VEGF-PET imaging is a non-invasive biomarker showing differential changes in the tumor during sunitinib treatment

Wouter B. Nagengast¹, Marjolijn N. Lub-de Hooge²,³, Sjoukje F. Oosting¹, Wilfred F.A. den Dunnen⁴, Frank-Jan Warnders¹, Adrienne H. Brouwers³, Johan R. de Jong³, Patricia M. Price⁶, Harry Hollema⁴, Geke A.P. Hospers¹, Philip H. Elsinga³, Jan Willem Hesselink⁵, Jourik A. Gietema¹, Elisabeth G.E de Vries¹.

Departments of Medical Oncology¹, Hospital and Clinical Pharmacy², Nuclear Medicine and Molecular Imaging³, Pathology⁴ and Surgery⁵, University of Groningen and University Medical Center Groningen The Netherlands; Academic Department of Radiation Oncology⁶, The Christie Hospital, Manchester, United Kingdom.

Address of correspondence:
Elisabeth G. de Vries, MD, PhD
Department of Medical Oncology
University Medical Center Groningen
Hanseplein 1
Postbus 30.001
9700 RB Groningen
E.G.E.de.Vries@int.umcg.nl
Phone +31 50 361 2821 / 1847

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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Abstract

Non-invasive imaging of angiogenesis could ease the optimization of anti-angiogenesis treatments for cancer. In this study we evaluated the role of VEGF-PET as a biomarker of dynamic angiogenic changes in tumors following treatment with the kinase inhibitor sunitinib. The effects of sunitinib treatment and withdrawal on the tumor was investigated using the new VEGF-PET tracer $^{89}$Zr-ranibizumab as well as 18F-FDG PET and 15O-water PET in mouse xenograft models of human cancer. The obtained imaging results were compared with tumor growth, bioluminescence imaging, VEGF plasma levels and immunohistological analyzes. In contrast to $^{18}$F-FDG and $^{15}$O-water PET, VEGF-PET demonstrated dynamic changes during sunitinib treatment within the tumor with a strong decline in signal in the tumor center and only minimal reduction in tumor rim, with a pronounced rebound after sunitinib discontinuation. VEGF-PET results corresponded with tumor growth and immunohistochemical vascular- and tumor-markers.

Our findings highlight the strengths of VEGF-PET imaging to allow serial analysis of angiogenic changes in different areas within a tumor.

Precis

A PET imageable marker for tumor angiogenesis could permit non-invasive monitoring of dynamic changes in patients, helping guide treatment strategies, optimal dose finding, and drug combination studies.
Introduction

Angiogenesis, the formation of new blood vessels, is one of the hallmarks of carcinogenesis. Vascular endothelial growth factor (VEGF) and its corresponding receptors (VEGFR) on endothelial cells are important players in the regulation of angiogenesis, providing targets for anti-angiogenic agents (1). When used either as single agents or combined with chemotherapy, anti-angiogenic drugs have improved disease outcome in several tumor types. However, this benefit is modest and often of limited duration. Further, unexplained paradoxical effects have been observed. For example, the VEGF tyrosine kinase inhibitor (TKI) sunitinib is used for the clinical treatment of metastatic renal cell cancers and gastrointestinal stromal tumors as it blocks angiogenesis in primary tumors (2), but its use may also lead to increased invasiveness at the tumor boundary and promotion of metastases (3,4). This complex and dynamic interaction between tumor cells and their micro-environment may be an important reason why previous investigation of potential biomarkers has failed to predict response to anti-angiogenic therapy. The search for biomarkers has been especially directed towards circulating markers and visualizing anatomic tumor changes during anti-angiogenic treatment (4). The biological responses that occur in response to anti-angiogenic treatment are presumably dynamic over time and likely to be heterogeneous within the tumor. Therefore molecular imaging, enabling visualization of biological processes, might provide a better insight into how tumors respond to anti-angiogenic treatment with agents such as sunitinib.

Well known techniques for molecular imaging include measurement of tissue glucose uptake with $^{18}$F-FDG positron emission tomography (PET) to assess metabolism and measurement of tissue perfusion with $^{15}$O-water PET. A new option is direct imaging the molecules involved...
in the promotion or inhibition of VEGF signaling using PET analysis, which may be achieved following molecular radiolabeling. Therapeutic inhibitors of the VEGF pathway, such as the monoclonal antibody bevacizumab and the antibody derivative ranibizumab, which bind and block VEGF-A and have proven clinical anti-angiogenic effectiveness, are attractive for this purpose (5, 6).

