Characterization of Melanocortin NDP-MSH Agonist Peptide Fragments at the Mouse Central and Peripheral Melanocortin Receptors

Carrie Haskell-Luevano,* Jerry Ryan Holder, Eileen K. Monck, and Rayna M. Bauzo

Department of Medicinal Chemistry, University of Florida, Gainesville, Florida 32610

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The central melanocortin receptors, melanocortin-4 (MC4R) and melanocortin-3 (MC3R), are involved in the regulation of satiety and energy homeostasis. The MC4R in particular has become a pharmaceutical industry drug target due to its direct involvement in the regulation of food intake and its potential therapeutic application for the treatment of obesity-related diseases. The melanocortin receptors are stimulated by the native ligand, α-melanocyte stimulating hormone (α-MSH). The potent and enzymatically stable analogue NDP-MSH (Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) is a lead peptide for the identification of melanocortin amino acids important for receptor molecular recognition and stimulation. We have synthesized nine peptide fragments of NDP-MSH, deleting N- and C-terminal amino acids to determine the "minimally active" sequence of NDP-MSH. Additionally, five peptides were synthesized to study stereochemical inversion at the Phe 7 and Trp 9 positions in attempts to increase tetra- and tripeptide potencies. These peptide analogues were pharmacologically characterized at the mouse melanocortin MC1, MC3, MC4, and MC5 receptors. This study has identified the Ac-His-DPhe-Arg-Trp-NH_2 tetrapeptide as possessing 10 nM agonist activity at the brain MC4R. The tripeptide Ac-DPhe-Arg-Trp-NH_2 possessed micromolar agonist activities at the MC1R, MC4R, and MC5R but only slight stimulatory activity was observed at the MC3R (at up to 100 μM concentration). This study has also examined to importance of both N- and C-terminal NDP-MSH amino acids at the different melanocortin receptors, providing information for drug design and identification of putative ligand−receptor interactions.

Introduction

The melanocortin-3 and -4 receptors (MC3R, MC4R) found in the brain have been identified in knockout mice to be involved in feeding behavior, obesity, metabolism, and energy homeostasis.[1−3] The most well-studied melanocortin receptor ligands are for the skin melanocortin-1 receptor (MC1R) which are involved in pigmentation and animal coat coloration.[4−6] Additionally, the melanocortin-5 receptor has been deleted from the mouse genome and identified as playing a role in exocrine gland function.[7] The melanocortin receptors belong to the superfamily of seven transmembrane spanning G-protein coupled receptors (GPCR's) and stimulate the cAMP signal transduction pathway.[8] The endogenous agonist ligands for these MCRs are derived by posttranslational processing of the pro-opiomelanocortin (POMC) gene transcript, which upon differential processing, results in the generation of the α-, β-, and γ-melanocyte stimulating hormones (MSH) and adrenocorticotropin (ACTH). All these melanocortin peptide agonists contain a core His-Phe-Arg-Trp tetrapeptide sequence that has been attributed to the ligand selectivity and stimulation of the melanocortin receptors.[9−11] The melanocortin receptor family also has two endogenous antagonists, agouti[12] and the agouti-related protein (AGRP),[13,14] which are the only known antagonists of GPCR's discovered to date.

Due to the importance of the melanocortin agonists resulting in decreased food intake,[15] design of MC4R specific therapeutic agents for the treatment of obesity-related diseases including heart disease, type II diabetes mellitus, stroke, and hypertension is being pursued by pharmaceutical industry, biotechnology companies, and academic laboratories.[16] One of the classical rational ligand design approaches involves identifying which amino acids are functionally important and what minimally sized peptide fragment of the parent hormone is needed to produce a pharmacological response. Structure−activity studies of the melanocortin peptides date back to the 1960s.[17,18] A linear tridecapeptide known as NDP-MSH, Ac-Ser-Tyr-Ser-Nle[4]-Glu-His-DPhe[7]-Arg-Trp-Gly-Lys-Pro-Val-NH_2,[19] was initially developed for investigations of the melanocortin system. Subsequent SAR studies established that the core "His-Phe-Arg-Trp" tetrapeptide and "Phe-Arg-Trp" tripeptide were active in generating a physiological response in the frog and lizard skin bioassays.[9−11] Further studies examining stereochemically modified tetra- and tripeptides established that Ac-His-DPhe-Arg-Trp-NH_2 bound and activated the human melanocortin receptor hMC1R and hMC4R at submicromolar concentrations.[20] Substructural and biological information gained from melanocortin-based peptides resulted in the generation of the hypothesis that the bioactive conformation of the MSH peptides involves a β-turn.[4] Upon the basis of this later hypothesis, a non-peptide small molecule combinatorial library based upon a β-turn mimetic template resulted

