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Evaluation of ^{99m}Tc -Labeled Cyclic RGD Dimers: Impact of Cyclic RGD Peptides and ^{99m}Tc Chelates on Biological Properties

Yang Zhou, Young-Seung Kim, Xin Lu, and Shuang Liu*

School of Health Sciences, Purdue University, West Lafayette, Indiana, USA

Abstract

The main objective of this study is to explore the impact of cyclic RGD peptides and ^{99m}Tc chelates on biological properties of ^{99m}Tc radiotracers. Cyclic RGD peptide conjugates, HYNIC-K(NIC)-RGD₂ (HYNIC = 6-hydrazinonicotinyl; RGD₂ = E[c(RGDfK)]₂ and NIC = nicotinyl) HYNIC-K(NIC)-3G-RGD₂ (3G-RGD₂ = Gly-Gly-Gly-E[Gly-Gly-Gly-c(RGDfK)]₂), and HYNIC-K(NIC)-3P-RGD₂ (3P-RGD₂ = PEG₄-E[PEG₄-c(RGDfK)]₂), were prepared. Macrocyclic ^{99m}Tc complexes [^{99m}Tc (HYNIC-K(NIC)-RGD₂)(tricine)] (**1**), [^{99m}Tc (HYNIC-K(NIC)-3G-RGD₂)(tricine)] (**2**) and [^{99m}Tc (HYNIC-K(NIC)-3P-RGD₂)(tricine)] (**3**) were evaluated for their biodistribution and tumor-targeting capability in athymic nude mice bearing MDA-MB-435 human breast tumor xenografts. It was found that **1**, **2** and **3** could be prepared with high specific activity (~111GBq/3mol). All three ^{99m}Tc radiotracers have two major isomers, which show almost identical uptake in tumors and normal organs. Replacing the bulky and highly charged [^{99m}Tc (HYNIC)(tricine)(TPPTS)] (TPPTS = trisodium triphenylphosphine-3,3',3''-trisulfonate) with a smaller [^{99m}Tc (HYNIC-K(NIC))(tricine)] resulted in less uptake in the kidneys and lungs for **3**. Surprisingly, all three ^{99m}Tc radiotracers shared a similar tumor uptake (**1**: 5.73 ± 0.40 %ID/g; **2**: 5.24 ± 1.09 %ID/g; and **3**: 4.94 ± 1.71 %ID/g) at 60 min p.i. The metabolic stability of ^{99m}Tc radiotracers depends on cyclic RGD peptides (3P-RGD₂ > 3G-RGD₂ ~ RGD₂) and ^{99m}Tc chelates ([^{99m}Tc (HYNIC)(tricine)(TPPTS)] > [^{99m}Tc (HYNIC-K(NIC))(tricine)]). Immunohistochemical studies revealed a linear relationship between the $\alpha_v\beta_3$ expression levels and tumor uptake or tumor/muscle ratios of **3**, suggesting that **3** is useful for monitoring the tumor $\alpha_v\beta_3$ expression. Complex **3** is a very attractive radiotracer for detection of integrin $\alpha_v\beta_3$ -positive tumors.

Keywords

integrin $\alpha_v\beta_3$; ^{99m}Tc -labeled cyclic RGD peptides; breast tumor imaging

INTRODUCTION

Over the last several years, we have been using a ternary ligand system (HYNIC, tricine and TPPTS) (Figure 1A: HYNIC = 6-hydrazinonicotinyl; TPPTS = trisodium triphenylphosphine-3,3',3''-trisulfonate) for ^{99m}Tc -labeling of small biomolecules (BMs), which include chemotactic peptides and LTB₄ receptor antagonists for imaging infection and inflammation,^{1, 2} integrin $\alpha_v\beta_3$ receptor antagonists for tumor imaging,^{3–7} and GPIIb/IIIa receptor antagonists for imaging thrombus.^{8–10} This ternary ligand system forms complexes [^{99m}Tc (HYNIC-BM)(tricine)(TPPTS)] (Figure 1A: BM = peptide and non-peptide receptor ligand) with extremely high specific activity.^{8, 10} These ternary

*Correspondence should be addressed to: Dr. Shuang Liu, School of Health Sciences, Purdue University, 550 Stadium Mall Drive, West Lafayette, IN 47907, Phone: 765-494-0236, Fax 765-496-1377, liu100@purdue.edu.

ligand ^{99m}Tc complexes are stable for >6 h, and are often formed as equal mixtures of two diastereomers if the biomolecule has a chiral center. The composition of ternary ligand ^{99m}Tc complexes has been determined to be 1:1:1:1 for Tc:HYNIC-BM:tricine:TPPTS through a series of mixed ligand experiments, and further confirmed by LC-MS at both the tracer (^{99m}Tc) and macroscopic (^{99}Tc) levels.^{11, 12} The utility of HYNIC for the ^{99m}Tc -labeling of small biomolecules has been reviewed extensively.^{13, 14}

In our previous communications,^{15, 16} we reported several phosphine and pyridine-containing HYNIC chelators and their corresponding macrocyclic ^{99m}Tc complexes. We found that the attachment site of the lysine (K) linker is critical for the formation of macrocyclic ^{99m}Tc complexes. For example, if the linker is attached to the 3-position of the pyridine ring, HYNIC derivatives form complexes [$^{99m}\text{Tc}(\text{HYNIC-K}(\text{NIC})\text{-BM})(\text{tricine})$] (Figure 1B: NIC = nicotinyl) in high yield (>95%). The HPLC data suggest that these complexes exist in solution as four isomers: two diastereomers and two conformational isomers, which interconvert rapidly at room temperature.^{15, 16} While conformational isomers are formed because of the rigidity of macrocycle, diastereomers are formed due to a combination of the chirality of the lysine linker and of the Tc chelate.^{15, 16}

