Synthesis and Evaluation of [^{18}F] Labeled Pyrimidine Nucleosides for Positron Emission Tomography Imaging of Herpes Simplex Virus 1 Thymidine Kinase Gene Expression

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Synthesis of three novel 2′-deoxy-2′-[^{18}F]fluoro-1-β-D-arabinofuranosyluracil derivatives [^{18}F]FPAU, [^{18}F]-FBrVAU, and [^{18}F]FTMAU is reported. The compounds were synthesized by coupling of 1-bromo-2-deoxy-2-fluoro sugars with corresponding silylated uracil derivatives. In vitro cell uptake indicated that all three compounds are taken up selectively in RG2TK+ cells with negligible uptake in RG2 cells. The results indicate that [^{18}F]FBrVAU and [^{18}F]FTMAU have better uptake profiles in comparison to [^{18}F]FPAU and have potential as PET probes for imaging HSV1-tk gene expression.

Introduction

The herpes simplex virus 1 thymidine kinase (HSV1-tk) gene is one of the most widely used reporter genes for imaging gene regulation and expression using positron emission tomography (PET). We initially demonstrated the feasibility of using HSV1-tk as a reporter gene, with a radiolabeled probe[1] and by γ camera[2] and PET imaging.[3] This was subsequently confirmed by reports from other labs.[4,5] Thymidine kinases are enzymes that phosphorylate thymidine to thymidine 5′-monophosphate. HSV-tk gene encodes the HSV1-TK enzyme. Unlike mammalian thymidine kinase 1 (TK-1), which is highly specific for thymidine, HSV1-TK has a fairly broad range of substrate specificity.[6,7] HSV1-TK can phosphorylate thymidine nucleoside analogues, which have modifications on the heterocyclic ring or on the sugar moiety, and various acyclic analogues of guanosine.[6,7] This property has been utilized in gene therapy protocols involving HSV1-TK enzyme activated prodrug therapy, wherein the target tissue is transfected or transduced with HSV1-tk gene. Cells expressing HSV-tk activate prodrugs such as gancyclovir or acyclovir by phosphorylation, and their accumulation in the cell leads to apoptosis, whereas normal cells remain unaffected by these drugs. This type of gene therapy has been used for selective destruction of malignant tissue.[8−11]

A number of different PET reporter probes for imaging HSV-tk gene expression are currently available. These include ^{11}C, ^{18}F, or ^{124}I radiolabeled nucleoside analogues such as [^{11}C]-FMAU,[12] [^{124}I]FIAU,[3] [^{18}F]FHBG, [13,14] [^{18}F]FHPG,[13,14] [^{18}F]-FMAU,[13,15] etc. Although each probe offers certain advantages over others, there are also certain limitations. For example, our group demonstrated that 2′-fluoro-2′-deoxy-1β-D-arabinofuranosyl-5-[^{124}I]iodouracil ([^{124}I]FIAU) has excellent imaging properties for studying HSV-tk gene expression in transduced animals.[1−3] However, the long half-life of ^{124}I ($t_{1/2}$ = 4.28 days) may be a disadvantage where sequential and repetitive imaging is necessary.

Alauddin and co workers[16] and more recently Shields and co workers[17] have developed a general procedure for the synthesis of ^{18}F labeled, 5-substituted-2′-deoxy-2′-[^{18}F]fluoro-arabinofuranosyluracil derivatives, such as [^{18}F]FMAU, [^{18}F]-FBAU, [^{18}F]FAU, etc., and are currently evaluating their potential as PET markers for cellular proliferation. The 5-substituted thymidine derivatives have also been shown to be good substrates for HSV1-TK.[3,18−24] Our aim was to synthesize 5-substituted derivatives of 2′-fluoro-2′-deoxythymidine, with a range of different hydrophobicities, and then to evaluate them as potential radiolabeled PET reporter probes for imaging HSV1-tk gene expression. Hydrophobic derivatives are of interest because the currently available probes are hydrophilic and do not readily cross the intact blood−brain barrier.

