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Synthesis and evaluation of bivalent ligands for binding to the human melanocortin-4 receptor

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Abstract

Membrane proteins, especially G-protein coupled receptors (GPCRs), are interesting and important theragnostic targets since many of them serve in intracellular signaling critical for all aspects of health and disease. The potential utility of designed bivalent ligands as targeting agents for cancer diagnosis and/or therapy can be evaluated by determining their binding to the corresponding receptors. As proof of concept, GPCR cell surface proteins are shown to be targeted specifically using multivalent ligands. We designed, synthesized, and tested a series of bivalent ligands targeting the over-expressed human melanocortin 4 receptor (hMC4R) in human embryonic kidney (HEK) 293 cells. Based on our data suggesting an optimal linker length of 25±10 Å inferred from the bivalent melanocyte stimulating hormone (MSH) agonist, the truncated heptapeptide, referred to as **MSH(7)**: Ac-Ser-Nle-Glu-His-*D*-Phe-Arg-Trp-NH_2 was used to construct a set of bivalent ligands incorporating a hMC4R antagonist, **SHU9119**: Ac-Nle-$_c$[Asp-His-2′-*D*-Nal-Arg-Trp-Lys]-NH_2 and another set of bivalent ligands containing the **SHU9119** antagonist pharmacophore on both side of the optimized linkers. These two binding motifs within the bivalent constructs were conjoined by semi-rigid $(Pro\text{-}Gly)_3$ units with or without the flexible poly(ethylene glycol) (PEGO) moieties. Lanthanide-based competitive binding assays showed bivalent ligands binds to the hMC4R with up to 240-fold higher affinity than the corresponding linked monovalent ligands.

Keywords

Bivalent ligands; linkers; melanorcortin receptors; Eu-binding assay; solid phase synthesis

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1. Introduction

Cancer is in its most aggressive state when it has metastasized to the entire body. Hence early detection is critical to the successful treatment of many human cancers. Therapies to treat cancer must selectively target these invading cells within healthy normal tissues. It is accepted that metastatic cancers include multiple genetic abnormalities that are currently targets for many bio-pharmaceutical companies. Most current drug therapies are not molecularly specific and are associated with side-effects and toxicities. There is a possibility of individually developing single molecules containing multiple pharmacophores for specific overexpressed proteins on a cancer cell that may have a differentially reduced expression on the normal cell.[1] These resulting multivalent molecules could display enhanced affinity for the targeted cells.[2-4] Targeting cell surface receptors can help inhibit cell surface receptor-ligand interactions or act as positive / negative effectors of downstream signal transduction. Multimeric ligands that contain a target specific agonist or antagonist pharmacophore can take toxic pay-loads directly into the tumors, destroying the cancer cells, while leaving the normal cells unharmed. An imaging agent on the multimeric ligand will be guided as a single molecule that will potently bind and image the infected area (overexpressed receptors) and can be used in non-invasive techniques for cancer detection e.g. early detection of adenomatous polyps in colon cancer.

The common feature of the bivalent ligand binding is that following the initial binding of one pharmacophore within a bivalent construct, succeeding binding is more favorable thanks to decreased loss of entropy.[5] As the result, the bivalent ligands can enhance binding affinity, agonist / antagonist potency and GPCR subtype selectivity. The central dogma of GPCR pharmacology has been the concept that unlike agonists, antagonist ligands display equivalent affinities for a given receptor, regardless of the cellular environment in which the affinity is assayed.[6]

As a proof of the concept, we chose to mimic the cancer cells through overexpression of MC4 receptor, which is one of five types of melanocortin receptors termed MC1–5R that exhibit about 40–60% homology.[7] Among the melanocortin receptors, the MC4R is of particular interest and a potential target in the research study as a major regulator of eating behavior and body weight, and suggestions have been made towards its role in the stimulation of male erectile activity.[8,9] Synthetically low molecular weight agonists and antagonists selective for the different subtypes are highly warranted as remedies for treatment of various dysfunctional states, such as obesity, anorexia, impotence, and autoimmune disorders.[10] Studies have suggested that bivalent ligands have receptor binding properties that differ substantially from those of the monovalent ligand, and the spacer used to link the two pharmacophores within the construct exerts a profound influence on the potency. The bivalent ACTH antagonists show potency enhancements up to approximately 25 times that of the monovalent constructs also demonstrating the role of spacer [bis(maleimide)cross-linking] and a peptide pharmacophore component within a bivalent construct.[11] Handl *et al.* demonstrated the potential of a series of MSH-7 agonist homobivalent ligands compared to its monovalent construct that can be utilized as targeting agents for cancer imaging.[3] The homobivalent ligands binds to hMC4R with increased affinity and apparent co-operativity compared to their monovalent analogues.[3] The increased

