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Synthesis, Transport and Mechanism of a Type I Prodrug: L-Carnitine Ester of Prednisolone

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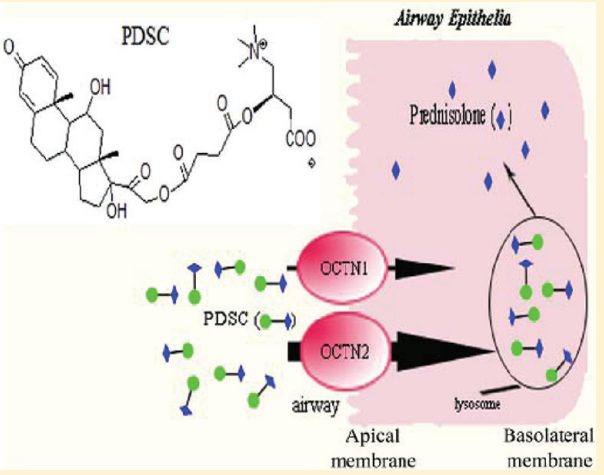
ABSTRACT: Aerosol glucocorticoid medications have become more and more important in treating BA (bronchial asthma). Although these agents are dosed to directly target airway inflammation, adrenocortical suppression and other systematic effects are still seen. To tackle this problem in a novel way, two L-carnitine ester derivatives of prednisolone (as the model drug), namely, PDC and PDSC, were synthesized to increase the absorption of prednisolone across the human bronchial epithelial BEAS-2B cells by the organic cation/carnitine transporter OCTN2 (SLC22A5) and then to slowly and intracellularly release prednisolone. The transport of prednisolone, PDC and PDSC into the human bronchial epithelial BEAS-2B cells was in the order PDSC > prednisolone > PDC at 37 °C. It was found that PDSC displayed 1.79-fold increase of uptake compared to prednisolone. Transport of PDSC by BEAS-2B was temperature-, time-, and Na^+-dependent and saturable, with an apparent K_m value of 329.74 μM, suggesting the involvement of carrier-mediated uptake. An RT-PCR study showed that organic cation/carnitine transporters OCTN1 and OCTN2 are expressed in BEAS-2B cells, but little in HEK293T cells. The order of uptake by HEK293T was prednisolone > PDC > PDSC. In addition, the inhibitory effects of organic cations such as L-carnitine, ergothioneine, TEA^+ and ipratropium on PDSC uptake in BEAS-2B cells were in the order L-carnitine > ipratropium > TEA^+ > ergothioneine, whereas their inhibitory effects on PDSC uptake in HEK293T cells were negligible. Finally, in vitro LPS-induced IL-6 production from BEAS-2B was more and longer suppressed by PDSC than prednisolone and PDC. All of these results suggested PDSC may be an attractive candidate for asthma treatment.



KEYWORDS: prednisolone, L-carnitine, prodrug, OCTN2 transporter, pulmonary absorption, BEAS-2B cells, ELISA, IL-6

INTRODUCTION

Organic cation/carnitine transporters (OCTNs) belong to the solute carrier family SLC 22A. Three distinct OCTN isoforms, namely, OCTN1−3, have been identified and mediate the entry of organic cations into cells.[1] The human SLC-family transporters hOCTN (1/2/3) are potential carrier systems which are involved in L-carnitine transport.[2−4] hOCTN1, a low-affinity carnitine transporter, is expressed in kidney, trachea, bone marrow, fetal liver, and some tumoral human cells.[5,6] hOCTN2 is 76% homologous to hOCTN1. This high-affinity carnitine transporter is expressed in kidney, trachea, spleen, bone marrow, skeletal muscle, heart, and placenta in adult humans. It has been reported that a mutation on the gene coding for hOCTN2 could be responsible for a primary systemic carnitine deficit.[7−9] Recently, hOCTN3 has been identified in patients presenting with Crohn's disease. It is an intermediate-affinity carnitine transporter, which has been suggested by kinetic studies in testis, muscle culture, renal tubular cells, and intestinal epithelial cells.[10,11]

L-Carnitine (β-hydroxy-γ-trimethylaminobutyrate), a small highly polar zwitterionic molecule, plays a crucial physiological role in β-oxidation and energy metabolism via translocation of long-chain fatty acids across the mitochondrial inner membrane.[12,13] L-Carnitine is endogenously produced in very few tissues (brain, kidney, and liver); therefore it is largely obtained from the diet (meat and dairy products) and is absorbed in the intestine before it is further distributed to some other tissues in the body via OCTN transporters.[14,15]

Bronchial asthma (BA) is characterized by airway inflammation, which results in hyperresponsiveness of the lower respiratory tackle to various environmental stimuli.[16] They are a major worry to the patient because various symptoms compromise school and work performance. Hospitalization of patients places a burden on society due to the inherent large economic costs.[17] BA is a reversible airway disease, since the abnormally low airflow rates can be partially or fully restored by prescription bronchodilator and anti-inflammatory medications, such as oral prednisolone, at various doses and for various numbers of days. This

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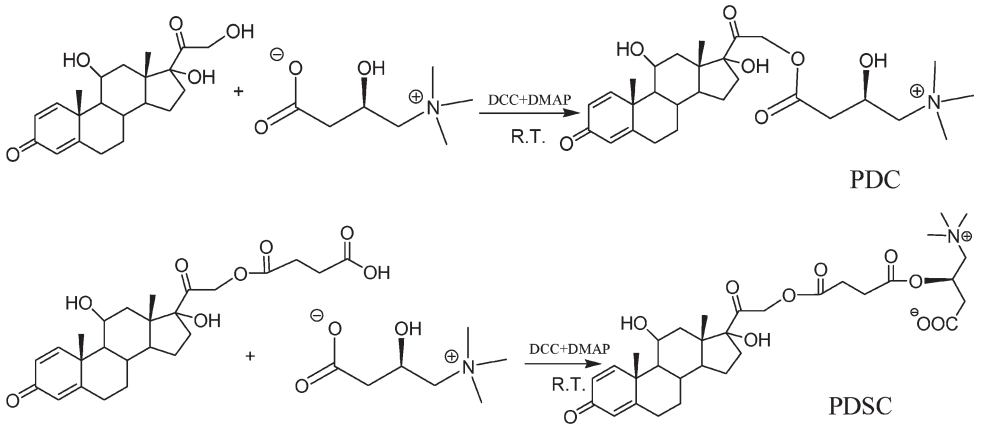
Scheme 1. Synthesis of PDC and PDSC



treatment is regarded as the "gold standard" for maximal anti-inflammatory and clinical efficacy, though at the price of potentially serious long-term and, occasionally, very severe systemic side effects.[18] During the past 10 years, the number of prescriptions of aerosol glucocorticoid medications has increased,[19] demonstrating that anti-inflammatory aerosol corticotherapy is gaining greater acceptance in BA management as recommended by international asthma treatment guidelines.[17] Today, beclomethasone dipropionate and budesonide and fluticasone propionate are the most commonly prescribed aerosol corticotherapies for adult BA.[20] However, even though these agents are dosed in aerosol rather than tablet form to directly target airway inflammation, adrenocortical suppression and other adverse effects are still seen.[21] The systemic absorption of inhaled corticosteroid medications and their potential to cause adrenocortical suppression depends on many factors,[22] especially drug lipophilicity.[23] Highly lipophilic aerosol corticosteroid medications, like fluticasone propionate and mometasone furoate, show great partitioning into the systemic tissue compartment and large volume of distribution, while ones that have low lipophilicity, like triamcinolone acetonide and budesonide, have small volume of distribution but limited permeation into bronchial epithelial cells.[23−25]

Due to the highly lipophilicity of prednisolone (as the model drug), its absorption into airway mucosa was rapid, but its diffusion from mucosa would be fast too, which could cause unwanted systematic effects and poor local anti-inflammatory effects. In the present study, two L-carnitine ester derivatives of prednisolone, namely, PDC and PDSC, were synthesized as described in Scheme 1. Because of highly polarity of L-carnitine, the two synthesized prodrugs will probably reduce the volume of distribution and improve the pulmonary residence time compared with prednisolone when administered by aerosols. Then we investigated the feasibility of these two conjugates to increase absorption of prednisolone across the human bronchial epithelial cells. We also explored the mechanisms for translocation of the two conjugates across the human bronchial cells in vitro and investigated their biological effects in reducing the LPS-induced release of IL-6 by BEAS-2B cells.

