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International Journal of Pharmaceutics 320 (2006) 104–113
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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# Simultaneous modulation of transport and metabolism of acyclovir prodrugs across rabbit cornea: An approach involving enzyme inhibitors

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Received 27 February 2006; received in revised form 8 April 2006; accepted 17 April 2006
Available online 23 May 2006

**Abstract**

The aim of this study is to identify the class of enzymes responsible for the hydrolysis of amino acid and dipeptide prodrugs of acyclovir (ACV) and to modulate transport and metabolism of amino acid and dipeptide prodrugs of acyclovir by enzyme inhibitors across rabbit cornea. L-Valine ester of acyclovir, valacyclovir (VACV) and L-glycine-valine ester of acyclovir, gly-val-acyclovir (GVACV) were used as model compounds. Hydrolysis studies of VACV and GVACV in corneal homogenate were conducted in presence of various enzyme inhibitors. $IC_{50}$ values were determined for the enzyme inhibitors. Transport studies were conducted with isolated rabbit corneas at 34 °C. Complete inhibition of VACV hydrolysis was observed in the presence of Pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl-fluoride) and PCMB (*p*-chloromercuribenzoic acid). Similar trend was also observed with GVACV in the presence of bestatin. $IC_{50}$ values of PCMB and bestatin for VACV and GVACV were found to be $3.81 \pm 0.94$ and $0.34 \pm 0.08$ μM respectively. Eserine, tetraethyl pyrophosphate (TEPP) and diisopropyl fluorophosphate (DFP) also produced significant inhibition of VACV hydrolysis. Transport of VACV and GVACV across cornea showed decreased metabolic rate and modulation of transport in presence of PCMB and bestain respectively. The principle enzyme classes responsible for the hydrolysis of VACV and GVACV were carboxylesterases and aminopeptidases respectively. Enzyme inhibitors modulated the transport and metabolism of prodrugs simultaneously even though their affinity towards prodrugs was distinct. In conclusion, utility of enzyme inhibitors to modulate transport and metabolism of prodrugs appears to be promising strategy for enhancing drug transport across cornea.


*Keywords:* Enzyme inhibitors; Corneal hydrolysis; Prodrugs; Transporters; Hydrolytic enzymes

## 1. Introduction

Prodrugs are designed to be therapeutically inactive until in vivo activation to the parent drug, hence reliable in vivo activation of the prodrug is considered critical for their pharmacological activity (Han and Amidon, 2000). Thus controlling the mechanism of in vivo activation of prodrugs can be critical for prodrug delivery. Enzymatic and chemical processes are known to play an important role in the hydrolysis of prodrugs and it is well known that enzymatic processes play dominant role than chemical process (Anand et al., 2003). It is also well known that hydrolytic enzymes are present ubiquitously in all biological fluids and tissues. For example, esterases are expressed throughout the body and can be utilized in the hydrolysis of an ester functional group. The prodrug to drug conversion can take place by various enzymes in different tissues, by peptidases in the intestine (Das and Radhakrishnan, 1976); by esterases in the skin (Kim et al., 1974; Roy and Manoukian, 1994); by phosphomonoesterases in plasma (Melby and St. Cyr, 1961); by β-glucuronidases at tumor tissue (Watanabe et al., 1981).

Cornea acts as the principal barrier for topically applied ophthalmic drugs. This role has generally been ascribed to the resistance offered by the corneal epithelium; however the cornea can further control the amount of active drug reaching the internal eye by virtue of its capacity to metabolize a prodrug during its transit. Studies by Lee et al. (1985) revealed that rank order of esterase activity is highest in the iris-ciliary body followed by cornea and aqueous humor. Even though the cornea is not as

*Abbreviations:* DFP, diisopropylfluorophosphate; E-64, *trans*-Epoxysuccinyl-L-leucylamido-(4-guanidino) butane; EDTA, ethylenediamine tetra acetic acid; PCMB, *p*-chloromercuricbenzoic acid; Pefabloc SC, 4-(2-aminoethyl)-benzenesulfonyl-fluoride; PMSF, phenyl methyl sulfonyl fluoride; TEPP, tetraethylpyrophosphate

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doi:10.1016/j.ijpharm.2006.04.010

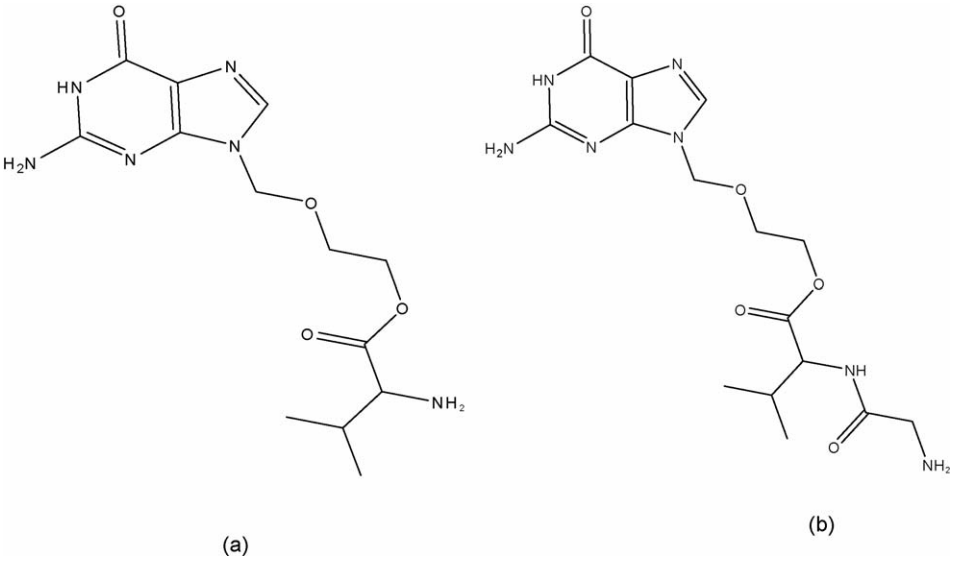


Fig. 1. Chemical structure of: (a) valacyclovir and (b) Gly-Val-acyclovir.

enzymatically active as iris-ciliary body, it is still in a strategic position to determine the amount of intact prodrug ultimately reaching the internal eye structures from topical dosing.

