Redistribution of Nucleoside Transporters to the Cell Membrane Provides a Novel Approach for Imaging Thymidylate Synthase Inhibition by Positron Emission Tomography

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Abstract
Thymidylate synthase (EC 2.1.1.45) is a key enzyme for the de novo synthesis of DNA and as such a target for anticancer drug development. There is a need to develop noninvasive methods for assessing thymidylate synthase inhibition in tumors. The aim of this study was to assess the potential of 3'-deoxy-3-[18F]fluorothymidine ([18F]FLT) positron emission tomography (PET) for early measurement of thymidylate synthase inhibition and to elucidate the cellular mechanisms involved. Radiation-induced fibrosarcoma-1 tumor-bearing mice were injected with a single i.p. dose of the thymidylate synthase inhibitor 5-fluorouracil (5-FU; 165 mg/kg) and imaged by [18F]FLT-PET at 1 to 2 hours after treatment. Deoxyuridine, thymidine kinase 1 (cytoplasmic thymidine kinase; EC2.7.1.21), and ATP levels in excised tumors were measured. Cellular assays for membrane transport were also done. There was a 1.8-fold increase in the 60-minute [18F]FLT tumor/heart radioactivity ratio in drug-treated mice compared with vehicle controls ($P = 0.0016$). Plasma and tumor deoxyuridine levels increased significantly but thymidine kinase and ATP levels were unchanged. Whole-cell assays implicated a (low level) functional role for the type-1 equilibrative nucleoside transporter (ENT). There was an increase in type-1 ENT-binding sites per cell from 49,110 in untreated cells to 73,142 ($P = 0.03$) in cells treated with 10 mg/mL 5-FU for 2 hours, without a change in transporter affinity ($P = 0.41$). We conclude that [18F]FLT-PET can be used to measure thymidylate synthase inhibition as early as 1 to 2 hours after treatment with 5-FU by a mechanism involving redistribution of nucleoside transporters to the plasma membrane. (Cancer Res 2006; 66(17): 8558-64)

Introduction
Thymidylate synthase (EC 2.1.1.45) is a critical enzyme for DNA replication because it catalyses the de novo synthesis of thymidine monophosphate (TMP), a key nucleotide precursor for DNA synthesis. Due to its central role in DNA synthesis, thymidylate synthase remains an exploitable target for anticancer drug development. Pyrimidines, such as 5-fluorouracil (5-FU) in combination with leucovorin, a reduced folate that stabilizes the binding of 5-FU to thymidylate synthase, have been used for several decades in the treatment of gastrointestinal and other cancers (1–3). More recently oral 5-FU prodrugs, such as capecitabine, have been developed (1, 4, 5). In addition to these pyrimidines, inhibitors that are structural analogues of 5,10-methylenetetrahydrofolate, a cosubstrate of thymidylate synthase, generally referred to as antifolates, have been developed. These include raltitrexed, permutrexed, nolatrexed, and BGC 9331 (ZD9931; refs. 6–9). Whereas the above agents inhibit both normal tissue and tumor thymidylate synthase, new antifolates, such as BGC 638 and BGC 945, are targeted to tumors via the α-folate receptor, leading to selective inhibition of tumor thymidylate synthase (6, 10, 11).

Quantification of thymidylate synthase inhibition in tumors currently relies on measurement of biochemical changes that accompany thymidylate synthase inhibition (2, 7). For instance, thymidylate synthase inhibition leads to an increase in intracellular pools of the thymidylate synthase substrate dUMP and its corresponding nucleoside 2'-deoxyuridine; thus, pharmacodynamic effects of thymidylate synthase inhibitors have been assessed by measuring elevation of this nucleoside in the patient’s plasma (7, 8). However, elevated plasma 2'-deoxyuridine is mainly a marker of global thymidylate synthase inhibition in proliferating cells (i.e., not tumor specific). Positron emission tomography (PET) imaging of thymidylate synthase biochemistry is a strategy that may provide more specific information at the tumor site and could have important use in the development of the next generation of thymidylate synthase inhibitors.