To demonstrate the utility of HYNIC-K(NIC) as a bifunctional coupling agent (BFC), we prepared three new cyclic RGD peptide conjugates, HYNIC-K(NIC)-RGD₂ (RGD₂ = E[c(RGDfK)]₂), HYNIC-K(NIC)-3G-RGD₂ (3G-RGD₂ = Gly-Gly-Gly-E[Gly-Gly-Gly-c(RGDfK)]₂), and HYNIC-K(NIC)-3P-RGD₂ (3P-RGD₂ = PEG₄-E[PEG₄-c(RGDfK)]₂). We also prepared their macrocyclic complexes [$^{99m}\text{Tc}(\text{HYNIC-K}(\text{NIC})\text{-RGD}_2)(\text{tricine})$] (**1**), [$^{99m}\text{Tc}(\text{HYNIC-K}(\text{NIC})\text{-3G-RGD}_2)(\text{tricine})$] (**2**) and [$^{99m}\text{Tc}(\text{HYNIC-K}(\text{NIC})\text{-3P-RGD}_2)(\text{tricine})$] (**3**), and evaluated them in the athymic nude mice bearing MDA-MB-435 human breast tumor xenografts for their tumor-targeting capability and biodistribution. For comparison purposes, we also evaluated [$^{99m}\text{Tc}(\text{HYNIC-3P-RGD}_2)(\text{tricine})(\text{TPPTS})$] (Figure 1: **4**) in the same tumor-bearing animal model. The main objective of this study is to explore the impact of RGD peptides (RGD₂, 3G-RGD₂ and 3P-RGD₂) and ^{99m}Tc chelates ([$^{99m}\text{Tc}(\text{HYNIC})(\text{tricine})(\text{TPPTS})$] versus [$^{99m}\text{Tc}(\text{HYNIC-K}(\text{NIC}))(\text{tricine})$]) on biological properties of ^{99m}Tc radiotracers.

EXPERIMENTAL SECTION

Materials and Instruments

Chemicals were purchased from *Sigma/Aldrich* (St. Louis, MO), and were used without purification. BOC-Lys(nicotinoyl)-OH was purchased from *Bachem Biosciences Inc.* (Prussia, PA). Cyclic RGD peptides G₃-E[G₃-c(RGDfK)]₂ (3G-RGD₂) and PEG₄-E[PEG₄-c(RGDfK)]₂ (3P-RGD₂) were custom-made by the Peptides International, Inc. (Louisville, KY). Sodium succinimidyl 6-(2-(2-sulfonatobenzaldehyde)hydrazono)nicotinate (HYNIC-OSu) was prepared according to the literature method.¹⁷ [$^{99m}\text{Tc}(\text{HYNIC-3P-RGD}_2)(\text{tricine})(\text{TPPTS})$] (**4**) was prepared using the procedure described in our previous reports.¹⁸ NMR spectra were recorded on a 300 MHz Bruker ARX FT NMR spectrometer and reported as ppm relative to TMS. The ESI (electrospray ionization) mass spectral data were collected on a Finnigan LCQ classic mass spectrometer, School of Pharmacy, Purdue University. Na $^{99m}\text{TcO}_4$ was obtained from a commercial $^{99}\text{Mo}/^{99m}\text{Tc}$ Technelite® generator (North Billerica, MA).

HPLC Methods

The semi-prep HPLC method (Method 1) used a LabAlliance HPLC system equipped with a UV/vis detector ($\lambda=254$ nm) and Zorbax C₁₈ semi-prep column (9.4 nm × 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 70% A (0.1% TFA in water) and 30% B (0.1% TFA in

methanol) at 0–5 min, followed by a gradient mobile phase going from 30% B at 5 min to 70% B at 20 min. HPLC Method 2 used the LabAlliance HPLC system equipped with a β -ram IN/US detector (Tampa, FL) and Zorbax C₁₈ column (4.6 mm $\times$ 250 mm, 300 Å pore size; Agilent Technologies, Santa Clara, CA). The flow rate was 1 mL/min. The gradient mobile phase started with 90% A (25 mM NH₄OAc, pH = 6.8) and 10% B (acetonitrile) to 85% A and 15% B at 5 min, followed by a gradient mobile phase going from 15% B at 5 min to 20% B at 20 min and to 60% B at 25 min. HPLC Method 3 started with 90% A (25 mM NH₄OAc, pH = 6.8) and 10% B (acetonitrile) to 85% A and 15% B at 5 min, followed by a gradient mobile phase going from 15% B at 5 min to 40% B at 20 min and to 60% B at 25 min.

BOC-Lys(nicotinoyl)-OSu (BOC-K(NIC)-OSu)

Dicycloheylcarbodiimide (DCC: 61.9 mg, 300 μ mol) was added to a solution containing BOC-Lys(nicotinoyl) (101 mg, 290 μ mol) and N-hydroxysuccinimide (NHS: 34.5 mg, 300 μ mol) in 3 mL of anhydrous DMF. The reaction mixture was stirred at room temperature for 5 h. The white precipitate was filtered and discarded. The filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in dichloromethane (3 mL). After filtration, the filtrate was concentrated to 1 mL. The solution was added dropwise into diethyl ether (20 mL) to give white precipitate. The solid was filtered, washed with diethyl ether (3 $\times$ 5 mL), and dried under vacuum to give the crude product BOC-K(NIC)-OSu. The yield was 75.8 mg (~60%). ¹H NMR (CDCl₃): 1.60 (s, 9H, (CH₃)₃CO), 1.75 (m, 2H, CH₂), 1.88 (m, 2H, CH₂), 2.12 (m, 2H, CH₂), 3.01 (s, 4H, COCH₂CH₂CO), 3.66 (t, 2H, CH₂CH₂NH), 5.10 (dd, H, NHCHCO), 7.54 (t, H, aromatic), 8.36 (d, H, aromatic), 8.88 (d, H, aromatic), and 9.15 (s, H, aromatic).

