Evaluation of $^{18}F$fluorothymidine as a biomarker for early therapy response in a mouse model of colorectal cancer

Reference:
Rapic Sara, Vangestel Christel, Verhaeghe Jeroen, Thomae David, Pauwels Patrick, van den Wyngaert Tim, Staelens Steven, Stroobants Sigrid.- Evaluation of $^{18}F$fluorothymidine as a biomarker for early therapy response in a mouse model of colorectal cancer
Full text (Publisher's DOI): http://dx.doi.org/doi:10.1007/S11307-016-0974-5
To cite this reference: http://hdl.handle.net/10067/1385190151162165141
Evaluation of $[^{18}\text{F}]$fluorothymidine as a biomarker for early therapy response in a mouse model of colorectal cancer

Sara Rapic$^a$, Christel Vangestel$^{a,b}$, Jeroen Verhaeghe$^a$, David Thomae$^{a,b}$, Patrick Pauwels$^{c,d}$, Tim Van den Wyngaert$^{a,b}$, Steven Staelens$^a$, Sigrid Stroobants$^{a,b}$

$^a$Molecular Imaging Center Antwerp (MICA), Faculty of Medicine and Health Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium
$^b$Department of Nuclear Medicine, Antwerp University Hospital, Wilrijkstraat 10, 2650 Edegem, Belgium
$^c$Center for Oncological Research (CORE), University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium
$^d$Department of Pathology, Antwerp University Hospital, Wilrijkstraat 10, 2650 Edegem, Belgium

**Corresponding author**
Prof. Dr. Sigrid Stroobants
E-mail: Sigrid.Stroobants@uza.be
Phone: +3238213696
Fax: +3238253308

**Manuscript category:** Original article

**Running title:** $[^{18}\text{F}]$FLT-PET for Early Therapy Response
Abstract

**Purpose.** In oncology, positron emission tomography imaging using dedicated tracers as biomarkers may assist in early evaluation of therapy efficacy. Using [\(^{18}\)F]fluorothymidine ([\(^{18}\)F]FLT), we investigated the early effects of chemotherapeutic treatment on cancer cell proliferation in a BRAF-mutated colorectal cancer xenograft model. **Procedures.** Colo205 subcutaneously inoculated animals underwent 90-min dynamic imaging before and 24 hours after treatment with vehicle (control), cetuximab (resistant) or irinotecan (sensitive). Total distribution volume was quantified from dynamic data and standardized uptake values as well as tumor-to-blood ratios were calculated from static images averaged over the last 20 minutes. *In vivo* imaging data was correlated with *ex vivo* proliferation and thymidine metabolism proteins. **Results.** All imaging parameters showed a significant post-treatment decrease from [\(^{18}\)F]FLT baseline uptake for the irinotecan group (*p*≤0.001) as compared with the cetuximab and vehicle group, and correlated strongly with each other (*p*≤0.0001). *In vivo* data were in agreement with Ki67 staining, showing a significantly lower percentage of Ki67-positive cells in the irinotecan group as compared with other groups (*p*≤0.0001). Tumor expression of thymidine kinase 1 phosphorylated on serine 13, thymidylate synthase and thymidine phosphorylase remained unaffected, while thymidine kinase 1 expression was, surprisingly, significantly higher in irinotecan-treated animals (*p*≤0.01). In contrast, tumor ATP levels were lowest in this group. **Conclusions.** [\(^{18}\)F]FLT-PET was found to be a suitable biomarker of early tumor response to anti-proliferative treatment, with static imaging not being inferior to full compartmental analysis in our xenograft model. The dynamics of thymidine kinase 1 protein expression and protein activity in low ATP environments merits further investigation.

**Keywords:** [\(^{18}\)F]-fluorothymidine, proliferation, colorectal cancer, positron emission tomography, kinetic modeling
Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies and one of the leading causes of cancer-related mortality worldwide [1]. Non-invasive positron emission tomography (PET) imaging of cancer hallmarks using dedicated tracers as predictive biomarkers may assist in the timely selection of an optimized and individualized treatment regimen. The PET tracer 3'-deoxy-3'-[\(^{18}\)F]fluorothymidine ([\(^{18}\)F]FLT), a thymidine analogue, is able to specifically visualize cell proliferation [2]. Cellular uptake of [\(^{18}\)F]FLT is determined by the activity of the thymidine salvage pathway (Fig. 1). In this pathway exogenous thymidine, regulated by thymidine phosphorylase (TP), is transported across the cell membrane by nucleoside transporters. Once inside the cell, it is phosphorylated to thymidine monophosphate by thymidine kinase-1 (TK1) in the presence of adenosine triphosphate (ATP), and finally incorporated into DNA as thymidine triphosphate [3, 4]. ATP also acts as a co-factor for TK1 activity, inducing a reversible oligomeric shift of TK1 during the S-phase from a dimeric to tetrameric form, with the dimer exhibiting low affinity to thymidine [5, 6]. [\(^{18}\)F]FLT traces the thymidine salvage pathway, except once phosphorylated by TK1, [\(^{18}\)F]FLT is intracellularly retained since the fluorine at the 3’ position prevents its incorporation into DNA. As a result, [\(^{18}\)F]FLT uptake can be considered as a measure of TK1 activity, which is closely linked to proliferation and peaks during the S-phase of the cell cycle [3, 7]. Recent studies have evaluated [\(^{18}\)F]FLT as potential early biomarker of treatment response in subcutaneous xenograft CRC models and have shown [\(^{18}\)F]FLT uptake to correlate with TK1 activity and/or proliferation [8, 9].

