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Comparison of D-[^{18}F]FMAU and L-[^{18}F]FMAU as PET Imaging Agents for HSV1-TK Gene Expression

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D-[^{18}F]FMAU and L-[^{18}F]FMAU are F-18 labeled nucleoside analogue which have been efficiently synthesized in order to be a PET imaging probe. D-[^{18}F]FMAU and L-[^{18}F]FMAU were compared as PET imaging agents using HSV1-TK gene expressing tumor-bearing mice. Their cellular uptake profiles were also compared using MCA and MCA-TK cell lines. D-[^{18}F]FMAU demonstrated higher cellular uptake and higher accumulation in MCA-TK tumor regions than L-[^{18}F]FMAU. On the other hand, L-[^{18}F]FMAU showed higher MCA-TK/MCA ratio of %ID/g than that of D-[^{18}F]FMAU. L-[^{18}F]FMAU can be utilized as a good candidate for HSV1-TK PET imaging. It can be used for antiviral drug evaluation.

Key Words: HSV1-TK, D-[^{18}F]FMAU, L-[^{18}F]FMAU, PET imaging, MicroPET

Introduction

Gene therapy is a technique that is developing rapidly for treatment of a number of different cancers. Among various gene therapy approaches, the prodrug strategy is the most popular.[1] This strategy involves the delivery of a suicide gene (or reporter gene) to the target cells and administration of prodrugs. The suicide gene encodes novel nonmammalian enzymes, such as herpes simplex virus type-1 thymidine kinase (HSV1-TK), which can convert a relatively nontoxic prodrug into a highly toxic agent.[2] HSV1-TK is one of the most commonly used effector gene systems for imaging gene expression. It can act as both a suicide gene and reporter gene. It is a nonspecific nucleoside kinase, making it effective against a variety of tumor models both *in vitro* and *in vivo*.[3-5]

Optimization of the therapeutic effect requires an adequate radiolabeled probe to image the reporter gene. Thus, these probes are of high interest in the fields of radiopharmaceuticals and nuclear medicine.[6]

Nucleosides with the unnatural L-configuration have been studied as potent chemotherapeutic agents against human immunodeficiency virus (HIV), hepatitis B virus (HBV), and certain forms of cancer.[7,8] Among these nucleosides, radionuclide labeled pyrimidine nucleosides analogues were developed as potential antitumor and antiviral agents.[9,10] C-11 labeled 2'-deoxy-2'-fluoro-5-[^{11}C-methyl]-1-β-D-arabinofuranosyluracil (D-[^{11}C]-FMAU) was developed as a radiotracer for cell proliferation by positron emission tomography (PET).[11] However, C-11 labeled radiopharmaceuticals are of limited clinical application due to their short half-life ($t_{1/2}$ = 20 min).[12] Therefore, the analogues labeled with longer half-life radioactive atom, such as fluorine-18 ($t_{1/2}$ = 110 min), are optimum for effective clinical application. Some of the fluorinated analogues of pyrimidine nucleosides have been studied as potential agents for imaging tumor cell proliferation or HSV1-TK reporter gene expression.[12-14] Accordingly, Alauddin *et al.* developed the F-18 labeled 2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyluracil (D-[^{18}F]FMAU) (Fig. 1).[9]

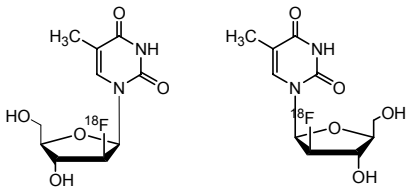
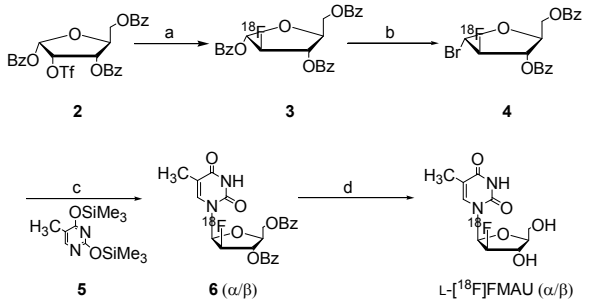


Figure 1. Structures of D-[^{18}F]FMAU and L-[^{18}F]FMAU.

There are four kinds of stereoisomers of FMAU (i.e. β-D, α-D, β-L, and α-L). Among them, L-FMAU has demonstrated high antiviral activity against HBV and Epstein Barr virus (EBV).[15,16] In the present investigation, we prepared D-[^{18}F]FMAU and L-[^{18}F]FMAU (Fig. 1), and evaluated their PET imaging properties in HSV1-TK gene expressing tumor-bearing mice. We also tested their *in vitro* cellular uptake profiles using MCA and MCA-TK cell lines. The synthetic and screening protocols are illustrated in details.