BOC-Lys(nicotinoyl)-E[c(RGDfK)]₂ (BOC-K(NIC)-RGD₂)

BOC-K(NIC)-OSu (8.9 mg, 20 μ mol) and E[c(RGDfK)]₂ (4.8 mg, 4 μ mol) were dissolved in anhydrous DMF (2 mL). Upon addition of 3 drops of diisopropylethylamine (DIEA), the resulting solution was stirred at room temperature overnight. After addition of water (2 mL), the pH in the mixture was adjusted to 6.0 – 7.0. The product was separated from the reaction mixture by HPLC (Method 1). Lyophilization of collected fractions at 20.7 min afforded the expected product BOC-K(NIC)-RGD₂. The yield was 3.1 mg (47%). ESI-MS: m/z = 1651.5 for [M + H]⁺ (M = 1650.8 calcd. for [C₇₆H₁₁₀N₂₂O₂₀]⁺).

Lys(nicotinoyl)-E[c(RGDfK)]₂ (K(NIC)-RGD₂)

Boc-K(NIC)-RGD₂ was dissolved in neat TFA (2.0 mL). After stirring at room temperature for 15 min, 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 collected fractions at 16.5 min afforded K(NIC)-RGD₂. The yield was 2.5 mg (~86%). ESI-MS: m/z = 1552 for [M + H]⁺ (M = 1550.8 calcd. for [C₇₁H₁₀₂N₂₂O₁₈]⁺).

HYNIC-Lys(nicotinoyl)-E[c(RGDfK)]₂ (HYNIC-K(NIC)-RGD₂)

HYNIC-OSu (3.5 mg, 8 μ mol) and K(NIC)-RGD₂ (2.5 mg, 1.6 μ mol) were dissolved in DMF (2 mL). After addition of DIEA (3 drops), the reaction mixture was stirred at room temperature for 24 hrs. Upon addition of 2 mL NH₄OAc buffer (100 mM, pH = 7.0), the product was separated from the mixture by HPLC (Method 1). Lyophilization of collected fraction at 18.7 min afforded HYNIC-K(NIC)-RGD₂. The yield was 1.1 mg (38%). ESI-MS: m/z = 1854.3 for [M + H]⁺ (M = 1553.8 calcd. for [C₈₄H₁₁₁N₂₅O₂₂S]⁺).

BOC-Lys(nicotinoyl)-Gly₃-E[Gly₃-c(RGDfK)]₂ (BOC-K(NIC)-3G-RGD₂)

BOC-K(NIC)-OSu (6.3 mg, 14 μ mol) and Gly₃-E[Gly₃-c(RGDfK)]₂ (5.1 mg, 2.8 μ mol) were dissolved in DMF (2 mL). To the mixture was added 3 drops of DIEA. The solution was stirred at room temperature overnight. After addition of water (2 mL), the pH was adjusted to 6.0 – 7.0. The product was separated from the reaction mixture by HPLC (method 1). Lyophilization of collected fractions at 19.3 min afforded BOC-K(NIC)-3G-RGD₂. The yield was 3.0 mg (50%). ESI-MS: m/z = 2163.9 for [M + H]⁺ (M = 2164 calcd. for [C₉₄H₁₃₇N₃₁O₂₉]⁺).

Lys(nicotinoyl)-Gly₃-E[Gly₃-c(RGDfK)]₂ (K(NIC)-3G-RGD₂)

Boc-K(NIC)-3G-RGD₂ was dissolved in 2.0 mL of anhydrous TFA. After stirring at room temperature for 15 min, excess TFA was removed. The residue was dissolved in 2 mL of 0.1 M NH₄OAc buffer (100 mM, pH = 7.0), and the resulting solution was subjected to HPLC purification (Method 1). The fractions at 16.7 min were collected. Lyophilization of collected fractions afforded K(NIC)-3G-RGD₂. The yield was 2.9 mg (~90%). ESI-MS: m/z = 2064.4 for [M + H]⁺ (M = 2063.9 calcd. for [C₈₉H₁₂₉N₃₁O₂₇]⁺).

HYNIC-Lys(nicotinoyl)-Gly₃-E[Gly₃-c(RGDfK)]₂ (HYNIC-K(NIC)-3G-RGD₂)

HYNIC-OSu (3.1 mg, 7 μ mol) and K(NIC)-3G-RGD₂ (2.9 mg, 1.4 μ mol) were dissolved in DMF (2 mL). After addition of DIEA (3 drops), the mixture was stirred at room temperature for 2 days. Upon addition of 2 mL NH₄OAc buffer (100 mM, pH = 7.0), the product was separated by HPLC (Method 1). Lyophilization of collected fraction at 15.5 min afforded HYNIC-K(NIC)-3G-RGD₂. The yield was 0.5 mg (15%). ESI-MS: m/z = 2366.9 for [M + H]⁺ (M = 2367 calcd. for [C₁₀₂H₁₃₈N₃₄O₃₁S]⁺).

BOC-Lys(nicotinoyl)-PEG₄-E[PEG₄-c(RGDfK)]₂ (BOC-K(NIC)-3P-RGD₂)

BOC-K(NIC)-OSu (5.4 mg, 12 μ mol) and PEG₄-E[PEG₄-c(RGDfK)]₂ (5.0 mg, 2.4 μ mol) were dissolved in DMF (2 mL). To the mixture was added DIEA (3 drops). The solution was stirred at room temperature overnight. After addition of water (2 mL), the pH was adjusted to 6.0 – 7.0. The product was separated from the reaction mixture by HPLC (method 1). Lyophilization of collected fractions at 21.5 min afforded the intermediate BOC-K(NIC)-3P-RGD₂. The yield was 3.4 mg (60%). ESI-MS: m/z = 2392.4 for [M + H]⁺ (M = 2392.2 calcd. for [C₁₀₉H₁₇₃N₂₅O₃₅]⁺).

Lys(nicotinoyl)-PEG₄-E[PEG₄-c(RGDfK)]₂ (K(NIC)-3P-RGD₂)

Boc-K(NIC)-3P-RGD₂ was dissolved in 2.0 mL of TFA. After stirring the solution at room temperature for 15 min, TFA was removed, and 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 collected fractions at 14.2 min afforded the expected product K(NIC)-3P-RGD₂. The yield was 3.0 mg (~90%). ESI-MS: m/z = 2294 for [M + H]⁺ (M = 2292.2 calcd. for [C₁₀₄H₁₆₅N₂₅O₃₃]⁺).

