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Novel targeting strategy based on multimeric ligands for drug delivery and molecular imaging: homooligomers of α-MSH

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Abstract—Homooligomers constructed with 4- and 6-amino acid fragments of melanocortin (α-MSH) bind with higher affinity and with apparent cooperativity to melanocortin receptor, compared to their constituent monomers. Individual ligands were tethered with various spacers of different length and rigidity and the influence of spacers on binding was studied. Binding assays were performed on cells transfected with the melanocortin receptor, hMC4R. There is a 5–7-fold decrease in the EC_{50} with the addition of each subunit, going from monomer to trimer. The Hill coefficient increases from 0.76 for the monomer to 1.12 for the dimer and 1.35 for the trimer. These data show a general trend of increasing avidity with increasing number of ligands in oligomers.

Multivalent interactions are characterized by the simultaneous binding of an entity that displays multiple copies of a molecular recognition element to multiple receptors contained on another entity. These multiple interactions enhance the binding of a multivalent molecule compared to the corresponding monovalent molecule. Multivalent ligands are important features of molecular recognition, as cells often encounter naturally occurring multiple arrays. The relevance of multivalent ligands as well as theoretical definitions of interactions (avidity, cooperativity, etc.) is well documented.[1] By combining multiple specific ligands into a single molecule it is possible to create compounds which will selectivity bind to cells bearing the appropriate mix of complementary receptors.[2]

Multimeric ligands have been developed to inhibit cell-surface–receptor interactions or to act as effectors of downstream signal transduction. A representative example of such inhibitors is pentavalent ligands that target the heat-labile enterotoxin LT-1. Five galactose residues are attached to a pentacyclen scaffold via a linker ranging in length from 32 to 83 atoms.[3] The best ligand displayed a 10^5-fold increase in IC_{50} compared to galactose. As another example, peptide ligands ([Nle[4], D-Phe[7]]α-MSH (NDP-MSH), dynorphin and substance P) were attached to polyvinylalcohol (PVA) using either disulphide or thioether linkages. The NDP-MSH agent (containing 10–15 copies) bound tightly and specifically to melanoma cell lines that displayed the corresponding melanocortin receptor. However, dynorphin and substance P agents did not bind to any cells.[4,5] Binding data indicated a significant hysteresis in binding kinetics with rapid on rates and immeasurably slow off rates. Such kinetics were ascribed to the phenomenon of cooperative affinity.[6]

The influence of linker length on binding affinity was investigated by Kessler et al.[7] using homodimers of somatostatin analogues. Dimers of cyclic D-Pro-Phe-Thr-Lys(*Z*)-Trp-Phe were tethered to hydrocarbons of different lengths, and dimers separated by 16–18 atom linkers displayed 150 times increased affinity over the monomer. As the linker length increased beyond this length, the apparent affinity of the dimers decreased. Insertion of a rigid acetylene moiety in linker 14 atoms in length increased affinity almost 100-fold in these dimers. Brandenburg et al.[8] synthesized peptidic oligomers containing up to six monomer units of NDP-MSH (4–10) conjugated through formation of oxime bonds to different templates. The observed affinity was 8 times higher than the native peptide and 62 times higher than monomeric $CH_3CH{=}N{-}O{-}CH_2{-}$CO-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2.

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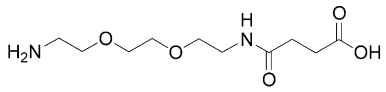
In this communication, we describe the stepwise, solid-phase synthesis and characterization of MSH-fragment homomultimers. The major advantage of stepwise synthesis lies in the flexibility provided for targeted design of multimeric ligands. Many features of such molecules may be fine-tuned after identification of parameters contributing to binding. Location of pharmacophores in multimeric ligands as well as the flexibility of scaffolds bearing these subunits may be modified systematically to generate multidimensional structure–activity relationships (SARs). The benefit of solid-phase synthesis is well controlled assembling of a single entity, and this will be especially important in the design of heteromeric ligands since conjugation methods lead always to ill-defined mixtures.

