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PET imaging of early response to the tyrosine kinase inhibitor ZD4190

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Abstract

Purpose—We evaluated noninvasive positron emission tomography (PET) imaging for monitoring tumor response to the VEGFR-2 tyrosine kinase (TK) inhibitor ZD4190 during cancer therapy.

Experimental design—Orthotopic MDA-MB-435 tumor-bearing mice were treated with ZD4190 (100 mg/kg orally per day for three consecutive days). Tumor growth was monitored by caliper measurement. During the therapeutic period, longitudinal PET scans were acquired using ^{18}F -FDG, ^{18}F -FLT and ^{18}F -FPPRGD2 as imaging tracers to evaluate tumor glucose metabolism, tumor cell proliferation, and angiogenesis, respectively. Imaging metrics were validated by immunohistochemical analysis of Ki67, GLUT-1, F4/80, CD31, murine integrin $\beta 3$, and human integrin $\alpha \nu \beta 3$.

Results—Three consecutive daily oral administrations of 100 mg/kg of ZD4190 were effective in delaying MDA-MB-435 tumor growth. A significant difference in tumor volume was observed on day 7 between the treatment group and the control group ($p < 0.01$). After the final treatment, tumor growth resumed after a short delay. In the control tumors, ^{18}F -FPPRGD2 uptake was stable between days 0 and 7. In ZD4190-treated tumors, ^{18}F -FPPRGD2 uptake had decreased significantly relative to baseline by $26.74 \pm 8.12\%$ ($p < 0.05$) on day 1 and by $41.19 \pm 6.63\%$ ($p < 0.01$) on day 3, then had returned to baseline on day 7. Tumor uptake of ^{18}F -FLT had also decreased on both day 1 and day 3 after initiation of ZD4190 treatment. No significant change in ^{18}F -FDG uptake in ZD4190-treated tumors was observed, however, compared with the control group. All of the imaging findings were supported by ex vivo analysis of related biomarkers.

Conclusion—The longitudinal imaging results demonstrated the usefulness of quantitative ^{18}F -FLT and ^{18}F -FPPRGD2 PET imaging in evaluating the early antiproliferative and antiangiogenic effects of ZD4190. The quantification data from the PET imaging were consistent with the pattern of initial growth inhibition with treatment, followed by tumor relapse after treatment cessation.

Keywords

Positron emission tomography (PET); Antiangiogenic treatment; ^{18}F -FDG; ^{18}F -FLT; ^{18}F -FPPRGD2; Integrin; Therapy response; Tyrosine kinase inhibitor

Introduction

As early as 1971, Folkman proposed that antiangiogenesis might be an effective anticancer strategy [1], based on the observation that tumor growth is associated with marked vascularity [2]. Recognition of the vascular endothelial growth factor (VEGF) pathway as a

key regulator of angiogenesis led to the development of several VEGF-targeting agents, including agents that prevent VEGF-A from binding to its receptors [3], antibodies that directly block VEGFR-2 [4, 5], and small molecules that inhibit the kinase activity of VEGFR-2, thereby blocking growth factor signaling [6–8]. Tyrosine kinase (TK) inhibitors compete with adenosine-5'-triphosphate (ATP) for binding within the intracellular domain of various wild-type and/or mutated receptor TKs, representing a new paradigm in anticancer therapy [9]. In addition, compared with the conventional cytotoxic agents, VEGFR TK inhibitors may be more tolerable as a treatment for a wide range of solid tumor types, either as monotherapy or combined with radiation and/or additional chemotherapy [7].

ZD4190 is a heteroaromatic substituted anilinoquinazoline, which is a potent inhibitor of VEGFR-2 TK activity [7]. Through the inhibition of VEGF-stimulated endothelial cell proliferation, ZD4190 has shown therapeutic efficacy against solid tumor types with diverse histology, including tumors originating from breast, lung, prostate, and ovary [10]. By chronic oral administration, ZD4190 has elicited significant and dose-dependent antitumor activity [7]. Prolonged tumor inhibition was further demonstrated in a PC-3 xenograft model with 10 weeks of ZD4190 treatment [7].

