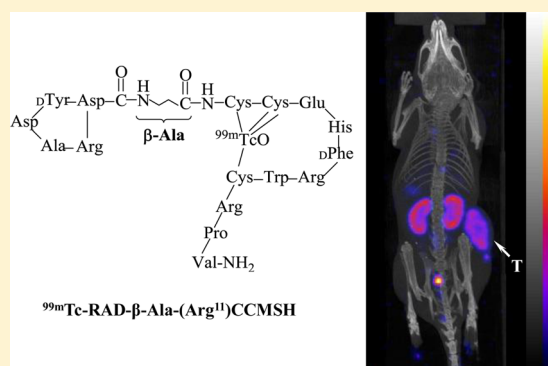


# Substitution of the Lys Linker with the $\beta$ -Ala Linker Dramatically Decreased the Renal Uptake of $^{99m}\text{Tc}$ -Labeled Arg-X-Asp-Conjugated and X-Ala-Asp-Conjugated $\alpha$ -Melanocyte Stimulating Hormone Peptides

Adam M. Flook,<sup>†</sup> Jianquan Yang,<sup>†</sup> and Yubin Miao<sup>\*,†,‡,§</sup>

<sup>†</sup>College of Pharmacy, <sup>‡</sup>Cancer Research and Treatment Center, and <sup>§</sup>Department of Dermatology, University of New Mexico, Albuquerque, New Mexico 87131, United States

**ABSTRACT:** The purpose of this study was to examine whether the substitution of the Lys linker with the  $\beta$ -Ala could reduce the renal uptake of  $^{99m}\text{Tc}$ -labeled Arg-X-Asp-conjugated and X-Ala-Asp-conjugated  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) peptides. RSD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH (1) {c[Arg-Ser-Asp-D-Tyr-Asp]- $\beta$ -Ala-Cys-Cys-Glu-His-D-Phe-Arg-Trp-Cys-Arg-Pro-Val-NH<sub>2</sub>}, RTD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH (2), RVD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH (3), RAD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH (4), NAD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH (5), and EAD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH (6) peptides were synthesized and evaluated for their melanocortin 1 (MC1) receptor binding affinities in B16/F1 melanoma cells. The biodistribution of their  $^{99m}\text{Tc}$ -conjugates were determined in B16/F1 melanoma-bearing C57 mice. The substitution of the Lys linker with  $\beta$ -Ala linker dramatically reduced the renal uptake of all six  $^{99m}\text{Tc}$ -peptides.  $^{99m}\text{Tc}$ -4 exhibited the highest melanoma uptake ( $15.66 \pm 6.19\%$  ID/g) and the lowest kidney uptake ( $20.18 \pm 3.86\%$  ID/g) among these  $^{99m}\text{Tc}$ -peptides at 2 h postinjection. The B16/F1 melanoma lesions could be clearly visualized by single photon emission computed tomography (SPECT)/CT using  $^{99m}\text{Tc}$ -4 as an imaging probe.



## INTRODUCTION

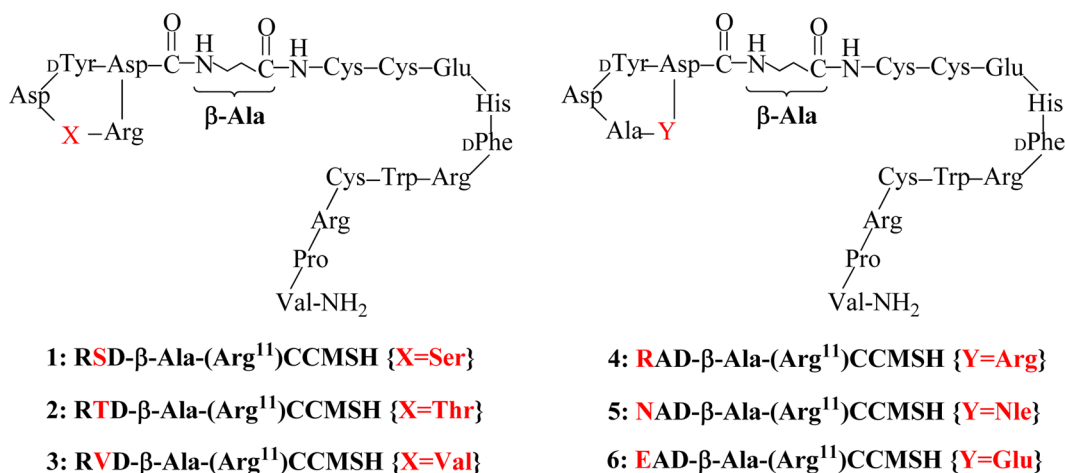
Melanocortin 1 (MC1) receptor is an attractive molecular target for melanoma imaging because of its overexpression on both murine and human melanoma cells.<sup>1–16</sup> Recently, we have identified a class of  $^{99m}\text{Tc}$ -labeled  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) peptides to target MC1 receptors for melanoma imaging. Specifically, the cyclic RXD motifs {Arg-X-Asp-D-Tyr-Asp, X = Gly, Ala, Val, Thr, Ser, Nle, Phe, and D-Phe} were attached to [Cys<sup>3,4,10</sup>, D-Phe<sup>7</sup>, Arg<sup>11</sup>] $\alpha$ -MSH<sub>3–13</sub> via a lysine linker to yield RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides. Interestingly, single amino acid at the X position yielded a profound impact on the melanoma targeting and clearance properties of  $^{99m}\text{Tc}$ -RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides. For instance, the substitution of Gly in  $^{99m}\text{Tc}$ -RGD-Lys-(Arg<sup>11</sup>)CCMSH with Ala, Thr, Val, and Ser improved the MC1 receptor binding affinities and enhanced the melanoma uptake in B16/F1 melanoma-bearing C57 mice.<sup>17–19</sup> On the other hand, the substitution of Gly in  $^{99m}\text{Tc}$ -RGD-Lys-(Arg<sup>11</sup>)CCMSH with Nle decreased the MC1 receptor binding affinity. Although the substitution of Gly in  $^{99m}\text{Tc}$ -RGD-Lys-(Arg<sup>11</sup>)CCMSH with Phe and D-Phe increased the MC1 receptor binding affinities, both  $^{99m}\text{Tc}$ -RFD-Lys-(Arg<sup>11</sup>)CCMSH and  $^{99m}\text{Tc}$ -RfD-Lys-(Arg<sup>11</sup>)CCMSH exhibited much higher liver uptake as compared to  $^{99m}\text{Tc}$ -RGD-Lys-(Arg<sup>11</sup>)CCMSH.<sup>18</sup>

Despite the promising melanoma targeting results, extremely high renal uptake (67–135% ID/g at 2 h postinjection) is a

common issue associated with  $^{99m}\text{Tc}$ -RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides.<sup>17–19</sup> Thus, it is desirable to reduce the nonspecific renal uptake of  $^{99m}\text{Tc}$ -RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides to facilitate their potential therapeutic applications. In our previous reports, L-lysine co-injection significantly reduced the renal uptake of  $^{99m}\text{Tc}$ -RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides by 37%–51% at 2 h postinjection without affecting their tumor uptake.<sup>17–19</sup> L-Lysine is a positively charged amino acid. The effect of L-lysine co-injection in reducing the renal uptake indicated that the overall positive charges of  $^{99m}\text{Tc}$ -RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides contributed to their nonspecific renal uptake. Obviously, the substitution of the positively charged Lys linker with a neutral amino acid can decrease the overall charges of  $^{99m}\text{Tc}$ -RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides. According to the effect of L-lysine co-injection in decreasing the renal uptake, we hypothesized that the substitution of the Lys linker with a neutral  $\beta$ -Ala linker would decrease the renal uptake of  $^{99m}\text{Tc}$ -RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides. To examine our hypothesis, we synthesized six peptides with  $\beta$ -Ala linkers, namely, peptides 1–6. The MC1 receptor binding affinities of these six peptides were examined in B16/F1 melanoma cells. The peptides were readily radiolabeled with  $^{99m}\text{Tc}$  using SnCl<sub>2</sub> as a reducing agent. We further determined

Received: July 22, 2014

Published: October 7, 2014



**Figure 1.** Schematic structures of RXD-β-Ala-(Arg<sup>11</sup>)CCMSH and XAD-β-Ala-(Arg<sup>11</sup>)CCMSH peptides.

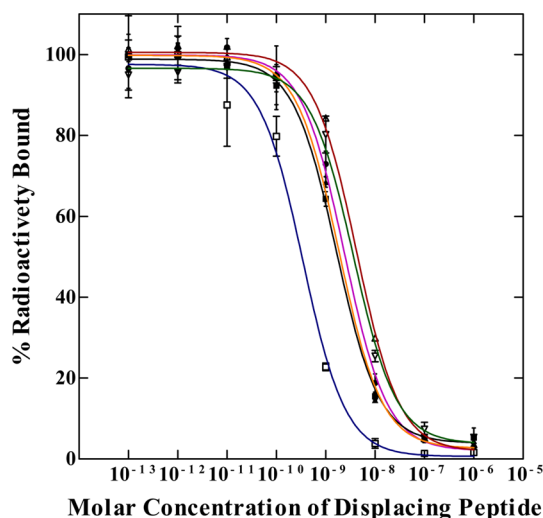
the biodistribution properties in B16/F1 melanoma-bearing C57 mice for these six <sup>99m</sup>Tc-peptides. Thereafter, we determined the imaging property of <sup>99m</sup>Tc-4 in B16/F1 melanoma-bearing C57 mice.

