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Design, synthesis and biological studies of efficient multivalent melanotropin ligands: tools towards melanoma diagnosis and treatment

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Abstract

In order to achieve early detection and specific cancer treatment we propose the use of multivalent interactions in which a series of binding events leads to increased affinity and consequently to selectivity. Using melanotropin (MSH) ligands, our aim is to target melanoma cells, which overexpress melanocortin receptors. In this study, we report the design and efficient synthesis of new trivalent ligands bearing MSH ligands. Evaluation of these multimers on a cell model engineered to overexpress melanocortin 4 receptors (MC4R) showed up to a 350-fold increase in binding compared to the monomer, resulting in a trivalent construct with nanomolar affinity starting from a micromolar affinity ligand. Cyclic adenosine monophosphate (cAMP) production was also investigated leading to more insights into the effects of multivalent compounds on transduction mechanisms.

Keywords

Binding; multiple interactions; multimeric ligand design; targeted therapy; cancer; melanoma

Introduction

Targeted therapy consists of drugs that will target specific patterns (e. g. proteins) involved in tumor spread and growth. Targeting tumor characteristics has the advantage of leading to specific action on the cancer cells, leaving normal cells intact^{1, 2}. G protein coupled receptors (GPCRs) are intensively studied as targets for many diseases since they are involved in cell signaling pathways and have been shown to be up-regulated in many cancers³. Melanoma is the most threatening skin cancer: once the metastatic stage is reached the prognosis is poor since the tumor is resistant to most cures. Therefore, early diagnosis and specific treatment could increase the chances of survival. The melanocortin 1 receptor

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(MC1R) is known to be overexpressed^{4–6} in most forms of melanoma, which made an important target^{7–10} since it can be targeted using a variety of ligands including well-studied melanotropin ligands. The cooperative multivalency effect is involved in many biological pathways^{11, 12}, and consists of the multiple and simultaneous binding of ligands to multiple targets leading to an increased affinity. Increases in specificity can also be provided by multivalency by a complex interaction between ligand affinity and receptor number^{13, 14}. Our strategy consists in seeking cooperative effects using multimers to bind with a high affinity and selectivity to cancer cells targets. Previously, our group and others have described the use of different templates to construct multimers^{15–24} resulting in relative enhancement, up to about 50-fold. Herein, we describe the synthesis of new dendritic based multimers that can bear up to three melanotropin ligands and the study of their affinity and activity towards a cancer cell model readily available overexpressing MC4R.

Results

Design

In order to seek multivalent interactions, different criteria have to be considered such as the target, the nature of the multimer backbone, the linker length, the valency and the ligands to be used. Different kinds of scaffold have been used in the literature such as peptides^{23, 24}, polymers^{19, 20} and dendrimers^{21, 22}. Our aim is to use a tetravalent scaffold that will be able to bear three peptides to seek a cooperative effect and one tag that could be used for imaging or therapeutic purposes. In 2006, Chassaing *et al.*, described the synthesis of a small tetravalent template²⁵, which appeared readily modifiable and hence fit our criteria. However, the distance between ligands is important,^{15–24} and the described template was too small to “adjust” concomitantly three ligands in the binding sites of three GPCRs. Thus, we modified the backbone of our scaffold on the *N*-terminal with a Boc-Gly, so that every added peptide of a trivalent construct would be equidistant from the scaffold quaternary carbon. An additional β -Ala spacer was placed on the *C*-terminal of the scaffold as a small spacer to the potential tag moiety. Previous studies have described the optimal length to achieve crosslinking of GPCRs and a potential increase in affinity as between 25 to 50 Å^{23, 24, 26}. The cooperative effect being a thermodynamic process, it is known that a compromise between linker properties such as flexibility and length are necessary. We have previously described PEGO-(Pro-Gly)_{3–6}-PEGO as a more functional semi-flexible spacer, compared to fully flexible PEGO, moderately flexible β -Ala and the more rigid (Pro-Gly) as linkers^{21, 22}.

In the current work, we explore the multivalent cooperative effect of a low affinity melanotropin ligand pharmacophore, MSH(4): His-DPhe-Arg-Trp-CONH₂ (Figure 1). This peptide was chosen as a model since it binds with micromolar affinity to both receptors MC1R and the MC4R. It can also be modified on its *N*-terminal without affecting the binding affinity and activity. Furthermore, the relatively low affinity provided a larger potential dynamic range with which to measure affinity enhancements¹³. We designed two different ligands (Figure 1): one with on its *N*-terminal a lysine bearing a triple bond on the side chain (peptide **A**) and one directly acylated on the *N*-terminal by a triple bond containing moiety (peptide **B**). To be able to have a strategic scheme that can be used for any other applications, the lysine modification was appropriate when modification within the peptide sequence is possible but not at the *N*- or *C*-terminus.

Scaffold synthesis

The synthesis of scaffold (**7**) is reported in Scheme 1. Compound (**1**) was synthesized as described in the literature by Chassaing *et al.*²⁵. Coupling of (**1**) with Boc-Glycine was achieved using *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate

(HCTU) in dimethylformamide (DMF) in the presence of diisopropylamine (DIEA) to afford the coupling product, which was then hydrolyzed into (**3**) with a 2N solution of lithium hydroxide in tetrahydrofuran (THF). Compound (**3**) was coupled to β -Alanine methyl ester (β -Ala-OMe) using (Benzotriazol-1-yloxy)-tris-dimethylaminophosphonium hexafluorophosphate (BOP) and DIEA in DMF and the resulting product (**4**) was saponified to yield (**5**), following the procedure described above, but at 0°C. Reduction of cyano groups into amines was done in a Parr apparatus under 50 psi of hydrogen with PtO₂ in a mixture of methanol and chloroform to afford (**6**), that was further treated with di-tert-butyl dicarbonate (Boc₂O) in a mixture of water and acetonitrile in the presence of triethylamine to generate (**7**). This scaffold (**7**) was used for the synthesis of the various trivalent ligands (Figure 2). Another scaffold with orthogonal protections was synthesized starting from compound (**6**), in order to selectively produce either the monovalent ligand or the divalent one (Figure 2). This orthogonally protected scaffold (**8**) was obtained after Fmoc protection of (**6**) using Fmoc-OSu in a mixture of water and acetonitrile in the presence of NaHCO₃ (Scheme 1).