The most popular way to evaluate therapeutic response is by the change in tumor dimension or volume. Clinical trials with conventional cytotoxic chemotherapeutic agents have mainly used morphological imaging, mostly computed tomography (CT) or magnetic resonance imaging (MRI), to provide indices of therapeutic response, according to the Response Evaluation Criteria in Solid Tumors (RECIST) introduced in the year 2000 [11]. However, antiangiogenic agents are typically cytostatic, rather than cytotoxic, and lead to a stop or delay of tumor progression rather than tumor shrinkage [12]. Thus, tumor volume is not a sensitive indicator of therapeutic efficacy and might require months to assess. Instead, microvessel density (MVD) is the most commonly used end-point for assessing antiangiogenic treatment in clinical studies. MVD is measured from biopsies taken before and at one or more times after treatment begins, using a variety of immunohistochemical vascular markers to identify the vessels [13]. However, measurement of MVD is problematic for assessing the vascular efficacy of antiangiogenic agents [14] since blocking angiogenesis may be accompanied by a proportional reduction in tumor growth that would not result in a net change in MVD. Besides, vessel counts and/or density may remain unchanged even in the face of effective therapy [15]. A similar problem has also been found with noninvasive imaging methods for measuring functional vascular volume and perfusion, such as PET studies with ^{15}O -water [16], ultrasonography [17] and dynamic contrast-enhanced MRI [18]; i.e., a negative effect on vascular volume and perfusion indicated by noninvasive imaging cannot be interpreted as absence of an antiangiogenic effect [19].

Currently available, specifically targeted molecular imaging probes for PET can noninvasively detect unique molecular and biological targets in living subjects that may provide exciting opportunities to monitor therapeutic response [20]. For example, PET imaging with ^{18}F -FDG can reflect accelerated glucose metabolism, which is one of the phenotypic or functional changes observed in cancer tissue [21]. The most extensively investigated tracer for imaging cell proliferation is 3'-deoxy-3'- ^{18}F -fluorothymidine (^{18}F -FLT), because thymidine kinase is regarded as the main factor responsible for the accelerated growth of malignant tissue [22]. Integrin $\alpha\text{v}\beta_3$, a marker of angiogenesis, is associated with the regulation of cellular activation, migration, proliferation, survival, and differentiation. It is highly expressed on proliferating and activated endothelial cells associated with neovascularization in both malignant tumors and normal tissue, but not in quiescent blood vessels. It is also preferentially expressed on several types of cancer cells, such as melanoma, glioma, and ovarian and breast cancers [23, 24]. PET imaging with peptides based on arginine-glycine-aspartic acid (RGD) has been used to visualize and

quantify integrin $\alpha v \beta 3$ expression levels in vivo and to further evaluate the efficacy of antiangiogenesis therapeutic agents [25, 26]. In this study, we evaluated three PET tracers, ^{18}F -FDG, ^{18}F -FLT and ^{18}F -FPPRGD2, for measuring tumor response to the VEGF RTK inhibitor ZD4190 in an MDA-MB-435 tumor model.

Materials and methods

General materials

Unless otherwise specified, all reagents were of analytical grade and were obtained from commercial sources. ZD4190, a substituted 4-anilinoquinazoline, was synthesized following a previously reported procedure [27]. ^{18}F -F⁻ radionuclide was obtained from the NIH Clinical Center cyclotron facility by proton irradiation of ^{18}O -enriched water. ^{18}F -FDG was purchased from the Nuclear Pharmacy of Cardinal Health, and reconstituted with sterile saline. Reversed-phase extraction C₁₈ Sep-Pak cartridges were obtained from Waters and pretreated with ethanol and water before use. The syringe filter and polyethersulfone membranes (pore size 0.22 μm , diameter 13 mm) were obtained from Nalge Nunc International. Analytical as well as semipreparative reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters 600 chromatography system with a Waters 996 photodiode array detector and a Beckman 170 radioisotope detector. The recorded data were processed using Empower software.

Synthesis of ^{18}F -FLT and ^{18}F -FPPRGD2

^{18}F -FLT was prepared manually according to the method of Lee et al. [28] with minor modification. In brief, aqueous ^{18}F -fluoride (50 mCi/0.2 ml) was mixed with 200 μl of CH_3CN and 10 μl of tetraethylammonium bicarbonate (TEABC, 1 mol/l) in the reaction vessel. Three sequences of heating (105°C) with a helium gas flow resulted in dry ^{18}F -fluoride/TEABC. The precursor 3-*N*-Boc-1-[5-*O*-(4,4'-dimethoxytrityl)-3-*O*-nosyl-2-deoxy- β -*D*-lyxofuranosyl] thymine (5 mg; ABX, Germany) was then added in 100 μl of CH_3CN and 300 μl of 2-methyl-2-butanol and reacted for 5 min at 105°C. The protecting group was removed from the labeled intermediate by acidic hydrolysis (1 mol/l HCl, 105°C, 5 min). The reaction mixture was neutralized with NaOH (2 mol/l) and purified on a semipreparative C₁₈ HPLC column eluted with 8% ethanol/H₂O (4 ml/min) and sterilized by filtration through a 0.2- μm membrane. Radiochemical yield (uncorrected for decay) was 73.5 $\pm$ 9.3% ($n=5$). Radiochemical purity was over 99% and specific activity was higher than 40 GBq/ μmol at the end of synthesis.

