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Characterization of 3-[^{125}I]iodo-α-methyl-L-tyrosine transport via human L-type amino acid transporter 1

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Abstract

We examined transport of 3-[^{125}I]iodo-α-methyl-L-tyrosine ([^{125}I]IMT) in *Xenopus laevis* oocytes co-expressing human L-type amino acid transporter 1 (a component of system L) and human 4F2hc. Human LAT1 mediated transport of [^{125}I]IMT. [^{125}I]IMT uptake was decreased by the presence of L-isomers of Cys, Leu, Ileu, Phe, Met, Tyr, His, Trp and Val and D-isomers of Leu, Phe and Met. Human LAT1-mediated [^{125}I]IMT uptake was highly stereoselective for the L-isomers of Tyr, His, Trp, Val and Ileu. To examine the effects of 3-iodination and α-methylation on IMT transport, kinetic parameters of IMT were compared with those of mother Tyr and 3-[^{125}I]iodo-L-tyrosine (3-I-Tyr). Uptake of Tyr, 3-I-Tyr and [^{125}I]IMT followed Michaelis-Menten kinetics, with K_m values of 29.0 ± 5.1, 12.6 ± 6.1 and 22.6 ± 4.1 μM, respectively. Neither the α-methyl group nor the size of the 3-iodinated Tyr residue was an obstacle to transport via hLAT1. Furthermore, affinity of IMT for hLAT1 is higher than that of the natural parent tyrosine. The level of efflux mediated by hLAT1 was highly stimulated by extracellularly applied L-Leu, suggesting exchange of [^{125}I]IMT and L-Leu via hLAT1.

Keywords: Membrane transport; 3-[^{123}I]Iodo-α-methyl-L-tyrosine; Artificial amino acid; L-type amino acid transporter 1; 4F2hc; *Xenopus laevis* oocyte

1. Introduction

3-[^{123}I]Iodo-α-methyl-L-tyrosine ([^{123}I]IMT, Fig. 1) was developed as a functional imaging agent for Tyr transport mechanisms in the brain [1] and pancreas [2]. It is a non-metabolizable artificial amino acid that has also been used clinically for SPECT imaging of tumors [3].

In cultured type 86HG-39 human glioma cells, membrane transport of [^{123}I]IMT is dominated by 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH)-sensitive transport; i.e., amino acid transport system L [4]. The kinetics of [^{123}I]IMT transport have also been studied in rat C6 glioma cells [5], human GOS3 glioma cells [6] and human Ewing's sarcoma cells [7]. The studies of [^{123}I]IMT uptake in glioma cells revealed that the majority of transport takes place via the Na^+-independent system L (> 70%) and that relatively minor uptake takes place via the Na^+-dependent system $B^{0,+}$ (< 20%). Lahoutte et al. reported that system T also mediates [^{123}I]IMT transport into U266 human myeloma cells [8].

The transport of large neutral amino acids with branched or aromatic side chains is mediated by amino acid transport system L [9]. System L is a Na^+-independent neutral amino acid transport system, and is thought to be a major route by which cells obtain branched or aromatic amino acids [9]. System L uptake has been defined as uptake of an amino acid in Na^+-free buffer which is subject to inhibition by an excess of BCH [10].

In 1998, by expression cloning from C6 rat glioma cells, we isolated cDNA encoding a Na^+-independent transporter that acts as a component of amino acid transport system L [11], and named the protein L-type amino acid transporter 1 (LAT1). This protein, which has 12 trans-membrane domains, preferentially transports large neutral amino acids such as Leu, Ile, Val, Phe, Tyr, Trp, Met and His [11]. In the performance of its function, it forms a heterodimer with a single membrane-spanning protein called 4F2hc, a type II membrane glycoprotein [11].

