

Supporting information

Peptoid-Peptide Hybrids as Potent Novel Melanocortin Receptor Ligands

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Experimental Section

Synthesis of peptoid-peptide hybrids

Fmoc-protected amino acids were purchased from Alexis (Switzerland). The following side chain protected- N^{α} -Fmoc-protected amino acids were used: Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH and Fmoc-Trp(Boc)-OH. HATU and HOAt were purchased from Perkin Elmer Biosystems (Nieuwerkerk a/d/ IJsel, The Netherlands). The peptoid-peptide hybrids were synthesized on ArgoGel™ Rink-NH-Fmoc resin, 0.32 mmol/g (Argonaut Technologies, Muttenz, Switzerland). Peptide grade NMP, DCE, and TFA and HPLC-grade CH_3CN , MTBE, and n-hexane were purchased from Biosolve B.V. (Valkenswaard, The Netherlands). All other solvents and reagents were acquired from commercial sources and used without further purification.

Fmoc-protected N-substituted glycines; Fmoc-NNle-OH, Fmoc-NLys(Boc)-OH, Fmoc-NArg(Boc)₂-OH and Fmoc-NhTrp(Boc)-OH were synthesized as described earlier^{1,2,3}. Fmoc-Trp(Boc)-OH was synthesized in four steps from indole-3-carboxaldehyde. Briefly, indole-3-carboxaldehyde was protected with a Boc-group by the method of Wolman⁴ using di-t-butyl dicarbonate instead of t-butyloxycarbonyl azide and sodium hydride in N,N-dimethylformamide followed by reductive amination of glycine ethyl ester with the protected aldehyde in the presence of sodium borohydride in methanol. Subsequently, the ethyl ester was saponified using 1.05 equivalent of sodium hydroxide (0.5 M) in dioxane and the amino group of the resulting sodium salt was protected with a Fmoc-group⁵ to provide Fmoc-Trp(Boc)-OH.

Fmoc-NhArg(Boc)₂-OH was synthesized in two steps from Fmoc-NLys(Boc)-OH: the Boc-group of Fmoc-NLys(Boc)-OH was removed by treatment with HCl/ether, followed by guanylation of the amino group with N,N'-bis-Boc-1-guanylpyrazole^{6,7}.

Peptoid-peptide hybrids were synthesized on a MultiSynTech Syro II Robot Synthesizer (MultiSynTech, Witten, Germany) employing the "monomer approach"⁸ in which Fmoc-protected N-substituted glycine monomers and Fmoc-protected amino acids are coupled. Hybrids were synthesized on 0.05 mmol scale in a polypropylene reaction tube equipped with a polypropylene frit. Syntheses were carried out on ArgoGel™ Rink-NH-Fmoc resin (0.32 mmol/g) to obtain C-terminal amides. Each synthesis started with 156 mg dry resin. This resin was swollen in DCE (twice with 2 mL, each for 4 min) followed by NMP (two times with 2 mL, each for 4 min). The Fmoc group was removed by a double treatment with 2 mL 20% piperidine in NMP during 8 min. Subsequently, the resin was extensively washed with NMP (five times with 2.5 mL each 2 min). To this deprotected resin, 1 mL of a 0.2 M Fmoc-protected N-substituted glycine monomer or Fmoc-protected amino acid solution in NMP was added followed by 0.75 mL of a 0.267 M solution of HATU/HOAt in NMP and 0.5 mL of a 0.4 M solution of DiPEA in NMP. After 45 min, the reaction tube was drained and the resin was washed with NMP (five times with 2.5 mL each 2 min). During the resin swelling and washing steps, Fmoc deprotection and coupling reactions, the resin suspension was magnetically mixed by levitation stirring for 15 s per min of coupling time. After the final Fmoc removal the free N-terminus was acetylated by a double treatment with 2.2 mL acetic anhydride capping solution (0.5 M Ac_2O , 0.125 DiPEA, and 0.015 M HOBt in NMP) for 21 min. Finally, the resin was washed with NMP (five times with 2.5 mL each 2 min) and DCE (three times with 2.5 mL each 2 min). The thus obtained anchored peptoid peptide hybrids were cleaved from the resin and deprotected by treatment with 2 mL TFA/ H_2O /TIS/EDT (90:5:2.5:2.5, v/v/v/v) solution for 2 h at room temperature, followed by precipitation with MTBE/n-hexane (1:1, v/v, 50 mL). After pelleting the precipitate by centrifugation (3000 rpm, 5 min), the supernatant was decanted,