^{18}F -FPPRGD2 was synthesized according to the method described in our previous report [29]. The purified and dried ^{18}F -NFP was reconstituted in dimethyl sulfoxide (DMSO, 200 μl), and added to a DMSO solution of PRGD2 (0.1 μmol) with DIPEA (20 μl). The peptide mixture was incubated at 105°C for 10 min. After dilution with 500 μl 5% acetic acid, the mixture was purified by semipreparative HPLC. The desired fractions were combined and evaporated in vacuo to remove the solvent. The ^{18}F -labeled peptide ^{18}F -FPPRGD2 was then formulated in normal saline and passed through a 0.22- μm Millipore filter. The radiochemical purity of ^{18}F -FPPRGD2 was more than 99%, based on analytical HPLC. The specific activity was over 30 GBq/ μmol at the end of synthesis.

Tumor growth, drug treatment and PET imaging

The MDA-MB-435 cell line was purchased from the American Type Culture Collection (ATCC) and grown in Leibovitz's L-15 medium supplemented with 10% (v/v) fetal bovine serum under a 100% air atmosphere at 37°C. The tumor model was developed in female athymic nude mice at 5–6 weeks of age (Harlan Laboratories) by orthotopic injection of 5×10^6 cells in the left mammary fat pad of each mouse. Tumor growth was followed by caliper

measurements made perpendicular to the tumor. The tumor volume was estimated from the formula: tumor volume = $a \times (b^2)/2$, where a and b are the tumor length and width, respectively, in millimeters. All procedures in this animal study were conducted under a protocol approved by the NIH Clinical Center's Animal Care and Use Committee (ACUC). Moreover, all mice were maintained in a specific pathogen-free facility in accordance with the requirements of the ACUC.

The detailed therapy and the longitudinal scan schedule are shown in Table 1. Once the tumor volumes had reached approximately 200 mm³, a total of 48 mice were randomized into six groups (eight per group): ¹⁸F-FPPRGD2 control, ¹⁸F-FPPRGD2 treatment, ¹⁸F-FLT control, ¹⁸F-FLT treatment, ¹⁸F-FDG control, and ¹⁸F-FDG treatment. After the baseline microPET imaging (day 0), three daily treatments with ZD4190 (100 mg/kg orally) were administered on days 1, 2 and 3 in the treatment group. DMSO vehicle was used in the control group. PET scans were repeated on day 1 (2 h after treatment), day 3 (2 h after treatment) and day 7. At each time point, one mouse from each group was killed, and the tumors were excised for histopathology.

PET scans and image analysis were performed using an Inveon microPET scanner (Siemens Medical Solutions). Each MDA-MB-435 tumor-bearing mouse, under isoflurane anesthesia, was injected via a tail vein with 3.7 MBq (100 µCi) of ¹⁸F-FPPRGD2, ¹⁸F-FDG or ¹⁸F-FLT. Five-minute static scans were acquired at 1 h (¹⁸F-FPPRGD2, ¹⁸F-FDG) or 1.5 h (¹⁸F-FLT) after injection. For the ¹⁸F-FDG scans, mice were fasted for 6 h before tracer injection and were maintained under isoflurane anesthesia during the injection, accumulation, and scanning periods. For the ¹⁸F-FPPRGD2 and ¹⁸F-FLT scans, mice were not fasted before tracer injection and were not anesthetized during the tracer accumulation period. The images were reconstructed using a two-dimensional ordered-subset expectation maximization algorithm, and no correction was applied for attenuation or scatter. For each microPET scan, regions of interest (ROIs) were drawn over the tumor using vendor software ASI Pro 5.2.4.0 on decay-corrected whole-body coronal images. The maximum radioactivity concentrations (accumulation) within a tumor were obtained from maximum pixel values within the multiple ROI volumes and then converted to megabecquerels per milliliter per minute using a conversion factor. These values were then divided by the administered activity to obtain (assuming a tissue density of 1 g/ml) an image ROI-derived maximum percent injected dose per gram (%ID/g_{max}).

