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Synthesis, physicochemical properties and antiviral activities of ester prodrugs of ganciclovir

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Abstract

The purpose of this study was to synthesize a series of diester prodrugs of ganciclovir (GCV), for improving ocular and oral bioavailability and therapeutic activity. Solubility, log *P*, pH stability profile, in vitro antiviral activity, cytotoxicity, inhibition profile and ocular tissue hydrolysis of the GCV prodrugs were measured. Val–Val–GCV and Val–Gly–GCV diesters were found to exhibit greater aqueous stability compared to Val–GCV and Gly–Val–GCV while ocular tissue hydrolysis demonstrated Val–Gly–GCV and Gly–Val–GCV to be more stable. Val–Val–GCV and Val–GCV diesters were the most lipophilic compounds and were predicted to possess a partition coefficient 295- and 12-fold greater than that of GCV, respectively. All the prodrugs possess much higher aqueous solubility than the parent drug GCV. Ex vivo uptake in the rabbit eye indicates that the prodrugs have high uptake potential. The prodrugs showed no increase in cytotoxicity compared to GCV, instead there was a marked increase in their potency against human cytomegalovirus (HCMV) as well as HSV-1 and HSV-2. This should allow therapeutic response to be seen at a lower concentration that can be achieved more easily, than the drugs currently being used. In conclusion, the diester GCV prodrugs demonstrated excellent chemical stability, high aqueous solubility and markedly enhanced antiviral potency against the herpes viruses without any increase in cytotoxicity.

Keywords: Ganciclovir; Peptide; Diester; Prodrug; Esterification; Antiviral activity

1. Introduction

Diseases that commonly affect the retina include bacterial endopthalmitis and cytomegalovirus (CMV) retinitis. Human cytomegalovirus (HCMV) is an opportunistic herpesvirus that causes serious infections

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in immunologically immature or compromised hosts such as neonates, organ transplant recipients, cancer and AIDS patients (Crowe et al., 1991; Drew, 1992). Cytomegalovirus retinitis develops in about 20–30% with AIDS patients especially with a low CD4 cell count (Luckie, 1995; Masur et al., 1996). HCMV retinitis occurs as a result of viral replication and virus induced inflammation in the retina. The infection is known to first appear in the inner layers of the retina, and then spread outward to all other layers including retinal pigmented epithelium (RPE). Viral replication within retinal endothelial cells is a key factor for progression of the disease and occurs in the retinal endothelial, RPE and retinal glial cells (Burd et al., 1996). Retinal endothelial cells are considered to be the primary sites of retinal infection leading to disruption of the blood–retinal-barrier, which facilitates the spread of the infection towards RPE (Bodaghi et al., 1999). Focal yellowish white granular patches, diffuse edema and retinal hemorrhage with necrosis are symptoms of advanced CMV retinitis, and if left untreated can lead to blindness.

Two main components of the barrier that prevent entry of molecules from the systemic circulation into the ocular compartments are the blood–retinal-barrier (BRB) in the posterior segment and the blood–aqueous-barrier (BAB) in the anterior segment. BRB and the BAB together form the blood–ocular-barrier. The BRB prevents the entry of the drugs from the choroids into the retina. Drug delivery to the posterior segment of the eye, comprising the retina, choroid, vitreous humor and sclera, is one of the most interesting and challenging tasks facing the pharmaceutical industry. The passage of drugs from the anterior chamber to the posterior chamber would not be an efficient process due to the continuous drainage of the aqueous humor.

So far, 9-(1,3-dihydroxy-2-propoxymethyl) guanine (GCV) is the drug of choice for the treatment of CMV retinitis (Laskin et al., 1987; Buhles et al., 1988). In fact, GCV has been reported to be 26 times more potent than acyclovir (ACV) against HCMV in vitro (Morse et al., 1993). However, the bioavailability of orally administered GCV in humans ranged from 2.6 to 7.3% (Spector et al., 1995). GCV, like other nucleoside analogs such as trifluridine (TFT) and ACV, is hydrophilic and possesses poor transcellular permeability across membranes (Kulikowski, 1994). Low ocular bioavailability of ganciclovir is due to its poor aqueous solubility and low lipophilicity (Kenley et al., 1986; Benjamin et al., 1987), which has resulted in formulation difficulties and poor delivery into the eye. The blood–ocular-barriers prevent permeation of therapeutic concentrations of GCV into the anterior and posterior chambers of the eye, following oral or intravenous administration (Arevalo et al., 1995; Cunha-Vaz, 2004). As a consequence, current treatment strategy calls for frequent administration of GCV by intravitreal injections or surgical placement of vitreal implants in conjunction with systemic administration (Henry et al., 1987; Morlet et al., 1996; Cadman, 1997a,b). But these techniques of drug delivery into the eye are associated with high risk of secondary infections, retinal detachment and vitreous hemorrhage (Martin et al., 1997).

Strategies enhancing permeation of GCV into the retinal and corneal tissues can lead to significant improvement in therapeutic options and efficacy, which in turn may lead to reduced frequency of drug administration and/or surgery. Lipophilic prodrug derivatization of hydrophilic compounds is a strategy which can lead to enhanced partitioning across cellular membranes. A series of both mono- and di-acyl ester GCV prodrugs were evaluated in an attempt to improve the ocular bioavailability and efficacy of this antiviral agent (Dias et al., 2002). Although the lipophilic acyl ester prodrugs of GCV had equivalent GCV in vitro antiviral activity, the enhanced lipophilicity and poor aqueous solubility constrained their use as topical, intravitreal or systemic agents. Therefore, transporter targeted prodrug derivatization is a new and exciting strategy currently being explored by researchers (Duvvuri et al., 2003). Under this strategy, prodrugs are designed to target membrane transporter(s) expressed on selected tissues. Following uptake, the prodrug is rapidly converted to the parent drug by cellular enzymes. This strategy may also lead to significant improvement in solubility of the parent drug depending on the promoiety.

For targeted drug delivery, peptide transporters (PepT1 and PepT2) are perhaps the drug transporters that have attracted the most attention due to their wide substrate specificity (Amasheh et al., 1997; Balimane et al., 1998). Several successes in drug delivery have resulted through peptide transporter targeted drug modeling. L-Valine ester prodrugs of

GCV and ACV, valganciclovir (Val–GCV) and valacyclovir (Val–ACV), respectively, demonstrated significant enhancement in intestinal absorption of the parent drug as a result of PepT1-mediated transport (Steingrimsdottir et al., 2000; Sugawara et al., 2000; Hoglund et al., 2001; Reusser, 2001). Since the peptide transporter (PepT1) is expressed on the corneal epithelium, and Val–Val–ACV being a substrate for this transporter, an increased corneal permeation was observed (Anand et al., 2003). The neural retina is a site of active replication of the CMV virus (Burd et al., 1996). Higher intracellular GCV concentrations in the retinal cells resulting from GCV prodrugs may significantly improve efficacy of GCV in CMV retinitis.

