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Identification of a Na^+-Dependent Cationic and Neutral Amino Acid Transporter, $B^{0,+}$, in Human and Rabbit Cornea

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Abstract: The purpose of this study was to identify and functionally characterize an active transport system for L-arginine on rabbit corneal epithelium and human cornea and study its interaction with the amino acid ester prodrugs of acyclovir (Anand, B. S.; Mitra, A. K. *Pharm. Res.* **2002**, *19*, 1194−1202). Transport characteristics of [^{3}H]-L-arginine across freshly excised rabbit corneas were determined at various concentrations, in the absence of sodium and chloride ions. Inhibition studies were conducted in the presence of other amino acids, ouabain, and amino acid ester prodrugs of acyclovir (glycine-ACV, phenylalanine-ACV and γ-glutamate-ACV). Reverse transcription-polymerase chain reaction (RT-PCR) for amino acid transporter $B^{0,+}$ was carried out on total RNA isolated from rabbit cornea, rabbit corneal epithelium, and human cornea. Transport of L-Arg across rabbit cornea was saturable ($K_m = 306 \pm 72$ μM and $V_{max} = 0.12 \pm 0.01$ nmol min^{-1} cm^{-2}) and was Na^+, Cl^-, and energy dependent. Transport was inhibited by neutral and cationic amino acids and a $B^{0,+}$ system specific inhibitor, BCH (Sloan, J. L.; Mager, S. *J. Biol. Chem.* **1999**, *274*, 23740−23745), but not by anionic amino acids. Amino acid prodrugs of ACV (Glu-ACV and Phe-ACV) also inhibited transport of [^{3}H]-L-Arg across rabbit cornea. Amino acid transporter $B^{0,+}$ was identified by RT-PCR and its identity confirmed by subcloning and sequencing in rabbit cornea, rabbit corneal epithelium, and human cornea. A Na^+-, Cl^--, and energy-dependent carrier for L-Arg, $B^{0,+}$, was identified on rabbit corneal epithelium and human cornea. Glu-ACV and Phe-ACV appear to be substrates for this transporter. The presence of such transporters on the corneal epithelium may provide new opportunities for transporter-targeted prodrug design for enhanced corneal absorption.

Keywords: Amino acid transport; system $B^{0,+}$; arginine; cornea; rabbit; prodrugs

1. Introduction

Amino acids require specialized transport systems to cross the plasma membrane.[3] In mammalian cells multiple systems operate to mediate the transport of amino acids, and these transport systems differ markedly in their substrate specificity, substrate affinity, driving forces, and tissue expression pattern.[4] Numerous amino acid transport systems have been characterized at the molecular level including L, y^+L, A, ASC, asc, $b^{0,+}$, $B^{0,+}$ and x^-, Gly, N, and T.[5−8] One such

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transport system, designated $B^{0,+}$, is defined by Na^+-dependent transport of both neutral and cationic amino acids.[9] System-$B^{0,+}$ transport has been reported in mouse blastocysts, *Xenopus* oocytes, a human intestinal cell line, rabbit small intestine, rabbit conjunctiva, rat pituitary gland, bullfrog lung, and human lung.[9−17]

The conjunctiva has been reported to express a large number of transporter proteins including amino acid transporters, monocarboxylic acid transporter (MCT), and nucleoside transporter which are believed to play a role in the absorption of drugs through conjunctiva following topical administration.[13,18,19] The corneal epithelium is composed of five to six layers of columnar epithelial cells. Its barrier properties arise from the high electrical resistance of both the outermost cell membranes and the zonulae occludens, which restrict the paracellular movement of molecules across these layers. Delivery of hydrophilic compounds, like acyclovir,[1] to the deeper corneal layers is a major challenge in ocular therapeutics.[20] There is limited information on the mechanisms of amino acid transport across corneal epithelium, the primary barrier for ocular drug absorption. Prodrug design aimed at increasing drug permeability via carrier-mediated transport mechanisms is a promising strategy that has been described previously.[21,22] The oligopeptide and large neutral amino acid[5] transport systems have been identified for the first time on the corneal epithelium in our laboratory.[1] Valacyclovir (VACV), the valine ester of ACV, was found to utilize the oligopeptide transport system present on the cornea for enhanced corneal permeation.[1] Thus, in order to enhance the corneal permeability of polar compounds a strategy can be adopted to utilize the transporters present on the corneal epithelium. In view of the high protein synthesis rate in the corneal epithelium, an integral part of continuous replacement of cells lost by desquamation, it is necessary to understand the processes by which amino acids are presented at the synthetic sites.[23] L-Arginine is present at a concentration of about 18 μM in human tear fluid.[18] It is a cationic, dibasic, semiessential amino acid with numerous roles in cellular metabolism. It serves as an intermediate in the urea cycle and a precursor for polyamine biosynthesis, and has both immunomodulatory and secretory activities.[22]

Infection with herpes simplex virus type 1 (HSV-1) causes corneal and stromal keratitis leading to blindness.[24] ACV, a guanosine analogue, provides effective treatment in superficial herpes keratitis.[25] However, ACV has poor permeation characteristics across the corneal epithelium. Prodrug design is an effective strategy to overcome the problem of poor permeation across biological membranes. The active nutrient transport systems have become key targets for prodrug

