International Journal of Pharmaceutics 399 (2010) 121–128

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Brain uptake of ketoprofen–lysine prodrug in rats

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ARTICLE INFO

Article history:
Received 13 July 2010
Received in revised form 4 August 2010
Accepted 13 August 2010
Available online 19 August 2010

Keywords:
Brain uptake
Prodrug
LAT1
Drug delivery

ABSTRACT

The blood–brain barrier (BBB) controls the entry of xenobiotics into the brain. Often the development of central nervous system drugs needs to be terminated because of their poor brain uptake. We describe a way to achieve large neutral amino acid transporter (LAT1)-mediated drug transport into the rat brain. We conjugated ketoprofen to an amino acid L-lysine so that the prodrug could access LAT1. The LAT1-mediated brain uptake of the prodrug was demonstrated with in situ rat brain perfusion technique. The ability of the prodrug to deliver ketoprofen into the site of action, the brain intracellular fluid, was determined combining in vivo and in vitro experiments. A rapid brain uptake from blood and cell uptake was seen both in in situ and in vivo experiments. Therefore, our results show that a prodrug approach can achieve uptake of drugs via LAT1 into the brain intracellular fluid. The distribution of the prodrug in the brain parenchyma and the site of parent drug release in the brain were shown with in vivo and in vitro studies. In addition, our results show that although lysine or ketoprofen are not LAT1-substrates themselves, by combining these molecules, the formed prodrug has affinity for LAT1.

1. Introduction

Brain penetration is essential for drugs intended to act within the central nervous system (CNS). The entry of drug molecules into the CNS is efficiently governed by the blood–brain barrier (BBB) (Begley, 2004; Pardridge, 2003). Many pharmacologically active drugs fail early in their development phase because they lack the structural features essential for crossing the BBB and distributing into the brain parenchyma. Therefore, CNS drugs should be designed with appropriate brain penetration properties (Liu et al., 2008). Good BBB penetration can be achieved by designing highly permeable compounds and by screening out efflux transporter substrates (Liu et al., 2008). In addition to BBB permeability, the non-specific interaction with brain tissue is also an important factor for uptake optimization (Summerfield et al., 2006) since high non-specific binding to brain lipids is not desirable in a CNS drug. Furthermore, in cases where the site of action is inside the cell, the cellular uptake of drugs will be as important as the BBB permeability (de Lange and Danhof, 2002). One attractive approach to achieve good BBB penetration and low brain non-specific tissue binding, without losing the pharmacological activity, is to utilize the prodrug approach (Rautio et al., 2008). The properties of a drug molecule can be altered in a bio-reversible manner by conjugating the drug with a suitable promoiety. Increasing the lipophilicity of a poorly permeable drug molecule often leads to increase in BBB permeability (Summerfield et al., 2007, 2006). However, at same time the increased lipophilicity may increase accumulation of the drug in peripheral tissues. On the other hand, the lowering of the lipophilicity of highly tissue bound drugs often results in poor BBB permeability. Due to the complex role of the drug lipophilicity in brain penetration, the traditional prodrug approach, which deals with the hydrophilicity and lipophilicity of the drugs, is often not suitable and instead a more sophisticated prodrug approach is needed.

Several specific endogenous influx transporters have been identified on the brain capillary endothelial cells which form the BBB, e.g., the large neutral amino acid transport system (LAT1). 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. Amino acid transporters are also expressed in the neuronal and other cells in the brain parenchyma (Boado et al., 1999; Duelli et al., 2000). The exploitation of the LAT1 with a prodrug approach could be an innovative way to enhance BBB permeation properties (Gynther et al., 2008; Killian et al., 2007; Sakaeda et al., 2001) and improve drug uptake into the brain intracellular compartment (Su et al., 1995; Wang and Welty, 1996) without increasing non-specific tissue binding. In addition, if one could achieve selective release of the active drug in the target tissue, this would result in a reduced plasma concentration of the drug and fewer peripheral effects. In our previous study, we were able to demonstrate with in situ rat brain perfusion technique that an amino acid

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doi:10.1016/j.ijpharm.2010.08.019

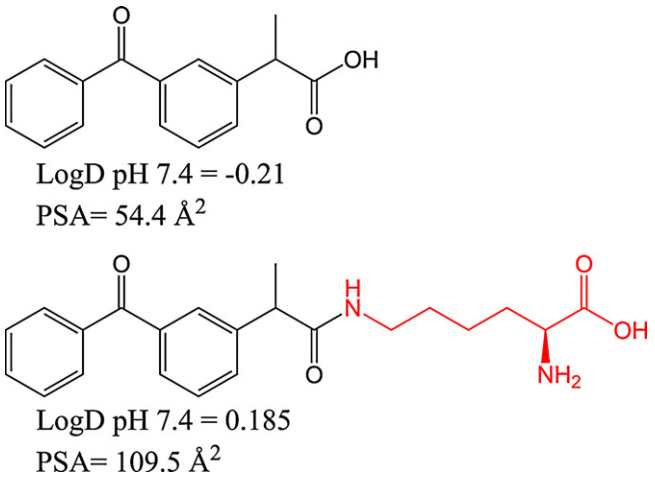

Fig. 1. Structures and selected properties of ketoprofen and ketoprofen–lysine amide (**1**). Lysine promoiety is indicated with red color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

L-tyrosine ketoprofen conjugate can cross the BBB via LAT1. However, the ester bond between the promoiety and parent drug was too labile in systemic circulation and in vivo brain uptake and the determination of brain distribution could not be achieved (Gynther et al., 2008). Therefore, our aim was to design a more stable prodrug using an amide bond instead of an ester bond. The prodrug was designed to meet requirements for LAT1-substrates based on the LAT1 binding site model (Smith, 2005; Uchino et al., 2002). Based on that model, a potential LAT1 substrate should have a positively charged amino group, a negatively charged carboxyl group and a hydrophobic side chain. L-Lysine was selected as the promoiety because the amine functional group in the side chain enables the formation of biodegradable linkage between this amino acid and the model drug of the present study, ketoprofen. L-Lysine is not a LAT1 substrate itself because of the hydrophilic amino group in the side chain. However, we hypothesized that by conjugating the amino group with ketoprofen via an amide bond, the prodrug formed would function as a LAT1 substrate since the hydrophilic amino group had been removed.

