Biochemical and Biophysical Research Communications **261,** 209–213 (1999)
Article ID bbrc.1999.0981, available online at http://www.idealibrary.com on IDEAL®

Analogs of MTII, Lactam Derivatives of α-Melanotropin, Modified at the N-Terminus, and Their Selectivity at Human Melanocortin Receptors 3, 4, and 5

Maria A. Bednarek,*[,1] Tanya Macneil,† Rubana N. Kalyani,† Rui Tang,† Lex H. T. Van der Ploeg,† and David H. Weinberg†

**Department of Medicinal Chemistry and †Department of Obesity Research, Merck Research Laboratories, Rahway, New Jersey 07065*

Received June 15, 1999

In search for selective agonists at human melanocortin-4 receptor, proline-substituted analogs of MTII, a potent nonselective agonist at melanocortin receptors, were prepared by solid-phase syntheses and evaluated for their ability to bind and activate human MC-3, MC-4, and MC-5 receptors. Replacement of Nle[4] with Pro resulted in [Pro[4]]MTII with affinity to and agonist potency at hMC-4R similar to MTII, but with about 400-fold lower potency at hMC-5R and about 20-fold lower potency at hMC-3R. The substantial increase in selectivity of [Pro[4]]MTII with respect to hMC-5R prompted us to investigate additional analogs of MTII with modified N-termini. The Ac-Nle[4] segment, not encompassed in the lactam ring, was substituted with flexible, hydrophobic, or hydrophilic substituents, and also, with residues resembling proline. The similar agonist potency of these peptides to that of MTII at hMC-4R but significantly lower activity of these compounds at hMC-5R demonstrated that the N-terminal fragment of MTII has virtually no effect on the binding affinity and activation at hMC-4R, but it is essential for full potency at hMC-5R. **© 1999 Academic Press**

Key Words: **melanotropin; melanocortin receptor; proline-scan; binding affinity; cAMP accumulation assay.**

The melanocortin peptides or melanotropins, α-MSH, β-MSH, γ-MSH and ACTH, act through the receptors expressed in the central nervous system and in various peripheral tissues (1–3). Five subtypes of the human receptor, hMC1-5R, mediating the effects of melanotropins have been identified (4–7). The receptors belong to the family of G protein-coupled receptors; their activation leads to elevation of cAMP. Although the physiological role of the individual receptors is not yet well understood, several previously known functions of melanotropins have already been associated with one of the receptors. For instance, the MC-1 receptor, with high affinity for α-MSH, is believed to play a role in pigmentation and inflammation (1–3). The MC-2 receptor, with high affinity for ACTH but not for other melanotropins, is implicated in regulation of steroid production in the adrenal glands (1, 6). The physiological role of the MC-3 receptor, found in the brain, the placenta, the gut and in the heart, is less well defined (8). Rapidly growing evidence suggests that the human MC-4 receptor, widely distributed in the brain, is involved in regulation of the energy balance and body weight. Thus, compounds active at the MC-4 receptor might be useful in the treatment of eating disorders (9–11). The MC-5 receptor, expressed in the brain and in peripheral tissues, is similarly not well characterized (12). For a better understanding and differentiation of the physiological functions of the melanocortin receptors expressed in the brain, hMC3-5R, new and receptor-subtype selective agonists and antagonists are needed, because the known natural ligands, melanotropins, have similar activation and binding affinities at the human MC-3, MC-4 and MC-5 receptors.

Extensive structure-activity studies on melanotropins, in particular on α-MSH, led to a small cyclic peptide, MTII, Ac-Nle[4]-cyclo(5β → 10ϵ)(Asp[5]-His[6]-D-Phe[7]-Arg[8]-Trp[9]-Lys[10])-amide[2] (13). The MTII compound is a potent but not selective agonist at the human MC3, MC4 and MC5 receptors (13). Encompassed by the lactam ring of MTII, the 6-9 fragment of α-MSH, His[6]-Phe[7]-Arg[8]-Trp[9], is regarded as an "active site" essential to interaction of melanotropins with the receptors (1, 14). Our structure-function studies on MTII (15) identified the side chains of amino acids critical for

[1] To whom correspondence should be addressed at Merck Research Laboratories, R50G-141, P.O. Box 2000, Rahway, NJ 07065. Fax: (732) 594-6449. E-mail: maria_bednarek@merck.com.

