Integrin $\alpha_v\beta_3$–Targeted Radiotracer $^{99m}$Tc-3P-RGD$_2$ Useful for Noninvasive Monitoring of Breast Tumor Response to Antiangiogenic Linifanib Therapy but not Anti-Integrin $\alpha_v\beta_3$ RGD$_2$ Therapy

Shundong Ji$^1$, Yumin Zheng$^{1,2}$, Guoqiang Shao$^1$, Yang Zhou$^1$ and Shuang Liu$^1$$^\ast$

1. School of Health Sciences, Purdue University, IN 47907, USA.
2. Department of Nuclear Medicine, China-Japan Friendship Hospital, Beijing, 100029, China.

$^\ast$Corresponding author: Dr. Shuang Liu, School of Health Sciences, Purdue University, 550 Stadium Mall Drive, West Lafayette, Indiana 47907. Phone: 765-494-0236; Fax 765-496-1377; Email: liu100@.purdue.edu.

© Ivyspring International Publisher. This is an open-access article distributed under the terms of the Creative Commons License (http://creativecommons.org/licenses/by-nc-nd/3.0/). Reproduction is permitted for personal, noncommercial use, provided that the article is in whole, unmodified, and properly cited.

Received: 2013.06.24; Accepted: 2013.08.06; Published: 2013.10.12

Abstract

**Purpose:** $^{99m}$Tc-3P-RGD$_2$ is a $^{99m}$Tc-labeled dimeric cyclic RGD peptide that binds to integrin $\alpha_v\beta_3$ with high affinity and specificity. The purpose of this study was to demonstrate the utility of $^{99m}$Tc-3P-RGD$_2$ SPECT/CT (single photon emission computed tomography/computed tomography) as a molecular imaging tool for noninvasive monitoring breast tumor early response to antiangiogenesis therapy with linifanib, and to illustrate its limitations in monitoring the efficacy of anti-$\alpha_v\beta_3$ treatment.

**Methods:** To support SPECT/CT imaging, biodistribution and therapy studies, the xenografted breast cancer model was established by subcutaneous injection of $5 \times 10^6$ MDA-MB-435 cells into the fat pad of each athymic nude mouse. Linifanib (ABT-869) was used as antiangiogenesis agent. The tumor volume was $180 \pm 90$ mm$^3$ on the day (-1 day) before baseline SPECT/CT. Each animal was treated twice daily with vehicle or 12.5 mg/kg linifanib. Longitudinal $^{99m}$Tc-3P-RGD$_2$ SPECT/CT imaging was performed on days -1, 1, 4 and 11. Tumors were harvested at each time point for pathological analysis of hematoxylin and eosin (H&E) and immunohistochemistry (IHC). Tumor uptake of $^{99m}$Tc-3P-RGD$_2$ was calculated from SPECT/CT quantification. When cyclic peptide E[c(RGDfK)]$_2$ (RGD$_2$) was used as the anti-$\alpha_v\beta_3$ agent, SPECT/CT images were obtained only at 7 and 21 days after last RGD$_2$ dose.

**Results:** The tumor uptake of $^{99m}$Tc-3P-RGD$_2$ from SPECT/CT quantification was almost identical to that from biodistribution. There was a dramatic reduction in both %ID and %ID/cm$^3$ tumor uptake of $^{99m}$Tc-3P-RGD$_2$ during the first 24 hours of linifanib therapy. The therapeutic effect of linifanib was on both tumor cells and vasculature, as determined by IHC analysis of integrin $\alpha_v\beta_3$ and CD31. Changes in tumor vasculature were further confirmed by pathological H&E analysis of tumor tissues. While its %ID tumor uptake increased steadily in vehicle-treated group, the %ID tumor uptake of $^{99m}$Tc-3P-RGD$_2$ decreased in linifanib-treated group slowly over the 11-day study period. The degree of tumor response to linifanib therapy correlated well to the integrin $\alpha_v\beta_3$ expression levels before linifanib therapy.

**Conclusion:** $^{99m}$Tc-3P-RGD$_2$ is an excellent radiotracer for monitoring integrin $\alpha_v\beta_3$ expression during and after linifanib therapy. $^{99m}$Tc-3P-RGD$_2$ SPECT/CT is an useful molecular imaging tool for patient selection before antiangiogenic and anti-$\alpha_v\beta_3$ therapy; but it would be difficult to use $^{99m}$Tc-3P-RGD$_2$ for accurate and noninvasive monitoring of early tumor response to anti-$\alpha_v\beta_3$ therapy.

Key words: Linifanib, monitoring antiangiogenic therapy, integrin $\alpha_v\beta_3$, and $^{99m}$Tc-3P-RGD$_2$.

Introduction

Solid tumor cells require higher levels of oxygen and metabolites to support their rapid uncontrolled growth than normal cells [1-5]. This is accomplished by increased angiogenesis at tumor site and is often
seen as the rate limiting step in tumor progression [3, 5]. Targeting tumor angiogenesis with small molecular inhibitors represents a promising strategy for controlling and eradicating tumor growth [6-8]. Linifanib (ABT-869) is a multi-targeted receptor tyrosine kinase inhibitor, specifically targeting vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) receptors [9-14]. In vitro, linifanib was shown to inhibit the phosphorylation of members of VEGF and PDGF receptor families [11]. Treatment with linifanib resulted in pronounced regression of tumors in vivo in a variety of pre-clinical tumor models [12-14]. Clinical studies showed promising results in breast cancer, metastatic non-small cell lung cancer (NSCLC), and liver cancer [15, 16]. As antiangiogenic therapies have become more common, finding suitable translational biomarkers for antiangiogenic modulation of the tumor vasculature has become critically important.

Microvessel density (MVD) has been proposed as a prognostic indicator of progression, overall survival, and disease-free survival in cancer patients [17, 18]. Evaluation of MVD 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 its invasive nature. DCE (dynamic contrast-enhanced) MRI (magnetic resonance imaging) has been used to measure tumor perfusion properties [19-22], but DCE-MRI is technically challenging and standardization is complicated. [18F]FDG PET was used to monitor linifanib therapy by determining reduced metabolic activity in the “treated” tumors [13], but glucose metabolism may not be an ideal biomarker for monitoring antiangiogenic therapy because most of metabolic activities take place in tumor cells rather than tumor vasculature.

