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Prodrug approach to improve absorption of prednisolone

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Abstract

Amino acid and dipeptide prodrugs have been developed to examine their potential in enhancing aqueous solubility and permeability as well as to bypass P-glycoprotein (P-gp) mediated cellular efflux of prednisolone. Prodrugs have been synthesized and identified with LC/MS/MS and NMR. Prodrugs displayed significantly higher aqueous solubility relative to prednisolone. These compounds also exhibited higher stability under acidic conditions relative to basic medium. [14]-Erythromycin uptake remained unaltered in the presence of valine-valine-prednisolone (VVP) indicating lower affinity towards P-gp. Moreover, VVP generated significantly higher transepithelial permeability across MDCK-MDR1 cells compared to prednisolone. Importantly, [3H]-GlySar uptake diminished significantly in the presence of VVP indicating high affinity towards peptide transporters. Moreover, prednisolone was regenerated from VVP due to enzymatic hydrolysis in SIRC cell homogenate. Results obtained from these studies clearly suggest that peptide transporter targeted prodrugs is a viable strategy to improve aqueous solubility and overcome P-gp mediated cellular efflux of prednisolone.

Keywords

Prodrugs; prednisolone; peptide transporters; efflux; homogenate

1. Introduction

Topical delivery of glucocorticoids is one of the most preferred and convenient method of administration to treat anterior ocular inflammatory conditions. Despite being highly patient compliant, topical administration faces numerous challenges such as rapid tear turnover, drainage to systemic circulation and non-specific absorption in other ocular tissues (Hariharan et al., 2009a). In addition, corneal epithelium expresses efflux proteins such as P-gp (Dey et al., 2003; Verstraelen and Reichl, 2013) and multidrug resistance associated proteins (MRPs) (Karla et al., 2007a; Karla et al., 2007b; Karla et al., 2009; Verstraelen and Reichl, 2014). These drug efflux pumps play an active role in exporting drug molecules

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from cornea to tear film resulting in poor drug accumulation in anterior ocular tissues (Dey et al., 2004; Hariharan et al., 2009a; Hariharan et al., 2009b). Cumulative effects of these barriers cause very poor ocular absorption (<5%) of topically applied therapeutic agents.

Prednisolone, a corticosteroid is currently administered to treat ocular inflammation (Thomas and Melton, 1992). Prednisolone generates anti-inflammatory effects by binding to glucocorticoid receptors, thereby triggering signal transduction pathways (De Bosscher and Haegeman, 2009). In spite of high efficacy, ocular absorption of this steroid is very limited from topical administration. In addition to poor aqueous solubility, prednisolone is a known substrate of P-gp (Hariharan et al., 2009a; Karssen et al., 2002; Nakayama et al., 1999; Oka et al., 2002; Troutman and Thakker, 2003; Van der Heyden et al., 2012). Moreover, an optimum balance between hydrophilicity and lipophilicity is required to permeate cornea, which is lacking in prednisolone. Hence, an effective strategy to address challenges associated with topical prednisolone administration has been presented in this article.

Transporter targeted prodrug delivery approach has received considerable attention to improve corneal absorption of poorly permeable drugs (Anand et al., 2004; Gunda et al., 2006; Katragadda et al., 2006; Majumdar et al., 2009; Suresh et al., 2010; Vadlapudi et al., 2013; Vadlapudi et al., 2012; Vooturi et al., 2012). This approach involves chemical derivatization of drugs with membrane (influx) transporter targeting ligands. Among the basis of targeting ligands selection, the solubility of parent drug can also be improved significantly (Majumdar et al., 2005). Peptide transporters (PEPT1 and PEPT2) have been extensively utilized for prodrug derivatization due to their high substrate affinity and broad specificity. These transporters are primarily responsible for the transport of small peptides such as di and tri-peptides. The structure, function, mechanisms and substrate specificity have been widely explored. Peptide transporters are highly expressed on cornea (Anand and Mitra, 2002; Kadam et al., 2013; Xiang et al., 2009). These transporters have been targeted to improve transcorneal absorption of poorly permeable drugs such as acyclovir (Anand et al., 2006; Anand and Mitra, 2002) and ganciclovir (Gunda et al., 2006; Majumdar et al., 2005) in our laboratory. Valine-valine-acyclovir and valine-valine-ganciclovir prodrugs demonstrated significantly higher aqueous solubility and transcorneal permeability compared to parent drugs i.e., acyclovir and ganciclovir (Anand et al., 2003; Majumdar et al., 2005). Moreover, valine based dipeptide prodrugs appear to substantially bypass P-gp mediated cellular efflux (Agarwal et al., 2008; Jain et al., 2005; Katragadda et al., 2006; Wang et al., 2012).