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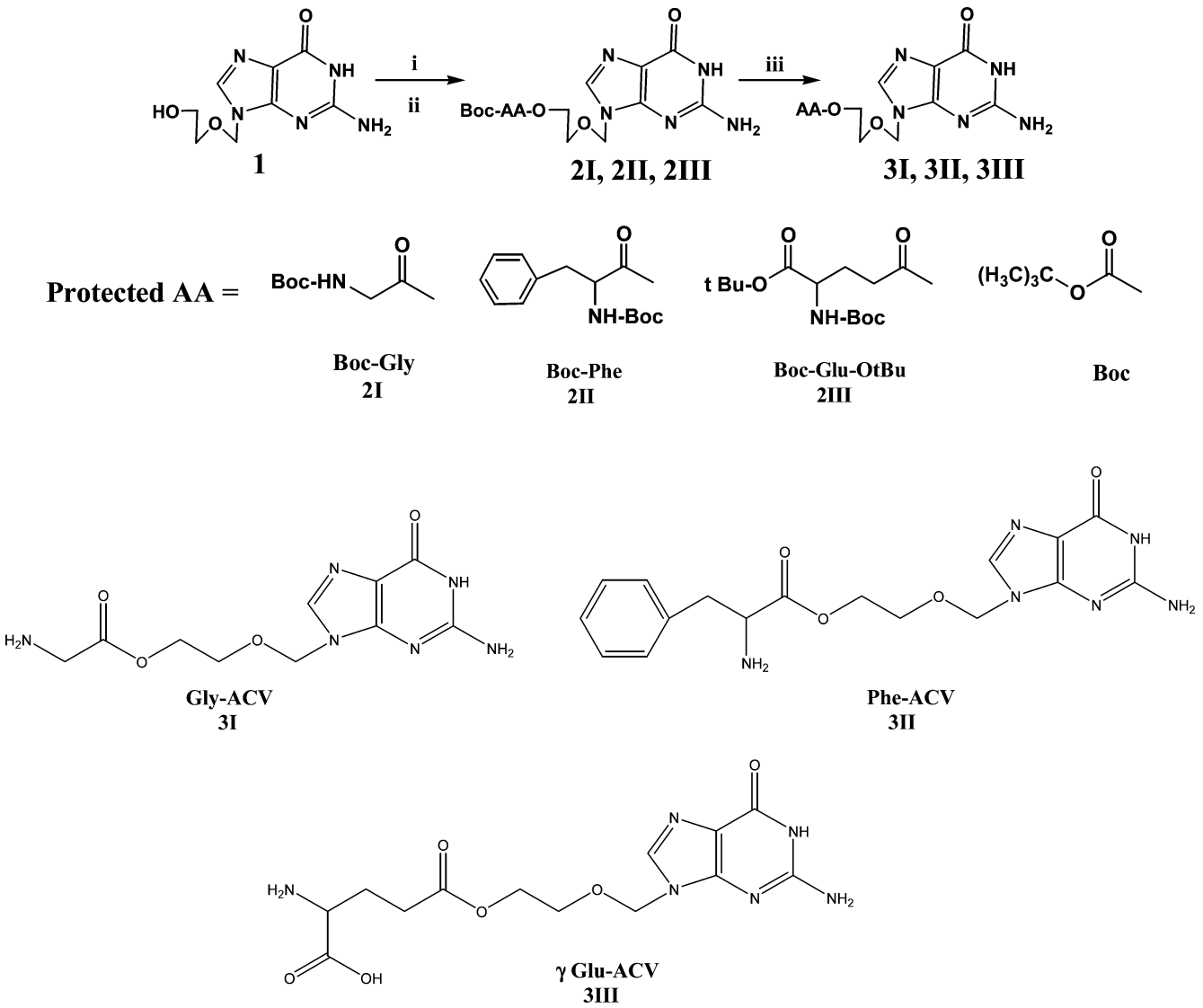

Figure 1. Steps involved in the synthesis of amino acid ester prodrugs of acyclovir: (i) Boc-AA, DCC, DMF, 0 °C, 1 h; (ii) DMAP, DMF, 24 h, rt; (iii) TFA, 0 °C, 30 min.

design. A few amino acid transporters have been utilized to improve the absorption of amino acid conjugated drugs. For example, phosphonoformate−L-tyrosine conjugate has been demonstrated to be actively transported through monolayers of porcine brain microvessel endothelial cells by active amino acid transporters.[26] The objectives of this study are (i) to examine the presence of any amino acid transport system for the permeation of L-Arg across rabbit cornea, (ii) to identify the transporter at the molecular level, and (iii) to study the feasibility of enhancing corneal drug absorption, using a prodrug approach, targeting this amino acid transporter. Three amino acid ester prodrugs of ACV, Gly-ACV, Phe-ACV, and γ-Glu-ACV, are synthesized and used as model prodrugs in these studies.

2. Experimental Section

2.1. Animals. New Zealand albino adult male rabbits weighing between 2 and 2.5 kg were obtained from Myrtle's Rabbitry, Thompson Station, TN. Studies involving these rabbits were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Synthesis of Gly-ACV, Phe-ACV, and γ-Glu-ACV. Synthesis of these amino acid ester prodrugs was carried out as depicted in Figure 1 and involved[1] (i) formation of N-protected amino acid anhydrides, (ii) coupling of the N-protected amino acid anhydride with ACV, and finally (iii) deprotection of the amino group of the amino acid ester of ACV. Synthesis of γ-Glu-ACV required not only the N-protection of the glutamic acid but also the protection of α-carboxyl group. A mixture of N-protected amino acid (Boc-AA) and dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF) at a ratio of 1:2 DCC/Boc-AA was stirred for 1 h at 0 °C under a nitrogen atmosphere. A

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Table 1. HPLC Assay Conditions and Retention Times for the Drugs

prodrug	mobile phase aq[a]:org[b]	retention times (min)	
		parent drug	amino acid prodrug
Gly-ACV	98:2	9.2	5.3
Phe-ACV[c]	88:12	3.0	7.1
γ-Glu-ACV	98:2	8.8	12.0

