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# Characterization of the system L amino acid transporter in T24 human bladder carcinoma cells

Do Kyung Kim [a,b], Yoshikatsu Kanai [a,c,*], Hye Won Choi [a], Sahatchai Tangtrongsup [a], Arthit Chairoungdua [a], Ellappan Babu [a], Kittipong Tachampa [a], Naohiko Anzai [a], Yuji Iribe [a], Hitoshi Endou [a]

[a] *Department of Pharmacology and Toxicology, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan*
[b] *Department of Oral Physiology, Chosun University College of Dentistry, Gwangju 501-759, South Korea*
[c] *PRESTO, Japan Science and Technology Corporation (JST), 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan*

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**Abstract**

System L is a major nutrient transport system responsible for the $Na^+$-independent transport of large neutral amino acids including several essential amino acids. In malignant tumors, a system L transporter L-type amino acid transporter 1 (LAT1) is up-regulated to support tumor cell growth. LAT1 is also essential for the permeation of amino acids and amino acid-related drugs through the blood–brain barrier. To search for in vitro assay systems to examine the interaction of chemical compounds with LAT1, we have investigated the expression of system L transporters and the properties of [$^{14}$C]L-leucine transport in T24 human bladder carcinoma cells. Northern blot, real-time quantitative PCR and immunofluorescence analyses have reveled that T24 cells express LAT1 in the plasma membrane together with its associating protein 4F2hc, whereas T24 cells do not express the other system L isoform LAT2. The uptake of [$^{14}$C]L-leucine by T24 cells is $Na^+$-independent and almost completely inhibited by system L selective inhibitor BCH. The profiles of the inhibition of [$^{14}$C]L-leucine uptake by amino acids and amino acid-related compounds in T24 cells are comparable with those for the LAT1 expressed in *Xenopus* oocytes. The majority of [$^{14}$C]L-leucine uptake is, therefore, mediated by LAT1 in T24 cells. Consistent with LAT1 in *Xenopus* oocytes, the efflux of preloaded [$^{14}$C]L-leucine is induced by extracellularly applied substrates of LAT1 in T24 cells. This efflux measurement has been proven to be more sensitive than that in *Xenopus* oocytes, because triiodothyronine, thyroxine and melphalan were able to induce the efflux of preloaded [$^{14}$C]L-leucine in T24 cells, which was not detected for *Xenopus* oocyte expression system. T24 cell is, therefore, proposed to be an excellent tool to examine the interaction of chemical compounds with LAT1.


*Keywords:* Amino acid transporter; System L; Malignant tumor; Blood–brain barrier

## 1. Introduction

System L is a plasma membrane amino acid transport system which mediates $Na^+$-independent transport of large neutral amino acids [1,2]. It is a major route through which living cells including tumor cells take up branched or aromatic amino acids. In addition, system L is present in the basolateral membrane of epithelial cells and plays important roles in the absorption of amino acids through the epithelial cells of small intestine and renal proximal tubules [1]. System L is also pivotal in the permeation of amino acids through the blood–tissue barriers such as blood–brain barrier and placenta barrier [1]. A remarkable feature of system L is its broad substrate selectivity [3]. Because of this property, system L is regarded as a drug transporter which transports not only naturally occurring amino acids but also amino acid-related drugs such as L-dopa, melphalan, thyroid hormones and gabapentin [1,4–8].

By expression cloning, we isolated the first isoform of system L amino acid transporter LAT1 (L-type amino acid

*Abbreviations:* LAT1, L-type amino acid transporter 1; LAT2, L-type amino acid transporter 2; 4F2hc, 4F2 heavy chain; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid

* Corresponding author. Department of Pharmacology and Toxicology, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan. Tel.: +81-422-47-5511x3453; fax: +81-422-79-1321.
*E-mail address:* ykanai@kyorin-u.ac.jp (Y. Kanai).

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transporter 1) from C6 rat glioma cells [9]. LAT1 is a 12-membrane-spanning protein which mediates a $Na^+$-independent amino acid exchange. It prefers large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine and histidine for its substrates [9–11]. We and other researchers further demonstrated that a single-membrane-spanning protein, the heavy chain of 4F2 antigen (4F2hc), is essential for the functional expression of LAT1. LAT1 and 4F2hc form a heterodimeric complex via a disulfide bond [9,10,12–15]. Although the expression of 4F2hc is ubiquitous, the expression of LAT1 is restricted to certain tissues such as brain, placenta and testis [9,10]. LAT1 is highly expressed in cultured cells and malignant tumors, presumably to support their continuous growth [9,10,16,17]. Recently, it was demonstrated that LAT1 and 4F2hc proteins are present in the luminal and abluminal membranes of brain capillary endothelial cells, components of the blood–brain barrier [18–21]. Following 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) [3,22]. These transporters include systems asc, $y^+L$, $x_c^-$ and $b^{0,+}$ as well as the second system L isoform, LAT2 [3,22]. LAT2 is more ubiquitously expressed than LAT1 and transports not only large neutral amino acids but also small neutral amino acids [23–25].

Based on what is mentioned above, it is proposed that the manipulation of system L activity, in particular that of LAT1, would have therapeutic implications. The inhibition of LAT1 activity in tumor cells could be effective in the inhibition of tumor cell growth by depriving tumor cells of essential amino acids [3]. The availability of in vitro assay systems for the interaction with LAT1 would facilitate the development of such drugs. It would, in addition, be beneficial to design drugs with efficient permeation through the blood–brain barrier utilizing LAT1 as a permeation path. The functional properties of LAT1 have been studied formerly by injecting LAT1 cRNA into *Xenopus* oocytes or by transiently transfecting LAT1 cDNA to cultured mammalian cells, which is not well-suited for the efficient screening of chemical compounds [9–12,15,26]. Therefore, we have characterized the system L-mediated amino acid transport in T24 human bladder carcinoma cells, which was previously shown to express high levels of messages for LAT1 and 4F2hc [10]. We report here that L-leucine uptake by T24 cells is almost exclusively mediated by LAT1, so that T24 cells would be an excellent tool to examine the interaction of chemical compounds with LAT1.

## 2. Materials and methods

### 2.1. Materials

[$^{14}$C]L-leucine was purchased from Perkin Elmer Life Sciences Inc. (Boston, MA). Gabapentin and droxidopa were kindly provided by Parke-Davis Pharmaceutical Research (Ann Arbor, MI) and Sumitomo Pharmaceutical Co. Ltd. (Osaka, Japan), respectively. Mouse anti-human CD98 (4F2hc) monoclonal antibody was purchased from Ancell (Bayport, MN). Affinity-purified rabbit anti-LAT1 polyclonal antibody was supplied by Kumamoto Immunochemical Laboratory, Transgenic Inc. (Kumamoto, Japan). Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated rabbit anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) and DAKO (Glostrup, Denmark), respectively. Other chemicals were purchased from Sigma (St. Louis, MO). T24 human bladder carcinoma cells were provided by the Health Science Research Resources Bank, Japan Health Sciences Foundation (Osaka, Japan).

