Synthesis and *in Vitro* Characterization of Drug Conjugates of l-carnitine as Potential Prodrugs That Target Human Octn2

LEI DIAO, JAMES E. POLLI

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland 21201

Received 19 August 2010; revised 21 January 2011; accepted 2 March 2011

Published online 31 March 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.22557

ABSTRACT: The objective was to evaluate the potential of drug conjugates with l-carnitine as prodrugs that target organic cation/carnitine transporter (OCTN2). Twenty-two l-carnitine analogues were evaluated for human organic cation/carnitine transporter (hOCTN2) inhibition; the 3′-hydroxyl group was found to be the only functional group not contributing to l-carnitine interaction with hOCTN2 among the three functional groups on l-carnitine (i.e., 3′-hydroxyl, amine, and carboxylate). The 3′-hydroxyl group on l-carnitine was therefore chosen as the conjugate site. Three drug–l-carnitine conjugates (i.e., valproyl–l-carnitine, naproxen–l-carnitine, and ketoprofen–l-carnitine) were synthesized along with two ketoprofen analogues that incorporated a linker group (glycolic acid or glycine) between ketoprofen and l-carnitine (i.e., ketoprofen–glycolic acid–l-carnitine and ketoprofen–glycine–l-carnitine). These potential prodrugs were evaluated for their *in vitro* inhibition, transport, and metabolism properties. All three drug–l-carnitine conjugates and ketoprofen–glycine–l-carnitine were OCTN2 inhibitors, as well as substrates. For valproyl–l-carnitine, $K_i = 155 \pm 19\,\mu M$, $K_m = 132 \pm 23\,\mu M$, and normalized $J_{max} = 0.467 \pm 0.028$; for naproxen–l-carnitine, $K_i = 5.97 \pm 0.81\,\mu M$, $K_m = 257 \pm 57\,\mu M$, and normalized $J_{max} = 0.141 \pm 0.012$; for ketoprofen–l-carnitine, $K_i = 82.2 \pm 5.3\,\mu M$, $K_m = 77.0 \pm 4.0\,\mu M$, and normalized $J_{max} = 0.412 \pm 0.015$; for ketoprofen–glycine–l-carnitine, $K_i = 14.4 \pm 1.4\,\mu M$, $K_m = 58.5 \pm 8.7\,\mu M$, and normalized $J_{max} = 0.0789 \pm 0.0037$. Ketoprofen–glycolic acid–l-carnitine was unstable in metabolic buffers and chemical buffers. On the contrary, naproxen–l-carnitine, ketoprofen–l-carnitine, and ketoprofen–glycine–l-carnitine were stable in chemical and metabolic buffers. The results demonstrate the potential of drug–l-carnitine conjugates to serve as prodrugs that target OCTN2. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:3802–3816, 2011

Keywords: prodrugs; transporters; site-specific delivery; renal reabsorption; stability; substrate; carnitine

INTRODUCTION

l-Carnitine (β-hydroxy γ-trimethylaminobutyrate) is a hydrophilic endogenous molecule that plays an essential role in the transfer of long- and medium-chain fatty acids into mitochondria for β-oxidation.[1] It plays a critical role in energy metabolism of peripheral tissues that derive metabolic energy from fatty acid oxidation such as heart, skeletal muscle, liver, and placenta.[2] l-Carnitine and its endogenous metabolite acetyl-l-carnitine can also cross the blood–brain barrier (BBB) and are considered to have different physiological roles in the brain, such as helping acetylcholine formation.[3] Acetyl-l-carnitine may be useful for the treatment of Alzheimer's disease.[3]

The organic cation/carnitine transporter (OCTN2) is a sodium-dependent high-affinity cation/carnitine transporter that is widely expressed in human tissues, including skeletal muscle, kidney, heart, placenta, and brain.[4] OCTN2 is responsible for l-carnitine tissue distribution and tubular reabsorption.[5–7] Mutations of the *OCTN2* gene in humans cause primary carnitine deficiency.[8–10] Patients with primary carnitine deficiency excrete carnitine in urine due to the defective tubular reabsorption; plasma and tissue levels of carnitine drop below 10% of normal values, eliciting clinical

Abbreviations used: HBSS, Hanks balanced salt solution; MDCK, Madin–Darby canine kidney; OCTN2, organic cation/carnitine transporter; SFB, sodium-free buffer.

Additional Supporting Information may be found in the online version of this article. Supporting Information

Correspondence to: James E. Polli (Telephone: +410-706-8292; Fax: +410-706-5017; E-mail: jpolli@rx.umaryland.edu)

Journal of Pharmaceutical Sciences, Vol. 100, 3802–3816 (2011)

significant symptoms that include hypoketotic hypoglycemia, cardiomyopathy, and skeletal myopathy.[10]

There is very limited information about the substrate requirements of OCTN2. Other than l-carnitine and acylcarnitines, such as acetyl-l-carnitine, propionyl-l-carnitine, and palmitoyl-l-carnitine,[11,12] few compounds or drugs have been shown to be OCTN2 substrates. Beta-lactam antibiotics are OCTN2 substrates.[13] One report demonstrated the transport of butyryl-l-carnitine by human organic cation/carnitine transporter (hOCTN2) in a high-affinity/low-capacity manner using the *Xenopus laevis* oocyte expression system.[14] Another report analyzed the transport efficiency of OCTN2 and found that OCTN2 did not uptake several drugs, although efficiently translocated mildronate, and concluded that OCTN2 was not a general drug transporter but a highly specific carrier for carnitine and closely related molecules.[15]

A drug conjugate of l-carnitine was hypothesized to be recognized and transported by OCTN2. Therefore, such conjugates may have potential to serve as prodrugs that target tissues expressing OCTN2. One *in vivo* report demonstrated that the conjugate of nipecotic acid with l-carnitine showed enhanced penetration of nipecotic acid across the mouse BBB compared with nipecotic acid, and significantly increased the latency to convulsions induced by pentylenetetrazole compared with either nipecotic acid or l-carnitine.[16] However, this study did not explore whether the conjugate was transported by OCTN2, how parent drug impacted transport properties, or how linker chemistry between parent drug and l-carnitine affected conjugate stability and release of the parent drug.

In this study, 22 l-carnitine analogues were evaluated for OCTN2 inhibition in order to find a suitable position on l-carnitine for drug conjugation. Three drug–l-carnitine conjugates (e.g., valproyl–l-carnitine, naproxen–l-carnitine, and ketoprofen–l-carnitine) were synthesized by conjugation of the carboxylate group on parent drug with the 3′-hydroxyl group of l-carnitine. Two analogues of ketoprofen-l-carnitine that incorporate a linker group (glycolic acid or glycine) between ketoprofen and l-carnitine were also synthesized. These potential prodrugs were evaluated for their *in vitro* inhibition, transport, and metabolic properties. Results demonstrated the potential value of drug conjugates with l-carnitine as prodrugs that target OCTN2.

Experimental

Materials

Fetal bovine serum, trypsin–EDTA, and Dulbecco's modified Eagle medium (DMEM) were purchased from Invitrogen Corporation (Carlsbad, California). l-[^{3}H]carnitine was purchased from American Radiolabeled Chemicals (St. Louis, Missouri). l-Carnitine hydrochloride and ketoprofen were purchased from Spectrum Chemicals & Laboratory Products (Gardena, California). Naproxen was purchased from Cayman Chemical (Ann Arbor, Michigan). *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was purchased from Alfa Aesar (Ward Hill, Massachusetts). 4-Dimethylaminopyridine (DMAP) was purchased from Novabiochem (Gibbstown, New Jersey). Dicyclohexylcarbodiimide (DCC) was purchased from Thermo Scientific (Franklin, Massachusetts). Rat plasma was purchased from Valley Biomedical (Winchester, Virginia). Rat kidney homogenate was purchased from Pel-Freez Biologicals (Rogers, Arkansas). Valproic acid, benzyloxyacetic acid, glycine tert butyl ester hydrochloride, l-carnitine analogues in Table 1 (except compounds **5–8**), mouse liver S9 fraction, mouse plasma, rat liver microsome, and esterase solution from porcine liver were purchased from Sigma–Aldrich (St. Louis, Missouri).