[2] Throughout this report, the numbering of the amino acid residues in α-MSH has been retained for all cyclic peptides.

0006-291X/99 $30.00
Copyright © 1999 by Academic Press
All rights of reproduction in any form reserved.

TABLE 1

Proline Analogs of MT-II [Ac-Nle^4-cyclo(5$\beta \rightarrow 10\epsilon$)(Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10})-amide]

		Binding IC_{50} (nM)[#]			cAMP EC_{50} (nM)[##]		
No.	Compound	hMC-3R	hMC-4R	hMC-5R	hMC-3R	hMC-4R	hMC-5R
	MT-II	1.6 ± 0.09	0.07 ± 0.02	0.89 ± 0.01	1.2 ± 0.07	0.17 ± 0.01	2.3 ± 0.29
1	Nle^4 → Pro	23 ± 6.2	0.4 ± 0.04	670 ± 233	17 ± 2.08	0.7 ± 0.15	342 ± 117
2	His^6 → Pro	4.3 ± 1.3	0.24 ± 0.08	0.9 ± 0.02	5.1 ± 0.88	0.34 ± 0.06	0.93 ± 0.09
3	D-Phe^7 → D-Pro	>5000	>5000	>5000	3%@5	9%@5	3%@5
4	Arg^8 → Pro	1177 ± 38	661 ± 325	297 ± 41.6	2%@2	7%@2	358 ± 104
5	Trp^9 → Pro	>5000	>5000	>5000	2%@10	7%@10	>10000

[#] Concentration of peptide at 50% specific binding. When peptide was not able to reach 50% specific binding, the percentage of ^{125}I-NDP-α-MSH displaced at a given peptide concentration (μM) was reported.

[##] Concentration of peptide at 50% maximum cAMP accumulation or, the percentage of cAMP accumulation (relative to α-MSH) observed at a given peptide concentration (μM).

interaction of MTII with the human MC-3, MC-4 and MC-5 receptors. Thus, the aromatic residues in position 7 (Phe) and 9 (Trp) appear to be critical for the efficient binding of MTII to the receptors, but the basic hydrophilic residues in positions 6 (His) and 8 (Arg) are less essential for the binding of MTII.

The present study is aimed at the synthesis of MTII-analogs of high potency at the human MC-4 receptor and improved selectivity with respect to the human MC-3 and MC-5 receptors. First, we evaluated analogs of MTII with each amino acid in turn substituted with proline, in binding assays and in intracellular cAMP accumulation assays at the human MC-3, MC-4 and MC-5 receptors. Incorporation into a peptide chain of a conformationally constrained amino acid, such as proline, often stabilizes various peptide conformations, some of them probably of biological significance. Not seldom pattern of the peptide-receptor recognition is altered in the systematic replacement process. The similarity between potency of [Pro^4]MTII and MTII with respect to agonist potency at the human MC-4 receptor but contrasted by the about 400-fold lower activity of [Pro^4]MTII at the human MC-5 receptor prompted us to further examine the role of the N-terminal segment of MTII in interactions with the human MC-3, MC-4 and MC-5 receptors. Here, we report the synthesis and biological evaluation of several analogs of MTII modified at the N-terminus, which are potent and selective agonists at the human MC-4 receptor.

MATERIALS AND METHODS

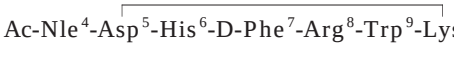
Peptide synthesis and purification. Building of peptide chains on p-methylbenzhydrylamine resin, formation of the lactam ring on a resin, deprotection and cleavage of peptides from a resin, and purification of the crude products by high-pressure liquid chromatography were performed as previously described in detail (15). The chromatographically homogenous compounds were analyzed by amino acid analysis and electrospray mass spectrometry.