A variety of 5-substituted thymidine derivatives were previously synthesized and evaluated for their potential as antiherpes agents.[18−24] The primary mechanism of action was based on the ability of herpes viral thymidine kinase to phosphorylate the thymidine derivatives, which in turn would be incorporated into viral DNA causing multistrand breaks leading to destruction of virus. We hypothesized that antiviral efficacy of a particular molecule is a good indicator of its rate of phosphorylation. Hence, the target molecules were chosen on the basis of the ability of the nonradioactive analogues to inhibit HSV1 viral replication. Here, we report the synthesis and in vitro evaluation of three novel radiolabeled probes 2′-[^{18}F]fluoro-2′-deoxy-5-bromovinyl-1β-D-arabinofuranosyluracil ([^{18}F]FBrVAU), 2′-[^{18}F]fluoro-2′-deoxy-5-propyl-1β-D-arabinofuranosyluracil ([^{18}F]-FPAU), and 2′-[^{18}F]fluoro-2′-deoxy-5-trifluoromethyl-1β-D-arabinofuranosyluracil ([^{18}F]FTMAU) for PET imaging of HSV-tk gene expression.

Materials and Methods

Chemicals. All reagents and solvents were purchased from Aldrich Chemical Co. (St. Louis, MO) and used without further purification, unless stated otherwise. The triflate sugar (2-*O*-(trifluoromethylsulfonyl)-1,3,5-tri-*O*-benzoyl-α-D-arabinofuranose) **1** was synthesized according to literature procedure[18] and used in the reactions. The silyl ethers 5-alkyl-2,4-bis-trimethylsilanyloxypyrimidine were synthesized in situ according to published procedure[19] and used in the reaction. Sep-Pak extraction cartridges were purchased from Waters Associates (Milford, MA). Radioactively labeled (^{3}H and ^{14}C) nucleosides were purchased from Moravek Biochemicals (Brea, CA).

Cell Lines. RG2 rat glioma cell line was kindly provided by Dr. Darell Bigner (Duke University Medical Center, Durham, NC). RG2 cells were transduced with the recombinant replication-deficient STK retrovirus containing the NeoR gene and HSV1-tk gene as previously described (see Supporting Information).[1] The

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Scheme 1. Synthesis of [^{18}F]FPAU, [^{18}F]FBrVAU, and [^{18}F]FTMAU

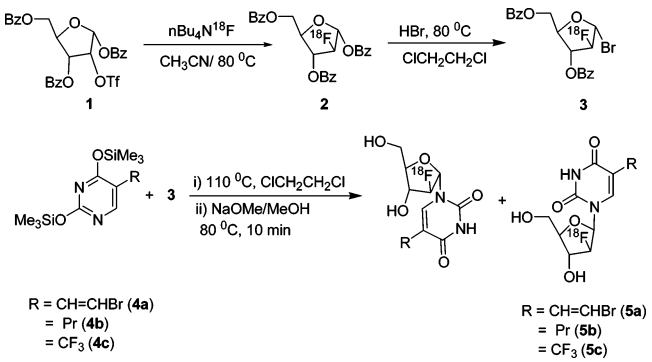

Table 1. Decay-Corrected Radiochemical Yields of [^{18}F]FBrVAU, [^{18}F]FPAU, and [^{18}F]FTMAU

compd	decay-corrected yield, %
[^{18}F]FBrVAU	12 ± 4
[^{18}F]FPAU	20 ± 3
[^{18}F]FTMAU	16 ± 3

transduced cell line, RG2TK+, has remained stable and has expressed constant levels of HSV1TK+ since 1994; this cell line has been characterized previously.[1,2]

