Vitreal Kinetics of Quinidine in Rabbits in the Presence of Topically Co-administered P-gp Substrates/Modulators

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a) **Running title**: Effect of Topically Co-administered P-gp Substrates/Modulators

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d) **ABBREVIATIONS**: P-gp, p-glycoprotein; PHS, prednisolone hemisuccinate sodium; $k_{\text{e}, \text{app}}$, apparent elimination rate constant; $t_{1/2}$, elimination half-life; MRT, mean retention time; AUC, area under curve; CL, clearance; RPE, retinal pigmented epithelium; IPBS, isotonic phosphate buffer saline; HPLC, high performance liquid chromatography.
ABSTRACT

The purpose of this study was to investigate whether topically administered P-glycoprotein (P-gp) substrates/modulators can alter vitreal kinetics of intravitreally administered quinidine. Male New Zealand rabbits were used under anesthesia. Vitreal kinetics of intravitreally administered quinidine (0.75 μg dose) was determined alone and in the presence of verapamil (co-administered topically/intravitreally) or prednisolone hemisuccinate sodium (PHS, co-administered topically). In the presence of topically instilled verapamil (1% w/v), elimination half-life (t1/2) (176 ± 7 min), apparent elimination rate constant (λz) (0.0039 ± 0.0001 min⁻¹) and mean retention time (MRT) (143 ± 30 min) of intravitreally administered quinidine were significantly different from that of the control (105 ± 11 min, 0.0066 ± 0.0007 min⁻¹ and 83 ± 13 min, respectively). A 2-fold increase in the t1/2 with a corresponding decrease in λz and a 1.5-fold increase in the MRT of quinidine was observed in the presence of topically co-administered 2% w/v PHS. Intravitreal co-administration of quinidine and verapamil resulted in a significant increase in the t1/2 (159 ± 9 min) and a decrease in the λz (0.0043 ± 0.0002 min⁻¹) of quinidine. The vitreal pharmacokinetic parameters of sodium fluorescein, alone or in the presence of topically instilled verapamil, did not show any statistically significant difference, indicating that ocular barrier integrity was not affected by topical verapamil administration. Results from this study suggest that topically applied P-gp substrates/modulators can alter vitreal pharmacokinetics of intravitreally administered P-gp substrates, possibly through the inhibition of P-gp expressed on the basolateral membrane of the retinal pigmented epithelium.
INTRODUCTION

The retina is the primary target for most posterior segment ocular disorders such as age-related macular degeneration, diabetic macular edema, retinitis pigmentosa, endophthalmitis and proliferative vitreoretinopathy (Kim et al., 2007). Drug delivery to the posterior chamber ocular tissues is, however, challenged by various physiological barriers such as the cornea, conjunctiva, sclera and the blood-ocular-barriers (Dey et al., 2003; Duvvuri et al., 2003b; Majumdar et al., 2003a; Majumdar et al., 2003b; Cunha-Vaz, 2004; Yasukawa et al., 2004). The retinal pigmented epithelium (RPE), which forms the outer blood-retinal-barrier, limits vitreal penetration of drugs administered by the systemic and trans-scleral routes (Duvvuri et al., 2003a; Ghate and Edelhauser, 2006; Janoria et al., 2007). P-glycoprotein (P-gp), a 170 kDa ATP dependent membrane bound efflux protein, expressed on the RPE plays a major role in restricting diffusion of P-gp substrates from the choroidal stroma into the neural retina across the RPE (Kennedy and Mangini, 2002; Steuer et al., 2005).

P-gp displays broad specificity, accepting many structurally, functionally and mechanistically unrelated compounds (Ambudkar et al., 2003), and its role in limiting drug penetration across biological barriers is well established. P-gp mediated drug efflux at the blood-brain-barrier is a major factor behind poor penetration of chemotherapeutic agents, that are P-gp substrates, into the brain following systemic administration (Golden and Pollack, 2003; Kemper et al., 2004). A number of reports also illustrate intestinal P-gp’s role in limiting systemic bioavailability of orally administered agents. Moreover, upregulation of P-gp expressed by tumor cells is considered to be a major mechanism behind multidrug resistance (Matheny et al., 2001; Fromm, 2003; Fromm,
2004; Kunta and Sinko, 2004). Additionally, it has also been demonstrated that P-gp expressed on the canalicular membrane of the hepatocytes and the luminal surface of the proximal kidney tubule cells, including nephrons, expedite hepatic and renal elimination of substrates.

Generally, P-gp is expressed on the apical membrane of epithelial cells, preventing drug transport from the lumen into the systemic circulation (e.g. intestinal epithelium) or from the systemic circulation into the brain (endothelial cells of the Blood-Brain-Barrier) (Matheny et al., 2001). An earlier report, however, suggests that P-gp is expressed on both apical as well as basal membranes of the RPE cells (Kennedy and Mangini, 2002). P-gp on the RPE cells may thus affect permeation of substrates from the vitreous humor into the systemic circulation, and vice versa (Dey et al., 2003; Duvvuri et al., 2003b), and could be a major factor behind the inability of systemic, periocular and trans-scleral routes of administration to generate and maintain therapeutic concentrations of P-gp substrates in the retina. Thus, factors/agents that can modulate the efflux activity of RPE P-gp could probably alter ocular pharmacokinetics of P-gp substrates.

In the past, a number of strategies attempting to modulate the activity or expression of efflux proteins on various mammalian tissues have been investigated. These include the use of chemosensitisers, prodrugs, polymers, nanoparticles, transcriptional regulators and monoclonal antibodies (Jain et al., 2004; Katragadda et al., 2005; Nobili et al., 2006). Surprisingly, there are only three studies, to our knowledge, investigating the effect of drug-drug interaction at the level of the RPE P-gp and its effect on ocular drug pharmacokinetics in vivo. These recent reports evaluated
the effect of systemic/systemic, systemic/intravitreal or intravitreal/intravitreal co-administration of substrates or inhibitors on ocular pharmacokinetics (Duvvuri et al., 2003a; Senthilkumari et al., 2008a; Senthilkumari et al., 2008b). However, so far, the effect of topically administered P-gp substrates/inhibitors on the functional activity of P-gp expressed on the RPE has not been reported.

