Published in final edited form as:

Sci Transl Med. 2018 November 07; 10(466): . doi:10.1126/scitranslmed.aau4445.

^{68}Ga -DOTA-GGNle-CycMSH_{hex} targets the melanocortin-1 receptor for melanoma imaging

Jianquan Yang¹, Jingli Xu¹, Rene Gonzalez², Thomas Lindner³, Clemens Kratochwil^{3,*}, and Yubin Miao^{1,*}

¹Department of Radiology, University of Colorado Denver, Aurora, CO 80045, USA

²Department of Medical Oncology, University of Colorado Denver, Aurora, CO 80045, USA

³Department of Nuclear Medicine, University Hospital Heidelberg, 69120 Heidelberg, Germany.

Abstract

Melanocortin-1 receptor (MC1R) is a molecular target for melanoma imaging and therapy due to its overexpression on rodent and human melanoma cells. Here we evaluated the MC1R targeting and specificity of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} with murine and human melanoma cells, and murine and xenografted tumors. ^{68}Ga -DOTA-GGNle-CycMSH_{hex} was used first-in-human as an imaging probe to evaluate the possibility of radionuclide therapy in advanced stage melanoma patients. ^{68}Ga -DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} displayed MC1R-specific targeting properties in murine and human melanoma cells, as well as in murine melanoma and human melanoma-xenografted tumors. Both B16/F10 and M21 melanoma lesions could be easily imaged by positron emission tomography (PET) using ^{68}Ga -DOTA-GGNle-CycMSH_{hex}. The first-in-human images of melanoma brain metastases in patients demonstrated the clinical relevance of MC1R as a molecular target for melanoma imaging, highlighting the potential of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} as a MC1R-targeting melanoma imaging probe and underscoring the need to develop MC1R-targeting therapeutic agents for treating patients with metastatic melanomas.

One Sentence Summary:

^{68}Ga -DOTA-GGNle-CycMSH_{hex}, a radiolabeled melanocortin-1 receptor-targeting imaging probe, detects melanoma in first-in-human testing.

*Corresponding author: yubin.miao@ucdenver.edu (Y.M.), Clemens.Kratochwil@med.uni-heidelberg.de (C.K.).

Author contributions: JY and JX were responsible for execution of experiments, data collection and statistical analysis. JY also contributed to manuscript preparation. RG contributed to research project organization and execution, and review and critique of the manuscript. TL contributed to human doses production. CK was responsible for first-in-human studies on melanoma patients, image data interpretation, and review and critique of the manuscript. YM was responsible for research project conception, organization, and execution, contributed to the statistical analysis design and execution, wrote the first draft manuscript, and reviewed and edited the final draft of the manuscript.

Competing interests: Dr. Yubin Miao is inventor on awarded US patents (US-8,986,651-B2; US-9,493,537-B2 and US-10,047,135-B2) held by University of New Mexico that covers compounds with reduced ring size for use in diagnosing and treating melanoma, including metastatic melanoma and methods related to same.

Data and materials availability: All data associated with this study are present in the paper or supplementary materials. The request for peptides will be handled by corresponding authors through a licensing agreement (for commercial purpose) or material transfer agreement (for research and noncommercial purposes).

INTRODUCTION

Malignant melanoma is the most lethal form of skin cancer with an increasing rate of incidence. Although malignant melanoma makes up less than 5% of skin cancer cases, it accounts for 75% of all skin cancer deaths (1). Approximately 76,380 new cases and 10,130 fatalities occurred in the United States in 2016 (1). High mortality of melanoma is due to the extreme aggressiveness associated with metastatic melanoma. Unfortunately, the success of traditional treatments, such as dacarbazine chemotherapy, Interleukin-2, and Interferon- α immunotherapy, is limited for metastatic melanoma, with a traditional median overall survival of 6–9 months (2–3).

Over the past several years, new molecular approaches including targeting BRAF-V600E mutation, cytotoxic T-lymphocyte antigen 4 (CTLA-4), and programmed death-1 receptor (PD-1) have shown promising results in treating metastatic melanoma (4–8). For instance, the treatments of vemurafenib (BRAF inhibitor), ipilimumab (targeting CTLA-4) and nivolumab (PD-1 inhibitor) have improved median overall survivals by months in metastatic melanoma patients (4–8). However, long-term survival of metastatic melanoma patients remains at <10%. Therefore, it is highly desirable to develop new treatments for metastatic melanoma.

Melanocortin-1 receptor (MC1R) is a G protein-coupled receptor over-expressed on both rodent and human melanoma cells (9–16). Greater than 80% of melanotic and amelanotic human metastatic melanomas over-express MC1R (9), making it an attractive molecular target for developing receptor-targeting radiopharmaceuticals for melanoma imaging and therapy. Meanwhile, α -melanocyte-stimulating hormone (α -MSH) and its analogues can bind to MC1Rs with nanomolar binding affinities, suggesting the feasibility of using α -MSH peptides to deliver diagnostic and therapeutic radionuclides to melanoma cells via peptide-MC1R binding for melanoma imaging and therapy (17–27). Upon MC1R binding, α -MSH peptides can be internalized into melanoma cells (28, 29).

Over several years, we have developed radiolabeled lactam-cyclized α -MSH peptides for melanoma targeting, built upon the construct of DOTA-GGNle-CycMSH_{hex} (1,4,7,10-tetraazacyclononane-1,4,7,10-tetraacetic acid-Gly-Gly-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-CONH₂) (24–25, 27, 30–34). DOTA-GGNle-CycMSH_{hex} was readily radiolabeled with ¹¹¹In and ⁶⁷Ga for melanoma imaging using single photon emission tomography (SPECT) (24, 30) and ¹⁷⁷Lu for potential targeted radionuclide therapy of melanoma (32). High B16/F1 melanoma uptake ($25.53 \pm 2.22\%$ ID/g), fast urinary clearance ($84.91 \pm 2.81\%$ ID) and high tumor to normal organ uptake ratios at 2 h post-injection of ⁶⁷Ga-DOTA-GGNle-CycMSH_{hex} (30) demonstrated its potential as an imaging probe for melanoma imaging using SPECT. High B16/F1 melanoma uptake ($21.63 \pm 6.27\%$ ID/g) at 2 h post-injection and prolonged melanoma retention ($8.24 \pm 1.51\%$ ID/g) at 24 h post-injection of ¹⁷⁷Lu-DOTA-GGNle-CycMSH_{hex} (32) highlighted its potential for targeted radionuclide therapy of melanoma.

Building upon the promising results of ⁶⁷Ga-DOTA-GGNle-CycMSH_{hex} and ¹⁷⁷Lu-DOTA-GGNle-CycMSH_{hex}, we developed positron emission tomography (PET) imaging probes for

melanoma, taking advantage of PET's high sensitivity and fast acquisition features. The combination of our MC1R-targeting peptide (DOTA-GGNle-CycMSH_{hex}) with PET may potentially offer a highly sensitive molecular approach to select MC1R-positive melanoma patients for targeted radionuclide therapy. Gallium-68 ($T_{1/2} = 68$ mins, 89% β^+ , β^+ $E_{\max} = 1.899$ MeV) is an attractive diagnostic PET radionuclide which can be readily obtained through a commercially available in-house $^{68}\text{Ge}/^{68}\text{Ga}$ generator, enabling the production of ^{68}Ga -radiopharmaceuticals independent of an on-site cyclotron. The short half-life of 68 min generates minimal radiation doses to patients over time. Meanwhile, the parent radionuclide of ^{68}Ge has a half-life of 270.8 d, making the shelf-life of $^{68}\text{Ge}/^{68}\text{Ga}$ generator approximately 6–12 months based on elution schedules. As such, the $^{68}\text{Ge}/^{68}\text{Ga}$ generator is affordable and offers flexibility to the preparation of ^{68}Ga -radiopharmaceuticals.