binding affinity and positive cooperativity were most likely not due to statistical binding, but rather to a receptor clustering mechanism, wherein multiple receptors are bound by the same multivalent ligand.[12] In this study, we used a combination of agonist and antagonist pharmacophores in the design of bivalent ligands and the results could help determine organizational features of the melanocortin receptor-GPCR. We chose to construct ligands containing one copy of **MSH(7)**, a truncated version of [Nle^4-*D*-Phe^7]-α-melanocyte stimulating hormone (NDP-α-MSH) and a very potent cyclic MC4R antagonist **SHU9119**.[13] These two MC4R pharmacophores were separated by a series of linkers, which are different in flexibility and length. Poly(ethylene glycol) (PEGO) and (Pro-Gly)$_3$ units were used either by themselves or by incorporations, as shown in Table 1.

It has been proposed that the first pharmacophore binding event serves to attach the multivalent ligand to the surface, here we have evaluated the use of a tight binding pharmacophore **SHU9119** in combination with a comparatively lower binding pharmacophore, **MSH(7)**.[14,15] We proposed that there would be effectively an additive enhancement of binding compared to homobivalent **MSH**(**7**) analogues, which we have shown in a previous publication, as the pharmacophore **SHU9119** should bind to the receptor tightly and then linkers should provide greater opportunity for the bivalent ligand to explore more volume and thus have a higher probability to bind multiple receptors at once, hence making them capable of cross-linking adjacent receptors.[3]

2. Results and Discussion

2.1. Synthesis

As shown in Figure 1, bivalent ligands **7-12** and **13-18** consisting of two **SHU9119** moieties and **MSH(7)** and **SHU9119,** respectively, with PEGO and/or (Pro-Gly)$_3$ linkers were synthesized by standard solid phase synthesis using Fmoc-chemistry successfully. Monovalent ligands **1-6** were also prepared as control ligands by the same procedure.

The cyclized heptapeptide **SHU9119** was constructed on Rink amide Tentagel S resin and PEGO linkers were attached to the resin. The PEGO attached resin was proportionally split for syntheses of control monovalent ligands **4-6**, bivalent ligands **10-12**, and **16-18.** For the synthesis of ligands **11** and **12**, the split resin was coupled with Fmoc-Lys(Alloc)-OH and the solid phase peptide synthesis continued to complete the second **SHU9119** sequence. Subsequently, part of the split resin was coupled with Fmoc-amino acids stepwise to attach the **MSH(7)** moiety for ligands **17-18**. Attachment of **SHU9119** or **MSH(7)** moiety in bivalent or monovalent ligands were performed routinely without difficulty by the procedures as mentioned above. Another portion of the resin was used to connect with (Pro-Gly)$_3$ and PEGO linkers and **SHU9119** or **MSH(7)** was attached to the resulting resin to afford longer length bivalent ligands **10** and **16**. The resin was previously split into three syringe reactor portions for the attachment of Pro-Gly linkers, giving monovalent ligand resins **1-3**. The attachment of the second moiety **SHU9119** or **MSH(7)** was carried out, affording **7-9** or **13-15**, respectively. The Fmoc-groups on all of the resin precursors were deprotected, and peptides were acylated, and cleaved by a mixture of TFA, EDT, thioanisole, and water (91/3/3/3) that afforded the desired ligands **1-18** as shown in Table 1.

Ligands **1- 18** were purified by preparative RP-HPLC and were characterized by ESI-MS and/or MALDI-TOF MS to confirm their structures.