Competitive binding assays. Binding activity of compounds was measured using membranes from Chinese hamster ovary (CHO) cells expressing the cloned melanocortin receptors. Binding reactions contained membranes, 200 pM [^{125}I]-NDP-a-MSH (New England Nuclear Corp.), and increasing concentrations of unlabelled test compounds. Reactions were incubated for 1.5 hrs and then filtered as described previously (15). Binding data were analyzed using GraphPad curve-fitting software. Active peptides were evaluated in 3 independent experiments.

cAMP assays. Agonist activity of compounds was measured using Chinese hamster ovary (CHO) cells expressing the cloned melanocortin receptors. Compounds together with 0.6 mM IBMX were incubated at room temperature with dissociated cells for 40 min., lysed with dilute HCl or by incubation at 100°C for 4 min. to terminate the assay as described previously (15). cAMP was quantitated by Amersham (Arlington Heights, IL) RPA556, cAMP SPA screening assay or by New England Nuclear (Boston, MA) SMP-001J Flashplate cAMP assay. All active peptides were characterized in 3 independent experiments. Tables 1 and 2 indicate the mean ± SEM (standard error of the mean). All analogs yielded >65% activation relative to α-MSH at hMC3-5R, suggesting that their activation abilities were very similar to that of α-MSH. One exception was compound **2,** [Pro^6]MTII, which produced only 15 % activation relative to α-MSH at the human MC-3 receptor.

RESULTS AND DISCUSSION

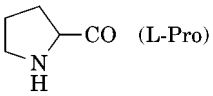
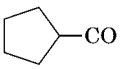
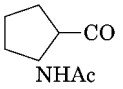
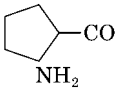
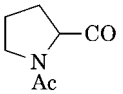
At the human MC-3, MC-4 and MC-5 receptors, the lactam derivative of α-MSH, Ac-Nle^4-cyclo(5$\beta \rightarrow 10\epsilon$)(Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10})-amide, MTII, which has a fairly large and flexible 23-membered ring, is about a 10-fold more potent agonist than the parent linear peptide, α-MSH, Ac-Ser^1-Tyr^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-amide, and is equipotent to NDP-α-MSH, Ac-Ser^1-Tyr^2-Ser^3-Nle^4-Glu^5-His^6-D-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-amide, at the same receptors (16). However, like α-MSH and NDP-α-MSH, the MTII peptide is also less selective at the melanocortin receptors, hMC3-5R.



Our previous NMR analysis (15) revealed no preferred conformation(s) for MTII in organic solutions.

TABLE 2

Analogs of MT-II Modified at the N-Termini [X-cyclo(5β → 10ϵ)(Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10})-amide]

