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Synthesis and Evaluation of Bivalent NDP- α -MSH(7) Peptide Ligands for Binding to the Human Melanocortin Receptor 4 (hMC4R)

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Abstract

We demonstrate the potential utility of multivalent ligands as targeting agents for cancer imaging or therapy by determining the binding of homobivalent ligands to their corresponding receptors. This manuscript details the synthesis and evaluation of a series of bivalent ligands containing two copies of the truncated heptapeptide version of [Nle⁴-D-Phe⁷]- α -melanocyte stimulating hormone (NDP- α -MSH), referred to as MSH(7). These were connected with various semirigid linkers containing Pro-Gly repeats, with or without flexible poly(ethylene glycol) (PEGO) moieties at their termini. Modeling data suggest a distance of 20–50 Å between the ligand binding sites of two adjacent G-protein coupled receptors, GPCRs. These bivalent ligands were observed to bind with higher affinity compared to their monovalent counterparts. Data suggest these ligands may be capable of cross-linking adjacent receptors. An optimal linker length of 25 ± 10 Å, inferred from these ligands, correlated well with the inter-receptor distance estimated through modeling. Although there was no difference in maximal binding affinities between the ligands constructed with the Pro-Gly repeats versus those constructed with the PEGO inserts, the PEGO-containing ligands bound with high affinities over a greater range of linker lengths.

INTRODUCTION

Current cancer treatments suffer from a lack of specificity for cancer cells. Using peptide ligands for specific delivery of a drug or a contrast agent to the site of action has been demonstrated for a number of cancers using many different ligand–receptor pairs (1). However, a limitation of this approach is that targeting an individual receptor requires that it be overexpressed or mutated in the cancer and nowhere else in the body. While such target receptors may be available for some cancers, they are not widely prevalent. Hence, alternative targeting strategies are required. We hypothesize that specific targeting of cancer cells could be achieved through the development of multivalent ligands directed toward unique combinations of cell surface proteins that are expressed on the target cancer cells compared to normal cells. By targeting combinations, these ligands can be more specific for the cells of interest while circumventing nonspecific interaction with healthy tissues. As a proof of the concept that such cell surface proteins can indeed be targeted specifically, we

chose to mimic the cancer cells through overexpression of MC4 receptors because of our considerable experience with MC4 receptors (2, 3). Thus, in order to demonstrate that multivalent peptide ligands bind to their target receptors with increased affinity and cooperativity, we have constructed ligands containing two copies of MSH(7),¹ a truncated version of [Nle^{4-D}-Phe⁷]- α -melanocyte stimulating hormone (NDP- α -MSH) (2), a peptide hormone analogue that binds specifically to the melanocortin receptors. These two MSH(7) ligands are separated by a linker, which is constructed with different amino acids and/or organic derivatives in order to provide differences in flexibility and length.

Previously, we described the synthesis and evaluation of a series of short homobivalent MSH(4) His-D-Phe-Arg-Trp-NH₂ and MSH(6) Nle-Glu-His-D-Phe-Arg-Trp-NH₂ ligands which bind to cells expressing the human melanocortin 4 receptor (hMC4R) with increased affinity and apparent cooperativity compared to their monovalent counterparts (3). However, the linkers used in these studies were likely too short to allow for cross-linking of multiple receptors. Thus, the increased binding affinity and positive cooperativity observed in these studies were most likely due to “statistical” binding, wherein affinity is apparently increased through increased local concentrations, and not receptor “clustering”, wherein multiple receptors are bound by the same multivalent ligand (4) (Figure 1). In order to improve the likelihood that multivalent ligands simultaneously cross-link receptors, we have constructed homobivalent MSH(7) ligands with linkers that are longer and more flexible than those previously investigated.

It has been documented that the hMC4 receptors can form homodimers (5) and that other melanocortin receptors can form homo- or heterodimers (6). The lack of high-resolution data for G-protein coupled receptors (GPCRs) has made it difficult to elucidate the 3D structure of these receptors, thus further complicating the task of rationally designing multivalent ligands that could span the distance between adjacent GPCRs. Early estimates suggest that this distance is at least 40 Å (7-9). The crystal structure of rhodopsin (10) has made it possible to model the structures of additional members of the GPCR family of proteins based on computer-aided modeling by homology. For example, the human melanocortin 4 receptor (hMC4R) has been modeled on the basis of sequence homology and the known rhodopsin structure (11). This model was used to assist in the determination of the linker lengths needed to span the distance between adjacent GPCR binding sites.