* Reprint requests should be addressed to Carrie Haskell-Luevano, University of Florida, Department of Medicinal Chemistry, PO Box 100485, Gainesville, FL 32610-0485. Phone: (352) 846-2722. E-mail: Carrie@cop.ufl.edu.

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in the identification of two compounds, EL1 [with racemic naphthylalanine (2′), in the $i + 1$ position, DPro in the $i + 2$ position, and Trp in the $i + 3$ position] and EL2 [Trp in the $i + 1$ position, DLys in the $i + 2$ position, and Phe in the $i + 3$ position] that possessed micromolar agonist activity at the mMC1R.[21] Interestingly, these compounds were less potent than the linear peptide fragments,[20] suggesting that the β-turn mimetic used in that study was not an optimal template.

Based on the above rationale, this study was undertaken to synthesize and pharmacologically characterize peptide fragments of NDP-MSH at the cloned murine melanocortin receptors MC1R, MC3R, MC4R, and MC5R. The overall objectives were (i) to identify melanocortin ligand amino acids that may be important for melanocortin receptor selectivity to aid in the design of receptor specific agonists and (ii) to reevaluate and validate the minimally active peptide fragment of NDP-MSH at the cloned mouse melanocortin receptors MC1R, MC3R, MC4R, MC5R. This is the first report of the fragment analysis of NDP-MSH at all the cloned melanocortin receptors (excluding the MC2R, which is only stimulated by ACTH)[5] in parallel. This information is important for identifying putative ligand−receptor interactions, using homology molecular modeling and the 2.8 Å resolution structure of the GPCR rhodopsin,[22] and the potential design of potent non-peptide melanocortin receptor agonists.

Results

Evaluation of NDP-MSH Fragment Peptides. Table 1 summarizes the NDP-MSH peptide fragment functional agonist pharmacology at the mouse melanocortin MC1, MC3, MC4, and MC5 receptors. Removal of the C-terminal Pro-Val amino acids (**1**) resulted in equipotent activity compared with NDP-MSH at the MC1, MC3, and MC4 receptors, whereas at the MC5R, 5-fold decreased potency resulted. Removal of the C-terminal Gly-Lys amino acids (**2**) resulted in equipotent activity compared with NDP-MSH at the MC1, MC3, MC4, and MC5 receptors, compared with **1**. Analogue **3** has the N-terminal Ser-Tyr-Ser residues deleted from NDP-MSH and resulted in equipotent activity compared with NDP-MSH at the MC1, MC3, and MC4 receptors, whereas at the MC5R, 5-fold decreased potency resulted. Peptide **4** has both the N-terminal Ser-Tyr-Ser and C-terminal Lys-Pro-Val residues omitted and resulted in equipotent activity compared with NDP-MSH at the MC1, MC3, and MC4 receptors (within the 3-fold inherent experimental error) and 5-fold decreased potency resulted at the MC5R. Analogue **5** differs from **4** only by the removal of the Gly^{10} amino acid (α-MSH numbering), and it is equipotent with **4** at the MCRs and within experimental error of the NDP-MSH potencies at these receptors. Analogue **6** further removes the Nle^4 amino acid of NDP-MSH and results in 300-, 50-, and 10-fold decreased potencies at the MC1R, MC3R, and MC4R, compared to NDP-MSH, respectively, but remains equipotent at the MC5R, compared with **5**. The removal of the N-terminal Ser-Tyr-Ser-Nle-Glu NDP-MSH amino acids (**7**) results in 16- and 70-fold decreased potencies as compared with NDP-MSH at the MC1R and MC3R, while possessing equipotency to NDP-MSH at the MC4R and MC5R. Analogue **8**, Ac-His-DPhe-Arg-Trp-NH_2, is down to the core putative

