Replacement of the Lys linker with an Arg linker resulting in improved melanoma uptake and reduced renal uptake of Tc-99m-labeled Arg-Gly-Asp-conjugated alpha-melanocyte stimulating hormone hybrid peptide

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ABSTRACT

The purpose of this study was to reduce the non-specific renal uptake of Arg-Gly-Asp (RGD)-conjugated alpha-melanocyte stimulating hormone (α-MSH) hybrid peptide through structural modification or L-lysine co-injection. The RGD motif {cyclic(Arg-Gly-Asp-DTyr-Asp)} was coupled to [Cys^{3,4,10}, D-Phe^7, Arg^{11}] α-MSH$_{3-13}$ {(Arg^{11})CCMSH} through the Arg linker (substituting the Lys linker) to generate a novel RGD-Arg-(Arg^{11})CCMSH hybrid peptide. The melanoma targeting and pharmacokinetic properties of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH were determined in B16/F1 melanoma-bearing C57 mice. The effect of L-lysine co-injection on the renal uptake was determined through the co-injection of L-lysine with ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH or ^{99m}Tc-RGD-Lys-(Arg^{11})CCMSH. Replacement of the Lys linker with an Arg linker exhibited a profound effect in reducing the non-specific renal uptake of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH, as well as increasing the tumor uptake of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH compared to ^{99m}Tc-RGD-Lys-(Arg^{11})CCMSH. ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH exhibited high tumor uptake ($21.41 \pm 3.74\%$ ID/g at 2 h post-injection) and prolonged tumor retention ($6.81 \pm 3.71\%$ ID/g at 24 h post-injection) in B16/F1 melanoma-bearing mice. The renal uptake values of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH were 40.14–64.08% of those of ^{99m}Tc-RGD-Lys-(Arg^{11})CCMSH ($p < 0.05$) at 0.5, 2, 4 and 24 h post-injection. Co-injection of L-lysine was effective in decreasing the renal uptakes of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH by 27.7% and ^{99m}Tc-RGD-Lys-(Arg^{11})CCMSH by 52.1% at 2 h post-injection. Substitution of the Lys linker with an Arg linker dramatically improved the melanoma uptake and reduced the renal uptake of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH, warranting the further evaluation of ^{188}Re-labeled RGD-Arg-(Arg^{11})CCMSH as a novel MC1 receptor-targeting therapeutic peptide for melanoma treatment in the future.

1. Introduction

High mortality of malignant melanoma is associated with the occurrence of metastatic melanoma due to its aggressiveness and resistance to current chemotherapy and immunotherapy regimens.[1] The survival times for patients with metastatic melanoma average 3–15 months.[2,3] Therefore, it is highly desirable to develop novel and effective therapeutic approaches for melanoma treatment. G protein-coupled melanocortin-1 (MC1) receptor is a distinct molecular target for developing diagnostic and therapeutic peptides[4–11] for melanoma due to its over-expression on human and mouse melanoma cells.[12–16] MC1 receptor-targeting α-MSH

peptides have been used to specifically deliver the therapeutic radionuclides into melanoma cells for treatment.[9–11] Promising preclinical therapeutic results of ^{212}Pb-, ^{177}Lu-, ^{188}Re-labeled metal-cyclized α-MSH peptides[9–11] in B16/F1 melanoma-bearing C57 mice demonstrated the potential of peptide-targeted radionuclide therapy as a novel effective approach for melanoma treatment.

Recently, we have reported a study on using a novel RGD-conjugated α-MSH hybrid peptide {RGD-Lys-(Arg^{11})CCMSH} for melanoma therapy.[17] The (Arg^{11})CCMSH peptide was used as an effective delivery vehicle to specifically transport the RGD motif (apoptosis inducer) into melanoma cells to induce apoptosis. Specifically, the RGD motif was conjugated to the (Arg^{11})CCMSH through the Lys linker to generate the RGD-Lys-(Arg^{11})CCMSH hybrid peptide. Technetium-99m-labeled RGD-Lys-(Arg^{11})CCMSH displayed rapid MC1 receptor-mediated internalization in B16/F1 melanoma cells. Furthermore, ^{99m}Tc-RGD-Lys-(Arg^{11})CCMSH

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exhibited rapid and high melanoma uptake (14.83 ± 2.94% ID/g at 2 h post-injection) and prolonged tumor retention (7.59 ± 2.04% ID/g at 24 h post-injection) in B16/F1 melanoma-bearing C57 mice.[17] RGD-Lys-(Arg[11])CCMSH showed remarkable clonogenic cytotoxic effect in B16/F1 melanoma cells. Since ^{99m}Tc and ^{188}Re are matched-pair diagnostic and therapeutic radionuclides sharing similar coordination chemistry, these results highlighted the potential of ^{188}Re-labeled RGD-conjugated α-MSH hybrid peptides as a novel class of hybrid peptides for melanoma therapy.

