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A Novel *In Vitro* Assay to Assess Phosphorylation of 3'- [¹⁸F]fluoro-3'-Deoxythymidine

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Abstract

Purpose—3'-[¹⁸F]fluoro-3'-deoxythymidine ([¹⁸F]FLT) is phosphorylated by thymidine kinase 1 (TK-1), a cell cycle regulated enzyme. Appropriate use of [¹⁸F]FLT tracer requires validation of the TK-1 activity. Here, we report development of a novel phosphoryl-transfer assay to assess phosphorylation of [¹⁸F]FLT both in tumor cell lysates and tumor cells.

Procedures—The intrinsic F-18 radioactivity was used to quantify both substrate and phosphorylated products using a rapid thin layer chromatography method. Phosphorylation kinetics of [¹⁸F]FLT in SW480 and DiFi tumor cell lysates and cellular uptake were measured.

Results—The apparent Michaelis–Menten kinetic parameters for [¹⁸F]FLT are $K_m = 4.8 \pm 0.3$ μ M and $V_{max} = 7.4$ pmol min⁻¹ per 1×10^6 cells with ~2-fold higher TK-1 activity in DiFi versus SW480 lysates.

Conclusions—The apparent K_m of [^{18}F]FLT was comparable to the value reported with purified recombinant TK-1. The uptake of [^{18}F]FLT by SW480 cells is inhibited by nitrobenzylthioinosine or dipyridamole indicating that uptake is mediated predominantly by the equilibrative nucleoside transporters in these tumor cells.

Keywords

3'-[^{18}F]fluoro-3'-deoxythymidine ([^{18}F]FLT); Thymidine kinase 1; Positron emission tomography (PET); PET tracers; Molecular imaging

Introduction

Molecular imaging with positron emission tomography (PET) is a unique, noninvasive imaging approach for both clinical research and diagnosis of cancer [1–3]. Importantly, 3'-[^{18}F]fluoro-3'-deoxythymidine ([^{18}F]FLT) has been proposed as the long sought-after agent for noninvasive PET imaging of cellular proliferation and monitoring tumor response to therapy [4, 5]. Recently, [^{18}F]FLT has been developed as a radiotracer targeted to the activity of thymidine kinase 1 (TK-1) for imaging tumor cellular proliferation, a potential indicator of response to therapy [6–9]. [^{18}F]FLT is one of the most promising ^{18}F PET tracers because of its *in vivo* stability, metabolic trapping in proliferating cells, and a favorable physical half-life (110 min) for PET imaging [1, 4, 10]. Recent clinical studies have also demonstrated that [^{18}F]FLT may be a promising tumor therapy response marker for lung cancer, as well as other kinds of cancers [4, 6, 10–14]. It has been shown that the metabolic mechanism of FLT is based on phosphorylation in the DNA synthesis pathway. Once [^{18}F]FLT enters into the cells by a specific transporter, it is phosphorylated to FLT 5'-monophosphate (FLTMP) by TK-1, a cytoplasmic enzyme responsible for converting thymidine (dT) to the corresponding 5'-monophosphate in rapidly dividing cells such as cancer cells [15, 16]. Subsequent phosphorylations lead to the diphosphate (FLTDP) by thymidylate kinase and to triphosphate (FLTTP) by nucleotide diphosphate kinase. Because of the negatively charged phosphate group, the 5'-monophosphates of various pyrimidine analogues would be retained intracellularly. Tumor uptake of [^{18}F]FLT correlates with the TK-1 activity [6, 17], the expression of which is tightly regulated during the cell cycle. TK-1 activity is very low in G1, increases at the G1/early S boundary, reaches maximum in late S phase/G2, and disappears during mitosis [16, 18]. While phosphate metabolites of nucleosides are predicted to accumulate preferentially in tumor tissue, the use of [^{18}F]FLT as a PET tracer requires a more complete understanding of the mechanisms of accumulation and retention in the tumor cells and tissues.

