Eur. J. Med. Chem. 36 (2001) 137–146

PII: S0223-5234(00)01200-9/FLA

Original article

Design of new small cyclic melanocortin receptor-binding peptides using molecular modelling: Role of the His residue in the melanocortin peptide core

Peteris Prusis, Ruta Muceniece, Ilze Mutule, Felikss Mutulis, Jarl E.S. Wikberg*

Department of Pharmaceutical Pharmacology, Uppsala University, SE-751 24 Uppsala, Sweden

Received 23 January 2000; revised 3 November 2000; accepted 10 November 2000

Abstract – The conserved core of melanocyte stimulating hormones (MSH), His-Phe-Arg-Trp, was probed by comparing a cyclic pentapeptide containing His-DPhe-Arg-Trp, with three structurally similar cyclic peptides, that lacked the His residue. All three peptides bound to the MC_1, MC_3, MC_4 and MC_5 receptors with similar affinities. Molecular modelling indicated that the 3D structure of the DPhe-Arg-Trp of all three peptides were closely similar. The data indicate that the His residue of the small rigid cyclic MSH core peptides does not participate in binding with the melanocortin receptors. © 2001 Éditions scientifiques et médicales Elsevier SAS

melanocortin receptors / melanocyte stimulating hormone / radioligand binding / molecular modelling

1. Introduction

Melanocortin receptors are members of the G-protein coupled receptor superfamilly. There are five subtypes of the melanocortin receptors (MC_1–MC_5), all which were cloned a few years ago [1–5]. The MC_1 receptor has been found in melanocytes where it controls skin pigmentation, as well as in the periaqueductal grey area of the brain [6]. More recent data indicated that the MC_1 receptor is also present in cells of the immune system [7]. MC_3, MC_4 and MC_5 receptors are localised in the central nervous system; the MC_3 and MC_5 receptors also in periphery [1, 3, 4, 8]. Recent studies indicate that the MC_4 receptor is involved in control of feeding behaviour [9] and the MC_5 receptor in control of exocrine gland function [10]. The MC_2 receptor has distinct structural and binding properties [11] and it is localised in the adrenal cortex [12].

Melanocyte stimulating hormones (MSH) are natural ligands for four of the melanocortin receptor subtypes, namely the MC_1, MC_3, MC_4 and MC_5 receptors. These hormones are peptides of more than 10 amino acids in length that bind with higher affinity to the MC_1 receptor, than to the MC_3, MC_4 and MC_5 receptors [13, 14], and which share a common core sequence motif: HFRW [15].

A number of artificial linear and cyclic MSH peptide analogues have been synthesised, most of which contain the core sequence. Most of these substances show quite similar selectivity profiles to the natural hormones [15]. Moreover, it was shown that exchange of the L-Phe residue in the core sequence to D-Phe generally increases the affinities for the melanocortin receptors [16].

Abbreviations: AcOH, acetic acid; Boc, *tert*-butoxycarbonyl; DIEA, diisopropylethylamine; DMF, dimethylformamide; DPPA, diphenylphosphoryl azide; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HMPB, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (or its residue); HPLC, high performance liquid chromatography; MeCN, acetonitrile; MeOH, methanol; NMM, *N*-methylmorpholine; PAC, 4-hydroxymethylphenoxy-acetic acid residue; PAL, 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)-valeric acid residue; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl; PEG-PS, polyethylene-graft polystyrene support; PyAOP, 7-azabenzotriazol-1-yloxytris(pyrrolidino) phosphonium hexafluorophosphate; TBTU, *O*-(7-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, trityl.

* Correspondence and reprints.
E-mail address: Jarl.Wikberg@farmbio.uu.se. (J.E.S. Wikberg).

An alanine scan of the α-MSH peptide indicated that the core sequence plays a key role in the binding of melanocortin hormones to their receptors, although the available data suggests that the importance of the His residue may be lower, compared to the other three core amino acids [17].

However, it has been shown that the minimal active α-MSH peptide fragment on lizard skin assay was the Ac-His-Phe-Arg-Trp-NH_2 peptide, which contains the His residue [18]. In a later study on small linear tripeptides containing D-amino acids in different positions, it was found that none of these peptides were able to bind to MC_1, MC_3 and MC_5 receptors, while two peptides (Ac-DPhe-Arg-Trp-NH_2 and Ac-DPhe-Arg-DTrp-NH_2) bound to the MC_4 receptor [19].

In our attempts to understand the pharmacophore of the melanocortin receptors, we recently made a 3D model of the MC_1 receptor using homology modelling, based on the rhodopsin footprint [20]. In these attempts, we also designed a small cyclic MSH peptide analogue that contained the core sequence with L-Phe exchanged to D-Phe. The smallest of these peptides was a pentapetide, cHdFRWG (**1**), which was found to bind with substantial affinity to the melanocortin receptors, the highest affinity being shown for the MC_1 receptor [21]. The peptide (**1**), which just contains the core sequence and glycine, was subsequently successfully docked into our MC_1 receptor model [18]. However, these docking studies indicated that only three of the core residues, namely Phe, Arg and Trp, were strongly involved in binding of the cHdFRWG (**1**) peptide to the receptor, while the interactions with the His residue appeared to be minor.

The present study was devoted to test the hypothesis that the His residue of the cyclic core peptides does not have a major role for the melanocortin receptor binding. For the purpose of these studies we designed three new cyclic peptides; one being a cHdFRWG analogue in which histidine was replaced by asparagine: cNdFRWG (**2**), and the other two being the cyclic peptides c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) which contain the D-Phe, Arg and Trp core residues, and Glu, which side chains were cyclised with the *N*-terminal of the D-Phe residue (see *figure 1*). In support of our hypothesis, we report here that these histidine-lacking peptides bind with equal or higher affinity to the melanocortin receptors, compared with cHdFRWG (**1**).

