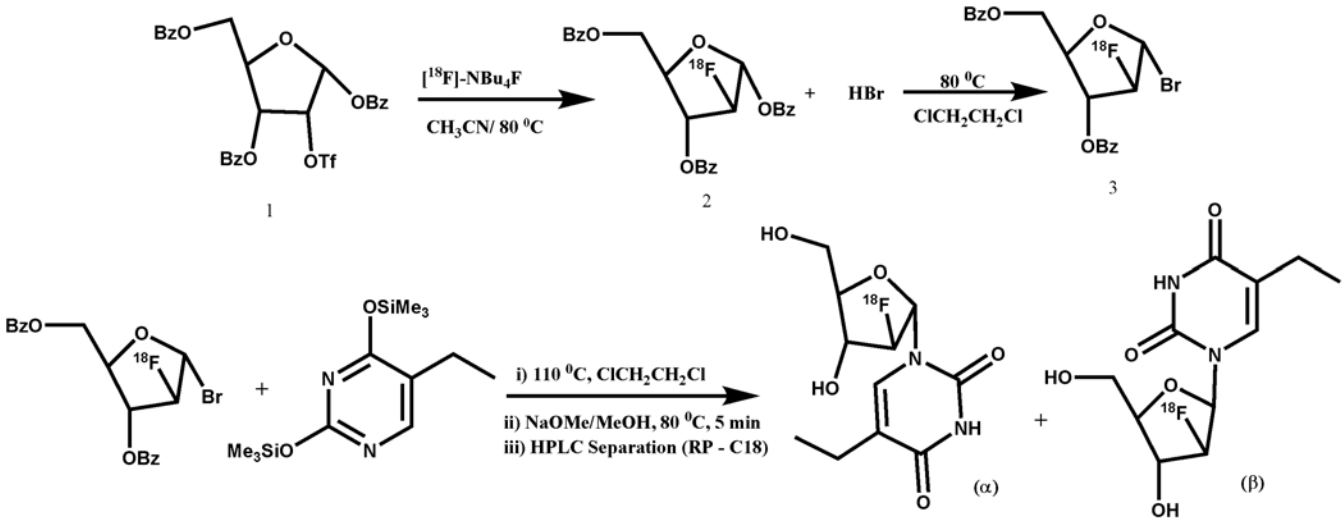
[^{18}F]FEAU Synthesis

With minor chemical modifications, [^{18}F]FEAU was synthesized by coupling the radiolabeled fluoro-sugar to the silylated pyrimidine derivative following a procedure reported by Alauddin and coworkers [**1**]. Briefly, ^{18}F produced from the proton reaction with an oxygen-18 enriched water target in the form of [^{18}F]HF and was absorbed on an anion exchange resin then subsequently eluted with 0.5 mL of 0.8% tetrabutylammonium bicarbonate into a v-vial. The solvent was evaporated azeotropically using acetonitrile by heating the vial to 80°C under reduced pressure. To the dry residue, 25 mg of 2-*O*-(trifluoromethylsulfonyl)-1,3,5-tri-*O*-benzoyl-α-D-arabinofuranose in 0.8 mL of anhydrous acetonitrile was added. The reaction mixture was heated at 80°C for 30 minutes. The reaction mixture was cooled to room temperature, passed through a silica Sep-Pak® Plus column (pre-conditioned with 5 mL of hexane) and eluted with ethyl acetate (2 × 1.5 mL). The ethyl acetate solution was dried at 80°C under reduced pressure. To the vial was added 0.4 mL of 1,2-dichloroethane and 0.1 mL 30% hydrogen bromide in acetic acid and the reaction mixture was heated at 80°C for 10 min. Toluene (1 mL) was added to the reaction mixture and the hydrogen bromide/acetic acid was removed azeotropically. After ensuring complete removal of both excess reagents and solvent, the freshly prepared 2,4-bis-*O*-trimethylsilyl-5-ethyl-thymine (40 mg) in 1 mL of 1,2-dichloroethane was added and the reaction mixture was heated at 110°C for 1 h. The reaction mixture was cooled to room temperature, passed through a silica Sep-Pak® Plus column (pre-conditioned with 5 mL of hexane) and eluted with 10% methanol in dichloromethane (2 × 1.25 mL). The solvent was removed as previously described and 0.4 mL of 0.5 M sodium methoxide in methanol was added, heated at 80°C for 5 min, with subsequent neutralization with 0.1 mL of 2 N HCl in MeOH. After the solvent was removed under reduced pressure, the product was diluted with HPLC solvent and purified by HPLC. Synthesis time was ~180-220

minutes and the yield varied between 10-30%. The specific activity of the product was ~37 GBq/μmol (~1 Ci/μmol) and radiochemical purity was >98% following purification by HPLC. The product was formulated in 0.9% NaCl containing 5% ethanol to a total volume based on about 10 mCi per mL and terminally sterilized by filtration. The final formulation was a clear colorless solution with a pH around 7.0.