Synthesis. [^{18}F]FBrVAU, [^{18}F]FPAU, and [^{18}F]FTMAU were synthesized by coupling the radiolabeled fluoro sugar with the corresponding silylated pyrimidine derivatives following a procedure reported by Alauddin and co-workers,[16] with minor modifications as shown in Scheme 1. Briefly, ^{18}F in the form of [^{18}F]HF, trapped on an anion-exchange resin, was eluted with 0.5 mL of 0.8% tetrabutylammonium bicarbonate into a flask containing 1 mL of acetonitrile. The water acetonitrile azeotrope was completely removed by heating the flask to 80 °C under reduced pressure while maintaining a slow stream of argon gas. To the flask, 1 mL of anhydrous acetonitrile was added, and the flask was dried as described above. The flask was cooled using dry ice−2-propanol slush, and 20 mg of 2-*O*-(trifluoromethylsulfonyl)-1,3,5-tri-*O*-benzoyl-α-D-arabinofuranose in 0.7 mL of anhydrous acetonitrile was added. The mixture was heated at 80 °C for 30 min. The mixture was cooled to room temperature and passed through a silica Sep-Pak plus column (preconditioned with 5 mL of hexane) under an argon atmosphere and eluted with ethyl acetate (2×1.5 mL). The ethyl acetate solution was dried as described earlier, and 0.4 mL of 1,2-dichloroethane and 0.1 mL 30% HBr in acetic acid were added. The reaction mixture was heated at 80 °C for 10 min, and 30% HBr in acetic acid was removed by azeotropic distillation with 1 mL of toluene. After ensuring complete removal of excess reagents and solvent, 50 mg of silyl ether derivative in 0.7 mL of 1,2-dichloroethane was added under an argon atmosphere, and the reaction mixture was heated to 110 °C for 1 h. Then reaction mixture was cooled and passed through a silica Sep-Pak plus column (preconditioned with 5 mL of hexane) under an argon atmosphere and eluted with 10% MeOH in CH_2Cl_2 (2×1.25 mL). The solvent was removed, and 0.4 mL of 0.5 M sodium methoxide in MeOH was added. The reaction mixture was heated at 80 °C for 10 min and then cooled to room temperature. The reaction mixture was neutralized with 0.1 mL of 2 N HCl in MeOH, and the solvent was removed under reduced pressure. The products, including the α and β anomers, were purified using reverse-phase HPLC using a C-18 column (250×10 mm) with an appropriate HPLC solvent (12% ethanol in 0.01 M saline solution for [^{18}F]-FBrVAU, 8% ethanol in 0.01 M saline solution for [^{18}F]FPAU, and 5% ethanol in 0.01 M saline solution for [^{18}F]FTMAU) at a flow rate of 7 mL/min. The radiochemical purity of the product was >98% with decay-corrected yields of 12−20% (see Table 1). The specific activity of the compounds was >58 GBq/μmol. The total time of synthesis was about 180 min. The nonradioactive analogues were prepared using a similar procedure and characterized using NMR and mass spectrometry (see Supporting Information).[20,21,24] The purified radioactive samples were co-injected with pure nonradioactive analogues and had very similar retention times, ensuring the chemical identity and purity of the compounds.

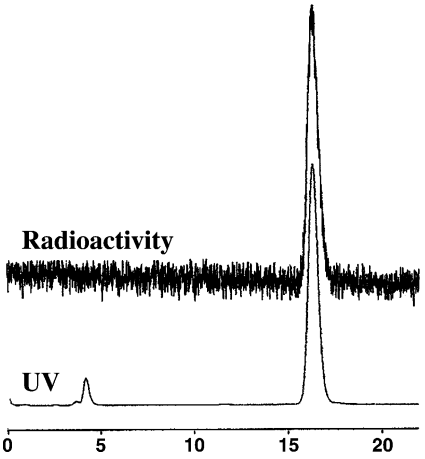

Figure 1. HPLC chromatogram of purified [^{18}F]FBrVAU co-injected with its nonradioactive analogue.

