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Linker Effects on Biological Properties of ^{111}In -Labeled DTPA Conjugates of a Cyclic RGDfK Dimer

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Abstract

In this report, we present *in vitro* and *in vivo* evaluation of three ^{111}In -labeled DTPA conjugates of a cyclic RGDfK dimer: DTPA-Bn-SU016 (SU016 = E[c(RGDfK)]₂; DTPA-Bn = 2-(*p*-isothiocyanobenzyl)diethylenetriaminepentaacetic acid), DTPA-Bn-E-SU016 (E = glutamic acid) and DTPA-Bn-Cys-SU016 (Cys = cysteic acid). The integrin $\alpha_v\beta_3$ binding affinities of SU016, DTPA-Bn-SU016, DTPA-Bn-E-SU016 and DTPA-Bn-Cys-SU016 were determined to be 5.0 ± 0.7 nM, 7.9 ± 0.6 nM, 5.8 ± 0.6 nM and 6.9 ± 0.9 nM, respectively, against ^{125}I -c(RGDyK) in binding to integrin $\alpha_v\beta_3$, suggesting that E or Cys residue has little effect on the integrin $\alpha_v\beta_3$ affinity of E[c(RGDfK)]₂. It was also found that the ^{111}In -labeling efficiency of DTPA-Bn-SU016 and DTPA-Bn-E-SU016 is 3–5 times better than that of DOTA analogs due to fast chelation kinetics and high-yield ^{111}In -labeling under mild conditions (e.g. room temperature). Biodistribution studies were performed using BALB/c nude mice bearing U87MG human glioma xenografts. ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 all displayed a rapid blood clearance. Their tumor uptake was comparable between 0.5 h and 4 h postinjection (p.i.) within experimental error. ^{111}In -DTPA-Bn-E-SU016 had a significantly lower ($p < 0.01$) kidney uptake than ^{111}In -DTPA-Bn-SU016 and ^{111}In -DTPA-Bn-Cys-SU016. The liver uptake of ^{111}In -DTPA-Bn-SU016 was 1.69 ± 0.18 %ID/g at 24 h p.i. while the liver uptake of ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 was 0.55 ± 0.11 %ID/g and 0.79 ± 0.15 %ID/g at 24 h p.i., respectively. Among the three ^{111}In radiotracers evaluated in this study, ^{111}In -DTPA-Bn-E-SU016 has the lowest liver and kidney uptake and the best tumor/liver and tumor/kidney ratios. Results from metabolism studies indicated that there is little (<10%) metabolism for three ^{111}In radiotracers at 1 h p.i. Imaging data showed that tumors can be clearly visualized at 4 h p.i. with good contrast in the tumor-bearing mice administered with ^{111}In -DTPA-Bn-E-SU016. It is concluded that using a glutamic acid linker can significantly improve excretion kinetics of the ^{111}In -labeled E[c(RGDfK)]₂ from liver and kidneys.

INTRODUCTION

Integrin $\alpha_v\beta_3$ plays a critical role in tumor angiogenesis and metastasis (1–5). The highly restricted expression of integrin $\alpha_v\beta_3$ during tumor growth, invasion and metastasis makes it

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an interesting molecular target for development of the integrin $\alpha_v\beta_3$ -targeted radiotracers (6–9). In the last decade, many radiolabeled cyclic RGD peptides have been evaluated as potential radiotracers for early detection of the integrin $\alpha_v\beta_3$ -positive tumors by single photon emission computed tomography (SPECT) or positron emission tomography (PET). [^{18}F]Galacto-RGD has become the first PET radiotracer under clinical investigation for noninvasive visualization of integrin $\alpha_v\beta_3$ expression in cancer patients (10–12). The integrin $\alpha_v\beta_3$ -targeted radiotracers have been reviewed extensively (13–18).

We and others have been using cyclic RGD dimers, such as E[c(RGDfK)]₂ (SU016), to develop integrin $\alpha_v\beta_3$ -targeted diagnostic (^{64}Cu , $^{99\text{m}}\text{Tc}$ and ^{111}In) and therapeutic (^{90}Y and ^{177}Lu) radiotracers (19–36). It has been found that the radiolabeled cyclic RGD dimers have much better tumor uptake than monomeric analogs due to their higher integrin $\alpha_v\beta_3$ binding affinity. Recently, we reported the impact of pharmacokinetic modifying (PKM) linkers on biological properties of the $^{99\text{m}}\text{Tc}$ -labeled HYNIC-PKM-SU016 (HYNIC= 6-hydrazinonicotinamide; PKM = glutamic acid (E), lysine (K) or PEG₄ (15-amino-4,7,10,13-tetraoxa-pentadecanoic acid)) (23). Even though they have minimal impact on integrin $\alpha_v\beta_3$ affinity of SU016, all three PKM linkers are able to reduce the radiotracer uptake in blood, kidneys, liver and lungs, and increase target-to-background (T/B) ratios. E and K have advantages over PEG₄ due to lower liver uptake and higher tumor/liver ratios of the $^{99\text{m}}\text{Tc}$ -labeled E[c(RGDfK)]₂ (23). For ^{111}In -labeled DOTA-PKM-E[c(RGDfK)]₂ (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), however, PEG₄ appears to be a better linker than E and K since ^{111}In -DOTA-PEG₄-E[c(RGDfK)]₂ has the highest tumor-to-blood ratio and lowest uptake in liver and kidneys (33).

