Published in final edited form as:

Bioorg Med Chem Lett. 2017 November 15; 27(22): 4952–4955. doi:10.1016/j.bmcl.2017.10.022.

Metastatic melanoma imaging using a novel Tc-99m-labeled lactam-cyclized alpha-MSH peptide

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Abstract

The purpose of this study was to determine the metastatic melanoma imaging property of ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex} {hydrazinonicotinamide-8-aminooctanoic acid-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-CONH₂}. HYNIC-Aoc-Nle-CycMSH_{hex} was synthesized using fluorenylmethyloxy carbonyl (Fmoc) chemistry. The IC₅₀ value of HYNIC-Aoc-Nle-CycMSH_{hex} was 0.78 ± 0.13 nM for B16/F10 melanoma cells. ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex} displayed significantly higher uptake (14.26 ± 2.74 and 10.45 ± 2.31% ID/g) in B16/F10 metastatic melanoma-bearing lung than that in normal lung (0.90 ± 0.15 and 0.53 ± 0.14% ID/g) at 2 and 4 h post-injection, respectively. B16/F10 pulmonary metastatic melanoma lesions were clearly visualized by SPECT/CT using ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex} as an imaging probe at 2 h post-injection, underscoring its potential as an imaging probe for metastatic melanoma detection.

Graphical abstract

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Keywords

Alpha-melanocyte stimulating hormone; ^{99m}Tc -labeled lactam-cyclized peptide; metastatic melanoma imaging

Malignant melanoma is the most deadly form of skin cancer with an increasing incidence. Approximately 76,380 new cases and 10,130 fatalities occurred in the United States in 2016.¹ Unfortunately, no curative treatment exists for metastatic melanoma. Molecularly targeted approaches, namely 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 over the past several years.^{2–8} 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, the long-term survival of metastatic melanoma patients remains less than 10%.

Melanocortin-1 (MC1) receptor is a distinct molecular target for developing radiolabeled peptides for melanoma imaging and therapy due to its over-expression on murine and human melanoma cells.^{9–13} Alpha-melanocyte stimulating hormone (α -MSH) peptides can bind to MC1 receptors with nanomolar binding affinities.^{14–22} We have developed a class of ^{99m}Tc -labeled lactam-cyclized α -MSH peptides to target MC1 receptors for melanoma imaging.^{17, 21, 22} ^{99m}Tc (140-keV γ -photon) is an attractive imaging radionuclide with 6 h half-life and is widely used in nuclear medicine. It is easy and convenient to obtain ^{99m}Tc via a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator. Meanwhile, ^{99m}Tc shares similar coordination chemistry with therapeutic ^{188}Re . Therefore, ^{99m}Tc -labeled lactam-cyclized α -MSH peptides could readily switch to ^{188}Re -labeled peptides for melanoma therapy. Our ^{99m}Tc -labeled lactam-cyclized α -MSH peptides build upon the construct of HYNIC-GGNle-CycMSH_{hex} {Hydrazinonicotinamide-Gly-Gly-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-CONH₂}.¹⁷ Interesting, we found that the replacement of -GG- linker with -Aoc- (8-aminooctanoic acid) linker substantially enhanced B16/F1 melanoma uptake. The B16/F1 melanoma lesions could be clearly visualized by single photon emission computed tomography (SPECT)/CT using ^{99m}Tc (EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex} as an imaging probe.²¹

In this study, we managed to evaluate the metastatic melanoma imaging property of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ in B16/F10 pulmonary metastatic melanoma-bearing C57 mice. Firstly, HYNIC-Aoc-Nle-CycMSH_{hex} (Figure 1) was synthesized using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and purified by reverse phase-high performance liquid chromatography (RP-HPLC). The chemical purity of HYNIC-Aoc-Nle-CycMSH_{hex} was greater than 90% after the HPLC purification. The identity of HYNIC-Aoc-Nle-CycMSH_{hex} was confirmed by electrospray ionization mass spectrometry. The found molecular weight (1257.3) matched the calculated molecular weight. Secondly, the MC1 receptor binding affinity of HYNIC-Aoc-Nle-CycMSH_{hex} was determined in B16/F10 melanoma cells. The IC₅₀ value of HYNIC-Aoc-Nle-CycMSH_{hex} was 0.78 ± 0.13 nM in B16/F10 melanoma cells (Figure 2).