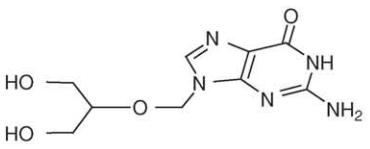
Hence, the overall objective of this study was to synthesize a series of highly water-soluble monopeptide and dipeptide diester GCV prodrugs (Fig. 1), and to delineate their physicochemical properties, pH stability profile, ocular tissue hydrolysis, in vitro antiviral activity and cytotoxicity. We selected Val–Val, Gly–Val and Val–Gly as candidates for the promoiety because we had earlier synthesized prodrugs with these promoieties and they were found to be good candidates (Anand et al., 2003).

2. Materials and methods

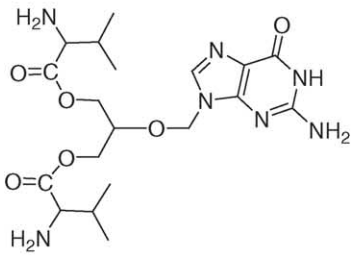
GCV was obtained as a gift from Hoffman La Roche (Nutley, NJ). Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (Ham) (1:1) (D-MEM/F12) and fetal bovine serum (FBS) were purchased from Gibco (Invitrogen Corporation, Grand Island, NY). All other chemicals and solvents were of reagent or analytical grade (Sigma Chemical Company, St. Louis, MO) and used without further purification. Distilled, deionized water was used in the preparation of the buffers and mobile phases. New Zealand male Albino rabbits, weighing 2–2.5 kg (Myrtle's Rabbitry, Thompson Station, TN) were used for ocular studies. pH measurements were made with a Corning Model 125 pH meter equipped with a combination electrode (Corning Science Products, Midfield, MA). Mass spectra of all compounds were obtained by Thermo-Finnigan LC/MS with ESI ion source. Both column and infusion injection method were used to obtain the mass spectra. NMR spectra were collected by Bruker 250 MHz NMR with sample dissolved in d_6-DMSO.

2.1. Synthesis of GCV prodrugs

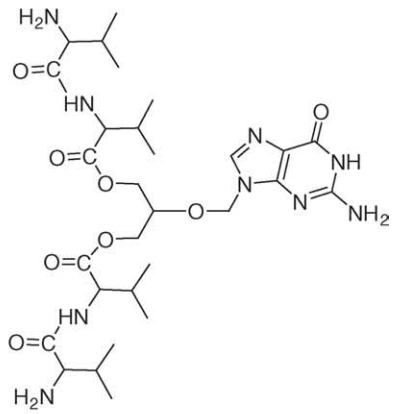
A method for GCV dipeptide synthesis is shown in Scheme 1. BOC–Valine (3.4 g, 15.7 mmol), DCC (1.6 g, 7.8 mmol) and 20 ml of anhydrous dimethyl formamide (DMF) were added to a 100 ml flask and the mixture was stirred for 1 h at 0 °C under nitrogen. The white precipitate of dicyclohexylurea (DCU) was generated during the stirring. To the reaction flask, a DMF solution of 1.0 g of GCV (3.9 mmol) and 0.24 g 4-dimethlyamino pyridine (DMAP, 2 mmol) was added drop-wise. The reaction mixture was kept at 0 °C for 1 h and was stirred continuously for 24 h at room temperature. The urea derivative was removed by filtration. After DMF was removed in vacuum, 30 ml of ethyl acetate was added to the residue. This solution was washed with 10 ml of saturated sodium bicarbonate solution and 20 ml of brine, respectively. The organic layer was collected and dried over $MgSO_4$. After removal of most of the solvent, the residue had been loaded onto the silica gel column and purified with 1:7 mixture of methanol/methylene chloride. The resulting white solid, BOC–Val–GCV diester was dissolved in 20 ml of TFA and stirred at 0 °C for 1 h to remove the BOC group. Following removal of the TFA in vacuum, 20 ml of ethyl acetate was added to the oily reside. The solution was then added drop-wise to the cold diethyl ether. After filtration, 2.5 g of Val–GCV diester (di-TFA salt) was collected as white solid with high purity (>95%). Four milliliters of triethylamine (TEA, 28 mmol) was added to Val–GCV diester and kept for 15 min before dissolving it in 15 ml DMF. Mixture was added drop-wise to the BOC–glycine anhydride solution prepared by the same method described previously and stirred overnight at room temperature. The same purification and deprotection procedure as mentioned previously was followed. Overall, 2.54 g of pure Gly–Val–GCV diester (di-TFA salt) in white power was collected with a yield of 82% (from GCV). The synthetic procedure for Val–Val–GCV was essentially the same as described for Gly–Val–GCV diester, whereas the method for Val–Gly–GCV diester only differed in that it was started with the synthesis of Gly–GCV diester as shown in Scheme 2 (Nashed and Mitra, 2003; Gao, 2000).



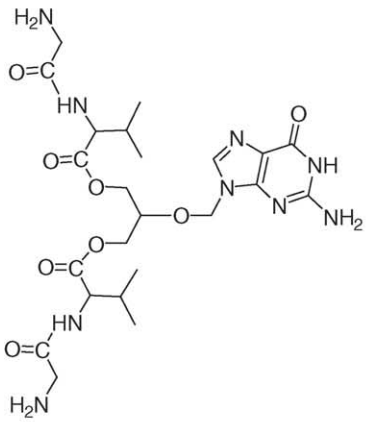
Ganciclovir (GCV)



