Evaluation of Amino Acid-Mustard Transport as L-Type Amino Acid Transporter 1 (LAT1)-Mediated Alkylating Agents

Ken-ichi HOSOYA,*[,a] Hirokazu KYOKO,[a] Naoki TOYOOKA,[b] Atsushi KATO,[c] Masahiro ORIHASHI,[d] Masatoshi TOMI,[a] and Masanori TACHIKAWA[a]

[a] *Department of Pharmaceutics, University of Toyama;* [b] *Organic Chemistry, University of Toyama;* [c] *Hospital Pharmacy, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan: and* [d] *Teika Pharmaceutical Co., Ltd.; 1–3–27 Arakawa, Toyama 930–0982, Japan.*

Received July 7, 2008; accepted September 4, 2008; published online September 5, 2008

The L-type amino acid transporter 1 (LAT1, SLC7A5) is an Na^+-independent neutral amino acid transporter the expression of which is located in retinal endothelial cells. Due to its broad substrate selectivity, LAT1 has been proposed to mediate the transport of amino acid-related drugs across the blood-tissue barriers. Here, we have investigated the transport screening of amino acid-mustards using a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2) which expresses LAT1. We synthesized 5 amino acid-mustards: tyrosine-mustard, phenylglycine-mustard, alanine-mustard, ornithine-mustard, and lysine-mustard. LAT1-mediated [^{3}H]L-phenylalanine (Phe) uptake by TR-iBRB2 cells was inhibited in a competitive manner by tyrosine-mustard and phenylglycine-mustard as well as melphalan (phenylalanine-mustard). Phenylglycine-mustard was able to induce the efflux of [^{3}H]Phe preloaded into the TR-iBRB2 cells expressing LAT1 through the obligatory exchange mechanism, although tyrosine-mustard, alanine-mustard, ornithine-mustard, lysine-mustard, and melphalan did not induce any significant efflux. These findings suggest that phenylglycine-mustard is a better substrate for LAT1 than melphalan and other amino acid-mustards.

Key words L-type amino acid transporter 1; amino acid-mustard; alkylating agent; transport; inner blood–retinal barrier

System L is an amino acid transport system that transports neutral amino acids, including several essential amino acids, in an Na^+-independent manner.[1)] System L plays a major role in providing essential branched and aromatic amino acids in living cells. Due to its broad substrate selectivity, system L is proposed to mediate not only the transport of naturally occurring amino acids but also amino acid-related drugs, such as melphalan, an anticancer drug, L-dopa, a therapeutic drug for Parkinsonism, and gabapentin, an anticonvulsant.[1—3)] L-Type amino acid transporter 1 (LAT1/SLC7a5)[4)] and LAT2 (SLC7a8)[5)] have been shown to encode as system L. These transporters are unique because they require an additional protein, the heavy chain of the 4F2 cell surface antigen (CD98/SLC3a2), for functional expression. The expression of LAT1 is localized at the blood-tissue barriers, such as brain capillary endothelial cells, syncytiotrophoblast cells, and retinal endothelial cells, while LAT2 is expressed ubiquitously.[6—8)] Therefore, LAT1 plays a key role in transporting amino acid-related drugs to the brain and retina from the circulating blood.

L-Dopa is the most widely used drug for Parkinson's disease, since L-dopa and its metabolite, 3-*O*-methyldopa, are able to be transported *via* LAT1 in the luminal and abluminal membranes of the blood–brain barrier.[3)] Melphalan has been thought to be mediated by system L because it is a phenylalanine derivative: phenylalanine-mustard.[2)] However, Uchino *et al.* reported that melphalan is not transported rapidly compared with L-dopa and gabapentin because melphalan did not induce the efflux of [^{14}C]phenylalanine (Phe) preloaded on to oocytes expressing LAT1 through the obligatory exchange mechanism.[9)] A number of alkylating agents, such as nitrogen-mustard, cyclophosphamide, and melphalan, but mostly melphalan, are used as cancer chemotherapy in patients with retinoblastoma. Melphalan is directly injected into the vitreous humor because it is not well transported from blood to the vitreous/retina through the blood–retinal barrier (BRB).[10)] Consequently, it is important to understand the substrate recognition of amino acid-mustards for LAT1 as far as development of a transportable alkylating agent for LAT1 at the inner BRB is concerned.

