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Impact of PKM Linkers on Biodistribution Characteristics of the ^{99m}Tc -Labeled Cyclic RGDfK Dimer

Shuang Liu^{*}, Zhengjie He, Wen-Yuan Hsieh, Young-Seung Kim, and Young Jiang

Abstract

This report describes synthesis of three new cyclic RGDfK peptide conjugates, HYNIC-PKM-SU016 (PKM = E, K and PEG4), and *in vivo* evaluation of the impact of PKM linkers on biodistribution characteristics of their ternary ligand complexes [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] in athymic nude mice bearing the MDA-MB-435 human breast cancer xenografts. Results from biodistribution studies show that PKM linkers have minimal impact on the integrin $\alpha_v\beta_3$ binding capability of radiotracers. Even though they have different charges under physiological conditions, all three linkers (E, K and PEG4) are able to reduce the uptake of ^{99m}Tc -labeled E[c(RGDfK)]₂ in blood, kidneys, liver and lungs, and increase target-to-background (T/B) ratios at >30 min postinjection. E and K may have advantages over PEG4 due to a combination of relatively low liver uptake, high tumor/liver and tumor/lung ratios of ternary ligand complexes [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = E and K).

INTRODUCTION

For the last several years, we and others have been using multimeric cyclic RGD peptides, such as E[c(RGDfK)]₂ (SU016), to develop integrin $\alpha_v\beta_3$ -targeted radiotracers for imaging rapidly growing and metastatic tumors (1–11). The RGD peptide serves as “vehicles” to carry radionuclide (^{99m}Tc , ^{18}F and ^{64}Cu) to the integrin $\alpha_v\beta_3$ overexpressed on tumor cells and activated endothelial cells of the tumor neovasculature. ^{99m}Tc is the choice of radionuclide for single photon computed tomography (SPECT) and planar imaging due to its optimal nuclear properties (half-life, abundance and energy of gamma photons) and easy availability at low cost (12–14). For the ^{99m}Tc -labeling, 6-hydrazinonicotinic acid (HYNIC) has been used as a bifunctional coupling agent while tricine and a water-soluble phosphine or pyridine analog are used as coligands to prepare ternary ligand ^{99m}Tc complexes [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{L})$] (L = ISONIC (isonicotinic acid), TPPTS (trisodium triphenylphosphine-3,3',3''-trisulfonate, and PDA (2,5-pyridinedicarboxylic acid)) (1–3).

Recently, we studied the effect of coligands on biodistribution characteristics of ternary ligand complexes [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{L})$] (Figure 1: L = ISONIC, TPPTS and PDA) in athymic nude mice bearing MDA-MB-435 human breast cancer xenografts (2). It was found that coligand has a significant impact on the radiotracer excretion kinetics from non-tumor organs, such as liver and lungs, while maintaining their tumor targeting capability. Similar results were observed for [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{L})$] (L = TPPTS and ISONIC) and [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{EDDA})$] (EDDA = ethylenediamine-N,N'-diacetic acid) in BALB/c mice bearing human U87MG glioma xenografts (3). Among several radiotracers evaluated, [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{TPPTS})$] shows a great potential as a new radiotracer for imaging the integrin $\alpha_v\beta_3$ -positive tumors.

^{*}To whom correspondence should be addressed. School of Health Sciences, Purdue University, 550 Stadium Mall Drive, West Lafayette, IN 47907. Phone: 765-494-0236; Fax 765-496-1377; Email: lius@pharmacy.purdue.edu.

It is highly desirable for the radiotracer to clear from non-cancerous organs, such as liver and lungs, so that a high target-to-background (T/B) ratio can be achieved and clinically useful images of tumor can be obtained in a short period of time. In the last several years, a variety of pharmacokinetic modifying (PKM) linkers have been used to improve excretion kinetics of radiolabeled cyclic RGD peptides. For example, a sugar moiety was used to increase T/B ratios of the ^{125}I - and ^{18}F -labeled cyclic RGD peptides (15–20). Small peptides sequences, such as di(cysteic acid), Asp₃ and Ser₃, have been used to improve blood clearance and to minimize liver accumulation of radiolabeled nonpeptide integrin $\alpha_v\beta_3$ antagonists (21,22) and ^{18}F -labeled c(RGDfK) (23,24). The hexaethylene glycol (HEG) linker has been used in the ^{18}F -labeled RGDfE dimer and tetramer (11,12,23,24). It was reported that introduction of the polyethylene glycol (PEG) linker can improve not only tumor uptake but also excretion kinetics of ^{125}I - and ^{18}F -labeled c(RGDyK) and ^{64}Cu -labeled E[c(RGDyK)]₂ (25–28). PKM linkers and their applications to improve the excretion kinetics of radiolabeled small biomolecules have been reviewed recently (13,24,29–31).

To explore the impact of PKM linkers on tumor uptake and excretion kinetics of the $^{99\text{m}}\text{Tc}$ -labeled E[c(RGDfK)]₂, we have prepared three HYNIC conjugates, HYNIC-PKM-SU016 (Figure 1: PKM = glutamic acid (E), lysine (K), and PEG4), and their ternary ligand complexes [$^{99\text{m}}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = E, K, and PEG4). These three PKM linkers were chosen due to their different charge under physiological conditions. In this report, we present synthesis and *in vivo* evaluation of biodistribution characteristics of complexes [$^{99\text{m}}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = E, K, and PEG4) in athymic nude mice bearing MDA-MB-435 human breast cancer xenografts. The main objective of this study is to select an optimal PKM linker (E, K or PEG4) for the $^{99\text{m}}\text{Tc}$ -labeled small biomolecules, which is very important in successful development of target-specific radiopharmaceuticals.