2. Results

2.1. Binding

The binding of (**2**), (**3**) and (**4**) was tested on intact CV-1 origin, SV40 (COS-1) cells expressing the human MC_1, MC_3, MC_4 and MC_5 receptors using competitive receptor-binding assays. The resulting K_i values, ob-

α-MSH: Ser-Tyr-Ser-Met-Glu-**His- Phe-Arg-Trp**-Gly-Lys-Pro-Val

β-MSH: Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-**His- Phe-Arg-Trp**-Gly-Ser-Pro-Pro-Lys-Asp

γ-MSH: Tyr-Val-Met-Gly-**His- Phe-Arg-Trp**-Asp-Arg-Phe

cHdFRWG (**1**): **His-dPhe-Arg-Trp**-Gly

cNdFRWG (**2**): Asn-**dPhe-Arg-Trp**-Gly

c(dFRWE)OH (**3**): **dPhe-Arg-Trp**-Glu-OH

c(dFRWE)NH_2 (**4**): **dPhe-Arg-Trp**-Glu-NH_2

Figure 1. Primary structures of the cHdFRWG (**1**), cNdFRWG (**2**), c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) peptides and natural MSH peptides.

Table I. K_i values (mean ± S.E.M) obtained from competition curves for cHdFRWG (**1**), cNdFRWG (**2**), c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) peptides on human MC_1, MC_3, MC_4 and MC_5 receptors.

Peptide	Receptor			
	MC_1 K_i (μmol/L)	MC_3 K_i (μmol/L)	MC_4 K_i (μmol/L)	MC_5 K_i (μmol/L)
1	3.2 ± 0.3	113.0 ± 21.0	71.4 ± 9.3	207.0 ± 45.0
2	0.8 ± 0.1	10.4 ± 5.7	4.6 ± 0.5	7.8 ± 1.0
3	1.6 ± 0.5	106.5 ± 78.6	48.5 ± 22.3	36.1 ± 15.3
4	4.58 ± 0.5	4.6 ± 0.2	17.1 ± 1.1	79.8 ± 34.5

tained from competition tests of these peptides, are summarised in *table I* along with the corresponding data for the cHdFRWG (**1**) peptide taken from our earlier study [21]. Competition curves for (**2**), (**3**) and (**4**) are shown in *figure 2*. The data show that all four peptides share similar affinities for the MC_1 receptor. However, the cNdFRWG (**2**) peptide showed 11-, 16- and 27-fold improved affinity on MC_3, MC_4 and MC_5 receptors, compared with the cHdFRWG (**1**) peptide, respectively. Moreover, c(dFRWE)NH_2 (**4**) showed 24-fold higher affinity for the MC_3 receptor compared with cHdFRWG (**1**), although its affinity for the MC_4 and MC_5 receptors were only slightly higher. The c(dFRWE)OH (**3**) peptide showed a similar affinity profile as the cHdFRWG (**1**) peptide. It must be noted that the c(dFRWE)NH_2 (**4**) peptide showed 23-fold higher affinity for the MC_3 receptor when compared with the c(dFRWE)OH (**3**) peptide.

2.2. Modelling of peptides

The bond missing in the initial stages (see section 4 for details) of the modelling of the cHdFRWG (**1**) and cNdFRWG (**2**) peptides was the peptide bond between the Gly and His/Asn residues, while for the c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) peptides it was the lactam bond between the Glu and Phe residues. Conformational analysis indicated that the models of peptides (**1**) and (**3**) could be grouped into four clusters, peptide (**4**) models could be grouped into three clusters and peptide (**2**) models could be grouped into five clusters. The largest clusters for (**1**), (**2**), (**3**) and (**4**) peptide models contained 47, 53, 79 and 57 molecules (out of 100 modelled for each peptide), respectively. The structure with the lowest energy was selected from each of the largest cluster for each peptide and their geometry was compared. The model of the cNdFRWG (**2**) peptide was very similar to the model of the cHdFRWG (**1**) peptide, the root mean square distance (RMSD) between C_α and C_β atoms of the core DPhe-Arg-Trp tripeptide being 0.06 Å. The models for the c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) peptides were highly similar, the RMSD between all common atoms being 0.01 Å. Moreover, the geometry of the c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) peptide models was also very similar to the cHdFRWG (**1**) peptide model, the RMSD between C_α and C_β atoms of the core DPhe-Arg-Trp tripeptide being 0.21 and 0.22 Å, respectively. The aligned 3D structures of the all four peptides are shown in *figure 3*.

3. Discussion

Our data show that the cNdFRWG (**2**), c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) peptides possess similar or even improved binding affinities for the melanocortin receptors compared to the cHdFRWG (**1**) peptide. Thus, the loss of the His residue does not result in any loss in binding affinities compared to the cHdFRWG (**1**) peptide; instead, a clear increase in binding affinity is seen for the cNdFRWG (**2**) peptide for the MC_3, MC_4 and MC_5 receptors, while for the c(dFRWE)NH_2 (**4**) peptide, an increase in affinity is seen for the MC_3 receptor. The data verify our hypothesis that the His residue is not important for the binding of the cyclic MSH-core peptides to the melanocortin receptors. Our data, thus, give strong support to our earlier MC_1 receptor–ligand complex model [20], where the His residue of the cHdFRWG (**1**) peptide was not involved in any important interactions.

