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Structure–Activity Relationship of Cyclic Peptide Penta-c[Asp-His^6-DPhe^7-Arg^8-Trp^9-Lys]-NH_2 at the Human Melanocortin-1 and -4 Receptors: His^6 Substitution

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Abstract—A series of MT-II related cyclic peptides, based on potent but non-selective hMC4R agonist (Penta-c[Asp-His^6-DPhe^7-Arg^8-Trp^9-Lys]-NH_2) was prepared in which His^6 residue was systematically substituted. Two of the most interesting peptides identified in this study are Penta-c[Asp-**5-ClAtc**-DPhe-Arg-Trp-Lys]-NH_2 and Penta-c[Asp-**5-ClAtc**-DPhe-**Cit**-Trp-Lys]-NH_2 which are potent hMC4R agonists and are either inactive or weak partial agonists (not tested for their antagonist activities) in hMC1R, hMC3R and hMC5R agonist assays.

In the last decade, five human melanocortin receptor subtypes (hMC1R-hMC5R) have been cloned and characterized.[1] The melanocortin receptors are G-protein coupled receptors (GPCRs) which mediate a wide range of physiological functions including pigmentation (MC1R), glucocorticoid production (MC2R), food intake and energy expenditure (MC3R and MC4R) as well as exocrine gland function (MC5R).[1] Recently, it was suggested that MC4R also plays a role in sexual function.[2] To better understand the physiological functions of the melanocortin receptors in different animal species, potent and highly subtype selective agonists and/or antagonists are required and the identification of these pharmacological agents remains one of the key challenges in the melanocortin field.

As previously reported, by using 5-substituted Atc (2-aminotetraline-2-carboxylic acid) amino acids as histidine surrogate, we identified linear pentapeptide **1** (Penta-5-BrAtc^6-DPhe^7-Arg^8-Trp^9-Gly^{10}-NH_2, α-MSH numbering) as a potent hMC4R agonist (EC_{50} = 35 nM), selective against hMC1R (50% activation at 50 μM), hMC3R (no agonist activity at 50 μM) and hMC5R (no agonist activity at 50 μM).[3] Due to the highly flexible nature of a linear peptide, we were unable to determine the bioactive conformation(s) of peptide **1**. In an attempt to restrict the number of possible conformations, we used potent but non-selective MT-II[4] analogue **2** (Fig. 1, Penta-c[Asp-His^6-DPhe^7-Arg^8-Trp^9-Lys]-NH_2) as the template for an extensive structure–activity relationship (SAR) study. This report summarizes our initial effort in replacing His^6 residue of peptide **2**.

All new cyclic peptides reported in this study were synthesized as follows: amino acids Fmoc-Lys(Boc); Fmoc-Trp; Fmoc-Arg(Pmc); Fmoc-DPhe; Fmoc-His(Trt) or other Fmoc protected histidine surrogates and Fmoc-Asp(O*t*Bu) were sequentially coupled onto NH_2-Rink resin using standard Fmoc methodology and HBTU activation.[5] After N-capping with valeric anhydride, trifluoroacetic acid treatment of the resin released the linear peptide as a primary amide at the C-terminus and also removed the side-chain protecting groups of Asp and Lys. The crude linear peptide in DMF was then cyclized between the unprotected side chains of Asp and Lys using BOP coupling. The resulting cyclic peptide was purified to homogeneity using reversed-phase HPLC and characterized by fast atom bombardment mass spectroscopy. Fmoc-protected substituted Atc amino acids were prepared as described previously[3] and other amino acids used were purchased from commercial sources (Fig. 2).

