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Further structure-activity studies of lactam derivatives of MT-II and SHU-9119: Their activity and selectivity at human melanocortin receptors 3, 4 and 5

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Abstract

Recently we have demonstrated that replacing His^6 by constrained amino acids in the well known antagonist SHU9119 resulted in potent and selective antagonist ligands especially at the *h*MC3R and *h*MC4 receptors. With the aim to further explore position 6 in the sequence of SHU9119 and MT-II, we have designed, synthesized, and pharmacologically characterized a series of peptide analogues of MT-II and SHU9119 at the human melanocortin receptors subtypes MC3R, MC4R and MC5R. All these peptides were modified at position 6 with constrained amino acids which are commercially available. In this study we have identified new selective ligands for the hMC4R, and an antagonist for the hMC3/hMC4 receptors. Additionally, we have discovered an interesting new selective antagonist at the *h*MC3R, Ac-Nle-c[Asp-βAla-DNal(2′)-Arg-Trp-Lys]-NH_2 (**2**, PG-106) which represents an important tool in further biological investigations of the *h*MC3R. PG-106 will be useful in further efforts to differentiate the substructural features responsible for selectivity at the *h*MC3R, *h*MC4R, and *h*MC5R.

Keywords

Melanocortin receptors; Melanotropins; Structure-activity relationships; Cyclic melanotropins; Receptor selective melanotropin antagonists

Introduction

Melanotropins (α-MSH, β-MSH, γ-MSH) are peptide hormones and neurotransmitters derived through a series of proteolytic cleavages from the precursor pro-opiomelanocortin (POMC). Over the past decade, molecular cloning of the five subtype receptors (MC1R to MC5R) for these peptides and ACTH has provided tools for systematic studies of their physiological effects [7,8,9,10,22,27,30]. The natural ligands for these receptors include α-MSH, β-MSH, γ-MSH as well as ACTH. α-MSH is a linear peptide with 13 residues (Ac-Ser-Tyr-Ser-Met-

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Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2). Recent discoveries have demonstrated that these peptides and their derivatives also display several other effects besides pigmentation and adrenal function including the regulation of feeding and sexual behaviour, and the modulation of the immune system [e.g. 1,5-8]. Substitution of DPhe-7 Phe-7 and Nle for Met-4 respectively yields NDP-α-MSH which was found to be a superagonist at melanocortin receptors [1,19, 26].

Intensive efforts are currently being made to develop selective ligands for the melanocortin receptors to understand the physiological roles played by these receptors [6,12,17,20]. In particular, clarification of the role of melanocortin receptor subtypes, *i.e*., MC3R, MC4R and MC5R [4,5,23,29] is particularly important specially after the discovery that MC3R and MC4R are involved in the regulation of feeding [11,21,28]. In fact, agonists of the MC4R cause a reduction of food intake by inducing satiety, while antagonists promote feeding [11,28]. Ligands at this receptor represent an attractive pharmaceutical target in development of drugs to the treatment of obesity and other eating disorders. Previous extensive structure-activity studies on melanotropins, in particular on α-MSH, led to the small cyclic peptide MT-II, Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2 (1), a potent and non-selective agonist at the human melanocortin receptors (18). Similarly, the lactam analogue Ac-Nle-c[Asp-His-*D*Nal(2′)-Arg-Trp-Lys]-NH_2, SHU-9119, is a potent antagonist at human melanocortin 3 and 4 receptors (19) and a partial agonist at the *h*MC5R (19,120). Recently, extensive structure-activity studies performed in our laboratory have shown that the replacement of His residue in position 6 in the sequence of MT-II and SHU9119 (Ac-Nle-[Asp-His-*D*Phe/*D*Nal(2)-Arg-Trp-Lys]-NH_2), with some dihedral constrained amino acids led to potent and highly hMC3R and hMC4R selective ligands [2,13,14,15]. Also, the introduction of β-modified proline in position 6 of MT-II sequence resulted in analogues with enhanced hMC5R selectivity [3]. In our effort to obtain new melanotropin peptides with improved potency and selectivity, we have designed and synthesized a series of novel cyclic MT-II and SHU9119 analogues in which we investigate the impact of additional constrained amino acids in position 6 on receptor selectivity.

