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⁶⁴Cu-Labeled Alpha-Melanocyte-Stimulating Hormone Analog for MicroPET Imaging of Melanocortin 1 Receptor Expression

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Abstract

The alpha-melanocyte-stimulating hormone (α -MSH) receptor (melanocortin type 1 receptor, or MC1R) plays an important role in the development and growth of melanoma cells. It was found that MC1R was overexpressed on most murine and human melanoma, making it a promising molecular target for melanoma imaging and therapy. Radiolabeled α -MSH peptide and its analogs that can specifically bind with MC1R have been extensively explored for developing novel agents for melanoma detection and radionuclide therapy. The goal of this study was to evaluate a ⁶⁴Cu-labeled α -MSH analog, Ac-Nle-Asp-His-D-Phe-Arg-Trp-Gly-Lys(DOTA)-NH₂ (DOTA-NAPamide), as a potential molecular probe for microPET imaging of melanoma and MC1R expression in melanoma xenografted mouse models. 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) conjugated NAPamide was synthesized and radiolabeled with ⁶⁴Cu ($t_{1/2}$ =12 h) in NH₄OAc (0.1 M; pH 5.5) buffered solution for 60 min at 50 °C. Cell culture studies reveal rapid and high uptake and internalization of ⁶⁴Cu-DOTA-NAPamide in B16F10 cells. Over 90% of receptor-bound tracer is internalized at 3 h incubation. A cellular retention study demonstrates that the receptor-bound ⁶⁴Cu-DOTA-NAPamide is slowly released from the B16F10 cells into the medium; 66% of the radioactivity is still associated with the cells even after 3 h incubation. The biodistribution of ⁶⁴Cu-DOTA-NAPamide was then investigated in C57BL/6 mice bearing subcutaneous murine B16F10 melanoma tumors with high capacity of MC1R and Fox Chase Scid mice bearing human A375M melanoma with a relatively low number of MC1R receptors. Tumor uptake values of ⁶⁴Cu-DOTA-NAPamide are found to be $4.63 \pm 0.45\%$ and $2.49 \pm 0.31\%$ ID/g in B16F10 and A375M xenografted melanoma at 2 h postinjection (pi), respectively. The B16F10 tumor uptake at 2 h pi is further inhibited to $2.29 \pm 0.24\%$ ID/g, while A375M tumor uptake at 2 h pi remains $2.20 \pm 0.41\%$ ID/g with a coinjection of excess α -MSH peptide. MicroPET imaging of ⁶⁴Cu-DOTA-NAPamide in B16F10 tumor mice clearly shows good tumor localization. However, low A375M tumor uptake and poor tumor to normal tissue

contrast were observed. This study demonstrates that ^{64}Cu -DOTA-NAPamide is a promising molecular probe for α -MSH receptor positive melanoma PET imaging as well as MC1R expression imaging in living mice.

INTRODUCTION

Malignant melanoma has emerged as a significant public health problem in western countries (1). Currently, effective therapies are not available for treatment of metastatic melanoma. Extensive efforts have been spent to develop novel strategies for melanoma systemic treatment. For example, immunotherapy, chemotherapy, and biological therapy have been investigated intensively; however, little success has been achieved. The most important approach responsible for the improvement of survival rate of melanoma still remains early diagnosis (2).

Recently, peptide receptor-targeted radionuclide therapy (PRRT) has been shown to be an attractive approach for treatment of receptor-expressing tumors in preclinical and clinical settings (3, 4). It was shown that the somatostatin analog, [tetraazacyclododecane-tetraacetic acid (DOTA¹), Tyr³] octreotide radiolabeled with ^{90}Y could effectively inhibit tumor growth in a rat subcutaneous tumor model (5). Complete or partial remissions were also achieved in a study using ^{177}Lu -labeled [DOTA, Tyr³] octreotate for treating patients with gastroenteropancreatic neuroendocrine tumors (6). The success of using radiolabeled somatostatin analogs for tumor radiotherapy has inspired researchers to develop other radiolabeled peptides for treatment of different tumors.

