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Large Amino Acid Transporter 1 (LAT1) Prodrugs of Valproic Acid: New Prodrug Design Ideas for Central Nervous System Delivery

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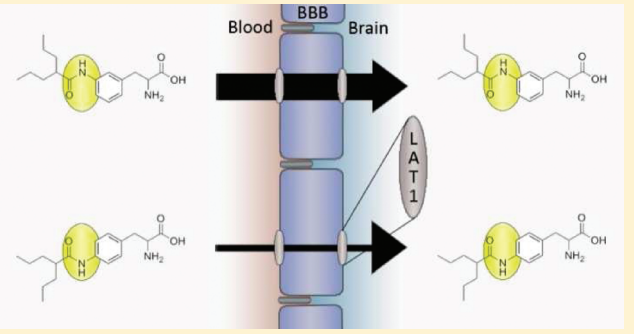
ABSTRACT: Central nervous system (CNS) drug delivery is a major challenge in drug development because the blood−brain barrier (BBB) efficiently restricts the entry of drug molecules into the CNS at sufficient amounts. The brain uptake of poorly penetrating drugs could be improved by utilizing the transporters at the BBB with a prodrug approach. In this study, we designed four phenylalanine derivatives of valproic acid and studied their ability to utilize a large amino acid transporter 1 (LAT1) in CNS delivery with an aim to show that the meta-substituted phenylalanine prodrugs bind to LAT1 with a higher affinity compared with the affinity of the para-substituted derivatives. All of the prodrugs crossed the BBB carrier mediatedly via LAT1 in *in situ* rat brain perfusion. For the first time, we introduced a novel meta-substituted phenylalanine analogue promoiety which improved the LAT1 affinity 10-fold and more importantly the rat brain uptake of the prodrug 2-fold compared with those of the para-substituted derivatives. Therefore, we have characterized a new prodrug design idea for CNS drug delivery utilizing a transporter-mediated prodrug approach.



KEYWORDS: central nervous system, blood-brain barrier, large amino acid transporter 1, brain drug delivery, prodrugs, valproic acid

INTRODUCTION

Currently, one of the most significant challenges in pharmaceutical research is the development of new drugs targeting the central nervous system (CNS). Despite recent advances in brain research, adequate efficient drug therapies against many CNS disorders such as Alzheimer's disease, Parkinson's disease, and brain tumors have not been discovered, which leads to the early hospitalization or institutionalization of patients. A successful treatment of the CNS diseases requires not only the identification of appropriate targets within the CNS but also strategies to improve the passage of therapeutics across the relatively insurmountable blood−brain barrier (BBB) into the CNS at sufficient levels to achieve the desired effect. It is estimated that not more than 2 % of the small molecular weight drugs and practically none of the large molecular weight drugs developed for the CNS disorders cross the BBB at adequate levels.[1]

The BBB expresses a number of specific carrier-mediated transport mechanisms, ensuring an adequate nutrient supply for the brain.[2] These transporters at the BBB involve transporters for nutrients, such as amino acids, glucose, and vitamins, which cannot pass the BBB by passive diffusion due to their unfavorable physicochemical properties. Of the transporter systems presented at the BBB, the carriers especially for glucose (glucose transporter 1, GluT1) and large amino acids (large neutral amino acid transporter 1, LAT1) have been found to have a sufficiently high transport capacity to hold promise for significant drug delivery to the brain.[3,4] In this study, we focused on LAT1, one of the four members of the LAT family.

It is highly promising to utilize LAT1 in CNS prodrug design. The brain uptake of the neutral L-amino acids (e.g., L-phenylalanine, L-tyrosine, and L-leucine) into the brain via LAT1 is efficient because LAT1 is expressed on both the luminal and the abluminal membrane of the capillary endothelial cells at the BBB with expression levels approximately 100-fold greater than in other tissues (e.g., placenta, retina, gut).[5,6] Furthermore, the LAT1 transporters at the BBB have a relatively higher affinity for the amino acids compared with the LAT transporters in the peripheral tissues.[5] Of the amino acid transporters presented at the BBB, LAT1 has been shown to have the prime role of transporting neutral amino acids into the brain.[7]

Consequently, several drug molecules in clinical use such as melfalan,[8] gabapentin,[9] and baclofen[10] mimic the endogenous substrates of LAT1. They all utilize LAT1 at the BBB in the brain uptake, demonstrating that LAT1 can be utilized in drug brain delivery. These drugs have a very close structural similarity with the endogenous LAT1 substrates because they have a negatively charged carboxyl group and a positively charged amino group at pH 7.4 and a lipophilic side chain.

A LAT1-mediated prodrug design has been proven to be suitable for drugs structurally resembling amino acids (e.g., dopamine[11] and

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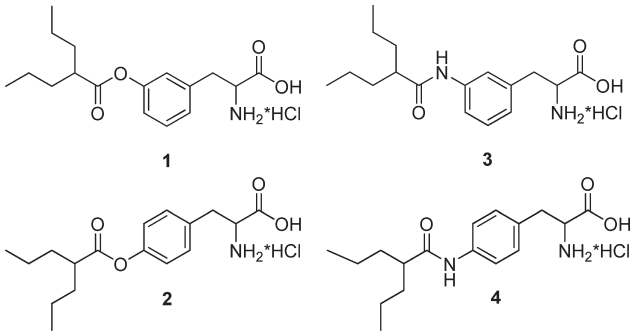


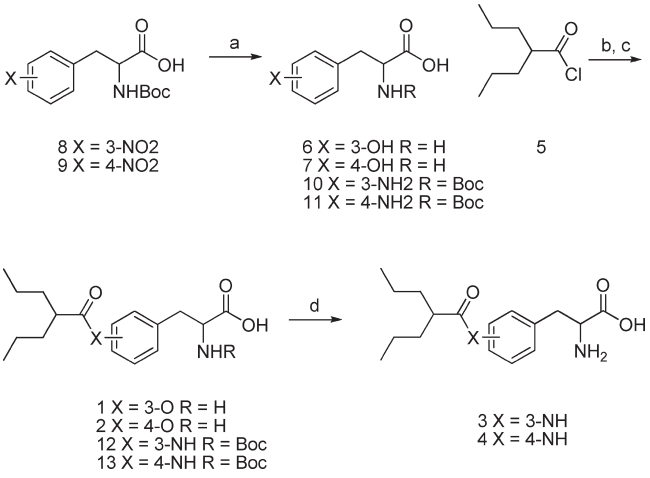
Figure 1. Chemical structures of the valproic acid prodrugs **1**−**4**. All prodrugs are racemic mixtures.