Topical eye drops containing antimicrobial and anti-inflammatory agents, steroids and other therapeutic compounds are routinely administered to treat various ocular infections and disorders. Many of these agents are P-gp substrates/inhibitors and can diffuse into the RPE. The objective of this study was to determine whether topically administered P-gp substrates could modulate the functional activity of RPE P-gp and alter the vitreal pharmacokinetics of another P-gp substrate, quinidine, administered intravitreally. Erythromycin (Matheny et al., 2001) prednisolone (P-gp substrates commonly applied topically) (Karssen et al., 2002) and verapamil (a P-gp inhibitor used in earlier reports investigating inhibition of RPE P-gp) were administered topically. Quinidine, used in an earlier study to evaluate functional activity of RPE P-gp (Duvvuri et al., 2003a), was used as a model P-gp substrate in this study and its pharmacokinetic parameters were evaluated.
Materials and Methods

Animals: New Zealand male albino rabbits (NZW) were procured from Myrtle’s Rabbitry (Thompson Station, TN, USA). Experiments conformed to the tenets of the Association for Research in Vision and Ophthalmology (ARVO) statement on the Use of Animals in Ophthalmic and Vision Research and followed the University of Mississippi IACUC approved protocols.

Materials: Microdialysis probes (CMA/20; 20,000 Dalton molecular weight cut-off and 10 mm shaft) were obtained from CMA/Micro-dialysis Inc (North Chelmsford, MA, USA). Erythromycin, prednisolone hemisuccinate sodium (PHS), verapamil hydrochloride, fluorescein sodium and quinidine hydrochloride were purchased from Sigma Chemical Co. (St Louis, MO, USA). Ketamine hydrochloride and Xylazine were procured from Fort Dodge Animal Health (Fort Dodge, IA, USA) and Lloyd Laboratories (Shenandoah, IA, USA), respectively. Pentobarbital was obtained from Virbac AH, Inc. (Fort Worth, TX, USA). Solvents used were purchased from Fisher Scientific (St. Louis, Missouri, USA).

In vitro probe recovery: Probe recovery was determined by placing the probe in an isotonic phosphate buffered saline (IPBS) solution, pH 7.4, at 37 ºC, containing a known concentration of quinidine (equivalent to an intravitreal dose of 0.75 µg) alone or in the presence of verapamil or PHS. The probe was perfused with sterile IPBS (with or without verapamil) at a flow rate of 2 µL/min, and the dialysate was collected every 20 minutes. Relative recovery was calculated using equation 1.

\[
\text{Recovery}_{\text{in vitro}} = \frac{C_d}{C_s}
\]
Cd = Dialysate quinidine concentration.
Cs = Quinidine concentration in IPBS.
The concentration of quinidine in the vitreous humor samples was calculated by dividing the dialysate concentration with the \textit{in vitro} recovery factor obtained as described above.

\textbf{Probe implantation:} Rabbits (weighing 2-2.5 Kg) were anesthetized using ketamine (35 mg/kg) / xylazine (3.5 mg/kg) administered intramuscularly and were maintained under anesthesia throughout the duration of the experiment (ketamine / xylazine administered intramuscularly every 40 minutes). Prior to probe implantation, 1% tropicamide was applied topically to dilate the pupil. A 22G needle was then inserted into the posterior chamber of the eye. The point of insertion was approximately 3 mm below the corneal-scleral limbus. The needle was withdrawn, and the vitreal probe was implanted immediately. The position of the probe was adjusted so that the semipermeable membrane was in the mid-vitreous section. The probes were continuously perfused with sterile IPBS (pH 7.4) at a flow rate of 2 \mu L/min using a CMA/100 microinjection pump (CMA/Micro-dialysis Inc). Following probe implantation, animals were allowed to stabilize for a period of 2 hours prior to drug administration. Vitreal samples were collected every 20 minutes for a period of 9 hours. Samples were collected in microcentrifuge tubes and stored at -20 °C until further analysis. At the end of the study, animals were euthanized, under deep anesthesia, with an overdose of sodium pentobarbital administered through the marginal ear vein.
Drug administration: Quinidine was administered intravitreally (0.75 µg dose in 50 µL of IPBS). Studies were carried out with quinidine administered alone (control) or in the presence of topically co-administered erythromycin (0.2% w/v, pH 7.4), verapamil (0.5% w/v and 1% w/v, pH 6.0) and PHS (1% w/v and 2% w/v, pH 7.4). One hundred microliters of the inhibitor solution was instilled in the conjunctival sac. In the preliminary studies, erythromycin (0.2% w/v) was applied topically at 0, 2 and 4 hours post intravitreal quinidine injection. Subsequently, the topical P-gp substrate/inhibitor administration time was modified to 2, 4 and 6 hours post quinidine administration, to prolong the residence of the topically applied agent in the RPE tissue. Further studies with verapamil and PHS were carried out with topical instillation at 2, 4 and 6 hours post intravitreal administration. All solutions were prepared in sterile IPBS.

Vitreal pharmacokinetics of quinidine (0.75 µg) was also studied in the presence of intravitreally administered verapamil (100 µg). In this study, verapamil was co-administered intravitreally with quinidine (injection volume 50 µL; co-dissolved), following the probe stabilization period. Additionally, IPBS (pH 7.4) containing verapamil (1 mg/mL) was continuously perfused through the concentric probes to maintain high verapamil levels in the vitreous humor throughout the duration of the experiment.

Fluorescein kinetics: Vitreal kinetics of intravitreally administered fluorescein (dose - 10µg, injection volume 50 µL), alone or in the presence of topically co-administered verapamil (1% w/v, IPBS pH 6.0), were studied to ensure preservation of the barrier properties of the RPE in the presence of topical verapamil (1% w/v). In these
experiments, 100µL of 1%w/v verapamil solution was applied at 2, 4 and 6 hours post intravitreal fluorescein administration.

