

RESEARCH ARTICLE

Synthesis and Preliminary Evaluation of 5-¹⁸F]fluoroleucine

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Abstract: Background: Amino acid transporters, such as LAT1, are overexpressed in aggressive prostate and breast carcinomas, directly influencing pathways of growth and proliferation.

Objective: The purpose of this study was to synthesize and characterize a novel ¹⁸F labeled leucine analog, 5-¹⁸F]fluoroleucine, as a potential imaging agent for aggressive tumors which may not be amenable to imaging by FDG PET.

Methods: 5-fluoroleucine was synthesized and characterized, and its ¹⁸F-labeled analog was synthesized from a mesylate precursor. First, breast cancer cell line assays were performed to evaluate uptake of ³H- or ¹⁴C-labeled L-leucine and other essential amino acids. Both L-leucine and 5-¹⁸F]fluoroleucine were tested for uptake and accumulation over time, and for uptake *via* LAT1. Biodistribution studies were performed to estimate radiation dosimetry for human studies. Small animal PET / CT studies of a breast cancer were performed to evaluate *in vivo* 5-¹⁸F]fluoroleucine tumor uptake.

Results: Breast cancer cell lines showed increasing high net accumulation of L-¹⁴C]leucine. Both L-leucine and 5-¹⁸F]fluoroleucine showed increasing uptake over time in *in vitro* tumor cell assays, and uptake was also shown to occur *via* LAT1. The biodistribution study of 5-¹⁸F]fluoroleucine showed rapid renal excretion, no significant *in vivo* metabolism, and acceptable dosimetry for use in humans. *In vivo* small animal PET / CT imaging of a breast cancer xenograft showed uptake of 5-¹⁸F]fluoroleucine in the tumor, which progressively increased over time.

Conclusion: 5-¹⁸F]fluoroleucine is a leucine analog which may be useful in identifying tumors with high or upregulated expression of amino acid transporters, providing additional information that may not be provided by FDG PET.

Keywords: Leucine, fluoroleucine, amino acid, tumor imaging, positron emission tomography, dosimetry.

INTRODUCTION

PET imaging with 2-fluoro-2-deoxyglucose (FDG) has established utility in identifying a wide number of malignancies, and in evaluating response to novel therapies in several tumor types. Several tumors, however, do not show high glycolytic activity, and may overexpress alternative metabolic pathways to support growth and altered metabolism. Upregulation of amino acid transport may be an alternative method to detect tumors and provide targets for novel therapies, irrespective of glycolytic activity. Imaging alternative metabolic pathways, including essential amino acid upregulation, has demonstrated clinical utility for several amino acid analogs [1, 2].

Recent studies have demonstrated the links between amino acid tumor metabolism with growth, proliferation and

survival [3-5]. Essential branched chain amino acids have shown a direct influence on critical growth and survival pathways through several mechanisms. Specifically, the essential amino acid L-leucine can directly stimulate the growth and survival of a tumor through activation of the critical mTORC1 pathway [3, 5]. Inhibiting essential amino acid uptake through targeting amino acid transporters, thereby inhibiting leucine uptake, may offer a new therapeutic opportunity for the treatment of cancers by diminishing tumor growth and metastasis through inhibition of M-phase cell cycle and mTORC1 signaling pathways [4]. Several studies have shown that aggressive, therapy resistant cancers with worse prognosis are associated with overexpression of amino acid transporters such as LAT1 [6-10]. A recent review article has concisely summarized the rationale for targeting the LAT transporter in cancer [11].

To assess the essential amino acid uptake in tumors, and to image tumors overexpressing amino acid transporters that cannot be imaged by FDG PET, we developed and validated a novel radiolabeled leucine analog, 5-¹⁸F]fluoroleucine,

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and assessed the feasibility of its translational use in pre-clinical studies. First, to determine the feasibility of imaging using an L-leucine analog we assessed the uptake of [^{14}C]leucine in breast cancer cells in comparison to several other essential amino acids. The novel leucine analog, 5- ^{18}F fluoroleucine, was subsequently developed and assessed for its uptake in tumor cell lines. Uptake of 5- ^{18}F fluoroleucine was at least partially LAT-mediated, as determined by an established LAT1 inhibitor and a competitive LAT1 substrate. The uptake of 5- ^{18}F fluoroleucine was also compared to that of L- ^{14}C leucine uptake in these cell lines. Further, biodistribution of 5- ^{18}F fluoroleucine was performed in normal mice to determine radiation dosimetry and assess translational potential. Finally, 5- ^{18}F fluoroleucine was evaluated *in vivo* by small animal PET / CT to assess feasibility of tumor detection.

MATERIALS AND METHODS

General

All chemicals were purchased from Sigma-Aldrich unless otherwise specified. Amino acids labeled with ^3H and ^{14}C were obtained from Perkin-Elmer (Waltham, MA). Aluminum-backed sheets (Silica gel 60 F254) used for analytical TLC and silica gel 60 for normal-phase column chromatography were obtained from EM Science (Gibbstown, NJ). Preparative thick layer chromatography was used for small-scale purification with plates obtained from Whatman (Clifton, NJ) or EM Science. High pressure liquid chromatography (HPLC) was performed using a Beckman Gold HPLC system equipped with a Model 126 programmable solvent module, a Model 166 NM variable wavelength detector, a Model 170 radioisotope detector and a Beckman System Gold remote interface module SS420X; data were acquired using the 32 Karat[®] software (Beckman Coulter, Inc., Brea, CA). Reversed-phase HPLC was performed using a Waters 4.6 \times 250-mm XTerra RP18 (5 μm) column. Radio TLC sheets were analyzed by cutting the sheet into small strips and counting them for ^{18}F activity in an automated gamma counter (LKB 1282, Wallac, Finland or Perkin Elmer Wizard II, Shelton, CT). Proton NMR spectra were obtained on a Varian 400 NMR spectrometer (Palo Alto, CA).

Uptake of Essential Amino Acids in Breast Cancer Cell Lines

Uptake of amino acids radiolabeled with ^3H or ^{14}C in breast cancer cell lines MDA-MB-231, MCF7 and SKBr3 was determined. Briefly, tumor cells (200,000 cells/well/ 2 ml medium) were plated and incubated overnight at 37 $^\circ\text{C}$. Cell culture supernatants were aspirated and the cells were washed with PBS, and resuspended in 2 ml of Hanks media. Cells were then incubated with the radiotracer {3.7 kBq (100nCi)/10 μL } at 37 $^\circ\text{C}$ for 1 h. At the end of the incubation period, cells were washed with PBS, lysed by incubating with 1.0 ml of 0.5N NaOH for 1 hour, and mixed with scintillation fluid (Ecolite, MP Biomedical). Radioactivity in the lysate was measured using a beta counter (LKB, 1282, Wallac, Finland) by counting for 1 min. Results were expressed as the percent of input radioactivity that was associated with the cells. Another experiment was performed to determine the uptake at various times ranging from 5 min to

3 hours of incubation. This was performed for our lead candidates (leucine and phenylalanine) to examine feasibility of delayed imaging. A minimum of 3 samples were used for all conditions. ^3H -FDG uptake in several breast cancer cell lines was relatively low compared to essential amino acid uptake (<3% in all cases; data not shown), likely due to glucose containing media; thus, a direct comparison with 5- ^{18}F fluoroleucine was not performed.

