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Cyclic analogs of α-melanocyte-stimulating hormone (αMSH) with high agonist potency and selectivity at human melanocortin receptor 1b

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ABSTRACT

α-Melanotropin (αMSH), 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]-NH_2,[1] has been long recognized as an important physiological regulator of skin and hair pigmentation in mammals. Binding of this peptide to the melanocortin receptor 1 (MC1R) leads to activation of tyrosinase, the key enzyme of the melanin biosynthesis pathway. In this study, interactions of the human MC1bR (an isoform of the receptor 1a) with the synthetic cyclic analogs of αMSH were studied. These ligands were analogs of MTII, Ac-Nle[4]-cyclo-(Asp[5]-His[6]-D-Phe[7]-Arg[8]-Trp[9]-Lys[10])-NH_2, a potent pan-agonist at the human melanocortin receptors (hMC1,3–5R). In the structure of MTII, the His[6]-D-Phe[7]-Arg[8]-Trp[9] segment has been recognized as "essential" for molecular recognition at the human melanocortin receptors (hMC1,3–5R). Herein, the role of the Trp[9] in the ligand interactions with the hMC1b,3–5R has been reevaluated. Analogs with various amino acids in place of Trp[9] were synthesized and tested *in vitro* in receptor affinity binding and cAMP functional assays at human melanocortin receptors 1b, 3, 4 and 5 (hMC1b,3–5R). Several of the new peptides were high potency agonists (partial) at hMC1bR (EC_{50} from 0.5 to 20 nM) and largely inactive at hMC3–5R. The bulky aromatic side chain in position 9, such as that in Trp, was found not to be essential to agonism (partial) of the studied peptides at hMC1bR.

1. Introduction

Physiological functions of melanotropins (αMSH, βMSH, γMSH and ACTH) are mediated by specific transmembrane-spanning proteins designated melanocortin receptors (MC1–5R) [9,12,13,16]. These receptors are coupled to G-proteins and their activation leads to the increase of cAMP production, protein kinase A activation and $[Ca^{2+}]_i$ elevation [12,14,15,22,29,30]. The melanocortin receptor 1 (MC1R) was initially cloned from mouse melanoma cells and normal human melanocytes [11,24]. It was subsequently detected in numerous other peripheral sites such as keratinocytes,

[1] Throughout this report, the numbering of the amino acid residues in αMSH, 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]-NH_2, has been retained for all linear and cyclic peptides.

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Abbreviations: Aic, 2-amino-indane-2-carboxylic acid; Bip(4,4′), 4,4′-biphenylalanine; Cha, cyclohexylalanine; Chg, cyclohexylglycine; Nal(2′), 2′-naphthylalanine; Phg, phenylglycine; Pip, pipecolic acid; Tic, tetrahydroquinoline-3-carboxylic acid.

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sebocytes, endothelial and immune cells (macrophages, fibroblasts, monocytes, mast cells, neutrophils and dendrite cells), the pituitary, testis, corpus luteum, placenta, and others [11,12,24]. In the last decade, various aspects of pharmacology of MC1R function in skin and hair of mammals have been intensively studied [12,14,17,23,25,26]. It was established that activation of MC1R by α-melanotropin (αMSH), and other melanocortin peptides, results in the proliferation of melanocytes in epidermis and in the stimulation of melanotropic enzymes that control the relative amount pheomelanin and photoprotective eumelanin [12,17,23]. Variants of MC1R that cause coat changes in rodents have been identified [10]. In humans, several MC1R mutants have been described which are associated with red hair and tendency to sunburn [17,23]. Additionally, loss-of-function variants of that receptor have been associated with increased risk of melanoma [17,23]. It was consequently suggested that appropriate MC1R ligands might offer protection against the damaging and mutagenic effects of UV radiation [14,29,30].

In structures of the endogenous MC1R agonists (αMSH, βMSH, γMSH and ACTH), a short segment, His^6-Phe^7-Arg^8-Trp^9,[2] was identified as critical for molecular recognition [20,27]. The same segment is also present in the structures of synthetic, peptidic hMC1R ligands [1–8,18–21,27]. Of those compounds, a linear peptide NDP-αMSH, 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}-NH_2, and a lactam derivative, Ac-Nle^4-cyclo-(Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10})-NH_2, designated MTII, have been most frequently used as research tools [1,27]. These more enzyme resistant analogs of αMSH are highly potent hMC1R agonists but not selective versus other melanocortin receptor subtypes. In the ''essential segment'' of NDP-αMSH and MTII, His^6-D-Phe^7-Arg^8-Trp^9, the aromatic residues in positions 7 (Phe) and 9 (Trp) were reported to be crucial for efficient interactions with the hMC1R and other receptor isoforms, while the basic residues, His^6 and Arg^8, were considered less essential [1–8,18–21,27].