## RESULTS

The schematic structures of six new peptides are presented in Figure 1. The peptides were synthesized and purified by reverse phase-high performance liquid chromatography (RP-HPLC) according to our previously published procedures.<sup>17,18</sup> The overall synthetic yields were 25–30% for all six peptides. The chemical purities of the peptides were greater than 95% after the HPLC purification (Table 1). The peptide identities were confirmed by electrospray mass spectrometry. The measured molecular weight was 2123 Da for peptide 1, 2137 Da for peptide 2, 2135 Da for peptide 3, 2107 Da for peptide 4, 2064 Da for peptide 5, and 2080 Da for peptide 6 (Table 1). The competitive binding curves of the peptides are shown in Figure 2. The IC<sub>50</sub> value was 2.76 ± 0.51 nM for peptide 1, 1.56 ± 0.63 nM for peptide 2, 1.99 ± 0.16 nM for peptide 3, 0.35 ± 0.01 nM for peptide 4, 3.34 ± 0.28 nM for peptide 5, and 3.84 ± 0.71 nM for peptide 6 in B16/F1 melanoma cells, respectively.

All six peptides were readily radiolabeled with <sup>99m</sup>Tc with greater than 95% radiolabeling yields. The <sup>99m</sup>Tc-peptides were separated from their excess nonlabeled peptides by RP-HPLC. The specific activities of <sup>99m</sup>Tc-1, <sup>99m</sup>Tc-2, <sup>99m</sup>Tc-3, <sup>99m</sup>Tc-4, <sup>99m</sup>Tc-5, and <sup>99m</sup>Tc-6 were 8.62 × 10<sup>9</sup>, 8.57 × 10<sup>9</sup>, 8.57 × 10<sup>9</sup>, 8.68 × 10<sup>9</sup>, 8.85 × 10<sup>9</sup>, 8.79 × 10<sup>9</sup> MBq/g, respectively. The retention times of <sup>99m</sup>Tc-1, <sup>99m</sup>Tc-2, <sup>99m</sup>Tc-3, <sup>99m</sup>Tc-4, <sup>99m</sup>Tc-5, and <sup>99m</sup>Tc-6 were 13.1, 13.0, 14.1, 13.7, 17.0, and 14.2 min, respectively. All six <sup>99m</sup>Tc-peptides were stable in mouse serum at 37 °C for 24 h (Figure 3).

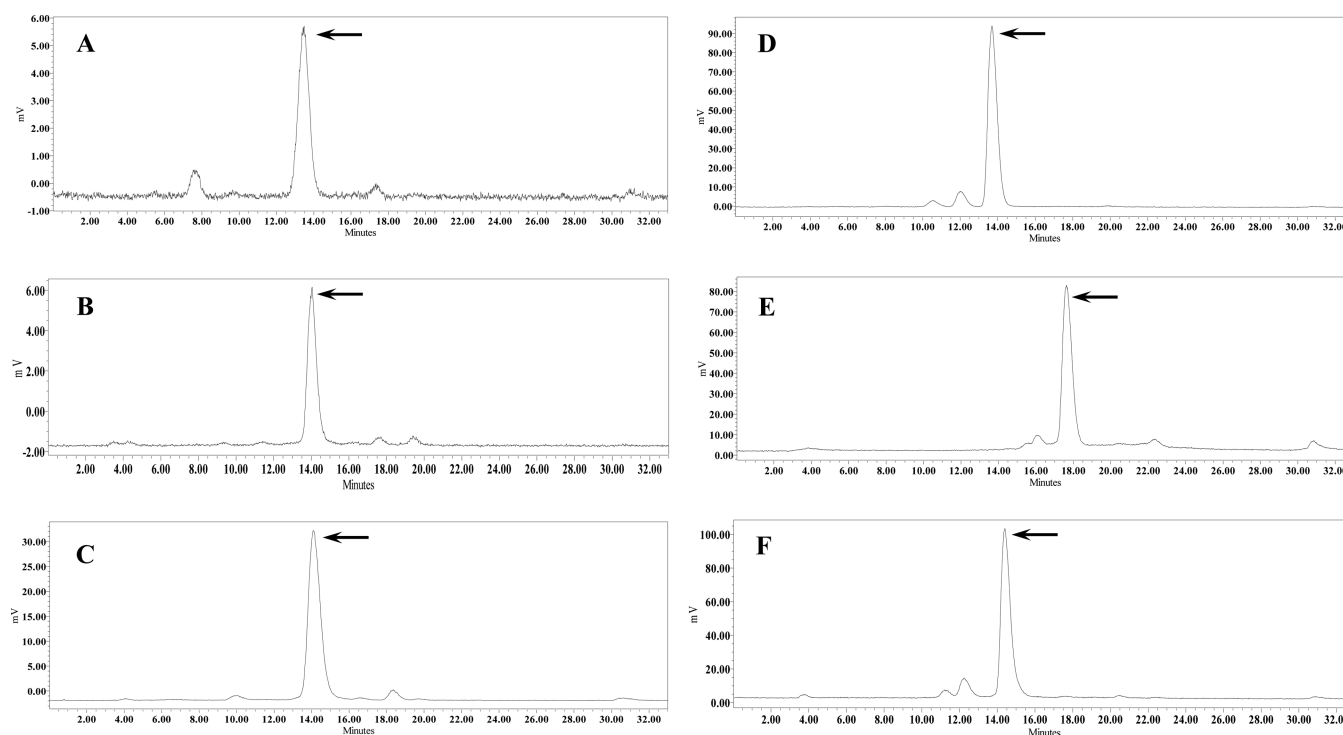
The melanoma targeting and pharmacokinetic properties of <sup>99m</sup>Tc-1, <sup>99m</sup>Tc-2, <sup>99m</sup>Tc-3, <sup>99m</sup>Tc-4, <sup>99m</sup>Tc-5, and <sup>99m</sup>Tc-6 are shown in Tables 2–7. All six <sup>99m</sup>Tc-peptides exhibited similar tumor uptake pattern in B16/F1 melanoma-bearing C57 mice. The highest tumor uptake appeared either at 2 or 4 h postinjection. Among these six <sup>99m</sup>Tc-peptides, <sup>99m</sup>Tc-4 showed the highest tumor uptake of 15.66 ± 6.19% ID/g at 2 h postinjection. The tumor uptake of <sup>99m</sup>Tc-4 gradually decreased to 14.67 ± 3.81 and 7.79 ± 2.68% ID/g at 4 and 24 h postinjection. Co-injection of 10 μg (6.1 nM) of nonradiolabeled NDP-MSH with <sup>99m</sup>Tc-4 decreased the tumor uptake to



**Figure 2.** Competitive binding curves of peptide 1 (●, pink), peptide 2 (■, black), peptide 3 (▲, orange), peptide 4 (□, blue), peptide 5 (▽, green), and peptide 6 (△, red) in B16/F1 murine melanoma cells. The IC<sub>50</sub> values were 2.76 ± 0.51 nM for peptide 1, 1.56 ± 0.63 nM for peptide 2, 1.99 ± 0.16 nM for peptide 3, 0.35 ± 0.01 nM for peptide 4, 3.34 ± 0.28 nM for peptide 5, and 3.84 ± 0.71 nM for peptide 6.

2.43 ± 0.53% ID/g at 2 h postinjection, demonstrating that the tumor uptake was MC1 receptor-mediated.