Synthesis of l-Carnitine Analogues

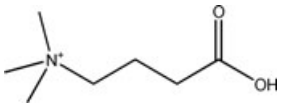
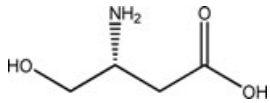
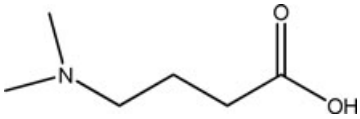
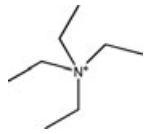
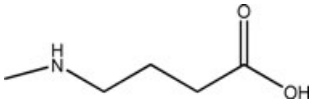
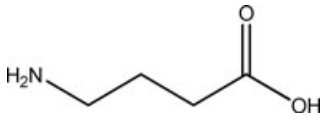
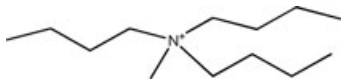
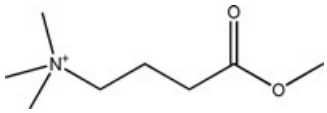
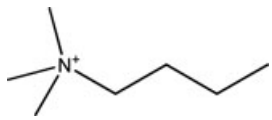
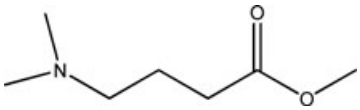
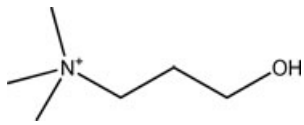
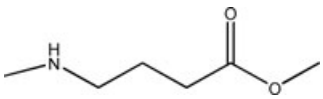
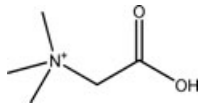
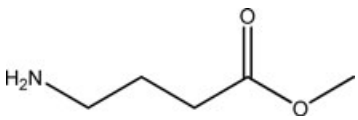
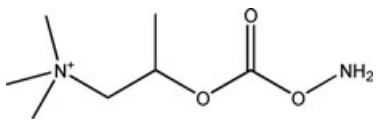
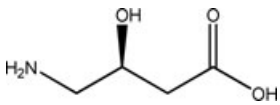
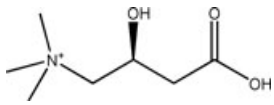
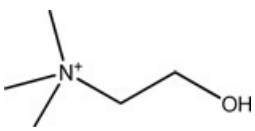
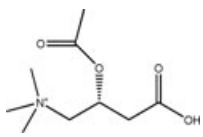
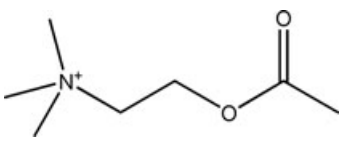
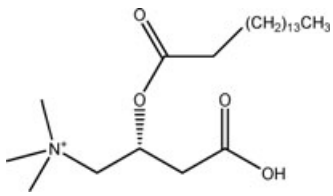
Compounds **5–8** in Table 1 were synthesized by esterification of compounds **1–4** (500 mg each) in MeOH (30 mL) and *p*-toluenesulfonic acid (100 mg) overnight at room temperature (RT). To purify compound **5**, the solvent was evaporated under vacuum and the reaction mixture was dissolved in acetone followed by filtration. The filtrate was dried under vacuum to yield the final compound **5**. To purify compounds **6–8**, the crude product was dissolved in 1 M NaOH (20 mL), extracted into 15 mL EtOAc (3×), and washed with 15 mL of 1 N $NaHCO_3$ (3×), 15 mL of water (3×), and 15 mL of brine (1×). The organic layer was dried over sodium sulfate and filtered. Removal of EtOAc under reduced pressure yielded compounds **6–8**.

Synthesis of Valproyl–l-Carnitine and Ketoprofen–l-Carnitine

Schemes 1–4 summarize the synthesis of valproyl–l-carnitine, ketoprofen–l-carnitine, naproxen–l-carnitine, ketoprofen–glycolic acid–l-carnitine, and ketoprofen–glycine–l-carnitine, respectively. Synthesis methods for all five compounds were modified from a previous report and also from applied protection chemistry.[17,18]

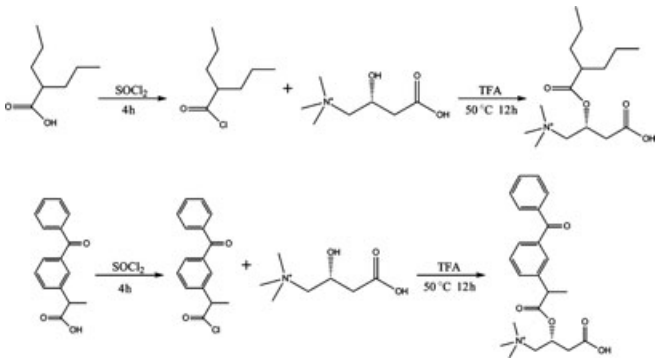
For valproyl–l-carnitine and ketoprofen–l-carnitine (Scheme 1), valproic acid or ketoprofen (1 g) was activated by oxalyl chloride (0.5 mL) for 4 h. Three drops of dimethylformamide (DMF) was added as a catalyst. l-Carnitine (1.1 equivalent) dissolved in **trifluoroacetic acid** (TFA; 10 mL) was then added to activated valproic acid or ketoprofen after solvent evaporation under vacuum. The reaction mixture was heated to 50°C for 12 h, after which the solvent

Table 1. List of 22 l-Carnitine Analogues Screened as Human Organic Cation/Carnitine Transporter Inhibitors

Number	l-Carnitine Analogue Structure	Percent Uptake	Number	l-Carnitine Analogue Structure	Percent Uptake
1		2.28 (± 0.09)	12		90.9 (± 4.5)
2		8.98 (± 0.06)	13		52.9 (± 1.3)
3		40.6 (± 1.2)	14		23.9 (± 0.4)
4		92.1 (± 3.4)	15		48.2 (± 1.1)
5		19.5 (± 0.5)	16		83.6 (± 2.1)
6		13.9 (± 0.3)	17		71.0 (± 2.5)
7		62.8 (± 1.6)	18		45.2 (± 0.2)
8		91.1 (± 3.4)	19		74.1 (± 2.6)
9		94.0 (± 4.9)	20		6.05 (± 0.09) using 100 μM
10		84.8 (± 2.5)	21		15.3 (± 0.3) using 100 μM
11		73.8 (± 4.2)	22		16.7 (± 0.6) using 100μM

Cis-inhibition studies of l-carnitine (2.5 μM with spiked l-[^{3}H] carnitine) uptake were carried out using l-carnitine analogue at a single concentration (500 μM unless indicated) in Hanks balanced salt solution. Results are expressed in terms of percent uptake of l-carnitine, compared with l-carnitine uptake without l-carnitine analogue present. Lower percent uptake of l-carnitine indicates higher inhibition potency.

 DOI 10.1002/jps

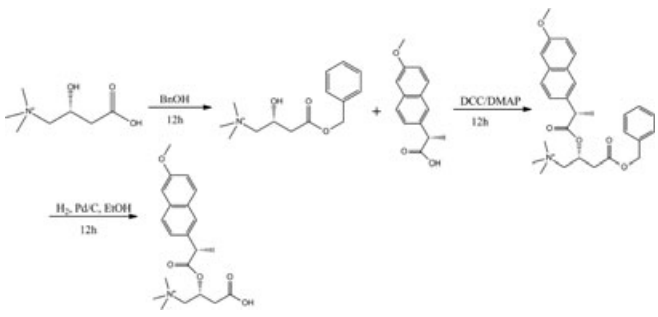


Scheme 1. Synthesis of valproyl–l-carnitine and ketoprofen–l-carnitine.

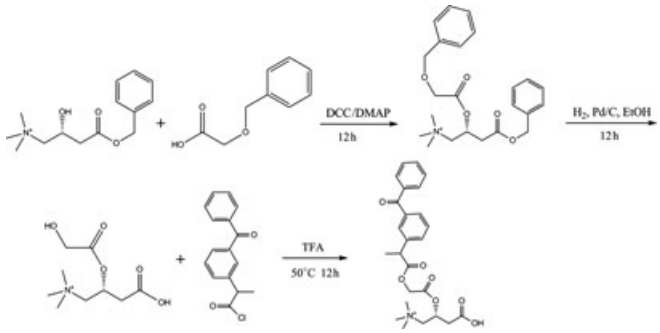
was evaporated under vacuum. Water was added and extracted with ether. Aqueous layer was kept and extracted with butanol saturated with water (3×). Butanol layer was combined and extracted with water saturated with butanol (3×). Finally, butanol fraction was dried with anhydrous sodium sulfate, filtered, and evaporated under vacuum to yield valproyl–l-carnitine or ketoprofen–l-carnitine. ^{1}H NMR [dimethyl sulfoxide (DMSO), 500 MHz] for valproyl–l-carnitine: δ 0.79–0.92 (t, 6H), 1.17–1.31 (m, 4H), 1.33–1.45 (m, 2H), 1.47–1.60 (m, 2H), 2.31–2.39 (m, 1H), 2.49–2.58 (m, 1H), 2.70–2.74 (m, 1H), 3.13 (s, 9H), 3.68–3.74 (d, 1H), 3.83–3.90 (q, 1H), 5.43–5.50 (m, 1H). ^{1}H NMR (DMSO, 500 MHz) for ketoprofen–l-carnitine: δ 1.42–1.51 (d, 3H), 3.27–3.69 (s, 9H), 3.68–3.88 (m, 1H), 3.93–4.06 (m, 2H), 5.30–5.40 (m, 1H), 7.52–7.76 (m, 9H).

Synthesis of Naproxen–l-Carnitine

Scheme 2 summarizes the synthetic approach for naproxen–l-carnitine. l-Carnitine hydrochloride (1 g) was dissolved in benzyl alcohol (10 mL). The carboxylate group of l-carnitine hydrochloride was protected by adding DCC (1 equivalent) and DMAP (0.2 equivalent). After stirring for 12 h, the reaction mixture was filtered and the solvent was evaporated. Acetonitrile (10 mL) was added and filtered to give benzyl-l-carnitine. Benzyl-l-carnitine was then dissolved in



Scheme 2. Synthesis of naproxen–l-carnitine.

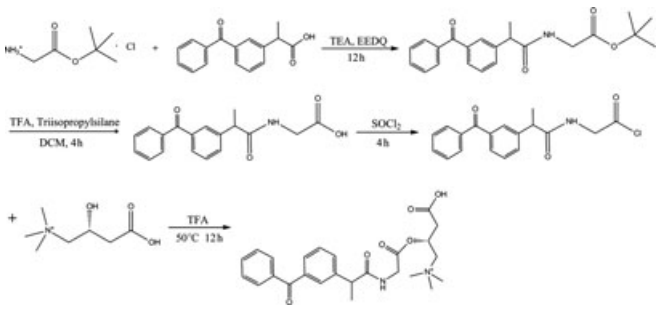


Scheme 3. Synthesis of ketoprofen–glycolic acid–l-carnitine.

DMF. Naproxen (1 equivalent), DCC (1 equivalent), and DMAP (0.2 equivalent) were then added to conjugate naproxen to carboxylate-protected l-carnitine over 12 h. The reaction mixture was filtered and evaporated. Naproxen–l-carnitine was obtained by catalytic hydrogenation to remove the benzyl ester (10% Pd/charcoal in EtOH at 40–50 psi for 12 h), followed by purification by preparative thin layer chromatography using solvent mixture ethanol and butanone (1:1). ^{1}H NMR (DMSO, 500 MHz): δ 1.42–1.59 (d, 3H), 2.73–2.88 (s, 2H), 3.04–3.19 (m, 11H), 3.84–4.01 (m, 4H), 5.31–5.51 (m, 1H), 7.11–7.26 (d, 1H), 7.30–7.38 (s, 1H), 7.38–7.51 (t, 1H), 7.68–7.94 (m, 3H).

