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Radioiodinated 4-iodo-L-*meta*-tyrosine, a system L selective artificial amino acid: molecular design and transport characterization in Chinese hamster ovary cells (CHO-K1 cells)

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Abstract

Introduction: High expression of the system L amino acid transporter has been observed in clinically important tissues including tumors and the blood-brain barrier. We examined amino acid transport system L selectivity of ^{14}C(U)-L-tyrosine (^{14}C-Tyr), ^{125}I-4-iodo-L-*meta*-tyrosine (4-^{125}I-*m*Tyr), ^{125}I-6-iodo-L-*meta*-tyrosine (6-^{125}I-*m*Tyr), ^{125}I-3-iodo-α-methyl-L-tyrosine (^{125}I-IMT) and ^{125}I-3-iodo-L-tyrosine (3-^{125}I-Tyr) using Chinese hamster ovary cells (CHO-K1).

Methods: Cells in the exponential growth phase were incubated with 18.5 kBq of labeled amino acid in 2 mL of phosphate-buffered saline-based uptake solution and an uptake solution with/without Na^+ at 37°C or 4°C. We examined the effects of the following compounds (1.0 mM) on transport: 2-(methylamino)isobutyric acid (a specific inhibitor of system A, in Na^+-containing uptake solution); 2-amino-bicyclo [2,2,1]heptane-2-carboxylic acid (a specific inhibitor of system L, in Na^+-free uptake solution); sodium azide and 2,4-dinitrophenol (NaN_3 and DNP, inhibitors of the generation of adenosine triphosphate); *p*-aminohippurate and tetraethylammonium (PAH and TEA, inhibitors of organic anion and cation transporters); and L- and D-isomers of natural amino acids.

Results: ^{14}C-Tyr exhibited affinity for systems L, A and ASC. 4-^{125}I-*m*Tyr and 3-^{125}I-Tyr exhibited high specificity for system L, whereas 6-^{125}I-*m*Tyr and ^{125}I-IMT exhibited affinity for both systems L and ASC. Uptake of 4-^{125}I-*m*Tyr was markedly reduced by incubation at 4 °C, and was not significantly inhibited by NaN_3, DNP, PAH or TEA. The inhibition profiles of the L- and D-isomers of natural amino acids indicated that system L mediates the transport of 4-^{125}I-*m*Tyr.

Conclusions: 4-^{125}I-*m*Tyr exhibited the greatest system L specificity (93.46±0.13%) of all of the tested amino acids.

Key words: 4-iodo-L-*meta*-tyrosine; System L; Neutral amino acid; Chinese hamster ovary cells

1. Introduction

Amino acid uptake by cells appears to be one of the most important processes involved in cellular functions. System L is an important neutral amino acid transport system, because it is a major route by which cells are supplied with large neutral amino acids, including branched and aromatic amino acids. Substrates of system L include some essential amino acids and precursors of neurotransmitters [1]. System L transporters are expressed at high levels in angioendothelial cells of the blood-brain barrier and the placenta barrier [2]. Alterations in amino acid transport systems in the brain are frequently associated with neuropsychiatric disorders, such as Parkinson's disease [3] and schizophrenia [4]. Also, system L is up-regulated in many kinds of tumor cells undergoing active growth and proliferation [2]. Because of its

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broad substrate selectivity, it has been proposed that system L transports not only naturally occurring amino acids but also amino-acid related compounds such as L-DOPA (therapeutic drug for Parkinson's disease), meluphalan (anticancer phenylalanine mustard), triiodothyronine (thyroid hormone), thyroxine (thyroid hormone), gabapentin (anticonvulsant), and *S*-(1,2-dichlorovinyl)-L-cysteine (neurotoxic cysteine conjugate) [5–10]. System L plays important roles in the metabolism and pharmacokinetics of the abovementioned drugs and compounds. Thus, the available evidence suggests that system-L-specific artificial amino acids would be useful as imaging agents for studies of tumors, cerebral functions and other important biological phenomena.

Systems L, A and ASC, which are the neutral amino acid transport systems of the plasma membrane, have different biochemical characteristics. System L is defined by its Na^+-independency and inhibition by 2-amino-bicyclo[2,2,1] heptane-2-carboxylic acid (BCH). System A is defined by its Na^+-dependency and inhibition by 2-(methylamino) isobutyric acid (MeAIB). System ASC is a Na^+-dependent transport system that is intolerant of *N*-methylation of substrates, but no specific substrate for system ASC has yet been found. These systems have been characterized in a study by Shotwell et al. using Chinese hamster ovary cells [11]. A well-characterized, popular cell line such as CHO-K1 may be a useful tool for the development of artificial amino acids, by facilitating comparisons of transport properties between different amino acid transport markers.