Azide moiety and protected peptide synthesis

The azido acetic acid was prepared with good yields by substitution of sodium azide on the corresponding bromoacetic acid in water. Adequately protected MSH(4) analogs were synthesized on solid support using a Sieber amide resin following the standard Fmoc strategy synthesis procedure. These peptides were functionalized on the *N*-terminal with a triple bond either by acylation with pentynoic acid, or by coupling a lysine modified on its side chain by pentynoic acid. Cleavage of the resins, a very critical to keep the protections on the side chains was successfully processed using 1% Trifluoroacetic acid (TFA) in dichloromethane (DCM) to give the protected peptides (**A**) and (**B**) (Figure 1), with 85% yields. These crude protected peptides (**A**) and (**B**) were used without purification for the coupling to the different scaffolds.

Assembly of multivalent constructs

The synthesis of the final trivalent constructs, described in Scheme 2, was achieved on MBHA resin using regular Boc strategy. The scaffold (**7**) plus the amino acids composing the linkers were successively attached on the resin, until the desired sequence was reached. The azidoacetic acid was finally coupled with equal amounts of coupling agent and DIEA. The resin was dried and the protected peptides (**A**) or (**B**) were then attached to the template *via* reaction of click chemistry²⁷, which was achieved in the presence of copper iodide, ascorbic acid and DIEA in a mixture of DMF and 2,6-lutidine either at room temperature or using microwave irradiation to accelerate the reaction rate. The monovalent and bivalent constructs NB 397 and NB 399, respectively, were synthesized, using the same strategy as above, starting from the scaffold (**8**) and by removing first the proper orthogonal protecting group, depending on the desired compound, *i.e.* monomeric or dimeric ligands (Figure 2). Analytical data is reported in Table 1.

Binding Assays

Ac-MSH(4) (Figure 1) has poor binding affinity for the MC4R (IC₅₀ 4900 nM, Table 3). The linker length effect on potency compared to Ac-MSH(4) was studied using two kinds of linker within the range of 25 to 60 Å (Table 2). A general trend can be observed: the shorter the linker, the better the affinity. Also, we can observe a difference depending on the type of linker used. When the semi-flexible Gly-Pro linker is used, the affinity was only increased from 8- to 20-fold compared to the monomer (IC₅₀ 600 to 200 nM). However, the use of the more flexible β -Ala linker showed an affinity enhancement from 40- to 70-fold depending on the length used. It is notable that the trivalent compounds, NB297 and NB342, are made of ligands (**A**) and (**B**), respectively, but the length from ligand to ligand is the same. These

two trivalent ligands show the same IC_{50} of about 72 nM, which is consistent with the importance of the length and the *N*-terminal modifiability of MSH(4). Although analogues containing different linkers were synthesized, the construct that showed the best affinity was the shortest one, NB341, ($24 \pm 5 \text{ \AA}$), which does not have any linker besides the scaffold bonds.

To get more insights on the valence effect, we synthesized and investigated monovalent and bivalent ligands of MSH(4), NB397 and NB399, respectively (Figure 2) and compared their affinities to the corresponding trivalent ligand (Table 3 and Figure 3). As predicted, the affinity of the monovalent ligand NB397, *i.e.* the ligand being directly attached to the scaffold was found similar to the affinity of acetylated MSH, Ac-MSH(4) (IC_{50} about 4700 nM \pm 200 nM). Surprisingly, the affinity of the bivalent ligand, NB399, (IC_{50} 310 nM) showed an order of magnitude improvement in binding compared to the monovalent ligand and with the trivalent ligand, NB341, another additional order of magnitude increase in the affinity was observed, leading to an IC_{50} of 14 nM. The fact that an order of magnitude is reached *per* additional ligand provides evidence that three receptors are involved in binding, which suggests a cooperative mechanism of binding rather than a statistic effect²⁶.

cAMP Assays

Melanotropin ligands are known to stimulate cAMP when bound to melanocortin receptors such as the human MC4R. cAMP measurements were made using the mono-, bi- and trivalent constructs (NB397, NB399 and NB341, respectively) to investigate how these ligands combinations affect cell signaling. cAMP production was activated by each of the constructs demonstrating that they all are agonists for the MC4R receptor. The sensitivity for activation of cAMP production was found to increase with the valency (Figure 4). In order to determine if the efficacy of signaling was similar between the ligands, the relationship between the number of ligands bound and the amount of cAMP released was evaluated. Receptor occupancy was calculated at different percentages of the maximal cAMP response, which was measured as the amount of cAMP produced (above control) compared to that observed in response of 10 μ M of forskolin. As shown in Table 4, the number of receptors occupied by each construct was within the same range, suggesting that the bivalent and trivalent constructs activate the transduction mechanisms as a monovalent construct. It is also important to remember that the bivalent NB399 and trivalent NB341 molecules showed orders of magnitude higher affinity, which supports the fact that two and three receptors are bound. However, since the occupancy was similar at the same concentration of cAMP for every construct, it provides evidence that the bivalent and trivalent ligands activate the transduction mechanisms with effectiveness similar to a monovalent construct.

Discussion

We have been engaged in a program to design ligands that might selectively target human cancers for diagnosis and therapeutic purposes. Multivalent interactions are part of numerous biological systems and high-affinity highly selective complexes may be formed starting from low-affinity ligands. In this context, our aim was to: i) design new tools for diagnosis and treatment of melanoma cells using novel multivalent melanotropin trivalent molecules; and ii) investigate their binding and activity properties. In the multivalent strategy, particular care should be taken in the choice/design of the scaffold and the linker, which should have the “right size” in order to avoid a too large cost in entropy^{11, 12}. Two types of linkers have been investigated within the proposed ideal range for spanning ligands for simultaneous binding ($25\text{--}50 \text{ \AA}$)^{23, 26}. “Bis-ornithine” represented a powerful scaffold to be explored for grafting ligands, since the χ_1 and χ_2 rotamers of this α, α' -amino acid side chains are locked, with a minimal to maximal distance between the two NH_2 around 5

to 9 Å. The third amino function involved in the grafting of the MSH(4) ligand is on a more flexible position, as the three rotamers (Φ , ψ , θ) of β -Ala might theoretically be accessible. Altogether this construct appeared as the best compromise for offering four anchoring points with a minimal mobility, a critical point in this strategy. The synthetic scheme was developed so diverse multivalent compounds can be easily prepared with high yields. Competitive binding experiments were performed by evaluating displacement of a potent Eu-NDP- α -MSH ligand (Figure 1). Although β -Ala linkers showed a 40- to 70-fold increase in affinity the best results were reached for multimers without any linker. The monovalent compound attached to the β -Ala- “bis-ornithine” scaffold led to a similar micromolar affinity as Ac-MSH(4), but increasing the ligand valency increased the affinity from the monovalent to the divalent and the trivalent constructs, as expected for successful concomitant binding to multiple receptors. Selectivity of this trivalent construct, which displayed a 350 times-fold increase in affinity when compared to the monovalent ligand, will be ensured when a high density of MSH receptors is reached as shown for many melanoma cell lines.

