Boronophenylalanine, a boron delivery agent for boron neutron capture therapy, is transported by ATB$^{0,+}$, LAT1 and LAT2

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Boron neutron capture therapy (BNCT) is a therapeutic modality for malignant tumors using the nuclear capture and fission reactions that occur when boron-10 ($^{10}$B) is irradiated with neutron beams. This reaction, in theory, only kills $^{10}$B-containing cells because the destructive effect of the alpha particles and lithium nuclei, which are produced by the reaction, is limited to the immediate vicinity of the reaction (5–9 μm, approximately one cell diameter). Thus, the efficacy of BNCT relies on the selective delivery of $^{10}$B to malignant cells. Although various boron delivery agents have been proposed, only two compounds are currently under consideration in clinical trials, p-boronophenylalanine (BPA) and sodium borocaptate. The latter is an anionic polyhedral boraneicosahedron containing 12 boron atoms. This compound is thought to enter tumor cells by passive diffusion through the plasma membrane. In contrast, BPA is localized to tumor cells by selective uptake mediated by transporters.

The efficacy of boron neutron capture therapy relies on the selective delivery of boron carriers to malignant cells. p-Boronophenylalanine (BPA), a boron delivery agent, has been proposed to be localized to cells through transporter-mediated mechanisms. In this study, we screened aromatic amino acid transporters to identify BPA transporters. Human aromatic amino acid transporters were functionally expressed in Xenopus oocytes and examined for BPA uptake and kinetic parameters. The roles of the transporters in BPA uptake were characterized in cancer cell lines. For the quantitative assessment of BPA uptake, HPLC was used throughout the study. Among aromatic amino acid transporters, ATB$^{0,+}$, LAT1 and LAT2 were found to transport BPA with $K_m$ values of 137.4 ± 11.7, 20.3 ± 0.8 and 88.3 ± 5.6 μM, respectively. Uptake experiments in cancer cell lines revealed that the LAT1 protein amount was the major determinant of BPA uptake at 100 μM, whereas the contribution of ATB$^{0,+}$ became significant at 1000 μM, accounting for 20–25% of the total BPA uptake in MCF-7 breast cancer cells. ATB$^{0,+}$, LAT1 and LAT2 transport BPA at affinities comparable with their endogenous substrates, suggesting that they could mediate effective BPA uptake in vivo. The high and low affinities of LAT1 and ATB$^{0,+}$, respectively, differentiate their roles in BPA uptake. ATB$^{0,+}$, as well as LAT1, could contribute significantly to the tumor accumulation of BPA at clinical dose.

Alterations of transporter expression in tumor cells, an adaption to altered tumor metabolism, offer opportunities for selective drug delivery. In this context, aromatic amino acid transporters upregulated in tumor cells are expected to play an important role in the delivery of BPA, a phenylalanine analogue. Previous studies have suggested that system L transporters, particularly LAT1, are involved in the transport of BPA. The expression of LAT1 is highly upregulated in various cancers, where it is thought to contribute to tumor growth by increasing amino acids supply. Since we found LAT1 as a first system L transporter, we have examined its functional properties and revealed that a PET tracer, 3-fluoro-L-α-methyl-tyrosine, is taken up by LAT1 into tumors. This study provides a BPA transport profile of human aromatic amino acid transporters. In previous studies, the mechanisms of BPA uptake were studied by using non-specific
transporter inhibitors and/or exploiting Na⁺-dependence of the uptake. However, such approaches cannot separate transporter isoforms. In the present study, we have expressed each transporter in *Xenopus* oocytes to examine whether it transports BPA. Here, we have identified transporters responsible for BPA uptake among aromatic amino acid transporters. We have, furthermore, characterized their transport kinetics and roles in BPA transport in cancer cells.