Fluorescence staining

The tumor-bearing animals were killed immediately after the PET scans and tumor samples were collected. Frozen tumor tissue sections (5 µm) were fixed with cold acetone for 20 min and dried in the air for 30 min at room temperature. After blocking with 2% BSA for 30 min, the sections were incubated with humanized anti-human integrin αvβ3 antibody Abegrin (1:200; MedImmune) for 2 h at room temperature and then visualized with Cy3-conjugated donkey anti-human secondary antibodies (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA). For the overlay staining of CD31 and CD61, slices were incubated with rat anti-mouse CD31 antibody (1:300; BD Biosciences) and hamster anti-mouse β3 CD61 antibody (1:200; BD Biosciences) and then visualized with Cy3-conjugated goat anti-rat and FITC-conjugated goat anti-hamster secondary antibody (1:400; Jackson ImmunoResearch Laboratories). For the staining of Ki67 and GLUT-1, slices were incubated with rabbit anti-human Ki67 antibody (1:100; BD Biosciences) and rabbit anti-human GLUT-1 antibody (1:300; Abcam) separately, and then visualized with Cy3-conjugated goat anti-rabbit and FITC-conjugated goat anti-rabbit secondary antibody (1:400; Jackson ImmunoResearch Laboratories). For the staining of F4/80, slices were incubated with rat anti-mouse F4/80 antibody (1:300; Abcam), and then visualized with Cy3-conjugated goat anti-rat secondary antibody (1:400; Jackson ImmunoResearch

Laboratories). After washing with PBS three times for 5 min each time, the whole slides were mounted with mounting medium containing 4'-6-diamidino-2-phenylindole (DAPI). Fluorescence images were acquired with an epifluorescence microscope (Olympus, X81) equipped with filter sets for DAPI (excitation 387/11 nm, emission 409 nm), ET-GFP (excitation 488/20 nm, emission 525/50 nm), and ET-DSRed (excitation 545/30 nm, emission 620/60 nm).

Fluorescence staining analysis

Image-Pro Plus (v. 6.0) software was used to assess the total number of DAPI-positive and Ki67-positive nuclei, the macrophage-infiltrated area, human $\alpha\beta 3$ fluorescence intensity, murine $\beta 3$ fluorescence intensity, and human GLUT-1 expression. The Ki67 staining index (SI) was defined as the percentage of positive nuclei in relation to the total number of nuclei. Human $\alpha\beta 3$ fluorescence intensity, murine $\beta 3$ fluorescence intensity, human GLUT-1 expression, and macrophage infiltration intensity were calculated by measuring the integrated optical density of images of equivalent area (millimeters squared). For each tumor section, ten random high-power images ($\times 20$ magnification) were analyzed.

Statistical analysis

Quantitative data are expressed as means $\pm$ SD. Means were compared using a Student's *t* test. *P* values <0.05 were considered statistically significant.

Results

ZD4190 treatment inhibited MDA-MB-435 tumor growth

Three consecutive daily oral administrations of 100 mg/kg ZD4190 were effective in delaying MDA-MB-435 tumor growth. A time-dependent increase in tumor volume was observed in the control group (Fig. 1a), and the average percentage tumor volume increases, expressed as $(V - V_0)/V_0$, were $21.8 \pm 14.6\%$ on day 1, $39.5 \pm 20.2\%$ on day 3, $94.3 \pm 53.5\%$ on day 7, and $219.0 \pm 68.1\%$ on day 14. In the ZD4190 treatment group, however, the percentage increases were $5.9 \pm 2.0\%$ on day 1, $6.9 \pm 6.0\%$ on day 3, $16.4 \pm 8.4\%$ on day 7, and $110.6 \pm 49.8\%$ on day 14. A significant difference in tumor volume was observed at day 7 between the treatment group and the control group ($p < 0.01$). After the final treatment, tumor growth resumed after a short delay, which is consistent with a previous report [7]. There was no significant body weight loss observed during the treatment period, nor any other obvious side effect at the dosage of ZD4190 used in this brief study.

ZD4190 treatment showed no significant effect on glucose metabolism

The tumor-bearing mice were scanned with ^{18}F -FDG PET on days 0, 1, 3 and 7, and typical coronal images are shown in Fig. 1b and c. Compared with the control group, no significant change in ^{18}F -FDG uptake in ZD4190-treated tumors was observed. The maximum tumor uptake values in the treatment group were 11.2 ± 0.5 , 11.8 ± 0.5 , 12.7 ± 3.8 and 10.6 ± 0.9 %ID/ g_{max} , respectively, on days 0, 1, 3 and 7, while those in the control group remained around 11.2 % ID/ g_{max} throughout the study. Reduced or no signal toward the center of larger tumors is often observed, indicating the development of central necrosis, and this was the reason for our use of the maximum instead of the mean tumor uptake as the quantitative parameter.