Despite the high melanoma uptake, ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH also exhibited high non-specific renal uptake in B16/F1 melanoma-bearing mice in our previous report.[17] Reduction of non-specific renal uptake will facilitate further evaluation of the α-MSH hybrid peptide for melanoma therapy in melanoma-bearing mice. Both structural modification and co-injection of L-lysine were effective in decreasing the renal uptake of ^{188}Re-labeled metal-cyclized α-MSH peptide.[15] For instance, the substitution of Lys with Arg at the 11th position of ^{188}Re-(Arg[11])CCMSH peptide dramatically reduced its renal uptake by 50% in B16/F1 melanoma-bearing mice.[15] Hence, we substituted the Lys linker in RGD-Lys-(Arg[11])CCMSH with an Arg linker to examine whether such replacement could exhibit the similar profound effect in reducing the renal uptake in this study. The RGD motif {cyclic(Arg-Gly-Asp-DTyr-Asp)} was coupled to [Cys[3,4,10], D-Phe[7], Arg[11]] α-MSH$_{3-13}$ {(Arg[11])CCMSH} through the Arg linker to generate a new RGD-Arg-(Arg[11])CCMSH hybrid peptide. We determined melanoma targeting and pharmacokinetic properties, single photon emission computed tomography (SPECT)/CT imaging of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH in B16/F1 melanoma-bearing mice. Meanwhile, we determined the effect of L-lysine co-injection on the renal uptakes of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH and ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH in B16/F1 melanoma-bearing mice. Furthermore, we determined clonogenic cytotoxic effect of RGD-Arg-(Arg[11])CCMSH in B16/F1 melanoma cells.

2. Materials and methods

2.1. Chemicals and reagents

Amino acids and resin were purchased from Advanced Chem-Tech Inc. (Louisville, KY) and Novabiochem (San Diego, CA). ^{99m}TcO$_4^-$ was purchased from Cardinal Health (Albuquerque, NM). ^{125}I-Tyr^2-[Nle^4, D-Phe^7]-α-MSH {^{125}I-(Tyr^2)-NDP-MSH} tracer was obtained from Perkin–Elmer, Inc. (Shelton, CT) for in vitro competitive binding studies. 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 Fischer Scientific (Waltham, MA) and used without further purification. B16/F1 murine melanoma cells were obtained from American Type Culture Collection (Manassas, VA).

2.2. Peptide synthesis

RGD-Arg-(Arg[11])CCMSH was synthesized according to our published procedure[17] with slight modification on Sieber amide resin by an Advanced ChemTech multiple-peptide synthesizer (Louisville, KY). Briefly, the linear peptide backbone was synthesized using standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry. Fmoc-Arg(pbf) was coupled between the RGD and (Arg[11])CCMSH motifs to generate the Arg linker during the synthesis of linear peptide backbone. The protected linear peptide backbone was cyclized and all protecting groups were removed using the reagents described in our published procedure.[17] Finally, the peptide was purified by reverse phase-high performance liquid chromatography (RP-HPLC) and characterized by LC–MS.

2.3. In vitro receptor binding assay

The receptor binding affinity (IC$_{50}$ value) of RGD-Arg-(Arg[11])CCMSH was determined according to our previously published procedure[17] with slight modification. Briefly, B16/F1 cells were incubated at room temperature (25 °C) for 2 h with approximately 40,000 counts per minute (cpm) of ^{125}I-(Tyr^2)-NDP-MSH in the presence of increasing concentrations (10^{-12}–10^{-5} M) of RGD-Arg-(Arg[11])CCMSH in 0.3 mL of binding medium. The IC$_{50}$ value of RGD-Arg-(Arg[11])CCMSH was calculated using Prism software (GraphPad Software, La Jolla, CA).

2.4. Biodistribution studies

RGD-Arg-(Arg[11])CCMSH was radiolabeled with ^{99m}Tc via a glucoheptonate transchelation reaction according to our previously published procedure[17] with slight modification. Firstly, the ^{99m}Tc^{7+} (as ^{99m}TcO$_4^-$ eluent) was easily reduced by SnCl$_2$ to ^{99m}Tc^{5+} (as ^{99m}TcO^{3+} metal center) at room temperature (25 °C). The ^{99m}TcO^{3+} metal center was stabilized by glucoheptonate to form the ^{99m}Tc-glucoheptonate intermediate at room temperature (25 °C). Then, RGD-Arg-(Arg[11])CCMSH was added to compete off glucoheptonate to yield ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH at 75 °C. The radiolabeled peptide was purified to a single species by Waters RP-HPLC (Milford, MA) on a Grace Vydac C-18 reverse phase analytic column (Deerfield, IL). The stability of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was determined by incubation in mouse serum at 37 °C for up to 24 h according to our published procedure.[18]

All of the animal studies were conducted in compliance with Institutional Animal Care and Use Committee approval. The pharmacokinetics of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was determined in B16/F1 melanoma-bearing C57 female mice (Harlan, Indianapolis, IN). B16/F1 melanoma-baring C57 mice were generated according to our published procedure.[17] Approximately 0.037 MBq of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was injected into each mouse via the tail vein. Groups of five 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. To determine the receptor specificity of the tumor uptake at 2 h post-injection, 10 μg (6.1 nmol) of unlabeled NDP-MSH or 3.5 μg (6.1 nmol) of RGD peptide was co-injected with 0.037 MBq of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH .

