0026-895X/02/6104-729–737$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2002 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 61:729–737, 2002

Vol. 61, No. 4
1329/972847
Printed in U.S.A.

Transport of Amino Acid-Related Compounds Mediated by L-Type Amino Acid Transporter 1 (LAT1): Insights Into the Mechanisms of Substrate Recognition

HIROSHI UCHINO, YOSHIKATSU KANAI, DO KYUNG KIM, MICHAEL F. WEMPE, ARTHIT CHAIROUNGDUA, EMIKO MORIMOTO, M. W. ANDERS, and HITOSHI ENDOU

Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan (H.U., Y.K., D.K.Y., A.C., E.M., H.E.); Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan (H.U.); Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation, Tokyo, Japan (Y.K.); and Department of Pharmacology and Physiology, School of Medicine and Dentistry, University of Rochester, Rochester, New York (M.F.W., M.W.A)

Received August 30, 2001; accepted January 7, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The L-type amino acid transporter 1 (LAT1) is an Na^+-independent neutral amino acid transporter subserving the amino acid transport system L. Because of its broad substrate selectivity, system L has been proposed to be responsible for the permeation of amino acid-related drugs through the plasma membrane. To understand the mechanisms of substrate recognition, we have examined the LAT1-mediated transport using a *Xenopus laevis* oocyte expression system. LAT1-mediated [^{14}C]phenylalanine uptake was strongly inhibited in a competitive manner by aromatic-amino acid derivatives including L-dopa, α-methyldopa, melphalan, triiodothyronine, and thyroxine, whereas phenylalanine methyl ester, *N*-methyl phenylalanine, dopamine, tyramine, carbidopa, and droxidopa did not inhibit [^{14}C]phenylalanine uptake. Gabapentin, a γ-amino acid, also exerted a competitive inhibition on LAT1-mediated [^{14}C]phenylalanine uptake. Although most of the compounds that inhibited LAT1-mediated uptake were able to induce the efflux of [^{14}C]phenylalanine preloaded to the oocytes expressing LAT1 through the obligatory exchange mechanism, melphalan, triiodothyronine, and thyroxine did not induce the significant efflux. Based on the experimental and semiempirical computational analyses, it is proposed that, for an aromatic amino acid to be a LAT1 substrate, it must have a free carboxyl and an amino group. The carbonyl oxygen closer to the amino group needs a computed charge of −0.55∼−0.56 and must not participate in hydrogen bonding. In addition, the hydrophobic interaction between the substrate side chain and the substrate binding site of LAT1 seems to be crucial for the substrate binding. A substrate, however, becomes a blocker once Connolly accessible areas become large and/or the molecule has a high calculated logP value, such as those for melphalan, triiodothyronine, and thyroxine.

System L is an amino acid transporter that transports large neutral amino acids in an Na^+-independent manner (Oxender and Christensen, 1963; Christensen, 1990). It is a major route through which living cells take up branched or aromatic amino acids from extracellular fluids. In addition, system L, as a basolateral membrane transport system, plays important roles in the absorption of amino acids through the epithelial cells of the small intestine and renal proximal tubules (Christensen, 1990). System L is also essential in the penetration of amino acids through the blood-brain barrier and the placenta barrier (Christensen, 1990). Because of its broad substrate selectivity, system L is proposed to transport not only naturally occurring amino acids but also amino acid-related compounds such as L-dopa, a therapeutic drug for Parkinsonism; melphalan, an anticancer phenylalanine mustard; triiodothyronine and thyroxine, two thyroid hormones; gabapentin, an anticonvulsant; and *S*-(1,2-dichlorovinyl)-L-cysteine, a neurotoxic cysteine conjugate (Goldenberg et al., 1979; Christensen, 1990; Lakshmanan et al., 1990; Blondeau et al., 1993; Patel et al., 1993; Su et al., 1995; Gomes and Soares-da-Silva, 1999). The precise examination on the interaction of these compounds with system L transporters, however, has been difficult using system L activity endogenous to cultured cells or tissue preparations, particularly for the compounds with low transport rate.

By means of expression cloning, we isolated a cDNA encoding the first isoform of system L transporter from C6 rat glioma cell cDNA library (Kanai et al., 1998). The transporter designated as L-type amino acid transporter 1 (LAT1) is a predicted 12-transmembrane protein and is unique because it requires an additional single membrane-spanning protein,

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science, the Promotion and Mutual Aid Corporation for Private Schools of Japan, the Japan Science and Technology Corporation, Japan Foundation for Applied Enzymology, and the Japan Health Sciences Foundation.

ABBREVIATIONS: LAT1, L-type amino acid transporter 1; 4F2hc, 4F2 heavy chain; AM1, Austin-model 1; ClogP, calculated logP.

the heavy chain of 4F2 cell surface antigen (4F2hc), for its functional expression in the plasma membrane (Kanai et al., 1998). LAT1 mediates Na^+-independent amino acid exchange and prefers large neutral amino acids with bulky or branched side chains for its substrates. Although the expression of 4F2hc is ubiquitous, the expression of LAT1 is restricted to certain tissues such as brain, placenta, and testis (Kanai et al., 1998). LAT1 is highly expressed in cultured cells and malignant tumors. presumably to support their continuous growth (Sang et al., 1995; Wolf et al., 1996; Kanai et al., 1998). Recently, we and others demonstrated that LAT1 and 4F2hc proteins are present in the luminal and abluminal membranes of brain capillary endothelial cells and mediate the permeation of amino acids through the blood-brain barrier (Duelli et al., 2000; Kageyama et al., 2000; Matsuo et al., 2000; Killian and Chikhale, 2001). After the identification of LAT1, transporters structurally related to LAT1 have been found to be associated with 4F2hc or another single membrane-spanning subunit rBAT (related to $b^{0,+}$ amino acid transporter) (Verrey et al., 2000). These transporters include systems asc, y^+L, x_C^- and $b^{0,+}$ as well as the second system L isoform, LAT2 (Fukasawa et al., 2000; Verrey et al., 2000). LAT2 is more ubiquitously expressed than LAT1 and transports not only large neutral amino acids but also small neutral amino acids (Pineda et al., 1999; Rossier et al., 1999; Segawa et al., 1999).

A remarkable characteristic of system L, as mentioned above, is its broad substrate selectivity, which enables the transporter to accept amino acid-related compounds. Because of this characteristic, system L is regarded as a drug transporter responsible for the determination of pharmacokinetics of amino acid-related drugs. To understand the mechanisms of substrate recognition, we have expressed system L transporter LAT1 in *Xenopus laevis* oocytes and examined LAT1-mediated transport of amino acids and amino acid-related compounds.

Experimental Procedures

Materials. L-[^{14}C]Phenylalanine, L-[^{14}C]tyrosine, [^{14}C]dopamine, [^{125}I]triiodothyronine, and [^{125}I]thyroxine were purchased from PerkinElmer (Boston, MA). L-[^{14}C]dopa was from American Radiolabeled Chemicals, Inc (St. Louis, MO). Gabapentin and droxidopa were provided by Parke-Davis Pharmaceutical Research (Ann Arbor, MI) and Sumitomo Pharmaceutical Co. Ltd (Osaka, Japan), respectively. Other chemicals were purchased from Sigma (St. Louis, MO). The chemical structures of amino acid related drugs used in the present investigation are shown in Fig. 1.