In addition to difficulties in estimating the size and location of the receptor binding pockets, it is also relatively difficult to estimate the actual length of the linkers in question. This difficulty arises due to the fact that the peptide type linkages used to construct these linkers will be flexible, thus permitting folding and secondary structure. We used the estimation that one amino acid residue is roughly equivalent to 1.2 Å, an estimation based on the average linear distance between atoms in a peptide bond and the assumption that at least one conformer of these linkers can exist in a stretched conformation with little or no secondary structure (12). Upon binding, the linker may lose its folding to be stretched in extended conformations.

¹Abbreviations: α -MSH, α -melanocyte stimulating hormone; NDP- α -MSH, Nle^{4-D}-Phe⁷- α -melanocyte stimulating hormone; hMC4R, human melanocortin 4 receptor; Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle Medium; ESI-MS, electrospray ionization-mass spectrometry; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethyloxycarbonyl; FT-ICR, Fourier transform-ion Cyclotron Resonance; GPCR, G-protein coupled receptor; HCTU, *O*-(1*H*-6-chlorobenzotriazol-1-yl) (dimethylamino)methylene]uranium hexafluorophosphate *N*-oxide; HOBt, 1-hydroxybenzotriazole; HOCl, 6-chloro-1-hydroxybenzotriazole; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MMFF, Merck molecular force field; MSH(7), des-{Ser¹-Tyr²-Gly¹⁰-Lys¹¹-Pro¹²-Val¹³}-Nle^{4-D}-Phe⁷- α -melanocyte stimulating hormone; PEGO, 19-amino-5-oxo-3,10,13,16-tetraoxa-6-azanonadecan-1-oic acid; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-yl sulfonyl; TFA, trifluoroacetyl; TRF, time-resolved fluorescence; Trt, triphenylmethyl (trityl).

This manuscript details the construction and evaluation of a family of homobivalent MSH(7) ligands with linkers ranging in length from 18 to 148 atoms that could span distances over 100 Å (Table 1). A series of control ligands also were constructed in which MSH(7) was attached to only one end of the various linkers (ligands **1–5**, Table 1) to allow for a direct determination of the influence of the linker on the binding affinity of the ligand to the receptor. Pro-Gly repeats were chosen as the building blocks for the linker unit, as this semirigid construct provided the best results in our initial studies (3). The Pro residues add rigidity to the overall linker structure, while the Gly residues provide for some flexibility. Five bivalent ligands were synthesized with these Pro-Gly repeat regions (ligands **6–10**, Table 1) directly corresponding to the monovalent ligands. As an added feature, PEGO units were either themselves used as linkers or were incorporated into the termini of Pro-Gly repeats (ligands **11–16**, Table 1). As it has been proposed that the first binding event serves to tether the multivalent ligand to the cell surface (13), we hypothesized that the addition of these flexible PEGO moieties to the ends of the linkers would provide a greater opportunity for the ligand to explore more volume and thus have greater opportunity to find and bind multiple receptors at once.

EXPERIMENTAL PROCEDURES

Chemical Materials

N- α -Fmoc-protected 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). For the *N*- α -Fmoc-protected amino acids, the following side chain protecting groups were used: Arg(*N*^ε-Pbf); Glu(*O*-*t*Bu); His(*N*^{im}-Trt); Ser(*O*-*t*Bu); Trp(*N*ⁱ-Boc). Peptide synthesis solvents, reagents, and acetonitrile for HPLC were reagent grade, were acquired from VWR (West Chester, PA) or Aldrich-Sigma (Milwaukee, WI), and were used without further purification unless otherwise noted. The solid-phase synthesis was performed in fritted syringes using Domino manual synthesizer obtained from Torviq (Niles, MI).

The purity of the final products was checked by analytical RP-HPLC using a Waters Alliance 2695 separation model with a Waters 2487 dual wavelength detector (220 and 280 nm) on a reverse-phase column (Waters Symmetry 4.6 $\times$ 75 mm, 3.5 μ m). Peptides were eluted with a linear gradient of aqueous CH₃CN/0.1% CF₃CO₂H at a flow rate of 1.0 mL/min. Purification of ligands was achieved on a Waters 600 HPLC using a reverse-phase column (Vydac C-18, 15–20 μ m, 22 $\times$ 250 mm). Peptides were eluted with a linear gradient of CH₃CN/0.1% CF₃CO₂H at a flow rate of 5.0 mL/min. Separation was monitored at 230 and 280 nm.

Size exclusion chromatography was performed on a borosilicate glass column (2.6 $\times$ 250 mm, Sigma, St. Louis, MO) filled with medium-sized Sephadex G-25 or G-10. The compounds were eluted with an isocratic flow of 1.0 M aqueous AcOH. Structures were characterized by ESI (Finnigan, Thermoquest LCQ ion trap instrument) or MALDI-TOF (Bruker Reflex-III, α -cyanocinnamic acid as a matrix). For internal calibration, an appropriate mixture of standard peptides was used with an average resolution of 8000–9000. High-resolution mass measurements were carried out on a FT-ICR IonSpec 4.7T instrument.