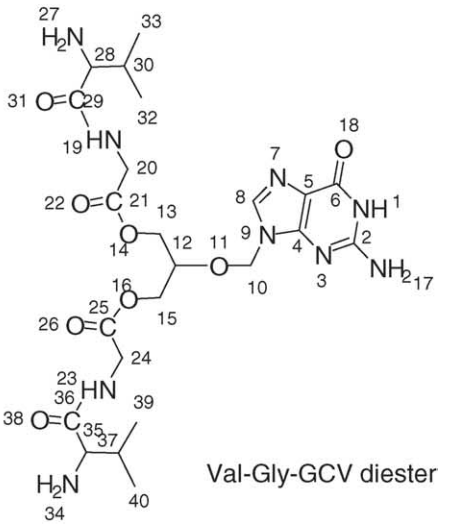
Val-GCV diester



Val-Val-GCV diester

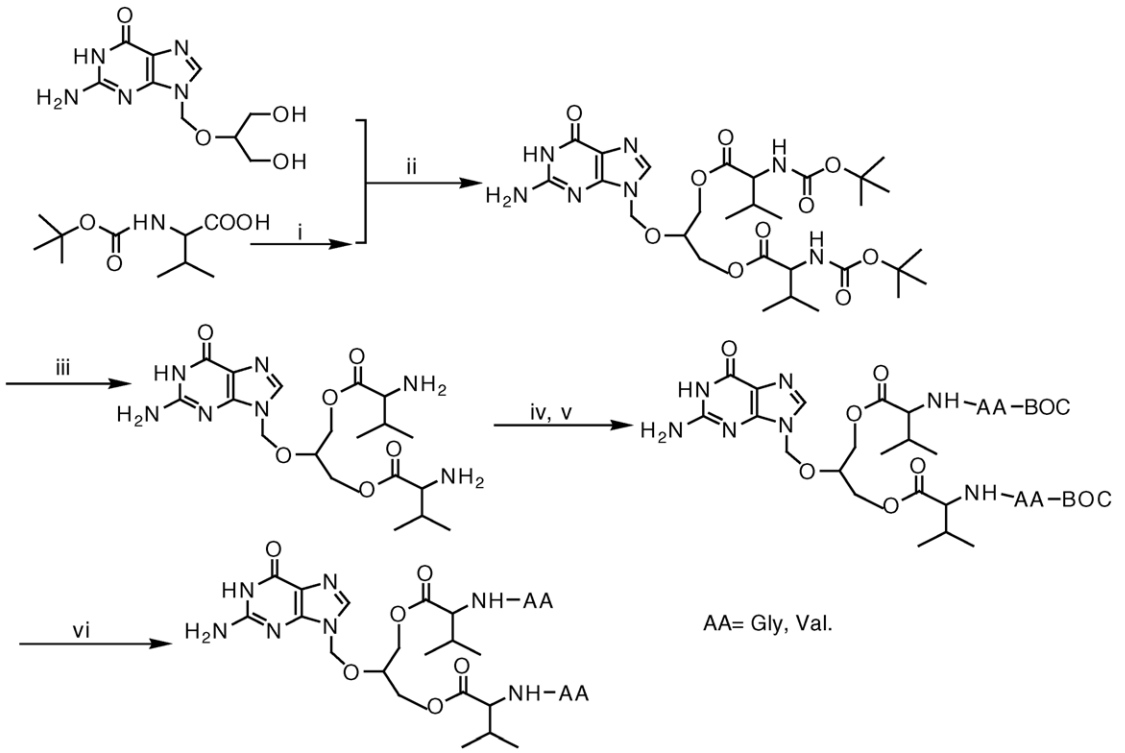


Gly-Val-GCV diester



Val-Gly-GCV diester

Fig. 1. Structures of ganciclovir (GCV) and its peptide diester prodrugs.



(i) DCC, DMF 0°C, 1h; (ii) DMAP, DMF, rt, 24 h; (iii) TFA, 0°C, 1h; (vi)TEA, 15 min; (v)BOC-AA anhydride, DMAP, rt, 24 h; (vi) TFA, 0°C, 1h.

(A)

Val-GCV diester

ESI-MS m/z (MH^+): 454.4; Calculated for $C_{19}H_{31}N_7O_6$: 453.2.

^{1}H NMR (DMSO-d_6): δ 0.92 (12H, d, C**H**$_3$), 2.09 (2H, m, C**H**$(CH_3)_2$), 3.80 (2H, m, C**H**NH_2), 4.21-4.37 (5H, m, C**H** + C**H**$_2$O-Val), 4.37 (2H, m, C**H**NH_2), 5.48 (2H, s, C**H**$_2$O), 6.65 (2H, s, N**H**$_2$), 7.83 (1H, s, C**H**), 8.55 (4H, bd, CHN**H**$_2$), 10.90 (1H, s, N**H**).

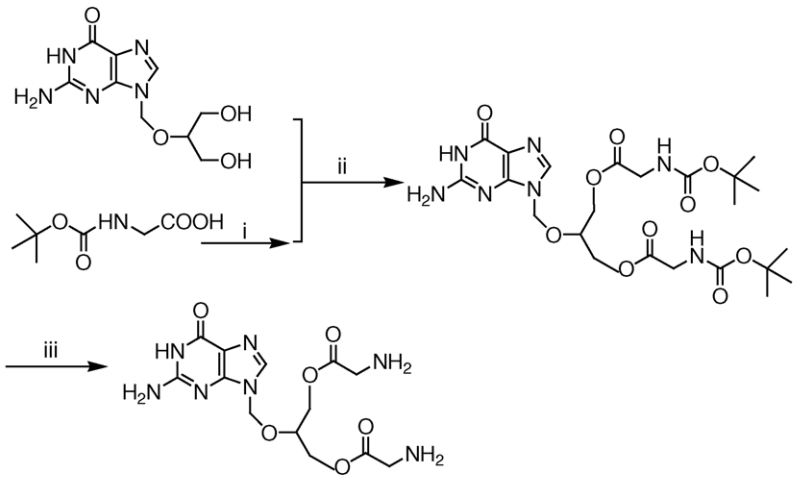
Val-Val-GCV diester

ESI-MS m/z (MH^+): 652.5; Calculated for $C_{29}H_{49}N_9O_8$: 651.4

^{1}H NMR (DMSO-d_6): δ 0.89 (24H, m, C**H**$_3$), 2.04 (4H, m, C**H**$(CH_3)_2$), 3.71 (4H, m, C**H**NH + C**H**NH_2), 4.07-4.22 (5H, m, C**H**$(CH_2)_2$ + C**H**$_2$O-Val), 5.44 (2H, s, C**H**$_2$O), 6.45 (2H, s, N**H**$_2$), 7.76 (1H, s, N**H**), 8.07 (4H, bd, CHN**H**$_2$). 8.52 (2H, bd, CHN**H**), 10.71 (1H, s, N**H**).

(B)

Scheme 1. Synthesis of GCV prodrugs (A) and NMR and mass spectrophotometer data (B).



(A) (i) DCC, DMF, 0°C, 1h; (ii) DMAP, DMF, rt, 24 h; (iii) TFA, 0°C, 1h.

Val-Gly-GCV diester

ESI-MS m/z (MH^+): 568.4; Calculated for $C_{23}H_{37}N_9O_8$: 567.3.