Materials and Methods

**Materials.** Chemicals and cell media were purchased from Wako Pure Chemicals (Osaka, Japan) unless otherwise specified. L-isomer of BPA was from Sigma-Aldrich (St Louis, MO, USA). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), a fluorescence-derivatization agent, was from Dojindo (Kumamoto, Japan). Pancreatic cancer MIA PaCa-2, cervical cancer HeLa S3, and breast cancer MCF-7 cell lines were from the American Type Culture Collection (Manassas, VA, USA). Pancreatic cancer cell line T3M4 was from Riken Cell Bank (Tsukuba, Japan). Hepatocarcinoma FLC-4 was a gift from Dr. Seishi Nagamoto. Asf104 serum-free medium was from Ajinomoto (Tokyo, Japan). Fetal bovine serum was from Gibco (Gibco Life Technologies, Grand Island, NY, USA).

**Transport assay in Xenopus oocytes.** The human cDNAs for transporters are listed in Table 1: the cDNAs of the transporters, collectrin and 4F2hc were cloned into pcDNA3.1(+) (Invitrogen Life Technologies, Carlsbad, CA, USA) by standard recombinant methods. Capped complementary RNAs (cRNA) were transcribed from linearized cDNA plasmid templates by T7 mMESSAGE mMACHINE kit (Ambion Life Technologies, Austin, TX, USA).

The transport assay was carried out as previously described. Briefly, oocytes were micro-injected with cRNA: 25 ng for equimolar mixture of collectrin and B₀AT₁, 4F2hc and LAT1 or LAT2; 12.5 ng for LAT4; and 25 ng for the others. B₀AT₁ cRNA was co-injected with that of collectrin for B₀,+ and LAT1 or LAT2; 12.5 ng for LAT4; and 25 ng for the others. B₀AT₁ cRNA was co-injected with that of collectrin for B₀,+. Na⁺ uptake buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂·2H₂O, 1 mM MgCl₂·6H₂O, and 5 mM HEPES, pH 7.5) or Na⁺-free uptake buffer was used. In Na⁺ free uptake buffer, NaCl was replaced with 96 mM choline chloride. Uptake was terminated by washing three times with an ice-cold uptake buffer.

**High performance liquid chromatography analysis.** Amino acid derivatization were reported elsewhere. Samples were prepared and separated by HPLC as described below. Each oocyte was homogenized in 100 µL water by sonication and spun at 15,000g at 4°C for 10 min. After carefully removing the yolk fraction by aspiration, 20 µL supernatant was vigorously mixed with 180 µL methanol for deproteinization, and spun at 15,000g at 4°C for 10 min. Cell samples were similarly prepared. Protein concentrations were determined by bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA) before deproteinizing. One hundred microliters of the supernatant was dried in a vacuum chamber. The sediment was resolved in 20 µL of 200 mM sodium borate (pH 8.0). For fluorescence derivatization, 5 µL of 40 mM NBD-F in acetonitrile (MeCN) was added and heated at 60°C for 2 min. Derivatizing reaction was terminated by adding 75 µL of 0.5% trifluoroacetic acid (TFA) aqueous solution. Twenty microliters of the sample was separated by a NANOSPACE SI-2 HPLC system (Shiseido, Tokyo, Japan) with a fluorescence detector. The analytical column was Capcell Pak C₁₈ MGII S5 (250 × 2.0 mm i.d.). The mobile phase was MeCN–TFA–water (27.5:0.05:72.5, vol/ vol/vol) with a flow rate of 200 µL/min. Isocratic elution was carried out for 15 min. The BPA in samples was quantified by comparing the peak height with that of a BPA standard of known amount.

**Cell culture.** MIA PaCa-2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM). HeLa S3 cells were in Eagle’s minimum essential medium. MCF-7 cells were in DMEM with insulin (0.01 mg/mL), sodium pyruvate (1 mM), and NEAA. FLC-4 cells were in ASF104 serum-free medium. T3M4 cells were in RPMI-1640 medium. Cell lines were cultured at 37°C with 5% CO₂. Media were supplemented with FBS (10%) and penicillin/streptomycin (100 µ/mL).

