Monitoring Tumor Response to Linifanib Therapy with SPECT/CT Using the Integrin $\alpha_v\beta_3$ -Targeted Radiotracer ^{99m}Tc -3P-RGD₂

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ABSTRACT

The objective of this study was to determine the utility of ^{99m}Tc -3P-Arg-Gly-Asp (RGD₂) single photon emission computed tomography (SPECT)/computed tomography (CT) for noninvasive monitoring of integrin $\alpha_v\beta_3$ -expression response to antiangiogenic treatment with linifanib. Linifanib or vehicle therapy was carried out in female athymic *nu/nu* mice bearing U87MG glioma (high $\alpha_v\beta_3$ expression) or PC-3 prostate (low $\alpha_v\beta_3$ expression) tumors at 12.5 mg/kg twice daily. The average tumor volume was $180 \pm 90 \text{ mm}^3$ the day prior to baseline SPECT/CT. Longitudinal ^{99m}Tc -3P-RGD₂ SPECT/CT imaging was performed at baseline (–1 day) and days 1, 4, 11, and 18. Tumors were harvested at all imaging time points for histopathological analysis with H&E and immunohistochemistry. A significant difference in tumor volumes between vehicle- and linifanib-treated groups was observed after

4 days of linifanib therapy in the U87MG model. The percent injected dose (%ID) tumor uptake of ^{99m}Tc -3P-RGD₂ peaked in the vehicle-treated group at day 11, while the %ID/cm³ tumor uptake decreased slowly over the whole study period. During the first 2 days of linifanib treatment, a rapid decrease in both %ID/cm³ tumor uptake and tumor/muscle ratios of ^{99m}Tc -3P-RGD₂ was observed, followed by a slow decrease until day 18. No decrease in tumor uptake of ^{99m}Tc -3P-RGD₂ or tumor volume was observed for either treatment group in the PC-3 model. Changes in tumor vasculature were confirmed by histopathological H&E analysis and immunohistochemistry. Longitudinal imaging using ^{99m}Tc -3P-RGD₂ SPECT/CT may be a useful tool for monitoring the downstream biologic effects of linifanib therapy.

Introduction

Inhibiting angiogenesis is a promising strategy for cancer therapy (Dunn et al., 2000; Bergers and Benjamin, 2003; Bergers et al., 2003; Ferrara and Kerbel, 2005). As antiangiogenic therapies have become more common, finding suitable translational biomarkers for antiangiogenic modulation of the tumor vasculature has become more important (Cao et al., 2011). Microvessel density has been proposed as a prognostic indicator of progression, overall survival, and disease-free survival in cancer patients. Evaluation of microvessel density is typically performed by immunostaining endothelial cells in tumor tissues and counting the number of vessels using a high power field microscope. This approach is not practical for routine monitoring of antiangiogenic therapy due to the invasive nature of the procedure. Dynamic contrast-enhanced

magnetic resonance imaging (DCE-MRI) has been used to measure perfusion properties of tumors, but this method can be technically challenging and standardization is complex (Jeswani and Padhani, 2005; O'Connor et al., 2007). Positron emission tomography (PET) and single photon emission computed tomography (SPECT) tracers have been developed to evaluate expression of certain integrins, particularly $\alpha_v\beta_3$, that are involved in angiogenesis. The need for a cyclotron and lack of simple kit preparation presents a significant challenge for current ^{18}F -labeled radiotracers for more routine imaging of integrin expression. Therefore, there is an unmet need for radiotracers that are readily available and clinically useful for early detection of integrin $\alpha_v\beta_3$ -positive tumors and metastases and for monitoring antiangiogenic therapy.