Previous reports indicated that esterase activity in the corneal epithelium is approximately twice than in the stroma and endothelium (Lee et al., 1982a,b). According to these reports the bulk of esterase-mediated hydrolysis takes place in the epithelium, so that the residence time of the drug in this tissue can have a significant impact on its ocular bioavailability. Previous studies also indicate that corneal epithelium possesses appreciable amount of aminopeptidase activity. Aminopeptidase activity is highest in the corneal epithelium and iris-ciliary body followed by conjunctiva and corneal stroma. The pattern of substrate hydrolysis indicates that a common dominant aminopeptidase isozyme is present in these tissues except the conjunctiva. Such enzymes are known to act as a significant metabolic barrier for topically applied enkephalins (Stratford and Lee, 1985).

Valacyclovir, L-valine ester prodrug of acyclovir showed three to five times higher systemic availability when administered orally (Curran and Noble, 2001; Perry and Faulds, 1996; Pescovitz et al., 2000; Smiley et al., 1996; Weller et al., 1993). Increased bioavailability of valacyclovir is attributed to its carrier-mediated intestinal absorption, via the hPEPT1 peptide transporter (Balimane et al., 1998; Ganapathy et al., 1998; Han et al., 1998). Topical application of VACV also showed higher permeability across cornea (Anand and Mitra, 2002). Recent studies from our laboratory also indicate that increased GVACV permeability across the cornea is due to the involvement of PEPT1 transporter (Anand et al., 2003).

VACV and GVACV are readily absorbed across corneal epithelium owing to their appreciable affinity for PEPT1 (Anand et al., 2004). Following absorption through the corneal epithelium the compounds undergo extensive hydrolysis to yield ACV thus causing the intact prodrugs to be unavailable for penetratation into the deeper tissues of the eye. Transport studies of VACV in Caco-2 cells by Han et al. (1998) also demonstrated that more than 90% of the drug in the receiver compartment was ACV, indicating extensive intracellular VACV hydrolysis. A strategy of using enzyme inhibitors to modulate the degradation rate of prodrugs of ACV may be helpful in treating HSV infections of the deeper cornea and other intraocular tissues (iris-ciliary body, lens, and uvea) without having to administer the drug quite frequently. We can also extend this strategy to target the various transporters present in deeper ocular tissues.

The present paper delineates the role of corneal esterases and peptidases in hydrolyzing ester and peptide bonds. Aminoacid and dipeptide ester prodrugs of ACV were selected as model compounds and their hydrolysis rates were determined in the presence of various enzyme inhibitors. We further investigated the inhibition capacity ($IC_{50}$) of these inhibitors towards these hydrolyzing enzymes and how such inhibitory activity can modulate the transport and metabolism of these prodrugs in the cornea.

## 2. Materials and methods

### 2.1. Animals

New Zealand albino adult male rabbits weighing between 2.0 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. Materials

VACV was a gift from GlaxoSmithKline (Research Triangle Park, NC, USA) and GVACV was synthesized in our laboratory (Fig. 1). Enzyme inhibitors, EDTA, PMSF, E-64, aprotinin, leupeptin, pepstatin, bestatin, eserine, PCMB, DFP and TEPP were purchased from Sigma Chemical Company and Pefabloc SC was purchased from Fluka Chemical Corp. (Milwaukee, WI). The solvents were of analytical grade and obtained from Fisher Scientific. The growth medium, minimum essential

medium, non-essential amino acids (NEAA) and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY). Penicillin, streptomycin, sodium bicarbonate, HEPES, amphotericin-B, and polymixin-B were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were obtained from Sigma Chemical Company.

### 2.3. Corneal tissue hydrolysis

The corneal tissue hydrolysis was carried out as described previously (Tak et al., 2001). The method is described briefly as follows.

#### 2.3.1. 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 isotonic phosphate buffered saline (IPBS) at pH 7.4. The cornea was removed after cutting along the scleral-limbus junction. The corneal tissue was homogenized in 5 ml chilled (4 °C) IPBS with a tissue homogenizer (Tissue Tearor Model 985-370) in an ice bath. The homogenate was centrifuged at 12,500 rpm for 30 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.

#### 2.3.2. Hydrolysis procedure

Prior to the initiation of an experiment, the supernatant was equilibrated at 34 °C for about 30 min after adding the enzyme inhibitor. The concentrations chosen for the enzyme inhibitors were well within the acceptable range. Hydrolysis was initiated by the addition of 0.2 ml of a 1 mM prodrug solution to 1.3 ml of the supernatant. The control consisted of 1.3 ml of IPBS instead of the supernatant. Aliquots (50 μl) were withdrawn at appropriate time intervals for up to 96 h. 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 12,500 rpm for 15 min prior to analysis by HPLC for the intact ester prodrug and the regenerated parent drug acyclovir. Apparent first order rate constants were calculated and corrected for any chemical hydrolysis observed with the negative control.

### 2.4. Uptake studies

The procedure for preparing primary corneal epithelial culture was reported previously from our laboratory (Dey et al., 2003). Uptake studies were performed on rPCEC (Rabbit Primary Corneal Epithelial Culture) using 12-well plates. All uptake studies were conducted on cultured primary rabbit corneal cells after 10–12 day seeding. The medium was removed, and cells were washed twice with Dulbecco Phosphate Buffered Saline (DPBS) at pH 7.4. In typical uptake experiments, cells were incubated with substrates, [$^3$H] GlySar (0.5 μCi/ml) prepared in DPBS for 10 min. Following incubation cells were washed three times with ice-cold HEPES buffer to terminate the uptake experiment. Then the cells were lysed overnight with 1 ml 0.05% (w/v) Triton X-100 in 1N NaOH at room temperature. Aliquots (500 μl) from each well were transferred to scintillation vials containing 5 ml scintillation cocktail (Fisher Scientific, Fairlawn, NJ). Samples were then analyzed by the liquid scintillation spectrophotometry using scintillation counter (Beckman Instruments Inc., Model LS-6500) and the rate of uptake was normalized to the protein content of each well. The amount of protein in the cell lysate was measured by BioRad protein estimation kit using bovine serum albumin as the standard (BioRad Protein estimation Kit, Hercules, CA).

