Pharmaceutical Research, Vol. 26, No. 5, May 2009 (© 2008)
DOI: 10.1007/s11095-008-9741-x

Research Paper

Enhanced Corneal Absorption of Erythromycin by Modulating P-Glycoprotein and MRP Mediated Efflux with Corticosteroids

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Received July 31, 2008; accepted September 29, 2008; published online October 29, 2008

Purpose. The objectives were (i) to test *in vivo* functional activity of MRP2 on rabbit corneal epithelium and (ii) to evaluate modulation of P-gp and MRP2 mediated efflux of erythromycin when co-administered with corticosteroids.
Methods. Cultured rabbit primary corneal epithelial cells (rPCECs) was employed as an *in vitro* model for rabbit cornea. Cellular accumulation and bi-directional transport studies were conducted across Madin-Darby Canine Kidney (MDCK) cells overexpressing MDR1 and MRP2 proteins to delineate transporter specific interaction of steroids. Ocular pharmacokinetic studies were conducted in rabbits following a single-dose infusion of erythromycin in the presence of specific inhibitors and steroids.
Results. Bi-directional transport of erythromycin across MDCK-MDR1 and MDCK-MRP2 cells showed significant difference between BL-AP and AP-BL permeability, suggesting that erythromycin is a substrate for P-gp and MRP2. Cellular accumulation of erythromycin in rPCEC was inhibited by steroids in a dose dependent manner. MK571, a specific MRP inhibitor, modulated the aqueous humor concentration of erythromycin *in vivo*. Even, steroids inhibited P-gp and MRP2 mediated efflux with maximum increase in k_a, $AUC_{0-\infty}$, C_{max} and C_{last} values of erythromycin, observed with 6α-methyl prednisolone.
Conclusion. MRP2 is functionally active along with P-gp in effluxing drug molecules out of corneal epithelium. Steroids were able to significantly inhibit both P-gp and MRP2 mediated efflux of erythromycin.

KEY WORDS: corneal epithelium; corticosteroids; erythromycin; microdialysis; multidrug resistance associated protein (MRP); ocular pharmacokinetics; P-glycoprotein (P-gp).

INTRODUCTION

ATP-binding cassette (ABC) transporters are transmembrane proteins involved in the translocation of a wide variety of substrates across biological membranes. Multidrug resistance is primarily caused due to cellular extrusion of drugs by P-glycoprotein (P-gp), multidrug resistance associated proteins (MRPs) and breast cancer resistance protein (BCRP) all of which belong to the ABC super family of membrane transporters. These proteins are known to limit absorption across many biological barriers and restrict entry into important pharmacological sanctuary sites (1). Such efflux proteins have also been reported to be expressed in various tissues of the eye such as retinal pigmented epithelium (2–4), retinal capillary endothelium (5,6), ciliary nonpigmented epithelium (7), conjunctival epithelium (8) and iris and ciliary muscle cells (5). Expression and functional activity of P-gp on the rabbit and human corneal epithelia have been reported (9). Recently, our laboratory has also demonstrated the expression and functional activity of multidrug resistance associated protein-2 (MRP2) on rabbit and human corneal epithelial cells (10,11).

Drug molecules which are pharmacologically and structurally unrelated often are substrates for these efflux proteins. Ocular therapeutic agents are no exceptions with macrolides (erythromycin, azythromycin) (12), fluoroquinolones (grepafloxacin, ciprofloxacin) (12–15), immuno-suppressive agents (cyclosporine A) (16,17) and steroids (hydrocortisone, dexamethasone) (18) classified as substrates for these efflux proteins. In addition, due to overlapping substrate specificities, multiple efflux pumps might be involved in effluxing out a drug molecule, simultaneously. For e.g., cyclosporine A which is used in keratoconjunctivitis sicca, has been reported to interact with P-gp, MRP1, MRP2 and BCRP (19).

Though topical administration of drugs is the most preferred and convenient route to treat ocular diseases, it suffers from extremely limited bioavailability (<5%). Drug loss is primarily because of pre-corneal factors such as solution drainage, tear turn over rate and absorption by

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ABBREVIATIONS: AP, apical; AUC, area under curve; BL, basolateral; CsA, cyclosporine A; Ery, erythromycin; MDCK, Madin-Darby Canine Kidney; MPL, 6α-methyl prednisolone; MRP, multidrug resistance associated protein; P_{app}, apparent permeability; P-gp, P-glycoprotein; PL, prednisolone; PS, prednisone; rPCEC, rabbit primary corneal epithelial cell.

other tissues resulting in the loss of drug to systemic circulation. Low ocular bioavailability has also been attributed to the lipoidal nature of the corneal epithelium and the water laden stroma which act as rate limiting barriers for hydrophilic and lipophilic molecules, respectively. In addition, efflux transporters on the corneal epithelium could also contribute to low ocular bioavailability by actively effluxing molecules from the cornea back into the tear film. A recently published report from our laboratory has shown the role of P-gp as a barrier to *in vivo* ocular drug absorption (20).

Hence, a viable strategy to improve the ocular absorption of topically administered drugs that are substrates for efflux proteins would be to inhibit efflux pumps on the cornea. Such inhibitors could significantly elevate the cellular concentration of the drug in the cornea as well as the aqueous humor. However, specific efflux modulators may cause significant toxicity at doses needed to cause efflux modulation and are not therapeutically acceptable (21). Hence, a dual advantage could be achieved if efflux inhibitors had a therapeutic effect which is relevant in the treatment regimen, in addition to their primary role of modulating efflux. Erythromycin, a broad spectrum antibiotic used to treat superficial bacterial infections of the cornea and conjunctiva (brand name: Ilotycin®) was selected as the drug substrate for our study (22,23). Bacterial infections are invariably associated with inflammation of the eye. For this reason, corticosteroids were chosen as inhibitors for our study. In addition to modulation of efflux, these compounds can also elicit anti-inflammatory action in a relevant anti-bacterial treatment regimen.

In vivo functional activity of P-gp has already been reported with testosterone as a model inhibitor (20). Though the functional activity of MRP2 on human and rabbit corneal epithelial cells has been reported, its ability to modulate drug concentrations across cornea has not been established in an *in vivo* setting. Moreover, it is essential to determine if therapeutically relevant corticosteroids when co-administered with erythromycin can inhibit both P-gp and MRP mediated efflux in the corneal epithelium. These objectives require determining the pharmacokinetics of erythromycin following topical co-administration with MK571 (a specific MRP inhibitor) and steroids. Unfortunately, there are few drawbacks associated while determining the disposition of drugs applied topically. Several pharmacokinetic models have been proposed to predict absorption and disposition of drugs applied topically to the eye, but all involve varying complexities with regard to numerical analyses (24–26). Another major constraint is the inaccessibility of aqueous humor for serial sampling. Conventional pharmacokinetic studies require sacrificing at least six animals per time point and as such the numbers would drastically increase depending on the number of time points required to develop a complete pharmacokinetic profile.

To simplify the approach and to estimate ocular disposition of topically applied drugs, we conceptualized the combination of a topical well infusion model and aqueous humor microdialysis sampling. In the topical well infusion model, a constant level of drug is maintained over the cornea with the help of a plastic cylindrical well such that the effect of tear dynamics is minimized and simpler equations can be applied independent of compartmental modeling (27). Absorption through tissues such as conjunctiva and lacrimal glands could be eliminated which helps estimating the corneal absorption rate constant, precisely. Problems involved with serial sampling of ocular fluids could be overcome by utilizing microdialysis which is superior over conventional sampling techniques in determining ocular pharmacokinetics by both reducing the number of subjects and providing statistically robust data (28).

Therefore, the objectives of this study were (i) to determine *in vitro* if steroids could inhibit both P-gp and MRP2 mediated efflux of erythromycin, (ii) to evaluate the role of MRP2 in modulating *in vivo* corneal drug absorption and (iii) to assess the role of steroids as potential co-administering agents to enhance corneal drug absorption of actively effluxed drugs, such as erythromycin.

MATERIALS AND METHODS

Materials

MPL, PL, PS and cyclosporine A (CsA) were purchased from Sigma-Aldrich (St. Louis, MO). MK-571, a specific inhibitor of MRP was procured from Biomol International (Plymouth Meeting, PA). GF120918 was a generous gift from GlaxoSmithKline Ltd. [^{14}C] Erythromycin (specific activity 48.8 mCi/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Stock solutions of steroids (20 mg/ml), CsA (1 mg/ml), GF120918 (1 mg/ml) and MK-571 (25 mg/ml) were prepared in dimethyl sulfoxide (DMSO) and aliquots were diluted in Dulbecco's phosphate-buffered saline (DPBS) to achieve the desired concentration.

MDCK cells transfected with the human MDR1 gene (MDCK MDR1) and human MRP2 gene (MDCK MRP2) were generously provided by Drs. A. Schinkel and P. Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands). Cell culture supplies which included minimum essential medium (MEM, for rPCEC), Dulbecco's modified Eagle's medium (DMEM, for MDCK MDR1 and MDCK MRP2 cells), Trypsin-EDTA solution, non-essential amino acids and fetal bovine serum were procured from Invitrogen (Carlsbad, CA). Penicillin, streptomycin, sodium bicarbonate, lactalbumin, HEPES, amphotericin B and polymyxin B sulfate were procured from Sigma-Aldrich. Culture flasks (75 cm^2 growth area) were procured from MidSci (St. Louis, MO). Twelve-well culture plates (3.8 cm^2 growth area per well) and polyester Transwell filter inserts (1.1 cm^2, 0.4 μm mean pore size) were obtained from Corning Costar Corp (Cambridge, MA).

