

## Enhanced targeting with heterobivalent ligands

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### Abstract

**A novel approach to specifically target tumor cells for detection and treatment is the proposed use of heteromultivalent ligands, which are designed to interact with, and noncovalently crosslink, multiple different cell surface receptors. Although enhanced binding has been shown for synthetic homomultivalent ligands, proof of cross-linking requires the use of ligands with two or more different binding moieties. As proof-of-concept, we have examined the binding of synthetic heterobivalent ligands to cell lines that were engineered to coexpress two different G-protein-coupled human receptors, i.e., the human melanocortin 4 receptor (MC4R) expressed in combination with either the human  $\delta$ -opioid receptor ( $\delta$ OR) or the human cholecystokinin-2 receptor (CCK2R). Expression levels of these receptors were characterized by time-resolved fluorescence saturation binding assays using Europium-labeled ligands; Eu-DPLCE, Eu-NDP- $\alpha$ -MSH, and Eu-CCK8 for the  $\delta$ OR, MC4R, and CCK2R, respectively. Heterobivalent ligands were synthesized to contain a MC4R agonist connected via chemical linkers to either a  $\delta$ OR or a CCK2R agonist. In both cell systems, the heterobivalent constructs bound with much higher affinity to cells expressing both receptors, compared with cells with single receptors or to cells where one of the receptors was competitively blocked. These results indicate that synthetic heterobivalent ligands can noncovalently crosslink two unrelated cell surface re-**

**ceptors, making feasible the targeting of receptor combinations. The *in vitro* cell models described herein will lead to the development of multivalent ligands for target combinations identified in human cancers. [Mol Cancer Ther 2009;8(8):2356–65]**

### Introduction

A major goal of cancer therapy is to kill or inhibit cancer cells, while minimizing deleterious effects on normal cells. Molecular biology and pharmacogenomics have revealed critical molecular differences between normal and tumor cells. These differences may allow for the development of agents that can be specifically targeted to cancer to inhibit signaling pathways for cell replication, differentiation, and survival. Such anticancer-targeted therapies include small-molecule tyrosine kinase inhibitors, antisense mRNA inhibitors, and antibodies, which are in various stages of clinical development (1–3).

Although these approaches have resulted in clinical benefit for selected cancer subtypes, there are a number of limitations to targeting only single genes or gene products (1, 4). An alternative approach examined here involves agents that are capable of delivering a payload directly and specifically to receptor targets that need not be overexpressed or essential for survival. We and others have proposed an approach (“molecular Velcro”), wherein heteromultivalent ligands are designed to target to cell surface receptor combinations that are unique to the target cell (5–7).

Multivalent ligands consist of multiple binding moieties (pharmacophores) that are tethered together via chemical linkers. It is well-known that multivalent binding can lead to high avidity and specificity in binding (6, 8, 9). A wide spectrum of binding moieties can be used, including small peptide fragments, truncated versions of antibodies, and carbohydrate analogues (10–13). Although monoclonal antibodies (mAbs) have found success in the clinic, the high molecular weight of mAbs is a drawback to their multimerization (14, 15). Small peptides, such as those used in our current study, do not share this limitation (7, 16).

Multivalent ligands can be homomultivalent, with multiple copies of the same ligand, or they can be heteromultivalent, with different types of ligands targeted to different types of receptors. Previous work has shown that homomultivalent ligands exhibit increased avidity or potency and that flexible linkers of 20 to 50 Å provide the greatest enhancement of binding affinities (6, 8, 13, 17–19). However, in addition to requiring overexpression of a single receptor, homomultivalent constructs cannot unequivocally distinguish statistical proximity effects from the noncovalent crosslinking (clustering) of receptors, which would be needed for heteromultivalent interactions. Thus, demonstration of receptor noncovalent crosslinking requires the use of heteromultivalent constructs.

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To evaluate the binding of heterobivalent ligands to their corresponding receptors, it was necessary to construct and stringently characterize cell lines that expressed one, or both, of the target receptors. In the current proof-of-concept studies, three different G-protein-coupled receptors (GPCR) were chosen as target gene products: the human  $\delta$ -opioid receptor ( $\delta$ OR), the human melanocortin receptor subtype 4 (MC4R), and the human cholecystokinin-2 receptor (CCK2R). These were coexpressed in combinations of MC4R +  $\delta$ OR and MC4R + CCK2R for testing of Deltorphin-MSH7 and MSH7-CCK6 heterobivalent structural constructs, respectively.

Here, Chinese hamster ovary (CHO) cell lines were engineered to transiently coexpress the MC4R and  $\delta$ OR receptors and were characterized by lanthanide-based time-resolved fluorescence saturation binding assay using Europium-labeled monomeric ligands: Eu-NDP- $\alpha$ -MSH and Eu-DPLCE, respectively. An Deltorphin II-MSH7 heterobivalent ligand was synthesized and binding affinity determined in cells expressing one or both receptors. In another system, stable coexpression of the MC4R and CCK2R receptors was successfully established in the Hek293 cell line. This engineered line and derivatives were tested for their ability to bind the corresponding monomeric ligands as well as a heterobivalent ligand containing both MSH7 and CCK6 pharmacophores. In both cell systems, we observed similar results demonstrating that heterobivalent constructs were bound to two different receptors with increased avidity.

These results show the feasibility of simultaneously targeting multiple receptors using heterobivalent ligands. Additionally, this study shows that cell lines can be constructed that are suitable for screening heterobivalent ligands in high-throughput mode. The methodology described and the dual receptor expression system will facilitate further development of novel ligands for targeting human cancers.

## Materials and Methods

### Cell Culture

The parental cell lines used in the experiments were the CHO-K1 (American Type Culture Collection; CRL-9618), Hek293 (American Type Culture Collection; CRL-1573) cell lines. The MC4R stable-transfected Hek293 cell line (Hek293/MC4R) was described previously (20). All cells were maintained at 37°C and 5% CO<sub>2</sub>. All cell lines except for the CHO cells were maintained in DMEM/Ham's Nutrient Mixture F-12 supplemented with 10% fetal bovine serum. CHO cells were maintained in Ham's F-12 media supplemented with 10% fetal bovine serum.

