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Kinetic analyses of *trans*-1-amino-3-[^{18}F]fluorocyclobutanecarboxylic acid transport in *Xenopus laevis* oocytes expressing human ASCT2 and SNAT2

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ABSTRACT

Introduction: *Trans*-1-amino-3-[^{18}F]fluorocyclobutanecarboxylic acid (*anti*-[^{18}F]FACBC) is a promising amino acid positron emission tomography (PET) radiotracer for visualizing prostate cancer. We previously showed that *anti*-FACBC is transported by amino acid transporters, especially by alanine-serine-cysteine transporter 2 (ASCT2), which is associated with tumor growth. We studied this affinity to assess the mechanism of *anti*-FACBC transport in prostate cancer cells.

Methods: Kinetic assays for *trans*-1-amino-3-fluoro-[1-^{14}C]cyclobutanecarboxylic acid ([^{14}C]FACBC) were performed in *Xenopus laevis* oocytes over-expressing either ASCT2 or sodium-coupled neutral amino acid transporter 2 (SNAT2), both of which are highly expressed in prostate cancer cells. We also examined the kinetics of [^{14}C]FACBC uptake using mammalian cell lines over-expressing system L amino acid transporter 1 or 2 (LAT1 or LAT2).

Results: ASCT2 and SNAT2 transported [^{14}C]FACBC with Michaelis–Menten kinetics K_m values of 92.0 ± 32.3 μM and 222.0 ± 29.3 μM, respectively. LAT1 and LAT2 transported [^{14}C]FACBC with Michaelis–Menten K_m values of 230.4 ± 184.5 μM and 738.5 ± 87.6 μM, respectively.

Conclusions: Both ASCT2 and SNAT2 recognize *anti*-FACBC as a substrate. *Anti*-FACBC has higher affinity for ASCT2 than for SNAT2, LAT1, or LAT2. The ASCT2-preferential transport of *anti*-[^{18}F]FACBC in cancer cells could be used for more effective prostate cancer imaging.

1. Introduction

The ^{11}C-labeled natural amino acid radiotracer most frequently used in the imaging of tumors by positron emission tomography (PET) is 2-amino-4-([^{11}C]methylthio)butyric acid ([^{11}C]Met) [1]. However, the short half-life of carbon-11 (^{11}C) is problematic for routine clinical use, and the rapid metabolism of ^{11}C-labeled natural amino acids typically generates radiolabeled metabolites that can confound kinetic analysis and lower the image quality. Several fluorine-18 (^{18}F)-labeled non-natural amino acid tracers have therefore been explored [1,2]. *Trans*-1-amino-3-[^{18}F]fluorocyclobutanecarboxylic acid (*anti*-[^{18}F]FACBC), one such non-natural amino acid, exhibits highly tumor-specific accumulation in glioblastoma multiforme, renal papillary cell cancer, and prostate cancer [3]. A recent phase 1 clinical study demonstrated *anti*-[^{18}F]FACBC to have favorable characteristics (e.g., low brain uptake, slow urinary excretion, and high *in vivo* stability) for imaging brain and prostate cancers [4]. The following phase 2a clinical study showed that *anti*-[^{18}F]FACBC rapidly accumulates in primary lesions, draining lymph nodes, and bone metastases of prostate cancer [5].

Our previous gene expression profile of amino acid transporters and their contributions to *anti*-FACBC accumulation in prostate cancer cells using a transporter knockdown assay [6,7] suggested a major role for Na^+-dependent transporters. Although both sodium-coupled neutral amino acid transporters, ASCT2 (encoded by *SLC1A5*) and SNAT2 (encoded by *SLC38A2*) [8], are highly expressed in prostate cancer cells, ASCT2 predominantly contributed to *anti*-FACBC accumulation into prostate cancer cells [6]. This may be attributed to different affinities of *anti*-FACBC for these amino acid transporters. To date, the kinetic properties of *anti*-FACBC transport by amino acid transporters have not been reported.

ASCT2, which is thought to be a primary contributor to *anti*-FACBC uptake, has been described in association with tumor growth and

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proliferation [9,10] in cancer cells. Enhanced ASCT2 expression was reported in a variety of human cancer cells, including hepatocellular carcinoma, colorectal and prostate cancer, glioma, and metastases [6,11]. Current evidence suggests such elevated expression of ASCT2 is linked to aggressive phenotype of cancer and poor prognosis in patients with prostate and colorectal adenocarcinomas [12,13]. ASCT2-mediated high affinity transport of glutamine has been discussed as a supplier of glutamine almost essential for tumor growth, which is in turn a key player in nutrient signaling for tumor cell metabolism [14]. Therefore, if *anti*-FACBC is proven as a high-affinity substrate of ASCT2, *anti*-FACBC could serve as a PET probe to detect or diagnose proliferative tumor tissues.

Na^+-independent system L amino acid transporter 1, or LAT1 (encoded by *SLC7A5*), transports branched or aromatic amino acids and is expressed in primary human cancers and several cancer cell lines [9]. LAT2 (encoded by *SLC7A8*), another subtype of system L amino acid transporter, is expressed more ubiquitously than LAT1 [15]. However, these transporters are thought to contribute only slightly to *anti*-FACBC uptake into human prostate cancer DU145 cells [6]. Demonstration of the affinities of *anti*-FACBC for these amino acid transporters would help to explain why their contributions to *anti*-FACBC transport differ.

In the present study, we aimed to clarify kinetic features of *anti*-FACBC transport mediated by ASCT2, SNAT2, LAT1, and LAT2 by employing *Xenopus laevis* oocytes or mammalian cell lines over-expressing amino acid transporters. This first detailed report of the kinetics of *anti*-FACBC uptake by these amino acid transporters demonstrates that *anti*-[^{18}F]FACBC could be used for more effective prostate cancer imaging.