The disadvantage of using DOTA as the bifunctional chelator (BFC) for ^{111}In -labeling of small biomolecules is its slow chelation kinetics and low radiolabeling efficiency (33,37,38). In contrast, DTPA has higher radiolabeling efficiency (37–39), and have been successfully used for ^{90}Y , ^{177}Lu and ^{111}In -labeling of biomolecules, including antibodies (40–44) and small peptides (39, 40, 45, 46). In this study, we report three new cyclic RGDfK dimer DTPA conjugates, DTPA-Bn-SU016 (DTPA-Bn = 2-(*p*-isothiocyanobenzyl) diethylenetriaminepentaacetic acid), DTPA-Bn-E-SU016 and DTPA-Bn-Cys-SU016 (Cys = cysteic acid), and the evaluation of their ^{111}In complexes (Figure 1) as the integrin $\alpha_v\beta_3$ -targeted radiotracers in the BALB/c nude mice bearing U87MG human glioma xenografts. ^{111}In is of our particular interest since it has been widely (second only to $^{99\text{m}}\text{Tc}$) used for SPECT imaging, and the ^{111}In -labeled E[c(RGDfK)]₂ can be used as the imaging surrogate to evaluate the potential of the ^{90}Y -labeled E[c(RGDfK)]₂ as integrin $\alpha_v\beta_3$ -targeted radiotracer for tumor radiotherapy. The main objective of this study is to explore the effect of linkers (E and Cys) on biodistribution characteristics of the ^{111}In -labeled E[c(RGDfK)]₂. The ultimate goal of using different linkers is to increase T/B ratios by minimizing radiotracer uptake in non-tumor organs while maintaining high tumor uptake.

EXPERIMENTAL

Materials

All chemicals were purchased from *Sigma/Aldrich*, unless specified. The cyclic pentapeptide, c(RGDfK), was obtained from Peptides International, Inc. (Louisville, KY). 2-(*p*-Isothiocyanobenzyl)diethylenetriaminepentaacetic acid (*p*-SCN-Bn-DTPA) was purchased from Macrocyclics (Dallas, TX). $^{111}\text{InCl}_3$ was obtained from Perkin-Elmer Life and Analytical Sciences (North Billerica, MA). E[c(RGDfK)]₂ and E-E[c(RGDfK)]₂ were prepared according to the procedure described in our previous communications (21,23). The reversed-phase C-18 SepPak cartridges were obtained from Waters (Milford, MA). The ammonium acetate buffer for ^{111}In -labeling studies was passed over a Chelex-100 column (1×15 cm) to minimize the trace metal contaminants.

HPLC and ITLC Methods

The HPLC Method 1 used a LabAlliance semi-prep HPLC system equipped with a UV/vis detector ($\lambda = 254$ nm) and a reverse phase Zorbax C₁₈ column (9.4 mm $\times$ 250 mm, 100 Å pore size). 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 a HP Hewlett® Packard Series 1100 HPLC system equipped with Radioflow Detector LB509 and Agilent Zorbax SB-C18 column (4.6 mm $\times$ 250 mm, 5 μ m). The flow rate was 1.0 mL/min. The mobile phase was isocratic with 90% solvent A (25 mM NaOAc 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, then to 10% solvent B at 25 min. The radio-ITLC method used GelmanSciences silica-gel paper strips and a 1:1 mixture of acetone and saline as the eluant. Using this method, the ¹¹¹In labeled cyclic RGDfK peptide dimer migrates to the solvent front while the “free” or “unchelated” ¹¹¹In remained at the origin.

DTPA-Bn-SU016

DTPA-Bn-SCN (2.7 mg, 4 μ mol) and SU016 (3.0 mg, 2 μ mol) were dissolved in 1 mL of anhydrous dimethylformamide (DMF). The pH in the reaction mixture was adjusted to 8.5–9.0 with DIEA. The reaction mixture was stirred at room temperature for ~6 h. After addition of 3 mL of 100 mM NH₄OAc buffer (pH = 7.0), the resulting solution was filtered, and the filtrate was subjected to HPLC-purification (Method 1). The fraction at ~15 min was collected. Lyophilization of the collected fractions afforded the expected product as a white power. The yield was 1.8 mg (48%). ESI-MS (positive mode) for DTPA-Bn-SU016: $m/z = 1859.62$ for $[M + H]^+$ ($M = 1858.82$ calcd. for C₈₁H₁₁₆N₂₃O₂₆S).

DTPA-Bn-E-SU016

DTPA-Bn-SCN (2.7 mg, 4 μ mol) and E-E[c(RGDfK)]₂ (3.0 mg, 2 μ mol) were dissolved with anhydrous DMF (1 mL). The mixture was stirred at room temperature for 10 min. DIEA was added to adjust pH to ~8.0. The reaction mixture was stirred at room temperature for 6 h. After addition of 100 mM NH₄OAc buffer (3 mL, pH = 7.0), the resulting solution was subjected to semi-prep HPLC purification (Method 1). The fractions at ~17.5 min were collected. Lyophilization of collected fractions gave the desired product as a white powder. The yield was 1.5 mg (38%). ESI-MS (positive mode) for DTPA-Bn-E-SU016: $m/z = 1989.68$ for $[M + H]^+$ ($M = 1988.87$ calcd. for C₈₆H₁₂₄N₂₄O₂₉S).