### Ligand Synthesis

Europium labeled ligands (Eu-NDP- $\alpha$ -MSH, Eu-CCK8, and Eu-DPLCE) and heterobivalent compounds DeltII-[PG]<sub>15</sub>-MSH7 and MSH7-Pego-[PG]<sub>6</sub>-Pego-CCK6 (Fig. 1) were prepared as previously described (20, 21) by solid-phase synthesis. Briefly, ligands were synthesized using a manual synthesizer (Torviq) with *N*<sup>α</sup>-Fmoc/*t*Bu chemistry. Polyethylene glycol units were introduced by first adding diglycolic anhydride to the free *N*<sup>α</sup>-terminal and then acti-

vating the free carboxylate as an imidazolide for attachment of 4,7,10-trioxo-1,13-tridecanediamine to form polyethylene glycol units (13). Diethylenetriaminepentaacetic acid chelator was attached to peptides on solid support using the HOBT ester method to decrease unwanted cross-linking and dimerization (21). [D-Pen<sup>2</sup>, L-Cys<sup>5</sup>] Enkephalin (DPLCE) requires a free *N*<sup>α</sup>-terminal to retain biological activity; therefore, the chelator was conjugated via the Alloc-protected  $\epsilon$ -side chain of lysine. Ligands were cleaved from the resin using a TFA-scavenger cocktail and purified by high performance liquid chromatography (HPLC). DPLCE was cyclized by air oxidation and then chelate ligands were labeled with Europium (III) chloride in neutral pH buffers. Excess Europium was removed by size exclusion chromatography (Sepak C18; Waters). Structures were characterized by Mass Spec (ESI, Termoquest, LCQ; MALDI-TOF, Bruker Reflex III) and quantitative HPLC.

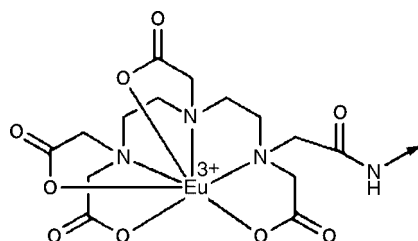
The Cy5-labeled ligand (MSH7-peg-[PG]<sub>3</sub>-K(Cy5)-peg-CCK6; Fig. 1) was synthesized by conjugation of Cy5 dye to the bivalent ligand using the lysine side chain. After incorporation of the first Pego linker, *N*<sup>α</sup>-Fmoc-*N*<sup>ε</sup>-Mtt, lysine was incorporated into the sequence and then peptide synthesis completed. Peptide was cleaved from the resin and purified by preparative HPLC. Purified peptide was dissolved in DMSO and Cy5-NHS ester (Amersham Biosciences) was added. The reaction was monitored using analytic HPLC at 280 nm. Labeled peptide was purified using size exclusion chromatography (C-18 Sep-Pak), lyophilized, and characterized using MALDI-MS.

### Construction of Expression Vector(s) for MC4R, $\delta$ OR, and CCK2R

MC4R and  $\delta$ OR cDNA were cloned into the pBudCE4.1 vector (Invitrogen V532-20), which is a dual promoter vector capable of expressing two independent recombinant proteins. Using pcDNA3.1-MC4R as a template, full-length MC4R was amplified using PCR primers with adapters containing Kpn I and Bgl II restriction enzyme sites. Primers were designed using the MC4R sequence: sense, 5'-TCA ATC GGT ACC ATG GTG AAC TCC-3'; and antisense, 5'-GGT ACC AGA TCT GCT TAA TAT CTG CT-3'. A Kozak sequence (A/G NN ATG G) was also generated at the 5' end of the MC4R PCR fragment. The fragment was digested with Kpn I/Bgl II and ligated into the Kpn I/Bgl II site between the EF-1  $\alpha$  promoter and BGH poly(A) of the pBudCE4.1 vector. The sequence of the recombinant plasmid was verified by sequencing both strands and named pBudCE-MC4R. The plasmid pcDNA3.1(+)/Hygro containing full-length  $\delta$ OR cDNA was provided by Henry Yamamura (University of Arizona, Tucson, AZ) and digested with Apa I and EcoR I, blunting the 5'- and 3'-protruding termini with T4 DNA polymerase, and subsequently subcloned into pBudCE4.1-MC4R, which was cut with Xba I, blunting the 5' end with T4 DNA polymerase and dephosphorylated. This  $\delta$ OR cDNA insert was subcloned between the cytomegalovirus promoter and SV40 poly(A) of pBudCE-MC4R. The resulting plasmid, termed pBudCE-MC4R- $\delta$ OR, was verified using restriction enzyme analysis and DNA sequencing.

Compound	Structure
Eu-NDP- $\alpha$ -MSH	<sup>1</sup> Eu-(DTPA)-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH <sub>2</sub>
Eu-DPLCE	H-Tyr- <sub>i</sub> [DPen-Gly-Phe-Cys]-Phe-Lys(Eu-(DTPA))-NH <sub>2</sub>
Eu-CCK8	Eu-(DTPA)-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH <sub>2</sub>
Delt II-[PG] <sub>15</sub> -MSH7	<sup>2</sup> H-Deltorphin II-[Pro-Gly] <sub>15</sub> -MSH7-NH <sub>2</sub>
MSH7-Pego-[PG] <sub>6</sub> -Pego-CCK6	<sup>3</sup> Ac-MSH7-PEGO-[Pro-Gly] <sub>6</sub> -PEGO-CCK6-NH <sub>2</sub>
MSH7-Pego-[PG] <sub>3</sub> -K(Cy5)-Pego-CCK6	Ac-MSH7-PEGO-[Pro-Gly] <sub>3</sub> -Lys(Cy5)-PEGO-CCK6-NH <sub>2</sub>

<sup>1</sup> Eu-(DTPA)



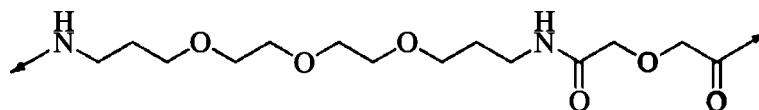
<sup>2</sup> Deltorphin II

Tyr-DAla-Phe-Glu-Val-Val-Gly

MSH7

Ser-Nle-Glu-His-DPhe-Arg-Trp

<sup>3</sup> PEGO



CCK6

Nle-Gly-Trp-Nle-Asp-Phe

**Figure 1.** List of compounds and their structures used in this study.

For the CCK2R construct, a 1,700-bp fragment containing the full-length CCK2 receptor gene was PCR amplified using a panel of human lymph cDNA (Clontech, K1426-1) as a template. The sequences of the forward and reverse primers were as follows: CAC CGG TCG ACC GGG GGC CAT GGA AGC TGC TAA AG and GCA GGT CGA CCC TTG TCA GAG, respectively. This purified PCR product was used as template DNA for a second PCR reaction using the following forward and reverse primers: CAC CAT GGA GCT GCT AAA GCT GAA CCG G and TCA GCC AGG GCC CAG TGT, respectively. These primers were designed with a CACC sequence at the 5' end so that the PCR product could be directly cloned into the pcDNA3.1 TOPO vector (Invitrogen; K4900-01). Subsequently, the CCK2R gene was cut out of the pcDNA3.1 TOPO vector using HindIII and XhoI, and inserted into

the pcDNA3.1/Zeo(+) vector at the HindIII and XhoI sites. The constructs mentioned above were verified via DNA sequencing.

