Large Neutral Amino Acid Transporter Enables Brain Drug Delivery via Prodrugs

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The blood−brain barrier efficiently controls the entry of drug molecules into the brain. We describe a feasible means to achieve carrier-mediated drug transport into the rat brain via the specific, large neutral amino acid transporter (LAT1) by conjugating a model compound to L-tyrosine. A hydrophilic drug, ketoprofen, that is not a substrate for LAT1 was chosen as a model compound. The mechanism and the kinetics of the brain uptake of the prodrug were determined with an in situ rat brain perfusion technique. The brain uptake of the prodrug was found to be concentration-dependent. In addition, a specific LAT1 inhibitor significantly decreased the brain uptake of the prodrug. Therefore, our results reveal for the first time that a drug−substrate conjugate is able to transport drugs into the brain via LAT1.

Introduction

The entry of drug molecules into the central nervous system is efficiently controlled by the blood−brain barrier (BBB).[a1] It is estimated that more than 98% of small molecular weight drugs and practically 100% of large molecular weight drugs developed for central nervous system disorders do not readily cross the BBB.[2] Many of the pharmacologically active drugs fail early in their development phase because these molecules lack the structural features essential for crossing the BBB.

The BBB endothelial cells differ from endothelial cells in the rest of the body by the presence of tight junctions, lack of fenestrae, and low occurrence of pinocytic vesicles.[3] There are also numerous enzymes[4] and efflux proteins present at the cerebral endothelial cells;[5,6] in addition, astrocytes,[7] pericytes,[8] and neurons take part in the formation of the BBB.[9] Because of these distinctive features of the BBB, it exhibits an efficient structural and functional barrier for the penetration of drugs into the central nervous system.[4,10] However, as each neuron in the human brain is perfused by its own blood vessel, a solute that is able to cross the BBB is distributed rapidly into the whole brain tissue.[11] Thus, vascular drug delivery holds potential in central nervous system drug delivery.

Several specific endogenous influx transporters have been identified at the brain capillary endothelium forming the BBB. These include transporters for nutrients, such as amino acids, glucose, and vitamins.[12] As many drug molecules have similar structural properties to endogenous substrates, it is clear that some membrane transporters can take part in drug transport as well.[13] The large neutral amino acid transport system (LAT1) is expressed on the luminal and abluminal membrane of the capillary endothelial cells, and it efficiently transports neutral L-amino acids (e.g., phenylalanine and leucine) into the brain.[14,15] Several clinically used amino acid mimeting drugs, such as L-dopa,[16] gabapentin,[17] and melphalan,[18] have been shown to be delivered into the brain predominantly via cerebrovascular LAT1-mediated transport, thus demonstrating the ability of LAT1 to be utilized in drug delivery. However, these drugs bear a very close structural resemblance to endogenous LAT1 substrates.

Chemical drug modification in a way that the drug can be recognized by specific transporters but still maintain therapeutic efficacy has proven to be very challenging. One attractive approach is to conjugate an endogenous transporter substrate to the active drug molecule in a bioreversible manner, that is, to utilize the prodrug approach. The prodrug should be designed in such a way that it is recognized by a specific transporter mechanism at the BBB. The endogenous transporter transports the prodrug across the BBB, and in brain tissue, the active drug is released from the prodrug to induce pharmacological action. By prodrug approach, the BBB penetration properties of a drug molecule can be enhanced without modifying its pharmacological properties.[19]

An amino acid L-tyrosine is a LAT1 substrate[20] that has a phenolic hydroxyl group suitable for the conjugation of various structurally different drug molecules with a biodegradable linkage and leaving both carboxyl and α-amino groups unsubstituted—a necessary feature for LAT1 recognition. In a study by Walker et al.,[21] a phosphonoformate L-tyrosine conjugate was able to inhibit the transport of [^{3}H]L-tyrosine in porcine brain microvessel endothelial cells. In another study, *p*-nitro- and *p*-chlorobenzyl ether conjugates of L-tyrosine inhibited the transport of [^{3}H]L-tyrosine in rabbit corneal cell line.[22] These results indicate that L-tyrosine conjugates are able to bind to the LAT1 transporter. However, the ability of these conjugates to cross the cell membrane has not yet been studied.

