**RESEARCH PAPER** 

# Design, Synthesis and Brain Uptake of LATI-Targeted Amino Acid Prodrugs of Dopamine

Lauri Peura • Kalle Malmioja • Kristiina Huttunen • Jukka Leppänen • Miia Hämäläinen • Markus M. Forsberg • Jarkko Rautio • Krista Laine

Received: 23 August 2012 / Accepted: 14 December 2012 © Springer Science+Business Media New York 2013

#### ABSTRACT

**Purpose** Drug delivery to the brain is impeded by the blood-brain barrier (BBB). Here, we attempted to enhance the brain uptake of cationic dopamine by utilizing the large amino acid transporter I (LATI) at the BBB by prodrug approach.

**Methods** Three amino acid prodrugs of dopamine were synthesized and their prodrug properties were examined *in vitro*. Their LATI-binding and BBB-permeation were studied using the *in situ* rat brain perfusion technique. The brain uptake after intravenous administration and the dopamine-releasing ability in the rat striatum after intraperitoneal administration were also determined for the most promising prodrug.

**Results** All prodrugs underwent adequate cleavage in rat tissue homogenates. The prodrug with phenylalanine derivative as the promoiety had both higher affinity for LATI and better brain uptake properties than those with an alkyl amino acid mimicking promoiety. The phenylalanine prodrug was taken up into the brain after intravenous injection but after intraperitoneal injection the prodrug did not elevate striatal dopamine concentrations above those achieved by corresponding L-dopa treatment.

**Conclusions** These results indicate that attachment of phenylalanine to a cationic drug via an amide bond from the meta-position of its aromatic ring could be highly applicable in prodrug design for LAT I -mediated CNS-delivery of not only anionic but also cationic polar drugs.

**KEY WORDS** blood-brain barrier · brain drug delivery · dopamine · large amino acid transporter I · prodrugs

M. Hämäläinen • M. M. Forsberg • J. Rautio • K. Laine

School of Pharmacy, University of Eastern Finland, Kuopio, Finland e-mail: lauri.peura@uef.fi

## e AADC aromatic L-amino acid decarboxylase

**ABBREVIATIONS** 

AUC	area under the curve
BBB	blood-brain barrier
BBN	9-borabicyclo[3.3.1]nonane
CNS	central nervous system
COMT	catechol-O-methyl transferase
EDC	I-ethyl-3-(3-dimethylaminopropyl)carbodiimide
GluT I	glucose transporter 1
HOBt	l-hydroxybenzotriazole
LATI	large neutral amino acid transporter 1
LLOQ	lower limit of quantification
PA	brain permeability-surface area
SVTC2	ascorbic acid transporter
TPSA	topological polar surface area

## INTRODUCTION

The blood-brain barrier (BBB) acts as a major impediment to hydrophilic drugs as they try to reach the brain making the central nervous system (CNS) drug development a complicated process (1). The majority of CNS-acting drug candidates do not readily enter the brain in pharmacologically relevant amounts, and the further development of those candidates has often been discontinued (2). As a consequence, in addition to the discovery of totally novel potential neurotherapeutic agents, the need for efficient drug delivery systems to enhance their brain distribution seems worthwhile while the general population is aging and the physicochemical properties of many novel drug molecules tend to be less favorable for brain uptake (3).

One promising strategy for improving the poor brain uptake of drug molecules is to adopt the prodrug approach which utilizes specific carrier-mediated transport mechanisms expressed at the BBB (4). These transporters ferry

L. Peura (🖂) • K. Malmioja • K. Huttunen • J. Leppänen •

polar pivotal nutrients, such as amino acids, glucose and vitamins, from blood to the brain across the BBB. In the carrier-mediated prodrug brain delivery strategy, a substrate of an endogenous transporter is conjugated to an active drug molecule in a bioreversible manner. This brain targeting strategy can transfer the active drug molecule to the site of action by the transporter without modifying the structure of the active drug and, thus, there is no loss of the biological activity of the drug. Especially glucose transporters (glucose transporter 1, GluT1, SLC2A1), ascorbic acid transporters (large neutral amino acid transporter 1, LAT1, SLC7A5) have been successfully utilized as drug carriers to the brain (5–13).

Levodopa (L-dopa), the only clinically used prodrug of dopamine, is transported into the brain via LAT1transporters as a "pseudonutrient" (14). The "pseudonutrient" structure of L-dopa is metabolically transformed to dopamine in the brain while its metabolic transformation in peripheral tissues is prevented by simultaneous administration of peripheral inhibitors of L-dopa metabolizing enzymes, *i.e.* aromatic L-amino acid decarboxylase (AADC) and catechol-O-methyl transferase (COMT). The need for these inhibitors complicates L-dopa treatment. Furthermore, chronic L-dopa treatment is confronted by many problematic pharmacokinetic obstacles, e.g. extensive metabolism, plasma fluctuations, erratic oral absorption and variability in the extent of the first-pass effect (15). In advanced Parkinson's disease, an extensive loss of nigrostriatal dopaminergic neurons leads to a significant decrease in striatal AADC activity (16) that is essential to convert Ldopa to dopamine. However, L-dopa with AADC- and COMT-inhibitors still remains the cornerstone of the drug treatment of Parkinson's disease.

Dopamine is a textbook example of a drug that has been used for brain targeting by prodrug technology because there are several pharmacokinetic problems encountered in its use to treat Parkinson's disease. First, both the amino terminal and the catechol hydroxyl groups of dopamine are rapidly and extensively metabolized after oral dosing (17). Furthermore, dopamine is largely ionized in the bloodstream and, thus, it is not able to cross the BBB via passive diffusion (17). Hence, several prodrugs providing better structural features for intestinal absorption, protection from premature metabolism and improved brain distribution have been investigated. The brain targeted prodrugs consist mainly of lipophilic analogs of dopamine intended to improve the passive delivery of dopamine especially across intestinal endothelial cells and the BBB (18–23). However, quite a few transporter-utilizing prodrugs of dopamine have also been described previously (24-26), and unfortunately, the in vivo pharmacokinetic properties have not been studied with all of these prodrugs, and none of the pharmacokinetic studies has compared a prodrug with the standard L-dopa therapy, *i.e.* L-dopa combined with the peripheral inhibitors of AADC and COMT.

In this study, we investigated three new CNS-targeted amino acid prodrugs of dopamine (Fig. 1) which have been designed as substrates of LAT1. We have previously reported that the meta-substituted phenylalanine is a potential promoiety for carrying anionic drugs into the brain via LAT1 (13). The model drugs used in all previous studies have been anionic. In the present study, we have studied three amino acid promoieties, aspartic acid (1), 2-aminoapidic acid (2) and meta-substituted carboxylic acid analogue of phenylalanine (3) (Fig. 1), as LAT1-targeted promoieties of a cationic dopamine with an aim to enhance its brain uptake. The model compounds used previously in LAT1-targeting (*i.e.*, nipecotic acid (11), mercaptopurine (10), ketoprofen (5,7) and valproic acid (13)) have also been relatively lipophilic especially after conjugation with the promoiety. Here, we studied the influence of more hydrophilic prodrugs on the LAT1-binding and determined their translocation across the BBB. The prodrugs 1-3 were synthesized, their physicochemical properties were determined in in vitro assays and their ability to bind to LAT1 and to cross the BBB were studied by using the in situ rat brain perfusion technique. Finally, the brain uptake of the selected prodrug 3 after intravenous administration in rats and its efficacy to release dopamine in the rat brain after intraperitoneal administration were determined.

#### MATERIALS AND METHODS

#### Materials

All materials used in this study were purchased from commercial sources unless otherwise noted. Their origin is specified in the text. All chemicals were of the highest purity available. All radio-labeled compounds used in the present studies were uniformly labeled. Compounds **1–3** were synthesized according to the published procedures. The manufacturers of the instruments are also specified in the text.

#### Animals

Adult male Wistar rats were obtained from the Laboratory Animal Centre Kuopio, Finland (originally from Harlan, the Netherlands). At the beginning of the studies, the rats were 7–9 weeks old and weighed 200–250 g. Pelleted food and tap water were supplied *ad libitum*, and the rats were housed in artificially light- and temperature-controlled environment (12 h light/dark cycle,  $22\pm1^{\circ}$ C). All procedures with the animals were performed according to the appropriate European Community Guidelines and reviewed by

Fig. 1 Chemical structures of dopamine prodrugs 1–3.



the Animal Ethics Committee at the University of Eastern Finland, and approved by the local provincial government (license numbers ESAVI/6317/04.10.03/2011 and ESHL-2009-06954/Ym-23). The animal welfare 3R principles (replacement, refinement and reduction) were followed.

#### **General Synthetic Procedures**

Prodrugs 1-3 were synthesized from Cbz-L-aspartic acid 1methyl ester (Aldrich, St. Louis, MO, USA), DL-2aminoadipic acid (Sigma, St. Louis, MO, USA) and Boc-3-cyano-L-phenylalanine (Aldrich, St. Louis, MO, USA), respectively, as shown in Scheme 1. Other reagents were purchased from commercial suppliers and were used without purification. Reactions were monitored by thin-layer chromatography using aluminum sheets coated with silica gel 60 F245 (0.24 mm) with UV and ninhydrin visualization. Microwave reactions were conducted with Biotage Iniator (Biotage, Sweden). Purifications by flash chromatography were performed either on a Combiflash Companion Instrument with RediSep Columns (Teledyne ISCO, Lincoln, CA, USA) or by normal column chromatography with silica gel 60 (0.063–0.200 mm mesh). <sup>1</sup>H and <sup>13</sup>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 the solvent peak 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). All compounds tested in the assays were characterized with combustion analysis to be at least 95% of purity unless otherwise stated.

