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Evaluation of ^{111}In -Labeled Cyclic RGD Peptides: Tetrameric Not Tetravalent

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Abstract

This report presents the synthesis and evaluation of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetracetic acid; 6G-RGD₄ = E{G₃-E[G₃-c(RGDfK)]₂}₂ and G₃ = Gly-Gly-Gly), $^{111}\text{In}(\text{DOTA-RGD}_4)$ (RGD₄ = E{E[c(RGDfK)]₂}₂) and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ (3G-RGD₂ = G₃-E[G₃-c(RGDfK)]₂) as new radiotracers for imaging integrin $\alpha_v\beta_3$ -positive tumors. The IC₅₀ values of DOTA-6G-RGD₄, DOTA-RGD₄ and DOTA-3G-RGD₂ were determined to be 0.4 ± 0.1 , 1.5 ± 0.2 and 1.3 ± 0.2 nM against ^{125}I -c(RGDyK) bound to integrin $\alpha_v\beta_3$ -positive U87MG human glioma cells. $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$, $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ were prepared by reacting $^{111}\text{InCl}_3$ with the respective DOTA conjugate in NH₄OAc buffer (100 mM, pH = 5.5). Radiolabeling could be completed by heating the reaction mixture at 100 °C for 15 – 20 min. The specific activity was ~1850 MBq/μmol for $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ and ~1480 MBq/μmol for $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$. The athymic nude mice bearing U87MG human glioma xenografts were used to evaluate tumor uptake and excretion kinetics of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$, $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$. The results from both the integrin $\alpha_v\beta_3$ binding assay and biodistribution studies suggest that the tetrameric cyclic RGD peptides, such as RGD₄ and 6G-RGD₄, are most likely bivalent in binding to the integrin $\alpha_v\beta_3$. Both $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-RGD}_4)$ had significantly higher tumor uptake than $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ at 24 – 72 h post-injection due to the extra RGD motifs in RGD₄ and 6G-RGD₄. $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ had very little metabolism while $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ had a significant metabolism during its excretion via both renal and hepatobiliary routes over the 2 h period, probably due to its much larger size. The combination of high tumor uptake with long tumor retention suggests that their corresponding ^{90}Y and ^{177}Lu analogs M(DOTA-6G-RGD₄) (M = ^{90}Y and ^{177}Lu) might be useful as therapeutic radiotracers for treatment of integrin $\alpha_v\beta_3$ -positive solid tumors.

Keywords

integrin $\alpha_v\beta_3$; ^{111}In -labeled cyclic RGD peptides; tumor imaging

INTRODUCTION

Radiolabeled cyclic RGD (Arg-Gly-Asp) peptides represent a new class of radiotracers that target the integrin $\alpha_v\beta_3$ overexpressed on both tumor cells and activated endothelial cells of the neovasculature during tumor growth, invasion and metastasis (1–9). Over last several years, many multimeric cyclic RGD peptides, such as E[c(RGDfK)]₂ (RGD₂) and E{E[c

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(RGDfK)]₂)₂ (RGD₄), have been used to maximize the integrin $\alpha_v\beta_3$ binding affinity and improve the radiotracer tumor uptake (10–30). The results from in vitro assays, ex-vivo biodistribution and in vivo imaging studies clearly demonstrated that radiolabeled (^{99m}Tc, ¹⁸F, ⁶⁴Cu and ⁶⁸Ga) cyclic RGD peptide tetramers, such as E{E[c(RGDxK)]₂}₂ (x = f and y), have better tumor targeting capability as evidenced by their higher tumor uptake and longer tumor retention times than their dimeric and monomeric counterparts (25,26,29,30). In our previous studies (31–37), we found that RGD₂ was monodentate while PEG₄-E[PEG₄-c(RGDfK)]₂ (3P-RGD₂: PEG₄ = 15-amino-4,7,10,13-tetraoxapentadecanoic acid) and (G₃-E[G₃-c(RGDfK)]₂) (3G-RGD₂: G₃ = Gly-Gly-Gly) and are bivalent due to the longer distance between two RGD motifs. However, it remains unclear if the cyclic RGD tetramers, such as RGD₄, are tetravalent in binding to integrin $\alpha_v\beta_3$.

To answer this fundamental question, we prepared a new RGD tetramer conjugate, DOTA-E{G₃-E[G₃-c(RGDfK)]₂}₂ (Figure 1: DOTA-6G-RGD₄: DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and its ¹¹¹In complex ¹¹¹In(DOTA-6G-RGD₄). A competitive displacement assay was used to determine the integrin $\alpha_v\beta_3$ binding affinity of DOTA-3G-RGD₂, DOTA-RGD₄ and DOTA-6G-RGD₄ against ¹²⁵I-c(RGDyK) bound to the integrin $\alpha_v\beta_3$ -positive U87MG glioma cells. Biodistribution and imaging studies were carried out in athymic nude mice bearing U87MG glioma xenografts. For comparison purposes, we also evaluated DOTA-RGD₄, DOTA-3G-RGD₂, ¹¹¹In(DOTA-RGD₄) and ¹¹¹In(DOTA-3G-RGD₂) using the same assays. The comparison of IC₅₀ values of DOTA-3G-RGD₂, DOTA-RGD₄ and DOTA-6G-RGD₄ and tumor uptake of ¹¹¹In(DOTA-6G-RGD₄), ¹¹¹In(DOTA-RGD₄) and ¹¹¹In(DOTA-3G-RGD₂) will allow us to determine the valency of RGD₄ and 6G-RGD₄.

EXPERIMENTAL SECTION

Materials and Instruments

Chemicals were purchased from *Sigma/Aldrich* (St. Louis, MO). Cyclic RGD peptide 3G-RGD₂ was purchased from the Peptides International, Inc. (Louisville, KY). DOTA-OSu (1,4,7,10-tetraazacyclododecane-4,7,10-triacetic acid-1-acetate(N-hydroxysuccinamide)) was purchased from Macrocyclics (Dallas, TX). DOTA-3G-RGD₂ and DOTA-RGD₄ were prepared according to the literature methods (14,26,33). MALDI (Matrix Assisted Laser Desorption Ionizations) Mass spectral data were collected using an Applied Biosystems Voyager DE PRO mass spectrometer (Framingham, MA), Department of Chemistry, Purdue University. ¹¹¹InCl₃ was obtained from Perkin-Elmer Life and Analytical Sciences (North Billerica, MA). The NH₄OAc buffer for ¹¹¹In-labeling studies was passed over a Chelex-100 column (1×15 cm) to minimize the trace metal contaminants.

HPLC Methods

Method 1 used a LabAlliance HPLC system equipped with a UV/vis detector (λ =254 nm) and Zorbax C₁₈ semi-prep column (9.4 mm × 250 mm, 100 Å pore size; Agilent Technologies, Santa Clara, CA). The flow rate was 2.5 mL/min and the mobile phase was isocratic with 90% A (0.1% TFA in water) and 10% B (0.1% TFA in acetonitrile) at 0–5 min, followed by a gradient mobile phase going from 10% B at 5 min to 40% B at 20 min. Method 2) used the LabAlliance HPLC system equipped with a β -ram IN/US detector (Tampa, FL) and Zorbax C₁₈ column (4.6 mm × 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% A (25 mM NH₄OAc, pH = 6.8) and 10% B (acetonitrile) at 0–5 min, followed by a gradient mobile phase from 10% B at 5 min to 20% B at 15 min and to 50% B at 20 min.

