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Stereoisomeric Prodrugs to Improve Prednisolone Absorption

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Abstract

A series of stereoisomeric prodrugs have been designed to examine their efficacy in generating higher absorption relative to prednisolone. Prodrugs have been synthesized and identified with LC/MS/MS and NMR analysis. Prodrugs have been characterized for their aqueous solubility, buffer stability and cell cytotoxicity properties. Cellular uptake and permeability studies have been conducted across MDCK-MDR1 cells to determine prodrug affinity towards P-glycoprotein (P-gp) and peptide transporters. Enzyme-mediated degradation of prodrugs has been determined using Statens Seruminstitut Rabbit Cornea (SIRC) cell homogenates. Prodrugs exhibited superior aqueous solubility relative to prednisolone. Prodrugs circumvented P-gp mediated cellular efflux and were recognized by peptide transporters. Prodrugs produced with D-isomers (D-valine) were significantly stable towards both chemical and enzymatic hydrolysis. Results obtained from this study clearly suggest that a stereoisomeric prodrug approach is an effective strategy to overcome P-gp mediated efflux and poor transcorneal permeability of prednisolone following topical administration.

INTRODUCTION

Prednisolone, a corticosteroid is generally indicated for the treatment of ocular inflammation, allergic reactions and anterior uveitis [1-5]. Topical drug delivery is the most preferable method of drug administration for the treatment of ocular diseases affecting anterior tissues of the eye. However, this method of drug administration faces numerous challenges including rapid tear turnover rate, drainage to systemic circulation and non-specific absorption resulting in lower drug absorption in anterior ocular tissues [6]. Moreover, the apical surface of the corneal epithelium expresses drug efflux proteins such as P-glycoprotein (P-gp) [7,8] and multidrug resistance associated proteins (MRPs) [9-12]. These efflux pumps actively transports drug molecules from the cornea back to the tear film [6,13, 14]. This process further limits drug accumulation in anterior ocular tissues. The cumulative effects of these obstacles result in poor ocular drug bioavailability following topical administration. Importantly, to generate high transcorneal permeability following topical administration, drugs need to possess an optimum balance between hydrophilicity and lipophilicity characteristics. Prednisolone is a poor water soluble drug and an excellent substrate of P-gp [6,15-19].

Transporter targeted prodrugs have been extensively employed to improve drug transport across poorly permeable membranes. Peptide transporters have been widely targeted due to their high substrate affinity, capacity, and broad specificity. These influx transporters have been demonstrated to be highly expressed on the apical surface of the corneal epithelium [20-22]. This transporter has been previously targeted with amino acid and dipeptide prodrugs to improve corneal transport of poorly permeable drugs in our laboratory [22-28].Amino acids employed to generate amino acid and dipeptide prodrugs possessed L-configuration as L-isomers possess high affinity and specificity towards peptide transporters [29,30].However, the major concern with L-isomers containing dipeptide prodrugs has been poor enzymatic stability. To modulate the rate of enzymatic degradation, incorporation of D-isomers at a definite position has been previously employed in our laboratory [23,31,32].

The main objective of the present study is to investigate the efficacy of stereoisomeric prodrug approach to improve aqueous solubility, corneal permeability and circumvent P-gp mediated cellular efflux of prednisolone. Several stereoisomeric prodrugs such as L-valine-D-valine-prednisolone (LDP), D-valine-L-valine-prednisolone (DLP) and D-valine-D-valine-prednisolone (DDP) were produced. Enzymatic stability of stereoisomeric prodrugs has been determined in SIRC cell homogenates. To examine P-gp and peptide transporter affinity of prednisolone and prodrugs, uptake and transport studies have been carried out usingMDCK-MDR1 cells.

MATERIALS AND METHODS

Materials

Prednisolone was purchased from Sigma-Aldrich (St. Louis, MO). Boc-L-valine and Boc-D-valine were purchased from Bachem. [3H]-GlySar(specific activity: 4 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA) and used at the concentration of 1 µCi/mL. [14C]-Erythromycin (specific activity:

51.3 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA) and used at the concentration of 0.5 μCi/mL. 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). The SIRC cell line was purchased from American Type Culture Collection (CCL-60; ATCC, Rockville, MD). The growth medium Dulbecco's modified Eagle's Medium (DMEM), trypsin/EDTA and non essential amino acids were obtained from Gibco (Invitrogen, Grand Island, NY). Minimum essential medium (MEM), was procured from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta biological. All remaining chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Culture flasks (75 and 25 cm^2), 12-well plates (3.8 cm^2 growth area/well) and Transwells® were obtained from Costar (Bedford, MA, USA). All chemical agents procured were of special reagent grade and utilized without any further purification.