The effects of L-lysine co-injection on the renal uptakes of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH and ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH was examined in B16/F1 melanoma-bearing C57 as well. Two groups of five mice were injected with an aqueous mixture of 0.037 MBq of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH and 15 mg of L-lysine or an aqueous mixture of 0.037 MBq of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH and 15 mg of L-lysine, respectively. The mice were sacrificed at 2 h post-injection, and tumors and kidneys were harvested, weighed and counted.

2.5. Imaging melanoma with ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH

Approximately 6.1 MBq of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was injected into a B16/F1 melanoma-bearing C57 mouse via the tail vein for melanoma imaging. The mouse was anesthetized with 1.5% isoflurane for small animal SPECT/CT (Nano-SPECT/CT®, Bioscan) imaging 2 h post-injection. It took 9 min for a whole-body CT scan and 45 min for the SPECT scan. Reconstructed SPECT and CT data were co-registered using InVivoScope (Bioscan, Washington DC).

2.6. Clonogenic cytotoxicity of RGD-Arg-(Arg[11])CCMSH

To determine whether the replacement of the Lys linker with an Arg linker affected the clonogenic cytotoxicity of the hybrid

peptide, we examined the clonogenic cytotoxic effect of RGD-Arg-(Arg[11])CCMSH in B16/F1 melanoma cells according to our published procedure[17] with slight modification. Briefly, B16/F1 cells in a six-well plate (200 cells/well) were incubated in the culture medium at 37 °C for 3 h with or without 0.1 µM of RGD-Arg-(Arg[11])CCMSH, (Arg[11])CCMSH or RGD, respectively. After the incubation, the cells were washed with PBS twice and returned to the culture medium to form colonies. After six days, the cells were fixed with methanol/glacial acetic acid (3:1), stained with hematoxylin and examined under a microscope for survivors. Colonies contained greater than 50 cells were determined to be survivors. The clonogenic survival percentages of peptide-treated groups were normalized taking the clonogenic survival percentage of untreated group (in culture medium) as 100%.

2.7. Statistical method

Student t-tests for unpaired data were conducted to determine the significant differences between the groups in the studies of biodistribution and clonogenic cytotoxicity. Differences at the 95% confidence level ($p < 0.05$) were considered significant.

3. Results

RGD-Arg-(Arg[11])CCMSH was synthesized and purified by RP-HPLC. The identity of peptide was confirmed by electrospray ionization mass spectrometry (molecular weight, 2178.2; calculated molecular weight, 2177.9). RGD-Arg-(Arg[11])CCMSH displayed greater than 95% purity with 30% overall synthetic yield. Figure 1 illustrates the schematic structure of RGD-Arg-(Arg[11])CCMSH, as well as the schematic structure of RGD-Lys-(Arg[11])CCMSH for comparison. The competitive binding curve of RGD-Arg-(Arg[11])CCMSH is shown in Figure 2. The IC$_{50}$ value of RGD-Arg-(Arg[11])CCMSH was 0.7 nM in B16/F1 cells. The peptide was readily labeled with ^{99m}Tc in greater than 95% radiolabeling yield. ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was completely separated from its excess non-labeled peptide by RP-HPLC. The retention times of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH and its non-labeled RGD-Arg-(Arg[11])CCMSH were 13.2 and 12.4 min, respectively. ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was stable in mouse serum at 37 °C for 24 h.

The melanoma targeting and pharmacokinetic properties of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH are shown in Table 1. ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH exhibited rapid and high tumor uptake in melanoma-bearing mice. The tumor uptake value was 14.09 ± 2.42% ID/g at 0.5 h post-injection. ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH reached its peak tumor uptake value of 21.41 ± 3.74% ID/g at 2 h post-injection. There was 16.05 ± 2.00% ID/g of the ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH radioactivity remained in the tumors at 4 h post-injection. The tumor uptake value of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH gradually decreased to 6.81 ± 3.71% ID/g at 24 h post-injection. In melanoma uptake blocking studies, the tumor uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH with 10 µg (6.1 nmol) of non-radiolabeled NDP-MSH co-injection was only 9.15% of the tumor uptake without NDP-MSH co-injection at 2 h after dose administration ($p < 0.01$),
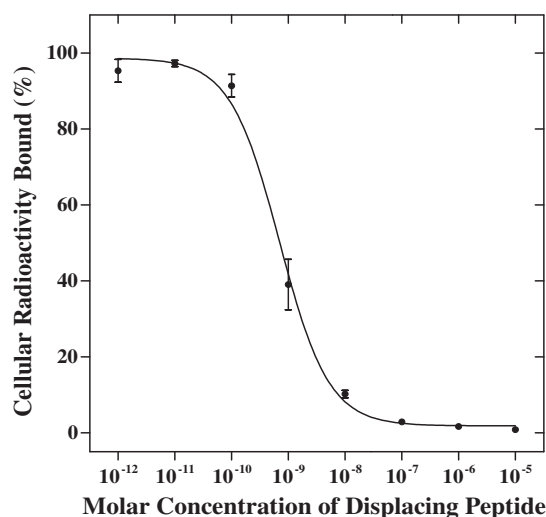
Figure 2. The competitive binding curve of RGD-Arg-(Arg[11])CCMSH in B16/F1 melanoma cells. The IC$_{50}$ value of RGD-Arg-(Arg[11])CCMSH was 0.7 nM.

demonstrating that the tumor uptake was specific and MC1 receptor-mediated. Compared to the tumor uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH, co-injection of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH with 3.5 µg (6.1 nmol) of RGD decreased 21.2% of the tumor uptake value. Whole-body clearance of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was rapid, with approximately 68% of the injected radioactivity cleared through the urinary system by 2 h post-injection (Table 1). Normal organ uptakes of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH were generally low (<3.6% ID/g) except for the kidneys after 2 h post-injection. High tumor/blood and tumor/muscle uptake ratios were demonstrated as early as 0.5 h post-injection (Table 1).