It has been reported that most murine and human melanoma metastases bear up-regulated alpha-melanocyte-stimulating hormone (α -MSH) receptors (melanocortin type 1 receptor, or MC1R) (7, 8). This suggests that α -MSH peptides may serve as vehicles for delivering radionuclides, fluorescent molecules, or chemotherapeutic drugs for targeting melanoma. Novel radiolabeled α -MSH analogs for melanoma imaging or therapy have been investigated, and significant progress has been achieved in this field. For example, a library of α -MSH analogs radiolabeled with $^{99\text{m}}\text{Tc}$ (9, 10), ^{111}In (11–14, 26, 27), ^{125}I (15), ^{67}Ga (16), ^{86}Y (17), and ^{64}Cu (17, 18) exhibited excellent tumor-targeting abilities and pharmacokinetic properties in xenografted or metastatic melanoma models, suggesting the potential application of these radiometals or radiohalogens labeled α -MSH peptides for melanoma detection. Most importantly, it was reported that an α -MSH peptide ReCCMSH(Arg¹¹) radiolabeled with therapeutic radionuclide, either ^{188}Re or ^{212}Pb , showed promising therapeutic effects in mice bearing either B16F1 murine or TXM13 human xenografted melanoma (19, 20). These results highlight the possibility of using α -MSH peptide tagged with therapeutic radionuclides for metastatic melanoma therapy.

However, it was found that on different human and murine melanoma cell lines the receptor number of MC1R ranged from as low as undetectable to around 10 000 (7). Considering the

¹Abbreviations: α -MSH, alpha-melanocyte-stimulating hormone; melanocortin type 1 receptor, MC1R; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; PET, positron emission tomography; SPECT, single-photon emission spectroscopy; HPLC, high-performance liquid chromatography; 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG); pi, postinjection.

density of MC1R is one of the major factors that ultimately determine the uptake of the MC1R ligand in melanoma, to develop a noninvasive imaging tool with an ability to visualize the MC1R expression in living subjects will be very useful. This type of molecular probe may eventually render us the power to not only diagnose melanoma early but also screen patients suitable for PRRT strategy. To this end, an α -MSH analog, Ac-Nle-Asp-His-D-Phe-Arg-Trp-Gly-Lys(DOTA)-NH₂ (DOTA-NAPamide, 16), was radiolabeled with a positron emitter ⁶⁴Cu ($t_{1/2}$ = 12.7 h, $E_{\beta^+ \text{ max}}$ = 656 keV, 19%) (Figure 1). The ability of ⁶⁴Cu-DOTA-NAPamide to image the MC1R expression using micropositron emission tomography (micro-PET) was further evaluated on two melanoma mice models with different MC1R densities.

MATERIALS AND METHODS

General

DOTA was obtained from Macrocyclics Inc. (Richardson, TX). NAPamide was synthesized with over 95% purity by the Stanford Protein and Nucleic Acid Biotechnology Facility using standard 9-fluorenylmethyloxycarbonyl (Fmoc)/O-benzotriazolyl-tetramethyluronium hexafluorophosphate (HBTU) chemistry on rink amide MBHA resin. All other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). ⁶⁴Cu was provided by the Washington University School of Medicine (St. Louis, MO). A CRC-15R PET dose calibrator (Capintec Inc., Ramsey, NJ) was used for all radioactivity measurements. Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on a Dionex Summit HPLC system (Dionex Corp., Sunnyvale, CA) equipped with a 170U 4-Channel UV-vis absorbance detector and radioactive detector (Carroll & Ramsey Associates, model 105S, Berkeley, CA). UV detection wavelengths were 218, 254, and 280 nm for all experiments. Both semipreparative (Zorbax SB-C18, 9.4 mm × 250 mm) and analytical (Dionex Acclaim120 C18, 4.6 mm × 250 mm) RP-HPLC columns were used. The mobile phase was solvent A, 0.1% trifluoroacetic acid (TFA)/H₂O, and solvent B, 0.1% TFA/acetonitrile. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a Perseptive Voyager-DE RP Biospectrometry instrument (Framingham, MA) by the Stanford Protein and Nucleic Acid Biotechnology Facility. Alpha-cyano-4-hydroxy-cinnamic acid (α -CHCA, prepared as 10 g/L in 33.3% CH₃CN:33.3% EtOH:33.3% H₂O:0.1% TFA) was used as solid matrix. A375M cell line was a generous gift from Dr. M. Kolodny; University of California, Los Angeles, CA, and B16/F10 murine melanoma cells were obtained from the American Type Tissue Culture Collection. C57BL/6 and Fox chase scid mice were purchased from Charles River Laboratories (Boston, MA).