A preferred method of PET data quantification is compartmental or kinetic modeling from which biological measures such as tracer net influx rate and distribution can be obtained [10, 11]. However, this approach is labor-intensive and time-consuming as it requires the plasma concentration over time acquired by invasive, continuous blood sampling during a dynamic scanning protocol that typically lasts up to 60-120 minutes. Although several studies
have shown the feasibility of the application of a blood input function (IF) derived directly from the PET image, the prolonged scanning remains a limitation [12]. Therefore, simplified albeit semi-quantitative methods such as standardized uptake value are preferred to measure tracer uptake, especially in a clinical setting as they require less complex scanning and data analysis protocols.

Using $^{18}$F]FLT, we evaluated the early effects on cancer cell proliferation of cetuximab and irinotecan in a subcutaneous, BRAF-mutated mouse model of CRC. Cetuximab binds to the human epidermal growth factor receptor (EGFR), which is overexpressed in many cancers including CRC, preventing its activation and resulting in the inhibition of the mitogen-activated protein kinase (MAPK) pathway, which is involved in DNA synthesis, cell proliferation and migration [13]. However, tumors with mutations in BRAF, an oncogene effector downstream of EGFR, are resistant to the effects of EGFR inhibitors [14, 15]. Irinotecan is a chemotherapeutic agent clinically used to treat metastatic CRC. As a prodrug, irinotecan needs to be activated by carboxylesterases to its active metabolite SN-38, a topoisomerase I inhibitor. Activated irinotecan causes single-strand DNA breaks, subsequent cell cycle arrest in the S-phase and indirect inhibition of TK1 [16, 17].

In order to investigate the feasibility of $^{18}$F]FLT as biomarker for early treatment response, we assessed changes in $^{18}$F]FLT uptake and correlated various in vivo imaging parameters with ex vivo tumor cell proliferation. We performed these studies in subcutaneous CRC xenografts treated with irinotecan (sensitive) and compared these results with cetuximab (resistant) and vehicle (control)-treated xenografts. Furthermore, compartmental analysis was used to characterize $^{18}$F]FLT kinetics in the tumors and correlate the obtained parameters to values acquired with simplified, semi-quantitative methods in order to evaluate whether simplified models can reliably be used as a surrogate for kinetic parameters.
Material and Methods

Radiotracer Preparation

$[^{18}F]FLT$ was produced on a Veenstra FluorSynthon II synthesis module that was adapted for fully automated synthesis of the tracer. Briefly, 25 mg of 3-N-Boc-5′-O-dimethoxytrityl-3′-O-nosyl-thymidine (ABX) dissolved in 0.5 ml anhydric acetonitrile was added to azeotropically dried $[^{18}F]$-F and the mixture was heated at 115°C for 8 min. The ACN was evaporated under reduced pressure for 1.5 min and 500 μl of 1N HCl was added and the mixture was heated to 105°C for 5 min. Next, the reaction mixture was quenched with 1 ml NaOAc 2M and loaded onto a HPLC loop through a preconditioned Alumina cartridge. $[^{18}F]FLT$ was purified using a Phenomenex Luna C18(2), 250×10 mm, 10 μm HPLC column and a mixture of sterile saline/ethanol (90/10, V/V) as mobile phase at a flow rate of 3 ml/min. The fraction containing $[^{18}F]FLT$ (Rt=12 min) was collected and transferred to a shielded LAF cabinet where it was diluted with 0.9 % NaCl to reduce the ethanol concentration to < 10 % and sterile filtered (VWR PES 25mm 0,22 μm syringe filter). To evaluate the chemical and radiochemical purity, reverse phase analytical HPLC analysis (Chromolith RP 18e 100×4,6mm) was performed using an isocratic elution of methanol/water (7,5/92,5, V/V) at a flow rate of 1 mL/min. Radiochemical purity was > 95% and specific activity amounted to 286±95 GBq/μmol.

In Vivo Experiments

Xenograft Tumor Model

Colo205 cells (2x10⁶ cells in 0.1 ml PBS per mouse) were used to grow subcutaneous tumors in the right hind limb of 5-7 week-old female CD1 athymic nude mice (Charles-River). Tumor diameters were measured with a digital caliper and volume was calculated by the formula: $\frac{1}{2}$ (length x width²). All animal experiments were approved by the local ethical committee and were performed in accordance with European and Belgian regulation (2012-58).