Materials and Methods

Materials

N^{α}-Fmoc-protected amino acids and resin were purchased from Advanced ChemTech (Louisville, KY). HBTU and HOBt were purchased from Quantum Biotechnologies (Montreal, Quebec, Canada). For the N^{α}-Fmoc-protected amino acids, the following side chain protecting groups were used: Lys(N^{ε}-Alloc); Arg(*N*gu-Pbf); Asp(β-Allyl); His(N^{im}-Trt) and Trp(N^{in}-Boc). All protected amino acid derivatives were analyzed for purity by thin-layer chromatography before use. Peptide synthesis solvents, reagents, as well as CH_3CN for HPLC were reagent grade and were acquired from commercial sources and used without further purification unless otherwise noted. TLC was done on Analtech, Inc. (Newark, DE) silica gel 60 F_{254} plates using the following solvent systems: (A) 1-butanol / acetic acid / pyridine / water (5:5:1:4); (B) ethyl acetate / pyridine / acetic acid / water (5:5:1:3); (C) 1-butanol / acetic acid / water (4:1:1). The peptides were detected on the TLC plates using iodine vapor. Amino acid analyses were performed at the University of Arizona Biotechnology Core Facility. The system used was an Applied Biosystems Model 420A amino acid analyzer with automatic hydrolysis (Vapor Phase at 160 °C for 1 h 40 min using 6 N HCl) and a precolumn phenylthiocarbamyl-amino acid (PTC-AA) analysis. No corrections are made for amino acid decomposition. FAB-MS analyses were performed at the University of Arizona Core Facility. The instrument was custom made in Breman, Germany, and consists of a LIQUID SIMS4 Sectors AMD mass spectrometer. The experimental conditions consisted of a glycerol matrix-scan of 200-2000 Da in the positive ion mode. The purity of the finished peptides was checked by TLC in three solvent systems and by analytical RP-HPLC at 230, 254, and 280 nm using a Hewlett-Packard

1090 Series II Liquid Chromatograph with a built-in diode array detector (Table 1). In all cases, the purity of the finished peptides was greater than 95%. The structures of the pure peptides were confirmed by FAB-MS. The analytical data for the peptide is given in Table 1.

General Method for Peptide Synthesis and Purification

The protected peptide resins used to make the cyclic melanotropins were prepared using 0.5 g of Rink amide resin (0.7 mmol of NH_2/g of resin) by first coupling N^{α}-Fmoc-Lys(N^{ε}-Alloc)-OH to the resin previously deprotected by a 25% piperidine solution in DMF for 30 min. In this strategy automated solid phase synthesis was performed on the Advanced ChemTech ACT 396 instrument. The following protected amino acids were then added stepwise N^{α}-Fmoc-Trp(N^{in}-Boc)-OH, N^{α}-Fmoc-Arg(N^{γ}-Pbf)-OH, N^{α}-Fmoc-DNal(2′)-OH, Fmoc-DPhe-OH, N^{α}-Fmoc-His-OH or N^{α}-Fmoc-Xaa-OH N^{α}-Fmoc-Asp(β-Allyl)-OH, and N^{α}-Fmoc-Nle-OH. Each coupling reaction was accomplished using a 3-fold excess of amino acid with HBTU and HOBt in the presence of diisopropylethyl amine (DIEA, 6-fold excess). The N^{α}-Fmoc protecting groups were removed by treating the protected peptide resin with 25% piperidine solution in DMF twice (1 × 5 min and 1 × 25 min). The peptide resin was then washed three times with DMF and the next coupling step was then initiated in a stepwise manner. All reactions were done under an Argon atmosphere. Following the assembly of the protected peptide resin, the terminal N^{α}-Fmoc group was removed in the usual manner. The N^{α} amino group was acetylated with 25% acetic anhydride in dichloromethane for 20 min. The next step was removal of the N^{γ}-Alloc group of Lys and the β-allyl group of Asp under well controlled conditions [24]. For this the peptide resin was washed with CH_2Cl_2 under Argon atmosphere and was added a solution of $PhSiH_3$ (24 equiv.) in 2 mL of CH_2Cl_2 all under Argon. Then a solution of $Pd(PPh_3)_4$ (0.25 equiv.) in 6 mL of CH_2Cl_2 was added and reaction allowed to proceed under Argon for 30 min. Then the peptide resin was washed with CH_2Cl_2 (3x), with DMF (3x) and with DCM (4x), and the deprotection process repeated. The macrocyclic lactam ring formation was the mediated by addition of HBTU (6 equiv.), HOBt (6 equiv.) and DIEA (12 equiv.) for 2 h. The process was repeated if necessary (Kaiser test was used to monitor completion). The peptide was then cleaved from the resin using trifluoracetic acid/triethylsilane/H_2O (9.0:0.5:0.5) for 3 h. The resin was removed by filtration and the crude peptide recovered by precipitation using chilled anhydrous ethyl ether to give a white powder which was purified by HPLC on a C18-bonded silica column (Vydac 218TPP1010, 1.0 × 25 cm) eluting with a linear gradient of acetonitrile in aqueous 0.1% TFA. The products were obtained by lyophilization of the appropriate fractions after removal of the acetonitrile by rotary evaporation. Analysis by analytical HPLC and TLC (3 solvents) showed the peptides to be pure (>98%) (Table 1). The structures were further confirmed by high resolution mass spectroscopy and amino acid analysis.