Kidneys were the excretion routes for all six <sup>99m</sup>Tc-peptides. Among these six <sup>99m</sup>Tc-peptides, <sup>99m</sup>Tc-4 showed the lowest renal uptake of 20.18 ± 3.86% ID/g at 2 h postinjection. The renal uptake of <sup>99m</sup>Tc-4 gradually decreased to 19.83 ± 6.34 and 3.92 ± 0.99% ID/g at 4 and 24 h postinjection. Co-injection of 10 μg (6.1 nM) of nonradiolabeled NDP-MSH with <sup>99m</sup>Tc-4 did not significantly reduce the renal uptake (*p* > 0.05) at 2 h postinjection, indicating that the renal uptake was nonspecific. The substitution of the positively charged Lys linker with the neutral β-Ala dramatically decreased the renal uptake of <sup>99m</sup>Tc-RXD-β-Ala-(Arg<sup>11</sup>)CCMSH peptides. Interestingly, further reduction of the overall positive charges of <sup>99m</sup>Tc-5 and <sup>99m</sup>Tc-6 did not decrease the renal uptake further as compared to <sup>99m</sup>Tc-4. All six <sup>99m</sup>Tc-peptides displayed fast urinary clearance. Approximately 68–78% of <sup>99m</sup>Tc-peptides cleared through the urinary system by 2 h postinjection, whereas approximately 77–86% of <sup>99m</sup>Tc-peptides washed out through the urinary system by 4 h postinjection. The effect of L-lysine



**Figure 3.** Radioactive HPLC profiles of  $^{99\text{m}}\text{Tc}$ -1 (A),  $^{99\text{m}}\text{Tc}$ -2 (B),  $^{99\text{m}}\text{Tc}$ -3 (C),  $^{99\text{m}}\text{Tc}$ -4 (D),  $^{99\text{m}}\text{Tc}$ -5 (E), and  $^{99\text{m}}\text{Tc}$ -6 (F) in mouse serum after incubation at 37 °C for 24 h. The arrows denote the original retention times of  $^{99\text{m}}\text{Tc}$ -1 (13.1 min),  $^{99\text{m}}\text{Tc}$ -2 (13.0 min),  $^{99\text{m}}\text{Tc}$ -3 (14.1 min),  $^{99\text{m}}\text{Tc}$ -4 (13.7 min),  $^{99\text{m}}\text{Tc}$ -5 (17.0 min), and  $^{99\text{m}}\text{Tc}$ -6 (14.2 min), prior to the incubation in mouse serum.

**Table 1.** Capacity Factors, Chemical/Radiochemical Purities, and Measured Molecular Weights of the RXD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH and XAD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH Peptides and Their  $^{99\text{m}}\text{Tc}$ -Conjugates<sup>a</sup>

peptide and $^{99\text{m}}\text{Tc}$ -peptide	capacity factor ( <i>k'</i> )	chemical/radiochemical purity (%)	measured molecular weight (Da)
1	1.35	95	2123
2	0.98	95	2137
3	1.94	96	2135
4	3.32	97	2107
5	3.17	96	2064
6	2.28	96	2080
$^{99\text{m}}\text{Tc}$ -1	3.34	99	ND
$^{99\text{m}}\text{Tc}$ -2	3.29	98	ND
$^{99\text{m}}\text{Tc}$ -3	3.40	99	ND
$^{99\text{m}}\text{Tc}$ -4	4.18	98	ND
$^{99\text{m}}\text{Tc}$ -5	4.63	98	ND
$^{99\text{m}}\text{Tc}$ -6	3.47	98	ND

<sup>a</sup>ND = not determined.

co-injection on the renal uptake of  $^{99\text{m}}\text{Tc}$ -4 at 2 h postinjection is presented in Figure 4. Co-injection of 15 mg of L-lysine significantly ( $p < 0.05$ ) reduced the renal uptake of  $^{99\text{m}}\text{Tc}$ -4 from  $20.18 \pm 3.86\%$  ID/g to  $13.06 \pm 3.62\%$  ID/g without significantly affecting the tumor uptake at 2 h postinjection.

Because  $^{99\text{m}}\text{Tc}$ -4 showed the highest tumor uptake and the lowest renal uptake than the other five  $^{99\text{m}}\text{Tc}$ -peptides at 2 h postinjection, we further determined the tumor imaging property, specificity of tumor uptake, and urinary metabolites of  $^{99\text{m}}\text{Tc}$ -4 in B16/F1 melanoma-bearing C57 mice. Whole-body SPECT/CT image at 2 h postinjection is presented in Figure 5. Flank B16/F1 melanoma lesions were clearly visualized by SPECT

using  $^{99\text{m}}\text{Tc}$ -4 as an imaging probe. The SPECT image of tumor accurately matched its anatomical location obtained in the CT image. The SPECT image showed high contrast of tumor to normal organ except for kidneys, which was consistent with the biodistribution results. As shown in Figure 5, the tumor uptake was blocked by unlabeled NDP-MSH, demonstrating that the tumor uptake was receptor-mediated. The urinary metabolites of  $^{99\text{m}}\text{Tc}$ -4 at 2 h postinjection are shown in Figure 6.  $^{99\text{m}}\text{Tc}$ -4 remained intact in the urine at 2 h postinjection.

## DISCUSSION

In our previous reports,<sup>17–19</sup> we have found the importance of single amino acid at the X position in the tumor targeting properties of  $^{99\text{m}}\text{Tc}$ -RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides in B16/F1 melanoma-bearing C57 mice. Specifically, the substitution of Gly in  $^{99\text{m}}\text{Tc}$ -RGD-Lys-(Arg<sup>11</sup>)CCMSH with Ala, Thr, Val, and Ser improved the MC1 receptor binding affinities and enhanced the melanoma uptake in B16/F1 melanoma-bearing C57 mice.<sup>17–19</sup> Despite the promising melanoma targeting results associated with  $^{99\text{m}}\text{Tc}$ -RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides, it is desirable to reduce the nonspecific renal uptake (67–135% ID/g at 2 h postinjection) of  $^{99\text{m}}\text{Tc}$ -RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides to facilitate their potential therapeutic applications. In this study, we substituted the positively charged Lys linker with the neutral  $\beta$ -Ala linker to determine whether such linker change could reduce the renal uptake of  $^{99\text{m}}\text{Tc}$ -RXD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH peptides. Furthermore, we replaced the RAD moiety with NAD and EAD moieties to examine whether the further reduction of the overall positive charges of  $^{99\text{m}}\text{Tc}$ -NAD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH and  $^{99\text{m}}\text{Tc}$ -EAD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH could decrease their renal uptake further.

The substitution of Lys linker with  $\beta$ -Ala linker slightly affected the receptor binding affinities of RXD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH peptides. The receptor binding affinities of RXD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH

Table 2. Biodistribution of  $^{99m}\text{Tc}$ -1 in B16/F1 Melanoma-Bearing C57 Mice<sup>a</sup>

tissue	0.5 h	2 h	4 h	24 h	2 h NDP
Percentage Injected Dose/Gram (% ID/g)					
tumor	9.64 ± 1.52	9.62 ± 1.53	12.15 ± 2.36	3.62 ± 1.10	1.12 ± 0.01 <sup>*b</sup>
brain	0.15 ± 0.01	0.02 ± 0.01	0.02 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
blood	2.66 ± 0.84	0.50 ± 0.14	0.06 ± 0.02	0.61 ± 0.46	0.03 ± 0.01
heart	1.56 ± 0.13	0.23 ± 0.04	0.10 ± 0.06	0.07 ± 0.03	0.17 ± 0.01
lung	3.85 ± 0.40	0.64 ± 0.15	0.34 ± 0.11	0.29 ± 0.12	0.48 ± 0.06
liver	1.41 ± 0.13	0.74 ± 0.08	0.86 ± 0.08	0.31 ± 0.08	0.57 ± 0.05
skin	3.07 ± 1.85	0.52 ± 0.40	0.32 ± 0.13	0.48 ± 0.24	0.29 ± 0.18
spleen	1.34 ± 0.15	0.32 ± 0.05	0.35 ± 0.05	0.18 ± 0.06	0.22 ± 0.06
stomach	1.83 ± 0.20	0.62 ± 0.17	0.77 ± 0.21	0.32 ± 0.16	0.62 ± 0.23
kidneys	30.5 ± 5.18	28.73 ± 3.40	28.70 ± 5.22	8.12 ± 2.34	13.67 ± 2.54 <sup>*b</sup>
muscle	0.25 ± 0.02	0.04 ± 0.03	0.02 ± 0.01	0.05 ± 0.02	0.05 ± 0.07
pancreas	0.68 ± 0.28	0.11 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	0.08 ± 0.01
bone	0.93 ± 0.45	0.19 ± 0.06	0.05 ± 0.01	0.42 ± 0.28	0.16 ± 0.09
Percentage Injected Dose (% ID)					
intestines	1.71 ± 0.21	1.11 ± 0.60	1.59 ± 0.41	0.63 ± 0.19	0.76 ± 0.23
urine	53.37 ± 4.23	68.46 ± 9.28	83.96 ± 0.88	84.33 ± 3.37	90.95 ± 0.59
Uptake Ratio of Tumor/Normal Tissue					
tumor/blood	3.62	19.24	202.50	5.93	37.33
tumor/kidneys	0.32	0.33	0.42	0.45	0.08
tumor/lung	2.50	15.03	35.74	12.48	2.33
tumor/liver	6.84	13.00	14.13	11.68	1.96
tumor/muscle	38.56	240.50	607.50	72.40	22.40

<sup>a</sup>The data are presented as percent injected dose/gram or as percent injected dose (mean ± SD,  $n = 4$ ). <sup>b</sup>(\*)  $p < 0.05$  for determining the significance of differences in tumor and kidney uptake between  $^{99m}\text{Tc}$ -1 with or without NDP-MSH peptide blockade at 2 h postinjection.