#### Construction of Cell Lines Coexpressing MC4R and $\delta$ OR (CHO/MC4R/ $\delta$ OR Cells)

Cell transfections were done using FuGENE 6 transfection reagent (Roche; 1814-443). For establishment of cell lines transiently expressing both MC4R and  $\delta$ OR receptors, CHO cells were plated on Wallac B&W Isoplate TC (Wallac/PerkinElmer; 1450-583) 96-well plates at a density of 20,000 cells per well, allowed to adhere for 24 h, and then transfected with pBudCE-MC4R- $\delta$ OR vector. Forty-eight hours after transfection, cells were tested for MC4R and  $\delta$ OR cell surface expression by MC4R and  $\delta$ OR binding assay using Eu-NDP- $\alpha$ -MSH and Eu-DPLCE, respectively, as described previously (20, 21).

Transient transfection efficiencies were determined by cotransfection of pBudCE-MC4R- $\delta$ OR vector and green fluorescent protein (GFP) reporter constructs (Invitrogen; A-150228) in CHO cells. Cells were harvested 48 h after transfection and analyzed by fluorescence activated cell sorting for GFP expression.

#### Construction of Stable Transfected Cell Lines (Hek293/CCK2R and Hek293/MC4R/CCK2R Cells)

Hek293 cells were transfected with the pcDNA3.1/Zeo (+)-CCK2R construct. After 48 h, replacement media contained 0.1 mg/mL zeocin. Single stable transfectants were observed in 3 to 4 wk. The CCK2R surface expression level for each clone was measured by ligand binding assay using Eu-CCK8. A stable dual expressing cell line was made by transfecting Hek293/MC4R cells with the CCK2R construct. After 48 h, dual selection media included both 0.1 mg/mL zeocin (Invitrogen; 450430) and 0.4 mg/mL geneticin (Life Technologies; 11811-031). Clones were tested for ligand binding at both receptors using Eu-CCK8 and Eu-NDP- $\alpha$ -MSH.

#### In Cyto Lanthanide-Based Time-Resolved Fluorescence Binding Assays

Lanthanide-based binding assays were done on whole CHO or Hek293 cells as previously described (20, 21). Saturation- and competitive-binding assays confirmed the functional expression of cell surface receptors using monomeric ligands. For saturation binding, increased concentration of labeled ligands Eu-NDP- $\alpha$ -MSH, Eu-DPLCE, and Eu-CCK8 were used for binding to the MC4R,  $\delta$ OR, and CCK2R respectively. Nonspecific binding was determined in the presence of 10  $\mu$ mol/L NDP- $\alpha$ -MSH, 10  $\mu$ mol/L Naloxone, or 1  $\mu$ mol/L CCK8, respectively. The competitive binding assay was used to evaluate heterobivalent ligand binding on engineered cells, i.e., increasing amounts of the Delt II-[PG]<sub>15</sub>-MSH7 ligand competed with known amounts of Eu-labeled ligand (10 nmol/L Eu-NDP- $\alpha$ -MSH or 10 nmol/L Eu-DPLCE), or the MSH7-Pego-[PG]<sub>6</sub>-Pego-CCK6 ligand competed with 10 nmol/L Eu-NDP- $\alpha$ -MSH or 0.1 nmol/L Eu-CCK8.

For screening, one-point ligand binding assays were done for each clone. Respectively, MC4R,  $\delta$ OR, or CCK2R expression was determined by incubation with 10 nmol/L Eu-NDP- $\alpha$ -MSH, 10 nmol/L Eu-DPLCE, or 0.1 nmol/L Eu-CCK8 in the presence or absence of unlabeled 10  $\mu$ mol/L NDP- $\alpha$ -MSH, 10  $\mu$ mol/L Naloxone, or 1  $\mu$ mol/L CCK8. Specific binding was determined as the difference between values in the absence (total) and presence (nonspecific) of competing unlabeled ligand.

#### Immunocytochemistry

Cells were grown on coverslips to ~70% confluence. The MC4R antibody (RDI, RTMC4Rabr) recognizes an epitope on the cell exterior. Hence, live cells were incubated with this antibody (10  $\mu$ g/mL) for 5 min, washed in antibody-free buffer (2  $\times$  5 min), and fixed with 2% paraformaldehyde. Before labeling with secondary antibody (anti-rabbit Alexa 488; Invitrogen; A-11008), fixed cells were incubated with 25 mmol/L glycine and permeabilized with 0.1% TritonX-100 (22). Antibodies for  $\delta$ OR

(mouse; Neuromics; RA10101) and CCK2R (rabbit; Abcam, Inc.; AB13173) recognize intracellular epitopes. Thus, cells were incubated with these antibodies following permeabilization.

For dual labeling of MC4R and  $\delta$ OR, MC4R-labeled coverslips were incubated sequentially with the primary antibody to  $\delta$ OR, washed, and then labeled with a Texas red-conjugated anti-mouse antibody (The Jackson Laboratory).

Because the antibodies for MC4R and CCK2R are both raised in rabbit, when dual labeling cells for these receptors, an intermediate blocking strategy was required to assure specificity of secondary antibodies for each specific primary (23). MC4R-labeled coverslips were incubated overnight with a 10 $\times$  concentration of nonlabeled anti-rabbit IgG to block free anti-rabbit IgG sites remaining on the MC4R-specific polyclonal antibody, washed (3  $\times$  5 min) in PBS, and incubated with the CCK2R antibody for 60 min followed by washing and incubation with a secondary anti-rabbit IgG labeled with Alexa480 (Invitrogen) for 45 min at 25°C. All processed coverslips were mounted onto glass slides using a 50% glycerol/saline solution containing the antibleach agent paraphenylenediamine (0.1%). To assure 100% blocking of rabbit anti-MC4R sites, a control was included in all batches, wherein the primary CCK2R antibody was omitted before incubation with secondary antibody. Any Texas red labeling on these coverslips indicated that the blocking step was ineffective and the entire batch was discarded.