## Methyl 2-(((Benzyloxy)Carbonyl)Amino)-4-((3,4-bis (Benzyloxy)Phenethyl)Amino)-4-Oxobutanoate (4)

To a solution of Cbz-L-Asp-OMe (0.76 g, 2.70 mmol), HOBt (0.40 g, 2.97 mmol) and 3,4-dibenzyloxyphenethylamine

(1.00 g, 2.70 mmol) in DCM (20 ml) was added EDC (0.84 g, 4.43 mmol) and triethylamine (1.51 ml, 10.81 mmol). The solution was stirred at room temperature overnight. The crude product was purified by flash-chromatography eluting with a gradient of DCM:MeOH mixture. After combining the appropriate fractions and evaporating the solvents, the title compound (1.42 g, 2.38 mmol) was obtained as a white solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  2.59;2.83 (2H, dd,  $\mathcal{J}$ =15.76, 4.41 Hz), 2.67 (2H, t,  $\mathcal{J}$ = 6.94 Hz), 3.34–3.46 (2H, m), 3.75 (3H, s), 4.56 (1H, dt,  $\mathcal{J}$ =8.43, 4.14 Hz), 5.13 (2H, s), 5.14 (2H, s), 5.16 (2H, s), 6.67 (1H, dd,  $\mathcal{J}$ =7.93, 1.83 Hz), 6.77 (1H, s), 6.87 (1H, d,  $\mathcal{J}$ =8.24 Hz), 7.30–7.38 (11H, m), 7.45 (4H, d,  $\mathcal{J}$ =7.02 Hz).

## L-2-Amino-4-((3,4-Dihydroxyphenethyl)Amino)-4-Oxobutanoic Acid (1)

Compound 4 (1.42 g, 2.38 mmol) in DCM (10 ml) was added to a solution of MeOH:H<sub>2</sub>O (1:1) (10 ml). Lithium hydroxide (0.30 g, 7.14 mmol) was added and the mixture was stirred for 2 h at room temperature. After completion, the solvents were evaporated and the crude product was carried to the next step without further purification.

The crude product from the previous step was dissolved in a 4:1 mixture of methanol and 3 M HCl (20 ml). Palladium activated on charcoal (10% Pd basis) (0.1 g) was added to the solution and the mixture was put in a reactor and pressurized with hydrogen (4 bar) and left to stir for 2 h at room temperature. The mixture was filtered through a pad of Celite to remove the catalyst, and the title compound (0.38 g, 1.42 mmol) was obtained after removing the solvents under reduced pressure as a yellowish solid. Analytically pure sample was obtained as a white solid after purification by column chromatography eluting using gradient of DCM:EtOH:H<sub>2</sub>O mixture. <sup>1</sup>H NMR (500 MHz, MeOD)  $\delta$  2.64 (2H, t, 7= 7.32), 2.82–2.96 (2H, m), 3.35 (2H, t, 7=7.32 Hz), 4.24 (1H, br. s.), 6.53 (1H, dd, 7=7.93, 1.53 Hz), 6.66 (1H, d, 7=1.83 Hz ), 6.67–6.70 (1H, m). 13C (125 MHz, MeOH) 33.88, 34.43, 41.02, 56.95, 115.02, 115.46, 119.68, 130.52,

Scheme I Synthetic pathways of the prodrugs of dopamine. Reaction conditions: (*a*) 3,4dibenzyloxyphenethylamine hydrochloride, HOBt, EDC, TEA, DCM; (*b*) LiOH, MeOH: H<sub>2</sub>O; (*c*) 3 M HCl, Pd, MeOH; (*d*) 9-BBN, Toluene, MW (27); (*e*) Ethylene diamine, THF; (*f*) 6 M HCl.



143.37, 144.61, 167.54, 169.45. MS calcd. for  $C_{12}H_{17}N_2O_5$ , 268.27; found 269.18. Anal. Calcd. for  $C_{12}H_{16}N_2O_5$ ·1.6 HCl: C, 44.13; H, 5.43; N, 8.58. Found: C, 43.94; H, 5.37; N, 8.48.

## 4'-(3-Carboxypropyl)-5'-Oxospiro[Bicyclo[3.3.1] Nonane-9,2'-[1,3,2]Oxazaborolidin]-2'-uide (5)

A mixture of B-methoxy-9-borabicyclo[3.3.1]nonane 1 M in hexanes (3.11 ml, 3.11 mmol) and DL-2aminoadipic acid (1.00 g, 6.20 mmol) were placed in a microwave vial with a stirring bar. Ten ml of toluene were added and the vial was capped. This mixture was irradiated in a microwave reactor at 140°C for 20 min. After the reaction was cooled down the clear solution was transferred to a flask and the solvent was removed under reduced pressure, which gave a yellowish solid. This solid was purified by flash-chromatography using hexane:ethyl acetate (1:1) as an eluent. After combining the appropriate fractions, the solvents were evaporated to afford the title compound (1.21 g, 2.18 mmol) as a white solid. 1H NMR (500 MHz, MeOD)  $\delta$  1.50 (2H, m), 1.65–1.98 (16H, m), 2.41 (2H, t,  $\mathcal{J}$ =7.09 Hz), 3.69 (1H, m).

## 4'-(4-((3,4-Bis(Benzyloxy)Phenethyl)Amino)-4-Oxobutyl)-5'-Oxospiro[Bicyclo[3.3.1]Nonane-9,2'-[1,3,2]Oxazaborolidin]-2'-uide (6)

To a solution of compound 5 (1.2 g, 4.32 mmol), HOBt (0.642 g, 4.75 mmol) and 3,4-dibenzyloxyphenethylamine (1.59 g, 4.23 mmol) in DCM (20 ml) was added EDC (1.34 g, 8.64 mmol) and triethylamine (2.41 ml, 17.28 mmol). This solution was stirred at room temperature overnight. The crude product was purified by flash-chromatography eluting gradiently with hexane:ethyl acetate mixture. After combining the appropriate fractions and evaporating the solvents, the title compound (2.08 g, 3.49 mmol) was obtained as a white solid. 1H NMR (500 MHz, MeOD)  $\delta$  1.50 (2H, m), 1.65–1.97 (16H, m), 2.33 (2H, t,  $\mathcal{J}$ =7.09 Hz), 2.73 (2H, t,  $\mathcal{J}$ =7.09 Hz), 3.39 (2H, t,  $\mathcal{J}$ =7.25 Hz), 3.63 (1H, m), 5.10 (2H, s), 5.13 (2H, s), 6.77 (1H, dd,  $\mathcal{J}$ =8.20, 1.89 Hz), 6.94 (1H, d,  $\mathcal{J}$ =1.89 Hz ), 6.96 (1H, s), 7.30–7.38 (6H, m), 7.46 (4H, t,  $\mathcal{J}$ =8.35 Hz).

## DL-2-Amino-6-((3,4-Dihydroxyphenethyl)Amino)-6-Oxohexanoic Acid (2)

Compound 6 (2.08 g, 3.49 mmol) was dissolved in THF (20 ml) in a 100 ml Erlenmeyer flask. Ethylene diamine (1.17 ml, 17.46 mmol) was then added and this solution was heated with a heat gun until the reaction was complete (1 to 2 min). The reaction was allowed to cool down and precipitate. The precipitate was filtered, washed with THF ( $3 \times 20$  ml) and dried in vacuum.

The crude product from previous step was dissolved in a 4:1 mixture of methanol and 3 M HCl (20 ml). Palladium active on charcoal (10% Pd basis) (0.1 g) was added to the solution and the mixture was placed in a hydrogen chamber (4 mbar) and left to stir for 2 h at room temperature. The mixture was filtered through a pad of Celite® (Sigma, St. Louis, MO, USA) to remove the catalyst.. The compound was obtained as a solid after removing the solvents under reduced pressure. To ensure the formation of HCl salt, the title compound was bubbled with HCl(g) in ACN. Removing of the solvent gave the title compound as a yellowish solid (0.31 g, 1.05 mmol). 1H NMR (500 MHz, D<sub>2</sub>O) δ 1.57 (2H, m), 1.75 (2H, m), 2.21 (2H, t), 2.67 (2H, t, 7=6.15 Hz), 3.39 (2H, t, 7=6.46 Hz), 3.71 (1H, t, 7=5.83 Hz), 6.69 (1H, d, 7=8.20 Hz), 6.77 (1H, s), 6.85 (1H, d, 7=7.57 Hz), 13C NMR (125 MHz, D<sub>2</sub>O) 21.14, 29.79, 33.83, 35.21, 40.59, 116.32, 116.79, 121.27, 131.16, 142.32, 143.85, 174.40, 176.61. MS calcd. for C<sub>14</sub>H<sub>20</sub>N<sub>2</sub>O<sub>5</sub>, 296.32; found 297.23. Anal. Calcd. for C<sub>14</sub>H<sub>20</sub>N<sub>2</sub>O<sub>5</sub>·1.5 ACN·0.5 HCl: C, 54.29; H, 6.70; N, 13.03. Found: C, 54.20; H, 7.09; N, 12.79.

## 3-(2-Amino-2-Carboxyethyl)Benzoic Acid (7)

Boc-3-cyano-L-phenylalanine (0.90 g, 3.10 mmol) was dissolved in 6 M HCl (20 ml) and refluxed overnight. The white precipitate was filtered, washed with cold water and dried under vacuum to afford the title compound (0.65 g, 3.10 mmol) as a white powder. 1H NMR (500 MHz, DMSO)  $\delta$  3.20 (2H, d), 4.18 (1H, t), 7.46 (1H, m), 7.54 (1H, d,  $\mathcal{J}$ =7.57 Hz), 7.85 (1H, d,  $\mathcal{J}$ =7.88 Hz), 7.87 (1H, s).