Experimental Setup
Our experimental design was based on an initial proof of concept study to determine the effect of irinotecan on tumor cell proliferation and apoptosis in this xenograft tumor model by means of immunochemistry. This showed a prominent decrease in proliferation 24 hours after administration of a single dose of irinotecan, while apoptosis levels remained unaffected (Supplemental Data, Fig. S1). Experiments started when tumors had reached an average size of approximately 500 mm$^3$ and mice were randomized into three treatment groups: control (saline, $n=8$), cetuximab (1 mg of clinical-grade 5 mg/ml per mouse, $n=7$) and irinotecan (100 mg/kg of clinical-grade 20 mg/ml, $n=8$). All agents were administered intraperitoneally as a single bolus in a maximum volume of 0.2 ml.

*Image Acquisition and Reconstruction*

Imaging was performed using two Inveon scanners with a spatial resolution of ~1.5 mm (Siemens) [18]. For every animal, a 90-min dynamic scan was acquired before and 24 hours after treatment. Prior to tracer administration, mice were anesthetized with 4% isoflurane (AbbVie) in oxygen and maintained with 1-2% during preparations and scan acquisition, while their body temperature was maintained at 37°C with a heating pad. The amount of delivered anesthesia was adjusted manually according to constant observation of the respiratory rate. First, a tail vein catheter was inserted to facilitate intravenous injections. To avoid image artifacts, a double-lumen urethral catheter optimized for molecular imaging was inserted next and connected to a clinical infusion pump (Fresenius) in order to rinse out radioactive urine from the animal’s bladder [19]. Finally, the animal was placed onto a multimodal scanner bed (Minerve) and positioned in the μPET/CT gantry. Acquisition of emission data on the Inveon scanners was started simultaneously with the intravenous injection of 9.25 MBq [$^{18}$F]FLT and with continuous bladder flushing at a rate of 15 ml/h.

With each PET scan, a computed tomography (CT) scan was acquired in 4x binning mode with a 50 μm spot size at 80 kVp and 500 μA in 120 projections for scatter and attenuation correction.
of the PET data. μPET and μCT images were intrinsically co-registered since they were acquired on a multimodal gantry (Minerve). The acquired μPET data were histogrammed in 39 time frames (12x10, 3x20, 3x30, 3x60, 3x150, 15x300 s) and reconstructed using three-dimensional ordered subset expectation maximization (OSEM3D, 16 subsets and 2 iterations) followed by 18 maximum a posteriori (MAP) iterations including scatter and attenuation correction. This resulted in an axial field of view of 12.7 cm and a 128x128x159 matrix of 0.77x0.77x0.79 mm voxels. μCT acquisitions were analytically reconstructed using the Feldkamp algorithm to a 352x352x606 matrix with 0.223 mm voxels.

Image Analysis

All μPET/CT images were analyzed using PMOD v3.3 (PMOD Technologies). To determine the optimal compartmental model for [18F]FLT in our animal model, we tested a two-tissue compartment model (2TCM) [11]. In this model, the kinetic parameters $K_1$ and $k_2$ represent the rate of forward and reverse [18F]FLT transport between plasma and tissue, respectively, and $k_3$ and $k_4$ describe TK1 phosphorylation and dephosphorylation rate, respectively (Fig. 1). The blood volume fraction was included in the modeling, and both 2TCM with reversible phosphorylation ($k_4 \neq 0$) and irreversible phosphorylation ($k_4 = 0$) were tested. Blood IFs were derived directly from the time activity curve (TAC) of the left ventricle (LV) of the heart in the dynamic images. For this purpose, a region of interest (ROI) was drawn in the LV in an early frame from the dynamic PET image and the LV 2D isocontour at 95% of the maximum was determined. To obtain a tumor TAC, the tumor was first localized on the CT image. Next, in late frames from the PET image, a 3D isocontour volume of interest (VOI) at 70% of the maximum PET pixel value was defined within the tumor. TACs were extracted and subsequently fitted using following initial values: 0.05 for the blood volume fraction, 0.1 for $K_1$ (ml/ccm/min), $k_2$ (1/min) and $k_3$ (1/min), and 0 or 0.01 for $k_4$ (1/min). From the fitted kinetic
parameters, the tracer net influx rate ($K_{\text{FLT}}$) and the total distribution volume ($DV_{\text{tot}}$) of $[^{18}\text{F}]$FLT were calculated using the formulas $(K1k3)/(k2+k3)$ or $(K1/k2)x(1+(k3/k4))$, respectively.

The kinetic measure with which we proceeded, depended on the optimal compartment model as determined on the basis of the Akaike information criterion (AIC) obtained from all animals (before and after treatment), with the lowest value representing the best fit. Correlations were examined between the kinetic measure obtained with the best model and the static measure calculated from different late time frames (50-60, 60-70, 70-80 and 80-90 min) in order to define the optimal time frames for static analysis. These frames were then averaged and the tumor VOI was applied onto the obtained static image to calculate the mean standardized uptake value ($SUV_{\text{mean}}$) by dividing the mean activity (in kBq/cc) in the tumor VOI by the corresponding injected radiotracer activity (in kBq) normalized to the body weight (in g) of the mouse at the time of the experiment. Additionally, mean tumor-to-blood ratios ($TBR_{\text{mean}}$) were calculated by dividing the mean activity in the tumor VOI by the mean activity in the LV ROI.