#### Cell Fluorescence Imaging

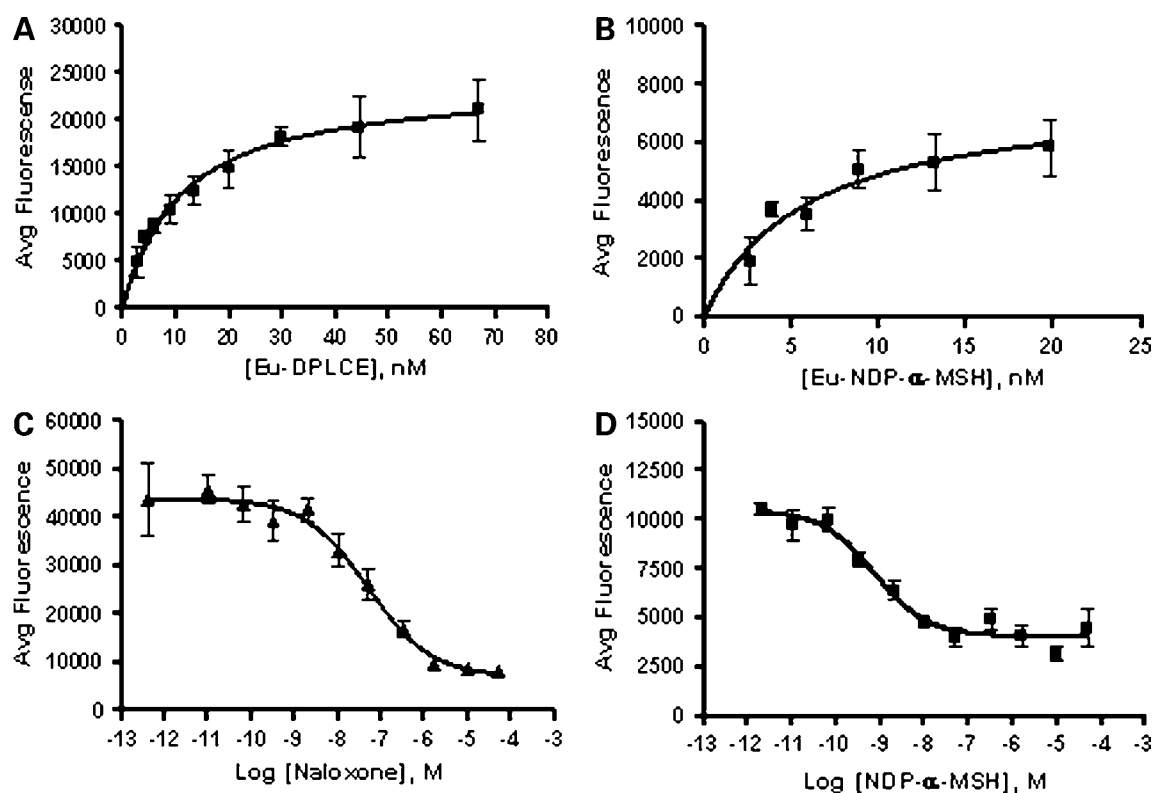
An inverted Olympus IX70 microscope equipped with a 40  $\times$  1.4 NA ultrafluor objective, and a 100 W Hg lamp as the excitation source was used for cell imaging experiments. For imaging dual receptor expressing cells using the Cy5 tagged ligand (Fig. 1), a # 1 coverslip bearing live Hek293/MC4R/CCK2R cells was placed in a 37°C chamber mounted on the microscope stage. Cy5 fluorescence was excited with a 20-nm BP filter centered at 640 nm and emitted light collected through a 30-nm filter centered at 690 nm. A liquid cooled CCD camera (Photometrics CH-250) was used to acquire images.

#### Cytotoxicity of Monovalent and Bivalent Ligands

Hek293 cells expressing MC4R and/or CCK2R were plated at a density of 20,000 cells per well into 96-well dishes and allowed to attach overnight. Cells were treated with 1 nmol/L or 1  $\mu$ mol/L of NDP- $\alpha$ -MSH, or CCK8 or MSH7-Pego-[PG]<sub>6</sub>-Pego-CCK6 for 24 or 48 h. Viable cell count was determined with a celltiter-Glo luminescent cell viability assay (Promega; G7571). Cytotoxicity was quantified as the percentage of treated cell viability relative to untreated controls, thus a higher relative viability corresponds to lower toxicity.

#### Data Analysis

Saturation and competition binding data were analyzed by nonlinear regression analysis using GraphPad Prism (GraphPad Software). Saturation binding data were fitted to a classic one site binding (hyperbola) equation and competitive binding data were fitted to a classic one site binding competition equation. For saturation binding assays,  $K_d$



**Figure 2.** MC4R and  $\delta$ OR saturation binding and competitive binding analysis. **A**, saturation binding of Eu-DPLCE ligand to  $\delta$ OR in CHO/MC4R/ $\delta$ OR cells. The curve shows  $\delta$ OR specific binding only (total-nonspecific). From these data, the  $K_d$  of  $10.5 \pm 2.6$  nmol/L and  $B_{max}$  of  $24,000 \pm 2,000$  AFU ( $R^2 = 0.91$ ). Points, average of four samples; bars, SE. **B**, saturation binding assay of Eu-NDP- $\alpha$ -MSH ligand to MC4R in CHO/MC4R/ $\delta$ OR cells. The curve shows MC4R-specific binding only. From these data, the  $K_d$  of  $5.6 \pm 2.7$  nmol/L and  $B_{max}$  of  $7,700 \pm 1,400$  AFU ( $R^2 = 0.83$ ). **C** and **D**, competitive binding to CHO cells coexpressing MC4R and  $\delta$ OR. **C**, increasing concentrations of Naloxone were added in the presence of 10 nmol/L Eu-DPLCE. From these data, the  $IC_{50}$  was 65 nmol/L with  $R^2 = 0.90$ . **D**, increasing concentration of NDP- $\alpha$ -MSH were added to cells in the presence of 10 nmol/L Eu-NDP- $\alpha$ -MSH. From these data,  $IC_{50}$  was 0.77 nmol/L with  $R^2$  of 0.89.

values were determined after correction for nonspecific binding as the concentration that yielded half-maximal binding. For competitive binding assays, the  $IC_{50}$  was determined after correction for nonspecific binding as the concentration of unlabeled ligand sufficient to compete off 50% of the labeled ligand.

## Results

### Creation and Characterization of Transient Systems Coexpressing MC4R and $\delta$ OR

Although tremendous efforts were made to construct a cell line stably expressing both MC4R and  $\delta$ OR, this was not achieved (data not shown). However, the MC4R and  $\delta$ OR were observed to coexpress well in CHO cells for a short period of time using the pBudCE-MC4R- $\delta$ OR vector. Hence, a transient dual receptor-expressing system using the same cell line and same vector was characterized. Maximal surface expression of both receptors in CHO cells was determined to be 48 hours after transfection by in cyto lanthanide-based time-resolved fluorescence binding assays (Supplementary Fig. S1). All subsequent binding assays were done at this time point. Saturation-binding

assays showed that Eu-DPLCE bound to the  $\delta$ OR with a  $K_d$  of  $10.5 \pm 2.6$  nmol/L and a  $B_{max}$  of  $24,000 \pm 2,000$  AFU (Average Fluorescence Unit; Fig. 2A); and that Eu-NDP- $\alpha$ -MSH bound to the MC4R with a  $K_d$  of  $5.6 \pm 2.7$  nmol/L and a  $B_{max}$  of  $7,700 \pm 1,400$  AFU (Fig. 2B).

