Modification of the Structure of a Metallopeptide: Synthesis and Biological Evaluation of ^{111}In-Labeled DOTA-Conjugated Rhenium-Cyclized α-MSH Analogues

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Rhenium-cyclized CCMSH analogues are novel melanoma-targeting metallopeptides with high tumor uptake, long tumor retention, and low background in normal tissues, which make these metallopeptides an ideal structural motif for designing novel melanoma-targeting agents. ReCCMSH has been derivatized with a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelate so that it can be labeled with a wide variety of radionuclides for imaging and therapeutic applications. This study involved optimization of the in vivo biological properties of DOTA-ReCCMSH (**S**), through modification of the structure of the metallopeptide. Several DOTA-ReCCMSH analogues, Ac-Lys(DOTA)-ReCCMSH (**4**) DOTA-ReCCMSH(Arg^{11}) (**6**), DOTA-ReCCMSH-OH (**8**), and DOTA-ReCCMSH-Asp-OH (**10**), were synthesized using solid phase peptide synthesis followed by rhenium cyclization. The IC_{50} values of the metallopeptides were determined through competitive binding assays against ^{125}I-(Tyr^2)-NDP. Radiolabeling of the DOTA-rhenium-cyclized peptides with ^{111}In was carried out in NH_4OAc (0.1 M; pH 5.5)-buffered solution for 30 min at 70 °C. The stability of the radiolabeled complexes was evaluated in 0.01 M, pH 7.4, phosphate-buffered saline/0.1% bovine serum albumin solution. After separation of the radiolabeled peptide from the unlabeled peptide by reverse phase high-performance liquid chromatography, the biodistribution of the radiolabeled complex was performed in C57 mice bearing B16/F1 murine melanoma tumors. All radiolabeled complexes showed fast blood clearance (2 h postinjection (pi): ^{111}In-**S**, 0.07 ± 0.03% ID/g; ^{111}In-**4**, 0.09 ± 0.06% ID/g; ^{111}In-**6**, 0.21 ± 0.08% ID/g; ^{111}In-**8**, 0.11 ± 0.10% ID/g; and ^{111}In-**10**, 0.05 ± 0.03% ID/g), and their clearance was predominantly through the urine (4 h pi: 93.5 ± 1.7, 87.8 ± 6.5, 89.8 ± 4.2, 93.3 ± 1.1, and 93.8 ± 1.8 (% ID) for ^{111}In-labeled **S**, **4**, **6**, **8**, and **10**, respectively). Tumor uptake values of 9.45 ± 0.90, 6.01 ± 2.36, 17.41 ± 5.61, 9.27 ± 0.68, and 7.32 ± 2.09 (% ID/g) for ^{111}In-labeled **S**, **4**, **6**, **8**, and **10**, respectively, were observed at 4 h pi. The kidney uptake was 9.27 ± 2.65% ID/g for ^{111}In-**S**, 19.02 ± 2.63% ID/g for ^{111}In-**4**, 7.37 ± 1.13% ID/g for ^{111}In-**6**, 8.70 ± 0.88% ID/g for ^{111}In-**8**, and 8.13 ± 1.47% ID/g for ^{111}In-**10** at 4 h pi. Complex **6** showed high melanoma uptake and lower kidney uptake than the corresponding Lys^{11} analogues, supporting **6** for further investigations as a potential therapeutic radiopharmaceutical.

Introduction

Malignant melanoma has become a significant public health problem especially in Europe and the U. S.[1−3] The high proliferation rate of melanoma cells, the rapid and extensive occurrence of metastases, and their resistance to most conventional therapies such as chemotherapy and external beam irradiation make the development of effective new methods for early detection and treatment of primary lesions and their metastases crucial.[4]

Extensive research has been performed on the development of drugs for melanoma imaging and therapy. Typically, the melanoma-targeting agents are subdivided into five classes:[5] (i) compounds that nonspecifically localize in melanoma such as [^{67}Ga]citrate[6] and 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG);[7,8] (ii) monoclonal antibodies and antibody fragments that can specifically bind to the melanoma-associated antigen;[9−11] (iii) compounds that mimic precursors in the melanin formation cycle;[12,13] (iv) compounds that have high affinities for melanin, such as 3,7-(dimethylamino)phenazathionium chloride [methylene blue (MTB)] radiolabeled with ^{211}At,[14] radiolabeled N-alkyl-*p*-iodobenzamides such as [^{123}I]N-(2-diethylaminoethyl)-4-iodobenzamide (^{123}I-BZA), [^{123}I]N-(2-diethylaminoethyl)-3-iodo-4-methoxybenzamide (^{123}I-IMBA),[15−18] and their derivatives radiolabeled with technetium;[19,20] and (v) peptides including α-melanocyte-stimulating hormone (α-MSH) analogues[21] and technetium-labeled RGD-containing peptides.[22] Although some agents in the first four classes have been successfully utilized in clinical melanoma localization[7,8,11] and some have great potential for melanoma therapy,[14] they all have some unfavorable properties such as high cost,[7,8] low tumor to nontumor tissues ratios,[9−13,15−18] poor pharmacokinetics,[12,13] short cellular retention in the tumor,[15−20] and modest accumulation of radioactivity in the tumor.[15−20]

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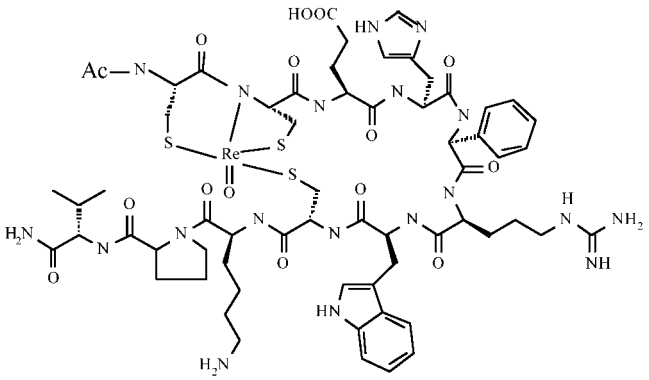


Figure 1. Structure of Ac-ReCCMSH.

Currently, some of the most promising agents for melanoma targeting are α-MSH analogues. It has been reported that most murine and human melanoma metastases bear α-MSH receptors.[23] This suggests that radiolabeling α-MSH peptides with appropriate radionuclides may allow melanoma targeting. α-MSH is a tridecapeptide (Ac-Ser[1]-Tyr[2]-Ser[3]-Met[4]-Glu[5]-His[6]-Phe[7]-Arg[8]-Trp[9]-Gly[10]-Lys[11]-Pro[12]-Val[13]-NH_2) that is primarily responsible for the regulation of skin pigmentation by controlling melanin synthesis and dispersal of melanocytes.[24] Structure−bioactivity studies have shown that His-Phe-Arg-Trp is the minimal receptor recognition sequence.[25] The replacement of Phe with D-Phe resulted in a 200 and 1500-fold increased potency in frog and lizard skin assays, respectively.[26] Several enzymatically stable, superpotent, and long-acting agonist analogues have been synthesized. For example, the linear α-MSH peptide analogue NDP (Ac-Ser-Tyr-Ser- Nle[4]-Glu-His-D-Phe[7]-Arg-Trp-Gly-Lys-Pro-Val-NH_2) is used routinely for biological assays of melanoma due to its high potency and prolonged activity.[27] It was reported that one or two NDP sequences conjugated to a DTPA chelate radiolabeled with ^{111}In showed modest tumor uptake [e.g., 1.26 ± 0.2% ID/g at 4 h postinjection (pi)]. However, most of the radioactivity had washed out of the tumor by 2 h pi, and extremely high amounts of nonspecific radioactivity had accumulated in the liver and kidneys. These results imply that this radiolabeled complex would not be suitable for routine clinical use.[38] Other strategies have been used to constrain the bioactive conformation of α-melanotropin, thereby decreasing the flexibility of the peptides and enhancing their resistance to proteolysis. These include side chain to side chain monocyclization, the N- to C-terminal monocyclization, and the bicyclization of the peptides. Ac-[Cys[4],Cys[10]]-α-MSH-NH_2, cyclized via a disulfide bond and Ac-Nle[4]-[Asp[5],D-Phe[7],Lys[10]]-α-MSH(4-10)-NH_2, cyclized via a lactam bridge, stand out as super potent and prolonged-acting peptides.[28] Another strategy used to control the conformation of the peptide is utilization of transition metals. It has been reported that transition metal ion complexation can form a well-defined rigid scaffold[29] and stabilize peptide α-helices and β-turns.[30,31] Our previous studies have incorporated the transition metals rhenium and technetium directly into α-MSH analogues through coordination to Cys sulfhydryls and amide nitrogens to generate novel cyclic α-MSH analogues (Figure 1).[32,33] Nuclear magnetic resonance (NMR) studies have shown that in the rhenium-cyclized peptide, ReCCMSH [ReO-(Ac-Cys[3]-Cys[4]-Glu-His-D-Phe[7]-Arg-Trp-Cys[10]-Lys-Pro-Val-NH_2)], the rhenium core was coordinated by three thiolate sulfurs from Cys[3,4,10] and one amide nitrogen from Cys[4] to form a square pyramidal coordination geometry about the Re(V) center with an oxo group at the apex. The in vivo biodistribution studies in both B16/F1 murine and TXM-13 human melanoma-bearing mouse models have shown that ^{99m}TcCCMSH and ^{188}ReCCMSH exhibit favorable biological properties (high tumor radioactivity uptake, high resistance to degradation, long cellular retention in the tumor, and rapid clearance from the normal organs).[32,34−35] These characteristics strongly suggest that ^{99m}TcCCMSH and ^{188}ReCCMSH could be excellent radioimaging/radiotherapy agents for melanoma. Moreover, it also suggests that ReCCMSH is an ideal structural motif for designing novel melanoma receptor targeting agents.

