This item is the archived peer-reviewed author-version of:

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

Molecular imaging and biology - ISSN 1536-1632 - 19:1(2017), p. 109-119

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 [¹⁸F]fluorothymidine as a biomarker for early therapy response in a mouse model of colorectal cancer

Sara Rasic^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}

^aMolecular Imaging Center Antwerp (MICA), Faculty of Medicine and Health Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

^bDepartment of Nuclear Medicine, Antwerp University Hospital, Wilrijkstraat 10, 2650 Edegem, Belgium

^cCenter for Oncological Research (CORE), University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

^dDepartment 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: [¹⁸F]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 ($p \leq 0.001$) as compared with the cetuximab and vehicle group, and correlated strongly with each other ($p \leq 0.0001$). *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 ($p \leq 0.0001$). 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 ($p \leq 0.01$). 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

[¹⁸F]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 [¹⁸F]-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. [¹⁸F]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 [¹⁸F]FLT (R_t=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 (2×10⁶ 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 mm³ 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 μ PET/CT gantry. Acquisition of emission data on the Inveon scanners was started simultaneously with the intravenous injection of 9.25 MBq [¹⁸F]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 μ m spot size at 80 kVp and 500 μ A in 120 projections for scatter and attenuation correction

of the PET data. μ PET and μ CT images were intrinsically co-registered since they were acquired on a multimodal gantry (Minerve). The acquired μ PET 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. μ CT acquisitions were analytically reconstructed using the Feldkamp algorithm to a 352x352x606 matrix with 0.223 mm voxels.

Image Analysis

All μ PET/CT images were analyzed using PMOD v3.3 (PMOD Technologies). To determine the optimal compartmental model for [18 F]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 [18 F]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_{FLT}) and the total distribution volume (DV_{tot}) of [^{18}F]FLT were calculated using the formulas $(K1 \times k3)/(k2 + k3)$ or $(K1/k2) \times (1 + (k3/k4))$, 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_{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_{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- μ m-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 μ l 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 13000 $\times$ g 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 $\pm$ 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 ($p \leq 0.05$). Asterisks on plots and graphs denote a statistically significant difference *versus* all other groups, unless otherwise stated, and are marked as follows: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ and **** = $p \leq 0.0001$.

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 DV_{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 DV_{tot} , which showed a significant decrease in post-treatment tracer uptake compared to baseline uptake in the irinotecan group ($-31\% \pm 7$, $p = 0.0007$) as opposed to the cetuximab ($-4\% \pm 3$, $p = 0.95$) and vehicle group ($-13\% \pm 6$, $p = 0.27$), where no significant changes from baseline values were seen (Fig. 2c).

DV_{tot} values from the animals in the irinotecan group were correlated with their SUV_{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 DV_{tot} and SUV_{mean} and were averaged to obtain a static image from which SUV_{mean} and TBR_{mean} were calculated.

SUV_{mean} changes from baseline showed a significant reduction in tumor [^{18}F]FLT uptake after treatment with irinotecan ($-20\% \pm 5$, $p = 0.0002$) while treatment with cetuximab ($-7\% \pm 3$, $p = 0.28$) or vehicle ($-8\% \pm 3$, $p = 0.12$) did not induce significant changes from baseline uptake (Fig. 3a). Similarly, TBR_{mean} remained unaffected by treatment with vehicle (Fig. 3b, $-7\% \pm 5$, $p = 0.24$) and cetuximab ($1\% \pm 3$, $p = 0.99$), while decreasing significantly after irinotecan treatment ($-21\% \pm 4$, $p \leq 0.0001$). μPET images obtained from representative vehicle, cetuximab and irinotecan-treated animals before and after treatment are shown in Fig. 3c.