### 2.2. Northern blot analysis

RNA was prepared form T24 cells maintained in the growth medium (minimum essential medium supplemented with 10% fetal bovine serum) at 37 °C by the guanidinium isothiocyanate method using cesium–trifluoroacetic acid (Amersham Pharmacia Biotech) in accordance with the manufacturer's instruction. Poly(A)$^+$RNA was selected by oligo(dT) cellulose chromatography (Amersham Pharmacia Biotech) [27]. Human kidney poly(A)$^+$RNA was purchased from Clontech. Poly(A)$^+$RNA (3 μg/lane) was separated on a 1% agarose gel in the presence of 2.2 M formaldehyde and was blotted onto a nitrocellulose filter (Schleicher and Schuell) as described elsewhere [27]. The *Sma*I fragment of human LAT1 cDNA corresponding to 649–1128 bp of human LAT1 nucleotide sequence (GenBank/EMBL/DDBJ accession no. AB018009), the *Pst*I fragment of human 4F2hc cDNA corresponding to 106–645 bp of human 4F2hc nucleotide sequence (GenBank/EMBL/DDBJ accession no. AB018010) and the *Bst*EII fragment of human LAT2 cDNA corresponding to 1001–1847 bp of human LAT2 nucleotide sequence (GenBank/EMBL/DDBJ accession no. AB037669) were labeled with [$^{32}$P]dCTP using a $^{T7}$Quick prime kit (Pharmacia). Hybridization was performed for 20 h at 42 °C as described elsewhere [27]. The filters were washed in 0.1× SSC/0.1% SDS at 65 °C [27].

### 2.3. Real-time quantitative RT-PCR

For real-time quantitative RT-PCR analysis, total RNA was prepared from T24 cells using an RNA preparation kit (Isogen, Nippon-Gene, Japan) following the manufacturer's instructions. The total RNA was treated with DNase I (Boehringer Mannheim; 1 unit per 10 mg of RNA) at 25 °C for 20 min. First-strand cDNA was produced using SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies) with an oligo dT primer [28]. Five micrograms of total RNA were used for a reverse transcription reaction (20 μl). For the real-time quantitative PCR, 1 μl out of the 20 μl reverse transcription reaction mixture was used.

Standard first-strand cDNAs for LAT1, LAT2 and 4F2hc were synthesized from each cRNA. LAT1 cRNA was obtained by in vitro transcription using T3 RNA polymerase from the LAT1 cDNA (GenBank/EMBL/DDBJ accession no. AB018009) in pBluescript II SK $^-$ (Stratagene) linearized with *Xho*I [29]. LAT2 and 4F2hc cRNAs were also obtained by in vitro transcription using SP6 RNA polymerase for LAT2 cDNA (GenBank/EMBL/DDBJ accession no. AB037669) in pSPORT 1 (Life Technologies) linearized with *Rsr*II and T7 RNA polymerase for 4F2hc cDNA (GenBank/EMBL/DDBJ accession no. AB018010) in pBluescript II SK $^-$ linearized with *Bam*HI, respectively [29]. The first-strand cDNAs were synthesized as described above using cRNAs for LAT1, LAT2 and 4F2hc as templates [28].

The 7700 Sequence Detector System (Perkin Elmer/Applied Biosystems) was used for real-time quantitative RT-PCR analyses [30]. The RT-PCR reaction mixture (50 μl) included reverse transcription products corresponding to 250 ng of total RNA or standard first-strand cDNAs corresponding to 25 fg–25 ng of cRNA, 25 μl of Taqman Universal PCR Master Mix (Perkin Elmer/Applied Biosystems), 0.5 μl of forward and reverse primer (10 μM) and 1 μl of corresponding Taqman probe (5 μM) [30]. RT-PCR cycle parameters were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The primers and the probes were designed using Primer Express software (Perkin Elmer/Applied Biosystems) and synthesized by Perkin Elmer/Applied Biosystems. For LAT1, the forward and reverse primers were 5′-GAT CCT GCT GGG CTT CGT-3′ and 5′-AGT TTG GTG CCT TCA AAT GAG AA-3′, respectively, and the Taqman probe was 6 FAM-AGA TCG GGA AGG GTG ATG TGT CCA ATC-TAMRA. For 4F2hc, the forward and reverse primers were 5′-CTC AGG CAA GGC TCC TGA CT-3′ and 5′-GGC AGG GTG AAG AGC ATC A-3′, respectively, and the Taqman probe was 6 FAM-TGC CGG CTC AAC TTC TTC GAC TCT AC-TAMRA. For LAT2, the forward and reverse primers were 5′-AAT GCA TTT GAG AAT TTC CAG GA-3′ and 5′-GAG CCC TGA AGG AAA GCC A-3′, respectively, and the Taqman probe was 6 FAM-CCT GAC ATC GGC CTC GTC GCA-TAMRA. The concentration of reverse and forward primers used was 900 nM for each. The concentration of probe was 250 nM.

### 2.4. Immunofluorescence of T24 cells

T24 cells on the culture plates were fixed with 4% paraformaldehyde for 1 h. The cells were collected by scraping and centrifugation at 1000 rpm for 5 min. The cells were then resuspended in warmed 2% agar solution, centrifuged at 3000 rpm for 5 min and cooled for 30 min at 4 °C. The cell pellets in solid agar were again fixed with 4% paraformaldehyde for 3 h, embedded in paraffin wax and processed for paraffin sectioning.

Sections (3 μm) were dewaxed, rehydrated and incubated with affinity-purified anti-LAT1 polyclonal antibody (1:100) overnight at 4 °C and then with mouse anti-human CD98 (4F2hc) monoclonal antibody (Ancell; diluted 1:100) for 2 h at room temperature [18]. Thereafter, they were treated with Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 1 h, and then, FITC-conjugated rabbit anti-mouse IgG (DAKO) for 1 h at room temperature. Images were acquired using a Carl Zeiss laser-scanning confocal microscope LSM 510 equipped with a X63 immersion objective [18]. An He–Ne laser beam was used for excitation at 543 nm for Cy3 visualization. Emission from Cy3 was detected via an LP585 filter. For FITC visualization, an argon laser beam was used for excitation at 488 nm. Emission from FITC was detected via a LP505 filter.

### 2.5. Uptake measurements in T24 cells

T24 cells were maintained in the growth medium (minimum essential medium supplemented with 10% fetal bovine serum) at 37 °C in 5% $CO_2$. The cells were collected and seeded on 24-well plates ($1 \times 10^5$ cells/well) in the fresh growth medium. The uptake measurements were performed at 48 h after seeding.

After the removal of the growth medium, the cells were washed three times with the standard uptake solution (125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, 1.2 mM $KH_2PO_4$, 5.6 mM glucose, pH 7.4) or $Na^+$-free uptake solution (125 mM choline-Cl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, 1.2 mM $KH_2PO_4$, 5.6 mM glucose, pH 7.4), and preincubated for 10 min at 37 °C. Then, the medium was replaced by the uptake solution containing [$^{14}$C]L-leucine. The uptake was terminated by removing the uptake solution followed by washing three times with ice-cold uptake solution [31,32]. Then, cells were solubilized with 0.1 N NaOH and radioactivity was counted by liquid scintillation spectrometry. The values are expressed as pmol/mg protein/min. For the measurement of the uptake of [$^{14}$C]L-leucine, four to six wells of T24 cells were used for each data point. To confirm the reproducibility of the results, three or four separate experiments were performed for each measurement. Results from the representative experiments are shown in the figures.

For the inhibition experiments, the uptake of 20 μM [$^{14}$C]L-leucine was measured in the presence or absence of 2 mM nonlabeled test compounds, unless otherwise indicated [9–11]. For triiodothyronine, thyroxine and melphalan, the effects of 100 μM nonradiolabeled compounds were examined on the uptake of 1 μM [$^{14}$C]L-leucine in the uptake solution containing a final concentration of 0.1% DMSO [10,11].