Solid-Phase Synthesis

The Rink resin was washed with DMF, and the *N*^α-Fmoc protecting group was removed with 1:1 or 1:4 piperidine in DMF, as the case may be (1 $\times$ 2 min and 1 $\times$ 20 min). The resin was washed successively with DMF, DCM, DMF, and then a solution of 1.0 M HOBt in

DMF, and DMF, and the next N^{α} -Fmoc amino acid was coupled using preactivated 0.3 M HOCT or HOBt esters in THF (3 equiv of N^{α} -Fmoc amino acid, 3 equiv of HOCT/HOBt, and 3 equiv of DIC). The resin slurry was stirred for 2 h or until the bromophenol test became negative. If the test failed, the resin was washed with DMF, and the amino acid was coupled again by the HCTU/2,4,6-lutidine procedure (0.3 M solution of 3 equiv of N^{α} -Fmoc amino acid, 3 equiv of HCTU, and 6 equiv of 2,4,6-lutidine in DMF) for 3 h or by a preformed symmetric anhydride (3 equiv of N^{α} -Fmoc amino acid and 1.5 equiv of DIC in a 1:1 DMF–DCM mixture) until the Kaiser test was negative. If the second coupling did not result in a negative Kaiser test, the resin was washed with DMF, and the free amino groups were capped with 50% Ac_2O in pyridine for 10 min.

After coupling Trp(N^{β} -Boc), Arg(N^{ϵ} -Pbf), D -Phe, His(N^{β} -Trt), Glu(O - t Bu), Nle, and Ser(O - t Bu) sequentially to the Rink amide resin, Gly and Pro were attached alternatively as many times as needed to synthesize the control ligands **1–5** (Scheme 1). Assembly of the second ligand, MSH(7), was carried out by the procedure described above to give ligands **6–10**. Similarly for the synthesis of ligands **11–16**, after the assembly of MSH(7) sequence on the resin, the flexible linker PEGO was attached, by first adding diglycolic anhydride and then activating the free carboxylate as an imidazolide for the attachment of 4,7,10-trioxa-1,13-tridecanediamine (**3**). This was followed by stepwise assembly of repetitive Pro-Gly linker units. Another PEGO attachment followed by the second MSH(7) sequence and N-terminal acetylation yielded resin bound protected precursors of compounds **11–16** (Scheme 1). A cleavage cocktail (10 mL per 1 g of the resin) consisting of $\text{CF}_3\text{CO}_2\text{H}$ (91%), H_2O (3%), $\text{HSCH}_2\text{CH}_2\text{SH}$ (3%), and PhSMe (3%) was injected into the resin, and the mixture was agitated at room temperature for 3 h. The solution was filtered off, the resin was washed with $\text{CF}_3\text{CO}_2\text{H}$ (2×3 min), the liquid phases were concentrated under a stream of nitrogen, and the product was precipitated using cold Et_2O . The products **1–16** were washed three times with cold Et_2O , lyophilized, purified, and characterized as described above. Mass spectral and HPLC characterization data are given in Table 2.

Molecular Modeling

Conformational searches and molecular dynamics were performed with *MacroModel* v 9.1 implemented under the Maestro 7.5 interface on a Linux workstation. The *MacroModel* implementations of Merck Molecular Force Field (MMFF), AMBER*, and OPLS all-atom force fields were used (14). AMBER* is a reparametrized AMBER force field containing a new set of torsional parameters that more closely reproduces *ab initio* calculations on the conformational preferences of simple peptides (15). For solution-phase calculations, the GB/SA continuum model for water was used. Amide bonds were required to be trans except in the case of proline whose imide bonds were intentionally sampled and accepted with either cis or trans geometry in the conformational searches.

Conformation Search

Conformational searches were performed with the systematic Monte Carlo method of Goodman and Still (16). For each search, 5000 starting structures were generated and minimized until the gradient was less than $(0.05 \text{ kJ/mol})/\text{\AA}$, using the truncated Newton–Raphson method implemented in *MacroModel*. Duplicate conformations and those with energy greater than 50 kJ/mol above the global minimum were discarded.

