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Synthesis and Characterization of Time-resolved Fluorescence Probes for Evaluation of Competitive Binding to Melanocortin Receptors

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ABSTRACT

Probes for use in time-resolved fluorescence competitive binding assays at melanocortin receptors based on the parental ligands MSH(4), MSH(7), and NDP-α-MSH were prepared by solid phase synthesis methods, purified, and characterized. The saturation binding of these probes was studied using HEK-293 cells engineered to overexpress the human melanocortin 4 receptor (hMC4R) as well as the human cholecystokinin 2 receptor (hCCK2R). The ratios of non-specific binding to total binding approached unity at high concentrations for each probe. At low probe concentrations, receptor-mediated binding and uptake was discernable, and so probe concentrations were kept as low as possible in determining K_d values. The Eu-DTPA-PEGO-MSH(4) probe exhibited low specific binding relative to non-specific binding, even at low nanomolar concentrations, and was deemed unsuitable for use in competition binding assays. The Eu-DTPA-PEGO probes based on MSH(7) and NDP-α-MSH exhibited K_d values of 27 ± 3.9 nM and 4.2 ± 0.48 nM, respectively, for binding with hMC4R. These probes were employed in competitive binding assays to characterize the interactions of hMC4R with monovalent and divalent MSH(4), MSH(7), and NDP-α-MSH constructs derived from squalene. Results from assays with both probes reflected only statistical enhancements, suggesting improper ligand spacing on the squalene scaffold for the divalent constructs. The K_i values from competitive binding assays that employed the MSH(7)-based probe were generally lower than the K_i values obtained when the probe based on NDP-α-MSH was employed, which is consistent with the greater potency of the latter probe. The probe based on MSH(7) was also competed with monovalent, divalent, and trivalent MSH(4) constructs that previously demonstrated multivalent binding in competitive binding assays against a variant of the probe based on NDP-α-MSH. Results from these assays confirm multivalent binding, but suggest a more modest increase in avidity for these MSH(4) constructs than was previously reported.

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1. Introduction

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 Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 [NDP-α-MSH], a superpotent ligand that binds to melanocortin receptors,[1,2] have been used for this purpose.[3,4] In such assays, one often assumes thermodynamic control, i.e., that the respective on-rates and off-rates of both the competing and competed ligands are similar so that all bound and unbound states are in equilibrium. 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 molecules to living cells is difficult since ligands and labeled

Abbreviations: BSA, bovine serum albumin; Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; CuAAC, copper(I)-catalyzed azide-alkyne cycloaddition; DCM, dichloromethane; DIC, diisopropyl carbodiimide; DIEA, diisopropylethylamine; DMEM, Dulbecco's Modified Eagle Medium; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; IC_{50}, half maximal inhibitory concentration; ESI MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethyoxycarbonyl; Fmoc-PEGO, 1-(9*H*-fluoren-9-yl)-3,19-dioxo-2,8,11,14,21-pentaoxa-4,18-diazatricosan-23-oic acid; FT-ICR MS, Fourier transform ion cyclotron resonance mass spectrometry; hMC4R, human melanocortin 4 receptor; HOBt, 1-hydroxybenzotriazole; HRMS, high resolution mass spectroscopy; MEM, Minimum Essential Medium; MSH(4), His-DPhe-Arg-Trp; MSH(7), Ser-Nle-Glu-His-DPhe-Arg-Trp; NDP-α-MSH, Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2; PEGO, 19-amino-5-oxo-3,10,13,16-tetraoxa-6-azanonadecan-1-oic acid; TBTA, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine; TEAA, triethylammonium acetate; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; TRF, time-resolved fluorescence.