MATERIALS AND METHODS

Chemicals and Animals. Ipratropium and ergothioneine were purchased from Sigma-Aldrich (St. Louis, MO). L-Carnitine was purchased from Advanced Technology & Industrial Co., Ltd. (Hongkong, China). Prednisolone succinate and prednisolone were purchased from Lihua Pharmaceutical Co., Ltd. (Henan, China). 4-Dimethylamino-pyridine (DMAP) was purchased from Tianyu (Dongyang, China). *N,N′*-Dicyclohexylcarbodiimide (DCC) and tetraethylammonium (TEA^+) were purchased from Kelong Chemical Reagent Factory (Chengdu, China). Lipopolysaccharide (LPS) was purchased from Sunbio Medical Biotechnology Co., Ltd. (Shanghai, China). Micro BCA protein assay kit was bought from Pierce Chemical Co. (Rockford, IL). Acetonitrile (HPLC purity) was purchased from Thermo Fisher Scientific Inc. (Fair Lawn, New Jersey). All other chemicals and reagents were commercial products of reagent grade.

Kunming mice, 22−25 g, were provided by West China Experimental Animal Center of Sichuan University (China). All protocols were approved by the Institutional Animal Care and Use Committee of Sichuan University and Project of Sichuan Animal Experiment Committee, license 045, China.

Cell Culture. BEAS-2B, HEK293T were obtained from American Type Culture Collection (Manassas, VA). DMEM and the serum-free medium LHC-8 were purchased from Gibco Ltd. (Paisley, U.K.). BEAS-2B cells are human bronchial epithelial cells transformed by SV40 large T antigen.[26] BEAS-2B cells were grown in the serum-free medium LHC-8 with 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco-Life Technologies, Gaithersburg, MD) in an atmosphere containing 5% CO_2, at 37 °C. HEK293T was cultured in 5% CO_2, at 37 °C, in DMEM supplemented with 10% heat-inactivated (30 min at 56 °C) fetal bovine serum (FBS) (Gibco-Life Technologies, Gaithersburg, MD), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco-Life Technologies, Gaithersburg, MD).

Synthesis of Prednisolone-L-carnitine and Prednisolone Succinate-L-carnitine. Prednisolone was monoesterified with carboxyl group of L-carnitine. Anhydrous *N,N*-dimethylformamide (DMF, 20 mL) was added to a flask containing prednisolone (432 mg, 1.2 mmol), a catalytic amount of 4-(dimethylamino)-pyridine (DMAP, 37 mg, 0.3 mmol), *N,N′*-dicyclohexylcarbodiimide (DCC, 790 mg, 3.1 mmol) and L-carnitine (161 mg, 1 mmol) at ambient temperature, under an atmosphere of nitrogen. The reaction mixture was stirred overnight at room temperature, and the reaction was monitored by thin layer chromatography [eluent dichloromethane (DCM)/methanol, 1:0.2 (v/v), prednisolone-L-carnitine, R_f 0.37]. After the reaction was finished, 5% acetic acid solution was added, the precipitated

dicyclohexylurea (DCU) was filtered off, and the filtrate was evaporated under reduced pressure. The residue was purified by column chromatography [eluent DCM/methanol, 1:0.2] to give prednisolone-L-carnitine (272.16 mg, 54%) as a white solid.

Prednisolone succinate was monoesterified with the hydroxyl group of L-carnitine by the same method mentioned above. The reaction was monitored by TLC [eluent DCM/methanol, 1:0.5 (v/v), prednisolone succinate-L-carnitine, R_f 0.3], and the crude product was purified by column chromatography [eluent DCM/methanol, 1:0.5 (v/v)] to get prednisolone succinate-L-carnitine (277.84 mg, 46%) as a white solid. All compounds were characterized by NMR and electrospray ionization mass spectrometry.

Prednisolone-L-carnitine: yield 54%; mp 222−224 °C; purity 98.2% according to HPLC; λ_{max} = 254.0 nm. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.81 (1H, s), 7.30−7.32 (1H, d, J = 10.4 Hz), 6.14−6.17 (1H, d, J = 11.6 Hz), 5.91 (1H, s), 5.18 (1H, s), 4.64−4.68 (2H, m), 4.46−4.52 (1H, dd, J = 6 Hz, J = 19.2 Hz), 4.27−4.28 (1H, s), 4.16 (1H, s), 4.03−4.10 (1H, dd, J = 6 Hz, J = 19.6 Hz), 3.17−3.26 (2H, m), 3.11 (1H, s), 2.50−2.59 (3H, m), 2.27−2.31 (1H, d, J = 16.4 Hz), 2.06−2.01 (2H, m), 1.93−1.98 (1H, d, J = 18.8 Hz), 1.74−1.80 (2H, d, J = 23.6 Hz), 1.60−1.68 (2H, m), 1.52−1.58 (1H, d, J = 22 Hz), 1.38 (1H, s), 1.26−1.36 (1H, m), 0.96−1.06 (1H, m), 0.86−0.90 (1H, d, J = 14 Hz), 0.76 (1H, s). ^{13}C NMR (400 MHz, DMSO-d_6), δ (ppm): 211.9, 185.5, 176.1, 170.8, 157.0, 127.3, 121.8, 88.7, 68.6, 66.0, 62.21, 55.6, 52.2, 51.3, 46.8, 39.7, 34.2, 33.1, 31.6, 23.8, 21.1, 17.2. High resolution mass spectrum (HRMS): calculated for $C_{28}H_{42}NO_7$, 504.65 Da; found, 504.65 Da.

Prednisolone succinate-L-carnitine: yield 46%; mp 218−220 °C; purity 97.6% according to HPLC; λ_{max} = 254.0 nm. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 7.59−7.61 (1H, d, J = 10 Hz), 6.37−6.40 (1H, d, J = 10 Hz), 6.16 (1H, s), 5.66−5.71 (1H, q J = 8 Hz), 5.02−5.20 (2H, 2d, J = 18.4 Hz), 4.53 (1H, s), 3.90−3.96 (1H, m), 3.65−3.69 (1H, d, J = 14.4 Hz), 3.23 (9H, s), 2.88−2.90 (2H, d, J = 6 Hz), 2.81−2.83 (2H, d, J = 5.6 Hz), 2.60−2.72 (3H, m), 2.44−2.56 (2H, m), 2.19−2.21 (2H, d, J = 9.2 Hz), 1.97−2.00 (1H, d, J = 11.2 Hz), 1.79−1.82 (2H, d, J = 12.8 Hz), 1.48−1.71 (2H, m), 1.46 (3H, s), 1.11−1.20 (2H, m), 0.88 (3H, s). ^{13}C NMR (400 MHz, DMSO-d_6), δ (ppm): 211.2, 185.8, 177.5, 168.8, 155.4, 124.2, 128.4, 89.7, 70.2, 66.7, 65.1, 64.2, 59.0, 54.7, 49.9, 44.4, 39.8, 38.8, 33.0, 32.0, 29.9, 25.7, 20.6, 14.0. High resolution mass spectrum (HRMS): calculated for $C_{32}H_{45}NO_{10}$, 605.41 Da; found, 605.56 Da.