BOC-Protected Cysteic acid (Boc-Cys-OH)

To a solution of cysteic acid hydrate (0.56 g, 3 mmol) and triethylamine (0.61 g, 6 mmol) in anhydrous DMF (10mL) was added a solution of di-tert-butyl dicarbonate (0.78 g, 3.6 mmol) in 5 mL of DMF. The mixture was stirred at room temperature for 48 h. Evaporation of solvent under vacuum gave colorless oil. The residue was dissolved in methylene chloride (10 mL). Upon addition of diethyl ether (30 mL) with vigorous steering, the white precipitate was formed. Solvents were decanted and discarded. The solid residue was washed with anhydrous diethyl ether (10 mL), and dried under vacuum to give a foam-like white solid. The yield was 0.89 g (80%). ¹H NMR (δ in ppm, CDCl₃): 6.60 (d, NH, J = 5.4 Hz), 3.78 (m, 1H, CH), 2.45 (m, 2H, CH₂), 1.15(s, 9H, t-Bu).

BOC-Protected Cysteic Acid Succinimidyl Ester (Boc-Cys-OSu)

To a solution of Boc-Cys-OH (as its triethylamine salt) (0.89 g, 2.4 mmol) and NHS (0.345 g, 3 mmol) in anhydrous DMF (20 mL) was added DCC (0.618 g, 3 mmol). The resulting mixture

was stirred at room temperature for 24 h. Acetic acid (1.0 mL) was added into the mixture above and the stirring was continued for 3 h. The precipitate DCU was filtered off. The filtrate was evaporated to dryness. The residue was dissolved in methylene chloride (15 mL). Upon removal of a small amount of DCU precipitate, the expected product was precipitated out by adding the filtrate into anhydrous diethyl ether (30 mL). The solid was washed with diethyl ether (20 mL), and dried under vacuum to give a white solid. The yield was 1.20 g (100%). ^1H NMR (δ in ppm, CDCl_3): 6.46(s, 1H, NH), 4.88(m, 1H, CH), 3.40(m, 2H, CH_2), 2.80(s, 4H, CH_2CH_2), 1.42(s, 9H, t-Bu).

Boc-Cys-SU016

To a solution of Boc-Cys-OSu (7.2 mg, 0.015 mmol) in DMF (1 mL) was added SU016 (13.2 mg, 0.01 mmol). DIEA was added to adjust pH in the reaction mixture to 8.5–9.0. The resulting mixture was stirred at room temperature for 30 min, and the pH was adjusted to ~8.5, if necessary. The reaction was stirred at room temperature overnight. The desired product was isolated from the reaction mixture by HPLC purification (Method 1). The collected fractions at ~15 min were combined. Lyophilization of the combined fractions afforded the expected product as a white powder. The yield was 6.4 mg (41%). ESI-MS (positive mode) for Boc-Cys-SU016: $m/z = 1570.75$ for $[\text{M} + \text{H}]^+$ ($M = 1569.71$ calcd. for $\text{C}_{67}\text{H}_{101}\text{N}_{20}\text{O}_{22}\text{S}$).

Cys-SU016

Boc-Cys-SU016 (5 mg, 0.003 mmol) was treated with 2 mL of anhydrous trifluoroacetate (TFA) at room temperature for 30 min. Volatiles were completely removed under vacuum. The residue was dissolved in 100 mM NH_4OAc buffer (3 mL). The resulting solution was filtered, and the filtrate was purified by HPLC (Method 1). The fractions at ~15.5 min were collected. Lyophilization of collected fractions gave the desired product as a white powder (3.5 mg). ESI-MS (positive mode) for Cys-SU016: $m/z = 1469.57$ for $[\text{M} + \text{H}]^+$ ($M = 1468.65$ calcd. for $\text{C}_{62}\text{H}_{92}\text{N}_{20}\text{O}_{20}\text{S}$).

DTPA-Bn-Cys-SU016

DTPA-Bn-SCN (2.7 mg, 4 μmol) and Cys-SU016 (3.0 mg, 2 μmol) were dissolved in anhydrous DMF (1 mL). The mixture was stirred at room temperature for 10 min. DIEA was used to adjust pH in the mixture to 8.5–9.0. The reaction mixture was stirred at room temperature for another 6 h while maintaining pH at 8.5–9.0. Upon addition of 100 mM NH_4OAc (3 mL, pH = 7.0), the resulting solution was filtered. The filtrate subjected to HPLC purification (Method 1). The fractions at ~15.5 min were collected. Lyophilization of collected fractions gave the desired product as a white powder. The yield was 1.5 mg (35%). ESI-MS (positive mode) for DTPA-Bn-Cys-SU016: $m/z = 2012.08$ for $[\text{M} + \text{H}]^+$ ($M = 2010.81$ calcd. for $\text{C}_{84}\text{H}_{122}\text{N}_{24}\text{O}_{30}\text{S}_2$).

^{111}In -Labeling and Solution Stability

To a clean Eppendorf tube were added 200 μL of DTPA-Bn-SU016 or DTPA-Bn-E-SU016 or DTPA-Bn-Cys-SU016 solution (0.2–0.5 mg/mL in 0.2 M NaOAc buffer, pH=5.5), and 15 μL of $^{111}\text{InCl}_3$ solution (1–5 mCi, in 0.05 M HCl). The reaction mixture was kept at room temperature for 20 min. A sample of the resulting solution was analyzed by radio-HPLC and ITLC. The solution stability of all three radiotracers was determined in saline and 6 mM EDTA solution (pH=5.0). Samples were analyzed by radio-HPLC at 2, 8, 12 and 24 h after Sep-Pak C-18 purification.