Integrins are receptors involved in cell adhesion and migration of endothelial cells (Desgrosellier and Cheresh, 2010). It is known that the cross-talk between receptor tyrosine kinases and integrin receptors is crucial for many cellular functions. Interactions between $\alpha_v\beta_3$ and vascular endothelial growth factor receptor (VEGFR)/platelet-derived growth factor receptor (PDGFR) seem to be particularly important for vascularization (Cybulsky et al., 1994; Jones et al., 1997; Heldin et al., 1998; Woodard et al., 1998; Clemmons et al., 1999;

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ABBREVIATIONS: %ID, percent injected dose; CT, computed tomography; ECM, extracellular matrix; HYNIC, 6-(2-(2-sulfonatobenzaldehyde)hydrazono)nicotiny; PDGFR, platelet-derived growth factor receptor; PET, positron emission tomography; PEG, polyethylene glycol; RGD, Arg-Gly-Asp tripeptide sequence; SPECT, single photon emission computed tomography; T/M, tumor-to-muscle; TPPTS, trisodium triphenylphosphine-3,3',3''-trisulfonate; VEGFR, vascular endothelial growth factor receptor.

Soldi et al., 1999; Borges et al., 2000; Mahabeleshwar et al., 2007; Somanath et al., 2009). This relationship regulates many cellular activities during angiogenesis, including endothelial cell migration, survival, and tube formation, and hematopoietic cell functions within the vasculature (Somanath et al., 2009; Desgrosellier and Cheresch, 2010). Thus, monitoring $\alpha_v\beta_3$ expression is of interest for antiangiogenic therapies.

Many extracellular matrix proteins interact with $\alpha_v\beta_3$ via the Arg-Gly-Asp (RGD) tripeptide sequence. Over the last several years, there has been interest in radiolabeled multimeric cyclic RGD peptides as radiotracers for tumor imaging by SPECT or PET (Shi et al., 2009a,b, 2011; Chakraborty et al., 2010; Zhou et al., 2012a). ^{99m}Tc -3P-RGD₂ is a ^{99m}Tc -labeled cyclic RGD peptide dimer. It is readily prepared from $^{99m}\text{TcO}_4^-$ with high specific activity using a kit formulation, making it easily accessible (Wang et al., 2009; Jia et al., 2011). In several preclinical models, ^{99m}Tc -3P-RGD₂ showed high tumor uptake, rapid renal clearance, and high metabolic stability (Wang et al., 2009; Jia et al., 2011; Zhou et al., 2011). ^{99m}Tc -3P-RGD₂ tumor uptake measured by percent injected dose (%ID)/cm³ and tumor-to-muscle (T/M) ratio had a linear correlation with tumor β_3 and CD31, a marker for tumor blood vessels (Zhou et al., 2011, 2012a). ^{99m}Tc -3P-RGD₂ SPECT/computed tomography (CT) was also used for quantification of tumor radioactivity accumulation and delineation of tumor necrotic regions (Zhou et al., 2012b; Shao et al., 2013). It is currently under clinical investigation as a new SPECT radiotracer for imaging carcinomas of breast and lung in cancer patients (Ma et al., 2011; Zhu et al., 2012).

Linifanib (ABT-869) is a multitargeted receptor tyrosine kinase inhibitor, specifically targeting VEGF and PDGF receptors (Albert et al., 2006; Zhou et al., 2009; Jiang et al., 2011). In vitro, linifanib was shown to inhibit the phosphorylation of members of the VEGF and PDGF receptor families (Shankar et al., 2007; Hernandez-Davies et al., 2011). Treatment with linifanib resulted in pronounced regression of tumors in vivo in a variety of preclinical tumor models (Albert et al., 2006). Phase II studies showed promising results in breast cancer, metastatic non-small cell lung cancer, and liver cancer (Tannir et al., 2011). Here we determined the utility of imaging ^{99m}Tc -3P-RGD₂ tumor uptake as a biomarker for antiangiogenic treatment with linifanib in the U87MG glioma xenograft model, which has a high vessel density and $\alpha_v\beta_3$ expression, and the PC-3 prostate cancer model, which has low vessel density and $\alpha_v\beta_3$ expression.

