Bioorganic & Medicinal Chemistry Letters 20 (2010) 2489–2492

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and characterization of a Eu-DTPA-PEGO-MSH(4) derivative for evaluation of binding of multivalent molecules to melanocortin receptors

Liping Xu [a], Josef Vagner [b], Ramesh Alleti [c], Venkataramanarao Rao [c], Bhumasamudram Jagadish [c], David L. Morse [a], Victor J. Hruby [b,c], Robert J. Gillies [a], Eugene A. Mash [c,*]

[a] H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL 33612, USA
[b] The Bio5 Institute, University of Arizona, Tucson, AZ 85721-0240, USA
[c] Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ 85721-0041, USA

ARTICLE INFO

Article history:
Received 20 January 2010
Revised 26 February 2010
Accepted 1 March 2010
Available online 4 March 2010

Keywords:
Multimeric ligands
Human melanocortin 4 receptor
NDP-α-MSH
MSH(4)

ABSTRACT

A labeled variant of MSH(4), a tetrapeptide that binds to the human melanocortin 4 receptor (hMC4R) with low μM affinity, was prepared by solid-phase synthesis methods, purified, and characterized. The labeled ligand, Eu-DTPA-PEGO-His-DPhe-Arg-Trp-NH_2, exhibited a K_d for hMC4R of 9.1 ± 1.4 μM, approximately 10-fold lower affinity than the parental ligand. The labeled MSH(4) derivative was employed in a competitive binding assay to characterize the interactions of hMC4R with monovalent and divalent MSH(4) constructs derived from squalene. The results were compared with results from a similar assay that employed a more potent labeled ligand, Eu-DTPA-NDP-α-MSH. While results from the latter assay reflected only statistical effects, results from the former assay reflected a mixture of statistical, proximity, and/or cooperative binding effects.

Early detection of many human cancers would be facilitated by the availability of imaging agents that selectively bind to cancer cells and report their existence and location by noninvasive techniques.[1–4] Development of such imaging agents involves covalently linking reporter moieties and multiple copies of ligands to produce 'multivalent molecules' that will cooperatively bind to receptors expressed on the surface of cancer cells.[5–11] Multivalent molecules constructed from weakly binding ligands should display avidity for such cells when compared with the affinity of a single copy of the parental ligand.[5–16]

The affinity of a molecule for binding to a receptor is often quantified by a competitive binding assay against a labeled ligand of known potency. For example, labeled forms of NDP-α-MSH, a superpotent ligand that binds to human melanocortin receptors,[17,18] have been used for this purpose.[19,20] In such assays one often assumes thermodynamic control, that is, that the respective on-rates and off-rates of the competing ligands are similar. If this is not the case, details of how the assay is carried out (order and timing of reagent addition, timing of measurements taken) can affect the outcome. Determination of on-rates and off-rates for binding of multivalent molecules to living cells is difficult since labeled probes may be taken up by the cells and receptors may aggregate and cycle to and from the cell surface. In the absence of knowledge of ligand on-rates and off-rates, a close match between the affinity of the ligands used in construction of the multivalent molecule and the labeled ligand used as the probe in the competitive binding assay would seem prudent.