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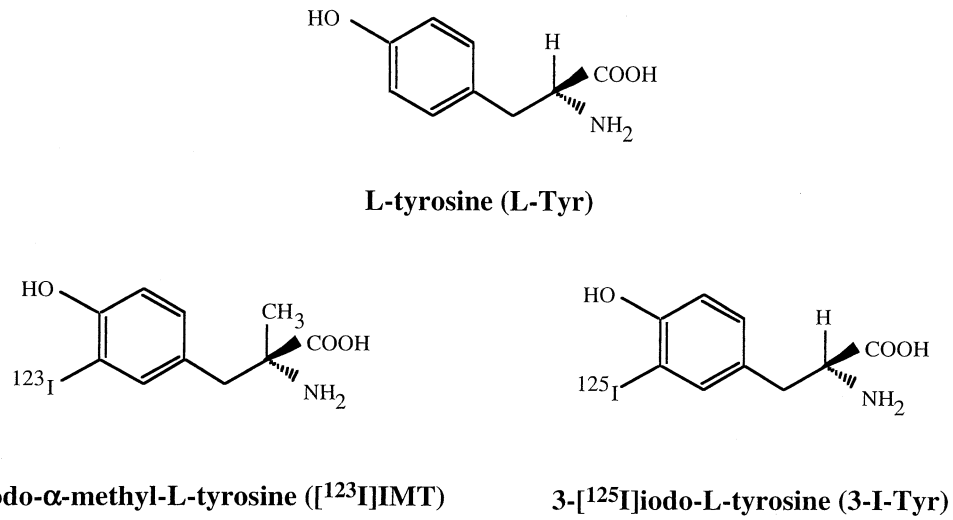

Fig. 1. Chemical structures.

It has been suggested that amino acid transporters are upregulated in transformed cells to support the high-level protein synthesis required for continuous cell growth and proliferation [9]. Some human tumor cell lines (e.g., Hela, HL-60, KG-1 and U937) express both human LAT1 (hLAT1) and the heavy chain of human 4F2 (h4F2hc) at high levels [12].

In the present study, we examined aspects of transport of [^{125}I]IMT via hLAT1 (uptake, inhibition and kinetics) using *Xenopus laevis* oocytes co-expressing hLAT1 and h4F2hc. To examine the effects of 3-iodination and α-methylation on IMT transport, kinetic parameters of IMT were compared with those of mother Tyr and 3-I-Tyr.

2. Materials and methods

All animal experiments were approved by the ethical committee of our university.

2.1. Preparation of amino acids

Reagent grade chemicals (Sigma-Aldrich, Germany) were used in this experiment. [^{125}I]NaI (8.1×10^{19} Bq/mol) was obtained from Amersham Pharmacia (U.K.). No-carrier-added [^{125}I]IMT and 3-I-Tyr were prepared using the conventional chloramine-T method, as described elsewhere [1,2]. Synthesis of inactive IMT was performed according to the method of Krummeich et al. [13]. L-[^{14}C(U)]-Tyr was obtained from American Radiolabeled Chemicals (USA).

2.2. Transport characterization

Human LAT1 (hLAT1) cDNA (GenBank/EMBL/DDBJ accession no. AB18009) was isolated from an oligo-dT-primed cDNA library prepared from human teratocarcinoma PA-1 cell poly(A)+ RNA (Clontech, USA), using a fragment corresponding to nucleotides 1135 to 1529 of rat LAT1 cDNA (GenBank/EMBL/DDBJ accession no. AB015432) as a probe, as described elsewhere [10,14]. The amino acid sequence of this hLAT1 cDNA was 100% identical to that of the hLAT1 cDNA sequence published by Prassad et al. [15].

For functional expression in *Xenopus laevis* oocytes, cRNAs for hLAT1 and human 4F2hc (h4F2hc) (GenBank/EMBL/DDBJ accession no. AB018010) were obtained by in vitro transcription using T3 RNA polymerase for hLAT1 in pBluescript II SK- (Stratagene, USA) linearized with XhoI and T7 RNA polymerase for human 4F2hc (h4F2hc) in pZL1 (Life Technologies Inc., USA) linearized with BamHI, as described elsewhere [16]. Examination of expression in *Xenopus laevis* oocytes was performed according to the methods of Kanai et al. [17], with minor modifications. *Xenopus laevis* oocytes were injected with 17.6 ng of hLAT1 cRNA and 7.4 ng of h4F2hc cRNA at a final molar ratio of 1 : 1. For expression of only hLAT1 or only h4F2hc in *Xenopus laevis* oocytes, 17.6 ng of hLAT1 cRNA or 7.4 ng of h4F2hc cRNA was injected. Two days after injection, the uptake of radio-labeled amino acids in Na^+-free uptake solution (100 mM choline-Cl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM Tris, pH 7.4) containing 18.5 to 74 kBq/ml ^{125}I-labeled amino acids was measured.

