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Advances in Brief

Monitoring of Herpes Simplex Virus Thymidine Kinase Enzyme Activity Using Positron Emission Tomography[1]

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Abstract

9-[(1-[^{18}F]Fluoro-3-hydroxy-2-propoxy)methyl]guanine ([^{18}F]FHPG) was evaluated as a tracer for noninvasive positron emission tomography (PET) imaging of herpes simplex virus type 1 thymidine kinase (HSV-tk) gene expression. C6 rat glioma cells with and without the HSV-tk gene were incubated with [^{18}F]FHPG for 2 h. The *in vitro* tracer uptake in HSV-tk-containing C6tk cells was 35 ± 5 times higher than that in control cells. In nude rats carrying both a C6 and a C6tk tumor, the average ratio of tracer accumulation between the tumors was 15 ± 5 at 2 h postinjection. The tracer is rapidly cleared from nontarget tissue into the urine because only the HSV-tk-expressing tumor, kidneys, and bladder remained visible on the late PET images. HPLC analysis revealed that three metabolites, tentatively assigned as FHPG mono-, di-, and triphosphate, were formed in the C6tk tumors only. In conclusion, we have demonstrated that [^{18}F]FHPG is a promising tracer for monitoring HSV-tk enzyme activity *in vivo* with PET.

Introduction

The HSV-tk[3] gene has been explored as a reporter and/or suicide gene by several groups (1, 2). Both gene therapy with HSV-tk and the use of this gene as a marker are currently applied in patients with various forms of cancer (3, 4). However, the conditions for clinical gene therapy protocols are not yet optimal.

A method to monitor the activity of HSV-tk *in vivo* would be extremely useful to optimize clinical gene therapy protocols.

Scintigraphic imaging, like PET and single photon emission computed tomography, can offer information about both the extent of gene expression and its distribution, provided that an appropriate reporter gene is included in the therapeutic cassette (5). As compared with single photon emission computed tomography, PET imaging offers a higher resolution and sensitivity and allows noninvasive quantification of biological processes (6).

Several radiolabeled thymidine and ganciclovir derivatives have been proposed as probes for imaging of HSV-tk enzyme activity with PET, including [^{124}I]FIAU (6), 8-[^{18}F]fluoroganciclovir (7), and [^{18}F]FHPG (8, 9). These radiopharmaceuticals are better substrates for the HSV-tk enzyme than for human thymidine kinases (10). After phosphorylation by HSV-tk, the tracer is trapped in HSV-tk-expressing cells only and can be visualized externally with a PET camera.

Preliminary studies showed [^{18}F]FHPG to be a potential tracer for HSV-tk imaging with PET. *In vitro*, high and selective accumulation of [^{18}F]FHPG in several HSV-tk-expressing cell lines was observed (8, 11, 12). The enantiomers of [^{18}F]FHPG did not exhibit any significant differences in cellular accumulation kinetics (13). The first *in vivo* data showed that tumor uptake was 3- and 6-fold higher in mice bearing a HSV-tk-expressing HT-29 human colon cancer than in mice with a control tumor at 2 and 5 h after injection of [^{18}F]FHPG, respectively (8). Despite these promising results, no *in vivo* [^{18}F]FHPG PET studies have yet been reported. In this study, we have performed biodistribution and PET studies on rats bearing both a HSV-tk-expressing tumor and a control tumor in their flanks to further substantiate the feasibility of monitoring HSV-tk enzyme activity using [^{18}F]FHPG PET.

Materials and Methods

Chemicals. DMEM (catalogue number 42430-025), G418, and FCS were purchased from Life Technologies, Inc. (Paisley, United Kingdom). Matrigel was from Becton Dickinson (Bedford, MA). Polyclonal rabbit anti-tk antibody was purchased from Dr. W. C. Summers (Yale University School of Medicine, New Haven, CT). The packaging cell PA317-tk was a gift from Dr. S. G. Marcus (Genetic Therapy Inc., Gaithersburg, MD).

