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BINDING AND cAMP STUDIES OF MELANOTROPIN PEPTIDES WITH THE CLONED HUMAN PERIPHERAL MELANOCORTIN RECEPTOR, hMC1R

Carrie Haskell-Luevano[1], Hiroto Miwa[2], Chris Dickinson[3], Victor J. Hruby[1*], Tadataka Yamada[2,4], and Ira Gantz[5*]

[1]Department of Chemistry, University of Arizona, Tucson, AZ 85721

Departments of [2]Internal Medicine, [3]Pediatrics, [4]Physiology, and [5]Surgery, University of Michigan Medical Center, Ann Arbor, MI

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Summary: Binding and stimulation of cAMP by the melanotropin peptides α-MSH (α-melanocyte-stimulating hormone) and its superpotent analogues [Nle^4, DPhe^7] α-MSH (MT-I) and Ac-[Nle^4, Asp^5, DPhe^7, Lys^{10}] α-MSH$_{4-10}$-NH$_2$ (MT-II) were undertaken to examine their respective properties on the human peripheral melanocyte melanocortin receptor, hMC1R. α-MSH was found to possess a binding IC_{50} value of $6.5 \pm 0.9 \times 10^{-9}$ M and cAMP EC_{50} value of $2.0 \pm 0.6 \times 10^{-9}$ M. MT-I possesses a binding IC_{50} value of $1.2 \pm 0.3 \times 10^{-9}$ M and a cAMP EC_{50} of $0.5 \pm 0.03 \times 10^{-9}$ M. MT-II possesses a binding IC_{50} of $0.57 \pm 0.08 \times 10^{-9}$ M and cAMP EC_{50} value of $0.20 \pm 0.05 \times 10^{-9}$ M.

α-Melanotropin (α-melanocyte-stimulating hormone, α-MSH) is a linear tridecapeptide consisting of the amino acid sequence Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH$_2$. This melanocortin hormone is derived from the pre-prohormone, pro-opiomelanocortin (POMC), is secreted from the *pars intermedia* of the pituitary gland of most vertebrates,(1, 2) and has been identified as activating adenylate cyclase, increasing intracellular levels of cyclic AMP (cAMP) in melanocyte membrane receptors (3). Although this hormone is thought to possess a variety of central and peripheral biological activities, its most recognized role is regulating skin pigmentation (4-8). Clinical applications of melanotropin peptides include detection and possible eradication of melanoma cancer (9, 10). The peripheral melanocortin receptor, designated MC1R, has been isolated from human melanoma (11, 12) and is the G-protein coupled receptor believed to be involved in regulating skin pigmentation.

Utilizing the classical frog (*Rana pipiens*) and lizard (*Anolis carolinensis*) skin bioassays (13, 14), extensive structure-function studies of a diverse number of melanotropin analogues have been examined to identify compounds possessing

* To whom correspondence should be addressed.

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increased biological potencies, as compared to the native hormone, α-MSH. Substitution of Nle (sidechain = CH_2-CH_2-CH_2-CH_3) for Met^4 resulted in peptides possessing more potent biological activity. Inversion of configuration of L-Phe^7 to D-Phe^7 led to the analogue [Nle^4, DPhe^7] α–MSH (hereafter referred to as "MT-I") which showed a substantial increase in potency as well as prolonged (or residual) activity (1, 15). The design of a cyclic lactam peptide, Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2 (hereafter referred to as "MT-II"), was based on conformational considerations and molecular dynamic simulations, and resulted in potencies up to 90 times greater than α-MSH (16, 17). Both MT-I and MT-II peptide analogues are in phase I clinical trials for use in the prevention of sun-induced melanoma (18-20). This study was undertaken to examine binding of the melanotropin peptides α-MSH, MT-I, and MT-II to the cloned human peripheral MC1 receptor, and their ability to stimulate generation of the second messenger, cAMP.