HYNIC-Lys(nicotinoyl)-PEG₄-E[PEG₄-c(RGDfK)]₂ (HYNIC-K(NIC)-3P-RGD₂)

HYNIC-OSu (2.9 mg, 6.5 μ mol) and K(NIC)-3P-RGD₂ (3.0 mg, 1.3 μ mol) were dissolved in DMF (2 mL). After addition of DIEA (3 drops), the mixture was stirred at room temperature for 3 days. The product was separated from the reaction mixture by HPLC (Method 1). Lyophilization of collected fraction at 18 min afforded the expected product HYNIC-K(NIC)-3P-RGD₂. The yield was 1.0 mg (30%). ESI-MS: m/z = 2595.3 for [M + H]⁺ (M = 2595.2 calcd. for [C₁₁₇H₁₇₄N₂₈O₃₇S]⁺).

^{99m}Tc-Labeling and Dose Preparation

To a 5 cc vial were added the HYNIC-peptide conjugate (25 µg), 0.4 mL of 0.25 M succinate buffer (pH = 4.8), and 0.4 mL of tricine solution (25 mg/mL in 0.25 M succinate buffer). After addition of 0.5 mL of ^{99m}TcO₄⁻ (370 – 740 MBq in saline) and 25 µL of SnCl₂ (1.0 mg/mL in 0.1 N HCl), the vial was heated at 100 °C for 20 min in a water bath. The vial was then allowed to stand at room temperature for ~5 min. A sample of the resulting solution was analyzed by radio-HPLC (Method 2). Log P values were determined according to the literature procedure.¹⁸ For biodistribution studies, ^{99m}Tc radiotracers were purified by HPLC. Volatiles in the mobile phase were removed under vacuum. Doses were prepared by dissolving the radiotracer in saline to ~1110 kBq/mL. For imaging studies, doses were prepared by dissolving the radiotracer in saline to ~37 MBq/mL. In the blocking experiment, RGD₂ was dissolved in the dose solution to a concentration of 3.5 mg/mL. The resulting solution was filtered with a 0.20 µm Millex-LG filter before being injected into animals. Each animal was injected with ~0.1 mL of the dose solution.

Interconversion of Isomers

Complex **2** was prepared according to the procedure above. Peak **A** and Peak **C** were collected into two separate round-bottom flasks. After complete removal of the HPLC mobile phases, the residue in each flask was re-dissolved in saline (1 mL). The resulting solution was analyzed by radio-HPLC (Method 2) at t = 0, 1, 2, 3, and 5 h post-purification for Peak **A** and t = 0.5, 1.5, 2.5, 3.5, and 5.5 h post-purification for Peak **C**.

Animal Model

Biodistribution and imaging studies were performed in compliance with the NIH animal experimentation guidelines (*Principles of Laboratory Animal Care*, NIH Publication No. 86-23, revised 1985). The protocol was approved by the Purdue University Animal Care and Use Committee (PACUC). All human tumor cell lines (U87MG, MDA-MB-435, A549 and PC-3) were obtained from ATCC (Manassas, VA). U87MG glioma cells were cultured in the Minimum Essential Medium, Eagle with Earle's Balanced Salt Solution (non-essential amino acids sodium pyruvate). PC-3 and A549 cancer cells were cultured in the F-12 medium (GIBCO, Grand Island, NY). MDA-MB-435 breast cancer cells were grown in the RPMI Medium 1640 with L-glutamine (GIBCO, Grand Island, NY). All tumor cell lines were supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin solution, and grown 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. Female athymic *nu/nu* mice were purchased from Harlan at 4 – 5 weeks of age, and were implanted with 5 × 10⁶ tumor cells in 0.1 mL of saline into the shoulder flanks. All procedures were performed in a laminar flow cabinet using aseptic techniques. Four to six weeks after inoculation, the tumor size was 0.1 – 0.5 g, and animals were used for biodistribution and imaging studies.

Biodistribution Protocol

Four tumor-bearing mice (20 – 25 g) were randomly selected, and each animal was administered with ~111 kBq of the ^{99m}Tc radiotracer by tail vein injection. The animals were sacrificed by sodium pentobarbital overdose (~200 mg/kg) at 60 min post-injection (p.i.). Blood was withdrawn from the heart of tumor-bearing mice. Tumors 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 Perkin Elmer Wizard – 1480 γ-counter (Shelton, CT). The organ uptake was calculated as the percentage of injected dose per gram of organ mass (%ID/g). For **3**, the biodistribution data were collected at 30, 60 and 120 min p.i., and four animals were used at each time

point. For the blocking experiment, four tumor-bearing athymic nude mice were used, and each animal was administered with ~111 kBq of **3** along with ~350 µg (~14 mg/kg) of RGD₂. Biodistribution data (%ID/g) and tumor-to-background (T/B) ratios are reported as an average plus the standard deviation on the basis of the results from four tumor-bearing mice (8 tumors). Comparison between two radiotracers or between two different tumor types was made using the one-way ANOVA test (GraphPad Prim 5.0, San Diego, CA). The level of significance was set at $p < 0.05$.

Planar Imaging

Planar imaging was performed by using athymic nude mice ($n = 3$) bearing U87MG, MDA-MB-435, A549 or PC-3 tumor xenografts. Each animal was administered with ~10 MBq of ^{99m}Tc radiotracer in 0.1 mL saline via tail vein injection. Animals were then anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg), and were then placed supine on a custom-made single head mini γ -camera (Diagnostic Services Inc., NJ) equipped with a parallel-hole, low-energy and high-resolution collimator. Anterior images were acquired at 15, 30, 60, 120 min p.i. and stored digitally in a 128 × 128 matrix. The acquisition count limits were set at 300 K. After completion of imaging, animals were sacrificed by sodium pentobarbital overdose (~200 mg/kg). The whole-body planar images were selected and reported without filtration.