Boc-E{G₃-E[G₃-c(RGDfK)]₂}₂ (Boc-6G-RGD₄)

The succinimidyl ester of Boc-protected glutamic acid (Boc-E(OSu)₂) was prepared according to the literature method (12). To a solution of Boc-E(OSu)₂ (2.1 mg, 4.75 μmol) in DMF (2 mL) was added 3G-RGD₂ (21 mg, 11.5 μmol). After addition of DIEA (3 drops), the reaction mixture was stirred for 2 h at room temperature. The reaction was terminated by addition of 3 mL NH₄OAc buffer (100 mM, pH = 7.0). The product was isolated by HPLC (Method 1). Lyophilization of the collected fractions at 15.8 min afforded desired product Boc-6G-RGD₄. The yield was 7.6 mg (~41%). MALDI-MS: m/z = 3877 for [M + H]⁺ (M = 3875 calcd. for [C₁₆₄H₂₄₁N₅₇O₅₄]⁺).

E{G₃-E[G₃-c(RGDfK)]₂}(6G-RGD₄)

Boc-6G-RGD₄ was dissolved in TFA (2 mL). After standing at room temperature for 5 min, excess TFA was removed. The residue was dissolved in 2 mL of 0.1 M NH₄OAc buffer (100 mM, pH = 7.0). The product was separated from the mixture by HPLC (Method 1). Lyophilization of the collected fractions at 14.2 min afforded 6G-RGD₄. The yield was 5.6 mg (~76%). MALDI-MS: m/z = 3777 for [M + H]⁺ (M = 3775 calcd. for [C₁₅₉H₂₃₃N₅₇O₅₂]⁺).

DOTA-E{G₃-E[G₃-c(RGDfK)]₂}(DOTA-6G-RGD₄)

DOTA-OSu (5.0 mg, 10.0 μmol) and 6G-RGD₄ (3.8 mg, 1.0 μmol) were dissolved in anhydrous DMF (1 mL). After addition of excess DIEA (3 drops), the reaction mixture was stirred for 2 h at room temperature. Upon addition of 2 mL NH₄OAc buffer (100 mM, pH = 7.0), the product was separated by HPLC (Method 1). Lyophilization of the collected fractions at 14.5 min afforded DOTA-6G-RGD₄ with >95% purity. The yield was 3.2 mg (~76%). MALDI-MS: m/z = 4163 for [M + H]⁺ (M = 4161 calcd. for [C₁₇₅H₂₅₉N₆₁O₅₉]⁺).

¹¹¹In-Labeling and Dose Preparation

To a clean Eppendorf tube were added 200–300 μL of the DOTA conjugate solution (0.1 mg/mL in 0.1 M NaOAc buffer, pH = 5.5) and 20 μL of ¹¹¹InCl₃ solution (~18.5 MBq in 0.05 M HCl). The vial was heated in a water bath at 100 °C for ~15 min. After heating, the Eppendorf tube was allowed to stand at room temperature for ~10 min. A sample of the resulting solution was analyzed by radio-HPLC (Method 2). For biodistribution studies, the ¹¹¹In radiotracer was purified by HPLC (Method 2). Volatiles in the mobile phase were removed under vacuum (~5 mmHg) at room temperature. Doses were prepared by dissolving the purified radiotracer in saline to a concentration of ~1.1 MBq/mL. For imaging studies, doses were prepared by dissolving the radiotracer in saline to ~37 MBq/mL. The resulting solution was filtered with a 0.20 micron Millex-LG filter before being injected into animals. Each animal was injected with ~0.1 mL of the dose solution.

Determination of Octanol/Water Partition Coefficients

The octanol/water partition coefficients were determined using the following procedure. In short, the ¹¹¹In radiotracer was purified by HPLC. Volatiles were removed completely under vacuum. The residue was dissolved in an equal volume (3 mL:3 mL) mixture of n-octanol and 25 mM phosphate buffer (pH = 7.4). After stirring vigorously for ~20 min, the mixture was centrifuged at a speed of 8,000 rpm for 5 min. Samples (in triplets) from both n-octanol and aqueous layers were counted in a Perkin Elmer Wizard – 1480 γ-counter (Shelton, CT). The partition coefficients were calculated. The log P values were measured three different times and reported as an average of three independent measurements plus the standard deviation.

In Vitro Whole-Cell Integrin $\alpha_v\beta_3$ Binding Assay

The in vitro integrin binding affinity and specificity of RGD peptides were assessed using ^{125}I -c(RGDyK) as the integrin-specific radioligand. Briefly, U87MG human glioma cells were grown in Gibco's Dulbecco's medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen Co, Carlsbad, CA), at 37 °C in humidified atmosphere containing 5% CO_2 . Filter multiscreen DV plates were seeded with 10^5 glioma cells in binding buffer and incubated with ^{125}I -c(RGDyK) in the presence of increasing concentrations of the RGD conjugate. After removing the unbound ^{125}I -c(RGDyK), hydrophilic PVDF filters were collected. The radioactivity was determined using a γ -counter (Packard, Meriden, CT). The IC_{50} values were calculated by fitting the data by nonlinear regression using GraphPad PrismTM (GraphPad Software, Inc., San Diego, CA). Experiments were carried out twice with triplicate samples. The IC_{50} values are reported as an average plus the standard deviation.

Animal Model

Biodistribution and imaging studies were performed using the athymic nude mice bearing U87MG human glioma xenografts in compliance NIH animal experiment guidelines (*Principles of Laboratory Animal Care*, NIH Publication No. 86-23, revised 1985). The animal protocol was approved by the Purdue University Animal Care and Use Committee. U87MG cells were cultured in the Minimum Essential Medium, Eagle with Earle's Balanced Salt Solution (non-essential amino acids sodium pyruvate) (ATCC, Manassas, VA) in humidified atmosphere of 5% carbon dioxide, and were supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin solution (GIBCO, Grand Island, NY). Female athymic nu/nu mice were purchased from Harlan (Indianapolis, IN) at 4 – 5 weeks of age. Each mouse was implanted subcutaneously with 5×10^6 tumor cells into the left and right upper flanks. In this way, one could assess the impact of tumor size on the radiotracer imaging quality in a single tumor-bearing mouse. All procedures will be performed in a laminar flow cabinet using aseptic technique. Three to four weeks after inoculation, the tumor size was 0.05 – 0.4 g, and animals were used for biodistribution and imaging studies.

Biodistribution Protocol

Twenty-five tumor-bearing mice (20 – 25 g) were randomly divided into five groups. Each tumor-bearing mouse was administered with ~111 KBq of the ^{111}In radiotracer by tail vein injection. Five animals were sacrificed by sodium pentobarbital overdose (~200 mg/kg) at 0.5, 1, 4, 24 and 72 h postinjection (p.i.). Blood samples were withdrawn from the heart of the tumor-bearing mice. Tumor and normal organs (brain, eyes, heart, spleen, lungs, liver, kidneys muscle and intestine) were harvested, washed with saline, dried with absorbent tissue, weighed, and counted on a γ -counter (Perkin Elmer Wizard – 1480, Shelton, CT). The organ uptake was calculated as the percentage of injected dose per gram of organ mass (%ID/g) or the percentage of injected dose per organ (%ID/organ). For the blocking experiment, five tumor-bearing athymic nude mice (20 – 25 g) were used, and each animal was administered with ~111 KBq of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ along with ~350 μg (~14 mg/kg) of RGD_2 . Such a high dose of blocking agent was used to make sure that the integrin $\alpha_v\beta_3$ binding was completely blocked. At 1 h p.i., all five tumor-bearing animals were sacrificed for organ biodistribution. Biodistribution data (%ID/g) and target-to-background (T/B) ratios are reported as an average plus the standard variation based on the results from five tumor-bearing mice (10 tumors) at each time point. Comparison between two radiotracers was made using the two-way ANOVA test (GraphPad Prim 5.0, San Diego, CA). The level of significance was set at $p < 0.05$.