Altered integrin αvβ3 expression has been detected in carcinomas of breast [23-25], prostate [26], ovary [27], lung [28], melanomas [29], and gliomas [30, 31]. The integrin αvβ3 expression has been correlated with an aggressive phenotype and metastatic dissemination [32-35]. It was reported that the integrin αvβ3 is overexpressed on both tumor cells and neovascularization in breast cancer xenografts [36], and is an interesting target to develop new imaging probes for early cancer detection [37-39]. It is well-established that the cross-talk between integrins and receptor tyrosine kinases (e.g. VEGFR and PDGFR) is crucial for many cellular functions [40-42]. Integrin αvβ3 and PDGFR-β co-precipitate and PDGFR activation increases endothelial cell migration and proliferation. The functional association between VEGFR2/PDGFR and integrin αvβ3 is of reciprocal nature since each is able to promote activation of its counterpart. This mutually beneficial relationship provides the conceptual basis to use radiolabeled cyclic RGD peptides as radiotracers for tumor imaging [43-52] and noninvasive monitoring of anti-αvβ3 and antiangiogenesis therapy [52, 53]. While they are appreciated for their role as noninvasive tools to monitor tumor response to antiangiogenesis therapy [43-46], it remains untested if integrin αvβ3-targeted radiotracers are useful for monitoring the tumor response to anti-αvβ3 treatment.

In this study, we evaluated 99mTc-3P-RGD2, a 99mTc-labeled cyclic RGD peptide dimer [54, 55], for its capability to monitor integrin αvβ3 expression during breast tumor growth and after treatment with linifanib and E[c(RGDfK)]2 (RGD2) in the xenografted MDA-MB-435 human breast tumor model. 99mTc-3P-RGD2 is of particular interest due to the linear relationship between its tumor uptake and integrin αvβ3 expression [56]. 99mTc-3P-RGD2 SPECT/CT has been successfully used for quantification of radioactivity accumulation, delineation of tumor necrosis [57], and monitoring progression of breast cancer lung metastases [58]. RGD2 was used as anti-αvβ3 agent due to its higher integrin αvβ3 binding affinity than that of c(RGDfK) because of dimerization [54]. MDA-MB-435 cell line was of interest due to its high potential for metastasis. Despite the controversy associated with its identity [59-61], more recent results confirmed that MDA-MB-435 is indeed a poorly differentiated and aggressive breast tumor cell line [62]. The main objective of this study was to demonstrate the utility of 99mTc-3P-RGD2 SPECT/CT as a molecular imaging tool for monitoring early tumor response to antiangiogenesis therapy in small animals, and to illustrate its limitations in monitoring the efficacy of anti-αvβ3 treatment.

MATERIALS AND METHODS

Chemicals and Analytical Method. Linifanib (ABT-869) was from AbbVie Inc. (North Chicago, IL). Trisodium triphenylphosphine-3,3′,3″-trisulfonate (TPPTS) and tricine were purchased from Sigma-Aldrich (St. Louis, Missouri), and were used without further purification. Cyclic RGD peptides, E[c(RGDfK)]2 (RGD2) and PEG2-E[c(RGDfK)]2 (3P-RGD2; PEG2 = 15-amino-4,7,10,13-tetraoxapentadecanoic acid), were made by Peptides International Inc. (Louisville, KY). HYNIC-3P-RGD2 (HYNIC = 6-(2-(2-sulfonatobenzaldehyde)hydrazono) nicotinyl) was prepared using the method described in our previous communication [55]. Na99mTcO4 was obtained from Cardinal HealthCare (Chicago, IL). The radio-HPLC method used a LabAlliance system.
equipped with a β-ram IN-US detector and Zorbax C18 column (4.6 mm x 250 mm, 300 Å pore size; Agilent Technologies, Santa Clara, CA). The flow rate was 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 min, followed by a gradient mobile phase from 10% B at 5 min to 40% B at 20 min.

**Radiolabeling and Dose Preparation.** ⁹⁹mTc-3P-RGD₂ was prepared according to the literature method [55] using lyophilized kit formulation containing 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. ⁹⁹mTc-labeling was accomplished by adding ~1.5 mL of Na₉⁹mTcO₄ solution (1,110 – 1,850 MBq). The reconstituted vial was heated in a 100 °C water bath for 20 – 30 min. Radiochemical purity determined by radio-HPLC must be >95% before being used for both imaging and biodistribution studies. Doses for biodistribution were prepared by dissolving the radiotracer in saline to a concentration of ~1.1 MBq/mL. For SPECT/CT imaging, doses were prepared by dissolving the radiotracer in saline to a concentration of 370 – 740 MBq/mL. The dose solution was filtered with a 0.20 µm Millex-LG filter before being injected into animals. Each animal was injected with 0.1 – 0.2 mL of the dose solution.

**Cell Culture.** MDA-MB-435 cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA). Tumor cells were grown at 37 °C in Minimal Essential Medium (Alpha Modification) supplemented with 10% fetal bovine serum (FBS, ATCC) and 1% penicillin and streptomycin solution (GIBCO Industries Inc., Langley, OK), and at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cells were grown as monolayers and were harvested or split when they reached 90% confluence to maintain exponential growth.

**Animal Model.** Biodistribution and imaging studies were performed using athymic nude mice bearing MDA-MB-435 breast cancer xenografts in compliance NIH animal experiment guidelines (Principles of Laboratory Animal Care, NIH Publication No. 86-23, revised 1985). The protocol for animal studies has been approved by the Purdue University Animal Care and Use Committee (PACUC). Female athymic nu/nu mice were purchased from Harlan (Indianapolis, IN) at 4 – 5 weeks of age. Each animal implanted with 5 × 10⁶ MDA-MB-435 cells into the fat pad. The tumor volume was measured every two days for the first two weeks, every day for next 3 weeks, and calculated by the formula: (length x width²)/2. Animals were subjected to biodistribution and imaging at specified times.

**Linifanib Treatment Protocol.** Groups (n = 5) were size-matched with an average tumor volume of 180 ± 90 mm³ one day before baseline SPECT/CT imaging. Vehicle (0.15% hydroxypropylmethylcellulose, 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 was dosed orally twice daily, with a time interval of >8 h between consecutive doses.