New Zealand albino male rabbits weighing between 2.0 and 2.5 kg were obtained from Myrtle's Rabbitry (Thompson Station, TN). Studies were performed according to the animal protocol in ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Linear microdialysis probes (MD-2000, 0.32×10 mm, polyacrylonitrile membrane and 0.22 mm tubing) employed for aqueous humor sampling were procured from Bioanalytical Systems (West Lafayette, IN). Microinjection pump (CMA/100) for perfusing the isotonic phosphate buffer saline was obtained from CMA/Microdialysis (Acton, MA). Ketamine HCl and Rompun (Xylazine) were purchased from Fort Dodge Animal Health (Fort Dodge, IA) and Bayer Animal Health (Shawnee Mission, KS), respectively. Topical wells were custom made by Hansen Ophthalmic Development Corporation (Iowa City, IA) according to specific

instructions (Fig. 1A). All other chemicals were obtained from Sigma-Aldrich. The solvents were of HPLC grade and obtained from Fisher Scientific Company (St. Louis, MO).

Methods

Cell Culture. The rabbit corneal epithelial cells were cultured according to a previously published method from our laboratory (9). Cells were nourished with culture medium comprising MEM, 10% FBS, HEPES, sodium bicarbonate, penicillin, streptomycin sulphate and 1% (*v*/*v*) non-essential amino acids, adjusted to pH 7.4. Cells were grown in 75 cm^2 culture flasks and maintained at 37°C, in a humidified atmosphere of 5% CO_2 and 90% relative humidity. Culture medium was replaced every alternate day. Cells were subcultured after 7 days (subculture ratio, 1:5) with 0.25% trypsin containing 0.53 mM EDTA and plated at a density of 250,000 cells/well on 12-well culture plates.

MDCK MDR1 and MDCK MRP2 cells were grown in culture medium comprising DMEM, 10% FBS, HEPES, sodium bicarbonate, penicillin, streptomycin sulphate and 1% (*v*/*v*) non-essential amino acid, adjusted to pH 7.4. Cells were grown in similar conditions as mentioned above and plated at a density of 250,000 cells/well on 12-well culture plates. Cells were also grown on collagen coated polyester Transwell filter inserts with a seeding density of 50,000 cells/cm^2. Integrity of monolayers formed on Transwell filter inserts was assessed by measuring the transepithelial electrical resistance (TEER) value with the help of an epithelial volt ohmmeter (EVOM; World Precision Instruments, Sarasota, FL). TEER values of the cell monolayer were approximately 250 Ωcm^2 after correcting for the resistance imparted by filters.

Cellular Accumulation Studies. Cellular accumulation studies were conducted on rPCEC (10–12 days post-seeding, passages 5 to 10), MDCK MDR1 and MDCK MRP2 cells (5–7 days post-seeding, passages 5 to 15). The medium was aspirated and cells were washed twice (once in 10 min) with Dulbecco's phosphate-buffered saline (DPBS; pH 7.4), Concentrated stock solutions of GF120918, MK-571 and steroids were prepared in DMSO. Test solutions were then prepared by adding aliquots from the stock solution and diluting with DPBS, pH 7.4, to achieve required concentration. DMSO concentration in the final solution did not exceed 1% (*v*/*v*). Cellular accumulation was then initiated by adding 1 ml of [^{14}C] erythromycin (0.25 μCi/ml) prepared in DPBS, pH 7.4, in the absence and presence of competing substrates/test compounds. Incubation with drug solution was carried out for 15 min at 37°C. Following incubation, the drug solution was aspirated and cell monolayers were washed twice with 1 ml of ice-cold stop solution (210 mM KCl, 2 mM HEPES) to arrest cellular accumulation. Finally, cells were lysed with 1 ml of 0.3% NaOH containing 0.1% Triton-X solution and stored overnight. Cellular radioactivity was quantified using a scintillation counter and the rate of cellular accumulation of [^{14}C] erythromycin was normalized to the protein content in each well.

Bi-directional Transport Studies. Bi-directional transport experiments were carried out with monolayers of MDCK MDR1 and MDCK MRP2 cells. Similar to cellular accumulation studies, solutions containing test compounds were prepared in DPBS, pH 7.4. The medium was aspirated and cells were washed twice (once in 10 min) with DPBS, pH 7.4. Apical (AP) to basolateral (BL) transport was initiated by adding 0.5 ml of test solution to the apical chamber while the

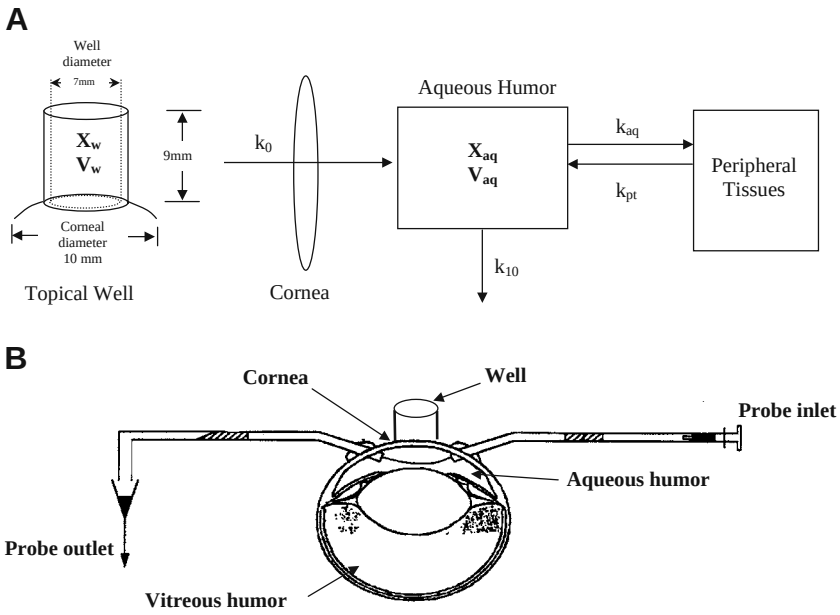


Fig. 1. A Schematic model representation of a single-dose continuous infusion to the rabbit eye. The cornea is assumed to act more like a barrier than a compartment. Elimination was assumed to be occurring primarily through the central (aqueous humor) compartment. From aqueous humor, drug may reversibly distribute to peripheral tissues. **B** Schematic representation of the topical well infusion model coupled with anterior segment microdialysis.

basolateral chamber contained DPBS, pH 7.4. BL to AP transport was initiated by adding 1.5 ml of the test solution to the BL chamber while the AP chamber contained DPBS, pH 7.4. Transport studies were conducted for a period of 3.5 h at 37°C. Samples (150 µl) were withdrawn from the receiver chamber at predetermined time points, i.e. 15, 30, 45, 60, 90, 120, 150, 180 and 210 min and replaced with equal volume of DPBS, pH 7.4, to maintain sink conditions. Radioactivity in samples containing [^{14}C] erythromycin was measured using a scintillation counter. Samples containing steroids were stored at −80°C until further analysis by HPLC. The net efflux was assessed by calculating the efflux ratio as shown in Eq. 1. An efflux ratio greater than 1.0 indicates net efflux.

$$\text{Efflux Ratio} = \left(P_{app}BL \rightarrow AP\right) / \left(P_{app}AP \rightarrow BL\right) \quad (1)$$

where, P_{app} BL→AP and P_{app} AP→BL indicates apparent drug permeability in the BL to AP and AP to BL direction, respectively.

Cell Proliferation Assay. Cell proliferation assay was performed on rPCEC, to examine the cytotoxicity of steroids at a concentration of 500 µM along with erythromycin for a period of 12 h. Specific efflux inhibitors such as GF120918 (2 µM) and MK-571 (50 µM) are noncytotoxic at the concentration used in our study. A commercial assay (CellTiter 96 AQ_{ueous} Non-radioactive Cell Proliferation Assay Kit; Promega, Madison, WI) based on a colorimetric method was employed for determining the number of proliferating cells. The experimental procedure followed was similar to a previously published report (20).

In Vivo Anterior Segment Microdialysis

Probe Implantation. Animals were anesthetized prior to surgery by means of ketamine HCl (35 mg/kg) and xylazine (3.5 mg/kg) administered intramuscularly. Anesthesia is maintained throughout the experiment with ketamine HCl and xylazine administered every 40 min. Prior to probe implantation, pupils were dilated with two drops of 1% tropicamide solution, instilled topically. A 25-gauge needle was first inserted across cornea, just above the corneal–scleral limbus and made to traverse right across the other end without damaging the iris-ciliary body and lens. The sample collecting end of the linear probe was inserted carefully into the bevel-end edge of the needle. The needle was slowly retracted leaving the probe with the dialysis membrane in the middle of the anterior chamber as shown in Fig. 1B. The probes were perfused with isotonic phosphate buffer saline (IPBS), pH 7.4, at a flow rate of 2.4 µl/min using a CMA/100 microinjection pump. After probe implantation, the animals were allowed to stabilize for 2 h before the initiation of any study. This duration has been shown to be sufficient for the restoration of intraocular pressure and replenishment of the aqueous humor originally lost during probe implantation (29).

