SUPPLEMENTAL INFORMATION

Materials and Methods

Synthesis of 2-iodo-α-methyl-L-phenylalanine methyl ester (2a):

Thionyl chloride ($SOCl_2$; 1.44 mL, 19.8 mmol) was added to methanol (CH_3OH; 20 mL) slowly at 0°C, and then 2-iodo-α-methyl-L-phenylalanine (1a; 500 mg, 1.53 mmol) was added to the solution. The resulting mixture was stirred for 2 h at room temperature (RT) and then refluxed for 56 h. The solvent was evaporated, and the residue was dissolved in MilliQ water (15 mL). The solution was adjusted to pH 8 with saturated $NaHCO_3$, and extracted with chloroform (20 mL × 3), then dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to provide compound (2a) as a light brown solid (442.5 mg, 90.9%). ^1H NMR ($CDCl_3$): δ1.43 (3H, s, CH_3), 1.60 (2H, s, NH_2), 3.14–3.29 (2H, dd, CH_2), 3.74 (3H, s, OCH_3), 6.90–7.87 (4H, aromatic). ESI-MS Calc'd for $C_{11}H_{14}INO_2$ $(M+H)^+$: m/z 320, found: 320.

Synthesis of *N*-trifluoroacetyl-2-iodo-α-methyl-L-phenylalanine methyl ester (3a):

To a solution of compound (2a) (442.5 mg, 1.39 mmol) in dry dichloromethane (CH_2Cl_2) (3 mL), triethylamine (TEA; 289 μL, 2.08 mmol) and trifluoroacetic anhydride ($(TFA)_2O$; 289 μL, 2.08 mmol) in CH_2Cl_2 (3 mL) were slowly added dropwise at 0°C, then stirred for 2 h at RT. The solution was washed with 10% citric acid (10 mL × 3) and 5% $NaHCO_3$ (10 mL × 3), then dried over anhydrous $MgSO_4$. The solvent was removed in vacuo, and the residue was subjected to column chromatography on a silica gel using a mixture of chloroform/hexane (1:1) as an eluent to

provide compound (3a) as a white solid (311.4 mg, 54.1%). ^{1}H NMR ($CDCl_3$): δ1.79 (3H, s, CH_3), 3.48–3.64 (2H, dd, CH_2), 3.78 (3H, s, OCH_3), 6.94–7.87 (4H, aromatic). ESI-MS Calc'd for $C_{13}H_{13}F_3INO_3$ $(M+Na)^+$: m/z 437, found: 437.

Synthesis of *N*-trifluoroacetyl-2-trimethylstannyl-α-methyl-L-phenylalanine methyl ester (4a):

To a solution of compound (3a) (100 mg, 0.24 mmol) in dry toluene (3 mL), tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$; 4.7 mg, 4.10 μmol) and hexamethylditin (323.6 mg, 0.99 mmol) in dry toluene (3 mL) were added, then stirred for 10 min at RT followed by refluxing for 4.5 h at 115°C. The reaction mixture was filtrated through Celite, and the solvent was removed in vacuo. The residue was subjected to column chromatography on a silica gel using a mixture of ethyl acetate/hexane (1:4) as an eluent to provide compound (4a) as a white solid (71.8 mg, 65.9%). ^{1}H NMR ($CDCl_3$): δ0.29–0.42 (9H, t, $SnMe_3$) 1.71 (3H, s, CH_3), 3.32–3.52 (2H, dd, CH_2), 3.73 (3H, s, OCH_3), 6.99–7.44 (4H, aromatic). ESI-MS Calc'd for $C_{16}H_{22}F_3NO_3Sn$ $(M-H)^-$: m/z 452, found: 452.