Chemistry and Radiochemistry

DOTA was conjugated with NAPamide in a manner similar to that described before (21). Briefly, DOTA, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), and *N*-hydroxysulfonosuccinimide (SNHS) at a molar ratio of 1:1:0.8 were mixed and incubated at 4 °C for 30 min (pH = 5.5). NAPamide was then added to the in-situ-prepared sulfosuccinimidyl ester of DOTA (DOTA-OSSu) in a theoretical stoichiometry of 5:1. The reaction solution was mixed and reacted at 4 °C overnight (pH 8.5–9.0). The resulting

DOTA–NAPamide conjugate was then purified by HPLC on a semipreparative C-18 column. The flow rate was 3 mL/min, with the mobile phase starting from 95% solvent A and 5% solvent B (0–3 min) to 35% solvent A and 65% solvent B at 33 min, then going to 85% solvent B and 15% solvent A (33–36 min), maintaining this solvent composition for another 3 min (36–39 min), and returning to initial solvent composition by 42 min. Fractions were collected and lyophilized. The target product was characterized by MALDI-TOF-MS and ready for use in the radiolabeling reactions. Analytic HPLC was performed on the same HPLC system using a C-18 analytical column and flow rate of 1 mL/min.

The DOTA–NAPamide was radiolabeled with ^{64}Cu by addition of 185 MBq (5 mCi) $^{64}\text{CuCl}_2$ (2 μg of peptide per 37 MBq ^{64}Cu) in 0.1 N NaOAc (pH 5.5) buffer followed by a 1 h incubation at 50 °C. The reaction was then terminated by adding 5 μL of TFA, and radiolabeled complex was purified by analytical radio-HPLC. The flow rate was 1 mL/min, with the mobile phase starting from 80% solvent A and 20% solvent B (0–3 min) to 70% solvent A and 30% solvent B at 33 min, then going to 85% solvent B and 15% solvent A (33–36 min), maintaining this solvent composition for another 3 min (36–39 min), and returning to initial solvent composition by 42 min. Purified radiolabeled peptide was dried by a rotary evaporator. The radiolabeled compound was reconstituted in phosphate-buffered saline (PBS) and passed through a 0.22 μm Millipore filter into a sterile vial for in vitro and animal experiments.

The nonradioactive $^{\text{nat}}\text{Cu}$ –DOTA–NAPamide was also synthesized by mixing DOTA–NAPamide (400 μg , 0.269 μmol) with high-purity natural copper chloride (CuCl_2 , 55 μg , 0.409 μmol) in 0.1 N NaOAc (pH 5.5) buffer followed by 2 h incubation at 70 °C. The product was purified by HPLC and confirmed by MALDI-TOF-MS.

Octanol/Water Partition Coefficient

To determine the lipophilicity of ^{64}Cu –DOTA–NAPamide, approximately 370 kBq of ^{64}Cu -labeled complex in 500 μL of PBS (pH 7.4) was added to 500 μL of octanol in an Eppendorf microcentrifuge tube. The resulting biphasic system was mixed vigorously for 10 min and left at room temperature for another 60 min. The two phases were then separated by centrifugation at 2000 g for 5 min (model 5415R Eppendorf microcentrifuge; Brinkman, Westbury, NY). From each layer an aliquot of 100 μL was removed and counted in a γ -counter (Packard Instruments). The partition coefficient ($\log P$) was then calculated as a ratio of counts in the octanol fraction to the counts in the water fraction. The experiment was repeated 3 times.

In Vitro Cell Assays

B16F10 murine and A375M human melanoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose medium, which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 48 mg of gentamicin. The cells were expanded in 75- cm^2 tissue culture flasks and kept in a humidified atmosphere of 5% CO_2 at 37 °C, with the medium changed every other day. A confluent monolayer was detached with trypsin and dissociated into a single-cell suspension for further cell culture.

The in vitro cell binding assays were performed with the murine melanoma cell line B16F10 as previously described (7–9). 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% bovine serum albumin (BSA), and 0.3 mM 1,10-phenanthroline], the cells were incubated at 25 °C for 2 h with either DOTA–NAPamide or ^{nat}Cu–DOTA–NAPamide (peptide concentration varying from 10^{–12} to 10^{–6} M) and approximately 50 000 counts per minute (cpm) of ¹²⁵I-(Tyr²)-[Nle,⁴_D-Phe⁷]-α-MSH [¹²⁵I-(Tyr²)-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 was analyzed using the ORIGIN6.0 computer program (Northampton, MA), and the IC₅₀ values, the concentration of competitor required to inhibit 50% of radioligand binding, of the DOTA–NAPamide and ^{nat}Cu–DOTA–NAPamide were calculated.