[a] Composition of aqueous phase (pH = 2.5): 25 mM KH_2PO_4. [b] Composition of organic phase: acetonitrile. [c] Taken from ref 37.

solution of acyclovir[1] and 4-(*N*,*N*-dimethylamino)pyridine (DMAP) was added to the reaction mixture, stirred for 24 h, and then filtered. The solvent within the filtrate was partially removed in vacuo, and the impure solution was added dropwise to cold diethyl ether. The resulting precipitate was filtered and dried, followed by acidolytic removal (treating the compound with trifluoroacetic acid "TFA" for 30 min at 0 °C) of Boc protecting group to yield the desired amino acid esters of ACV.[1] The NMR measurements of the final products of these prodrugs are consistent with previously reported measurements.[27]

2.3. Stability of Prodrugs in Transport Medium. The buffer used in transport studies was DPBS, pH 7.4, containing 130 mM NaCl, 2.5 mM KCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 5 mM glucose. A stock solution of the prodrug (1 mg/mL) was prepared in DPBS buffer and used immediately. Aliquots of the buffer (4.75 mL) were placed in screw-capped vials and allowed to equilibrate at 34 °C. Prodrug stock solution (0.25 mL) was subsequently added to the buffer. The vials were placed in a constant shaker bath set at 34 °C and 60 rpm. Samples (0.1 mL) were collected at appropriate time intervals for up to 48 h and were stored at −80 °C until further analysis. All experiments were conducted at least in triplicate.

2.4. Analytical Procedure. All stability samples were assayed using HPLC. The system comprised a Waters 1525 binary HPLC pump and a Waters 474 scanning fluorescence detector. Sample fluorescence was measured at an excitation λ of 265 nm and an emission λ of 380 nm. The stationary phase was a C18 column, 4.6 × 250 mm. The mobile phase consisted of a mixture of buffer (25 mM KH_2PO_4) and acetonitrile. The percentage of acetonitrile (organic phase) was varied to elute the compounds of interest. Table 1 summarizes the mobile phase composition, flow rate, and retention times.

2.5. Corneal Transport Studies. Transport of L-Arg across freshly excised rabbit cornea was carried out according to the method of Tak et al.[28] Briefly, New Zealand albino rabbits weighing 2.0−2.5 kg were euthanized by an overdose of pentobarbital through a marginal ear vein. Eyes were then carefully enucleated and washed with ice-cold DPBS pH 7.4 to remove any traces of blood. Subsequently, a small incision was made to the sclera, and the cornea was carefully excised, leaving some portion of the sclera attached to it for mounting on the diffusion apparatus. After separation of the lens and iris ciliary body, the cornea was washed with ice-cold DPBS, pH 7.4. It was then mounted on a Side-Bi-Side diffusion apparatus maintained at 34 °C (corneal temperature in vivo). Amino acid solutions,[15] 3.0 mL, were added on the epithelial side of the cornea (donor chamber). To the other half chamber (receiver chamber) 3.2 mL of DPBS (pH 7.4) was added and the solutions in both the chambers were stirred continuously using magnetic stirrer bars. The receiver chamber volume of DPBS was maintained slightly higher to generate hydrostatic pressure in order to maintain the curvature of the cornea throughout the experiment. Sink conditions prevailed during the entire experiment. Samples (100 μL) were removed from the receiver chamber at appropriate time intervals and replaced with an equal volume of DPBS. The samples were transferred to scintillation vials containing 5 mL of scintillation cocktail, and radioactivity was measured using a scintillation counter (Beckman Instruments Inc., model LS-9000). [^{14}C]Mannitol, a paracellular marker, was added to the donor solutions, and its transport was determined in a similar manner to assess the integrity of the cornea during the experiment.

(a) Saturation Kinetics of L-Arg Transport. Transport of L-Arg across rabbit cornea was carried out as described previously with L-Arg solutions at different concentrations (0.5−1000 μM) in DPBS, pH 7.4, spiked with [^{3}H]-L-Arg (2 μCi/mL). Michaelis−Menten parameters K_m and V_{max} were determined by nonlinear regression.

(b) Inhibition Studies. Transport of [^{3}H]-L-Arg (2 μCi/mL) was studied in the presence of various amino acids (1 mM) to determine the substrate specificity of the system responsible for L-Arg translocation.

(c) Na^+, Cl^-, and Energy Dependence. When the effect of Na^+ on amino acid transport was studied, NaCl and Na_2HPO_4 in the buffer were substituted with equimolar quantities of choline chloride and K_2HPO_4, respectively. Similarly, when the effect of Cl^- on amino acid transport was studied, NaCl, KCl, and $CaCl_2$ in the buffer were substituted with equimolar quantities of NaH_2PO_4 and KH_2PO_4 and calcium acetate. Energy dependency studies were performed by preincubating the cornea with buffer alone (control), or 500 μM ouabain for 30 min. Transport of [^{3}H]-L-Arg (2 μCi/mL) was then determined at pH 7.4 as described previously.

(d) pH Dependence. Buffer pH was varied from 6.0 to 7.4 for pH dependency studies. Transport of [^{3}H]-L-Arg (2.0 μCi/mL) was conducted according to the procedure described previously.

(e) Inhibition with Prodrugs. To determine whether the amino acid ester prodrugs of ACV utilized the same transport system involved in the transport of L-Arg across cornea,

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transport of [^{3}H]-L-Arg (2 μCi/mL) was studied in the presence and absence of the three amino acid ester prodrugs (1 mM).