$K_m$ and $V_{max}$ values were determined using Eadie–Hofstee plots based on the [$^{14}$C]L-leucine uptake measured for 1 min at 1, 3, 10, 20, 30, 100, 300 and 1000 μM. To determine the $K_i$ values, the uptake rates of [$^{14}$C]L-leucine were measured for 1 min at varied con-

centrations with or without addition of inhibitors. The $K_i$ values were determined by double-reciprocal plot analysis in which 1/uptake rate of [$^{14}$C]L-leucine was plotted against 1/L-leucine concentration. The $K_i$ values were calculated from the following equation when competitive inhibition was observed: $K_i$ = concentration of inhibitor/((($K_m$ of L-leucine with inhibitor/$K_m$ of L-leucine without inhibitor) − 1) [11,33].

### 2.6. Efflux measurements

T24 cells were incubated for 10 min in the uptake solution containing 1 μM [$^{14}$C]L-leucine (2 μCi/ml) to load the T24 cells with [$^{14}$C]L-leucine [9–11]. The T24 cells were then washed three times with uptake solution and incubated in 500 μl uptake solution with or without addition of test compounds. After incubation, 500 μl of incubation medium was removed from the well. Then, cells were solubilized with 0.1 N NaOH and the radioactivity was counted by liquid scintillation spectrometry. The radioactivity in the medium and the remaining radioactivity in the T24 cells were measured. The values were expressed as % radioactivity (radioactivity of medium or T24 cells/(radioactivity of medium + radioactivity of T24 cells)) [9,11]. $K_m$ values of the [$^{14}$C]L-leucine efflux induced by extracellularly applied L-leucine was determined using the Eadie–Hofstee plot based on the [$^{14}$C]L-leucine efflux measured for 1 min at 1, 10, 30, 100, 300, 1000 and 3000 μM of extracellularly applied L-leucine [11].

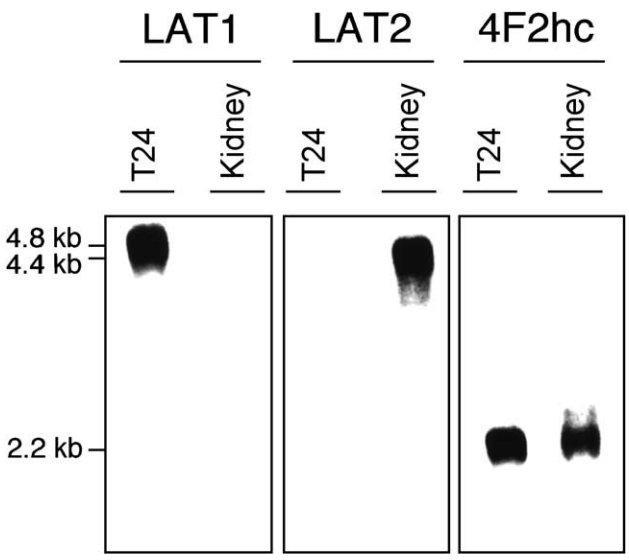


Fig. 1. Expressions of LAT1 and 4F2hc in T24 cells. Northern blots of poly(A)$^+$ RNA isolated from T24 cells (T24) were compared with those of human kidney poly(A)$^+$ RNA (Kidney). High stringency Northern blot analysis of poly(A)$^+$ RNA (3 μg) from T24 cells and human kidney were performed using $^{32}$P-labeled LAT1, LAT2 and 4F2hc cDNAs as probes. In T24 cells, strong hybridization was detected for LAT1 (4.8 kb) and 4F2hc (2.2 kb), but not for LAT2. Human kidney poly(A)$^+$ RNA was used as a positive control for LAT2 (4.4 kb).

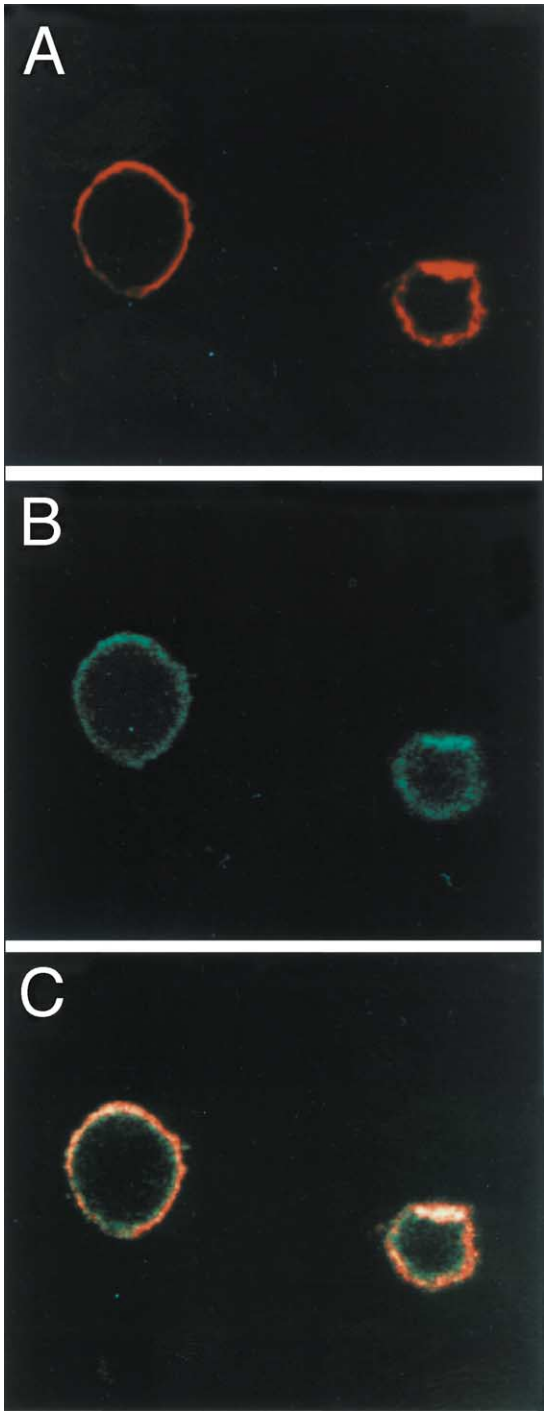


Fig. 2. Immunodetection of LAT1 and 4F2hc in T24 cells by confocal laser-scanning microscopy. Confocal immunofluorescence microscopic analysis was performed on T24 cells as described in Materials and methods. Both LAT1 immunoreactivity (red; A) and 4F2hc immunoreactivity (green; B) were detected on the plasma membrane of T24 cells. LAT1 immunostaining (red) and 4F2hc immunostaining (green) were merged and shown in (C), indicating the coexistence of LAT1 and 4F2hc in the plasma membrane of T24 cells.

### 2.7. Data analysis

All experiments were performed in triplicate. Results are presented as mean ± S.E. Statistical significance was analyzed by using Student's *t*-test for two groups and one-way analysis of variance for multi-group comparisons. $P < 0.05$ is considered statistically significant.

## 3. Results

### 3.1. Detection of LAT1 as the system L transporter in T24 cells

In Northern blot analyses, mRNAs for LAT1 and its associating protein 4F2hc were detected in T24 cells (Fig. 1). The LAT2 message was not detected in T24 cells, whereas human kidney poly(A)$^+$ RNA used as a positive control for LAT2 [23,24] exhibited high levels of expression of LAT2 as well as 4F2hc (Fig. 1). Consistent with the previous report, LAT1 message was not detected in human kidney [10].