In the present study we designed and synthesized a prodrug that is ketoprofen–lysine amide (**1**) (Fig. 1), and determined its ability to deliver unbound ketoprofen into the brain intracellular compartment whose site of action is in the brain ICF, where the target protein cyclooxygenase-2 (COX-2) is expressed (Spencer et al., 1998; Teismann et al., 2003). We found that the prodrug was indeed able to cross the BBB via LAT1-mediated uptake. In addition, the prodrug was rapidly taken up, most likely by active transport, into the brain cells where a significant amount of the parent drug was released. These results indicate that this prodrug approach represents a potential way to increase the brain uptake of small molecular weight CNS drugs.

2. Materials and methods

2.1. Synthetic methods and materials

All the reactions were performed with reagents of commercial high purity quality without further purification unless otherwise mentioned. Reactions were monitored by thin-layer chromatography using aluminium sheets coated with silica gel 60 F_{245} (0.24 mm) with suitable visualization. Purifications by flash chromatography were performed on silica gel 60 F_{245} (50 mm). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance AV500 spectrometer (Bruker Biospin, Fällanden, Switzerland) operating at 500.13 and 125.75 MHz, respectively, using TMS as an internal standard. Furthermore, the products were characterized by elemental analysis (C, H, N) with a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer (CE Instruments, Milan, Italy) as well by mass spectroscopy with a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionization source. The structure of prodrug **1** was confirmed by ^{1}H and ^{13}C NMR spectroscopy, which showed no organic impurities other than water and NMR solvent. The purity of **1** was verified by elemental analysis and the result was that the compound contains 2.75 water molecules per one prodrug molecule. The elemental analysis result matches to the commonly used criteria of purity required, which allows 0.4% variation in the analysis result.

2.2. Synthesis procedures and characterization data

2.2.1. N(α)-boc-lysine methyl ester

N(α)-boc-N(ε)-CBz-lysine methyl ester (5.0 g, 12.7 mmol) was dissolved in dry EtOAc (50 mL) and Pd/C 10% (70 mg) was added to the solution. The mixture was stirred under H_2 (3 bar) at ambient temperature for 3 h and filtered on celite. The filtrate was evaporated to yield 3.23 g (98%) of the product, which was used without further purification. ^{1}H NMR (CD_3Cl) δ 1.38 (m, 2H), 1.44 (s, 9H), 1.59 (m, 2H), 1.80 (m, 2H), 3.21 (q, 2H), 3.74 (s, 3H), 4.30 (m, 1H), 5.04 (br, 2H).

2.2.2. N(α)-boc-N(ε)-ketoprofen–lysine methyl ester

Ketoprofen (5.13 g, 21.2 mmol), DMAP (0.24 g, 1.9 mmol) and N(α)-boc-lysine methyl ester (5.0 g, 19.2 mmol) were dissolved in dry dichloromethane (30 mL) and cooled in an ice–water bath before adding DCC (4.65 g, 22.1 mmol). The mixture was stirred for 48 h at the ambient temperature and filtered. The filtrate was evaporated and purified on normal phase silica using EtOAc:petroleum ether (1:1) as an eluent, yielding white solid, 2.74 g (29%). ^{1}H NMR (CD_3Cl) δ 1.30 (m, 2H), 1.43 (s, 9H), 1.46 (m, 2H), 1.54 (d, 3H), 1.61 (m, 1H), 1.76 (m, 1H), 3.21 (m, 2H), 3.61 (m, 1H), 3.71 (s, 3H), 4.24 (m, 1H), 7.48 (m, 3H), 7.59 (m, 2H), 7.66 (m, 1H), 7.74 (m, 1H), 7.80 (m, 2H).

2.2.3. Ketoprofen–lysine hydrochloride (*1*)

N(α)-boc-N(ε)-ketoprofen–lysine methyl ester (2.74 g, 5.53 mmol) was dissolved in methanol–water (20 mL + 10 mL) and $LiOH{\cdot}H_2O$ (290 mg, 6.9 mmol) was added to the solution. The mixture was stirred for 16 h and solvents were evaporated. The white solid product was dried in high vacuum for overnight. The solid was dissolved in dry ACN (150 mL) and treated with hydrochloric acid gas for 10 min and with N_2 for 5 min. The purification on normal phase silica using DCM:MeOH (80:20) as an eluent yielded white solid, 1.27 g (55%). ^{1}H NMR (CD_3OD) δ 1.41 (br, 1H), 1.47 (d, 3H, 7.1 Hz), 1.54 (m, 2H), 1.86 (m, 1H), 1.93 (m, 1H), 3.19 (m, 2H), 3.35 (s, 3H), 3.77 (m, 1H, 7.1 Hz), 3.94 (m, 1H), 7.5 (m, 3H), 7.64 (m, 3H), 7.77 (m, 3H). ^{13}C NMR (CD_3OD) δ 18.8, 23.3, 29.8, 29.8, 31.1, 39.9, 47.2, 49.9, 53.8, 129.6, 129.7, 129.7, 129.9, 129.9, 131.0, 132.9, 133.9, 138.7, 139.0, 143.7, 171.7, 176.6, 198.5. ESI–MS: 383.28 [M+1].