The purpose of present study was to test the substrate recognition of synthetic amino acid-mustards: tyrosine-mustard, phenylglycine-mustard, alanine-mustard, ornithine-mustard, and lysine-mustard. In the present study, we have used a conditionally immortalized rat retinal endothelial cell line (TR-iBRB2), which expresses LAT1,[8)] to examine LAT1-mediated transport of [^{3}H]Phe and amino acid-mustards.

MATERIALS AND METHODS

Reagents L-[2,6-^{3}H]Phenylalanine ([^{3}H]Phe, 54 Ci/mmol) and D-[1-^{14}C]mannitol ([^{14}C]D-mannitol, 56 mCi/mmol) were purchased from Amersham Life Science (Buckinghamshire, U.K.) and American Radiolabeled Chemicals (St. Louis, MO, U.S.A.), respectively. All other chemicals were of reagent grade and available commercially.

Synthesis of Amino Acid-Mustards Tyrosine-mustard,[11)] phenylglycine-mustard,[12)] ornithine-mustard,[13)] and lysine-mustard[14)] were prepared according to the literature procedures, and alanine-mustard was prepared from commercially available Boc-Dap-O*t*Bu by the same procedure as that used for ornithine-mustard (Fig. 1).[13)] The structures were confirmed from the ^{1}H-NMR spectra (500 MHz, D_2O) δ 3.62 (2H, m), 3.75—3.82 (8H, m), 4.44 (1H, m).

Cell Culture TR-iBRB2 cells possess endothelial markers and L-type amino acid transporter 1 (SLC7a5/LAT1), facilitative glucose transporter 1 (SLC2a1/GLUT1), P-glycoprotein (ABCB1a/mdr1a), creatine transporter (SLC6a8/CRT), taurine transporter (SLC6a6/TauT), and scavenger receptor class B, type1 (SR-BI) which are expressed at the

* To whom correspondence should be addressed. e-mail: hosoyak@pha.u-toyama.ac.jp

Fig. 1. Structure of Amino Acid-Mustards

inner BRB *in vivo*.[8,15—18] Thus, TR-iBRB2 cells maintain certain *in vivo* functions and are a suitable *in vitro* model for the inner BRB. DMEM containing 100 U/ml benzylpenicillin potassium, 100 μg/ml streptomycin sulfate, and 10% FBS was used as the culture medium for TR-iBRB2 cells. TR-iBRB2 cells (passage number 27—35) were seeded onto rat tail collagen type I-coated tissue culture plates (BD Biosciences, Franklin Lakes, NJ, U.S.A.) and cultured at 33 °C in a humidified atmosphere of 5% CO_2/air. The permissive-temperature for TR-iBRB2 cell culture is 33 °C due to the presence of temperature-sensitive SV 40 large T-antigen.[15]

[^{3}H]Phe Uptake by TR-iBRB2 Cells TR-iBRB2 cells (5×10^4 cells/cm^2) were cultured at 33 °C for 2 d on rat tail collagen type I-coated 24-well plates (BD Biosciences) and washed with extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose, and 10 mM HEPES (pH 7.4) at 37 °C. Uptake was initiated by applying 200 μl ECF buffer (pH 7.4) containing 0.25 μCi [^{3}H]Phe (23.1 nM) and 0.01 μCi [^{14}C]D-mannitol (0.893 μM) to estimate the volume of adhering water at 37 °C in the presence or absence of inhibitors. Na^+-free ECF buffers were prepared in two different ways: the choline ECF buffer was prepared by equimolar replacement of NaCl and $NaHCO_3$ with choline chloride and choline bicarbonate, respectively, while the Li ECF buffer was prepared by equimolar replacement of NaCl and $NaHCO_3$ with LiCl and $KHCO_3$, respectively. Cl^--free ECF buffer was prepared by replacement with equimolar gluconate. After a predetermined period, uptake was terminated by removing the solution, followed by immersing cells in ice-cold uptake buffer to stop uptake. The cells were then solubilized in 1 N NaOH and subsequently neutralized with 1 N HCl. Cell-associated radioactivity and protein content were assayed by liquid scintillation spectrometry and detergent compatible protein assay (a DC protein assay kit, Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as a standard.