Preparation of [^{18}F]FHPG. [^{18}F]FHPG was prepared as described by Alauddin *et al.* (14). However, the deprotection was performed at 90°C for 5 min (15), and the radiopharmaceutical was purified by HPLC over a semi-preparative Alltima C_{18} reverse-phase column (5 μ, 10 × 250 mm; Alltech) using 2% ethanol and 10 mM NaH_2PO_4 in 0.9% NaCl as the eluent (flow rate, 6 ml/min, R_t, 21 min). The product was obtained with a specific activity of >20 TBq/mmol and a radiochemical purity of >99% (HPLC).

Cell Lines. C6 rat glioma cells obtained from the American Type Culture Collection were cultured in monolayers in DMEM supplemented with 10% FCS in a humidified atmosphere with 5% CO_2 at 37°C. HSV-tk-positive C6 cells (C6tk+) were obtained by transfection of the C6 cells with supernatant of PA317-tk packaging cells containing replication incompetent retroviruses carrying the HSV-tk gene and the NeoR gene. Subsequent G418 selection resulted in the C6tk+ cell line. Stable resistance in the C6tk+ cells was assured by culturing this cell line in the presence of G418 (0.5 mg/ml).

Ganciclovir Sensitivity Assay. Ganciclovir sensitivity was tested *in vitro* with a cytotoxicity assay. In a 6-well plate, 500 cells per well were incubated with ganciclovir. The concentration range of ganciclovir was 10^{-3} to 10^3 μg/ml. On day 7, the number of cells was counted. Three independent experiments were performed, each in triplicate. From the survival curves, the ganciclovir concentrations that inhibited cell survival by 50% (IC_{50}) were determined.

Animal Model. Female nude rats (HSD Ham RNU rnu; Harlan, the Netherlands; body weight, 140–200 g; age, 6 weeks) were injected with tumor cells. Before injection, C6 and C6tk+ cells (5 × 10^6cells/0.1 ml of DMEM/10% FCS) were mixed with 0.1 ml of Matrigel. Subsequently, the C6 and C6tk+ cell suspensions were injected s.c. into the right and left flank close to the forelegs of the nude rat, respectively. Ten to 14 days after injection of the tumor cells, a solid tumor nodule of 0.7–3 cm in diameter had grown in each flank. In this experimental setting, the animal carried both the HSV-tk-containing tumor and the control tumor, which minimizes the effects of biodiversity because each animal serves as its own control. All studies were carried out in compliance with the local ethical guidelines for animal experiments. The protocols were approved by the local Animal Ethics Committee.

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[3] The abbreviations used are: HSV-tk, herpes simplex virus type 1 thymidine kinase; FHPG, 9-[(1-fluoro-3-hydroxy-2-propoxy)methyl]guanine; FIAU, 2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-5-iodo-uridine; HPLC, high-performance liquid chromatography; PET, positron emission tomography; ROI, region of interest; tk, thymidine kinase; p.i., post-injection.

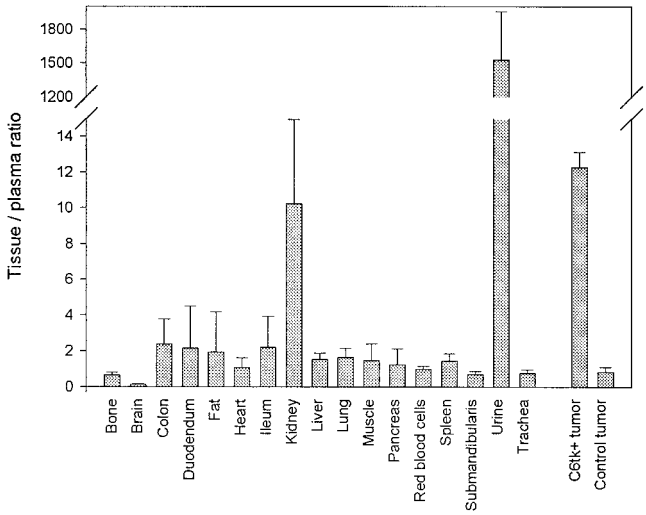

Fig. 1. Biodistribution of [^{18}F]FHPG 2 h after i.v. injection.