To further investigate these dual expressing cells, competitive binding assays were done (Fig. 2C and D). Naloxone or NDP- $\alpha$ -MSH effectively displaced Eu-DPLCE or Eu-NDP- $\alpha$ -MSH with  $IC_{50}$  values of 65 nmol/L ( $R^2 = 0.90$ ) or 0.77 nmol/L ( $R^2 = 0.89$ ), respectively. Importantly, testing of heterobivalent ligand binding using this cell system required that blocking one receptor would not interfere with the affinity of the other receptor and vice versa. To examine this, competitive binding studies of each monomeric ligand to its receptor were done in the presence of excess competitor for the heterologous receptor. The  $IC_{50}$  value for NDP- $\alpha$ -MSH to displace Eu-NDP- $\alpha$ -MSH was 0.65 nmol/L when  $\delta$ OR was blocked with Naloxone, which is similar to the  $IC_{50}$  value of 0.77 nmol/L measured in the absence of naloxone (as shown in Fig. 2D). Similarly, the  $IC_{50}$  of Naloxone to displace Eu-DPLCE from the  $\delta$ OR was 55 nmol/L in the presence of excess NDP- $\alpha$ -MSH, compared with an  $IC_{50}$  value of 65 nmol/L in the absence of NDP- $\alpha$ -MSH (Fig. 2C).

Hence, blocking the  $\delta$ OR did not interfere with the binding affinity of the MC4R and vice versa.

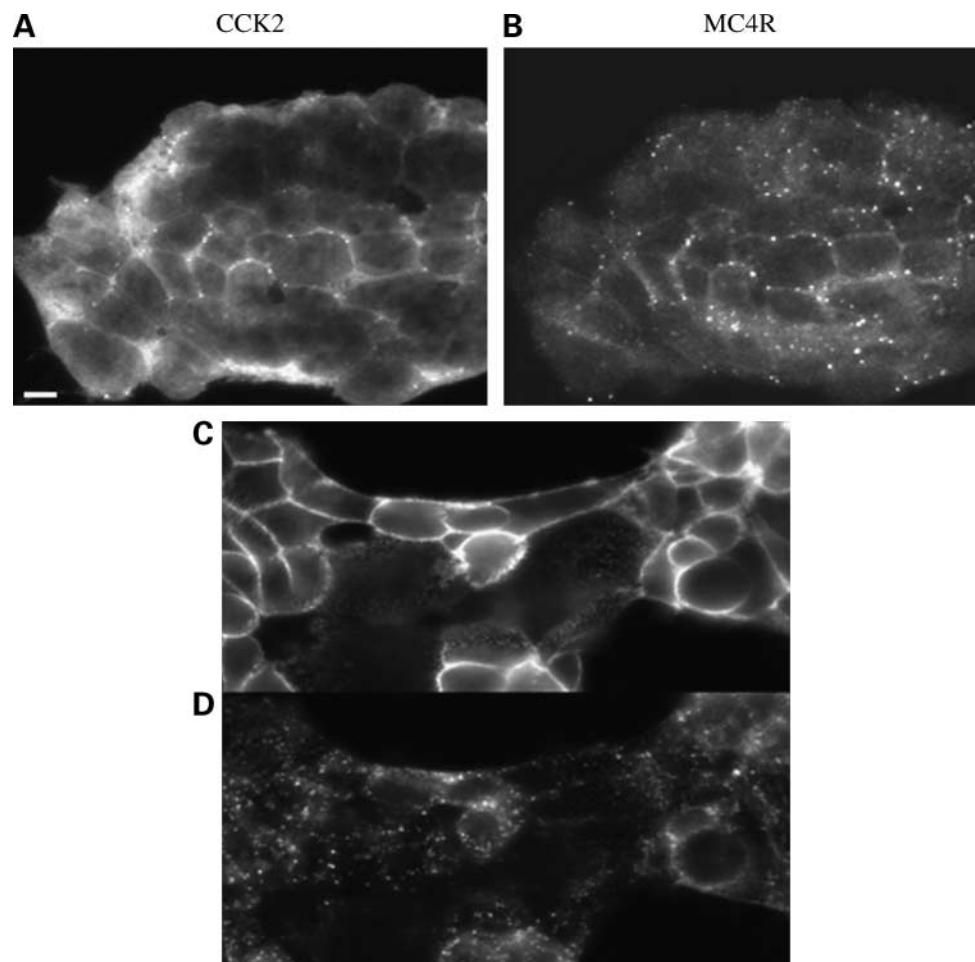
Transfection efficiency was determined for this transient dual expression system using GFP because it can be quantified at the single-cell level by flow cytometry and the efficiency of target gene expression roughly corresponds with GFP expression (24–26). CHO cells were cotransfected with GFP and pBudCE-MC4R- $\delta$ OR plasmid and the percentage of cells expressing GFP was measured 48 hours after transfection. Transfection efficiency was  $33 \pm 4\%$  ( $n = 9$ ), which is similar to that typically achieved using lipid-based transfection reagents.

Expression of  $\delta$ OR and MC4R simultaneously in the same cells within the transfected population was also examined using immunocytochemistry (Supplementary Fig. S2). Only surface resident MC4 receptors were labeled and a peripheral distribution was clearly observed. Because the procedure for labeling the  $\delta$ OR allowed access to intracellular compartments, a significant amount of  $\delta$ OR was observed within the cell and determination of surface expression was less clear. But, overlap of signal from both antibodies at the cell periphery was observed within individual cells, and we conclude that the transfected cells are expressing

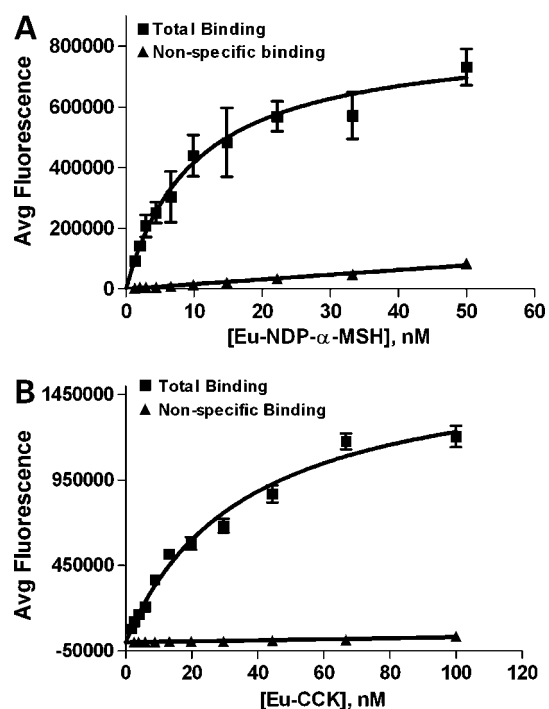
both receptors types. Control samples without primary antibodies were devoid of signal (data not shown).