Our present data also show that the mode of cyclisation of the c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) peptides does not induce any negative influences in the binding to the melanocortin receptors. These findings, along with the modelling results showing that all four peptides essentially retain the same backbone

conformation for their core residues, indicate that it is mainly the core DPhe-Arg-Trp tripeptide that form the major interactions in MSH-peptides with the melanocortin receptors. However, the higher affinity of the peptide (**2**) for the MC_3, MC_4 and MC_5 receptors compared with (**1**) is interesting and might indicate the presence of a specific interaction between the Asn sidechain and the receptors. Moreover, differences in the binding of the c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) peptides to the MC_3 receptor might indicate the presence of a specific interaction of the *C*-terminal of these peptides with the MC_3 receptor.

Several earlier structural studies on the melanocortin peptides were aimed at large MSH peptides [15, 22–28]. Interestingly, these studies indicate that the conformation for large MSH peptide analogues deviate considerably, even in their core region [22–28]. Moreover, these large and flexible peptides may change their conformation during binding to the MCR's.

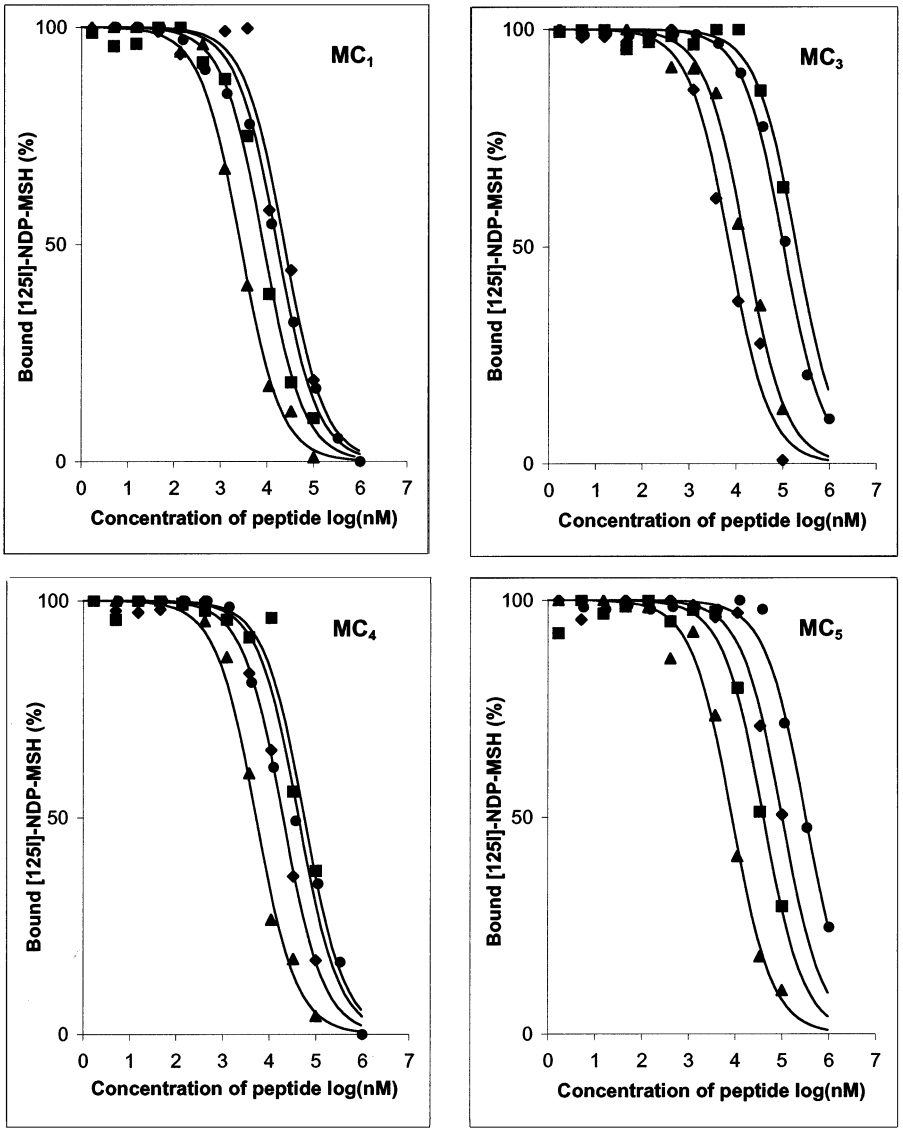

Figure 2. Competition curves of cHdFRWG (●), cNdFRWG (▲), c(dFRWE)OH (■) and c(dFRWE)NH_2 (◆) peptides on recombinant human melanocortin receptors.

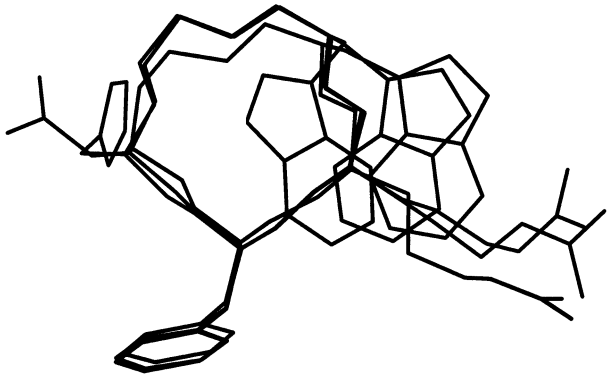

Figure 3. Aligned 3D structures of the cHdFRWG (**1**), cNdFRWG (**2**), c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) peptides. The graph was made by using MOLSCRIPT [36].