EXPERIMENTAL SECTION

Materials

Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS), succinic acid, trisodium triphenylphosphine-3,3',3''-trisulfonate (TPPTS), and tricine were purchased from *Sigma-Aldrich* (St. Louis, MO). Boc-Glu(OSu)OH and lys(Boc)-OH were from Bachem Biosciences Inc. (Prussia, PA). The Boc-protected PEG4 was obtained from Quanta BioDesign, Ltd (Powell, OH). Cyclic pentapeptide, c(RGDfK), was obtained from Peptides International, Inc. (Louisville, KY). The cyclic RGDfK dimer, E[c(RGDfK)]₂ (SU016), was prepared according to the procedure described in our previous communication (2). Sodium succinimidyl 6-(2-(2-sulfonatobenzaldehyde)hydrazono)nicotinate (HYNIC-NHS) was prepared according to literature procedure (32). Na $^{99\text{m}}\text{TcO}_4$ was obtained from a DuPont Pharma $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generator, N. Billerica, MA.

Instrument and Methods

The ^1H NMR data were obtained using a Bruker DRX 300 MHz FT NMR spectrometer and chemical shifts as δ are reported in ppm relative to TMS. Mass spectral data were collected on a Finnigan LCQ classic mass spectrometer, School of Pharmacy, Purdue University. The semi-prep HPLC method (Method 1) used a LabAlliance semi-prep HPLC system equipped with a UV/vis detector ($\lambda = 254$ nm) and Zorbax C₁₈ semi-prep column (9.4 mm $\times$ 250 mm, 100 Å pore size) for peptide purification. The flow rate was 2.5 mL/min. The mobile phase was isocratic with 90% solvent A (0.1% acetic acid in water) and 10% solvent B (0.1% acetic acid in acetonitrile) at 0 – 5 min, followed by a gradient mobile phase going from 90% solvent A and 10% solvent B at 5 min to 60% solvent A and 40% solvent B at 20 min. The radio-HPLC method (Method 2) used the LabAlliance HPLC system equipped with a β -ram INUS detector and Zorbax C₁₈ column (4.6 mm $\times$ 250 mm, 300 Å pore size). The flow rate was 1 mL/min.

The mobile phase was isocratic with 90% solvent A (25 mM ammonium acetate buffer, pH = 5.0) and 10% solvent B (acetonitrile) at 0 – 2 min, followed by a gradient mobile phase going from 10% solvent B at 2 min to 15% solvent B at 5 min and to 20% solvent B at 20 min. The ITLC method used GelmanSciences silica-gel paper strips and a 1:1 mixture of acetone and saline as eluant. By this method, ^{99m}Tc complexes migrate to the solvent front while $^{99m}\text{TcO}_4^-$ and $[^{99m}\text{Tc}]$ colloid remain at the origin.

Boc-E(OBu-t)-SU016

To a solution of Boc-E(OBu-t)-NHS (8.0 mg, 0.02 mmol) in DMF (1.0 mL) was added diisopropylethylamine (DIEA) to adjust pH of the reaction mixture to 8.0 – 8.5. SU016 (13.8 mg, 0.01 mmol) was added, and the resulting mixture was stirred for 30 min. The reaction was stirred at room temperature overnight, and the pH was maintained at 8.0 – 8.5 with DIEA. The crude product was dissolved in 2 mL of 0.5 N NH_4OAc buffer (pH = 7.0), and was purified by HPLC (Method 1). The fraction at ~16 min was collected. Lyophilization of the collected fractions afforded Boc-E(OBu-t)-SU016. The yield was 10 mg (~60%). ESI-MS (positive mode): m/z = 1603.9 for $[\text{M} + \text{H}]^+$ (1603.82 calcd. for $[\text{C}_{73}\text{H}_{111}\text{N}_{20}\text{O}_{21}]^+$).

E-SU016

Boc-E(OBu-t)-SU016 (10 mg) was treated with 1.0 mL of trifluoroacetic acid (TFA), and the resulting mixture was stirred at room temperature for 30 min. Excess TFA was removed. The crude product was dissolved in 0.5 N NH_4OAc buffer (pH = 7.0) and purified by HPLC (Method 1). The fraction at ~11 min was collected. Lyophilization of the combined collections afforded E-SU016. The yield was 6.4 mg (~71%). ESI-MS (positive mode): m/z = 1447.5 for $[\text{M} + \text{H}]^+$ (1447.70 calcd. for $[\text{C}_{64}\text{H}_{95}\text{N}_{20}\text{O}_{19}]^+$).

HYNIC-E-SU016

HYNIC-NHS (1.3 mg, 3 μmol) and E-SU016 (3.1 mg, 2 μmol) were dissolved in 1.0 mL of DMF. The mixture was stirred at room temperature for 12 h with pH being maintained at 8.0 – 8.5. The resulting mixture was subjected to HPLC purification (Method 1). The fraction at ~14 min was collected. Lyophilization of the collected fractions afforded HYNIC-E-SU016 as a white powder. The yield was 2.0 mg (~60%). ESI-MS (positive mode): m/z = 1750.5 for $[\text{M} + \text{H}]^+$ (1750.74 calcd. for $[\text{C}_{77}\text{H}_{103}\text{N}_{23}\text{O}_{23}\text{S}]^+$).