#### Construction and Characterization of Cell Lines Stably Expressing both MC4R and CCK2R

A cell line that stably expressed both the MC4R and CCK2R (Hek293/MC4R/CCK2R) was constructed as described in the Materials and Methods section. Immunocytochemistry was used to evaluate the pattern of expression in the derived lines. As shown in Fig. 3A and B, and Supplementary Fig. S6, many cells express both receptors simultaneously. These cells were evaluated for surface expression levels by saturation binding assays. Saturation binding analysis showed that Eu-NDP- $\alpha$ -MSH bound to Hek293/MC4R/CCK2 cells with a  $K_d$  of  $8.3 \pm 1.9$  nmol/L and a  $B_{max}$  of  $732,000 \pm 59,000$  AFU, as shown in Fig. 4A. This  $K_d$  value is consistent with our previous results using this ligand in MC4 single receptor-expressing cells (20). Similarly, saturation analysis of Eu-CCK binding to CCK2R was achieved with a  $K_d$  of  $34.6 \pm 3.9$  nmol/L, and a  $B_{max}$  of  $1,600,000 \pm 83,000$  AFU (Fig. 4B). This  $K_d$  value was consistent with that observed in the cell line expressing the CCK2R alone, e.g.,  $K_d$  of  $38.6 \pm 3.8$  nmol/L using this same Eu-CCK ligand.



**Figure 3.** Immunolabeling of MC4R and CCK2R and the distribution of heterobivalent ligand labeling in MC4R and CCK2R-expressing cells. **A** and **B**, images of Hek293 cells, which stably expressed both the CCK2R and MC4R, labeled with antibodies against the MC4R and CCK2R receptors. **C** and **D**, images of cells incubated for 3 min with 0.8 nmol/L of Cy5-labeled MSH-CCK ligand, then washed with ligand-free media. The ligand distribution was determined immediately following the rinse (**C**), and 7 min thereafter (**D**). Scale bar, 20  $\mu$ mol/L for both images pairs.



**Figure 4.** MC4R and CCK2R saturation binding analysis. **A**, saturation curve of Eu-NDP- $\alpha$ -MSH obtained from the MC4R and CCK2 dual expression cell line. The figure shows total binding ( $\blacksquare$ ) and binding in the presence of 10  $\mu$ mol/L NDP- $\alpha$ -MSH ( $\blacktriangle$ ). From these data, the  $K_d$  of  $8.3 \pm 1.9$  nmol/L, and  $B_{max}$  of  $732,000 \pm 59,000$  AFU. Lines, the computer modeled best fit of the data using GraphPad Prism software using the non-linear regression, one site-binding equation, with a  $R^2$  value of 0.81. Points, the average of four samples; bars, SE. **B**, saturation curve of Eu-CCK8 obtained from the MC4R and CCK2 dual expression cell line. The figure shows total binding ( $\blacksquare$ ) and binding in the presence of 1  $\mu$ mol/L CCK8 ( $\blacktriangle$ ). From these data, the  $K_d$  of  $34.6 \pm 3.9$  nmol/L, and  $B_{max}$  of  $1,600,000 \pm 83,000$  AFU, with a  $R^2$  value of 0.96.

#### Determination of Receptor Numbers on Cells

Standard curves for Eu-labeled ligands were generated by adding increasing amounts of Eu-NDP- $\alpha$ -MSH, Eu-DPLCE, or Eu-CCK8 to quadruplicate wells of a 96-well plate. These were used to generate a relationship between fluorescence intensities and ligand concentrations (Supplementary Fig. S3). These calibration data allowed for the determination of the amount of ligand present at the  $B_{max}$  obtained from the saturation binding data shown in Fig. 2A and B and Fig. 4A and B. For binding to the MC4 receptor on CHO/MC4R/ $\delta$ OR cells, the  $B_{max}$  was  $7,700 \pm 1,400$  AFU, which corresponds to  $0.76 \pm 0.14$  fmol per well. For  $\delta$ OR binding, the  $B_{max}$  was  $24,000 \pm 2,000$  AFU which corresponds to  $4.30 \pm 0.36$  fmol per well. The average cell number per well was  $75,000 \pm 2,300$  cells ( $n = 4$ ). After correcting for transfection efficiency ( $33 \pm 4\%$ ), it was calculated that there were  $18,000 \pm 3,300$  MC4R/cell and  $100,000 \pm 8,600$   $\delta$ OR/cell. Thus,  $\delta$ OR outnumbered MC4R by a ratio of  $\sim 6:1$ .

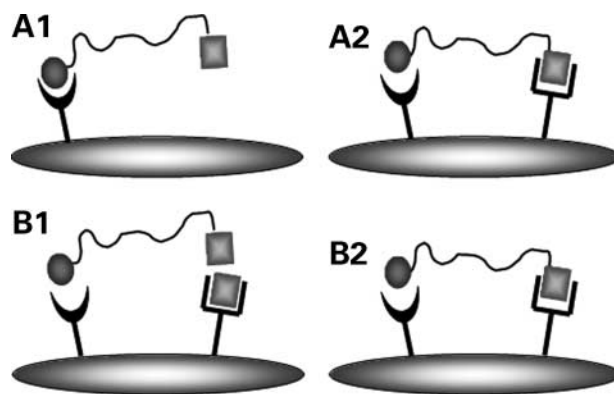
Taking a similar approach, receptor numbers were determined in Hek293/MC4R/CCK2R cells. It was calculated that there were  $1,100,000 \pm 56,000$  CCK2 receptors/cell and  $640,000 \pm 52,000$  MC4R receptors/cell in this stably ex-

pressing line. Hence, the CCK2 receptors outnumbered MC4R by a ratio of  $\sim 2:1$ .