Monte Carlo/Stochastic Dynamics

All simulations were performed at 310 K with Monte Carlo/stochastic dynamics (MC/SD) hybrid simulation algorithm (17) with either the AMBER* all-atom force field or the new OPLS-2005 force field in *MacroModel 9.1*. A time step of 1.5 fs was used for the stochastic dynamics (SD) part of the algorithm. The MC part of the algorithm used random torsional

rotations between $\pm 60^\circ$ and $\pm 180^\circ$ that were applied to all rotatable bonds except the proline amide C–N bond where the random rotations between $\pm 0^\circ$ and $\pm 180^\circ$ were applied. No torsion rotations were applied to bonds in the pyrrolidine ring of proline, as the barriers are low enough to permit adequate sampling from the SD part of the simulation. The total simulation time was 1000 ps, and samples were taken at 1 ps interval, yielding 1000 conformations for analysis.

G-Protein Coupled Receptor Size Estimations

The PDB file of a recently described bovine rhodopsin protein in a trigonal crystal form (1GZM) (18) was loaded into the *DeepView* program (Swiss-Pdb Viewer Program, <http://www.expasy.org/spdbv/>). This program allows distances between different residues on the GPCR structure to be measured. Several residues on the outer edges of the transmembrane domain were chosen, and distances across the GPCR were measured. The “width” of the GPCR was taken as the average of eight of these measured distances.

Cell Culture

HEK293 cells overexpressing the human melanocortin 4 receptor (hMC4R) were used to assess the binding at the hMC4R. The hMC4R vector was originally received from Dr. Ira Gantz, University of Michigan (19). 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.

Lanthanide-Based Binding Assays

Lanthanide-based competitive binding assays were conducted according to the method which has been previously described (20). In brief, HEK293/hMC4R cells were plated in black and white 96-well isoplates (Wallac, 1450–584) at a density of 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 μ L of nonlabeled ligand and 50 μ L 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 μ L wash buffer (50 mM Tris–HCl, 0.2% BSA, 30 mM NaCl). Enhancement solution (Perkin-Elmer; 1244–105) was added (100 μ L/well), and the plate was incubated for at least 30 min at 37 °C prior to reading. The plates were read on a Wallac VICTOR³ instrument using the standard Eu TRF measurement (340 nm excitation, 400 μ s delay, and emission collection for 400 μ s at 615 nm).

Data Analysis

Data from at least five independent binding experiments were compiled into a single competition curve and were analyzed with *GraphPad Prism* software using the sigmoidal dose–response (variable slope) classical equation for nonlinear regression analysis. The total number of data points used to generate each binding curve is indicated by *n* in Table 1.

RESULTS

Synthesis

The synthesis of ligand dimers **6–16** consisting of two MSH(7) ligands connected in a head-to-tail fashion by PEGO and/or Pro-Gly linkers and monovalent controls **1–5** is depicted in Scheme 1. The heptapeptide MSH(7) was constructed on Rink amide Tentagel S resin (initial loading 0.2 mmol/g). Resin **17** retained all side chain protecting groups, except for

the *N*-terminal Fmoc protecting group. PEGO linkers were attached to the *N*-terminal of **17**, giving [PEGO]_{*m*} resins **18** and **19**. The resin **18** was proportionally split for synthesis of compounds **11** and **12** or for synthesis of longer ligands **13–16**. For the ligands **11** and **12**, the free amine groups of resins **18** and **19** were coupled with Fmoc-Trp(*N*^ε-Boc)-OH, and solid-phase peptide synthesis continued to complete the second MSH(7) sequence, then acetylated, giving resins **20** and **21**. To the second portion of resin **18** already split into four reactors, Pro-Gly linkers were alternatively coupled to the *N*-terminus of **18**, giving resins **22–25**. PEGO linker was attached, and the second ligand was built up similar to **20** and **21**, giving acetylated resins **26–29**. Analogously, resin **17** was split into five portions for the attachment of Pro-Gly linkers, giving resins **30–34**. For the synthesis of monomers **1–5** and dimers **6–10**, each of the above resins **30–34** was split. The synthesis of second ligand was carried out, giving resins **35–39**. All resin precursors **20** and **21** and **26–39** were simultaneously deprotected and cleaved from the Rink resin using a mixture of trifluoroacetic acid, 1,2-ethanedithiol, thioanisole, and water (91/3/3/3) that produced the desired compounds **1–16**. Peptides **1–16** were purified by size exclusion chromatography and reverse-phase C₁₈ preparative HPLC and were characterized by ESI-MS and/or MALDI-TOF mass spectrometry, and/or FT-ICR (Table 2).

Linker Length Estimate

The 3D structure of the hMC4R has been modeled on the basis of the sequence homology and crystal structure of rhodopsin (11). We used the crystal structure of a recently published bovine rhodopsin protein, with two protein molecules per asymmetric unit (18) to determine the width of the hMC4R and to estimate the distance between two adjacent ligand binding sites. Using the *DeepView/Swiss-Pdb Viewer* program to visualize the 3D structure of rhodopsin, we measured the “width” of the GPCR dimer and found that the average distance from edge to edge was ~70 Å at the widest point (Figure 2A).

