Articles

Discovery of Prototype Peptidomimetic Agonists at the Human Melanocortin Receptors MC1R and MC4R

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[Nle^4,D-Phe^7]-α-MSH (NDP-MSH), a highly potent analogue of α-melanocyte-stimulating hormone (α-MSH), possesses nanomolar efficacies at all the melanocortin receptor subtypes except the MC2R. Evaluation of the melanocortin "message" sequence of [Nle^4,D-Phe^7]-α-MSH was performed on the human melanocortin receptor subtypes designated hMC1, hMC3R, hMC4R, and hMC5R. Tetrapeptides and tripeptides were stereochemically modified to explore topochemical preferences at these receptors and to identify lead peptides possessing agonist activity and subtype selectivity. Four peptides were discovered to only bind to the hMC1 and hMC4 receptor subtypes. The tetrapeptide Ac-His-D-Phe-Arg-Trp-NH_2 (**1**) possessed 0.6 μM binding affinity at the hMC1R, 1.2 μM binding affinity at the hMC4R, and agonist activity at both receptors. The tripeptides Ac-D-Phe-Arg-Trp-NH_2 (**6**) and Ac-D-Phe-Arg-D-Trp-NH_2 (**7**) possessed 2.0 and 9.1 μM binding affinities, respectively, only at the hMC4R, and both compounds effected agonist activity. The tetrapeptide Ac-His-Phe-Arg-D-Trp-NH_2 (**4**) possessed 6.3 μM affinity and full agonist activity at the hMC1R, while only binding 7% at the hMC3R, 36% at the hMC4R, and 11% at the hMC5R at a maximal concentration of 10 μM. These data demonstrate that the His-Phe-Arg-Trp message sequence of the melanocortin peptides does not bind and stimulate each melanocortin receptor in a similar fashion, as previously hypothesized. Additionally, this study identified the simplest structural agonists for the hMC1R and hMC4R receptors reported to date.

Introduction

The melanotropin peptides include α-, β-, γ-melanocyte-stimulating hormones (MSH) and adrenocorticotropin (ACTH). All of these hormones are derived by posttranslational processing of the pro-opiomelanocortin (POMC) gene transcript, and all possess a central His-Phe-Arg-Trp sequence (Table 1), which is also referred to as the "message" sequence for melanotropin hormones due to its conservation in these peptides. Before the melanocortin receptors were cloned, primarily two classical pigmentation assays were utilized to study the efficacy of natural and synthetically derived melanotropin peptides. The frog (*Rana pipiens*)[1] and lizard (*Anolis carolinensis*)[2,3] skin bioassays monitored peptide activity by quantitating skin darkening in response to concentration and time. Studies utilizing these bioassays have identified Ac-His-Phe-Arg-Trp-NH_2 as the minimal synthetic fragment to elicit a melanotropic response. Relative to α-MSH, Ac-His-Phe-Arg-Trp-NH_2 was 400000-fold less potent in the frog skin bioassay[4] and approximately 7000-fold less potent in the lizard skin bioassay,[5] and neither α-MSH nor this tetrapeptide possessed prolonged (residual) melanotropic activity in either assay. Truncation studies of the highly potent, enzymatically stable, and prolonged acting agonist Ac-Ser-Tyr-Ser-Nle^4-Glu-His-D-Phe^7-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (NDP-MSH)[6−8] identified the prolonged acting tripeptide Ac-D-Phe-Arg-Trp-NH_2 (**6**) as the minimally active NDP-MSH fragment. This tripeptide resulted in a 30 μM EC_{50} value (2000000-fold less potent than NDP-MSH) in the frog skin bioassay[9,10] but possessed only 5000-fold decreased potency (1 μM EC_{50} value) compared to NDP-MSH, in the lizard skin bioassay.[9] Further stereochemical modifications of this tripeptide resulted in analogues Ac-D-Phe-D-Arg-Trp-NH_2 (**9**) and Ac-D-Phe-Arg-D-Trp-NH_2 (**7**) with increased potencies of 5−20-fold in the frog skin bioassay simply by changing the chirality at positions 8 and 9, respectively (Table 2).[10] These studies led to the proposal of MSH-based agonist pharmacophore models at the frog skin melanocortin receptor and aided in proposing specific ligand−receptor interactions at hMC1R.[11]