In the [^{3}H]Phe efflux studies,[8] TR-iBRB2 cells were washed three times with ECF buffer and incubated with 0.25 μCi [^{3}H]Phe (23.1 nM) in ECF buffer for 5 min at 37 °C in order to preload [^{3}H]Phe. Cells were then washed three times with ice-cold ECF buffer and incubated with ECF buffer in the absence (control) or presence of amino acids/amino acid-mustards at 37 °C. After 4 min, incubated ECF buffer was taken to measure the efflux of preloaded [^{3}H]Phe from cells. The radioactivity in the cells was measured as described above.

Data Analysis The uptake of [^{3}H]Phe by TR-iBRB2 cells was expressed as the cell-to-medium (cell/medium) ratio using the following equation (Eq. 1):

$$\begin{aligned}\text{cell/medium ratio} = &([^3\text{H}]\text{ dpm per cell protein (mg)})/\\ &([^3\text{H}]\text{ dpm per }\mu\text{l medium})\\ &-([^{14}\text{C}]\text{ dpm pser cell protein (mg)})/\\ &([^{14}\text{C}]\text{ dpm per }\mu\text{l medium})\end{aligned} \quad (1)$$

The efflux of [^{3}H]Phe by TR-iBRB2 cells was expressed as follows:

$$\begin{aligned}\text{fractional outflow (\%)} = &([^3\text{H}]\text{ dpm in the medium})/\\ &([^3\text{H}]\text{ dpm in the cells}\\ &+[^3\text{H}]\text{ dpm in the medium})\times100\end{aligned} \quad (2)$$

For kinetic studies, the Michaelis–Menten constant (K_m), the maximal uptake rate (V_{max}) and nonsaturable uptake rate (K_d) of Phe uptake were calculated from the following equation (Eq. 3) using the nonlinear least-square regression analysis program, MULTI.[19]

$$V = V_{max}\times S/(K_m+S)+K_d\times S \quad (3)$$

where V and S are the uptake rate of Phe for 1 min and the concentration of Phe, respectively.

Unless otherwise indicated, all data represent means± S.E.M. Statistical significance of differences among means of several groups was determined by one-way analysis of variance (ANOVA) followed by modified Fisher's least squares difference method.

RESULTS

Transport Activity To examine LAT1-mediated transport activity, [^{3}H]Phe uptake was performed using TR-iBRB2 cells as an *in vitro* model expressing LAT1 of the inner BRB. The time-course of [^{3}H]Phe uptake by TR-iBRB2 cells is shown in Fig. 2A. [^{3}H]Phe uptake increased linearly for 2 min and the initial uptake rate was 124 μl/(min·mg protein). Therefore, all subsequent uptake measurements were conducted for 1 min. The inhibitory effect of Na^+-free conditions on [^{3}H]Phe uptake by TR-iBRB2 cells was examined under two different sets of conditions. Both choline ECF buffer and Li ECF buffer had little effect on [^{3}H]Phe uptake. Cl^--free conditions did not have any effect on [^{3}H]Phe uptake (Fig. 2B). This confirms that [^{3}H]Phe uptake by TR-iBRB2 cells is mediated by an Na^+- and Cl^--independent process. As shown in Fig. 2C, the intracellular Phe uptake was saturable, and nonlinear least-squares regression analysis showed that the K_m, V_{max}, and K_d were 86.8± 34.4 μM, 11.2±1.3 nmol/(min·mg protein), and 2.66±0.31 μl/(min·mg protein) (mean±S.D.), respectively. A *cis*-inhibition study was performed to characterize the [^{3}H]Phe uptake by TR-iBRB2 cells (Table 1). [^{3}H]Phe uptake was inhibited by 2 mM L-phenylalanine, L-leucine, and 2-aminobicyclo-(2,2,1)-heptane-2-carboylic acid (BCH) by more than 85%. However, it was only inhibited by less than 30% in the presence of a substrate of LAT2, such as L-alanine, and the basic amino acid, L-arginine, at a concentration of 2 mM. These results support the evidence that [^{3}H]Phe uptake by TR-iBRB2 is mainly mediated *via* LAT1 in TR-iBRB2 cells.[4,8]