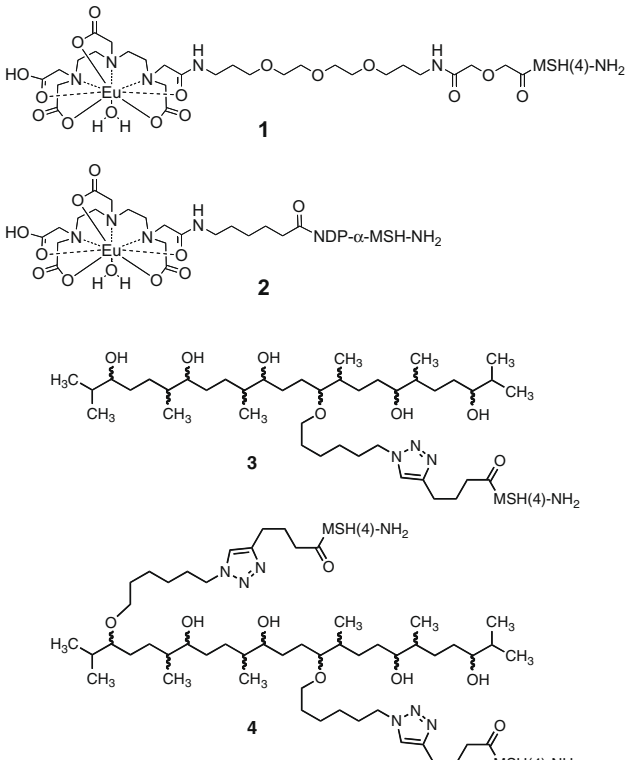
As we are currently investigating the behavior of multivalent molecules derived from the weakly binding ligand MSH(4),[21–23] we wished to prepare and test a probe based on this ligand for use in competitive binding assays. We report herein the synthesis of the Eu-DTPA labeled derivative **1**; studies of the binding affinity of **1** with the human melanocortin 4 receptor (hMC4R); and the use of **1** in a competitive binding assay involving previously reported monovalent and divalent MSH(4) constructs derived from squalene.[8]

*Synthesis of Eu-DTPA-PEGO-MSH(4) (**1**).* The synthesis of **1** is depicted in Scheme 1. MSH(4) was synthesized manually using an

Abbreviations: Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; DCM, dichloromethane; DIC, diisopropyl carbodiimide; DIEA, diisopropylethyl amine; DMEM, Dulbecco's Modified Eagle Medium; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; EC_{50}, effective concentration, 50%; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethyoxycarbonyl; FT-ICR MS, Fourier transform ion cyclotron resonance mass spectrometry; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; hMC4R, human melanocortin 4 receptor; HOBt, 1-hydroxybenzotriazole; MSH(4), His-DPhe-Arg-Trp; NDP-α-MSH, Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val; Pbf, 2,2,4,6,7-pentamethyldihydro-benzofuran-5-ylsulfonyl; PEGO, 19-amino-5-oxo-3,10,13,16-tetraoxa-6-azanonadecan-1-oic acid; *t*-Bu, *tert*-butyl; TEAA, triethylammonium acetate; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TRF, time-resolved fluorescence; Trt, triphenylmethyl.

* Corresponding author. Tel.: +1 520 621 6321; fax: +1 520 621 8407.
E-mail address: emash@u.arizona.edu (E.A. Mash).

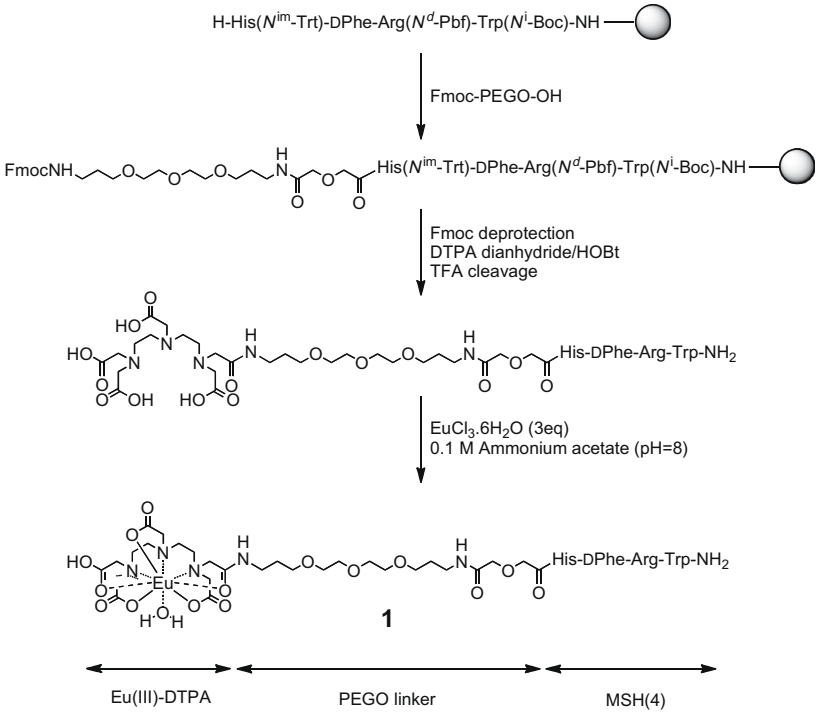
0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.03.007