2.2. Binding of monovalent and bivalent ligands

The binding affinities were evaluated in a lanthanide based competitive assay (Dissociation Enhanced Lanthanide FluoroImmuno Assay: DELFIA) using optimized 10 nM standard agonist Eu-DTPA-NDP-α-MSH chelate in HEK293 cells overexpressing hMC4R. As shown in Table 2, EC_{50} values were calculated after computing the hill slope using the GraphPad Prism software and compared with ligands **MSH(7)** with EC_{50} ∼50 nM and **SHU9119** with EC_{50} ∼60 pM; these binding affinities were consistent with the ones obtained from previously published data using radiolabeled binding assay.[16]

The conjugation of the linkers to the monovalent antagonist-**SHU9119** reduced its high affinity (EC_{50} = 59 pM) up to 400 nM by 6800-fold in ligand **6**. As the different lengths of linkers were attached to the antagonist pharmacophore, there was a trend observed in binding to the receptor. Apparently, the analogues containing the semi rigid $(Pro\text{-}Gly)_3$ linker retained higher binding affinity than those with the flexible linker PEGO, and longer length of PEGO linker resulted in the loss of binding affinity (EC_{50} = 400 nM) in ligand **6**. However, the low affinity was reversed to high affinity (EC_{50} = 3.7 nM, 108-fold) by the insertion of a semi rigid linker $(Pro\text{-}Gly)_3$ in ligand **4.** Ligand **1** with a shorter length of linker (10-20 Å) showed higher binding affinity (EC_{50} = 4.0 nM) than ligands **2** (EC_{50} = 8.1 nM) and **3** (EC_{50} = 13 nM) with a longer length of linker. This is coincident with our previous result showing linker effects on the binding affinities of MSH(7) monovalent ligands.[3] It is clear that the attachment of a semi rigid $(Pro\text{-}Gly)_3$ linker, which may assist in reducing entropy of the ligand, results in the high binding affinity of the monovalent ligands.

Contrary to the effect of a linker on monovalent ligands **1-6,** there was no such clear effect observed on the bivalent ligands **8-18**. Interestingly, bivalent ligands **12** and **18**, which contain a flexible longer linker PEGO-PEGO like monovalent ligand **6** (EC_{50} = 400 nM) retained the same high binding affinities (EC_{50} = 1.9 nM and 1.7 nM, respectively) as the other bivalent ligands. The increases of binding affinities in **12** and **18** were more than 200-fold relative to the monovalent ligand. It may not be simple to explain how the flexible linkers did not cause disturbances of the binding affinities in the bivalent ligands, but it is possible that the attachment of the second pharmacophore help in reducing the effects of flexibility of the linker but increasing cooperative effects between two pharmacophores.

Use of the optimized linker of 15 – 30 Å, obtained from our previous experiments, fixed the translational and rotational entropies associated with the linker types for the hMC4R.[3] The incorporation of a potent antagonist **SHU9119** with the optimized length of a linker $(Pro\text{-}Gly)_9$ to monovalent ligand **2** (EC_{50} = 8.1 nM) improved the binding affinity slightly in bivalent ligands **8** (EC_{50} = 1.6 nM, 5-fold) and **14** (EC_{50} = 2.0 nM, 4-fold) (Figure 3).

In general, the homo-bivalent ligands (**8-11**) and hetero-bivalent ligands (**13-17**) bound with slightly increased (2-13 fold) affinity for the receptor compared to the monovalent linker-**SHU9119** analogues (**1-5**). Interestingly, all of the homo- and hetero-bivalent ligands showed the same high range of binding affinities regardless of their linkers and

pharmacophores. This result may be explained simply by the first tight binding of **SHU9119** that can assist crosslinking of the second pharmacophore in the vicinity. The increase of binding affinities in hetero-bivalent ligands (**13-15**) was shown to be more pronounced (20-118 fold) comparing to the monovalent linker MSH(7) analogues (**19-21**). Consistent with the results obtained in previous works, it is clear that the more pronounced enhancements can be achieved with lower affinity pharmacophores in bivalent ligands.[17]