A typical saturation binding experiment was performed to determine the MC1R densities on the A375M and B16F10 melanoma cells. The cells (1 × 10⁶) were incubated in 0.15 mL of binding medium in the presence of ¹²⁵I-(Tyr²)-NDP ranging from approximately 2 × 10³ to 2 × 10⁶ cpm per tube with and without 10^{–6} M NDP for 2 h at 37 °C. The radioactivity of bound and free radioligand was counted. The data were analyzed by ORIGIN6.0, and the binding capacities of the B16F10 and A375M were calculated.

Cell uptake, internalization, and cellular retention of the ⁶⁴Cu–DOTA–NAPamide were examined in B16F10 cells using the method described previously (15). Briefly, in 24-well tissue plates prepared as described above, approximately 170 000 dpm of ⁶⁴Cu-labeled peptide (76.5 nCi, 0.283 ng, 0.38 nM) in 0.5 mL of binding media were added to each well containing cells and incubated at 37 °C for 5 min to 3 h. The nonspecific binding was determined by coinubation with nonradiolabeled NDP at a final concentration of 10 μM. Internalization of the radiolabeled peptide was determined by washing the cells with acidic buffer [40 mM sodium acetate (pH 4.5) containing 0.9% NaCl and 0.2% BSA] to remove the membrane-bound radiocomplex and then measuring the remaining internalized radioactivity. The cellular retention properties of the membrane-bound and internalized ⁶⁴Cu-labeled peptides were determined by incubating B16F10 cells in binding media at 37 °C with the radiolabeled complexes for 3 h. The binding media were then removed, the cells were washed twice with cold 0.01 M PBS (pH 7.4)/0.2% BSA, 0.5 mL of culture media was added, and radioactivity released into the media was monitored at various time points over a 3-h incubation period.

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 shoulder with 1 × 10⁶ cultured B16/F10 murine melanoma cells. Nine to ten days postinoculation, the tumors grew to a weight of ~500 mg and the tumor-bearing mice were subject to in vivo biodistribution and imaging studies, while 6 week old Fox chase ICR Scid mice were inoculated with 4 × 10⁶ A375M human

melanoma cells. Three to four weeks postinoculation, the mice bearing A375M tumors were ready for further studies.

For biodistribution studies the C57 BL/6 mice bearing B16F10 mouse melanoma and Fox chase mice bearing A375M human melanoma ($n = 4$ for each group) were injected with 703–777 KBq (19–21 μCi , 70–78 ng) of ^{64}Cu –DOTA–NAPamide with (blocking group, $n = 3$) or without coinjection of 200 μg of NDP through the tail vein and sacrificed at different time points from 30 min to 24 h postinjection (pi). Tumor and normal tissues of interest were removed and weighed, and their radioactivity was measured in a gamma counter. The radioactivity uptake in the tumor and normal tissues was expressed as a percent of the injected radioactive dose per gram of tissue (% ID/g).

MicroPET Imaging

PET imaging of tumor-bearing mice was performed on a microPET R4 rodent model scanner (Concorde Microsystems Inc., Knoxville, TN). The mice bearing B16F10 and A375M melanoma were injected with about 4.44 MBq (120 μCi) of ^{64}Cu –DOTA–NAPamide via the tail vein. At 120 min postinjection, the mice were anesthetized with 2% isoflurane and placed in the prone position and near the center of the field of view of microPET. The 10-min static scans were obtained, and the images were reconstructed by a two-dimensional ordered subsets expectation maximum (OSEM) algorithm. Regions of interest (ROIs) were then drawn over the tumor on decay-corrected whole-body coronal images. The counts per pixel per minute were obtained from the ROI and converted to counts per milliliter per minute using a calibration constant. By assuming a tissue density of 1 g/mL, the ROIs were converted to counts/g/min. An image ROI-derived % ID/g of tissue was then determined by dividing counts per gram per minute with 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 groups, with $P < 0.05$ being significantly different.