Metabolism

Normal athymic nude mice ($n = 3$) were used for metabolism studies. Each mouse was administered with ~3.7 MBq of **1**, **2** or **3** 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 filtered. The filtrate was analyzed by HPLC. Feces samples were collected at 120 min p.i. and suspended in 20% acetonitrile aqueous solution after homogenization. The resulting mixture was vortexed for ~5 min. After centrifuging at 8,000 rpm, the supernatant was collected and filtered. The filtrate was analyzed by HPLC. The percentage radioactivity recovery was determined by γ -counting for urine and feces samples.

Immunohistochemistry

Tumor tissues were harvested, snap-frozen immediately in the OCT (optimal cutting temperature compound) solution, and cut into slices (5 µm). After drying thoroughly, the slides were fixed with ice-cold acetone for 10 min, and dried in air for 20 min. Sections were blocked with 10% goat serum for 30 min, and then were incubated with the hamster anti-integrin β_3 antibody (1:100, BD Biosciences, San Jose, CA) and rat anti-CD31 antibody (1:100, BD Biosciences, San Jose, CA) for 1 h at room temperature. After incubating with Cy3-conjugated goat anti-hamster and FITC-conjugated goat anti-rat secondary antibodies (1:100, Jackson ImmunoResearch Inc., West Grove, PA) and washing with PBS. The fluorescence was visualized with an Olympic BX51 microscope (Olympus America Inc., Center Valley, PA). All pictures were taken under 200x magnification using the same exposure time. The average area of positively stained CD31 or β_3 on cryostat sections from at least 15 randomly selected fields in each group was calculated to assess the relative expression in tumor tissues by the NIH *ImageJ* software. The fluorescent density data were expressed as a percentage of the total area, presented as the mean $\pm$ S.D., and plotted against the %ID/g tumor uptake (radioactivity density) and T/B ratios of complex **3**. Experiments were performed three times. Statistical analysis was performed by Newman-Keuls multiple comparison. The level of significance was set at $p < 0.05$.

Supporting Information Available

Biodistribution data of **1**, **2**, **3** and **4** (Table SI1) in the athymic nude mice ($n = 4$ with 8 tumors) bearing MDA-MB-435 tumor xenografts at 1 h p.i., biodistribution data (Table SI2) of **3** in the athymic nude mice ($n = 4$ with 8 tumors) bearing four different human tumor xenografts at 1 h p.i., the radio-HPLC chromatograms of **2** (Figure SI1) to show the inter-conversion from **A** to **D** (top) and **C** to **B** (bottom) at room temperature, radio-HPLC chromatograms of **1**, **2** and **3** (Figure SI2) in the saline (**A**) before injection, in the urine at 30 min p.i. (**B**) and 120 min p.i. (**C**), and in the feces (**D**) at 120 min p.i., and representative microscopic fluorescence images (Figure SI3) of the xenografted tumor tissues (U87MG, MDA-MB-435, A549 and PC-3), are all in word document. These materials are available free of charge at <http://pubs.acs.org>.

RESULTS

HYNIC-Conjugate Synthesis

As illustrated in Chart I, HYNIC-K(NIC)-RGD₂, HYNIC-K(NIC)-3G-RGD₂, and HYNIC-K(NIC)-3P-RGD₂ were prepared by reacting K(NIC)-RGD₂, K(NIC)-3G-RGD₂ and K(NIC)-3P-RGD₂, respectively, with excess HYNIC-OSu in DMF in the presence of DIEA. All new cyclic RGD peptide conjugates were purified by semi-prep HPLC and characterized by ESI-MS. Their HPLC purities were > 95% before being used for ^{99m}Tc-labeling.

Radiochemistry

^{99m}Tc complexes **1** – **3** were prepared according to Chart I from the reaction of the HYNIC conjugate with ^{99m}TcO₄[−] in the presence of excess tricine and stannous chloride. ^{99m}Tc-labeling was completed by heating the reaction mixture at 100 °C for 20 – 30 min. The [^{99m}Tc]-colloid formation was minimal (<0.5%) if more than 10 mg tricine was used in each vial. Complexes **1**, **2** and **3** could be prepared with high radiochemical purity (RCP >90%) and very high specific activity (~111 GBq/μmol). Their partition coefficients were determined in an equal volume mixture of 1-octanol and 25 mM phosphate buffer (pH = 7.4). The calculated log P values of complexes **1** – **3** along with their HPLC retention times are summarized in Table 1.

Isomerism

Figure 2 illustrates representative radio-HPLC chromatograms of macrocyclic ^{99m}Tc complexes **1** – **3**. As expected, there were two pairs of peaks (designated as **A** – **D**) for **1** and **2**. We isolated **A** and **C** for complex **2**, and studied their relationship with **B** and **D**. It was found that **A** was able to convert to **D** while **C** was converted to **B** even at the room temperature (Figure SI1). There was no inter-conversion between **A** and **B** or **C** and **D**, suggesting that **A** and **D** (or **C** and **B**) are conformational isomers due to restricted rotations of the macrocycle (Figure 1B). Similar results were also obtained in our previous studies.^{15, 16} However, we were not able to separate the four isomers of **3** despite of many attempts to change chromatographic conditions by using different HPLC columns, flow rates and ionic strength of the mobile phase. It is possible that the lipophilicity of the four isomers in **3** is so close that it always shows one single radiometric peak in its radio-HPLC chromatogram.