Results and Discussion

Chemistry. L-[^{18}F]FMAU was prepared by coupling the radiolabeled fluoro-sugar with the corresponding silylated thymine



Scheme 1. Reagents and conditions: (a) *n*-$Bu_4N^+.{}^{18}F^-$, CH_3CN; (b) HBr, CH_3COOH, $ClCH_2CH_2Cl$; (c) **5**, $ClCH_2CH_2Cl$, reflux; (d) CH_3ONa, CH_3OH

Table 1. *In vitro* cellular uptake ratio of D-[^{18}F]FMAU and L-[^{18}F]FMAU between MCA-TK and MCA cells

MCA-TK/MCA	15 min	30 min	60 min	120 min	240 min
ratio of D-[^{18}F]FMAU	419	357	146	121	70
ratio of L-[^{18}F]FMAU	39	42	46	77	103

following the preparation procedure for D-[^{18}F]FMAU reported by Alauddin *et al.*[9] but starting with the protected sugar triflate (L-form). The tribenzoyl triflate sugar **2** (L-form) was used as a precursor because sometimes the sulfonyl imidazole precursor could not be detected by radio-TLC or gave low labeling yields. Use of similar radiofluorination conditions with the tribenzoyl triflate **2**, however, showed evidence of product formation about > 85% (by radio-TLC). F-18 Fluoride was eluted from QMA cartridge using 50 μL of 4% $TBAHCO_3$. The solvent was completely removed by azeotrope with acetonitrile. To the reaction v-vial, the solution of triflate precursor **2** in acetonitrile was added and the reaction mixture was heated to 80 °C then cooled to room temperature. Unreacted F-18 was removed with two silica Sep-pak (light) and eluted with ethyl acetate. This mixture was checked by reverse phase HPLC system with authentic compound. Compound **3** was converted into the corresponding 1-*α*-bromo derivative **4** using 33% HBr/AcOH. 3,5-Di-*O*-benzoyl nucleoside compound **6** (*α*/*β* anomers) was synthesized by refluxing a mixture of compounds **4** and **5** in 1,2-dichloroethane. The benzoyl groups of **6** were hydrolyzed using methanolic solution of sodium methoxide to give L-[^{18}F]FMAU (Scheme 1). The product was purified by reverse phase HPLC. The radiochemical purity of the product (*β*-form) was more than 98% with decay-corrected yields of 25 - 35%.[17] D-[^{18}F]FMAU was prepared according to the literature method illustrated by Alauddin *et al.*[9]

Biology. The *in vitro* cellular uptake testing was determined in two cell lines, the MCA hepatoma cell line (derived from the Morris hepatoma RH 7777 cell line) and the MCA-TK cell line (derived from HSV1-TK-expressing cells using a retroviral vector). Both D-[^{18}F]FMAU and L-[^{18}F]FMAU showed little accumulation in the wild type MCA cells. The uptake of both compounds was significantly increased in the HSV1-TK expressing cells (MCA-TK) up to 240 min, depending on the time elapsed. D-[^{18}F]FMAU uptake in MCA-TK was higher than

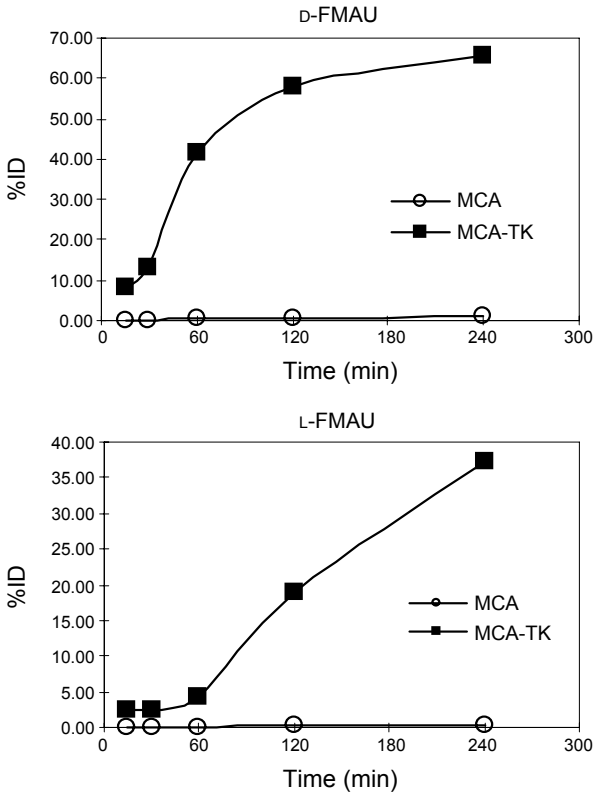


Figure 2. *In vitro* cellular uptake of D-[^{18}F]FMAU (20 μCi/well) and L-[^{18}F]FMAU (20 μCi/well) in MCA-TK and MCA cells.