***X. laevis* Oocyte Expression.** Capped cRNAs for rat LAT1 and rat 4F2hc were synthesized in vitro using T7 RNA polymerase, as described elsewhere (Kanai et al., 1998). *X. laevis* oocyte expression studies were performed as described elsewhere with minor modifications (Kanai and Hediger, 1992; Utsunomiya-Tate et al., 1996). Briefly, oocytes were treated with collagenase A (2 mg/ml) (Roche Molecular Biochemicals, Mannheim Germany) for 30 to 50 min at room temperature in Ca^{2+}-free medium (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5) to remove follicular layer and then maintained in modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 10 mM HEPES, pH 7.5). For coexpression of LAT1 and 4F2hc in *X. laevis* oocytes, defolliculated oocytes were injected with LAT1 cRNA (15 ng) and 4F2hc cRNA (10 ng) to give a molar ratio of 1:1 (Kanai et al., 1998). After injection of cRNAs, the oocytes were incubated in the modified Barth's solution at 18°C until uptake was measured.

Uptake Measurement. Uptake measurements were performed 2 days after injection of cRNA. Groups of six to eight oocytes were washed in the uptake solution and then incubated in 500 μl of uptake solution (100 mM choline chloride, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 5 mM Tris, pH 7.4) containing 0.5 to 2.0 μCi/ml of radiolabeled compounds for 15 min at 22°C (Kanai et al., 1998). The oocytes were then washed five times with ice-cold uptake solution. The radioactivity was counted by liquid scintillation spectrometry, and the values are expressed in picomoles per oocyte per minute.

For measurement of the uptake of radiolabeled amino acids, six to eight oocytes were used for each data point. To confirm the reproducibility of the results, three separate experiments were performed for each measurement with different batches of oocytes and in vitro transcribed cRNA, except for K_m and K_i determination. Results from the representative experiments are shown in figures.

For the inhibition experiments, the uptake of 20 μM L-[^{14}C]phenylalanine by the oocytes expressing LAT1 and 4F2hc was measured in the presence or absence of 2 μM nonlabeled test compounds, unless otherwise indicated. For triiodothyronine, thyroxine, and melphalan, the effects of 50 or 100 mM nonradiolabeled compounds were examined on the uptake of 1 mM L-[^{14}C]phenylalanine in the uptake solution containing a final concentration of 1% DMSO.

K_m values were determined with the Eadie-Hofstee equation based on the LAT1-mediated L-leucine uptake measured at 3, 10, 30, 100, 300, and 1000 μM. LAT1-mediated amino acid uptake was calculated as the difference between the mean of uptake into the oocytes that had been injected with cRNAs for LAT1 and 4F2hc and those of control oocytes injected with water instead of cRNA.

To measure the K_i values for transport, oocytes expressing LAT1 and 4F2hc were incubated for 15 min in uptake solution with various

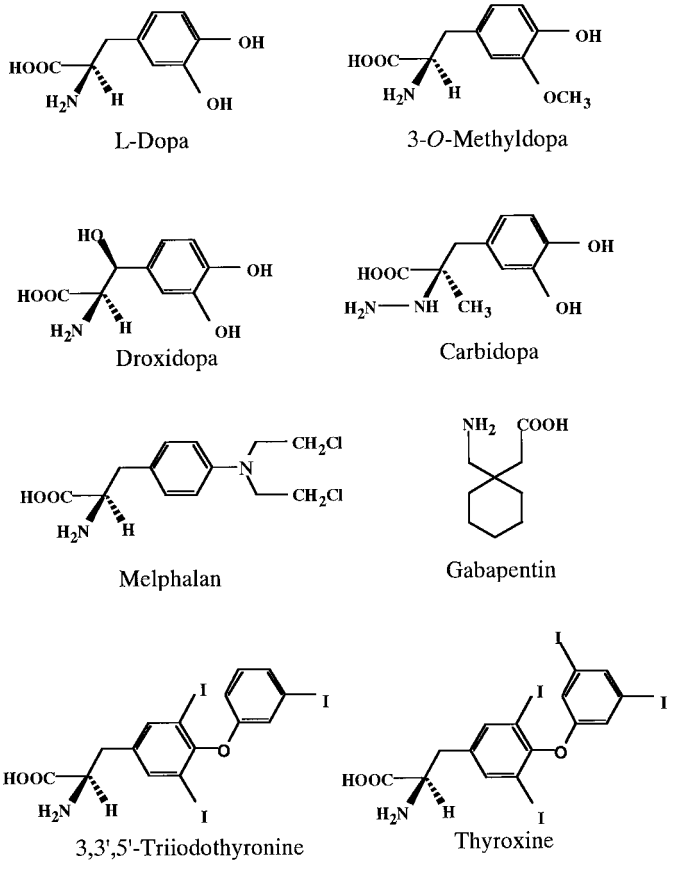

Fig. 1. Chemical structures of amino acid-related drugs.

concentration of L-[^{14}C]phenylalanine with or without addition of inhibitors. The K_i values were determined by double-reciprocal-plot analysis in which 1/uptake rate of L-[^{14}C]phenylalanine was plotted against 1/L-[^{14}C]phenylalanine concentration. The K_i values were calculated from the following equation when competitive inhibition was observed: K_i = concentration of inhibitor / [(K_m of phenylalanine with inhibitor/K_m of phenylalanine without inhibitor) − 1] (Apiwattanakul et al., 1999).

Efflux Measurements. Oocytes expressing LAT1 and 4F2hc were incubated for 30 min in the uptake solution containing 20 μM L-[^{14}C]phenylalanine (2 μCi/ml) to load the oocytes with L-[^{14}C]phenylalanine (Kanai et al., 1998). The oocytes were then washed five times with ice-cold uptake solution and transferred individually to separate wells of 48-well plates containing 150 μl of uptake solution with or without addition of 100 μM test compounds. After 5 min of incubation, 125 μl of incubation medium was removed from each well and mixed with an equal volume of 20% SDS. The oocytes were transferred to scintillation vials and solubilized with 10% SDS. The radioactivity in the medium and the remaining radioactivity in the oocytes were measured. The values were expressed as percentage radioactivity (radioactivity of medium or oocytes / (radioactivity of medium + radioactivity of oocytes oocytes) (Kanai et al., 1998).

Computational Analysis. Chemical structures were drawn with CS Chem-Draw Ultra (version 6.0; Cambridge Soft Corporation, Cambridge, MA) and copied into CS Chem3D Pro (version 6.0.1; Cambridge Soft Corporation). For each molecule, a molecular mechanics minimization was performed with a root-mean-square of 0.001. The CS MOPAC PRO application was used to compute, at the semiempirical Austin-model 1 (AM1) level of theory with a root-mean-square of 0.001, a theoretical dipole (Debye) and charge for the various atoms. Upon which, the property server was used to compute calculated logP (ClogP) and the Connolly accessible area (Å^2).

Statistical analysis. Values are shown as means ± S.E.M. (n = 6–8). Statistical differences were analyzed by Student's unpaired t test.

Results

Transport Activity. As shown in Fig. 2A, *X. laevis* oocytes that express LAT1 and 4F2hc exhibited a high level of L-[^{14}C]phenylalanine uptake compared with water-injected control oocytes. Uptake was time-dependent and exhibited a linear dependence on incubation time up to 30 min; all subsequent uptake measurements were conducted for 15 min and the values are expressed as picomoles per oocyte per minute. LAT1-mediated transport was calculated as the difference between uptake by oocytes expressing LAT1 and 4F2hc and that by water-injected control oocytes. As shown in Fig. 2B, LAT1-mediated L-[^{14}C]phenylalanine uptake was saturable and followed Michaelis-Menten kinetics with K_m values of 12.5 ± 3.1 μM (mean ± SEM of four separate experiments). Because the transport of L-[^{14}C]phenylalanine was not dependent on Na^+ or Cl^- (data not shown), transport measurements were performed under sodium-free conditions in subsequent experiments.