The positron emitting radiotracer 2-[11C]thymidine, a substrate for thymidine kinase 1 (TK1; cytoplasmic thymidine kinase; EC2.7.1.21 ref. 12), a key enzyme in salvage pathway for producing TMP (13), has been assessed for its potential for imaging thymidylate synthase inhibition (14). In that study, patients treated with nolatrexed and scanned 1 hour after drug administration had an increase in tumor 2-[11C]thymidine-related radioactivity levels that correlated with area under the plasma nolatrexed concentration versus time curve (14). It was hypothesized that inhibition of the de novo pathway will lead to enhanced use of TMP produced via the salvage pathway and hence 2-[11C]thymidine uptake. The radiotracer 2-[11C]thymidine is, however, not an ideal PET marker because it is rapidly metabolized in vivo (15). In the present report, we investigated the feasibility of the more metabolically stable thymidine analogue, 3'-deoxy-3-[18F]fluorothymidine ([18F]FLT; ref. 16), to image thymidylate synthase inhibition. We showed for the first time that [18F]FLT-PET can be used very early to image inhibition of thymidylate synthase in vivo by a mechanism involving redistribution of nucleoside transporters to the plasma membrane.
Materials and Methods

Radiochemicals. [18F]FLT was produced on-site by Hammersmith Imanet Ltd. (Medical Research Council Cyclotron Building, Hammersmith Hospital, London, United Kingdom). It was prepared by radiofluoridation of the 2′,3′-anhydro-5-O-(4′,4′-dimethoxytrityl)-thymidine precursor as has been described previously (17). [Methyl-3H]thymidine ([3H]Tdr; 925 GBq/mmol, 37 MBq/mL) and [2-3H]adenine ([2H]Adenine; 777 GBq/mmol, 37 MBq/mL) were purchased from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, United Kingdom). [18F]FLT ([18F]FLT; 174 GBq/mmol, 37 MBq/mL) and [3H]nitrobenzylmercaptopurine ribonucleoside (NMPR), also known as [3H]-(p-nitrobenzyl)-6-thioinosine (555 MBq/mmol, 37 MBq/mL) were purchased from Moravek Biochemicals, Inc. (Brea, CA).

Cell lines and radiation. Induction-induced fibrosarcoma-1 (RIF-1) cells (18) were cultured in RPMI 1640 (Life Technologies, Strathclyde, United Kingdom) supplemented with 10% fetal bovine serum (Sigma, Poole, United Kingdom) and antibiotics in a 5% CO2 incubator at 37°C. Exponentially growing cells were used for the in vitro radiotracer studies or inoculated into 6- to 8-week-old male C3H/HeJ mice obtained from Harlan UK Ltd. (Bicester, United Kingdom). Tumors were induced by inoculation of 5 × 106 cells s.c. on the back of the mice. All animal work was done by licensed investigators in accordance with the UK Animals (Scientific Procedures) Act 1986” (HMSO, London, United Kingdom, 1990) and in full compliance with government regulations and guidelines on the welfare of animals in experimental neoplasia (19). Tumors were selected for PET imaging studies when they reached 5 to 8 mm in diameter.

PET scanning and image analysis. Mice were scanned on a dedicated small animal PET scanner (quad-HIDAC; Oxford Positron Systems, Weston-on-the-Green, United Kingdom; refs. 18, 20, 21). Before scanning, the mice were treated with either PBS (control) or 5-FU at a single i.p. dose of 165 mg/kg body weight and scanned at 1 to 2 hours after drug injection. For scanning, anesthesia was induced with isoflurane/O2/N2O and the tail veins of the mice were cannulated. The animals were placed within a thermostatically controlled bed and positioned prone within the scanner. The bed was calibrated to provide a mouse rectal temperature of ~37°C. A bolus injection of [18F]FLT (2.96-3.7 MBq; 80-100 μCi) was given i.v. via the tail vein cannula and scanning commenced. Dynamic emission scans were acquired in list-mode format over 60 minutes. The acquired data were then sorted into 0.5-mm sinogram bins and 19 time frames (0.5 × 0.5 × 0.5 mm voxels; 4 × 15, 4 × 60, and 11 × 300 seconds) for image reconstruction, which was done by filtered back projection using a two-dimensional Hamming filter (cutoff 0.6). The image data-sets obtained were transferred to a SUN workstation (Ultra 10; SUN Microsystems, Santa Clara, CA) and visualized using the Analyze software (version 6.0; Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). Cumulative images of the dynamic data composed of 0 to 1 minute after injection and 30 to 60 minutes after injection were used for visualization of radiotracer uptake and to define the regions of interest (ROI) on heart (mainly blood pool) and tumors, respectively. ROIs were defined on five tumor and five heart slices (each 0.5 mm thickness). The count densities were averaged for each ROI at each of the 19 time points to obtain a time versus radioactive curve (TAC) for the ROIs. Tumor TAC was normalized to that of heart at each of the time points to obtain the normalized uptake value (NUV). The [18F]FLT-PET data from heart were used as internal input function for normalizing tumor data because it comprises mainly of blood radioactivity (18). The NUV at 60 minutes after injection (NUV60), the area under the NUV curve (AUC) calculated as the integral of NUV from 0 to 60 minutes, and the fractional retention of tracer (FRT), the radioactivity at 60 minutes relative to that at 2.5 minutes, were used for comparisons. FRT is a useful variable in that it indicates the proportion of radiotracer delivered to the tumor that is retained. It therefore normalizes tumor [18F]FLT uptake to delivery.