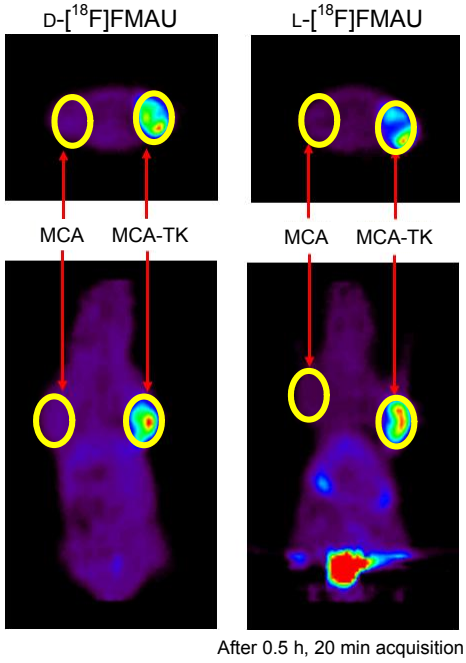


Figure 3. MicroPET images of D-[^{18}F]FMAU and L-[^{18}F]FMAU in MCA (Morris hepatoma RH 7777 cell line) and MCA-TK (HSV1-TK gene transduced) tumor-bearing nude mice. Each image represents the same transverse and coronal plane.

L-[^{18}F]FMAU (Fig. 2).[17] Table 1 illustrates the cellular uptake ratios of D-[^{18}F]FMAU and L-[^{18}F]FMAU between MCA-TK and MCA cells. *in vitro* cellular uptake ratio of L-[^{18}F]FMAU

Table 2. Quantification of D-[^{18}F]FMAU and L-[^{18}F]FMAU in mice bearing MCA-TK or MCA tumors

	%ID/g		MCA-TK/MCA ratio	D-FMAU/ L-FMAU ratio
	MCA	MCA-TK		
D-[^{18}F]FMAU	3.53	11.57	3.28	2.77
L-[^{18}F]FMAU	1.00	4.18	4.18	

was continuously increased up to 240 min. On the contrary, ratio of D-[^{18}F]FMAU was continuously decreased.

MicroPET images (coronal and transverse) of D-[^{18}F]FMAU and L-[^{18}F]FMAU using MCA-TK (right shoulder) and MCA (left shoulder) tumor-bearing mice are shown in Figure 3 to examine the *in vivo* effects. Each image represents the same transverse and coronal planes. High level radioactivity accumulation of D-/L-[^{18}F]FMAU was shown in MCA-TK tumor region. But there was little radioactivity accumulation in the wild MCA tumor region.

The %injected dose/g of tissue (%ID/g) values of D-[^{18}F]FMAU and L-[^{18}F]FMAU in MCA and MCA-TK tumor region were calculated from the microPET images (Table 2). The region of interest (ROI) was placed on MCA and MCA-TK tumor regions in the microPET images and %ID/g was estimated by pre-determined calibration factors through phantom study.[14] The %ID/g values for D-[^{18}F]FMAU were higher than that of L-[^{18}F]FMAU in both MCA and MCA-TK tumor regions. The D-[^{18}F]FMAU/L-[^{18}F]FMAU ratio of MCA-TK %ID/g (D-FMAU/L-FMAU ratio) was found to be 2.77. This means that D-[^{18}F]FMAU is accumulated in the MCA-TK tumor region higher than L-[^{18}F]FMAU by 2.77 times.

Conclusion

In conclusion, D-[^{18}F]FMAU and L-[^{18}F]FMAU were prepared and compared as PET imaging agents using HSV1-TK gene expressing tumor-bearing mice. Their cellular uptake profiles were compared using MCA and MCA-TK cell lines. D-[^{18}F]FMAU demonstrated higher cellular uptake and higher accumulation in MCA-TK tumor regions than L-[^{18}F]FMAU. On the contrary, L-[^{18}F]FMAU showed higher MCA-TK/MCA ratio of %ID/g than that of D-[^{18}F]FMAU. L-[^{18}F]FMAU can be utilized as a good candidate for HSV1-TK PET imaging. It can be used for antiviral drug evaluation.