Inhibition of LAT1-Mediated Uptake by Amino Acid-Related Compounds. LAT1-mediated L-[^{14}C]phenylalanine uptake (20 μM) was measured in the presence of 2 mM concentrations of nonlabeled compounds. As shown in Fig. 3A, L-[^{14}C]phenylalanine uptake was markedly inhibited by tyrosine, L-dopa, 3-*O*-methyldopa, α-methylphenylalanine, α-methyltyrosine, α-methyldopa, and gabapentin. Triiodothyronine, thyroxine, and melphalan also inhibited L-[^{14}C]phenylalanine uptake (Fig. 3B). In contrast, *N*-methylphenylalanine, phenylalanine methyl ester, carbidopa, droxidopa, tyramine, and dopamine did not inhibit L-[^{14}C]phenylalanine uptake (Fig. 3A). As shown in Fig. 4A and B, tyrosine and L-dopa were competitive inhibitors of L-[^{14}C]phenylalanine uptake. 3-*O*-Methyldopa, α-methyldopa, α-methyltyrosine, gabapentin, triiodothyronine, thyroxine, and melphalan were also competitive inhibitors of LAT1-mediated L-[^{14}C]phenylalanine uptake (data not shown). The K_i values for these compounds are provided in Table 1. The semiempirical (AM1) computational data for

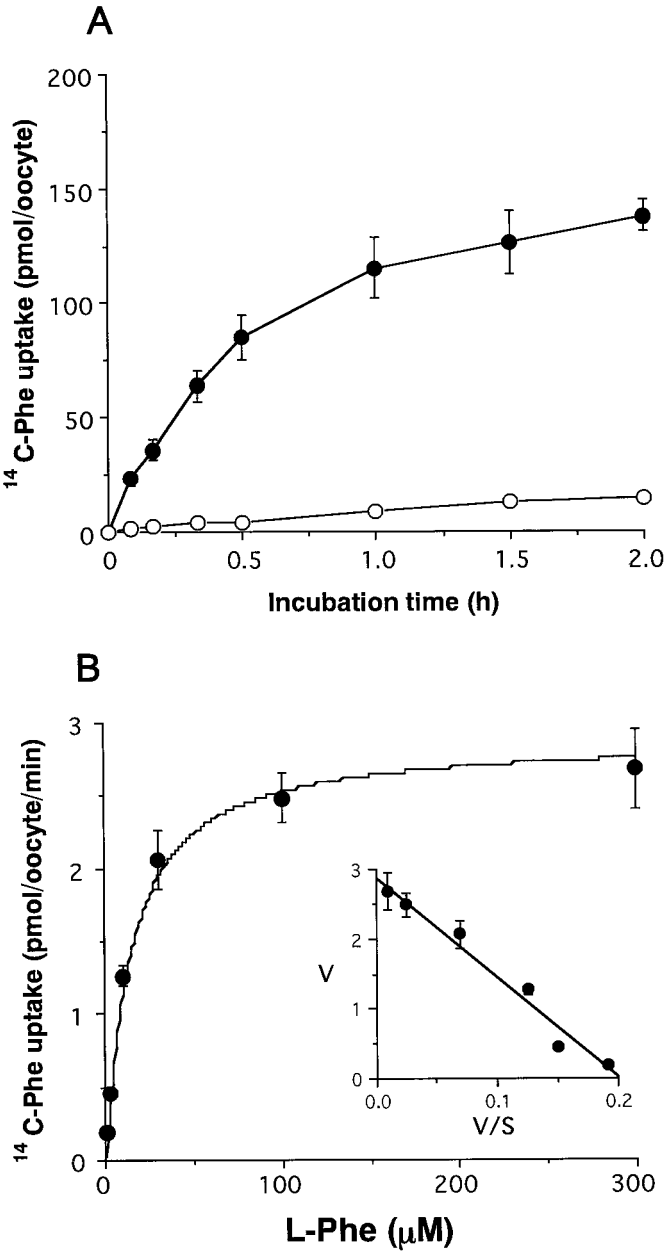

Fig. 2. L-[^{14}C]Phenylalanine uptake by the *X. laevis* oocytes coexpressing LAT1 and 4F2hc. A, time course of L-[^{14}C]phenylalanine (50 μM) uptake by oocytes that express both LAT1 and 4F2hc (●) and by the control oocytes injected with water instead of cRNAs (○). B, concentration dependence of LAT1-mediated L-[^{14}C]phenylalanine uptake. LAT1-mediated uptake of 1, 3, 10, 30, 100, and 300 μM L-phenylalanine was measured in oocytes coexpressing LAT1 and 4F2hc and is plotted against L-phenylalanine concentration. L-Phenylalanine uptake was saturable and was fit to the Michaelis-Menten equation (K_m = 14.2 μM; V_{max} = 2.88 pmol/oocyte/min). The inset shows an Eadie-Hofstee plot of L-phenylalanine uptake that was used to determine the kinetic parameters.

amino acids and amino acid-related compounds examined in the present study are provided in Table 2.

Transport of Amino Acid-Related Compounds. Among the compounds that inhibited LAT1-mediated L-[^{14}C]phenylalanine uptake, we examined whether L-dopa, triiodothyronine, and thyroxine, which are available as radiolabeled compounds, are transported by LAT1. As shown in Fig. 5A, L-[^{3}H]dopa as well as L-[^{14}C]tyrosine were transported by LAT1 as high-affinity substrates (Table 1). The K_m value for each coumpound was roughly close to the K_i value (Table 1). In contrast, [^{14}C]dopamine was not a substrate for LAT1. As shown in Fig. 5B, oocytes expressing LAT1 exhibited significantly higher uptake of [^{125}I]triiodothyronine and [^{125}I]thyroxine than control oocytes, although the levels of uptake were low compared with amino acid substrates. Uptake of [^{125}I]triiodothyronine and [^{125}I]thyroxine by LAT1 was not detected when uptake measurements were performed on ice, confirming the transporter-mediated uptake of these compounds (data not shown).

Characteristics of LAT1-Mediated Amino-Acid Efflux. In experiments in which oocytes were loaded with L-[^{14}C]phenylalanine and the efflux of loaded radioactivity induced by extracellularly applied nonlabeled phenylalanine was measured, we detected efflux of radioactivity confirming the previous observation indicating the obligatory exchange of substrate amino acids mediated by LAT1 (Fig. 6A). Almost half of the loaded radioactivity appeared in the extracellular medium in 30 min in the presence of 100 μM phenylalanine in the extracellular medium. The efflux of loaded L-[^{14}C]phenylalanine induced by 100 μM phenylalanine applied extracellularly was almost linear for up to 15 min (Fig. 6A); hence, efflux was measured for 10 min, and values are expressed as percentage of radioactivity loaded into oocytes in subsequent measurements. The efflux of loaded L-[^{14}C]phenylalanine induced by extracellularly applied phenylalanine followed Michaelis-Menten kinetics with a K_m value of 14.0 μM (Fig. 6B). In addition, the efflux of L-[^{14}C]phenylalanine was not dependent on extracellular Na^+ or Cl^- (data not shown).