Receptor Binding Assay

Competition binding experiments were carried out using whole HEK293 cells stably expressing human MC3, MC4, and MC5 receptors. HEK293 cells transfected with hMCRs [3] were seeded on 96-well plates 48 hours before assay (100,000 cells/well). For the assay, the cell culture medium was aspirated and cells were washed twice with a freshly prepared binding buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1mM 1,10-phenanthrolone, 0.5 mg/L leupeptin, 200 mg/L bacitracin. Next, cells were incubated with different concentrations of unlabeled peptide and labeled [^{125}I]-[Nle^4,*D*-Phe^7]-α-MSH (Perkin-Elmer Life Science, 100,000 cpm/well, 0.1386 nM) for 40 min at 37°C. The assay medium was subsequently removed and each well was washed twice with the binding buffer. The cells were then lysed by the addition of 250 μL of 0.1M NaOH and 250 μL of 1% Triton X-100. The lysed cells were transferred to 12×75 mm glass tubes and the radioactivity was measured by a Wallac 1470 WIZARD Gamma Counter.

Adenylate Cyclase Assay

HEK 293 cells transfected with human melanocortin receptors [3] were grown to confluence in MEM medium (GIBCO) containing 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 96-well plates 48 hours before assay (100,000 cells/well). For the assay, the cell culture medium was removed and the cells were rinsed with 1 mL of MEM buffer (GIBCO) or with Earle's balanced salt solution (EBSS, GIBCO). An aliquot (0.4 mL) of the Earle's balanced salt solution was placed in each well along with 5 μL 0.5 mM isobutylmethylxanthine (IBMX) for 1 min at 37°C. Next, varying concentration of aliquots of melanotropin peptides (0.1 mL) were added, and the cells were incubated for 3 min at 37°C. The reaction was stopped by aspirating the assay buffer and adding 0.15 mL ice-cold Tris/EDTA buffer to each well. After dislodging the cells with the help of trypsin, the cells were transferred to polypropylene micro-centrifuge tubes and placed in a boiling water bath for 15 min. The cell lysate was then centrifuged for 2 min at 6500 rpm, and 50 μL of the supernatant was aliquoted into an Eppendorf tube. The total cAMP content was measured by competitive binding assay according to the TRK 432 assay kit instructions (Amersham Corp., Piscataway, NJ).

Data Analysis

IC_{50} and EC_{50} values represent the mean of two experiments performed in triplicate. IC_{50} and EC_{50} estimates and their associated standard errors were determined by fitting the data using a nonlinear least squares analysis, with the help of GraphPad Prism 4 (GraphPad Software, San Diego, CA).