Planar Imaging

The athymic nude mice ($n = 3$) bearing U87MG glioma xenografts were used for imaging studies of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$. Each tumor-bearing mouse was administered with ~ 3.7 MBq of ^{111}In radiotracer via tail vein injection. Animals were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (19 mg/kg), and were then placed supine on a single head mini γ -camera (Diagnostic Services Inc., NJ) equipped with a parallel-hole, medium-energy and high-resolution collimator. Anterior images were acquired at 0.5, 1, 4, 24 and 72 h p.i. and stored digitally in a 128×128 matrix. The acquisition count limits were set at 200 K. After completion of imaging, animals were sacrificed by sodium pentobarbital overdose (~ 200 mg/kg).

Metabolism

Normal nude mice were used for metabolism studies. Each mouse was administered with ~ 3.7 MBq of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ and $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ via tail vein. The urine samples were collected at 30 min and 120 min p.i. by manual void, and were mixed with equal volume of 50% acetonitrile aqueous solution. The mixture was centrifuged at 8,000 rpm. The supernatant was collected and passed through a $0.20 \mu\text{m}$ Millex-LG filter unit. The filtrate was analyzed by HPLC (Method 2). Feces, liver and kidney samples were collected at 120 min p.i. and suspended in 20% acetonitrile aqueous solution after homogenization. The resulting mixture was vortexed for 5 – 10 min. After centrifuging at 8,000 rpm, the supernatant was collected and passed through a $0.20 \mu\text{m}$ Millex-LG filter unit. The filtrate was analyzed by radio-HPLC (Method 2). The percentage radioactivity recovery was $>95\%$ (by γ -counting) for both urine and feces samples.

RESULTS

DOTA-6G-RGD₄

Synthesis of DOTA-6G-RGD₄ was straightforward. The key step was the deprotection of Boc-6G-RGD₄. The excess TFA must be removed within 10 min of the reaction. Otherwise, the major product was the hydrolyzed product E-3G-RGD₂ ($\text{E-G}_3\text{-E}[\text{G}_3\text{-c(RGDfK)}]_2$) not 6G-RGD₄. Conjugation of 6G-RGD₄ with excess DOTA-OSu in DMF in the presence of excess DIEA afforded DOTA-6G-RGD₄ in high yield (76%). Its identity has been confirmed by MALDI-MS.

In Vitro Integrin $\alpha_v\beta_3$ Binding Affinity

The integrin $\alpha_v\beta_3$ binding affinities of c(RGDyK), DOTA-3G-RGD₂, DOTA-RGD₄ and DOTA-6G-RGD₄ were determined using the integrin $\alpha_v\beta_3$ -positive U87MG human glioma cells. Figure 2 shows displacement curves of ^{125}I -c(RGDyK) by c(RGDyK), DOTA-3G-RGD₂, DOTA-RGD₄ and DOTA-6G-RGD₄. Their IC_{50} values were calculated to be 42.2 ± 5.2 , 1.3 ± 0.2 , 1.5 ± 0.2 and 0.4 ± 0.1 nM, respectively.

Radiochemistry

$^{111}\text{In}(\text{DOTA-6G-RGD}_4)$, $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ were prepared by reacting $^{111}\text{InCl}_3$ with DOTA-6G-RGD₄, DOTA-RGD₄ and DOTA-3G-RGD₂, respectively, in NH_4OAc buffer (100 mM, pH = 5.5). Radiolabeling was completed by heating the reaction mixture at 100°C for ~ 15 min. The radiochemical purity was $>92\%$ for all three ^{111}In radiotracers. The specific activity was ~ 925 MBq/mg or (~ 1850 MBq/ μmol) for ^{111}In (DOTA-3G-RGD₂). They were analyzed using the same method (Method 2). Their HPLC retention times and log P values were listed in Table 1.

Biodistribution Characteristics

Selected biodistribution data are listed in Tables 2–4 for $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$, $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$. Figure 3 compares their uptake (%ID/g) in the tumor and intestine, as well as their tumor/kidney and tumor/liver ratios in the athymic nude mice bearing U87MG human glioma xenografts. The comparison of their tumor uptake in combination with the IC_{50} values of cyclic RGD peptide DOTA conjugates will allow us to determine if they are actually tetravalent in binding to the integrin $\alpha_v\beta_3$.

In general, $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ excreted predominantly via the renal route with >75% of the injected radioactivity recovered from urine and <10% of the injected radioactivity recovered from feces at 2 h p.i. $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ had a rapid blood clearance with high tumor/blood ratios (Table 2). It had a high initial tumor uptake (12.66 ± 1.25 %ID/g at 0.5 h p.i.) with a slow washout (11.45 ± 2.46 , 8.99 ± 2.61 , 6.68 ± 1.42 and 5.59 ± 0.54 %ID/g at 1, 4, 24 and 72 h p.i., respectively). The liver uptake of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ was low (3.04 ± 1.38 , 3.77 ± 0.79 , 3.05 ± 0.14 , 2.46 ± 0.36 and 2.01 ± 0.45 %ID/g at 0.5, 1, 4, 24 and 72 h p.i., respectively), and its tumor/liver ratios were high (4.16 ± 0.41 , 3.04 ± 0.65 , 2.95 ± 0.86 , 3.53 ± 0.58 and 2.09 ± 1.41 at 0.5, 1, 4, 24 and 72 h p.i., respectively). The kidney and muscle uptake of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ was also low with high tumor/kidney and tumor/muscle ratios (Table 2). The tumor uptake of $^{111}\text{In}(\text{DOTA-RGD}_4)$ was high (8.97 ± 0.52 , 7.98 ± 2.36 , 7.32 ± 2.66 , 5.84 ± 1.09 and 3.16 ± 0.43 %ID/g at 0.5, 1, 4, 24 and 72 h p.i., respectively), but its tumor retention time was not as long as that of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ (Figure 3). $^{111}\text{In}(\text{DOTA-RGD}_4)$ had a rapid blood clearance with high tumor/blood ratios over the 72 h period (Table 3). The initial kidney uptake (Figure 3) of $^{111}\text{In}(\text{DOTA-RGD}_4)$ was significantly higher than that of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$. However, there was no significant difference between $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ in their kidney uptake at >1 h p.i. $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ shared very similar liver uptake and tumor/liver ratio (Figure 3). $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ also had a rapid blood clearance with high tumor/blood ratios (Table 4). Its initial tumor uptake (10.19 ± 3.61 %ID/g at 0.5 h p.i.) was comparable to that of $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ within the experimental error; but its tumor uptake was significantly ($p < 0.01$) lower (Figure 3: tumor uptake = 5.59 ± 0.54 %ID/g for $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$, 3.16 ± 0.43 %ID/g for $^{111}\text{In}(\text{DOTA-RGD}_4)$ and 1.99 ± 0.39 %ID/g for $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ at 72 h p.i.). The uptake of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ in intestine, kidneys and liver was also significantly ($p < 0.05$) lower than that of $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$. As a result, the tumor/kidney and tumor/liver ratios of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ were higher than that of both $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ (Figure 3).

