Mechanism of Corneal Permeation of L-Valyl Ester of Acyclovir: Targeting the Oligopeptide Transporter on the Rabbit Cornea

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Purpose. To delineate mechanisms associated with the corneal transport of a L-valine prodrug of an antiviral agent, acyclovir.

Method. The permeability and enzymatic hydrolysis of L-Val-ACV were evaluated using freshly excised rabbit cornea. Transport mechanism across rabbit cornea was investigated through a competitive inhibition study of L-Val-ACV with other substrates of human peptide transporter (hPepT1).

Results. L-Valyl ester of Acyclovir (L-Val-ACV) was approximately threefold more permeable across the intact rabbit cornea than acyclovir (ACV). Dipeptides, β-lactam antibiotics, and angiotensin converting enzyme (ACE) inhibitors, strongly inhibited the transport of L-Val-ACV indicating that a carrier mediated transport system specific for peptides is primarily responsible for the corneal permeation of L-Val-ACV. L-Val-ACV transport was found to be saturable (K_m = 2.26 ± 0.34 mM, J_{max} = 1.087 ± 0.05 nmoles cm^{-2} min^{-1}), energy and pH dependent.

Conclusions. Functional evidence of an oligopeptide transport system present on the rabbit cornea has been established. The peptide transporter on the corneal epithelium may be targeted to improve the ocular bioavailability of poorly absorbed drugs.

KEY WORDS: corneal permeability; oligopeptide transporter; ACE inhibitors; β-lactam antibiotics; glycylsarcosine; glycylproline; acyclovir; valacyclovir.

INTRODUCTION

Infection with herpes simplex virus (HSV) is the single most frequent cause of corneal opacities in developed countries (1). Currently available therapy for HSV keratitis involves the use of trifluorothymidine, idoxuridine, and vidarabine. However, one of the major problems associated with these drugs is their poor ocular absorption and cytotoxicity restricting their use in long-term treatment. In superficial herpes keratitis, the efficacy of a 3% ophthalmic ointment of acyclovir (ACV) applied 5 times a day for up to 14 days has been reported (2). However, due to the various side effects associated with the use of ointments in the eye ACV has not been approved for clinical use in HSV keratitis in the United States. In addition, ACV ointment is not effective against stromal keratitis or when deeper ocular tissues are involved (3). Main constraints to topical ocular delivery of ACV in the treatment of HSV-1 keratitis include rapid precorneal elimination, conjunctival absorption, tear turnover, nasolacrimal drainage, and poor corneal permeability of ACV due to its hydrophilic nature (4). The cornea is composed of 5 to 6 layers of columnar epithelium with tight junctions presenting a barrier to the hydrophilic compounds, followed by the stroma, which contains more than 90% water and hence presents a barrier to hydrophobic compounds. To circumvent the problem of poor permeation of ACV, the effect of highly lipophilic ACV acyl ester prodrugs on the corneal permeation and regeneration of ACV has been studied (4). But, due to their enhanced lipophilicity these compounds are poorly soluble in water and hence their formulation into 1–3% eye drops is not feasible. For a compound to be effective topically and to be formulated into eye drops, it must possess sufficient hydrophilicity and at the same time exhibit sufficient permeability across the cornea to reach 3–5 times the minimum inhibitory concentration levels.

Amino acid ester prodrugs of nucleoside antiviral drugs have been employed recently to increase oral bioavailability of the parent drugs (5,6). Valacyclovir, L-Valyl-ACV (Fig. 1b) is such a prodrug, which is derived from acyclovir (ACV) (Fig. 1a) by esterifying L-valine to the hydroxyl group of ACV (7). Upon administration, valacyclovir (L-Val-ACV) is rapidly and completely converted to acyclovir, the active parent drug by enzymatic hydrolysis. The prodrug increases the oral bioavailability of acyclovir three- to fivefolds in humans (5–7). Enhanced oral absorption of acyclovir has been attributed to the human peptide transporter (hPEPT1) mediated transport of valacyclovir. Valacyclovir is recognized as a peptidyl derivative and absorbed by peptide transporters, even though there is no peptide bond in its structure (8–10).

Recently, a significant amount of work has been reported on substrate specificities of membrane transporters and receptors. (11–16). This knowledge can be utilized to target drugs to a particular transporter through prodrug design. Therefore, utilizing transporters present on the corneal epithelium may enhance corneal permeability of polar compounds. Very little is known about the presence of various transporters and receptors on the corneal epithelium. Several studies have been carried out to identify and characterize the presence of peptide transporters on various mammalian cell lines and their involvement in the transport of peptidyl substrates (11,12). The presence of an oligopeptide transport system on the corneal epithelium has been recently reported (17,18).

In the present study, we have investigated the mechanism of corneal permeation of the antiviral agent L-Val-ACV, which has been shown to be a substrate for the oligopeptide transport system in the intestinal epithelium (8–10,19). We have also delineated the transport mechanisms of various other substrates to determine the functional expression of a carrier system specific for the transport of peptidyl substrates across intact rabbit cornea.

MATERIALS AND METHODS

Materials

VACV was a gift from Glaxo Wellcome Inc, RTP, NC, USA. [^{3}H] glycylsarcosine (specific radioactivity 4 Ci/mmol) was obtained from Moravek Biochemicals, Brea, CA, USA. [^{14}C] mannitol was obtained from ICN Biochemicals, Inc., Irvine, CA, USA. All other chemicals were obtained from

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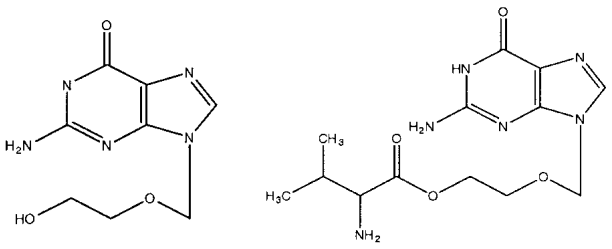

(a) (b)

Fig. 1. Structure of (a) Acyclovir and (b) L-Val-ACV.