Anal. Calcd. for $C_{22}H_{27}N_2O_4Cl{\cdot}(2.75H_2O)$ C, 56.40; H, 6.99; N, 5.98, Found: C, 56.06; H, 6.62; N, 6.08.

2.3. Analytical methods

The quantitative determination of **1** and ketoprofen in plasma and brain samples was performed using liquid chromatography–electrospray tandem mass spectrometry (LC–MS) (Agilent 1200 Series Rapid Resolution LC System, Agilent Technologies,

Waldbronn, Germany) coupled with an electrospray ionization (ESI) triple quadrupole mass spectrometer (Agilent 6410 Triple Quadrupole LC/MS, Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation was performed on a reversed phase column by gradient method with acetonitrile and water, both containing 1% formic acid at a flow-rate of 0.3 mL/min. Positive electrospray ionization and selected reaction monitoring was used for the detection of **1** (m/z 383 → 84) and ketoprofen (m/z 255 → 209).

1 and ketoprofen concentrations in in vitro samples were analyzed by Agilent 1100 HPLC system (Agilent Technologies Inc., Waldbronn, Karlsruhe, Germany) that consisted of a binary pump, a vacuum degasser, an automated injector system autosampler Hewlett Packard 1050, an UV-detector Hewlett Packard 1050 variable wavelength detector. The chromatographic separation was performed on a reversed phase column by isocratic method with 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. The detector wavelength was set at 256 nm

2.4. Brain and plasma sample preparation

Ketoprofen was isolated from the rat brain homogenate samples by liquid–liquid extraction. 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. Ethyl acetate (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 LC–MS analysis, the samples were reconstituted in 50% (v/v) acetonitrile in water, and filtered.

Brain homogenate was prepared as described above and **1** in the brain homogenate was quantitated. The samples were acidified with 200 μl of 5 M hydrochloric acid and vortexed for 5 min. Water (2.0 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 three times and the supernatants were combined. The supernatants applied to preconditioned and equilibrated C18 solid phase extraction cartridges (Speedisk SPE C18 50 mg/6 mL, Mallinckrodt Baker Inc., Phillipsburg, NJ, USA), which had first been washed with 5.0 mL of 5% (v/v) methanol in water solution. The analytes were eluted with 9.0 mL of methanol, and evaporated to dryness under a nitrogen stream at 40 °C. Prior to LC–MS analysis, samples were reconstituted in 400 μL of 30% (v/v) acetonitrile in water, and filtrated.

Ketoprofen and **1** were isolated from the plasma samples by protein precipitation. Plasma (100 μL) was acidified with 50 μL of 1 M hydrochloric acid and vortexed for 5 min. Acetonitrile (1.0 mL) was added and the samples were vortexed for 2 min, after which time the samples were centrifuged for 5 min (5500 × g). The supernatants were collected, and the samples were evaporated to dryness under a nitrogen stream at 40 °C. Prior to LC–MS analysis, samples were reconstituted in 400 μL of 30% (v/v) acetonitrile in water, and filtrated.

2.5. Equations

The unbound drug volume of distribution in brain ($V_{u,brain}$) describes the relationship between the total drug concentration in the brain and the unbound drug concentration in brain ECF (Hammarlund-Udenaes et al., 2008). $V_{u,brain}$ is measured in mL/g $_{brain}$:

$$V_{u,brain} = \frac{AUC_{brain}}{AUC_{u,brain\,ECF}} \quad (1)$$

where AUC_{brain} (nmol/g_{brain} min) comprises the amount of unbound drug in the ECF and the amount of drug associated with the cells:

$$AUC_{brain} = V_{brain\ ECF} \times AUC_{u,brain\,ECF} + V_{cell} \times AUC_{cell} \quad (2)$$

$V_{brain\ ECF}$ and V_{cell} are the physiological fractional volumes of the brain ECF and brain cells, respectively (mL/g_{brain}), and AUC_{cell} is the amount of drug associated with the cells (nmol/mL_{cell} min). The distribution volume of unbound drug in the cell is described by $V_{u,cell}$ (mL_{ICF}/mL_{cell}) and the intracellular concentration of unbound drug is described by $AUC_{u,cell}$ (nmol/mL_{ICF} min):

$$AUC_{cell} = V_{u,cell} + AUC_{u,cell} \quad (3)$$

$V_{u,cell}$, describes the affinity of the drug for physical binding inside the cells (Friden et al., 2007) and it was estimated using the brain homogenate binding experiment and taking V_{cell} into account in the dilution factor:

$$V_{u,cell} = 1 + \frac{d}{V_{cell}}\left(\frac{1}{f_{u,h,D}} - 1\right) \quad (4)$$

A previously described approach to account for the effect of tissue dilution on unbound fraction was used to calculate the brain unbound fraction (Kalvass and Maurer, 2002).

$$f_{u,brain} = \frac{f_{u,homogenate}}{D - (D-1)f_{u,homogenate}} \quad (5)$$

where D represents the fold dilution of brain tissue, and $f_{u,homogenate}$ is the ratio of concentrations determined from the buffer and brain homogenate samples.

2.6. Animals

Adult male Wistar rats (250 g) were supplied by the National Laboratory 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.).

2.7. In situ rat brain perfusion technique

In situ rat brain perfusion technique was used to evaluate 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 publication (Gynther et al., 2008). [^{14}C]Sucrose (0.2 μCi/mL) was used as a vascular marker to determine V_v and to demonstrate the integrity of the BBB during brain perfusion. The in situ rat perfusion technique is described in more detail in an article by Gynther et al. (2008).