Although PET imaging using MC1R-targeting peptide (^{68}Ga -DOTA-GGNle-CycMSH_{hex}) may facilitate the accurate imaging of malignant melanoma, optical fluorescence imaging is a powerful complementary tool to visualize tumor lesions which can potentially provide an opportunity to improve surgical outcome via imaging-guided surgery. Therefore, we developed an MC1R-targeting fluorescence imaging probe in parallel with the MC1R-targeting PET imaging probe (^{68}Ga -DOTA-GGNle-CycMSH_{hex}). By replacing the DOTA chelator with fluorescence Cyanine5.5 carboxylic acid (Cy5.5) and conjugating to GGNle-CycMSH_{hex}, we generated Cy5.5-GGNle-CycMSH_{hex}. The combination of MC1R-targeted PET and fluorescence imaging probes could potentially provide opportunities for accurate detection and imaging-guided surgical removal of melanoma lesions, as well as identification of MC1R-positive melanoma patients for targeted radionuclide therapy.

Here DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} were synthesized using fluorenylmethyloxycarbonyl (Fmoc) chemistry. Their receptor binding affinities were determined on B16/F10 murine (metastatic) and M21 (low MC1R density) human melanoma cells (16, 17, 34–35). The MC1R expression in B16/F10 and M21 melanoma cells and tumor lesions were stained by Cy5.5-GGNle-CycMSH_{hex}. The biodistribution and imaging properties of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} were examined on B16/F10 flank and pulmonary melanoma models and M21 human melanoma xenografts. ^{68}Ga -DOTA-GGNle-CycMSH_{hex} PET was used first-in-human on metastatic melanoma patients who were considered suitable for the evaluation of experimental approaches.

RESULTS

Peptide synthesis and in vitro competitive binding assay

DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} (Fig. 1) were synthesized and purified by reverse phase-high performance liquid chromatography (RP-HPLC). After purification, DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} displayed greater than 90% purity. The identities of DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} were confirmed by electrospray ionization mass spectrometry. The measured molecular weights matched with calculated molecular weights. The half maximal inhibitory concentration (IC_{50}) values of DOTA-GGNle-CycMSH_{hex} were 2.1 ± 0.3 nM and 1.1 ± 0.2 nM on B16/F10 and M21 melanoma cells, respectively. The IC_{50} values of Cy5.5-GGNle-

CycMSH_{hex} were 7.1 ± 0.1 nM and 2.6 ± 0.3 nM on B16/F10 and M21 melanoma cells, respectively (Fig. 1).

Radiochemistry, specific binding and fluorescence staining

⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} was readily prepared with greater than 95% radiolabeling yield, and was completely separated from excess non-labeled peptide by RP-HPLC. The retention time of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} and DOTA-GGNle-CycMSH_{hex} was 16.9 and 15.7 min, respectively. ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} was stable in mouse serum at 37 °C for 1 h. Urine analysis revealed that 80% of the ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} excreted renally also remained intact in urine 2 h post-injection. ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} displayed receptor-mediated binding on B16/F10 and M21 cells. Approximately 95% and 84% of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} uptake was blocked on B16/F10 and M21 melanoma cells, respectively ($P < 0.05$) (Fig. 2). The MC1R expression in B16/F10 and M21 cells and in melanoma lesions was examined with fluorescence staining using Cy5.5-GGNle-CycMSH_{hex}. As demonstrated in Fig. 3, Cy5.5-GGNle-CycMSH_{hex} showed receptor-mediated binding on B16/F10 and M21 cells, B16/F10 and M21 flank melanoma lesions, as well as on B16/F10 pulmonary metastatic melanoma lesions. The uptake of Cy5.5-GGNle-CycMSH_{hex} was substantially inhibited by peptide blockade of MC1R.

Biodistribution

Tumor targeting and biodistribution properties of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} were determined in B16/F10 flank melanoma-bearing C57 mice. The biodistribution results of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} are presented in Table 1. ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} displayed rapid melanoma uptake. The B16/F10 tumor uptake was 18.63 ± 4.97 and 24.27 ± 3.74 % ID/g at 0.5 and 1 h post-injection, respectively. ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} exhibited prolonged tumor retention, with 11.61 ± 2.87 % ID/g of tumor uptake at 2 h post-injection. The co-injection of non-radioactive NDP-MSH blocked 92% of the tumor uptake at 1 h post-injection, demonstrating that the tumor uptake was MC1R-mediated. Whole-body clearance of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} was rapid, with 85% of the injected dose being washed out of the body via the renal system by 1 h post-injection, and 92% of the injected dose cleared by 2 h post-injection. Kidneys have the highest uptake of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} of normal organs, and renal uptake was 9.22 ± 1.38 , 7.78 ± 0.63 , and 6.01 ± 0.91 ID/g at 0.5, 1 and 2 h post-injection, respectively. The co-injection of NDP-MSH did not significantly ($P > 0.05$) reduce the renal uptake, indicating that the renal uptake of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} was not receptor-mediated. The accumulation of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} in other normal organs was much lower than kidneys. High tumor/blood and tumor/normal organ uptake ratios were demonstrated as early as 0.5 h post-injection.

The tumor targeting and biodistribution properties of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} were also determined in M21 human melanoma-xenografted nude mice (Table 2). As compared to the biodistribution results in B16/F10 melanoma-bearing mice, ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} displayed a similar distribution pattern with fast tumor uptake, prolonged tumor retention, and quick urinary clearance in M21 melanoma-xenografted mice. The M21 tumor

uptake was 6.05 ± 0.83 , 6.07 ± 0.68 , 5.15 ± 0.78 % ID/g at 0.5, 1 and 2 h post-injection, respectively. The tumor uptake was MC1R-specific because the co-injection of non-radioactive NDP-MSH blocked 83% of the tumor uptake at 1 h post-injection. M21 tumor uptake was lower than B16/F10 tumor uptake, which is likely attributed to lower MC1R receptor density (34), as well as differences in tumor morphology. The M21 tumor is solid unlike the soft and vascularized B16/F10 tumor. Kidneys exhibit the highest uptake of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} of all normal organs, however the renal uptake of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} wasn't MC1R-specific since the co-injection of NDP-MSH did not significantly ($P > 0.05$) reduce renal uptake. Similarly, the accumulation of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} in other normal organs was much lower than in the kidneys. Thus, ^{68}Ga -DOTA-GGNle-CycMSH_{hex} reached high tumor/blood and tumor/normal organ uptake ratios as early as 0.5 h post-injection.

Melanoma imaging in tumor-bearing mice

Both flank B16/F10 tumors and M21 xenografted tumors could be visualized by PET using ^{68}Ga -DOTA-GGNle-CycMSH_{hex} as an imaging probe at 0.5, 1 and 2 h post-injection (Fig. 4). ^{68}Ga -DOTA-GGNle-CycMSH_{hex} displayed the best imaging contrast between tumor and other normal organs at 1 h post-injection for both B16/F10 and M21 flank tumor models, consistent with the biodistribution results. In the B16/F10 pulmonary metastatic melanoma model, ^{68}Ga -DOTA-GGNle-CycMSH_{hex} exhibited substantial uptake in the melanoma-bearing lung instead of sharp images of metastatic melanoma lesions (Fig. 5).