RESULTS

Chemistry and Radiochemistry

Synthesis of DOTA–NAPamide conjugate (Figure 1) was achieved through coupling of in-situ-prepared DOTA–OSSu ester with the ϵ -amino group of the lysine residue in the NAPamide. The desired product was purified by semipreparative HPLC, and the purity of the target compound was generally obtained in 70% yield with over 95% purity. The retention time on analytical HPLC for DOTA–NAPamide was found to be 19.1 min. The purified DOTA–NAPamide was characterized by MALDI-TOF-MS. The measured molecular weight (MW) was consistent with the expected MW: $m/z = 1485.97$ for $[\text{M} + \text{H}]^+$ ($\text{C}_{68}\text{H}_{101}\text{N}_{20}\text{O}_{18}$, calcd MW = 1485.75). For $^{\text{nat}}\text{Cu}$ –DOTA–NAPamide, MW was measured as 1546.36 for $[\text{M} + \text{H}]^+$ ($\text{C}_{68}\text{H}_{99}\text{CuN}_{20}\text{O}_{18}$, calcd MW = 1546.67).

DOTA–NAPamide conjugate was then successfully labeled with ^{64}Cu at 50 °C for 1 h incubation. The purification of radiolabeling solution using RP-HPLC afforded ^{64}Cu –DOTA–NAPamide with >95% radiochemical purity. Under several of HPLC conditions tested in this study, separation of ^{64}Cu –DOTA–NAPamide and unlabeled DOTA–NAPamide was not achieved. A modest specific activity of 0.4 Ci/ μmol (14.8 MBq/nmol, 270 $\mu\text{Ci}/\mu\text{g}$) ^{64}Cu –DOTA–NAPamide was obtained as determined by analytic HPLC (decay corrected). The specific activity of the tracer prepared in this research is very close to that of another ^{64}Cu -labeled α -MSH analog, ^{64}Cu –DOTA–ReCCMSH(Arg¹¹) (300 $\mu\text{Ci}/\mu\text{g}$) which was reported recently (17). From the octanol–water partition coefficient measurements the log *P* value of tracer was determined to be -1.15 ± 0.01 , indicating intermediate hydrophilicity of this compound.

In Vitro Receptor-Binding Assay and Biological Activity

A typical saturation binding experiment was performed to determine the MC1R densities on A375M and B16F10 melanoma cells. The cells (1×10^6) were incubated in 0.15 mL of binding medium in the presence of ^{125}I -(Tyr²)-NDP ranging from approximately 2×10^3 to 2×10^6 cpm per tube with and without 10^{-6} M NDP for 2 h at 37 °C. The radioactivity of bound and free radioligand was counted. The data were analyzed by ORIGIN6.0, and the binding capacity of B16F10 and A375M were calculated to be $21\,687 \pm 4171$ and 400 ± 93 (sites/cell $\pm$ SD), respectively.

The receptor-binding affinity study of peptides for MC1R was performed using B16F10 cells. The IC₅₀ values of DOTA–NAPamide and ^{nat}Cu –DOTA–NAPamide were determined to be 2.67 ± 0.23 and 3.66 ± 0.29 nM, respectively, from the competitive receptor binding assay (Figure 2A). The IC₅₀ value of DOTA–NAPamide is very close to what is reported in the literature (1.19 ± 0.19 nM, determined by using B16F1 cells) (14). Furthermore, the binding affinity of DOTA–NAPamide is only slightly reduced by chelation with Cu. Figure 2B shows the cell binding and internalization of ^{64}Cu –DOTA–NAPamide in B16F10 cells at 37 °C over a 3 h incubation period. The cell binding observed for the radiolabeled peptide steadily increased over the 3-h incubation time, and it reached a plateau 4.7% at 3 h. The ^{64}Cu –DOTA–NAPamide showed rapid internalization with approximately 73% internalization of the receptor-bound radiolabeled complexes within 5 min of incubation, while around 94% of receptor-bound tracer was internalized at 3-h incubation. Cellular retention of the radiolabeled complex as a function of time is shown in Figure 2C. The receptor-bound ^{64}Cu –DOTA–NAPamide slowly released from the cells into the medium, with 66% of the radioactivity still associated with the cells even after 3 h incubation.

Biodistribution Studies

The in vivo biodistribution of ^{64}Cu –DOTA–NAPamide was examined with both the B16F10 mouse melanoma and the A375M human melanoma tumor-bearing mouse model. Biodistribution of the radiolabeled peptide at 1, 2, 4, and 24 h was obtained in the B16F10 mouse model (Figure 3A), while 2 and 24 h biodistribution data was obtained in the A375M melanoma model (Figure 3B). In both tumor models the radiolabeled complex displayed rapid blood clearance; $0.97 \pm 0.47\%$ and $1.33 \pm 0.34\%$ ID/g blood uptake was observed at 2 h pi for B16F10 and A375M tumor bearing mice, respectively. Rapid and moderate activity