For the Na^+-containing uptake solution, the choline-Cl present in the Na^+-free uptake solution was replaced with NaCl. For the Cl^- -free uptake solution, the Cl^- present in the Na^+-containing uptake solution was replaced with gluconate anion. Preliminary experiments to determine the time-course of no–carrier-added (n.c.a.) [^{125}I]IMT uptake into oocytes expressing hLAT1 indicated that the uptake was linearly dependent on incubation time up to 45 min (data not shown). Therefore, for all subsequent experiments,

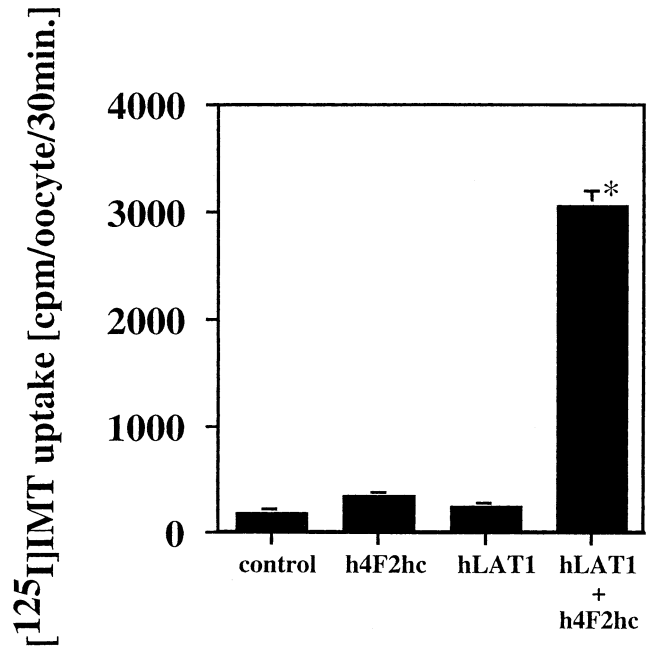

Fig. 2. [^{125}I]IMT uptake into *Xenopus laevis* oocytes co-expressing hLAT1 and h4F2hc. Co-expression of hLAT1 and h4F2hc resulted in high uptake of [^{125}I]IMT. *, $P<0.001$.

amino acid uptake rate was measured for 30 min and expressed as pmol/oocyte/min.

K_m and V_{max} of the substrates Tyr, 3-I-Tyr and IMT were determined by double reciprocal plot analysis of hLAT1-mediated amino acid uptake measured at 1, 3, 10, 30, 100 and 300 μM. hLAT1-mediated amino acid uptake was calculated as the difference between mean uptake of oocytes injected with both hLAT1 and h4F2hc cRNAs and mean uptake of control oocytes injected with water. For the inhibition study, the oocytes were incubated in the presence of 3 mM of 1 of 21 different natural L- and D-amino acids: Gly, Ala, Ser, Thr, Cys, Leu, Ileu, Phe, Met, Tyr, His, Trp, Val, Asn, Gln, Asp, Glu, Lys, Arg, Pro and Cyst.

For the efflux measurement, 100 nl (74 Bq) of [^{125}I]IMT was injected into oocytes using a fine-tipped glass micropipette, as described elsewhere [18,19]. Oocytes were individually incubated for 5 min in ice-cold Na^+-free uptake solution, and then transferred to Na^+-free uptake solution with or without 20 μfM nonradiolabeled L-leucine maintained at room temperature (18–22°C). The radioactivity of the medium and the residual radioactivity of the oocytes were measured. The efflux values were expressed as percent radioactivity (radioactivity of medium/[radioactivity of medium + radioactivity of oocytes] × 100%) [18,19].

For the uptake and efflux measurements, 7 to 8 oocytes were used for each data point. Each data point in the figures represents the mean±standard deviation of uptake (n = 7–8). To confirm the reproducibility of results, 3 separate experiments using different batches of oocytes and *in-vitro*–transcribed cRNA were performed for each measurement, except for K_m and V_{max} determination. Results from the representative experiments are shown in the figures.