Tumor Size vs. Tumor Uptake

As illustrated in Figure 4A, there was a linear relationship between the %ID tumor uptake of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ at 60 min p.i. and tumor size (0.03 – 0.60 g; $n = 12$) with $R^2 = 0.959$. As the tumor became larger, the total tumor uptake of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ increased. In contrast, the %ID/g tumor uptake (Figure 4B) of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ decreased as the tumor size increased. For example, $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ has the tumor uptake in the range of 8.0 – 15 %ID/g with the tumor size being 0.05 – 0.30 g. When the tumor size is in the range of 0.5 – 0.6 g, the tumor uptake of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ is only ~6.0 %ID/g.

Integrin $\alpha_v\beta_3$ Specificity

Figure 5 compares the 60-min organ uptake of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ in the absence/presence of RGD₂. Clearly, co-injection of RGD₂ significantly blocked the tumor uptake of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ (1.23 ± 0.26 %ID/g with RGD₂ vs. 11.45 ± 2.36 %ID/g without RGD₂ at 1 h p.i.). Its uptake in normal organs was also significantly blocked by co-injection

of excess RGD₂. For example, the uptake of ¹¹¹In(DOTA-6G-RGD₄) in eyes, heart, intestine, liver, lungs, and spleen was 1.54 ± 0.22 , 1.49 ± 0.29 , 9.25 ± 1.27 , 3.77 ± 0.79 , 3.44 ± 1.86 and 2.38 ± 0.49 %ID/g, respectively, without RGD₂, while its uptake in the same organs was only 0.33 ± 0.06 , 0.66 ± 0.18 , 0.79 ± 0.10 , 0.79 ± 0.20 , 2.06 ± 1.12 , and 0.54 ± 0.09 %ID/g, respectively, in the presence of excess RGD₂.

Planar Imaging

Figure 6 illustrates the planar images of the glioma-bearing mice administered with ~3.7 MBq of ¹¹¹In(DOTA-6G-RGD₄) (top) ¹¹¹In(DOTA-3G-RGD₂) (bottom) and at 1, 4, 24 and 72 h p.i. The tumors could be clearly visualized with excellent T/B contrast as early as 1 h p.i. in the glioma-bearing mice administered with ¹¹¹In(DOTA-6G-RGD₄) and ¹¹¹In(DOTA-3G-RGD₂). Both ¹¹¹In(DOTA-6G-RGD₄) and ¹¹¹In(DOTA-3G-RGD₂) had a very long tumor retention time ($t_{1/2} > 30$ h), which was consistent with the results obtained from biodistribution studies (Tables 2 and 3). In addition, we also observed that the tumor distribution of ¹¹¹In(DOTA-3G-RGD₂) is quite heterogeneous most likely due to the heterogeneity of integrin $\alpha_v\beta_3$ overexpressed on tumor tissues.

Metabolism

Figure 7 shows representative radio-HPLC chromatograms of ¹¹¹In(DOTA-6G-RGD₄) and ¹¹¹In(DOTA-3G-RGD₂) in saline before injection, in urine at 30 min and 120 min p.i., and in feces at 120 min p.i. The percentage radioactivity recovery from the urine and feces samples was >95% for both radiotracers. Attempts to extract radioactivity from the liver and kidneys were not successful due to limited radioactivity accumulation in both organs. There was very little metabolite detected in either urine or feces samples over the 2 h period for ¹¹¹In(DOTA-3G-RGD₂). For ¹¹¹In(DOTA-6G-RGD₄), however, there was a significant metabolism during its excretion via both renal and hepatobiliary routes. Only ~25% of ¹¹¹In(DOTA-6G-RGD₄) remained intact in the feces sample.

DISCUSSION

In this study, we have prepared ¹¹¹In complexes of DOTA-6G-RGD₄, DOTA-RGD₄ and DOTA-3G-RGD₂. It was found that the integrin $\alpha_v\beta_3$ binding affinity follows the order of DOTA-6G-RGD₄ > DOTA-RGD₄ ~ DOTA-3G-RGD₂ >> c(RGDyK). Two factors contribute to the higher integrin $\alpha_v\beta_3$ binding affinity of DOTA-6G-RGD₄, DOTA-RGD₄ and DOTA-3G-RGD₂ as compared to c(RGDyK). Previously, we have clearly demonstrated that 3G-RGD₂ binds to the integrin $\alpha_v\beta_3$ in a bivalent fashion while RGD₂ is only monovalent due to the short distance (6 bonds excluding K residues) between two RGD motifs (31,33,37). The fact that DOTA-RGD₄ (IC₅₀ = 1.5 ± 0.2 nM) and DOTA-3G-RGD₂ (IC₅₀ = 1.3 ± 0.2 nM) shared almost identical IC₅₀ values strongly suggests that DOTA-RGD₄ is bivalent in binding to integrin $\alpha_v\beta_3$. This conclusion is supported by their similar tumor uptake (Figure 3), and is completely consistent with the biodistribution data obtained for the ^{99m}Tc-labeled cyclic RGD peptides 3G-RGD₂ and RGD₄ (31,37). Were DOTA-RGD₄ tetravalent, it would have had significantly higher integrin $\alpha_v\beta_3$ binding affinity than DOTA-3G-RGD₂ whereas ¹¹¹In(DOTA-RGD₄) would have had higher initial tumor uptake than ¹¹¹In(DOTA-3G-RGD₂).

DOTA-6G-RGD₄ (IC₅₀ = 0.4 ± 0.1 nM) has higher integrin $\alpha_v\beta_3$ binding affinity than DOTA-RGD₄ (IC₅₀ = 1.5 ± 0.2 nM). As a result, ¹¹¹In(DOTA-6G-RGD₄) has slightly higher initial tumor uptake (Figure 3) than ¹¹¹In(DOTA-RGD₄). Previously, we found that both bivalency and the “locally enhanced RGD concentration” contribute to the higher integrin $\alpha_v\beta_3$ binding affinity of 3G-RGD₂ and 3P-RGD₂ as compared to that of c(RGDfK) (31–37). We also found that the contribution from the bivalency to integrin $\alpha_v\beta_3$ binding affinity and radiotracer tumor uptake are much more significant than that from the “locally enhanced RGD

