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Technetium-99m-Labeled Arg-Gly-Asp-Conjugated Alpha-Melanocyte Stimulating Hormone Hybrid Peptides for Human Melanoma Imaging

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Abstract

Introduction—The purpose of this study was to examine whether ^{99m}Tc-labeled Arg-Gly-Asp (RGD)-conjugated alpha-melanocyte stimulating hormone (α -MSH) hybrid peptide targeting both melanocortin-1 (MC1) and $\alpha_v\beta_3$ integrin receptors was superior in melanoma targeting to ^{99m}Tc-labeled α -MSH or RGD peptide targeting only the MC1 or $\alpha_v\beta_3$ integrin receptor.

Methods—RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} were designed to target both MC1 and $\alpha_v\beta_3$ integrin receptors, MC1 receptor only and $\alpha_v\beta_3$ integrin receptor only, respectively. The MC1 or $\alpha_v\beta_3$ integrin receptor binding affinities of three peptides were determined in M21 human melanoma cells. The melanoma targeting properties of ^{99m}Tc-labeled RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} were determined in M21 human melanoma-xenografted nude mice. Meanwhile, the melanoma uptake of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH was blocked with various non-radiolabeled peptides in M21 melanoma xenografts.

Results—RGD-Lys-(Arg¹¹)CCMSH displayed 2.0 and 403 nM binding affinities to both MC1 and $\alpha_v\beta_3$ integrin receptors, whereas RAD-Lys-(Arg¹¹)CCMSH or RGD-Lys-(Arg¹¹)CCMSH_{scramble} lost their $\alpha_v\beta_3$ integrin receptor binding affinity by greater than 248-fold or MC1 receptor binding affinity by more than 100-fold, respectively. The melanoma uptake of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH was 2.49 and 2.24 times ($p < 0.05$) the melanoma uptakes of ^{99m}Tc-RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH_{scramble} at 2 h post-injection, respectively. Either RGD or (Arg¹¹)CCMSH peptide co-injection could block 42% and 57% of the tumor uptake of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH, whereas the co-injection of RGD+(Arg¹¹)CCMSH peptide mixture could block 66% of the tumor uptake of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH.

Conclusions—Targeting both MC1 and $\alpha_v\beta_3$ integrin receptors enhanced the melanoma uptake of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH in M21 human melanoma xenografts. Flank M21 human

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melanoma tumors were clearly visualized by SPECT/CT imaging using ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH as an imaging probe, highlighting its potential use as a dual-receptor-targeting imaging probe for human melanoma detection.

Keywords

Arg-Gly-Asp-conjugated; alpha-melanocyte stimulating hormone hybrid peptide; dual receptor-targeting human melanoma imaging

INTRODUCTION

Malignant melanoma is the most lethal form of skin cancer with an increasing incidence. In the year 2009, approximately 68,720 newly diagnosed cases and 8,650 fatalities were predicted to occur in the United States [1]. Since no curative treatment exists for metastatic melanoma, early diagnosis and prompt surgical removal is a patient's best opportunity for a cure. Melanocortin-1 (MC1) receptor is a G protein-coupled transmembrane receptor which is over-expressed on human and mouse melanoma cells [2–6], making it an attractive molecular target for developing melanoma-specific imaging probes. Meanwhile, radiolabeled α -melanocyte stimulating hormone (α -MSH) peptides can specifically bind the MC1 receptors with nanomolar binding affinities, underscoring the feasibility of selective delivery of the diagnostic radionuclides by radiolabeled α -MSH peptides to melanoma cells for imaging [7–11].

Integrins are a family of adhesion receptors composed of two non-covalently bound transmembrane glycoprotein subunits (α and β). Integrin receptors are involved in tumor metastasis and angiogenesis, and mediate a variety of cell adhesion activities [12–15]. The $\alpha_v\beta_3$ integrin receptor is known to play a key role in melanoma tumor growth, invasiveness and metastases [14–17], and is over-expressed in invasive melanoma [18]. The Arg-Gly-Asp (RGD) peptide can specifically bind to the $\alpha_v\beta_3$ integrin receptor. Several radiolabeled cyclic RGD peptides have been successfully used to target the $\alpha_v\beta_3$ integrin receptors presented on M21 human melanoma cells for melanoma imaging [19–26].