Previously, we developed $^{89}\text{Zirconium}$-labeled bevacizumab ($^{89}\text{Zr}$-bevacizumab) as a biomarker for the PET analysis of VEGF levels, with the aim of providing insight into the available target for VEGF-dependent anti-angiogenic therapy and thus assist in tumor response prediction (6). This approach proved promising, as tumor uptake of $^{89}\text{Zr}$-bevacizumab mediated by VEGF-A binding significantly higher than control antibody was demonstrated, providing a potential new tracer for non-invasive imaging of VEGF signaling in the microenvironment of the tumor. Further, the $^{89}\text{Zr}$ label, with its half life of 78 hours, proved valuable for antibody imaging allowing high resolution PET over at least 24 hours. However, maximum uptake did not occur until 4-7 days post injection (6), likely due to the 21 day serum half-life of bevacizumab (7). To gain more dynamic insight into tumor response during anti-angiogenic treatment, we have since developed the PET tracer $^{89}\text{Zr}$-ranibizumab for potential use as non-invasive biomarker of VEGF signaling. Ranibizumab, a monoclonal antibody fragment (Fab) derivative of bevacizumab, is used to treat macular degeneration (8). It has a higher affinity for all soluble and matrix bound human VEGF-A isoforms than bevacizumab (7). In addition, it allows fast and sequential follow-up PET scans, as its serum half-life is only 2-6 hours.

In this study, we investigated the biological effects of sunitinib treatment and subsequent withdrawal in human xenograft tumor models, using tumor growth assays,
immunohistochemistry, and PET analyses (18F-FDG, 15O-water and 89Zr-ranibizumab). A secondary aim was to determine the utility of 89Zr-ranibizumab-PET analysis as a biomarker for anti-angiogenic treatment.
Methods

Cell lines and in vitro experiments. The human ovarian tumor cell lines A2780 (provided by Dr TC Hamilton, Fox Chase Cancer Center, USA). The human ovarian cancer cell line SKOV-3 and the human colon cancer cell line Colo205 were obtained from the ATCC. Cell lines were quarantined until screening for microbial contamination and mycoplasma were performed and proven to be negative. Meanwhile a reproducible supply of cells was established by cryopreservation. All experiments were performed within a predefined number of passages. Key features of the cell lines were routinely checked. Growth and morphology of both cell lines was observed and noted to be consistent with prior descriptions of the lines; no further genetic characterization was performed. A2780 and Colo205 were cultured in RPMI 1640 (Invitrogen) with 10% heat inactivated fetal calf serum (FCS) (Bodinco BV) and 2 mM L-glutamine (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO₂. SKOV-3 in Dulbecco's Modified Eagles Medium (DMEM) with 4.5 g/mL glucose and 10% FCS. Cells were subcultured three times per week.

For in vitro experiments sunitinib (LC Laboratories) was dissolved in dimethyl sulfoxide (DMSO) at 40 mg/mL and stored at -80 °C. The MTT-assay was used to determine cytotoxicity of sunitinib in A2780 and Colo205 cells (9). Cells were seeded (A2780 3,750 cells/well, Colo205 3,000 cells/well) in a 96-wells plate in quadruplicate for each sunitinib concentration (0–20,000 nM) and cultured for 4 days. No cytotoxicity occurred at sunitinib levels up to 5,000 nM, which is a relevant plasma level in mice (supplement 1).
18F-FDG and 15O-water synthesis. 18F-FDG was produced using the coincidence 18F-FDG synthesis module (10). Carrier-added [15O]O2 was produced by irradiation of a mixture of nitrogen and 1% oxygen gas with 7 MeV deuterons from a Scanditronix MC-17 cyclotron. [15O]O2 was reacted with hydrogen gas at 400 °C to generate 15O-water. The 15O-water was trapped in a sterivial with 2.5 mL of 0.9% NaCl and sterilized by filtration (22 µm Millex GP filter).

Conjugation and 89Zr-labeling of ranibizumab and control Fab-IgG. Conjugation and labeling of ranibizumab (Lucentis®, Novartis Pharma) and Fab-IgG were executed as described for U36 (6, 11). The chelate desferrioxamine B (Df) (Novartis Pharma) was succinylated (N-sucDf), and coupled to lysine residues of ranibizumab by means of a tetrafluorophenol-N-sucDf ester. Conjugation was performed at room temperature for 30 minutes at pH 9.5-9.7. Hereafter, the mixture was adjusted to pH 4.2-4.4 (0.1 mol/L H2SO4) and 50 µL of 25 mg/mL ethylenediaminetetraacetic acid (EDTA)(Calbiochem) was added to remove Fe(III). The resultant solution was then purified by ultrafiltration, diluted in water for injection (5 mg/mL) and stored at -20°C. Labeling was performed with 89Zr produced by Cyclotron BV. 89Zr-oxalate was adjusted to pH 3.9-4.2 and mixed for 3 minutes, then adjusted to pH 6.7-6.9 using HEPES buffer. N-sucDf-ranibizumab was added and incubated for 45 min at 20 °C. 89Zr-ranibizumab labeling (specific activity (SA) 1500 MBq/mg) resulted in yields of >95%. 24 hours storage in 37 °C serum displayed no measurable decrease in protein-bound radioactivity and revealed adequate VEGF-A binding, comparable to unlabeled ranibizumab using a VEGF-coated enzyme-linked immunosorbent assay as described previously for bevacizumab (supplement 2)(6). Control 89Zr-Fab-IgG is a humanized Fab-fragment.
comparable in size to ranibizumab. It was similarly prepared and showed no binding affinity towards VEGF-A.