It has been reported that there is a close relationship between ^{18}F -FDG accumulation and the grading of immunohistochemical expression of GLUT-1 glucose transporter in tumor tissues [30]. On immunofluorescence staining of the tumor sections, the intensity of GLUT-1 staining had increased minimally on day 1 and day 3 after initiation of ZD4190 treatment (Supplementary Fig. 1), which was consistent with the ^{18}F -FDG imaging result. We also

stained the tumor sections against a macrophage-specific marker, F4/80, to evaluate ZD4190-induced inflammation, since tumor inflammation may increase ^{18}F -FDG accumulation. Compared with control tumors, a slight inflammatory reaction was observed on day 3 after initiation of ZD4190 treatment ($p>0.05$; Supplementary Fig. 2).

ZD4190 treatment inhibited tumor cell proliferation

To evaluate ZD4190-mediated changes in cell proliferation, MDA-MB-435 tumor-bearing mice were scanned with the thymidine-based PET tracer ^{18}F -FLT. As shown in Fig. 2a, tumor uptake of ^{18}F -FLT was decreased on both day 1 and day 3 after initiation of ZD4190 treatment, indicating that the drug inhibited tumor cell proliferation. The maximum tumor uptake values ($\% \text{ID}/\text{g}_{\text{max}}$) calculated from PET images for the two groups are shown in Fig. 2b. Treated tumors showed relative reductions in FLT uptake of approximately $8.1 \pm 2.9\%$, compared to baseline on day 1 ($p<0.05$) and $21.0 \pm 8.5\%$ on day 3 ($p<0.01$). On day 7, i.e. 4 days after the final treatment, FLT uptake had returned to the baseline level, as reflected by resumed tumor growth. The tumors in the control group, however, showed a constant FLT uptake at all time points.

To confirm the PET observations on tumor cell proliferation, we performed Ki67 staining on tumor sections. Example images of anti-Ki67-stained sections for control and ZD4190-treated tumors on days 1, 3 and 7 are shown in Fig. 3a. Untreated tumors showed a relatively high proliferation rate with a Ki67 SI of more than 70%, independent of tumor size. ZD4190 treatment significantly slowed down tumor cell proliferation with Ki67 SIs of $45 \pm 6\%$ and $30 \pm 3\%$ on day 1 and day 3 after initiation of treatment, respectively ($p<0.01$). The Ki67 SI had returned to the baseline level by day 7 (Fig. 3b).

ZD4190 treatment downregulated integrin $\alpha\text{v}\beta 3$ expression

To determine the effect of ZD4190 treatment on tumor vasculature and tumor cell integrin expression, we performed PET scans utilizing the high-affinity integrin radio-ligand ^{18}F -FPPRGD2. As shown in Fig. 4a, MDA-MB-435 tumors were clearly visualized on ^{18}F -FPPRGD2 PET images and had clean backgrounds. In the control tumors, ^{18}F -FPPRGD2 uptake was stable between days 0 and 7. In ZD4190-treated tumors, ^{18}F -FPPRGD2 uptake had decreased significantly on day 1 and day 3 by $26.7 \pm 8.1\%$ ($p<0.05$) and $41.2 \pm 6.6\%$ ($p<0.01$), respectively, relative to the baseline, then had returned to the baseline level on day 7 (Fig. 4b).

Since integrin $\alpha\text{v}\beta 3$ is the main target for ^{18}F -FPPRGD2 imaging, we stained tumor sections with both anti-murine integrin $\beta 3$ and anti-human integrin $\alpha\text{v}\beta 3$ antibodies. As shown in Fig. 5a, untreated MDA-MB-435 tumors had positive human integrin $\alpha\text{v}\beta 3$ staining. The fluorescence intensity had decreased significantly on day 1 ($p<0.05$) and day 3 ($p<0.01$) after initiation of ZD4190 treatment, and had returned to the control level on day 7 ($p>0.05$; Fig. 5b). Murine $\beta 3$ (CD61) was predominantly colocalized with murine CD31, a vascular endothelial marker, indicating that murine integrin $\beta 3$ is expressed mainly on the tumor vascular endothelial cells. Following ZD4190 treatment, the median murine $\beta 3$ fluorescence intensity had decreased significantly on day 1 and day 3 ($p<0.05$) and had returned to the control level on day 7 (Fig. 6).