The present approach for evaluating the structure of the melanocortin peptides was instead devoted to small cyclic core peptides whose conformational space is comparatively limited. Of course the loss of interaction points outside of the core is expected to lead to reductions in affinities [17, 18, 29]. However, our core peptides retained substantial affinities for the melanocortin receptors, which made the present analysis feasible.

An additional goal of the present study was to try to create molecules with even smaller ring size in order to induce additional rigidity. The modelling of (**3**) and (**4**) showed that the geometry of their DPhe-Arg-Trp cores is very similar to that of (**1**). Moreover, both (**3**) and (**4**) have smaller cycle sizes of 14 atoms, compared with the 15 atoms in (**1**), giving additional rigidity to the cycle structure. The increased rigidity is the most likely reason why the majority of the models of the c(dFRWE)OH (**3**) and c(dFRWE)NH_2 (**4**) peptides were populated into single large clusters.

The rigidity of the small cyclic peptides investigated in this study indicates that their backbone conformation might remain intact during binding to the receptor. There presently exists a great interest in the design of drugs for the melanocortin receptors, e.g. for the control of feeding behaviour and the present small rigid cyclic MSH-core peptides might serve as excellent templates for further drug design.

4. Experimental protocols

4.1. Synthesis and characterisation of cyclic peptides

Reagents were purchased from Aldrich, Fluka and Applied Biosystems. Solid phase synthesis of peptides was made on a Pioneer peptide synthesis system (Applied Biosystems), as is detailed further below. LC/MS was performed on a Perkin Elmer instrument PE SCIEX API 150EX with Turboionspray Ion Source and Vydac 214 MS 52 C4 MASS SPEC 2.1×250 mm high performance liquid chromatography (HPLC) column using a gradient formed from water and acetonitrile with 0.02% trifluoroacetic acid (TFA) additive (HPLC A). Analytical HPLC was performed on a Waters' system equipped with a Millenium[32] Workstation, 2690 Separation Module and 996 Photodiode Array Detector. Protected peptides were analysed on a Hichrom LiRP18-5-4076 (3.2×100 mm) column, eluent 60% acetonitrile (MeCN):40% 0.02 M aqueous NaH_2PO_4 (HPLC B). Endproducts were analysed on a Vydac 201HS52 RPC18 (2.1×250 mm) column, eluent – linear gradient in 40 min from 10% MeCN:90% 0.01 M aqueous triethylammonium acetate pH 6.0 to 50% MeCN:50% 0.01 M aqueous triethylammonium acetate pH 6.0 (HPLC C). Thin layer chromatography (TLC) was performed using precoated silica gel plates F254 (Merck) in the following solvent systems: 1: $CHCl_3$-*n*-butanol-ethanol-ethylacetate-water (10:6:4:3:1), 2: *n*-butanol-acetic acid (AcOH)-water (4:1:1), 3: ($CHCl_3$-methanol (MeOH)-17% NH_3, 2:2:1). Semipreparative HPLC was carried out on a LKB system consisting of a 2150 HPLC pump, 2152 LC controller and 2151 variable wavelength monitor. Freeze-drying was performed at 0.1 bar on a Lyovac GT2 freeze-dryer (Finn-Aqua) equipped with a Busch 010-112 vacuum pump and a liquid nitrogen trap. The Aminosyraanalyscentralen of the Biomedical Centre, Uppsala, Sweden, performed amino acid analysis as a service.

4.1.1. Synthesis of cHdFRWG (**1**) *peptide ditrifluoroacetate (see figure 1)*

4.1.1.1. HO-HMPB-PEG-PS (i)

PEG-PS×HCl (1.0 g, 0.19 mmol) was suspended in 50 mL dimethylformamide (DMF) and 5.0 mL diisopropylethylamine (DIEA) was added and the suspension filtered through a sintered glass filter. The polymer on the filter was suspended in 20 mL DMF and filtered

off again. The last operation was repeated 5 times. HMPB (183 mg, 0.76 mmol), *O*-(7-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate (TBTU) (244 mg, 0.76 mmol) and DIEA (0.196 mL, 1.14 mmol) were dissolved in 10 mL DMF and then immediately added to the wet polymer on the filter with gentle stirring. The solution was allowed to pass slowly through the filter. The collected filtrate was again introduced onto the polymer layer and after 1 h the solution was removed by filtration. Finally, the polymer (*i*) retained on the filter was washed with 200 mL DMF and used in the next step.

4.1.1.2. Fmoc-Gly-O-HMPB-PEG-PS (ii)

Fmoc-Gly-OH (3.57 g, 2.28 mmol) was dissolved in 100 mL CH_2Cl_2 and dicyclohexylcarbodiimide (235 mg, 1.14 mmol) was added and the mixture shaken for 10 min at room temperature. The *N*,*N*′-dicyclohexylurea precipitate formed was filtered off and washed on the filter with CH_2Cl_2. The solvent was then evaporated from the filtrate at 0 °C. The residue was dissolved in a minimal amount of DMF (10 mL) and the support (*i*) and 4-dimethylaminopyridine (23 mg, 0.19 mmol) were immediately added and the mixture shaken until dissolution of the 4-dimethylaminopyridine. The mixture was then left for 1.5 h at 20 °C whereafter it was filtered. The resin (*ii*) retained on the filter was washed with 100 mL DMF and applied to the next step.