		Binding assay, IC_{50} (nM)[#]			cAMP assay, EC_{50} (nM)[##]		
No.	X	hMC-3R	hMC-4R	hMC-5R	hMC-3R	hMC-4R	hMC-5R
MTII	Ac-L-Nle	1.6 ± 0.09	0.07 ± 0.02	0.89 ± 0.01	1.2 ± 0.07	0.17 ± 0.01	2.3 ± 0.29
6	H	218 ± 54	10 ± 5.1	1900 ± 321	169 ± 36	2.1 ± 0.2	3367 ± 857
7	Ac	40 ± 6.4	3 ± 1.2	646 ± 99	21 ± 3	0.83 ± 0.03	703 ± 239
8	L-Nle	0.8 ± 0.2	0.1 ± 0.06	1.3 ± 0.18	0.4 ± 0.1	0.23 ± 0.03	5.37 ± 1.7
9	L-NMe-Nle	1.2 ± 0.3	0.5 ± 0.1	1.5 ± 0.3	0.4 ± 0.2	0.19 ± 0.02	1.3 ± 0.3
10	L-Ala	30 ± 7	1.2 ± 0.25	367 ± 1.28	20 ± 2.7	1.23 ± 0.38	930 ± 378
11	Ac-L-Ala	30 ± 4	0.6 ± 0.22	321 ± 44	19 ± 5.4	0.83 ± 0.09	260 ± 46
12	$NH_2(CH_2)_5CO$	22 ± 7.1	1.4 ± 0.25	300 ± 64	5 ± 0.58	0.22 ± 0.1	182 ± 46
13	$Ac\text{-}NH(CH_2)_5CO$	12 ± 1.8	0.6 ± 0.21	274 ± 53	11 ± 1.2	0.43 ± 0.09	263 ± 48
14	$CH_3(CH_2)_4CO$	2.3 ± 0.3	0.1 ± 0.05	2.6 ± 1.2	0.5 ± 0.1	0.15 ± 0.03	5.5 ± 0.4
15	$CH_3OOC(CH_2)_3CO$	22 ± 7.1	1.4 ± 0.52	297 ± 64	10 ± 3.3	0.35 ± 0.13	156 ± 30
16	$HOOC(CH_2)_3CO$	525 ± 67	14 ± 4.2	>5000	223 ± 15	3.1 ± 0.19	1700 ± 252
17	Ac-L-Glu	2860 ± 345	57 ± 12	>5000	429 ± 104	6.1 ± 1.9	1647 ± 259
18	Ac-L-Lys	26 ± 3.8	2.9 ± 1.1	154 ± 33	9.1 ± 1	0.3 ± 0.1	139 ± 25
19	 (L-Pro)	13 ± 1.2	0.3 ± 0.06	120 ± 50	32 ± 14	1 ± 0.17	370 ± 62
20		5.9 ± 0.9	0.2 ± 0.08	41 ± 31	1.8 ± 0.46	0.17 ± 0.04	40 ± 4.7
21*		12 ± 3.8	0.6 ± 0.32	86 ± 8.9	5 ± 1.6	0.12 ± 0.03	64 ± 10
22**		52 ± 3 4.9 ± 1.4	2.5 ± 1.3 0.06 ± 0.02	337 ± 95 50 ± 25	42 ± 10 1.7 ± 0.21	1.3 ± 0.28 0.09 ± 0.003	433 ± 18 42 ± 8
1	 (Ac-L-Pro)	23 ± 6.2	0.4 ± 0.04	670 ± 233	17 ± 2.1	0.7 ± 0.15	294 ± 168

[#,##] As for Table 1.

* Tested as a mixture of two *cis* compounds.

** Two *cis* isomers tested separately.

We anticipated, therefore, that incorporation of conformationally constrained proline into the lactam ring might stabilize bioactive conformations and might improve selectivity of MTII at the melanocortin receptors.

Proline analogs of MTII listed in Table 1 were prepared as described in reference 15, and were tested for their binding to human MC-3, MC-4 and MC-5 receptors in competitive binding assays, and for their signal transduction efficacies in cells expressing these receptors, see Materials and Methods, and Table 1. Substitution of Phe^7 or Trp^9 with proline resulted in inactive compounds even at micromolar concentrations, confirming our previous observations (15) that the aromatic side chains at positions 7 (Phe) and 9 (Trp) are necessary for interaction of MTII with the mentioned receptors.

Replacement of Arg^8 with proline also resulted in a compound devoid of agonist activity at the human MC-4 receptor (at micromolar peptide concentrations). From the Ala-scan of MTII, we had already learned that omission of arginine side chain at position 8 results in a 100- to 2000-fold reduction of activation and binding affinities at the human MC-3, MC-4 and MC-5 receptors (15). However, the analog of MTII with Pro instead of Arg^8 exhibited an even larger decrease in agonist potency. This suggests that the steric constraints introduced by proline at position 8 severely disturbed the conformation(s) required for recognition by the human MC-4 receptor.

Substitution of His at position 6 with proline led to compound **2,** with the agonist potency at the human MC-4 receptor similar to that of MTII (see Materials and Methods, not a fully effective agonist at hMC-3R). This corroborated our earlier observations that the imidazole group of histidine is not essential for the binding of MTII with hMC-4R (15, 17). Moreover, the restriction of conformational freedom at position of histidine did not affect interactions of MTII with hMC-4R.

Interestingly, replacement of the hydrophobic norleucine residue in position 4, located on the outside of the lactam ring, by the similarly hydrophobic proline residue yielded compound **1,** [Pro^4]MTII, as potent agonist as MTII at the human MC-4 receptor, but 400 times less active at the human MC-5 receptor and 20 times less potent at the human MC-3 receptor, see Table 1. These results implay that acetyl-proline at the N-terminus of MTII interferes with the binding of the agonist to the human MC-5 receptor, either because of its bulkiness or because it lacks a free αNH, perhaps required for interactions via hydrogen-bonds.