## 4'-(3-Carboxybenzyl)-5'-Oxospiro[Bicyclo[3.3.1] Nonane-9,2'-[1,3,2]Oxazaborolidin]-2'-uide (8)

A mixture of B-methoxy-9-borabicyclo[3.3.1]nonane 1 M in hexanes (3.11 ml, 3.11 mmol) and compound 7 were placed in a microwave vial with a stirring bar. Ten ml of toluene were added and the vial was capped. This mixture was irradiated in a microwave reactor at 140°C for 20 min. After the reaction was cooled down, the clear solution was transferred to a flask and the solvent was removed under reduced pressure, which resulted in a yellowish solid. This solid was purified by flash-chromatography

eluting with hexane:ethyl acetate (1:1). After combining the appropriate fractions, the solvents were evaporated to afford the title compound (1.00 g, 3.05 mmol) as a white solid. 1H NMR (500 MHz, MeOD)  $\delta$  1.42–1.87 (12H, m), 3.13 (1H, dd,  $\mathcal{J}$ =14.66, 8.04 Hz), 3.37 (1H, dd,  $\mathcal{J}$ =14.66, 4.89 Hz), 4.01 (1H, t,  $\mathcal{J}$ =6.46 Hz), 7.59 (1H, d,  $\mathcal{J}$ =7.57 Hz), 7.86 (1H, t,  $\mathcal{J}$ =7.57 Hz), 7.94 (1H, d,  $\mathcal{J}$ =7.57 Hz), 8.03 (1H, s).

## 4'-(3-((3,4-Bis(Benzyloxy)phenethyl)Carbamoyl)Benzyl)-5'-Oxospiro[Bicyclo[3.3.1]Nonane-9,2'-[1,3,2] Oxazaborolidin]-2'-uide (9)

To a solution of compound 8 (0.8 g, 2.44 mmol), HOBt (0,33 g, 2.44 mmol) and 3,4-dibenzyloxyphenethylamine (0.82 g, 2.22 mmol) in DCM (20 ml) were added EDC (0.69 g, 4.43 mmol) and triethylamine (1.24 ml, 8.86 mmol). This solution was stirred overnight at room temperature. The crude product was purified by flash-chromatography, eluting gradiently with a hexane:ethyl acetate mixture. After combining the appropriate fractions and evaporating the solvents, the title compound (0.98 g, 1.52 mmol) was obtained as a white solid. 1H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  1.30–1.80 (12H, m), 2.81 (2H, m), 3.08 (1 H, dd,  $\mathcal{J}$ =14.98, 9.30 Hz), 3.37 (1H, dd,  $\mathcal{J}$ =14.66, 4.26 Hz), 3.60 (2H, m), 3.94 (1H, m), 5.07 (2H, s), 5.10 (2H, s), 6.73 (1H, d,  $\mathcal{J}$ = 7.88 Hz), 6.82 (1H, s), 6.89 (1H, d,  $\mathcal{J}$ =8.20 Hz), 7.28–7.42 (11H, m), 7.47 (1H, d,  $\mathcal{J}$ =4.10 Hz), 7.55 (1H, s).

## L-2-Amino-3-(3-((3,4-DihydroxyphenethylCarbamoyl)) Phenyl)Propanoic Acid (3)

Compound 9 (0.98 g, 1.52 mmol) was dissolved in THF (20 ml) in a 100 ml Erlenmeyer flask. Ethylene diamine (0.46 ml, 6.85 mmol) was then added and this solution was heated with a heat gun until the reaction was complete (1 to 2 min). The reaction was allowed to cool down and precipitate. The precipitate was filtered, washed with THF ( $3 \times 20$  ml) and dried in vacuum.

The white solid from previous step was dissolved in a 4:1 mixture of methanol and 6 M HCl (20 ml). Palladium activated on charcoal (10% Pd basis) (0.1 g) was added to the solution and the mixture was put in a hydrogen chamber (4 mbar) and left to stir for 2 h at room temperature. The mixture was filtered through a pad of Celite to remove the catalyst. The title compound (0.19 g, 0.54 mmol) was obtained after removing the solvents under reduced pressure as a yellowish solid. 1H NMR (500 MHz, DMSO)  $\delta$  2.64 (2H, t), 3.03 (1H, m), 3.15 (1H, m), 3.44 (2H, m), 3.51 (1H, t, J=5.67 Hz), 6.44 (1H, d, J=7.88 Hz), 6.61 (1H, d, J=7.25 Hz), 7.78 (1H, s). 13C (125 MHz, DMSO)  $\delta$ 34.67, 35.55, 41.27, 55.15, 115.25, 116.28, 119.00, 125.50,

128.11, 130.52, 132.11, 135.03, 137.18, 143.75, 145.40, 152.73, 166.38, 171.96. MS calcd. for  $C_{18}H_{20}N_2O_5$  344.36; found 345.13. Anal. Calcd. for  $C_{18}H_{20}N_2O_5$ ·H<sub>2</sub>O: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.88; H, 5.99; N, 7.61.

#### **Analytical Procedures**

The amounts of the prodrugs **1–3** and dopamine in analytical and biological samples were analyzed by the Agilent HPLC 1100 Series system (Agilent Technologies Inc., Little Falls, Wilmington, DE, USA) that consisted of a binary gradient pump G1312A, a vacuum degasser G1322A, an autosampler G1313A, a thermostatically controlled column compartment G1316A and ChemStation software (Rev. B.04.03; Agilent Technologies Inc., Palo Alto, CA, USA). The prodrugs and dopamine were detected by an electrochemical (EC) detector ESA CouloChem III detector (ESA Inc., Chelmsford, MA, USA) with an amperometric analytical cell model 5041 with a glassy carbon-ceramic target and 0.0005" gasket. The applied potential was +150 mV and the range 20 nA. Injection volume was 2.5 µl for analyzing dopamine and 5  $\mu$ l for the prodrugs. The analytes were separated on a reversed phase column (Zorbax SB-Aq, 100×2.1 mm, 3.5 µm, AgilentTechnologies Inc., Little Falls, Wilmington, DE, USA) with a Zorbax-Aq guard column (AgilentTechnologies Inc., Little Falls, Wilmington, DE, USA) with isocratic elution. The mobile phase consisted of acetonitrile (20%, v/v) and 150 mM monobasic sodium phosphate containing 4.8 mM citric acid monohydrate, 3 mM 1-octanesulfonic acid sodium salt and 50 µM disodium EDTA (80%, v/v). The pH of the mobile phase was adjusted to 5.60 with sodium hydroxide (10 M). The column temperature was maintained at 30°C with mobile phase flowing at 0.3 ml/min. The method was shown to be linear between dopamine concentrations of 0.05-20 pmol/ injection and prodrug concentrations of 0.01-1.0 nmol/ brain hemisphere, respectively.

#### **Brain and Plasma Sample Preparation Procedures**

The prodrugs **2** and **3** were separated from brain tissue (a whole brain hemisphere) by protein precipitation followed by liquid-liquid extraction and solid-phase extraction (SPE). First, the thawed brain hemisphere was homogenized with 2.5 ml of water. To precipitate the proteins, the samples were then acidified with 200 $\mu$ l of 5 M hydrochloric acid and vortexed for 5 min. MeOH (1.5 ml) was added and the homogenates were vortexed for 2 min. After standing for 10 min, the samples were centrifuged for 10 min (14,000 g at 7°C) after which the supernatants were collected. The supernatant was evaporated to 3 ml under a stream of nitrogen at 40°C. The washing of the pellet was repeated two times with water (1.5 ml) and the supernatants were

combined. Water was added up to 8 ml to the combined supernatants after which they were centrifuged (14,000 g at 7°C). The supernatants were loaded into preconditioned and equilibrated C18 SPE-columns (Supelco Discovery DSC-18 3 ml, Supelco Park Bellefonte, PA, USA). After the supernatants had been added, 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 eluted out from the columns two times with 0.5 ml of 80% (v/v) MeOH in water, and the solution was evaporated to dryness under a stream of nitrogen at 40°C. Prior to analysis, the samples were reconstituted in 150 µl of 0.1 M perchloric acid and analyzed by HPLC-EC. The prodrugs were shown to remain intact during the whole sample preparation procedure (data not shown). External standards were used for the brain samples. The calibration curves of the brain methods were linear over a range of 0.4–2.0 nmol/brain hemisphere for 2 and 0.01-2.0 nmol/brain hemisphere for 3. The lower limits of quantification (LLOQ) for spiked samples were 0.4 nmol of prodrug/brain hemisphere for 2 and 0.01 nmol of prodrug/brain hemisphere for 3.

Dopamine levels in rat striatal samples were assayed by homogenizing the tissue in 0.1 M perchloric acid (1:10, w/v) with MSE Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, UK). The homogenates were centrifuged for 15 min at 16,000 g at 4°C. The supernatants were then filtered by 0.45  $\mu$ m GHP Acrodisc Syringe Filters (Pall Gelman Laboratory, Ann Arbor, MI, USA) and diluted (1:1) with 0.1 M perchloric acid. Dopamine concentrations in tissue homogenates of both striata were determined by the HPLC method described above. External standards were used for the striatal samples. The calibration curve of this method was linear over a range of 0.05–10.0 pmol/injection. The LLOQ was 0.01 pmol of dopamine/injection.