Microdialysis. After 2 h stabilization period, the rabbit eyelids were mechanically retracted with Colibri retractors and the topical well was placed over the eye such that the well was right on top of the cornea. It was fixed to the corneal surface during the infusion period with the help of a surgical adhesive. Care was taken to avoid contact with the entry and exit ports of the aqueous humor microdialysis probe as shown in Fig. 1B. Subsequent to placing the well, the animals were allowed to stabilize for another 45 min. After this time period, 200 µl of IPBS containing [^{14}C] erythromycin (10 µCi/ml, 207 µM) alone and in combination with test compounds was added to the well. The drug solution was allowed to diffuse for a period of 120 min after which it was completely aspirated from the well. The well was subsequently removed and the corneal surface was washed clean with two drops of distilled water. Samples were collected every 20 min throughout the infusion and post-infusion phases over a period of 7 h. At the end of the experiment, euthanasia was performed under deep anesthesia with an intravenous injection of sodium pentobarbital through the marginal ear vein. Radioactivity in the samples was measured by a scintillation counter.

In Vitro Probe Calibration. Microdialysis probe recovery was determined in IPBS containing a known concentration of [^{14}C] erythromycin (0.5 µCi/ml) maintained at 34°C. The concentration of [^{14}C] erythromycin used in the probe recovery experiments was decided based on the assumption that not more than 5% of the topically administered drug reaches the aqueous humor. The probe was continuously perfused at a constant flow rate of 2.4 µl/min and samples were collected every 20 min for a period of 3 h. The ratio between the concentrations of a substance in solution to the outside of the probe is defined as "recovery", usually expressed as a ratio or percentage (30). The recovery of a compound is calculated according to Eq. 2.

$$\text{Recovery} = C_{out}/C_{in} \quad (2)$$

C_{out} represents the concentration in the outflow solution and C_{in} denotes the concentration in the medium. With the help of Eq. 2, the dialysate concentrations were transformed into actual aqueous humor concentrations by Eq. 3.

$$C_{aq} = C_{dl}/\text{Recovery} \quad (3)$$

C_{aq} is the concentration of the analyte in the aqueous humor and C_{dl} represents the concentration of the analyte in dialysate. The recovery of the linear probe was between 10% and 20% for erythromycin which did not alter in the presence of inhibitors. Recovery of the probe was also ascertained after the completion of an animal experiment. The difference in recovery before and after the experiment was not statistically significant (data not shown), suggesting that there was no significant variation in the recovery of the probes over the experimental time period. However, an average of the recovery values determined before and after the experiment was considered for calculating aqueous humor concentrations.

Mathematical Data Treatment

Ocular disposition of a drug administered by topical infusion with the help of a plastic well over the cornea could be represented by a model shown in Fig. 1A. Drug from the reservoir reaches the aqueous humor by passive diffusion through cornea and then reversibly distributes to other

peripheral tissues such as the iris and ciliary bodies and lens. Aqueous outflow is considered as the primary drug elimination pathway from the aqueous humor. Based on this model, the disposition of drug in the aqueous humor can be represented by Eq. 4.

$$\frac{dX_{\mathrm{aq}}}{dt} = k_0 - \sum_{i=1}^{x} k_{aq}X_{aq} + \sum_{i=1}^{y} k_{PT}X_{PT} - k_{10}X_{aq} \qquad (4)$$

A zero order rate constant, k_0 represents the constant infusion rate of the drug to the cornea; k_{aq} and k_{pt} are the apparent first order rate constants for the transfer of drug from aqueous humor to the peripheral tissues and vice versa; X_{aq} and X_{pt} represent the amounts of drug in the aqueous humor and peripheral tissues, respectively; k_{10} denotes apparent first order elimination rate constant of the drug from the aqueous humor through the aqueous outflow pathway; y is the number of peripheral tissues in equilibrium with aqueous humor and x is the number of tissues receiving drug from the aqueous humor that is greater than or equal to y ($x \geq y$). It is also possible that elimination from peripheral tissues may or may not occur. However it will not significantly alter drug disposition in the aqueous humor.

Drug infusion rate constant is related to the drug present in the precorneal reservoir by Eq. 5.

$$k_0 = k_{\mathrm{a}}C_{\mathrm{w}}V_{\mathrm{w}} \qquad (5)$$

where, k_{a} represents the first order rate constant for drug absorption through the cornea; C_{w} and V_{w} are the concentration and volume of the drug in the precorneal reservoir. During the initial infusion period, the concentration of the drug in the precorneal reservoir is much greater than the concentration of the drug in the aqueous humor ($C_{\mathrm{w}} \gg C_{\mathrm{aq}}$). Thus, apart from constant infusion rate term (k_0), which could be substituted by Eq. 5, other terms in Eq. 4 could be neglected. Accounting for this correction during the initial infusion period and making the substitution for k_0, Eq. 6 is obtained.

$$\left(\frac{dC_{\mathrm{aq}}}{dt}\right)_{\mathrm{I}} = \frac{k_{\mathrm{a}}C_{\mathrm{w}}V_{\mathrm{w}}}{V_{\mathrm{aq}}} \qquad (6)$$

V_{aq} denotes the physiological volume of the aqueous humor. Subscript I refers to the initial rate which can be determined from the initial slope of C_{aq} *vs t*. Equation 6 can be rearranged to estimate the first order corneal absorption rate constant, k_{a} according to Eq. 7.

$$k_{\mathrm{a}} = \frac{\left(\frac{dC_{\mathrm{aq}}}{dt}\right)_{\mathrm{I}} * V_{\mathrm{aq}}}{C_{\mathrm{w}}V_{\mathrm{w}}} \qquad (7)$$

Thus, the topical infusion method represented mathematically by Eqs. 4 to 7 permits a rational and reliable determination of ocular pharmacokinetics whereby absorption, distribution and elimination can be estimated without any complex compartmental analysis.

Analytical Procedure

Radioactivity in cellular accumulation, bi-directional transport and *in vivo* anterior segment microdialysis experiments with [^{14}C] Erythromycin was measured by a scintillation counter (Model LS 6500; Beckman Instruments Inc., Fullerton, CA). Samples of steroids from bi-directional transport experiments were assayed with reverse phase-high performance liquid chromatography (RP-HPLC). The system comprised of a Waters 515 HPLC pump, Alcott 795 UV/Vis detector and an Alcott autosampler Model 718 AL HPLC. A C8 Luna column 4.6×250 mm (Phenomenex, Torrance, CA) was employed. The mobile phase was composed of acetonitrile:water:trifluoroacetic acid (70:30:0.1% *v/v*). Flow rate was maintained at 1 ml/min and the detection wavelength was set at 254 nm. Elution times for 6α-methyl prednisolone, prednisolone and prednisone were 5.7, 4.4 and 4.7 min, respectively. The limit of quantification for all three steroids was found to be 15 ng/ml. This method generated rapid and reproducible results.

Data Treatment

Cumulative amounts transported in bi-directional transport experiments across cell monolayers were plotted as a function of time. Linear regression of the amounts transported as a function of time yielded the rate of transport across the cell monolayer (dM/dt). Rate divided by the cross-sectional area available for transport (A) generated steady-state flux as shown in Eq. 8.

$$\mathrm{Flux} = (\mathrm{dM/dt})/\mathrm{A} \qquad (8)$$

Slopes were then obtained from the linear portion of the curve to calculate apparent permeability (P_{app}) through normalization of the steady-state flux to the donor concentration (C_{d}) according to Eq. 9.

$$P_{\mathrm{app}} = \mathrm{Flux}/C_{\mathrm{d}} \qquad (9)$$

Dose-dependent inhibition data were fitted to a relationship described in Eq. 10.

$$Y = \min + \frac{\max - \min}{1 + 10^{(LogIC_{50} - x)\cdot H}} \qquad (10)$$

X denotes the logarithm of the concentration of corticosteroid used, Y is the cellular accumulation of [^{14}C] erythromycin, IC_{50} represents the inhibitor concentration where the efflux of [^{14}C] erythromycin is inhibited by 50% and H is the Hill constant. Y starts at a minimum (min) value (at low inhibitor concentration) and then plateaus at a maximum (max) value (at high inhibitor concentration) resulting in a sigmoidal shape. Data were fitted to Eq. 10 using a transformed nonlinear regression curve analysis program (GraphPad Prism version 4.0, GraphPad Software Inc., San Diego, CA).

All relevant pharmacokinetic parameters were calculated using noncompartmental analyses of the aqueous humor concentration-time profiles of erythromycin with a pharmacokinetic software package, WinNonlin, v5.0 (Pharsight, Mountain View, CA). The data was fitted to a noncompartmental model, with a constant infusion rate. The fit was examined by observing correlation coefficient (r), coefficient of determination (R^2), coefficient of variance (CV) and residual plots. The slopes of the terminal phase of

concentration-time profiles were estimated by log-linear regression and the terminal rate constant (λ_z) was calculated from the slope. The terminal half-lives were calculated from the equation: $t_{1/2}=0.693/\lambda_z$. Absorption rate constant (k_a) was obtained from Eq. 7.

Statistical Analysis

In vivo anterior segment microdialysis and cellular accumulation studies were conducted at least in quadruplicate and bi-directional transport experiments were conducted at least in triplicate. Results from *in vivo* experiments are expressed as mean ± standard error (S.E). All other results are expressed as mean ± standard deviation (S.D). The Student *t* test was applied to determine statistical significance between two groups, with $p<0.05$ being considered to be statistically significant.