4.1.1.3. H-His(Trt)-D-Phe-Arg(Pbf)-Trp(Boc)-Gly-O-HMPB-PEG-PS (iii)

The resin (*ii*) was placed into a peptide synthesis column and applied to four consecutive synthesis cycles; each cycle consisting of: (1) circulation through the column with a 20% solution of piperidine in DMF (5 min); (2) washing of the support with DMF (2 min); (3) circulation with a solution of Fmoc-amino acid (0.76 mmol), TBTU (0.29 g, 0.76 mmol) and DIEA (0.65 mL, 3.8 mmol) in 10 mL DMF (30–60 min); (4) washing with DMF; (5) treating with 0.3 M acetic anhydride in DMF for 5 min and (6) washing again with DMF. At the end of the four synthesis cycles, steps (1) and (2) were applied to remove the *N*-terminal Fmoc group. The resulting peptidylpolymer was additionally washed with MeOH, followed by CH_2Cl_2 and dried for 2 h at 0.1 mm Hg.

4.1.1.4. H-His(Trt)-D-Phe-Arg(Pbf)-Trp(Boc)-Gly-OH·HCl (iv)

The peptidylpolymer (*iii*) (0.19 mmol) was placed into a glass column equipped with a sintered glass disk and stopcock at the bottom. A 10 mL portion of 1% TFA in CH_2Cl_2 was added to the column and the resin was left to react for 3 min at room temperature, followed by filtering under vacuum into a flask containing a stirred solution of $NaOCOCH_3 \cdot 3H_2O$ (3.6 g, 26 mmol) in 100 mL water. This procedure was repeated 10 times by adding the TFA solution and filtering into the same flask. The organic phase from the filtering flask was then separated and placed at −20 °C for 10 h. The residual water then formed ice, which was removed by filtration. The filtrate was then dried over $MgSO_4$. The mixture was once again filtered to remove the $MgSO_4$ and the filtrate was evaporated and the residue dissolved in 20 mL MeOH. This solution was twice slowly passed through a column (5.5×50 mm) with Dowex 1 (Sigma) in Cl^- form. The column was washed with 5 mL MeOH and the eluate was evaporated and the residue treated with dry ether. The crystalline precipitate formed was filtered off. R_f (1), 0.22. k′(B), 12.5; λ_{max}: 204 nm (100% absorption), 234 nm (32%, shoulder), 248 nm (19%, shoulder), 264 nm (11%, shoulder), 276 nm (6%, shoulder), 285 nm (6%), 293 nm (4%). Yield, 85 mg (33%).

4.1.1.5. Cyclo-[His(Trt)-D-Phe-Arg(Pbf)-Trp(Boc)-Gly] (v)

The linear precursor (*iv*) (83 mg, 0.062 mmol) was dissolved in 60 mL DMF, cooled to 0 °C, and a 0.23 M solution of *N*-methylmorpholine (NMM) in DMF (0.26 mL, 0.062 mmol) and a 0.20 M solution of diphenylphosphorylazide (DPPA) in DMF (0.62 mL, 0.124 mmol) were added. The mixture was left at 0 °C for 4 days. Additionally, every day 0.26 mL of the NMM and 0.31 mL of the DPPA solutions were added. The mixture was then brought to room temperature and left for 1 day, whereafter the solvent was evaporated and the residue triturated with ether. The crystalline precipitate formed was filtered off, washed on the filter with dry ether, then washed with 5% aqueous citric acid, water, 5% $NaHCO_3$, water again and then dried under vacuum in the presence of P_2O_5. R_f (1), 0.91. k′(B), 1.3; λ_{max}: 203 nm (100% absorption), 257 nm (4%, shoulder), 262 nm (5%), 270 nm (4%, shoulder). Yield, 24 mg (30%).

4.1.1.6. Cyclo-(His-DPhe-Arg-Trp-Gly).2 TFA (**1**)

The protected cyclopeptide (*v*) (24 mg, 0.019 mmol) was dissolved in 2 mL of a deprotection mixture (TFA-phenol-anisole-1,2-ethanedithiol-water, 92:2:2:2:2) and left at room temperature for 2 h. The solution was then evaporated at 0 °C, dry ether was added and the precipitate formed was filtered off and washed with ether, then dried in vacuo over KOH. This raw product was dissolved in 0.5 mL of 60% MeCN in water, the solution was divided into 3 portions and placed into centrifuge tubes and each portion was diluted with 0.1% aqueous TFA to 1.5 mL volume. The mixtures were centrifuged and the clear centrifugates were applied onto an HPLC semipreparative column (10×250 mm, Vydac RP C_{18}, 90 A, 201HS1010), eluate – 13% MeCN in water+0.1% TFA, detection at 220 nm. Fractions of the eluate containing pure (**1**) were pooled and lyophilised. A white powder formed. Yield, 15.6 mg (91%). R_f (2), 0.50; R_f (3), 0.53. k′(A), 10.1. k′(C), 6.2; λ_{max}: 216 nm (100% absorption), 272 nm (12%, shoulder), 279 nm (13%), 287 nm (11%). Turboionspray mass spectrometry, positive ionisation: *m*/*e* 684.4 $[M+H]^+$; negative ionisation: *m*/*e* 682.4 $[M-H]^-$, 796.4 $[M-H+TFA]^-$, 911.5 $[M-H+2TFA]^-$. Amino acid analysis: Gly, 1.04; Phe, 0.94; His, 1.00; Trp, 0.95; Arg, 1.02.