The TK-1 assay using [γ - ^{32}P]ATP as a phosphate donor has been widely used for the characterization of TK-1 activity of dT analogs by using purified recombinant human thymidine kinase [18–23]. However, a long time (overnight) is required for the development of the PEI-cellulose thin layer chromatography (TLC) plate prior to quantifying the radioactivity of products on the plate. An alternative approach to measure the TK-1 activity uses competition experiments measuring [^3H]dT phosphorylation in the presence of various nonradiolabeled dT analogs [18, 24]. Pioneering work to correlate TK-1 activity and cell uptake of FLT used a ^3H -FLT TK assay in A549 carcinoma cells [15]. As a substrate of

TK-1, [^{18}F]FLT accumulates in proliferating cells after 5'-monophosphorylation. The specificity for cancer cells depends on the selective phosphorylation of [^{18}F]FLT by TK-1 expressed in dividing cells with a high phosphorylation rate leading to high tumor uptake. Kinetic analysis of N-substituted analogues of dT with purified recombinant human TK-1 enzyme yields Michaelis–Menten kinetic parameters, K_m , ranging from 3.4–90 μM [18].

Targeting nucleoside transporters (NTs) by nucleoside analogues have therapeutic and imaging applications in cancer and other diseases [25–28]. Because nucleosides are relatively hydrophilic molecules, their passive diffusion across cell membranes is limited. The transport of nucleosides, such as [^{18}F]FLT, across plasma membranes or between intracellular compartments is mediated primarily by specialized NT proteins. In a fibrosarcoma cell line, Perumal et al. [29] showed by inhibition studies that the uptake of [^{18}F]FLT is mediated predominantly by the human equilibrative nucleoside transporter (hENT1) since it was markedly inhibited by nitrobenzyl thioguanosine or dipyridamole with less inhibition by dT, uridine, adenine, and thymine.

Biochemical evaluation of putative TK-1 substrates for *in vivo* PET imaging requires measurement of their uptake and phosphorylation kinetic parameters in tumor cells. Here, we report development of a novel F-18 phosphoryl-transfer assay to assess phosphorylation of [^{18}F]FLT by tumor cell lysates. Both substrate and phosphorylated products were separated using a rapid TLC method and quantified using the intrinsic F-18 radioactivity. This allowed measurement of apparent Michaelis–Menten kinetic constant (K_m) values directly in cell lysates. Using this method, phosphorylation kinetics of [^{18}F]FLT were measured in both SW480 and DiFi tumor cell lysates. A variation of the assay was used to demonstrate that the uptake of [^{18}F]FLT into SW480 tumor cells, measured in the absence and presence of known inhibitors of NTs, is mediated predominantly by the equilibrative NTs.

Materials and Methods

Reagents

3'-Fluoro-3'-deoxythymidine was purchased from Sigma-Aldrich (St. Louis, MO, USA). FLT cyclic precursor (2,3'-anhydro-5'-O-benzoyl-2'-deoxythymidine) was purchased from ABX Advanced Biochemical Compounds (Radeberg, Germany). Anion exchange cartridges (QMA Sep-Pak) were obtained from Waters (Milford, MA). Unless specifically indicated, all other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical grade or better. Thin layer silica gel-60 plates (Alltech Uniplates, 10×20 cm, pre-scored) were purchased from Grace Davison Discovery Sciences, Deerfield IL.

[^{18}F]FLT Radiotracer Synthesis

[^{18}F]FLT was prepared from the benzoate protected cyclic precursor (2,3'-anhydro-5'-O-benzoyl-2'-deoxythymidine) applying methods as described by Machulla et al. [30, 31] adapted to a commercial GE TRACERlab® FFX-N module [32]. Specific activity was measured by analytical reverse phase high performance liquid chromatography (HPLC) (Dynamax Microsorb 60-8 C18, 250×4.6 mm, Varian, Walnut Creek, CA) eluting with 10%

ethanol/water at 1 mL/min and UV detection at 235 nm, compared with a standard curve of reference FLT. The product was obtained with radiochemical purity >96% and with a specific radioactivity of about 1,000 Ci/mmol in a number of preparations. Mass concentration of [^{18}F]FLT in assays (usually 0.5–1.0 μM) was calculated from the specific activity and radioactivity dose.