Animal experiments. Animal experiments were performed with isoflurane inhalation anesthesia (induction 3%, maintenance 1.5%). Tumor cells for xenografting were harvested by trypsinization and resuspended in culture medium and Matrigel™ (BD Bioscience). In vivo imaging and ex vivo biodistribution experiments were conducted using male nude HSD athymic mice (Harlan). 6-8 weeks old mice were injected subcutaneously with $1.0 \times 10^5$ SKOV3, $5 \times 10^6$ A2780, $5 \times 10^6$ A2780luc+ or $5 \times 10^6$ Colo205 cells mixed with 0.1 mL Matrigel™. In vivo studies were commenced when resulting tumors measured 6-8 mm in diameter. All animal experiments were approved by the animal experiments committee of the University of Groningen.

MicroPET imaging and ex-vivo biodistribution. All tracers were injected intravenously into the penile vein. $^{18}$F-FDG (5.0 ± 1.0 Mbq) microPET images (Focus 220 rodent scanner (CTI Siemens)) were obtained 1 hour post injection. Animals were fasted for 12 h before $^{18}$F-FDG administration. 10 minutes dynamic PET imaging was taken after $^{15}$O-water (78 ± 8.9 Mbq) administration followed by microCT imaging using a MicroCAT II (CTI Siemens) for anatomic localization. $^{89}$Zr-ranibizumab (3.5 ±1.5 Mbq) images were taken 0, 3, 6 and 24 hours post injection. Static images of 30 minutes acquisition time were obtained each time.

Following image reconstruction, quantification was performed with AMIDE Medical Image Data Examiner software (version 0.9.1, Stanford University)(12). To quantify radioactivity within the tumor, 3D volumes of interest (VOIs) were drawn. $^{18}$F-FDG is presented as standardized uptake value (SUV), using mean tumor uptake per cm$^3$ divided by mean body
uptake. \(^{15}\)O-water is presented as percentage uptake per cm\(^3\) relative to tumor uptake in the first frame (30 seconds) of the scan. For \(^{89}\)Zr-ranibizumab, the total injected dose was calculated by decay correction of total activity present at 0 hour after injection in the animal. The data was quantified as percentage injected dose per gram (%ID/g), assuming a tissue density of 1. Data is presented as percentage uptake compared to baseline and corrected for tumor volume. Following sacrifice, organs and tissues were excised, rinsed for residual blood and weighed. Samples and primed standards were counted for radioactivity in a well-type LKB-1282-Compu-gamma system (LKB Wallac) and corrected for physical decay. Harvested tumors were divided, immediately frozen at -80°C and paraffin embedded for further analysis.

**Dose finding study and pharmacokinetics of \(^{89}\)Zr-ranibizumab.** Based on available \(^{85}\)Zr-bevacizumab data for comparison (5) we firstly used the SKOV-3 xenograft model to evaluate \(^{89}\)Zr-ranibizumab characteristics. In 4 groups of animals, 4 protein doses (3, 8 and 40 \(\mu\)g (all \(n = 4\)) and 350 \(\mu\)g (\(n = 1\)) ranibizumab labeled with a fixed amount (MBq) of \(^{89}\)Zr were administered and biodistribution was determined 24 hours post injection. Subsequently, four groups (\(n = 4\)) of mice were injected with 5 \(\mu\)g of \(^{89}\)Zr-ranibizumab and 1, 3, 6 and 24 hours thereafter a group was sacrificed and ex-vivo biodistribution performed. One group of mice (\(n = 4\)) was injected with 5 \(\mu\)g of \(^{89}\)Zr-Fab-IgG. Ex vivo biodistribution followed 24 h post injection.

**Sunitinib treatment in A2780 and Colo205 models.** Sunitinib malate was dissolved in DMSO at 75 mg/mL. Before administration, sunitinib was diluted in phosphate-saline buffer (PBS; 140 mmol/L NaCl, 9 mmol/L Na\(_2\)HPO\(_4\), 1.3 mmol/L NaH\(_2\)PO\(_4\); pH = 7.4) and administrated...
once daily ip at 60 mg/kg (6.5 mL/kg) or placebo (vehicle). Sixty mg/kg sunitinib daily previously demonstrated anti-tumor efficacy in xenograft bearing mice and changes in tumor derived human VEGF plasma levels with minimal toxicity (13). Four different treatment schedules with once daily intraperitoneal (ip) treatment were used. 18F-FDG imaging was performed in A2780 bearing animals at baseline (n = 4), day 7 (n = 4) of sunitinib treatment and following a drug-free week (n = 5). Serial 15O-water PET was carried out in A2780 bearing animals (n = 5) at baseline and following 7 days sunitinib. Serial 89Zr-ranibizumab imaging (5 ± 1 µg) was performed in A2780 bearing mice treated with sunitinib for 1 week followed by a drug-free week (n = 4), sunitinib ip for 2 weeks (n = 4), sunitinib for 1 week (n = 4) or placebo treatment (n = 4) and Colo205 tumor bearing mice with sunitinib for 1 week followed by a drug-free week (n = 5) at baseline, day 7 and day 14. To assess relative attribution of non-VEGF driven uptake, serial 89Zr-Fab-IgG imaging was performed in A2780 bearing animals (n = 5) at baseline, day 7 of sunitinib and following a drug-free week. Tumor volumes were assessed by external calibration along the longest axis (x-axis) and the axis perpendicular to the longest axis (y-axis). Tumor volume was calculated by the formula: 