2.8. In vivo bolus injections

1 and ketoprofen were dissolved in 0.9% NaCl solution pH 7.4 resulting in 30 mM concentration. A 0.2 mL bolus injection of either drug was administered into cannulated jugular vein of the rats, and plasma and brain concentration were taken at following time: 10, 30, 60, 120 and 300 min. For V_d and CL determinations the plasma samples were taken in following time-points: 2, 5, 10, 15, 30, 45, 60, 90 and 120 min. The plasma samples were drawn from the cannulated jugular vein at different time-points and the brain were removed after the rats were sacrificed. Ketoprofen and **1** levels in brain parenchyma were calculated by correcting the measured brain sample concentration for the cerebral vascular space component, which was determined to be 0.0116 mL/g.

2.9. In vivo microdialysis

A microdialysis guide cannula (MAB 6.10.IC, AgnTho's AB, Lidingö, Sweden) was implanted into the striatum (coordinates from bregma: AP +0.5 mm; L −3.0 mm; DV −3.8 mm) under chloral hydrate anesthesia (350 mg/kg i.p.). After a 1 week recovery period, the rats were again anesthetized and a concentric microdialysis probe (MAB 9.10.4; 4 mm exposed membrane, 6 kDa cut-off, AgnTho's AB) was inserted into the striatum through the guide cannula. An intravenous microdialysis probe (MAB 11.20.10; 10 mm exposed membrane, 6 kDa cut-off, AgnTho's AB) was inserted into the left femoral vein, and both probes were perfused with Krebs Ringer solution (consisting of 138 mM NaCl, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2{\cdot}6H_2O$, 11 mM $NaHCO_3$, 1 mM $NaH_2PO4{\cdot}H_2O$, and 11 mM D-glucose, pH 7.4) at a flow-rate of 2 μL/min. For drug administration, the right femoral vein was cannulated with polyethylene tubing filled with saline and 20 IU/mL dalteparin. Microdialysis probes were perfused for 80 min before intravenous drug administration, and the dialysate was collected as 10 or 20-min fractions for 5 h into polypropylene vials (AgnTho's AB). All dialysate drug concentrations were corrected with recovery values. The samples were frozen at −20 °C and stored at −80 °C until analyzed.

2.10. In vitro recovery of microdialysis probes

To estimate the true free drug concentration in brain ECF and the blood probe calibration was performed in vitro for both probe types. The recovery was determined as the ratio of a drug in the dialysate to the drug concentration (1 μM) in a non-stirred bulk Krebs Ringer solution at 37 °C (C_{dial}/C_{bulk}; $n = 3$ probes for each drug, three determinations per probe; flow-rate 2 μL/min, collection time 20 min).

2.11. In vitro brain tissue and plasma protein binding

Drug-naive animals were sacrificed, the brain was removed, and three volumes of a phosphate buffered saline (pH 7.4) were added. The brains were homogenized on ice with an ultrasonic probe after which the drug was added. Blood was recovered from anesthetized animal with heart puncture and plasma was separated by centrifugation. Equilibrium dialysis of 400 μL of homogenate or plasma and 600 μL of buffer was performed in triplicate for 4 h at 37 °C in Single-Use RED Plate with an 8-kDa cut-off dialysis membrane (Thermo Scientific, Rockford, IL). The buffer to homogenate or plasma concentration ratio was calculated, which was used to calculate the in vitro brain tissue and plasma protein binding (Friden et al., 2007; Kalvass and Maurer, 2002).

2.12. Apparent partition coefficients

The apparent partition coefficients (log D) of ketoprofen and **1** were determined at room temperature by a 1-octanol–phosphate buffer system at pH 7.4. Before use, the 1-octanol was saturated with phosphate buffer for 24 h by stirring vigorously. A known concentration of compound in phosphate buffer was shaken for 30 min (pH 7.4) with a suitable volume of 1-octanol. After shaking, the phases were separated by centrifugation at 14,000 rpm for 4 min. The concentrations of the compounds in the buffer phase before and after partitioning were determined by HPLC.

2.13. Polar surface area

Polar surface areas of **1** and ketoprofen were calculated using the method by Ertl et al. (2000), implemented in molecular operating environment.

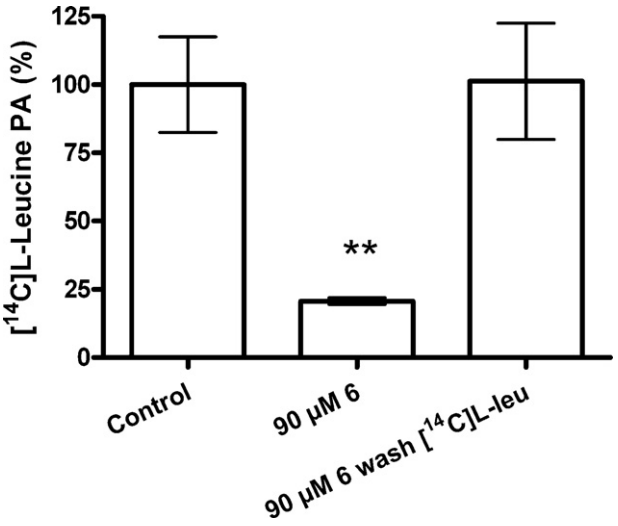

Fig. 2. Mechanism of **1** rat brain uptake. The PA product of 0.2 μCi/mL [^{14}C]L-leucine in the absence or presence of **1**. The control PA product 0.02059 ± 0.00323 mL/s/g is decreased to 0.00426 ± 0.00019 mL/s/g in the presence of 90 mM **1**. The PA product of 0.2 μCi/mL [^{14}C]L-leucine recovered after washing the prodrug from the brain capillaries, demonstrating the PA product of 0.02084 ± 0.0031 mL/s/g. The data is presented as mean ± SD ($n = 3$). Asterisks denote a statistically significant difference from the respective control (**$P < 0.01$, one-way ANOVA, followed by Dunnett t-test).

