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5-[¹⁸F]Fluoroalkyl Pyrimidine Nucleosides: Probes for PET Imaging of Herpes Simplex Virus Type-1 Thymidine Kinase Gene Expression

Ann-Marie Chacko^a, Eric Blankemeyer^b, Brian P. Lieberman^b, Wenchao Qu^b, and Hank F. Kung^{b,c,*}

^aInstitute for Environmental Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

^bRadiology, University of Pennsylvania, Philadelphia, PA 19104, USA

^cPharmacology, University of Pennsylvania, Philadelphia, PA 19104, USA

Abstract

Introduction—The preliminary *in vivo* evaluation of novel 5-[¹⁸F]fluoroalkyl-2'-deoxyuridines ([¹⁸F]FPrDU, [¹⁸F]FBuDU, [¹⁸F]FPeDU; [¹⁸F]**1a–c**, respectively) and 2'-fluoro-2'-deoxy-5-[¹⁸F]fluoroalkyl-1-β-D-arabinofuranosyl uracils ([¹⁸F]FFPrAU, [¹⁸F]FFBuAU, [¹⁸F]FFPeAU; [¹⁸F]**1d–f**, respectively) as probes for imaging herpes simplex virus type-1 thymidine kinase (HSV1-*tk*) gene expression are described.

Methods—[¹⁸F]**1a–f** were successfully synthesized by a rapid and efficient two step one-pot nucleophilic fluorination reaction using 5-*O*-mesylate precursors and [¹⁸F]F[−]. For *in vivo* studies, tumor xenografts were grown in nude mice by implanting RG2 cells stably expressing HSV1-*tk* (RG2TK+) and wild-type cells (RG2).

Results—Biodistribution studies at 2 h p.i. revealed that the uptake of [¹⁸F]**1a–b** and [¹⁸F]**1d–e** in RG2TK+ tumors was not significantly different from control tumors. However, [¹⁸F]**1c** and [¹⁸F]**1f** had an average 1.6 and 1.7-fold higher uptake in RG2TK+ tumors than control RG2 tumors. Blood activity curves for [¹⁸F]**1c** and [¹⁸F]**1f** highlight rapid clearance of radioactivity in the blood. Dynamic small animal PET (A-PET) imaging studies of tumor-bearing mice with [¹⁸F]**1c** and [¹⁸F]**1f** showed higher initial uptake (3.5-fold and 1.4-fold, respectively) in RG2TK+ tumors than control tumors, with continued washout of activity from both tumors over time.

Conclusions—Biological evaluations suggest that [¹⁸F]**1c** and [¹⁸F]**1f** may have limited potential for imaging HSV1-*tk* gene expression due fast washout of activity from the blood thus significantly decreasing sensitivity and specificity of tracer accumulation in HSV1-*tk* expressing tumors.

Keywords

5-[¹⁸F]Fluoroalkyl pyrimidine nucleosides; HSV1-*tk*; PET; Reporter gene imaging

*To whom correspondence should be addressed: Hank F. Kung, Ph.D., Department of Radiology, University of Pennsylvania, 3700 Market Street, Room 305, Philadelphia, PA 19104. Tel: (215) 662-3096; Fax: (215) 349-5035; E-mail: E-mail: kunghf@gmail.com.

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1. Introduction

The molecular imaging of herpes simplex virus type-1 thymidine kinase (HSV1-*tk*) gene expression is an exciting avenue for directly or indirectly assessing the success of therapeutic gene delivery and expression by gene therapy, and for following treatment outcomes [1,2]. Imaging of HSV1-*tk* has also been useful in the study of basic biological mechanisms by acting as an efficient readout of cell trafficking [3] and endogenous gene regulation [4,5], for example.

HSV1-*tk* gene expression is indirectly monitored *via* its gene product, the HSV1-TK enzyme. HSV1-TK levels can be imaged by administration of radiolabeled reporter probes that are transported into the cell, selectively phosphorylated by the HSV1-TK enzyme, and therefore “trapped” for subsequent detection by such imaging modalities as positron emission tomography (PET). An ideal candidate for PET imaging HSV1-*tk* gene expression should have significant cellular trapping in HSV1-TK positive tissue due specific phosphorylation by HSV1-TK, negligible uptake in nontarget tissue typically associated with host mammalian TK activity, *in vivo* stability, and desirable pharmacokinetics *in vivo*, including fast washout from nontarget organs. Just as desirable is the facile preparation of the PET radionuclide-labeled probe by a method that affords high yields of the radiotracer quickly and efficiently.