Table 1. Functional Activity of the NDP-MSH Peptide Agonists at the Mouse melanocortin receptors[a]

		mMC1R		mMC3R		mMC4R		mMC5R	
peptide	structure	EC_{50} (nM)	fold diff	EC_{50} (nM)	fold diff	EC_{50} (nM)	fold diff	EC_{50} (nM)	fold diff
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.23 ± 0.06		0.73 ± 0.19		3.07 ± 0.57		0.41 ± 0.09	
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.030 ± 0.14	1	0.10 ± 0.02	1	0.32 ± 0.11	1	0.086 ± 0.028	1
1	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-NH_2	0.018 ± 0.007	1	0.16 ± 0.004	1	0.44 ± 0.07	1	0.46 ± 0.25	5
2	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-NH_2	0.036 ± 0.016	1	0.29 ± 0.09	3	0.78 ± 0.02	2	0.53 ± 0.31	6
3	Ac-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.021 ± 0.0003	1	0.11 ± 0.02	1	0.32 ± 0.03	1	0.46 ± 0.25	5
4	Ac-Nle-Glu-His-DPhe-Arg-Trp-Gly-NH_2	0.045 ± 0.01	1	0.39 ± 0.02	4	0.94 ± 0.34	3	0.59 ± 0.29	7
5	Ac-Nle-Glu-His-DPhe-Arg-Trp-NH_2	0.034 ± 0.010	1	0.26 ± 0.03	3	0.45 ± 0.17	1	0.39 ± 0.29	5
6	Ac-Glu-His-DPhe-Arg-Trp-NH_2	8.94 ± 0.39	**300**	15.2 ± 2.66	**150**	4.31 ± 0.15	**13**	0.42 ± 0.13	5
7	Ac-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.49 ± 0.06	**16**	6.82 ± 0.12	**68**	0.45 ± 0.12	1	0.17 ± 0.08	2
8	Ac-His-DPhe-Arg-Trp-NH_2	25.6 ± 4.70	**850**	195 ± 44.6	**2000**	10.2 ± 1.44	**32**	3.46 ± 0.33	**40**
9	Ac-DPhe-Arg-Trp-NH_2	4540 ± 454	**150000**	slight agonist @ 100 μM		2320 ± 227	**7300**	1,660 ± 257	**19000**
10	Ac-His-Phe-Arg-Trp-NH_2	7690 ± 3585	**260000**	4370 ± 535	**44000**	2110 ± 243	**6600**	103 ± 28	**1200**
11	Ac-Phe-Arg-Trp-NH_2	5870 ± 2040	**195000**	>100000		>100000		slight agonist @ 100 μM	
12	Ac-His-Phe-Arg-DTrp-NH_2	14500 ± 1270	**480000**	1730 ± 243	**17000**	6620 ± 1250	**21000**	317 ± 31	**3700**
13	Ac-His-DPhe-Arg-DTrp-NH_2	97.2 ± 39.4	**3200**	2440 ± 1320	**24000**	154 ± 29.5	**480**	61.3 ± 12.1	**700**
14	Ac-DPhe-Arg-DTrp-NH_2	slight agonist @ 100 μM		>100000		11200 ± 3720	**35000**	6880 ± 2730	**80000**

[a] The indicated errors represent the standard error of the mean determined from at least three independent experiments. Slight agonist denotes that some stimulatory response was observed but not enough to determine an EC_{50} value.

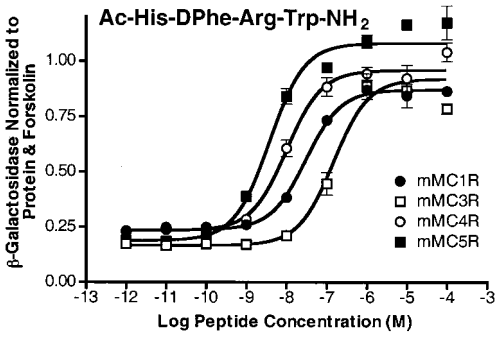

Figure 1. Stimulatory agonist activity of the tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 (analogue **8**) at the mouse melanocortin receptors MC1R, MC3R, MC4R, and MC5R. This peptide possess 25 nM (MC1R), 195 nM (MC3R), 10 nM (MC4R), and 3.5 nM (MC5R) EC_{50} values. These values were generated from six independent experiments in parallel at the melanocortin receptors.