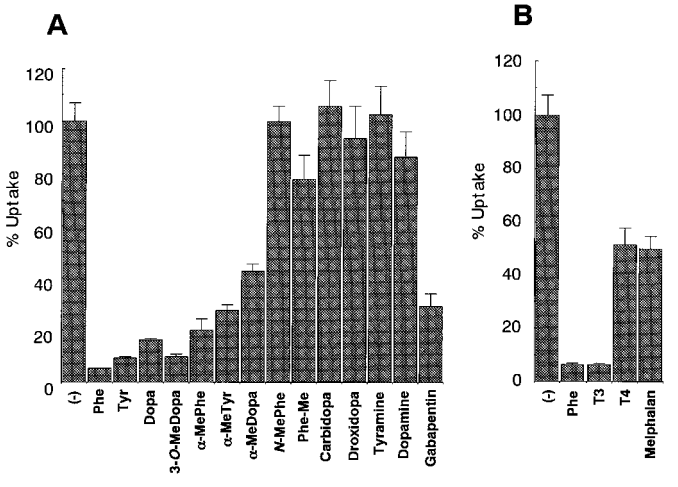

Fig. 3. Inhibition of L-[^{14}C]phenylalanine transport by amino acids and analogs. A, LAT1-mediated L-[^{14}C]phenylalanine uptake (20 μM) was measured in the absence (−) and presence of 2 mM nonradiolabeled phenylalanine (Phe), tyrosine (Tyr), L-dopa, 3-*O*-methyldopa (3-*O*-MeDopa), α-methylphenylalanine (α-MePhe), α-methyltyrosine (α-MeTyr), α-methyldopa (α-MeDopa), *N*-methylphenylalanine (*N*-MePhe), phenylalanine methyl ester (Phe-Me), carbidopa, droxidopa, tyramine, dopamine, and gabapentin. Uptake is expressed as percentage of L-[^{14}C]phenylalanine uptake measured in the absence of inhibitors (−). B, the effects of L-phenylalanine (Phe, 100 μM), triiodothyronine (T3, 50 μM), thyroxine (T4, 50 μM), and melphalan (100 μM) on the uptake of L-[^{14}C]phenylalanine (1 μM). Uptake is expressed as a percentage of L-[^{14}C]phenylalanine uptake measured in the absence (−) of inhibitors.

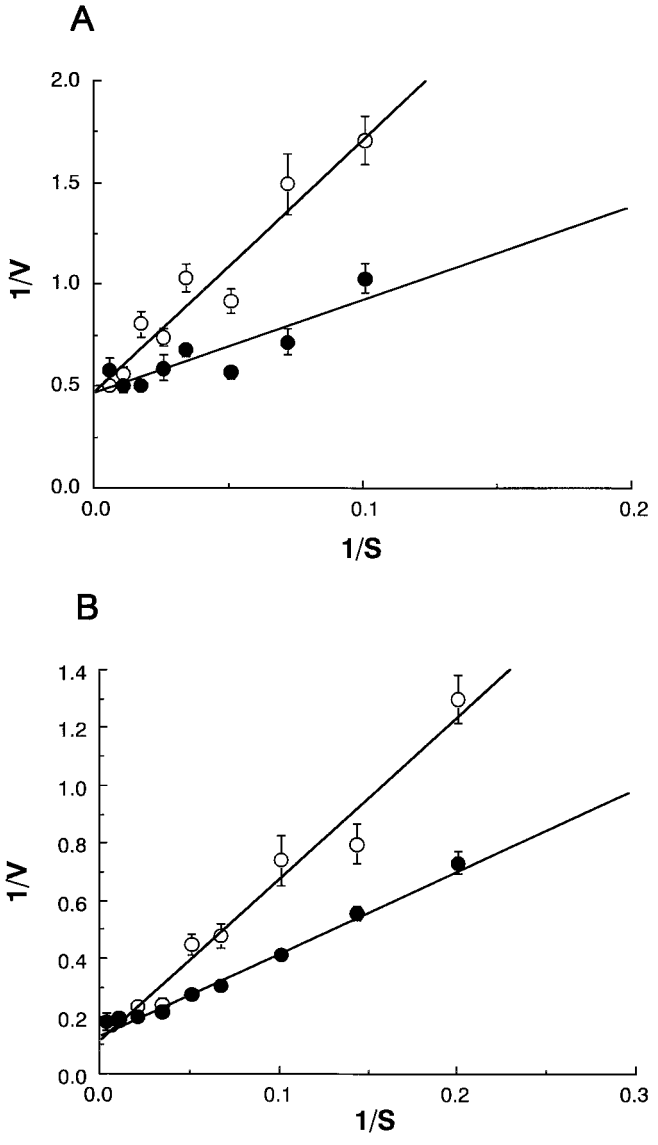

Fig. 4. Inhibitory effects of tyrosine and L-dopa on LAT1-mediated phenylalanine uptake. LAT1-mediated uptake of various concentrations of L-[^{14}C]phenylalanine was measured in the presence (○) or absence (●) of 20 μM tyrosine (A) or 50 μM L-dopa (B), and double-reciprocal-plot analyses were performed. K_i values were calculated as described under *Experimental Procedures*. Lines were fitted by the least squares method.

TABLE 1.
Kinetic parameters for amino-acid related compounds

Compounds	K_i	K_m
	μM	
Phenylalanine		12.5
Tyrosine	31.1	13.2
L-Dopa	67.2	34.2
3-*O*-Methyldopa	56.4	
α-Methyltyrosine	107	
α-Methyldopa	405	
Gabapentin	340	
Triiodothyronine	5.8	
Thyroxine	90.3	
Melphalan	49.1	

LAT1-Mediated Efflux Induced by Amino Acid-Related Compounds. The amino acid-related compounds that inhibited LAT1-mediated L-[^{14}C]phenylalanine uptake were investigated to determine whether they induce the efflux of loaded L-[^{14}C]phenylalanine when applied extracellularly. As shown in Fig. 7A, the efflux of loaded L-[^{14}C]phenylalanine was induced by the extracellularly applied tyrosine, L-dopa, 3-*O*-methyldopa, α-methylphenylalanine, α-methyltyrosine, α-methyldopa, and gabapentin (100 μM). Significant efflux was not induced by carbidopa, triiodothyronine, thyroxine, and melphalan (Fig. 7, A and B).

Discussion

LAT1 is selective for large neutral α-amino acids with branched or aromatic side chains as substrates (Kanai et al., 1998). Because *N*-methylphenylalanine and phenylalanine methyl ester had little effect on LAT1-mediated L-[^{14}C]phenylalanine uptake, it is suggested that both α-amino and α-carboxyl groups are recognized by the substrate-binding site of LAT1 (Fig. 3A). In agreement with this conclusion, tyramine and dopamine, both of which lack α-carboxyl groups, and carbidopa, an *N*-amino derivative of L-α-methyldopa (Fig. 1), failed to inhibit LAT1-mediated transport (Fig. 3A).

The semiempirical (AM1) computational data in Table 2 provides additional insight and support to the experimental conclusions. In the examination of the results for carbidopa and phenylalanine methyl ester, it is observed that the relative charges (Besler et al., 1990) residing on the carbonyl oxygen atoms (−0.3811 and −0.3924; −0.2805 and −0.3608, respectively) are substantially different from those for the molecules that inhibit L-[^{14}C]phenylalanine uptake, such as tyrosine and gabapentin (−0.5635 and −0.5948, respectively). Because all of the molecules investigated possess a chiral center, except gabapentin, the two resonance-stabilized carbonyl oxygens in the computed gas-phase molecules reside in chemically distinct environments and are therefore enantiotopic. Hence, from the calculated values in Table 2, it can be concluded that the resonance stabilized carbonyl oxygen closer in proximity to the amino group must have a computed charge of (−0.55 ∼ −0.56), except for gabapentin (−0.5948); not only is gabapentin a nonchiral γ-amino acid but it also contains a cyclohexane ring that experience ring-flips (Fig. 1) (Su et al., 1995), unlike all of the aromatic amino acid analogs that possess a weak field effect on the amino nitrogen (Wempe, 2001). In the case of *N*-methyl phenylalanine, the amino charge of −0.22 is far from ideal (−0.27) for the aromatic amino acids and therefore not recognized. Hence, both carbonyl oxygen charge (−0.55 ∼ −0.56) and amino charge (−0.27) are required for an aromatic amino acid to be a substrate of LAT1. This is in contrast to the system T transporter TAT1, which recognizes aromatic amino acids as anions (Kim et al., 2001).