A variety of nucleoside analogues have been developed as probes for the PET imaging of intracellular HSV1-TK levels. These probes include the pyrimidine nucleoside analogue of the natural substrate thymidine, [^{124}I]-labeled or [^{18}F]-labeled FIAU [6,7]. Acycloguanosine nucleoside analogues such as [^{18}F]FHBG [8,9], and [^{18}F]FHPG [10,11] have also had success in imaging HSV1-*tk* gene expression although they would seem better suited for imaging the HSV1-*tk* mutant, HSV-*sr39tk* [12]. [^{124}I]FIAU has long been considered the probe of choice due to the straightforward incorporation of ^{124}I into the nucleoside structure and the highly sensitive and selective accumulation in HSV1-*tk* expressing tissue. However, [^{124}I]FIAU has significant non-specific phosphorylation by mammalian TKs which can be confounding when trying to detect lower levels of reporter gene expression. The fairly long radioactive half-life of ^{124}I ($t_{1/2}$ 4.2 d), has allowed for imaging later time points, although [^{124}I]FIAU is susceptible to deiodination *in vivo*. Owing to the limitations of [^{124}I]FIAU there has been a need to develop improved HSV1-TK PET reporter probes. Again, the ideal probe should have high sensitivity and selectivity, with minimal background activity, and allow for repetitive imaging more frequently and within a more dynamic range of reporter gene expression. The fairly short half-life of ^{18}F ($t_{1/2}$ 110 min) makes [^{18}F]-labeled HSV1-TK reporter probes the goal of choice for successfully designing and synthesizing PET reporter probes targeting HSV1-*tk* reporter gene expression.

In recent years, [^{18}F]FEAU (2'-[^{18}F]fluoro-2'-deoxy-5-ethyl-1- β -D-arabinofuranosyluracil) has emerged as having improved sensitivity and selectivity for HSV1-*tk* expressing cells [13-16], with the additional benefit of having potential for HSV-*sr39TK* [17]. However, [^{18}F]-labeled pyrimidine nucleosides with the radiolabel incorporated in the sugar at C-2' (e.g., [^{18}F]FEAU and [^{18}F]FIAU) suffer from cumbersome radiochemistry and long procedure times (>180 min) and afford the probes in low to moderate radiochemical yields due to multiple steps [18-21]. To address the radiosynthetic challenges of [^{18}F]FEAU, while maintaining the sensitivity and selectivity of [^{18}F]FEAU and [^{124}I]FIAU, [^{18}F]FFEAU (2'-fluoro-2'-deoxy-5-(2-[^{18}F]fluoroethyl)-1- β -D-arabinofuranosyluracil) has been designed for the radiolabel to be introduced more easily at the end of the C-5 alkyl chain at the last stages of radiosynthesis to decrease synthesis times and increase radiochemical yields [22]. Although there is an improvement in synthesis times (90–120 min) for [^{18}F]FFEAU, the extremely poor labeling yield (~0.2%) of limits its routine use for basic research studies and translation to clinical studies [16,22]. Nonetheless, *in vitro* and *in vivo* studies demonstrate that [^{18}F]FFEAU

has comparable characteristics to [^{18}F]FEAU and are indicative of the *in vivo* potential of [^{18}F]FFEAU for imaging HSV1-*tk* gene expression [16,22].

We have since identified two novel series of 5- ^{18}F fluoroalkyl pyrimidine nucleosides, [^{18}F] **1a–f**, as depicted in Fig. 1, that are easily prepared in good radiochemical yields (17–35%) in 60 min and have favorable *in vitro* properties as summarized in Table 1 [23,24]. Series A, with a C-2' hydrogen, are the 5- ^{18}F fluoroalkyl-2'-deoxyuridines. Nucleosides within this series have names reflective of the C-5 substitution and sugar moiety: 5-(3- ^{18}F fluoropropyl)-2'-deoxyuridine ([^{18}F]FPrDU, [^{18}F] **1a**), 5-(4- ^{18}F fluorobutyl)-2'-deoxyuridine ([^{18}F]FBuDU, [^{18}F] **1b**), and 5-(5- ^{18}F fluoropentyl)-2'-deoxyuridine ([^{18}F]FPeDU, [^{18}F] **1c**). Series B compounds are the 2'-fluoro-2'-deoxy-5- ^{18}F fluoroalkylarabinouridines with a C-2' fluorine: 2'-fluoro-2'-deoxy-5-(3- ^{18}F fluoropropyl)-1- β -D-arabinofuranosyluracil ([^{18}F]FFPrAU, [^{18}F] **1d**), 2'-fluoro-2'-deoxy-5-(4- ^{18}F fluorobutyl)-1- β -D-arabinofuranosyluracil ([^{18}F]FFBuAU, [^{18}F] **1e**), and 2'-fluoro-2'-deoxy-5-(5- ^{18}F fluoropentyl)-1- β -D-arabinofuranosyluracil ([^{18}F]FFPeAU, [^{18}F] **1f**). These nucleosides are a logical extension to [^{18}F]FFEAU with its 5-fluoroethyl group.

The purpose of this study was to further validate these novel probes [^{18}F] **1a–f** with the HSV1-*tk* reporter gene system. Herein we report results of preliminary *in vivo* biodistribution and small animal PET (A-PET) imaging studies of our 5- ^{18}F fluoroalkylpyrimidine nucleosides [^{18}F] **1a–f** using a nude mouse model with tumor xenografts derived from murine glioma cells stably expressing HSV1-*tk* (RG2TK+) and non-transduced wild-type glioma cells (RG2). Results were also directly compared [^{125}I]FIAU to determine the relative sensitivity and selectivity of our tracers in imaging HSV1-*tk* expressing tumors.

