Characterization of Amino Acid Transport System L in HTB-41 Human Salivary Gland Epidermoid Carcinoma Cells

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Abstract. *Background: The amino acid transport system L is a major nutrient transport system that is responsible for transport of neutral amino acids, including several essential amino acids. The current study attempted to investigate the expression and functional characterization of amino acid transport system L in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells. Materials and Methods: RT-PCR analysis, Western blot analysis and amino acid transport measurements were used. Results: The HTB-41 cells expressed the L-type amino acid transporter 1 (LAT1) together with its associating protein heavy chain of 4F2 antigen (4F2hc) in the plasma membrane, whereas the HTB-41 cells did not express the L-type amino acid transporter 2 (LAT2). The uptakes of [^{14}C]L-leucine were Na^+-independent and completely inhibited by a system L selective inhibitor, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH). The affinity of [^{14}C]L-leucine uptake and the inhibition profile of [^{14}C]L-leucine uptake by various L-amino acids in the HTB-41 cells were comparable with those for the LAT1 expressed in Xenopus oocytes. Conclusion: The transport of neutral amino acids including several essential amino acids into the HTB-41 human submaxillary salivary gland epidermoid carcinoma cells are mediated by LAT1.*

Amino acids are indispensable for protein synthesis, which is essential for cell growth and proliferation in both normal and transformed cells (1, 2). Amino acid transport across the plasma membrane is mediated *via* amino acid transporters located on the plasma membrane (1, 2). Among these systems, the amino acid transport system L, which is a Na^+-independent neutral amino acid transport system, is a major route for providing living cells, including tumor cells, with neutral amino acids, including several essential amino acids (1, 3).

The system L-type amino acid transporters 1 and 2 (LAT1 and LAT2), the first and second isoforms of system L, have been isolated (4-6). There are predicted to be a total twelve membrane-spanning proteins that mediate Na^+-independent amino acid exchange (4-6). They require an additional single membrane-spanning protein, a heavy chain of 4F2 antigen (4F2hc), for their functional expression in the plasma membrane (4-10). LAT1 mRNA is only expressed in restricted organs such as the brain, spleen, placenta and testis (4, 5, 10, 11). However, the mRNAs of LAT2 and 4F2hc are ubiquitously expressed in all normal embryonic and normal adult tissues (4-6, 10). In addition, LAT1 is highly expressed in malignant tumors, presumably to support their continuous growth and proliferation (4, 5, 12, 13). LAT1 prefers large neutral amino acids as its substrates (4, 5, 14), while LAT2 transports not only large neutral amino acids, but also small neutral amino acids, in a fashion that appears to have broader substrate selectivity than LAT1 (6, 15-17).

It has been proposed that the manipulation of system L activity, in particular that of LAT1, could have antitumor therapeutic implications. The inhibition of LAT1 activity could suppress the growth of tumor cells by depriving them of essential amino acids.

Salivary gland tumors are a morphologically and clinically diverse group of neoplasms, which may present considerable diagnostic and management challenges to the pathologist or surgeon (18). Salivary gland tumors are rare with an overall incidence in the Western world of about 2.5-3.0 per 100,000 per year and among salivary gland tumors, squamous cell carcinomas are rare (19). However, although this tumor has a poor prognosis, unlike most other salivary gland

Abbreviations: LAT1/2, L-type amino acid transporter 1/2; 4F2hc, heavy chain of 4F2 antigen; RT-PCR, reverse transcription-polymerase chain reaction; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid.

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malignancies, survival at 5 years is prognostically significant (19). Furthermore, the expression and functional characterization of amino acid transporters such as amino acid transport system L in salivary gland tumors, including squamous carcinomas, are not known at all.

In the present study, therefore, the expression and functional characterization of the amino acid transport system L were investigated in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells.

Materials and Methods

Materials. [^{14}C]L-leucine was purchased from PerkinElmer Life Sciences Inc. (Boston, MA, USA). Affinity-purified rabbit anti-LAT1, anti-LAT2 and anti-4F2hc polyclonal antibodies were supplied by Kumamoto Immunochemical Laboratory, Transgenic Inc. (Kumamoto, Japan). The 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) and other chemicals were purchased from Sigma (St Louis, MO, USA).