7-chlorokynurenic acid[12]), but importantly, LAT1-mediated prodrug design has also been shown to be suitable for drugs having other chemical structures than amino acid structures. L-Cysteine derivatives of the anticancer agent 6-mercaptopurine and a model compound 2-methyl-1-propanethiol have been shown to be able to bind to LAT1 when using an *in situ* rat brain perfusion technique.[13] In addition, Walker et al.,[14] Bonina et al.,[15] and Balakrishnan et al.[16] have shown that L-tyrosine as a promoiety of prodrugs demonstrates an affinity for LAT1 in cell lines. However, the ability of these conjugates to cross the cell membrane or the BBB has not been studied. Recently, in our laboratory, both L-tyrosine and L-lysine prodrugs of ketoprofen have been proven to be uptaken both rapidly and efficiently into the rat brain via LAT1 in *in situ* rat brain perfusion.[4,17]

So far, the promoieties of the published prodrugs utilizing LAT1 have been natural amino acids. In the present study, we have further developed the studies to achieve carrier-mediated drug transport into the rat brain via LAT1 by conjugating a model compound to L-phenylalanine and to L-phenylalanine analogues, which are not natural amino acids. However, limited information is available on the specific dimensions of the LAT1 at the BBB. It is known that the LAT1 binding of a phenylalanine analogue substituted at the meta-position of the phenol hydroxyl group is much more potent transporter substrate than that of the analogue substituted at the para-position.[18] To show the actual potential of amino acid analogues as carriers for the drug targeting to the CNS via LAT1, both amide and ester prodrugs of valproic acid (**1**−**4**) (Figure 1) were synthesized and evaluated. The mechanism of the binding of the prodrugs into LAT1 and the amount of **1**−**4** transported into the brain were investigated by using the sophisticated *in situ* rat brain perfusion technique.[19,20] Both ester and amide analogues were studied to optimize the systemic pharmacokinetics of the prodrugs.

In this study, valproic acid has been used as a model compound for CNS delivery. By improving the CNS delivery of valproic acid, the side effects elsewhere in the body, which are rare but severe (i.e., teratogenicity and hepatotoxicity), can be decreased. Valproic acid is also a suitable model compound because of its appropriate properties for prodrug derivatization, analytics, and pharmacological studies. According to our *in situ* rat brain perfusion studies, valproic acid is not a substrate for LAT1 (unpublished data). Instead of being a LAT1 substrate, valproic acid is a substrate for fatty acid transporters at the BBB, most likely for MCT1 (monocarboxylic acid transporter 1), and the brain uptake of valproic acid is supposed to be both diffusion- and MCT-mediated.[21−23] However, the efflux clearance of valproic acid from brain to plasma is much higher than the influx clearance because valproic acid is a substrate for efflux transporters at the BBB.

Scheme 1. Synthetic Pathways to the Prodrugs of Valproic Acid[a]



[a] Reaction conditions: (a) 10% Pd−C H_2, MeOH; (b) (for 1,2) TFA; (c) (for 13,14) 1 M NaOH, DCM; (d) HCl, ACN.

The purpose of the present study was to show that meta-substituted phenylalanine prodrugs are more potent LAT1 substrates than those substituted at the para-position of phenylalanine using valproic acid as a model drug. This prodrug approach has not been employed previously. In addition, prior to this study, amino acid analogues have not been utilized as prodrug promoieties in drug brain delivery.

EXPERIMENTAL SECTION

Materials. All materials used were purchased from commercial sources unless otherwise noted. Their origin is specified in the text. All chemicals were of the highest purity available. All radiolabeled compounds used in the studies were uniformly labeled. Compounds **1**−**4** were synthesized according to published procedures.

Animals. Adult male Wistar rats (200−230 g) were obtained from the National Laboratory Animal Centre (Kuopio, Finland; originally from Harlan, The Netherlands). Animal care and handling were performed in accordance with the Finnish law (26/2006) and regulation (36/EEO/2006) for laboratory animal experiments and with the European Communities Council Directive of Nov. 24, 1986 (86/609/EEC). In particular, the number of animals and their suffering has been minimized and approved by the Animal Ethics Committee. The rats were fed *ad libitum* and housed in an artificial-light and temperature-controlled environment (12 h light/dark cycle, 22 ± 1 °C). All of the experiments were carried out during the light phase.

General Synthetic Procedures. Prodrugs **1**−**4** were synthesized from DL-*m*-tyrosine (**6**) (Fluka, St. Louis, MO, USA), L-tyrosine (**7**) (Sigma, St. Louis, MO, USA), Boc-3-nitro-L-phenylalanine (**8**) (Aldrich, St. Louis, MO, USA), and Boc-4-nitro-L-phenylalanine (**9**) (Aldrich, St. Louis, MO, USA), respectively, as shown in Scheme 1. Valproic acid chloride (**5**) was synthesized from 2-propylpentanoic acid (Acros Organics, Waltham, MA, USA). Reactions were monitored by thin-layer chromatography using aluminum sheets coated with silica gel 60 F245 (0.24 mm) (Merck, Darmstadt,

Germany) with suitable visualization. Purifications by flash chromatography were performed using Combiflash Companion with RediSep Columns (Teledyne ISCO, Lincoln, USA). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin, Fallanden, Switzerland) operating at (500.13 and 125.75) MHz, respectively, using tetramethylsilane (TMS) or deuterated solvent as an internal standard. Furthermore, the products were characterized by mass spectrometry with a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization source, and the purity was determined by elemental analysis (C, H, N) with a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer (CE Instruments, Milan, Italy).

Valproic Acid Chloride (5). 2-Propylpentanoic acid (Acros Organics, Waltham, MA, USA) (1 g, 6.93 mmol) was dissolved in DCM (30 mL), and the mixture was cooled in an ice-water bath. Thionyl chloride (Merck, Darmstadt, Germany) (0.554 mL, 7.63 mmol) was then added slowly. The mixture was refluxed until chloride formation was complete (4 h). Solvents were evaporated using a rotary evaporator and vacuum pump, and the crude product was used as such without further purification.

***m*-Valproylphenylalanine (1).** DL-*m*-Tyrosine (**6**) (Fluka, St. Louis, MO, USA) (1 g, 5.52 mmol) was dissolved in trifluoroacetic acid (TFA) (30 mL) in an ice-water bath, and the mixture was stirred for 0.5 h. **5** (1.08 g, 6.62 mmol) was added slowly to the mixture. The reaction was brought to room temperature and stirred overnight. Solvents were evaporated using a rotary evaporator and vacuum pump. The crude product was purified with a column chromatography on silica gel (MeOH/DCM = 1:9) to give product as a TFA salt. The crude product was dissolved in acetonitrile (ACN; 20 mL), and dry HCl gas was introduced to the solution for 0.5 h. Excess HCl was flushed with N_2, and ACN was evaporated with a rotary evaporator and vacuum pump to give a white solid 0.32 g (30%). ^{1}H NMR (DMSO): δ 0.93 (t, 6H), 1.38 (m, 4H), 1.52 (m, 2H), 1.63 (m, 2H), 2.59 (m, 1H), 3.14 (d, 2H), 4.16 (t, 1H), 7.00 (d, 1H), 7.03 (s, 1H), 7.18 (d, 1H), 7.37 (t, 1H). ^{13}C NMR (DMSO): δ 13.9, 22.5, 34.1, 35.3, 44.4, 53.0, 120.6, 122.6, 127.0, 129.6, 136.9, 150.4, 170.2, 174.1. MS: calcd. for $C_{17}H_{26}NO_4$ 307.38 $[M]^+$, found 308.18 $[M + 1]^+$. Anal. ($C_{17}H_{25}NO_4 \cdot HCl \cdot 6/5H_2O \cdot$ ACN) calcd (%): C, 56.14; H, 7.79; N, 6.89; found: C, 55.8; H, 7.8; N, 6.72.