Materials and Methods

Chemicals and Analytical Method. Trisodium triphenylphosphine-3,3',3''-trisulfonate (TPPTS) and tricine were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. HYNIC-3P-RGD₂ (HYNIC, 6-(2-(2-sulfonatobenzaldehyde)hydrazono)nicotiny); 3P-RGD₂, polyethylene glycol (PEG)₄-E [PEG₄-c(RGDfK)]₂; and PEG₄, 15-amino-4,7,10,13-tetraoxapentadecanoic acid) were prepared according to previously reported methods (Wang et al., 2009; Jia et al., 2011). Na $^{99m}\text{TcO}_4$ was obtained from Cardinal HealthCare (Chicago, IL). The radio-high-performance liquid chromatography method used a LabAlliance system (Scientific Systems, Inc., State College, PA) equipped with a β -ram IN-US detector and Zorbax C₁₈ column (4.6 mm $\times$ 250 mm, 300-Å pore size) with a flow rate of 1 ml/min. The mobile phase was isocratic with 90% solvent A (25 mM NH₄OAc buffer, pH = 5.0) and 10% solvent B (acetonitrile)

at 0–5 minutes, followed by a gradient mobile phase from 10% B at 5 minutes to 40% B at 20 minutes.

^{99m}Tc -3P-RGD₂ Synthesis and Dose Preparation. ^{99m}Tc -3P-RGD₂ was prepared according to a previously published method (Wang et al., 2009; Jia et al., 2011) using a lyophilized kit formulation prepared in-house, which contains 20 μg HYNIC-3P-RGD₂, 5 mg TPPTS, 6.5 mg tricine, 40 mg mannitol, 38.5 mg disodium succinate hexahydrate, and 12.7 mg succinic acid. ^{99m}Tc -labeling was accomplished by adding 1–1.5 ml of Na $^{99m}\text{TcO}_4$ solution (1110–1850 MBq) to the HYNIC-3P-RGD₂ mixture. The reconstituted vial was then heated at 100°C for 30 minutes. The resulting solution was analyzed by radio-high-performance liquid chromatography. The radiochemical purity was >90%. Doses were prepared by dissolving the radiotracer in saline to a concentration of 370–525 MBq/ml for imaging studies.

Animal Models. The animal protocol was reviewed and approved by the Purdue University Animal Care and Use Committee and by the AbbVie Institutional Animal Care and Use Committee. All studies were conducted in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities. U87MG and PC-3 cell lines were obtained from American Type Culture Collection (Manassas, VA). U87MG cells were cultured in the minimum essential medium while PC-3 cells were cultured in the F-12 medium [Gibco (Life Technologies), Grand Island, NY]. All cells were supplemented with 10% fetal bovine serum (American Type Culture Collection, Manassas, VA) and 1% penicillin and streptomycin solution (Gibco Industries Inc., Langley, OK) at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were grown as a monolayer and were harvested or split when they reached 90% confluence to maintain exponential growth. Female athymic *nu/nu* mice were purchased from Harlan (Indianapolis, IN) at 4–5 weeks of age. Each mouse was implanted subcutaneously near the shoulder with 5×10^6 cells. The tumor volume was measured every 2 days for the first 2 weeks, and every day for the next 3 weeks with digital calipers. The tumor volume was calculated using the formula:

$$\text{Tumor Volume} = \frac{\text{Length} \times \text{Width}^2}{2} \quad (1).$$

Treatment Protocol. Groups ($n = 8$ –10) were size-matched with an average tumor volume of $180 \pm 90 \text{ mm}^3$ 1 day before baseline SPECT/CT imaging. This occurred 3 weeks after inoculation for U87MG tumors and 4 weeks after PC-3 inoculation. Vehicle [0.15% hydroxypropylmethyl cellulose, 2% ethanol, 5% Tween 80, 20% PEG400, 73% saline] or linifanib treatment was initiated at a dose of 12.5 mg/kg on day 0 after baseline imaging. Linifanib (Abbott Laboratories, Abbott Park, IL) was dosed orally twice daily, with a time interval of >8 hours between consecutive doses.

