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Metastatic Melanoma Imaging with an ^{111}In -labeled Lactam Bridge-cyclized Alpha-Melanocyte Stimulating Hormone Peptide

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Abstract

Introduction—The purpose of this study was to examine whether a novel lactam bridge-cyclized ^{111}In -labeled 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid-Gly-Glu-c[Lys-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Arg-Pro-Val-Asp] {DOTA-GlyGlu-CycMSH} could be an effective imaging probe for metastatic melanoma detection.

Methods— ^{111}In -DOTA-GlyGlu-CycMSH was prepared and purified by reverse phase high performance liquid chromatography (RP-HPLC). The internalization and efflux of ^{111}In -DOTA-GlyGlu-CycMSH were examined in B16/F10 melanoma cells. The biodistribution of ^{111}In -DOTA-GlyGlu-CycMSH was determined in B16/F10 pulmonary metastatic melanoma-bearing and normal C57 mice. Pulmonary metastatic melanoma imaging was performed by small animal SPECT/CT (Nano-SPECT/CT[®]) using ^{111}In -DOTA-GlyGlu-CycMSH as an imaging probe and compared with ^{18}F -FDG PET imaging.

Results— ^{111}In -DOTA-GlyGlu-CycMSH was readily prepared with greater than 95% radiolabeling yield. ^{111}In -DOTA-GlyGlu-CycMSH displayed rapid internalization and extended efflux in B16/F10 cells. ^{111}In -DOTA-GlyGlu-CycMSH exhibited significantly ($p < 0.05$) higher uptakes (2.00 ± 0.74 %ID/g at 2 h post-injection and 1.83 ± 0.12 %ID/g at 4 h post-injection) in metastatic melanoma-bearing lung than that in normal lung (0.08 ± 0.08 %ID/g and 0.05 ± 0.05 %ID/g at 2 and 4 h post-injection, respectively). The activity accumulation in normal organs were low (< 0.5 %ID/g) except for the kidneys 2 and 4 h post-injection. B16/F10 pulmonary melanoma metastases were clearly visualized with ^{111}In -DOTA-GlyGlu-CycMSH 2 h post-injection rather than with ^{18}F -FDG 1 h post-injection.

Conclusions— ^{111}In -DOTA-GlyGlu-CycMSH exhibited favorable metastatic melanoma targeting and imaging properties, highlighting its potential as an effective imaging probe for metastatic melanoma detection.

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Keywords

Metastatic melanoma imaging; radiolabeled lactam bridge-cyclized peptide; alpha-melanocyte stimulating hormone

Introduction

Skin cancer is the most commonly diagnosed cancer in the United States. Malignant melanoma is the most lethal form of skin cancer and the most commonly diagnosed malignancy among young adults with an increasing incidence. It is predicted that there will be 62,940 cases of malignant melanoma newly reported and 8,420 fatalities in 2008 (1). Melanoma metastases are highly aggressive and the survival time for patients with metastatic melanoma averages 3-15 months (2). Unfortunately, no curative treatment exists for metastatic melanoma. Early diagnosis and prompt surgical removal are a patient's best opportunity for a cure. Single photon emission tomography (SPECT) and positron emission tomography (PET) techniques are attractive non-invasive imaging modalities due to their high sensitivity (10^{-10} to 10^{-11} M for SPECT and 10^{-11} to 10^{-12} M for PET) and spatial resolution (1-2 mm) (3,4). Currently, 2- ^{18}F fluoro-2-deoxy-D-glucose (^{18}F FDG) PET imaging is commonly used for the diagnosis and staging of melanoma. However, ^{18}F FDG is not a melanoma-specific imaging probe since the elevated uptake of ^{18}F FDG in tumor is due to the higher metabolism and energy consumption in tumor cells than that in normal cells. ^{18}F FDG PET imaging only detects 23% melanoma metastases smaller than 5 mm (5). Meanwhile, some melanoma cells are not detected by ^{18}F FDG PET imaging since they use substrates other than glucose as energy sources (6,7). Therefore, it is highly desirable to develop novel effective imaging probes to detect primary, metastatic and recurrent melanomas.

G protein-coupled melanocortin-1 (MC1) receptors have been used as targets to develop melanoma-specific imaging probes due to their over-expression on human and mouse melanoma cells (8-12). Radiolabeled α -melanocyte stimulating hormone (α -MSH) peptide analogues, derived from wild-type α -MSH, are very promising candidates for melanoma imaging and therapy due to their nanomolar MC1 receptor binding affinities and high receptor-mediated tumor uptakes in murine melanoma-bearing mice and human melanoma xenografts (13-17). Novel ^{111}In -labeled 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-conjugated lactam bridge-cyclized α -MSH peptides were developed to target MC1 receptors for melanoma imaging in our previous report (18). Lactam bridge-cyclization was employed to improve the stabilities of the peptides against the proteolytic degradations *in vivo* and enhance the binding affinities of the peptides through stabilizing their secondary structures such as beta turns (19-22). DOTA was coupled to the lactam bridge-cyclized peptides directly or through a negatively-charged amino acid linker (-Gly-Glu-) to determine the effect of a negatively-charged linker in reducing the renal uptakes of ^{111}In -labeled lactam bridge-cyclized peptides. Introduction of a negatively-charged amino acid linker (-Gly-Glu-) into the peptide sequence decreased the renal uptakes by 44% without affecting the tumor uptakes 4 h post-injection. ^{111}In -labeled DOTA-GlyGlu-CycMSH (DOTA-Gly-Glu-c[Lys-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Arg-Pro-Val-Asp]) exhibited high receptor-mediated tumor uptake ($10.40 \pm 1.40\%$ ID/g at 2 h post-injection) in flank B16/F1 murine melanoma-bearing mouse model (18), highlighting the potential of using ^{111}In -labeled DOTA-GlyGlu-CycMSH as a melanoma-specific imaging probe for metastatic melanoma detection.