**Imaging Protocol for SPECT/CT.** Longitudinal SPECT/CT imaging was performed at -1, 1, 4 and 11 days after initiation of linifanib treatment using a u-SPECT-II/CT scanner (Milabs, Utrecht, The Netherlands) equipped with 0.6 mm multi-hole collimators. Linifanib was dosed two hours prior to SPECT/CT. One hour before SPECT/CT imaging, ⁹⁹mTc-3P-RGD₂ (37 – 55.5 MBq) was administered via the lateral tail vein. Anesthesia was induced using an air flow rate of 350 mL/min and approximately 3.0% isoflurane. After induction of anesthesia, the air flow rate was reduced to 250 mL/min with approximately 2.0% isoflurane. 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. SPECT reconstruction, data processing and quantification of organ uptake were performed according to literature methods [56, 57]. Reconstructed images were visualized as both orthogonal slices and maximum intensity projections. The tumor uptake of ⁹⁹mTc-3P-RGD₂ was reported as the percentage of injected dose (%ID) and percentage of injected dose per unit volume (%ID/cm³), and was compared with that obtained from biodistribution.

**RGD₂ Treatment Protocol.** Each mouse was implanted subcutaneously with 5 × 10⁶ MDA-MB-435 cells into the fat pads. Seven days after inoculation of MDA-MB-435 cells, the tumor-bearing mice were randomly divided into 2 groups: the vehicle-treated group (n = 5) and RGD₂-treated group (n = 5) administered intravenously by tail-vein injection of RGD₂ at a dose of 50 mg/kg. The RGD₂-treatment was continued for 10 days (a dose every two days with a total of 5 doses). Animals in the vehicle-treated group were administered with equal volume of saline. SPECT/CT imaging was performed at week 1 or week 3 after the
formed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. The level of significance was set at \( p < 0.05 \). GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) was used for linear and nonlinear regression analysis.

**RESULTS**

Monitoring of Breast Tumor Growth. Figure 1A shows 3D and transverse views of SPECT/CT images of a tumor-bearing mouse at 5, 7, 14, 21, 28 and 35 days after inoculation of MDA-MB-435 breast tumor cells. Small tumors (Figure 1A: D5) were readily detected with excellent contrast. When the tumor size was 0.15 – 0.40 \( \text{cm}^3 \), the radioactivity distribution was relatively uniform. After week 3, the tumors were >0.50 \( \text{cm}^3 \), and the radioactivity distribution heterogeneity was significant due to tumor necrosis (Figure 1A: D28). By week 5, there was extensive necrosis in MDA-MB-435 breast tumors. Necrotic regions had little radioactivity accumulation (Figure 1A: D5) due to lack of integrin \( \alpha_v \beta_3 \) expression (Figure 1B).

Comparison of Breast Tumor Uptake Values from SPECT/CT and Biodistribution. To validate \( \text{u-SPECT-II/CT} \) as a tool for quantification of the tumor uptake of \( 99mTc-3P-RGD_2 \), we performed a biodistribution study using animals with tumor size being 0.01 – 1.2 \( \text{cm}^3 \), and compared the tumor uptake values to those from SPECT/CT quantification. Figure 2 exhibits the relationship between the tumor size (\( \text{cm}^3 \)) and the tumor uptake (\%/ID for total radioactivity and \%/ID/g or \%/ID/cm\textsuperscript{3} for radioactivity concentration) determined by SPECT/CT (A and C) and biodistribution (B and D). Apparently, the tumor uptake of \( 99mTc-3P-RGD_2 \) from SPECT/CT was almost identical to that from biodistribution. Similar results were also seen in the U87MG model [64]. u-SPECT-II/CT is excellent imaging platform for accurate quantification of tumor uptake of \( 99mTc-3P-RGD_2 \) in small animals.

Tumor Uptake Changes during Tumor Growth. When the tumors were very small (<0.1 \( \text{cm}^3 \)), their uptake of \( 99mTc-3P-RGD_2 \) were low due to lack of integrin \( \alpha_v \beta_3 \) contribution from neovasculature. Between weeks 2 and 3, the tumor size was in the range of 0.15 – 0.30 \( \text{cm}^3 \), and the \%/ID/g tumor uptake of \( 99mTc-3P-RGD_2 \) peaked (Figure 2: B and D). Between weeks 4 and 5, the tumor size was >0.5 \( \text{cm}^3 \). During this period, the % ID tumor uptake of \( 99mTc-3P-RGD_2 \) was enhanced steadily with increased tumor size (Figure 2: A and C), but its % ID/g uptake decreased slowly due to necrosis (Figure 1B), maturation of blood vessels, and larger interstitial space [63]. The overall relationship between tumor volume and % ID uptake of \( 99mTc-3P-RGD_2 \) was linear (Figure 2: A: \( R^2 = \)
The relationship between tumor size and %ID/cm³ tumor uptake of ⁹⁹mTc-3P-RGD₂ was best described as quadratic polynomial (Figure 2: B and D).

Fig 1. A: The 3D (upper panel) and transverse (lower panel) views of SPECT/CT images of a mouse with bearing MDA-MB-435 breast cancer xenografts at 5, 7, 14, 21, 28 and 35 days after inoculation of MDA-MB-435 human breast cancer cells. The animal was administered with 37 – 55.5 MBq of ⁹⁹mTc-3P-RGD₂ via the lateral tail vein. B: Microscopic images (Original magnification: ×200) of tumor slice selected from the necrotic and viable regions after immunohistochemical staining for integrin β₃ and CD31. The tumor tissue was harvested on day 28 days after inoculation of MDA-MB-435 cells. CD31 was used as a biomarker for endothelial cells on blood vessels (both mature vasculature and neovascularity). Integrin β₃ was visualized with Cy3 (red) and CD31 was visualized with FITC (green). The yellow color in overlay images indicates the presence of integrin β₃ on neovasculature.

Fig 2. Left: Linear relationship between the tumor size (cm³) and the tumor uptake (%ID for total radioactivity accumulation) determined by SPECT/CT quantification (A) and biodistribution (C). Right: Relationship between the tumor size (g) and tumor uptake (%ID/g or %ID/cm³ for radioactivity concentration) obtained from SPECT/CT quantification (B) and biodistribution (D: n = 26).
Integrin $\alpha_\text{v}\beta_3$ Expression Changes during Tumor Growth. Figure 3 displays selected microscopic images of the slices obtained from the xenografted MDA-MB-435 tumors (0.05 g, 0.13 g, 0.28 g, 0.55 g and 0.88 g) after immunohistochemical staining for integrin $\beta_3$ and CD31. As expected, small tumors (<0.05 g) had few microvessels with very little integrin $\alpha_\text{v}\beta_3$ expression. When tumors became larger (0.2 – 0.5 g), the blood vessel density was increased, as illustrated by the enhanced yellow and orange colors on overlay images. When tumors were > 0.5 g, the tumor blood vessel density decreased while the total integrin $\alpha_\text{v}\beta_3$ expression level was increased due to more contributions from MDA-MB-435 tumor cells, which was completely consistent with the %ID tumor uptake values of $^{99m}$Tc-3P-RGD$_2$ (Figure 2: B and D).

