Direct comparison of 2-amino[3-^{11}C]isobutyric acid and 2-amino[^{11}C]methyl-isobutyric acid uptake in eight lung cancer xenograft models

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Received May 21, 2018; Accepted October 5, 2018

DOI: 10.3892/ijo.2018.4596

Abstract. The non-natural amino acid positron emission tomography tracers, 2-amino[3-^{11}C]isobutyric acid ([3-^{11}C]AIB) and 2-amino[^{11}C]methyl-isobutyric acid ([^{11}C]MeAIB), are metabolically stable *in vivo* and accumulate in tumors. [3-^{11}C]AIB is transported into cells mainly via the amino acid transport system A and partially via systems L and ASC, whereas [^{11}C]MeAIB is transported into cells specifically via system A. How transport via the different systems affects the tumor uptake of these tracers, however, is unclear. In the present study, the tumor uptake of the two tracers was directly compared in eight lung cancer models (A549, H82, H441, H460, H1299, H1650, PC14, and SY), and the correlation of tumor uptake with several factors (amino acid transporter expression, contribution of amino acid transport systems to AIB uptake and tumor proliferation indices) was analyzed. Biodistribution analyses revealed that the tumor uptake of [3-^{11}C]AIB (4.9 to 19.2% injected dose per gram [ID/g]) was higher than that of [^{11}C]MeAIB (3.1 to 15.9% ID/g) in all eight tumors, with a statistically significant difference in three tumors ($P<0.01$ in H441 and H460 tumors, $P<0.05$ in H82 tumors). A significant correlation was observed between the tumor uptake of the two tracers ($r=0.95$, $P<0.01$). The mRNA expression levels of the amino acid transporters of system A (SLC38A1 and SLC38A2), system L (SLC7A5) and system ASC (SLC1A5) were higher in all eight tumors than in the normal lung, with widely varying expression patterns. Although the contributions of the amino acid transport systems, Ki-67 indices and tumor doubling times greatly differed among the eight models, these factors did not correlate with the tumor uptake of either tracer. The higher tumor uptake of [3-^{11}C]AIB and the correlation of tumor uptake between [3-^{11}C]AIB and [^{11}C]MeAIB warrant further investigation in clinical studies in order to clarify the role of [3-^{11}C]AIB PET in oncology imaging.

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Key words: 2-aminoisobutyric acid, 2-(methylamino)isobutyric acid, positron emission tomography, imaging, lung cancer, amino acid transporter

Introduction

Positron emission tomography (PET) provides functional information of regions of interest and is widely used in oncology, for staging, prognosis and evaluating therapeutic efficacy (1). PET can reveal a therapeutic response before a change in tumor size is detected by morphological imaging modes, such as computed tomography and magnetic resonance imaging (2). ^{18}F-labeled fluorodeoxyglucose ([^{18}F]FDG) PET is most commonly used to assess therapeutic efficacy in clinical practice, despite having several limitations; e.g., [^{18}F]FDG also accumulates in areas of inflammation induced by anticancer therapy (3). Several attempts have been made to develop a new PET probe to compensate for the limitations of [^{18}F]FDG (3). An amino acid-based PET probe is a promising candidate as amino acid transporters are upregulated in many types of cancer, but are limited in inflammatory cells (3). ^{11}C-labeled methyl-L-methionine ([^{11}C]Met) is used most often among amino acid-based PET probes and accumulates less in areas of inflammation than [^{18}F]FDG (3). Therefore, [^{11}C]Met is useful for accurately evaluating therapeutic efficacy. In clinical practice, the application of [^{11}C]Met is limited to brain tumor imaging, however, due to its instability *in vivo* and its high accumulation in several healthy organs (4). Therefore, there is a strong need to develop novel amino acid-based PET tracers that are stable *in vivo*.

Non-natural amino acids, 2-aminoisobutyric acid (AIB) and its derivative 2-(methylamino)isobutyric acid (MeAIB), are metabolically stable *in vivo* and their ^{14}C-labeled analogs accumulate in tumors (5,6). AIB and MeAIB are transported

mainly by amino acid transport system A, in contrast to [^{11}C]Met, which is transported via system L (7). Therefore, these positron-labeled analogs are expected to play a different role than [^{11}C]Met in oncology imaging.

^{11}C-labeled AIB was synthesized as [1-^{11}C]AIB (8), and its usefulness has been demonstrated in patients with melanoma and soft tissue sarcomas (5,9,10). The clinical applications of [1-^{11}C]AIB are quite limited, however, as the labeling procedure for [1-^{11}C]AIB is complex and inefficient (11). Recently, a simple and efficient synthetic method for [3-^{11}C]AIB was established in our previous study, in which C-11 is located at the 3-position instead of the 1-position (12). Although [3-^{11}C]AIB has not yet been clinically evaluated, several preclinical studies have demonstrated that [3-^{11}C]AIB highly accumulates in tumors, but much less so in inflammatory lesions, and is useful for evaluating early metabolic changes following radiation therapy in mouse models (13-15). The low uptake of [3-^{11}C]AIB in the lung (13-15) suggests the potential usefulness of [3-^{11}C]AIB for clinical application in lung cancer diagnostic imaging.