2.14. Data analyses

Statistical differences between groups were tested using one-way ANOVA, followed by two-tailed Dunnett t-test (Fig. 2). Data analyses for the dose–uptake curve (Fig. 3) were calculated as non-linear regressions using GraphPad Prism 4.0 for Windows. The normality of the data was tested using Shapiro-Wilk test. The area under the concentration curves was calculated as 10–300 min, because there were too few data points at the elimination phase to be extrapolated as 0–∞. The area under the concentration curves was calculated with GraphPad Prism 4.0 for Windows. All statistical analyses were performed using SPSS 14.0 for Windows.

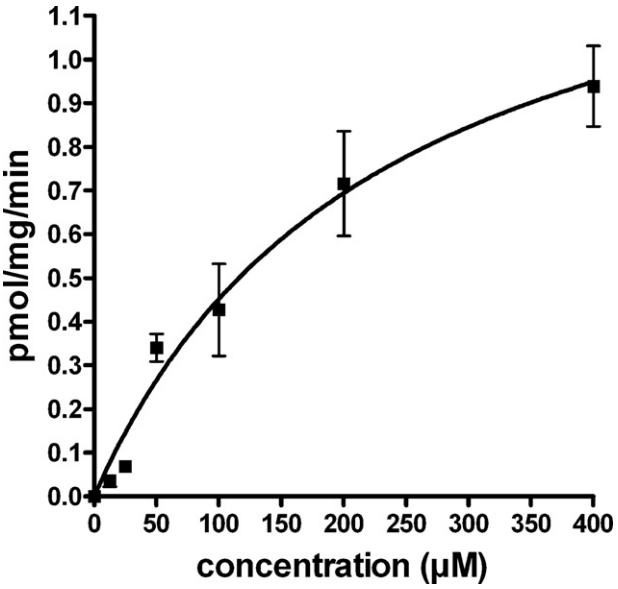

Fig. 3. Kinetics of **1** rat brain uptake. Relationship between the concentration of **1** in the perfusion medium and the brain uptake of **1**. K_m and V_{max} are 231.6 ± 60.4 μM and 1.50 ± 0.20 pmol/mg/min, respectively. The possible fraction passive diffusion of the brain uptake was not determined. The data is presented as mean ± SD ($n = 3$).

Table 1
Brain uptake kinetics and brain distribution of ketoprofen, **1**, and ketoprofen released from **1**.

	Ketoprofen	Ketoprofen from **1**	**1**	Ketoprofen from **1** and **1** compared to ketoprofen[a]
$V_{u,brain}$	1.9 mL/g	–	5.0 mL/g	–/2.6
$f_{u,brain\ in\ vitro}$	0.244	–	0.193	–/0.79
$f_{u,\ plasma\ in\ vitro}$	0.042	–	0.281	–/7
AUC_{brain}[b]	134.1 nmol/g × min	113.7 nmol/g × min	203.3 nmol/g × min	0.8/1.5
$AUC_{brain\ ECF}$[b]	70.8 nmol/mL × min	0.5 nmol/mL × min	40.5 nmol/mL × min	0.007/0.6
AUC_{plasma}[b]	14,507 nmol/mL × min	2128 nmol/mL × min	3162 nmol/mL × min	0.15/0.22
$AUC_{u,plasma}$[b]	605.0 nmol/mL × min	1.5 nmol/mL × min	455.7 nmol/mL × min	0.002/0.8
AUC_{cell}[b]	199.9 nmol/mL_{cell} × min	189.3 nmol/mL_{cell} × min	325.3 nmol/mL_{cell} × min	0.95/1.63
$AUC_{u,cell}$[b]	33.5 nmol/mL_{ICF} × min	31.8 nmol/mL_{ICF} × min	48.7 nmol/mL_{ICF} × min	0.95/1.45
$AUC_{u,brainECF}/AUC_{u,plasma}$[b]	0.12	0.33	0.09	2.75/0.75
$AUC_{u,cell}/AUC_{u,brainECF}$[b]	0.47	63.6	1.20	135/2.6
$AUC_{u,cell}/AUC_{u,plasma}$[b]	0.06	21.8	0.11	363/1.8

[a] Ratio of values of ketoprofen released from **1** and values of **1** compared to values of ketoprofen itself.
[b] AUC-values are calculated as 10–300 min.

3. Results

3.1. Prodrug 1 is able to cross the rat BBB via LAT1-mediated uptake

The ability of **1** to bind to LAT1 was studied with the in situ rat brain perfusion technique (Gynther et al., 2008). The permeability–surface area (PA) product, which estimates the rate of transport of a solute across the BBB, was determined for radiotracer [^{14}C]L-leucine. The PA product of the radiotracer was significantly decreased in a LAT1 inhibition assay, resulting in 79.3% inhibition of its uptake. This is a clear evidence of significant binding of **1** to the LAT1 (Fig. 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 90 μM for 30 s, followed by washing of the prodrug from the brain capillaries with 30 s perfusion of prodrug-free perfusion medium and finally perfusing the rat brain with [^{14}C]L-leucine for 30 s. The PA product of [^{14}C]L-leucine recovered after the wash, indicating that the binding of **1** to the LAT1 has been reversible (Fig. 2). After demonstrating affinity for LAT1, the in situ rat brain uptake of **1** was determined to be saturable and concentration dependent in the dose range from 12.5 to 400 μM (Fig. 3), indicative of transporter-mediated uptake. The uptake exhibited a typical saturation curve of the Michaelis–Menten type and the data was used to determine the K_m and V_{max} values for **1**. The possible fraction of passive permeation was not determined, because there is no specific LAT1 inhibitor available which could inhibit the activity of LAT1 entirely. The brain uptake of 25 μM **1** was 0.068 ± 0.000 pmol/mg/min which significantly decreased to 0.011 ± 0.02 pmol/mg/min after addition of 2 mM L-phenylalanine, a LAT1 substrate (Kageyama et al., 2000) to the perfusion medium, evidence that the uptake was LAT1-mediated. In addition, the calculated polar surface area (PSA) of **1** is 109.5 Å^2, which is higher than the proposed maximum PSA of molecules that can penetrate cell membrane via passive diffusion (Pajouhesh and Lenz, 2005).