N^{α}-Fmoc/*t*-Bu solid-phase peptide synthesis strategy and standard DIC/HOBt or HBTU/DIEA activations on Rink amide Tentagel resin.[24] Attachment of the PEGO linker was performed using DIC/HOBt activation (3 equiv Fmoc-PEGO, 3 equiv of HOBt, and 3 equiv of DIC). Next, the DTPA chelator was attached to the N-terminus of the resin-bound PEGO-MSH(4) construct as follows. After Fmoc removal, the resin was washed with DMSO. DTPA dianhydride (10 equiv) and HOBt (30 equiv) were dissolved in dry DMSO (1 mL) at 50 °C and then stirred for 20 min at room temperature. This mixture was injected into the syringe reactor which was shaken overnight, then the resin washed with DMSO, THF, 20% aqueous THF, THF, 5% DIEA in THF (5 min), THF, DMF, THF, and DCM. A cleavage mixture (10 mL/g of resin) consisting of trifluoroacetic acid (91%), water (3%), 1,2-ethanedithiol (3%), and thioanisole (3%) was injected into the syringe reactor containing the resin and the mixture shaken for 4 h at room temperature. The solution was then filtered off, and the resin was washed with TFA (2×3 min). Filtrates were collected and concentrated under a stream of nitrogen, and the product was precipitated by addition of cold ether to the residue. The peptide pellet was washed three times with cold ether, dried, dissolved in 1.0 M acetic acid, and lyophilized. The lyophilized DTPA-PEGO-MSH(4) construct was purified by preparative HPLC and characterized by analytical HPLC and FT-ICR MS.[25]

The metal-free precursor to **1** was dissolved in 0.1 M ammonium acetate, the pH was adjusted to 8 with aqueous 0.1 M NH_4OH, and 3 equiv of $EuCl_3{\cdot}6H_2O$ in water were added. The reaction mixture was stirred at room temperature overnight. The excess $EuCl_3$ and ammonium salts were removed using a SEPAC C-18 reverse-phase column with repetitive washing (20 mL of HPLC grade water). The final product was eluted using 50% aqueous acetonitrile (4 mL), concentrated, lyophilized, and characterized by analytical HPLC and FT-ICR MS. Data appear in Table 1.

*Saturation binding of **1** to hMC4R.* Hek293 cells overexpressing hMC4R were used to assess ligand binding. Quantitative receptor-binding assays were carried out following a previously described method.[19] Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Cells were seeded in a 96-well plate at a density of 15,000 cells per well and were allowed to reach 80–90% confluence. On the day of the experiment, media was aspirated from all wells. Probe **1** diluted in binding buffer (DMEM, 1 mM 1,10-phenanthroline, 200 mg/L bacitracin, 0.5 mg/L leupeptin, 0.3 BSA) was added to the wells to result in fi-



Scheme 1. Synthesis of Eu-DTPA-PEGO-MSH(4) (**1**).