The primary objective of this study is to develop prodrugs of prednisolone to improve aqueous solubility, corneal permeability and circumvent P-gp mediated cellular efflux. Valine-valine-prednisolone (VVP) and valine-prednisolone (VP) have been synthesized. Interactions of VVP, VP and prednisolone with P-gp have been examined by uptake and transport studies across MDCK-MDR1 cells. This transfected cell line has been selected as it has been extensively employed to delineate P-gp interaction and transport of a wide range of compounds in our laboratory (Jain et al., 2004; Luo et al., 2011; Minocha et al., 2012; Patel et al., 2014a; Patel et al., 2014b; Patel et al., 2014c; Vadlapatla et al., 2011). The regeneration of prednisolone from VVP has been examined by conducting SIRC cell homogenate study.

2. Materials and Methods

2.1. Materials

Prednisolone and Boc-L-valine were purchased from Sigma-Aldrich (St. Louis, MO) and Bachem respectively. [3H]-Glysar (specific activity: 4Ci/mmol) and [14C]-Erythromycin (specific activity: 51.3 mCi/mmol) were obtained from Moravek Biochemicals (Brea, CA, USA). MDCK cells, retrovirally transfected with the human MDR1 cDNA (MDCKII-MDR1) were generously donated by Drs. A. Schinkel and P. Borst (Netherlands Cancer Institute, Amsterdam, Netherlands). SIRC cell line was purchased from American Type Culture Collection (CCL-60; ATCC, Rockville, MD). Growth medium Dulbecco's modified Eagle's Medium (DMEM), Triple Express Trpsin®, non-essential amino acids, minimum essential medium (MEM), were procured from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta biological. Penicillin, triton X-100, HEPES, D-glucose, streptomycin, sodium bicarbonate, cyclosporine A, GF 120918, 4-(N,N-dimethylamino) pyridine (DMAP), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), triethyl amine (TEA), trifluoroacetic acid (TFA), sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate (Na₂HPO₄), potassium phosphate (KH₂PO₄), calcium chloride (CaCl₂), magnesium sulfate (MgSO₄), glucose and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Culture flasks (75 and 25 cm²), 12-well plates (3.8 cm² growth area/well) and Transwells® (Costar) were obtained from Fisher Scientific (Houston, TX, USA). All chemical agents procured were of special reagent grade and utilized without any further purification.

2.2. Methods

2.2.1. Synthesis of VVP—VVP was synthesized according to a procedure previous published from our laboratory (Fig. 1) (Agarwal et al., 2008). To synthesize VVP, VP was first synthesized by conjugating L-valine to prednisolone with an ester coupling agent such as EDC and DMAP. VP was then conjugated to L-valine to generate VVP using amide coupling agents, EDC and triethylamine (TEA). In a round bottom flask, Boc-Val-OH (346mg, 1.59mmol) and EDC (304 mg, 1.59 mmol) was dissolved in anhydrous dimethyl formamide (DMF) and stirred at 0 °C for 45 min under nitrogen atmosphere (mixture 1). In a second round bottom flask, prednisolone (300 mg, 0.83 mmol) and DMAP (120 mg, 0.98 mmol) were dissolved in DMF at room temperature for 30 min to activate the terminal hydroxyl group of prednisolone (mixture 2). Mixture 2 was then added dropwise to the mixture 1 with the help of a syringe under constant stirring. The mixture was stirred for 24 h under nitrogen atmosphere. The reaction mixture was filtered and DMF was evaporated under reduced pressure to obtain crude product. The product (Boc-VP) was purified with silica based column chromatography by 5% methanol in dichloromethane (DCM) as eluent. Boc-VP was deprotected by the addition of 1:1 TFA/DCM at 0 °C over 50 min. VP was further purified by recrystallization in cold diethyl ether. The solvent was evaporated under reduced pressure to obtain a final dried product. The yield obtained was 86%.

VVP was generated by conjugating L-valine to VP with amide coupling agents such as EDC and TEA under similar conditions. TEA was added in the reaction medium to activate the amine group in VP. The product Boc-VVP was purified with column chromatography with

3% methanol in DCM as eluent. VVP was deprotected with 1:1 TFA/DCM at 0 °C for 50 min. The final product (VVP) was purified by recrystallization with cold diethyl ether. The yield obtained was 78%.

