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

Baseline [^{18}F] *FMISO* μPET as a predictive biomarker for response to *HIF* – 1α inhibition combined with 5-FU chemotherapy in a human colorectal cancer xenograft model

Reference:

De Bruycker Sven, Vangestel Christel, van den Wyngaert Tim, wyffels Leonie, Wouters An, Pauwels Patrick, Staelens Steven, Stroobants Sigrid.- Baseline [^{18}F] *FMISO* μPET as a predictive biomarker for response to *HIF* – 1α inhibition combined with 5-FU chemotherapy in a human colorectal cancer xenograft model

Molecular imaging and biology - ISSN 1536-1632 - (2016), p. 1-11

Full text (Publishers DOI): <http://dx.doi.org/doi:10.1007/s11307-015-0926-5>

To cite this reference: <http://hdl.handle.net/10067/1320780151162165141>

*Baseline [^{18}F]FMISO μPET as a
Predictive Biomarker for Response to
HIF-1 α Inhibition Combined with 5-FU
Chemotherapy in a Human Colorectal
Cancer Xenograft Model*

**Sven De Bruycker, Christel Vangestel,
Tim Van den Wyngaert, Leonie wyffels,
An Wouters, Patrick Pauwels, Steven
Staelens, et al.**

Molecular Imaging and Biology

ISSN 1536-1632

Mol Imaging Biol

DOI 10.1007/s11307-015-0926-5

Your article is protected by copyright and all rights are held exclusively by World Molecular Imaging Society. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at link.springer.com".

RESEARCH ARTICLE

Baseline [^{18}F]FMISO μPET as a Predictive Biomarker for Response to HIF-1 α Inhibition Combined with 5-FU Chemotherapy in a Human Colorectal Cancer Xenograft Model

Sven De Bruycker,¹ Christel Vangestel,^{1,2} Tim Van den Wyngaert,^{1,2} Leonie wyffels,^{1,2} An Wouters,³ Patrick Pauwels,³ Steven Staelens,¹ Sigrid Stroobants^{1,2}

¹Molecular Imaging Center Antwerp (MICA), University of Antwerp, Universiteitsplein 1, Wilrijk, Antwerp, Belgium

²Department of Nuclear Medicine, Antwerp University Hospital, Wilrijkstraat 10, 2650, Edegem, Antwerp, Belgium

³Center for Oncological Research (CORE), University of Antwerp, Universiteitsplein 1, Wilrijk, Antwerp, Belgium

Abstract

Purpose: The purpose of this study was to characterize imaging biomarkers for the potential benefit of hypoxia-inducible factor-1 (HIF-1) α inhibition (by PX-12) during 5-fluorouracil (5-FU) chemotherapy in the treatment of colorectal cancer (CRC).

Procedures: Therapy response to 5-FU $\pm$ PX-12 was assessed with baseline [^{18}F]fluoromisonidazole ([^{18}F]FMISO) and longitudinal 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG) positron emission computed tomography ($\mu\text{PET}/\text{CT}$) in CRC xenograft model ($n=36$) during breathing of a hypoxic (10 % O_2) or normoxic (21 % O_2) atmosphere. *Ex vivo*, immunohistochemistry was performed.

Results: Baseline [^{18}F]FMISO uptake and relative tumor volume (RTV) 2 days after 5-FU or 5-FU + PX-12 administration correlated significantly ($p \leq 0.01$). Under hypoxic breathing conditions, [^{18}F]FDG uptake (-53.1 ± 8.4 %) and Ki67 expression (-16 %) decreased and RTV stagnated in the 5-FU + PX-12 treatment group, but not in 5-FU alone-treated tumors. Under normoxic breathing, [^{18}F]FDG uptake (-23.5 ± 15.2 % and -72.8 ± 7.1 %) and Ki67 expression (-5 % and -19 %) decreased and RTV stagnated in both the 5-FU and the combination treatment group, respectively.

Conclusion: Baseline [^{18}F]FMISO μPET may predict the beneficial effect of HIF-1 α inhibition during 5-FU chemotherapy in CRC.

Key words: [^{18}F]fluoromisonidazole, Tumor hypoxia, 5-fluorouracil, PX-12, 2-deoxy-2-[^{18}F]fluoro-D-glucose

Introduction

Hypoxia, defined as reduced availability of oxygen (O_2), frequently occurs in a variety of solid tumors, including colorectal cancer (CRC) [1]. Hypoxic areas are the result of an imbalance between the supply and consumption of O_2 , due to immature, chaotic, or leaking tumor microvasculature

Electronic supplementary material The online version of this article (doi:10.1007/s11307-015-0926-5) contains supplementary material, which is available to authorized users.

Correspondence to: Sigrid Stroobants; e-mail: sigrid.stroobants@uza.be

Published online: 04 January 2016

or an increase in diffusion distance. Since hypoxia may result in a more aggressive phenotype, it is associated with a worse clinical outcome. Moreover, it has an undesirable impact on radiotherapy efficacy and it also reduces the effectiveness of certain chemotherapeutics [2, 3]. Furthermore, hypoxic regions within a tumor are less penetrable to cytotoxic agents due to malfunctioning vasculature, further diminishing drug efficacy [2–4].

One of the most important molecular mechanisms behind this tolerance is the activation of the transcription factor hypoxia-inducible factor-1 (HIF-1). HIF-1, an $\alpha\beta$ heterodimer, stimulates the production of numerous proteins that are necessary for the cellular reaction to hypoxia. The HIF-1 α subunit is rapidly degraded by the proteasome under normoxic conditions, but stabilizes under hypoxic conditions. HIF-1 transcriptional activity is regulated by heterodimerization of HIF-1 α with the constitutively expressed HIF-1 β and binding to the hypoxia response elements in the promoters of genes that regulate a variety of biological processes. These include genes involved in tumor survival, progression, and proliferation. HIF-1 upregulation will increase the fraction of mutated cells that are less susceptible to apoptosis, which may explain the frequently observed resistance to conventional chemotherapy [2–4]. For instance, a recent *in vitro* study showed the importance of HIF-1 in the resistance of HCT116 CRC cells to 5-fluorouracil (5-FU) [5], a major chemotherapeutic in the treatment of CRC [6]. Research has recently focused on new therapeutic possibilities specifically aimed at targeting the HIF-1 pathway in the hypoxic tumor microenvironment [7, 8].