**Membrane fractionation and Western blot analysis.** Cultured cells were collected and pelleted at 5000g for 3 min at 4°C. The cell pellet was sonicated in 500 µL ice-cold PBS (pH 7.4) containing Complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). Homogenate was clear at 1000g for 10 min. The supernatant was centrifuged at 10,000g for 10 min. The resultant supernatant was ultracentrifuged at 391,000g for 30 min. The membrane pellet was washed twice with ice-cold PBS and re-suspended in 50 µL PBS with 1% NP-40. The protein concentrations were determined by bicinchoninic acid assay. Protein samples were separated on a 10% polyacrylamide gel. The gel was blotted onto a PVDF membrane at 100 V for 30 min. The membrane was probed in TBST buffer (10 mM Tris, 150 mM, pH 7.6 adjusted with HCl) containing 5% skim milk with anti-SLC6A14/ATB⁰⁺ (1:1000; MBL, Nagoya, Japan) followed by goat anti-rabbit IgG HRP-conjugated (1:5000; Jackson, West Grove, PA, USA), anti-LAT1 antibody (1:5000; Trans Genic Inc., Kumamoto, Japan) followed by goat anti-rabbit IgG HRP-conjugated (1:5000; Jackson). Na⁺-K⁺ ATPase was detected as loading control by anti-Na⁺-K⁺ ATPase antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by goat anti-mouse IgG HRP-conjugated (1:5000; Jackson). The antibody-treated membrane was developed by ECL Prime Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) and imaged by LAS-4000 mini version 2.0 (Fujifilm, Tokyo, Japan).

**Transport assay in cultured cells.** The FLC-4, MCF-7, MIA PaCa-2, HeLa S3 and T3M4 cells were seeded on a 24-well

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**Table 1. p-Borophenylalanine (BPA) uptake by human aromatic amino acid transporters**

| Transport system | Gene | Protein | Na⁺ dependence | BPA transport |
|------------------|------|---------|----------------|---------------|
| g⁻ | SLC6A19 | B₀AT₁ | Dependent | – |
| g⁺ | SLC6A14 | ATB⁰⁺ | Dependent | + |
| L₁ | SLC7A5 | LAT1 | Independent | + |
| L₂ | SLC43A1 | LAT3 | Independent | – |
| T | SLC6A10 | LAT1 | Independent | – |

*Xenopus* oocytes expressing each transporter were incubated for 30 min in uptake buffer containing 100 µM BPA with or without Na⁺ on the basis of Na⁺ dependence. Oocyte lysate was separated by HPLC for BPA detection. +, positive for BPA uptake; –, negative for BPA uptake.
plate at $1.2 \times 10^5$, $2 \times 10^5$, $0.8 \times 10^5$, $1.2 \times 10^5$ and $1 \times 10^5$ cells/well. Cells were incubated for 2 days. The uptake experiment was carried out as described\(^{(18)}\) in Hanks’ balanced salt solution or Na’-free Hanks’ balanced salt solution in which NaCl was replaced with equivalent concentration of choline chloride.

For RNA interference, MCF-7 cells were seeded at $1.2 \times 10^5$ cells/well on a 24-well plate 24 h prior to transfection with RNAiMax (Invitrogen Life Technologies). The cells were subjected to uptake experiments after 48 h of transfection. The siRNAs used in this study were: non-targeting controls siRNA #1 (D-001810-01-05) and #2 (D-001810-01-20) from Thermo Scientific; and LAT1 siRNA #3 (s15653), #4 (s15654), and #5 (s15655) from Ambion Life Technologies.

**Statistics.** Student’s $t$-test was used for statistical analyses. $P < 0.05$ was taken as significant. Each experiment was carried out in triplicate. Representative results are shown in figures.

**Results**

**Determination of transporter-mediated BPA uptake.** Standard BPA and amino acid mixture were separated by HPLC after pre-column derivatization with NBD-F. NBD-BPA was eluted between NBD-alanine and NBD-proline (Fig. 1a,b). NBD-BPA (0.1 pmol/injection) was detected with our HPLC specifications. BPA standards were linearly quantified from 0.1 to 12 pmol with a correlation coefficient $r = 0.9995$. To establish HPLC determination of BPA in biological sample, BPA transported into *Xenopus* oocytes by LAT1 was separated by HPLC (Fig. 1c,d). The BPA peak of LAT1-expressing oocyte was higher than that of the control, indicating that BPA uptake by LAT1 was detected by this analytical procedure. Quantitative estimation of BPA in biological sample was carried out when the BPA peak height was more than two times higher than the background. To assess the recovery of BPA, samples prepared from oocytes were exogenously supplemented with 1 pmol of BPA before homogenization. The recovery was estimated to be $97 \pm 1.25\%$ (mean $\pm$ SD, $n = 3$).