A synthetic method for [^{11}C]MeAIB was also established, and several clinical studies have reported that a high uptake of [^{11}C]MeAIB is observed in patients with lung cancer, head and neck cancer and lymphoma (6,16,17,18). [^{11}C]MeAIB has higher specificity than [^{18}F]FDG for differentiating between benign and malignant chest diseases (18).

Both AIB and MeAIB are transported into cells by the amino acid transport system A and have similar affinities for system A (19). In contrast to MeAIB, which is a highly selective substrate of system A, AIB is also partially transported by systems L and ASC (7). These findings suggest that the additional contribution of systems L and ASC may increase the tumor uptake of [3-^{11}C]AIB compared with [^{11}C]MeAIB, but no direct comparisons of tumor uptake between these two PET probes have been reported. In the present study, the tumor uptake of [3-^{11}C]AIB and [^{11}C]MeAIB in eight lung cancer models was compared, and possible correlations of tumor uptake with several factors, such as the expression of amino acid transporters associated with systems A, L and ASC, and the contribution of the amino acid transport systems to AIB uptake and tumor proliferation indices, were explored.

Materials and methods

Cell culture. Six human lung cancer cell lines A549 (CCL-185), H82 (HTB-175), H441 (HTB-174), H460 (HTB-177), H1299 (CRL-5803) and H1650 (CRL-5883) were obtained from ATCC (Manassas, VA, USA). SY was obtained from Immuno-Biological Laboratories (Takasaki, Japan) and PC14 (RCB0446) was obtained from RIKEN Cell Bank (Tsukuba, Japan). The cells were maintained in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) in a humidified incubator maintained at 37˚C with 5% CO_2.

Tumor models. The protocols used for the animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences (Chiba, Japan), and all animal experiments were conducted in accordance with the institutional guidelines regarding animal care and handling. Mice (BALB/c-nu/nu, 6 weeks old) were obtained from CLEA Japan (Tokyo, Japan) and weighed 18-20 g. The animals were maintained in an environment with a controlled temperature and humidity, under a 12 h-light/dark cycle. The animals were provided with food and water ad libitum. The human lung cancer cells were subcutaneously inoculated into both shoulders of male nude mice (12 mice/each model) under isoflurane anesthesia (1.5%). We employed mice in which subcutaneous tumors reached a diameter of approximately 9 mm, and the maximum tumor diameter was 12 mm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from subcutaneous tumors was purified using an RNeasy Plus Universal Mini kit (Qiagen, Hilden, Germany). Human lung total RNA was purchased from Clontech (Mountain View, CA, USA). First-strand cDNA was synthesized using a SuperScript First-Strand Synthesis system for RT-PCR (Life Technologies, Carlsbad, CA, USA). Real-time (quantitative) PCR (qPCR) was conducted in triplicate with TaqMan probes to detect amino acid transporters (SLC38A1, Hs01562168_m1; SLC38A2, Hs00255854_m1; SLC38A4, Hs00215989_m1; SLC1A5, Hs01056542_m1 and SLC7A5, Hs00185826_m1) and 18S rRNA (4319413E, Thermo Fisher Scientific, Waltham, MA, USA) using Premix Ex Taq reagent (Takara Bio, Otsu, Japan) and Mx3000P qPCR systems (Agilent Technologies, Santa Clara, CA, USA). Gene expression levels of the amino acid transporters were normalized to 18S rRNA expression in each sample.

PET imaging. [3-^{11}C]AIB was synthesized by combining iodo[^{11}C]methane and methyl *N*-(diphenylmethylene)-D,L-alaninate in the presence of tetrabutylammonium fluoride, followed by alkaline hydrolysis and acidic deprotection, as described previously (12). [^{11}C]MeAIB was synthesized by methylating AIB methyl ester hydrochloride using [^{11}C]methyl triflate in the presence of 1,2,2,6,6-pentamethyl-piperidine, followed by alkaline hydrolysis, as previously described (16). The 10-min PET scans of tumor-bearing mice (n=1 per tumor model and per tracer) began at 30 min after the intravenous injection of approximately 12 MBq of [3-^{11}C]AIB or [^{11}C]MeAIB in saline using a small-animal PET system (Inveon, Siemens Medical Solutions, Malvern, PA, USA) under isoflurane anesthesia (1.5%). Body temperature was maintained at ~37˚C with a heat lamp and a heated bed during scans. Images were reconstructed using a 3D maximum *a posteriori* (18 iterations with 16 subsets, β=0.2) without attenuation correction.