To expand the chelation ability of ReCCMSH to a wider variety of radionuclides for imaging and therapeutic applications, DOTA was selected for conjugation to the N terminus of ReCCMSH because of its strong coordination ability with a variety of γ, β, and α radiometal emitters. High tumor uptake and retention were observed for ^{111}In-DOTA-ReCCMSH.[36,37] DOTA conjugation increased the whole body clearance of ^{111}In-DOTA-ReCCMSH due to its increased hydrophilicity, resulting in a high tumor/blood ratio for ^{111}In-DOTA-ReCCMSH as compared to ^{99m}TcCCMSH.[36,37] Unfortunately, slightly elevated levels of radioactivity remained in the kidney, which may result in radionephrotoxicity and limit its use in therapeutic applications.

Here, we report on the chemistry, radiochemistry, biochemistry, and biology (including methods to minimize kidney retention) of a number of ^{111}In-DOTA-derivatized ReCCMSH analogues. We synthesized several new DOTA-ReCCMSH analogues, namely, Ac-Lys(DOTA)-ReCCMSH (**4**), DOTA-ReCCMSH(Arg[11]) (**6**), DOTA-ReCCMSH-OH (**8**), and DOTA-ReCCMSH-Asp-OH (**10**). Our objective was to optimize the in vivo biological properties of DOTA-ReCCMSH through modification of the structure of DOTA-ReCCMSH. The in vitro binding affinities of the peptides for the melanoma receptor were determined in the B16/F1 cell line. The in vivo biodistributions of the ^{111}In-labeled DOTA-ReCCMSH analogues were compared to that of ^{111}In-DOTA-ReCCMSH (**S**) in the B16/F1 murine melanoma subcutaneous C57 mouse model.

Results

Synthesis of 4. The reaction scheme for the synthesis of peptide **4** is shown in Figure 2. There are two Lys residues in Ac-Lys[1]-ReCCMSH, one in position 1 and the other in position 11. Specific conjugation of DOTA to the ϵ-amine group on Lys[1] required the use of Boc and Z protecting groups for the Lys[1] and Lys[11] residues, respectively. Because decomposition of ReCCMSH under high pH was observed (unpublished data), an acid labile protecting group for Lys[11] was preferred. Base labile protecting groups such as Dde for Lys[11] would not be useful. Fmoc/HBTU chemistry combined with TFA cleavage was used for the peptide synthesis; therefore, the protecting group for Lys[11] should possess sufficient acid stability to withstand conditions for the cleavage of the other protecting groups. The Z group is a very stable protecting group in base, dilute acid, and cold

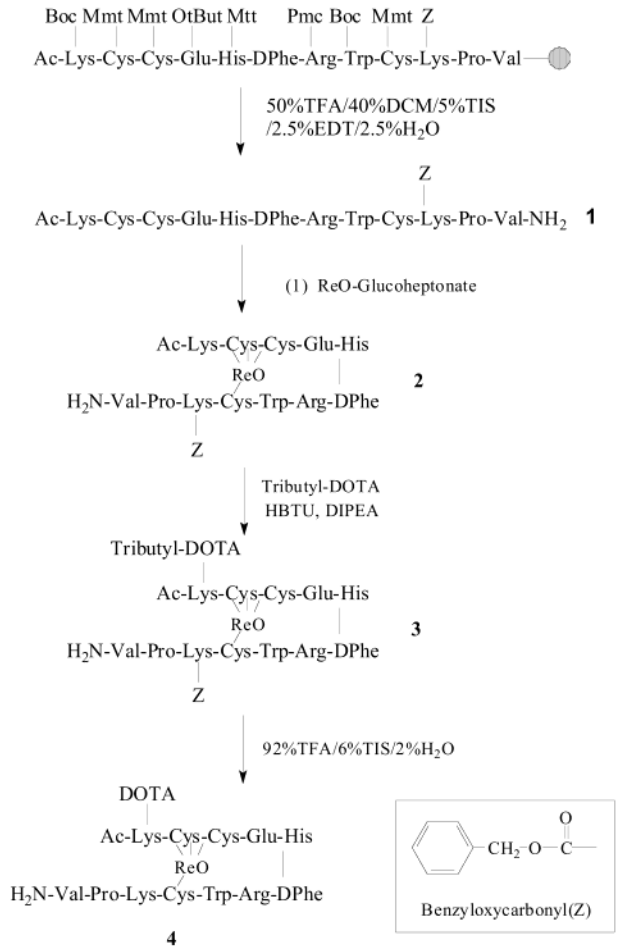


Figure 2. Reaction scheme for the synthesis of **4**.

TFA. Cleavage is usually accomplished with the strong acid HBr−AcOH, HF, or hydrogenation with palladium as the catalyst.[40] Therefore, the Z group was selected for protection of Lys,[11] and the acid labile Boc group was used for protection of Lys[1]. Although it was reported that deprotection of the Z group should be carried out in strong acid (HBr or HF), we found that greater than 90% cleavage occurred at room temperature using TFA and that the Z group was quite stable at low concentrations of TFA. Thus, 50% TFA was selected to cleave the protecting group in the first step in Figure 2, and a partially protected peptide **1** was successfully isolated. HPLC analysis showed its purity was greater than 90% after purification. The purified peptide was then reacted with Re-glucoheptonate for cyclization to yield complex **2**. Complex **2** was then conjugated with tributyl-protected DOTA under HBTU activation in mild base, and complex **3** was generated in greater than 70% yield. The final product **4** resulted after 4 h from cleavage of **3** in 92% TFA/6% TIS/2% water. HPLC analysis showed the purity of the peptide to be over 80%, suitable for ^{111}In labeling. Because four steps and three HPLC purifications were carried out during this synthesis, the isolated yield for the synthesis of peptide **4** was about 1−2%.

Synthesis of 6, 8, and 10. Peptides **6**, **8**, and **10** were synthesized using the same approach as for **S**, through a two step reaction involving linear peptide synthesis and rhenium cyclization, which is shown in Figure 3. Linear peptides **5**, **7**, and **9** were generally obtained in

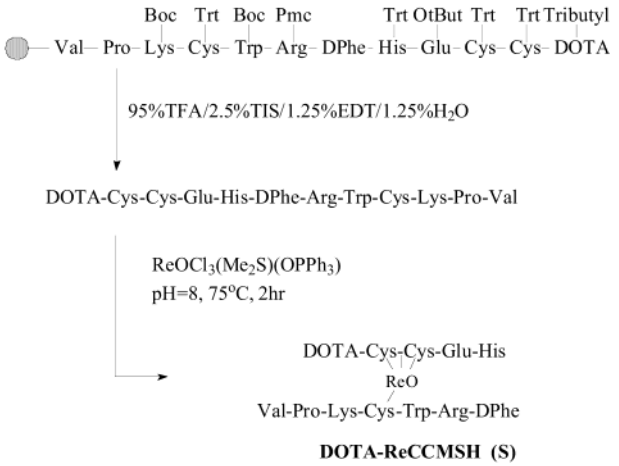


Figure 3. Reaction scheme for the synthesis of **S**.

40−60% yield and >95% purity. The rhenium-cyclized peptides were isolated in 20% yield. The rhenium precursor $ReOCl_3(Me_2S)(OPPh_3)$ was used for peptide cyclization. Complexes **6**, **8**, and **10** were produced with 95% purity in 10−30% overall yield.

All peptides synthesized above were characterized by electrospray ionization mass spectrometry (ESIMS). The measured molecular weight (MW) was consistent with the expected MW in all cases (Table 1). The HPLC gradients and retention times for the above peptides are given in Table 2.

Preparation of Indium-111-DOTA-Rhenium-Cyclized α-MSH Analogues. Using the gradients listed in Table 2, the radiolabeled peptides were completely separated from their nonradiolabeled counterparts by RP-HPLC. The radiochemical purity of In-111-labeled **4**, **6**, **8**, and **10** was >99%, and their specific activities were greater than 46 500 Ci/mmol. ^{111}In-labeled DOTA peptides were stable for over 24 h at room temperature in 0.01 M PBS (pH 7.4)/0.1% BSA.