Synthesis of Ketoprofen–Glycolic Acid–l-Carnitine

Scheme 3 summarizes the synthetic approach for ketoprofen–glycolic acid–l-carnitine. Benzyl-l-carnitine (1 g) and benzyloxyacetic acid (1 equivalent) were coupled by DCC (1 equivalent) and DMAP (0.2 equivalent) in DMF for 12 h. Reaction mixture was then filtered and dissolved in water after solvent removal under vacuum. The aqueous solution was then extracted with butanol saturated with water (3×). Butanol fraction was combined and dried by anhydrous sodium sulfate. After catalytic hydrogenation to remove the benzyl esters (10% Pd/charcoal in EtOH at 40–50 psi for 12 h), EtOH was vacuum evaporated and glycolic acid–l-carnitine conjugate was dissolved in TFA (10 mL). Oxalyl chloride-activated ketoprofen (1 equivalent) was added and the reaction mixture was heated to 50°C for 12 h, after which the solvent was evaporated under vacuum. Water was added and extracted with ether. Aqueous layer was kept and extracted with butanol saturated with water (3×). Butanol layer was combined and extracted with water saturated with butanol (3×). Finally, butanol fraction was dried with anhydrous sodium sulfate, filtered, and evaporated under vacuum to yield ketoprofen–glycolic acid–l-carnitine. ^{1}H NMR (DMSO, 500 MHz): δ 1.36–1.57 (d, 3H), 2.91–3.18 (m, 9H), 3.63–3.74 (m, 2H), 4.04–4.15 (m, 1H), 5.42–5.58 (m, 1H), 7.38–8.14 (m, 9H).



Scheme 4. Synthesis of ketoprofen–glycine–l-carnitine.

Synthesis of Ketoprofen–Glycine–l-Carnitine

Scheme 4 summarizes the synthetic approach for ketoprofen–glycine–l-carnitine. Glycine tert butyl ester hydrochloride (1 g) was dissolved in EtOAc. Triethylamine (1 equivalent) was added and stirred for 30 min. Ketoprofen (1 equivalent) and EEDQ (1 equivalent) were then added to conjugate ketoprofen to glycine tert butyl ester. After 12 h, the solvent was evaporated under vacuum and ketoprofen–glycine conjugate was obtained by deprotection of tert-butyl group in dichloromethane (DCM) (10 mL) with TFA (10 mL) and triisopropylsilane (3 mL) for 4 h. The crude product was further purified by silica gel column chromatography using a mobile phase of DCM and MeOH (90:10). Ketoprofen–glycine was then activated by oxalyl chloride (0.5 mL) for 4 h. Three drops of DMF was added as catalyst. l-Carnitine (1.1 equivalent) dissolved in TFA (10 mL) was then added to activated ketoprofen–glycine after solvent evaporation under vacuum. The reaction mixture was heated to 50°C for 12 h, after which the solvent was evaporated under vacuum. Water was added and extracted with ether. Aqueous layer was kept and extracted with butanol saturated with water (3×). Butanol layer was combined and extracted with water saturated with butanol (3×). Finally, butanol fraction was dried with anhydrous sodium sulfate, filtered, and evaporated under vacuum to yield ketoprofen–glycine–l-carnitine. ^{1}H NMR (DMSO, 500 MHz): δ 1.30–1.42 (d, 3H), 2.61–2.69 (d, 2H), 3.00–3.16 (s, 9H), 3.70–3.92 (m, 4H), 5.43–5.55 (m, 1H), 7.49–7.78 (m, 9H), 8.66–8.75 (m, 1H).

Cell Culture

Stably transfected hOCTN2–Madin–Darby canine kidney (MDCK) and Calu-3 cells were cultured at 37°C, 90% relative humidity, and 5% CO_2 atmosphere and fed every 2 days, as previously described.[19] Media for hOCTN2–MDCK cells was composed of DMEM supplemented with 10% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin. Cells were passaged after reaching 80% confluence. Calu-3 cells were maintained in DMEM with 10% fetal bovine serum, 0.1 mM nonessential amino acid solution, 50 units/mL penicillin, and 50 μg/mL streptomycin.

Inhibition Study

Inhibition studies of l-carnitine analogues and conjugates were conducted as previously described.[19] Briefly, after reaching 90% confluence, cells were seeded in 12-well cluster plates at a density of 1.5 million cells per well and cultured for 4 days. The culture medium was changed every 48 h. Inhibition studies were performed on the fourth day and were conducted in presence of Hank's balance salts solution (HBSS), which contains 137 mM sodium chloride. Cells were exposed to donor solution containing 2.5 μM l-carnitine (spiked with l-[^{3}H]-carnitine) in the presence or absence of potential inhibitor (i.e., l-carnitine analogue or conjugate) at 37°C and 50 rpm orbital shaking for 10 min. The donor solution was removed and the cells were washed thrice with ice-cold sodium-free buffer (SFB) wherein sodium chloride was replaced with 137 mM tetraethylammonium chloride. Subsequently, cells were lysed using 0.25 mL of 1 M NaOH for 2 h at RT and neutralized with 0.25 mL of 1 M HCl. Cell lysate was then counted for the associated radioactivity using a liquid scintillation counter. J_{max} of l-carnitine was measured on each inhibition study occasion. Unless otherwise noted, data are summarized as mean (±SEM) of three measurements.

To measure K_i of conjugate, inhibition studies were performed as described above, where a range of drug concentrations were applied to inhibit l-carnitine uptake. The following inhibition model was applied:

$$J = \frac{J_{max} \times S}{K_t\left(1 + I/K_i\right) + S} + P_p \times S \qquad (1)$$

where K_i is the inhibition coefficient, I is the concentration of inhibitor, and S is the concentration of l-carnitine (2.5 μM). In applying Eq. 1, only K_i was estimated using nonlinear regression fitting performed by WinNonlin 4.1 (Pharsight, Mountain View, California). The other three parameters (i.e., J_{max}, K_t, and P_p) were estimated from l-carnitine uptake studies without inhibitor.

Uptake Study

Human organic cation/carnitine transporter–Madin–Darby canine kidney and Calu-3 cells were seeded in 12-well plates (Corning; Corning, New York). Seeding density was 1.5 million cells/well. On day 4 after seeding, uptake studies were performed for l-carnitine (spiked with l-[^{3}H]-carnitine) or conjugate over a range of concentrations in HBSS and SFB. Nontransporter-mediated passive uptake was

assessed by measuring l-carnitine or conjugate uptake in SFB.

Uptake was determined to be linear over 10 min. At the end of the assay (10 min), cells were washed thrice with chilled SFB. Acetonitrile was then applied and, after its evaporation, cells were incubated with a mixture of acetonitrile and water (1:1, containing internal standard compound) for 30 min on a plate shaker at 50 oscillations per minute.[20] Conjugate was quantified by liquid chromatography–tandem mass spectrometry (LC–MS/MS). l-Carnitine was quantified by scintillation counting.

Uptake data in SFB were fitted to passive transport model (Eq. 2):

$$J = P\mathrm{p} \times S \tag{2}$$

where J is uptake, P_p is the passive permeability, and S is conjugate concentration. Uptake data in HBSS were fitted to the Michaelis–Menten model (Eq. 3):

$$J = \frac{J_{\max} \times S}{K_\mathrm{t} + s} + P_\mathrm{p} \times S \tag{3}$$

where $J_{\max}$ and K_t are the Michaelis–Menten coefficients. Equations 2 and 3 were applied sequentially to mock and transporter-expressing cell data to estimate P_p, K_t, and $J_{\max}$. The P_p estimate from mock cells was applied to Eq. 3. Nonlinear curve fitting was performed using WinNonlin 4.1 (Pharsight).

In Vitro Chemical and Metabolic Stability Studies

The following matrices were used to assess the *in vitro* chemical and metabolic stability of naproxen–l-carnitine, ketoprofen–l-carnitine, ketoprofen–glycolic acid–l-carnitine, or ketoprofen–glycine–l-carnitine: mouse plasma (lyophilized; reconstituted with 1 mL water and diluted to 90% with 50 mM Tris–HCl buffer, pH 7.5), mouse liver S9 fraction (20 mg/mL) in the presence of 1 mM nicotinamide adenine dinucleotide phosphate, rat liver microsome (2 mg/mL in phosphate buffered saline), 90% rat plasma, rat kidney homogenate (quickly thawed and centrifuged at 4°C for 10 min at 10,000 × *g* and supernatant was used for stability study), esterase solution from porcine liver (50 units/mL in 10 mM borate buffer, pH 8.0), HBSS (pH 6.8), PBS (pH 7.5), 50 mM Tris–HCl buffer (pH 7.5), and 0.1 M HCl. Conjugate was incubated in metabolic and chemical buffers at 37°C. At a designated time point, 100 μL aliquot was taken and quenched immediately with 400 μL acetonitrile, followed by centrifugation at 14,000 × *g* for 10 min at 4°C. Supernatants were frozen and maintained at −80°C prior to LC–MS/MS analysis.

Analytical Methods

l-[^{3}H]-carnitine was quantified by scintillation counting. Conjugate samples were quantified by LC–MS/MS. The LC–MS/MS instrumental system consisted of Finnigan Surveyor® Plus Autosampler, Finnigan Surveyor® LC Pump Plus, and Finnigan TSQ® Quantum Discovery MAX™ mass spectrometer with an electrospray ionization source and triple-quadrupole mass analyzer (Thermo Fisher Scientific Inc.; Waltham, MA). The column was a SynergiTM Polar-RP column (4 μm, 50 × 2.00 mm; Phenomenex, Torrance, California). A gradient of acetonitrile with 0.1% formic acid and water with 0.1% formic acid was used as the mobile phase. The gradient was an initial 50% organic phase for 1 min, a linear increase to 80% organic phase over 3 min, a plateau of 80% organic phase for 1 min, a linear decrease to 50% organic phase over 1 min, and a plateau of 50% organic phase for 1 min, with a flow rate of 0.4 mL/min. Compounds were monitored by selective reaction monitoring in positive mode: valproic acid–l-carnitine (288.2 → 144.1), naproxen–l-carnitine (374.0 → 194.1), ketoprofen–l-carnitine (398.2 → 209.0), ketoprofen–glycolic acid–l-carnitine (456.2 → 209.0), ketoprofen–glycine–l-carnitine (455.2 → 209.0), and ketoprofen (255.3 → 209.0). The assay was linear ($r^2 > 0.999$) over 20–1000 nM for each compound.