Supplement Figure 1. Radiosynthesis of [^{18}F]FEAU

[^{18}F]FFEAU Synthesis

The preparation and radiolabeling of 2'-[^{18}F]fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-(2-fluoroethyl)-uracil (FFEAU) with ^{18}F was recently described [**2**] and a short summary follows. Fluorine-18 was either produced from enriched [^{18}O]H_2O by the ^{18}O(p,n)^{18}F nuclear reaction using the MSKCC CS-15 cyclotron or was purchased from a commercial source. The aqueous [^{18}F]HF was resolubilized as tetrabutylammonium [^{18}F]fluoride, by mixing aliquots of [^{18}F]fluoride ion with tetrabutylammonium hydroxide (0.5 μL, 1.0 M solution in water) and tetrabutylammonium fluoride (0.5 μL, 1.0 M solution in THF), followed by acetonitrile addition,

then azeotropic distillation was carried out at 110°C under nitrogen gas flow. Further azeotropic distillation with acetonitrile was conducted to complete the drying of the [^{18}F]fluoride ion.

The dried activity was redissolved in anhydrous acetonitrile (200 μL) and transferred to a Vacutainer® containing 2'-fluoro-2'-deoxy-3',5'-di-*O*-benzoyl-1-β-D-arabinofuranosyl-3-*N*-benzoyl-5-(2-(2,2,2-trifluoroethanesulfonyloxy)ethyl)-uracil (0.5-1.0 mg). The Vacutainer® was capped, vortexed, then heated for 30 min at 85°C. After cooling, the reaction mixture was treated in one of two ways. Method A was to load the reaction mixture onto a Silica Gel Sep-Pak® Plus cartridge and elute with 5 mL of dichloromethane/methanol (9:1). Method B was to load the reaction mixture onto a Accell Plus QMA Sep-Pak® (either Plus or Light size) that was pre-conditioned with saturated sodium bicarbonate solution and then elute with acetonitrile (10 mL). The eluted solutions were evaporated under reduced pressure and in most cases were analyzed by radio-HPLC to check for $^{18}F^-$ incorporation.

The evaporated product mixture was redissolved in anhydrous methanol (300 μL) and transferred to a Vacutainer®, then 4 μL of sodium methoxide (0.5 M in methanol) was added. The Vacutainer® was capped, vortexed, then heated for 5 min at 70°C. After cooling briefly, 4.0-4.5 μL of aqueous HCl (0.5 N) solution was added to neutralize the mixture. The methanol was evaporated under a stream of nitrogen gas flow with minimal heating. The product residue was dissolved in HPLC mobile phase and injected onto a C-18 semi-preparative column. The product fraction was collected and concentrated by rotary evaporation with mild heating. The residue was formulated either in ethanol (for *in vitro* studies) or in 0.9% sodium chloride solution, with 5% ethanol added, for *in vivo* studies. Specific activity values were estimated to be in the order of 1.5-2.6 GBq/μmol (0.04-0.07 Ci/μmol), based on carrier *n*-

Bu_4NF added and the radiochemical yields obtained, and radiochemical purity was 95% as determined by radio-TLC [**2**].