Cell Growth and Preparation of Crude Cell Lysates

SW480 colon carcinoma cells were derived from a primary Duke's stage B colon carcinoma resected from a 50-year-old male patient. DiFi cells were originally established from a rectal tumor resected from a patient with familial polyposis. Both cell lines were obtained through American Type Culture Collection and maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) at 37°C and with 5% CO_2 as described [33]. Lysates were prepared from cells growing exponentially ~24 h after re-plating (at 30–50% confluence) by a modification of established methods [34, 35]. In brief, trypsinized cells were washed twice with TBS-EDTA and lysed (4°C) at $\sim 5 \times 10^6$ cells/ml in 10 mM Tris-HCl, pH 7.5, 0.5% P-40 (v/v) 1 mM DTT, 5 mM NaF, 5 mM MgCl_2 , with protease inhibitors, 1 $\mu\text{g}/\text{ml}$ aprotinin and 1 mM phenylmethylsulfonylfluoride. After centrifugation (15,000 $\times g$, 20 min), the lysate supernate was used immediately or stored (0.5 ml aliquots) at –80°C. Since the lysing buffer is likely to also disrupt the mitochondrial membrane, the lysate may also contain mitochondrial TK-2, though in dividing cells TK-2 is reported to be 1–5% of the total TK activity [36] and FLT is a poor substrate for TK-2 [38].

[^{18}F]FLT Phosphoryl-Transfer Assay and Kinetic Analysis

A published method [35] was modified. The standard reaction mixture contained 30 μl lysate in 20 mM KPO_4 , pH 7.6, 40 mM KCl, 5 mM MgCl_2 , 1 mM DTT, 5 mM NaF, 5 mM ATP, and 1.85– 3.70 MBq (50–100 μCi) [^{18}F]FLT in a total volume of 90 μl (for four time points). The NaF was included to inhibit endogenous protein phosphatases in the cell lysate preparation so as to minimize depletion of added ATP via futile kinase/phosphatase cycling. The enzymic reaction at 37°C was usually initiated with [^{18}F]FLT and assays were run in duplicate or triplicate and repeated at least three times. After incubation for 15, 30, 60, or 120 min, 20 μl aliquots were removed, 5 μl 5% SDS added, cooled (ice bath), and immediately spotted (3–5 μl) on a 10 cm silica gel TLC plate that was rapidly (~10 min) developed in 100% acetonitrile and scanned for ^{18}F radioactivity using a Bioscan AR-2000 TLC Scanner (Bioscan, Washington DC, USA). The zero time was defined as the time of addition of [^{18}F]FLT to the reaction mix and the initial zero time point was quenched within 30 s of initiating the reaction. Controls lacked either lysate or ATP. Inhibitors tested included 2 mM dT, 2 mM adenine and 2 mM FLT. For each reaction mixture, the TK-1 activity was calculated from the rate of decrease in substrate over time measured by quantification of ^{18}F compounds separated by TLC from which the percentage of ^{18}F remaining as substrate was determined. TK-1 activities were also measured in lysates from cells harvested at two stages of growth to vary the S phase percentage. To measure the apparent K_m for FLT, the reactions were run with addition of varying concentrations of cold FLT (1 to 60 μM) with data analyzed by nonlinear regression according to the Michaelis–Menten equation using GraphPad Prism 4 software.

Cell Uptake Kinetics and Inhibition

Cell uptake of [^{18}F]FLT was measured at a concentration range of 0.1 to 60 μM FLT with or without pre-treating cells with 2 mM thymidine (dT) inhibitor for 30 min. After incubation of either 30 or 60 min at 37°C with FLT, the cells were rinsed three times with cold DMEM and collected by 1.0 mL trypsin/EDTA. Aliquots of cells were used to count the radioactivity. Each cell uptake assay was performed in triplicate and the uptake rate was calculated as F-18 uptake/min/ 10^6 cells.