Octanol/Water Partition Coefficient Study. Octanol/water partition coefficients were determined for [^{18}F]FBrVAU, [^{18}F]-FPAU, and [^{18}F]FTMAU at four different pH values of 6.4, 7.0, 7.4, and 8.4 by measuring the distribution of radiolabeled compound in 1-octanol and phosphate buffered saline (PBS). A 20 μL sample of radiolabeled compound ([^{18}F]FBrVAU, [^{18}F]FPAU, or [^{18}F]-FTMAU) in saline was added to a vial containing 0.5 mL each of 1-octanol and PBS. After vortexing for 1 min, the vial was centrifuged for 5 min to ensure complete separation of layers. Then 20 μL of each layer was taken in a preweighed vial and ^{18}F counts (400−550 keV energy range) were measured using an AutoGamma 5550 spectrometer (Packard Instruments, Meriden, CT). Counts per unit weight of sample were calculated, and log *P* values were calculated using the formula

$$\log_{10} P = \log_{10}\left(\frac{\text{counts in 1g of octanol}}{\text{counts in 1 g of water}}\right)$$

In Vitro Studies. RG2TK+ and RG2 cells in culture were used to compare the accumulation of [^{14}C]FIAU, [^{3}H]thymidine, and each of the thymidine analogues [^{18}F]FBrVAU, [^{18}F]FPAU, and [^{18}F]-FTMAU, as previously described.[1] Paired time-course radiotracer accumulation experiments were run over 120 min, and the data were normalized to [^{3}H]TdR accumulation. The incubation medium contained [^{14}C]FIAU (0.37 kBq/mL), [^{18}F]FBrVAU, [^{18}F]FPAU (10 kBq/mL), or [^{18}F]FTMAU and [^{3}H]TdR (3.7 kBq/mL). The radioactivity assay for the in vitro tissue culture studies involved ^{18}F γ-counting (AutoGamma 5550 spectrometer; Packard Instruments, Meriden, CT) immediately after the experiment, followed 24−36 h later by ^{3}H and ^{14}C β-isotope counting (Tri-Carb liquid scintillation analyzer, model 1600TR; Packard Instruments) using external standard quench correction and standard dual-counting techniques. All data were expressed as disintegrations per minute (dpm)/g cells (or dpm/mL medium). The net accumulation rate was calculated from the slope of the probe accumulation versus time plot ((dpm/g cells)/(dpm/mL medium) vs time; units = (mL medium/min)/g cells − a medium clearance constant).

Results

Synthesis. The synthesis of [^{18}F]FBrVAU was carried out with minor modifications to the procedure previously reported.[16] Although the reaction time was almost the same as that reported previously,[16] the application of a vacuum resulted in complete drying of the products during the intermediate steps, and this ensured consistent yields. Both α and β anomers were obtained during the synthesis, and the biologically useful β anomer was

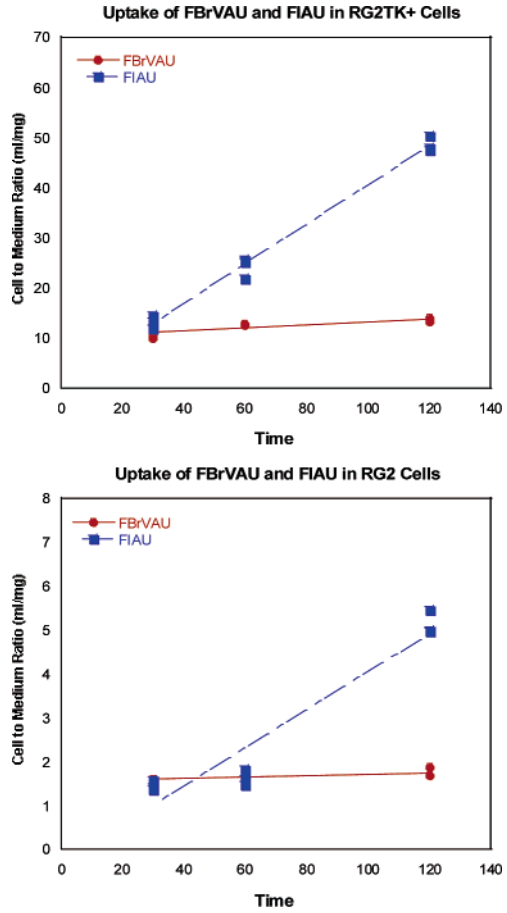

Figure 2. Typical in vitro uptake profile of [^{14}C]FIAU and [^{18}F]-FBrVAU in RG2TK+ and RG2 cells.