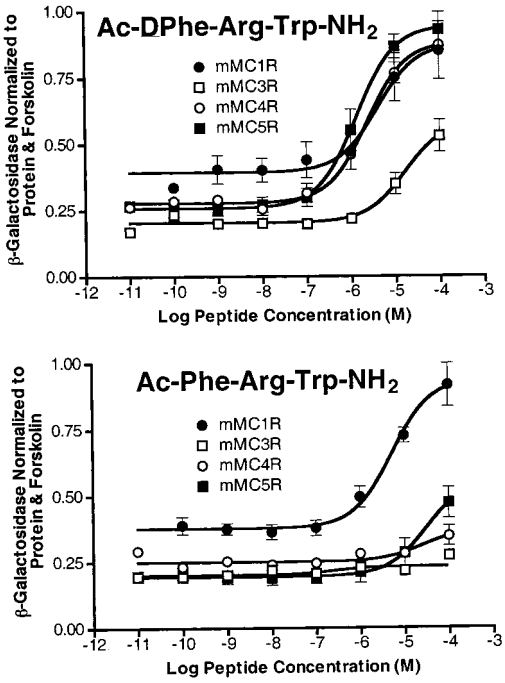

Figure 2. Functional activities of the Ac-DPhe-Arg-Trp-NH_2 (analogue **9**) and Ac-Phe-Arg-Trp-NH_2 (analogue **11**) peptides which differ by inversion of chirality at the Phe^7 position. Analogue **9** ($DPhe^7$) possessed micromolar agonist EC_{50} values at the MC1R, MC4R, and MC5R, while analogue **11** ($LPhe^7$) only possessed micromolar agonist EC_{50} activity at the MC1R. These values were generated from at least three independent experiments in parallel at the melanocortin receptors.

melanocortin message amino acids and results in 850- and 2000-fold decreased potency at the MC1R and MC3R while possessing only 32- and 40-fold decreased potency at the MC4 and MC5 receptors, respectively, as compared to NDP-MSH. Figure 1 illustrates the nanomolar agonist EC_{50} values and dose−response curves of **8** at the MC1, MC3, MC4, and MC5 receptors. The tripeptide Ac-DPhe-Arg-Trp-NH_2 (**9**) lost ability to stimulate the MC3R to 50% maximal response at up to 100 μM concentrations (Figure 2) and possessed 150000-, 7300-, and 19000-fold decreased potencies at the MC1R, MC4R, and MC5R, respectively, compared to NDP-MSH.

Stereochemical and Modifications of NDP-MSH (6−9 and 7−9) Sequence. Previous studies of modifying the stereochemistry at the 7 and 9 positions in the tetrapeptide Ac-His-$DPhe^7$-Arg-Trp^9-NH_2 and tripeptide Ac-$DPhe^7$-Arg-Trp^9-NH_2 sequences resulted in increased activity.[11] Table 1 summarizes the results of stereochemical modifications of these tetra- and tripeptides at the 7 and 9 positions. The core Ac-His-Phe-Arg-Trp-NH_2 amino acids of the melanocortin agonist peptide (**10**), possessed 256000-, 43700-, 6600-, and 1200-fold decreased potency compared to NDP-MSH and resulted in 33000-, 6000-, 690-, and 250-fold decreased potency compared to α-MSH at the MC1, MC3, MC4, and MC5 receptors, respectively. The tripeptide Ac-Phe-Arg-Trp-NH_2 (**11**), resulted in loss of stimulatory activity at the MC3R and MC4R at up to 100 μM concentrations and possessed an MC1R EC_{50} value equal to 5.8 μM and slight agonist activity at the and MC5R (Figure 2). Peptide **12**, Ac-His-Phe-Arg-DTrp-NH_2, resulted in 482000-, 17000-, 21000-, and 3700-fold decreased potencies as compared with NDP-MSH at the MC1, MC3, MC4, and MC5 receptors, respectively. Analogue **13**, Ac-His-DPhe-Arg-DTrp-NH_2, resulted in 3200-, 24000-, 490-, and 700-fold decreased potencies as compared with NDP-MSH at the MC1, MC3, MC4, and MC5 receptors, respectively. The Ac-DPhe-Arg-DTrp-NH_2 tripeptide (**14**) possessed slight agonist activity at 100 μM, lost ability to stimulate the MC3R at up to 100 μM concentrations, and possessed 35000- and 80000-fold decreased potencies at the MC4 and MC5 receptors, respectively.