Table 3. Biodistribution of  $^{99m}\text{Tc}$ -2 in B16/F1 Melanoma-Bearing C57 Mice<sup>a</sup>

tissue	0.5 h	2 h	4 h	24 h	2 h NDP
Percentage Injected Dose/Gram (% ID/g)					
tumor	13.29 ± 2.36	13.85 ± 1.43	9.40 ± 2.86	5.23 ± 1.81	1.19 ± 0.30 <sup>*b</sup>
brain	0.20 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
blood	7.53 ± 0.99	1.48 ± 0.02	0.27 ± 0.17	0.06 ± 0.05	3.69 ± 0.01
heart	2.38 ± 1.75	0.27 ± 0.07	0.14 ± 0.05	0.09 ± 0.02	0.22 ± 0.01
lung	4.61 ± 3.16	0.53 ± 0.13	0.30 ± 0.01	0.16 ± 0.03	0.39 ± 0.14
liver	1.36 ± 0.10	0.78 ± 0.02	0.73 ± 0.07	0.43 ± 0.11	0.61 ± 0.08
skin	6.44 ± 2.64	1.33 ± 0.59	0.22 ± 0.15	0.22 ± 0.04	0.38 ± 0.08
spleen	1.19 ± 0.58	0.31 ± 0.15	0.27 ± 0.14	0.23 ± 0.06	0.21 ± 0.07
stomach	2.01 ± 0.72	0.83 ± 0.23	0.66 ± 0.20	0.37 ± 0.26	0.92 ± 0.37
kidneys	35.79 ± 2.11	28.60 ± 5.14	26.17 ± 4.83	11.50 ± 2.81	18.09 ± 1.89 <sup>*b</sup>
muscle	0.46 ± 0.19	0.06 ± 0.05	0.02 ± 0.01	0.03 ± 0.03	0.04 ± 0.01
pancreas	1.25 ± 1.00	0.11 ± 0.09	0.02 ± 0.02	0.05 ± 0.03	0.07 ± 0.03
bone	0.90 ± 0.90	0.29 ± 0.20	0.09 ± 0.07	0.11 ± 0.06	0.11 ± 0.04
Percentage Injected Dose (% ID)					
intestines	2.28 ± 1.24	0.93 ± 0.21	1.29 ± 0.47	2.03 ± 1.42	0.84 ± 0.22
urine	53.46 ± 7.42	77.83 ± 2.83	84.94 ± 2.10	90.45 ± 3.66	90.63 ± 2.07
Uptake Ratio of Tumor/Normal Tissue					
tumor/blood	1.76	9.36	34.81	87.17	0.32
tumor/kidneys	0.37	0.48	0.36	0.45	0.07
tumor/lung	2.88	26.13	31.33	32.69	3.05
tumor/liver	9.77	17.76	12.88	12.16	1.95
tumor/muscle	28.89	230.83	470.00	174.33	29.75

<sup>a</sup>The data are presented as percent injected dose/gram or as percent injected dose (mean ± SD,  $n = 4$ ). <sup>b</sup>(\*)  $p < 0.05$  for determining the significance of differences in tumor and kidney uptake between  $^{99m}\text{Tc}$ -2 with or without NDP-MSH peptide blockade at 2 h postinjection.

peptides were approximately 2-fold weaker than RXD-Lys-(Arg<sup>11</sup>)CCMSH peptides, respectively. Despite the fact that the -His-DPhe-Arg-Trp- motif is the binding moiety for the MC1 receptor, the decrease in receptor binding affinity with the  $\beta$ -Ala linker substitution indicated that the linker might somehow

interact with the receptor binding moiety. Such interaction might be related to the side chain of the Lys linker. The decrease in receptor binding affinities of RXD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH peptides also resulted in the reduction in tumor uptake of  $^{99m}\text{Tc}$ -RXD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH peptides by 21–45% in



Table 4. Biodistribution of  $^{99m}\text{Tc}$ -3 in B16/F1 Melanoma-Bearing C57 Mice<sup>a</sup>

tissue	0.5 h	2 h	4 h	24 h	2 h NDP
Percentage Injected Dose/Gram (% ID/g)					
tumor	12.49 ± 2.10	13.11 ± 4.78	11.18 ± 2.76	4.66 ± 1.92	1.30 ± 0.46 <sup>a,b</sup>
brain	0.14 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.03 ± 0.02	0.02 ± 0.01
blood	6.11 ± 0.56	0.78 ± 0.06	0.14 ± 0.08	0.06 ± 0.01	0.43 ± 0.01
heart	1.99 ± 0.11	0.35 ± 0.07	0.15 ± 0.02	0.08 ± 0.02	0.32 ± 0.06
lung	2.61 ± 0.26	1.13 ± 0.09	0.41 ± 0.13	0.11 ± 0.02	0.64 ± 0.12
liver	2.22 ± 0.05	1.36 ± 0.33	1.62 ± 0.34	0.50 ± 0.06	1.34 ± 0.23
skin	4.86 ± 2.81	0.98 ± 0.44	0.16 ± 0.07	0.34 ± 0.05	0.82 ± 0.18
spleen	1.36 ± 0.38	0.27 ± 0.14	0.31 ± 0.09	0.08 ± 0.02	0.34 ± 0.08
stomach	2.82 ± 0.60	1.02 ± 0.48	0.85 ± 0.29	0.39 ± 0.15	1.74 ± 0.27
kidneys	30.77 ± 2.40	30.37 ± 6.04	27.03 ± 0.71	7.58 ± 1.78	17.96 ± 5.92
muscle	0.44 ± 0.11	0.17 ± 0.05	0.02 ± 0.02	0.14 ± 0.04	0.12 ± 0.07
pancreas	0.76 ± 0.36	0.14 ± 0.01	0.10 ± 0.06	0.08 ± 0.06	0.10 ± 0.11
bone	0.46 ± 0.12	0.60 ± 0.38	0.09 ± 0.09	0.17 ± 0.15	0.10 ± 0.05
Percentage Injected Dose (% ID)					
intestines	1.89 ± 0.32	0.87 ± 0.22	1.33 ± 0.47	1.00 ± 0.59	0.90 ± 0.17
urine	49.29 ± 2.66	74.19 ± 7.90	84.10 ± 4.44	84.47 ± 6.30	86.59 ± 4.94
Uptake Ratio of Tumor/Normal Tissue					
tumor/blood	2.04	16.81	79.86	77.67	3.02
tumor/kidneys	0.41	0.43	0.41	0.61	0.07
tumor/lung	4.79	11.60	27.27	42.36	2.03
tumor/liver	5.63	9.64	6.90	9.32	0.97
tumor/muscle	28.39	77.12	559.00	33.29	10.83

<sup>a</sup>The data are presented as percent injected dose/gram or as percent injected dose (mean ± SD, *n* = 4). <sup>b</sup>(\*) *p* < 0.05 for determining the significance of differences in tumor and kidney uptake between  $^{99m}\text{Tc}$ -3 with or without NDP-MSH peptide blockade at 2 h postinjection.