The prodrug **3** was separated from the thawed plasma samples simply by protein precipitation. First, the plasma samples (100  $\mu$ l) were acidified with 40 $\mu$ l of 5 M hydrochloric acid and vortexed for 2 min. MeOH (100  $\mu$ l) was added and the plasma samples were vortexed for 2 min. After standing for 10 min, the samples were centrifuged for 5 min (16,000 g at 7°C). The supernatants were analyzed by the HPLC-EC method. The prodrug was shown to remain intact during the whole sample preparation procedure. External standards were used for the plasma samples. The calibration curves of the plasma methods were linear over a range of 0.01–15 nmol/100  $\mu$ l of plasma. The LLOQ of spiked samples were 0.01 nmol of prodrug/100  $\mu$ L of plasma.

## Aqueous Solubility and Apparent Partition Coefficients

The aqueous solubilities of 2 and 3 were determined at room temperature (21°C) in phosphate buffer (160 mM) at

pH 7.4. An excess amount of **2** and **3** was added to 0.5 ml of buffer, the mixtures were shaken for 2 h, filtered (0.45  $\mu$ m, Millipore, Billerica, MA, USA) and analyzed by the HPLC-EC method described above. The pH of the mixtures was checked during the shaking, and adjusted if needed.

Apparent partition coefficients (log D) of **1–3** and dopamine were determined in mutually saturated 1-octanol– phosphate buffer at pH 7.4 at room temperature. **1–3** or dopamine was first dissolved in the phosphate buffer, combined with 1-octanol (1:10), and the octanol–phosphate buffer mixtures were shaken for 180 min to reach an equilibrium distribution. The concentrations of the prodrug or dopamine in the phosphate phase after the shaking were analyzed by the HPLC-EC method described above, and log D values were calculated.

#### **Topological Polar Surface Area**

Topological polar surface areas (Å<sup>2</sup>) of **1–3**, dopamine and L-dopa were calculated with MOE-2011.10 [*Molecular Operating Environment (MOE)*, 2011.10; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, **2011]** using group contributions to approximate the polar surface area only from connection table information. The parameterization is that of Ertl *et al.* 2000 (28).

#### In Vitro Stability Studies

To demonstrate the chemical and enzymatic stabilities of 1-3 under physiological conditions, and to demonstrate the bioconversion of 1-3 to dopamine, the prodrugs were exposed to degradation in various media, including phosphate buffer (pH 7.4, 0.16 M,  $\mu = 0.5$ ), 75% (v/v in isotonic phosphate buffer pH 7.4) rat 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 1-3 was incubated in a thermostatically controlled water bath at 37°C, and 100 µl aliquots were withdrawn at regular time intervals. The withdrawn biological samples were analyzed by protein precipitation with 200 µl of acetonitrile, and after mixing, the samples were centrifuged for 10 min (16,000 g at 7°C). The samples from the phosphate buffer were analyzed immediately without protein precipitation. The HPLC-EC analysis was performed to determine the amounts of intact prodrug and dopamine in the supernatant.

#### In Situ Rat Brain Perfusion Technique

The *in situ* rat brain perfusion method of Takasato *et al.* (29) is a very useful and sensitive method for evaluating the BBB transport mechanisms and the brain uptake of drugs. In

this study, transport mechanisms, LAT1-affinity and brain uptake of the prodrugs 1-3 were determined by the modified in situ rat brain perfusion technique (13,29,30). Briefly, the rats were anesthetized with ketamine (90 mg/kg, i.p., Intervet International B.V., Boxmeer, Netherlands) and xylazine (8 mg/kg, i.p., Intervet International B.V., Boxmeer, Netherlands), and their right carotid artery system was exposed. The right external carotid artery was ligated, and the right common carotid artery was cannulated with heparinized PE-50 catheters. The blood flow to the rat brain was terminated, and the right brain hemisphere was then perfused for 30-60 min with perfusion solution containing prodrug **1–3** or L-dopa. The rat was decapitated and the brain samples were collected for further analysis. Validation, description and discussion of the in situ rat brain perfusion technique used in this study have been described in more detail in our previous paper (13).

#### Determination of the LATI-Affinity for I-3

To determine the ability of the prodrugs or L-dopa to bind to LAT1 using the *in situ* rat brain perfusion, 0.2  $\mu$ Ci/ml [<sup>14</sup>C]leucine (i.e. 0.64 µM), an endogenous substrate of LAT1, was added into the perfusion fluid with 100 µM concentration of the prodrug **1–3** or L-dopa. The permeability-surface area of [<sup>14</sup>C]-leucine was determined after 30 s co-perfusion, and the LAT1-binding of the LAT1-substrates was evaluated by the reduction in the permeability-surface area of  $[^{14}C]$ -leucine caused by competitive binding of the substrate to LAT1. The permeability-surface area of 0.2  $\mu$ Ci/ml [<sup>14</sup>C]-leucine after the co-perfusion with a prodrug or L-dopa was compared with the 100% permeability-surface area of 0.2  $\mu$ Ci/ml [<sup>14</sup>C]leucine without any competitive substrates. The 100% permeability-surface area product of  $[^{14}C]$ -leucine has been determined to be  $19.0\pm3.6 \ \mu l/s/g$  after 30 s perfusion of  $0.2 \,\mu \text{Ci/ml} \,[^{14}\text{C}]$ -leucine solution (13).

#### Brain Uptake Studies of 2 and 3

The brain uptake studies of prodrugs **2** and **3** were also performed by using the *in situ* rat brain perfusion technique. To quantify their brain uptake, the right brain hemisphere was perfused for 60 s with 37°C perfusion medium containing 25–600  $\mu$ M concentrations of the prodrugs **2** or **3**. 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 potential efflux activity of the transporters.

In addition to the uptake studies of 2 and 3, our interest was to evaluate whether 3 could cross the BBB by a nonsaturable brain transport mode or by carrier-mediated transport via LAT1. In an attempt to delineate the role of the nonsaturable transport mode, the rat brain capillaries were first washed for 30 s with cold prodrug-free perfusion medium  $(5^{\circ}C)$  to decrease the activity of the transporters. To maintain the low activity of LAT1, also the 60 s perfusion with 100  $\mu$ M of the prodrug was performed at 5°C. Finally, the brain hemisphere 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 ensure the reduced activity of the transporter. The cold-perfusion study of **3** was also performed with an endogenous competitor, 2 mM L-phenylalanine, to determine the activity of LAT1 at 5°C. A competition study with 2 mM L-phenylalanine was also conducted at 5°C to clarify whether **3** was being taken up into the brain in a carrier-mediated manner.

## Pharmacokinetic Studies of 3 in Rats After Intravenous Bolus Injections

The brain uptake of **3** from blood to the brain and its basic pharmacokinetic parameters were determined after intravenous bolus injection in rats. The rats were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). Their right jugular vein was exposed and cannulated with a PE-50 catheter. The rats were treated with buprenorphine (0.12 mg/kg, i.p.) as an analgesic for 24 h after the cannulation. The cannulated rats were allowed to recover until the following day. A total of 25.36 µmol/kg of prodrug 3 were administered into the cannulated jugular vein of the rats as a 0.5 ml bolus injection. The rats were pretreated with intraperitoneal injection of carbidopa (30 mg/kg) (Orion Pharma, Espoo, Finland) and entacapone (10 mg/kg) (Orion Pharma, Espoo, Finland) 30 min before the administration of **3** in order to compare **3** to the standard L-dopa treatment of Parkinson's disease. Carbidopa and entacapone were used to prevent the premature conversion of L-dopa to dopamine in peripheral tissues. Carbidopa, entacapone and 3 were administered as suspensions containing a few drops of Tween 80 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 0.9% NaCl solution at pH 7.4. Plasma samples were collected 2, 5, 10, 30, 60 and 80 min after the prodrug administration and the brain samples (a whole brain hemisphere) were collected at 10, 30, 60 and 180 min after the prodrug administration. The collected brain and plasma samples were immediately frozen, and stored at -70°C. Rat brain and plasma levels of **3** were analyzed after the sample preparation by the HPLC-EC method described above. The brain levels were corrected by the measured brain sample concentration at the cerebral vascular space component, which was previously determined to be 0.0149 ml/g (13).

## Dopamine Release from 3 in Rat Striatum After Intraperitoneal Injections

The ability of prodrug 3 to release dopamine in the rat striatum after intraperitoneal administration was

investigated by treating the rats with 50.71  $\mu$ mol/kg of **3**. Before the intraperitoneal administration, the rats were allowed to adapt to the handling and animal room for 5 days. The dopamine levels after the intraperitoneal administration of 3 plus carbidopa and entacapone were compared to those after an equivalent intraperitoneal dose Ldopa (free base) (Sigma-Aldrich) combined with carbidopa and entacapone. Both L-dopa and prodrug 3 were administered as suspensions containing a few drops of Tween 80 in 0.9% NaCl solution. Carbidopa (30 mg/kg) and entacapone (10 mg/kg) were administered intraperitoneally in the same formulation 30 min before the administration of L-dopa or 3. The rats were sacrificed at 10, 30, 60, and 180 min after administration of L-dopa or 3, and their dissected striata were frozen immediately in liquid nitrogen and stored at -70°C until sample preparation and HPLC-EC analyses.

#### **Data Analyses**

The figures were created and all statistical analyses were performed using nonlinear regression by GraphPad Prism 5.0 for Windows (GraphPad Software Inc., La Jolla, USA). Statistical significance of differences between different groups were analyzed using one-way ANOVA followed by a two-tailed Dunnett's test. The significance is reported as \*\*\*p<0.001 or as \*p<0.05 for all tests. The pharmacokinetic data analysis of **3** after intravenous administration was carried out by WinNonlin® Professional 5.3 (Pharsight Corparation, St. Louis, MO, USA) by using non-compartmental model for the intravenous bolus injections, and with 1/Yhat weighting.