2.6. RT-PCR and Sequencing. Reverse transcription-PCR was performed on the basis of the method of Sugawara et al.,[29] with slight modifications, using 1 μg of total RNA isolated from human cornea (kindly provided by Alcon Labs, Fort Worth, TX). The forward and reverse primers were 5′ GAA GGA GAA AGT GTC GGC TTC A 3′ and 5′ TAC CAC CTT GCC AGA CGA TTT G 3′, respectively. These primers correspond to the nucleotide positions 42−64 and 774−796 in human amino acid transporter $B^{0,+}$ ($ATB^{0,+}$) sequence, respectively. RT-PCR was carried out using the GeneAmp RNA PCR Reagent kit from Applied Biosystems. The conditions for reverse transcription were as follows: denaturation of the template RNA for 10 min at 70 °C and reverse transcription for 60 min at 42 °C. The conditions for PCR amplification were as follows: denaturation for 1 min at 94 °C; annealing for 1 min at 58 °C and extension for 1 min at 72 °C; 37 cycles; final extension for 10 min at 72 °C. The resultant product (~754 bp) was subcloned in pGEM-T Easy vector (Promega) and sequenced from both T7 and SP6 directions utilizing an automated Perkin-Elmer Applied Biosystems 377 Prism DNA sequencer to establish its molecular identity. The sequence was analyzed using the GCG version 10 (Genetics Computer Group, Inc., Madison, WI). Under identical conditions, RT-PCR was also conducted on total RNA purified (acid guanidinium thiocyanate−phenol−chloroform extraction method) from freshly excised rabbit corneal epithelium.[30] The sense and antisense primers selected were 5′ CTG GGA CAA TTT GCT AGC TTA 3′ and 5′ AGA GCT GTA AAA TAT ACC ACC 3′, respectively. These primers correspond to the nucleotide positions 286−307 and 783−804, respectively, in rat $ATB^{0,+}$ sequence and were designed on the basis of two highly conserved regions among rat $ATB^{0,+}$, mouse $ATB^{0,+}$, and human $ATB^{0,+}$ sequences. The resultant product (~518 bp) was subcloned in pGEM-T Easy vector and sequenced from both T7 and SP6 directions to establish its molecular identity.

2.7. Computer Analysis. A nucleotide sequence homology search was performed with a basic local alignment tool (BLAST) via on-line connection to the National Center of Biotechnology Information Database. Multiple nucleotide sequence comparisons were made with the CLUSTAL W (1.81) multiple sequence alignment tool from SwissProt.

2.8. Data Treatment. In the case of corneal transport of L-Arg, steady-state flux values and permeability coefficients were calculated.[28] The flux data was then fitted to the classical Michaelis−Menten equation denoted by eq 1, which represents the carrier-mediated process. V is the total rate of uptake, V_{max} is the maximum uptake rate for the carrier-mediated process, and K_m (Michaelis−Menten constant) is the concentration at half the maximum velocity. Data was fitted to eq 1 using a nonlinear least-squares regression analysis program (KaleidaGraph V3.09).

$$V = \frac{V_{max}[C]}{K_m + [C]} \quad (1)$$

2.9. Statistical Analysis. All experiments were conducted at least in triplicate, and results are expressed as mean ± standard deviation. Michaelis−Menten parameters K_m and V_{max} are expressed as mean ± SE. Statistical analysis between two groups was carried out with Student's t-test. A difference between mean values was considered significant if the p-value was ≤ 0.05.

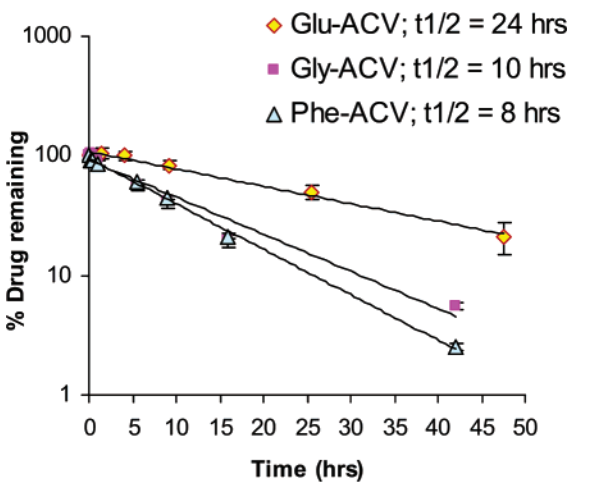

Figure 2. First-order chemical hydrolysis of the prodrugs in DPBS buffer. Each value represents the mean ± standard deviation ($n = 3$).

Table 2. Apparent First-Order Rate Constants and Half-Lives of Chemical Hydrolysis of Prodrugs in DPBS Buffer, pH 7.4

prodrug	rate constant (k) (h^{-1})	half-life ($t_{1/2}$) (h)
Gly-ACV	0.0653 ± 0.002	10.6 ± 0.3
Phe-ACV	0.0877 ± 0.0025	7.9 ± 0.2
γ-Glu-ACV	0.0291 ± 0.0034	23.8 ± 2.9

3. Results

3.1. Stability of Prodrugs in DPBS. The stability of amino acid (glycine, L-phenylalanine, and L-glutamate) esters of ACV was determined in transport buffer DPBS, pH 7.4. Degradation half-lives ranged from 8 to 24 h, and among the three prodrugs, γ-glutamate ester was found to be the most stable at 34 °C in DPBS, pH 7.4 (Figure 2 and Table 2). More than 90% of the prodrugs remained intact in DPBS for 1 h. Therefore subsequent corneal transport studies using these prodrugs as inhibitors were conducted for a period of 1 h.