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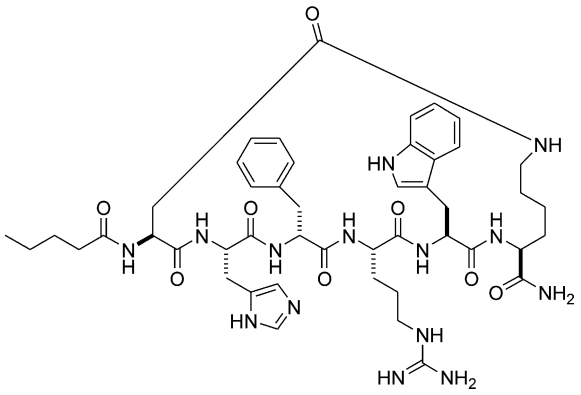

Figure 1. Structure of peptide **2** (Penta-c[Asp-His[6]-DPhe[7]-Arg[8]-Trp[9]-Lys]-NH_2).

Agonist assays were performed using HEK293 cells transfected with hMC1R-hMC5R as reported in detail elsewhere.[5,6] The EC_{50} values reported in Tables 1–4 are the average of at least two separate experiments. Binding assays were performed using radiolabeled NDP-MSH as reported in detail elsewhere.[6] The IC_{50} values reported in Table 4 are the average of at least two separate experiments.

As shown in Table 1, MT-II[4] (Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2) is a potent hMC4R agonist ($EC_{50} = 0.6$ nM) but is not selective against hMC1R ($EC_{50} = 0.6$ nM). Substitution of His in MT-II with Ala, carried out in our laboratories (data not shown) and previously reported by Bednarek et al.[8] gave a peptide which retained most of its hMC1R and hMC4R agonist activities, compared to MT-II. Replacement of His in MT-II with Gln gave peptide **3** which is similar to MT-II (the standard error in our assays is about 2-fold) in both hMC1R and hMC4R agonist potencies. The agonist activities of the above [Ala[6]]- and [Gln[6]]-MT-II analogues indicate that the basic imidazole side chain of His is not required for MT-II's activation of hMC1R and hMC4R.

When N-cap (Ac-Nle) of MT-II was replaced with a *n*-pentanoyl group, the resulting peptide **2** is essentially

Table 1. Agonist activity of various cyclic peptides at the human melanocortin receptors

Peptide	Amino acid sequence	hMC4R EC_{50} (nM)[a]	hMC1R EC_{50} (nM)[a]
MT-II	Ac-Nle-c[Asp-**His**-DPhe-Arg-Trp-Lys]-NH_2[b]	0.6	0.6
3	Ac-Nle-c[Asp-**Gln**-DPhe-Arg-Trp-Lys]-NH_2	0.3	0.3
2	Penta-c[Asp-**His**-DPhe-Arg-Trp-Lys]-NH_2[b]	0.9	0.4
4	Penta-c[Asp-**4-CF_3Phe**-DPhe-Arg-Trp-Lys]-NH_2	2	12
5	Penta-c[Asp-**Bip**-DPhe-Arg-Trp-Lys]-NH_2	6	53
6	Penta-c[Asp-**Tic**-DPhe-Arg-Trp-Lys]-NH_2	3	5
7	Penta-c[Asp-**Disc**-DPhe-Arg-Trp-Lys]-NH_2 (1st isomer)[c]	2	3
8	Penta-c[Asp-**Disc**-DPhe-Arg-Trp-Lys]-NH_2 (2nd isomer)[c]	118	56
9	Penta-c[Asp-**Atc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	65	900
10	Penta-c[Asp-**Aic**-DPhe-Arg-Trp-Lys]-NH_2	65	370

[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.
[b]Ac stands for $CH_3C(=O)$, Penta stands for $CH_3CH_2CH_2CH_2C(=O)$.
[c]1st isomer and 2nd isomer refer to the order in which the two diastereomers eluted under our HPLC conditions.[7]
[d]Tested as a 1:1 mixture of diastereomers.