Over-expressions of both the MC1 and $\alpha_v\beta_3$ integrin receptors on the melanoma highlight the potential application of the radiolabeled RGD-conjugated α -MSH hybrid peptides as dual-receptor-targeting imaging probes for melanoma imaging. We hypothesized that the unique radiolabeled RGD-conjugated α -MSH hybrid peptide targeting both the MC1 and $\alpha_v\beta_3$ integrin receptors would be superior in melanoma targeting to the radiolabeled α -MSH or RGD peptide targeting only the MC1 or $\alpha_v\beta_3$ integrin receptor. In this study, we designed three hybrid peptides with similar molecular weights, namely RGD-Lys-(Arg¹¹)CCMSH {cyclic(Arg-Gly-Asp-DTyr-Asp)-Lys-[Cys^{3,4,10}, D-Phe⁷, Arg¹¹] α -MSH_{3–13}}, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble}, to evaluate our hypothesis. The peptides were radiolabeled with ^{99m}Tc using a glucoheptonate transchelation reaction. The melanoma targeting and pharmacokinetic properties of the ^{99m}Tc -labeled RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} were determined in M21 human melanoma-xenografted nude mice. Meanwhile, the melanoma uptake and biodistribution of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH with various non-radiolabeled peptide blockades were examined in M21 human melanoma-xenografted nude mice. Furthermore, single photon emission computed tomography (SPECT)/CT imaging was performed in a M21 human melanoma-xenografted nude mouse to demonstrate the feasibility of using ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH as an imaging probe for human melanoma detection.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

Amino acid and resin were purchased from Advanced ChemTech Inc. (Louisville, KY) and Novabiochem (San Diego, CA). $^{99m}\text{TcO}_4^-$ was purchased from Cardinal Health (Albuquerque, NM). ^{125}I -Tyr²-[Nle⁴, DPhe⁷]- α -MSH { ^{125}I -(Tyr²)-NDP-MSH} and ^{125}I -Echistatin were obtained from PerkinElmer, Inc. (Shelton, CT). Cyclo(Arg-Gly-Asp-DPhe-Val) {RGD} peptide was purchased from Enzo Life Sciences (Plymouth Meeting, PA) for peptide blocking studies. All other chemicals used in this study were purchased from Thermo Fisher Scientific (Waltham, MA) and used without further purification. M21 human melanoma cells were supplied by Dr. David A. Cheresh from the Department of Pathology, Moores University of California–San Diego Cancer Center.

MC1 and $\alpha_v\beta_3$ Integrin Receptor Quantitation Assay

The MC1 and $\alpha_v\beta_3$ integrin receptor densities were determined on M21 human melanoma cells. Briefly, 0.2 million M21 cells were incubated at 37°C for 2 h in the presence of an increasing concentration of ^{125}I -(Tyr²)-NDP-MSH (2.8, 5.5, 11.1, 22.2, 44.4, 110.9, 221.7, 443.4, 886.8 nCi) or ^{125}I -Echistatin (2.3, 4.5, 9.1, 18.2, 36.4, 90.9, 181.8, 363.6, 727.2 nCi) in 0.5 mL of binding medium {Minimum Essential Medium (MEM) with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.2% bovine serum albumin (BSA), 0.3 mM 1,10-phenanthroline}. The reaction medium was aspirated after incubation. Cells were rinsed with 0.5 mL of ice-cold pH 7.4, 0.2% BSA / 0.01 M phosphate buffered saline (PBS) twice, and then the levels of activity associated with the cells were measured in a Wallac 1480 automated gamma counter (PerkinElmer, NJ). Non-specific binding was determined by incubating the cells and ^{125}I -(Tyr²)-NDP-MSH with 10 μM non-radioactive NDP-MSH or incubating the cells and ^{125}I -Echistatin with 10 μM non-radioactive RGD peptide. Specific binding was obtained by subtracting the nonspecific binding from total binding. Maximum specific binding (B_{max}) was estimated from nonlinear curve fitting of specific binding (dpm) versus the concentration of ^{125}I -(Tyr²)-NDP-MSH (fmol/mL) or ^{125}I -Echistatin (fmol/mL) using the Prism software (GraphPad Software, La Jolla, CA).

Peptide Synthesis

The RGD-Lys-(Arg¹¹)CCMSH was synthesized according to our published procedure [27]. RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} were prepared and characterized using the method described previously [27] with modifications. Briefly, intermediate scaffolds of H₂N-Arg(Pbf)-Ala-Asp(OtBu)-DTyr(tBu)-Asp(O-2-phenylisopropyl)-Lys(Boc)-Cys(Trt)-Cys(Trt)-Glu(OtBu)-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-Cys(Trt)-Arg(Pbf)-Pro-Val and H₂N-Arg(Pbf)-Gly-Asp(OtBu)-DTyr(tBu)-Asp(O-2-phenylisopropyl)-Lys(Boc)-Cys(Trt)-Cys(Trt)-Glu(OtBu)-Arg(Pbf)-His(Trt)-Trp(Boc)-DPhe-Cys(Trt)-Arg(Pbf)-Pro-Val were synthesized on Sieber amide resin using standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry by an Advanced ChemTech multiple-peptide synthesizer (Louisville, KY). The protecting groups of 2-phenylisopropyl were removed and the peptides were cleaved from the resin treating with a mixture of 2.5% of trifluoroacetic acid (TFA) and 5% of triisopropylsilane. After the precipitation with ice-cold ether and characterization by liquid chromatography-mass spectroscopy (LC-MS), the protected peptides were dissolved in H₂O/CH₃CN (50:50) and lyophilized to remove the reagents such as TFA and triisopropylsilane. The protected peptides were further cyclized by coupling the carboxylic group from the Asp with the alpha amino group from the Arg at the N-terminus. The cyclization reaction was achieved by overnight reaction in dimethylformamide (DMF) using benzotriazole-1-yl-oxytris-pyrrolidino-phosphonium-hexafluorophosphate (PyBOP) as a coupling agent in the presence of N,N-diisopropylethylamine (DIEA). After characterization by LC-MS, the cyclized protected

