



## [<sup>14</sup>C]Fluciclovine (alias *anti*-[<sup>14</sup>C]FACBC) uptake and ASCT2 expression in castration-resistant prostate cancer cells



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### ABSTRACT

**Introduction:** *trans*-1-Amino-3-[<sup>18</sup>F]fluorocyclobutanecarboxylic acid ([<sup>18</sup>F]fluciclovine, also known as *anti*-[<sup>18</sup>F]FACBC), is a tracer for positron emission tomography (PET) imaging for detection of tumors such as prostate cancer (PCa). Our previous study showed that ASCT2 (Na<sup>+</sup>-dependent amino acid transporter (AAT)) mediates fluciclovine uptake in androgen-dependent PCa cells; its expression is influenced by androgen, a key hormone in the progression of primary PCa and castration-resistant prostate cancer (CRPC). In this study, we investigated the uptake mechanisms and feasibility of [<sup>18</sup>F]fluciclovine for CRPC in the androgen-dependent PCa cell line LNCaP and LNCaP-derivatives LNCaP-SF and LN-REC4.

**Methods:** LNCaP-SF was established after long-term cultivation of LNCaP in steroid-free conditions, and LN-Pre and LN-REC4 were established from LNCaP inoculated in intact and castrated severe combined immunodeficient mice, respectively. Uptake and competitive inhibition experiments were performed with *trans*-1-amino-3-fluoro[1-<sup>14</sup>C]cyclobutanecarboxylic acid ([<sup>14</sup>C]fluciclovine) to characterize the involvement of AATs in androgen-dependent PCa (LNCaP and LN-Pre) and CRPC-like (LNCaP-SF and LN-REC4) cell lines. AAT expression was analyzed by Western blotting, and [<sup>14</sup>C]fluciclovine uptake in androgen-dependent PCa and CRPC-like cell lines were investigated in the presence or absence of dihydrotestosterone (DHT).

**Results:** The contribution of Na<sup>+</sup>-dependent AATs to [<sup>14</sup>C]fluciclovine uptake in all cell lines was 88–98%, and [<sup>14</sup>C]fluciclovine uptake was strongly inhibited by L-glutamine and L-serine, the substrates for Na<sup>+</sup>-dependent alanine-serine-cysteine (system ASC) AATs, in the presence of Na<sup>+</sup>. DHT enhanced ASCT2 expression in LNCaP, LN-Pre, and LN-REC4, but not in LNCaP-SF, and the responses of ASCT2 expression to DHT correlated with [<sup>14</sup>C]fluciclovine uptake.

**Conclusions:** System ASC, especially ASCT2, could play a major role in [<sup>14</sup>C]fluciclovine uptake into CRPC-like and androgen-dependent PCa cells, suggesting [<sup>18</sup>F]fluciclovine-PET is applicable to the detection of CRPC as well as androgen-dependent PCa.

**Advance in knowledge:** [<sup>18</sup>F]fluciclovine-PET may be applied for the detection of CRPC.

**Implication for patient care:** [<sup>18</sup>F]fluciclovine-PET may permit early intervention for CRPC treatment.

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### 1. Introduction

For more than 40 years since Huggins et al. published their study [1], androgen and androgen receptor (AR) signaling have been considered key regulators of carcinogenesis and progression of prostate cancer (PCa) [2]. In primary PCa, cancer cell proliferation depends on androgens secreted from the testis and adrenal glands. Chemical castration

targeting androgens and androgen receptor (AR), known as androgen deprivation therapy (ADT), is often chosen in locally advanced PCa as well as metastatic PCa [3], and therapeutic efficacy is monitored by serum prostate-specific antigen (PSA). Although ADT is a standard treatment, more than half of PCa patients develop castration-resistant prostate cancer (CRPC), an acquired androgen-independent behavior, within several years. CRPC is one of the most aggressive recurrent PCa types and often has poor prognosis [4]. Preclinical studies using androgen-independent LNCaP sublines [5] demonstrated the association between CRPC progression and androgen-dependent mechanisms (e.g. AR overexpression, AR mutation, AR-hypersensitive variants,

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alteration of intratumor androgen metabolism) as well as androgen-independent pathways (e.g. growth factor, cytokine) [6]. Therefore, the characteristics of CRPC may shift in response to the surrounding environment. Various drugs targeting androgens and AR have been proven effective in CRPC patients (e.g. enzalutamide [7] and abiraterone [8]). It is believed that AR signaling remains after PCa converts to CRPC [9].

