Published in final edited form as:

Bioconjug Chem. 2012 November 21; 23(11): 2300–2312. doi:10.1021/bc300509k.

A novel, cysteine-modified chelation strategy for the incorporation of $[M^I(CO)_3]^+$ ($M = \text{Re}, {}^{99m}\text{Tc}$) in an α -MSH peptide

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Abstract

Engineering peptide-based targeting agents with residues for site specific and stable complexation of radionuclides is a highly desirable strategy for producing diagnostic and therapeutic agents for cancer and other diseases. In this report, a model N-S-N_{Py} ligand (**3**) and a cysteine-derived alpha-melanocyte stimulating hormone (α -MSH) peptide (**6**) were used as novel demonstrations of a widely applicable chelation strategy for incorporation of the $[M^I(CO)_3]^+$ ($M = \text{Re}, {}^{99m}\text{Tc}$) core into peptide-based molecules for radiopharmaceutical applications. The structural details of the core ligand-metal complexes as model systems were demonstrated by full chemical characterization of *fac*- $[\text{Re}^I(\text{CO})_3(\text{N},\text{S},\text{N}_{\text{Py}}-\mathbf{3})]^+$ (**4**) and comparative high performance liquid chromatography (HPLC) analysis between **4** and $[\text{Re}^I(\text{CO})_3(\text{N},\text{S},\text{N}_{\text{Py}}-\mathbf{3})]^+$ (**4a**). The α -MSH analogue bearing the N-S-N_{Py} chelate on a modified cysteine residue (**6**) was generated and complexed with $[M^I(CO)_3]^+$ to confirm the chelation strategy's utility when applied in a peptide-based targeting agent. Characterization of the $\text{Re}^I(\text{CO})_3$ -**6** peptide conjugate (**7**) confirmed the efficient incorporation of the metal center, and the ${}^{99m}\text{Tc}^I(\text{CO})_3$ -**6** analogue (**7a**) was explored as a potential single photon emission computed tomography (SPECT) compound for imaging the melanocortin 1 receptor (MC1R) in melanoma. Peptide **7a** showed excellent radiolabeling yields and *in vitro* stability during amino acid challenge and serum stability assays. *In vitro* B16F10 melanoma cell uptake of **7a** reached a modest value of $2.3 \pm 0.08\%$ of applied activity at 2 h at 37 °C while this uptake was significantly reduced by coincubation with a nonlabeled α -MSH

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Supporting Information Available

Radiolabeling yields for **4a**. Stability of **4a** and **5** during *in vitro* amino acid challenge assays. HPLC chromatogram of purified **6**. Normalized and offset HPLC chromatograms of **6** and **7**. *In vitro* stability of **7a**. X-ray crystallography experimental procedures and tables with crystal data, structure refinement, bond lengths, and bond angles for **4** (CCDC deposition number: 877545). This material is available free of charge via the Internet at <http://pubs.acs.org>.

analogue, NAPamide (3.2 μM) ($P < 0.05$). *In vivo* SPECT/X-ray computer tomography (SPECT/CT) imaging and biodistribution of **7a** were evaluated in a B16F10 melanoma xenografted mouse model. SPECT/CT imaging clearly visualized the tumor at 1 h post injection (p.i.) with high tumor-to-background contrast. Blocking studies with coinjected NAPamide (10 mg per kg of mouse body weight) confirmed the *in vivo* specificity of **7a** for MC1R-positive tumors. Biodistribution results with **7a** yielded a moderate tumor uptake of 1.20 ± 0.09 percentage of the injected radioactive dose per gram of tissue (% ID/g) at 1 h p.i. Relatively high uptake of **7a** was also seen in the kidneys and liver at 1 h p.i. ($6.55 \pm 0.36\%$ ID/g and $4.44 \pm 0.17\%$ ID/g, respectively), although reduced kidney uptake was seen at 4 h p.i. ($3.20 \pm 0.48\%$ ID/g). These results demonstrate the utility of the novel $[\text{M}^{\text{I}}(\text{CO})_3]^+$ chelation strategy when applied in a targeting peptide.

Introduction

A wide variety of radionuclides exist for generating diagnostic radiopharmaceutical agents. Among these, $^{99\text{m}}\text{Tc}$ is often the preferred radiometal for single photon computed tomography (SPECT) imaging applications due to its favorable low energy γ -emission (140 keV), 6.02 h half life and wide availability from commercial ^{99}Mo - $^{99\text{m}}\text{Tc}$ generators at low cost. The $[\text{M}^{\text{I}}\text{Tc}^{\text{I}}(\text{CO})_3(\text{OH}_2)_3]^+$ core is a particularly attractive choice for radiolabeling biomolecules due to its low molecular volume, straightforward aqueous preparation from non-toxic reagents and facile coordination to a variety of both hard and soft donor atoms.^{1–3}

For targeted molecular imaging applications, the radiometal must form kinetically and thermodynamically stable complexes with the targeting molecule at residues which are not essential for interacting with the biological target. A bifunctional chelator is often used for site specific and stable complexation of the $[\text{M}^{\text{I}}\text{Tc}^{\text{I}}(\text{CO})_3]^+$ center in peptide-based targeting agents. Traditionally, a separately optimized chelate system is covalently attached through an extension or linker to the *N*- or *C*-terminus or to an inner amino acid residue of the peptide.^{4–6} Modified amino acids containing the desired chelate (e.g. single amino acid chelate)^{7, 8} or containing orthogonal chemical moieties (e.g. azides, alkynes) for chelate generation or attachment^{9–11} can be introduced at specific locations within a peptide sequence during solid phase production strategies. Such site specific chelate incorporation is more difficult in larger, recombinantly produced proteins (e.g. antibodies, antibody fragments, affibodies) as multiple solvent-exposed residues with similar chemical reactivity are usually available on these molecules.

Another desirable method for $[\text{M}^{\text{I}}\text{Tc}^{\text{I}}(\text{CO})_3]^+$ chelate formation is to engineer the targeting molecule with specific residues which can selectively coordinate the radiometal directly. Such a strategy offers the advantage of not requiring additional synthetic steps or modifications for chelate incorporation after the initial production and purification of the peptide sequence. Furthermore, it can be applied to ligands produced by either solid phase or recombinant methods. This approach has been demonstrated in peptides bearing the hexahistidine-tag (H_6 -tag)^{12–23} or a variant sequence containing alternating histidine and glutamic acid residues $[(\text{HE})_3\text{-tag}]$.²⁴ Both sequences have been shown to successfully complex the $[\text{M}^{\text{I}}\text{Tc}^{\text{I}}(\text{CO})_3]^+$ core during radiolabeling. Unfortunately, animal biodistribution studies with these radiolabeled peptides have yielded relatively high nonspecific uptake of the radiotracer in some normal organs, such as the liver and intestines, suggesting possible transchelation of the metal *in vivo*.^{13, 14, 17, 20, 24} Furthermore, the exact coordination modes around the metal center in these chelation strategies remain undefined. Additional chelate engineering strategies with well defined and stable coordination spheres and which are applicable to both recombinant and synthetic peptide production strategies

would be of great value for generating clinically relevant, $^{99m}\text{Tc}^{\text{I}}(\text{CO})_3$ -based radiopharmaceutical agents.

In this report, we demonstrate a widely applicable method to introduce a site specific chelate group for the $[\text{M}^{\text{I}}(\text{CO})_3]^+$ core ($\text{M} = \text{Re}, ^{99m}\text{Tc}$) into peptide-based molecules for radiopharmaceutical applications *in vitro* and *in vivo*. Our strategy is to introduce a non-essential cysteine residue containing an *S*-alkylated 2-(methyl)pyridine moiety at the *N*-terminus of a targeting peptide (Figure 1). As discussed below, this chelate structure can be incorporated during solid phase production of the peptide. Therefore, we envision this method can be applied to a large number of targeting peptides since these molecules are commonly produced by solid phase techniques. Such a strategy is expected to minimally affect the nature of the free peptide compared to alternative chelating methods while generating an efficient N-S- N_{Py} chelate group for the $[\text{M}^{\text{I}}(\text{CO})_3]^+$ core consisting of the derivatized cysteine's α -amine, thioether, and pyridal nitrogen.^{3, 25–27} We chose to incorporate our ligand design into a melanoma-targeting peptide as the first demonstration of our chelation strategy for complexing $[\text{M}^{\text{I}}(\text{CO})_3]^+$ in peptide-based radiopharmaceutical agents.