resuspended in MTBE/n-hexane (1:1, v/v) and centrifuged again. Finally, the pellet was washed twice with MTBE (50 mL), dissolved in *tert*-BuOH/water (1:1, v/v, 5-10 mL) and lyophilized to give the peptoid peptide hybrids as white fluffy solids. The identity and purity of the synthesized hybrids were verified by HPLC and LC-ESI-MS. The mass of each hybrid was measured and the observed monoisotopic ($M + H$)⁺ values were correlated with the calculated ($M + H$)⁺ values. The measured mass value was consistent with the calculated mass value in all cases.

HPLC runs were performed on two HPLC systems: a Gilson automated HPLC workstation with UV detector system operating at 214 nm and 278 nm, or a Shimadzu automated HPLC system with a UV detector system operating at the same wavelengths. Analytical HPLC was conducted by using an Alltech Adsorbosphere XL C18 (300 Å, 5 µm, 250 x 4.6 mm) column at a flow rate of 1.0 mL min⁻¹. Elution was effected with a gradient from 0.1% TFA in water/acetonitrile (90/10) to 0.1% TFA in acetonitrile/water (60/40) over 40 minutes. Electrospray ionization mass spectrometry (ESI-MS) was measured on a Shimadzu LCMS-QP8000 single quadrupole benchtop mass spectrometer operating in a positive ionization mode. For the MS full scan spectra, data were acquired in continuum mode over the range m/z 200 to 1500 in 1 second at unit mass resolution. A deflector voltage of 50 V was set, which produces predominantly ($M + H$)⁺ ions. Instrumental control, data acquisition and data processing were carried out using the CLASS 8000 software package (version 1.10).

Binding assay

For binding experiments, the human MC3 or MC4 receptors or the mouse MC5 receptor were transiently expressed in HEK293 cells. IC₅₀ and K_i values were determined with iodinated (Nle⁴;Phe⁷)- α -MSH (NDP-MSH) as tracer. NDP-MSH was iodinated using bovine lactoperoxidase (Calbiochem, La Jolla, CA) and ¹²⁵I-Na (ICN, Aurora, OH) according to Oosterom et al⁹ and subsequently high pressure liquid chromatography-purified on a C18 column (mBondapak 3.9 X 300 mm, Waters, Milford, MA). Cells growing in 24-wells plates were washed with tris-buffered saline (TBS) supplemented with 2.5 mM calcium chloride and incubated for 30 min at room temperature with peptides and tracer diluted in Ham's F10 medium (Gibco, Paisley, Scotland) supplemented with 2.5 mM calcium chloride, 0.25% bovine serum albumine (BSA) (ICN, Aurora, USA) and 200 KIU/ml aprotinin (Sigma, Steinheim, Germany). After two washes with ice-cold TBS (+ 2.5 mM calcium chloride) to remove non-bound tracer, the cells were lysed in 1M sodium hydroxide and samples were counted in a γ -counter.

In each binding experiment, IC₅₀ values of non-iodinated NDP-MSH were determined to allow calculation of K_i values using the formula: K_i = (IC₅₀(compound)/IC₅₀(NDP-MSH)) * Kd(NDP-MSH). The K_d values of ¹²⁵I-NDP-MSH for the receptors were calculated from saturation isotherms of ¹²⁵I-NDP-MSH (two independent experiments for calculation of K_i values for each receptor).