Table 5. Biodistribution of  $^{99m}\text{Tc}$ -4 in B16/F1 Melanoma-Bearing C57 Mice<sup>a</sup>

tissue	0.5 h	2 h	4 h	24 h	2 h NDP
Percentage Injected Dose/Gram (% ID/g)					
tumor	12.55 ± 3.37	15.66 ± 6.19	14.67 ± 3.81	7.79 ± 2.68	2.43 ± 0.53 <sup>a,b</sup>
brain	0.19 ± 0.04	0.05 ± 0.01	0.03 ± 0.01	0.05 ± 0.05	0.02 ± 0.02
blood	3.82 ± 0.62	1.01 ± 0.42	0.60 ± 0.29	0.46 ± 0.44	0.51 ± 0.10
heart	2.28 ± 0.29	0.90 ± 0.34	0.69 ± 0.33	0.31 ± 0.21	0.73 ± 0.23
lung	7.22 ± 1.60	3.15 ± 0.79	2.34 ± 1.19	1.01 ± 0.48	3.12 ± 1.13
liver	2.39 ± 0.62	1.09 ± 0.13	1.05 ± 0.26	0.56 ± 0.23	1.17 ± 0.33
skin	5.50 ± 0.60	1.38 ± 0.41	0.70 ± 0.13	0.42 ± 0.24	1.29 ± 0.42
spleen	1.39 ± 0.51	0.80 ± 0.31	0.55 ± 0.34	0.60 ± 0.38	0.64 ± 0.43
stomach	7.11 ± 2.20	2.61 ± 1.04	1.88 ± 0.63	0.44 ± 0.20	2.88 ± 0.65
kidneys	25.64 ± 4.06	20.18 ± 3.86	19.83 ± 6.34	3.92 ± 0.99	19.82 ± 9.39
muscle	0.90 ± 0.48	0.23 ± 0.14	0.18 ± 0.11	0.34 ± 0.32	0.17 ± 0.08
pancreas	0.84 ± 0.35	0.29 ± 0.09	0.12 ± 0.08	0.20 ± 0.14	0.14 ± 0.05
bone	1.60 ± 0.27	0.59 ± 0.16	0.24 ± 0.20	0.50 ± 0.42	0.33 ± 0.20
Percentage Injected Dose (% ID)					
intestines	2.93 ± 0.44	2.58 ± 1.25	1.90 ± 0.39	0.89 ± 1.10	1.46 ± 0.26
urine	49.54 ± 2.65	78.28 ± 2.36	83.80 ± 3.67	94.25 ± 1.90	83.61 ± 5.94
Uptake Ratio of Tumor/Normal Tissue					
tumor/blood	3.29	15.50	24.45	16.93	4.76
tumor/kidneys	0.49	0.78	0.74	1.99	0.12
tumor/lung	1.74	4.97	6.27	7.71	0.78
tumor/liver	5.25	14.37	13.97	13.91	2.08
tumor/muscle	13.94	68.09	81.50	22.91	14.29

<sup>a</sup>The data are presented as percent injected dose/gram or as percent injected dose (mean ± SD, *n* = 4). <sup>b</sup>(\*) *p* < 0.05 for determining the significance of differences in tumor and kidney uptake between  $^{99m}\text{Tc}$ -4 with or without NDP-MSH peptide blockade at 2 h postinjection.

B16/F1 melanoma-bearing C57 mice. Specifically, the tumor uptake of  $^{99m}\text{Tc}$ -4 was 79% of the tumor uptake of  $^{99m}\text{Tc}$ -RAD-Lys-(Arg<sup>11</sup>)CCMSH at 2 h postinjection.<sup>19</sup>

The substitution of Lys linker with  $\beta$ -Ala linker dramatically decreased the renal uptake of  $^{99m}\text{Tc}$ -RXD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH

peptides by 64–79% in B16/F1 melanoma-bearing C57 mice. For instance, the renal uptake of  $^{99m}\text{Tc}$ -4 was only 22% of the renal uptake of  $^{99m}\text{Tc}$ -RAD-Lys-(Arg<sup>11</sup>)CCMSH at 2 h postinjection.<sup>19</sup> It is worthwhile to note that there is a positively charged Arg residue in the RAD moiety of  $^{99m}\text{Tc}$ -4. Thus, we

Table 6. Biodistribution of  $^{99m}\text{Tc}$ -5 in B16/F1 Melanoma-Bearing C57 Mice<sup>a</sup>

tissue	0.5 h	2 h	4 h	24 h	2 h NDP
Percentage Injected Dose/Gram (% ID/g)					
tumor	9.93 ± 2.37	11.38 ± 1.29	10.81 ± 5.48	4.17 ± 1.14	2.06 ± 0.85 <sup>a,b</sup>
brain	0.25 ± 0.04	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.05 ± 0.01
blood	6.12 ± 2.22	1.02 ± 0.48	0.87 ± 0.43	0.14 ± 0.02	1.22 ± 0.01
heart	3.34 ± 0.47	0.62 ± 0.03	0.56 ± 0.31	0.11 ± 0.03	0.48 ± 0.25
lung	8.93 ± 0.54	2.31 ± 0.55	1.28 ± 0.27	0.74 ± 0.30	1.92 ± 0.41
liver	3.11 ± 0.82	1.67 ± 0.39	1.37 ± 0.45	0.50 ± 0.08	1.93 ± 0.77
skin	6.11 ± 1.06	1.14 ± 0.23	0.62 ± 0.10	0.16 ± 0.04	1.47 ± 0.68
spleen	2.56 ± 0.86	0.76 ± 0.21	0.55 ± 0.08	0.20 ± 0.13	0.33 ± 0.20
stomach	4.23 ± 0.38	1.14 ± 0.37	0.75 ± 0.06	0.25 ± 0.09	2.11 ± 0.84
kidneys	35.68 ± 2.10	37.23 ± 9.69	32.68 ± 6.55	8.39 ± 2.56	22.48 ± 9.23
muscle	1.37 ± 0.37	0.06 ± 0.06	0.15 ± 0.04	0.05 ± 0.01	0.39 ± 0.29
pancreas	1.43 ± 0.76	0.22 ± 0.08	0.01 ± 0.01	0.01 ± 0.01	0.25 ± 0.14
bone	2.09 ± 0.64	0.35 ± 0.29	0.03 ± 0.01	0.09 ± 0.07	0.19 ± 0.08
Percentage Injected Dose (% ID)					
intestines	2.40 ± 0.36	2.84 ± 1.98	4.77 ± 3.46	0.59 ± 0.18	2.28 ± 0.78
urine	31.78 ± 3.81	73.27 ± 5.41	77.67 ± 5.88	93.77 ± 1.03	74.78 ± 10.44
Uptake Ratio of Tumor/Normal Tissue					
tumor/blood	1.62	11.16	12.43	29.79	1.69
tumor/kidneys	0.28	0.31	0.33	0.50	0.09
tumor/lung	1.11	4.93	8.45	5.64	1.07
tumor/liver	3.19	6.81	7.89	8.34	1.07
tumor/muscle	7.25	189.67	72.07	83.40	5.28

<sup>a</sup>The data are presented as percent injected dose/gram or as percent injected dose (mean ± SD,  $n = 4$ ). <sup>b</sup>(\*)  $p < 0.05$  for determining the significance of differences in tumor and kidney uptake between  $^{99m}\text{Tc}$ -5 with or without NDP-MSH peptide blockade at 2 h postinjection.

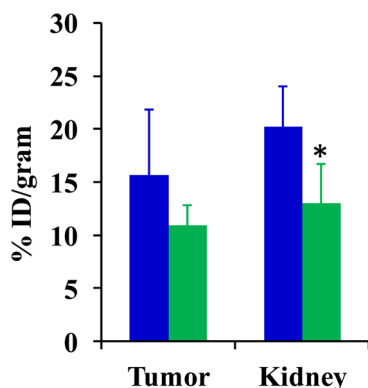
Table 7. Biodistribution of  $^{99m}\text{Tc}$ -6 in B16/F1 Melanoma-Bearing C57 Mice<sup>a</sup>

tissue	0.5 h	2 h	4 h	24 h	2 h NDP
Percentage Injected Dose/Gram (% ID/g)					
tumor	12.25 ± 1.86	10.13 ± 3.60	13.36 ± 4.12	3.71 ± 1.59	1.20 ± 0.25 <sup>a,b</sup>
brain	0.15 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
blood	5.94 ± 1.42	0.86 ± 0.04	0.38 ± 0.12	0.08 ± 0.01	0.47 ± 0.01
heart	1.73 ± 0.35	0.44 ± 0.08	0.35 ± 0.03	0.14 ± 0.08	0.29 ± 0.15
lung	3.50 ± 1.96	2.57 ± 1.00	1.35 ± 0.51	0.35 ± 0.08	1.73 ± 0.74
liver	2.06 ± 0.73	1.08 ± 0.41	0.93 ± 0.16	0.26 ± 0.08	0.86 ± 0.03
skin	5.92 ± 0.76	0.55 ± 0.03	0.64 ± 0.05	0.24 ± 0.02	0.86 ± 0.15
spleen	1.95 ± 1.15	0.59 ± 0.25	0.49 ± 0.18	0.14 ± 0.11	0.26 ± 0.05
stomach	2.91 ± 1.36	0.70 ± 0.12	0.89 ± 0.06	0.12 ± 0.02	1.16 ± 0.30
kidneys	25.65 ± 8.27	25.25 ± 9.26	24.37 ± 7.66	4.12 ± 1.14	25.93 ± 1.62
muscle	0.85 ± 0.26	0.05 ± 0.03	0.03 ± 0.01	0.10 ± 0.08	0.04 ± 0.04
pancreas	0.72 ± 0.39	0.14 ± 0.07	0.06 ± 0.04	0.02 ± 0.01	0.07 ± 0.02
bone	1.43 ± 0.32	0.27 ± 0.12	0.07 ± 0.03	0.14 ± 0.06	0.10 ± 0.05
Percentage Injected Dose (% ID)					
intestines	2.19 ± 0.72	1.38 ± 0.92	2.29 ± 0.66	0.55 ± 0.16	2.51 ± 0.34
urine	42.67 ± 21.25	75.09 ± 11.87	86.51 ± 3.43	92.30 ± 1.33	85.18 ± 0.53
Uptake Ratio of Tumor/Normal Tissue					
tumor/blood	2.06	11.78	35.16	46.38	2.55
tumor/kidneys	0.48	0.40	0.55	0.90	0.05
tumor/lung	3.50	3.94	9.90	10.60	0.69
tumor/liver	5.95	9.38	14.37	14.27	1.40
tumor/muscle	14.41	202.60	445.33	37.10	30.00