HYNIC-K(Boc)OH

To a solution of HYNIC-NHS (200 mg, 0.5 mmol) in 10 mL of DMF were added K(Boc)OH (123 mg, 0.5 mmol) and triethylamine (~60 μL , 0.5 mmol). The mixture was stirred at room temperature for 24 h. After filtration, the filtrate was evaporated to dryness. The residue was washed with diethyl ether. The crude product was purified by HPLC (Method 1). Fractions at ~14 min were collected. Lyophilization of the combined collections gave an off-white powder. The yield was 200 mg (~73%). ^1H NMR ($\text{DMSO}-d_6$, chemical shifts in ppm relative to TMS): 1.34 (s, 9H, Boc group); 1.36 (m, 4H, CH_2); 1.78 (m, 2H, CH_2); 2.90 (q, 2H, $\text{CH}_2\text{NH-Boc}$); 4.35 (m, 1H, CH); 6.75 (t, 1H, NH-Boc); 7.10 – 8.50 (m, 8H, aromatic); 8.52 (s, 1H, $=\text{CH}$); 8.75 (bs, 1H, NHCO); and 11.90 (s, 1H, $\text{NHN}=\text{CH}$). ESI-MS (positive mode): m/z = 550.2 for $[\text{M} + \text{H}]^+$ (549.19 calcd. for $[\text{C}_{24}\text{H}_{32}\text{N}_5\text{O}_8\text{S}]^+$).

HYNIC-K(Boc)-NHS

To a solution of HYNIC-K(Boc)OH (170 mg, 0.31 mmol) and NHS (41 mg, 0.36 mmol) in DMF (5 mL) was added DCC (74 mg, 0.36 mmol). The mixture was stirred at room temperature for 24 h. To the mixture was added 100 μL of acetic acid, and the solution was stirred for 5 h. After filtration, the filtrate was evaporated to dryness. The residue was dissolved in CH_2Cl_2 (10 mL), and the mixture was filtered to remove insoluble solid. To the filtrate was added 20

mL diethyl ether to give an off-white precipitate, which was collected, washed with diethyl ether, and dried under vacuum. The yield was 165 mg (~82%). ¹H NMR (DMSO-*d*₆, chemical shifts in ppm relative to TMS): 1.35 (s, 9H, Boc group); 1.50 (m, 4H, CH₂); 1.80 (m, 2H, CH₂); 3.32 (m, 2H, CH₂NH-Boc); 4.30 (m, 1H, CH); 7.20 – 8.10 (m, 8H, aromatic); 8.30 (t, 1H, NH-Boc); 8.58 (d, 1H, NHCO); 9.00 (s, 1H, =CH); and 11.31 (s, 1H, NH). ESI-MS (positive mode): *m/z* = 647.2 for [M + H]⁺ (647.21 calcd. for [C₂₈H₃₅N₆O₁₀S]⁺).

HYNIC-K(Boc)-SU016

HYNIC-K(Boc)-NHS (1.3 mg, 2 μmol) and SU016 (3.1 mg, 2 μmol) were dissolved in 1.0 mL of anhydrous DMF. The pH was maintained at 8.0 – 8.5 with DIEA, and the reaction mixture was stirred at room temperature for 12 h. After addition of 2 mL of 0.5 N NH₄OAc buffer (pH = 7.0), the product was isolated by HPLC (Method 1). The fraction at ~16 min was collected. Lyophilization of the combined collections afforded the expected product, HYNIC-K(Boc)-SU016. The yield was 6.4 mg (~60%). ESI-MS (positive mode): *m/z* = 1849.9 for [M + H]⁺ (1849.85 calcd. for [C₈₃H₁₁₇N₂₄O₂₃S]⁺).

HYNIC-K-SU016

HYNIC-K(Boc)-SU016 (2 mg) was treated with 1.0 mL of TFA, and the mixture was stirred at room temperature for ~30 min. Excess TFA was removed under vacuum. The residue was dissolved in 3 mL of 0.5 N NH₄OAc buffer (pH = 7.0). The product was purified by HPLC (Method 1). Lyophilization of the collected fractions at ~11 min afforded the expected product, HYNIC-K-SU016. The yield was 1.2 mg (~63%). ESI-MS (positive mode): *m/z* = 1749.9 for [M + H]⁺ (1749.78 calcd. for [C₇₈H₁₀₉N₂₄O₂₁S]⁺).

Boc-PEG4-SU016

To a solution of Boc-PEG4-OH (5.5 mg, 0.015 mmol) in 1 mL of DMF were added NHS (3.5 mg, 0.03 mmol) and DCC (6.2 mg, 0.03 mmol). After the mixture was stirred at room temperature for 2 h, SU016 (26.3 mg, 0.02 mmol) was added into the mixture above. The pH was adjusted 8.0 – 8.5 using DIEA (~8 μL). The reaction mixture was stirred at room temperature overnight. To the reaction mixture was added 3 mL of 0.5 M NH₄OAc buffer (pH = 7.0). The cloudy solution was filtered, and the filtrate was subjected to HPLC purification (Method 1). The fraction at ~14 min was collected. Lyophilization of the combined fractions afforded Boc-PEG4-SU016. The yield was 7.5 mg (~30%). ESI-MS (positive mode): *m/z* = 1665 for [M + H]⁺ (1665.85 calcd. for [C₇₅H₁₁₇N₂₀O₂₃]⁺).