**Identification of human amino acid transporters mediating BPA uptake.** The HPLC-based uptake assay was applied to screen over human aromatic amino acid transporters to identify BPA transporters. The transporters listed in Table 1 were selected based on the substrate selectivity in which phenylalanine or tyrosine is preferred.\(^{(19)}\) The selected amino acid transporters were functionally expressed in *Xenopus* oocytes (Fig. S1). The experimental and control oocytes were subjected to uptake experiments followed by HPLC detection. As shown in Fig. 2 and Table 1, ATB0,+, LAT1 and LAT2 were found to transport BPA.

**Time- and concentration-dependent uptake of BPA by ATB0,+, LAT1 and LAT2.** The dependence of BPA transport on uptake time and BPA concentration was examined. The uptake of BPA by each transporter was linear over 30 min (Fig. 3). Thus, uptakes were measured for 30 min to determine kinetic parameters. The BPA transports by ATB0,+, LAT1 and LAT2 were saturable and followed the Michaelis–Menten kinetics (Fig. 4). The Michaelis–Menten constants ($K_m$) and $V_{max}$ values were determined by the Lineweaver–Burk plot (Fig. 4) and summarized in Table 2.

**Boronophenylalanine uptake in cancer cell lines and expression of LAT1 and ATB0,+.** LAT1 expression is associated with normal tissues, whereas LAT1 and ATB0,+ are known for their rele-
To assess the contribution of LAT1 and ATB0,+, in BPA uptake, uptake rates were measured in cancer cell lines with various expression levels of LAT1 and ATB0,+. The LAT1 protein amounts were compared in Western blot analysis: low in FLC-4, medium in MCF-7 and MIA PaCa-2, and high in HeLa S3 and T3M4 cells (Fig. 5a). In contrast, ATB0,+ protein was strongly detected in MCF-7 and also weakly in T3M4 cells, but not in the other cell lines (Fig. 5b). The BPA uptake rates were proportional to the LAT1 protein levels (Fig. 5a,c). We examined the Na+-dependence of BPA uptake, because ATB0,+ is Na+-dependent whereas LAT1 is not. There were no significant differences in the uptakes at 100 μM BPA between Na+-free and normal Na+ conditions (Fig. 5c).

Increased contribution of ATB0,+ at higher BPA concentration. ATB0,+, has a lower affinity to BPA than LAT1 (Table 2). Thus, ATB0,+, is proposed to work at higher BPA concentra-
tions. Consistent with this, a significant Na$^{+}$-dependent uptake of BPA was observed at 1000 $\mu$M in MCF-7 cells (Fig. 6a). To reduce the contribution of LAT1 to BPA uptake in MCF-7 cells, the LAT1 expression was silenced by RNA interference. Treatment of MCF-7 cells with siRNAs depleted the LAT1 protein amount to ~20% (Fig. 6b). Among the siRNAs tested, control siRNAs and LAT1 siRNAs had a similar impact on the LAT1 protein amount (Fig. 6b) and BPA uptake (Fig. S2). After LAT1 was knocked down in 1000 $\mu$M BPA, the residual component was further decreased by removing Na$^{+}$ and also inhibited by lysine, a high-affinity substrate of ATB$^{0,+}$ (21) but not interactive with LAT1 (12) (Fig. 6c). This Na$^{+}$-dependent and lysine-inhibitable component is proposed to be mediated by ATB$^{0,+}$. Such component was only found at 1000 $\mu$M, accounting for at least 20–25% of the total BPA uptake (Fig. 6c). In 100 $\mu$M of BPA, the residual component after LAT1 knockdown was not dependent on Na$^{+}$ (Fig. 6c).