Synthesis of Unlabeled 5-fluoroleucine

This compound and the mesylate precursor **7** were synthesized essentially following a procedure reported for synthesis of (2*S*,4*S*)- and (2*S*,4*R*)-5-fluoroleucine and (2*S*,4*S*)-[5,5- $^2\text{H}_2$]-5-fluoroleucine [12].

Synthesis of 5- ^{18}F fluoroleucine

Fluorine-18 was obtained either by in house cyclotron irradiation of [^{18}O]H $_2\text{O}$ or from PET-NET solutions (Durham, NC). For labeling reactions, ^{18}F activity trapped in a QMA cartridge (Waters, Milford, MA) was eluted with a mixture of Kryptofix (28 mg) and potassium carbonate (2.4 mg) in 0.75 ml of 95% acetonitrile, and dried by azeotroping with acetonitrile three times. A solution of the mesylate precursor (**7**) in acetonitrile (5 mg; 1 ml) was added to the above dried ^{18}F activity, and the mixture heated at 110 $^\circ\text{C}$ for 15-20 minutes. Acetonitrile was evaporated, and the radioactivity reconstituted in the HPLC solvent and injected on to the reversed-phase HPLC column that was eluted at 1 ml/min with a gradient consisting of 0.1% TFA in each water (solvent A) and acetonitrile (solvent B); the proportion of B was increased linearly from 30% to 100% over a period of 30 min. The HPLC fractions corresponding to [^{18}F] **8** ($t_R = 17.9$ min) were collected, diluted with water and extracted with diethyl ether. Diethyl ether was evaporated and trifluoroacetic acid (TFA; 0.5 mL) was added, and the mixture left at 20 $^\circ\text{C}$ for 5 minutes to remove Boc and *tert*-butyl ester groups from [^{18}F] **8**. The TFA was evaporated with a stream of argon, and the resultant 5- ^{18}F fluoroleucine ([^{18}F] **5**) was reconstituted in PBS for use in biological assays.

Determination of Specificity of Uptake of L- ^{14}C leucine and 5- ^{18}F fluoroleucine in MCF7 Cells

To determine whether the uptake of 5- ^{18}F fluoroleucine is mediated by LAT [13], uptake assays were performed as described above but in the presence of increasing concentrations (0.5mM, 1mM and 5mM) of D-methionine, an amino acid competitive inhibitor, and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), an LAT inhibitor. A minimum of 3 samples were used for all conditions.

Biodistribution of 5- ^{18}F fluoroleucine in Normal Mice

All animal studies, including the below described small animal PET/CT imaging, were performed under an approved protocol by our Institutional Animal Use and Care Committee. Biodistribution of 5- ^{18}F fluoroleucine was determined in C57/B6 mice at 1, 2, and 4 hours (n=5 / time point) after administration of 222 kBq (6 μCi) of the radiotracer *via* tail vein. Mice were humanely euthanized at the 3 specified time points, organs of interest excised, weighed, and counted in a gamma well counter along with injection standards. Decay corrected uptake in organs was expressed as a percentage of

injected dose per gram (%ID/g). Urine at 1 h was analyzed by TLC to determine radioactivity associated with intact tracer. For this, filtered urine sample was applied to a silica gel TLC plate and eluted with 40:10:16:5 solution of n-butanol: water: acetic acid: EtOH. With this mobile phase, 5-fluoroleucine and free fluoride elute with an R_f values of 0.61 and 0.1, respectively.

Radiation Dosimetry

Tissue radioactivity values from the above biodistribution study in mice were used to estimate the radiation dose in humans for 5-¹⁸F]fluoroleucine. For most organs, the radioactivity content of the whole organ was used in the estimates. For the skeleton, the radioactivity in a weighed bone sample was measured and the total organ activity was computed from the percent injected dose per gram and an estimate of the fraction of body weight contributed by the skeleton.

The average value ($n = 5$) of percent injected activity per organ was computed for each time point. The following tissues were used as “source” organs in the MIRD schema: liver, spleen, heart wall, kidneys, thyroid, skeleton, muscle, brain and lungs. The fractional activities for the mouse source organs were allometrically scaled to the human phantoms by multiplying by the ratio $f_{\text{mouse}}/f_{\text{human}}$, where f = the fraction of the organ weight per total body weight. In computing f , the actual mouse organ weights and body weights were used, and for humans the reference values were obtained from the ICRP Report 89 [14]. For each tissue, the fraction of injected activity per whole organ $A(t)$ versus scaled time following administration were fit to a single-exponential function. The “residence time” (RT) of the MIRD schema was calculated for each of the above source organs as follows:

$$RT = A0 / (\lambda_b + \lambda_p)$$

where λ_p = physical decay constant for ¹⁸F (hr^{-1}), and λ_b = biological decay constant. To determine the residence time for the urinary bladder contents, the radioactivity in all measured tissues, including that in the gastrointestinal tract contents, was summed and fit to a single exponential to estimate the rate of clearance from the total body due to urinary excretion. The biological half-life (32 hr) computed for 78% of the radioactivity (total body radioactivity minus approximate contents of the GI tract) and used as input to the OLINDA bladder model, assuming a 4 hour voiding interval.

The contents of the stomach at one hour (fraction dose = 1.4×10^{-3}) was used as input to the OLINDA ICRP GI tract model to obtain the residence times for stomach, small intestine and large intestine. For the “remainder of the body”, the remainder weight was taken to be the total body weight minus the sum of the weights of the above source organs. The average fraction of injected dose per gram for the non-source organs (skin, blood, small/large intestines, muscle, tail) was multiplied by the remainder weight to give the fractional “remainder” activity. The activity in the skeleton was assumed to be associated with trabecular bone. The residence times for the source tissues were used as input into OLINDA/EXM [15] to calculate estimates of radiation absorbed dose to various target organs in the adult male and female.