**Fig 3.** Microscopic images of frozen tumor slices from the xenografted MDA-MB-435 tumors (0.05 g, 0.13 g, 0.28 g, 0.55 g and 0.88 g) after immunohistochemical staining for integrin $\beta_3$ and CD31. CD31 was used as a biomarker for tumor endothelial cells. Integrin $\beta_3$ was visualized with Cy3 (red), and CD31 with FITC (green). Yellow color indicates the integrin $\beta_3$ on new blood vessels.
Monitoring Tumor Response to Linifanib Therapy. Figure 4A compares tumor volume changes in both vehicle (n = 5) and linifanib (n = 5) treated groups. A significant difference in tumor volume (Figure 4A: p < 0.05) was seen between vehicle and linifanib-treated groups after 4 days of linifanib treatment. This effect is further illustrated by SPECT/CT images (Figure 4B) obtained at -1, 1, 4 and 11 days after initiation of linifanib therapy. Figure 5 shows SPECT/CT quantification data to illustrate early breast tumor response to linifanib angiogenesis therapy. In the linifanib-treated group, there was a dramatic reduction in both %ID and %ID/cm³ tumor uptake of ⁹⁹mTc-3P-RGD₂ as early as 24 h. It was clear that ⁹⁹mTc-3P-RGD₂ SPECT can detect much earlier biological changes (decrease in integrin αvβ3 expression level) as a result of linifanib therapy. The % ID tumor uptake of ⁹⁹mTc-3P-RGD₂ increased steadily in the vehicle-treated group (Figure 5A), while its %ID/cm³ tumor uptake decreased slowly over 11 day study period (Figure 5B).

Fig 4. A: Comparison of tumor volumes in vehicle and linifanib-treated in the MDA-MB-435 models. Tumor volume was determined by caliper measurements. *: p < 0.05, significantly different from the vehicle-treated group. #: p < 0.01, significantly different from the vehicle-treated group from that specific date until the end of study. B: Transverse views of selected SPECT/CT images from athymic nude mice bearing MDA-MB-435 breast tumors in the vehicle (upper panel) and linifanib-treated groups (lower panel). SPECT/CT images were obtained at -1, 1, 4 and 11 days after initiation of linifanib therapy to illustrate the early tumor response in terms of tumor uptake and integrin αvβ3 expression levels. Arrows indicate the presence of tumors.

http://www.thno.org
Comparison of Tumor Response in Different Animal Models. Figure 6 compares the changes in %ID/cm³ tumor uptake and T/M ratios of ⁹⁹mTc-3P-RGD₂ after linifanib treatment in three different xenografted tumor-bearing animal models (U87MG: high integrin αᵥβ₃ expression on tumor cells and neovasculature; MDA-MB-435: moderate integrin αᵥβ₃ expression on tumor cells and neovasculature; PC-3: low integrin αᵥβ₃ expression on tumor cells and neovasculature). The %ID/cm³ tumor uptake and T/M ratios of ⁹⁹mTc-3P-RGD₂ in the U87MG and PC-3 models were obtained from our previous report [64]. The changes in %ID/cm³ tumor uptake and T/M ratios were calculated by subtracting the value on a specific date (+1, +4 and +11) from the value on day -1. *: p < 0.05, significantly different from those in the U87MG and PC-3 models. #: p < 0.01, significantly different from those in the U87MG and MDA-MB-435 models.

Effects of Linifanib on Tumor Vasculature and Integrin αᵥβ₃ Expression. Immunohistochemical staining was performed to study the impact of linifanib treatment on integrin αᵥβ₃ and CD31 expression in MDA-MB-435 tumors. Figure 7 (top) shows overlay images of the tumor slices stained with hamster anti-integrin β₃ (red color) or rat anti-CD31 (green color) antibody in both vehicle and linifanib-treated groups. The yellow color in overlay images indicates the presence of integrin β₃ on new blood vessels. It was quite clear that the effect of linifanib therapy was on both tumor cells and tumor neovasculature as indicated by the disappearance of red (breast tumor cells) and yellow/orange (neovasculature) colors for integrin αᵥβ₃ in the overlay images. After 24 h of linifanib therapy, the integrin αᵥβ₃ on neovasculature disappeared almost completely while the tumor cell integrin αᵥβ₃ expression level decreased steadily during the 11-day period (Figure 7: top). This was further supported by the pathological H&E staining data of tumor slices in vehicle and linifanib-treated groups (Figure 7: bottom) at -1 and 1 days. Within 24 h after initiation of linifanib therapy, the blood vessel density was dramatically reduced. These data clearly
show that linifanib treatment had a strong impact on both breast tumor cells and neovasculature.

**Relationship between Tumor Response and Integrin α\_vβ\_3/CD31 expression.** Figure 8 shows plots of the %ID/cm³ tumor uptake changes after linifanib treatment against integrin α\_vβ\_3 (left) and CD31 (right) expression levels in the U87MG, MDA-MB-435 and PC-3 models at the time of diagnosis. The tumor uptake change was calculated by deducting the %ID/cm³ tumor uptake of ⁹⁹mTc-3P-RGD\_2 on days 1, 4 and 11 from its %ID/cm³ tumor uptake on -1 day for the same animal. Integrin α\_vβ\_3 and CD31 expression levels were obtained from the tumors (0.1 - 0.3 g) without significant necrosis. Obviously, there was an excellent linear relationship between %ID/cm³ tumor uptake changes (the tumor response or therapeutic effect) and the total integrin α\_vβ\_3 expression levels (integrin α\_vβ\_3 density) at the time of diagnosis (-1 day). The same linear relationship was also seen between the tumor response and the CD31 expression levels (blood vessel density).