We describe a feasible strategy to achieve carrier-mediated drug transport into the rat brain via LAT1 by conjugating a model compound to L-tyrosine. A hydrophilic nonsteroidal anti-inflammatory drug, ketoprofen, was chosen as a model compound, and an amino acid prodrug 2-amino-3-{4-[2-(3-benzoylphenyl)propionyloxy]phenyl}propionic acid (**1**) (Figure 1) was synthesized with the aim of utilizing the LAT1 system to bypass the BBB. By conjugating L-tyrosine, a known LAT1 substrate, from the phenolic hydroxyl group to the carboxyl group of

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[a] Abbreviations: BBB, blood−brain barrier; BCH, 2 mM 2-aminobicyclo(2, 2, 1)heptane-2-carboxylic acid; DMSO, dimethyl sulfoxide; F, cerebral perfusion flow rate; LAT1, large neutral amino acid transporter; PA, permeability−surface area; V_d, volume of distribution; V_v, vascular volume.

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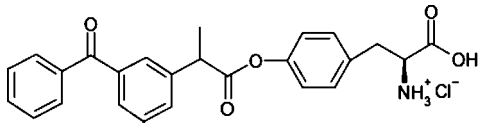

Figure 1. Chemical structure of prodrug **1**.

ketoprofen via an ester linkage, a zwitterionic prodrug that LAT1 recognizes was formed.[23]

Results and Discussion

Chemical and Enzymatic Stability of Prodrug 1. The degradation of **1** was studied in aqueous buffer solution of pH 7.4 at 37 °C. The degradation followed pseudofirst-order kinetics with a half-life of 22.5 ± 1.4 h (mean ± SD, $n = 2$). Therefore, **1** demonstrated sufficient chemical stability in aqueous solutions for further evaluation.

If the target for a prodrug design is the site-selective drug delivery to the central nervous system, the prodrug must not only be delivered to its intended site of action, but its bioconversion should also be selective.[4] In the present study, **1** was highly susceptible to enzymatic hydrolysis as it was quantitatively cleaved to ketoprofen and L-tyrosine in 80% rat serum, in 20% rat brain homogenate, and in 50% rat liver homogenate. The enzymatic hydrolysis followed pseudofirst-order kinetics, the half-lives being 10.2 ± 0.4, 4.4 ± 1.8, and 2.8 ± 0.6 min (mean ± SD, $n = 3$) in serum, brain homogenate, and liver homogenate, respectively. Therefore, **1** undergoes rapid bioconversion to ketoprofen and L-tyrosine in the brain tissue. However, **1** is also highly susceptible to enzymatic hydrolysis in both rat serum and liver, which may compromise its effective brain drug delivery.

In Situ Rat Brain Perfusion Technique. In the present study, an in situ rat brain perfusion technique was used for the determination of the brain uptake mechanism of the prodrug, since it has advantages over both in vivo and in vitro techniques. The experimental conditions can be easily manipulated to study saturable processes such as carrier-mediated uptake, and moreover, experimental conditions that would be toxic in the in vivo situation can be used. The most important advantage over in vivo experiments is the simplicity of the pharmacokinetics that makes the accurate determination of the uptake mechanism of the prodrug possible.[24–26] In vivo techniques take into account not only BBB penetration but also binding to plasma proteins, metabolism, and clearance, and there is significant value in removing some of this complexity and assessing brain penetration at the level of the BBB in situ. Furthermore, in situ techniques provide further insight into the molecular descriptors that are crucial for BBB penetration.[26] The most important advantage over in vitro techniques is that the BBB is in its normal physiological state when an in situ technique is used.