^{1}H NMR (DMSO-d_6): δ 0.97 (12H, d, C**H**$_3$), 2.10 (2H, m, C**H**$(CH_3)_2$), 3.66-3.94 (6H, m, C**H**NH_2 + **C**H_2NH_2), 4.10-4.19 (5H, m, C**H**$(CH_2)_2$ + CH_2O-Gly), 5.47 (2H, s, CH_2O), 6.62 (2H, s, NH_2), 7.84 (1H, s, CH), 8.17 (4H, bd, CHNH_2), 8.89 (2H, d, CH_2N**H**), 10.84 (1H, s N**H**).

Gly-Val-GCV diester

ESI-MS m/z (MH^+): 568.5; Calculated for $C_{23}H_{37}N_9O_8$: 567.3.

^{1}H NMR (DMSO-d_6):δ 0.88 (12H, d, C**H**$_3$), 2.06 (2H, m, C**H**$(CH_3)_2$), 3.68 (6H, m, C**H**NH + CH_2NH_2), 4.12-4.29 (5H, m, C**H**$(CH_2)_2$ + CH_2O-Val), 5.48 (2H, s, CH_2O), 6.65 (2H, s, NH_2), 7.79 (1H, s, C**H**), 8.23 (4H, bd, CH_2NH_2), 8.70 (2H, d, CHN**H**), 10.98 (1H, s, NH).

(B)

Scheme 2. Synthesis of Gly–GCV diester (A) and NMR and mass spectrophotometer data (B).

2.2. Physicochemical properties

2.2.1. Aqueous solubility studies

Aqueous solubility studies were carried out at pH 3.4, in phthalate buffer system of strength 62.5 mM. Excess amount of drug was added to 1 ml buffer in glass vials and placed in a shaking water bath at 25 °C and 60 rpm for 24 h. At the end of this time period solutions were centrifuged at 12,500 rpm for 10 min and the supernatant filtered through 0.45 μm Nalgene syringe filter membrane. The pH was again determined to check for any change but there was only a slight change in the pH. The analysis of the samples was done within 15–20 min of taking the sample to prevent any precipitation. Initial 0.5 ml of the filtrate was discarded as an added precaution to avoid adsorption of the prodrugs onto the filter membrane. Solubility studies were carried out in duplicate.

Drug concentration was determined using HPLC analysis.

2.2.2. Partition coefficient

log *P* (*n*-octanol/water) values were estimated using ACD Labs Software, ACD/I-Lab Web Service (ACD/LogP 7.04).

2.2.3. pH stability studies

Chemical stability of the GCV prodrugs was examined at different pH values: 1, 3, 5–9. Phthalate (pH 3 and 5), phosphate (pH 6 and 7) and borate (pH 8 and 9) buffers were used for this study. The pH of solution was adjusted to within ±0.02 units of the target with sodium hydroxide/hydrochloric acid in all cases. Hydrochloric acid was used for preparing buffer of pH 1. The degradation profile of each prodrug was studied at three different buffer strengths (25, 50 and 62.5 mM). Ionic strength of all the buffers was adjusted to 0.1 M with KCl. Aliquots (9.9 ml) of the buffers were taken in glass vials and allowed to equilibrate to 37 °C in a water bath. Stock solutions (10 mg/ml) of each of the prodrugs were prepared in water immediately before initiation of an experiment. One hundred microliters of the stock solution was then added to 9.9 ml of buffer (previously warmed to 37 °C) to yield a final prodrug concentration of 100 μg/ml. The vials were placed in a shaking water bath at 37 °C and 60 rpm for the duration of the experiment. Samples (100 μl) were withdrawn at predetermined time intervals and immediately stored at −80 °C until further analysis. The frozen samples were thawed and analyzed immediately by HPLC. All experiments were carried out in triplicate.

2.2.4. HPLC analysis

Stability and solubility samples were analyzed by an HPLC system comprising of HP 1050 series quaternary gradient pump, Alcott 718AL refrigerated autosampler, HP 1100 series fluorescence detector (λ excitation 265 nm and λ emission 380 nm), HP 3395 integrator, Phenomenex C18 (4.6 × 250) column and C18 guard column. Mobile phase comprised of 15 mM phosphate buffer (pH 2.5) and acetonitrile. Proportion of acetonitrile was varied depending upon the prodrug being analyzed. The analytical method was validated with respect to precision (%R.S.D. < 1) linearity ($r^2 > 0.998$), reproducibility (%R.S.D. < 2) and selectivity (no interfering peaks were observed).

2.3. Ocular tissue hydrolysis studies

2.3.1. Preparation of ocular tissues

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 physiological saline and blotted dry. An incision was made on the scleral–limbus junction, to separate the posterior segment of the eye. Vitreous humor was withdrawn using a tuberculin syringe. The neural retina, retinal pigmented epithelium–choroid and sclera were subsequently removed. All the tissues were stored at −80 °C prior to use. The tissues were homogenized in 10 ml chilled (4 °C) isotonic phosphate buffer solution (IPBS) for about 4 min with a tissue homogenizer in an ice bath. The homogenate was centrifuged at 12,500 rpm for 25 min at 4 °C to remove debris, and the supernatant thus obtained was used for hydrolysis studies. Protein content of each of the supernatant was determined by using a BioRad assay with bovine serum albumin as the standard.

2.3.2. Hydrolysis procedure

The supernatant was equilibrated at 37 °C in a water bath for about 30 min prior to an experiment. The study was initiated by adding 0.6 ml of prodrug in IPBS solution (0.1 mM) to 2.4 ml of the supernatant. The control consisted of 0.6 ml prodrug solution and 2.4 ml of IPBS instead of the supernatant. Samples (50 μl) were withdrawn at appropriate time intervals, immediately diluted with 50 μl chilled methanol to terminate the reaction and then stored at −80 °C until further analysis. The samples were thawed and centrifuged at 10,000 rpm for 10 min prior to analysis by HPLC for the intact diester prodrug, the regenerated monoester and the parent drug GCV. Apparent first-order rate constants were calculated and corrected for any chemical hydrolysis observed with the control.

2.4. Cell proliferation assay

Cytotoxicity of the GCV prodrugs was further evaluated by cell proliferation assay employing ARPE-19 cells. This cell line of human RPE origin, has been widely used as an in vitro model for native

RPE (Dunn et al., 1996). Cell titer 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI) was used for this purpose. ARPE-19 cells were plated at passages 24–30 in 96-well plates. Solutions of GCV and the prodrugs (100 μM–2 mM) were prepared in culture medium. Aliquots of prodrugs (100 μl) were added in culture medium in each well and cells were incubated along with the drug solution in a humidified, 5% CO_2 atmosphere for 24 h. The effects of GCV prodrugs on the proliferation of ARPE-19 cells were observed as a function of drug concentration. Proliferation of ARPE-19 cells in the presence of different concentrations of GCV and its prodrugs were compared with a positive control (without drug). Wells containing only medium were used as a blanks (negative control). Color determination was carried out at 485 nm (reference at 590 nm) using a 96-well microtiter plate reader (SpectraFluor Plus, Tecan, Switzerland).