\[(x \cdot y^2)/2.\]

Plasma human VEGF analysis and tumor immunohistochemistry. Plasma VEGF levels of sacrificed A2780 bearing mice were determined with the human VEGF ELISA kit (R&D Systems). Paraffin-embedded tumors (n=4 per treatment group) were stained with hematoxylin and eosin and antibodies against von Willebrand factor (vWf), Ki67 anti-human and anti-mouse, VEGFR2, GLUT-1, HIF1α (Dako), human VEGF-A (sc-152, Santa Cruz). The mean vascular density (MVD) and VEGFR2 were scored in 3 defined hot spot areas that contained the maximum number of micro vessels, as previously described (6). To assess relative attribution of non-VEGF driven uptake, serial 89Zr-Fab-IgG imaging was performed in A2780 bearing animals (n = 5) at baseline, day 7 of sunitinib and following a drug-free week.
changes in tumor vessel diameter, the shortest diameter of the largest vessels (≥ 5 per slide) were measured using computerized Aperio Image Software (Aperio Technologies Inc). Blood vessel proliferation was determined by calculating the percentage blood vessels with Ki67 positive endothelial cells. The tumor proliferation index was calculated by percentage of Ki67 positive cells in at least 4 high power fields (200x). MVD, tumor vessel diameter, VEGFR2, blood vessel proliferation and tumor proliferation were obtained for tumor rim (area < 500 µm of tumor margin) and center (area > 500 µm of tumor margin). Glut-1 and HIF1α staining was scored manually as percentage positive of total tumor tissue per slide.

Statistical analysis. Data is presented as means ± standard error of the mean (SEM). Statistical analysis was performed using the Mann-Whitney U test for unpaired data and the Wilcoxon Matched Pairs test for paired data (SPSS, version 14). Tumor growth curves (trends) were analyzed using linear regression analyses. All tests were two sided and the significance level was taken as ≤ 0.05.
Results

*Sunitinib reduces tumor growth, but rapid regrowth occurs after discontinuation.* Sunitinib treatment was evaluated in the A2780 and Colo205 model, which were chosen for their angiogenic profile and constant growth rate (2, 14). In A2780 bearing mice, tumor growth diminished after 7 days of daily sunitinib treatment. Tumors in treated animals increased 123.7 ± 16.0% from baseline, compared with 182.0 ± 18.1% in non-treated animals (Fig. 1A). Thereafter sunitinib was withdrawn for 7 days and tumor growth accelerated to 190 ± 10.8% on day 14. Tumor growth tended to be slower when sunitinib was continued, resulting in a tumor volume of 140.0 ± 16.1% at day 14 compared with day 0 ($P = 0.067$). In the Colo205 model, sunitinib induced significant tumor growth stabilization at day 7 after daily sunitinib treatment. When sunitinib was withdrawn, tumors showed regrowth, from an average volume of 86.6 ± 12.9% on day 7 to 132.9 ± 26.8% on day 14 ($P = 0.096$), with a significant tumor growth curve from zero ($P = 0.0006$).

*Molecular imaging with $^{18}$F-FDG PET and $^{15}$O-water PET.* At baseline, $^{18}$F-FDG PET showed a homogeneous tumor uptake in A2780 tumor bearing animals (Fig. 1B). Seven days of sunitinib treatment resulted in a 55% homogeneous decrease in $^{18}$F-FDG uptake compared with non-treated animals. When sunitinib treatment was stopped, $^{18}$F-FDG uptake on day 14 was slightly higher than on day 7.

$^{15}$O-water PET in A2780 tumors showed a 3-8 fold lower uptake at baseline than well perfused organs such as heart and kidneys, resulting in suboptimal tumor visualization (Fig. 1C). Further, $^{15}$O-water PET tumor uptake at baseline was 15.8% higher in the center compared with the tumor rim. By day 7 of sunitinib treatment, tumor uptake tended to
decrease in the center compared to baseline (-16.8 ± 7.7%), though not significantly (P = 0.11) and no difference between tumor rim and center was observed.