PEG4-SU016

Boc-PEG4-SU016 (10 mg, 6 μmol) was treated with TFA (3.0 mL) at room temperature for 30 min. The TFA was removed under the reduced pressure. The residue was dissolved in 2 mL of ammonium acetate buffer (pH = 7.0), and was then purified by HPLC to afford the expected product PEG4-SU014. The yield was 7.0 mg (~74%). ESI-MS (positive mode): *m/z* = 1565.2 for [M + H]⁺ (1565.80 calcd. for [C₇₀H₁₀₉N₂₀O₂₁]⁺).

HYNIC-PEG4-SU016

In a 5 cc vial, HYNIC-NHS (1.3 mg, 3 μmol) and PEG4-SU016 (3.0 mg, 2 μmol) were mixed with 1 mL of anhydrous DMF. After the mixture was stirred at room temperature for 10 min, the pH was adjusted to 8.0 – 8.5 with DIEA. The reaction mixture was stirred for 6 h at room temperature, and was then mixed with 3 mL of 0.5 M NH₄OAc buffer (pH = 7.0). The resulting solution was subjected to HPLC purification. The desired product HYNIC-PEG4-SU016 was collected after lyophilization. The yield was 2.0 mg (~54%). ESI-MS (positive mode): *m/z* = 1869.0 for [M + H]⁺ (1868.83 calcd. for [C₈₃H₁₁₈N₂₃O₂₅S]⁺).

General Procedure for Preparation of Ternary Ligand ^{99m}Tc Complexes: [$^{99m}\text{Tc}(\text{HYNIC-Linker-SU016})(\text{tricine})(\text{TPPTS})$] (Linker = E, K and PEG4)

To a 5 cc vial filled with 1.0 mL solution (pH = 4.8) containing 5 mg of TPPTS, 6.5 mg of tricine, 40 mg of mannitol, 38.5 mg of disodium succinate hexahydrate, and 12.7 mg of succinic acid, were added 0.2 mL of the solution containing HYNIC-PKM-SU016 (100 $\mu\text{g/mL}$ in water) and 0.3 mL of $\text{Na}[^{99m}\text{TcO}_4]$ solution (30 – 100 mCi/mL) in saline. The vial was heated at 100 $^{\circ}\text{C}$ for 20 min in a lead-shielded water bath. After radiolabeling, the vial was placed back into the lead pig, and allowed to stand at room temperature for 5 – 10 min. A sample of the resulting solution was analyzed by the radio-HPLC (Method 2) and TLC. The radiochemical purity (RCP) was >95%. The [^{99m}Tc]colloid was minimal (<0.5%) using the non- SnCl_2 formulation.

Dose Preparation for Animal Studies

For biodistribution studies, ternary ligand complexes [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = E, K and PEG4) were first prepared, and then purified by radio-HPLC (Method 2). Volatiles in the HPLC mobile phases were completely removed under the reduced pressure. Doses were made by dissolving the HPLC-purified radiotracer in saline to give a concentration of $\sim 10 \mu\text{Ci/mL}$. Each tumor-bearing mouse was administered intravenously with 0.1 mL of the prepared dose solution.

Determination of Log P Values

Log P values of ternary ligand ^{99m}Tc complexes [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = E, K and PEG4) were determined using the following procedure: the radiotracer was prepared and purified by HPLC (Method 2). Volatiles were completely removed under the reduced pressure. The residue was dissolved in a mixture of equal volume (3 mL:3 mL) n-octanol and 25 mM phosphate buffer (pH = 7.4). After stirring vigorously for 20 – 25 min, the mixture was centrifuged for 5 min at a speed of 8,000 rpm. Samples (in triplets) from n-octanol and aqueous layers were obtained, and were counted in a gamma counter (Beckman Gama 8000). The partition coefficient was calculated using the following equation: $P = (\text{activity concentration in n-octanol})/(\text{activity concentration in aqueous layer})$. The log P values were reported as an average of three different measurements plus the standard variation.

Animal Studies

Biodistribution and metabolism studies were performed using the athymic nude mice bearing MDA-MB-435 human breast cancer xenografts in compliance NIH animal experiment guidelines (*Principles of Laboratory Animal Care*, NIH Publication No. 86-23, revised 1985). The animal protocol for animal studies has been approved by the Purdue University Animal Care and Use Committee (PACUC).

Tumor-Bearing Mice

Female athymic nu/nu mice were purchased from Harlan (Indianapolis, IN) at 4 – 5 weeks of age. Tumor cells were grown at 37 $^{\circ}\text{C}$ in Minimal Essential Medium (Alpha Modification) containing 3.7 g of sodium bicarbonate/L, 10% fetal bovine serum v/v, in a humidified atmosphere of 5% carbon dioxide. The nude mice were implanted with $\sim 5 \times 10^6$ cells of MDA-MB-435 estrogen receptor-negative human breast cancer cells into the mammary fat pad. Four weeks after inoculation, animals were used for biodistribution and metabolism studies.

Biodistribution

Sixteen tumor-bearing mice (20 – 25 g) were anesthetized with IP injection of Ketamine (40 – 100 mg/kg) and Xylazine (2 – 5 mg/kg). Once the animal was in surgical plane of anesthesia, as noted by lack of pain response, the radiotracer ($\sim 1 \mu\text{Ci}$) dissolved in 0.1 mL saline was

administered via tail vein. Four animals were sacrificed by exsanguinations and opening of thoracic cavity at 5, 30, 60, and 120 min postinjection. Organs (tumor, blood, brain, eyes, spleen, lungs, liver, kidneys, muscle and intestine) were excised, washed with saline, weighed, and counted on a gamma counter. The organ uptake was calculated as a percentage of the injected dose per organ (%ID/organ) and a percentage of the injected dose per gram of wet tissue (%ID/g).