$^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ was readily prepared according to our published procedure²¹ with greater than 95% radiolabeling yield, and was completely separated from the excess non-labeled peptide by RP-HPLC. The retention time of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ and HYNIC-Aoc-Nle-CycMSH_{hex} was 20 and 18 min, respectively. $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ displayed MC1 receptor-mediated binding in B16/F10 melanoma cells. Approximately 97% of the cellular uptake of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ was blocked by 1 μM of HYNIC-Aoc-Nle-CycMSH_{hex} ($p < 0.05$) (Figure 3).

The biodistribution property of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ was determined in B16/F10 pulmonary metastatic melanoma-bearing C57 mice and compared with normal C57 mice. The biodistribution results of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ are presented in Table 1. $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ exhibited significantly ($p < 0.05$) higher uptake in metastatic melanoma-bearing lung than that in normal lung. The uptake values of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ radioactivity in the metastatic melanoma-bearing and normal lungs were 14.26 ± 2.74 and $0.90 \pm 0.15\%$ ID/g, 10.45 ± 2.31 and $0.53 \pm 0.14\%$ ID/g at 2 and 4 h post-injection, respectively. The uptake values of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ radioactivity in the metastatic melanoma-bearing and normal lungs were 4.48 ± 0.76 and $0.20 \pm 0.03\%$ ID/g at 24 h post-injection. Whole-body clearance of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ was rapid, with approximately 86% of the injected radioactivity cleared through the urinary system by 2 h post-injection (Table 1). Approximately 95% of the injected radioactivity cleared out the body at 24 h post-injection.

Generally, cancer cells in primary tumor can develop distant metastases through blood circulation and lymphatic system. In this study, B16/F10 melanoma cells were injected into mice via tail veins to mimic the development of pulmonary melanoma metastases via blood circulation. The imaging property of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ was evaluated in B16/F10 pulmonary metastatic melanoma-bearing C57 mice at 12, 14 and 16 days post-injection of B16/F10 melanoma cells. The representative maximum intensity projection SPECT/CT images are presented in Figure 4. The pulmonary metastatic melanoma lesions were clearly visualized by SPECT/CT using $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ as an imaging probe at 2 h post-injection, highlighting the potential

of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ as an imaging probe to identify MC1 receptor-positive melanoma metastases.

In our previous work, we have demonstrated the advantage of radiolabeled MC1 receptor-targeting $\alpha\text{-MSH}$ peptide in identifying melanoma metastases in B16/F10 pulmonary metastatic melanoma model as compared to $^{18}\text{F}\text{-FDG}$.²³ Therefore, it is possible to use $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ SPECT as an alternative approach for $^{18}\text{F}\text{-FDG}$ PET to identify melanoma metastases. Furthermore, it is feasible to radiolabel HYNIC-Aoc-Nle-CycMSH_{hex} with ^{188}Re for potential therapy of melanoma metastases since both ^{99m}Tc and ^{188}Re share similar coordination chemistry. Essentially, ^{99m}Tc and ^{188}Re are matched-pair theranostic radionuclides that can be readily and cost-effectively obtained from commercial $^{99}\text{Mo}/^{99m}\text{Tc}$ and $^{188}\text{W}/^{188}\text{Re}$ generators. Therefore, it is practically feasible to prepare $^{99m}\text{Tc}/^{188}\text{Re}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ with onsite generators. The combination use of $^{99m}\text{Tc}/^{188}\text{Re}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ would be attractive because the ^{99m}Tc -peptide could be used for tumor imaging to select MC1 receptor-positive patients for therapy of ^{188}Re -peptide. The combination of $^{99m}\text{Tc}/^{188}\text{Re}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ could potentially provide personalized diagnosis and treatment to melanoma patients.

The experimental details are presented in References and notes.^{24–28}

In conclusion, $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ exhibited significantly ($p<0.05$) higher uptake in metastatic melanoma-bearing lung than that in normal lung. B16/F10 pulmonary metastatic melanoma lesions could be clearly visualized by SPECT/CT using $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ as an imaging probe, underscoring its potential as an imaging agent for metastatic melanoma detection in the future.