2. Materials and Methods

2.1 General

Reagents used for radiosyntheses were purchased from commercial sources and were used without further purification. Radiolabeling precursors were synthesized according to previously published procedure [24,25]. Animal experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) and Radiation Safety Committees of University of Pennsylvania. All procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2 Radiochemistry

2.2.1 Preparation of 5- ^{18}F fluoroalkyl pyrimidines, [^{18}F] **1a–f—[^{18}F] **1a–f** were prepared as reported previously (Fig. 2) [23,24]. Briefly, [^{18}F]Fluoride, purchased from IBA Molecular (Sterling, VA, USA), was obtained as an [^{18}O]-enriched aqueous solution and passed through a Sep-Pak Light QMA cartridge (Waters, Milford, MA, USA). The ^{18}F activity was eluted with a Kryptofix 222 (K[2,2,2])/K₂CO₃/CH₃CN/H₂O solution, dried under an N₂ stream, and reacted with the 5-*O*-mesylate precursor **2a–f** [24], in DMF at 135 °C for 5 min. Anhydrous 0.5 N NaOMe/MeOH was added and the mixture heated at 135 °C for 5 min. After neutralization with 10% HCl water was added, and the solution passed through a preconditioned Oasis HLB cartridge (Waters, Milford, MA, USA). The cartridge was washed with water, and the radiolabeled compound eluted with MeOH and purified by semi-preparative HPLC [Phenomenex Gemini C₁₈ semi-preparative column (10 mm×250 mm, 5 μm), MeOH/H₂O (3:7) (for compounds [^{18}F] **1a–b**, **d–e**) or MeOH/H₂O (4:6) (for compounds [^{18}F] **1c** and **1f**), flow rate 2–4 mL/min, *t*_R = 10.5–23.3 min]. Radiochemical purity (RCP) and specific activity (SA) of the HPLC-purified materials were determined by analytical HPLC [Phenomenex Gemini C₁₈ analytical column (4.6 mm×250 mm, 5 μm), MeOH/H₂O (3:7) (for compounds [^{18}F] **1a–b**, **d–e**) or MeOH/H₂O (4:6) (for compounds [^{18}F] **1c** and **1f**), flow rate 1**

mL/min, t_R = 6.0–21.6 min]. SA was estimated by comparing the UV peak intensity of the purified [^{18}F]-labeled compound with the calibration curve for the nonradioactive standard of known concentration.

2.2.2 Preparation of 2'-fluoro-2'-deoxy-5-[^{125}I]iodo-1- β -D-arabinofuranosyluracil ([^{125}I]FIAU)—

[^{125}I]NaI (0.1 N NaOH) was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA) in no-carrier-added form. [^{125}I]FIAU was prepared by the iododestannylation method as reported previously [24,26]. Briefly, to a solution of 5-tri-*n*-butylstannyl derivative of FIAU in MeOH was added [^{125}I]NaI, followed by the addition of 30% H_2O_2 /acetic acid. The reaction was left at room temperature for 30 min. The reaction was quenched with a saturated sodium bisulfite solution, followed by saturated sodium bicarbonate. The reaction mixture was loaded onto a preconditioned C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA). The C_{18} cartridge was eluted with water and followed by MeOH to isolate [^{125}I]FIAU. [^{125}I]FIAU was purified by analytical HPLC [Phenomenex Gemini C_{18} analytical column (4.6 mm $\times$ 250 mm, 5 μm), MeOH/ H_2O (2:8), flow rate 1 mL/min, t_R = 8.2 min]. The iodinated products are prepared under no-carrier added conditions and so it is likely that the specific activity (SA) is close to the theoretical maximum value of 81.4×10^4 GBq/mmol (2200 Ci/mmol) for ^{125}I .

2.3 Cell Lines

Rat glioma cells RG2, expressing the HSV1-tk gene (RG2TK+), were used for evaluation as reported [6]. Non-transduced (wild-type) RG2 cells (RG2) obtained from ATCC were used as controls. RG2 cells were cultured in DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin (GIBCO BRL, Grand Island, NY). Geneticin (G418, 250 mg/L) was added to the RG2 medium for selecting HSV1-tk positive cells (RG2TK+). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 /95% air.

2.4 Animals

Subcutaneous xenografts were produced in nu/nu CD-1 male nude mice (Charles River Laboratories, Inc. Wilmington, MA) by a subcutaneous injection of 2×10^6 tumor cells in 100 μL of serum-free cell culture media under anesthesia (a mixture of ketamine and xylazine, intraperitoneally). The transduced RG2TK+ cells were positioned in the left shoulder, while in the same animal wild-type RG2 cells were injected in the right shoulder as a negative control. Animals were studied when the subcutaneous tumor xenografts reached a diameter of 1 cm (normally occurring on 14-21 days after the subcutaneous implantation) [26].