Biodistribution. Approximately 12 MBq of [^{11}C]AIB or [^{11}C]MeAIB in saline were administered to tumor-bearing mice via the tail vein. At 30 min post-injection, the mice were euthanized by isoflurane inhalation, and tumors, blood and major organs were removed and weighed. The radioactivity was measured with an auto-well gamma counter (Perkin-Elmer, Waltham, MA, USA). Each mouse had two tumors (one in each shoulder), and two mice were injected for each tumor model (two with each PET tracer); i.e., 4 data, tumor uptake of each tracer for each tumor model; and 16 data, organ uptake of each tracer, were collected. Data are expressed as the percentage of injected dose per gram wet tissue weight (% ID/g).

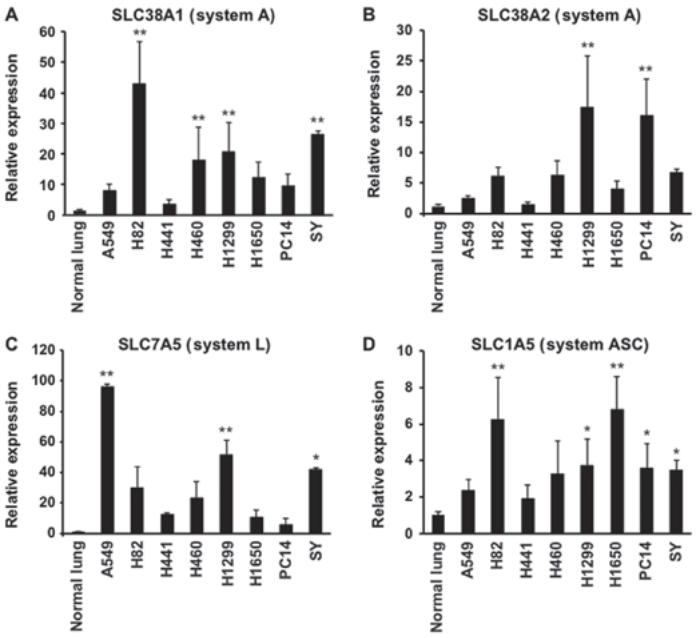

Figure 1. The mRNA expression of the amino acid transporters, (A) SLC38A1, (B) SLC38A2, (C) SLC7A5, and (D) SLC1A5, in eight xenograft tumors and normal lung. Data represent the means ± standard deviation (SD) from three independent experiments. *P<0.05 and **P<0.01 vs. normal lung analyzed by ANOVA with the Dunnett's multiple comparison test.

Contribution of amino acid transport systems to AIB uptake in vitro. The cultured cells were rinsed three times with Dulbecco's phosphate-buffered saline (PBS) containing 5 mM $MgCl_2$ and 9 mM $CaCl_2$ (PBS) or choline phosphate buffer (137 mM choline-Cl, 2.7 mM KCl, 8.1 mM K_2HPO_4, 1.47 mM KH_2PO4, 5 mM $MgCl_2$ and 9 mM $CaCl_2$; pH 7.4). As competitive inhibitors, 10 mM 2-amino-2-norbornanecarboxylic acid (BCH) and 10 mM MeAIB were used. The cells washed with PBS or choline phosphate buffer were incubated with 92.5 kBq of [^{14}C]AIB (American Radiolabeled Chemicals, St. Louis, MO, USA) in 500 μl PBS or choline phosphate buffer and incubated for 15 min at 37˚C. Following incubation, the cells were washed three times with ice-cold PBS or ice-cold choline phosphate buffer and lysed in 50 mM NaOH aqueous solution. The radioactivity was measured with a liquid scintillation counter (Perkin-Elmer). The total protein concentration in the cell lysate was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Each experiment was conducted in triplicate. The contribution of amino acid transport systems A, L and ASC to [^{14}C]AIB uptake was calculated as previously described (20). Briefly, the total cell uptake of [^{14}C]AIB was divided into the contribution of each system as follows: System A, the portion of the sodium-dependent uptake that was inhibited by MeAIB; system ASC, the portion of the sodium-dependent uptake that was not inhibited by MeAIB; system L, sodium-independent uptake that was inhibited by BCH; and the non-saturable fraction, the remaining uptake. Data are expressed as a percentage of the total uptake and three independent experiments were conducted.

Ki-67 immunohistochemical staining. As a separate experiment, mice were subcutaneously injected with human lung cancer cells (3 tumors/each tumor model). Subcutaneous tumors were resected from mice and fixed in 10% neutral-buffered formalin and embedded in paraffin. The tumor sections (1-μm-thick) were deparaffinized and stained with mouse anti-Ki-67 antibody (MIB-1, Dako Cytomation, Glostrup, Denmark) at a dilution of 1:100, as previously described (21). The Ki-67 index (%) was calculated by counting the percentage of Ki-67-positive cell nuclei per 2,500-6,000 cells in 5 regions with a high density of stained cells.