Sigma Chemical Company (St. Louis, MO, USA). The solvents were of analytical grade and obtained from Fisher Scientific (St. Louis, MO, USA).

Animals

Adult male New Zealand albino rabbits weighing between 2–2.5 kg were obtained from Myrtle's Rabbitry, TN, USA. This research was conducted strictly according to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Prodrug Stability in Transport Medium

The transport buffer Delbecco's phosphate buffer saline (DPBS) was prepared at different pH values (5–7.4). Stock solution of the prodrug (1 mM) was prepared in DPBS buffer and used immediately. Aliquots (9.8 ml) of the buffer were placed in screw-capped vials and allowed to equilibrate at 34°C. Prodrug stock solution (0.2 ml) was subsequently added to the buffer. The vials were placed in a constant shaker bath set at 34°C and 60 rpm. Samples (0.2 ml) were collected at appropriate time intervals for up to 36 h. The samples were immediately stored at –80°C until further analysis. All experiments were conducted at least in triplicate.

Corneal Tissue Hydrolysis

The corneal tissue hydrolysis was carried out as described previously (20). The method is described briefly as follows.

Preparation of Corneal Tissue

New Zealand albino male rabbits were used for this study. Animals were euthanized by a lethal injection of sodium pentobarbital through the marginal ear vein. Each eye was immediately enucleated and the ocular surface was rinsed with ice cold pH 7.4 isotonic phosphate buffer saline (IPBS) to remove any trace of blood. After a small incision to the sclera, vitreous humor was aspirated using a 1 ml tuberculin syringe. The cornea, lens, and iris cilary body were sequentially removed after cutting along the scleral-limbus junction. The corneal tissue was homogenized in 5 ml chilled (4°C) IPBS for about 4 min with a tissue homogenizer (Tissue Tearor Model 985-370) in an ice bath. The homogenate was centrifuged at 12,500 rpm for 25 min at 4°C to remove cellular debris, and the supernatant was used for hydrolysis studies. Protein content of each supernatant was determined with a BioRad assay using bovine serum albumin as the standard.

Hydrolysis Procedure

The supernatant was equilibrated at 34°C for about 30 min prior to an experiment. Hydrolysis was initiated by the addition of 0.2 ml of a 1 mM prodrug solution to 0.8 ml of the supernatant. The control consisted of 0.8 ml of IPBS instead of the supernatant. Aliquots (50 μl) were withdrawn at appropriate time intervals for up to 24 h. The samples were immediately diluted with 50 μl chilled methanol to quench the reaction and stored at –80°C until further analysis. Subsequently, these were thawed and centrifuged at 10,000 rpm for 10 min prior to analysis by HPLC for the intact ester prodrug and the regenerated parent ACV. Apparent first order rate constants were calculated and corrected for any chemical hydrolysis observed with the control.

Corneal Permeation Studies

Permeation of ACV, its ester prodrug L-Val-ACV and cephalexin was studied using side-bi-side™ diffusion cells (type VSC-1, Crown Glass Company, Inc., Somerville, NJ, USA). New Zealand albino rabbits were used for determination of *in vitro* corneal permeability. The rabbits were euthanized by injecting an overdose of pentobarbital into the marginal ear vein. The eyes were proptosed and immediately enucleated followed by washing with ice-cold Dulbecco's Phosphate Buffer Saline (DPBS) to remove any trace of blood. The cornea was removed in a similar fashion as described in the preparation of ocular tissues section previously, except that some scleral portion was left adhered to the cornea. The scleral part attached to the cornea helped to hold the membrane in place between the half cells during the course of a transport study (21). After removal, the cornea was washed with ice cold DPBS and mounted on the side-bi-side diffusion half cell with the epithelial side facing the donor chamber. Temperature of the half cells was maintained at 34°C by circulating water through the jacketed chambers of the diffusion apparatus.

DPBS (3.2 ml) was added to the receiver chamber and the other half chamber (donor chamber) was filled with appropriate drug or prodrug solutions (3 ml). A slight excess volume in the receiver chamber helped to maintain the corneal shape by a marginally elevated hydrostatic pressure. The contents of both the chambers were stirred continuously with magnetic stirrers. Aliquots (100 μl) were removed from the receptor chamber at appropriate time points over a 3 h period and were replaced with an equal volume of DPBS. The samples were stored at –80°C until further HPLC analysis. Control experiments using [^{14}C] mannitol, a paracellular marker, were carried out in a similar way to assess corneal integrity during the course of the experiment.

Competitive Inhibition Studies

Transport of L-Val-ACV (1 mM) was studied in presence of various dipeptide substrates (10 mM) as inhibitors. Similar inhibition studies were also conducted with cephalexin and glycylsarcosine to establish the substrate specificity of the peptide transporter.

Concentration Dependent Transport of L-Val-ACV

Concentration dependent transport of L-Val-ACV was determined with varying concentrations (0.25–20 mM) of the compound and Michaelis Menten parameters K_m and J_{max} were calculated.

Transport of VACV as a Function of pH

The pH effect on the interaction of L-Val-ACV (1 mM) with the peptide transporter was also characterized. The buffer used was DPBS and the pH range studied was within 5–7.4.

Energy Dependent Transport of L-Val-ACV

Inhibitor of Na^+/K^+-ATPase (ouabain –5 mM) was used to further characterize the transport process. In these experiments the corneal membrane was pre-incubated with the inhibitor for 30 min before the initiation of transport study. Because ouabain has been known to be toxic, the integrity and viability of the corneal tissue was tested. Transport of [^{14}C] mannitol in presence of 5 mM ouabain was carried out to test the membrane integrity. Viability of the tissue in presence of 5 mM ouabain was determined by carrying out cytotoxicity assay. For this assay CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA) was used. This assay is a colorimetric method for quantitatively determining lactate dehydrogenase, a stable cytosolic enzyme that is released upon cell lysis.