RESULTS

Bi-directional Transport of [^{14}C] Erythromycin Across MDCK-MDR1 and MDCK-MRP2 Cells

Apparent permeability (P_{app}) values of [^{14}C] erythromycin in both directions across MDCK cell monolayers over-expressing MDR1 and MRP2 proteins were calculated. In both cell types, the BL-AP permeability of [^{14}C] erythromycin was significantly higher than AP-BL permeability (Table I). Across MDCK-MDR1 cells, the BL-AP and AP-BL permeabilities of [^{14}C] erythromycin were found to be $4.50\pm0.25\times10^{-6}$ and $0.94\pm0.14\times10^{-6}$ cm/s, respectively, yielding an efflux ratio of 4.81. Similarly, BL-AP and AP-BL permeabilities of [^{14}C] erythromycin across MDCK-MRP2 cells were $3.88\pm0.32\times10^{-6}$ and $0.62\pm0.01\times10^{-6}$ cm/s, respectively, yielding an efflux ratio of 6.28. However, in the presence of specific P-gp inhibitor (GF120918, 2 μM) and MRP inhibitor (MK571, 50 μM), the BL-AP and AP-BL permeabilities were almost similar (Table I), resulting in efflux ratio values of 1.31 and 1.05 in MDCK-MDR1 and MDCK-MRP2 cells, respectively.

Cellular Accumulation of [^{14}C] Erythromycin in rPCEC in the Presence of Specific Inhibitors

Cellular accumulation of [^{14}C] erythromycin was significantly elevated in the presence of GF120918 and MK571, relative to control; 170% and 310%, respectively (Fig. 2). A cumulative rise of ~390% in the cellular accumulation of [^{14}C] erythromycin was obtained when the two inhibitors were added together (Fig. 2).

Bi-directional Transport of Corticosteroids Across MDCK-MDR1 and MDCK-MRP2 Cells

MPL, PL and PS were the selected corticosteroids chosen as inhibitors for our study. Bi-directional transport of these steroids was evaluated across MDCK cells overexpressing MDR1 and MRP2 to delineate if they were efficiently transported by P-gp and MRP2. In both the cell lines, the BL-AP permeability of MPL, PL and PS was significantly higher than AP-BL permeability. Bi-directional permeability values of these steroids across both cell lines are listed in Table II. Amongst the steroids studied, MPL exhibited the highest efflux ratio of 3.49 and 3.88 across MDCK-MDR1 and MDCK-MRP2 cells, respectively. PL and PS followed MPL in terms of effective transport by P-gp and MRP2. These results with MDCK-MDR1 cells substantiate the fact that steroids are good substrates for P-gp. Moreover, results with MDCK-MRP2 cells clearly demonstrate the MRP2 mediated efflux of steroids.

Dose-Dependent Inhibition of [^{14}C] Erythromycin Efflux in rPCEC in the Presence of Corticosteroids

To determine the nature and potency as an inhibitor, dose-dependent inhibition of [^{14}C] erythromycin efflux, in the presence of various concentrations of MPL, PL and PS was carried out. As shown in Fig. 3, cellular accumulation of [^{14}C] erythromycin increased in a dose-dependent fashion in the presence of all three steroids. A modified log [dose]—response curve was applied to fit the data in order to obtain

Table I. Permeability Values of [^{14}C] Erythromycin (0.1 μCi/ml) Across MDCK-MDR1 and MDCK-MRP2 Cells

Cell line	Drug ± inhibitor	Permeability ($\times 10^6$) cm/s		Efflux ratio
		AP-BL	BL-AP	
MDCK-MDR1	Ery	0.94±0.14	4.50±0.25	4.81
	Ery + GF120918	2.31±0.08	3.03±0.15	1.31
	Ery + MPL	2.25±0.13	2.51±0.11	1.12
	Ery + PL	1.36±0.03	1.95±0.12	1.43
	Ery + PS	1.26±0.07	2.12±0.22	1.68
MDCK-MRP2	Ery	0.62±0.01	3.88±0.32	6.28
	Ery + MK571	2.43±0.48	2.55±0.30	1.05
	Ery + MPL	1.87±0.22	2.24±0.05	1.20
	Ery + PL	1.58±0.24	2.13±0.13	1.35
	Ery + PS	1.41±0.10	2.27±0.35	1.61

Efflux ratio of a compound is the difference between the BL-AP and AP-BL permeability and which signifies the extent to which the compound is effluxed. Efflux ratios of erythromycin were found to be 4.81 and 6.28 in MDCK-MDR1 and MDCK-MRP2 cells respectively, suggesting, that it is significantly effluxed by P-gp and MRP2, respectively. In the presence of specific inhibitors (GF120918, 2 μM and MK571, 50 μM) and steroids (MPL, PL and PS, each at 500 μM), efflux ratio of erythromycin reduced to ~1, suggesting inhibition of efflux. Data are expressed as mean ± S.D. ($n=3$)

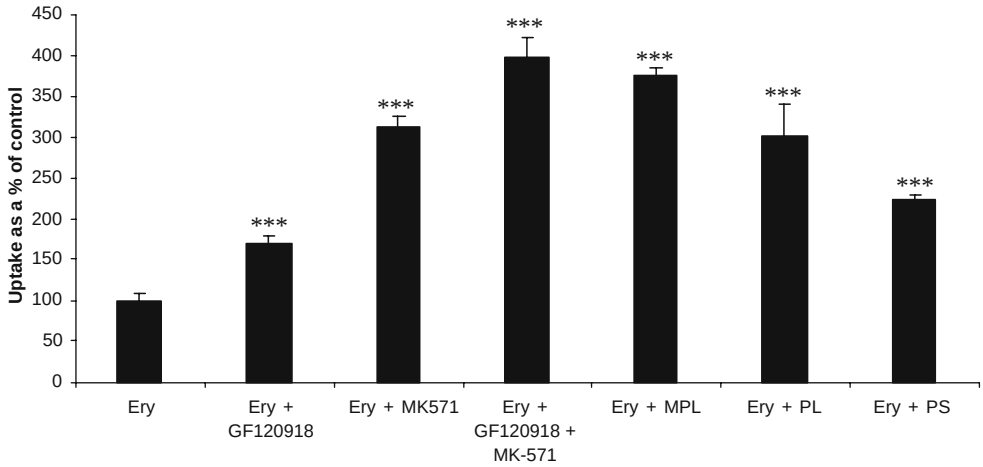


Fig. 2. Cellular accumulation of [^{14}C] erythromycin (0.25 μCi/ml) by rPCEC cells in the presence of a specific P-gp inhibitor (GF120918, 2 μM), MRP inhibitor (MK571, 50 μM), 6α-methyl prednisolone (MPL, 500 μM), prednisolone (PL, 500 μM) and prednisone (PS, 500 μM). Cellular accumulation of [^{14}C] erythromycin in the absence of any inhibitor was considered as control. Both, GF120918 and MK571 caused significant inhibition, suggesting that erythromycin is a good substrate for P-gp and MRP2. Statistically significant increase in the cellular accumulation of [^{14}C] erythromycin was observed in the presence of MPL, PL and PS, suggesting that these compounds can act as inhibitors of P-gp and MRP2. Each data point represents mean ± S.D. (n=4). (***) represents significant difference from control (p<0.005).

IC_{50} values. IC_{50} values for MPL, PL and PS were 99.05 μM, 324.9 and 236.6 μM, respectively. A relatively low IC_{50} value for MPL, implied a stronger affinity towards efflux proteins as compared to PL and PS. A high concentration of inhibitor is usually not recommended and hence we limited the inhibitory concentration of all steroids to 500 μM for further studies. At this concentration, MPL provided complete inhibition where as PL and PS showed ~80% inhibition of erythromycin efflux.

Estimation of Cell Cytotoxicity of Corticosteroids by Cell Proliferation Assay in rPCEC

Cell proliferation assay was carried out to evaluate the cellular toxicity of MPL, PL and PS at a concentration of 500 μM. Percentage of viable cells treated with steroids after 12 h was not statistically different from control (without steroid treatment), suggesting that MPL, PL and PS were not toxic to cells at the concentration used (data not shown).

Cellular Accumulation and Bi-directional Transport of [^{14}C] Erythromycin in the Presence of Corticosteroids Across MDCK-MDR1 and MDCK-MRP2 Cells

To examine the inhibitory potential of these steroids with respect to individual transporters i.e., P-gp and MRP2, cellular accumulation and bi-directional transport of [^{14}C] erythromycin in the presence of MPL, PL and PS were performed. All three steroids inhibited both P-gp and MRP2 mediated efflux of erythromycin, but to different extents. In MDCK-MDR1 cells, MPL, PL and PS demonstrated a 178%, 145% and 151% rise in the cellular accumulation of [^{14}C] erythromycin, relative to control (Fig. 4A). Comparatively, a significantly higher inhibition was observed in MDCK-MRP2 cells in the presence of these steroids. MPL, PL and PS enhanced the cellular accumulation of [^{14}C] erythromycin with an increase greater than 300% relative to control (Fig. 4B). Similar results were obtained with bi-directional transport experiments. In MDCK-MRP2 cells, AP-BL permeability of [^{14}C] erythromycin increased significantly relative to control ($0.62\pm0.01\times10^{-6}$ cm/s), in the presence of MPL, PL and PS, suggesting an inhibition of MRP2 mediated efflux from the apical side. BL-AP permeabilities were correspondingly lower resulting in an efflux ratio of 1.20, 1.35 and 1.61 for [^{14}C] erythromycin in the presence of MPL, PL and PS, respectively (Table I). In MDCK-MDR1 cells, the efflux ratio diminished from 4.28 (control) to 1.12, 1.43 and 1.68 in the presence of MPL, PL and PS, respectively (Table I). However, it is interesting to note that in the presence of PL and PS, the AP-BL permeability did not increase significantly relative to control ($0.94\pm0.14\times10^{-6}$ cm/s).