For the hMC4R, four transmembrane domains (TM), viz., TM3, TM4, TM5 and TM6, are integral in forming the ligand-binding pocket of this GPCR. On the basis of this arrangement of the TM domains, the center of the binding pocket is estimated to be located ~12 Å from the nearest edge of the receptor (Figure 2A). If two adjacent receptors have their binding pockets facing each other in a head-to-head arrangement (Figure 2B), the distance between the centers of these binding pockets would be ~25 Å. If the binding pockets are arranged in a head-to-tail fashion or a tail-to-tail fashion, the distance between the binding sites would be expected to increase (Figure 2C,D, respectively). Additionally, it must be noted that annular lipids may be associated with receptors and thus would be expected to add an additional 8 Å to the distance based on an estimated cross-sectional area of ~45 Å² (21–23). Furthermore, these estimations assumed that, upon dimerization, each receptor maintained its overall dimensions and that the two receptors did not participate in domain swapping. With these assumptions, the length needed to span the distance between these binding sites was estimated to be between 20 and 50 Å. This estimation implies that linkers shorter than 20 Å (e.g., (3)) cannot cross-link multiple GPCRs.

Modeling of Linkers and Homobivalent Ligands

The compounds **7**, **8**, and **14** (Table 1) displayed the highest affinity in our lanthanide-based binding assays. As is evident, the linker lengths in terms of atoms do not bear any correlation with activity, as a whole range of compounds having linker lengths from 36 to 76 atoms displayed similar affinities, irrespective of the nature of the linkers. This indicates either a secondary structure formation or a large degree of inherent flexibility of the linkers such that the bivalent mode of binding of MSH epitopes is preserved but the overall conformation of the linker region is reasonably different among compounds. Modeling of the Pro-Gly repeats and PEGO linkers bears out both of these possibilities. These maximum

entropy methods indicated that a semirigid helical structure for Pro-Gly-based linkers and a very flexible conformation for PEGO spacers were allowable conformations.

Initially, Monte Carlo conformational searches were performed separately on model rigid linker peptides $[PG]_n$ ($n = 3, 6, 9$) and the flexible linker PEGO. As can be seen from Figure 3a, an 18-mer Pro-Gly (PG) sequence folded into a helical structure in the lowest-energy conformer with a triangular shape (viewed perpendicular to axis) similar to a polyproline helix type II (24). However, the Pro-Gly helix was broader with a pitch of 5 Å per seven residues. This lowest-energy conformer was further investigated using Monte Carlo/stochastic dynamics simulations at 310 K for 1000 ps of simulation time. This revealed that this 18-mer could span a distance of 5–10 Å per seven-residue turn with an average of 7 Å from the population sample set, while still retaining a significant helical shape. Therefore, the PG repeat is predicted to be stabilized in a semirigid conformation as was anticipated in the initial design of our rigid linkers. Additionally, the per-residue span of 0.8–1.6 Å indicated here roughly correlates with the span of 1.2 Å unit from linear sequence estimations that were used initially. However, it must be noted here that the distance ranges from dynamic simulations are not linear distances measured along the C_α backbone but instead are spatial distances based upon the pitch of modeled helix. Further experimental verification of these results is presently being investigated in our group.

A similar study set of conformational searches and MD simulations on the flexible PEGO linker revealed that it could assume a bent conformation at the global minimum, as one would expect (Figure 3b) and that the population contained many low-energy conformers with linear (open) shapes. This indicates that a 20-atom PEGO spacer can take any number of conformations with a distance range 4–18 Å long. The fact is evident, as the solvent medium can easily replace most of the hydrogen bonds in the open conformation that it forms with itself in the bent conformation.

We also investigated a model ligand, Ac-MSH(7)-PEGO-[PG]₉-PEGO-MSH(7)-NH₂ to investigate the conformation and dynamics of this compound with respect to linker mobility and space interrogation by the attached ligands. Figure 3c shows the ligand along with a ruler in angstrom units. The MD simulation results included populations of both open and closed conformations (MSH epitopes either further apart or closer together). Although the difference in energy between these two conformations was quite large, we believe the binding of MSH epitopes to the receptors could relieve this energy difference. Therefore, the open conformation may more closely resemble the binding mode. Upon the basis of these models, the lengths spanned by the linkers in the ligand library were estimated, as shown in Table 1. It is worth noting that these linker lengths correlated quite well with the inter-receptor distances of 25 ± 10 Å estimated from the modeled GPCR dimers reported above and in the literature (25).