3. Results

3.1. Preparation of amino acids

Labeling of α-methyl-L-tyrosine and L-tyrosine produced [^{125}I]IMT and 3-I-Tyr, respectively, at labeling efficiency of more than 80% for both compounds. After purification, no-carrier-added [^{125}I]IMT and 3-I-Tyr with radiochemical purities greater than 95% were obtained. For both compounds, purification by reverse-phase HPLC yielded inactive IMT at a rate of 68%.

3.2. Transport characterization

As shown in Fig. 2, hLAT1 requires h4F2hc for functional ([^{125}I]IMT uptake) expression in *Xenopus laevis* oocytes. The uptake of [^{125}I]IMT by *Xenopus laevis* oocytes expressing only hLAT1 was 16-fold greater ($p<0.001$) than that of the control oocytes (injected with water instead of cRNA). Uptake of [^{125}I]IMT by *Xenopus laevis* oocytes co-expressing h4F2hc and hLAT1 was 13-fold greater than that of oocytes expressing only hLAT1.

The uptake of [^{125}I]IMT by co-expressing oocytes in the presence and absence of Na^+ is compared in Fig. 3.

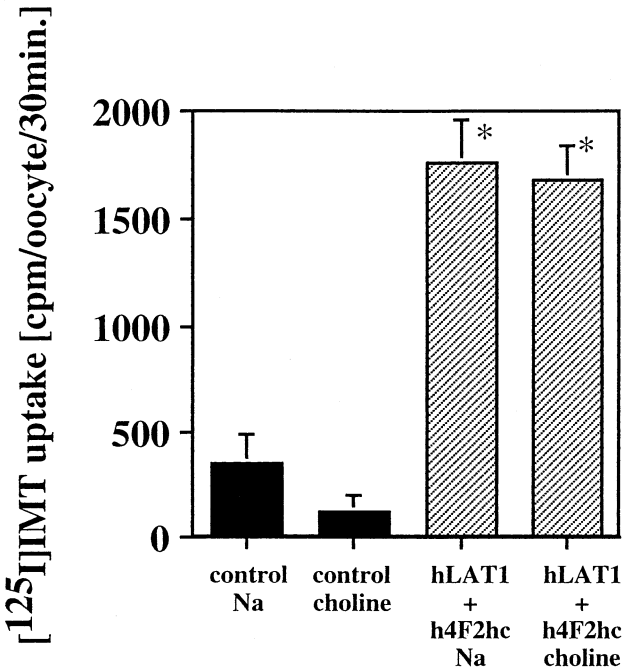

Fig. 3. Na^+ independence of [^{125}I]IMT transport in oocytes expressing hLAT1 and h4F2hc. There was no significant difference in uptake of [^{125}I]IMT (n.c.a.) between the standard uptake solution and the Na^+-free uptake solution, in which Na^+ was replaced with the same concentration of choline. *, $P<0.001$.