Cell line and cell culture. HTB-41 human submaxillary salivary gland epidermoid carcinoma cells were provided by the American Type Culture Collection (ATCC, Rockville, MD, USA). The HTB-41 cells were grown in modified McCoy's media with 1.5 mM L-glutamine adjusted to contain 2.2 g/l sodium bicarbonate with 10% FBS in accordance with the ATCC's instructions. The cells were maintained as monolayers in plastic culture plates at 37˚C in a humidified atmosphere containing 5% CO_2.

RT-PCR analysis. Total RNA was prepared from the HTB-41 cells by an RNA preparation kit (Isogen, Nippon-Gene, Japan) in accordance with the manufacturer's instructions. For RT-PCR analysis, the first-strand cDNA was prepared from the HTB-41 cell total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies Inc., CA, USA) with an oligo dT primer, and used as a template for PCR amplification. The PCR amplification was performed using Taq polymerase Amplitaq Gold (Roche Molecular Systems, Inc., Germany) in the following protocol: 94˚C for 12 min, followed by 25 cycles of 94˚C for 30 s, 60˚C for 30 s and 72˚C for 45 s and a final extension step of 72˚C for 10 min. Pairs of primers, 5'-TTCATCGCAG TACATCGTGG-3' (491-510 bp) and 5'-CCCAGGTGATA GTTCCCGAA-3' (1008-1027 bp) for human LAT1, 5'-AGCCCTGAAGAAAGAGATCG-3' (811-830 bp) and 5'-TGCAT ATCTGTACAATCCCC-3' (1321-1340 bp) for human LAT2 and 5'-TCGATTACCTGAGCTCTCTG-3' (551-570 bp) and 5'-GGGA TTTTGTATGCTCCCCA-3' (1041-1060 bp) for human 4F2hc were used for PCR amplification.

Western blot analysis. To investigate the expressions of LAT1, LAT2 and 4F2hc proteins in the HTB-41 cells, Western blot analyses were performed as described elsewhere (20, 21). The membranes were treated with anti-LAT1 (1:1,000), anti-LAT2 (1:1,000) or anti-4F2hc (1:1,000) affinity-purified primary antibodies.

Uptake measurements. To characterize the function of the endogenously expressed amino acid transport system L in the HTB-41 cells, uptake experiments were performed as described elsewhere (22). The HTB-41 cells were collected and seeded on 24-well plates (1×10^5 cells/well) in fresh growth medium. The uptake measurements were performed when the cells were approximately 85-95% confluent on 24-well plates.

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 containing [^{14}C]L-leucine, followed by washing three times with ice-cold Na^+-free uptake solution. The cells were then solubilized with 0.1 M NaOH and the 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 HTB-41 cells were used for each data point. To confirm the reproducibility of the results, three or four separate experiments were performed for each measurement and the results from representative experiments are shown in the figures.

The K_m (the concentration of L-leucine which yields one-half of maximum velocity) and V_{max} (the maximum velocity of [^{14}C]L-leucine uptake) values were determined using Eadie-Hofstee plots based on the [^{14}C]L-leucine uptakes measured for 1 min at 1, 3, 10, 30, 100, 300 and 1000 μM. The IC_{50} values for BCH on the L-leucine transport were determined on the 1 μM [^{14}C]L-leucine uptake measured for 1 min in the presence of 0, 1, 3, 10, 30, 100, 300, 1000 and 3000 μM BCH. To measure the K_i (the dissociation constant of the L-leucine and BCH) values for BCH, the uptake rates of [^{14}C]L-leucine were measured for 1 min at various concentrations of [^{14}C]L-leucine (1, 3, 10, 30, 100, 300 and 1000 μM) with or without the addition of 100 μM BCH. The K_i values were determined by double reciprocal plot analysis where 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) (14, 21). For the inhibition experiments, the uptake of 30 μM [^{14}C]L-leucine was measured in the presence or absence of the 3 mM non-labeled L-amino acids and BCH.

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

Results

Detection of system L amino acid transporters in HTB-41 cells. In the RT-PCR analysis, the PCR products for LAT1 and its associating protein 4F2hc were detected, whereas the LAT2 was not detected in HTB-41 cells (Figure 1). Western blot analysis was performed on the membrane fractions prepared from the HTB-41 cells. The antibodies raised against LAT1 and 4F2hc recognized the 40 kDa protein band and 85 kDa protein band under reducing conditions, respectively (Figure 2). The protein band of LAT2 was not recognized on the membrane fractions (Figure 2).