### 2.5. Corneal transport studies

Transport of VACV and GVACV across freshly excised rabbit cornea was performed according to the method of Tak et al. (2001). Briefly, New Zealand albino rabbits weighing 2.0–2.5 kg were killed by an overdose of pentobarbital through a marginal ear vein. Eyes were then carefully enucleated and washed with ice-cold DPBS (pH 7.4). Subsequently, a small incision was made in the sclera and the cornea was carefully excised, leaving some portion of the sclera attached to it for mounting on the diffusion apparatus. It was then mounted on a diffusion apparatus (Side-bi-Side) maintained at 34 °C (corneal temperature in vivo). Prodrug solutions (3 ml) were added with/without enzyme inhibitor on the epithelial side of the cornea (donor chamber). In the other half-chamber (receiver chamber), 3.2 ml of DPBS (pH 7.4) was added with or without enzyme inhibitor and solutions in both the chambers were stirred continuously using magnetic stirrer bars. Receiver chamber volume of DPBS was maintained slightly higher to generate hydrostatic pressure to maintain the curvature of the cornea throughout the experiment. Sink conditions prevailed during the entire experiment. Transport experiments were conducted for a period of 3 h. One hundred microlitres aliquots were removed from the receiver chamber at appropriate intervals and replaced with an equal volume of DPBS. Samples were stored at −80 °C until further HPLC analysis.

### 2.6. Analytical procedure

VACV and GVACV samples were assayed by reversed phase high-performance liquid chromatography (HPLC). The HPLC system comprised of a Rainin Dynamax Pump SD-200, Rainin Dynamax UV Detector UV-C at 254 nm and an Alcott autosampler Model 718 AL HPLC. The column used was a C18 Luna column 4.6 × 250 mm (Phenomenex). The mobile phase consisted of 20 mM phosphate buffer (pH adjusted to 2.5) and acetonitrile as the organic modifier. The percentage of organic phase was varied in order to elute compounds of interest. This method gave rapid and reproducible results. Limits of quantification were found to be: ACV, 100 ng/ml; valacyclovir, 250 ng/ml; Gly-val-acyclovir, 500 ng/ml. Intra and inter day precision (measured by coefficient of variation, CV%) was less than 3%, and 4% respectively.

### 2.7. 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 versus time graph and expressed per unit of corneal surface area as described by Eq. (1). Cumulative amount of drug transported is the sum of the receptor cell prodrug and the regenerated drug:

$$\text{Flux}\,(J) = \frac{(\mathrm{d}M/\mathrm{d}t)}{A} \quad (1)$$

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, $C_\mathrm{d}$ according to Eq. (2).

$$\text{Permeability}\,(P_\mathrm{app}) = \frac{\text{Flux}}{C_\mathrm{d}} \quad (2)$$

### 2.8. Affinity measurement

For dose–response studies in which VACV and GVACV hydrolytic rate was inhibited, the inhibitory effect of PCMB and bestatin was described by the model illustrated by Eq. (3):

$$Y = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + 10^{\log \mathrm{IC50} - X}} \quad (3)$$

in which $X$ is the logarithm of inhibitor concentration, $Y$ the hydrolysis rate and top and bottom denote maximum and minimum hydrolysis rates, respectively. The $IC_{50}$ value was estimated using Eq. (3).

### 2.9. Metabolic capacity

Garren et al. (1989) proposed a mathematical model based on Fickian diffusion with first order enzymatic reaction. This model was similar to the model proposed by Ando et al. (1977) for diffusion with chemical reaction in the skin, which is mathematically complex. Simpler model was developed by Repta et al. by choosing appropriate experimental conditions. This simpler model estimates the relative contribution of diffusion and metabolism processes across tissue layer; only when assumptions are valid. This model was used in this project by assuming corneal epithelium as the barrier layer and underlying tissues such as endothelium and stroma provide significantly less diffusional resistance and enzymatic activity. The equation developed by Repta et al. can be described as Eq. (4)

$$\frac{(F_\mathrm{u} - F_\mathrm{r})}{F_\mathrm{u}} = (\alpha L)\mathrm{csc}h(\alpha L) \quad (4)$$

where $F_\mathrm{u}$, flux of prodrug into the receiver reservoir, represents the situation where no metabolism occurs. $F_\mathrm{r}$ is the flux of drug into the receiver chamber. Determination of $F_\mathrm{u}$, and $F_\mathrm{r}$ under appropriate experimental conditions allowed estimations of $\alpha L$ by iteratively solving Eq. (4). Values of $(\alpha L)^2$ give an indication of the magnitude of the ratio of metabolic capacity to diffusion rate. $\alpha$ is represented as $\alpha = \sqrt{k/D_\mathrm{r}}$ where $k$ is first-order reaction rate constant and $D_\mathrm{r}$ is diffusivity of prodrug across corneal epithelium. Detailed explanation for the derivation of the above Eq. (4) was given by Garren et al. (1989).

### 2.10. Statistical analysis

All experiments were conducted at least in triplicate and results are expressed as mean ± S.D. Statistical significance testing was done with 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 was $\leq 0.05$. The method used to discriminate among the means is Fisher's least significance difference (LSD). $IC_{50}$ value was estimated using software PRISM. $K_{12}$, $K_{13}$, and $K_{23}$ parameters were estimated using WINNONLIN program by fitting the amount of amino acid intermediate generated versus time to a one compartmental model with first order elimination.

## 3. Results

### 3.1. VACV degradation rate in the presence of enzyme inhibitors

Corneal homogenate hydrolysis of VACV was done in presence of various classes of enzyme inhibitors to delineate the enzyme class responsible for the degradation of VACV (Fig. 2). Hydrolysis of VACV was inhibited neither by 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml E-64, 0.5 mM PMSF and 2 μg/ml aprotinin, nor by the peptidase inhibitor bestatin (0.5 mM). VACV hydrolysis was partially inhibited by the choline esterase reversible inhibitor eserine (1 mM) and irreversible inhibitors DFP (1 μl/ml), TEPP (1 μl/ml). VACV hydrolysis was completely inhibited by irreversible serine hydrolase inhibitor, Pefabloc SC (1 mg/ml) and a carboxylesterase inhibitor, PCMB (1 mM).

### 3.2. GVACV degradation rate in the presence of enzyme inhibitors

Corneal homogenate hydrolysis of GVACV was done in presence of various classes of enzyme inhibitors to delineate the enzyme class responsible for the degradation of GVACV (Fig. 3). Hydrolysis of GVACV was not inhibited by 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml E-64, 0.5 mM PMSF and 2 μg/ml aprotinin, nor was it inhibited by the choline esterase reversible inhibitor eserine (1 mM) and irreversible inhibitors DFP and TEPP (1 μl/ml). GVACV hydrolysis was partially inhibited by irreversible serine hydrolase inhibitor Pefabloc SC (1 mg/ml), and a carboxylesterase inhibitor, PCMB (1 mM). GVACV hydrolysis was almost completely inhibited by specific aminopeptidase inhibitor, bestatin (0.5 mM), and significantly inhibited by a metalloprotease inhibitor, EDTA (5 mM).