Bivalent and trivalent ligands showed orders of magnitude increases in affinity, which supports the premise that when bound, these ligands occupy two and three receptors, respectively. Activity experiments established that starting from AcMSH(4), which is a weak agonist of MSH receptors, cAMP production increased with affinity of the multivalent ligand, the trivalent construct exhibiting an EC₅₀ value in the nanomolar range close to its IC₅₀ value. However, the occupancy is similar at the same concentration of cAMP for every construct, even though two or three receptors are involved in binding. This suggests that the bivalent and trivalent ligands activate the transduction mechanisms much as a monovalent ligand. G protein coupled receptors are known to dimerize, either in the presence of the ligand and/or at the basal level^{28, 29}. Dimers of receptors first identified on cells overexpressing receptors, probably as a consequence of the high density of receptors, have been shown to exist in tissues. However, very recently^{28, 29} it has been demonstrated that the dimers of GPCRs were in fact asymmetric and that only one G protein in the complex presented the expected orientation in the complex to be activated. Thus, we can speculate that even though MSH divalent and trivalent constructs must recruit two to three receptors, respectively as ascertained by the gain in affinity, in the final ligand-receptor complex formed with G protein only one G protein can be activated.

Conclusion

New multimeric ligands were designed for multivalent interactions with MSH receptors. The optimal construct showed a 350-fold increase in the affinity vs. the corresponding micromolar affinity of the monovalent construct and Ac-MSH(4). Activity studies confirmed that starting from a weak agonist the final trivalent construct yielded an agonist with nanomolar potency. The pharmacokinetic properties of this highly potent melanotropin trivalent ligand will be explored to ascertain the sensitivity and selectivity to detect and target melanoma cells overexpressing MC1R. From a more fundamental point of view, these multimeric ligands should constitute powerful tools to explore the transduction mechanism of G protein coupled receptors.

Experimental Section

Chemicals and Materials

Reagents and solvents used were reagent grade quality from commercial sources and used without further purification unless noted otherwise. Purifications by flash chromatography were performed on EMD chemicals silica gel 60 (0.04–0.063mm). *p*-Methylbenzhydramine-resin (MBHA resin), (0.7 mmol/g) and Sieber amide resin

(0.5mmol/g) were purchased from ChemPep, and amino acids were purchased from Aapptec and Iris Biotech GmbH. Other starting materials and chemicals were mostly purchased from Sigma Aldrich and from Acros for platinum dioxide. NMR spectra were recorded on Bruker ARX 250 or 500 MHz and are referenced in ppm (δ), and coupling constants (J) are expressed in Hz. Melting points were measured using a Kofler apparatus and are uncorrected. Mass spectra were obtained with an ESI-mass spectrometer (Finnigan, thermoelectron, lcq classic), and high-resolution fast atom bombardment spectrometer (JEOL HX110 sector instrument) or by MALDI-TOF (Voyager DE-Pro). HPLC was performed on a Hewlett-Packard 1100 series liquid chromatograph (Agilent technologies) with a C8 column (SymmetryPrep, 0.78 \times 30cm) or C18 column (Vydac, 1.0 \times 25cm), separations were monitored at 230 and 280 nm and final purity was determined as 95% using a C18 Vydac column (0.46 \times 25 cm).

Methyl 2-(2-(*tert*-butoxycarbonylamino-acetamido)-4-cyano-2-(2-cyanoethyl-butanoate) (2)

To compound (1) (5.85 g, 30 mmol, 1eq.) dissolved in 50 mL of dry DMF, were added Boc-Gly (37 mmol, 1.2 eq.), HCTU (30 mmol, 1eq.) and DIEA (33 mmol, 1.1 eq.). After overnight stirring at room temperature under argon atmosphere, the mixture was poured into a saturated solution of ammonium hydrochloride. After extraction with EtOAc, the organic phase was washed with 5% citric acid, sat. NaCl and 10% NaHCO₃, dried over MgSO₄ and concentrated *in vacuo* leading to (2), which was purified by silica gel chromatography EtOAc:Cy (6:4), crystallization from EtOAc afforded (2) as a white solid, (9.8 g, 28 mmoles, 92% yield); R_f 0.52 EtOAc:Cy (8:2); mp 102–103°C; ¹H NMR (CDCl₃) δ (ppm): 3.91 (s, 3H), 3.75 (m, 2H), 2.95 (m, 2H), 2.32 (m, 4H), 2.17 (m, 2H), 1.48 (s, 9H); ¹³C NMR (CDCl₃) δ (ppm): 12.1 (CH₂), 28.2 (CH₂), 30.2 (CH₃), 45.2 (CH₂), 53.9 (CH₃), 62.5 (C), 80.8 (C), 118.4 (CN), 156.2 (CO), 165.6 (CO), 171.7 (CO); MS (HRMS) m/z calc. for C₁₆H₂₄N₄O₅Na 375.1644, found 375.1638.

2-(2-(*tert*-Butoxycarbonylamino-acetamido)-4-cyano-2-(2-cyanoethyl) butanoic acid (3)

A solution of (2) (8.8 g, 25 mmol, 1 eq.) in THF (100 mL) was treated by slow addition of 25 mL of LiOH 2N (51 mmol, 2 eq.). The solution was stirred for 1 hr and a solution of NaHSO₄ (1N) was added until pH 3. The product was extracted using EtOAc and the organic phase was dried over MgSO₄ and concentrated *in vacuo* to afford (3) as a white solid (8g, 24 mmoles, 95 % yield). R_f 0.38 DCM:MeOH:CH₃COOH (8:2:0.1); mp 74–75°C; ¹H NMR (CDCl₃) δ (ppm): 3.88 (m, 2H), 2.96 (m, 2H), 2.37 (t, J 8Hz, 4H), 2.23 (m, 2H), 1.46 (s, 9H); ¹³C NMR (CDCl₃) δ (ppm): 12.2 (CH₂), 28.4 (CH₂), 30.1 (CH₃), 45.3 (CH₂), 53.9 (CH₃), 64.5 (C), 80.8 (C), 118.4 (CN), 156.2 (CO), 158.2 (CO), 171.2 (CO); MS (HRMS) m/z calc. for C₁₅H₂₂N₄O₅Na 361.1488, found 361.1482.