Discussion

This report extends previous structure−activity studies focused on the active site of the melanocortin agonist ligands[9−11,20] by performing a comprehensive structure−function study of the melanocortin agonist NDP-MSH peptide fragments at the MC1, MC3, MC4, and MC5 melanocortin receptors. The most noteworthy results of this study are that the NDP-MSH based tetrapeptide contains nanomolar agonist activities at the mouse MC1 (25.6 nM EC_{50}), MC4 (10.2 nM EC_{50}), and MC5 (3.5 nM EC_{50}) receptors (Figure 1). During the preparation of this report, a publication examining NDP-MSH peptide fragments at the human MC4R was reported,[23] and for the tetrapeptide His-DPhe-Arg-Trp-NH_2, 8.7 nM ligand efficacy was observed. Additionally, the data reported by Yang et al.[23] for the His-DPhe-Arg-Trp-NH_2 tetrapeptide are in agreement with the values reported herein, validating these results as reproducible by different laboratories using melanocortin receptors from different species (human versus mouse) and different functional assays (intracellular cAMP accumulation assay versus β-galactosidase reporter gene assay).

No significant changes in ligand potency were observed at any of the melanocortin receptors until the Nle^4 (α-MSH numbering) was deleted from the NDP-MSH (4−9) peptide fragment, **5** and **6**, respectively. Removal of the Nle^4 residue from the Ac-Nle-Glu-His-DPhe-Arg-Trp-NH_2 analogue resulted in 300- and 51-fold decreased potencies at the skin MC1R and brain MC3R, comparing **6** with **5**. The Nle side chain consists of a $CH_2CH_2CH_2CH_3$ alkyl chain that is hydrophobic. As homology molecular modeling studies of the melanocortin receptors[24,25] have primarily focused upon the identification of putative ligand DPhe-Arg-Trp amino acids with the corresponding receptor residues involved

in molecular interactions, not many studies have been pursued examining possible Nle^4−receptor interactions. These data, however, suggest that identifying these potential ligand−receptor interactions at the MC1R and MC3R may aid in the design of small molecules with enhanced potency, and perhaps the utilization of the potential ancillary binding site may result in novel MC1R and MC3R ligands. This hypothesis is further supported by comparison of **3** and **7** which differ by the absence of the Nle^4 and Glu^5 amino acids and result in 16- and 68-fold decreased potencies at the MC1R and MC3R, while being equipotent at the MC3R and MC4R. Removal of the His^6 residue from the tetrapeptide to the Ac-DPhe-Arg-Trp-NH_2 (**9**) resulted in significant decreases in ligand potency at the melanocortin receptors and lost ability to maximally stimulate the MC3R at up to 100 μM concentrations (Figure 2). A 180- to 480-fold decrease in ligand potency resulted at the MC1R, MC4, and MC5 receptors upon deletion of this His^6 amino acid, implying that significant melanocortin receptor interactions are occurring with this ligand amino acid and are potentially important for the design of potent and selective small molecule melanocortin peptide mimetics. Finally, any modification by amino acid deletion of the NDP-MSH peptide resulted in at least approximately 5-fold decreased potency at the mMC5R (Table 1).