Molecular tumor imaging using $^{89}$Zr-ranibizumab PET. $^{89}$Zr-ranibizumab was first evaluated in the SKOV-3 model. Within 3 hours pi of $^{89}$Zr-ranibizumab clear tumor visualization was seen (Fig. 2A), with a plateau at 24 h (Fig. 2B). At 24 h, $^{89}$Zr-ranibizumab tumor uptake was significantly 2.54 fold higher than $^{89}$Zr-Fab-IgG (3.96 ± 1.00 %ID/g versus 1.56 ± 0.38 %ID/g, P = 0.034), signifying VEGF-A specificity of $^{89}$Zr-ranibizumab uptake. Increasing doses of unlabeled ranibizumab, blocked tumor uptake of $^{89}$Zr-ranibizumab to the uptake level of $^{89}$Zr-Fab-IgG was reached (Fig. 2B). Furthermore, organ biodistribution revealed rapid blood clearance of $^{89}$Zr-ranibizumab from 8.44 ± 2.19 %ID/g 1 h pi to 0.38 ± 0.38 %ID/g 24 h pi resulting in tumor/blood ratios higher than 10 (supplement 3).

Sunitinib decreases $^{89}$Zr-ranibizumab tumor uptake, especially in the tumor center, with a marked rebound after discontinuation. In the next experiments $^{89}$Zr-ranibizumab quantification was performed 24 hours pi unless otherwise stated. At baseline, $^{89}$Zr-ranibizumab uptake was 8.75% lower in the tumor rim than in the center (P = 0.05), comparable to findings seen with $^{15}$O-water PET. In A2780 placebo treated mice, $^{89}$Zr-ranibizumab (%ID/g) tumor uptake remained constant between baseline and 1 week (Δ 8%, P = 0.229). However after 7 days of sunitinib treatment in A2780 and Colo205 xenografts, there was a pronounced reduction in $^{89}$Zr-ranibizumab tumor uptake in the tumor center but minimal effect in the tumor rim, whereas $^{18}$F-FDG decreased homogeneously (Fig. 2D). In A2780 tumors, $^{89}$Zr-ranibizumab uptake decreased by only 19.5 ± 5.1% in the rim, whereas a decrease of 45.4 ± 5.9% was found in the center compared with baseline (Fig. 3B). Control experiments with $^{89}$Zr-Fab-IgG, showed only a minimal decrease in uptake at day 7 following
sunitinib treatment in A2780 tumors, indicating minimal changes in non-specific uptake of Fab fragments following sunitinib treatment. In contrast, decreased $^{89}$Zr-ranibizumab uptake was 3.2 fold greater than control $^{89}$Zr-Fab-IgG experiments (Fig. 3D). Interestingly, in A2780 tumors treated for 14 days, $^{89}$Zr-ranibizumab uptake in the rim increased 46% at day 14 compared with day 7 (Fig. 3C), while in the center the uptake remained low compared to baseline.

PET scans performed 7 days after stopping sunitinib treatment showed higher $^{89}$Zr-ranibizumab tumor uptake in both models, which exceeded baseline values. In A2780, $^{89}$Zr-ranibizumab tumor rim uptake increased 69.5 ± 18.3% versus 7 days sunitinib, and 34.6 ± 11.4% ($P = 0.056$) versus baseline (Fig. 3B). Likewise, uptake in the tumor center increased and returned to baseline. Also in Colo205 tumors, $^{89}$Zr-ranibizumab uptake in the tumor center exceeded baseline values (31.7 ± 9.9%, $P = 0.033$) 7 days after discontinuation (supplement 4 and 5). Control experiments using $^{89}$Zr-Fab-IgG showed some enhancement in passive tumor uptake, but were 2.83 fold lower than with $^{89}$Zr-ranibizumab.

Differential tumor response on $^{89}$Zr-ranibizumab-PET scan between tumor rim and center corresponds with microscopic changes. Histological examination also revealed a differential effect of sunitinib on the tumor rim and center. After 7 days of sunitinib treatment, non-affected blood vessels were mainly present in the tumor rim. Tumor tissue surrounding these vessels retained a high proliferation rate, and low HIF1$\alpha$ and GLUT-1 expression (Fig. 4A). Tumor tissue surrounding affected vessels in the center showed a low tumor proliferation rate, high HIF1$\alpha$ and GLUT-1 expression. The center contained predominantly vital areas with some areas of necrosis (supplement 7). Seven days of sunitinib treatment resulted in a reduction in all vascular markers and decreased tumor cell proliferation, which
was most pronounced in the tumor center (supplement 8). Interestingly, at day 14 the tumor blood vessel diameter returned to baseline and increased tumor proliferation rate was observed in the tumor rim, which coincided with increased VEGF-A staining. Interestingly, no changes in vascular markers or tumor and endothelial proliferation were observed in the tumor center compared to day 7.