concentration” (31,32). For example, the tumor uptake of the ^{99m}Tc -labeled 3G-RGD₂ was almost 3x higher than that of the ^{99m}Tc -labeled c(RGDfK) in the same model (31). Thus, we believe that the differences in the integrin $\alpha_v\beta_3$ binding affinity of DOTA-6G-RGD₄ and DOTA-RGD₄ and the initial tumor uptake of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-RGD}_4)$ are most likely caused by the number of possibilities to achieve simultaneous integrin $\alpha_v\beta_3$ binding for 6G-RGD₄ and RGD₄. The concentration factor exists in both DOTA-6G-RGD₄ and DOTA-RGD₄. The difference is that there are only two possibilities for DOTA-RGD₄ to achieve bivalency while any two of the four RGD motifs in DOTA-6G-RGD₄ can achieve bivalency due to the longer distance between RGD motifs. If DOTA-6G-RGD₄ were tetravalent or trivalent, it would have had much higher integrin $\alpha_v\beta_3$ binding affinity than DOTA-RGD₄ whereas $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ would have shown much higher initial tumor uptake than $^{111}\text{In}(\text{DOTA-RGD}_4)$. This conclusion is supported by the fact that $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ share a similar initial tumor uptake (Figure 3: 12.66 ± 1.25 %ID/g and 10.19 ± 3.61 %ID/g, respectively) at 0.5 h p.i. Even though DOTA-6G-RGD₄ and DOTA-RGD₄ may not be tetravalent, the extra cyclic RGD motifs in DOTA-6G-RGD₄ and DOTA-RGD₄ contribute significantly to the higher tumor uptake of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-RGD}_4)$ than that of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ at 24 – 72 h p.i. (Figure 3). The concentration factor might also explain why $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-RGD}_4)$ had higher uptake than $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ (Figure 3) in the intestine, liver and kidneys, all of which are integrin $\alpha_v\beta_3$ -positive (15,16). From this point of view, we believe that $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ is most likely bivalent in binding to the integrin $\alpha_v\beta_3$. However, the IC₅₀ values and ex-vivo biodistribution data cannot completely eliminate the possibility of trivalency or tetravalency for $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$.

For diagnostic radiotracers, the tumor retention time may not be as critical as tumor uptake and T/B ratios. For the ^{90}Y and ^{177}Lu radiotracers, however, the longer tumor retention time will result in higher radiation dose to the integrin $\alpha_v\beta_3$ -positive tumors during the same period of time. It must be noted that the ability of a multimeric RGD peptide to achieve bivalency also depends on the integrin $\alpha_v\beta_3$ density. If the tumor integrin $\alpha_v\beta_3$ density is high, the distance between two neighboring integrin $\alpha_v\beta_3$ sites will be short, and it is easier for the multimeric RGD peptide to achieve bivalency. If the integrin $\alpha_v\beta_3$ density is very low, the distance between two neighboring integrin $\alpha_v\beta_3$ sites will be long, and it might be more difficult for the same multimeric RGD peptide to achieve simultaneous integrin $\alpha_v\beta_3$ binding.

The integrin $\alpha_v\beta_3$ -specificity of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ is demonstrated by the “blocking experiment” (Figure 5). The blockage of radiotracer uptake strongly suggests that its glioma localization is indeed integrin $\alpha_v\beta_3$ -mediated. The partial uptake blockage in the eyes, heart, intestine, lungs, liver and spleen indicates that the accumulation of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ in these organs is in large part integrin $\alpha_v\beta_3$ -mediated. This conclusion is supported by the immunohistopathological studies (15,16), which showed a strong positive staining of endothelial cells of small glomeruli vessels in kidneys and weak staining in branches of the hepatic portal vein. Similar results were also obtained for radiolabeled (^{99m}Tc and ^{64}Cu) cyclic RGD peptide dimers, such as 3G-RGD₂ and 3P-RGD₂ (31–33,36,37).

It is well established that multimerization of cyclic RGD peptides can significantly improve the integrin $\alpha_v\beta_3$ -targeting capability as evidenced by their higher integrin $\alpha_v\beta_3$ binding affinity and better tumor uptake with better tumor uptake than their monomeric counterparts (26,30–37). However, this also leads to more radioactivity accumulation of radiolabeled multimeric cyclic RGD peptides in normal organs, as evidenced by the higher uptake of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-RGD}_4)$ than $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ in the intestine, liver and kidneys. As a result, the tumor/liver and tumor/kidney ratios of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-RGD}_4)$ are significantly ($p < 0.01$) lower than those of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ at >1 h p.i. This is consistent with more accumulation in the abdominal region of tumor-

bearing mice administered with $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ (Figure 6: top) than that with $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ (Figure 6: bottom). Thus, $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ is a better radiotracer than $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-RGD}_4)$ for diagnostic purposes. For the integrin $\alpha_v\beta_3$ -targeted therapeutic radiotracers, however, 6G-RGD₄ has advantages over RGD₄ and 3G-RGD₂ because $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ has a longer tumor retention time than both $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ and $^{111}\text{In}(\text{DOTA-RGD}_4)$. At this moment, it is not clear why $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ is able to maintain its integrity during its excretion from both renal and hepatobiliary routes while $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ has significant degradation during its excretion from the hepatobiliary route (Figure 7). It is likely that the metabolic instability of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ during its hepatobiliary excretion is caused by its much larger size (M.W. = 4,269 Daltons) than that of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ (M.W. = 2,326 Daltons).

The %ID tumor uptake reflects the total integrin $\alpha_v\beta_3$ expression level on tumor cells and tumor neovasculature while the %ID/g tumor uptake reflects the integrin $\alpha_v\beta_3$ density. When tumor is small (<0.05 g or 50 m³), there is little tumor-mass and angiogenesis. As a result, $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ has low %ID tumor uptake (Figure 4A). As tumor grows, the integrin $\alpha_v\beta_3$ level becomes higher, and the %ID tumor uptake of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ increases (Figure 4A). However, the microvessel density decreases due to the maturation of blood vessels, and the integrin $\alpha_v\beta_3$ density also decreases due to larger interstitial space and higher collagen concentrations (38). In addition, parts of the tumor may become necrotic, leading to lower integrin $\alpha_v\beta_3$ density. It is not surprising that $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ had significantly less %ID/g uptake in larger tumors (Figure 4B). Similar results were also reported for $^{64}\text{Cu}(\text{DOTA-3P-RGD}_2)$ (33). The linear relationship between the tumor size and %ID tumor uptake suggests that $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ might have the potential for monitoring tumor growth or shrinkage. However, the relationship between the radiotracer uptake and integrin $\alpha_v\beta_3$ levels needs to be further established in our future studies. The ability to non-invasively quantify integrin $\alpha_v\beta_3$ level will provide a useful tool to select the patients more appropriately for anti-angiogenic therapy (39, 40).

CONCLUSION

In this study, we evaluated $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$, $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ for their potential as new integrin $\alpha_v\beta_3$ -targeted radiotracers. On the basis of results from the integrin $\alpha_v\beta_3$ binding assay and biodistribution studies, it is concluded that (1) tetrameric RGD peptides 6G-RGD₄ and RGD₄ are not tetravalent; (2) 6G-RGD₄, RGD₄ and 3G-RGD₂ most likely bind to the integrin $\alpha_v\beta_3$ in a bivalent fashion; (3) two extra cyclic RGD motifs in 6G-RGD₄ and RGD₄ contribute significantly to the longer tumor retention times of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-RGD}_4)$ than that of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$; (4) further increase of multiplicity from cyclic RGD dimers, such as 3G-RGD₂, has a adverse impact on T/B ratios due to the increased radioactivity accumulation in normal organs; and (5) the combination of high tumor uptake and prolonged tumor retention of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ suggests that its ^{90}Y and ^{177}Lu analogs, $\text{M}(\text{DOTA-6G-RGD}_4)$ (M = ^{90}Y and ^{177}Lu), might have to potential for the targeted radiotherapy of glioblastoma.