Table 1
FT-ICR MS and HPLC characterization of compound **1**

Formula	Calculated masses [ion]	Masses found (error)	t_R[a]
$C_{60}H_{85}N_{16}O_{19}{}^{151}Eu$	743.27604 $[M+2]^{2+}$	743.27683 (1.1 ppm)	11.36
$C_{60}H_{85}N_{16}O_{19}{}^{153}Eu$	744.27718 $[M+2]^{2+}$	744.27971 (3.4 ppm)	

[a] Analyzed on a 3 × 150 mm 3.5 Å Waters C18 XBridge column, flow rate 0.3 mL/min, linear gradient from 10% to 60% B in A over 30 min, where A is 0.1% TEAA in water and B is 90% acetonitrile and 10% A, detection at 220 and 280 nm. Purity >95%.

Table 2
Binding constants for Eu-DTPA-PEGO-MSH(4) (**1**) and Eu-DTPA-NDP-α-MSH (**2**) with hMC4R

Compounds	K_d	n[a]
1	9.1 ± 1.4 μM	4
2	18.8 ± 1.7 nM	(Ref. 19)

[a] The value given is the average of n independent binding experiments, each done in quadruplicate.

Table 3
Competitive binding of MSH(4), **3**, and **4** to hMC4R

Compounds	Probe **1** K_i[a]	Probe **2** K_i[b]
MSH(4)	0.76 ± 0.03 μM (n = 4)	0.72 ± 0.09 μM (n = 4)
3	5.0 ± 0.95 μM (n = 4)	0.85 ± 0.13 μM (n = 5)
4	0.27 ± 0.02 μM (n = 4)	0.27 ± 0.02 μM (n = 4)

[a] The K_i was calculated using the equation $K_i = EC_{50}/(1 + ([ligand]/K_d))$, where [ligand] refers to the concentration of probe **1** used as the labeled competed ligand (0.5 μM).
[b] Here the [ligand] refers to the concentration of probe **2** (10 nM).

nal concentrations ranging from 2 to 30 μM. In wells used to test nonspecific binding, cells were incubated with various concentrations of probe **1** in the presence of 100 μM of unlabeled MSH(4) at 37 °C for 1 h. The cells were washed with wash buffer, enhancement solution was added, and fluorescence measured on a Wallac VICTOR[3] instrument using standard Eu TRF measurement conditions (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm). Saturation curves were analyzed with GraphPad Prism software using the one site binding (hyperbola) classical equation for nonlinear regression analysis.

As shown in Table 2, compound **1** possessed a lower binding affinity (K_d = 9.1 ± 1.4 μM) at hMC4R when compared with compound **2** (K_d = 18.8 ± 1.7 nM),[19] well within the expected range in a saturation binding assay for an MSH(4)-based fluorescent probe.

*Binding assays using **1** and **2**.* Eu-DPTA-PEGO-MSH(4) (**1**) and Eu-DTPA-NDP-α-MSH (**2**) were each used as the probe in a previously described competitive binding assay[19] to characterize the interactions of the monovalent and divalent constructs **3** and **4** with hMC4R.[†]

In brief, Hek293 cells overexpressing hMC4R (6 × 10^5 receptors/cell on the cell surface) were plated in a 96-well plate. On the day of the assay, the cells had reached 80–90% confluence. Media was aspirated from all wells, and 50 μL of the compounds to be tested (dilutions ranging from 2 × 10^{-4} to 1 × 10^{-11} M) and 50 μL of Eu-labeled ligand (probe **1**, 0.5 μM or probe **2**, 10 nM) were added to each well. Cells were incubated in the presence of unlabeled and labeled ligands at 37 °C for 1 h. The cells were washed with wash buffer, enhancement solution was added, and fluorescence measured as described above. Competitive binding data were analyzed by nonlinear regression analysis using GraphPad Prism software and fitted to a classic one site binding competition equation. Results are given in Table 3.

Using probe **1**, the K_i values for compounds **3** [scaffold + 1 × MSH(4)] and **4** [scaffold + 2 × MSH(4)] were approximately sevenfold higher and threefold lower, respectively, than the values for the parental MSH(4) ligand. These results indicate that attachment of MSH(4) to the squalene-derived scaffold had a modest detrimental effect on monovalent ligand binding, and that some combination of statistical, proximity, and/or cooperative effects resulted in enhancement of affinity in the divalent ligand, despite attachment to the scaffold. The K_i for **4** relative to **3** showed an 18-fold enhancement.