After a 7 day drug-free period, all vascular makers recovered and tumor vessel diameter even exceeded baseline. In addition, HIF1α and Glut-1 expression decreased while an increased VEGF-A expression and tumor proliferation were observed (Fig. 4 and Fig. 5). Furthermore, large areas of randomly distributed multiple blood-filled regions (pelioses) were present at baseline and after discontinuation in parallel with high endothelial proliferation (Fig. 4D). Together these findings indicate rapid tumor revascularization after stopping sunitinib, which closely matches *in vivo* PET findings.

*Changes in tumor derived human plasma VEGF levels.* After 7 days of sunitinib treatment, tumor derived human VEGF plasma levels decreased by 60% (Fig. 5B), while after 14 days of sunitinib treatment there was a 6.61 fold increase compared with day 7. After the 7 day drug-free period a 4.79 fold increase was observed.
Discussion

This study is the first to investigate the effect of sunitinib treatment and its withdrawal on tumor biology using the molecular imaging techniques $^{89}$Zr-ranibizumab-PET, $^{18}$F-FDG PET and, $^{15}$O-water PET in human xenograft tumor models. $^{89}$Zr-ranibizumab-PET corresponded best with findings observed by tumor proliferation and vascularization assays, histology and immunohistochemistry. Strikingly, sunitinib treatment caused a clear decline in $^{89}$Zr-ranibizumab-PET signal in the tumor center, with only minimal reduction of uptake in the tumor rim, with a pronounced rebound after discontinuation. These findings demonstrate that sunitinib treatment effectively inhibits proliferation, neo-vascularization and VEGF signaling and secondly that $^{89}$Zr-ranibizumab-PET is a valuable molecular imaging technique with utility as a biomarker for the determination of intratumoral VEGF status and/or angiogenesis.

Currently MRI and PET imaging, are used to visualize anti-angiogenic treatment effects mainly by measuring changes in blood flow and permeability (15-17). Preclinically, MRI demonstrated decreased transfer constant $K_{\text{trans}}$ following anti-VEGF treatment in a xenografts model, especially in the tumor center (18). High variability in MRI results during follow up of anti-angiogenic treatment hampers the predictive value for individual patients (19, 20). PET imaging has a higher sensitivity compared with MRI and allows whole body imaging (21, 22). In this study, we have demonstrated the utility of $^{89}$Zr-ranibizumab-PET as a valuable imaging biomarker of the VEGF pathway. Interestingly, if used in patients this PET tracer could also provide insight in changes in VEGF levels in normal organs during sunitinib treatment, which might influence distant invasion after treatment with sunitinib (3).
Rapid blood clearance of $^{89}$Zr-ranibizumab and VEGF driven tissue uptake resulted in high tumor to background ratios which enables more rapid tumor follow up with maximal uptake of $^{89}$Zr-ranibizumab in the tumor within 24 hours post injection compared to 4 days for $^{89}$Zr-bevacizumab. Relatively high kidney uptake of $^{89}$Zr-ranibizumab, as seen with other metal labeled proteins, is a consequence of glomerular filtration of the tracer followed by tubular reabsorption and subsequent lysosomal degradation (23, 24). $^{89}$Zr-ranibizumab binds with 20-100 fold higher affinity compared to bevacizumab to all human VEGF-A isoforms, including matrix bound isoforms (6). Most likely, $^{89}$Zr-ranibizumab binds like bevacizumab in vivo to VEGF located on the cell surface and the extra cellular matrix as shown by Stollman et al. using $^{111}$In-bevacizumab in VEGF$^{165}$ and VEGF$^{189}$ over expressing melanoma xenografts (25). Moreover, changes in $^{89}$Zr-ranibizumab tumor uptake were 3 fold higher compared to control $^{89}$Zr-Fab-IgG, indicating VEGF mediated tumor uptake. One of our most important findings however, is that $^{89}$Zr-ranibizumab tumor uptake can be followed dynamically within the tumor at different time points during and after sunitinib treatment.

$^{89}$Zr-ranibizumab does not bind to murine VEGF and therefore gives no information about the contribution of mouse VEGF to tumor angiogenesis in our xenografts model. However, our results corresponded highly with the angiogenic state of the tumors shown by other assays, indicating human VEGF to be largely responsible for the angiogenesis effects observed. There was a striking difference between tumor rim and center as uptake demonstrated with VEGF-PET. In concordance with reduced tumor growth, sunitinib treatment resulted in a decrease of angiogenic markers like microvessel density (MVD), as seen in other tumor models (2), vessel diameter, VEGFR2 expression. Further, reduced endothelial proliferation and the absence of pelioses showed true inhibition of angiogenesis.
The inhibition of angiogenesis was also associated with high HIF1α and Glut-1 expression and decreased Ki67 staining, indicating increased hypoxia and cellular stress and reduced tumor cell proliferation. All effects were more pronounced in the center than in the tumor rim, as highlighted by $^{89}$Zr-ranibizumab-PET. The $^{89}$Zr-ranibizumab-PET signal is the sum of perfusion of the tracer into the tumor followed by binding to VEGF, it is therefore a resultant of changed perfusion, MVD and VEGF expression, reflecting VEGF biodistribution and bioavailability and allowing in vivo insight in overall tumor angiogenesis.

