Title: Effects of the Arg-Pro and Gly-Gly-Nle Moieties on Melanocortin-1 Receptor Binding Affinities of α-MSH Peptides

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SUPPORTING INFORMATION

Experimental details for peptide synthesis, in vitro competitive binding assay, radiolabeling, biodistribution and imaging studies, and urinary metabolites analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

Chemicals and Reagents

Amino acids and resin were purchased from Advanced ChemTech Inc. (Louisville, KY) and Novabiochem (San Diego, CA). ^{125}I-Tyr^{2}-[Nle^{4}, DPhe^{7}]-α-MSH {^{125}I-(Tyr^{2})-NDP-MSH} was obtained from PerkinElmer, Inc. (Waltham, MA) for receptor binding assay. $^{99m}TcO_4^-$ was purchased from Cardinal Health (Albuquerque, NM). All other chemicals used in this study were purchased from Thermo Fischer Scientific (Waltham, MA) and used without further purification. B16/F1 murine melanoma cells were obtained from American Type Culture Collection (Manassas, VA).

Peptide Synthesis and *In Vitro* Competitive Binding Assay

The Ac-GGNle-CCEHdFRWC-NH_2, Ac-GGNle-CCEHdFRWCRP-NH_2, Ac-CCEHdFRWC-NleGG-NH_2 and Ac-CCEHdFRWCRP-NleGG-NH_2 peptides were synthesized using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry according to the published procedure[14] with slight modification on Sieber amide resin by an Advanced ChemTech multiple-peptide synthesizer (Louisville, KY). Briefly, 70 μmol of Sieber amide resin and 210 μmol of Fmoc-protected amino acids were used for the synthesis. Each peptide was purified by reverse phase-high performance liquid chromatography (RP-HPLC) and characterized by liquid chromatography-mass spectroscopy (LC-MS).

The IC_{50} values of Ac-GGNle-CCEHdFRWC-NH_2, Ac-GGNle-CCEHdFRWCRP-NH_2, Ac-CCEHdFRWC-NleGG-NH_2 and Ac-CCEHdFRWCRP-NleGG-NH_2 peptides for the MC1 receptor were determined in B16/F1 melanoma cells. The receptor binding assay was replicated in triplicate for each peptide. The B16/F1 cells were seeded into a 24-well cell culture plate at a density of 2.5×10^5 cells/well 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) (HEPES), pH 7.4, 0.2% bovine serum albumin (BSA), 0.3 mM 1,10-phenathroline}, the cells were incubated at 25 °C for 2 h with approximately 30,000 counts per minute (cpm) of ^{125}I-(Tyr^2)-NDP-MSH in the presence of increasing concentrations (10^{-12} M to 10^{-5} M) of each peptide in 0.3 mL of binding medium. The reaction 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) to remove any unbound radioactivity and lysed in 0.5 mL of 1 M NaOH for 5 min. The activities associated with the cells were measured in a Wallac 1480 automated gamma counter (PerkinElmer, NJ). The IC_{50} value for each peptide was calculated using Prism software (GraphPad Software, La Jolla, CA).

Peptide Radiolabeling

Only Ac-GGNle-CCEHdFRWCRP-NH_2 and Ac-CCEHdFRWCRP-NleGG-NH_2 exhibited low nanomolar receptor binding affinities, we further radiolabeled them with ^{99m}Tc. Ac-GGNle-CCEHdFRWCRP-NH_2 and Ac-CCEHdFRWCRP-NleGG-NH_2 peptides were labeled with ^{99m}Tc via a direct reduction reaction with $SnCl_2$. Briefly, 10 μL of 1 mg/mL $SnCl_2$ in 0.1 M HCl, 40 μL of 0.5 M NH_4OAc (pH 5.2), 100 μL of 0.2 M Na_2tartate (pH 9.2), 100 μL of fresh $^{99m}TcO_4^-$ solution (37-74 MBq), and 10 μL of 1 mg/mL of each peptide in aqueous solution were added