## RESULTS

## In Vitro Studies of I-3

The aqueous solubilities of **2** and **3** at pH 7.40 were determined to be higher than 10 mg/ml. The maximum solubilities of **2** and **3**, and the aqueous solubility of **1** were not determined because of the small amount of the prodrugs available. However, the apparent partition coefficients of **1–3** at pH 7.40 (log D) were determined to be negative,  $-2.25 \pm 0.12$ ,  $-1.54 \pm 0.16$  and  $-1.57 \pm 0.16$  (mean  $\pm$  sd, n=3) respectively, indicating that the prodrugs are highly hydrophilic at pH 7.40. As a comparison, the log D of dopamine at pH 7.40 was determined to be  $0.20 \pm 0.11$  (mean  $\pm$  sd, n=3). In addition, topological polar surface areas (TPSA) of **1–3**, L-dopa and dopamine were calculated with MOE-2011.10. TPSA of the prodrugs was calculated to be  $132.9 \text{ Å}^2$ , and that of L-dopa and dopamine was  $103.8 \text{ Å}^2$  and  $66.5 \text{ Å}^2$ , respectively (Table I).

The stabilities of **1–3** and their bioconversion to dopamine were studied in aqueous buffer (pH 7.4), in 20% rat

Prodrug	Buffer (pH 7.4) (h)	75% rat plasma (h)	20% rat brain homogenate (h)	50% rat liver homogenate (h)	TPSA (Å <sup>2</sup> )
	24.4±3.3	4.8±1.0	24. I ± 8. I	4.4±0.9	132.9
2	$9.9 \pm 0.04$	$3.2 \pm 0.2$	30.58ª	5.3±1.1	132.9
3	$19.9 \pm 2.6$	$1.5 \pm 0.1$	$28.4 \pm 5.5$	$2.7 \pm 0.4$	132.9
L-dopa	n.a	n.a	n.a.	n.a.	103.8
Dopamine	n.a	n.a	n.a	n.a	66.5

**Table I** Hydrolysis Rates of **I**–**3** in Phosphate Buffer (pH 7.4), in Rat Plasma, in Rat Brain and Rat Liver Homogenates (37°C) ( $T_{1/2}$ , mean  $\pm$  sd; n=3) and Topological Polar Surface Areas (TPSA) for **I**–**3**, L-dopa and Dopamine

<sup>a</sup> Mean (n=2)

brain homogenate, 50% rat liver homogenate and in 75% rat plasma at 37°C (Table I). The chemical and enzymatic degradation of **1–3** followed pseudo-first-order kinetics, and the half-lives are presented in Table I. The release of dopamine was also verified quantitatively by the HPLC-EC method. The prodrugs appeared to be reasonably stable in the aqueous buffer at pH 7.4 having half-lives of approximately 10 h (**2**) or longer (**1** and **3**). The half-lives of the prodrugs in the rat plasma and in the rat liver homogenates were notably shorter than those determined in the aqueous buffer solution (Table I) suggesting that the prodrugs released the parent drug predominantly enzymatically. According to the *in vitro* data, **1–3** were suitable for further evaluation by the *in situ* and *in vivo* methods in rats.

## Transporter-Mediated Brain Uptake of 1–3 by In Situ Rat Brain Perfusion

Strong inhibition in the permeability-surface area (PA) product of  $[^{14}C]$ -leucine was apparent when perfusing 0.2  $\mu$ Ci/ml  $[^{14}C]$ -leucine (*i.e.* 0.64  $\mu$ M) in the presence of 100  $\mu$ M concentration of the prodrug **3** (Fig. 2) as the PA



**Fig. 2** LATI-binding data of prodrugs  $100 \,\mu$ M **I–3**,  $100 \,\mu$ M L-dopa and  $90 \,\mu$ M lysine-ketoprofen (7). The PA product of  $0.2 \,\mu$ Ci/ml [<sup>14</sup>C]-leucine,  $19.0 \pm 3.6 \,\mu$ ]/s/g, was reduced to  $11.93 \pm 1.10 \,\mu$ ]/s/g (38.8% inhibition) in the presence of **I**, to  $12.68 \,\mu$ ]/s/g (35.0% inhibition) in the presence of **2** and to  $2.76 \pm 0.72 \,\mu$ ]/s/g (85.5% inhibition) in the presence of **3** (means  $\pm$  sd, n=3). As a comparison, the PA product [<sup>14</sup>C]-leucine decreased to  $4.1 \pm 0.26 \,\mu$ ]/s/g (66.3% inhibition) in the presence of L-dopa and to  $4.26 \pm 0.19 \,\mu$ ]/s/g (79.3% inhibition) in the presence of lysine-ketoprofen (7) (means  $\pm$  sd, n=3). The asterisk denotes a statistically significant difference from the respective control (\*\*\*P < 0.001, one-way ANOVA, followed by Dunnett t-test).

product of  $[^{14}C]$ -leucine decreased from  $19.0\pm3.6 \,\mu$ l/s/g to  $2.76 \pm 0.72 \,\mu$ l/s/g (mean  $\pm$  sd, n=3), respectively. This observation *i.e.* about 85% inhibition in  $[^{14}C]$ -leucine brain uptake by 3, confirms our previous results which showed that metasubstituted phenylalanine was a potential promoiety and carrier of anionic drugs into the brain (13). In the present study, the promoiety has been modified also for cationic drugs, and this modification was shown to be capable of retaining its LAT1-targeting action. In addition, this study demonstrates for the first time that hydrophilic prodrugs can also have high binding affinity for LAT1. Moreover, the inhibition caused by **3** was significantly stronger than that observed after *in situ* rat brain perfusion of an equivalent concentration of L-dopa (Fig. 2) which was  $66.26 \pm 1.39\%$  (mean  $\pm$  sd, n=3). This result indicates that 3 has better possibilities to compete with the endogenous LAT1-substrates than L-dopa when being taken up into the brain by LAT1. This observation encouraged us to study the brain uptake of **3** in more detail by using the *in situ* rat brain perfusion to complement the  $[^{14}C]$ -leucine brain uptake inhibition determination. In contrast, prodrugs 1 and 2 did not possess as strong affinity for LAT1 as 3, and they were less potent than L-dopa in their ability to inhibit the brain uptake of leucine (Fig. 2). **1** was able to decrease the PA product of  $[^{14}C]$ leucine with  $38.80 \pm 5.61\%$  and **2** with  $34.95 \pm 10.54\%$ (mean  $\pm$  sd, n=3), respectively. This observation was not consistent with our previous studies where the lysine derivative of ketoprofen, a structurally-related prodrug to 1 and 2, achieved almost 80% inhibition of leucine brain uptake at the equivalent concentration (7) (Fig. 2).

We also studied the total amounts of the prodrugs 2 and 3 in the rat brain after the *in situ* rat brain perfusion to confirm that the prodrugs do not only bind to LAT1 but are also translocated across the BBB, because the entry of a molecule into the brain does not necessarily occur even though the prodrug might bind very tightly to the transporter (31). As illustrated in Fig. 3, only prodrug 3 possessed the ability to cross the rat BBB and gain entry into the brain tissue during the *in situ* rat brain perfusion. In contrast, prodrug 2, which showed only low affinity for LAT1 (Fig. 2), was not shown to cross the rat BBB even at as high as 600  $\mu$ M concentration in the perfusion fluid (data not



**Fig. 3** Kinetics of the rat brain uptake of **3**.  $K_m$  and  $V_{max}$  are 227.9 ± 52.0  $\mu$ M and 0.99 ± 0.09 pmol/mg/min (mean ± sd, n = 3) respectively.

shown). The poor brain uptake of **2** may indicate the fact that very high affinity to LAT1 is needed for the brain uptake of LAT1-targeted prodrugs. Since **1** and **2** lack affinity for LAT1 and **2** was not taken up into the brain at detectable amounts, we decided to perform more comprehensive *in vivo* brain uptake studies only for **3**.

Roles of reduced temperature and simultaneous LAT1 inhibition in the rat brain uptake of **3** at 100  $\mu$ M concentration were investigated by using cold-perfusion (5°C) and 2 mM L-phenylalanine as a competitive endogenous LAT1 substrate (Fig. 4) to demonstrate the role of LAT1 in the brain uptake of **3**. A lower perfusion fluid temperature

decreased the brain uptake of 3 from about 0.307 pmol/ mg/min to 0.095 pmol/mg/min, respectively. This corresponded to approximately 69% inhibition and was a clear evidence of carrier-mediated brain uptake of 3. A similar reduction of about 61% inhibition in the brain uptake of **3** was shown when co-perfusing the rat brain with  $100 \,\mu M$  of 3 and 2 mM L-phenylalanine, and this further confirmed the involvement of LAT1 in the brain uptake of **3**. A competition study with 2 mM L-phenylalanine was also conducted at 5°C to demonstrate that 3 was being taken up into the brain by a carrier-mediated manner also at 5°C (Fig. 4). A significant decrease in the brain uptake of **3** was observed when 3 was co-perfused with 2 mM L-phenylalanine at a reduced temperature, when compared to the results of the same experiment conducted at 37°C. However, there still remains a non-saturable, cold-insensitive part in the brain uptake of 3.