In this study, ^{111}In -labeled DOTA-GlyGlu-CycMSH was further evaluated in B16/F10 pulmonary metastatic melanoma mouse model to validate its feasibility as an effective melanoma-specific imaging probe for melanoma metastases detection. The biodistribution of ^{111}In -labeled DOTA-GlyGlu-CycMSH was determined in the B16/F10 pulmonary

metastatic melanoma-bearing C57 mice and compared with that in the normal C57 mice. Dual-modality small animal SPECT/CT (Nano-SPECT/CT[®]) was used to detect different-stage pulmonary melanoma metastases using ¹¹¹In-labeled DOTA-GlyGlu-CycMSH as an imaging probe to monitor the development of melanoma metastases. The imaging properties on melanoma metastases between ¹⁸F-FDG PET imaging and ¹¹¹In-DOTA-GlyGlu-CycMSH SPECT/CT imaging were compared by injecting ¹⁸F-FDG and ¹¹¹In-DOTA-GlyGlu-CycMSH with a time interval of 26 h in a pulmonary metastatic melanoma-bearing mouse, respectively.

Materials and Methods

Chemicals and Reagents

Amino acid and resin were purchased from Advanced ChemTech Inc. (Louisville, KY) and Novabiochem (San Diego, CA). DOTA-tri-t-butyl ester was purchased from Macrocyclics Inc. (Richardson, TX). ¹¹¹InCl₃ was purchased from Trace Life Sciences, Inc. (Dallas, TX). ¹²⁵I-Tyr²-[Nle⁴, D-Phe⁷]-α-MSH {¹²⁵I-(Tyr²)-NDP-MSH} was obtained from PerkinElmer, Inc. (Shelton, CT). All other chemicals used in this study were purchased from Thermo Fischer Scientific (Waltham, MA) and used without further purification. B16/F10 murine melanoma cells were obtained from American Type Culture Collection (Manassas, VA).

MC1 Receptor Quantitation Assay

The MC1 receptor density was determined on B16/F10 melanoma cells. One million B16/F10 cells were incubated at room temperature (25°C) for 2 h in the presence of an increasing concentration (2.5, 5.0, 10, 15, 20, 40, 60, 100 nCi) of ¹²⁵I-(Tyr²)-NDP-MSH in 0.5 mL of binding media {Minimum Essential Medium (MEM) with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.2% bovine serum albumin (BSA), 0.3 mM 1,10-phenanthroline}. The reaction media were aspirated after incubation. Cells were rinsed with 0.5 mL of ice-cold pH 7.4, 0.2% BSA / 0.01 M phosphate buffered saline (PBS) twice, and then the levels of activity associated with the cells were measured in a Wallac 1480 automated gamma counter (PerkinElmer, NJ). Non-specific binding was determined by incubating the cells and ¹²⁵I-(Tyr²)-NDP-MSH with non-radioactive NDP-MSH at a final concentration of 10 μM. Specific binding was obtained by subtracting the nonspecific binding from total binding. Maximum specific binding (B_{max}) was estimated from nonlinear curve fitting of specific binding (dpm) versus the concentration of ¹²⁵I-(Tyr²)-NDP-MSH (fmole/mL) using Prism software (GraphPad Software, La Jolla, CA).

In vitro Competitive Binding Assay

The IC₅₀ value of DOTA-GlyGlu-CycMSH was determined in B16/F10 cells. The cells were harvested and seeded into a 24-well cell culture plate (5×10⁵/well) and incubated at 37°C overnight. After being washed once with binding media, the cells were incubated at 25°C for 2 h with approximately 50,000 cpm of ¹²⁵I-(Tyr²)-NDP-MSH in the presence of increasing concentrations of DOTA-GlyGlu-CycMSH (10⁻¹² to 10⁻⁵ M) in 0.3 mL of binding media. The reaction media were aspirated after incubation. Cells were rinsed with 0.5 mL of ice-cold pH 7.4, 0.2% BSA / 0.01 M PBS twice, and then lysed in 0.5 mL of 1 N NaOH for 5 min. The activities associated with the cells were measured in a gamma counter. The IC₅₀ value was calculated by using Prism software.