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References

1. Weber WA, Haubner R, Vabulienė E, Kuhnast B, Wester HJ, Schwaiger M. Tumor angiogenesis targeting using imaging agents. *Q J Nucl Med* 2001;45:179–182. [PubMed: 11476168]
2. Costouros NG, Diehn FE, Libutti SK. Molecular imaging of tumor angiogenesis. *J Cell Biochem Suppl* 2002;39:72–78. [PubMed: 12552605]
3. Liu S, Robinson SP, Edwards DS. Integrin $\alpha_v\beta_3$ directed radiopharmaceuticals for tumor imaging. *Drugs of Future* 2003;28:551–564.
4. Haubner R, Wester HJ. Radiolabeled tracers for imaging of tumor angiogenesis and evaluation of antiangiogenic therapies. *Curr Pharm Des* 2004;10:1439–1455. [PubMed: 15134568]
5. Liu S. Radiolabeled multimeric cyclic RGD peptides as integrin $\alpha_v\beta_3$ -targeted radiotracers for tumor imaging. *Mol Pharm* 2006;3:472–487. [PubMed: 17009846]
6. Chen X. Multimodality imaging of tumor integrin $\alpha_v\beta_3$ expression. *Mini-Rev Med Chem* 2006;6:227–234. [PubMed: 16472190]
7. Cai W, Chen X. Multimodality Molecular imaging of tumor angiogenesis. *J Nucl Med* 2008;49:113S–128S. [PubMed: 18523069]
8. Cai W, Niu G, Chen X. Imaging of integrins as biomarkers for tumor angiogenesis. *Curr Pharmaceut Des* 2008;14:2943–2973.
9. Liu S. Radiolabeled cyclic RGD peptides as integrin $\alpha_v\beta_3$ -targeted radiotracers: Maximizing Binding Affinity via Bivalency. *Bioconjugate Chem (ASAP)*. 2009
10. Thumshirn G, Hersel U, Goodman SL, Kessler H. Multimeric cyclic RGD peptides as potential tools for tumor targeting: solid-phase peptide synthesis and chemoselective oxime ligation. *Chem Eur J* 2003;9:2717–2725.
11. Poethko T, Schottelius M, Thumshirn G, Herz M, Haubner R, Henriksen G, Kessler H, Schwaiger M, Wester HJ. Chemoselective pre-conjugate radiohalogenation of unprotected mono- and multimeric peptides via oxime formation. *Radiochimica Acta* 2004;92:317–327.
12. Chen X, Liu S, Hou Y, Tohme M, Park R, Bading JR, Conti PS. MicroPET imaging of breast cancer α_v -integrin expression with ^{64}Cu -labeled dimeric RGD peptides. *Mol Imag Biol* 2004;6:350–359.
13. Chen X, Tohme M, Park R, Hou Y, Bading JR, Conti PS. MicroPET imaging of breast cancer α_v -integrin expression with ^{18}F -labeled dimeric RGD peptide. *Mol Imaging* 2004;3:96–104. [PubMed: 15296674]
14. Wu Y, Zhang X, Xiong Z, Cheng Z, Fisher DR, Liu S, Gambhir SS, Chen X. MicroPET imaging of glioma integrin $\alpha_v\beta_3$ expression using ^{64}Cu -labeled tetrameric RGD peptide. *J Nucl Med* 2005;46:1707–1718. [PubMed: 16204722]
15. Zhang X, Xiong Z, Wu Y, Cai W, Tseng JR, Gambhir SS, Chen X. Quantitative PET imaging of tumor integrin $\alpha_v\beta_3$ expression with ^{18}F -FRGD2. *J Nucl Med* 2006;47:113–121. [PubMed: 16391195]
16. Wu Z, Li Z, Chen K, Cai W, He L, Chin FT, Li F, Chen X. Micro-PET of tumor integrin $\alpha_v\beta_3$ expression using ^{18}F -labeled PEGylated tetrameric RGD peptide (^{18}F -FPRGD4). *J Nucl Med* 2007;48:1536–1544. [PubMed: 17704249]
17. Li ZB, Chen K, Chen X. ^{68}Ga -labeled multimeric RGD peptides for microPET imaging of integrin $\alpha_v\beta_3$ expression. *Eur J Nucl Med Mol Imaging* 2008;35:1100–1108. [PubMed: 18204838]
18. Liu S, Edwards DS, Ziegler MC, Harris AR, Hemingway SJ, Barrett JA. $^{99\text{m}}\text{Tc}$ -Labeling of a hydrazinonicotinamide-conjugated vitronectin receptor antagonist useful for imaging tumor. *Bioconjugate Chem* 2001;12:624–629.
19. Liu S, Cheung E, Ziegler MC, Rajopadhye M, Edwards DS. ^{90}Y - and ^{177}Lu -labeling of a DOTA-conjugated vitronectin receptor antagonist for tumor therapy. *Bioconjugate Chem* 2001;12:559–568.
20. Janssen M, Oyen WJG, Massuger LFAG, Frielink C, Dijkgraaf I, Edwards DS, Rajopadhye M, Corstens FH, Boerman OC. Comparison of a monomeric and dimeric radiolabeled RGD-peptide for tumor targeting. *Cancer Biother Radiopharm* 2002;17:641–646. [PubMed: 12537667]
21. Janssen M, Oyen WJG, Dijkgraaf I, Massuger LF, Frielink C, Edwards DS, Rajopadhye M, Boonstra H, Corstens FH, Boerman OC. Tumor targeting with radiolabeled integrin $\alpha_v\beta_3$ binding peptides in a nude mice model. *Cancer Res* 2002;62:6146–6151. [PubMed: 12414640]