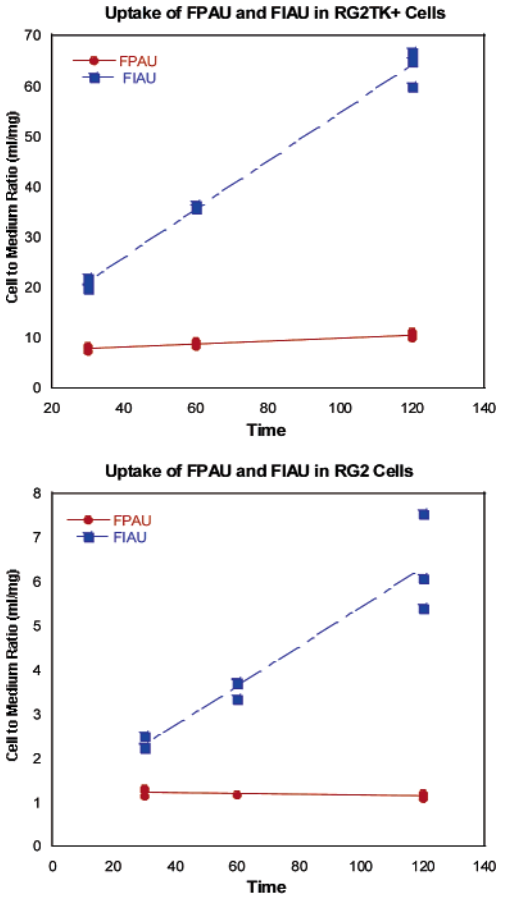

Figure 3. Typical in vitro uptake profile of [^{14}C]FIAU and [^{18}F]FPAU in RG2TK+ and RG2 cells.

obtained as the major product. The ratio of anomers obtained varied slightly between 1:4 and 1:5 (α/β). This method was easily extended for the synthesis of [^{18}F]FPAU and [^{18}F]FTMAU and did not result in any significant difference in the ratio of the anomers. The yields varied slightly, but the method has not been optimized for each individual compound. The nonradioactive (^{19}F) analogues were synthesized using a similar procedure, and they were characterized by NMR and mass spectrometry (see Supporting Information). The results corresponded to the reported values.[20,21] The purified compounds were used as reference standards for HPLC analysis. Figure 1 shows the HPLC chromatogram of purified [^{18}F]FBrVAU co-injected with its nonradioactive analogue.

Octanol/Water Partition Coefficient Studies. The log of the octanol/water partition coefficients (log *P*) of [^{18}F]FBrVAU, [^{18}F]FPAU, and [^{18}F]FTMAU are 1.02 ± 0.06, 0.50 ± 0.03, and 0.03 ± 0.03 at pH 7.4, respectively. The corresponding value for FIAU is −0.10 ± 0.01 at pH 7.4. The higher log *P* values for [^{18}F]FBrVAU and [^{18}F]FPAU compared to FIAU is in accordance with the observed trend of increasing alkyl chain length corresponding with increasing lipophilicity. The low log *P* value of [^{18}F]FTMAU probably reflects the high polarizing effect of the trifluoromethyl group, which can impart a slight ionic character to the molecule.