peptides were dissolved in H₂O/CH₃CN (50:50) and lyophilized to remove the reagents such as PyBOP and DIEA. The protecting groups were totally removed by treating with a mixture of trifluoroacetic acid (TFA), thioanisole, phenol, water, ethanedithiol and triisopropylsilane (87.5:2.5:2.5:2.5:2.5:2.5) for 2 h at room temperature (25 °C). The peptides were precipitated and washed with ice-cold ether for four times, purified by reverse phase-high performance liquid chromatography (RP-HPLC) and characterized by LC-MS.

***In vitro* Competitive Binding Assay**

The IC₅₀ values of RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} for the MC1 receptor were determined according to our previously published procedure [27] with modifications. Briefly, the M21 cells were harvested and seeded into a 24-well cell culture plate (1.5 × 10⁵ cells/well) and incubated at 37 °C overnight. After being washed with binding medium {Modified Eagle's medium with 25 mM N-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid), pH 7.4, 0.2% bovine serum albumin (BSA), 0.3 mM 1,10-phenanthroline}, the cells were incubated at 37 °C for 2 h with approximately 30,000 counts per minute (cpm) of ¹²⁵I-(Tyr²)-NDP-MSH in the presence of increasing concentrations (10⁻¹² to 10⁻⁵ M) of each peptide in 0.3 mL of binding medium. The reaction medium was aspirated after the incubation. The cells were rinsed twice with 0.5 mL of ice-cold pH 7.4, 0.2% BSA / 0.01 M phosphate buffered saline (PBS) and lysed in 0.5 mL of 1 N NaOH for 5 minutes. The radioactivities associated with cells were measured in a Wallac 1480 automated gamma counter (PerkinElmer, NJ). The IC₅₀ values of the peptides for the MC1 receptor were calculated using the Prism software (GraphPad Software, La Jolla, CA).

The IC₅₀ values of RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} for the α_vβ₃ integrin receptor were determined according to the published procedure [28] with modifications. The M21 cells were harvested, washed twice with PBS, and resuspended (2 × 10⁶ cells/mL) in binding buffer (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 1 mmol/L MnCl₂, 0.1% bovine serum albumin). The M21 cells (1 × 10⁵ cells/well) were seeded in Millipore 96-well filter multiscreen DV plates (0.65 μm pore size) and incubated at 25 °C for 2 h with approximately 30,000 cpm of ¹²⁵I-Echistatin in the presence of increasing concentrations (10⁻¹¹ to 10⁻⁴ M) of each peptide in 0.2 mL of binding medium. After the incubation, the plates were filtered through a multiscreen vacuum manifold and rinsed twice with 0.5 mL of ice-cold pH 7.4, 0.2% BSA / 0.01 M PBS. The hydrophilic polyvinylidenedifluoride (PVDF) filters were collected and the radioactivities were measured in a Wallac 1480 automated gamma counter (PerkinElmer, NJ). The IC₅₀ values of the peptides for the α_vβ₃ integrin receptor were calculated using the Prism software (GraphPad Software, La Jolla, CA).

Peptide Radiolabeling

RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} were radiolabeled with ^{99m}Tc via a glucoheptonate transchelation reaction using methods described previously [27]. Briefly, 100 μL of 2 mg/mL SnCl₂ in 0.2 M glucoheptonate aqueous solution and 200 μL of fresh ^{99m}TcO₄⁻ solution (37–74 MBq) were added into a reaction vial and incubated at room temperature (25 °C) for 20 min to form ^{99m}Tc-glucoheptonate. Then, 10 μL of 1 mg/mL each peptide aqueous solution was added into the reaction vial and the pH of the reaction mixture was adjusted to 8.5 with 0.1 M NaOH. The reaction mixture was incubated at 75 °C for 40 min. The radiolabeled peptide was purified to single species by Waters RP-HPLC (Milford, MA) on a Grace Vydac C-18 reverse phase analytic column (Deerfield, IL) using a 20 min gradient of 16–26% acetonitrile in 20 mM HCl aqueous solution at a flow rate of 1 mL/min. The purified peptide

samples were purged with N₂ gas for 20 min to remove the acetonitrile. The pH of the final solution was adjusted to 5 with 0.1 N NaOH and normal saline for animal studies.

