.

Quantification of loperamide in biological tissue matrix

The liquid chromatographic system was an Agilent Series 1290 comprising of the following modular components: binary pump, a vacuum solvent microdegasser, an autosampler with 100-well tray and a thermostatically controlled column compartment. Separation was achieved on an Agilent Zorbax SB C8 (2.1x100 mm, 1.8 μ) column. The mobile phase consisted of water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B) at a flow rate of 0.23 mL/min, which were applied in the following gradient elution: 0 min, 80 % A: 20% B, in next 7 min to 100% B. Each run was followed by a 3 min wash with 100 % acetonitrile, and an equilibration period of 5 min. Two microliter of sample was injected. The column temperature was set at 35 °C.

The mass spectrometric analysis was performed with a Q-TOF-MS/MS (Model #G6530A, Agilent Technologies, Palo Alto, CA, USA) equipped with an ESI source with Jet Stream

technology using the following parameters: drying gas (N₂) flow rate, 10.0 L/min; drying gas temperature, 250 °C; nebulizer, 30 psig, sheath gas temperature, 350 °C; sheath gas flow, 9 L/min; capillary, 3000 V; skimmer, 65V; Oct RF V, 750V; fragmentor voltage, 125V. All the operations, acquisition and data analysis were controlled by Agilent MassHunter Acquisition Software Ver. A.05.00 and processed with MassHunter Qualitative Analysis Software Ver. B.07.00. Each sample was analyzed in positive mode in the range of m/z = 100-1000. Accurate mass measurements were obtained by means of reference ion correction using reference masses at m/z 121.0509 (protonated purine) and 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921] in positive ion mode. The compounds were confirmed in each spectrum. For this purpose, the reference solution was introduced into the ESI source via a T-junction using an Agilent Series 1200 isocratic pump (Agilent Technologies, Santa Clara, CA, USA) using a 100:1 splitter set at a flow rate of 20 µL/min. In the positive ion mode, the protonated species $[M+H]^+$ at m/z 477.2317 (calculated m/z 477.2303) for compound LOP and m/z 531.1574 (calculated m/z 531.156) for internal standard (ketoconazole) were observed.

Data interpretation

Drug distribution into the AH and VH from plasma was calculated according to equations 1 & 2 respectively,

$$D_{AH} = \frac{\text{Concentration of Loperamide in AH (ng / mL)}}{\text{Concentration of Loperamide in Plasma (ng / mL)}} \quad (\text{Eq. 1})$$

$$D_{VH} = \frac{\text{Concentration of Loperamide in VH (ng / mL)}}{\text{Concentration of Loperamide in Plasma (ng / mL)}} \quad (\text{Eq. 2})$$

Where, D_{AH} and D_{VH} represent the distribution ratios of LOP from plasma into AH and VH, respectively.

Electroretinography

To evaluate the LOP-EQ or TQ induced toxicity to retinal functions, electroretinography (ERG) analyses were performed in 3 SD rats from the same cohort utilized in ocular distribution studies, as per the standard protocols.³² The rats (n=3 in a group) were placed in 2 treatment groups, so that each group receives only one treatment, and were accordingly administered with LOP (0.5 mg/kg) + EQ (2.5 mg/kg) and LOP (0.5 mg/kg) + TQ (2.5 mg/kg) separately, through the tail vein. The rats were injected with P-gp inhibitor (EQ (2.5 mg/kg) or TQ (2.5 mg/kg)) 30 min prior to the LOP (0.5 mg/kg) administration. The ERG waves were recorded before the administration of 2.5 mg/kg EQ and/or TQ as well as at the end of 2 h, and 1 day post-LOP (0.5 mg/kg) administration. Briefly, dark-adapted rats (overnight prior to commencement) were anesthetized with an I.P. injection of ketamine and xylazine combination. Gold ring electrodes of 2-3 mm diameter (Roland Consult, Stasche & Finger GmbH, Germany), were positioned on both corneas after anesthetizing them with topical 0.5% proparacaine hydrochloride followed by topical application of 1% tropicamide for pupil dilation. The stainless steel surgical needles were inserted subcutaneously into the