Doses Preparation

All ^{111}In radiotracers for animal studies were purified with Sep-Pak C-18 cartridge. To chelate free ^{111}In , 30 μL of EDTA (6 mM, pH=5.5) was added to the reaction mixture 5 min before

purification. The cartridge was activated with ethanol (10 mL), followed by H₂O (10 mL). After radiotracer was loaded, the cartridge was washed with saline (10 mL). The radiotracer was eluted with 80% ethanol (0.4 mL). Volatiles were completely removed under vacuum. Doses for animal studies were made by dissolving the purified radiotracer in saline to 80 μ Ci/mL for biodistribution studies, and 2.0 mCi/mL for metabolism or imaging studies. For the blocking experiment, E[c(RGDfK)]₂ was used as the blocking agent. Doses were prepared by dissolving E[c(RGDfK)]₂ in saline to give a final concentration of 3.0 mg/mL. Each tumor-bearing mouse was injected with 0.1 mL of solution (~15 mg/kg).

Water-Octanol Partition Coefficient

All three ¹¹¹In radiotracers were purified with Sep-Pak C-18 cartridge to avoid interference from radioimpurities. The purified radiotracer was dissolved in a equal volume mixture of 25 mM phosphate buffer (3 mL, pH = 7.4) and n-octanol (3 mL). After stirring for ~20 min at room temperature, the mixture was centrifuged at a speed of 8,000 rpm for 5 min. Samples (in triplets) from aqueous and n-octanol layers were counted in a γ -counter (Wallac 1470-002, Perkin Elmer, Finland). Partition coefficients were calculated. The log P value was measured three times and reported as an average of three independent measurements plus the standard deviation.

Integrin $\alpha_v\beta_3$ Receptor Binding Assay

The *in vitro* integrin-binding affinities and specificities of cyclic RGD peptides were determined in a whole-cell displacement assay using ¹²⁵I-c(RGDyK) as the integrin-specific radioligand. ¹²⁵I-c(RGDyK) was prepared in high specific activity (~1200Ci/mmol) according to the literature method (55). The receptor binding experiments were performed using U87MG human glioma cells. In short, the U87MG glioma cells were seeded in 24-well plates (2 $\times$ 10⁵ cells/well) and incubated overnight at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) to allow adherence. During the cell-binding experiments, the plates were rinsed twice with binding buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, 0.1% BSA). The glioma cells were incubated with ¹²⁵I-c(RGDyK) in the presence of a cyclic RGD peptide (0–1000 nM). The total incubation volume was then adjusted to 300 μ L. After the glioma cells were incubated at 4°C for 2 h, the supernatant was removed. The glioma cells were washed three times with cold binding buffer, were then dissolved with 2 M NaOH at 37°C. The cell-associated radioactivity was collected and determined using a γ -counter (Wallac 1470-002, Perkin Elmer, Finland). The same experiments were carried out twice with quadruple samples for each. The IC₅₀ values were calculated by fitting the data by nonlinear regression using GraphPad Prism (GraphPad Software, San Diego, CA). The IC₅₀ values are reported as an average of samples plus the standard deviation.

Animal Model

The human U87MG human glioma cells from Professor Jingde Zhu (Shanghai Cancer Research Institute) were maintained at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Female BALB/c nude mice (4–5 weeks of age) were purchased from Department of Animal Experiment, Peking University Health Center. The U87MG cells (5 $\times$ 10⁶) were implanted subcutaneously into the right upper flanks of mice. When tumors reached ~0.8 cm in mean diameter, the tumor-bearing mice were used to biodistribution and imaging studies.

Biodistribution

Biodistribution studies were performed by Dr. Wang's group in accordance with guidelines of Peking University Health Science Center Animal Care Committee. Sixteen tumor-bearing mice

were randomly divided into four groups. Each animal was administered intravenously with ~8 μCi of ^{111}In radiotracer via tail-vein. Four animals were anesthetized with intraperitoneal injection of sodium pentobarbital (45.0 mg/kg), and sacrificed by cervical dislocation at 0.5, 1, 4 and 24 h p.i. Blood, heart, liver, spleen, kidney, stomach, intestine, muscle, bone and tumor were harvested, weighed, and measured for radioactivity in a γ -counter (Wallac 1470-002, Perkin Elmer, Finland). Organ uptake was calculated as a percentage of injected dose per gram of tissue (%ID/g). In the blocking experiment, each animal was administered with excess E[c(RGDfK)]₂ 30 min prior to injection of ^{111}In -DTPA-Bn-E-SU016. Four animals were sacrificed at 4 h p.i. for biodistribution using the same procedure.

Gamma Imaging

The imaging study was performed by administering with 200 μCi of ^{111}In -DTPA-Bz-E-SU016 in 0.1 mL saline into each animal via tail vein. Animals were anesthetized with intraperitoneal injection of sodium pentobarbital at a dose of 45.0 mg/kg, then were placed supine on a two-head γ -camera (SIEMENS, E. CAM) equipped with a parallel-hole, middle-energy, and high-resolution collimator. Anterior images were acquired at 4 h and 24 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 cervical dislocation.

Metabolism

The metabolic stability of all three ^{111}In radiotracers was evaluated in normal BALB/c mice. Each mouse was administered with 200 μCi of the radiotracer in 0.1 mL saline via intravenous injection. Urine samples were collected at 1 h postinjection, and were mixed with 100 μL of 6 mM EDTA (pH = 5.0). The mixtures were centrifuged at a speed of 1500 rpm for 15 min. The supernatants were collected and filtered through a 0.22 μm Millex-LG syringe driven filter unit. The filtrates were analyzed by radio-HPLC (Method 2).

Statistical Analysis

Biodistribution data and target-to-background ratios are reported as an average plus the standard variation. Comparison between two different radiotracers was also made using the two-way ANOVA test (GraphPad Prism 4.0 □ GraphPad Software, San Diego, CA). The level of significance was set at $p < 0.05$.