In Vitro Uptake Studies. In vitro uptake studies of [^{18}F]-FBrVAU, [^{18}F]FPAU, and [^{18}F]FTMAU were performed concurrently in HSV1-TK expressing RG2TK+ cells and in wild-type RG2 cells (negative control). Two reference tracers, [^{3}H]-TdR (for cell viability) and [^{14}C]FIAU (for HSV1-TK expression), were also included in the incubation medium for paired comparisons. The results of these uptake studies are shows in Figures 2−4. It is apparent from the plots that [^{18}F]FBrVAU, [^{18}F]FPAU, and [^{18}F]FTMAU are accumulated only in RG2TK+ cells and not in RG2 cells. The uptake rates for [^{18}F]FBrVAU, [^{18}F]FPAU, and [^{18}F]FTMAU in RG2TK+ and RG2 cells were calculated from the slopes of the plots and normalized to that of TdR[1] (Table 2). The rate of uptake follows the order [^{18}F]-FTMAU > [^{18}F]FBrVAU > [^{18}F]FPAU. The corresponding values for FIAU, which is considered as a reference standard, are also included in Table 2.

Discussion

Medical imaging (radionuclide, magnetic resonance, and optical) has undergone extraordinary changes in the past decade. This has largely been due to improved imaging technology and to convergence with complementary advances in molecular biology and cell biology. Noninvasive reporter gene imaging is now widely applied in preclinical studies,[1−3,25−27] and initial clinical applications are now being reported.[28,29] Reporter gene constructs driven by constitutive promoter elements can be used to monitor transduced cells in adoptive therapies and gene therapy vectors. Inducible promoters function as sensor elements and can be used to provide information about endogenous gene regulation,[30,31] mRNA stabilization, and specific protein−protein interactions.[32−34] In addition, the promoter can also be cell-specific, restricting expression of the transgene to certain tissue and organs. These "molecular images" can provide spatial, temporal, and functional information about various biological processes at the cellular level, averaged over the resolution element of the acquired image.

Radiotracer-based reporter imaging (γ camera or PET) involves a complementary "reporter gene" and "reporter probe".

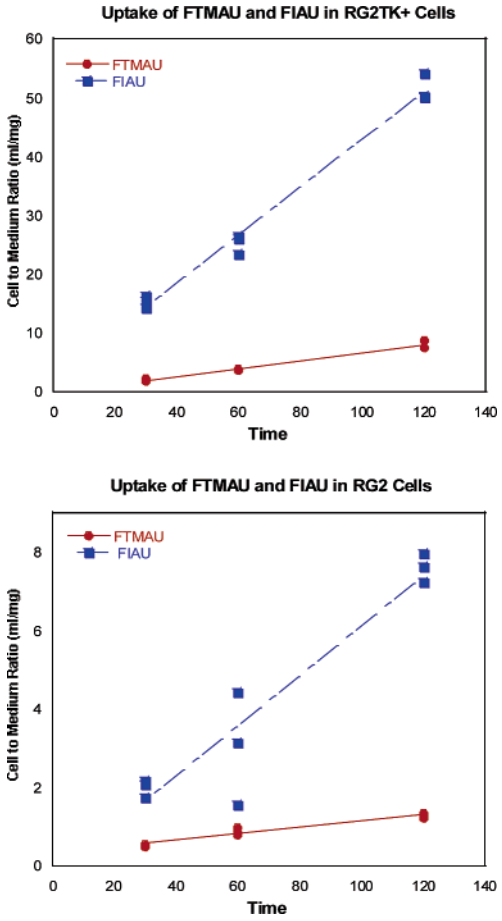

Figure 4. Typical in vitro uptake profile of [^{14}C]FIAU and [^{18}F]-FTMAU in RG2TK+ and RG2 cells.

Table 2. Comparison of Normalized Accumulation Rates in RG2TK+ and RG2 Cell Lines

compd	RG2TK+, mL mg^{-1} min^{-1}	RG2, mL mg^{-1} min^{-1}	RG2TK+/ RG2	*n* (no. expts)
[^{14}C]FIAU	0.274 ± 0.095	0.048 ± 0.036	~5.7	10
[^{18}F]FBrVAU	0.035 ± 0.017	−0.001 ± 0.001		4
[^{18}F]FPAU	0.014 ± 0.003	−0.001 ± 0.001		3
[^{18}F]FTMAU	0.051 ± 0.005	0.004 ± 0.001	~13	3

The reporter gene product is frequently an enzyme that converts a radiolabeled substrate (the "reporter probe") to a metabolite that is selectively trapped within transduced cells. Alternatively, the reporter gene product can be a receptor or transporter that "irreversibly" traps the radiolabeled probe in transduced cells during the period of image acquisition.