Tumor doubling time. Subcutaneous tumor size (n=8/tumor model) was measured twice a week using a caliper. Tumor volume was calculated according to the following formula: Tumor volume (mm^3) = (length x $width^2$)/2. Tumor volume doubling time during the logarithmic growth phase was calculated according to the following formula: Doubling time (days) = $t\ln 2/\ln(V_1/V_0)$, where t is the time interval between the initial and second measurement, V_0 is the tumor volume at the initial measurement and V_1 is the tumor volume at the second measurement (22).

Statistical analysis. The uptake of [3-^{11}C]AIB and [^{11}C]MeAIB was analyzed by a Student's t-test. The mRNA expression levels in normal lung and tumors were analyzed by ANOVA with Dunnett's multiple comparison test. The correlation among tumor uptake and potential factors was examined using Pearson's correlation coefficient (r). A value of P<0.05 was considered to indicate a statistically significant difference.

Results

mRNA expression of amino acid transporters in xenograft tumors. The mRNA expression levels of amino acid transporters of system A (SLC38A1, SLC38A2 and SLC38A4), system L (SLC7A5) and system ASC (SLC1A5) in xenograft tumors were determined by RT-qPCR. The SLC38A1, SLC38A2, SLC7A5 and SLC1A5 mRNA levels were detected and upregulated in all eight types of tumors (Fig. 1A-D),

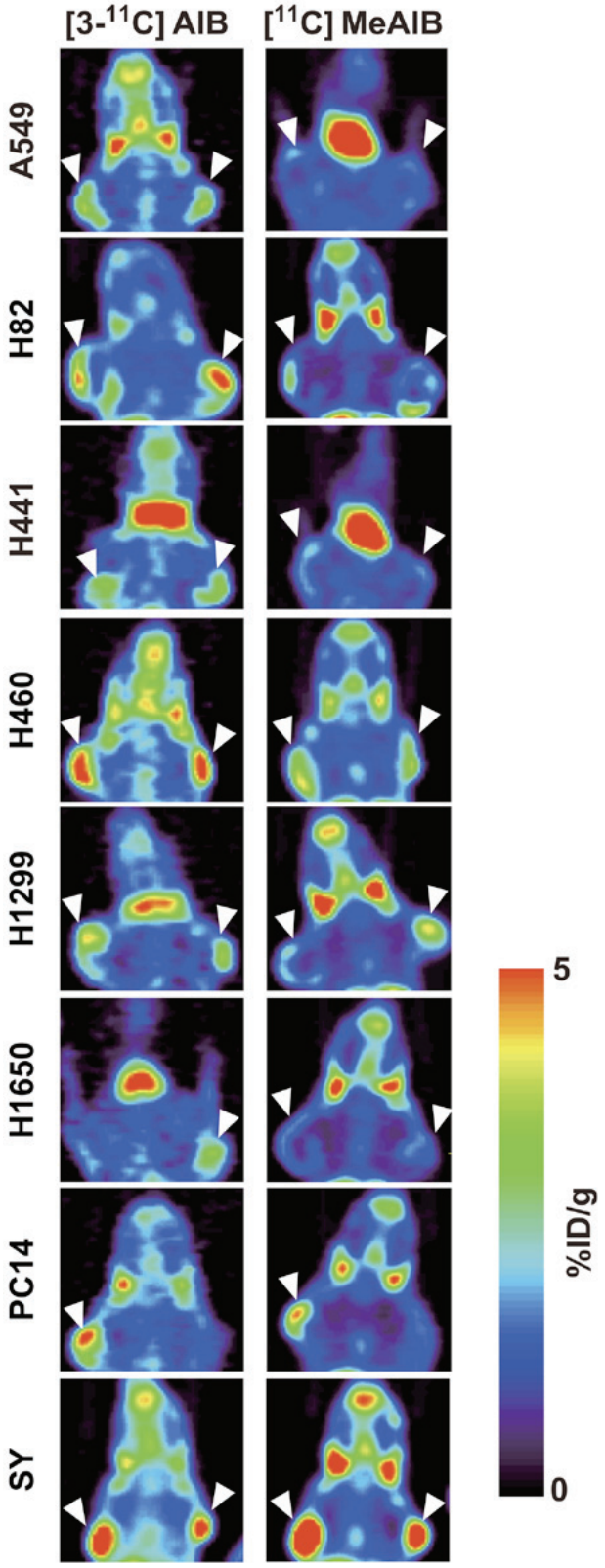

Figure 2. Representative coronal images of PET with [3-^{11}C]AIB and [^{11}C]MeAIB at 30-40 min after intravenous injection in tumor-bearing mice. Arrowheads indicate tumors.