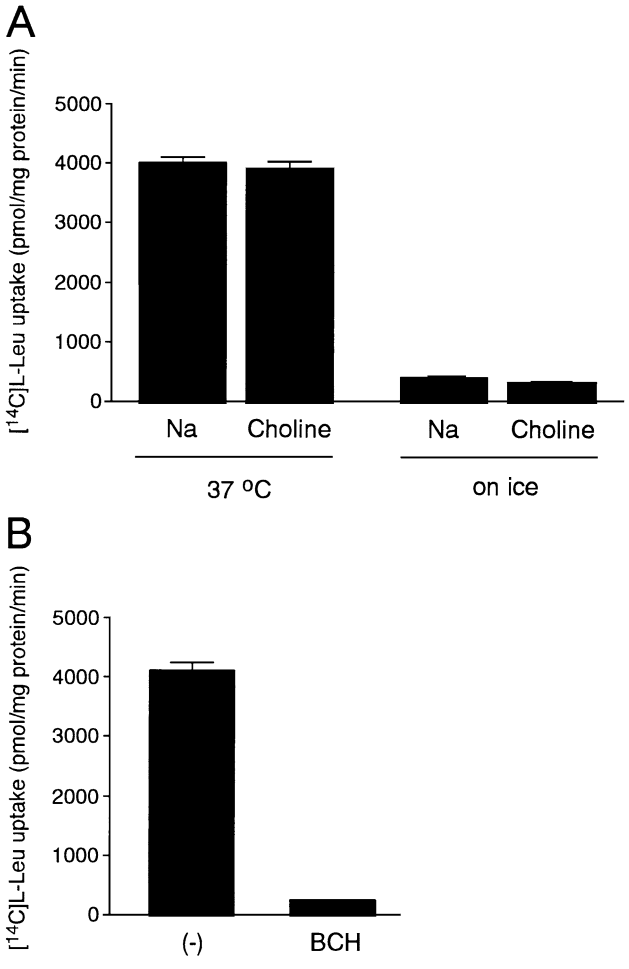


Fig. 3. [$^{14}$C]L-Leucine uptake by T24 cells. (A) Ion dependence of [$^{14}$C]L-leucine transport. [$^{14}$C]L-Leucine (20 μM) uptake measured in the standard uptake solution (Na) was compared with that measured in the $Na^+$-free uptake solution (Choline). The [$^{14}$C]L-leucine transport measurements were performed at 37 °C and on ice. (B) Inhibition of [$^{14}$C]L-leucine transport by BCH, a specific inhibitor of system L. The [$^{14}$C]L-leucine (20 μM) uptake was measured in the presence (BCH) or absence (−) of 2 mM BCH.

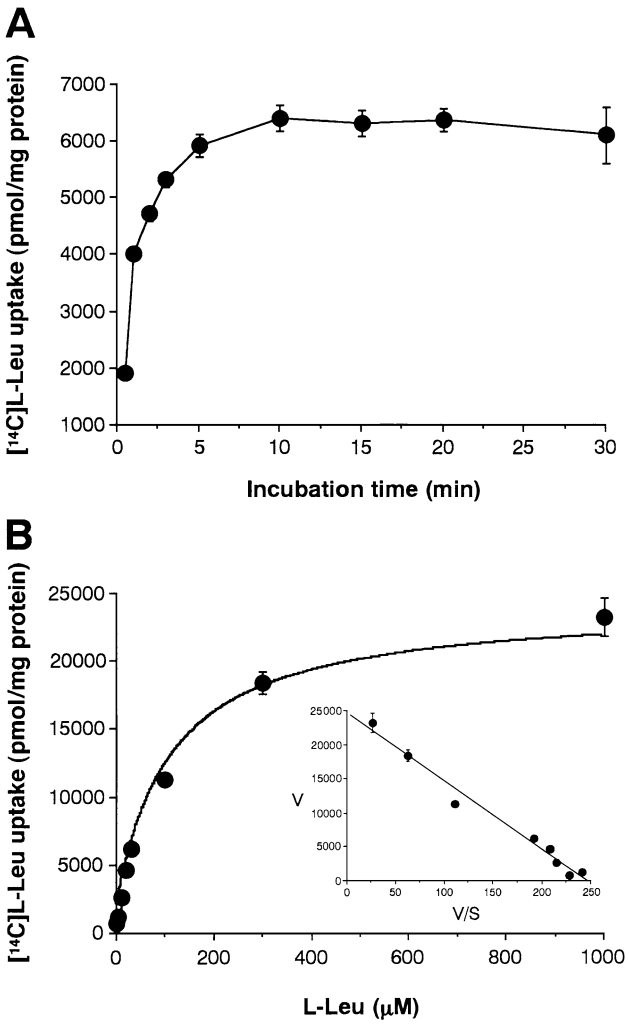


Fig. 4. Time course and concentration dependence of [$^{14}$C]L-leucine uptake by T24 cells. (A) The dependence of [$^{14}$C]L-leucine uptake on the incubation time. T24 cells were incubated in the $Na^+$-free uptake solution containing 20 μM [$^{14}$C]L-leucine for 0.5–30 min. (B) Concentration dependence of [$^{14}$C]L-leucine uptake in T24 cells. The uptake of [$^{14}$C]L-leucine by T24 cells was measured for 1 min and plotted against L-leucine concentration. L-Leucine uptake was saturable and fit to the Michaelis–Menten curve ($K_m = 100.5$ μM; $V_{max} = 23{,}878$ pmol/mg protein/min). The inset shows an Eadie–Hofstee plot of L-leucine uptake that was used to determine the kinetic parameters.

The presence of LAT1 and 4F2hc in T24 cells was further examined by real-time quantitative RT-PCR. In the real-time quantitative RT-PCR, LAT1 and 4F2hc were detected in T24 cells (data not shown). The amount of LAT1 was found to be ~1.5-fold higher than that of 4F2hc in T24 cells ($10.44 \pm 0.97\ 10^{-18}$ mol/μg total RNA vs. $6.15 \pm 0.45\ 10^{-18}$ mol/μg total RNA). Consistent with the result form the Northern blot analysis (Fig. 1), LAT2 was

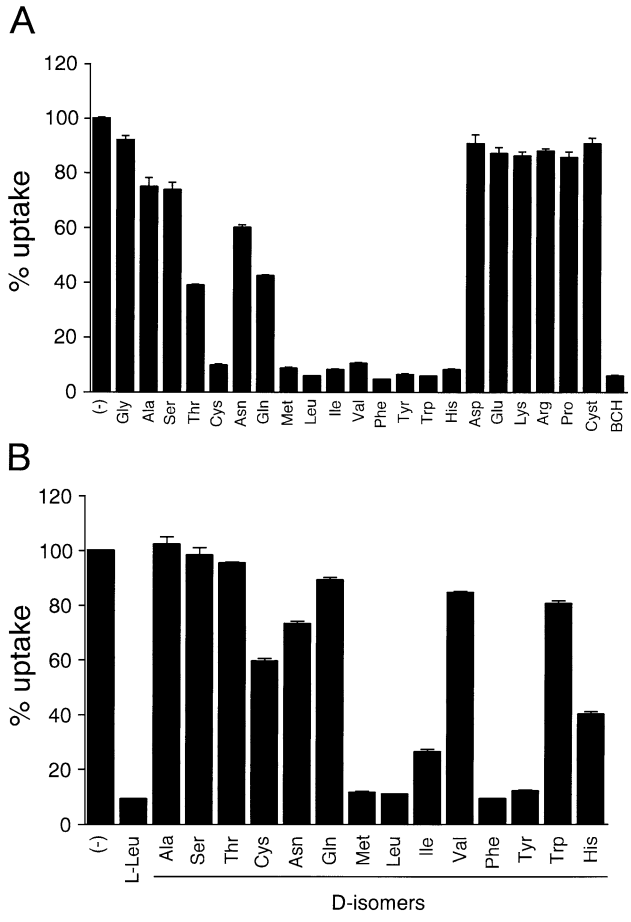


Fig. 5. Inhibition of [$^{14}$C]L-leucine uptake by amino acids in T24 cells. The [$^{14}$C]L-leucine uptake (20 μM) was measured in the presence of 2 mM nonradiolabeled indicated L-amino acids and system L specific inhibitor BCH (A) and D-amino acids (D-isomers) (B).

not detected in T24 cells in real-time quantitative RT-PCR (data not shown).