Analytical Procedures

All samples were assayed using HPLC. The system comprised of a Rainin Dynamax Pump SD-200 and a Rainin Dynamax UV Detector UV-C at 254 nm. The column used was a C18 Luna column 4.6 × 250 mm (Phenomenex). The mobile phase consisted of a mixture of buffer and an organic modifier. The percentage of organic phase was varied to elute the compounds of interest. This method gave rapid and reproducible results. HPLC conditions for the various compounds have been summarized in Table I.

The HPLC conditions of L-Val-ACV have been reported (22), and it was found that the metabolites of nucleosides did not interfere with the retention times of the prodrug and the parent drug, acyclovir.

Steady State Flux and Permeability Measurements across Intact Rabbit Cornea

Steady state fluxes (SSF) were determined from the slope of the cumulative amount of drug transported vs. time graph and expressed per unit of corneal surface area as described by Eq. (1). The cumulative amount of drug transported is the sum of the receptor cell prodrug and the regenerated drug:

$$\text{Flux } (J) = (dM/dt)/A \quad (1)$$

where M is the cumulative amount of drug transported and A is the corneal surface area exposed to permeant. Corneal membrane permeabilities (CMP) are determined by normalizing the SSF to the donor concentration, C_d according to Eq. (2).

$$\text{Permeability } (P_{app}) = \text{Flux}/C_d \quad (2)$$

Determination of K_m and V_{max}

The transport data for L-Val-ACV was fitted to the classical Michaelis Menten equation:

$$J = \frac{J_{max} * C}{K_m + C} \quad (3)$$

where J_{max} is the maximum rate of drug transport and K_m is the permeant concentration where half the maximal rate is reached. K_m and J_{max} of L-Val-ACV permeation across cornea were determined using a nonlinear least square regression analysis program (KaleidaGraph V3.09). The quality of the fit was determined by evaluating the coefficient of determination (r^2), the standard error of parameter estimates, and by visual inspection of the residuals.

Statistical Analysis

All experiments were conducted at least in triplicate and the results are expressed as mean ±SD except in the case of Michaelis Menten parameters K_m and V_{max} where the values are presented as mean ±SE. Student's t test was used to detect statistical significance and $p < 0.05$ was considered to be statistically significant.

RESULTS

Stability in Transport Buffer

The stability of the L-valyl ester prodrug of ACV was determined both in the transport buffer, DPBS (pH 5–7.4)

Table I. HPLC Assay Conditions and Retention Times for the Drugs

Drug	Composition of aqueous phase (pH = 2.5)	Composition of organic phase	Mobile phase Aq: Org	Retention times (min.)[b] Parent Drug	Prodrug
ACV	25 mM $NH_4H_2PO_4$	Acetonitrile	98:2	10.0	—
Val-ACV	25 mM $NH_4H_2PO_4$	Acetonitrile	95:5	5.3	9.7
Cephalexin	20 mM KH_2PO_4[a]	Methanol	72:28	7.4	—

[a] pH = 4.4.
[b] UV detection at λmax = 254 nm.

and IPBS (pH 7.4) for a period of 36 h (Table II and Fig. 2A). The pH of maximal stability within the range of pH studied was around pH 5.0. The $t_{1/2}$ ranged from 17–105 h indicating that the chemical hydrolysis may cause regeneration of the parent drug during the course of a transport experiment (Fig. 2A, inset). Therefore, for these studies the total amount of transport across cornea was determined by adding the amount of parent drugs generated from prodrugs to the amount of intact prodrug transported across the cornea since the drug in the receptor phase must have originated from already permeated prodrug.

Corneal Tissue Hydrolysis

Enzymatic hydrolysis of the prodrug was assessed using the corneal tissue. The L-valyl ester prodrug of ACV was readily converted to the active parent drug, ACV (Fig. 2B). The half life ($t_{1/2}$) was observed to be 85 min indicating that the major contribution to the regeneration of the parent drug ACV during the course of a transport study (3 h) was due to enzymatic hydrolysis. Therefore, the total amount of drug permeating through the corneal membrane was taken as the sum of the prodrug and the regenerated parent drug.

Corneal Permeation of ACV and L-Val-ACV

A cumulative amount of drug transported (the sum of the prodrug and the regenerated parent drug) was plotted as a function of time (Fig. 3A). Apparent permeabilities (P_{app}) of ACV and L-Val-ACV across rabbit cornea were determined from the linear portion of the time vs. the cumulative amount (moles) plot (Fig. 3, inset). The estimated P_{app} values for ACV and L-Val-ACV were found to be $4.24 \pm 1.41 \times 10^{-6}$ cm s^{-1} and $1.20 \pm 0.044 \times 10^{-5}$ cm s^{-1}, respectively. L-Val-ACV was approximately three times more permeable across the corneal epithelium than ACV (Fig. 3B).

Inhibition of Glycylsarcosine and Cephalexin Transport

Small dipeptides and β-lactam antibiotics have been shown to be substrates for the dipeptide transporters (14,23,24). Glycylsarcosine, one of the well-known nonhydrolyzable peptide substrates was used to characterize the substrate specificity of the peptide transporter on the rabbit cornea. A significant inhibition ($p < 0.05$) in the transport of [^{3}H] glycylsarcosine across excised cornea (Fig. 4A) in the presence of 10 mM glycylsarcosine was observed. Moreover the transport of cephalexin across cornea was inhibited (approximately 85%) in the presence of glycylsarcosine and glycylproline (Fig. 4B), indicating the presence of a peptide carrier mediated transport mechanism on the corneal epithelium.

Table II. First Order Rate Constants of L-Val-ACV in Buffers at Different pH

Buffer	Rate constant (k* 10^2 h^{-1})	Half life (hr)
DPBS 5.0	[a]	—
DPBS 6.0	0.66 ± 0.079	105
DPBS 7.4	3.80 ± 0.34	18
IPBS 7.4	4.07 ± 0.21	17

[a] No measurable degradation during the period of a 36 h experiment.