### 3.3. VACV and GVACV degradation rate chemical versus enzymatic

VACV and GVACV degradation rates in the IPBS and in corneal homogenate were compared to identify the contribu-

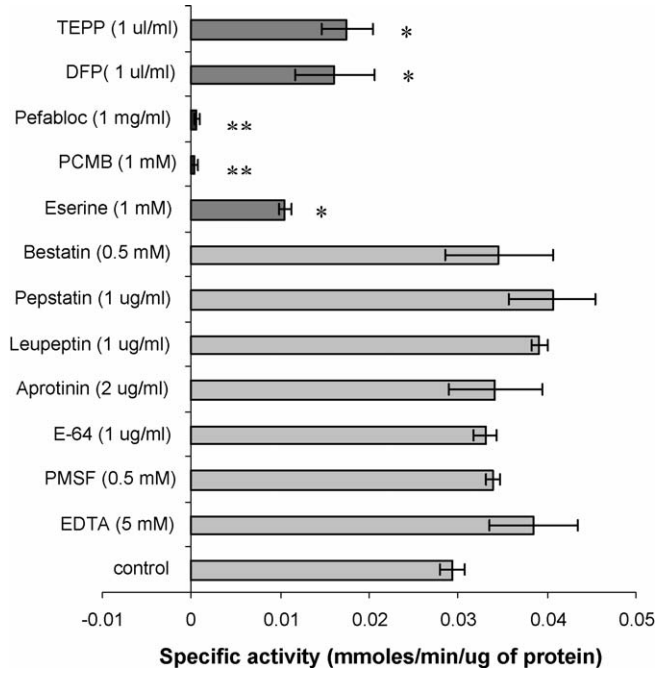


Fig. 2. Effect of enzyme inhibitors on VACV hydrolysis by corneal homogenates; $^{*}p<0.05$, $^{**}p<0.01$. Values are means ± S.D. ($n=3$).

tion of the enzyme hydrolysis compared to chemical hydrolysis. The results indicate that enzymatic processes play dominant role than chemical process (Fig. 4). The rate of degradation in the corneal homogenate was significantly higher than in the IPBS both for VACV and GVACV. Results also indicate that VACV is more susceptible to hydrolysis than GVACV, both in IPBS and in corneal homogenate (Fig. 4).

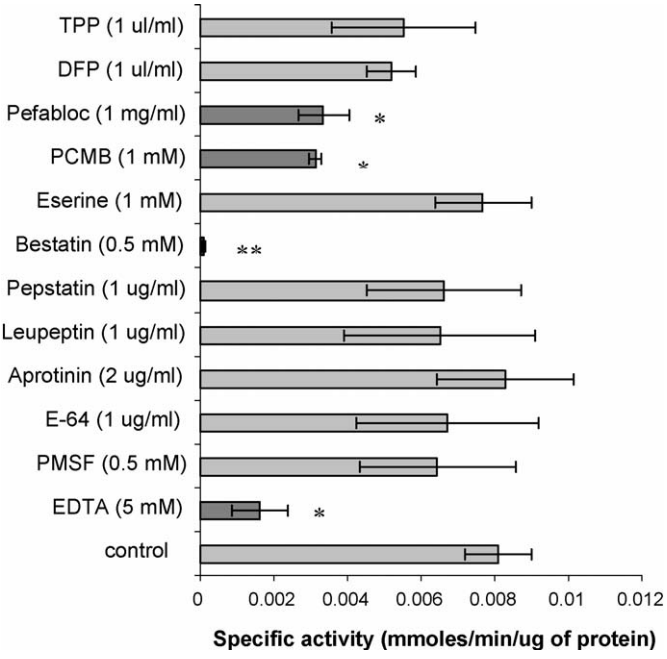


Fig. 3. Effect of enzyme inhibitors on GVACV hydrolysis by corneal homogenates; $^{*}p<0.05$, $^{**}p<0.01$. Values are means ± S.D. ($n=3$).

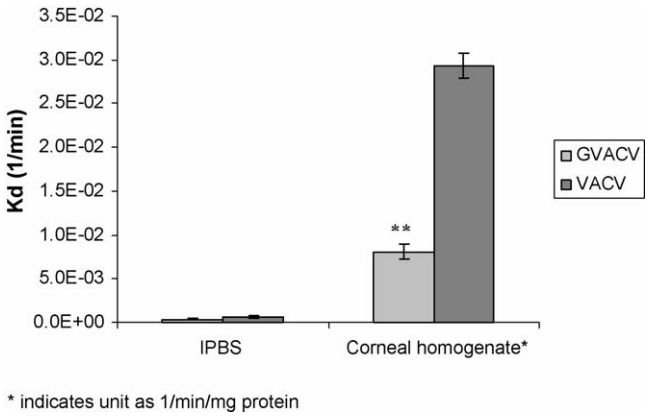


Fig. 4. Comparison of chemical vs. enzymatic hydrolysis rates for both the prodrugs VACV and GVACV; $^{**}p<0.01$. Values are means ± S.D. ($n=3$).

### 3.4. $IC_{50}$ values of PCMB and bestatin

Degradation rate of VACV and GVACV in the presence of various concentrations of PCMB and bestatin was done to characterize the inhibition capacity of these inhibitors towards these hydrolyzing enzymes. Concentration dependent inhibition was observed for VACV and GVACV hydrolysis in presence of PCMB and bestatin respectively. Concentrations used for PCMB were ranging from 0.01 to 1000 μM and bestatin were ranging from 0.0005 to 500 μM respectively. The inhibitory effect of PCMB and bestatin in corneal homogenate appeared to be dose dependent and saturable at higher concentrations (Figs. 5 and 6). $IC_{50}$ values of PCMB and bestatin from the dose–response curve were estimated by fitting the data to the nonlinear equation Eq. (3) and were found to be $3.81 \pm 0.94$ and $0.34 \pm 0.08$ μM for VACV and GVACV respectively.