First-in-human melanoma imaging

Similar to preclinical studies, first-in-human imaging of two melanoma patients demonstrated moderate to strong uptake in metastatic melanoma lesions (Fig. 6). Three melanoma metastases in brain, one metastasis in lung, one metastasis in connective tissue, and bulky metastases in small intestine of patient 1 (A and C in Fig. 6) were easily visualized by PET using ^{68}Ga -DOTA-GGNle-CycMSH_{hex}. The routine staging per ^{18}F -FDG PET/CT (B and D in Fig. 6) was non-inferior, and therefore also confirmative for true positive findings regarding the detection of extra-cerebral lesions. However, due to the high physiological uptake of ^{18}F -FDG in the brain, an additional MRI (BM1 and BM2 in Fig. 6) was performed to confirm the brain metastases using conventional means. For patient 2, massive melanoma metastases on the left leg and groin lymph node were visualized by PET using either ^{68}Ga -DOTA-GGNle-CycMSH_{hex} or ^{18}F -FDG (E and F in Fig. 6). As compared to more homogenous uptake of ^{18}F -FDG in those metastases, the uptake of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} was more heterogeneous, suggesting different MC1R expressions among those metastases.

DISCUSSION

Malignant melanoma is the most lethal form of skin cancer due to the extreme aggressiveness associated with metastasis. We have been developing radiolabeled α -MSH peptides to target MC1Rs for melanoma imaging and therapy because MC1R is expressed on more than 80% of melanotic and amelanotic human melanoma metastases (9). Here we have demonstrated MC1R-targeting and specificity of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} and

Cy5.5-GGNle-CycMSH_{hex} using murine and human melanoma cells, as well as murine melanoma and human melanoma-xenografted tumors. Both B16/F10 and M21 melanoma lesions could be easily imaged by PET using ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex}. MC1R expression in B16/F10 and M21 melanoma lesions could be specifically stained by Cy5.5-GGNle-CycMSH_{hex}. The promising results on MC1R-specific imaging and staining on both flank melanoma and pulmonary metastatic melanoma lesions suggested the potential of using Cy5.5-GGNle-CycMSH_{hex} for imaging-guided surgery after identifying melanoma lesions using ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex}. The combined use of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} may potentially improve the surgical outcomes of melanoma patients via imaging-guided surgery.

We and others have been using the B16/F10 pulmonary metastatic melanoma model to evaluate the targeting and imaging properties of radiolabeled α -MSH peptides (16, 17, 34) due to the high metastatic nature of the B16/F10 cell line. B16/F10 melanoma cells were injected into mice via tail veins to generate pulmonary melanoma metastases. This inoculation route mimics the development of melanoma metastases through blood circulation, whereas sentinel lymph node is the first organ to develop metastasis in a melanoma patient. Both B16/F10 flank melanoma lesions and B16/F10 pulmonary metastatic melanoma lesions were positively stained by Cy5.5-GGNle-CycMSH_{hex}, indicating that the metastasis process did not result in a substantial change in the MC1R expression. PET images showed substantial uptake of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} in B16/F10 pulmonary metastatic melanoma lesions. In our previous work, we demonstrated the success of SPECT imaging of B16/F10 pulmonary melanoma metastases using ^{99m}Tc- and ⁶⁷Ga-labeled lactam-cyclized α -MSH peptides as imaging probes (34, 36). The difference in imaging quality between SPECT and PET images in this B16/F10 pulmonary metastatic melanoma model was likely attributed to the lower resolution of animal PET as compared to animal SPECT.

After diagnosis of melanoma, it is important to determine whether the melanoma has metastasized to lymph nodes and other distant organs such as liver, lung and brain. Approximately 60% of patients with metastatic melanoma develop brain metastases during their course of disease (37). Patients with brain metastases have much shorter life expectancies than patients without brain metastases. Meanwhile, it is challenging to effectively treat brain metastases without side effects. Here we could identify the melanoma metastases in brain, as well as in lung, connective tissue and small intestine of patients by PET using ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex}. The sharp images of these metastases in patients demonstrated the clinical relevance of MC1R as a valid and attractive molecular target for melanoma imaging, highlighting the potential of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} as a MC1R-targeting probe for melanoma imaging. This underscores the need to develop MC1R-targeting therapeutic agents for treating patients with metastatic melanomas.

The PET images of melanoma metastases suggest that MC1R is not only a valid molecular target for melanoma during early development but is also present in advanced stages of melanoma and later generations of daughter metastases. Moreover, the heterogeneous intensity of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} uptake among melanoma metastases in patient 2 indicates that the MC1R density was different in these metastatic melanoma lesions. From

the therapeutic point of view, it is important to note that receptor-targeted radionuclide therapy is much more effective when the receptors are overexpressed in all or at least nearly all tumor lesions. Therefore, it is crucial to identify such patients for potential successful receptor-targeted radionuclide therapy. The PET images of melanoma patients demonstrate the potential of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} PET to select patients with sufficient MC1R expression in all metastases before tailoring therapies for these patients toward or against MC1R-targeted radionuclide therapy. We have previously demonstrated the promising melanoma targeting property of ^{177}Lu -DOTA-GGNle-CycMSH_{hex} (32). ^{177}Lu is an attractive theranostic radionuclide. When ^{177}Lu decays, it generates medium-energy (0.497 MeV) β -particles for treatment, as well as γ -rays (113 and 208 keV) for SPECT imaging. The short tissue penetration of β -particles makes ^{177}Lu a suitable radionuclide for treating small tumors and metastases due to its fairly localized radiation emission. Although we have yet to examine the therapeutic efficacy of ^{177}Lu -DOTA-GGNle-CycMSH_{hex}, the combination of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} and ^{177}Lu -DOTA-GGNle-CycMSH_{hex} may potentially offer an attractive approach to receptor-targeted imaging and radionuclide therapy for melanoma.

MC1R is a melanoma-specific molecular target without relevant physiological expression on normal tissues. Despite DOTA-GGNle-CycMSH_{hex} exhibiting low nanomolar affinity to MC1R, the relatively low number of MC1R copies per cell could be a potential limiting factor for MC1R-targeted radionuclide therapy. The human imaging doses of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} were prepared with 7.5 μg of DOTA-GGNle-CycMSH_{hex} in this study. However, it is possible that higher amount of GGNle-CycMSH_{hex} would be needed to deliver more ^{177}Lu activity to melanoma cells for MC1R-targeted radionuclide therapy. Therefore, there is a potential scenario in which increased GGNle-CycMSH_{hex} mass could partially saturate MC1Rs on melanoma cells. More research is needed to determine the threshold amount of GGNle-CycMSH_{hex} without MC1R blocking effect, as well as to achieve high specific activity during the radiolabeling or through additional purification process. Non-specific renal uptake of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} could be another potential limiting factor for MC1R-targeted radionuclide therapy. Non-specific renal uptake of radiolabeled peptide is due to the electrostatic interaction between positively-charged peptide molecules and negatively-charged surface of tubule cells as the peptides are filtered in the glomerulus and reabsorbed in the cells of the proximal tubule (38). Positively-charged lysine has been effective in reducing the renal uptake of radiolabeled peptide by shielding such electrostatic interaction (39–41), suggesting co-injection of lysine could be used to further reduce renal uptake if needed.

In conclusion, we demonstrated the MC1R-targeting and specificity of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} with murine and human melanoma cells, as well as in murine melanoma and human melanoma-xenografted tumors. Both B16/F10 and M21 melanoma lesions could be imaged by PET using ^{68}Ga -DOTA-GGNle-CycMSH_{hex} as an imaging probe. The combination of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} may potentially improve surgical outcome via imaging-guided surgery. The first-in-human images of melanoma metastases in patients demonstrated the clinical relevance of MC1R as a valid and attractive molecular target for melanoma imaging, as well as highlighted the potential of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} as a MC1R-targeting

melanoma imaging probe. Furthermore, the successful imaging of melanoma metastases in patients underscored the need to develop MC1R-targeting therapeutic peptides for treating patients with metastatic melanomas.

