Evaluation of $\alpha_v\beta_3$ -Mediated Tumor Expression with a ^{99m}Tc -Labeled Ornithine-Modified RGD Derivative During Glioblastoma Growth *In Vivo*

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Abstract

In this study, a novel way of distinguishing the intrinsic relationship between $\alpha_v\beta_3$ integrin targeting and detection of tumor growth by using a radiolabeled tracer based on a cyclic Arg-Gly-Asp (RGD) peptide was provided. The potential of the *in vivo* scintigraphic imaging of the developing vasculature from the early stage of tumor growth was evaluated. Alongside with the scintigraphic images, biodistribution studies were performed at distinct time points to validate this noninvasive imaging approach. The ability to noninvasively assess the tumor growth of $\alpha_v\beta_3$ integrin-positive glioblastoma tumors provides a method to better understand tumor angiogenesis *in vivo* and allows for a direct assessment of anti-integrin treatment efficacy.

Key words: angiogenesis, cRGDfK, glioblastoma, scintigraphic imaging, ^{99m}Tc , tumor growth

Introduction

Angiogenesis, the sprouting of new capillaries from existing microvasculature, is a crucial process in the pathogenesis of a large number of nonrelated diseases, such as tumor growth, rheumatoid arthritis, ocular diseases, and psoriasis.^{1–3} In cancer, early angiogenesis is essential for tumor growth and provides important targets for diagnosis and therapy.^{4,5} Specifically, the formation of new blood vessels is a key requirement for solid tumor growth beyond the size of 2–3 mm³, since diffusion is no longer sufficient to supply the tissue with oxygen and nutrients.⁴

A number of molecular imaging approaches have been applied to visualize angiogenesis such as radiotracer-based imaging (single-photon emission computed tomography [SPECT] or positron emission tomography [PET]), magnetic resonance imaging (MRI), ultrasound, and optical imaging.^{6–8} Among the most widely used molecules for angiogenesis imaging studies are vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) and integrin receptors.⁹

Peptides based on the Arg–Gly–Asp (RGD) sequence have been identified as an essential binding motif to some integrin receptors that mediate cell adhesion between cells and proteins of the extracellular matrix.^{10,11} They are widely used as specific ligands for the $\alpha_v\beta_3$ integrin receptors, which are abundantly expressed on several tumors, including osteosarcomas, melanomas, lung carcinomas, breast cancer, and glioblastoma multiforme (GBM).^{12–14} GBM is among the most highly vascularized tumors. It is also the most common primary brain tumor and one of the most lethal cancers.^{15,16} Current standard treatment of GBM consists of surgery and combined radiation therapy and chemotherapy.^{17,18}

The relatively high sensitivity of radiotracer-based imaging systems SPECT and PET provides an advantage in the practical application of molecular imaging techniques for the evaluation of angiogenesis.^{6,19,20} In the past decade, many RGD peptides and nonpeptide RGD mimetics have been labeled with different radioisotopes to develop angiogenesis-targeting radiocompounds for both diagnosis and

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therapy (^{99m}Tc , ^{125}I , ^{111}In , ^{90}Y , ^{18}F , ^{177}Lu , ^{89}Zr , ^{64}Cu , and ^{68}Ga), and some of them have already been introduced into clinical trials.^{21–31}

Recently, the authors focused on the biological evaluation of an RGD derivative, cRGDfK-Orn₃-CGG, with promising tumor uptake and retention in a GBM tumor mouse model.³¹ They designed this novel RGD peptide sequence, relying on the previous studies of this group with high prostate cancer uptake in subcutaneous PC-3 tumor-bearing mice, in which the chelating moiety, comprising the amino acids Gly-Gly-Cys,^{32–34} was N-terminally conjugated to a gastrin-releasing peptide analogue, bombesin, through amino acid spacer groups.^{32,34} In relation to the tri-peptide chelating ligand, of the so-called N₃S type, it has been shown that it forms a well-defined and stable complex with $[\text{Tc}(\text{V})\text{O}_3]^+$, which is a widely studied metal core.³⁵ As far as the tri-amino acid spacer of ornithines might be concerned, by maximizing the effect of the charge with the introduction of this polar linker, the hydrophilic character of the final compound influenced the targeting efficiency of $\alpha_v\beta_3$ integrin receptors and the overall biodistribution profile. More specifically, c(RGDfK)-(Orn)₃-[CGG- ^{99m}Tc] showed high and stable labeling with Tc-99m, high tumor uptake, and rapid blood clearance, while the main route of its excretion was through the urinary pathway. Moreover, this new RGD derivative was also conjugated with magnetic nanoparticles with promising theranostic applications.³⁶