RESULTS

DTPA Conjugates

DTPA-Bn-SU016 was prepared by reacting E[c(RGDfK)]₂ with excess *p*-SCN-Bn-DTPA in DMF under slightly basic conditions (pH = 8.0–9.0). The same procedure was used for preparation of DTPA-Bn-E-SU016 and DTPA-Bn-Cys-SU016. All three conjugates were purified by HPLC with the purity >98% before being used for radiolabeling and animal studies. The ESI-MS data were completely consistent with the proposed formula for DTPA-Bn-SU016, DTPA-Bn-E-SU016 and DTPA-Bn-Cys-SU016.

Integrin $\alpha_v\beta_3$ Binding Affinity

The integrin $\alpha_v\beta_3$ binding affinities of DTPA-Bn-SU016, DTPA-Bn-E-SU016, and DTPA-Bn-Cys-SU016 were determined using the integrin $\alpha_v\beta_3$ -positive U87MG human glioma cells. For comparison purposes, c(RGDyK) and SU016 were also evaluated in the same assay. Figure 2 shows the displacement curves of c(RGDyK), SU016, DTPA-Bn-SU016, DTPA-Bn-E-SU016 and DTPA-Bn-Cys-SU016 against the binding of ^{125}I -c(RGDyK) to integrin $\alpha_v\beta_3$ on U87MG glioma cells. The IC₅₀ values were calculated to be 31.9 ± 4.0 nM for c(RGDyK), 5.0

± 0.7 nM for SU016, 7.9 ± 0.6 nM for DTPA-Bn-SU016, 5.8 ± 0.6 nM for DTPA-Bn-E-SU016 and 6.9 ± 0.9 nM for DTPA-Bn-Cys-SU016.

Radiochemistry

^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 were prepared by reacting $^{111}\text{InCl}_3$ with the DTPA conjugate in NH_4OAc buffer (200 mM, pH = 5.5). Radiolabeling was almost instantaneous at room temperature, particularly at higher concentrations of the DTPA conjugate. Their radiochemical purity (RCP) was >99% after Sep-Pak purification. The specific activity was in the range of 100–150 mCi/ μmole , which is consistent with that obtained for the ^{111}In -labeled DTPA conjugate of a cyclic RGDfK monomer (40). The specific activity obtained at room temperature for all three ^{111}In radiotracers in this study was 3–5 time better than that of the ^{111}In -labeled DOTA conjugates (~ 30 mCi/ μmole obtained at 100°C) (33). It should be noted that the radiolabeling conditions used in this study are not optimized. The specific activity of ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 could be further improved at elevated temperatures. All three ^{111}In radiotracers were analyzed using the same HPLC method (Method 2). Their HPLC retention times were 18.2 min, 11.3 min and 11.7 min, respectively. Their log P values were determined in an equal volume mixture of n-octanol and 25 mM phosphate buffer (pH = 7.4), and were calculated to be -2.7 ± 0.1 ($n = 3$), -3.4 ± 0.1 ($n = 3$) and -3.3 ± 0.2 ($n = 3$), respectively. Both HPLC retention time and log P values suggest that incorporation of E and Cys amino acid linkers increases hydrophilicity of the ^{111}In -labeled cyclic RGDfK dimer.

Solution Stability

High solution stability is a requirement for both diagnostic and therapeutic radiotracers. In this study, we studied solution stability of ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 both in saline and in the presence of EDTA challenge. Figure 3 shows their solution stability data in saline (a) and 6 mM EDTA solution (b). It was clear that all three ^{111}In radiotracers remained stable for >24 h in saline and in aqueous solution of 6 mM EDTA.

Biodistribution Characteristics

Biodistribution properties of ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 were evaluated using the BALB/c nude mice bearing the U87MG human glioma xenografts. Selected biodistribution data are listed in Tables 1–3. Figure 4 shows the comparison of excretion kinetics and T/B ratios between ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016. Since they share the same cyclic RGDfK dimer (SU016) and the ^{111}In -DTPA chelate, their difference in biodistribution and excretion characteristics is caused by the linker (E and Cys).

In general, all three ^{111}In radiotracers displayed a rapid blood clearance predominantly via the renal route. Their blood clearance curves were almost identical (Figure 4), and their bone uptake was relatively low (Tables 1–3). The tumor uptake of ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 was comparable between 0.5 h and 4 h p.i. within the experimental error. At 24 h p.i., however, ^{111}In -DTPA-Bn-SU016 had the tumor uptake (1.69 ± 0.18 %ID/g) that was significantly higher ($p < 0.05$) than that of ^{111}In -DTPA-Bn-E-SU016 (0.89 ± 0.17 %ID/g) and ^{111}In -DTPA-Bn-Cys-SU016 (0.98 ± 0.29 %ID/g). The kidney uptake of ^{111}In -DTPA-Bn-E-SU016 was much lower ($p < 0.001$) than that of ^{111}In -DTPA-Bn-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 over the 24 h study period. The liver uptake of ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 was comparable between 0.5 h and 1 h p.i. (Figure 4); but it was significantly higher ($p < 0.001$) for ^{111}In -DTPA-Bn-SU016 than that for ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 at 24 h p.i. For example, the liver uptake of ^{111}In -DTPA-Bn-SU016

was 1.69 ± 0.18 %ID/g at 24 h p.i. and the liver uptake of ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 was only 0.55 ± 0.11 %ID/g and 0.79 ± 0.15 %ID/g at 24 h p.i., respectively. Among the three ^{111}In radiotracers evaluated in this animal model, ^{111}In -DTPA-Bn-E-SU016 has the lowest uptake in liver and kidneys, and the best tumor/liver and tumor/kidney ratios (Figure 4) at 24 h p.i.