Results

The melanotropin analogues listed in Table 1, were prepared by solid-phase peptide synthesis and evaluated for their binding affinities to the human melanocortin receptors 3–5 in competitive binding assays using the radiolabeled ligand [^{125}I]-NDP-α-MSH and for their agonist or antagonist potency in cAMP assays employing the HEK293 cells expressing these receptors (Table 2).. Replacement of His^6 with a βAla residue in the MT-II sequence, yielded analogue **1** (Ac-Nle-c[Asp-βAla-*D*Phe-Arg-Trp-Lys]-NH_2, PG-105), with partial agonist activity at the *h*MC3R, but with 140-fold lower in binding affinity and 170-fold lower cAMP activity (EC_{50} = 320 and 1.85 nM, respectively) compared to MT-II. Analogue **1** was found to have no binding affinity for the *h*MC4 and *h*MC5 receptors at 10^{-5}M. Analogue **2** (Ac-Nle-c[Asp-βAla-*D*Nal(2′)-Arg-Trp-Lys]-NH_2, PG-106), which differs from **1** by having a *D*-Nal (2′) in position 7, was a selective antagonist at the *h*MC3R and totally inactive as a ligand at the *h*MC4R and *h*MC5R. These results confirm that simple, unconstrained amino acid residues in the 6 position of MT-II could generate a selective ligand at *h*MC3R.

On the other hand , the analogue **3** (Ac-Nle-[Asp-tBuGly-*D*Phe-Arg-Trp-Lys]-NH_2, PG-107) with a tButGly residue in position 6, that is, a residue more bulky and constrained in the side chain moiety, was found to have affinity for all melanocortin receptors tested in this study. Analogue **3** was a full and selective agonist at the *h*MC3R, almost 13-fold more selective for the *h*MC3R than the *h*MC4R, and 5-fold selective with respect to the *h*MC5R (IC_{50} = 41 nM, 550 nM, and 200 nM, respectively). Surprisingly, the analogue **4** (Ac-Nle-[Asp-tBuGly-*D*Nal (2′)-Arg-Trp-Lys]-NH_2, PG-108) which differs from 3 by having a *D*Nal(2′) in position 7, was found to have slight agonist activity at all melanocortin receptors tested. In fact, analogue **4** has a similar high affinity for both the *h*MC3R and *h*MC4R, and is slightly selective for the *h*MC5R (IC_{50s} = 18.4 nM, 25.7 nM, and 6.3 nM, respectively). However, analogue **4** was 37-fold more potent at the *h*MC3R than the *h*MC4R, and 15-fold more with respect to *h*MC5R (EC_{50} = 66 nM, 2400 nM, and 1000 nM, respectively) in functional cAMP assays. Analogue **5** (Ac-Nle-c[Asp-Hyp(Bzl)-*D*Phe-Arg-Trp-Lys]-NH_2, PG-103) which substitutes in position

6, a more hydrophilic Pro derivative, was found to be highly potent but slightly less potent than MT-II, exhibiting similar binding affinity at the *h*MC3R and the *h*MC4R (IC_{50} = 2.8 nM and 4.6 nM, respectively) as for the *h*MC5R. These results suggest that the Bzl residue does not significantly affect binding affinity at melanocortin receptors. Interestingly, analogue **6** (Ac-Nle-c[Asp-Hyp(Bzl)-*D*Nal(2′)-Arg-Trp-Lys]-NH_2, PG-104) was found to be a potent antagonist at the *h*MC4R (IC_{50} = 17 nM) and the *h*MC3R (IC_{50} = 15 nM), but a partial agonist at the *h*MC5R (IC_{50} = 15 nM, EC_{50} = 70 nM). Analogue **7** (Ac-Nle-c[Asp-Mamb-*D*Phe-Arg-Trp-Lys]-NH_2, PG-135) with a 3-aminomethyl-benzoic acid (Mamb) residue in position 6 instead of His, had very weak binding at the *h*MC3R, the *h*MC4R and the *h*MC5R. Interestingly, analogue **8** (Ac-Nle-c[Asp-Mamb-*D*Nal(2′)-Arg-Trp-Lys]-NH_2, PG-135) which differs from **7** by having *D*Nal(2′) in position 7, was a potent and highly selective antagonist for the *h*MC3R. These data suggest that the Mamb residue has a considerable impact in the formation of ligand-receptor complexes but only for antagonists at the *h*MC3R. It is possible that the same residue could destabilize the ligand-receptor interactions for other melanocortin receptors when *D*Phe^7 is present in position 7. Analogue **9** (Ac-Nle-c[Asp-Tic-*D*Phe-Arg-Trp-Lys]-NH_2, PG-943) containing the Tic residue in position 6, resulted in an agonist at *h*MC3R, *h*MC4R and *h*MC5R with a very high binding affinity for all the receptor subtypes (IC_{50} = 2.6 nM, 15 nM and 10 nM respectively). This was in contrast with our earlier findings [15] where substitution of DNal(2')^7 was done in place of DPhe^7. Interestingly, this analogue was a very potent antagonist at hMC3R and hMC4R and a full agonist at hMC5R.