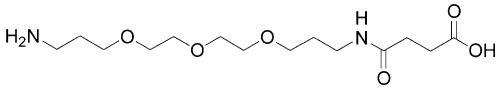
To initiate a systematic study of multimeric interactions, we have chosen to evaluate binding of α-melanocyte stimulating hormone (α-MSH) to the human melanocortin receptor. The biological functions and SAR of α-MSH analogues have been extensively researched and reviewed in our group.[8] Many potent analogues were investigated, and studies indicate that residues in the β-turn region 6–9 (His-Phe-Arg-Trp) are critical for agonist activity. By replacing the Met in position 4 with a Nle and the Phe in position 7 with a D-Phe, a potent α-MSH analogue was obtained (NDP-α-MSH, Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2).[9,10] We selected two analogues: the tetrapeptide Ac-His-D-Phe-Arg-Trp-NH_2 and the hexapeptide Ac-Nle-Glu-His-D-Phe-Arg-Trp-NH_2. These compounds should have low nanomolar binding activity and while still maintaining the opportunity to detect enhancement of avidity through oligomerization. We have also tried to keep the molecular weight of ligands as low as possible for eventual in vivo applications.

The structure of the spacer (indifferent connector of ligands) will play an important role in the orientation of the pharmacophores and must be chosen with care. The ideal spacer should not interfere with the binding affinities of the ligand to the receptor, and preferably should be both hydrophilic and small. As an obvious choice we included polyethylene glycol (PEG) spacers, combined with polyamides.[11] The spacer built from PEG complies with all requirements for an ideal molecule, except for its very high flexibility. Such a flexible construct would pay an entropic cost proportional to its flexibility and could erase the avidity effect of multimeric binding. We implemented PEG knowing its limitations because we have developed a solid-phase procedure for attachment of relatively small triethylene glycol units to the peptide backbone (Fig. 1, Scheme 1). The combination of this procedure with polyamide chains may serve as a tool for modular solid-phase synthesis and fine-tuning of spacer properties, especially flexibility.

The combinations of amino acids and PEG spacers allow us to build tethers of various length and rigidity. PEG spacers represent one side of the rigidity scale offering a highly flexible and hydrophilic tether. To decrease conformational mobility of the spacer, we implemented more rigid amide linkages built by alternation of β and α amino acids (Table 1, compounds **5**, **8**, and **11**). These linkers are more rigid than ethylene glycols, but more flexible than those containing constrained proline (compound **4**) or 4-aminomethylbenzoic acid (compounds **3**, **7**, and **10**). Trimers (A1, A2, A3) were constructed with an intervening lysine providing an *N*-linked bridge to the third ligand. Hence, linker lengths in Table 3 are expressed as atom lengths between A1–A2, A2–A3 and A1–A3.



PEG-Su-14



PEG-Su-19

Figure 1. Diethylene glycol succinimidyl and triethylene glycol succinimidyl units.

Table 1. Binding affinities of homodimers of α-MSH tetrapeptides Ac-His-DPhe-Arg-Trp-**Spacer**-His-DPhe-Arg-Trp-NH_2

Compd	Spacer[a]	Length[b]	EC_{50} (nM)	Hill coeff.
1	No spacer	0	264	0.9
2	βAla	4	119	1.39
3	AMB[c]	7	38	1.05
4	Pro-Gly-Pro	9	14	0.84
5	βAla-Gly-βAla	11	143	0.99
6	PEG-Su-14	14	234	1.10
7	$(AMB)_2$	14	123	1.12
8	$(βAla-Gly)_2$-βAla	18	231	1.09
9	PEG-Su-19	19	662	1.83
10	$(AMB)_3$	21	164	1.05
11	$(βAla-Gly)_3$-βAla	25	571	0.88

[a] Spacer is incorporated into peptide via amide bond.
[b] Length of spacer in atoms.
[c] 4-Aminobenzoic acid.

Table 2. Binding affinities of homodimers of α-MSH hexapeptides Ac-Nle-Glu-His-DPhe-Arg-Trp-**Spacer**-Nle-Glu-His-DPhe-Arg-Trp-NH_2