VEGF-A staining was performed with an antibody which binds to human all VEGF-A isoforms and to a lesser extent to murine VEGF. The VEGF-A expression, was less intense in the center although we had expected an increase as a result of hypoxia caused by VEGFR inhibition. When we analyzed the plasma level of tumor derived human VEGF in these mice, we observed an initial decrease after 7 days of sunitinib treatment, which matches the results assessed with $^{89}$Zr-ranibizumab-PET and underlines the striking finding of initial drop in VEGF expression by tumor cells during sunitinib treatment that has not been reported before. A potential explanation for the initial decrease of VEGF could be an off target effect of sunitinib. Apart from blocking VEGFR signaling, sunitinib binds over seventy other kinases (26). For instance, it inhibits STAT3, an activator of VEGF transcription, which could explain the initial VEGF reduction (27, 28). Ebos et al. measured already elevated human plasma VEGF levels after 1 week sunitinib treatment in a PC-3 xenograft model (13). We however observed just after 2 weeks of sunitinib an enhanced VEGF-PET signal in the tumor rim, which corresponded microscopically with more intense VEGF-A staining, a raise in blood vessel diameter, tumor cell proliferation and raise in plasma VEGF levels. These results suggest transient anti-tumor effects as well as dynamic changes in the VEGF pathway.
resulting in tumor adaptation to sunitinib treatment. These data are in concordance with vessel normalization already 8 days following initiation of the pan-VEGFR-TKI cediranib in a glioblastoma model (19, 29). These results demonstrate the need for non-invasive follow up in continuous as well as intermittent dosing schedules in the clinic to understand the effect of anti-angiogenic treatment.

An explanation for the differences observed between the tumor rim and center might be that tumor cells located at the tumor rim are likely not only dependent on tumor blood vessels, but also benefit from adjacent peritumoral blood vessels. Indeed, we did not observe changes in peritumoral blood vessels during sunitinib (supplement 6), while others even observed a large increase in peritumoral blood vessel diameter following cediranib in their glioblastoma model (19). Taken together, our findings emphasize that differences exist in response to anti-angiogenic therapy among tumor areas. The interest for biological processes occurring at the edge of the tumor is also attracted by the phenomenon of epithelial mesenchymal transition (EMT) in tumors. EMT contributes to tumor progression and enhances the metastatic potential of tumor cells. It has been proposed that induction of hypoxia is one of the instigators (3, 30, 31). Indeed, sunitinib has showed to increase invasiveness at the tumor boundary and enhanced the forming of metastases (3). VEGF-PET enables non-invasive follow up of this process in the primary tumor as well as in all metastatic lesions.

The strong rebound in $^{89}$Zr-ranibizumab-PET signal and rapid tumor regrowth after sunitinib discontinuation reflects what is seen in the clinic. Indeed, comparably to our findings preclinical immunohistochemical analyses have demonstrated rapid revascularization after stopping the VEGFR-TKI AG-013736 (32). Moreover, regrowth of lymph node metastases and
flare up of clinical symptoms have been reported during the sunitinib free period or rapidly after discontinuation (33, 34).

In our study, the clinically most frequently used tracer $^{18}$F-FDG did not reflect intratumoral differences or rapid tumor revascularization and regrowth after stopping sunitinib. Furthermore, the homogeneous reduction in $^{18}$F-FDG uptake did not correspond with the vitality seen with bioluminescence imaging and histology. In addition, no differences between tumor rim and center were observed in the present study with $^{15}$O-water after sunitinib despite the clinical feasibility of $^{15}$O-water PET to assess baseline tumor perfusion status (35). $^{15}$O-water PET performance is partly hampered by a relative low tumor perfusion compared to well perfused organs. For example, in patients 2-fold lower perfusion rates were found in primary renal cell cancers versus normal kidneys (36).

The newly developed VEGF tracer can be clinically used and translate preclinical findings. Recently the first clinical trials have been initiated to visualize VEGF. $^{111}$In-bevacizumab visualized all known melanoma lesions in a feasibility study, even very small (1 cm) lesions (37). At this moment serial $^{89}$Zr-bevacizumab VEGF-PET scans are performed to study the role of these scans during anti-angiogenic treatment in renal cell carcinoma patients (38). When more rapid insight in the dynamic changes in VEGF response in the tumors is required at shorter intervals $^{89}$Zr-ranibizumab offers an additional investigative opportunity.