Data Analysis

Data were expressed as mean ± SEM derived from three independent wells. Statistical significance was evaluated using Graphpad Prism (Graphpad Software, Inc.; La Jolla, California).

RESULTS AND DISCUSSION

Inhibition Screening of l-Carnitine Analogues

Twenty-two l-carnitine analogues were evaluated for their inhibition of l-carnitine uptake into hOCTN2–MDCK cells (Table 1). These studies were conducted in order to explore the structural requirements for compound interacting with OCTN2 and elucidate a possible suitable position on l-carnitine to conjugate drug for prodrug design. In Table 1, results are expressed in terms of percent uptake of l-carnitine compared with l-carnitine uptake without l-carnitine analogue present. Lower percent uptake indicates higher inhibition potency.

Results indicate that the 3′-hydroxyl group of l-carnitine is not necessary for OCTN2 inhibition because (3-carboxylpropyl)trimethylammonium (**1**) was a potent inhibitor. The potent inhibition by 4-dimethylaminobutyric acid (**2**) further indicates that the 3′-hydroxyl group is not necessary. Additionally, d-carnitine (**20**), acetyl-l-carnitine (**21**), and

palmitoyl-l-carnitine (**22**) each lack 3′-hydroxyl group in the R-configuration, yet were potent inhibitors. Second, results indicate that inhibition potency depended on the type of amine. Potency showed quaternary amine > tertiary amine > secondary amine > primary amine, as evident from the results of compounds (3-carboxylpropyl)trimethylammonium (**1**), 4-dimethylaminobutyric acid (**2**), 4-methylaminobutyric acid (**3**), and gamma-aminobutyric acid (**4**). Results from compounds **5–8**, which are methyl esters of compounds **1–4**, showed the similar trend. The lack of inhibition by S-4-amino-3-hydroxybutanoic acid (**9**) also supports the need for an amine that is not a primary amine. Third, the carboxylic acid group of l-carnitine contributes to OCTN2 binding affinity. For example, methyl esterification reduced inhibitory potency. Compounds **5–8** exhibited reduced inhibition potency compared with acid counterparts **1–4**. N-butyltrimethylammonium (**16**) and (3-hydroxypropyl)trimethylammonium (**17**) are analogues of compound **1** but lack the carboxylate and were not potent inhibitors. Tetraethylammonium (**13**), tetramethylammonium (**14**), and tributylmethylammonium (**15**) are simple quaternary amines that lack a carboxylate and exhibited less potency than compound **1**. Interestingly, betaine (**18**) possesses a carboxylic acid group, but was less potent than compound **1**, suggesting that too close a proximity of the amine and carboxylate is detrimental to binding affinity.

These findings of the favorable contributions of the amine and carboxylate, and the lack of the need for the 3′- hydroxyl group, identified the 3′- hydroxyl on l-carnitine as a suitable position for conjugation. Hence, the 3′- hydroxyl was chosen to conjugate parent drug (or linker) with l-carnitine. This approach is consistent with prior results wherein acetyl-l-carnitine and palmitoyl-l-carnitine were found to be transported by OCTN2.[11,12]

Synthesis of l-Carnitine Prodrugs

Three drug–l-carnitine conjugates were synthesized and depicted in Figures 1a–1c, and were valproyl–l-carnitine, naproxen–l-carnitine, and ketoprofen–l-carnitine. Additionally, two ketoprofen conjugates that employ glycolic acid and glycine as linkers were subsequently synthesized in order to promote more rapid release of the drug compared with ketoprofen–l-carnitine, as discussed below. These two additional conjugates were ketoprofen–glycolic acid–l-carnitine and ketoprofen–glycine–l-carnitine (Figs. 1d and 1e).

Inhibition of l-Carnitine Uptake by Drug–l-Carnitine Conjugates

Valproyl–l-carnitine, naproxen–l-carnitine, and ketoprofen–l-carnitine were evaluated for their inhibition of l-carnitine uptake into hOCTN2–MDCK cells in HBSS. Each inhibited l-carnitine uptake into hOCTN2–MDCK monolayers in a concentration-dependent manner. Figure 2 plots their inhibition profiles. Kinetic parameters are listed in Table 2. All three conjugates were hOCTN2 inhibitors, with K_i of $155 \pm 19\,\mu M$ for valproyl–l-carnitine, $5.97 \pm 0.81\,\mu M$ for naproxen–l-carnitine, and $82.2 \pm 5.3\,\mu M$ for ketoprofen–l-carnitine.

Uptake of Drug-l-Carnitine Conjugates by OCTN2

Figure 3 represents the concentration-dependent uptake of the valproyl–l-carnitine, naproxen–l-carnitine, and ketoprofen–l-carnitine into hOCTN2–MDCK monolayers in the presence and absence of sodium. Uptake of each conjugate was greater in the presence of sodium than in the absence of sodium, indicating active uptake by the transporter.

Kinetic uptake parameters are listed in Table 2. For valproyl–l-carnitine, $K_m = 132 \pm 23\,\mu M$ and $J_{max} = 0.413 \pm 0.023$ pmol/(s cm^2). For naproxen–l-carnitine, $K_m = 257 \pm 57\,\mu M$ and $J_{max} = 0.159 \pm 0.013$ pmol/(s cm^2). For ketoprofen–l-carnitine, $K_m = 77.0 \pm 4.0\,\mu M$ and $J_{max} = 0.439 \pm 0.006$ pmol/(s cm^2). In Table 2, the normalized J_{max} value is J_{max} of the conjugate normalized for functional hOCTN2 expression. Specifically, conjugate J_{max} is divided by l-carnitine J_{max} from the same study occasion to give normalized J_{max}. The normalized J_{max} of valproyl–l-carnitine, naproxen–l-carnitine, and ketoprofen–L-carnitine were 0.467 ± 0.028, 0.141 ± 0.012, 0.412 ± 0.015, respectively. Overall, the ketoprofen conjugate was perhaps the most promising as a substrate, in that it exhibited the most favorable K_m and showed a J_{max} that was about half that of l-carnitine.

To further confirm that the active transport was mediated by OCTN2, the l-carnitine inhibition of the uptake of naproxen and ketoprofen conjugates was investigated. Results are plotted in Figure 4. In Figure 4a, l-carnitine (50 and $100\,\mu M$) significantly inhibited the uptake of naproxen–l-carnitine ($50\,\mu M$) into hOCTN2–MDCK cells in HBSS ($p < 0.05$). In the presence of l-carnitine, naproxen–l-carnitine uptake in HBSS was comparable to its uptake in SFB (99% inhibition), indicating that OCTN2-mediated active uptake was abolished. In Figure 4b, l-carnitine (5–$20\,\mu M$) significantly inhibited the uptake of ketoprofen–l-carnitine (50–$1000\,\mu M$) in HBSS ($p < 0.05$). Ketoprofen–l-carnitine uptake was reduced by 95% in the presence of $1000\,\mu M$ l-carnitine. Therefore, the active transport of conjugates by OCTN2 was confirmed.

Furthermore, ketoprofen–l-carnitine uptake in a second cell line, Calu-3 cell line, was performed. Calu-3 cell line is a human bronchial epithelial cell line and widely used as a transport model to study drug delivery to the respiratory epithelium.[21,22] Calu-3 cells also express OCTN2, but not in an

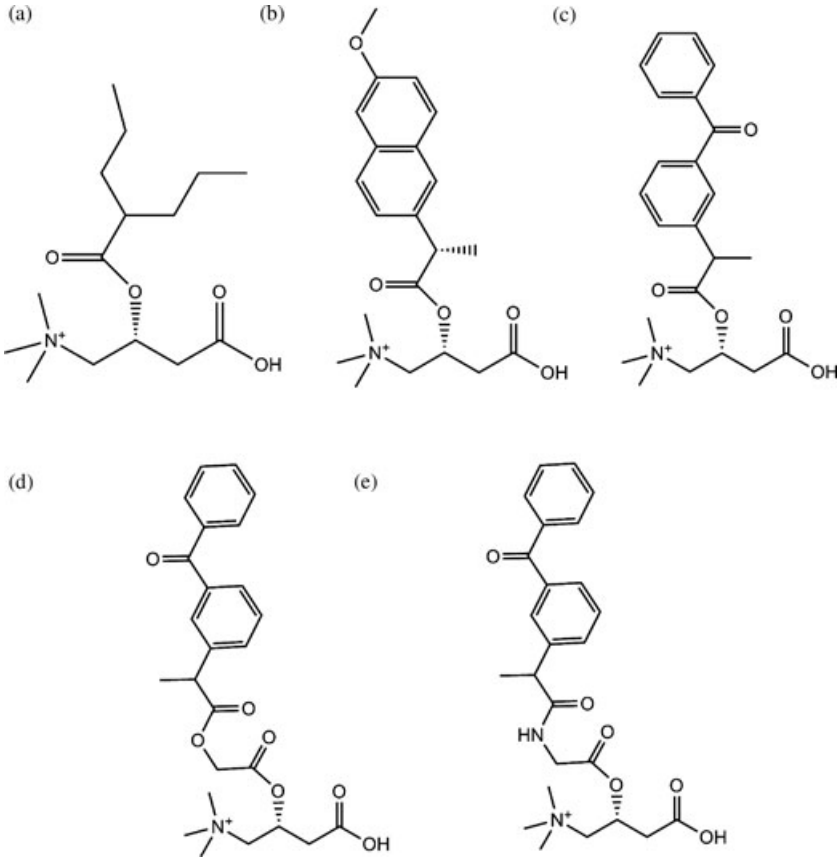


Figure 1. Structures of the prodrugs. (a) Valproyl–l-carnitine, (b) naproxen–l-carnitine, (c) ketoprofen–l-carnitine, (d) ketoprofen–glycolic acid–l-carnitine, and (e) ketoprofen–glycine–l-carnitine.

overexpression fashion as hOCTN2–MDCK cells.[23] As demonstrated in Supplemental Figure 1, both uptake of l-carnitine and ketoprofen–l-carnitine into Calu-3 cells were higher in the presence of sodium than in the absence of sodium, supporting the active uptake in Calu-3 cells.