Small Animal PET / CT Imaging

BALB/c mice ($n=4$) were implanted with 4T1 breast cancer cells in the left flank and allowed to grow until palpable tumors were detected. Mice were first anesthetized with 2% isoflurane (Isoflo; Abbott Laboratories) in 100% oxygen. The animals were placed on a heated PET/CT animal holder, which provided anesthesia through a nose cone, and administered 3.8 ± 1.5 MBq (102.8 ± 41.2 μCi) of [¹⁸F]fluoroleucine in approximately 100 μl PBS. Mice were then immediately transferred for dynamic list-mode PET imaging over 70 min. Images were reconstructed with an iterative reconstruction algorithm into image pixel size of 0.4 x 0.4 x 0.4 mm. The image reconstruction software (Inveon Acquisition Workplace 1.5SP1; Siemens Medical Solutions USA, Inc.) provided for correction of radioactivity decay, random coincidences, dead-time losses, and photon attenuation. Regions of interest (ROI) were drawn on the co-registered CT image for large organs to determine the uptake and washout over the dynamic imaging period. After imaging, tumors were excised, weighed and counted for radioactivity to determine the decay corrected %ID/gm.

RESULTS

Uptake of Essential Amino Acids in Breast Cancer Cell Lines *in vitro*

As shown in Fig. 1A, uptake of [¹⁴C]leucine in MCF7, SKBR3, and MDA231 breast cancer cell lines was $32.7 \pm 1.3\%$, $30.0 \pm 1.1\%$, and $36.1 \pm 1.1\%$, respectively after an hour of incubation. Except for histidine, a number of other amino acids tested including phenylalanine, a molecule of interest to us, demonstrated a relatively high uptake in these cell lines. Uptake of both L-leucine and L-phenylalanine increased with time as shown in Fig. 1B. This provided supporting evidence that delayed imaging using ¹⁸F-labeled versions of these compounds could result in high contrast images.

Synthesis of 5-fluoroleucine and its ¹⁸F-labeled Analog

An unlabeled standard of 5-fluoroleucine was synthesized by adaptation of the procedure reported in literature and the Scheme for this is shown (Fig. 2A). Commercially available (SigmaAldrich) (*S*)-1-(*tert*-butoxycarbonyl)-5-oxopyrrolidine-2-carboxylic acid (**1**) was converted to its *tert*-butyl ester (**2**) by DCC-mediated esterification with *tert*-butanol in 85% yield. This intermediate was methylated using methyl triflate with lithium bis(trimethylsilyl)amide as the base to yield (2*S*,4*S*)-di-*tert*-butyl 4-methyl-5-oxopyrrolidine-1,2-dicarboxylate (**3**) in 63% yield. This lactam was hydrolyzed by treatment with lithium hydroxide to yield (2*S*,4*S*)-5-*tert*-butoxy-4-(*tert*-butoxycarbonylamino)-2-methyl-5-oxopentanoic acid (**4**) in 83%. The acid (**4**) was reduced to the corresponding alcohol (**5**) by first converting it to a mixed anhydride with isobutyl chloroformate, and subsequent reduction with sodium borohydride in an overall yield of 56%. Conversion of this alcohol to mesylate (**6**; 95%) and introduction of another Boc protecting group on nitrogen (42%) delivered the precursor compound **7**. Displacement of the mesylate function in the precursor with fluorine using tetrabutyl ammonium fluoride (55%), and removal of all protecting groups by treatment with TFA

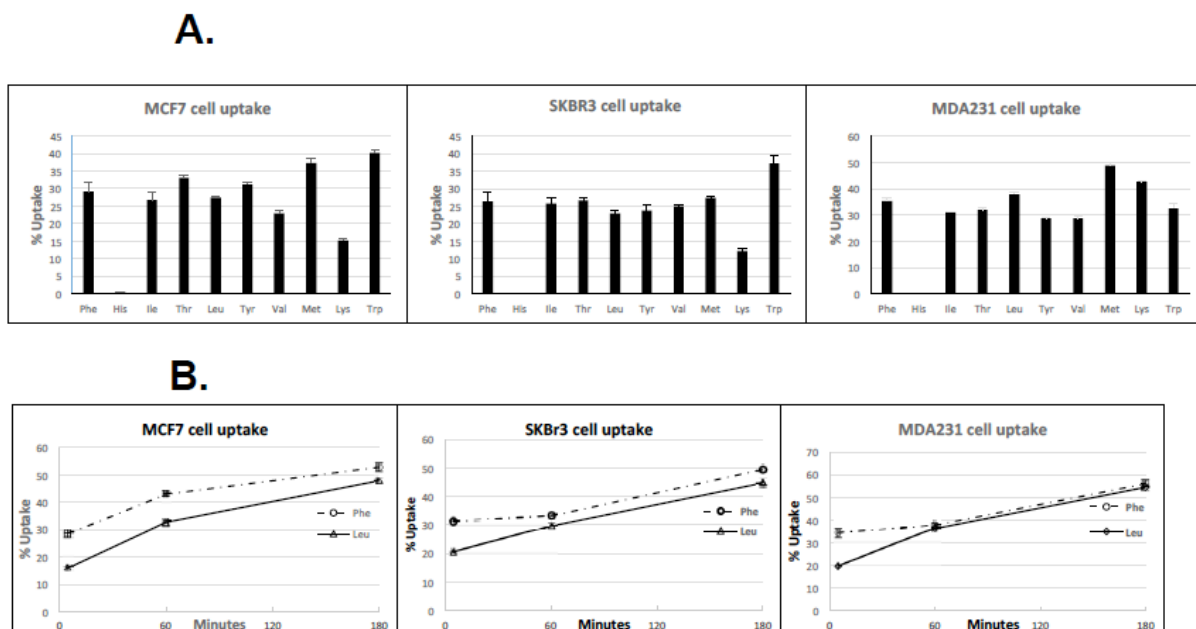


Fig. (1). Essential amino acid uptake in breast cancer cell lines. A) (left) MCF7, (middle) SKBR3, and (right) MDA231. Note relatively good uptake of leucine within all breast tumor cell lines. (Phe= phenylalanine; His= histidine; Ile= isoleucine; Thr= threonine; Leu= leucine; Val= valine; Met= methionine; Lys= lysine; Trp= tryptophan) B) In all breast cancer cell lines, our 2 primary essential amino acid candidates (phenylalanine and leucine) show *increasing* uptake over the 3-hour time period. (**Leu=L-leucine**; **Phe=phenylalanine**).