In the confocal immunofluorescence microscopic analyses, LAT1 and 4F2hc proteins were detected in T24 cells (Fig. 2A and B). They were found to be mainly colocalized in the plasma membrane (Fig. 2C). The results from Northern blot analysis, real-time quantitative RT-PCR and confocal immunofluorescence microscopic analysis indicate that LAT1 but not LAT2 is present together with 4F2hc in the plasma membrane of T24 cells.

### 3.2. The properties of [$^{14}$C]L-leucine uptake by T24 cells

The properties of [$^{14}$C]L-leucine transport were examined in T24 cells. As shown in Fig. 3A, the level of [$^{14}$C]L-leucine uptake (20 μM) by T24 cells measured in the standard uptake solution was not altered by replacing NaCl of the uptake solution with choline-Cl, indicating that L-leucine uptake by T24 cells is largely $Na^+$-independent. In the subsequent experiments, the transport measurements were performed under $Na^+$-free conditions. As shown in Fig. 3B, the uptake of [$^{14}$C]L-leucine (20 μM) was almost completely inhibited by 2 mM BCH, indicating that system L is responsible for the [$^{14}$C]L-leucine uptake by T24 cells. The [$^{14}$C]L-leucine uptake was not detected when the uptake measurements were performed on ice, confirming that the [$^{14}$C]L-leucine uptake by T24 cells was due to the transporter-mediated transport (Fig. 3A).

To determine the time course of [$^{14}$C]L-leucine uptake by T24 cells, the level of uptake was measured for 0.5–30 min (Fig. 4A). Uptake of [$^{14}$C]L-leucine (20 μM) was time-dependent and exhibited a linear dependence on the incubation time up to 1 min (Fig. 4A); all subsequent uptake measurements were conducted for 1 min and the values are expressed as pmol/mg protein/min. As shown in Fig. 4B, [$^{14}$C]L-leucine uptake was saturable and followed Michaelis–Menten kinetics with a $K_m$ values of 100.5 ± 11.7 μM

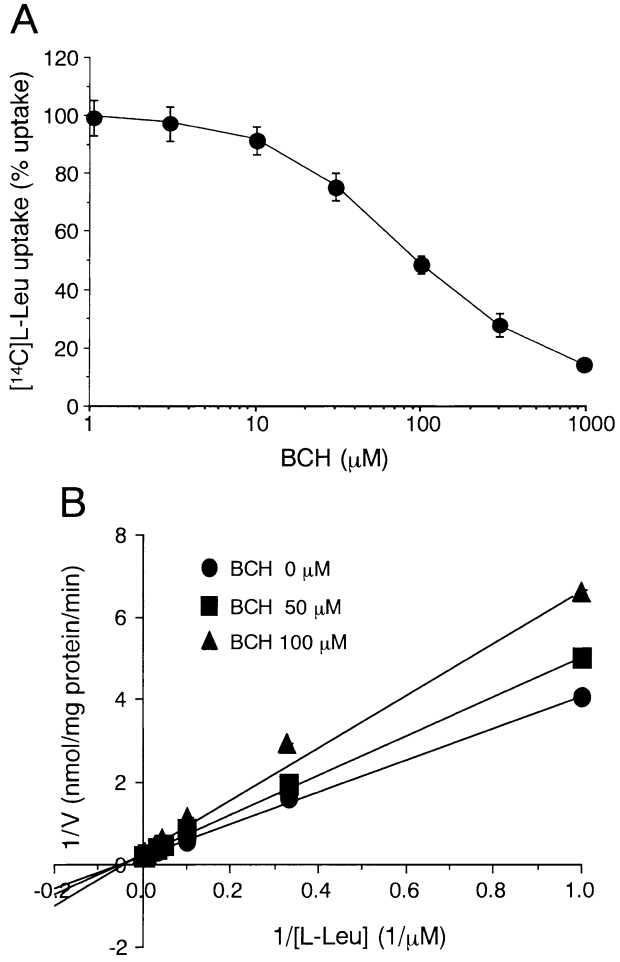


Fig. 6. The inhibitory effect of BCH on [$^{14}$C]L-leucine uptake in T24 cells. (A) The concentration-dependent inhibition of [$^{14}$C]L-leucine uptake by BCH. The [$^{14}$C]L-leucine uptake (1 μM) was measured for 1 min in the $Na^+$-free uptake solution in the presence of varied concentrations of BCH and expressed as a percentage of control L-leucine uptake in the absence of BCH. (B) Double reciprocal plot analyses of the inhibitory effect of BCH on [$^{14}$C]L-leucine uptake. The [$^{14}$C]L-leucine uptake (1, 3, 10, 30, 100, 300 and 1000 μM) was measured in the $Na^+$-free uptake solution in the presence (50 μM, filled square; 100 μM, filled triangle) or absence (filled circle) of BCH.