Positron Emission Tomography

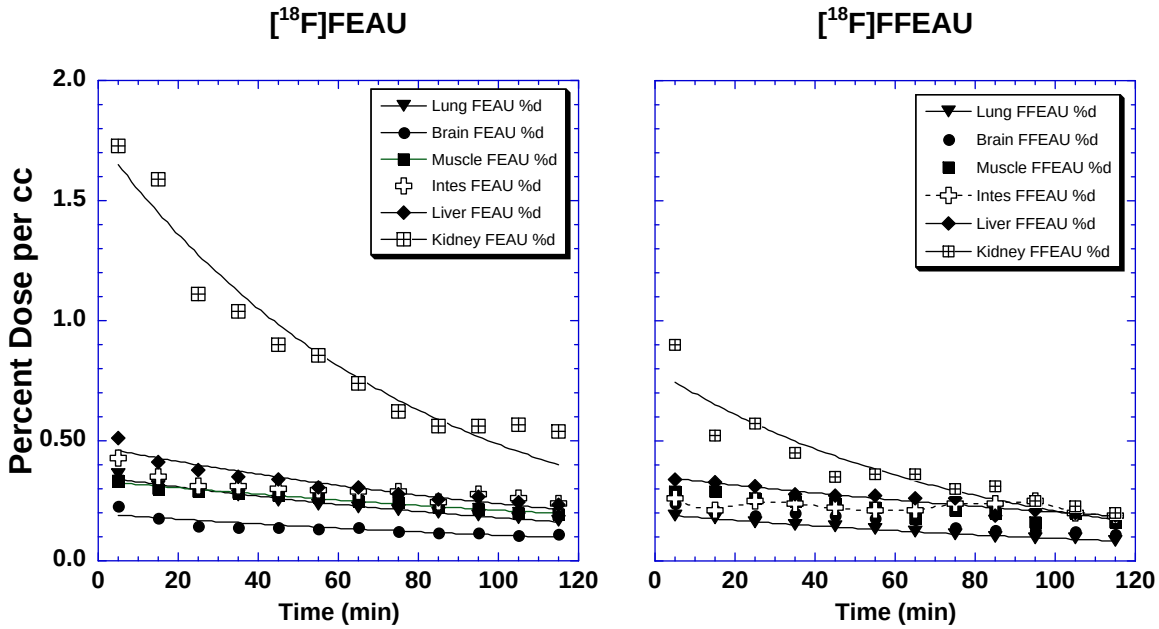
PET imaging was performed using the GE Advance® Tomograph (General Electric, Milwaukee, WI); with a spatial resolution of 5mm FWHM (full width half maximum) at the center of the field of view (FOV). The camera was previously cross-calibrated with the AutoGamma 5550 spectrometer (Packard, CT). Measured attenuation correction was performed using a 7-minute duration transmission scan with two 9 mCi germanium transmission sources. 2-D emission scans were performed for all studies.

Dynamic emission data was obtained from 5 minutes post injection; acquisition frames were 10 minutes in duration. Emission counts were corrected for random co-incidences, dead time, scatter and decay-corrected to time of injection. Emission scans were reconstructed using an iterative reconstruction method with measured attenuation correction, smoothed with an 8 mm Gaussian filter. The reconstruction parameters were 28 subsets, 2 iterations in a 256x256 matrix using a loop filter of 2.15mm FWHM and a post filter of 3.0 mm FWHM. Regional tumor radioactivity concentrations (% ID/cc) were estimated from the maximum pixel within regions of interest drawn around the tumor on transaxial slices of the reconstructed image sets and the radioactivity measurement was plotted at the acquisition time midpoint.

Additional Results

The uptake and clearance profiles of [^{18}F]FEAU- and [^{18}F]FFEAU-derived radioactivity in various organ structures were obtained from the dynamic PET imaging data (Fig. 1). The clearance half-times were estimated and the values are given in Table 1. The half-times for [^{18}F]FEAU and [^{18}F]FFEAU clearance were similar (~2 hours) in most organs, except for intestine, and were slightly more rapid than that from the RG2 tumors. Renal clearance was

rapid and bladder radioactivity was high; increasing from 2 to 20 %dose/cc for FEAU and from 3 to 21 %dose/cc for FFEAU over the 120 minute experimental period.



Supplement Figure 2. Activity-time profiles of [^{18}F]FEAU and [^{18}F]FFEAU in different organs

Supplement Table 1

Clearance of Radioactivity from Different Organs

	[^{18}F]FEAU	[^{18}F]FFEAU
	half-time	**half-time**
Organ	(min)	(min)
RG2 tumor	126 ± 7	149 ± 9
Brain	105 ± 2	114 ± 19
Lung	103 ± 5	93 ± 2
Muscle	152 ± 7	129 ± 15
Liver	100 ± 10	128 ± 11
Intestine	163 ± 26	-
Kidney	54 ± 4	51 ± 7

Values are estimates from a single exponential fit of mean values shown in Fig. 5, ± standard error of the estimate.