Both static imaging parameters, SUV_{mean} and TBR_{mean} , showed a very strong correlation with the dynamic imaging parameter DV_{tot} (Fig. 4a, SUV_{mean} : $r=0.90$, $p=0.002$ and TBR_{mean} : $r=0.99$, $p\leq 0.0001$). 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, SUV_{mean} : $r=0.89$, $p=0.003$; TBR_{mean} : $r=0.88$, $p=0.004$ and DV_{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, $64\%\pm 2$ versus $77\%\pm 1$ and $78\%\pm 1$, respectively, $p\leq 0.0001$), yet no significant correlation was seen with the *in vivo* imaging parameters (Ki67 versus SUV_{mean} : $r=0.24$, $p=0.57$, versus TBR_{mean} : $r=0.15$, $p=0.73$ and versus DV_{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}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}F]FLT uptake. Our results showed [^{18}F]FLT tumor uptake, as quantified by DV_{tot} , SUV_{mean} and TBR_{mean} , to be significantly decreased from baseline values in the irinotecan-treated tumors. In vehicle and cetuximab-treated animals, DV_{tot} , SUV_{mean} and TBR_{mean} remained unaffected and did not differ from baseline uptake. Our *in vivo* observations indicate the ability of [^{18}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}F]FLT-PET studies [8, 21, 22].

The [^{18}F]FLT response was quantified by dynamic (DV_{tot}) as well as static imaging parameters (SUV_{mean} , TBR_{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}F]FLT can undergo dephosphorylation. Since k_4 was found to be of the same order of magnitude as k_3 ($k_3/k_4=0.9\pm0.3$), calculating K_{FLT} was not sensible and only DV_{tot} was considered from the obtained kinetic parameters. SUV_{mean} and TBR_{mean} were calculated from late static [^{18}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, DV_{tot} showed a very strong correlation with both static imaging parameters, implying that SUV_{mean} as well as TBR_{mean} can be used to evaluate [^{18}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}F]FLT tumor uptake was reflected by a significantly lower presence of the proliferation marker Ki67 in the irinotecan-treated tumors, confirming that [^{18}F]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}F]FLT tumor uptake [12, 24, 25]. However, Ki67 does not always accurately reflect differences in [^{18}F]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}F]FLT uptake and TK1, since [^{18}F]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}F]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}F]FLT tumor uptake, the so-called [^{18}F]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: [¹⁸F]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 [¹⁸F]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 [¹⁸F]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

1. Ferlay J, Soerjomataram I, Dikshit R, et al (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136:E359–86
2. Shields AF, Grierson JR, Dohmen BM, et al (1998) Imaging proliferation in vivo with [F-18]FLT and positron emission tomography. *Nat Med* 4:1334–1336
3. Bading JR, Shields AF (2008) Imaging of Cell Proliferation: Status and Prospects. *J Nucl Med* 49:64S–80S
4. Hu C-M, Chang Z-F (2007) Mitotic control of dTTP pool: a necessity or coincidence? *J Biomed Sci* 14:491–497
5. Munch-Petersen B, Cloos L, Jensen HK, Tyrsted G (1995) Human thymidine kinase 1. Regulation in normal and malignant cells. *Adv Enzyme Regul* 35:69–89
6. Li C-L, Lu C-Y, Ke P-Y, Chang Z-F (2004) Perturbation of ATP-induced tetramerization of human cytosolic thymidine kinase by substitution of serine-13 with aspartic acid at the mitotic phosphorylation site. *Biochem Biophys Res Commun* 313:587–593
7. Soloviev D, Lewis D, Honess D, Aboagye E (2012) [(18)F]FLT: an imaging biomarker of tumour proliferation for assessment of tumour response to treatment. *Eur J Cancer* 48:416–424
8. Mudd SR, Holich KD, Voorbach MJ, et al (2011) Pharmacodynamic Evaluation of Irinotecan Therapy by FDG and FLT PET/CT Imaging in a Colorectal Cancer Xenograft Model. *Mol Imaging Biol* 14:617–624
9. McKinley ET, Smith RA, Zhao P, et al (2013) 3'-Deoxy-3'-[18F]-Fluorothymidine PET Predicts Response to V600EBRAF-Targeted Therapy in Preclinical Models of Colorectal Cancer. *J Nucl Med* 54:424–430
10. Tomasi G, Turkheimer F, Aboagye E (2011) Importance of Quantification for the Analysis of PET Data in Oncology: Review of Current Methods and Trends for the Future. *Mol Imaging Biol* 14:131–146
11. Gunn RN, Gunn SR, Cunningham VJ (2001) Positron emission tomography