MATERIALS AND METHODS

Binding Assays. The coding region of hMC1R, cloned from a human genomic EMBL3 phage library (Clontech, Palo Alto, CA) was placed into the eukaryotic expression vector, CMVneo, and stably transfected into L-cells. Transfected cells were grown to confluence in 12-well (2.4 x 1.7 cm) tissue culture plates. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 4.5 g/100 mL glucose, 10% fetal calf serum, 100 units/mL penicillin and streptomycin, 1 mM sodium pyruvate, and 1 mg/mL Geneticin. For the assays, this medium was removed and cells were washed twice with a freshly prepared binding buffer consisting of 97% minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthrolone, 0.5 mg/L leupeptin and 200 mg/L bacitracin. A 400 µL dilution of the peptide being tested was added to the well, with the concentrations ranging between 10^{-11} and 10^{-6} M. Next, 100 µL solution of [^{125}I-Tyr^2, Nle^4, DPhe^7] α-MSH (20), (100,000 cpm / well) was added to each well and the cells were incubated at 37 °C for 40 min. The medium was subsequently removed and each well was washed twice with binding buffer. The cells were lysed by the addition of 0.5 mL 0.1 M NaOH and 0.5 mL 1%Triton X-100. The mixture was left to react for 5 min, and the contents of each well transferred to labeled 16 x 150-mm glass tubes and quantified in a γ-counter. [^{125}I-Tyr^2, Nle^4, DPhe^7] α-MSH was prepared and purified by methods described previously (21).

cAMP Assays. A commercially available cAMP assay kit (TRK 432, Amersham Corp.) was employed. L-cells transfected with the human MC1 receptors were grown to confluence in 12-well (2.4 x 1.7 cm) tissue culture plates. The cells were maintained in Dulbecco's modified Eagles's medium (DMEM, GIBCO) containing 4.5 g/100 mL glucose, 10% fetal calf serum, 100 units/mL penicillin and streptomycin, 1 mM sodium pyruvate, and 1 mg/mL Geneticin. For the assays, the medium was removed and cells were washed twice with Earle's balanced salt solution containing 10 mM HEPES (pH 7.4), 1 mM glutamine, 26.5 mM sodium bicarbonate, and 100 mg/mL bovine serum albumin. An aliquot (0.5 mL) of Earle's balanced salt solution (EBSS, GIBCO) was placed into each well along with 5 µL of 2 x 10^{-2} M isobutylmethylxanthine. Varying concentrations of melanotropins were added, and the cells incubated for 30 min at 37 °C. Ice-cold 100% ethanol (1.0 mL / well) was added to stop the reaction. The

incubation medium and scrapped cells were transferred to 16 x 150-mm glass tubes, then placed on ice for 30 min. The precipitate was then centrifuged for 10 min at 1,900 x g, the supernatant was dried under a nitrogen stream and resuspended in 50 mM Tris, 2 mM EDTA (pH 7.5). The cAMP content was measured by competitive binding assay according to the assay instructions.

Peptide Synthesis. Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2 was synthesized by solid phase synthetic methods, as previously described (17, 22). The other melanotropins, α-MSH and [Nle^4, $DPhe^7$] α-MSH, were obtained from Peninsula Laboratories, Inc. (Belmont, CA).

RESULTS AND DISCUSSION

The human peripheral melanocortin receptor, hMC1R, was transfected successfully into L-cells (mouse fibroblast-like cell line) and used to evaluate both their competitive binding (Figure 1) and their ability to generate cAMP (Figure 2) for the melanotropin peptides α-MSH, MT-I, and MT-II. Tables 1 and 2 summarize the binding IC_{50}, cAMP EC_{50}, and relative potency values, respectively, for each melanotropin peptide examined in this study.

It has been found previously that the classical lizard skin bioassay relative potencies correlate with the mammalian melanoma tyrosinase system (9, 23, 24). Table 3 summarizes the previous peripheral bioassay relative potencies for both the frog and lizard skin assay systems. It is interesting to note that the relative potencies reported for MT-I in the lizard skin assay are in close agreement with the relative potency of hMC1R binding, Table 1. MT-II, based on the reported relative potency of 90.0 (17) does not appear to correlate well. However, due to the potential clinical relevance of this compound, it subsequently has been resynthesized and re-evaluated for the past several years. These biological evaluations have resulted in relative potencies between

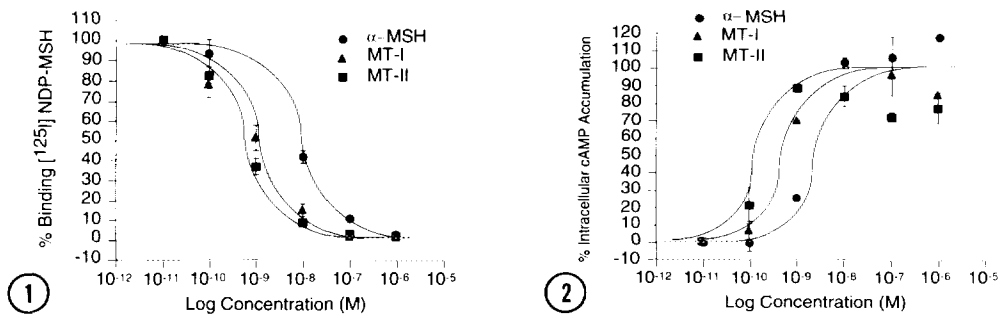

Figure 1. Inhibition of α-MSH, MT-I, and MT-II of [^{125}I] NDP-MSH binding to L-cells transfected with the hMC1 receptor. Binding is depicted as a percentage of total specific binding.