Acknowledgments

We thank Dr. Fabio Gallazzi for his technical assistance. This work was partially supported by University of Colorado Denver start-up fund and the NIH grant R15CA173516.

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24. *In vitro* competitive binding assay: Amino acid and resin were purchased from Advanced ChemTech Inc (Louisville, KY) and Novabiochem (San Diego, CA) for peptide synthesis All other chemicals used in this study were purchased from Thermo Fischer Scientific (Waltham, MA) and used without further purification B16/F10 melanoma cells were obtained from American Type Culture Collection (Manassas, VA) and cultured according to the instruction of American Type Culture Collection HYNIC-AocNle-CycMSH_{hex} was synthesized and purified by reverse phase-high performance liquid chromatography (RP-HPLC) and characterized by liquid chromatography-mass spectrometry (LC-MS) according to our published procedure²¹The receptor binding affinity of HYNIC-AocNle-CycMSH_{hex} was determined in B16/F10 melanoma cells by *in vitro* competitive receptor binding assay The receptor binding assay was replicated in triplicate ¹²⁵I-Tyr²-[Nle⁴, D-Phe⁷]-α-MSH { ¹²⁵I-(Tyr²)-NDP-MSH } was obtained from PerkinElmer, Inc (Waltham, MA) for receptor binding assay B16/F10 cells (2×10⁵ cells/well, n = 3) were incubated at 37 °C for 2 h with approximately 30,000 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 {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 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 Wallac 1480 automated gamma counter (PerkinElmer, NJ) The IC₅₀ value was calculated using the Prism software (GraphPad Software La Jolla, CA, USA).
25. Specific binding of ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex}: ^{99m}Tc was purchased from Cardinal Health (Denver, CO) for peptide radiolabeling. ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex} was prepared according to our published procedure.²¹ Briefly, 50 μL of ^{99m}TcO₄⁻ 37–74 MBq, 10 μL of 1 mg/mL SnCl₂ in 0.1 N HCl solution, 200 μL of a mixture of 5 mg/mL of EDDA (Ethylenediaminediacetic acid) and 25 mg/mL of Tricine aqueous solution and 10 μL of 1 mg/mL peptide aqueous solution were added into 400 μL of 0.5 M NH₄OAc (pH 5.44) in a reaction vial and incubated at 95°C for 30 min. The radiolabeled peptide was purified to

a single species by Waters RP-HPLC on a Grace Vydac C-18 reverse phase analytic column using a 20-min gradient of 22–32% acetonitrile in 20 mM HCl aqueous solution at a flow rate of 1 mL/min. The purified peptide was purged with N₂ gas for 20 min to remove the acetonitrile. The pH of the final solution was adjusted to 5 with 0.1 N NaOH and normal saline for cell binding and animal studies. B16/F10 cells (2×10⁵ cells/well, n = 3) were incubated at 37 °C for 2 h with approximately 150,000 counts per minute (cpm) of ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex} with or without 1 μM of HYNIC-Aoc-Nle-CycMSH_{hex} 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 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 Wallac 1480 automated gamma counter (PerkinElmer, NJ). The experiment was replicated in triplicate.

26. Biodistribution of ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex}: All 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 biodistribution of ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex} was determined in B16/F10 pulmonary metastatic melanoma-bearing C57 mice and compared with normal C57 mice. HPLC-purified 0.037 MBq of ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex} 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.
27. Pulmonary metastatic melanoma imaging: B16/F10 pulmonary metastatic melanoma-bearing mice (12, 14 and 16 days post cell injections) were used for SPECT/CT imaging using ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex} as an imaging probe. Each mouse was injected with 8.6–15.9 MBq of ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSH_{hex} via the tail vein. The mice were euthanized at 2 h post-injection for small animal SPECT/CT (Nano-SPECT/CT[®], Bioscan) imaging. The 9-min CT imaging was immediately followed by the whole-body SPECT scan. The SPECT scans of 24 projections were acquired. Reconstructed SPECT and CT data were visualized and co-registered using InVivoScope (Bioscan, Washington DC).
28. Statistical analysis was performed using the Student's t-test for unpaired data. A 95% confidence level was chosen to determine the significance of difference between the activity distribution in pulmonary metastatic melanoma-bearing and normal C57 mice, with p<0.05 being significantly different.

Figure 1.
Schematic structure of HYNIC-Aoc-Nle-CycMSH_{hex}.