**Ex Vivo Validation Experiments**

**Biodistribution**

Immediately after their post-treatment scan animals were euthanized through cervical dislocation, tumors were removed and weighed, and radioactivity was counted in an automatic gamma counter (2480 Wizard², PerkinElmer). Tumor uptake was expressed as percentage of injected dose per gram of tissue (%ID/g). After gamma counting, tumors were cut in half: one half was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry, while the other half was snap-frozen in liquid nitrogen and used for protein studies.

**Immunohistochemical Analysis**

Formalin-fixed, paraffin-embedded tumor tissue was cut in 5-µm-thick sections for immunostaining. Sections were deparaffinized in xylene and rehydrated in graded ethanol.
Antigen retrieval was performed by maintaining the sections in 10 mM citrate buffer (pH 6.0) at sub-boiling temperature. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Sections were incubated overnight at 4°C with primary antibodies against cleaved caspase-3 (CC3), Ki67, TK1, TK1 phosphorylated on serine 13 (pTK1), TS and TP. On the next day, sections were incubated at room temperature for 30 minutes with either secondary goat anti-rabbit antibody (DAKO) or SignalStain Boost Detection Reagent (Cell Signaling) and treated with diaminobenzidine (DAKO). Finally, the sections were counterstained with Mayer’s hematoxylin (Sigma-Aldrich) and mounted with water based mounting medium (Aquatex, Merck). An overview of primary and secondary antibodies is given in Table 1.

Stained sections were examined and analyzed under a light microscope (CX-31 RBSF, Olympus) at 200x magnification for CC3 or at high power field (x400 magnification), excluding areas of necrosis. Two independent observers including a certified pathologist performed all staining analysis. CC3-positive cells were quantified using dedicated software (NIS-Elements, Nikon), calculating the average percentage of positive cell fragments. Ki67 staining was evaluated by assigning to each section a percentage of positively stained nuclei. Evaluation of TK1, pTK1, TS and TP expression was assessed by multiplying the intensity score (0, 1, 2 or 3) with the percentage of positively stained (0-100%) cells at each intensity and calculating their sum [20]. All scoring values were obtained from 10 equally sized ROIs chosen in one representative tumor section and averaged to achieve the final score for each staining.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

TK1 protein concentrations were determined in vehicle (n=8), cetuximab (n=7) and irinotecan (n=7)-treated tumor homogenates using a commercially available ELISA kit (SEC823Hu, USCN Life Science Inc.). Snap-frozen tumor tissue was minced to small pieces and homogenized in 10 ml PBS on ice with a glass homogenizer (VWR). The resulting suspension was sonicated (Elma Ultrasonic) and homogenates were centrifuged for 5 min at 5000xg at 4°C.
Supernatants were removed and stored at -80°C until processing. The assay was performed in duplicate and according to manufacturer’s instructions. The measured TK1 concentrations were normalized to total protein content (BCA Protein Assay Kit, Pierce, Thermo Fischer Scientific) and expressed in picograms per milligram.

The tumor ATP content from vehicle (n=8), cetuximab (n=7) and irinotecan (n=7)-treated animals was quantified using the ATP Colorimetric Assay Kit (ab83355, Abcam), providing a reaction mix containing 4% ATP probe, 4% ATP converter and 4% developer in ATP Assay Buffer. Snap-frozen tumor tissue was homogenized in 100 μl ATP buffer using a 3-mm Polytron mixing homogenizer (PT 1200 E, Kinematica AG) at highest speed for 30-60 sec. Homogenates were centrifuged for 5 min at 13000xg at 4°C, supernatants were collected and kept on ice at all times. ATP standards and collected samples were placed in duplicate into a 96-well plate following manufacturer’s instructions. ATP concentrations in the samples were calculated by plotting the measured absorbance at 570 nm against the standard curve, normalized to total protein content (BCA Protein Assay Kit, Pierce, Thermo Fischer Scientific) and expressed in nanomoles per milligram.

**Statistical Analysis**

All statistical analyses were performed using GraphPad Prism software version 6.0c. Statistical significance was determined for all data, expressed as mean ± SEM, using a non-directional paired or unpaired Student’s t test to compare two groups, a one-way ANOVA to compare more than two unpaired groups or a two-way ANOVA with repeated measures (followed by Tukey’s multiple comparisons test) to compare therapy effect. Correlations were tested using the Pearson’s correlation coefficient. Statistical significance was set at a 5% level (p≤0.05). Asterisks on plots and graphs denote a statistically significant difference versus all other groups, unless otherwise stated, and are marked as follows: * = p≤0.05, ** = p≤0.01, *** = p≤0.001 and **** = p≤0.0001.
Results