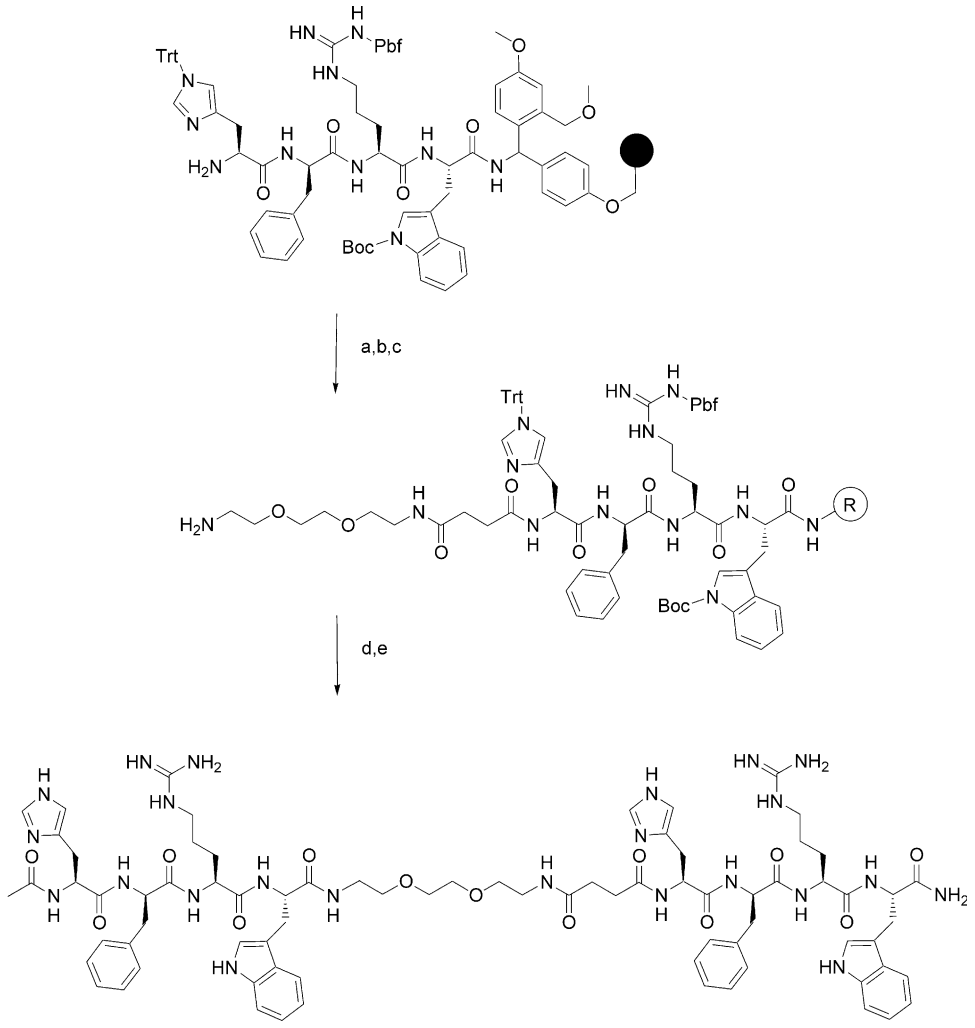
Compd	Spacer[a]	Length[b]	EC_{50} (nM)	Hill coeff.
12	βAla-Gly-βAla	11	7	0.91
13	PEG-Su-14	14	9	1.23
14	PEG-Su-19	19	33	1.59
15	$(PEG-Su-19)_2$	38	122	0.97

[a] Spacer is incorporated into peptide via amide bond.
[b] Length of spacer in atoms.

Table 3. Binding affinities of homotrimers of α-MSH tetrapeptides Ac-His-DPhe-Arg-Trp-**Spacer**-Lys(Ac-His-DPhe-Arg-Trp)-**Spacer**-His-DPhe-Arg-Trp-NH_2

Compd	Spacer[a]	Length[b]	EC_{50} (nM)	Hill coeff.
16	βAla	11-11-11	15	1.13
17	βAla-Gly-βAla	18-25-18	24	1.56

[a] Spacer is incorporated into peptide via amide bond.
[b] Length of spacer in atoms.



Scheme 1. The example of the incorporation of diethylene glycol unit into a growing peptide chain. Reagents: (a) succinic anhydride, DIEA, HOBt; (b) 1.0 M CDI in DMF; (c) 50% ($-CH_2OCH_2CH_2NH_2)_2$ [2,2′-(ethylenedioxy)bis(ethylamine)] in DMF, 24 h; (d) peptide synthesis followed by acetylation; (e) 91% TFA, 3% water, 3% thioanisole, 3% EDT, 5 h.

All ligands (Tables 1–3) were synthesized by standard solid-phase technology using a manual synthesizer (Torviq, Tucson, AZ, USA) with N^{α}-Fmoc/*tert*-butyl chemistry.[12] Polyethylene glycol parts were attached as shown in Scheme 1.