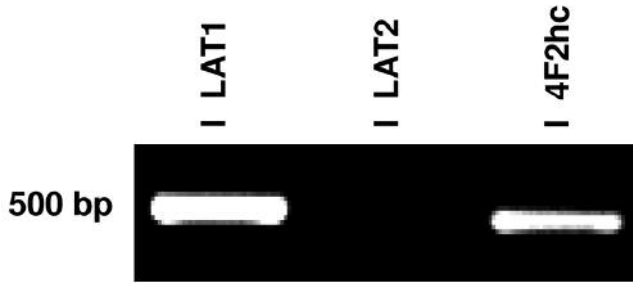

Figure 1. *Detection of LAT1, LAT2 and 4F2hc by RT-PCR in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells. The PCR products were subjected to electrophoresis on a 1.2% agarose gel and visualized with ethidium bromide. The 536 bp LAT1-specific PCR product and 509 bp 4F2hc-specific PCR product were obtained.*

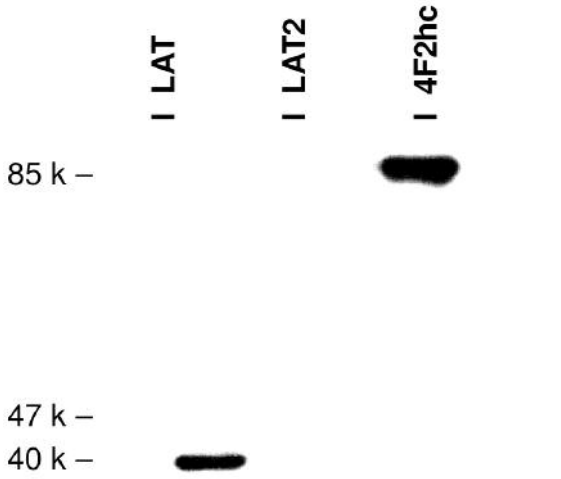

Figure 2. *Western blot analysis of LAT1, LAT2 and 4F2hc in HTB-41 cells. Western blot analyses were performed on the membrane fractions prepared from HTB-41 cells in the presence of 2-mercaptoethanol using anti-LAT1, anti-LAT2 and anti-4F2hc antibodies.*

Properties of [^{14}C]L-leucine uptake by HTB-41 cells. As shown in Figure 3A, the level of [^{14}C]L-leucine uptake (30 μM) by the HTB-41 cells measured in the standard uptake solution (Na) was not altered by replacing NaCl of the uptake solution with choline-Cl, indicating that L-leucine uptake by HTB-41 cells was primarily Na^+-independent. In the subsequent experiments, the transport measurements were performed under Na^+-free conditions. When the uptake measurements were performed on ice, [^{14}C]L-leucine uptake was not detected, confirming that the [^{14}C]L-leucine uptake by the HTB-41 cells was due to transporter-mediated transport (Figure 3A). As shown in Figure 3B, the uptake of [^{14}C]L-leucine (30 μM) was almost completely inhibited by 3 mM BCH, a specific inhibitor of system L amino acid transporters, indicating that the system L amino acid transporters were responsible for [^{14}C]L-leucine uptake in the HTB-41 cells.

To determine the time-course of [^{14}C]L-leucine uptake by HTB-41 cells, the level of [^{14}C]L-leucine (30 μM) uptake was measured for 0.5, 1, 2, 2.5, 5, 10, 15 and 20 min. The uptake of [^{14}C]L-leucine was time-dependent and exhibited a linear dependence on the incubation time up to 1 min (Figure 4). Hence all the subsequent uptake measurements were conducted for 1 min and the values are expressed as pmol/mg protein/min. As shown in Figure 5, the [^{14}C]L-leucine uptake was saturable and followed Michaelis-Menten kinetics with a K_m value of 47.7±5.2 μM (mean±S.E.M. of three separate experiments).

Inhibition of [^{14}C]L-leucine uptake by BCH in HTB-41 cells. As shown in Figure 6, BCH (1-3,000 μM) inhibited the [^{14}C]L-leucine (1 μM) uptake in a concentration-dependent manner with an IC_{50} value of 65.3±5.2 μM (mean±S.E.M. of three separate experiments). In the HTB-41 cells, 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 41.1±3.8 μM (mean±S.E.M. of four separate experiments) (Figure 7).