Tumor Histology. To allow collection of histological data from the C6 and C6tk+ tumors, these tumors were excised and frozen in liquid nitrogen. Four-μm slices were cut from the frozen tissue. The cell type and viability were determined on H&E-stained slides.

Immunohistochemistry for HSV-tk Expression. The HSV-tk expression in cell lines and tumor tissue was tested immunohistochemically with the HSV-tk-directed antibody. Frozen tissue sections were fixed in acetone for 10 min and air-dried. After three washes with PBS, sections were incubated in 0.075% hydrogen peroxide in PBS [140 mM NaCl, 9 mM Na_3PO_4, 1.3 mM NaH_2PO_4 (pH 7.4)] for 30 min and washed again. The sections were reacted for 1 h at room temperature with rabbit antiserum against the HSV-tk protein in 1% BSA in PBS at a 1:100 dilution and the cell lines at a dilution of 1:500. The sections were reacted with swine anti-rabbit biotinylated IgG (1:300 dilution; DAKO, Glostrup, Denmark) and streptavidin (1:300; DAKA, Denmark) each for 30 min. 3-Amino-9-ethyl-carborole was used as the chromogen, which provided a red stain color in areas of HSV-tk expression. Sections were counterstained with hematoxylin.

Accumulation of [^{18}F]FHPG in Tumor Cells. Monolayers of C6 and C6tk+ cells in 3 ml of DMEM supplemented with 10% FCS were incubated with 3–4 MBq of [^{18}F]FHPG for 2 h at 37°C. Thereafter, the culture medium was removed, and the monolayers were washed three times with 1.5 ml of ice-cold PBS. The cells were harvested from the culture plates by treatment with 0.3 ml of 0.5% trypsin for 5 min and resuspended in 1.5 ml of culture medium to neutralize the trypsin. The cellular accumulation of radioactivity was measured in a gamma counter (LKB Wallac, Turku, Finland) and normalized to the number of viable cells in the monolayer.

Biodistribution. Ten to 14 days after inoculation of the HSV-tk-transduced C6tk+ tumor cells and the wild-type C6 cells, the tumor-bearing nude rats were anesthetized with sodium pentobarbital (60 mg/kg). [^{18}F]FHPG (7–9 MBq in 0.4–0.9 ml of HPLC eluent) was injected in the tail vein of the rats. After 2 h, the rats were sacrificed by extirpation of the heart. Tumors and several organs were dissected, and the radioactivity accumulated in these tissues was measured with a gamma counter.

Analysis of [^{18}F]FHPG Metabolites in Tumors and Plasma. The tumors were homogenized in 1 ml of PBS with 20 μl of 70% $HClO_4$. The homogenate was centrifuged (5 min, 1000 $\times$ *g*), and the pellet was washed twice with 1 ml of PBS with 20 μl of $HClO_4$. The radioactivity in the acid-soluble and acid-insoluble fractions was measured with a gamma counter. The acid-soluble fraction of the tumor and plasma samples was analyzed by gradient ion-exchange HPLC [Nucleosil 100–5 SB column (5 μ, 4 $\times$ 250 mm); flow rate, 1.5 ml/min; mobile phase ammonium phosphate (pH 6.5); gradient, 0.1 M (0–5 min), 0.1–0.5 M (linear gradient; 5–25 min), and 0.5 M (25–35 min)]. The eluate was collected in fractions of 1.5 ml during a 35-min period and counted in a gamma counter. More than 95% of the radioactivity injected on the HPLC column was recovered in the eluate.