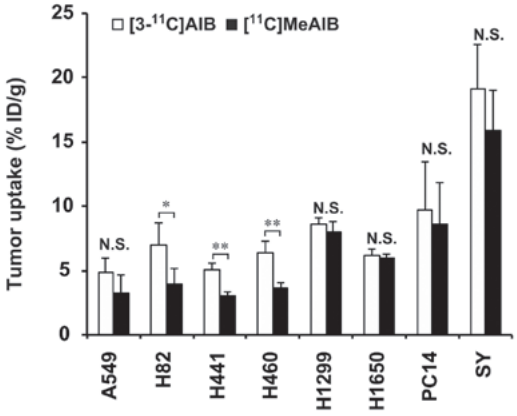

Figure 3. Tumor uptake of [3-^{11}C]AIB and [^{11}C]MeAIB in tumor-bearing mice. Data represent the means ± SD (n=4). *P<0.05 and **P<0.01 analyzed by the Student's t-test. N.S., not significant.

whereas SLC38A4 mRNA was not detected in normal lung or in any of the tumors (data not shown). The SLC38A1 and SLC38A2 expression levels in the tumors were 3.6- to 42.8-fold and 1.4- to 17.3-fold higher than those in the normal lung, respectively (Fig. 1A and B). The expression of SLC38A1 in the H82, H460, H1299 and SY tumors, and that of SLC38A2 in the H1299 and PC14 tumors was significantly higher than that in the normal lung (P<0.01, Fig. 1A and B). The expression levels of SLC7A5 were 5.8- to 95.9-fold higher in the tumors than in the normal lung, with significant differences between A549, H1299 or SY and the normal lung (P<0.01 in the A549 and H1299 tumors, and P<0.05 in the SY tumors, Fig. 1C). The expression levels of SLC1A5 were 1.9- to 6.8-fold higher than those in the normal lung, with significant differences obtained between the H82, H1299, H1650, PC14 or SY tumors and the normal lung (P<0.01 in H82 and H1650 tumors; P<0.05 in H1299, PC14 and SY tumors; Fig. 1D). The eight xenograft tumors exhibited various expression patterns of the amino acid transporters involved in AIB and MeAIB uptake.

PET imaging with [3-^{11}C]AIB and [^{11}C]MeAIB in tumor-bearing mice. Coronal images revealed a higher uptake of [3-^{11}C]AIB than of [^{11}C]MeAIB in the eight tumors (Fig. 2). Both tracers accumulated at low levels in the lungs and at high levels in the salivary glands, consistent with previous reports (6,13,23).

Biodistribution of [3-^{11}C]AIB and [^{11}C]MeAIB in tumor-bearing mice. The mean tumor uptake of [3-^{11}C]AIB at 30 min after injection ranged from 4.9 to 19.2% ID/g, and that of [^{11}C]MeAIB ranged from 3.1 to 15.9% ID/g in the eight lung cancer mouse models (n=4/tracer, Fig. 3). The tumor uptake of [3-^{11}C]AIB in the H82, H441 and H460 tumors was significantly higher than that of [^{11}C]MeAIB (P<0.05 in the H82 tumors, and P<0.01 in the H441 and H460 tumors, Fig. 3). Although the difference was not statistically significant, [3-^{11}C]AIB uptake in the other tumors was higher than that of [^{11}C]MeAIB in the corresponding tumors (Fig. 3). [3-^{11}C]AIB uptake in the blood, lung, pancreas, kidney and muscle was significantly higher than that of [^{11}C]MeAIB (n=16/tracer, Table I).

Contribution of amino acid transport systems to AIB cell uptake. As the transport of AIB into cells has been reported to occur via amino acid transport systems A, L and ASC (7), the contribution of each system to uptake in the eight different

Table I. Normal organ uptake (% ID/g) of [3-^{11}C]AIB and [^{11}C]MeAIB at 30 min after intravenous injection in tumor-bearing mice.

Organ	[3-^{11}C]AIB (n=16)	[^{11}C]MeAIB (n=16)
Blood	2.27±0.32	1.48±0.18[a]
Lung	4.38±0.39	3.19±0.52[a]
Liver	12.76±2.42	11.97±2.05
Spleen	11.23±1.18	12.05±2.50
Pancreas	62.87±7.10	43.85±6.29[a]
Intestine	7.77±1.61	6.74±1.64
Kidney	19.62±4.48	14.88±3.07[a]
Muscle	1.26±0.20	1.01±0.28[a]

[a]P<0.01 vs. [3-^{11}C]AIB.