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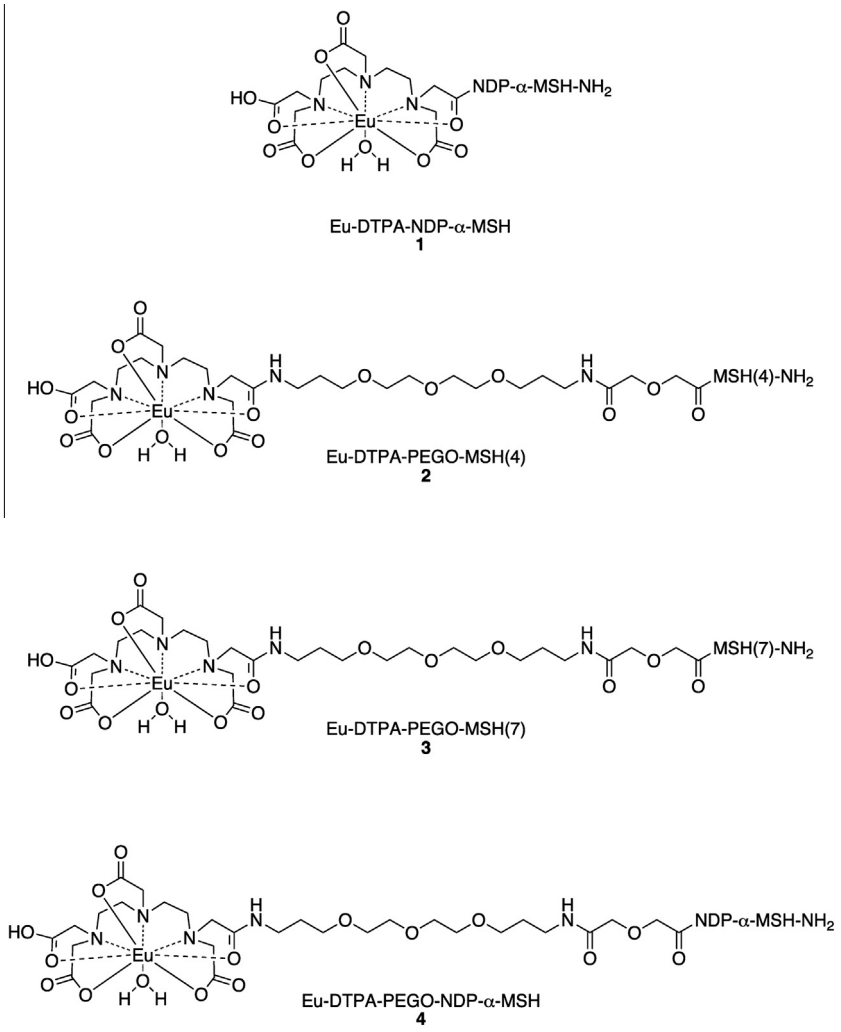
probes may be taken up by the cells and because receptors may cycle to and from the cell surface. In the absence of knowledge of ligand and probe on-rates and off-rates, a close match between the affinities of the competed probe and the competing ligand in a competitive binding assay would seem prudent—but is it necessary? This issue was examined experimentally as reported herein.

We have investigated the binding of multivalent molecules, including several derived from the weak ligand Ac-His-DPhe-Arg-Trp-NH_2 [MSH(4)][5–7], to human melanocortin 4 receptors (hMC4R).[8] MSH(4) was selected because synergistic effects are generally more easily detected for multivalent constructs of low-affinity ligands.[9–13] Probe **1** is based on NDP-α-MSH and was used to determine the K_i values for many of our multivalent constructs. However, we became concerned that competitions between superpotent probes such as **1** and multivalent constructs based on much weaker ligands such as MSH(4) were inherently unbalanced, and that perhaps the measured avidity of a competing multivalent construct depended on the affinity of the competed fluorescent probe. In a preliminary study, we prepared the Eu-DTPA-PEGO-MSH(4) probe **2** and tested it in saturation and competitive binding assays.[14] The K_d of **2**, determined by saturation binding to HEK-293 cells overexpressing hMC4R, was 9.1 μM, compared with a reported K_d for **1** of 8.3 nM.[15] We report herein syntheses of the structurally related probes Eu-DTPA-PEGO-MSH(7) **3** and Eu-DTPA-PEGO-NDP-α-MSH **4**, studies of the saturation binding of **2–4** with hMC4R, and the use of **3** and **4** in competitive binding assays involving monovalent and divalent MSH(4), MSH(7), and NDP-α-MSH constructs derived from squalene[16], as well as monovalent, divalent, and trivalent MSH(4) constructs that previously exhibited multivalent binding in competitive binding assays against a variant of probe **4**.[17]



2. Materials and methods

2.1. Chemical synthesis

2.1.1. General experimental

Dichloromethane (DCM), diethyl ether, and tetrahydrofuran (THF) were dried by passage through activated alumina. Other solvents and commercial reagents were used as supplied. For moisture sensitive reactions, glassware was flame-dried under argon. Analytical thin-layer chromatography (TLC) was carried out on

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