Inhibition of [^{14}C]L-leucine uptake by various amino acids. In order to examine which L-amino acids interacted with the L-leucine uptake mechanism in the HTB-41 cells, the [^{14}C]L-leucine (30 μM) uptake was measured in the presence of 3 mM nonlabeled L-amino acids in the Na^+-free uptake solution. The [^{14}C]L-leucine uptake was strongly inhibited by the L-isomers of methionine, leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan and histidine (Figure 8). Threonine, cysteine, asparagine and glutamine exhibited weaker inhibitory effects on [^{14}C]L-leucine transport and glycine, alanine, serine, aspartate, glutamate, lysine, arginine, proline and cystine did not reveal any inhibitory effects on [^{14}C]L-leucine transport (Figure 8).

Discussion

By both RT-PCR and Western blot analysis of the membrane fraction, the HTB-41 cells were shown to express LAT1, together with its associating protein 4F2hc, but not to express the other system L isoform LAT2 (Figures 1 and 2). In a previous study, it was reported that KB human oral cancer cells expressed LAT1 and 4F2hc, but not LAT2, and that the transport of neutral amino acids into the KB cells was mediated mainly by LAT1 (20). In addition, LAT1 is highly expressed in malignant tumors, presumably to support their continuous growth and proliferation (4, 5, 12, 13).