Imaging Protocol for SPECT/CT. Longitudinal SPECT/CT imaging was performed at baseline (–1), 1, 4, 11, and 18 days after treatment initiation using a micro-SPECT-II/CT scanner (Milabs, Utrecht, The Netherlands) equipped with 0.6-mm multipinhole collimators. One hour prior to SPECT/CT imaging, 37–55.5 MBq ^{99m}Tc -3P-RGD₂ in 0.1–0.2 ml of saline was administered intravenously via the lateral tail vein. Linifanib was dosed approximately 2 hours prior to SPECT/CT imaging. Anesthesia was induced using an airflow rate of 350 ml/min and approximately 3.0% isoflurane. After induction of anesthesia, the airflow rate was reduced to 250 ml/min with approximately 2.0% isoflurane. Animals were maintained at 37°C during image acquisition. SPECT images were acquired using 75 projections over 30 minutes. After SPECT acquisition, CT imaging was performed using “normal” acquisition settings (2-degree intervals) at 45 kV and 500 μA .

Image Reconstruction and Data Processing. SPECT images were reconstructed using pixel-based ordered subset expectation maximization (POSEM) algorithm with 6 iterations and 16 subsets. CT data were reconstructed using a cone-beam filtered back-projection algorithm (NRecon v1.6.3; Skyscan). After reconstruction, the SPECT and CT data were automatically co-registered and

resampled to equivalent voxel sizes. Co-registered images were further rendered and visualized using PMOD software (PMOD Technologies, Zurich, Switzerland). A 3D-Gaussian filter (0.8 mm full-width half-maximum) was applied to smooth noise and the look-up tables were adjusted for good visual contrast. Reconstructed images were visualized as both orthogonal slices and maximum intensity projections. All images presented are normalized for injected dose for visual comparison.

Radioactivity Quantification. Radiation sources of known radioactivity were imaged and reconstructed using the same scanning protocol described above. A standard curve was generated to correlate the pixel intensities in the reconstructed images to the radioactivity as measured by a γ -counter. The regions of interest were drawn manually to cover the entire tumor based on transverse view of the CT image. For tumor delineation with SPECT, a threshold of 50% or more of the maximum pixel value on the SPECT image was chosen. Tumor volume and radioactivity counts were generated by using the PMOD software and the amount of radioactivity in each tumor was calculated according to the above mentioned standard curve. Tumor uptake of ^{99m}Tc -3P-RGD₂ was expressed as %ID and %ID/cm³. Reference regions of interest were drawn over muscle as background radioactivity for T/M ratio calculations.

Protocol for Tumor Immunostaining. Tumors were cut into two pieces for immunostaining and H&E staining ($n = 3$ –5 tumors per time point). After harvesting tumors, the tumor sections for immunostaining were immediately snap-frozen in optical cutting temperature solution (Sakara, Torrance, CA). Tumors were then cut into 5- μm slices. After thorough drying at room temperature, slides were fixed with ice-cold acetone for 10 minutes, and air dried for 20 minutes at room temperature. The sections were blocked with 10% goat serum (Jackson ImmunoResearch Inc., West Grove, PA) for 30 minutes at room temperature and then incubated with hamster anti-integrin β_3 antibody (1:100; BD Biosciences, San Jose, CA) and rat anti-CD31 antibody (1:100; BD Biosciences) for 1 hour at room temperature. A β_3 antibody was chosen to represent $\alpha_v\beta_3$ because the only other integrin with a β_3 subunit besides $\alpha_v\beta_3$ is expressed on platelets. The majority of β_3 in the tumor sections is likely to be on the vasculature, and tumor cells (Shattil, 1995). After incubating with Cy3-conjugated goat antihamster (1:100; Jackson ImmunoResearch Inc.) and fluorescein isothiocyanate-conjugated goat anti-rat secondary antibodies (1:100; Jackson ImmunoResearch Inc.), the sections were washed with phosphate-buffered saline. Fluorescence was visualized with a Nikon fluorescence microscope (Nikon Instruments, Melville, NY).