Activation assay

Activation of human MC3 or MC4 receptors (and rat MC4 receptor) or the mouse MC5 receptors was determined using LacZ as a reporter gene¹⁰. HEK293 cells growing in 10-cm dishes were co-transfected with 100-200 ng receptor DNA and 7 µg of CRE-LacZ construct. After transfection, the cells were plated into 96-wells plates (Becton Dickinson). Two days after plating, the cells were incubated with peptides at the appropriate concentrations in serum-free medium (DMEM containing 0.2% BSA (ICN, Aurora, USA) glutamine (Gibco, Paisley,

Scotland) and non-essential amino acids (Gibco, Paisley, Scotland)). After 5-6 hours of incubation, the assay medium was aspirated and 40 ml of lysis buffer (PBS containing 0.1% triton-X-100 (Boehringer, Mannheim, Germany)) was added. The plates were stored at -20 °C and after thawing 80 μ l of substrate mix (0,1 M phosphate buffer, pH7.4 containing 1.6 g/l o-Nitrophenyl β -D-galactopyranoside (ONPG, Molecular probes, Leiden, the Netherlands), 67.5 μ M b-mercaptoethanol (Merck, Darmstadt, Germany) and 1.5 mM magnesium chloride) was added. Absorbance at 405 nm was determined in a Victor² microplate reader (PerkinElmer, Brussels, Belgium).

Grooming assay

Male Wistar rats weighing 200-240 g at the start of the study were used. Animals were housed in groups of 2-3 in plastic cages on a sawdust bedding and kept at a 12/12hr light/dark cycle, with food and water available ad libitum. All testing procedures in this experiment were approved of by the Ethics Committee on Animal Experiments of the Utrecht University. For grooming assays, rats received an intra-cerebroventricular (i.c.v.) cannula as described in Brakkee et al.¹¹, and were allowed to recover for three days. Before the experiment, the rats were kept for at least one hour in the experimenting room to reduce novelty-induced grooming. Grooming assays were performed as described in Gispen et al.¹². Briefly, observation started 10 minutes after i.c.v. injection of compounds diluted in saline. Grooming behaviour was scored at timepoints with intervals of 15 seconds for a period of 60 minutes. The grooming score per rat is expressed as the number of timepoints that the rat showed grooming behaviour. For i.c.v. injections, 3 μ l of fluid was used.