into a reaction vial and incubated at 25 °C for 20 min to form ^{99m}Tc-labeled peptide. Each ^{99m}Tc-peptide was purified to a single species by Waters RP-HPLC (Milford, MA) on a Grace Vydac C-18 reverse phase analytic column (Deerfield, IL) using a 20-min gradient of 16-26% acetonitrile in 20 mM HCl aqueous solution at a flow rate of 1 mL/min. Each purified peptide was purged with N_2 gas for 20 mins to remove the acetonitrile. The pH of final peptide solution was adjusted to 7.4 with 0.1 N NaOH and sterile normal saline for biodistribution and imaging studies.

Biodistribution Studies

All the animal studies were conducted in compliance with Institutional Animal Care and Use Committee approval. The biodistribution properties of ^{99m}Tc-Ac-GGNle-CCEHdFRWCRP-NH_2 and ^{99m}Tc-Ac-CCEHdFRWCRP-NleGG-NH_2 were determined in B16/F1 melanoma-bearing C57 female mice (Harlan, Indianapolis, IN). Each C57 mouse was subcutaneously inoculated on the right flank with 1×10^6 B16/F1 cells. The weight of tumors reached approximately 0.2 g 10 days post cell inoculation. Each melanoma-bearing mouse was injected with 0.037 MBq of ^{99m}Tc-Ac-GGNle-CCEHdFRWCRP-NH_2 or ^{99m}Tc-Ac-CCEHdFRWCRP-NleGG-NH_2 via the tail vein. Groups of 5 mice were sacrificed at 0.5, 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 body weight. The specificity of tumor uptake was determined by co-injecting ^{99m}Tc-Ac-GGNle-CCEHdFRWCRP-NH_2 or ^{99m}Tc-Ac-CCEHdFRWCRP-NleGG-NH_2 with 10 μg (6.1 nmol) of unlabeled NDP-MSH at 2 h post-injection.

Melanoma Imaging with ^{99m}Tc-Ac-GGNle-CCEHdFRWCRP-NH_2

We further determined the melanoma imaging property of ^{99m}Tc-Ac-GGNle-CCEHdFRWCRP-NH_2 due to its higher tumor uptake and faster urinary clearance than ^{99m}Tc-Ac-CCEHdFRWCRP-NleGG-NH_2. Approximately 7.4 MBq of ^{99m}Tc-Ac-GGNle-CCEHdFRWCRP-NH_2 was injected into a B16/F1 melanoma-bearing C57 mouse via the tail vein. The mouse was euthanized for small animal SPECT/CT (Nano-SPECT/CT®, Bioscan, Washington DC) imaging 2 h post-injection. The 9-min CT imaging was immediately followed by the SPECT imaging of whole-body. The SPECT scans of 24 projections were acquired. Reconstructed data from SPECT and CT were visualized and co-registered using InVivoScope (Bioscan, Washington DC).

Urinary Metabolites of ^{99m}Tc-Ac-GGNle-CCEHdFRWCRP-NH_2

We also examined the urinary metabolites of ^{99m}Tc-Ac-GGNle-CCEHdFRWCRP-NH_2. Approximately 3.7 MBq of ^{99m}Tc-Ac-GGNle-CCEHdFRWCRP-NH_2 was injected into a B16/F1 melanoma-bearing C57 mouse via the tail vein to determine the urinary metabolites. The mouse was euthanized to collect urine at 2 h post-injection. The collected urine sample was centrifuged at 16,000 g for 5 min before the HPLC analysis. Thereafter, an aliquot of the urine was injected into the HPLC. A 20-minute gradient of 16-26% acetonitrile / 20 mM HCl with a flow rate of 1 mL/min was used for urine analysis.

Statistical Analysis

Statistical analysis was performed using the Student's t-test for unpaired data to determine the significance of differences in tumor and kidney uptake with/without peptide blockade in

biodistribution studies described above. Differences at the 95% confidence level ($p<0.05$) were considered significant.