Homobivalent Ligands with Longer Linkers Bind with an Increased Affinity

Ligand binding was evaluated via the lanthanide-based binding assay that has been described previously (20). In all cases, the bivalent constructs of MSH(7) bound with more than 10-fold higher affinities compared to the ligands composed of monovalent MSH(7) plus the linker (Figure 4A). In order to assess the enhancement in binding affinity of the bivalent ligand relative to the monovalent ligand, the EC_{50} of the monovalent ligand was divided by twice the EC_{50} for the bivalent ligand, accounting for the presence of two binding subunits in the bivalent ligand compared to only one in the monovalent ligand. Even following this correction, Figure 4B indicates that a significant enhancement is still detected when observing bivalent versus monovalent ligand binding. The trend relating linker length to average EC_{50} followed a similar pattern for the control monomers and the dimers.

Ligands **7** and **8**, with linker lengths of 36 and 54 atoms (10–20 Å and 15–30 Å, respectively), showed the highest binding affinity of all compounds in the Pro-Gly series. The estimated linker lengths for these compounds (25 ± 10 Å), were identical to the estimated inter-receptor distance obtained through modeling.

Addition of Flexible Ends to Overall Rigid Linkers Provides no Further Increase in Affinity

Although compounds **11–14** have similar and appreciable activity, the flexibility of the PEGO linkers precluded using these compounds for distance estimation (*vide infra*; see also Figure 3B). Nonetheless, it was notable that, while the maximum affinities of the PEGO series were not higher than those of the Pro-Gly series, they retained high-affinity binding over a wider range of ligand sizes (Table 1, Figure 5A). Notably, 20-atom compound **11** and 76-atom compound **14** bound with significantly higher affinity ($p < 0.001$) compared to the similarly sized, 18-atom compound **6** and 72-atom compound **9**, respectively. To directly compare compounds with and without PEGO units, binding data were plotted as a function of Pro-Gly units. In this analysis (Figure 5B), only the shortest PEGO compound showed significant improvement in binding over PG alone. It is also noteworthy that, for both series, the affinity decreases with increasing linker length, after the minimum, optimal length criterion of 2 PG repeats is met.

DISCUSSION

We have synthesized 16 MSH(7)-containing ligands and confirmed their binding to the hMC4R in whole cell assays. All 11 of the homobivalent ligands bound with at least a 10-fold greater affinity than the corresponding monovalent controls. The conjugation of linkers to monovalent MSH(7) did not significantly decrease the binding affinity of the ligand when linkers were shorter than 40 atoms. As the linker lengths increased beyond 40 atoms, significant decreases in the binding affinities were observed. This can be attributed to the increased translational and rotational entropies associated with longer linkers (26). Thus, the linker is interfering with the ligand receptor interactions leading to a decreased affinity.

Bivalent ligands bound with an enhanced affinity compared to monovalent ligands. On the basis of our modeling studies, linkers longer than ~25 Å allowed both ligands to reach and simultaneously bind adjacent receptors. Although we hypothesize that the enhanced affinity observed with the bivalent ligands is a result of the receptor clustering mechanism, we cannot conclusively demonstrate this in this experimental system. Such a demonstration will require a heterobivalent system, which is currently under investigation.

We originally hypothesized that the addition of flexible PEGO moieties to the ends of the otherwise relatively rigid linker would enhance ligand binding by allowing the dimer to investigate more space, thus increasing the likelihood of finding and binding to a second receptor. While this may be the case, the results are not dramatic. The 76-atom PEGO-decorated dimer **14** bound with an EC_{50} of 11 ± 2 nM, while the corresponding 72-atom [PG] dimer **9** bound with a significantly higher 22 ± 3 nM. However, the 112-atom compound **15** (19 nM) and the 90-atom compound **10** (22 nM) were not significantly different. This would suggest that either the structures or the distance spans of these linkers are similar enough that they do not demonstrate any effects on ligand binding. The effect of PEGO was most evident in the short linkers. The 20-atom PEGO-linked dimer **11** bound with significantly higher affinity ($EC_{50} = 16 \pm 2$ nM) compared to the corresponding 18-atom PG-linked dimer **6** (39 ± 8 nM). Both Pro-Gly and PEGO-[PG]_n-PEGO-based linkers were expected to possess a certain degree of inherent flexibility, especially the PEGO-based linker. Computational modeling revealed that linkers could assume multiple conformations with a range of distance due to this inherent flexibility. Whereas Pro-Gly may maintain a more or less rigid structure (as evident from the helical structure in MD simulations, the

PEGO-[PG]_n-PEGO was more free to take a linear or bent shape overall and could assume a wider range of distances. For example, compound **14** had a predicted linker length of 18–56 Å, which was a larger range compared to compound **7**, which had a range of only 10–20 Å. Thus, it is predicted that these two linker types can assume different overall geometries. Because of lower flexibility, the Pro-Gly series provided a clearer picture of the distance between two receptors. Notably, the PEGO series maintained high-affinity binding, even out to lengths of 100 atoms, beyond which there appeared to be a decrease in binding affinity.