LAT1 accepts α-methyl amino acids as substrates (Figs. 3A and 7A; Table 1), indicating that the binding site of LAT1 can accommodate methyl substituents on the α-carbon. It is therefore suggested that binding to the substrate binding site is dependent on the interaction of positive and negative charges of α-amino and α-carboxyl groups with the substrate-binding site and that an interaction between the α-carbon and the binding site is not essential. This is consistent with the observation that, as mentioned above, gabapentin, which is not an α-amino acid, is a substrate for LAT1 (Figs. 3A and 7A). Comparison of the charges on the α-carbons of L-dopa versus α-methyldopa (−0.2904 and −0.1990), and tyrosine versus α-methyltyrosine (−0.2872 and −0.1952) (Table 2) shows that the relative values are consistent with the conclusion that the α-carbon binding interaction is not the most essential interaction. In fact, the K_i values provided in Table 1 have the trend tyrosine < L-dopa < α-methyltyrosine < α-methyldopa, which is the same trend observed for the carbonyl oxygen's charge (−0.5635 < −0.5633 < −0.5579 < −0.5558) but not for the α-carbons.

Droxidopa, a β-hydroxy derivative of L-dopa (Fig. 1), failed to interact with LAT1 (Fig. 3A). A possible explanation for this is that a loss in electron-density associated with the

TABLE 2
Summary of computational analysis
The compounds are ordered according to calculated logP (ClogP).

Compound	ClogP	Connolly Accessible Area	Dipole	Charge				
				α-Carbon	Carbonyl C	Carbonyl O [a]		Nitrogen
		Å²	*Debye*					
Droxidopa	−1.573	357.98	8.586	−0.3317	0.4412	−0.4994[b]	−0.5432	−0.2635
L-Dopa	−0.4986	355.09	9.862	−0.2904	0.4440	−0.4701	−0.5633	−0.2709
Carbidopa [c]	−0.4448	383.65	3.239	−0.0055	0.3553	−0.3811	−0.3924	−0.3351
α-Methyldopa	−0.1896	372.95	9.697	−0.1990	0.4492	−0.4697	−0.5558	−0.2707
3-*O*-Methyldopa	−0.0524	394.53	10.645	−0.2883	0.4429	−0.4682	−0.5625	−0.2734
Tyrosine	0.0984	348.38	10.882	−0.2872	0.4420	−0.4691	−0.5635	−0.2742
Dopamine	0.2090	320.01	19.645	−0.2552				−0.2918
α-Methyltyrosine	0.4074	365.89	10.936	−0.1952	0.4473	−0.4704	−0.5579	−0.2711
Phenylalanine	0.7654	340.89	11.225	−0.2853	0.4417	−0.4700	−0.5628	−0.2755
Tyramine	0.8060	312.90	17.222	−0.2551				−0.2918
Phenylalanine methyl ester	1.006	385.02	7.209	−0.1536	0.3349	−0.2805	−0.3608	−0.2780
α-Methyl phenylalanine	1.074	358.13	10.749	−0.1951	0.4482	−0.4707	−0.5572	−0.2715
N-Methyl phenylalanine	1.521	369.57	11.510	−0.2807	0.4396	−0.4744	−0.5620	−0.2206
Gabapentin	1.642	336.33	10.446	−0.3378	0.3653	−0.5996	−0.5948	−0.3139
Melphalan	2.114	506.75	11.286	−0.2908	0.4430	−0.4719	−0.5653	−0.2696
3,3′,5′-Triiodothyronine	5.672	580.63	10.120	−0.2949	0.4461	−0.4664	−0.5545	−0.2775
Thyroxine	6.795	621.19	8.883	−0.3003	0.4466	−0.4645	−0.5534	−0.2752

[a] The second oxygen charge is the oxygen atom closer in proximity to the amino group.
[b] The oxygen atom interacting as a H-bond with the β-hydroxy group.
[c] Computed as the protonated carboxylic acid; -NH-NH2 (−0.3351 and −0.2804).

carboxylic acid (due to the intramolecular hydrogen bonding interaction between β-hydroxyl group and α-carboxyl group) decreases droxidopa's ability to interact with the binding site, although it is still possible that the β-hydroxy group apparently interferes with the substrate binding.

The affinity of LAT1 for phenylalanine, tyrosine, and L-dopa, which have zero, one, or two phenolic hydroxyl groups (Fig. 1), varies with the number of phenolic hydroxyl groups. As shown in Table 1, the K_m value of L-dopa is higher than those of phenylalanine and tyrosine. Consistent with this, the K_i value of L-dopa is higher than that of tyrosine (Table 1). This cannot be attributed to the bulkiness of the 3′-position of tyrosine, because 3-*O*-methyldopa, a methoxy derivative of L-dopa with more bulky moiety at the 3′-position, inhibited LAT1-mediated transport more strongly than L-dopa (Fig. 3A). The K_i values provided in Table 1, instead, have the trend tyrosine < 3-*O*-methyldopa < L-dopa, which is the same trend observed for calculated ClogP values (0.0984 > −0.0524 > −0.4986) (Table 2). Therefore, it is suggested that hydrophobicity is an important determinant for the binding of amino acid side chains to the substrate-binding site of LAT1 (Yunger and Cramer, 1981; Chollet et al., 1997). Hydrophobic interactions between substrate side chains and the substrate-binding site apparently play critical roles in the stability of the substrate binding.

Consistent with the observations on system L in cultured cells or membrane-vesicle preparations, LAT1-mediated

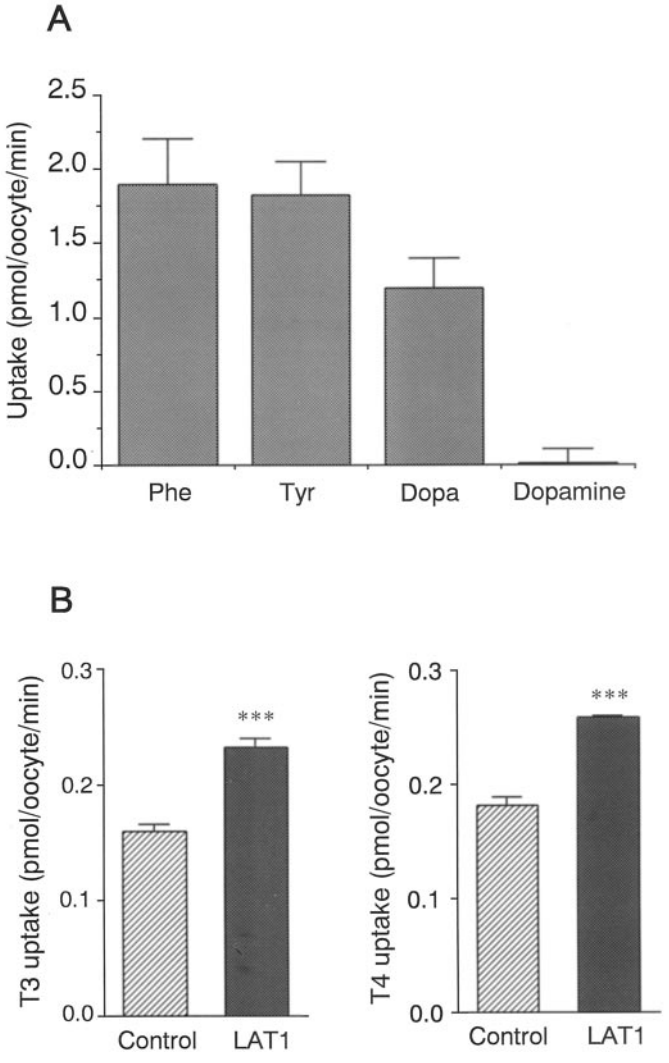

Fig. 5. LAT1-dependent uptake of amino acid-related compounds. A, LAT1-mediated uptake of L-[^{14}C]phenylalanine (Phe, 100 μM), L-[^{14}C]tyrosine (Tyr, 100 μM), L-[^{14}C]dopa (Dopa, 100 μM) and [^{14}C]dopamine (Dopamine, 100 μM). B, uptake of 30 μM [^{125}I]triiodothyronine (T3, left) and 30 μM [^{125}I]thyroxine (T4, right) was compared between control oocytes and LAT1-expressing oocytes.