An in situ rat brain perfusion technique was used to evaluate the intravascular volume (V_v) of the rat brain, the cerebral perfusion flow rate (F), and the brain capillary permeability–surface area (PA) product of [^{14}C]L-leucine and [^{14}C]urea. The presence of functional LAT1 transporters was evaluated based on recent publications.[25,27–29] A 0.2 μCi/mL amount of [^{14}C]sucrose was used as a vascular marker to determine V_v and to demonstrate the integrity of the BBB during brain perfusion.[29] The value of V_v was determined to be 0.0116 ± 0.0013 mL/g (mean ± SD, $n = 3$). The integrity of the brain capillaries was tested with 0.2 μCi/mL [^{14}C]sucrose after the addition of 1% (v/v) dimethyl sulfoxide (DMSO). The value of V_v was 0.01032 ± 0.00098 mL/g (mean ± SD, $n = 3$) indicating that the addition of 1% (v/v) DMSO does not compromise the integrity of the tight junctions between the endothelial cells.

A highly diffusible and lipophilic solute [^{3}H]diazepam was used to determine F under the test conditions.[27] The value of F for 0.2 μCi/mL [^{3}H]diazepam was determined to be 0.02846 ± 0.00395 mL/s/g (mean ± SD, $n = 4$). The value of F for [^{3}H]diazepam was also determined using 5 °C perfusion medium, and it was 0.02561 ± 0.0045 mL/s/g (mean ± SD, $n = 2$). Similar F values indicate that the low temperature of the perfusion medium did not have an effect on the passive diffusion of [^{3}H]diazepam across the BBB.

The permeability of rat BBB in the test system was studied with [^{14}C]urea, a polar molecule with low BBB permeability.[27,28] The PA product of 0.2 μCi/mL [^{14}C]urea was determined to be 0.00018 ± 0.000056 mL/s/g (mean ± SD, $n = 3$). The low permeability of urea was in agreement with previous studies.[27,28]

The presence of functional LAT1 transporters in rat BBB was confirmed with L-leucine, which is an endogenous substrate for the LAT1.[14] The PA product of 0.2 μCi/mL [^{14}C]L-leucine was determined to be 0.02059 ± 0.00323 mL/s/g (mean ± SD, $n = 5$). The PA product was also determined using 5 °C perfusion medium with PA product of 0.0037 ± 0.00023 mL/s/g (mean ± SD, $n = 3$), suggesting that the uptake of L-leucine in the in situ rat brain perfusion test method was carrier-mediated, since the carrier-mediated uptake is reduced when the temperature is lowered.[30] In addition, the determination of cerebrovascular LAT1 functional expression was carried out with a competition assay by perfusing [^{14}C]L-leucine (0.2 μCi/mL) with 2 mM concentration of another known LAT1 substrate, L-phenylalanine.[28] This coperfusion resulted in almost complete inhibition (99%) of [^{14}C]L-leucine brain uptake, thereby demonstrating functional expression of cerebrovascular LAT1, which is in agreement with the literature.[28]

Determination of the Brain Uptake Mechanism for Prodrug 1. The ability of **1** to bind into LAT1 was studied with the in situ rat brain perfusion technique. In the presence of **1**, the [^{14}C]L-leucine PA product was significantly decreased from 0.02059 ± 0.00323 mL/s/g (mean ± SD, $n = 5$) to 0.000317 ± 0.000136 mL/s/g (mean ± SD, $n = 3$) (98.5% inhibition). This is a clear evidence of significant binding of **1** to the LAT1 (Figure 2). To further study the binding kinetics of **1** to LAT1, the PA product of [^{14}C]L-leucine was determined after perfusing rat brain first with **1** at 60 μM 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 rat brain with 0.2 μCi/mL [^{14}C]L-leucine for 30 s. The PA product of [^{14}C]L-leucine (0.01902 ± 0.0058 mL/s/g mean ± SD, $n = 3$) recovered, indicating that the binding of **1** to the LAT1 is reversible (Figure 2).