In summary, the optimal linker length to span two GPCRs in our model was 25 ± 10 Å, and this generally fits with the binding data for the Pro-Gly series. However, the significant bioactivity of compound **6** suggests that a distance may be less than 25 Å. This may indicate either a statistical effect of two epitopes or domain swapping among receptors. The present study cannot make a conclusive statement with respect to this mode, and additional work will need to be completed to further assess the actual optimal linker length and differentiation in binding modes. Moreover, this distance will most likely vary depending on the exact receptor evaluated and may change when evaluating heterovalent binding which involves different types of receptors. Finally, the only way to demonstrate actual cross-linking of multiple receptors using our experimental system requires the evaluation of heterovalent ligands. Such work is currently underway, and the results from the experiments reported here will be used to design linkers for our future work involving heterovalent ligands for direct targeting of cancer cells.

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Figure 1. Two of the four potential modes of multivalent ligand binding. A. The *cluster effect* results in recruitment of receptors on the cell surface to allow for multiple receptor–ligand interactions. B. The *statistical effect* allows ligands to bind to single binding sites on a receptor based on increased local effective concentration of the ligand.

Scheme 1. Solid-Phase Synthesis of MSH(7) Peptides 1–16^a

^a Reagents and conditions: (a) 1:1 or 1:4 piperidine in DMF. (b) Fmoc/tBu solid-phase synthesis. (c) PEGO attachment (ref 3). (d) Fmoc/tBu solid-phase synthesis of Pro-Gly repeats. (e) pyridine/Ac₂O (50/50). (f) CF₃CO₂H/HSCH₂CH₂SH/PhSMe/H₂O (91/3/3/3).

Figure 2.

The distance estimations between the binding sites of two adjacent GPCRs (modeled from pdb 1GZM). A. The binding site is estimated to be ~12 Å from the nearest edge of the receptor. When the receptors are arranged in a head-to-head (B), tail-to-head (C), or tail-to-tail fashion (D), the estimated distances between the binding pockets are 25, 35, and 45 Å, respectively. These distances do not account for the presence of additional lipids or domain swapping as was discussed in the text.

Figure 3.

Computational study of -[Pro-Gly]- and -[PEGO]- linkers and a model homobivalent ligand to investigate the conformation, distance span of the linkers, and overall linker moiety. Part A (top left) shows a [PG]₉ repeat taking a 6₁₇-helical conformation. MD simulation of this conformation at 310 K shows a significant helical pattern in the entire sample set of 1000 conformations with length ranging from 15 to 29 Å. Part B (top right) shows the PEGO linker in a bent conformation at global minimum. As expected, the linker shows a high degree of flexibility in MD simulations, with distance span between two ends ranging from 4 to 18 Å. Part C (bottom) shows a hypothetical homobivalent ligand, with ruler above, to indicate the approximate distance span of different subunits. The distances calculated from the GPCR dimer study and the modeling of linkers correlate quite well with the binding data.

Figure 4. Binding results of monovalent ligands versus bivalent ligands. Binding of the ligands was tested via competition with Eu-NDP- α -MSH. A. Bivalent ligands (■) bind with a higher affinity than their corresponding monovalent ligands (▲). B. The affinity enhancement was calculated by the equation: $EC_{50}^{\text{monovalent}}/2(EC_{50}^{\text{bivalent}})$. Results represent the average of at least five independent experiments, each done in quadruplicate, and error bars indicate the standard deviation.

Figure 5.

A. Binding results of the two classes of bivalent MSH(7) ligands. Binding of the ligands was tested via competition with Eu-NDP- α -MSH. The bivalent ligands constructed with the PEGO inserts (▲) do not bind with higher affinity compared to the bivalent ligands constructed primarily from Pro-Gly repeats (■). Results represent the average of at least five independent experiments, each done in quadruplicate, and error bars indicate the standard deviation. B. Comparison of compounds with the same number of PG repeats. Only one set of compounds showed increased binding affinity upon incorporation of PEGO inserts.