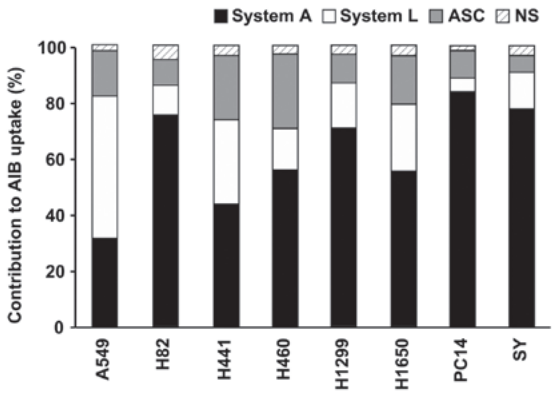

Figure 4. Contributions of each amino acid transport system to AIB uptake *in vitro*. Data represent percentage of the total [^{14}C]AIB uptake. Data show the means from three independent experiments. NS, non-saturable fraction.

types of lung cancer cells was evaluated. The contributions ranged from 31 to 84% for system A, from 5 to 50% for system L and from 9 to 27% for system ASC (Fig. 4). In the seven cell lines other than A549, the contribution of system A was higher than that of system L, whereas in the A549 cells, the contribution of system L was 1.6-fold higher than that of system A (Fig. 4).

Tumor proliferation. To evaluate the association between tumor proliferation and the tumor uptake of each tracer, the Ki-67 index based on tumor sections immunostained with the anti-Ki-67 antibody and tumor doubling time by measuring tumor size in the eight tumor models were determined. The Ki-67 index was 14.0 to 62.1% (Fig. 5A), and the doubling time during the logarithmic growth phase was 2.4 to 5.5 days (Fig. 5B).

Correlation analysis. The tumor uptake of [3-^{11}C]AIB significantly and positively correlated with that of [^{11}C]MeAIB in the eight tumor models (r=0.95, P<0.01, Fig. 6 and Table II). The tumor uptakes of [3-^{11}C]AIB and [^{11}C]MeAIB were quantified in PET images, and there were significant correlations of tumor uptake in the PET analyses with that in the biodistribution

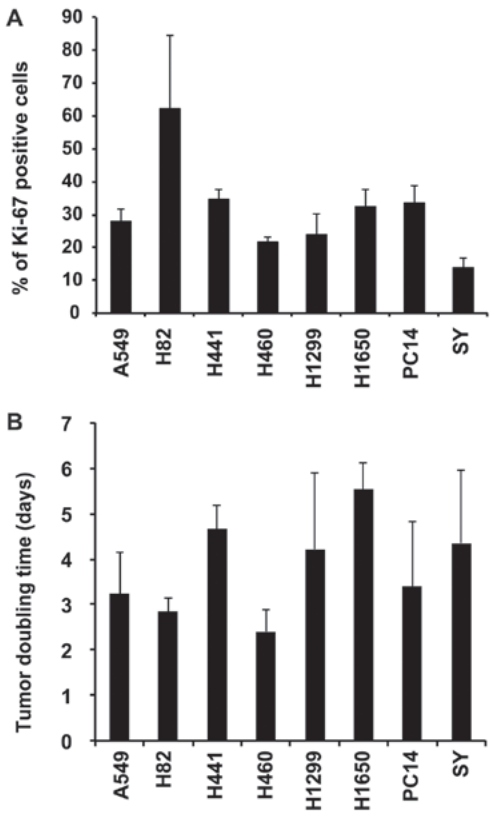

Figure 5. (A) Ki-67 index (%) in eight xenograft tumors. Data represent the means ± SD (n=3). (B) Tumor volume doubling time in eight xenograft tumors. Data represent the means ± SD (n=8).

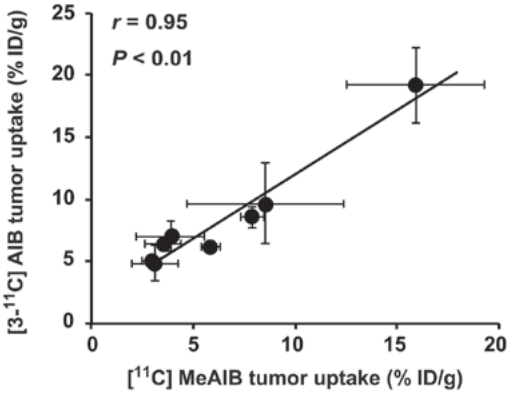

Figure 6. Correlation analysis between [3-^{11}C]AIB and [^{11}C]MeAIB tumor uptake.

analyses (r=0.69, P<0.05 for [3-^{11}C]AIB; r=0.81, P<0.05 for [^{11}C]MeAIB). The expression of the individual amino acid transporter mRNAs did not correlate with the uptake of [3-^{11}C]AIB or that of [^{11}C]MeAIB (Table II). The contribution of system A to uptake significantly and positively correlated with the expression of SLC38A2 mRNA (r=0.71, P<0.05, Table II), whereas there no significant correlation was observed between the contributions of the other systems to uptake and the mRNA expression of the other amino acid transporters, or between the contributions of the other systems and tumor uptake (Table II). The proliferation indices (Ki-67 index and the tumor doubling time) did not correlate with tumor uptake (Table II).