Five human melanocortin receptor subtypes (hMC1R−hMC5R) have been cloned and characterized.[12−17] All of these receptors respond to all of the melanotropin peptides (Table 1), with the exception of the hMC2R,

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Table 1. Primary Sequence of the Melanocortin Peptides Derived from POMC

ACTH(1−39)	SYSME**HFRW**GKPVGKKRRPVKVYPNGAEDESAEAFPLEF
α-MSH	Ac-SYSME**HFRW**GKPV-NH_2
β-MSH	DEGPYKME**HFRW**GSPPKD
γ-MSH	YVMG**HFRW**DRFG

Table 2. Biological Activity of Stereochemically Modified Melanotropin Tetra- and Tripeptides on the Frog Skin MCR

peptide	structure	frog MCR[a,b] EC_{50} (μM)
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.00015
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.000015
1	Ac-His-DPhe-Arg-Trp-NH_2	0.2
2	Ac-His-DPhe-DArg-DTrp-NH_2	1.9
3	Ac-His-DPhe-DArg-Trp-NH_2	10.0
4	Ac-His-Phe-Arg-DTrp-NH_2	2.0
5	Ac-Gly-DPhe-Arg-DTrp-NH_2	4.2
6	Ac-DPhe-Arg-Trp-NH_2	30.0
7	Ac-DPhe-Arg-DTrp-NH_2	1.5
8	Ac-DPhe-DArg-DTrp-NH_2	>100
9	Ac-DPhe-DArg-Trp-NH_2	5.5
10	Ac-Phe-Arg-DTrp-NH_2	>100
11	Ac-Phe-DArg-DTrp-NH_2	>100

[a] Values reported by Haskell-Luevano et al.[10] which were tested at a range of concentrations (10^{-4}−10^{-12} M). [b] Errors for individual data points in the assay are less than 25%.

which only responds to ACTH,[13] and thus has been deleted from this study. The rationale behind this study is that since all the melanotropin peptides contain the His-Phe-Arg-Trp sequence, this sequence interacts with similar melanocortin receptor residues in a "common" fashion (hence the term message sequence), with the variations of the hormone N- and C-terminus responsible for receptor selectivity and additional differences in potency. The goal of this study was to examine stereochemically modified tripeptides and tetrapeptides on the human melanocortin receptors to determine selectivity and functional properties (i.e. agonism) and to correlate with recent frog skin melanocortin studies.[10] Such information is expected to provide a basis for future structure-based design studies that are focused on the discovery of MSH peptidomimetic agonists.