The enhanced binding affinity of the bivalent ligands is attributed to apparent cooperativity. Evaluation of the Hill coefficients resulting from the monovalent and bivalent ligand bindings confirms that the bivalent ligands bind with a cooperative effect. The obtained Hill coefficient for monovalent analogues on an average was less than 1 (0.88) while for the bivalent analogues, the average was greater than 2, even with exceptionally low coefficients for mixed bivalent ligands **13** and **14**. These low Hill coefficients (0.7 and 0.8 for **13** and **14**, respectively) may indicate that there is no cooperativity between the two pharmacohpores due to the short and rigid linkers. Comparing to ligands **13** and **14**, ligand **15** possess higher Hill coefficient (2.0), which could assist in the increase of binding affinity (EC_{50} = 1.6 nM, 8-fold). It was observed that bivalent ligands with higher Hill coefficient showed much more increased binding affinity than those with lower Hill coefficient. The enhanced Hill coefficient that arises from the transition from monovalent binding to bivalent binding suggested that the additional binding motif increased the probability that the bivalent ligand will find and bind more tightly to its corresponding receptor or due to increased local concentration of subsequent ligands at receptor sites.[18] We have concluded the improved likelihood that multivalent ligands simultaneously cross-link receptors through combination of a low affinity pharmacophore and a high affinity pharmacophore.

3. Experimental Procedures

3.1. Materials

N^{α}-Fmoc-amino acids were purchased from SynPep (Dublin, CA) or from Novabiochem (San Diego, CA). Rink amide Tentagel S resin was acquired from Rapp Polymere (Tubingen, Germany). HCTU, HOBt, and HOCt were purchased from IRIS Biotech (Marktredwitz, Germany). Solvents and reagents were reagent grade and were used without further purification unless otherwise noted. The solid-phase synthesis was performed manually in fritted syringes purchased from Torviq (Niles, MI). Purification of ligands was achieved on a Waters 600 HPLC using a reverse-phase column (Vydac C-18, 15-20 μm, 22 × 250 mm). The purity of the ligands was checked by analytical PR-HPLC using a Waters Alliance 2695 separation model with a Waters 2487 dual wavelength detector (220 and 280 nm) on a reverse-phase column (Jupiter 5U C18 300A; 2.2 × 2.5 cm). The mass of the ligands was confirmed by ESI method (Finnigan, Thermoelectron, LCQ classic).

3.2. Solid-Phase Synthesis

The Tentagel Rink amide resin (0.22 mmol/g) was washed with DMF 3 times and the N^{α}-Fmoc group was removed with 50% or 25% piperidine in DMF for 2 min or 20 min, respectively. The resin was washed successively with DMF, DCM, DMF, and then a solution of 1.0 M HOBt in DMF, and DMF. N^{α}-Fmoc amino acid was coupled using

preactivated 0.3 M HOBt esters in THF (3 eq. of N^{α}-Fmoc-amino acid, 3 eq. of HOBt and 3 eq. of DIEA) for 2 h and confirmed by Kaiser test (negative). If the coupling was not completed during 2 h (positive), the resin was washed with DMF and coupled again by the HCTU/ 2,4,6-lutidine procedure (3 eq. of N^{α}-Fmoc-amino acid, 3 eq. of HCTU, and 6 eq. of 2,4,6-lutidine in DMF) for 3 h or by preformed symmetric anhydride (3 eq. of N^{α}-Fmoc-amino acid and 1.5 eq. of DIC in DMF/DCM (1/1)) until the Kaiser test was negative. If the second coupling was not completed, the resin was washed with DMF, and the free amino group was capped with 50% Ac_2O in pyridine for 10 min.

After sequential couplings of Lys(*N*-Alloc), Trp(N^{i}-Boc), Arg(N^{g}-Pbf), 2′-*D*-Nal, His(N^{im}-Trt), and Asp(*O*-Allyl), the Alloc and Allyl groups were orthogonally deprotected from Lys and Asp side chains, respectively, using Pd(0) chemistry without cleaving the terminal Fmoc group on Asp(*O*-Allyl). 3 eq. of palladium tetrakis-triphenyl phosphine [$Pd(PPh_3)_4$] and $CHCl_3$/AcOH/*N*-methylmorpholine (37:2:1) (15 mL/g resin) was mixed under Ar.[19] The catalyst was dissolved by bubbling a stream of Ar through the solution and immediately pulled up by the syringe containing the dried tentagel resin. The reaction mixture was put on the shaker and the reaction carried out for 2 h. The palladium-based reaction mixture was then drained and the resin was washed thoroughly with 3 eq. DIEA in DMF, 3 eq. sodium di-thiocarbamate in DMF, and 3 eq. HOBt in DMF. The resin was then subjected to DMF/ DCM washes to prepare the resin for lactam cyclization under microwave condition. The lactam cyclization was carried out using 3 eq. DIEA/ 3 eq. HOBt/ 3 eq. HBTU in DMF (conventional microwave; 4 sec; 4 times) until the syringe got hot; the syringe was vortexed to dissipate the heat in-between individual microwave heating. The solution changed color from pale-yellow to light orange indicating the use of the reactants and the completion of reaction. After routine DMF/DCM washing, the Fmoc-group was deprotected for next coupling with N^{α}-Fmoc-Nle-OH.