Metabolism

Two tumor-bearing mice were used for each radiotracer. Each tumor-bearing mouse was administered with the radiotracer at a dose of 100 μ Ci/mice. The urine samples were collected at 2 h postinjection by manual void, and were mixed with equal volume of acetonitrile. The mixture was centrifuged at 8,000 rpm. The supernatant was collected and filtered through a 0.20 micron Millex-LG syringe driven filter unit to remove any precipitate and large proteins. The filtrate was analyzed by radio-HPLC. The feces samples were collected at ~120 min postinjection. The sample was suspended in a mixture of 50% acetonitrile aqueous solution, and the resulting mixture was vortexed for 5 – 10 min. After centrifuging at 8,000 rpm for 5 min, the supernatant was collected and passed through a 0.20 micron Millex-LG syringe driven filter unit. The filtrate was analyzed by radio-HPLC.

Data and Statistical Analysis

The biodistribution data and T/B ratios are reported as an average plus the standard variation based on the results from four animals at each time point. Comparison between two different radiotracers was also made using the one-way ANOVA test. The level of significance was set at $p = 0.05$.

RESULTS

HYNIC-PKM-SU016 Conjugates

HYNIC-E-SU016 was prepared (Chart I) by conjugation of Boc-E(OBu-t)-NHS with one equivalent of E[c(RGDfK)]₂ (SU016), followed by deprotection of the Boc-protecting group in TFA, and conjugation with HYNIC-NHS in DMF. HYNIC-K-SU016 was prepared (Chart I) by conjugation of HYNIC-NHS with one equivalent of K(Boc)OH, followed by activation of the carboxylic group in HYNIC-K(Boc)OH, conjugation of HYNIC-K(Boc)-NHS with SU016 in DMF, and deprotection of the Boc-protecting group. For HYNIC-PEG4-SU016 (Chart I), Boc-PEG4 was first activated to form the activated ester Boc-PEG4-NHS, which was then allowed to react with SU016 to afford Boc-PEG4-SU016. The Boc-protecting group was removed by treating Boc-PEG4-SU016 with anhydrous TFA. Conjugation of PEG4-SU016 with HYNIC-NHS in DMF produced HYNIC-PEG4-SU016. The HYNIC-linker-SU016 (linker = E, K and PEG4) conjugates were purified by a semi-prep HPLC method. Their HPLC purity was >95% before being used for the ^{99m}Tc-labeling.

Radiochemistry

[^{99m}Tc(HYNIC-PKM-SU016)(tricine)(TPPTS)] (PKM = E, K and PEG4) were prepared according to Chart II. TPPTS was chosen as the coligand due to the high solution stability of its ternary ligand ^{99m}Tc complexes (1–4), and the availability of the non-SnCl₂ formulation (33–35). The radiochemical purity (RCP) of all three radiotracers was >95% with minimal amount (<0.5%) of [^{99m}Tc]colloid using non-SnCl₂ formulation. It was also found that all three radiotracers remained stable in 25 mM ammonium acetate buffer (pH = 5) for more than 12 h, which is consistent with the results obtained from our previous studies (1,2).

All three radiotracers were analyzed by the same HPLC method (Method 2). Figure 2 shows their representative HPLC chromatograms in the kit matrix. Small radioimpurities were formed during the radiolabeling process. No identification was made for these radioimpurities. The HPLC retention times for [$^{99m}\text{Tc}(\text{HYNIC-E-SU016})(\text{tricine})(\text{TPPTS})$], [$^{99m}\text{Tc}(\text{HYNIC-K-SU016})(\text{tricine})(\text{TPPTS})$] and [$^{99m}\text{Tc}(\text{HYNIC-PEG4-SU016})(\text{tricine})(\text{PDA})$], were 12.2, 15.0 and 13.5 min, respectively, under identical chromatographic conditions. It is interesting to note that [$^{99m}\text{Tc}(\text{HYNIC-K-SU016})(\text{tricine})(\text{TPPTS})$] shows two peaks, which are often observed for ternary ligand complexes [$^{99m}\text{Tc}(\text{HYNIC-biomolecule})(\text{tricine})(\text{TPPTS})$] due to chirality of the Tc chelate and presence of chiral center(s) in the targeting biomolecule (35–38). However, [$^{99m}\text{Tc}(\text{HYNIC-E-SU016})(\text{tricine})(\text{TPPTS})$] and [$^{99m}\text{Tc}(\text{HYNIC-PEG4-SU016})(\text{tricine})(\text{TPPTS})$] both show one radiometric peak under the same chromatographic conditions (Figure 2). Attempts to separate the two diastereomers in [$^{99m}\text{Tc}(\text{HYNIC-E-SU016})(\text{tricine})(\text{TPPTS})$] and [$^{99m}\text{Tc}(\text{HYNIC-PEG4-SU016})(\text{tricine})(\text{TPPTS})$] were not successful. It is obvious that the introduction of E or PEG4 linker between HYNIC and $\text{E}[\text{c}(\text{RGDfK})]_2$ makes it much more difficult to separate these two diastereomers.