Malignant melanoma is a serious public health problem due to its increasing incidence and high mortality rate.²⁸ The latter factor results mainly from limitations in the early detection and treatment of melanoma metastases.^{29–31} Identification of melanoma cell signaling pathways and biomarkers in recent years has allowed the development of molecular imaging agents such as radiolabeled antibodies³² and antibody fragments³³ for diagnosing melanoma metastases at earlier stages.³⁴ Unfortunately, many of these agents have met with limited success due to their antigenicity,³⁵ reduced rates of tumor penetration,³⁶ and slow circulation.³⁷ α -melanocyte stimulating hormone (α -MSH) peptide analogues, which target the melanocortin 1 receptor (MC1R) on melanoma cells, are an emerging class of molecular agents with promise for imaging and treating metastatic melanoma.

Melanocortin receptors, including MC1R and its variants, fall within the membrane bound, G-protein-coupled receptor (GPCR) family. Of these receptors, MC1R is the most prominent member in regulating normal melanocyte function.³⁸ The overexpression of MC1R (~400 to 22,000 receptors/cell) observed in both murine and human melanoma compared to normal melanocytes presents this receptor as an attractive biomarker for primary and metastatic melanoma in both clinical settings and animal models.^{30, 39–42} Due to their favorable pharmacokinetics and high affinity for MC1R, low molecular weight α -MSH peptides are nearly ideal compounds for targeting melanoma and its metastases.⁴³

Several general strategies have been employed to incorporate PET (e.g., ^{18}F ⁴⁴, ^{64}Cu ⁴²) and SPECT (e.g., ^{99m}Tc ³⁰, ^{111}In ⁴⁵, and ^{188}Re ⁴⁶) imaging radionuclides into α -MSH peptides for targeting MC1R in melanoma models. In one approach, ligands or bifunctional chelators for metal complexation are appended to the termini or side chains of α -MSH peptides with linear^{42, 44, 47–53} or cyclic (via lactam rings^{54–56} or disulfide bridges^{57, 58}) conformations.^{59, 60} A second strategy takes advantage of coordination bonds between the metal and the peptide to convert a linear peptide into a metal cyclized version.^{30, 58, 61, 62} The majority of these α -MSH complexes contain the M^{V} oxo core, which generally improves the overall system's resistance to chemical and proteolytic degradation.^{60, 61, 63} Many of the metal cyclized peptides contain non-radioactive rhenium for structural purposes only and are functionalized with ligands for incorporating alternative radionuclides similar to the other cyclic and linear α -MSH analogs.^{58, 62–65} The cyclized α -MSH peptides typically show higher *in vivo* potencies than linear analogs, although the *in vitro* MC1R affinities of both peptide conformations generally fall within the low to sub-nanomolar IC_{50} range.^{59, 60} Of the various radiolabeled α -MSH peptides, the metal cyclized analogs usually

demonstrate the most favorable *in vivo* stabilities and tumor uptake values with high kidney clearance.^{58, 60, 62, 64}

To address affinity and to minimize the additional mass and potential negative impacts of bifunctional chelate attachment, we report a novel strategy to incorporate the $[M^I(CO)_3]^+$ core into a peptide framework by modification of a terminal cysteine residue to generate a potent tridentate chelate. Initial studies presented here demonstrated the feasibility of the N-S-N_{Py} ligand system with the $[M^I(CO)_3]^+$ core through structural, complexation efficiency and metal-chelate stability studies. A linear α -MSH analogue [Ac-Nle-Asp-His-D-Phe-Arg-Trp-Gly-Lys-NH₂ (NAPamide)] with excellent MC1R targeting ability and pharmacokinetic properties^{42, 44, 49} was selected to implement this chelate strategy. The cysteine-modified NAPamide analogue bearing the N-S-N_{Py} chelate system was successfully synthesized and complexed with the $[M^I(CO)_3]^+$ core. The ^{99m}Tc-radiolabeled peptide was evaluated both *in vitro* to determine its stability and uptake in B16F10 melanoma cells and *in vivo* to assess its biodistribution in B16F10 mouse xenograft models.

Experimental Procedures

All reagents and solvents were of reagent grade or higher from commercial suppliers (Aldrich, Fluka, Acros, Fisher) and used as received unless noted otherwise. *fac*-[Re^I(CO)₃(OH₂)₃](SO₃CF₃) and *S*-(pyridin-2-ylmethyl)-L-cysteine (ligand **1**) were prepared as previously described.^{25, 26} ^{99m}Tc-pertechnetate (^{99m}TcO₄⁻) was purchased from Cardinal Health in Spokane, WA or obtained from Stanford Nuclear Medicine Clinic. ¹²⁵I-(Tyr²)-[Nle⁴,D-Phe⁷]- α -MSH [¹²⁵I-(Tyr²)-NDP] was purchased from Perkin Elmer (Waltham, MA). Elemental analysis was performed by Quantitative Technologies, Inc. (NJ). Nuclear magnetic resonance (NMR) spectra were recorded at 293 K on a Varian Mercury Vx 300 spectrometer using 5 mm NMR tubes. ¹H and ¹³C NMR spectra peak positions were referenced using residual solvent signals or trimethylsilane as an internal standard. Spectra were processed using Varian VNWR 6.1 software. Mass spectra were obtained on a Thermo-Finnigan LCQ Advantage instrument for electrospray ionization mass spectrometry (ESI-MS) or a Perseptive Voyager-DE RP Biospectrometry instrument (Framingham, MA) for matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). Infrared (IR) spectra were recorded on a Thermo Nicolett 6700 FTIR with an ATR cell and analyzed with OMNIC 7.1a software. UV/Vis spectra were recorded on a Varian Cary 50 Bio spectrophotometer and analyzed with Cary WinUV 3.00 software. Solutions of the compounds were prepared in UV/Vis quality methanol and measured in quartz cuvettes. Analytical separation and identification of small molecules (**1**, **2**, **3**, **4**, **4a**, **5**; Scheme 1 and Chart 1) and the Re^I(CO)₃-conjugated peptide (**7**; Chart 1) by reversed-phase high performance liquid chromatography (RP-HPLC) were conducted using a Varian Pursuit XR column (C18, 5 μ m, 4.6 $\times$ 250 mm) with a Phenomenex security guard cartridge (C18, 4.0 $\times$ 3.0 mm). A Perkin-Elmer Series 200 analytical chromatography system and a Hitachi D-7000 series analytical chromatography system (L-7100 pump, L-7400 UV detector) were used for the above compounds. Unless noted otherwise, elutions performed on these systems used a gradient profile as previously described (Gradient 1)²⁶ with 0.1% TFA in water (solvent A) and MeOH (solvent B). Chromatograms were plotted using OriginPro 8.5.1 (OriginLab Corporation, MA). Preparatory HPLC separations and purifications used an Agilent Zorbax column (SB-C18, 7 μ m, 21.2 $\times$ 250 mm) on a Hitachi D-7000 series semi-preparative chromatography system (L-7150 pump, L-7400 UV detector). Chromatographic purification and analysis of the non-labeled peptide (**6**; Chart 1) was performed on a Dionex Ultimate 300 HPLC system (Dionex Corp.) using both semi-preparative (Vydac; 218TP510-C18, 5 μ m, 10 $\times$ 250 mm) and analytic (Vydac; 214TP54-C18, 5 μ m, 4.6 $\times$ 250 mm) peptide RP-HPLC columns. The mobile phase gradients used 0.1% TFA in water and 0.1% TFA in acetonitrile (MeCN). Radio-HPLC purification and

analysis with the radiolabeled peptide (**7a**; Chart 1) was conducted with the semi-preparative RP-HPLC Vydac peptide column on a Dionex 680 chromatography system with a UVD 170U absorbance detector and model 105S single-channel radiation detector (Carroll & Ramsey Associates, Berkley, CA). The recorded data were processed with use of Chromeleon version 6.50 software (Sunnyvale, CA). Phosphate buffered saline (PBS, 0.01 M, pH 7.4) was obtained from Gibco/Invitrogen (Carlsbad, CA). The B16F10 murine melanoma cell line was obtained from American Type Culture Collection (Manassas, VA). Female C57BL/6 mice were purchased from Charles River Laboratory (Wilmington, MA).