Three novel pyrimidine analogues were developed with the goal to attain higher lipophilicity than FIAU to facilitate passive diffusion across cell membranes and across the intact blood−brain barrier while retaining selective phosphorylation by HSV1-tk. [^{18}F]FBrVAU, [^{18}F]FPAU, and [^{18}F]FTMAU were synthesized in good yields following a procedure developed by Alauddin et al. The yields obtained for each of the compounds were consistent (Table 1). The α/β anomeric ratio of the compounds varied between 1:4 to 1:5. The total time for synthesis was about 3 h from EOB.

As expected, the log *P* values of [^{18}F]FBrVAU and [^{18}F]-FPAU indicate that both of these compounds are significantly more hydrophobic compared to FIAU (log $P = -0.10$) and reflects the longer carbon chain at the 5-position. On the basis of log *P*, FBrVAU and FPAU would be expected to cross cell membranes and the blood−brain barrier more readily by passive diffusion (noncarrier mediated transport) than FIAU.

Table 3. Comparison of Normalized Accumulation Rates in RG2TK+ and RG2 Cell Lines

compd	RG2TK+, mL mg^{-1} min^{-1}	RG2, mL mg^{-1} min^{-1}	RG2TK+/ RG2
[^{14}C]FIAU	0.31 ± 0.07	0.036 ± 0.012	~8.6
[^{18}F]FFEAU	0.29 ± 0.05	0.00053 ± 0.00058	~550
[^{18}F]FHPG	0.0077 ± 0.0003	0.0004 ± 0.0003	~19
[^{18}F]FHBG	0.022 ± 0.001	0.0032 ± 0.0026	~6.9

All three compounds demonstrate rapid equilibration across cell membranes and selective accumulation in HSV1-tk transduced RG2 cells compared to wild-type RG2 cells. Although the net accumulation rates were not as high as that reported for FIAU[1] and FFEAU,[35] they are substantially higher or comparable to that obtained with FHBG and FHPG in the same cell lines[14] (Table 3). Importantly, all three compounds have very low uptake in wild-type RG2 cells in comparison to that obtained with FIAU. The uptake ratio of FTMAU (RG2TK+/RG2) is comparable to that obtained with FIAU and with FHBG and FHPG (Tables 2 and 3). The uptake ratio (transduced to nontransduced cells) of FBrVAU and FPAU is likely to be even higher (more comparable to that of FFEAU) because of the very low uptake in wild-type RG2 cells. Thus, background activity due to phosphorylation by endogenous mammalian thymidine kinase in rapidly proliferating tissue would be expected to be low in comparison to that with FIAU, and this will contribute to higher specificity for HSV1 thymidine kinase activity.

Conclusion

These results demonstrate that the synthetic procedure developed by Alauddin et al. for the synthesis of 2′-deoxy-2′-[^{18}F]fluoro-1β-D-arabinofuranosyl-5-methyluracil can easily be extended to other 5-alkyl substituted analogues without a significant loss in yields or changes in the anomeric ratio. The in vitro experiments reveal that [^{18}F]FBrVAU and [^{18}F]FTMAU are good substrates for the HSV1-tk expressing RG2TK+ cells. The higher hydrophobicity (log $P = 1.02$ at pH 7.4) of [^{18}F]-FBrVAU indicates that it has a better chance of passively diffusing across cell membranes and crossing an intact blood−brain barrier than FIAU or the other radiolabeled HSV1-TK probes. The hydrophilicity and pH dependency of [^{18}F]FTMAU may limit its use as a PET probe for imaging HSV1-tk gene expression in non-CNS tissue.

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Supporting Information Available: Spectroscopic characterization data of nonradioactive analogues of **5a**−**c** and details on cell cultures, transduction, and in vitro uptake study. This material is available free of charge via the Internet at http://pubs.acs.org.

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