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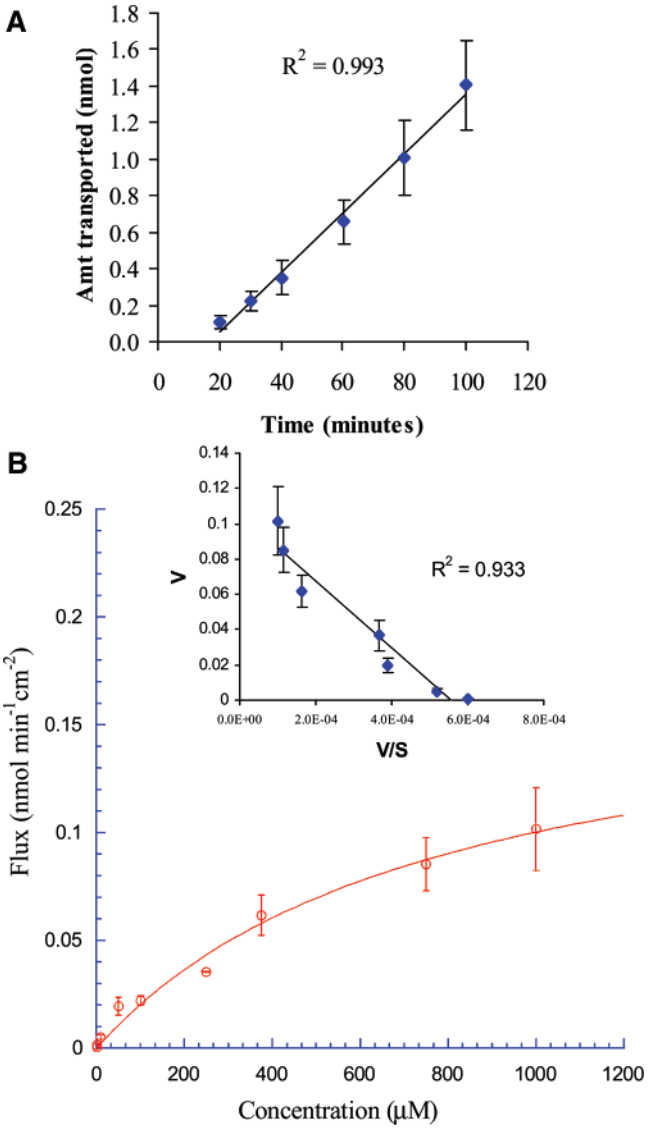

Figure 3. (A) Time course of cumulative amount of 50 μM L-Arg transport across freshly excised rabbit cornea, 34 °C, pH 7.4. Each value represents the mean ± standard deviation ($n = 3$). (B) Concentration dependent transport of L-Arg across freshly excised rabbit cornea at 34 °C, pH 7.4. Inset shows Eadie−Hofstee transformation of the data. Each value represents the mean ± standard deviation ($n = 3$).

3.2. Corneal Permeation Studies. (a) Saturation Kinetics of L-Arg Transport. Transport experiments were conducted for a period of 100 min with donor concentrations of L-Arg ranging from 0.5 to 1000 μM. Flux and apparent permeability (P_{app}) values were calculated from a plot of cumulative amount of Arg transported across rabbit cornea as a function of time (Figure 3A). Transport of L-Arg across the cornea was found to be concentration dependent and saturable at higher concentrations (Figure 3B). The data was fitted to a classical Michaelis−Menten equation (eq 1), and the kinetic parameters K_m and V_{max} were calculated to be 306 ± 72 μM (mean ± SE) and 0.12 ± 0.01 nmol min^{-1} cm^{-2} (mean ± SE), respectively. Eadie−Hofstee transformation of the data suggested the involvement of a single carrier in the transport process (Figure 3B inset).

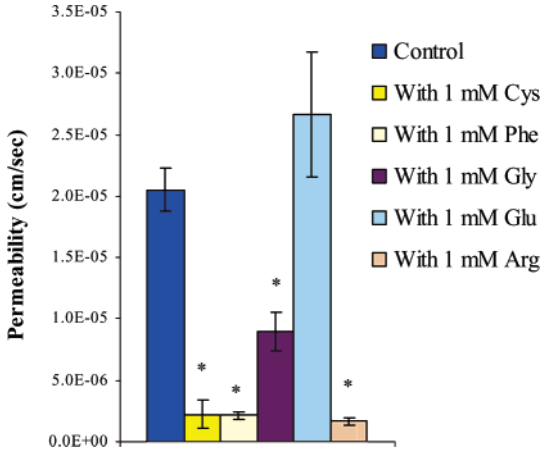

Figure 4. Transport of [^{3}H]-L-Arg (2 μCi/mL) across freshly excised rabbit cornea in the presence of different amino acids (1 mM) and inhibitors (1 mM). Each value represents the mean ± standard deviation ($n = 3$). The asterisk symbol (*) represents significant difference from control ($p \leq 0.05$).