Glycine and Proline were attached alternatively as many times as needed to synthesize the synthetic ligands **1-3** (Figure 1). Assembly of the second peptide, **SHU9119** or **MSH(7)**, in conjunction with the Pro-Gly linker, was carried out by the solid phase peptide synthesis procedure described above to afford ligands **7-9** or ligands **13-15**. Similarly, for the synthesis of ligands **5** and **6**, the flexible PEGO linker was attached by first adding diglycolic anhydride and then activating the free carboxylate as an imidazole for the attachment of 4,7,10-trioxa-1,13-tridecanediamine.[20] In synthetic ligands **4, 10,** and **16**, the PEGO was followed by repetitive Pro-Gly linker units in a sequential manner. Another PEGO attachment followed by the second **SHU9119** or **MSH(7)** and the *N*-terminal acetylation, yielded resin bound protected precursors of ligands **11-12** or ligands **17-18**. The resin was cleaved with a cleavage cocktail (10 mL/g resin) consisting of TFA, EDT, thioanisole, and water (91:3:3:3) for 3 h at room temperature. The solution was filtered off and the resin was washed with TFA (2 × 3 min). Combined solution was concentrated under N_2 and triturated by cold Et_2O. The precipitate was washed with cold Et_2O three times, dried, and dissolved in water for lyophilization. The lyophilized product was dissolved in water and purified by preparative RP-HPLC and characterized.

3.3. Homogeneity tests of the monovalent, bivalent synthetic peptide analogues

The purity of each peptide was evaluated by analytical RP-HPLC with a 30 min linear gradient of 10% to 40% of acetonitrile containing 0.1% TFA (Column: Jupiter 5U C18 300A; 2.2 × 2.5 cm; flow rate 1 mL/min). The same condition was employed to calculate the average concentration of the peptide ligands by the tryptophan assay. A standard solution (10 μL of 0.5 mM *D*-Trp in DMSO) was co-injected with 5 μL and 10 μL sample in two separate runs and the absorbance (area under peak) of the sample against *D*-Trp was measured at 280 nm. The peptide concentration was then calculated by the formula described in our earlier publication.[21]

3.4. Cell Culture

HEK293 cells overexpressing the hMC4R were used for binding assay. The hMC4R vector was originally received from Dr. Ira Gantz, University of Michigan.[22] The coding region of the hMC4R gene was expressed in pcDNA3.1 (Invitrogen, V790-20). HEK293/hMC4R cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS.

3.5. Lanthanide Based Binding Assays

Lanthanide based competitive binding assays were conducted according to the method which has been previously described.[23] In brief, HEK293/hMC4R cells were plated in black and white 96-well isoplates (Wallac, 1450-584) at a density 12,000 cells/well and were allowed to grow for 3 days. On the day of the experiment, media was aspirated from all wells. 50 μL of non-labeled ligand and 50 μL of Eu-labeled ligand (final concentration of 10 nM for Eu-NDP-α-MSH) were added to each well. Ligands were diluted in binding media (DMEM, 1 mM 1, 10-Phenanthroline, 200 mg/L Bacitracin, 0.5 mg/L Leupeptin, 0.3% BSA) and samples were tested in quadruplicate, unless otherwise noted. Cells were incubated in the presence of ligand for 1 h at 37 °C. Following the incubation, cells were washed 3 times with 250 μL wash buffer (50 mM Tris-HCl, 0.2% BSA, 30 mM NaCl). Enhancement solution (Perkin Elmer; 1244-105) was added (100 μL/well) and the plate was incubated for at least 30 min at 37 °C prior to reading. The plates were read on a Wallac VICTOR^3 instrument using the standard Eu TRF measurement (340 nm excitation, 400 μsec delay, and emission collection for 400 μsec at 615 nm).