Table 2. Agonist activity of Atc containing cyclic peptides at the human melanocortin receptors

Peptide	Amino acid sequence	hMC4R EC_{50} (nM)[a]	hMC1R EC_{50} (nM)[a]
11	Penta-c[Asp-**5-BrAtc**-DPhe-Arg-Trp-Lys]-NH_2 (1st isomer)[b,c]	41	70% @ 50 μM[e]
12	Penta-c[Asp-**5-BrAtc**-DPhe-Arg-Trp-Lys]-NH_2 (2nd isomer)[c]	15	80% @ 50 μM[e]
13	Penta-c[Asp-**6-BrAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	37	60% @ 50 μM[e]
14	Penta-c[Asp-**7-BrAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	10	140
15	Penta-c[Asp-**8-BrAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	176	60% @ 50 μM[e]
16	Penta-c[Asp-**5-ClAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	9	30% @ 50 μM[e]
17	Penta-c[Asp-**6-ClAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	33	50% @ 50 μM[e]
18	Penta-c[Asp-**5-MeOAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	5	735
19	Penta-c[Asp-**5-EtOAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	35	1275
20	Penta-c[Asp-**5-iPrOAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	7	1330
21	Penta-c[Asp-**5-MeAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	25	80% @ 50 μM[e]
22	Penta-c[Asp-**5-EtAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	8	420
23	Penta-c[Asp-**5-iPrAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	7	480
24	Penta-c[Asp-**5-Me_2NAtc**-DPhe-Arg-Trp-Lys]-NH_2 (mixture)[d]	45	480

[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.
[b]Penta stands for $CH_3CH_2CH_2CH_2C(=O)$.
[c]1st isomer and 2nd isomer refer to the order in which the two diastereomers eluted under our HPLC conditions.[7]
[d]Tested as a 1:1 mixture of diastereomers.
[e]Not tested for antagonist activities.

Table 3. Agonist activity of various cyclic peptides at the human melanocortin receptors

Peptide	Amino acid sequence	hMC1R EC_{50} (nM)[a]	hMC3R EC_{50} (nM)[a]	hMC4R EC_{50} (nM)[a]	hMC5R EC_{50} (nM)[a]
MT-II	Ac-Nle-c[Asp-**His**-DPhe-Arg-Trp-Lys]-NH_2	0.6	25	0.6	25
2	Penta-c[Asp-**His**-DPhe-Arg-Trp-Lys]-NH_2	0.4	6	0.9	33
25	Penta-c[Asp-**5-ClAtc**-DPhe-Arg-Trp-Lys]-NH_2 (1st isomer)[c]	60% @ 50 μM[b]	0% @ 50 μM[b]	40	38% @ 50 μM[b]
26	Penta-c[Asp-**5-ClAtc**-DPhe-Arg-Trp-Lys]-NH_2 (2nd isomer)[c]	20% @ 50 μM[b]	25% @ 50 μM[b]	4	75% @ 50 μM[b]
27	Penta-c[Asp-**5-ClAtc**-DPhe-**Cit**-Trp-Lys]-NH_2 (1st isomer)[c]	0% @ 50 μM[b]	0% @ 50 μM[b]	105	0% @ 50 μM[b]
28	Penta-c[Asp-**5-ClAtc**-DPhe-**Cit**-Trp-Lys]-NH_2 (2nd isomer)[c]	0% @ 50 μM[b]	0% @ 50 μM[b]	48	0% @ 50 μM[b]

[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.
[b]Not tested for antagonist activities.
[c]1st isomer and 2nd isomer refer to the order in which the two diastereomers eluted under our HPLC conditions.[7]

Table 4. Binding and agonist activities of various cyclic peptides at the human melanocortin receptors

Peptide	hMC1R IC_{50} (nM)[a]	hMC1R EC_{50} (nM)[b]	hMC4R IC_{50} (nM)[a]	hMC4R EC_{50} (nM)[b]
MT-II	1	0.6	2	0.6
25	Not determined	60% @ 50 μM[c]	Not determined	40
26	5815	20% @ 50 μM[c]	15	4
27	14,790	0% @ 50 μM[c]	675	105
28	13,090	0% @ 50 μM[c]	410	48

