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Bioorganic & Medicinal Chemistry Letters 13 (2003) 649–652

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Structure–Activity Relationship of Linear Peptide Bu-His^6-DPhe^7-Arg^8-Trp^9-Gly^{10}-NH_2 at the Human Melanocortin-1 and -4 Receptors: DPhe^7 and Trp^9 Substitution

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Received 8 October 2002; accepted 22 November 2002

Abstract—A series of pentapeptides, based on hMC4R pentapeptide agonist (Bu-His^6-DPhe^7-Arg^8-Trp^9-Gly^{10}-NH_2), was prepared in which either DPhe^7 or Trp^9 residue was systematically substituted. A number of interesting DPhe surrogates (D-Thi, D-3-CF_3Phe, D-2-Nal and D-3,4-diClPhe) as well as Trp surrogates (2-Nal and Bta) were identified in this study.

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] One of the main goals of our laboratories is to identify potent and selective human melanocortin-4 receptor (hMC4R) agonists for the treatment of obesity.[2]

As previously reported, our lead pentapeptide **1** (Bu-His^6-DPhe^7-Arg^8-Trp^9-Gly^{10}-NH_2, α-MSH numbering) is 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 not selective against hMC1R ($EC_{50} = 10$ nM).[3] 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 agonist activity and to maintain or improve hMC4R agonist activity. We previously reported our results in replacing His and Arg residues of pentapeptide **1**,[3,4] this report summarizes our effort in replacing DPhe and Trp residues of peptide **1**. During the preparation of this manuscript, a report appeared in which DPhe residue in a linear tetrapeptide template (Ac-His-DPhe-Arg-Trp-NH_2) was replaced with 26 different amino acids and the resulting peptides were characterized in *mouse* clone MC1R–MC5R.[5,6] On the other hand, while there are a number of reports in which Trp residue was replaced by Ala, Pro, DTrp or 2-Nal[7–11] in different cyclic peptide templates, the effect of Trp substitution in a linear peptide template has not been extensively studied.

All new peptides reported in this study and NDP-MSH were synthesized on solid phase from suitably protected amino acids using standard Fmoc or Boc methodology.[12] The crude peptides were purified to homogeneity using reversed-phase HPLC and characterized by fast atom bombardment mass spectroscopy. Peptide α-MSH and all amino acids used in this study were purchased from commercial sources.

Agonist assays were performed using HEK293 cells transfected with hMC1R–hMC5R as reported in detail elsewhere.[12,13] The EC_{50} values reported in Tables 1 and 2 are the average of at least two separate experiments. Binding assays were performed using radiolabeled NDP-MSH as reported in detail elsewhere.[13] The IC_{50} values reported in Tables 1 and 2 are the average of at least two separate experiments.

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doi:10.1016/S0960-894X(02)01052-1

Table 1. Binding and agonist activities of DPhe[7] modified pentapeptides at the human melanocortin receptors

Peptide	Amino acid sequence	hMC1R IC_{50} (nM)[a]	hMC1R EC_{50} (nM)[b]	hMC4R IC_{50} (nM)[a]	hMC4R EC_{50} (nM)[b]
1	Bu-His-**DPhe**-Arg-Trp-Gly-NH_2[c]	575	10	150	20
2	Bu-His-**D-4-CH_3Phe**-Arg-Trp-Gly-NH_2	Not determined	55	Not determined	77
3	Bu-His-**D-4-ClPhe**-Arg-Trp-Gly-NH_2	300	20	80	20
4	Bu-His-**D-4-BrPhe**-Arg-Trp-Gly-NH_2	300	54	22	13
5	Bu-His-**D-4-HOPhe**-Arg-Trp-Gly-NH_2	Not determined	180	Not determined	1150
6	Bu-His-**D-4-MeOPhe**-Arg-Trp-Gly-NH_2	70	0.4	120	60
7	Bu-His-**D-4-EtOPhe**-Arg-Trp-Gly-NH_2	16	0.1	100	16
8	Bu-His-**D-1-Nal**-Arg-Trp-Gly-NH_2	4000	30	350	65
9	Bu-His-**D-2-Nal**-Arg-Trp-Gly-NH_2	5000	48	30	0% @ 50 μM[d]
10	Bu-His-**D-3,4-diClPhe**-Arg-Trp-Gly-NH_2	250	10	4	0% @ 50 μM[d]
11	Bu-His-**DTha**-Arg-Trp-Gly-NH_2	Not determined	4	Not determined	8
12	Bu-His-**D-3-CF_3Phe**-Arg-Trp-Gly-NH_2	9600	611	300	45

[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]Ac stands for $CH_3C(=O)$, Bu stands for $CH_3CH_2CH_2C(=O)$.
[d]Not tested for antagonist activities.