**Distribution of prednisolone hemisuccinate sodium (PHS) and verapamil in ocular tissues following topical or intravitreal application:** In a separate set of studies, verapamil (1% w/v, pH 6.0) or PHS (2% w/v, pH 7.4) was applied topically in the cul-de-sac of the rabbit's eye at 2, 4, and 6 hours post probe stabilization. At the end of 7 hours (for the verapamil studies) and 9 hours (for the PHS studies), rabbits were euthanized, eyes were enucleated and ocular tissues were collected and analyzed for drug content using an HPLC system. Additionally, ocular tissue concentrations of verapamil at the end of 7 hours, following intravitreal administration, as described earlier, was also determined.

**Bioreversion of PHS to prednisolone:** PHS, a hemisuccinate ester prodrug of prednisolone, (Augustijns et al., 1998) requires hydrolysis (chemical or enzymatic) of the ester bond to generate free prednisolone. The presence of esterase activity in rabbit ocular tissues has been demonstrated and well documented with ester prodrugs of pilocarpine, dipivefrin, gancyclovir and acyclovir (Tsuji et al., 1987; Majumdar et al., 2006; Majumdar et al., 2008). Bioreversion of PHS was studied in vitreous humor and in the ocular tissues such as the cornea, iris-ciliary body and RPE/choroid as previously described (Majumdar et al., 2008). Vitreous humor was centrifuged, and the supernatant was used. All the other ocular tissues were homogenized in 5 mL chilled IPBS with a tissue homogenizer (Tissuemiser, Fisher Scientific, St Louis, USA) for
periods of 30 seconds, with 1 minute intervals, in an ice bath. The homogenates were centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatants were used for the PHS enzymatic hydrolysis studies. Protein content in the supernatant was measured using the Bradford Protein estimation kit (Sigma Chemical Co, St Louis, MO), and the final protein content was adjusted to 1 mg/mL with IPBS.

Hydrolysis studies were carried out in triplicate at 37°C in a shaking water bath (75 reciprocations per min). One Hundred (100) µL of PHS stock solution was added to the required volume of the tissue homogenates and to vitreous humor to obtain a final PHS concentration of 10 µg/mL. At predetermined time points, 100 µL samples were withdrawn, and an equal volume of ice cold methanol was immediately added to the sample to arrest the enzymatic degradation process. Stability of PHS (10 µg/mL) in IPBS was also studied as a control.

Analytical procedures

Sample preparation: For studies involving distribution of PHS and verapamil in ocular tissues following topical or intravitreal application, enucleated eyes were rinsed with ice cold IPBS, to remove any traces of blood, and blotted dry using Kimwipes®. Aqueous and vitreous humor samples were collected using a 27G needle attached to a 1 mL tuberculin syringe. Eyes were then dissected and iris-ciliary bodies, lenses and retina/choroid tissues were isolated and weighed. Tissues were homogenized in ice cold IPBS using a Tissuemiser. Homogenates were diluted with an equal volume of ice cold acetonitrile:methanol (50:50) mixture, and centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was analyzed for drug content. Aqueous and vitreous humor
samples were used as such, or diluted with IPBS, and taken for analysis. Extraction efficiency of PHS and verapamil from the ocular tissues was almost 100%.

**Chromatography:** Quinidine and verapamil were analyzed using an HPLC system comprised of a Waters 717 Plus autosampler, Waters 2475 multi λ Fluorescence detector, Waters 600 controller pump and Agilent 3395 integrator. A Symmetry® C18 (4.6 x 250 mm) column was used, and the flow rate was set at 1 mL/min for both the compounds. Quinidine analysis was carried out using 20 mM phosphate buffer (pH 2.5) with 20% acetonitrile as the mobile phase, at an excitation wavelength of 250 nm and emission wavelength of 440 nm. Verapamil quantification was performed at an excitation wavelength of 280 nm and emission wavelength of 320 nm using acetonitrile and 0.07% v/v o-phosphoric acid in deionized water (33:67) as the mobile phase. Fluorescein and prednisolone analyses were performed using reversed phase HPLC procedures as previously described (Macha and Mitra, 2001; Chang Y. Cho, 2003)

**Data analysis:** Vitreal pharmacokinetic parameters of quinidine were determined by non-compartmental analysis using WinNonlin, version 5.2 (Pharsight; Mountain View, CA). Terminal slopes of the vitreous concentration-time profile were estimated by the log-linear regression, and the apparent elimination rate constant (λz) was derived from the slope. Elimination half-life (t1/2) was calculated from the equation: \( t_{1/2} = \frac{0.693}{\lambda_z} \). The area under the vitreal concentration-time curve from time “0” to time “t” and from time “300” to time “540” was calculated by linear trapezoidal method and extrapolated to infinity.
according to Eq 2.
\[ \text{AUC}_{0-\infty} = \text{AUC}_{0-t} + \frac{C_v}{\lambda_z} \]  

(2)

The area under the statistical moment curve (AUMC_{0-\infty}) was calculated using Eq 3.
\[ \sum_{t_{n-1}}^{t_n} \left( \frac{C_{n-1} \cdot t_{n-1} + C_n \cdot t_n}{2} \right) \left( t_n - t_{n-1} \right) + \frac{C_{v2} \cdot t_{last}}{\lambda_z} + \frac{C_v}{\lambda_z^2} \]  

(3)

where, \( C_v \) is the quinidine concentration at last time point (540 min). The mean retention time (MRT) was calculated using the equation: \( \text{MRT} = \frac{\text{AUMC}_{0-\infty}}{\text{AUC}_{0-\infty}} \). The total clearance was calculated as: \( \text{CL} = \frac{\text{Dose}}{\text{AUC}_{0-\infty}} \). \( \text{CL}_{300-540} = \frac{\text{Dose}}{\text{AUC}_{300-540}} \). The apparent volume of distribution at steady-state \( V_{ss} = \frac{(\text{Dose} \times \text{AUMC}_{0-\infty})}{(\text{AUC}_{0-\infty})^2} \). The time course of fluorescein after a single intravitreal bolus dose was described by a biexponential profile (Macha and Mitra, 2001a) as expressed in Eq 4.
\[ C = A e^{-\alpha t} + B e^{-\beta t} \]  

(4)