Our recent structure–function studies on NDP-αMSH and MTII have shown that the side chain of Arg^8 is not critical for molecular recognition at the hMC1bR and hMC5R (the receptors found mainly in peripheral sites) but is required for the interactions of these peptides with the hMC3R and hMC4R (the receptors present predominantly in the CNS) [5–7]. The human melanocortin 1b receptor, hMC1bR, possesses pharmacological properties virtually identical to that of its isoform, human melanocortin receptor 1a, hMC1aR [28]. In several compounds studied, sterically constrained cyclic amino acids were incorporated in position 8 (Arg), and most of the new analogs showed high agonist potency at hMC1b,5R but were poor ligands for hMC3,4R, even at 10 μM peptide concentration [5,6]. Structures of these new, dual hMC1b,5R agonists subsequently served as templates for the design of potent hMC5R agonists, selective with respect to hMC1bR and also hMC3,4R [5,6].

In the present study, a role of the Trp^9 residue of the ''essential core'' of MTII in molecular recognition at the MC receptors was evaluated. Analogs with conformationally constrained amino acids such as Pro, Pip, Tic and others in position 9 (Trp) were tested at hMC1b,3–5R in affinity binding and cAMP activation assays [2]. It was anticipated that a cyclic amino acid in position 9 might stabilize some bioactive conformations of the lactam ring and perhaps alter selectivity of MTII for the MC receptors. Several new compounds lacking the indole side chain of Trp^9 displayed high agonist potency at hMC1bR (EC_{50} from 1 to 20 nM) and were largely inactive at hMC3–5R. Their syntheses and pharmacological evaluation *in vitro* at hMC1b,3–5R are reported.

2. Materials and methods

2.1. Peptide synthesis, purification and characterization

Elongation of peptidyl chains on *p*-methylbenzhydrylamine polystyrene resin, 0.20 mmol/g loading (Boc-synthesis, 431A ABI peptide synthesizer), deprotection and cleavage of peptides from the resin with HF, and purification of the crude products by high-pressure liquid chromatography were performed as previously described in detail [2]. A standard gradient system of 10–100% buffer B in 30 min (G1) was used for analysis; buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% trifluoroacetic acid in acetonitrile. The second gradient system used for analysis was 0–100% buffer B in 30 min (G2); buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% trifluoroacetic acid in methanol. The chromatographically homogenous compounds were analyzed by electrospray mass spectrometry (Hewlett Packard series 1100 MSD spectrometer).

2.2. Competitive binding assays

Binding activity of compounds was measured using membranes from Chinese hamster ovary (CHO) cells expressing the cloned melanocortin receptors. Binding assay wells contained membranes, 200 pM [^{125}I]NDP-αMSH (New England Nuclear Corp.), and increasing concentrations of unlabeled test compounds from 0.05 to 20 μM. Reactions were incubated for 1.5 h and then filtered as described previously [2]. Nonspecific binding was determined in the presence of 1 uM NDP-αMSH. Binding data were analyzed using GraphPad curve fitting software. Active peptides were evaluated in three or more independent experiments.

2.3. cAMP assays

Agonist activities of all compounds were measured using Chinese hamster ovary cells expressing the cloned melanocortin receptors (see Ref. [2] for details). Cells were detached from tissue culture flasks, collected by 5 min centrifugation, and resuspended in Earle's Balanced Salt solution (Life Technologies, Gaithersburg, MD) with addition of 10 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM glutamine, and 1 mg/ml bovine serum albumin. Compounds from 0.003 to 5000 nM concentration together with 0.6 mM isobutylmethylxanthine

[2] Throughout this report, the numbering of the amino acid residues in αMSH, 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}-NH_2, has been retained for all linear and cyclic peptides.

(IBMX) were incubated at room temperature with dissociated cells for 40 min and lysed with 0.1 M HCl to terminate the assay (SMP-001J) or with PerkinElmer detection buffer (SMP-004). cAMP was quantitated by PerkinElmer Life Sciences (NEN) (Boston, MA) SMP-001J or SMP-004 Flashplate cAMP assay. Activation by compounds was compared to the maximum response to αMSH. Active peptides were evaluated in three or more independent experiments. Data analysis of cAMP assays was performed with GraphPad Prism curve fitting software from GraphPad, San Diego, CA.

3. Results

Analogs of MTII listed in Tables 1 and 3 and analogs of NDP-αMSH listed in Table 2 were prepared by solid phase syntheses as previously described (see Ref. [2] and Section 2). They were evaluated for their binding affinities to the human melanocortin receptors 1b, 3, 4 and 5 in the competitive binding assays using the radiolabeled ligand [^{125}I]-NDP-αMSH and for their agonist potency in cAMP assays employing CHO cells expressing these receptors [2]. The human melanocortin 1b receptor possesses pharmacological properties virtually identical to that of its isoform, human melanocortin receptor 1a [28]. Functional antagonism of peptides discussed in this study was not determined.