Analysis of tumor 2′-deoxyuridine levels. To confirm that thymidine synthase was inhibited by 5-FU, 2′-deoxyuridine levels were assessed by high-performance liquid chromatography (HPLC). Plasma and RIF-1 tumors were taken from mice treated with PBS or 165 mg/kg 5-FU at 2 hours after injection and immediately snap frozen in liquid nitrogen. This time point was selected to coincide with the imaging assay. Plasma samples (150 μL) were initially prepared using perchlorate protein precipitation: after neutralization and centrifugation, the supernatant was purified by solid-phase extraction. Whole (weighed) tumor samples were homogenized and the homogenate was treated similar to plasma samples. After evaporation to dryness using a vacuum centrifuge, the samples were reconstituted for analysis by reverse-phase HPLC as described previously (22). 2′-Deoxyuridine was separated on a C18 stationary phase with a mobile phase comprising 0.1% trifluoroacetic acid and detected at 267 nm using a UV photodiode array detector. 2′-Deoxyuridine losses from sample preparation were estimated by measuring the recovered activity of [5-3H]2′-deoxyuridine spiked into blank plasma or tumor samples. Chromatographic peak areas for the samples were compared with a calibration curve; data were expressed as micromole per liter and nanomole per gram for plasma and tumors, respectively.

Analysis of tumor TK1 protein and cofactor levels. TK1 protein and cofactor (ATP) were assessed by Western blot and bioilluminescence assays, respectively. RIF-1 tumors were excised from mice treated with PBS or 165 mg/kg 5-FU at 2 hours after injection and snap frozen in liquid nitrogen. Tumors were pulverized in liquid nitrogen and homogenized in ice-cold PBS using an Ultra-Turrax homogenizer (IKA, Staufen, Germany). The samples were then centrifuged at 800 × g (for 30 minutes at 4°C). Supernatants were analyzed for protein content using a commercial bicinchoninic acid (BCA) protein assay kit (Perbio Science, Cheshire, United Kingdom). Aliquots of the supernatants (containing 30 μg protein) were mixed with equivalent volumes of native Tris-glycine sample buffer (Invitrogen Life Science, Paisley, United Kingdom) and separated on a precast Tris-glycine (4-20%) gel (Invitrogen). Western blot analysis was done as described previously (18, 20). For quantification of band intensities, the films were scanned using a GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hertfordshire, United Kingdom) and analyzed with the Quantity One software (version 4.0.3; Bio-Rad Laboratories). ATP levels were determined by a bioilluminescence assay as described previously (18, 20) using the same supernatants used for the Western blots above. A calibration curve was prepared from ATP standards, which were provided with the ATP assay kit.

Incorporation of radioactivity into DNA. RIF-1 cells were cultured in full growth medium as above and treated with 1 or 10 μg/mL 5-FU dissolved in PBS for an equal volume of PBS for 2 hours. After drug treatment, the cells were washed and incubated with 0.185 MBq tritiated thymidine ([3H]Tdr) or [3H]FLT for 1 hour. The cells were washed and scraped into plastic tubes. The cellular protein content of whole cells was assayed by a commercial BCA protein assay kit and used for normalization of radioactivity levels in DNA (assuming no losses in the DNA assay) on different days. The cells were centrifuged. DNA was isolated from whole-cell pellet using the AquaPure Genomic Isolation kit (Bio-Rad Laboratories, Hercules, CA). Hydrated DNA samples were placed in opaque 24-well plates (Perkin-Elmer, Beaconsfield, United Kingdom) and left to dry. Aliquots (500 μL) of scintillation (Microscint-40; Perkin-Elmer) were added to each well and the radioactivity in each well was counted on a β-counter (TopCount NXT, Packard Instruments, Meriden, CT). Data were quench corrected and expressed as counts per minute (cpm)/mg protein.