Small Molecule Synthesis and Radiolabeling

S-(pyridin-2-ylmethyl)-Fmoc-L-cysteine, 2—This compound was prepared using procedures shown previously for *N*-terminal Fmoc-protection of amino acids.⁶⁶ A solution of Fmoc-Cl (0.3 g, 0.974 mmol) in 7 mL of dioxane was added dropwise over 2 h to an ice cooled, stirred solution of **1** (0.464 g, 1.16 mmol) in 15 mL of 10% Na₂CO₃. The solution was then warmed to room temperature and stirred for an additional 2 h. Water (130 mL) was added to quench the reaction and the solution was extracted with diethyl ether (3 × 15 mL). The aqueous layer was cooled in an ice-bath and the pH was adjusted to 4–5 with 6 M HCl. The resulting white precipitate was collected by extraction with ethyl acetate (5 × 50 mL). The combined organic layers were concentrated to ~70 mL by rotary evaporation and washed with water (3 × 25 mL), then brine (1 × 15 mL), and dried with Na₂SO₄. The solvent was removed by rotary evaporation, and residual solvent was removed by several rounds of adding diethyl ether followed by rotary evaporation. The resulting solid was dried under vacuum to yield 0.37 g of **2** (82%). Anal. Calcd for C₂₄H₂₄N₂O₅S: C, 63.70; H, 5.35; N, 6.19. Found: C, 63.70; H, 5.14; N, 6.08. ¹H NMR [δ(ppm), CDCl₃]: 8.61 (d, C_{Py}H, 1 H), 7.82 (dd, C_{Py}H, 1 H), 7.71 (m, C_{Ar}H, 2 H), 7.60 (dd, C_{Ar}H, 2 H), 7.43 (d, C_{Py}H, 1 H), 7.38–7.23 (m, C_{Ar}H, 5 H), 6.37 (d, NH, 1 H), 4.62 (m, N-CH, 1 H), 4.38 (m, O-CH₂, 2 H), 4.20 (t, CH, 1 H), 4.08 (d, J=13.8 Hz, Py-CH_{2a}-S, 1 H), 3.91 (d, J=14.1 Hz, Py-CH_{2b}-S, 1 H), 3.09 (dd, S-CH₂, 2H). ¹³C NMR [δ(ppm), CDCl₃]: 173.4 (COO), 157.3 (CO), 156.1 (C_{Ar}), 147.0 (C_{Ar}H), 144.0 (C_{Ar}H), 141.3 (C_{Ar}H), 139.3 (C_{Ar}H), 127.7 (2 C_{Ar}H), 127.2 (2 C_{Ar}H), 125.3 (2 C_{Ar}H), 124.7 (C_{Ar}H), 123.1 (C_{Ar}H), 120.0 (2 C_{Ar}H), 67.0 (CH₂, Fmoc), 54.7 (CH), 47.3 (CH, Fmoc), 37.0 (CH₂), 35.0 (CH₂). MS (–ESI): 433.0; calcd for [M–H][–]: 433.1.

2-((pyridin-2-ylmethyl)thio)ethanamine, 3—Ligand **3** has previously been synthesized and characterized.^{67, 68} However, an alternative synthetic strategy using aqueous conditions which are amenable for selective *S*-alkylation of peptide-based molecules was used for the current report. Cysteamine hydrochloride (0.085 g, 0.75 mmol) and 2-(bromomethyl)pyridine hydrobromide (0.189 g, 0.75 mmol) were dissolved in 5 mL of water. The solution was brought to pH 7–8 with 1 M NaHCO₃ and stirred at room temperature overnight. The reaction mixture was concentrated by rotary evaporation, acidified with 0.1% aqueous TFA, and purified by preparatory HPLC using the following gradient system (Gradient 2) with a flow rate of 10 mL/min: 0–5 min 100% A; step to 90% A/10% B and hold from 5–15 min; 15–21.5 min linear gradient to 75% A/25% B; 21.5–25 min linear gradient to 100% B; 25–28 min 100% B; linear gradient to 100% A by 30 min. Fractions with the product were pooled, solvent was removed by rotary evaporation, and the residue was dried under vacuum to yield 0.161 g of **3** (54%). ¹H NMR [δ(ppm), CD₃CN]: 8.65 (d, 1 H), 8.19 (dd, 1 H), 7.74 (d, 1 H), 7.65 (dd, 1 H), 6.89 (broad s, 3 H), 4.08 (s, 2 H), 3.22 (t, 2 H), 2.91 (t, 2 H). ¹³C NMR [δ(ppm), CD₃CN]: 156.0, 145.2, 144.5, 127.4, 125.8, 39.5, 33.5, 29.4. MS (+ESI): 169.0; calcd for [M+H]⁺: 169.1. The spectral data were consistent with those reported previously for **3**.^{67, 68}

fac-[Re^I(CO)₃(N,S,N_{Py}-3)](C₂F₃O₂), 4—Ligand **3** (0.030 g, 0.076 mmol) was dissolved in 1.3 mL of water, 0.1 M *fac*-[Re^I(CO)₃(OH₂)₃](SO₃CF₃) (0.677 mL, 0.068 mmol) was added, and the solution's pH was brought to 3–4 with 0.1 M HCl. The solution was stirred and heated with a condenser at 75 °C for 26 h while the pH was kept at 3–5 with the addition of 0.1 M HCl or 0.1 M NaHCO₃ as needed. The solution was purified by preparatory HPLC using the following gradient system (Gradient 3) with a flow rate of 12 mL/min: 0–3 min 100% A; step to 75% A/25% B and hold from 3–9 min; 9–25 min linear gradient to 100% B; 25–32 min 100% B; linear gradient to 100% A by 33 min. Fractions with the product were pooled, solvent was removed by rotary evaporation, and the residue was dried under vacuum to yield 0.024 g of **4** (64%). X-ray quality crystals were obtained by slow diffusion of benzene into an MeCN solution of **4**. X-ray crystallography experimental procedures, crystal data and structure refinement parameters can be found in the Supporting Information. Anal. Calcd for C₁₃H₁₂F₃N₂O₅ReS: C, 28.31; H, 2.19; N, 5.08. Found: C, 29.16; H, 2.44; N, 5.15. ¹H NMR [δ(ppm), CD₃CN]: 8.98 (d, C_{Py}H, 1 H), 8.05 (dd, C_{Py}H, 1 H), 7.73 (d, C_{Py}H, 1 H), 7.47 (dd, C_{Py}H, 1 H), 4.83 (d, *J* = 17.7 Hz, Py-CH_{2a}-S, 1 H), 4.58 (broad s, NH, 1 H), 4.48 (d, *J* = 17.7 Hz, Py-CH_{2b}-S, 1 H), 4.02 (broad s, NH, 1 H), 2.96 (m, CH, 1 H), 2.85 (m, CH, 1 H), 2.65 (m, CH, 1 H), 2.25 (m, CH, 1 H). ¹³C NMR [δ(ppm), CD₃CN]: 184.9 (COO, TFA), 161.0 (C_{Py}), 156.0 (C_{Py}H), 142.7 (CF₃, TFA), 141.1 (C_{Py}H), 126.3 (C_{Py}H), 125.3 (C_{Py}H), 45.4 (CH₂), 44.2 (CH₂), 36.5 (CH₂). MS (+ESI): 439.2; calcd for [M]⁺: 439.0. IR (solid, cm⁻¹): 2030, 1921, 1668, 1199, 1125. UV/Vis ε_{max} (251 nm): 9400 M⁻¹cm⁻¹.