In Vitro Cell Assays. The IC_{50} values of peptides **4**, **6**, **8**, and **10** were determined to be 3.3, 2.1, 15.8, and 78.6 nM, respectively, from competitive receptor binding assays (Table 1). The results indicate that coupling a DOTA chelate through the ϵ-amine of an N terminus Lys group or substituting Lys[11] with Arg only slightly decreases the receptor binding affinity as compared to **S** (1.2 nM). However, changing the C terminus from an amide to a carboxylate group or adding aspartic acid to the C terminus significantly decreases the affinity of the resultant peptide (10−100-fold decrease).

In Vivo Studies. Biodistribution data in B16/F1 murine melanoma-bearing C57 mice for ^{111}In-labeled peptides **4**, **6**, **8**, and **10** are listed in Table 3 (the biodistribution data for ^{111}In-labeled **S**, from ref 37, are also included for comparison). The Student's *t*-test for unpaired data was performed to compare ^{111}In-**S** with ^{111}In-labeled **4**, **6**, **8**, and **10**. Only *p* values of less than 0.05 are listed. As compared to ^{111}In-**S**, substitution of a single Lys[11] residue with Arg[11] significantly increased the in vivo tumor-targeting ability of ^{111}In-**6** at all investigated time points ($p < 0.05$) except 4 h (Table 3, Figure 4A). Moreover, the kidney uptake of ^{111}In-**6** was lower than ^{111}In-**S** at the 0.5 h pi time point with significance ($p < 0.05$) and without significance at 2 and 4 h pi (Table 3, Figure 4B).

Table 1. IC_{50} Values of the α-MSH Analogues and Their Expected and Measured MWs by ESIMS[a]

peptides	IC_{50}	sequence	expected (MW)	measured (MW)
S	1.2×10^{-9}	DOTA-ReCCEHdFRWCKPV-NH_2	1991	1991
1	*	Ac-Lys-CCEHdFRWCK(Z)PV-NH_2	1712.0	1711
2	*	Ac-Lys-ReCCEHdFRWCK(Z)PV-NH_2	1908.2	1909
3	*	Ac-Lys(tri-*tert*-butoxy-DOTA)-ReCCEHdFRWCK(Z)PV-NH_2	2462.9	2463
4	3.3×10^{-9}	Ac-Lys(DOTA)-ReCCEHdFRWCKPV-NH_2	2160.5	2161
5	*	DOTA-CCEHdFRWCRPV-NH_2	1820.1	1820
6	2.1×10^{-9}	DOTA-ReCCEHdFRWCRPV-NH_2	2018.3	2018
7	*	DOTA-CCEHdFRWCKPV-OH	1795.1	1794
8	1.6×10^{-8}	DOTA-ReCCEHdFRWCKPV-OH	1991.3	1992
9	*	DOTA-CCEHdFRWCKPVD-OH	1910.2	1909
10	7.9×10^{-8}	DOTA-ReCCEHdFRWCKPVD-OH	2106.4	2107

[a] Nle, norleucine; dF, D-phenylalanine; ND, not determined; *, IC_{50} values of the precursor were not determined.

Table 2. HPLC Gradients and Retention Times for DOTA-Peptide Synthesis and Analysis[a]

peptide	0−3 min (%)	3−23 min (%)	23−26 min (%)	26−29 min (%)	29−32 min (%)	RT (min)
1	38	48	90	90	38	11.9
2	37	47	90	90	37	18.8
3	47	57	90	90	47	14.5
4	21	31	90	90	18	23.3
5	21	31	90	90	21	7.5
6	15	25	90	90	15	19.7
7	23	33	90	90	23	8.6
8	24	34	90	90	24	16.5
9	21	31	90	90	21	9.0
10	24	34	90	90	24	12.8
^{111}In-**4**	18	28	90	90	18	15.9
^{111}In-**6**	16	21	90	90	16	19.2
^{111}In-**8**	19	25	90	90	19	18.6
^{111}In-**10**	19	25	90	90	19	12.1

[a] Note: solvent A, 0.1%TFA/H_2O; solvent B, 0.1%TFA/CH_3CN. The percentage of B in A for each sample is listed.

A Lys residue was added to the N terminus of ReCCMSH as a spacer, and DOTA was coupled to the ϵ-amino group of Lys[1] to form **4**. The tumor uptake of ^{111}In-**4** was lower than that of ^{111}In-**S**, without significance, at 0.5, 2, and 24 h pi and significantly at 4 h pi ($p < 0.05$). ^{111}In-**4** also exhibited high kidney uptake at all investigated time points ($p < 0.05$) (Table 3, Figure 4).

In-111-labeled rhenium-cyclized DOTA peptides **8** and **10**, which contain carboxylic acid end groups, exhibited lower tumor uptake at most time points. In addition, the kidney uptake of ^{111}In-**8** and ^{111}In-**10** are close to or higher than that of ^{111}In-**S**, giving no advantage to these two radiolabeled complexes in vivo as compared with ^{111}In-**S**.

The four new radiolabeled peptide complexes all showed fast blood clearance, as did ^{111}In-**S**. The blood uptake was only 0.07 ± 0.03, 0.09 ± 0.06, 0.21 ± 0.08, 0.11 ± 0.10, and 0.05 ± 0.03% ID/g for ^{111}In-labeled peptides **S**, **4**, **6**, **8**, and **10**, respectively, at 2 h pi, resulting in high tumor/blood ratios at this time point, as well as the later time points (Table 4). Although the concentration of radioactivity in the blood for ^{111}In-**6** was higher than that of ^{111}In-**S** at 2 h pi, the difference was not significant ($p > 0.05$). The radioactivity that had accumulated in the other normal tissues for these four radiolabeled peptides was very low, suggesting low radiation doses for these tissues when therapeutic radionuclides were used for peptide labeling. Like ^{111}In-**S**, the radioactivity of the four radiolabeled analogues was quickly and predominantly cleared through the kidneys into the urine. At 0.5 h pi, more than 75% of the administered radioactivity was excreted into the urine, and by 2 h pi, over 90% ID had cleared into the urine (Figure 5A). At the same time, low levels of radioactivity (only about 1% ID) were observed for these complexes in the gastrointestinal (GI) tract (Figure 5B).

The specificity of ^{111}In-**6** was determined in vivo in B16/F1 murine melanoma-bearing C57 mice 2 h pi in the presence and absence of 10 mg NDP (Table 5). Of the complexes evaluated, only ^{111}In-**6** showed improved biological properties as compared to ^{111}In-**S** (i.e., higher tumor uptake and retention and lower kidney uptake); so, it was the only one tested for in vivo specificity of tumor uptake. The blocking studies for ^{111}In-**S** were previously reported.[37] The results in Table 5 show that the tumor uptake of ^{111}In-**6** is specific and can be blocked with NDP.

Discussion

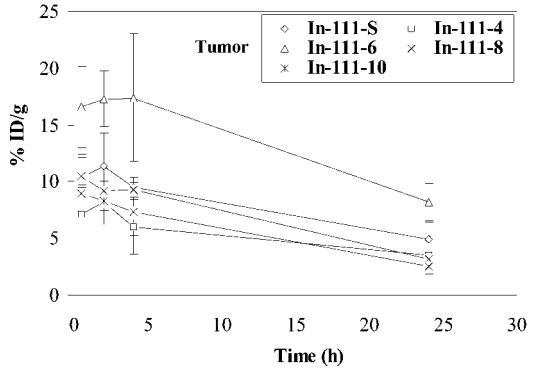
The presence of α-MSH receptors on most murine and human melanomas and internalization of receptor agonists suggested that radiolabeled α-MSH analogues might be used as therapeutic agents for melanoma. The ultimate objective of our research is to develop such an agent based on α-MSH analogues. Previously, we reported that ^{99m}TcCCMSH exhibited high melanoma tumor uptake, high tumor retention values, and low accumulation in the normal organs.[34,36] The macrocyclic chelate DOTA was conjugated to the amino terminus of ReCCMSH to facilitate labeling with a wider variety of radionuclides for therapeutic purposes including, but not limited to, Y-90 and the radiolanthanides. It was hypothesized that β- or α-emitting radiolabeled DOTA peptides would have the same biodistribution pattern as ^{111}In-labeled DOTA peptides. This assumption has been widely used in the development of peptide radiopharmaceuticals, especially for ^{90}Y-labeled peptide studies. Therefore,^{111}In-labeled rhenium-cyclized analogues were evaluated in primary studies to characterize the best candidate suitable for radiotherapy investigations.