Stability and Hydrolysis Studies. *a. Stability in Mice Plasma.* A stock solution of PDSC was aliquoted, dried under N_2 flow, and reconstituted to 10 μM with mouse plasma previously preheated to 37 °C. Aliquot samples were collected at different time points. Samples were quenched in 5-fold volumes of ice-cold methanol and centrifuged at 2500g for 5 min. Supernatant was removed and analyzed by HPLC (Waters Co. Ltd., Milford, MA, USA). Rate constants of hydrolysis were determined by pseudofirst-order kinetic models.

b. Chemical Stability. The nonenzymatic hydrolysis of PDSC in different pH phosphate buffers (pH 1.2, 4.5, 6.8, 7.4) was determined as described above.

c. Enzymatic Stability. Enzymatic stability experiments were performed in triplicate with some modifications as described previously.[27] Confluent BEAS-2B cells were washed with phosphate buffer saline (PBS, pH 7.4) and then harvested with 0.05% trypsin−EDTA at 37 °C for 5−20 min. Trypsin was neutralized by adding DMEM. The cells were washed off the plate and spun down by centrifugation. The pelleted cells were washed twice with pH 7.4 PBS (10 mM), and resuspended in pH 7.4 PBS (10 mM) to obtain a final concentration of approximately 5 × 10^6 cells/mL. The cells were then lysed by three freeze/thaw cycles. The resultant was resolved in 0.2 M acetate buffer (pH 4.5). The hydrolysis reactions were carried out in 24-well plates (Corning, NY). BEAS-2B cells suspension (960 μL) was placed in triplicate wells, and the reactions were started with the addition of substrate stock solution (40 μL) and incubated at 37 °C. The final concentration of the test compound in the reaction mixtures was 5 μM. At various time points, 20 μL aliquots were removed and added to 2 volumes of 10% ice-cold TFA. The mixtures were centrifuged for 30 min at 1800g and 4 °C, and the supernatant was filtered through a 0.45 μm filter. The recovered filtrate was then analyzed by HPLC.

d. BALF Stability. Mice were sacrificed, and a bronchoalveolar lavage (BAL) was immediately performed using 4 × 0.5 mL of pH 7.4 PBS (10 mM) as previously described.[28,29] The bronchoalveolar lavage fluid (BALF) was collected and lyophilized. The resultant was redissolved with 190 μL of pH 7.4 PBS (10 mM) in triplicate wells of 24-well plates (Corning, Corning, NY).The hydrolysis reactions were started with the addition of substrate stock solution (10 μL) in 190 μL of pH 7.4 PBS (10 mM) and incubated at 37 °C. The hydrolysis rate of PDSC in BALF was determined as (c) described.

Transport Experiments with HEK293T and BEAS-2B Cells. Transport experiments were performed in triplicate with some modifications as described previously.[30] BEAS-2B and HEK293T cells (1 × 10^5 cells/well) were seeded into 24-well plates and then cultured for 2 days. In general, uptake was initiated by adding 1 mL of transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES, adjusted to pH 7.4) containing test substrate to cells of interest, and cells were incubated for 30 min to evaluate prednisolone and PDC, PDSC uptake by BEAS-2B and HEK293T respectively at 4 or 37 °C, unless the period of time was indicated. L-Carnitine, ergothioneine, TEA^+ or ipratropium was also added in to assess their inhibitory potential. At the end of the uptake reaction, cells were washed with ice-cold transport buffer twice, and then, after cells were digested with 0.25% trypsin solution, three freeze/thaw cycles of the suspensions were carried out to release the intracellular prednisolone or prodrug. Prodrug was hydrolyzed to release prednisolone by adding 30 μL of 1 M NaOH to each well, and after incubation at 30 °C for 5 min, 30 μL of 1 M HCl was added in to neutralize the excess NaOH. The resultant cell lysate was lyophilized and reconstructed with 100 μL of H_2O. The amount of prednisolone in the solution was measured using HPLC. Part of the solution was used for determination of total protein amount with a BCA assay kit as previously described.[31] Na^+ free transport medium was prepared by replacing 125 mM NaCl in the standard transport medium with 125 mM *N*-methyl-D-glucamine (NMG) Cl and was used to assess the uptake in the absence of Na^+.

Analytical Methods. The kinetic parameters K_m (Michaelis constant) and V_{max} (maximal uptake rate) of PDSC uptake by BEAS-2B cells were calculated by nonlinear least-squares regression analysis using OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA), according to the following Michaelis−Menten saturation kinetics equations, where v and s are the uptake rate of substrate and substrate concentration,

Table 1. Gene-Specific Oligonucleotide Primer Pairs for qRT-PCR

gene	primer sequence (5′ to 3′)
OCTN1	
sense	CTGCTATTGCGAACCCTGCC
antisense	CAGCATGACCAGACCAATGGATAAG
OCTN2	
sense	CAGCCATCCTCACCTTGTTTC
antisense	TGTGGGCCTTTCTTGACCATC
β-actin	
sense	GCTATCCAGGCTGTGCTATC
antisense	TGTCACGCACGATTTCC

respectively.

$$v = V_{max}s/(K_m + s) \quad (1)$$

$$v = V_{max1}s/(K_{m1} + s) + V_{max2}s/(K_{m2} + s) \quad (2)$$

The data were fitted to eq 2 with two saturable transport components, where the indices 1 and 2 indicate the high- and low-affinity components, respectively. Results were presented as the mean ± SEM. The statistical significance of differences between two means was determined by Student's *t* test, and that among means of more than two groups was determined by one-way analysis of variance (ANOVA) followed by a Tukey test using OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA). Differences with a *p*-value of 0.05 or less were considered as statistically significant.

Reverse Transcription Polymerase Chain Reaction. RT-PCR experiments were performed with some modifications as described previously.[30] In general total RNA was prepared from BEAS-2B and HEK293T cells using RNAprep pure Cell/Bacteria Kit according to the manufacturer's protocol (Tiangen Biotech co., Ltd. Beijing, China). Single-strand cDNAs were synthesized with a TINAScript RT Kit (Tiangen Biotech Co., Ltd. Beijing, China), and the expression levels of OCTN1 and OCTN2 mRNAs were analyzed by RT-PCR. The PCR products were separated by electrophoresis in 2% agarose gel and visualized using Light Capture (Bio-Rad, Hercules, CA). Relative quantification of OCTN1 and OCTN2 mRNA expression was performed with an iCycler IQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using RealMasterMix (SYBR Green) (Tiangen Biotech Co., Ltd. Beijing, China) with 50 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. The expression level was normalized to that of β-actin. Gene-specific primers used for RT-PCR are listed in Table 1.