Cellular Internalization and Efflux of ¹¹¹In-DOTA-GlyGlu-CycMSH

DOTA-GlyGlu-CycMSH was synthesized as described previously (18) and identified by mass spectrometry. ¹¹¹In-DOTA-GlyGlu-CycMSH was prepared in a 0.5 M NH₄OAc-buffered solution at pH 5.4 according to the published procedure (18). Briefly, 50 μl of ¹¹¹InCl₃ (37-74 MBq in 0.05 M HCl), 10 μl of 1 mg/ml DOTA-GlyGlu-CycMSH aqueous solution and 400

μl of 0.5 M NH_4OAc (pH 5.4) were added into a reaction vial and incubated at 75°C for 45 min. After the incubation, 20 μl of 0.5% EDTA aqueous solution was added into the reaction vial to scavenge potential unbound ^{111}In . The radiolabeled complex was purified to single species by Waters RP-HPLC (Milford, MA) on a Grace Vadc C-18 reverse phase analytical column (Deerfield, IL) using a 20-minute gradient of 16-26% acetonitrile in 20 mM HCl aqueous solution with a flowrate of 1 ml/min. Cellular internalization and efflux of ^{111}In -DOTA-GlyGlu-CycMSH were evaluated in B16/F10 cells. After being washed once with binding media, B16/F10 cells in cell culture plates were incubated at 25°C for 20, 40, 60, 90 and 120 min ($n=4$) in the presence of approximately 200,000 counts per minute (cpm) of HPLC-purified ^{111}In -DOTA-GlyGlu-CycMSH (0.019 pmol). After incubation, the reaction media were aspirated and the cells were rinsed with 2×0.5 mL of ice-cold pH 7.4, 0.2% BSA / 0.01 M PBS. Cellular internalization of ^{111}In -DOTA-GlyGlu-CycMSH was assessed 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 radioactivity. The remaining internalized radioactivity was obtained by lysing the cells with 0.5 mL of 1 N NaOH for 5 min. Membrane-bound and internalized ^{111}In activities were counted in a gamma counter. Cellular efflux of ^{111}In -DOTA-GlyGlu-CycMSH was determined by incubating B16/F10 cells with ^{111}In -DOTA-GlyGlu-CycMSH for 2 h at 25°C , removing non-specific-bound activity with 2×0.5 mL of ice-cold pH 7.4, 0.2% BSA / 0.01 M PBS rinse, and monitoring radioactivity released into cell culture media. At time points of 20, 40, 60, 90 and 120 min, the radioactivities on the cell surface and in the cells were separately collected and counted in a gamma counter.

Biodistribution Studies

All the animal studies were conducted in compliance with Institutional Animal Care and Use Committee approval. B16/F10 pulmonary metastatic melanoma model was established by injecting 0.2 million of cultured B16/F10 cells into each C57 mouse (Harlan, Indianapolis, IN) through the tail vein. The B16/F10 pulmonary metastatic melanoma-bearing C57 mice were used for biodistribution studies 16 days after the cell injection. The pharmacokinetics of ^{111}In -DOTA-GlyGlu-CycMSH was determined in B16/F10 pulmonary metastatic melanoma-bearing and normal C57 mice. HPLC-purified 0.037 MBq of ^{111}In -DOTA-GlyGlu-CycMSH was injected into each mouse through the tail vein. Groups of 5 mice were sacrificed at 2, 4 and 24 h post-injection, and tumors and organs of interest were harvested, weighed and counted. Blood values were taken as 6.5% of the whole-body weight. Statistical analysis was performed using the Student's t-test for unpaired data. A 95% confidence level was chosen to determine the significance between the activity distribution in pulmonary metastatic melanoma-bearing and normal mice, with $p<0.05$ being significantly different.

Metastatic Melanoma Imaging

A pulmonary metastatic melanoma-bearing mouse was used for [^{18}F]FDG PET imaging 16 days after the cell injection and used for ^{111}In -DOTA-GlyGlu-CycMSH SPECT/CT imaging on next day (a time interval of 26 h) to compare the difference in detection efficiency between [^{18}F]FDG and ^{111}In -DOTA-GlyGlu-CycMSH. The mouse was injected with 35.15 MBq of commercial [^{18}F]FDG (Biotech, NM) via the tail vein. At 1 h post-injection, the mouse was anesthetized with 1.5% isoflurane and placed in the prone position near the center of the field of view of small animal PET (LabPETTM) (Gamma Medica-Ideas, Sherbrooke, Quebec). A 30-min static scan was acquired, and the image was reconstructed by maximum likelihood estimation method (MLEM). The mouse was returned to the cage for recovery after the ^{18}F -FDG PET imaging study was completed. The same mouse was injected with 24.05 MBq of ^{111}In -DOTA-GlyGlu-CycMSH via the tail vein 24 h after the ^{18}F -FDG PET imaging study. The mouse was anesthetized with 1.5% isoflurane for small animal SPECT/CT (Nano-SPECT/CT[®], Bioscan) imaging 2 h post-injection. The 6-min CT imaging was immediately followed by the focused SPECT imaging of lung. The SPECT scans of 24 projections were acquired

and total acquisition time was 45 min. After the focused lung imaging, the mouse was sacrificed with CO₂ inhalation for whole-body SPECT/CT imaging. The 9-min CT imaging was immediately followed by the SPECT imaging of whole-body. Reconstructed data from SPECT and CT were visualized and co-registered using InVivoScope (Bioscan, Washington DC). Necropsy analysis of the mouse was performed to confirm the pulmonary metastatic melanoma lesions after the SPECT/CT imaging studies were completed. To monitor the development of pulmonary melanoma metastases with ¹¹¹In-DOTA-GlyGlu-CycMSH, a pulmonary metastatic melanoma-bearing mouse was injected with 10.36 MBq of ¹¹¹In-DOTA-GlyGlu-CycMSH through the tail vein for small animal SPECT/CT imaging 20 days after the cell injection. The mouse was anesthetized with 1.5% isoflurane for SPECT/CT imaging 2 h post-injection as described above. The SPECT/CT images of pulmonary melanoma metastases developed 20 days after the cell injection were compared with the SPECT/CT images of pulmonary melanoma metastases developed 17 days after the cell injection. Necropsy analysis of the mouse was performed after the SPECT/CT imaging studies were completed. The metastatic melanoma-bearing lung was taken out for a SPECT imaging to confirm the uptake of ¹¹¹In-DOTA-GlyGlu-CycMSH activity.