In which A and B are zero time concentration coefficients, \( t \) the time (min) and \( \alpha \) and \( \beta \) are the disposition rate constants of the initial and terminal phase, respectively. Vitreous humor was considered as part of the apparent central compartment and all other exchanging compartments including, the anterior chamber, were considered as part of the apparent peripheral compartment. Elimination was assumed to take place through plasma from the apparent central compartment. This model is illustrated as follows:

\[
\begin{align*}
\text{X1} & \xleftarrow{k_{10}} \text{X1} & \xleftarrow{k_{21}} \text{X2} & \xrightarrow{k_{12}} \text{X2}
\end{align*}
\]

Quinidine is administered into the vitreous humor (X1), and the variables X1 and X2 represent the amount of the drug in the vitreous humor and other exchanging compartments.
compartments, respectively. A similar model has been used to describe the kinetics of intravitreally administered fluorescein and other drugs (Macha and Mitra, 2001b; Macha and Mitra, 2001a; Macha and Mitra, 2002). In the open two-compartment model represented by equation 4, the rate constant of drug transfer from the apparent peripheral to the apparent central compartment \( (k_{21}) \) was calculated according to Eq 5.

\[
k_{21} = \frac{\alpha B + \beta A}{A + B}
\]  

(5)

The elimination rate constant of fluorescein from the apparent central compartment \( (k_{10}) \) was determined using Eq 6.

\[
k_{10} = \frac{\alpha \beta}{k_{91}}
\]  

(6)

Rate constant of fluorescein transfer from the apparent central compartment to the apparent peripheral compartment \( (k_{12}) \) was calculated using Eq 7.

\[
k_{12} = \alpha + \beta - (k_{21} + k_{10})
\]  

(7)

Data obtained was subjected to statistical analysis using Students t-test. A p value \( \leq 0.05 \) was considered to denote a statistically significant difference.
RESULTS

Effect of topically applied erythromycin on vitreal kinetics of intravitreally administered quinidine: Vitreal kinetics of intravitreally administered quinidine (0.75 µg dose, 50 µL injection volume) was studied either alone or in the presence of topically co-administered erythromycin (100 µL of a 0.2% w/v solution in IPBS, pH 7.4). The erythromycin solution was administered either at 0, 2 and 4 hours or at 2, 4 and 6 hours following intravitreal quinidine administration. When erythromycin was applied at 0, 2 and 4 hours, vitreal pharmacokinetic parameters i.e. \( t_{1/2} \) (104 ± 7 min), \( CL \) (0.0086 ± 0.0030 mL.min\(^{-1}\)) and \( \lambda_2 \) (0.0066 ± 0.0004 min\(^{-1}\)) were not significantly different from that of the control values (105 ± 11 min, 0.0048 ± 0.0012 mL.min\(^{-1}\) and 0.0066 ± 0.0007 min\(^{-1}\), respectively). A change in the topical erythromycin dosing times to 2, 4 and 6 hours following intravitreal quinidine administration also did not produce a significant difference in the pharmacokinetic parameters. Higher doses of erythromycin, in a solution form, could not be administered due to the limited aqueous solubility (2 mg/mL) of erythromycin.

Effect of topically co-administered verapamil on intravitreal kinetics of quinidine: Effect of topically co-administered verapamil (100 µL applied at 2, 4 and 6 hours) on the intravitreal kinetics of quinidine (0.75 µg) was examined at two different verapamil concentrations (0.5% w/v & 1% w/v). Verapamil had limited solubility at pH 7.4 (1 mg/mL) (Duvvuri et al., 2003a). Thus, verapamil solutions used in this study were prepared in IPBS pH 6.0 ± 0.1. The concentration-time profiles of quinidine following intravitreal administration, alone or in the presence of topically co-administered
verapamil (0.5% w/v & 1% w/v), are illustrated in Figures 1A and 1B. The vitreal pharmacokinetic parameters have been provided in Table 1. At a concentration of 0.5% w/v, verapamil did not produce any significant change in the vitreal kinetics of quinidine. However, at 1% w/v, topical verapamil produced a 1.7-fold decrease in the $\lambda_z$ (from $0.0066 \pm 0.0007$ to $0.0039 \pm 0.0001$ min$^{-1}$), a 1.7-fold increase in $t_{1/2}$ and a 1.7-fold increase in the MRT. Statistically significant differences between the mean vitreal quinidine concentrations of the 1% verapamil treated group and the control group was observed from the 360 minute time point onwards (Fig. 1B). Significant differences in AUC$_{0-\infty}$ and CL were not observed from those of the control. However, when partial areas were taken into account a 1.7-fold increase in AUC$_{300-540}$ and a 1.7-fold decrease in CL$_{300-540}$ were observed.

Vitreal kinetics of quinidine in the presence of intravitreally administered verapamil: The effect of intravitreal co-administration of verapamil (100 µg) on the vitreal kinetics of quinidine was also examined. In these studies the microdialysis probe perfusion solution contained verapamil, (1 mg/mL) to maintain a significantly higher verapamil/quinidine ratio in the vitreous humor. Figure 2 represents the vitreous concentration-time profile of quinidine in the presence of intravitreally co-administered verapamil. A 1.6-fold increase in MRT (from $83 \pm 13$ min to $131 \pm 16$ min), a 1.5-fold decrease in $\lambda_z$ (from $0.0066 \pm 0.0007$ min$^{-1}$ to $0.0043 \pm 0.0002$ min$^{-1}$) and a corresponding 1.5-fold increase in $t_{1/2}$ (from $105 \pm 11$ min to $159 \pm 9$ min) of quinidine were observed in the presence of intravitreally co-administered verapamil (Table 2). Statistically significant differences between the mean vitreal quinidine concentrations of
the treated and control groups were observed from the 100 minute time point onwards (Fig. 2).