3.6. Data Analysis

Data from independent binding experiments were analyzed with GraphPad Prism Software using the sigmoidal dose-response (variable slope) classical equation for non-linear regression analysis. The dose response curves obtained were used to compute the Hill coefficients for the individual synthetic ligands and their corresponding EC_{50} values using the above mentioned lanthanide-based (Eu-NDP-α-MSH) competition binding assay.

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Abbreviations

Ac	acetyl
ACTH	adrenocorticotropic hormone
Alloc	allyloxycarbonyl
Boc	t-butoxycarbonyl
BSA	bovine serum albumin

DIC	N,N′,-diisopropylcarbodiimide
DIEA	diisopropylethylamine
DMEM	Dulbecco's modified eagle medium
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
EDT	ethanedithiol
Fmoc	9-fluorenylcarboxy
HCTU	2-(6-chloro-1H-benzotrialzole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HEK	human embryonic kidney
HOBt	*N*-hydroxybenzotriazole
HOCt	
hMC4R	human melanocortin 4 receptor
MSH	melanocyte stimulating hormone
Nal	naphthylalanine
Pbf	2,2,4,6,7-pentamethlydihydro-benzofuran-5-sulfonyl
PEGO	poly(ethylene glycol)
RP-HPLC	reverse phase high performance liquid chromatography
TFA	trifluoroacetic acid
Trt	trityl

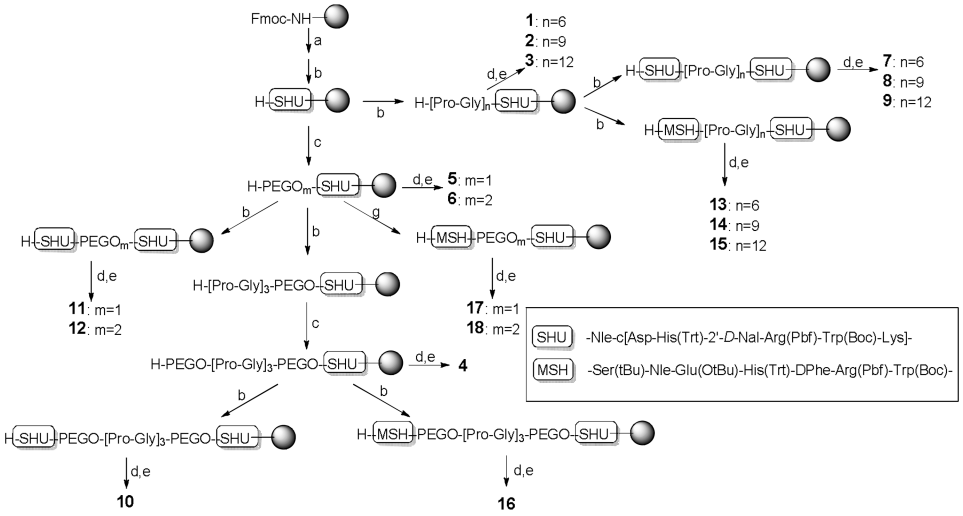


Figure 1.

Preparation of monovalent and bivalent ligands. Reagents and conditions: (a) 1:1 or 1:4 piperidine in DMF; (b) Standard solid phase synthesis using Fmoc-chemistry; (c) PEGO attachment (Ref. 3); (d) Ac_2O/pyridine (50/50); (f) TFA/EDT/thioanisole/water (91/3/3/3).

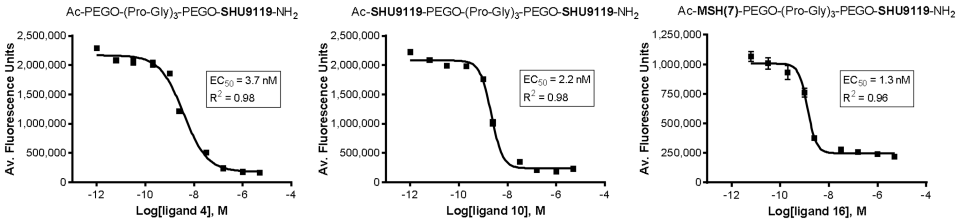


Figure 2.