Methods

Synthesis: LDP, DLP and DDP Prodrugs were synthesized according to the procedure previous published from our laboratory [37]. To synthesize LDP, DP was first synthesized by conjugating D-valine to prednisolone with an ester coupling agent such as EDC and DMAP. DP was then conjugated to L-valine to generate LDP using amide coupling agents, EDC and triethylamine (TEA). Briefly, Boc-D-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). Prednisolone (300 mg, 0.83 mmol) and DMAP (120 mg, 0.98 mmol) were dissolved in DMF at room temperature for 30 min. Mixture 2 was then added drop wise to the mixture 1 drop wise under constant stirring. The resulting mixture was stirred for 24 h under nitrogen atmosphere. Crude mixture was filtered and DMF was evaporated under reduced pressure. The product (Boc-DP) was purified with silica based column chromatography using 5% methanol in dichloromethane (DCM). Boc-DP was deprotected with 1:1 TFA/DCM at 0°C for 50 min. DP was then purified by recrystallization in cold diethyl ether. Ether was evaporated under reduced pressure to obtain a final dried product with a yield obtained of 86%.

For LDP, same procedure was followed except the starting material was DP and TEA instead of Prednisolone and DMAP. The product Boc-LDP was purified with column chromatography with 3% methanol in DCM as eluent. LDP was deprotected with 1:1 TFA/DCM at 0°C for 50 min and purified by recrystallization with cold diethyl ether. The yield obtained was about78%.

Reagents and reactions conditions were similar for all stereoisomeric prednisolone prodrugs. To synthesize stereoisomeric prodrugs, D-valine has been inserted at definite position in dipeptide conjugates. D-valine-prednisolone (DP) was synthesized similar to L-valine-prednisolone (LP). To synthesize DDP, D-valine was conjugated to DP, respectively. For DLP prodrug, D-valine was conjugated to LP.

Identification: Prodrugs were identified using LCMS and NMR analysis.LC/MS (m/z) for LP and DP was +460.4. LC/MS (m/z) for LLP, LDP, DLP and DDP was +559.5.

^{1}H NMR analysis for LP (400MHz, DMSO-d_6) 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) ^{13}C NMR analysis for VP (100MHz, DMSO-d_6): 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.

^{1}H NMR analysis for DP (400MHz, DMSO-d_6) 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), 4.21 (s, 1H), 5.23 (s, 2H), 5.90 (s, 1H), 6.17 (d, 1H), 7.25 (d, 1H), 8.05 (s, 2H).^{13}C NMR analysis for DP (100MHz, DMSO-d_6): 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, 57.0, 67.5, 90.5, 121.5, 127.6, 152.4, 167.2, 169.0, 184.1, 203.7.

^{1}H NMR analysis for DLP and LDP (400MHz, DMSO-d_6) 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), 4.18 (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) ^{13}C NMR analysis for DLP (100MHz, DMSO-d_6): 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, 56.4, 59.9, 67.5, 90.5, 121.5, 127.6, 152.4, 167.2, 169.0, 172.5, 184.1, 203.7.

^{1}H NMR analysis for DDP (400MHz, DMSO-d_6) 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), 4.21 (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). ^{13}C NMR analysis for DDP (100MHz, DMSO-d_6): 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.

Cell Culture: MDCK-wild type (MDCK-WT) and MDCK-MDR1 cells (passage 6–10) were cultivated in DMEM medium supplemented with 10% FBS (heat inactivated), penicillin (100 units/mL) and streptomycin (100 μg/mL), pH at 7.4 in 75 cm^2 tissue culture flask. SIRC cells were grown in MEM supplemented with 10% calf serum, lactalbumin (1.76 mg/mL), HEPES (1.3 mg/mL), and penicillin-streptomycin (100 μg/mL) at pH 7.4. Plates were placed in an incubator at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. The medium was changed on alternate day until cells reached 90% confluency (6–7 days for MDCK-MDR1 cells and 8-10 days for SIRC cells).

Aqueous solubility studies: Aqueous solubility was determined by adding 5 mg of each 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. Following centrifugation at 12,500 rpm for 10 min, the supernatant was collected and filtered through 0.45 μm Nalgene syringe filter membrane. Appropriate dilutions were made to the supernatant and drug/prodrug concentration was determined by a reversed phase HPLC.

Buffer stability studies: To study chemical hydrolysis, prodrugs (20 μg/mL) were dissolved in approximately 5 mL of buffer in centrifuge tubes and placed in shaking water bath (PolyScience®) at 37°C and 60 rpm. At predetermined time points, samples (100 μL) were withdrawn and stored at −80° C until further analysis with HPLC. Prodrug concentrations remaining were plotted versus time in order to determine the degradation rate constants.

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 density of 20,000 cells per well and incubated at37°C in an atmosphere of 5% CO_2 and 90% relative humidity. Following incubation, medium was removed and 200 µL assay medium (serum free DMEM) containing different drug/prodrug concentrations (5-250 µM) was added into each well. Cells were incubated with drug/prodrug solution for 4 h. Assay medium and 1% Triton x-100 was selected as controls. Absorbance was determined at a 450 nm with a microplate reader (Biorad, Hercules, CA).