To obtain cells with different populations in S phase, SW480 cells were grown to confluence, starved for 24 h (cultured in serum-free medium), and then split into 6-well plates at ~50% confluence. At 6 and 24 h after plating, the cell populations were 19% and 30% in S phase, respectively, as determined by flow cytometry. The uptake assays were performed as described above, except in the absence or presence of various inhibitors: dT, adenine, uridine, (each at 2 mM), or 20 μM nitrobenzylthioinosine (NBMPR) and 20 μM dipyrnidole.

Cell Cycle Analysis

SW480 cells, at various S phases, were harvested by trypsinization and resuspended with PBS/0.1% BSA to a concentration of 1×10^6 cell/ml. Cells were then fixed with cold ethanol and stained with propidium iodide (3.8 mM sodium citrate, 50 $\mu\text{g/ml}$ PI, 0.5 mg/ml RNase A in PBS) at a cell concentration of 1×10^6 cell/ml. Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences, Mountain View, CA) with CellQuest software (BD Biosciences).

Results

A Novel Assay for Measurement of TK-1 Activity and Inhibition in Cell Lysates

[^{18}F]FLT metabolism by SW480 colon cancer cell lysate was evaluated after 30 min and 2 h incubation with ATP. Analysis of putative metabolic products of phosphorylation of [^{18}F]FLT in cancer cell lysates by radio-TLC at three time points is shown in Fig. 1. After the reaction mixture was spotted on the TLC plate, it was rapidly developed in 100% acetonitrile (~10 min). The substrate [^{18}F]FLT migrated at an R_f of 0.51 ± 0.03 . After incubation with SW480 lysate, F-18 radioactivity was detected in three additional bands (Fig. 1), which were assigned to FLTMP, FLTDP, and FLTTP by analogy to products detected by HPLC [16, 37]. Controls either without lysate or ATP showed only a single peak corresponding with the [^{18}F]FLT substrate. After 30 min, about 70% of initial FLT was converted to metabolites (Fig. 1a, middle trace). After 120 min incubation, there was almost complete conversion of [^{18}F]FLT to FLTDP/FLTTP metabolites (Fig. 1a, upper trace). In DiFi cell lysates (Fig. 1b), ~95% FLT was converted to metabolic products after 30 min incubation. However, conversion from the FLTMP to FLTDP/FLTTP was slower than in the SW480 cell lysates with ~40% FLTMP remaining after 120 min.

Estimation of Kinetic Parameters K_m and V_{max}

The new assay was applied to estimate the quantitative rate parameters for phosphorylation of [^{18}F]FLT in SW480 cell lysates. Conversion of [^{18}F]FLT was measured by TLC scan at

0, 15, 30, 60 min, respectively, using a constant concentration of [^{18}F]FLT titrated with increasing concentrations of cold FLT ranging up to 56 μM (Fig. 2). The concentration of metabolic products increases and the reaction time courses are linear during the first 30 to 60 min under these experimental conditions. Beyond 60 min., the rate tends to decrease (not shown) towards the end-point of complete conversion to products (see Fig. 1). The initial rates of phosphorylation of [^{18}F]FLT were determined from the slope of the increase in products over 60 min reaction.

The calculated initial rates of phosphorylation of [^{18}F]FLT, as a function of FLT, yield a saturation curve and Eadie–Hofstee plot indicative of the TK-1 kinetics (Fig. 3). The average of apparent Michaelis–Menten constant (K_m) which was calculated from three independent series of measurements is $4.8 \pm 0.3 \mu\text{M}$ (mean $\pm$ standard error of the mean) with mean values of a maximum velocity (V_{max}) for the lysate of $7.4 \pm 0.24 \text{ pmol min}^{-1}$ per 1×10^6 cells.