Synthesis of 4-iodo-α-methyl-L-phenylalanine methyl ester (2b):

$SOCl_2$ (0.358 mL, 4.94 mmol) was added to CH_3OH (10 mL) slowly at 0°C, then 4-iodo-α-methyl-L-phenylalanine (1b; 502 mg, 1.65 mmol) was added to the solution. The resulting mixture was stirred for 23 h at RT and then refluxed for 23 h. The solvent was evaporated, and the residue was dissolved in MilliQ water (15 mL). The solution was adjusted to pH 8 with saturated

$NaHCO_3$, and extracted with chloroform (20 mL × 3), then dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to provide compound (2b) as a white solid (414.1 mg, 78.9%). ^1H NMR ($CDCl_3$): δ1.37 (3H, s, CH_3), 2.72–3.07 (2H, dd, CH_2), 3.70 (3H, s, OCH_3), 6.89–7.61 (4H, aromatic).

Synthesis of *N*-trifluoroacetyl-4-iodo-α-methyl-L-phenylalanine methyl ester (3b):

To a solution of compound (2b) (414.1 mg, 1.30 mmol) in dry CH_2Cl_2 (3 mL), TEA (270 μL, 1.95 mmol) and $(TFA)_2O$ (270 μL, 1.95 mmol) in CH_2Cl_2 (3 mL) were slowly added dropwise at 0°C, then stirred for 2 h at RT. The solution was washed with 10% citric acid (10 mL × 3) and 5% $NaHCO_3$ (10 mL × 3), then dried over anhydrous $MgSO_4$. The solvent was removed in vacuo, and the residue was subjected to column chromatography on a silica gel using a mixture of chloroform/hexane (1:1) as an eluent to provide compound (3b) as a colorless oil (278.4 mg, 53.5%). ^1H NMR ($CDCl_3$): δ1.78 (3H, s, CH_3), 3.13–3.64 (2H, dd, CH_2), 3.84 (3H, s, OCH_3), 6.71–7.61 (4H, aromatic).

Synthesis of *N*-trifluoroacetyl-4-trimethylstannyl-α-methyl-L-phenylalanine methyl ester (4b):

To a solution of compound (3b) (30 mg, 0.072 mmol) in dry toluene (1 mL), $Pd(PPh_3)_4$ (1.42 mg, 1.23 μmol) and hexamethylditin (97.1 mg, 0.30 mmol) in dry toluene (1.2 mL) were added, then stirred for 10 min at RT followed by refluxing for 3 h at 115°C. The reaction mixture was filtrated through Celite, and the solvent was removed in vacuo. The residue was subjected to column

chromatography on a silica gel using a mixture of chloroform/hexane (1:1) as an eluent to provide compound (4b) as a colorless oil (19.9 mg, 60.9%). ^{1}H NMR ($CDCl_3$): δ0.20–0.34 (9H, t, $SnMe_3$) 1.79 (3H, s, CH_3), 3.16–3.62 (2H, dd, CH_2), 3.85 (3H, s, OCH_3), 6.94–7.40 (4H, aromatic). ESI-MS Calc'd for $C_{16}H_{22}F_3NO_3Sn$ $(M+Na)^+$: m/z 476, found: 476.

Chiral Chromatography Analysis

The chirality of 2-^{77}Br-BAMP was checked by chiral chromatography. After purification by RP-HPLC (system 1), chiral chromatography analysis was performed with a chiral column (CHIRALPAK ZWIX(+), 3.0 × 250 mm, Daicel Chemical Industries) eluted with methanol/acetonitrile/water (18:80:2) containing 100 mM formic acid and 50 mM diethylamine at the flow rate of 0.3 mL/min. Non-radioactive 2-BAMP and 4-BAMP (NAGASE & Co.) were also analyzed under the same conditions.