To further explore the linker effect on lipophilicity of ternary ligand complexes [$^{99m}\text{Tc}(\text{HYNIC-linker-SU016})(\text{tricine})(\text{TPPTS})$] (linker = E, K and PEG4), we also determined their partition coefficients (P values) in an equal volume mixture of n-octanol and 25 mM phosphate buffer (pH = 7.4). The log P values are -3.13 ± 0.20 , -3.41 ± 0.14 and -3.56 ± 0.16 for [$^{99m}\text{Tc}(\text{HYNIC-E-SU016})(\text{tricine})(\text{TPPTS})$], [$^{99m}\text{Tc}(\text{HYNIC-K-SU016})(\text{tricine})(\text{TPPTS})$] and [$^{99m}\text{Tc}(\text{HYNIC-PEG4-SU016})(\text{tricine})(\text{TPPTS})$], respectively. As expected, complexes [$^{99m}\text{Tc}(\text{HYNIC-linker-SU016})(\text{tricine})(\text{TPPTS})$] (linker = E, K and PEG4) are more hydrophilic than [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{TPPTS})$] (log P = -2.66 ± 0.25) (2). There is no significant difference in log P values of all three new radiotracers within experimental error, even though their HPLC retention times suggest that [$^{99m}\text{Tc}(\text{HYNIC-E-SU016})(\text{tricine})(\text{TPPTS})$] might be more hydrophilic than [$^{99m}\text{Tc}(\text{HYNIC-K-SU016})(\text{tricine})(\text{TPPTS})$] and [$^{99m}\text{Tc}(\text{HYNIC-PEG4-SU016})(\text{tricine})(\text{TPPTS})$] (Figure 2).

Biodistribution Characteristics

For biodistribution studies, all three radiotracers were purified by HPLC to remove excess “unlabeled” HYNIC-PKM-SU016 conjugate that may compete with the radiotracer in binding to the integrin $\alpha_v\beta_3$. Biodistribution data for complexes [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = E, K and PEG4) are summarized in Tables 1 – 3, respectively. Figure 3 shows direct comparison of their tumor uptake and excretion kinetics of from blood, liver and kidneys at 5, 30, 60 and 120 min postinjection. The T/B ratios in selected organs are illustrated in Figure 4. The biodistribution data and T/B ratios for [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{TPPTS})$] were obtained from our previous studies (2).

In general, [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = K and PEG4) have the tumor uptake (3 – 4% ID/g) very similar to that of [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{TPPTS})$] (Figure 3). Their tumor uptake remains constant over the 2 h study period. The tumor uptake of [$^{99m}\text{Tc}(\text{HYNIC-E-SU016})(\text{tricine})(\text{TPPTS})$] is comparable to that of [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = K and PEG4) and [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{TPPTS})$] over the first 30 min; but it increases from 4.07 ± 0.19 %ID/g at 30 min postinjection to 6.33 ± 0.55 %ID/g at 60 min postinjection, and remains almost unchanged between 60 – 120 min postinjection. Compared to [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{TPPTS})$], all three new radiotracers show faster clearance from blood, liver, muscle, lungs, and kidneys (Tables 1 – 3). As a result, there is a steady increase in T/B ratios over the 2 h study period. Among the three radiotracers evaluated in this study, [$^{99m}\text{Tc}(\text{HYNIC-K-SU016})(\text{tricine})(\text{TPPTS})$] has the best tumor/liver and tumor/lung ratios at 120 min postinjection while [$^{99m}\text{Tc}(\text{HYNIC-E-SU016})(\text{tricine})(\text{TPPTS})$] has the best tumor/blood and tumor/muscle ratios (Figure 4). The

tumor/muscle ratio of [$^{99m}\text{Tc}(\text{HYNIC-PEG4-SU016})(\text{tricine})(\text{TPPTS})$] are almost the same as that of [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{TPPTS})$]. Its tumor/liver ratio is comparable to that of [$^{99m}\text{Tc}(\text{HYNIC-PEG4-SU016})(\text{tricine})(\text{TPPTS})$] within experimental error over the 2 h study period.

Metabolism

We performed metabolism studies on all three new radiotracers using athymic tumor-bearing nude mice. Since they are excreted from renal and hepatobiliary (to a much less degree) routes, we tried to collect samples from both feces and urine samples, and to determine if these radiotracers retain their chemical integrity at 2 h postinjection. Figure 5 shows radio-HPLC chromatograms of [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = E, K and PEG4) in the urine at 120 min postinjection. It is quite clear that these three radiotracers have different metabolic patterns. For example, [$^{99m}\text{Tc}(\text{HYNIC-E-SU016})(\text{tricine})(\text{TPPTS})$] had no significant metabolism (<1.0%) over the 2 h study period. The percentage of “metabolized component” (at ~ 3 min) for [$^{99m}\text{Tc}(\text{HYNIC-K-SU016})(\text{tricine})(\text{TPPTS})$] was <10% at 120 min postinjection, and >70% of [$^{99m}\text{Tc}(\text{HYNIC-PEG4-SU016})(\text{tricine})(\text{TPPTS})$] remained intact over 2 h postinjection (Figure 5). The presence of two overlapped peaks in the HPLC chromatogram of [$^{99m}\text{Tc}(\text{HYNIC-PEG4-SU016})(\text{tricine})(\text{TPPTS})$] is probably due to chromatographic artifact. We also tried to extract radioactivity from feces samples; but the activity level in feces samples was so low that we were unable to detect either intact ternary ligand ^{99m}Tc complexes or their metabolites.