forehead and back leg to serve as reference and ground electrodes respectively. The dark-adapted ERG responses were recorded from both the eyes simultaneously, exposing them to lights flashes of various intensities (−4.0 Log units to 2.88 Log units) delivered by a Ganzfeld light source. The a- wave and b-wave amplitudes in SD rats obtained before dosing P-gp inhibitors (EQ and/or TQ) were taken as baseline to compare the amplitudes of a and b waves recorded at the end of 2h and 1 day post dosing (0.5mg/kg LOP) and were reported as % amplitude change.

Statistical analysis

The data obtained from ocular distribution and ERG studies were subjected to one-way analysis of variance (ANOVA) and Tukey's post hoc HSD (GraphPad Software, San Diego California, USA), to estimate the statistical significance. The $p < 0.05$ were considered statistically significant.

Results

All intravenous solutions (LOP, EQ and TQ) were clear with pH of 6.8 -7.0 and showed an assay of 98.5-103.4%. No significant loss of drug ($< 1\%$) was noted during filter validation (passing these solutions through 0.22 μm nylon filter). Moreover, no precipitation was seen after introducing these solutions into phosphate buffer saline (pH 7.4), ruling out the possibility of precipitation at the injection site. All these formulations were stored at room temperature for 1-8 weeks and were found to be stable with 2% change in the assay during this period. The excipient concentrations utilized in the formulations were within the limits noted in the inactive ingredients (IIG) database of US Food and Drug Administration (US-FDA) and/or below reported intravenous LD_{50} values in rats, to ensure safety and biocompatibility of these parenteral solutions.^{33,34}

The LC/MS method adopted for the analysis of LOP was sensitive with a limit of detection (LOD) of 0.1 ng/mL, and a limit of quantitation (LOQ) of 0.5 ng/mL in the ocular tissues and was linear over the range of concentrations tested with r^2 values of 0.993, 0.990 and 0.994 in plasma, AH and VH, respectively. Greater than 98% of LOP was extracted from all tissues by protein precipitation technique using ice cold acetonitrile. The bioanalytical method was specific to LOP and IS, without any interference. The % recovery of LOP from the samples stored at 4°C and freshly obtained samples were 98 % and 96.3%, respectively, suggesting low or no drug loss during the storage of the samples.

P-gp protein expression analysis in ocular tissues

The western blot demonstrated a protein band with MW ~170 kDa in both IC and RC tissue samples (Fig. 1A) confirming the expression of P-gp in these samples. However, the P-gp band in RC sample was faint compared to IC samples thereby proving lower expression of P-gp in RC. (Fig. 1A). The protein bands were further quantified by blot analysis by using ImageJ. The relative protein intensity in the western blot was higher for the IC compared to that in RC (Fig. 1B). The loading control (β -actin) bands of same thickness with MW ~ 45 kDa in the IC and RC samples confirmed uniform sample loading (Fig. 1A).

Ocular distribution studies

The intravenous formulations of LOP, EQ and TQ were freshly prepared for the ocular distribution studies. The plasma LOP levels in SD rats, from different treatment groups, are represented in Figure 2. The concentration of LOP in the plasma of rats co-administered with LOP and EQ or TQ, at both doses of inhibitor/s, was slightly higher compared to the rats treated with LOP alone, however, the difference was not statistically significant (Fig. 2).

Figure 3A delineates the AH LOP levels in the rats from different treatment groups. Significant LOP levels were observed in the AH of rats treated with EQ or TQ, at both doses of inhibitor/s ($p < 0.05$), while the LOP concentrations were below LOD in the AH of the rats administered with LOP alone (Fig. 3A). The concentration of LOP in the AH of rats administered with the lower dose (1 mg/kg) of EQ or TQ were ~ 4.5 to 5.0-folds higher compared to the rats treated with the higher dose (2.5 mg/kg) of EQ or TQ ($p < 0.05$) (Fig. 3A). Interestingly, in the rats administered with the lower dose of EQ (1 mg/kg), the AH LOP concentrations were ~ 5.0, 7.0 and 1.5 folds higher compared to that noted in the rats treated with, higher dose of EQ (2.5 mg/kg), higher dose of TQ (2.5 mg/kg) and lower dose of TQ (1 mg/kg), respectively ($p < 0.05$) (Fig. 3A).