Binding affinity and activation data for Pro analogs of MTII (**1–5**) at hMC3–5R have previously been reported by us [3]. Results of their testing in the hMC1bR assays are summarized in Table 1. Peptide **1** with Pro in place of Nle^4 (external to the lactam ring) was a high potency agonist at hMC3–5R and at hMC1bR (EC_{50} = 5.4 nM). Similarly, the analog of MTII with His^6 of the lactam ring replaced by Pro (**2**) showed high agonism at hMC3–5R and hMC1bR as well. In contrast, peptide **4** with Pro in position 8 (Arg) of the lactam ring was a weak, micromolar agonist at hMC1bR, and also, a rather low affinity ligand for hMC3,4R (IC_{50}s = 1.18 and 0.66 μM, respectively), and a moderately potent agonist at hMC5R (IC_{50} = 300 nM, EC_{50} = 360 nM) [3]. Replacement of the aromatic residues of MTII, Phe^7 or Trp^9, with proline yielded compounds nearly inactive at hMC3–5R [3]. At hMC1bR, the D-Pro^7 analog (**3**) was not able to form stable ligand–receptor complexes but, peptide with the Trp^9 → Pro substitution (**5**) elicited a moderate, partial agonist response (EC_{50} = 48 nM, ca. 60% maximum cAMP accumulation at 10 μM). Unlike the other tested Pro peptides, compound **5**, Pro^9-MTII, displayed therefore an enhanced selectivity for hMC1bR.

In parallel studies, Pro analogs of NDP-αMSH (**6**) were evaluated in receptor affinity binding and cAMP accumulation assays at hMC1b,3–5R, data compiled in Table 2. NDP-αMSH shares with MTII a common structural motif: His^6-D-Phe^7-Arg^8-Trp^9, which is critical for efficient interactions of these peptides with the melanocortin receptors [21,27]. The amino acid residues external to this ''essential core'' have been considered less crucial to molecular recognition. In this study, analogs of NDP-αMSH (**7–11**, **16–18**) in which residues 1 through 5 and 10 through 13 were replaced with Pro, one at a time, showed agonist potency at hMC1b,3–5R equal or up to 80-fold lower than that of NDP-αMSH. Pro^4-NDP-αMSH, **10**, was about 100-fold weaker agonist at hMC5R than the parent compound. Interestingly, Pro^4-MTII was previously reported [3] to be also an about 150–750-fold less effective activator at hMC5R than MTII (**19**).

Single replacement of ''the essential residues'' of NDP-αMSH (6 through 9) with Pro resulted in compounds **12–15** which elicited diverse effects at hMC1bR. Hence, an analog modified in position 6 (His^6 → Pro) was a highly potent agonist at hMC1bR and at the other counter screened MC receptors (**12**). It generated about 30% and 60% maximum cAMP response at hMC3/4R, respectively. In contrast, Pro in position 7 (Phe) or 8 (Arg) of NDP-αMSH was deleterious to the formation of stable ligand–peptide complexes with all studied isoforms of the melanocortin receptor (compounds **13** and **14**). The next compound, with Pro in place of Trp^9 (**15**), was previously shown to be practically inactive at hMC3–5R at micromolar concentrations [3]. Yet in this study, it was measured as a full 2.9 nM agonist at hMC1bR and thus displayed a rather high selectivity for this receptor (greater than 2000-fold).

Together, from the first part of our structure–function studies on analogs of MTII and NDP-αMSH, compounds with Pro in position 9 (Trp), **5** and **15**, emerged as hMC1bR agonists with enhanced selectivity for this receptor. Their structures have subsequently served as scaffolds for the design of other agonists of more desirable pharmacological properties at hMC1bR. Syntheses of the NDP-αMSH analogs with modified

Table 1 – Proline analogs of MTII

No.	Compound*	hMC1bR	
		Binding assay, IC_{50} (nM)≠	cAMP assay, EC_{50} (nM)≠≠
	MTII	0.36 ± 0.1	0.31 ± 0.3
1	Nle^4 → Pro	7.8 ± 1.5	5.4 ± 2
2	His^6 → Pro	5.7 ± 2	3.8 ± 1
3	D-Phe^7 → D-Pro	4%@10 μM	12%@5 μM
4	Arg^8 → Pro	2500 ± 310 (64%@ 10 μM)	130 ± 35 (63%@5 μM)
5	Trp^9 → Pro	470 ± 100 (73%@10 μM)	48 ± 15 (56%@5 μM)

*Ac-Nle^4-cyclo(Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10})-NH_2; ≠concentration of peptide at 50% specific binding or, the percentage of inhibition (relative to [^{125}I]-NDP-αMSH) observed at a given peptide concentration (μM); ≠≠concentration of peptide at 50% maximum cAMP accumulation (and the percentage of cAMP accumulation relative to [^{125}I]-NDP-αMSH at the concentration > 5 μM), or the percentage of cAMP accumulation observed at the designated μM concentration. Data are the average of 3 or more independent determination together with the standard error of the mean.