Table 1The Ligands Synthesized^a

Compound	No of atoms present in the linker	Estimated linker length (Å)	EC ₅₀ (nM)	n
(1)	18	5-10	99±13	244
(2)	36	10-20	82±12	306
(3)	54	15-30	236±24	295
(4)	72	20-40	188±25	303
(5)	90	25-50	428±55	276
(6)	18	5-10	39±8	230
(7)	36	10-20	11±2	236
(8)	54	15-30	15±3	238
(9)	72	20-40	22±3	225
(10)	90	25-50	22±4	257
(11)	20	4-18	16±2	261
(12)	40	8-36	13±1	279
(13)	58	13-46	16±2	277
(14)	76	18-56	11±2	256
(15)	112	28-76	19±13	259
(16)	148	38-96	34±8	272

= MSH (7); = (Pro-Gly)₃; = Pego linker

^aLigands **1–5** represent the control monovalent ligands which are similar in structure to the bivalent ligands **6–10**. Ligands **11–16** have the PEGO units incorporated into the linker structure for added flexibility. *n* indicates the total number of data points used to generate the binding curve and the resulting EC₅₀ value.

Table 2

Analytical Data for Synthetic Peptide Conjugates

compound	mass calculated ^a	mass found	<i>t_R</i> (purity %)	<i>K'</i>
1 (C ₆₇ H ₉₄ N ₂₀ O ₁₆)	718.3657 (M+2) ²⁺	718.3658(M+2) ²⁺	12.6 ^b (98.8)	18.5
2 (C ₈₈ H ₁₂₄ N ₂₆ O ₂₂)	633.3206 (M+3) ³⁺	633.3193(M+3) ³⁺	13.2 ^b (100)	11.35
3 (C ₁₀₉ H ₁₅₄ N ₃₂ O ₂₈)	787.3948(M+3) ³⁺	787.3972(M+3) ³⁺	13.4 ^b (98.4)	9.62
4 (C ₁₃₀ H ₁₈₄ N ₃₈ O ₃₄)	706.3538 (M+4) ⁴⁺	706.354(M+4) ⁴⁺	13.6 ^b (98.5)	11.42
5 (C ₁₅₁ H ₂₁₄ N ₄₄ O ₄₀)	821.9094 (M+4) ⁴⁺	821.9074(M+4) ⁴⁺	13.7 ^b (82.3)	11.58
6 (C ₁₃₁ H ₁₈₀ N ₃₈ O ₃₂)	700.3485(M+4) ⁴⁺	700.3455(M+4) ⁴⁺	22.6 ^c (100)	15.44
7 (C ₁₃₈ H ₁₉₀ N ₄₀ O ₃₄)	984.8201(M+3) ³⁺	984.8166(M+3) ³⁺	22.4 ^c (100)	17.62
8 (C ₁₅₉ H ₂₂₀ N ₄₆ O ₄₀)	683.7397(M+5) ⁵⁺	683.7428(M+5) ⁵⁺	22.3 ^c (100)	15.4
9 (C ₁₈₀ H ₂₅₀ N ₅₂ O ₄₆)	646.9882(M+6) ⁶⁺	646.9911(M+6) ⁶⁺	22.2 ^c (97.5)	19.94
10 (C ₂₀₁ H ₂₈₀ N ₅₈ O ₅₂)	724.0253(M+1) ⁶⁺	724.0284(M+1) ⁶⁺	22.2 ^c (100)	19.92
11 (C ₁₀₈ H ₁₅₃ N ₂₉ O ₂₇)	573.0451 (M+4) ⁴⁺	573.0441 (M+4) ⁴⁺	20.0 ^b (92.9)	9.43
12 (C ₁₂₂ H ₁₇₉ N ₃₁ O ₃₃)	869.7839(M+3) ³⁺	869.7818(M+3) ³⁺	20.4 ^b (83.7)	8.26
13 (C ₁₄₃ H ₂₀₉ N ₃₇ O ₃₉)	768.1455(M+4) ⁴⁺	768.1487(M+4) ⁴⁺	20.0 ^b (83.1)	8.27
14 (C ₁₆₆ H ₂₄₂ N ₄₄ O ₄₆)	897.9566(M+4) ⁴⁺	897.9605(M+4) ⁴⁺	22.6 ^c (100)	15.72
15 (C ₂₀₈ H ₃₀₂ N ₅₆ O ₅₈)	645.6136(M+7) ⁷⁺	645.6156(M+7) ⁷⁺	22.3 ^c (100)	17.44
16 (C ₂₅₀ H ₃₆₂ N ₆₈ O ₇₀)	680.5935(M+8) ⁸⁺	680.5965(M+8) ⁸⁺	22.2 ^c (100)	15.33

^aThe exact molecular mass of the structure, calculated using the atomic masses of the most common isotope for the element.^bLinear gradient of 10–40% B in 30 min.^cLinear gradient of 5–45% B in 30 min. 0.1% aqueous TFA (A) and CH₃CN (B).