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Pharmacokinetics of Erythromycin in Rabbit Corneas Following Single-Dose Infusion. Role of P-Glycoprotein as a Barrier to *in vivo* Ocular Drug Absorption

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Running Title: Functional P-glycoprotein Expression in Rabbit Cornea

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Abbreviation: MDCK, Madin-Darby Canine Kidney; P-gp, P-glycoprotein; DPBS, Dulbecco's phosphate- buffered saline; RT-PCR, Real Time-Polymerase Chain Reaction; AUC, Areas Under the Curve; CsA, Cyclosporine A.

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ABSTRACT

Efflux pump like P-glycoprotein (P-gp) is known to be a major barrier to drug delivery. Functional P-glycoprotein has been recently identified in cornea and corneal cell lines. Thus it is probable that P-glycoprotein may restrict *in vivo* ocular drug absorption resulting in low ocular bioavailability. Experiments were designed using New Zealand Albino (NZW) rabbits to assess inhibitors of P-gp efflux to increase drug absorption. Anesthetized rabbits were given constant topical infusions of [^{14}C]-erythromycin in the presence and absence of inhibitors. Testosterone, verapamil, quinidine and cyclosporine A (CsA) were selected as P-gp inhibitors. Transport experiments were conducted in MDCK-MDR1 cells. Erythromycin exhibited significant efflux out of MDCK-MDR1 cells suggesting that erythromycin is a good substrate for P-gp. Ocular pharmacokinetic studies were conducted using a topical single-dose infusion method. Maximum inhibition of P-gp mediated efflux was observed with 500 μM testosterone. $AUC_{0-\infty}$ of erythromycin with 500 μM testosterone was almost 4 times higher than $AUC_{0-\infty}$ without any inhibitor. Rate of elimination (k_{10}) for erythromycin and those with inhibitors was found to be similar (141 ± 23 min) suggesting that elimination pathways were not altered. All the inhibitors were found to be non-toxic. Verapamil also inhibited the efflux pump with moderate change in $AUC_{0-\infty}$ and C_{max} compared to control. Thus, P-gp is found to be active *in vivo* and it restricts topical erythromycin absorption across the cornea which can be inhibited by known P-gp inhibitors. Therefore ocular bioavailability of P-gp substrates can be significantly enhanced by proper selection of P-gp inhibitors.

INTRODUCTION

Multidrug resistance to absorption of drugs is mainly due to three proteins, P-glycoprotein (P-gp), lung resistance related protein (LRP) and multidrug resistance associated protein-1 (MRP1) (Cole et al., 1992; Scheper et al., 1993). A variety of structurally and pharmacologically unrelated drugs and compounds are substrates for P-gp. These include anticancer drugs like doxorubicin and daunorubicin (Shtil et al., 2000), steroids like hydrocortisone and dexamethasone (Ueda et al., 1992), HIV protease inhibitors, such as ritonavir (Perloff et al., 2001) and saquinavir (Huisman et al., 2003) and cardiac drugs like digoxin and quinidine (Duvvuri et al., 2003).

Such efflux pumps have been identified in the eye. In the eye, P-gp is expressed in the retinal capillary endothelial cells (Holash and Stewart, 1993), retinal pigmented epithelial cells (Schlingemann et al., 1998), ciliary non-pigmented epithelium (Wu et al., 1996), conjunctival epithelial cells (Saha et al., 1998), and iris and ciliary muscle cells (Holash and Stewart, 1993). More recently, P-gp has been identified in the human and rabbit cornea for the first time in our laboratory (Dey et al., 2003). Functional expression of P-gp has been demonstrated by studying Rhodamine-123 efflux in primary rabbit corneal cultures and cell lines.

Topical administration is the preferred mode to treat diseases that affect the anterior chamber of the eye. A majority of topically applied drugs including steroids (Kupferman and Leibowitz, 1974), β-blockers (Schoenwald and Huang, 1983), antibiotics (Barza et al., 1983) and non-steroidal anti-inflammatory agents (Agata et al., 1984) enter the eye through the cornea. Unfortunately, the disposition of drugs administered in this manner is not well understood, although it is generally agreed that bioavailability of

topically applied drugs is extremely limited (<5%). Low ocular bioavailability so far has been attributed primarily to the inability of drug molecules to cross the lipoidal membrane and not attributed to any efflux proteins that might efflux molecules out of corneal epithelium into the precorneal fluid.

Although functional P-gp in cornea and corneal cell lines has been discovered, it has not yet been established that this efflux pump can function *in vivo* to modulate drug concentration and activity in the eye. Several pharmacokinetic models of varying complexity have been proposed to predict absorption and disposition of drugs applied topically to the eye (Lee and Robinson, 1979; Makoid and Robinson, 1979; Miller et al., 1981). A classical pharmacokinetic approach predicts aqueous humor levels reasonably well; however transcorneal absorption rate constant is very difficult to determine due to the complexity of the model. Pharmacokinetics of topically applied pilocarpine in the albino rabbit eye has been described using a four-compartment classical model represented by a four exponential equation yielding eight equation parameters (Makoid and Robinson, 1979). Although the experimental data fit the equation quite well, the calculated parameters could not be assigned to absorption, distribution or elimination very accurately. Another pharmacokinetic model has been applied to pilocarpine pharmacokinetics that utilizes a physiological based model (Lee and Robinson, 1979; Miller et al., 1981). However both modeling approaches are complex with regard to numerical analyses.

In order to simplify the approach and correctly estimate ocular absorption and absorption rate constant, a “topical infusion” model has been described (Eller et al., 1985). In this model, a constant concentration of the drug is maintained on the cornea so

that the effect of tear dynamics is minimized and simpler equations can be applied independent of modeling. During constant input, absorption, distribution, and elimination can be determined independent of the number of the peripheral compartments that are operative. Constant concentration was maintained through the use of a plastic cylindrical well containing the drug solution.

We conceptualized the use of a combination of the topical well infusion model and aqueous humor microdialysis sampling for precise prediction of ocular absorption. This method will also reduce inter-individual variability and minimize the number of animals required. In this report, we have discussed the use of this novel technique to determine the ocular pharmacokinetics of erythromycin (a P-gp substrate). Molecular identification and functional characterization of P-gp in human and rabbit cornea has already been reported from our laboratory (Dey et al., 2003). This study discusses the functional nature of P-gp in an *in vivo* rabbit model and delineates the role of P-gp in lowering ocular bioavailability of topically applied drugs. In addition, a partial protein sequence has been reported for the first time which would help in further cloning and characterization of rabbit P-gp.

MATERIALS AND METHODS

MATERIALS

Cyclosporin A (CsA), quinidine, verapamil and testosterone were purchased from Sigma Chemical Company (St. Louis, MO). [^{14}C]-Mannitol (specific activity: 55 mCi/mmol) and [^{3}H]-Diazepam (specific activity: specific activity: 85 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{14}C]-Erythromycin (specific activity: 48.8 mCi/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA). [^{14}C]-Diazepam (specific activity: 56 mCi/mmol) and [^{3}H]-Mannitol (specific activity: 20 Ci/mmol) were purchased from Amersham (Piscataway, NJ) and ICN Biomedicals Inc. (Irvine, CA) respectively. CsA (2 mM) was first dissolved in ethanol (Fisher Scientific, Fair Lawn, NJ), and then aliquots were diluted in DPBS to a final concentration of either 10 or 20 μM for inhibition studies. Testosterone (10 mM) was dissolved in ethanol, and aliquots were diluted with DPBS (as described above) to the desired concentration.

Madin-Darby canine kidney (MDCK) cells transfected with the human *mdr1* gene (MDCK-MDR1) was a gift from The Netherlands Cancer Institute (Professor Piet Borst, Amsterdam, The Netherlands). The growth medium, Minimum Essential Medium (MEM) (for rabbit corneal epithelial cells) and Dulbecco's modified Eagle's medium (DMEM) (for MDCK-MDR1 cells) were obtained from Life Technologies (Grand Island, NY). Penicillin, streptomycin, sodium bicarbonate, lactalbumin, amphotericin B, polymyxin B sulfate, non-essential amino acids and HEPES were purchased from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum (FBS) was procured from JRH Biosciences (Lenexa, KS). The buffer used in transport studies was Dulbecco's

phosphate buffered saline (DPBS), containing 129 mM sodium chloride, 2.5 mM potassium chloride, 7.4 mM disodium hydrogen phosphate, 1.3 mM potassium dihydrogen phosphate, 1 mM calcium chloride, 0.7 mM magnesium sulfate, and 5.3 mM glucose at pH 7.4. Culture flasks (75 cm^2 growth area), polyester Transwells® (pore size 0.4 μm, diameter 6.5 mm), 12 well culture plates (1.1 cm^2 growth area) and polyester membranes (pore size 0.4 μm, diameter 1cm) were obtained from Costar (Bedford, MA). p-GEM-T-Easy vector and EcoRI were obtained from Promega (Madison, WI, USA)

Linear probes (MD-2000, 0.32 X 10 mm, polyacronitrile membrane, 0.22 mm tubing) for aqueous humor sampling were obtained from BioAnalytical Systems (West Lafayette, IN). A microinjection pump (CMA/100), for pumping isotonic buffer was purchased from CMA Microdialysis (Acton, MA). Ketamine hydrochloride was obtained from Fort Dodge Animal Health. Rompun® (Xylazine) was obtained from Bayer Animal Health. Nembutal sodium solution was procured from Abbott Laboratories (Abbott Park, IL). Topical wells were custom made by Hansen Ophthalmic Development Corporation (Iowa City, IA) according to specific instructions. The dimensions of the plastic well are depicted in Fig. 1A.

Cell Culture

MDCK-MDR1 cells were maintained at 37º C in a humidified 5% CO_2, 95% air atmosphere. Cells were seeded at 50,000 cells/cm^2 on polyester Transwell® filter inserts (12 well). Experiments were conducted on these cells between 6-8 days of culture. Expression of P-gp efflux pump in these transfected cells was examined with Western Blotting and the cells were split twice a week (subculture ratio 1:5). Since the test

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compound (erythromycin) was [^{14}C]-labeled, it was possible to analyze the transport of the test compound and monitor the membrane integrity simultaneously using [^{3}H]-mannitol and [^{3}H]-diazepam.