Results

The MC1 receptor density of B16/F10 cell was determined by saturation binding assay using commercial ¹²⁵I-(Tyr²)-NDP-MSH as a radioactive tracer. The saturation curve and scatchard plot are presented in Figure 1. The Bmax of B16/F10 cells was 23394 dpm/million cells (2884 receptors/cell). DOTA-GlyGlu-CycMSH was synthesized, purified by RP-HPLC and identified by electrospray ionization mass spectrometry. Figure 2 illustrates the competitive binding curve of DOTA-GlyGlu-CycMSH in B16/F10 cells. The IC₅₀ value of DOTA-GlyGlu-CycMSH was 2.43 nM in B16/F10 cells. The peptide was easily labeled with ¹¹¹In using a 0.5 M NH₄OAc-buffered solution at pH 5.4 with greater than 95% labeling yield. ¹¹¹In-DOTA-GlyGlu-CycMSH was completely separated from its excess non-labeled peptide by RP-HPLC. The retention time of ¹¹¹In-DOTA-GlyGlu-CycMSH was 18.1 min using a 20 min gradient of 16-26% acetonitrile in 20 mM HCl aqueous solution with a flow rate of 1 mL/min (Fig. 3). The specific activity of ¹¹¹In-DOTA-GlyGlu-CycMSH was 8.23×10⁸ MBq/g. ¹¹¹In-DOTA-GlyGlu-CycMSH was stable in mouse serum at 37°C for 24 h. The schematic structure of ¹¹¹In-DOTA-GlyGlu-CycMSH is shown in Figure 3.

Cellular internalization and efflux of ¹¹¹In-DOTA-GlyGlu-CycMSH were evaluated in B16/F10 cells. Figure 4 illustrates cellular internalization and efflux of ¹¹¹In-DOTA-GlyGlu-CycMSH. ¹¹¹In-DOTA-GlyGlu-CycMSH exhibited rapid cellular internalization and extended cellular retention. There was 68.7±10.8% of ¹¹¹In-DOTA-GlyGlu-CycMSH activity internalized in the B16/F10 cells 20 min post incubation. There was 79.7±10.1% of ¹¹¹In-DOTA-GlyGlu-CycMSH activity internalized in the cells after 2 h incubation. Cellular efflux experiments demonstrated that 90.6±5.3% of ¹¹¹In-DOTA-GlyGlu-CycMSH activity remained inside the cells 2 h after incubating cells in culture media.

The pharmacokinetics and tumor targeting properties of ¹¹¹In-DOTA-GlyGlu-CycMSH were determined in B16/F10 pulmonary metastatic melanoma-bearing C57 mice and compared with normal C57 mice at 2, 4 and 24 h post-injection. The biodistribution results of ¹¹¹In-DOTA-GlyGlu-CycMSH are shown in Table 1. ¹¹¹In-DOTA-GlyGlu-CycMSH exhibited significantly (*p*<0.05) higher uptake value in metastatic melanoma-bearing lung than that in normal lung. The uptake values of ¹¹¹In-DOTA-GlyGlu-CycMSH radioactivity in the metastatic melanoma-bearing and normal lungs were 2.00±0.74 and 0.08±0.08 %ID/g, 1.83±0.12 and 0.05±0.05 %ID/g at 2 and 4 h post-injection, respectively. The uptake values of ¹¹¹In-DOTA-GlyGlu-CycMSH radioactivity in the metastatic melanoma-bearing and normal lungs were 0.29±0.06 and 0.03±0.02 %ID/g at 24 h post-injection. The ¹¹¹In-DOTA-

GlyGlu-CycMSH displayed higher lung/normal organ uptake ratios in pulmonary metastatic melanoma-bearing mice than that in normal mice (Table 1). Whole-body clearance of ^{111}In -DOTA-GlyGlu-CycMSH was rapid, with approximately 84% of the injected radioactivity cleared through the urinary system by 2 h post-injection (Table 1). Approximately 96% of the injected radioactivity cleared out the body at 24 h post-injection.