2. Materials and methods

2.1. General

Trans-1-amino-3-fluoro-[1-^{14}C]cyclobutanecarboxylic acid ([^{14}C]FACBC; 2.08 GBq/mmol) and unlabeled *anti*-FACBC were synthesized by EaglePicher Pharmaceutical Services and EIWEISS, respectively [7]. [1-^{14}C]2-amino-4-(methylthio)butyric acid ([^{14}C]Met; 2.04 GBq/mmol) was purchased from American Radiolabeled Chemicals. [^{14}C(U)]2-aminopropionic acid ([^{14}C]Ala; 6.26 GBq/mmol) and [1-^{14}C]2-(methylamino)-2-methylpropionic acid ([^{14}C]MeAIB; 2.20 GBq/mmol) were purchased from PerkinElmer. The full-length cDNA clones of human ASCT2 and human SNAT2 were purchased from Origene and Open Biosystems, respectively.

2.2. Cell cultures

The LAT1 and LAT2 transport studies were performed at Fuji Biomedix Co., Ltd. (Tokyo, Japan) using mouse renal proximal tubule cell line S2 cells stably expressing human LAT1 (S2-LAT1) or human LAT2 (S2-LAT2). Cell clones transfected with empty pcDNA3.1 plasmid vector were named S2-mock. These cells were cultured in RITC 80-7 medium (IWAKI) containing 5% FBS, 10 μg/mL transferrin, 0.08 U/mL insulin, and 10 ng/mL epidermal growth factor [16].

2.3. Transport study using Xenopus laevis oocytes

ASCT2 and SNAT2 cRNA molecules were synthesized using the SP6 mMESSAGE mMACHINE kit (Life Technologies) from linearized cDNAs digested with the restriction enzymes *Xba*I and *Xho*I, respectively. The cRNAs were quantified by ultraviolet absorption spectroscopy.

The transport assay was performed as previously described [17]. Briefly, defolliculated oocytes were injected with 15 ng of hASCT2 cRNA, 30 ng of hSNAT2 cRNA, or the same volume (50 nL) of water. The cRNA-injected oocytes were incubated in modified Barth's solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4) containing 50 μg/mL gentamicin at 18 °C for 3 days. The oocytes were then incubated in a 24-well plate with a Na^+-containing buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.4) containing 3 μM of a ^{14}C-amino acid at 25 °C for the designated time. In the Na^+-free buffer, the NaCl was replaced with an equivalent concentration of *N*-methyl-D-glucamine (NMDG). The pH levels of the Na^+-containing and Na^+-free buffers were adjusted to 7.4 with NaOH and 4-morpholineethanesulfonic acid, respectively. For the competitive inhibition assay, nonradiolabeled L-alanine was used as the inhibitor. Uptake was terminated by washing the oocytes 3 times with an ice-cold uptake buffer. The oocytes were solubilized with 5% sodium dodecyl sulfate. Radioactivity was measured using a liquid scintillation counter (LSC-5100 or LSC-6100; Aloka).

2.4. Analytical method

Uptake, expressed as the cell-to-medium ratio (i.e., μL/oocyte), was obtained by dividing the amount of uptake by the concentration of substrate in the uptake medium. Initial transporter-mediated uptake rates were obtained by subtracting the uptake by the water-injected oocytes from the uptake by the ASCT2 or SNAT2 cRNA-injected oocytes. To evaluate the kinetic parameters, the uptake rates were fitted by nonlinear least-squares regression analysis using KaleidaGraph software (Synergy Software).

2.5. Transport study using LAT1- and LAT2-expressing cell lines

The uptake measurement methods were described by Wiriyasermkul et al. [18]. Cellular [^{14}C]FACBC uptake was measured in Na^+-free Hanks balanced salt solution (HBSS; 125 mM choline chloride, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1 mM KH_2PO_4, 1.3 mM $CaCl_2$, 5.6 mM D-glucose, and 25 mM *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid), pH 7.4) containing [^{14}C]FACBC and unlabeled *anti*-FACBC at the concentrations indicated in each experiment. At the end of the assay, the cells were washed with ice-cold uptake buffer and lysed with 0.2 N NaOH. Radioactivity was measured using a liquid scintillation counter (LSC-6100; Aloka). An aliquot of the lysate was used to determine the protein concentration using the bicinchoninic acid assay (Thermo Fisher Scientific). Intracellular [^{14}C]FACBC accumulation was normalized to the protein content and is presented as the ratio of accumulation to the initial concentration of *anti*-FACBC (i.e., the cell-to-medium ratio [μL/mg protein/min]). The kinetics of *anti*-FACBC uptake by LAT1 and LAT2 were analyzed using KaleidaGraph software.

2.6. Statistical analysis

The 2 groups were compared using a 2-tailed paired Student's *t* test. A P value of <0.05 was considered significant.

3. Results

3.1. Time course of the uptake of [^{14}C]FACBC by ASCT2 and SNAT2

We first examined [^{14}C]FACBC uptake by oocytes expressing ASCT2 or SNAT2, both of which are highly expressed in prostate cancer cells [6]. The uptake of [^{14}C]FACBC by ASCT2 and SNAT2 increased linearly over 30 min and was significantly greater ($P < 0.01$) in cRNA-injected than in water-injected oocytes (Fig. 1A and B).

[^{11}C]Met is a representative PET radiotracer for imaging amino acid uptake. Therefore, [^{14}C]Met uptake by ASCT2 and SNAT2 was also examined to compare the mechanisms of uptake. The uptake of [^{14}C]Met by both transporters increased linearly over 30 min (Fig. 1C and