Predictive biomarkers are critically needed to identify patients with a worse prognosis due to tumor hypoxia that may benefit from appropriate hypoxia-targeting therapies. To overcome the shortcomings of invasive O_2 probe measurements [9], the main focus for assessing tumor oxygenation has been on non-invasive imaging methods, such as positron emission tomography/computed tomography (PET/CT). The most promising PET markers for hypoxia are F-18 labeled 2-nitroimidazole-based tracers. Under hypoxic conditions, 2-nitroimidazoles undergo a series of reduction reactions into reactive intermediate metabolites, which covalently bind to cellular macromolecules and consequently are trapped within viable hypoxic cells. In contrast, under normoxic conditions, the first step in this reduction series is reversible and no metabolites will accumulate. Numerous 2-nitroimidazole agents have been developed and validated both preclinically and clinically. [^{18}F]fluoromisonidazole ([^{18}F]FMISO) was the first 2-nitroimidazole tracer developed for hypoxia imaging and still remains the most studied hypoxia PET tracer [10].

2-Deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG), a F-18 labeled glucose analog and hence an indicator of glucose metabolism, is the most commonly used PET tracer for cancer detection, disease prognostication, and therapy response monitoring. [^{18}F]FDG enters the cell via glucose

transporters (most importantly GLUT-1), becomes phosphorylated by hexokinase (HK), but does not enter further glycolysis. In this way, the intracellular-accumulated [^{18}F]FDG molecules can be detected with PET [11]. Under hypoxic conditions, oxidative phosphorylation ceases and from that moment onwards, cells are dependent on anaerobic glycolysis for their energy supply. This boost in anaerobic glycolysis is better known as the Pasteur effect [12]. Nevertheless, increased demand for glucose may also occur in aerobic conditions, known as the Warburg effect [12]. Moreover, the expression of HIF-1 α , which is an important transcription factor for glucose transporters and glycolytic enzymes, such as HK, has also been observed in non-hypoxic tumor tissue [13, 14]. All these limit the potential of [^{18}F]FDG as a specific tracer for hypoxia [15, 16]. For instance, it was shown in a mammary carcinoma xenograft model that only [^{18}F]FMISO uptake was significantly altered under O_2 modification conditions; [^{18}F]FDG uptake remained stable [17]. In spite of that, significant increases in [^{18}F]FDG uptake were observed following chemical activation of HIF-1 α in an *in vivo* model of human CRC [18]. However, the value of [^{18}F]FDG for cancer imaging is irrefutable, as it is a measure of overall tumor burden and metabolic activity. Sequential [^{18}F]FDG imaging during treatment may be an excellent tool for predicting therapy outcome [15].

In the present study, we investigated the value of [^{18}F]FMISO small-animal PET (μPET) as a predictive biomarker for a hypoxic sensitizing treatment schedule in a human CRC xenograft model during breathing of a hypoxic (10 % O_2) or normoxic (21 % O_2) atmosphere. 1-Methylpropyl 2-imidazolyl disulfide (PX-12), a thioredoxin inhibitor [19], has been shown to decrease HIF-1 α expression *in vitro* and *in vivo* [20, 21]. We made use of the HIF-1 α inhibiting potential of PX-12 to sensitize hypoxic Colo205 tumors to the therapeutic effects of 5-FU and investigated whether baseline [^{18}F]FMISO μPET can predict the potential beneficial effect of PX-12. Tumor response to the treatment regimen was assessed using [^{18}F]FDG μPET /CT imaging and immunohistochemistry (IHC).

Materials and Methods

In Vitro Evaluation

First, the therapy regimen was investigated *in vitro* under both normoxic and hypoxic conditions. The effects of 5-FU (Sigma-Aldrich, Diegem, Belgium), PX-12 (Tocris Bioscience, Abingdon, United Kingdom) and the combined therapy on cell proliferation were determined using a Quick Cell Proliferation Assay Kit, according to manufacturer's instructions (BioVision, Milpitas, CA, USA). Low-passage Colo205 CRC cells (PerkinElmer, Maltham, MA, USA) were cultured in RPMI medium enriched with 10 % fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate,

and 1 % penicillin-streptomycin (Invitrogen, Merelbeke, Belgium) at 37 °C and 5 % CO_2 in a humidified incubator.

For cytotoxicity experiments, cells were harvested by trypsinization with 0.05 % trypsin-EDTA (Invitrogen) and counted with the Muse Cell Count and Viability Assay (Merck Millipore, Overijse, Belgium). 3×10^4 cells were seeded per well in 96-well plates. Cells were allowed to attach by overnight incubation of the plates at 37 °C in a humidified 5 % CO_2 /95 % air atmosphere (normoxia). Hypoxic conditions were achieved in a Bactron IV anaerobic chamber (Shel Lab, Cornelius, OR, USA) [22]. In the anaerobic chamber, measurements with a ToxiRAE II oxymeter (RAE BeNeLux, Hoogstraten, Belgium) confirmed that the O_2 partial pressure was stable at ≤ 0.2 %. Stock solutions of 5-FU and PX-12 were made in dimethylsulfoxide (DMSO; Aca Pharma, Waregem, Belgium). From these stock solutions, serial dilutions were made with sterile water. Plated cells were treated with 5-FU alone (0–120 μM for 24 h), PX-12 alone (0–500 μM for 24 h), or with a combination of both drugs, where PX-12 was used at a fixed concentration of 150 μM , while the concentration range of 5-FU was added. Following the treatment period, WST-1 reagent was added and absorbance was measured at 450 nm using a Powerwave X microplate scanning spectrophotometer (Bio-Tek, Bad Friedrichshall, Germany). Optical density values from control cells, treated with vehicle, were considered as 100 % of cellular viability. Experiments were carried out in quintuplicate in triplicate plates.