Diagnostic imaging techniques such as SPECT and PET are useful tools for detection of PCa [10]. For example, tracers such as [<sup>111</sup>In] capromab pendetide [11], [<sup>11</sup>C]/[<sup>18</sup>F]-labeled choline [12,13], and [<sup>11</sup>C]acetate [14] are used to detect PCa. *trans*-1-Amino-3-[<sup>18</sup>F] fluorocyclobutanecarboxylic acid ([<sup>18</sup>F]Fluciclovine, also known as *anti*-[<sup>18</sup>F]FACBC) is a synthetic leucine-analog amino acid PET tracer that accumulates in PCa cells [15]. A phase I clinical trial of [<sup>18</sup>F] fluciclovine in Japan demonstrated the favorable pharmacokinetics for PCa detection such as slow excretion in urine and high stability [16]. In several studies, [<sup>18</sup>F]fluciclovine detected PCa lesions including primary PCa [17], local recurrence after radical prostatectomy [15], and metastasis in pelvic lymph nodes and bone [15,18,19]. These results suggest that [<sup>18</sup>F]fluciclovine is a useful PET tracer for the initial staging and restaging of PCa patients.

[<sup>18</sup>F]Fluciclovine accumulation is mediated by two neutral amino acid transporters (AATs), Na<sup>+</sup>-dependent system ASC transporters (especially ASCT2), and Na<sup>+</sup>-independent system L transporters (especially LAT1), which are upregulated in various cancers including PCa [20–22] and are thought to be involved in fluciclovine uptake into prostate cancer cells [23,24]. Moreover, the affinity of fluciclovine for human ASCT2 is similar to that of natural neutral amino acids such as L-alanine and L-serine [25]. We have also demonstrated that androgen enhances ASCT2 expression, while bicalutamide, an anti-androgen drug, inhibits androgen-induced ASCT2 expression in an androgen-sensitive cell line, LNCaP [26]. The responses of ASCT2 to androgen and bicalutamide correlate with *trans*-1-amino-3-fluoro[1-<sup>14</sup>C]cyclobutanecarboxylic acid ([<sup>14</sup>C]fluciclovine, also known as *anti*-[<sup>14</sup>C]FACBC) uptake [26]. These results suggest that changes in fluciclovine accumulation in PCa might reflect changes in AAT expression and amino acid requirements in androgen-dependent PCa cells.

Although a number of preclinical studies of PCa have been conducted, the relationship between AAT expression and [<sup>18</sup>F]fluciclovine uptake in CRPC cells has not been addressed. If AR-related signaling remains intact in CRPC cells, [<sup>18</sup>F]fluciclovine might be used to detect CRPC, because ASCT2 expression is regulated by androgen [26,27]. In this study, we investigated the uptake mechanisms of [<sup>14</sup>C]fluciclovine through AATs in CRPC-like cells, and considered the feasibility of [<sup>18</sup>F] fluciclovine in CRPC diagnosis.

## 2. Materials and methods

### 2.1. Reagents and radioisotope-labeled tracers

All reagents were purchased from commercial suppliers (Wako Pure Chemical Industries, Osaka, Japan; Sigma-Aldrich Japan, Tokyo, Japan; and Nacalai Tesque, Kyoto, Japan) unless otherwise described.

The <sup>14</sup>C-labeled tracer was used because its long half-life (5,700 years) makes it more convenient for in vitro experiments than <sup>18</sup>F (110 min). [<sup>14</sup>C]fluciclovine (2.09 GBq/mmol) was synthesized [23] by Sekisui Medical (Tokyo, Japan). The radiochemical purity was confirmed by thin-layer chromatography, and found to be >95%.

### 2.2. Cell culture

LNCaP cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The LNCaP-derived sublines (LNCaP-SF, LN-Pre, and LN-REC4) were established and cultured as described [5]. Briefly, LNCaP-SF cells, maintained in steroid-free media in vitro, proliferate only in androgen ablation conditions, but PSA mRNA was induced in the presence of dihydrotestosterone (DHT) in a concentration-

dependent manner. LN-Pre and LN-REC4 cells, which were developed in intact and castrated severe immunodeficient mice, exhibit androgen-sensitive PSA expression and cell growth [5].

### 2.3. Uptake study

Uptake experiments were performed as described [28] with minor modifications. Cells (5×10<sup>4</sup> per well) were suspended in medium and seeded in 24-well flat-bottom tissue culture plates (Becton Dickinson, East Rutherford, NJ). After 3 days, cells were washed twice with sodium buffer (140 mM NaCl, 5 mM KCl, 5.6 mM D-glucose, 0.9 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM HEPES, pH 7.3 at 37 °C) or choline buffer (sodium chloride was replaced with an equivalent concentration of choline chloride), and then incubated with [<sup>14</sup>C]fluciclovine at a final concentration of 10 μM for 5, 15, 30, and 60 min at 37 °C in air. Uptake was stopped by removing the tracer solution and rapidly washing the cells twice with ice-cold buffer. The cells were lysed in 0.1 N NaOH, and radioactivity was measured with a Tri-Carb 2910TR liquid scintillation counter (PerkinElmer, Waltham, MA) and Ultima Gold (PerkinElmer). The protein concentration of the cell lysate was determined with a VersaMax microplate reader (Nihon Molecular Device K.K., Osaka, Japan) using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Tracer uptake is expressed as nmol/mg protein.