Results

Table 3 summarizes the binding affinities and signal transduction efficacy (intracellular cAMP accumulation) of stereochemically modified tri- and tetrapeptides. The α-MSH and NDP-MSH tridecapeptides have been included for reference. Intracellular cAMP accumulation bioassays were only performed on analogues which possessed greater than 50% competitive displacement of [^{125}I] NDP-MSH at 10 μM. α-MSH cAMP values were not determined simultaneously in these experiments, but correlation with competitive binding values have been reported extensively in initial reports of the human receptor cloning and characterization.[12−17] α-MSH has the greatest affinity for the hMC1R, with 8.3- and 6.5-fold decreased binding affinities at the hMC3R and hMC4R, respectively, and 93-fold decreased potency at the hMC5R. NDP-MSH maintains nanomolar affinities and potencies at all the human melanocortin receptors, excluding the hMC2R which does not respond to other melanotropin hormones except ACTH.[13] Tetrapeptide **1**, Ac-His-DPhe-Arg-Trp-NH_2, possessed binding affinities of 0.6 μM at the hMC1R and 1.1 μM at the hMC4R and were 1200- and 990-fold less potent than NDP-MSH, respectively. This peptide, at a 10 μM concentration, was unable to competitively displace [^{125}I]NDP-MSH at either the hMC3R or the hMC5R. Analogue **4**, Ac-His-Phe-Arg-DTrp-NH_2, was the only other tetrapeptide that was able to competitively displace [^{125}I]NDP-MSH in a dose−response manner at the hMC1R and resulted in 6 μM binding affinity. The tripeptides examined in this study were only able to generate dose-response competitive binding curves at the hMC4R (Figure 1). Analogue **6** (Ac-DPhe-Arg-Trp-NH_2) differs from analogue **1** only by the deletion of the His^6 residue and resulted in a 1.8-fold decreased potency compared with that of analogue **1**. Of particular note, however, is that analogue **1** (Ac-His-DPhe-Arg-Trp-NH_2) was able to generate the maximum intracellular cAMP accumulation observed for NDP-MSH, albeit at sub-micromolar efficacy, but the tripeptide Ac-DPhe-Arg-Trp-NH_2 (analogue **6**) resulted in only 40% generation of maximal cAMP at 10 μM concentration (Figure 2). Analogue **7**, Ac-DPhe-Arg-DTrp-NH_2, resulted in a 9 μM binding affinity but was only able to generate 50% maximal cAMP accumulation at 10 μM concentration. Table 3 reports analogue binding values that were able to competitively displace [^{125}I]NDP-MSH greater than 50% at 10 μM concentrations. Figure 3 summarizes the percentage that the ligands were able to displace [^{125}I]-NDP-MSH and competitively bind to the various human melanocortin receptors at 10 μM concentration.

Discussion

Stereochemical modifications of MSH peptides have previously been shown to increase affinity and/or sustained agonist activity.[10] In this study, a series of "active site" tripeptides and tetrapeptides were evaluated on human melanocortin receptors to identify prototypic leads for MSH-based peptidomimetic drug discovery. These tetrapeptides are based upon the message sequence of melanocortin peptides that consists of His-Phe-Arg-Trp (Table 1) and is common to all melanotropin peptides including ACTH. Since all of the melanocortin receptors recognize the melanotropin peptides (except MC2R), and contain positionally conserved residues in the putative binding pocket, it is postulated that the ligand message residues would interact with

Table 3. Binding and Intracellular cAMP Accumulation of Stereochemically Modified Melanotropin Tetra- and Tripeptides

peptide	structure	hMC1R		hMC3R		hMC4R		hMC5R	
		binding[a] IC_{50} (nM)	cAMP[b] EC_{50} (nM)	binding[a] IC_{50} (nM)	cAMP[b] EC_{50} (nM)	binding[a] IC_{50} (nM)	cAMP[b] EC_{50} (nM)	binding[a] IC_{50} (nM)	cAMP[b] EC_{50} (nM)
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	5.97 ± 0.33	ND	50.04 ± 10.1	ND	38.7 ± 1.44	ND	557 ± 198	ND
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.51 ± 0.11	0.19 ± 0.04	1.17 ± 0.14	4.10 ± 0.62	1.16 ± 0.09	0.72 ± 0.04	0.86 ± 0.04	0.58 ± 0.17
		IC_{50} (μM)	EC_{50} (μM)	IC_{50} (μM)	EC_{50} (μM)	IC_{50} (μM)	EC_{50} (μM)	IC_{50} (μM)	EC_{50} (μM)
1	Ac-His-DPhe-Arg-Trp-NH_2	0.615 ± 0.041	0.228 ± 0.088	>10		1.153 ± 0.209	0.793 ± 0.437	>10	
2	Ac-His-DPhe-DArg-DTrp-NH_2	>10		>10		>10		>10	
3	Ac-His-DPhe-DArg-Trp-NH_2	>10		>10		>10		>10	
4	Ac-His-Phe-Arg-DTrp-NH_2	6.347 ± 0.619	1.428 ± 0.207	>10		>10		>10	
5	Ac-Gly-DPhe-Arg-DTrp-NH_2	>10		>10		>10		>10	
6	Ac-DPhe-Arg-Trp-NH_2	>10		>10		2.081 ± 0.438	*40%	>10	
7	Ac-DPhe-Arg-DTrp-NH_2	>10		>10		9.157 ± 0.767	*50%	>10	
8	Ac-DPhe-DArg-DTrp-NH_2	>10		>10		>10		>10	
9	Ac-DPhe-DArg-Trp-NH_2	>10		>10		>10		>10	
10	Ac-Phe-Arg-DTrp-NH_2	>10		>10		>10		>10	
11	Ac-Phe-DArg-DTrp-NH_2	>10		>10		>10		>10	