One B16/F10 pulmonary melanoma-bearing C57 mouse was injected with ^{18}F FDG and ^{111}In -DOTA-GlyGlu-CycMSH via the tail vein (with a time interval of 26 h) to compare melanoma metastases imaging properties 16 and 17 days post the cell injection, respectively. The focused three-dimensional and transaxial SPECT/CT and PET images are presented in Figures 5A1-5A5, respectively. The pulmonary metastatic melanoma lesions were visualized by SPECT/CT using ^{111}In -DOTA-GlyGlu-CycMSH as an imaging probe (Figs. 5A1 and 5A2) rather than ^{18}F FDG PET imaging (Figs. 5A4 and 5A5). ^{111}In -DOTA-GlyGlu-CycMSH clearly identified individual metastatic melanoma deposits (Figs. 5A1 and 5A2). Melanoma metastases were confirmed by necropsy picture (Fig. 5A3). Whole-body SPECT/CT and PET images are also presented in Figures 5B1 and 5B2. Pulmonary metastatic melanoma lesions were visualized with ^{111}In -DOTA-GlyGlu-CycMSH by whole-body SPECT/CT imaging (Fig. 5B1). ^{111}In -DOTA-GlyGlu-CycMSH was mainly excreted through the urinary system. The accumulation of ^{111}In -DOTA-GlyGlu-CycMSH activity in normal organs was extremely low except for the kidneys in the whole-body SPECT/CT image, which was consistent with the biodistribution results. ^{18}F FDG exhibited much higher normal organ accumulation than ^{111}In -DOTA-GlyGlu-CycMSH except for the kidneys (Fig. 5B2). Another pulmonary melanoma-bearing C57 mouse was injected with ^{111}In -DOTA-GlyGlu-CycMSH via the tail vein 20 days post the cell injection to monitor the development of melanoma metastases. The focused three-dimensional and transaxial tumor images are presented in Figures 5C1 and 5C2. Compared to the pulmonary melanoma metastases developed 17 days post the cell injection, more and bigger metastatic melanoma lesions appeared in the lung 20 days post the cell injection. ^{111}In -DOTA-GlyGlu-CycMSH clearly identified both distinct metastatic melanoma deposits and bigger metastatic melanoma lesions (Figs. 5C1 and 5C2). The lung was taken out for necropsy examination to confirm the melanoma metastases after the SPECT/CT imaging studies were completed. Furthermore, the metastatic melanoma-bearing lung was examined by SPECT imaging to confirm the uptake of ^{111}In -DOTA-GlyGlu-CycMSH activity. The necropsy picture and SPECT image of the melanoma metastases-bearing lung are presented in Figures 5C3 and 5C4. Melanoma metastases were confirmed by both necropsy picture and SPECT image (Figs. 5C3 and 5C4).

Discussion

High mortality of malignant melanoma is associated with the occurrence of metastatic melanoma due to its aggressiveness and resistance to current chemotherapy and immunotherapy regimens. Early diagnosis and prompt surgical removal of the malignant melanoma provide the patients the best opportunities for cures or prolonged survival. Despite the clinical use of ^{18}F FDG in melanoma staging and melanoma metastases identification, ^{18}F FDG is not a melanoma-specific imaging agent and is also not effective in imaging small melanoma metastases (< 5 mm) and melanomas that have primary energy sources other than glucose (5-7). Alternatively, ^{111}In -labeled lactam bridge-cyclized α -MSH peptide (^{111}In -DOTA-GlyGlu-CycMSH) was reported as a potential melanoma-specific SPECT imaging probe targeting the MC1 receptor for melanoma detection in our previous report (18), highlighting its potential as an effective imaging probe for metastatic melanoma detection. Hence, ^{111}In -DOTA-GlyGlu-CycMSH was further evaluated in B16/F10 pulmonary metastatic melanoma mouse model in this report.

B16/F10 melanoma cell line is a murine melanoma cell line with high metastatic potential (23). Therefore, B16/F10 cells were injected into the tail vein to generate an experimental metastatic melanoma mouse model to evaluate the tumor targeting and imaging properties of ^{111}In -DOTA-GlyGlu-CycMSH in this report. The number of experimental pulmonary melanoma metastases was reported to be proportional to the number of melanoma cells intravenously injected (23). The pulmonary metastatic melanoma increased in size and number with time after the melanoma cell injection (24). More and bigger metastatic melanoma lesions appeared in lung 17 days post the cell injection than that in lung 14 days post the cell injection (24). Besides the lung metastases, intravenous injection of B16/F10 melanoma cells also induced extra-pulmonary metastases such as bone, adrenal gland, liver and muscle metastases (25-31). In this report, the mouse was injected with 2×10^5 B16/F10 cells to generate pulmonary melanoma metastases to evaluate the tumor targeting and imaging properties of ^{111}In -DOTA-GlyGlu-CycMSH. The pulmonary metastatic melanoma-bearing mice were used for the biodistribution studies 16 days post the cell injection. All the mice used in the biodistribution studies developed pulmonary melanoma metastases. Recently, small animal CT has been investigated to follow the development and progression of melanoma metastases after the intravenous injection of B16/F10 melanoma cells (25). Among the pulmonary and extra-pulmonary melanoma metastases, the small animal CT imaging was reported to be best suited to detect the lesions in the lung and bone that provide high contrast between the tumor lesions and normal lung or bone tissues. Pulmonary metastatic melanoma deposits were initially detectable by small animal CT approximately 15-18 days post tail vein inoculation of B16/F10 melanoma cells (25). The growth of melanoma metastases in the lung could be monitored with additional CT studies over time (25). In this report, small animal CT imaging was performed 15 days post the cell injection to select pulmonary metastatic melanoma-bearing mice for PET and SPECT/CT imaging studies 16 and 20 days post the cell injection, respectively.