2.5. Ex vivo uptake studies in rabbit retina

Uptake studies were conducted using rabbit retina ex vivo (Majumdar et al., 2004). Male albino rabbits weighing 2–2.5 kg were used for the studies. Animals were anesthetized with Ketamine HCl (35 mg/kg) and Xylazine (5 mg/kg) administered intramuscularly and then euthanized by a lethal injection of sodium pentobarbital to the marginal ear vein. Each eye was immediately enucleated, and the ocular surface was rinsed with Dulbecco's phosphate buffered saline (DPBS), pH 7.4 (130 mM NaCl, 0.03 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), to remove any blood, vitreous or extraneous debris. For separating the posterior segment of the eye, an incision was made on the scleral–limbus junction. The posterior segment, with retina facing inside, was then placed in a small glass well such that the tissue assumed the shape of a cup, where the drug could be placed and the uptake study could be conducted. Ex vivo experiments were conducted at 25 °C since higher temperatures resulted in retinal detachment (Majumdar et al., 2004). The radioactive test solution (300 μl) was then placed into the cup and uptake was allowed for 5 min, after which the test solution was removed and the tissue was completely immersed in ice cold stop solution (50 mM KCl and 25 mM HEPES) for 2 min. This step was repeated three times using fresh stop solution, to remove any unbound radioactive material. Then, the neural retina and RPE/choroid were separated from the sclera and were lysed overnight with 1 ml of 0.1% Triton-X solution in 1N NaOH. Aliquots (500 μl) were then transferred to scintillation vials containing 5 ml scintillation cocktail (Fisher Scientific, Fair Lawn, NJ). Samples were analyzed for radioactivity using a scintillation counter (Beckman Instruments Inc., Model LS-6500). Uptake was normalized to protein content of each well. Cell lysate protein content was measured by the method of Bradford (1976) using bovine serum albumin as the standard (Bio-Rad protein estimation kit, Hercules, CA).

2.6. In vitro antiviral activity and cytotoxicity testing

2.6.1. Preparation of human foreskin fibroblast cells

Newborn human foreskins were obtained as soon as possible after circumcisions were performed and had been placed in minimal essential medium (MEM) containing 50 μg vancomycin/ml, 3 μg fungizone/ml, 100 units penicillin/ml and 25 μg gentamicin/ml, for 4 h at room temperature. The medium was then removed, the foreskin minced into small pieces and washed repeatedly until red cells were no longer present. The tissue was then trypsinized using trypsin at 0.25% with continuous stirring for 15 min at 37 °C in a CO_2 incubator. At the end of each 15 min period, the tissue was allowed to settle to the bottom of the flask. The supernatant containing cells was poured through sterile cheesecloth into a flask containing MEM and 10% fetal bovine serum. The flask containing the medium was kept on ice throughout the trypsinizing procedure. After each decanting of cells, the cheese cloth was washed with a small amount of MEM containing serum. Fresh trypsin was added each time to the foreskin pieces and the procedure repeated until no more cells became available. The cell-containing medium was then centrifuged at 1000 rpm at 4 °C for 10 min. The supernatant liquid was discarded and the cells resuspended in a small amount of MEM with 10% FBS. The cells were counted using a Coulter Counter and then placed in an appropriate number of 25 cm^2 tissue culture flasks. As cells became confluent and needed trypsinization,

they were gradually expanded into 175 cm^2 flasks. The cells were maintained on vancomycin and fungizone to passage three. Cell lines were tested periodically for the presence of mycoplasma contamination using the Hoechst fluorescent stain for mycoplasma DNA. Cells were utilized only until passage 10.

2.6.2. Plaque reduction assay

Two days prior to use, Human Fibroblast Foreskin (HFF) cells were trypsinized, counted and plated into six-well plates and incubated at 37 °C with 5% CO_2 and 90% humidity. On the date of assay, the drug was made up at the desired concentration in 2% MEM and then serially diluted 1:5 in 2% MEM to give six concentrations of drug. The drug concentrations utilized were usually 100 μM down to 0.03 μM. The virus to be used was diluted in MEM containing 10% FBS to a desired concentration which will give 20–30 plaques per well. The media was then aspirated from the wells and 0.2 ml of virus was added to each well in triplicate with 0.2 ml of media being added to drug toxicity wells. The plates were then incubated for 1 h with shaking every 15 min. After the incubation period, the drug was applied to each well in 2 ml volume and the plates were incubated for 3 days for HSV-1 and HSV-2, 8 days for HCMV, 7 days for MCMV or 10 days for VZV, after which the cells were stained with a 1.5% solution of neutral red. At the end of the 4–6 h incubation period, the stain was aspirated, and plaques counted using a stereomicroscope at 10× magnification. The cytotoxic concentration which was toxic to 50% of the cells tested (CC_{50}) and effective concentration which produces effect in 50% of the cells tested (EC_{50}) values were determined.

3. Results

3.1. Physicochemical properties

3.1.1. Solubility and log P

Solubility studies were carried out pH 3.4 instead of pH 7.4 because all the prodrugs demonstrated maximum stability at this pH. At pH 7.4, there would be a high amount of degradation of the prodrugs within 24 h. All solubility determinations were carried out after a 24 h equilibration period. Val–GCV diester was observed to possess the highest aqueous solubility amongst all the prodrugs studied (Table 1). All the diester GCV prodrugs demonstrated drastic increase in aqueous solubility compared to GCV. Table 1 summarizes the log *P* and solubility values for the GCV prodrugs. Val–Val–GCV diester was predicted to be the most lipophilic compound of the prodrugs synthesized. The results indicated an almost 295-fold increase in *n*-octanol/water partition coefficient relative to GCV and an almost 12-fold increase over Val–GCV diester. Val–GCV diester resulted in a 23-fold increase in *n*-octanol/water partition coefficient over GCV. However, addition of a glycine (Gly) moiety significantly reduced the lipophilicity of the prodrug with no significant increase in partition coefficient over GCV. There might have been an increase in octanol solubilities compared to GCV, for Gly–Val and Val–Gly diesters as well, which the software program may not have been able to accurately predict.

3.1.2. pH stability profile

Effect of pH on the stability of the GCV prodrugs was examined within the pH range of 1–9. Buffer-free degradation rate constants at each pH, for each GCV prodrug, were determined by back-extrapolating the plots of degradation rate constants versus buffer concentration to the *y*-axis, i.e. zero buffer concentration (Fig. 2). The buffer-free degradation rate constants, indicating rate of loss of the prodrug, have been summarized in Table 2. Apparent first-order degradation kinetics was exhibited by all the prodrugs and ester hydrolysis was observed to be the predominant degradation mechanism.