In Vivo Experiments

The lowest AIC value was found when 2TCM included a non-zero \( k_4 \) during curve fitting (Table 2). Using 2TCM, compartmental analysis of \([^{18}F]FLT\) kinetics was performed and the kinetic measure \( D_{V_{tot}} \) was calculated. The averaged image-derived input function obtained from all animals showed a fast uptake of \([^{18}F]FLT\) and a rapid clearance (Fig. 2a). The averaged post-treatment tumor TACs displayed significantly lower \([^{18}F]FLT\) retention in irinotecan-treated tumors as compared with the cetuximab and vehicle group from 30 minutes after tracer injection (Fig. 2b, \( p \leq 0.05 \)). This was confirmed by the changes in \( D_{V_{tot}} \), which showed a significant decrease in post-treatment tracer uptake compared to baseline uptake in the irinotecan group (-31%±7, \( p = 0.0007 \)) as opposed to the cetuximab (-4%±3, \( p = 0.95 \)) and vehicle group (-13%±6, \( p = 0.27 \)), where no significant changes from baseline values were seen (Fig. 2c).

\( D_{V_{tot}} \) values from the animals in the irinotecan group were correlated with their \( S_{V_{mean}} \) values calculated from different time frames during the last 40 minutes of acquisition (Table 3). The last time frames, from 70 to 90 min, from the dynamic images showed the best correlation between \( D_{V_{tot}} \) and \( S_{V_{mean}} \) and were averaged to obtain a static image from which \( S_{V_{mean}} \) and \( T_{B_{R_{mean}}} \) were calculated.

\( S_{V_{mean}} \) changes from baseline showed a significant reduction in tumor \([^{18}F]FLT\) uptake after treatment with irinotecan (-20%±5, \( p = 0.0002 \)) while treatment with cetuximab (-7%±3, \( p = 0.28 \)) or vehicle (-8%±3, \( p = 0.12 \)) did not induce significant changes from baseline uptake (Fig. 3a). Similarly, \( T_{B_{R_{mean}}} \) remained unaffected by treatment with vehicle (Fig. 3b, -7%±5, \( p = 0.24 \)) and cetuximab (1%±3, \( p = 0.99 \)), while decreasing significantly after irinotecan treatment (-21%±4, \( p \leq 0.0001 \)). \( \mu PET \) images obtained from representative vehicle, cetuximab and irinotecan-treated animals before and after treatment are shown in Fig. 3c.
Both static imaging parameters, $\text{SUV}_{\text{mean}}$ and $\text{TBR}_{\text{mean}}$, showed a very strong correlation with the dynamic imaging parameter $\text{DV}_{\text{tot}}$ (Fig. 4a, $\text{SUV}_{\text{mean}}$: $r=0.90$, $p=0.002$ and $\text{TBR}_{\text{mean}}$: $r=0.99$, $p<0.0001$). Furthermore, all *in vivo* imaging parameters were significantly correlated with the radioactivity counted in the irinotecan-treated tumor *ex vivo* as represented by %ID/g (Fig. 4b, $\text{SUV}_{\text{mean}}$: $r=0.89$, $p=0.003$; $\text{TBR}_{\text{mean}}$: $r=0.88$, $p=0.004$ and $\text{DV}_{\text{tot}}$: $r=0.90$, $p=0.003$).

**Ex Vivo Validation Experiments**

Tumor cell proliferation was assessed *ex vivo* through evaluation of the proliferation marker Ki67 and of proteins involved in thymidine metabolism (TK1, TS and TP, Fig. 5a). The percentage of Ki67-positive cells was significantly lower in the irinotecan group as compared with the cetuximab and vehicle group (Fig. 5b, 64%±2% _versus_ 77%±1% and 78%±1%, respectively, $p<0.0001$), yet no significant correlation was seen with the *in vivo* imaging parameters (Ki67 _versus_ $\text{SUV}_{\text{mean}}$: $r=0.24$, $p=0.57$, _versus_ $\text{TBR}_{\text{mean}}$: $r=0.15$, $p=0.73$ and _versus_ $\text{DV}_{\text{tot}}$: $r=0.19$, $p=0.65$). In contrast, TK1 expression was elevated in the irinotecan group in comparison with the cetuximab and vehicle group, while pTK1, TS and TP expression did not differ between groups (Fig 5b, Table 4).