2.2.2. Identification of VP and VVP—VP and VVP were identified by LCMS and NMR analysis. LC/MS (M/z) for VP and VVP was +460.4 and +559.5, respectively. ¹H NMR analysis for VP (400MHz, DMSO-d₆) δ: 0.80 (s, 3H), 0.90–0.95 (m, 1 H), 0.91–1.02 (m, 9H) 1.18–1.65 (m, 14H) 1.68–2.40 (m, 4H), 4.18 (s, 1H), 5.23 (s, 2H), 5.90 (s, 1H), 6.17 (d, 1H), 7.25 (d, 1H), 8.05 (s, 2H) ¹³C NMR analysis for VP (100MHz, DMSO-d₆): 16.7, 17.5, 19.0, 20.1, 23.8, 27.6, 31.2, 31.8, 32.0, 32.5, 32.8, 33.2, 42.1, 47.2, 48.3, 49.8, 56.4, 67.5, 90.5, 121.5, 127.6, 152.4, 167.2, 169.0, 184.1, 203.7. ¹H NMR for VVP (400MHz, DMSO-d₆) δ: 0.80 (s, 3H), 0.90–0.95 (m, 1 H), 0.91–1.02 (m, 15H) 1.18–1.65 (m, 14H) 1.68–2.40 (m, 5H), 3.60 (m, 1H), 3.80 (s, 1H), 5.11(d, 2H), 5.23 (s, 2H), 5.90 (s, 1H), 6.17 (d, 1H), 7.25 (d, 1H), 8.05 (s, 1). ¹³C NMR for VVP: (100MHz, DMSO-d₆): 16.7, 17.5, 18.5, 19.0, 20.1, 23.8, 27.6, 30.5, 31.2, 31.8, 32.0, 32.5, 32.8, 33.2, 42.1, 47.2, 48.3, 49.8, 57.0, 59.9, 67.5, 90.5, 121.5, 127.6, 152.4, 167.2, 169.0, 172.5, 184.1, 203.7.

2.2.3. Cell Culture—MDCK-wild type (MDCK-WT) and MDCK-MDR1 cells (passage 6–10) were cultivated in 75 cm² tissue culture flask in DMEM medium supplemented with 10% non-essential amino acids, 10% FBS (heat inactivated), HEPES (20mM), penicillin (100 units/mL) and streptomycin (100 µg/mL), pH 7.4. SIRC cells were cultured according to the protocol provided by ATCC. Cells were grown in MEM supplemented with 10% calf serum, lactalbumin (1.76 mg/mL), HEPES (1.3 mg/mL), penicillin (100 units/mL) and streptomycin (100 µg/mL), pH 7.4. Cells were maintained at 37 °C in an atmosphere of 5% CO₂ and 90% relative humidity. Growth medium was replaced on every alternate day until cells reached 90% confluency (6–7 days for MDCK-MDR1 cells and 8–10 days for SIRC cells).

2.2.4. Aqueous solubility studies—Aqueous solubility studies were carried out by adding 5 mg of the compound to 0.5 mL distilled deionized water (DDW) and vortexed vigorously to generate a saturated solution. Tubes were placed in shaking water bath (60 rpm) at 25 °C for 4 h. Then the tubes were centrifuged at 12,500 rpm for 10 min to separate undissolved compound. The supernatant was carefully collected and filtered through 0.45 µm Nalgene syringe filter membrane. Appropriate dilutions were made to the supernatant and concentration was determined by reversed phase HPLC analysis.

2.2.5. Buffer stability studies—Chemical hydrolysis of prodrugs was determined under various pH conditions (3.4, 5.4 and 7.4). Briefly, prodrugs (20 µg/mL) were dissolved in approximately 5 mL of buffer solutions in 15 mL centrifuge tubes. Tubes were placed in shaking water bath (PolyScience®) set at 37 °C and 60 rpm. Samples (100 µL) were withdrawn at predetermined time points and stored at – 80° C until further analysis. Prodrug concentrations remaining were plotted against time in order to calculate degradation rate constants.

2.2.6. Cell cytotoxicity—Cytotoxicity of drug/prodrug was determined in MDCK-WT cells using lactate dehydrogenase (LDH) based cytotoxicity detection kit (Takara Bio Co. St

Louis, MO). Briefly, cells were seeded in 96 well culture plates at a density of 20,000 cells per well in DMEM medium supplemented with 10% non-essential amino acids, 10% FBS (heat inactivated), HEPES (20mM), penicillin (100 units/mL) and streptomycin (100 µg/mL), pH 7.4 and incubated for 12 h at 37°C prior to drug/prodrug treatment. Following incubation, the medium was removed and 200 µL assay medium (serum free DMEM) containing five different drug/prodrug concentrations (5–250 µM) was added into each well. Cells were incubated with drug/prodrug solution for 4 h at 37 °C, 90% humidity and 5% CO₂. The assay medium and 1% Triton x-100 were selected as negative and positive control, respectively. LDH released from cells was determined according to a manufacturer's protocol. Absorbance was measured with the help of a microplate reader (Biorad, Hercules, CA) at a wavelength of 450 nm.