Discussion

Previously published reports suggested that system L transporters mediate the entry of BPA into tumor cells.(5,7,8) The present study clarified this by separately expressing transporters in Xenopus oocytes; LAT1 and LAT2 transport BPA whereas LAT3 and LAT4 do not. In addition, ATB$^{0,+}$ was proved to be a BPA transporter. ATB$^{0,+}$ has been shown to be upregulated in malignant tumors as discussed later and proposed to be a potential therapeutic target. (6,20,22,23) ATB$^{0,+}$ transports all amino acids except glutamate and aspartate by using a Na$^{+}$ and Cl$^{-}$/C0 gradient.(21) This broad selectivity of ATB$^{0,+}$ holds potential to be used for targeted drug delivery.(6)

The range of $K_{m}$ values for large neutral amino acids is 10–30 $\mu$M in LAT1 and 40–120 $\mu$M in LAT2.(13,24) ATB$^{0,+}$ transports 18 amino acids at various affinities with $K_{m}$ values ranging from 10 to 600 $\mu$M (21). The BPA $K_{m}$ values for these transporters fall within the endogenous substrate ranges, suggesting that these transporters could mediate effective BPA uptake in vivo (Table 2). LAT1 and ATB$^{0,+}$ differentially uptake BPA, depending on its concentration. At 100 $\mu$M BPA,
LAT1 appears to be a key BPA transporter because the BPA uptake rate was Na+-independent and positively correlated with the amount of LAT1 protein (Fig. 5). At 1000 μM, ATB0,+ measurably transports BPA, accounting for 20–25% of total uptake in MCF-7 cells (Fig. 6c). This ratio is comparable to the ATB0,+ contribution in the uptake of iodine-123-α-methyl tyrosine, a single photon emission computed tomography tracer, in glioma cells. Clinically, blood BPA concentrations reach ~2000 μM when administering 250 mg BPA/kg body weight, suggesting that the contribution of ATB0,+ could be significant at the clinical dose of BPA if the tumors express ATB0,+.

From the Michaelis–Menten curves in Figure 4, both LAT1 and ATB0,+ are extrapolated to be almost saturated to transport BPA at the clinical concentrations. Despite the saturated kinetics, an animal study using a regimen of increasing dose from 250 to 1000 mg BPA/kg body weight resulted in an increase in tumor boron concentration. Also, the extension of infusion time increased boron accumulation in tumor. Wyss et al. observed that intratumor blood flow is heterogeneous in low-grade glioma and so is the uptake of an amino acid PET tracer. Such heterogeneous vasculature possibly generates various local BPA concentrations within the tumor mass. Higher-dose and longer-infusion protocol might help raise the

**Fig. 5.** Transporter expression and p-boronophenylalanine (BPA) uptake in cancer cell lines. (a) Expression level of LAT1 was analyzed by western blotting using crude membrane fraction. The cell lines were ordered in increasing LAT1 amounts. Na+/K+ ATPase was used as loading control. (b) Similarly, the expression of ATB0,+ was analyzed. ATB0,+ was expressed in MCF-7 cells, to a lesser degree in T3M4 cells, and not detected in the other cell lines. (c) Uptake of BPA in cell lines was measured for 5 min in the presence or absence of Na+. The presence of Na+ did not significantly affect the BPA uptakes in the cell lines. A representative result was shown with the mean ± SEM (n = 4).

**Fig. 6.** Differential roles of ATB0,+ and LAT1 in p-boronophenylalanine (BPA) uptake. (a) Uptake of BPA in MCF-7 cells was measured for 10 min at 100 μM (left) and 1000 μM (right) BPA. At 1000 μM, the uptake rate in the presence of Na+ increased by ~1.4-fold from the Na+-free uptake. (b) MCF-7 cells were transfected with non-targeting control siRNA #1 and #2, and LAT1-targeting siRNA #3, #4 and #5. The siRNAs #3–#5 achieved a ~80% reduction of LAT1 protein amount. (c) Uptake by ATB0,+ was separated by LAT1 knockdown and lysine inhibition. The uptakes in the presence of Na+ and with mock knockdown were set at 1.0 at each BPA concentration (bars 1 and 4). At 100 μM BPA, the uptake levels with LAT1 knockdown did not differ regardless of Na+ (bars 2 and 3). At 1000 μM, LAT1 knockdown left a significant Na+-dependent component (bars 5 and 6). Overall, although with various statistical significances, the BPA uptake in bar 5 was inhibited by 5 mM lysine to a level similar to bar 6 (bar 7). This Na+-dependent and lysine-inhibitable component accounted for at least 20–25% of the total uptake. siRNA#1 was used for control siRNA. n.s., not significant.