Table 1. Yields, purities and mass spectral data of used peptoid-peptide hybrids

	Yield (mg)	Purity [#] (%)	[M+H] ⁺ calculated	[M+H] ⁺ found
1 : Ac-Nle-Gly-Lys-D-Phe-Arg-Trp-Gly-NH ₂	52.4	97	904.52	904.25
3 : Ac- <u>NNle</u> -Gly-Lys-D-Phe-Arg-Trp-Gly-NH ₂	53.0	95	904.52	904.35
4 : Ac-Nle-Gly- <u>NLys</u> -D-Phe-Arg-Trp-Gly-NH ₂	53.5	92	904.52	904.25
5 : Ac-Nle-Gly-Lys- <u>NPhe</u> -Arg-Trp-Gly-NH ₂	48.8	78	904.52	904.43
6 : Ac-Nle-Gly-Lys-D-Phe- <u>NArg</u> -Trp-Gly-NH ₂	40.5	87	904.52	904.20
7 : Ac-Nle-Gly-Lys-D-Phe-Arg- <u>NhTrp</u> -Gly-NH ₂	45.2	82	918.53	918.15
8 : Ac- <u>NNle</u> -Gly- <u>NLys</u> -D-Phe-Arg-Trp-Gly-NH ₂	53.3	92	904.52	904.25
9 : Ac- <u>NNle</u> -Gly- <u>NPhe</u> -Arg-Trp-Gly-NH ₂	54.8	86	904.52	904.30
10 : Ac- <u>NNle</u> -Gly-Lys-D-Phe- <u>NArg</u> -Trp-Gly-NH ₂	50.5	87	904.52	904.15
11 : Ac- <u>NNle</u> -Gly-Lys-D-Phe-Arg- <u>NhTrp</u> -Gly-NH ₂	53.4	79	918.53	918.25
12 : Ac-Nle-Gly- <u>NLys</u> - <u>NPhe</u> -Arg-Trp-Gly-NH ₂	53.0	95	904.52	904.25
13 : Ac-Nle-Gly- <u>NLys</u> -D-Phe- <u>NArg</u> -Trp-Gly-NH ₂	50.3	89	904.52	904.40
14 : Ac-Nle-Gly- <u>NLys</u> -D-Phe-Arg- <u>NhTrp</u> -Gly-NH ₂	52.5	80	918.53	918.15
15 : Ac-Nle-Gly-Lys- <u>NPhe</u> - <u>NArg</u> -Trp-Gly-NH ₂	49.1	94	904.52	904.30
16 : Ac-Nle-Gly-Lys- <u>NPhe</u> -Arg- <u>NhTrp</u> -Gly-NH ₂	50.6	68	918.53	918.20
17 : Ac-Nle-Gly-Lys-D-Phe- <u>NArg</u> - <u>NhTrp</u> -Gly-NH ₂	47.3	92	918.53	918.25
18 : Ac- <u>NNle</u> -Gly- <u>NLys</u> - <u>NPhe</u> -Arg-Trp-Gly-NH ₂	53.9	87	904.52	904.35
19 : Ac- <u>NNle</u> -Gly- <u>NLys</u> -D-Phe- <u>NArg</u> -Trp-Gly-NH ₂	53.0	85	904.52	904.20
20 : Ac- <u>NNle</u> -Gly- <u>NLys</u> -D-Phe-Arg- <u>NhTrp</u> -Gly-NH ₂	53.8	86	918.53	918.25
21 : Ac-Nle-Gly- <u>NLys</u> - <u>NPhe</u> - <u>NArg</u> -Trp-Gly-NH ₂	48.5	94	904.52	904.25
22 : Ac-Nle-Gly- <u>NLys</u> - <u>NPhe</u> -Arg- <u>NhTrp</u> -Gly-NH ₂	51.3	90	918.53	918.25
23 : Ac-Nle-Gly-Lys- <u>NPhe</u> - <u>NArg</u> - <u>NhTrp</u> -Gly-NH ₂	50.7	98	918.53	918.25