Table II. Pearson's correlation coefficients (*r*) among tumor uptake ([3-^{11}C]AIB and [^{11}C]MeAIB) and potential factors.

	Tumor uptake		Amino acid transporter expression				Contribution of amino acid transport system			Proliferation indices	
	[3-^{11}C] AIB	[^{11}C] MeAIB	SLC 38A1	SLC 38A2	SLC 7A5	SLC 1A5	System A	System L	System ASC	Ki-67 index	Tumor doubling time
[3-^{11}C]AIB	1.00	**0.95**[a]	0.35	0.28	-0.01	-0.02	0.63	-0.46	-0.69	-0.45	0.14
[^{11}C]MeAIB		1.00	0.21	0.36	-0.04	0.03	0.65	-0.44	-0.69	-0.50	0.29
SLC38A1			1.00	NA	NA	NA	0.55	NA	NA	0.44	-0.34
SLC38A2				1.00	NA	NA	**0.71**[b]	NA	NA	-0.13	-0.15
SLC7A5					1.00	NA	NA	0.67	NA	-0.22	-0.24
SLC1A5						1.00	NA	NA	-0.25	0.51	0.21
System A							1.00	NA	NA	NA	NA
System L								1.00	NA	NA	NA
System ASC									1.00	NA	NA
Ki-67 index										1.00	0.22
Tumor doubling time											1.00

Significant correlations ([a]P<0.01 and [b]P<0.05) are highlighted in bold font. NA, not applicable.

Discussion

Non-natural amino acid PET tracers [3-^{11}C]AIB and [^{11}C]MeAIB are expected to play a new role in oncology imaging that differs from that of [^{11}C]Met, which is currently the most widely used amino acid PET tracer in clinical practice (5-7,13). MeAIB is transported by amino acid transport system A, whereas AIB is transported by systems L and ASC in addition to system A. How the difference in transport affects tumor uptake of these tracers is unclear, however, as there have been no direct comparisons of the uptake of the two PET probes. In the present study, tumor uptake of the two tracers was directly compared in eight lung cancer models, and possible correlations of the tumor uptake with several factors were analyzed.

First, the mRNA expression levels of amino acid transporters associated with uptake of AIB and MeAIB in xenograft tumors derived from eight lung cancer cell lines (A549, H82, H441, H460, H1299, H1650, PC14, and SY) were determined. The mRNAs involved in system A (SLC38A1 and SLC38A2), system L (SLC7A5) and system ASC (SLC1A5) were overexpressed in all the tumors compared with normal lung, with the amplitude of overexpression varying among the eight tumors, indicating that these tumor models are suitable for evaluating differences in tumor uptake of [3-^{11}C]AIB and [^{11}C]MeAIB.

Next, PET and biodistribution analyses with [3-^{11}C]AIB and [^{11}C]MeAIB were conducted in the eight tumor models. PET images revealed a high accumulation of [3-^{11}C]AIB in all eight tumors. Moreover, the tumor uptake of [3-^{11}C]AIB was higher than that of [^{11}C]MeAIB. The biodistribution experiments of [3-^{11}C]AIB and [^{11}C]MeAIB confirmed these findings, i.e., the tumor uptake of [3-^{11}C]AIB was higher than that of [^{11}C]MeAIB, and the differences in uptake were statistically significant in three tumors (P<0.01 in H441 and H460 tumors, and P<0.05 in H82 tumors). Several clinical studies of [^{11}C]MeAIB PET have reported high tumor uptake in patients (6,17) and high specificity in the differential diagnosis between benign and malignant disease (18). In the present study, tumor uptake between the two tracers was significantly correlated (r=0.94, P<0.01). Taken together, [3-^{11}C]AIB PET is expected to visualize tumors in patients more efficiently than [^{11}C]MeAIB PET and to be useful for imaging malignancies.