To confirm the required properties of LAT1 substrate reported by Uchino et al.,[23] we synthesized two ketoprofen prodrugs with phenylalanine promoiety (**2** and **3**) and two prodrugs with leucine promoiety (**4** and **5**). The synthesis and structural characterization of these prodrugs are described in the Supporting Information. Ketoprofen was conjugated with the carboxyl group by diethylester linker or the amino group to delete the zwitterionic properties of the amino acids that are necessary for LAT1 recognition. None of these four prodrugs were able to bind to LAT1 (Supporting Information), which is in correlation with the literature.[23] Therefore, our results confirm that the potential substrate should have a positively charged amino group, a negatively charged carboxyl group, and a hydrophobic side chain.

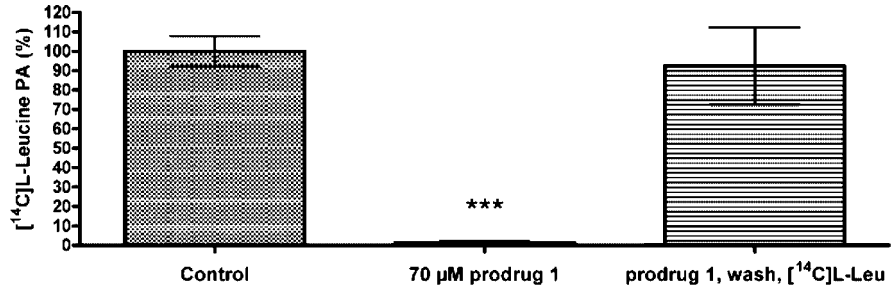

Figure 2. Mechanism of prodrug **1** rat brain uptake. The PA product of 0.2 μCi/mL [^{14}C]L-leucine in the absence or presence of **1**. The control PA product 0.02059 ± 0.00323 mL/s/g (mean ± SD, $n = 5$) is decreased to 0.000317 ± 0.000136 mL/s/g (mean ± SD, $n = 3$) in the presence of 70 μM **1** (98.5% inhibition). The uptake of [^{14}C]L-leucine is recovered after washing the prodrug from the brain capillaries, demonstrating the PA product of 0.01902 mL/s/g. An asterisk denotes a statistically significant difference from the respective control (***$P < 0.001$, one-way ANOVA, followed by Dunnett's t test).

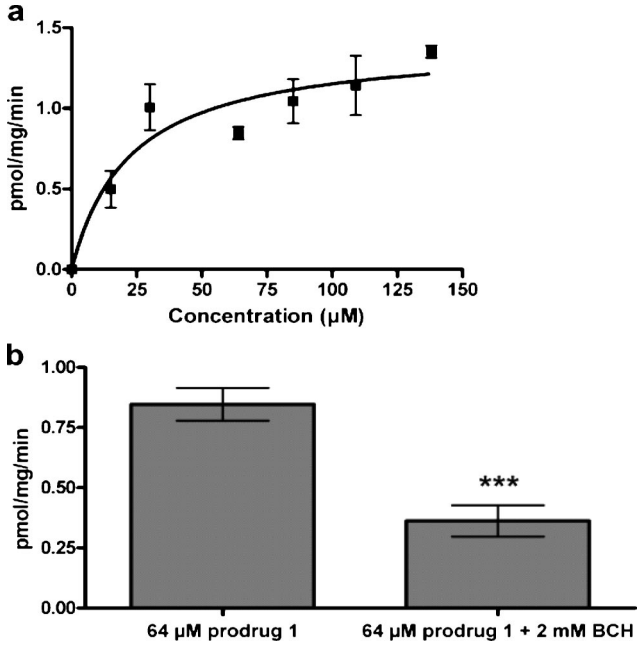

Figure 3. Kinetics of prodrug **1** rat brain uptake. (a) Relationship between **1** concentration of the perfusion medium and **1** brain uptake. K_m and V_{max} are 22.49 ± 9.18 μM and 1.41 ± 0.15 pmol/mg/min (mean ± SD, $n = 3$), respectively. (b) The uptake of prodrug **1** with 64 μM concentration is 0.846 ± 0.069 pmol/mg/min (mean ± SD, $n = 3$), whereas in the presence of 2 mM BCH, the uptake of **1** is decreased to 0.362 ± 0.065 pmol/mg/min (mean ± SD, $n = 3$). The decrease of the uptake is statistically significant (***$P = 0.001$, t test).