**Effect of topical prednisolone hemisuccinate sodium (PHS) on vitreal kinetics of quinidine:** Prednisolone is practically insoluble in water (Karssen et al., 2002), and therefore its water soluble derivative PHS was used in this study. The effect of topically administered PHS (1% w/v and 2% w/v) on the vitreal kinetics of intravitreally administered quinidine was studied (Table 3 and Figs. 3A & 3B). One hundred microliters of a 1% or 2% w/v PHS solution were instilled at 2, 4 and 6 hours following quinidine administration. Topical co-administration of PHS 1% w/v resulted in a 1.4-fold increase in the t1/2 and a 1.4-fold decrease in the λz of quinidine. However, statistically significant changes in AUC300-540 and CL300-540 were not observed at this dose. Co-administration of 2% w/v PHS produced a 2.0-fold increase in the t1/2 and a 2-fold decrease in the λz of quinidine. A 1.4-fold increase in CL300-540 and AUC300-540 were also observed. Moreover, a 1.6-fold increase in MRT of quinidine was observed when compared to that of the control. Statistically significant differences between the mean vitreal quinidine concentrations of the 2% PHS treated group and the control group was noted from the 360 minute time point onwards (Fig. 3B).

**Ocular tissue distribution of verapamil and PHS:** Table 4 represents ocular tissue concentrations of verapamil (post topical and intravitreal administration) and PHS sodium (following topical administration). Topical and intravitreal routes of administration generated similar verapamil concentrations in the RPE/choroid at the end
of 7 hours. The 7 hour time point was selected for verapamil since the effect of topical verapamil on the vitreal quinidine kinetics became evident at around this time (significant change in the mean vitreal concentrations between the treated and control groups) in the pharmacokinetic profile (Fig. 1A). Very low/insignificant verapamil concentrations were observed in the vitreous humor following topical verapamil administration, indicating that the 2 hours stabilization period was sufficient to seal the scleral port created during probe implantation. PHS tissue concentrations were evaluated at the end of the experiment (9 h). Interestingly, following topical administration, the fraction of PHS sodium appearing in the vitreous humor (concentration in the vitreous humor as a percentage of the topically administered dose) was significantly higher than that observed with verapamil.

Bioreversion of PHS

Table 5 depicts the apparent pseudo-first-order degradation rate constants and half-lives of PHS in ocular tissue homogenates (1 mg/mL protein content), including vitreous humor. Tissue homogenates were prepared in IPBS pH 7.4. PHS was hydrolyzed to the parent drug, prednisolone, suggesting the role of esterases in the bioreversion of PHS. Degradation rate constants were obtained from log concentration of PHS remaining versus time plots. Hydrolysis rate constant obtained from the control (PHS in IPBS) were subtracted from the overall observed rate constants to estimate rate constants for the enzyme mediated hydrolytic process.
Intravitreal kinetics of fluorescein in the presence of topically applied verapamil:

Figure 4 illustrates the vitreous concentration-time profile of fluorescein, alone or in the presence of topically co-administered verapamil. Vitreal fluorescein concentration-time data could be best fitted to a two-compartment open model. One hundred microliters of verapamil (1% w/v, pH 6.0) was applied topically at 2, 4 and 6 hours after fluorescein administration to evaluate the effect of topical verapamil administration on the barrier properties of the RPE. The vitreal pharmacokinetic parameters of fluorescein, such as elimination half-life, CL, AUC, steady-state volume of distribution and apparent elimination rate constant, remained unchanged in the presence of topically co-administered verapamil (Table 6).
DISCUSSION

The goal of this novel study was to evaluate whether a drug-drug interaction could occur between intravitreally and topically co-administered P-gp substrates and its effect on the ocular pharmacokinetics of the intravitreally administered compound.

Duvvuri et al demonstrated that intravitreal co-administration of verapamil and quinidine resulted in increased vitreal elimination of quinidine. Moreover, when quinidine was administered systemically and verapamil was co-administered intravitreally, the vitreal AUC of quinidine increased significantly (Duvvuri et al., 2003a). In another in vivo study, Senthikumari et al. reported a significant increase in the ocular tissue concentrations of intravitreally administered rhodamine-123 in the presence of a P-gp inhibitor applied intravenously (Senthilkumari et al., 2008a). The authors hypothesized that the increased vitreal rhodamine-123 concentrations were probably the result of inhibition of efflux mediated by P-gp expressed on the ocular tissues. In a subsequent study, the investigators studied systemic co-administration of both compounds (rhodamine-123 and the inhibitor) but did not observe an increase in vitreal rhodamine-123 concentrations, probably because of inadequate inhibitor concentrations at the target site (Senthilkumari et al., 2008b).

The above three studies, to our knowledge the only published reports investigating in vivo RPE P-gp mediated efflux, evaluated the effect of either intravitreal or systemically co-administered inhibitors on vitreal kinetics of P-gp substrates. From a therapeutic point of view, with respect to delivery of P-gp substrates to the posterior chamber ocular tissues, the use of an intravitreal inhibitor is not feasible considering that high intravitreal levels of the inhibitor can only be maintained through multiple
intravitreal injections. On the other hand, the use of systemic inhibitors is not attractive because of nonspecific systemic exposure to the inhibitor and the limited, clinically relevant, inhibitor dose that can be administered.

A hitherto uninvestigated and novel alternative approach that could be therapeutically effective as well as minimize systemic exposure would be modulation of efflux mediated by P-gp expressed on the RPE through topical substrate/inhibitor application. As discussed earlier, several therapeutic agents that are P-gp substrates are currently administered topically. Literature also suggests that a fraction of topically administered agents may reach the RPE (Salminen and Urtti, 1984; Ozturk et al., 1999; Ozturk et al., 2000; Acheampong et al., 2002; Tan et al., 2002). Taking both factors into consideration, this study was undertaken to evaluate the feasibility of modulating ocular kinetics of intravitreally administered substrates through local application of P-gp substrates/inhibitors.

Erythromycin, 0.2% w/v, applied at 0, 2 and 4 hours or at 2, 4 and 6 hours following intravitreal quinidine administration, did not significantly affect the pharmacokinetic parameters of quinidine. Higher doses of erythromycin were not tested because of limited aqueous solubility. Topical verapamil, at a concentration of 0.5% w/v, also did not produce any significant change in the vitreal quinidine kinetics. The inability of 0.5% w/v verapamil and 0.2% w/v erythromycin to affect the pharmacokinetic parameters of quinidine could be due to insufficient inhibitor concentrations at the RPE, at these doses. Co-administration of topical verapamil 1% w/v resulted in a significant decrease in the apparent elimination rate constant and an increase in the vitreal half-life and mean retention time of quinidine in the posterior chamber (Table 1). Significant
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differences in AUC$_{0-\infty}$ and CL, from those of the control, were not observed possibly because the inhibitory effect of the topically administered agents becomes significant only when vitreal quinidine concentrations reduce to the low levels observed at the midpoint of the study, or, because adequate inhibitor concentrations are achieved at the RPE at that point of time. Consistent with the findings with verapamil and erythromycin, PHS demonstrated a dose-dependent effect on the vitreal pharmacokinetic parameters of quinidine (Table 3). At a concentration of 2% w/v, PHS produced a much more significant change compared to 1% w/v, probably because of higher concentrations of prednisolone generated at the target site, the RPE.