In Vivo Imaging

Radiotracers were synthesized at the Department of Nuclear Medicine of the Antwerp University Hospital. [^{18}F]FMISO (specific activity 170–423 $\text{GBq}/\mu\text{mol}$, radiochemical purity >99 %) was synthesized in a FluorSynthon I automated synthesis module (Comacer Netherlands, The Netherlands) by modification of a previously described method [23]. [^{18}F]FDG was produced using a GE FASTlab synthesizer (GE Healthcare, Diegem, Belgium).

Female CD-1 athymic nude mice ($n=36$; Charles Rivers, Calco, Italy) were purchased at an age of 6 weeks. The experimental protocol was approved by the Antwerp University Ethical Committee for Animal Experiments (2012–69), and all applicable institutional and European guidelines for the care and use of animals were followed. Animals were group-housed (6–8 per cage) in individually ventilated cages under a 12:12 dark/light cycle, controlled temperature (20–23 °C) and humidity (50–60 %) with *ad libitum* access to standard laboratory chow and water. Colo205 cells were harvested by trypsinization, washed two times with sterile phosphate-buffered saline (PBS), counted using the Muse Cell Count and Viability Assay, and resuspended in sterile PBS at a concentration of 2×10^7 viable cells per ml. Mice were inoculated with 100 μl of cell

suspension in the right hind leg. Tumor growth was evaluated three times a week with digital caliper measurements from the moment the tumors became palpable. Tumor volume was calculated with the formula $V=0.5 \times (\text{length} \times \text{width}^2)$. Experiments were initiated 2 to 3 weeks after inoculation, when tumors reached a volume of $265 \pm 25 \text{ mm}^3$ (mean $\pm$ SEM) on average. To be able to discriminate between hypoxic and normoxic tumors, we made use of our validated model of hypoxic and normoxic breathing [24].

A schematic representation of the set-up of the imaging study can be found in Fig. 1a. Tumor-bearing mice were randomly divided into six groups ($n=6$ per group) and exposed to short-term (230 min 10 % O_2) hypoxic or normoxic breathing conditions (ambient air, 21 % O_2) [25], whereupon a baseline [^{18}F]FMISO $\mu\text{PET}/\text{CT}$ scan was performed to determine baseline tumor hypoxia. Details of the scan protocol are represented in Fig. 1b. Approximately 18.5 MBq of [^{18}F]FMISO in a final volume of 200 μl 0.9 % saline was administered as a bolus intravenous injection via the tail vein. During [^{18}F]FMISO $\mu\text{PET}/\text{CT}$ acquisition, mice were anesthetized with isoflurane (induction 5 %, maintenance 1–2 %) and medical O_2 (100 %) and body temperature was kept constant by a heating bed. Respiration rate was continuously monitored during image acquisition. Static PET acquisition (20 min), followed by an anatomical CT acquisition (7 min), was performed on an Inveon $\mu\text{PET}/\text{CT}$ scanner (Siemens Preclinical Solution, Knoxville, TN, USA), with a spatial resolution of approximately 1.4 mm in the center of the field of view.

The next day, a [^{18}F]FDG $\mu\text{PET}/\text{CT}$ scan was performed to determine baseline tumor metabolism, under normoxic breathing conditions for all animals (Fig. 1b). For all [^{18}F]FDG experiments, animals were fasted overnight. Approximately 18.5 MBq of [^{18}F]FDG in a final volume of 200 μl was injected via the tail vein of awake mice 30 min before start of the scan. Prior to tracer administration, whole blood glucose levels were measured from a blood drop obtained from the contralateral tail vein using an OneTouch Ultra2 glucose meter (LifeScan, Tilburg, The Netherlands). Glucose measurements were performed in duplicate. Mice were allowed to recover from anesthesia after scanning, whereupon they were re-exposed to hypoxic or normoxic breathing conditions during 4 h. Next, animals were treated with a single dose of 5-FU (150 mg/kg; intraperitoneally (i.p.)) [26, 27] with or without PX-12 (12 mg/kg; i.p.) and re-exposed to their respective breathing conditions for four additional hours. Since the maximal effect of 5-fluoro-2'-deoxyuridine-5'-monophosphate (5-FdUMP), the active metabolite of 5-FU, is seen after 2 h in mice [28], we expected this breathing protocol to be sufficient for observing a different therapy effect in our hypoxic model. Control animals received an i.p. injection with vehicle (0.9 % saline) under the same breathing conditions (Fig. 1c). Two and 6 days post-therapy, an additional [^{18}F]FDG $\mu\text{PET}/\text{CT}$ scan

Fig. 1 Study design. Exposure to hypoxic or normoxic breathing conditions is represented by the *thick black lines*. **a** Imaging study. After μPET scans, μCT was performed for accurate tumor delineation and follow-up of tumor volume. At the end of the study, animals were sacrificed for *ex vivo* validations on tumor tissue. Arrows indicate the timing of the μPET scans. **b** Detail of the [^{18}F]FMISO (*upper*) and [^{18}F]FDG (*lower*) imaging protocols. **c** Overview of the different therapy groups ($n=6$ per group).

(under normoxic breathing conditions for all animals) was performed to determine individual treatment response.

PET images were reconstructed using four iterations $\times$ 16 subsets of a three-dimensional (3D)-ordered subset expectation maximization (OSEM3D) algorithm following Fourier rebinning. Dead time, normalization, randoms, scatter, and attenuation correction were applied.