2.5 Biodistribution studies with mouse tumor model

Biodistribution studies were carried out in RG2TK+ and RG2 tumor-bearing nude mice. Approximately 20 μCi of 5-[^{18}F]fluoroalkylpyrimidine nucleosides [^{18}F]1a–f with 5 μCi of [^{125}I]FIAU in 150 μL saline was co-injected into the lateral tail vein. The animals were sacrificed at 2 h post-injection (p.i.) by cardiac excision under isoflurane anesthesia. Several tissues and organs, HSV1-tk-expressing and non-transduced parental tumors were removed and weighed. The radioactivity in each tissue was measured using a gamma counter (Packard Cobra, Meridian, CT). Results were expressed as the percentage of the injected dose (I.D.) per gram of tissue (% I.D./g). Each value represents the mean $\pm$ S.D. of 6 mice unless otherwise noted.

2.6 In vivo blood activity study

Blood activity curves were obtained from RG2TK+ and RG2 tumor-bearing nude mice. Approximately 20 μCi of 5-[^{18}F]fluoroalkylpyrimidine nucleosides [^{18}F]1a–f with 5 μCi of

[¹²⁵I]FIAU in 150 μ L saline was co-injected into the lateral tail vein. Blood samples were collected from the contralateral tail vein at 2, 5, 15, 30, 60, and 120 min p.i. Blood samples were weighed and the radioactivity measured using a gamma counter (Packard Cobra, Meridian, CT). Results were expressed as % I.D./g of blood. Each value represents the mean $\pm$ S.D. of 3 mice unless otherwise noted.

2.7 Small animal PET imaging in mice

All scans were performed on an in-house Philips Mosaic Animal PET (A-PET) imaging system [27] which has an imaging field of view of $\sim$ 11.5 cm. A-PET studies were done in two nude mice with an RG2TK+ tumor xenograft in the left shoulder, and in the same animal, a wild-type RG2 tumor in the right shoulder as a negative control. Tumor-bearing mice were injected with $\sim$ 500 μ Ci (in 150 μ L saline) of [¹⁸F]**1c** ([¹⁸F]FPeDU) or [¹⁸F]**1f** ([¹⁸F]FFPeDU) *via* the tail vein. Data acquisition commenced $\sim$ 5 min post radiotracer injection and dynamic scans were carried out over a period of 3 h (5 min per frame; image voxel size 0.5 mm³). Region-of-interest (R.O.I.) analysis was performed using AMIDE (<http://amide.sourceforge.net>) on reconstructed images.

2.8 Statistics

Experimental values are reported as the mean $\pm$ S.D. of *n* experiments (where noted). Descriptive statistics of tracer accumulation were performed using GraphPad Prism 4.0 and Microsoft Excel 2004. Data were compared using an unpaired Student's *t*-test; results were considered statistically significant when $0.01 < P < 0.05$, very significant when $0.001 < P < 0.01$, and extremely significant when $P < 0.001$.

3. Results

3.1 Biodistribution

The results of the biodistribution studies for [¹⁸F]**1a–f** in comparison to [¹²⁵I]FIAU in tumor-bearing CD-1 *nu/nu* mice are summarized in Table 2. The mice were sacrificed at 2 h post-injection (p.i.) and selected organs were isolated and counted in a well counter. [¹⁸F]**1a–f** cleared very rapidly from the blood at 2 h p.i. with a blood retention of only 0.09–0.31% injected dose (I.D.)/g as compared to 1.04% I.D./g for [¹²⁵I]FIAU. Furthermore, all six compounds had generally quite low organ accumulation as compared to [¹²⁵I]FIAU. An apparent trend follows that blood and organ retention are higher for the 5-[¹⁸F]fluoroalkyl-2'-deoxyuridines, [¹⁸F]**1a–c** as compared to their 2'-fluoro-2'-deoxy-5-[¹⁸F]fluoroalkyl-1- β -D-arabinofuranosyluracil analogues, [¹⁸F]**1d–f**, respectively. Furthermore, accumulation looks to be highest for the 5-fluoropropyl nucleosides [¹⁸F]**1a,d**, followed by the 5-fluorobutyl nucleosides [¹⁸F]**1b,e**, and finally the 5-fluoropentyl nucleosides [¹⁸F]**1c,f**.

RG2TK+ tumor uptake values of the 5-[¹⁸F]fluoroalkyl pyrimidines was quite low as compared to [¹²⁵I]FIAU (0.16–0.35% I.D./g *versus* 4.37% I.D./g). Uptake of [¹⁸F]**1a–b** and [¹⁸F]**1d–e** demonstrated a lack of specificity for RG2TK+ tumors over control tumors and normal organs. In comparison, only the 5-fluoropentyl nucleosides [¹⁸F]**1c** and [¹⁸F]**1f**, had significant selectivity ($P < 0.05$) for RG2TK+ tumors, with an average 1.55 and 1.74-fold higher uptake in RG2TK+ tumors than control RG2 tumors, respectively (Fig. 3).

Notable tracer concentrations were observed in the liver and kidneys. The higher kidney values suggested that these hydrophilic nucleosides emptied *via* the renal-urinary system as has been observed with many [¹⁸F]-labeled pyrimidine nucleosides [15,22]. Activity in bone was also observed with [¹⁸F]**1a–f**, with bone activity significantly increasing with increasing fluoroalkyl chain length.