# Brain Uptake and Basic Pharmacokinetic Parameters of 3 After Intravenous Administration

Encouraged by the *in situ* results, the brain uptake of **3** *in vivo* was determined after intravenous administration in rats. A total of 25.35  $\mu$ mol/kg solution of **3** was administered into the right jugular vein of the rats. The results indicate that **3** was able to cross the BBB *in vivo* in the presence of competitive endogenous substrates (Fig. 5). The area under the curve for **3** during the 180 min after the administration in the brain (AUC<sub>brain</sub>) was 13.39 nmol\*min/g and in plasma (AUC<sub>plasma</sub>) 1,481 nmol\*min/ml (Table II). The half-life of **3** in plasma during the distribution phase was as short as 3.10 min indicating that the prodrug **3** is excreted



Fig. 4 Rat brain uptake of **3** after cold perfusion and co-perfusion with 2 mM L-phenylalanine. The uptake of **3** with 100  $\mu$ M concentration significantly decreased from 0.307 ± 0.02 pmol/mg/min to 0.095 ± 0.01 pmol/mg/min (mean ± sd, n = 3) (69.1% inhibition) in cold perfusion medium (5°C). A comparable inhibition (60.8%) was observed during the co-perfusion of 100  $\mu$ M of **3** and 2 mM of L-phenylalanine. When conducting the study with 2 mM L-phenylalanine at 5°C the uptake of **3** inhibited 78.6%. There was a statistically significant difference between the column 100  $\mu$ M **3** (37°C) and the other columns (\*\*\* P < 0.001 one-way ANOVA, followed by Dunnett t-test). There was also a significant difference between columns 100 $\mu$ M **3** + 2 mM Phe (37°C) and 100 $\mu$ M **3** + 2 mM Phe (5°C) (\* P < 0.05 one-way ANOVA, followed by Dunnett t-test).





rapidly as a hydrophilic compound or distributed rapidly into the rest of the rat body (Table II).

## Release of Dopamine from 3 in the Rat Striatum After Intraperitoneal Administration

In addition to the brain uptake studies *in vivo*, we evaluated whether **3** was able to elevate the striatal dopamine concentrations in the rats. In this study, we compared the dopamine concentrations in striatal samples after intraperitoneal administration of the prodrug **3** with those induced after intraperitoneal administration of an equivalent dose of Ldopa. A dose of 50.71  $\mu$ mol/kg of **3** or L-dopa suspension was administered intraperitoneally into the rats, which were pretreated with AADC- and COMT-inhibitors (*i.e.*, carbidopa (30 mg/kg) and entacapone (10 mg/kg), respectively) to prevent peripheral metabolism of L-dopa to dopamine. The results indicate that **3** does not increase the striatal dopamine concentrations *in vivo*, whereas the striatal concentrations of dopamine were significantly increased 60 min after the L-dopa administration (Fig. 6).

#### DISCUSSION

The brain distribution of polar or high molecular weight drugs is effectively limited by the BBB, leading to a fact that

**Table II** The Basic Pharmacokinetic Parameters of **3** After Intravenous Bolus Injection into Rats (mean  $\pm$  SD, n=4)

Parameters	Prodrug 3
Dose (µmol/kg)	25.35
AUC <sub>brain</sub> (nmol*min/g) (10–180 min)	13.39
AUC <sub>plasma</sub> (nmol*min/ml) (2–180 min)	1481
CL (ml/min/kg)	13.52±3.92
T <sub>1/2</sub> (min)	3.10±0.60

the therapeutically relevant brain uptake of a drug candidate is more of an exception than the rule (2). The CNSacting drugs have been traditionally designed to cross the BBB by simple passive transcellular diffusion. Thus, for decades only relatively lipophilic and low molecular weight compounds have been thought to have the ideal physicochemical properties to allow the drugs to reach the brain. However, the novel therapeutically active drug candidates tend to have higher molecular mass (32) and to possess less favourable physicochemical characteristics for BBBpermeation (3) leading to the urgent need to develop better drug brain delivery strategies.

In recent years, transporter-mediated drug brain delivery has shown substantial promise as a strategy to enhance transmembrane permeability of polar compounds, which are not otherwise able to cross the cell membranes (4). Prodrugs in which the parent drug molecule has been conjugated in a bioreversible manner with an endogenous substrate of a transporter expressed at the BBB have been



**Fig. 6** Effect of **3** and L-dopa on rat striatal dopamine concentrations after equivalent intraperitoneal doses (50.71  $\mu$ mol/kg). The rats were pre-treated with carbidopa (30 mg/kg) and entacapone (10 mg/kg) 30 min before the administration of L-dopa or **3**. The asterisks denote a statistically significant difference from the respective control (\*\*\**P* < 0.001, one-way ANOVA, followed by Dunnett t-test).

reported in many prior studies with the aim being to enhance the brain uptake of the parent drug (4). The most suitable transporters at the BBB for CNS-targeting of drugs by the prodrug approach seem to be LAT1 and GluT1. SVCT1, which is mainly expressed at the choroid plexus, could also be utilized as a drug carrier to the brain.

Tyrosine derivatives of *e.g.* nipecotic acid, phosphonoformate, ketoprofen and valproic acid have been shown to possess therapeutically significant affinity for LAT1 (8,11,13,33). Most studies of LAT1-targeted prodrugs have focused only on the measurement of the LAT1-affinity of the prodrugs, and the actual brain uptake of those compounds has rarely been evaluated. Only the LAT1-targeted ketoprofen and valproic acid derivatives have been shown to be taken up into the brain in a LAT1-mediated manner in the *in situ* rat brain perfusion in addition to binding with high affinity to that transporter (7, 8, 13). Furthermore, prodrugs substituted at the meta-position of phenylalanine have been shown to bind to LAT1 with brain uptake significantly superior to the corresponding para-substituted prodrugs (13). Those meta-substituted phenylalanine prodrugs with an unnatural amino acid promoiety are most probably able to compete with the endogenous amino acids of the systemic circulation while utilizing LAT1, because of their high LAT1-affinity, which is even greater than that of the endogenous substrates.

In addition to LAT1-targeting, some drugs such as dopamine, L-dopa, ketoprofen and 7-chlorokyurenic acid have been conjugated with a glucose promoiety (5,6,22,23,34) in order to modify those structures to make them into substrates for GluT1. Those derivatives possess affinity for GluT1 but there is no clear evidence that these prodrugs had been taken up from blood into the brain after in vivo administration. Nipecotic acid, kynurenic acid and diclofenamic acid conjugated with ascorbic acid have been shown to act as substrates for SVCT1 and they are believed to be taken up into the brain via that transporter (35,36). However, none of the previous reports has so far investigated the brain uptake of the SVCT1-targeted prodrugs in vivo. Though there are several transporters which can be considered in brain drug targeting, LAT1 is viewed as the most attractive transporter for carrier-mediated brain drug delivery approaches (4).

In addition to the LAT1-targeted amino acid "pseudonutrient" prodrug of dopamine, L-dopa, a series of glycosyl derivatives of dopamine has been previously investigated in order to determine the ability of the glucose or galactose promoiety to enhance the brain uptake of dopamine (23). The galactosylated derivatives of dopamine have been shown to bind to GluT1 and to be active in models of Parkinson's disease in rats. However, the *in vivo* pharmacokinetic properties of the GluT1-targeted dopamine derivatives have not been studied with all these prodrugs, and none of the pharmacokinetic studies has compared a prodrug with L-dopa combined with peripheral inhibitors of Ldopa metabolizing enzymes, *e.g.* carbidopa and entacapone, the mainstay therapy in treating the symptoms of Parkinson's disease. Another published study with a transporter-mediated dopamine prodrug utilized glutathione transporters at the BBB (24). This dopamine derivative has been shown to possess high affinity for the glutathione transporter in a MDCK cell monolayer and to be able to release dopamine in brain homogenate *in vitro* but the *in vivo* brain uptake capability of that derivative has not been clarified.

In this study, we have introduced one promising prodrug strategy, the utilization of LAT1 as a prodrug carrier at the BBB, which could be capable of enhancing the penetration of polar cationic drugs into the brain. Dopamine prodrugs **1–3** were designed, synthesized and characterized, their physicochemical properties were studied *in vitro*, and their ability to utilize LAT1 was evaluated with the *in situ* rat brain perfusion technique. In addition, the brain uptake of **3** was determined after an intravenous bolus injection in rats, and the release of dopamine from **3** in the rat striatal tissue was determined after an intraperitoneal bolus injection.

In the present study, the dopamine prodrugs **1–3** showed good aqueous solubility at pH 7.40, and their apparent log D -values at the same pH were negative. In addition, the topological polar surface areas (TPSA) of the prodrugs were much higher than the proposed maximum PSA ( $<90 \text{ Å}^2$ ) for molecules that can cross the BBB by passive diffusion (37), indicating that the prodrugs are not very likely penetrated into the brain by passive diffusion. After evaluation of the in vitro data, it was assumed that 1-3 permeate through the biological membranes via transporters or paracellularly at physiological pH (38). However, it is unlikely that the paracellular pathway takes any major role in the brain permeation of polar solutes (1). On the other hand, it is worthwhile to bear in mind that the hydro- or lipophilicity of a drug molecule is not the only parameter that could be used as a surrogate for drug brain penetration (3) even though the log D -values of the majority of CNS-acting drugs were shown to be 0-4 (37). It is also worthwhile mentioning that lipophilic drugs are usually potential substrates of efflux transporters, extruding neurotherapeutics from the brain back to the bloodstream (3), and lipophilic derivatives are more likely to possess high unspecific brain tissue binding and plasma protein binding (37). Both of these properties, *i.e.* being a substrate for an efflux transporter and binding to the brain tissue and plasma proteins, can decrease the unbound brain concentration of an active drug compound. In addition, lipophilic compounds possess a high volume of distribution as they are able to cross the biological membranes in general, and the specific fraction taken up into the brain might be relatively low. Thus, the influx transporter utilizing prodrug strategies, in which the parent molecules are relatively hydrophilic and the prodrug specific for certain influx transporter specific, are very attractive and promising in drug targeting into the brain.