The blocking experiment was used to demonstrate tumor-specificity of the radiotracer. In this experiment, $\text{E}[\text{c}(\text{RGDfK})]_2$ (15 mg/kg or ~ 300 $\mu\text{g}/\text{mouse}$) was used as the blocking agent. Such a high dose was used to make sure that the integrin $\alpha_v\beta_3$ is almost completely blocked. Figure 5 illustrates biodistribution data of ^{111}In -DTPA-Bn-E-SU016 at 4 h p.i. in the presence of $\text{E}[\text{c}(\text{RGDfK})]_2$. Apparently, pre-injection of excess $\text{E}[\text{c}(\text{RGDfK})]_2$ significantly reduced the tumor uptake of ^{111}In -DTPA-Bn-E-SU016, suggesting that its tumor uptake is indeed integrin $\alpha_v\beta_3$ -mediated. However, its uptake in liver, spleen, stomach, guts and kidneys, was also reduced significantly, most likely due to the blockage of integrin $\alpha_v\beta_3$ in these organs.

Metabolism

Metabolism studies on ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 were performed using normal BALB/c nude mice. Urine samples were analyzed to determine if they are able to retain their chemical integrity at 1 h p.i. We also tried to analyze feces samples at 1 h p.i., but the radioactivity level was too low to be detected. Figure 6 shows HPLC chromatograms of ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 in the urine at 1 h p.i. It is quite clear that there is little metabolite (<10%) in urine during the 1 h study period.

Imaging Study

A planar image study was performed on ^{111}In -DTPA-Bn-E-SU016 using the BALB/c nude mice bearing U87MG human glioma xenografts. Figure 7 illustrates representative scintigraphic images of the tumor-bearing mouse administered with ~ 200 μCi ^{111}In -DTPA-Bn-E-SU016 at 4 h and 24 h p.i. The tumor was visualized as early as 1 h p.i. (data not shown). The tumor/muscle ratio was 5.6 ± 1.2 ($n = 3$) at 4 h p.i. and 6.1 ± 1.1 ($n = 3$) at 24 h p.i., which were in good agreement with that obtained from direct tissue sampling (Tables 1–3). Because of high kidney and liver activity, the tumor/kidney and tumor/liver ratios were relatively low (0.4 ± 0.1 and 1.0 ± 0.1 at 4 h, 1.9 ± 0.4 and 2.3 ± 0.3 at 24 h, respectively).

DISCUSSION

Many factors may influence the tumor uptake and tumor retention of a radiotracer (13–15). These include binding affinity, receptor population, protein binding, and pharmacokinetics. The fact that [^{18}F]Galacto-RGD has been successfully used as the first PET radiotracer for noninvasive visualization of integrin $\alpha_v\beta_3$ in cancer patients strongly suggests that there are sufficient integrin $\alpha_v\beta_3$ expressed in tumor for PET or SPECT imaging. While the radiolabeled cyclic RGD peptide monomers, such as $\text{c}(\text{RGDfK})$, are useful for imaging the integrin $\alpha_v\beta_3$ expression in tumors, they will have limited clinical utility in visualizing tumors in the lower abdomen region due to their relatively low tumor uptake and partial hepatobiliary excretion. Therefore, there is a continuing need to further improve the tumor targeting capability and excretion kinetics of radiotracers.

To improve integrin $\alpha_v\beta_3$ binding affinity, the multivalent concept has been successfully used to prepare multimeric cyclic RGD peptides, such as $\text{E}[\text{c}(\text{RGDfK})]_2$ and $\text{E}\{\text{E}[\text{c}(\text{RGDfK})]_2\}_2$, for development of the integrin $\alpha_v\beta_3$ -targeted diagnostic (^{64}Cu , $^{99\text{m}}\text{Tc}$ and ^{111}In) and therapeutic (^{90}Y and ^{177}Lu) radiotracers (19–36). Different PKM linkers have been used to improve excretion kinetics of the radiolabeled peptides. For example, a sugar moiety was used

to increase T/B ratios of ^{125}I and ^{18}F -labeled cyclic RGD peptides (47–50). The sugar moiety has also been used to improve tumor uptake and TB ratios of the ^{111}In -labeled DOTA-somatostatin peptide conjugates (51). Small peptides, such as di(cysteic acid), have been used to improve blood clearance and to minimize liver accumulation of radiolabeled peptides and nonpeptide integrin $\alpha_v\beta_3$ antagonists (38,52–54). It has been 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 (55,56). PEG₄ and amino acid linkers, such as E and K, have also been used to improve excretion kinetics of ^{111}In and $^{99\text{m}}\text{Tc}$ -labeled E[c(RGDfK)]₂ (23,33). PKM linkers and their applications to improve excretion kinetics of radiolabeled small biomolecules have been reviewed recently (15,16, 37).

In this study, we prepare ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 with high specific activity (100–150 mCi/ μmole). We are particularly interested in DTPA as the BFC because of its high ^{111}In -labeling efficiency. The specific activity obtained for these three ^{111}In radiotracers was 3–5 time better than that of the ^{111}In -labeled DOTA-PKM-E[c(RGDfK)]₂ (PKM = E, K and PEG₄) conjugates (33). We also examined the effect of E and Cys linkers on integrin $\alpha_v\beta_3$ binding affinity of the cyclic RGDfK dimer DTPA conjugate, and found that the integrin $\alpha_v\beta_3$ binding affinity of DTPA-Bn-SU016, DTPA-Bn-E-SU016 and DTPA-Bn-Cys-SU016 was almost identical. This suggests that introduction of the E and Cys linker does not affect the integrin $\alpha_v\beta_3$ binding affinity of E[c(RGDfK)]₂. Similar conclusion has been made for the HYNIC and DOTA conjugates of E[c(RGDfK)]₂ (23).