The biodistribution data of ^{111}In-labeled **S** demonstrated that DOTA conjugation did not decrease the tumor-targeting capacity of the molecule. In fact, ^{111}In-**S** exhibited high tumor accumulation and retention as compared to ^{99m}TcCCMSH/^{188}ReCCMSH.[36] The clearance of ^{111}In-**S** from the body was rapid (92% of the administrated radioactivity cleared through the urine at 2 h pi). Although the accumulation of the radioactivity in the other normal tissues was low, ^{111}In-**S** showed high nonspecific kidney uptake, which seriously impedes the efficiency of **S** labeled with α or β radionuclides for therapeutic use.[36]

Table 3. Biodistribution Comparison for **S**, **4**, **6**, **8**, and **10** in B16/F1 Murine Melanoma-Bearing C57 Mice at 0.5, 2, 4, and 24 h pi (% ID/g)[a]

time (h)	complex	tumor	blood	muscle	heart	lungs	liver	spleen	pancreas	kidneys
0.5	**S**	10.36 ± 1.75	1.57 ± 0.37	0.42 ± 0.17	0.72 ± 0.21	1.70 ± 0.55	0.59 ± 0.15	0.59 ± 0.23	0.62 ± 0.31	13.0 ± 3.21
	4	7.15 ± 2.60	1.99 ± 0.59	0.42 ± 0.15	0.86 ± 0.20	1.51 ± 0.35	0.66 ± 0.10	0.51 ± 0.23	0.48 ± 0.18	23.41 ± 2.81[c]
	6	16.60 ± 3.62[b]	1.70 ± 0.14	0.39 ± 0.14	0.76 ± 0.18	1.66 ± 0.40	0.66 ± 0.05	0.78 ± 0.12	0.41 ± 0.03	9.31 ± 0.98[b]
	8	10.44 ± 1.98	1.56 ± 0.46	0.39 ± 0.11	0.68 ± 0.34	1.21 ± 0.38	0.45 ± 0.09	0.40 ± 0.16	0.39 ± 0.05	11.47 ± 3.22
	10	8.96 ± 0.49	1.33 ± 0.17	0.25 ± 0.07	0.44 ± 0.24	1.16 ± 0.09	0.43 ± 0.05	0.29 ± 0.11[b]	0.27 ± 0.01[b]	11.53 ± 0.66
2	**S**	11.45 ± 2.89	0.065 ± 0.026	0.047 ± 0.033	0.066 ± 0.054	0.18 ± 0.07	0.23 ± 0.01	0.090 ± 0.107	0.048 ± 0.026	8.98 ± 0.82
	4	8.15 ± 1.90	0.090 ± 0.062	0.020 ± 0.018	0.080 ± 0.069	0.18 ± 0.07	0.24 ± 0.04	0.11 ± 0.07	0.074 ± 0.065	18.45 ± 3.20[c]
	6	17.29 ± 2.49[c]	0.21 ± 0.08	0.030 ± 0.032	0.070 ± 0.091	0.30 ± 0.09	0.38 ± 0.04[d]	0.27 ± 0.19	0.080 ± 0.076	8.72 ± 1.34
	8	9.19 ± 1.24	0.11 ± 0.10	0.054 ± 0.010	0.058 ± 0.057	0.18 ± 0.05	0.15 ± 0.01[d]	0.20 ± 0.13	0.030 ± 0.019	8.80 ± 1.76
	10	8.29 ± 0.91	0.050 ± 0.029	0.13 ± 0.08	0.070 ± 0.059	0.16 ± 0.05	0.16 ± 0.02	0.23 ± 0.19	0.029 ± 0.020	8.07 ± 1.81
4	**S**	9.46 ± 0.90	0.034 ± 0.033	0.093 ± 0.056	0.12 ± 0.10	0.11 ± 0.05	0.20 ± 0.04	0.22 ± 0.10	0.043 ± 0.036	9.27 ± 2.65
	4	6.01 ± 2.36[b]	0.082 ± 0.041	0.017 ± 0.024	0.029 ± 0.056	0.18 ± 0.11	0.29 ± 0.09	0.19 ± 0.11	0.057 ± 0.023	19.02 ± 2.63[c]
	6	17.41 ± 5.61	0.085 ± 0.029	0.086 ± 0.071	0.054 ± 0.052	0.13 ± 0.04	0.30 ± 0.04[c]	0.27 ± 0.26	0.015 ± 0.021	7.37 ± 1.13
	8	9.27 ± 0.68	0.056 ± 0.024	0.035 ± 0.009	0.12 ± 0.11	0.10 ± 0.03	0.14 ± 0.02[b]	0.15 ± 0.14	0.074 ± 0.042	8.70 ± 0.88
	10	7.32 ± 2.09	0.051 ± 0.028	0.047 ± 0.012	0.054 ± 0.039	0.097 ± 0.067	0.16 ± 0.03	0.16 ± 0.08	0.031 ± 0.032	8.13 ± 1.47
24	**S**	4.86 ± 1.52	0.010 ± 0.019	0.031 ± 0.011	0.13 ± 0.12	0.060 ± 0.047	0.16 ± 0.03	0.14 ± 0.06	0.027 ± 0.029	5.64 ± 1.31
	4	3.54 ± 1.09[b]	0.072 ± 0.123	0.11 ± 0.14	0.13 ± 0.11	0.14 ± 0.11	0.28 ± 0.04[c]	0.21 ± 0.15	0.18 ± 0.20	13.39 ± 1.68[c]
	6	8.19 ± 1.63[b]	0.060 ± 0.042	0.14 ± 0.10	0.36 ± 0.16	0.24 ± 0.14	0.32 ± 0.04[c]	0.32 ± 0.25	0.081 ± 0.053	5.64 ± 0.52
	8	3.14 ± 0.37	0.10 ± 0.10	0.14 ± 0.04	0.15 ± 0.14	0.11 ± 0.12	0.17 ± 0.02	0.22 ± 0.05	0.064 ± 0.084	7.35 ± 0.56
	10	2.48 ± 0.57	0.018 ± 0.022	0.082 ± 0.135	0.017 ± 0.028	0.035 ± 0.027	0.19 ± 0.02	0.043 ± 0.086	0.017 ± 0.029	6.28 ± 0.82

[a] $n = 4$ for complexes **4**, **6**, **8**, and **10** and 5 for complex **S**; mean ± SD. [b] $0.05 > p > 0.01$. [c] $0.01 > p > 0.001$. [d] $p < 0.001$.



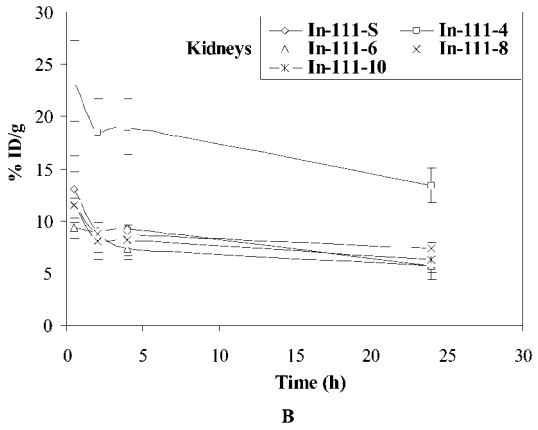


Figure 4. Tumor and kidney uptake (% ID/g) curves for In-111-labeled **S**, **4**, **6**, **8**, and **10**. Bars, SD.

Two strategies are generally used to decrease the renal uptake of the radiolabeled peptides. One is the pre- or coadministration of amino acids and their derivatives. The second is modification of the structure of the peptide to optimize the in vivo biological properties of the radiolabeled peptide. The renal accumulation of monoclonal antibodies or peptides can be reduced in both mice and patients through coinjection or infusion of amino acids.[39] The administration of lysine reduces the renal dose about 5-fold and increases the maximum tolerable dose (MTD) by 25% for radiolabeled Fab fragments.[41] We demonstrated that a single coinjection of 30 mg of L-lysine decreased kidney uptake by 48, 55, and 70% at 30 min, 1, and 4 h pi, respectively, without affecting the tumor uptake of ^{99m}TcCCMSH.[34]

Although administration of L-lysine has proved to be an effective method for reducing renal uptake in our system, synthesis of rationally designed peptides that optimize in vivo biological properties such as higher tumor uptake and lower background for normal tissues is still a goal. Thus, four peptides were designed and evaluated in this study for their ability to reduce kidney retention.

The structure of **S** is fairly rigid, and the macrocyclic group DOTA is in close proximity to the rhenium core. This may influence the binding affinity of the peptide and result in some unfavorable properties. One lysine residue was added as a spacer to increase the distance between the macrocyclic group DOTA and the rhenium-cyclized core. We also hoped that it would act as a pharmacokinetic modifier. However, in vitro studies showed that the IC_{50} value of **4** is approximately two times larger (lower affinity) than that of **S** (Table 1). The in vivo biodistribution data showed that in comparison to ^{111}In-**S**, ^{111}In-**4** had lower tumor uptake at all time points investigated. Moreover, as compared with ^{111}In-**S** and the other three peptides investigated in this study, significantly higher kidney accumulation and retention were observed for ^{111}In-labeled **4**. Radiolabeled antibodies and peptide in which the bifunctional chelate is conjugated through a lysine ϵ-amino group (or other amino acid residue) have shown high kidney uptake and retention.[46−50] In these studies, the ^{111}In-DTPA-ϵ-amino-lysine, ^{67}Ga-2-(p-SCN-Bz)-NOTA-ϵ-amino-lysine/methionine, and ^{111}In-DOTA-ϵ-amino-lysine were observed as the major metabolites in the kidneys.[46−50] However, injection of ^{111}In-DOTA-ϵ-amino-lysine directly into mice resulted in rapid urinary clearance without kidney retention, suggesting that the radiolabeled antibody was most likely responsible for the kidney retention.[50] Our results for ^{111}In-**4**, in which the DOTA is conjugated through an ϵ-amino group of Lys[1],

