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Structure–Activity Relationship of Linear Peptide Bu-His-DPhe-Arg-Trp-Gly-NH_2 at the Human Melanocortin-1 and -4 Receptors: Arginine Substitution

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Abstract—A series of pentapeptides, based on Bu-His^6-DPhe^7-Arg^8-Trp^9-Gly^{10}-NH_2 and modified at the Arg^8 position, was prepared and pharmacologically characterized. Peptides containing either cyanoguanidine or acylguanidine, two substantially less basic arginine surrogates, were found to retain the agonist activity of the parent peptide at both hMC1R and hMC4R. This study unequivocally shows that the positive charge of Arg^8 is not essential for efficient interactions of our pentapeptide with both hMC1R and hMC4R.

In the last decade, five human melanocortin receptor subtypes (hMC1R-hMC5R) have been cloned and characterized.[1] The melanocortin receptors belong to the superfamily of G-protein coupled receptors (GPCRs) mediating a wide range of physiological functions: pigmentation (MC1R), glucocorticoid production (MC2R), food intake and energy expenditure (MC3R and MC4R) as well as exocrine gland function (MC5R).[1] Our laboratories are interested in the identification of potent and selective human melanocortin-4 receptor (hMC4R) agonists for the treatment of obesity.

Adopting a ligand based approach, we first carried out a truncation study of NDP-MSH (Ac-Ser^1-Tyr^2-Ser^3-Nle^4-Glu^5-His^6-DPhe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2),[2] in a manner similar to that reported by Hruby et al.,[3] which led to the identification of pentapeptide **1** (Bu-His^6-DPhe^7-Arg^8-Trp^9-Gly^{10}-NH_2, α-MSH numbering) as a potent hMC4R agonist (EC_{50} = 20 nM), selective against hMC3R (no agonist activity at 50 μM) and hMC5R (no agonist activity at 50 μM) but non-selective against hMC1R (EC_{50} = 10 nM).[4] In an extensive structure–activity relationship (SAR) study of pentapeptide **1**, we systematically replaced each of the five amino acids of peptide **1** by other coding or non-coding amino acids in an effort to dial out hMC1R activity and to maintain or improve hMC4R activity. This report summarizes our effort in replacing the Arg^8 residue of pentapeptide **1**.

At the initiation of this study, the reported SAR of Arg^8 substitution in melanotropin derivatives was obtained from either amphibian skin, reptilian skin or mammalian melanoma cell assays (mediated by MC1R).[5] For example, Hruby et al. substituted Arg in Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2 with Nle and observed a 1400-fold drop in potency in the frog skin assay and a 4500-fold drop in potency in the lizard skin assay.[6] In a later study, Hruby et al. replaced Arg in Ac-His-DPhe-Arg-DTrp-NH_2 with Lys, Gln, Ala, and Gly, and the resulting peptides suffered a drop of 2- to 43-fold in EC_{50} values compared to the parent in a frog skin assay.[3] Pouton et al. carried out an Ala scan of α-MSH and found that replacement of Arg^8 with Ala led to a 100-fold reduction in EC_{50} value in a mouse melanoma tyrosinase assay.[7] These results clearly demonstrate the importance of Arg^8 in the activation of MC1R by Arg^8-containing melanotropin derivatives in various species. No information about the influence of Arg^8 substitution on hMC4R agonist activity was available at the start of this study.

It is widely accepted that analogues containing highly basic guanidine functionality ($pK_a \sim 14$)[8] possess poor

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oral bioavailability and blood brain barrier penetration. Since our target hMC4R is centrally located, the main goals of our arginine substitution study were to discover novel arginine mimetics with reduced basicity and to identify any selectivity pocket which might be present in the arginine binding site.

All new peptides (**1**–**11**) and NDP-MSH were synthesized on solid phase from suitably protected amino acids using standard Fmoc and Boc methodology.[9] The crude peptides were purified to homogeneity using reversed-phase HPLC and characterized by fast atom bombardment mass spectroscopy.

Peptide α-MSH; amino acids Fmoc-Arg(PMC)-OH, Fmoc-Cit-OH, Fmoc-His(Trt)-OH, Fmoc-Orn(Boc)-OH, Fmoc-Lys(Boc)(*i*Pr)-OH and Fmoc-Arg(NO_2)-OH were purchased from commercial sources. Boc-Orn(CH_3)$_2$-OH,[10] Boc-Lys(CH_3)$_2$-OH[10] and Boc-Arg(CN)-OH[11] were prepared according to literature procedures. Boc-Lys(Fmoc)(Bn)-OH was prepared by mono-benzylation of Boc-Lys-OH followed by Fmoc protection of the side chain secondary amine.[12]

The acylguanidine analogue **11** was prepared as follows: pentapeptide Bu-His(Trt)-DPhe-Glu(Oall)-Trp-Gly-resin was prepared on solid phase using standard Fmoc methodology. With the peptide still on resin, the allyl ester of Glu was removed using $PdCl_2$, PPh_3, and $(Bu_3Sn)_2O$. The free side chain carboxylic acid of Glu was then coupled with BocNH-C(=NH)-NH_2.HCl[13] using HBTU as the coupling agent. Trifluoroacetic acid treatment released the peptide from the resin and removed all side chain protecting groups.