System L1 and L2 (components of system L) genes are classified into two different families of solute carriers (SLCs), the SLC7 and SLC43 families, respectively, based on the standards established by the Human Gene Nomenclature Committee [12–15]. The SLC7 family is further divided into the cationic amino acid transporter family and the L-type amino acid transporter (LAT) family [12–14]. The two known isoforms of system L1 belong to the LAT family and are named type 1 (LAT1) and type 2 (LAT2). The LAT1 and LAT2 transporters of system L1, but not the LAT3 and LAT4 transporters of system L2, form a heterodimeric complex with an additional protein, the heavy chain of the 4F2 antigen (CD98; 4F2hc/SLC3A2), which is required for LAT1/2 transport function. LAT1/2 and LAT 3/4 also differ in that LAT1 and LAT2 selectively transport neutral amino acids by obligatory exchange mechanisms, whereas LAT3 and LAT 4 did not display obligatory exchange mechanisms. Although these transporters show partly overlapping expression patterns and substrates, some differences in their expression and substrates have been observed [12–15].

Since tumors require increased uptake of amino acids for their enhanced growth and proliferation, it is of interest that LAT1 is strongly up-regulated in many cancer cells both in vitro and in vivo [16].

The amino acid Tyr is important biologically, not only as a component of proteins and a precursor of thyroid hormones but also (in its hydroxylated form dopa) as a precursor of catecholamines and melanin [17]. Previous findings suggested that Tyr derivatives could be used to measure neutral amino acid transport.

We previously investigated the biodistribution and excretion of the following compounds in mice, using metabolite analysis: ^{125}I-4-iodo-L-*meta*-tyrosine (4-^{125}I-*m*Tyr), ^{125}I-6-iodo-L-*meta*-tyrosine (6-^{125}I-*m*Tyr), ^{125}I-3-iodo-α-methyl-L-tyrosine (^{125}I-IMT), naturally occurring ^{14}C(U)-L-tyrosine (^{14}C-Tyr), and ^{125}I-3-iodo-L-tyrosine (3-^{125}I-Tyr) (Fig. 1). We have presented our findings regarding the in vivo pharmacological features of those compounds in several reports [18–21]. The artificial amino acid 4-^{125}I-*m*Tyr exhibited high metabolic stability, rapid blood clearance, rapid urinary excretion and a biodistribution similar to that of ^{125}I-IMT [21]. However, the pharmacological characteristics of 4-^{125}I-*m*Tyr differed greatly from those of ^{125}I-IMT, particularly in renal handling [21].

Mechanisms of accumulation of the above compounds (except for ^{125}I-IMT) have not been fully clarified. In the present study, using CHO-K1 cells and a modification of the method of Shotwell et al., we examined the mechanisms of uptake of iodinated tyrosine analogues, with particular emphasis on 4-^{125}I-*m*Tyr. The present findings should be

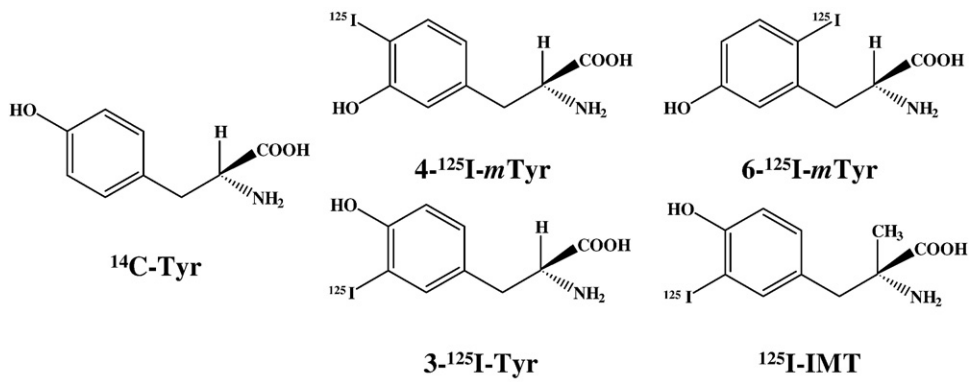

Fig. 1. Chemical structures of ^{14}C(U)-L-tyrosine (^{14}C-Tyr), ^{125}I-4-iodo-L-*meta*-tyrosine (4-^{125}I-*m*Tyr), ^{125}I-6-iodo-L-*meta*-tyrosine (6-^{125}I-*m*Tyr), ^{125}I-3-iodo-L-tyrosine (3-^{125}I-Tyr) and ^{125}I-3-iodo-α-methyl-L-tyrosine (^{125}I-IMT).

useful for the development of radiopharmaceuticals that target system L amino acid transport [16].