3.2 Blood activity curve

The blood activity curves for [^{18}F]**1c**, [^{18}F]**1f** and [^{125}I]FIAU in tumor-bearing mice ($n=3$) are shown in Fig 4. Blood activity of [^{18}F]**1c** was highest at 2 min p.i. with a value of $10.64 \pm 4.11\%$ I.D./g. Nearly half the activity rapidly cleared from the blood within 5 min. Remaining activity slowly cleared thereafter, leaving $0.27 \pm 0.20\%$ I.D./g at 2 h p.i. The blood clearance profile of [^{18}F]**1f** was similar to that of [^{18}F]**1c**, except the initial blood activity was lower at $4.18 \pm 0.17\%$ I.D./g. Only $0.16 \pm 0.05\%$ I.D./g was remaining in the blood after 2 h. In comparison, [^{125}I]FIAU had a similar profile to [^{18}F]**1c**, although it had a slightly slower clearance time than both [^{18}F]tracers with $0.69 \pm 0.26\%$ I.D./g left in the blood at 2 h p.i.

3.3 PET Imaging

Dynamic small animal PET (A-PET) imaging studies of tumor-bearing mice were carried out with the 5-fluoropentyl nucleosides [^{18}F]**1c** and [^{18}F]**1f**. A-PET images of selected coronal projections (30, 60, 120, and 180 min) with [^{18}F]**1c** and [^{18}F]**1f** are shown in Fig. 5. Tumors were grown with RG2 cells stably expressing the HSV1-*tk* gene (RG2TK+) on the left shoulder and the control tumor was grown with non-transduced RG2 cells on the right shoulder. As the images demonstrate in Fig. 5, [^{18}F]**1c** and [^{18}F]**1f** did have higher accumulation in the RG2TK+ tumor *versus* the RG2 tumor soon after injection. However, RG2TK+ tumor activity was only slightly higher than background activity throughout the entire study. The tracers both concentrated nearly immediately in the kidneys and bladder. However, clearance of activity from the kidneys was much faster for [^{18}F]**1c** than [^{18}F]**1f**. After 60 min with [^{18}F]**1c**, activity in the kidneys cleared with near all activity pooling in the urine. Little to no radioactivity is seen in the lungs, heart, liver, and intestines.

Regions of interest (R.O.I.) were drawn around the RG2TK+ and RG2 tumors and time-activity curves generated for [^{18}F]**1c** and [^{18}F]**1f** during the 2-h dynamic scan as shown in Fig. 6. Rapid initial uptake into both target and control tumors was observed for [^{18}F]**1c** and [^{18}F]**1f**. For [^{18}F]**1c**, radioactivity in the target RG2TK+ tumor peaked at 15 min p.i. (Fig. 6A, left), whereas for [^{18}F]**1f** tumor uptake was measured to be highest at the start of image acquisition (~ 8 min p.i.) (Fig. 6B, left). For both compounds, radioactivity steadily declined after maximum uptake indicating washout of the probes from both target and control tumors. RG2TK+ to RG2 tumor ratios for [^{18}F]**1c** were 2.2 to 3.6 and highest between 5–45 min p.i. (Fig 6A, left). For [^{18}F]**1f**, there was 1.1– to 1.4-fold higher uptake in RG2TK+ tumors to RG2 tumors (Fig 6B, right). Visually, RG2TK+ and RG2 tumors were nearly indistinguishable at later time points, and this was confirmed by R.O.I. analysis.

4. Discussion

The PET imaging of HSV1-*tk* gene expression is gaining more prominence as a powerful tool for the assessment of suicide gene therapy. Hence, there is a need to develop HSV1-TK reporter probes that are highly sensitive and selective to HSV1-*tk* expressing tissues. [^{18}F]FEAU [15-17,28] and [^{18}F]FFEAU [16,22] have emerged in recent years as very promising *in vivo* HSV1-TK PET reporter probes. The time consuming, multi-step synthesis of [^{18}F]FEAU (>180 min) [15,17] and very low radiochemical yield of [^{18}F]FFEAU ($<0.2\%$) [16,22] make them less practical for routine use in clinical studies. We have recently reported a novel synthetic methodology towards C-5 alkylated pyrimidine nucleosides [25]. By employing this methodology we have synthesized two novel series of 5-fluoroalkyl pyrimidines. These probes were designed to allow for improved binding and subsequent phosphorylation by HSV1-TK while having an easily accessible handle to introduce ^{18}F in high radiochemical yields [24]. Indeed, *via* a two-step one pot method, we were able to synthesize two novel series of six [^{18}F]-labeled pyrimidine nucleosides, [^{18}F]**1a-f**, within 60 min from end of bombardment (E.O.B.) with moderate overall radiochemical yields of 17.5–35.2% (decay-corrected) in high