Stability and characterization of BAMPs

For the evaluation of in vitro stability, 20 μL of each ^{77}Br-BAMP (20 kBq, <5 pmol) was added to 180 μL of freshly prepared murine serum, and the solution was incubated at 37°C for 48 h. The radioactivity of the sample was analyzed by TLC (Silica gel 60, Merck) developed with 10% ammonium chloride/methanol [1:1]. After the samples were filtered through a 10-kDa cut-off ultrafiltration membrane (Vivaspin 500; Sartorius), the radioactivity of the samples was analyzed by RP-HPLC (system 2). For the evaluation of in vivo stability, blood was drawn from the hearts of

mice and urine was collected at 6 h after the administration of each ^{77}Br-BAMP (250 kBq, <50 pmol) to normal mice. After centrifugation of blood samples at 3,000 rpm for 10 min at 4°C, urine samples and the resultant plasma samples were analyzed by TLC. After being filtered through a 10-kDa cut-off ultrafiltration membrane, the radioactivity of the samples was analyzed by RP-HPLC (system 2).

Lipophilicity Measurement

The lipophilicity of BAMPs was estimated by measuring the coefficients of partition between 1-octanol and 0.1 M of phosphate buffer (pH 7.4) as follows: a 10-μL aliquot of each ^{77}Br-BAMP (20 kBq, <5 pmol) was mixed with 3 mL each of 1-octanol and 0.1 M of phosphate buffer in a test tube. The mixture was vortexed (3 × 1 min), and this mixture was then left to stand for 20 min. After the procedure had been repeated three times, the mixture was centrifuged for 5 min. Two 1-mL aliquots of each phase were removed, and their radioactivity was measured with a well-type gamma counter. The partition coefficient at pH 7.4 was determined by calculating the ratio of radioactivity of 1-octanol to that of buffer and then expressed as a common logarithm ($\log D_{7.4}$).

In Vitro Protein Binding of BAMPs

The plasma protein binding of the BAMPs was measured according to the procedure of Kuga et al. (ref. *13*) with slight modification. Each ^{77}Br-BAMP (30 kBq, <5 pmol in 20 μL saline) was added to 180 μL of murine plasma, and the mixture was then centrifuged at 3,000 rpm for 5 min at RT with a

10-kDa cutoff ultrafiltration membrane. The radioactivity of the part of the initial solutions (10 μL) and the filtrates (10 μL) was measured with a well-type gamma counter. The free fraction and protein-binding ratio were determined as follows:

Free fraction (%) = (radioactivity of filtrate) / (radioactivity of initial solution) × 100.

Protein-binding rate (%) = 100 – free fraction.

Cell Culture

A human colon adenocarcinoma cell line, LS180, was routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM) at 37°C in 5% CO_2, 95% air. HEK293-hLAT1, HEK293-hLAT2 and HEK293-mock cells were routinely maintained in Eagle's minimal essential medium (EMEM) containing 10% heat-inactivated FBS, penicillin (100 units/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM) supplemented with Non-Essential Amino Acids (Wako Pure Chemical Industries) at 37°C in 5% CO_2, 95% air.

Cellular Uptake Studies

Alpha-methyl-L-tyrosine (AMT) and α-methyl-aminoisobutyric acid (MeAIB) were purchased from Sigma-Aldrich. 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) was obtained from NARD Institute. LS180 (1.0×10^5 cells/well) cells were plated in 24-well plates, and HEK293-mock, HEK293-hLAT1 and HEK293-hLAT2 cells (1.0×10^5 cells/well) were plated in

poly-D-lysine coated 24-well plates (BD Biosciences). After incubating in the growth medium for 24 h, the cells were washed three times with sodium-free Hank's balanced salt solution (HBSS) (Na^+-free HBSS; 137 mM choline chloride, 5.3 mM KC1, 1.3 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 0.35 mM K_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $KHCO_3$, 5.6 mM D-glucose (pH 7.4)) and then incubated in Na^+-free HBSS for 10 min. For the uptake studies, cells were incubated with ^{18}F-FAMT (100 kBq, final concentration: 1.5 μM), 2-^{77}Br-BAMP (20 kBq, final concentration: 1.5 μM) or 4-^{77}Br-BAMP (20 kBq, final concentration: 1.5 μM) at 37°C for 1 min.