2.2.7. Uptake studies—Cellular uptake studies were performed according to a previously published protocol from our laboratory with little modifications (Patel et al., 2012). Briefly, cells were plated at a density of 3×10^6 in 12 well culture plates. On reaching confluency, medium was removed and cell monolayer was washed with 2 mL of Dulbecco's phosphate buffered saline (DPBS) for three times (each wash of 10 min). Uptake studies were initiated by incubating cells with radioactive solution at 37 °C for 20 min. Following incubation, radioactive solution was quickly removed and plates were rinsed three times with ice-cold stop solution (210 mM KCl, 2 mM HEPES, pH of 7.4) to terminate further uptake. One mL of lysis buffer (0.1% Triton-X solution in 0.3% NaOH) was added to each well and cells were allowed to lyse overnight at room temperature. About 500 µL solutions were transferred to scintillation vial containing 3 mL of scintillation cocktail and vortexed vigorously. The radioactivity associated with cells was analyzed using a scintillation counter (Beckman Instruments Inc., Model LS-6500; Fullerton, CA). Uptake rate was normalized to protein count which was measured using BioRad protein estimation kit (BioRad protein; Hercules, CA).

2.2.8. Transport studies—Transepithelial transport studies were conducted across MDCK-MDR1 cells according to the procedure previously published from our laboratory with minor modifications (Agarwal et al., 2008). Briefly, MDCK-MDR1 cells were seeded at the density of 3 million in 12-well Transwell® plates. Monolayer integrity was determined by measuring transepithelial electric resistance (TEER), with an EVOM (epithelial volt ohmmeter from World Precision Instruments, Sarasota, FL). TEER values obtained for each cell monolayer were in the range of 200–250 Ω cm². Prior to the initiation of the study, cell monolayers were washed thoroughly with DBPS (pH 7.4) at 37 °C, three times (each wash of 10 min). For determining A-B permeability, 0.5 mL drug/prodrug solution (100 µM) was added in the apical chamber of 12-well Transwell® plates and 1.5 mL of drug/prodrug free DPBS was added to the receiving chamber. To determine B-A transport, 1.5 mL drug/prodrug solution (100 µM) was added in the basolateral chamber and 0.5 mL drug/prodrug free DPBS was added in the apical chamber. Transport study was performed for a period of 3 h at 37 °C. At predetermined time points, approximately 200 µL of sample was withdrawn from the receiving chamber and replaced with equal volumes of fresh DPBS to maintain sink conditions. Samples were stored at –80 °C until further analysis by LC/MS/MS.

2.2.9. SIRC cell homogenate study—SIRC cell homogenate study was carried out according to the method previously published from our laboratory with little modifications (Agarwal et al., 2008). Briefly, SIRC cells were washed three times with DPBS (pH 7.4) at 37 °C (ten min each wash). Cells were collected with mechanical scrapper in 2 volumes of DPBS and homogenized in Multipro variable speed homogenizer (DREMEL, Racine, WI). Tubes were centrifuged at 12,500 rpm for 10 min and supernatant was collected. The supernatant was diluted with DPBS (pH 7.4) to obtain a final protein concentration of 0.5 mg/mL. To 5 mL of supernatant solution, about 15 µg/mL of prodrug was dissolved and placed in shaking water bath (60 rpm) at 37 °C. Samples (100 µL) were withdrawn at predetermined time points and equal volume of methanol:acetonitrile (1:3) mixture was added to each sample to terminate enzymatic activity. Samples were stored at –80 °C until further analysis with HPLC technique.

3. Data analysis

3.1. HPLC analysis

Solubility, buffer and cell homogenate stability samples were analyzed by a reversed phase HPLC technique. The system consisted of Waters 515 HPLC pump, Alcott 718 AL HPLC antosampler, Pynamax® UV Absorbance detector. A C-8 Luna Column (250mm × 4.6 mm; Phenomenex, Torrance, CA) was pumped with a mixture of 60% methanol and 40% 16 mM potassium dihydrogen phosphate buffer (PH 4.0) containing 0.1% TFA at the flow rate of 0.8 mL/min. Absorbance wavelength was set at 254 nm. Prednisolone, VP and VVP eluted at 7.9, 9.8 and 14.1 min, respectively.