Cellular uptake studies: Cellular uptake studies were performed according to a protocol published from our laboratory with little modifications [33]. Briefly, cells were seeded at a density of 3×10^6 cells in 12 well plates and maintained at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity till confluency. Before initiation of uptake studies, medium was removed and cell monolayers were washed with 2 mL of Dulbecco's Phosphate Buffered Saline (DPBS, pH 7.4) for three times at 37°C (each wash of 10 min). Cells were incubated with radioactive solution in DPBS at 37°C for 20 min. Following incubation, radioactive solution was quickly removed and plates were washed with ice-cold stop solution (210 mM KCl, 2 mM HEPES, pH of 7.4) to Arrest uptake process. About, 1 mL of lysis buffer (0.1% Triton-X solution in 0.3% NaOH) was added to each well and plates were stored overnight for cell lysis. On the following day, 500 µL solutions were added to 3 mL of scintillation cocktail and radioactivity was determined with a scintillation counter (Beckman Instruments Inc., Model LS-6500; Fullerton, CA). Uptake rate was normalized to protein count which was quantified using BioRad protein estimation kit (BioRad protein; Hercules, CA).

Transport studies: Transepithelial transport studies were conducted across MDCK-MDR1 cells according to a method previously published from our laboratory [34]. Briefly, MDCK-MDR1 cells were seeded in 12-well Transwell® plates. Before initiation of transport study, monolayer integrity was determined by measuring transepithelial electric resistance (TEER), with an EVOM (epithelial volt ohmmeter from World Precision Instruments, Sarasota, FL) and found to be approximately 230 Ωcm^2. 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. About 1.5 mL drug/prodrug solution (100 µM) was added in the basolateral chamber to determine B-A transport. Transport study was performed for a period of 3 h at 37°C. Samples (200 µL) were withdrawn at predetermined time points from the receiving chamber and replaced with fresh DPBS to maintain sink conditions. Samples were stored at −80°C until further analysis.

SIRC cell homogenate study: SIRC cell homogenate study was carried out according to the method previously published from our laboratory with little modifications [34]. Briefly, SIRC cells were rinsed 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 using Multipro variable speed homogenizer (DREMEL, Racine, WI) in eppendroff tubes. Tubes were centrifuged at 12,500 rpm for 10 min and the supernatant was collected. Appropriate dilutions were made with DPBS (pH 7.4) to obtain a final protein concentration of 0.5 mg/mL. About 15 µg/mL of prodrug was dissolved in 5 mL supernatant solution and placed in shaking water bath (60 rpm) at 37 °C. Samples (100 µL) were withdrawn at predetermined time points and proteins were precipitated with an equal volume of a mixture of methanol: acetonitrile (1:3). Samples were stored at −80°C until further analysis.

Data analysis

HPLC analysis: Solubility, buffer and cell homogenate samples were analyzed with reversed phase HPLC. The HPLC system consisted of Waters 515 HPLC pump connected to Alcott 718 AL HPLC antosampler and Pynamax® UV Absorbance detector. A C(8) Luna Column (250mm x 4.6 mm; Phenomenex, Torrance, CA) was pumped with 60% methanol and 40% 16 mM potassium dihydrogen phosphate buffer (pH 4.0) containing 0.1% TFA at 0.8 mL/min (isocratic flow). Absorbance wavelength was 254 nm. Prednisolone and LP were detected at 7.9 and 9.8 min, respectively. DP, DLP, LDP and DDP eluted at 10.2, 15.2, 15.8, 12.7 min, respectively.

LC/MS/MS analysis: Transport samples were analyzed with LC/MS/MS. QTrap ® LC/MS/MS mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with Agilent 1100 series quaternary pump (Agilent G1311A), vacuum degrasser (Agilent G1379A) and autosampler (Agilent G1367A) was employed. A C18 XTerra® column(2.1mm x 50 mm) was pumped with mobile phase (80% acetonitrile and 20% water containing 0.1% FA) at a flow rate of 0.25 mL/min and chromatographs were obtained for 4 min. Hydrocortisone was used as an internal standard (IS). Drug/prodrug and IS were detected around 2.5-3 min.

Electro spray ionization in the positive mode was applied for the sample introduction and analytes were detected using multiple-reaction monitoring (MRM) method. Precursor and product ions obtained for LDP, DLP and DDP were +559.5/171.2. Precursor and product ions obtained for LP and DP were +460.4/442.1, prednisolone +360.9/342.9 and IS+ 363.3/121.2. 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 showed significant linearity up to nanomolar range.

- **Sample preparation for LC/MS/MS:** To determine drug/prodrug concentrations, a liquid-liquid extraction technique was employed. Briefly, 50 µL of IS (500 ng/mL) was added to each sample and 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. Tubes were placed in −80°C for 30 min and TBME was collected. The organic solvent was evaporated under reduced pressure. The residue obtained was reconstituted in 100 µL of LCMS/MS mobile phase. Subsequently, 20µL of reconstituted solution was injected into a LC/MS/MS.

Permeability Analysis: Cumulative amount transported was plotted against time to obtain permeability rates. Linear regression of the amount transported was plotted as a function of time to generate rate of the transport (dM/dt). Transport

rates were divided by the cross-section area (A) to obtain steady state flux [Flux = (dM/dt)/A].Steady state flux was normalized with donor concentration (C_d) to determine permeability rates (Permeability = Flux/C_d).

Statistical analysis: All experiments were performed at least in quadruplicates and results are expressed as mean±standard deviation (S.D) unless or otherwise specified. Statistical comparison of mean values was performed with student t-test. A value of $p < 0.05$ was selected as statistically significant.