Biodistribution Studies

All the animal studies were conducted in compliance with Institutional Animal Care and Use Committee approval. The pharmacokinetics of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH was determined in M21 melanoma-xenografted nude mice (Harlan, Indianapolis, IN). The nude mice were subcutaneously inoculated on the right flank with 5 × 10⁶ M21 cells. The weight of tumors reached approximately 0.1–0.2 g 21 days post cell inoculation. Each melanoma-xenografted nude mouse was injected with 0.037 MBq of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH via the tail vein. Groups of 5 mice were sacrificed at 0.5, 2, 4 and 24 h post-injection, and tumors and organs of interest were harvested, weighed and counted. Blood values were taken as 6.5% of the whole-body weight. The biodistribution of ^{99m}Tc-RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH_{scramble} were determined in M21 melanoma-xenografted nude mice at 2 h post-injection for comparison.

Peptide Blocking Studies

The receptor specificity of the tumor uptake was determined at 2 h post-injection by co-injecting 0.037 MBq of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH with unlabeled (Arg¹¹)CCMSH (6.1 nmol), RGD (6.1 nmol) or (Arg¹¹)CCMSH + RGD (6.1 nmol + 6.1 nmol) peptides, respectively. Each melanoma-xenografted nude mouse was injected with 0.037 MBq of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH with different peptide blockade via the tail vein. Groups of 5 mice were sacrificed at 2 h post-injection, and tumors and organs of interest were harvested, weighed and counted. Blood values were taken as 6.5% of the whole-body weight.

Imaging Human Melanoma with ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH

A M21 human melanoma-xenografted nude mouse was injected with 7.4 MBq of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH via the tail vein. The mouse was euthanized at 2 h post-injection for small animal SPECT/CT (Nano-SPECT/CT®, Bioscan) imaging. The 9-min CT scan was immediately followed by the whole-body SPECT scan. The SPECT scans of 24 projections were acquired over 90 mins. Reconstructed data from SPECT and CT were visualized and co-registered using the InVivoScope software (Bioscan, Washington DC).

Statistical Methods

Statistical analysis was performed using the Student's t-test for unpaired data to determine the significant differences between the groups in the biodistribution studies and peptide blocking studies described above. Differences at the 95% confidence level (p<0.05) were considered significant.

RESULTS

The MC1 and α_vβ₃ integrin receptor densities of the M21 human melanoma cells were determined by saturation binding assay using commercial ¹²⁵I-(Tyr²)-NDP-MSH and ¹²⁵I-Echistatin as radioactive tracers, respectively. The saturation curves and scatchard plots are presented in Figure 1. The B_{max} values of the M21 cells were 10,395 dpm/million cells (1,281 receptors/cell) for the MC1 receptor and 783,410 dpm/million cells (96,555 receptors/cell) for the α_vβ₃ integrin receptor.

RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} were synthesized, purified by RP-HPLC and characterized by electrospray ionization mass spectrometry. All three peptides displayed greater than 95%

purity with 25–30% overall synthetic yields. The peptide structures are presented in Figure 2. The competitive binding curves of the peptides for the MC1 or $\alpha_v\beta_3$ integrin receptor are shown in Figure 3. The IC_{50} values of RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} were 2.0, 0.3, 216 nM for the MC1 receptor. The IC_{50} values of RGD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} were 403 and 504 nM for the $\alpha_v\beta_3$ integrin receptor, RAD-Lys-(Arg¹¹)CCMSH exhibited greater than 100,000 nM binding affinity for the $\alpha_v\beta_3$ integrin receptor.

The peptides were readily labeled with ^{99m}Tc using a glucoheptonate transchelation reaction with greater than 95% radiolabeling yields. ^{99m}Tc-labeled RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} were completely separated from their excess non-labeled peptides by RP-HPLC. ^{99m}Tc-labeled RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} showed greater than 98% radiochemical purities after the HPLC purification. The retention times of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH, ^{99m}Tc-RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH_{scramble} were 12.2, 13.4 and 16.1 mins, respectively (Fig. 4).

The melanoma targeting and pharmacokinetic properties of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH were determined in M21 melanoma-xenografted nude mice at 0.5, 2, 4 and 24 h post-injection. The melanoma targeting and pharmacokinetic properties of ^{99m}Tc-RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH_{scramble} were determined in M21 melanoma-xenografted nude mice at 2 h post-injection for comparison. The biodistribution results of ^{99m}Tc-labeled RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} are shown in Table 1. ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH exhibited rapid and substantial tumor uptake in melanoma-xenografted nude mice. The tumor uptake value was $2.67 \pm 1.13\%$ ID/g at 0.5 h post-injection. There were 2.69 ± 0.78 and $2.51 \pm 1.14\%$ ID/g of the ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH activity remaining in the tumors 2 and 4 h post-injection. Even at 24 h post-injection, the tumor retention of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH was $1.49 \pm 0.86\%$ ID/g. Whole-body clearance of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH was rapid, with approximately 66% of the injected radioactivity cleared through the urinary system by 2 h post-injection (Table 1). Normal organ uptakes of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH were generally low (<2% ID/g) except for the kidneys and liver after 2 h post-injection. High tumor/blood and tumor/muscle uptake ratios were demonstrated as early as 2 h post-injection (Table 1). The renal uptake of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH reached its peak value of $90.80 \pm 19.35\%$ ID/g at 4 h post-injection. The renal uptake decreased to $27.91 \pm 10.03\%$ ID/g at 24 h post-injection.