Cellular Accumulation of [^{14}C] Erythromycin in rPCEC in the Presence of Corticosteroids

Cellular accumulation of [^{14}C] erythromycin in rPCEC significantly elevated in the presence of steroids used at a concentration of 500 μM (Fig. 2). P-gp and MRP2 has been previously reported to be functionally active in rPCEC (9,10). Cellular accumulation of [^{14}C] erythromycin increased by 375%, 300% and 225%, relative to control, in the presence of MPL, PL and PS, respectively. This observation clearly demonstrates the inhibition of both P-gp and MRP2 mediated efflux of erythromycin by steroids.

In Vivo Ocular Absorption of [^{14}C] Erythromycin in the Presence of CsA, MK571 and Corticosteroids

Ocular absorption of [^{14}C] erythromycin was determined in rabbits in the presence of CsA (20 μM), MK571 (50 μM)

Table II. Permeability Values of 6α-Methyl Prednisolone (MPL, 10 μM), Prednisolone (PL, 10 μM) and Prednisone (PS, 10 μM) Across MDCK-MDR1 and MDCK-MRP2 Cells

Cell line	Drug	Permeability ($\times 10^6$) cm/s		Efflux ratio
		AP-BL	BL-AP	
MDCK-MDR1	MPL	6.07±0.48	21.2±0.57	3.49
	PL	6.63±0.07	19.3±0.89	2.92
	PS	6.97±0.21	17.7±0.47	2.54
MDCK-MRP2	MPL	4.99±0.61	19.4±0.33	3.88
	PL	4.95±0.67	15.8±0.36	3.18
	PS	6.04±0.15	17.8±0.22	2.95

Efflux ratio of a compound is the ratio of BL-AP to AP-BL permeability and which signifies the extent to which the compound is effluxed. Efflux ratios of MPL, PL and PS were significantly greater than 1.0 in both MDCK-MDR1 and MDCK-MRP2 cells, suggesting, that these steroids are transported by P-gp and MRP2. Data are expressed as mean ± S.D. (n=3)

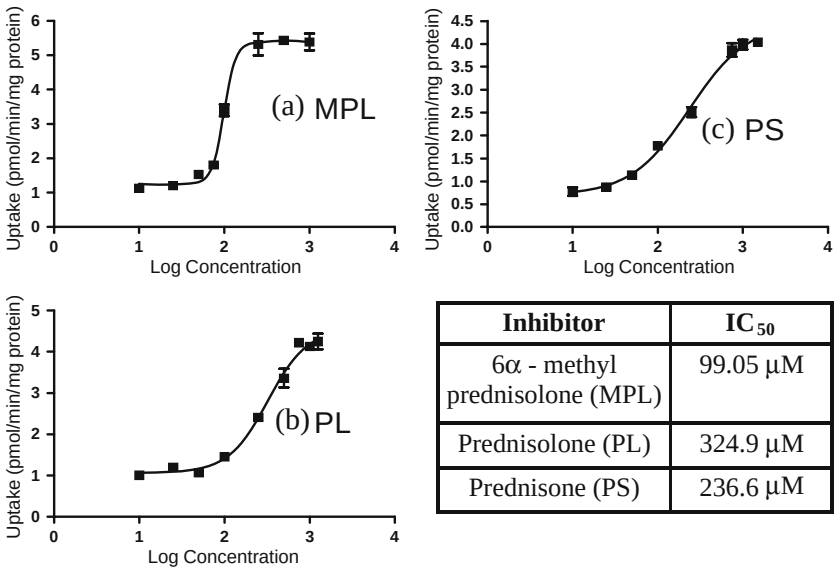
and all three steroids (500 μM). CsA and MK571, model inhibitors of P-gp and MRP, respectively, were used to test the *in vivo* functional activity of the efflux pumps on the rabbit cornea. Moreover, ocular absorption of [^{14}C] erythromycin was also studied in the presence of MPL, PL and PS to investigate if the steroids can significantly elevate the aqueous humor concentration of erythromycin by inhibiting the P-gp and MRP mediated efflux. Aqueous humor concentration-time profile data was significantly higher relative to control, when [^{14}C] erythromycin was topically administered along with CsA, MK571 and steroids (Figs. 5 and 6).

All pharmacokinetic parameters have been listed in Table III. Corneal absorption rate constant (k_a) of erythromycin when administered in the absence of an inhibitor was $4.54\pm0.62\times10^{-5}$ min^{-1}. Absorption rate constant increased by two-fold in the presence of CsA and PL and by three-fold in the presence of MK571, MPL and PS. Such improved absorption resulted in higher C_{max} and $AUC_{0-\infty}$ values of erythromycin in the presence of the inhibitors. Even the C_{last} values of erythromycin observed at 480 min were significantly higher in the presence of all three steroids relative to control (0.11±0.03 μg/ml).

The first order terminal elimination rate constants (λ_z) were calculated for erythromycin when administered in the absence and presence of inhibitors to elucidate whether the inhibitors cause any change in the aqueous elimination pathway. The terminal elimination rate constant values (λ_z) did not alter significantly in presence of inhibitors. The λ_z values of erythromycin ranged from $4.85\pm0.42\times10^{-3}$ to $5.80\pm0.90\times10^{-3}$ min, resulting in terminal elimination half-lives ranging between 120 and 143 min.

DISCUSSION

Low ocular bioavailability of topically administered drugs is primarily due to a sequence of events occurring in the pre-corneal area. Upon instillation, majority of the dose is



Inhibitor	IC_{50}
6α - methyl prednisolone (MPL)	99.05 μM
Prednisolone (PL)	324.9 μM
Prednisone (PS)	236.6 μM

Fig. 3. Dose-dependent inhibition of [^{14}C] erythromycin efflux by **a** 6α-methyl prednisolone (MPL), **b** prednisolone (PL) and **c** prednisone (PS). Cellular accumulation of [^{14}C] erythromycin in the presence of these inhibitors was fitted to a sigmoidal log [dose]–response curve. MPL was comparatively potent as an inhibitor with an IC_{50} of ~100 μM. PL and PS had a higher IC_{50} value suggesting that higher concentrations are required for inhibition. Each data point represents mean ± S.D. (n=4).

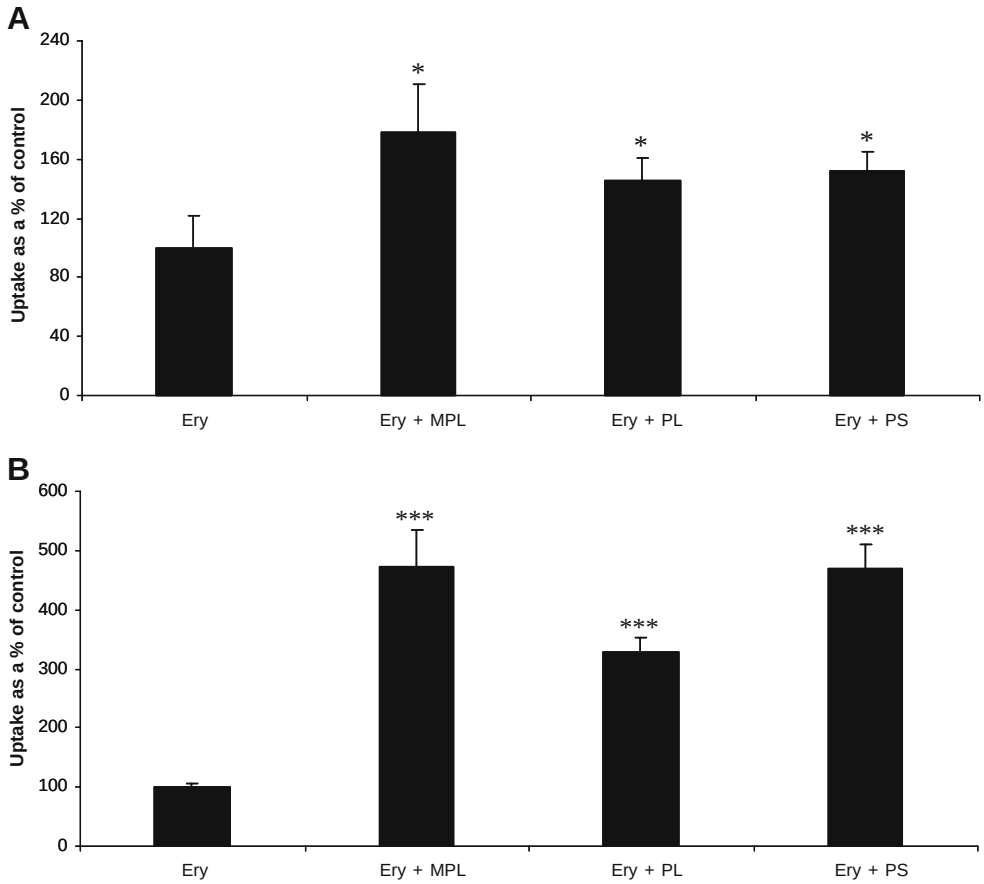


Fig. 4. A, **B** Cellular accumulation of [^{14}C] erythromycin (0.25 μCi/ml) in the presence of 6α-methyl prednisolone (MPL, 500 μM), prednisolone (PL, 500 μM) and prednisone (PS, 500 μM) in **A** MDCK-MDR1 cells and **B** MDCK-MRP2 cells. Cellular accumulation of [^{14}C] erythromycin in the absence of any inhibitor was considered as control. Statistically significant increase in the cellular accumulation of [^{14}C] erythromycin was observed in the presence of MPL, PL and PS in both MDCK-MDR1 and MDCK-MRP2 cells, suggesting that these compounds can act as inhibitors of both efflux proteins. Each data point represents mean ± S.D. (n=4). (*) and (***) represents significant difference from control (p<0.05) and (p<0.005), respectively.

lost due to drug solution drainage, continuous turn over of the tear film, blinking and absorption by adjacent tissues resulting in drug loss to systemic circulation. Under normal physiological conditions, the volume of the precorneal tear film is about 10 μl with an average tear turn over rate of 1.2 μl/min (31). There is instant dilution of the drug by the tear volume upon instillation, with rapid decrease in drug concentration because of continuous turn over of the tear film. Irritation and external stimuli could further accelerate drug loss from the precorneal area. It is reported that an average tear turn over rate of 1.2 μl/min could result in a ten-fold decrease in the concentration of fluorescein within 5–10 min of instillation (31). Consequently, the concentration of the drug in the pre-corneal area decreases significantly which brings the efflux transporters on the corneal epithelium into play. Apart from the pre-corneal factors, low ocular bioavailability so far has been attributed to the inability of molecules to cross the lipoidal membrane until it was recently shown that P-gp in the corneal epithelium can act as a barrier to *in vivo* drug absorption though cornea (20). MRP2 has also been shown to be functionally active in rabbit and human corneal epithelial cells (10,11). The mRNA expression levels of BCRP and other isoforms of MRPs: MRP1 and MRP3 have been reported in the human cornea, though functional activity and localization still remains to be assessed (32). Since, a majority of the topically applied drugs are substrates for one or more of these efflux pumps, it is vital to evaluate their role in drug absorption across cornea.