For the inhibition assay, cells were incubated in Na^+-free HBSS with various inhibitors (amino acids and their analogs) at 1 mM for 1 min. After the incubation, cells were washed three times with ice-cold Na^+-free HBSS and then lysed with 500 μL of 0.1 M NaOH. The radioactivity in the cell lysate was measured by a well-type gamma counter.

Quantification of mRNA

A real-time polymerase chain reaction (RT-PCR) analysis was performed to determine the expressions of LAT1, LAT2, LAT3, and LAT4 mRNA in LS180 cells. Total RNA was isolated from LS180 cells using a NucleoSpin RNA II kit (Macherey-Nagel). The first-strand complement DNA (cDNA) was synthesized from 0.5 μg of total RNA with PrimeScript Reverse Transcriptase (Takara Bio). Full-length DNA of LAT1, LAT2, LAT3 and LAT4 was synthesized from each cDNA with KOD-Plus-Neo (Toyobo) under the following conditions: 98°C for 60 s; 5 cycles of 98°C for 10 s, 74°C for 60 s; 5 cycles of 98°C for 10 s, 72°C for 60 s; 5 cycles of 98°C for 10 s, 70°C for 60 s; 20

cycles of 98°C for 10 s, 68°C for 60 s; final extension at 72°C for 10 min. After purification with High Pure PCR Product Purification Kit (Roche Applied Science), PCR products were analyzed using agarose gel electrophoresis, and a single band of each full-length DNA of LATs was detected. The concentrations of the full-length DNA of the LATs were determined with a BioPhotometer (Eppendorf). The RT-PCR carried out using serial dilutions of known concentrations of full-length DNA of the LATs as templates and generated standard curves. The RT-PCR was performed by first incubating each cDNA sample with the primers (0.5 μM each) and Thunderbird SYBR qPCR Mix (Toyobo). Amplification was carried out for 40 cycles (95°C for 15 s, 60°C for 30 s) with a Piko-Real thermal cycler (Thermo Fisher Scientific). The quantities of LAT1, LAT2, LAT3, and LAT4 mRNA in LS180 cells were determined from the standard curves. The sequences of the specific primers used for the analysis are shown in Table S1.

Supplemental Table 1. Primers used for the real-time PCR analysis

	Accession No.	Primer sequences		Expected size (bp)
LAT1	AB018009	sense	ATCGGGAAGGGTGATGTGTCCAAT	103
		antisense	CAAAGAGGCCGCTGTATAATGCCA	
LAT2	AB037669	sense	TTTCCTTCAGGGCTCCTTTGCCTA	98
		antisense	AGATGGCTCTGGGAAGGTTCTTGT	
LAT3	AB103033	sense	TTTGTGACCTTTGTCCTGCACACC	85
		antisense	TGGATGGGAACACTGCAGCATAGA	
LAT4	BC027923	sense	ACCGAGCCAGAGAATGTCACCAAT	118
		antisense	AATTTAGCATCTCGTCCTGGGCCT	