These findings gave rise to the possibility of extending applications of cRGDfK-Orn₃-[CGG- ^{99m}Tc] from targeted imaging of $\alpha_v\beta_3$ expression to noninvasively monitoring of tumor growth over a prolonged period of time (35 days) after subcutaneous inoculation of GBM cells (U87MG) at the left front flank of severe combined immunodeficient (SCID) mice. Along with the scintigraphic images, biodistribution studies were performed to validate this noninvasive imaging approach.

To the best of knowledge, only two studies based on the noninvasive nuclear imaging techniques for visualizing the angiogenic activity during tumor growth are found in literature. The first one refers to a monomeric cyclic RGD derivative labeled with F-18 [^{18}F -FB-c(RGDyK)] that was applied for the microPET imaging of brain tumor growth in an orthotopic U87MG glioblastoma xenograft model.²⁴ The second published work deals with a dimeric ^{99m}Tc -HYNIC-labeled c(RGDfK) with three PEG4 chain spacer groups (^{99m}Tc -3P-RGD₂) that was used as an imaging SPECT/CT platform to monitor $\alpha_v\beta_3$ expression and tumor necrosis during glioma growth in a subcutaneous U87MG athymic nu/nu mouse model.³⁷ The same group has also used the ^{99m}Tc -3P-RGD₂ derivative for the monitoring progression of breast cancer lung metastases.³⁸

The novelty of this study relies on the use of a ^{99m}Tc -labeled monomeric cyclic RGD derivative, c(RGDfK)-(Orn)₃-[CGG- ^{99m}Tc], which even if it disposes lower tumor accumulation in relation to multimeric ^{99m}Tc -RGD peptides^{39–43} was able to clearly detect integrin expression variations during glioblastoma tumor growth. Nevertheless, apart from dimerization that contributes to a higher integrin $\alpha_v\beta_3$ binding affinity, a direct comparison of tumor uptake among ^{99m}Tc -labeled RGD derivatives is somehow difficult due to the wide variety of (1) the linkers and chelating groups^{44–46} and (2) the tumor cell lines with different $\alpha_v\beta_3$ receptor density levels that are used.^{6,47–49}

Materials and Methods

Materials

The RGD derivative, cRGDfK-Orn₃-CGG, was designed by this group³¹ and was consequently obtained from Caslo Laboratory at the Technical University of Denmark. All other chemicals were of reagent grade and used without further purification. Technetium-99m, in the form of $^{99m}\text{NaTcO}_4$ in saline, was eluted from a commercial ^{99}Mo - ^{99m}Tc generator (Drytec; GE Healthcare). High-glucose Dulbecco's modified Eagle Medium (DMEM) was purchased from Sigma-Aldrich. Trypsin-EDTA, L-glutamine, penicillin-streptomycin solution, and heat-inactivated fetal bovine serum (FBS) were obtained from Biocrom KG.

Methods

The ^{99m}Tc -complexes were identified by comparative RP-HPLC analysis on a Waters 600 chromatography system coupled to both a Waters 2487 Dual λ absorbance detector and a Gabi γ detector from Raytest. Radiochromatographic analysis was performed on a C-18 RP (25.4 $\times$ 2.5 cm, 5 μm porosity) column eluted with a binary gradient system at a 1 mL/min flow rate.

Scintigraphic images were acquired on a small mouse-sized camera manufactured by this team.⁵⁰ The detection device is based on two Position Sensitive Photomultiplier Tubes (H85000; Hamamatsu), a parallel hole collimator, and a NaI(Tl) pixelated scintillator. The spatial resolution of the systems is ~ 1.5 mm at 0 mm distance from the collimator's surface.