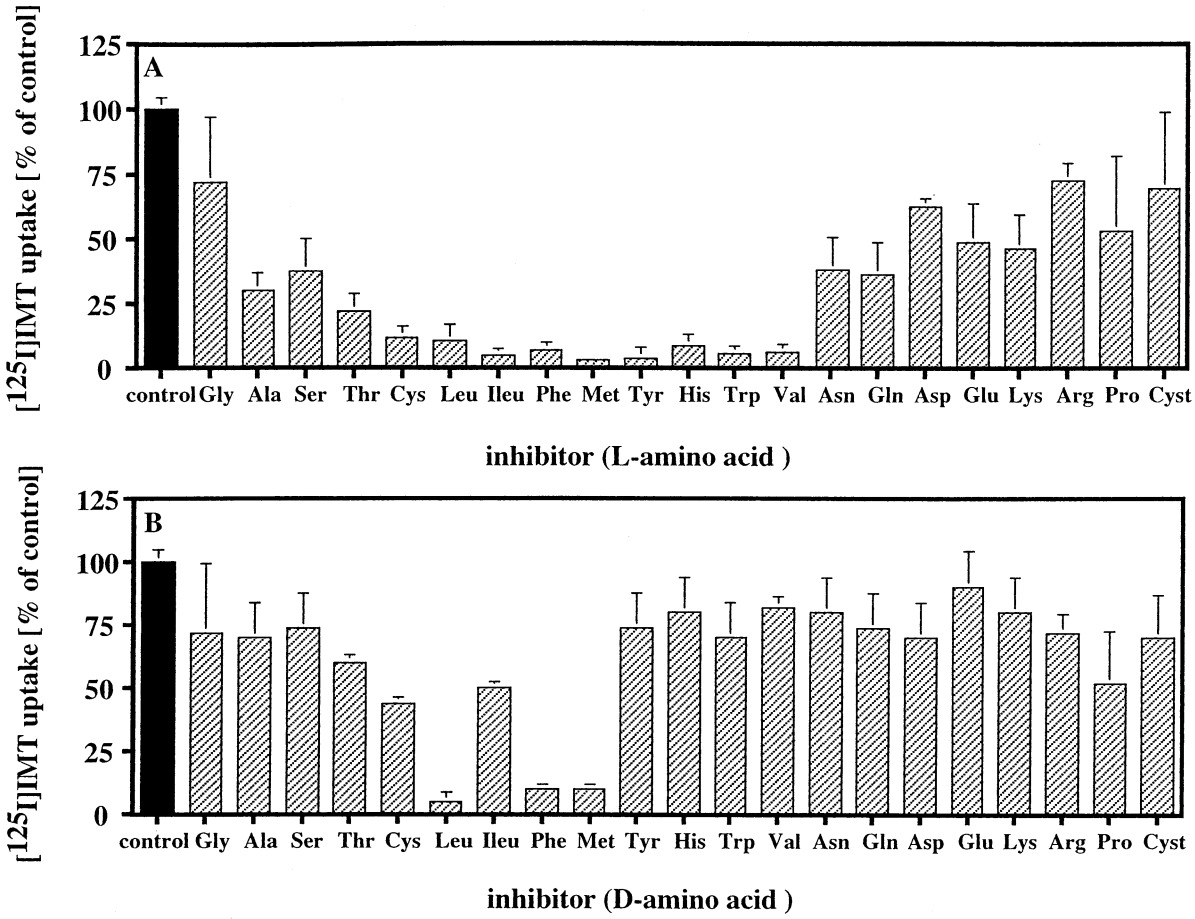

Fig. 4. The effects of L- and D-amino acids on hLAT1-mediated transport of [^{125}I]IMT. (a) Inhibition of hLAT1-mediated [^{125}I]IMT (n.c.a.) uptake by L-amino acids in Na^+-free uptake solution. Human LAT1-mediated [^{125}I]IMT (n.c.a.) uptake was measured in the presence of 3 mM nonradiolabeled L-amino acids. Values are expressed as percentage of the control value ([^{125}I]IMT uptake in the absence of inhibitors). (b) Inhibition of hLAT1-mediated [^{125}I]IMT (n.c.a.) uptake by D-amino acids. Same experimental procedures as described in (a).

[^{125}I]IMT uptake was not dependent on Na^+ or Cl^- (data not shown).

As shown in Fig. 4 (A), the uptake of [^{125}I]IMT was markedly inhibited by the presence of Cys, Leu, Ileu, Phe, Met, Tyr, His, Trp and Val. Ala, Ser, Thr, Asn and Gln had relatively weaker inhibitory effects on hLAT1-mediated [^{125}I]IMT uptake. As shown in Fig. 4 (B), D-isomers of Leu, Phe and Met strongly inhibited hLAT1-mediated [^{125}I]IMT uptake. In contrast, hLAT1-mediated [^{125}I]IMT uptake was highly stereoselective for the L-isomers of Tyr, His, Trp, Val and Ileu.

The uptake was saturable and fit the Michaelis-Menten curve (Fig. 5). The kinetic parameters obtained are shown in Table 1. Uptake of Tyr, 3-I-Tyr and IMT followed Michaelis-Menten kinetics with K_m values of 29.0 ± 5.1, 12.6 ± 6.1 and 22.6 ± 4.1 μM (mean ± standard deviation of 4 separate experiments), respectively.

The level of [^{125}I]IMT efflux mediated by hLAT1 was fairly low. However, as shown in Fig. 6, L-Leu applied extracellularly greatly stimulated the efflux of [^{125}I]IMT (preloaded substrate). This suggests amino acid exchange via hLAT1.