ELISA for the determination of TK1 protein concentration showed a threefold higher signal in the irinotecan group as compared with the cetuximab and vehicle group (Fig. 6, 28.8±5.63 _versus_ 11.26±1.94 pg/mg, $p=0.0007$ and 8.36±0.64 pg/mg, $p=0.003$, respectively). ATP levels were found to be lowest in the irinotecan group, albeit not significantly different from the cetuximab and vehicle group (Fig. 6, 0.092±0.010 _versus_ 0.347±0.239 nmol/mg, $p=0.48$ and 0.259±0.117 nmol/mg, $p=0.71$, respectively).
Discussion

This study assessed the feasibility of $[^{18}\text{F}]$FLT-PET as biomarker for early treatment response in an irinotecan (sensitive) and cetuximab (resistant)-treated mouse model of colorectal cancer. Furthermore, we evaluated the reliability of a simplified, semi-quantitative method as a surrogate for full compartmental quantification of $[^{18}\text{F}]$FLT uptake. Our results showed $[^{18}\text{F}]$FLT tumor uptake, as quantified by $\text{DV}_{\text{tot}}$, $\text{SUV}_{\text{mean}}$ and $\text{TBR}_{\text{mean}}$, to be significantly decreased from baseline values in the irinotecan-treated tumors. In vehicle and cetuximab-treated animals, $\text{DV}_{\text{tot}}$, $\text{SUV}_{\text{mean}}$ and $\text{TBR}_{\text{mean}}$ remained unaffected and did not differ from baseline uptake. Our in vivo observations indicate the ability of $[^{18}\text{F}]$FLT-PET to provide an early assessment of tumor response to anti-proliferative treatment and are in line with previously reported preclinical and clinical $[^{18}\text{F}]$FLT-PET studies [8, 21, 22].

The $[^{18}\text{F}]$FLT response was quantified by dynamic ($\text{DV}_{\text{tot}}$) as well as static imaging parameters ($\text{SUV}_{\text{mean}}$, $\text{TBR}_{\text{mean}}$). Kinetic modeling of dynamic data is considered to be the most quantitative method since it makes use of a measured arterial input function and a tissue time activity curve. In line with previous studies, the 2TCM with reversible phosphorylation best described the tumor TAC as assessed by the AIC [12, 23]. This model, together with an image-derived left ventricular IF, was therefore used to perform all further compartmental analysis. The fact that $k_4$, the parameter describing TK1 dephosphorylation rate, could not be excluded from the kinetic model, indicates that phosphorylated $[^{18}\text{F}]$FLT can undergo dephosphorylation. Since $k_4$ was found to be of the same order of magnitude as $k_3$ ($k_3/k_4 \approx 0.9 \pm 0.3$), calculating $K_{\text{FLT}}$ was not sensible and only $\text{DV}_{\text{tot}}$ was considered from the obtained kinetic parameters. $\text{SUV}_{\text{mean}}$ and $\text{TBR}_{\text{mean}}$ were calculated from late static $[^{18}\text{F}]$FLT images (70-90 min post-injection) and can be considered as simplified models for irreversible and reversible kinetics, respectively [23]. In this study, $\text{DV}_{\text{tot}}$ showed a very strong correlation with both static imaging parameters, implying that $\text{SUV}_{\text{mean}}$ as well as $\text{TBR}_{\text{mean}}$ can be used to evaluate $[^{18}\text{F}]$FLT uptake response.
Similar observations were made in patients with non-small cell lung cancer [23] as well as in mice bearing subcutaneous human epithelial carcinoma and Lewis lung carcinoma tumors [12].

The decrease in tracer uptake in irinotecan-treated tumors was not due to induction of apoptosis (cell death) since histologic evaluation of CC3 showed no post-treatment increase of the percentage of apoptotic cells (Supplemental Data). Instead, reduced $[^{18}\text{F}]{\text{FLT}}$ tumor uptake was reflected by a significantly lower presence of the proliferation marker Ki67 in the irinotecan-treated tumors, confirming that $[^{18}\text{F}]{\text{FLT}}$-PET is able to accurately assess changes in tumor cell proliferation. In our study, no significant correlation was found between the percentage of Ki67-positive cells and the (semi-) quantified imaging parameters, although several preclinical and clinical studies have found Ki67 staining to correlate with $[^{18}\text{F}]{\text{FLT}}$ tumor uptake [12, 24, 25]. However, Ki67 does not always accurately reflect differences in $[^{18}\text{F}]{\text{FLT}}$ uptake, most likely because Ki67 is not S-phase specific, but is expressed in all phases of the cell cycle with the exception of G0 [26, 27].