radiochemical purity and reasonable specific activity. It is likely that our method could be easily adapted for automation to attain higher radiochemical yields in even shorter preparation times. The 5- ^{18}F fluoroalkyl-2'-deoxyuridines ^{18}F **1a–c** and 2'-fluoro-2'-deoxy-5- ^{18}F fluoroalkylarabinouridines ^{18}F **1d–f** have 5-(2-fluoropropyl), 5-(2-fluorobutyl), and 5-(2-fluoropentyl) moieties, respectively. *In vitro* studies demonstrated that ^{18}F **1a–c** had higher sensitivity than ^{18}F **1d–f** for glioma cells expressing HSV1-*tk* (RG2TK+), with the accumulation of the probe generally decreasing with increasing fluoroalkyl chain length following a 2 h incubation. Using uptake in control RG2 cells as the nontarget reference, ^{18}F **1a–f** exhibited a 5.0– to 20.5-fold for HSV1-*tk* expressing cells *in vitro*, with ^{125}I FIAU having a selectivity of 9.9 (Table 1). We considered all six compounds as candidates for HSV1-TK PET reporter probes so we continued with the *in vivo* characterization of compounds ^{18}F **1a–f** as described in this paper.

In vivo biodistribution studies and dynamic small animal PET (A-PET) studies were carried out in a tumor-bearing nude mouse model to assess the sensitivity and selectivity of reporter probe-trapping in HSV1-*tk* expressing tissue due to monophosphorylation by the HSV1-TK enzyme. Furthermore, we wanted to understand the pharmacokinetic behavior of these nucleosides *in vivo*. Surprisingly, dual-isotope biodistribution studies revealed there was very low uptake of ^{18}F **1a–f** in the RG2TK+ tumors at 2 h p.i. ($\leq 0.35 \pm 0.12\%$ I.D./g) as compared to ^{125}I FIAU ($4.37 \pm 0.93\%$ I.D./g) (Table 2). Furthermore, only the 5-fluoropentyl pyrimidines ^{18}F **1c** and ^{18}F **1f** had specificity for RG2TK+ tumors, with maximum RG2TK+/RG2 tumor uptake ratios (target-to-nontarget ratio) of 1.55 ± 0.44 and 1.74 ± 0.53 , respectively (Fig. 3). The selectivity values were much lower than ^{125}I FIAU (5.87 ± 1.31). These results are in stark contrast to the *in vitro* data which showed that ^{18}F **1c** had very high selectivity for RG2TK+ cells in comparison to the lower selectivities of ^{18}F **1f** and ^{125}I FIAU (i.e., 19.9 ± 6.9 versus 5.5 ± 2.3 and 9.9 ± 4.6 , respectively). Activity in the thoracic and abdominal organs was quite low, albeit slightly higher kidney activities would suggest that route of clearance was predominantly renal. And although log *P* values were high in comparison to ^{125}I FIAU at pH 7.4 (Table 1), there was low to negligible uptake into the brain as was observed with ^{125}I FIAU (Table 2).

In hopes of understanding the discrepancies between *in vitro* and *in vivo* results we looked at bone uptake as a pseudomarker of defluorination/metabolism of the ^{18}F -labeled probes. It has been often reported that introduction of a C-2' fluorine in the sugar-portion of the nucleoside limits the metabolic degradation of the nucleoside due to stabilization of the glycosidic bond. Bone uptake was observed for all compounds, with bone activity significantly increasing with increasing fluoroalkyl chain length. Bone uptake of ^{18}F **1a–f** was not as high as that reported for some radiofluorinated nucleosides [29]. Interestingly, the results did not highlight major metabolic differences between the 5- ^{18}F fluoroalkyl-2'-deoxyuridines ^{18}F **1a–c** and the 2'-fluoro-2'-deoxy-5- ^{18}F fluoroalkyl-1- β -D-arabinofuranosyl uracils ^{18}F **1d–f** with their “protective” fluorine. ^{125}I FIAU had significant loss of the radioiodide resulting in high dose to the thyroid ($355.41 \pm 227.357\%$ I.D./organ) while still maintaining high sensitivity and selectivity for HSV1-*tk* positive tissues. Hence, we looked to circulating blood-activity levels in hopes of understanding the limited uptake of the probes in RG2TK+ tumors.

At 2 h p.i. there was little ^{18}F activity remaining in the blood ($\leq 0.35 \pm 0.12\%$), with ^{18}F **1a–c** having slightly higher values as compared to their analogous counterparts ^{18}F **1d–f**. ^{125}I FIAU had blood circulating pools almost 3-fold higher than ^{18}F **1a–f**. Detailed studies on blood clearance of ^{18}F **1c** and ^{18}F **1f** compared to ^{125}I FIAU (Fig. 3) revealed that the ^{18}F nucleosides cleared from the blood much faster than ^{125}I FIAU and that there was still a significant dose of ^{125}I FIAU remaining in the blood for potential recirculation to the tumor. Detailed metabolite analysis was not conducted on these isolated blood fractions.

Dynamic A-PET imaging of [^{18}F]**1c** and [^{18}F]**1f** revealed that within 8 min of administration of the radiotracers most of the radioactivity was pooled in the kidneys and bladder (urine). This would suggest that the majority of radiotracer is removed from the blood pool so quickly that there is not enough radiolabeled probe remaining for sensitive and selective accumulation in the RG2TK+ tumor. Although we can visualize uptake of radiotracers [^{18}F]**1c** and [^{18}F]**1f** into RG2TK+ tumors (Fig. 5), and there is a measurable difference between uptake in RG2TK+ and RG2 tumors (3.6-fold and 1.4-fold at its maximum for [^{18}F]**1c** and [^{18}F]**1f**, respectively) (Fig. 6, right), there is no significant contrast between the tumor and the surrounding tissue. This can be due to the steady washout of activity from both the target and nontarget tumor at a rate constant if not faster than the surrounding tissue.