Transport Screening LAT1-mediated [^{3}H]Phe uptake by TR-iBRB2 cells was measured in the presence of 100 μM concentrations of synthetic amino acid-mustards and melphalan (Fig. 1). Aromatic amino acid-mustards, melphalan,

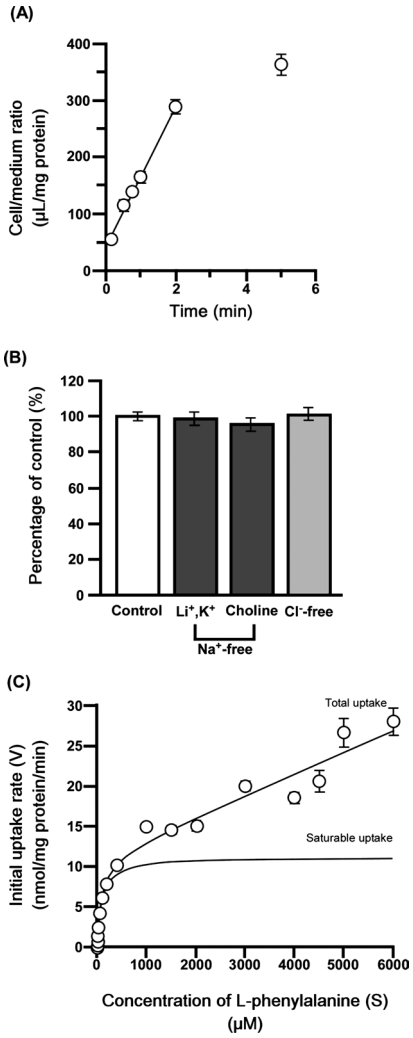

Fig. 2. Time-Course (A), Na^+- and Cl^--Dependence (B) and Concentration-Dependence (C) of [^{3}H]Phe Uptake by TR-iBRB2 Cells

The uptake of [^{3}H]Phe (0.25 μCi) was measured in TR-iBRB2 cells at 37 °C. Each point represents the mean±S.E.M. (n=4).

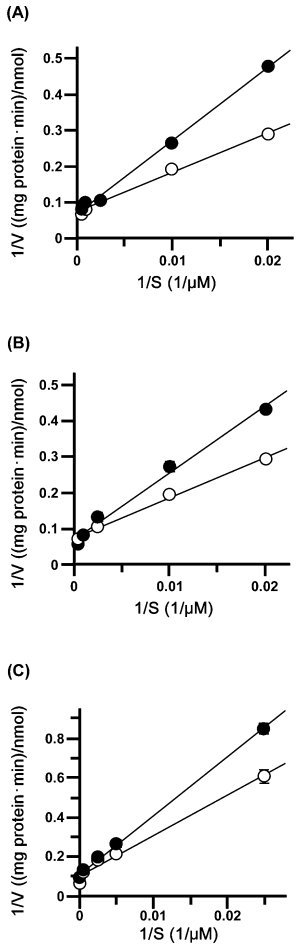

Fig. 3. Lineweaver–Burk Plot Analysis of [^{3}H]Phe Uptake by TR-iBRB2 Cells in the Presence (●) and Absence (○) of Unlabeled Amino Acid-Mustards

The uptake of [^{3}H]Phe uptake with L-phenylalanine (10, 50, 100, 400, 1000, 2000, or 2500 μM) was measured in the absence (control) or presence of 100 μM melphalan (A), 100 μM tyrosine-mustard (B), and 100 μM phenylglycine-mustard (C) at 37 °C. Each point represents the mean±S.E.M. (n=4).