The D_{AH} in rats treated with LOP alone was zero since LOP did not penetrate into the AH from the plasma (Fig. 3B). On the other hand, a significantly higher D_{AH} in rats co-administered with LOP and EQ or TQ, at both inhibitor/s doses, was noticed ($p < 0.05$) (Fig. 3B). The D_{AH} in rats administered with lower dose (1 mg/kg) of either EQ or TQ were ~ 5.0 to 5.5-folds higher with respect to that observed in the rats treated with higher dose (2.5 mg/kg) of either of the inhibitors ($p < 0.05$) (Fig. 3B). Overall, ~ 1.7-8.5-folds higher D_{AH} was observed in the rats treated with the lower dose of EQ compared to that seen in the rats from other treatment group ($p < 0.05$) (Fig. 3B).

The LOP concentrations in the VH of rats from different treatment groups are presented in Figure 4A. The VH LOP levels in rats administered with LOP alone were below the LOD, whereas, in the rats treated with LOP and inhibitors, significant VH LOP levels were observed ($p < 0.05$) (Fig. 4A). Hence, the plasma to VH LOP distribution ratio (D_{VH}) in rats receiving EQ or TQ, at both the doses of inhibitor/s, was significantly higher compared to that of the rats receiving LOP alone ($p < 0.05$) (Fig. 4B). The VH LOP levels and D_{VH} in rats administered with lower dose of EQ or TQ were slightly higher than that of the rats administered with higher dose of inhibitors but were not statistically different (Fig. 4).

Electroretinography (ERG)

Electroretinography (ERG) was performed to understand LOP-EQ or TQ interactions with the retina thereby affecting the functioning of photoreceptors cells and inner retinal cells. The ERG measures the functioning of photoreceptor cells and bipolar/Müller cells as a-wave and b-wave, respectively (Fig. 5A). Changes in the a-wave recording suggest a light or chemical induced alterations in the functioning of photoreceptors cells in the outer segment of the retina, while the variations in the b-wave recordings suggest damage to the inner retina³⁵⁻³⁷. Hence, the % change in the amplitude of a- and b-wave were examined to understand the cytocompatibility of LOP-EQ/TQ interactions with retina of SD rats.

Figure 5B illustrates the % change in the amplitude of a- and b- waves recorded from the ERG studies. At 2h post treatment with LOP, the a-wave amplitude measured in rats pretreated with EQ, changed significantly with respect to baseline, however, no changes were noticed in the b-wave amplitude ($p<0.05$) (Fig. 5B). At 1day post LOP treatment, no significant change from baseline in the a- and b- wave amplitude was noticed in the rats pretreated with EQ (Fig. 5B). In the rats pretreated with TQ, the a- and b-wave amplitudes recorded at 2h and 1day post-dosing with LOP showed no noticeable difference from the baseline (Fig. 5B). Since the ERG studies in rats co-administered with LOP and higher dose (2.5 mg/kg) of EQ or TQ did not show any toxic events (changes in b-wave) to the retinal functioning, ERG studies were not conducted at the lower dose of EQ or TQ.