[^{18}F]FLT Uptake Kinetics in SW480 Cells

With this new assay, the concentration dependency of FLT cellular uptake was characterized in SW480 colon cancer cells at 37°C using a combination of [^{18}F]FLT radiotracer (0.7 μM) and 0.2–50 μM carrier FLT (Fig. 4). FLT uptake was not saturable at even up to 100 μM FLT (not shown) with an apparent Michaelis–Menten K_t calculated from linear regression of the Eadie–Hofstee plot (Fig. 4b) to be $27 \pm 2 \mu\text{M}$. The calculated maximum uptake velocity (V_{max}) was $97 \pm 6 \text{ pmol} \cdot \text{min}^{-1}$ per 1×10^6 cells. Analysis of the cell lysates, prepared from cells at each of the time points, showed by TLC, almost complete conversion of the trapped F-18 to phosphorylated products (not shown) analogous to those exhibited in Fig. 1a and b (upper traces) where all the [^{18}F]FLT is phosphorylated to FLTMP, FLTDP and FLTTP.

[^{18}F]FLT Cell Uptake Mechanism— Membrane Transport Inhibition

To elucidate the transport mechanism of [^{18}F]FLT, uptake of [^{18}F]FLT in SW480 cells was studied at two time points (30 and 60 min) in the presence of known inhibitors of nucleoside transport systems, including dT, adenine, uridine at 2.0 mM, and NBMPR, dipyridamole at 20 μM . In SW480 cells either in the absence or presence of inhibitors, the total uptake of [^{18}F]FLT at 60 min was about twice that at 30 min. (Fig. 5). The uptake of [^{18}F]FLT (0.8 and 1.7 μCi per 10^6 cells at 30 and 60 min) was almost completely inhibited by dT (96%) or dipyridamole (91%) and partly inhibited by adenine (51%), uridine (70%), or NBMPR (84%) with comparable inhibition observed at either 30 or 60 min. The cell uptake kinetics measured at a lower percentage of S phase cells (19%) was markedly lower than with 30% S phase cells, although the effect of inhibitors was comparable (not shown).

Discussion

[^{18}F]FLT has recently been developed as a PET tracer conceived to image cell proliferation in tumors based on its uptake into cells followed by phosphorylation [4, 5]. After being transported across the cell membrane, [^{18}F]FLT is phosphorylated by TK-1, with TK-1 activity governing FLT uptake in tissue as measured by PET [15]. However, the level of TK-1 expression within tumor cells varies and although correlated with the rate of cellular

proliferation, such variation confounds prediction of the utility of [^{18}F]FLT for PET imaging in particular types of tumors. Based on an interest in FLT or its analogs as substrates for TK-1 in the context of PET tumor imaging, we report here the development of a novel rapid biological assay for measuring the phosphorylation of [^{18}F]FLT *in vitro* and have applied the method to assessing the TK-1 activity in cancer cell lysates and for measuring [^{18}F]FLT uptake by tumor cells.