The agonist assays were performed using HEK293 cells transfected with hMC1R or hMC4R as reported in detail elsewhere.[9,14] The EC_{50} values reported in Table 1 are the average of at least two separate experiments.

The agonist potencies (EC_{50} values) of the peptide analogues towards hMC1R and hMC4R are summarized in Table 1. The lead pentapeptide **1** (Bu-His-DPhe-Arg-Trp-Gly-NH_2) is a potent hMC4R agonist (EC_{50} = 20 nM) but non-selective against hMC1R (EC_{50} = 10 nM). For comparison purpose, known agonists α-MSH and NDP-MSH were determined in our assays to have EC_{50} values of 0.8 nM and 0.5 nM at hMC1R and 25 nM and 1 nM at hMC4R, respectively. When Arg in peptide **1** was replaced with sterically similar but non-basic Cit (see Fig. 1), the resulting peptide **2** showed a 38-fold drop in potency at hMC4R and a 95-fold decreased potency at hMC1R, compared with peptide **1**. This significant drop (well beyond the two fold experimental error) in agonist potency of peptide **2** could be due to the lack of a positive charge in Cit and/or that both of the terminal NHs of Arg are involved in interacting with the receptors (vide infra). Peptide **3**, containing His,[15] was 70-fold less potent at hMC4R and 170-fold less potent at hMC1R,

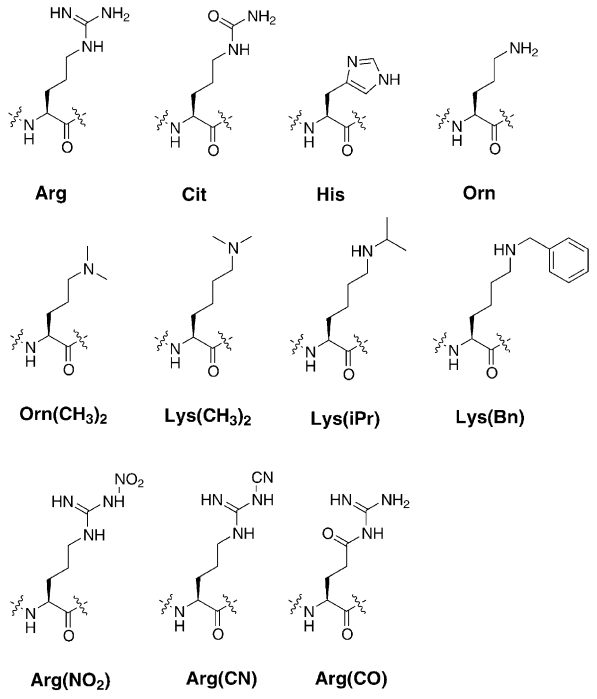

Figure 1. Structures of amino acids.

Table 1. Agonist activity of the Arg[8] modified pentapeptides at the human melanocortin receptors

Peptide	Amino acid sequence	hMC4R EC_{50} (nM)[a]	hMC1R EC_{50} (nM)[a]
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	25	0.8
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	1	0.5
1	Bu-His-DPhe-**Arg**-Trp-Gly-NH_2	20	10
2	Bu-His-DPhe-**Cit**-Trp-Gly-NH_2	760	950
3	Bu-His-DPhe-**His**-Trp-Gly-NH_2	1420	1700
4	Bu-His-DPhe-**Orn**-Trp-Gly-NH_2	2500	900
5	Bu-His-DPhe-**Orn(CH_3)$_2$**-Trp-Gly-NH_2	3280	350
6	Bu-His-DPhe-**Lys(CH_3)$_2$**-Trp-Gly-NH_2	1670	60
7	Bu-His-DPhe-**Lys(iPr)**-Trp-Gly-NH_2	25% @ 50 μM	255
8	Bu-His-DPhe-**Lys(Bn)**-Trp-Gly-NH_2	4800	85
9	Bu-His-DPhe-**Arg(NO_2)**-Trp-Gly-NH_2	85% @ 50 μM	40% @ 50 μM
10	Bu-His-DPhe-**Arg(CN)**-Trp-Gly-NH_2	70	15
11	Bu-His-DPhe-**Arg(CO)**-Trp-Gly-NH_2	10	1

[a]Concentration of peptide at 50% maximum cAMP accumulation or the % of cAMP accumulation (relative to NDP-MSH) observed at the highest peptide concentration tested.

compared with peptide **1**. A follow-up study using different modified His with different chain length and point of attachment of the imidazole side chain might give peptides of improved potencies or selectivity.