***p*-Valproylphenylalanine (2).** Compound **2** was synthesized from L-tyrosine (**7**) (Sigma, St. Louis, MO, USA) (1 g, 5.52 mmol) and **5** (1.08 g, 6.62 mmol) with the same procedures as **1** to give a white solid (0.43 g, 23%). ^{1}H NMR ($CDCl_3$): δ 0.93 (t, 6H), 1.38 (m, 4H), 1.50 (m, 2H), 1.67 (m, 2H), 2.54 (t, 1H), 3.26 (d, 2H), 4.23 (t, 1H), 6.94 (d, 2H), 7.35 (d, 2H). ^{13}C NMR (MeOD): δ 14.3, 21.7, 35.8, 36.6, 46.5, 55.1, 123.4, 131.7, 133.4, 151.9, 171.1, 176.9. MS: calcd. for $C_{17}H_{26}NO_4$ 307.38 $[M]^+$, found 308.18 $[M + 1]^+$. Anal. ($C_{17}H_{25}NO_4 \cdot 7/5HCl \cdot 2/5ACN$) calcd (%): C, 57.03; H, 7.42; N, 5.23; found: C, 57.08; H, 7.41; N, 5.32.

Boc-3-amino-phenylalanine (10). Pd (10% on activated charcoal) (0.12 g) was added carefully to Boc-3-nitro-L-phenylalanine (**8**) (Aldrich, St. Louis, MO, USA) (1 g, 3.22 mmol) in MeOH (30 mL). The reaction was stirred in H_2-atmosphere until complete. The mixture was filtered through Celite, and the solvent was evaporated by a rotary evaporator and vacuum pump. A pink solid formed in vacuum to give 0.9 g (100%) of the title compound. ^{1}H NMR ($CDCl_3$): δ 1.41 (s, 9H), 3.02 (d, 2H), 4.48 (dd, 1H), 6.63 (m, 3H), 7.08 (t, 1H).

Boc-4-amino-phenylalanine (11). Compound **11** was synthesized from Boc-4-nitro-L-phenylalanine (**9**) (Aldrich, St. Louis, MO, USA) (1.15 g, 3.71 mmol) using the same method as for **10** to give a pink solid (1 g, 96%). ^{1}H NMR ($CDCl_3$): δ 1.41 (s, 9H), 3.00 (d, 2H), 4.44 (s, 1H), 5.13 (s, 1H), 6.21 (s, 2H), 6.62 (d, 2H), 6.96 (d, 2H).

***m*-Valpromidoyl-Boc-phenylalanine (12).** The cooled mixture of **10** (0.9 g, 3.21 mmol) in 1 M NaOH (5 mL) and DCM (20 mL) was stirred vigorously, and **5** in DCM (0.87 g, 5.35 mmol) was added dropwise to the solution. The reaction was stirred overnight in room temperature. The aqueous layer was separated and extracted with ethyl acetate (3 × 5 mL). The combined organic layers were washed first with a saturated aqueous $NaHCO_3$ and then with H_2O. The separated organic phase was dried over Na_2SO_4 and evaporated to dryness under reduced pressure. Purified with flash chromatography (MeOH/DCM = 1:99 to 1:1) to give an off-white solid of 0.79 g (61%) of the title compound. ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 6H), 1.32 (m, 4H), 1.36 (s, 9H), 1.65 (m, 2H), 1.63 (m, 2H), 2.59 (m, 1H), 3.14 (d, 2H), 4.16 (t, 1H), 7.00 (d, 1H), 7.03 (s, 1H), 7.18 (d, 1H), 7.37 (t, 1H).

***p*-Valpromidoyl-Boc-phenylalanine (13).** Compound was synthesized starting from **11** (1 g, 3.57 mmol) and **5** (0.78 g, 4.82 mmol) with the similar method as **12**, yielding an off-white solid of 0.79 g (55%). Flash chromatography (MeOH/DCM = 3:97 to 1:1). ^{1}H NMR ($CDCl_3$): δ 0.89 (t, 6H), 1.35 (m, 4H), 1.40 (s, 9H), 1.46; 1.68 (m, 4H), 2.27 (m, 1H), 3.12 (d, 2H), 4.59 (d, 1H), 5.02 (d, 1H) 7.08 (d, 2H), 7.53 (d, 2H), 7.70 (s, 1H).

***m*-Valpromidoylphenylalanine (3).** Compound **12** was dissolved in ACN (30 mL), and HCl gas was introduced to the mixture for 0.5 h. The excess of the HCl was flushed with N_2, and the ACN was evaporated with rotary evaporator and vacuum pump to give the crude product. The product was recrystallized from water/ethanol (pH = 1−4) to give a white solid of 0.55 g (83%). ^{1}H NMR (DMSO): δ 0.86 (t, 6H), 1.24 (m, 4H), 1.30 (m, 4H), 1.53 (m, 2H), 2.43 (m, 1H), 3.04 (dd, 2H), 3.88 (t, 1H), 6.96 (d, 1H), 7.21 (t, 1H), 7.53 (d, 1H), 7.56 (s, 1H), 9.95 (s, 1H). ^{13}C NMR (DMSO): δ 14.0, 20.2, 34.9, 36.3, 46.1, 53.9, 117.9, 120.2, 124.0, 128.7, 136.2, 139.5, 170.2, 174.3 MS: calcd. for $C_{17}H_{26}N_2O_3$ 306.4 $[M]^+$, found 307.28 $[M + 1]^+$. Anal. ($C_{17}H_{26}N_2O_3 \cdot HCl \cdot 1/2EtOH \cdot 2/5ACN$) calcd (%): C, 59.06; H, 8.23; N, 8.79; found: C, 59.26; H, 8.59; N, 9.14.

***p*-Valpromidoylphenylalanine (4).** Compound **4** was synthesized from **13** with a similar method as **3** to yield a white solid 0.48 g (81%). ^{1}H NMR (DMSO): δ 0.86 (t, 6H), 1.23 (m, 4H), 1.30 (m, 2H), 1.52 (m, 2H), 2.43 (m. 1H), 3.07 (d, 2H), 4.08 (t, 1H), 7.17(d, 2H), 7.58 (d, 2H), 8.43 (s, 3H), 10.01 (s, 1H). ^{13}C NMR (DMSO): 14.0, 20.2, 34.9, 36.3, 46.0, 54.0, 117.9, 120.2, 124.0, 128.7, 136.2, 139.5, 170.2, 174.3. MS: calcd. for $C_{17}H_{26}N_2O_3$ 306.4 $[M]^+$, found 307.19 $[M + 1]^+$. Anal. ($C_{17}H_{26}N_2O_3 \cdot HCl \cdot 7/10H_2O \cdot 3/10ACN$) calcd (%): C, 57.48; H, 8.03; N, 8.76; found: C, 57.32; H, 8.33; N, 8.96.

Analytical Procedure. The amount of the prodrugs **1**−**4** in analytical and biological samples was analyzed by the Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Karlsruhe, Germany) that consisted of a binary pump G1312A, a vacuum degasser G1379A, an automated injector system autosampler Hewlett-Packard 1050, an UV detector Hewlett-Packard 1050 variable wavelength detector, and an analyst software Agilent ChemStation for LC Systems Rev. A.10.02. An reverse-phase high performance liquid chromatography (RP-HPLC) C-8 column (Agilent Technologies, Little Falls Wilmington,

DE, USA) was used with a Zorbax C-8 guard column (Agilent Technologies, Little Falls, Wilmington, DE, USA). The UV-detector was set at 254 nm. The mobile phase consisted of a mixture of acetonitrile (12%) and a 0.05 M phosphate buffer solution of pH 3.0 (88%) having the flow rate at 1.1 mL/min. The amount of valproic acid in analytical and biological samples was analyzed by the Agilent gas chromatography system (6890 N gas chromatography, 7683 ALS autosampler and 5973 mass selective detector, Agilent Technologies, Palo Alto, CA, USA). The data were processed by the Agilent Enhanced Chemstation software (version 2.00). A cross-linked 5% phenyl methyl siloxane capillary column (HP-5MS; 30 m × 0.25 mm × 0.25 μm, Agilent Technologies) was used with helium as the carrier gas (0.9 mL/min). A total of 0.05 μL of the sample was injected into the system (1 μL split injection, ratio 1:20). The temperature program was as follows: at 90 °C, followed by an increase of 2 °C/min to 100 °C, followed by 40 °C/min to 300 °C. Selected monitoring at *m*/*z* 201 for silylated valproic acid and octanoid acid (internal standard) was used to quantify the compounds.