Our cellular accumulation studies on rPCEC showed a significant accumulation of erythromycin in the presence of specific P-gp and MRP inhibitors than when tested alone, suggesting that rPCEC expresses both P-gp and MRP which are functionally active (Fig. 2). It is also evident that erythromycin acts as a good substrate for both P-gp and MRP. As a result, a cumulative increase in the cellular accumulation of erythromycin was observed when both GF120918 and MK571 were concomitantly administered, suggesting that both P-gp and MRP act synergistically in drug efflux. In the past, MDCK cells transfected with MDR1 and MRP2 proteins have been extensively employed to screen drug candidates for potential efflux (33–36). Bi-directional transport of erythromycin across MDCK-MDR1 and MDCK-MRP2 cells also corroborated well with our cellular accumulation results, with an efflux ratio of 4.81 and 6.28, respectively, in the absence of inhibitors. The efflux ratio is reduced to 1.31 and 1.05 in the presence of GF120918 and MK-571, respectively, suggesting that the efflux

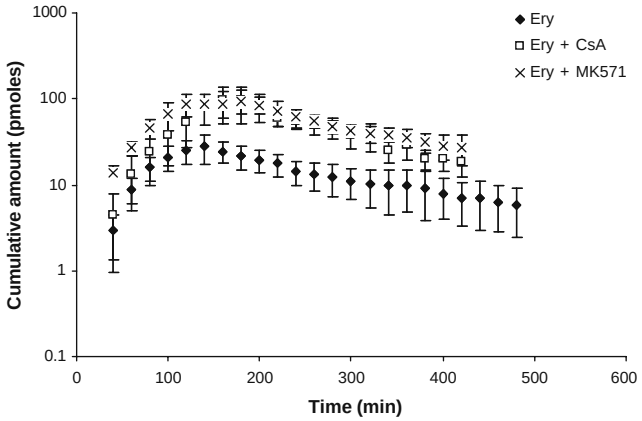


Fig. 5. Aqueous humor concentration-time profile data of [^{14}C] erythromycin (10 μCi/ml) in the absence (*filled diamond*) and presence of cyclosporine A (*unfilled square*, 20 μM) and MK571 (*ex symbol*, 50 μM). Aqueous humor concentration of [^{14}C] erythromycin increased significantly in the presence of P-gp and MRP inhibitors, suggesting that both the efflux pumps are functionally active in effluxing out the substrate. [^{14}C]Erythromycin was given as a topical single-dose infusion in a plastic cylindrical well placed over the cornea. The well was removed at 120 min and samples were collected for a period of 8 h. Each data point represents mean ± S.D. (n=4).

has been effectively shut down by these specific inhibitors (Table I). This further confirms erythromycin as a substrate for both P-gp and MRP2 and justifies the rationale to be used as a model compound to study the effects of cumulative efflux.

Inhibition of efflux pumps by co-administering a drug with a specific inhibitor is one of the viable strategies to improve drug absorption. However, specific inhibitors such as verapamil is toxic in humans at doses effective to cause P-gp modulation (37). Moreover, such compounds appear to alter the pharmacokinetic properties of the co-administered drug by nonspecific interactions thereby requiring dosage adjustments (38). In addition, many modulators lack an inherent pharmacological action to be indicated in the same disease state as the primary drug candidate. Therefore, if the inhibitors should be selected in such a manner so as to have a therapeutic importance in the treatment regimen, then a dual advantage could be realized by inhibition of efflux and elicitation of an additive or synergistic pharmacological action of the inhibitor. Having selected erythromycin, which is used to treat bacterial infections of the eye, it is relevant to evaluate corticosteroids which are potent anti-inflammatory agents, as potential inhibitors for our study. MPL, PL and PS were narrowed down from the list of corticosteroids that are indicated, on the basis of a preliminary competitive inhibition study on rPCEC (data not shown). These steroids significantly increased the cellular accumulation of erythromycin *in vitro*, by inhibiting efflux. These steroids are well known to be efficiently transported by P-gp (39). But their affinity to MRP family has not been yet reported, although structurally similar compounds such as estradiol and sulphated steroids have been reported to be substrates of MRPs (40–43). Bi-directional transport studies across MDCK-MRP2 cells clearly shows significant difference between BL-AP and AP-BL permeabilities with efflux ratios of 3.88, 3.18 and 2.95 for MPL, PL and PS, respectively (Table II). This result confirms the role of MRP2 in the transport of these steroids. Interaction of these steroids with MDR1 protein is once again consistent, with the efflux ratios of 3.49, 2.92 and 2.54 for MPL, PL and PS, respectively (Table II). Moreover, rank order of these steroids in terms of efflux ratio in MDR1 transfected cells, is comparable with a previous report which showed MPL to have a better affinity towards P-gp, possibly due to the presence of a methyl group at the 6α-position (44). Since, these agents exhibited similar order of efflux ratios in MDCK-MRP2 cells, it may be conceived that a similar affinity pattern exists for these compounds towards both the efflux pumps.

Dose dependent inhibition studies on rPCEC evaluated the role of these steroids as potential efflux modulators. As illustrated in Fig. 3, all three steroids inhibited the efflux of erythromycin in a dose dependent manner with MPL being effective at a lower concentration relative to PL and PS. This result once again corroborates the fact that MPL has a stronger affinity to the efflux pumps relative to PL and PS. Also, the inhibitory effects of these steroids were significantly different between MDCK-MDR1 and MDCK-MRP2 cells (Figs. 4A, B). In MDCK-MRP2 cells, the accumulation of erythromycin drastically increased by 450%, 300% and 400% in the presence of MPL, PL and PS, respectively, compared to 160%, 140% and 150% increase in MDCK-MDR1 cells. The possibilities for such different extents of inhibition could be that (a) these steroids are better inhibitors for MRP2 than P-gp mediated efflux of erythromycin (b) erythromycin could be a better substrate for MRP2 than P-gp and (c) expression levels of MRP2 in MDCK-MRP2 cells could be higher than the levels of MDR1 in MDCK-MDR1 cells. In addition, permeability values of steroids in AP-BL direction are lower (though not significant) across MDCK-MRP2 cells compared to MDCK-MDR1 cells (Table II). This result further confirms the analysis that these steroids have a better affinity to MRP2.

Bi-directional transport results are consistent with the cellular accumulation studies suggesting that all three steroids inhibit both P-gp and MRP2, but to different extents. Efflux

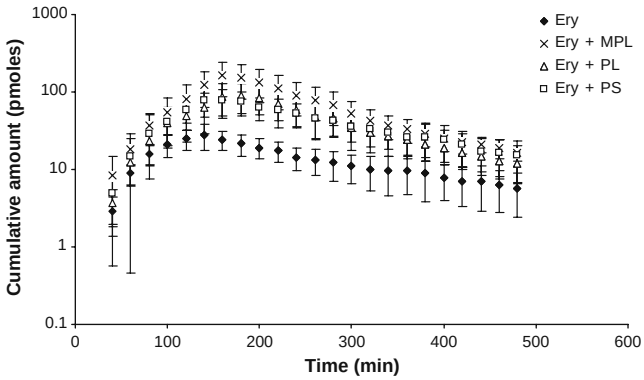


Fig. 6. Aqueous humor concentration-time profile data of [^{14}C] erythromycin (10 μCi/ml) in the absence (*filled diamond*) and presence of 6α-methyl prednisolone (*ex symbol*, MPL 500 μM), prednisolone (*unfilled triangle*, PL 500 M) and prednisone (*unfilled square*, PS 500 μM). Aqueous humor concentration of [^{14}C] erythromycin increased significantly in the presence of MPL, PL and PS, suggesting that these steroids inhibit both P-gp and MRP2 mediated efflux to varying extents. [^{14}C]Erythromycin was given as a topical single-dose infusion in a plastic cylindrical well placed over the cornea. The well was removed at 120 min and samples were collected for a period of 8 h. Each data point represents mean ± S.D. (n=4).