All compounds exhibited similar rate profiles in the pH range studied rate. All the diester prodrugs studied did not exhibit any appreciable degradation in the

Table 1
Solubility and log *P* values of GCV and its diester prodrugs

Test compound	Solubility (mM)	Predicted log *P*[a]
GCV	7.73 ± 0.20	−2.07 ± 0.68
Val–GCV diester	329.02 ± 16.67	−0.70 ± 0.81
Val–Val–GCV diester	123.45 ± 9.80	0.40 ± 0.94
Gly–Val–GCV diester	87.22 ± 3.34	−2.05 ± 0.94
Val–Gly–GCV diester	203.11 ± 5.25	−2.05 ± 0.94

[a] Predicted value of log *P* was obtained using the ACD/I-Lab Web Service (ACD/LogP 7.04).

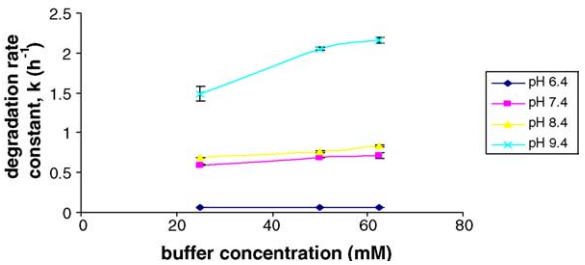


Fig. 2. Buffer independent degradation rate constant for Gly–Val–GCV diester.

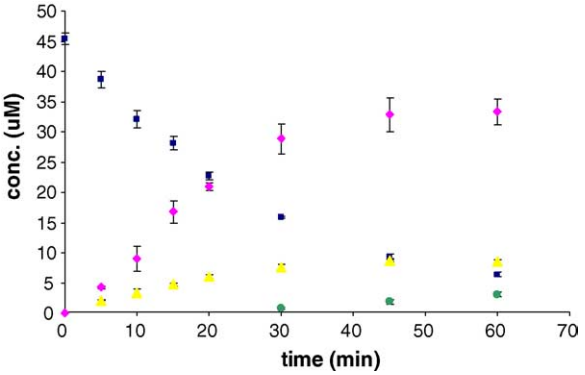


Fig. 3. Gly–Val–GCV diester hydrolysis in neural retina homogenate: (■) Gly–Val–GCV diester, (♦) Gly–Val–GCV monoester, (▲) Val–GCV diester and (●) GCV ($n = 3$).

pH range of 1–5 even after 7 days at 37 °C (data not shown). An increased susceptibility to hydrolysis was observed as the pH was raised towards the alkaline range. The smooth line fitted to the data shows a curvature indicates a greater stability of the protonated form compared to the unprotonated species. These results are consistent with our previous observations with monoacyl ester and monopeptide prodrugs of GCV and ACV (Dias et al., 2002; Anand et al., 2003).

3.2. Ocular tissue hydrolysis

The pseudo-first-order rate constants for the loss of the diester prodrugs in vitreous humor, neural retina, RPE–choroid and sclera have been summarized in Table 3. The dipeptide diester prodrug was initially degraded to dipeptide monoester and monopeptide diester which then further degraded to regenerate the parent drug GCV. A mass balance with respect to concentration of total GCV containing moieties was maintained throughout the experiment. The formation of monoester dipeptide, diester monopeptide metabolites and the parent drug GCV indicate the role of esterase and peptidase enzymes in the metabolism of dipeptide diester prodrugs. While with Val–GCV diester (monopeptide) only monopeptide monoester was formed which on further hydrolysis leads to regeneration of the parent drug GCV. Fig. 3 depicts the concentration profile for the Gly–Val–GCV diester hydrolysis in neural retina homogenate (others not shown).

Table 2
Buffer-free degradation rate constant k ($10^2 \times h^{-1}$) for various prodrugs, representing the loss of prodrug, at different pH values ($n = 3$)

Test compound	pH 6.4	pH 7.4	pH 8.4	pH 9.4
Val–GCV diester	8.21 ± 0.11	24.5 ± 0.81	24.95 ± 0.17	33.91 ± 0.59
Val–Val–GCV diester	0.3 ± 0.001	2.19 ± 0.86	2.48 ± 0.36	15.22 ± 0.03
Gly–Val–GCV diester	6.3 ± 0.16	50.91 ± 1.8	58.28 ± 0.57	104.95 ± 3.6
Val–Gly–GCV diester	0.57 ± 0.02	2.26 ± 0.05	2.56 ± 0.09	4.42 ± 0.15

Table 3
First-order rate constants ($10^2 \times min^{-1}$) for the hydrolysis of diester prodrugs, representing the loss of prodrug, in ocular tissue homogenate of Albino rabbits at 37 °C and pH 7.4 ($n = 3$)

Test compound	Vitreous humor	Neural retina	RPE–choroid	Sclera
Val–GCV diester	0.93 ± 0.38	1.27 ± 0.49	4.62 ± 1.08	1.78 ± 0.56
Val–Val–GCV diester	1.13 ± 0.87	4.35 ± 0.44	5.68 ± 1.39	3.90 ± 1.04
Gly–Val–GCV diester	0.36 ± 0.02	2.13 ± 0.11	2.55 ± 0.91	1.77 ± 0.16
Val–Gly–GCV diester	0.31 ± 0.11	0.77 ± 0.03	2.41 ± 0.4	1.19 ± 0.36

The pseudo-first-order rate constants summarized in Table 3 have been corrected for the chemical hydrolysis determined from the control.

3.3. Cell proliferation

GCV and all the diester prodrugs of GCV tested for cytotoxicity by cell proliferation assay in ARPE-19 cell line did not demonstrate any effect in the concentration range tested (0.1–2 mM). Methotrexate was used as a positive control at 5, 10 and 20 μM concentrations as it is known to be cytotoxic within this range. Table 4 summarizes the percent cytotoxicity of all prodrugs along with those of methotrexate, ACV and GCV. All the dipeptide diester prodrugs were determined to be less cytotoxic than GCV.

3.4. Ex vivo uptake studies

Glycylsarcosine (Gly–Sar) is known to be a model substrate for both peptide transporters PepT1 and Pept2. We have selected [^{3}H]Gly–Sar as a control in all the uptake studies. Various diester prodrugs had been used at 1 mM initial concentration as a competitive inhibitor. All the prodrugs tested showed inhibition (Fig. 4) of Gly–Sar uptake. GCV itself did not show any significant inhibition of Gly–Sar uptake, indicating that it does not involve any peptide transporter.