Figure 2. Comparison of the cAMP response produced in L-cells transfected with the hMC1 receptor in response to α-MSH, MT-I, and MT-II .

Table 1. Comparative Binding Activities of Melanotropin Peptides on the hMC1 Receptor

Peptide	Structure	Peptide Activity IC_{50}[a] (nM) ± SE	Peptide Activity Relative[b] Potency
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	6.5 ± 0.9	1.0
MT-I	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	1.2 ± 0.3	5.4
MT-II	Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2	0.57 ± 0.08	11.4

[a]IC_{50} = concentration of peptide at 50% specific binding, with the standard error (SE) listed (N = 4 to 6). The peptides were tested at a range of concentrations (10^{-11} to 10^{-6} M). [b]Relative Potencies were calculated based on the IC_{50} values relative to α-MSH.

0.5 to 1.0 that of α-MSH in the frog skin bioassay, and between 10 to 90 times more potent than α-MSH in the lizard skin bioassay (unpublished results). If we accept the more conservative value of relative potency in the lizard skin bioassay as 10, then this value also correlates well with the binding data presented in Table 1. These results are in agreement with the previous reports suggesting that the lizard bioassay potencies are more comparable to the mammalian system than are the frog skin potencies. The close correlation between relative potencies in the lizard skin bioassay and hMC1R binding data also suggests that a conserved structural homology may exist between the mammalian hMC1R and lizard receptors in that the peptide interactions with each receptor possess similar results.

Previously reported values of α-MSH and MT-I (NDP-MSH) in competitive binding assays have been reported as α-MSH (IC_{50} = 2.0 x 10^{-9} M), MT-I (IC_{50} = 2.8 x

Table 2. Comparative cAMP Activities of Melanotropin Peptides on the hMC1 Receptor

Peptide	Structure	Peptide Activity EC_{50}[a] (nM) ± SE	Peptide Activity Relative[b] Potency
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	2.0 ± 0.06	1.0
MT-I	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.5 ± 0.03	4.0
MT-II	Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2	0.2 ± 0.05	10.0

[a]EC_{50} = concentration of peptide at 50% maximal cAMP generation, with the standard error (SE) listed (N = 4). The peptides were tested at a range of concentrations (10^{-11} to 10^{-6} M). [b]Relative Potencies were calculated based on the EC_{50} values relative to α-MSH.

Table 3. Comparative Biological Activities of the Melanotropin Peptides in the Frog and Lizard Skin Bioassays

Peptide	Structure	Relative Peptide Potencies: Frog Skin	Relative Peptide Potencies: Lizard Skin
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	1.0	1.0
MT-I	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	60.0[a]	5.0
MT-II	Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2	0.8[b]	10.0 - 90.0*

[a] Relative potencies from Sawyer et al. (25).
[b] Relative potencies from Al-Obeidi et al. (17).
* Indicates results tested in various experiments, over several years, in our laboratory and those of others; see Results and Discussion section for discussion.

10^{-11} M) in the hMC1R transfected into 293 cells (11), and α-MSH (IC_{50} = 9.2 x 10^{-11} M), MT-I (IC_{50} = 2.3 x 10^{-11} M) in the hMC1 receptor transfected into COS-7 cells (12). The relative potencies of MT-I compared to α-MSH (1.0) is 71 in the 293 cells and 4 in the COS-7 cells. The latter relative potencies are more consistent with the values reported in this study.

The functional cAMP assay results, Table 2, also demonstrate relative potencies approximately equivalent to receptor binding (Table 1), and the lizard skin bioassay (Table 3). Interestingly, the trend in relative potencies, MT-II > MT-I > α-MSH, for both ligand binding and cAMP generation is approximately the same. This may indicate that the available conformations of the peptide can both increase receptor-ligand binding affinity and induce a receptor conformational change that effects the generation of cAMP by the same relative orders of magnitude.

We conclude from this study that the melanotropin peptides examined, interact with the hMC1 receptor and generate cAMP in the order of increasing potency, MT-II > MT-I > α-MSH. The observation that the relative potencies found from this study may correlate with the relative potencies reported previously for the classical lizard skin bioassay suggests a functional similarity between the two assay systems.

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