DISCUSSION

For a new integrin $\alpha_v\beta_3$ -targeted radiotracer to be successful, it should be able to have a high tumor uptake with diagnostically useful T/B ratios in a short period of time (28,29). Since most high-incidence tumors (namely lung, colorectal and breast cancers metastatic to the lymphatic system) occur in torso, it is necessary to avoid radioactivity accumulation in the gastrointestinal tract, which may interfere with interpretation of tumor activity in the abdominal region. Therefore, efforts have been directed towards optimization of the radiotracer excretion kinetics so that the radiotracer has a fast clearance from non-tumor organs (such as blood, liver, and lungs) while maintaining its high tumor uptake.

There are several approaches to increase the renal clearance of radiotracers (13,29,30). These include: (1) the use of hydrophilic amino acid residues in targeting biomolecules; (2) the use of hydrophilic chelators or coligands for the ^{99m}Tc -labeling; and (3) the use of charged PKM linkers between the ^{99m}Tc -chelator and the targeting biomolecule (namely E[c(RGDfK)]₂ or SU016 in this study). Incorporation of different amino acid residues into small biomolecules, such as cyclic RGD pentapeptides, often leads to alteration of their receptor binding affinity. Thus, it is common to use PKM linkers and chelators or coligands to modify pharmacokinetics of radiotracers while keeping the cyclic pentapeptide framework intact so that the integrin $\alpha_v\beta_3$ binding affinity is not adversely affected.

Recently, we examined the coligand effect on biodistribution characteristics of [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{L})$] (L = ISONIC, TPPTS, and PDA) in athymic nude mice bearing MDA-MB-435 human breast cancer xenografts, and found that the coligand has a significant impact on excretion kinetics of the ^{99m}Tc -labeled E[c(RGDfK)]₂ from blood, liver and lungs (2). In this study, we studied the impact of three PKM linkers (E, K and PEG4) on lipophilicity, tumor uptake, and excretion kinetics of the ^{99m}Tc -labeled E[c(RGDfK)]₂. It was found that all three radiotracers [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = E, K and PEG4) (log P = -3.13 ± 0.20 , -3.41 ± 0.14 and -3.56 ± 0.16 , respectively) are more hydrophilic than [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{TPPTS})$] (log P = -2.66 ± 0.25). Based on biodistribution characteristics, it seems that PKM linkers have minimal impact on tumor-targeting capability

as indicated by their similar tumor uptake (Figure 3). Even though they may have different charges under physiological conditions, all three PKM linkers (E, K and PEG4) are able to reduce the uptake of ^{99m}Tc -labeled $\text{E}[\text{c}(\text{RGDfK})]_2$ from blood, liver and lungs, and increase their T/B ratios, particularly at >60 min postinjection (Figure 4). E and K are especially useful to minimize radioactivity accumulation in liver and lungs.

Small peptides in blood plasma are filtered through glomerular capillaries in the kidneys and subsequently reabsorbed by proximal tubular cells by carrier-mediated endocytosis. The membranes of renal tubular cells contain negatively charged sites to which the positively charged groups, such as guanidine moieties in cyclic RGD peptides, are expected to bind. In this respect, the negatively charged small peptides, such as E, can be used as PKM linkers to reduce renal uptake of ^{99m}Tc -labeled $\text{E}[\text{c}(\text{RGDfK})]_2$ since the extra carboxylic group are expected to be deprotonated under physiological conditions. In this way, the interaction between membranes of renal tubular cells and the positively charged guanidine groups in $\text{E}[\text{c}(\text{RGDfK})]_2$ can be minimized; thereby reducing renal uptake of the ^{99m}Tc -labeled $\text{E}[\text{c}(\text{RGDfK})]_2$. This assumption is supported by the lower kidney uptake of $[\text{HYNIC-E-SU016}(\text{tricine})(\text{TPPTS})]$ than that of $[\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{TPPTS})]$ (Figure 3). The use of negatively charged E linker also decreases the radiotracer accumulation in blood and liver (Figure 3), which results in high tumor/blood and tumor/liver ratios for $[\text{HYNIC-E-SU016}(\text{tricine})(\text{TPPTS})]$ (Figure 4). Similar results were also reported for ^{99m}Tc -DKCK-c(RGDfK) (39). Therefore, the negatively charged PKM linkers are particularly useful to enhance the radiotracer elimination from the body without prolonged retention in kidneys.

Harris et al reported the use of a PEG4 linker to improve T/B ratios of the ^{99m}Tc -labeled nonpeptide integrin $\alpha_v\beta_3$ antagonists (21,22). Chen et al also found that introduction of the PEG linker (M.W. = 2000 or 3400 Daltons) can improve tumor uptake and excretion kinetics of the radiolabeled RGD peptides (25–28). In this study, we found that the PEG4 linker significantly enhance the radiotracer excretion from blood, liver and kidneys. Among three radiotracers evaluated in this study, $[\text{HYNIC-PEG4-SU016}(\text{tricine})(\text{TPPTS})]$ has the lowest kidney uptake (Figure 3). Even though PEG linkers are neutral, they are useful tools for optimization of pharmacokinetic parameters of target-specific radiotracers.