Brain Uptake Determination of Prodrug 1. The brain uptake of **1** was determined with the in situ rat brain perfusion technique using seven concentrations of **1** ranging from 0 to 138 μM at 60 s of perfusion time (Figure 3a). The uptake of **1** was found to be concentration-dependent and exhibited a typical saturation curve of the Michaelis–Menten type with a K_m value of 22.49 ± 9.18 μM and a V_{max} value of 1.41 ± 0.15 pmol/mg/min (mean ± SD) (Figure 3a). The brain uptake of 64 μM **1** was 0.846 ± 0.069 pmol/mg/min, and addition of 2 mM 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH), a specific LAT1 substrate,[30] to the perfusion medium significantly decreased the brain uptake to 0.362 ± 0.065 pmol/mg/min (mean ± SD, $n = 3$) (Figure 3b). The brain uptake of **1** was below the lower limit of detection when 10 and 15 μM concentrations of **1** were coperfused with BCH. These results confirm the hypothesis that the brain uptake of **1** is LAT1-mediated. Finally, a nonsaturable component of **1** brain uptake was determined using a 60 μM concentration of **1** in a 5 °C perfusion medium.[30] The concentration of **1** in brain tissue was below the lower limit of detection, indicating that there is no passive diffusion of **1** across the BBB. A lower perfusion medium temperature did not have any effect on passive diffusion of [^{3}H]diazepam across the BBB.

Table 1. Capillary Depletion Analysis after 60 s of Perfusion of **1** with 85 μM Concentration, Followed by Washing the Prodrug from the Capillaries with 30 s of Perfusion[a]

right cerebrum	V_d (mL/g)
whole brain	0.0123 ± 0.0016
supernatant	0.0118 ± 0.0008
pellet	*b*

[a] Mean ± SD, $n = 3$. [b] Below the lower limit of detection.

Capillary Depletion Analysis. Capillary depletion analysis of brain samples from perfused brain showed that **1** is present in the supernatant fraction, which consists of brain parenchyma (Table 1). The concentration of **1** in the endothelial cell-enriched pellet fraction was below the lower limit of detection. However, because the uptake of **1** determined from the whole brain is higher as compared to brain parenchyma, a fraction of **1** is captured into the endothelial cells.

Conclusion

In this study, an L-tyrosine prodrug **1** demonstrated significant reversible inhibition of brain uptake of the radiotracer [^{14}C]L-leucine in the in situ rat brain perfusion model, indicating that **1** binds to the LAT1. More importantly, **1** is able to cross the endothelial cells and penetrate into the brain parenchyma; the brain uptake of **1** was both concentration-dependent and saturable. The uptake of **1** was also significantly decreased when 5 °C perfusion medium was used, indicating that the brain uptake of **1** is carrier-mediated. In addition, the LAT1 inhibitor BCH significantly decreased the brain uptake of **1**. When put together, these results strongly suggest that the rat brain uptake of **1** is LAT1-mediated. Importantly, **1** is not only recognized but also transported across the rat BBB by LAT1; results that have not been earlier reported for any drug−substrate prodrug. In the prodrug structure, L-tyrosine acts as a carrier that possesses the essential structural features needed for LAT1 binding. The systemic pharmacokinetics of **1** is unknown, but the ester bond between ketoprofen and L-tyrosine is probably cleaved mostly by esterases present in the peripheral tissues. Therefore, this prodrug technology demands further development before being fully applicable to oral drug delivery. In summary, conjugation of therapeutically active drug with L-tyrosine, with biodegradable linkage, may represent a strategy to achieve

transport of hydrophilic neuropharmaceuticals into the brain by utilizing carrier-mediated transport and prodrugs.