RESULTS AND DISCUSSION

Aqueous solubility studies

Aqueous solubility of prednisolone was 0.31±0.03 mg/mL. Aqueous solubility of LP was 4.24±0.36 mg/mL. Saturated aqueous solubility generated by DP prodrug was approximately 5.2±0.2. DP exhibited approximately 17-fold higher aqueous solubility relative to prednisolone. LDP, DLP and DDP displayed saturated aqueous solubility of 5.2±0.3, 4.6±0.2 and 3.4±0.4 mg/mL, respectively. LDP, DLP and DDP produced about 17, 15 and 11-fold higher aqueous solubility relative to prednisolone. Aqueous solubility of all prodrugs was observed to be significantly higher relative to prednisolone. Poor aqueous solubility is one of the major factors that pose difficulties in formulating ophthalmic solutions of prednisolone. Significant improvement in aqueous solubility by amino acid and dipeptide prodrugs may offer tremendous formulation related benefits over prednisolone for topical administration. Results obtained from aqueous solubility studies for prodrugs have been observed to be consistent with previously published reports from our laboratory [35-37].

Buffer stability studies

Chemical hydrolysis of stereoisomeric prodrugs was determined at various pH values (3.4, 5.4 and 7.4). Degradation rate constants and half-life values obtained for prodrugs are presented in Table 1. Prodrugs were observed to be stable at acidic pH compared to slightly neutral conditions. DP generated lower degradation rate constant value at pH 3.4 relative to 7.4. Degradation rate constants of DP at pH 3.4, 5.4 and 7.4 were 0.22±0.04, 0.43±0.08 and 2.6± 0.5 x 10^{-3} min^{-1}, respectively. Degradation rate constant at pH 7.4 was 6-fold higher relative to pH 3.4. Degradation half-life of DP observed at pH 3.4, 5.4 and 7.4 was 55±10 h, 28±5 h and 4.5±0.9 h, respectively. This result indicated that DP is highly susceptible to alkaline hydrolysis relative to acidic hydrolysis. Similar results have been observed for a wide range of amino acid prodrugs developed in our laboratory [35,38,39]. Lower degradation half-life at pH 7.4 suggests that a large amount of DP may degrade in intracorneal tear fluid upon topical administration to regenerate prednisolone.

LDP and DLP stereoisomeric prodrugs exhibited no significant degradation at acidic PHS (3.4 and 5.4). However, these compounds degraded rapidly with rise in pH (7.4). The degradation rate constant value displayed by LDP and DLP prodrugs at pH 7.4 was 0.31± 0.02 and 0.19±0.07 x 10^{-3} min^{-1}, respectively. Degradation half life produced by LDP and DLP at pH 7.4 was 37±2 and 65±19 h, respectively. DDP prodrug was observed to be highly stable with no apparent degradation at all pH values. Degradation half life values generated by LLP at pH 5.4 and 7.4 were 48±10 h and 27±5 h, respectively. Interestingly,

Table 1: Degradation rate constant and half-life values of prodrugs at various pH conditions. Results are expressed as mean±standard deviation (n=3).

Prodrugs	pH	Degradation rate constant x 10^{-3} (min^{-1})	Half Life (h)
DP	3.4	0.22±0.04	55±10
	5.4	0.43±0.08	28±5
	7.4	2.6±0.5	4.5±0.9
LDP	3.4	ND	-
	5.4	ND	-
	7.4	0.31±0.02	37±2
DLP	3.4	ND	-
	5.4	ND	-
	7.4	0.19±0.07	65±19
DDP	3.4	ND	-
	5.4	ND	-
	7.4	ND	-

LDP and DLP prodrugs displayed significantly higher half life at pH 7.4 relative to LLP. Particularly, DLP prodrug displayed more than 2-fold higher degradation half life value compared to LLP at pH 7.4. Results obtained from this study clearly suggested that the incorporation of D-isomers in the dipeptide prodrug structure significantly enhances the stability towards chemical hydrolysis. Moreover, the position of D-isomers in the prodrug structure plays an important role in promoting chemical stability. It was observed that the presence of D-isomer as a terminal moiety (DLP) imparted more chemical stability to prodrugs relative to L-isomer (LDP).These results were also observed with stereoisomeric dipeptide prodrugs of saquinavir [32].

Cytotoxicity studies

To determine cellular cytotoxicity of stereoisomeric prodrugs in MDCK-WT cells, LDH assay was employed. Assay medium with no serum and drug/prodrug was selected as negative control (NC). Results obtained from this study are depicted in Fig. 2. Triton X-100 displayed approximately 5-fold increase in absorbance relative to control indicating significant cytotoxicity to MDCK-WT cells (Figure 1). Prednisolone did not exhibit any cytotoxic effects in the concentration range 50-250 µM. Similarly, DP displayed no significant cytotoxicty in the concentration range of 50-250 µM (data not shown). However, dipeptide prodrugs were observed to be slightly toxic at relatively higher concentrations. At 250 µM concentration, dipeptide prodrugs generated approximately 2-fold higher absorbance relative to NC. Interestingly, prodrugs displayed no cell cytotoxicity in the concentration range of 50-150 µM. Hence, to avoid cytotoxic effects of prodrugs, uptake and transport experiments were carried out at 100 µM concentration.