24 : Ac- <u>NNle</u> -Gly-Lys- <u>NPhe</u> - <u>NArg</u> -Trp-Gly-NH ₂	53.9	94	904.52	904.50
25 : Ac- <u>NNle</u> -Gly-Lys- <u>NPhe</u> -Arg- <u>NhTrp</u> -Gly-NH ₂	47.4	79	918.53	918.40
26 : Ac- <u>NNle</u> -Gly-Lys-D-Phe- <u>NArg</u> - <u>NhTrp</u> -Gly-NH ₂	50.8	89	918.53	918.40
27 : Ac-Nle-Gly- <u>NLys</u> -D-Phe- <u>NArg</u> - <u>NhTrp</u> -Gly-NH ₂	54.6	89	918.53	918.25
28 : Ac-Nle-Gly- <u>NLys</u> - <u>NPhe</u> - <u>NArg</u> - <u>NhTrp</u> -Gly-NH ₂	51.3	94	918.53	918.55
29 : Ac- <u>NNle</u> -Gly-Lys- <u>NPhe</u> - <u>NArg</u> - <u>NhTrp</u> -Gly-NH ₂	56.0	94	918.53	918.25
30 : Ac- <u>NNle</u> -Gly- <u>NLys</u> -D-Phe- <u>NArg</u> - <u>NhTrp</u> -Gly-NH ₂	52.4	88	918.53	918.45
31 : Ac- <u>NNle</u> -Gly- <u>NLys</u> - <u>NPhe</u> -Arg- <u>NhTrp</u> -Gly-NH ₂	50.8	86	918.53	918.25
32 : Ac- <u>NNle</u> -Gly- <u>NLys</u> - <u>NPhe</u> - <u>NArg</u> -Trp-Gly-NH ₂	53.9	87	904.52	904.50
2 : Ac- <u>NNle</u> -Gly- <u>NLys</u> - <u>NPhe</u> - <u>NArg</u> - <u>NhTrp</u> -Gly-NH ₂	57.4	90	918.53	918.35
33 : Ac-Nle-Gly-Lys-D-Phe-Arg- <u>NTrp</u> -Gly-NH ₂	35.8	86	904.52	904.60
34 : Ac- <u>NNle</u> -Gly-Lys-D-Phe-Arg- <u>NTrp</u> -Gly-NH ₂	38.5	83	904.52	904.55
35 : Ac-Nle-Gly- <u>NLys</u> -D-Phe-Arg- <u>NTrp</u> -Gly-NH ₂	40.0	83	904.52	904.75
36 : Ac-Nle-Gly-Lys-D-Phe- <u>NArg</u> - <u>NTrp</u> -Gly-NH ₂	37.8	89	904.52	904.50
37 : Ac- <u>NNle</u> -Gly- <u>NLys</u> -D-Phe-Arg- <u>NTrp</u> -Gly-NH ₂	31.1	73	904.52	904.55
38 : Ac- <u>NNle</u> -Gly-Lys-D-Phe- <u>NArg</u> - <u>NTrp</u> -Gly-NH ₂	36.3	73	904.52	904.40
39 : Ac-Nle-Gly- <u>NLys</u> -D-Phe- <u>NArg</u> - <u>NTrp</u> -Gly-NH ₂	39.5	92	904.52	904.40
40 : Ac- <u>NNle</u> -Gly- <u>NLys</u> -D-Phe- <u>NArg</u> - <u>NTrp</u> -Gly-NH ₂	40.1	91	904.52	904.50
41 : Ac-Nle-Gly-Lys-D-Phe- <u>NhArg</u> -Trp-Gly-NH ₂	31.9	99	918.53	919.25
42 : Ac-Nle-Gly-Lys-D-Phe- <u>NhArg</u> - <u>NhTrp</u> -Gly-NH ₂	41.6	96	932.55	932.85