Table 4. Comparison in Uptake Ratio of Tumor/Normal Tissues (Each in % ID/g) Among **S**, **4**, **6**, **8**, and **10** in B16/F1 Murine Melanoma-Bearing C57 Mice at 0.5, 2, 4, and 24 h pi[a]

time (h)	complex	blood	muscle	kidneys	liver	lungs
0.5	**S**	6.9 ± 2.0	27.8 ± 10.2	0.85 ± 0.28	18.4 ± 5.0	6.6 ± 2.1
	4	3.8 ± 1.7	17.6 ± 4.8	0.30 ± 0.06	10.7 ± 3.0	4.7 ± 1.2
	6	9.7 ± 1.4	47.4 ± 23.0	1.8 ± 0.3	25.2 ± 6.4	11.1 ± 5.8
	8	6.9 ± 1.2	27.9 ± 6.4	0.95 ± 0.22	23.1 ± 1.2	9.0 ± 1.6
	10	6.8 ± 0.8	38.2 ± 13.3	0.78 ± 0.01	20.7 ± 1.5	7.8 ± 0.5
2	**S**	196.4 ± 77.7	318.9 ± 148.1	1.3 ± 0.3	49.9 ± 11.2	69.4 ± 25.1
	4	175.1 ± 193.0	468.0 ± 360.2	0.46 ± 0.16	33.3 ± 6.9	48.6 ± 18.0
	6	93.7 ± 36.9	2100 ± 3435	2.0 ± 0.4	45.7 ± 1.8	65.4 ± 30.2
	8	142.9 ± 105.4	174.4 ± 29.4	1.1 ± 0.1	63.0 ± 9.3	52.8 ± 13.8
	10	223.6 ± 132.5	101.9 ± 77.9	1.1 ± 0.2	51.3 ± 4.9	56.6 ± 21.2
4	**S**	489.8 ± 304.4	159.3 ± 122.7	1.1 ± 0.3	49.4 ± 12.2	100.3 ± 39.6
	4	88.7 ± 49.8	3404.8 ± 5680.2	0.28 ± 0.11	20.8 ± 6.0	48.4 ± 43.4
	6	254.2 ± 130.0	193.2 ± 90.3	2.5 ± 1.3	60.2 ± 29.2	122.1 ± 55.9
	8	199.6 ± 101.6	277.2 ± 70.6	1.1 ± 0.1	69.1 ± 11.5	92.0 ± 20.4
	10	199.3 ± 151.9	156.1 ± 33.5	0.91 ± 0.28	46.8 ± 16.1	104.3 ± 68.0
24	**S**	601.0 ± 215.0	173.2 ± 84.2	0.88 ± 0.26	32.7 ± 13.3	168.6 ± 160.2
	4	67.2 ± 72.2	13.3 ± 0.5	0.27 ± 0.09	12.7 ± 3.5	22.9 ± 16.3
	6	95.0 ± 24.1	42.2 ± 8.4	1.5 ± 0.5	25.6 ± 1.6	43.7 ± 23.5
	8	22.4 ± 11.1	26.8 ± 6.4	0.43 ± 0.05	18.5 ± 3.7	85.1 ± 105.1
	10	65.5 ± 24.4	27.3 ± 26.1	0.40 ± 0.11	12.9 ± 4.1	105.3 ± 60.0

[a] $n = 4$ for complexes **4**, **6**, **8**, and **10** and 5 for complex **S**.

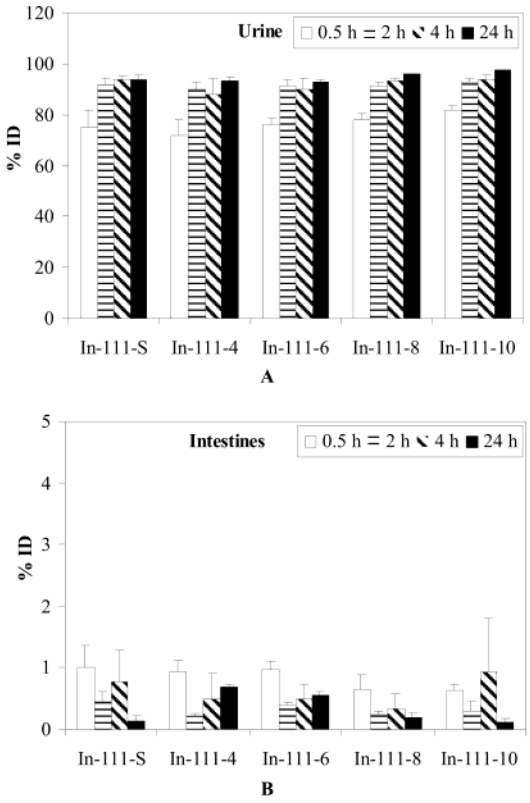


Figure 5. Clearance for In-111-labeled **S**, **4**, **6**, **8**, and **10** in B16/F1 murine melanoma-bearing C57 mice. Bars, SD.

are consistent with the literature reports (Table 3, Figure 4B). However, comparing ^{111}In-**4** with the other four compounds reported in this study suggests that the Ac-Lys^1 is not useful for conjugating the DOTA to this peptide.

Two peptides, **8** and **10**, were designed to investigate the influence of the overall charge of the complexes on their biodistribution. Theoretically, under physiological conditions, the overall charges for ^{111}In-labeled **S**, **8**, and **10** are 0, −1, and −2, respectively, assuming the histidine is uncharged at physiologic pH (7.4). Biodistribution studies show that the tumor uptake increased in the order ^{111}In-**10** < ^{111}In-**8** < ^{111}In-**S**. It is possible

Table 5. Uptake of ^{111}In-**6** in Various Tissues (% ID/g) at 2 h pi in the Presence and Absence of 10 mg NDP

	unblocked	blocked
tumor	17.29 ± 2.49	1.95 ± 0.46
blood	0.21 ± 0.08	0.27 ± 0.16
muscle	0.03 ± 0.03	0.18 ± 0.24
heart	0.07 ± 0.09	0.33 ± 0.35
lung	0.30 ± 0.09	0.44 ± 0.11
liver	0.38 ± 0.04	1.61 ± 0.38
spleen	0.27 ± 0.19	0.47 ± 0.21
pancreas	0.08 ± 0.08	0.11 ± 0.01
kidney	8.72 ± 1.34	11.23 ± 6.97

that the decreased tumor-targeting abilities of ^{111}In-**10** and ^{111}In-**8** in vivo result from both the lower in vitro binding affinities of peptides **8** and **10** and the increased total charge and hydrophilic properties of the radiolabeled complexes. Additionally, the kidney uptake of ^{111}In-**10** and ^{111}In-**8** is close to that of ^{111}In-**S** at 30 min, 2, 4, and 24 h pi (Table 3), suggesting that the increased negative charge of these radiolabeled peptides does not affect the in vivo renal uptake and handling. It is the local distribution of the charge in peptide, not the overall charge, that is probably responsible for binding with the luminal membrane of the kidney. This result is also in agreement with our hypothesis that the positively charged amine group of the Lys^{11} residue in the ReCCMSH sequences is primarily responsible for the nonspecific retention of radioactivity in the kidneys. The positively charged amine group in lysine or the guanidino group in arginine can bind with the negatively charged sites on the renal tubular cells after glomerular filtration, thus increasing the renal uptake of radiolabeled peptides.[51,52] High kidney accumulation of radioactivity is observed for ^{111}In-DTPA-octreotide, but a recent study shows that substitution of the Phe^1 residue in ^{111}In-DTPA-octreotide with the basic amino acid residue Lys significantly increased the renal uptake, while substitution with an Asp residue decreased the renal uptake.[53,54]

Our previous results showed that replacement of Lys^{11} with Nle^{11} or Gly^{11} in ^{99m}TcCCMSH significantly reduced the kidney uptake and altered its clearance from urinary to the GI tract, suggesting that substitu-

tion of Lys^{11} with an appropriate amino acid residue might generate a peptide with good pharmacokinetic properties. Although only the "His-Phe-Arg-Trp" sequence is reported essential for receptor binding, we found that the Nle^{11}- or Gly^{11}-substituted ^{99m}TcCCMSH peptides exhibited significantly lower tumor uptake, demonstrating that Lys^{11} is an important component for in vivo tumor targeting in this metal-cyclized molecule.[5] Arginine was, therefore, substituted for Lys^{11} since its side chain structure is similar to that of lysine. Arginine is also positively charged at physiologic pH; however, the positive charge in arginine is more delocalized. The IC_{50} value of **6** was found to be slightly higher (lower affinity) than that of **S** (2.1 vs 1.2 nM, Table 1). The in vivo studies, however, showed that ^{111}In-**6** had significantly higher tumor uptake at almost all of the time points investigated and lower kidney uptake with significance at 30 min and without significance at 2 and 4 h as compared to ^{111}In-**S**. The tumor uptake for ^{111}In-**6** was determined to be specific for the α-MSH receptor, as it was for ^{111}In-**S**,[37] through blocking studies using the superpotent α-MSH receptor agonist NDP. Of the four ^{111}In-labeled complexes investigated, ^{111}In-**6** shows the highest tumor and lowest kidney uptake at all time points investigated and thus has the highest tumor/kidney ratio (Table 4). This finding suggests that a guanidino side chain may be a better fit for the receptor than the ϵ-amino side chain in the rhenium-cyclized CCMSH system. This result suggests that the in vitro receptor binding affinity of a complex alone does not determine its in vivo tumor uptake. The in vivo tumor-targeting process is much more complex than in vitro cell binding, and complex stability, hydrophobicity, and pharmacokinetics play important roles in tumor targeting.