- compartmental models. *J Cereb Blood Flow Metab* 21:635–652
12. Kim SJ, Lee JS, Im KC, et al (2008) Kinetic Modeling of 3'-Deoxy-3'-¹⁸F-Fluorothymidine for Quantitative Cell Proliferation Imaging in Subcutaneous Tumor Models in Mice. *J Nucl Med* 49:2057–2066
 13. Jonker DJ, O'Callaghan CJ, Karapetis CS, et al (2007) Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 357:2040–2048
 14. Di Nicolantonio F, Martini M, Molinari F, et al (2008) Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 26:5705–5712
 15. Pietrantonio F, Petrelli F, Coinu A, et al (2015) Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: A meta-analysis. *Eur J Cancer* 51:587–594
 16. Rothenberg ML (2001) Irinotecan (CPT-11): recent developments and future directions--colorectal cancer and beyond. *Oncologist* 6:66–80
 17. Voeller DM, Grem JL, Pommier Y, et al (2000) Identification and proposed mechanism of action of thymidine kinase inhibition associated with cellular exposure to camptothecin analogs. *Cancer Chemother Pharmacol* 45:409–416
 18. Visser EP, Disselhorst JA, Brom M, et al (2009) Spatial resolution and sensitivity of the Inveon small-animal PET scanner. *J Nucl Med* 50:139–147
 19. Deleye S, Heylen M, Deiteren A, et al (2014) Continuous flushing of the bladder in rodents reduces artifacts and improves quantification in molecular imaging. *Mol Imaging* 13:1–12
 20. Brockenbrough JS, Morihara JK, Hawes SE, et al (2009) Thymidine kinase 1 and thymidine phosphorylase expression in non-small-cell lung carcinoma in relation to angiogenesis and proliferation. *J Histochem Cytochem* 57:1087–1097
 21. Lee W-C, Chang C-H, Ho C-L, et al (2011) Early Detection of Tumor Response by FLT/MicroPET Imaging in a C26 Murine Colon Carcinoma Solid Tumor Animal Model. *J Biomed Biotechnol* 2011:1–7

22. Troost EGC, Bussink J, Hoffmann AL, et al (2010) 18F-FLT PET/CT for early response monitoring and dose escalation in oropharyngeal tumors. *J Nucl Med* 51:866–874
23. Frings V, Yaqub M, Hoyng LL, et al (2014) Assessment of Simplified Methods to Measure 18F-FLT Uptake Changes in EGFR-Mutated Non-Small Cell Lung Cancer Patients Undergoing EGFR Tyrosine Kinase Inhibitor Treatment. *J Nucl Med* 55:1417–1423
24. Brockenbrough JS, Souquet T, Morihara JK, et al (2011) Tumor 3'-Deoxy-3'-18F-Fluorothymidine (18F-FLT) Uptake by PET Correlates with Thymidine Kinase 1 Expression: Static and Kinetic Analysis of 18F-FLT PET Studies in Lung Tumors. *J Nucl Med* 52:1181–1188
25. Vesselle H, Grierson J, Muzi M, et al (2002) In vivo validation of 3'-deoxy-3'-[(18F)]fluorothymidine ([18F]FLT) as a proliferation imaging tracer in humans: correlation of [18F]FLT uptake by positron emission tomography with Ki-67 immunohistochemistry and flow cytometry in human lung tumors. *Clin Cancer Res* 8:3315–3323
26. McKinley ET, Ayers GD, Smith RA, et al (2013) Limits of [18F]-FLT PET as a Biomarker of Proliferation in Oncology. *PLoS ONE* 8:e58938
27. Zhang CC, Yan Z, Li W, et al (2012) [18F]FLT-PET Imaging Does Not Always "Light Up" Proliferating Tumor Cells. *Clin Cancer Res* 18:1303–1312
28. Keen H, Pichler B, Kukuk D, et al (2011) An Evaluation of 2-deoxy-2-[18F]Fluoro-D-Glucose and 3'-deoxy-3'-[18F]-Fluorothymidine Uptake in Human Tumor Xenograft Models. *Mol Imaging Biol* 14:355–365
29. Lee SJ, Kim SY, Chung JH, et al (2010) Induction of thymidine kinase 1 after 5-fluorouracil as a mechanism for 3'-deoxy-3'-[18F]fluorothymidine flare. *Biochem Pharmacol* 80:1528–1536
30. Kenny LM, Contractor KB, Stebbing J, et al (2009) Altered tissue 3'-deoxy-3'-[18F]fluorothymidine pharmacokinetics in human breast cancer following capecitabine treatment detected by positron emission tomography. *Clin Cancer Res* 15:6649–6657
31. Barthel H, Cleij MC, Collingridge DR, et al (2003) 3'-deoxy-3'-[18F]fluorothymidine