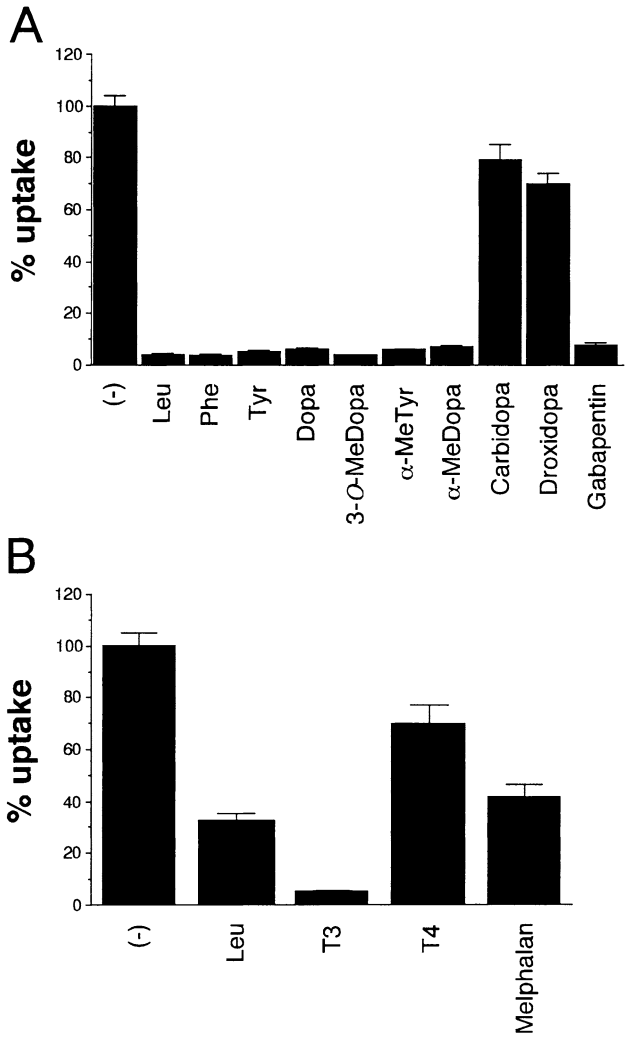


Fig. 7. Inhibition of [$^{14}$C]L-leucine uptake by amino acid analogues in T24 cells. (A) The [$^{14}$C]L-leucine uptake (20 μM) was measured for 1 min in the presence of 2 mM nonradiolabeled L-leucine (Leu), L-tyrosine (Tyr), L-dopa (Dopa), 3-*O*-methyldopa (3-*O*-MeDopa), α-methyltyrosine (α-MeTyr), α-methyldopa (α-MeDopa), carbidopa, droxidopa or gabapentin. The values are expressed as a percentage of control [$^{14}$C]L-leucine uptake in the absence of inhibitors (−). (B) The [$^{14}$C]L-leucine uptake (1 μM) was measured for 1 min in the presence of 100 μM nonradiolabeled L-leucine (Leu), triiodothyronine (T3), thyroxine (T4) and melphalan. The values are expressed as a percentage of control [$^{14}$C]L-leucine uptake measured in the absence of inhibitors (−).

(mean ± S.E. of three separate experiments) for [$^{14}$C]L-leucine uptake.

### 3.3. Inhibition of [$^{14}$C]L-leucine uptake by amino acids and amino acid-related compounds

In order to examine which compounds interact with L-leucine uptake by T24 cells, the [$^{14}$C]L-leucine uptake (20 μM) was measured in the presence of 2 mM nonlabeled amino acids and amino acid-related compounds in the $Na^+$-free uptake solution. The [$^{14}$C]L-leucine uptake was highly inhibited by L-isomers of cysteine, methionine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, histidine and BCH (Fig. 5A). Threonine, asparagine and glutamine and some other L-amino acids exhibited weaker inhibitory effects on [$^{14}$C]L-leucine transport (Fig. 5A).

As shown in Fig. 5B, [$^{14}$C]L-leucine uptake was markedly inhibited by D-isomers of methionine, leucine, isoleucine, phenylalanine and tyrosine, and less strongly by D-cysteine, D-asparagine and D-histidine, which is, in principle, consistent with the properties of LAT1 expressed in the *Xenopus* oocytes [9].

BCH (1−1000 μM) inhibited [$^{14}$C]L-leucine uptake (1 μM) in a concentration-dependent manner (Fig. 6A) with a $IC_{50}$ value of 131.5 ± 11.4 μM (mean ± S.E. of four separate experiments) (Fig. 6A). The inhibition of [$^{14}$C]L-leucine uptake by BCH was shown to be competitive in a double reciprocal plot analysis with a $K_i$ value of 156.2 ± 7.9 μM (mean ± S.E. of three separate experiments) (Fig. 6B and Table 1).

The effects of amino acid-related compounds on the L-leucine transport in T24 cells were investigated by measuring the [$^{14}$C]L-leucine uptake in the presence of the nonlabeled amino acid-related compounds. As shown in Fig. 7A, [$^{14}$C]L-leucine uptake by T24 cells was markedly inhibited by L-dopa, 3-*O*-methyldopa, α-methyltyrosine, α-methyldopa and gabapentin as well as leucine phenylalanine and tyrosine. Triiodothyronine, thyroxine, and melphalan also inhibited [$^{14}$C]L-leucine uptake (Fig. 7B). In contrast, carbidopa and droxidopa did not inhibit [$^{14}$C]L-leucine uptake (Fig. 7A). The inhibition of [$^{14}$C]L-leucine uptake by phenylalanine, tyrosine, L-dopa, 3-*O*-methyldopa, α-methyltyrosine, α-methyldopa, gabapentin, triiodothyronine, thyroxine and melphalan was in a competitive manner (data not shown). The $K_i$ values for these compounds are provided in Table 1.

### 3.4. The properties of [$^{14}$C]L-leucine efflux from T24 cells

To examine the properties of [$^{14}$C]L-leucine efflux from T24 cells, T24 cells were loaded with [$^{14}$C]L-leucine by

Table 1
$K_i$ values of unlabeled amino acid-related compounds on the uptake of [$^{14}$C]L-leucine into T24 cells

| Compounds | $K_i$ (μM)[a] |
|---|---|
| Phenylalanine | 55.2 ± 1.90 |
| Tyrosine | 60.4 ± 3.74 |
| Dopa | 138 ± 6.90 |
| 3-*O*-Methyldopa | 96.5 ± 7.80 |
| α-Methyltyrosine | 153 ± 23.5 |
| α-Methyldopa | 216 ± 18.7 |
| Gabapentin | 191 ± 16.7 |
| Triiodothyronine | 7.30 ± 0.80 |
| Thyroxine | 162 ± 19.7 |
| Melphalan | 75.3 ± 6.84 |
| BCH | 156 ± 7.90 |

[a] $K_i$ values were determined as described in Materials and methods. The table was constructed based on three separate experiments.

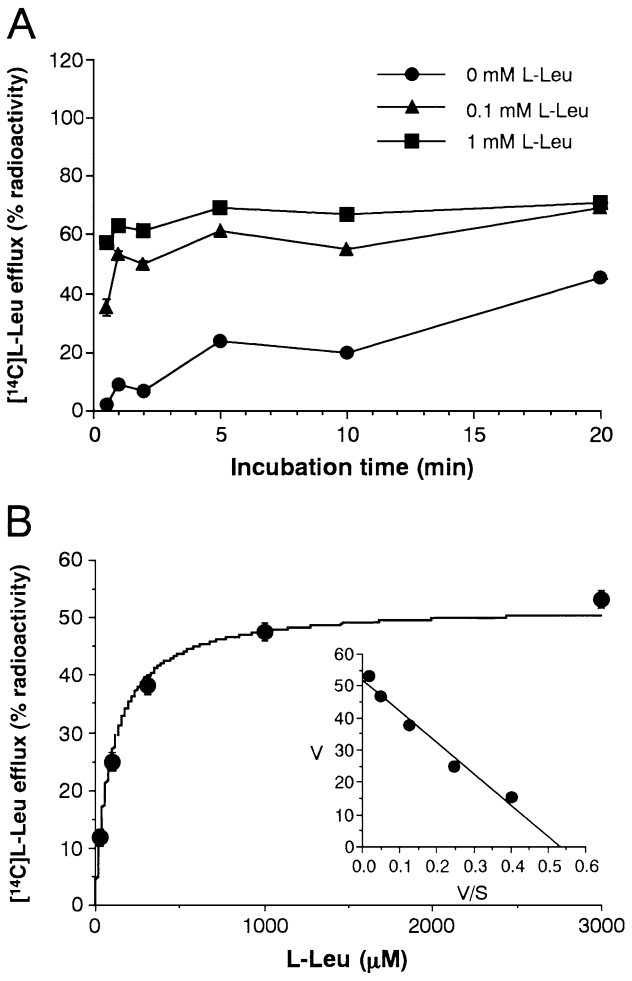


Fig. 8. [$^{14}$C]L-Leucine efflux from T24 cells. (A) Time course of [$^{14}$C]L-leucine efflux from T24 cells preloaded with [$^{14}$C]L-leucine. The efflux was measured in the presence (0.1 mM L-Leu; 1 mM L-Leu) or absence (0 mM L-Leu) of L-leucine in the extracellular medium. The values are expressed as a percentage of the total radioactivity loaded to the T24 cells (see Materials and methods). (B) The dependence of [$^{14}$C]L-leucine efflux on the concentration of extracellular L-leucine. T24 cells were preloaded with [$^{14}$C]L-leucine by being incubated in the uptake solution containing 1 μM [$^{14}$C]L-leucine for 10 min. The efflux of loaded [$^{14}$C]L-leucine induced by extracellularly applied L-leucine was measured for 1 min and plotted against extracellular L-leucine concentration. Inset: Eadie–Hofstee plot of the [$^{14}$C]L-leucine efflux used to determine kinetic parameters. The L-leucine efflux was saturable and fit to a Michaelis–Menten curve. The $K_m$ and $V_{max}$ values were 103.8 ± 8.0 μM and 54.2 ± 0.4% radioactivity, respectively.