### 2.4. Competitive inhibition uptake study

Experimental procedures were performed as described [28]. Briefly, after cells were cultured in 24-well flat-bottom tissue culture plates, they were incubated in sodium or choline buffer containing 10 μM [<sup>14</sup>C] fluciclovine for 5 min at 37 °C in air in the presence or absence of 2 mM inhibitors. Synthetic and natural amino acids were used as inhibitors: 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH; for system L in the absence of Na<sup>+</sup>); 2-(methylamino)-isobutyric acid (MeAIB; for system A, IMINO and PAT in the presence of Na<sup>+</sup>); N-ethylmaleimide (NEM; for system y<sup>+</sup> and LAT2/3/4 in the absence of Na<sup>+</sup>); L-glutamine (Gln; for system A, ASCT2, B<sup>0</sup>, N, y<sup>+</sup>L, L, and B<sup>0,+</sup> in the presence of Na<sup>+</sup>); L-serine (Ser; for system A, ASCT1/2, B<sup>0</sup>, N, y<sup>+</sup>L, asc, L and B<sup>0,+</sup> in the presence of Na<sup>+</sup>).

[<sup>14</sup>C]Fluciclovine uptake in sodium and choline buffers without inhibitors was normalized to 100%, and the inhibitory effects of synthetic and natural amino acids on [<sup>14</sup>C]fluciclovine uptake were calculated as a percentage of the control in each buffer.

### 2.5. Western blotting

After cells were lysed in lysis buffer (Cell Signaling Technology Japan, Tokyo, Japan) according to manufacturer protocols, 10 μg total protein was loaded in Any-kD PROTEAN TGX precast gels (Bio-Rad, Hercules, CA) and transferred to PVDF membrane using Transblot Turbo (Bio-Rad). The membranes were incubated in ImmunoBlock (DS Pharma biomedical, Osaka, Japan) for 60 min at room temperature and then incubated with primary antibodies overnight at 4 °C as follows: rabbit anti-ASCT2 (D7C12) monoclonal antibody (1:1000, Cell Signaling Technology Japan); rabbit anti-androgen receptor pAb (ChIP grade, 1:2000; Abcam, Cambridge, MA); goat anti-actin pAb (C-11) (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). The next day, the membranes were washed three times (10 min each) with Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Then, membranes were incubated with anti-rabbit or anti-goat secondary antibody conjugated to horseradish peroxidase (1:10000, Sigma-Aldrich, St. Louis, MO) for 60 min at room temperature. After washing three times with TBS-T, proteins were detected with ECL Prime detection reagent (GE Healthcare Japan, Tokyo, Japan) then chemiluminescence was developed using Hyperfilm ECL (GE Healthcare Japan), Hi-Rendol, and Hi-Renfix solution (FUJIFILM, Tokyo, Japan). The protein concentration of the cell lysate was determined as described in Section 2.3. The molecular mass of each protein band was determined according to the

manufacturer's product information sheet. Band density was quantitated using ImageJ 1.42 software (National Institutes of Health, Bethesda, MD), and each density ratio was normalized to the loading control (actin).

## 2.6. Flow cytometry

Experiments were performed as described elsewhere [24], with minor modifications. Cells were fixed in 90% methanol with  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline for 30 min on ice. Then, the fixed cells were suspended in 0.1% Triton X-100 dissolved in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS with 0.5% (w/v) bovine serum albumin and 0.5 mM ethylenediamine tetraacetic acid for 15 min on ice for permeation of the membrane. This step was followed by immunostaining with anti-ASCT2 primary antibody, (which recognizes the intracellular epitope, described in Section 2.5) for 30 min on ice. Then, the cells were stained with phycoerythrin (PE)-labelled goat anti-rabbit secondary antibody for 30 min on ice. Rabbit monoclonal antibody (DA1E) IgG (Cell Signalling Technology Japan) was used as isotype control. Data were acquired using a FACSCalibur flow cytometer (Becton Dickinson) and analyzed with WinMDI 2.8 software (The Scripps Institute).