Therefore, many studies also investigated the relationship between $[^{18}\text{F}]{\text{FLT}}$ uptake and TK1, since $[^{18}\text{F}]{\text{FLT}}$ uptake is considered to be a direct measure of TK1 activity as it peaks during the S-phase, and found both strong [24, 28] and weak correlations [27, 29]. It is anticipated that therapeutic agents that, directly or indirectly, block the thymidine salvage pathway and thus TK1 activity, decrease $[^{18}\text{F}]{\text{FLT}}$ uptake. On the other hand, blocking the de novo pathway (Fig. 1) through thymidylate synthase (TS) inhibitors is reported to upregulate the thymidine salvage pathway, increasing TK1 activity to regulate intracellular TTP and resulting in an unexpected increase in $[^{18}\text{F}]{\text{FLT}}$ tumor uptake, the so-called $[^{18}\text{F}]{\text{FLT}}$ “flare” effect [29, 30]. In our study, we observed a treatment effect of irinotecan on TK1 expression but not on TS and TP expression, suggesting that irinotecan indeed plays a role in the thymidine salvage pathway without interfering with the de novo pathway as reported by Voeller and colleagues [17]. Surprisingly, however, our quantification of TK1 expression by means of ELISA and IHC demonstrated higher levels of TK1 in irinotecan-treated tumors as compared with vehicle and
cetuximab-treated tumors. Barthel et al. observed the same effect on TK1 expression in a RIF-1 tumor model after 5-FU treatment: \([^{18}F]\)FLT tumor uptake was markedly decreased 48h after treatment as was the number of proliferating tumor cells as assessed by PCNA staining, whereas TK1 levels were significantly higher than those in control tumors [31]. They focused on tumor ATP levels to explain their unexpected result, since ATP is an important cofactor for TK1 activity, transforming TK1 to a dimer in its absence and to a tetramer in its presence, with the dimer representing the low-efficiency form of TK1 [5, 6]. Indeed, their findings showed significantly lower ATP levels in 5-FU-treated tumors as compared with untreated tumors. Another factor that can cause TK1 dimerization is the phosphorylation of TK1 on serine-13. Although phosphorylated TK1 retains its enzymatic activity, it has a decreased affinity for thymidine and reduced catalytic efficiency, leading to the cellular accumulation of inactive TK1 [6, 32, 33]. In our study, phosphorylated TK1 did not differ between the treatment groups. ATP levels, however, were found to be lowest in the irinotecan group, albeit not significantly different from the vehicle and cetuximab groups due to large within-group variations. Taken together, we conclude that the decreased \([^{18}F]\)FLT tumor uptake despite increased TK1 expression could be explained by the reduced ATP levels in irinotecan-treated tumors, i.e. the dimeric, inactive form of TK1 accumulated within the cell. Our results discourage the use of total TK1 expression when examining the effect of drugs inducing cell cycle arrest as \([^{18}F]\)FLT cellular retention is likely reflected in the oligomeric status of TK1 rather than total protein expression.
Conclusions

In a CRC xenograft model, we have shown that PET using the proliferation marker $[^{18}F]$FLT is able to detect early response to anti-proliferative treatment. A significant decrease of $[^{18}F]$FLT tumor uptake was detectable already 24h after irinotecan treatment and was accompanied by reduced tumor cell proliferation as measured by Ki67 despite an increase in total TK1 expression, suggesting that tumor ATP levels play an important role in regulating $[^{18}F]$FLT uptake. Furthermore, we have demonstrated the reliability of a simplified, semi-quantitative method as a surrogate for full compartmental quantification of $[^{18}F]$FLT uptake which can be applied in future preclinical oncological studies.

Acknowledgements

The authors thank Philippe Joye and Caroline Berghmans of the Molecular Imaging Center Antwerp for their valuable technical assistance.

This work was funded by the University of Antwerp through a Bijzonder Onderzoeksfond (BOF27327). D.T. is funded by the Research Foundation Flanders (FWO) through a postdoctoral grant (1211313N). Si.S and C.V. are supported by the Innovative Medicines Initiative Joint Undertaking (www.imi.europa.eu) under grant agreement number 115151, resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies’ in-kind contribution.

Conflict of interest

The authors declare that they have no conflict of interest.
References


Tables

**Table 1.** Antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Supplier</th>
<th>Host</th>
<th>Reactivity</th>
<th>Dilution factor</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>Cell Signaling no. 9027</td>
<td>Rabbit</td>
<td>Human</td>
<td>1:300</td>
<td>SignalStain Boost Detection Reagent</td>
</tr>
<tr>
<td>Thymidine kinase 1</td>
<td>Abcam ab76495</td>
<td>Rabbit</td>
<td>Human</td>
<td>1:200</td>
<td>Goat anti-rabbit antibody</td>
</tr>
<tr>
<td>Phosphorylated Thymidine kinase 1</td>
<td>Abcam ab59266</td>
<td>Rabbit</td>
<td>Human</td>
<td>1:100</td>
<td>Goat anti-rabbit antibody</td>
</tr>
<tr>
<td>Thymidylate synthase</td>
<td>Abcam ab108995</td>
<td>Rabbit</td>
<td>Human</td>
<td>1:100</td>
<td>Goat anti-rabbit antibody</td>
</tr>
<tr>
<td>Thymidine phosphorylase</td>
<td>Proteintech 12383-1-AP</td>
<td>Rabbit</td>
<td>Human</td>
<td>1:50</td>
<td>Goat anti-rabbit antibody</td>
</tr>
<tr>
<td>Cleaved caspase-3</td>
<td>Cell Signaling no. 9664</td>
<td>Rabbit, mouse, rat</td>
<td>1:400</td>
<td>Goat anti-rabbit antibody</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Comparison of kinetic models using AIC

<table>
<thead>
<tr>
<th></th>
<th>Before treatment</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2TCM ($k_4=0$)</td>
<td>-21.9 ± 7.1</td>
<td>-5.9 ± 2.9</td>
</tr>
<tr>
<td>2TCM ($k_4≠0$)</td>
<td>-49.1 ± 8.1£</td>
<td>-40.7 ± 3.0£</td>
</tr>
</tbody>
</table>