Both ^{99m}Tc-RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH_{scramble} displayed lower melanoma uptake values than that of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH. The tumor uptake values of ^{99m}Tc-RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH_{scramble} were 1.08 ± 0.47 and $1.20 \pm 0.72\%$ ID/g at 2 h post-injection, respectively (Table 1). The tumor uptake value of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH was 2.49 and 2.24 times ($p < 0.05$) the tumor uptake values of ^{99m}Tc-RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH_{scramble}, respectively.

The specificity of the melanoma uptake of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH was determined by peptide blocking studies. The results are presented in Figure 5. The tumor uptake of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH was significantly ($p < 0.05$) blocked by either (Arg¹¹)CCMSH (6.1 nmol), RGD (6.1 nmol) or (Arg¹¹)CCMSH + RGD (6.1 nmol + 6.1 nmol) peptide at 2 h post-injection. The tumor uptake of ^{99m}Tc-RGD-Lys-(Arg¹¹)CCMSH was reduced from $2.69 \pm 0.78\%$ ID/g to $1.15 \pm 0.51\%$ ID/g when co-injected with (Arg¹¹)CCMSH peptide. Co-injection of RGD peptide decreased the tumor uptake

of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH to $1.56 \pm 0.54\%$ ID/g. Co-injection of (Arg¹¹)CCMSH + RGD peptide mixture exhibited the best blocking effect and reduced the tumor uptake of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH to $0.91 \pm 0.29\%$ ID/g.

To examine the human melanoma imaging property of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH, one M21 human melanoma-xenografted nude mouse was injected with ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH through the tail vein. The whole-body, coronal and transversal SPECT/CT images are presented in Figure 6. Flank human melanoma tumors were visualized clearly by ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH at 2 h post-injection. ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH exhibited high tumor to normal organ uptake ratios except for the kidneys and liver. Radioactivity in the bladder demonstrated the urinary clearance of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH, which was consistent with the biodistribution results.

DISCUSSION

Both MC1 and $\alpha_v\beta_3$ integrin receptors have been attractive molecular targets for the development of receptor-targeting peptide radiopharmaceuticals due to their over-expression on melanoma cells. Radiolabeled α -MSH peptides have been used to target the MC1 receptors for melanoma imaging [7–11], whereas radiolabeled RGD peptides have been utilized to targeting the $\alpha_v\beta_3$ integrin receptors for melanoma detection [16–20], respectively. We are interested in developing novel radiolabeled hybrid peptides to target both MC1 and $\alpha_v\beta_3$ integrin receptors for melanoma imaging. We hypothesized that the unique radiolabeled RGD-conjugated α -MSH hybrid peptide targeting both the MC1 and $\alpha_v\beta_3$ integrin receptors would be superior in melanoma targeting to the radiolabeled α -MSH or RGD peptide targeting only the MC1 or $\alpha_v\beta_3$ integrin receptor. Hence, we determined the MC1 and $\alpha_v\beta_3$ integrin receptor densities in M21 human melanoma cells to demonstrate the presence of both receptors. Our results on receptor density indicated that 1,281 MC1 receptors/cell and 96,555 $\alpha_v\beta_3$ integrin receptor/cell presented in M21 melanoma cells, making M21 human melanoma xenografts a suitable animal model to examine our hypothesis.

We designed three hybrid peptides with similar molecular weights to examine our hypothesis in this study. Firstly, the cyclic RGD motif {cyclic(Arg-Gly-Asp-DTyr-Asp)} was coupled to the (Arg¹¹)CCMSH through a Lys linker to generate RGD-Lys-(Arg¹¹)CCMSH to target both MC1 and $\alpha_v\beta_3$ integrin receptors. Secondly, it is known that the switch from the RGD to RAD sacrifices the binding affinity of the RGD to the $\alpha_v\beta_3$ integrin receptor. We thus replaced the Gly in the RGD motif with Ala to yield RAD-Lys-(Arg¹¹)CCMSH to target the MC1 receptor only. Thirdly, it is also known that the His-DPhe-Arg-Trp sequence is critical for high MC1 receptor binding. We therefore scrambled the His-DPhe-Arg-Trp sequence into the Arg-His-Trp-DPhe sequence to generate RGD-Lys-(Arg¹¹)CCMSH_{scramble} to target the $\alpha_v\beta_3$ integrin receptor only. The IC₅₀ values of RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} peptides for the MC1 and $\alpha_v\beta_3$ integrin receptors were determined in the M21 cells. The receptor binding affinities of the three hybrid peptides strongly supported the rationale of our peptide design. RGD-Lys-(Arg¹¹)CCMSH exhibited 2.0 and 403 nM binding affinities to the MC1 and $\alpha_v\beta_3$ integrin receptors, respectively. As we anticipated, RAD-Lys-(Arg¹¹)CCMSH maintained nanomolar MC1 receptor binding affinity (0.3 nM) but dramatically lost its $\alpha_v\beta_3$ integrin receptor binding affinity (>100,000 nM) by greater than 248-fold compared to RGD-Lys-(Arg¹¹)CCMSH. Not surprisingly, RGD-Lys-(Arg¹¹)CCMSH_{scramble} maintained 504 nM $\alpha_v\beta_3$ integrin receptor binding affinity but lost its MC1 receptor binding affinity by more than 100-fold compared to RGD-Lys-(Arg¹¹)CCMSH.