Discussion

Numerous investigations in the past have successfully demonstrated the impact of third-generation small molecule inhibitors (SMIs) (EQ and/or TQ) in improving blood to organ penetration of P-gp substrates by modulating the P-gp mediated efflux activity in the plasma membranes of various organs.^{8,9,38,39} In the present study, LOP was selected as a model specific substrate of P-gp, but not BCRP and MRP, expressed in BOB.^{8,39} EQ and TQ were considered as model inhibitor molecules to determine the effectiveness of these potential P-gp inhibitors in modulating the BOB's P-gp activity and the cytocompatibility of LOP – EQ/TQ interactions with the retina in SD rats. It has been reported that TQ inhibits P-gp and BCRP with equal specificity, while EQ binds to P-gp with more specificity, than BCRP. Thus, these molecules inhibit the P-gp and/or BCRP mediated efflux transport of most of the lipophilic drugs, but not multidrug resistance protein (MRP) mediated efflux.^{21,39} Literature reports demonstrated the upregulation of other efflux transporters like BCRP or MRP to compensate the lack of P-gp expression in *mdr1a* knockout mice.⁴⁰ However, to the best of our knowledge no literature reports have discussed the upregulation of other efflux transporters following the inhibition of P-gp activity. In the current experiment, if there was upregulation of any other transporters due to the inhibition of P-gp activity in the BOB they would not interact with loperamide as it specifically binds to P-gp, but not to another transporter.^{8,39}

Literature reports describe the utility of the western blotting, reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry techniques in determining the P-gp protein expression in the ocular tissues.⁴¹ In the current study, the western blot results portrayed a protein band with MW ~170 kDa in the IC and RC samples obtained from the SD rats, confirming the existence of the P-gp protein in the BAB and the BRB, respectively (Fig. 1A). The higher relative P-gp protein intensity in the IC compared to that in the RC demonstrated higher P-gp expression in the IC than in the RC, suggesting greater MDR 1a efflux activity in the BAB over that in the BRB (Fig. 1B). These findings were consistent with our pharmacokinetic data. Moreover, a few reports in the literature also demonstrated the variations in the P-gp mediated efflux of substrates at the BAB and the BRB, in rat models, confirming the differential expression of P-gp in the BAB and the BRB.^{11,23,42}

The doses of inhibitors were selected from the literature reports which demonstrated effective inhibition of P-gp activity at different blood organ interface.²¹⁻²⁵ In the ocular distribution studies, the rats co-administered with the LOP and EQ or TQ, at both doses of inhibitor/s, exhibited the characteristic central opiate effect of LOP such as restricted movements, erected tail, respiratory depression, eye protrusion, pupil constriction and lacrimation due to the increased brain penetration of LOP as a result of inhibition of the BBB's P-gp activity, as reported earlier in the literature.^{8,38} These effects were prominent and for a longer duration in the rats treated with higher EQ or TQ dose, but decreased significantly by the end of 2h in the rats administered with the lower EQ or TQ dose. No central opiate effects were noticed in the rats administered with LOP alone, confirming the P-gp mediated efflux at the BBB in these rats. The mean plasma LOP concentration in the rats pretreated with EQ or TQ, at both inhibitor/s doses, was slightly higher (not statistically significant) compared to the untreated rats, which could be attributed to reduction in renal clearance due to the inhibition of P-gp activity in the kidneys (Fig. 2).^{39,43}

The insignificant, or zero, plasma to AH penetration of LOP, in rats receiving LOP alone, confirmed the P-gp mediated active efflux of substrates across the BAB. These results are consistent with the previous reports and validate our western blot analysis results.^{11,23,42} On the other hand, the higher LOP levels in the AH of rats pretreated with EQ or TQ, at both inhibitor/s doses, indicate enhanced plasma to ocular tissue penetration of LOP, suggesting effective modulation of P-gp in the BAB by the inhibitors ($p < 0.05$) (Figs. 3 and 4). The above findings corroborate with the literature reports which demonstrate the interaction of EQ and TQ with the MDR 1a efflux transporter function. This modulation of P-gp efflux activity is either due to the blocking the drug/substrate binding site alone by EQ, or blocking both drug binding and ATP binding site by TQ, in the P-gp structure.^{21,38} The high affinity of EQ ($IC_{50} < 0.1 \mu M$) and TQ ($IC_{50} < 0.05 \mu M$) for the P-gp transporter is responsible for effective inhibition of the efflux mechanism.²¹