***In Vitro* Stability Studies.** To demonstrate the chemical and enzymatic stability of **1**−**4** in physiological circumstances, and to show the bioconversion of **1**−**4** to valproic acid, the prodrugs were exposed to degradation in phosphate buffer (pH 7.4, 0.16 M, μ = 0.5), 75% (v/v in isotonic phosphate buffer, pH 7.4) human plasma, 50% (v/v in isotonic phosphate buffer, pH 7.4) rat liver homogenate, and 20% (v/v in isotonic phosphate buffer, pH 7.4) rat brain homogenate. Each prodrug **1**−**4** was incubated in a thermostatically controlled water bath at 37 °C, and 100 μL aliquots were withdrawn at regular time intervals. The biological samples were exposed to protein precipitation by 200 μL of acetonitrile, and after mixing, the samples were centrifuged. The HPLC analysis was performed to determine the amount of intact prodrug in the supernatant. To determine the released valproic acid in the samples, 100 μL aliquots were precipitated by 200 μL of acetonitrile, and 100 μL of octanoid acid (concentration 35 μM) as an internal standard was added. After mixing, 1200 μL of H_2O was added, and the sample was centrifuged. The supernatant was loaded into preconditioned and equilibrated C18 SPE-columns (Supelco Discovery DSC-18 1 mL, Supelco Park Bellefonte, PA, USA). The column was first washed with 1 mL of 5% (v/v) MeOH in water and then with 1 mL of MeOH to wash out the intact prodrug. Finally, valproic acid and octanoid acid were diluted from the columns two times with 0.5 mL of 50% (v/v) acetonitrile/acetone and evaporated to dryness under nitrogen stream at 40 °C. Prior to analysis, the samples were diluted into 50 μL of pyridine and derivatized with 50 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) (Sigma, St. Louis, MO, USA) for 30 min at 40 °C. The amount of released valproic acid as well as octanoid acid in the sample was determined by gas chromatography/mass spectroscopy (GC-MS).

***In Situ* Rat Brain Perfusion Technique.** The most precise and widely used method for evaluating the BBB transport mechanisms and the BBB transport of drugs is the *in situ* rat brain perfusion method of Takasato et al.[19] In this study, the modified *in situ* rat brain perfusion technique was used to determine the transport mechanisms, affinity, and brain uptake of the prodrugs **1**−**4**.

In our studies, the rats were anesthetized with ketamine (90 mg/kg intraperitoneal (i.p.)) and xylazine (8 mg/kg i.p.). Their body temperature was maintained at 37 °C during the operation. In our modified method, after exposure of the right common carotid artery system, the right external carotid artery was ligated, and the right common carotid artery was catheterized with a PE-50 catheter containing 100 IE/mL heparin. The right occipital and the right pterygopalatine arteries were left open. Before perfusion, the perfusion buffer (composition in mM: 128 NaCl, 24 $NaHCO_3$, 4.2 KCl, 2.4 $NaH_2PO_4 \cdot H_2O$, 1.5 $CaCl_2 \cdot 2H_2O$, 0.9 $MgCl_2 \cdot 6H_2O$ and 9 D-glucose) was gassed to pH 7.4 with 95% O_2/5% CO_2 to attain blood steady state gas levels. The perfusate was then filtered with a 0.45 μm Millex-HV filter and warmed to 37 °C. Just immediately before the perfusion, the blood flow from the systemic circulation was stopped by severing the cardiac ventricles to eliminate the contralateral blood flow contribution. Hypoxic brain damage, while the common carotid artery was cannulated, was prevented via the crossover of blood from the contralateral carotid artery at the circle of Willis.[20] During the perfusion, the perfusion flow rate in the common carotid artery was kept constant at 10 mL/min using a Harvard PHD 22/2000 syringe pump (Harvard Apparatus Inc., Holliston, MA, USA). The flow of the perfusion fluid was controlled so that the perfusion was relegated only to the right brain hemisphere and to prevent pressure harming to the BBB. After 30−60 s perfusions the animal was decapitated, the skull was opened, and the right brain hemisphere was immediately deep-frozen or excised from six different regions (frontal, parietal, occipital, hippocampus, thalamus, and striatum). The brain samples from the regions (10−20 mg) were digested in 0.5 mL of Solvable (PerkinElmer, Boston, MA, USA) for 3 h at 50 °C, 1.5 mL of scintillation cocktail (Ultima Gold, PerkinElmer, Boston, MA, USA) was added, and the radioactivity was analyzed on a liquid scintillation spectrometer (Wallac 1450 MicroBeta; Wallac Ltd., Finland). The frozen samples were stored at −80 °C until sample preparation.

The *in situ* rat brain technique was validated by determining the intravascular volume of the rat brain (V_v) using 0.2 μCi/mL [^{14}C]-sucrose (PerkinElmer, Boston, MA, USA) as a marker as well as by determining the cerebral perfusion flow rate (*F*) of 0.2 μCi/mL [^{3}H]-diazepam (PerkinElmer, Boston, MA, USA) and the brain capillary permeability surface area (PA) of 0.3 μCi/mL [^{14}C]-urea (PerkinElmer, Boston, MA, USA). The PA of 0.2 μCi/mL [^{14}C]-leucine was also determined to validate the technique. The results of the validation studies were compared with the results reported previously[4,13,24] (Table 1). The sucrose perfusion demonstrates the integrity of the BBB capillaries and tight junctions during the perfusion and represents the vascular volume of the rat brain. The diazepam perfusion represents the maximum allowable transition of the solute from brain capillaries to the parenchyma under the particular test conditions. These two validation results were used in all of the brain uptake calculations. The PA value of a relatively low penetrable, polar 0.3 μCi/mL [^{14}C]-urea was also determined to be consistent with the values reported earlier (Table 1). Finally, the PA value of 0.2 μCi/mL [^{14}C]-leucine, a known endogenous substrate of LAT1, was observed to be similar to the values reported earlier (Table 1). This is the value of the 100% leucine BBB uptake which has been used as a control in competition studies.