3.5. In vitro antiviral activity and selectivity

Antiviral potencies of the GCV prodrugs have been summarized in Table 5. All the GCV diesters synthesized exhibited greater potency against HSV-1, HSV-2 and HCMV compared to the drugs ACV and GCV, which are the drugs of choice for HSV-1, HSV-2 and HCMV. There was no increase in potency of these prodrugs against VZV. In fact, Val–Gly–GCV was five times more potent against HSV-1 than ACV. The other GCV prodrugs also demonstrated significant improvement in potency against HSV-1.

Table 4
Percentage cytotoxicity profile of methotrexate, ACV, GCV and various diester prodrugs of GCV at three different concentrations

Compounds	100 μM	1 mM	2 mM
Methotrexate	22.82 (5 μM)	59.46 (10 μM)	100.00 (20 μM)
GCV	24.95	32.93	41.84
ACV	10.75	21.79	39.76
Val–GCV diester	7.43	24.50	33.76
Val–Val–GCV diester	0.58	2.99	5.03
Val–Gly–GCV diester	0.69	1.67	12.32
Gly–Val–GCV diester	0.73	1.54	11.66

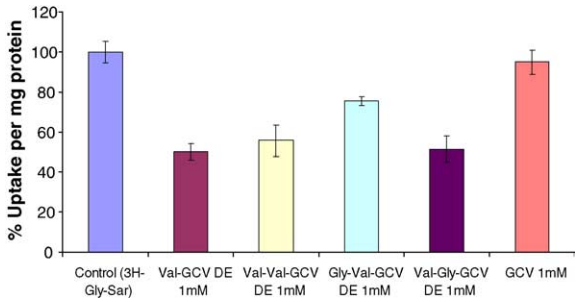


Fig. 4. Ex vivo uptake of 3H–Gly–Sar in rabbit retina ex vivo in the presence of 1 mM of Val–GCV diester, Val–Val–GCV diester, Gly–Val–GCV diester, Val–Gly–GCV diester and GCV.

All the prodrugs are at least six to seven times more potent in their activity than ACV against HSV-2. Diester GCV prodrugs are almost one to two times more potent against HCMV compared to GCV. There was no significant change in the antiviral efficacy against VZV. Although selectivity indices (CC_{50}/EC_{50}) of Val–GCV, Val–Val–GCV, Gly–Val–GCV and Val–Gly–GCV against HSV-1, HSV-2 and HCMV are not much higher than the drugs currently used, yet the CC_{50} values are at least 40–50-fold higher than EC_{50} values, indicating that these diester prodrugs are not cytotoxic to non-virus infected cells.

4. Discussion

The objective of this study was to synthesize a series of mono- and dipeptide diester prodrugs of GCV and to investigate their in vitro antiviral activity, cytotoxicity, peptide transporter affinity, stability and physicochemical properties. The overall aim was to investigate whether these GCV prodrugs are effective in various ocular infections, that are currently seen in immunocompromised patients and to compare the differences in characteristics of the prodrugs synthesized.

NMR data recorded in this research clearly indicated the structure of the prodrugs. The BOC–Valine anhydride could react with GCV at either CH_2–OH or

Table 5
Effective and cytotoxic concentration (μM) at 50% level of ACV, GCV and various prodrugs and their selectivity indices (SI)

Prodrug	HSV-1	HSV-2	HCMV	VZV
ACV	$EC_{50} = 1.3$ $CC_{50} \geq 444$ $SI \geq 341$	$EC_{50} = 4.9$ $CC_{50} \geq 444$ $SI \geq 90$	– – –	$EC_{50} = 3.4$ $CC_{50} \geq 444$ $SI \geq 130$
GCV	– – –	– – –	$EC_{50} = 7.1$ $CC_{50} \geq 392$ $SI \geq 55$	– – –
Val–GCV diester	$EC_{50} = 0.29$ $CC_{50} \geq 147$ $SI \geq 506$	$EC_{50} = 0.44$ $CC_{50} \geq 147$ $SI \geq 334$	$EC_{50} = 2.9$ $CC_{50} \geq 147$ $SI \geq 50$	$EC_{50} = 11.3$ $CC_{50} \geq 147$ $SI \geq 13$
Val–Val–GCV diester	$EC_{50} = 0.56$ $CC_{50} \geq 113$ $SI \geq 201$	$EC_{50} = 0.34$ $CC_{50} \geq 113$ $SI \geq 332$	$EC_{50} = 4.0$ $CC_{50} \geq 113$ $SI \geq 28$	$EC_{50} = 18.4$ $CC_{50} \geq 113$ $SI \geq 6$
Gly–Val–GCV diester	$EC_{50} = 0.38$ $CC_{50} \geq 126$ $SI \geq 331$	$EC_{50} = 0.5$ $CC_{50} \geq 126$ $SI \geq 252$	$EC_{50} = 3.6$ $CC_{50} \geq 126$ $SI \geq 35$	ND[a] ND[a] ND[a]
Val–Gly–GCV diester	$EC_{50} = 0.25$ $CC_{50} \geq 126$ $SI \geq 504$	$EC_{50} = 0.25$ $CC_{50} \geq 126$ $SI \geq 504$	$EC_{50} = 4.6$ $CC_{50} \geq 126$ $SI \geq 27$	$EC_{50} = 4.3$ $CC_{50} \geq 126$ $SI \geq 29$

[a] Not done.

NH_2 sites. However, only the ester linkage was formed in this research, which was supported by the change of chemical shift of CH_2–OH from 3.35 in GCV to 4.1 (after the reaction). The chemical shift of amino acid and peptide were also coincident with predication. It was also observed that the peaks of α-H of amino acid are shift to lower field after the formation of peptide bond by acylation with other amino acid. For example, the chemical shift of α-H for Valine moiety in BOC–Val–Gly (Bachem, King of Prussia, PA 19406), Val–Val and Gly–Val–GCV are very similar with values of 3.8, 3.7 and 3.7, respectively, which are about 0.3 ppm higher than Valine. NH_2 was observed in low field (>8) because all of the prodrugs synthesized in this research were in TFA salt.