MATERIALS AND METHODS

Study design

The goal of this work was to assess whether MC1R-targeting ^{68}Ga -DOTA-GGNle-CycMSH_{hex} could be used to evaluate the possibility of radionuclide therapy on advanced stage melanoma patients. First, the MC1R-targeting and specificity of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} were examined with murine and human melanoma cells, as well as in murine melanoma and human melanoma-xenografted tumors. Then ^{68}Ga -DOTA-GGNle-CycMSH_{hex} was used first-in-human as an imaging probe to evaluate the possibility of radionuclide therapy on metastatic melanoma patients who were considered suitable for the evaluation of experimental approaches (in agreement with the Declaration of Helsinki, 37: Unproven interventions in clinical practice) because they had already exhausted all other available treatment options.

Chemicals and reagents

Amino acids and resin were purchased from Advanced ChemTech Inc. and Novabiochem. DOTA-tri-*t*-butyl ester and Cyanine5.5 carboxylic acid (Cy5.5) were purchased from Macrocyclics Inc. and Lumiprobe Corporation for peptide synthesis, respectively. The ^{68}Ge - ^{68}Ga generator was purchased from RadioMedix Inc. All other chemicals used in this study were purchased from Thermo Fischer Scientific and used without further purification. Four percent Paraformaldehyde (PFA) in PBS was obtained from Alfa Aesar, xylene was obtained from Fisher Chemical, 4',6-diamidino-2-phenylindole (DAPI) Fluoromount-G mounting medium was obtained from SouthernBiotech. Prolong Diamond anti-fade mounting reagent with DAPI was obtained from Life Technologies. B16/F10 murine melanoma cells were obtained from American Type Culture Collection. M21 human melanoma cells were supplied by Dr. David A. Cheresch from the Department of Pathology, Moores University of California-San Diego Cancer Center.

Peptide synthesis

DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} were synthesized using standard fluorenylmethyloxycarbonyl (Fmoc) chemistry according to our published procedure (23) with modifications. Briefly, linear peptide backbones of (tBu)₃DOTA-Gly-Gly-Nle-Asp(O-2-PhiPr)-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-Lys(Dde) and Cy5.5-Gly-Gly-Nle-Asp(O-2-PhiPr)-His(Trt)-DPhe-Arg(Pbf)-Trp(Boc)-Lys(Dde) were synthesized on Sieber Amide resin by an Advanced ChemTech multiple-peptide synthesizer. Seventy micromoles of resin, 210 μmol of each Fmoc-protected amino acid, 210 μmol of (tBu)₃DOTA and Cy5.5 were used for the synthesis. The protecting group of Dde was removed by 2% hydrazine for peptide cyclization. The protecting group of 2-phenylisopropyl was removed and the protected peptide was cleaved from the resin treating with a mixture of 2.5% of trifluoroacetic acid (TFA) and 5% of triisopropylsilane. After the precipitation with ice-cold ether, each protected peptide was characterized by liquid chromatography-mass

spectroscopy (LC-MS). Then, each protected peptide was further cyclized by coupling the carboxylic group from the Asp with the ϵ -amino group from the Lys. The cyclization reaction was achieved by an overnight reaction in dimethylformamide (DMF) using benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium-hexafluorophosphate (PyBOP) as a coupling agent in the presence of *N,N*-diisopropylethylamine (DIPEA). After characterization by LC-MS, each cyclized protected peptide was dissolved in H₂O/CH₃CN (50:50) and lyophilized. The protecting groups were totally removed by treatment with a mixture of trifluoroacetic acid (TFA), thioanisole, phenol, water, ethanedithiol and triisopropylsilane (87.5:2.5:2.5:2.5:2.5:2.5) for 2 h at room temperature (25 °C). Each peptide was precipitated and washed with ice-cold ether four times, purified by RP-HPLC and characterized by LC-MS.

In vitro competitive binding assay of DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex}

The MC1R binding affinities of DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} were determined on B16/F10 and M21 melanoma cells by in vitro competitive receptor binding assay. The receptor binding assay was replicated in triplicate. B16/F10 and M21 cells (2×10^5 cells/well, $n = 3$) were seeded separately in 24-well cell culture plates and incubated at 37 °C overnight. After being washed with binding medium [Modified Eagle's medium with 25 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid), pH 7.4, 0.2% bovine serum albumin (BSA), 0.3 mM 1,10-phenanthroline], the cells were incubated at 37 °C for 2 h with approximately 45,000 counts per minute (cpm) of ¹²⁵I-(Tyr²)-NDP-MSH in the presence of 10^{-12} to 10^{-5} M of the peptide in 0.3 mL of binding medium. The binding medium was aspirated after the incubation. The cells were rinsed twice with 0.5 mL of ice-cold pH 7.4, 0.2% BSA/0.01 M phosphate buffered saline (PBS) and lysed in 0.5 mL of 1 N NaOH for 5 min. The cells were harvested and measured in a PerkinElmer Wallac 1480 automated γ -counter. The IC₅₀ value was calculated using Prism from GraphPad Software.

Preparation, serum stability and urinary metabolites of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex}

The ⁶⁸Ge-⁶⁸Ga generator was eluted with 4 mL of 0.05 M HCl via fractional elutions (1 mL/fraction) to obtain ⁶⁸GaCl₃. ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} was prepared in a 2 M NH₄OAc-buffered solution (pH 5). Briefly, 300 μ L of ⁶⁸GaCl₃ (37–74 MBq in 0.05 M HCl aqueous solution), 20 μ L of 1 mg/mL peptide aqueous solution and 40 μ L of 2 M NH₄OAc (pH 5) were added into a reaction vial and incubated at 95°C for 5 mins. The pH of the reaction mixture was 4.5. After the incubation, 10 μ L of 0.5% EDTA (ethylenediaminetetraacetic acid) aqueous solution was added into the reaction vial to scavenge potentially unbound ⁶⁸Ga³⁺ ions. The radiolabeled complexes were purified to single species by a Waters RP-HPLC on a Grace Vydac C-18 reverse phase analytical column using the following gradient at a 1 mL/min flowrate. The mobile phase consisted of solvent A (20 mM HCl aqueous solution) and solvent B (100% CH₃CN). The gradient was initiated and kept at 82:18 A/B for 3 min followed by a linear gradient of 82:18 A/B to 72:28 A/B over 20 min. Then, the gradient was changed from 72:28 A/B to 10:90 A/B over 3 min followed by an additional 5 min at 10:90 A/B. Thereafter, the gradient was changed from 10:90 A/B to 82:18 A/B over 3 mins. The purified peptide sample was purged with N₂ gas for 15 min to remove the acetonitrile. The pH of the final solution was adjusted to 7.4 with

0.1 N NaOH and sterile saline for animal studies. In vitro serum stability of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} was determined by incubation in mouse serum at 37°C for 1 h and monitored for degradation by RP-HPLC. To determine urine metabolites, HPLC-purified ^{68}Ga -DOTA-GGNle-CycMSH_{hex} (0.74 MBq, 100 μL) was injected into a normal C57 mouse through the tail vein. At 2 h post-injection, the mouse was sacrificed and the urine was collected. The radioactive metabolites in the urine were analyzed by HPLC. A 20-min gradient of 18–28% CH_3CN / 20 mM HCl was used for the urine analysis.