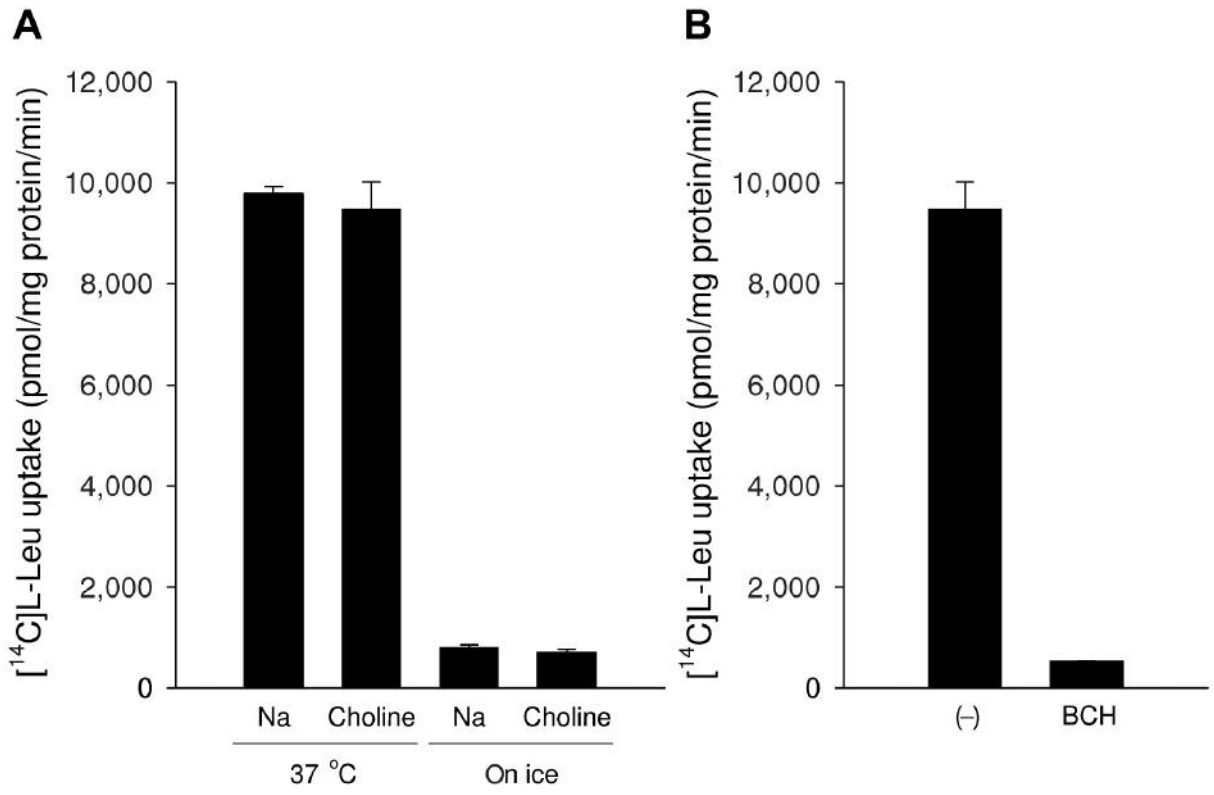

Figure 3. *[14C]L-leucine uptake by HTB-41 cells. (A) lon dependence of [14C]L-leucine transport. [14C]L-leucine (30 μM) uptake measured in the standard uptake solution (Na) was compared with that measured in the Na+-free uptake solution (Choline). The [14C]L-leucine transport measurement was performed at 37°C and on ice. (B) Inhibition of [14C]L-leucine transport by BCH, a specific inhibitor of amino acid transport system L. The [14C]L-leucine (30 μM) uptake was measured in the presence (BCH) or absence (–) of 3 mM BCH.*

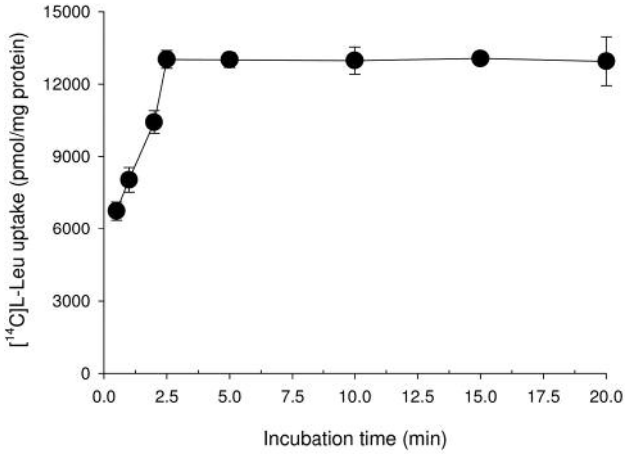

Figure 4. *Time-course of [14C]L-leucine uptake by HTB-41 cells. The HTB-41 cells were incubated in Na+-free uptake solution containing 30 μM [14C]L-leucine for 0.5, 1, 2, 2.5, 5, 10, 15 and 20 min.*

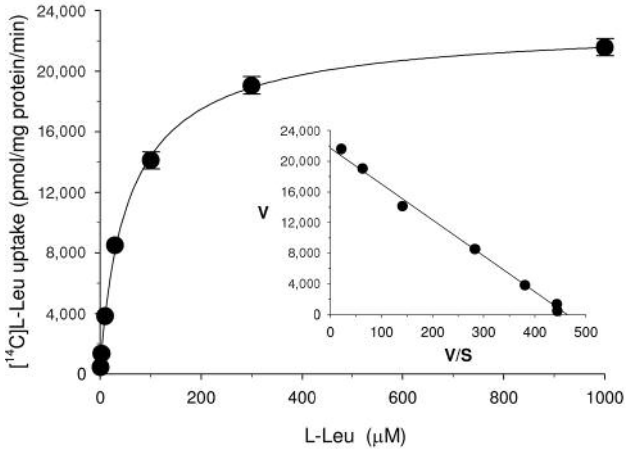

Figure 5. *Concentration dependence of [14C]L-leucine uptake by HTB-41 cells. The uptake of [14C]L-leucine by HTB-41 cells was measured for 1 min and plotted against L-leucine concentration. The L-leucine uptake was saturable and fitted the Michaelis-Menten curve (K_m=47.7 μM). The inset shows an Eadie-Hofstee plot of L-leucine uptake that was used to determine the kinetic parameters.*

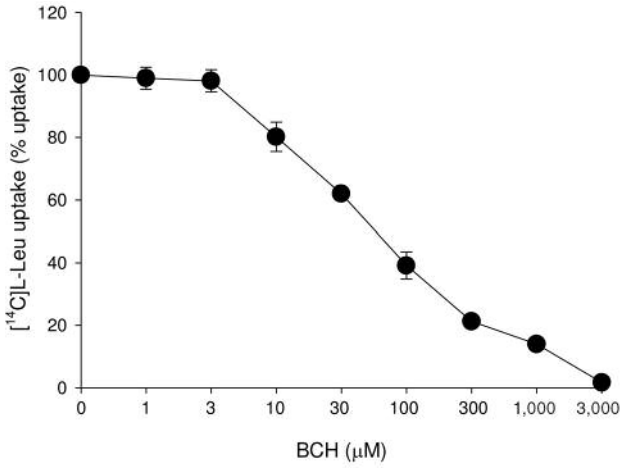

Figure 6. *Concentration-dependent inhibition of [^{14}C]L-leucine uptake by BCH in HTB-41 cells. The [^{14}C]L-leucine uptake (1 μM) was measured for 1 min in the presence of various BCH concentrations and was expressed as a percentage of the control L-leucine uptake in the absence of BCH.*