Table III. Pharmacokinetic Parameters after a Single-Dose Infusion of [^{14}C] Erythromycin (10 μCi/ml) in the Absence and Presence of Cyclosporine A (CsA, 20 μM), MK571 (50 μM), 6α-Methyl Prednisolone (MPL, 500 μM), Prednisolone (PL, 500 μM) and Prednisone (500 μM)

Drug ± inhibitor	C_{max} (μg/ml)	$AUC_{0-\infty}$ (μg min/ml)	$C_{last\ (480\ min)}$ (μg/ml)	k_a ($\times 10^5$ min^{-1})	λ_z ($\times 10^3$ min^{-1})
Ery[a]	0.55±0.07	140.5±23.7	0.11±0.03	4.54±0.62	4.85±0.42
Ery + CsA	1.93±0.25*	381.7±46.6**	ND	10.87±2.07*	5.63±0.43
Ery + MK571	2.10±0.23*	433.8±35.9**	ND	14.11±1.18*	4.96±0.51
Ery + MPL	2.99±0.64*	567.3±96.3**	0.29±0.03**	15.90±3.92*	5.28±0.36
Ery + PL	1.66±0.34*	355.6±68.5*	0.22±0.05	10.33±2.73	5.57±0.17
Ery + PS	1.57±0.21*	358.1±53.8*	0.26±0.05*	12.49±2.30*	5.80±0.90

As evident from the data, C_{max} and $AUC_{0-\infty}$ values of erythromycin were significantly increased in the presence of CsA and MK571 relative to control; suggesting inhibition of P-gp and MRP2 mediated efflux. MPL, PL and PS also inhibited the efflux of erythromycin to different extents. Corneal absorption rate constant (k_a) significantly increased in the presence of MPL, PL and PS leading to higher C_{max} and $AUC_{0-\infty}$ values relative to control. Data are expressed as mean ± S.E. (n=4)

C_{max} maximum concentration, $AUC_{0-\infty}$ area under the curve, $C_{last\ (480\ min)}$ concentration at the last time point i.e., 480 min, k_a corneal absorption rate constant, λ_z terminal elimination rate constant

*$p<0.05$; **$p<0.01$ (significant difference from control)

[a] Control

ratio of erythromycin across MDCK-MDR1 cells drastically reduced from 4.81 (control) to 1.12, 1.43 and 1.68 in the presence of MPL, PL and PS, respectively (Table I). But, AP-BL permeability of erythromycin in the presence of steroids, especially PL and PS, did not increase significantly compared to control. However, a reduction in efflux ratios of erythromycin is due to significant decrease in BL-AP permeability. Thus, from the cellular accumulation and the bi-directional transport studies, it is reasonable to conclude that PL and PS are weak modulators of P-gp. In contrast, a significant increase in the AP-BL permeability of erythromycin with substantial reduction of efflux ratio across MDCK-MRP2 cells from 6.28 (control) to 1.20, 1.35 and 1.61 in the presence of MPL, PL and PS, respectively, clearly indicate that these steroids are good inhibitors of MRP2.

Pharmacokinetics of erythromycin in the presence of these steroids was further evaluated in rabbits with a topical well infusion model coupled with anterior segment microdialysis. The principal advantage with the current 'topical single-dose infusion' model is that it eliminates the effect of tear dynamics and other precorneal losses thus obviating the need for compartmental modeling. Eliminating precorneal losses helps to predict corneal absorption rate constant precisely without complications from noncorneal absorptive processes such as conjunctival and nasolacrimal duct drainage. The current model has been previously established for the corneal absorption and ocular pharmacokinetics of topically applied drugs (20,45–47).

A significant elevation of $AUC_{0-\infty}$, C_{max} and three-fold difference in corneal absorption rate constant (k_a) of erythromycin with MK571 (50 μM), a specific MRP inhibitor, clearly confirms the functional activity of MRP2 (Table III). Since, MK-571 is not specific for MRP2, it could be possible that the increase in aqueous humor concentration of erythromycin might be due to a collective inhibition of other isoforms of MRPs as well. But at this point, we do not have any information on the functional activity of other isoforms of MRPs in rabbit cornea, though mRNA expression levels of MRP1 and MRP3 are reported in the human cornea (32). Similarly, higher aqueous humor concentration of erythromycin in the presence of cyclosporine A (CsA, 20 μM), a P-gp inhibitor, resulting in significant increase of k_a, $AUC_{0-\infty}$ and C_{max} suggest the role of P-gp in modulating drug absorption across cornea (Table III). Thus, both P-gp and MRP2 present on the rabbit corneal epithelium appear to be functionally active and can pose a barrier to drug absorption.

Potential inhibitory effects of steroids were then evaluated *in vivo*. Pharmacokinetic parameters of erythromycin such as k_a, $AUC_{0-\infty}$, C_{max} and C_{last} significantly increased in the presence of steroids suggesting that these compounds are effective in inhibiting both the P-gp and MRP2 mediated efflux across rabbit cornea. Once again, such increase in pharmacokinetic parameters in the presence of steroids may be due to a collective inhibition of other isoforms of MRPs as well. Better affinity of MPL towards both P-gp and MRP2 as witnessed in our *in vitro* experiments is again observed *in vivo* with maximum inhibition of erythromycin efflux obtained, when co-administered with MPL. Moreover, k_a, $AUC_{0-\infty}$, C_{max} and C_{last} values of erythromycin were significantly higher in the presence of MPL compared to a single specific efflux inhibitor which clearly suggests that MPL inhibits both P-gp and MRP2.

There was no significant difference in the elimination rate constant of erythromycin when administered alone and in the presence of specific inhibitors and steroids. Aqueous humor is presumed to be the primary route of elimination. In the rabbit eye, the turnover rate of aqueous humor equals a bulk flow of ~1.5% (4.2 μl/min) of the volume of the anterior chamber per minute (48). Hence the half life a drug, whose elimination is exclusively by the aqueous humor elimination pathway, would be near about 30 min. Terminal half lives of erythromycin ranged from 120 to 143 min, which is longer than the aqueous humor turnover rate, suggesting that tissue binding may have an influence on the elimination of erythromycin.

Thus, our studies clearly indicate that both P-gp and MRP2 are functionally active *in vivo* and steroids can modulate the efflux mediated by both these pumps, resulting in higher aqueous humor concentration of erythromycin. Out of the three corticosteroids studied, MPL exhibited most promising results in terms of efflux inhibition, both *in vitro* and *in vivo*. Concomitant administration of corticosteroids, if

relevant in a treatment regimen, might provide a viable strategy where the primary drug of choice suffers potential efflux by P-gp and/or MRP. A significant increase in pharmacokinetic parameters such as C_{max} and $AUC_{0-\infty}$ in the aqueous humor during concurrent administration of an inhibitor could be seen as an opportunity to reduce the frequency of dosing or to lower the effective dose resulting in better patient compliance. Depending on the disease condition that is being treated, other potential therapeutic inhibitors could be used adopting a similar strategy modulating efflux.