Table 2 – Proline analogs of NDP-αMSH*

No.	Compound	Binding assay≠							cAMP assay≠≠						
		IC_{50} (nM)				Selectivity			EC_{50} (nM)				Selectivity		
		MC1bR	hMC3R	hMC4R	hMC5R	3/1 b	4/1 b	5/1 b	MC1bR	hMC3R	hMC4R	hMC5R	3/1 b	4/1 b	5/1 b
6	NDP-αMSH	0.39 ± 0.01	0.78 ± 0.03	1.6 ± 0.03	0.27 ± 0.01	2	4	0.7	0.87 ± 0.04	0.29 ± 0.006	0.58 ± 0.01	0.37 ± 0.008	0.3	0.6	0.4
7	Ser^1 → Pro	0.2 ± 0.09	0.4 ± 0.18	0.8 ± 0.41	0.2 ± 0.07	2	4	1	0.2 ± 0.11	0.15 ± 0.09	0.52 ± 0.3	0.23 ± 0.15	1	2	1
8	Tyr^2 → Pro	1.2 ± 0.6	3 ± 0.5	3.3 ± 2	2 ± 0.2	2.5	2.7	1.6	0.92 ± 0.53	0.73 ± 0.43	3.1 ± 1.8	1.7 ± 0.5	0.8	3	2
9	Ser^3 → Pro	0.52 ± 0.2	0.95 ± 0.4	1.5 ± 0.7	0.46 ± 0.22	1.8	2.8	0.9	0.26 ± 0.15	0.29 ± 0.17	0.96 ± 0.4	0.75 ± 0.4	1	3.5	3
10	Me^4 → Pro	5.2 ± 0.23	4.8 ± 0.68	10 ± 0.5	48 ± 2.4	0.9	2	9	6.9 ± 0.4	1.7 ± 0.2	2.8 ± 1.3	33 ± 2	0.2	0.4	4
11	Glu^5 → Pro	0.2 ± 0.09	1.6 ± 0.3	1.1 ± 0.18	12 ± 2.4	8	5	60	0.4 ± 0.05	1.5 ± 0.09	1.3 ± 0.5	8.2 ± 3	4	3	20
12	His^6 → Pro	1.5 ± 0.1	2.3 ± 0.27	2.1 ± 0.3	0.43 ± 0.04	1.5	1.4	0.3	0.7 ± 0.4	1.1 ± 0.5 (36%)	9.9 ± 5 (67%)	0.6 ± 0.2	1.5	14	1
13	D-Phe^7 → D-Pro	16%@2.5 μM	5%@2.5 μM	4%@2.5 μM	3%@2.5 μM				7%@5 μM	0%@5 μM	1%@5 μM	0%@5 μM			
14	Arg^8 → Pro	>1000	>10000	>10000	>10000				9%@5 μM	3%@10 μM	9%@5 μM	21%@10 μM			
15	Trp^9 → Pro	5.3 ± 0.4	25%@5 μM	17%@5 μM	33%@5 μM				2.9 ± 0.5	>1500 (31%)	6%@2.5 μM	15%@2.5 μM			
16	Gly^{10} → Pro	3.9 ± 0.2	15 ± 1.9	24 ± 3	19 ± 0.45	3.8	6	5	2.7 ± 0.9 (78%)	4.1 ± 0.2 (70%)	5.8 ± 0.6	28 ± 8 (41%)	1.5	2	10
17	Lys^{11} → Pro	2.4 ± 0.2	4 ± 0.9	6.2 ± 0.2	1.1 ± 0.02	1.6	3	0.4	1.3 ± 0.4	2.3 ± 0.3 (70%)	1.4 ± 0.8	1.3 ± 0.2 (78%)	1.7	1	1
18	Val^{13} → Pro	0.7 ± 0.5	0.7 ± 0.2	1.6 ± 0.07	0.34 ± 0.24	1	2	0.5	0.64 ± 0.1	0.61 ± 0.2	0.42 ± 0.18	0.61 ± 0.09	1	0.6	1

*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}-NH_2; ≠concentration of peptide at 50% specific binding or, the percentage of inhibition (relative to [^{125}I]-NDP-αMSH) observed at a given peptide concentration (μM); ≠≠concentration of peptide at 50% maximum cAMP accumulation (and the percentage of cAMP accumulation relative to [^{125}I]-NDP-αMSH at the concentration > 5 μM), or the percentage of cAMP accumulation observed at the designated μM concentration. Data are the average of 3 or more independent determination together with the standard error of the mean.