2. Materials and methods

2.1. Materials

The radiolabeled precursors (D,L-*m*Tyr, α-methyl-L-tyrosine and L-tyrosine) were purchased from Sigma-Aldrich Japan Co. ^{125}I-NaI was purchased from Amersham Pharmacia Biotech. ^{14}C-Tyr was purchased from American Radiolabeled Chemicals Inc. Chinese hamster ovary cells (CHO-K1) were purchased from the RIKEN Bioresource center cell bank. Plastic tissue culture dishes (diameter, 60 mm) were purchased from Falcon. Plastic culture flasks (surface area, 25 cm^2) were purchased from Nalge Nunc International. 2-amino-bicyclo[2,2,1]heptane-2-carboxylic acid (BCH, a system-L-specific inhibitor), 2-(methylamino) isobutyric acid (MeAIB, a system-A-specific inhibitor), natural L/D-amino acids and other chemicals of reagent grade were purchased from Kanto Chemical.

2.2. Labeled Amino acids

^{125}I-3-iodo-α-methyl-L-tyrosine (^{125}I-IMT) and ^{125}I-3-iodo-L-tyrosine (3-^{125}I-Tyr) were prepared using conventional methods described elsewhere [18–21]. The methods used to prepare the other labeled amino acids are described in detail elsewhere, and are described here briefly. For ^{125}I-4-iodo-L-*meta*-tyrosine (4-^{125}I-*m*Tyr) and ^{125}I-6-iodo-L-*meta*-tyrosine (6-^{125}I-*m*Tyr), we separated L-*m*Tyr from D, L-*m*Tyr by high-performance liquid chromatography (HPLC) using a Crownpak CR(−) chiral column (Daicel Chemical Industries). The separated L-*m*Tyr was labeled with ^{125}I-NaI and Chloramine-T (Kanto Chemical). Radioiodination of L-*m*Tyr produces two major geometric isomers: 6-iodo- and 4-iodo-L-*m*Tyr [20]. To divide and purify those two isomers, we performed HPLC with a Nova-Pak C18 column (Waters). Radiochemical yield was checked using a silica gel thin-layer chromatography kit (Merck). The labeled compounds were used in their non-carrier-added condition.

2.3. Cell line

We performed the cellular uptake experiment using a modification of the method of Shotwell et al. [11]. CHO-K1 cells were maintained by serial passage in 25-cm^2 cell culture flasks. The cells were fed with Dulbecco's modified Eagle's medium (Sigma-Aldrich Japan K.K.) supplemented with L-glutamine (2 mM) and 10% fetal bovine serum without antibiotics, in an atmosphere of 5% CO_2 and 95% air at 37°C (pH 7.4). Subculture was performed every 5 days using 0.02% ethylenediamine tetra-acetic acid (EDTA) and 0.05% trypsin.

CHO-K1 cells were seeded on 60-mm dishes at a density of 5×10^5 cells/cm^2 in 5.0 ml of the complete culture medium. The growth curve was plotted based on growth up to 6 days after inoculation. Cells were used for experiments on the third or fourth day after inoculation.

2.4. Time course of uptake of labeled amino acids

After removal of the culture medium, each of the above 60-mm dishes was washed once with 5 mL of incubation medium for 10 min at 37°C. The incubation medium consisted of 137 mM NaCl, 2.7 mM KCl, 8.0 mM $Na_2HPO_4\cdot12H_2O$, 1.5 mM KH_2PO_4, 1.8 mM $CaCl_2\cdot2H_2O$, 1.0 mM $MgCl\cdot6H_2O$ and 5 mM 2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, with a final pH of 7.4. The cells were then incubated with 2 mL of incubation medium containing 18.5 kBq of labeled amino acids, for 5, 10, 15 or 30 min at 37°C. The incubation medium was then aspirated, and the cell monolayers were rapidly rinsed twice using 5 mL of ice-cold incubation medium. The cells were then solubilized in 1.5 mL of 1 N NaOH, and the radioactivity of each aliquot was counted. We measured the radioactivity associated with solubilized cells using an ARC-380 well-type scintillation counter (Aloka) for ^{125}I-labeled compounds, and using an LS6500 liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA) for ^{14}C-labeled compounds.