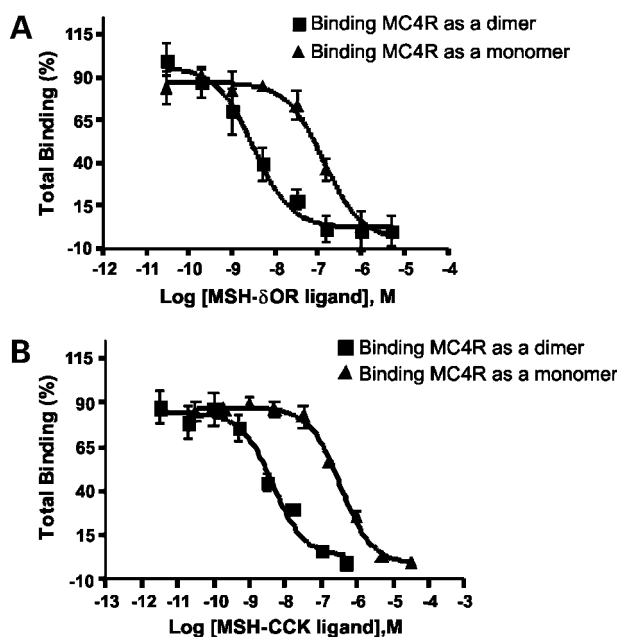
#### Evaluation of Heterobivalent Ligand Binding

Ligand binding assays for heterovalent ligands are more complicated than for monovalent ligands, as binding to multiple different receptor types must be validated. For the purpose of testing heterobivalent constructs, there are two ways that binding assays may be designed, as illustrated in Fig. 5. In one type of experiment, heterodimers are tested on two different cell lines: one that expresses a single complementary receptor (Fig. 5A1) and another that expresses both of the complementary receptors (Fig. 5A2). This system was used to test the binding of the heterobivalent MSH-CCK ligand. Alternatively, heterodimeric binding can be assessed using a single cell line that expresses both complementary receptors in the presence (Fig. 5B1) or absence (Fig. 5B2) of receptor blockage using excess unlabeled ligand. This system was used to test the binding of the heterobivalent MSH- $\delta$ OR ligand.

Heterobivalent ligands were prepared to target the MC4R- $\delta$ OR and MC4R-CCK2R combinations. DeltII-[PG]<sub>15</sub>-MSH7 was constructed and tested for binding to the MC4R and  $\delta$ OR receptors as described above. This ligand competed Eu-NDP- $\alpha$ -MSH with an  $IC_{50}$  value of  $3.3 \pm 1.8$  nmol/L and  $159.6 \pm 46.3$  nmol/L in the absence and presence of naloxone (an  $\delta$ OR antagonist), respectively (number of replicates,  $n = 5$ ,  $P < 0.01$ ). Figure 6A shows a representative binding curve in competition with Eu-NDP- $\alpha$ -MSH in the absence (dimer) and presence (monomer) of naloxone. The DeltII-[PG]<sub>15</sub>-MSH7 ligand competed with Eu-DPLCE with  $IC_{50}$  values of  $230 \pm 74$  nmol/L and  $500 \pm 90$  nmol/L in the absence and presence of excess NDP- $\alpha$ -MSH ( $n = 5$ ,  $P > 0.05$ ), respectively. From the hMC4R data, the heterodimer bound with much higher affinity when both complementary receptors are available, compared with its binding when the  $\delta$ OR was blocked. In



**Figure 5.** Evaluation of heterobivalent ligand binding. There are two different approaches by which heterobivalent binding can be evaluated. One approach uses two different cell types (**A1** and **A2**); one that expresses a single complementary receptor and another that expresses both of the complementary receptors, whereas another approach uses the same cell type but blocks binding at one receptor by addition of an agent (**B1** and **B2**).



**Figure 6.** Representative competitive binding assay at the MC4R. **A**, increasing concentrations of the MSH-Deltorphin heterobivalent ligand were added to CHO/MC4R/ $\delta$ OR cells in the presence of 10 nmol/L Eu-DTPA-NDP- $\alpha$ -MSH and the absence (dimer) and presence (monomer) of competing naloxone. For dimer binding (absence of naloxone), the  $IC_{50}$  of 3.2 nmol/L with  $R^2$  of 0.83; for monomer binding (presence of naloxone), the  $IC_{50}$  of 134.0 nmol/L with  $R^2$  of 0.93. **B**, increasing concentrations of the MSH-CCK heterobivalent ligand were added to cells in the presence of 10 nmol/L Eu-DTPA-NDP- $\alpha$ -MSH. For dimer binding, Hek293/CCK2R/MC4R cells were used and for monomer binding, Hek293/MC4R cells were used. For dimer binding, the  $IC_{50}$  of 4.5 nmol/L with  $R^2$  of 0.88; for monomer binding, the  $IC_{50}$  of 349.5 nmol/L with  $R^2$  of 0.96.

contrast, binding to the  $\delta$ OR did not seem to be affected by the availability of the second receptor (MC4R). These results were interpreted to indicate that the enhancement of divalent binding was only apparent at the less abundant of the two receptors. In other words, all of the MC4 receptors can be engaged in a heterobivalent complex, whereas only 1 of 6 of the  $\delta$ OR can be thus occupied. The remaining 5 of 6 of the  $\delta$ OR receptors thus must bind monovalently. In all, these data are consistent with receptor cross-linking and heterobivalent interactions (Supplementary Fig. S4). Notably, a short linker ligand, DeltII-[PG]<sub>3</sub>-MSH7 (18-atom long linker) showed no significant increase of binding affinity between MC4R monovalent ( $IC_{50}$ , 110  $\pm$  9 nmol/L) and bivalent ( $IC_{50}$ , 180  $\pm$  30 nmol/L) binding modes. The observation that smaller compounds show no enhancement suggests that the enhancement with longer linkers was not simply due to a local concentration (statistical) effect.

In the alternative system, an MSH-CCK heterodimer, MSH7-Pego-[PG]<sub>6</sub>-Pego-CCK6, was constructed. This ligand was tested using the competitive binding assay in the MC4R or CCK2R single receptor cell lines (i.e., "monomeric" binding) as well as the dual receptor MC4R + CCK2R cell line (i.e., "dimeric" binding), as outlined in Fig. 5A1 and A2. This ligand competed with Eu-NDP- $\alpha$ -

MSH as a monomer with an  $IC_{50}$  value of 251.4  $\pm$  38.8 nmol/L ( $n = 5$ ) and as a dimer with an  $IC_{50}$  value of 3.1  $\pm$  0.3 nmol/L ( $n = 5$ ; \*,  $P < 0.01$ ); thus, an 81-fold enhancement was observed. Figure 6B shows a representative competitive binding curve for dimeric and monomeric binding to the MC4R. For the CCK2 receptor, this ligand competed with Eu-CCK with an  $IC_{50}$  value of 190.0  $\pm$  51.3 nmol/L and 167.5  $\pm$  20.2 nmol/L for monomeric and dimeric binding, respectively ( $n = 5$ , not significant). These results were consistent with those presented above; i.e., that the less abundant receptor showed significant enhancement for divalent, compared with monovalent interactions, whereas the more abundant receptors did not.