## 2.7. Dihydrotestosterone (DHT) exposure

Cells were suspended in DMEM with 5% Charcoal/Dextran-treated FBS (CSS; Thermo Fisher Scientific) and seeded in 6-well flat-bottom tissue culture plates ( $5 \times 10^5$  per well). The following day, DHT dissolved in dimethylsulfoxide (DMSO) was added to each well and incubated for three days (final concentration of DHT: 10 nM, DMSO: 0.1%). The control cells were treated with DMSO without DHT at the same concentration. Exposure conditions including the final concentration of DHT and incubation time were determined based on the alteration of ASCT2 mRNA expression in response to DHT in LNCaP as measured by PCR (data not shown). The uptake experiment (uptake buffer, sample preparation, and measurement of protein concentration) was conducted as described in Section 2.3.

## 2.8. Data and statistical analysis

All data are presented as the mean  $\pm$  standard deviation (SD). Statistical analyses were performed in SAS software version 5.0 (SAS Institute Japan, Tokyo, Japan) using the two-tailed unpaired *t*-test or Dunnett's multiple comparison tests.  $P < 0.05$  was considered significant.

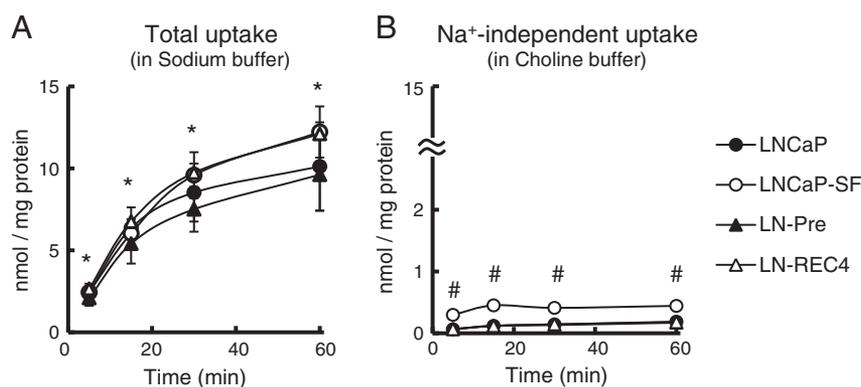
## 3. Results

### 3.1. Comparison of [ $^{14}\text{C}$ ]fluciclovine uptake between androgen-dependent PCa and CRPC-like cell lines

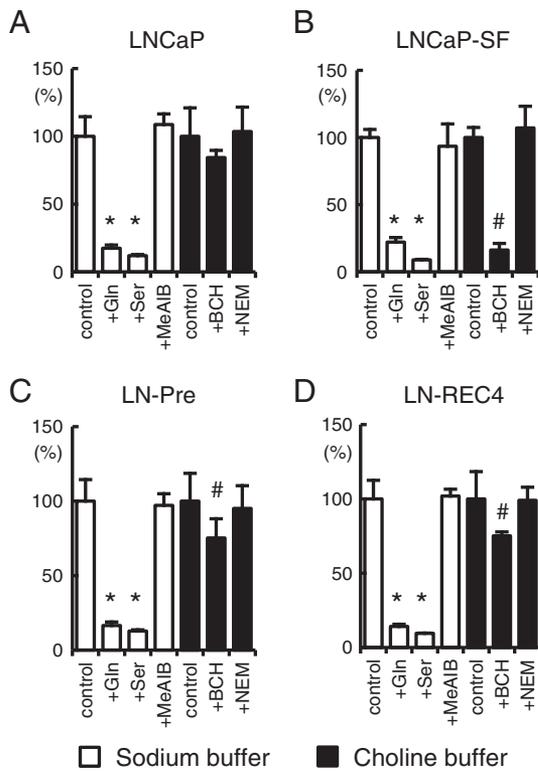
We compared [ $^{14}\text{C}$ ]fluciclovine uptake in androgen-dependent PCa (LNCaP and LN-Pre) and CRPC-like (LNCaP-SF and LN-REC4) cell lines. As shown in Fig. 1A, total [ $^{14}\text{C}$ ]fluciclovine uptake did not significantly differ between androgen-dependent PCa and CRPC-like cell lines, except between LN-Pre and LN-REC4 cells. The uptake of LNCaP, LNCaP-SF, LN-Pre, and LN-REC4 was as follows:  $2.5 \pm 0.5$ ,  $2.4 \pm 0.3$ ,  $2.1 \pm 0.5$ , and  $2.6 \pm 0.4$  nmol/mg protein at 5 min;  $6.4 \pm 1.2$ ,  $6.0 \pm 0.9$ ,  $5.4 \pm 1.2$ , and  $6.7 \pm 1.0$  nmol/mg protein at 15 min;  $8.5 \pm 1.8$ ,  $9.6 \pm 1.4$ ,  $7.5 \pm 1.4$ , and  $9.7 \pm 1.4$  nmol/mg protein at 30 min; and  $10.1 \pm 2.7$ ,  $12.2 \pm 1.6$ ,  $9.6 \pm 2.2$ , and  $12.1 \pm 1.7$  nmol/mg protein at 60 min, respectively. The contribution of  $\text{Na}^+$ -dependent AATs to [ $^{14}\text{C}$ ]fluciclovine uptake was 88–98% of total uptake of [ $^{14}\text{C}$ ]fluciclovine in all cell lines; the contribution of  $\text{Na}^+$ -independent AATs to [ $^{14}\text{C}$ ]fluciclovine uptake was minimal (2–12%), although  $\text{Na}^+$ -independent [ $^{14}\text{C}$ ]fluciclovine uptake in LNCaP-SF was substantially higher than in other cell lines (Fig. 1B). These results suggest  $\text{Na}^+$ -dependent AATs are largely involved in [ $^{14}\text{C}$ ]fluciclovine uptake into LNCaP and its derivative sublines, regardless of androgen responsiveness.