Figure 2.

The *in vitro* competitive binding curve of HYNIC-Aoc-Nle-CycMSH_{hex} in B16/F10 melanoma cells. The IC₅₀ value of HYNIC-Aoc-Nle-CycMSH_{hex} was 0.78 ± 0.13 nM.

Figure 3.

Radioactive HPLC profile of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ (left). The retention time of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ was 20 min. Specific binding of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ on B16/F10 melanoma cells with (□) or without (■) the presence of 1 μM of HYNIC-Aoc-Nle-CycMSH_{hex} (right).

Figure 4. Representative maximum intensity projection SPECT/CT images of $^{99m}\text{Tc}(\text{EDDA})\text{-HYNIC-Aoc-Nle-CycMSH}_{\text{hex}}$ at 2 h post-injection in B16/F10 pulmonary metastatic melanoma-bearing C57 mice at 12, 14 and 16 days post-injection of B16/F10 melanoma cells (A, B and C), respectively. Pulmonary melanoma metastases are highlighted with arrows on the images.

Biodistribution of ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSh_{hex} in B16/F10 pulmonary melanoma-bearing (Lung Mets) and normal C57 mice (Normal). The data were presented as percent injected dose/gram or as percent injected dose (Mean ± SD, n = 5)

Table 1

Tissues	2 h		4 h		24 h	
	Lung Mets	Normal	Lung Mets	Normal	Lung Mets	Normal
Percent injected dose/gram (%ID/g)						
Lung	14.26 ± 2.74	0.90 ± 0.15 *	10.45 ± 2.31	0.53 ± 0.14 *	4.48 ± 0.76	0.20 ± 0.03 *
Brain	0.07 ± 0.02	0.06 ± 0.01	0.04 ± 0.02	0.03 ± 0.03	0.03 ± 0.02	0.01 ± 0.01
Blood	0.81 ± 0.06	0.91 ± 0.24	0.46 ± 0.26	0.55 ± 0.25	0.12 ± 0.09	0.02 ± 0.01
Heart	0.48 ± 0.14	0.38 ± 0.08	0.41 ± 0.22	0.16 ± 0.05	0.12 ± 0.05	0.09 ± 0.04
Liver	1.43 ± 0.09	1.82 ± 0.47	1.52 ± 0.30	1.17 ± 0.33	0.64 ± 0.08	0.56 ± 0.07
Spleen	0.59 ± 0.28	0.52 ± 0.14	0.27 ± 0.14	0.27 ± 0.06	0.22 ± 0.11	0.13 ± 0.09
Stomach	1.67 ± 0.71	2.23 ± 0.66	1.42 ± 0.09	1.79 ± 0.71	0.66 ± 0.46	0.90 ± 0.76
Kidneys	7.31 ± 0.91	7.70 ± 2.29	4.57 ± 0.59	3.59 ± 0.59	1.52 ± 0.41	1.34 ± 0.27
Muscle	0.20 ± 0.12	0.18 ± 0.10	0.13 ± 0.08	0.14 ± 0.09	0.08 ± 0.04	0.09 ± 0.03
Pancreas	0.39 ± 0.09	0.44 ± 0.15	0.54 ± 0.09	0.38 ± 0.07	0.15 ± 0.07	0.07 ± 0.02
Bone	0.46 ± 0.29	0.30 ± 0.10	0.41 ± 0.27	0.21 ± 0.17	0.11 ± 0.02	0.18 ± 0.05
Skin	1.12 ± 0.27	0.75 ± 0.41	0.74 ± 0.28	0.95 ± 0.50	0.58 ± 0.22	0.53 ± 0.40
Percent injected dose (%ID)						
Intestines	2.59 ± 0.26	2.73 ± 1.05	4.61 ± 0.80	2.78 ± 0.89	2.42 ± 0.97	1.78 ± 0.83
Urine	76.17 ± 4.04	86.33 ± 2.60	78.47 ± 4.26	85.94 ± 4.25	90.94 ± 3.94	94.52 ± 1.56

* p<0.05 significance of difference in lung uptake between ^{99m}Tc(EDDA)-HYNIC-Aoc-Nle-CycMSh_{hex} in B16/F10 pulmonary metastatic melanoma-bearing and normal C57 mice.