being incubated for 10 min in the uptake solution containing 1 μM [$^{14}$C]L-leucine. The efflux of loaded radioactivity was measured in the presence or absence of nonlabeled L-leucine in the extracellular medium. Nearly 60% of the loaded radioactivity appeared in the extracellular medium in 1 min in the presence of extracellular 0.1 or 1 mM L-leucine (Fig. 8A). The extracellularly applied L-leucine induced the efflux of preloaded [$^{14}$C]L-leucine in a concentration-dependent manner (Fig. 8B). The $K_m$ value of extracellularly applied L-leucine to induce the preloaded [$^{14}$C]L-leucine efflux was 103.8 ± 8 μM. The efflux of [$^{14}$C]L-leucine was not dependent on the extracellular $Na^+$ (data not shown).

### 3.5. [$^{14}$C]L-leucine efflux induced by amino acid-related compounds

The amino acid-related compounds that inhibited [$^{14}$C]L-leucine uptake in T24 cells were investigated to examine

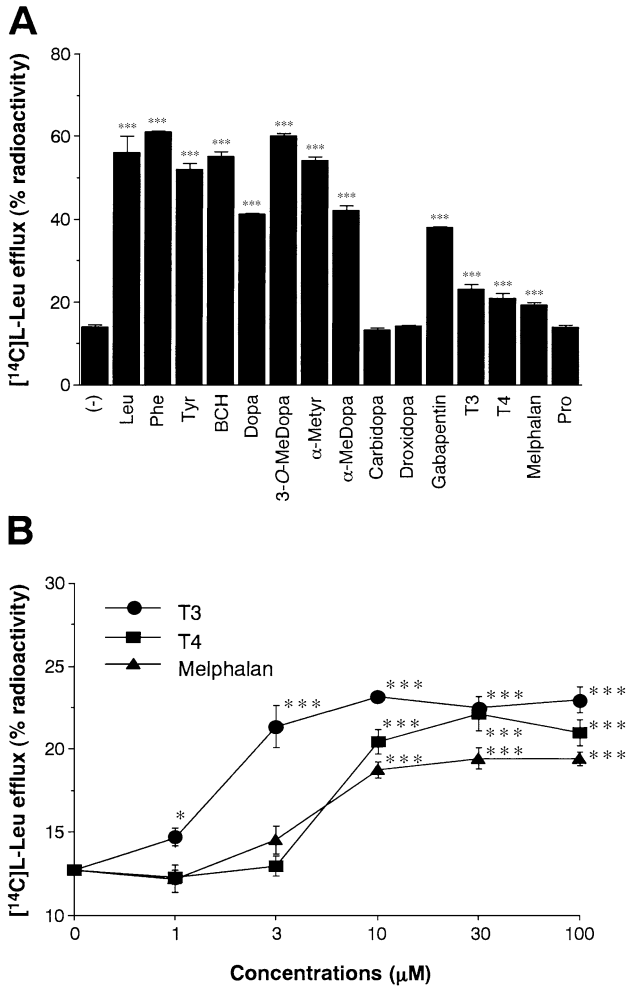


Fig. 9. Efflux of [$^{14}$C]L-leucine by amino acid-related compounds. (A) Efflux of preloaded [$^{14}$C]L-leucine induced by amino acid-related compounds. The efflux of preloaded [$^{14}$C]L-leucine from the T24 cells was measured for 1 min in the presence of 100 μM L-leucine (Leu), L-phenylalanine (Phe), L-tyrosine (Tyr), BCH, L-dopa (Dopa), 3-*O*-methyldopa (3-*O*-MeDopa), α-methyltyrosine (α-Metyr), α-methyldopa (α-MeDopa), carbidopa, droxidopa, gabapentin, triiodothyronine (T3), thyroxine (T4), melphalan or proline (Pro) in the medium and compared with that measured in the absence of these compounds (−). (B) The concentration dependence of the effect of extracellular triiodothyronine, thyroxine and melphalan to induce [$^{14}$C]L-leucine efflux. The efflux of preloaded [$^{14}$C]L-leucine from the T24 cells was measured for 1 min in the presence of varied concentration of triiodothyronine (T3, filled circle), thyroxine (T4, filled square) and melphalan (filled triangle) in the medium. * $P<0.05$ vs. control (the efflux measured in the absence of triiodothyronine, thyroxine or melphalan), *** $P<0.001$ vs. control (the efflux measured in the absence of triiodothyronine, thyroxine or melphalan).

whether they induce efflux of preloaded [$^{14}$C]L-leucine when applied extracellularly. As shown in Fig. 9A, extracellularly applied L-leucine, L-phenylalanine, L-tyrosine, BCH, L-dopa, 3-*O*-methyldopa, α-methyltyrosine, α-methyldopa and gabapentin induced high levels of efflux of preloaded [$^{14}$C]L-leucine. Triiodothyronine, thyroxine and melphalan induced lower but significant efflux of the preloaded [$^{14}$C]L-leucine (Fig. 9A). The efflux of preloaded [$^{14}$C]L-leucine induced by triiodothyronine, thyroxine and melphalan was shown to be dependent on the concentration of extracellularly applied triiodothyronine, thyroxine and melphalan (Fig. 9B). Significant efflux was not induced by carbidopa and droxidopa (Fig. 9A).

## 4. Discussion

In the present study, we investigated the expression of system L transporters and the properties of L-leucine transport in T24 human bladder carcinoma cells. We demonstrated by Northern blot and real-time quantitative PCR that T24 cells express LAT1, an isoform of system L transporter, at high levels together with its associating protein, 4F2hc. T24 cells do not express the other system L isoform, LAT2 (Fig. 1). In the Western blot analysis which we performed in the previous study on the membrane fraction prepared from T24 cells, we detected proteins for LAT1 and 4F2hc connected with each other via a disulfide bond [10]. In the present study, we performed an immunofluorescence microscopic analysis on T24 cells and showed that LAT1 protein is present in the plasma membrane of T24 cells with 4F2hc protein (Fig. 2). Therefore, it is concluded that T24 cells express LAT1 but not LAT2 in the plasma membrane as a partner of 4F2hc.