Primary Culture of Rabbit Corneal Epithelial Cells (rPCEC)

Primary culture of rabbit corneal epithelial cells was performed according to our previous report (Dey et al., 2003). Corneas from New Zealand White (NZW) rabbits were excised, washed thoroughly with DPBS, blotted dry and transferred to sterile culture dishes containing 0.5 mL trypsin (0.25%) or 1.2 U/mL protease (Dispase II, Roche Molecular Biochemical). Corneas were placed upside down (with the concave surface touching the protease solution), and incubated at 37º C for 30 minutes. The epithelial cells were stripped off with gentle scraping from peripheral areas (1-1.5 mm from the limbus) to the center. Care was taken to peel only the epithelial layer and not the underlying stromal layer as that would contaminate the epithelial cells with keratinocytes. Cells were washed with MEM and placed in culture dishes. After 12 hours (when most of the cells have attached to the bottom), MEM medium was removed and fresh MEM medium supplemented with insulin (5 μg/mL), transferrin (5 μg/mL), sodium selenite (5 ng/mL), amphotericin B (0.25 μg/mL), polymyxin B sulfate (0.5 μg/mL), penicillin (100U/ml), streptomycin (100 μg/mL), human recombinant epidermal growth factor (10 ng/mL) and bovine pituitary extract (50 μg/mL) were added. The media was changed twice a week and the cells were subcultured every 7-10 days (subculture ratio 1:5). Cells reached senescence by passages 7-10, so passages 2-6 were used for all further experiments.

Transport Experiments

Bidirectional transport of [^{14}C]-erythromycin across MDCK-MDR1 was carried out as described previously (Gao et al., 2000) with some modifications. The purpose of the transport experiment was to determine that erythromycin was a good substrate for P-gp with a good efflux potential which could be employed to test the functionality of P-gp in rabbit corneas. Transepithelial electrical resistance (TEER) of rPCEC cells was measured to elucidate tight junction properties. TEER was measured with EVOM™ Chopstick Electrodes (World Precision Instruments, Sarasota, FL). Cell layers had TEER values ranging from 150-250 Ω.cm^2. MDCK-MDR1 cells with TEER ≥ 200 Ω.cm^2 were used for transport studies. Both the apical (AP) and the basolateral (BL) chambers of each insert were washed twice with 37º C –DPBS for 15 min. [^{14}C]-erythromycin (with either [^{3}H]-mannitol or [^{3}H]-diazepam) at a concentration of 0.05 µCi/ml was added to the donor side (0.5 ml for the AP chamber and 1.5 ml for the BL chamber) and fresh DPBS was placed in the receiver side. To inhibit the efflux activity of P-gp, cell monolayers were incubated with DPBS containing CsA (10µM) for an additional 20 min. The test compound was added along with CsA (10µM) and fresh DPBS containing the inhibitor was placed in the receiver side. Sink conditions were maintained throughout the experiment. At specified time points, 100 µl aliquots were removed from the receiver chamber and replaced with an equal volume of DPBS. Samples were transferred to scintillation vials containing 5 ml scintillation cocktail and its radioactivity measured. [^{3}H]-mannitol, a paracellular marker, was used simultaneously to assess the integrity of the cell layer for the duration of the experiment. The net efflux of a test compounds was assessed by calculating the ratio of apparent permeability (P_{app}) in the BL-to AP direction

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vs. P_{app} in the AP-to BL direction ($P_{app\ B\rightarrow A}/P_{app\ A\rightarrow B}$). A ratio greater than 1.0 indicates net efflux.

Estimation of Cell Cytotoxicity using Cell Proliferation Assay

Cell proliferation assay was carried out to examine the cytoxicity of erythromycin and the inhibitors. For this assay CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI) was used. This assay is a colorimetric method for determining the number of viable cells in proliferation. The method comprises of a novel tetrazolium compound (3-(4,5-dimethlythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium, MTS) and an electron coupling reagent (phenazine methosulfate, PMS). MTS is bioreduced by cells into a formazan that is soluble in tissue culture medium. The conversion of MTS into the water soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan produced, as measured by the amount at 490 nm absorbance, is directly proportional to the number of viable cells in culture.

rPCEC cells were plated at passage 3 in 96-well plates and the seeding density was optimized at 1.25 X 10^4 cells/well. This density was chosen based on the standard Beer-Lambert plot (data not shown) obtained with different seeding densities of the cells at the time points required. A seeding density of 1.25 X 10^4 cells/well produced measurable optical density values that were in the linear range for all the time points.

Solutions of erythromycin and the various inhibitors (at various inhibitory concentrations) were made in the culture medium and appropriate volumes were added to make up to a final volume of 100 µl medium in each well. A plate was seeded for each

time point. The cells were incubated with the solution in a humidified 5% CO_2 atmosphere. The effect of the compounds on the proliferation of these cells was observed with a change in the drug concentration and the time of exposure. Proliferation of rPCEC cells in the presence of different concentrations of erythromycin and the inhibitors was compared with the positive control (without drug) at each time point and these values were all corrected for, with a negative control (without cells). Exactly 24 hrs and 48 hrs after incubating the cells with the drug solutions, 20 µl of the MTS/PMS solution was added to each well. The plate was then incubated for another 4 hrs at 37^{o} C in a humidified 5% CO_2 atmosphere to allow for the dye to interact with the cells. The absorbency of the solutions was read at 490 nm with an automated 96-well microplate reader. Since the amount of formazan produced is directly proportional to the number of viable cells in culture, percentage of viable cells was calculated in each of the drug treated wells compared to the control.

Animal Model

New Zealand albino male rabbits weighing between 5.0 – 5.5 lbs were obtained from Myrtle's Rabbitry (Thompson Station, TN). All studies involving rabbits were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were kept under anesthesia throughout an experiment with ketamine HCl (35 mg/kg) and xylazine (3.5 mg/kg) given intramuscularly every hour. Prior to the implantation of the microdialysis probes, pupils were dilated with two drops of 1% tropicamide. The linear probe was implanted in the anterior chamber using a 25-guage needle. It was inserted

across the cornea just above the corneal scleral limbus so that it traverses through the center of the anterior chamber to the opposite end of the cornea as evidenced by microscopic examination. The sample-collecting end of the linear probe was inserted carefully into the bevel edge end of the needle. The needle was slowly retracted leaving the probe with the dialyzing membrane in the middle of the anterior chamber. The outlets of both the probes were fixed to prevent any disturbances during sample collection. The probes were perfused with isotonic phosphate buffer saline (pH 7.4) at a flow rate of 2 µl/min using a CMA/100 microinjection pump. After probe implantation, the animals were allowed to stabilize for 2 h prior to 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 (Macha and Mitra, 2001). Following the two hour stabilization, the eyelids of the rabbits 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. This positioning allows the drug solution to be in direct contact with the cornea and exclude the sclera. The outer flange of the topical well was coated with a surgical adhesive to prevent its movement. Subsequent to placing the well, the animals were allowed to stabilize for another 45 min to maintain proper intraocular pressure. Following this time period, 150 µl of IPBS containing the radiolabeled compounds, [^{14}C]-erythromycin (10µCi/ml), [^{14}C]-diazepam (10µCi/ml) or [^{3}H]-mannitol (10µCi/ml) were added to the well. The compounds were allowed to diffuse for a period of 75 min following which the drug solution was aspirated from the well which was subsequently removed. The corneal surface was washed clean with a few 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 an experiment, euthanasia was performed under deep anesthesia with an intravenous injection of sodium pentobarbital through the marginal ear vein. Samples obtained in the study were analyzed by a scintillation counter (LS 6500, Beckman Instruments Inc., Fullerton, CA).

In vitro probe calibration

Microdialysis probe recovery was determined in an aqueous solution containing a known concentration of the compound maintained at physiological temperature. The probe was continuously perfused at a constant flow rate of 2 µl/min, and samples were collected every 20 min. The ratio between the concentration of a substance in solution outside the probe is defined as "recovery", usually expressed as a ratio or percentage (Zetterstrom et al., 1982). The recovery factor of the probes is an important factor in determining the extracellular concentration of the drug. The recovery of a compound of interest is calculated according to equation (1).

$$\text{Recovery} = C_{out}/C_{in} \qquad \text{Equation (1)}$$

C_{out} is the concentration in the outflow solution and C_i the concentration in the medium. The dialysate concentrations were transformed into the actual anterior concentrations by equation (2).

$$C_i^* = C_{out}^*/\text{Recovery} \qquad \text{Equation (2)}$$

C_i^* is the substance concentration in the aqueous humor, and C_{out}^* is the concentration of the compound in dialysate.

The recovery of the linear probe was between 10-20% for both erythromycin and the inhibitors studied. There was no significant variation in the recovery of the probes with time over the experimental time period.

Mathematical Data Treatment

A model in which drug is administered at a constant rate to the corneal surface from the reservoir has been developed (Figure 1). Drugs are then transported passively across the cornea into the aqueous humor from where it may reversibly distribute to the adjacent tissues, namely, the iris and ciliary bodies and the lens. Drug is eliminated primarily from the anterior chamber via the aqueous humor pathway (Figure 1). Based on this model, the disposition of drug in the aqueous humor can be written as follows:

$$\frac{dX_{aq}}{dt} = k_0 - \sum_{i=1}^{x} k_{aq} X_{aq} + \sum_{i=1}^{y} k_{PT} X_{PT} - k_{10} X_{aq} \qquad \text{Equation (3)}$$

k_0 is the constant-input rate from the precorneal area to the aqueous humor, X_{aq} and X_{PT} are the amounts of drug in the aqueous humor compartment and peripheral compartments respectively, k_{10} represents the overall elimination from the aqueous humor, k_{aq} and k_{PT} are the first order rate constants for the transfer of drug from the aqueous humor to the peripheral tissues and vice versa, y is the number of peripheral tissues returning drug to aqueous humor and x is the number of tissues receiving drug from the aqueous humor which is greater than or equal to y ($x \geq y$). Peripheral tissues include lens, iris-ciliary body etc. It is also possible that elimination from peripheral tissues may or may not occur, but will not affect the drug disposition in the aqueous humor.