Table 2. Binding and agonist activities of Trp[9] modified pentapeptides at the human melanocortin receptors

Peptide	Amino acid sequence	hMC1R IC_{50} (nM)[a]	hMC1R EC_{50} (nM)[b]	hMC4R IC_{50} (nM)[a]	hMC4R EC_{50} (nM)[b]
1	Bu-His-DPhe-Arg-**Trp**-Gly-NH_2[c]	575	10	150	20
13	Bu-His-DPhe-Arg-**DTrp**-Gly-NH_2	Not determined	110	Not determined	110
14	Bu-His-DPhe-Arg-**1-Nal**-Gly-NH_2	1200	150	600	140
15	Bu-His-DPhe-Arg-**2-Nal**-Gly-NH_2	600	14	50	25
16	Bu-His-DPhe-Arg-**Bta**-Gly-NH_2	2880	274	1000	100
17	Bu-His-DPhe-Arg-**3,4-diClPhe**-Gly-NH_2	Not determined	1	Not determined	194

[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]Bu stands for $CH_3CH_2CH_2C(=O)$.

As shown 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 is not selective against hMC1R ($EC_{50} = 10$ nM). For comparison purpose, known linear peptide agonist NDP-MSH[14] was determined in our assays to have EC_{50} values of 0.5 nM (hMC1R) and 1 nM (hMC4R) while α-MSH showed EC_{50} values of 0.8 nM (hMC1R) and 25 nM (hMC4R). When DPhe in peptide **1** was replaced with D-4-CH_3Phe, the resulting peptide **2** showed about a 4-fold drop (the standard error in our assays is about 2-fold) in agonist potency at hMC4R and a 5.5-fold drop in agonist potency at hMC1R, compared with peptide **1**. Use of D-4-ClPhe gave peptide **3**, which possessed similar agonist potency at both hMC1R and hMC4R, compared with peptide **1**. Consistent with our results, Hruby et al. reported that substitution of DPhe with D-4-ClPhe using cyclic peptide MTII (Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2)[15,16] as the template gave SHU9203 which was 4-fold more potent at hMC1R agonist assay and equipotent at hMC4R agonist assay, compared with the parent MTII.[17] The D-4-BrPhe containing peptide **4** showed the same agonist potency at hMC4R but displayed a 5-fold drop in agonist potency at hMC1R, compared with peptide **1**.

The D-4-HOPhe containing peptide **5** suffered a 58-fold drop in hMC4R agonist potency and 18-fold drop in hMC1R agonist potency, compared with peptide **1**. Linear tetrapeptide Ac-His-D-4-HOPhe-Arg-Trp-NH_2 also showed a 160- and 140-fold drop in agonist potencies at mouse MC1R and MC4R, respectively.[5] Since peptides containing other substituents at the 4-position of D-Phe, such as peptides **2**–**4**, are significantly more potent in hMC1R and hMC4R agonist assays than peptide **5**, we speculate that the unfavorable interaction between a polar phenol group in peptide **5** and the hydrophobic residues within the putative DPhe binding pocket[18,19] might be responsible for the loss in agonist potency of peptide **5** at both receptors. Consistent with the above theory, capping of the polar phenol group with methyl or ethyl groups, yielded peptides **6** and **7** which showed significantly improved agonist activities at both hMC1R and hMC4R, compared with peptide **5**. Unexpectedly, peptides **6** and **7** both showed high selectivity (>100 fold) towards hMC1R over hMC4R.[20]

The D-1-Nal containing peptide **8** showed a 3-fold loss in agonist potencies at both hMC1R and hMC4R, compared with peptide **1**. A similar substitution in a linear tetrapeptide gave Ac-His-D-1-Nal-Arg-Trp-NH_2

which showed a 18-fold drop in agonist potencies at both mouse MC1R and MC4R,[5] a finding which is consistent with our results. Use of D-2-Nal gave peptide **9** which was unable to activate hMC4R at up to 50 μM concentration but was a potent and full agonist at hMC1R. Linear tetrapeptide Ac-His-D-2-Nal-Arg-Trp-NH_2 has been reported to be a full agonist at mouse MC1R and an antagonist at mouse MC4R,[5] similar to peptide **9** pharmacologically.

Use of D-3,4-diClPhe gave peptide **10**, which was unable to activate hMC4R but was equipotent to peptide **1** in the hMC1R agonist assay. Peptide **11** containing DTha with a thiophene side chain was more potent than DPhe containing peptide **1** in both hMC1R and hMC4R agonist assays. It might be interesting to investigate other DPhe surrogates with five membered heterocycles (e.g., thiazole) at the side chain. Peptide **12**, containing D-3-CF_3Phe, retained good hMC4R agonist potency ($EC_{50} = 45$ nM) but suffered a 61-fold drop in hMC1R agonist potency, an unexpected but desirable outcome. D-Tha and D-3-CF_3Phe represent two novel DPhe surrogates which might be useful for future design of melanocortin peptide or small molecule agonists.

The more interesting peptides discussed above were subsequently tested in the hMC1R and hMC4R binding assays and the data are shown in Table 1. Although the binding affinities and the agonist activities of these DPhe modified peptides are not linearly correlated, they do track with each other in a qualitative sense. For example, the most potent hMC1R agonists within the series, peptides **6** and **7**, were the best hMC1R binders; on the other hand, the least potent hMC1R agonist within the series, peptide **12**, was also the weakest hMC1R binder. Similarly, the most potent hMC4R agonist within the series, peptide **4**, was the best hMC4R binder; on the other hand, the least potent hMC4R agonist within the series, peptide **8**, was also the weakest hMC4R binder.