Immunoblotting

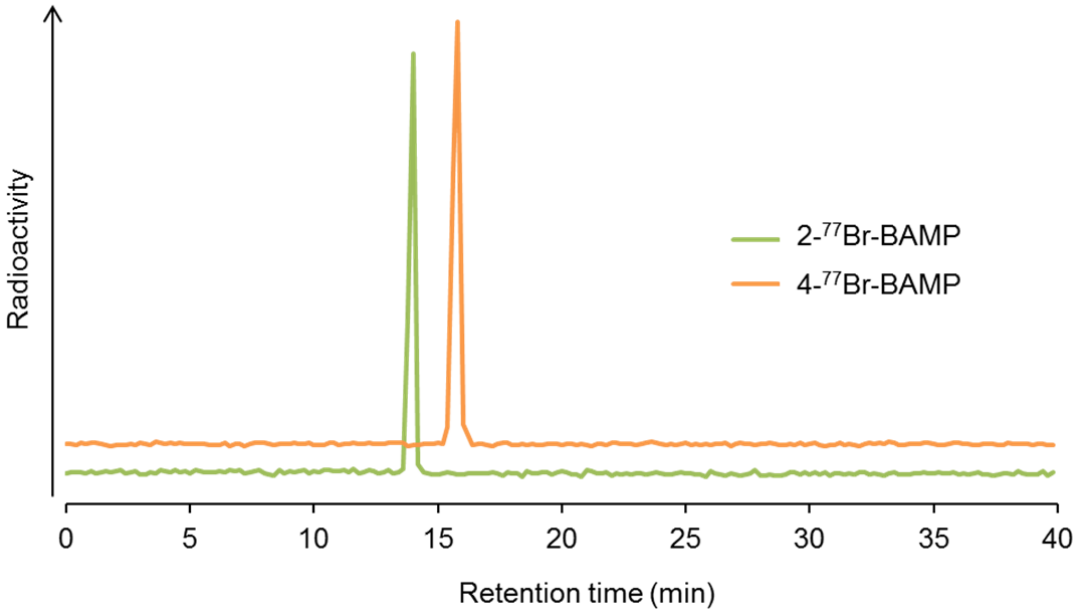
Cells were dissolved in sample buffer (25% glycerin, 1% sodium dodecyl sulfate [SDS], 62.5 mM Tris-Cl, 10 mM dithiothreitol) and incubated at 65°C (LAT1 and LAT2) or 95°C (CD98 and β-actin) for 15 min. Aliquots of samples containing 20 μg of protein were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride membrane. Blots were incubated overnight at 4°C in 10 mM Tris–HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.5 (TBST), with 5% skim milk and then with rabbit anti-LAT1 C-terminus antibody (1:5,000; according to the procedure of Morimoto et al.*), rabbit anti-LAT2 N-terminus antibody (1:5,000; according to the procedure of Morimoto et al.*), rabbit anti-CD98 antibody (1:200; Santa Cruz Biotechnology) or rabbit anti-actin antibody (1:1,000; Cell Signaling Technology, Beverly, MA) overnight at 4°C. After being washed with TBST, the blots were incubated with goat horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:20,000; Cell Signaling Technology) for 1.5 h at RT. The blots were further washed with TBST, and specific proteins were visualized by using enhanced chemiluminescence western blotting detection reagents (GE Healthcare).

*Morimoto E, Kanai Y, Kim do K, et al. Establishment and characterization of mammalian cell lines stably expressing human L-type amino acid transporters. J Pharmacol Sci. 2008 ;108:505-16.