The constant-input rate is related to the drug in the precorneal area (well) by equation 4:

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$$k_0 = k_a C_w V_w \qquad \text{Equation (4)}$$

k_a is the first-order rate constant for drug transport across the cornea, C_w is the concentration of drug in the well, and V_w is the volume of the drug solution in the well. Equation 4 can be substituted into equation 3 and along with the relationship $C_{aq} = \frac{X_{aq}}{V_{aq}}$, can be written as:

$$\frac{dC_{aq}}{dt} = \frac{k_a C_w V_w}{V_{aq}} - \sum_{i=1}^{x} \frac{k_{aq} X_{aq}}{V_{aq}} + \sum_{i=1}^{y} \frac{k_{PT} X_{PT}}{V_{aq}} - \frac{k_{10} X_{aq}}{V_{aq}} \qquad \text{Equation (5)}$$

Equation 5 can be further simplified as,

$$\frac{dC_{aq}}{dt} = \frac{k_a C_w V_w}{V_{aq}} - \sum_{i=1}^{x} k_{aq} C_{aq} + \sum_{i=1}^{y} \frac{k_{PT} C_{PT} V_{PT}}{V_{aq}} - k_{10} C_{aq} \qquad \text{Equation (6)}$$

Since at initial times of the infusion, concentration of drug in the well is much greater than concentration of drug in the aqueous humor ($C_w >> C_{aq}$), the first term on the right side of equation 6 predominates over the second, third and the fourth terms. Thus equation 6 can be rewritten as,

$$\left(\frac{dC_{aq}}{dt}\right)_I = \frac{k_a C_w V_w}{V_{aq}} \qquad \text{Equation (7)}$$

where V_{aq} is the physiological volume of aqueous humor (250 µl). Subscript I in equation 7 refers to the initial rate which can be determined from the initial slope of C_{aq} versus t. Equation 7 can be rearranged as follows to allow estimation of the corneal absorption rate (k_a).

$$k_a = \frac{\left(\frac{dC_{aq}}{dt}\right)_I * V_{aq}}{C_w V_w} \qquad \text{Equation (8)}$$

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If the topical infusion is allowed to continue until steady state is reached, then integration of equation 3 yields equation 9:

$$\lim_{t\to\infty} C_{aq} \equiv C_{ss} = \frac{k_0}{k_{10} V_{aq}} = \frac{k_a V_w C_w}{k_{10} V_{aq}} \qquad \text{Equation (9)}$$

Thus the topical infusion method along with equations 3-8 permit a rational and reliable determination of ocular pharmacokinetics whereby absorption, distribution and elimination can be characterized without using complex compartmental analysis.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Corneal epithelial tissue (rabbit) and corneal epithelial cells were collected and snap frozen in liquid nitrogen. Total RNA was extracted from these tissues and cells using standard protocol. Briefly, the cells and tissue were taken and 800 µl of Tri-LS® was added. The cells and tissue were homogenized and transferred to eppendorf tubes. RNA was extracted by phenol-$CHCl_3$-isopropranolol method. Then it is purified and dissolved in 20µl of RNAse-DNAse free water.

The forward and reverse primer designed for rabbit MDR1 was 5'-CGG ACC ACC ATT GTG ATA GC-3' and 5'-GGT CGG GTG GGA TAG TTG AA-3' respectively. RT-PCR was performed based on the method of (Sugawara et al., 2000) with modifications using 1 µg of total RNA. RT-PCR was carried out using the GeneAmp® RNA PCR Kit (Applied Biosystems). The conditions for reverse transcription were as follows: denaturation of the template RNA for 10 min at 70 ^{0}C and reverse transcription for 60 min at 42 ^{0}C. The conditions for PCR amplification were as follows: denaturation for 1 min at 94 ^{0}C; annealing for 1 min at 58 ^{0}C and extension for 1 min at 72 ^{0}C; 37 cycles;

final extension for 10 min at 72 ^{0}C. The resultant PCR product (~ 1106 bp) was electrophoresed at 120 V for 30 min in 1% agarose gel (with ethidium bromide).

Analytical Method and Data Treatment

Apparent permeability (P_{app}) was calculated using the following equation 10

$$P_{app} = \left(\frac{dC}{dt}\right) / \left(\frac{V_c}{A} * C_0 * 60\right) \qquad \text{Equation (10)}$$

P_{app} denotes the apparent permeability in cm/sec, dC/dt is the slope of plot of concentration (mM) vs. time (min); C_0 is the initial donor concentration of the drug; V_c is the volume of the receiver chamber and A is the surface area available for diffusion (in polyester Transwells, the surface area for diffusion is 1.1 cm^2).

Dose – dependent inhibition data was fitted to a dose-response relationship given by equation (11)

$$Y = \min + \frac{\max - \min}{1 + 10^{(\mathrm{Log}IC_{50} - x) * H}} \qquad \text{Equation (11)}$$

IC_{50} is the inhibitor concentration where the rate of ocular absorption is doubled and H is the Hill constant. Data was fitted to equation (4) using a transformed non-linear regression curve analysis program (GraphPad Prism Version 3.03).

The rate constants for elimination from aqueous chamber were determined by nonlinear regression analysis of the concentration-time data (Winnonlin, version 2.1, Pharsight Corporation, Mountain View, CA). The best fit model for the aqueous concentration-time data of erythromycin was selected based on the coefficient of variation percentage, Akaike's information criterion, F-test, Run-test, and residual plots.

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Statistical Testing

All experiments were conducted at least in triplicate and results are expressed as mean ± standard deviation. E_{max} and IC_{50} values are expressed as mean ± S.E. Statistical significance testing was done using a two-level factorial analysis of variance (ANOVA) (Statgraphics Plus Version 5.1). A difference between mean values was considered significant if the p-value obtained was ≤ 0.05. The method used to discriminate among the means is Fisher's least significance difference (LSD).

RESULTS

Transport of [^{14}C]-Erythromycin in MDCK-MDR1 Cells

Transport experiments were conducted for a period of 180 minutes with [^{3}H]-erythromycin. The morphology of MDCK-MDR1 cells suggests that these cells do not form well-defined tight junctions (reflected by TEER value of 200-250 $\Omega.cm^2$). [^{3}H]-erythromycin was used at a concentration of 0.05 μCi/ml. Flux and apparent permeability (P_{app}) values were calculated from a plot of cumulative amount of erythromycin transported as a function of time. The integrity of MDCK-MDR1 cell layer was not compromised during transport as evident by [^{14}C]-mannitol flux (~ 0.5%/hour). There was no polarity observed of [^{14}C]-mannitol transport suggesting that mannitol was indeed being transported by paracellular pathway (data not shown). The basolateral-to-apical (BL→AP) transport of CsA was significantly higher than the apical-to-basolateral transport (AP→BL) (Figure 2A). The BL→AP and AP→BL permeabilities were found to be 4.79 (± 0.32) X 10^{-5} cm/sec and 9.83 (± 0.66) X 10^{-6} cm/sec respectively yielding a ratio (BL→AP/ AP→BL) of about 5. In the presence of 10 μM of CsA (inhibitor) both BL→AP and AP→BL permeabilities became similar. The BL→AP and AP→BL permeabilities in presence of 500 μM verapamil were 2.74 (± 0.29) X 10^{-6} cm/sec and 3.19 (± 0.42) X 10^{-6} cm/sec respectively (Figure 2B).

Simultaneous transport of [^{3}H]-mannitol was also monitored during the transport of erythromycin. Both BL→AP and AP→BL permeabilities remained unaltered indicating no change in membrane characteristics during transport. The mannitol permeability did not change in the presence of 10 μM CsA (Figure 3).

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Dose Dependent Inhibiton of [^{14}C] Erythromycin Absorption Across Rabbit Cornea.

To investigate the nature and potency as an inhibitor, dose dependant inhibition of [^{14}C]-erythromycin absorption in presence of testosterone was carried out. Testosterone was chosen since it is a very selective inhibitor with no substrate potential for P-gp mediated efflux. As seen in Figure 4A, testosterone inhibited ocular absorption of [^{14}C]-erythromycin in a dose-dependent manner. Inhibitory concentration (IC_{50} - concentration needed to inhibit the efflux by 50%), values was calculated for the inhibitor. The data was fitted to a modified log [Dose]-response curve (curve not shown) fit to yield IC_{50} values. IC_{50} value for testosterone was calculated as 241 ± 27.6 μM. Hill factor was chosen to be 1.

***In vivo* ocular absorption of Mannitol and Diazepam Across Rabbit Cornea.**

To investigate the nature of paracellular and transcellular permeation characteristics of the cornea, *in vivo* ocular absorption of [^{3}H] mannitol (a paracellular marker) and [^{14}C] diazepam was carried out. In addition, [^{3}H] mannitol uptake was carried out simultaneously in the presence of erythromycin and the P-gp inhibitors. As shown in figure 5, there was a 3-fold increase in the corneal absorption rate (k_a) indicating that diazepam traverses through the transcellular pathway of the corneal epithelium (Table 2). As illustrated in figure 5, similar elimination rates were found for both these compounds. Elimination half-life of mannitol was found to be 93 min whereas the relatively lipophilic compound diazepam had an elimination half-life of 119 min. In addition, the absorption of mannitol was not altered in the presence of erythromycin and the inhibitors suggesting

that the inhibitors used at the specific concentration do not cause any cell damage or change tight junctional properties of the cornea.

***In vivo* ocular absorption of Erythromycin in presence of Inhibitors.**

Ocular absorption of erythromycin in presence of the inhibitors was carried out to determine the functionality of P-gp. Dose dependent inhibition of [^{14}C] erythromycin was observed with testosterone. However no significant difference was noted in corneal absorption rate or $AUC_{0-\infty}$ when testosterone was used at 100 µM or 150 µM. However a significant inhibition of P-gp efflux (determined by increased absorption) was observed at 250 µM with the highest inhibition found at 500 µM. At 500 µM of testosterone, a 4-fold increase in $AUC_{0-\infty}$, 5-fold increase in maximum aqueous concentration ($C_{aq,max}$) and a 9-fold increase in the corneal absorption rate were found as compared to control (erythromycin without any testosterone) (Table 1). Higher concentrations of testosterone were not studied because of the difficulty in formulation due to very low aqueous solubility of testosterone. Higher concentration can be achieved by increasing the ethanol concentration; however that is reported to cause damage to cell morphology.

Other model P-gp inhibitors including CsA, verapamil and quinidine were also studied. CsA (20µM) caused significant increase in $AUC_{0-\infty}$ (2-fold), k_a (3 fold) and $C_{aq,max}$ (2.5 fold) as compared to control (Figure 4B and Table 2). Quinidine (200 µM) also caused inhibition of the P-gp mediated efflux pump. The first order elimination rates (k_{10}) was calculated for all the inhibitors studied to elucidate if the inhibitors cause any change in the aqueous elimination pathway. There was no difference observed in the elimination rates for all the inhibitors as compared to erythromycin alone. Elimination rate constant

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(k_{10}) ranged from 4.1 – 5.7 (X 10^{-3} min^{-1}) with elimination half-lives ranging from 122 – 169 minutes.

Estimation of Cell Cytotoxicity by Cell Proliferation Assay.

Cell proliferation assay was carried out to examine the toxicity of erythromycin and the various inhibitors. For this assay CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI) was employed.

Results indicate that erythromycin did not inhibit cell growth (Figure 6). Methotrexate was selected as a positive control since it is known to inhibit cell proliferation and cause cytotoxicity. Methotrexate (100 µM) caused significant cell death (43.2% cells viable). The inhibitors used in the *in vivo* studies were all used in the same concentration used.