(b) Substrate Specificity. To investigate the substrate specificity of the carrier involved in the transport of L-Arg, various amino acids were examined as potential inhibitors. As depicted in Figure 4, transport of [^{3}H]-L-Arg across rabbit cornea was inhibited markedly by L-isomers of neutral (Phe, Cys, and Gly) and basic (Arg) amino acids. Inhibition by acidic amino acid (L-glutamate) was negligible. Also, transport of [^{3}H]-L-Arg was attenuated in the presence of a system-$B^{0,+}$ specific inhibitor, BCH.[2]

(c) Effects of Ouabain, Cl^-, Na^+, and pH. The effect of ouabain, an inhibitor of Na^+/K^+-ATPase, on the transport of L-Arg across cornea was studied to determine whether the process required energy expenditure. Treatment with ouabain significantly inhibited transport of [^{3}H]-L-Arg (Figure 5A) across cornea, indicating that energy was required for L-Arg transport. Moreover, [^{3}H]-L-Arg transport was also reduced in sodium free buffer (Figure 5A), suggesting that the transport was mediated by a Na^+-dependent system. The kinetics remained unaltered with a change in buffer pH, indicating that the transport of L-Arg across rabbit cornea was independent of pH (Figure 5B). Also, a reduced transport of [^{14}C]-L-Arg was observed in the absence of chloride ion free buffer (Figure 5C). These results are consistent with the $B^{0,+}$ amino acid transport system, indicating that L-Arg might be utilizing this transport system for its permeation across the cornea.

(d) Effect of Prodrugs. Glu-ACV and Phe-ACV (1 mM) inhibited the transport while Gly-ACV (1 mM) did not inhibit the transport of [^{3}H]-L-Arg across freshly excised rabbit cornea (Figure 6). These results indicate that both γ-Glu-ACV and Phe-ACV are probably recognized as substrates by the L-Arg transporter on the corneal epithelium while Gly-ACV is not.

3.3. RT-PCR and Sequencing. PCR products were analyzed by gel electrophoresis on 0.8% agarose. cDNA was

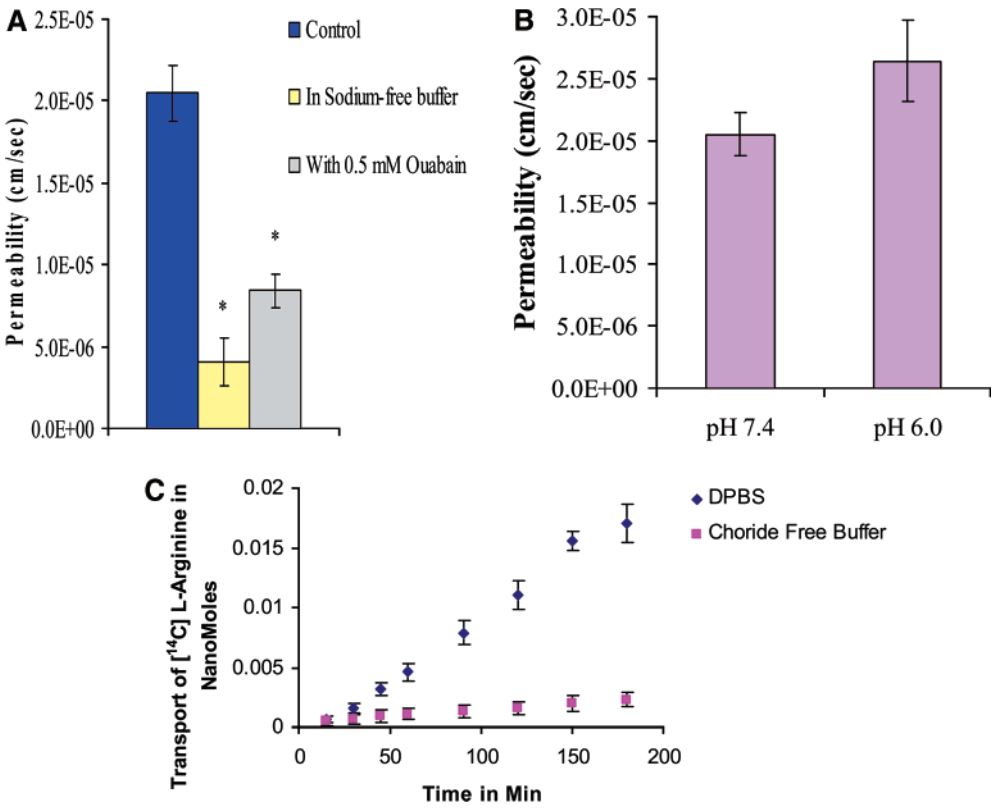

Figure 5. (A) Transport of [^{3}H]-L-Arg (2 μCi/mL) across freshly excised rabbit cornea in the absence and presence of ouabain and in a sodium-free buffer. Each value represents the mean ± standard deviation ($n = 3-4$). The asterisk symbol (*) represents significant difference from control ($p \leq 0.05$). (B) Transport of [^{3}H]-L-Arg (2 μCi/mL) across freshly excised rabbit cornea showing pH independence. The differences are not statistically significant. Each value represents the mean ± standard deviation ($n = 3-4$). (C) Transport of [^{14}C]-L-Arg (2 μCi/mL) across freshly excised rabbit cornea in DPBS and in chloride-free buffer. Each value represents the mean ± standard deviation ($n = 3-4$). Values are significantly different ($p \leq 0.05$).