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local concentrations in poorly perfused regions, resulting in homogeneous BPA distribution. Clinically, a high-dose and longer-infusion regimen (900 mg BPA/kg, 6-h infusion) is reported to extend patients’ survival. (1,30)

In addition to the transporter kinetics, expression levels of LAT1 and ATB0,+ in head and neck cancers, melanoma and glioma profiles of these transporters over other tumors (Fig. S3). Cancers and melanomas appeared to have higher expression expressed sequence tag analysis showed a negative association were compared using data from the Human Protein Atlas project (http://www.proteinatlas.org) (Fig. S3). (32) Head and neck cancers and melanomas to appear to have higher expression profiles of these transporters over other tumors (Fig. S3). Recent successful applications of BNCT to head and neck cancers may rely on the abundance of LAT1 and ATB0,+ in situ. LAT1 or ATB0,+ was moderately or weakly detected in the glioma specimens in Figure S3. However, in two other studies, LAT1 expression was detected in all glioma specimens with increasing immunostaining intensity over grade (Table S1). (33,34) In contrast, LAT2 is expressed in normal tissues. (11) An expressed sequence tag analysis showed a negative association of LAT2 with tumor tissues. (9) Based on these findings, the main role of LAT2 in the BPA pharmacokinetics is proposed to lower the tumor-to-normal (T/N) ratio of BPA accumulation. This hypothesis could be appropriately tested by using primary specimens containing both tumor and adjacent normal tissues, not by cultured cells.

On a methodological note, HPLC was extensively used throughout this study to detect and quantify BPA, instead of direct-current plasma atomic emission spectrometry (DCP-AES). Our HPLC method derivatizes BPA with NBD-F and detects NBD-BPA with a detection limit of 0.1 pmol per injection, whereas DCP-AES requires a minimum of 16 nmol BPA/sample for detection. (36) Di Pierro et al. (36) reported an HPLC application with α-phthalaldehyde derivatization and a similar detection limit. Such derivatization methods are applicable to compounds with amino groups with which the derivatizing reagents react.

p-Borophenylalanine is well accumulated in tumor cells with a low T/N ratio, whereas sodium borocaptate has a high T/N ratio with weak accumulation. (4) The T/N ratio of BPA might be improved by modifying BPA to be more LAT1-specific. Recently, we reported that the methylation of the α-carbon in tyrosine confers LAT1 selectivity. (15) The PET tracer 3-fluoro-L-α-methyl-tyrosine is a successful example of modifying an existing substrate for LAT1 selectivity. (15)

In conclusion, the screening and kinetic analysis of aromatic amino acid transporters revealed that LAT1, LAT2 and ATB0,+ transport BPA at high, medium and low affinity, respectively. Their affinities for BPA are comparable with those of the endogenous substrate, suggesting that they could mediate effective BPA uptake in vivo. Uptake experiments in cancer cell lines revealed differential roles of LAT1 and ATB0,+ in BPA uptake depending on the concentrations. It is proposed that ATB0,+ as well as LAT1, could contribute significantly to the tumor accumulation of BPA at clinical dose.

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Disclosure Statement
The authors have no conflict of interest.

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**Supporting Information**

Additional supporting information may be found in the online version of this article:

**Fig. S1.** Functional expression of aromatic amino acid transporters.

**Fig. S2.** Equivalent effectiveness of LAT1 siRNAs.

**Fig. S3.** Expression profile of ATB\(^{0,+}\) and LAT1 in cancers.

**Table S1.** LAT1 expression in gliomas.