4.1.2. Synthesis of c(dFRWE)OH (**3**) *peptide (see figure 1)*

Fmoc-Glu(OAl)-OH (245 mg, 0.6 mmol) was dissolved in 10 mL CH_2Cl_2, dicyclohexylcarbodiimide (62 mg, 0.3 mmol) was added and the mixture shaken for 10 min at room temperature. The *N*,*N*′-dicyclohexylurea precipitate formed was filtered off and washed on the filter with CH_2Cl_2. The filtrate was evaporated in vacuo at 0 °C. The oily residue obtained was immediately dissolved in a minimal amount of DMF (5 mL) and 4-hydroxymethylphenoxy-acetic acid residue (PAC)-PEG-PS (0.29 g, 0.05 mmol) was added followed by 4-dimethylaminopyridine (6 mg, 0.05 mmol), under shaking, until its dissolution. The mixture was left for 1.5 h at 20 °C and then filtered. The polymer was washed on the filter with 100 mL DMF and then placed into a peptide synthesis column. The Fmoc group was removed by 20% piperidine in DMF (5 min), followed by washing with DMF. Fmoc-Trp(Boc)-OH (79 mg, 0.15 mmol), *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU; 57 mg, 0.15 mmol) and DIEA (0.17 mL, 1.0 mmol) were dissolved in 4 mL DMF and circulated through the column for 30 min. The support was then washed with DMF, treated with 20% piperidine in DMF (5 min) and washed again. Fmoc-Arg(Pbf)-OH (97 mg, 0.15 mmol), HATU (57 mg, 0.15 mmol) and DIEA (0.17 mL, 1.0 mmol) were dissolved in 4 mL DMF and circulated through the column for 60 min. The support was washed with DMF, treated with 20% piperidine in DMF (5 min) and washed again. Fmoc-DPhe-OH (58 mg, 0.15 mmol), HATU (57 mg, 0.15 mmol) and DIEA (0.17 mL, 1.0 mmol) were dissolved in 4 mL DMF and circulated through the column for 60 min. The support was washed with DMF, treated with 20% piperidine in DMF (5 min) and washed again. The support was washed with 5% AcOH+2.5% NMM in chloroform. Tetrakis(triphenyl-phosphine)-palladium(0) (173 mg, 0.15 mmol) was dissolved in 4 mL of the above mentioned mixture and circulated through the column for 2 h. The support was washed with 0.5% DIEA+0.5% Na diethyldithiocarbamate in DMF, and then washed with DMF. 7-Azabenzotriazol-1-yloxytris(pyrrolidino) phosphonium hexafluorophosphate (PyAOP) (78 mg, 0.15 mmol) and DIEA (0.17 mL, 1.0 mmol) were dissolved in 4 mL DMF and circulated through the column for 8 h. The support was washed with DMF, followed by methanol, and then followed by dichloromethane, where after it was dried in vacuo. The resin was treated with 3 mL of a deprotection mixture (TFA-water-1,2-ethanedithiol-triisopropylsilane (TIS), 92.5:2.5:2.5:2.5) at room temperature for 3 h and filtered. The filtrate was evaporated at 0 °C, dry ether was added, the precipitate was filtered off and washed on the filter with ether, then dried in vacuo over KOH. The raw product obtained was dissolved in 0.5 mL of 60% MeCN in water, the solution was divided into 3 portions and placed into centrifuge tubes, each being diluted with 0.1% aqueous TFA to a volume of 1.5 mL. The tubes were centrifuged and the clear centrifugates applied onto an HPLC semipreparative column (10×250 mm, Vydac RP C_{18}, 90 A, 201HS1010), eluated with 12% MeCN in water+0.1% TFA, with detection at 220 nm. Fractions containing pure peptide (**3**) were pooled and lyophilised. A white powder formed. Yield, 11.6 mg (37%). R_f (2), 0.49; R_f (3), 0.34. k′(A), 8.9. k′(C), 5.3; λ_{max}: 216 nm (100% absorption), 272 nm (15%, shoulder), 279 nm (16%), 287 nm (14%). Turboionspray mass spectrometry, positive ionisation: *m*/*e* 619.3 $[M+H]^+$; negative ionisation: *m*/*e* 617.3 $[M-H]^-$, 731.4 $[M-H+TFA]^-$, 845.5 $[M-H+2TFA]^-$. Amino acid analysis: Glu, 1.04; Phe, 0.97; Trp, 1.05; Arg, 1.00.

4.1.3. Synthesis of cNdFRWG peptide (**2**) *trifluoroacetate (see figure 1)*

Fmoc-PAL-PEG-PS (333 mg, 0.05 mmol) was placed into a peptide synthesis column. The Fmoc group was removed by 20% piperidine in DMF (5 min) and the support was washed with DMF. Fmoc-Asp(OH)-OAl (59 mg, 0.15 mmol), HATU (57 mg, 0.15 mmol) and DIEA (0.17 mL, 1.0 mmol) were dissolved in 4 mL DMF and circulated through the column for 30 min. The support was washed with DMF, treated with 20% piperidine in DMF (5 min) and washed again. Further synthesis was made as described for peptide (**3**). These steps included the attachments of the following amino acid derivatives, in sequence: Fmoc-Gly-OH, Fmoc-Trp(Boc)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-DPhe-OH. Yield, 5.3 mg (14%). R_f (2), 0.46, R_f (3), 0.46. k′(A), 8.6. k′(C), 5.3; λ_{max}: 217 (100% absorption), 272 nm (13%, shoulder), 279 nm (16%), 287 nm (14%). Turboionspray mass spectrometry, positive ionisation: *m*/*e* 661.4 $[M+H]^+$; negative ionisation: *m*/*e* 659.4 $[M-H]^-$, 773.4 $[M-H+TFA]^-$, 887.5 $[M-H+2TFA]^-$. Amino acid analysis: Asp, 1.04; Gly, 1.04; Phe, 0.96; Trp, 1.00; Arg, 0.98.