Values are expressed as mean AIC ± SEM

# statistically significant at $p≤0.0001$ versus 2TCM ($k_4=0$)

**Table 3.** Identification of the optimal time point for SUV$_{\text{mean}}$ and TBR$_{\text{mean}}$ calculations

<table>
<thead>
<tr>
<th></th>
<th>50'-60'</th>
<th>60'-70'</th>
<th>70'-80'</th>
<th>80'-90'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson’s $r$</td>
<td>0.76</td>
<td>0.78</td>
<td>0.79</td>
<td>0.81</td>
</tr>
<tr>
<td>$p$ value</td>
<td>0.0006</td>
<td>0.0004</td>
<td>0.0003</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

**Table 4.** IHC score of enzymes involved in thymidine metabolism

<table>
<thead>
<tr>
<th></th>
<th>TK1</th>
<th>pTK1</th>
<th>TS</th>
<th>TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>48.8 ± 5.2</td>
<td>122.4 ± 10.4</td>
<td>125.3 ± 20.9</td>
<td>145.3 ± 10.7</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>72.1 ± 6.6</td>
<td>136.6 ± 7.2</td>
<td>102.0 ± 15.7</td>
<td>181.4 ± 10.4</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>104.8 ± 18.3£</td>
<td>136.6 ± 13.7</td>
<td>141.6 ± 19.1</td>
<td>163.7 ± 14.7</td>
</tr>
</tbody>
</table>

Values are expressed as mean IHC score ± SEM

# statistically significant at $p=0.007$ versus vehicle
**Figure 1.** Schematic diagram of thymidine and $[^{18}F]$FLT uptake and metabolism in the cell. 

*ENT1*, equilibrative nucleoside transporter 1; *dR-1P*, 2´-deoxyribose-1-phosphate; *dUMP*, deoxyuridine monophosphate; *FLT*, fluorothymidine; *KI* and *k2*, forward and reverse rate of $[^{18}F]$FLT transport, respectively; *k3* and *k4*, TK1 phosphorylation and dephosphorylation rate, respectively; *MAPK*, mitogen-activated protein kinase; *TDP*, thymidine diphosphate; *TK1*, thymidine kinase, *TMP*, thymidine monophosphate; *TP*, thymidine phosphorylase; *TS*, thymidylate synthase; *TTP*, thymidine triphosphate.
Figure 2. Kinetic modeling of $[^{18}\text{F}]\text{FLT}$. (a) Mean $[^{18}\text{F}]\text{FLT}$ image-derived blood time activity curve ± SEM of all animals. Inset displays the IF over the first 10 minutes. (b) Mean tumor TAC ± SEM after treatment with vehicle (○), cetuximab (×) or irinotecan (Δ). * statistically significant versus vehicle and cetuximab group at $p<0.05$. (c) Mean $\text{DV}_{\text{tot}}$ changes (%) from baseline ± SEM in vehicle, cetuximab and irinotecan group. *** statistically significant versus baseline at $p<0.001$. 
Figure 3. Semi-quantification of $[^{18}\text{F}]{\text{FLT}}$. Averaged SUV$_{\text{mean}}$ (a) and TBR$_{\text{mean}}$ (b) changes (%) from baseline ± SEM in vehicle, cetuximab and irinotecan-treated group. *** statistically significant versus baseline at $p<0.001$, **** statistically significant versus baseline at $p<0.0001$. (c) μPET SUV images averaged over 70-90 min after injection showing $[^{18}\text{F}]{\text{FLT}}$ tumor uptake in a representative vehicle, cetuximab and irinotecan-treated animal (from left to right) before (upper panel) and after (lower panel) treatment. Tumors are indicated by white squares.
**Figure 4.** Correlations between the dynamic parameter $DV_{tot}$ and the static parameters $SUV_{mean}$ and $TBR_{mean}$ (a) and between *ex vivo* tumor radioactivity expressed as %ID/g and $DV_{tot}$, $SUV_{mean}$ and $TBR_{mean}$ (b) in the irinotecan-treated tumors.
Figure 5. Immunohistochemical validation of imaging data. (a) Photographs (x400) of representative slides from Ki67, TK1, pTK1, TS and TP (from top to bottom)-stained tumor treated with vehicle, cetuximab or irinotecan (from left to right). Positively stained tumor cells are colored brown. (b) Bars represent the averaged ± SEM IHC score for each staining in tumors from animals treated with vehicle, cetuximab or irinotecan. ** statistically significant versus vehicle group at p<0.01, **** statistically significant versus vehicle and cetuximab group at p<0.0001.

Figure 6. Assessment of TK1 and ATP protein expression. Averaged TK1 (left y-axis) and ATP (right y-axis) concentration ± SEM in tumors of animals treated with vehicle (n=8), cetuximab (n=7) or irinotecan (n=7). ** statistically significant versus cetuximab group at p<0.01, *** statistically significant versus vehicle group at p<0.001.