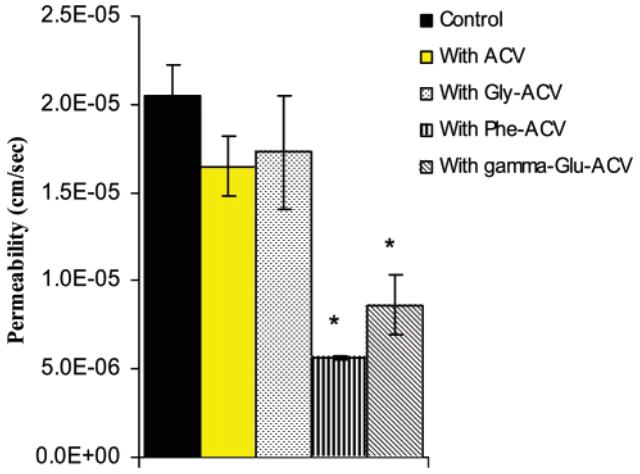

Figure 6. Transport of [^{3}H]-L-Arg (2 μCi/mL) across freshly excised rabbit cornea in the presence of ACV and amino acids prodrugs of ACV (1 mM). Each value represents the mean ± standard deviation ($n = 3$). The asterisk symbol (*) represents significant difference from control ($p \leq 0.05$).

generated from the total RNA isolated from (a) rabbit cornea and (b) rabbit corneal epithelium and was then PCR amplified using the primers specific for rat $ATB^{0,+}$ sequence (primers designed on the basis of multiple sequence alignment between rat, mouse, and human homologues of $ATB^{0,+}$). A 518-bp product was obtained (Figure 7A) in both cornea and corneal epithelium. The 518-bp fragment from rabbit corneal epithelial cDNA was ligated into pGEM-T Easy vector, subcloned, and sequenced in both directions. A BLAST search (NCBI) was performed using this as the query sequence. Among the sequences producing significant alignments was the human amino acid transporter $B^{0,+}$ (GenBank accession number AF151978). As the rabbit $ATB^{0,+}$ full length gene sequence was not available, a comparison at the nucleotide or protein level could not be made directly.

In a previous study,[31] the primary structure of mouse $ATB^{0,+}$ was found to be highly homologous to human $ATB^{0,+}$ with 88% identity between the sequences of the two proteins. In the future, rabbit $ATB^{0,+}$ could be cloned and its sequence compared to that of $hATB^{0,+}$. This would then give us an idea about the similarity and differences in the sequence and hence the function of $ATB^{0,+}$ across the species. A major ~754-bp band was obtained when PCR was performed using primers specific for $hATB^{0,+}$ sequence and cDNA from human cornea (Figure 7B). This DNA

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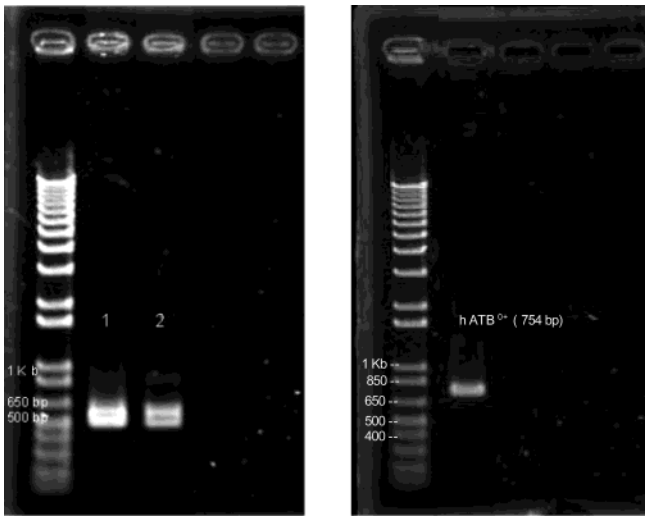

Figure 7. (A) RT-PCR amplification of $ATB^{0,+}$ using total RNA from rabbit cornea (1) and rabbit corneal epithelium (2). Aliquots of PCR products were analyzed by gel electrophoresis on 0.8% agarose. A single ~518-nt PCR product was obtained. A 1 kbp DNA ladder is shown on the left. (B) RT-PCR amplification of $ATB^{0,+}$ using total RNA from human cornea. Aliquots of PCR products were analyzed by gel electrophoresis on 0.8% agarose. A single ~754-nt PCR product was obtained. A 1 kbp DNA ladder is shown on the left.

fragment was ligated into pGEM-T Easy vector, subcloned, and sequenced. The sequence obtained from both directions showed maximum sequence similarity to human $ATB^{0,+}$ sequence. Thus the broad specificity, Na^+-dependent amino acid transporter, $ATB^{0,+}$, was found to be present on both rabbit and human corneas.

4. Discussion

In recent years, numerous nutrient transporters (amino acid, peptide, glucose) have been identified and cloned. Na^+-dependent amino acid transport processes have been demonstrated in the bullfrog alveolar pigmented epithelium (L-Lys),[16] rabbit small intestine,[12] and pigmented rabbit conjunctiva (L-Arg).[13,18] A Na^+-dependent transporter of neutral and basic amino acids, characteristic of system $B^{0,+}$, appears to be involved in the transport of L-Arg in pigmented rabbit conjunctiva. The same transport system $B^{0,+}$ has also been found to be responsible for the conjunctival transport of L-NA (N^G-nitro-L-arginine), a potent nitric oxide synthase (NOS) inhibitor.[14] There is no evidence, however, for the presence of this transport system in rabbit or human corneal epithelium. In this study, we have identified a carrier-mediated transport process for L-Arg in rabbit cornea. Transport of L-Arg across rabbit cornea was concentration dependent with a K_m of 306 μM and showed the involvement of a single carrier in the process (Figure 3B). The transport of L-Arg across rabbit cornea was reduced significantly in the presence of ouabain, an inhibitor of Na^+/K^+-ATPase, and in sodium- and chloride- free buffers (Figure 5A,C). These results suggest that L-Arg permeates the corneal epithelial cells through an energy-, sodium-, and chloride-dependent carrier-mediated transport system.