Biodistribution

Table 2 lists biodistribution data of **3** in athymic nude mice bearing MDA-MB-435 human breast tumor xenografts at 30, 60 and 120 min p.i. We also obtained 60-min biodistribution data (Table SI1) of **1**, **2** and **4**. The 60-min data were used because the blood radioactivity accumulation was relatively low at this time point (Figure 3A). The tumor uptake of **3** was

3.90, 4.94, and 4.85 %ID/g at 30, 60 and 120 min p.i., respectively (Table 2). Its liver uptake was 2.45, 1.77, and 2.05 %ID/g with tumor/liver ratios being 1.64, 2.88, and 2.38 at 30, 60, and 120 min p.i. respectively. Replacing [$^{99m}\text{Tc}(\text{HYNIC})(\text{tricine})(\text{TPPTS})$] with [$^{99m}\text{Tc}(\text{HYNIC-K}(\text{NIC}))(\text{tricine})$] resulted in lower uptake in the kidneys and lungs for **3** (Figure 3A: $p < 0.05$). Complex **1** also had high tumor uptake (5.73 ± 0.40 %ID/g), which was comparable to that of **3** (4.94 ± 1.71 %ID/g) within the experimental error. Complex **2** had the best tumor/liver ratio (Figure 3B: 4.1 ± 0.97).

Bioequivalence of Isomers

We compared the 60-min organ uptake of the purified Peak **A** and Peak **C** of complex **2** in athymic nude mice bearing MDA-MB-435 breast cancer xenografts, and found that these two isomers had almost identical biodistribution patterns (Table S1). For example, the tumor uptake was 5.24 ± 1.09 %ID/g for Peak **A** and 4.55 ± 1.44 %ID/g for Peak **C**. The blood radioactivity was 0.60 ± 0.15 %ID/g for Peak **A** and 0.56 ± 0.20 %ID/g for Peak **C**. Their uptake values in normal organs were also comparable within the experimental error except that in the intestine (Table S1). Since there was an interconversion between isomer **A** and **D** or **C** and **B**, we believe that different isomers in macrocyclic complexes **1** and **3** will also have equivalent tumor uptake.

Integrin $\alpha_v\beta_3$ Specificity

Figure 3C compares the organ uptake of **3** in the absence/presence of RGD₂ at 60 min p.i. Co-injection of excess RGD₂ significantly blocked its tumor uptake (1.48 ± 0.42 %ID/g with RGD₂ vs 4.94 ± 1.71 %ID/g without RGD₂). The normal organ uptake was also blocked by RGD₂. For example, the uptake of **3** in the intestine, lungs and spleen was 5.56 ± 1.73 , 2.19 ± 0.94 , and 1.09 ± 0.44 %ID/g, respectively, without RGD₂, while its uptake in the same organs was 1.82 ± 0.86 , 1.52 ± 0.27 , and 0.65 ± 0.07 %ID/g, respectively, in the presence of RGD₂.

Metabolism

We examined the metabolic stability of complexes **1** – **3** using normal athymic nude mice. It was found that the radioactivity recovery from the urine and feces samples was $> 95\%$ for all three radiotracers. There were metabolites detected in both urine and feces samples over the 2 h study period for **1** (Figure SI2). Peak **A** was completely metabolized while Peak **C** still remained intact. There was also significant metabolism of **2** during its excretion via both renal and hepatobiliary routes (Figure SI2). However, very little metabolite was detected in urine samples for **3** over the 2 h period and $\sim 65\%$ of **3** remained intact in the feces sample.

Comparison of Tumor Uptake in Different Animal Models

We compared the uptake of **3** in the xenografted U87MG, MDA-MB-435, A549 and PC-3 tumors (Table SI2). As illustrated in Figure 4A, U87MG gliomas had the highest uptake and the uptake of PC-3 tumor was the lowest. Its uptake in MDA-MB-435 and A549 tumors was comparable within experimental errors. The tumor uptake of **3** followed a general trend: U87MG (8.43 ± 3.14 %ID/g) $>$ MDA-MB-435 (4.94 ± 1.71 %ID/g) $\sim$ A549 (3.47 ± 0.88 %ID/g) $\gg$ PC-3 (1.52 ± 0.42 %ID/g). To further illustrate this trend, we obtained whole-body images (Figure 4B) of athymic nude mice bearing U87MG, MDA-MB-435, A549 and PC-3 tumor xenografts. Once again, the U87MG glioma had the highest uptake with the best contrast. PC-3 tumors were not clearly visualized. The T/B contrast in the planar images for MDA-MB-435 and A549 tumors was not as good as that of U87MG gliomas; but it was much better than that of the PC-3 tumors.

Comparison of Tumor $\alpha_v\beta_3$ Expression Levels

Immunohistochemistry was performed to determine the $\alpha_v\beta_3$ levels in tumor tissues according to the literature method.²⁸ Figure SI3 illustrates microscopic fluorescence images of the xenografted tumor tissues (U87MG, MDA-MB-435, A549 and PC-3) labeled with anti-integrin β_3 (red) and anti-CD31 (green) antibodies. In the overlay images, the green color indicates the presence of blood vessels, red color indicates the presence of β_3 , and yellow color indicates the β_3 expressed on the tumor vasculature. Figure 5 shows the quantitative analysis of β_3 (A) and CD31 (B) for U87MG, MDA-MB-435, A549 and PC-3 tumor tissues. The β_3 expression level (fluorescence density) was defined by the percentage of red-colored area over the total area in each slice of tumor tissue. Since $\alpha_{IIIB}\beta_3$ is expressed almost exclusively on activated platelets, the relative fluorescent intensity in tumor tissue is predominantly from the contribution of $\alpha_v\beta_3$. Blood vessel density (CD31 expression level) was quantified by measuring the percentage of green-colored area over the total area in each slice of tumor tissue. We found that the $\alpha_v\beta_3$ expression level was the highest in U87MG glioma and the lowest in PC-3 prostate tumor tissues. It followed the trend: U87MG > MDA-MB-435 ~ A549 > PC-3 (Figure 5A), which is completely consistent with the tumor uptake data (Figure 4). The CD31 expression levels (blood vessel density) followed the same order: U87MG > MDA-MB-435 ~ A549 > PC-3 (Figure 5B). Similar results were also obtained for **4** in our previous studies.¹⁹

Relationship between $\alpha_v\beta_3$ Expression and Tumor Uptake or Tumor/Muscle Ratios

Figure 5C shows plot of the %ID/g tumor uptake (radioactivity density) of **3** and $\alpha_v\beta_3$ levels (fluorescence density) in U87MG, MDA-MB-435, A549 and PC-3 tumor tissues.