The main difference between ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 is the linker group. Both HPLC and log P data suggest that the use of E and Cys linker groups increases hydrophilicity of the ^{111}In radiotracer. The tumor targeting capability of ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 has been evaluated by direct tissue sampling and gamma imaging in BALB/c nude mice bearing U87MG human glioma xenografts. Results from biodistribution studies show that there is no significant difference in their tumor uptake during the first 4 h p.i. (Figure 5), suggesting that E and Cys linkers have little impact on targeting capability of the ^{111}In -labeled E[c(RGDfK)]₂. This conclusion is supported by similar integrin $\alpha_v\beta_3$ binding affinity of SU016, DTPA-Bn-SU016, DTPA-Bn-E-SU016 and DTPA-Bn-Cys-SU016 (Figure 2). The blood clearance patterns of ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 are almost identical over the 24 h study period, indicating that introduction of E and Cys linkers did not significantly affect their protein binding. In addition, ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 are also able to retain their chemical integrity with <10% metabolism detected in the urine samples at 1 h p.i. The fact that very little radioactivity was detected in feces samples from the mice administered with ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 suggest that they are excreted mainly via the renal route.

Despite their similarities, E and Cys linkers have a significant impact on organ uptake and excretion kinetics of the ^{111}In -labeled cyclic RGDfK dimer. For example, the kidney uptake follows the order of ^{111}In -DTPA-Bn-E-SU016 < ^{111}In -DTPA-Bn-SU016 < ^{111}In -DTPA-Bn-Cys-SU016 (Figure 5). The tumor/kidney ratios of ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 are significantly lower ($p < 0.05$) than that of ^{111}In -DTPA-Bn-SU016 at 24 h p.i. In general, small biomolecules in the blood plasma are filtered through glomerular capillaries in kidneys and may be subsequently reabsorbed by the proximal tubular cells (33). Membranes of renal tubular cells contain negatively charged sites to which the positively charged groups (such as guanidine in the RGD sequence) are expected to bind. The carboxylic group of the glutamic acid residue is deprotonated under physiological conditions. Therefore,

it is not surprising that the use of E linker in ^{111}In -DTPA-Bn-E-SU016 results in the reduced kidney uptake (Figure 5). However, it is not clear why ^{111}In -DTPA-Bn-Cys-SU016 has higher kidney uptake than ^{111}In -DTPA-Bn-E-SU016 even though their log P value and HPLC retention times (Figure 6) are very similar.

Another significant difference is the liver uptake. Both ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016 have lower liver uptake and better tumor/liver ratios ($p < 0.05$) than ^{111}In -DTPA-Bn-SU016 at 24 h p.i. These data strongly suggest that the liver uptake could be reduced by introducing the negatively charged amino acid residues due to increased hydrophilicity of the radiotracer. Similar conclusion has been made for the $^{99\text{m}}\text{Tc}$ -labeled HYNIC-PKM-SU016 (PKM = E, K or PEG₄) conjugates (23). However, this conclusion seems to contradict to that made by Dijkgraaf and coworkers (33) for the ^{111}In -labeled DOTA-PKM-E[c(RGDfK)]₂ (PKM = E, K and PEG₄) conjugates. Apparently, the impact of PKM linkers on both liver uptake and liver clearance kinetics of the radiotracer may vary with the change of bifunctional chelator and its radiometal chelate.

CONCLUSION

In this study, we evaluated the integrin $\alpha_v\beta_3$ binding affinities of SU016, DTPA-Bn-SU016, DTPA-Bn-E-SU016 and DTPA-Bn-Cys-SU016. It was found that introduction of the glutamic acid and cysteic acid linkers did not affect the integrin $\alpha_v\beta_3$ binding affinity of the cyclic RGDfK dimer DTPA conjugate. Another important finding of this study is that the ^{111}In -labeling efficiency of all three DTPA conjugates (DTPA-Bn-SU016, DTPA-Bn-E-SU016 and DTPA-Bn-Cys-SU016) is significantly better than that of DOTA analogs. In this regard, DTPA has the advantage over DOTA due to its fast chelation kinetics and high-yield ^{111}In -labeling under mild conditions (e.g. room temperature). All three radiotracers (^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016 and ^{111}In -DTPA-Bn-Cys-SU016) have high in vitro and in vivo solution stability. Results from biodistribution studies show that incorporation of the glutamic acid linker between DTPA and E[c(RGDfK)]₂ increases the clearance of ^{111}In -labeled E[c(RGDfK)]₂ from the liver and kidneys. Among the three radiotracers evaluated in this study, ^{111}In -DTPA-Bn-E-SU016 has the best tumor/liver and tumor/kidney ratios.

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Figure 1.

Schematic presentation of ^{111}In -labeled cyclic RGDfK dimer DTPA conjugates: $^{111}\text{In}(\text{DTPA-Bn-PKM-SU016})$ (PKM = direct bond, glutamic acid (E) and cysteine (Cys); SU016 = E [c(EGDfK)]₂).

Figure 2.

Inhibition of ^{125}I -c(RGDyK) binding to $\alpha_v\beta_3$ integrin on U87MG human glioma cells by c(RGDyK) (●), SU016 (■), DTPA-Bn-SU016 (○), DTPA-Bn-E-SU016 (△), and DTPA-Bn-Cys-SU016 (×). IC_{50} values were calculated to be 31.9 ± 4.0 nM for c(RGDyK), 5.0 ± 0.7 nM for SU016, 7.9 ± 0.6 nM for DTPA-Bn-SU016, 5.8 ± 0.6 nM for DTPA-Bn-E-SU016 and 6.9 ± 0.9 nM for DTPA-Bn-Cys-SU016, respectively.