[^{99m}Tc^I(CO)₃(N,S,N_{Py}-3)]⁺, 4a—The [^{99m}Tc^I(CO)₃(OH₂)₃]⁺ solution was prepared from an Isolink kit (Tyco, Inc.) by injecting ^{99m}TcO₄⁻ solution into the vial and heating at 95 °C for 25 min, cooling the vial on ice for 15 min after acidifying to pH 3–4 with 1 M HCl, and neutralizing to pH 7 with 1 M NaOH. Solutions of **3** in water (1 × 10⁻³ – 1 × 10⁻⁵ M) were added to the appropriate buffer (10 mM sodium phosphate pH 6.0 or pH 7.4; 10 mM sodium acetate pH 4.5) in a sealable labeling vial. The vial was sealed and de-gassed with nitrogen for 7–10 min and [^{99m}Tc^I(CO)₃(OH₂)₃]⁺ solution was added to bring the final ligand concentration to values of 1 × 10⁻⁴ – 1 × 10⁻⁶ M (Table S1) in final volumes of 200–300 μL. The vials were heated at 70 °C for 30 min, cooled on ice, and analyzed or purified by radio-HPLC on the Perkin-Elmer Series 200 chromatography system described above equipped with a Perkin-Elmer Radiomatic 610TR detector.

Amino Acid Challenge Assays with ^{99m}Tc^I(CO)₃-ligand Complexes—Complex **5** (Chart 1), described previously as [^{99m}Tc^I(N,S,N_{Py}-1)(CO)₃]⁺,²⁶ was produced by reacting **1** at 1 × 10⁻⁴ M (final concentration) with [^{99m}Tc^I(CO)₃(OH₂)₃]⁺ at pH 7.4 using analogous conditions as for generating **4a** above. Solutions of HPLC-purified **4a** or **5** (100 μL) were mixed with solutions containing L-cysteine or L-histidine at 2 mM in 10 mM sodium phosphate buffer pH 7.4 (200 μL) and incubated at 37 °C. Aliquots were periodically monitored by radio-HPLC up to 4 h to determine complex resistance to transchelation in the presence of the competing amino acids.

NAPamide Analogue Synthesis, Complexation Reactions, and Biological Evaluations

(S-(pyridin-2-ylmethyl)-Cys)-Nle-Asp-His-D-Phe-Arg-Trp-Gly-Lys-NH₂, 6—The NAPamide derivative was synthesized on an automated peptide synthesizer (CS Bio, CS 336X). Briefly, Rink Amide LS resin (200 mg, 40 μmol, Advanced ChemTech, 0.2 mmol/g loading) was swollen in *N,N*-dimethylformamide (DMF) for 30 min. With the exception of **2** (synthesized above), Fmoc-protected amino acids were purchased from Novabiochem/EMD Chemicals Inc. Fmoc groups were removed with 20% piperidine in DMF. The aliquots of amino acids (0.20 mmol) were activated in a solution containing 0.20 mmol of *N*-hydroxybenzotriazole hydrate and 0.5 M diisopropylcarbodiimide in DMF. Peptide

deprotection and cleavage were carried out by a 3-h incubation in a mixture of TFA/thioanisole/ethanedithiol/water (92.5:2.5:2.5:2.5). The mixture was filtered, and the peptide in solution was precipitated with anhydrous diethyl ether. The resulting peptide was washed four times with ice-cold anhydrous diethyl ether, dried, and dissolved in water. The peptide was purified by RP-HPLC on a C-4 column. Fractions were collected and lyophilized to yield the target product, **6**, in 55% yield. The purified peptide was analyzed by RP-HPLC (C-18) using the following gradient system (Gradient 4) with solvent A and 0.1% TFA in MeCN (solvent C): 0–3 min 95% A/5% C, 3–33 min linear gradient to 35% A/65% C, 33–36 min linear gradient to 5% A/95% C, 36–39 min hold at 5% A/95% C, 39–42 min linear gradient to 95% A/5% C, equilibrate in 95% A/5% C. MS (MALDI-TOF): 1251.4; calcd for $[M+H]^+$: 1251.6.

fac-[Re^I(CO)₃(N,S,N_{Py}-6)]⁺, **7**—To a solution of **6** (750 μg) in 185 μL of PBS (adjusted to pH 6.0 with 1 M HCl) was added 1 molar equivalent of 0.1 M *fac*-[Re^I(CO)₃(OH₂)₃](SO₃CF₃) (5.7 μL). The mixture was initially mixed by vortex (10 seconds) and was then heated at 60 °C for 6 h with periodic mixing by vortex. The solution was purified by analytical RP-HPLC using the following gradient system (Gradient 5) with a flow rate of 1 mL/min: 0–3 min 100% A; step to 75% A/25% B and hold from 3–12 min; 12–28.5 min linear gradient to 100% B; 28.5–33.5 min 100% B; step to 100% A at 33.6 min and equilibrate in 100% A. The fraction with the desired product was bubbled with nitrogen to evaporate MeOH. The solution was frozen and lyophilized to yield 600 μg of **7** (74%). MS (+ESI): 761.6, 1521.2/1519.4; calcd for $[M+2H]^{2+}$: 761.8, calcd for $[M]^+$: 1521.6/1519.6.

[^{99m}Tc^I(CO)₃(N,S,N_{Py}-6)]⁺, **7a**—A solution of **6** (50 μg) in water was added to PBS (adjusted to pH 6.0 with 1 M HCl) in a sealable labeling vial. [^{99m}Tc^I(CO)₃(OH₂)₃]⁺ solution (3.4 mCi) was added to bring the final volume to 200–300 μL. The vial was heated at 70 °C for 30 min, cooled in an ice bath, and analyzed by radio-HPLC on the Dionex 680 chromatography system described above. Purification used the following gradient system (Gradient 6): 0–2 min 72% A/28% C, 2–32 min linear gradient to 67% A/33% C, 32–35 min linear gradient to 15% A/85% C, 35–38 min linear gradient to 72% A/28% C, 38–42 min hold at 72% A/28% C.) Subsequent analysis of **7a** used the following gradient system (Gradient 7): 0–3 min 95% A/5% C, 3–33 min linear gradient to 5% A/95% C, 33–36 min hold at 5% A/95% C, 36–39 min linear gradient to 95% A/5% C, 39–42 min hold at 95% A/5% C.

In Vitro Stability Analysis

Serum Stability Assay—7a (100 μCi) in 50 μL of PBS was added to 500 μL of mouse serum (Sigma). After incubation at 37 °C for 4 h, the solution was filtered through a NanoSep device (10 K; Pall Corp.). The filtrate was then injected into the radio-HPLC column under conditions identical to those used for analyzing the purified radiolabeled peptide (Gradient 7). The fractions eluted from HPLC were collected into plastic tubes every 30 s. The radioactivity of each tube was measured by a γ-counter (Perkin-Elmer model 1470), and the radio-HPLC chromatogram was plotted using Origin, version 6.0 (MicroCal).

Amino Acid Challenge Assays—^{99m}Tc radiolabeled NAPamide analogue was subject to transchelation in the presence of histidine and cysteine (1 mM) as an assay of label stability. Solutions of HPLC-purified **7a** (400 μL) were added to solutions containing L-cysteine or L-histidine at 1 mM in 10 mM sodium phosphate buffer pH 7.4 (600 μL). The solutions were stirred and incubated at 37 °C for 4 h. Aliquots were periodically monitored by radio-HPLC (Gradient 7) to determine complex stability.