Specific binding of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} on B16/F10 and M21 melanoma cells

The specific binding of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} was determined using B16/F10 and M21 melanoma cells. The B16/F10 and M21 cells (1×10^6 cells/tube, $n = 3$) were incubated at 37 °C for 2 h with approximately 0.037 MBq of ^{68}Ga -DOTA-GGNle-CycMSH_{hex} with or without 10 μg (6.07 nmol) of unlabeled NDP-MSH in 0.3 mL of binding medium [Modified Eagle's medium with 25 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid), pH 7.4, 0.2% bovine serum albumin (BSA), 0.3 mM 1,10-phenanthroline]. The binding medium was aspirated after the incubation. The cells were rinsed three times with 0.5 mL of ice-cold pH 7.4, 0.2% BSA/0.01 M phosphate buffered saline (PBS) and measured in a PerkinElmer Wallac 1480 automated γ -counter

B16/F10 murine melanoma and M21 human melanoma-xenografted melanoma models

All animal studies were conducted in compliance with Institutional Animal Care and Use Committee approval. B16/F10 murine melanoma and M21 human melanoma-xenografted melanoma models were generated for fluorescence staining, biodistribution and imaging studies. For the B16/F10 flank melanoma model, each C57 mouse (Charles River Labs, 4–6 weeks, female) was subcutaneously inoculated with 1×10^6 B16/F10 cells on the right flank. Ten days post inoculation, tumor weights reached approximately 0.2 g. The flank tumor-bearing mice were used for fluorescence staining, biodistribution studies, and imaging studies. For the B16/F10 pulmonary metastatic melanoma model, each C57 mouse was intravenously injected with 2×10^5 B16/F10 cells via the tail vein. The mice were used for fluorescence staining 16 d post-injection, and imaging studies 21 d post-injection. For M21 human melanoma xenografts, each nude mouse (Charles River Labs, 4–6 weeks, female) was subcutaneously inoculated with 5×10^6 M21 cells on the right flank. Fourteen days post inoculation, the tumor weights reached approximately 0.2 g and these mice were used for fluorescence staining, biodistribution studies, and imaging studies.

Fluorescence staining of melanoma cells and tumor lesions using Cy5.5-GGNle-CyMSH_{hex}

B16/F10 and M21 melanoma cells (1×10^5 cells per well) were seeded in a 4-well Lab-Tek Chamber Glass Slide System from Thermo Scientific and incubated at 37° overnight. After 24 h, the cells were fixed with 4% PFA in PBS and incubated at room temperature for 15 min, washed with PBS 3 times, treated with 0.5% Triton X-100 at room temperature for 15 mins and washed with PBS for 3 times. The cells were incubated with 1 μM of Cy5.5-GGNle-CyMSH_{hex} with/without 1 μM of NDP-MSH peptide blockade at room temperature for 1 h and washed with PBS 3 times. Then the cells were stained for nuclei and mounted with DAPI Fluoromount-G mounting medium from SouthernBiotech and stayed in

dark at room temperature for 24 h. The fluorescent signal was observed and recorded at 100 × magnification under an Olympus FV1000 confocal microscope.

Paraffin embedded B16/F10 and M21 melanoma sections (5 μm thickness) were incubated with 1 μM of Cy5.5-GGNle-CycMSH_{hex} with/without 1 μM of NDP-MSH peptide blockade at room temperature for 1 h after deparaffinization with xylene. Tissue samples were then washed with PBS three times and stained and mounted with Prolong Diamond anti-fade mounting reagent with DAPI from Life Technologies. The fluorescent signal was observed and recorded at 100 × magnification under an Olympus FV1000 confocal microscope.

Biodistribution of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex}

All animal studies were conducted in compliance with Institutional Animal Care and Use Committee approval. The biodistribution properties of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} were determined on B16/F10 murine melanoma-bearing C57 mice and M21 human melanoma-xenografted nude mice. Each melanoma-bearing mouse was injected with 0.37 MBq of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} via the tail vein. Groups of 4 mice were sacrificed at 0.5, 1 and 2 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. The specificity of the tumor uptake of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} was determined by co-injecting 10 μg (6.07 nmol) of unlabeled NDP-MSH which is a linear α-MSH peptide analogue with sub-nanomolar MC1R binding affinity.

PET imaging of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} in melanoma-bearing mice

The melanoma imaging property of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} was determined on B16/F10 flank melanoma- and B16/F10 pulmonary metastatic melanoma-bearing C57 mice, and on M21 human melanoma-xenografted nude mice. The melanoma-bearing mice were generated as described above and used for imaging studies. The B16/F10 pulmonary melanoma metastases were generated by injecting B16/F10 cells into the tail vein of each C57 mouse, mimicking the development of metastases via blood circulation. Each melanoma-bearing mouse was injected with 7.4 MBq of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} via the tail vein. PET imaging studies of live flank melanoma-bearing mice were performed at 0.5, 1 and 2 h post-injection, whereas the imaging study of live pulmonary metastatic melanoma-bearing mice was conducted at 0.5 and 1 h post-injection. Reconstructed PET data were visualized using Vivoquant from Invivo.

First-in-human PET imaging of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} in metastatic melanoma patients

To assess the eventual possibility of MC1R-targeting radionuclide therapy, ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} PET/CT was offered to two metastatic melanoma patients. These patients had already failed other available melanoma treatments including chemotherapy (dacarbazine and paclitaxel/carboplatin), immunotherapy (ipilimumab) and BRAF inhibitor (vemurafenib). PD-1 therapy wasn't available yet at the time of the study. After exhausting all other available treatment options, it was well in line with the updated Declaration of Helsinki (37: Unproven interventions in clinical practice) to consider non-standard

approaches for these individual patients. The patients were informed about the experimental character of the procedure which was offered according to the German Pharmaceutical Act §13(2b), and given written informed consent. The ethics committee of the University of Heidelberg approved the retrospective evaluation of the obtained data as an observational study.

^{68}Ga -DOTA-GGNle-CycMSH_{hex} was prepared at pH 3.5 in a 2.5 M NaOAc-buffered solution (pH 9). Briefly, 1 mL of $^{68}\text{GaCl}_3$ (600–1000 MBq in 0.6 M HCl aqueous solution), 5 µL of 1 mM peptide aqueous solution, 10 µL of 20% ascorbic acid aqueous solution and 300 µL of 2.5 M NaOAc were added into a reaction vial and incubated at 95°C for 15 mins. The pH of the reaction mixture was 3.5. After the incubation, the mixture was purified by solid phase extraction (Varian Bond Elut Plexa from Agilent Technologies) before injection. Approximately 300–600 MBq of the purified ^{68}Ga -DOTA-GGNle-CycMSH_{hex} were obtained in 6 mL of 10% ethanol and saline.

The patients underwent imaging on a Siemens Biograph 6 scanner 1 h after intravenous injection of approximately 300 MBq of ^{68}Ga -DOTA-GGNle-CycMSH_{hex}. First, a non-contrast-enhanced low-dose CT-scan (130 keV, 30 mAs, reconstruction with soft tissue kernel to a slice thickness of 5 mm) was performed, and later used for attenuation correction of the related PET scan. Then, 3D emission scans from head to mid-thigh were obtained by 8 bed-positions (15.5 cm field of view; 3 min acquisition time, respectively). Images were reconstructed with an ordered subset expectation maximization (OSEM) algorithm correcting for randoms, scatter and decay using 2 iterations/ 16 subsets and Gauss-filtered to a transaxial resolution of 5 mm at full-width at half maximum (FWHM).