determined by HPLC

* monoisotopic mass

Table 2. EC₅₀ values and their range (M) of compounds on MC receptors

	MC3		MC4		mMC5	
	EC50	range	EC50	range	EC50	range
α -MSH	1,46E-09	1,03E-09 2,06E-09	1,21E-08	9,22E-09 1,68E-08	7,01E-10	4,60E-10 1,05E-09
1	7,77E-09	6,40E-09 9,45E-09	5,13E-10	2,77E-10 9,50E-10	2,98E-08	2,55E-08 3,48E-08
2	>1.0E-05		>1.0E-05		>1.0E-05	
3	2,50E-08	1,79E-08 3,55E-08	2,80E-09	5,15E-10 1,57E-08	7,80E-07	6,76E-07 8,94E-07
4	3,50E-07	2,61E-07 4,60E-07	1,80E-08	6,91E-09 4,69E-08	5,70E-07	4,73E-07 6,94E-07
5	>1.0E-05		1,40E-06	8,57E-07 2,28E-06	>1.0E-05	
6	4,10E-06	2,15E-06 7,88E-06	5,80E-07	8,95E-08 3,79E-06	2,80E-07	2,39E-07 3,38E-07
7	2,20E-06	1,78E-06 2,65E-06	3,00E-07	1,69E-07 5,28E-07	2,20E-06	1,54E-06 3,02E-06
8	2,00E-06	1,47E-06 2,64E-06	6,70E-08	3,67E-08 1,23E-07	8,50E-06	5,55E-06 1,29E-05
9	1,30E-06	4,45E-06 3,79E-05	1,80E-06	8,42E-07 3,98E-06	>1.0E-06	
10	5,28E-06	3,88E-06 7,17E-06	7,86E-07	4,99E-07 1,24E-06	6,85E-06	4,24E-06 1,11E-05
11	3,48E-06	2,87E-06 4,22E-06	2,83E-07	1,81E-07 4,43E-07	1,13E-05	7,11E-06 1,78E-05
12	>1.0E-05		>1.0E-05	1,44E-06 2,70E-06	>1.0E-05	
13	>1.0E-05		1,80E-06	1,22E-06 2,64E-06	5,14E-07	3,91E-07 6,77E-07
14	>1.0E-05		1,79E-06	1,06E-06 3,01E-06	>1.0E-05	
15	0,00E+00		0,00E+00		0,00E+00	
16	0,00E+00		0,00E+00		1,65E-08	1,43E-08 1,90E-08
17	1,46E-07	1,21E-07 1,78E-07	8,40E-08	5,50E-08 1,28E-07	>1.0E-05	
18	>1.0E-05		>1.0E-05		6,11E-06	5,38E-06 6,94E-06
19	>1.0E-05		3,31E-06	2,66E-06 4,12E-06	>1.0E-05	
20	>1.0E-05		5,77E-06	2,27E-06 1,47E-05	1,96E-19	1,39E+05
21	>1.0E-05		>1.0E-05		>1.0E-05	
22	>1.0E-05		>1.0E-05		2,56E-08	4,78E-05
23	1,88E-05	7,03E-06 5,05E-05	9,40E-04	4,88E-36 3,20E+29	8,22E-06	7,00E-06 9,64E-06
24	0,00E+00		0,00E+00		0,00E+00	
25	0,00E+00		0,00E+00		3,18E-07	2,62E-07 3,86E-07
26	1,67E-07	1,38E-07 2,02E-07	1,07E-07	6,28E-08 1,81E-07	9,84E-07	8,49E-07 1,14E-06
27	4,44E-06	3,55E-06 5,56E-06	3,79E-07	2,13E-07 6,74E-07	0,00E+00	
28	0,00E+00		0,00E+00		0,00E+00	
29	0,00E+00		0,00E+00		1,63E-05	2,84E-06 9,37E-05
30	5,86E-06	5,14E-06 6,69E-06	7,53E-07	5,54E-07 1,02E-06	0,00E+00	
31	0,00E+00		0,00E+00		>1.0E-05	
32	>1.0E-05		>1.0E-05		2,57E-07	2,22E-07 3,13E-07
33	3,00E-07	1,90E-07 4,84E-07	1,58E-07	8,27E-08 2,94E-07	3,97E-06	2,91E-06 5,50E-06
34	7,13E-07	6,31E-07 8,19E-07	5,76E-07	3,73E-07 8,95E-07	2,01E-06	1,56E-06 2,57E-06
35	5,25E-06	3,28E-06 8,43E-06	1,30E-06	1,12E-06 1,49E-06	9,11E-08	7,41E-08 1,11E-07
36	6,38E-07	5,40E-07 7,58E-07	1,58E-07	9,17E-08 2,84E-07	1,28E-05	7,33E-06 2,22E-05
37	1,26E-05	4,92E-06 3,19E-05	4,37E-06	3,56E-06 5,33E-06	1,00E-06	8,52E-07 1,19E-06
38	9,57E-07	7,58E-07 1,19E-06	4,74E-07	3,50E-07 6,53E-07	3,04E-06	2,55E-06 3,62E-06
39	6,00E-06	4,83E-06 7,35E-06	2,04E-06	1,88E-06 2,31E-06	4,67E-05	4,30E-07 5,04E-03
40	1,35E-05	5,07E-06 3,55E-05	5,48E-06	4,74E-06 6,31E-06		

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