Table 1. Effect of Several Inhibitors on [^{3}H]Phe Uptake by TR-iBRB2 Cells

Inhibitors	Percentage of control
Control	100±3
2 mM L-phenylalanine	4.53±0.14*
2 mM L-leucine	11.3±1.1*
2 mM BCH	14.6±0.4*
2 mM L-alanine	71.2±2.0**
2 mM L-arginine	77.2±4.6**
100 μM melphalan	42.6±3.7*
100 μM tyrosine-mustard	48.6±1.6*
100 μM phenylglycine-mustard	52.3±3.6*
100 μM alanine-mustard	95.1±5.9
100 μM ornithine-mustard	86.1±7.1
100 μM lysine-mustard	58.9±3.6*

[^{3}H]Phe uptake by TR-iBRB2 cells was performed at 37 °C for 1 min in the absence (control) or presence of inhibitors. Each value represents the mean±S.E.M. (n=4—7). * p<0.01, ** p<0.05, significantly different from control. BCH: 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid.

tyrosine-mustard, and phenylglycine-mustard, inhibited [^{3}H]Phe uptake by 57.7%, 51.4%, and 47.7%, respectively, whereas aliphatic amino acid-mustards had weaker inhibitory effects on [^{3}H]Phe uptake: lysine-mustard, ornithine-mustard, and alanine-mustard inhibited [^{3}H]Phe uptake by 41.1%, 13.9%, and 4.9%, respectively (Table 1). Lineweaver–Burk plot analysis of [^{3}H]Phe uptake in the presence of 100 μM melphalan, tyrosine-mustard, and phenylglycine-mustard showed competitive inhibition with an inhibition constant (K_i) of 101 μM, 132 μM, and 231 μM, respectively (Fig. 3). The amino acid-mustards that inhibited LAT1-medaited [^{3}H]Phe uptake were investigated to determine whether they could induce the efflux of loaded [^{3}H]Phe when applied extracellularly. As shown in Fig. 4, the efflux of loaded [^{3}H]Phe was induced by 100 μM phenylglycine-mustard as well as LAT1 substrates, such as 2 mM phenylalanine, L-leucine, and BCH. No significant efflux was induced by melphalan, tyrosine-mustard, lysine-mustard, ornithine-mustard, and alanine-mustard nor an LAT1-nonsubstrate, L-arginine.

DISCUSSION

The present study demonstrates transport screening of synthetic amino acid-mustards and melphalan in TR-iBRB2 cells which express LAT1. Phenylglycine-mustard is a potent

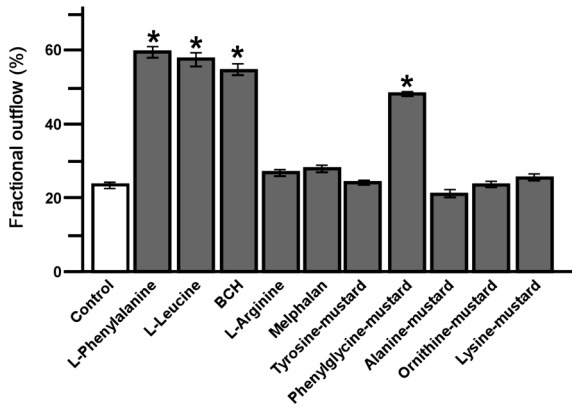

Fig. 4. Efflux of [^{3}H]Phe by Amino Acids and Amino Acid-Mustards

The efflux of loaded [^{3}H]Phe from TR-iBRB2 cells was measured in the absence (control) or presence of amino acids at a concentration of 2 mM, and melphalan and amino acid-mustards at a concentration of 100 μM at 37 °C for 4 min. Each column represents the mean±S.E.M. (n=4). $*p<0.01$, significantly different from control.