2.5. Determination of transport system selectivity of labeled amino acids

We examined the system-L specificity of transport of the labeled amino acids in CHO-K1 cells. One of the following compounds was added to cell cultures at a final concentration of 1 mM: BCH in incubation medium without Na^+; or MeAIB in incubation medium with Na^+. The cells were then incubated in 2 mL of incubation medium with/without Na^+ containing 18.5 kBq of one of the labeled amino acids for 5 min at 37°C. In the medium without Na^+, NaCl was replaced with the same concentration of choline-Cl. The other experimental conditions were the same as those of the time course experiment.

2.6. Characterization of 4-^{125}I-mTyr transport

For the inhibition experiment, we added one of the following compounds (final concentration, 1.0 mM) to cell cultures in incubation medium containing Na^+ and 18.5 kBq of 4-^{125}I-*m*Tyr [22]: *p*-aminohippurate (PAH, inhibitor of organic anion transporter), tetramethylammonium (TEA, inhibitor of organic cation transporter), 2,4-dinitrophenol (DNP, inhibitor of adenosine triphosphate generation) or sodium azide (NaN_3, inhibitor of adenosine triphosphate generation). Because it has previously been demonstrated that both enantiomers of amino acids are endogenously present in mammalian tissues [23,24], we tested the transport-inhibitory properties of both D- and L-amino acids, at a final concentration of 1.0 mM. The cells were incubated with the above compounds for 5 min at 37°C. To examine effects of low temperature, we incubated cells for 5 min at 4°C.

2.7. Statistical analysis

Data were collated as the mean±S.D. of three to five measurements, and each experiment was performed in duplicate. Results were analyzed using Student's *t* test. Probability levels of $P<.0001$ were considered to indicate statistical significance.

3. Results

Fig. 2 shows the growth curve of the CHO-K1 cells. It took 5 days for the cells to reach confluence. On the second to fourth day after inoculation, the cells were in the exponential growth phase. Cells were used for experiments on the third or fourth day after inoculation.

In the time course analysis of uptake of labeled amino acids into CHO-K1 cells, steady-state levels were reached about 10 min after the start of incubation (Fig. 3). An incubation time of 5 min was used for all uptake measurements. Mother ^{14}C-Tyr had the highest rate of uptake at steady-state (Fig. 3A). Uptake of 4-^{125}I-*m*Tyr was much greater than that of 6-^{125}I-*m*Tyr or 3-^{125}I-Tyr for up to 15 min after the start of incubation (Fig. 3B).

Fig. 4 shows the contributions of individual transport systems to total uptake of the 5 labeled amino acids. Systems A, ASC and L mediated uptake of mother ^{14}C-Tyr. Systems ASC and L mediated uptake of ^{125}I-IMT and 6-^{125}I-*m*Tyr. The labeled amino acids 4-^{125}I-*m*Tyr and 3-^{125}I-Tyr exhibited high specificity for Na^+-independent uptake mediated by system L.

Uptake of 4-^{125}I-*m*Tyr was markedly reduced by incubation at 4 °C (3.3%). None of the chemical inhibitors significantly inhibited uptake of 4-^{125}I-*m*Tyr (Fig. 5). Neutral amino acids (Ala, Ser, Thr, Cys, Leu, Ile, Phe, Met, Tyr, His, Trp Val and Asn) inhibited uptake of 4-^{125}I-*m*Tyr (Fig. 6). However, D-amino acids (Ala, Ser, Thr and Cys) had weak or statistically insignificant inhibitory effects on uptake of 4-^{125}I-*m*Tyr. L-isomers had stronger inhibitory effects than D-isomers.

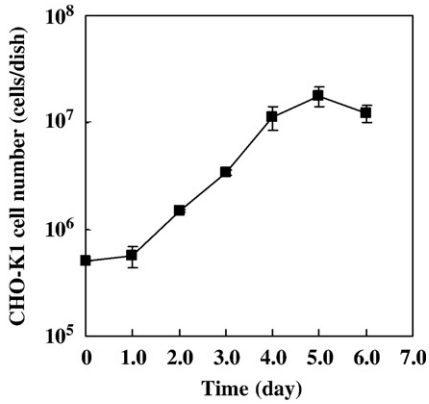

Fig. 2. Growth curve of CHO-K1 cells.