3.2. Sample preparation

3.2.1. LC/MS/MS analysis—Uptake and transport samples were analyzed with LC/MS/MS technique. QTrap® LC/MS/MS mass spectrometer (Applied Biosystems, Foster City, CA, USA) was equipped with Agilent 1100 series quaternary pump (Agilent G1311A), vacuum degasser (Agilent G1379A) and autosampler (Agilent G1367A). A C18 XTerra® column (2.1mm × 50 mm) was pumped with mobile phase (80% acetonitrile and 20% water containing 0.1% formic acid) at a flow rate of 0.2 mL/min. Chromatographs were obtained for 5 min. Hydrocortisone was used as an internal standard (IS). Drug, prodrug and internal standard eluted between 2.5–3 min.

Electrospray ionization in the positive mode was applied for the sample introduction. Analytes of interest were detected using multiple-reaction monitoring (MRM) method. Precursor ions were subjected to collision-induced dissociation to generate daughter ions. Precursor and product ions obtained were VVP +559.5/171.2, VP +460.4/442.1, prednisolone +360.9/342.9 and hydrocortisone + 363.3/121.2. The turbo ion spray setting and collision gas pressure were also optimized (IS Voltage: 5500 V, temperature: 300 °C, nebulizer gas: 40 psi, curtain gas: 40 psi). MS/MS was performed with nitrogen as the collision gas. Other ion source parameters include declustering potential (56 V), collision energy (22 V), entrance potential (5.5 V) and collision cell exit potential (4 V). Analytical data for drug/prodrug obtained with MRM method showed significant linearity up to nanomolar range.

3.2.2. Sample preparation for LC/MS/MS—Liquid-liquid extraction technique was employed for sample preparation. Briefly, 50 μL of IS (500 ng/mL) was added to each sample. Drug/prodrug and IS were extracted with 600 μL of ice-cold tert-butyl methyl ether (TBME). Samples were vortexed for 2 min and centrifuged at 10,000 rpm for 7 min to separate aqueous phase from the organic phase. Tubes were placed in $-80\text{ }^{\circ}\text{C}$ for 30 min. TBME was separated and evaporated under reduce pressure (45 min). The residue obtained was reconstituted in 100 μL DDW containing 0.1% formic acid. Subsequently, 20 μL of reconstituted solution was injected into the LC/MS/MS system.

3.3. Permeability Analysis

Cumulative amount of drug/prodrug transported was plotted against time to determine cell permeability. Linear regression of the amounts transported as a function of time yielded the rate of the transport (dM/dt) which was divided by the cross-section area (A) to obtain steady state flux [$\text{flux} = (dM/dt)/A$]. Steady state flux was normalized with donor concentration (C_d) to generate permeability ($\text{permeability} = \text{flux}/C_d$).

3.4. Statistical analysis

All experiments were performed at least in quadruplicate unless specified and results are expressed as mean $\pm$ standard deviation (S.D). Statistical comparison of mean values was performed with student t-test against control. A value of $p < 0.05$ was considered to be statistically significant.

4. Results and discussion

4.1. Aqueous solubility studies

Saturated aqueous solubility of prednisolone and prodrugs was determined in DDW. Aqueous solubility of VP and VVP was found to be approximately 4.24 ± 0.36 and 3.11 ± 0.59 mg/mL, respectively. Aqueous solubility of prednisolone was determined to be 0.31 ± 0.03 mg/mL. VP and VVP displayed about 13 and 10-fold higher aqueous solubility relative to prednisolone. One of the major drawbacks in formulating ophthalmic solution (drops) of prednisolone is the inherent poor aqueous solubility of the parent drug. Significant enhancement in aqueous solubility by prodrug derivatization may provide aqueous topical ophthalmic solution at the desired concentration.

4.2. Buffer stability studies

Buffer stability studies of VP and VVP were carried out to determine degradation rate constant and half-life under various pH conditions. Results obtained from this study are presented in Table 1. Prodrugs exhibited significantly higher stability towards chemical hydrolysis at lower pH. However, these compounds degraded rapidly with rise in pH suggesting that alkaline hydrolysis of VP and VVP is relatively rapid relative to acidic hydrolysis. The degradation rate constant of VP at pH 3.4 was found to be 220-fold lower relative to pH 7.4. Degradation half-life for VP at pH 3.4 and 7.4 was observed to be 129 ± 26 and 0.62 ± 0.18 h, respectively. Data obtained from this result demonstrated that amino acid prodrug of prednisolone is significantly unstable at pH 7.4. As the pH of tear fluid ranges from 6.5 to 7.6, VP may degrade significantly upon topical instillation.