Using PMOD v3.3 software (PMOD Technologies, Zürich, Switzerland), an elliptic volume of interest (VOI) that enclosed the entire tumor was positioned manually and was centered on the tumor area that showed maximal uptake. Then, 3D isocontours at 60 % of the maximum pixel value within this elliptic VOI were generated automatically. Mean standardized uptake values (SUV_{mean}) were calculated within the isocontour. For [^{18}F]FDG images, glucose correction was applied to the SUV_{mean} by normalizing to a value of 100 mg/dl: glucose corrected $\text{SUV}_{\text{mean}} = (\text{SUV}_{\text{mean}} \times \text{glucose level}) / 100 \text{ mg/dl}$.

For tumor volume determinations at the different time points (days 0, 2, and 6), tumors were manually delineated on CT images. Relative tumor volumes (RTV) of individual mice were calculated by dividing the tumor volume 2 and 6 days after therapy administration by its baseline volume as

determined on CT images and were used as the basis for response assessment.

Ex Vivo Validation

At the end of the study, animals were sacrificed and tumor tissue was resected, formalin-fixed, and paraffin-embedded for histological examination by IHC for HIF-1 α , Ki67 (proliferation marker), and HK1 (enzyme involved in the metabolic trapping of [^{18}F]FDG). Tissue sections of 3 μm thick were mounted on SuperFrost microscope slides (Menzel-Glaser, Braunschweig, Germany). IHC was performed according to manufacturer's instructions. The following antibodies were used: HIF-1 α , #NB100-654 (Novus Biologicals, Cambridge, United Kingdom; 1/500; overnight incubation); Ki67, #9027 (Cell Signaling Technology, Leiden, The Netherlands; 1/300; overnight incubation); HK1, #H00003098-M01 (Novus Biologicals; 1/500; 1 h incubation). After washing, sections were incubated with anti-rabbit horseradish peroxidase (HRP)-labeled SignalStain Boost IHC detection reagent (Cell Signaling Technology; ready-to-use; for HIF-1 α and Ki67), or anti-mouse HRP-labeled secondary antibody (Dako; ready-to-

use; for HK1). Negative controls were incubated with antibody diluent instead of primary antibody.

Slices were examined by means of a CX31 microscope (Olympus, Aartselaar, Belgium). Two observers, including one certified pathologist, scored the percentage of Ki67-positive tumor cells independently. For HK1, the percentage of positive tumor cells were scored as follows: 0 %, score 0; 1–20 %, score 1; 21–40 %, score 2; 41–60 %, score 3; 61–80 %, score 4; 81–100 %, score 5. Intensities of staining were categorized as absent, score 0; faint, score 1; moderate, score 2; or strong, score 3 [18]. A final histological score (H-score) was calculated after counting 10 high-power fields ($\times 200$) as follows: $\text{H-score} = [(a_1 \times i_1) + (a_2 \times i_2)]/2$, with a and i being the score of amount and the intensity of positive-stained tumor cells, respectively, and 1 and 2 refer to two independent observers. Since active HIF-1 α is translocated to the nucleus, only nuclear HIF-1 α staining was quantified using the Allred method [29]. Areas of high necrosis and edges of the tumor tissue were avoided.

Statistics

Statistical analyses were performed using SPSS software v20 (IBM Corp., Chicago, IL, USA). Simple linear regression analysis was performed to predict therapy effect based on the baseline [^{18}F]FMISO μPET scan. Correlation analysis was performed using Spearman's method. Differences between unmatched groups were analyzed using Kruskal-Wallis tests. Differences between matched groups were analyzed using Friedman tests. Post-hoc analysis with the Mann-Whitney U tests (unmatched) and Wilcoxon signed-rank tests (matched) was conducted. $p \leq 0.05$ was considered statistically significant. All data are expressed as mean $\pm$ SEM.

Results

In Vitro Evaluation

Results of the *in vitro* experiments are summarized in Fig. S1. Under normoxic conditions, the half maximal inhibitory concentration (IC_{50}) after 24 h of exposure of Colo205 cells to 5-FU therapy was $2.77 \pm 1.10 \mu\text{M}$. When the IC_{20} (150 μM , also determined via the WST-1 assay, data not shown) of PX-12 was added to the treatment regimen, a decrease in cellular proliferation below 50 % was observed even with the lowest concentrations of 5-FU (0.1 μM , when given in monotherapy resulting in only 18 % inhibition of proliferation). Under hypoxic conditions, no IC_{50} was reached after 24 h for 5-FU concentrations up to 120 μM . However, a decreasing trend of Colo205 cell proliferation (up to 40 % inhibition) was seen when PX-12 was applied in combination with increasing concentrations of 5-FU, as compared to the administration of 5-FU alone ($p = 0.05$).

In Vivo Imaging

Baseline [^{18}F]FMISO SUV_{mean} was significantly higher in the animals exposed to hypoxic breathing compared to normoxic breathing (0.72 ± 0.05 vs. $0.37 \pm 0.04 \text{ g/cm}^3$; $p < 0.001$; Fig. 2a). Baseline [^{18}F]FDG SUV_{mean} was comparable in both groups (0.74 ± 0.03 vs. $0.76 \pm 0.05 \text{ g/cm}^3$; $p = 0.6$; Fig. 2b), as well as baseline tumor volume (333 ± 49 vs. $348 \pm 48 \text{ mm}^3$; $p = 0.9$; Fig. 2c).