3.2. Prodrug 1 is able to cross rat BBB in vivo and is rapidly distributed from the brain extracellular compartment

Encouraged by the in situ results, we determined the ability of the prodrug to cross the BBB in vivo and compared its uptake to that of ketoprofen. **1** [0.2 mL of 30 mM (0.6 μmol)] or ketoprofen solution was administered into the left jugular vein in rats. Samples were collected with microdialysis probes from femoral vein and striatum for 300 min: first at 10 min intervals for 60 min and then at 20 min intervals. We were able to detect **1** from the brain extracellular fluid (ECF), evidence that **1** is able to cross the BBB in vivo. The area under the concentration curve for unbound drug (AUC_u 10–300 min) in brain ECF and plasma was determined for ketoprofen, **1**, and ketoprofen released from **1**. The results are shown in Table 1. The apparent maximum concentration of **1** in brain ECF was achieved rapidly, this was followed by a rapid elimination. In contrast, ketoprofen achieved the apparent maximum concentration in the brain slower (Fig. 4a and b). The high free concentration of **1** compared to total plasma concentration suggests that **1** is not highly bound to plasma proteins. This was confirmed by measuring the in vitro free fraction ($f_{u,plasma}$) of ketoprofen and **1** in rat plasma (Table 1). However, the $f_{u,plasma}$ of ketoprofen after ketoprofen administration is approximately 60-fold higher compared to the $f_{u,plasma}$ of ketoprofen released from **1**. The $f_{u,plasma}$ in vitro data is consistent with the ketoprofen in vivo data. Therefore, it is possible that the lower $f_{u,plasma}$ of ketoprofen released from **1** is an artifact caused by analytical difficulties due to low ketoprofen concentrations in rat plasma. It is now clear that **1** is able to penetrate the brain. However, it was still unclear why **1** is so rapidly eliminated from the ECF. After i.v. injection of **1** only a small concentration of released ketoprofen was detected in plasma and the levels were barely detectable in ECF (Table 1), indicating that the rapid elimination of **1** from the ECF is not due to metabolism of **1**.

3.3. Prodrug 1 is actively transported into the brain cells

The microdialysis results indicated that there was either active efflux of **1** from brain to blood, or active influx from ECF to brain cells. In addition, high non-specific binding to the brain tissue may also have accounted for the rapid decrease in the ECF concentrations. In an attempt to elucidate which of these mechanisms was involved, the concentrations of **1** and ketoprofen were determined from whole brain tissue and blood after an i.v. bolus injection of **1** or ketoprofen (Fig. 4c and d). After the injection, the rats were sacrificed at specific time-points ranging from 10 to 300 min. Blood and brain samples were analyzed for **1** and ketoprofen concentrations (Table 1). The results support the proposal that **1** is transported to the brain. In addition, the results indicate that ketoprofen is released from **1** within the brain tissue. When whole tissue results are combined with the result acquired from microdialysis studies, the distributions of **1** and ketoprofen inside the BBB, the value of $V_{u,brain}$, can be determined (Table 1). The higher $V_{u,brain}$ of **1** compared to that of ketoprofen indicates that **1** is rapidly removed from the ECF into the cells, and not effluxed into the blood circulation. However, the high $V_{u,brain}$ may also suggest that **1** is highly bound non-specifically within the brain tissue, which is not desired. Therefore, the free fractions in brain tissue of **1** and ketoprofen were determined in vitro (Table 1). **1** and ketoprofen were found to have quite similar free fractions in brain tissue in vitro, indicating that the larger $V_{u,brain}$ of **1** is not due to non-specific binding to brain tis-