Cellular membrane transport inhibition assays. To determine the mechanism of whole-cell plasma membrane transport, cell uptake studies were done with [18F]FLT in the presence or absence of various known inhibitors (23). RIF-1 cells were cultured as above and treated with full growth medium containing PBS or 10 μg/mL of 5-FU for 2 hours. After drug treatment, the cells were washed and trypsinned. The effect of trypsin was blocked with full growth medium. The cells were washed again and suspended in HBSS (Sigma). Cells (2 × 105) were preincubated for 15 minutes in HBSS buffer containing PBS (control) or an inhibitor of nucleoside or nucleobase transport (and phosphorylation), including 20 μmol/L nitrobenzylthioguanosine (NBTG), 20 μmol/L dipyridamole, 2 μmol/L thymidine, 2 μmol/L uridine, 2 μmol/L adenine, and 2 μmol/L thymine; total incubation volume of 1 mL. The cells were then incubated with 1.8 to 3.7 MBq [18F]FLT at room temperature (~ – 20°C) for 1 hour with mild shaking. At the end of the incubation period, the reaction was quickly stopped by adding 5 mL ice-cold PBS and centrifuged at 500 × g for 5 minutes. The supernatant was removed; the cells were washed once and resuspended in 100 μL of 2.5% lysis buffer (10% sodium carbonate,
Kinetics of nucleoside transport. In these studies, exponentially growing RIF-1 cells were treated for 2 hours with PBS or 5-FU at concentrations of 1 or 10 μg/mL. Single cells prepared by trypsinization were suspended in HBSS with mild shaking. To assess plasma membrane NBPMP-sensitive nucleoside transporter-binding sites on RIF-1 cells, 2 × 10^6 cells were incubated with 0.1, 0.25, 0.5, 1, 2, 4, 8, 16 mmol/L [3H]NBMPR in HBSS. The total incubation volume was 400 μL and the studies were carried out at room temperature (~ 20°C) for 60 minutes (equilibrium binding). In parallel experiments to assess nonspecific binding, equal numbers of cells were preincubated with 20 mmol/L of the specific inhibitor, NBTF, for 15 minutes and then incubated with [3H]NBMPR at the same concentration range. At the end of the incubation, the reaction was quickly stopped by adding 5 mL ice-cold Na+ buffer [150 mmol/L NaCl, 5 mmol/L Tris-HCl (pH 7.4)] and the mixture was centrifuged at 500 × g for 5 minutes. The supernatant was removed and the cells were washed once and resuspended in 100 μL lysis buffer until they were fully disintegrated. The solution was carefully transferred into opaque 24-well plates and air dried, and the radioactivity was counted (TopCount). Specific binding (binding sites per cell) was defined as the difference in [3H]NBMPR content in the presence and absence of NBTF. The maximal binding (B_{max}; the number of transporters) and the concentration of ligand required to reach half-maximal binding (K_{IC} affinities) were determined by curve fitting using the Prism software, version 4.03 (GraphPad, San Diego, CA). Data were fitted to the equation: \( Y = B_{max} \times X / (K_{IC} + X) \), where Y and X are the specific binding and [3H]NBMPR concentration in nmol/L, respectively.

Statistics. Statistical analyses were done using the Prism software, version 4.03 (GraphPad). Differences between PBS- and 5-FU-treated groups were tested for significance using the Student’s t test for unpaired data. Two-tailed P ≤0.05 were considered significant.

### Results

The accumulation of [18F]FLT in RIF-1 tumors increases early after 5-FU treatment. We have shown previously that [18F]FLT uptake decreases at 24 and 48 hours after a single i.p. dose of 5-FU (165 mg/kg), whereby the post-5-FU [18F]FLT uptake correlated with histologically assessed proliferation. Figure 1A and B shows the localization of [18F]FLT in RIF-1 tumors 1 to 2 hours after treatment with PBS or 5-FU at the same dose used previously. Localization of radioactivity was seen in the PBS-treated tumor (Fig. 1A) that was qualitatively enhanced by 5-FU treatment at 1 to 2 hours (Fig. 1B). Quantitative assessment of the time course of [18F]FLT uptake in RIF-1 tumors after i.v. injection of the radiotracer also showed increased uptake (Fig. 1C). The two TACs in this case were visually different after ~ 15 minutes. A summary of the kinetic components calculated from the TACs are depicted in Table 1. All variables, including the NUV_{60}, FRT, and AUC, were significantly higher in the 5-FU-treated mice compared with vehicle controls (Table 1). We also examine whether thymidylate synthase was inhibited under the conditions used. Both plasma and tumor

### Table 1. Summary of PET pharmacokinetics for PBS- and 5-FU-treated mice together with corresponding deoxyuridine levels