The biodistribution results of ^{99m}Tc -labeled RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} in M21 human melanoma xenografts strongly supported our hypothesis. ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH exhibited superior melanoma targeting properties to ^{99m}Tc -RAD-Lys-(Arg¹¹)CCMSH or ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH_{scramble}. The melanoma uptake value of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH was 2.49 and 2.24 times the tumor uptake value of ^{99m}Tc -RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH_{scramble}, respectively. The tumor blocking studies demonstrated that either RGD or (Arg¹¹)CCMSH peptide co-injection could block 42% and 57% of the tumor uptake of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH, whereas the co-injection of RGD+(Arg¹¹)CCMSH peptide mixture could block 66% of the tumor uptake of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH. Moreover, the melanoma uptake value of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH was higher than the sum of the melanoma uptake values of ^{99m}Tc -RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH_{scramble}, indicating a synergistic (beyond additive) effect between both receptors in M21 human melanoma for ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH. Compared to ^{99m}Tc -RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH_{scramble}, the enhanced melanoma uptake of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH was likely due to the over-expression of both MC1 and $\alpha_v\beta_3$ integrin receptors on M21 melanoma cells. Potential synergistic effect between the MC1 and $\alpha_v\beta_3$ integrin receptors might promote the elevation of the regional peptide concentration of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH in the proximity of the MC1 and $\alpha_v\beta_3$ integrin receptors. For instance, ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH molecule bound to either MC1 or $\alpha_v\beta_3$ integrin receptor would be in the close proximity of other available MC1 or $\alpha_v\beta_3$ integrin receptors, facilitating the further binding of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH molecule somewhat dissociated from the current binding receptor to other available MC1 or $\alpha_v\beta_3$ integrin receptors in the close proximity.

It is worthwhile to note that the Student's t-test p value was 0.025 for the tumor uptakes of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH with or without RGD blockade, whereas the Student's t-test p values were 0.0061 and 0.0023 for the tumor uptakes of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH with or without (Arg¹¹)CCMSH blockade and RGD+(Arg¹¹)CCMSH blockade, respectively. Therefore, we performed a GraphPad one-way ANOVA followed by Dunnett's post test, which is a more sophisticated statistical analysis method than the Student's t-test. The ANOVA results confirmed the significant difference between ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH with or without (Arg¹¹)CCMSH blockade, as well as between ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH with or without RGD+(Arg¹¹)CCMSH blockade. However, the difference was not significant between ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH with or without RGD blockade. We used 6.1 nmol of (Arg¹¹)CCMSH, 6.1 nmol of RGD or a mixture of 6.1 nmol of (Arg¹¹)CCMSH and 6.1 nmol of RGD in the peptide blocking studies, respectively. Although the co-injection of 6.1 nmol of (Arg¹¹)CCMSH significantly blocked the tumor uptake of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH, it was likely that greater than 6.1 nmol of RGD blockade would be needed to significantly block the tumor uptake of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH.

As showed in Figure 6, flank M21 human melanoma tumors were clearly visualized by SPECT/CT imaging using ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH as an imaging probe 2 h post injection, highlighting the potential use of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH for human melanoma imaging. ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH displayed high tumor to normal organ uptake ratios except for the kidneys and liver, which was coincident with the biodistribution results (Table 1). It is worthwhile to note that the epsilon amino group on the side chain of the Lys was available in ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH and added a positive charge to the overall charge of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH, that might contribute to the high renal uptake value of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH due to the electrostatic interaction between positively-charged peptide molecules and negatively-charged tubule cells. Co-

injection of positively-charged lysine or arginine effectively reduced the renal uptakes of ^{188}Re -labeled metal-cyclized CCMSH by 50% [29]. Accordingly, co-injection of lysine or arginine would be an option to reduce the renal uptake of $^{99\text{m}}\text{Tc}$ -RGD-Lys-(Arg 11)CCMSH by reducing the electrostatic interaction between positively-charged peptide molecules and negatively-charged tubule cells. Replacement of Lys with Asp reduced the renal and liver uptakes of ^{111}In -DOTA-Lys-NOC by 84% and 45% [30]. Accordingly, substitution of Lys with Asp in $^{99\text{m}}\text{Tc}$ -RGD-Lys-(Arg 11)CCMSH would be another strategy to decrease the renal and liver uptakes of $^{99\text{m}}\text{Tc}$ -RGD-Lys-(Arg 11)CCMSH in further studies.