4. Discussion

In the first step of their metabolism, neutral amino acids are taken into cells by membrane transport and pooled as free amino acids. They are then used as monomers of structural proteins, materials for biosynthesis of neurotransmitters, or substrates for energy metabolism [9]. Because of its high metabolic stability, [^{125}I]IMT is not metabolized and remains a free amino acid *in vivo* [1]. This artificial amino acid [^{125}I]IMT has generated much interest since it was demonstrated that its uptake specifically reflects increased amino acid membrane transport in gliomas [3,20].

Membrane transport of [^{123}I]IMT has been studied using cultured human 86HG-39 glioma cells [4], rat C6 glioma cells [5], human GOS3 glioma cells [6], and human Ewing's sarcoma cells [7]. The majority of transport of [^{123}I]IMT in glioma cells involves the Na^+-independent system L (>

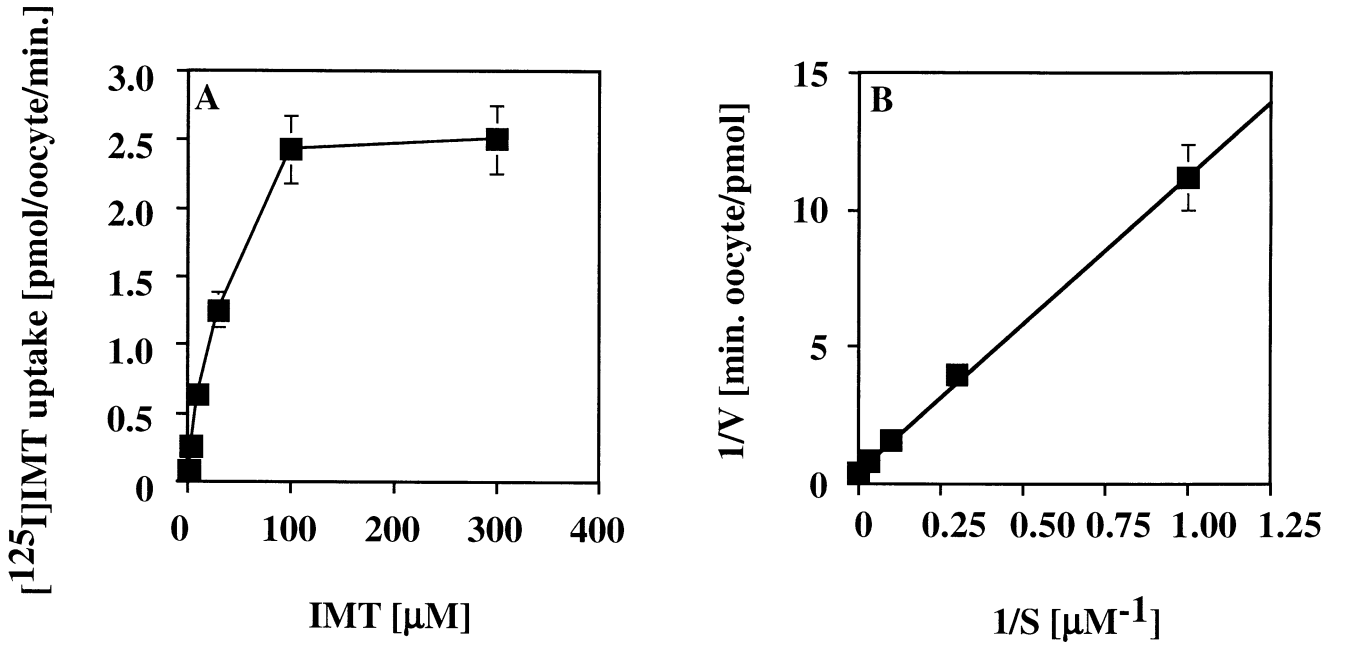

Fig. 5. Concentration dependence of hLAT1-mediated [^{125}I]IMT uptake. The hLAT1-mediated [^{125}I]IMT uptake in oocytes co-expressing hLAT1 and h4F2hc was measured for 1, 3, 10, 30, 100 and 300 μM nonradiolabeled IMT in Na^+-free uptake solution. The uptake was plotted against IMT concentration. (b) Double reciprocal plot of the IMT uptake, from which kinetic parameters were determined.