In Vitro Stabilities of Naproxen–l-Carnitine and Ketoprofen–l-Carnitine

Naproxen–l-carnitine and ketoprofen–l-carnitine were subjected to *in vitro* metabolic and chemical stabilities. Results are plotted in Figure 5. Over 5 h, naproxen–l-carnitine (1 μM) was stable in porcine liver esterase solution (50 units/mL), but degraded gradually to about 60% in rat plasma (Fig. 5a). Meanwhile, ketoprofen–l-carnitine (1 μM) was stable in rat plasma, as well rat kidney homogenate and rat liver microsomes (2 mg/mL; Fig. 5b). Ketoprofen–l-carnitine stability is supported by the observation that no ketoprofen was released in these three metabolic buffers. On the contrary, ketoprofen–l-carnitine was susceptible to alkaline hydrolysis. In 1 h, ketoprofen–l-carnitine fully degraded to ketoprofen in 0.2 M KOH in methanol incubated at 50°C.

Table 2. Human Organic Cation Carnitine Transporter Inhibition and Uptake Kinetic Parameters of Prodrugs

Prodrug	Inhibitor Constant K_i (μM)	Michaelis Constant K_m (μM)	Michaelis Constant J_{max} [pmol/(s cm^2)]	Normalized J_{max}[a]	$P_p \times 10^6$ (cm/s)
Valproyl–l-carnitine	155 ± 19	132 ± 23	0.413 ± 0.023	0.467 ± 0.028	0.0257 ± 0.033
Naproxen–l-carnitine	5.97 ± 0.81	257 ± 57	0.159 ± 0.013	0.141 ± 0.012	0.141 ± 0.003
Ketoprofen–l-carnitine	82.2 ± 5.3	77.0 ± 4.0	0.439 ± 0.006	0.412 ± 0.015	0.0751 ± 0.0082
Ketoprofen–glycine–l-carnitine	14.4 ± 1.4	58.5 ± 8.7	0.0485 ± 0.0020	0.0789 ± 0.0037	0.0723 ± 0.0025

Data were summarized as mean (±SEM) of three measurements.
[a] To accommodate variation in organic cation/carnitine transporter expression levels across study occasions, J_{max} of each prodrug was normalized against l-carnitine J_{max} from the same study occasion, yielding normalized J_{max}.

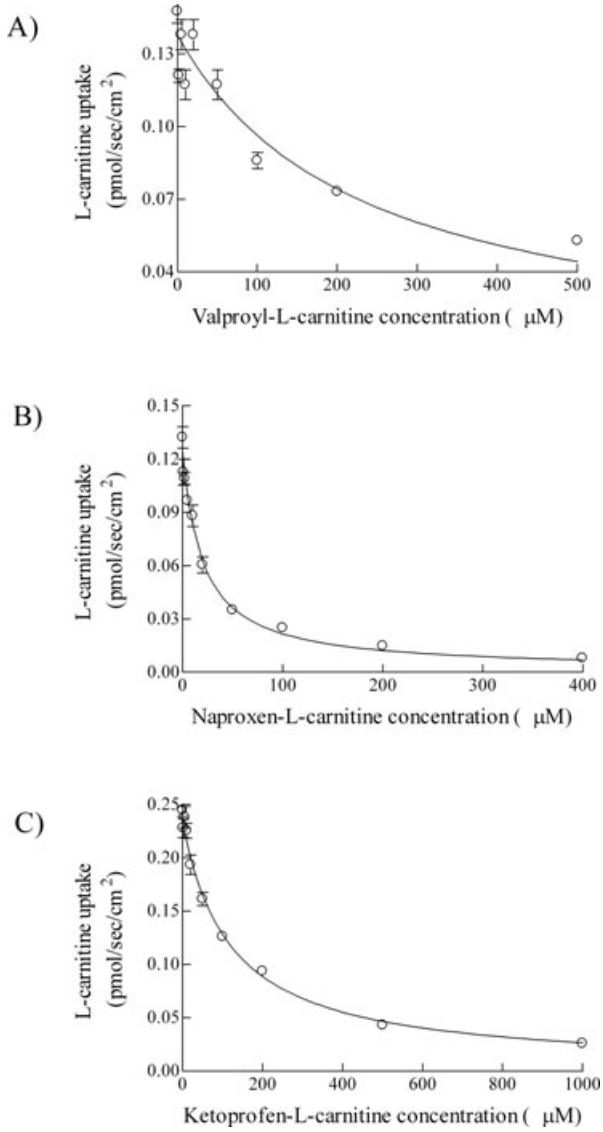


Figure 2. Inhibition of l-carnitine uptake into human organic cation/carnitine transporter–Madin–Darby canine kidney cells by (a) valproyl–l-carnitine, (b) naproxen–l-carnitine, and (c) ketoprofen–l-carnitine. Error bars denote SEM.

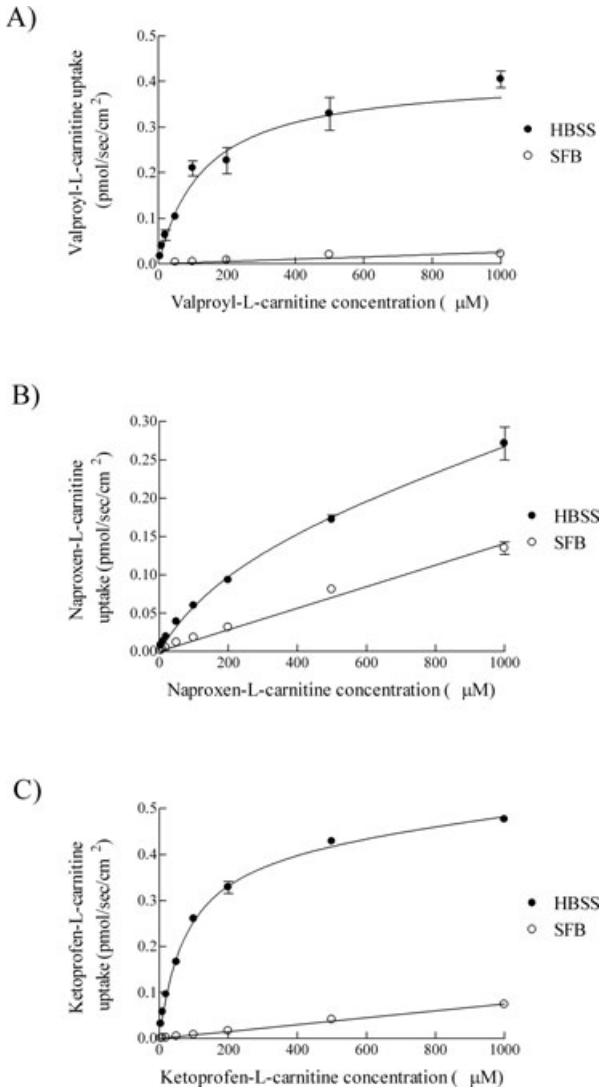


Figure 3. Uptake of valproyl–l-carnitine, naproxen–l-carnitine, and ketoprofen–l-carnitine into human organic cation/carnitine transporter–Madin–Darby canine kidney cells in the presence and absence of sodium. (a) Valproyl–l-carnitine uptake was greater in Hanks balanced salt solution (HBSS) than in sodium-free buffer (SFB), (b) naproxen–l-carnitine uptake was greater in HBSS than in SFB, (c) ketoprofen–l-carnitine uptake was greater in HBSS than in SFB. Error bars denote SEM.

Design of Ketoprofen–Glycolic Acid–l-Carnitine and Its *in Vitro* Stability

Because ketoprofen–l-carnitine exhibited favorable OCTN2 transport, but insufficient enzymatic activation by rat plasma, rat kidney homogenate, or rat liver microsomes, analogues of ketoprofen–l-carnitine that incorporate a linker group between ketoprofen and l-carnitine were subsequently synthesized and evaluated. A linker that afforded a less sterically hindered ester was hypothesized to be more susceptible to enzymatic hydrolysis.[24] Glycolic acid was selected as a linker because it possesses a carboxylate that can be conjugated via the 3′-hydroxyl group of l-carnitine, as well an alcohol that can be conjugated via ketoprofen's acid.