The [$^{14}$C]L-leucine uptake measured in T24 cells was $Na^+$-independent and almost completely inhibited by the system L selective inhibitor BCH [1,34] (Fig. 3A and B). This suggests that the majority of L-leucine uptake by T24 cells is mediated by system L. Because, as already mentioned, T24 cells express LAT1, one of the two system L transporters, LAT1 is proposed to be responsible for the L-leucine uptake by T24 cells. The [$^{14}$C]L-leucine uptake by T24 cells is saturable and followed Michaelis–Menten kinetics (Fig. 4B). The $K_m$ value for the [$^{14}$C]L-leucine uptake was ~ 100 μM, relatively higher than those of human LAT1 and rat LAT1 expressed in *Xenopus* oocytes (~ 20 μM), which may be due to different cell systems and conditions used [9,10]. The profiles of inhibition of [$^{14}$C]L-leucine uptake by L- and D-amino acids in T24 cells were basically identical to those determined for human LAT1 and rat LAT1 expressed in *Xenopus* oocytes, except that [$^{14}$C]L-leucine uptake was more strongly inhibited by L-cysteine, L-threonine, D-isoleucine, D-tyrosine and D-histidine in T24 cells [9,10] (Fig. 5A and B).

BCH is an amino acid-related compound which has been used as a selective inhibitor of system L [1,34]. It was, however, shown that the transport mediated by a $Na^+$-dependent neutral and basic amino acid transporter $ATB^{0,+}$ with the characteristics of system $B^{0,+}$ is also inhibited by BCH [35]. In the $Na^+$-free condition, however, it is still correct that BCH selectively inhibits system L transport. [$^{14}$C]L-leucine uptake by T24 cells was inhibited by BCH in a concentration-dependent fashion (Fig. 6A). The inhibition was shown to be competitive with a $K_i$ value of ~ 150 μM (Fig. 6B).

Consistent with the properties of rat LAT1 expressed in *Xenopus* oocytes, [$^{14}$C]L-leucine uptake by T24 cells was strongly inhibited by L-dopa, 3-*O*-methyldopa, α-methyltyrosine, α-methyldopa and gabapentin, whereas carbidopa and droxidopa did not exert remarkable inhibitory effects on the [$^{14}$C]L-leucine uptake (Fig. 7A). The $K_i$ values for these compounds were comparable with those determined in rat LAT1 expressed in *Xenopus* oocytes (Table 1 and Ref. [11]). As shown in Fig. 7B, [$^{14}$C]L-leucine uptake by T24 cells was inhibited by triiodothyronine, thyroxine and melphalan, consistent with human LAT1 and rat LAT1 expressed in *Xenopus* oocytes [10,11]. As already shown for rat LAT1 expressed in *Xenopus* oocytes, triiodothyronine exhibited the lowest $K_i$ value (~ 7 μM for T24 cells (Table 1); ~ 6 μM for rat LAT1 expressed in *Xenopus* oocytes Ref. [11]) among the compounds tested.

The profiles of inhibition of [$^{14}$C]L-leucine uptake by amino acids and amino acid-related compounds in T24 cells are, thus, in principle, comparable to those for human LAT1 and rat LAT1 expressed in *Xenopus* oocytes [9–11]. As already discussed, the examinations on the expression of messages and proteins have revealed that LAT1 is the predominant system L transporter in T24 cells. Taken together, it is concluded that the majority of [$^{14}$C]L-leucine uptake is mediated by LAT1 in T24 cells. This indicates that the T24 cell system is an excellent tool to screen the compounds which interact with LAT1 by measuring the effects on the [$^{14}$C]L-leucine uptake.

It was already shown that LAT1 mediates the exchange of amino acids [9–11]. In rat LAT1 expressed in *Xenopus* oocytes, we previously showed that the measurement of the efflux of preloaded radiolabeled substrates can be a means to evaluate whether the compounds that inhibit LAT1-mediated transport are transportable substrates or nontransportable blockers [11]. This strategy is, in particular, useful for the compounds whose radiolabeled forms are not available [33,36,37]. In the present study, we performed efflux measurements in T24 cells. T24 cells were preloaded with [$^{14}$C]L-leucine by being incubated for 10 min in the uptake solution containing 1 μM [$^{14}$C]L-leucine. As shown in Fig. 8A, by responding to the extracellularly applied 100 μM L-leucine, ~ 60% of preloaded radioactivity was effluxed from T24 cells to the medium in 1 min. The rate of the efflux of radioactivity was dependent on the concentration of the extracellularly applied L-leucine with the $K_m$ value of ~ 100 μM (Fig. 8B), comparable with that of L-leucine uptake by T24 cells (~ 100 μM). This is consistent with the

concept of obligatory exchange for the transport mediated by LAT1 [9–11]. We thus concluded that the efflux measurement procedure can also be applicable to T24 cells.

By performing the efflux measurements in T24 cells, we examined whether the amino acid-related compounds induce the efflux of preloaded [$^{14}$C]L-leucine. Consistent with the efflux measurements performed on rat LAT1 expressed in *Xenopus* oocytes, L-dopa, 3-*O*-methyldopa, α-methyltyrosine, α-methyldopa and gabapentin induced high level of efflux of preloaded [$^{14}$C]L-leucine (Fig. 9A). In addition, we examined BCH in the present study to show that BCH also induces the efflux of preloaded [$^{14}$C]L-leucine (Fig. 9A). These compounds are supposed to be transported by LAT1 in T24 cells. In rat LAT1 expressed in *Xenopus* oocytes, triiodothyronine, thyroxine and melphalan did not induce detectable levels of efflux of preloaded [$^{14}$C]L-phenylalanine although they exhibited inhibitory effects on [$^{14}$C]L-phenylalanine uptake, consistent with the low transport rates for [$^{125}$I]triiodothyronine and [$^{125}$I]thyroxine via rat LAT1 expressed in *Xenopus* oocytes [11]. In the present study, however, the significant efflux of preloaded [$^{14}$C]L-leucine was detected by the application of triiodothyronine, thyroxine and melphalan to the T24 cells, which was dependent on the concentration of extracellularly applied triiodothyronine, thyroxine and melphalan (Fig. 9A and B). This indicates that the efflux measurements in T24 cells is more sensitive than those in *Xenopus* oocytes.

For thyroid hormones triiodothyronine and thyroxine, it was already shown that they are transported by LAT1 although the transport rate is low in spite of their high affinity to LAT1. However, it was not determined whether melphalan is in fact transported by LAT1 or not. In this study, we showed that melphalan induces significant efflux of preloaded [$^{14}$C]L-leucine from T24 cells (Fig. 9A and B). This is the first to demonstrate that melphalan is transported by LAT1. Greig et al.[38] reported that melphalan is transported across the blood–brain barrier via system L, which is presumably mediated by LAT1 expressed in the blood–brain barrier. Melphalan was, in addition, shown to be transported by system L in in vivo preparation [39]. The level of system L activity in tumor cells was reported to be correlated with melphalan sensitivity [40,41]. Because LAT1 is up-regulated in tumor cells, LAT1 is proposed to be at least one of the major routes through which melphalan permeates the plasma membrane to enter the tumor cells.

In the present study, we have characterized the [$^{14}$C]L-leucine uptake by T24 cells and shown that it is mediated by LAT1 a isoform of system L transporter. We propose, therefore, that the T24 cell system is an excellent tool to examine the interaction of chemical compounds with LAT1. Because LAT1 is up-regulated in tumor cells to support their continuous growth, the inhibition of LAT1 would be a new rationale to suppress tumor cell growth. T24 cell would be useful to screen the chemical compounds for the inhibitors of LAT1. In addition, we showed, in the present study, that the efflux of preloaded [$^{14}$C]L-leucine from T24 cells is a sensitive means to evaluate whether the compounds which inhibited LAT1-mediated transport is in fact transported by LAT1 or not. The T24 cell system is, therefore, also useful to develop drugs with efficient permeation through the blood–brain barrier utilizing LAT1 as a permeation path.

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