Measurement of Interleukin-6 (IL-6). Cell treatment experiments were performed with some modifications as described previously.[32] In general BEAS-2B cells treatment experiments used 24-well polystyrene plates (NEST, Biotech Co., Ltd.) containing 1 mL of cell culture per well, with cells seeded at an initial density of 5×10^5/well. After one day we applied uptake buffer containing 10 μM concentrations of the treatments for 30 min, followed by incubation with drug-free culture containing 1 μg/mL LPS; at different times we took 1 μL of the medium for cytokine assays and measured viable cell count by MTT (Sigma, USA) after treatment experiments. Experiments used five wells for each treatment level and allocated five wells per culture plate as controls. Positive controls were included to monitor changes in the BEAS-2B cell response. All experiments were replicated with at least two independent cell passages.

Commercially available enzyme-linked immunosorbent assay (ELISA) kits (Pierce-Endogen, Rockford, IL, USA) were used to determine IL-6 levels in cell culture supernatants. IL-6 standard curves (range 0.5−60 pg/mL) and blank buffer wells were included in duplicate in each assay. IL-6 concentrations were expressed as picograms of cytokine per milliliter.

Table 2. The Stability Results of PDSC at 37°C in Phosphate Buffers of Different pH, in Mouse Plasma and Bronchoalveolar Lavage Fluid and in BEAS-2B Cell Homogenates[a]

medium	pH	$t_{1/2}$ (h)
phosphate buffer	1.2	20.38 ± 2.160
	4.5	12.38 ± 1.213
	6.8	9.90 ± 1.030
	7.4	9.49 ± 1.006
mouse plasma		0.07 ± 0.005
bronchoalveolar lavage fluid	7.4	8.45 ± 0.946
bEAS-2B cell homogenates	4.5	9.91 ± 0.971

[a] Results represent mean ± SEM (n = 3).

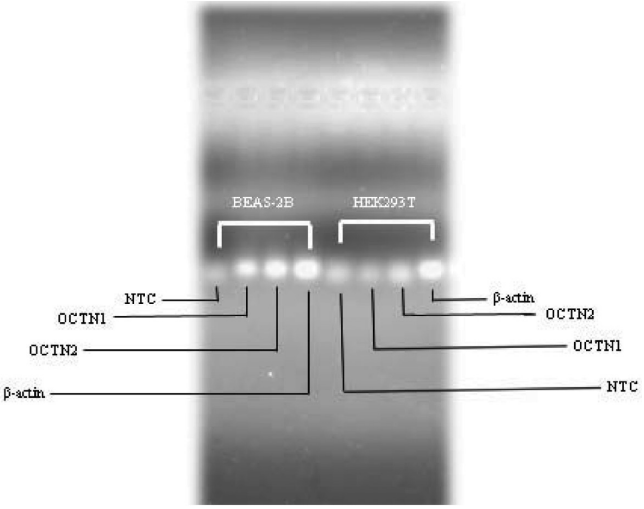


Figure 1. Expressions of OCTN1 and OCTN2 mRNAs in BEAS-2B cells and HEK293T cells. The reactions using specific primer sets were conducted as described in Materials and Methods. NTC stands for no template control (negative control). As positive controls, we used cDNAs derived from BEAS-2B cells and HEK293T cells for β-actin. The PCR reaction was applied for the amplification of each gene as described in Materials and Methods.

RESULTS

Synthesis of Prednisolone Prodrugs. The L-carnitine ester prodrugs of prednisolone and prednisolone succinate were obtained as white fluffy powders. The yield for prodrug analogue was approximately 40−55%, the purity of all prodrugs was at least 95% as determined by HPLC. The molecular weights and ^{1}H NMR and ^{13}C NMR spectra for all prodrugs were in agreement with those required by their structures.

Stability and Hydrolysis Study. The experiments were performed at 37 °C in different pH phosphate buffers, mice plasma,

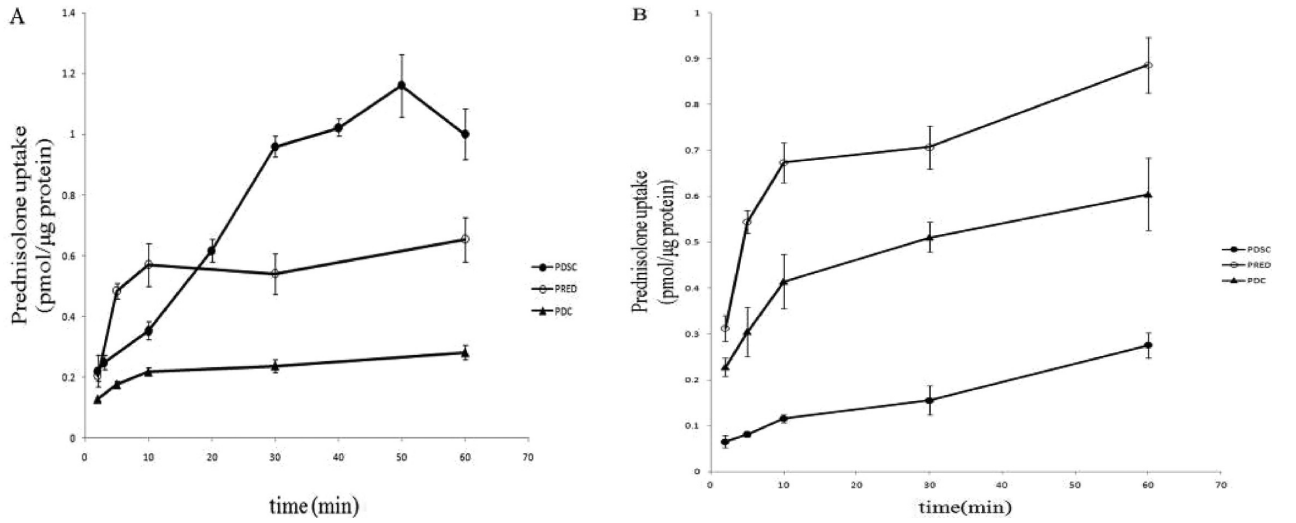


Figure 2. Time course of prednisolone, PDC and PDSC uptake by BEAS-2B cells (A) and HEK293T cells (B). Uptake of prednisolone, PDC and PDSC (10 μM) by BEAS-2B cells and HEK293T cells was measured over 60 min at pH 7.4 and 37 °C. Data are shown as mean ± SEM (n = 3).

BALF and BEAS-2B cell homogenates. The estimated half-lives ($t_{1/2}$) obtained from linear regression of pseudo-first-order plots of PDSC concentration vs time are listed in Table 2. It could be observed that the chemical hydrolysis of PDSC was a pH-dependent process and PDSC was more stable at acidic pH than at neutral pH. The data also indicate that enzymes from mice plasma markedly increase the rate of hydrolysis as compared to that from BALF and BEAS-2B cell homogenates.

Expression of OCTN1 and OCTN2 in BEAS-2B and HEK293T Cells. We examined expression of organic cation transporters in BEAS-2B and HEK293T cells. As shown in Figure 1, mRNA expression of OCTN1, and OCTN2 was studied by means of RT-PCR. OCTN1 and OCTN2 mRNAs were detected in BEAS-2B cells, whereas OCTN1 and OCTN2 mRNAs were not (up to 35 PCR cycles) in HEK293T cells.