Apparently, there is a linear relationship between the %ID/g tumor uptake of **3** and $\alpha_v\beta_3$ levels with R^2 being 0.9536. The same linear relationship was also seen between the T/M ratios of **3** and $\alpha_v\beta_3$ levels with R^2 being 0.9452 (Figure 5D).

DISCUSSION

In this study, we evaluated the impact of cyclic RGD peptides and ^{99m}Tc chelates on biodistribution properties and metabolic stabilities of ^{99m}Tc radiotracers. We found that **1**, **2** and **3** all have two major isomers (Figure 2), which have almost identical tumor uptake and biodistribution properties (Table SI2). Replacing [$^{99m}\text{Tc}(\text{HYNIC})(\text{tricine})(\text{TPPTS})$] with a smaller [$^{99m}\text{Tc}(\text{HYNIC}-\text{K}(\text{NIC}))(\text{tricine})$] resulted in less uptake in kidneys and lungs for **3** (Figure 3A: $p < 0.05$). The blocking experiment (Figure 3C) showed that their tumor uptake is $\alpha_v\beta_3$ -specific. This conclusion is completely consistent with our previous findings.¹⁸

In principle, HYNIC can be used for ^{99m}Tc -labeling of any small biomolecule.^{13, 14} However, problems may arise when biomolecules contain one or more disulfide linkages, which are often vital to keep the rigid cyclic conformation and to maintain their high receptor binding affinity. The use of a large amount of TPPTS in combination with high temperature heating may destroy the S-S disulfide bonds and cause adverse effect on biological properties of ^{99m}Tc -labeled small biomolecules. When HYNIC-K(NIC) is used as the BFC, SnCl_2 is the reducing agent, and tricine is used to stabilize the ^{99m}Tc -HYNIC core.¹⁶ There is no need for TPPTS either as reducing agent for $^{99m}\text{TcO}_4^-$ or coligand to stabilize the ^{99m}Tc -HYNIC core. In this respect, HYNIC-K(NIC) offers significant advantages over HYNIC as the BFC for ^{99m}Tc -labeling of small biomolecules with one or more disulfide linkages.

Recently, we found that 3P-RGD₂ and 3G-RGD₂ (Figure 1) have a significant advantage over RGD₂ with respect to their $\alpha_v\beta_3$ binding affinity and the tumor uptake of their radiotracers in the xenografted U87MG human glioma.^{20–26} However, this advantage

disappeared in the xenografted MDA-MB-435 breast tumor model because all three ^{99m}Tc radiotracers share a similar tumor uptake (**1**: 5.73 ± 0.40 %ID/g; **2**: 5.24 ± 1.09 %ID/g; and **3**: 4.94 ± 1.71 %ID/g) at 60 min p.i. A possible explanation is the fact that the $\alpha_v\beta_3$ level in xenografted MDA-MB-435 breast tumors is significantly lower than that in the U87MG glioma xenografts.^{19, 26} If the $\alpha_v\beta_3$ density is high, the distance between two neighboring $\alpha_v\beta_3$ sites will be short, which makes it easier for a multimeric RGD peptide to achieve the bivalency. If the $\alpha_v\beta_3$ density is low, the distance between two neighboring $\alpha_v\beta_3$ sites will be long, and it might be more difficult for the same multimeric cyclic RGD peptide to achieve the simultaneous $\alpha_v\beta_3$ binding.

Among the ^{99m}Tc radiotracers evaluated in this study, **3** has the highest metabolic stability, but it was not as good as that of **4**.¹⁸ To determine if the metabolic instability of **3** affects the linear relationship between its %ID/g tumor uptake and the $\alpha_v\beta_3$ expression levels, we performed biodistribution studies to quantify the tumor uptake of **3**, and immunohistochemical staining studies to quantify the $\alpha_v\beta_3$ and CD31 expression levels in the xenografted U87MG, MDA-MB-435, A549 and PC-3 tumors. We found that **3** had the highest uptake in U87MG glioma and lowest uptake in PC-3 tumors, and its tumor uptake followed the trend of U87MG > MDA-MB-435 ~ A549 > PC-3 (Figure 4A), which was further confirmed by planar imaging studies (Figure 4B). The $\alpha_v\beta_3$ levels follow the order of U87MG > MDA-MB-435 ~ A549 > PC-3 (Figure 5A). The linear relationship between the $\alpha_v\beta_3$ expression levels and its tumor uptake (Figure 5C) or tumor/muscle ratios (Figure 5D) suggests that **3** is useful for monitoring the tumor $\alpha_v\beta_3$ expression, and its metabolic instability didn't affect the linear relationship between its tumor uptake and the $\alpha_v\beta_3$ levels.

The ability to non-invasively estimate the $\alpha_v\beta_3$ level is important for the selection of the appropriate cancer patients who will benefit most from the anti- $\alpha_v\beta_3$ and antiangiogenesis therapy.^{27–32} For example, if the cancer patient has high tumor uptake of **3**, he/she would be most likely responsive to the anti- $\alpha_v\beta_3$ treatment. Conversely, if the cancer patient shows little tumor uptake of **3**, the anti- $\alpha_v\beta_3$ therapy would not be effective regardless of the amount of anti- $\alpha_v\beta_3$ drug administered to the cancer patient. It is important to realize that the $\alpha_v\beta_3$ levels obtained from imaging studies are used only in relative terms (high, medium and low). In clinical settings, it is possible to compare the $\alpha_v\beta_3$ levels obtained from two control levels: one from highly aggressive glioma with very high $\alpha_v\beta_3$ expression, and the other obtained from much less aggressive tumors with low $\alpha_v\beta_3$ expression to determine more precisely whether the tested level is high, low or intermediate.