Experimental Section

General Synthetic Methods. ^{1}H (500.13 MHz) and ^{13}C (125.77 MHz) NMR spectra were recorded on a Bruker Avance 500 spectrometer in CD_3OD. Chemical shifts (δ) are reported in ppm relative to CD_3OD (3.31 for ^{1}H and 49.0 for ^{13}C). Electrospray ionization mass spectra (ESI-MS) were obtained on a LCQ ion trap mass spectrometer equipped with an electrospray ionization source (Finnigan MAT, San Jose, CA). Elemental analyses (CHN) were carried out with a Thermo Quest CE Instruments EA 1110 CHNS-O elemental analyzer. Flash chromatography was performed on J. T. Bakers silica gel for chromatography (pore size 60 Å, particle size 50 μm).

Synthesis of 2-Amino-3-{4-[2-(3-benzoyl-phenyl)propionyl-oxy]phenyl}propionic Acid (1). L-Tyrosine (0.150 g, 0.828 mmol) was dissolved in trifluoroacetic acid (15 mL) and cooled to 0 °C. 2-(3-Benzoyl-phenyl)propionyl chloride (1.13 g, 4.14 mmol) was added to the solution, and the mixture was stirred at room temperature overnight. The solvent was evaporated, and the crude product was purified by flash chromatography (hexane:ethyl acetate 1:1 gradually increasing to methanol). Combined fractions were evaporated and dissolved to acetonitrile (20 mL), and HCl gas was added to the mixture for 5 min. The formed clear solution was evaporated and dried in vacuo. The yield of white solid was 0.346 g (92%).

^{1}H NMR (CD_3OD): δ 1.63 (3H, d, J = 7.2 Hz, CH_3), 3.12–3.35 (2H, m, CH_2), 4.16 (^{1}H, q, J = 7.2 Hz, C*H*), 4.24 (^{1}H, dd, J = 7.9 Hz, J = 5.4, C*H*), 7.03 (2H, d, J = 8.6 Hz, Ar*H*), 7.32 (2H, d, J = 8.5 Hz, ArH), 7.51–7.83 (9H, m, Ar*H*). ^{13}C NMR (CD_3OD): δ 18.8 (1H, *C*H$_3$), 36.6 (1C, *C*H$_2$),46.5 (1C, *C*H), 55.1 (1C, CH), 123.2 (2C, Ar*C*H), 129.6 (2C, Ar*C*H), 130.0 (1C, Ar*C*H), 130.1 (1C, Ar*C*H), 130.2 (1C, Ar*C*H), 131.0 (2C, Ar*C*H), 131.7 (2C, Ar*C*H), 133.0 (1C, Ar*C*H), 133.5 (1C, Ar*C*), 133.9 (1C, Ar*C*H), 138.7 (1C, Ar*C*), 139.4 (1C, Ar*C*), 142.1 (1C, Ar*C*), 151.9 (1C, Ar*C*), 171.1 (1C, CO), 174.6 (1C, *C*O), 198.3 (1C, *C*O). MS: *m*/*z* calcd for $C_{25}H_{23}NO_5$, 417.5; found, 418.1. Anal. calcd for ($C_{25}H_{23}NO_5 \cdot HCl \cdot H_2O$): C, 63.63; H, 5.55; N, 2.97. Found: C, 63.73; H, 5.29; N, 3.35.

HPLC Assay. The prodrug concentration in rat brain samples was analyzed by Agilent 1100 HPLC system (Agilent Technologies Inc., 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. The detector wavelength was set at 256 nm. A mixture of acetonitrile (50%) and a 0.02 M phosphate buffer solution of pH 2.5 (50%) at a flow rate of 1 mL/min was used as a mobile phase. The lower limit of quantification was 1 μM, and the method was linear over the range of 1–50 μM. In vitro samples were analyzed by HPLC system consisting of a Merck Hitachi L-6200A intelligent pump, Merck Hitachi L-4500 diode array detector, a Merck Hitachi D-6000A interface module, and a Merck Hitachi AS-2000 autosampler (Merck LaChrom, Hitachi, Tokyo, Japan). A mixture of 30% acetonitrile (v/v) and a 0.02 M 70% phosphate buffer solution of pH 6.0 (v/v) at a flow rate of 1 mL/min was used as a mobile phase. The lower limit of quantification was 0.4 μM, and the method was linear over the range of 0.4–211 μM. Reversed-phase HPLC was conducted with a Zorbax RP-18 column (150 mm × 4.6 mm, 5 μm, Agilent Technologies, Little Falls Wilmington, DE).