Conclusions

Several DOTA-rhenium-cyclized peptides were synthesized and evaluated. ^{111}In-**6** showed higher melanoma tumor uptake and lower kidney uptake than the other compounds that were investigated. The tumor uptake of ^{111}In-**6** was determined to be specific for the α-MSH receptor. This suggests that **6** is a superior agent for further radiotherapeutic investigations.

Experimental Section

Abbreviations. The abbreviations of the amino acids are in accordance with the recommendation of IUPAC-IUB.[42] Additional abbreviations are as follows: NDP, [Nle,^{4}D-Phe^7]α-MSH; ReCCMSH, ReO[$Cys^{3,4,10}$,D-Phe^7]α-MSH_{3-13}; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DOTA-ReCCMSH, DOTA-ReO[$Cys^{3,4,10}$,D-Phe^7]α-MSH_{3-13}; Fmoc, 9-Fluorenylmethyloxycarbonyl; HBTU, O-benzotriazolyl-tetramethyluronium hexafluorophosphate; Boc, *tert*-Butyloxycarbonyl; Z, benzyloxycarbonyl; Dde, 4,4-dimethyl-2,6-dioxocyclohexa-1-ylidene; Mmt, 4-methoxytrityl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Mtt, 4-methyltrityl; DIPEA, N-diisopropylethylamine; TFA, trifluoroacetic acid; DCM, dichloromethane; TIS, thioanisole; EDT, ethanedithiol; DTT, dithiothreitol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RP-HPLC, reverse phase high performance liquid chromatography; SPPS, solid phase peptide synthesis; % ID/g, percent injected dose per gram; % ID, percent injected dose.

General Aspects. DOTA-tri-*tert*-butyl ester was obtained from Macrocyclics Inc. (Richardson, TX). Fmoc amino acids and resins were purchased from Nova-biochem Co. (San Diego, CA) and Advanced Chemtech (Louisville, KY). All other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Solutions were made using distilled deionized water (>18 MW resistivity). $^{111}InCl_3$ (specific activity 1720 GBq/mmol) was purchased from Mallinckrodt Medical, Inc. (St. Louis, MO). The Synergy 432A desktop solid phase peptide synthesizer was obtained from Applied Biosystems (Foster City, CA). HPLC was performed on an ISCO chromatography system equipped with an UV−vis absorbance detector (ISCO Inc., Lincoln, NE). Both semipreparative (Vydac 218TP54 C18, 4.6 mm × 250 mm) and analytical (Vydac 218TP54 C18, 4.6 mm × 250 mm) RP-HPLC columns were used. The mobile phase was solvent A, 0.1% TFA/H_2O, and solvent B, 0.1%TFA/acetonitrile. ESIMS was performed on a Vestec 201 mass spectrometer at low potential at MASS Consortium Co. (San Diego, CA). B16/F1 murine melanoma cells were obtained from the American Type Tissue Culture Collection. C57 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN).

Synthesis of 4. The preparation of **4** is shown in Figure 2. The partially protected peptide Ac-Lys-CCMSH(Lys^{11}-(Z)) (**1**) was synthesized by conventional SPPS techniques on an automatic peptide synthesizer, using standard Fmoc/HBTU chemistry on rink amide MBHA resin. The peptides were N-terminal-acetylated by activation with 75 mmol acetic acid after deprotection of the N-terminal amino acid residues. Peptide cleavage and deprotection were carried out by a 3 h incubation in a mixture of TFA/DCM/TIS/EDT/H_2O (50:45:2.5:1.25:1.25). The mixture was filtered, and the peptide in solution was precipitated with diethyl ether. The resulting peptide was washed four times with ice-cold diethyl ether, dried, and dissolved in 1 mM DTT. The peptide was purified by RP-HPLC on a C-18 column (Table 2). Fractions were collected and lyophilized. The target product was characterized by ESIMS and ready for use in the next step in the reaction (Table 1).

The rhenium cyclization of peptide **1** was carried out using glucoheptonate as a transfer ligand. Re(V)O-glucoheptonate was prepared using the procedure reported previously.[43] Briefly, 50−100 mg of Na-glucoheptonate and 5 mg of NH_4ReO_4 were dissolved in a 250 mL 5% Na_2EDTA solution. Then, 6.4 mg of $SnCl_2 \cdot 2H_2O$ in 10 mL of 0.1 M HCl was added to the solution, and the pH of the solution was adjusted to 5 with 1 M NaOH. After the solution sat at room temperature for 10 min, the color of the solution changed to blue-purple from colorless, indicating the formation of the complex.

An aqueous solution containing 2 mg/mL of peptide **1** was prepared. Then, freshly prepared Re-glucoheptonate (2:1 molar ratio of Re-glucoheptonate:peptide) was added, and the pH was adjusted to 8 using 1.0 M NaOH. The solution was heated at 75 °C for 2 h, during which time the color of the solution changed to light brown. The solution was purified by RP-HPLC. Fractions containing Ac-Lys-ReCCMSH(Lys^{11}-(Z)) (**2**) were collected and lyophilized.

A solution containing 26.7 μL of HBTU (10.8 μg/μL DMF), 26.2 μL of tri-*tert*-butoxy-DOTA (16.6 μg/μL DMF), and 0.5 μL of N,N-diisopropylethylamine (DIPEA) was prepared and incubated for 10 min at room temperature (RT) in a small vial. Then, peptide **2** (58 μL, 5 μg/μL DMF) was added and the resulting solution was incubated for another 15−30 min at RT. The reaction mixture was injected into a RP-HPLC for purification, and the main product Ac-Lys(Tri-*tert*-butoxy-DOTA)-ReCCMSH(Lys^{11}-(Z)) (**3**) was collected and lyophilized (Table 2).

Deprotection of the tri-*tert*-butoxy and benzyloxycarbonyl (Z) groups from peptide **3** was achieved after a 4 h of incubation in a mixture of TFA/TIS/H_2O (92:6:2) at RT. The peptide was precipitated with diethyl ether from the solution and washed four times with ice-cold diethyl ether. The final product **4** was dried by gently blowing off the diethyl ether with N_2. Peptide **4** was dissolved in H_2O and analyzed by RP-HPLC (Table 2).

Synthesis of 6, 8, and 10. The approach for the synthesis of peptides **6**, **8**, and **10** is the same as for the **S** synthesis, which is shown in Figure 3. The linear peptides DOTA-CCMSH(Arg^{11}) (**5**), DOTA-CCMSH-OH (**7**), and DOTA-CCMSH-Asp-OH (**9**) were prepared using the SPPS technique

with rink amide MBHA resin (for peptide **5**), Fmoc-Val-Wang resin (for peptide **7**), or Fmoc-Asp(OtBu)-Wang resin (for peptide **9**). Following purification by RP-HPLC (Table 2), they were cyclized by rhenium coordination using the method described previously to make the final products **6**, **8**, and **10**.[44] Briefly, the peptides **5**, **7**, or **9** and $ReOCl_3(Me_2S)(OPPh)_3$ were dissolved in 60% MeOH/40% H_2O solution with a molar ratio of 1:1.5, and the pH was adjusted to approximately 8 with 1.0 M NaOH. The solution turned to orange-brown upon heating at 70 °C for 60 min. The mixture was centrifuged to remove an insoluble precipitate, and the rhenium−peptide complex in the supernatant was purified by RP-HPLC on a C18 semipreparative column (Table 2). Product elution was monitored at either 280 or 412 nm using a UV−vis detector. The product peak was collected, lyophilized, and identified by ESIMS (Table 1).

Preparation of Indium-111-DOTA Rhenium-Cyclized α-MSH Analogues. Radiolabeled complexes of ^{111}In with the DOTA-ReCCMSH analogues were prepared using a 0.1 M NH_4OAc-buffered solution at pH 5.5. Briefly, 20 μL of $^{111}InCl_3$ (5 mCi/500 μL in 0.04 M HCl), 80 μL of 0.1 M NH_4OAc (pH 5.5), and 10 μg of peptide were mixed and incubated at 70 °C for 45 min. The radiolabeled complex was purified by RP-HPLC (Table 2). Purified radiolabeled peptides were flushed with N_2 to remove the acetonitrile, and the pH was adjusted to neutral with 0.2 M sodium phosphate (pH 8.0)/15 mM NaCl.