The renal uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH reached its peak value of 43.01 ± 8.14% ID/g at 2 h post-injection. The renal uptake decreased to 16.16 ± 4.01% ID/g at 24 h post-injection. The effects of L-lysine co-injection on the renal and tumor uptakes of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH or ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH at 2 h post-injection are presented in Figure 3. Co-injection of 15 mg of L-lysine significantly ($p < 0.05$) reduced the renal uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH from 43.01 ± 8.14% ID/g to 31.10 ± 6.42% ID/g without affecting the tumor uptake, as well as significantly ($p<0.05$) decreased the renal uptake of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH from 67.12 ± 8.79% ID/g to 32.20 ± 5.98% ID/g without affecting the tumor uptake at 2 h post-injection.

A whole-body SPECT/CT image of a B16/F1 melanoma-bearing mouse at 2 h post-injection is presented in Figure 4. Flank melanoma tumors were visualized clearly by SPECT using ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH as an imaging probe at 2 h post-injection. The SPECT image of tumor accurately matched its anatomical information from a corresponding CT image. ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH displayed high tumor to normal organ uptake ratios except for the kidneys, which was consistent with the biodistribution results. Figure 5 illustrates the clonogenic cytotoxic effect of
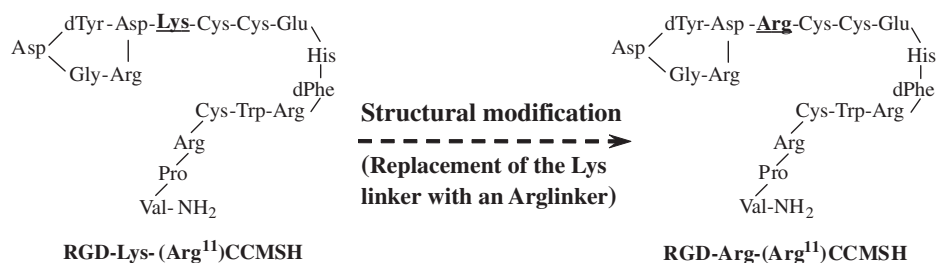
Figure 1. Schematic Structures of RGD-Arg-(Arg[11])CCMSH and RGD-Lys-(Arg[11])CCMSH.

Table 1
Biodistribution of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH in B16/F1 melanoma-bearing C57 mice

Tissue	0.5 h	2 h	4 h	24 h	2 h NDP Blockade	2 h RGD Blockade
Percent injected dose/gram (%ID/g)						
Tumor	14.09 ± 2.42	21.41 ± 3.74	16.05 ± 2.00	6.81 ± 3.71	1.96 ± 0.71*	16.88 ± 2.69*
Brain	0.16 ± 0.02	0.06 ± 0.02	0.04 ± 0.03	0.06 ± 0.02	0.06 ± 0.01	0.04 ± 0.01*
Blood	3.64 ± 0.69	0.55 ± 0.47	0.71 ± 0.41	0.24 ± 0.14	0.47 ± 0.29	0.04 ± 0.01*
Heart	2.36 ± 0.37	0.73 ± 0.27	0.53 ± 0.19	0.34 ± 0.07	0.61 ± 0.21	0.55 ± 0.22
Lung	7.55 ± 1.29	2.05 ± 0.52	1.01 ± 0.41	0.76 ± 0.14	1.42 ± 0.31*	1.21 ± 0.50*
Liver	4.21 ± 1.04	3.58 ± 1.08	4.93 ± 0.38	1.89 ± 0.36	2.87 ± 0.34	2.30 ± 0.22*
Skin	4.55 ± 1.01	1.29 ± 0.32	0.82 ± 0.22	0.75 ± 0.13	1.26 ± 0.14	0.95 ± 0.38
Spleen	2.73 ± 0.80	1.17 ± 0.57	1.75 ± 0.55	1.45 ± 0.37	1.53 ± 0.12	0.67 ± 0.38
Stomach	4.30 ± 1.78	2.75 ± 1.71	3.44 ± 0.83	0.47 ± 0.17	2.40 ± 0.49	2.03 ± 1.39
Kidneys	37.22 ± 4.72	43.01 ± 8.14	36.65 ± 11.68	16.16 ± 4.01	34.79 ± 8.46	38.48 ± 7.45
Muscle	1.79 ± 1.14	0.21 ± 0.12	0.32 ± 0.25	0.25 ± 0.05	0.50 ± 0.12*	0.28 ± 0.20
Pancreas	1.02 ± 0.36	0.36 ± 0.20	0.37 ± 0.12	0.19 ± 0.09	0.37 ± 0.22	0.32 ± 0.17
Bone	2.01 ± 0.19	0.57 ± 0.15	0.97 ± 0.22	0.90 ± 0.39	0.98 ± 0.39	0.52 ± 0.42
Percent injected dose (%ID)						
Intestines	3.19 ± 0.22	2.57 ± 0.58	2.62 ± 0.57	1.01 ± 0.11	2.34 ± 0.52	1.79 ± 0.58
Urine	43.10 ± 5.62	67.81 ± 6.28	75.13 ± 3.61	88.33 ± 1.64	76.85 ± 3.01	71.79 ± 5.07
Uptake ratio of tumor/normal tissue						
Tumor/blood	3.87	38.93	22.61	28.38	4.17	422.00
Tumor/kidneys	0.38	0.50	0.44	0.42	0.06	0.44
Tumor/lung	1.87	10.44	15.89	8.96	1.38	13.95
Tumor/liver	3.35	5.98	3.26	3.60	0.68	7.34
Tumor/muscle	7.87	101.95	50.16	27.24	3.92	60.29