[a] IC_{50} = concentration of peptide at 50% specific binding ($N = 4-6$). In cases where the peptide was unable to reach 50% specific binding, the percentage of bound ligand that was able to competitevily displace [^{125}I]NDP-MSH at 10 μM, >10 is listed. [b] EC_{50} = effective concentration of peptide at 50% maximal cAMP accumulation ($N = 4-6$). An asterisk (*) indicates that only partial intracellular accumulation was observed at a 10 μM peptide concentration. ND signifies that these values were not determined simultaneously in these experiments. These peptides were tested at a range of concentrations ($10^{-5}-10^{-11}$ M).

Figure 1. Binding dose−response curves of the analogues listed at the hMC4R. IC_{50} values were determined from the value at 50% of the total binding. The curves shown represent the average of duplicate experiments performed in triplicate. The errors associated with each compound is listed in Table 3.

Figure 2. Functional assay dose−response curves of the analogues listed at the hMC4R. The curves shown represent the average of two experiments performed in duplicate. The errors associated with each compound are listed in Table 3. The analogues Ac-DPhe-Arg-DTrp-NH_2 and Ac-DPhe-Arg-Trp-NH_2 were only able to generate partial intracellular cAMP accumulation at 10 μM, while the tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 was able to generate a maximal response.

the conserved receptor residues in a similar manner. Diastereomeric peptides containing D-Phe and/or Trp residues were examined since the all-L-tetrapeptide was only weakly potent, and the all-L-tripeptide Phe-Arg-Trp possessed no activity in either the frog or lizard skin bioassays.[4,5,9,10] N-Terminal acetylation and C-terminal amidation are present on all these analogues as they create the presence of two additional amide bonds that extend the backbone past the central residues, provide increased enzymatic stability, and extend putative ligand−receptor interactions.

At the hMC1R, which has been reported as only being expressed in the skin (dermal melanocytes and melanosomes),[12,13] analogue **4** (LPhe^7) was only 10-fold less potent than analogue **1** (DPhe^7). This result is especially interesting since DPhe^7-containing analogues have been found to increase potency significantly at the frog and lizard skin receptors.[18] Additionally, this particular tetrapeptide (**4**) was selective for the hMC1R in that only at this subtype did a dose−response competitive displacement binding of [^{125}I]NDP-MSH occurr. At a concentration of 10 μM, analogue **4** only bound 7%, 36%,

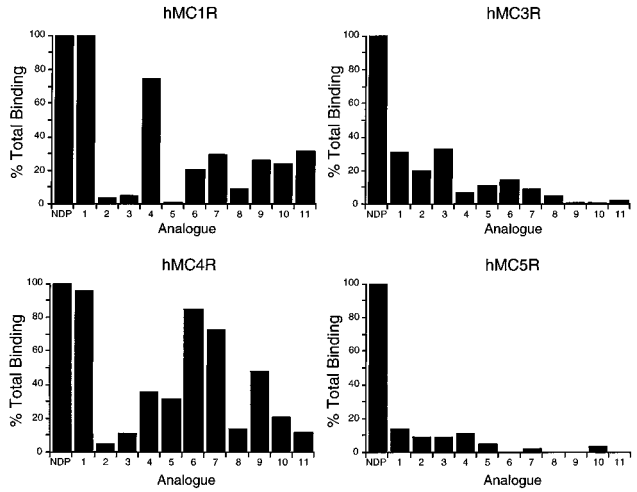

Figure 3. Summary of analogue binding to each human MCR subtype at 10 μM concentration. Only analogues possessing greater than 50% total binding were analyzed to determine IC_{50} values.