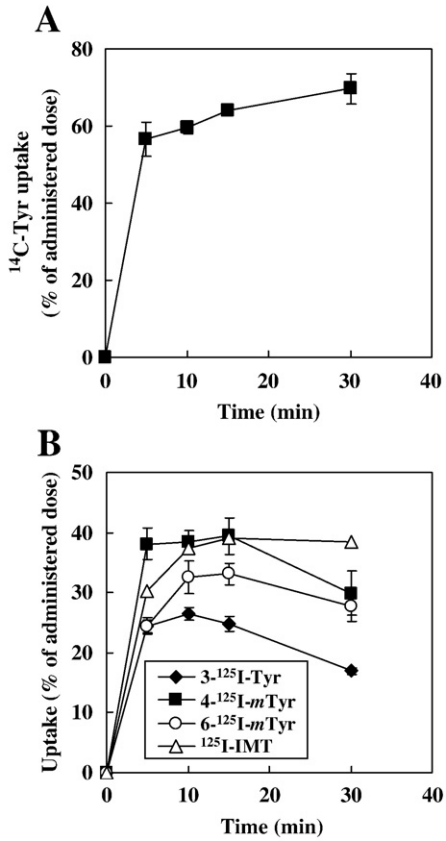

Fig. 3. Time course analysis of uptake of ^{14}C-Tyr (A) and the ^{125}I-labeled tyrosine analogues (B) into CHO-K1 cells. Each bar represents the mean± S.D. of five monolayers.

4. Discussion

In experiments using Chinese hamster ovary cells, Shotwell et al. have determined the individual contributions of transport systems L, A and ASC to the total uptake of naturally occurring neutral amino acids [11]. They reported that most neutral amino acids enter cells via multiple transport systems. System L was named for its "leucine-preferring" characteristics, although it also transports other large neutral amino acids [15]. In a review article, Christensen concluded that leucine and phenylalanine should not be considered specific substrates of system L [1]. BCH, which is a non-metabolizable amino acid analogue, is commonly used (in place of naturally occurring neutral amino acids) as a specific model compound for system L transport in uptake solution without Na^+ [1].

Radioiodination has been extensively used to label compounds of medical/biologic interest. However, the chemical structure of BCH suggests that it is difficult to radiolabel, especially with a single photon emission nuclide. Tyr derivatives labeled with a γ-emitter (^{125}I or ^{123}I) or a positron emitter (^{124}I) are suitable radiopharmaceuticals for studies of system L transport specificity. The phenol ring is a convenient site for labeling of Tyr and its derivatives