Stereochemical and Modifications of NDP-MSH (6−9 and 7−9) Sequence. Previous studies of stereochemical inversion from L to D chirality at the α-carbon of the Phe-Arg-Trp α-MSH 7−9 sequence[11,20] resulted in the identification of human melanocortin receptor selective compounds and prolonged activity after the ligands had been removed from the assay media in the frog skin bioassay. In the study presented herein, tetrapeptides **8**, **10**, **12**, and **13** have stereochemical modifications at the 7 and 9 positions. Modifications at the 7 position (comparing **10** with **8**) result in 300-, 22-, 210-, and 30-fold decreased potencies at the mouse MC1R, MC3R, MC4R, and MC5R, respectively. These data suggest that the DPhe^7 stereochemistry at the MC1 and MC4 mouse receptors are important for enhanced agonist potency. These results indicate that further modifications at the 7 position of similar analogues may result in increased melanocortin receptor selectivity in receptors expressed either centrally or peripherally. This concept is further illustrated by the Ac-*x*Phe-Arg-Trp-NH_2 tripeptides **9** and **11** which differ in the x = L or D configuration of Phe^7. Analogue **9** (Ac-DPhe-Arg-Trp-NH_2) was unable to stimulate a MC3R functional response at up to 100 μM concentrations, yet it possessed micromolar potencies at the MC1, MC4, and MC5 receptors (Table 1, Figure 2). Peptide **11** (Ac-Phe-Arg-Trp-NH_2) lost functional MC3R and MC4R activity at up to 100 μM concentrations, yet it possessed some agonist activities at the MC1 and MC5 receptors (Figure 2).

Stereochemical modifications at the DTrp^9 position (**12**, **13**, and **14**) generally resulted in decreased potencies compared with the Trp^9 containing peptides (**10**, **8**, and **9**, respectively). Interestingly, comparison of Trp^9 (**10**) and DTrp^9 (**12**) resulted in a 2.5-fold increased MC3R potency of the DTrp^9 analogue, whereas at the other melanocortin receptors, 2- to 3-fold decreased potencies were observed (Table 1). The combination of DPhe^7 and DTrp^9 containing peptides (**13** and **14**) resulted in 4- to 20-fold decreased potencies at the melanocortin receptors as compared to the peptides containing only DPhe^7 (**8** and **9**), respectively. These data suggest the stereochemical modification of Trp^9 to the D configuration in these tetra- and tripeptides results in decreased potencies, with the exception of **12** at the MC3R.

Comparison of Mouse versus Human MC4R NDP Fragment SAR. Reports of MC4R in vitro mutagenesis studies[23,26] comparing different ligands at similar receptor amino acid side chain mutations suggested that there might be significant pharmacological and perhaps physiologically important differences between the human and mouse forms of the MC4 receptors. At the human MC4R, nine fragments of des-acetyl NDP-MSH were evaluated. Three des-acetyl analogues were similar to peptides **6**, **8**, and **9** reported herein, with the difference that the peptides reported herein were all N-terminal acetylated. Direct comparison of the functional activities of the peptides Ac-Glu-His-DPhe-Arg-Trp-NH_2, Ac-His-DPhe-Arg-Trp-NH_2, and the des-acetyl forms at the mouse and human MC4Rs, respectively, resulted in identical functional EC_{50} values within the errors reported. This comparison demonstrates that the bioassays evaluating intracellular cAMP accumulation and using the β-galactosidase reporter gene bioassay result in identical results and may be compared directly from the literature for the melanocortin-4 receptor. Additionally, these data suggest that the N-terminal acetyl group does not add any increased ligand potency and may only serve as a role in protection of the ligand from enzymatic degradation by peptidases. However, the Ac-DPhe-Arg-Trp-NH_2 peptide possessed a 2.3 μM EC_{50} value at the mouse MC4R, while the DPhe-Arg-Trp-NH_2 peptide possessed a 17.7 nM EC_{50} value at the human MC4R.[23] These data imply that the role of the NDP-MSH His^6 residue may possess different interactions with the mouse and human MC4 receptors. In support of this hypothesis, melanocortin receptor mutagenesis studies to date have not conclusively identified putative MCR residues that interact with the His^6 ligand amino acid, although melanocortin receptor homology molecular modeling studies have identified putative receptor residues that may be interacting with the His^6 moiety.[24,27]

Conclusions

A structure−function study of N- and C-terminal deleted amino acid peptide fragments derived from the agonist NDP-MSH peptide has been performed and pharmacologically characterized at the mouse melanocortin receptors MC1R, MC3R, MC4R, and MC5R in parallel. This study has identified the NDP-MSH tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 as possessing 10 nM agonist activity at the central MC4R involved in feeding behavior and food intake, 25 nM agonist activity at the peripheral MC1R involved in pigmentation and animal coat coloration, 195 nM agonist activity at the central and peripheral MC3R involved in energy homeostasis, and 3 nM agonist activity at the peripheral MC5R involved in exocrine gland regulation.