Using probe **2**, the K_i values for compounds **3** and **4** were approximately equal to and threefold lower than the values for the parental MSH(4) ligand, respectively. The latter result is consistent with the prior study[8] and with statistical probability. However, compound **3** exhibited significantly greater affinity for hMC4R than was previously reported.[8] The reason for this difference is unknown. The current results using the high affinity competing probe **2** indicate that, to the extent that an MSH(4) ligand can compete with **2** for hMC4R, ligand bound to scaffold is as potent as the parental ligand.

This study has demonstrated that compound **1** binds specifically to hMC4R and is useful as a fluorescent probe in assays of competitive binding at that receptor. Probe **1** is easier and less expensive to prepare than is probe **2**. In addition, probe **1** appears to be more sensitive than probe **2** to the binding of low affinity ligands to hMC4 receptors. Characterizations of the interactions of other multivalent MSH(4) constructs with melanocortin receptors using probes **1** and **2** are in progress.

Acknowledgments

The authors thank Renata Patek for laboratory assistance. This work was supported by Grants R33 CA 95944, RO1 CA 97360, RO1 CA 123547, and P30 CA 23074 from the National Cancer Institute.

References and notes

1. Weissleder, R.; Mahmood, U. *Radiology* **2001**, *219*, 316.
2. Gillies, R. J.; Hruby, V. J. *Expert Opin. Ther. Targets* **2003**, *7*, 137.
3. Gillies, R. J.; Hoffman, J. M.; Lam, K. S.; Menkens, A. E.; Piwnica-Worms, D. R.; Sullivan, D. C.; Weissleder, R. *Mol. Imaging* **2005**, *4*, 98.
4. Cassidy, P. J.; Radda, G. K. *J. R. Soc. Interface* **2005**, *2*, 133.
5. Handl, H. L.; Vagner, J.; Han, H.; Mash, E.; Hruby, V. J.; Gillies, R. J. *Expert Opin. Ther. Targets* **2004**, *8*, 565.
6. Vagner, J.; Handl, H. L.; Monguchi, Y.; Jana, U.; Begay, L. J.; Mash, E. A.; Hruby, V. J.; Gillies, R. J. *Bioconjugate Chem.* **2006**, *17*, 1545.
7. Bowen, M. E.; Monguchi, Y.; Sankaranarayanan, R.; Vagner, J.; Begay, L. J.; Xu, L.; Jagadish, B.; Hruby, V. J.; Gillies, R. J.; Mash, E. A. *J. Org. Chem.* **2007**, *72*, 1675.
8. Jagadish, B.; Sankaranarayanan, R.; Xu, L.; Richards, R.; Vagner, J.; Hruby, V. J.; Gillies, R. J.; Mash, E. A. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3310.
9. Handl, H. L.; Sankaranarayanan, R.; Josan, J. S.; Vagner, J.; Mash, E. A.; Gillies, R. J.; Hruby, V. J. *Bioconjugate Chem.* **2007**, *18*, 1101.
10. Vagner, J.; Xu, L.; Handl, H. L.; Josan, J. S.; Morse, D. L.; Mash, E. A.; Gillies, R. J.; Hruby, V. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 1685.
11. Xu, L.; Vagner, J.; Josan, J. S.; Lynch, R. M.; Morse, D. L.; Baggett, B.; Han, H.; Mash, E. A.; Hruby, V. J.; Gillies, R. J. *Mol. Cancer Ther.* **2009**, *8*, 2356.
12. Mammen, M.; Chio, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754.
13. Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E. *Angew. Chem., Int. Ed.* **2006**, *45*, 2348.
14. Carlson, C. B.; Mowery, P.; Owen, R. M.; Dykhuizen, E. C.; Kiessling, L. L. *ACS Chem. Biol.* **2007**, *2*, 119.
15. Trouche, N.; Wieckowski, S.; Sun, W.; Chaloin, O.; Hoebeke, J.; Fournel, S.; Guichard, G. *J. Am. Chem. Soc.* **2007**, *129*, 13480.
16. Diestler, D. J.; Knapp, E. W. *Phys. Rev. Lett.* **2008**, *100*, 178101(4).
17. For the 'high-affinity' ligand NDP-α-MSH (Ser-Tyr-Ser-Nle-Glu-His-ᴅPhe-Arg-Trp-Gly-Lys-Pro-Val) see: Sawyer, T. K.; Sanfilippo, P. J.; Hruby, V. J.; Engel, M.