#### Imaging of Cy5-Labeled Heterobivalent Ligand Binding and Ligand-Induced Receptor Internalization

Binding of the heterobivalent MSH-CCK ligand tagged with the fluorophore Cy5 to the dual CCK2R/MC4R-expressing line was also evaluated at the cell level. Immediately following incubation with the ligand (15 seconds with 0.8 nmol/L), significant binding to the cell surface was observed. After 3 minutes, the ligand was washed from the cell chamber, and receptor distribution was followed (Fig. 3C). By 10 minutes, all ligand was observed to be in punctate structures assumed to be receptor capping zones (Fig. 3D). To determine if the receptor/ligand complex was significantly internalized, CCK ligand (50  $\mu$ mol/L) was added to the chamber in an attempt to compete ligand from receptors remaining on the surface. No significant loss of ligand was observed with this strategy, indicating that the receptor/ligand complex had been substantially internalized.

#### Cytotoxicity of Monovalent and Bivalent Ligands

Synthetic heterobivalent constructs are novel, and may have unanticipated effects on cell behavior, such as proliferation or survival. Viability was assayed in cells expressing MC4R or CCK2R or both following incubation with either monovalent ligands or bivalent ligands. None of the monovalent or bivalent ligands had a toxic effect on any of the cell lines at a 1 nmol/L concentration. However, at 1  $\mu$ mol/L, there was reduced viability in dual receptor expressing cells following 24 and 48 hours of treatment with either CCK or the heterobivalent ligands (Supplementary Fig. S5A-F).

#### Discussion

Expressing pairs of GPCRs in heterologous cell systems is a useful approach to investigate and characterize the binding of bivalent pharmacophores (27). Pairs of functionally related GPCR subtypes have been coexpressed in the same cell, such as the  $\beta_2$ - and  $\beta_3$ -adrenoceptors (28), and the CXC chemokine receptors, CXCR1 and CXCR2 (29). The current work used heteroexpression of GPCRs as system for proof-of-concept studies to evaluate heterobivalent ligand binding.

In the current study, we readily developed a stable cell line expressing both the MC4R and the CCK2R receptor in the Hek293 cell line. It seems reasonable to speculate that MC4R and CCK2R receptors may have a functional



relationship as both are involved in the control of meal size and food intake (30, 31). Although the other receptor pair MC4R and  $\delta$ OR are expressed in the brain, they are unlikely expressed in the same cells, as their expression profiles are unique, and they are functionally unrelated. Establishing stable cell lines expressing both MC4R and  $\delta$ OR receptors was a particularly elusive challenge. Several different expression systems, including two COOH-terminal truncated receptors (32) and a pDisplay vector (33), were investigated in six cell lines with different biochemistries (34–36) without success. However, it was interesting to observe that one cell clone achieved high expression levels of both receptors detected by ligand binding assay, but this clone underwent a distinctive morphologic change, which led to cell death after 20 days. These observations have led us to surmise that cross-talk prevented coexpression of these heterologous receptors in the same cell. Numerous recent studies have indicated that cross-talk can occur between GPCR classes, which may give rise to synergistic or more complicated effects (37–40). For the receptor pair MC4R and  $\delta$ -opioid, it is possible that there may be cross-talk between the receptors that results in a proapoptotic signal. Further experiments will be needed to confirm this molecular mechanism.

To test the dual expressing systems, heterobivalent ligands with MSH7-deltorphin and CCK6-MSH7 were synthesized by the solid phase method. The constructs tested were part of larger families of heterobivalent constructs that are described elsewhere (41). These were connected by proline-glycine repeats as the core of the linker unit (Fig. 1). In one case (MC4R- $\delta$ OR), these were tested in the same cell line in the presence and absence of heterologous blockade, and in the other (CCK2R-MC4R), these were tested in cell lines that expressed either one or both receptors. It is interesting to observe that, in both cases, the receptor with lower abundance (MC4R) showed significant enhancement in the binding affinity when both receptors were available (dimer) compared with cases where only the single receptor was available (monomer), which indicated the heterobivalent ligands were engaged and bound to both receptors. The finding that no enhancement was observed when binding was determined at the higher abundance receptor is expected (Supplementary Fig. S4). After the lower abundance receptors are saturated by bivalent interactions, the remaining higher abundance receptors are only available to bind the ligands as monomers, which is weak binding. This finding is in agreement with the mathematical model that the absolute number of receptors and the ratio of receptor expression on the target cell is critical to achieving specificity (42).

Previous work from our laboratory using homobivalent ligands with relatively short linker lengths showed enhancement by “statistical” binding, where affinity is increased simply via increased local concentration and not receptor “cross-linking” (13, 20, 21, 43). To discriminate receptor cross-linking effects from statistical effects, a series of MSH7-deltorphin heterobivalent ligands with variable linker lengths and rigidities were designed, synthesized, and screened for binding affinities using a cell system that expressed both the MC4R and the  $\delta$ OR as described in this

study (41). A construct with the optimal linker length for binding enhancement, i.e., DeltII-[PG]<sub>15</sub>-MSH7 with a 90-atom linker, was used in the current study. Short linker constructs evidenced no enhancement. A similar series of CCK6-MSH7 heterobivalent ligands were also designed, synthesized, and screened. The optimally binding construct, i.e., MSH7-Pego-[PG]<sub>6</sub>-Pego-CCK6 (76 atoms), was used herein and exhibited potent binding compared with compounds with shorter linkers, i.e., MSH-Pego-CCK6 (20 atoms) and MSH-Pego-Pego-CCK6 (40 atoms), which exhibited no significant binding enhancement between single and dual expressing cells (data not shown).

In addition to the efficient cell binding of these heterobivalent ligands, the internalization of these ligand-receptor complexes will be important for drug targeting, imaging, and eventual delivery of therapies. Internalization of these heterobivalent constructs would amplify any potential imaging signal, and could equally allow for payloads to be delivered to the cell nuclei. In addition to delivering a therapeutic payload, it was equally important to assess whether these novel agents induced any toxicity of their own. Although cytotoxicity may often be a goal in cancer, these agents may also be useful to deliver positive therapies, such as gene replacements to the nucleus (vide supra). Notably, the bivalent MSH7-CCK6 ligand used in this study showed no reduction in viability at either low (1 nmol/L) or high doses (1  $\mu$ mol/L) in the nontargeted cells, yet there was toxicity in the target cells. This toxicity was also observed with CCK alone, suggesting that the effect was not specific to receptor cross-linking and was a product of engaging the CCK-R. This is an area that warrants further investigation.

In summary, we have developed a system to investigate the effects of the binding of heterobivalent ligands to two different cell surface receptors in target cells (i.e., those that coexpress both receptors). Such an approach may be useful for the development of potent ligands for targeting physiologically relevant receptor combinations, especially in cancers, such as Her2/Her3, Her2/Her4, etc. Using the *in vitro* cell systems described herein, developing the multivalent ligands for target combinations in pancreatic cancer (44) are under way.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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