PET Studies. PET studies were performed using a Siemens Exact HR+ positron camera (Siemens/CTI, Knoxville, TN) with a resolution of about 5 mm (FWMH). Transmission and emission scans were obtained in two-dimensional mode. The tumor-bearing rats were anesthetized with sodium pentobarbital (60 mg/kg) and positioned in the PET camera with their long axis parallel to the transaxial plane of the tomograph. A 20-min transmission scan was obtained to correct for attenuation of 511 keV photons by tissue. A $H_2{}^{15}O$ PET scan was performed to identify differences in perfusion between the tumors that could influence tracer accumulation. Because both tumors were present in the same animal, correction for the $H_2{}^{15}O$ input was not necessary. Thus, approximately 20 MBq of $H_2{}^{15}O$ in 0.3 ml of 0.9% NaCl was injected into the tail vein, and data acquisition was started. At least 15 min after the $H_2{}^{15}O$ PET data acquisition was completed, about 8 MBq of [^{18}F]FHPG was injected into the tail vein. For 2 h, data were acquired. Emission scans were corrected for radioactive decay, attenuation, random counts, and dead time. Data analysis was performed using Siemens ECAT 7.1 software. In frame 10 (3.5 min p.i.) of the [^{18}F]FHPG PET images and frame 8 (2.5 min p.i.) of the $H_2{}^{15}O$ PET images, ROIs were drawn around both tumors in all coronal slices where the tumor was visible. The ROI activities were summed, and time-activity curves were calculated. Tracer accumulation is expressed as standardized uptake values (*SUV*), which can be defined as:

$$SUV = \frac{\text{Tissue radioactivity concentration (MBq/ml)}}{\text{Injected dose (MBq)/body weight (g)}}$$

The radioactivity concentration in tissue was calculated by dividing the tissue activity in the ROI by the volume of the ROI.

Statistical Analysis. Differences in tracer accumulation between C6 and C6tk+ tumor cells were analyzed using an unpaired Student's *t* test for *in vitro* studies and a paired Student's *t* test for *in vivo* experiments. $P < 0.05$ was considered significant.

Results

Ganciclovir Sensitivity. The HSV-tk+-expressing cells were more sensitive to ganciclovir than the control cells because the IC_{50} was 39 $\pm$ 18 μg/ml for C6 and 0.059 $\pm$ 0.018 μg/ml for C6tk+. As a result, the ganciclovir resistance factor of C6 compared with C6tk+ was 650.

HSV-tk Expression and Histology. HSV-tk expression in the C6tk+ cell line and the C6 control cell line was tested with the HSV-tk-directed antibody. A heterogenous HSV-tk expression was detected in the C6tk+ cell line. Counting 1000 cells on cytospins revealed staining in all these cells, with 28% of the cells being more

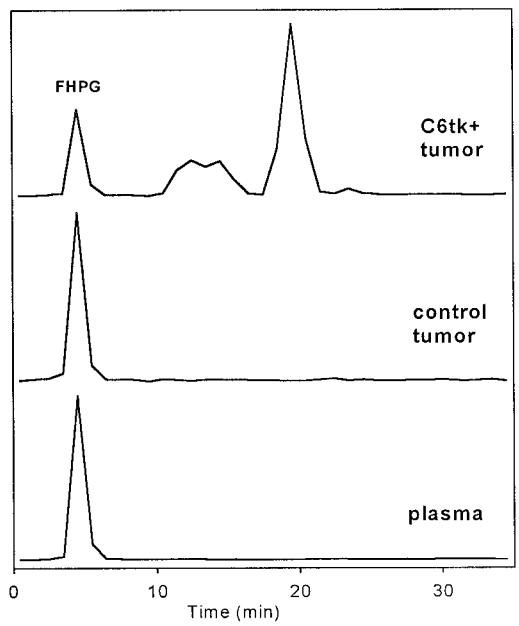

Fig. 2. [^{18}F]FHPG metabolite analysis in the plasma and the acid-soluble fraction of the tumors using anion exchange HPLC (120 min p.i.).