ACKNOWLEDGEMENTS

This work was supported by NIH grants R01EY09171-14 and R01EY10659-12

REFERENCES

1. A. H. Schinkel, and J. W. Jonker. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv. Drug Deliv. Rev.* **55**:3–29 (2003).
2. J. V. Aukunuru, G. Sunkara, N. Bandi, W. B. Thoreson, and U. B. Kompella. Expression of multidrug resistance-associated protein (MRP) in human retinal pigment epithelial cells and its interaction with BAPSG, a novel aldose reductase inhibitor. *Pharm. Res.* **18**:565–572 (2001).
3. P. A. Constable, J. G. Lawrenson, D. E. Dolman, G. B. Arden, and N. J. Abbott. P-Glycoprotein expression in human retinal pigment epithelium cell lines. *Exp. Eye Res.* **83**:24–30 (2006).
4. B. G. Kennedy, and N. J. Mangini. P-glycoprotein expression in human retinal pigment epithelium. *Mol. Vis.* **8**:422–430 (2002).
5. J. A. Holash, and P. A. Stewart. The relationship of astrocyte-like cells to the vessels that contribute to the blood-ocular barriers. *Brain Res.* **629**:218–224 (1993).
6. J. Shen, S. T. Cross, D. D. Tang-Liu, and D. F. Welty. Evaluation of an immortalized retinal endothelial cell line as an *in vitro* model for drug transport studies across the blood-retinal barrier. *Pharm. Res.* **20**:1357–1363 (2003).
7. J. Wu, J. J. Zhang, H. Koppel, and T. J. Jacob. P-glycoprotein regulates a volume-activated chloride current in bovine non-pigmented ciliary epithelial cells. *J. Physiol.* **491**(Pt 3):743–755 (1996).
8. P. Saha, J. J. Yang, and V. H. Lee. Existence of a p-glycoprotein drug efflux pump in cultured rabbit conjunctival epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **39**:1221–1226 (1998).
9. S. Dey, J. Patel, B. S. Anand, B. Jain-Vakkalagadda, P. Kaliki, D. Pal, V. Ganapathy, and A. K. Mitra. Molecular evidence and functional expression of P-glycoprotein (MDR1) in human and rabbit cornea and corneal epithelial cell lines. *Invest. Ophthalmol. Vis. Sci.* **44**:2909–2918 (2003).
10. P. K. Karla, D. Pal, and A. K. Mitra. Molecular evidence and functional expression of multidrug resistance associated protein (MRP) in rabbit corneal epithelial cells. *Exp. Eye Res.* **84**:53–60 (2007).
11. P. K. Karla, D. Pal, T. Quinn, and A. K. Mitra. Molecular evidence and functional expression of a novel drug efflux pump (ABCC2) in human corneal epithelium and rabbit cornea and its role in ocular drug efflux. *Int. J. Pharm.* **336**:12–21 (2007).
12. C. Seral, S. Carryn, P. M. Tulkens, and F. Van Bambeke. Influence of P-glycoprotein and MRP efflux pump inhibitors on the intracellular activity of azithromycin and ciprofloxacin in macrophages infected by Listeria monocytogenes or Staphylococcus aureus. *J. Antimicrob. Chemother.* **51**:1167–1173 (2003).
13. J. M. Michot, C. Seral, F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. *Antimicrob. Agents Chemother.* **49**:2429–2437 (2005).
14. K. Naruhashi, I. Tamai, N. Inoue, H. Muraoka, Y. Sai, N. Suzuki, and A. Tsuji. Involvement of multidrug resistance-associated protein 2 in intestinal secretion of grepafloxacin in rats. *Antimicrob. Agents Chemother.* **46**:344–349 (2002).
15. I. Tamai, J. Yamashita, Y. Kido, A. Ohnari, Y. Sai, Y. Shima, K. Naruhashi, S. Koizumi, and A. Tsuji. Limited distribution of new quinolone antibacterial agents into brain caused by multiple efflux transporters at the blood-brain barrier. *J. Pharmacol. Exp. Ther.* **295**:146–152 (2000).
16. D. A. Hesselink, R. M. van Hest, R. A. Mathot, F. Bonthuis, W. Weimar, R. W. de Bruin, and T. van Gelder. Cyclosporine interacts with mycophenolic acid by inhibiting the multidrug resistance-associated protein 2. *Am. J. Transplant.* **5**:987–994 (2005).
17. A. Sakata, I. Tamai, K. Kawazu, Y. Deguchi, T. Ohnishi, A. Saheki, and A. Tsuji. *In vivo* evidence for ATP-dependent and P-glycoprotein-mediated transport of cyclosporin A at the blood-brain barrier. *Biochem. Pharmacol.* **48**:1989–1992 (1994).
18. K. Ueda, N. Okamura, M. Hirai, Y. Tanigawara, T. Saeki, N. Kioka, T. Komano, and R. Hori. Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *J. Biol. Chem.* **267**:24248–24252 (1992).
19. E. Mannermaa, K. S. Vellonen, and A. Urtti. Drug transport in corneal epithelium and blood-retina barrier: emerging role of transporters in ocular pharmacokinetics. *Adv. Drug Deliv. Rev.* **58**:1136–1163 (2006).
20. S. Dey, S. Gunda, and A. K. Mitra. Pharmacokinetics of erythromycin in rabbit corneas after single-dose infusion: role of P-glycoprotein as a barrier to *in vivo* ocular drug absorption. *J. Pharmacol. Exp. Ther.* **311**:246–255 (2004).
21. S. Katragadda, B. Budda, B. S. Anand, and A. K. Mitra. Role of efflux pumps and metabolising enzymes in drug delivery. *Expert. Opin. Drug Deliv.* **2**:683–705 (2005).
22. R. P. Kowalski, L. M. Karenchak, and E. G. Romanowski. Infectious disease: changing antibiotic susceptibility. *Ophthalmol. Clin. North Am.* **16**:1–9 (2003).
23. C. Queille-Roussel, M. Poncet, S. Mesaros, A. Clucas, M. Baker, and A. M. Soloff. Comparison of the cumulative irritation potential of adapalene gel and cream with that of erythromycin/tretinoin solution and gel and erythromycin/isotretinoin gel. *Clin. Ther.* **23**:205–212 (2001).
24. V. H. Lee, and J. R. Robinson. Mechanistic and quantitative evaluation of precorneal pilocarpine disposition in albino rabbits. *J. Pharm. Sci.* **68**:673–684 (1979).
25. M. C. Makoid, and J. R. Robinson. Pharmacokinetics of topically applied pilocarpine in the albino rabbit eye. *J. Pharm. Sci.* **68**:435–443 (1979).
26. S. C. Miller, K. J. Himmelstein, and T. F. Patton. A physiologically based pharmacokinetic model for the intraocular distribution of pilocarpine in rabbits. *J. Pharmacokinet. Biopharm.* **9**:653–677 (1981).
27. M. G. Eller, R. D. Schoenwald, J. A. Dixson, T. Segarra, and C. F. Barfknecht. Topical carbonic anhydrase inhibitors IV: Relationship between excised corneal permeability and pharmacokinetic factors. *J. Pharm. Sci.* **74**:525–529 (1985).
28. K. D. Rittenhouse, and G. M. Pollack. Microdialysis and drug delivery to the eye. *Adv. Drug Deliv. Rev.* **45**:229–241 (2000).
29. S. Macha, and A. K. Mitra. Ocular pharmacokinetics in rabbits using a novel dual probe microdialysis technique. *Exp. Eye Res.* **72**:289–299 (2001).
30. T. Zetterstrom, L. Vernet, U. Ungerstedt, U. Tossman, B. Jonzon, and B. B. Fredholm. Purine levels in the intact rat brain. Studies with an implanted perfused hollow fibre. *Neurosci. Lett.* **29**:111–115 (1982).
31. S. Mishima, A. Gasset, S. D. Klyce Jr., and J. L. Baum. Determination of tear volume and tear flow. *Invest. Ophthalmol.* **5**:264–276 (1966).
32. T. Zhang, C. D. Xiang, D. Gale, S. Carreiro, E. Y. Wu, and E. Y. Zhang. Drug transporter and cytochrome P450 mRNA expression in human ocular barriers: implications for ocular drug disposition. *Drug Metab. Dispos.* **36**:1300–1307 (2008).
33. S. Agarwal, D. Pal, and A. K. Mitra. Both P-gp and MRP2 mediate transport of Lopinavir, a protease inhibitor. *Int. J. Pharm.* **339**:139–147 (2007).

34. D. J. Brayden, and J. Griffin. Avermectin transepithelial transport in MDR1- and MRP-transfected canine kidney monolayers. *Vet. Res. Commun.* **32**:93–106 (2008).
35. S. D. Flanagan, C. L. Cummins, M. Susanto, X. Liu, L. H. Takahashi, and L. Z. Benet. Comparison of furosemide and vinblastine secretion from cell lines overexpressing multidrug resistance protein (P-glycoprotein) and multidrug resistance-associated proteins (MRP1 and MRP2). *Pharmacology.* **64**:126–134 (2002).
36. M. Horio, I. Pastan, M. M. Gottesman, and J. S. Handler. Transepithelial transport of vinblastine by kidney-derived cell lines. Application of a new kinetic model to estimate *in situ* Km of the pump. *Biochim. Biophys. Acta.* **1027**:116–122 (1990).
37. R. Krishna, and L. D. Mayer. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *Eur. J. Pharm. Sci.* **11**:265–283 (2000).
38. R. Krishna, and L. D. Mayer. Liposomal doxorubicin circumvents PSC 833-free drug interactions, resulting in effective therapy of multidrug-resistant solid tumors. *Cancer Res.* **57**: 5246–5253 (1997).
39. T. Mizutani, M. Masuda, E. Nakai, K. Furumiya, H. Togawa, Y. Nakamura, Y. Kawai, K. Nakahira, S. Shinkai, and K. Takahashi. Genuine functions of P-glycoprotein (ABCB1). *Curr. Drug Metab.* **9**:167–174 (2008).
40. Z. S. Chen, Y. Guo, M. G. Belinsky, E. Kotova, and G. D. Kruh. Transport of bile acids, sulfated steroids, estradiol 17-beta-D-glucuronide, and leukotriene C4 by human multidrug resistance protein 8 (ABCC11). *Mol. Pharmacol.* **67**:545–557 (2005).
41. X. Y. Chu, S. E. Huskey, M. P. Braun, B. Sarkadi, D. C. Evans, and R. Evers. Transport of ethinylestradiol glucuronide and ethinylestradiol sulfate by the multidrug resistance proteins MRP1, MRP2, and MRP3. *J. Pharmacol. Exp. Ther.* **309**:156–164 (2004).
42. D. W. Loe, K. C. Almquist, S. P. Cole, and R. G. Deeley. ATP-dependent 17 beta-estradiol 17-(beta-D-glucuronide) transport by multidrug resistance protein (MRP). Inhibition by cholestatic steroids. *J. Biol. Chem.* **271**:9683–9689 (1996).
43. N. Zelcer, G. Reid, P. Wielinga, A. Kuil, I. van der Heijden, J. D. Schuetz, and P. Borst. Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4). *Biochem. J.* **371**:361–367 (2003).
44. C. R. Yates, C. Chang, J. D. Kearbey, K. Yasuda, E. G. Schuetz, D. D. Miller, J. T. Dalton, and P. W. Swaan. Structural determinants of P-glycoprotein-mediated transport of glucocorticoids. *Pharm. Res.* **20**:1794–1803 (2003).
45. B. S. Anand, S. Katragadda, S. Gunda, and A. K. Mitra. *In vivo* ocular pharmacokinetics of acyclovir dipeptide ester prodrugs by microdialysis in rabbits. *Mol. Pharm.* **3**:431–440 (2006).
46. S. Gunda, S. Hariharan, and A. K. Mitra. Corneal absorption and anterior chamber pharmacokinetics of dipeptide monoester prodrugs of ganciclovir (GCV): *in vivo* comparative evaluation of these prodrugs with Val-GCV and GCV in rabbits. *J. Ocul. Pharmacol Ther.* **22**:465–476 (2006).
47. S. Katragadda, S. Gunda, S. Hariharan, and A. K. Mitra. Ocular pharmacokinetics of acyclovir amino acid ester prodrugs in the anterior chamber: Evaluation of their utility in treating ocular HSV infections. *Int. J. Pharm.* **359**:15–24 (2008).
48. P. R. McMaster, and F. J. Macri. The rate of aqueous humor formation in buphthalmic rabbit eyes. *Invest. Ophthalmol.* **6**:84–87 (1967).