To expand on these observations, additional analogs of MTII with modified N-termini were synthesized and tested at hMC3-5R, as outlined in Materials and Methods, see also Table 2. Analogs of MTII without an acetyl group, compounds **8** and **9,** were as potent as MTII at the human MC-3, MC-4 and MC-5 receptors, but also were not selective, whereas, the analog without both acetyl group and norleucine, compound **6,** was at least 200-fold less active at the studied receptors, Table 2.

Replacement of Ac-Nle^4 with a substantially less lipophilic Ala or Ac-Ala yielded compounds **10** and **11,** respectively, with agonist potencies at hMC-4R similar to that of MTII, but with at least 200-fold lower activities at hMC-5R. Apparently, a longer hydrophobic side chain (as in norleucine) in position 4 is required for the full agonist potency of MTII at hMC-5R.

In compounds **12, 13** and **15,** acetyl-norleucine at position 4 was replaced by $NH_2(CH_2)_5COOH$, Ac-$NH(CH_2)_5COOH$ and $CH_3OOC(CH_2)_3COOH$, respectively. These substituents were chosen to mimic the extended hydrophobic side chain of norleucine, but they also contained polar groups (amino, amide or ester group) to allow interactions through the ionic- and hydrogen-bonds. The analogs were as potent as MTII at the human MC-4 receptor but were at least 500-fold less active at the human MC-5 receptor. Thus, the polar groups of compounds **12, 13** and **15** had fairly negligible influence on binding to and activation of the human MC-4 receptor. At the human MC-5 receptor, the polar groups either interfered with hydrophobic interactions or, were unable to assume the role of $^{\alpha}$NH of Nle in formation of H-bonds (presumably because of their larger distances from the peptide backbone), or they were engaged in other ionic-or hydrogen-bonds unfavorable for agonist activity at this receptor.

Interestingly, compounds **16** and **17** with negatively charged substituents, $HCOO(CH_2)_3COOH$ and Ac-Glu, replacing acetyl-norleucine, were only one order of magnitude less potent than MTII in the cAMP activation assay at hMC-4R. The modest effect of the carboxyl groups on activation and binding affinities at the human MC-4 receptor was in a sharp contrast with their effect on the recognition of the same peptides by other receptors. Compounds **16** and **17** were about 50-fold less potent at hMC-3R and practically inactive at hMC-5R at micromolar peptide concentrations.

Replacement of Ac-Nle^4 with substituents similar to proline (or Ac-Pro): cyclopentanecarboxylic acid, 2-aminoacetyl-cyclopentanecarboxylic acid or 2-amino-cyclopentanecarboxylic acid (compounds **20, 21** or **22,** respectively) yielded peptides as potent as [Pro^4]MTII at hMC4-R but at least 100-fold less active at hMC-5R.

These results therefore, demonstrate that the N-terminal segment of MTII (Ac-Nle^4, not included in the lactam ring) is one of the principle structural features determining potency and selectivity of MTII at the melanocortin receptors, hMC3-5R. The Ac-Nle^4 fragment seems to have no effect on binding affinity and activation at the human MC-4 receptor but, it appears to be indispensable for the full potency at the human MC-5 receptor. Flexible and hydrophobic residues are required at position of Ac-Nle^4 for maximum activity at hMC-5R, whereas, hydrophobic and bulky substituents or, hydrophilic or ionic modifications, drastically diminish agonist activities of MTII analogs at human MC-5 receptor. Interestingly, the same fragment (Ac-Nle^4) in the non-selective antagonist at hMC-4R, SHU9119 peptide, Ac-Nle^4-cyclo(5$\beta \rightarrow$ 10ϵ)(Asp^5-His^6-D-2′-Nal^7-Arg^8-Trp^9-Lys^{10})-amide (18), seemed to be of a little importance in defining selectivity of this peptide for the melanocortin receptors, hMC3-5R. For example, the [Pro^4]SHU9119 analog, as an antagonist at the human MC-3 and MC-4 receptors, was as potent as the parent compound and, was only 5-fold less potent as an agonist at the human MC-5 receptor (data not included).