Replacement of Arg with Orn (peptide **4**) led to a 125-fold loss at hMC4R and a 90-fold reduction at hMC1R potency, compared with peptide **1**. *N,N*-Dimethylation of Orn (peptide **5**) did not significantly change the agonist potencies at both receptors, compared with peptide **4**. The three pentapeptides containing *N*-alkylated Lys (peptides **6**–**8**) all showed modest selectivity towards hMC1R over hMC4R, with peptide **7** showing only 25% activation at 50 μM at hMC4R.

Another strategy of lowering the basicity of arginine involves the introduction of electron withdrawing groups into or adjacent to the guanidino group. Nitro- and cyano- groups are known to lower the pK_a of guanidine by 14 units.[16,17] Peptide **9** containing nitroguanidine was inactive at both hMC1R and hMC4R. On the other hand, peptide **10** containing cyanoguanidine retains good potency at both hMC4R (70 nM) and hMC1R (15 nM). Acylguanidine,[18] with p$K_a \sim 7.6$, was incorporated into peptide **11** which is roughly equipotent to peptide **1** as a hMC4R agonist (within the two fold experimental error) but is 10-fold more potent than peptide **1** as a hMC1R agonist. The observed improvement in agonist activity of peptide **11** at hMC1R might be due to intramolecular H-bonding interaction between the acylguanidine carbonyl and other H-bond donor of the peptide (e.g., terminal NH_2 of the acylguanidine group) thus rigidifying the peptide or it might be due to a favorable interaction between the acylguanidine carbonyl and the receptors. The agonist activities of peptide **10** and **11**, taken together with that of peptide **2**, suggest that the two terminal NHs and **not** the positive charge of Arg^8 is essential for efficient interactions of our pentapeptide with both hMC1R and hMC4R.

In recent years, substitution of Arg^8 with Lys, Ala, Pro, Glu, (2)Nal and Nle in various cyclic/linear peptides has been reported by different laboratories,[19–24] generally resulting in decreased peptide potency/affinity for the cloned melanocortin receptors. Cyanoguanidine and acylguanidine, identified in this study, are the first two arginine mimetics to replace Arg^8 in a melanotropin derivative and retain the efficacy in both hMC1R and hMC4R.

Based on homology modeling of hMC1R and site-directed mutagenesis data,[25,26] it was proposed that Arg^8 of NDP-MSH interacts with a hydrophilic binding pocket composed of several negatively charged residues in the transmembrane region of the receptor. A recent study examined binding affinities and potencies of substituted NDP-MSH at receptor mutants of hMC4R (generated by site-directed mutagenesis) and Arg^8 of NDP-MSH was hypothesized to interact at a molecular level with hMC4R residue D122 in the third transmembrane region.[24] The results of this study (using linear peptides) and that of Bednarek et al. (using cyclic peptides)[20,22] clearly demonstrate that ionic interaction between Arg^8 residue of melanotropin ligands with hMC1R and hMC4R is relatively **unimportant** for receptor activation. The above two models of hMC1R and hMC4R were constructed without extensive SAR knowledge of Arg^8 and studying peptides containing arginine mimetics identified in this study at the above receptor mutants should provide further insight into the molecular interactions between ligand and receptor.

In summary, a study was carried out in which Arg^8 in a linear pentapeptide was replaced by 10 arginine surrogates and peptides containing cyanoguanidine (**10**, p$K_a \sim 0$) or acylguanidine (**11**, p$K_a \sim 7$), two substantially less basic arginine surrogates, were found to retain the agonist activity of the parent peptide (**1**) at both hMC1R and hMC4R. This study unequivocally shows that the positive charge of Arg^8 is not essential for efficient interactions of pentapeptide Bu-His-DPhe-Arg-Trp-Gly-NH_2 with both hMC1R and hMC4R. Use of the two newly discovered arginine mimetics as the anchor or needle for *N*-terminal and *C*-terminal exploration should be a promising approach for identifying low molecular weight, peptidomimetic melanocortin receptor agonists or antagonists; this strategy has previously been successfully used in the discovery of thrombin and factor Xa inhibitors.[27,28] It should also be interesting and informative to study the pharmacological effect of replacing Arg^8 with cyanoguanidine and acylguanidine moieties in Arg^8-containing melanocortin peptide agonists and antagonists known in the literature (e.g., MTII, SHU9119, HS014, and HP228 etc.).[29]

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