### 3.2. Competitive inhibition of transport in [ $^{14}\text{C}$ ]fluciclovine in androgen-dependent PCa and CRPC-like cell lines

To estimate the involvement of AATs in [ $^{14}\text{C}$ ]fluciclovine uptake into androgen-dependent PCa and CRPC-like cell lines, competitive inhibition experiments were performed with natural/synthetic amino acid inhibitors (Fig. 2A–D). Gln and Ser strongly inhibited [ $^{14}\text{C}$ ]fluciclovine transport in the presence of  $\text{Na}^+$  (9–22% of control), and BCH moderately inhibited [ $^{14}\text{C}$ ]fluciclovine uptake in choline buffer (16% of control in LNCaP-SF, 75–84% of control in other cells). In contrast, [ $^{14}\text{C}$ ]fluciclovine uptake was not inhibited by MeAIB in sodium buffer or by NEM in choline buffer in all cell lines. As described in Section 3.1, the contribution of  $\text{Na}^+$ -independent (i.e. system L AATs-mediated) uptake for [ $^{14}\text{C}$ ]fluciclovine in the PCa cell lines used in this study was 12% at most. Considering our previous reports on fluciclovine recognition [23–25], these results suggest systems ASC, especially ASCT2, mediates fluciclovine transport into CRPC-like cells as well as androgen-dependent PCa cells (see Section 2.4. for the characteristics of inhibitors for AATs).



**Fig. 1.** (A) Time-course of total [ $^{14}\text{C}$ ]fluciclovine uptake into PCa (LNCaP and LN-Pre) and CRPC-like (LNCaP-SF and LN-REC4) cells in sodium buffer over 60 min. Each point represents the mean  $\pm$  SD ( $n=9$ ). \* $P < 0.05$  between LN-Pre and LN-REC4. (B) Time-course of  $\text{Na}^+$ -independent [ $^{14}\text{C}$ ]fluciclovine uptake into PCa (LNCaP and LN-Pre) and CRPC-like (LNCaP-SF and LN-REC4) cells in choline buffer over 60 min. Each point represents the mean  $\pm$  SD ( $n=6$ ). # $P < 0.05$  between LNCaP and LNCaP-SF.



**Fig. 2.** Competitive inhibition percentages of [<sup>14</sup>C]fluciclovine uptake for 5 min into (A) LNCaP (PCa), (B) LNCaP-SF (CRPC-like), (C) LN-Pre (PCa) and (D) LN-REC4 (CRPC-like) cells. Cells were incubated in sodium buffer (white bar) or choline buffer (black bar) with or without the indicated inhibitors (2 mM). [<sup>14</sup>C]fluciclovine uptake without inhibitors (control) in sodium or choline buffer was normalized to 100%. Each value represents the mean±SD (n=4–18). \*P<0.05 vs. control in sodium buffer, #P<0.05 vs. control in choline buffer.