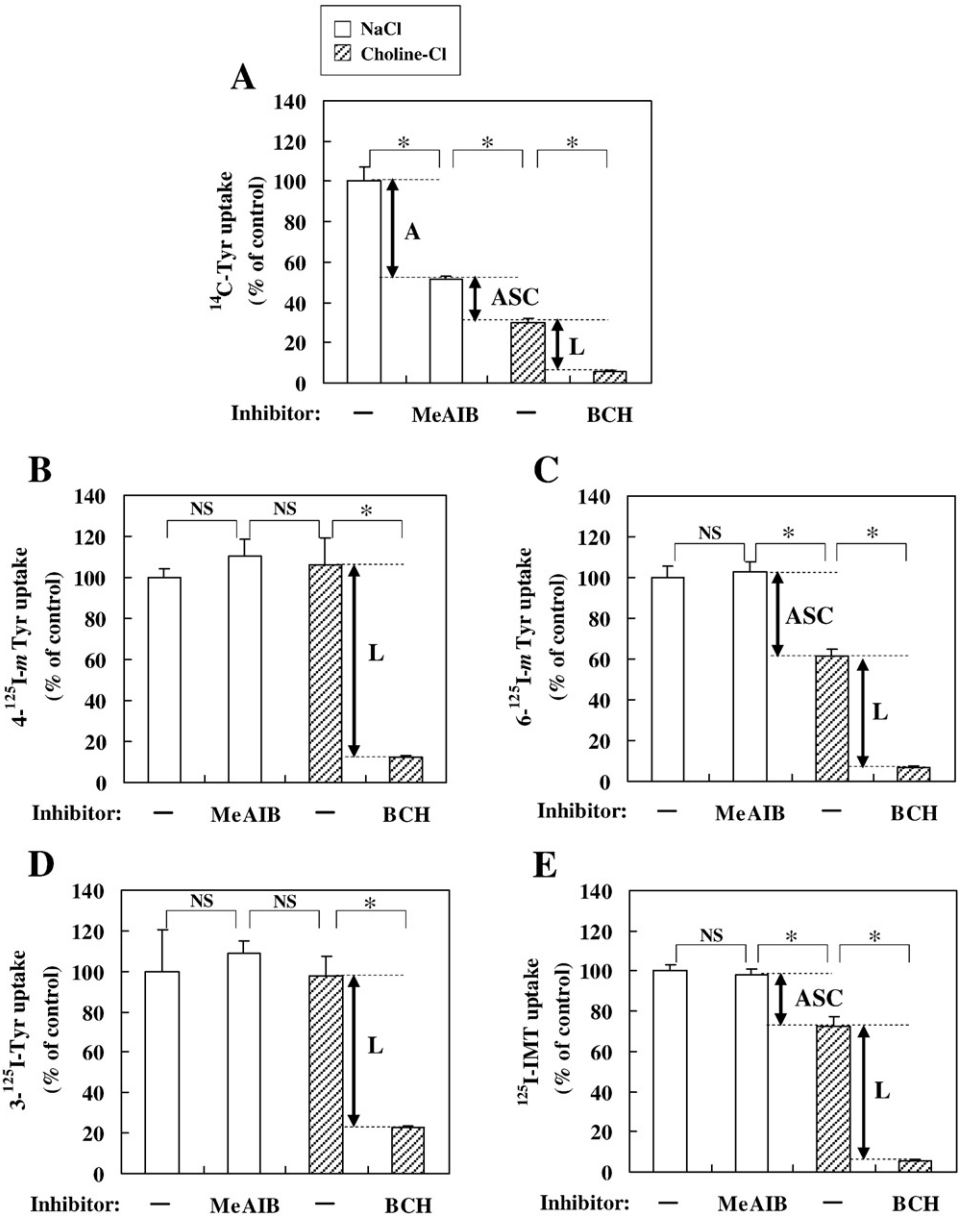

Fig. 4. Experimental definition and comparison of contributions of neutral amino acid transport systems to uptake of ^{14}C-Tyr (A), 4-^{125}I-*m*Tyr (B), 6-^{125}I-*m*Tyr (C), 3-^{125}I-Tyr (D) and ^{125}I-IMT (E) into CHO-K1 cells. Each bar represents the mean±S.D. of five monolayers. *P<.0001; NS, not significant.

(Fig. 1). In the present study, we prepared ^{125}I-labeled compounds by performing direct electrophilic radioiodination using the Chloramine-T method, because it is an easy way to prepare radioiodinated compounds [20].

In the present study, transport of mother ^{14}C-Tyr was mediated by systems A, ASC and L, and ^{14}C-Tyr is therefore not a specific substrate of a single neutral amino acid transport system in CHO-K1 cells (Fig. 4). We speculated that the increase in weight or bulkiness caused by introduction of iodine into the phenol ring of Tyr could change the affinity of Tyr for these amino acid transport systems. The molecular weight of Tyr (or *m*Tyr) and α-methyl tyrosine is about 181 and 195, respectively, and the atomic weight of the stable isotope of iodine is about 127. Thus, the atomic weight of iodine accounts for approximately 41% and 38% of the molecular weight of $C_9H_{11}NO_3I$ (for 3-I-Tyr, 4-I-*m*Tyr and 6-I-*m*Tyr) and $C_{10}H_{13}NO_3I$ (for IMT), respectively. The present results indicate that these molecular alterations caused changes in their specificity for amino acid transport systems (Fig. 4). Also, differences in the position of the iodine and hydroxyl group in the phenol ring and in the presence of an alpha-methyl group caused differences in specificity for amino acid systems.

In the present study, mono-iodination of the phenol ring caused a lack of affinity for system A and an increase in specificity for system L (Fig. 4B–E), compared to ^{14}C-Tyr (Fig. 4A). For example, 3-^{125}I-Tyr exhibited no affinity for system A transport.