### 3.5. [$^{14}$C] Mannitol transport

In order to assess the integrity of the cornea in the presence of enzyme inhibitors, [$^{14}$C] mannitol transport across cornea was also determined simultaneously. Integrity of the cornea was not compromised in presence of 1 mM PCMB and 0.05 mM

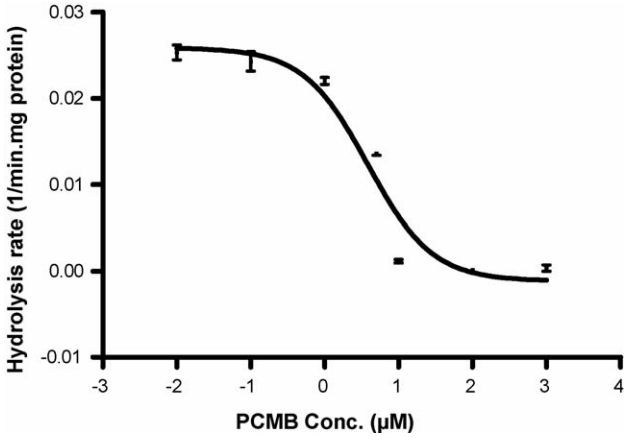


Fig. 5. Dose dependent inhibition of VACV corneal homogenate hydrolysis rate in presence of PCMB. Values are mean ± S.D. ($n=3$).

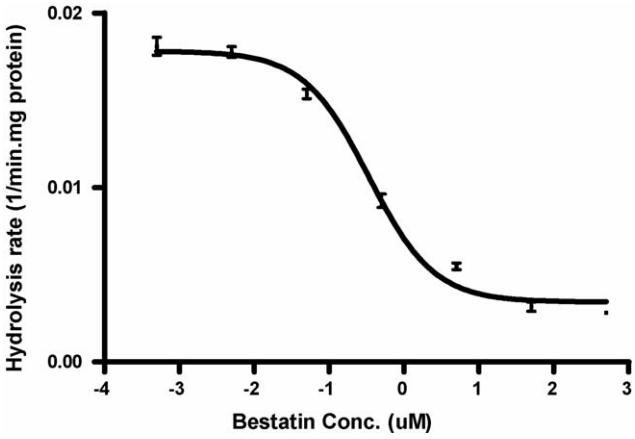


Fig. 6. Dose dependent inhibition of GVACV corneal homogenate hydrolysis rate in presence of bestatin. Values are mean ± S.D. ($n = 3$).

bestatin, since cumulative amount of [$^{14}$C] mannitol transported was not changed significantly compared to control. In presence of 0.5 mM bestatin, cumulative amount of [$^{14}$C] mannitol transported was significantly increased indicating disruption of paracellular junctions. Therefore all the remaining experiments were conducted with 0.05 mM bestatin (Fig. 7A).

### 3.6. [$^3$H] Diazepam transport

Diazepam is considered as a transcellular marker and its transport across cornea was also determined simultaneously in the presence of enzyme inhibitors. Cumulative amount of [$^3$H] diazepam transported did not change significantly relative to control indicating cell membrane integrity (Fig. 7B).

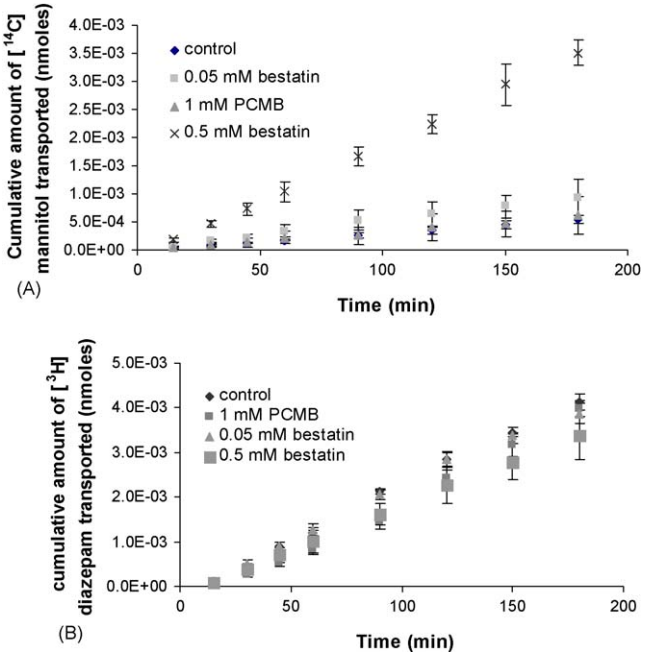


Fig. 7. (A) Amount of [$^{14}$C]mannitiol transported (0.5 μCi/ml) across isolated rabbit cornea in presence of 1 mM PCMB, 0.5 mM bestatin and 0.05 mM bestatin. Values are mean ± S.D. ($n = 4$). (B) Amount of [$^3$H] diazepam transported (0.5 μCi/ml) across isolated rabbit cornea in presence of 1 mM PCMB, 0.5 mM bestatin and 0.05 mM bestatin. Values are mean ± S.D. ($n = 4$).

### 3.7. Transport of VACV in presence of PCMB

Transport of 1 mM VACV was studied across rabbit cornea in the presence of 1 mM PCMB to evaluate the effect of enzyme inhibitor on the transport pattern. PCMB was added to solutions on both sides of the cornea. Transport of VACV in presence of PCMB generated higher amounts of VACV in the receiver chamber compared to control but amounts of ACV generated in the receiver chamber was similar to control (Fig. 8B). Transport of VACV (total) across cornea showed around 2 times enhancement in permeability value in presence of PCMB (Fig. 8A).

### 3.8. Transport of GVACV in presence of bestatin

Transport of 1 mM GVACV was performed across rabbit cornea in the presence of 0.05 mM bestatin to evaluate the effect of enzyme inhibitor on the transport pattern. Bestatin was placed in solutions on both sides of the cornea. Transport of GVACV in presence of bestatin generated similar amounts of GVACV in the receiver chamber relative to control. However the amounts of VACV and ACV generated from GVACV were less than control (Fig. 9B). Transport of GVACV (total) across cornea showed around 4.5 times decrease in permeability value in the presence of 0.05 mM bestain (Fig. 9A).

