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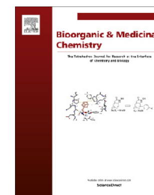

Synthesis and evaluation of bivalent ligands for binding to the human melanocortin-4 receptor

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ABSTRACT

Membrane proteins, especially G-protein coupled receptors (GPCRs), are interesting and important theragnostic targets since many of them serve in intracellular signaling critical for all aspects of health and disease. The potential utility of designed bivalent ligands as targeting agents for cancer diagnosis and/or therapy can be evaluated by determining their binding to the corresponding receptors. As proof of concept, GPCR cell surface proteins are shown to be targeted specifically using multivalent ligands. We designed, synthesized, and tested a series of bivalent ligands targeting the over-expressed human melanocortin 4 receptor (hMC4R) in human embryonic kidney (HEK) 293 cells. Based on our data suggesting an optimal linker length of 25 ± 10 Å inferred from the bivalent melanocyte stimulating hormone (MSH) agonist, the truncated heptapeptide, referred to as **MSH(7)**: Ac-Ser-Nle-Glu-His-*D*-Phe-Arg-Trp-NH_2 was used to construct a set of bivalent ligands incorporating a hMC4R antagonist, **SHU9119**: Ac-Nle-$_c$[Asp-His-2′-*D*-Nal-Arg-Trp-Lys]-NH_2 and another set of bivalent ligands containing the **SHU9119** antagonist pharmacophore on both side of the optimized linkers. These two binding motifs within the bivalent constructs were conjoined by semi-rigid $(Pro-Gly)_3$ units with or without the flexible poly(ethylene glycol) (PEGO) moieties. Lanthanide-based competitive binding assays showed bivalent ligands binds to the hMC4R with up to 240-fold higher affinity than the corresponding linked monovalent ligands.

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1. Introduction

Cancer is in its most aggressive state when it has metastasized to the entire body. Hence early detection is critical to the successful treatment of many human cancers. Therapies to treat cancer must selectively target these invading cells within healthy normal tissues. It is accepted that metastatic cancers include multiple genetic abnormalities that are currently targets for many bio-pharmaceutical companies. Most current drug therapies are not molecularly specific and are associated with side-effects and toxicities. There is a possibility of individually developing single molecules containing multiple pharmacophores for specific overexpressed proteins on a cancer cell that may have a differentially reduced expression on the normal cell.[1] These resulting multivalent molecules could display enhanced affinity for the targeted cells.[2–4] Targeting cell surface receptors can help inhibit cell surface receptor-ligand interactions or act as positive/negative effectors of downstream signal transduction. Multimeric ligands that contain a target specific agonist or antagonist pharmacophore can take toxic payloads directly into the tumors, destroying the cancer cells, while leaving the normal cells unharmed. An imaging agent on the multimeric ligand will be guided as a single molecule that will potently bind and image the infected area (overexpressed receptors) and can be used in non-invasive techniques for cancer detection, for example, early detection of adenomatous polyps in colon cancer.

The common feature of the bivalent ligand binding is that following the initial binding of one pharmacophore within a bivalent

Abbreviations: Ac, acetyl; ACTH, adrenocorticotropic hormone; Alloc, allyloxycarbonyl; Boc, *t*-butoxycarbonyl; BSA, bovine serum albumin; DIC, *N*,*N*′,-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMEM, Dulbecco's Modified Eagle Medium; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; EDT, ethanedithiol; Fmoc, 9-fluorenylcarboxy; HCTU, 2-(6-chloro-1H-benzotrialzole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; HEK, human embryonic kidney; HOBt, *N*-hydroxybenzotriazole; hMC4R, human melanocortin 4 receptor; MSH, melanocyte stimulating hormone; Nal, naphthylalanine; Pbf, 2,2,4,6,7-pentamethlydihydro-benzofuran-5-sulfonyl; PEGO, poly(ethylene glycol); RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; Trt, trityl.

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construct, succeeding binding is more favorable thanks to decreased loss of entropy.[5] As the result, the bivalent ligands can enhance binding affinity, agonist/antagonist potency and GPCR subtype selectivity. The central dogma of GPCR pharmacology has been the concept that unlike agonists, antagonist ligands display equivalent affinities for a given receptor, regardless of the cellular environment in which the affinity is assayed.[6]

As a proof of the concept, we chose to mimic the cancer cells through overexpression of MC4 receptor, which is one of five types of melanocortin receptors termed MC1–5R that exhibit about 40–60% homology.[7] Among the melanocortin receptors, the MC4R is of particular interest and a potential target in the research study as a major regulator of eating behavior and body weight, and suggestions have been made towards its role in the stimulation of male erectile activity.[8,9] Synthetically low molecular weight agonists and antagonists selective for the different subtypes are highly warranted as remedies for treatment of various dysfunctional states, such as obesity, anorexia, impotence, and autoimmune disorders.[10] Studies have suggested that bivalent ligands have receptor binding properties that differ substantially from those of the monovalent ligand, and the spacer used to link the two pharmacophores within the construct exerts a profound influence on the potency. The bivalent ACTH antagonists show potency enhancements up to approximately 25 times that of the monovalent constructs also demonstrating the role of spacer [bis(maleimide)cross-linking] and a peptide pharmacophore component within a bivalent construct.[11] Handl et al. demonstrated the potential of a series of MSH-7 agonist homobivalent ligands compared to its monovalent construct that can be utilized as targeting agents for cancer imaging.[3] The homobivalent ligands binds to hMC4R with increased affinity and apparent co-operativity compared to their monovalent analogues.[3] The increased binding affinity and positive cooperativity were most likely not due to statistical binding, but rather to a receptor clustering mechanism, wherein multiple receptors are bound by the same multivalent ligand.[12] In this study, we used a combination of agonist and antagonist pharmacophores in the design of bivalent ligands and the results could help determine organizational features of the melanocortin receptor-GPCR. We chose to construct ligands containing one copy of **MSH(7)**, a truncated version of [Nle^4-*D*-Phe^7]-α-melanocyte stimulating hormone (NDP-α-MSH) and a very potent cyclic MC4R antagonist **SHU9119**.[13] These two MC4R pharmacophores were separated by a series of linkers, which are different in flexibility and length. Poly(ethylene glycol) (PEGO) and (Pro-Gly)$_3$ units were used either by themselves or by incorporations, as shown in Table 1.