As shown in Figure 6, the *in vitro* chemical and metabolic stability of ketoprofen–glycolic acid–l-carnitine was evaluated using mouse liver S9 fraction, mouse plasma, rat plasma, rat kidney homogenate, and esterase solution from porcine liver, as well as various chemical buffers including HBSS (pH 6.8), PBS (pH 7.5), SFB (pH 6.8), Tris–HCl (50 mM, pH 7.5), water, and 0.1 M HCl. Broadly, ketoprofen–glycolic acid–l-carnitine was not stable. In mouse liver S9 fraction (Fig. 6a), the conjugate completely degraded

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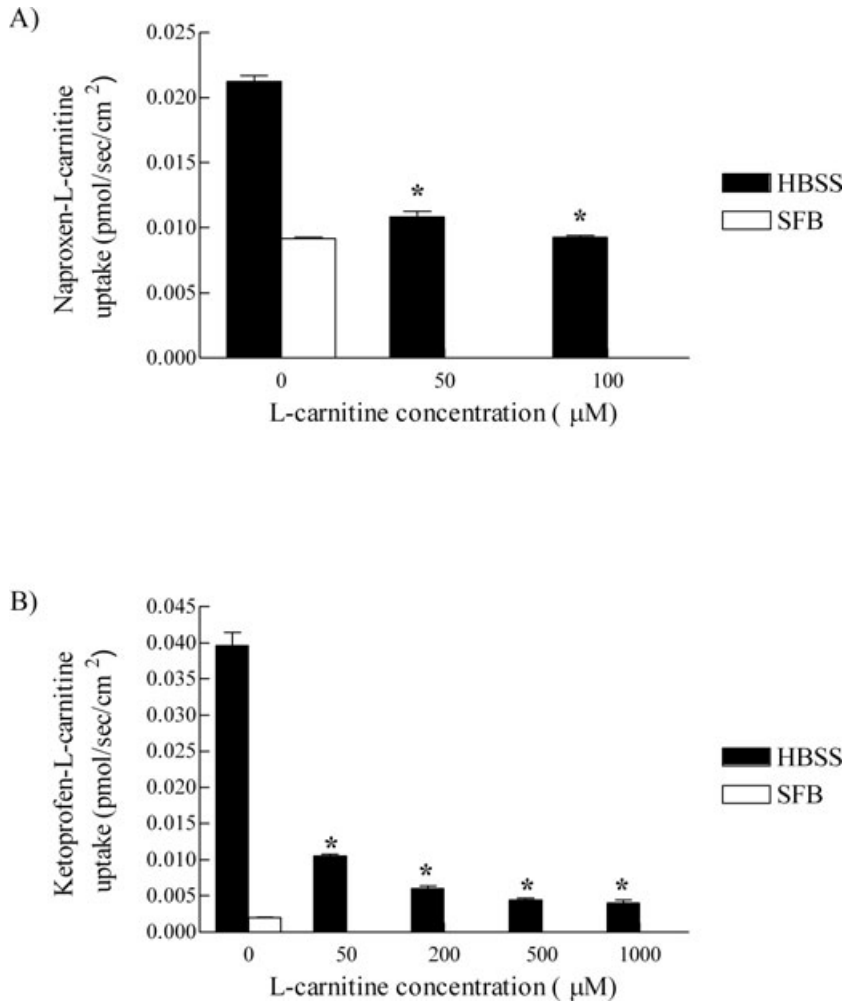

Figure 4. Inhibition of naproxen–l-carnitine or ketoprofen–l-carnitine uptake into human organic cation/carnitine transporter–Madin–Darby canine kidney (hOCTN2–MDCK) cells by l-carnitine. (a) Inhibition of naproxen–l-carnitine uptake (50 μM) into hOCTN2–MDCK cells by 50 and 100 μM l-carnitine, (b) inhibition of ketoprofen–l-carnitine uptake (50 μM) into hOCTN2–MDCK cells by 0–1000 μM l-carnitine. ∗, $p < 0.05$ compared with control in the absence of l-carnitine. Error bars denote SEM.

in 30 min. In mouse plasma, rat plasma, rat kidney homogenate, and porcine liver esterase solution (Figs. 6b–6e), ketoprofen–glycolic acid–l-carnitine showed degradation over 5 h. Qualitatively, ketoprofen release was observed in all metabolic buffers except rat plasma, where no generated ketoprofen was observed. Quantitatively, only small amounts of ketoprofen were apparently generated. For example, in mouse liver S9 fraction (Fig. 6a), at 30 min, when loss of conjugate was complete, only about 10% of conjugate was converted to, or at least present as, ketoprofen. By 120 min, and through to 300 min, about 20% of conjugate was converted to, or at least present as, ketoprofen.

This rapid hydrolysis was also evident even in chemical buffers (Fig. 6f). In 3 h, ketoprofen–glycolic acid–l-carnitine degraded to 47% in HBSS, 58% in PBS, 69% in SFB, 65% in Tris–HCl buffer, and 72% in water. It was stable in 0.1 M HCl, as perhaps could be expected, as hydroxide concentration in 0.1 M HCl is low. Because of its poor stability in HBSS and SFB, inhibition and uptake studies of ketoprofen–glycolic acid–l-carnitine were not conducted.

Design of Ketoprofen–Glycine–l-Carnitine and Its Inhibition, Uptake, and Stability Profiles

Because ketoprofen–l-carnitine was too stable in various metabolic buffers, whereas ketoprofen–glycolic acid–l-carnitine was too unstable in chemical and metabolic buffers, ketoprofen–glycine–l-carnitine was devised. Glycine contains an α-amino, rather than an α-hydroxyl, as is the case for glycolic acid. Hence, ketoprofen–glycine–l-carnitine possesses an amide between ketoprofen and the linker, whereas still affording a less hindered ester compared with ketoprofen–l-carnitine.

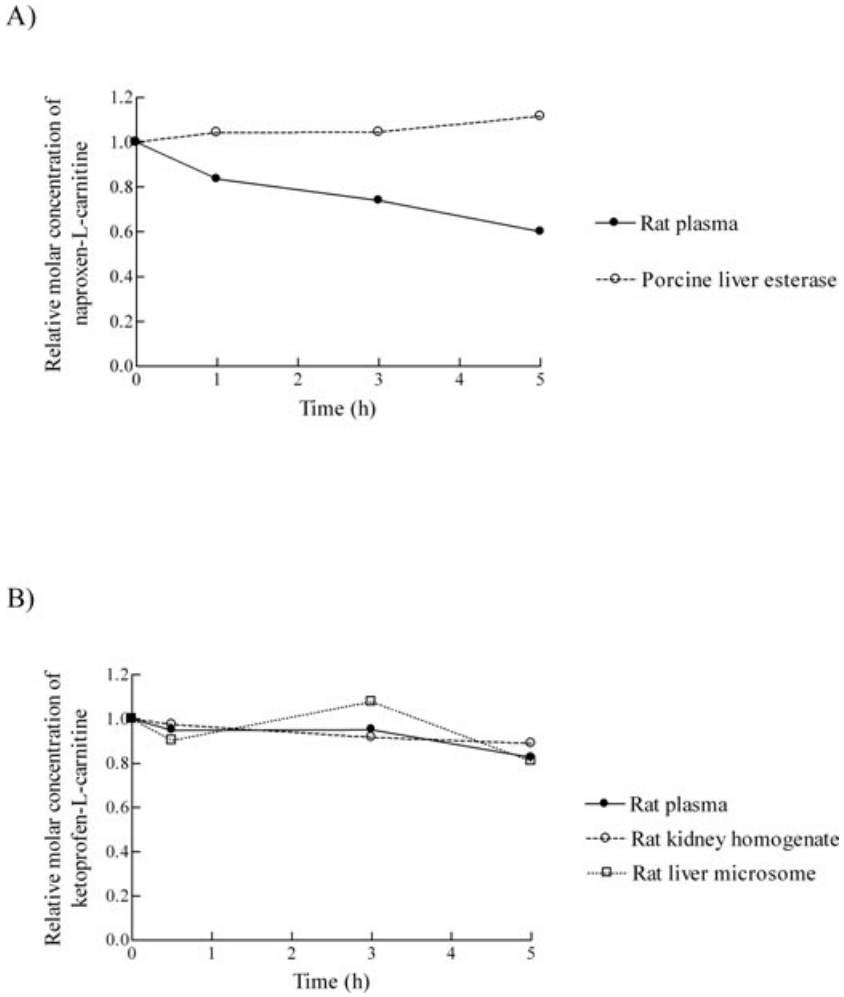

Figure 5. *In vitro* metabolic stabilities of naproxen–l-carnitine and ketoprofen–l-carnitine. (a) Stability of naproxen–l-carnitine (1 μM) in rat plasma and porcine liver esterase solution (50 units/mL), (b) stability of ketoprofen–l-carnitine (1 μM) in rat plasma, rat kidney homogenate, and rat liver microsome (2 mg/mL).

Figure 7 shows inhibition, uptake, and stability results for ketoprofen–glycine–l-carnitine. In Figure 7a, the conjugate inhibited l-carnitine uptake into hOCTN2–MDCK monolayers in a concentration-dependent manner. $K_i = 14.4 \pm 1.4\,\mu M$ (Table 2), which is lower (i.e., more potent) than ketoprofen–l-carnitine's K_i value.

In Figure 7b, ketoprofen–glycine–l-carnitine uptake into hOCTN2–MDCK cells was greater in the presence of sodium than in the absence of sodium, indicating active uptake. Kinetic uptake parameters for uptake of ketoprofen–glycine–l-carnitine are listed in Table 2 with $K_m = 58.5 \pm 8.7\,\mu M$, $J_{max} = 0.0485 \pm 0.0020$ pmol/(s cm^2), and normalized $J_{max} = 0.0789 \pm 0.0037$. Hence, its K_m was comparable to ketoprofen–l-carnitine's K_m, but its J_{max} is several folds lower than that of ketoprofen–l-carnitine. In Figure 7c, l-carnitine (50–1000 μM) significantly inhibited the ketoprofen–glycine–l-carnitine (50 μM) uptake in HBSS ($p < 0.05$), further confirming that ketoprofen–glycine–l-carnitine was actively transported by hOCTN2. Ketoprofen–glycine–l-carnitine uptake was reduced by 83% in the presence of 1000 μM l-carnitine.

Ketoprofen–glycine–l-carnitine was substantially more stable than ketoprofen–glycolic acid–l-carnitine. In Figure 7d, over 90% of conjugate was remaining in mouse plasma and rat kidney homogenate after 5 h. In HBSS and mouse liver S9 fraction, it degraded to 84% and 79%, respectively, after 5 h.