Strong CD31 staining was observed in all untreated MDA-MB-435 tumor sections, indicating relatively abundant vasculature within this tumor. After ZD4190 treatment, the staining pattern of CD31 changed dramatically, suggesting potential morphological changes in tumor vasculature induced by the drug. For example, the blood vessels in the control tumors were much dilated, and microvessel cavities could be identified, whereas the

microvessel cavities had collapsed in ZD4190-treated tumors, especially on day 1 and day 3 after treatment (Fig. 6).

Discussion

VEGF-mediated angiogenesis has been shown to be a key process in the growth of solid tumors [2]. Inhibition of the cognate TK insert domain-containing receptor (KDR/Flk1) has been reported to modulate the signaling event efficiently and specifically [6–8]. Many small molecular inhibitors, such as SU11248 (sunitinib), BAY 43–9006 (sorafenib) and AG-012736 (axitinib), have progressed to clinical use or advanced clinical trials [20]. The objective rate of response to these TK inhibitors was usually less than 50% in the selected patients [31]. Thus, noninvasive visualization and quantification of antitumor and antiangiogenesis potency would be of great value for patient selection, and for optimization of dosage and dose intervals of compounds in this category. In this study, we evaluated the early response of MDA-MB-435 tumors to ZD4190 with multiplexing PET imaging probes.

ZD4190 has good intrinsic activity against the KDR enzyme (IC_{50} 0.03 μ M) and higher selectivity over the related kinase Flt-1 (IC_{50} 0.708 μ M) and FGFR-1 (IC_{50} 5.3 μ M) [27]. It can inhibit VEGF-stimulated proliferation of endothelial cells at only 50 nM via a specific effect on KDR- or Flt-1-mediated signal transduction [7]. With a therapy regimen of three consecutive daily oral doses of 100 mg/kg, ZD4190 inhibited MDA-MB-435 tumor growth without evident tumor regression (Fig. 1), which is consistent with the effects of other antiangiogenesis therapeutic agents [20].

^{18}F -FDG PET has been widely used in the clinic for tumor diagnosis, cancer staging and monitoring of therapeutic response. However, ^{18}F -FDG PET studies of tumors treated with antiangiogenic drugs may show a contradictory increase in activity, which makes evaluation of post-therapeutic studies using this technique difficult [32]. Even though ZD4190 effectively inhibited tumor growth in this study, we did not observe significant changes in ^{18}F -FDG uptake at all time points. Thus, ^{18}F -FDG PET is not suitable for monitoring therapeutic responses to ZD4190. On day 3 after treatment, ^{18}F -FDG uptake showed a slight increase compared with that in the control group, but without statistical significance. Consistently, minimal increases in GLUT-1 staining intensity and macrophage infiltration were also observed in sections of ZD4190-treated tumors compared to control tumors.

^{18}F -FLT is the most extensively studied probe for imaging cellular proliferation in vivo. ^{18}F -FLT PET is more specific than ^{18}F -FDG PET in evaluating tumor proliferation and tumor response, with fewer false-positive findings in inflammatory lesions [33]. A series of animal studies have indicated that ^{18}F -FLT uptake decreases rapidly in response to radiotherapy [34], cytotoxic chemotherapy [35], and various protein kinase inhibitors [36]. Initial patient studies have further emphasized the potential of ^{18}F -FLT PET for monitoring tumor response to therapy [37, 38]. In our study, ^{18}F -FLT uptake in the untreated tumors remained unchanged throughout the study. In ZD4190-treated tumors, the uptake had decreased by 8.1% on day 1 and by 21% on day 3, suggesting that ZD4190 has a potent antiproliferative effect, which was confirmed by Ki67 staining of tumor sections. We also noticed that the magnitude of the decrease in Ki67 SI was larger than that of ^{18}F -FLT on PET imaging (52.8% vs. 21%). We speculate that the nonspecific background of ^{18}F -FLT PET uptake in nonproliferating cells might be partially responsible for this discrepancy. Another possibility is that Ki67 staining may not accurately reflect the response of the whole tumor even though we collected data from multiple regions of different tumor sections.