Discussion

Structure-Activity Relationships

Our previous results have demonstrated that incorporation of the conformationally constrained proline residue and its analogues into the lactam bridge might stabilize bioactive conformations, and can improve selectivity of melanotropin peptides at the MCRs [13,15]. In fact, replacing His^6 by Pro^6 in the well known antagonist SHU9119 we have obtained a potent agonist at the *h*MC5R (EC_{50} = 0.072 nM) with full antagonist activity at *h*MC3 and *h*MC4 receptors. We also have demonstrated that the same substitution in MT-II led to a compound with agonist activity at the *h*MCRs substantially similar to that of MT-II. Based on these and other results [2,18] we have come to the conclusion that the imidazole group of histidine is not essential for the binding of MT-II and SHU9119 at the *h*MC4R, and that the restriction of conformational freedom at histidine position by proline and related analogues did not affect interactions of MT-II and SHU-9119 at the *h*MC4R. To expand on these important observations, additional analogues of these melanotropins substituted in position 6 with other unconventional amino acid residues were synthesized and tested at the *h*MC3R, *h*MC4R and *h*MC5R (Table 2).

These results demonstrate that the presence of an appropriate residue in position 6 can change the biological profile of melanocortin peptides at the hMC3–5 receptors. The structure–activity relationships information provided by this set of synthetic melanocortin analogues supports further the hypothesis that the position 6 could be a factor for selectivity and potency at central melanocortin receptors.

In conclusion, structure-activity studies on synthetic melanocortin analogues at the human MC3, MC4 and MC5 receptors have identified new ligands as antagonists at the *h*MC3R/*h*MC4R (analogues **2** and **6**) but most importantly we have discovered a new selective antagonist for the *h*MC3R (analogue **2**) which is potentially useful in further biological investigations of the *h*MC3R. Since it is selective for the *h*MC3R over the *h*MC4R and hMC5R, it can help us to differentiate the substructural features responsible for selectivity at melanocortin receptors.

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The following additional abbreviations are used:

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in J. Biol. Chem. **1972**, 247, 977-983.

AAA, amino acid analysis; Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; Bzl, benzyl; *t*Bu, *tert*-butyl; cAMP, adenosine 3',5'-cyclic monophosphate; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Et_3SiH, triethylsilane; FAB-MS, fast-atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, *N*-hydroxybenzotriazole; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid); Pbf, 2,2,4,6,7-pentamethyldihydrobenzo-furan-5-sulfonyl; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Trt, triphenylmethyl (trityl)..
Amino acid symbols denote *L*-configuration unless indicated otherwise. Mamb, 3-aminomethyl-benzoic acid.