[a]Concentration of peptide at 50% radiolabeled NDP-MSH displacement.
[b]Concentration of peptide at 50% maximum cAMP accumulation or the % of cAMP accumulation (relative to NDP-MSH) observed at the highest peptide concentration tested.
[c]Not tested for antagonist activities.

equipotent to MT-II in both hMC1R and hMC4R agonist assays. Our results with peptide **2** are consistent with those of Bednarek et al. who reported that substitution of Ac-Nle in MT-II with a *n*-hexanoyl group led to no change in hMC4R binding and agonist activities (its effect on hMC1R binding and agonist activities were not reported).[9] Peptide **2** was chosen as the template in which its His residue was systematically replaced by a number of coding or non-coding amino acids in an effort to dial out hMC1R activity and/or to improve hMC4R activity.

Replacement of His in peptide **2** with 4-CF_3Phe or Bip (Fig. 1) gave peptides **4** and **5** which are both potent hMC4R agonists (EC_{50} = 2 and 6 nM, respectively) and slightly selective against hMC1R (6- and 7-fold, respectively). It is possible that further analoguing of peptides **4** and **5** using other substituted Phe as His surrogates might give peptides of better hMC4R potency and/or selectivity.

Bednarek et al. showed that His in MT-II could be replaced by conformationally constrained Pro with only a 2-fold drop in hMC4R agonist activity (its effect on hMC1R was not reported).[9] A number of constrained amino acids were scanned at the His position using peptide **2** as the template and the results of the more interesting analogues are discussed below. Tic-containing peptide **6** is a potent hMC4R agonist but is not selective against hMC1R. Racemic Disc amino acid, structurally related to Pro, gave a separable mixture of two diastereomers, peptides **7** and **8**. Peptide **7** is a potent hMC4R agonist with EC_{50} = 2 nM but is not selective against hMC1R. Racemic Atc amino acid gave peptide **9**, which when tested as a mixture of two diastereomers, showed moderate selectivity against hMC1R (~14 fold). It should be noted that linear pentapeptides containing Atc (Penta-Atc-DPhe-Arg-Trp-Gly-NH_2) also displayed moderate selectivity against hMC1R.[3] Amino acid Aic, a five-membered ring analogue of Atc, gave peptide **10** which is equipotent to peptide **9** in hMC4R but is less selective against hMC1R as peptide **9**. Encouraged by the moderate hMC4R selectivity of peptide **9**, we prepared a series of cyclic peptides based on peptide **2** in which His was replaced by substituted Atc. The agonist activities of the resulting peptides are summarized in Table 2.

The four positional isomers of BrAtc were incorporated into cyclic peptide **2** giving peptides **11**–**15**. As shown in Table 2, peptides **11**–**15** displayed hMC4R EC_{50} values within the range of 10–176 nM. The SAR of BrAtc in cyclic peptide is very different from that in the linear peptide (Penta-His-DPhe-Arg-Trp-Gly-NH_2) where only 5-BrAtc gave potent and selective hMC4R agonists while 6-BrAtc, 7-BrAtc or 8-BrAtc gave peptides which are either inactive (unable to stimulate any agonist response at 50 μM) or are weak partial agonists (unable to stimulate the maximum agonist response at 50 μM) in hMC4R agonist assay.[3] Interestingly, peptides **11** and **12** containing 5-BrAtc and peptide **13** containing 6-BrAtc are almost identical in their hMC1R and hMC4R agonist activities. Similarly, 6-ClAtc gave peptide **17** which has a similar agonist profile compared to 5-ClAtc containing peptide **16**. It is reasonable to speculate that further modification of peptides **13** and **17** by using other 6-substituted Atc might yield more potent and/or more selective hMC4R agonists. Despite the above possibility, we were encouraged by the good hMC4R potency and selectivity of peptides **12** and **16** and equipped with a panel of various 5-substituted Atc from our linear peptide study, we prepared peptides **18**–**24**. Although a number of these peptides are of similar or slightly improved hMC4R agonist activities compared to peptide **16**, none of them was able to dial out hMC1R activity as effectively as peptide **16**.