RT-PCR and Protein Translation.

PCR products were analyzed by gel electrophoresis on 0.8% agarose. The forward and reverse primers designed for rabbit MDR1 were 5'-CGG ACC ACC ATT GTG ATA GC-3' and 5'-GGT CGG GTG GGA TAG TTG AA-3' respectively. The forward and reverse primers were designed from human, rat and mouse cDNA sequences as discussed in our earlier report (Dey et al., 2003). PCR amplification of specific rabbit MDR1 sequences was carried out. When rPCEC and intact rabbit corneal epithelial RNA was PCR amplified, both gave one single product, which is ~ 1100 bp long (Figure 7). The ~ 1100 bp fragment was subcloned in pGEM-T-Easy vector and grown in competent DH5α™ *E. Coli* cells. The cloned insert was sequenced from both T7 and SP6 promoter regions and the sequence matched. The final protein sequence was obtained using Protein

BLAST and the resultant amino acid sequence matched with any known sequences in GenBank. The sequence match showed >89% homology with human MDR1 protein (Figure 8). This partial protein fraction suggests that rabbit corneal epithelial cells express P-gp at the molecular level with high protein homology to human P-gp.

DISCUSSION

P-gp has been demonstrated to be an important determinant of the pharmacokinetics of lipophilic compounds in various body tissues. However, little is known about the functional expression of P-gp in the eye, especially in the corneal epithelium. Moreover clinical studies are not feasible because of the invasive nature of surgeries required for such pharmacokinetic studies. Substrates recognized by P-gp include many different classes of drugs, some of which are used topically in the eye for the management of ocular diseases.

A majority of topically applied drugs enter the eye through the cornea. Topical delivery is, by far, the most common route of ocular drug delivery. The process of corneal drug absorption has proven to be extremely inefficient. Poor absorption is largely due to precorneal loss and the resistance exerted by the corneal epithelium to drug penetration. Only about 1-10% of the topically applied dose actually is absorbed intraocularly. Other restrictive mechanisms include solution drainage, lacrimation and a highly selective corneal barrier to exclude exogenous compounds from the internal eye structures. Low ocular bioavailability so far was attributed primarily to the inability of drug molecules to cross the lipoidal corneal membrane and not attributed to any efflux proteins that might efflux molecules out of corneal epithelium into the precorneal fluid.

In this present study we report for the first time the role of P-gp in restricting ocular drug absorption. P-gp has been shown to be present functionally in corneal epithelial cells in culture; however *in vivo* determination of P-gp has not been made previously in ocular tissues. We hypothesize that the corneal epithelium is the rate limiting barrier for most of the drugs administered as topical solution and the presence of

P-gp would definitely modulate drug concentration in the aqueous and would directly be responsible for low ocular bioavailability. Thus it was imperative to choose a proper P-gp substrate that not only has a higher efflux potential (to test the functionality of the pump) but also has a clinical application. Erythromycin was chosen because it is a broad spectrum antiobiotic, a large, lipophilic molecule (MW 733.9) which can be a selective substrate of P-gp. Also erythromycin (brand name Ilotycin®) is being currently used to treat superficial bacterial infection of the cornea/conjunctiva (Queille-Roussel et al., 2001; Kowalski et al., 2003). Radiolabeled erythromycin transport was studied both from the AP→BL and BL→AP directions in MDCK-MDR1 cells to examine whether erythromycin is a good substrate of P-gp. These cells are known to express MDR1 (P-gp) protein in excess. In the past, MDCK cells have been used extensively as models of the intestinal epithelium, to screen drug candidates for efflux potential (Horio et al., 1990; Hunter et al., 1993a; Hunter et al., 1993b; Collett et al., 1996; Doppenschmitt et al., 1999). Transport of [^{14}C]-erythromycin across MDCK-MDR1 cell layers confirms the polarized efflux characteristics of P-gp. In absence of any inhibitor BL→AP permeability was 4.9 fold higher than AP→BL permeability. In the presence of 10 μM of CsA, the permeabilities from both sides became approximately equal. This suggests clearly that P-gp is functional and present in the corneal epithelium. Morphological studies have shown that MDCK-MDR1 do not form well defined tight junctions (data not shown). This is reflected in its low TEER values.

So far, the biggest challenge in ocular pharmacokinetic studies of drugs is the inaccessibility of ocular fluids for serial sampling. Microdialysis has been a valuable sampling technique in evaluating drug disposition and pharmacokinetics. The main

feature of this sampling procedure is that it does not induce any changes in the physiological volume of the sampling compartment. Ocular microdialysis is a relatively recent event first reported in the late 1980s (Gunnarson et al., 1987) . The application of this technique in ocular drug kinetics has had a major impact on the study of drug disposition in the eye (Sato et al., 1996; Rittenhouse et al., 1999). It has been used in both the aqueous and vitreous chambers in several laboratories (Hughes et al., 1996; Waga and Ehinger, 1997). In our laboratory, we have successfully used this technique to study the disposition of drugs in the aqueous and vitreous chambers simultaneously (Macha and Mitra, 2001).

Along with aqueous humor microdialysis, we proposed to use a modified ocular model to study the pharmacokinetics of erythromycin which was employed earlier to study topical carbonic anhydrase inhibitors (Eller et al., 1985). Previous ocular pharmacokinetic models have been utilized with very limited success (Makoid and Robinson, 1979). These models predict aqueous humor levels reasonably well; however it fails to measure the transcorneal absorption rate accurately. This is due to the presence of precorneal kinetic events which complicate a compartmental modeling approach (Patton, 1980). The topical model utilizes constant concentration of the drug on the cornea by a “topical single-dose infusion” method. This eliminated the effect of tear dynamics and other precorneal losses and simpler equations can be applied independent of modeling. A principal advantage of this topical infusion method is that it obviates the need for compartmental modeling and does not require knowledge of the precorneal factors in estimating k_a. The overall goal of this model is to a) predict corneal absorption rate constants without complications from parallel loss due to non absorptive processes

(tear turnover and scleral/conjunctival absorption) and b) compare absorption kinetics of erythromycin with and without inhibitors to elucidate the functional nature of P-gp in the cornea. Ocular disposition of mannitol and diazepam was also studied to compare the paracellular and transcellular permeability changes respectively. The corneal absorption rate for diazepam was found to be 3 fold higher than mannitol. This is because diazepam is relatively more lipophilic than mannitol since the corneal epithelium is more permeable to lipophilic compounds than hydrophilic compounds (Hughes et al., 1993).

Pharmacokinetic parameters from erythromycin absorption were compared with its inhibitors. Erythromycin was found to have a very low absorption constant. This suggests that erythromycin is actively being effluxed out and its permeability is much lower than the transcellular component. However with increasing concentration of the inhibitor (testosterone), the corneal absorption is significantly improved. At 500 μM of testosterone, k_a is 9-fold higher than without inhibitor. All other known inhibitors of P-gp also increased the corneal absorption rate with significant increases in the aqueous humor-time $AUC_{0-\infty}$ and the maximum aqueous concentration. These results clearly suggest that as P-gp is progressively inhibited; more drug molecules enter the aqueous compartment with increasing rates of absorption and produces higher concentrations in the aqueous. This observation has high clinical significance in the management of ocular diseases. So far poor ocular bioavailability of topically applied drugs was attributed to precorneal losses and no attempt has been made to increase bioavailability. With this new finding, we could incorporate inhibitors in the ophthalmic formulation which would inhibit P-gp and produce higher concentration inside the eye. In this study we have also tested the inhibitors (same concentration as *in vivo* studies) for their cytotoxic potential if

used along with drugs. None of the inhibitors were found to be cytotoxic (as evident by its inability to stop cell proliferation) and hence can be used along with ophthalmic drugs.

Aqueous humor is presumed to be the primary route of elimination of drug from the eye. In the rabbit eye, the turnover rate of aqueous humor equals a bulk flow rate of 1.5% of the volume of the anterior chamber per minute ($t_{1/2}$= 46 min) (Maurice, 1987). Half-lives of erythromycin and its inhibitors ranged from 122-169 minutes, which is much longer than the aqueous humor turnover rate, suggesting that tissue binding may have an influence on elimination of drug from the eye. Systemic uptake thorough the highly vascular anterior uvea has been proposed for compounds having very short half lives. This route of elimination occurs for fluorescein at a rate equal to 10% of the aqueous humor turnover (Jones and Maurice, 1966). Therefore, this route may not be the predominant route of elimination for the compounds used in this study.

In conclusion, this report provides functional evidence of the existence of P-gp in rabbit cornea. Molecular studies and protein sequence deduction have proven that rabbit MDR1 and human MDR1 share a high sequence homology (89%) which is very much expected since the sequence of genes are conserved within the mammalian system. In the future, cloning and expression of rabbit MDR1 will help us gain valuable insights into the characteristics of this efflux pump. Drug delivery strategies to the cornea and to the inner chambers of the eye will certainly change with this new finding and new ways to by-pass P-gp to increase ocular bioavailability will be implemented.

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Footnotes:

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Legends for figures:

Figure 1. Mathematical model representation of the single dose-continuous infusion to the rabbit eye. The dimensions of the plastic cylindrical well (used to simulate single-dose infustion) are also given. Open boxes represent individual compartments with the elimination occurring primarily through the central (aqueous humor) compartment. Drug distribution occurs which is in dynamic equilibrium with the other tissue compartments. The cornea is assumed to act more like a barrier than a compartment. The topical well is designed according to an earlier report and custom made.

Figure 2. Polarized transport of [^{14}C] erythromycin across MDCK-MDR1 cells.

A. Bidirectional transport of [^{14}C] erythromycin as a function of time. BL→AP (■) transport was found to be approximately 5 times high than AP→BL (▲) transport.
B. Comparison of AP→BL and BL→AP permeabilities with and without 10 μM CsA.

Each point represents mean ± S.D. (n=4).

Figure 3. Simultaneous transport of [^{3}H]-mannitol across MDCK-MDR1 cells in the presence of erythromycin. There is no statistical significance difference between the AP→BL (■) transport versus the BL→AP (♦) transport. The transport is also not altered in the presence of 10 μM of the inhibitor, CsA (▲).

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Figure 4A. Concentration-time profile of 1.5 µCi of erythromycin (▲) with and without different concentrations (100 µM, 150 µM, 250 µM and 500 µM) of the inhibitor (testosterone). 100 µM (×) and 150 µM (◊) testosterone did not inhibit P-gp mediated efflux of erythromycin. However, 250 µM (Δ) and 500 µM (□) of testosterone inhibited P-gp mediated efflux to a significant extent (*p<0.05, **p<0.01). [^{14}C]-erythromycin was given as a topical single-dose infusion in a plastic cylindrical well placed on top of the cornea. The well was removed at 75 min and samples were taken for 7 hours.
Data are expressed as mean ± S.D. (n=4).