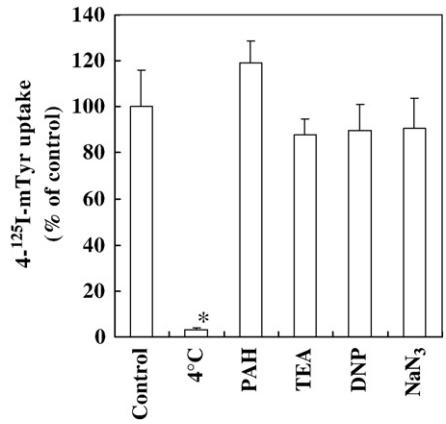

Fig. 5. Effects of inhibitors on accumulation of 4-^{125}I-*m*Tyr in CHO-K1 cells incubated in uptake solution with Na^+. Each column represents the mean±S. D. of five monolayers. *P<.0001.

The position of iodine in the phenol ring appears to be an important factor in the system selectivity of radioiodinated tyrosine derivatives. In the present study, 3-^{125}I-Tyr and 4-^{125}I-*m*Tyr exhibited high specificity for system L transport (Fig. 4 B and D), whereas 6-^{125}I-*m*Tyr exhibited affinity for both system L and system ASC (Fig. 4C). Mono-iodination at the *meta*/*para* position of the phenol ring resulted in specificity for system L. Mono-iodination at the *ortho* position of the ring resulted in affinity for systems ASC and L. Also, alpha-methylation of 3-^{125}I-Tyr conferred affinity for system ASC, as indicated by the results for alpha-methylated 3-^{125}I-Tyr (IMT) (Fig. 4 D and E).

We further examined in vitro transport of 4-^{125}I-*m*Tyr, which exhibited the greatest selectivity for system L. PAH and TEA did not inhibit uptake of 4-^{125}I-*m*Tyr into CHO-K1 cells, indicating that transport of 4-^{125}I-*m*Tyr was not mediated by organic anion or cation transporters. DNP and