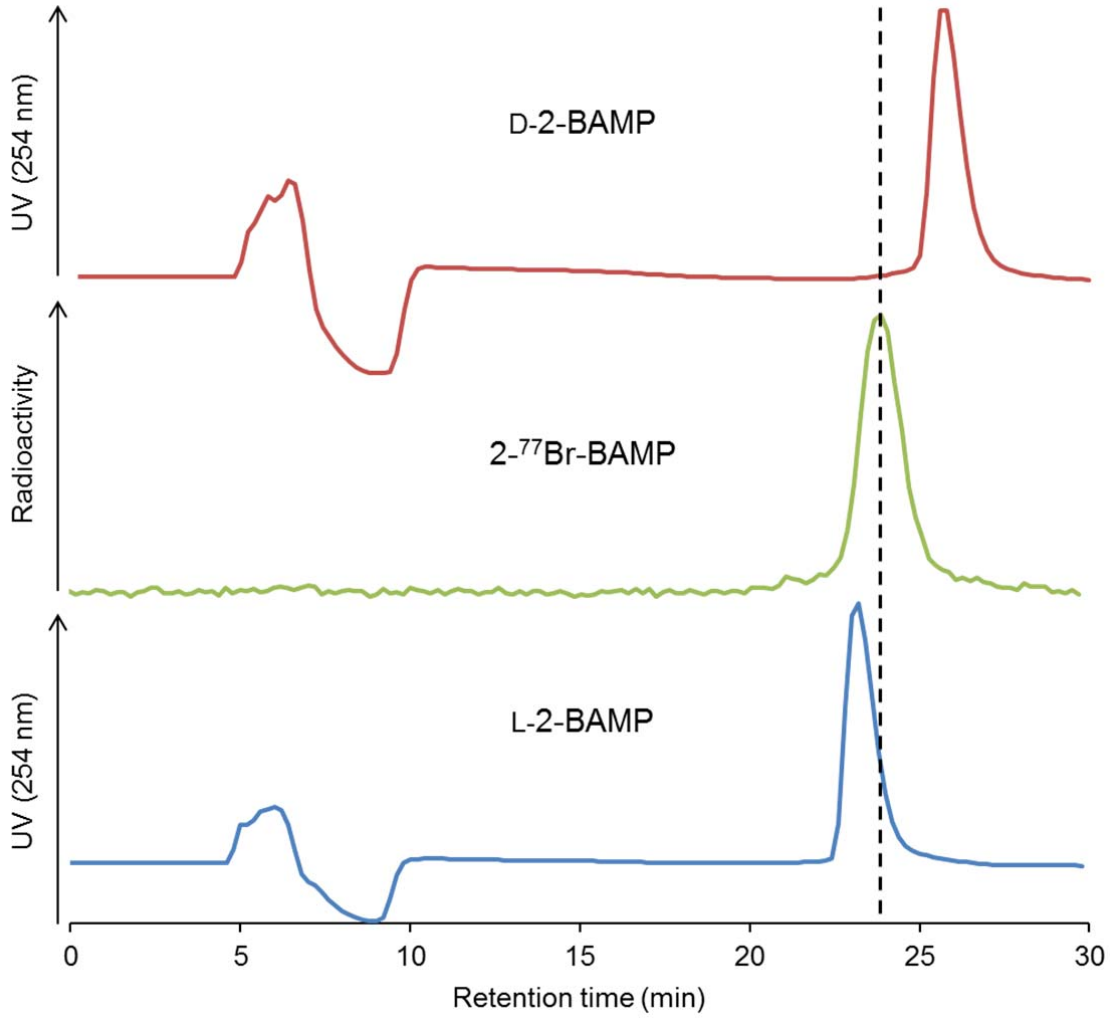
Protein Incorporation Studies

LS180 cells were incubated with 2-^{77}Br-BAMP or 4-^{77}Br-BAMP (500 kBq, <100 pmol) at 37°C for 6 h, and then the cells were washed with PBS and detached using trypsin. Cells were suspended in a Tris–HCl buffer (pH 7.5) containing 0.2 M NaCl, 5 mM $CaCl_2$ and 0.1% Triton X-100, and then homogenized (Vibra-cell VC-750, Sonics & Materials). The sample was analyzed with size-exclusion HPLC (SE-HPLC; TSKgel Super SW2000 column, 4.6 × 300 mm, Tosoh) at flow rate of 0.2 mL/min eluted with 0.1 M phosphate buffer (pH 6.8). MW-marker (HPLC) (Oriental Yeast Co.) was used as a molecular weight marker of SE-HPLC. After being filtered through a 10-kDa cut-off ultrafiltration membrane, the radioactivity of the samples was analyzed by RP-HPLC (system 2). For the evaluation in vivo, tumors dissected from LS180-tumor-bearing mice were cut into small pieces and homogenized in a Tris–HCl buffer (pH 7.5) containing 0.2 M NaCl, 5 mM $CaCl_2$ and 0.1% Triton X-100. After centrifugation at 8000×g for 10 min at 4°C, the supernatant was analyzed by SE-HPLC. After the samples were filtered through a 10-kDa cut-off ultrafiltration membrane, the radioactivity of the samples was analyzed by RP-HPLC (system 2).