Cell Culture and Animal Model

B16F10 murine melanoma cells were cultured in Dulbecco's modified Eagle's high-glucose medium (GIBCO, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator containing 5% CO₂ at 37 °C. A 70%–80% confluent monolayer was detached with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and dissociated into a single-cell suspension for further cell culture and assays. All animal studies were carried out in compliance with federal and local institutional rules for the conduct of animal experimentation. Approximately 1×10^6 cultured B16F10 cells were suspended in 100 μ L of PBS and subcutaneously implanted in the right shoulders of C57BL/6 mice. Tumors were grown to a size of 0.5–1 cm in diameter (1~2 weeks) prior to imaging and biodistribution studies.

In Vitro B16F10 Cell Assays

IC₅₀ Cell Binding Assay—The receptor binding affinity of **7** for MC1R in B16F10 cells was performed as previously described.⁶⁹ Briefly, 5×10^5 cells were re-suspended in Dulbecco's Modified Eagle's Medium containing 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), 0.2% bovine serum albumin, and 0.3 mM 1,10-phenanthroline. The cells were then incubated at 37 °C for 90 min with **7** (peptide concentration varying from 10^{-11} – 10^{-5} M) and approximately 20,000 counts per min (cpm) of ¹²⁵I-(Tyr²)-NDP. Cells were washed three times with PBS and the radioactivity of the cells was measured. The experiment was performed in triplicate. Data were analyzed using Graphpad Prism 5.0 (Northampton, MA), and the IC₅₀ value (the concentration of competitor required to inhibit 50% of the radioligand binding) of the peptide was calculated.

Cell Uptake Assay—Cell uptake studies of **7a** B16F10 cells were performed as previously described.⁴² Briefly, 3×10^5 cells were seeded in 12-well tissue culture plates and allowed to attach overnight. After a wash with PBS, the cells were incubated with **7a** (1 μ Ci per well, in culture medium) with or without NAPamide (3.2 μ M/well) at 37 °C or 4 °C for 0.5, 1 and 2 h. The cells were then washed 3 times with PBS and lysed in 1 mL of 1.0 M NaOH and 0.1% sodium dodecyl sulfate. Radioactivity was measured by a γ -counter (Perkin-Elmer model 1470). Cell uptake was expressed as the percentage of added radioactivity. Experiments were performed twice with triplicate wells.

Small-Animal SPECT/CT Imaging

Small animal SPECT/X-ray computed tomography (SPECT/CT) imaging was performed on a combined SPECT/CT scanner for small animals (X-SPECT; Gamma Medica). Mice bearing B16F10 xenografts were injected via the tail vein with approximately 200 μ Ci of **7a** with or without 10 mg of NAPamide per kg of mouse body weight. At 1 h post injection (p.i.), mice were anesthetized with 2% isoflurane and placed in the prone position near the center of the field of view (FOV) of the scanner. For micro-CT image acquisition, 512 images (170 μ m slice thickness) were acquired in 5 min at 0.4 mA and 80 kVp. SPECT was performed using a 1 mm multi pinhole collimator (single head, 360° rotation, 64 projections, 30 s/projection and a 5 cm FOV). CT images were reconstructed by using a cone-beam filtered backprojection algorithm into a $512 \times 512 \times 512$ matrix with a voxel size of 170 μ m. The SPECT images were reconstructed using a 2-dimensional ordered-subsets expectation maximization (OSEM) algorithm with 8 subsets and 10 iterations into a $64 \times 64 \times 60$ matrix size with a voxel size of 1.5 mm. All data were imported into Amira (Mercury Computing Systems, Chelmsford) for processing and visualization.

Biodistribution Studies

For biodistribution studies, nude mice bearing B16F10 xenografts ($n = 4$ per group) were injected via the tail vein with approximately 200 μCi of **7a** and were euthanized at 1 and 4 h p.i. Tumor and normal tissues of interest were removed and weighed, and their radioactivity was measured in a γ -counter. Radioactivity uptake was expressed as a percentage of the injected radioactive dose per gram of tissue (% ID/g).

Statistical Methods

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

Results and Discussion

Model Ligand Synthesis, Rhenium Complexation, and Characterization

Although several N-S- Npy chelate structures incorporating the $[\text{M}^{\text{I}}(\text{CO})_3]^+$ core have been shown in the literature,^{26, 70, 71} full structural characterization of the underivatized 2-*S*-(pyridin-2-ylmethyl)ethanamine- $\text{M}^{\text{I}}(\text{CO})_3$ complexes is shown here for the first time. The synthetic route for producing these compounds is outlined in Scheme 1. Ligand **3** was generated by mild alkylation of 2-(bromomethyl)pyridine with cysteamine at room temperature in a pH 7–8 bicarbonate solution. Following preparatory HPLC purification of the reaction, ligand **3** was isolated in a moderate yield (54%) with spectral properties corresponding to those reported previously for this known compound.⁶⁷ An aqueous solution of **3** was subsequently heated with *fac*- $[\text{Re}^{\text{I}}(\text{CO})_3(\text{OH}_2)_3](\text{SO}_3\text{CF}_3)$ at 75 °C while keeping the pH moderately acidic (pH 3–5). The reaction was monitored by analytical HPLC and after 26 h showed a single major peak at 18.5 min, indicating completion of the reaction. The reaction solution was cooled to room temperature and allowed to sit undisturbed for several days, during which crystals spontaneously formed. Although they were not suitable for X-ray analysis, NMR characterization showed these crystals to be the desired complex, **4**. The singlet for the methylene hydrogens (*S*- CH_2 -Py) in ligand **3** yielded characteristic H_a - H_b splitting ($J = 17.7$) and downfield shifts (from 4.08 ppm to 4.83 and 4.48 ppm) upon coordination to the $[\text{Re}^{\text{I}}(\text{CO})_3]^+$ center.²⁶ Similarly, the other hydrogen atoms along the ligand backbone (*S*- CH_2 - CH_2 - NH_2) became diastereotopic in **4** compared to **3** with unique NMR resonances and intense splitting patterns for each atom. The complex splitting patterns could be explained by the presence of overlapping or interconverting diastereomeric structures in solution. Diastereomeric products are expected upon complexation to the metal center due to the prochiral thioether of the free ligand,^{25, 26} and such sulfur-rhenium bonds have been shown to readily undergo pyramidal inversion to yield interconverting isomers at room temperature.^{72, 73} Preparatory HPLC was used to purify the crude crystals and reaction solution, yielding **4** with TFA as the counterion. NMR characterization of this purified product matched that of the crystals isolated from the crude reaction mixture. IR analysis showed two strong bands at 2030 and 1921 cm^{-1} , indicating tridentate ligand coordination to yield a *fac*- $[\text{Re}^{\text{I}}(\text{CO})_3]^+$ complex.

X-ray quality crystals of the purified product were obtained by slow diffusion of benzene into a MeCN solution containing **4**. The structure revealed the tridentate coordination of N-S- Npy to *fac*- $[\text{Re}^{\text{I}}(\text{CO})_3]^+$ (Figure 2, Table 1). The facial orientated ligand formed two strained five-membered coordination rings on the metal center. The rhenium-nitrogen bond lengths (Re-NH₂, 2.218(4) Å; Re-N_{py}, 2.206(4) Å) and rhenium-sulfur bond length (2.4488(11) Å) were similar to those observed for several analogous N-S- Npy cysteine-based ligand complexes of *fac*- $[\text{Re}^{\text{I}}(\text{CO})_3]^+$ (Re-NH₂, 2.219–2.227 Å; Re-N_{py}, 2.205–2.218 Å; Re-S, 2.447–2.453 Å).²⁶ However, the rhenium-sulfur bond in **4** was slightly shorter than

observed in other ligands with thioether donors coordinated to $\text{fac-}[\text{Re}^{\text{I}}(\text{CO})_3]^+$ (2.455–2.504 Å).^{25, 72} Similarly, the bond angles of the ligand relative to the metal observed in **4** (NPy-Re-NH_2 , 83.34(14)°; $\text{NH}_2\text{-Re-S}$, 80.01(11)°; NPy-Re-S , 79.83(10)°) were equally constricted as other five-membered coordination rings found with thioether pyridine ligands with $\text{fac-}[\text{Re}^{\text{I}}(\text{CO})_3]^+$ (NPy-Re-NH_2 , 82.0–85.26°; $\text{NH}_2\text{-Re-S}$, 79.97–81.29°; NPy-Re-S , 79.23–80.52°).^{26, 71} However, the NPy-Re-S angle in **4** was more restricted than the corresponding six-membered ring (88.93°) in a comparable $\text{fac-}[\text{Re}^{\text{I}}(\text{CO})_3]^+$ complex with *S*-(2-(2'-pyridyl)ethyl)cysteamine.⁷¹ The Re-CO bond angles of 87.3–90.2° in **4** were comparable to those of other distorted octahedral complexes (86.5–90.3°)^{25, 26, 71} and confirmed the facial coordination of the ligand.