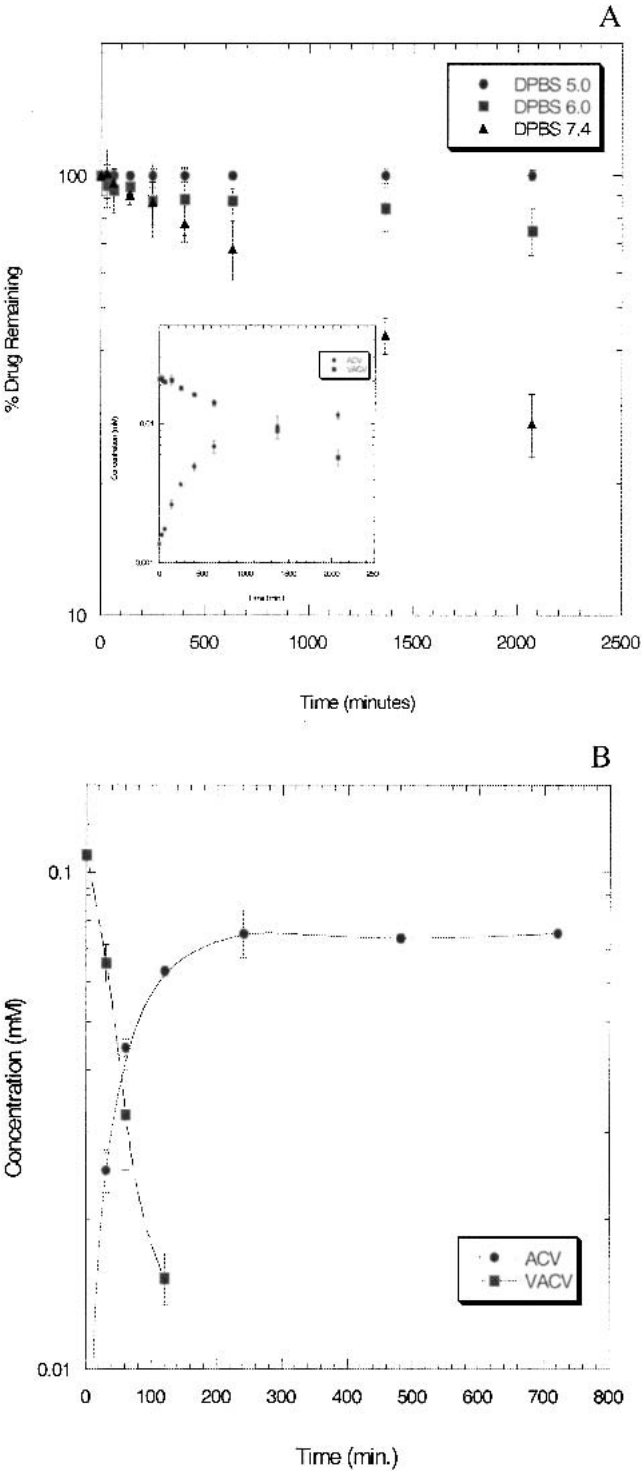

Fig. 2. (A) First order chemical hydrolysis of L-Val-ACV in DPBS buffer: (●) pH 5.0; (■) 6.0; (▲) 7.4). Inset, regeneration of ACV from L-Val-ACV upon chemical hydrolysis (DPBS, pH 7.4); (■) L-Val-ACV; (●) ACV. (B) Regeneration of ACV from L-Val-ACV upon hydrolysis in isolated rabbit cornea. (■) L-Val-ACV; (●) regenerated parent drug ACV. (Mean ± SD, n = 3).

Competitive Inhibition of L-Val-ACV Transport

Inhibition of L-Val-ACV transport was studied in the presence of several inhibitors to further characterize the transport mechanism. Dipeptides (gly-sar, gly-pro), ACE in-