Cellular uptake studies

Drug efflux pumps such as P-gp have been reported to be highly expressed on the apical surface of the corneal epithelium [7,8,40]. Due to apical localization in the corneal epithelium, P-gp might play an important role in ocular disposition of therapeutic agents following topical administration (Figure 2). Previously, P-gp has been demonstrated to play a major role in limiting the transcorneal permeability of various therapeutic

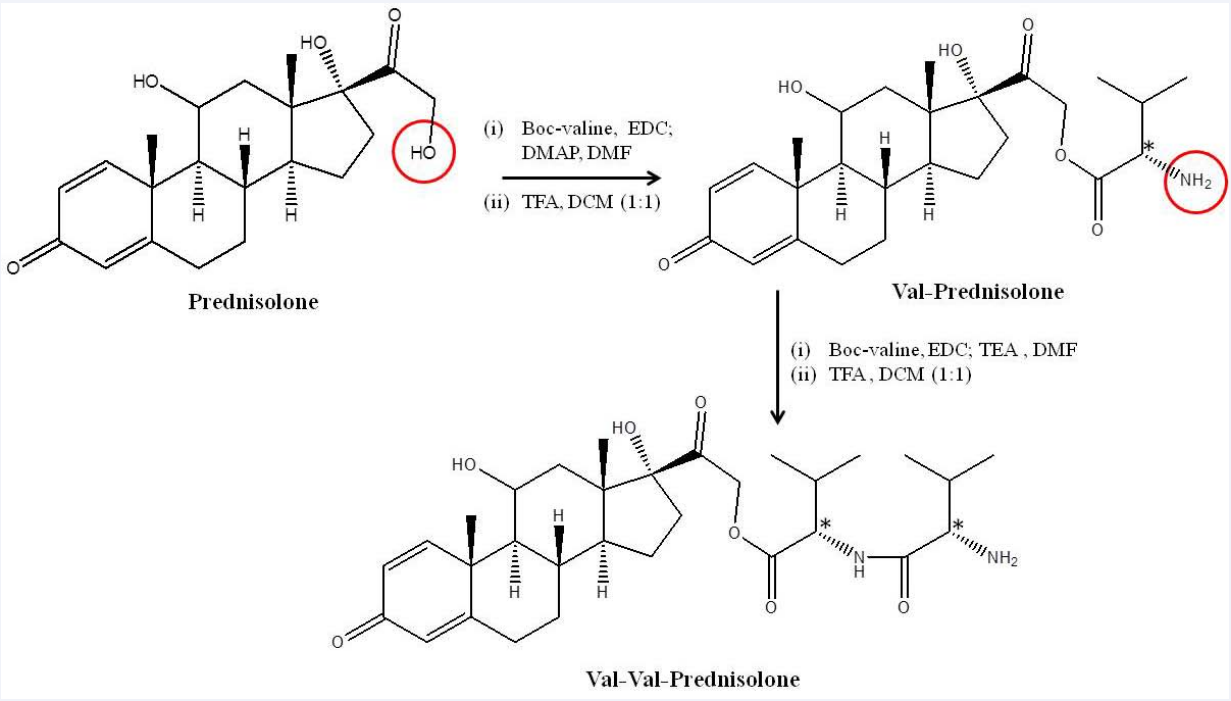

Figure 1 Synthesis of Val-P (A) and Val-Val-P (B) prodrug of prednisolone. Note: Different stereoisomers of valine (Val) were used to produce LDP, DLP and DDP.* Sign represents carbon which is chiral.
Reagents and conditions for Val-P: (i) Boc-valine, EDC in DMF: 45 min at 0°C; prednisolone, DMAP in DMF: 30 min at RT and mixture stirred for 24 h at RT. (ii) TFA in DCM (1:1): 50 min at 0°C.
Reagents and conditions for Val-Val-P: (i) Boc-valine, EDC in DMF: 45 min at 0°C; VP, TEA in DMF: 30 min at RT and mixture stirred for 24 h at RT. (ii) TFA in DCM (1:1): 50 min at 0°C.

agents [6,8,14,26,41]. Hence, evasion of this efflux pump at corneal epithelium might significantly improve transcorneal drug permeation and accumulation in anterior ocular tissues following topical administration.

The potential of stereoisomeric prodrugs to circumvent P-gp mediated cellular efflux was studied. [14C]-Erythromycin was selected as a model substrate of P-gp. Cellular uptake studies were performed in MDCK-MDR1 cells as this cell line is widely employed to study P-gp interaction with substrate drugs [29,34-36,42]. As observed in Figure 3, cellular uptake of [14C]-Erythromycin was significantly elevated in the presence of prednisolone. About, 2.3-fold enhancement in the uptake process was observed indicating that prednisolone has high substrate affinity towards P-gp. Previously, P-gp has been demonstrated to play a significant role in limiting cellular uptake and transport of prednisolone [6, 15-19]. Results obtained from this study were consistent with these reports.

Interestingly, [14C]-Erythromycin uptake remained unaltered in the presence of DP. This result suggested that DP may not have substrate affinity towards P-gp. Similarly, no significant rise in the uptake process was observed in the presence of LDP, DLP and DDP. This result suggested that stereoisomeric prodrugs may not be recognized as substrates by P-gp relative to prednisolone. Significant evasion of P-gp renders these prodrugs potential candidates for generating higher corneal absorption following topical administration.