In the studies involving topical application of 1% verapamil (Figure 1B) and 2% PHS (Figure 2B), statistically significant difference between the mean vitreal quinidine concentrations of the control and the treated groups were observed from the 360 minute time point. In the case of 1% PHS a statistically significant difference between the means of the control and treated groups was observed only after 460 minutes. When partial areas were taken into account, significant differences in AUC$_{300-540}$ and CL$_{300-540}$ were observed in the 1% w/v verapamil and 2% w/v PHS treated groups, from that of control, demonstrating an interaction of topically administered P-gp substrates with RPE P-gp. These results illustrate the importance of the permeability kinetics of the topically applied agents.

Fluorescein has been used as a marker compound to monitor the integrity and tightness of the blood-retinal barriers. The vitreal pharmacokinetic parameters of fluorescein were not affected by topical co-administration of 1% w/v verapamil (Table 6), and the values obtained in this study were consistent with previously published values.
(Macha and Mitra, 2001a). The results thus strongly suggest that the observed effect of topical verapamil on the vitreal kinetics of quinidine is a result of verapamil interacting with RPE P-gp.

Since prednisolone rather than PHS is known to interact with P-gp, bioreversion of PHS to prednisolone is necessary. In vitro metabolism studies confirmed bioreversion of PHS, to generate free prednisolone, in the ocular tissues (Table 5). At higher topical doses of PHS, greater quantities of PHS would be reaching the RPE, and thus greater concentrations of prednisolone would be generated. Interestingly, the ocular tissue distribution studies, following topical administration of verapamil and PHS, revealed that significantly higher fractions of the topically administered PHS dose reached the vitreous humor (almost 5-fold higher) compared to verapamil. Cheruvu et al. indicated that compounds with high logP values demonstrate lower trans-scleral permeability, possibly because of an interaction with the proteins expressed on the Bruch’s membrane (Cheruvu and Kompella, 2006). The higher vitreal PHS concentrations observed could be a result of greater aqueous solubility of PHS at physiological pH ranges, favoring greater diffusion across the Bruch’s membrane and/or because of changes in the binding affinity with the Bruch’s membrane proteins. The ocular tissue concentration data (Table 4) also suggests that verapamil and PHS migrate laterally along the cornea/aqueous humor route, and possibly also across the conjunctiva, into the sclera. The results further demonstrate that following topical administration, sufficient verapamil and PHS concentrations can accumulate in the choroid/RPE tissue and inhibit P-gp on the basolateral membrane of RPE. In fact, the concentration of verapamil in the RPE/choroid tissue 7 hours post topical verapamil instillation was
similar to that obtained after intravitreal administration. Insignificant vitreal concentrations, however, suggest that very little verapamil could traverse across the RPE into the neural retina.

The elimination rate of quinidine from the vitreous humor was observed to decrease in the presence of intravitreally administered verapamil (Table 2), suggesting functional involvement of P-gp expressed on the basolateral membrane of the RPE, or apical membrane of the retinal endothelial cells. These results are contrary to that of Duvvuri et al., wherein quinidine elimination was observed to increase in the presence of intravitreal verapamil. The authors had suggested P-gp expressed on the neural retina, facing the vitreous, probably influences elimination of P-gp substrates from the vitreous humor. However, there are no other reports corroborating P-gp expression on the neural retina. Besides P-gp expression on the apical membrane of the retinal endothelial cells, most *in vitro and ex vivo* studies suggest functional activity of P-gp is localized on the basolateral membrane of the RPE. A study by Steuer et al. demonstrated higher permeability of verapamil (2.6-fold) and rhodamine (3.5-fold) across isolated RPE in the neural retina to choroid direction compared to the choroid to neural retina direction, demonstrating the significance of P-gp localized on the basolateral membrane (choroidal side) of the RPE (Steuer et al., 2005). The results by Senthilkumari et al. also suggest functional expression of P-gp on the basolateral membrane of the RPE (Senthilkumari et al., 2008a). Biochemical and functional studies carried out by Kennedy et al., is the only study, to our knowledge, that suggests expression of P-gp on the apical membrane of the RPE. However, the authors postulated that P-gp localized on the apical RPE probably serves additional functions.
such as modulation of volume sensitive chloride efflux or functions as lipid translocase, etc. (Kennedy and Mangini, 2002). However, since the intravitreal verapamil dose administered was not mentioned by the authors (Duvvuri et al., 2003a), there is a possibility that the verapamil dose administered in the earlier study was sufficient to inhibit P-gp on the neural retina but not interact with P-gp expressed on the basolateral membrane of the RPE.

Taken together, the above results demonstrate that topically administered P-gp substrates can migrate along the corneal-scleral pathway and possibly across the conjunctiva into the sclera and significantly alter elimination profiles of intravitreally administered P-gp substrates. This interaction can be used to modulate drug elimination from the posterior chamber of the eye. The impact of this strategy might be significantly more marked with topical and systemic co-administration (currently under investigation), considering the low plasma concentrations of the P-gp substrates generated compared to the vitreous humor concentrations obtained following intravitreal administration. This technique may also help modulate efflux activity of other transporters, such as the multidrug resistant proteins, expressed on the RPE. Further investigation of this novel approach is warranted.

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REFERENCES


Footnote:

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LEGENDS FOR FIGURES

Figure 1A
Vitreal concentration-time profile of quinidine (0.75 µg) alone (control) or in the presence of topically co-administered verapamil 0.5% w/v (100 µL administered at 2, 4 and 6 h after quinidine administration). Data points represent mean ± standard deviation of four determinations.