Formation of the cRGDfK-Orn₃-[CGG- $^{99m}\text{Tc}(\text{v})$] complex. The RGD derivative, cRGDfK-Orn₃-CGG radiolabeled with ^{99m}Tc , was previously evaluated as a tumor-imaging agent with regard to its radiochemical, radiobiological, and imaging characteristics.³¹ Radiolabeling of cRGDfK-Orn₃-CGG was performed according to an already published method through the ^{99m}Tc -gluconate precursor. Briefly, sodium gluconate is used as an intermediate exchange ligand for ^{99m}Tc , and stannous chloride is the reducing agent. Radiochemical analysis as well as *in vitro* stability and metabolic studies showed high radiochemical yield and stability of the radiolabeled derivative.

Cell culture. Human glioblastoma cancer cells (U87MG) were maintained in DMEM high glucose supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 1% GlutaMAX. The cells were incubated in a controlled humidified atmosphere containing 5% CO_2 at 37°C and

TABLE 1. MEAN VALUES OF ANIMAL AND TUMOR WEIGHT DURING TUMOR DEVELOPMENT ($N=3-5$)

	Animal weight (g)	Tumor weight (g)
Day 0	23.62 $\pm$ 0.7	0.00 $\pm$ 0.0
Day 3	24.73 $\pm$ 0.8	0.03 $\pm$ 0.01
Day 9	23.98 $\pm$ 0.5	0.04 $\pm$ 0.02
Day 21	25.67 $\pm$ 0.9	0.05 $\pm$ 0.01
Day 29	23.17 $\pm$ 0.8	0.63 $\pm$ 0.02
Day 35	25.73 $\pm$ 0.9	1.86 $\pm$ 0.03

FIG. 1. Time course profile of GBM growth in U87MG tumor-bearing mice ($n=3/\text{day}$) during the 35-day experimental period. GBM, glioblastoma multiforme.

were subcultured weekly. They were detached from the flask surface by using a trypsin/EDTA solution (0.25%).

Animal models. SCID mice from the same colony weighing 20–25 g were obtained from the breeding facilities of the Institute of Biology NCSR “Demokritos.” Under sterile conditions, mice were subcutaneously inoculated with 100 μL of cell suspension ($\sim 10^7$ U87MG cells/animal) just above the left anterior leg. Tumors overexpressing the $\alpha_v\beta_3$ receptors were allowed to grow up to 35 days postinoculation. Mice did not demonstrate any abnormal behavior (hyperactivity, weight loss, dehydration of skin, fur standing up, or hunched posture) that would urge us to sacrifice them earlier than day 35. All *in vivo* studies were performed in compliance with European legislation, while this Institution has approved of all animal protocols presented in the current study.

Quantitative evaluation of integrin expression. The authors performed a quantitative evaluation of the $\alpha_v\beta_3$ -mediated tumor expression during the early stages of U87MG glioblastoma differentiation using the radiolabeled RGD peptide,

FIG. 3. Graphical presentation of tumor-to-tissue ratios, at 1 hour p.i. of cRGDfK-Orn₃-[CGG-^{99m}Tc], on different days of tumor growth ($n=3-5$).

cRGDfK-Orn₃-[CGG-^{99m}Tc]. They evaluated the *in vivo* profile of tumor angiogenesis on days 0, 3, 9, 21, and 35 after cancer cell inoculation. Taking into consideration the findings of a similar study by Shao et al.,³⁷ where the glioma growth rate was slow up to the 3rd week of cell inoculation, and by correlating them with tumor measurements taken by a caliper, the authors excluded the time window 9–21 from the imaging study, since tumor dimensions were not high enough to be detected. Dynamic imaging studies were performed in different groups of SCID mice ($n=3$) bearing U87MG tumors at each of the above-mentioned time points. Biodistribution studies were also performed on these mice after the imaging studies were completed. SCID mice were injected through the tail vein with 100 μL (~ 3.70 MBq/animal) of cRGDfK-Orn₃-[CGG-^{99m}Tc] diluted in saline (pH 7.0), and the spatiotemporal localization of the ^{99m}Tc-labeled RGD was calculated.