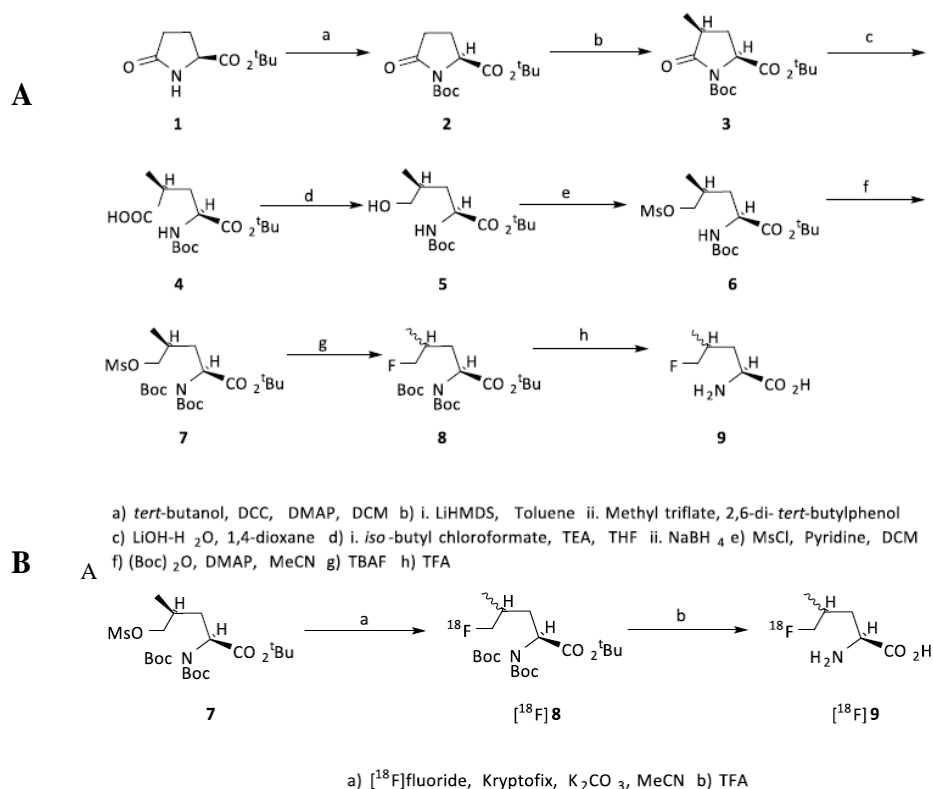


Fig. (2). Scheme for the synthesis of 5-fluoroleucine and its ¹⁸F-labeled analog 5-[¹⁸F]fluoroleucine. **A.** Scheme for the synthesis of 5-fluoroleucine and its mesylate precursor. **B.** Scheme for the synthesis of 5-[¹⁸F]fluoroleucine from the mesylate precursor.

(96%) gave the final product 5-fluoroleucine. NMR data of this compound and the intermediates were consistent with their structure and the data reported [12]. However, unlike in this reported procedure, we did not use 2-mesitylene sulfonic acid in the conversion of **7** to **8**. Thus, the product **8** was a diastereomeric mixture as indicated by its NMR spectrum.

The overall decay-corrected radiochemical yield for the synthesis of 5-[¹⁸F]fluoroleucine (Fig. **2B**) from aqueous [¹⁸F]fluoride was 30.0 ± 14.3% (n = 14). Radio TLC showed essentially one product corresponding to authentic fluoroleucine with more than 95% of radioactivity associated with this spot. Specific activity was not determined per se; how-

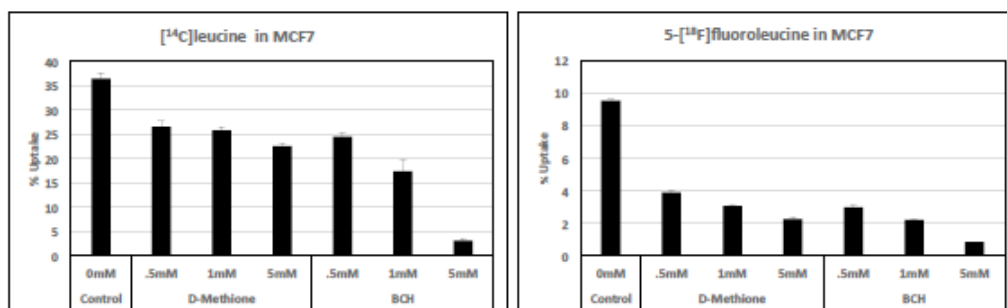


Fig. (3). Uptake of 5-¹⁸F]fluoroleucine is LAT-mediated. Inhibition of cellular uptake of [¹⁴C]leucine (left) and 5-¹⁸F]fluoroleucine (right) is dose-dependent for D-methionine, and for the LAT1 inhibitor BCH. (minimum n=3 samples per experimental conditional; 1 STD is too small to display).

Table 1. Biodistribution of 5-¹⁸F]fluoroleucine in C57/B6 mice.

| Organ | 1hr | | | 2hr | | | 4hr | | |
|----------|-------|---|------|-------|---|------|-------|---|------|
| Liver | 3.78 | ± | 0.62 | 3.10 | ± | 0.30 | 2.55 | ± | 0.20 |
| Spleen | 3.71 | ± | 0.52 | 3.10 | ± | 0.81 | 2.29 | ± | 0.37 |
| Lungs | 3.11 | ± | 0.30 | 2.69 | ± | 0.85 | 2.00 | ± | 0.17 |
| Heart | 3.39 | ± | 0.32 | 2.64 | ± | 0.45 | 1.94 | ± | 0.18 |
| Kidneys | 5.84 | ± | 0.89 | 4.75 | ± | 0.53 | 3.60 | ± | 0.67 |
| Stomach | 3.38 | ± | 0.77 | 2.69 | ± | 1.07 | 2.94 | ± | 0.99 |
| Sm. Int. | 4.76 | ± | 1.06 | 3.99 | ± | 0.49 | 3.49 | ± | 0.41 |
| Lg. Int. | 2.07 | ± | 0.27 | 2.44 | ± | 0.29 | 3.08 | ± | 0.28 |
| Thyroid | 1.26 | ± | 0.48 | 1.47 | ± | 0.42 | 2.10 | ± | 0.50 |
| Muscle | 2.52 | ± | 0.20 | 1.93 | ± | 0.31 | 1.20 | ± | 0.12 |
| Blood | 2.78 | ± | 0.68 | 3.63 | ± | 3.36 | 1.75 | ± | 0.14 |
| Pancreas | 31.94 | ± | 4.69 | 21.54 | ± | 4.42 | 13.19 | ± | 2.57 |
| Bone | 7.68 | ± | 1.48 | 9.81 | ± | 2.36 | 11.36 | ± | 2.71 |
| Skin | 2.41 | ± | 0.18 | 1.58 | ± | 0.16 | 1.22 | ± | 0.20 |
| Brain | 2.58 | ± | 0.27 | 2.44 | ± | 0.29 | 1.91 | ± | 0.16 |

All values expressed as %ID/gm (mean ± 1STD)

ever, assuming all of the mesylate is converted to unlabeled fluoro-leucine, and based on the maximum of 0.6 GBq (16 mCi) synthesized, a specific activity of 60 GBq (1.6 Ci)/mmol is estimated.

Specificity of Uptake of [¹⁴C]leucine and 5-¹⁸F]fluoroleucine in MCF7 Breast Cancer Cells *in vitro*

The uptake of L-¹⁴C]leucine and 5-¹⁸F]fluoroleucine in MCF7 cells decreased with increasing concentrations of D-methionine and BCH¹³. These data confirm that the uptake of L-¹⁴C]leucine in MCF7 cells is at least partially LAT-mediated (Fig. 3). Further, this data demonstrates that 5-fluoro-leucine is a substrate for LAT.