Histopathological H&E Staining. Histopathological analysis was performed by H&E staining of tumors according to previously published methods (Zhou et al., 2011). Briefly, all the tissues were

fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin and 4- μm sections were deparaffinized and rehydrated through graded alcohols. Sections were stained for H&E to evaluate the morphology and then examined under light microscope. Aperio's ImageScope Viewer (Vista, CA) was used to visualize the whole-slide digital scans and capture images.

Statistical Analysis. All data were expressed as the mean plus or minus the standard error of the mean. Statistical analyses were performed by two-way analysis of variance followed by the Newman-Keuls test for multiple comparisons to compare treatment groups. The level of significance was set at $P < 0.05$. A one-way analysis of variance was performed to determine changes over time. GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) was used for linear and nonlinear regression analysis.

Results

In this study, ^{99m}Tc -3P-RGD₂ SPECT/CT was used to monitor linifanib therapy in the xenografted U87MG glioma and PC-3 prostate cancer models. ^{99m}Tc -3P-RGD₂ was prepared in high yield and radiochemical purity (RCP > 90%) with high specific activity (>185 MBq/ μg or 370 GBq/ μmol). Linifanib and ^{99m}Tc -3P-RGD₂ were well tolerated in all studies.

Linifanib Therapy in the U87MG Model. Figure 1A compares the tumor volumes for both vehicle- ($n = 5$ –8) and linifanib- ($n = 5$ –10) treated groups in the U87MG model. There was a significant decrease in tumor volume with linifanib therapy compared with vehicle treatment beginning at day 4 ($P < 0.05$). The %ID tumor uptake of ^{99m}Tc -3P-RGD₂ peaked in the vehicle-treated group ($n = 8$) at day 11 (Fig. 2A), while the %ID/cm³ tumor uptake decreased slowly over the whole study period (Fig. 2B). In the linifanib-treated group ($n = 10$), there was a significant reduction in %ID/cm³ and tumor-to-muscle ratio of ^{99m}Tc -3P-RGD₂ compared with vehicle as early as 24 hours after linifanib treatment ($P < 0.05$), and both remained decreased compared with vehicle at subsequent time points until day 18. The difference between linifanib- and vehicle-treated groups was not significant until day 4 with %ID (Fig. 2A). The reduction in ^{99m}Tc -3P-RGD₂ tumor uptake observed with linifanib treatment is further illustrated by SPECT/CT images in Fig. 3.

Linifanib Therapy in the PC-3 Tumor Model. As shown in Fig. 1B, there was no significant difference in tumor

Fig. 1. Comparison of tumor volumes in vehicle ($n = 8$ for both U87MG and PC-3) and linifanib-treated ($n = 10$ for U87MG and $n = 8$ for PC-3) groups in the U87MG (A) and PC-3 (B) models. Tumor volumes were determined by caliper measurements.

Fig. 2. Comparison of the %ID tumor uptake (A and D), %ID/cm³ tumor uptake (B and E), and tumor/muscle ratios (C and F) of ^{99m}Tc-3P-RGD₂ in vehicle- ($n = 8$ for both U87MG and PC-3) and linifanib-treated ($n = 10$ for U87MG and $n = 8$ for PC-3) groups. Tumor uptake values and T/M ratios were calculated from quantification of SPECT/CT images obtained for U87MG (left) and PC-3 (right) models. Significance is indicated by an asterisk (*).

volumes between vehicle- ($n = 8$) and linifanib-treated ($n = 8$) groups. No significant difference in ^{99m}Tc-3P-RGD₂ tumor uptake was observed between these two groups by %ID (Fig. 2D), %ID/cm³ (Fig. 2E), or T/M ratio (Fig. 2F) at any of the five imaging time points.