substrate for LAT1, better than melphalan, tyrosin-mustard, and aliphatic amino acid-mustards. System L transports neutral amino acids in an Na^+-independent manner and is classified as LAT1 and 2. Recently, LAT3 (SLC43a1)[20] and 4 (SLC43a2)[21] have been identified as a lower affinity transporter for system L. LAT1 and 2 offer higher affinity for Phe transport (<100 μM) than that of LAT3 and 4 (>1 mM).[4,5,20,21] [^{3}H]Phe uptake by TR-iBRB2 cells was Na^+-independent and saturable with a K_m of 86.8 μM (Fig. 2). This uptake had only a minor effect in the presence of 2 mM L-alanine (Table 1).[4,5] These results are very consistent with LAT1-mediated [^{3}H]Phe uptake by TR-iBRB2 cells.

Uchino *et al.*, investigated the mechanism of substrate recognition for LAT1 and found that LAT1 accepts α-methyl amino acids as substrates.[9] In order to recognize amino acid derivatives that are substrates for LAT1, they most probably needed to have positive and negative charges at the α-carbon and hydrophobic moiety.[9] Therefore, we selected α-amino acids which have an aromatic moiety, or β-hydroxyl, δ- and ε-amino groups. Aromatic amino acids introduce a mustard moiety to an aromatic ring: tyrosine-mustard and phenylglycine-mustard. The β-hydroxyl group of L-serine is substituted by a mustard moiety, alanine-mustard, and the δ- and ε-amino group of L-ornithine and L-lysine are substituted by a mustard moiety, ornithine-mustard and lysine-mustard, respectively (Fig. 1).

Aliphatic amino acid-mustards, such as alanine-mustard and ornithine-mustard, failed to inhibit [^{3}H]Phe uptake by TR-iBRB2 cells, suggesting that these compounds do not interact with LAT1 (Table 1). Lysine-mustard and aromatic amino acid-mustards inhibited [^{3}H]Phe uptake by TR-iBRB2 cells by more than 40%, suggesting that the amino acid with hydrophobic moiety provides higher affinity binding site for LAT1. LAT1 is an amino acid exchange transporter which makes it possible to evaluate whether compounds accepted by the binding site of LAT1 are transported or not by examining their ability to induce the efflux of loaded radiolabeled substrate.[8,9] This strategy is particularly useful for compounds with no readily available radiolabeled form. Of the aromatic amino acid-mustards, although melphalan has the highest binding affinity for LAT1 (Fig. 3), melphalan failed to induce the efflux of loaded [^{3}H]Phe as is in the case of L-arginine which is not a substrate for LAT1 (Fig. 4). In addition to melphalan, tyrosine-mustard, alanine-mustard, ornithine-mustard, and lysine-mustard did not induce any significant efflux, suggesting that these compounds are faint substrates of LAT1. In contrast, phenylglycine-mustard induced the efflux of loaded [^{3}H]Phe as is the case with transportable substrates of LAT1, such as L-phenylalanine, L-leucine, and BCH. This suggests that phenylglycine-mustard is a better substrate for LAT1 than other amino acid-mustards. Although further studies are needed to determine the anti-tumor activities for phenylglycine-mustard, phenylglycine-mustard is a more potent substrate of LAT1 than melphalan, tyrosine-mustard, and lysine-mustard.

Based on the results of the present study, we propose a model for a transportable alkylating agent for LAT1. An aromatic amino acid as a backbone is produced with greater affinity for LAT1 than an aliphatic amino acid since the aromatic moiety is more hydrophobic than the aliphatic moiety. Of the aromatic amino acid-mustards, the glycine moiety of phenylglycine-mustard is one carbon shorter than the alanine moiety of melphalan and tyrosine-mustard. Phenylglycine-mustard may adapt to the binding site of LAT1 as a transportable form because introduction of the mustard moiety may be sufficiently marked to interrupt the transport of phenylalanine and tyrosine. LAT1 is expressed not only at the inner BRB[8] but also in malignant tumors.[22] These pieces of evidence suggest that phenylglycine-mustard is transported from the circulating blood to the retina across the inner BRB. Subsequently, phenylglycine-mustard may be taken up by retinoblastoma.