accumulation in the high MC1R expressing B16F10 tumors was observed at early time points ($3.69 \pm 0.28\%$ ID/g at 1 h pi). Tumor uptake slowly increased to $4.63 \pm 0.45\%$ ID/g at 2 h pi and $4.43 \pm 0.94\%$ ID/g at 4 h pi, then the activity was slowly washed out from the tumor, and uptake dropped to $1.67 \pm 0.58\%$ ID/g over 24 h (Figure 3A). While for the A375M tumor with low MC1R expression, tumor uptake was $2.49 \pm 0.31\%$ ID/g at 2 h pi (Figure 3B), which was significantly lower than the uptake of ^{64}Cu -DOTA-NAPamide in the B16F10 tumor model ($P < 0.05$). Furthermore, the in vivo specificity of ^{64}Cu -DOTA-NAPamide tumor uptake was investigated by coinjection of 200 μg of nonradiolabeled NDP with the radiolabeled peptide complex to the A375M melanoma-bearing Fox Chase ICR Scid mice and B16F10 melanoma-bearing C57 mice at 2 h. In the B16F10 tumor model, coinjection of NDP specifically reduced the tumor uptake of ^{64}Cu -DOTA-NAPamide from 4.63 ± 0.45 to 2.29 ± 0.24 (% ID/g; $P < 0.05$) without changing the distribution of ^{64}Cu -DOTA-NAPamide in most of the normal tissues studied (Figure 4). However, coinjection of NDP with radiolabeled peptide did not reduce uptake of ^{64}Cu -DOTA-NAPamide in the A375M tumor ($2.49 \pm 0.31\%$ ID/g for nonblocking versus $2.20 \pm 0.41\%$ ID/g for blocking) at 2 h pi.

For both tumor models ^{64}Cu -DOTA-NAPamide displayed high accumulation and retention in the liver. It was observed that $12.17 \pm 2.00\%$ and $4.53 \pm 1.19\%$ ID/g remaining was still in the liver at 24 h pi for A375M and B16F10 tumor mice model, respectively. High renal activity accumulation was also observed at all times in both tumor models (Figure 3A and B).

MicroPET Imaging

Decay-corrected coronal (top) and transaxial (bottom) microPET images of mouse bearing A375M tumor on the right shoulder and mouse bearing B16F10 tumor at 1, 2, and 4 h after tail vein injection of 4.44 MBq (120 μCi) ^{64}Cu -DOTA-NAPamide are shown in the Figure 5A and C, respectively. Although the radioactivity accumulation in the liver and kidney are quite high, the B16F10 tumor is still clearly visualized with good tumor to contralateral background contrast at 1–4 h pi. However, lower uptake in A375M tumor was observed, resulting in poor tumor to normal tissue contrast. Quantification of activity accumulation in the A375M and B16F10 melanoma and major organs (Figure 5B and D) agreed well with the biodistribution results.

DISCUSSION

Molecular imaging of cancer is a fast-growing research field (22). Molecular imaging technologies have demonstrated great benefits for better understanding cancer biology as well as facilitating cancer drug development and cancer early detection (22, 23). Development of novel imaging methods and molecularly targeted probes will allow us to not only locate a tumor but also visualize the expression and activity of specific molecular targets and biological processes in a tumor. Currently, 2-deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG) is widely used in malignant melanoma diagnosis and staging in clinic. The molecular targets for ^{18}F FDG are glucose transporters (e.g., GLUT1), and hexokinase. ^{18}F FDG only provides the glucose metabolic activity of a tumor (24). MC1R plays an

important role in the proliferation and differentiation of melanocytes as well as in the development and growth of melanoma cells. It is also considered as a genetic linker to skin cancer (25). Overexpression of MC1R in melanoma cells makes it a promising molecular target for melanoma imaging and therapy. Thus, it is important to develop a PET molecular probe for imaging MC1R expression in living subjects.