The integrin $\alpha v\beta 3$ receptor is upregulated on both tumor cells and the endothelium, and it plays important roles in metastasis and angiogenesis. Noninvasive visualization and

quantification of integrin $\alpha\beta 3$ expression levels in vivo opens a new window on monitoring integrin expression, facilitating the understanding of angiogenesis mechanisms and monitoring of antiangiogenic treatment [39]. We and others have developed a series of radiolabeled RGD monomers and multimers for imaging integrin $\alpha\beta 3$ expression. Among them, ^{18}F -AH111585 [40, 41], ^{18}F -galacto-RGD [25, 42] and ^{18}F -FPPRGD2 [29, 43] have been tested in humans. Compared with ^{18}F -AH111585 and ^{18}F -galacto-RGD, the dimeric peptide ^{18}F -FPPRGD2 showed the highest tumor integrin-specific accumulation and most favorable in vivo kinetics [29].

There have been several reports of the use of these RGD peptide tracers to monitor chemotherapy and/or antiangiogenic treatment efficacy. Paclitaxel therapy in Lewis lung carcinoma-bearing mice significantly retarded tumor growth and was accompanied by a corresponding reduction in tumor glucosamino $^{99\text{m}}\text{Tc}$ -D-c(RGDfK) and ^{18}F -AH111585 uptake [40, 44]. Morrison et al. [40] used ^{18}F -AH111585 to detect the therapeutic response after three doses (100 mg/kg) of ZD4190 in Calu-6 tumors. They found that the uptake of ^{18}F -AH111585 was reduced significantly after ZD4190 treatment. Consistent with the previous reports, we also observed decreased ^{18}F -FPPRGD2 uptake in ZD4190-treated tumors, especially on day 1 and day 3 after initiation of treatment, while the ^{18}F -FPPRGD2 uptake in untreated tumors remained the same throughout the period of the experiment.

Since integrin $\alpha\beta 3$ is expressed on both MDA-MB-435 tumor cells of human origin and tumor vascular endothelial cells of mouse origin, we performed immunostaining against both human integrin $\alpha\beta 3$ and murine $\beta 3$. Down-regulated integrin levels on both tumor cells and vasculature were consistent with the decreased tumor uptake of ^{18}F -FPPRGD2, supporting the ^{18}F -FPPRGD2 findings. However, the underlying mechanism is still unclear and will need further investigation. It is worth noting that different drugs may have distinct effects on tumor vasculature and integrin expression. For example, it has been reported that dasatinib, a Src family kinase (SFKs) inhibitor, does not decrease tumor vascularization, or $\alpha\beta 3$ expression, even though it markedly inhibits tumor growth and decreases uptake of ^{64}Cu -DOTA-c(RGDfK) in U87MG xenografts, as observed with PET imaging [45]. With the same tumor model, we have found that only murine integrin expression on tumor vasculature is down-regulated after treatment with Abraxane, but that on tumor cells it is almost unaffected [46].

The promising imaging results observed in this longitudinal study suggest the usefulness of quantitative ^{18}F -FLT and ^{18}F -FPPRGD2 PET imaging in measuring early responses to the antiproliferative and antiangiogenic effects of ZD4190. The quantification data from the PET imaging are also consistent with the pattern of initial growth inhibition after treatment initiation, followed by tumor relapse after treatment cessation. Moreover, changes in ^{18}F -FPPRGD2 and ^{18}F -FLT uptake accurately predicted the tumor response, despite the absence of measurable tumor size change. Compared with ^{18}F -FLT PET imaging, ^{18}F -FPPRGD2 imaging showed lower background and higher tumor/muscle ratio. In addition, after ZD4190 treatment the magnitude of the changes in tumor uptake of ^{18}F -FPPRGD2 was also higher than that of ^{18}F -FLT. From this point of view, ^{18}F -FPPRGD2 is advantageous over ^{18}F -FLT as a PET tracer for imaging the early response of tumor to ZD4190. The reproducibility of imaging should also be considered for data analysis and comparison. As reported previously, both ^{18}F -FDG small-animal PET and ^{18}F -FPPRGD2 mouse xenograft studies were reproducible with moderately low variability. The coefficient of variation for the mean tumor uptake values between ^{18}F -FDG small-animal PET scans performed on the same day 6 h apart was $15.4 \pm 12.6\%$ [47] and that of ^{18}F -FPPRGD2 was $11.1 \pm 7.6\%$ [48]. We also observed a similar coefficient of variation of the maximum tumor uptake values in both ^{18}F -FDG and ^{18}F -FPPRGD2 PET imaging quantification. On day 0, the coefficient of variation of the maximum tumor uptake values quantified by ^{18}F -FLT PET imaging was 11.9%. In

this study, we used a relatively large number of animals (eight per group) to minimize the effect of the reproducibility of imaging on data analysis.