Figure 4B. Concentration-time profile of 1.5 µCi of erythromycin (▲) with and without 20µM of CsA (■). As evident from the graph, CsA inhibited P-gp mediated efflux to a significant extent (*p<0.05). Data are expressed as mean ± S.D. (n=4)

Figure 5. Concentration-time profile of [^{3}H]-mannitol (▲) (1.5 µCi) and [^{14}C]-diazepam (■) (1.5 µCi) after a topical single-dose infusion. The rate of corneal absorption for diazepam was 3-fold higher than mannitol but the elimination rates were similar. Data are expressed as mean ± S.D. (n=4).

Figure 6. Cell Proliferation Assay in rPCEC cells in the presence of various P-gp inhibitors using CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay Kit. The cells were grown for a period of 24 hours and were incubated with the inhibitors for 48 hours. Cells grown in identical conditions without the presence of any drug/inhibitor are considered as control. All the data were corrected for absorbance resulting from blank

media (negative control). Statistical significance testing is done using a two-level factorial analysis of variance (ANOVA). Data are expressed as mean ± S.D. (n=8)

Figure 7. RT-PCR analysis of rabbit *mdr1* gene expression in rabbit corneal epithelium (lane 1) and rPCEC (lane 2). PCR products (5 µl) were analyzed by gel electrophoresis on 0.8% agarose. Ethidium bromide staining showed a 1106 bp band that corresponded to rabbit *mdr1*. A 1 Kbp DNA ladder is shown on the left. PCR products were verified by subcloning and sequencing.

Figure 8. Partial amino acid sequence of rabbit P-gp as obtained from Protein BLAST. The protein sequence was aligned with human P-gp sequence to determine the percent homology with human P-gp. The rabbit sequence that does not have an amino acid match with human P-gp is shown in parenthesis. From the obtained alignment, rabbit P-gp was found to have 89% protein homology with human P-gp.

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Table 1. Effect of various P-gp inhibitors on the $AUC_{0-\infty}$ and maximum aqueous concentration ($C_{aq,max}$) of [^{14}C]-erythromycin in rabbit cornea. As evident from the data, 250 µM and 500 µM of testosterone inhibited P-gp mediated efflux to a significant extent (*p<0.05, **p<0.01). Among the other inhibitors studied, CsA and quinidine also inhibited P-gp mediated efflux. Each point represents mean ± S.D.(n=4).

Drug and/or Inhibitor	**$AUC_{0-\infty}$** **µg.min/ml**	**$C_{aq,max}$** **µg/ml**
Erythromycin	31.67 (± 4.99)	0.136 (0.009)
Erythromycin + 100 µM Test	40.21 (± 5.29)	0.151 (± 0.021)
Erythromycin + 150 µM Test	43.51 (± 7.75)	0.183 (± 0.033)
Erythromycin + 250 µM Test	88.58 (± 6.34)*	0.489 (± 0.095)**
Erythromycin + 500 µM Test	118.76 (± 10.72)**	0.672 (± 0.095)**
Erythromycin + 20 µM CsA	65.18 (± 7.09)*	0.356 (± 0.060)*
Erythromycin + 200 µM Quin	52.63 (± 7.79)*	0.286 (± 0.052)*
Erythromycin + 500 µM Vera	43.56 (± 6.99)	0.205 (± 0.039)

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Table 2. Effect of various P-gp inhibitors on the corneal absorption rate and first order elimination rate of [^{14}C]-erythromycin in rabbit cornea.

Control corneal absorption rate for erythromycin is 1.61 (±0.21) X 10^{-5} min^{-1} without inhibitor. 250 μM and 500 μM testosterone, CsA and quinidine inhibited P-gp efflux significantly (*p<0.05, **p<0.01). The initial rates were determined from the initial slope of C_{aq} versus time. For comparison purposes, the rates for mannitol (a paracellular marker) and diazepam (a transcellular marker) are also given.

ND: Not determined. Each point represents mean ± S.D. (n=4).

Drug and/or Inhibitor	**k_a (X 10^5 min^{-1})**	**k_{10} (X 10^3 min^{-1})**
Erythromycin	1.61 (±0.21)	4.3 (±0.21)
Erythromycin + 100 μM Test	1.59 (±0.19)	4.1 (±0.29)
Erythromycin + 150 μM Test	1.46 (±0.29)	4.3 (±0.50)
Erythromycin + 250 μM Test	6.22 (±0.77)*	4.9 (±0.33)
Erythromycin + 500 μM Test	9.71 (±0.99)**	5.1 (±0.69)
Erythromycin + 20 μM CsA	4.47 (±0.67)*	5.0 (±0.46)
Erythromycin + 200 μM Quin	2.99 (±0.39)*	5.3 (±0.71)
Erythromycin + 500 μM Vera	1.78 (±0.20)	4.7 (±0.55)
[^{3}H]-Mannitol	3.31 (±0.89)	ND
[^{14}C]-Diazepam	9.91 (±1.83)	5.7 (±0.82)

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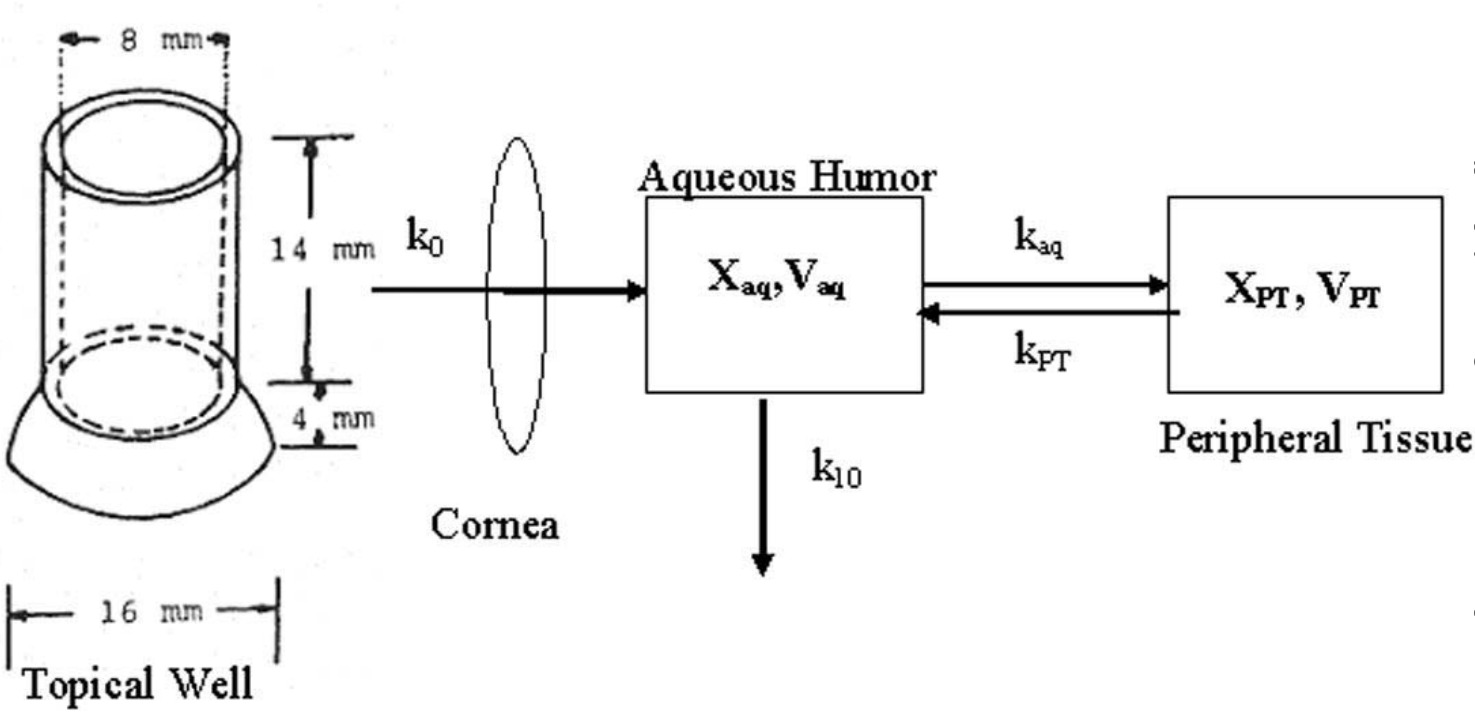

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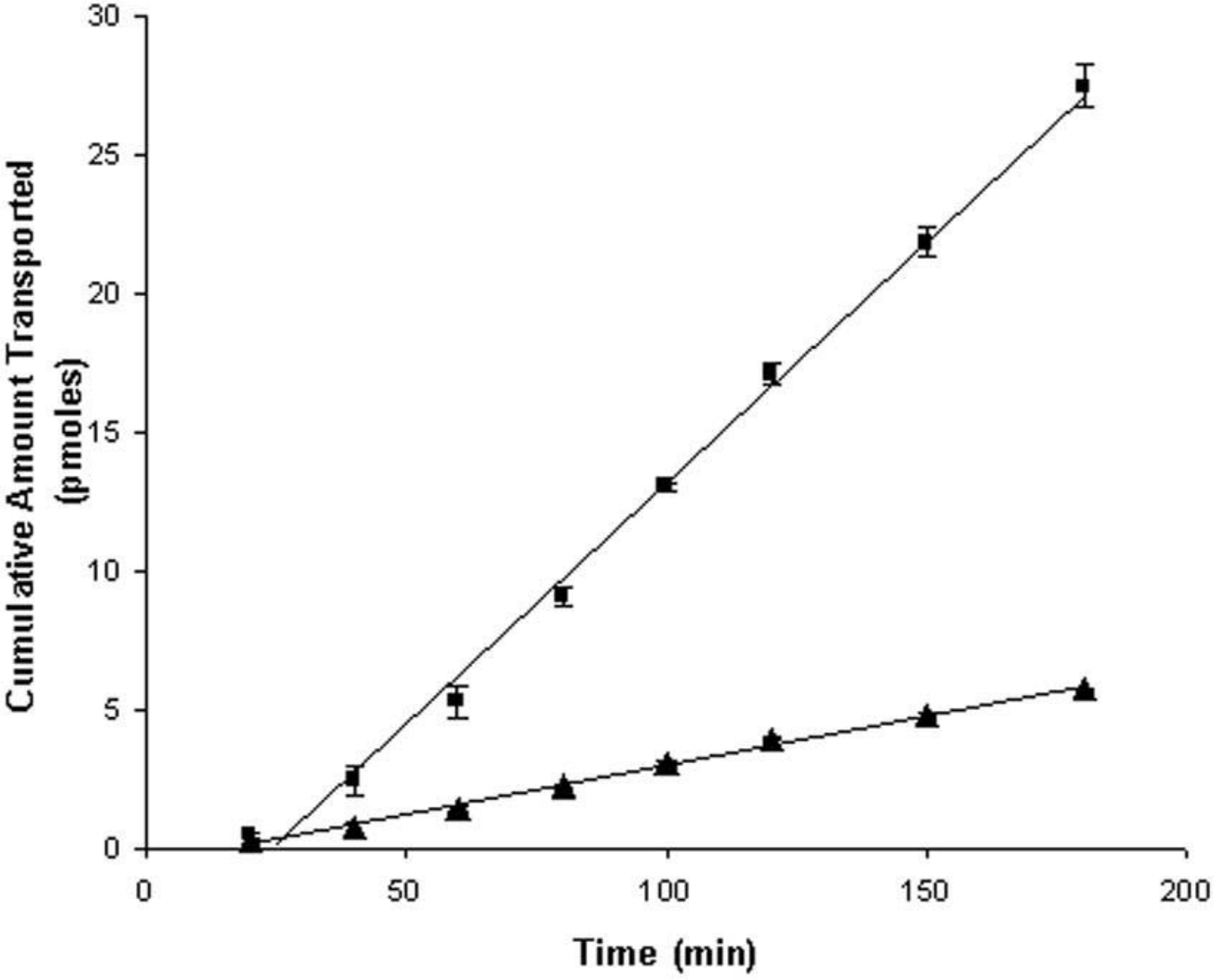