CONCLUSION

In conclusion, we found that HYNIC-K(NIC) is a useful BFC for routine ^{99m}Tc -labeling of small biomolecules. Replacing the highly charged [$^{99m}\text{Tc}(\text{HYNIC})(\text{tricine})(\text{TPPTS})$] with a much smaller [$^{99m}\text{Tc}(\text{HYNIC-K(NIC)})(\text{tricine})$] resulted in less uptake in the kidneys and lungs. The metabolic stability of ^{99m}Tc radiotracers depends on both RGD peptides (3P-RGD₂ > 3G-RGD₂ ~ RGD₂) and ^{99m}Tc chelates ([$^{99m}\text{Tc}(\text{HYNIC})(\text{tricine})(\text{TPPTS})$] > [$^{99m}\text{Tc}(\text{HYNIC-K(NIC)})(\text{tricine})$]). However, the metabolic instability of **3** has little effects on its tumor-targeting capability, and the linear relationship between the tumor $\alpha_v\beta_3$ expression levels and its tumor uptake or tumor/muscle ratios. We believe that **3** is a very attractive radiotracer for early cancer detection and noninvasive monitoring of the tumor $\alpha_v\beta_3$ expression before and after anti- $\alpha_v\beta_3$ therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structure of cyclic RGD peptide dimers and their ^{99m}Tc complexes. Structure **A** represents the ternary ligand system, in which tricine and TPPTS are used as co-ligands to stabilize the ^{99m}Tc -HYNIC core. Structure **B** represents the chelating HYNIC system with tricine as the only co-ligand to complete the octahedral coordination sphere of technetium. In this way, the bulky and highly charged TPPTS is eliminated. The small letter “f” represents D-phenylalanine.

Figure 2. Representative radio-HPLC chromatograms of **1** (top), **2** (middle) and **3** (bottom). The two pairs of radiometric peaks were designated as **A – D** for complexes **1** and **2**.

Figure 3. Comparison of biodistribution data (A) and the selected T/B ratios (B) of **1**, **2**, **3** and **4** at 60 min p.i. in athymic nude mice ($n = 4$) bearing MDA-MB-435 breast tumor xenografts. **C**: Comparison of biodistribution data **3** in the absence/presence of excess RGD₂ (350 μ g/mouse) at 60 min p.i. in the athymic nude mice ($n = 4$) bearing MDA-MB-435 breast tumor xenografts.

Figure 4.

A: Comparison of the %ID/g tumor uptake of **3** in the athymic nude mice bearing U87MG, MDA-MB-435, A549 and PC-3 tumor xenografts at 60 min p.i. The tumor uptake data are expressed as %ID/g $\pm$ S.D (*: $p < 0.05$, significantly different from all other groups; †: $p < 0.05$, significantly different from U87MG group; #: $p < 0.05$, significantly different from PC-3 group). **B:** Planar images of the athymic nude mice bearing U87MG, MDA-MB-435, A549 and PC-3 tumor xenografts at 60 min p.i.

Figure 5.

Top: Quantitative analysis of β_3 (A) and CD31 (B) immunostaining from the xenografted tumor tissues (U87MG, MDA-MB-435, A549 and PC-3). The CD31 expression was represented by the percentage of green area over the total area in each slice of tissue. The total integrin β_3 expression (tumor cells and neovasculature) was represented by the percentage of red area over the total area in each slice of tumor tissue. Each data point was derived from at least 15 different areas of the same tissue (100X magnification). Experiments were repeated three times independently with similar results. Values are means $\pm$ S.D. (*: $p < 0.05$, significantly different from all the other groups; †: $p < 0.05$, significantly different from the U87MG group; #: $p < 0.05$, significantly different from the PC-3 group). **Bottom:** Relationship between the relative β_3 expression (fluorescence density) obtained from tissue staining and the %ID/g tumor uptake (C: radioactivity density) or tumor/muscle ratios (D) of **3** in four different xenografted tumors (U87MG, MDA-MB-435, A549 and PC-3).

Chart I.

Representative synthetic scheme for preparation of HYNIC-conjugated cyclic RGD peptides and their corresponding macrocyclic ^{99m}Tc complexes.

Table 1

Radiochemical purity (RCP), HPLC retention time and log P values for $^{99\text{m}}\text{Tc}$ -labeled cyclic RGD peptides.

Radiotracer	RCP (%)	Retention Time (min)		Log P Value
		A	C	
1	> 90	14.2	16.1	-3.39 ± 0.06
2	> 95	10.9	13.4	-3.25 ± 0.08
3	> 95	13.7		-2.25 ± 0.07
4	> 95	12.9		-4.35 ± 0.10

Table 2

Biodistribution data of **3** in athymic nude mice (n = 4) bearing MDA-MB-435 human breast tumor xenografts.

Organ	30 min	60 min	120 min
Blood	0.99 ± 0.07	0.84 ± 0.09	0.49 ± 0.15
Brain	0.18 ± 0.05	0.14 ± 0.03	0.10 ± 0.05
Eyes	0.98 ± 0.28	1.64 ± 0.38	0.85 ± 0.30
Heart	1.21 ± 0.43	0.93 ± 0.37	0.83 ± 0.27
Intestine	6.22 ± 1.00	5.56 ± 2.73	7.26 ± 4.55
Kidneys	11.32 ± 2.87	6.41 ± 2.78	7.04 ± 3.30
Liver	2.45 ± 0.62	1.77 ± 0.70	2.05 ± 0.68
Lungs	3.34 ± 0.54	2.19 ± 0.94	2.05 ± 0.58
Muscle	1.07 ± 0.43	0.74 ± 0.32	0.53 ± 0.09
Spleen	1.84 ± 0.43	1.09 ± 0.44	1.37 ± 0.41
MDA-MB-435	3.90 ± 0.54	4.94 ± 1.71	4.85 ± 1.73
Tumor/Blood	3.19 ± 0.98	5.84 ± 1.75	9.64 ± 1.21
Tumor/Liver	1.64 ± 0.32	2.88 ± 0.83	2.38 ± 0.76
Tumor/Lung	1.17 ± 0.14	2.36 ± 0.66	2.31 ± 0.46
Tumor/Muscle	3.93 ± 1.01	7.07 ± 2.72	9.48 ± 4.15