Representative binding curves resulting from a typical competitive binding assay for monovalent ligand **4** (left), bivalent ligands **10** (middle) and **16** (right). Increasing concentration of ligands were competed using 10 nM Eu-α-NDP-MSH and hMC4R. Each data point represents the average of quadruplicate sample wells, with error bars indicating the standard error of means.

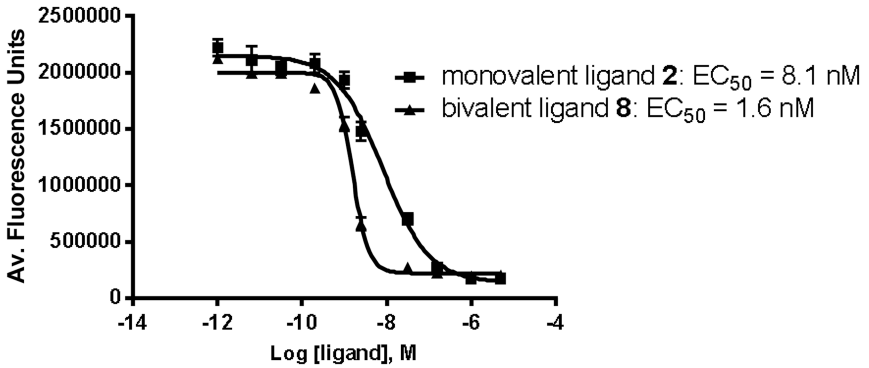
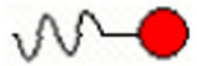
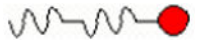
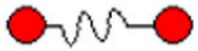


Figure 3. Comparison of the binding of monovalent ligand 2 and bivalent ligand 8 to hMC4R

Table 1

Analytical data of monovalent and bivalent ligands for hMC4R

	ligand[a]	molecular formula	no of atoms present in the linker	Estimated linker length (Å)	MS[b]		HPLC[c] (t_R, min)
					calcd	observed	
1		$C_{96}H_{131}N_{27}O_{21}$	36	10-20	1999.2	2000.1[a]	11.9
2		$C_{117}H_{161}N_{33}O_{27}$	54	15-30	2460.2	2461.2[a]	11.9
3		$C_{140}H_{196}N_{39}O_{33}$	72	20-40	2922.5	2924.1[a]	11.9
4		$C_{103}H_{153}N_{25}O_{27}$	58	13-46	2173.5	2174.4[a]	12.3
5		$C_{68}H_{97}N_{17}O_{15}$	20	4-18	1392.5	1393.6[b]	12.5
6		$C_{82}H_{123}N_{19}O_{21}$	40	8-36	1709.9	1710.6[b]	12.5
7		$C_{148}H_{197}N_{41}O_{29}$	36	10-20	3014.4	3016.4[a]	N.D
8		$C_{169}H_{227}N_{47}O_{35}$	54	15-30	3476.9	3478.5[a]	13.2
9		$C_{194}H_{267}N_{53}O_{41}$	72	20-40	3997.5	3940.9[a]	13.1
10		$C_{155}H_{219}N_{39}O_{35}$	58	13-46	3186.6	3188.1[a]	13.6
11		$C_{120}H_{163}N_{31}O_{23}$	20	4-18	2407.7	2407.3[a]	14.0
12		$C_{134}H_{189}N_{33}O_{29}$	40	8-36	2726.1	2727.0[a]	13.9
13		$C_{144}H_{197}N_{40}O_{31}$	36	10-20	2955.3	2955.0[a]	12.0
14		$C_{165}H_{227}N_{46}O_{37}$	54	15-30	3417.0	3417.0[a]	12.0

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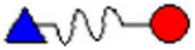
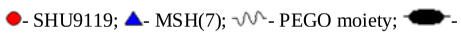
	ligand[a]	molecular formula	no of atoms present in the linker	Estimated linker length (Å)	MS[b]		HPLC[c] (t_R, min)
					calcd	observed	
15		$C_{186}H_{257}N_{52}O_{43}$	72	20-40	3880.2	3880.7[a]	12.0
16		$C_{151}H_{219}N_{38}O_{37}$	58	13-46	3129.4	3129.2[a]	12.2
17		$C_{116}H_{163}N_{30}O_{25}$	20	4-18	2348.6	2348.6[a]	12.4
18		$C_{130}H_{189}N_{32}O_{31}$	40	8-36	2665.4	2666.8[a]	12.5



[a] *N*-terminus: acetylated; *C*-terminus: amidated.