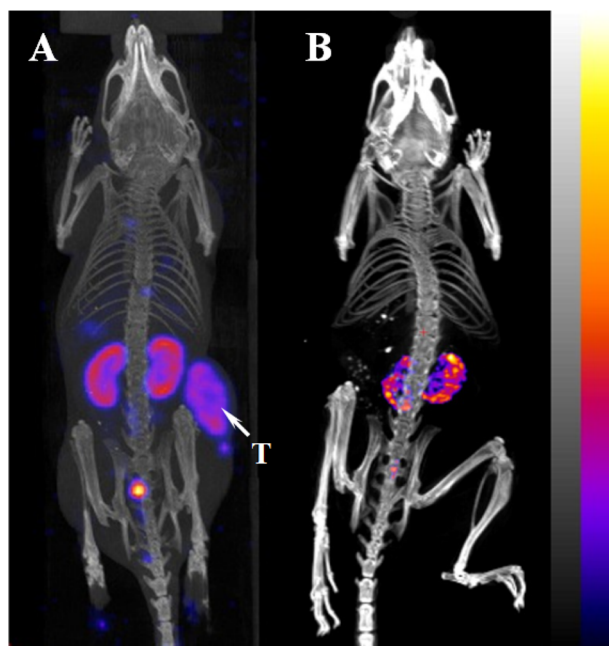
<sup>a</sup>The data are presented as percent injected dose/gram or as percent injected dose (mean ± SD,  $n = 4$ ). <sup>b</sup>(\*)  $p < 0.05$  for determining the significance of differences in tumor and kidney uptake between  $^{99m}\text{Tc}$ -6 with or without NDP-MSH peptide blockade at 2 h postinjection.

were interested in whether the replacement of Arg with Nle (neutral) and Glu (negatively charged) could further decrease the renal uptake of  $^{99m}\text{Tc}$ -5 and  $^{99m}\text{Tc}$ -6 as compared to  $^{99m}\text{Tc}$ -4. Interestingly, the replacement of Arg with Nle and Glu did not further decrease the renal uptake of  $^{99m}\text{Tc}$ -5 and  $^{99m}\text{Tc}$ -6 as compared to  $^{99m}\text{Tc}$ -4. Clearly,

the tumor targeting and clearance properties of  $^{99m}\text{Tc}$ -4 were more favorable than the other  $^{99m}\text{Tc}$ -peptides investigated in this study. Thus, we further examined its melanoma imaging property and urinary metabolites. The B16/F1 melanoma lesions could be clearly visualized by SPECT/CT using  $^{99m}\text{Tc}$ -4 as an imaging probe.



**Figure 4.** Effect of L-lysine co-injection on the tumor and kidney uptake of  $^{99m}\text{Tc}$ -4 at 2 h postinjection. The blue and green columns represent the tumor and renal uptake of  $^{99m}\text{Tc}$ -4 without and with L-lysine co-injection: (\*)  $p < 0.05$  for determining the significance of differences in tumor and kidney uptake between  $^{99m}\text{Tc}$ -4 without and with L-lysine co-injection.



**Figure 5.** Representative whole-body SPECT/CT image of B16/F1 melanoma-bearing C57 mice 2 h after injection of  $^{99m}\text{Tc}$ -4 without (A) and with (B) peptide blockade. Flank melanoma lesions (T) are highlighted with an arrow on the image.

At the present time,  $^{99m}\text{Tc}$ -(Arg<sup>11</sup>)CCMSH and  $^{99m}\text{Tc}$ (EDDA)-HYNIC-GGNle-CycMSH<sub>hex</sub> were reported as promising cyclic imaging probes for melanoma.<sup>12,20</sup> Remarkably,  $^{99m}\text{Tc}$ -4 displayed

comparably high melanoma uptake ( $14.67 \pm 3.81\%$  ID/g) as  $^{99m}\text{Tc}$ -(Arg<sup>11</sup>)CCMSH and  $^{99m}\text{Tc}$ (EDDA)-HYNIC-GGNle-CycMSH<sub>hex</sub> at 4 h postinjection. However, the renal uptake of  $^{99m}\text{Tc}$ -4 was higher than those of  $^{99m}\text{Tc}$ -(Arg<sup>11</sup>)CCMSH ( $11.66 \pm 1.44\%$  ID/g) and  $^{99m}\text{Tc}$ (EDDA)-HYNIC-GGNle-CycMSH<sub>hex</sub> ( $7.52 \pm 0.96\%$  ID/g). The difference in renal uptake was likely due to the structural differences among these  $^{99m}\text{Tc}$ -peptides. Interestingly, we found that L-lysine co-injection significantly reduced the renal uptake of  $^{99m}\text{Tc}$ -4 by 35% at 2 h postinjection without affecting its tumor uptake significantly (Figure 4). Therefore, L-lysine co-injection could be utilized to further decrease the renal uptake of  $^{188}\text{Re}$ -RAD-Lys-(Arg<sup>11</sup>)CCMSH to facilitate its potential therapeutic application.

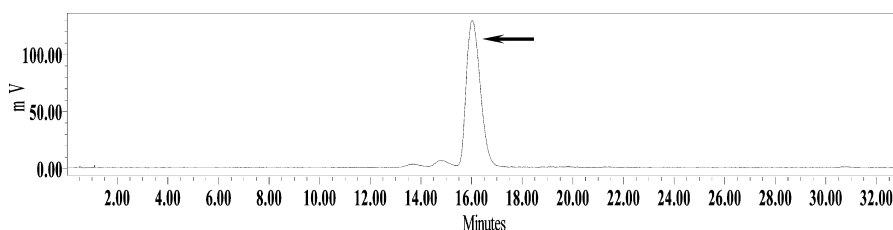
## CONCLUSIONS

The substitution of the Lys linker with the  $\beta$ -Ala linker dramatically decreased the renal uptake of  $^{99m}\text{Tc}$ -RXD- $\beta$ -Ala-(Arg<sup>11</sup>)CCMSH peptides. Among these six  $^{99m}\text{Tc}$ -peptides,  $^{99m}\text{Tc}$ -4 exhibited the highest tumor uptake and the lowest renal uptake at 2 h postinjection. The replacement of Arg with Nle and Glu did not further decrease the renal uptake of  $^{99m}\text{Tc}$ -5 and  $^{99m}\text{Tc}$ -6 as compared to  $^{99m}\text{Tc}$ -4. The tumor targeting and clearance properties of  $^{99m}\text{Tc}$ -4 highlighted it as a lead peptide for future studies.

## EXPERIMENTAL SECTION

**Chemicals and Reagents.** Amino acids and resin were purchased from Advanced ChemTech Inc. (Louisville, KY) and Novabiochem (San Diego, CA).  $^{125}\text{I}$ -Tyr<sup>2</sup>-[Nle<sup>4</sup>,D-Phe<sup>7</sup>]- $\alpha$ -MSH [ $^{125}\text{I}$ -(Tyr<sup>2</sup>)-NDP-MSH] was obtained from PerkinElmer, Inc. (Waltham, MA) for receptor binding assay.  $^{99m}\text{TcO}_4^-$  was purchased from Cardinal Health (Albuquerque, NM). 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).