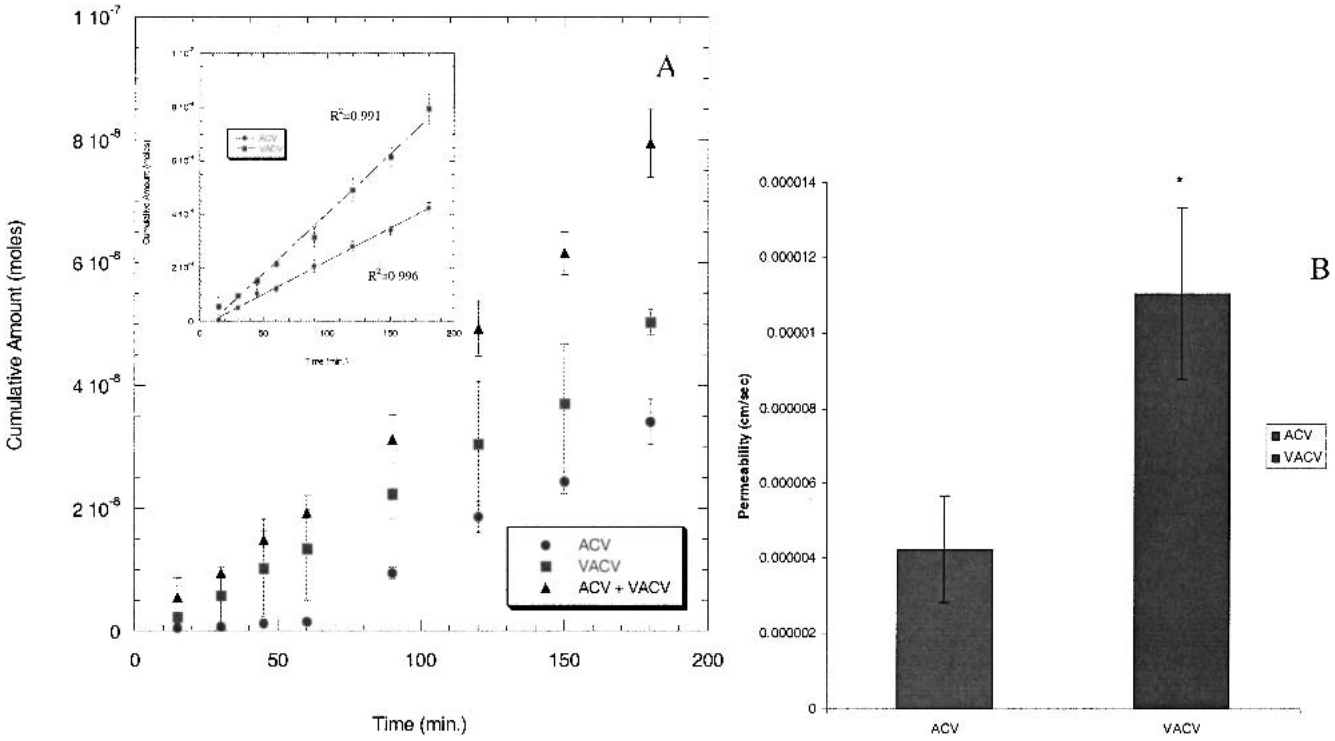

Fig. 3. (A) Permeation profile of the prodrug L-Val-ACV across isolated rabbit cornea as a function of time: (●) regenerated parent drug-ACV and the (■) L-Val-ACV; (▲) cumulative amount of L-Val-ACV. Inset, time course of transport of cumulative amount of (●) ACV ($R^2 = 0.996$) and (■) L-Val-ACV ($R^2 = 0.991$) across rabbit cornea. (B) Permeability of ACV and L-Val-ACV across isolated rabbit cornea. (Mean ± SD, n = 4). * Represents $p < 0.05$.

hibitors (enalapril, captopril), and β-lactam antibiotics (cephalexin, cephazolin) all of which are known to be substrates for PEPT1 transporter, significantly ($p < 0.05$) reduced the transport of L-Val-ACV across rabbit cornea (Fig. 5A). The transport of L-Val-ACV was almost completely inhibited (95%) in the presence of 10 mM glycylsarcosine (Fig. 5B), suggesting that all the significant transport activity was probably PEPT1 mediated.

Concentration Dependent Transport of L-Val-ACV

The transport of L-Val-ACV across the corneal epithelium was found to be concentration dependent and saturable

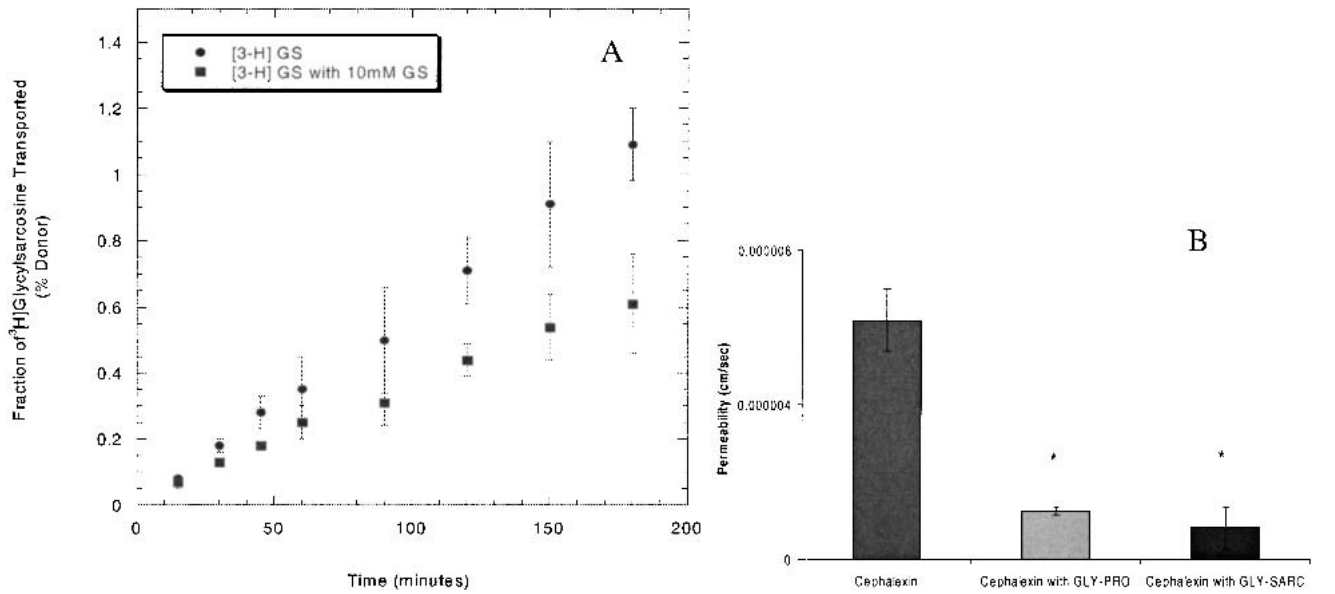

Fig. 4. (A) Inhibition of [^{3}H] Glycylsarcosine transport (0.5 μCi/ml) across freshly excised rabbit cornea: (●) [^{3}H] GS (12.5 μM); (■) [^{3}H] GS (12.5 μM) in presence of glycylsarcosine (10 mM). (B) Inhibitory effect of various dipeptides on the permeability of cephalexin. (Mean ± SD, n = 4). * Represents $p < 0.05$.