V_v (mL/g) was calculated from eq 1.

$$V_v = \frac{q_{tot}}{C_{pf}} \quad (1)$$

where q_{tot} represents the [^{14}C]-sucrose amount in the brain (μCi/g) after a short perfusion, and C_{pf} represents the

Table 1. Validation Results for the *In Situ* Rat Brain Perfusion Technique (Mean ± SD)

parameter	our results (n = 4)	Killian et al.[13]	Gynther et al.[4]	Rouselle et al.[24]
V_v (mL/mg)	14.9 ± 2.0		11.6 ± 1.3	~8
F (mL/(s·mg))	73.6 ± 11.9	51.9 ± 2.2	28.46 ± 4.0	60−80
urea PA (mL/(s·mg))	0.24 ± 0.05	0.34 ± 0.3	0.18 ± 0.06	~0.1−0.2
leucine PA (mL/(s·mg))	19.0 ± 3.6	51.8 ± 3.2	20.59 ± 3.2	

[^{14}C]-sucrose concentration in the perfusion fluid (μCi/mL). F (mL/(g·min)), which demonstrates the maximum allowable transition of the solute from brain capillaries to the parenchyma, was calculated from eq 2.

$$F = \frac{q_{tot}}{T^x C_{pf}} \quad (2)$$

where q_{tot} represents the [^{3}H]-diazepam amount in the brain (μCi/g) after a perfusion, C_{pf} represents [^{3}H]-diazepam concentration in the perfusion fluid (μCi/mL), and T (min) represents the perfusion time minus the delay time when the perfusate reaches the brain once the perfusion pump is started. PA values (mL/(g·min)) were calculated using eqs 3 and 4.

$$PA = -F^* n\left(1 - \frac{K_{in}}{F}\right) \quad (3)$$

$$K_{in} = \frac{(q_{tot}/C_{pf}) - V_v}{T} \quad (4)$$

In eq 3, F is calculated from eq 2, and K_{in} (unidirectional transfer constant) is calculated from eq 4. In eq 4, q_{tot} represents the amount of the radio-labeled solute (e.g., [^{14}C]-leucine) in the rat brain (μCi/g) after a perfusion, C_{pf} represents the concentration of the radio-labeled solute in the perfusion fluid (μCi/mL), V_v is calculated from the eq 1, and T (min) represents the perfusion time minus the delay time when the perfusate reach the brain once the perfusion pump is started.

Determination of the Brain Uptake Mechanism for Prodrugs 1−4. To determine the ability of the prodrugs to bind into LAT1 using the *in situ* rat brain perfusion, a known amount of [^{14}C]-leucine, an endogenous substrate of LAT1, was added into the perfusion fluid with different concentrations of the prodrug **1**−**4**. The PA of ^{14}[C]-leucine was determined, and the LAT1 binding of the LAT1 substrates is shown by decreased PA of ^{14}[C]-leucine caused by a competitive binding of the substrate to LAT1 in a saturable manner. By these competition studies, the inhibition value (K_i) was determined. The K_i value is the concentration where the prodrug inhibits the brain uptake of the [^{14}C]-leucine half of the maximum. Consequently, the K_i value is a version of K_m-value, but it is illustrating only the inhibition of the competing molecule, not brain uptake of the prodrug. To determine the interaction of **1**−**4** with the LAT1 at the BBB, the prodrugs were coperfused for 30 s with [^{14}C]-leucine (0.2 μCi/mL). The concentrations were from 0.5 to 100 μM for **1** and **3** and from 0.5 to 700 μM for **2** and **4**. The 100% PA product of [^{14}C]-leucine was determined after 30 s of perfusion of 0.2 μCi/mL [^{14}C]-leucine solution. The PA of 0.2 μCi/mL [^{14}C]-leucine coperfused with the prodrug was compared with the 100% PA of 0.2 μCi/mL [^{14}C]-leucine.

To determine whether the binding of the prodrugs **1**−**4** to LAT1 is reversible or irreversible, the PA product of [^{14}C]-leucine was studied after perfusing the rat brain first with **1**, **2**, **3**, or **4** at the concentration of 100% [^{14}C]-leucine inhibition for 30 s, followed by washing the prodrug from the brain capillaries with 30 s of perfusion of prodrug-free perfusion medium and finally perfusing the brain with 0.2 μCi/mL [^{14}C]-leucine for 30 s. If the endogenous substrate is taken into the brain in an amount that is comparable to that of the first perfusion, the binding of the prodrug is reversible.

Brain Uptake Studies of the Prodrugs. The brain uptake of the prodrugs **1**−**4** was also performed using the *in situ* rat brain perfusion technique. To quantify the brain uptake of the prodrugs, the rat brain vasculature was perfused for 60 s with 37 °C perfusion medium containing 600 μM concentration of prodrug **1**, **2**, **3** or **4**. After the perfusion, the brain vasculature was washed with cold prodrug-free perfusion medium (5 °C) for 30 s to wash out the remaining prodrug from the brain vasculature and to decrease the efflux activity of the transporters. To study the role of the other amino acid transporters at the BBB in the brain uptake of the prodrugs, the uptake studies of **3** and **4** at 600 μM concentration for 60 s were also performed by using 1 mM concentration of the substrates of those transporters as inhibitors. L-lysine (Sigma, St. Louis, MO, USA) for the transporters of system y^+ and L-glutamic acid (Sigma, St. Louis, MO, USA) for the transporters of system X^- were used. L-lysine and L-glutamic acid are not substrates of LAT1.[7]

In addition to the uptake studies of the prodrugs **1**−**4**, our interest was to show whether the prodrugs cross the BBB by passive diffusion (nonsaturable component) or by carrier-mediated transport via LAT1. To show the role of the passive diffusion, the brain capillaries were first washed for 30 s with cold prodrug-free perfusion medium (5 °C) to decrease the activity of the transporters. To maintain the low activity of LAT1, also the 60 s perfusion with 50 μM of the prodrug was studied at 5 °C. Finally, the brain vasculature was washed with the cold prodrug-free perfusion medium (5 °C) for 30 s to wash out the remaining prodrug from the brain vasculature and to maintain the decreased efflux activity of the transporter.

Brain Sample Preparation. The prodrugs **1**−**4** were separated from the brain by protein precipitation followed by liquid−liquid extraction and solid-phase extraction (SPE). The brain hemisphere was homogenized with 2.5 mL of 20% (v/v) MeOH in water. For precipitating the proteins, the samples were then acidified with 200 μL of 5 M hydrochloric acid and vortexed for 5 min. MeOH in water (2 mL of 20% v/v) was added, and the homogenates were vortexed for 2 min. After standing for 10 min, the samples were centrifuged for 10 min (7500 g at 7 °C) after which the supernatants were collected. This was repeated three times, and the supernatants were combined. The combined supernatants were loaded into preconditioned and equilibrated C18 SPE-columns (Supelco Discovery DSC-18 3 mL, Supelco Park Bellefonte, PA, USA). The columns were first washed with 2 mL of 5% (v/v) MeOH in water and then with 2 mL of water. Finally, the prodrugs were diluted from the columns three times with 3 mL of MeOH and evaporated to dryness under nitrogen stream at 40 °C. Prior to analysis, the samples were reconstituted

Table 2. Hydrolysis Rates of the Prodrugs 1−4 in Phosphate Buffer (pH 7.4), in Rat Brain and Liver Homogenates and in Human Plasma (37 °C) ($T_{1/2}$, mean, n = 2)

prodrug	buffer (pH 7.4) (degraded in 3 weeks)	20% rat brain homogenate $T_{1/2}$ (min)	50% rat liver homogenate $T_{1/2}$ (min)	75% human plasma $T_{1/2}$ (min)
1	20%	75	1.00	708
2	15%	86	1.38	696
3		4%[a]	7%[a]	0%[a]
4		6%[a]	10%[a]	3%[a]

[a] Degraded in 24 h.

in 200 μL of 20% (v/v) acetonitrile in water and analyzed by ultraviolet high-performance liquid chromatography with ultraviolet detector (HPLC-UV). External standards were used for the brain samples. The calibration curves of the brain methods were linear over a range of 0.4−16 nmol/brain hemisphere. The lower limits of quantification for spiked samples were 0.4 nmol of prodrug/brain hemisphere.