Biodistribution studies. Biodistribution studies were performed in combination with scintigraphic imaging of radiolabeled RGD peptides. When dynamic scintigraphic imaging was completed at 1 hour postinjection (p.i.), the tumor-bearing SCID mice were sacrificed by ether anesthesia. Groups of SCID mice ($n=3-5$) were sacrificed on each day

FIG. 2. Biodistribution results of cRGDfK-Orn₃-[CGG-^{99m}Tc] in U87MG tumor-bearing mice ($n=3-5$) at 1 hour p.i. (% ID/g, injected dose per gram; p.i., postinjection).

FIG. 4. Representative planar γ images of U87MG tumor-bearing mice injected with 3.70 MBq of cRGDFK-Orn₃-[CGG- ^{99m}Tc] at 1 hour p.i. on different days of tumor growth ($n=3$). The arrows indicate the presence of the tumor, kidneys, and bladder.

designated for assessment of tumor growth. The main organs were subsequently removed and, together with samples of muscles and urine, were weighed and counted in a NaI well counter. Stomach and intestines were not emptied before the measurements. Uptake of the radiotracer in each tissue was calculated and expressed as the percentage of injected dose per gram of the tissue (% ID/g). The % ID in whole blood was estimated assuming a whole-blood volume of 6.5% of the total body weight. Biodistribution data are reported as % ID/g and are presented as mean $\pm$ SD ($n=3-5$).

Imaging studies. Determination of the spatiotemporal localization of cRGDFK-Orn₃-[CGG- ^{99m}Tc] was performed in different groups of SCID mice bearing U87MG tumors by dynamic scintigraphic imaging on different days of angiogenesis. After injecting 100 μL (~ 3.70 MBq) of radiolabeled peptide intravenously into the tail vein, the animals were anesthetized. Immediately after radiotracer injection, anesthesia was induced by intraperitoneal administration of 100 μL /10 g body weight of a stock solution containing 0.5 mL ketamine-hydrochloride (100 mg/mL), 0.25 mL xylazine-hydrochloride (20 mg/mL), and 4.25 mL NaCl 0.9%. Dynamic images of

the injected mice were obtained from 4 to 10 minutes up to 1 hour p.i.

Data were continuously acquired and sequential images of 2-minutes frames were stored for all animals. All images were planar, scintigraphic, and no tomographic reconstruction was performed. The raw data obtained by the imaging system have a size of 50×100 pixels, which corresponds to an area of 50×100 mm. The images were stored in raw format and processed with ImageJ open source software (version 1.47c; NIH). Then, they were linearly interpolated in ImageJ, to provide a smoother final image with 250×500 pixels. No smoothing algorithm was used. ImageJ is also used to select the colormap and enhance image contrast at a certain level where the organs/structures of interest can be distinguished. All successive 2-minute frames were summed to achieve an image with high statistics, on which regions of interest (ROIs) were drawn. Then, those ROIs were applied to the 2-minute frames to provide semiquantitative time-activity curves. The percentage of the injected dose per organ (%ID/org) was calculated. Accordingly, the %ID/g in tumors was estimated based on the weight of the organs.

Statistical analyses

The biodistribution data are presented as mean %ID/g $\pm$ SD ($n=3-5$) and were statistically analyzed with Student's *t*-test. Analyses were two-tailed, with a *p*-value < 0.05 being considered statistically significant and $p = 0.1$ as not significant.

Results and Discussion

Evaluation of angiogenesis with biodistribution and imaging techniques

The new RGD derivative was efficiently labeled with ^{99m}Tc through the CGG chelating moiety with a high-specific activity of 5.55 GBq/ μmol . The assessment of GBM tumor growth as a function of time in a U87MG GBM tumor-bearing mouse model was studied by using the radiolabeled peptide cRGDFK-Orn₃-[CGG- ^{99m}Tc] from the day of

TABLE 2. COMPARISON OF TUMOR UPTAKE OF cRGDFK-Orn₃-[CGG- ^{99m}Tc] IN U87MG TUMOR-BEARING MICE AT 1 HOUR POSTINJECTION

	Imaging (ID/g [%])	Biodistribution (ID/g [%])
Day 0	0 $\pm$ 0	0 $\pm$ 0.0
Day 3	3.0 $\pm$ 0.4	2.3 $\pm$ 0.3
Day 9	5.0 $\pm$ 1.5	3.8 $\pm$ 1.7
Day 21	7.4 $\pm$ 2.0	11.6 $\pm$ 2.1
Day 29	3.8 $\pm$ 0.4	5.3 $\pm$ 0.2
Day 35	3.4 $\pm$ 0.4	3.9 $\pm$ 0.5

Data obtained from imaging ($n=3$) and biodistribution ($n=3-5$) studies.