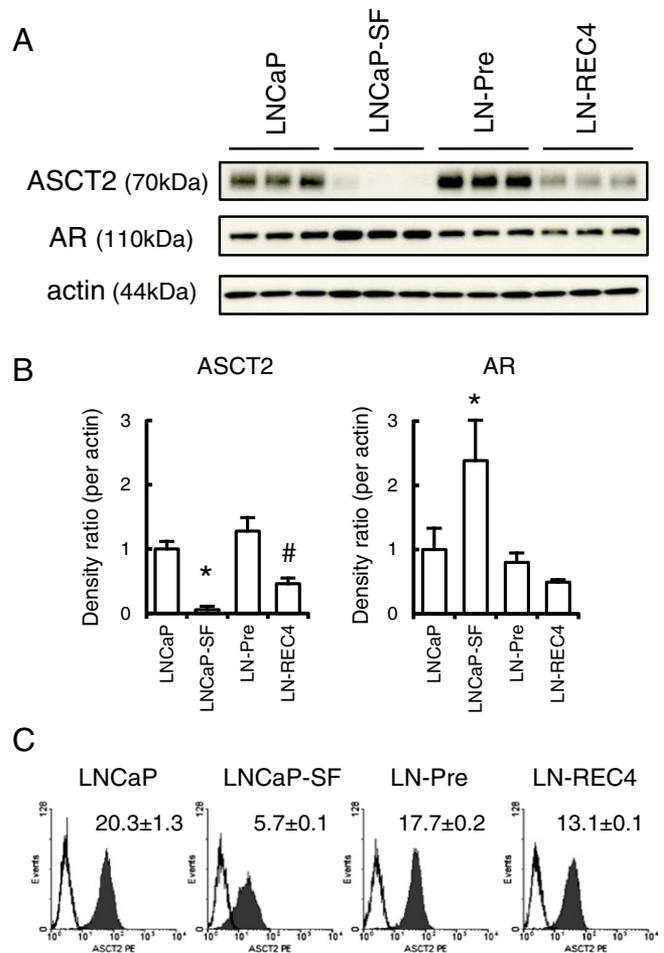
### 3.3. Expression of ASCT2 in androgen-dependent PCa and CRPC-like cell lines

Protein expression of ASCT2 and AR in androgen-dependent PCa and CRPC-like cell lines was analyzed by Western blotting (Fig. 3). As shown in Fig. 3A–B, ASCT2 expression in CRPC-like cells, especially in LNCaP-SF, was lower than in androgen-dependent PCa cells (P<0.01 between LNCaP and LNCaP-SF; P=0.011 between LN-Pre and LN-REC4). In addition, the protein expression of AR was higher in LNCaP-SF cells, but tended to decrease in LN-REC4 cells relative to that in the parental cell lines (P=0.042 vs. LNCaP, and P=0.055 vs. LN-Pre, respectively) (Fig. 3A–B). Furthermore, the same tendency of ASCT2 expression was observed by flow cytometry (Fig. 3C).

### 3.4. Responses of ASCT2 expression to DHT in androgen-dependent PCa and CRPC-like cell lines

ASCT2 expression in response to DHT was investigated to characterize androgen dependency in CRPC-like cell lines. DHT enhanced ASCT2 expression 2.8-fold in LNCaP, 2.2-fold in LN-Pre, and 1.7-fold in LN-REC4, but was unaltered in LNCaP-SF cells (Fig. 4A–B).

Finally, we investigated the effect of DHT on [<sup>14</sup>C]fluciclovine uptake in androgen-dependent PCa and CRPC-like cell lines. [<sup>14</sup>C]fluciclovine uptake tended to be lower in CRPC-like cells than in androgen-dependent PCa (Fig. 5). Further [<sup>14</sup>C]fluciclovine uptake was increased by DHT stimulation (1.2–1.3 fold over the controls) in LNCaP, LN-Pre, and LN-REC4, but not LNCaP-SF cells. The uptake without and with DHT was as follows: 8.2±0.8 and 9.6±1.5 nmol/mg protein in LNCaP (n=9, P=0.032); 5.9±0.5 and 6.4±0.8 nmol/mg protein in LNCaP-SF (n=12, P=0.092); 7.0±0.5 and 8.1±1.1 nmol/mg protein in LN-Pre



**Fig. 3.** Protein expression profiles of PCa (LNCaP and LN-Pre) and CRPC-like (LNCaP-SF and LN-REC4) were confirmed by Western blotting (A, B) and flow cytometry (C). In Western blotting (A), each cell lysate (10 µg/lane) was fractionated on SDS-PAGE and incubated with the indicated antibodies. Actin was used as a loading control. Band density ratios (B) are shown as (the band density of each protein)/(the band density of actin). Each ratio represents the mean±SD (n=3, \*P<0.05 vs. LNCaP, #P<0.05 vs. LN-Pre). Results of flow cytometry analysis (C), with the fluorescence intensity (X-axis) and cell number (Y-axis) shown as histograms. The values are the T/N ratio calculated from the mean fluorescence intensity of ASCT2 expression (closed histogram: T) and isotype control (open histogram: N), respectively (n=3).