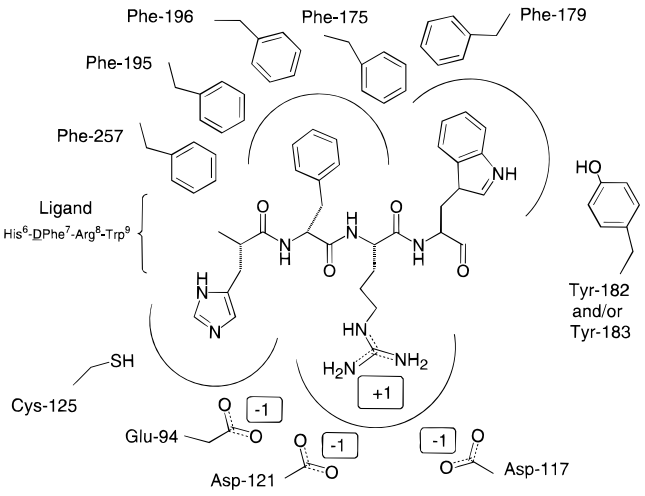

Figure 4. Schematic representation of the putative hMC1R residues interacting with the message sequence His-DPhe-Arg-Trp of NDP-MSH. These interactions are based on 3D homology molecular modeling of the hMC1R as previously described.[11] The specific hMC1 receptor residues are labeled and positioned in the same proximity as the ligand residues (center of figure) that they are proposed to interact with.

and 11% at the hMC3R, hMC4R, and hMC5R, respectively (Figure 3). This is a hMC1R selective ligand, albeit weakly potent, but nevertheless provides a structural template that may be modified to increase potency.

Tripeptides **6** (Ac-DPhe-Arg-Trp-NH_2) and **7** (Ac-DPhe-Arg-DTrp-NH_2) only bind to the hMC4R at up to 10 μM. These peptides also possessed micromolar activity on the frog skin melanocortin receptor (Table 2). Recently, the physiological role of MC4R has been unequivocally determined to be involved in feeding and obesity.[18,19] Antagonists of this receptor have been previously reported and include the *agouti* protein[20,21] and the peptide Ac-Nle-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-NH_2 (SHU-9119).[22] The two tripeptides **6** and **7** are strong candidates for focused structure-based drug design to generate non-peptide-related molecules with enhanced potencies.

Removal of His^6 from analogue **1** (Ac-His-DPhe-Arg-Trp-NH_2) afforded analogue **6** (Ac-DPhe-Arg-Trp-NH_2), which was only able to bind to the hMC1R at 20% of the total binding at a concentration of 10 μM (Figure 3). The absence of His^6 residue resulted in 150-fold decreased potency compared to the presence of this residue in the tetrapeptide examined on the frog skin MCR.[10] This previous data is consistent with current observations. These data clearly illustrate the importance of the His^6 residue at the skin melanocortin receptor (hMC1R). At the hMC4R, however, the absence of His^6 in analogue **6** resulted in only a 2-fold decrease in potency. This suggests that the His^6 residue is not critical for binding to the hMC4R. Interestingly, none of the tetrapeptides examined in this study were able to competitively displace 50% of the total binding of [^{125}I]NDP-MSH to the hMC3R and hMC5R. This result was unexpected as these message residues, and in particular the stereochemical combination of His-DPhe-Arg-Trp (derived from NDP-MSH), were hypothesized to bind similarly to all the melanocortin receptors, except the MC2R which only is activated by ACTH.