Table 2. Analytical Data for the Peptides Synthesized in This Study[a]

peptide	structure	HPLC k' (system 1)	HPLC k' (system 2)	purity (%)	mass spectral analysis (M + 1)
1	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-NH_2	3.6	9.7	>95	1449.1
2	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-NH_2	3.9	10.3	>95	1266.5
3	Ac-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	3.8	9.7	>99	1310.3
4	Ac-Nle-Glu-His-DPhe-Arg-Trp-Gly-NH_2	3.5	9.7	>99	986.3
5	Ac-Nle-Glu-His-DPhe-Arg-Trp-NH_2	3.9	9.7	>99	928.6
6	Ac-Glu-His-DPhe-Arg-Trp-NH_2	3.5	7.4	>99	816.1
7	Ac-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	2.7	7.7	>99	1068.1
8	Ac-His-DPhe-Arg-Trp-NH_2	2.7	6.9	>99	685.7
9	Ac-DPhe-Arg-Trp-NH_2	4.4	8.2	>99	549.9
10	Ac-His-Phe-Arg-Trp-NH_2	4.1	8.2	>98	687.1
11	Ac-Phe-Arg-Trp-NH_2	4.7	9.1	>99	550.1
12	Ac-His-Phe-Arg-DTrp-NH_2	3.7	7.3	>99	686.7
13	Ac-His-DPhe-Arg-DTrp-NH_2	3.3	6.7	>99	687.1
14	Ac-DPhe-Arg-DTrp-NH_2	4.1	7.7	>99	550.0

[a] HPLC k' = [(peptide retention time − solvent retention time)/solvent retention time] in solvent system 1 (10% acetonitrile in 0.1% trifluroacetic acid/water and a gradient to 90% acetonitrile over 40 min) or solvent system 2 (10% methanol in 0.1% trifluroacetic acid/water and a gradient to 90% methanol over 40 min). An analytical Vydac C_{18} column (Vydac 218TP104) was used with a flow rate of 1.5 mL/min. The percentage peptide purity is determined by HPLC at a wavelength of 214 λ.