The enzymatic hydrolysis of the prodrugs released dopamine (Table I) indicating that the molecules can, indeed, act as prodrugs. Enzymatic hydrolysis rates of **1–3** in rat plasma appeared to be relatively high when compared with those in the rat liver and brain homogenates. This may be due to the fact that the plasma hydrolysis studies were conducted in 75% rat plasma whereas the rat liver and brain tissue homogenates were diluted by isotonic buffer (pH 7.4) to concentrations of 50% or 20%, respectively. Additionally, the differences between the hydrolysis rates of the prodrugs in rat brain and liver homogenates probably correspond also to the distinct tissue distribution of the prodrughydrolyzing esterases. Amide prodrugs in general may be too stable for releasing the parent drug but the present dopamine prodrugs seem to belong to those amide prodrugs which are capable of releasing the parent drug, such as lisdextroamphetamine (39), a prodrug of dextroamphetamine, lysine-ketoprofen, a prodrug of ketoprofen (7), and 9-[P-(N,N-dipropylsulfamide)] benzoylamino-1,2,3,4-4Hacridine, a prodrug of tacrine (40).

The inhibition of  $[^{14}C]$ -leucine brain uptake in the *in situ* rat brain perfusion evoked by **3** was significantly higher than that of **1** and **2** and the corresponding inhibition achieved by L-dopa (Fig. 2). The potency of meta-substituted phenylalanine promoiety as a LAT1-targeted drug brain carrier was in agreement with our earlier findings (13). In this paper, we have shown that the promoiety could also be modified as a carboxylic acid derivative for cationic drug candidates with poor brain penetration. Surprisingly, 1 and **2** did not possess as high affinity for LAT1 as shown by the lysine-ketoprofen (7), which has the promoiety that structurally resembles the promoieties of 1 and 2. Therefore, the alkyl promoiety in general does not seem to act as favorable a LAT1-substrate as its aromatic counterpart, and the alkyl amino acid promoiety cannot be generalized to be able to function as a high-affinity LAT1-promoiety for every parent drug. However, the whole prodrug molecule can act as a high-affinity LAT1 substrate as shown by the lysineketoprofen prodrug.

The results of the *in situ* uptake studies showed that the penetration of **3** across the rat BBB was significantly better than that of **2** (Fig. 3). In fact, **2** did not cross the rat BBB at concentrations above the lower limit of quantification (*i.e.* 0.4 nmol/brain hemisphere). The reason why **2**, and most probably also **1**, were not taken up into the brain in detectable amounts is most probably due to their low affinity for the transporter. This was the first time when the translocation of the LAT1 prodrugs across the BBB was shown to be dependent on their high affinity to LAT1. This observation

with the concentration dependent brain uptake of 3 also further confirmed the proposal that  $\mathbf{3}$  is being taken up into the brain in a carrier-mediated manner. The role of nonsaturable brain transport mode of **3** was investigated by using cold-perfusion  $(5^{\circ}C)$  and by competitively inhibiting LAT1 with co-perfusion of 2 mM L-phenylalanine (Fig. 4). The lowered temperature of the perfusion fluid decreased the brain uptake of the prodrug **3** significantly. This significant decrease in the brain uptake is clear evidence of carrier-mediated passage of 3 across the BBB. A similar decrease was shown after the co-perfusion with 2 mM Lphenylalanine. However, in both experiments the uptake of 3 was not totally inhibited. Thus, we conducted an additional competitive inhibition study with 2 mM L-phenylalanine and **3** at 5°C and found that the brain uptake was further decreased by about 20%. This observation may indicate that the activity of LAT1 is not totally inhibited even in a cold perfusion medium. Alternatively, some part of **3** may be transferred in our study through the BBB by a non-saturable, cold-insensitive manner, which is the part of **3** that crossed the BBB most probably by passive diffusion. On the other hand, the physicochemical properties of **3**, *i.e.* high aqueous solubility (over 10 mg/ml), low log D (-1.57)and high TPSA (132.9  $\text{Å}^2$ ), do not indicate that **3** is likely to be taken up by passive diffusion.

To summarize the *in vitro* and *in situ* data, it seems that meta-substituted phenylalanine can be an efficient and potential promoiety for improving the brain uptake of small molecular weight cationic drugs that are not readily transported into the brain by passive diffusion. **3** was taken up into the rat brain in a carrier-mediated manner, rapidly and at significant amounts via LAT1 whereas the alkyl amino acid derivatives (**1** and **2**) did not possess sufficiently high affinity for LAT1 and, hence, were not taken up into the brain in appreciable amounts. Thus, it seemed reasonable to perform more comprehensive *in vivo* brain uptake studies with **3**.

Although the *in vitro* and *in situ* studies indicated that **3** was a promising LAT1-transported prodrug, it did not evoke desirable pharmacological effects *i.e.* it could not elevate the striatal dopamine levels in the in vivo situation (Fig. 6), even though it was taken up into the rat brain after intravenous administration (Fig. 5). As a hydrophilic compound, 3 may not reach the brain parenchyma in order to release dopamine enzymatically. Most probably 3 may be cleared out from the brain too rapidly to release dopamine in pharmacologically significant amounts. However, LAT1 and other amino acid transporters are known to be expressed also in the glia cells and astrocytes (41, 42), and thus the rapid CNS elimination after intravenous administration was unexpected because those amino acid transporters would be expected to carry **3** into the cells and trap the prodrug or the active molecule within the cells. As a

consequence, the time period for 3 to release dopamine at the site of action may be too short to achieve any favorable elevating effect on the striatal dopamine levels. In order to achieve relevant pharmacological actions, the CNS-acting drug must also gain access to its site of action within the brain tissue after its passage into brain.

Higher amounts of L-dopa were taken up into the brain than **3** and this drug was able to elevate the striatal dopamine supply even though its affinity for LAT1 was relatively weak (Figs. 2 and 6). It is believed that the brain uptake of Ldopa occurs in both passive and carrier-mediated manners (43). That may explain why L-dopa was superior in the *in vivo* situation when compared to **3**. The part of L-dopa being taken up into the brain by passive diffusion is considered to increase when it is metabolized into 3-O-met-dopa. This metabolite binds to LAT1 with much higher affinity than Ldopa itself, and acts as a competitive inhibitor for L-dopa transport across the BBB (44). This significantly higher LAT1-binding affinity of 3-O-met-dopa makes the competing hypothesis more relevant even though the plasma levels of 3-O-met-dopa are normally low.

#### CONCLUSIONS

In the present study, we explored amino acid prodrugs (1-3) of dopamine and found a new promoiety to carry this cationic drug molecule into the brain via the LAT1 transporter. Firstly, we showed that these prodrugs possess affinity for LAT1 at the rat BBB during the in situ rat brain perfusion and they evoked a sustained release of dopamine in *in vitro* stability studies, thus fulfilling the specifications required for tissue-targeted prodrugs. Secondly, only the meta-substituted phenylalanine promoiety attached to dopamine via an amide bond (prodrug 3) exhibited adequate affinity for LAT1 for carrier-mediated brain uptake. The aspartic acid (prodrug 1) and adipidic acid (prodrug 2) prodrugs did not possess as favourable properties as LAT1 substrates as 3 and this was the first time when the translocation of the LAT1 prodrugs across the BBB was shown to be dependent on their high affinity to LAT1. In addition to the *in situ* studies, we showed that prodrug **3** was also taken up into the rat brain after its intravenous administration in vivo in rats. Unfortunately, we still could not find enhanced dopamine concentrations in the rat striata after intraperitoneal administration of the prodrug **3** when compared with corresponding equimolar dose of L-dopa. This may be because 3 is too quickly eliminated from the brain due to its high polarity. Our *in vivo* data also highlighted the problem that a prodrug that shows potential in vitro and in situ, may not necessarily work well in in vivo studies.

Taken together, these findings imply that LAT1 is a promising target for brain drug delivery of poorly brain penetrating drugs, and the attachment of phenylalanine to the parent drug via an amide bond from the meta-position of its aromatic ring is a feasible promoiety for LAT1targeting of cationic drugs. However, further work needs to be conducted to establish whether this promoiety can be used as a drug carrier to the brain also in conjunction with other cationic and poorly brain penetrating parent drugs.

## ACKNOWLEDGMENTS AND DISCLOSURES

Lauri Peura, Kalle Malmioja, Jarkko Rautio and Krista Laine share equal contribution to this work.

The authors would like to express their profound gratitude to laboratory technicians Helly Rissanen and Jaana Leskinen for their skillful assistance. Marko Lehtonen, M.Sc., is also acknowledged for his skillful help in the HPLC-EC-analytics, Tiina Kääriäinen, Ph.D., for her skillful help in the animal studies and Ewen MacDonald, Ph.D., for refining the English of this paper. We also thank Henna Härkönen, M.Sc., for the polar surface area calculations. The work was financially supported by the Graduate School of Pharmaceutical Research, the Academy of Finland (# 132637), the Orion-Farmos foundation, the Finnish Pharmaceutical Society, the Finnish Parkinson Foundation and the Emil Aaltonen Foundation.