ID/g, injected dose per gram.

inoculation of cancer cells (day 0) until tumors reached a volume of $\sim 3 \text{ cm}^3$ (day 35). In Table 1, the mean values of animal weight and tumor weight on various angiogenesis stages during tumor development are presented. Figure 1 shows the time course profile of glioblastoma growth in U87MG tumor-bearing mice for the experimental period of 35 days.

In this study, biodistribution studies (Fig. 2) were performed on different days of U87MG tumor growth. The significantly high tumor uptake of the radiolabeled RGD derivative at 1 hour p.i. on day 21 of angiogenesis ($11.60\% \pm 2.05\%$ ID/g) can be attributed to the peak of angiogenesis.^{34,51,52} Significantly, ($p < 0.05$) low tumor uptake on days 3 and 9 of the study in relation to day 21 can be attributed to the early vascular stage of the tumor.^{51–53} On the other hand, reduced tumor uptake on days 29 ($p = 0.1$) and 35 ($p < 0.05$) relative to day 21 can be attributed to the presence of hypoxic/necrotic regions in the tumor resulting in reduced expression of $\alpha_v\beta_3$ receptors as well as to the maturation of the vascular bed network, elimination, and other factors affecting late angiogenesis development. High kidney values were observed during the whole study, reflecting the elimination route of the radiolabeled compound. Uptake in all other organs such as the liver, stomach, intestines, and muscles was insignificant.

Relative tracer uptake expressed as tumor-to-blood, tumor-to-muscle, tumor-to-intestines, tumor-to-heart, and tumor-to-liver ratios at 1 hour p.i. was plotted versus days of tumor growth (Fig. 3). The high tumor-to-background ratios were mainly attributed to high tumor uptake in combination with low background. More specifically on day 21 of the study, the tumor-to-muscle and tumor-to-intestines contrast ratios were very high with values of 10.5 and 6.2, respectively.

Dynamic imaging studies of anesthetized U87MG tumor-bearing mice were performed on a high-resolution, dedicated small animal γ -camera, up to 1 hour p.i. versus several days of tumor development (Fig. 4). The experimental tumor at the left anterior leg was much more clearly delineated between 21 and 35 days of the under-study tumor growth period. ROI analysis was performed on the collected planar images to obtain semiquantitative information and the relative ID/g (%) tumor values were calculated. Imaging and biodistribution studies were in good agreement and indicated that the highest tumor uptake of cRGDfK-Orn₃-[CGG-^{99m}Tc] in U87MG tumor-bearing mice was observed ~ 3 weeks after inoculation (day 21) (Table 2).

According to the *in vivo* imaging and biodistribution studies, the predominant excretion route of the cRGDfK-Orn₃-[CGG-^{99m}Tc] was through the urinary system, which is in agreement with the initial study of the radiopeptide.³¹ This hydrophilic RGD derivative had been appropriately designed to improve excretion kinetics and consequently to reduce accumulation of radioactivity at the upper abdominal area.

A method of understanding GBM tumor growth and its relationship with $\alpha_v\beta_3$ integrin expression by using a high-resolution γ -camera has been demonstrated. Tumor growth assessment by this kind of noninvasive imaging could be useful in directing physicians to continue or modify the

therapeutic protocol of choice. Experimentally, the application of this radiotracer for tracking the evolution of cancer in different types of malignant tumors remains to be explored without necessitating animal euthanization as was confirmed by these findings. Nevertheless, a correlation of imaging with histochemical analysis of tumor tissues for distinguishing the vascular growth from integrin expression or for examining the heterogeneity during tumor growth would be undoubtedly very useful as a complementary tool.