**Fig 7. A**: Overlay images of MDA-MB-435 tumor tissues after immunohistochemical staining for integrin β\_3 and CD31 in vehicle (upper panel) and linifanib-treated groups (lower panel) to illustrate the changes in integrin β\_3 expression level and blood vessel density during linifanib therapy. Tumor tissues were harvested at -1, 1, 4 and 11 days after initiation of linifanib therapy. CD31 was the marker for tumor blood vessels (both mature and newly formed), which was visualized with FITC (green color). Integrin β\_3 was visualized with Cy3 (red color). The yellow color in overlay images indicates the presence of integrin β\_3 on new blood vessels. **B**: Images of selected histological slice (H&E stained) of tumor tissues from animals in the vehicle (upper panels) and linifanib-treated (lower panels) groups to illustrate vascular density changes after only one day of linifanib therapy. Red color indicates the presence of blood vessels.
Fig 8. Linear relationship between the %ID/cm³ tumor uptake change at days 1 (top), 4 (middle) and 11 (bottom) after linifanib therapy and the expression levels of integrin αvβ3 (left) and CD31 (right) in three animal models. The %ID/cm³ tumor uptake values of 99mTc-3P-RGD2 were calculated from SPECT/CT quantification, and reported as the mean plus/minus standard error of the mean based on results from five animals (n = 5). The %ID/cm³ tumor uptake values and integrin αvβ3/CD31 expression levels in the U87MG and PC-3 tumors were obtained from our previous report [64]. The %ID/cm³ tumor uptake change was calculated by deducting the %ID/cm³ tumor uptake of 99mTc-3P-RGD2 on days 1, 4 and 11 from its original value on -1 day (before linifanib therapy) in the same animal. The average %ID/cm³ tumor uptake change is used as an indicator of tumor response to linifanib antiangiogenesis treatment.

Effects of RGD2 Therapy on Tumor Volume.
Figure 9A shows the tumor growth curves during 42 days of study period for both vehicle (n = 5) and RGD2-treated (n = 5) groups. No significant difference in tumor volumes was found over first 3 days after initiation of RGD2 therapy. After two doses of RGD2 (4 days after initiation of RGD2 therapy), there was significant tumor volume reduction in RGD2-treated group. By the time of the final dose (10 days after initiation of RGD2 therapy), the tumor volume in the vehicle-treated group was almost 3x larger than that in RGD2-treated group. Obviously, there was a significant therapeutic effect from RGD2 treatment on the basis of tumor volume change. However, RGD2 treatment had to be stopped after five doses of RGD2 because of toxicity, as indicated by significant weight loss (Figure 9B).

Fig 9. Tumor growth curves (A) and body weight changes (B) for vehicle (n = 5) and RGD2-treated (n = 5) groups in the xenografted MDA-MB-435 breast tumor model. Tumor size was calculated on the basis of caliper measurements according the formula (length × width²)/2. Body weight was measured by balance. RGD2 treatment had to be stopped after five doses of RGD2 because of toxicity, as indicated by significant weight loss. The body weight recovered after termination of RGD2 treatment.
Effects of RGD2 Therapy on Tumor Uptake. SPECT/CT images (Figure 10: top) were obtained before RGD2 treatment, and at 21 and 35 days after inoculation of MDA-MB-435 cells. Tumor volumes (A), tumor uptake (B: %ID and C: %ID/cm³) and T/M ratios (D) were calculated from SPECT/CT quantification. Since the tumor retention time of RGD2 was ~15 h [65], a 7-day interval between the final RGD2 dosing and the first SPECT/CT imaging study was used to make sure that there was no significant blockage of ⁹⁹mTc-3P-RGD2 binding to integrin αvβ3. As expected, there was significant reduction in tumor volume (Figure 10A) and %ID tumor uptake (Figure 10B) of ⁹⁹mTc-3P-RGD2 between the vehicle and RGD2-treated animals. However, its %ID/cm³ tumor uptake was comparable in vehicle and RGD2-treated groups within experimental errors (Figure 10C: p > 0.05) at 7 days post RGD2 treatment. By 21 days after RGD2 therapy, the %ID/cm³ uptake in RGD2-treated group was significantly higher than that in vehicle group (Figure 10C: p < 0.05) while T/M ratios were almost identical at both time points (Figure 10D). RGD2 therapy resulted in significant reduction in both the tumor volume and %ID tumor uptake (the tumor radioactivity) of ⁹⁹mTc-3P-RGD2 (Figure 10: A and B); but it also led to the enhanced %ID/cm³ tumor uptake (the radioactivity density) of ⁹⁹mTc-3P-RGD2 (Figure 10C).

Fig 10. Top: The 3D and transverse views of SPECT/CT images of tumor-bearing mice before RGD2 treatment, as well as on day 21 and 35 after inoculation of MDA-MB-435 cells (or on day 7 and 21 after final dose of RGD2). SPECT image at day 7 was used only for comparison purposes. Bottom: SPECT/CT quantification data, including tumor volumes (A), %ID tumor uptake (B), %ID/cm³ tumor uptake (C), and T/M ratios (D) in vehicle (n = 5) and RGD2-treated (n = 5) groups. *p < 0.05 and #p < 0.01: significantly different from the vehicle-treated group.
Discussion

In this study, we found that 99mTc-3P-RGD₂ is an excellent radiotracer for imaging MDA-MB-435 tumors (Figure 1A). As shown in Figure 2, the uptake values (%ID and %ID/cm³) of 99mTc-3P-RGD₂ from SPECT/CT is almost identical to those from biodistribution. There was also a linear relationship between tumor size and %ID uptake of 99mTc-3P-RGD₂ (Figure 2: A and C). These results strongly suggested that the tumor uptake obtained from SPECT/CT images can accurately reflect the total tumor integrin αvβ3 expression. 99mTc-3P-RGD₂ is useful for monitoring integrin αvβ3 expression changes during tumor growth (Figure 1A).

[18F]AH111585 has been used to monitor sunitinib therapy response [43-45], and its tumor uptake was significantly reduced 2 days after sunitinib therapy. It was believed that [18F]AH111585 was better than [18F]FDG for imaging angiogenesis [45]. ⁶⁴Cu-DOTA-RGD was used to monitor dasatinib therapy [46]. It was also found that dasatinib was able to significantly reduce the uptake of ⁶⁴Cu-DOTA-RGD in the “treated” tumors. A significant challenge for [¹⁸F]AH111585 and many ¹⁸F-based PET radiotracers to assume a widespread clinical utility is their poor clinical availability. In contrast, ⁹⁹mTc-3P-RGD₂ is readily prepared with very high specific activity using a kit formulation [54, 55]. From availability point of view, ⁹⁹mTc-3P-RGD₂ has significant advantages of the corresponding ¹⁸F PET radiotracers.