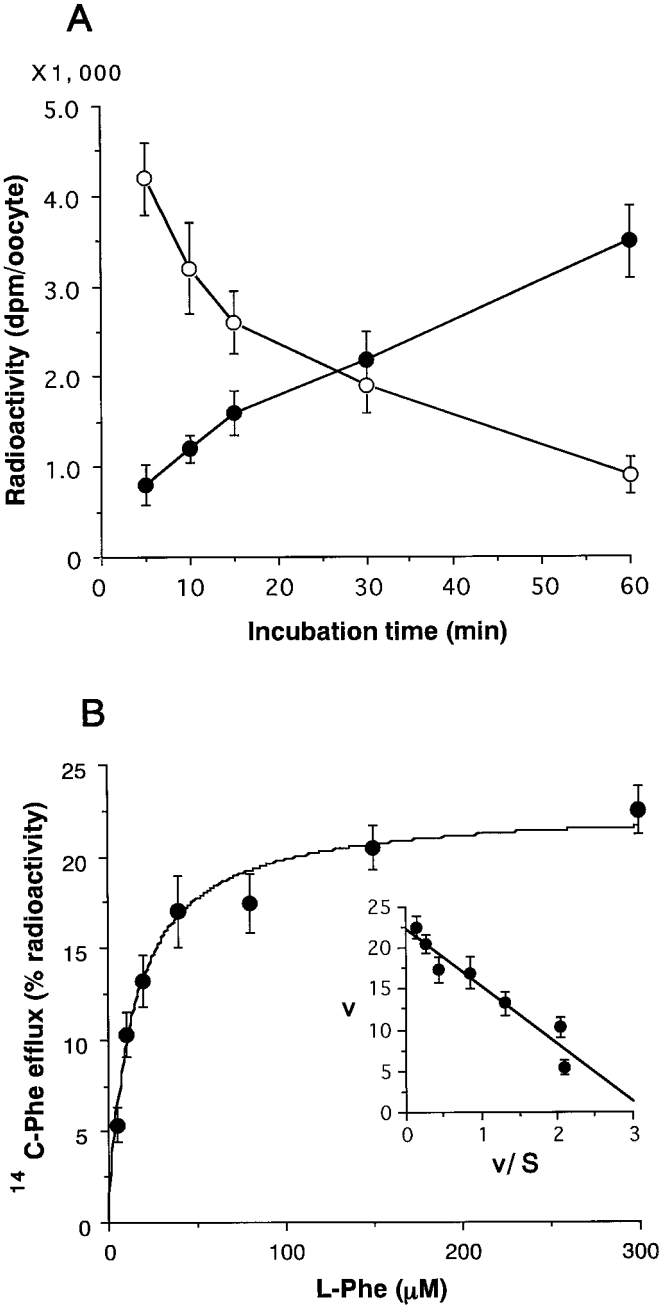

Fig. 6. LAT1-dependent L-[^{14}C]phenylalanine efflux. A, time course of L-[^{14}C]phenylalanine efflux (●) from the oocytes expressing LAT1 in the presence of 100 μM L-phenylalanine in the extracellular medium. The radioactivity remaining in the oocytes is shown with ○. B, the dependence of L-[^{14}C]phenylalanine efflux on the concentration of extracellular L-phenylalanine. The efflux of loaded L-[^{14}C]phenylalanine was induced by extracellularly applied L-phenylalanine in a concentration-dependent manner. Inset, Eadie-Hofstee plot of the L-[^{14}C]phenylalanine efflux used to determine kinetic parameters.

transport is inhibited by thyroid hormones, such as triiodothyronine and thyroxine, and by melphalan (Fig. 3B) (Goldenberg et al., 1979; Vistica, 1980; Lakshmanan et al., 1990; Blondeau et al., 1993; Prasad et al., 1994). It was previously reported that IU12/ASUR4, a *X. laevis* homolog of LAT1, accepts thyroid hormones as substrates (Ritchie et al., 1999). These amino acids with bulky side chains are competitive inhibitors of LAT1-mediated transport with K_i values comparable with those of L-dopa and other phenylalanine derivatives (Table 1). Therefore, the binding site of LAT1 can accommodate bulky side chains, such as those of thyroid hormones and melphalan. Interestingly, the K_i value of triiodothyronine is among the lowest of the compounds tested. Hydrophobic interactions between the side chain of triiodothyronine and the substrate-binding site of LAT1 are proposed to be strong enough to promote this high affinity.

In contrast to substrate amino acids, which exhibit high levels of uptake, the transport rates of [^{125}I]triiodothyronine and [^{125}I]thyroxine are low despite their high affinity for the binding site of LAT1 (Fig. 5). As discussed below, the transport rate of melphalan is also low. With the organic anion transporter OAT1, it was reported that the transport rate of nonsteroidal anti-inflammatory drugs is inversely correlated with the hydrophobicity of the compounds (Apiwattanakul et al., 1999). For OAT1, hydrophobic interactions are also regarded as important determinants of substrate binding (Apiwattanakul et al., 1999). Compounds with strong hydrophobic interactions with the binding site possess high affinity for the binding site, whereas the transport rate would, in contrast, be retarded, perhaps because of slow dissociation from the substrate binding site. Another possible explanation for their slow rate of transport is the bulkiness of their side chains. Thyroid hormones and melphalan have bulky side chains, which may interfere with conformational changes of the transporter protein associated with the translocation of substrates.

To evaluate whether compounds that inhibit LAT1-mediated transport are also transportable substrates or nontransportable blockers, we performed efflux measurements. Phenylalanine applied to the outside of oocytes induced the efflux of loaded L-[^{14}C]phenylalanine (Fig. 6). Efflux was dependent on incubation time and on the concentration of extracellularly applied L-phenylalanine (Fig. 6). The K_m value of extracellular L-phenylalanine required to induce the efflux of loaded L-[^{14}C]phenylalanine was close to that of L-[^{14}C]phenylalanine uptake, which is consistent with the concept of obligatory exchange for transport mediated by LAT1. Taking advantage of this exchange property, it is possible to evaluate whether compounds accepted by the binding site of LAT1 are transported or not by examining their ability to induce the efflux of loaded L-[^{14}C]phenylalanine. This strategy is, in particular, useful for compounds for which radiolabeled forms are not available (Apiwattanakul et al., 1999; Fukasawa et al., 2000; Kanai et al., 2000). As shown in Fig. 7A, phenylalanine, tyrosine, and L-dopa, which are transportable substrates of LAT1 (Fig. 5A), induce the efflux of loaded L-[^{14}C]phenylalanine, whereas carbidopa, which does not inhibit L-[^{14}C]phenylalanine uptake (Fig. 3A), does not induce efflux of loaded L-[^{14}C]phenylalanine. 3-*O*-Methyldopa, α-methylphenylalanine, α-methyltyrosine, α-methyldopa, and gabapentin also induced the efflux of loaded L-[^{14}C]phenylalanine, indicating that these compounds are transported by LAT1 (Fig. 7A).