Possible Target Sites for l-Carnitine Prodrugs

Conjugates of naproxen and ketoprofen were evaluated, with naproxen and ketoprofen serving as model drugs (e.g., medicinal agents with a single carboxylate). Naproxen and ketoprofen were also chosen because they are nonsteroidal anti-inflammatory drugs (NSAIDs) that may benefit from selective delivery to

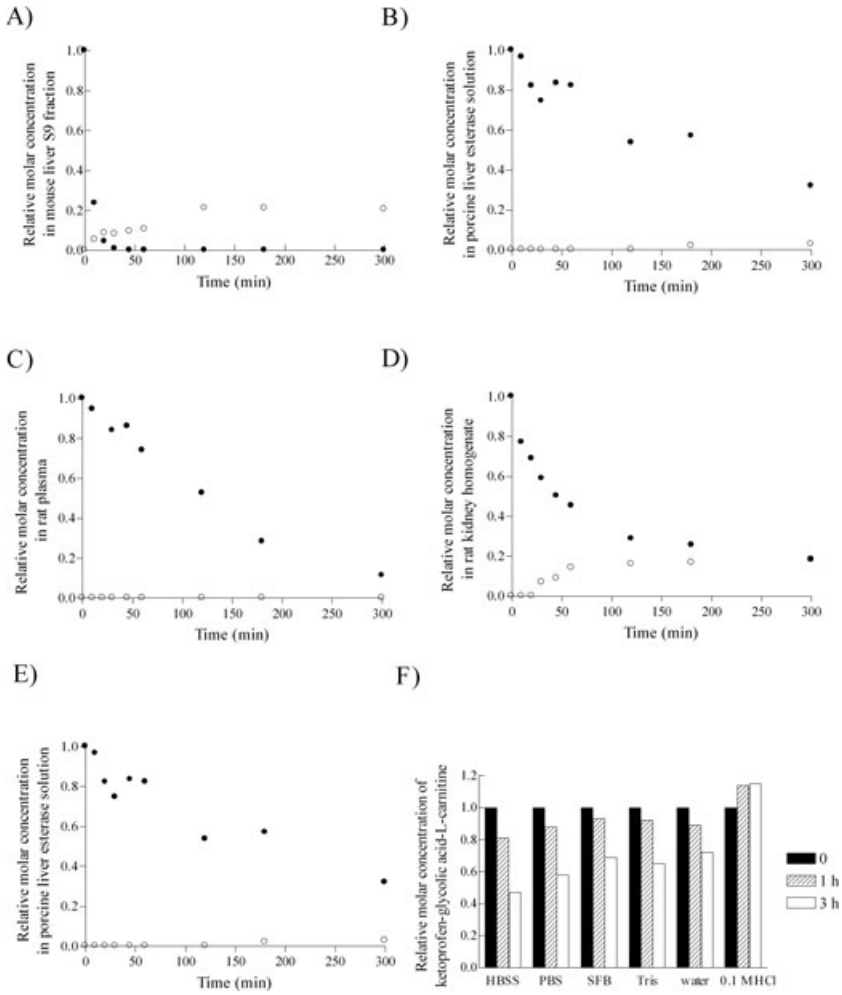

Figure 6. *In vitro* chemical and metabolic stability of ketoprofen–glycolic acid–l-carnitine (5 μM), along with the generation of ketoprofen. Filled circles (•) indicate ketoprofen–glycolic acid–l-carnitine and open circles (○) indicate ketoprofen. (a) Mouse liver S9, (b) mouse plasma, (c) rat plasma, (d) rat kidney homogenate, (e) porcine liver esterase solution, and (f) chemical stability of ketoprofen–glycolic acid–l-carnitine in various buffers.

the kidney. The kidney is a potential target site for an l-carnitine prodrug strategy.

l-Carnitine and acetyl-l-carnitine are not bound to plasma proteins and filtered at the glomerulus in the kidney.[25] l-Carnitine and acetyl-l-carnitine are reabsorbed by OCTN2 that is expressed in the brush border membranes of the proximal tubule cells.[6] Therefore, drug conjugate of l-carnitine may be reabsorbed into proximal tubular cells, achieving targeted delivery to the kidney. After OCTN2-mediated reabsorption, drug could be released via prodrug degradation by various hydrolases because proximal tubular cells are the most metabolically active cells in the kidney.[26] Naproxen and ketoprofen were chosen because they are NSAIDs that are used to treat renal protein excretion via hemodynamic effects.[26] However, these NSAIDs can be toxic in nonrenal tissues, including causing serious gastrointestinal and central side effects.[27]

Additionally, targeting NSAIDs to the proximal tubular cell may aid in the prevention of tubulointerstitial inflammation, as well as some tubular defect diseases such as Fanconi and Bartter's syndrome.[26] The study by Haas et al.[28] demonstrated that naproxen–lysozyme prevented furosemide-induced renal synthesis of prostaglandin E2, whereas an equimolar amount of free naproxen was not effective.[28] This reduction in prostaglandin E2 synthesis indicates that targeting to the proximal tubular cell of the kidney could enhance the renal efficacy of NSAIDs, as well as possibly reduce extrarenal side effects because of the lower dose.

Another potential site for targeted delivery of l-carnitine prodrug is the brain, such that a valproic acid prodrug was evaluated in this study. The administration of l-carnitine or acetyl-l-carnitine for the treatment of Alzheimer's disease[3] suggested that l-carnitine and acetyl-l-carnitine are transported

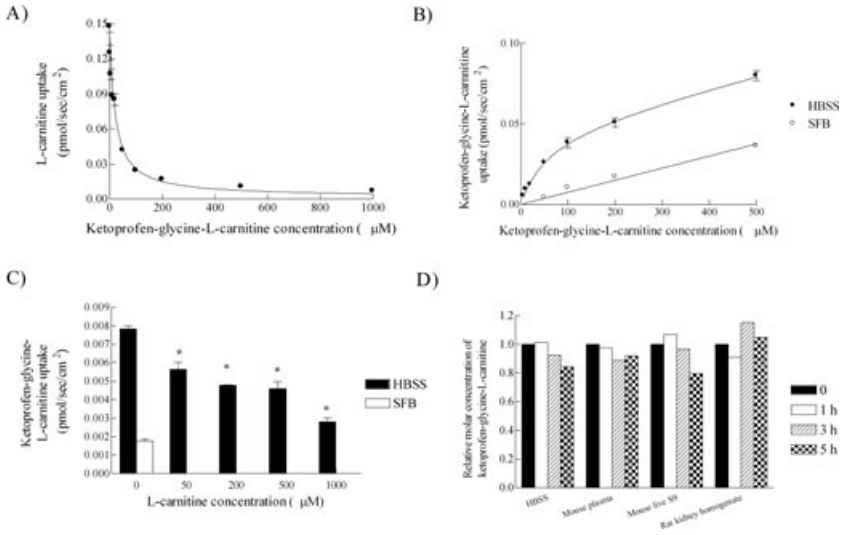

Figure 7. Inhibition, uptake, and stability profiles of ketoprofen–glycine–l-carnitine. (a) Inhibition of l-carnitine uptake into organic cation/carnitine transporter–Madin–Darby canine kidney (MDCK) cells by ketoprofen–glycine–l-carnitine, (b) uptake of ketoprofen–glycine–l-carnitine into human organic cation/carnitine transporter (hOCTN2)–MDCK cells in the presence and absence of sodium, (c) inhibition of ketoprofen–glycine–l-carnitine uptake (50 μM) into hOCTN2–MDCK cells by 0–1000 μM l-carnitine. ∗, $p < 0.05$ compared with control in the absence of l-carnitine. (d) The stability of ketoprofen–glycine–l-carnitine (5 μM) in Hanks balanced salt solution or metabolic buffers.

from the circulating blood into the brain across the BBB. *In vitro* and *ex vivo* studies using brain capillary endothelial cells and microdialysis method in mouse have demonstrated that l-carnitine and acetyl-l-carnitine permeate the BBB.[29,30] One report demonstrated the enhanced delivery and successive release of nipecotic acid in mouse brain after intraperitoneal injection of nipecotic acid–l-carnitine conjugate compared with nipecotic acid itself.[16] Valproic acid was chosen here because it is used for the treatment of convulsions, migraines, and bipolar disorder.[31]

Prodrug Inhibition and Transport Properties

The inhibition K_i value follows the order of naproxen–l-carnitine < ketoprofen–glycine–l-carnitine < ketoprofen–l-carnitine < valproyl–l-carnitine, whereas the Michaelis constant for uptake K_m follows the order of ketoprofen–glycine–l-carnitine < ketoprofen–l-carnitine < valproyl–l-carnitine < naproxen–l-carnitine. For valproyl–l-carnitine and ketoprofen–l-carnitine, K_i and K_m values are essentially identical, indicating that substrate binding was the rate-limiting step in each translocation by hOCTN2; for naproxen–l-carntine and ketoprofen–glycine–l-carnitine, K_m values are apparently larger than corresponding K_i values, implicating postbinding event(s) as the rate-limiting step(s) in the uptake of naproxen–l-carnitine and ketoprofen–glycine–l-carnitine. The normalized J_{max} values follow the order of ketoprofen–glycine–l-carnitine < naproxen–l-carnitine < ketoprofen–l-carnitine < valproyl–l-carnitine. The J_{max} value of ketoprofen–glycine–l-carnitine is about one-fifth of ketoprofen–l-carnitine, indicating that even the small changes in the substrate structure might cause significant changes in the transport capacity. The J_{max} values of ketoprofen–l-carnitine and valproyl–l-carnitine are similar, indicating that OCTN2 can transport l-carnitine conjugates efficiently as large as ketoprofen–l-carnitine, not limited to small l-carnitine conjugates such as valproyl–l-carnitine or butyryl–l-carnitine.

l-Carnitine Prodrug Considerations

An ideal l-carnitine prodrug would be stable in plasma, selectively taken up into target tissue by OCTN2, and then hydrolyzed to release the parent drug in target tissue. All three drug–l-carnitine conjugates and ketoprofen–glycine–l-carnitine are OCTN2 substrates. Ketoprofen–l-carnitine, and to some extent naproxen–l-carnitine, are stable in metabolic buffers, which perhaps is in agreement with Brass,[32] who suggested the relative *in vivo* stability of pivaloyl–l-carnitine. Ketoprofen–glycolic acid–l-carnitine is unstable in metabolic buffers, as well as chemical buffers such as HBSS and SFB; release of ketoprofen from ketoprofen–glycolic acid–l-carnitine was observed in metabolic buffers. Ketoprofen–glycine–l-carnitine showed greater stability than ketoprofen–glycolic acid–l-carnitine in chemical buffer and metabolic buffers. From *in vitro* data, it appears that none of these five conjugates

possess ideal prodrug properties for targeted delivery. However, *in vitro* stability results do not necessarily predict *in vivo* stability. Conjugation of l-carnitine with nipecotic acid showed enhanced exposure of nipecotic acid within mouse brain compared with nipecotic acid.[16] Therefore, nipecotic acid conjugate of l-carnitine definitely degraded and released nipecotic acid *in vivo*. Further *in vivo* studies are being conducted to investigate the targeting potential of prodrugs. It is expected that drug conjugates with l-carnitine might degrade and release drug *in vivo*, preferably in target tissue.