Inhibition of VEGF and PDGF receptors with linifanib normalizes tumor vasculature [9-11, 13, 14]. Since growth factors (e.g. VEGF) increase integrin αvβ3 expression [40-42], inhibition of VEGF and PDGF receptors could lead to a decrease of integrin αvβ3 expression. This makes integrin αvβ3 a mechanistically relevant biomarker to assess the tumor response to linifanib therapy. In this study, we found significant decreases in the tumor uptake (%ID and %ID/cm³) of ⁹⁹mTc-3P-RGD₂ prior to tumor volume changes after linifanib treatment (Figures 4). Changes in tumor vasculature were confirmed by IHC staining data (Figure 7A) and pathological H&E analysis (Figure 7B).

It is interesting to note that the therapeutic effect of linifanib is not limited to tumor blood vessels. It is well-established that linifanib is a multi-targeted receptor tyrosine kinase inhibitor [9-14]. Since VEGF and PDGF receptors are overexpressed on tumor cells and neovasculature [41, 42], it is not surprising that linifanib has inhibitory effects on both tumor cells and tumor vasculature. There are two phases for the tumor response to linifanib therapy (Figure 5): a dramatic decrease in the tumor uptake (%ID and %ID/g) of ⁹⁹mTc-3P-RGD₂ within the first 24 h after initiation of linifanib therapy, and a steady tumor uptake reduction over the next 10 days of study period. The IHC and H&E staining data (Figure 7) suggest that the rapid decrease in tumor uptake of ⁹⁹mTc-3P-RGD₂ is most likely induced by antiangiogenic effect of linifanib on tumor neovasculature while the slow tumor uptake reduction is caused by either its direct inhibitory effect on tumor cells or slow tumor cell apoptosis as a result of normalization of tumor vasculature [9-11, 13, 14]. This explanation is supported by the sharp decrease of CD31 signals (Figure 7A) and tumor microvessel density (Figure 7B) over the first 24 h after initiation of linifanib treatment, followed by slow disappearance of fluorescence intensity from integrin β3 on tumor cells over the next 10 days of study period (Figure 7A).

In our previous study [64], we found that there was a significant decrease in tumor uptake (%ID and %ID/cm³) and T/M ratios of ⁹⁹mTc-3P-RGD₂ in the xenografted U87MG model while no significant changes in tumor volume or tumor uptake of ⁹⁹mTc-3P-RGD₂ were seen in the xenografted PC-3 model after linifanib treatment. In this study, we found that the changes in tumor volume (Figure 4) and tumor uptake of ⁹⁹mTc-3P-RGD₂ (Figure 6A) in the MDA-MB-435 model were between those observed in the U87MG and PC-3 models. This is consistent with the relative integrin αvβ3 expression levels on three xenografted tumors [56, 66], and reciprocal relationship between integrin αvβ3 and VEGFR2 [40-42]. We also found that there is a linear relationship between the integrin αvβ3/CD31 expression levels and the tumor response, as indicated by changes in %ID/cm³ tumor uptake and T/M ratios of ⁹⁹mTc-3P-RGD₂ (Figure 8). Highly vascularized tumors (e.g. U87MG) with higher expression level of integrin αvβ3 and CD31 have better tumor response to linifanib therapy than poorly vascularized tumors (e.g. PC-3) with low expression levels of integrin αvβ3 and CD31. ⁹⁹mTc-3P-RGD₂ has the potential as a pre-treatment screening tool to select appropriate patients who will benefit most from the antiangiogenic linifanib treatment. If the tumor in cancer patient has a high expression level of integrin αvβ3, as indicated by the high tumor uptake of ⁹⁹mTc-3P-RGD₂ in SPECT/CT images at the time of diagnosis, antiangiogenic linifanib therapy would more likely be effective. If the tumor has very little integrin αvβ3 expression (low tumor uptake of ⁹⁹mTc-3P-RGD₂), the antiangiogenic therapy would not be effective regardless the amount of linifanib administered into each cancer patient.

It is important to note that there are no accurate techniques to quantify absolute integrin αvβ3 expression levels. Western blotting was used to determine...
the total integrin αβ3 protein concentration in tumor tissues [44, 45, 56, 67, 68]; but the percentage of integrin αβ3 recovery from the tumor tissue and its activation state remained unknown. We have been using IHC staining with anti-integrin β3 antibodies to determine the relative expression levels of integrin αβ3 in the tumor tissues [56, 66]. This technique is a better fit to reflect the activation state of integrin αβ3 in acetonet-fixed tumor tissues since only the activated integrin αβ3 is able to bind the fluorescence-labeled anti-integrin β3 monoclonal antibody. We also found that the primary antibody is very important for IHC staining of tumor tissues. In this study, we use hamster anti-human integrin β3 antibody for staining of integrin αβ3 on both tumor cells and neovasculature mainly because of the murine nature of xenografted tumor tissues. It must be noted that quantification of absolute fluorescent intensity is operator-dependent and subjected to individual interpretation. Tumors of different sizes might have different integrin αvβ3 expression patterns because of tumor necrosis. The integrin αvβ3 expression pattern is heterogeneous even in the same tumor tissue. Thus, the relative expression levels of integrin αβ3 and CD31 only represent the status under specific experimental conditions, such as the primary integrin αβ3 monoclonal antibody, animal species, inoculation location of tumor cells, tumor types, and tumor size.

As illustrated in this study, there is significant challenge in using 99mTc-3P-RGD2 to monitor early tumor response to RGD2 therapy. Since 99mTc-3P-RGD2 and RGD2 target the same receptor, there must be a waiting period to allow RGD2 to dissociate completely from integrin αβ3 sites before using 99mTc-3P-RGD2 to determine integrin αβ3 level changes after RGD2 therapy. Otherwise, it would be difficult to determine if changes in tumor uptake are actually caused by its blocking effect or therapeutic effect. It is also important to note that the waiting period also depends on tumor retention time of the anti-αβ3 agent. If its tumor retention time is short (2 - 3 h), it is possible to image tumors with 99mTc-3P-RGD2 24 h (~10 half-lives) after anti-αβ3 therapy. Since its tumor retention time is ~15 h [65], it would take 6 - 7 days for RGD2 to dissociate completely from integrin αβ3 sites. During this period, the tumor might undergo significant biological changes. Therefore, it is difficult to use the integrin αβ3-targeted radiotracers (e.g. 99mTc-3P-RGD2) to accurately monitor early tumor response to anti-αβ3 therapy.