Effects of Linifanib Treatment on $\alpha_v\beta_3$ Expression. Figure 4 shows images of histologic tumor slices from both U87MG (upper panel) and PC-3 (lower panel) models. U87MG tumors exhibited a high density of blood vessels while the

PC-3 tumors had a low density of blood vessels prior to linifanib therapy, as indicated by the high integrin β_3 and CD31 expression on U87MG tumors and low expression of β_3 and CD31 on PC-3 tumors prior to starting treatment (Fig. 5). After linifanib therapy, both β_3 and CD31 expression in the U87MG tumors diminished while there was little change in the PC-3 tumors (Fig. 5).

Immunohistochemical staining was performed to explore the expression patterns of CD31 and $\alpha_v\beta_3$ in the U87MG and

Fig. 3. Transverse views of selected SPECT/CT images from athymic nude mice bearing U87MG glioma (top) and PC-3 prostate cancer (bottom) tumors in the vehicle- (upper panel) and linifanib-treated groups (lower panel). SPECT/CT images were obtained at baseline (–1), 1, 4, 11, and 18 days (D) after initiation of linifanib therapy. Tumors at baseline are indicated by arrows.

PC-3 tumors during treatment and to understand the differences in tumor uptake of ^{99m}Tc -3P-RGD₂. Figure 5 shows overlay images of U87MG tumors harvested at –1, 1, 4, 11, and 18 days after initiation of linifanib therapy. Within the first 24 hours after the initiation of linifanib therapy, the main effect was on tumor neovasculature as indicated by the disappearance of β_3 on tumor vasculature. By day 4, β_3 on tumor vasculature disappeared completely while the intensity of β_3 on tumor cells decreased steadily (Fig. 5). By day 18, there was extensive necrosis in tumors from both vehicle- and linifanib-treated groups. Very little CD31 and β_3 expression was detected in necrotic regions, while CD31 and β_3 were highly expressed in the viable region.

Maximal Decrease in $\alpha_v\beta_3$ Expression and Minimal Time to Achieve Maximal Decrease in $\alpha_v\beta_3$ Expression. A slow reduction ($P < 0.05$) compared with baseline in both tumor uptake and T/M ratio of ^{99m}Tc -3P-RGD₂ in the vehicle group

(Fig. 6) was observed during the first 4 days. The tumor-response curve for the vehicle-treated group was modeled by a linear regression with R^2 being 0.921 for the %ID/cm³ tumor uptake and 0.976 for T/M ratios. During the first 4 days after initiation of linifanib therapy, there was a very fast decrease compared with baseline in both the %ID/cm³ tumor uptake (Fig. 6A) and T/M ratios (Fig. 6B) of ^{99m}Tc -3P-RGD₂ followed by a slow decrease over the next 14 days ($P < 0.05$). The cross-point of the two phases indicates the maximal decrease in $\alpha_v\beta_3$ expression and minimal time to achieve the maximum decrease in $\alpha_v\beta_3$ expression for this dose of linifanib in the U87MG model. The tumor-response curve was modeled by an exponential decay, with R^2 being 0.985 for the %ID/cm³ tumor uptake and 0.932 for T/M ratio. The maximal tumor decrease in $\alpha_v\beta_3$ expression was calculated to be $70 \pm 7\%$ from the %ID/cm³ tumor uptake plot and $66 \pm 14\%$ from T/M ratio plot. The calculated time to achieve maximal decrease in $\alpha_v\beta_3$ expression was 1.65 days for the %ID/cm³ tumor uptake plot and 1.52 days for T/M ratio plot (Fig. 6).

Fig. 4. Selected histologic slices (H&E stained) of tumor tissues (U87MG versus PC-3) from animals before treatment (left) and after 18 days (D) of linifanib-treatment (right) groups to illustrate vascular density changes after linifanib therapy.