Table 3 – Analogs of MTII

No.	Compound*	Binding assay IC_{50} (μM)≠				Selectivity			cAMP assay EC_{50} (μM)≠≠			
		MC1bR	hMC3R	hMC4R	hMC5R	3/1b	4/1b	5/1 b	MC1bR	hMC3R	hMC4R	hMC5R
19	MTII	0.36 ± 0.1	3.1 ± 0.094	0.18 ± 0.05	1.2 ± 0.15	9	0.5	3.3	0.31 ± 0.3	1.1 ± 0.06	0.26 ± 0.01	2.3 ± 0.06
20	Ala^{9}	380 ± 16	12%@10 μM	31%@10 μM	25%@19 μM				80 ± 7 (60%)	2%@10 μM	21%@10 μM	10%@10 μM
21	Me^{9}	36 ± 21	8300 ± 500	2900 ± 860	>10000	230	80		5.5 ± 2 (75%)	470 ± 120 (60%)	86%@ 5 μM	>5000 (37%)
22	Cha^{9}	2.8 ± 2	240 ± 130	240 ± 60	560 ± 190	85	85	200	3.4 ± 1 (65%)	15 ± 7	390 ± 75	410 ± 130 (45%)
23	Tyr(Me)^{9}	3 ± 0.5	170 ± 70	2 ± 0.6	145 ± 30	56	1	48	16 ± 4 (45%)	110 ± 50 (68%)	6 ± 0.8	130 ± 80 (54%)
24	D-Nal(2′)^{7} (SHU9119)	0.62 ± 0.11	0.23 ± 0.04	0.07 ± 0.01	0.65 ± 0.01	0.4	0.1	1	0.36 ± 0.02 (54%)	5%@1 μM	3%@1 μM	0.088 ± 0.04
25	D-Nal(2′)^{7}, Pro^{9}	190 ± 30	>1000 (36%)	1330 ± 90 (71%)	610 ± 59		7	3	30 ± 11 (46%)	6%@5 μM	0%@10 μM	3400 ± 50 (50%)
26	D-Tyr(Me)^{7}, Pro^{9}	12 ± 3	>10000	29%@10 μM	>10000				18 ± 6 (70%)	13%@5 μM	>20000	19%@5 μM
27	D-Tyr(Me)^{7}, Pip^{9}	13 ± 1.6	>4000	2910 ± 150 (55%)	2760 ± 660		220	210	7.7 ± 4 (71%)	18%@4 μM	54%@2 μM	1650 ± 500 (51%)
28	D-Tyr(Me)^{7}, Tic^{9}	1.2 ± 0.3	230 ± 160	220 ± 23	90 ± 22	190	180	75	1.3 ± 1 (86%)	108 ± 32 (75%)	220 ± 36 (73%)	230 ± 75
29	D-Tyr(Me)^{7}, Sar^{9}	7 ± 2	>10000	>7000	1370 ± 570 (83%)			200	7.7 ± 1 (71%)	17%@5 μM	12%@5 μM	1700 ± 200
30	D-Tyr(Me)^{7}, Aib^{9}	23 ± 3	>20000	1530 ± 260 (77%)	>10000		66		20 ± 0 (58%)	10%@5 μM	58%@5 μM	>5000
31	D-Tyr(Me)^{7}, Phg^{9}	1.9 ± 0.8	>10000	6200 ± 2160 (60%)	3950 ± 2850 (72%)		3260	2100	20 ± 8 (90%)	>3000	12%@5 μM	>3500
32	D-Tyr(Me)^{7}, Chg^{9}	11 ± 3	12%@2 μM	1720 ± 108 (37%)	1260 ± 200 (51%)		150	120	7.4 ± 2 (69%)	24%@1 μM	41%@1 μM	>500
33	D-Tyr(Me)^{7}, Cha^{9}	1 ± 0.8	160 ± 86	43 ± 3	105 ± 37	160	43	110	2.1 ± 0.7 (62%)	40 ± 20 (19%)	140 ± 60 (50%)	49 ± 20 (79%)
34	D-Tyr(Me)^{7}, Aic^{9}	1.2 ± 0.2	220 ± 110	16 ± 6	240 ± 180	180	13	200	14 ± 1 (62%)	940 ± 270 (35%)	130 ± 20 (80%)	230 ± 20 (82%)
35	D-Bip(4,4′)^{7}	1.2 ± 0.39	3.3 ± 0.31	0.98 ± 0.25	0.54 ± 0.22	3	0.8	0.5	0.7 ± 0.06 (64%)	1.2 ± 0.12 (43%)	8%@2.5 μM	0.42 ± 0.03