In summary, we report here a family of potent agonists at the human MC-4 receptor which have improved selectivity with respect to the human MC-5 and MC-3 receptors. These compounds might be useful in further studies on physiological functions of the melanocortin receptors. The new family of MTII analogs with modified N-termini provides insight into the structural requirements necessary for efficient binding to and activation of the melanocortin receptors, hMC3-5R. The experience gained in the study should be useful in the design of further analogs of melanotropins.

REFERENCES

1. Eberle, A. N., (1988) The Melanotropins: Chemistry, Physiology and Mechanism of Action. Karger, Basel.
2. Hadley, M. E. (1989) The Melanotropic Peptides: Source, Synthesis, Chemistry, Secretion and Metabolism. CRC Press, Boca Raton, FL.
3. Vaudry, H., and Eberle, A. N. (1993) The Melanotropic Peptides. New York Academy of Science, New York.
4. Chhajlani, V., Muceniece, R., and Wikberg, J. E. S. (1993) *Biochem. Biophys. Res. Commun.* **195,** 866–873.
5. Gantz, I., Shimoto, Y., Konda, Y., Miwa, H., Dickson, C. J., and Yamada, T. (1994) *Biochem. Biophys. Res. Commun.* **200,** 1007–1014.

6. Roselli-Rehfuss, L., Mountjoy, K. G., Robbins, L. S., Mortrud, M. T., Low, M. J., Tatro, J. B., Entwistle, M. L., Simerly, R. B., and Cone, R. D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8856–8860.
7. Schioth, H. B., Chhajlani, V., Muceniece, R., Klusa, V., and Wikberg, J. E. S. (1996) *Life Sci.* **59,** 797–801.
8. Gantz, I., Konda, Y., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Warson, S. J., Del Valle, J., and Yamada, T. (1993) *J. Biol. Chem*. **268,** 8246–8250.
9. Mountjoy, K. G., Mortrud, M. T., Low, M. J., Simerly, R. B., and Cone, R. D. (1994) *Mol. Endocrinol.* **8,** 1298–1308.
10. Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., and Cone, R. D. (1997) *Nature* **385,** 165–168.
11. Mountloy, K. G., and Wong, J. (1997) *Mol. Cell. Endocrinol.* **128,** 171–177.
12. (a) Labbe, O., Desarnaud, F., Eggerickx, D., Vassart, G., and Parmentier, M. (1994) *Biochemistry* **33,** 4543–4549. (b) Chen, W., Kelly, M. A., Opiz-Araya, X., Thomas, R. E., Low, M. J., and Cone, R. D. (1997) *Cell* **91,** 789–798.
13. Al-Obeidi, F., de L. Castrucci, A. M., Hadley, M. E., and Hruby, V. J. (1989) *J. Med. Chem*. **32,** 2555–2561.
14. Hruby, V. J., Wilkes, B. C., Hadley, M. E., Al-Obeidi, F., Sawyer, T. K., Staples, D. J., Devaux, A., Dym, O., Castrucci, A. M., Hintz, M. F., Riehm, J. P., and Rao, K. R. (1987) *J. Med. Chem*. **30,** 2126–2130.
15. Bednarek, M. A., Silva, M. V., Arison, B., MacNeil, T., Kalyani, R. N., Huang, R-R. C., and Weinberg, D. H. (1999) *Peptides* **20,** 401–409.
16. Sawyer, T. K., Sanfilippo, P. J., Hruby, V. J., Engel, M. H., Heward, C. B., Burnett, J. B., and Hadley, M. E. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5754–5758.
17. Analog [Pro^6]MTII has been recently reported (abstract) by V. J. Hruby and co-workers at the 217th National Meeting of American Chemical Society, Anaheim, California, March 21–25, 1999.
18. Al-Obeidi, F., Hruby, V. J., Hadley, M. E., Sawyer, T. K., and de L. Castrucci, A. M. (1990) *Int. J. Peptide Protein Res.* **35,** 228–234.