Results of the longitudinal [^{18}F]FDG $\mu\text{PET/CT}$ study are summarized in Fig. 3. In the 5-FU monotherapy cohort, [^{18}F]FDG SUV_{mean} did not change under hypoxic breathing conditions 2 days post-therapy ($-4.7 \pm 8.4 \%$), and a small increase was observed 6 days post-therapy ($8.5 \pm 7.4 \%$; Fig. 3a). RTV increased significantly in those animals at both time points ($20.9 \pm 7.0 \%$; $p = 0.04$ and $36.0 \pm 12.3 \%$; $p = 0.03$, respectively; Fig. 3b). Under normoxic breathing conditions, a significant reduction in [^{18}F]FDG uptake occurred 2 days after therapy administration ($-28.4 \pm 5.6 \%$; $p = 0.03$), and this reduction persisted 6 days post-therapy ($-23.5 \pm 15.2 \%$; Fig. 3a). No significant changes in RTV were seen at both time points ($5.2 \pm 6.0 \%$ and $7.7 \pm 15.0 \%$, respectively; Fig. 3b).

Addition of PX-12 resulted in a reduction of [^{18}F]FDG SUV_{mean} in the hypoxic breathing cohort both 2 days ($-33.2 \pm 15.3 \%$; $p = 0.05$) and 6 days (-53.1 ± 8.4 ; $p = 0.03$) post-therapy (Fig. 3c), without significant changes in tumor volume ($-4.4 \pm 4.5 \%$ and $-5.1 \pm 9.5 \%$, respectively; Fig. 3d). Similar results were obtained in the normoxic breathing group: [^{18}F]FDG uptake decreased significantly at both post-therapy time points ($-17.7 \pm 8.2 \%$; $p = 0.05$ and $-72.8 \pm 7.1 \%$; $p = 0.04$; Fig. 3c), without significant changes in RTV ($6.9 \pm 4.5 \%$ and $0.2 \pm 3.6 \%$, respectively; Fig. 3d).

In the control group, a non-significant increase in [^{18}F]FDG SUV_{mean} in the hypoxic breathing cohort was detected 2 days ($14.5 \pm 4.5 \%$) and 6 days ($21.8 \pm 9.8 \%$) after saline administration (Fig. 3e). RTV, measured on μCT images, was significantly increased at both time points (29.9 ± 1.9 ; $p = 0.03$ and $79.0 \pm 7.5 \%$; $p = 0.03$, respectively; Fig. 3f). In tumors of the normoxic breathing cohort, [^{18}F]FDG SUV_{mean} was also non-significantly increased compared to baseline 2 days ($19.4 \pm 10.6 \%$) and 6 days ($13.7 \pm 10.2 \%$) after saline administration (Fig. 3e), which was reflected by an increase in RTVs ($23.7 \pm 6.4 \%$; $p = 0.05$ and $55.7 \pm 17.4 \%$; $p = 0.03$, respectively; Fig. 3f).

Using simple linear regression pre-therapy [^{18}F]FMISO SUV_{mean} could significantly predict RTV 2 days after therapy administration in the monotherapy cohort ($R^2 = 0.6$; $p = 0.002$; Fig. 4a). This suggests that [^{18}F]FMISO is a negative predictor for 5-FU therapy response. However, significance was lost 6 days post-therapy (Fig. 4b). When 5-FU was applied in combination with the HIF-1 α inhibitor PX-12, [^{18}F]FMISO became a positive predictor of therapy response 2 days after therapy administration ($R^2 = 0.5$;

Fig. 2 Baseline parameters in the two breathing groups. **a** [^{18}F]FMISO SUV_{mean} . **b** Glucose-corrected [^{18}F]FDG SUV_{mean} . **c** Tumor volume. Asterisks indicate significant ($p \leq 0.001$) differences between the hypoxic and normoxic breathing group.

$p=0.006$; Fig. 4c), and even 6 days after therapy administration ($R^2=0.5$; $p=0.02$; Fig. 4d). In the control group, [^{18}F]FMISO SUV_{mean} did not predict RTV. No correlations

were found between baseline [^{18}F]FMISO and baseline [^{18}F]FDG SUV_{mean} , nor between baseline [^{18}F]FDG SUV_{mean} and RTV.

Fig. 3 *In vivo* longitudinal imaging study. Graphic representation of relative glucose-corrected [^{18}F]FDG SUV_{mean} (left panel) and tumor growth curves (right panel) before and after 5-FU, the combination of 5-FU and PX-12, or saline administration under hypoxic (dotted lines) or normoxic (full lines) breathing conditions. **a–b** 5-FU monotherapy group. **c–d** 5-FU and PX-12 combination therapy group. **e–f** Control group. Asterisks indicate significant ($p \leq 0.05$) intra-animal changes.

Fig. 4 Scatterplots of baseline [^{18}F]FMISO SUV_{mean} vs. relative tumor volume (RTV). **a** Two days after 5-FU monotherapy. **b** Six days after 5-FU monotherapy. **c** Two days after 5-FU and PX-12 combination therapy. **d** Six days after 5-FU and PX-12 combination therapy. Asterisks indicate significant (** $p \leq 0.01$; *** $p \leq 0.001$) Spearman's correlations.

Representative [^{18}F]FMISO and [^{18}F]FDG $\mu\text{PET/CT}$ images of two animals treated with the combination therapy are shown in Fig. 5.

Ex Vivo Validation

Six days after therapy administration, the mice were euthanized immediately after their last [^{18}F]FDG $\mu\text{PET/CT}$ scan. *Ex vivo*, the expression of Ki67 was slightly lower in 5-FU-treated tumors as compared to control tumors under both hypoxic (72 ± 4 vs. 79 ± 2 %) and normoxic (69 ± 1 vs. 74 ± 4 %; $p = 0.02$) breathing conditions (Fig. 6a). In 5-FU+PX-12-treated tumors, Ki67 expression was considerably lower than in 5-FU-treated tumors under both hypoxic (63 ± 3 vs. 72 ± 4 %) and normoxic (55 ± 4 vs. 69 ± 1 %; $p = 0.006$) breathing conditions (Fig. 6a). No differences were found between the two breathing cohorts within a therapy group. Significant correlations were found between [^{18}F]FDG SUV_{mean} and Ki67 ($\rho = 0.7$; $p < 0.001$; Fig. 6b) and between RTV and Ki67 ($\rho = 0.5$; $p = 0.001$; Fig. 6c). A significant correlation was found between HK1 and [^{18}F]FDG SUV_{mean} ($\rho = 0.4$; $p = 0.02$; Fig. 6d).