22. Liu S, Hsieh WY, Kim YS, Mohammed SI. Effect of coligands on biodistribution characteristics of ternary ligand ^{99m}Tc complexes of a HYNIC-conjugated cyclic RGDfK dimer. *Bioconjugate Chem* 2005;16:1580–1588.
23. Jia B, Shi J, Yang Z, Xu B, Liu Z, Zhao H, Liu S, Wang F. ^{99m}Tc -labeled cyclic RGDfK dimer: initial evaluation for SPECT imaging of glioma integrin $\alpha_v\beta_3$ expression. *Bioconjugate Chem* 2006;17:1069–1076.
24. Liu S, He ZJ, Hsieh WY, Kim YS, Jiang Y. Impact of PKM linkers on biodistribution characteristics of the ^{99m}Tc -labeled cyclic RGDfK dimer. *Bioconjugate Chem* 2006;17:1499–1507.
25. Liu S, Hsieh WY, Jiang Y, Kim YS, Sreerama SG, Chen X, Jia B, Wang F. Evaluation of a ^{99m}Tc -labeled cyclic RGD tetramer for noninvasive imaging integrin $\alpha_v\beta_3$ -positive breast cancer. *Bioconjugate Chem* 2007;18:438–446.
26. Dijkgraaf I, Kruijtz JAW, Liu S, Soede A, Oyen WJG, Corstens FHM, Liskamp RMJ, Boerman OC. Improved targeting of the $\alpha_v\beta_3$ integrin by multimerization of RGD peptides. *Eur J Nucl Med Mol Imaging* 2007;34:267–273. [PubMed: 16909226]
27. Dijkgraaf I, Liu S, Kruijtz JAW, Soede AC, Oyen WJG, Liskamp RMJ, Corstens FH, Boerman OC. Effect of linker variation on the in vitro and in vivo characteristics of an ^{111}In -labeled RGD Peptide. *Nucl Med Biol* 2007;34:29–35. [PubMed: 17210459]
28. Jia B, Liu Z, Shi J, Yu ZL, Yang Z, Zhao HY, He Z, Liu S, Wang F. Linker effects on biological properties of ^{111}In -labeled DTPA conjugates of a cyclic RGDfK dimer. *Bioconjugate Chem* 2008;19:201–210.
29. Wang JJ, Kim YS, Liu S. ^{99m}Tc -labeling of HYNIC-conjugated cyclic RGDfK dimer and tetramer using EDDA as coligand. *Bioconjugate Chem* 2008;19:634–642.
30. Liu S, Kim YS, Hsieh WY, Sreerama SG. Coligand effects on solution stability, biodistribution and metabolism of ^{99m}Tc -labeled cyclic RGDfK tetramer. *Nucl Med Biol* 2008;35:111–121. [PubMed: 18158950]
31. Shi J, Wang L, Kim YS, Zhai S, Liu Z, Chen X, Liu S. Improving tumor uptake and excretion kinetics of ^{99m}Tc -labeled cyclic Arginine-Glycine-Aspartic (RGD) dimers with triglycine linkers. *J Med Chem* 2008;51:7980–7990. [PubMed: 19049428]
32. Wang L, Shi J, Kim YS, Zhai S, Jia B, Liu Z, Zhao H, Liu Z, Wang F, Chen X, Liu S. Improving tumor targeting capability and pharmacokinetics of ^{99m}Tc -labeled cyclic RGD dimers with PEG₄ linkers. *Mol Pharm* 2009;6:231–245. [PubMed: 19067525]
33. Shi J, Kim YS, Zhai S, Liu Z, Chen X, Liu S. Improving tumor uptake and pharmacokinetics of ^{64}Cu -labeled cyclic RGD dimers with G₃ and PEG₄ Linkers. *Bioconjugate Chem* 2009;20:750–759.
34. Liu Z, Niu G, Shi J, Liu SL, Wang F, Liu S. ^{68}Ga -Labeled cyclic RGD dimers with Gly₃ and PEG₄ linkers: promising agents for tumor integrin $\alpha_v\beta_3$ PET imaging. *Eur J Nucl Med Mol Imaging* 2009;36:947–957. [PubMed: 19159928]
35. Liu Z, Liu S, Wang F, Liu S, Chen X. Non-invasive imaging of tumor integrin expression using ^{18}F -labeled RGD dimer peptide with PEG₄ linkers. *Eur J Nucl Med Mol Imaging* 2009;36:1296–1307. [PubMed: 19296102]
36. Shi J, Kim YS, Chakraborty S, Jia B, Wang F, Liu S. 2-Mercaptoacetylglucylglycyl (MAG₂) as a bifunctional chelator for ^{99m}Tc -labeling of cyclic RGD dimers: effects of technetium chelate on tumor uptake and pharmacokinetics. *Bioconjugate Chem* 2009;20:1559–1568.
37. Shi J, Wang L, Kim YS, Zhai S, Jia B, Zhao H, Wang F, Liu S. $^{99m}\text{TcO}(\text{MAG}_2\text{-3G}_3\text{-dimer})$: a new integrin $\alpha_v\beta_3$ -targeted SPECT radiotracer with high tumor uptake and favorable pharmacokinetics. *Eur J Nucl Med Mol Imaging* 2009;36:1874–1884. [PubMed: 19484236]
38. Jain RK. Transport of molecules in the tumor interstitium: a review. *Cancer Res* 1987;47:3039–3051. [PubMed: 3555767]
39. Cai W, Rao J, Gambhir SS, Chen X. How molecular imaging is speeding up antiangiogenic drug development. *Mol Cancer Ther* 2006;5:2624–2633. [PubMed: 17121909]
40. Niu G, Chen X. Has molecular and cellular imaging enhanced drug discovery and drug development? *Drugs in R&D* 2008;9:351–368.

Figure 1.

Structure of a new cyclic RGD tetramer conjugate: DOTA-6G-RGD₄.

Figure 2. Competitive inhibition curves of ^{125}I -c(RGDyK) bound to the U87MG human glioma cells by c(RGDyK), DOTA-3G-RGD₂, DOTA-RGD₄ and DOTA-6G₃RGD₄. Their IC₅₀ values were calculated to be 42.2 ± 5.2 , 1.1 ± 0.1 , 1.4 ± 0.3 and 0.4 ± 0.1 nM, respectively.

Figure 3.

Direct comparison of the tumor and intestine uptake (%ID/g), tumor/kidney and tumor/liver ratios between $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$, $^{111}\text{In}(\text{DOTA-RGD}_4)$ and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ in the athymic nude mice ($n = 5$) bearing U87MG human glioma xenografts.

Figure 4. The relationship between the tumor size and tumor uptake of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ at 60 min p.i. in the athymic nude mice (n = 12) bearing the U87MG glioma xenografts.

Figure 5. Comparison of the 60-min biodistribution data of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ in athymic nude mice (n = 5) bearing U87MG glioma xenografts in the absence/presence of excess RGD₂ to demonstrate its integrin $\alpha_v\beta_3$ -specificity.

Figure 6.

The whole-body planar images of the tumor-bearing mice administered with $\sim 100 \mu\text{Ci}$ of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ (top) and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ (bottom) at 1, 4, 24 and 72 h p.i.

Figure 7.

Typical radio-HPLC chromatograms of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ (left) and $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ (right) in saline before injection, in urine at 30 min and 120 min p.i., and in feces at 120 min p.i. Each mouse was administered with ~100 μCi radiotracer.

Table 1

Radiochemical purity (RCP), HPLC retention time and log P values for ^{111}In -labeled cyclic RGD peptides.

Compound	RCP (%)	Retention Time (min)	Log P Value
$^{111}\text{In}(\text{DOTA-3G-RGD}_2)$	>95	13.9	-4.13 ± 0.08
$^{111}\text{In}(\text{DOTA-6G-RGD}_4)$	> 92	17.1	-4.40 ± 0.04
$^{111}\text{In}(\text{DOTA-RGD}_4)$	> 92	19.1	-3.41 ± 0.13

Table 2

Selected biodistribution data of $^{111}\text{In}(\text{DOTA-6G-RGD}_4)$ in athymic nude mice ($n = 5$) bearing U87MG human glioma xenografts.