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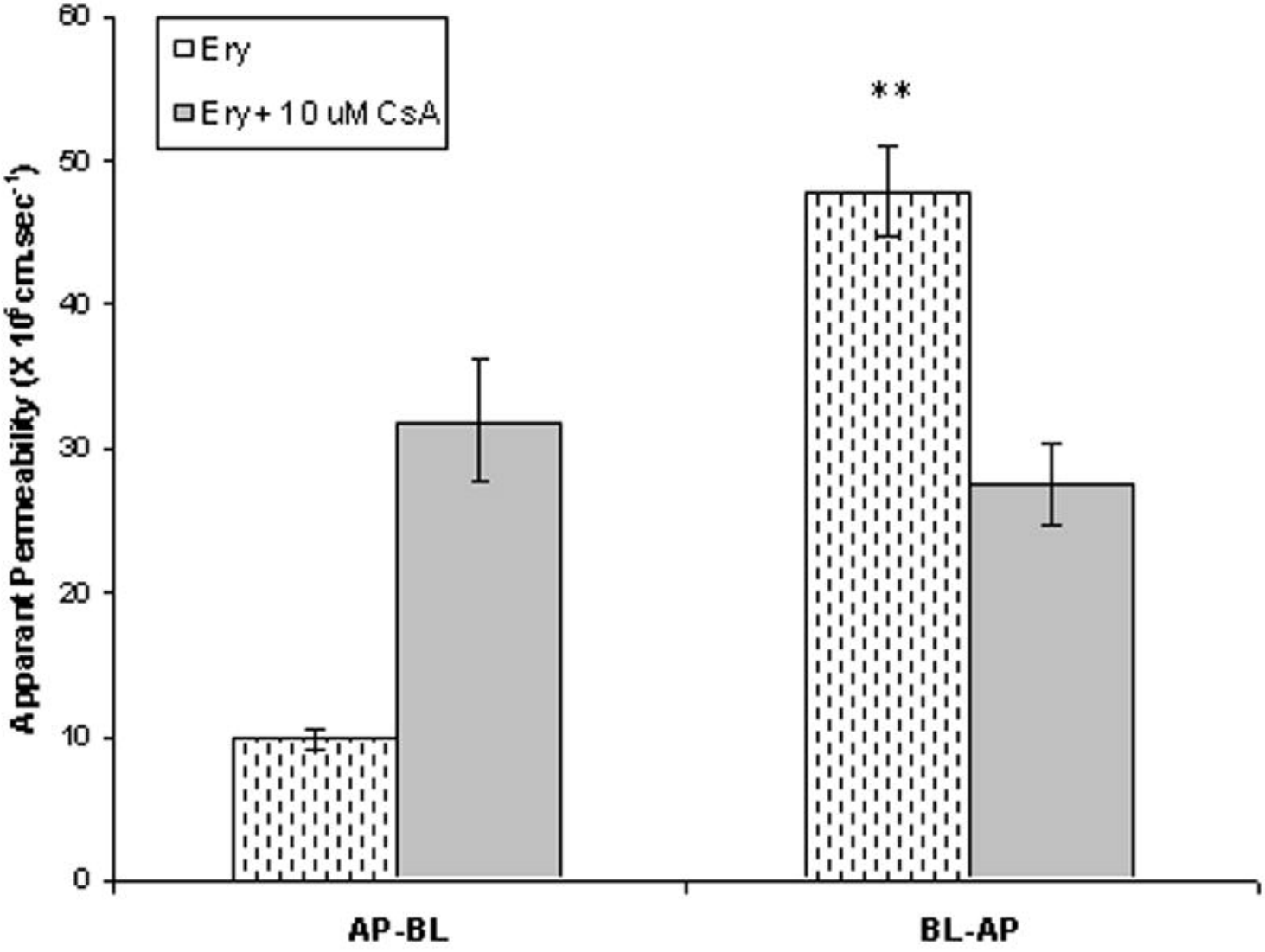

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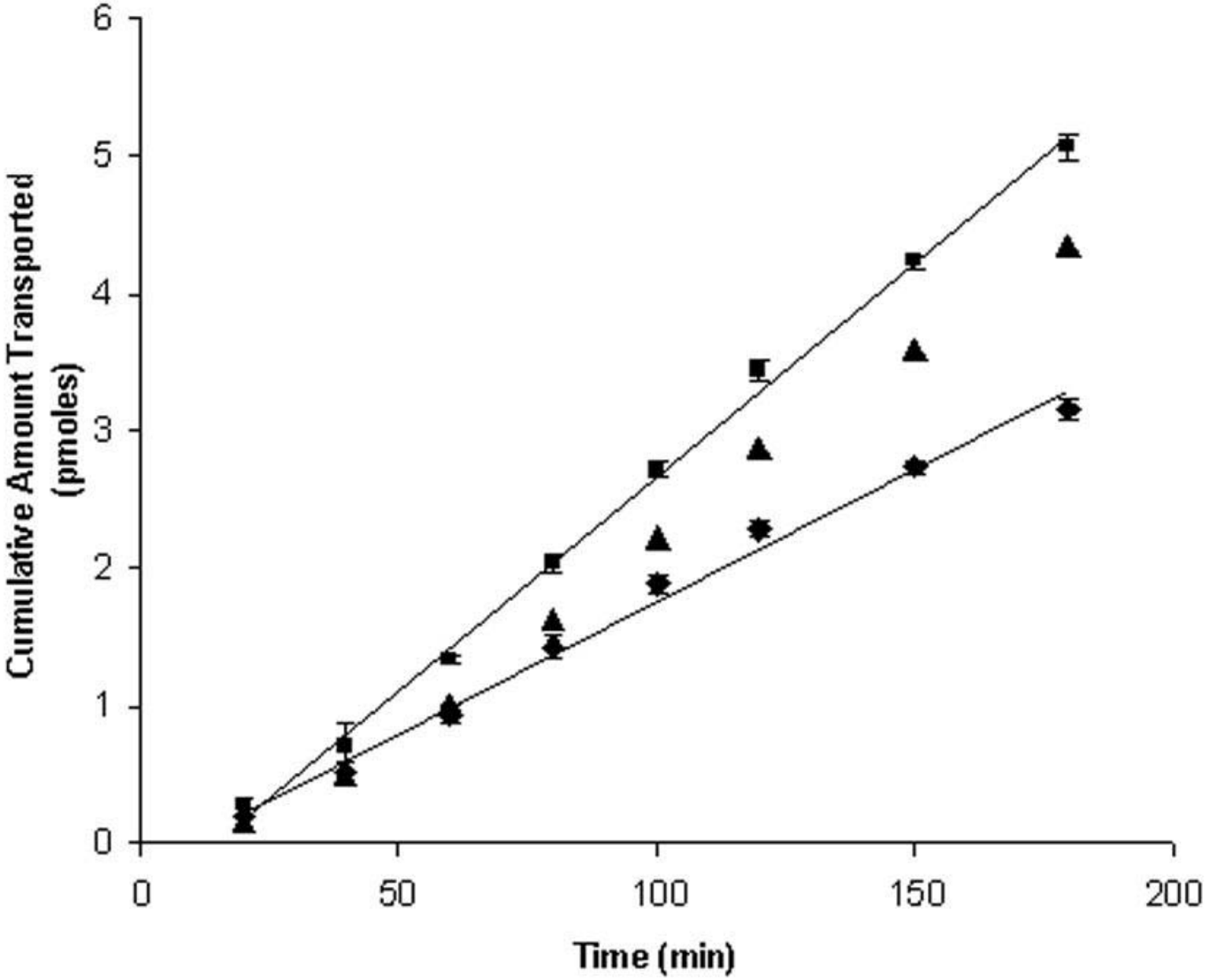