The data was presented as percent injected dose/gram or as percent injected dose (mean ± SD, *n* = 5).
* *p* <0.05, significance comparison between ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH with or without peptide blockade at 2 h post-injection.
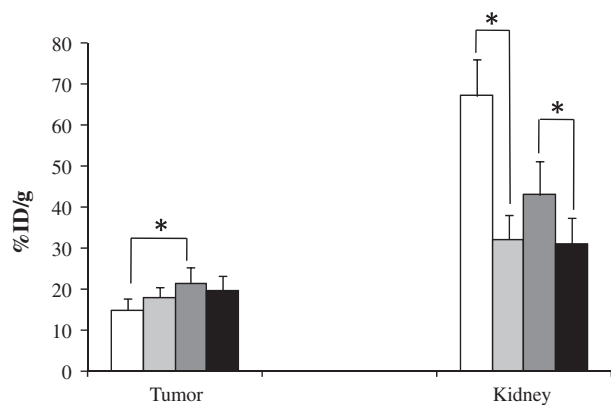

Figure 3. Effect of L-lysine co-injection on the tumor and kidney uptakes of ^{99m}Tc-RGD-Lys-(Arg^{11})CCMSH and ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH at 2 h post-injection. The white (□) and light grey (▨) columns represented the tumor and renal uptakes of ^{99m}Tc-RGD-Lys-(Arg^{11})CCMSH with or without L-lysine co-injection. The heavy grey (▨) and black (■) columns represented the tumor and renal uptakes of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH with or without L-lysine co-injection. L-lysine co-injection significantly (p <0.05) reduced the renal uptakes of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH by 27.7% and ^{99m}Tc-RGD-Lys-(Arg^{11})CCMSH by 52.1% at 2 h post-injection without affecting the tumor uptakes. Meanwhile, the tumor uptake value of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH was 1.44 times (* p <0.05) the tumor uptake value of ^{99m}Tc-RGD-Lys-(Arg^{11})CCMSH at 2 h post-injection.

Figure 4. Whole-body SPECT/CT image of ^{99m}Tc-RGD-Arg-(Arg^{11})CCMSH in a B16/F1 melanoma-bearing C57 mouse at 2 h post-injection. Tumor (T), kidneys (K) and bladder (BL) were highlighted with arrows on the image.

RGD-Arg-(Arg^{11})CCMSH in B16/F1 melanoma cells. RGD-Arg-(Arg^{11})CCMSH exhibited remarkable cytotoxic effect in B16/F1 melanoma cells, with 62% decrease (p <0.05) in clonogenic survival in comparison with the untreated group. Although incubation with (Arg^{11})CCMSH and RGD peptides reduced 11% and 7% of clonogenic survival compared with the untreated group, the differences were not significant (p >0.05).