4.1.4. Synthesis of c(dFRWE)NH_2 (**4**) *peptide trifluoroacetate (see figure 1)*

Fmoc-PAL-PEG-PS (333 mg, 0.05 mmol) was placed into a peptide synthesis column. The Fmoc group was removed by 20% piperidine in DMF (5 min) and the support was washed with DMF. Fmoc-Glu(OAl)-OH (61 mg, 0.15 mmol), HATU (57 mg, 0.15 mmol) and DIEA (0.17 mL, 1.0 mmol) were dissolved in 4 mL DMF and circulated through the column for 30 min. The support was washed with DMF, treated with 20% piperidine in DMF (5 min) and washed again. Further synthesis was made as described for peptide (**3**). Yield, 12 mg (33%). R_f (2), 0.56; R_f (3), 0.37. k′(A), 8.8. k′(C), 6.3; λ_{max}: 217 nm (100% absorption), 272 nm (16%, shoulder), 279 nm (18%), 287 nm (15%). Turboionspray mass spectrometry, positive ionisation: *m*/*e* 618.3 $[M+H]^+$; negative ionisation: *m*/*e* 616.3 $[M-H]^-$, 730.4 $[M-H+TFA]^-$, 844.5 $[M-H+2TFA]^-$. Amino acid analysis: Glu, 1.01; Phe, 0.97; Trp, 1.00; Arg, 0.99.

4.2. Pharmacology

4.2.1. Expression of receptor clones

The human MC_1 and MC_5 receptors [1, 2] had been cloned into the expression vector pRc/CMV (InVitrogen). The human MC_3 and MC_4 receptors [3, 4] had been cloned into the expression vector pCMV/neo. For receptor expression COS-1 cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty per cent confluent cultures were transfected with the DNA mixed with liposomes in serum-free medium [11]. After transfection, the serum-free medium was replaced by serum-containing medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged and used for radioligand binding.

4.2.2. Binding studies

The transfected cells were washed with binding buffer [14] and distributed into 96-well non-culture-coated plates, which were centrifuged and the binding buffer was removed. The cells were then immediately incubated in the well plates for 2 h at 37 °C with 0.05 mL binding buffer in each well containing a constant concentration of [^{125}I]NDP-MSH and appropriate concentrations of the competing unlabelled ligand. After incubation, the cells were washed with 0.2 mL of ice-cold binding buffer and detached from the plates with 0.2 mL of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data were analysed with the BindAid package (Melacure Therapeutics AB, Uppsala, Sweden), suitable for radioligand-binding data analysis. Data were analysed by fitting to formulas derived from the law of mass action by the method generally referred to as computer modelling. The K_d values for [^{125}I]NDP-MSH for the melanocortin receptors were taken from Schiöth et al. [13, 14]. The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding for [^{125}I]NDP-MSH.

4.2.3. [^{125}I]NDP-MSH

The [^{125}I]NDP-MSH used in the binding studies were made by radioiodination of [Nle^4, D-Phe^7]α-MSH (NDP-MSH) [30] by the chloramine T method and purified by HPLC [31].

4.3. Molecular modelling

4.3.1. Software and parameters

For molecular modelling purposes we used XPLOR [32] and CHARMM [33] softwares running on a Digital PWS 600au computer. We used all atom CHARMM22 force field [34]. Throughout the calculations a switching function was applied to both van der Waals and electrostatic potentials between 8 and 12 Å, non-bonded neigh-

bour list generated within a radius of 13 Å and dielectrical constant set to 80. Conformational analysis was performed by using XPLOR [32]. The results obtained from calculations were explored by using the Sybyl [35] program running on SGI Indigo^2 workstation. Figures showing models of peptides were created using the MOLSCRIPT program [36].

4.3.2. Methodology

The modelling procedure was essentially the same as described previously [20]. Thus, we initially generated a set of 100 initial models with randomised ϕ and ψ torsion angles by using CHARMM [33]. During this initial generation of cyclic peptide models, the molecules were treated as if they had been linear; that is by assuming that one bond in the cycle was missing. This was made in order to allow the coverage of the full range of torsional space. The rest of calculations were performed using XPLOR [32]. Each structure from the set of randomly generated molecules was subjected to brief minimisation followed by high temperature (1000 K) dynamics. During this stage, the peptide was treated as if it was linear, but a gradually increasing distance restraint was applied in order to achieve cyclisation. For the rest of the calculations, the missing bond in the cyclic peptide was restored and the molecule slowly annealed to 300 K. Finally, the energy of the molecule was minimised. We also performed an analysis of the conformational space on the set of molecules obtained from these calculations. We first measured the RMSD for the C_α and C_β atoms of the DPhe-Arg-Trp tripeptide for each pair of the molecules in the set. The values thus obtained were used to group the molecules into clusters. Thus, into each cluster, molecules were included whose average RMSD to all other molecules in the cluster was less than 0.5 Å.

Acknowledgements

Support was obtained from the Swedish MRC (04X-05957).

References

[1] V. Chhajlani, R. Muceniece, J.E.S. Wikberg, 195 (1993) 866–873.

[2] Chhajlani V., Wikberg J.E.S., FEBS Lett. 309 (1992) 417–420.

[3] Gantz I., Konda Y., Tashiro T., Shimoto Y., Miwa H., Munzert G., Watson S.J., DelValle J., Yamada T., J. Biol. Chem. 268 (1993) 8246–8250.

[4] Gantz I., Miwa H., Konda Y., Shimoto Y., Tashiro T., Watson S.J., DelValle J., Yamada T., J. Biol. Chem. 268 (1993) 15174–15179.