Methyl 9,9-bis(2-cyanoethyl)-2,2-dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatetradecan-14-oate (4)

To a solution of (3) (4g, 12 mmol, 1eq.), β -Ala-OMe (14 mmol, 1.2 eq.) and BOP (12 mmol, 1eq.) in 24 mL of dry DMF was added DIEA (24 mmol, 2 eq.). After overnight stirring at room temperature under argon atmosphere, the mixture was poured into a saturated solution of ammonium hydrochloride. After extraction with EtOAc, the organic phase was washed with 5% citric acid, sat. NaCl and 10% NaHCO₃. The organic phase was dried over MgSO₄ and concentrated *under vacuum* to yield compound (3) as an oil, which was purified by silica gel chromatography, EtOAc:Cy (6:4), leading to an oil, which was crystallized in EtOAc to afford (4) as white crystals (4.3g, 10 mmoles, 85% yield); R_f 0.56 EtOAc:Cy (8:2); mp 138–139°C; ¹H NMR (CDCl₃) δ (ppm): 3.75 (m, 2H), 3.71 (s, 3H), 3.57 (q, 2H), 3.01 (m, 2H), 2.61 (t, 2H), 2.27 (m, 4H), 1.92 (m, 2H), 1.46 (s, 9H); ¹³C NMR (CDCl₃) δ (ppm): 12.1 (CH₂), 28.3 (CH₂), 30.9 (CH₃), 32.85 (CH₂), 36.05 (CH₂), 52.2 (CH₂), 52.2

(CH₃), 61.9 (C), 80.7 (C), 118.9 (CN), 160.63 (CO), 169.63 (CO), 169.76 (CO), 173.34 (CO); MS (HRMS) m/z calc. for C₁₉H₂₉N₅O₆Na 446.2010, found 446.2017.

9,9-bis(2-Cyanoethyl)-2,2-dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatetradecan-14-oic acid (5)

To a solution of (4) (6.38g, 16 mmol, 1eq.) in THF:MeOH (5:1) cooled at 4° C, LiOH (2N) (20 mL, 40 mmol, 2.5 eq.) was added dropwise. After stirring 25 min at 4°C, the reaction was quenched by addition of water and decreasing the pH to 3 with a 1N NaHSO₄ solution. After extraction with EtOAc (5x), the organic phases were assembled, dried over MgSO₄ and concentrated *in vacuo* to yield the pure compound (5) as a white solid (5.9 g, 14 mmoles, 90% yield). *R*_f0.45 DCM:MeOH:CH₃COOH (8:2:0.1); mp 124–125°C; ¹H NMR (MeOD) δ (ppm): 3.69 (s, 2H), 3.52 (t, J 3.5 Hz, 2H), 2.77 (m, 2H), 2.61 (t, J 2.6 Hz, 2H), 2.39 (m, 4H), 2.17 (m, 2H), 1.49 (s, 9H); ¹³C NMR (CDCl₃) δ (ppm): 12.1 (CH₂), 28.3 (CH₂), 30.4 (CH₃), 33.6 (CH₂), 36.6 (CH₂), 54.5 (CH₂), 57.4 (CH₃), 62.22 (C), 80.9 (C), 119.7 (CN), 156.68 (CO), 170.3 (CO), 173.4 (CO), 176.57 (CO); MS (HRMS) m/z calc. for C₁₈H₂₈N₅O₆ 410.2034, found 410.2040.

4-(2-(tert-Butoxycarbonylamino-acetamido)-4-((2-carboxyethylcarbamoyl) heptane-1,7-diaminium chloride (6)

Compound (5) (2g, 4.8 mmol, 1 eq.) in 15 mL of MeOH:CHCl₃ (5:0.15) was reduced in the presence of PtO₂ (0.228 g, 0.77mmol, 0.16 eq.) for 2 hours in a Parr apparatus under 4 bars of H₂. The suspension was filtered through celite and evaporated *under vacuum* to give (6) as a white powder (1.6 g, 3.9 mmoles, 80 % yield), which was used in the next step without further purification since the NMR showed no detectable impurities. mp 116–117°C; ¹H NMR (D₂O) δ (ppm): 3.68 (s, 2H), 3.39 (m, 2H), 2.88 (m, 4H), 2.42 (m, 2H), 2.06 (m, 2H), 1.81 (m, 2H), 1.44 (m, 4H), 1.36 (s, 9H); ¹³C NMR (D₂O) δ (ppm): 21.5 (CH₂), 28.1 (CH₃), 31.3 (CH₂), 36.4 (CH₂), 39.2 (CH₂), 44.1 (CH₂), 52.6 (CH₂), 62.3 (C), 81.3 (C), 158.5 (CO), 171.2 (CO), 173.7 (CO), 175.8 (CO); MS (HRMS) m/z calc. for C₁₈H₃₆N₅O₆ 418.2660, found 418.2664.

9,9-bis(3-(tert-Butoxycarbonylamino-propyl)-2,2-dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatetradecan-14-oic acid (7)

To a solution of (6) (10.18 g, 16.5 mmol, 1eq.) in 80 mL H₂O:MeCN, (1:1) Boc₂O (50 mmol, 3 eq.) and triethylamine (6 eq.) were added. The solution was stirred at room temperature for 24 hours. The pH was then decreased to 3 with 1N NaHSO₄. The solution was extracted with CH₂Cl₂. The organic phase was dried over MgSO₄ and then concentrated *in vacuo* to afford an oil, which was purified by silica gel chromatography in EtOAc:Cy:MeOH:acetic acid (50:50:2.5:1) to afford (7) as a white solid (8.2g, 13.2 mmol, 80% yield). *R*_f0.27 EtOAc:Cy:MeOH:acetic acid (50:46:3:1); mp 140–141°C; ¹H NMR (CDCl₃) δ (ppm): 3.72 (m, 2H), 3.51 (m, 2H), 2.99 (m, 4H), 2.57 (m, 2H), 2.39 (m, 2H), 1.65 (m, 2H), 1.42 (s, 9H), 1.39 (s, 18H), 1.38 (m, 2H); ¹³C NMR (CDCl₃) δ (ppm): 21.6 (CH₂), 24.6 (CH₂), 28.4 (CH₃), 32.5 (CH₂), 34.1 (CH₂), 40.4 (CH₂), 44.9 (CH₂), 63.7 (C), 79.6 (C), 156.7 (CO), 169.1 (CO), 172.7 (CO), 173.4 (CO); MS (HRMS) m/z calc. for C₂₈H₅₁N₅O₁₀Na 640.3534, found 640.3528.