Experimental Section

Peptide synthesis was performed using standard Fmoc methodology[28] on an automated synthesizer (Advanced ChemTech 440MOS, Louisville, KY). The amino acids Fmoc-Ser-(tBu), Fmoc-Tyr(tBu), Fmoc-Nle, Fmoc-Glu(OtBu), Fmoc-His-(Trt), Fmoc-Arg(Pbf), Fmoc-DPhe, Fmoc-Trp(Boc), Fmoc-DTrp-(Boc), Fmoc-Gly, Fmoc-Lys(Boc), Fmoc-Pro, Fmoc-Val, and Fmoc-Phe were purchased from Peptides International (Louisville, KY). The peptides were assembled on rink-amide-MBHA resin (0.40 mequiv/g substitution), purchased from Peptides International (Louisville, KY). All reagents were ACS grade or better. The synthesis was performed using a 40-well Teflon reaction block with a course Teflon frit. Approximately 200 mg of resin (0.08 mmol) was added to each reaction block well. The resin was allowed to swell for 2 h in dimethylformamide (DMF) and deprotected using 25% piperidine in DMF for 5 min followed by a 20 min 25% piperidine incubation at 500 rpms. A positive Kaiser[29] test resulted, indicating free amine groups on the resin. The growing peptide chain was added to the amide resin using the general amino acid cycle as follows: 500 μL of DMF is added to each reaction well to "wet the frit," 3-fold excess amino acid starting from the C-terminus is added (500 μL of 0.5 M amino acid solution containing 0.5 M HOBt in DMF) followed by the addition of 500 μL of 0.5 M DIC in DMF, and the reaction well volume is brought up to 3 mL using DMF. The coupling reaction is mixed for 1 h at 500 rpms, followed by emptying of the reaction block by positive nitrogen gas pressure. A second coupling reaction is performed by the addition of 500 μL of DMF to each reaction vessel, followed by the addition of 500 μL of the respective amino acid (3-fold excess), 500 μL of 0.5 M HBTU, and 400 μL of 1 M DIEA. The reaction well volume is brought up to 3 mL with DMF and mixed at 500 rpm for 1 h. After the second coupling cycle, the reaction block is emptied, and the resin-Nα-protected peptide is washed with DMF (4.5 mL, 5 times). Nα-Fmoc deprotection is performed by the addition of 4 mL of 25% piperidine in DMF and mixed for 5 min at 500 rpms followed by a 20 min deprotection at 500 rpms. The reaction well is washed with 4.5 mL of DMF, and the next coupling cycle is performed as described above. Deprotection of the amino acid side chains and cleavage of the amide peptide from the resin was performed by incubating the peptide resin with 3 mL of cleavage cocktail (95% TFA, 2.5% water, 2.5% triisopropylsilane) for 3 h at 500 rpms. The cleavage product was emptied from the reaction block into a cleavage block containing 7 mL of collection vials under nitrogen gas pressure. The resin was washed with 1.5 mL of cleavage cocktail for 5 min and 500 rpms and added to the previous cleavage solution. The peptides were transferred to preweighted 50 mL conical tubes and precipitated with cold (4 °C) anhydrous ethyl ether (up to 50 mL). The flocculent peptide was pelleted by centrifugation (Sorval Super T21 high-speed centrifuge using the swinging bucket rotor) at 2000 rpm for 3 min, the ether was decanted off, and the peptide was washed one time with cold anhydrous ethyl ether and pelleted. The crude peptide was dried in vacuo for 48 h. The crude peptide yields ranged from 60% to 90% of the theoretical yields. A 7 to 15 mg sample of crude peptide was purified by RP-HPLC using a Shimadzu chromatography system with a photodiode array detector and a semipreparative RP-HPLC C_{18} bonded silica column (Vydac 218TP1010, 1.0 × 25 cm) and lyophilized. The purified peptide was >95% pure as determined by analytical RP-HPLC (214 λ) and had the correct molecular mass (University of Florida protein core facility), Table 2.

Cell Culture and Transfection. Briefly, HEK-293 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum and seeded 1 day prior to transfection at 1 to 2 × 10^6 cells per100 mm dish. Melanocortin receptor DNA in the $pCDNA_3$ expression vector (20 μg) were transfected using the calcium phosphate method. Stable receptor populations were generated using G418 selection (1 mg/mL) for subsequent bioassay analysis.

Functional Bioassay. HEK-293 cells stably expressing the melanocortin receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene as previously described.[26,30,31] Briefly, 5000−15000 posttransfection cells were plated into 96-well Primera plates (Falcon) and incubated overnight. Forty-eight hours posttransfection the cells were stimulated with 100 μL of peptide (10^{-4}−10^{-12} M) or forskolin (10^{-4} M) control in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media was aspirated, and 50 μL of lysis buffer (250 mM Tris-HCl pH = 8.0 and 0.1% Triton X-100) was added. The plates were stored at −80 °C overnight. The plates containing the cell lysates were thawed the following day. Aliquots of 10 μL were taken from each well and transferred to another 96-well plate for relative protein determination. To the cell lysate plates, 40 μL of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 200 mg of ONPG) was added to each well, and the plates were incubated at 37 °C. The sample absorbance, OD_{405}, was measured using a 96-well plate reader (Molecular Devices). The relative protein was determined by adding 200 μL of 1:5 dilution Bio Rad G250 protein dye:water to the 10 μL cell lysate sample taken previously, and the OD_{595} was measured on a 96-well plate reader (Molecular Devices). Data points were normalized both to the relative protein content and nonreceptor dependent forskolin stimulation.

Data Analysis. EC_{50} values represent the mean of duplicate experiments performed in triplicate, quadruplet, or more independent experiments. EC_{50} estimates and their associated standard errors were determined by fitting the data to a

nonlinear least-squares analysis using the PRISM program (v3.0, GraphPad Inc.). The results are not corrected for peptide content.

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