Extensive metabolic degradation has been observed for the ^{99m}Tc -labeled c(RGDfK) (39), $[\text{HYNIC-SU016}(\text{tricine})(\text{TPPTS})]$ (2), and the ^{64}Cu -labeled c(RGDfK) tetramer (9). In this study, we found that all three radiotracers have a different degree of metabolism. For example, $[\text{HYNIC-E-SU016}(\text{tricine})(\text{TPPTS})]$ has little metabolism (<1.0%) while $[\text{HYNIC-K-SU016}(\text{tricine})(\text{TPPTS})]$ has <10% metabolism during the 2 h study period. Majority (>70%) of $[\text{HYNIC-PEG4-SU016}(\text{tricine})(\text{TPPTS})]$ remained intact at 2 h postinjection (Figure 5). Since all three radiotracers used in this study share the same Tc chelate and the same cyclic RGDfK dimer, the difference in their metabolic patterns is likely related to the PKM linker.

The pharmacokinetic requirement for an optimal radiotracer depends largely on its medical applications (diagnostic or therapeutic). For therapeutic radiopharmaceuticals, the high uptake in kidneys and prolonged kidney retention of radiotracers will impose a serious challenge for the radiolabeled cyclic RGD peptides (12,30,31). For tumor imaging, however, the most important factor in selecting an optimal radiotracer is its excretion kinetics from the liver and lungs (29,30). Although all three linkers are able to increase the excretion rate of ^{99m}Tc -labeled $\text{E}[\text{c}(\text{RGDfK})]_2$ from non-tumor organs, E and K may have advantages over PEG4 due to a combination of low liver uptake, high tumor/liver and tumor/lung ratios (Figure 4) of $[\text{HYNIC-PKM-SU016}(\text{tricine})(\text{TPPTS})]$ (PKM = E and K).

CONCLUSION

In this report, we described the *in vivo* evaluation of biodistribution characteristics of complexes [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = E, K and PEG4) in nude mice bearing MDA-MB-435 human breast cancer xenografts. The results from biodistribution studies show that excretion kinetics of the ^{99m}Tc -labeled $\text{E}[\text{c}(\text{RGDfK})]_2$ can be modified with various PKM linker. The fact that [$^{99m}\text{Tc}(\text{HYNIC-PKM-SU016})(\text{tricine})(\text{TPPTS})$] (PKM = E, K and PEG4) have a tumor uptake comparable to that of [$^{99m}\text{Tc}(\text{HYNIC-SU016})(\text{tricine})(\text{TPPTS})$] during 2 h study period suggests that changing PKM linkers has no adverse effect on the integrin $\alpha_v\beta_3$ binding capability of the ^{99m}Tc -labeled $\text{E}[\text{c}(\text{RGDfK})]_2$. Among the three linkers, E and K linkers are especially useful in minimizing the radioactivity accumulation in liver and lungs. For other ^{99m}Tc -labeled small biomolecules, the choice of PKM linker must be confirmed by their distribution characteristics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HYNIC, 6-hydrazinonicotinamide; PKM, pharmacokinetic modifier; E, glutamic acid; K, lysine; PEG4, 15-amino-4,7,10,13-tetraoxa-pentadecanoic acid; SQ168, [2-[[[5-[carbonyl]-2-pyridinyl]hydrazono]methyl]-benzenesulfonic acid]-Glu(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe}; TPPTS, trisodium triphenylphosphine-3,3',3''-trisulfonate.

Figure 1.

Structures of HYNIC-PKM-SU016 conjugates and their ternary ligand complexes [^{99m}Tc (HYNIC-PKM-SU016)(tricine)(TPPTS)] (PKM = E, K and PEG4).

Figure 2.

Radio-HPLC chromatograms of [$^{99m}\text{Tc}(\text{HYNIC-E-SU016})(\text{tricine})(\text{TPPTS})$] (top), [$^{99m}\text{Tc}(\text{HYNIC-K-SU016})(\text{tricine})(\text{TPPTS})$] (middle), and [$^{99m}\text{Tc}(\text{HYNICPEG4-SU016})(\text{tricine})(\text{TPPTS})$] (bottom) in the kit matrix.

Figure 3. Comparison of organ uptake (%ID/g) between [^{99m}Tc (HYNIC-SU016)(tricine)(TPPTS)], [^{99m}Tc (HYNIC-E-SU016)(tricine)(TPPTS)], [^{99m}Tc (HYNIC-K-SU016)(tricine)(TPPTS)], and [^{99m}Tc (HYNIC-PEG4-SU016)(tricine)(TPPTS)] in athymic nude mice bearing MDA-MB-435 human breast cancer xenografts.

Figure 4.

Comparison of T/B ratios between [^{99m}Tc (HYNIC-SU016)(tricine)(TPPTS)], [^{99m}Tc (HYNIC-E-SU016)(tricine)(TPPTS)], [^{99m}Tc (HYNIC-KSu016)(tricine)(TPPTS)], and [^{99m}Tc (HYNIC-PEG4-SU016)(tricine)(TPPTS)] in athymic nude mice bearing MDA-MB-435 human breast cancer xenografts.

Figure 5.

Radio-HPLC chromatograms of [$^{99m}\text{Tc}(\text{HYNIC-E-SU016})(\text{tricine})(\text{TPPTS})$] (top), [$^{99m}\text{Tc}(\text{HYNIC-K-SU016})(\text{tricine})(\text{TPPTS})$] (middle), and [$^{99m}\text{Tc}(\text{HYNICPEG4-SU016})(\text{tricine})(\text{TPPTS})$] (bottom) in the urine at 120 min postinjection. Each tumor-bearing mouse was administered with 100 – 120 μCi of radiotracer. Two tumor-bearing mice were used for each radiotracer.

Chart I.
Synthesis of HYNIC-Conjugated Cyclic RGDfK Conjugates

Chart II.
Synthesis of Ternary Ligand ^{99m}Tc Complexes