Transporter-Mediated PDSC Uptake by BEAS-2B Cells. To investigate the mechanisms of prednisolone, PDC and PDSC transportation into airway epithelium, prednisolone, PDC and PDSC uptake was initially examined in BEAS-2B and HEK293T cells (Figure 2), after exposing the cells to 10 μM prednisolone, PDC and PDSC at 37 °C. Uptake of prednisolone and PDC by BEAS-2B and HEK293T cells was rather quick and reached plateau within 10 min, while uptake of PDSC in epithelial cells increased in a time-dependent manner, and was linear with time up to at least 30 min. To characterize the saturable uptake system in airway epithelial cells, temperature dependence of prednisolone, PDC and PDSC uptake was examined. Figure 3 showed the temperature dependence of prednisolone, PDC and PDSC uptakes after exposing the cells from 1 μM to 800 μM substrates for 30 min at 37 and 4 °C, respectively. Lowering temperature from 37 to 4 °C caused 80% decrease in absorption of PDSC by BEAS-2B cells and 15% decrease by HEK293T cells. By contrast, decreasing temperature to 4 °C resulted in approximately 35% inhibition of the transportations of prednisolone and PDC by BEAS-2B and HEK293T cells. A much greater decrease in absorption observed at 4 °C for PDSC indicated the involvement of process(es) other than passive diffusion through paracellular pathways. We compared PDSC uptake by BEAS-2B Cells and HEK293T cells (Figure 3). Its uptake by BEAS-2B Cells was significantly higher than that by HEK293T cells, and increased time-dependently. In particular, the order of uptake by BEAS-2B was PDSC > prednisolone > PDC, whereas the order of uptake by HEK293T was prednisolone > PDC > PDSC. These results suggested that a carrier-mediated transport mediated PDSC uptake by BEAS-2B cells. To evaluate the uptake rate, we then quantified intracellular accumulation of PDSC for 30 min, which was in the linear range of uptake. PDSC uptake by BEAS-2B cells, which was obtained by subtraction of the uptake at 4 °C and fitted to the Michaelis−Menten equation, had two components with K_{m1} of 1.67 ± 0.03, K_{m2} of 427.15 ± 109.18 μM, V_{max1} of 0.02 ± 0.001, and V_{max2} of 0.12 ± 0.001 pmol/μg/30 min (Figure 4). The first-order rate constant (k_d) for the apparently nonsaturable component was $1.94 \times 10^{-5} \pm 3.09 \times 10^{-6}$ pmol/μg/30 min (obtained by nonlinear least-squares regression analysis).

Characterization of PDSC Uptake by BEAS-2B Cells and HEK293T Cells. The inhibitory effects of L-carnitine, ergothioneine, TEA^+, ipratropium and Na^+ on PDSC uptake by BEAS-2B cells and HEK293T cells are shown in Figure 5. PDSC uptake by BEAS-2B cells was significantly reduced to 23.21% of control in the presence of L-carnitine (Figure 5A), while the inhibitory effect of ergothioneine was marginal (Figure 5B). As a percentage of the uninhibited control, the order of inhibitory effects was L-carnitine (23.21%) > ipratropium (61.31%) > TEA^+ (75.11%) > ergothioneine (84.46%) (Figure 5A−D). When Na^+ in the transport buffer was replaced with *N*-methyl-D-glucamine at equimolar concentration, the uptake of PDSC by BEAS-2B cells was significantly decreased (Figure 5E). Interestingly under the same conditions, the inhibitory effects of these organic cations and Na^+ on PDSC uptake by HEK293T cells were trifling (Figure 5).

IL-6 Production. To evaluate the potential potency of PDSC on asthma, the effect of prednisolone, PDC or PDSC on LPS-induced IL-6 production from BEAS-2B cells was examined at 2, 4, 8, 16, 24 h by ELISA, respectively.

Figure 6 clearly showed that, in the positive control group, LPS strongly stimulated the release of IL-6 from BEAS-2B cells in a time-dependent manner. This result was in agreement with previous studies.[33] The release of IL-6 from the negative control group, which was the standard uptake buffer, was insignificant up

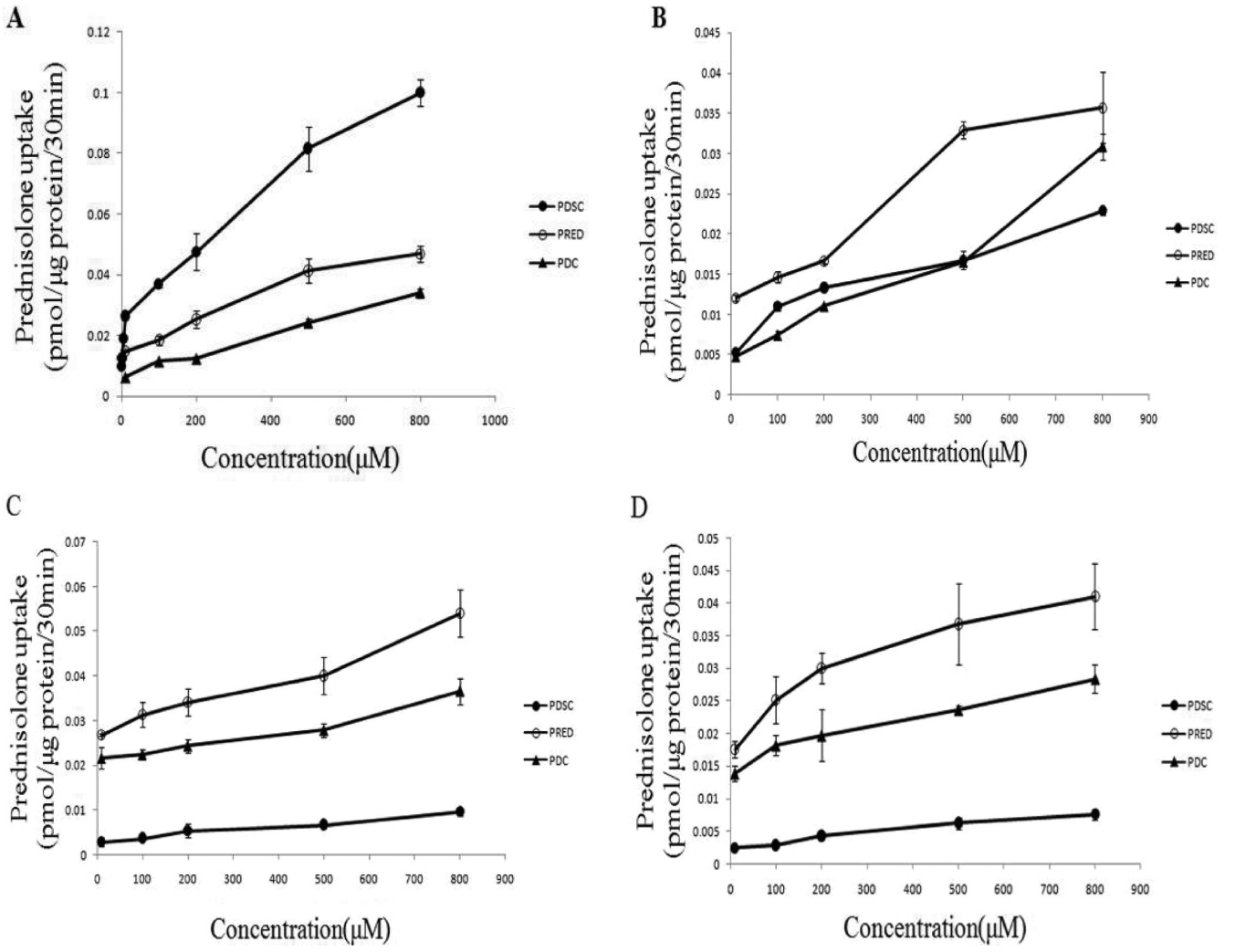


Figure 3. Effect of temperature on uptake after exposing the BEAS-2B cells (A and B) and HEK293T cells (C and D) to prednisolone, PDC and PDSC for 30 min at different concentrations. (A) BEAS-2B cells were incubated at 37 °C. (B) BEAS-2B cells were incubated at 4 °C. (C) HEK293T cells were incubated at 37 °C. (D) HEK293T cells were incubated at 4 °C. Each point represents the mean ± SEM (n = 3).

to 24 h. Among the three treatment groups, the release of IL-6 from the PDSC group was the significantly least at 8 and 16 h. The release of IL-6 from the prednisolone group was significantly less than that of PDC treatment and positive control groups within 24 h. On the other hand, the release of IL-6 was not significantly different between PDC treatment and positive control groups. After the experiment, all of the cells had more than an 80% survival rate (data not shown).