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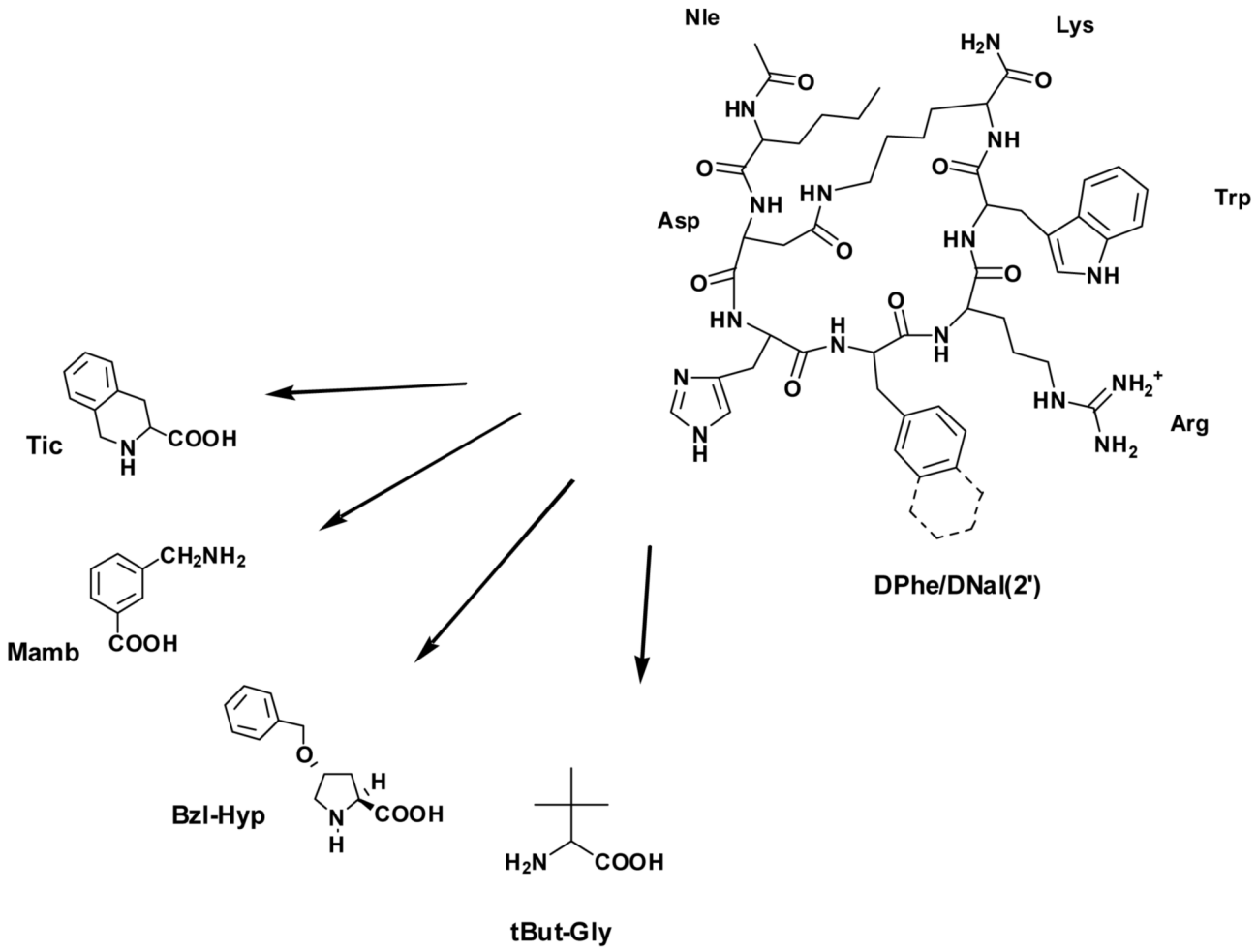

Ac-Nle-c[Asp-*His*-DPhe-Arg-Trp-Lys]-NH_2 **MT-II**

Ac-Nle-c[Asp-*His*-DNal(2')-Arg-Trp-Lys]-NH_2 **SHU9119**

Fig 1.
Structure of the potent monocyclic peptides MT-II and SHU9119, and the conformationally constrained amino acids considered for the modification at position 6.

Table 1

Physicochemical properties of the melanocortin peptides

Code	Sequence	Solv. A[a]	Solv. B[a]	Solv. C[a]	M.W.	MS	HPLC[b]
PG105	Ac-Nle-[Asp-βAla-DPhe-Arg-Trp-Lys]-NH_2	0.82	0.04	0.41	958.09	959.1	4.47
PG106	Ac-Nle-[Asp-βAla-DNal(2′)-Arg-Trp-Lys]-NH_2	0.78	0.04	0.38	1008.5	1009.1	4.15
PG107	Ac-Nle-[Asp-tBuGly-DPhe-Arg-Trp-Lys]-NH_2	0.85	0.08	0.43	1000.8	1001.2	5.13
PG108	Ac-Nle-[Asp-tBuGly-DNal(2′)-Arg-Trp-Lys]-NH_2	0.82	0.04	0.41	1050.6	1051.1	5.47
PG103	Ac-Nle-[Asp-Hyp(Bzl)-DPhe-Arg-Trp-Lys]-NH_2	0.80	0.04	0.39	1074.2	1074.8	5.44
PG104	Ac-Nle-[Asp- Hyp(Bzl)-DNal(2′)-Arg-Trp-Lys]-NH_2	0.92	0.10	0.55	1124.4	1124.6	5.23
PG135	Ac-Nle-c[Asp-Mamb-DPhe-Arg-Trp-Lys]-NH_2	0.90	0.12	0.48	1020.4	1020.7	5.74
PG-136	Ac-Nle-c[Asp-Mamb-DNal(2′)-Arg-Trp-Lys]-NH_2	0.92	0.13	0.51	1070.6	1070.9	5.81
PG-943	Ac-Nle-c[Asp-Tic-DPhe-Arg-Trp-Lys]-NH_2	0.90	0.12	0.49	1046.3	1046.8	5.61