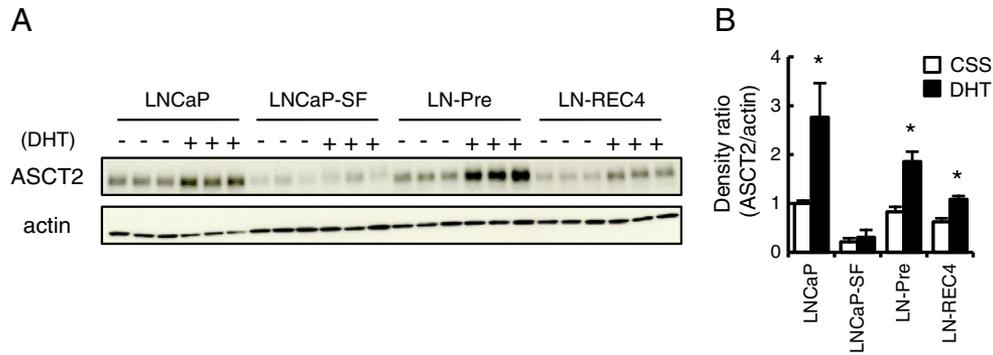
(n=8–9, P=0.025); and 5.9±0.2 and 7.8±0.3 nmol/mg protein in LN-REC4 (n=8, P<0.01), respectively. These results suggest that the alteration of [<sup>14</sup>C]fluciclovine uptake correlates with the degree of ASCT2 protein expression in LNCaP, LN-Pre, and LN-REC4 cell lines.

## 4. Discussion

This is the first study to investigate the differences between androgen-dependent PCa and CRPC-like cells in the context of fluciclovine uptake, AAT involvement with inhibitors, and androgen dependency *in vitro*.

We previously reported that Na<sup>+</sup>-dependent AATs, especially ASCT2, mediate [<sup>14</sup>C]fluciclovine transport in androgen-dependent PCa cells [23,24]. In the present study, total uptake of [<sup>14</sup>C]fluciclovine and inhibition profiles was similar in CRPC-like cells and androgen-dependent LNCaP cells under normal culture conditions (Figs. 1 and 2), suggesting that [<sup>18</sup>F]fluciclovine-PET can be used to visualize lesions in patients with androgen-dependent PCa as well as CRPC [16,19,29].

As shown in Fig. 3, ASCT2 expression in CRPC-like cells was lower than that in the parent cells, but the inhibition profile in the presence



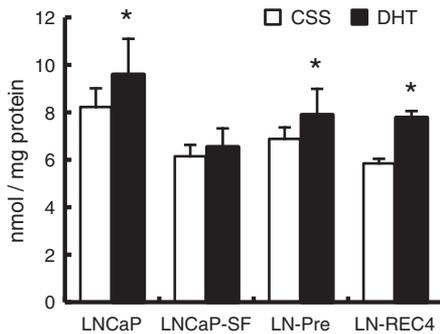
**Fig. 4.** The effect of androgen on ASCT2 protein expression in PCa (LNCaP and LN-Pre) and CRPC-like (LNCaP-SF and LN-REC4) cells. Cells were incubated with steroid-free media (CSS) for 24 h, and then CSS without DHT (white bar) or with DHT (black bar) was exposed for 72 h. (A) Total ASCT2 protein expressions were analyzed using Western blotting (10  $\mu$ g/lane). The band of actin is shown as a loading control. (B) The ASCT2 expression ratio were calculated as (the band density of ASCT2)/(the band density of actin). Each ratio represents the mean  $\pm$  SD (n=3). \*P<0.05 vs. CSS.

of Gln and Ser (Fig. 2) was almost same between parent and CRPC-like cells. This discrepancy could be attributed to the binding sites of ASCT2 in CRPC-like cells and their parent cells being saturated, regardless of the expression level, because the concentration of inhibitors (2 mM) used in this experiment was approximately 100-times the Km value of ASCT2 (Km value of Gln 23.8  $\mu$ M, Ser 18.8  $\mu$ M) [30]. We also believe that Gln partially inhibited LAT1 activity in LNCaP-SF because LAT1 recognizes Gln with low affinity (Km=1.64 mM) [31]. In the case of LNCaP-SF, ASCT2 as well as LAT1 (only slightly) may be involved in their observation because the expression of ASCT2 did not disappear completely. Similarly, in the case of LN-REC4, ASCT2 may be involved in their inhibition because ASCT2 activity was strongly inhibited by Gln/Ser because of the high concentration of the inhibitors. Furthermore, considering substrate recognition, we suggest that not only ASCT2 but also other sodium-dependent AATs, such as system N, may contribute to fluciclovine uptake in CRPC-like cells because Gln and Ser are also substrates of SNAT5 [24]. Another possible explanation for this could be that post-translational modifications of ASCT2, such as glycosylation level [32] and interaction with scaffold protein [33], may control the transport function of ASCT2. Although there are some discrepancies between our results and the characteristics of each cell line, we believe the molecular mechanisms revealed here could be beneficial for future research projects because several mechanisms including androgens, growth factors, and/or cytokines are thought to be involved in CRPC progression [5].