[†] Compounds **3** and **4** are mixtures of stereoisomers and regioisomers; the sites of ligand attachment shown are arbitrary; see Ref. 8.

H.; Heward, C. B.; Burnett, J. B.; Hadley, M. E. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5754.
18. Hadley, M. E.; Anderson, B.; Heward, C. B.; Sawyer, T. K.; Hruby, V. J. *Science* **1981**, *213*, 1025.
19. Handl, H. L.; Vagner, J.; Yamamura, H. I.; Hruby, V. J.; Gillies, R. J. *Anal. Biochem.* **2004**, *330*, 242.
20. De Silva, C. R.; Vagner, J.; Lynch, R. M.; Gillies, R. J.; Hruby, V. J. *Anal. Biochem.* **2010**, *398*, 15.
21. For the 'low-affinity' ligand MSH(4) (His-D-Phe-Arg-Trp) see: Hruby, V. J.; Wilkes, B. C.; Hadley, M. E.; Al-Obeidi, F.; Sawyer, T. K.; Staples, D. J.; de Vaux, A. E.; Dym, O.; de Lauro Castrucci, A. M.; Hintz, M. F.; Riehm, J. P.; Rao, K. R. *J. Med. Chem.* **1987**, *30*, 2126.
22. Castrucci, A. M. L.; Hadley, M. E.; Sawyer, T. K.; Wilkes, B. C.; Al-Obiedi, F.; Staples, D. J.; de Vaux, A. E.; Dym, O.; Hintz, M. F.; Riehm, J. P.; Rao, K. R.; Hruby, V. J. *Gen. Comp. Endocrinol.* **1989**, *73*, 157.
23. Haskell-Luevano, C.; Hendrata, S.; North, C.; Sawyer, T. K.; Hadley, M. E.; Hruby, V. J.; Dickinson, C.; Gantz, I. *J. Med. Chem.* **1997**, *40*, 2133.
24. The Rink resin (0.23 mmol/g) was swollen in DMF for 1 h, washed with DMF, and the N^{α}-Fmoc protecting groups were removed using 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). The resin slurry was shaken for 2 h or until a Kaiser test became negative. If the test failed, the resin was washed with DMF and the amino acid was coupled again using the 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 remaining amino groups were capped using 50% acetic anhydride in pyridine for 10 min. When the coupling reaction was finished, the resin was washed with DMF, and the coupling procedure repeated for the next amino acid until all the amino acids in the sequence were attached.
25. DTPA-PEGO-MSH(4) was purified on a 22 × 250 mm 10–22 Å Vydac 218TP1022 column, flow rate 5 mL/min, linear gradient from 0% to 30% B in A over 30 min, where A is 0.1% TFA in water and B is 0.08% TFA in acetonitrile, detection at 230 nm, and analyzed on a 3 × 150 mm 3.5 Å Waters C18 XBridge column, flow rate 0.3 mL/min, linear gradient from 10% to 60% B in A over 30 min, where A is 0.1% TFA in water and B is 0.08% TFA in acetonitrile, detection at 220 nm, t_R = 12.06 min, purity >95%. ESI-MS: calculated $[M+2]^{2+}$ 669.3; found 669.2.