Several α -MSH analogs which can recognize the MC1R have been labeled with different radionuclides for melanoma detection and radiotherapy (9–20, 26–28). The most promising melanoma-targeting peptides are linear peptide NAPamide (16) and rhenium-cyclized CCMSH peptides (9, 11, 15). NAPamide is a short peptide with only eight amino acid residues. It can be easily synthesized in high yield and purity using the conventional solid-phase peptide synthesis and Fmoc/HBTU chemistry. Conjugating the peptide with metal chelate, DOTA, can also be easily achieved through reaction between the Lys⁸ ϵ -amino group in the NAPamide and in-situ-activated DOTA in buffered solution. The resulting DOTA–NAPamide conjugate as well as Cu-coordinated ligand (^{nat}Cu–DOTA–NAPamide) displays high binding affinity to MC1R (Figure 2A). ¹¹¹In- and ⁶⁷Ga-labeled DOTA–NAPamide exhibit high tumor, moderate kidney, and low blood uptake at 4 h pi (16). ⁶⁴Cu is a positron emitter that can be readily produced using a medical cyclotron. Its intermediate half-life makes it suitable for small peptide radiolabeling (29–33). Considering all of these advantages, we hypothesized that radiolabeling DOTA–NAPamide with ⁶⁴Cu would generate a novel molecular probe for routine PET imaging of melanoma and MC1R expression.

Subsequently, ⁶⁴Cu–DOTA–NAPamide was prepared and its MC1R targeting ability evaluated in cell culture and in vivo. Murine melanoma cell line B16F10 and human melanoma cell line A375M were then used in biological studies because they express different levels of MC1R ($B_{\max} = 2.2 \times 10^4$ and 400 receptors/cell for B16F10 and A375M, respectively). In vitro B16F10 cell studies show that ⁶⁴Cu–DOTA–NAPamide specifically binds to MC1R and reaches a plateau at 3 h incubation. The majority of receptor-bound radiolabeled complexes are rapidly internalized from 5 min to 3 h incubation (Figure 2B). A cellular retention study demonstrates that the receptor-bound ⁶⁴Cu–DOTA–NAPamide is slowly washed out from cells, with 66% of the radioactivity still associated with the cells even after 3 h incubation. These results are quite similar to the results of cell uptake, internalization, and retention of ^{99m}Tc–CCMSH reported previously (9). The high MC1R binding and internalization property of ⁶⁴Cu–DOTA–NAPamide in cell culture justifies its further evaluation in animal models.

In vivo biodistribution studies show that ⁶⁴Cu–DOTA–NAPamide possesses reasonable uptake in high MC1R expression B16F10 melanoma at 1–24 h pi (Figure 3A) and that the maximum uptake of $4.63 \pm 0.45\%$ ID/g is achieved at 2 h pi. When coinjected with a large amount of NDP peptide, accumulation of the tracer at 2 h pi is reduced by 50% (Figure 4), demonstrating the specific accumulation of the tracer. More interestingly, the tracer uptake in low MC1R expression A375M tumor at 2 h pi is around 50% uptake of B16F10 tumor. Coinjection of NDP cannot inhibit tracer uptake in A375M tumor. These data indicate that the 2.49% ID/g uptake of A375M at 2 h pi is likely nonspecific accumulation. We also observe increased liver and kidney uptake values in the B16F10 blocked mice group

compared to B16F10 group in this research. It is possible that the large amount of NDP peptide used in this study (200 vs 20 μ g used in ref 17) may play a role in altering the biodistribution of the tracer in the kidney and liver. Further studies are needed to confirm and elucidate this phenomenon.

High accumulation and retention of ^{64}Cu -DOTA-NAPamide in the liver is observed in both B16F10 and A375M tumor models (Figure 3A and B). This can be explained by the release of uncoordinated ^{64}Cu from radiolabeled peptide by decomposition in the blood or transchelation in the liver. Better chelation systems for ^{64}Cu such as TETA (1,4,8,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid) or cross-bridged cyclam ligands may improve the metal-chelate stability and subsequently improving the biodistribution (34–35). Similar to other radiometal-labeled peptides (9, 11, 17, 21, 30), high renal activity accumulation and retention of ^{64}Cu -DOTA-NAPamide is also observed in both tumor models (Figure 3A and B). This could possibly be decreased by the use of L- or D-lysine without affecting tumor retention (9, 15) in order to achieve a better kidney to normal tissue ratio and reduce radiation dose to the tested subject.