We hypothesized that additional contributions of systems L and ASC to the uptake of AIB may increase AIB uptake in tumors expressing systems A, L and ASC. Therefore, several correlation analyses were conducted to find factor(s) associated with the observed difference in tumor uptake between [3-^{11}C]AIB and [^{11}C]MeAIB. There is no correlation between tumor uptake and the expression of amino acid transporters. Kagawa *et al* reported that MeAIB cell uptake correlated with the expression of amino acid transport system A (24). This discrepancy could be due to the different cell sets used [they used A431, LS180, H441 and PC14 (24)] and the difference between the *in vitro* cellular uptake (24) and *in vivo* tumor uptake (the present study). Next, a correlation analysis was performed between tumor uptake and the contribution of each amino acid transport system (A, L, and ASC) to AIB uptake. As mentioned above, AIB is transported via systems L and ASC in addition to system A, and the contributions to uptake by these systems in the eight tumor cell lines were determined and compared with the *in vivo* tumor uptake. There was no correlation between the contribution of each system and tumor uptake. Of note, the contribution of each transport system substantially varied among cell lines, and the contribution of system A was not large in several tumors, particularly in A549 tumors, in which the contribution of system A was only 31.4% and that of system L was 50.0%. To our knowledge, although

there are many reports that AIB is transported mainly via system A (20,25,26), there is no report that some tumor cells transport it mainly via the other amino acid transport system(s). On the other hand, tumor uptake was significantly correlated between [3-^{11}C]AIB and [^{11}C]MeAIB, as shown in the present biodistribution study. These findings suggest that the injected amount of [3-^{11}C]AIB for the *in vivo* study (PET and biodistribution) was very small and the affinity of AIB to system A is higher than that to the other systems; therefore, AIB would be transported into tumors *in vivo* mainly via system A. In other words, the difference in the contributions of the transport systems would produce no prominent differences in tumor uptake *in vivo*. Finally, a correlation analysis of tumor uptake and proliferation indices was conducted as some studies have reported that upregulation of amino acid transport system A is associated with prognosis in several tumors (27,28) and expression of the system A transporter SLC38A1 correlates with the cell proliferation index Ki-67 in breast cancer (28). Although the Ki-67 index and tumor doubling time in the eight xenograft tumors were determined in the eight models, these proliferation indices were not correlated with the tumor uptake of the two PET tracers. Unfortunately, we cannot provide direct evidence to address the difference of AIB and MeAIB tumor uptake in the present study. There are several potential reasons: i) Blood flow and/or blood volume (29,30); ii) variations in amino acid metabolism (31-33); iii) alterations in transporter activity (31-33); and iv) differences in the expression levels between mRNA and protein (34). In general, xenograft tumor models cannot completely reproduce the characteristics of human cancer, and system A activity is reported to change with the levels of intracellular amino acid pools, cell density, cell division and hormonal stimulation (31-33). These potential explanations could account for the lack of correlation detected, but clinical PET studies are needed to clarify these points.

Although the present study showed the potential of PET with [3-^{11}C]AIB for lung cancer diagnosis, there are several limitations as follows: First, the PET analyses were conducted only a mouse per tumor model. However, there are significant correlations of tumor uptake in the PET analyses with that in the biodistribution studies (n=4/tumor model), supporting the results of PET analyses. To provide additional evidence of our results, there is a need for further PET analyses with a large number. Second, the present study employed only male mice since lung cancer is more common in men compared with women (35). However, lung cancer develops also in women (35), and further studies with female animals are required before clinical studies.

In conclusion, the direct comparison of the tumor uptake of [3-^{11}C]AIB and [^{11}C]MeAIB in eight lung cancer xenograft models revealed greater accumulation of [3-^{11}C]AIB than [^{11}C]MeAIB in all eight tumors. Although the amino acid transporter expression levels, the contribution of transport systems to AIB uptake, and proliferation indices showed substantial variations among the eight models, tumor uptake was not correlated with those factors. The higher tumor uptake of [3-^{11}C]AIB and the correlation of tumor uptake between [3-^{11}C]AIB and [^{11}C]MeAIB warrant further investigation in clinical studies to clarify a role of [3-^{11}C]AIB PET in oncology imaging.

Acknowledgements

The authors would like to thank the staff of the Cyclotron Operation section for producing the radioisotope; Mr. Yusuke Kurihara, Mr. Masanao Ogawa and Mr. Nobuki Nengaki for the technical support for the PET tracer synthesis; Mr. Hidekatsu Wakizaka for operation and quality control of the animal PET system; and Ms. Yuriko Ogawa and Ms. Naoko Kuroda for technical assistance with the animal experiments.

Funding

This study was supported by KAKENHI (15K09976 and 16K10304), AMED-CREST, and the National Institutes for Quantum and Radiological Science and Technology.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HS was involved in the data design, data collection, data analysis and interpretation, and in the writing of the manuscript. ABT was involved in research design, data design, data collection, data analysis and interpretation, and in the writing of the manuscript. AS was involved in data collection. MO was involved in data interpretation and PET tracer production. KK was involved in data interpretation and in the writing of the manuscript. MRZ was involved in data interpretation. TS was involved in data interpretation and in the writing of the manuscript. TH was involved in data interpretation and in the writing of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The protocols used for the animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences (Chiba, Japan), and all animal experiments were conducted in accordance with the institutional guidelines regarding animal care and handling.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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