The novel RGD derivative, cRGDfK-Orn₃-CGG, is considered to be appropriately designed for radiolabeling with ^{99m}Tc for monitoring of $\alpha_v\beta_3$ integrin-positive glioblastoma tumor growth *in vivo*. Furthermore, a work on the conjugation of this peptide to magnetic nanoparticles, which will lead to a dual-modality SPECT/MRI contrast agent that can also act as a drug nanocarrier as well as a hypothermia therapeutic agent, has been recently accepted for publication.^{54–56}

Conclusions

A novel way of distinguishing the intrinsic relationship between $\alpha_v\beta_3$ integrin targeting and detection of tumor growth by using a radiolabeled tracer based on a cyclic RGD peptide was provided. The potential of the *in vivo* scintigraphic imaging of the developing vasculature from the early stage of tumor growth was evaluated. The ability to noninvasively assess the tumor growth of $\alpha_v\beta_3$ integrin-positive glioblastoma tumors provides a method to better understand tumor angiogenesis *in vivo* and allows for a direct assessment of anti-integrin treatment efficacy.

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Disclosure Statement

No conflicting financial interests exist.

References

1. Eliceiri BP, Cheresh DA. The role of α_v integrins during angiogenesis: Insights into potential mechanisms of action and clinical development. *J Clin Invest* 1999;103:1227.
2. Folkman J. Angiogenesis and apoptosis. *Semin Cancer Biol* 2003;13:159.
3. Harris AL. Angiogenesis as a new target for cancer control. *Eur J Cancer* 2003;Supplements 1:1.
4. Folkman J. Tumor angiogenesis: Therapeutic implications. *N Engl J Med* 1971;285:1182.
5. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr Rev* 2003;24:389.
6. Lee HY, Jeong JM. Multimodality imaging of angiogenesis. *Open Nucl Med J* 2010;2:166.