Experimental Section

General. All reagents and solvents were purchased from Aldrich Chemical Co. and used without further purification. The solid phase extraction cartridge (Sep-pak, silica) was purchased from Waters Associates. The QMA cartridge (SPE cartridge Chromafix 30-PS-HCO_3) was obtained from Macherey-Nagel Inc. (USA). 2-*O*-[(Trifluoromethyl)sulfonyl]-1,3,5-tri-*O*-benzoyl-α-L-ribofuranose (**2**) and thymine-2,5-bis-trimethylsilyl ether (**5**) were prepared following reported methods with a little modification. Thin layer chromatography (TLC) was performed on Merck 60 F254 silica plates. Radio-TLC was monitored on a Bioscan AC-3000 scanner (Washington D.C., USA) and HPLC was performed on a Waters system using a 515 pump, 2487 UV detector (254 nm), and Raytest GABI γ-detector using a semi-preparative C18 reverse phase column (Waters, Xterra C18, 7.9 × 250 mm) and an analytical C18 column (Waters, mbondapak-C18, 3.9 × 300 mm). F-18 was produced with MC-50 cyclotron by irradiation of $H_2{}^{18}O$ at Korea Institute of Radiological and Medical Sciences (KIRAMS). The MCA-RH7777 rat hepatoma (MCA) cell line and thymidine kinase (TK)-transduced MCA cells (MCA-TK)[1] were kindly provided by Dr. Kwon, the Molecular Oncology Laboratory of KIRAMS. All animal experiments were approved by the pertinent committees of our institutions and performed in compliance with institutional guidelines for the conduct of animal experimentation.

Purification of L-[^{18}F]FMAU.[17] The mixture containing α and β anomers was purified through reverse phase HPLC using a semi-preparative Xterra C18 column (7.9 × 250 mm) with 5% CH_3CN/H_2O at a flow rate of 3.0 mL/min. The fraction eluting at 12 - 14 min was collected. The α/β anomeric ratio of the synthetic compounds was about 1:9 ratio. Finally, the collected sample was identified using analytical HPLC system by co-injection with authentic compound (L-[^{19}F]FMAU).

Cellular uptake test.[17] MCA and MCA-TK cells were grown to 5 × 10^5 cells/well in 6-well culture plates and incubated at 37 °C for 24 h. [^{18}F]-D- or L-FMAU was added to each well (20 μC^3/2 mL) and the mixture was incubated for 15, 30, 60, 120, 240 min at 37 °C in 5% CO_2 atmosphere. After that, the media were removed, the cells were rinsed with Dulbecco's phosphate-buffered saline, and adherent cells were harvested. Finally, the radioactivity was determined by gamma counter.

Tumor xenograft model. Five to six-week-old female BALB/c nu/nu mice (SLC, Hamamatsu, Japan) were used for establishment of the tumor model. MCA (0.5 - 1.0 × 10^6 cells) and MCA-TK cells (0.5 - 1.0 × 10^6 cells) suspended in 100 μL of serum-free cell culture medium were subcutaneously transplanted into both shoulders of mice. In order to prepare a mouse model carrying two different xenografts, the transduced MCA-TK cells suspension was transplanted into the right shoulder while the wild-type MCA cells suspension was transplanted into the left shoulder as a negative control in the same mouse. Tumor growth was assessed by measuring the bidimensional diameters using calipers. Mice carrying subcutaneous tumors that reached volumes of approximately 1,000 mm^3 were used for *in vivo* imaging experiments.

Radionuclide imaging of tumor xenograft mice. Tumors reached an approximate volume of 1,000 mm^3 about 3 weeks after subcutaneous implantation. 300 μCi of D- or L-[^{18}F]FMAU was administered to each mouse carrying the tumor through the tail vein. Mice were anesthesized with 2% isoflurane (Choongwae, Seoul, Korea) before injection with the test compounds and with 1.5% isoflurane during scanning for imaging. For thyroid-blocked images, 1 mg solution of sodium perchlorate (Sigma, St. Louis, MO) was intraperitoneally injected into the mice before i.v. injection of D- or L-[^{18}F]FMAU. Gamma camera (ZLC3700S, Siemens, Nunich, Germany) images of mice were taken at 0.5 h post-injection. Images were acquired up to 200,000 counts with the pinhole collimators having a focal length of 9 cm and 4 mm diameter. MicroPET images were obtained using a microPET system (microPET-R4; Concorde Microsystems,

Inc., Knoxville, TN). The acquired images were reconstructed according to pre-determined calibration factors.

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