Firstly, the assay is fast and simple enabling simultaneous assay of multiple reactions that provide facile determination of relative TK-1 activities and kinetic parameters for the initial rate of phosphorylation of [^{18}F]FLT by TK-1 in tumor cell lysates. Secondly, the method quantitatively measures the metabolized products of [^{18}F]FLT in a short time. In this assay, the reaction mix was treated with SDS to terminate the reaction and the radioactive metabolized products of [^{18}F]FLT including FLTMP, FLTDP, and FLTTP were detected by a simple radio-TLC plate which was developed in acetonitrile in less than 10 min. The initial rate of FLT phosphorylation is determined by the rate of decrease in substrate as calculated from the percentage of FLT in the total [^{18}F]-labeled metabolic products. The total amount of metabolized products in a reaction mixture at any time point can be calculated from the percentage of FLT substrate remaining (FLT%) and the initial FLT concentration in the reaction. The FLT% is measured by scanning the radioactivity of TLC plate and taking the reasonable assumption that the percentage of hot (labeled) [^{18}F]FLT equals that of cold (unlabeled) FLT. In this study, the relative rate of FLT phosphorylation in each cell type, ~2-fold faster in DiFi versus SW480 lysates, can be appreciated by comparing the rate of decrease in substrate (Fig. 1c). Interestingly, the rates of phosphorylation of FLT, as measured by depletion of FLT over time, were found to be essentially linear for at least 60 min (Fig. 2), with >50% conversion to phosphorylated products. In part, this can be accounted for by the relatively high level of ATP (5 mM) used in the assay and also by the further phosphorylation of FLTMP, the product formed by TK-1-mediated phosphorylation of FLT to FLTDP and FLTTP. Since FLTMP does not significantly accumulate over time, particularly in assays with SW480 cell lysates (Fig. 1a), this abrogates potential product inhibition or significant de-phosphorylation back-reaction [37] that might otherwise complicate the observed simple kinetics for TK-1 phosphorylation of FLT.

We included 5 mM NaF in the assay as a general protein phosphatase inhibitor to minimize depletion of ATP by the cell lysate. Under conditions of the assay, excess ATP drives sequential phosphorylation of the FLTMP product obtained from the initial reaction mediated by TK-1 to predominantly FLTDP and FLTTP. As is shown in Fig. 3, the measured apparent Michaelis–Menten constant K_m (4.8 μM) for phosphorylation of [^{18}F]FLT is comparable to, though somewhat higher than, that reported by others (2.1 μM) for FLT phosphorylation using purified TK-1 [38]. Although the cell lysates are likely to also contain the mitochondrial TK-2, TK-1 predominates in proliferating cells [36]. In addition, TK-2 fails to phosphorylate FLT [38], supporting the interpretation that the phosphorylation of [^{18}F]FLT in cell lysates is mediated by TK-1. Since both SW480 and DiFi human cell lines were cultured in DMEM (devoid of dT) supplemented with 10% FCS, reported to have dT concentrations ranging up to ~2 μM [39], and intracellular and extracellular dT are approximately in equilibrium [40], the maximal intracellular

concentration of dT is estimated to be $\sim 0.2 \mu\text{M}$. Thus the endogenous dT in the cell lysates ($\sim 5 \times 10^6$ cells/ml and cell volume $\sim 2\text{--}4 \mu\text{l}/10^6$ cells) is estimated to be $<1 \text{ nM}$, a concentration with negligible effect on either the lysate assay for FLT phosphorylation or FLT uptake assays reported here in which FLT concentrations in the μM range are used. However, the presence of other nucleosides and nucleotides in the lysate, though diluted ~ 100 -fold from intracellular concentrations, may compete in the assay, an effect that may contribute to the somewhat higher apparent K_m for phosphorylation of FLT by TK-1 in cell lysates as compared with the K_m for FLT measured by others using the purified enzyme.