These negative *in vivo* results would suggest that there are at least three factors limiting the targeted uptake and retention of [^{18}F]**1a–f** in HSV1-*tk* expressing tumors in nude mice: 1) Membrane permeability of these tracers to enter tumor cells (*via* the nucleoside transporters) may be too slow and thus is the predominant factor in limiting the intracellular signal; 2) *in vivo* elimination of these radiotracer from the circulating blood pool is too fast; 3) the kinetics of phosphorylation by the HSV1-TK enzyme is not sufficiently fast to overcome the very fast blood clearance.

It should be noted that [^{18}F]**1a–f** are closest in chemical structure and site of radiolabeling to the successful 5-(2-fluoroethyl) pyrimidine nucleoside [^{18}F]FFEAU. As such, it is surprising that our *in vivo* results do not even compare to those obtained with [^{18}F]FFEAU [16,22]. However, a recent comparison of the current literature suggests that a potential point of inconsistency between studies is the choice of animal model which may be reflective of interspecies differences (e.g., *rnu/rnu* rat versus athymic nude mouse) [16]. *In vivo* studies of [^{18}F]FFEAU were conducted in *rnu/rnu* rats and ours in *nu/nu* mice.

5. Conclusions

The successful synthesis of [^{18}F]**1a–f** in good yields and short preparation times, and the very promising *in vitro* results highlights the potential of these novel 5- ^{18}F fluoroalkyl pyrimidine nucleosides for imaging HSV1-*tk* reporter gene expression *in vivo*. Yet, biodistribution studies of [^{18}F]**1a–f** followed by PET imaging studies [^{18}F]**1c** and [^{18}F]**1f** in a tumor-bearing nude mouse model emphasizes the lack of sensitivity and lack of, if not low selectivity of these probes for HSV1-*tk* expressing RG2TK+ tumors. These results thus far demonstrate the limited potential of these 5- ^{18}F fluoroalkyl nucleosides in evaluating HSV1-*tk* gene expression *in vivo*. The contrasting results of sensitivity and selectivity between *in vitro* and *in vivo* studies indicates the need to further study the metabolic fate of these nucleoside *in vivo* and determine whether interspecies differences between small animal tumor models play a role in determining the *in vivo* pharmacokinetics and resultant accumulation of radioactive probes in HSV1-*tk* expressing tissues.

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Figure 1.

Chemical structures of 5-[¹⁸F]fluoroalkyl pyrimidine nucleosides [¹⁸F]1a-f.

Figure 2.

Radiosynthesis of [¹⁸F]**1a–f** achieved by following two-step one-pot method using tri-benzoyl-protected 5-*O*-mesylate precursors **2a–f**. Obtained radiochemical yields of 17.5–35.2%, radiochemical purity >99%, and specific activity of 240–29400 mCi/μmol [24].

Figure 3.

In vivo uptake selectivity of [^{18}F]1a–f compared to [^{125}I]FIAU in RG2TK+ tumors as compared to muscle, blood, and RG2 tumors 2 h post i.v. injection in CD-1 *nu/nu* glioma-bearing mice. Data are expressed as mean $\pm$ S.D. as calculated from respective % I.D./g values. Statistical significance of RG2TK+/RG2 tumor uptake selectivity of [^{18}F]1c and [^{18}F]1f versus [^{18}F]1a–b and [^{18}F]1d–e determined by unpaired Student's *t* test: *, $P < 0.02$.

Figure 4. Blood activity curves in tumor-bearing nude mice: (A) $[^{18}\text{F}]\mathbf{1c}$; (B) $[^{18}\text{F}]\mathbf{1f}$; (C) $[^{125}\text{I}]\text{FIAU}$. Values are the mean $\pm$ S.D. of $n=3$ for $[^{18}\text{F}]\mathbf{1c}$ and $[^{18}\text{F}]\mathbf{1f}$. Values are the mean $\pm$ S.D. of $n=6$ for $[^{125}\text{I}]\text{FIAU}$.

Figure 5.

Small animal PET (A-PET) images of: (A) $[^{18}\text{F}]\mathbf{1c}$ and (B) $[^{18}\text{F}]\mathbf{1f}$ at 30, 60, 120, and 180 min p.i. Coronal projections. While under isofluorane anesthesia, 500 μCi of $[^{18}\text{F}]\mathbf{1c}$ or $[^{18}\text{F}]\mathbf{1f}$ was injected into the tail vein.

A. [^{18}F] 1c B. [^{18}F] 1f **Figure 6.**

Time-activity curves for RG2TK+ and RG2 tumors generated from R.O.I. drawn on A-PET images using AMIDE software. (A) [^{18}F] 1c and (B) [^{18}F] 1f. Injection of ~500 mCi [^{18}F] 1c or [^{18}F] 1f with peak uptake 0–10 min p.i. (left). RG2TK+ to RG2 tumor uptake ratio (right).