Interestingly, VVP demonstrated significant stability at all studied pH compared to VP. VVP was highly stable at pH 3.4 with no apparent degradation. Degradation rate constant at pH 5.4 was approximately 2-fold higher compared to pH 7.4. Half life obtained for VVP at pH 5.4 and 7.4 were 48 ± 10 and 27 ± 5 h, respectively. Importantly, degradation half life displayed by VVP was more than 40 times higher relative to VP at pH 7.4. Such significant stability at pH 7.4 along with higher aqueous stability suggests that VVP could be a promising candidate for developing a stable ophthalmic aqueous drop formulation for treating anterior ocular inflammation.

4.3. Cytotoxicity studies

Cytotoxicity of prednisolone and prodrugs was determined by LDH assay in MDCK-WT cells. Assay medium devoid of serum or any drug/prodrug was selected as negative control. Triton x-100 (1%) was considered as positive control. Results obtained from this study are presented in Fig. 2. Absorbance generated by prednisolone and prodrugs was compared to negative control (Assay medium). Triton x-100 displayed significant cytotoxicity to cells. About 5-fold increase in absorbance was observed in the presence of Triton x-100. Interestingly, prednisolone and VP did not generate any cytotoxic effects in the concentration range of 5–250 μ M. Similarly, VVP did not exhibit any cytotoxicity within the concentration range of 5–150 μ M. However, at 250 μ M, VVP produced cytotoxic effects. About 2-fold increase in absorbance was observed in presence of 250 μ M concentration of VVP. Based on these observations, 100 μ M concentration of prednisolone and prodrugs was selected for uptake and transport studies to avoid cytotoxic effects of these compounds.

4.4. Uptake studies

Cellular uptake studies in MDCK-MDR1 cells was carried out to examine the efficacy of prodrugs to overcome P-gp mediated cellular efflux of prednisolone. Prednisolone has been demonstrated to be an excellent substrate of P-gp (Hariharan et al., 2009a; Karssen et al., 2002; Nakayama et al., 1999; Oka et al., 2002; Troutman and Thakker, 2003; Van der Heyden et al., 2012). P-gp is a major drug efflux pump that is expressed on corneal epithelium (Dey et al., 2003; Verstraelen and Reichl, 2013). This efflux pump has been demonstrated to play an active role in limiting corneal permeability of various therapeutic agents (Dey et al., 2004; Dey et al., 2003; Hariharan et al., 2009a; Katragadda et al., 2006; Kawazu et al., 2006). Extensive efflux of therapeutic agents at corneal epithelium may result in poor drug efficacy and emergence of resistance. Hence, circumvention of P-gp may significantly enhance permeation of prednisolone in anterior ocular tissues following topical administration. Erythromycin was selected as a model substrate to assess P-gp mediated efflux and interaction with prednisolone and prodrugs. Erythromycin is indicated in the treatment of corneal/conjunctival bacterial infection which further supports its selection as P-gp substrate (Dey et al., 2004; Queille-Roussel et al., 2001). Moreover, uptake studies were carried out at pH 7.4 which is within the range of normal human tear pH (6.5–7.6) (Abelson et al., 1981; Fischer and Wiederholt, 1982).

[¹⁴C]-Erythromycin uptake was conducted in the presence and absence of prednisolone and prodrugs in MDCK-MDR1 cells. Results obtained from this study are presented in Fig. 3. Erythromycin uptake was elevated dramatically in the presence of prednisolone in MDCK-

MDR1 cells. A 2.3-fold increase in the uptake was observed in the presence of prednisolone. Such elevated uptake of [14C]-Erythromycin indicates that prednisolone is an excellent substrate of P-gp. Similarly, [14C]-Erythromycin uptake increased significantly in the presence of VP. A 1.7-fold increase in the uptake process was observed in the presence of VP. Results obtained from chemical hydrolysis studies suggested that VP degrades rapidly at pH 7.4. Hence, the increase in the uptake process might be due to weaker inhibition of P-gp activity by regenerated prednisolone from VP. Interestingly, erythromycin uptake did not alter significantly in the presence of VVP. This data suggested that VVP may not have considerable substrate affinity towards P-gp relative to prednisolone. Lower affinity towards P-gp may significantly improve the corneal absorption of VVP following topical administration.