There is no doubt that RGD2 can significantly reduce the tumor size (Figure 10A) and %ID tumor uptake (Figure 10B) of 99mTc-3P-RGD2; but its %ID/cm³ tumor uptake in RGD2-treated group was higher (p < 0.05) than that in vehicle-treated group (Figure 10C). It is possible that RGD2 was able to block the integrin αβ3 sites in RGD2-treated group, resulting in tumor growth inhibition, as indicated by tumor volume reduction. Once RGD2 therapy stopped, RGD2 slowly dissociated from tumor cells and neovasculature. As a result, integrin αβ3 is activated again. Recently it was reported that low concentrations of integrin αβ3 and αvβ3 inhibitors (e.g. Cilengitide) can stimulate tumor growth and angiogenesis by altering αβ3 integrin and VEGFR-2 trafficking, thereby promoting endothelial cell migration to VEGF [69]. That might explain why the %ID/g tumor uptake of 99mTc-3P-RGD2 in RGD2-treated group was higher than that in vehicle-treated group 3 weeks after RGD2 therapy (Figure 10C). More studies are needed to understand the integrin αβ3 expression or activation state changes after anti-αβ3 RGD2 therapy, as well as their relationship with the %ID/g tumor uptake of 99mTc-3P-RGD2.

CONCLUSION

In this study, we clearly show that 99mTc-3P-RGD2 SPECT/CT is a useful molecular imaging tool for monitoring the integrin αβ3 expression changes during breast tumor growth or during/after linifanib antiangiogenic therapy. 99mTc-3P-RGD2 SPECT/CT is also a useful pre-treatment screening tool to identify the cancer patient who will benefit best from antiangiogenic therapy with linifanib or related antiangiogenic drugs of the same or similar mechanism. However, it is difficult to use the integrin αβ3-targeted radiotracers, such as 99mTc-3P-RGD2, for monitoring the early tumor response to anti-αβ3 therapy with Cilengitide or RGD2. This conclusion may also apply to other radiotracers and anti-cancer drugs that target the same receptor.

ACKNOWLEDGEMENTS

Authors thank AbbVie Inc. (North Chicago, IL) for the use of Linifanib. This work was supported by Purdue University, the Challenge Research Award from Purdue Cancer Center, the Indiana Clinical and Translational Sciences Institute funded in part by grant Number TR000006 (Clinical and Translational Award) from the National Institutes of Health, National Center for advancing Translational Science, and R01 CA115883 (S.L.) from the National Cancer Institute.

COMPETING INTERESTS

The authors have declared that no competing interest exists.
REFERENCES

1. Folkman J. Role of angiogenesis in tumor growth and metastasis. Semin Oncol 2002;29:15-18.

2. Moussa SA. Anti-integrin as novel drug-discovery targets: potential therapeu-
tic and diagnostic implications. Curr Opin Chem Biol 2002;6:534-541.

3. Guo W, Giancotti FG. Integrin signalling during tumour progression. Nat Rev Mol Cell Biol 2004;5:816-826.

4. Fielding-Habermann B. Integrin adhesion receptors in tumor metastasis. Clin Exp Metastasis 2003;20:203-213.

5. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;3:401-410.

6. Fielding-Habermann B, Song S, Meyer-Morse N, Bergland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 2003;111:1287-1295.

7. Ferrara N, Kerbéls RJ. Angiogenesis as a therapeutic target. Nature 2001;438:967-974.

8. Haddley K. Linifanib Receptor tyrosine kinase inhibitor Oncolytic. Drug Future 2010;35:106-112.

9. Zhou J, Goh BC, Albert DH, Chen CS. ABT-869, a promising multi-targeted tyrosine kinase inhibitor. J Hematol Oncol 2009;2:33-45.

10. Jiang F, Albert DH, Luo Y, Tapang P, Zhang K, Davidsson SK, Fox GB, Le-

11. Shankar DB, Li J, Tapang P, Owen McCaul J, Pease LJ, Dai Y, Wei RJ, Albert

12. Hernandez-Davies JE, Zape JP, Landavaz EM, Tan X, Pressnell A, Griffith D, Heinrich MC, Glaser KB, Alford KM, Glaser KB. ABT-869, a multi-targeted receptor tyrosine kinase inhibitor, reduces tumor microvascularity and improves vascular wall integ-

13. Total lesion glycolysis and FDG SUV as early readouts of tumor response to

14. Tannir NM, Wong YN, Kollmannsberger CK, Ernstoff MS, Perry DJ, Appleman LJ, Posadas EM, Cho D, Choiuei TK, Coates A, Gupta N, Pradhan R, Humerickhouse R, Goh BC. Phase I and biomarker study of

15. Foote RL, Weidner N, Harris J, Hammond E, Lewis JF, Viong T, Ang WK, Fu KK. Evaluation of tumor angiogenesis measured with microsized density (D0) as a prognostic indicator in nasopharyngeal carcinoma: results of RT0C 9505. Int J Radiat Oncol Biol Phys 2005;61:745-753.

16. El-Assal ON, Yamamoto A, Soda Y, Yamaguchi M, Igarashi M, Yamamoto A, Niu G, Chen X. Has molecular and cellular imaging enhanced drug discovery

17. Cooper CR, Chay GH, Pienaar KJ. The role of αβ3 in prostate cancer progres-

18. Carreira T, Tietze A, Metz R, Merger MR, Goodman SL, Semmler W. Cileniglode inhibits progression of experimental breast cancer bone metastases as a single agent significantly using VCT, MBL and DCE-MRI in a longitudinal in vivo study. Int J Cancer 2011;128:2453-2462.

19. Liapi H, Flath A, Kitazawa S. Integrin αβ3 expression by bone-resident breast cancer metastases. Diag Mol Pathol 1996;5:127-135.

20. Niu G, Chen X. Has molecular and cellular imaging enhanced drug discovery and
drug development? Drugs R D 2008;9:351-365.

21. Tateshii U, Oka T, Inose T. Radiolabeled RGD Peptides as Integrin αβ3-targeted PET Tracers. Curr Med Chem 2012;19:3301-3309.