In Vitro Cell Assays. The in vitro cell binding assays were performed with the murine melanoma cell line B16/F1 as previously described.[36,45] Briefly, cells were seeded at a density of 0.2 million/well in 24 well tissue culture plates and allowed to attach overnight. Following a wash with the binding medium (Modified Eagle's Media with 25 mM HEPES, 0.2% BSA, and 0.3 mM 1,10-phenanthroline), the cells were incubated at 25 °C for 2 h with NDP (peptide concentration varying from 10^{-14}−10^{-6} M) and approximately 50 000 counts per minute (cpm) of ^{125}I-(Tyr^2)-NDP in 0.5 mL of binding media. The cells were rinsed twice with 0.01 M PBS (pH 7.4)/0.2% BSA and lysed in 0.5 mL of 1.0 M NaOH for 5 min, and the radioactivity of the cells was measured. The data were analyzed using the RADLIG computer program (Biosoft, Ferguson, MO), and the IC_{50} values, the concentration of competitor required to inhibit 50% of the radioligand binding, of the DOTA-coupled α-MSH peptide analogues were calculated.

Animal Biodistribution Studies. All animal studies were carried out in compliance with Federal and local institutional rules for the conduct of animal experimentation. C57 BL/6 female mice, 7−8 weeks old, were inoculated subcutaneously in the right flank with 1×10^6 cultured B16/F1 murine melanoma cells. Nine to ten days postinoculation, when the tumors had grown to a weight of ∼500 mg, the mice were injected with 2 mCi of ^{111}In-labeled peptide through the tail vein and housed separately, and their urine and feces were collected. The mice were sacrificed at different time points from 30 min to 24 h pi. Tumor and normal tissues of interest were removed and weighed, and their radioactivity was measured in a γ-counter. The radioactivity uptake in the tumor and normal tissues was expressed as a percentage of the injected radioactive dose per gram of tissue (% ID/g) or percentage of the injected dose (% ID).

Statistical Method. Statistical analysis was performed using the Student's *t*-test for unpaired data. A 95% confidence level was chosen to determine the significance between compounds, with $p < 0.05$ being significantly different.

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References

(1) Rigel, D. S.; Carucci, J. A. Malignant melanoma: prevention, early detection, and treatment in the 21st century. *CA Cancer J. Clin.* **2000**, *50*, 215−236.
(2) Boring, C. G.; Squires, T. S.; Tong, T. Cancer Statistics 1994. *CA Cancer J. Clin.* **1994**, *44*, 7−26.
(3) Mackie, R. M. The march of melanoma. *Clin. Exp. Dermatol.* **1993**, *18*, 2.
(4) McLaughlin, W. H.; Thramann, W. M.; Lambrecht, R. M.; Milius, R. A,; Bloomer, W. D. Preliminary observation of malignat melanoma therapy using radiolabeled alpha-methyltyrosine. *J. Surg. Oncol.* **1988**, *37*, 192−197.
(5) Link, E. M.; Carpenter, R. N.; Hansen, G. [^{211}At] Methylene blue for targeted radiotherapy of human melanoma xenografts: dose fraction in the treatment of cutaneous tumors. *Eur. J. Cancer* **1996**, *32A*, 1240−1247.
(6) Kagan, R.; Witt, T.; Bines, S. Gallium-67 scanning for malignant melanoma. *Cancer* **1988**, *61*, 272−274.
(7) Delbeke, D. Oncological application of FDG PET imaging: brain tumor, colorectal cancer lymphoma and melanoma. *J. Nucl. Med.* **1999**, *40*, 591−603.
(8) Ruhlmann, J.; Oehr, P.; Stegemann, G.; Steen, K.; Menzel, C.; Biersack, H. J. FDG-PET compared to conventional diagnostic methods in malignant melanoma. *J. Nucl. Med.* **1999**, *40*, 20P.
(9) Cornelius, E. A.; Neumann, R. D.,; Zoghbi, S. S. Intrapatient and interpatient comparison of tumor size and monoclonal antibody uptake in melanoma. *Clin. Nucl. Med.* **1988**, *13*, 243−249.
(10) Serafini, A. N.; Kotler, J.; Feun, L.; Dewanjee, M.; Robinson, D.; Salk, D.; Sfakianakis, G.; Abrams, P.; Savaraj, N.; Goodwin, D. Technetium-99m labeled monoclonal antibodies in the detection of metastatic melanoma. *Clin. Nucl. Med.* **1989**, *14*, 580−597.
(11) Eary, J. F.; Schroff, R. W.; Abrams, P. G.; Fritzberg, A. R.; Morgan, A. C.; Kasina, S.; Reno, J. M.; Srinivasan, A.; Woodhouse, C. S.; Wilbur, D. S.; Natale, R. B.; Collins, C.; Stehlim, J. S.; Mitchell, M.; Nelp, W. B. Successful imaging of malignant melanoma with technetium-99m-labeled monoclonal antibodies. *J. Nucl. Med.* **1989**, *30*, 25−32.
(12) Kloss, G.; Leven, M. Accumulation of radioiodinated tyrosine derivatives in the adrenal medulla and in melanomas. *Eur. J. Nucl. Med.* **1979**, *4*, 179−186.
(13) Bubeck, B.; Eisenhut, M.; Heimke, U.; Zum, W. K. Melanoma affine radiopharmaceuticals. I. A comparative study of ^{131}I labeled quinoline and tyrosine derivatives. *Eur. J. Nucl. Med.* **1981**, *6*, 227−233.
(14) Link, E. M.; Carpenter, R. N. ^{211}At-Methylene blue for targeted radiotherapy of human melanoma xenografts: treatment of micrometastases. *Cancer Res.* **1990**, *50*, 2963−2967.
(15) Moreau, M. F.; Michelot, J.; Papon, J.; Bayle, M.; Labarre, P.; Madelmont, J. C.; Parry, D.; Boire, J. Y.; Moins, N. Synthesis, radiolabeling, and preliminary evaluation in mice of some (N-diethylaminoethyl)-4-iodobenzamide derivatives as melanoma imaging agents. *Nucl. Med. Biol.* **1995**, *22*, 737−747.
(16) Moreau, M. F.; Madelmont, J. C.; Michelot, J.; Labarre, P.; Veyre, A.; Papon, J.; Bayle, M.; Boire, J. Y.; Pesplanch, G.; Meyniel, G. New ^{125}I-radiopharmaceuticals for diagnosis and treatment for malignant melanoma. *Eur. J. Nucl. Med.* **1993**, *18*, 538.
(17) Michelot, J. M.; Moreau, M. F. C.; Labarre, P. G.; Madelmont, J. C.; Veyre, A. J.; Papon, J. M.; Parry, D. F.; Bonafous, J. F.; Boire, J. Y. P.; Desplanches, G. G.; Bertrand, S. J.; Meyniel, G. Synthesis and evaluation of new iodine-125-radiopharmaceuticals as potential tracers for malignant melanoma. *J. Nucl. Med.* **1991**, *32*, 1573−1580.
(18) Eisenhut, M.; Hull, W. E.; Mohammed, A.; Mier, W.; Lay, D.; Just, W.; Gorgas, K.; Lehmann, W. D.; Haberkorn, U. Radioiodinated N-(2-Diethylaminoethyl)benzamide derivatives with high melanoma uptake: structure-affinity relationships, metabolic fate, and intracellular localization. *J. Med. Chem.* **2000**, *43*, 3913−3922.
(19) Auzeloux, P.; Papon, J.; Azim, E. M.; Borel, M.; Pasqualini, R.; Veyre, A.; Madelmont, J.-C. A potential melanoma tracer: synthesis, radiolabeling, and biodistribution in mice of a new nitridotechnetium Bis(aminothiol) derivative pharmacomodulated by a N-(Diethylaniboethyl)benzamide. *J. Med. Chem.* **2000**, *43*, 190−198.
(20) Friebe, M.; Mahmood, A.; Spies, H.; Berger, R.; Johannsen, B.; Mohammed, A.; Eisenhut, M.; Bolzati, C.; Davison, A.; Jones, A. G. '3+1' mixed ligand oxotechnetium(V) complexes with affinity for melanoma: synthesis and evaluation in vitro and in vivo. *J. Med. Chem.* **2000**, *43*, 2745−2752.
(21) Bard, D. R.; Wraight, E. P.; Knight, C. G. BisMSH-DTPA. A potential imaging agent for malignant melanoma. *Ann. N. Y. Acad. Sci.* **1993**, *680*, 451−453.