ASCT2 expression is upregulated in various tumors [34], and androgen regulates ASCT2 expression as well as cell growth [26]. Clinical reports suggest ASCT2 expression decreases gradually with PCa

progression from normal prostate to primary PCa, to PCa with ADT. Moreover, ASCT2 expression in CRPC is maintained as it is in PCa with ADT [27,35]. Our results support this observation: ASCT2 expression gradually decreased with increasing androgen independency, i.e. LNCaP (androgen-dependent PCa) > LN-REC4 (androgen-dependent CRPC-like) > LNCaP-SF (androgen-independent CRPC-like). In addition, [ $^{14}$ C]fluciclovine uptake correlated with ASCT2 expression in the androgen-dependent PCa and CRPC-like cells in the absence of androgen, mimicking ADT (Figs. 4 and 5). These results suggest that [ $^{18}$ F]fluciclovine-PET reflects the androgen-sensitivity of PCa and is useful for monitoring tumor response to therapy in patients with androgen-dependent PCa and in CRPC patients treated with alternative anti-androgen therapy [36] or second generation anti-androgen drugs [7,8].

Wang et al. [37] reported an inverse relationship between LAT1 and LAT3 gene expression during the progression from primary PCa to CRPC. To apply their putative scheme to our study, ASCT2 and LAT3 expression is stimulated by androgen signaling in androgen-dependent PCa, and the mammalian/mechanistic target of rapamycin (mTOR) pathway is activated by the intracellular abundance of leucine and glutamine, transported by LAT3 and ASCT2, respectively. In contrast, ADT would suppress LAT3 and ASCT2 expression in the primary PCa, leading to inactivation of mTOR due to a shortage of intracellular leucine and glutamine [37]. The activated transcription factor-4 (ATF4) then upregulates LAT1 expression, followed by restoration of intracellular leucine [37]. As a result, mTOR signaling is restored and the PCa cells recur as a CRPC. AAT expression is also related to the cancer microenvironment in interesting ways. Intratumoral cell density generally increases with tumor progression, and the transport activity of system ASC, but not of system L, decreases with increasing cell density [38]. We recently demonstrated that [ $^{18}$ F]fluciclovine accumulation is positively correlated with cell density in a rat glioma model [39]. In addition, the pH in progressive tumor tissue is comparatively acidic [40], and the transport activities of [ $^{14}$ C]fluciclovine by Na $^{+}$ -dependent AATs including system ASC are diminished, whereas the activity of Na $^{+}$ -independent AATs including system L increased under low pH conditions [24]. Thus, the contribution of Na $^{+}$ -independent AATs for [ $^{18}$ F]fluciclovine in CRPC may increase in the PCa microenvironment. As also shown in Fig. 2B, LNCaP-SF will be the more suitable CRPC model for proving the mechanism of [ $^{18}$ F]fluciclovine uptake because the contribution of LAT1 increases with increasing malignancy [21,22,37]. If our hypothesis and the proposal of Wang et al. are correct, [ $^{18}$ F]fluciclovine transport is mediated by ASCT2 during early PCa growth (e.g. androgen-dependent PCa); then, the contribution of LAT1 gradually increases after tumor progression (e.g. CRPC) because ASCT2 and LAT1 recognize fluciclovine [23–25]. If so, [ $^{18}$ F]fluciclovine-PET could be a feasible PET tracer for detecting all stages of PCa progression. Further investigation is needed to assess the clinical application of these findings in CRPC patients.



**Fig. 5.** The effect of androgen on [ $^{14}$ C]fluciclovine uptake over 5 min in PCa (LNCaP and LN-Pre) and CRPC-like (LNCaP-SF and LN-REC4) cells. Cells were incubated in steroid-free media (CSS) for 24 h, and then in CSS without DHT (white bar) or with DHT (black bar) for 72 h. Each value represents the mean  $\pm$  SD (n=8–12). \*P<0.05 vs. CSS.

## 5. Conclusion

System ASC, especially ASCT2, probably determine [<sup>18</sup>F]fluciclovine uptake into CPRC-like as well as androgen-dependent PCa cells, suggesting that [<sup>18</sup>F]fluciclovine-PET is useful for detection of CRPC as well as androgen-dependent PCa.

## Conflict of interest

Masahiro Ono, Shuntaro Oka, Hiroyuki Okudaira, and Yoshifumi Shirakami are employees of Nihon Medi-Physics Co., Ltd. Mark M. Goodman and Emory University have patent rights for [<sup>18</sup>F]fluciclovine and are eligible to receive royalties on [<sup>18</sup>F]fluciclovine from Nihon Medi-Physics Co., Ltd. David M. Schuster, Mark M. Goodman and Keiichi Kawai have an ongoing research collaboration with Nihon Medi-Physics Co., Ltd. Takeo Nakanishi, Atsushi Mizokami and Masato Kobayashi have no conflict of interest.

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