**Peptide Synthesis and in Vitro Competitive Binding Assay.** Six new peptides were synthesized using fluorenylmethyloxycarbonyl (Fmoc) chemistry according to our previously published procedures<sup>17–19</sup> with slight modification on Sieber amide resin by an Advanced ChemTech multiple-peptide synthesizer (Louisville, KY). Briefly, 70  $\mu\text{mol}$  of Sieber amide resin and 210  $\mu\text{mol}$  of Fmoc-protected amino acids were used for the synthesis. Fmoc- $\beta$ -Ala was used to generate a  $\beta$ -Ala linker in each peptide. Intermediate scaffolds of H<sub>2</sub>N-Arg(Pbf)-Ser/Thr/Val-Asp(OtBu)-D-Tyr(tBu)-Asp(O-2-phenylisopropyl)- $\beta$ -Ala-Cys(Trt)-Cys(Trt)-Glu(OtBu)-His(Trt)-D-Phe-Arg(Pbf)-Trp(Boc)-Cys(Trt)-Arg(Pbf)-Pro-Val and H<sub>2</sub>N-Arg(Pbf)/Nle/Glu(OtBu)- $\beta$ -Ala-Asp(OtBu)-D-Tyr(tBu)-Asp(O-2-phenylisopropyl)- $\beta$ -Ala-Cys(Trt)-Cys(Trt)-Glu(OtBu)-His(Trt)-D-Phe-Arg(Pbf)-Trp(Boc)-Cys(Trt)-Arg(Pbf)-Pro-Val were synthesized on Sieber amide resin. The protecting group of 2-phenylisopropyl of each scaffold was removed, and each peptide was 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



**Figure 6.** Radioactive HPLC profiles of urinary metabolites at 2 h postinjection of  $^{99m}\text{Tc}$ -4. The arrow denotes the original retention time of  $^{99m}\text{Tc}$ -4 prior to tail vein injection.

characterization by MS, each protected peptide was dissolved in  $\text{H}_2\text{O}/\text{CH}_3\text{CN}$  (50:50) and lyophilized to remove the reagents such as TFA and triisopropylsilane. Each protected peptide was further cyclized by coupling the carboxylic group from the Asp with the  $\alpha$  amino group from the Arg, Nle, or Glu at the N-terminus. The cyclization reaction was achieved by overnight reaction in dimethylformamide (DMF) using benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) as a coupling agent in the presence of *N,N*-diisopropylethylamine (DIPEA). The protecting groups were totally removed by treating with a mixture of 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). Each peptide was precipitated and washed with ice-cold ether four times, purified by RP-HPLC, and characterized by liquid chromatography–mass spectrometry (LC–MS). The chemical purity of each peptide was determined by Waters RP-HPLC instrument (Milford, MA) on a Grace Vydac C-18 reverse phase analytic column (Deerfield, IL) using a 20 min gradient of 18–28% acetonitrile in 20 mM HCl aqueous solution at a flow rate of 1 mL/min. The purities of all six peptides were greater than 95%.

The  $\text{IC}_{50}$  values of the peptides for the MC1 receptor were determined in B16/F1 melanoma cells. The competitive receptor binding assay was replicated in triplicate for each peptide. The B16/F1 cells were seeded into a 24-well cell culture plate at a density of  $2.5 \times 10^5$  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) (HEPES), pH 7.4, 0.2% bovine serum albumin (BSA), 0.3 mM 1,10-phenanthroline}, the cells were incubated at 25 °C for 2 h with approximately 30 000 counts per minute (cpm) of  $^{125}\text{I}$ -(Tyr<sup>2</sup>)-NDP-MSH in the presence of increasing concentrations ( $10^{-13}$ – $10^{-6}$  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) to remove any unbound radioactivity and lysed in 0.5 mL of 1 M NaOH for 5 min. The activities associated with the cells were measured in a Wallac 2480 automated  $\gamma$  counter (PerkinElmer, NJ). The  $\text{IC}_{50}$  value for each peptide was calculated using Prism software (GraphPad Software, La Jolla, CA). The standard deviation of  $\text{IC}_{50}$  value was generated by two independent experiments in triplicate for each peptide.

**Peptide Radiolabeling.** The peptides were labeled with  $^{99\text{m}}\text{Tc}$  via a direct reduction reaction using  $\text{SnCl}_2$  as a reducing agent. Briefly, 10  $\mu\text{L}$  of 1 mg/mL  $\text{SnCl}_2$  in 0.1 M HCl, 40  $\mu\text{L}$  of 0.5 M  $\text{NH}_4\text{OAc}$  (pH 5.2), 100  $\mu\text{L}$  of 0.2 M  $\text{Na}_2$  tartate (pH 9.2), 100  $\mu\text{L}$  of fresh  $^{99\text{m}}\text{TcO}_4^-$  solution (37–74 MBq), and 10  $\mu\text{L}$  of 1 mg/mL of each peptide in aqueous solution were added into a reaction vial and incubated at 25 °C for 20 min to form the  $^{99\text{m}}\text{Tc}$ -labeled peptide. Each  $^{99\text{m}}\text{Tc}$ -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) using a 20 min gradient of 18–28% acetonitrile in 20 mM HCl aqueous solution at a flow rate of 1 mL/min. Each purified peptide was purged with  $\text{N}_2$  gas for 20 min to remove the acetonitrile. The pH of final peptide solution was adjusted to 7.4 with 0.1 N NaOH and sterile normal saline for stability, biodistribution, and imaging studies. The serum stabilities of  $^{99\text{m}}\text{Tc}$ -1,  $^{99\text{m}}\text{Tc}$ -2,  $^{99\text{m}}\text{Tc}$ -3,  $^{99\text{m}}\text{Tc}$ -4,  $^{99\text{m}}\text{Tc}$ -5, and  $^{99\text{m}}\text{Tc}$ -6 were determined by incubation in mouse serum at 37 °C for 24 h and monitored for degradation by RP-HPLC. Briefly, 100  $\mu\text{L}$  of each HPLC-purified peptide solution ( $\sim 7.4$  MBq) was added into 100  $\mu\text{L}$  of mouse serum (Sigma-Aldrich Corp, St. Louis, MO) and incubated at 37 °C for 24 h. After the incubation, 200  $\mu\text{L}$  of a mixture of ethanol and acetonitrile (v:v = 1:1) was added to precipitate the serum proteins. The resulting mixture was centrifuged at 16000g for 5 min to collect the supernatant. The supernatant was purged with  $\text{N}_2$  gas for 30 min to remove the ethanol and acetonitrile. The resulting sample was mixed with 500  $\mu\text{L}$  of water and injected into RP-HPLC for analysis using the gradient described above.

**Biodistribution Studies.** All the animal studies were conducted in compliance with Institutional Animal Care and Use Committee approval (12-100851-HSC). The biodistribution properties of the  $^{99\text{m}}\text{Tc}$ -peptides were determined in B16/F1 melanoma-bearing C57 female mice (Harlan, Indianapolis, IN). Each C57 mouse was

subcutaneously inoculated on the right flank with  $1 \times 10^6$  B16/F1 cells. The weight of tumors reached approximately 0.2 g at 10 days after cell inoculation. Each melanoma-bearing mouse was injected with 0.037 MBq of each  $^{99\text{m}}\text{Tc}$ -peptide via the tail vein. Groups of four mice were sacrificed at 0.5, 2, 4, and 24 h postinjection, and tumors and organs of interest were harvested, weighed, and counted. Blood values were taken as 6.5% of the body weight. The specificity of tumor uptake was determined by co-injecting each  $^{99\text{m}}\text{Tc}$ -peptide with 10  $\mu\text{g}$  (6.1 nmol) of unlabeled NDP-MSH at 2 h postinjection.

The effect of L-lysine co-injection on the renal uptake of  $^{99\text{m}}\text{Tc}$ -4 was examined in B16/F1 melanoma-bearing C57. A group of four mice were injected with an aqueous mixture of 0.037 MBq of  $^{99\text{m}}\text{Tc}$ -4 and 15 mg of L-lysine. The mice were sacrificed at 2 h postinjection, and tumor and kidneys were harvested, weighed, and counted.

**Melanoma Imaging with  $^{99\text{m}}\text{Tc}$ -4.**  $^{99\text{m}}\text{Tc}$ -4 was the lead peptide because of its highest tumor uptake and the lowest renal uptake. Thus, we further determined the melanoma imaging property of  $^{99\text{m}}\text{Tc}$ -4 and the specificity of melanoma uptake. Approximately 3.7–4.1 MBq of  $^{99\text{m}}\text{Tc}$ -4 with or without 10  $\mu\text{g}$  (6.1 nmol) of unlabeled NDP-MSH was injected into B16/F1 melanoma-bearing C57 mice via the tail vein, respectively. The mice were euthanized for small animal SPECT/CT (Nano-SPECT/CT, Bioscan, Washington, DC) imaging 2 h postinjection. The 9 min CT imaging was immediately followed by the SPECT imaging of whole body. The SPECT scans of 24 projections were acquired. Reconstructed data from SPECT and CT were visualized and co-registered using InVivoScope (Bioscan, Washington, DC).