Normal Mice Biodistribution and Radiation Dosimetry of 5-¹⁸F]fluoroleucine

The data obtained from the biodistribution of 5-¹⁸F]fluoroleucine in normal mice are shown in Table 1 and Fig. 4. Pancreas uptake was high and decreased over time,

but remained higher than other normal background structures. Genitourinary tract activity was expectedly high as the primary route of excretion. At 1 hour, high and rapid renal clearance (kidney=5.8% injected dose/gm), low background (blood=2.8%; muscle =2.5%; skin=2.4%; %ID/gm), and relatively high bone uptake (7.7 %ID/gm) were seen. Given the high LAT expression in bone marrow [13] and the fact that there was little or no free [¹⁸F]fluoride in urine by TLC analysis, the bone uptake was considered to represent normal biodistribution of 5-¹⁸F]fluoroleucine. Most of the other organs (liver, spleen, lungs, heart, stomach, large intestine, muscle, blood, and skin) showed a mean uptake of approximately 2-4 %ID/gm at 1-4 hours. Brain uptake of 2.6 %ID/gm at 1 hour was in agreement with high LAT expression in normal human brain [13].

Radiation Dosimetry and Small Animal 5-¹⁸F]fluoroleucine PET/CT Tumor Imaging

The radiation dose estimates based on the above normal biodistribution data are summarized in Table 2. The whole

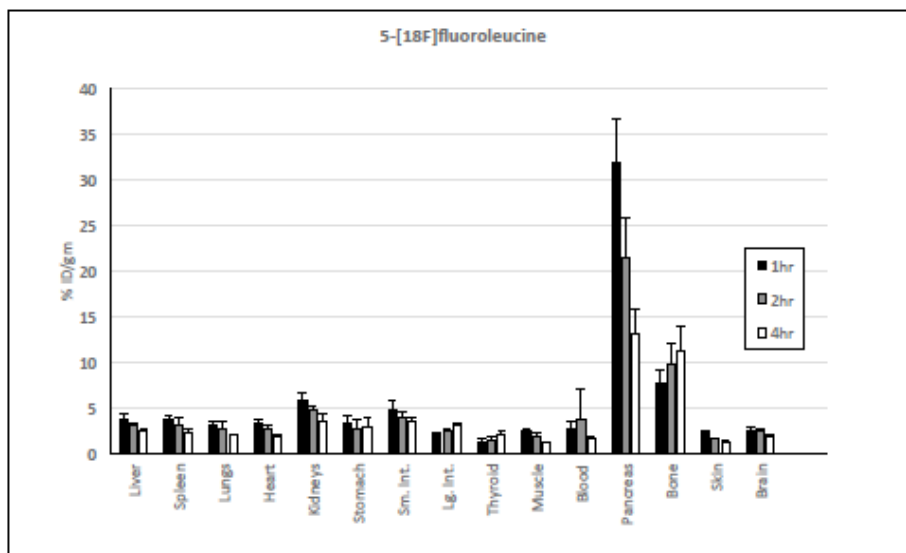


Fig. (4). Biodistribution of 5-^[18F]fluoroleucine in normal mice. Pancreas activity is relatively high. Excretion is *via* urine. Not shown is very high urine activity at 1 and 2 hours (257.0±59.6, and 141.3±87.4 %ID/gm, respectively).

Table 2. Organ dosimetry for 5-^[18F]fluoroleucine.

| Target Organ | Adult Male | | Adult Female | |
|------------------|-------------------------------|-------------------|-------------------------------|-------------------|
| | Organ Absorbed Dose (mSv/MBq) | Dose Error (1 SD) | Organ Absorbed Dose (mSv/MBq) | Dose Error (1 SD) |
| Adrenals | 9.48E-03 | 3.92E-03 | 1.19E-02 | 4.93E-03 |
| Brain | 7.31E-03 | 1.37E-03 | 8.55E-03 | 1.60E-03 |
| Female Breasts | | | 7.40E-03 | 3.06E-03 |
| Gallbladder Wall | 8.86E-03 | 3.67E-03 | 1.06E-02 | 4.39E-03 |
| LLI Wall | 1.18E-02 | 4.89E-03 | 1.48E-02 | 6.13E-03 |
| Small Intestine | 9.42E-03 | 3.90E-03 | 1.12E-02 | 4.64E-03 |
| Stomach Wall | 8.52E-03 | 3.53E-03 | 1.07E-02 | 4.43E-03 |
| ULI Wall | 8.87E-03 | 3.67E-03 | 1.13E-02 | 4.68E-03 |
| Heart Wall | 4.27E-02 | 1.01E-02 | 5.52E-02 | 1.30E-02 |
| Kidneys | 1.20E-02 | 3.64E-03 | 1.38E-02 | 4.18E-03 |
| Liver | 9.28E-03 | 1.38E-03 | 1.21E-02 | 1.80E-03 |
| Lungs | 6.60E-03 | 2.39E-03 | 8.43E-03 | 3.05E-03 |
| Muscle | 7.92E-03 | 3.28E-03 | 9.78E-03 | 4.05E-03 |
| Ovaries | | | 1.45E-02 | 6.00E-03 |
| Pancreas | 5.05E-02 | 1.77E-02 | 5.66E-02 | 1.98E-02 |
| Red Marrow | 2.54E-02 | 1.05E-02 | 2.83E-02 | 1.17E-02 |
| Osteogenic Cells | 4.72E-02 | 2.04E-02 | 6.21E-02 | 2.68E-02 |
| Skin | 5.75E-03 | 2.38E-03 | 7.08E-03 | 2.93E-03 |
| Spleen | 8.86E-03 | 5.44E-03 | 1.09E-02 | 6.69E-03 |
| Testes | 8.86E-03 | 3.67E-03 | | |
| Thymus | 7.81E-03 | 3.23E-03 | 9.84E-03 | 4.07E-03 |

Table 2. contd...

| Target Organ | Adult Male | | Adult Female | |
|----------------------|-------------------------------|-------------------|-------------------------------|-------------------|
| | Organ Absorbed Dose (mSv/MBq) | Dose Error (1 SD) | Organ Absorbed Dose (mSv/MBq) | Dose Error (1 SD) |
| Thyroid | 6.78E-03 | 3.14E-03 | 7.67E-03 | 3.18E-03 |
| Urinary Bladder Wall | 1.40E-01 | 5.80E-02 | 1.95E-01 | 9.03E-02 |
| Uterus | | | 1.92E-02 | 7.95E-03 |
| Lens of Eye | 2.85E-03 | | 2.85E-03 | |
| EDE (mSv/MBq) | 2.49E-02 | 4.18E-03 | 3.17E-02 | 6.08E-03 |
| ED (mSv/MBq) | 1.79E-02 | 3.41E-03 | 2.30E-02 | 4.99E-03 |