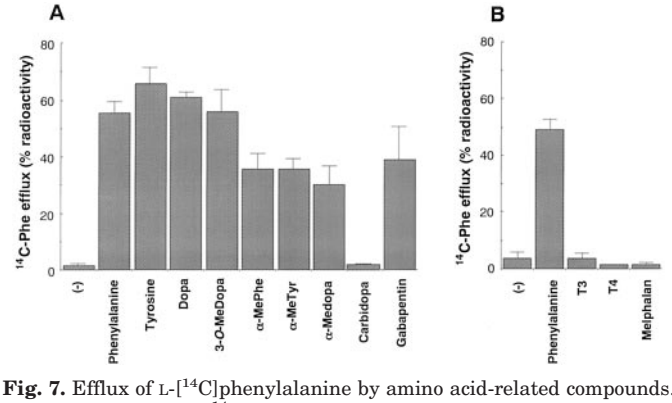

Fig. 7. Efflux of L-[^{14}C]phenylalanine by amino acid-related compounds. A, efflux of loaded L-[^{14}C]phenylalanine from the LAT1-expressing oocytes was measured in the absence (−) and presence of 100 μM phenylalanine, tyrosine, L-dopa, 3-*O*-methyldopa (3-*O*-MeDopa), α-methylphenylalanine (α-MePhe), α-methyltyrosine (α-MeTyr), α-methyldopa (α-Medopa), carbidopa, and gabapentin. B, efflux of loaded L-[^{14}C]phenylalanine was measured in the absence (−) and presence of L-phenylalanine (100 μM), triiodothyronine (T3, 50 μM), thyroxine (T4, 50 μM), or melphalan (100 μM).

In contrast, triiodothyronine, thyroxine, and melphalan did not induce detectable levels of efflux of loaded L-[^{14}C]phenylalanine (Fig. 7B). This observation is consistent with the results from the uptake measurement in which the transport rates of [^{125}I]triiodothyronine and [^{125}I]thyroxine were low compared with amino acid substrates (Fig. 5B). It is proposed that melphalan, along with thyroid hormones, is not transported at high rates. Triiodothyronine, thyroxine, and melphalan are thus not regarded as good substrates; indeed, they behave more like blockers even though they may be transported at low rates. It is interesting to note that melphalan, triiodothyronine, and thyroxine have relatively large Connolly accessible areas (>500Å^2), and have computed ClogP values greater than 1.80: 2.114, 5.672, and 6.795, respectively (Table 2).

Based on the results from the present study, we propose a model for the substrate-binding site of LAT1 (Fig. 8). Because both positive and negative charges at the α-carbon are required for transport, the substrate-binding site of LAT1 is proposed to possess the recognition sites that depend on electronic interactions with the peptide backbone of LAT1 (Fig. 8). The side chain-binding site is presumably associated with hydrophobic residues because hydrophobic interaction is critical for the binding of substrate amino acid side chains (Fig. 8). We proposed previously a model for the substrate binding for the system y^+L transporter y^+LAT1 which is structurally related to LAT1 and mediates Na^+-dependent transport of neutral amino acids and Na^+-independent transport of basic amino acids (Kanai et al., 2000). In the model, the side chain binding site is equipped with a positive charge recognition site. It is proposed that, although the substrate binding site of y^+LAT1 possesses similar spatial profile to that of LAT1, y^+LAT1 has acquired the additional mechanism for positive charge recognition in the course of evolution (Kanai et al., 2000).

Although our present study is based on the correlative relationships obtained in the somewhat nonphysiological *X. laevis* oocyte expression system, the structure-activity relationship data still allow the use of semiempirical (AM1) computational analysis to predict LAT-mediated transport. For

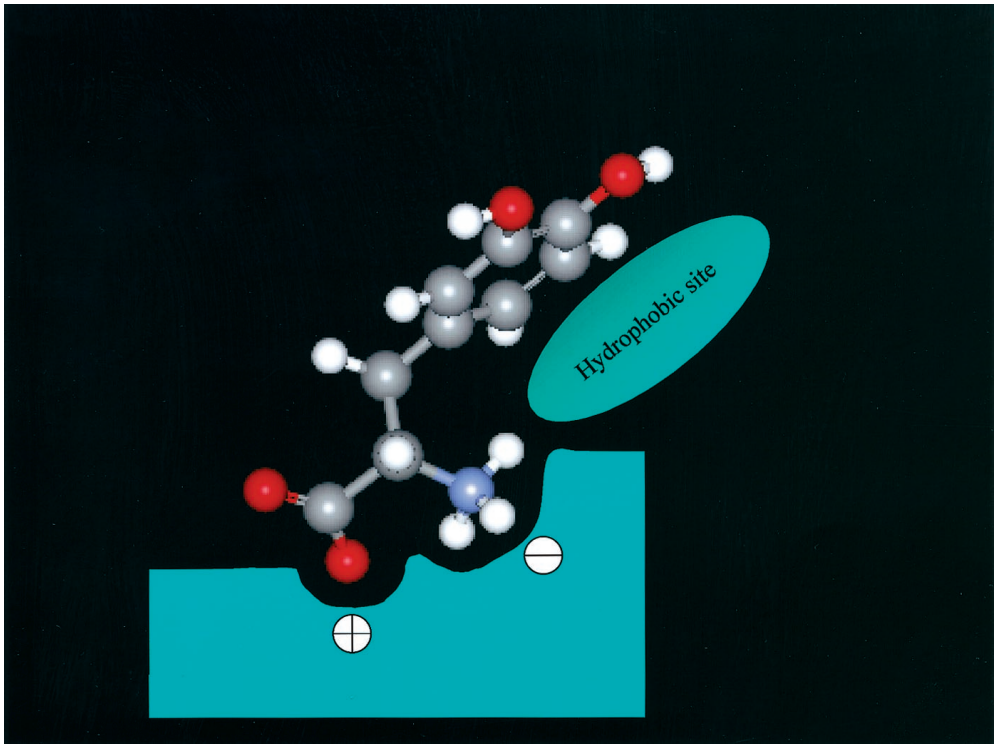

Fig. 8. Proposed model for the substrate-binding site of LAT1. The proposed mechanisms of substrate recognition are shown schematically for LAT1 and L-dopa as a model substrate. The binding site of LAT1 shown with green color is proposed to be composed of two sites: one for the binding of the positively charged α-amino group and the negatively charged α-carboxyl group (indicated by + and - symbols in the biding site), and the other for the binding of the substrate amino acid side chains (indicated as "Hydrophobic site"). The side chain binding site of LAT1 is proposed to accept large hydrophobic moieties. Carbon, hydrogen, oxygen, and nitrogen atoms are shown in gray, white, red, and blue, respectively.

an aromatic amino acid to be a LAT1 substrate, it must have a free carboxyl group. The carbonyl oxygen nearer the amino group needs a computed charge of ($-0.55 \sim -0.56$) and must not participate in hydrogen bonding. Furthermore, the molecule must contain an amino group with a computed nitrogen charge of ~ -0.27. These factors seem to be required for a molecule to be a substrate, whereas a substrate becomes a blocker once Connolly accessible areas become large (>500 Å^2) and/or the molecule has a calculated ClogP > 2.0, such as those for melphalan, triiodothyronine, and thyroxine.

Acknowledgments

We are grateful to Hisako Ohba and Michi Takahashi for technical assistance.

References

Apiwattanakul N, Sekine T, Chairoungdua A, Kanai Y, Nakajima N, Sophasan S, and Endou H (1999) Transport properties of nonsteroidal anti-inflammatory drugs by organic anion transporter 1 expressed in *Xenopus laevis* oocytes. *Mol Pharmacol* **55:**847–854.