Both receptor density and receptor binding affinity are important factors to take into account in the development of an imaging probe targeting two receptors (MC1 and $\alpha_v\beta_3$ integrin receptors) over-expressed on M21 human melanoma cells. Potential synergistic effect between two receptors and delicate balance in dynamic ligand-receptor binding play roles in the success of a dual receptor-targeting peptide as well. Despite the relatively low MC1 receptor density (1,281 receptors/cell) and high nanomolar $\alpha_v\beta_3$ integrin receptor binding affinity (403 nM), high $\alpha_v\beta_3$ integrin receptor density (96,555 receptor/cell) and low nanomolar MC1 receptor binding affinity (2.0 nM) might substantially contribute to the successful visualization of M21 human melanoma using $^{99\text{m}}\text{Tc}$ -RGD-Lys-(Arg 11)CCMSH as imaging probe. Enhanced melanoma uptake of $^{99\text{m}}\text{Tc}$ -RGD-Lys-(Arg 11)CCMSH (Table 1) and successful human melanoma imaging (Figure 6) using $^{99\text{m}}\text{Tc}$ -RGD-Lys-(Arg 11)CCMSH as an imaging probe provided a new insight into the design of novel dual receptor-targeting radiolabeled peptides for melanoma detection.

CONCLUSIONS

Targeting both MC1 and $\alpha_v\beta_3$ integrin receptors enhanced the melanoma uptake of $^{99\text{m}}\text{Tc}$ -RGD-Lys-(Arg 11)CCMSH in M21 human melanoma xenografts. Flank M21 human melanoma tumors were clearly visualized by SPECT/CT imaging using $^{99\text{m}}\text{Tc}$ -RGD-Lys-(Arg 11)CCMSH as an imaging probe, highlighting its potential use as a dual-receptor-targeting imaging probe for human melanoma detection.

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Figure 1.

The $\alpha_v\beta_3$ integrin (A) and MC1 (B) receptor densities in M21 human melanoma cells. The B_{max} values were 783,410 dpm/million cells (96,555 receptor/cell) for the $\alpha_v\beta_3$ integrin receptor and 10,395 dpm/million cells (1,281 receptor/cell) for the MC1 receptor.

Figure 2.

The structures of RGD-Lys-(Arg¹¹)CCMSH (A), RAD-Lys-(Arg¹¹)CCMSH (B) and RGD-Lys-(Arg¹¹)CCMSH_{scramble} (C) hybrid peptides. The receptor binding sequences were highlighted with dashed half circles.

Figure 3.

The $\alpha_v\beta_3$ integrin (A) and MC1 (B) receptor competitive binding curves of RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} hybrid peptides in M21 human melanoma cells. The IC₅₀ values of RGD-Lys-(Arg¹¹)CCMSH, RAD-Lys-(Arg¹¹)CCMSH and RGD-Lys-(Arg¹¹)CCMSH_{scramble} were 403, >100,000, 504 nM for the $\alpha_v\beta_3$ integrin receptor, and 2.0, 0.3, 216 nM for the MC1 receptor, respectively.

Figure 4. Radioactive HPLC profiles of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH (A), ^{99m}Tc -RAD-Lys-(Arg¹¹)CCMSH (B) and ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH_{scramble} (C). The retention times of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH, ^{99m}Tc -RAD-Lys-(Arg¹¹)CCMSH and ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH_{scramble} were 12.2, 13.4 and 16.2 min, respectively.

Figure 5. Biodistribution of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH with or without RGD blockade (6.1 nmol), (Arg¹¹)CCMSH blockade (6.1 nmol) and RGD+(Arg¹¹)CCMSH blockade (6.1 nmol + 6.1 nmol) in M21 human melanoma-xenografted nude mice at 2 h post-injection. * $p < 0.05$, significance comparisons on tumor, liver and kidneys between ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH with or without peptide blockade.

Figure 6. Three-dimensional (A), coronal (B) and transversal (C) SPECT/CT images of M21 human melanoma 2 h post-injection of 7.4 MBq of ^{99m}Tc -RGD-Lys-(Arg¹¹)CCMSH. The mouse was euthanized for small animal SPECT/CT (Nano-SPECT/CT®, Bioscan) imaging. The 9-min CT scan was immediately followed by the 90-min whole-body SPECT scan. Flank melanoma lesions (T) were highlighted with arrows on the images.