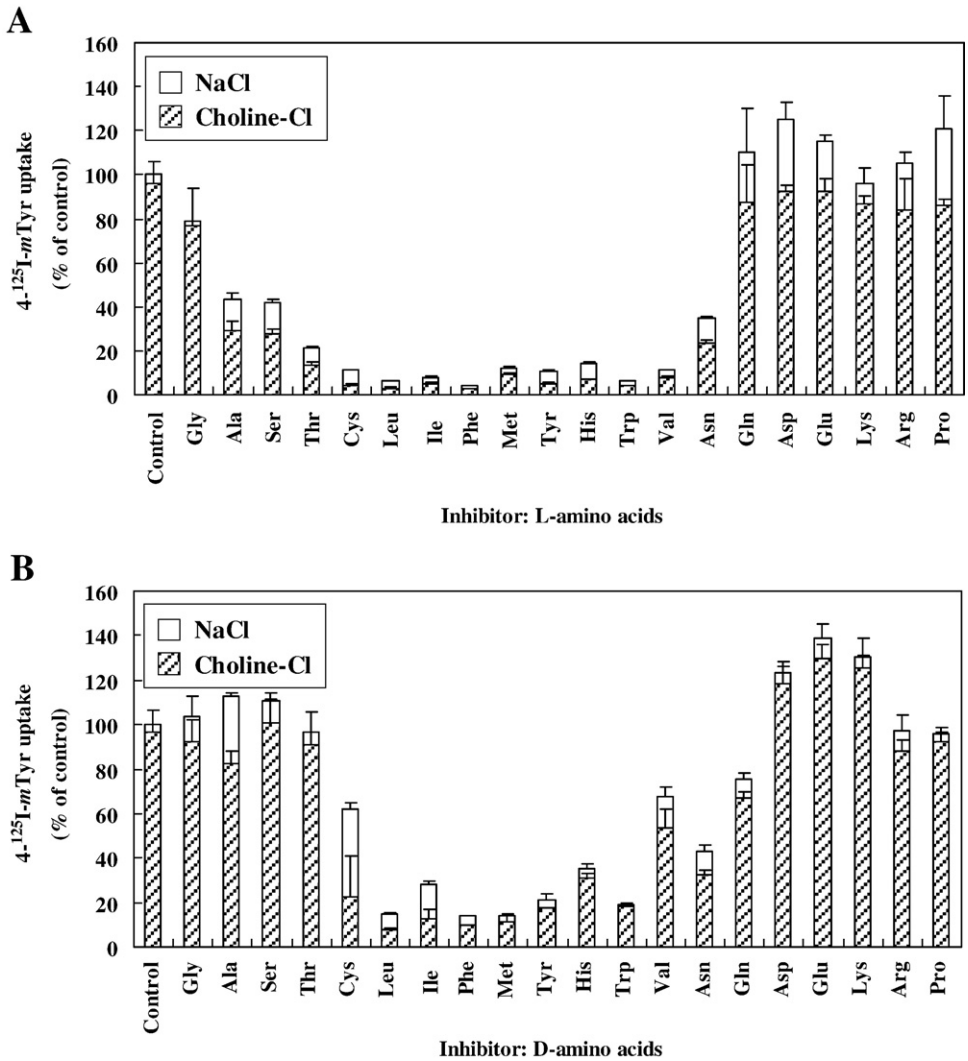

Fig. 6. The effects of L- and D-amino acids on uptake of 4-^{125}I-*m*Tyr into CHO-K1 cells.

NaN_3 did not inhibit uptake of 4-^{125}I-*m*Tyr, indicating that uptake of 4-^{125}I-*m*Tyr did not involve active transport. However, uptake of 4-^{125}I-*m*Tyr was markedly reduced by incubation at 4°C, indicating that transport of 4-^{125}I-*m*Tyr was carrier-mediated ($P<.0001$). There was no significant difference in uptake of 4-^{125}I-*m*Tyr between uptake solutions with and without Na^+, indicating that transport of 4-^{125}I-*m*Tyr was Na^+-independent (Fig. 4B). Because previous studies indicate that D-amino acids are endogenously present in mammalian tissues [23,24], we used both D- and L-amino acids in the present inhibition experiment. Neutral D- and L-amino acids inhibited uptake of 4-^{125}I-*m*Tyr (Fig. 6). The inhibition profiles in Fig. 6 are similar to those of the L-type amino acid transporter 1 (LAT1), which is a member of the system L transporter family. A similar inhibition profile has previously been obtained using the porcine kidney epithelial cell line LLC-PK1, which expresses LAT1 [25–27]. Thus, the present results indicate that uptake of 4-^{125}I-*m*Tyr is mediated by a Na^+-independent neutral amino acid transporter, most likely LAT1 (which is a neutral amino acid exchanger).

We believe there is a need for novel radiopharmaceuticals that are useful for assessment of system L amino acid transport function in clinical studies. Targeting of system L transport in tumor cells would greatly facilitate imaging of tumors, cerebral functions and other important biological phenomena in vivo.

To produce a useful radioiodinated in vivo radiotracer, the radioiodine must be attached in such a way that the resultant radiotracer has affinity for system L transport and in vivo stability. It is important that the radioiodinated compound is not broken down quickly via general metabolic pathways, because a complicated metabolic fate of a radioiodinated compound would require a complicated kinetic model for quantitative study [28]. Of the five tyrosine derivatives used in the present study, ^{125}I-IMT, 4-^{125}I-*m*Tyr and 6-^{125}I-*m*Tyr display metabolic stability, rapid blood clearance, rapid urinary excretion and rapid biodistribution, and are excreted as intact amino acids [19–21]. In contrast, natural ^{14}C-Tyr and 3-^{125}I-Tyr are not stable in vivo, and they have complex metabolic fates [18]. Stability against deiodination is essential, in order to prevent internal irradiation of organs that accumulate radioiodine, such as the thyroid and stomach.

In conclusion, 4-^{125}I-*m*Tyr shows promise as a novel system-L-selective amino acid transport marker. Further investigation is needed to develop methods for using 4-^{125}I-*m*Tyr in SPECT, and to clarify the behavior of 4-^{125}I-*m*Tyr in each target organ and tissue.

We examined the system L specificity of transport of iodinated tyrosine derivatives in CHO-K1 cells. Differences in the position of the iodine and hydroxyl group in the phenol ring and in the presence of an alpha-methyl group caused differences in the amino acid transport system selectivity of the tyrosine derivatives. Transport of mother ^{14}C-Tyr was mediated by systems A, ASC and L. 4-^{125}I-*m*Tyr and 3-^{125}I-Tyr exhibited high specificity for system L, whereas 6-^{125}I-*m*Tyr and ^{125}I-IMT exhibited affinity for both system L and system ASC. Of the 5 tyrosine derivatives used in the present study, 4-^{125}I-*m*Tyr appears to be most suitable for imaging of system L transport. Further study is needed to examine transport of 4-^{125}I-*m*Tyr via other transport systems.

Acknowledgments

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