Physicochemical data clearly indicate that the lipophilicity of GCV can be significantly enhanced, through peptide prodrug approach, without compromising solubility. In fact, aqueous solubility of all the GCV prodrugs was significantly higher than its parent drug, GCV. As a result, significant increase in transcellular permeability of GCV may be expected upon administration of these prodrugs. Although lipophilic ester prodrug derivatization, employing acyl ester GCV prodrugs, generated similar increase in log *P* values, there was marked decrease in solubility compared to GCV, which caused significant formulation and administration problems (Dias et al., 2002). However the Val–Val–GCV diester, whose predicted log *P* value indicates it to be a lipophilic drug, shows a 16-fold increase in aqueous solubility than the parent drug GCV suggesting greater hydrophilicity, yet it is not more potent or selective than Gly–Val–GCV or Val–Gly–GCV against HSV-1. We believe that the increase in the lipophilicity alone, did not have any significant effect on the antiviral activity but it was the result of the combined effect of transporter affinity, stability in buffer and tissue and other factors that resulted in higher activity for Val–Gly–GCV and Gly–Val–GCV diesters. All the diester prodrugs synthesized have demonstrated much higher solubility compared to GCV, with Val–GCV having the highest solubility (329.02 mM), which is 42 times higher than GCV.

Such an increase in the solubility is in part due to the increase in TFA molecules associated with the diester compared to the monoester. Each diester prodrug is a di cation with 2 molecules of TFA associated with a single molecule of the prodrug, whereas in monoesters there is only one molecule of TFA. There is a

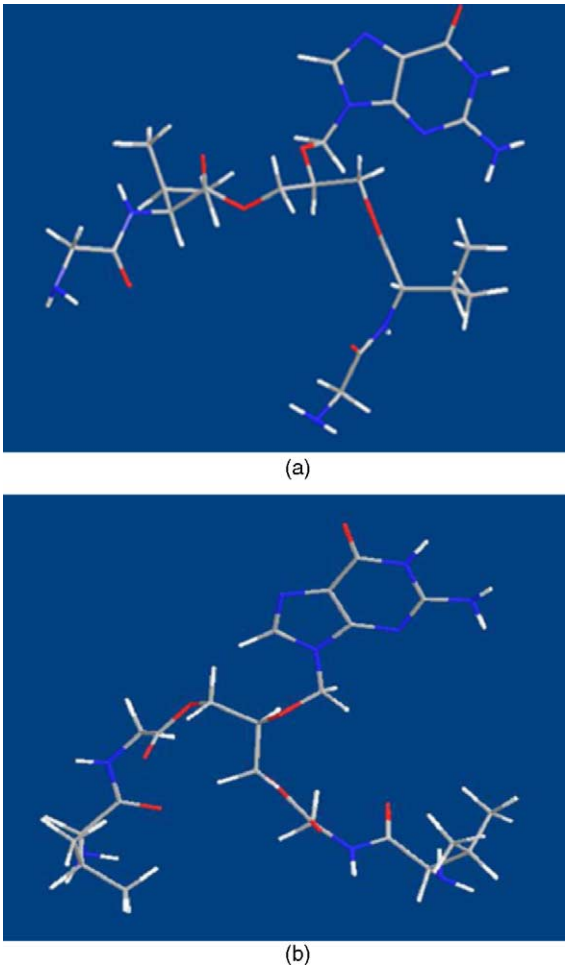


Fig. 5. Three-dimensional representation of the diester prodrugs: (a) Gly–Val–GCV and (b) Val–Gly–GCV.

significant decrease in the solubility of Gly–Val–GCV (87.22 mM) compared to Val–Gly–GCV (203.11 mM) which cannot be attributed to the number of TFA molecules. This is either due to absence of *iso*-propyl moieties at position 20 and 24 of Val–Gly–GCV diester or due to structural confirmation change in the solution form. To confirm our assumption we did a three-dimensional modeling of the two compounds (Fig. 5) and the data obtained indicated there was a difference in their Weiner index value (Wiener, 1947). Val–Gly–GCV had a higher Weiner index value (6404) compared to Gly–Val–GCV (5870). It has been observed that the Weiner index is inversely proportional to molecular compactness (van de Waterbeemd et al., 1995; Fabiana Alves de Lima Ribeiro, 2003). Thus, when the molecule is less compact it is more accessible to the water molecules and hence has higher solubility.

All the peptide diester GCV prodrugs were observed to interact with the ocular peptide transporter with varying extent. It may thus be expected that a marked increase in intracellular drug concentration may be achieved due to the combined effect of higher transcellular diffusion due to enhanced lipophilicity and significant carrier-mediated transport by peptide transporters.

The prodrugs are susceptible to chemical hydrolysis and would regenerate GCV. Moreover cellular enzymes present will also hydrolyze the prodrugs to regenerate GCV. In fact, a hydroxyl group at position 14 or 16 is essential for the GCV's anti-CMV activity (Harnden et al., 1990). Therefore, at least one of the two ester bonds attached to GCV in a diester must be broken to generate a monoester, which can thereby produce antiviral action through phosphorylation or it may be further hydrolyzed into the parent drug, GCV. Thus, the diester prodrugs lead to the generation of monoester GCV and which is further converted to parent drug GCV.

The hydrolysis of the diesters exhibited apparent first-order kinetics. Time-course of various species formation during the enzymatic hydrolysis of the diesters is shown in Fig. 3. A similar hydrolysis scheme was shown for alkyl (Benjamin et al., 1987) and benzoate diesters of GCV (Bundgaard et al., 1991; Jensen and Bundgaard, 1991). The kinetic data suggest that the enzymatic degradation of the ester depends greatly on the structure of the amino substituents, as has been previously observed for ACV (Bundgaard et al., 1991) and corticosteroids (Bundgaard et al., 1989).

Although there was no significant increase in the selective indices of the diester prodrugs against HCMV, yet these prodrugs were found to be more potent in their activity, which indicates that therapeutic concentrations can be easily reached. The diester GCV prodrugs may thus also improve ganciclovir's effectiveness in HCMV retinitis. Moreover, from the drug delivery point of view diesters may possess higher aqueous solubility and binding affinity for the peptide transporter.

These prodrugs have also shown to be more potent in their activity against HSV-1 and HSV-2. The prodrugs

did not show any improvement in potency as well as selectivity for VZV. Therefore, further investigations regarding their use in ocular viral infections should be carried out. These prodrugs appear to be good therapeutic candidates since the compounds exhibited minimal cytotoxicity and high potency.

In conclusion, several of the peptide ester prodrugs were found to interact with the peptide transporter PepT1. Val–Val–GCV and Val–GCV diesters were observed to possess both greater solubility and lipophilicity compared to the parent drug, GCV. Moreover, Val–Val–GCV, Val–GCV and Val–Gly–GCV diesters exhibited greater aqueous stability compared to Gly–Val–GCV diester and did not demonstrate any degradation at low pH values even after 7 days at 37 °C. Cytotoxicity of the prodrugs were comparable to that of the parent drug GCV indicating that prodrug derivatization did not lead to increased cytotoxicity. Considering the physicochemical parameters, aqueous stability, peptide transporter affinity and antiviral activity, diester prodrugs appear to be ideal candidates for drug delivery following oral and ocular administrations.

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