Besler BH, Merz KM Jr, and Kollman PA (1990) Atomic charges derived from semiempirical methods. *J Comput Chem* **11:**431–439.

Blondeau JP, Beslin A, Chantoux F, and Francon J (1993) Triiodothyronine is a high-affinity inhibitor of amino acid transport system L1 in cultured astrocytes. *J Neurochem* **60:**1407–1413.

Chollet JF, Deletage C, Faucher M, Miginiac L, and Bonnemain JL (1997) Synthesis and structure-activity relationships of some pesticides with an alpha-amino acid function. *Biochim Biophys Acta* **1336:**331–341.

Christensen HN (1990) Role of amino acid transport and countertransport in nutrition and metabolism. *Physiol Rev* **70:**43–77.

Duelli R, Enerson BE, Gerhart DZ, and Drewes LR (2000) Expression of large amino acid transporter LAT1 in rat brain endothelium. *J Cereb Blood Flow Metab* **20:**1557–1562.

Fukasawa Y, Segawa H, Kim JY, Chairoungdua A, Kim DK, Matsuo H, Cha SH, Endou H, and Kanai Y (2000) Identification and characterization of a Na^+-independent neutral amino acid transporter that associates with the 4F2 heavy chain and exhibits substrate selectivity for small neutral D- and L- amino acids. *J Biol Chem* **275:**9690–9698.

Goldenberg GJ, Lam HY, and Begleiter A (1979) Active carrier- mediated transport of melphalan by two separate amino acid transport systems in LPC-1 plasmacytoma cells in vitro. *J Biol Chem* **254:**1057–1064.

Gomes P and Soares-da-Silva P (1999) L-DOPA transport properties in an immortalised cell line of rat capillary cerebral endothelial cells, RBE 4. *Brain Res* **829:**143–150.

Kageyama T, Nakamura M, Matsuo A, Yamasaki Y, Takakura Y, Hashida M, Kanai Y, Naito M, Tsuruo T, Minato N, et al. (2000) The 4F2hc/LAT1 complex transports L-DOPA across the blood-brain barrier. *Brain Res* **879:**115–121.

Kanai Y, Fukasawa Y, Cha SH, Segawa H, Chairoungdua A, Kim DK, Matsuo H, Kim JY, Miyamoto K, Takeda E, et al. (2000) Transport properties of a system y+L neutral and basic amino acid transporter: insights into the mechanisms of substrate recognition. *J Biol Chem* **275:**20787–20793.

Kanai Y and Hediger MA (1992) Primary structure and functional characterization of high-affinity glutamate transporter. *Nature (Lond)* **360:**467–471.

Kanai Y, Segawa H, Miyamoto K, Uchino H, Takeda E, and Endou H (1998) Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). *J Biol Chem* **273:**23629–23632.

Killian DM and Chikhale PJ (2001) Predominant functional activity of the large, neutral amino acid transporter (LAT1) isoform at the cerebrovasculature. *Neurosci Lett* **306:**1–4.

Kim DK, Kanai Y, Chairoungdua A, Matsuo H, Cha SH, and Endou H (2001) Expression cloning of a Na^+-independent aromatic amino acid transporter with structural similarity to H^+/monocarboxylate transporters. *J Biol Chem* **276:**17221–17228.

Lakshmanan M, Goncalves E, Lessly G, Foti D, and Robbins J (1990) The transport of thyroxine into mouse neuroblastoma cells, NB41A3: the effect of L-system amino acids. *Endocrinology* **126:**3245–3250.

Matsuo H, Tsukada S, Nakata T, Chairoungdua A, Kim DK, Cha SH, Inatomi J, Yorifuji H, Fukuda J, Endou H, et al. (2000) Expression of a system L neutral amino acid transporter at the blood-brain barrier. *Neuroreport* **11:**3507–3511.

Oxender DL and Christensen HN (1963) Evidence for two types of mediation of neutral amino acid transport in Ehrlich cells. *Nature (Lond)* **197:**765–767.

Patel NJ, Fullone JS, and Anders MW (1993) Brain uptake of S - (1,2-dichlorovinyl)glutathione and *S*-(1,2-dichlorovinyl)-L-cysteine, the glutathione and cysteine *S*-conjugates of the neurotoxin dichloroacetylene. *Mol Brain Res* **17:**53–58.

Pineda M, Fernandez E, Torrents D, Estevez R, Lopez C, Camps M, Lloberas J, Zorzano A, and Palacin M (1999) Identification of a membrane protein, LAT-2, that co-expressed with 4F2 heavy chain, an L-type amino acid transport activity with broad specificity for small and large zwitterionic amino acids. *J Biol Chem* **274:**19738–19744.

Prasad PD, Leibach FH, Mahesh VB, and Ganapathy V (1994) Relationship between

thyroid hormone transport and neutral amino acid transport in JAR human choriocarcinoma cells. *Endocrinology* **134:**574–581.
Ritchie JW, Peter GJ, Shi YB, and Taylor PM (1999) Thyroid hormone transport by 4F2hc-IU12 heterodimers expressed in *Xenopus* oocytes. *J Endocrinol* **163:**R5–R9.
Rossier G, Meier C, Bauch C, Summa V, Sordat B, Verrey F, and Kuhn LC (1999) LAT2, a new basolateral 4F2hc/CD98-associated amino acid transporter of kidney and intestine. *J Biol Chem* **274:**34948–34954.
Sang J, Lim Y-P, Panzia M, Finch P, and Thompson NL (1995) TA1, a highly conserved oncofetal complementary DNA from rat hepatoma, encodes an integral membrane protein associated with liver development, carcinogenesis, and cell activation. *Cancer Res* **55:**1152–1159.
Segawa H, Fukasawa Y, Miyamoto K, Takeda E, Endou H, and Kanai Y (1999) Identification and functional characterization of a Na^+-independent neutral amino acid transporter with broad substrate selectivity. *J Biol Chem* **274:**19745–19751.
Su TZ, Lunney E, Campbell G, and Oxender DL (1995) 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* **64:**2125–2131.
Utsunomiya-Tate N, Endou H, and Kanai Y (1996) Cloning and functional characterization of a system ASC-like Na^+-dependent neutral amino acid transporter. *J Biol Chem* **271:**14883–14890.
Verrey F, Meier C, Rossier G, and Kuhn LC (2000) Glycoprotein- associated amino acid exchangers: broadening the range of transport specificity. *Pflueg Arch Eur J Physiol* **440:**503–512.
Vistica DT (1980) Cytotoxicity as an indicator for transport mechanism: evidence that murine bone marrow progenitor cells lack a high-affinity leucine carrier that transports melphalan in murine L1210 leukemia cells. *Blood* **56:**427–429.
Wempe MF (2001) Quaternary ammonium ions can externally block voltage-gated K channels. Establishing a theoretical and experimental model that predicts KDS and the selectivity of K over Na ions. *J Mol Struct* **562:**63–78.
Wolf DA, Wang S, Panzia MA, Bassily NH and Thompson NL (1996) Expression of a highly conserved oncofetal gene, TA1/E16, in human colon carcinoma and other primary cancers: homology to *Schistosoma mansoni* amino acid permease and *Caenorhabditis elegans* gene products. *Cancer Res* **56:**5012–5022.
Yunger LM and Cramer RD,3rd (1981) Measurement of correlation of partition coefficients of polar amino acids. *Mol Pharmacol* **20:**602–608.

Address correspondence to: Dr. Yoshikatsu Kanai, Department of Pharmacology and Toxicology, Kyorin University School of Medicine, 6–20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan. E-mail: ykanai@kyorin-u.ac.jp