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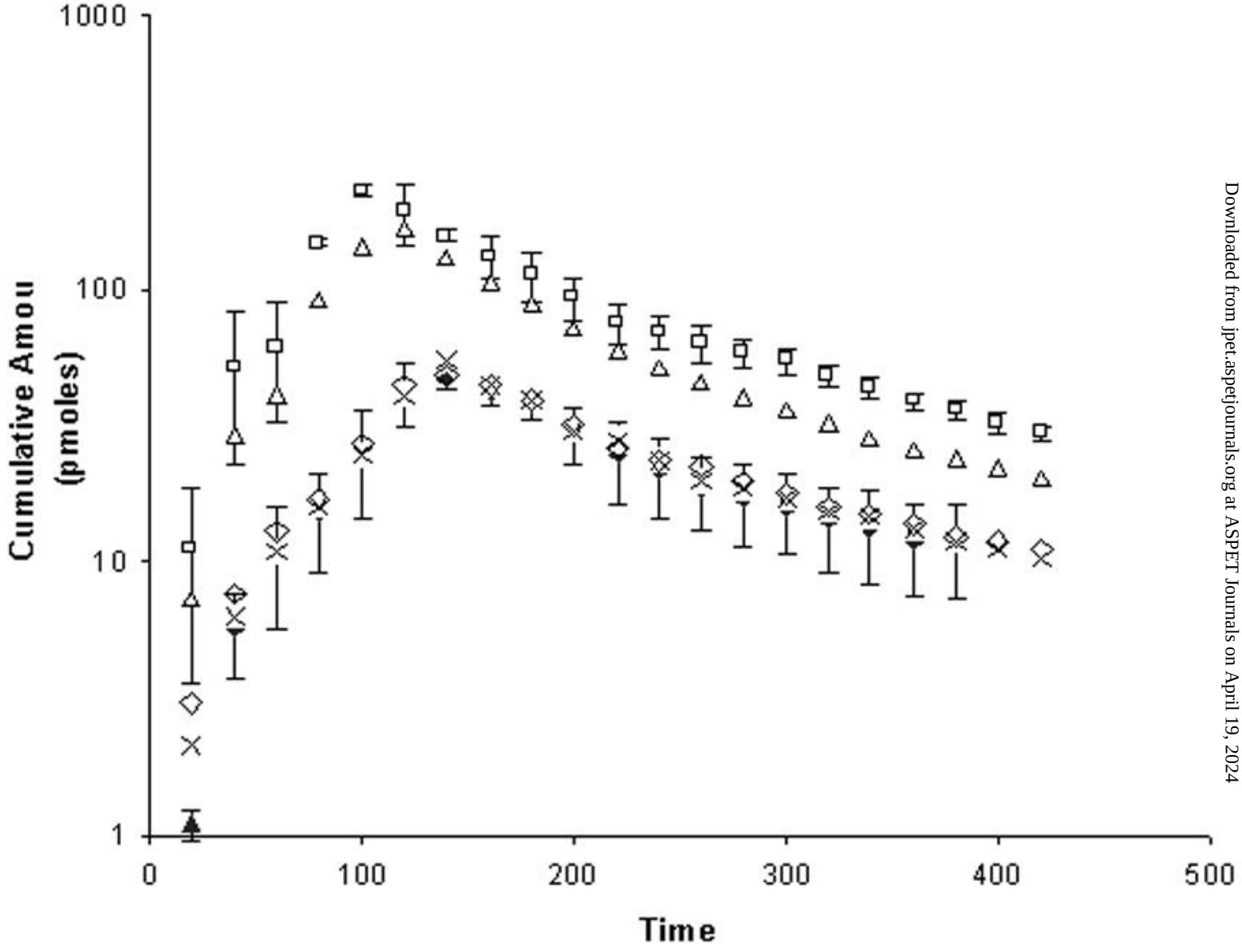

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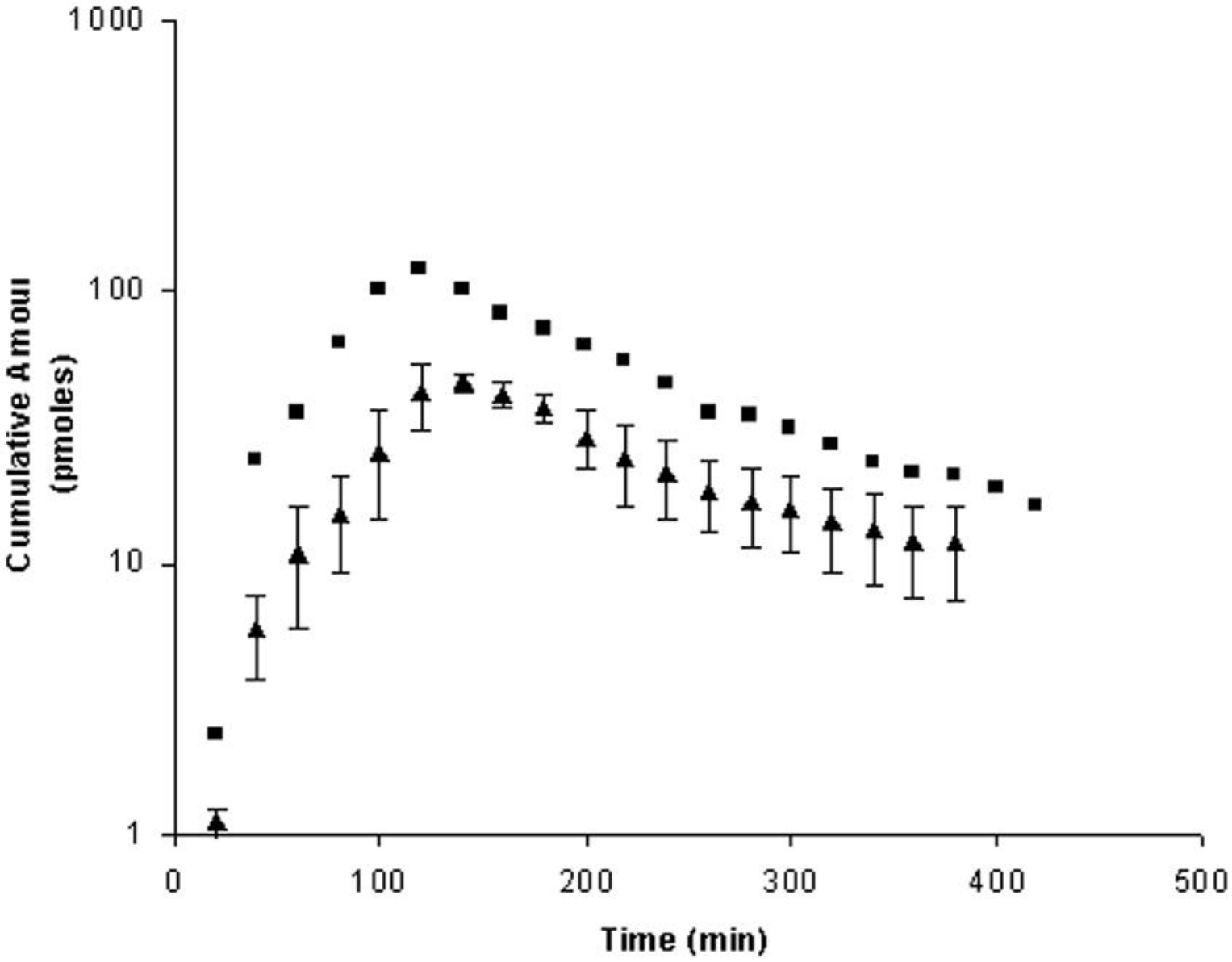

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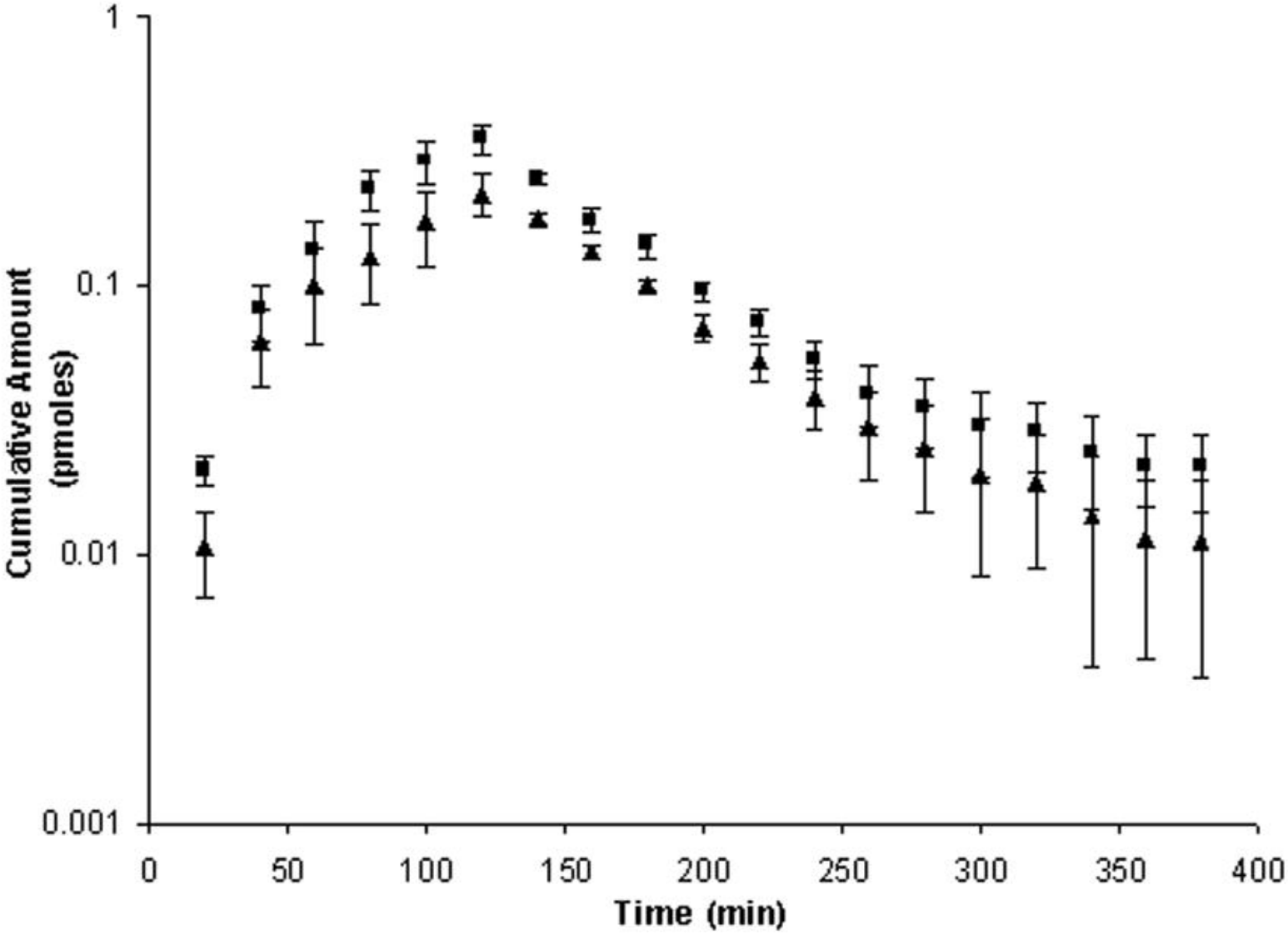