## REFERENCES

- Begley DJ. Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. Pharmacol Ther. 2004;104(1):29–45.
- Pardridge WM. Crossing the blood-brain barrier: are we getting it right? Drug Discov Today. 2001;6(1):1–2.
- Wager TT, Hou X, Verhoest PR, Villalobos A. Moving beyond rules: the development of a central nervous system multiparameter optimization (CNS MPO) approach to enable alignment of druglike properties. ACS Chem Neurosci. 2010;1(6):435–49.
- Pavan B, Dalpiaz A. Prodrugs and endogenous transporters: are they suitable tools for drug targeting into the central nervous system? Curr Pharm Des. 2011;17(32):3560–76.
- Gynther M, Ropponen J, Laine K, Leppänen J, Haapakoski P, Peura L, *et al.* Glucose promoiety enables glucose transporter mediated brain uptake of ketoprofen and indomethacin prodrugs in rats. J Med Chem. 2009;52(10):3348–53.
- Bonina F, Puglia C, Rimoli MG, Melisi D, Boatto G, Nieddu M, et al. Glycosyl derivatives of dopamine and L-dopa as anti-Parkinson prodrugs: Synthesis, pharmacological activity and *in vitro* stability studies. J Drug Target. 2003;11(1):25–36.
- Gynther M, Jalkanen A, Lehtonen M, Forsberg M, Laine K, Ropponen J, *et al.* Brain uptake of ketoprofen-lysine prodrug in rats. Int J Pharm. 2010;399(1–2):121–8.
- Gynther M, Laine K, Ropponen J, Leppänen J, Mannila A, Nevalainen T, *et al.* Large neutral amino acid transporter enables brain drug delivery via prodrugs. J Med Chem. 2008;51(4):932–6.
- Hokari M, Wu H, Schwarcz R, Smith QR. Facilitated brain uptake of 4-chlorokynurenine and conversion to 7-chlorokynurenic acid. Neuroreport. 1997;8(1):15–8.

- Killian DM, Chikhale PJ. A bioreversible prodrug approach designed to shift mechanism of brain uptake for amino-acidcontaining anticancer agents. J Neurochem. 2001;76(4):966–74.
- Bonina FP, Arenare L, Palagiano F, Saija A, Nava F, Trombetta D, *et al.* Synthesis, stability, and pharmacological evaluation of nipecotic acid prodrugs. J Pharm Sci. 1999;88(5):561–7.
- Balakrishnan A, Jain-Vakkalagadda B, Yang C, Pal D, Mitra AK. Carrier mediated uptake of L-tyrosine and its competitive inhibition by model tyrosine linked compounds in a rabbit corneal cell line (SIRC)—strategy for the design of transporter/receptor targeted prodrugs. Int J Pharm. 2002;247(1–2):115–25.
- Peura L, Malmioja K, Laine K, Leppänen J, Gynther M, Isotalo A, *et al.* Large amino acid transporter 1 (LAT1) prodrugs of valproic acid: new prodrug design ideas for central nervous system delivery. Mol Pharm. 2011;8(5):1857–66.
- Gomes P, Soares-Da-Silva P. L-DOPA transport properties in an immortalised cell line of rat capillary cerebral endothelial cells, RBE 4. Brain Res. 1999;829(1–2):143–50.
- Nutt JG. Pharmacokinetics and pharmacodynamics of levodopa. Mov Disord. 2008;23(S3):S580–4.
- Olanow C, Obeso J, Stocchi F. Drug insight: continuous dopaminergic stimulation in the treatment of Parkinson's disease. Nat Clin Pract Neurol. 2006;2(7):382–92.
- Chemuturi NV, Donovan MD. Role of organic cation transporters in dopamine uptake across olfactory and nasal respiratory tissues. Mol Pharm. 2007;4(6):936–42.
- Borgman RJ, McPhillips JJ, Stitzel RE, Goodman IJ. Synthesis and pharmacology of centrally acting dopamine derivatives and analogs in relation to Parkinson's disease. J Med Chem. 1973;16(6):630–3.
- Bodor N, Farag HH, Brewster III ME. Site-specific, sustained release of drugs to the brain. Science. 1981;214(4527):1370–2.
- Bodor N, Farag HH. Improved delivery through biological membranes. 11. A redox chemical drug-delivery system and its use for brain-specific delivery of phenylethylamine. J Med Chem. 1983;26 (3):313–8.
- Simpkins JW, Bodor N, Enz A. Direct evidence for brain-specific release of dopamine from a redox delivery system. J Pharm Sci. 1985;74(10):1033–6.
- Carelli V, Liberatore F, Scipione L, Impicciatore M, Barocelli E, Cardellini M, *et al.* New systems for the specific delivery and sustained release of dopamine to the brain. J Control Release. 1996;42(3):209–16.
- Denora N, Laquintana V, Lopedota A, Serra M, Dazzi L, Biggio G, *et al.* Novel L-Dopa and dopamine prodrugs containing a 2phenyl-imidazopyridine moiety. Pharm Res. 2007;24(7):1309–24.
- Fernández C, Nieto O, Fontenla JA, Rivas E, De Ceballos ML, Fernandez-Mayoralas A. Synthesis of glycosyl derivatives as dopamine prodrugs: interaction with glucose carrier GLUT-1. Org Biomol Chem. 2003;1(5):767–71.
- Fernández C, Nieto O, Rivas E, Montenegro G, Fontenla JA, Fernández-Mayoralas A. Synthesis and biological studies of glycosyl dopamine derivatives as potential antiparkinsonian agents. Carbohydr Res. 2000;327(4):353–65.
- More SS, Vince R. Design, synthesis and biological evaluation of glutathione peptidomimetics as components of anti-Parkinson prodrugs. J Med Chem. 2008;51(15):4581–8.
- Dent III WH, Erickson WR, Fields SC, Parker MH, Tromiczak EG.
  9-BBN: an amino acid protecting group for functionalization of amino acid side chains in organic solvents. Org Lett. 2002;4(8):1249–51.

- Ertl P, Rohde B, Selzer P. Fast calculation of molecular polar surface area as a sum of fragment-based contributions and its application to the prediction of drug transport properties. J Med Chem. 2000;43(20):3714–7.
- Takasato Y, Rapoport SI, Smith QR. An in situ brain perfusion technique to study cerebrovascular transport in the rat. Am J Physiol. 1984;247(3 Pt 2):H484–93.
- Smith QR, Allen DD. Methods in molecular medicine: the bloodbrain barrier: biology and research protocols. In: Sukriti Nag, editors. Totowa, NJ: Humana Press Inc.; 2003. p. 209–218.
- Krupka RM. Expression of substrate specificity in facilitated transport systems. JMB. 1990;117:69–78.
- 32. Liu X, Smith BJ, Chen C, Callegari E, Becker SL, Chen X, et al. Use of a physiologically based pharmacokinetic model to study the time to reach brain equilibrium: an experimental analysis of the role of blood-brain barrier permeability, plasma protein binding, and brain tissue binding. J Pharmacol Exp Ther. 2005;313 (3):1254–62.
- Walker I, Nicholls D, Irwin WJ, Freeman S. Drug delivery via active transport at the blood-brain barrier: affinity of a prodrug of phosphonoformate for the large amino acid transporter. Int J Pharm. 1994;104(2):157–67.
- 34. Ruocco LA, Viggiano D, Viggiano A, Abignente E, Rimoli MG, Melisi D, *et al.* Galactosylated dopamine enters into the brain, blocks the mesocorticolimbic system and modulates activity and scanning time in Naples high excitability rats. Neuroscience. 2008;152(1):234–44.
- 35. Dalpiaz A, Pavan B, Scaglianti M, Vitali F, Bortolotti F, Biondi C, *et al.* Transporter-mediated effects of diclofenamic acid and its ascorbyl pro-drug in the in vivo neurotropic activity of ascorbyl nipecotic acid conjugate. J Pharm Sci. 2004;93(1):78–85.
- 36. Manfredini S, Pavan B, Vertuani S, Scaglianti M, Compagnone D, Biondi C, *et al.* Design, synthesis and activity of ascorbic acid prodrugs of nipecotic, kynurenic and diclophenamic acids, liable to increase neurotropic activity. J Med Chem. 2002;45(3):559–62.
- van de Waterbeemd H, Smith DA, Jones BC. Lipophilicity in PK design: methyl, ethyl, futile. J Comput Aided Mol Des. 2001;15 (3):273–86.
- Levin VA. Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. J Med Chem. 1980;23(6):682–4.
- Elia J, Easley C, Kirkpatrick P. Lisdexamfetamine dimesylate. Nat Rev Drug Discov. 2007;6(5):343–4.
- Gong T, Huang Y, Zang Z, L L. Synthesis and characterization of 9-[P-(N, N-dipropylsulfamide)] benzoylamino-1,2,3,4–4H-acridine—a potential prodrug for the CNS delivery of tacrine. J Drug Target. 2004;12(3):177–82.
- Thurlow RJ, Hill DR, Woodruff GN. Comparison of the uptake of [3H]-gabapentin with the uptake of L-[3H]-leucine into rat brain synaptosomes. Br J Pharmacol. 1996;118(3):449–56.
- 42. Su T, Lunney E, Campbell G, Oxender DL. Transport of gabapentin, a  $\gamma$ -amino acid drug, by system L  $\alpha$ -amino acid transporters: a comparative study in astrocytes, synaptosomes, and CHO cells. J Neurochem. 1995;64(5):2125–31.
- Nutt JG, Fellman JH. Pharmacokinetics of levodopa. Clin Neuropharmacol. 1984;7(1):35–49.
- Gomes P, Soares-da-Silva P. Interaction between L-DOPA and 3-O-methyl-I-DOPA for transport in immortalised rat capillary cerebral endothelial cells. Neuropharmacology. 1999;38(9):1371–80.