Three-dimensional homology molecular modeling of the hMC1R predicted specific ligand−receptor interactions of the DPhe-Arg-Trp NDP-MSH tripeptide residues.[11,23] With the exception of the hMC2R, NDP-MSH was observed to bind to all the human melanocortin receptors with nanomolar affinities and functional activities. Figure 4 summarizes these putative interactions of His-DPhe-Arg-Trp. All of these particular melanocortin receptor residues which are proposed to interact with the His-DPhe-Arg-Trp (ligand) residues are positionally conserved (based upon the "Baldwin" alignment)[24] with the notable exceptions of F175 and Y183, which are only present in the hMC1R. The F195, F196, L197 "motif" of the hMC1R is also shifted by one residue to give the homologous three-residue positional combination of MFF in the hMC3R, hMC4R, and hMC5R (see box in Table 4). An original premise of such receptor homology modeling was that since the central sequence His-Phe-Arg-Trp is conserved in all the naturally occurring melanocortin peptides (Table 1), and since NDP-MSH possessed nanomolar affinities and efficacies at all the cloned melanocortin receptors studied to date, the His-DPhe-Arg-Trp sequence would interact with all the melanocortin subtypes in similar binding modes. Thus, the Ac-His-DPhe-Arg-Trp-NH_2 (**1**) peptide was predicted to possess similar interactions with these receptors. However, these predictions appear to be incorrect as based on the data presented in this study. Receptor homology between the hMCR family is only about 50−60%, which is considered low homology for a GPCR "family". Interestingly, however, tetrapeptide **1** was able to competitively displace [^{125}I]NDP-MSH only at the hMC1R and hMC4R (Table 3). Evaluating the hMC3R and hMC5R primary structure (Table 4), however, revealed significant residue changes in the putative binding pocket and neighboring the proposed specific receptor residues which are proposed to interact with tetrapeptide **1** (Figure 4). These residue functional changes are indicated by arrows and involve changes in hydrophobicity and/or electrostatic properties which may modify the local binding environment.

Two general theories to explain general mechanisms of signal transduction include conformational induction "which involves a receptor conformation never found in the absence of agonist" and conformational selection which "involves a choice from a library of conforma-

Table 4. Sequence Alignment of the Human Melanocortin Receptors Examined in This Study[a]