Statistics. The figures were created, and all statistical analyses were performed using nonlinear regression analyses in GraphPad Prism 4.0 for Windows (GraphPad Software Inc., La Jolla, USA). Statistical differences were calculated using one-way ANOVA followed by a two-tailed Dunnett's test. The differences were considered significant for $p < 0.01$, indicative of a significant difference.

RESULTS AND DISCUSSION

***In Vitro* Stability Studies.** The stability of the prodrugs **1**−**4** in aqueous buffer (pH 7.4) and their bioconversion to valproic acid were determined in 20% rat brain homogenate and 50% rat liver homogenate as well as in 75% human plasma (Table 2). All of the four prodrugs demonstrated adequate chemical stability in aqueous solutions for further evaluation. The ester derivatives **1** and **2** hydrolyzed rapidly to valproic acid in the tissue homogenates and in plasma. On the other hand, the enzymatic hydrolysis studies of the amides **3** and **4** in both the brain and the liver homogenates and in plasma resulted only in slight degradation of the prodrugs *in vitro*. Therefore, the half-lives of **3** and **4** remain unidentified. Although the biodegradation of **3** and **4** was determined to be quite slow, it is reasonable to determine their transporter-binding mechanism and the brain uptake. In some earlier cases, amide prodrugs have been reported to release the active drug only *in vivo*.[17,25] In fact, the slow release of an active drug in the CNS might provide sustained therapeutic effect for a prodrug.

There are several tissue-targeted bioreversible prodrugs having an amide bond between the parent drug and the promoiety which already are in clinical use or undergoing experimental phases.[27] Traditionally, most prodrugs have been esters[26] which are well-suited for improved intestinal absorption when a rapid hydrolysis after absorption by enzymes with high catalytic efficiencies is required. However, the ester prodrugs are not often practical for tissue targeting because of the premature nonspecific hydrolysis by carboxylesterases in plasma and liver before the prodrug reaches the targeted tissue. Instead, amide derivatives of the esterified prodrugs with or without linkers have recently been used as more feasible promoieties in the tissue targeting due to their slower enzymatic and chemical hydrolysis. Although amides have often been considered as too slow and insufficiently hydrolyzed derivatives, there are several tissue-targeted bioreversible amide prodrugs in clinical use or undergoing experimental phases.[27] Amide prodrug design has been recently applied successfully, for example, in enhancing the oral bioavailability of gemcitabine by using valproic acid as a promoiety,[28] in improving the BBB penetration of ketoprofen via LAT1 with lysine as a promoiety,[17] and improving the brain distribution of tacrine as a 9-[*P*-(*N*,*N*-dipropylsulfamide)]benzoylamino-1,2,3,4-4*H*-acridine prodrug.[29] An example of recent amide prodrugs in clinical use is lisdextroamfetamine (Vyvanse, New River Pharmaceuticals/Shire).[30] Consequently, amide derivation is a valuable alternative for esterification in the tissue targeting by the prodrug approach.

***In Situ* Rat Brain Perfusion Technique.** In this study, the transport mechanisms across the BBB and the LAT1 affinity of the prodrugs **1**−**4** were determined by using the modified *in situ* rat brain perfusion technique.[19,20] In addition, the *in situ* rat brain perfusion technique was used to determine the amount of the prodrug uptaken into the brain.

The *in situ* rat brain perfusion method is widely used to assess a drug's brain uptake and its mechanism. The technique has advantages over *in vitro* and *in vivo* techniques which demonstrate the BBB uptake of molecules.[19,20] The *in situ* rat brain perfusion technique is versatile and sensitive for studying brain uptake and its mechanism especially when utilizing the transporters at the BBB. The main advantage over the *in vitro* studies is that by the *in situ* technique the brain uptake can be represented while the BBB is located in its normal physiological state.[20] Additionally, the peripheral pharmacokinetics, such as binding to plasma proteins, absorption, metabolism, and clearance, which can have an extensive effect on the BBB penetration of drugs, are simplified. Thus, the *in situ* rat brain perfusion demonstrates quite reliably only the brain uptake. The perfusate is not metabolized in or distributed to other tissues than the brain. In addition, the *in situ* brain perfusion technique mimics the *in vivo* situation, but the competition of endogenous amino acids is minimized when using the brain perfusion technique with the Krebs buffer as perfusion solution. Experimental conditions that could be toxic *in vivo* can also be used in the *in situ* perfusion technique.

However, the *in situ* method has some overall drawbacks and assumptions.[19,20,31] First, the whole brain tissue has to be analyzed instead of the extracellular fluid leading to the development of extraction methods and massive analytical assays. Additionally, disruption in the physiological function of the BBB may occur during the experiment caused by, for example, analgesics, air bubbles, particulate matter, or hypoxia. One assumption of the *in situ* rat brain perfusion technique is that the efflux of the uptaken perfusate from the brain back to the blood during the perfusion is not taken into account. Another assumption related to this method is that the most limiting step