Model Ligand Radiolabeling and Stability Assessment

Following structural analysis of the metal-chelate complex with rhenium, the corresponding radioactive complex was generated. Ligand **3** was complexed with $[\text{}^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3(\text{OH}_2)_3]^+$ at 70 °C for 30 min at various ligand concentrations (10^{-4} – 10^{-6} M) and reaction pH values (4.5, 6.0, 7.4) to investigate chelation efficiency. All conditions explored led to a single new peak after analysis by radio-HPLC with a retention time of 18.6 min. The identity of this species was confirmed as the desired complex **4a** as its retention time closely matched that of **4** (18.5 min) under similar HPLC conditions (Figure 3). Excellent yields were obtained for **4a** at all pH values explored at 10^{-4} M ligand concentrations, although yields declined slightly at 10^{-5} M ligand concentration and substantially below this (Table S1). Reaction pH was found to affect labeling yields at these lower concentrations, giving higher yields at lower pH values. Although this result was anticipated based on previous results seen with ligand **3** during $^{99\text{m}}\text{Tc}$ radiolabeling,⁷⁰ the corresponding cysteine derived complex, **5**, (Chart 1) showed the opposite trend between pH and labeling yields.²⁶ This could be due to ionic attractions between the cationic $[\text{}^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3(\text{OH}_2)_3]^+$ species and the anionic carboxylate in ligand **1** (Chart 1) which would become more pronounced at higher pH values. Such ionic interactions would not occur with **3** since this ligand lacks a carboxyl group.

While **4a** was previously seen to be stable when challenged with cysteine or glutathione for 1 h,⁷⁰ the stability of **5** under challenge conditions has not yet been shown. For this report, challenge experiments were performed with **4a** and **5** using a large excess of histidine or cysteine, which are high affinity, biologically relevant competitive ligands for the $[\text{}^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3]^+$ core.⁷⁴ Purified, carrier-free **4a** and **5** showed no disassociation or trans-chelation in the presence of the competing ligands under biologically relevant temperature and pH values (37 °C, pH 7.4) up to 4 h as determined by radio-HPLC analysis (Table S2). The encouraging radiolabeling yields and stabilities seen in these studies confirmed the N-S- NPy donor system to be a favorable tridentate chelate group for $[\text{M}^{\text{I}}(\text{CO})_3]^+$ centers. This prompted us to incorporate this moiety within a targeting peptide for radiopharmaceutical applications.

NAPamide Analogue Synthesis, Rhenium Complexation, and Characterization

α -MSH analogs are favorable peptides for targeted radiopharmaceutical applications as they can accommodate a variety of amino acid residues or linkers for radionuclide integration while maintaining high affinity for MC1R and rapid clearance from normal tissues *in vivo*.^{44, 54, 64} While a variety of α -MSH peptides have incorporated rhenium and technetium as M^{VO} cores by either chelate attachment or peptide cyclization methods,^{30, 59, 62–65, 69, 75, 76} examples of these peptides with the $[\text{M}^{\text{I}}(\text{CO})_3]^+$ core have been much more limited.^{50, 53, 54} We hypothesized that introducing the N-S- NPy chelate group via attachment of an *S*-functionalized cysteine at the *N*-terminus of a high affinity NAPamide analogue would allow efficient complexation of the $[\text{M}^{\text{I}}(\text{CO})_3]^+$ core for

generating a novel radiolabeled peptide for melanoma detection. Such an analogue would be the first demonstration of a biomolecule bearing the N-S-N_{P_y} chelate for [M^I(CO)₃]⁺ incorporation.

To attach the chelate structure within the peptide under standard solid phase synthesis conditions, the amino group of ligand **1** was protected with Fmoc following procedures used previously with other amino acids.⁶⁶ Both ¹H and ¹³C NMR analysis of the resulting compound **2** (Chart 1) confirmed the attachment of the Fmoc group due to the anticipated increased number of resonances in the aromatic regions of the spectra. Mass spectral and elemental analysis also confirmed this compound's identity. Compound **2** was subsequently incorporated at the *N*-terminus of the NAPamide sequence during standard Fmoc solid phase peptide production. The resulting peptide, **6** (Chart 1), was isolated in moderate overall yield (55%) following purification. MALDI-TOF-MS analysis confirmed the successful production of the peptide as the mass obtained (1251.4 m/z) matched the anticipated mass for **6**. RP-HPLC analysis (Gradient 4) of **6** yielded a single peak with a retention time of 15.6 min in greater than 98% purity (Figure S1), indicating it was ready for use in subsequent reactions.

The [Re^I(CO)₃]⁺ conjugate of peptide **6**, peptide **7**, was produced to characterize the incorporation of the [M^I(CO)₃]⁺ core into the chelate bearing NAPamide. Heating **6** at pH 6.0 for 6 h at 60 °C with one equivalent of *fac*-[Re^I(CO)₃(OH₂)₃](SO₃CF₃) led to efficient conversion to **7**. Periodic RP-HPLC analysis of the reaction mixture using an aqueous/MeOH gradient (Gradient 1) showed the disappearance of the starting peptide (17.1 min) and the appearance of a single new peak with a later retention time (18.1 min) as would be expected for complexation of the [Re^I(CO)₃]⁺ core to the peptide (Figure S2). Following RP-HPLC purification of the reaction mixture and lyophilization of the product peak, **7** was isolated in good yield (>70%) and its identity confirmed by ESI-MS. The base peak observed during MS analysis (761.6 m/z) correlated with the expected mass of the doubly protonated peptide-Re^I(CO)₃ conjugate ([M+2H]²⁺). The spectrum also showed a prominent set of ions for the parent peak, [M]⁺, with m/z values of 1521.2 and 1519.4, representing the characteristic isotopic ratio for naturally abundant ¹⁸⁷Re and ¹⁸⁵Re. These results confirmed the complexation of a single [Re^I(CO)₃]⁺ center to the peptide as anticipated for **7** and encouraged us to pursue radiolabeling studies with ^{99m}Tc.

NAPamide Analogue Radiolabeling and *In Vitro* Stability

To generate a novel peptide labeled with a SPECT isotope for imaging melanoma, the NAPamide analogue, **6**, was complexed with [^{99m}Tc^I(CO)₃(OH₂)₃]⁺ at pH 6.0 and heated at 70 °C for 30 min. Initial analysis and purification of the reaction mixture by RP-HPLC using an aqueous/MeCN gradient (Gradient 6) showed a single major peak with a retention time of 14.1 min, assigned as **7a** (Chart 1). Complex **7a** was obtained in decay-corrected yields above 71% with high specific activities (1010–1900 mCi/μmol), demonstrating the excellent radiolabeling efficiency of the peptide. **7a** was then analyzed by radio-HPLC using the same conditions as for analyzing **7** above (Gradient 1). Its retention time of 18.5 min correlated closely to the 18.2 min retention time observed with purified **7** (Figure 4), indicating similar [M^I(CO)₃]⁺ complexation between the two analogs and confirming the identity of **7a**. Subsequent radio-HPLC analysis of **7a** using an alternative aqueous/MeCN gradient (Gradient 7) showed a single peak with a retention time of 18.7 min (Figure S3A). This alternative gradient was used for further analysis of **7a** in the studies below.