In general, Fig. 6e shows that protein expression of HIF-1 α was low to moderate in all cohorts. However, HIF-1 α expression was higher in tumors of animals treated with 5-FU than in control tumors both under hypoxic (4.3 ± 0.4 vs. 2.8 ± 0.2 ; $p = 0.01$) and normoxic (4.4 ± 0.3 vs. 2.9 ± 0.3 ; $p = 0.01$) breathing conditions. In 5-FU+PX-12-treated tumors,

HIF-1 α expression was slightly, but not significantly, lower than in 5-FU-treated tumors, but still higher than in control tumors both under hypoxic (3.7 ± 0.2 vs. 2.8 ± 0.2 ; $p = 0.02$) and normoxic (4.2 ± 0.3 vs. 2.9 ± 0.3 ; $p = 0.02$) breathing conditions (Fig. 6e).

Representative examples of a Ki67, HK1, and HIF-1 α immunohistochemical staining are shown in Fig. 6f–h, respectively.

Discussion

In the present study, we showed in a human CRC xenograft model the potential of baseline [^{18}F]FMISO PET as a predictive biomarker for therapy regimens which effects are depending on the oxygenation status of the tumor. A significant positive correlation was found between the baseline degree of tumor hypoxia, determined with [^{18}F]FMISO and the RTV 2 days after 5-FU chemotherapy administration. This implies that the growth of hypoxic tumors was influenced to a lesser extent than normoxic tumors by the effects of the cytostatic agent, and that we could predict this with baseline [^{18}F]FMISO μPET . When the effects of hypoxia were alleviated by adding the HIF-1 α inhibitor PX-12 to the treatment regimen, this trend was reversed to a significant negative correlation, up to 6 days post-therapy. This implies that, although therapy was effective in both breathing cohorts, [^{18}F]FMISO PET could predict that the combination therapy effect was more pronounced in hypoxic than in normoxic tumors. Similar

Fig. 5 Representative [^{18}F]FMISO and [^{18}F]FDG $\mu\text{PET}/\text{CT}$ SUV-corrected images (coronal view) of a mouse exposed to hypoxic breathing (upper half) and normoxic breathing (lower half), before (Day $_{-1}$, Day $_0$), and after (Day $_2$, Day $_6$) 5-FU and PX-12 therapy administration. Arrows indicate tumors.

results were observed in EMT6 breast cancer xenografts treated with the radiosensitizer tirapazamine and radiotherapy, where significant growth delay was only observed in hypoxic tumors with higher [^{18}F]fluoroazomycin-arabino-side ([^{18}F]FAZA) uptake [30]. As expected, no correlation was found between baseline [^{18}F]FMISO SUV_{mean} and the RTV in the control cohort.

By exposing half of the animals to hypoxic breathing conditions, we artificially modulated tumor oxygenation, which may not mimic the ideal physiological situation where intrinsic variation can be observed within a certain tumor type [30, 31] or between xenografts derived from different cell lines [32, 33]. However, this breathing technique allowed us to detect significant differences in tumor oxygenation status and consequently purely hypoxia-driven

differences in therapy response in relatively small groups of animals.

It has been noted that hypoxia may also increase [^{18}F]FDG uptake, although there is much debate about this phenomenon and most correlations are rather weak [34, 35]. We also investigated the potential of [^{18}F]FDG as a predictive biomarker. Within the two therapy groups, no correlations were found between baseline [^{18}F]FDG and baseline [^{18}F]FMISO SUV_{mean} nor between baseline [^{18}F]FDG and RTV on days 2 and 6. It should be noted that our study design limited the feasibility to validate [^{18}F]FDG as a predictive biomarker for hypoxia-sensitizing therapy regimens as, although therapy was administered in breathing regimens, these [^{18}F]FDG scans were acquired in normoxic conditions.

For this reason, we merely focused on the use of [^{18}F]FDG PET as a surrogate marker for therapy response. Under normoxic breathing conditions, [^{18}F]FDG uptake and Ki67 expression decreased and tumor growth stagnated in Colo205 xenografts up to 6 days after therapy administration. This was however not observed in mice breathing the hypoxic gas indicating therapy resistance in hypoxic CRC xenograft tumors *in vivo*. Up to 6 days after 5-FU+PX-12 combination therapy administration, [^{18}F]FDG SUV_{mean} , and Ki67 staining decreased and RTV stagnated under both hypoxic and normoxic breathing conditions, suggesting that PX-12 had indeed a sensitizing capacity in hypoxic tumors. Inhibition of HIF-1 α by PX-12 could no longer be observed by *ex vivo* IHC at the end of our study, which may not be surprising since the effect of PX-12 is fast and may not last longer than 48 h [20, 36, 37]. Moreover, it has been observed that xenograft tumors are more hypoxic post 5-FU treatment [38], which may explain the higher HIF-1 α expression in treated tumors compared to control tumors.

Within the two therapy groups, no significant correlations were found between the [^{18}F]FDG changes and the RTV. However, several factors should be considered. First, the animals were treated with only a single dose of 5-FU, and follow-up imaging was performed at two set time points, which may not have been ideal for detecting therapy response with [^{18}F]FDG μPET . In general the metabolic response occurs earlier than the anatomical response [39], so our follow-up [^{18}F]FDG imaging might have been too late to measure the initial metabolic therapy response optimally. This was especially the case in the 5-FU monotherapy cohorts: 6 days post-therapy, the observed anatomical response was still pronounced, whereas the metabolic response tended towards normalization. Second, the full effect of PX-12 on glucose regulation in tumor cells, and thus on [^{18}F]FDG incorporation, has not been investigated to date but may be influenced by HIF-1 α inhibition.