<table>
<thead>
<tr>
<th></th>
<th>NUV_{60}</th>
<th>FRT</th>
<th>AUC (activity ratio × min)</th>
<th>Plasma deoxyuridine level (μmol/L)</th>
<th>Tumor deoxyuridine level (nmol/g)</th>
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<tbody>
<tr>
<td>PBS (n)</td>
<td>1.08 ± 0.03 (8)</td>
<td>2.62 ± 0.17 (8)</td>
<td>57.42 ± 1.48 (8)</td>
<td>2.38 ± 0.37 (4)</td>
<td>10.66 ± 2.50 (4)</td>
</tr>
<tr>
<td>5-FU (n)</td>
<td>1.93 ± 0.11 (5)</td>
<td>5.85 ± 0.46 (5)</td>
<td>85.68 ± 3.69 (5)</td>
<td>4.21 ± 0.47 (4)</td>
<td>24.82 ± 3.14 (4)</td>
</tr>
<tr>
<td>Significance (5-FU vs PBS)</td>
<td>0.0016</td>
<td>0.0016</td>
<td>0.0016</td>
<td>0.02</td>
<td>0.01</td>
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NOTE: Data are mean ± SE (number of tumors). NUV_{60}, FRT, and AUC were determined from [18F]FLT-PET TACs obtained at 1 to 2 hours after 5-FU administration as described in Materials and Methods. Deoxyuridine levels were determined by HPLC as described in Materials and Methods.
2'-deoxyuridine levels, surrogate measures of thymidylate synthase inhibition (7, 8), were significantly increased after 5-FU treatment by ~2-fold (Table 1).

The early 5-FU-induced accumulation of $^{[18F]}$FLT in tumors is not due to enhanced TK1 protein, ATP levels, or incorporation of $^{[18F]}$FLT into DNA. We have attributed previously an increase in radiolabeled thymidine uptake after treatment with different thymidylate synthase inhibitors to enhanced salvage kinetics in an attempt of cells to overcome the block in TMP production via the de novo pathway (14, 24). Because tumor uptake of $^{[18F]}$FLT increased in vivo after thymidylate synthase inhibition, we examined key salvage pathway events, including TK1 levels, ATP levels, and incorporation of radioactivity into DNA, which might explain the increased $^{[18F]}$FLT uptake. Figure 2A and B shows TK1 and ATP levels in PBS- and 5-FU-treated tumor homogenates. These variables did not increase as expected. In contrast, there were nonsignificant decreases in both protein and cofactor levels. This indicates that the increased uptake of $^{[18F]}$FLT into tumors after thymidylate synthase inhibition occurs under normal levels of TK1 and ATP. Examination of thymidine incorporation into DNA was done with tritiated FLT $^{[3H]}$FLT using $^{[3H]}$TdR as a positive control. For $^{[3H]}$TdR, a significant increase in radiotracer uptake into cells was seen at two 5-FU concentrations ($P \leq 0.003$; Fig. 2C). The amount of radioactivity in DNA was 26.5% of that in whole cells. In contrast, there was no change in $^{[18F]}$FLT uptake into DNA (Fig. 2D); the amount of radioactivity in DNA was 0.2% of that in whole cells. This indicates that thymidylate synthase inhibition does not increase $^{[18F]}$FLT uptake into the DNA pool.

**Inhibitor studies indicate a role for equilibrative nucleoside transporter type 1.** To examine the role of membrane transport, the uptake of $^{[18F]}$FLT was studied in the presence or absence of known inhibitors of nucleoside or nucleobase transport. As in RIF-1 tumors, in vitro $^{[18F]}$FLT uptake in RIF-1 cells increased significantly after 5-FU treatment (Fig. 3A). The increased uptake was reduced by pretreatment with equilibrative nucleoside transport (ENT) inhibitors NBTG (type 1 ENT (ENT1) selective) and 5-FU treatment with different thymidylate synthase inhibitors to enhanced salvage kinetics in an attempt of cells to overcome the block in TMP production via the de novo pathway (14, 24). Because tumor uptake of $^{[18F]}$FLT increased in vivo after thymidylate synthase inhibition, we examined key salvage pathway events, including TK1 levels, ATP levels, and incorporation of radioactivity into DNA, which might explain the increased $^{[18F]}$FLT uptake. Figure 2A and B shows TK1 and ATP levels in PBS- and 5-FU-treated tumor homogenates. These variables did not increase as expected. In contrast, there were nonsignificant decreases in both protein and cofactor levels. This indicates that the increased uptake of $^{[18F]}$FLT into tumors after thymidylate synthase inhibition occurs under normal levels of TK1 and ATP. Examination of thymidine incorporation into DNA was done with tritiated FLT $^{[3H]}$FLT using $^{[3H]}$TdR as a positive control. For $^{[3H]}$TdR, a significant increase in radiotracer uptake into cells was seen at two 5-FU concentrations ($P \leq 0.003$; Fig. 2C). The amount of radioactivity in DNA was 26.5% of that in whole cells. In contrast, there was no change in $^{[18F]}$FLT uptake into DNA (Fig. 2D); the amount of radioactivity in DNA was 0.2% of that in whole cells. This indicates that thymidylate synthase inhibition does not increase $^{[18F]}$FLT uptake into the DNA pool.