4.5. Interaction with peptide transporter

[3H]-GlySar uptake studies were carried out in MDCK-MDR1 cells to study affinity of prodrugs towards peptide transporters. Peptide transporters have been reported to be highly expressed on corneal epithelium (Anand and Mitra, 2002; Kadam et al., 2013; Xiang et al., 2009). These transporters can be targeted with prodrugs due to their broad substrate specificity, affinity and capacity. Peptide transporter targeted dipeptide prodrugs of acyclovir and ganciclovir have demonstrated significant transcorneal permeability relative to parent drugs (Anand et al., 2003; Anand et al., 2006; Anand and Mitra, 2002; Gunda et al., 2006; Majumdar et al., 2005). Previously we have identified and functionally characterized peptide transporters with Gly-Sar as a model substrate for MDCK-MDR1 cells (Agarwal et al., 2007). [3H]-GlySar uptake diminished significantly to 40 and 30% in the presence of 1 mM and 2 mM cold Gly-Sar, respectively (Fig. 4). This result clearly demonstrates that peptide transporters are highly expressed and functionally active in MDCK-MDR1 cells. [3H]-GlySar uptake was weakly inhibited in the presence of VP indicating weaker affinity towards peptide transporters. Lower inhibition of [3H]-GlySar uptake in the presence of VP could be due to poor chemical and enzymatic stability. On the other hand, VVP significantly inhibited [3H]-GlySar uptake relative to prednisolone and VP. The uptake process diminished to 40% in the presence of 100 μ M concentration of VVP, comparable to 1 mM cold GlySar. No significant inhibition was observed in the presence of 100 μ M prednisolone. These results suggest that VVP is recognized as an excellent substrate by peptide transporters in MDCK-MDR1 cells. Based on these observations, it can be anticipated that VVP will generate higher corneal absorption by translocating through peptide transporters, which are expressed on corneal epithelium.

4.6. Transepithelial transport study

Transepithelial bidirectional transport across MDCK-MDR1 cells was carried out to compare permeability rates of prednisolone and prodrugs. P-gp is highly expressed on the apical surface of MDCK-MDR1 cells. Hence a typical substrate of this efflux pump would generate lower transport rates in the absorptive direction (apical to basolateral, A-B). As shown in Fig. 5, prednisolone transport in the absorptive direction is significantly lower compared to secretive direction (basolateral to apical, B-A). Apparent A-B and B-A permeabilities of prednisolone are found to be $1.3 \pm 0.2 \times 10^{-5}$ and $2.9 \pm 0.5 \times 10^{-5}$ cm/s, respectively. Efflux ratio (B-A permeability/A-B permeability) obtained for prednisolone

was approximately 2.2 which clearly indicates that the transport of this parent drug may be significantly limited by P-gp. Since P-gp is expressed on the apical membrane of corneal epithelium, transcorneal permeation of prednisolone may significantly hindered following topical administration.

Apparent A-B permeability of VP and particularly VVP was significantly higher relative to prednisolone (Fig. 6). VP and VVP generated 1.7 and 2.6-fold higher transport in the absorptive direction relative to prednisolone. Such enhancement in transepithelial transport may be due to circumvention of P-gp as well as peptide transporter mediated influx of prodrugs. Based on these results, it can be calculated that VVP may generate higher levels of prednisolone in anterior ocular tissues following topical administration. Such enhancement in the transcorneal permeation may result in superior efficacy and lower possibility of tolerance development.

4.7. SIRC cell homogenate study

Enzymatic hydrolysis of prodrugs was determined in SIRC cell homogenate at pH 7.4 for a period of 12 h. Results obtained from this study are displayed in Fig. 7A–B. VP degraded very rapidly to regenerate prednisolone. Degradation rate constant and half life of VP were found to be $0.0416 \pm 0.0005 \text{ min}^{-1}$ and $16.6 \pm 0.2 \text{ min}$, respectively. This result is consistent with chemical hydrolysis of VP indicating lower stability of the amino acid prodrug. However, degradation half-life of VP in cell homogenates is significantly lower relative to chemical hydrolysis (approximately 30 min). This observation suggests that VP undergoes enzymatic as well as chemical degradation to regenerate prednisolone. Regeneration of prednisolone is essential in order to generate anti-inflammatory effects. However, rapid degradation of VP may generate significantly higher amounts of prednisolone in tear fluid as well as corneal epithelial cells. Regenerated prednisolone in corneal epithelial cells may be transported back into the tear fluid by P-gp. This process may result in significant drug loss leading to poor anti-inflammatory efficacy.