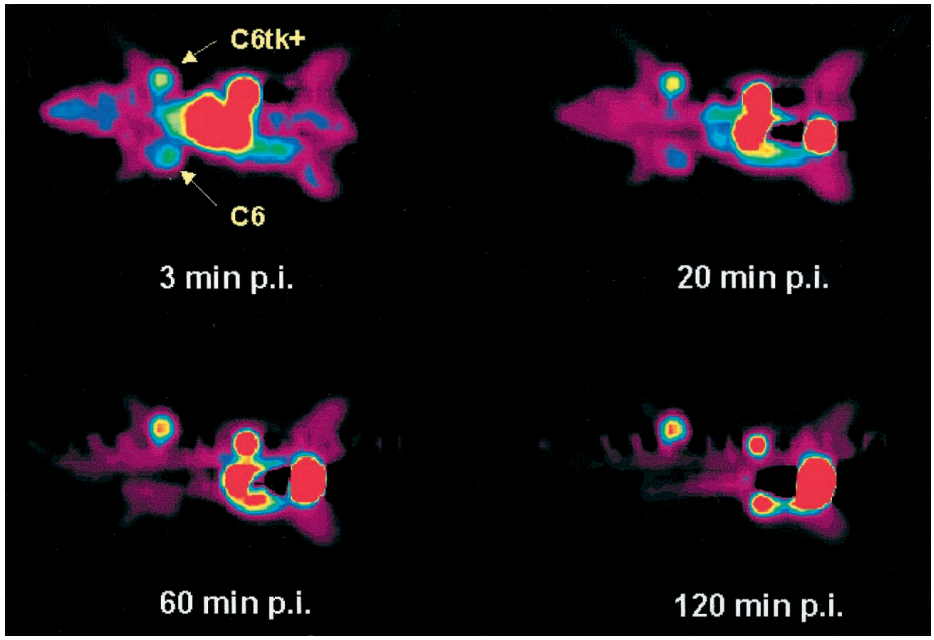

Fig. 3. [^{18}F]FHPG PET images of the same rat bearing a C6 and a C6tk+ tumor in its flanks at 3, 20, 60, and 120 min after injection of the tracer. Each image represents the same coronal plane shown from the ventral side of the rat (head on the *left side*).

clearly stained. The control C6 cells were negative. H&E staining showed growing tumor cells in both the s.c. C6 and C6tk+ tumor nodules. The HSV-tk directed antibody clearly stained the cells in the C6tk+ tumors, whereas the control C6 tumors were negative.

Cellular Accumulation of [^{18}F]FHPG. To assess the feasibility of [^{18}F]FHPG as a tracer for HSV-tk activity, *in vitro* accumulation studies were performed. After 2 h of incubation, *in vitro* cellular accumulation of [^{18}F]FHPG in C6 and C6tk+ rat glioma cells was 0.016 ± 0.002 and 0.56 ± 0.07% injected dose/10^6 cells, respectively. Thus, uptake in HSV-tk-containing C6tk+ cells was 35 ± 5 times higher than that in C6 control cells ($P < 0.0005$).

Biodistribution. Two h after injection of the tracer, the [^{18}F]FHPG content in several tissues was determined by *ex vivo* counting. As shown in Fig. 1, the level of radioactivity in both proliferative and nonproliferative tissues was similar to blood plasma levels. High levels of radioactivity were found in the kidneys and the urine as a result of the rapid renal excretion of the tracer. In contrast to the C6 control tumor, high levels of [^{18}F]FHPG had accumulated in the HSV-tk-containing C6tk+ tumor (tissue:plasma ratio, 12.3 ± 0.8). After only 2 h of biodistribution, the uptake of radioactivity in the C6tk+ tumor already exceeded the uptake in the C6 control tumor by a factor of 15 ± 5 ($n = 4$; $P < 0.0005$).