All compounds were purified by HPLC, then by SEC.[13] The purity of product was checked by thin-layer chromatography and HPLC.[14] Structures were characterized by ESI or MALDI-TOF.[15] Ligand binding was evaluated via competitive binding assays using ^{125}I-NDP-α-MSH as the competed ligand (Fig. 2). Binding assays were performed on Hek293 Human Embryonic Kidney cells transfected with hMC4R (human melanocortin receptor, subtype 4).[16]

As expected, the binding affinities of the hexapeptides were greater than those of the tetrapeptides due to the

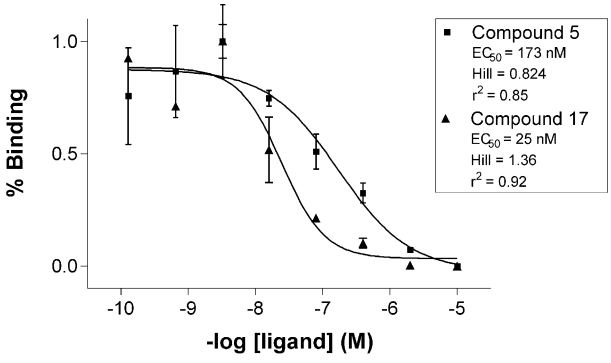


Figure 2. Representative binding curves for the competitive binding assay between ^{125}I-NDP-α-MSH and the NDP-α-MSH tetrapeptide dimer **5** and the trimer **17**. Data were fit using GraphPad Prism[17] (GraphPad Prism Software Inc., San Diego, CA, USA) using the sigmoidal dose–response (variable slope) classical equation for non-linear regression analysis. Each data point represents the normalized average of each assay run in duplicate.

increased number of binding resides. For the tetrapeptides, compound **4** displayed a low EC_{50} value, indicating that a rigid linker with length of **7** atoms is optimal for this set of dimers. The AMB family of linkers provides interesting results in terms of linker rigidity and its effects on ligand binding. Compounds **6** and **7** both have a linker length of 14 atoms, but compound **7**, with its more rigid AMB linker, has a binding affinity about 2 times greater than **6**, containing a flexible PEG linker. In agreement with this trend, compound **10**, with the longer AMB linker, has enhanced affinity compared with compounds **9** and **11** (both of similar lengths).

The enhanced binding affinity of the dimers and trimers is attributed to apparent cooperativity. Evaluation of the Hill coefficients resulting from ligand binding provides added evidence that our multimeric ligands bind with cooperative affinity. On average, the Hill coefficient for the tetrapeptide dimers is 1.12 while that for the trimers is 1.35. For the hexapeptide dimers, the average Hill coefficient is 1.18, while the average Hill coefficient for monomers was 0.78 (data not shown). The enhanced Hill coefficient that arises from the transition from monomer up to trimer suggests that additional ligands increase the probability that a ligand will find and bind to its corresponding receptor. The first binding event serves to tether the multimeric ligand to the surface of the cell, and a subsequent ligand will bind to another receptor. This occurs within the off rate of the first, tethering ligand, thus increasing the likelihood that another ligand of the multimer will bind to a receptor. The increased Hill coefficient therefore likely results not from actual cooperativity, but in part due to the increased local concentration of subsequent ligands at receptor sites.

In summary, we have synthesized and tested the binding affinities of a series of homodimeric and trimeric ligands containing truncated versions of NDP-α-MSH. Our results indicate that these ligands bind with enhanced affinity and apparent cooperativity compared to the monomers. Further optimization of linker length and rigidity will allow us to take full advantage of cooperative affinity and lead to a greater enhancement of binding, thus allowing the construction of multimeric ligands with enhanced selectivity for cells with complementary receptors.