7. Kagadis GC, Loudos G, Katsanos K, et al. In vivo small animal imaging: Current status and future prospects. *Med Phys* 2010;37:6421.
8. Munley MT, Kagadis GC, McGee KP, et al. An introduction to molecular imaging in radiation oncology: A report by the AAPM Working Group on Molecular Imaging in Radiation Oncology (WGMIR). *Med Phys* 2013;40:101501.
9. Jeswani T, Padhani AR. Imaging tumour angiogenesis. *Cancer Imaging* 2005;5:131.
10. Ruoslahti E, Pierschbacher MD. Arg-Gly-Asp: A versatile cell recognition signal. *Cell* 1986;44:517.
11. Zitzmann S, Ehemann V, Schwab M. Arginine-glycine-aspartic acid (RGD)-peptide binds to both tumor and tumor-endothelial cells *in vivo*. *Cancer Res* 2002;62:5139.
12. Barczyk M, Carracedo S, Gullberg D. Integrins. *Cell Tissue Res* 2010;339:269.
13. Schottelius M, Laufer B, Kessler H, et al. Ligands for mapping alphavbeta3-integrin expression *in vivo*. *Acc Chem Res* 2009;42:969.
14. Weis SM, Cheresh DA. alphaV integrins in angiogenesis and cancer. *Cold Spring Harb Perspect Med* 2011;1:a006478.
15. DeAngelis LM. Brain tumors. *N Engl J Med* 2001;344:114.
16. Kim KW. Brain angiogenesis in developmental and pathological processes. *FEBS J* 2009;276:4621.
17. Jo J, Schiff D, Purow B. Angiogenic inhibition in high-grade gliomas: Past, present and future. *Expert Rev Neurother* 2012;12:733.
18. Puduvalli VK, Yung AW. New frontiers in therapy of malignant gliomas. *Forum (Genova)* 1998;8:261.
19. Haubner R, Wester HJ. Radiolabeled tracers for imaging of tumor angiogenesis and evaluation of anti-angiogenic therapies. *Curr Pharm Des* 2004;10:1439.
20. Lim EH, Danthi N, Bednarski M, et al. A review: Integrin alphavbeta3-targeted molecular imaging and therapy in angiogenesis. *Nanomedicine* 2005;1:110.
21. Bach-Gansmo T, Bogsrud TV, Skretting A. Integrin scintimammography using a dedicated breast imaging, solid-state gamma-camera and (99m)Tc-labelled NC100692. *Clin Physiol Funct Imaging* 2008;28:235.
22. Beer AJ, Haubner R, Goebel M, et al. Biodistribution and pharmacokinetics of the alphavbeta3-selective tracer 18F-galacto-RGD in cancer patients. *J Nucl Med* 2005;46:1333.
23. Chakraborty S, Sarma HD, Vimalnath KV, et al. Tracer level radiochemistry to clinical dose preparation of (177)Lu-labeled cyclic RGD peptide dimer. *Nucl Med Biol* 2013;40:946.
24. Chen X, Park R, Khankaldyyan V, et al. Longitudinal microPET imaging of brain tumor growth with F-18-labeled RGD peptide. *Mol Imaging Biol* 2006;8:9.
25. Chen X, Park R, Shahinian AH, et al. Pharmacokinetics and tumor retention of 125I-labeled RGD peptide are improved by PEGylation. *Nucl Med Biol* 2004;31:11.
26. Hua J, Dobrucki LW, Sadeghi MM, et al. Noninvasive imaging of angiogenesis with a 99mTc-labeled peptide targeted at alphavbeta3 integrin after murine hindlimb ischemia. *Circulation* 2005;111:3255.
27. Jacobson O, Zhu L, Niu G, et al. MicroPET imaging of integrin alphavbeta3 expressing tumors using 89Zr-RGD peptides. *Mol Imaging Biol* 2011;13:1224.
28. Janssen M, Frielink C, Dijkgraaf I, et al. Improved tumor targeting of radiolabeled RGD peptides using rapid dose fractionation. *Cancer Biother Radiopharm* 2004;19:399.
29. Jeong JM, Hong MK, Chang YS, et al. Preparation of a promising angiogenesis PET imaging agent: 68Ga-labeled c(RGDyK)-isothiocyanatobenzyl-1,4,7-triazacyclononane-1,4,7-triacetic acid and feasibility studies in mice. *J Nucl Med* 2008;49:830.
30. Liu S. Radiolabeled cyclic RGD peptides as integrin alpha(v)beta(3)-targeted radiotracers: Maximizing binding affinity via bivalency. *Bioconjug Chem* 2009;20:2199.
31. Tsiapa I, Loudos G, Varvarigou A, et al. Biological evaluation of an ornithine-modified (99m)Tc-labeled RGD peptide as an angiogenesis imaging agent. *Nucl Med Biol* 2013;40:262.
32. Fragogeorgi EA, Zikos C, Gourni E, et al. Spacer site modifications for the improvement of the in vitro and in vivo binding properties of (99m)Tc-N(3)S-X-bombesin[2–14] derivatives. *Bioconjug Chem* 2009;20:856.
33. Gourni E, Bouziotis P, Benaki D, et al. Structural assessment and biological evaluation of two N3S bombesin derivatives. *J Med Chem* 2009;52:4234.
34. Liolios CC, Fragogeorgi EA, Zikos C, et al. Structural modifications of (9)(9m)Tc-labelled bombesin-like peptides for optimizing pharmacokinetics in prostate tumor targeting. *Int J Pharm* 2012;430:1.
35. Wong E, Fauconnier T, Bennett S, et al. Rhenium(V) and Technetium(V) Oxo complexes of an N(2)N'S peptidic chelator: Evidence of interconversion between the Syn and anti conformations. *Inorg Chem* 1997;36:5799.
36. Tsiapa I, Efthimiadou EK, Fragogeorgi EA, et al. 99mTc-Labeled aminosilane-coated iron oxide nanoparticles for molecular imaging of $\alpha v \beta 3$ -mediated tumor expression and feasibility for hyperthermia treatment. *J Colloid Interface Sci* 2014;433:163.
37. Shao G, Zhou Y, Wang F, et al. Monitoring glioma growth and tumor necrosis with the U-SPECT-II/CT scanner by targeting integrin alphavbeta3. *Mol Imaging* 2013;12:39.
38. Zhou Y, Shao G, Liu S. Monitoring breast tumor lung metastasis by U-SPECT-II/CT with an integrin alpha(v)beta(3)-targeted radiotracer(99m)Tc-3P-RGD(2). *Theranostics* 2012;2:577.
39. Liu S. Radiolabeled multimeric cyclic RGD peptides as integrin alphavbeta3 targeted radiotracers for tumor imaging. *Mol Pharm* 2006;3:472.
40. Liu S, Hsieh WY, Jiang Y, et al. Evaluation of a (99m)Tc-labeled cyclic RGD tetramer for noninvasive imaging integrin alpha(v)beta3-positive breast cancer. *Bioconjug Chem* 2007;18:438.
41. Wang J, Kim YS, Liu S. 99mTc-labeling of HYNIC-conjugated cyclic RGDfK dimer and tetramer using EDDA as coligand. *Bioconjug Chem* 2008;19:634.
42. Wang L, Shi J, Kim YS, et al. Improving tumor-targeting capability and pharmacokinetics of (99m)Tc-labeled cyclic RGD dimers with PEG(4) linkers. *Mol Pharm* 2009;6:231.
43. Zhou Y, Kim YS, Chakraborty S, et al. 99mTc-labeled cyclic RGD peptides for noninvasive monitoring of tumor integrin alphaVbeta3 expression. *Mol Imaging* 2011;10:386.
44. Decristoforo C, Santos I, Pietzsch HJ, et al. Comparison of in vitro and in vivo properties of [99mTc]cRGD peptides labeled using different novel Tc-cores. *Q J Nucl Med Mol Imaging* 2007;51:33.
45. Jia B, Liu Z, Shi J, et al. Linker effects on biological properties of 111In-labeled DTPA conjugates of a cyclic RGDfK dimer. *Bioconjug Chem* 2008;19:201.