9,9-bis(3-(9-Fluorenylmethoxy-carbonyl-aminopropyl)-2,2-dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatetradecan-14-oic acid (8)

To a solution, at 0°C, of (6) (1.7 g, 4 mmol, 1eq.) in 20 mL H₂O:MeCN (1:1) is added NaHCO₃ (1.36g, 16 mmol, 4eq.) and dropwise Fmoc-OSu (3 g, 9 mmol, 2.2eq.) dissolved in 6mL of MeCN. The temperature was then raised at room temperature and the solution was stirred at room temperature for 24 hours. The pH was then decreased to 3 with 1N NaHSO₄

and the solution was extracted with DCM. The organic phase was dried over MgSO_4 and then concentrated *in vacuo* to afford an oil, which was purified by silica gel chromatography in EtOAc:Cy:MeOH:acetic acid (50:50:2.5:1) to afford (**8**) as a white solid (2.2 g, 2.6 mmol, 65% yield). R_f 0.35 EtOAc:Cy:MeOH:acetic acid (50:46:3:1); mp 159–160°C. ^1H NMR (CDCl_3) δ (ppm): 7.75 (m, 4H), 7.58 (m, 4H), 7.39 (m, 4H), 7.31 (m, 4H), 4.35 (d, J 8 Hz, 4H), 4.19 (m, 2H), 3.75 (m, 2H), 3.53 (m, 2H), 3.13 (m, 4H), 2.59 (m, 2H), 2.47 (m, 2H), 1.64 (m, 2H), 1.44 (s, 9H), 1.40 (m, 2H), 1.27 (m, 2H); ^{13}C NMR (CDCl_3) δ (ppm): 24.4 (CH_2), 28.6 (CH_3), 32.8 (CH_2), 33.7 (CH_2), 36.21 (CH_2), 40.86 (CH_2), 44.85 (CH), 63.59 (CH_2), 80.6 (C), 120.1 (CH-Ar), 125.2 (CH-Ar), 127.34 (CH-Ar), 129.15 (CH-Ar), 141.4 (C-Ar), 143.8 (C-Ar), 157.2 (CO), 172.5 (CO), 172.86 (CO), 174.76 (CO); MS (HRMS) m/z calc. for $\text{C}_{48}\text{H}_{56}\text{N}_5\text{O}_{10}$ 862.4022, found 862.4036.

Azidoacetic acid

To a solution of sodium azide (8.2 g, 126 mmol, 2 eq.) in 42 mL of water bromoacetic acid (10 g, 63 mmol, 1 eq.) was slowly added. The solution was stirred at room temperature overnight. The reaction was then quenched by dropwise addition of 35 mL conc. HCl (37%). After extraction with Et_2O (4×50 mL), the organic phase was dried over MgSO_4 and concentrated *in vacuo* to afford azidoacetic acid, as an oil (59 mmol, 95% yield). ^1H NMR (CDCl_3) δ (ppm): 10.16 (s, 1H), 3.97 (s, 2H). IR: 2108.45cm^{-1} . MS (GC) m/z calc. for $\text{C}_2\text{H}_3\text{N}_3\text{O}_2$ 101.02, found 101.02.

Synthesis of the protected peptides (A), (B)

The protected peptides (**A**) and (**B**) were synthesized starting from 1 g of Sieber amide resin (0.5 mmol/g) using Fmoc chemistry in Torviq fritted syringes. The resin was swollen in DMF:DCM (1:1) for 1 hour. The resin was first deprotected using 25 % piperidine in DMF (1×5 min and 1×20 min). The amino acids were coupled to the resin with an elongation from the C-terminal to the N-terminal with Fmoc-Trp(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-D-Phe-OH, Fmoc-His(Trt) and either the modified lysine bearing a triple bond or pentynoic acid. Coupling was achieved for one hour using 3 equivalents (relative to resin loading) of HBTU and HOBT and 6 equivalents of DIEA. Coupling completion was confirmed using Kaiser test (negative test: yellow beads). Fmoc deprotections were performed as described above for the first deprotection step. After each step (coupling and deprotection) the resin was washed using DMF (3 × 2 min) and DCM (3 × 2 min) washings. Once the sequence was achieved the peptidyl-resin was thoroughly washed with DMF, DCM and finally MeOH before drying *under vacuum*. The resin was swollen in dry DCM and cleavage was performed by incubating (15 × 2 min) 1g of resin with a solution of distilled DCM (10 mL) containing 1% TFA and then pouring into 10% pyridine in methanol (30mL). The solution was then evaporated under reduced pressure and the resulting oil was dissolved in DCM (10 mL), which was washed with water to remove the trifluoroacetate pyridinium salt. The DCM solution was then transferred into a centrifuge tube and the protected peptide was precipitated with cold diethyl ether. The precipitate was isolated by centrifugation. The peptide was then triturated with ether and the pellet dried in the air to afford 85% yield of crude peptide, which was used for the next step without purification.

Assembly of the multivalent constructs

Multivalent constructs were synthesized on MBHA resin (20 mg, 0.7 mmol/g) using Boc chemistry in Torviq fritted syringes. The resin was swollen in DCM and then treated with 100% TFA (3 × 1 min), then rinsed with DCM following another rinse with 20 % DIEA in DCM. The amino acids were coupled to the resin with an elongation from C-ter to N-ter.

Trivalent constructs: NB297, NB298, NB299, NB300, NB301, NB302, NB341, NB342.**General procedure**

The scaffold (**7**) was first coupled to the resin followed by the linkers either Boc- β Ala-OH, Boc-Pro-OH or Boc-Gly-OH. Couplings were done using 3 equivalents of HCTU and 6 equivalents of DIEA. Boc removal was achieved using 100% TFA (3 \times 1min) following by DCM wash and 20% DIEA in DCM wash. Completion was monitored using Kaiser test. Azidoacetic acid was finally coupled using HBTU (3 eq) and DIEA (3 eq). The reaction was monitored using Kaiser test and a modified version of Kaiser test containing 5% PPh₃ to unveil the presence of the azido group, (regular Kaiser test gives yellow beads and the Kaiser test containing the phenyl phosphine gives blue beads³⁰). The resin was finally washed with DCM and MeOH and dried *under vacuum* for click chemistry. The resin was used unswollen, the “click reaction” was performed using *per* arm of template a 2-fold excess of protected peptides in the presence of CuI (5-fold excess), ascorbic acid (5-fold excess) and DIEA (7-fold excess) in a mixture of DMF:2,6-lutidine (7:3), either at room temperature for 4 days or with microwave activation at 250W, T max = 50°C for 60 \times 5 sec with liquid nitrogen cooling between each cycle. The resin was then rinsed using DMF (5 \times 2 min), 0.5% of diethylthiocarbamate in DMF (2 \times 10 min) and finally DMF, DCM and MeOH. The resin was dried *under vacuum* then transferred into a Teflon reactor, for 20 mg of resin, 30 μ L of anisole was added and HF cleavage was processed for 2 hours at 0°C. The resin was first filtered then washed with ether. The multivalent constructs were washed off from the resin using 10% acetic acid in H₂O, transferred into a centrifuge tube for lyophilization to afford the crude multivalent ligands, which were purified by HPLC. Final purity was determined as 95% by analytical HPLC (0–100% acetonitrile in 30 min) with a C18 column Vydac (0.46 \times 25 cm).