**Figure 2.** TK1 protein levels (A) and ATP concentration (B) in excised PBS- and 5-FU-treated tumors. TK1 protein was analyzed by Western blot and densitometry and expressed as absorbance units (arbitrary); control levels were not significantly different from treated levels; $P = 0.89$ and 0.40 for the 1- and 2-hour treatment groups, respectively. ATP concentration was analyzed by a bioluminescence method; control levels were not significantly different from treated levels; $P = 0.11$ and 0.06 for the 1- and 2-hour treatment groups, respectively. DNA content of $^{[3H]}$TdR (C) and $^{[18F]}$FLT (D) in PBS- and 5-FU-treated cultured RIF-1 cells. The cells were treated with 1 or 10 $\mu$g/mL 5-FU for 2 hours and washed off of drug. This was followed by 1 hour of incubation with $^{[3H]}$TdR or $^{[18F]}$FLT and extraction of DNA as described in Materials and Methods. Columns, mean cpm normalized to whole-cell protein content ($n = 4$); bars, SE (*, $P < 0.03$). Note the different Y axis scales.

**Figure 3.** Mechanism of $^{[18F]}$FLT cell membrane transport in RIF-1 cells growing in culture. Exponentially growing adherent cells that were treated with 5-FU at 10 $\mu$g/mL or with PBS (control) for 2 hours were washed and trypsinized, and 2 million cells were incubated with $^{[18F]}$FLT, $^{[3H]}$TdR, or $^{[18F]}$adenine in HBSS buffer for 1 hour in the presence or absence of nucleoside or nucleobase transport inhibitors. For inhibitor studies, cells were preincubated with the membrane transport inhibitors for 15 minutes. The cells were washed to remove free radioactivity with ice-cold PBS and assayed for the amount of radioactivity by scintillation counting ($n = 3-4$ experiments for each condition). A, effect of membrane transport inhibitors on $^{[18F]}$FLT uptake: columns A and B, untreated and 5-FU-treated cells, respectively; columns C, D, E, F, G, and H, 5-FU-treated cells that were preincubated with 20 $\mu$mol/L NBTG, 20 $\mu$mol/L dipyridamole, 2 $\mu$mol/L thymidine, 2 $\mu$mol/L uridine, 2 $\mu$mol/L adenine, and 2 $\mu$mol/L thymine, respectively. Columns, mean $^{[18F]}$FLT uptake per 2 million cells expressed as a percentage of radioactivity of the dose solution; bars, SE. B, effect of selected inhibitors on $^{[18F]}$FLT uptake in drug-free PBS-treated cells: columns A, I, J, and K, control, 20 $\mu$mol/L NBTG, 2 $\mu$mol/L thymidine, and 2 $\mu$mol/L adenine, respectively. Columns, mean $^{[18F]}$FLT uptake per 2 million cells expressed as a percentage of radioactivity of the dose solution; bars, SE. C, comparison of $^{[18F]}$FLT and $^{[3H]}$TdR with respect to inhibition of nucleoside transport with and without 5-FU treatment. Columns, mean radiotracer uptake; bars, ± SE. Each uptake data set is expressed as a percentage of the respective mean uptake in the absence of the inhibitor NBTG (columns L, N, P, and R). Columns L and M and columns N and O, uptake values for $^{[18F]}$FLT in the absence and presence of 5-FU, respectively; columns P and Q and columns R and S, uptake values for $^{[3H]}$TdR in the absence and presence of 5-FU, respectively. D, comparison of $^{[18F]}$FLT and $^{[3H]}$TdR uptake in PBS-treated (columns T and U) and 5-FU-treated cells (columns V and W) in the absence (columns T and V) and presence (columns U and W) of thymidine, respectively. Data are expressed as a percentage of the mean uptake in column T. $P = 0.05$, statistically significant for PBS or 5-FU controls versus inhibitor-treated comparisons in (A and O), for adenine and thymidine comparisons in (B), and for PBS versus 5-FU, PBS versus PBS-thymidine pretreated, and 5-FU versus 5-FU-thymidine pretreated comparisons in (D).
and dipyridamole [ENT1 and type 2 ENT (ENT2) selective]. The lack of a differential in \( ^{18}\text{F}\)[FLT uptake between these two inhibitors suggested that only ENT1 was inhibited or that the cells lacked ENT2. Thymidine and uridine, which (in addition to ENT) are substrates for the concentrative sodium-dependent nucleoside transporters, also decreased the 5-FU-induced \( ^{18}\text{F}\)[FLT uptake. A significant decline in uptake was also seen with the nucleoside transport inhibitors, adenine and thymine. Without 5-FU treatment, decreases in \( ^{18}\text{F}\)[FLT uptake were seen with thymidine and adenine (Fig. 3D); there was a slight decrease with NBTG but that was not statistically significant. This indicates that the decreases in 5-FU-induced \( ^{18}\text{F}\)[FLT uptake seen in Fig. 3A may in part be due to a decrease in baseline (uninduced) uptake. To confirm that ENT1 was involved in the 5-FU-induced \( ^{18}\text{F}\)[FLT uptake, we examined induced versus uninduced uptake of the natural ENT1 substrate \( ^{3}\text{H}\)TdR in the presence or absence of NBTG (Fig. 3C). The uptake of \( ^{3}\text{H}\)TdR in RIF-1 cells was almost completely blocked by NBTG in uninduced and 5-FU-induced cells (Fig. 3C). Figure 3C also shows that ENT1-dependent transport was lower in magnitude for \( ^{18}\text{F}\)[FLT compared with \( ^{3}\text{H}\)TdR. Thymine can be converted to thymidine and mask uptake studies. Adenine uptake can be confounded by TK1-dependent phosphorylation. Hence, we studied \( ^{3}\text{H}\)adenine uptake in uninduced and 5-FU-induced cells using thymidine to saturate TK1-dependent phosphorylation. Figure 3D shows that \( ^{3}\text{H}\)adenine uptake decreased after 5-FU treatment. In thymidine preinhibited cells, there was no difference in \( ^{3}\text{H}\)adenine uptake between PBS- and 5-FU-treated cells. These studies indicate that nucleoside transport may not be important in 5-FU-induced \( ^{18}\text{F}\)[FLT uptake.