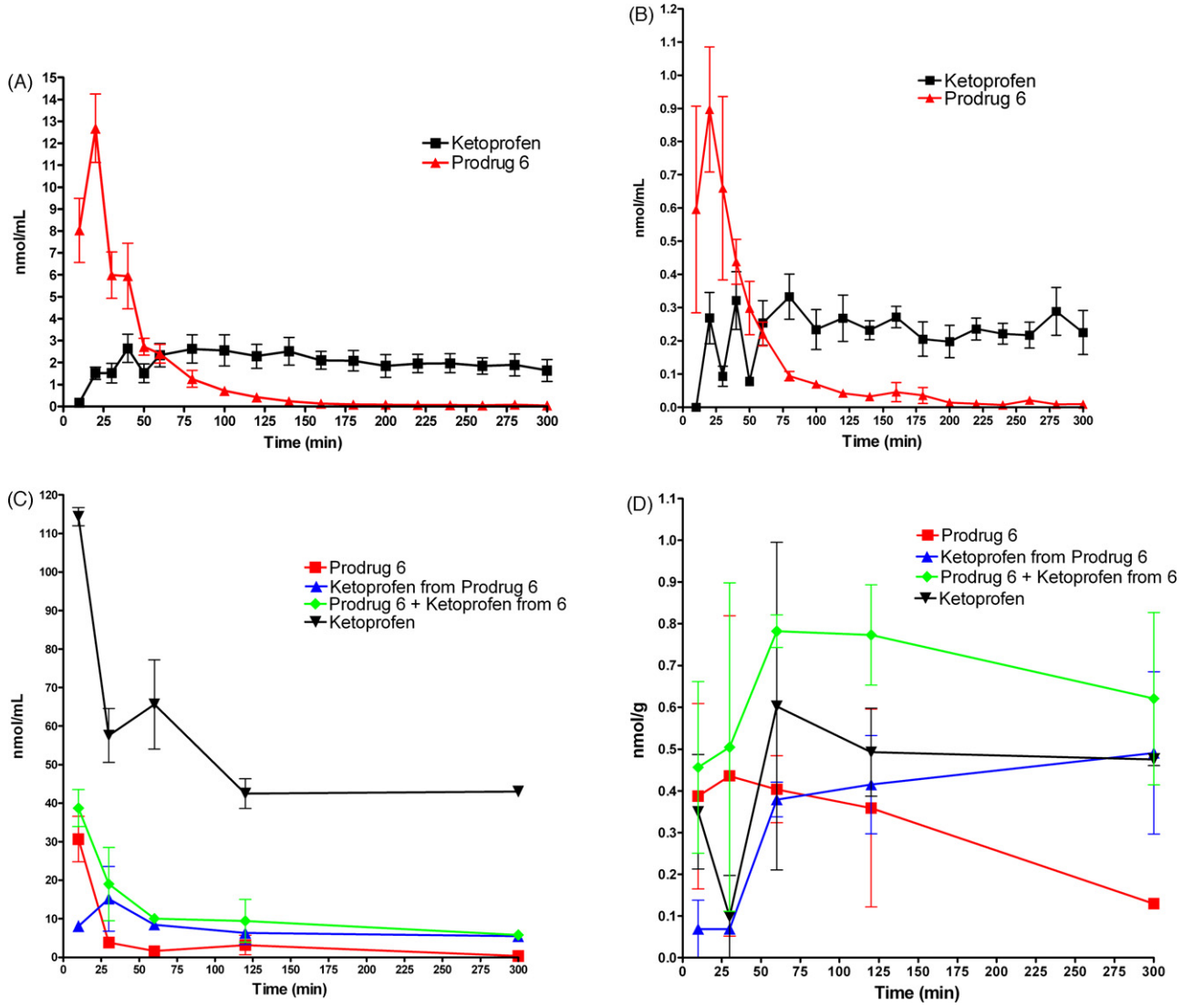

Fig. 4. Concentration–time profiles of **1** and ketoprofen obtained from plasma and brain after i.v. bolus injection. (a) Free concentration of **1** and ketoprofen in rat blood after 6 μmol i.v. bolus injection of **1** or ketoprofen. (b) Free concentration of **1** and ketoprofen in rat brain ECF after 6 μmol i.v. bolus injection of **1** or ketoprofen. (c) The plasma concentration of **1**, ketoprofen released from **1** and ketoprofen after administration of **1** or ketoprofen. (d) The brain concentration of **1**, ketoprofen released from **1** and ketoprofen after administration of **1** or ketoprofen. The concentrations are presented as mean ± SE (n = 3–7).

sue. In addition, the concentration ratio of unbound drug between ECF and brain intracellular fluid (ICF) was calculated (Table 1).

3.4. *Prodrug **1** releases a significant amount of the parent drug at the site of action*

The results indicating, that **1** is able to cross the BBB via LAT1 and is subsequently actively transported into brain cells in vivo, encouraged us to compare the abilities of **1** and ketoprofen to deliver unbound ketoprofen into the brain ICF. The area under the concentration curve of unbound drug in the ICF ($AUC_{u,cell}$) was determined for ketoprofen, **1** and ketoprofen released from **1** (Table 1). The $AUC_{u,cell}$ values of ketoprofen and ketoprofen released from **1** were almost identical. However, at 300 min there was still **1** present in the ICF, which could release more ketoprofen at the later time-points. In addition, the distribution of ketoprofen originating from **1** and ketoprofen between ICF and ECF was calculated (Table 1). Since ketoprofen is released from **1** intracellularly in the brain, the ICF-to-ECF ratio was significantly (135 times) higher for **1** as compared to ketoprofen administration. Furthermore, it has been reported that the relationship between brain unbound ECF concentration and unbound plasma concentration is the most useful parameter in the evaluation of the extent of the brain drug delivery (Hammarlund-Udenaes et al., 2008; Liu et al., 2008). However, when there is active transport present from ECF to ICF, it cannot be assumed that the ECF and ICF concentrations are equal. In addition, the target protein of ketoprofen resides within the cell (Spencer et al., 1998; Teismann et al., 2003). Therefore, the more appropriate parameter, in this case, is the unbound concentration ratio between ICF and plasma. The results show that, when **1** is administered the released ketoprofen ICF-to-unbound plasma concentration ratio is 363 times larger than that obtained after ketoprofen administration. This is due to low ketoprofen concentration released from **1** in plasma. However, as the $AUC_{u,cell}$ of **1** is not significantly higher compared to that of ketoprofen, the 363-fold difference in unbound ICF-to-plasma ratio has to be due to lower ketoprofen concentrations in plasma. The lower plasma concentration of ketoprofen after **1** administration is due to higher volume of distribution (V_d) and faster elimination of **1** from central compartment compared to ketoprofen. The V_d and clearance (CL) were calculated for both **1** and ketoprofen after i.v. bolus injection. The V_d and CL were 0.046 L and 0.017 L/min for **1** and, 0.028 L and 0.001 L/min for ketoprofen, respectively.