		N-terminal	
hMC1R		MAVQGSQRRLLGSLNSTPTAIPQLGLAANQTGARCLEV	
hMC4R		MVNSTHRGMHTSLHLWNRSSYRLHSNASESLGKGYSDGGCYEQL	
hMC3R		MSIQKKYLEGDFVFPVSSSSFLRTLLEPQLGSALLTAMNASCCLPSVQPTLPNGSEHLQAPFFSNQSSSAFCEQV	
hMC5R		MNSSFHLHFLDLNLNATEGNLSGPNVKNKSSPCEDM	
		TM1	**loop 1**
hMC1R	39	SISDGLFLSL GLVSLVENAL VVATIA	KNRNLH
hMC4R	45	FVSPEVFVTL GVISLLENIL VIVAIA	KNKNLH
hMC3R	76	FIKPEIFLSL GIVSLLENIL VILAVV	RNGNLH
hMC5R	37	GIAVEVFLTL GVISLLENIL VIGAIV	KNKNLH
		TM2	**loop 2**
hMC1R	71	SPMYCFICCL ALSDLLVSGT NVL**E**TA	VILLLEAGALVARAAVLQQL
hMC4R	77	SPMYFFICSL AVADMLVSVS NGS**E**TI	IITLLNSTDTDAQSFTVNI
hMC3R	108	SPMYFFLCSL AVADMLVSVS NAL**E**TI	MIAIVHSDYLTFEDQFIQHM
hMC5R	69	SPMYFFVCSL AVADMLVSMS SAW**E**TI	TIYLLNNKHLVIADAFVRHI
		TM3	**loop 3**
hMC1R	117	**D**NVI**D**VIT**C**S SMLSSLCFLG AIAVDRY	ISIFYALRYHSIVTL
hMC4R	122	**D**NVI**D**SVI**C**S SLLASICSLL SIAVDRY	FTIFYALQYHNIMTV
hMC3R	154	**D**NIF**D**SMI**C**I SLVASICNLL AIAVDRY	VTIFYALRYHSIMTV
hMC5R	115	**D**NVF**D**SMI**C**I SVVASMCSLL AIAVDRY	VTIFYALRYHHIMTA
		↑ ↑ ↑	
		TM4	**loop 4**
hMC1R	159	PRARQAVAAI WVASVV**F**STL **F**IA**Y**	YDHVAV
hMC4R	164	KRVGIIISCI WAACTVSGIL **F**II**Y**	SDSSAV
hMC3R	196	RKALTLIVAI WVCCGVCGVV **F**IV**Y**	SESKMV
hMC5R	157	RRSGAIIAGI WAFCTGCGIV **F**IL**Y**	SESTYV
		↑	
		TM5	**loop 5**
hMC1R	189	LLCLVV**FF**LA MLVLMAVLYV HML	ARACQHAQGIARLHKRQRPVHQGFGLKG
hMC4R	194	IICLIT**MF**FT MLALMASLYV HMF	LMARLHIKRIAVLPGTGAIRQGANMKG
hMC3R	226	IVCLIT**MF**FA MMLLMGTLYV HMF	LFARLHVKRIAALPPADGVAPQQHSCMKG
hMC5R	187	ILCLIS**MF**FA MLFLLVSLYI HMF	LLARTHVKRIALPGASSARQRTSMQG
		TM6	**loop 6**
hMC1R	240	AVTLTILLGI FFLCWGP**FF**L HLTLIVL	CPEHPTCGCIFK
hMC4R	244	AITLTILIGV FVVCWAP**FF**L HLIFYIS	CPQNPYCVCFMS
hMC3R	278	AVTITILLGV FIFCWAP**FF**L HLVLIIT	CPTNPYCICYTA
hMC5R	237	AVTVTMLLGV FTVCWAP**FF**L HLTLMLS	CPQNLYCSRFMS
		TM7	**C-terminal**
hMC1R	279	NFNLFLALII CNAIIDPLIY AFHSQ	ELRRTLKEVLTCSW
hMC4R	283	HFNLYLILIM CNSIIDPLIY ALRSQ	ELRKTFKEIICCYPLGGLCDLSSRY
hMC3R	317	HFNTYLVLIM CNSVIDPLIY AFRSL	ELRNTFREILCGCNGMNLG
hMC5R	276	HFNMYLILIM CNSVMDPLIY AFRSQ	EMRKTFKEIICCRGFRIACSFPRRD

[a] Residues highlighted in bold are proposed to directly interact with the His-DPhe-Arg-Trp ligand residues. The arrows represent functional residue changes which may affect ligand−receptor interactions and differentiate the pharmacological discrepancies observed between the two groups of the hMC1R, hMC4R and the hMC3R, hMC5R. The residues indicated in the box show the sequence shift of the FF residues of the hMC1R compared with the other receptors. The transmembrane domains were determined according to the "Baldwin" alignment.[24]

tions".[25−27] The latter theory is also referred to as the "ternary complex, or two-state" model (see refs 28 and 29 and references therein). In this model, the receptor is in differential populations between an "inactive" conformation, and an "active" conformation, with the latter state coupled to a G-protein in the absence of ligand. Schwartz et al. have proposed that for some GPCRs, the role of the ligand is to stabilize a particular receptor population.[29−31] Interestingly, at the hMC4R, analogue **6** (Ac-DPhe-Arg-Trp-NH_2) was only able to generate 40% of the intracellular cAMP accumulation as that of the His^6-containing peptide (Ac-His-DPhe-Arg-Trp-NH_2, analogue **1**), at the highest concentration tested. Nevertheless, from the dose−response curve (Figure 2) of analogue **6**, it seems probable that at yet higher concentrations the cAMP levels may be increased. However, the fact remains that these two analogues exhibit a difference in binding affinities of

only 2-fold, thus implicating a critical role of His^6 in ligand−receptor signaling (adenylate cyclase activation) but not binding. We speculate that the molecular interactions of the His-DPhe-Arg-Trp residues with the hMC4R are key for the aforementioned "conformational induction" mechanism of signal transduction.