Table 1

Comparison of Log *P* and *in vitro* uptake of tracers after 2 h incubation with RG2TK+ and RG2 cells [24].

Tracer	Log <i>P</i> ^a	% Uptake per 10 ⁶ Cells ^b		<i>In Vitro</i> Uptake Selectivity Index (RG2TK+/RG2)
		RG2TK+	RG2	
[¹⁸ F]1a ([¹⁸ F]FPrDU)	-0.57	8.2±1.5	0.41±0.07	20.5±4.4
[¹⁸ F]1b ([¹⁸ F]FBuDU)	-0.24	8.4±0.7	0.59±0.16	14.9±4.0
[¹⁸ F]1c ([¹⁸ F]FPeDU)	0.23	4.4±1.1	0.23±0.07	19.9±6.9
[¹⁸ F]1d ([¹⁸ F]FFPrAU)	-0.12	1.9±0.3	0.38±0.03	5.0±0.7
[¹⁸ F]1e ([¹⁸ F]FFBuAU)	0.27	3.7±0.7	0.66±0.13	7.8±4.3
[¹⁸ F]1f ([¹⁸ F]FFPeAU)	0.74	1.2±0.3	0.24±0.01	5.5±2.3
[¹²⁵ I]FIAU	0.05	29.4±7.4	3.4±2.0	9.9±4.6

^aLog *P* = log([1-octanol]/[0.1 M NaH₂PO₄ pH 7.47])

^bData are expressed as the mean±S.D. of three or more independent experiments performed in duplicate.

Table 2
Biodistribution of [¹⁸F]1a-f and [¹²⁵I]FIAU at 2 h in tumor-bearing nude mice^a (% I.D./g).

Tissue	5-[¹⁸ F]Fluoroalkyl Pyrimidine						
	[¹⁸ F]1a	[¹⁸ F]1b	[¹⁸ F]1c	[¹⁸ F]1d	[¹⁸ F]1e	[¹⁸ F]1f	[¹²⁵ I]FIAU ^b
Blood	0.31±0.11	0.24±0.06	0.16±0.05	0.15±0.08	0.11±0.02	0.09±0.03	1.00±0.14
Heart	0.28±0.09	0.24±0.07	0.14±0.02	0.15±0.08	0.11±0.02	0.07±0.04	0.41±0.10
Lung	0.31±0.10	0.27±0.07	0.19±0.03	0.14±0.08	0.13±0.03	0.12±0.03	0.77±0.14
Kidney	0.48±0.24	0.58±0.10	0.55±0.12	0.32±0.17	0.33±0.11	0.36±0.06	0.94±0.29
Spleen	0.31±0.11	0.24±0.05	0.13±0.03	0.16±0.09	0.11±0.02	0.11±0.04	0.71±0.21
Liver	0.39±0.12	0.45±0.11	0.38±0.05	0.21±0.14	0.24±0.04	0.27±0.05	0.55±0.15
Skin	0.33±0.13	0.23±0.04	0.12±0.05	0.18±0.15	0.13±0.03	0.07±0.03	0.86±0.25
Muscle	0.49±0.19	0.35±0.13	0.21±0.12	0.29±0.14	0.21±0.05	0.10±0.03	0.35±0.09
Bone	0.45±0.16	0.62±0.10	1.79±0.67	0.77±0.27	1.14±0.11	1.57±0.41	0.44±0.11
Brain	0.12±0.04	0.08±0.01	0.04±0.01	0.02±0.01	0.04±0.01	0.02±0.01	0.09±0.03
RG2 Tumor	0.30±0.13	0.30±0.10	0.19±0.04	0.19±0.10	0.16±0.04	0.10±0.03	0.73±0.17
RG2TK+ Tumor	0.35±0.12	0.29±0.09	0.29±0.05*	0.20±0.11	0.16±0.03	0.17±0.03**	4.30±0.92***
RG2TK+ tumor to nontarget ratios calculated from % I.D./g values							
RG2TK+/Muscle	0.70±0.37	0.84±0.41	1.37±0.79	0.68±0.51	0.77±0.23	1.66±0.55	12.96±3.11
RG2TK+/ Blood	1.13±0.57	1.19±0.47	1.78±0.60	1.33±1.03	1.43±0.34	1.81±0.55	4.29±0.80
RG2TK+/RG2	1.16±0.64	0.95±0.42	1.55±0.44	1.04±0.82	1.02±0.33	1.74±0.53	5.97±1.36

^a 20 µCi of [¹⁸F]1a-f and 5 µCi of [¹²⁵I]FIAU was co-injected *via* tail-vein while under isofluorane anesthesia; [¹⁸F]1a-f values are an average of n=6 and [¹²⁵I]FIAU values are an average of n=36; Statistical significance of RG2TK+ tumor uptake *versus* RG2 tumor uptake determined by unpaired Student's *t* test: *, *P*<0.05; **, *P*<0.005; ***, *P*<0.001.

^b Thyroid uptake in tumor-bearing nude mice was 355.41±227.357% I.D./organ.