4. Discussion

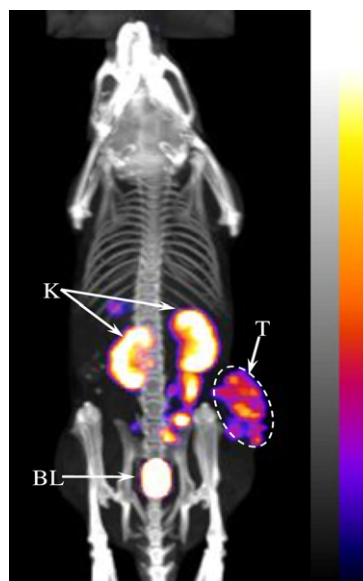Malignant melanoma is the most lethal form of skin cancer with an increasing incidence. It was predicted that 68,720 cases would be newly diagnosed and 8650 fatalities would occur in the year 2009.[19] Unfortunately, there is no curative treatment available for metastatic melanoma patients. Clearly, novel and effective treatments are urgently needed to fulfill the desperate need for melanoma treatment. The RGD-containing peptide could induce cell apoptosis after entering the cells without any requirement for integrin-mediated cell clustering or signals,[20] indicating the potential of the RGD motif as an intracellular apoptosis inducer. The RGD motif was coupled to either a somatostatin peptide or a bombesin peptide to generate the hybrid peptides.[21–26] RGD-Lys(111In-DTPA)-Tyr^3-Octreotate showed enhanced tumoricidal effects than ^{111}In-DTPA-Tyr^3-octreotate and ^{111}In-DTPA-RGD due to elevated tumor cell apoptosis,[21] underscoring the feasibility of using the
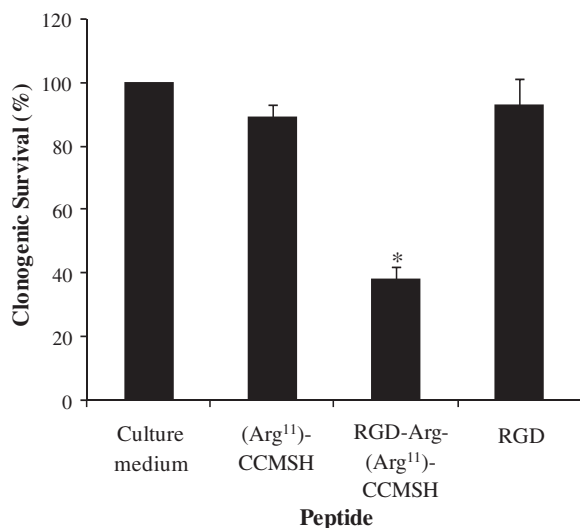
Figure 5. Clonogenic cytotoxic effect of RGD-Arg-(Arg[11])CCMSH in B16/F1 melanoma cells. The cells were visually examined under microscope for survival. Colonies contained more than 50 cells were scored as survivors. *$p <0.05$, significance comparison between RGD-Arg-(Arg[11])CCMSH treated cells and untreated cells (culture medium).

receptor-targeting peptides to transport the RGD motif (apoptosis inducer) into cancer cells to enhance the synergistic therapeutic effectiveness of the radiolabeled hybrid peptides. Recently, we have developed a novel RGD-Lys-(Arg[11])CCMSH hybrid peptide with remarkable clonogenic cytotoxicity in B16/F1 melanoma cells.[17] Meanwhile, [188]Re-(Arg[11])CCMSH treatment effectively prolonged the mean survival times of mice bearing B16/F1 and TXM13 melanomas.[9] These results highlighted the potential of [188]Re-RGD-Lys-(Arg[11])CCMSH hybrid peptide as a novel therapeutic peptide for melanoma treatment. However, the relative high non-specific renal uptake of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH (69.29 ± 14.34%ID/g at 4 h post-injection) needs to be reduced to facilitate further evaluation of [188]Re-labeled α-MSH hybrid peptides for melanoma treatment in melanoma mouse model.

A single amino acid change in the peptide sequence had a profound effect in reducing the non-specific renal uptake of the radiolabeled metal-cyclized α-MSH peptide.[15] The replacement of Lys with Arg at the 11th position of [188]Re-(Arg[11])CCMSH dramatically reduced its renal uptake by 50% ($p <0.05$) in B16/F1 melanoma-bearing C57 mice.[15] We demonstrated in our previous work[17] that the Lys linker between the RGD motif and the (Arg[11])CCMSH moiety played an important role in the high non-specific renal uptake of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH. Therefore, we replaced the Lys linker with an Arg linker to determine whether such single amino acid change could substantially decrease the renal uptake of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH in this study. The replacement of the Lys linker with an Arg linker resulted in greater MC1 receptor binding affinity of the hybrid peptide. New RGD-Arg-(Arg[11])CCMSH hybrid peptide displayed 0.7 nM MC1 receptor binding affinity in B16/F1 cells (Fig. 2), whereas RGD-Lys-(Arg[11])CCMSH exhibited 2.1 nM MC1 receptor binding affinity.[17] Favorable receptor binding results warranted the evaluation of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH in B16/F1 melanoma-bearing C57 mice.

The replacement of the Lys linker with an Arg linker dramatically enhanced the tumor uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH. The tumor uptake values of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH were 1.27, 1.44 and 1.28 times the tumor uptake values of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH at 0.5, 2 and 4 h post-injection, respectively. The improved melanoma uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was likely due to its stronger MC1 receptor binding affinity compared to ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH (0.7 nM vs 2.1 nM). Mean-

while, the tumor uptake values of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH were 1.44 and 2.05 times the tumor uptake values of [99m]Tc-(Arg[11])CCMSH at 4 and 24 h post-injection.[7] Moreover, ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH displayed prolonged tumor retention. The tumor uptake value at 4 h post-injection (16.05 ± 2.00% ID/g) was 75% of the tumor uptake value at 2 h post-injection (21.41 ± 3.74% ID/g). From the therapeutic perspective, high melanoma uptake and prolonged retention of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH could facilitate potential long-lasting synergistic therapeutic effects of apoptosis and targeted radiation from its [188]Re-labeled RGD-Arg-(Arg[11])CCMSH. Tumor uptake blocking studies with NDP-MSH or RGD in B16/F1 melanoma-bearing mice (Table 1) showed that 90.9% of the tumor uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was blocked by 6.1 nmol of NDP-MSH, whereas 21.2% of the tumor uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was blocked by 6.1 nmol of RGD, indicating that the majority of melanoma uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was MC1 receptor-mediated.