Statistical analysis

Statistical analysis was performed using the Student's t-test for unpaired data. A 95% confidence level was chosen to determine the significant difference between groups in cellular binding of ^{68}Ga -DOTA-GGNle-CycMSH_{hex}, and difference in tumor and renal uptakes between ^{68}Ga -DOTA-GGNle-CycMSH_{hex} with/without NDP-MSH co-injection. The differences at the 95% confidence level ($P < 0.05$) were considered significant.

ACKNOWLEDGMENTS

We thank Drs. Fabio Gallazzi, Lina Cheuy, Natalie Serkova and Ms. Kendra Huber for their technical assistance, and Dr. Eric R. Prossnitz for helpful discussions.

Funding: This work was supported in part by the NIH grant R01CA225837 and University of Colorado Denver start-up fund. Microscopy imaging experiments were performed in the University of Colorado Anschutz Medical Campus Advance Light Microscopy Core supported in part by NIH/NCATS Colorado CTSI Grant Number UL1 TR001082. PET imaging experiments were conducted in the University of Colorado Anschutz Medical Campus Animal Imaging Shared Resources supported in part by the University of Colorado Cancer Center (NCI P30 CA046934) and the Colorado Clinical and Translational Sciences Institute (NIH/NCATS UL1 TR001082).

REFERENCES AND NOTES

1. Siegel RL, Miller KD, Jemal A, Cancer Statistics, 2016. CA Cancer J Clin 66, 7–30 (2016). [PubMed: 26742998]
2. Tsao H, Atkins MB, Sober AJ, Management of cutaneous melanoma. N Engl J Med 54, 8–29 (2004).

3. Atallah E, Flaherty L, Treatment of metastatic malignant melanoma. *Curr Treat Options Oncol* 6, 185–193 (2005). [PubMed: 15869730]
4. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA; BRIM-3 Study Group. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 364, 2507–2516 (2011). [PubMed: 21639808]
5. Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, McArthur GA, Hutson TE, Moschos SJ, Flaherty KT, Hersey P, Kefford R, Lawrence D, Puzanov I, Lewis KD, Amaravadi RK, Chmielowski B, Lawrence HJ, Shyr Y, Ye F, Li J, Nolop KB, Lee RJ, Joe AK, Ribas A, Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med* 366, 707–714 (2012). [PubMed: 22356324]
6. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen, Gonzalez JBR, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ, Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363, 711–723 (2010). [PubMed: 20525992]
7. Weber JS, O'Day S, Urba W, Powderly J, Nichol G, Yellin M, Snively J, Hersh E, Phase I/II study of ipilimumab for patients with metastatic melanoma. *J Clin Oncol* 26, 5950–5956 (2008). [PubMed: 19018089]
8. Topalian SL, Sznol M, McDermott DF, Kluger HM, Carvajal RD, Sharfman WH, Brahmer JR, Lawrence DP, Atkins MB, Powderly JD, Leming PD, Lipson EJ, Puzanov I, Smith DC, Taube JM, Wigginton JM, Kollia GD, Gupta A, Pardoll DM, Sosman JA, Hodi FS, Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. *J Clin Oncol* 32, 1020–1030 (2014). [PubMed: 24590637]
9. Tatro JB, Wen Z, Entwistle ML, Atkins MB, Smith TJ, Reichlin S, Murphy JR, Interaction on an α -melanocyte stimulating hormone-diphtheria toxin fusion protein with melanotropin receptors in human metastases. *Cancer Res* 52, 2545–2548 (1992). [PubMed: 1314697]
10. Siegrist W, Solca F, Stutz S, Giuffrè L, Carrel S, Girard J, Eberle AN, Characterization of receptors for alpha-melanocyte-stimulating hormone on human melanoma cells. *Cancer Res* 49, 6352–6358 (1989). [PubMed: 2804981]
11. Tatro JB and Reichlin S, Specific receptors for alpha-melanocyte-stimulating hormone are widely distributed in tissues of rodents. *Endocrinology* 121, 1900–1907 (1987). [PubMed: 2822378]
12. Slominski A, Moellmann G, Kuklinska E. MSH inhibits growth in a line of amelanotic hamster melanoma cells and induces increases in cyclic AMP levels and tyrosinase activity without inducing melanogenesis. *J Cell Sci* 92, 551–559 (1989). [PubMed: 2557357]
13. Sniegocka M, Podgórska E, Płonka PM, Elas M, Romanowska-Dixon B, Szczygieł M, Zmijewski MA, Cichorek M, Markiewicz A, Brożyna AA, Slominski AT, Urbanska K. Transplantable melanomas in hamsters and gerbils as models for human melanoma. Sensitization in melanoma radiotherapy-from animal models to clinical trials. *Int J Mol Sci* 19, 1048 (2018).
14. Chen J, Cheng Z, Hoffman TJ, Jurisson SS, Quinn TP, Melanoma-targeting properties of ^{99m}Tc -labeled cyclic α -melanocyte-stimulating hormone peptide analogues. *Cancer Res* 60, 5649–5658 (2000). [PubMed: 11059756]
15. Miao Y, Whitener D, Feng W, Owen NK, Chen J, Quinn TP, Evaluation of the human melanoma targeting properties of radiolabeled alpha-melanocyte stimulating hormone peptide analogues. *Bioconj Chem* 14, 1177–1184 (2003). [PubMed: 14624632]
16. Guo H, Shenoy N, Gershman BM, Yang J, Sklar LA, Miao Y, Metastatic melanoma imaging with an ^{111}In -labeled lactam bridge-cyclized alpha-melanocyte stimulating hormone peptide. *Nucl Med Biol* 36, 267–276 (2009). [PubMed: 19324272]
17. Miao Y, Benwell K, Quinn TP, ^{99m}Tc and ^{111}In labeled alpha-melanocyte stimulating hormone peptides as imaging probes for primary and pulmonary metastatic melanoma detection. *J Nucl Med* 48, 73–80 (2007). [PubMed: 17204701]
18. Miao Y, Shelton T, Quinn TP, Therapeutic efficacy of a ^{177}Lu labeled DOTA conjugated α -melanocyte stimulating hormone peptide in a murine melanoma-bearing mouse model. *Cancer Biother Radiopharm* 22, 333–341 (2007). [PubMed: 17651039]