Results

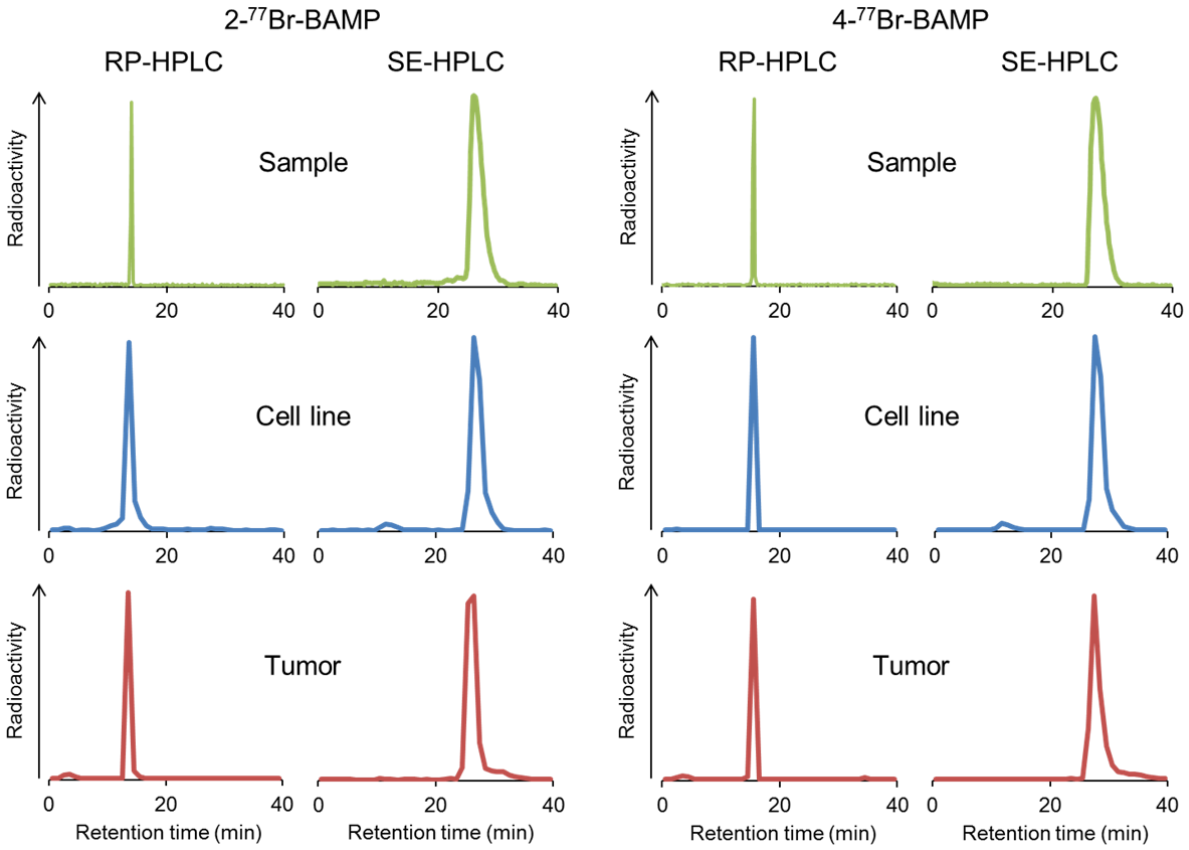


Supplemental Fig. 1. Analytical RP-HPLC (system 2) profiles of 2-^{77}Br-BAMP and 4-^{77}Br-BAMP.