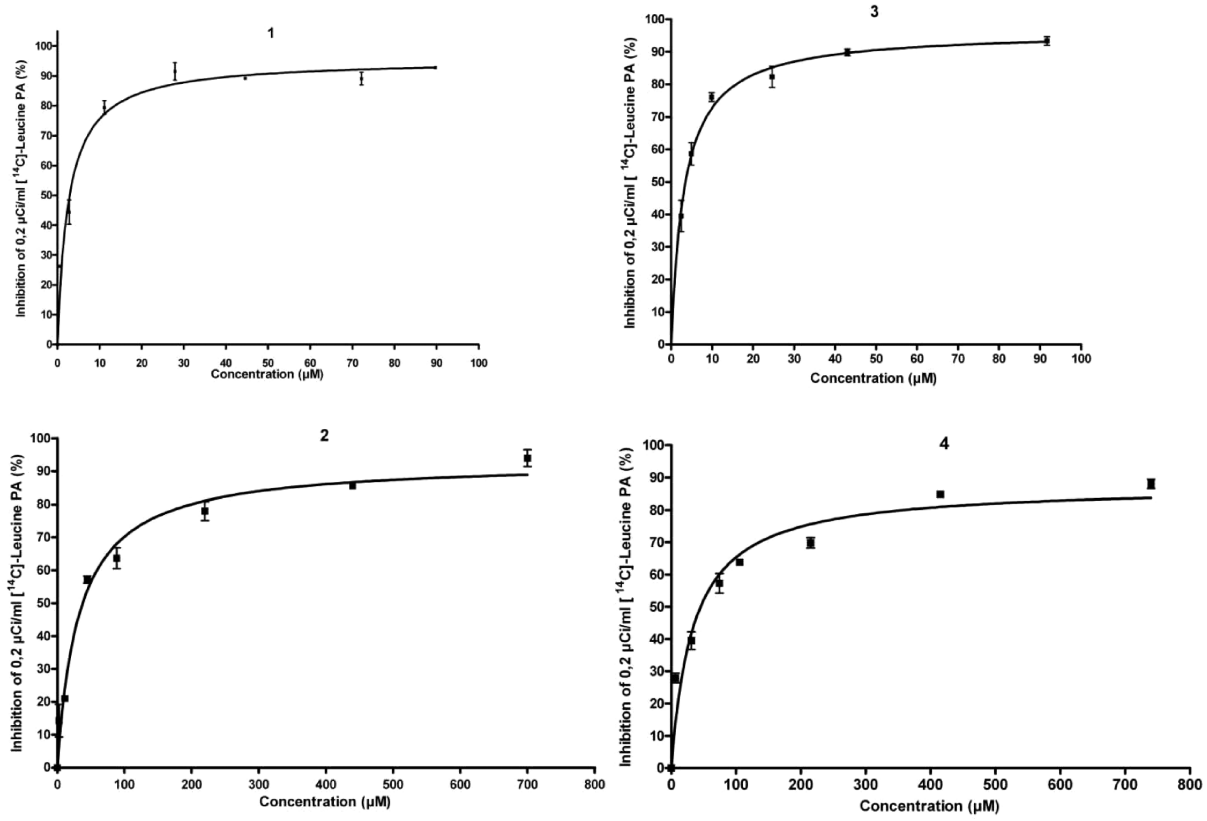


Figure 2. Inhibition of 0.2 μCi/mL [^{14}C]-leucine uptake across the BBB by valproate prodrugs **1**−**4**. The K_i-values are 2.7, 32.4, 3.2, and 34.1 μM (mean, n = 2), respectively.

of the brain penetration is the permeability across the BBB. To reduce the effects of these assumptions, the *in situ* data can be completed in some cases with the data of certain *in vitro* studies or other animal studies, such as *in vivo* bolus injection or infusion or microdialysis.[17] In spite of the assumptions of the *in situ* method, it is currently the most reliable and precise technique to evaluate the carrier-mediated transport across the BBB.

Transporter-Mediated Brain Uptake of Prodrugs 1−4. To identify the BBB uptake mechanism and the structure−activity relationships between LAT1 and the prodrugs **1**−**4**, the *in situ* perfusion technique described above was used. LAT1 is one of the most interesting transporters for improving the CNS delivery of small drug compounds by prodrug approach because it is expressed with greater amounts at the BBB compared with the other tissues; the binding affinities for LAT1 at the cerebrovasculature can be even 1000-fold higher than that for LAT1 at the peripheral cells.[5,6] Moreover, LAT1 has a relatively broad substrate specificity for prodrug design.[32,33] The other two influx transporters at the BBB are transporters of system y^+ (CAT1) and X^-,[7] and amino acid mimicking prodrugs can also have affinity for those transporters. Thus, the role of these other transporters has to be ruled out while studying LAT1-mediation of drug brain uptake (see below).

Strong inhibition of the PA product of [^{14}C]-leucine was found when perfusing 0.2 μCi/mL [^{14}C]-leucine in the presence of the prodrug **1**, **2**, **3**, or **4** (Figure 2). All of the prodrugs were able to inhibit the uptake of [^{14}C]-leucine in a concentration-dependent and saturable manner, indicating that the prodrugs were able to bind to the LAT1 at the BBB. In fact, of the two other influx amino acid transporters expressed at the BBB, also the transporters of system y^+ transports leucine into the brain. However, the transport of leucine via the system y^+ transporters is inefficient (millimolar K_m-value) and sodium-dependent, whereas the transport of the prime substrates of the transporter (i.e., L-lysine, L-arginine and L-ornithine) is efficient and sodium-independent, and they bind to the transporter with K_m-values between 70 and 200 μM.[34] Leucine is not a substrate for system X^- transporters. In general, the affinity of the neutral amino acids for LAT1 expressed at the BBB can be more than 10-fold higher than that of the other amino acid transporters at the BBB.[7] For the reasons mentioned above, leucine has been widely used as a LAT1-spesific substrate while studying the role of LAT1 as the BBB transport mode.[4,13,17,35−37]

In addition to LAT1-binding, there were significant differences between K_i-values of the prodrugs **1**−**4**, which were 2.7, 32.4, 3.2, and 34.1 μM (mean, n = 2), respectively (Figure 2). This observation illustrates clearly that the regioselective positioning of valproate in the L-phenylalanine structure significantly affects the binding of prodrug to LAT1. There were no significant differences between the affinities to LAT1 whether valproic acid was linked to the promoiety by amide or ester bond. However, a very significant difference between the affinities of the prodrugs to LAT1 was observed when valproic acid was linked to the para- or meta-position of the phenylalanine promoiety. We have shown that the affinities of **1** and **3** are more than 10-fold compared with those of **2** and **4**. The results confirm the LAT1 substrate binding properties reported by Takada et al.[18] who have shown that meta-isomer of melfalan is taken up more rapidly to the rat brain than para-isomer of melfalan in the *in situ* rat brain perfusion. Our results represent that the earlier findings of

Takada et al. could also be utilized in the prodrug approach to improve the brain uptake of polar and hydrophilic drug molecules. Because the prodrugs were racemic mixtures and LAT1 is stereospecific for L-isomers,[38] the affinity of the prodrugs having a pure L-amino acid promoiety might be even stronger.

The reversibility of the binding of the prodrugs to rat LAT1 was also examined to confirm that the prodrugs **1**−**4** do not inhibit the LAT1 transport as irreversible inhibitors. The reversibility studies were organized by determining the PA product of [^{14}C]-leucine after perfusing the rat brain first with a prodrug at 400 μM (**2** and **4**) or 93 μM (**1** and **3**) concentration for 30 s, followed by washing the prodrug from the brain capillaries with 30 s perfusion of prodrug-free perfusion medium, and finally perfusing the rat brain with 0.2 μCi/mL [^{14}C]-leucine for 30 s. The rat brain PA product of the [^{14}C]-leucine after the prodrug perfusion and wash resulted in 80−90% of the original PA product of the control demonstrating the binding to be reversible (Figure 3). The reversibility is an essential requirement, obviously, for the brain uptake of prodrugs via specific transporters.

Brain Uptake Studies of the Prodrugs 1−4. In addition, we studied the total amounts of the prodrugs **1**−**4** in the brain after the *in situ* rat brain perfusion to confirm that the prodrugs **1**−**4** do not only bind to LAT1 but are also transported across the BBB into the brain tissue. The entry of a molecule into the brain does not necessarily occur even if the prodrug binds very tightly to the transporter. As illustrated in Figure 4, the valproate prodrugs **1**−**4** are able to cross the rat BBB and gain entry into the brain tissue. The amides (**3** and **4**) were shown to cross the rat BBB more efficiently than the esters. However, the difference between these two groups of compounds may be due to the exposure of the esters to the degrading enzymes at the BBB during the perfusion. This confirms the reasonability to perform more comprehensive *in vivo* brain uptake studies for amide prodrugs, which are currently ongoing in our laboratory. The improvement in the brain uptake compared with meta- and para-substituted phenylalanine derivatives is also clearly shown in Figure 4. The PA product of prodrug **3** at 600 μM is almost 29 pmol/(mg·min), which is more than 2-fold higher than that of prodrug **4**. Thus, **3** both binds to LAT1 and crosses the BBB significantly more efficiently than **4**. The difference between the rate of the uptake of **3** and **4** is not as great as could be explained according to their binding affinities, and the reason for the reduced uptake capacity of the prodrug **3** may be steric restrictions. In other words, the prodrug **3** may bind to LAT1 so tightly that it does not leave the transporter relatively as easily as prodrug **4**.