Interestingly, the AH LOP concentration and the D_{AH} , in the rats treated with the higher dose of EQ or TQ was significantly lower compared to that observed in rats administered with the lower inhibitor dose (EQ or TQ) ($p < 0.05$) (Fig. 3). This could probably be due to the strong opioid agonist activity of LOP. It has been reported that the μ -opioid agonists increase the AH outflow and modifies the IC function.⁴³ Dortch-Carnes and Russell, (2006) reported that the binding of morphine, a μ -opioid agonist, to the μ -opioid receptors in the IC bodies, reduces the intraocular pressure by increasing AH outflow through the dilated trabecular meshwork and Sclemm's canal.⁴⁴ LOP, besides inducing central opiate effects, could also increase AH outflow through local opioid agonist action, like morphine, resulting in faster clearance of LOP from the AH. In our earlier investigation, it was noticed that in the P-gp knock out SD rats, administered with the high intravenous dose, the AH LOP levels were low due to the increased clearance of LOP from the AH.⁴⁵ In the current study, the increased AH outflow would have overshadowed the increased permeability of LOP across the BAB, and thus resulting in lower LOP concentrations in the AH, and D_{AH} , in rats treated with the higher dose of EQ or TQ (Fig. 3). Despite the higher P-gp inhibitory potential of TQ ($IC_{50} < 0.05 \mu M$), compared to EQ, significantly higher AH LOP concentration, were seen in rats treated with EQ compared to that in the rats administered with TQ at the lower

dose ($p < 0.05$) (Fig. 3). This could probably be attributed to TQ producing higher LOP levels in the brain compared to EQ, due to more efficient inhibition of P-gp by TQ, and consequent stronger central opiate effect resulting in lower LOP levels in the AH.^{21,44} Nonetheless, further studies are needed to confirm the suggested changes in the AH outflow associated with the interaction of intravenously administered LOP with the μ -opioid pathway, e.g. determining the reduction in intraocular pressure (IOP).

The impact of P-gp on the penetration of LOP through the BRB was apparent in the control (no inhibitor) rats where LOP levels were not detected in the VH. In contrast, the rats pretreated with EQ or TQ showed significant LOP levels in the VH ($p < 0.05$) (Fig. 4). The LOP concentrations and D_{VH} in the rats receiving EQ or TQ treatment, at any given inhibitor/s dose, were significantly higher demonstrating effective modulation of P-gp activity in the BRB ($p < 0.05$) (Fig. 4). These results agree with the previous literature reports that demonstrate improved penetration of substrates across the BRB on modulating P-gp activity.^{25,41,44} Surprisingly, no significant changes were seen in the LOP levels in the VH, and D_{VH} , in the rats treated with either EQ or TQ, both doses. This indicates complete inhibition of P-gp in the BRB (Fig. 4). Recently, a study demonstrated that TQ completely inhibits P-gp activity in the BRB but not in the BBB suggesting the differential expression of P-gp in BBB and BRB.⁴⁶ These reports, and the Western Blot data from the current study (Fig 1), suggest that the nearly similar LOP levels in the VH, and D_{VH} , in rats treated with EQ or TQ at any given dose, could be as a result of complete inhibition of P-gp activity on both apical and basolateral membranes of the RPE.^{26,27} A combination of EQ and TQ, both at 0.5 mg/kg dose, was also tested in the study, however, the rats pretreated with this combination of inhibitors did not show significant changes in the blood ocular penetration of loperamide compared to that seen in rats treated with loperamide alone thereby suggesting no effective modulation of P-gp activity in the BOB (data not shown).

Since P-gp protects the deeper ocular tissues from toxic substances, it is critical to estimate the substrate/inhibitor effect on the retinal functions, during and after the study period. No significant variation from baseline was noticed in the ERG a- and b-wave patterns recorded in the rats from different treatment groups, at 2h and 1 day, except for the a-wave of rats treated with LOP+EQ at the 2h study point only. This change in the a-wave amplitude ($p < 0.05$) (Fig. 5) could be attributed to the variations in the photoreceptor cell function as a result of LOP's opioid agonist behavior.^{43,44,45} The electroretinography, thus confirmed that the LOP-EQ/TQ did not show any acute or chronic toxic interactions with the retinal function at the doses and combinations used. Moreover, it also confirmed that the drug levels in the VH of rats pretreated with EQ and TQ were not because of structural damage to retinal tissues - which should have resulted in a change in b-wave.