**ENT1 levels increase with 5-FU treatment.** Because of a 1:1 stoichiometry between inhibitor and membrane-associated transporter levels, kinetic analysis of cell uptake with \( ^{3}\text{H}\)[NBMPR permits the number of membrane-associated ENT (predominantly ENT1) transporters and affinity of the transporter to be measured (25–27). Figure 4 shows the kinetics of \( ^{3}\text{H}\)[NBMPR uptake in PBS- and 5-FU-treated RIF-1 cells. The curves revealed a 5-FU concentration-dependent increase in the specific uptake of \( ^{3}\text{H}\)[NBMPR (i.e., the difference between the uptake of \( ^{3}\text{H}\)[NBMPR in the presence and absence of NBTG). A summary of the number of transporters (\( B_{\text{max}} \)) and transporter affinity (\( K_{D} \)) derived from this kinetic analysis is shown in Table 2. There was a 1.5-fold increase in number of transporters on the membrane after treatment of cells with 10 \( \mu \)g/mL 5-FU for 2 hours, without a change in transporter affinity. A nonsignificant increase in \( B_{\text{max}} \) was seen at 1 \( \mu \)g/mL 5-FU; \( K_{D} \) was not significantly different from control values in this case too.

**Discussion**

We have shown for the first time that \( ^{18}\text{F}\)[FLT-PET can be used to measure thymidylate synthase inhibition *in vivo* and defined the biochemical mechanism at the cellular level. Thymidylate synthase is a key enzyme for the *de novo* synthesis of DNA and as such a target for anticancer drug development. Currently the most widely established pharmacodynamic marker used for monitoring the efficacy of thymidylate synthase inhibition is an increase in plasma 2'-deoxyuridine, a consequence of increased intracellular dUMP in proliferating tissues throughout the body (7, 8, 28). Although easily accessible, this marker does not measure thymidylate synthase inhibition specifically at the tumor site and thus may be insensitive for the next generation of tumor-specific thymidylate synthase inhibitors. A PET imaging approach may overcome this limitation. We have developed \( ^{18}\text{F}\)[FLT-PET for this purpose. \( ^{18}\text{F}\)[FLT is a stable analogue of thymidine. 2-[\( ^{11}\text{C}\)]thymidine uptake has been shown to increase following AG337 administration (14). We subsequently showed that this phenomenon occurred in different cell types and following AG337 and 5-FU treatment but not after treatment with cisplatin that does not inhibit thymidylate synthase (24). In that study, the thymidylate synthase induced increases of \( ^{3}\text{H}\)TdR uptake occurred within 1 to 6 hours and classic decreases in \( ^{3}\text{H}\)TdR uptake resulting from inhibition of cell proliferation were observed at 24 and 48 hours after treatment (24). In a recent study by Gibbs et al. (11), tumor \( ^{[25]}\text{I}\)2'-deoxyuridine uptake was shown to increase after treatment of mice with the novel thymidylate synthase inhibitors BGC 638 and BGC 945. Thus, increase in nucleoside uptake after thymidylate synthase inhibition may be a general phenomenon that could be exploited to assess pharmacodynamics of thymidylate synthase inhibitors. Furthermore, it is not cell type dependent as the effects are seen in mouse and human cells, including RIF-1, HT29, and KB cells (11, 24).