*Ac-Nle^{4}-cyclo(Asp^{5}-His^{6}-D-Phe^{7}-Arg^{8}-Trp^{9}-Lys^{10})-NH_2; ≠concentration of peptide at 50% specific binding or, the percentage of inhibition (relative to [^{125}I]-NDP-αMSH) observed at a given peptide concentration (μM); ≠≠concentration of peptide at 50% maximum cAMP accumulation (and the percentage of cAMP accumulation relative to [^{125}I]-NDP-αMSH at the concentration > 5 μM), or the percentage of cAMP accumulation observed at the designated μM concentration. Data are the average of 3 or more independent determination together with the standard error of the mean.

residue 9 and their *in vitro* biding affinity and activation characteristics were summarized in a separate manuscript [8]. Herein, analogs of MTII with altered position 9 (Trp) are discussed, listed in Table 3. Most of the new cyclic peptides were poor ligands for the hMC3–5R (virtually inactive at micromolar peptide concentration), yet of noticeably high agonist potency at hMC1bR (about 50–90% maximal cAMP response).

Replacement of Trp^9 in MTII (**19**) with conformationally flexible amino acids, Ala, Nle or Cha, led to partial agonists at the hMC1bR, **20–22** (EC_{50} from 3 to 80 nM, about 60–80% activation). Also, peptide with Tyr(Me) in position 9, compound **23**, was not able to activate fully the hMC1bR (EC_{50} = 16 nM, 45% activation).

The subsequent two analogs were Pro^9 peptides with D-Nal(2′) and D-Tyr(Me) in positions 7, **25** and **26**, respectively. The D-$Nal(2')^7$,Pro^9 compound (**25**) elicited a partial agonist response at hMC1bR, similarly to D-$Nal(2')^7$-MTII (designated SHU9119, **24**) [19]. This peptide, however, was about 100-fold weaker ligand at hMC1bR than SHU9119. Compound **25** was also a micromolar ligand for hMC3–5R, whereas SHU9119 (**24**) was a sub-nanomolar binder to those receptors.

The Pro^9 analog with D-Tyr(Me) in position 7 (**26**) displayed about 10–40-fold higher binding affinity for hMC1bR than its MTII and SHU9119 counterparts (Pro^9-MTII, **5**, and Pro^9-SHU9119, **25**). This suggests that D-Tyr(Me) in position 7 of Pro^9-MTII (**26**) allowed for stronger interactions with hMC1bR than D-Phe or D-Nal(2′) in the same position [**26**, D-$Tyr(Me)^7$,-Pro^9-MTII at hMC1bR, EC_{50} = 18 nM, about 70% maximum cAMP response at 10 μM]. Yet similarly to **5** and **25**, this compound was ineffective as a ligand for hMC3–5R, hence, of higher receptor subtype selectivity.

As a result, the subsequent analogs of MTII (**27–34**) were designed to include D-Tyr(Me) in position 7. Their structures also encompassed various hydrophobic residues in place of Trp^9. Peptide **27** with pipecolic acid in position 9, D-$Tyr(Me)^7$,Pip^9-MTII, displayed a pharmacological profile at the melanocortin receptors 1b, 3, 4 and 5 similar to that of the D-$Tyr(Me)^7$,Pro^9 analog, **26**. Peptide **27** was a 13 nM ligand at hMC1bR and practically inactive at hMC3–5R. Interestingly, incorporation of the aromatic tetrahydroquinoline-3-carboxylic acid (Tic) moiety in position 9 resulted in **28**, D-$Tyr(Me)^7$,Tic^9-MTII, which was about 10-fold more potent at hMC1bR than the Pro^9 and Pip^9 analogs, **26** and **27**, respectively. The Tic^9 compound showed enhanced agonist potency at hMC3–5R as well (EC_{50} = 100–250 nM), and therefore was of lower receptor subtype selectivity.

To test the impact of the steric constrains in position 9 on the receptor selectivity of D-$Tyr(Me)^7$-MTII analogs, small amino acids, Sar and Aib, were incorporated in place of Trp (**29**, **30**). In the structure of the Sar^9 peptide (**29**), a conformationally constrained side chain and the free Nα-H in position 9 were absent. Yet this analog (**29**) displayed about two-fold higher agonist potency at hMC1bR than that of the Pro^9 peptide (**26**) and was practically inactive at hMC3–5R (**29**: at hMC1bR EC_{50} = 7.7 nM, 71% maximum cAMP release at 10 μM). Another analog lacking a conformationally constrained cycle in position 9 but with the free Nα-H in the same position, the Aib^9 peptide (**30**) was about as potent at hMC1bR as the Pro^9 peptide (**25**).

In the last group of D-$Tyr(Me)^7$ compounds (**31–34**), amino acids with a primary alpha-amino group and a hydrophobic, sterically constrained side chain were incorporated in position 9. The new compounds (**31–34**) were potent agonists at hMC1bR (EC_{50} from 2 to 20 nM, 60–90% maximum cAMP release). At the counter screened melanocortin receptors, the Phg^9 and Chg^9 analogs (**31**, **32**) were rather weak ligands or almost inactive, while those with Cha^9 or Aic^9 (**33**, **34**) displayed moderate binding affinity and hence were less receptor subtype selective.