Chemical and Enzymatic Stability of the Prodrug 1. The rate of chemical hydrolysis of **1** was studied in aqueous phosphate buffer solution of pH 7.4 (0.16 M, ionic strength 0.5) at 37 °C. An appropriate amount of **1** was dissolved in 10 mL of preheated buffer, and the solutions were placed in a thermostatically controlled water bath at 37 °C. At appropriate intervals, samples were taken and analyzed for remaining **1** by HPLC. Pseudofirst-order half-time ($t_{1/2}$) for the hydrolysis of **1** was calculated from the slope of the linear portion of the plotted logarithm of remaining **1** vs time.

The rate of enzymatic hydrolysis of **1** was studied at 37 °C in rat serum, which was diluted to 80% (v/v) with 0.16 M phosphate buffer of pH 7.4; in rat brain homogenate, which was diluted to 20% (v/v) with isotonic 0.16 M phosphate buffer of pH 7.4; and in rat liver homogenate, which was diluted to 50% (v/v) with isotonic 0.16 M phosphate buffer of pH 7.4. The concentration of esterase enzymes in the homogenates was not determined. The solutions were kept in a water bath at 37 °C, and 0.2 mL of serum/buffer or homogenate/buffer mixture was withdrawn and added to 0.2 mL of acetonitrile to precipitate protein from the sample. After immediate mixing and centrifugation, the supernatant was analyzed for remaining prodrug and released ketoprofen by the HPLC. The pseudofirst-order half-time ($t_{1/2}$) for the hydrolysis of **1** was calculated from the slope of the linear portion of the plotted logarithm of the remaining prodrug against time.

In Situ Rat Brain Perfusion Technique. A modified in situ rat brain perfusion technique [25,29] was used to quantify the brain uptake and the transport mechanism of **1** at the BBB. Adult male Wistar rats (200–230 g) were supplied by the National Laboratoty Animal Centre (Kuopio, Finland) for the rat brain perfusion studies. Rats were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (8 mg/mL, i.p.), and their right carotid artery systems were exposed. The right external carotid artery was ligated, and the right common carotid artery was cannulated with PE-50 catheters filled with 100 IE/mL heparin. The right occipital and the right pterygopalatine arteries were left open. The blood flow of the rats was stopped by severing the cardiac ventricles. The perfusion fluid was infused through the common carotid artery at the rate of 10 mL/min for 30–60 s using a Harvard PHD 22/2000 syringe pump (Harvard Apparatus Inc., Holliston, MA). The skull was opened, and brain sections (10–30 mg) were excised from six different regions of the right brain hemisphere (frontal cortex, parietal cortex, occipital cortex, hippocampus, caudate nucleus, and thalamus). The left parietal cortex was used as a control. All samples were analyzed for radioactivity by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Oy, Finland). Brain samples were dissolved in 0.5 mL of Solvable (PerkinElmer, Boston) overnight at 50 °C, and liquid scintillation cocktail (Ultima Gold, PerkinElmer, Boston) was added before the samples were analyzed. The perfusion medium consisted of a pH 7.4 bicarbonate-buffered physiological saline (128 mM NaCl, 24 mM $NaHCO_3$, 4.2 mM KCl, 2.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 9 mM D-glucose). The solution was filtered, heated to 37 °C, and bubbled with 95% O_2, 5% CO_2 to attain steady-state gas levels within the solution. The in situ rat brain technique was validated by measuring the V_v as estimated by [^{14}C]sucrose (PerkinElmer, Boston) and by quantifying the brain uptake of [^{3}H]diazepam (PerkinElmer, Boston), [^{14}C]urea (PerkinElmer, Boston), and [^{14}C]L-leucine (PerkinElmer, Boston). All radiolabeled compounds used in the in situ rat brain perfusion technique were uniformly labeled.