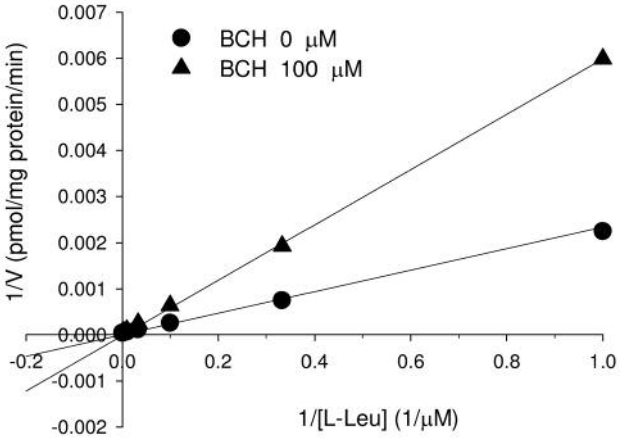

Figure 7. *Double reciprocal plot analysis of inhibitory effect of BCH on the [^{14}C]L-leucine uptake in HTB-41 cells. The [14C]L-leucine uptakes (1, 3, 10, 30, 100, 300 and 1,000 μM) were measured in the Na$^+$-free uptake solution in the presence (filled triangle) or absence (filled circle) of 100 μM BCH.*