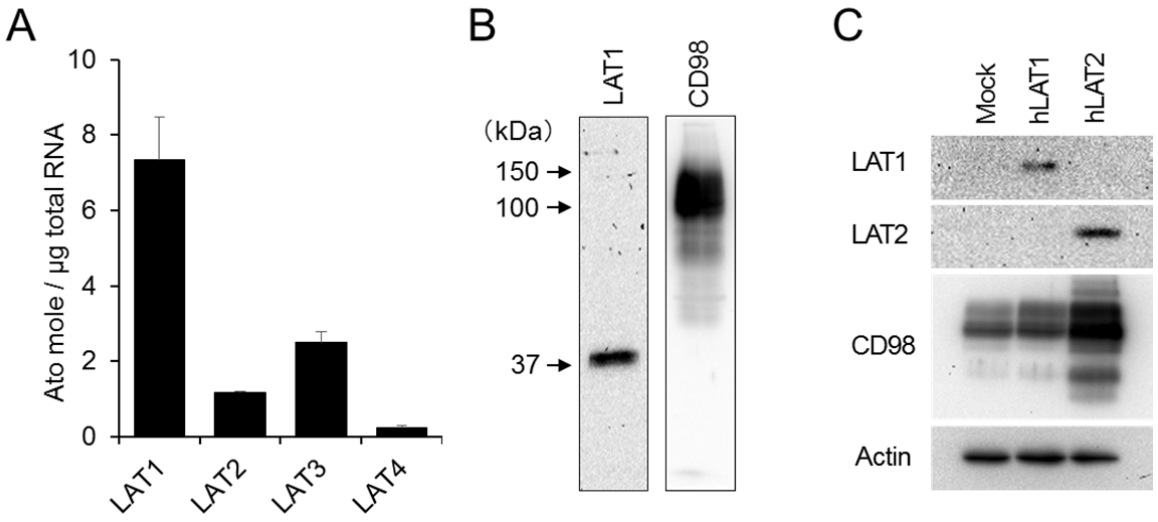


Supplemental Fig. 2. Chiral chromatography analysis of L-2-BAMP, D-2-BAMP and 2-^{77}Br-BAMP. The dotted line represents the retention time of 2-^{77}Br-BAMP.

Since the radio-detector was connected after the UV-detector, the retention time of radioactivity would be delayed by approx. 0.3–0.4 min. Thus, all of the 2-^{77}Br-BAMP was considered to be the L-form, indicating that 2-^{76}Br-BAMP could be prepared without racemization using our synthesis and radiobromination conditions.



Supplemental Fig. 3. Analytical RP-HPLC and SE-HPLC profiles of cell lysate after incubation with 2-^{77}Br-BAMP or 4-^{77}Br-BAMP at 37°C for 6 h, and the homogenate of a tumor dissected 6 h after the administration of 2-^{77}Br-BAMP or 4-^{77}Br-BAMP. The retention times of molecular weight markers were as follows. Glutamate dehydrogenase (MW 290,000): 13.2, Lactate dehydrogenase (MW 140,000): 14.8, Enolase (MW 67,000): 16.5, Myokinase (MW 32,000): 18.2, and Cytochrome c (MW 12,400): 21.9, respectively by SE-HPLC.



Supplemental Fig. 4. Analysis of LAT expression in cell lines. (A) The expressions of the LATs' mRNA in LS180 cells were determined by RT-PCR. Expressions of (B) LAT1 and CD98 protein in LS180 cells and (C) LAT1, LAT2 and CD98 protein in HEK293-mock, HEK293-hLAT1 and HEK293-hLAT2 cells by immunoblotting.

The expression level of LAT1 mRNA in LS180 cells was higher than that of the other LATs. Proteins of LAT1 and CD98, which covalently associate with LAT1 in the plasma membrane, were expressed in LS180 cells. Expression of LAT1 or LAT2 protein was not detected in HEK293-mock cells, whereas the HEK293-hLAT1 and HEK293-hLAT2 cells showed high expressions of LAT1 and LAT2, respectively. Thus, the difference between the uptake of tracers in HEK293-hLAT1 or HEK293-hLAT2 cells and that in HEK293-mock cells indicates the specific uptake via LAT1 or LAT2, respectively.