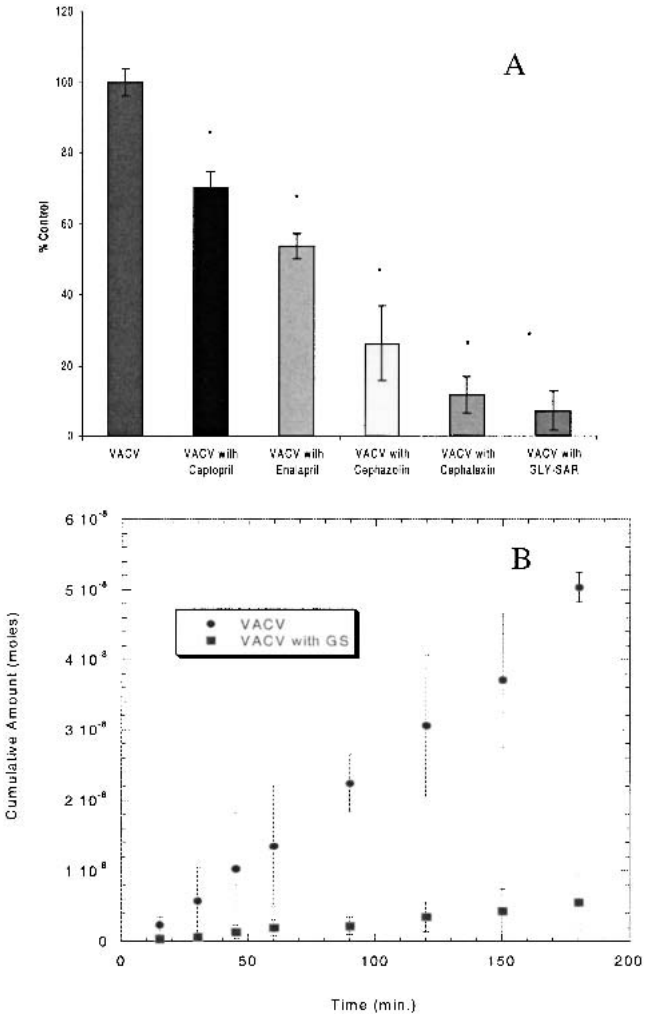

Fig. 5. (A) Inhibitory effect of 10 mM dipeptide and ACE inhibitors on the permeability of 1 mM L-Val-ACV. * Represents $p < 0.05$. (B) L-Val-ACV transport across rabbit cornea as a function of time: (●) L-Val-ACV; (■) L-Val-ACV in presence of 10 mM glycylsarcosine. Almost complete inhibition (95%) of L-Val-ACV transport in presence of glycylsarcosine was noticed, indicating that the major component is the saturable one and the linear component has negligible contribution to the overall transport of L-Val-ACV. (Mean ± SD, n = 4).

at higher concentrations (Fig. 6). The measurements were done at concentrations in the range of 0.25–20 mM L-Val-ACV. Using the KaleidaGraph program, the Michaelis Menten constant and the maximum velocity values for L-Val-ACV transport were determined as 2.26 ± 0.34 mM and 1.087 ± 0.05 nmoles cm^{-2} min^{-1}, respectively. Transformation of the data from the transport of L-Val-ACV resulted in a Woolf-Augustinsson-Hofstee plot ($R^2 = 0.934$) (Fig. 6, inset). The kinetics of L-Val-ACV transport matched a single, saturable carrier model.

Transport of L-Val-ACV as a Function of pH

Transport of L-Val-ACV was measured at variable pH's and the pH of maximum transport was found to be 7.4 (Fig. 7A). Transport of L-Val-ACV at pH 7.4 was approximately 10-fold higher than that at pH 5.0 and approximately 3-fold

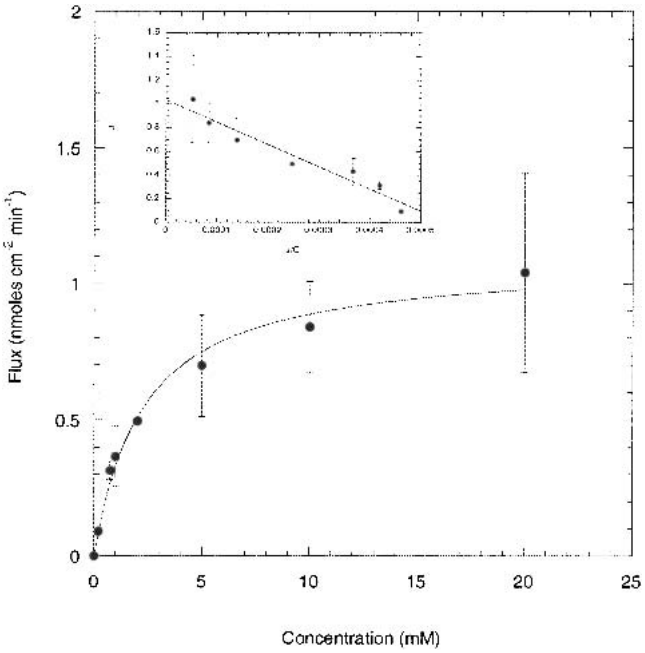

Fig. 6. Concentration dependent transport of L-Val-ACV across isolated rabbit cornea ($R^2 = 0.987$). Inset, Woolf-Augustinsson-Hofstee linear transformation ($R^2 = 0.934$) of the data [fluxes of L-Val-ACV, J (nmoles cm^{-2} min^{-1}) vs. fluxes of L-Val-ACV/concentration, J/S (μl cm^{-2} min^{-1})]. Values are mean ± SD (n = 4–6).

higher than that at pH 6.0. Compared to the prototypical PEPT1 substrate, glycylsarcosine, the transport of L-Val-ACV was different as a function of pH. Glycylsarcosine exhibited maximal uptake at pH 5.5, which was proton dependent (22).

Energy Dependent Transport of L-Val-ACV

Transport of small peptides via the oligopeptide transporter is energy dependent involving the transmembrane electrochemical H^+ gradient (25). The transport of L-Val-ACV was significantly inhibited, in presence of ouabain (Fig. 7B), which is a Na^+-K^+-ATPase inhibitor.