Importantly, the replacement of the Lys linker with an Arg linker significantly ($p <0.05$) reduced the non-specific renal uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH as well. The renal uptake values of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH were 53.65%, 64.08%, 52.89% and 40.14% of the renal uptake values of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH at 0.5, 2, 4 and 24 h post-injection, respectively. Considering the structural difference between ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH and ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH, the reduced non-specific renal uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH was likely associated with the side chain of the Arg linker. The decreased renal uptake and improved tumor uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH increased the tumor/kidney uptake ratios of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH. The tumor/kidney uptake ratios of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH were 2.38, 2.27, 2.44 and 2.21 times the tumor/kidney uptake ratios of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH at 0.5, 2, 4 and 24 h post-injection, respectively.

The strategy of infusing basic amino acids such as L-lysine has been successfully employed to decrease the renal uptakes of radiolabeled metal-cyclized α-MSH peptides by shielding the electrostatic interaction between positively-charged peptides and negatively-charged surface of tubule cells.[15,27,28] Hence, 15 mg of L-lysine was co-injected with either ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH or ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH at 2 h post-injection to determine whether the non-specific renal uptakes of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH and ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH were associated with the electrostatic interaction between the peptide and kidney cells. As we anticipated, co-injection of L-lysine reduced the renal uptake of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH by 52.1% ($p <0.05$) and decreased the renal uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH by 27.7% ($p <0.05$) without affecting the tumor uptakes, demonstrating that the electrostatic interaction played an important role in the non-specific renal uptakes of ^{99m}Tc-RGD-Lys-(Arg[11])CCMSH and ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH. The effect of L-lysine co-injection in reducing the renal uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH demonstrated it as another effective approach to further decrease the renal uptake if needed.

The replacement of the Lys linker with an Arg linker maintained the clonogenic cytotoxicity of RGD-Arg-(Arg[11])CCMSH hybrid peptide. RGD-Arg-(Arg[11])CCMSH exhibited similar promising cytotoxic effects compared to RGD-Lys-(Arg[11])CCMSH in B16/F1 cells.[17] Three-hour incubation with 0.1 μM of RGD-Arg-(Arg[11])CCMSH and RGD-Lys-(Arg[11])CCMSH decreased 62% and 65% of the clonogenic survival of B16/F1 cells compared to untreated control cells six days post the treatment. The cytotoxic effect of RGD-Arg-(Arg[11])CCMSH hybrid peptide was due to the apoptotic effect of the RGD motif coupled to the hybrid peptide because neither treatment with 0.1 μM of (Arg[11])CCMSH nor 0.1 μM of RGD peptide significantly ($p >0.05$) reduced the clonogenic survival of B16/F1 cells.

The remarkable clonogenic cytotoxic effect of RGD-Arg-(Arg[11])CCMSH warrants further evaluation of [188]Re-labeled RGD-Arg-(Arg[11])CCMSH for melanoma treatment.

Flank B16/F1 melanoma tumors were clearly visualized by SPECT/CT imaging using ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH as an imaging probe at 2 h post-injection (Fig. 4), demonstrating the feasibility of using ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH SPECT imaging to identify the MC1 receptor expression on human melanoma. The combination of using the matched-pair ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH and [188]Re-RGD-Arg-(Arg[11])CCMSH could potentially enhance the success of [188]Re-RGD-Arg-(Arg[11])CCMSH treatment. Imaging patients with ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH prior to the therapy would not only help the physicians to choose the right patients for effective treatments, but also allow the physicians to determine patient-specific dosimetries. Accurate patient-specific dosimetries would guide the physicians to determine the safe and efficacious doses for the patients. Furthermore, follow-up imaging with ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH during the therapy duration could monitor the response to the treatment, as well as provide the physicians critical information to modify the therapy regimens accordingly.

5. Conclusions

The replacement of the Lys linker with an Arg linker exhibited a profound effect in reducing the non-specific renal uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH, as well as increasing the tumor uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH. Co-injection of L-lysine was effective in decreasing the renal uptakes of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH and ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH. Compared to ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH, improved melanoma uptake and reduced renal uptake of ^{99m}Tc-RGD-Arg-(Arg[11])CCMSH warrants the further evaluation of [188]Re-labeled RGD-Arg-(Arg[11])CCMSH as a novel MC1 receptor-targeting therapeutic peptide for melanoma treatment in the future.