[5] Mountjoy K.G., Robbins L.S., Mortrud M.T., Cone R.D., Science 257 (1992) 1248–1251.

[6] Xia Wikberg J.E.S., Chhajlani V., NeuroReport 6 (1995) 2193–2196.

[7] Wikberg J.E.S, Eur. J. Pharmacol. 375 (1999) 295–310.

[8] M.J. Low, R.B. Simerly, R.D. Cone, Current Science (1994) 79–88.

[9] Fan W., Boston B.A., Kesterson R.A., Hruby V.J., Cone R.D., Nature 385 (1997) 165–168.

[10] Chen W., Kelly M.A., Opitz-Araya X., Thomas R.E., Low M.J., Cone R.D., Cell 91 (1997) 789–798.

[11] Schiöth H.B., Chhajlani V., Muceniece R., Klusa V., Wikberg J.E.S., Life Sci. 59 (1996) 797–801.

[12] Xia Y., Wikberg J.E.S., Cell Tissue Res. 286 (1996) 63–68.

[13] Schioth H.B., Muceniece R., Wikberg J.E.S., Pharmacol. Toxicol. 79 (1997) 161–165.

[14] Schioth H.B., Muceniece R., Wikberg J.E.S., Chhajlani V., Eur. J. Pharmacol. 288 (1995) 311–317.

[15] A.N. Eberle, The melanotropins: chemistry, physiology, and mechanisms of action. Basel, Karger, 1988.

[16] Sugg E.E., Cody W.L., Abdel-Malek Z., Hadley M.E., Hruby V.J., Biopolymers 25 (1987) 2029–2042.

[17] Sahm U.G., Olivier G.W., Branch S.K., Moss S.H., Pouton C.W., Peptides 15 (1995) 1297–1302.

[18] Castrucci A.M., Hadley M.E., Sawyer T.K., Wilkes B.C., al-Obeidi F., Staples D.J., de Vaux A.E., Dym O., Hintz M.F., Riehm J.P., Rao K.R., Hruby V.J., Gen. Comp. Endocrinol. 73 (1989) 157–163.

[19] Haskell-Luevano C., Hendrata S., North C., Sawyer T.K., Hadley M.E., Hruby V.J., Dickinson C., Gantz I., J. Med. Chem. 40 (1997) 2133–2139.

[20] Prusis P., Schioth H.B., Muceniece R., Herzyk P., Afshar M., Hubbard R.E., Wikberg J.E.S., J. Mol. Graph. Model 15 (1998) 307–317, 334.

[21] Schioth H.B., Muceniece R., Larsson M., Mutulis F., Szardenings M., Prusis P., Lindeberg G., Wikberg J.E.S., Eur. J. Pharmacol. 319 (1997) 369–373.

[22] Haskell-Luevano C., Nikiforovich G., Sharma S.D., Yang Y.K., Dickinson C., Hruby V.J., Gantz I., J. Med. Chem. 40 (1997) 1738–1748.

[23] Haskell-Luevano C., Shenderovich M.D., Sharma S.D., Nikiforovich G.V., Hadley M.E., Hruby V.J., J. Med. Chem. 38 (1995) 1736–1750.

[24] Nikiforovich G.V., Sharma S.D., Hadley M.E., Hruby V.J., Biopolymers 46 (1998) 155–167.

[25] Al-Obeidi F., O'Connor S.D., Job C., Hruby V.J., Pettitt B.M., J. Pept. Res. 51 (1998) 420–431.

[26] Prabhu N.V., Perkyns J.S., Pettitt B.M., Hruby V.J., Biopolymers 50 (1999) 255–272.

[27] Prabhu N.V., Perkyns J.S., Pettitt B.M., J. Pept. Res. 54 (1999) 394–407.

[28] Li S.Z., Lee J.H., Lee W., Yoon C.J., Baik J.H., Lim S.K., Eur. J. Biochem. 265 (1999) 430–440.

[29] Schioth H.B., Mutulis F., Muceniece R., Prusis P., Wikberg J.E.S., Eur. J. Pharmacol. 349 (1998) 359–366.

[30] Sawyer T.K., Sanfilippo P.J., Hruby V.J., Engel M.H., Heward C.B., Burnett J.B., Hadley M.E., Proc. Natl. Acad. Sci. USA 77 (1980) 5754–5758.

[31] Hunter W.M., Greenwood F.C., Nature 194 (1962) 495–496.

[32] A.T. Brünger, X-PLOR Version 3.1. A system for X-ray crystallography and NMR. London, Yale University Press, 1992.

[33] Brooks B.R., Bruccoleri R.E., Olafson B.D., States D.J., Swaminathan S., Karplus M., J. Comput. Chem. 4 (1983) 187–217.

[34] MacKerell J.A.D., Bashford D., Bellott M., Dunbrack Jr R.L., Evanseck J., Field M.J., Fischer S., Gao J., Guo H., Ha S., Joseph D., Kuchnir L., Kuczera K., Lau F.T.K., Mattos C., Michnick S., Ngo T., Nguyen D.T., Prodhom B., Reiher I.W.E., Roux B., Schlenkrich M., Smith J., Stote R., Straub J., Watanabe M., Wiorkiewicz-Kuczera J., Yin D., Karplus M., J. Phys. Chem. B102 (1998) 3586–3616.

[35] SYBYL 6.5, Tripos Inc., 1699 South Hanley Road, St. Louis, Missouri, 63144, USA.

[36] Kraulis P.J., J. Appl. Crystallogr. 24 (1991) 946–950.