**Urinary Metabolites of  $^{99\text{m}}\text{Tc}$ -4.** We also examined the urinary metabolites of  $^{99\text{m}}\text{Tc}$ -4. Approximately 3.7 MBq of  $^{99\text{m}}\text{Tc}$ -4 was injected into a B16/F1 melanoma-bearing C57 mouse via the tail vein to determine the urinary metabolites. The mouse was euthanized to collect urine at 2 h postinjection. The collected urine sample was centrifuged at 16000g for 5 min before the HPLC analysis. Thereafter, an aliquot of the urine was injected into the HPLC. A 20 min gradient of 16–26% acetonitrile/20 mM HCl with a flow rate of 1 mL/min was used for urine analysis.

**Statistical Analysis.** Statistical analysis was performed using the Student's *t* test for unpaired data to determine the significance of differences in tumor and kidney uptake with/without peptide blockade in biodistribution studies described above. Differences at the 95% confidence level ( $p < 0.05$ ) were considered significant.

## AUTHOR INFORMATION

### Corresponding Author

\*Phone: 505-925-4437. E-mail: ymiao@salud.unm.edu.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We appreciate Dr. Fabio Gallazzi for his technical assistance. This work was supported in part by NIH Grant NM-INBRE P20RR016480/P20GM103451 and UNM RAC Award. The images were generated by the KUSAIR established with funding from the W. M. Keck Foundation and the UNM Cancer Research and Treatment Center (Grant NIH P30 CA118100).

## ABBREVIATIONS USED

MC1, melanocortin 1;  $\alpha$ -MSH,  $\alpha$ -melanocyte stimulating hormone; RXD motif, Arg-X-Asp-D-Tyr-Asp; Fmoc, fluorenylmethyloxycarbonyl; TFA, trifluoroacetic acid; DMF, dimethylformamide; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate; DIPEA, *N,N*-diisopropylethylamine; LC–MS, liquid chromatography–mass spectrometry; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); BSA, bovine serum albumin; PBS, phosphate buffered saline; SPECT, single photon emission computed tomography



## ■ REFERENCES

- (1) Siegrist, W.; Solca, F.; Stutz, S.; Giuffrè, L.; Carrel, S.; Girard, J.; Eberle, A. N. Characterization of receptors for alpha-melanocyte-stimulating hormone on human melanoma cells. *Cancer Res.* **1989**, *49*, 6352–6358.
- (2) Tatro, J. B.; Atkins, M.; Mier, J. W.; Hardarson, S.; Wolfe, H.; Smith, T.; Entwistle, M. L.; Reichlin, S. Melanotropin receptors demonstrated in situ in human melanoma. *J. Clin. Invest.* **1990**, *85*, 1825–1832.
- (3) Miao, Y.; Whitener, D.; Feng, W.; Owen, N. K.; Chen, J.; Quinn, T. P. Evaluation of the human melanoma targeting properties of radiolabeled alpha-melanocyte stimulating hormone peptide analogues. *Bioconjugate Chem.* **2003**, *14*, 1177–1184.
- (4) Guo, H.; Shenoy, N.; Gershman, B. M.; Yang, J.; Sklar, L. A.; Miao, Y. Metastatic melanoma imaging with an  $^{111}\text{In}$ -labeled lactam bridge-cyclized alpha-melanocyte-stimulating hormone peptide. *Nucl. Med. Biol.* **2009**, *36*, 267–276.
- (5) Yang, J.; Guo, H.; Miao, Y. Technetium-99m-labeled Arg-Gly-Asp-conjugated alpha-melanocyte stimulating hormone hybrid peptides for human melanoma imaging. *Nucl. Med. Biol.* **2010**, *37*, 873–883.
- (6) Giblin, M. F.; Wang, N.; Hoffman, T. J.; Jurisson, S. S.; Quinn, T. P. Design and characterization of alpha-melanotropin peptide analogs cyclized through rhenium and technetium metal coordination. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12814–12818.
- (7) Froidevaux, S.; Calame-Christe, M.; Tanner, H.; Sumanovski, L.; Eberle, A. N. A novel DOTA-alpha-melanocyte-stimulating hormone analog for metastatic melanoma diagnosis. *J. Nucl. Med.* **2002**, *43*, 1699–1706.
- (8) Froidevaux, S.; Calame-Christe, M.; Schuhmacher, J.; Tanner, H.; Saffrich, R.; Henze, M.; Eberle, A. N. A gallium-labeled DOTA- $\alpha$ -melanocyte-stimulating hormone analog for PET imaging of melanoma metastases. *J. Nucl. Med.* **2004**, *45*, 116–123.
- (9) McQuade, P.; Miao, Y.; Yoo, J.; Quinn, T. P.; Welch, M. J.; Lewis, J. S. Imaging of melanoma using  $^{64}\text{Cu}$ - and  $^{86}\text{Y}$ -DOTA-ReCCMSH-(Arg $^{11}$ ), a cyclized peptide analogue of alpha-MSH. *J. Med. Chem.* **2005**, *48*, 2985–2992.
- (10) Wei, L.; Butcher, C.; Miao, Y.; Gallazzi, F.; Quinn, T. P.; Welch, M. J.; Lewis, J. S. Synthesis and biologic evaluation of  $^{64}\text{Cu}$ -labeled rhenium-cyclized alpha-MSH peptide analog using a cross-bridged cyclam chelator. *J. Nucl. Med.* **2007**, *48*, 64–72.
- (11) Cheng, Z.; Xiong, Z.; Subbarayan, M.; Chen, X.; Gambhir, S. S.  $^{64}\text{Cu}$ -labeled alpha-melanocyte-stimulating hormone analog for micro-PET imaging of melanocortin 1 receptor expression. *Bioconjugate Chem.* **2007**, *18*, 765–772.
- (12) Miao, Y.; Benwell, K.; Quinn, T. P.  $^{99\text{m}}\text{Tc}$ - and  $^{111}\text{In}$ -labeled alpha-melanocyte-stimulating hormone peptides as imaging probes for primary and pulmonary metastatic melanoma detection. *J. Nucl. Med.* **2007**, *48*, 73–80.
- (13) Miao, Y.; Figueroa, S. D.; Fisher, D. R.; Moore, H. A.; Testa, R. F.; Hoffman, T. J.; Quinn, T. P.  $^{203}\text{Pb}$ -labeled alpha-melanocyte-stimulating hormone peptide as an imaging probe for melanoma detection. *J. Nucl. Med.* **2008**, *49*, 823–829.
- (14) Miao, Y.; Gallazzi, F.; Guo, H.; Quinn, T. P.  $^{111}\text{In}$ -labeled lactam bridge-cyclized alpha-melanocyte stimulating hormone peptide analogues for melanoma imaging. *Bioconjugate Chem.* **2008**, *19*, 539–547.
- (15) Guo, H.; Yang, J.; Gallazzi, F.; Miao, Y. Reduction of the ring size of radiolabeled lactam bridge-cyclized alpha-MSH peptide resulting in enhanced melanoma uptake. *J. Nucl. Med.* **2010**, *51*, 418–426.
- (16) Guo, H.; Yang, J.; Gallazzi, F.; Miao, Y. Effects of the amino acid linkers on melanoma-targeting and pharmacokinetic properties of indium-111-labeled lactam bridge-cyclized  $\alpha$ -MSH peptides. *J. Nucl. Med.* **2011**, *52*, 608–616.
- (17) Flook, A. M.; Yang, J.; Miao, Y. Evaluation of new Tc-99m-labeled Arg-X-Asp-conjugated alpha-melanocyte stimulating hormone peptides for melanoma imaging. *Mol. Pharmaceutics* **2013**, *10*, 3417–3424.
- (18) Flook, A. M.; Yang, J.; Miao, Y. Effects of amino acids on melanoma targeting and clearance properties of Tc-99m-labeled Arg-X-Asp-conjugated alpha-melanocyte stimulating hormone peptides. *J. Med. Chem.* **2013**, *56*, 8793–8802.
- (19) Yang, J.; Miao, Y. Substitution of Gly with Ala enhanced the melanoma uptake of technetium-99m-labeled Arg-Ala-Asp-conjugated alpha-melanocyte stimulating hormone peptide. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 1541–1545.
- (20) Guo, H.; Fabio Gallazzi, F.; Miao, Y. Design and evaluation of Tc-99m-labeled lactam bridge-cyclized alpha-MSH peptides for melanoma imaging. *Mol. Pharmaceutics* **2013**, *10*, 1400–1408.