The D-$Bip(4,4')^7$ peptide (**35**) was measured in this study to be a potent, yet partial agonist at the hMC1bR (about 60% activation) and not selective with respect to hMC3–5R.

4. Discussion and conclusions

α-Melanotropin is a highly potent agonist at the human MC1bR, but of modest selectivity with respect to hMC3–5R (about 5–20-fold). Its two best known synthetic analogs: NDP-αMSH and MTII are also not receptor subtype selective [1,27]. Described herein structure–function studies on MTII yielded the Pro^9 analog—an agonist of enhanced hMC1bR selectivity. Its structure served as a scaffold for the design of other compounds with more desirable *in vitro* pharmacological properties at hMC1bR.

Briefly, binding and functional characteristics at hMC3–5R of analogs of MTII in which each amino acid in turn was replaced with Pro were reported by us several years ago [3]. It was expected that incorporation into the peptide chain of a conformationally constrained amino acid such as Pro might stabilized some lower energy conformers and, consequently, alter a pattern of the peptide-receptor recognition. In the course of this study, we tested the Pro analogs in the binding and functional assays at hMC1bR. With one exception, all evaluated peptides displayed similar agonist properties at hMC1bR to those previously reported at hMC3–5R, and hence they were not receptor subtype selective.

The exception was peptide **5** with Pro in place of Trp^9; compound previously shown to be nearly inactive at hMC3–5R even at micromolar concentrations [3]. Yet in this study, Pro^9-MTII was found to act as a moderate potency, partial agonist at hMC1bR. The ability of this peptide to elicit an agonist response at hMC1bR indicated that alterations to the side chain in position 9 are not deleterious to molecular recognition. In contrast, such changes clearly precluded activation of hMC3–5R [3]. Similar conclusions were drawn from the parallel studies on the Pro analogs of NDP-αMSH. Pro^9-NDP-αMSH (**15**) was found to elicit a full, 2.9 nM agonist response at hMC1bR, but was measured to be largely inactive at hMC3–5R. This compound was somewhat less potent at hMC1bR than NDP-αMSH (about 10–20-fold), but of a high selectivity with respect to hMC3–5R (greater than 2000-fold). Subsequent detailed structure–function studies around position 9 of NDP-αMSH have been summarized separately [8].

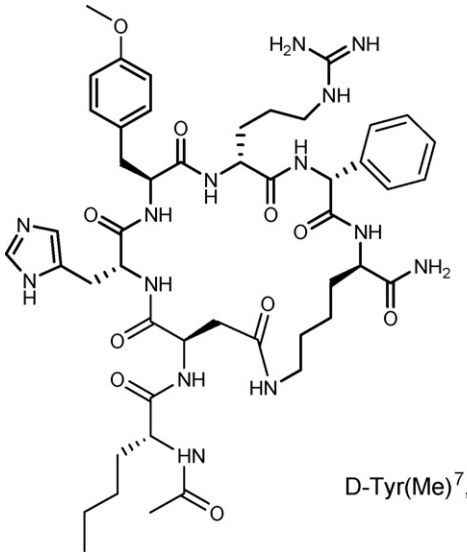
The Pro^9 analog of MTII (**5**) was an approximately 150–1500-fold less effective ligand at the hMC1bR than MTII. Unlike the parent compound, this lead peptide was not able to active fully the hMC1bR at the concentrations tested. This suggested that steric constrains imposed on the lactam cycle by the Pro ring

disturbed peptide conformations required for full activation of the hMC1bR. Absent from the structure of this peptide was the bulky, hydrophobic side chain of Trp^9, which might be required for the stabilization of biologically active conformers at the receptor site, and/or for hydrophobic interactions with the receptor. However, the side chain of Trp^9 was clearly not necessary for the full agonism of Pro^9-NDP-αMSH (**15**) at the hMC1bR. Perhaps the rather ''flexible'', linear peptide chain of this compound, unlike the constrained lactam ring of MTII, may adopt conformations which evoke full agonist response at hMC1bR, regardless of the type of the side chain in position 9. Such ''active'' conformers might also be stabilized by interactions of the hMC1bR with the residues other than those of the ''essential core'' of NDP-αMSH (located at the N-terminal and/or C-terminal ends of the His^6-D-Phe^7-Arg^8-Trp^9 segment). Those residues, 1 through 3 and 11 through 13, are not present in the structures of the MTII peptides. Interestingly, analogs of MTII with aliphatic, rather flexible moieties, Ala, Nle or Cha, in position 9 (Trp) displayed partial agonism at hMC1bR as well (**20–22**). At this receptor, their pharmacological profiles were similar to that of Pro^9-MTII (with the sterically constraining amino acid in position 9) but different from that of MTII (with Trp in position 9). Inability of the new peptides to activate fully the hMC1bR suggested that the bulky aromatic side chain of Trp is necessary for stabilization of the lactam conformers which are required for the full agonist response at hMC1bR, and/or for interactions with the receptor. Consistent with this suggestion were the *in vitro* functional characteristics of Tyr(Me)^9-MTII at hMC1b,3–5R. This peptide, with a ''smaller'' aromatic group in position 9 than that of Trp, like the analogs with non-aromatic moieties in the same position, was a partial agonist at hMC1bR. It was also a moderately potent, partial agonist at the hMC3,5R but a full 6 nM agonist at the hMC4R.