4. Discussion

The brain-to-blood ratio has been a backbone of CNS drug delivery optimization although it only predicts inert partitioning of drugs into lipid material (Jeffrey and Summerfield, 2007; van de Waterbeemd et al., 2001). Optimization of the brain-to-plasma ratio has lead to optimization of CNS drug uptake by lipophilization. However, after lipophilization, the concentration of unbound drug in the CNS is usually not elevated. The unbound fraction of the drug is the pharmacologically active fraction, and it has been postulated that the ratio of unbound drug in brain and plasma describes the extent of drug uptake more accurately (Hammarlund-Udenaes et al., 2008; Liu et al., 2008). In addition, recent studies have revealed that the transporter-mediated uptake of drugs might in fact be more widespread than previously assumed (Dobson and Kell, 2008). This can open new possibilities for improved brain uptake as our knowledge of these transporters increases. It may be possible to exploit transporter-mediated uptake to obtain drugs with good BBB permeation properties which are still able to avoid extensive non-specific brain tissue binding. In addition, with active uptake across the BBB and further into the brain cells, the drug concentrations in the brain may exceed those in the systemic blood circulation. Furthermore, by utilizing a prodrug approach, the unbound concentration ratio of the active drug between brain ICF and peripheral tissues can be further elevated.

The designed prodrug **1** was stable in aqueous medium and in brain and liver homogenates (data not shown). However, **1** was susceptible to enzymatic bioconversion in vivo and ketoprofen was detected from rat plasma and brain tissue after i.v. bolus injection. The PSA of **1** (Fig. 1) suggests that the prodrug is not able to cross the BBB by passive diffusion. In addition, the in situ LAT1 inhibition studies (Fig. 2) suggest that the prodrug has affinity for LAT1. Whereas, the in situ brain uptake results (Fig. 3) show that the uptake of **1** concentration dependent, saturable and the uptake is inhibited by LAT1 substrate. When the results from Figs. 2 and 3 are combined, there is significant evidence that **1** is able to cross the BBB via LAT1. There is the possibility that **1** is also a LAT2 substrate. However, as LAT1 is the transporter that is the functionally predominant isoform expressed at the BBB and LAT2 is mainly found in the small intestine and kidney, we concluded that the brain uptake of **1** is LAT1-mediated (Killian and Chikhale, 2001; Morimoto et al., 2008). Although human and rat both express LAT1 at the BBB, there are differences in the amino acid sequence of the transporters (Prasad et al., 1999). Both species share the same LAT1-substrates which is not unreasonable considering the availability of these amino acids in the diet. However, the ability of both human and rat LAT1 to recognize the same natural amino acids does not mean that both LAT1 variations are able to recognize synthesized amino acids such as **1**. Therefore, the LAT1-mediated brain uptake of **1** in rats does not necessary mean that **1** is able to utilize human LAT1. In addition, the uptake studies were performed with healthy animals and some CNS-disorders may have an effect on the expression and function of BBB transporters, such as LAT1. Therefore, the brain uptake of **1** could be different in an animal that has a CNS-disorder.

The microdialysis results show that **1** is able to cross the brain endothelial cells into the brain ECF in the prodrug form (Fig. 4). In addition, the prodrug is rapidly removed from the ECF and only small concentrations of ketoprofen can be detected in the brain ECF after the injection of **1**. However, both **1** and ketoprofen were detected when whole brain tissue concentrations were analyzed. By combining the in vivo microdialysis and whole tissue data with in vitro brain homogenate binding data, we were able to calculate the unbound concentrations of **1** and ketoprofen in the brain ICF, where the target protein COX-2 is expressed (Spencer et al., 1998; Teismann et al., 2003). In addition, the results indicate that the uptake from the brain ECF into the brain ICF is due to an active transporter. In fact, **1** acts rather similarly to another LAT1 substrate gabapentin, a drug which has low lipophilicity and is extensively distributed into brain cells (Friden et al., 2007). In addition, several authors have reported that gabapentin is actively transported into neuronal cells (Su et al., 1995; Wang and Welty, 1996). The uptake of **1** into the brain cells may be mediated by the same transporter as gabapentin. Unfortunately the unbound concentration ketoprofen in the ICF was not significantly higher after **1** injection compared to ketoprofen injection. However, the mechanism of **1** brain uptake and distribution in the brain tissue indicates that this prodrug strategy could be used for poorly permeable CNS drugs that have their site of action in the brain ICF without increasing their lipohilicity and non-specific tissue binding.

5. Conclusion

In the present study we were able to demonstrate that ketoprofen–lysine amide is able to cross the BBB LAT1-mediatedly although neither lysine nor ketoprofen are LAT1-substrates themselves. The prodrug was taken up into the brain intracellular compartment, where significant amount of ketoprofen was released. The high free fraction of **1** in plasma and the rapid cellular uptake into the brain cells creates a steep concentration between plasma and brain ECF, which enables LAT1-mediated uptake of **1** across the BBB. Although, the present study evaluated only the brain uptake of one ketoprofen prodrug and no significant enhancement in the brain uptake of ketoprofen was achieved, the strategy may offer a potential way to achieve brain uptake of other small molecular weight CNS drugs. Especially drugs whose site of action is in the brain ICF and have extremely low brain uptake. In conclusion, conjugating ketoprofen with L-lysine, LAT1-mediated brain uptake and the delivery ketoprofen into the brain ICF can be achieved.

Acknowledgments

This study was supported by the National Technology Agency of Finland (TEKES), Graduate School in Pharmaceutical Research, the Finnish Parkinson Foundation, Finnish Cultural Foundation, North Savo Regional fund, Kuopio University Foundation, and the Academy of Finland (projects 108569, 214334 and 114721). We also thank Helly Rissanen and Katja Hötti for their technical assistance.

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