VVP exhibited much superior enzymatic stability relative to VP. VVP degraded slowly to generate VP and prednisolone. Degradation rate constant and half-life of VVP were found to be $0.61 \pm 0.08 \times 10^{-3} \text{ min}^{-1}$ and $19 \pm 2 \text{ h}$, respectively. A finite amount of regenerated VP was observed which however, may degraded rapidly to reproduce prednisolone. Prednisolone appeared to regenerate from VVP at all selected time points. Sustained regeneration of prednisolone from VVP may be highly advantageous as this process will render parent drug to be readily available to elicit anti-inflammatory effects. Results obtained from this study suggest that VVP undergoes enzymatic degradation by both peptidase and esterase class of enzymes. Esterase-mediated degradation of VVP and VP produced prednisolone whereas; peptidase-mediated degradation of VVP generated VP in cell homogenate samples. Based on the levels of prednisolone regenerated in the homogenate samples, it is apparent that prodrugs undergo rapid enzymatic cleavage by esterase class of enzymes relative to peptidase type of enzymes.

5. Conclusion

Amino acid and dipeptide prodrugs were developed to improve corneal absorption of prednisolone following topical administration. VVP possessed excellent aqueous solubility and chemical stability at physiological pH (7.4). VVP demonstrated higher efficacy to circumvent Pgp mediated cellular efflux of prednisolone. Prodrugs were recognized by peptide transporters. Interestingly, VVP degraded slowly over a longer period of time to regenerate prednisolone. Results obtained from this study indicate that dipeptide prodrugs may be an effective strategy to improve transcorneal permeation of prednisolone. Furthermore, ophthalmic aqueous solution of VVP may be produced at higher concentrations to improve prednisolone absorption in anterior ocular tissues.

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Fig. 1.

Synthesis of VVP. To synthesize VVP, VP was first produced by conjugating valine to prednisolone. Another valine was then conjugated to VP to generate VVP. Reagents and conditions used for synthesis are explained in detail in the section 2.2.1.

Fig. 2.

Cytotoxicity of prednisolone (striped bar), VP (light gray bar) and VVP (dark gray bar) in MDCK-WT cells. Each data point is expressed as mean $\pm$ standard deviation ($n = 6$).

Absorbance is represented as percentage of control (no serum and drug in medium). Asterisk (**) represents significant difference from the control ($p < 0.01$).

Fig. 3.

Cellular uptake of [14C]-Erythromycin in MDCK-MDR1 cells in the absence and presence of prednisolone (P) and prodrugs in DPBS (pH 7.4) at 37 °C. Each data point is represented as mean $\pm$ standard deviation (n=4). Uptake is expressed as percentage of control ([14C]-Erythromycin). Asterisk (**) represents significant difference from the control ($p < 0.05$).

Fig. 4.

Cellular uptake of [3H]-GlySar in MDCK-MDR1 cells in the absence and presence of prednisolone (P) and prodrugs in DPBS (pH 7.4) at 37 °C. Each data point is represented as mean $\pm$ standard deviation (n=4). Uptake is expressed as percentage of control ([3H]-GlySar). Asterisk (**) represents significant difference from the control ($p < 0.05$).

Fig. 5. Transepithelial A-B (filled triangle) and B-A (empty triangle) transport of prednisolone across MDCK-MDR1 cells in DPBS (pH 7.4) at 37 °C for 180 min. Each data point is expressed as mean $\pm$ standard deviation (n = 4).

Fig. 6. A–B permeability rates of prednisolone and prodrugs across MDCK-MDR1 cells. Each point is expressed as mean $\pm$ standard deviation. Asterisk** indicates significant difference from control (P, prednisolone, $p < 0.05$).

Fig. 7.

SIRC cell homogenate study of VP (A) and VVP (B) in DPBS (pH 7.4) at 37 °C.

(A): ($\blacktriangle$) represents regenerated prednisolone from VP ($\blacktriangle$). (x) Represents total concentration (VP + prednisolone) at each time point.

(B): Degradation profile of VVP in SIRC cell homogenate in DPBS (pH 7.4) at 37 °C. ($\blacktriangle$) represents regenerated prednisolone from VVP ($\circ$) and/or VP ($\blacksquare$). (x) Represents total concentration (VVP + VP + prednisolone) at each time point.

Each data point is expressed as mean $\pm$ standard deviation ($n = 3$).

Table 1

Degradation rate constant and half-life values of VP and VVP at various pH conditions. Results are expressed as mean $\pm$ standard deviation (n=4).

pH	VP		VVP	
	Degradation rate constant $\times 10^{-3}$ (min ⁻¹)	Half-life (h)	Degradation rate constant $\times 10^{-3}$ (min ⁻¹)	Half-life (h)
3.4	0.09 $\pm$ 0.002	129 $\pm$ 26	ND	ND
5.4	0.89 $\pm$ 0.08	13 $\pm$ 1	0.25 $\pm$ 0.05	48 $\pm$ 10
7.4	20 $\pm$ 8	0.62 $\pm$ 0.18	0.44 $\pm$ 0.09	27 $\pm$ 5

Note: ND represents no degradation