(a)

(b)

Figure 3. Solution stability data of ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016, and ^{111}In -DTPA-Bn-Cys-SU016 in saline (a) and 6 mM EDTA solution (b).

Figure 4. Direct comparison of the excretion kinetics and tumor-to-background ratios between ^{111}In -DTPA-Bn-SU016, ^{111}In -DTPA-Bn-E-SU016, and ^{111}In -DTPA-Bn-Cys-SU016 in selected organs of the BALB/c nude mice bearing the U87MG human glioma xenografts.

Figure 5.

Biodistribution data of ^{111}In -DTPA-Bn-E-SU016 at 4 h p.i. with/without the presence of excess $\text{E}[\text{c}(\text{EGDfK})]_2$ in BALB/c nude mice bearing the U87MG human glioma xenografts.

Figure 6. Radio-HPLC chromatograms of ^{111}In -DTPA-Bn-SU016 (top), ^{111}In -DTPA-Bn-E-SU016 (middle), and ^{111}In -DTPA-Bn-Cys-SU016 (bottom) in the kit matrix and urine at 1 h p.i. Each normal BALB/c mouse was administered with 200 μCi of radiotracer. Two mice were used for each radiotracer.

Figure 7. A representative scintigraphic image of the tumor-bearing mouse administered with $\sim 200\ \mu\text{Ci}$ of ^{111}In -DTPA-Bn-E-SU016. The arrow indicates presence of tumor.

Table 1

Selected biodistribution data of ^{111}In -DTPA-Bn-SU016 in BALB/c nude mice bearing U87MG human glioma xenografts. The organ uptake is expressed %ID/g. Each data point represents an average of biodistribution data in four animals.

Organ	0.5 h	1 h	4 h	24 h
Tumor	2.53±0.63	2.21±0.65	1.86±0.34	1.69±0.18
Blood	0.69±0.02	0.22±0.04	0.09±0.06	0.05±0.02
Heart	1.07±0.09	0.95±0.40	0.49±0.05	0.43±0.09
Liver	2.48±0.22	2.49±0.38	2.86±0.53	1.93±0.28
Spleen	2.04±0.13	2.56±1.11	1.83±0.37	1.94±0.41
Kidney	9.07±1.29	7.34±0.70	7.05±1.49	6.14±1.34
Muscle	0.49±0.10	0.35±0.04	0.24±0.06	0.21±0.04
Gut	6.46±0.25	5.08±0.92	3.18±0.19	4.16±0.77
Stomach	3.88±0.39	2.86±0.15	1.78±0.15	1.55±0.24
Bone	0.93±0.07	0.76±0.14	0.52±0.23	0.51±0.11

Selected biodistribution data of ¹¹¹In-DTPA-Bn-E-SU016 in BALB/c nude mice bearing U87MG human glioma xenografts. The organ uptake is expressed %ID/g. Each data point represents an average of biodistribution data in four animals.

Table 2

Organ	0.5 h	1 h	4 h	24 h	4.0 h/Block
Tumor	2.45±0.43	2.33±0.55	1.86±0.15	0.89±0.17	0.25±0.06
Blood	0.71±0.10	0.21±0.07	0.06±0.01	0.03±0.01	0.04±0.01
Heart	1.06±0.14	0.72±0.10	0.47±0.05	0.20±0.04	0.06±0.00
Liver	2.94±0.38	3.12±0.38	2.15±0.09	0.55±0.11	0.29±0.04
Spleen	2.13±0.26	2.01±0.43	1.60±0.22	0.79±0.25	0.18±0.03
Kidney	6.04±1.40	5.29±0.51	3.80±0.33	1.37±0.16	2.58±0.42
Muscle	0.52±0.06	0.37±0.06	0.22±0.03	0.12±0.02	0.04±0.00
Gut	5.36±1.98	5.36±0.49	4.31±0.52	1.73±0.22	0.40±0.09
Stomach	3.92±0.28	3.00±0.30	1.64±0.11	0.70±0.06	0.22±0.07
Bone	0.97±0.20	0.77±0.23	0.40±0.08	0.34±0.04	0.08±0.02

Table 3

Selected biodistribution data of ^{111}In -DTPA-Bn-Cys-SU016 in BALB/c nude mice bearing U87MG human glioma xenografts. The organ uptake is expressed %ID/g. Each data point represents an average of biodistribution data in four animals.

Organ	0.5 h	1 h	4 h	24 h
Tumor	2.72±0.28	1.78±0.09	1.53±0.64	0.98±0.29
Blood	0.83±0.16	0.24±0.06	0.06±0.01	0.03±0.01
Heart	1.19±0.16	0.72±0.18	0.46±0.03	0.29±0.02
Liver	3.08±0.60	2.41±0.15	2.32±0.38	0.79±0.15
Spleen	2.08±0.19	1.74±0.62	1.58±0.11	1.08±0.24
Kidney	14.78±2.31	12.20±1.76	10.70±1.19	4.96±0.62
Muscle	0.57±0.04	0.37±0.05	0.23±0.03	0.14±0.02
Gut	7.09±0.86	5.03±0.81	3.48±0.44	3.41±0.82
Stomach	4.12±0.25	2.77±0.44	1.61±0.16	1.10±0.07
Bone	1.01±0.26	0.54±0.12	0.53±0.23	0.35±0.03