The role of the other amino acid transporters at the BBB (i.e., system y^+ and X^-) in the brain uptake of the prodrugs **3** and **4** was studied by perfusing the rat brain with **3** and **4** at 600 μM concentration for 60 s with 1 mM L-lysine (a substrate of the system y^+) and L-glutamic acid (a substrate of the system X^-) as inhibitors for the other amino acid transporters. There was no statistically significant difference at the brain uptakes of the prodrugs while coperfused with the substrates of the other amino acid transporters at the BBB. Therefore, the role of the other influx amino acid transporters at the BBB in the brain uptake of prodrugs was ruled out. Hence, in addition to the concentration-dependent inhibition of the brain uptake of L-leucine (Figure 2), this is another clear confirmation that it is LAT1 which ferries the prodrugs across the BBB.

The role of passive diffusion in the rat brain uptake of **3** and **4** at a 50 μM concentration was investigated by using cold perfusion (5 °C). The lower temperature decreased the brain uptake of the prodrugs from about 1 pmol/(mg·min) (**4**) and

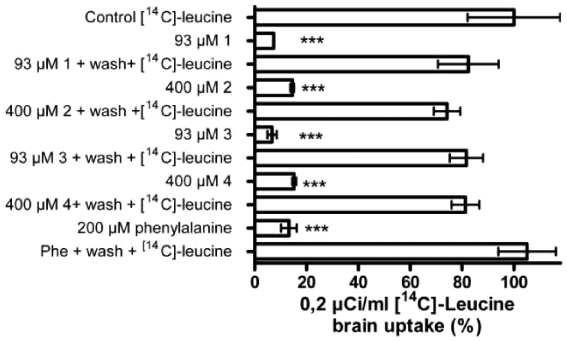


Figure 3. Reversibility of the LAT1 binding of the prodrugs **1**−**4**. The brain uptake of 0.2 μCi/mL [^{14}C]-leucine is inhibited in the presence of the prodrugs at 400 μM (**2** and **4**) or 93 μM (**1** and **3**) to 5−20% of the 0.2 μCi/mL [^{14}C]-leucine. When the brain vasculature is washed after perfusing the vasculature with the prodrug, the uptake of leucine is not inhibited more than 10%. The 200 μM phenylalanine is used as a positive control. The reversibility of the binding of phenylalanine to LAT1 is similar to that of the prodrugs. An asterisk denotes a statistically significant difference from the respective control (***P < 0.01, one-way ANOVA, followed by a Dunnett T3-test).

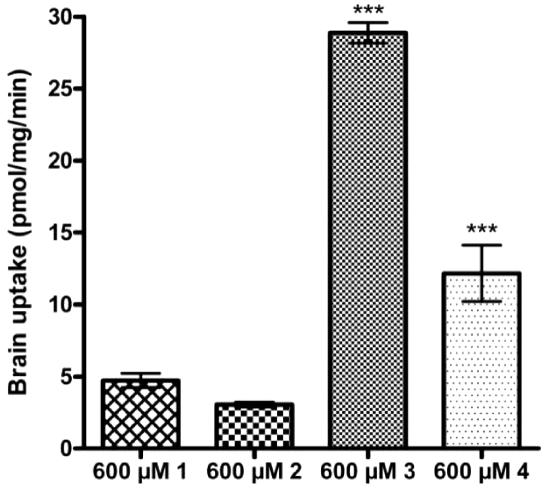


Figure 4. Rat brain uptake of **1**−**4** *in situ*. The brain uptakes of the ester prodrugs **1** and **2** at a concentration of 600 μM are 4.7 ± 0.5 and 3.1 ± 0.2 pmol/(mg·min) (mean ± SD, n = 3), respectively. The uptakes of the amide prodrugs **3** and **4** at concentration 600 μM are 28.9 ± 1.3 and 12.2 ± 2.0 pmol/(mg·min) (mean ± SD, n = 3), respectively. An asterisk denotes a statistically significant difference from the respective control (***P < 0.01, one-way ANOVA, followed by the Dunnett t-test). Column 600 μM **1** was used as a control in the Dunnett t-test. There is also a *** significant difference if column 600 μM **4** is used as a control in the Dunnett t test for column 600 μM **3**.

2 pmol/(mg·min) (**3**) to concentrations of below or near the limit of detection (Figure 5). This is a clear evidence of the carrier-mediated passage of the prodrugs across the BBB. In our previous studies, a lower perfusion medium temperature did not have any effect on the passive diffusion of [^{3}H]-diazepam across the BBB.[4]

In summary, these results show that both para- and especially meta-substituted phenylalanine derivatives can be efficient and potential promoieties for improving the brain uptake of drugs that cannot cross the BBB passively. The valproate prodrugs

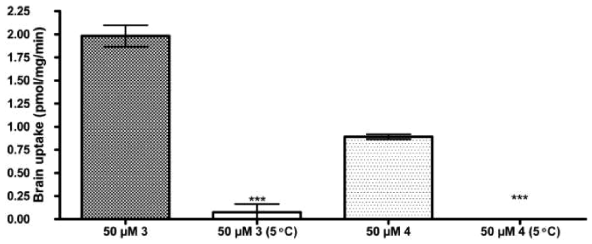


Figure 5. Rat brain uptake of the prodrugs **3** and **4** during cold perfusion. Perfusion medium at 5 °C decreased significantly the uptake of 50 μM **3** and **4** from 2.0 ± 0.1 and 0.9 ± 0.03 pmol/(mg·min) to almost zero (mean ± sd, n = 3), respectively. An asterisk denotes a statistically significant difference from the respective control (***P < 0.01, one-way ANOVA, followed by the Dunnett t-test). Columns 50 μM **3** and 50 μM **4** were used as controls in the Dunnett t-test. The cold perfusion was not performed for the ester prodrugs because of their high exposure for enzymatic degradation.

conjugated with the phenylalanine derivative were uptaken into the rat brain carrier-mediatedly, rapidly, and in significant amounts via LAT1.

CONCLUSION

In the present study, we showed that the phenylalanine derivative prodrugs **1**−**4** of valproic acid have an affinity for LAT1 at the rat BBB, their binding into LAT1 is reversible, they are able to release the active drug in the brain, and they are also uptaken into the brain parenchyma in a carrier-mediated manner. In addition, the role of the other influx amino acid transporters at the BBB in the brain uptake of prodrugs was ruled out. Consequently, the prodrugs **1**−**4** satisfy all of the requirements of brain-targeted prodrugs, and the brain uptake of those is LAT1-mediated. The amide derivatives were relatively stable in the *in vitro* studies, and so, more comprehensive *in vivo* studies are ongoing to prove their real biotransformation to valproic acid in rat brain tissue.

The most important finding of the study is the identification of a new potential promoiety for CNS drug delivery. This is the first time that amino acid derivatives but not natural amino acids have been reported as promoieties in the LAT1-mediated prodrug approach. Moreover, the phenylalanine derivative substituted from meta-position of the phenolic group is bound to LAT1 and uptaken into brain significantly more efficiently than the corresponding para-substitued analogue. Thus, phenylalanine derivatives substituted at the meta-position of the phenolic group could be highly usable in prodrug design for the LAT1-mediated CNS delivery of small molecular weight drugs with poor brain penetration properties.

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ABBREVIATIONS USED

AUC,area under curve; BBB,blood-brain barrier; CAT1,cationic amino acid transporter 1; CNS,central nervous system; DMSO, dimethyl sulfoxide; F,cerebral perfusion flow rate; GluT1,glucose transporter 1; LAT1,large neutral amino acid transporter 1; MCT1, monocarboxylic acid transporter 1; PA,brain permeability−surface area; V_v,brain intravascular volume

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