Discussion

Because growth factors, including VEGF, increase the expression of $\alpha_v\beta_3$ (Distler et al., 2003), inhibition of VEGF and PDGF receptors could lead to decreased $\alpha_v\beta_3$ expression. This makes $\alpha_v\beta_3$ a mechanistically relevant biomarker for assessing downstream biologic effects of linifanib therapy, which specifically targets VEGF and PDGF receptors. Vascular changes in response to linifanib have been previously demonstrated by dynamic contrast-enhanced magnetic resonance imaging preclinically and clinically, but changes in $\alpha_v\beta_3$ expression have not been reported (Wong et al., 2009; Jiang et al., 2011; Tannir et al., 2011; Luo et al., 2012). In this study, decreases in the tumor uptake (%ID/cm³ and T/M ratio) of ^{99m}Tc -3P-RGD₂ were observed prior to tumor volume changes in the U87MG model after linifanib treatment (Fig. 2). No

Fig. 5. Overlay images of U87MG (top) and PC-3 (bottom) tumor tissues after immunohistochemical staining for integrin β_3 and CD31 in vehicle- and linifanib-treated groups to illustrate the changes in integrin β_3 expression level and blood vessel density during therapy. Tumor tissues were harvested at -1, 1, 4, 11, and 18 days (D) after initiation of linifanib therapy. CD31 was used as the marker for tumor blood vessels (both mature and newly formed), which was visualized with fluorescein isothiocyanate (FITC; green color). Integrin β_3 was visualized with Cy3 (red color). The yellow color in overlay images indicates the integrin β_3 expression on new blood vessels. Magnification was $\times 200$ in all images.

significant changes in tumor uptake of ^{99m}Tc -3P-RGD₂ or corresponding tumor volume changes were seen in the PC-3 model (Figs. 1 and 2), indicating a lack of efficacy of linifanib therapy in this animal model. The changes in tumor vasculature were further confirmed by histopathological H&E analysis

(Fig. 4) after the last SPECT/CT imaging time point and further support the use of ^{99m}Tc -3P-RGD₂-SPECT as a biomarker for linifanib. The time to achieve maximal decrease in $\alpha_v\beta_3$ (Fig. 6) expression could be used to provide an indication of early pharmacodynamic response and as a guide to design of biomarker aspects of clinical studies.

The efficacy observed with linifanib therapy in the U87MG model (high $\alpha_v\beta_3$ and CD31 expression) but not in the PC-3 model (low $\alpha_v\beta_3$ and CD31 expression) is consistent with the reciprocal relationship between $\alpha_v\beta_3$ and VEGFR2. Highly vascularized tumors have higher expression levels of $\alpha_v\beta_3$ and VEGFR2 than do poorly vascularized tumors (Provias et al., 1997; Perrone et al., 2004; Tsutsui et al., 2005; Li et al., 2008). However, the predictive value of treatment outcome with initial VEGFR expression has had mixed results (Jain et al., 2009). Nevertheless, $\alpha_v\beta_3$ -expression level should be explored as a patient selection biomarker. Due to the limited number of animal models used in the present study, additional studies in models with intermediate levels of $\alpha_v\beta_3$ expression are warranted to further investigate if ^{99m}Tc -3P-RGD₂ SPECT/CT is useful as a noninvasive biomarker for patient selection.

Linifanib targets multiple tyrosine kinases, of which VEGFR2 and PDGFR directly interact with $\alpha_v\beta_3$. Inhibiting VEGFR2 and PDGFR may cause endothelial cells to undergo apoptosis, which would directly decrease the number of $\alpha_v\beta_3$ -expressing cells. Flt1 and CSF-1 (members of the VEGF and PDGF receptor families, respectively), which are inhibited by linifanib (Albert et al., 2006), regulate the production and activation of macrophages that accelerate the breakdown of the extracellular matrix (ECM). Breakdown of the ECM can cause exposure of sites recognized by $\alpha_v\beta_3$ and can likely allow newly sprouting vessels to interact with the remodeled ECM (Weis and Cheresh, 2011), which may also influence tracer uptake. Decreasing the leakiness of the vasculature by inhibiting VEGFR may also lead to a decrease in tumor uptake of ^{99m}Tc -3P-RGD₂ by limiting the amount available for both specific and nonspecific binding. However, the decrease in $\alpha_v\beta_3$ expression observed post-treatment with linifanib by immunohistochemistry indicates this scenario is less likely.