DISCUSSION

The oligopeptide transporters display broad substrate specificity. Dipeptides and tripeptides, but not free amino acids, are the primary substrates. These transporters not only carry nutrients across absorptive cell membranes but also function in the transport of exogenous compounds that have peptide like structures. Small dipeptides, ACE inhibitors, β-Lactam antibiotics are known substrates for intestinal PEPT1 and renal PEPT2. However, differential recognitions of these drugs by these two transporters have been observed (26). A wide range of substrates and peptidomimetics make the peptide transporters a versatile and robust target for transport of drugs and prodrugs. Results from this study aim at identifying a peptide transport mechanism for L-Val-ACV and various other peptidyl substrates across the rabbit cornea.

Prodrugs are designed to overcome the undesirable properties of drugs and prodrug is itself biologically inactive. Therefore it is essential for these prodrugs to yield the active parent drug upon administration to have drug efficacy as soon

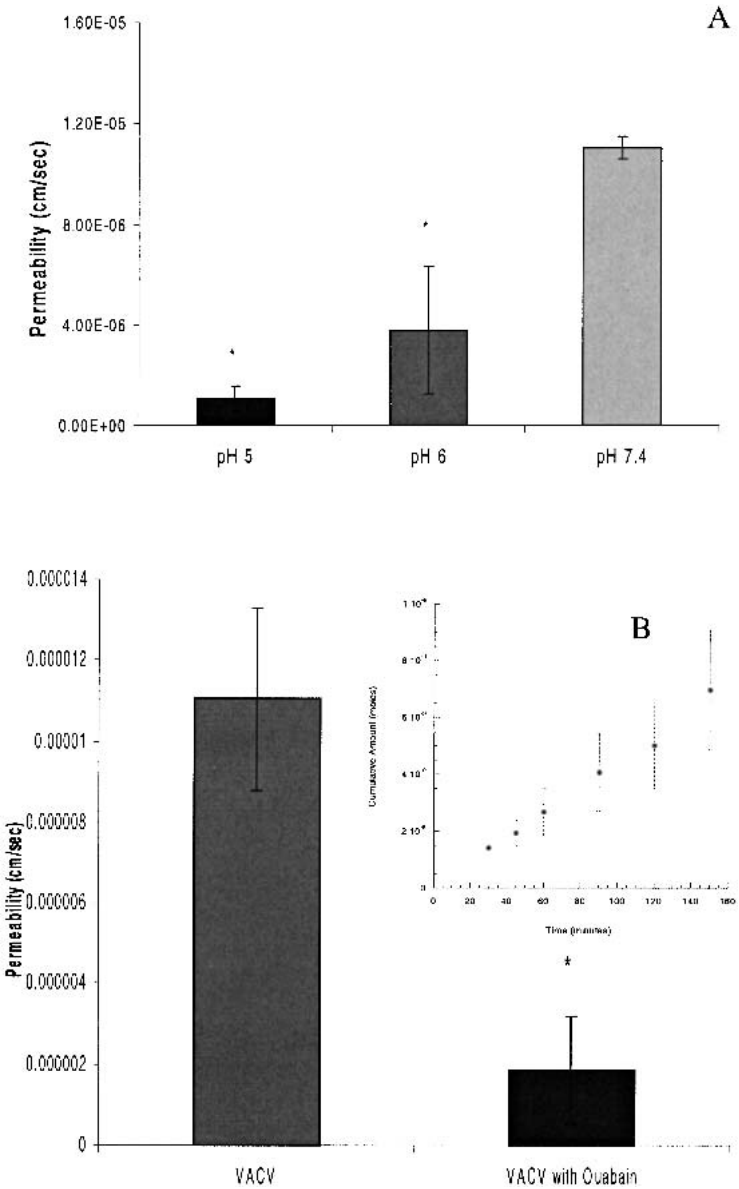

Fig. 7. Transport of L-Val-ACV across rabbit cornea (A) as a function of pH and (B) in presence of 5 mM ouabain. Inset time course of transport of cumulative amount of (●) L-Val-ACV ($R^2 = 0.99$) in presence of 5 mM ouabain. (Mean ± SD, n = 4). * Represents $p < 0.05$.

as their goals are achieved. In our tissue hydrolysis studies using rabbit cornea we demonstrated that L-Val-ACV was hydrolyzed to the parent drug ACV (Fig. 2B). Therefore, the amino acid prodrug of ACV is recognized by the ocular esterases and is cleaved to the parent drug, upon hydrolysis.

L-Valyl ester of antiviral nucleoside ACV demonstrates a three- to fivefold higher oral bioavailability than ACV. This increased bioavailability has been attributed to the carrier-mediated absorption of L-Val-ACV, which is recognized as a PEPT1 substrate (9,10,19). Results from our study indicate the permeability of L-Val-ACV was approximately threefold higher than that of ACV (Fig. 3B). The major barrier to the transport of drugs across the cornea is the lipoidal epithelium. The aqueous solubility of L-Val-ACV has been reported to be 174 mg/ml (27) in comparison to 2.5 mg/ml for Acyclovir (28). Higher permeability of L-Val-ACV than ACV suggested the involvement of a carrier mediated transport mechanism for the L-valyl ester prodrug of ACV. To establish the identity of the carrier mediated transport mechanism, glycylsarcosine and glycylproline were employed as prototypical peptide substrates to inhibit the transport of [^{3}H] glycylsarcosine (Fig. 4A) and cephalexin (Fig. 4B). A significant inhibition of transport of both glycylsarcosine and cephalexin indicated the presence of a carrier mediated transport mechanism specific for peptides on the rabbit cornea. Control experiments with [^{14}C] mannitol suggested that the corneal membrane integrity was maintained throughout the course of the experiment for all the transport experiments. The percent transport per hour of [^{14}C] mannitol in presence of different drugs was found to be: 0.32% (control); 0.27% (1 mM VACV); 0.31% (20 mM VACV); 0.25% (10 mM glycylsarcosine); 0.30% (10 mM glycylproline); 0.26% (10 mM capto-

pril); 0.30% (10 mM enalapril); 0.26% (10 mM cephalexin); 0.29% (10 mM cephazolin).