Bivalent construct: NB399

The scaffold (**8**) was coupled to the resin, followed by Boc group removal and acetylation of the resulting free amine. Fmoc groups were then removed and the azido acetic acid was coupled onto the two remaining amines to afford a bivalent construct with two azides. The azido group was coupled to protected peptide (**B**) *via* a click reaction. After HF cleavage the bivalent construct NB399 was obtained and purified by HPLC with a final purity 95% by analytical HPLC (0–100% acetonitrile in 30 min). All reactions were performed with the same protocols as described for the synthesis of the trivalent constructs.

Monovalent construct: NB397

The scaffold (**8**) was coupled to the resin, followed by Boc group removal and coupling of the azido acetic acid on the free amine. The azido group was then coupled to protected peptide (**B**) *via* a click reaction. The Fmoc groups were finally removed and the resulting free amines were acetylated. After HF cleavage the monovalent construct NB397 was purified by HPLC with a final purity 95% by analytical HPLC (0–100% acetonitrile in 30 min). All reactions were performed with the same protocols as described for the synthesis of the trivalent constructs.

Cell lines

HEK293 cells overexpressing the human MC4R were used to assess the affinity and the activity at the hMC4R. The coding region of the human MC4R gene was expressed in pcDNA3.1 (Invitrogen, V790-20). Hek293/hMC4R cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% SCS and 1% Penicillin-Streptomycin and were kept under standard conditions (37°C and 5% CO₂).

Time resolved fluorescence binding assay

The HEK293 cells were seeded at 20000 cells *per* well into 96 well Costar 3603 plates 3 days before the experiment. Competition assays were performed in quadruplicate unless noted otherwise using a fixed concentration of Eu-NDP- α -MSH (10 nM, 50 μ L *per* well) and 8 to 10 different concentrations of ligand. The day of the experiment, media was aspirated. Ligands were diluted in binding medium (DMEM, 1mM 1,10-phenantroline, 200 mg/L bacitracin, 0.5 mg/L leupeptin, and 0.2% bovine albumin serum (BSA)) and added to the cells that were incubated for 2 h at 37°C. Following the incubation, cells were washed three times with wash buffer (DMEM, 20 μ M EDTA, 0.2% BSA, 0.01% Tween 20), enhancement solution (Delfia, Perkin Elmer) was added to the plates (100 μ L *per* well) and incubated for 30 min at 37°C. The plates were read on a Wallac Victor instrument using the standard Eu(III) TRL measurement (340 nm excitation, 400s delay and emission collection for 400s at 615 nm). Competitive binding data were analyzed with GraphPad Prism software using nonlinear regression analysis and fitted to a classic one site binding competition equation.

cAMP biological response immunoassay

The HEK293 cells were seeded at 20000 cells *per* well into 96 well Costar 3598 plates 3 days before the experiment and incubated at 37°C in 5% CO₂. Assays were performed in triplicate in 3 different experiments. Media was aspirated and cells were pre-incubated with 1mM 3-isobutyl-1-methylxanthine (IBMX) in DMEM at 37°C for 10 min. 30 μ L of compounds and standards were added to the cells and 8 concentrations of ligand were tested (10⁻⁷ to 10⁻¹²) and plates were incubated for 15 min at 37°C. cAMP production was measured using a chemiluminescent immunoassay kit as described in detail in the user manual (Invitrogen C10558). Briefly, cells were incubated in 60 μ L of lysis buffer (provided with the cAMP assay kit) for 25 min at 37°C then transferred to precoated 96 well plates. To these plates were added 30 μ L of cAMP-AP (alkaline phosphatase conjugate) and 60 μ L of anti-cAMP antibody. The plates were incubated at room temperature for 1 hr and washed 5 times with wash buffer. Enhancement solution (CSPD substrate / Sapphire II enhancer) was added to the plates following by incubation at room temperature in the dark for 30 min. Intensity of the signal was measured using Wallac Victor instrument and the concentrations of cAMP were calculated after the average value of the basal condition was subtracted and based on a standard curve that was acquired during the same assay.

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Abbreviations

cAMP	cyclic adenosine monophosphate
β-Ala	β -alanine, NH ₂ -CH ₂ -CH ₂ -COOH
Boc₂O	di- <i>ter</i> -butyl-carbonate BOP, (Benzotriazol-1-yloxy)-tris-dimethylaminophosphonium hexafluorophosphate
BSA	bovine serum albumin
Cy	cyclohexane
DCM	dichloromethane

DIEA	diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
Fmoc-OSu	<i>N</i> -(9-Fluorenylmethoxycarbonyloxy) succinimide
GPCRs	G protein coupled receptors
HBTU	<i>O</i> -(Benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HCTU	<i>O</i> -(6-Chlorobenzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
MC1R	melanocortin 1 receptor
MC4R	melanocortin 4 receptor
MSH	melanotropin, melanocyte stimulating hormone
MSH(4)	His-DPhe-Arg-Trp-CONH ₂
TFA	trifluoroacetic acid
THF	tetrahydrofuran

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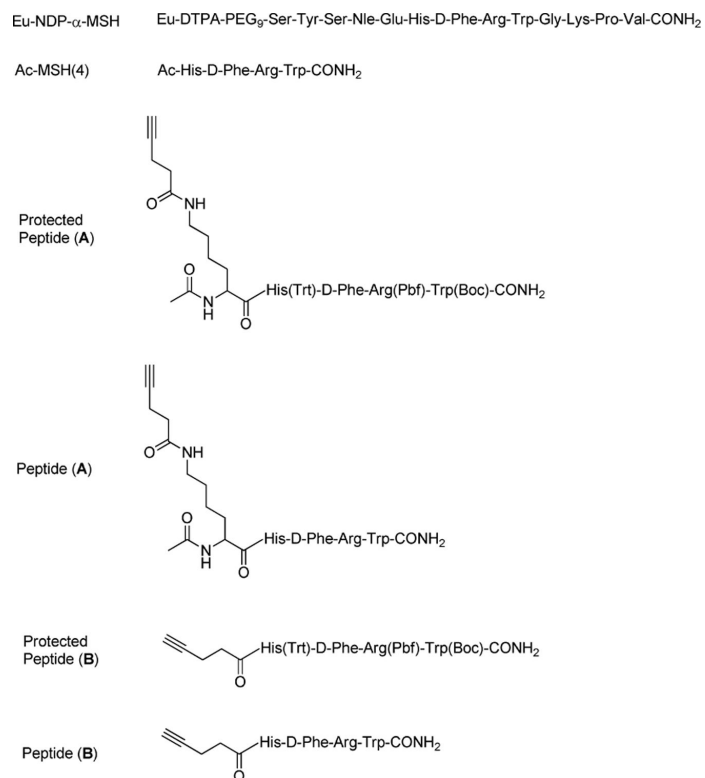


Figure 1. Sequences of Eu-NDP- α -MSH, Ac-MSH(4), protected peptides (A) and (B) and peptides (A) and (B).