Acknowledgments

References and notes

1. Marghood, A. A.; Slade, J.; Salopek, T. G.; Kopf, A. W.; Bart, R. S.; Rigel, D. S. Cancer **1995**, 75, 707.
2. Balch, C. M.; Soong, S. J.; Gershenwald, J. E.; Thompson, J. F.; Reintgen, D. S.; Cascinelli, N.; Urist, M.; McMasters, K. M.; Ross, M. I.; Kirkwood, J. M.; Atkins, M. B.; Thompson, J. A.; Coit, D. G.; Byrd, D.; Desmond, R.; Zhang, Y.; Liu, P. Y.; Lyman, G. H.; Morabito, A. J. Clin. Oncol. **2001**, 19, 3622.
3. Anderson, C. M.; Buzaid, A. C.; Legha, S. S. Oncology **1995**, 9, 1149.
4. Giblin, M. F.; Wang, N. N.; Hoffman, T. J.; Jurisson, S. S.; Quinn, T. P. Proc. Natl. Acad. Sci. U.S.A. **1998**, 95, 12814.
5. Froidevaux, S.; Calame-Christe, M.; Tanner, H.; Sumanovski, L.; Eberle, A. N. J. Nucl. Med. **2002**, 43, 1699.
6. Froidevaux, S.; Calame-Christe, M.; Schuhmacher, J.; Tanner, H.; Saffrich, R.; Henze, M.; Eberle, A. N. J. Nucl. Med. **2004**, 45, 116.
7. Miao, Y.; Benwell, K.; Quinn, T. P. J. Nucl. Med. **2007**, 48, 73.
8. Miao, Y.; Figueroa, S. D.; Fisher, D. R.; Moore, H. A.; Testa, R. F.; Hoffman, T. J.; Quinn, T. P. J. Nucl. Med. **2008**, 49, 823.
9. Miao, Y.; Owen, N. K.; Fisher, D. R.; Hoffman, T. J.; Quinn, T. P. J. Nucl. Med. **2005**, 46, 121.
10. Miao, Y.; Hylarides, M.; Fisher, D. R.; Shelton, T.; Moore, H.; Wester, D. W.; Fritzberg, A. R.; Winkelmann, C. T.; Hoffman, T. J.; Quinn, T. P. Clin. Cancer Res. **2005**, 11, 5616.
11. Miao, Y.; Shelton, T.; Quinn, T. P. Cancer Biother. Radiopharm. **2007**, 22, 333.
12. Tatro, J. B.; Reichlin, S. Endocrinology **1987**, 121, 1900.
13. Siegrist, W.; Solca, F.; Stutz, S.; Giuffre, L.; Carrel, S.; Girard, J.; Eberle, A. N. Cancer Res. **1989**, 49, 6352.
14. Chen, J.; Cheng, Z.; Hoffman, T. J.; Jurisson, S. S.; Jurisson, S. S.; Quinn, T. P. Cancer Res. **2000**, 60, 5649.
15. Miao, Y.; Owen, N. K.; Whitener, D.; Gallazzi, F.; Hoffman, T. J.; Quinn, T. P. Int. J. Cancer **2002**, 101, 480.
16. Miao, Y.; Whitener, D.; Feng, W.; Owen, N. K.; Chen, J.; Quinn, T. P. Bioconjug. Chem. **2003**, 14, 1177.
17. Yang, J.; Guo, H.; Gallazzi, F.; Padilla, R. S.; Berwick, M.; Miao, Y. Bioconjug. Chem. **2009**, 20, 1634.
18. Aloj, L.; Panico, M.; Caraco, C.; Del Vecchio, S.; Arra, C.; Affuso, A.; Accardo, A.; Mansi, R.; Tesauro, D.; De Luca, S.; Pedone, C.; Visentin, R.; Mazzi, U.; Morelli, G.; Salvatore, M. Cancer Biother. Radiopharm. **2004**, 19, 93.
19. Jemal, A.; Siegel, R.; Ward, E.; Hao, Y.; Xu, J.; Thun, M. J. C. A. Cancer J. Clin. **2009**, 59, 225.
20. Buckley, C. D.; Pilling, D.; Henriquez, N. V.; Parsonage, G.; Threlfall, K.; Scheel-Toellner, D.; Simmons, D. L.; Akbar, A. N.; Lord, J. M.; Salmon, M. Nature **1999**, 397, 534.
21. Capello, A.; Krenning, E. P.; Bernard, B. F.; Breeman, W. A.; van Hagen, M. P.; de Jong, M. J. Nucl. Med. **2004**, 45, 1716.
22. Bernard, B.; Capello, A.; van Hagen, M.; Breeman, W.; Srinivasan, A.; Schmidt, M.; Erion, J.; van Gameren, A.; Krenning, E.; de Jong, M. Cancer Biother. Radiopharm. **2004**, 19, 173.
23. Hofland, L. J.; Capello, A.; Krenning, E. P.; de Jong, M.; van Hagen, M. P. J. Nucl. Med. **2005**, 46, 191S.
24. Capello, A.; Krenning, E. P.; Bernard, B. F.; Breeman, W. A.; Erion, J. L.; de Jong, M. J. Nucl. Med. **2006**, 47, 122.
25. Li, Z. B.; Wu, Z.; Chen, K.; Ryu, E. K.; Chen, X. J. Nucl. Med. **2008**, 49, 453.
26. Liu, Z.; Yan, Y.; Liu, S.; Wang, F.; Chen, X. Bioconjug. Chem. **2009**, 20, 1016.
27. Wei, L.; Butcher, C.; Miao, Y.; Gallazzi, F.; Quinn, T. P.; Welch, M. J.; Lewis, J. S. J. Nucl. Med. **2007**, 48, 64.
28. Miao, Y.; Fisher, D. R.; Quinn, T. P. Nucl. Med. Biol. **2006**, 33, 723.