DISCUSSION

L-Carnitine has long been known for its role in fatty acid oxidation, and recently it has been shown that the OCTN1 and OCTN2 are responsible for the transport of L-carnitine in many organs including the brain, placenta, intestine, and kidney.[4,35−37] Strategies based on targeting a specific transporter by designing prodrugs may be used to improve the amount of transportation of some drugs.[37,38] In the present study, we synthesized two L-carnitine ester prodrugs and then we provided evidence for an active absorption mechanism for PDSC in the human airway epithelial cell line BEAS-2B, accomplished by expression of the organic cation transporters OCTN1 and OCTN2. In contrast to the uptake of PDSC by OCTN2 in a high affinity manner, the uptake of PDSC by OCTN1 is a low affinity process. Thus the BEA-2B cells absorption of PDSC appears to involve transport by OCNT2 and possibly OCTN1. Our findings also suggested that inhaled β_2-adrenergic agonists such as ipratropium might inhibit the absorption of PDSC when the two drugs are administered in combination. Finally, our studies indicated that the PDSC could cause sustained reduction in the LPS-induced release of IL-6 by BEAS-2B cells in vitro.

As shown in Figure 1, the expression profile of organic cation transporters determined by RT-PCR in BEAS-2B and HEK293T cells was in good agreement with the previous reports,[30] showing abundant expression of OCTN1 and OCTN2, in human airway epithelial BEAS-2B cells but little in HEK 293T. Therefore, these two cell lines were chosen for the transport experiments.

Two L-carnitine ester prodrugs (PDC and PDSC) were synthesized with the aim to improve the intracellular accumulation of prednisolone. As shown in Figure 2A, at different time points, the uptake of PDC was always less efficient than that of PDSC. The underlying reason probably was that the carboxyl

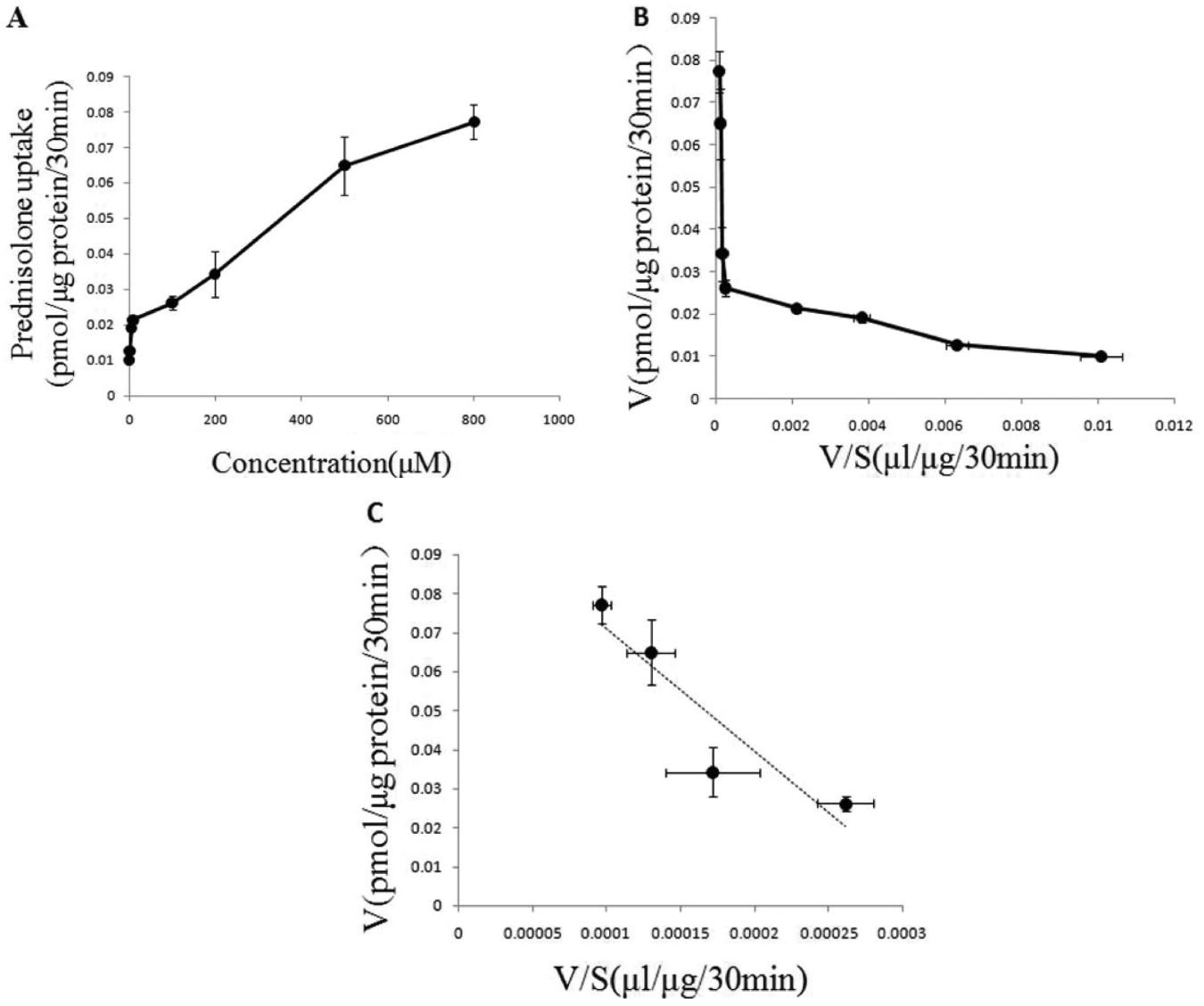


Figure 4. Concentration dependence for uptake of PDSC by BEAS-2B cells. (A) Cultured cells were incubated at 37 °C for 30 min. The uptake of PDSC by BEAS-2B cells was measured within the concentration range between 1 μM and 800 μM. (B) Eadie−Hofstee plots of the uptake of PDSC by BEAS-2B cells. (C) Eadie−Hofstee plots of the uptake of PDSC at low concentration in Figure 3B. These results were obtained by subtraction of PDSC uptake at 4 °C as the nonspecific uptake from the apparent uptake. Each point represents the mean ± SEM (n = 3).

moiety of L-carnitine was important for substrate recognition by OCTN2 transporter.[39] PDC, lacking the carboxyl moiety of L-carnitine, cannot be recognized by OCTN2 transporter. Thus, PDC could not be actively transported by OCTN2 transporter, while PDSC probably could be actively uptaken through OCTN2 transporter.