In addition to efflux pumps, peptide transporters are also reported to be highly expressed on the apical surface of corneal epithelial cells [20-22]. Dipeptide prodrugs of acyclovir and ganciclovir have displayed significant improvements in transcorneal permeability relative to unmodified parent drugs [22,24,25,28,43]. For investigating substrate affinity towards peptide transporters, cellular uptake studies of [3H]- GlySar were conducted in the presence and absence of prodrugs in MDCK-MDR1 cells. This cell line was selected as it highly expresses peptide transporters [44].Results obtained from this study are depicted in Figure 4. [3H]-GlySar uptake was significantly inhibited in the presence of cold GlySar (2 mM). This inhibition suggested that the peptide transporter is functionally active in MDCK-MDR1 cells. The uptake process remained unaltered in the presence of prednisolone (100 μM) indicating no affinity towards peptide transporters. Similarly, DP did not generate any significant inhibition in the uptake process. This result indicated that DP may not have high substrate affinity towards peptide transporters.

Interestingly, LDP and DLP prodrugs produced significant inhibition of [3H]-GlySar uptake. LDP and DLP generated about 55 and 36% inhibition in the uptake process. This result suggested that LDP and DLP were recognized by peptide transporters as excellent substrates. However, DDP displayed no significant inhibition of the uptake process. This data indicated that DDP might not have substrate affinity towards peptide transporters. Based on these results, it was observed that prodrug recognition by peptide transporter was highly dependent on the configuration of amino acids in the peptide structure. For

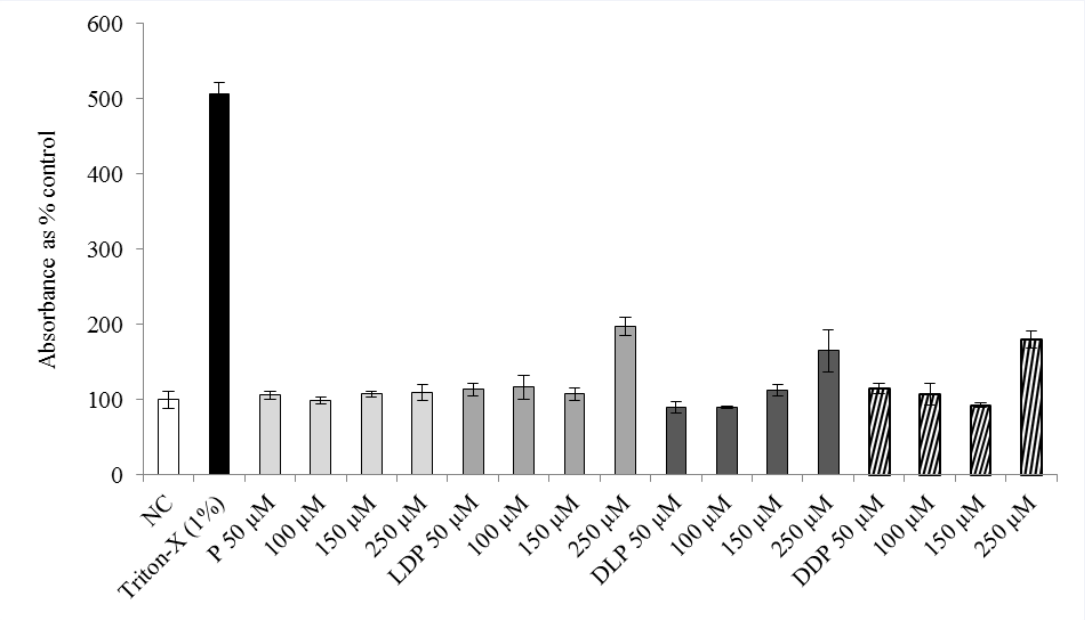

Figure 2 Cytotoxicity of prednisolone (P) and prodrugs in MDCK-WT cells. Each data point is expressed as mean±standard deviation (n=6). Absorbance is represented as percentage of control (no serum and drug in medium). Asterisk (**) represents significant difference from the control ($p < 0.01$).

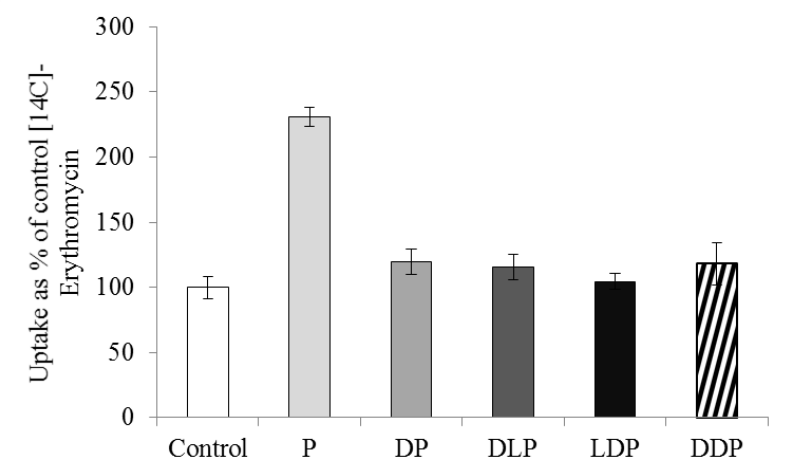

Figure 3 Cellular uptake of [14C] -Erythromycin in MDCK-MDR1 cells in the absence and presence of prednisolone (P) and prodrugs in DPBS (pH 7.4) at 37°C. Each data point is represented as mean ± standard deviation (n=4). Uptake is expressed as percentage of control ([14C]-Erythromycin). Asterisk (**) represents significant difference from the control ($p < 0.05$).