The characteristics of the cells played important roles in the development of melanoma metastases (23). Hence, the MC1 receptor density of B16/F10 cell and the binding affinity of DOTA-GlyGlu-CycMSH were determined in this report. The B_{max} of the B16/F10 cell was 2884 receptors/cell, which was in the range of receptor densities of human melanoma cell lines (12). DOTA-GlyGlu-CycMSH exhibited 2.43 nM MC1 receptor binding affinity in B16/F10 cells, making the receptor-targeting B16/F10 melanoma imaging possible. ^{111}In -DOTA-GlyGlu-CycMSH exhibited rapid cellular internalization and extended cellular retention in B16/F10 cells, with approximately 70% of the activity internalized in the cells 20 min post incubation and 90% of internalized activity remained in the cells after 2 h incubation in culture media. Efficient cellular internalization coupled with extended retention made the diagnostic and therapeutic radionuclide-labeled DOTA-GlyGlu-CycMSH suitable for melanoma imaging and therapy (10,32). The comparison of biodistribution results of the ^{111}In -DOTA-GlyGlu-CycMSH in pulmonary metastatic melanoma-bearing and normal mice demonstrated the feasibility of using ^{111}In -DOTA-GlyGlu-CycMSH to identify the pulmonary melanoma metastases. The uptake values of ^{111}In -DOTA-GlyGlu-CycMSH radioactivity in the metastatic melanoma-bearing lung (16 days post the cell injection) were 25.0 and 36.6 times the lung uptake values in normal lung at 2 and 4 h post-injection, respectively. Even 24 h post-injection, the uptake value of ^{111}In -DOTA-GlyGlu-CycMSH radioactivity in the metastatic melanoma-bearing lung was 9.7 times the lung uptake value in normal lung. The comparison of biodistribution results of the ^{111}In -DOTA-GlyGlu-CycMSH in pulmonary metastatic melanoma-bearing and normal mice also indicated higher blood, heart and spleen uptakes at 2 h post-injection in melanoma-bearing mice versus normal mice. The higher uptake values in blood, heart and spleen were likely due to either contamination of melanoma metastases or existence of melanoma metastases. The higher blood uptake value was likely due to the contamination of pulmonary melanoma metastases. The mice were euthanized by cervical dislocation and blood samples were collected by syringes from the chest cavities in the biodistribution studies. Since the B16/F10 tumors developed in the lung consisted of highly

vascularized dense gelatinous masses, it was likely that the cervical dislocation resulted in the release of gelatinous B16/F10 tumors into the chest cavity. The higher heart uptake value might be due to the existence of melanoma metastases in the heart since the heart was directly connected with the lung, which consisted of gelatinous melanoma metastases. The higher uptake value in spleen was also likely due to the existence of melanoma metastases. The differences in appearance among spleens from some (not all) melanoma-bearing mice and normal mice were easily observed during the biodistribution studies. Some spleens obtained from melanoma-bearing mice had black spots that didn't appear in any lungs obtained from normal mice.

MC1 receptor-targeting radiolabeled α -MSH peptides were reported to be effective in detecting experimental melanoma metastases (15,16,33). For instance, ^{111}In -DOTA-MSH_{oct} and ^{67}Ga -DOTA-NAPamide were able to image the B16/F1 lung or liver melanoma metastases by tissue autoradiograph (15,16). ^{111}In -DOTA-MSH_{oct} and ^{67}Ga -DOTA-NAPamide identified both melanotic and amelanotic melanoma metastases in lung (15,16), demonstrating the possibilities of radiolabeled linear NDP derivatives for metastatic melanoma detection. Recently, $^{99\text{m}}\text{Tc}$ - and ^{111}In -labeled metal-cyclized α -MSH peptides were reported to be successful in visualizing B16/F10 pulmonary melanoma metastases (24 days post the cell injection) by small animal SPECT/CT (Micro-CAT II SPECT/CT) in sacrificed melanoma-bearing mice (33), highlighting the potential of using radiolabeled α -MSH peptides for non-invasive melanoma metastases imaging. In this report, lactam bridge-cyclized ^{111}In -DOTA-GlyGlu-CycMSH was evaluated in live B16/F10 pulmonary metastatic melanoma-bearing mice (17 and 20 days post the cell injection) to demonstrate its ability for non-invasive imaging melanoma metastases and monitoring the development of melanoma metastases in live mice. Dual-modality small animal SPECT/CT (Nano-SPECT/CT[®]) was used to detect the pulmonary melanoma metastases using ^{111}In -DOTA-GlyGlu-CycMSH as an imaging probe to target the MC1 receptors on the melanoma metastases. Nano-SPECT/CT[®] is a powerful tool combining the high spatial resolution of CT and high sensitivity of SPECT. Co-registration of the CT data with the SPECT data allowed accurate identification and localization of the melanoma metastases which improves the detection of metastatic deposits in the body cavity where small animal CT alone faces challenges without the addition of a contrast agent. Pulmonary metastatic melanoma lesions in live mice were clearly imaged by Nano-SPECT/CT[®] with ^{111}In -DOTA-GlyGlu-CycMSH 2 h post-injection (Figs. 5A1 and 5A2). The SPECT/CT images were coincident with the biodistribution results in B16/F10 pulmonary metastatic melanoma model (Table 1). ^{111}In -DOTA-GlyGlu-CycMSH clearly identified the individual metastatic deposits developed in the lung 17 days post the cell injection (Figs. 5A1 and 5A2). The comparison of ^{111}In -DOTA-GlyGlu-CycMSH images and [^{18}F]FDG images (Figs. 5A4 and 5A5) in the same melanoma-bearing mouse demonstrated that ^{111}In -DOTA-GlyGlu-CycMSH was a superior imaging probe for pulmonary melanoma metastases detection. [^{18}F]FDG failed in identifying the pulmonary melanoma metastases that were clearly imaged with ^{111}In -DOTA-GlyGlu-CycMSH. Compared with the SPECT/CT images of pulmonary metastatic melanoma-bearing mouse 17 days post the cell injection (Figs. 5A1 and 5A2), more and bigger metastatic melanoma lesions were observed in the SPECT/CT images of pulmonary metastatic melanoma-bearing mouse 20 days post the cell injection (Figs. 5C1 and 5C2). Both individual metastatic foci and bigger lesions (20 days post the cell injection) developed in the lung were clearly visualized with ^{111}In -DOTA-GlyGlu-CycMSH (Figs. 5C1 and 5C2), demonstrating the feasibility of using ^{111}In -DOTA-GlyGlu-CycMSH as an imaging probe to monitor tumor response to therapy. Although more studies need to be conducted, the imaging of metastatic melanoma foci in the earlier stage of development (< 17 days) seems possible since the spatial resolution of Nano-SPECT with multiple-pinhole collimator is approximately 0.8 mm for Jaszczak phantom filled with ^{111}In aqueous solution.