However, [^{18}F]FDG SUV_{mean} correlated significantly with HK1 expression, an enzyme involved in the metabolic trapping of [^{18}F]FDG which is highly expressed in Colo205 cells [40]. Decreased HK1 expression after 5-FU therapy had already been observed *in vitro* in SW620 CRC cells

Fig. 6 Immunohistochemistry results. **a** Ki67 staining results. **b** Scatterplot of [^{18}F]FDG SUV_{mean} vs. Ki67. **c** Scatterplot of RTV vs. Ki67. **d** Scatterplot of [^{18}F]FDG SUV_{mean} vs. HK1. **e** HIF-1 α staining results. Asterisks indicate significant (* $p \leq 0.05$; ** $p \leq 0.01$) differences between groups (**a**, **e**) or significant (* $p \leq 0.05$; *** $p \leq 0.001$) Spearman's correlations (**b**–**d**). **f** Example of a Ki67 staining. Arrows indicate positively staining nuclei. **g** Example of a HK1 staining. Arrows indicate positively stained cytoplasm. **h** Example of a HIF-1 α staining. Arrows indicate positively stained nuclei.

[41]. In addition to this, a significant correlation between [^{18}F]FDG SUV_{mean} and Ki67 was observed. Therefore, despite the lack of correlation between [^{18}F]FDG changes and RTV within the therapy groups, which may question the additional value of this glucose analog within our set-up, our *ex vivo* data suggest that the observed [^{18}F]FDG trends may still be valid and useful as surrogate markers for therapy response.

Taken together, our results contribute to the current search for predictive biomarkers, which may enable patient stratification and therapy individualization. Compared with measurements made on biopsies, the sensitivity and non-invasiveness, the absence of sampling bias, and the possibility of longitudinal assessment are substantial advantages of the use of molecular imaging biomarkers.

Conclusions

We have shown in a human CRC xenograft model that baseline [¹⁸F]FMISO PET is able to predict the beneficial effect of HIF-1 α inhibition on 5-FU chemotherapy response. Our results meet the urgent need for non-invasive predictive imaging biomarkers.

Acknowledgments. We gratefully acknowledge Philippe Joye and Caroline Berghmans of the Molecular Imaging Center Antwerp (MICA) and Christophe Hermans of the Center for Oncological Research (CORE) for their excellent technical assistance, and Thomas Verbruggen of MICA for the [¹⁸F]FMISO productions. This work was funded by the University of Antwerp, Belgium through a PhD grant for Sven De Bruycker, an associate professor position for Patrick Pauwels and Steven Staels, and a full professor position for Sigrid Stroobants; by Antwerp University Hospital, Belgium, through a postdoctoral position for Christel Vangestel (Innovative Medicines Initiative, Quic-Concept), a full-time position for Leonie Wyffels, and a departmental position for Tim Van den Wyngaert, Patrick Pauwels, and Sigrid Stroobants; and by the Research Foundation Flanders, Belgium (FWO Vlaanderen) through a postdoctoral fellowship for An Wouters.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