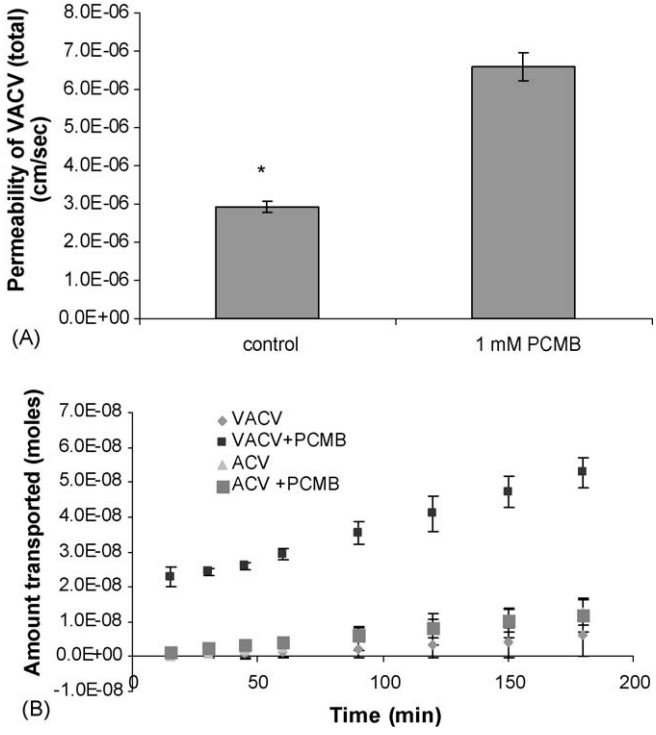


Fig. 8. (A) Permeability values of 1 mM VACV (total) in presence of 1 mM PCMB; $^*p < 0.05$. (B) Amount of prodrug (VACV) and parent drug (ACV) transported across isolated rabbit cornea in the absence and in the presence of 1 mM PCMB. Values are mean ± S.D. ($n = 4$).

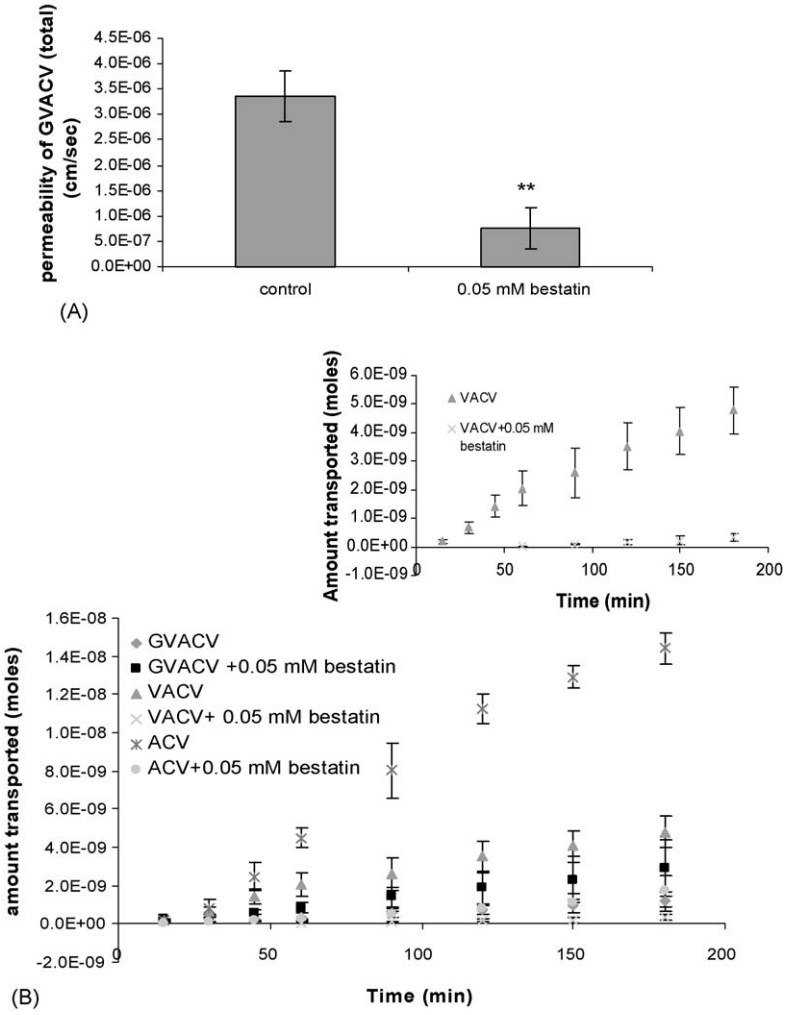


Fig. 9. (A) Permeability values of 1 mM GVACV (total) in presence of 0.05 mM bestatin; $^{**}p < 0.01$. (B) Amount of prodrug (GVACV), amino acid intermediate (VACV) and parent drug (ACV) transported across isolated rabbit cornea in the absence and in the presence of 0.05 mM bestatin. (Inset) Amount of amino acid intermediate (VACV) transported across isolated rabbit cornea in the absence (▲) and in the presence (×) of 0.05 mM bestatin. Values are mean ± S.D. ($n = 4$).

### *3.9. Affinity of VACV and GVACV towards PepT in presence of enzyme inhibitors*

[$^3$H] Glycylsarcosine (GS) was chosen as a model peptide transporter (PEPT) substrate. Uptake of [$^3$H] GlySar was studied in rPCEC cells in the presence of 5 mM VACV, 5 mM VACV along with 1 mM PCMB, 5 mM GVACV and 5 mM GVACV along with 0.05 mM bestatin, to identify the changes in the affinity of these prodrugs towards PEPT in presence of enzyme inhibitors. Uptake of [$^3$H] GlySar was significantly inhibited in the presence of prodrugs as well as prodrugs with the inhibitors (Fig. 10).