$[^{18}\text{F}]\text{FLT}$ PET has been considered as a noninvasive method for quantitating cellular proliferation in tumor and may be useful for monitoring tumor response to therapy. However, low uptake of $[^{18}\text{F}]\text{FLT}$ can cause missed detection of the tumor and limit the ability to image proliferation *in vivo*. Quantitative characterization and comparison of the cellular membrane transport efficiency of FLT analogs in specific tumor cells is a key step to the successful development of a sensitive imaging agent. Therefore, we applied the new assay to measure FLT uptake into SW480 human colon tumor cells using a combination of $[^{18}\text{F}]\text{FLT}$ radiotracer ($0.7 \mu\text{M}$) and up to $50 \mu\text{M}$ carrier FLT and showed efficient uptake (V_{max} of $97 \pm 6 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{per } 10^6 \text{ cells}$) that was markedly inhibited by dT. By contrast, a much smaller FLT uptake ($\sim 5 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{per } 10^6 \text{ cells}$) has been reported in HL-60 cells [41], with the FLT uptake being about half as fast as the uptake of dT. Likewise HPLC analysis of $[^{18}\text{F}]\text{FLT}$ metabolites in actively growing pancreatic cancer cell lines, such as SW-979 and BxPc-3, showed time-dependent uptake and conversion to FLTMP that was associated with the levels of TK-1 expression; further phosphorylation to FLTDP or FLTTP was not observed and there was minimal uptake of $[^{18}\text{F}]\text{FLT}$ in growth-arrested HT1080 fibroblasts and no significant uptake in isolated pancreatic lobules [16]. However, the uptake of FLT or of two other fluoronucleoside probes, FMAU and FIAU that could be incorporated into DNA, were each less than 15% of dT uptake as measured in human lung A549 adenocarcinoma cells [37]. Interestingly, in that study, FLTMP was also the predominant intracellular metabolite with minimal FLTDP and only partial conversion to FLTTP even after 2 h [37], whereas in the studies reported here, after 2 h either with SW480 or DiFi lysates (Fig. 1) or with SW480 cells (not shown), FLTTP and/or FLTDP predominated with little FLTMP that was observed only at earlier time points. The efficient uptake of $[^{18}\text{F}]\text{FLT}$ by SW480 cells that we observe, is consistent with high TK-1 expression in this colon cancer cell line. Similar uptake kinetics were observed with an essentially linear reciprocal plot without apparent saturation up to $100 \mu\text{M}$ FLT, a result consistent with a low-affinity and high-capacity system for the transport of FLT across the cell membrane. Combined with the results of phosphorylation kinetics above, the FLT uptake data suggests that metabolic trapping of FLT through phosphorylation by TK-1 is the rate-limiting step for cellular uptake and thus suggests that designing high-affinity substrates for TK-1 has potential for developing imaging agents with enhanced sensitivity as compared with FLT.

Due to their hydrophilicity, nucleosides do not traverse cellular membranes by diffusion but are transported by either equilibrative or concentrative transport. Such transporters for dT are up-regulated in tumors. In our studies, the specificity of FLT transport in SW480 human

colon cells was evaluated by co-incubating the FLT substrate with potential inhibitors with largest inhibition (96%) being effected by dT (2 mM) and only partial inhibition by the same concentration of uridine. The substituent at five positions of dT, such as methyl group strongly affects the interaction between the substrate and transporters. The inhibition by dT likely reflects both inhibition of transport and competitive inhibition of FLT phosphorylation by TK-1 since self-inhibition experiments revealed that unlabeled FLT substrate and dT each gave similar inhibition of radiolabeled [^{18}F]FLT uptake (not shown). The role of equilibrative nucleoside transporter for the uptake of [^{18}F]FLT in RIF-1 tumors was explored by Peruma et al [29] using known inhibitors of nucleoside transport and indicated that in RIF-1 fibrosarcoma cells, [^{18}F]FLT is transported by an equilibrative nucleoside transporter (ENT1), although at low levels compared with dT. In the studies reported here, the marked inhibition by ENT inhibitors NBMPR (ENT1 selective) or by dipyrda-mole (ENT1 and ENT2 selective) indicates that the equilibrative nucleoside transporters (ENT1 and/or ENT2) are major contributors to FLT uptake (>80%). As found by others in RIF-1 cells [29], our results with SW480 colon cancer cells are consistent with ENT1, in particular, serving to transport [^{18}F]FLT into tumors..

Several pyrimidine nucleoside analogs have been reported as potential agents for imaging tumor proliferation [42]. Such substituted derivatives are phosphorylated by human and other mammalian nucleoside kinases, such as TK-1. For example, ^{18}F radiolabeled FMAU is currently undergoing clinical studies in multiple centers for imaging tumor proliferation in a variety of cancer types. *in vitro* quantitative assessment and characterization of phosphorylation of fluorinated derivatives of pyrimidine nucleoside analogs in tumor cells is a necessary step for development of PET imaging agents for imaging nucleoside uptake. The new method for quantitative measurement of TK-1 activity of the radiotracers in tumor cells described here has utility for both testing new radiolabeled analogs directly and screening potential enzyme substrates by competitive assay against [^{18}F]FLT.