Conclusion

We showed that ^{18}F -FLT and ^{18}F -FPPRGD2 are superior to ^{18}F -FDG in monitoring tumor response to ZD4190, based on a correlation between quantitative imaging studies and ex vivo analyses of related biomarkers. The results also showed that a single targeted therapeutic agent can have multiple functional outcomes in tumor cells and tumor vasculature. Further, the results reinforce the notion that preclinical PET can make an important contribution to the development of molecularly targeted anticancer therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

a Antitumor activity of ZD4190 in MDA-MB-435 breast cancer model. Three consecutive doses of ZD4190 (100 mg/kg on days 1, 2, and 3), administered by gavage, inhibited tumor growth. **b** Representative decay-corrected whole-body coronal microPET images of mice bearing MDA-MB-435 breast cancer at 1 h after intravenous injection of ^{18}F -FDG (1.85 MBq/mouse) on days 0, 1, 3 and 7 after treatment was initiated. No significant increased uptake of ^{18}F -FDG is apparent on day 1 and day 3. The tumors are indicated by *arrows*. **c** Tumor uptake of ^{18}F -FDG quantified by ROI analysis

Fig. 2.
a Representative decay-corrected whole-body coronal microPET images of mice bearing MDA-MB-435 breast cancer at 1.5 h after intravenous injection of ^{18}F -FLT (1.85 MBq per mouse) on days 0, 1, 3 and 7 after treatment was initiated. The tumors are indicated by arrows. Decreased uptake of ^{18}F -FLT is apparent on day 1 and day 3, but it has returned to baseline level on day 7. **b** Quantitative microPET ROI analysis of tumor uptake for ^{18}F -FLT

Fig. 3. Proliferation of tumor cells in MDA-MB-435 tumors after ZD4190 treatment. **a** Tumor cell proliferation as assessed by Ki67 immunofluorescence staining. **b** Ki67 SI on days 1, 3 and 7 after initiation of ZD4190 treatment. Significant decreases in Ki67 SI are apparent on day 1 and day 3 after initiation of treatment compared with the control tumors (* $p < 0.05$, ** $p < 0.01$). (Three samples were used from each group, and the results were confirmed with a duplicate experiment.)

Fig. 4.

a Representative decay-corrected whole-body coronal microPET images of mice bearing MDA-MB-435 breast cancer at 1 h after intravenous injection of ^{18}F -FPPRGD2 (1.85 MBq per mouse) on days 0, 1, 3 and 7 after initiation of treatment was initiated. The tumors are indicated by *arrows*. Decreased tumor uptake of ^{18}F -FPPRGD2 is apparent on day 1 and day 3, but has returned to the baseline level on day 7. **b** Quantitative microPET ROI analysis of tumor uptake of ^{18}F -FPPRGD2

Fig. 5.

a Immunofluorescence staining of human integrin $\alpha v \beta 3$ for MDA-MB-435 tumor tissues. **b** Integrated optical density of human integrin $\alpha v \beta 3$ is decreased on both day 1 and day 3 after initiation of ZD4190 treatment compared with control group (* $p < 0.05$, ** $p < 0.01$)

Fig. 6. Immunofluorescence staining of tumor vasculature and murine integrin $\alpha\text{v}\beta 3$ expression with antibodies against CD31 (*red*) and CD61 (integrin $\beta 3$, *green*)

Table 1
Experimental design for longitudinal ¹⁸F-FDG, ¹⁸F-FLT and ¹⁸F-FPPRGD2 imaging of ZD4190 treatment efficacy, and ex vivo histopathology

	Day 0	Day 1	Day 2	Day 3	Day 7
¹⁸ F-FPPRGD2	Control	Vehicle treatment, microPET imaging	Vehicle treatment	Vehicle treatment, microPET imaging	microPET imaging
	ZD4190	ZD4190 treatment, microPET imaging	ZD4190 treatment	ZD4190 treatment, microPET imaging	microPET imaging
¹⁸ F-FDG	Control	Vehicle treatment, microPET imaging	Vehicle treatment	Vehicle treatment, microPET imaging	microPET imaging
	ZD4190	ZD4190 treatment, microPET imaging	ZD4190 treatment	ZD4190 treatment, microPET imaging	microPET imaging
¹⁸ F-FLT	Control	Vehicle treatment, microPET imaging	Vehicle treatment	Vehicle treatment, microPET imaging	microPET imaging
	ZD4190	ZD4190 treatment, microPET imaging	ZD4190 treatment	ZD4190 treatment, microPET imaging	microPET imaging
Histology		Tumor tissue sampling		Tumor tissue sampling	Tumor tissue sampling