The successful detection of both flank primary melanoma (18) and pulmonary melanoma metastases using ^{111}In -DOTA-GlyGlu-CycMSH as an imaging probe highlighted the potential application of radiolabeled DOTA-GlyGlu-CycMSH for peptide-targeted radionuclide therapy of metastatic melanoma if the non-specific renal uptake could be further reduced through the administration of positively-charged amino acids. The introduction of a negatively-charged amino acid linker (-Gly-Glu-) successfully reduced the renal uptake of ^{111}In -DOTA-GlyGlu-CycMSH by 44% without affecting the tumor uptake (18), demonstrating that the electrostatic interaction played an important role in the renal uptake of ^{111}In -DOTA-GlyGlu-CycMSH. Positively-charged amino acids such as lysine and arginine were successful in reducing the renal uptake of ^{188}Re -labeled metal-cyclized α -MSH peptides by up to 50% (11). It is highly likely that co-injection of lysine or arginine will further decrease the renal uptake of ^{111}In -DOTA-GlyGlu-CycMSH. Since DOTA can form stable complexes with a variety of radiometals including diagnostic and therapeutic radionuclides, DOTA-GlyGlu-CycMSH can be potentially labeled with a variety of therapeutic radionuclides such as alpha- and beta-emitters (14,34-37) to treat the metastatic melanoma at the different stage. For instance, high-energy β -emitters such as ^{90}Y appear appropriate for the treatment of larger tumors or large tumor burdens (35). Medium- and lower-energy β -emitters, such as ^{177}Lu (37), may be more suitable for treating smaller tumors or metastatic deposits. Alpha-emitters such as $^{212}\text{Pb}/\text{Bi}$ (14) are attractive for treating small tumor and metastases due to their short path-length and high linear energy transfer (LET). The efficient internalization and extended retention of ^{111}In -DOTA-GlyGlu-CycMSH (Fig. 4) in melanoma cells could potentially maximize the therapeutic effects of alpha- and beta-emitter-labeled DOTA-GlyGlu-CycMSH due to the shortened distance between the radiation generated from the radionuclide and the target cell nucleus. Furthermore, the toxicity of targeted radionuclide therapy would also be potentially decreased by the efficient internalization and extended retention since the cytotoxic radiation could be selectively and specifically delivered to the tumor cells in an efficient fashion. The combined utilization of diagnostic and therapeutic DOTA-GlyGlu-CycMSH could potentially improve the success of the peptide-targeted radionuclide therapy of melanoma. Imaging patients with ^{111}In -DOTA-GlyGlu-CycMSH prior to the therapy would allow clinicians to accurately determine accurate patient-specific dosimetry, which would improve the safe and efficacious application of peptide-targeted radionuclide therapy of melanoma.

In conclusion, ^{111}In -DOTA-GlyGlu-CycMSH exhibited favorable metastatic melanoma targeting and imaging properties, highlighting its potential as an effective imaging probe for metastatic melanoma detection.

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Figure 1. MC1 receptor density in B16/F10 murine melanoma cells. The $B_{\max}$ value was 23394 dpm/million cells (2884 receptors/cell).

Figure 2.
The competitive binding curve of DOTA-GlyGlu-CycMSH in B16/F10 murine melanoma cells. The IC_{50} value of DOTA-GlyGlu-CycMSH was 2.43 nM.

Figure 3. Schematic structure and radioactive HPLC profile of ^{111}In -DOTA-GlyGlu-CycMSH. The retention time of ^{111}In -DOTA-GlyGlu-CycMSH was 18.1 min using a 20 min gradient of 16-26% acetonitrile in 20 mM HCl aqueous solution with a flow rate of 1 mL/min.

Figure 4. Cellular internalization and efflux of ^{111}In -DOTA-GlyGlu-CycMSH (A and B) in B16/F10 murine melanoma cells at 25°C. Total bound radioactivity (◆), internalized activity (■) and cell membrane activity (▲) were presented as counts per minute (cpm).

Figure 5.