Conclusions

This study reveals that the central tetrapeptide (Ac-His-DPhe-Arg-Trp-NH_2) of NDP-MSH does not possess similar receptor binding affinities and functional activities as previously predicted for the melanocortin receptor subtypes (hMC1R, hMC3R, hMC4R, and hMC5R). We have identified Ac-His-Phe-Arg-DTrp-NH_2 as a lead analogue to advance the discovery of a selective hMC1R peptidomimetic agonist. Two tripeptides possessing agonist activities at the hMC4R provide lead analogues for further structure-based drug design studies focused at this melanocortin receptor subtype and for studies involving the regulation of feeding and obesity. Furthermore, the importance of the His^6 residue for recognition at the hMC1R and for signal transduction at the hMC4R has been established.

Experimental Section

Peptide Chemical Synthesis and Characterization. These tri- and tetrapeptides were synthesized and characterized as described in a previous study.[10]

Binding Assays. The coding region of the human melanocortin receptors 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 as previously described.[14,16,32] Transfected cells (0.6×10^6 cells/well) were grown to confluence in 12-well (2.4×1.7 cm) tissue culture plates. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 4.5 g/100 mL of glucose, 10% fetal calf serum, 100 units/mL of penicillin and streptomycin, 1 mM sodium pyruvate, and 1 mg/mL of Geneticin. For the assays, this medium was removed, and cells were washed twice with a freshly prepared binding buffer consisting of minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 1% bovine serum albumin, and 200 mg/L bacitracin. A 450 μL solution of the peptide concentration being tested was added to the well, with the concentrations ranging between 10^{-11} and 10^{-5} M. Next, a 50 μL solution of [^{125}I]NDP-MSH (100 000 cpm/well) was added to each well, and the cells were incubated at 37 °C for 1 h. The medium was subsequently removed, and each well was washed twice with assay buffer. The cells were lysed by the addition of 0.5 mL of 0.1 M NaOH and 0.5 mL of 1% Triton X-100. The mixture was left to lyse the cells for 10 min, and the contents of each well were transferred to labeled 16×150-mm glass tubes and quantified in a γ-counter. [^{125}I]-NDP-MSH was prepared and purified by methods described previously.[33]

cAMP Assays. A commercially available cAMP assay kit (TRK 432, Amersham Corp.) was employed. L-cells transfected with the human melanocortin receptors were grown to confluence in 12-well (2.4×1.7 cm) tissue culture plates. The cells were maintained in Dulbecco's modified Eagles's medium (DMEM, GIBCO) containing 4.5 g/100 mL of glucose, 10% fetal calf serum, 100 units/mL of penicillin and streptomycin, 1 mM sodium pyruvate, and 1 mg/mL of 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 2×10^{-2} M isobutylmethylxanthine. Varying concentrations of melanotropins were added, and the cells were incubated for 1 h at 37 °C. Ice-cold 100% ethanol (0.5 mL/well) was added to stop the reaction. The incubation medium and scraped cells were transferred to 16×150-mm glass tubes and then placed at 4 °C for at least 30 min. The precipitate was then centrifuged for 10 min, and 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.

Data Analysis. IC_{50} and EC_{50} values represent the mean of duplicate experiments performed in triplicate. IC_{50} estimates, EC_{50} estimates, and their associated standard errors were determined by fitting the data using nonlinear least-squares analysis.[34] The results are not corrected for peptide content.

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