To delineate the structural requirements of this carrier and also to identify its substrate specificity, the inhibitory effects of selected amino acids on [^{3}H]-L-Arg transport were investigated. The transport was inhibited significantly by neutral amino acids like L-Phe, L-Cys, and Gly (Figure 4). Inhibition of transport of [^{3}H]-L-Arg was also seen in the presence of cationic amino acid L-Arg. A negligible effect of L-Glu on transport of [^{3}H]-L-Arg most likely indicates that this amino acid carrier system is broadly specific for neutral and cationic amino acids and does not accept anionic amino acids as substrates.

Cationic amino acid transport systems have been defined as transporters, exhibiting affinities and translocation rates for cationic amino acids that are higher than or equivalent to those for other types of amino acids.[8] Four such transport systems have been classified as y^+, $b^{0,+}$, y^+L, and $B^{0,+}$. Of these, the y^+ system is specific for only cationic amino acids.[32,33] The other three systems ($b^{0,+}$, y^+L, and $B^{0,+}$) accept a wider range of substrates, including cationic and neutral amino acids; they differ, however, in their interactions with inorganic monovalent ions. System $B^{0,+}$ is Na^+ dependent and does not operate at an appreciable rate, unless this cation is present.[9] System $b^{0,+}$ is Na^+ independent and functions in the presence or absence of K^+, choline, or Li^+ salts.[34] Finally, system y^+L exhibits a more complex pattern in its cation interaction; whereas the transport of lysine through this system is unaffected by Na^+ replacement, the affinity of system y^+L toward neutral amino acids is dramatically decreased if Na^+ in the medium is substituted with K^+.[35] Also, BCH is transported via system $B^{0,+}$ but not by any of the other three systems.[8]

On the basis of the results presented in this report and the fact that the transport of [^{3}H]-L-Arg was inhibited significantly by BCH, and found to be energy, sodium ion, and chloride ion dependent (Figures 4, 5), it appears that the primary carrier involved in L-Arg transport across corneal epithelium is the $B^{0,+}$ system.

Finally, the RT-PCR results confirmed the presence of Na^+-dependent, broadly specific, $B^{0,+}$ amino acid transporter on rabbit cornea, rabbit corneal epithelium, and human

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cornea. An ~518-bp product was obtained, which, when subcloned and sequenced, exhibited maximum sequence homology to $ATB^{0,+}$ amino acid transporter. This article reports for the first time the identification of $ATB^{0,+}$ in rabbit corneal epithelium. We also carried out RT-PCR on the RNA extracted from human cornea. Subcloning and sequencing of a major ~754-bp band confirmed the presence of $hATB^{0,+}$ in human cornea.

The Na^+-coupled amino acid transport systems on the conjunctiva are known to regulate Na^+ absorption from the tear fluid into the superficial epithelial cells of the conjunctiva.[18] It has also been suggested that these transporters may be involved in the regulation of conjunctival cell volume and maintenance of fluid balance in the tear film.[36] On the basis of these reports, a similar role can be proposed for the Na^+-coupled amino acid transport systems in the cornea, especially the corneal epithelium. Thus, the $B^{0,+}$ transport system on the corneal epithelium, along with other Na^+-coupled amino acid transport systems, may be involved in the regulation of corneal epithelial cell volume, continuous cell renewal, and maintenance of fluid balance in the tear film.

A previous report from our lab showed enhanced nasal absorption of acyclovir, from the L-aspartate β-ester prodrug of ACV, probably due to the involvement of an active transport system.[37] In this study, we identified and functionally characterized an active transport system for L-arginine on rabbit cornea and studied the interaction of the three amino acid ester prodrugs of ACV with that transporter. Among the three amino acid ester prodrugs of acyclovir that were studied, the γ-L-glutamate and L-phenylalanyl esters of ACV appear to be competing with L-arginine for their transport across the cornea. Inhibition of corneal L-Arg transport by these two prodrugs (Figure 6) raises the interesting possibility that they themselves may be substrates for the Na^+-coupled L-Arg transport processes, i.e., they might be utilizing the same $B^{0,+}$ amino acid transporter for their permeation across the cornea. Such a possibility would be interesting to explore. The stability of the glutamate ester in DPBS, pH 7.4, was found to be the best of the three esters studied with a half-life of 24 h. This chemical stability is even higher than that of Val-acyclovir, the L-valyl ester of ACV, a well-known antiviral drug. Further, in an in vitro screening for antiviral activity against the herpes group of viruses, all three compounds exhibited similar EC_{50} values as ACV against herpes simplex virus type 1 infections (data not shown). Therefore, molecular modification might be an effective strategy to enhance the corneal permeation of ACV as these prodrugs appear to be utilizing an amino acid carrier for their transport and possess excellent in vitro antiviral activity. An in vivo screening for antiviral activity against HSV-1 will further confirm our results. The glycyl ester did not show significant inhibition of transport of L-Arg across cornea. It is possible that it utilizes a different amino acid transport system for its corneal permeation. Further transport and inhibition studies with these ACV esters will help delineate the structural requirements for the transport systems and their relative affinities.

In conclusion, this study demonstrates, for the first time, functional evidence of a high affinity, Na^+-dependent L-Arg carrier system with characteristics similar to those of the $B^{0,+}$ carrier in rabbit cornea. Biochemical evidence for the presence of this carrier in human cornea and rabbit corneal epithelium has also been presented. In the future, cloning and expression of $B^{0,+}$ from rabbit corneal epithelium will help us gain valuable insights into the substrate specificity and affinity of this corneal transporter and its species differentiation. As corneal epithelium is the primary barrier to the absorption of drugs after topical administration, the presence of such transporters on the corneal epithelium may provide new opportunities for transporter-targeted prodrug design for enhanced corneal absorption.

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