In summary, we have developed a new ^{18}F phosphorylation assay for the measurement of TK-1 activity in cell lysates with rapid analysis by TLC. Results from each assay can be obtained rapidly (<10 min) and multiple reactions can be assayed simultaneously. The assay not only provides a fast and simple method to measure TK-1 kinetic parameters, including the apparent Michaelis–Menten kinetic parameters (K_m and V_{max}) and cell uptake rates, but also has potential for testing the phosphorylation of new analog tracers mediated by TK-1. There is a relatively high TK-1 activity in cell lysates (V_{max} of $7.4 \text{ pmol min}^{-1}$ per 1×10^6 SW480 cells) with an apparent K_m for [^{18}F]FLT of $4.8 \pm 0.3 \text{ }\mu\text{M}$, slightly higher than the value reported with purified recombinant TK-1 [38]. Uptake kinetics into intact SW480 tumor cells yielded a value of K_t of $27 \pm 2 \text{ }\mu\text{M}$, with maximum uptake velocity V_{max} of $97 \pm 6 \text{ pmol min}^{-1}$ per 1×10^6 cells, data consistent with a low-affinity and high-capacity system. The present study on colon cancer SW480 cells provides functional evidence for the role of both equilibrative (ENT1 and ENT2) and concentrative nucleoside transport systems for FLT uptake, with most of the transport (>80% FLT) into SW480 cells being mediated by one or both of the equilibrative transporters.

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Fig. 1. Analysis of putative metabolic products of phosphorylation of $[^{18}\text{F}]\text{FLT}$ in cancer cell lysates (1×10^6 cells) by radio-TLC. **A** SW480 and **B** DiFi cancer cell lysates, each at 0, 30, and 120 min reaction; **C** time course of $[^{18}\text{F}]\text{FLT}$ composition in each cell line. TLC was developed in 100% CH_3CN . $[^{18}\text{F}]\text{FLT}$ 3'-deoxy-3'- $[^{18}\text{F}]\text{fluorothymidine}$, FLTMP FLT monophosphate, FLTDP FLT diphosphate, FLTTP FLT triphosphate.

Fig. 2.

The time courses of phosphorylation of [^{18}F]FLT during the first 60 min for different values of the FLT concentration **a** 0.42 μmol , $R^2=0.99$, **b** 2.62 μmol , $R^2=0.99$, **c** 4.62 μmol , $R^2=0.99$, **d** 7.32 μmol , $R^2=0.99$, **e** 11.52 μmol , $R^2=0.98$, **f** 17.12 μmol , $R^2=0.99$, **g** 28.22 μmol , $R^2=0.99$, and **h** 56.42 μmol , $R^2=0.97$. Error bars depict SD ($n=3$).

Fig. 3. Phosphorylation of $[^{18}\text{F}]\text{FLT}$. Direct (A) and reciprocal Eadie-Hofstee (B) plots for phosphorylation of $[^{18}\text{F}]\text{FLT}$ in SW480 cancer cell lysates in the presence of 5 mM ATP measured by radio-TLC developed in 100% CH_3CN . Regression analysis is consistent with a linear relationship ($R^2=0.98$).

Fig. 4.

Effect of concentration on the uptake of FLT. Concentration dependence (A) and reciprocal Eadie-Hofstee plot (B) for [^{18}F]FLT (0.05 to 50 μM) uptake by SW480 cancer cells at 37°C. Regression analysis is consistent with a linear relationship ($R^2=0.96$).

Fig. 5. Nucleoside and pharmacological inhibition of $[^{18}\text{F}]\text{FLT}$ uptake by S480 cells growing in culture at 30% S phase. *Grey* 30 min, *black* 60 min.