Determination of the Brain Uptake Mechanism for Prodrug 1. The ability of **1** to bind into LAT1 was studied with the in situ rat brain perfusion technique. The 100% PA product of [^{14}C]L-leucine was determined after 30 s of perfusion of 0.2 μCi/mL [^{14}C]L-leucine solution. In a competition study, [^{14}C]L-leucine (0.2 μCi/mL) was coperfused with 70 μM concentration of **1** for 30 s. To study whether the binding of **1** to LAT1 was reversible, the PA product of [^{14}C]L-leucine was determined after perfusing rat brain first with **1** at 60 μM 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]L-leucine for 30 s.

Brain Uptake Studies of Prodrug 1. The prodrug **1** brain uptake studies were performed with the in situ rat brain perfusion technique described above. Because of relatively low water solubility of **1**, it was first dissolved in DMSO and then added to the perfusion medium resulting in 1% (v/v) DMSO solution. After the pH was adjusted to 7.4, the solution was filtered with 0.45 μm Millex-HV filters. The rat brains were perfused for 60 s with 37 °C perfusion medium containing prodrug **1**. After perfusion, the remaining prodrug was washed from the brain vasculature with cold prodrug-

free perfusion medium (5 °C) for 30 s. The prodrug concentration of the perfusion medium was analyzed by HPLC after each perfusion to confirm that the prodrug had stayed intact. To study the role of passive diffusion (nonsaturable component) in the brain uptake of **1**, the brain capillaries were first washed for 30 s with cold prodrug-free perfusion medium (5 °C), followed by perfusion for 60 s with cold **1** solution (5 °C), and washed again with cold prodrug-free perfusion medium (5 °C).

Capillary Depletion Analysis. Capillary depletion analysis was carried out as previously described by Triguero et al.[31] Brain samples (right brain hemisphere) were weighed and homogenized in a glass homogenizer with 1.5 mL of physiological buffer. After homogenization, 2 mL of 26% dextran solution was added and the mixture was further homogenized. The homogenate was separated into two microcentrifuge tubes and centrifuged for 15 min (5400*g*, 4 °C). The resulting supernatant consisting of the brain parenchyma and the pellet rich in cerebral capillaries were separated and prepared for analysis with HPLC. The volume of distribution (V_d) values for the homogenate, supernatant fraction, and the capillary pellet were calculated.

Brain Sample Preparation. Because of the short half-life of **1** in the rat brain, the quantification of **1** from the rat brain samples was performed by analyzing the total concentration of formed ketoprofen after its enzymatic release from **1**. Ketoprofen was isolated from the rat brain samples by protein precipitation. A complete brain hemisphere was homogenated with 2.5 mL of water to produce 3.0 mL of homogenate. The samples were acidified with 300 μL of 2 M hydrochloric acid and vortexed for 5 min. Ethylacetate (1.0 mL) was added, and aliquots were vortexed for 2 min and centrifuged for 10 min (7500*g*) after which the supernatants were collected. This was repeated four times, and the supernatants were combined. The supernatants were evaporated to dryness under a nitrogen stream at 40 °C. Prior to analysis, the samples were reconstituted in 50% (v/v) acetonitrile in water and filtered. External standards were used for the brain samples. The calibration curve of the brain method was linear over a range of 0.2–2.0 nmol/brain hemisphere. The lower limit of quantification for spiked samples was 0.2 nmol of prodrug/brain hemisphere.

Data Analyses. The results from the brain uptake experiments are presented as means ± SD of at least three independent experiments. Data analyses for the dose−uptake curves were calculated as nonlinear regressions using GraphPad Prism 4.0 for Windows. Statistical differences between groups were tested using one-way ANOVA, followed by a two-tailed Dunnett's *t* test (Figure 2). In Figure 3b, a two-tailed independent samples *t* test was used. $P < 0.05$ was considered as statistically significant. The normality of the data was tested using a Shapiro−Wilk test. All statistical analyses were performed using SPSS 14.0 for Windows.

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Supporting Information Available: General synthetic procedures and in situ inhibition results for compounds **2–5** and the combustion analysis data for compounds **1–5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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