There were many ways for drug transportation into cells including passive diffusion,[40] active transport[41] and endocytosis.[42] Liposolubility, molecule size, and pK_a-dependent ionization were major factors in drug passive transport.[43−45] However, it was becoming increasingly clear that physicochemical properties cannot be considered as the only major factors of the extent of drug absorption.[30,46] Many cells possessed specialized membrane transport mechanisms for the entry of physiologically important molecules, such as sugars, amino acids, and neurotransmitters.[47,48] The SLC22 transporter family including OCTN1 and OCTN2 had been identified to handle cellular influx of organic cations such as L-carnitine and ipratropium.[30,49]

The uptake of prednisolone was better than that of PDC by BEAS-2B and HEK293T cells since the liposolubility of prednisolone was better than that of PDC. Prior to the work presented here, salbutamol and ipratropium bromide had been shown to be actively absorbed from human airway epithelium,[30,46] through the OCTN1 and OCTN2. Since there were similarities between salbutamol, ipratropium and PDSC in physicochemical properties, such as lipophilicity and membrane permeability, they may share the same transporter.[50] Here, we demonstrated for the first time that the uptake of PDSC was also carrier-mediated in airway epithelial cells by showing the transport's saturable kinetics, temperature sensitivity, Na^+ dependence and inhibition with some organic cationic compounds. Thus, although the liposolubility of PDSC was poor, the uptake efficiency of PDSC in BEAS-2B cells at 30 min was still better than that of prednisolone, whose liposolubility was fairly good. On the other hand, because of little expression of OCTN1 and OCTN2 transporter on HEK293T cell membrane, the cellular entry of

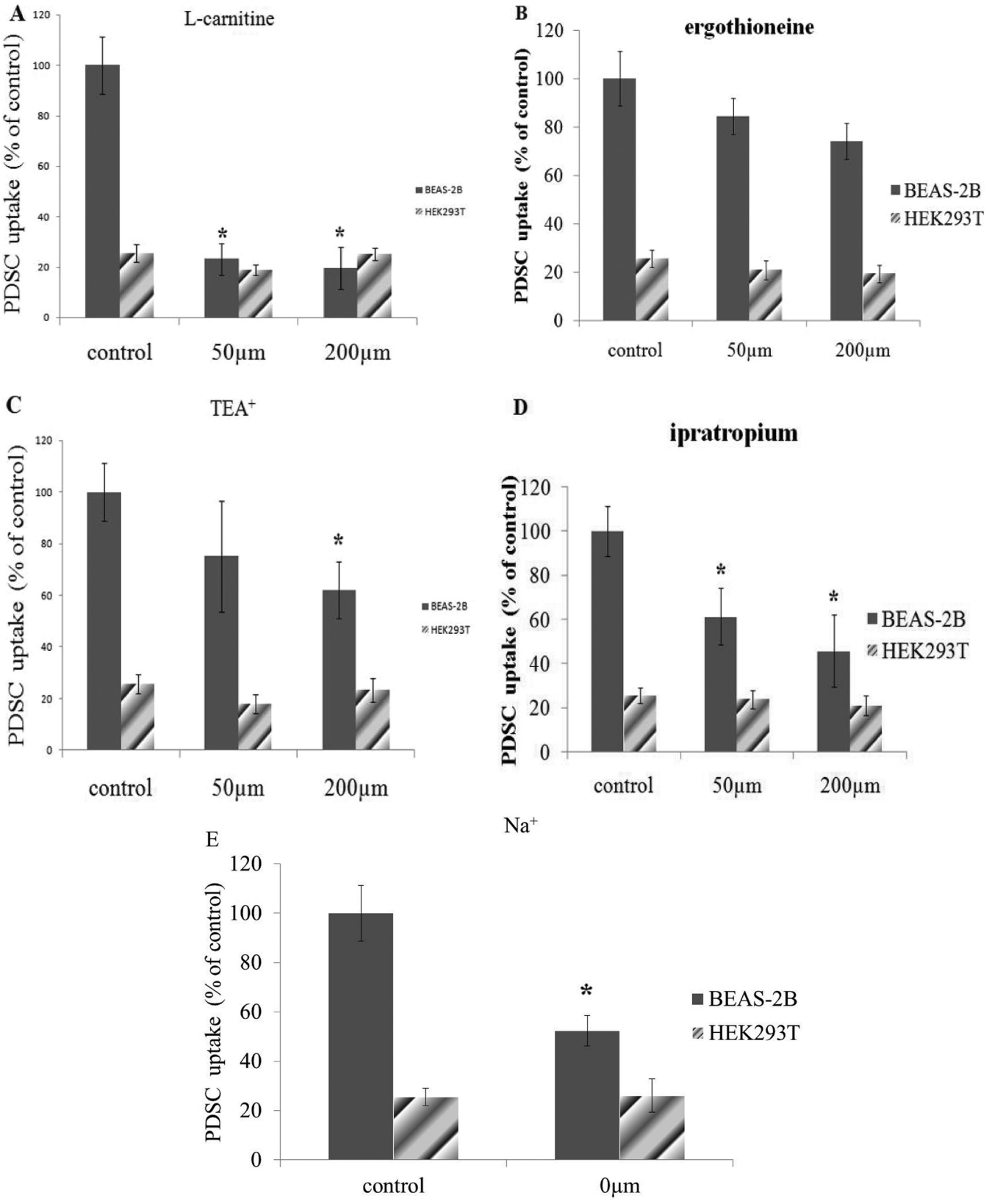


Figure 5. Effect of organic cation transporter inhibitors, including tetraethylammonium (TEA^+), ipratropium, L-carnitine, and ergothioneine, on PDSC uptaken by BEAS-2B cells or HEK293T cells (A—D). Effect of extracellular Na^+ on PDSC uptaken by BEAS-2B cells or HEK293T cells (E). For the Na^+-free experiment, NaCl was replaced by an equimolar concentration *N*-methyl-D-glucamine. Cells were incubated with 5 μM PDSC for 30 min at 37 °C in all experiments in the presence or absence of the inhibitors. Results represent mean ± SEM (n = 3). *P < 0.05 versus control.

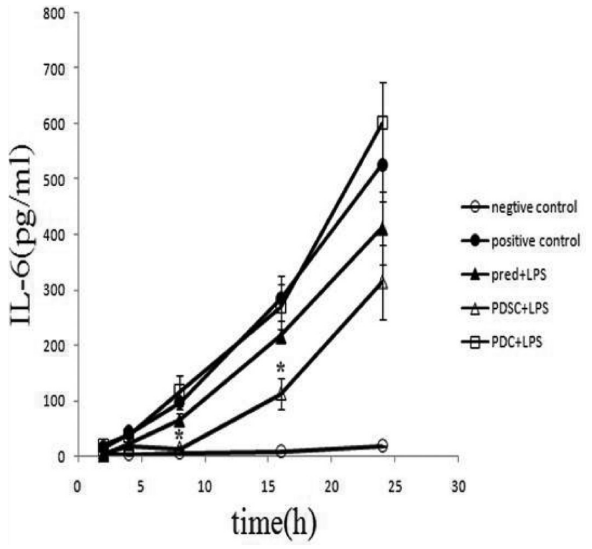


Figure 6. Effect of prednisolone, PDC and PDSC on lipopolysaccharide (LPS)-induced interleukin-6 (IL-6) release in BEAS-2B cells. Confluent monolayers were incubated in uptake buffer containing 10 μM concentrations of the treatments for 30 min, followed by incubation with drug-free culture containing 1 μg/mL LPS for up to 24 h. After stimulation, the supernatants of the cell culture were collected for the quantification of IL-6 by ELISA. The data shown are mean values from five independent experiments, each done in five times. Error bars represent mean ± SEM (n = 5). *P < 0.05 versus prednisolone treatment group.

the three drugs was largely dependent on passive transport. Therefore, PDSC cell uptake was minimal among the three drugs due to its high polarity. Then, PDSC was chosen for further investigations of transport mechanisms.