22. Haubner R, Wester HJ. Radiolabeled tracers for imaging of tumor angiogen-

23. Wang CL, Koh TS, Sze B, Hartono S, Thng CH, McKeegan E, Yong WP, Chen CS, Lee SC, Wong J, Lim R, Sukri N, Lim SE, Ong AB, Steinberg J, Gupta N, Pradhan R, Humerickhouse R, Goh BC. Phase I and biomarker study of

24. Stupp R, Bischoff J, Blasko J, Suki W, Berthelemy J, Rosen B, Kamineni A, Hendrix W, Costa E, Rose J, Lally L, Chau T, Guo J, Jin X, Chiorean G, Bredesen D, Cohen-Gersdorff G, Reardon D, Thomas C, Reardon D, Thomas C, Reardon D, Thomas C. Phase I and II trial of intravenous administration of a novel,

25. Gasparetti G, Brooks PC, Bignozzi E, Vermeulen BP, Bonoldi E, Dirix LY, Ranieri G, Miceli R, Cherehs DA. Vascular integrin αβ3: a new prognostic indi-
cator in breast cancer. Clin Cancer Res 1998;4:2625-2634.

26. Cooper CR, Chay GH, Pienaar KJ. The role of αβ3 in prostate cancer progres-

27. Carreira T, Tietze A, Metz R, Merger MR, Goodman SL, Semmler W. Cileniglode inhibits progression of experimental breast cancer bone metastases as a single agent significantly using VCT, MBL and DCE-MRI in a longitudinal in vivo study. Int J Cancer 2011;128:2453-2462.

28. Liapi H, Flath A, Kitazawa S. Integrin αβ3 expression by bone-resident breast cancer metastases. Diag Mol Pathol 1996;5:127-135.

29. Niu G, Chen X. Has molecular and cellular imaging enhanced drug discovery and
drug development? Drugs R D 2008;9:351-365.

30. Tateshii U, Oka T, Inose T. Radiolabeled RGD Peptides as Integrin αβ3-targeted PET Tracers. Curr Med Chem 2012;19:3301-3309.

31. Haubner R, Wester HJ. Radiolabeled tracers for imaging of tumor angiogen-

32. Wang CL, Koh TS, Sze B, Hartono S, Thng CH, McKeegan E, Yong WP, Chen CS, Lee SC, Wong J, Lim R, Sukri N, Lim SE, Ong AB, Steinberg J, Gupta N, Pradhan R, Humerickhouse R, Goh BC. Phase I and biomarker study of

33. Stupp R, Bischoff J, Blasko J, Suki W, Berthelemy J, Rosen B, Kamineni A, Hendrix W, Costa E, Rose J, Lally L, Chau T, Guo J, Jin X, Chiorean G, Bredesen D, Cohen-Gersdorff G, Reardon D, Thomas C, Reardon D, Thomas C. Phase I and II trial of intravenous administration of a novel,
54. Wang L, Shi J, Kim YS, Zhai S, Jia B, Zhao H, Liu Z, Wang F, Chen X, Liu S. Improving tumor-targeting capability and pharmacokinetics of 99mTc-labeled cyclic RGD dimers with PEG linkers. Mol Pharm 2009; 6:231-245.
55. Jia B, Liu Z, Zhu Z, Shi J, Jin X, Zhao H, Li F, Liu S, Wang F. Blood clearance kinetics, biodistribution and radiation dosimetry of a kit-formulated integrin αvβ3-selective radiotracer 99mTc-3PRGD2 in non-human primates. Mol Imaging Biol 2011;13:730-736.
56. Zhou Y, Kim YS, Chakraborty S, Shi J, Gao H, Liu S. 99mTc-labeled cyclic RGD peptides for noninvasive monitoring of tumor integrin αvβ3 expression. Mol Imaging 2011;10:386-397.
57. Shao G, Zhou Y, Wang F, Liu S. Monitoring glioma growth and tumor necrosis with the U-SPECT-II/CT scanner by targeting integrin αvβ3. Mol Imaging 2013;12:39-48.
58. Zhou Y, Shao G, Liu S. Monitoring breast tumor lung metastasis by U-SPECT-II/CT with an integrin αvβ3-targeted radiotracer 99mTc-3PRGD2. Theranostics 2012;2:577-588.
59. Suzuki M, Mose ES, Montel V, Tarin D. Dormant cancer cells retrieved from metastasis-free organs regain tumorigenic and metastatic potency. Am J Pathol 2006;169:673-681.
60. Selipanoff S, Grijalva R, Zhou X, Yang W, Eli MB, Mills GB, Yu D. Lineage infidelity of MDA-MB-435 cells: expression of melanocyte proteins in a breast cancer cell line. Cancer Res 2004;64:3479-3485.
61. Ellison G, Klinowska T, Westwood RF, Docter E, French T, Fox JC. Further evidence to support the melanocytic origin of MDA-MB-435. Mol Pathol 2002;55:294-299.
62. Chambers AF. MDA-MB-435 and M14 cell lines: identical but not M14 melanoma? Cancer Res 2009; 69:5292-5293.
63. Jain RK. Transport of molecules in the tumor interstitium: a review. Cancer Res 1987;47:3039-3051.
64. Ji S, Zhou Y, Voorbach MJ, Shao G, Zhang Y, Fox GB, Albert D, Luo Y, Liu S, Mudd S. Monitoring tumor response to linifanib therapy with SPECT/CT using the αvβ3-targeted radiotracer 99mTc-3PRGD2. J Pharmaceut Exp Ther 2013;346:251-258.
65. Shi J, Zhou Y, Chakraborty S, Kim YS, Jia B, Wang F, Liu S. Evaluation of 111In-labeled cyclic RGD peptides: effects of peptide and PEG, multiplicity on their tumor uptake, excretion kinetics and metabolic stability. Theranostics 2011;1:322-340.
66. Zhou Y, Kim YS, Lu X, Liu S. Evaluation of 99mTc-labeled cyclic RGD dimers: impact of cyclic RGD peptides and 99mTc chelates on biological properties. Bioconjug Chem 2012;23:586–595.
67. Wu Y, Zhang X, Xiong Z, Cheng Z, Fisher DR, Liu S, Gambhir SS, Chen X. MicroPET imaging of glioma integrin αvβ3 expression using 18F-labeled tetrameric RGD peptide. J Nucl Med 2005;46:1707-1718.
68. Wu Z, Li ZB, Chen K, Cai W, He L, Chin FT, Li F, Chen X. MicroPET of tumor integrin αvβ3 expression using 18F-labeled PEGylated tetrameric RGD peptide (18F-FPRGD4). J Nucl Med 2007;48:1536-1544.
69. Reynolds AR, Hart IR, Watson AR, Welti JC, Silva RG, Robinson SD, Vialante GD, Couraquesen M, Salih M, Jones MC, Jones DT, Saunders G, Kostourou V, Perron-Sierra F, Norman JC, Tucker GC, Hodivala-Dilke KM. Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. Nat Med 2009;15:392-400.