Another aromatic side chain of the ''essential core'' of MTII, that of D-Phe^7, has been long recognized as critical to agonism at the MC receptors [1–4,19]. Yet for the efficient activation of hMC1bR, opposite steric requirements seem to apply to the aromatic residue in position 7 compared to the one in position 9. In position 9, a bulky aromatic side chain of Trp appears to be preferable (discussed above), but in position 7, a rather ''small'' aromatic group such as that of D-Phe is required. MTII with D-Phe^7 is a full 0.3 nM agonist at hMC1bR, whereas its analog with the bulky naphthyl side chain in position 7 [D-Nal(2′)^7-MTII, designated SHU9119, **24**] is a partial agonist (about 50% activation, EC_{50} = 0.3 nM), in spite of its high binding affinity for hMC1bR (IC_{50} = 0.3 nM). The naphthyl group in position 7 also renders this peptide inactive for agonism at hMC3,4R; SHU9119 is a highly effective antagonist at these receptors.

Similar steric requirements for amino acid in position 7 appeared to govern interactions of yet another analog of MTII with hMC1bR, the D-(4,4′)Bip^7 peptide, **35** (EC_{50} = 0.7 nM, 64% activation). Our results indicate that the rather ''longish'' biphenyl side chain of D-Bip(4,4′)^7, like the naphthyl side chain in position 7 of SHU9119, allowed only for a partial activation at hMC1bR. The D-Bip(4,4′)^7 peptide elicited a partial agonist response at hMC3R as well, and was a full agonist at hMC5R, but basically inactive as a ligand for hMC4R at micromolar concentrations.

From the Pro^9 analogs of MTII with different aromatic residues in position 7 tested in this study (**5**, **25**, **26**), D-Tyr(Me)^7,Pro^9-MTII (**26**) showed the highest agonist potency at hMC1bR, about two-fold higher than that of its D-Phe^7 or D-Nal(2′)^7 counterparts, **5** and **25** (these three Pro^9 peptides were not able to reach 100% activation at the concentrations tested). Several analogs of D-Tyr(Me)^7, Pro^9-MTII with the flexible or constrained residues in position 9 were also potent (EC_{50} from 1 to 30 nM) but partial agonists (50–90% activation) at hMC1bR. With the exception of the Tic^9, Cha^9 and Aic^9 analogs (**28**, **33** and **34**) those peptides were poor ligands for the other counter screened receptors (hMC3–5R). The most interesting compound was D-Tyr(Me)^7, Phg^9-MTII (**31**) which reached almost 90% activation at hMC1bR (IC_{50} = 1.9 nM, EC_{50} = 20 nM).



D-Tyr(Me)^7,Phg^9-MTII (**31**)

Previously, we discussed analogs of MTII modified in position 8 (Arg) which were dual agonist at hMC1b,5R [5,6]. Compounds presented in this study showed that the appropriate changes at position 9 of MTII may lead to hMC1bR agonists of high selectivity versus the hMC5R and hMC3,4R as well (greater than 1000-fold). Taken together, our structure–function studies on MTII indicate that the side chains in positions 8 (Arg) and 9 (Trp) of the ''essential core'' of MTII affect selectivity of this peptide at the MC receptors. These moieties are required for the agonist potency at hMC3,4R, yet are less critical to agonism at hMC1b,5R (receptors found mainly in the peripheral tissues). Similarly, Holder et al. indicated [18] that appropriate modifications of the Trp^9 in the tetrapeptide Ac-His^6-D-Phe^7-Arg^8-Trp^9-NH_2 may yield melanocortin ligands of higher selectivity for the peripherally expressed mouse MC1R and MC5R versus the centrally expressed mouse MC3R and MC4R.

In summary: We report herein synthesis and biological evaluation *in vitro* of several analogs of MTII of high agonist potency (partial) at hMC1bR and improved selectivity with respect to hMC3–5R. These compounds could be useful in evaluation of the physiological role of hMC1bR in the peripheral tissues and in the design of new compounds.

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