Comparison of HSV1-*tk* and HSV1-*sr39tk* imaging probes

A major problem with comparing different radiolabeled pyrimidine nucleoside and guanosine-based probes is the absence of any reference or normalization to the amount of HSV1-*tk* or HSV1-*sr39tk* gene product (enzyme) present in the transduced cells or tumors used in the comparative studies. Most studies only report a comparison between transduced and wild-type cells or tumors, and present no independent measure of HSV1-TK enzyme activity in the transduced cell line or tumor that is used to test efficacy. This is illustrated by one study [**3**], where the uptake of 4 radiolabeled probes (FEAU, FIAU, FMAU and PCV) are compared in 3 transduced and non-transduced cell lines (C6tk/C6, C6sr39tk/C6 and MH3924A-*tk*+/ MH3924A). A comparison of the results from this study presented in Table 2 suggests that the levels of tk and sr39tk expression in the two transduced C6 cell lines are quite different, and that the results do not reflect an accurate comparison between the 4 radiolabeled probes for imaging tk and sr39tk expression.

Our *in vivo* results were also compared to other recent studies that assessed the uptake of different pyrimidine and acycloguanosine radiotracer in different HSV1-*tk* and HSV1-*tksr39* transduced tumors (Table 2). Despite the differences between these studies, including the use of adenoviral transduced cell lines and tumors, it is clear that FEAU shows consistent high levels of accumulation in transduced cells and tumors, with little or no accumulation in wild-type cells and tumors. The picture is less consistent in a comparison of the animal models. This may reflect differences in the host animal (rnu/rnu rat vs athymic nude mouse), radionuclide and site of labeling (eg., [^{18}F]FEAU vs [^{3}H]FEAU), or other factors (e.g., image roi measures vs tissue sampling and well counting).

Supplement Table 2

	In vitro **nKi ratio**	***In vivo*** (rat) (image roi) **2 h uptake ratio**	***In vitro*** [§] **2 h uptake ratio**	***In vivo*** [§] (mouse) (tissue sample) **2 h uptake ratio**	***In vitro*** [‡] **2 h uptake ratio**	***In vitro**** **2 h uptake ratio**		
Thymidine Analogs[†]	(RG2TK+ / RG2)	(RG2TK+ / RG2)	(HT29tk / HT29)	(HT29tk / HT29)	(C6tk / C6)	(C6tk / C6)	(C6 sr39tk / C6)	(MH3924A-*tk*+ / MH3924A
2'fluoro arabinosyl								
[^{18}F]FEAU	660 ± 335	11.5 ± 1.5			85			
[^{3}H]FEAU			121	5.0		~1.8*	~340*	~360*
[^{18}F]FFEAU°	192 ± 56	12.2 ± 1.4						
[^{18}F]FTMAU[+]	39 ± 21							
[^{18}F]FIAU					10.3			
[^{14}C]FIAU	8.2 ± 2.2		43	6.8		~2.8*	~13*	~26*
[^{124}I]FIAU		4.3 ± 1.1 (65 ± 21)*						
[^{18}F]FBrAU				7.9				
[^{3}H]FBrAU	2.8 ± 1.3							
[^{18}F]FMAU					2.4			
[^{3}H]FMAU	3.3 ± 0.9							
[^{14}C]FMAU			14-16^	4.9		~2.1*	~3.9*	~12*
[^{3}H]FFAU	3.9 ± 0.6		81[#]	9.8				
2' fluoro ribosyl								
[^{3}H]FIRU	78 ± 43							
[^{3}H]FMRU	24 ± 5							

3'fluoro ribosyl								
[^{18}F]FLT					1.6			
[^{14}C]FLT	0.55 ± 0.05							
ribosyl								
[^{3}H]BrUdR	1.14 ± 0.13							
[^{3}H]IUdR	1.20 ± 0.03							
Acyclo Guanosine Analogs								
[^{18}F]FHBG$^{\#}$	11.0 ± 8.8	2.4 ± 0.3	9-14^		~41			
[^{18}F]FHPG$^{\#}$	7.6 ± 2.4	1.2 ± 0.4	2-3^					
[^{3}H]GCV^	6.4 ± 1.9							
[^{3}H]ACV^	1.8 ± 0.2				~5.5			
[^{3}H]PCV						~1.9*	~316*	~14*

Values are the mean, ± standard deviation

† Analogs are listed in the same order as in Table 2

° Includes three separate experiments previously reported [**15**] Balatoni

^ From [**4**]

From [**5**]

+ From [**6**]

§ From **[7]**

‡ From **[8]**

* From **[3]**

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