Metabolites. The acid-insoluble fraction of the C6 and C6tk+ tumors, which contains the nucleic acids and proteins, comprised <10% of the total radioactivity in the tumor. The acid-soluble fraction (representing unbound radioactivity in plasma) of both tumors was analyzed by anion-exchange HPLC at 2 h postinjection. More than 98% of the radioactivity in the acid-soluble fraction in control tumors and in plasma consisted of unmetabolized [^{18}F]FHPG (Fig. 2). However, in the acid-soluble fraction of C6tk+ tumors, only 17 ± 6% ($n = 3$) of tissue radioactivity represented the parent compound. In addition to [^{18}F]FHPG, three negatively charged metabolites were found. The elution pattern of these metabolites and published data (8, 11) suggest that these metabolites could be [^{18}F]FHPG-monophosphate, [^{18}F]FHPG-diphosphate, and [^{18}F]FHPG-triphosphate. Like ganciclovir (10), [^{18}F]FHPG is expected to be converted by HSV-tk to FHPG-monophosphate, which will then be further phosphorylated by host cellular kinases. These phosphates are negatively charged and cannot cross the cell membrane. Thus, accumulation of radioactivity in the tumor represents HSV-tk enzyme activity.

PET Studies. From the $H_2{}^{15}O$ PET scan, the perfusion of the tumors was assessed. The $H_2{}^{15}O$ time-activity curves clearly showed that the perfusion is similar in both tumors (data not shown), which precludes differences in perfusion from being responsible for differences in tracer accumulation. In the [^{18}F]FHPG PET images, both the C6 and the C6tk+ tumor could clearly be seen as hot spots at 3 min postinjection, indicating that [^{18}F]FHPG rapidly enters neoplastic cells (Fig. 3). The consecutive PET images show that the tracer is rapidly cleared from nontarget tissues into the urine. After 120 min, the control tumor and other nontarget tissues were no longer visible, whereas the C6tk+ tumor, the kidneys, and the bladder were clearly delineated. Even a C6tk+ tumor weighing only 0.2 g could clearly be visualized with [^{18}F]FHPG PET. The [^{18}F]FHPG time-activity curves showed that the accumulation of radioactivity in both tumors reaches a maximum at about 4 min postinjection (Fig. 4). Thereafter, the level of radioactivity in the HSV-tk-containing tumor remained virtually constant. The radioactivity in the control tumor, on the other hand, decreased exponentially with a half-life of 28.1 ± 1.4 min. The ratio

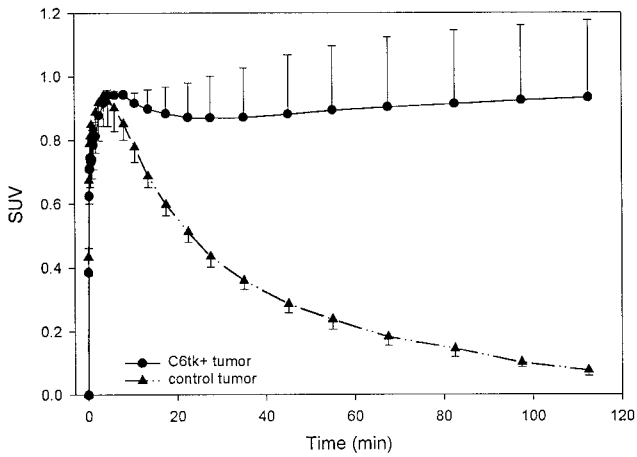

Fig. 4. [^{18}F]FHPG accumulation in the C6tk+ tumor and control tumor in nude rats.

between the activity in the C6tk+ tumor and the activity in the control tumor, as determined by PET, was 12 ± 4 after 2 h of tracer distribution. This ratio is slightly lower than the ratio determined by *ex vivo* counting (15 ± 5) because of spill-over and partial volume effects. From the time-activity curves, it is clear that the difference in uptake between control and C6tk+ tumors will increase with time and is not yet maximal at 120 min.