The contribution from tumor cells and vasculature to the uptake of ^{99m}Tc -3P-RGD₂ is unknown. Because of limitations in spatial resolution, it cannot be directly determined if changes in $\alpha_v\beta_3$ expression are due to changes in neovascularity, tumor

Fig. 6. Biologic response evaluated by tumor time-activity curves for $\% \text{ID}/\text{cm}^3$ (top) and T/M ratios (bottom) of ^{99m}Tc -3P-RGD₂ uptake in the vehicle- ($n = 8$) and linifanib- ($n = 10$) treated groups of the U87MG tumor model. Tumor $\% \text{ID}/\text{cm}^3$ values and T/M ratios were calculated based on SPECT/CT quantification. Curves were fitted using a linear regression model for the vehicle-treated group and a two-phase exponential decay model (nonlinear regression) for the linifanib-treated group.

cells, or both. It was reported that linifanib was able to normalize >75% of tumor vasculature by day 4, as evidenced by the disappearance of leaky microvessels (Jiang et al., 2011). If linifanib therapy had minimal impact on tumor cells compared with vasculature at day 4, it can be estimated that the percentage contribution from neovasculature to the $\alpha_v\beta_3$ expression and tumor uptake of ^{99m}Tc-3P-RGD₂ is ~60% in the U87MG model. This estimation is supported by the fact that the tumor uptake of ^{99m}Tc-3P-RGD₂ (Fig. 2) at day 4 was ~40% of its value at -1 day.

¹⁸F-labeled RGD peptides, such as ¹⁸F-fluciclatide and ¹⁸F-FPPRGD₂ have been used as PET radiotracers to monitor tumor response to antiangiogenic therapy with sunitinib, ZD4190, and paclitaxel preclinically (Morrison et al., 2009; Battle et al., 2011; Sun et al., 2011). Results from this study agree with previously published reports providing evidence for imaging the $\alpha_v\beta_3$ in response to therapy. However, imaging integrin expression has yet to be used to monitor treatment response clinically. Considering the kinetics, biodistribution, radiation dosimetry, and clinical availability, we believe that ^{99m}Tc-3P-RGD₂ has significant advantages over the ¹⁸F-labeled cyclic RGD peptide radiotracers. The linear relationship between T/M ratios of ^{99m}Tc-3P-RGD₂ and expression levels of $\alpha_v\beta_3$ and CD31 (Zhou et al., 2011, 2012a) could potentially make semiquantitative SPECT imaging of ^{99m}Tc-3P-RGD₂ more practical clinically. Therefore, further investigations are warranted to better understand how changes in tumor uptake and T/M ratios of ^{99m}Tc-3P-RGD₂ reflect the tumor response to antiangiogenic treatment before it can be used clinically to monitor investigational therapy.

Conclusion

An early decrease in tumor ^{99m}Tc-3P-RGD₂ uptake, which corresponded to efficacy, was observed with linifanib therapy in the U87MG xenograft glioma model, but not in the PC-3 xenograft model of prostate cancer. Vascular changes in response to linifanib treatment were confirmed by H&E analysis and immunohistochemistry. SPECT/CT imaging with ^{99m}Tc-3P-RGD₂ is a promising method for noninvasive monitoring of early downstream biologic response to linifanib therapy. Additional studies in tumor models with intermediate levels of $\alpha_v\beta_3$ expression are warranted to further verify ^{99m}Tc-3P-RGD₂ SPECT/CT as a screening tool for patient selection and to better understand how changes in tumor uptake reflect tumor response to antiangiogenic treatment before ^{99m}Tc-3P-RGD₂ can be used clinically to monitor investigational therapy.

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Authorship Contributions

Participated in research design: Zhang, Fox, Albert, Luo, Liu, Mudd.

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