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12. Peptides were synthesized by solid-phase technology using a manual synthesizer. The Rink resin was swollen in DMF for an hour. The resin was washed with DMF, and N^{α}-Fmoc protecting groups were removed with 50% piperidine in DMF (1 × 2 min and 1 × 20 min). The resin was washed again with DMF, 1.0 M HOBt in DMF, DMF, and the next N^{α}-Fmoc amino acid was coupled using preactivated 0.3 M HOBt ester in THF (3 equiv of N^{α}-Fmoc amino acid, 3 equiv of HOBt, and 3 equiv of DIC). Fmoc-Gly and Fmoc-His(Trt) need 50% THF/DMF solvent mixture to be dissolved. The resin slurry was stirred for 2 h or until Kaiser test became negative. If the test failed, the resin was washed with DMF and the amino acid was coupled again with HBTU/DIEA procedure (0.3 M solution of 3 equiv of N^{α}-Fmoc amino acid, 3 equiv of HBTU, and 6 equiv of DIEA in DMF) for 3 h. If the second coupling did not result in a negative Kaiser test, the resin was washed with DMF, and the rest of amino groups were capped with 50% acetic anhydride in pyridine for 10 min. When the coupling reaction was finished, the resin was washed with DMF, and the same procedure was repeated for the next amino acid until all the amino acids in the sequence were attached. After the last amino acid was incorporated, the Fmoc group was deprotected and the free amine groups was acetylated with 50% acetic anhydride in pyridine for 10 min, if needed. The resin was washed with DMF, THF and DCM. A cleavage mixture (10 mL per 1 g of the resin) consisting of trifluoroacetic acid (91%), water (3%), 1,2-ethandithiol (3%), and thioanisole (3%) was injected into the resin and the cleavage cocktail was stirred for 3 h at room temperature. The solution was filtered off, and the resin was washed with TFA (2×3 min), concentrated by a stream of nitrogen and the product was precipitated by cold ether. The peptide pellets were washed three times with cold ether, then lyophilized.
13. Purification of the compounds was achieved using a Hewlett-Packard 1100 series HPLC instrument with a reverse-phase column (Vydac, 10 mm × 220 mm, 10 μm, 300 Å). Peptides were eluted with a linear acetonitrile/0.1% aqueous TFA gradient at a flow rate of 3.0 mL/min. Separations were monitored at 230 and 280 nm with Hewlett-Packard 110 series UV detector and integrated with Hewlett-Packard 3396 series III integrator. The size exclusion chromatography (SEC) was performed on borosilicate glass column (2.6 × 50 cm, Sigma, St. Louis, MO, USA) filled with medium size Sephadex G-25

(Aldrich, Milwaukee, WI, USA). The compound was eluted with isocratic flow of 1.0 M aqueous acetic acid.
14. Thin-layer chromatography was performed in three different solvent systems and analytical reverse-phase C-18 HPLC using YMC ODS AM032 column (4.6 × 150 mm, 5 μm, 120 Å) at 220 and 280 nm. The compounds were eluted with a linear acetonitrile in 0.1% aqueous TFA gradient.
15. MALDI-TOF (Bruker Reflex-III, α-cyanocinnamic acid as a matrix) or ESI (Finnigan, Thermoquest LCQ ion trap instrument). For internal calibration an appropriate mixture of standard peptides was used with an average resolution of 8000–9000.
16. Cells were plated at a concentration of 100,000 cells/well in 24-well plates, and incubated for 48 h at 37 °C. On the assay day, cells were washed twice with 0.5 mL buffer (MEM, 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-Phenanthroline, 0.5 mg/L Leupeptin, 200 mg/L Bacitracin). All ligands were tested in duplicate in a total volume of 0.5 mL/well. Ligands were diluted in buffer and added to the wells resulting in final concentrations ranging from 1e-5 to 1.28e-10 M. ^{125}I-NDP-α-MSH (Perkin–Elmer, NEX352) was used as the competing ligand with 0.2 Ci (~0.017 nM, final concentration) being added to each well. Non-specific binding was tested in the presence of 10^{-4} M NDP-α-MSH. Cells were incubated in the presence of ligands at 37 °C for 40 min. Following the incubation, media was aspirated, cells were washed twice with 0.5 mL buffer, and lysed by addition of 0.4 mL 0.1 M NaOH and 0.4 mL of 1% Triton X-100. Cell lysates were transferred to 12×75 mm disposable culture tubes and radioactivity was counted using a Cobra II Auto-Gamma Counting System (Packard). Hill coefficients and EC_{50} values were determined using GraphPad Prism Software (GraphPad Software Inc., San Diego, CA, USA) using the sigmoidal dose–response (variable slope) classical equation for non-linear regression analysis. Data sets with an r^2 value <0.75 were excluded from further analysis. The EC_{50} values and Hill coefficients reported are an average of at least three separate binding assays all done in duplicate. One data point for compound **3** was excluded, as it was an obvious outlier with a Hill coefficient >8.
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