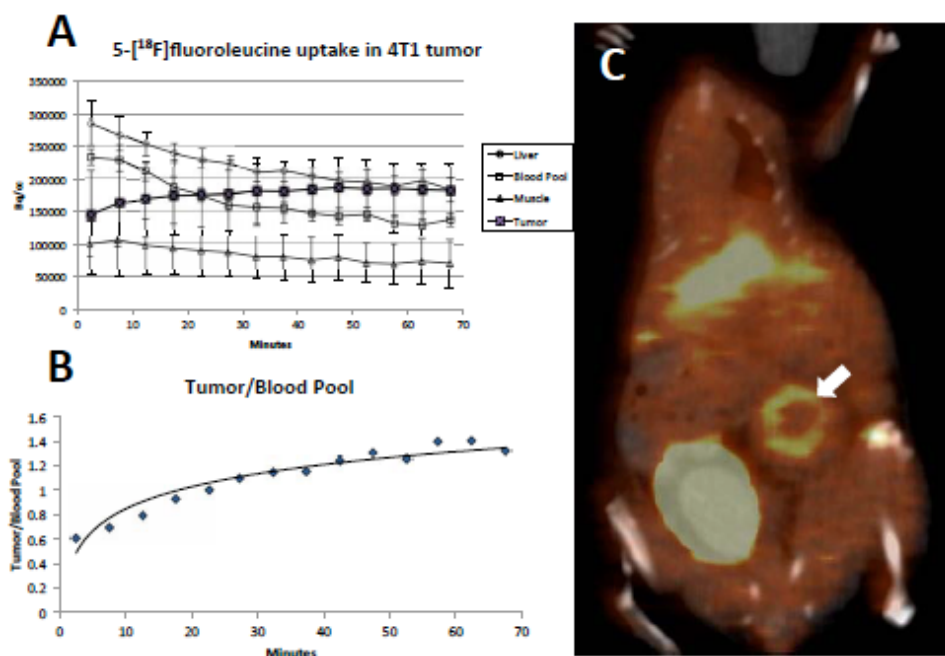


Fig. (5). Uptake of 5-¹⁸F] fluoroleucine in 4T1 breast tumor implanted into BALB/C mice. (A) Time activity curves 5-¹⁸F] fluoroleucine uptake in liver (open circle), blood pool (open square), muscle (open triangle), and 4T1 breast tumor (solid square) show increasing uptake in tumor, and decreasing background blood pool over time. (B) Tumor / background blood pool increases over time. (C) PET/CT image at 70 minutes post-injection demonstrates renal excretion with high bladder activity, and sufficient tumor uptake to identify the tumor above the background (solid arrow).

body effective dose equivalent for 5-¹⁸F]fluoroleucine is approximately 0.09, and 0.12 rem/mCi (2.49E-02 and 3.17E-02 mSv/MBq) for males and females, respectively. These estimates are quite comparable to current FDG PET dosimetry calculations. In the 4T1 tumor bearing BALB/c mice, the average 5-¹⁸F]fluoroleucine uptake was $4.1 \pm 1.8\%$ (%ID/gm \pm 1STD) for the tumors (n=4) ranging in size from 0.2-0.4 gm. The uptake in the tumor increased over time, and the background blood pool decreased over time (Fig. 5).

DISCUSSION

In this work, 5-¹⁸F]fluoroleucine was developed as a novel PET analog of leucine. Radiotracer assays using the LAT specific inhibitor BCH, and the competitive amino acid inhibitor D-methionine together confirm 5-¹⁸F]fluoroleucine uptake was at least partially *via* LAT. In both *in vitro* and

preclinical *in vivo* studies, 5-¹⁸F]fluoroleucine showed increasing tumor uptake over time, providing evidence that delayed imaging could improve contrast. *In vivo* metabolism could not be detected, and thus, this also likely contributes to improved image contrast. The biodistribution study also demonstrated rapid renal excretion, further facilitating high image contrast. The biodistribution study also provides evidence of acceptable radiation dosimetry, supporting the feasibility of translational human imaging. Small animal PET / CT imaging demonstrated tumor targeting with higher tumor to background contrast at delayed time points as predicted. Collectively, this data supports further investigation of this PET radiotracer for detection of tumors overexpressing amino acid transporters which may not be amenable to detection with FDG PET.

The biodistribution data showed normal organ uptake similar to that seen with other amino acid analogs [16-22]. In

these reports, relatively high uptake is seen in the pancreas, and consistent with this, 5-¹⁸F]fluoroleucine also demonstrated a very high uptake at 1 hour. The highest normal organ uptake at 1 hour was seen in the pancreas (31.9 %ID/gm), and this progressively decreased over time (13.2 %ID/gm at 4 hours). Another amino acid analog, with uptake primarily *via* SLC1A5, (2S,4R)-4-[¹⁸F]fluoroglutamine, has shown a similar biodistribution in mice [21]. (2S,4R)-4-[¹⁸F]fluoroglutamine showed highest uptake in pancreas at 1 hour (17.5 %ID/gm), and this progressively decreased over time (5.9 %ID/gm at 4 hours) [21]. This high uptake in the pancreas has been hypothesized due to the amino acid precursor requirement for exocrine hormone synthesis, and high protein turnover [21].

Relatively high activity in bone was seen at 1 hour (7.7 %ID/gm), which slightly increased over time (11.4 %ID/gm at 4 hours), as shown in Table 1. Again, this is quite similar to (2S,4R)-4-[¹⁸F]fluoroglutamine biodistribution in mice, which demonstrated high uptake in bone at 1 hour (7.9% ID/gm), and even higher uptake at 4 hours (19.4 %ID/gm) [21]. Despite this, (2S,4R)-4-[¹⁸F]fluoroglutamine has shown promising preliminary clinical results in the evaluation of brain tumors [23]. One hypothesis for the high uptake of 5-[¹⁸F]fluoroleucine in bone is that this could represent an index of protein synthesis in bone marrow. [³H] leucine has been shown to be an *in vitro* biomarker of index of protein synthesis, and [¹¹C] leucine is an *in vivo* PET imaging index of protein synthetic rate [24, 25]. In our study, free [¹⁸F]fluoride or other metabolites of 5-[¹⁸F]fluoroleucine were not seen in TLC of the urine, suggesting that the observed radioactivity in bone might represent the normal bone uptake due to protein synthesis. A previous report has shown high expression of human LAT in bone, as well as its moderate expression in brain [13]. An additional motivation for investigating this analog of L-leucine is its potential to serve as an index of protein synthesis. Although ¹¹C-leucine has been reported as an index of protein synthesis, there is no validated ¹⁸F index of protein synthesis currently available. A more detailed characterization of the metabolites is needed to confirm the absence of *in vivo* metabolism, and further characterization is needed to determine its correlation with protein synthetic rate in bone.