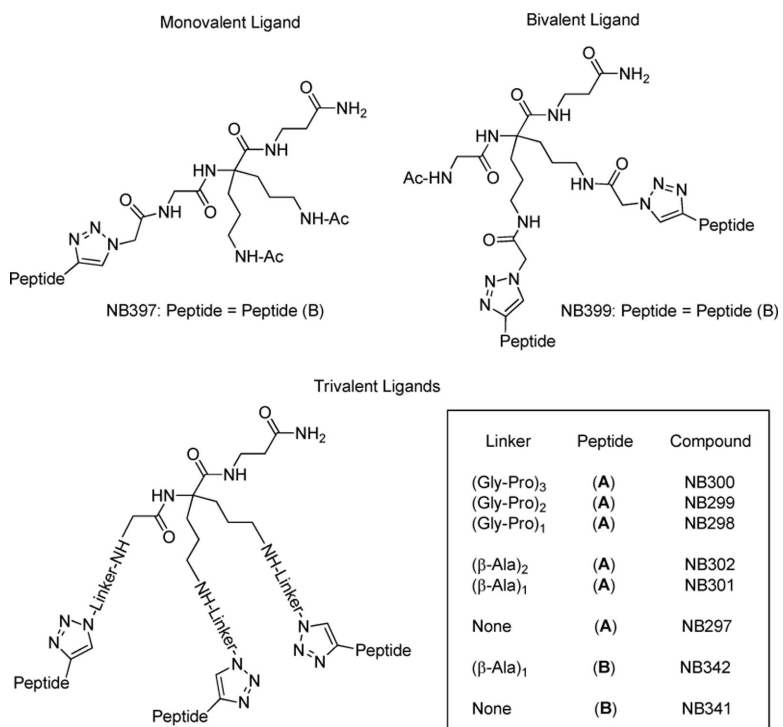


Figure 2. Structures of monovalent, bivalent and trivalent ligands.

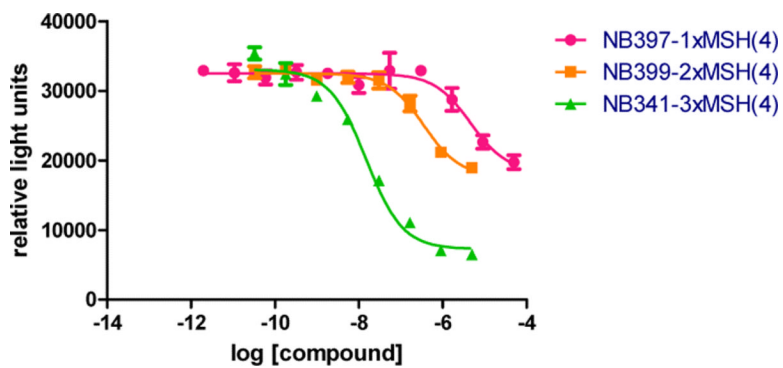


Figure 3. Time resolved fluorescence assay³¹. Competitive binding assay was performed by competing 10nM of Eu-NDP- α -MSH with various concentrations of multimers.

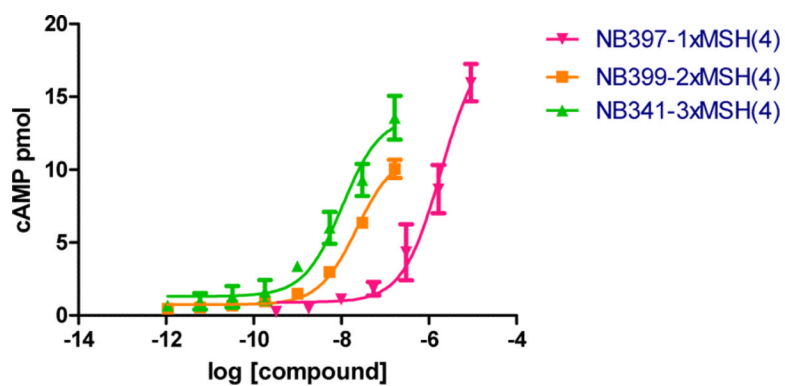
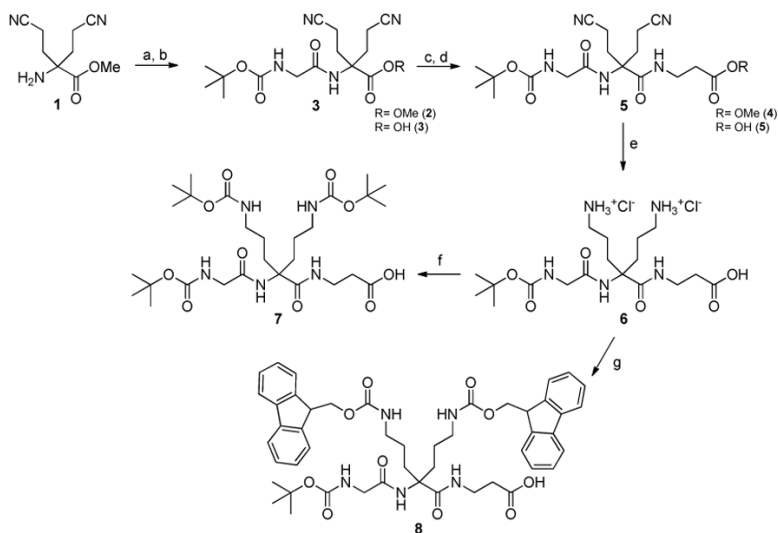
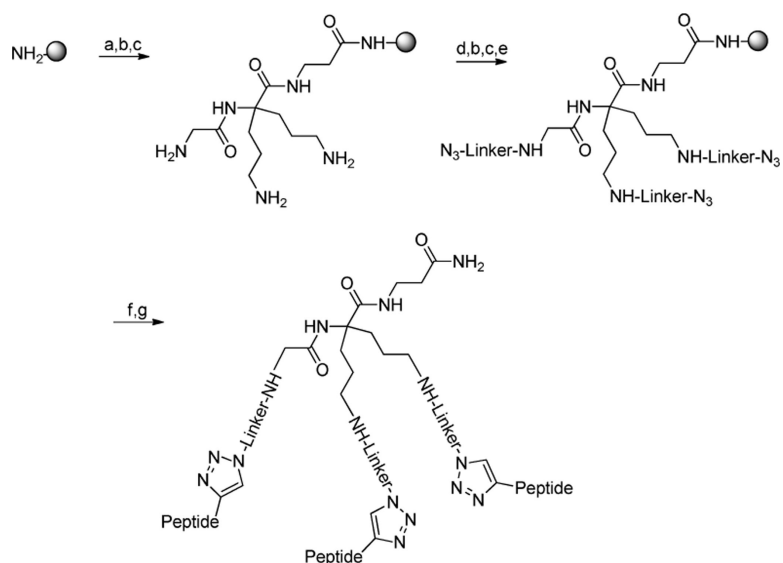