%ID/gram	0.5 h	1 h	4 h	24 h	72 h
Blood	0.97 ± 0.10	0.25 ± 0.04	0.05 ± 0.00	0.06 ± 0.02	0.03 ± 0.01
Bone	2.26 ± 0.71	2.35 ± 0.64	1.99 ± 0.15	1.41 ± 0.29	0.90 ± 0.41
Brain	0.13 ± 0.08	0.14 ± 0.03	0.13 ± 0.07	0.12 ± 0.07	0.10 ± 0.04
Eyes	1.20 ± 0.60	1.54 ± 0.22	1.61 ± 1.38	0.83 ± 0.20	0.53 ± 0.12
Heart	1.50 ± 0.76	1.49 ± 0.29	1.03 ± 0.23	0.80 ± 0.22	0.51 ± 0.16
Intestine	7.15 ± 3.70	9.25 ± 1.27	8.79 ± 2.98	10.91 ± 1.94	8.31 ± 2.14
Kidney	8.35 ± 0.52	8.42 ± 0.86	6.21 ± 0.65	4.44 ± 0.45	3.45 ± 0.90
Liver	3.04 ± 1.38	3.77 ± 0.79	3.05 ± 0.14	2.46 ± 0.36	2.01 ± 0.45
Lungs	3.65 ± 0.87	3.44 ± 1.86	2.72 ± 0.38	1.51 ± 0.22	0.91 ± 0.08
Muscle	1.23 ± 0.42	1.14 ± 0.23	0.80 ± 0.11	0.93 ± 0.54	0.45 ± 0.06
Spleen	2.63 ± 0.45	2.38 ± 0.49	2.35 ± 0.23	1.96 ± 0.31	2.07 ± 0.24
U87MG	12.66 ± 1.25	11.45 ± 2.46	8.99 ± 2.61	8.68 ± 1.42	5.59 ± 0.54
Tumor/Blood	13.06 ± 1.29	44.92 ± 9.66	198.34 ± 57.67	143.01 ± 23.40	152.37 ± 102.84
Tumor/Bone	5.60 ± 0.55	4.88 ± 1.05	4.53 ± 1.32	6.16 ± 1.01	4.67 ± 3.15
Tumor/Kidney	1.52 ± 0.15	1.36 ± 0.29	1.45 ± 0.42	1.95 ± 0.32	1.21 ± 0.82
Tumor/Liver	4.16 ± 0.41	3.04 ± 0.65	2.95 ± 0.86	3.53 ± 0.58	2.09 ± 1.41
Tumor/Lungs	3.46 ± 0.34	3.33 ± 0.72	3.30 ± 0.96	5.74 ± 0.94	4.59 ± 3.10
Tumor/Muscle	10.30 ± 1.02	10.08 ± 2.17	11.20 ± 3.26	9.30 ± 1.52	9.21 ± 6.22

Table 3

Selected biodistribution data of $^{111}\text{In}(\text{DOTA-RGD}_4)$ in athymic nude mice ($n = 5$) bearing U87MG human glioma xenografts.

%ID/gram	0.5 h	1 h	4 h	24 h	72 h
Blood	0.80 ± 0.18	0.37 ± 0.08	0.12 ± 0.05	0.04 ± 0.01	0.07 ± 0.02
Bone	1.64 ± 0.17	0.93 ± 0.13	0.86 ± 0.20	0.53 ± 0.27	0.75 ± 0.09
Brain	0.15 ± 0.04	0.12 ± 0.01	0.10 ± 0.02	0.07 ± 0.02	0.14 ± 0.07
Eyes	1.56 ± 0.15	1.23 ± 0.10	0.92 ± 0.46	0.64 ± 0.10	0.78 ± 0.46
Heart	1.45 ± 0.53	1.23 ± 0.11	0.90 ± 0.18	0.54 ± 0.26	1.01 ± 0.76
Intestine	10.79 ± 1.46	8.42 ± 1.65	9.19 ± 1.58	7.03 ± 2.06	3.37 ± 0.30
Kidney	11.18 ± 1.22	7.28 ± 0.57	5.83 ± 0.70	5.11 ± 0.52	3.48 ± 1.10
Liver	2.98 ± 0.51	2.67 ± 0.32	2.75 ± 0.29	2.15 ± 0.57	1.62 ± 0.40
Lungs	4.29 ± 1.26	3.43 ± 0.54	2.06 ± 0.39	1.50 ± 0.39	0.62 ± 0.36
Muscle	1.79 ± 0.38	1.22 ± 0.45	0.63 ± 0.11	0.78 ± 0.19	0.68 ± 0.31
Spleen	2.65 ± 1.36	2.40 ± 0.51	1.40 ± 0.95	1.15 ± 0.84	1.16 ± 0.63
U87MG	8.97 ± 0.52	7.98 ± 2.36	7.32 ± 2.66	5.84 ± 1.09	3.16 ± 0.43
Tumor/Blood	10.61 ± 1.97	19.00 ± 3.60	42.74 ± 5.65	151.67 ± 57.32	31.78 ± 4.57
Tumor/Bone	5.35 ± 0.60	8.58 ± 1.46	9.22 ± 4.79	28.78 ± 16.18	4.49 ± 0.04
Tumor/Kidney	0.84 ± 0.05	1.09 ± 0.25	1.26 ± 0.36	1.15 ± 0.74	0.96 ± 0.22
Tumor/Liver	2.69 ± 0.29	2.85 ± 0.67	2.50 ± 0.60	2.91 ± 1.17	1.68 ± 0.23
Tumor/Lungs	2.32 ± 1.03	2.77 ± 1.14	4.06 ± 0.83	4.71 ± 1.79	5.45 ± 1.61
Tumor/Muscle	5.37 ± 0.20	6.50 ± 1.22	14.07 ± 5.93	9.31 ± 6.36	4.87 ± 2.24

Table 4

Biodistribution data of $^{111}\text{In}(\text{DOTA-3G-RGD}_2)$ in athymic nude mice ($n = 5$) bearing U87MG human glioma xenografts.

%ID/gram	0.5 h	1 h	4 h	24 h	72 h
Blood	0.87 ± 0.13	0.30 ± 0.07	0.09 ± 0.00	0.03 ± 0.01	0.01 ± 0.00
Brain	0.13 ± 0.03	0.09 ± 0.00	0.07 ± 0.00	0.05 ± 0.01	0.04 ± 0.00
Eyes	1.20 ± 0.24	0.84 ± 0.13	0.51 ± 0.04	0.32 ± 0.05	0.16 ± 0.03
Heart	1.03 ± 0.12	0.60 ± 0.12	0.44 ± 0.02	0.28 ± 0.04	0.15 ± 0.04
Intestine	4.83 ± 1.15	4.81 ± 1.01	5.09 ± 1.75	3.77 ± 0.07	1.89 ± 0.19
Kidney	6.74 ± 0.79	3.88 ± 0.48	2.69 ± 0.10	2.08 ± 0.16	1.48 ± 0.14
Liver	2.45 ± 0.14	2.25 ± 0.04	2.23 ± 0.05	1.52 ± 0.15	0.85 ± 0.09
Lungs	3.13 ± 0.47	1.71 ± 0.48	1.22 ± 0.08	0.75 ± 0.02	0.32 ± 0.06
Muscle	1.33 ± 0.24	0.70 ± 0.03	0.39 ± 0.05	0.30 ± 0.09	0.20 ± 0.05
Spleen	1.63 ± 0.08	1.36 ± 0.13	1.18 ± 0.20	0.91 ± 0.10	0.88 ± 0.17
U87MG	10.19 ± 3.61	10.69 ± 1.82	6.72 ± 2.06	4.83 ± 1.19	1.99 ± 0.39
Tumor/Blood	11.74 ± 3.82	36.93 ± 9.74	72.50 ± 23.09	300.15 ± 29.08	215.91 ± 56.80
Tumor/Kidney	1.51 ± 0.47	2.77 ± 0.51	2.49 ± 0.75	2.36 ± 0.69	1.86 ± 0.30
Tumor/Liver	4.16 ± 1.43	4.74 ± 0.81	3.01 ± 0.87	3.25 ± 1.01	2.35 ± 0.54
Tumor/Lungs	3.35 ± 1.36	6.59 ± 1.86	5.60 ± 1.93	6.38 ± 1.49	6.43 ± 1.66
Tumor/Muscle	8.03 ± 3.56	15.31 ± 2.44	17.61 ± 5.95	17.86 ± 7.44	10.79 ± 3.69