Our data indicate that there may be some potential limitations for 5-[¹⁸F]fluoroleucine in the current form. Overall, its tumor uptake was relatively modest in cells *in vitro*, and *in vivo* in mice as demonstrated by the small animal PET / CT study. Stereochemical purity of 5-[¹⁸F]fluoroleucine that we used in the current study has not yet been determined. Given the results we obtained in the synthesis of unlabeled **8** and **9**, it is highly likely that the final labeled fluoroleucine was mixture of two diastereomers. In general, L-isomers of amino acids are better substrates for enzymes and transporters. However, there are conflicting reports on this, and the modest uptake could be due to the presence of considerable amounts of D-isomer in our preparations [22]. To address this, in future studies, we will separate the two isomers and evaluate them individually. Another reason for the low uptake might be the presence of excess carrier in our preparations due to its not so high specific activity. Much higher specific activity may be achievable starting with higher amounts of [¹⁸F]fluoride activity and possibly lowering amounts of mesylate. The relatively modest uptake for

amino acid analogs in general, such as that seen for ¹⁸F fluoroethytyrosine (FET), has been the impetus for an alternative strategy to image the LAT1 receptor with monoclonal antibodies [18]. Finally, a complete characterization of 5-[¹⁸F]fluoroleucine uptake *via* all amino acid transporters was not performed. Similar to other amino acid analogs, multiple transport mechanisms are likely to contribute to net uptake, with variable contributions depending upon specific cell line, pH and media conditions [20]. Due to the structural similarity of 5-[¹⁸F]fluoroleucine to leucine, only a primary known mechanism was tested, the LAT system; other amino acid transport mechanism may contribute to its cellular uptake. Recently, the specific uptake *via* LAT1 been demonstrated by 3-¹⁸F-L- α -methyl-tyrosine ([¹⁸F]FAMT) [26] with the potential clinical utility of increased specificity currently being investigated. Despite these general limitations of amino acid analogs, they have shown promising results for detection of a number of tumors including brain [23] and prostate cancers [28]. Tumor evaluation by uptake of labeled amino acids by transporters such as LAT, is emerging as an imaging strategy which may provide prognostic information in a variety of cancers [9, 11, 17, 29 30].

CONCLUSION

We have developed a method for the synthesis of 5-[¹⁸F]fluoroleucine. This radiotracer has demonstrated increasing accumulation in tumors over time in both *in vitro* and *in vivo* studies, rapid washout from background *in vivo*, minimal defluorination, and increasing tumor to background ratio over time. Radiation dosimetry calculations support safety and feasibility in clinical studies. Further studies are needed to more fully characterize amino acid transport mechanisms, and to assess the utility of this novel radiotracer in other tumor imaging applications.

CONFLICTS OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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REFERENCES

- [1] Schuster DM, Savir-Baruch B, Nieh PT, Master VA, Halkar RK, Rossi PJ, Lewis MM, Nye JA, Yu W, Bowman FD, Goodman MM. Detection of recurrent prostate carcinoma with anti-1-amino-3-18f-fluorocyclobutane-1-carboxylic acid PET/CT and ¹¹¹in-capromab pentetide SPECT/CT. *Radiology*. 2011; 259: 852-861.
- [2] Lapa C, Linsenmann T, Monoranu CM, Samnick S, Buck AK, Bluemel C, Czernin J, Kessler AF, Homola GA, Ernestus R-I, Löh M, Herrmann K. Comparison of the amino acid tracers ¹⁸F-FET and ¹⁸F-DOPA in high-grade glioma patients. *Journal of Nuclear Medicine*. 2014; 55: 1611-1616.
- [3] Nicklin P, Bergman P, Zhang BL, Triantafellow E, Wang H, Nyfeler B, Yang HD, Hild M, Kung C, Wilson C, Myer VE, MacKeigan JP, Porter JA, Wang YK, Cantley LC, Finan PM, Murphy LO.