## 4. Discussion

Enzymes responsible for the hydrolysis of VACV can be categorized as choline esterases and carboxyl esterases. Of these, carboxylesterases appear to be principle esterases in the hydrolysis of VACV, which is indicated by complete inhibition of hydrolysis by a carboxylesterase inhibitor, PCMB (Holmes and Masters, 1967) compared to choline esterase inhibitors like eserine, DFP, and TEPP (Lee, 1983). This pattern of inhibition of esterase activity is similar to that in liver and other organs, in which carboxylesterase has been found to be major esterase

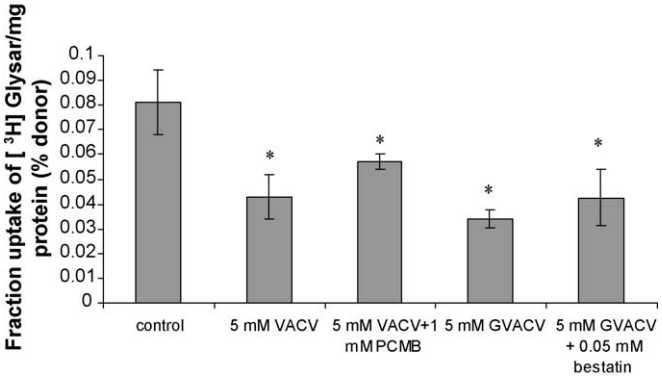


Fig. 10. Uptake of [$^3$H] Glysar in the presence of 5 mM VACV, 5 mM VACV + 1 mM PCMB, 5 mM GVACV and 5 mM GVACV + 0.05 mM bestatin at pH 6.0. Values are mean ± S.D. ($n = 4$). $^*p < 0.05$ from control.

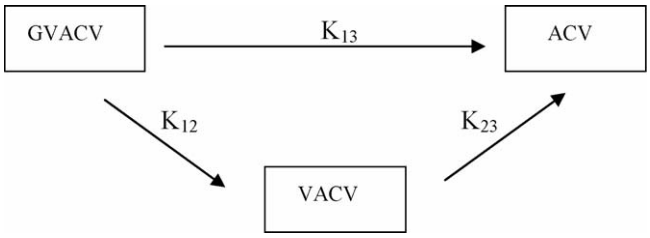


Fig. 11. Mechanism of bioreversion of Gly-Val-ACV to acyclovir. Rate constants $K_{12}$ indicates prodrug GVACV sequential hydrolysis to amino acid intermediate VACV, $K_{23}$ indicates intermediate to parent drug ACV and $K_{13}$ indicates prodrug GVACV to parent drug ACV.

(Okuda and Horiguchi, 1980). VACV hydrolysis was completely inhibited by an irreversible serine hydrolase inhibitor, Pefabloc SC (Fig. 2). This observation is consistent with a previous report which identified a novel serine hydrolase class enzyme, BPHL (biphenyl hydrolase like protein) for hydrolysis of the amino acid ester nucleoside prodrugs, valacyclovir and valganciclovir (Kim et al., 2003). As carboxyl esterases and serine hydrolases are classified under the same enzyme family (EC 3.1.), results from this study suggest that may be more than one type of esterases involved in the hydrolysis of VACV.

GVACV can get hydrolyzed at two positions, either at the ester bond or peptide bond. Usually peptide bonds were hydrolysed by chymotrypsin, trypsin and all other proteases. Results from this study indicate that enzymes responsible for the hydrolysis of GVACV can be categorized as aminopeptidases and esterases. Of these, aminopeptidases appear to be principle enzymes in the hydrolysis of GVACV, as indicated by complete inhibition of hydrolysis by an aminopeptidase inhibitor, bestatin. Partial inhibition by irreversible serine hydrolase inhibitor, Pefabloc SC, and carboxylesterase inhibitor, PCMB, indicate the involvement of esterases in an underlined manner (Fig. 3). These results can be explained by observing GVACV hydrolysis in a sequential manner (Fig. 11, Table 1). Negligible $K_{13}$ and pronounced $K_{23}$ values indicated that GVACV was less prone to esterase hydrolysis compared to VACV. So the participation of esterases in prodrug activation will not only depend on its linker to the parent drug but also on the pro-moiety itself. Even though EDTA showed significant inhibition in GVACV hydrolysis it produced no appreciable change in $K_{12}$, $K_{13}$, and $K_{23}$ values. Further studies are necessary to confirm whether EDTA may be forming coordination complexes preferably with the esterases than with aminopeptidases. One more observation indicated that $K_{23}$ values were slightly higher than $K_{vacv}$ values although both represent VACV degradation rate. This result can be explained by the low amount of VACV generated from GVACV, thereby undergoing fast subsequent hydrolysis to ACV. Studies by Lee et al. suggested that decreasing the dose of an ester prodrug may result in increase in its hydrolytic rate.

Chemical hydrolysis of GVACV was 1.8 times less compared to VACV indicating that dipeptide prodrug by virtue has more stability than aminoacid prodrug (Fig. 4). One reason offered for the instability of these amino acid esters in aqueous media is the strongly electron withdrawing affect of the protonated amine which makes the ester linkage susceptible to a nucleophile attack (Bundgaard et al., 1989). One more study also revealed that an increase in the hydrophobicity of the N-terminal aminoacid decreases the chemical hydrolysis rate (Nichifor and Schacht, 1997). Enzymatic hydrolysis rate of GVACV was 3.6 times lower relative to VACV (Fig. 4). Enzyme specificity towards a particular substrate is based on preferred residues adjacent to the scissile bond. Stability of the dipeptide depends on the N-terminal amino acid, the configuration of the C-terminal substituted amino acid (characterized by optical rotation), and the presence of substituents at the amino and carboxyl end groups of the dipeptides. Aminopeptidase-catalysed hydrolysis rates diminished in the order Ala $\geq$ Leu > Phe > Gly as the N-terminal amino acid whereas endopeptidase activity decreased in the order Ala > Gly > Phe $\approx$ Leu at the N-terminus (Nichifor and Schacht, 1997). This further confirms that aminopeptidases are playing major part in the hydrolysis of GVACV.

Interestingly, the flux of VACV in presence of PCMB was significantly higher than VACV intrinsic flux through the cornea, which suggests that metabolism of prodrug across the cornea is the rate-limiting step for overall corneal permeation of VACV (Fig. 8B). Permeation data compiled with and without the