\[ ^{18}\text{F}\][FLT has superior *in vivo* stability compared with 2-[\( ^{11}\text{C}\)]thymidine and \( ^{[25]}\text{I}\)iododeoxyuridine (29). Hence, we assessed the feasibility of using this radiotracers for imaging thymidylate synthase inhibition. Marked increase in \( ^{18}\text{F}\)[FLT-PET imaging end points together with marked increases in both plasma and tumor 2'-deoxyuridine were seen 1 to 2 hours after 5-FU treatment *in vivo*. We have proposed in previous studies that the mechanism of

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**Table 2. The effect of 5-FU on \( ^{[3]}\text{H}\)NBMPR kinetics in RIF-1 cells growing in culture**

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>5-FU (1 ( \mu )g/mL)</th>
<th>5-FU (10 ( \mu )g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( B_{\text{max}} ) (no. binding sites per cell)</td>
<td>49,101 ± 4,013</td>
<td>54,980 ± 3,811</td>
<td>73,142 ± 6,849</td>
</tr>
<tr>
<td>( K_{D} ) (nmol/L)</td>
<td>1,031 ± 0.16</td>
<td>1,263 ± 0.43</td>
<td>1,198 ± 0.13</td>
</tr>
<tr>
<td>Significance (5-FU versus PBS)</td>
<td>—</td>
<td>( B_{\text{max}}, 0.28; K_{D}, 1.00 )</td>
<td>( B_{\text{max}}, 0.03; K_{D}, 0.41 )</td>
</tr>
</tbody>
</table>

**NOTE:** Under the conditions used, \( ^{[3]}\text{H}\)NBMPR kinetics reflect the number and affinity of ENT1 nucleoside transporters on the cell membrane. Data are mean ± SE.
increased radiolabeled thymidine uptake following thymidylate synthase inhibition may involve increased use of the salvage pathway for producing DNA in an attempt of cells to overcome the block in TMP production via the de novo pathway (14, 24). This assertion was based on the use of radiolabeled thymidine, which is readily incorporated into DNA. In the present study, we showed that [3H]Tdr incorporation into DNA was enhanced by 5-FU treatment and that the DNA component comprised a substantial part of whole-cell uptake (26.5%). The proportion of [3H]FLT in DNA was only 0.2% that of whole cell and did not change after 5-FU treatment. This finding indicated that the mechanisms of retention of the two radiotracers were different. It also indicated that enhanced incorporation of thymidine analogues into DNA to overcome the block of the de novo pathway per se was not responsible for the general increase in nucleoside uptake. Examination of TK1 and ATP levels, the enzyme system responsible for the initial commitment of thymidine into the salvage pathway, showed no change in levels that could account for the increase in [18F]FLT uptake early after 5-FU treatment. Lastly, we examined membrane transport of [18F]FLT and showed that [18F]FLT was transported by ENT1, albeit at low levels compared with [3H]Tdr. The low substrate specificity of [18F]FLT for ENT1 even under conditions of 5-FU induction may be due to the lack of a 3’-OH group, which seems to be important for the transport of nucleoside analogues by ENT1 (30, 31).

Interestingly, we discovered that treatment of RIF-1 cells with 10 µg/mL 5-FU increased plasma membrane NBMPR-sensitive nucleoside transporter (predominantly ENT1) binding sites from 49,101 in untreated cells to 73,142 (P = 0.03). Thus, although a poor substrate, [18F]FLT is transported by ENT1, albeit at low levels compared with [3H]Tdr. The low substrate specificity of [18F]FLT for ENT1 even under conditions of 5-FU induction may be due to the lack of a 3’-OH group, which seems to be important for the transport of nucleoside analogues by ENT1 (30, 31).

M. Perumal, R.G. Pillai, E.O. Aboagye, unpublished data.

References
Redistribution of Nucleoside Transporters to the Cell Membrane Provides a Novel Approach for Imaging Thymidylate Synthase Inhibition by Positron Emission Tomography

Meg Perumal, Radhakrishna G. Pillai, Henryk Barthel, et al.


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