46. Shi J, Kim YS, Chakraborty S, et al. 2-Mercaptoacetylglucylglycyl (MAG2) as a bifunctional chelator for ^{99m}Tc -labeling of cyclic RGD dimers: Effect of technetium chelate on tumor uptake and pharmacokinetics. *Bioconjug Chem* 2009;20:1559.
47. Alves S, Correia JD, Gano L, et al. In vitro and in vivo evaluation of a novel $^{99m}\text{Tc}(\text{CO})_3$ -pyrazolyl conjugate of cyclo-(Arg-Gly-Asp-d-Tyr-Lys). *Bioconjug Chem* 2007;18:530.
48. Oliveira EA, Faintuch BL, Nunez EG, et al. Radiotracers for different angiogenesis receptors in a melanoma model. *Melanoma Res* 2012;22:45.
49. Psimadas D, Fani M, Gourni E, et al. Synthesis and comparative assessment of a labeled RGD peptide bearing two different (9)(9) ^{m}Tc -tricarboxyl chelators for potential use as targeted radiopharmaceutical. *Bioorg Med Chem* 2012;20:2549.
50. Loudos G, Majewski S, Wojcik R, et al. Performance evaluation of a dedicated camera suitable for dynamic radiopharmaceuticals evaluation in small animals. *IEEE Trans Nucl Sci* 2007;54:454.
51. Katsanos K, Karnabatidis D, Diamantopoulos A, et al. Thrombin promotes arteriogenesis and hemodynamic recovery in a rabbit hindlimb ischemia model. *J Vasc Surg* 2009;49:1000.
52. Vajkoczy P, Schilling L, Ullrich A, et al. Characterization of angiogenesis and microcirculation of high-grade glioma: An intravital multicolor fluorescence microscopic approach in the athymic nude mouse. *J Cereb Blood Flow Metab* 1998;18:510.
53. Wen S, Stolarov J, Myers MP, et al. PTEN controls tumor-induced angiogenesis. *Proc Natl Acad Sci U S A* 2001;98:4622.
54. de Barros AB, Tsourkas A, Saboury B, et al. Emerging role of radiolabeled nanoparticles as an effective diagnostic technique. *EJNMMI Res* 2012;2:39.
55. Ting G, Chang CH, Wang HE, et al. Nanotargeted radio-nuclides for cancer nuclear imaging and internal radio-therapy. *J Biomed Biotechnol* 2010;2010.
56. Loudos G, Kagadis GC, Psimadas D. Current status and future perspectives of *in vivo* small animal imaging using radiolabeled nanoparticles. *Eur J Radiol* 2011;78:287.