Table 1
Sequential hydrolysis rate constants from prodrug to amino acid intermediate to parent drug

| | $K_{12} \times 10^2$ (min$^{-1}$) | $K_{13}$ (min$^{-1}$) | $K_{23} \times 10^2$ (min$^{-1}$) | $K_{VACV} \times 10^2$ (min$^{-1}$) |
|---|---|---|---|---|
| Control | 3.6 ± 1.3 | – | 4.5 ± 1.3 | 2.9 ± 0.1 |
| EDTA (5 mM) | 3.3 ± 0.7 | – | 5.5 ± 0.8 | 3.8 ± 0.5 |
| PMSF (0.5 mM) | 4.5 ± 0.4 | – | 4.8 ± 0.4 | 3.4 ± 0.1 |
| E-64 (1 ug/ml) | 3.9 ± 0.9 | – | 5.0 ± 0.9 | 3.3 ± 0.1 |
| Aprotinin (2 ug/ml) | 3.8 ± 1.4 | – | 4.9 ± 1.4 | 3.4 ± 0.5 |
| Leupeptin (1 ug/ml) | 5.9 ± 1.1 | – | 6.6 ± 1.1 | 3.9 ± 0.1 |
| Pepstatin (1 ug/ml) | 3.4 ± 0.3 | – | 4.1 ± 0.3 | 4.1 ± 0.5 |
| Bestatin (0.5 mM) | – | – | – | 3.5 ± 0.6 |
| Eserine (1 mM) | 3.5 ± 1.5 | – | 3.9 ± 1.5 | 1.1 ± 0.1 |
| PCMB (1 mM) | 1.0 ± 0.1 | – | 1.2 ± 0.1 | 0.03 ± 0.02 |
| Pefabloc (1 mg/ml) | 2.2 ± 0.3 | – | 2.3 ± 0.3 | 0.06 ± 0.03 |
| DFP(1 ul/ml) | 2.0 ± 0.1 | – | 1.9 ± 0.1 | 1.6 ± 0.7 |
| TPP (1 ul/ml) | 5.5 ± 0.3 | – | 5.9 ± 0.4 | 1.7 ± 0.4 |

$K_{VACV}$, prodrug VACV degradation rate constant to parent drug ACV; –, no measurable degradation was observed.

Table 2
Metabolic capacity of enzyme inhibitors PCMB and bestatin for VACV and GVACV respectively

| $(\alpha L)^2$ | VACV → ACV | GVACV → VACV |
|---|---|---|
| Control | 8.63 | 1.49 |
| In presence of PCMB | 1.87 | – |
| In presence of 0.05 mM bestatin | – | 0.57 |

The value of $(\alpha L)^2$ indicate metabolic rate of the prodrug to intermediate or parent drug; –, not determined.

esterase inhibitor suggest significant contribution of metabolic barrier on the permeation rate of VACV. Amount of ACV generated was similar in the presence and absence of esterase inhibitor. This observation may result from significant VACV permeation in presence of PCMB thereby leading to more parent drug in the receiver chamber. As mentioned in the methods section, the parameter $(\alpha L)^2$ give an indication of the magnitude of the ratio of metabolic capacity to diffusion rate. Values of $(\alpha L)^2$ are 8.63 for VACV and 1.87 for VACV in presence of PCMB indicating that metabolic rate is significantly diminished in presence of PCMB (Table 2).

GVACV (total) showed decrement in total flux in presence of bestatin compared to control but surprisingly, the flux of GVACV treated with bestatin was similar to GVACV intrinsic flux. VACV generated from GVACV in presence of bestatin was less compared to control, thereby limiting the total flux (sum of GVACV, VACV and ACV fluxes) of GVACV across cornea. GVACV is proven to be less prone to ester hydrolysis so formation of VACV is a limiting factor (Fig. 9B inset). This result also indicates that GVACV by virtue has less permeability than VACV thereby showing GVACV degradation to VACV is the critical step for its permeability. The value of $(\alpha L)^2$ is 1.49 for GVACV and 0.57 for GVACV in presence of bestatin indicating metabolic rate is not significantly decreased relative to VACV (Table 2). Even though $IC_{50}$ values indicate that bestatin is a very potent inhibitor of GVACV hydrolysis relative to PCMB as an inhibitor of VACV hydrolysis, VACV produced high permeability in presence of PCMB compared to GVACV in presence of bestatin. So GVACV does not seem to be a good candidate to modulate its transport or metabolism using inhibitors.

While useful as screening tools, results from homogenate studies may have limited applicability to intact tissues. Free access to all cell surfaces and contents in tissue homogenates may lead to overestimation of the enzymatic activity encountered by a compound diffusing through the intact mucosa. Subcellular distribution of esterases in the bovine eye indicates that esterase activity in the corneal epithelium is 80% due to microsomal fraction and the remaining is present in the mitochondrial and cytoplasmic fractions (Lee et al., 1982a,b). Cornea is a multilayered and complex tissue in nature and epithelium and stroma can retain the compounds depending upon their physicochemical properties like log $P$ or log $D$. Further studies are needed to identify the distance/concentration profiles of the drug/prodrug traversing each layer of the cornea.

Utility of enzyme inhibitors seems to be promising as significant increase in VACV permeability was observed in the presence of PCMB. Uptake profile of glysar in presence of prodrugs and enzyme inhibitors indicate prodrugs interaction with the peptide transporter is not changed in presence of inhibitors. The above results also indicate that the utility of an enzyme inhibitor not only depends on its affinity towards the hydrolyzing enzyme but also on the chemical structure of the prodrug that is being targeted. In conclusion we can evaluate the utility of these enzyme inhibitors to other prodrugs in other tissues by carefully following the results and taking precautions not to overstate the results.

## Acknowledgements

This work was supported by NIH grants RO1 EY09171 and RO1 EY10659. We thank Glaxo Smithkline for their generous gift of VACV.

## Appendix A

1. Aprotinin is a competitive serine protease inhibitor.
2. Bestatin is a competitive and specific inhibitor of leucine aminopeptidase, aminopeptidase B, and triamino peptidase. It shows no inhibition of aminopeptidase A, trypsin, chymotrypsin, elastase, papain, pepsin, or themolysin. It does not inhibit carboxypeptidases.
3. E-64 is an irreversible cysteine protease inhibitor. E-64 will not inhibit serine proteases (except trypsin) like other cysteine protease inhibitors, leupeptin and antipain.
4. Zinc-dependent metalloproteinases, as well as other proteases that are stabilized by calcium, can be effectively inhibited by chelation of divalent metal ions with EDTA.
5. Leupeptin is a reversible, competitive inhibitor of serine and cysteine proteases. It has been reported to inhibit calpain, cathepsin B, H and L, and trypsin.
6. Pepstatin A is an inhibitor of acid proteases (aspartyl peptidases). The inhibitor is highly selective and does not inhibit thiol proteases, neutral proteases, or serine proteases.
7. PMSF is a competitive serine, cysteine protease inhibitor.
8. Pefabloc SC is a irreversible serine protease inhibitor.
9. Eserine is choline esterase reversible inhibitor and TEPP and DFP are choline esterase irreversible inhibitors.
10. PCMB is a carboxyl esterase inhibitor and sulfhydryl group modifier.

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