19. Raposinho PD, Correia JD, Alves S, Botelho MF, Santos AC, Santos I, A ^{99m}Tc(CO)₃-labeled pyrazolyl- α -melanocyte-stimulating hormone analog conjugate for melanoma targeting. *Nucl Med Biol* 35, 91–99 (2008). [PubMed: 18158948]
20. Morais M, Oliveira BL, Correia JD, Oliveira MC, Jiménez MA, Santos I, PD. Raposinho, Influence of the bifunctional chelator on the pharmacokinetic properties of ^{99m}Tc(CO)₃-labeled cyclic α -melanocyte stimulating hormone analog. *J Med Chem* 56, 1961–1973 (2013). [PubMed: 23414214]
21. Jiang H, Kasten BB, Liu H, Qi S, Liu Y, Tian M, Barnes CL, Zhang H, Cheng Z, Benny PD, Novel, cysteine-modified chelation strategy for the incorporation of [MI(CO)₃]⁺ (M = Re/^{99m}Tc) in an α -MSH peptide. *Bioconjug Chem* 23, 2300–2312 (2012). [PubMed: 23110503]
22. Kasten BB, Ma X, Liu H, Hayes TR, Barnes CL, Qi S, Cheng K, Bottorff SC, Slocumb WS, Wang J, Cheng Z, Benny PD, Clickable, hydrophilic ligand for fac-[MI(CO)₃]⁺ (M = Re/^{99m}Tc) applied in an S-functionalized α -MSH peptide. *Bioconjug Chem* 25, 579–592 (2014). [PubMed: 24568284]
23. Guo H, Yang J, Gallazzi F, Miao Y, Reduction of the ring size of radiolabeled lactam bridge-cyclized alpha-MSH peptide resulting in enhanced melanoma uptake. *J Nucl Med* 51, 418–426 (2010). [PubMed: 20150256]
24. Guo H, Yang J, Gallazzi F, Miao Y, Effects of the amino acid linkers on melanoma-targeting and pharmacokinetic properties of indium-111-labeled lactam bridge-cyclized α -MSH peptides. *J Nucl Med* 52, 608–616 (2011). [PubMed: 21421725]
25. Guo H, Gallazzi F, Miao Y, Design and evaluation of new Tc-99m-labeled lactam bridge-cyclized alpha-MSH peptides for melanoma imaging. *Mol Pharm* 10, 1400–1408 (2013). [PubMed: 23418722]
26. Froidevaux S, Calame-Christe M, Schuhmacher J, Tanner H, Saffrich R, Henze M, Eberle AN. A gallium-labeled DOTA- α -melanocyte-stimulating hormone analog for PET imaging of melanoma metastases. *J Nucl Med* 45, 116–123 (2004). [PubMed: 14734683]
27. Guo H, Miao Y, Introduction of an aminooctanoic acid linker enhances uptake of Tc-99m-labeled lactam bridge-cyclized alpha-MSH peptide in melanoma. *J Nucl Med* 55, 2057–2063 (2014). [PubMed: 25453052]
28. Slominski A, Wortsman J, Luger T, Paus R, Solomon S. Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. *Physiol Rev* 80, 979–1020 (2000). [PubMed: 10893429]
29. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol Rev* 84, 1155–1228 (2004). [PubMed: 15383650]
30. Guo H, Gallazzi F, Miao Y, Ga-67-labeled lactam bridge-cyclized alpha-MSH peptides with enhanced melanoma uptake and reduced renal uptake. *Bioconjug Chem* 23, 1341–1348 (2012). [PubMed: 22621181]
31. Guo H, Miao Y, Cu-64-labeled lactam bridge-cyclized alpha-MSH peptides for PET imaging of melanoma. *Mol Pharm* 9, 2322–2330 (2012). [PubMed: 22780870]
32. Guo H, Miao Y, Melanoma targeting property of a Lu-177-labeled lactam bridge-cyclized alpha-MSH peptide. *Bioorg Med Chem Lett* 23, 2319–2323 (2013). [PubMed: 23473679]
33. Liu L, Xu J, Yang J, Feng C, Miao Y, Imaging human melanoma using a novel Tc-99m-labeled lactam bridge-cyclized alpha-MSH peptide. *Bioorg Med Chem Lett* 26, 4724–4728 (2016). [PubMed: 27568083]
34. Guo H, Yang J, Shenoy N, Miao Y, Gallium-67-labeled lactam bridge-cyclized alpha-melanocyte stimulating hormone peptide for primary and metastatic melanoma imaging. *Bioconjug Chem* 20, 2356–2363 (2009). [PubMed: 19919057]
35. Yang J, Guo H, Miao Y, Technetium-99m-labeled Arg-Gly-Asp-conjugated alpha-melanocyte stimulating hormone hybrid peptides for human melanoma imaging. *Nucl Med Biol* 37, 873–883 (2010). [PubMed: 21055617]
36. Liu L, Xu J, Yang J, Feng C, Miao Y, Metastatic melanoma imaging using a novel Tc-99m-labeled lactam-cyclized alpha-MSH peptide. *Bioorg Med Chem Lett* 27, 4952–4955 (2017). [PubMed: 29054361]

37. Amer MH, Al-Sarraf M, Baker LH, Vaitkevicius VK. Malignant melanoma and central nervous system metastases: incidence, diagnosis, treatment and survival. *Cancer* 42, 660–668 (1978). [PubMed: 679158]
38. Mørgenson CE, Sjølling K. Studies on renal tubular protein reabsorption: partial and near complete inhibition by certain amino acids. *Scan J Clin Lab Invest* 37, 477–486 (1977).
39. Miao Y, Fisher DR, Quinn TP. Reducing renal uptake of ⁹⁰Y and ¹⁷⁷Lu labeled alpha-melanocyte stimulating hormone peptide analogues. *Nucl Med Biol* 33, 723–733 (2006). [PubMed: 16934691]
40. Miao Y, Owen NK, Whitener D, Gallazzi F, Hoffman TJ, Quinn TP. In vivo evaluation of ¹⁸⁸Re-labeled alpha-melanocyte stimulating hormone peptide analogs for melanoma therapy. *Int J Cancer* 101, 480–487 (2002). [PubMed: 12216078]
41. Yang J, Hu CA, Miao Y. Tc-99m-labeled RGD-conjugated alpha-MSH hybrid peptides with reduced renal uptake. *Amino Acids* 47, 813–823 (2015). [PubMed: 25557051]

Fig. 1. Schematic structures and in vitro competitive binding curves.

(A) Schematic structures of DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex}. (B) In vitro competitive binding curves of DOTA-GGNle-CycMSH_{hex} and Cy5.5-GGNle-CycMSH_{hex} using B16/F10 and M21 melanoma cells. The IC₅₀ values of DOTA-GGNle-CycMSH_{hex} were 2.1 ± 0.3 nM (*, black) and 1.1 ± 0.2 nM (•, pink) on B16/F10 and M21 melanoma cells, respectively. The IC₅₀ values of Cy5.5-GGNle-CycMSH_{hex} were 7.1 ± 0.1 nM (□, green) and 2.6 ± 0.3 nM (○, red) on B16/F10 and M21 melanoma cells, respectively.

Fig. 2. UV and radioactive HPLC profiles.

(A) UV HPLC profile of DOTA-GGNle-CycMSH_{hex} ($T_R = 15.7$ min) and (B) radioactive HPLC profile of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} ($T_R = 16.9$ min). (C) Serum stability of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} at 1 h post incubation ($T_R = 16.4$ min). (D) Radioactive HPLC profile of the urine sample of a normal C57 mouse at 2 h post-injection of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} ($T_R = 16.9$ min). Arrows indicate the original compounds of DOTA-GGNle-CycMSH_{hex} (A) and ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} (B, C, D). Specific binding of ⁶⁸Ga-DOTA-GGNle-CycMSH_{hex} on (E) B16/F10 and (F) M21 melanoma cells with or without peptide blockade. ($p < 0.05$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3. Fluorescence staining of MC1R. Fluorescence staining of (A) B16/F10 murine melanoma cells, (B) B16/F10 flank melanoma lesions, (C) B16/F10 pulmonary metastatic melanoma lesions, (D) M21 human melanoma cells, and (E) M21-xenografted flank melanoma lesions. Cells and melanoma tissues were stained with 1 μ M of Cy5.5-GGNle-CycMSH_{hex} (red) in the presence or absence of 100 μ M of peptide blockade. The nuclei were stained with DAPI (blue). The microscopic images were acquired by confocal laser microscopy at 100 $\times$ magnification. Scale bar, 20 μ m.

Fig. 4. PET imaging of flank melanoma.

Representative PET imaging of a live B16/F10 murine melanoma-bearing mouse (upper panel) and a live M21 human melanoma-xenografted nude mouse (lower panel) using ^{68}Ga -DOTA-GGNle-CycMSH_{hex} as an imaging probe at 0.5, 1, and 2 h post-injection. Melanoma lesions are highlighted with arrows on the images. T = tumor.

Fig. 5. PET imaging of pulmonary metastatic melanoma.