Figure 1B
Vitreal concentration-time profile of quinidine (0.75 µg) alone (control) or in the presence of topically co-administered verapamil 1% w/v (100 µL administered at 2, 4 and 6 h after quinidine administration). Data points represent mean ± standard deviation of four determinations.

Figure 2
Vitreal concentration-time profile of quinidine (0.75 µg) alone (control) or in the presence of intravitreally co-administered verapamil (100 µg, administered along with quinidine). Data points represent mean ± standard deviation of four determinations.

Figure 3A
Vitreal concentration-time profile of quinidine (0.75 µg) alone (control) or in the presence of topically co-administered prednisolone hemisuccinate sodium (1% w/v, 100 µL administered at 2, 4 and 6 h after quinidine administration). Data points represent mean ± standard deviation of four determinations.

Figure 3B
Vitreal concentration-time profile of quinidine (0.75 µg) alone (control) or in the presence of topically co-administered prednisolone hemisuccinate sodium (2% w/v, 100 µL administered at 2, 4 and 6 h after quinidine administration). Data points represent mean ± standard deviation of four determinations.

Figure 4
Vitreal concentration-time profile of fluorescein (10.0 µg) alone or in the presence of topically co-administered verapamil (1%w/v, 100 µL administered at 2, 4 and 6 h after intravitreal fluorescein administration). Data points represent mean ± standard deviation of four determinations.
Table 1: Vitreal pharmacokinetic parameters of intravitreally administered quinidine (0.75 µg dose) alone or in the presence of topically co-administered verapamil (0.5% w/v or 1% w/v). Verapamil was administered at 2, 4 and 6 hours after intravitreal quinidine administration.

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Quinidine</th>
<th>Quinidine + Verapamil (0.5% w/v)</th>
<th>Quinidine + Verapamil (1% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_e$ (min$^{-1}$)</td>
<td>0.0066± 0.0007</td>
<td>0.0066 ± 0.0010</td>
<td>0.0039 ± 0.0001 ***</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (µg x min/mL)</td>
<td>168 ± 56</td>
<td>180 ± 41</td>
<td>158 ± 37</td>
</tr>
<tr>
<td>CL (mL/min)</td>
<td>0.0048 ± 0.0012</td>
<td>0.0043 ± 0.0008</td>
<td>0.0049 ± 0.0012</td>
</tr>
<tr>
<td>$V_{ss}$ (mL)</td>
<td>0.41 ± 0.14</td>
<td>0.37 ± 0.12</td>
<td>0.72 ± 0.30</td>
</tr>
<tr>
<td>MRT$_\infty$ (min)</td>
<td>83 ± 13</td>
<td>84 ± 13</td>
<td>143 ± 30 **</td>
</tr>
<tr>
<td>AUC$_{300-540}$ (µg x min/mL)</td>
<td>7.73 ± 1.32</td>
<td>9.05 ± 0.38</td>
<td>12.86 ± 1.25*</td>
</tr>
<tr>
<td>CL$_{300-540}$ (mL/min)</td>
<td>0.099 ± 0.017</td>
<td>0.080 ± 0.004</td>
<td>0.060 ± 0.013*</td>
</tr>
</tbody>
</table>

Values represented as mean ± standard deviation (n=4). *p<0.05, **p<0.01, ***p<0.001
Table 2: Vitreal kinetic parameters of quinidine (0.75 µg dose) alone or in the presence of intravitreally co-administered verapamil (100 µg). Verapamil solution (1 mg/mL in IPBS) was used as the perfusate.

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Quinidine</th>
<th>Quinidine + Intravitreal Verapamil</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_2$ (min$^{-1}$)</td>
<td>0.0066 ± 0.0007</td>
<td>0.0043 ± 0.0002 ***</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (µg x min/mL)</td>
<td>168 ± 56</td>
<td>180 ± 72</td>
</tr>
<tr>
<td>CL (mL/min)</td>
<td>0.0048 ± 0.0012</td>
<td>0.0046 ± 0.0014</td>
</tr>
<tr>
<td>V$_{SS}$ (mL)</td>
<td>0.41 ± 0.14</td>
<td>0.63 ± 0.27</td>
</tr>
<tr>
<td>MRT$_{\infty}$ (min)</td>
<td>83 ± 13</td>
<td>131 ± 16 **</td>
</tr>
<tr>
<td>AUC$_{100-540}$ (µg x min/mL)</td>
<td>40 ± 0.03</td>
<td>56 ± 11*</td>
</tr>
<tr>
<td>AUC$_{300-540}$ (µg x min/mL)</td>
<td>7.73 ± 1.32</td>
<td>13.31 ± 2.28**</td>
</tr>
<tr>
<td>CL$_{300-540}$ (mL/min)</td>
<td>0.099 ± 0.017</td>
<td>0.057 ± 0.009**</td>
</tr>
</tbody>
</table>

Values represented as mean ± standard deviation (n=4). *p<0.05, **p<0.01, ***p<0.001
Table 3: Vitreal pharmacokinetic parameters of quinidine (0.75 µg dose), following intravitreal administration in the presence and absence of topically co-administered prednisolone hemisuccinate sodium (PHS).

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Quinidine</th>
<th>Quinidine + PHS 1% w/v</th>
<th>Quinidine + PHS 2% w/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>λz (min⁻¹)</td>
<td>0.0066± 0.0007</td>
<td>0.0048 ± 0.0004**</td>
<td>0.0033 ± 0.0004***†</td>
</tr>
<tr>
<td>AUC₀-∞ (µg x min/mL)</td>
<td>168 ± 56</td>
<td>191 ± 42</td>
<td>151 ± 21.6</td>
</tr>
<tr>
<td>CL (mL/min)</td>
<td>0.0048 ± 0.0012</td>
<td>0.0040 ± 0.0009</td>
<td>0.0050 ± 0.0007</td>
</tr>
<tr>
<td>V₃₃ (mL)</td>
<td>0.41 ± 0.14</td>
<td>0.37 ± 0.14</td>
<td>0.65 ± 0.20</td>
</tr>
<tr>
<td>MRT ∞ (min)</td>
<td>83 ± 13</td>
<td>87 ± 16</td>
<td>129 ± 21**†</td>
</tr>
<tr>
<td>AUC₃₀₀-₅₄₀(µg x min/mL)</td>
<td>7.73 ± 1.32</td>
<td>8.70 ± 0.79</td>
<td>11.76 ± 0.40*†</td>
</tr>
<tr>
<td>CL₃₀₀-₅₄₀(mL/min)</td>
<td>0.099 ± 0.017</td>
<td>0.087 ± 0.008</td>
<td>0.063 ± 0.002*†</td>
</tr>
</tbody>
</table>

Values represented as mean ± standard deviation (n=4).