Functional characterization of the peptide transporter on the rabbit cornea was further carried out. In the competitive inhibition studies, substrates of a peptide transporter, such as small dipeptides- glycylsarcosine and glycylproline, β-lactam antibiotics- cephalexin and cephazolin, and ACE inhibitors-enalapril and captopril resulted in competitive inhibition of L-Val-ACV transport (Fig. 5A). These results strongly suggest that the peptide carrier is involved in the membrane transport of L-Val-ACV, although the parent drug ACV is transported by passive diffusion (29,30). Interestingly the two β-lactam antibiotics (cephalexin and cephazolin) exhibited very high levels of inhibition: approximately 75% inhibition compared with the control. Cephalexin differs from cephazolin by an α-amino group, which is known to render high specificity for PEPT2 in comparison to non-α-amino cephalosporins (31). Therefore, a significant inhibition by both antibiotics strongly indicates the presence of a peptide transporter on the rabbit cornea. Almost complete inhibition (95%) of L-Val-ACV transport in presence of glycylsarcosine (Fig. 5B), indicated that the process comprised primarily of the saturable component (carrier mediated process). Concentration dependent studies (Fig. 6) demonstrated a carrier mediated transport of L-Val-ACV with a low affinity (K_m = 2.26 ± 0.34 mM), which is consistent with the previously published affinities for PEPT1 (8,9). These findings are consistent with a report on the presence of PepT1 steady-state mRNA in the corneal epithelial cells (18). However, the involvement of renal oligopeptide transporter cannot be ruled out because L-Val-ACV has been reported to be a substrate of PEPT2 (24).

Cellular transport of L-Val-ACV was observed to be pH dependent. The human intestinal peptide transporter has been reported as a H^+-dependent transporter (32). However, L-Val-ACV transport as a function of pH was observed to be different from that of a prototypical dipeptide substrate, glycylsarcosine. Differential effect of pH on the transport of Gly-Sar and L-Val-ACV in hPEPT1-transfected CHO cells has been previously reported (19). Influence of luminal pH on the transport of positively and negatively charged dipeptides using an intact preparation of rat small intestine showed that the transport of neutral and negatively charged dipeptides was stimulated by lowering the pH to 6.8, whereas increasing the pH to 8.0 strongly stimulated the transport of positively charged dipeptide but inhibited that of negatively charged dipeptide (33). The pH of maximal transport of L-Val-ACV was found to be 7.4 (Fig. 7A), whereas the pH of maximal uptake for glycylsarcosine has been reported as 5.5 (19). L-Val-ACV has three pK_a values i.e., 1.90, 7.47, and 9.43. Based on these values, it appears that the molecule can exist in solution as four different species: dicationic, cationic, anionic, and neutral (34). L-Val-ACV would predominantly exist as a cationic moiety at lower pH (6 or lower). As the pH is raised above 6.0, the fraction of cationic species becomes progressively less, reaching almost neutrality as the extracellular pH reaches 8.0. In the present study the maximal transport of L-Val-ACV occurs at 7.4, owing to the existence of a mixture of cationic and neutral species at that pH. The present study results are consistent with reports of interactions of positively charged dipeptides with PEPT1 (33). Transport of L-Val-ACV in presence of ouabain is significantly reduced (85%) (Fig. 7B). The permeation profile of L-Val-ACV in presence of 5 mM ouabain followed a linear trend (Fig. 7B inset) with no change in slopes suggesting that the corneal integrity was maintained throughout the experiment. Percent transport per hour of [^{14}C] mannitol in presence of 5 mM ouabain (0.32% as compared to 0.34% of control) also suggested that the corneal membrane integrity was maintained in presence of 5 mM ouabain. The tissue viability at the end of the experiment was found to be 98.3 ± 2.5% of the control (no ouabain), suggesting that the inhibition of L-Val-ACV transport in presence of 5 mM ouabain was a result of the inhibition of the Na^+/K^+ ATPase pump and not due to any other factors such as loss of integrity or viability of the tissue.

The inhibitory effect of ouabain can be explained on the basis of the transmembrane H^+ gradient essential for transport of peptides across membranes. A significant H^+ electrochemical gradient across the intestinal membrane is required to provide the proton driving force for uphill transport of small peptides via PEPT1 into enterocytes. This process is governed by the difference in pH between the unstirred brush border aqueous layer (pH 5.5–6.0) and the intracellular pH of intestinal epithelial cells (pH 7.0) (35). The luminal pH in the unstirred layer is established by the brush border Na^+/H^+ exchanger, which catalyzes the influx of Na^+ from the lumen into the cell in exchange for the H^+ efflux. The inwardly directed Na^+ gradient that is required to drive this process is generated by the action of Na^+/K^+ ATPase located on the basolateral membrane (36). Ouabain being a Na^+/K^+ ATPase inhibitor hindered the exchange and thus decreased the permeability of L-Val-ACV across cornea.

In conclusion, the results of the present study provide functional evidence for the presence of an oligopeptide transport system on the intact cornea. This study describes a carrier mediated transport mechanism for a non-peptidic drug L-Val-ACV, a prodrug of ACV, across freshly excised rabbit cornea. Transport of L-Val-ACV was found to be higher than that of the parent drug ACV, which is saturable at higher concentrations, pH dependent, and inhibited competitively by other known hPEPT1 substrates. Strategies such as targeting of a specific molecule towards a transporter/receptor by chemical modification may be employed to improve tissue bioavailability. The above findings are valuable in that they can serve the purpose of increasing the bioavailability of topically applied ocular drugs by designing peptidomimetics to target the oligopeptide transporter on the cornea.

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