- Nagaraju GP, Bramhachari PV, Raghu G, El-Rayes BF (2015) Hypoxia inducible factor-1 α : its role in colorectal carcinogenesis and metastasis. *Cancer Lett* 366:11–18
- Vaupel P, Mayer A (2007) Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev* 26:225–239
- Bertout JA, Patel SA, Simon MC (2008) The impact of O₂ availability on human cancer. *Nat Rev Cancer* 8:967–975
- Ruan K, Song G, Ouyang G (2009) Role of hypoxia in the hallmarks of human cancer. *J Cell Biochem* 107:1053–1062
- Ravizza R, Molteni R, Gariboldi MB et al (2009) Effect of HIF-1 modulation on the response of two- and three-dimensional cultures of human colon cancer cells to 5-fluorouracil. *Eur J Cancer* 45:890–898
- Gustavsson B, Carlsson G, Machover D et al (2015) A review of the evolution of systemic chemotherapy in the management of colorectal cancer. *Clin Colorectal Cancer* 14:1–10
- Semenza GL (2006) Development of novel therapeutic strategies that target HIF-1. *Expert Opin Ther Targets* 10:267–280
- Semenza GL (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 3:721–732
- Krohn KA, Link JM, Mason RP (2008) Molecular imaging of hypoxia. *J Nucl Med* 49:129S–148S
- Rajendran JG, Krohn KA (2015) F-18 fluoromisonidazole for imaging tumor hypoxia: imaging the microenvironment for personalized cancer therapy. *Semin Nucl Med* 45:151–162
- Gambhir SS (2002) Molecular imaging of cancer with positron emission tomography. *Nat Rev Cancer* 2:683–693
- Gatenby RA, Gillies RJ (2004) Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 4:891–899
- Kim J-W, Gao P, Dang CV (2007) Effects of hypoxia on tumor metabolism. *Cancer Metastasis Rev* 26:291–298
- Denko NC (2008) Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nat Rev Cancer* 8:705–713
- Wijnsman R, Kaanders JHAM, Oyen WJG, Bussink J (2013) Hypoxia and tumor metabolism in radiation oncology: targets visualized by positron emission tomography. *Q J Nucl Med Mol Imaging* 57:244–256
- Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324:1029–1033
- Bentzen L, Keiding S, Horsman MR et al (2000) Feasibility of detecting hypoxia in experimental mouse tumours with 18F-fluorinated tracers and positron emission tomography. *Acta Oncol* 39:629–637
- Mees G, Dierckx R, Vangestel C et al (2013) Pharmacologic activation of tumor hypoxia: a means to increase tumor 2-deoxy-2-[¹⁸F]fluoro-D-glucose uptake? *Mol Imaging* 12:49–58
- Powis G, Kirkpatrick DL (2007) Thioredoxin signaling as a target for cancer therapy. *Curr Opin Pharmacol* 7:392–397
- Welsh SJ, Williams RR, Birmingham A et al (2003) The thioredoxin redox inhibitors 1-methylpropyl 2-imidazolyl disulfide and pleurotin inhibit hypoxia-induced factor 1 α and vascular endothelial growth factor formation. *Mol Cancer Ther* 2:235–243
- Kim YH, Coon A, Baker AF, Powis G (2010) Antitumor agent PX-12 inhibits HIF-1 α protein levels through an Nrf2/PMF-1-mediated increase in spermidine/spermine acetyl transferase. *Cancer Chemother Pharmacol* 68:405–413
- Wouters A, Pauwels B, Lambrechts HAJ et al (2009) Chemoradiation interactions under reduced oxygen conditions: cellular characteristics of an in vitro model. *Cancer Lett* 286:180–188
- Adamsen TCH, Grierson JR, Krohn KA (2005) A new synthesis of the labeling precursor for [¹⁸F]-fluoromisonidazole. *J Label Compd Radiopharm* 48:923–927
- De Bruycker S, Vangestel C, Verbruggen T et al (2014) Baseline [¹⁸F]FMISO and early [¹⁸F]FDG μ PET changes as imaging biomarkers for HIF-1 α inhibition combined with 5-FU in colorectal cancer [abstract]. *European Molecular Imaging Meeting*
- Dubois L, Lieuwes NG, Janssen MH et al (2011) Preclinical evaluation and validation of [¹⁸F]HX4, a promising hypoxia marker for PET imaging. *Proc Natl Acad Sci U S A* 108:14620–14625
- Babsky AM, Zhang H, Hekmatyar SK et al (2007) Monitoring chemotherapeutic response in RIF-1 tumors by single-quantum and triple-quantum-filtered ²³Na MRI, 1H diffusion-weighted MRI and PET imaging. *Magn Reson Imaging* 25:1015–1023
- 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
- Kamm YJL, Peters GJ, Hull WE et al (2003) Correlation between 5-fluorouracil metabolism and treatment response in two variants of C26 murine colon carcinoma. *Br J Cancer* 89:754–762
- Choudhury KR, Yagle KJ, Swanson PE et al (2010) A robust automated measure of average antibody staining in immunohistochemistry images. *J Histochem Cytochem* 58:95–107
- Beck R, Roper B, Carlsen JM et al (2007) Pretreatment 18F-FAZA PET predicts success of hypoxia-directed radiochemotherapy using tirapazamine. *J Nucl Med* 48:973–980
- Wyss MT, Honer M, Schubiger PA, Ametamey SM (2005) NanoPET imaging of [¹⁸F]fluoromisonidazole uptake in experimental mouse tumours. *Eur J Nucl Med Mol Imaging* 33:311–318
- Troost EGC, Laverman P, Kaanders JHAM et al (2006) Imaging hypoxia after oxygenation-modification: comparing [¹⁸F]FMISO autoradiography with pimonidazole immunohistochemistry in human xenograft tumors. *Radiother Oncol* 80:157–164
- Campanile C, Arlt MJE, Kramer SD et al (2013) Characterization of different osteosarcoma phenotypes by PET imaging in preclinical animal models. *J Nucl Med* 54:1362–1368
- Busk M, Horsman MR, Kristjansen PEG et al (2008) Aerobic glycolysis in cancers: implications for the usability of oxygen-responsive genes and fluorodeoxyglucose-PET as markers of tissue hypoxia. *Int J Cancer* 122:2726–2734
- Rajendran JG, Mankoff DA, O'Sullivan F et al (2004) Hypoxia and glucose metabolism in malignant tumors: evaluation by [¹⁸F]fluoromisonidazole and [¹⁸F]fluorodeoxyglucose positron emission tomography imaging. *Clin Cancer Res* 10:2245–2252
- Jordan BF, Runquist M, Raghunand N et al (2005) The thioredoxin-1 inhibitor 1-methylpropyl 2-imidazolyl disulfide (PX-12) decreases vascular permeability in tumor xenografts monitored by dynamic contrast enhanced magnetic resonance imaging. *Clin Cancer Res* 11:529–536
- Ramanathan RK, Kirkpatrick DL, Belani CP et al (2007) A phase I pharmacokinetic and pharmacodynamic study of PX-12, a novel inhibitor of thioredoxin-1, in patients with advanced solid tumors. *Clin Cancer Res* 13:2109–2114
- Dupuis NP, Kusumoto T, Robinson MF et al (1995) Restoration of tumor oxygenation after cytotoxic therapy by a perflubron emulsion/carbogen breathing. *Artif Cells Blood Substit Immobil Biotechnol* 23:423–429

S. De Bruycker et al.: [^{18}F]FMISO as a predictive biomarker in a CRC xenograft model

39. Grassetto G, Capirci C, Marzola MC et al (2011) Colorectal cancer: prognostic role of ^{18}F -FDG-PET/CT. *Abdom Imaging* 37:575–579
40. Keen H, Pichler B, Kukuk D et al (2011) An evaluation of 2-deoxy-2- ^{18}F -fluoro-D-glucose and 3'-deoxy-3'- ^{18}F -fluorothymidine uptake in human tumor xenograft models. *Mol Imaging Biol* 14:355–365
41. Sharma RI, Smith TAD (2008) Colorectal tumor cells treated with 5-FU, oxaliplatin, irinotecan, and cetuximab exhibit changes in ^{18}F -FDG incorporation corresponding to hexokinase activity and glucose transport. *J Nucl Med* 49:1386–1394