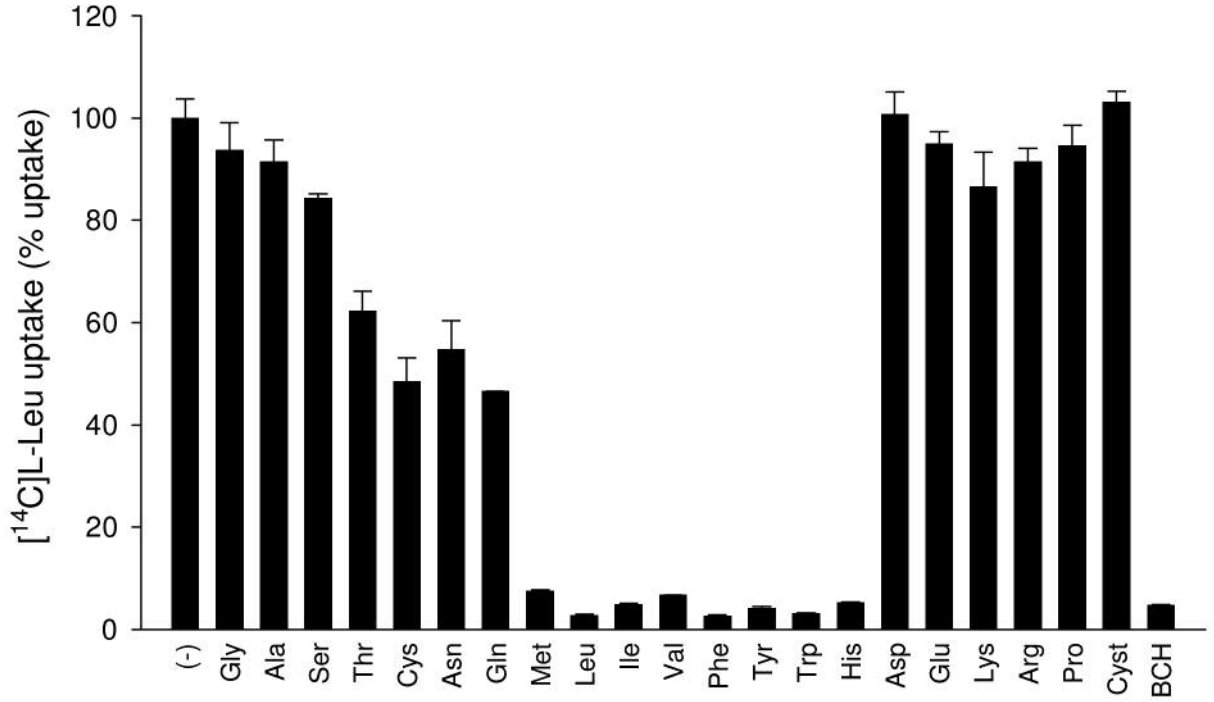

Figure 8. *Inhibition of [^{14}C]L-leucine uptake by amino acids in HTB-41 cells. The [^{14}C]L-leucine (30 μM) uptake was measured in the presence of 3 mM nonradiolabeled L-amino acids indicated and system L-specific inhibitor BCH in the Na$^+$-free uptake solution.*

The [^{14}C]L-leucine uptake measured in the HTB-41 cells was Na$^+$-independent and was almost completely inhibited by the selective inhibitor of the system L amino acid transporters, BCH (Figure 3). This suggested that the majority of L-leucine uptake by the HTB-41 cells was mediated by the amino acid transport system L, since the HTB-41 cells expressed LAT1.

The [^{14}C]L-leucine uptake by the HTB-41 cells was saturable and followed Michaelis-Menten kinetics (Figure 5). The K_m value of the HTB-41 cells for the [^{14}C]L-leucine uptake was approximately 48 μM. The K_m value of the HTB-41 cells was higher than that for human LAT1 (20 μM) (5) and rat LAT1 (18 μM) (4, 14) expressed in

Xenopus oocytes. This difference may be due to the different cell systems and conditions used and is considered to be small. In addition, when the K_m values of the HTB-41 cells (48 μM) and KB cells (65 μM) (20) for [^{14}C]L-leucine uptake were compared, they were more similar. This result also suggested that the majority of L-leucine uptake by the HTB-41 cells was mediated by LAT1.

BCH is an amino acid-related compound that has been used as a selective inhibitor of the system L amino acid transporters, including LAT1 and LAT2 (1, 23). Although BCH is a selective inhibitor of system L, BCH also inhibits the transport of amino acids mediated by a Na^+-dependent neutral and basic amino acid transporter, $ATB^{0,+}$ (24). Under Na^+-free conditions, however, BCH selectively inhibits the system L-mediated transport of amino acids (1, 23, 24). In this study, BCH completely inhibited the [^{14}C]L-leucine uptake measured in the HTB-41 cells (Figures 3, 6 and 7) in a concentration-dependent fashion (Figure 6). The inhibition was shown to be competitive, with a K_i value of 41 μM in the HTB-41 cells (Figure 7). The K_i value for the inhibition of [^{14}C]L-leucine uptake by BCH was very similar to the K_m value for [^{14}C]L-leucine uptake in the HTB-41 cells.

The [^{14}C]L-leucine uptake by the HTB-41 cells was only markedly inhibited by the L-isomers of the large neutral amino acids (Figure 8). The profiles of inhibition of [^{14}C]L-leucine uptake by L-amino acids in the HTB-41 cells are, thus, in principle, comparable to the previous reports for human LAT1 (5) and rat LAT1 (4, 14) expressed in *Xenopus* oocytes and those performed for the profiles of inhibition of [^{14}C]L-leucine uptake by L-amino acids in KB cells (20). It was thus concluded that the majority of [^{14}C]L-leucine uptake was mediated by LAT1 with its associating protein 4F2hc in the HTB-41 cells. These results also suggest that the HTB-41 cell system would be an excellent tool for investigating the transport properties of various compounds *via* LAT1.

The LAT1 is up-regulated in tumor cells to support their continuous growth and proliferation (4, 5, 12, 13) and is a major route through which the HTB-41 cells gather up large neutral amino acids. Even if the LAT1 activity in tumor cells were completely blocked, the growth and proliferation of normal cells would be possible because LAT2 is present. Therefore, the specific inhibition of LAT1 would be a new rationale for the suppression of tumor cell growth.

Overall, the majority of transport of neutral amino acids into HTB-41 human submaxillary salivary gland epidermoid carcinoma cells is mediated by LAT1 with its associating protein 4F2hc. The HTB-41 cell system would be an excellent cell system to investigate the characterization of LAT1 and LAT1 could be a new target for inhibiting salivary gland tumor cell growth.

Acknowledgements

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