Conclusion

In this study, the effect EQ/TQ in modulating P-gp activity in ocular tissue sites were investigated. The expression of P-gp in the BOB was confirmed by western blot analysis. An improvement in the penetration of LOP into the AH and the VH from the systemic circulation in the presence of EQ/TQ pointed to effective inhibition of P-gp mediated efflux activity at the BAB and BRB. Both inhibitors, modulated P-gp activity effectively in the

BRB at both doses tested. Interestingly, the inhibition of P-gp activity in the BAB was more pronounced at a lower EQ or TQ dose, due to minimal interference of LOP induced central/local opioid effects at the lower dose. The ERG studies confirmed that the LOP-EQ/TQ exposure did not show any toxic effect on the retinal functions. However, for clinical use of these inhibitors, consideration of more safety aspects is quintessential. Future studies, confirming the involvement of the μ -opioid pathway in the ocular kinetics of therapeutic agents, with a focus on AH dynamics and ocular blood-flow, are necessary.

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Non-Standard Abbreviations:

ABC	ATP binding cassette
AH	Aqueous humor
BAB	Blood aqueous barrier
BM	Bruch's membrane
BOB	Blood ocular barrier
BRB	Blood retinal barrier
<i>D_{AH}</i>	Distribution ratio of looperamide from plasma to aqueous humor
<i>D_{VH}</i>	Distribution ratio of looperamide from plasma to vitreous humor
EE	Extraction efficiency
EQ	Elacridar
ERG	Electroretinography
I.C.	Iris-ciliary body
I.P.	Intraperitoneal
IIG	Inactive ingredients
IV	intravenous
LOD	Limit of detection
LOP	Loperamide
LOQ	Limit of quantification
Mdr1a	Multidrug resistance 1a gene
P-gp	P-glycoprotein

PG	pigmented granules
Q-TOF	Quadrupole time of flight
R.C.	Retina Choroid
SD	Sprague Dawley
TQ	Tariquidar
UHPLC	Ultra high performance liquid chromatography
VH	Vitreous humor

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Figure 1.

A) Western-blot displaying the P-gp protein and β -actin bands in the IC and RC samples obtained from SD rats. B) The relative P-gp protein intensity was determined from the ratio of area obtained from P-gp protein band and β -actin band using ImageJ.

Figure 2.

The plasma loperamide (LOP) concentration levels in SD rats from different treatment groups. EQ (1) or (2.5): Elacridar (1 or 2.5 mg/kg); TQ (1) or (2.5): Tariquidar (1 or 2.5 mg/kg) (mean ± SEM, n=6).

Figure 3.

A) The aqueous humor (AH) loperamide (LOP) levels and B) AH to plasma Distribution ratio of LOP (D_{AH}) in the SD rats from different treatment groups. EQ (1) or (2.5): Elacridar (1 or 2.5 mg/kg); TQ (1) or (2.5)- Tariquidar (1 or 2.5 mg/kg) (mean $\pm$ SEM, n=6). *- indicates statistically significant difference at $p < 0.05$.

Figure 4.

A) The vitreous humor (VH) loperamide (LOP (0.5 mg/kg)) levels and B) VH to plasma Distribution ratio of LOP (D_{VH}) in the SD rats from different treatment groups. EQ (1) or (2.5): Elacridar (1 or 2.5 mg/kg); TQ (1) or (2.5): Tariquidar (1 or 2.5 mg/kg). (mean $\pm$ SEM, n=6). *- indicates statistically significant difference at $p < 0.05$.

Figure 5. Electoretinography results depicting A) representative a- and b- wave pattern and B) the % change from the baseline in the amplitude of a- and b-wave recorded at 2h and 1day post LOP (0.5 mg/kg) treatment in the SD rats pretreated with EQ or TQ (2.5 mg/kg) (mean $\pm$ SEM, n=3).