Figure 4. cAMP accumulation in hMC4R cells in response to multivalent ligands. cAMP was quantified using a chemiluminescent immunoassay described on the Experimental section. cAMP is expressed as the amount released from treated cells above that observed for control cells.



Scheme 1. Scaffold Synthesis

Reagents and conditions: (a) Boc-Gly-OH, HCTU, DIEA, DMF, rt, 92 %; (b) LiOH (2N), THF, rt, 95 %; (c) β -Ala-OMe, BOP, DMF, DIEA, rt, 85 %; (d) LiOH (2N), THF:MeOH, 0°C, 90 %; (e) PtO₂, H₂, 50 psi, MeOH:CHCl₃, rt, 80%; (f) Boc₂O, DIEA, H₂O:MeCN, rt, 80%; (g) Fmoc-OSu, H₂O:MeCN, NaHCO₃ 0°C to rt, 65 %.



Scheme 2. Assembly of the trivalent construct on solid support

Reagents and conditions: (a) **7**, HCTU, DIEA, DMF; (b) 100% TFA; (c) 20% DIEA in DCM; (d) Boc-linker-OH, HCTU, DIEA, DMF; (e) $\text{N}_3\text{CH}_2\text{CO}_2\text{H}$, HBTU, DIEA, DMF; (f) CuI, Ascorbic acid, DIEA, peptide (**A**) or (**B**), (Cf. Figure 2 for the sequences), DMF:2,6-Lutidine; (g) HF.

Table 1

Analytical data of synthesized multimers.

Compound ^a	Linker	Mass Spectrum ^b		HPLC ^c , t _R (min)
		Ion	Calc. Obsd.	
Ac-MSH(4)	-	(M+1) ⁺	686.3482 686.6389	9.75
NB297	No linker	(M+5) ²⁺	650.1385 650.1376	12.37
NB298	(Gly-Pro) ₁	(M+6) ⁶⁺	647.4978 647.4982	12.97
NB299	(Gly-Pro) ₂	(M+6) ⁶⁺	724.5349 724.5359	12.90
NB300	(Gly-Pro) ₃	(M+6) ⁶⁺	801.5720 801.5709	12.86
NB301	β-Ala	(M+6) ⁶⁺	577.4685 577.4683	12.90
NB302	(β-Ala) ₂	(M+6) ⁶⁺	612.9871 612.9861	12.83
NB341	No linker	(M+5) ²⁺	548.0752 548.0752	12.94
NB342	β-Ala	(M+5) ²⁺	590.6974 590.6969	12.90
NB397	No linker	(M+2) ²⁺	689.3679 689.3666	11.58
NB399	No linker	(M+4) ⁴⁺	578.8050 578.8053	12.62

^a NB297 to NB302 were synthesized using peptide (A) and NB341 to NB399 were synthesized using peptide (B). NB397 is the monovalent ligand and NB399 is the bivalent ligand.

^b Maldi-TOF or ESI-MS.

^c Peptides were eluted with a linear gradient from 0 to 100% acetonitrile containing 0.1% TFA in 30 min at 1 mL/min. The purity of all peptides determined by HPLC was 95%.

Table 2

Influence of linker length on affinity.

Compound	Linker	Estimated length between ligands (Å) ^a	IC ₅₀ (nM)	Relative potency to Ac-MSH(4)
Ac-MSH(4)	-	-	4600 ± 790	-
NB300	(Gly-Pro) ₃	60±20	620 ± 51	7
NB299	(Gly-Pro) ₂	52±20	410 ± 70	11
NB298	(Gly-Pro) ₁	45±20	220 ± 59	21
NB302	(β-Ala) ₂	48±10	120 ± 10	38
NB301	β-Ala	43±10	88 ± 12	52
NB297	No linker	35±10	71 ± 5.3	64
NB342	β-Ala	35±5	73 ± 3.5	63
NB341	No linker	24±5	14 ± 1.5	330

Competition experiments were carried out using time resolved fluorescence³¹. Multimers were competed against Eu-NDP-α-MSH. The IC₅₀ values are the average of 4 experiments each done in quadruplicate.

^aLinker length was estimated from an energy-minimized structure using MacroModel.

Table 3

Influence of valency on affinity.

Compound	IC50 (nM)	Relative potency to NB397
NB397-1×MSH(4)	4900 ± 760	-
NB399-2×MSH(4)	310 ± 73	16
NB341-3×MSH(4)	14 ± 1.5	350

Competition experiments were carried out using time resolved fluorescence³¹. Multimers were competed against Eu-NDP- α -MSH. The IC50 values are the average of 4 experiments each done in quadruplicate.

Table 4

Receptor occupancy.

Compound	[RL] at 5% of forskolin response	[RL] at 10% of forskolin response	[RL] at 25% of forskolin response
NB397-1×MSH(4)	13000 ± 1000	60000 ± 4900	420000 ± 34000
NB399-2×MSH(4)	12000 ± 1300	45000 ± 4100	400000 ± 22000
NB341-3×MSH(4)	14000 ± 1000	75000 ± 5400	450000 ± 33000

Receptor occupancy was calculated for the concentration of each ligand required to activate cAMP increase at 5%, 10% and 25% of the maximum observed with forskolin (10 μ M) treatment using the following established equation: $[RL] = B_{max} \times [L] / [L] + K_d$. The K_d values used for this calculation were estimated from the competition binding assays (Figure 3). The numbers of receptors was assumed to be constant at 7.5×10^5 receptors *per* cell, as previously measured for this cell line³².