It has been proposed that the first pharmacophore binding event serves to attach the multivalent ligand to the surface, here we have evaluated the use of a tight binding pharmacophore **SHU9119** in combination with a comparatively lower binding pharmacophore, **MSH(7)**.[14,15] We proposed that there would be effectively an additive enhancement of binding compared to homobivalent **MSH(7)** analogues, which we have shown in a previous publication, as the pharmacophore should bind to the receptor tightly and then linkers should provide greater opportunity for the bivalent ligand to explore more volume and thus have a higher probability to bind multiple receptors at once, hence making them capable of cross-linking adjacent receptors.[3]

2. Results and discussion

2.1. Synthesis

As shown in Figure 1, bivalent ligands **7**–**12** and **13**–**18** consisting of two **SHU9119** moieties and **MSH(7)** and **SHU9119**, respectively, with PEGO and/or (Pro-Gly)$_3$ linkers were synthesized by standard solid phase synthesis using Fmoc-chemistry successfully. Monovalent ligands **1**–**6** were also prepared as control ligands by the same procedure.

The cyclized heptapeptide **SHU9119** was constructed on Rink amide Tentagel S resin and PEGO linkers were attached to the resin. The PEGO attached resin was proportionally split for syntheses of control monovalent ligands **4**–**6**, bivalent ligands **10**–**12**, and **16**–**18** (Fig. 2). For the synthesis of ligands **11** and **12**, the split resin was coupled with Fmoc-Lys(Alloc)-OH and the solid phase peptide synthesis continued to complete the second **SHU9119** sequence. Subsequently, part of the split resin was coupled with Fmoc-amino acids stepwise to attach the **MSH(7)** moiety for ligands **17**–**18**. Attachment of **SHU9119** or **MSH(7)** moiety in bivalent or monovalent ligands were performed routinely without difficulty by the procedures as mentioned above. Another portion of the resin was used to connect with (Pro-Gly)$_3$ and PEGO linkers and **SHU9119** or **MSH(7)** was attached to the resulting resin to afford longer length bivalent ligands **10** and **16**. The resin was previously split into three syringe reactor portions for the attachment of Pro-Gly linkers, giving monovalent ligand resins **1**–**3**. The attachment of the second moiety **SHU9119** or **MSH(7)** was carried out, affording **7**–**9** or **13**–**15**, respectively. The Fmoc-groups on all of the resin precursors were deprotected, and peptides were acylated, and cleaved by a mixture of TFA, EDT, thioanisole, and water (91/3/3/3) that afforded the desired ligands **1**–**18** as shown in Table 1. Ligands **1**–**18** were purified by preparative RP-HPLC and were characterized by ESI-MS and/or MALDI-TOF MS to confirm their structures.

2.2. Binding of monovalent and bivalent ligands

The binding affinities were evaluated in a lanthanide based competitive assay (Dissociation Enhanced Lanthanide FluoroImmuno Assay: DELFIA) using optimized 10 nM standard agonist Eu-DTPA-NDP-α-MSH chelate in HEK293 cells overexpressing hMC4R. As shown in Table 2, EC_{50} values were calculated after computing the hill slope using the GraphPad Prism software and compared with ligands **MSH(7)** with EC_{50} ~50 nM and **SHU9119** with EC_{50} ~60 pM; these binding affinities were consistent with the ones obtained from previously published data using radiolabeled binding assay.[16]

The conjugation of the linkers to the monovalent antagonist-**SHU9119** reduced its high affinity (EC_{50} = 59 pM) up to 400 nM by 6800-fold in ligand **6**. As the different lengths of linkers were attached to the antagonist pharmacophore, there was a trend observed in binding to the receptor. Apparently, the analogues containing the semi rigid (Pro-Gly)$_3$ linker retained higher binding affinity than those with the flexible linker PEGO, and longer length of PEGO linker resulted in the loss of binding affinity (EC_{50} = 400 nM) in ligand **6**. However, the low affinity was reversed to high affinity (EC_{50} = 3.7 nM, 108-fold) by the insertion of a semi rigid linker (Pro-Gly)$_3$ in ligand **4.** Ligand **1** with a shorter length of linker (10–20 Å) showed higher binding affinity (EC_{50} = 4.0 nM) than ligands **2** (EC_{50} = 8.1 nM) and **3** (EC_{50} = 13 nM) with a longer length of linker. This is coincident with our previous result showing linker effects on the binding affinities of **MSH(7)** monovalent ligands.[3] It is clear that the attachment of a semi rigid (Pro-Gly)$_3$ linker, which may assist in reducing entropy of the ligand, results in the high binding affinity of the monovalent ligands.

Contrary to the effect of a linker on monovalent ligands **1**–**6**, there was no such clear effect observed on the bivalent ligands **8**–**18**. Interestingly, bivalent ligands **12** and **18**, which contain a flexible longer linker PEGO-PEGO like monovalent ligand **6** (EC_{50} = 400 nM) retained the same high binding affinities (EC_{50} = 1.9 nM and 1.7 nM, respectively) as the other bivalent ligands. The increases of binding affinities in **12** and **18** were more

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