Table 1

Biodistribution of ^{99m}Tc -RGD-Lys-(Arg 11)CCMSH (A), ^{99m}Tc -RAD-Lys-(Arg 11)CCMSH (B) and ^{99m}Tc -RGD-Lys-(Arg 11)CCMSHscramble (C) in M21 human melanoma-xenografted nude mice. The data was presented as percent injected dose/gram or as percent injected dose (mean $\pm$ SD, n=5)

Tissue	A			B		C		
	0.5 h	2 h	4 h	24 h	2 h	2 h	2 h	2 h
	Percent injected dose/gram (%ID/g)							
Tumor	2.67 $\pm$ 1.13	2.69 $\pm$ 0.78	2.51 $\pm$ 1.14	1.49 $\pm$ 0.86	1.08 $\pm$ 0.47*		1.20 $\pm$ 0.72*	
Brain	0.19 $\pm$ 0.06	0.05 $\pm$ 0.02	0.05 $\pm$ 0.02	0.04 $\pm$ 0.01	0.05 $\pm$ 0.01		0.03 $\pm$ 0.01	
Blood	2.70 $\pm$ 0.81	0.23 $\pm$ 0.13	0.15 $\pm$ 0.03	0.03 $\pm$ 0.01	0.80 $\pm$ 0.15		0.20 $\pm$ 0.04	
Heart	2.72 $\pm$ 0.86	0.43 $\pm$ 0.14	0.45 $\pm$ 0.09	0.21 $\pm$ 0.04	0.62 $\pm$ 0.15		0.37 $\pm$ 0.16	
Lung	5.06 $\pm$ 1.08	1.03 $\pm$ 0.22	0.85 $\pm$ 0.13	0.33 $\pm$ 0.09	1.13 $\pm$ 0.48		0.75 $\pm$ 0.30	
Liver	4.74 $\pm$ 0.99	3.16 $\pm$ 0.75	4.32 $\pm$ 0.68	2.58 $\pm$ 0.39	10.24 $\pm$ 1.65*		4.59 $\pm$ 1.36	
Skin	5.46 $\pm$ 0.80	1.35 $\pm$ 0.41	1.35 $\pm$ 0.20	0.82 $\pm$ 0.12	0.85 $\pm$ 0.16		0.62 $\pm$ 0.15	
Spleen	2.88 $\pm$ 0.66	1.14 $\pm$ 0.29	1.26 $\pm$ 0.31	0.88 $\pm$ 0.26	1.55 $\pm$ 0.28		0.81 $\pm$ 0.13	
Stomach	2.69 $\pm$ 2.27	1.84 $\pm$ 1.05	1.95 $\pm$ 0.29	0.73 $\pm$ 0.47	2.44 $\pm$ 0.62		0.76 $\pm$ 0.38	
Kidneys	83.61 $\pm$ 32.29	67.06 $\pm$ 16.53	90.80 $\pm$ 19.35	27.91 $\pm$ 10.03	81.92 $\pm$ 19.86		51.01 $\pm$ 11.82	
Muscle	0.75 $\pm$ 0.49	0.14 $\pm$ 0.06	0.13 $\pm$ 0.07	0.02 $\pm$ 0.01	0.09 $\pm$ 0.03		0.13 $\pm$ 0.11	
Pancreas	0.77 $\pm$ 0.30	0.23 $\pm$ 0.08	0.22 $\pm$ 0.10	0.15 $\pm$ 0.06	0.32 $\pm$ 0.19		0.19 $\pm$ 0.07	
Bone	1.19 $\pm$ 0.82	0.44 $\pm$ 0.18	0.32 $\pm$ 0.28	0.19 $\pm$ 0.12	0.73 $\pm$ 0.05		0.22 $\pm$ 0.05	
	Percent injected dose (%ID)							
Intestines	3.46 $\pm$ 0.62	2.50 $\pm$ 1.38	2.92 $\pm$ 1.02	1.33 $\pm$ 0.48	2.63 $\pm$ 1.00		1.55 $\pm$ 0.10	
Bladder	33.27 $\pm$ 13.86	66.36 $\pm$ 3.43	59.70 $\pm$ 4.25	83.55 $\pm$ 2.44	53.67 $\pm$ 8.41		75.13 $\pm$ 3.35	
	Uptake ratio of tumor/normal tissue							
Tumor/Blood	0.99	11.70	16.73	49.67	1.35		6.00	
Tumor/Kidneys	0.03	0.04	0.03	0.05	0.01		0.02	
Tumor/Lung	0.53	2.61	2.95	4.52	0.96		1.60	
Tumor/Liver	0.56	0.85	0.58	0.58	0.11		0.26	
Tumor/Muscle	3.56	19.21	19.31	74.50	12.00		9.23	

* p<0.05, significance comparisons on tumor, liver and kidneys between ^{99m}Tc -RGD-Lys-(Arg 11)CCMSH (A) and ^{99m}Tc -RAD-Lys-(Arg 11)CCMSH (B), between ^{99m}Tc -RGD-Lys-(Arg 11)CCMSH (A) and ^{99m}Tc -RGD-Lys-(Arg 11)CCMSHscramble (C) at 2 h post-injection.