Focused 3-dimensional (A1) and coronal (A2) SPECT/CT images of metastatic melanoma-bearing lung (17 days post the cell injection) 2 h post-injection of 24.05 MBq of ^{111}In -DOTA-GlyGlu-CycMSH. The mouse was anesthetized with 1.5% isoflurane, positioned prone, immobilized and imaged. Six-min CT scan and 45-min SPECT scan were performed; Necropsy picture (A3) of the metastatic melanoma-bearing lung (17 days post the cell injection); Focused 3-dimensional (A4) and sagittal (A5) PET images of metastatic melanoma-bearing lung (16 days post the cell injection) 1 h post-injection of 35.15 MBq of ^{18}F FDG. The mouse was anesthetized with 1.5% isoflurane, positioned prone, immobilized and imaged. A 30-min static PET scan was performed; Whole-body SPECT/CT and PET images (B1 and B2) using ^{111}In -DOTA-GlyGlu-CycMSH and ^{18}F FDG as imaging probes, respectively; Focused 3-dimensional (C1) and coronal (C2) SPECT/CT images of metastatic melanoma-bearing lung (20 days post the cell injection) 2 h post-injection of 10.36 MBq of ^{111}In -DOTA-GlyGlu-CycMSH. The mouse was anesthetized with 1.5% isoflurane, positioned prone, immobilized and imaged. Six-min CT scan and 45-min SPECT scan were performed; Necropsy picture (C3) and 3-dimensional (C4) SPECT/CT image of the metastatic melanoma-bearing lung (20 days post the cell injection).

Biodistribution of ^{111}In -DOTA-GlyGlu-CycMSH in B16/F10 pulmonary metastatic melanoma-bearing and normal C57 mice. The data was presented as percent injected dose/gram or as percent injected dose (Mean $\pm$ SD, n=5).

Table 1

Tissues	2 h			4 h			24 h		
	Lung Met.	Normal		Lung Met.	Normal		Lung Met.	Normal	
Lung	2.00 $\pm$ 0.74*	0.08 $\pm$ 0.08		1.83 $\pm$ 0.12*	0.05 $\pm$ 0.05		0.29 $\pm$ 0.06*	0.03 $\pm$ 0.02	
Blood	0.12 $\pm$ 0.05*	0.01 $\pm$ 0.01		0.03 $\pm$ 0.03	0.02 $\pm$ 0.02		0.03 $\pm$ 0.03	0.03 $\pm$ 0.02	
Bone	0.02 $\pm$ 0.01	0.03 $\pm$ 0.01		0.03 $\pm$ 0.03	0.06 $\pm$ 0.04		0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	
Brain	0.01 $\pm$ 0.01	0.02 $\pm$ 0.01		0.01 $\pm$ 0.01	0.01 $\pm$ 0.01		0.03 $\pm$ 0.03	0.01 $\pm$ 0.01	
Heart	0.20 $\pm$ 0.09*	0.04 $\pm$ 0.03		0.03 $\pm$ 0.01	0.03 $\pm$ 0.02		0.03 $\pm$ 0.02	0.05 $\pm$ 0.04	
Kidneys	16.24 $\pm$ 0.62	14.11 $\pm$ 2.23		14.46 $\pm$ 0.21	14.92 $\pm$ 1.50		15.13 $\pm$ 0.86	13.54 $\pm$ 1.48	
Liver	0.42 $\pm$ 0.07	0.41 $\pm$ 0.09		0.45 $\pm$ 0.05	0.38 $\pm$ 0.01		0.13 $\pm$ 0.02	0.12 $\pm$ 0.01	
Muscle	0.04 $\pm$ 0.03	0.03 $\pm$ 0.03		0.03 $\pm$ 0.02	0.03 $\pm$ 0.02		0.03 $\pm$ 0.02	0.02 $\pm$ 0.01	
Skin	0.26 $\pm$ 0.22	0.25 $\pm$ 0.17		0.36 $\pm$ 0.08	0.23 $\pm$ 0.08		0.06 $\pm$ 0.03	0.06 $\pm$ 0.01	
Spleen	0.26 $\pm$ 0.07*	0.06 $\pm$ 0.04		0.11 $\pm$ 0.05	0.06 $\pm$ 0.04		0.04 $\pm$ 0.03	0.04 $\pm$ 0.03	
Stomach	0.13 $\pm$ 0.05	0.14 $\pm$ 0.07		0.05 $\pm$ 0.04	0.08 $\pm$ 0.06		0.05 $\pm$ 0.03	0.06 $\pm$ 0.02	
Pancreas	0.05 $\pm$ 0.01	0.09 $\pm$ 0.07		0.10 $\pm$ 0.07	0.04 $\pm$ 0.03		0.04 $\pm$ 0.02	0.06 $\pm$ 0.01	
Percent injected dose (%ID)									
Intestine	7.70 $\pm$ 2.05	7.08 $\pm$ 1.54		5.97 $\pm$ 0.48	4.65 $\pm$ 0.85		0.11 $\pm$ 0.03	0.11 $\pm$ 0.08	
Urine	84.07 $\pm$ 2.32	84.74 $\pm$ 1.97		85.67 $\pm$ 1.66	89.02 $\pm$ 0.92		95.61 $\pm$ 0.59	96.06 $\pm$ 0.53	
Uptake ratio of tumor/normal tissue									
Lung/Blood	16.67	8.0		61.0	2.5		9.67	1.0	
Lung/Kidney	0.12	0.01		0.13	0.01		0.02	0.002	
Lung/Liver	4.76	0.20		4.07	0.13		2.23	0.25	
Lung/Muscle	50	2.67		61.0	1.67		9.67	1.5	

* P<0.05, significance comparison between ^{111}In -DOTA-GlyGlu-Cyc(Arg 11)CCMSH in pulmonary metastatic melanoma-bearing and normal C57 mice.