Peptide **16**, made from racemic 5-ClAtc, was carefully separated into diastereomeric peptides **25** and **26**. One

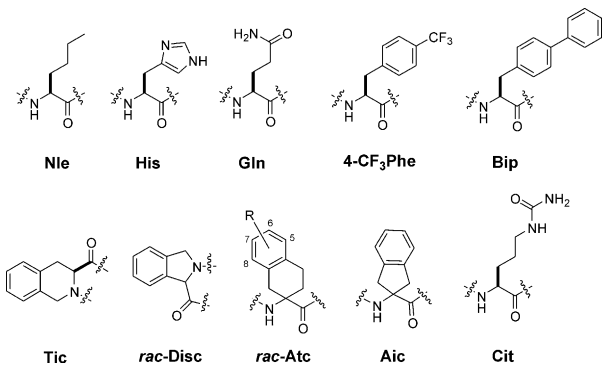

Figure 2. Structures of amino acids.

of the most interesting peptides discovered from this initial optimization study is peptide **26** (Penta-c[Asp-5-ClAtc-DPhe-Arg-Trp-Lys]-NH_2), which has an EC_{50} value of 4 nM in hMC4R and was unable to stimulate the maximum agonist response at 50 μM in hMC1R, hMC3R and hMC5R (Table 3). Although peptide **26** is about 7 fold less potent than MT-II as a hMC4R agonist, it possesses significantly improved selectivity against the other melanocortin receptor subtypes compared to MT-II. Substitution of Arg^8 in peptides **25** and **26** with non-basic[10] Cit gave a separable mixture of peptides **27** and **28**. Cyclic peptide **28** possessed good hMC4R agonist potency ($EC_{50} = 48$ nM), was unable to stimulate any agonist response in hMC1R, hMC3R and hMC5R, and is free of any basic side-chain. Comparing the agonist profiles of peptides **2** and **26** (Table 3), it is interesting that a simple change from His to 5-ClAtc while preserving the excellent hMC4R agonist potency, at the same time reduced hMC1R, hMC3R and hMC5R agonist potencies in a dramatic fashion.

Peptides **25**–**28** were also tested in hMC1R and hMC4R binding assays (Table 4). Peptide **26** is 8-fold less potent in hMC4R binding affinity and 7-fold less potent in hMC4R agonist activity, compared to MT-II; peptide **26** is >5000-fold less potent in hMC1R binding affinity, compared to MT-II, which is consistent with the very low hMC1R agonist response of peptide **26**. Peptide **28** is 27-fold less potent in hMC4R binding affinity and 12-fold less potent in hMC4R agonist activity, compared to peptide **26**; peptide **28** is 2-fold less potent in hMC1R binding affinity relative to peptide **26** which might account for **28**'s lack of hMC1R agonist response.

In summary, a series of cyclic peptides, based on potent but non-selective Penta-c[Asp-His^6-$DPhe^7$-Arg^8-Trp^9-Lys]-NH_2 and modified at His^6 residue, was prepared and pharmacologically characterized. Two very interesting peptides discovered from this initial study are Penta-c[Asp-5-ClAtc-DPhe-Arg-Trp-Lys]-NH_2 and Penta-c[Asp-5-ClAtc-DPhe-Cit-Trp-Lys]-NH_2. Both peptides are potent hMC4R agonists and are either inactive or are weak partial agonists in hMC1R, hMC3R and hMC5R agonist assays. Further modification of Atc amino acid, generation of pharmacophore model from NMR studies of the most selective cyclic peptides and in vivo studies using these potent and selective peptides would be reported in due course.[11–13]

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