[b] $(M + H)^+$, ESI method (Finnigan, Thermoelectron, LCQ classic).

[c] Performed on a Waters Alliance 2695 HPLC using a reverse-phase column (Jupiter 5U C18 300A; 2.2 × 2.5 cm) in gradient system (10-40% of acetonitrile containing 0.1% TFA within 30 min, 1 mL/min).

Table 2
Binding affinities of monovalent and bivalent ligands for hMC4R

ligand	Structure	[a]LogEC$_{50}$	[b]EC$_{50}$ (nM)	Hill coefficient
1	Ac-(Pro-Gly)$_6$-**SHU9119**-NH$_2$	-8.40 ± 0.06	4.0	1.1
2	Ac-(Pro-Gly)$_9$-**SHU9119**-NH$_2$	-8.09 ± 0.05	8.1	0.8
3	Ac-(Pro-Gly)$_{12}$-**SHU9119**-NH$_2$	-7.88 ± 0.17	13	1.1
4	Ac-PEGO-(Pro-Gly)$_3$-PEGO-**SHU9119**-NH$_2$	-8.43 ± 0.06	3.7	0.9
5	Ac- PEGO-**SHU9119**-NH$_2$	-7.60 ± 0.51	24	0.6
6	Ac- PEGO-PEGO-**SHU9119**-NH$_2$	-6.39 ± 0.46	400	0.8
7	Ac-**SHU9119**-(Pro-Gly)$_6$-**SHU9119**-NH$_2$	N.D	N.D	-
8	Ac-**SHU9119**-(Pro-Gly)$_9$-**SHU9119**-NH$_2$	-8.81 ± 0.02	1.6	2.2
9	Ac-**SHU9119**-(Pro-Gly)$_{12}$-**SHU9119**-NH$_2$	-8.71 ± 0.02	2.0	3.0
10	Ac-**SHU9119**-PEGO-(Pro-Gly)$_3$-PEGO-**SHU9119**-NH$_2$	-8.67 ± 0.02	2.2	1.9
11	Ac-**SHU9119**-PEGO-**SHU9119**-NH$_2$	-8.74 ± 0.06	1.8	2.2
12	Ac-**SHU9119**-PEGO-PEGO-**SHU9119**-NH$_2$	-8.73 ± 0.02	1.9	1.9
13	Ac-**MSH(7)**-(Pro-Gly)$_6$-**SHU9119**-NH$_2$	-8.39 ± 0.12	4.1	0.7
14	Ac-**MSH(7)**-(Pro-Gly)$_9$-**SHU9119**-NH$_2$	-8.71 ± 0.08	2.0	0.8
15	Ac-**MSH(7)**-(Pro-Gly)$_{12}$-**SHU9119**-NH$_2$	-8.80 ± 0.04	1.6	2.0
16	Ac-**MSH(7)**-PEGO-(Pro-Gly)$_3$-PEGO-**SHU9119**-NH$_2$	-8.86 ± 0.03	1.3	2.4
17	Ac-**MSH(7)**-PEGO-**SHU9119**-NH$_2$	-8.73 ± 0.02	1.4	2.7
18	Ac-**MSH(7)**-PEGO-PEGO-**SHU9119**-NH$_2$	-8.77 ± 0.02	1.7	2.7
19	Ac-(Pro-Gly)$_6$- **MSH(7)**-NH$_2$		82 ± 12[c]	
20	Ac-(Pro-Gly)$_9$- **MSH(7)**-NH$_2$		236 ± 24[c]	
21	Ac-(Pro-Gly)$_{12}$- **MSH(7)**-NH$_2$		188 ± 25[c]	
22	Ac-**MSH(7)**-(Pro-Gly)$_6$- **MSH(7)**-NH$_2$		11 ± 2[c]	

[a] The logEC$_{50}$ ± SEM are logarithmic values determined from the nonlinear regression analysis of data collected from 4 independent concentration range.

[b] Determined from Dissociation Enhanced Lanthanide FluoroImmuno Assay (DELFIA) using optimized 10 nM standard agonist Eu-DTPA-NDP-α-MSH chelate.

[c] see reference 3