* , ** , *** indicates statistical significant difference between quinidine (control) and treated groups (Quinidine+ PHS 2% w/v and Quinidine + PHS 1% w/v); *p<0.05, **p<0.01, ***p<0.001

† indicates statistical significant difference between Quinidine + PHS 2% w/v and Quinidine + PHS 1% w/v; †p<0.05
Table 4: Ocular distribution of verapamil and PHS. Verapamil tissue concentrations were determined 7h after topical (100 µL of a 1% w/v solution applied 2, 4 and 6 h post intravitreal quinidine administration) and intravitreal administration (dose: 100 µg). Ocular distribution of topically-applied PHS (100 µL of a 2% w/v solution applied at 2, 4 and 6 h post intravitreal quinidine administration) was determined 9 h after intravitreal quinidine administration, and the values have been reported for both intact PHS and free prednisolone concentrations observed. Values represent mean ± standard deviation (n=4)

* Generated as a result of bioreversion of PHS in the ocular tissues.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Verapamil concentration</th>
<th>PHS Topical application</th>
<th>Concentration of free Prednisolone *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Topical Application</td>
<td>Intravitreal Administration</td>
<td>Intact PHS concentration</td>
</tr>
<tr>
<td>Aqueous Humor (µg/mL)</td>
<td>7.9 ± 3.2</td>
<td>1.5 ± 0.3</td>
<td>10.2 ± 1.1</td>
</tr>
<tr>
<td>Iris-Ciliary Body (µg/gm)</td>
<td>4.4 ± 1.6</td>
<td>27.0 ± 10.0</td>
<td>7.0 ± 1.5</td>
</tr>
<tr>
<td>Lens (µg/gm)</td>
<td>6.3 ± 1.2</td>
<td>45.1 ± 4.2</td>
<td>-</td>
</tr>
<tr>
<td>Vitreous Humor (µg/mL)</td>
<td>0.086 ± 0.003</td>
<td>31.5 ± 1.4</td>
<td>0.64 ± 0.21</td>
</tr>
<tr>
<td>Retina-Choroid (µg/gm)</td>
<td>47.0 ± 8.1</td>
<td>52.0 ± 12.2</td>
<td>8.8 ± 1.2</td>
</tr>
</tbody>
</table>
Table 5 Apparent first order degradation rate constants (k) x 10³, min⁻¹ and half-lives (t₁/₂, min), of PHS (prednisolone hemisuccinate sodium) in ocular tissue homogenates (1 mg/mL protein content). Values represent mean ± standard deviation (n=4).

<table>
<thead>
<tr>
<th>Drug / Kinetic parameters</th>
<th>Control</th>
<th>Cornea</th>
<th>Vitreous humor</th>
<th>Iris-ciliary</th>
<th>RPE/Choroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>k</td>
<td>0.33 ± 0.05</td>
<td>0.81 ± 0.07</td>
<td>3.06 ± 0.34</td>
<td>2.00 ± 0.06</td>
<td>1.20 ± 0.05</td>
</tr>
<tr>
<td>t₁/₂</td>
<td>2118 ± 333</td>
<td>852 ± 74</td>
<td>228 ± 28</td>
<td>347 ± 10</td>
<td>578 ± 27</td>
</tr>
</tbody>
</table>
Table 6: Vitreal kinetics of intravitreally administered fluorescein (10 µg).

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Fluorescein</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{10}$ (min$^{-1}$)</td>
<td>0.021 ± 0.01</td>
</tr>
<tr>
<td>$k_{12}$ (min$^{-1}$)</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>$k_{21}$ (min$^{-1}$)</td>
<td>0.0135 ± 0.004</td>
</tr>
<tr>
<td>AUC (µg x min/mL)</td>
<td>3272 ± 632</td>
</tr>
<tr>
<td>$K_{10}$ t $\frac{1}{2}$ (min)</td>
<td>38.6 ± 20.3</td>
</tr>
<tr>
<td>CL (mL/min)</td>
<td>0.003 ± 0.0005</td>
</tr>
<tr>
<td>MRT $\infty$ (min)</td>
<td>124 ± 49</td>
</tr>
<tr>
<td>$V_{SS}$ (mL)</td>
<td>0.37 ± 0.1</td>
</tr>
<tr>
<td>$\beta$ (min$^{-1}$)</td>
<td>0.0049 ± 0.0009</td>
</tr>
<tr>
<td>$\beta$ t $\frac{1}{2}$ (min)</td>
<td>122 ± 39</td>
</tr>
</tbody>
</table>

Values represent mean ± standard deviation (n=4).
Fig: 1A

![Graph showing the effect of Quinidine and Quinidine + Topical Verapamil on Vitreal Concentration over time.

- **Quinidine**
- **Quinidine + Topical Verapamil 0.5% w/v**

**Axes:**
- **Y-axis:** Vitreal Concentration (μg/mL)
- **X-axis:** Time (minutes)
Fig: 2

- △ Quinidine
- ■ Quinidine + Intravitreal Verapamil

Vitreal Concentration (μg/mL) vs Time (minutes)
Fig: 3A

Vitreal Concentration (µg/mL) vs Time (minutes)

- Quinidine
- Quinidine + Topical Prednisolone 1% w/v
Fig: 3B

- Quinidine
- Quinidine + Topical Prednisolone 2% w/v

Vitreal Concentration (µg/mL) vs. Time (minutes)
Fig: 4

- **Fluorescein**
- **Fluorescein + Topical Verapamil 1% w/v**

Vitreal Concentration (µg/mL) vs. Time (minutes)