The biodistribution of ^{111}In - and ^{67}Ga -labeled DOTA-NAPamide was evaluated in the study using B6D2F1 mice bearing B16F1 melanoma (16). Both ^{111}In - and ^{67}Ga -labeled DOTA-NAPamide exhibit high tumor uptake and rapid clearance from normal tissue. For example, ^{111}In - and ^{67}Ga -labeled DOTA-NAPamide display 7.56 ± 0.51 and $9.43 \pm 1.06\%$ ID/g tumor uptake at 4 h pi, respectively. The uptake values for normal tissues such as liver, lung, spleen, and muscle are all below 0.45% ID/g at 4 h pi (16). In contrast, ^{64}Cu -labeled DOTA-NAPamide shows lower B16F10 tumor uptake (for example, $4.43 \pm 0.94\%$ ID/g at 4 h pi) but higher uptake and retention in normal tissues when used in C57 BL/6 mice bearing B16F10 murine melanoma. The observation that the α -MSH peptide labeled with different radiometals can change their in vivo properties has been reported before (17). It is generally believed that the charge of the radiopeptide and the in vivo stability of the metal-chelate are responsible for their different in vivo behaviors. Our results reported here further highlight the importance of selecting the appropriate radiometal for peptide labeling so that the best tracer can be developed for molecular target imaging.

MicroPET imaging and quantification analysis of ^{64}Cu -DOTA-NAPamide in mice bearing A375M or B16F10 tumor 1, 2, and 4 h after tail vein injection reveal moderate B16F10 tumor-to-background, while the A375M tumor-to-background ratio is low (Figure 5). These results are consistent with the finding obtained from the biodistribution studies. Therefore, in addition to early detection of α -MSH receptor positive melanoma, it is also possible to differentiate melanoma with different MC1R densities in living subjects using the molecular probe ^{64}Cu -DOTA-NAPamide in conjunction with quantitative PET imaging technology. Future research is needed to further evaluate whether this tracer can help in selection of patients for anti-MC1R treatment.

In conclusion, ^{64}Cu -DOTA-NAPamide demonstrates high cell uptake, internalization, and retention in MC1R high expression melanoma. Biodistribution and microPET imaging studies show moderate specific uptake in a B16F10 melanoma model with high MC1R density while a low uptake in A375M melanoma with low MC1R capacity. ^{64}Cu -DOTA-

NAPamide is a promising molecular probe for PET imaging MC1R expression in living subjects.

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Figure 1.
Schematic structures of DOTA-NAPamide and ⁶⁴Cu-DOTA-NAPamide.

Figure 2.

(A) In vitro inhibition of ^{125}I -(Tyr 2)-NDP binding to MC1R on murine B16F10 melanoma cell line by DOTA-NAPamide ($\text{IC}_{50} = 2.67 \pm 0.23$ nmol/L) and ^{nat}Cu -DOTA-NAPamide ($\text{IC}_{50} = 3.66 \pm 0.29$ nmol/L). Results expressed as percent of binding are mean of triplicate measurement $\pm$ standard deviation. (B) Cell binding and internalization over time for ^{64}Cu -DOTA-NAPamide with B16/F10 cells at 37 °C. Surface-bound radioactivity and internalized activity are expressed as a percent of total applied activity. (C) Percent retention of internalized ^{64}Cu -DOTA-NAPamide radioactivity in B16/F10 cells over time at 37 °C.

Figure 3.

(A) Biodistribution data for ^{64}Cu -DOTA-NAPamide in C57 mice bearing subcutaneously xenotransplanted B16F10 murine melanoma (A) and Fox Chase ICR Scid mice bearing A375M human melanoma (B). Data are expressed as the percent administered activity (injected dose) per gram of tissue (% ID/g) ($n = 4$). ^{64}Cu -DOTA-NAPamide exhibited significantly higher uptake in B16F10 at 2 h postinjection than in A375M ($P < 0.05$).

Figure 4.

Receptor-blocking study: biodistributions of ⁶⁴Cu-DOTA-NAPamide in A375M melanoma-bearing Fox Chase ICR Scid mice and B16F10 melanoma-bearing C57 mice at 2 h with and without coinjection of 200 μ g of NDP as a blocking agent. Significant inhibition of uptake in B16F10 melanoma ($P < 0.0015$), while not in A375M melanoma ($P > 0.05$), was observed. Data are expressed as normalized accumulation of activity in % ID/g $\pm$ SD ($n = 3$ for each group).

Figure 5. Decay-corrected coronal (top) and transaxial (bottom) microPET images of Fox Chase ICR Scid mouse bearing A375M tumor on the right shoulder (A) and C57 mouse bearing B16F10 tumor (C) at 1, 2, and 4 h after tail vein injection of $(4.44 \text{ MBq}, 120 \mu\text{Ci})$ ^{64}Cu -DOTA-NAPamide. Arrows indicate location of tumors (T). Time activity curves derived from the multitime point microPET imaging of mice bearing A375M (B) and B16F10 (D) melanoma. The regions of interest (ROIs) are shown as mean % ID/g $\pm$ SD ($n = 3$).