70%), and the Na^+-dependent system $B^{0,+}$ is responsible for a relatively minor amount of transport (< 20%). The Michaelis constant K_m of [^{125}I]IMT was found to be quite high (20−45 μM) in the above-mentioned cell line studies [4,5,6,7]. In U266 human myeloma cells, system L contributes 39.0% of [^{123}I]IMT influx, and system T contributes 43.8% [8]. System L transports amino acids with bulky and/or branched chains, and has a broad substrate specificity [9,11,12]. System T is more specific, and favors aromatic amino acids [9]. System T has only been found on the membrane of red blood cells and hepatocytes, whereas system L is present in most cell types [9]. The transport of most neutral amino acids involves several distinct systems with overlapping substrate specificities [9]. The main systems transporting neutral amino acids can be divided into Na^+-dependent transport systems (systems A, ASC, $B^{0,+}$ and B^0) and Na^+-independent transport systems (systems L and T) [9].

Cell line studies have revealed that systems L, A, T and $B^{0,+}$ (and/or B^0) play a role in [^{123}I]IMT transport [7,8]. However, it has also been shown that the Na^+-independent carrier systems $b^{0,+}$ and y^+ do not play a role in the [^{123}I]IMT uptake process [7,21]. It is unclear whether system y^+L is involved in [^{123}I]IMT uptake [7].

In the present study, the kinetic parameters determined in Table 1 reveal correlation between structure and transport

Table 1
Kinetic parameters of amino acid substrates

Amino acid	K_m (μM)	V_{max}
Tyr	29.0 ± 5.1	(1.00)
3-I-Tyr	12.6 ± 6.1	1.60
IMT	22.6 ± 4.1	0.81

K_m values = mean ± standard deviation of 4 separate experiments.

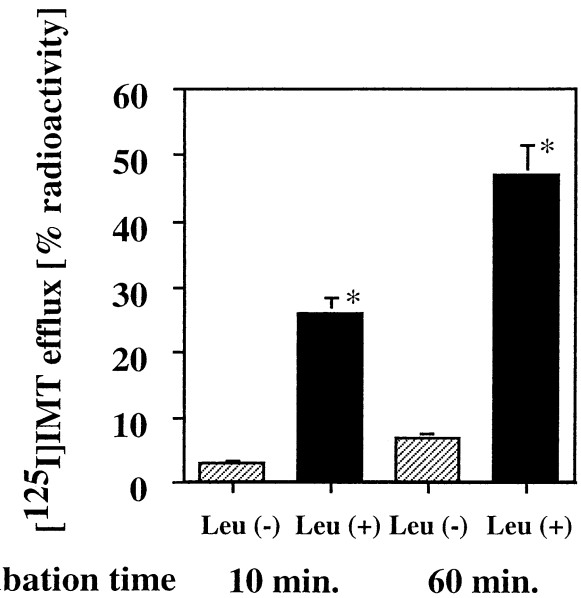

Fig. 6. [^{125}I]IMT efflux via hLAT1. Efflux of radioactivity from oocytes expressing hLAT1 and h4F2hc in Na^+-free uptake solution containing 0 μM (hatched columns) or 100 μM (black columns) of nonlabeled L-Leu was measured. Values are expressed as percentage of total radioactivity loaded into the oocytes. *, $P<0.001$.

function. The molecular weight of Tyr is about 181, and the atomic weight of the stable isotope of iodine is 127. The atomic weight of iodine accounts for approximately 41% and 38% of the molecular weight of 3-I-Tyr and IMT, respectively. Although hLAT1 preferentially transports neutral amino acids with bulky hydrophobic residues [11, 12], we assumed that the weight added by introducing iodine into the benzene ring of Tyr would decrease the affinity of [^{125}I]IMT membrane transport via hLAT1. However, our results were clearly contrary to this prediction. Not only did hLAT1 exhibit high degrees of affinity (K_m=10–30 μM) for all 3 substrates, Tyr, 3-I-Tyr and IMT, but it exhibited higher affinity for [^{125}I]IMT than for mother Tyr. The present K_m value for [^{125}I]IMT agrees with the values obtained in previous cell line studies. Thus, hLAT1 mediates transport of [^{125}I]IMT as well as Tyr and 3-I-Tyr, indicating that this transporter has adequate space for the α-methyl group of [^{125}I]IMT to pass through the transmembrane domains of the protein.