instance, prodrugs with L-isomer amino acid in the terminal (LDP) displayed significant interaction with peptide transporter. Whereas, prodrug with D-isomer in the terminal (DLP) generated lower affinity relative to L-isomer (LDP). The presence of two D-isomers in the peptide prodrug structure (DDP) resulted in loss of affinity towards peptide transporters. Our findings suggest that prodrugs containing L-isomers (DLP and LDP) could be translocated by peptide transporters and simultaneously circumvent recognition by P-gp. On the other hand, DDP prodrug may efficiently bypass P-gp mediated cellular efflux but might not be transported by peptide transporters. Based on these observations, LDP and DLP could be anticipated to generate significant corneal absorption following topical administrations. Moreover, results obtained from this study were consistent with those observed for stereoisomeric prodrugs of saquinavir and acyclovir [31,32].

Transepithelial transport study

Transepithelial apical to basolateral (A-B, absorptive direction) transport across MDCK-MDR1 cells was carried out to examine the potential of stereoisomeric prodrugs to overcome P-gp mediated cellular efflux. Apparent A-B and B-A permeability values generated by prednisolone were 1.3±0.2 x 10^{-5} and 2.9±0.5 x 10^{-5} cm/s, respectively. Efflux ratio was found

to be approximately 2.2, indicating significant role of P-gp in modulating prednisolone transport. Comparison of permeability rates generated by prednisolone and prodrugs in the absorptive direction (A-B) are depicted in (Figure 5). DP and DDP generated approximately 1.3 and 1.4-fold higher permeability in the absorptive direction relative to prednisolone. As observed in cellular uptake studies, DP and DDP were recognized as poor substrates of peptide transporters (Figure 4). However, these compounds displayed no substrate affinity towards P-gp transporter (Figure 3), which may have resulted in slight increase in the A-B transport. This result supports our assumption that the configuration of amino acids in the prodrug structure might play an important role in transport recognition particularly peptide transporters. Although DDP exhibited superior aqueous solubility and buffer stability, poor substrate affinity towards peptide transporter might be a crucial factor resulting in poor transport in the absorptive direction.

LDP and DLP prodrugs exhibited significantly higher transport rates in the A-B direction across MDCK-MDR1 cells. Apparent A-B permeability produced by LDP and DLP were 1.98 fold and 1.63 fold higher relative to prednisolone. This significant enhancement in the transport process was apparent due to both P-gp circumvention and peptide transporter recognition of LDP and DLP. Based on these results, it can be anticipated that LDP and DLP prodrugs might produce higher concentrations in the anterior tissues following topical administration. Hence, amino acid configuration (D/L-isomers) in the dipeptide prodrug

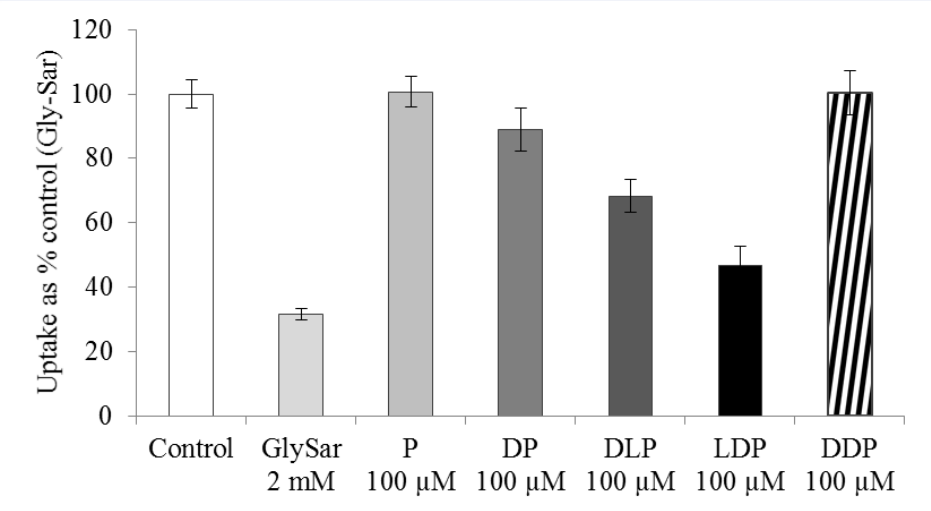

Figure 4 Cellular uptake of [3H]-GlySar in MDCK-MDR1 cells in the absence and presence of prednisolone (P) and prodrugs in DPBS (pH 7.4) at 37°C. Each data point is represented as mean±standard deviation (n=4). Uptake is expressed as percentage of control ([3H]-GlySar). Asterisk (**) represents significant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