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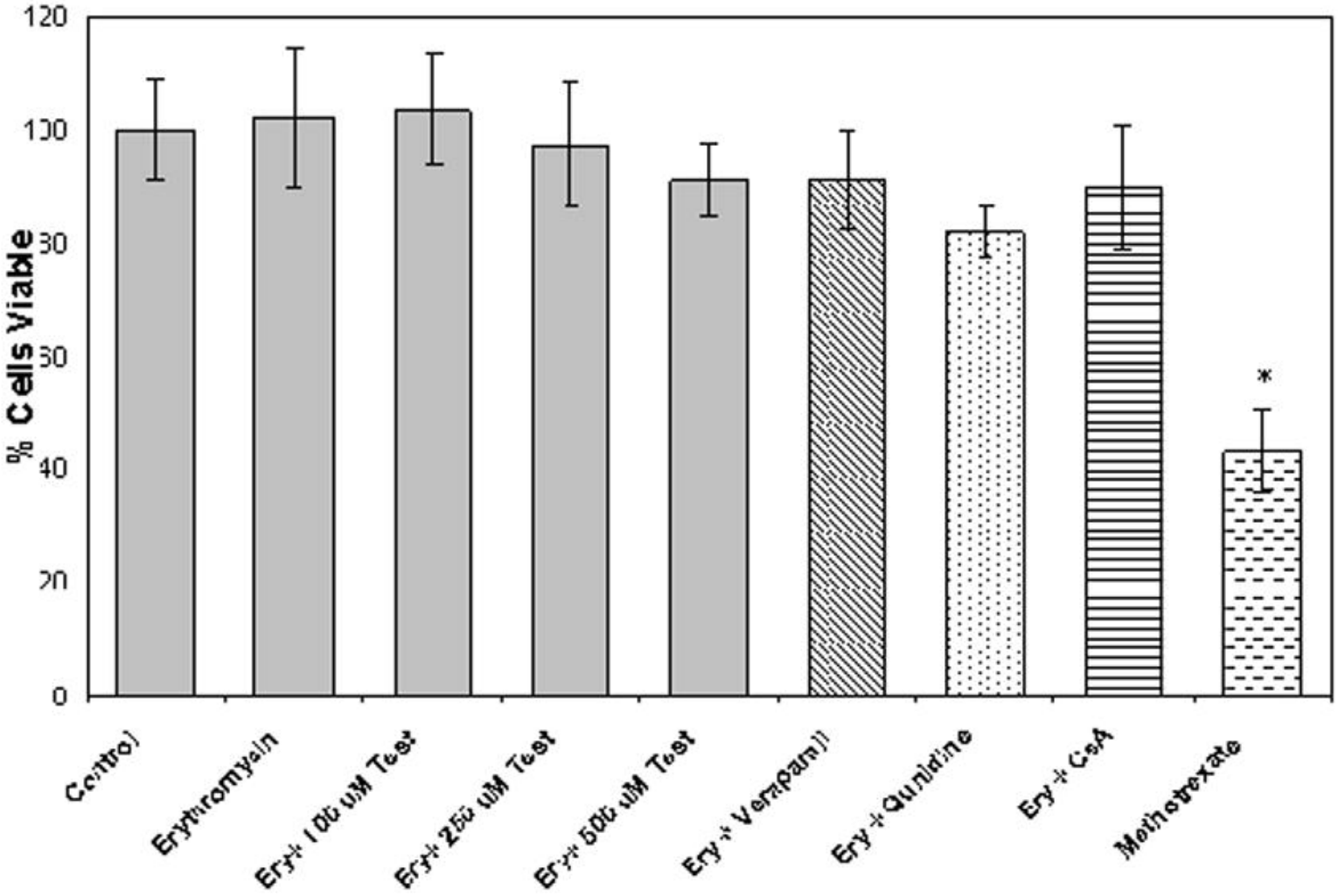

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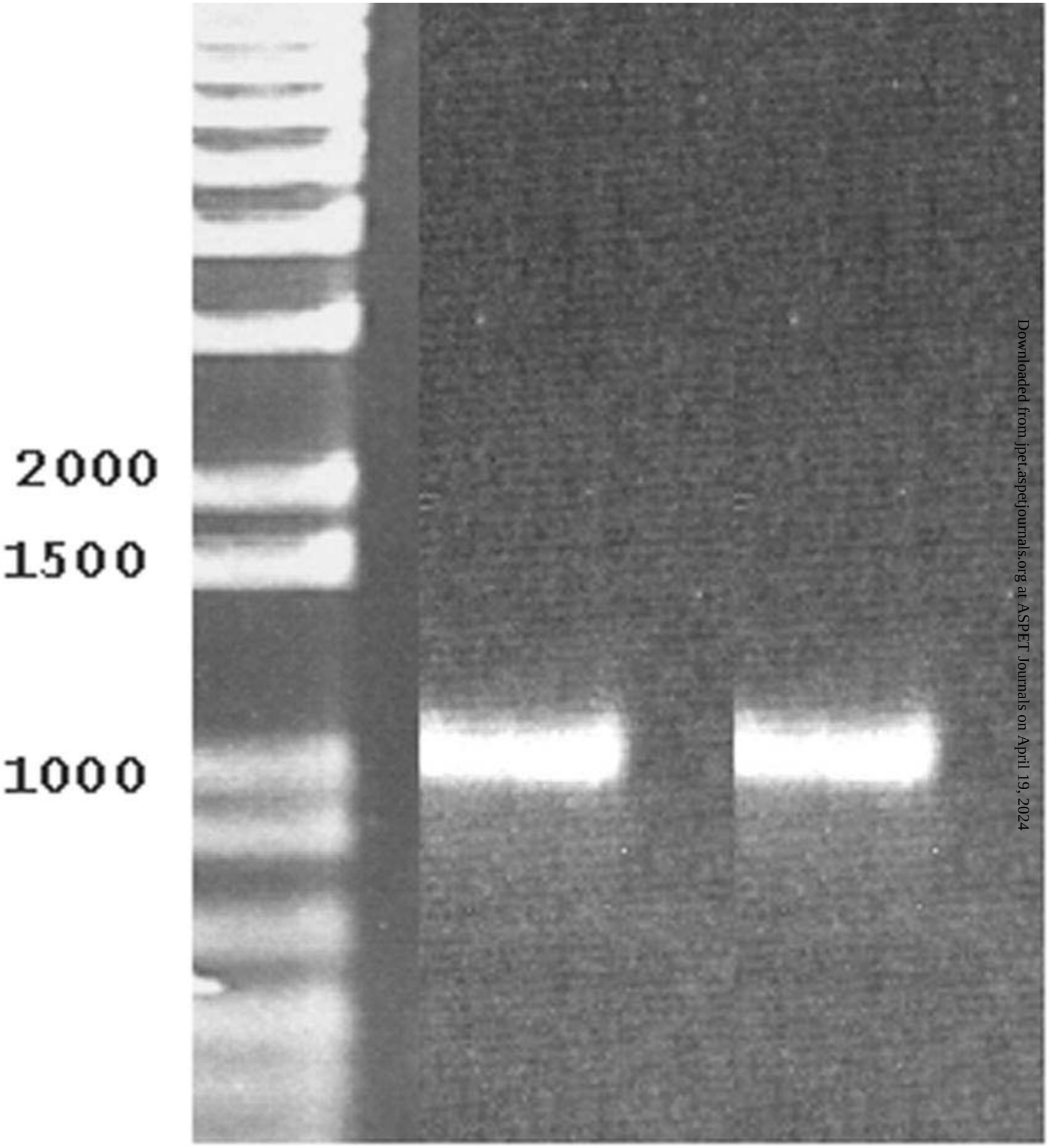

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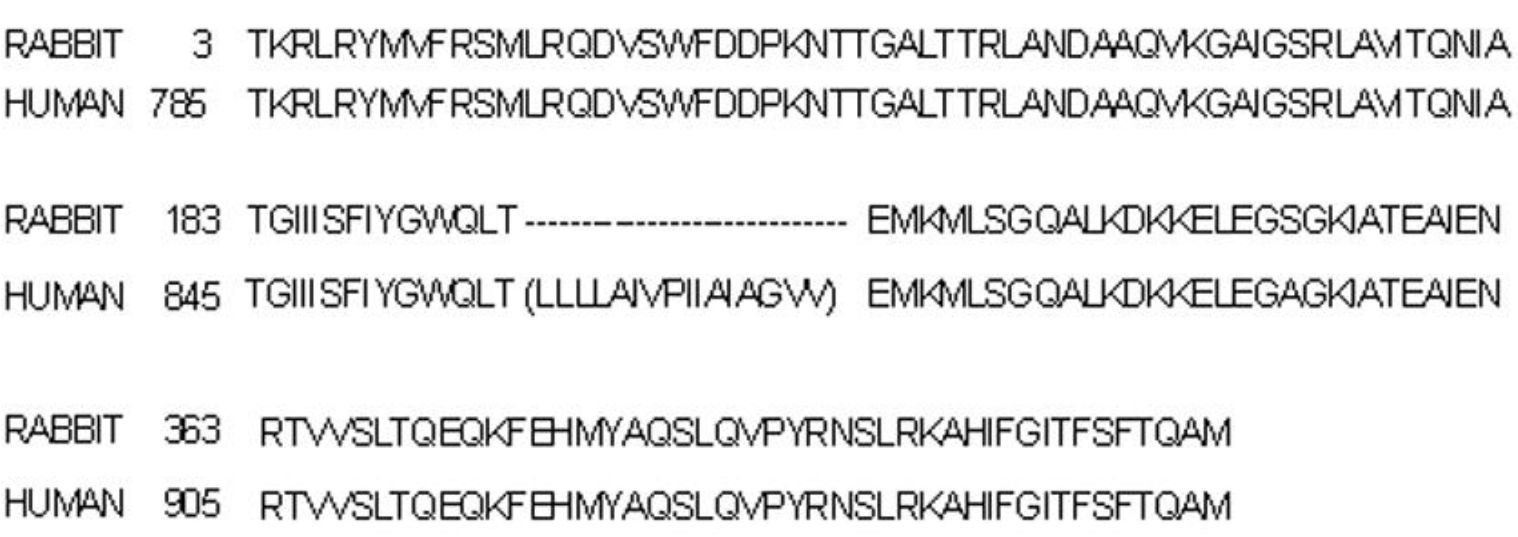

Correction to "Pharmacokinetics of Erythromycin in Rabbit Corneas after Single-Dose Infusion: Role of P-Glycoprotein as a Barrier to in Vivo Ocular Drug Absorption"

In the above article [Dey S, Gunda S, Mitra AK (2004) **311:**246-255], the last sentence in the first paragraph of "Transport of [^{14}C]Erythromycin in MDCK-MDR1 Cells" under *Results* should read as follows: The BL→AP and AP→BL permeabilities in the presence of 10 μM CsA verapamil were $2.74 \pm 0.29 \times 10^{-5}$ and $3.19 \pm 0.42 \times 10^{-5}$ cm/s, respectively.

We regret this error and apologize for any confusion or inconvenience it may have caused.