(22) Sivolapenko, G. B.; Skarlos, D.; Pectasides, D.; Stathopoulou, E.; Milonakis, A.; Sirmalis, G.; Stuttle, A.; Courtenay-Luck, N. S.; Konstantinides, K.; Epenetos, A. A. Imaging of metastatic melanoma utilizing a technetium-99m labeled RGD-containing synthetic peptide. *Eur. J. Nucl. Med.* **1998**, *25*, 1383−1389.
(23) Siegrist, W.; Solca, F.; Stutz, S.; Giuffre, L.; Carrel, S.; Girard, J.; Eberle, A. N. Characterization of receptor for a-melanocyte stimulating hormone on human melanoma cells. *Cancer Res.* **1989**, *49*, 6352−6358.
(24) Cone, R. D.; Mountjoy, K. G.; Robbins, L. S.; Nadeau, J. H.; Johnson, K. R.; Roselli-Rehfuss, L.; Mortrud, M. T. Cloning and functional characterization of a family of receptors for the melanotropin peptides. *Ann. N. Y. Acad. Sci.* **1993**, *680*, 342−363.
(25) Hruby, V. J.; Sharma, S. D.; Toth, K.; Jaw, J. Y.; Al-Obeidi, F.; Sawyer, T. K.; Hadley, M. E. Design, synthesis, and conformation of superpotent and prolonged acting melanotropin. *Ann. N. Y. Acad. Sci.* **1993**, *680*, 51−63.
(26) Sawyer, T. K.; Castrucci, A. M.; Staples, D. J.; Affholter, J. A.; De Vaux, A. E.; Hruby, V. J.; Hadley, M. E. Structure−activity relationship of [Nle,^{4} D-Phe^7] α-MSH: discovery of a tripeptidyl agonist exhibiting sustained bioactivity. *Ann. N. Y. Acad. Sci.* **1993**, *680*, 597−599.
(27) Sawyer, T. K.; Sanfilippo, P. J.; Hruby, V. J.; Engel, M. H.; Heward, C. B.; Burnett, J. B.; Hadley, M. E. 4-Norleucine, 7-D-Phenylalaine-α-melanotropin-stimulating hormone: a highly potent a-melanotropin with ultra long biological activity. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5754−5758.
(28) Haskell-Luevano, C.; Nikiforovich, G.; Sharma, S. D.; Yang, Y. K.; Dickinson, C.; Hruby, V. J.; Gantz, I. Biological and conformational examination of stereochemical modifications using the template melanotropin peptide, Ac-Nle-c[Asp-His-Phe-Arg-Trp-Ala-Lys]-NH_2, on human melanocortin receptors. *J. Med. Chem.* **1997**, *40*, 1738−1748.
(29) Shi, Y.; Sharma, S. Metallopeptide approach to the design of biologically active ligands: design of specific human neutrophil elastase inhibitors. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1469−1474.
(30) Ghadiri, M. R.; Soares, C.; Choi, C. Design of an artificial four-helix bundle metalloprotein via a noval ruthenium(II)-assisted self-assembly process. *J. Am. Chem. Soc.* **1992**, *114*, 4000.
(31) Imperiali, B.; Kapoor, T. M. The reverse turn as a template for metal coordination. *Tetrahedron* **1997**, *49*, 3501.
(32) Giblin, M. F.; Wang, N.; Hoffman, T. J.; Jurisson, S. S.; Quinn, T. P. Design and characterization of α-melanotropin peptide analogues cyclized through rhenium and technetium metal coordination. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12814−12818.
(33) Chen, J. Q.; Wang, N.; Jurisson, S. S.; Quinn, T. P. Biodistribution properties of linear and cyclic ^{99m}Tc-labeled a-melanotropin peptides. In *Technetium, Rhenium and Other Metals in Chemistry and Nuclear Medicine*; Nicolini, M., Mazzi, U., Eds.; SG Editorial: Padova, Italy; 1998; pp 457−463.
(34) Chen, J. Q.; Cheng, Z.; Hoffman, T. J.; Jurisson, S. S.; Quinn, T. P. Melanoma-targeting properties of ^{99m}Technetium-labeled cyclic a-melanocyte-stimulating hormone peptide analogues. *Cancer Res.* **2000**, *60*, 5649−5658.
(35) Wang, N.; Miao, Y.; Chen, J. Q.; Jurisson, S. S.; Quinn, T. P. Biodistribution properties of linear and cyclic 188-Re-labeled α-melanotropin peptides. Manuscript in preparation.
(36) Chen, J. Q.; Cheng, Z.; Miao, Y.; Jurisson, S. S.; Quinn, T. P. α-Melanocyte-Stimulating Hormone Peptide Analogues Labeled with Technetium-99m and Indium-111 for Malignant Melanoma Targeting. *Cancer* **2002**, *94*, 1196−1201.
(37) Chen, J. Q.; Cheng, Z.; Jurisson, S. S.; Quinn, T. P. Evaluation of an ^{111}In-DOTA-Rhenium Cyclized α-MSH Analog: A Novel Cyclic-Peptide Analogue with Improved Tumor Targeting Properties. *J. Nucl. Med.* **2001**, 42, 1847−1855.
(38) Wraight, E. P.; Bard, D. R.; Maughan, C. G.; Knight, C. G.; Page-Thomas, D. P. The use of a chelating derivative of alpha-melanocyte stimulating hormone for the clinical imaging of malignant melanoma. *Br. J. Radiol.* **1992**, *65*, 112−118.
(39) Behr, M. B.; Goldenberg, D. M.; Becker, W. Reducing the renal uptake of radiolabeled antibody fragments and peptides for diagnosis and therapy: present status, future prospects and limitations. *Eur. J. Nucl. Med.* **1998**, *25*, 201−212.
(40) Gross, E.; Meienhofer, J. *The peptides: analysis, synthesis, biology.* Protection of Functional Groups in Peptide Synthesis, Vol. 3; Academic Press Inc.: New York, NY, 1981.
(41) Behr, T. M.; Sharkey, R. M.; Sgouros, G.; Blumenthal, R. D.; Dunn, R. M.; Kolbert, K.; Griffiths, G.; Siegel, J. A.; Becker, W. S.; Goldenberg, D. M. Overcoming the nephrotoxicity of radiometal-labeled immunoconjugates: improved cancer therapy administered to a nude mouse model in relation to the internal radiation dosimetry. *Cancer* **1997**, *80*, 2591−2610.
(42) IUPAC-IUB Commission of Biochemical Nomenclature (CBN) Symbols for Amino-Acid Derivatives and Peptides, Recommendations 1971. *Eur. J. Biochem.* **1972**, *27*, 201−207.
(43) Kieviet, W. Technetium radiopharmaceuticals: chemical characterization and tissue distribution of Tc-glucoheptonate using Tc-99m and carrier Tc-99. *J. Nucl. Med.* **1981**, *22*, 703−709.
(44) Giblin, M. F.; Jurisson, S. S.; Quinn, T. P. Synthesis and characterization of rhenium-complexed a-melanotropin analogues. *Bioconjugate Chem.* **1997**, *8*, 347−353.
(45) Siegrist, W.; Oestreicher, M.; Stutz, S.; Girard, J.; Eberle, A. E. Radio-receptor assay for α-MSH using mouse B16 melanoma cells. *J. Receptor Res.* **1988**, *8*, 323−343.
(46) Duncan, J. R.; Welch, M. J. Intracellular metabolism of indium-111 DTPA labeled receptor targeted protein. *J. Nucl. Med.* **1993**, *34*, 1728−1738.
(47) Franano, F. N.; Edwards, W. B.; Welch, M. J.; Duncan, J. R. Metabolism of receptor targeted ^{111}In-DTPA-Glycoproteins: identification of ^{111}In-DTPA-ϵ-lysine as the primary metabolic and excretory product. *Nucl. Med. Biol.* **1994**, *21*, 1023−1034.
(48) Rogers, B. E.; Franano, F. N.; Duncan J. R.; Edwards, W. B.; Anderson, C. J.; Connett J. M.; Welch, M. J. Identification of metabolites of ^{111}In-diethylenetriaminepentaacetic acid-monoclonal antibodies and antibody fragments in vivo. *Cancer Res.* **1995**, *55*, 5714s−5720s.
(49) Wu, C.; Jagoda, E.; Brechbiel, M.; Webber, K. O.; Pastan, I.; Gansow, O.; Eckelman, W. C. Biodistribution and catabolism of Ga-67-labeled anti-Tac dsFv fragment. *Bioconjugate Chem.* **1997**, *8*, 365−369.
(50) Tsai, S. W.; Li, L.; Williams, L. E.; Anderson, A.-L.; Raubitschek, A. A.; Shively, J. E. Metabolism and renal clearance of ^{111}In-labeled DOTA-conjugated antibody fragments. *Bioconjugate Chem.* **2001**, *12*, 264−270.
(51) Arano, Y. Strategies to reduce renal radioactivity levels of antibody fragments. *Q. J. Nucl. Med.* **1998**, *42*, 262−270.
(52) Behr, T. M.; Goldenberg, D. M.; Becker, W. Reducing the renal uptake of radiolabeled antibody fragments and peptides for diagnosis and therapy: present status, future prospects and limitations. *Eur. J. Nucl. Med.* **1998**, *25*, 201−212.
(53) Akizawa, H.; Arano, Y.; Mifune, M.; Iwado, A.; Saito, Y.; Mukai, T.; Uehara, T.; Ono, M.; Fujioka, Y.; Ogawa, K.; Kiso, Y.; Saji, H. Effect of molecular charges on renal uptake of ^{111}In-DTPA-conjugated peptides. *Nucl. Med. Biol.* **2001**, *28*, 761−768.
(54) Bernard, B. F.; Krenning, E. P.; Breeman, W. A. P.; Rolleman, E. J.; Bakker, W. H.; Visser, T. J.; Mäcke, H.; De Jong, M. D-lysine reduction of indium-111 octreotide and yttrium-90 octreotide renal uptake. *J. Nucl. Med.* **1997**, *38*, 1929−1933.

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