Thus, introduction of hydrophobic iodine into the benzene ring of Tyr results in higher transport affinity. Neither the α-methyl group nor the size of the 3-iodinated Tyr residue was an obstacle to transport via hLAT1. In contrast, hLAT1 was unable to transport a 3,5-diodinated Tyr residue (data not shown).

It has been reported that system L-mediated [^{123}I]IMT transport and h4F2hc antigen expression significantly correlate with each other, and that both correlate with the proliferation rate of human glioma cells *in vitro* [22]. As shown in Fig. 2, hLAT1 requires h4F2hc for functional ([^{125}I]IMT uptake) expression in *Xenopus laevis* oocytes. The results in Fig. 3 show that the transport of [^{125}I]IMT was Na^+-independent. Radioactivity of oocytes injected with cRNA significantly increased after uptake incubation, compared to control oocytes. These findings are strong evidence for the occurrence of hLAT1-mediated transport of [^{125}I]IMT in *Xenopus laevis* oocytes injected with hLAT1 cRNA.

Because it has been shown that D-amino acids are endogenously present in mammalian tissues [23], both D- and L-amino acids were used as inhibitors. Fig. 4 shows that [^{125}I]IMT competed for transport with L-isomers of Cys, Leu, Ileu, Phe, Met, Tyr, His, Trip and Val and D-isomers of Leu, Phe and Met. High stereoselectivity for Tyr, His, Trip, Val and Ileu was observed. L-isomers of small neutral amino acids (Gly, Ala, Ser and Thr) slightly inhibited [^{125}I]IMT uptake. Neither basic amino acids (Lys and Arg) nor acidic amino acids (Glu and Asp) inhibited [^{125}I]IMT uptake.

As shown in Fig. 6, L-Leu applied extracellularly induced the efflux of [^{125}I]IMT (preloaded substrate). It has been suggested that hLAT1 acts as an intracellular Gln exchanger in the influx of substrates [12]. It has been reported that Asn and Gln are transported with relatively low affinity [12]. However, it has also been reported that the pools of free Asn and Gln in cells are considerably larger than those of other amino acids, such as Leu, Ileu, Phe, Met, Tyr, His, Trip and Val [24]. Other amino acid exchange transporters have been reported [25]; e.g., the cysteine/glutamate exchange transporter xCT, the cDNA of which has been cloned [26]. The free Glu pools in cells are also large [24].

Human LAT1 is expressed in a wide variety of organs. Its highest expression levels are in the brain, placenta, testis, bone marrow and fetal liver, followed by peripheral leukocytes, lymph nodes and the thymus [12]. In a previous study, rat LAT1 was found to act as a system L transporter at the blood-brain barrier [27].

Given present and previous findings of experiments using [^{123}I]IMT, this molecule can be expected to facilitate future research on tumors and cerebral function, and be useful in SPECT research into amino acid transport.

Of the genes identified thus far by the human genome project, about 3% are believed to function as membrane or intracellular transporters. Furthermore, about 30% of currently available clinical drugs target transporters or channels. The goals of membrane transport research include isolation of all human genes involved in drug transport, clarification of sites of expression of these genes and distribution of their gene products, identification of the gene product substrates, and characterization of inhibitory mechanisms. Further studies of transport of IMT via L-type amino acid transporters are currently being conducted by the present authors.

5. Conclusions

We have clarified characteristics of membrane transport of [^{125}I]IMT via the amino acid transporter hLAT1 (a component of system L) using molecular biological methods and hLAT1 cDNA. [^{125}I]IMT was confirmed to be the substrate transported by hLAT1. Uptake and inhibition of this compound were examined by co-expressing hLAT1 and h4F2hc in *Xenopus laevis* oocytes. [^{125}I]IMT uptake was decreased by the presence of L-isomers of Cys, Leu, Ileu, Phe, Met, Tyr, His, Trp and Val and D-isomers of Leu, Phe and Met. Human LAT1-mediated [^{125}I]IMT uptake was highly stereoselective for the L-isomers of Tyr, His, Trp, Val and Ileu. The uptake was Na^+-independent, and was activated by h4F2hc. The affinity of hLAT1-mediated transport for [^{125}I]IMT (K_m=22.6 μM) was greater than for mother Tyr (K_m=29.0 μM).

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