Peptide **9**, which showed weaker binding affinity than peptides **1** and **10** at hMC1R, also displayed lower agonist activity than peptides **1** and **10**. While peptides **9** and **10** were better binders than peptide **1** to hMC4R, peptides **9** and **10** were not able to trigger an agonist response at hMC4R even at the highest concentration of 50 μM. Functional antagonist assay was not carried out on peptides **9** and **10**. Since a number of D-2-Nal containing antagonists (e.g., SHU9119,[17] HS024,[21] MBP10,[9] etc.) and D-3,4-diClPhe containing antagonists (e.g., HS028[22]) are known in the literature, it might be interesting to investigate the pharmacological consequences of replacing the D-2-Nal or D-3,4-diClPhe residue in the above mentioned peptide antagonists with other 3,4-disubstituted DPhe amino acids (e.g., D-3,4-diBrPhe).

A number of pentapeptides with modified Trp were also prepared and the hMC1R and hMC4R agonist activities of these analogues are summarized in Table 2. When Trp in peptide **1** was replaced with D-Trp, the resulting peptide **13** showed a 5.5-fold drop in agonist potency at hMC4R and 11-fold drop in agonist potency at hMC1R. Inversion of chirality from Trp to DTrp in a cyclic peptide has been reported to have no effect on the binding affinity at hMC3R-hMC5R.[9] The 1-Nal containing peptide **14** showed a 7- and 15-fold drop in agonist potencies at hMC1R and hMC4R respectively, compared with peptide **1**. Use of 2-Nal gave peptide **15** which retained the same potency at both hMC1R and hMC4R, compared with peptide **1**. Successful replacement of Trp with 2-Nal has been demonstrated in a cyclic peptide agonist template at hMC4R[10] and in a cyclic peptide antagonist template at mouse MC1R and MC4R.[11] Peptide **16**, which contains Bta with a sulfur atom instead of a nitrogen atom in the side chain (Fig. 1), showed a 5-fold drop in agonist potency at hMC4R and 27-fold drop in agonist potency at hMC1R. Use of 3,4-diClPhe gave peptide **17** which unexpectedly showed good selectivity towards hMC1R over hMC4R (>150-fold).

The more interesting peptides discussed above were subsequently tested in the hMC1R and hMC4R binding assays and the data are shown in Table 2. While there is no linear correlation between the binding affinities and agonist activities of these Trp modified peptides, the stronger binders (peptides **1** and **15**) were indeed more potent agonists, compared with peptides **14** and **16**, at both hMC1R and hMC4R. Peptides **1** and **15** were virtually identical in their binding and agonist activities at both hMC1R and hMC4R demonstrating the equivalency of Trp and 2-Nal based on the template of Bu-His-DPhe-Arg-Trp-Gly-NH_2.[23]

In summary, a series of pentapeptides, based on Bu-His-DPhe-Arg-Trp-Gly-NH_2 and modified at either DPhe or Trp residue, was prepared and pharmacologically characterized. A number of pentapeptides containing novel DPhe surrogates, such as D-Thi and D-3-CF_3Phe, were found to be potent and full hMC1R and hMC4R agonists. On the other hand, pentapeptides containing D-2-Nal and D-3,4-diClPhe as DPhe surrogates were full agonists at hMC1R but were unable to stimulate an agonist response at hMC4R despite excellent binding

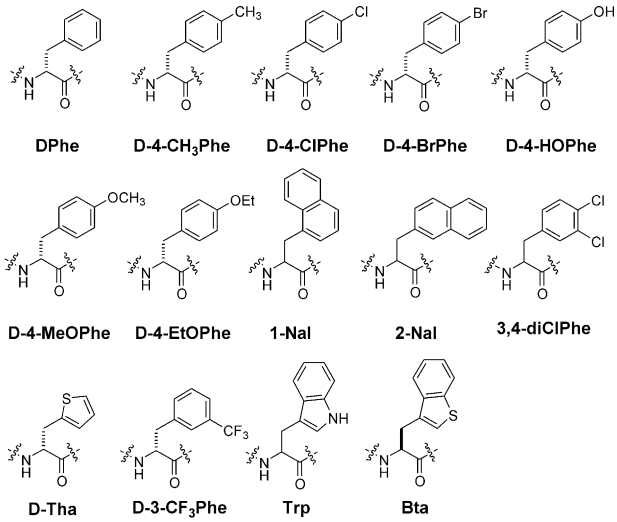

Figure 1. Structures of amino acids.

affinity. In addition, pentapeptide containing 2-Nal as Trp surrogate was found to be equipotent to the parent peptide at both hMC1R and hMC4R. Incorporation of the above DPhe and Trp surrogates into hMC4R selective linear and cyclic peptides would be the topic of future communication.

Acknowledgements

The authors are grateful to Roche Physical Chemistry Department for spectroscopic measurements and interpretations. We would also like to thank Dr. Jefferson Tilley for critical reading of the manuscript.

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