CONCLUSIONS

In this study, drug conjugates with l-carnitine were investigated for the potential as prodrugs that target OCTN2. Twenty-two l-carnitine analogues were evaluated for OCTN2 inhibition and 3′-hydroxyl group was found to be the only functional group not contributing to l-carnitine interaction with hOCTN2 among three functional groups on l-carnitine (i.e., 3′-hydroxyl, amine, and carboxylate). The 3′-hydroxyl group was subsequently chosen as the conjugate site on l-carnitine. Three drug–l-carnitine conjugates (i.e., valproyl–l-carnitine, naproxen–l-carnitine, and ketoprofen–l-carnitine), as well as two analogues of ketoprofen–l-carnitine that incorporate a linker group (glycolic acid or glycine) between ketoprofen and l-carnitine, were synthesized. These five potential prodrugs were evaluated for their *in vitro* inhibition, transport, and metabolism properties. All three drug–l-carnitine conjugates and ketoprofen–glycine–l-carnitine were OCTN2 substrates. The results demonstrate the potential of drug conjugates with l-carnitine to serve as prodrugs that target tissues with OCTN2 expression including the kidney and brain.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health Grant DK67530. The authors kindly acknowledge Dr. Xin Ming and Dr. Dhiren R. Thakker (University of North Carolina-Chapel Hill) for providing the hOCTN2–MDCK cell line used in this study.

REFERENCES

1. Bieber LL. 1988. Carnitine. Annu Rev Biochem 57:261–283.
2. Rebouche CJ, Paulson DJ. 1986. Carnitine metabolism and function in humans. Annu Rev Nutr 6:41–66.
3. Pettegrew JW, Levine J, McClure RJ. 2000. Acetyl-l-carnitine physical–chemical, metabolic, and therapeutic properties: Relevance for its mode of action in Alzheimer's disease and geriatric depression. Mol Psychiatry 5:616–632.
4. Koepsell H, Lips K, Volk C. 2007. Polyspecific organic cation transporters: Structure, function, physiological roles, and biopharmaceutical implications. Pharm Res 24:1227–1251.
5. Iwata D, Kato Y, Wakayama T, Sai Y, Kubo Y, Iseki S, Tsuji A. 2008. Involvement of carnitine/organic cation transporter OCTN2 (SLC22A5) in distribution of its substrate carnitine to the heart. Drug Metab Pharmacokinet 23:207–215.
6. Tamai I, China K, Sai Y, Kobayashi D, Nezu J, Kawahara E, Tsuji A. 2001. Na(+)-coupled transport of l-carnitine via high-affinity carnitine transporter OCTN2 and its subcellular localization in kidney. Biochim Biophys Acta 1512:273–284.
7. Yokogawa K, Higashi Y, Tamai I, Nomura M, Hashimoto N, Nikaido H, Hayakawa J, Miyamoto K, Tsuji A. 1999. Decreased tissue distribution of l-carnitine in juvenile visceral steatosis mice. J Pharmacol Exp Ther 289:224–230.
8. Nezu J, Tamai I, Oku A, Ohashi R, Yabuuchi H, Hashimoto N, Nikaido H, Sai Y, Koizumi A, Shoji Y, Takada G, Matsuishi T, Yoshino M, Kato H, Ohura T, Tsujimoto G, Hayakawa J, Shimane M, Tsuji A. 1999. Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter. Nat Genet 21:91–94.
9. Wang Y, Ye J, Ganapathy V, Longo N. 1999. Mutations in the organic cation/carnitine transporter OCTN2 in primary carnitine deficiency. Proc Natl Acad Sci U S A 96:2356–2360.
10. Tein I. 2003. Carnitine transport: Pathophysiology and metabolism of known molecular defects. J Inherit Metab Dis 26:147–169.
11. Ohashi R, Tamai I, Yabuuchi H, Nezu JI, Oku A, Sai Y, Shimane M, Tsuji A. 2009. Na(+)-dependent carnitine transport by organic cation transporter (OCTN2): Its pharmacological and toxicological relevance. J Pharmacol Exp Ther 291:778–784.
12. Wu X, Huang W, Prasad PD, Seth P, Rajan DP, Leibach FH, Chen J, Conway SJ, Ganapathy V. 1999. Functional characteristics and tissue distribution pattern of organic cation transporter 2 (OCTN2), an organic cation/carnitine transporter. J Pharmacol Exp Ther 290:1482–1492.
13. Ganapathy ME, Huang W, Rajan DP, Carter AL, Sugawara M, Iseki K, Leibach FH, Ganapathy V. 2000. Beta-lactam antibiotics as substrates for OCTN2, an organic cation/carnitine transporter. J Biol Chem 275:1699–1707.
14. Srinivas SR, Prasad PD, Umapathy NS, Ganapathy V, Shekhawat PS. 2007. Transport of butyryl-l-carnitine, a potential prodrug, via the carnitine transporter OCTN2 and the amino acid transporter $ATB^{0,+}$. Am J Physiol Gastrointest Liver Physiol 293:G1046–G1053.
15. Grigat S, Fork C, Bach M, Golz S, Geerts A, Schömig E, Gründemann D. 2009. The carnitine transporter SLC22A5 is not a general drug transporter, but it efficiently translocates mildronate. Drug Metab Dispos 37:330–337.
16. Napolitano C, Scaglianti M, Scalambra E, Manfredini S, Ferraro L, Beggiato S, Vertuani S. 2009. Carnitine conjugate of nipecotic acid: A new example of dual prodrug. Molecules 14:3268–3274.
17. Pourfarzams M, Bartlett K. 1991. Synthesis, characterisation and high-performance liquid chromatography of C6-C16 dicarboxylyl-mono-coenzyme A and -mono-carnitine esters. J Chromatogr 570:253–276.
18. Wuts PGM, Greene TW. 2006. Greene's protective groups in organic synthesis. 4th edn. New Jersey: Wiley-Interscience.
19. Diao L, Ekins S, Polli JE. 2009. Novel inhibitors of human organic cation/carnitine transporter (hOCTN2) via computational modeling and *in vitro* testing. Pharm Res 26:1890–1900.
20. Rais R, Gonzalez PM, Zheng X, Wring SA, Polli JE. 2008. Method to screen substrates of apical sodium-dependent bile acid transporter. AAPS J 10:596–605.
21. Florea BI, Cassara ML, Junginger HE, Borchard G. 2003. Drug transport and metabolism characteristics of the human airway epithelial cell line Calu-3. J Control Release 87:131–138.

22. Forbes B, Ehrhardt C. 2005. Human respiratory epithelial cell culture for drug delivery applications. Eur J Pharm Biopharm 60:193–205.
23. Endter S, Francombe D, Ehrhardt C, Gumbleton M. 2009. RT-PCR analysis of ABC, SLC and SLCO drug transporters in human lung epithelial cell models. J Pharm Pharmacol 61:583–591.
24. Buchwald P. 2001. Structure-metabolism relationships: Steric effects and the enzymatic hydrolysis of carboxylic esters. Mini Rev Med Chem 1:101–111.
25. Marzo A, Arrigoni ME, Mancinelli A, Cardace G, Corbelletta C, Bassani E, Solbiati M. 1991. Protein binding of l-carnitine family components. Eur J Drug Metab Pharmacokinet 3:364–368.
26. Haas M, Moolenaar, F, Meijer DK, de Zeeuw D. 2002. Specific drug delivery to the kidney. Cardiovasc Drugs Ther 16:489–496.
27. Franssen EJ, Moolenaar F, de Zeeuw D, Meijer DK. 1993. Low molecular weight proteins as carriers for renal drug targeting: Naproxen coupled to lysozyme via the spacer l-lactic acid. Pharm Res 10:963–969.
28. Haas M, Moolenaar F, Meijer DK, de Jong PE, de Zeeuw D. 1998. Renal targeting of a non-steroidal anti-inflammatory drug: Effects on renal prostaglandin synthesis in the rat. Clin Sci 95:603–609.
29. Inano A, Sai Y, Nikaido H, Hasimoto N, Asano M, Tsuji A, Tamai I. 2003. Acetyl-l-carnitine permeability across the blood-brain barrier and involvement of carnitine transporter OCTN2. Biopharm Drug Dispos 24:357–365.
30. Kido Y, Tamai I, Ohnari A, Sai Y, Kagami T, Nezu J, Nikaido H, Hashimoto N, Asano M, Tsuji A. 2001. Functional relevance of carnitine transporter OCTN2 to brain distribution of l-carnitine and acetyl-l-carnitine across the blood-brain barrier. J Neurochem 79:959–969.
31. Monti B, Polazzi E, Contestabile A. 2009. Biochemical, molecular and epigenetic mechanisms of valproic acid neuroprotection. Curr Mol Pharmacol 2:95–109.
32. Brass EP. 2002. Pivalate-generating prodrugs and carnitine homeostasis in man. Pharmacol Rev 54:589–598.