In conclusion, the data presented here show that phenylglycine-mustard is a potent substrate of LAT1 more than melphalan, tyrosine-mustard, and aliphatic amino acid-mustards. These findings provide important information to increase our understanding of the substrate binding and transport mechanisms of LAT1 and help in the development of a transportable alkylating agent for LAT1.

Acknowledgements This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and Research and Development Grants for Scientific Research from the Hokuriku Industrial Advancement Center.

REFERENCES

1) Christensen H. N., *Physiol. Rev.*, **70**, 43—77 (1990).
2) Goldenberg G. J., Lam H. Y., Begleiter A., *J. Biol. Chem.*, **254**, 1057—1064 (1979).
3) Gomes P., Soares-da-Silva P., *Brain Res.*, **829**, 143—150 (1999).
4) Kanai Y., Segawa H., Miyamoto K., Uchino H., Takeda E., Endou H., *J. Biol. Chem.*, **273**, 23629—23632 (1998).
5) Segawa H., Fukasawa Y., Miyamoto K., Takeda E., Endou H., Kanai Y., *J. Biol. Chem.*, **274**, 19745—19751 (1999).
6) Matsuo H., Tsukada S., Nakata T., Chairoungdua A., Kim D. K., Cha S. H., Inatomi J., Yorifuji H., Fukuda J., Endou H., Kanai Y., *Neuroreport*, **11**, 3507—3511 (2000).
7) Ritchie J. W., Taylor P. M., *Biochem. J.*, **356**, 719—725 (2001).
8) Tomi M., Mori M., Tachikawa M., Katayama K., Terasaki T., Hosoya K., *Invest. Ophthalmol. Vis. Sci.*, **46**, 2522—2530 (2005).
9) Uchino H., Kanai Y., Kim D. K., Wempe M. F., Chairoungdua A., Morimoto E., Anders M. W., Endou H., *Mol. Pharmacol.*, **61**, 729—737 (2002).
10) Kaneko A., Suzuki S., *Jpn. J. Clin. Oncol.*, **33**, 601—607 (2003).

11) Dall'Asta L., Ferrario P., *Helv. Chim. Acta*, **45**, 1065—1067 (1962).
12) Davis W., Roberts J. J., Ross W. C. J., *J. Chem. Soc.*, **1955**, 890—895 (1955).
13) Ishidate M., Sakurai Y., Aiko I., *Chem. Pharm. Bull.*, **8**, 732—737 (1960).
14) Vita G., Monti L., Angelucci R., *J. Med. Chem.*, **7**, 468—471 (1964).
15) Hosoya K., Tomi M., Ohtsuki S., Takanaga H., Ueda M., Yanai N., Obinata M., Terasaki T., *Exp. Eye Res.*, **72**, 163—172 (2001).
16) Nakashima T., Tomi M., Katayama K., Tachikawa M., Watanabe M., Terasaki T., Hosoya K., *J. Neurochem.*, **89**, 1454—1461 (2004).
17) Tomi M., Terayama T., Isobe T., Egami F., Morito A., Kurachi M., Ohtsuki S, Kang Y. S., Terasaki T., Hosoya, K., *Microvasc. Res.*, **73**, 100—106 (2007).
18) Tachikawa M., Okayasu S., Hosoya K., *Mol. Vis.*, **13**, 2041—2047 (2007).
19) Yamaoka K., Tanigawara Y., Nakagawa T., Uno T., *J. Pharmacobiodyn.*, **4**, 879—885 (1981).
20) Babu E., Kanai Y., Chairoungdua A., Kim D. K., Iribe Y., Tangtrongsup S., Jutabha P., Li Y., Ahmed N., Sakamoto S., Anzai N., Nagamori S., Endou H., *J. Biol. Chem.*, **278**, 43838—43845 (2003).
21) Bodoy S., Martín L., Zorzano A., Palacín M, Estévez R., Bertran J., *J. Biol. Chem.*, **280**, 12002—12011 (2005).
22) Wolf D. A., Wang S., Panzica M. A., Bassily N. H., Thompson N. L., *Cancer Res.*, **56**, 5012—5022 (1996).