In conclusion, $^{89}$Zr-ranibizumab-PET allows non-invasive dynamic and spatial in-vivo visualization and quantification of VEGF signaling. $^{89}$Zr-ranibizumab-PET therefore has potential use for preclinical and clinical follow-up, guidance of new treatment strategies, optimal dose finding and exploration of drug combinations, to increase the benefit of anti-angiogenic therapies.
Conflict of interest: The authors declare that no conflict of interest exists.

Acknowledgments

We would like to thank TH Oude Munnink, T Jones, EME van Straten and M Green for technical assistance and helpful comments on the manuscript.

Grant support

A personal grant to WB Nagengast and grants RUG 2007-3739 and RUG 2009-4273 from the Dutch Cancer Society.
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38 Clinical Trial Registration Database; www.clinicaltrials.gov, registration number NCT00831857.
Legends to the figures

Figure 1  A. Tumor volumes of A2780 and Colo205 treated tumors. Tumor volumes were normalized to the start of sunitinib treatment. B. Representative coronal $^{18}$F-FDG microPET images of non-treated A2780 bearing mice, following 1 week of sunitinib treatment and after 1 week of sunitinib treatment plus a stop week (Non-paired animals are presented) and $^{18}$F-FDG uptake quantification in control A2780 treated tumors, after 1 week of sunitinib treatment and after 1 week of sunitinib treatment plus a stop week. C. Representative fused transversal and coronal $^{15}$O-water PET/CT image after 1 week sunitinib in A2780 bearing mice and normalized $^{15}$O-water PET quantification at baseline and following 1 week of sunitinib. * $P < 0.05$, ** $P < 0.01$. Data presented as SEM.

Figure 2  A. Representative transversal and coronal microPET images of $^{89}$Zr-ranibizumab 1, 3, 6 and 24 hours pi in SKOV-3 xenograft. B. Relative tumor uptake versus injected protein dose of $^{89}$Zr-ranibizumab in SKOV-3 xenograft model (dotted line shows $^{89}$Zr-Fab-IgG tumor uptake) and time distribution curve of $^{89}$Zr-ranibizumab uptake in SKOV-3 xenograft model. C. Transversal and coronal microPET image of $^{18}$F-FDG (1 h pi) and $^{89}$Zr-ranibizumab (24 hours pi) at baseline in A2780 xenograft (paired sample) with clear tumor visualization. D. Following 1 week sunitinib $^{18}$F-FDG uptake in the tumor decreases homogeneously whereas $^{89}$Zr-ranibizumab uptake is still high in the tumor rim (paired sample). Tumor indicated by arrow.

Figure 3  A. Transversal and coronal microPET images of $^{89}$Zr-ranibizumab at 24 hours post injection of the tracer. High tumor to background ratios clearly visualize a more pronounced
reduction in the tumor center compared with the tumor rim after 7 day of sunitinib treatment. After 7 days of discontinuation, $^{89}$Zr-ranibizumab uptake increases (paired samples). At day 14 of sunitinib treatment, $^{89}$Zr-ranibizumab uptake returns to baseline in the tumor rim while remaining low in the tumor center. B. $^{89}$Zr-ranibizumab quantification of tumor rim and center of mice treated with sunitinib for 1 week at day 7 and following a stop week and after 14 days (C) of sunitinib treatment. Individual tumor uptake values are normalized relative to baseline. D. Change in average tumor uptake (both rim and center) of $^{89}$Zr-ranibizumab and $^{89}$Zr-Fab-IgG after 7 days of sunitinib and after a stop week in A2780 xenograft model. * $P < 0.05$, ** $P < 0.01$. Data presented as SEM.

Figure 4 A. Representative examples of A2780 illustrating affected and non-affected tumor vessels following 7 days of sunitinib treatment. Non-affected vessels demonstrate clear vWF staining with low Glut-1 expression, high Ki67 staining and low HIF1$\alpha$ expression in the surrounding tumor tissue which is the opposite for affected tumor vessels. B. Tumor vessel diameter of tumor rim and center and MVD quantification of the tumor rim and center. C. Representative VEGF-A staining. D. Percentage of tumor vessels containing Ki67 positive endothelial cells in tumor rim and center. * $P < 0.05$, ** $P < 0.01$. Data presented as SEM.

Figure 5 A. VEGFR2 staining in the tumor rim and center. B. Human VEGF plasma levels of sacrificed A2780 bearing animals. C. Tumor proliferation rate at the tumor rim and center. After 7 days of sunitinib overall tumor proliferation decreases, though it remains high at the rim. D. Tumor HIF1$\alpha$ expression is high after 1 and 2 weeks of sunitinib treatment. After the
stop week, HIF1α decreases towards baseline. * $P < 0.05$, ** $P < 0.01$. Data presented as SEM.
VEGF-PET imaging is a non-invasive biomarker showing differential changes in the tumor during sunitinib treatment

Wouter B. Nagengast, Marjolijn N. Lub-de Hooge, Sjoukje F. Oosting, et al.

Cancer Res Published OnlineFirst November 17, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-1088

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/11/17/0008-5472.CAN-10-1088.DC1

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