- Bidirectional transport of amino acids regulates mtor and autophagy. *Cell*. 2009; 136: 521-534.
- [4] Wang Q, Tiffen J, Bailey CG, Lehman ML, Ritchie W, Fazli L, Metierre C, Feng YJ, Li E, Gleave M, Buchanan G, Nelson CC, Rasko JE, Holst J. Targeting amino acid transport in metastatic castration-resistant prostate cancer: Effects on cell cycle, cell growth, and tumor development. *J Natl Cancer Inst*. 2013; 105: 1463-1473
- [5] Dang CV. Links between metabolism and cancer. *Gene Dev*. 2012; 26: 877-890.
- [6] Kaira K, Oriuchi N, Otani Y, Shimizu K, Tanaka S, Imai H, Yanagitani N, Sunaga N, Hisada T, Ishizuka T, Dobashi K, Kanai Y, Endou H, Nakajima T, Endo K, Mori M. Fluorine-18-alpha-methyltyrosine positron emission tomography for diagnosis and staging of lung cancer: A clinicopathologic study. *Clinical Cancer Research*. 2007; 13: 6369-6378.
- [7] Imai H, Kaira K, Oriuchi N, Yanagitani N, Sunaga N, Ishizuka T, Kanai Y, Endou H, Nakajima T, Mori M. L-type amino acid transporter 1 expression is a prognostic marker in patients with surgically resected stage I non-small cell lung cancer. *Histopathology*. 2009; 54: 804-813.
- [8] Kaira K, Oriuchi N, Imai H, Shimizu K, Yanagitani N, Sunaga N, Hisada T, Ishizuka T, Kanai Y, Endou H, Nakajima T, Mori M. L-type amino acid transporter 1 (lat1) is frequently expressed in thymic carcinomas but is absent in thymomas. *Journal of Surgical Oncology*. 2009; 99: 433-438.
- [9] Kaira K, Oriuchi N, Shimizu K, Imai H, Tominaga H, Yanagitani N, Sunaga N, Hisada T, Ishizuka T, Kanai Y, Oyama T, Mori M, Endo K. Comparison of l-type amino acid transporter 1 expression and l-[3-f-18]-alpha-methyl tyrosine uptake in outcome of non-small cell lung cancer. *Nuclear Medicine and Biology*. 2010; 37: 911-916.
- [10] Fuchs BC, Bode BP. Amino acid transporters asct2 and lat1 in cancer: Partners in crime? *Seminars in Cancer Biology*. 2005; 15: 254-266.
- [11] Wang Q, Holst J. L-type amino acid transport and cancer: Targeting the mtorc1 pathway to inhibit neoplasia. *American Journal of Cancer Research*. 2015; 5: 1281-1294.
- [12] Charrier J-D, Hadfield DS, Hitchcock PB, Young DW. Synthesis of (2s,4s)- and (2s,4r)-5-fluoroleucine and (2s,4s)-[5,5-2h2]-5-fluoroleucine. *Organic & Biomolecular Chemistry*. 2004; 2: 474-482.
- [13] Yanagida O, Kanai Y, Chairoungdua A, Kim DK, Segawa H, Nii T, Cha SH, Matsuo H, Fukushima J-i, Fukasawa Y, Tani Y, Takeda E, Uchino H, Kim JY, Inatomi J, Okayasu I, Miyamoto K-i, Takeda E, Goya T, Endou H. Human l-type amino acid transporter 1 (lat1): Characterization of function and expression in tumor cell lines. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. 2001; 1514: 291-302.
- [14] Protection ICoR. Basic anatomical and physiological data for use in radiological protection: Reference values. 2002; ICRP Report 89.
- [15] Stabin MG, Sparks RB, Crowe E. Olinda/exm: The second-generation personal computer software for internal dose assessment in nuclear medicine. *Journal of nuclear medicine* 2005; 46: 1023-1027.
- [16] Kuik W-J, Kema IP, Brouwers AH, Zijlma R, Neumann KD, Dierckx RAJO, DiMaggio SG, Elsinga PH. *In vivo* biodistribution of no-carrier-added 6-18f-fluoro-3,4-dihydroxy-l-phenylalanine (18f-dopa), produced by a new nucleophilic substitution approach, compared with carrier-added 18f-dopa, prepared by conventional electrophilic substitution. *Journal of Nuclear Medicine*. 2015; 56: 106-112.
- [17] Wiriyaerkmul P, Nagamori S, Tominaga H, Oriuchi N, Kaira K, Nakao H, Kitashoji T, Ohgaki R, Tanaka H, Endou H, Endo K, Sakurai H, Kanai Y. Transport of 3-fluoro-l- α -methyl-tyrosine by tumor-upregulated l-type amino acid transporter 1: A cause of the tumor uptake in pet. *Journal of Nuclear Medicine*. 2012; 53: 1253-1261.
- [18] Ikotun OF, Marquez BV, Huang C, Masuko K, Daiji M, Masuko T, McConathy J, Lapi SE. Imaging the l-type amino acid transporter-1 (lat1) with zr-89 immunopet. *PLoS one*. 2013; 8: e77476.
- [19] Tsukada H, Sato K, Fukumoto D, Nishiyama S, Harada N, Kakiuchi T. Evaluation of d-isomers of o-11c-methyl tyrosine and o-18f-fluoromethyl tyrosine as tumor-imaging agents in tumor-bearing mice: Comparison with l- and d-11c-methionine. *Journal of Nuclear Medicine*. 2006; 47: 679-688.
- [20] Tsukada H, Sato K, Fukumoto D, Kakiuchi T. Evaluation of d-isomers of o-18f-fluoromethyl, o-18f-fluoroethyl and o-18f-fluoropropyl tyrosine as tumour imaging agents in mice. *European Journal of Nuclear Medicine and Molecular Imaging*. 2006; 33: 1017-1024.
- [21] Lieberman BP, Ploessl K, Wang LM, Qu WC, Zha ZH, Wise DR, Chodosh LA, Belka G, Thompson CB, Kung HF. PET imaging of glutaminolysis in tumors by f-18-(2s,4r)4-fluoroglutamine. *Journal of Nuclear Medicine*. 2011; 52: 1947-1955.
- [22] Wang L, Lieberman BP, Plössl K, Qu W, Kung HF. Synthesis and comparative biological evaluation of l- and d-isomers of 18f-labeled fluoroalkyl phenylalanine derivatives as tumor imaging agents. *Nuclear Medicine and Biology*. 2011; 38: 301-312.
- [23] Venetti S, Dunphy MP, Zhang HW, Pitter KL, Zanzonico P, Campos C, Carlin SD, La Rocca G, Lyashchenko S, Ploessl K, Rohle D, Omuro AM, Cross JR, Brennan CW, Weber WA, Holland EC, Mellinger IK, Kung HF, Lewis JS, Thompson CB. Glutamine-based pet imaging facilitates enhanced metabolic evaluation of gliomas *in vivo*. *Sci Transl Med*. 2015; 7(274): 1-10.
- [24] Schmidt KC, Cook MP, Qin M, Kang J, Burlin TV, Smith CB. Measurement of regional rates of cerebral protein synthesis with l-[1-c-11]leucine and pet with correction for recycling of tissue amino acids: I. Kinetic modeling approach. *Journal of Cerebral Blood Flow and Metabolism*. 2005; 25: 617-628.
- [25] Smith CB, Schmidt KC, Qin M, Burlin TV, Cook MP, Kang J, Saunders RC, Bacher JD, Carson RE, Channing MA, Eckelman WC, Herscovitch P, Laveran P, Vuong BK. Measurement of regional rates of cerebral protein synthesis with l-[1-c-11]leucine and pet with correction for recycling of tissue amino acids: Ii. Validation in rhesus monkeys. *Journal of Cerebral Blood Flow and Metabolism*. 2005; 25: 629-640.
- [26] Wei L, Tominaga H, Ohgaki R, Wiriyaerkmul P, Hagiwara K, Okuda S, Kaira K, Oriuchi N, Nagamori S, Kanai Y. Specific transport of 3-fluoro-l-alpha-methyl-tyrosine by lat1 explains its specificity to malignant tumors in imaging. *Cancer Sci*. 2016; 107: 347-352.
- [27] Lieberman BP, Ploessl K, Wang L, Qu W, Zha Z, Wise DR, Chodosh LA, Belka G, Thompson CB, Kung HF. PET imaging of glutaminolysis in tumors by 18f-(2s,4r)4-fluoroglutamine. *Journal of Nuclear Medicine* 2011; 52: 1947-1955.
- [28] Turkbey B, Mena E, Shih J, Pinto PA, Merino MJ, Lindenberg ML, Bernardo M, McKinney YL, Adler S, Owenius R, Choyke PL, Kurdziel KA. Localized prostate cancer detection with ¹⁸F FACBC PET/CT: Comparison with mr imaging and histopathologic analysis. *Radiology*. 2014; 270: 849-856.
- [29] Burger IA, Zitzmann-Kolbe S, Pruijm J, Friebe M, Graham K, Stephens A, Dinkelborg L, Kowal K, Schibli R, Luurtsema G, Maas B, Horn-Tutic M, Haerle SK, Wieggers J, Schaefer NG, Hany TF, von Schulthess GK. First clinical results of (d)-18f-fluoromethyltyrosine (bay 86-9596) pet/ct in patients with non-small cell lung cancer and head and neck squamous cell carcinoma. *Journal of Nuclear Medicine*. 2014; 55: 1778-1785.
- [30] Sakata T, Ferdous G, Tsuruta T, Satoh T, Baba S, Muto T, Ueno A, Kanai Y, Endou H, Okayasu I. L-type amino-acid transporter 1 as a novel biomarker for high-grade malignancy in prostate cancer. *Pathology International*. 2009; 59: 7-18.