Representative PET imaging of a live B16/F10 pulmonary metastatic melanoma-bearing mouse using ^{68}Ga -DOTA-GGNle-CycMSH_{hex} as an imaging probe at 0.5 and 1 h post-injection. Pulmonary metastatic melanoma lesions are highlighted with arrows on the images.

Fig. 6. PET imaging of melanoma patients.

Representative first-in-human PET studies on two melanoma patients. Melanoma metastases in brain, lung, connective tissue and small intestine of patient 1 (A and C) were visualized by PET using ^{68}Ga -DOTA-GGNle-CycMSH_{hex}. Melanoma metastases on the left leg of patient 2 (E) were imaged by PET using ^{68}Ga -DOTA-GGNle-CycMSH_{hex}. ^{18}F -FDG PET (B, D, and F) studies were performed on the same patients for comparisons. The melanoma metastases in brain (BM1 and BM2) were confirmed by MRI and the metastasis in

connective tissue (CM1) was confirmed by CT. Melanoma metastases are highlighted with arrows on the images.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1.

Biodistribution of ^{68}Ga -DOTA-GGNle-CyCMSh_{hex} on B16/F10 murine melanoma-bearing C57 mice. The data are presented as percent injected dose/gram or as percent injected dose (Mean $\pm$ SD, n = 4)

Tissues	0.5 h	1 h	2 h	1 h NDP-MSH blockade
Percent injected dose/gram (%ID/g)				
Tumor	18.63 $\pm$ 4.97	24.27 $\pm$ 3.74	11.61 $\pm$ 2.87	1.94 $\pm$ 0.51 *
Brain	0.13 $\pm$ 0.02	0.07 $\pm$ 0.01	0.03 $\pm$ 0.01	0.09 $\pm$ 0.06
Blood	2.27 $\pm$ 0.32	0.83 $\pm$ 0.1	0.31 $\pm$ 0.06	0.98 $\pm$ 0.45
Heart	0.94 $\pm$ 0.06	0.36 $\pm$ 0.04	0.12 $\pm$ 0.02	0.35 $\pm$ 0.04
Lung	1.91 $\pm$ 0.22	0.87 $\pm$ 0.12	0.29 $\pm$ 0.02	0.73 $\pm$ 0.19
Liver	1.21 $\pm$ 0.08	0.79 $\pm$ 0.08	0.61 $\pm$ 0.06	0.69 $\pm$ 0.08
Skin	2.26 $\pm$ 0.12	1.23 $\pm$ 0.33	0.54 $\pm$ 0.19	0.8 $\pm$ 0.17
Spleen	0.75 $\pm$ 0.08	0.36 $\pm$ 0.05	0.33 $\pm$ 0.15	0.22 $\pm$ 0.15
Stomach	1.67 $\pm$ 0.87	0.91 $\pm$ 0.11	0.54 $\pm$ 0.16	0.49 $\pm$ 0.36
Kidneys	9.22 $\pm$ 1.38	7.78 $\pm$ 0.63	6.01 $\pm$ 0.91	5.98 $\pm$ 0.69
Muscle	0.44 $\pm$ 0.03	0.3 $\pm$ 0.05	0.13 $\pm$ 0.08	0.36 $\pm$ 0.24
Pancreas	0.44 $\pm$ 0.07	0.24 $\pm$ 0.05	0.11 $\pm$ 0.07	0.4 $\pm$ 0.19
Bone	0.98 $\pm$ 0.27	0.6 $\pm$ 0.13	0.24 $\pm$ 0.15	0.32 $\pm$ 0.22
Percent injected dose (%ID)				
Intestines	1.32 $\pm$ 0.51	0.68 $\pm$ 0.11	0.59 $\pm$ 0.31	0.68 $\pm$ 0.31
Urine	71.62 $\pm$ 11.72	85.39 $\pm$ 3.76	91.73 $\pm$ 0.26	91.83 $\pm$ 1.01
Uptake ratio of tumor/normal tissue				
Tumor/blood	8.21	29.24	37.45	1.98
Tumor/kidney	2.02	3.12	1.93	0.32
Tumor/lung	9.75	27.90	40.03	2.66
Tumor/liver	15.40	30.72	19.03	2.81
Tumor/muscle	42.34	80.90	89.31	5.39

* $P < 0.05$, tumor uptake with or without peptide blockade at 1 h post-injection.

Table 2.

Biodistribution of ^{68}Ga -DOTA-GGNle-CyCMSh_{hex} on M21 human melanoma-xenografted nude mice. The data are presented as percent injected dose/gram or as percent injected dose (Mean $\pm$ SD, n = 4).

Tissues	0.5 h	1 h	2 h	1 h NDP-MSH blockade
Percent injected dose/gram (%ID/g)				
Tumor	6.05 $\pm$ 0.83	6.07 $\pm$ 0.68	5.15 $\pm$ 0.78	1.02 $\pm$ 0.54 *
Brain	0.09 $\pm$ 0.02	0.06 $\pm$ 0.01	0.04 $\pm$ 0.02	0.05 $\pm$ 0.02
Blood	1.78 $\pm$ 0.44	0.69 $\pm$ 0.19	0.41 $\pm$ 0.21	0.52 $\pm$ 0.12
Heart	0.80 $\pm$ 0.17	0.31 $\pm$ 0.03	0.17 $\pm$ 0.02	0.24 $\pm$ 0.07
Lung	1.86 $\pm$ 0.28	0.8 $\pm$ 0.1	0.3 $\pm$ 0.04	0.57 $\pm$ 0.09
Liver	0.97 $\pm$ 0.15	0.57 $\pm$ 0.03	0.45 $\pm$ 0.07	0.5 $\pm$ 0.03
Skin	2.0 $\pm$ 0.34	0.94 $\pm$ 0.21	0.62 $\pm$ 0.12	0.37 $\pm$ 0.23
Spleen	0.54 $\pm$ 0.04	0.36 $\pm$ 0.05	0.21 $\pm$ 0.09	0.28 $\pm$ 0.23
Stomach	0.80 $\pm$ 0.24	0.32 $\pm$ 0.07	0.46 $\pm$ 0.4	0.25 $\pm$ 0.07
Kidneys	6.61 $\pm$ 1.11	4.09 $\pm$ 0.49	3.74 $\pm$ 0.55	3.76 $\pm$ 0.39
Muscle	0.37 $\pm$ 0.1	0.15 $\pm$ 0.03	0.11 $\pm$ 0.03	0.08 $\pm$ 0.02
Pancreas	0.43 $\pm$ 0.16	0.2 $\pm$ 0.03	0.14 $\pm$ 0.05	0.19 $\pm$ 0.08
Bone	1.06 $\pm$ 0.3	0.37 $\pm$ 0.1	0.35 $\pm$ 0.11	0.26 $\pm$ 0.24
Percent injected dose (%ID)				
Intestines	1.24 $\pm$ 0.36	0.68 $\pm$ 0.13	0.61 $\pm$ 0.27	0.51 $\pm$ 0.04
Urine	77.35 $\pm$ 4.03	88.91 $\pm$ 0.66	92.75 $\pm$ 1.13	93.4 $\pm$ 1.34
Uptake ratio of tumor/normal tissue				
Tumor/blood	3.4	8.80	12.56	1.96
Tumor/kidney	0.92	1.48	1.38	0.27
Tumor/lung	3.25	7.59	17.17	1.79
Tumor/liver	6.24	10.65	11.44	2.04
Tumor/muscle	16.35	40.47	46.82	12.75

* $P < 0.05$, tumor uptake with or without peptide blockade at 1 h post-injection.