Discussion

The aim of this study was to develop a noninvasive PET method for monitoring the HSV-tk activity. In the present study, we demonstrated in a double tumor-bearing rat model that [^{18}F]FHPG is a suitable tracer to detect HSV-tk expression with PET.

[^{124}I]FIAU was the first radiopharmaceutical that allowed successful imaging of HSV-tk expression using PET (6). Using this tracer, different levels of gene expression could be discriminated. However, widespread use of [^{124}I]FIAU PET is not expected because of (*a*) the slow clearance of the tracer from nontarget tissue, which prevents 1-day protocols; (*b*) the ability of human thymidine kinases to phosphorylate FIAU (16), which may give rise to significant nonspecific uptake in rapidly dividing cells; (*c*) the poor physical properties of ^{124}I, which hamper quantification and high spatial resolution (17); and (*d*) the limited availability of ^{124}I. A radiopharmaceutical labeled with ^{18}F instead of ^{124}I might overcome these restrictions.

Ganciclovir has been radiolabeled with ^{18}F in two positions: (*a*) at C-8 of the purine ring (8-[^{18}F]fluoroganciclovir; Ref. 18); and (*b*) in the side-chain ([^{18}F]FHPG; Refs. 13 and 17). 8-[^{18}F]Fluoroganciclovir has been applied to image adenovirus-directed hepatic HSV-tk expression in mice with PET (7). Although the tracer was rapidly cleared from most of the nontarget tissues into the urine, significant troublesome background accumulation was found in the intestines.

In the present study, we have demonstrated in rats with both a HSV-tk-expressing tumor and a control tumor in their flanks that [^{18}F]FHPG was rapidly and selectively trapped in the HSV-tk-expressing tumors due to phosphorylation of the tracer by the HSV-tk enzyme. This observation was supported by the presence of three negatively charged metabolites in HSV-tk-expressing tumors only. Hardly any accumulation of radioactivity in rapidly proliferating tissues, such as intestines, bone marrow, and control tumors, was observed, indicating that FHPG is not significantly phosphorylated by host kinases. The tracer was rapidly cleared from nontarget tissues into the urine. As a result, high target:background ratios were obtained after only 2 h of tracer distribution, which easily allows 1-day protocols. Although the sensitivity of this imaging technique remains to be determined, these excellent target:background ratios suggest that clinically relevant levels of HSV-tk expression can be monitored as well. Prolonged distribution times can improve the sensitivity of the technique.

In contrast to 8-[^{18}F]fluoroganciclovir (18), [^{18}F]FHPG can be prepared with high specific activity (14, 19), which allows low-mass injections (<5 μg per 400 MBq). Because of the low dose administered, the rapid clearance, the absence of significant tracer accumulation in nontarget tissues, and the absence of recirculating metabolites, application of [^{18}F]FHPG as a PET tracer is not expected to give rise to any cytotoxic effects. Pharmacokinetic studies in monkeys have shown that the radiation dose of [^{18}F]FHPG is acceptable, with the bladder wall receiving the highest burden due to the rapid renal excretion (20). The radiation dose and background accumulation in the bladder can significantly be reduced by urination or by applying a catheter.

In conclusion, we have demonstrated that we can noninvasively image HSV-tk expression in a double tumor-bearing rat model using [^{18}F]FHPG PET. From the high target:background ratios observed, this imaging method is expected to have a clinically relevant sensitivity. The relatively short half-life of ^{18}F allows sequential imaging of gene expression with [^{18}F]FHPG PET. Imaging of the level and the distribution of gene expression over time will provide new and useful information for monitoring clinical gene therapy protocols in the future.

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