High stability of radiolabeled complexes toward competitive ligands is essential for developing biologically relevant imaging compounds. The *in vitro* stability of **7a** was assessed for a 4 h period at 37 °C in mouse serum or in histidine or cysteine solutions at pH

7.4. More than 90% of the radiolabeled complex remained unaltered after 4 h incubation in mouse serum (Figure S3B). Similar to the results seen with **4a** and **5**, the radiolabeled peptide remained stable after 4 h incubation with excess histidine or cysteine (Figure S3C and S3D, respectively). These results showed the inertness of **7a** towards transchelation or degradation and warranted its further exploration for targeting MC1R *in vitro* and *in vivo*.

***In Vitro* Cell Analyses of the $M^I(\text{CO})_3$ -NAPamide Complexes**

The MC1R binding affinity of **7** in B16F10 cells was determined by a competitive binding study with ^{125}I -(Tyr²)-NDP as a radioligand and yielded an IC_{50} value of 2.0 ± 0.9 nM. The low nanomolar affinity of the rhenium-conjugated peptide indicated that incorporation of the cysteine-based ligand system and subsequent $[M^I(\text{CO})_3]^+$ complexation of the functionalized peptide did not hinder the peptide's ability to bind to MC1R. This encouraging result suggested the $M^I(\text{CO})_3$ -NAPamide complex to potentially be a useful *in vitro* MC1R targeting peptide and prompted further cell uptake and MC1R specificity studies with **7a** in B16F10 cells. Levels of cell uptake of **7a** observed after 0.5 h, 1 h and 2 h incubation at 37 °C or 4 °C are shown in Figure 5. **7a** exhibited moderate accumulation in B16F10 cells at 37 °C, reaching $1.2 \pm 0.08\%$, $1.7 \pm 0.01\%$ and $2.3 \pm 0.08\%$ of applied activity at 0.5, 1 and 2 h, respectively. This uptake was significantly reduced at 4 °C, reaching only $0.3 \pm 0.05\%$, $0.5 \pm 0.08\%$ and $0.4 \pm 0.09\%$ of applied activity at 0.5, 1 and 2 h, respectively. To further confirm whether the binding of **7a** with B16F10 cells was specific for MC1R, the cells were incubated with solutions containing both **7a** and excess amounts of unlabeled NAPamide for 0.5, 1 and 2 h at 37 °C. As shown in Figure 5, radiolabel uptake at 2 h decreased from $2.3 \pm 0.08\%$ to $1.4 \pm 0.1\%$ ($P < 0.05$) under these conditions. Thus, the unlabeled NAPamide reduced the binding of **7a** to B16F10 cells to a statistically significant degree, indicating that the uptake of the radiolabeled peptide was mediated by the MC1R. At 4 °C, however, there were no significant differences in B16F10 cell uptake of **7a** when administered alone or when coincubated with unlabeled NAPamide. This suggests that the pathway of receptor-mediated ligand binding and internalization is no longer active at 4 °C and only non-specific uptake and internalization occurs at this temperature.

***In Vivo* Imaging and Biodistribution Studies with the Radiolabeled NAPamide Analogue**

In vivo studies were then performed in a mouse model of melanoma to determine the imaging potential and biodistribution of the radiolabeled peptide. SPECT/CT images of a B16F10 tumor-bearing mouse at 1 h p.i. of **7a** are shown in Figure 6. The tumor was clearly visible at 1 h p.i., and high tumor-to-background contrast was achieved. The radiolabeled peptide showed low accumulation in most normal organs, resulting in high tumor imaging quality. Relatively high accumulation of radioactivity was observed in the kidneys and liver. Co-injection of excess NAPamide as a blocking agent considerably reduced tumor uptake, confirming the *in vivo* tumor-targeting specificity of **7a**.

The *in vivo* biodistribution of **7a** was examined in B16F10 tumor-bearing mice at 1 and 4 h p.i. Biodistribution studies confirmed that **7a** had modest uptake in tumors and low accumulation in most normal organs (Figure 7) and were consistent with the results observed during SPECT imaging. Tumor uptake of **7a** was $1.20 \pm 0.09\%$ ID/g at 1 h p.i. and decreased slightly to $0.84 \pm 0.10\%$ ID/g at 4 h p.i., although this decrease was not statistically significant ($P > 0.2$). The accumulation of **7a** within muscle, blood and most other normal tissues, however, was remarkably lower at 4 h compared to 1 h. High tumor-to-normal tissue ratios were observed 4 h after injection, including a tumor-to-blood ratio of 2.02 ± 0.58 and a tumor-to-muscle ratio of 14.09 ± 0.58 (Figure 7B). This finding demonstrated that there was good retention of the radiolabeled peptide in tumors compared to the other organs.

Some nonspecific accumulation in normal organs was found during *in vivo* analysis. Relatively high uptake of **7a** was found in the kidneys, reaching $6.55 \pm 0.36\%$ ID/g at 1 h p.i. This initially high renal accumulation was expected since the clearance of low molecular weight compounds, such as small peptides, from the body is mainly through the kidneys. The uptake of **7a** within the kidneys reduced to $3.20 \pm 0.48\%$ ID/g at 4 h p.i., demonstrating the relatively rapid clearance of **7a** from these organs. The liver uptake of **7a** remained relatively high at both time points ($4.44 \pm 0.17\%$ ID/g and $4.11 \pm 0.07\%$ ID/g at 1 h and 4 h after injection, respectively). The undesirable increased liver retention observed may be attributed to the changes in lipophilicity associated with the $[\text{}^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3]^+$ core or the pyridal group leading to hepatobiliary rather than renal clearance of the peptide from circulation. This general trend with $^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3$ -appended systems has been observed with other peptides incorporating chelators with dipyridal groups.⁸

The overall *in vivo* performance of **7a**, which employed the labeling strategy of incorporating the chelate and radiometal into the peptide backbone, indicated this complex had similar properties to other linear MSH analogues with the NAP-amide sequence using alternative radiolabeling strategies. In a chelate appended strategy, DOTA-conjugated peptides labeled with ^{111}In ^{49, 51} and ^{67}Ga ⁴⁸ showed fairly low uptake in most normal tissues compared to the melanoma tumors at 4 h p.i., while a ^{64}Cu ⁴² DOTA-conjugated analogue yielded greater non-specific retention in normal tissues compared to **7a**. While these DOTA-functionalized conjugates showed greater overall uptake in melanoma tumors at 4 h p.i. ($>2\%$ ID/g) compared to **7a**, they also had greater kidney retention ($>3.9\%$ ID/g) than did **7a**. A non-metal analog, an ^{18}F -fluorobenzoate α -MSH peptide,⁴⁴ had similar melanoma uptake at 1 h p.i. (1.19% ID/g) and lower melanoma uptake at 4 h p.i. (0.52% ID/g) compared to **7a**. Comparable linear α -MSH peptide analogues for targeting MC1R in melanoma applications have employed a ligand appended pyrazolyl-diamine (pz) chelate for $[\text{M}^{\text{I}}(\text{CO})_3]^+$ incorporation.^{50, 53, 54} While **7a** demonstrated lower uptake in B16F10 tumor xenografts in mice at 1 h p.i. ($1.20 \pm 0.09\%$ ID/g) compared to these other $^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3$ -labeled peptides ($>1.9\%$ ID/g), **7a** also had lower non-tumor accumulation in major excretory organs (liver, kidneys, intestines). The liver showed the highest retention of radioactivity ($4.11 \pm 0.07\%$ ID/g) at 4 h p.i. for **7a** in contrast with the intestines^{50, 54} or kidneys⁵³ for the pz- $^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3$ analogs. These variations in biodistribution patterns are likely due to variations in peptide sequences, inherent chelate properties, and locations of chelate incorporation employed for radiolabeling; these factors have been shown to impact the *in vivo* performance of radiolabeled peptides.^{8, 77}