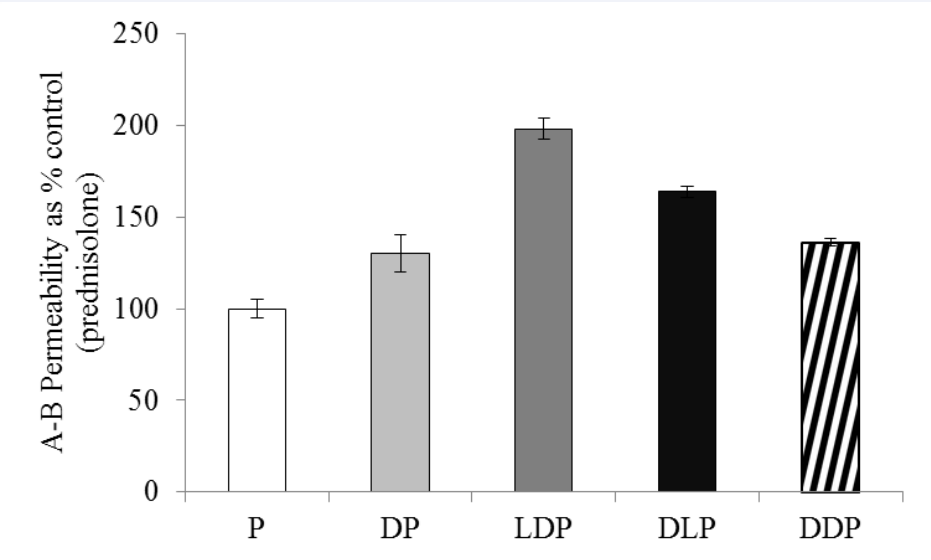

Figure 5 A-B permeability rates of prednisolone and prodrugs across MDCK-MDR1 cells. Each point is expressed as mean ± standard deviation (n=4). Asterisk** indicates significant difference from control (prednisolone, $p < 0.05$).

structure plays a crucial role in determining the transport of prodrugs. Prodrugs produced using a single L-amino acid isomer (LDP and DLP) might generate superior transport rate relative to prodrugs containing with a single (DP) or two (DDP) D-amino acid isomers.

SIRC cell homogenate study

SIRC cell homogenate study was carried out to determine the effect of D-amino acid isomers in diminishing enzymatic-mediated degradation of stereoisomeric prodrugs. DP was observed to degrade rapidly to regenerate prednisolone. Degradation rate constant and half life generated by DP were 0.004±0.0003 min^{-1} and 2.7±0.2 h, respectively. Degradation half life displayed by DP in cell homogenate was significantly lower relative to chemical hydrolysis at pH 7.4 ($t_{1/2}$ = 4.5±0.9 h). This result indicated that DP is highly susceptible to degradation by esterase class of enzymes. Degradation half life of LP was approximately 16 min. Results obtained from this study demonstrated that DP is significantly stable to enzymatic degradation relative to LP. Degradation half life of DP (160 min) was 10-fold higher compared to that of LP (16 min). Significant rise in degradation half-life suggest that prodrug developed using D-amino acid isomer may be significantly stable to enzymatic hydrolysis compared to L-isomers.

Enzymatic stability of LDP and DLP in SIRC cell homogenates was also determined. In comparison to DP, peptide prodrugs degraded significantly slowly. Degradation rate constant of LDP and DLP were found to be 0.5±0.03x 10^{-3} and 0.39 ±0.04 x 10^{-3} min^{-1}, respectively. Degradation half-life generated by LDP and DLP were approximately 23 ±1 and 29±2 h, respectively. Interestingly, DDP was observed to be significantly stable with no apparent degradation throughout the length of study in cell homogenates.DLP prodrug could undergo enzyme-mediated degradation at two positions: a) ester bond and b) peptide bond. Degradation of ester bond will result in regeneration of prednisolone, whereas cleavage of amide bond will regenerate LP. A large amount of prednisolone was regenerated throughout the length of study, indicating enzymatic hydrolysis of DLP. This result indicates that DLP significantly might be undergoing esterase-mediated degradation to produce parent drug. Similarly, LDP degraded enzymatically to reproduce prednisolone at all time points of the study. Regeneration of prednisolone is extremely crucial as the parent drug will be freely available to elicit anti-inflammatory responses in anterior ocular tissues. However, further studies are in process to delineate the exact mechanism of enzyme-mediated degradation of LDP and DLP prodrugs. In these studies, degradation half-life of LDP and DLP will be determined in the presence of various peptidase and esterase inhibitors.

CONCLUSION

The present study suggests that stereoisomeric prodrugs could be a feasible strategy to improve the corneal absorption of prednisolone following topical administration. These compounds displayed superior aqueous solubility and bypassed P-gp mediated cellular efflux of prednisolone. Moreover, these compounds were recognized by peptide transporters, which are highly expressed on the apical surface of the corneal epithelium. Based on the results obtained from this study, stereoisomeric prodrugs could be a feasible approach to improve accumulation of prednisolone into anterior ocular tissues following topical administration.

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