- as a new marker for monitoring tumor response to antiproliferative therapy in vivo with positron emission tomography. *Cancer Res* 63:3791–3798.
32. Chang ZF, Huang DY, Chi LM (1998) Serine 13 is the site of mitotic phosphorylation of human thymidine kinase. *J Biol Chem* 273:12095–12100.
 33. Segura-Peña D, Lutz S, Monnerjahn C, et al (2007) Binding of ATP to TK1-like enzymes is associated with a conformational change in the quaternary structure. *J Mol Biol* 369:129–141

Tables

Table 1. Antibodies used for immunohistochemistry

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

Table 2. Comparison of kinetic models using AIC

	Before treatment	After treatment
2TCM ($k_4=0$)	-21.9 ± 7.1	-5.9 ± 2.9
2TCM ($k_4 \neq 0$)	$-49.1 \pm 8.1^{\#}$	$-40.7 \pm 3.0^{\#}$

Values are expressed as mean AIC $\pm$ SEM

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

Table 3. Identification of the optimal time point for SUV_{mean} and TBR_{mean} calculations

	50'-60'	60'-70'	70'-80'	80'-90'
Pearson's r	0.76	0.78	0.79	0.81
p value	0.0006	0.0004	0.0003	0.0002

Table 4. IHC score of enzymes involved in thymidine metabolism

	TK1	pTK1	TS	TP
Vehicle	48.8 ± 5.2	122.4 ± 10.4	125.3 ± 20.9	145.3 ± 10.7
Cetuximab	72.1 ± 6.6	136.6 ± 7.2	102.0 ± 15.7	181.4 ± 10.4
Irinotecan	$104.8 \pm 18.3^{\#}$	136.6 ± 13.7	141.6 ± 19.1	163.7 ± 14.7

Values are expressed as mean IHC score $\pm$ SEM

$\#$ statistically significant at $p=0.007$ versus vehicle

Figures

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; k_1 and k_2 , forward and reverse rate of [^{18}F]FLT transport, respectively; k_3 and k_4 , *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 $\pm$ SEM of all animals. Inset displays the IF over the first 10 minutes. **(b)** Mean tumor TAC $\pm$ SEM after treatment with vehicle (○), cetuximab (×) or irinotecan (Δ). * statistically significant *versus* vehicle and cetuximab group at $p < 0.05$. **(c)** Mean DV_{tot} changes (%) from baseline $\pm$ SEM in vehicle, cetuximab and irinotecan group. *** statistically significant *versus* baseline at $p < 0.001$.

Figure 3. Semi-quantification of [^{18}F]FLT. Averaged SUV_{mean} (**a**) and TBR_{mean} (**b**) changes (%) from baseline $\pm$ 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}F]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 $\pm$ 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 $\pm$ 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 $p<0.001$.