Notes:

[a] *Solvent systems*:(A) 1-butanol/HOAc/pyridine/H_2O (5:5:1:4); (B) EtOAc/pyridine/AcOH/H_2O (5:5:1:3); (C) 1-butanol/AcOH/H_2O (4:1:1)

[b] HPLC *k*' = [(peptide retention time − solvent retention time)/solvent retention time] in a solvent system of 10% CH_3CN in 0.1% TFA and a gradient to 90% CH_3CN over 40 min. An analytical Vydac C_{18} (218TP104) **column was used with a flow rate of 1 mL/min.**

Table 2

Binding and intracellular cAMP accumulation of the α-melanotropin analogues at human melanocortin receptors

No	Peptide Code	Sequence	hMC3R			hMC4R			hMC5R		
			IC_{50} (nM)	EC_{50} (nM)	% Activity	IC_{50} (nM)	EC_{50} (nM)	% Activity	IC_{50} (nM)	EC_{50} (nM)[b]	% Activity
1	**PG105**	Ac-Nle-[Asp-βAla-DPhe-Arg-Trp-Lys]-NH_2	170±20	320±50	30	n.b.	1500 ±200	/	n.b.	n.a.	/
2	**PG106**	Ac-Nle-[Asp-βAla-DNal (2′)-Arg-Trp-Lys]-NH_2	210±30	n.a.	/	n.b.	9900	/	n.b.	n.a.	/
3	**PG107**	Ac-Nle-[Asp-tBuGly-DPhe-Arg-Trp-Lys]-NH_2	41±5	160±22	100	550±60	740±80	/	200.±30	1100 ±150	100
4	**PG108**	Ac-Nle-[Asp-tBuGly-DNal(2′)-Arg-Trp-Lys]-NH_2	18±2	66±7	5	26±3	2400 ±300	/	6.3±1	1000 ±100	100
5	**PG103**	Ac-Nle-[Asp-Hyp(Bzl)-DPhe-Arg-Trp-Lys]-NH_2	2.8±3	19±2	34	4.6±0.5	470±50	17	13±1	110±12	105
6	**PG104**	Ac-Nle-[Asp- Hyp(Bzl)-DNal(2′)-Arg-Trp-Lys]-NH_2	15±2	n.a.	/	17±2	>5000	/	15±5	70±10	23
7	**PG-135**	Ac-Nle-c[Asp-Mamb-DPhe-Arg-Trp-Lys]-NH_2	670±250	800±69	83	930±69	n.a.	4	1800 ±100	710±100	50
8	**PG-136**	Ac-Nle-c[Asp-Mamb-DNal(2)-Arg-Trp-Lys]-NH_2	67±13	n.a.	1.7	>3000	n.a.	/	2500 ±200	n.a.	/
9	**PG-943**	Ac-Nle-c[Asp-Tic-DPhe-Arg-Trp-Lys]-NH_2	2.6±0.4	20±2	102	15±2	6±1	99	10±2	1000	100
	MTII	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]- NH_2	1.25 ±0.20	1.85 ±0.20	100	1.07±0.3	2.87 ±0.52	100	7.47±0.2	3.3±0.7	100
	SHU9119	Ac-Nle-c[Asp-His-DNal (2)-Arg-Trp-Lys]- NH_2	0.23 ±0.02	-	0	0.06 ±0.01	-	0	0.09 ±0.02	0.12 ±0.01	97

IC_{50} = concentration of peptide at 50% specific binding (N = 4); EC_{50} = effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation (N = 4). The peptides were tested at a range of concentration from 10^{-10} to 10^{-5} M; n.b.= no binding affinity at the receptor indicated at 10^{-5}M conc. of ligand; n.a. = no cAMP activity at the receptor indicated at 10^{-5}M conc. of ligand.