Kinetic analysis of PDSC uptake by the BEAS-2B cells exhibited the presence of two saturable components, with high (1.67 μM) and low (427.15 μM) affinities. The K_m value of the high-affinity component was similar to that reported for rOCTN2 (2.38 μM).[51]On the other hand, the K_m value of the low-affinity component was closer to that of rOCTN1 (500 μM).[6]The probable reason for this was that the linker succinic acid conjugated with the 21-hydroxyl group of prednisolone made prednisolone long enough to avoid interfering L-carnitine combination with the transporters. So the OCTN2 transporter recognized PDSC as a high-affinity substrate. Further we reported mutual inhibition between PDSC and organic cations in OCTN2-mediated transport. As shown in Figure 5A, PDSC uptake by BEAS-2B was most potently inhibited by L-carnitine, a specific OCTN2 substrate, among the compounds tested. On the other hand PDSC uptake by BEAS-2B was seldom inhibited by ergothioneine (Figure 5B), which is a specific substrate for OCTN1.[52] In addition, the cellular uptake of PDSC was significantly decreased in HEK293T cells, whose OCTN1 and OCTN2 expression was little (Figure 3C). Thus, the present work provides the first evidence that OCTN2 contributes at least partly to the delivery of PDSC across pulmonary airway epithelium.

An ideal prodrug should exhibit good chemical stability but must be enzymatically converted to active parent drug following transport across the biological membrane. The enzymatic stability of PDSC in BEAS-2B cell homogenates, a suitable surrogate representing bioconversion in the human bronchus, revealed that the rates of hydrolysis were affected by the amount and the type of the enzymes. The hydrolysis rates were about 110-fold higher in plasma than in BEAS-2B cell homogenates. This difference may be mostly, if not completely, attributable to the two reasons: (i) a lower pH value used in the BEAS-2B cell homogenates studies (4.5), compared with the pH value for the blood hydrolysis experiments (7.4); (ii) the levels of esterases in the cell homogenates are lower than those in blood.

As a primary interface between pathogens and the environment, epithelial cells lining the mammalian airways and the alveolar surface area were a crucial site for innate immune responses.[53] Lipopolysaccharide (LPS) as a major component of the outer membrane of Gram-negative bacteria stimulates various cells including BEAS-2B cells to initiate a signaling cascade which ultimately leads to cell expression of inflammatory cytokines such as interleukin-6 (IL-6).[54] IL-6 is involved in the pathogenesis of asthma via its broad effects on immune and inflammatory responses.[55] PDSC could reduce IL-6 after it was intracellularly hydrolyzed to release prednisolone. Our results had shown that PDSC could be uptaken more efficiently by BEAS-2B cells. Due to PDSC's high hydrophilicity and one-way transportation by OCTN2, it would be "locked in" the cells, and gradually be hydrolyzed by intracellular lipases to free prednisolone. Release of IL-6 at 16 h was decreased 1.92-fold for treatment with PDSC, compared with prednisolone. These data corresponded with the hydrolysis rate of PDSC in BEAS-2B cell homogenates, suggesting that PDSC could be used for a once-daily treatment regimen for certain patient groups.

In the treatment of patients with asthma, an ideal inhaled corticosteroid would maximize the amount of active drug present at receptors in the lung while minimizing delivery of the drug to other systemic, adverse event-producing receptors.[56] After inhalation of an active corticosteroid, a part of the drug is deposited in the mouth and oropharynx, leading to side effects such as oral candidiasis. Administration of an inactive prodrug may reduce the incidence of local side effects in the mouth and oropharynx region, because the inactive drug deposited in that region would be swallowed before activation can occur. Here we exploited a novel way to avoid these adverse effects. Nearly all polyspecific organic cation transporters are expressed in the lung.[57] Human OCTN1 and OCTN2 are expressed in the respiratory epithelium of trachea and bronchi.[58] Horvath et al.[59]detected OCTN1 and OCTN2 expressions in the apical membrane of human lung airway epithelial cells with OCTN2 also showing relatively stronger expression at the surface of the alveolar type I epithelium. But in bronchial and vascular smooth muscle cells, mRNA expression levels for OCTN1, and OCTN2 were low compared with those in airway epithelial cells. Although we have not found any literature on whether there are OCTN1 and OCTN2 expressions in human mouth and oropharynx region, it is implausible for PDSC to be uptaken by the putative transporters, even if there are lots of them in the region, because PDSC is highly hydrophilic and would be quickly swallowed before it was uptaken by the transporters. Meanwhile, saliva is a colorless, dilute liquid composed of 98% water with pH around 6.64. Saliva composition consists of various enzymes such as α-amylase, lingual lipase and antimicrobial enzymes. But the total concentrations of most of these enzymes are much lower in saliva when compared with serum or cell homogenates.[60] So PDSC is unlikely to degrade in the nearby oropharyngeal bronchoalveolar locations. Therefore, PDSC would be beneficial in reducing the oropharyngeal adverse events (eg, pharyngitis and candidiasis)

that result from deposition of active corticosteroid in the oropharynx. When PDSC is deposited in lung (including bronchi, bronchioles and alveoli, where most asthma attacks[61]), it will be uptaken by OCTN1 and OCTN2 which express in the apical portion of human lung airway epithelial cells and at the surface of the alveolar type I epithelium. After entering cells, as an exotic, PDSC will be trapped in lysosomes that contain some 40 different types of hydrolytic enzymes,[62] where major PDSC conversion probably will slowly occur to release prednisolone and L-carnitine. The released prednisolone will combine with intracellular corticosteroid receptors to exert its potency, and the resulting L-carnitine will participate in transportation of fatty acids for energy production in mammalian mitochondria. Hence after PDSC is broken, it would unlikely be harmful to the cells, but may help to stabilize and even fuel the cells to thrive. This assumption was in agreement with our in vitro cytotoxicity experiments that BEAS-2B cells were respectively incubated with prednisolone, PDC and PDSC in three concentrations (50 μM, 100 μM, 200 μM) for 24 h, the viability of BEAS-2B cells determined by MTT assay were of insignificant difference compared with negative control ($p > 0.05$) (data not shown). Above all, PDSC probably would be safe and efficient to treat patients with asthma.

In conclusion, PDSC was identified as drug substrate of OCTN1 and OCTN2. Our results indicated that OCTN2 was the predominant contributor to carrier-mediated uptake of PDSC by BEAS-2B cells and after uptaken by the cells PDSC could convert into the parent drug and then reduced LPS-induced IL-6 production from BEAS-2B. Further studies are necessary to establish whether or not other inhaled glucocorticoids conjugated with L-carnitine can achieve similar outcomes. Finally, our findings indicated that prodrug of prednisolone with L-carnitine, making use of the carrier-mediated mechanisms, may be an attractive candidate for better drug delivery.

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ABBREVIATIONS USED

PDC, prednisolone-L-carnitine; PDSC, prednisolone succinate-L-carnitine

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