Overall, the *in vivo* performance of **7a** falls within the spectrum of ranges observed for similar metal and non-metal based radiolabeling strategies with linear α -MSH peptides, although its tumor uptake was lower and its liver retention was higher than for several $^{99\text{m}}\text{Tc}$ -cyclized α -MSH peptides.^{30, 58, 62} **7a** yielded modest tumor uptake 4 h p.i. with reasonable clearance from other organs (e.g., lungs, pancreas, intestines, spleen) correlated with melanoma metastases.⁷⁸ Additional optimization is needed to reduce the nonspecific accumulation of **7a** in the liver and gastrointestinal tract, which are also common sites of melanoma metastases.⁷⁸ Although the clearance pathway of **7a** may have been undesirably affected by the lipophilicity of the $[\text{}^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3]^+$ core, the advantages associated with the $[\text{}^{99\text{m}}\text{Tc}^{\text{I}}(\text{CO})_3]^+$ core such as small molecular size, stability and reproducible formation may outweigh lipophilicity shortcomings in alternative peptide applications with the labeling strategy employed here. These initial studies demonstrate the feasibility of the N-S-N_{py} chelate strategy for functionalization of the N-terminus of peptides. This chelate system can also be readily extended to peptide side chains with free amines, small molecules or peptidomimetic molecules for incorporation of $[\text{M}^{\text{I}}(\text{CO})_3]^+$ centers in radiopharmaceutical applications.

Conclusions

In summary, this study presents the first application of the novel, cysteine-derived, N-S-N_{py} chelate strategy for [M^I(CO)₃]⁺ complexation within an α-MSH peptide to generate a targeted imaging radiopharmaceutical. The model ligand systems in this study demonstrated the core ligand-metal interactions and the stability of the radiolabeled complex *in vitro*. The NAPamide analogue, **7a**, was prepared as a model peptide system and evaluated for MC1R binding properties, tumor uptake and tissue distribution in B16F10 melanoma. The radiolabeled peptide showed high stability and MC1R binding specificity *in vitro*. Biodistribution and small animal SPECT/CT imaging studies with **7a** yielded promising *in vivo* properties such as rapid tumor targeting, tumor retention, and generally good tumor-to-normal tissue ratios. These favorable results demonstrate the utility of the novel chelation strategy when applied in a targeting peptide and suggest its extension to other biomolecules. We expect this strategy to be applicable to a broad range of peptide-based radiopharmaceutical agents due to its ease in incorporation via solid phase synthesis and its efficiency for coordinating the widely used [M^I(CO)₃]⁺ core.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Mary Dyszlewski of Covidien, Inc. for the Isolink kits. This research was funded in part by the NIH Biotechnology Training Program at Washington State University (T32 GM008336), the National Cancer Institute (NCI) *In Vivo* Cellular Molecular Imaging Center (ICMIC) grant P50 CA114747 and Melanoma Research Alliance (ZC), the Zhejiang Provincial Natural Science Foundation of China (Z2110230), the Health Bureau of Zhejiang Province (2010ZA075, 2011ZDA013), the National Science Foundation of China (NSFC) (No. 81101023, 81170306, 81173468), the Ministry of Science and Technology of China (2011CB504400) and the National Science Technology Support Program (2012BAI13B06, 2012BAI13B06).

Abbreviations

CT	X-ray computed tomography
cpm	counts per minute
DMF	<i>N,N</i> -dimethylformamide
ESI-MS	electrospray ionization mass spectrometry
Fmoc	9-Fluorenylmethoxycarbonyl
FOV	field of view
HPLC	high performance liquid chromatography
¹²⁵I-(Tyr²)-NDP	¹²⁵ I-(Tyr ²)-[Nle ₄ , <i>p</i> -Phe ⁷]-α-MSH
% ID/g	percent injected dose per gram of tissue
IR	infrared
MALDI-TOF-MS	matrix assisted laser desorption/ionization time of flight mass spectrometry
MC1R	melanocortin 1 receptor
MeCN	acetonitrile
MeOH	methanol

α-MSH	alpha-melanocyte stimulating hormone
NAPamide	Ac-Nle-Asp-His-D-Phe-Arg-Trp-Gly-Lys-NH ₂
NMR	nuclear magnetic resonance
p.i.	post injection
pz	pyrazolyl-diamine tridentate chelate group
RP-HPLC	reversed-phase HPLC
SPECT	single photon emission computed tomography
TFA	trifluoroacetic acid

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Figure 1.

A general strategy for introducing a cysteine-based N-S-N_{py} chelate system for the [M^I(CO)₃]⁺ core to generate peptide-based targeted radiopharmaceuticals.

Figure 2. Crystal structure of **4** with thermal ellipsoids shown at the 30% probability level. Hydrogen atoms and counter ion (TFA) omitted for clarity. Atoms are colored as follows: C, gray; N, light blue; O, red; Re, dark blue; S, yellow.

Figure 3. Normalized and offset HPLC chromatograms for purified solutions of **4** (UV absorbance, 254 nm; lower blue trace) at 18.5 min and **4a** (radiodetector, counts per minute (cpm); higher red trace) at 18.6 min.

Figure 4. Normalized and offset HPLC chromatograms for purified solutions of **7** (UV absorbance, 254 nm; lower blue trace) at 18.2 min and **7a** (radiodetector, cpm; higher red trace) at 18.5 min.

Figure 5.

In vitro B16F10 cell uptake assay. B16F10 cells were incubated with 1 μ Ci of **7a**, a cysteine-derived MSH peptide, for 0.5, 1 and 2 h at 37 °C and 4 °C, with or without NAPamide as a blocking agent. Data represent the mean percentage of total radioactivity added. The experiment was performed in triplicate, and the error bars represent standard deviation. (● **7a** only at 37 °C, ■ **7a** with unlabeled NAPamide at 37 °C; ◆ **7a** only at 4 °C, ▴ **7a** with unlabeled NAPamide at 4 °C)

Figure 6. SPECT/CT imaging of **7a** in C57BL/6 mice bearing B16F10 tumor xenografts (n = 4 per group). Coronal SPECT/CT image of B16F10 tumor-bearing mice at 1 h post injection of approximately 200 μ Ci of **7a** alone (left image) or of **7a** coinjected with 10 mg NAPamide per kg of mouse body weight (right image) at 1 h after injection. Arrows indicate the location of tumors.

Figure 7. Biodistribution (A) and tumor-to-normal tissue ratios (B) of **7a** in B16F10 tumor-bearing C57BL/6 mice 1 and 4 h after injection of approximately 200 μ Ci of **7a**. Data are expressed as % ID/g $\pm$ SD (n = 4 per group).

Scheme 1.

Ligand **3** synthesis and coordination reactions. (a) aq. NaHCO₃; (b) aq. *fac*-[M^I(CO)₃(OH₂)₃]⁺, 70–75 °C.

Chart 1.
Structures and abbreviated designations of cysteine-based molecules and peptides.

Table 1Selected bond distances (Å) and angles (°) for compound **4**.

Bond Distances [Å]		Bond angles [°]	
Re-NH ₂	2.218(4)	N _{Py} -Re-NH ₂	83.34(14)
Re-N _{Py}	2.206(4)	NH ₂ -Re-S	80.01(11)
Re-S	2.4488(11)	N _{Py} -Re-S	79.83(10)
Re-C(O)	1.922(5)	C(O)-Re-C(O)	87.3(2)
	1.923(5)		87.8(2)
	1.926(5)		90.2(2)
S-CH ₂ (N _{Py})	1.806(5)	CH ₂ -S-CH ₂	104.3(3)
S-CH ₂ (CH ₂)	1.810(5)	Re-NH ₂ -CH ₂	114.6(3)