Enhanced targeting with heterobivalent ligands

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Abstract
A novel approach to specifically target cancer cells for
detection and treatment is the proposed use of hetero-
multivalent ligands, which are designed to interact with,
and noncovalently crosslink, multiple different cell sur-
face receptors. Although enhanced binding has been
shown for synthetic homomultivalent ligands, proof of
cross-linking requires the use of ligands with two or more dif-
ferent binding moieties. As proof-of-concept, we have ex-
amined 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 melan-
ocortin 4 receptor (MC4R) expressed in combination with ei-
er the human δ-opioid receptor (δOR) or the human
cholecystokinin-2 receptor (CCK2R). Expression levels of
these receptors were characterized by time-resolved fluores-
cence saturation binding assays using Europium-
labeled ligands; Eu-DPCLCE, Eu-NDP-α-MSH, and Eu-CCK8
for the δOR, MC4R, and CCK2R, respectively. Heterobiva-
 lent ligands were synthesized to contain a MC4R agonist
connected via chemical linkers to either a δOR or a CCK2R
agonist. In both cell systems, the heterobivalent constructs
bind with much higher affinity to cells expressing both re-
ceptors, 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 combina-
tions. The in vitro cell models described herein will lead to
the development of multivalent ligands for target combina-
tions 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 inhibi-
tors, and antibodies, which are in various stages of clinical
development (1–3).

Although these approaches have resulted in clinical ben-
efit for selected cancer subtypes, there are a number of limi-
tations 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 combi-
nations 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 anti-
bodies (mAbs) have found success in the clinic, the high
molecular weight of mAbs is a drawback to their multimer-
ization (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 multi-
ple copies of the same ligand, or they can be heteromultiva-
 lent, with different types of ligands targeted to different
types of receptors. Previous work has shown that homo-
 multivalent 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,
heteromultivalent constructs cannot unequivocally distin-
guish statistical proximity effects from the noncovalent
crosslinking (clustering) of receptors, which would be
needed for heteromultivalent interactions. Thus, demon-
stration of receptor noncovalent crosslinking requires the
use of heteromultivalent constructs.
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 δ-opioid receptor (δOR), the human melanocortin receptor subtype 4 (MC4R), and the human cholecystokinin-2 receptor (CCK2R). These were coexpressed in combinations of MC4R + δOR 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 δOR receptors and were characterized by lanthanide-based time-resolved fluorescence saturation binding assay using Europium-labeled monomeric ligands: Eu-NDP-α-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% CO₂. All cell lines except (Hek293/MC4R) was digested with Kpn I/Bgl II and ligated into the Kpn I/Bgl II restriction enzyme sites. A Kozak sequence (A/G NNN 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 α 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 δOR cDNA was provided by Henry Yamamura (University of Arizona, Tucson, AZ) and digested with Apa I and Eco R 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 δOR cDNA insert was subcloned between the cytomegalovirus promoter and SV40 poly(A) of pBudCE-MC4R. The resulting plasmid, termed pBudCE-MC4R-δOR, was verified using restriction enzyme analysis and DNA sequencing.
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/Zeoc(+) vector at the HindIII and XhoI sites. The constructs mentioned above were verified via DNA sequencing.

**Construction of Cell Lines Coexpressing MC4R and δOR (CHO/MC4R/δOR Cells)**

Cell transfections were done using FuGENE 6 transfection reagent (Roche; 1814-443). For establishment of cell lines transiently expressing both MC4R and δOR 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-δOR vector. Forty-eight hours after transfection, cells were tested for MC4R and δOR cell surface expression by MC4R and δOR binding assay using Eu-NDP-α-MSH and Eu-DPLCE, respectively, as described previously (20, 21).

### List of compounds and their structures used in this study.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
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<tr>
<td>Eu-NDP-α-MSH</td>
<td>Eu-(DTPA)-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH₂</td>
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<tr>
<td>Eu-DPLCE</td>
<td>H-Tyr-[DPen-Gly-Phe-Cys]-Phe-Lys(Eu-(DTPA))-NH₂</td>
</tr>
<tr>
<td>Eu-CCK8</td>
<td>Eu-(DTPA)-Asp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH₂</td>
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<td>Delt II-[PG]₃-MSH7</td>
<td>²H-Deltorphin II-[Pro-Gly]₃-MSH7-NH₂</td>
</tr>
<tr>
<td>MSH7-Pego-[PG]₃-Pego-CCK6</td>
<td>³Ac-MSH7-Pego-[Pro-Gly]₃-Pego-CCK6-NH₂</td>
</tr>
<tr>
<td>MSH7-Pego-[PG]₃-K(Cy5)-Pego-CCK6</td>
<td>Ac-MSH7-Pego-[Pro-Gly]₃-Lys(Cy5)-Pego-CCK6-NH₂</td>
</tr>
</tbody>
</table>

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

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Transient transfection efficiencies were determined by cotransfection of pBudCE-MC4R-6OR 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 transfecteds 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; 40430) and 0.4 mg/mL genetin (Life Technologies; 11811-031). Clones were tested for ligand binding at both receptors using Eu-CCK8 and Eu-NDP-α-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-α-MSH, 10 μmol/L Naloxone, or 1 μmol/L CCK8, respectively. Non-specific binding was determined in the presence of 10 nmol/L NDP-α-MSH, 10 μmol/L Naloxone, or 1 μmol/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]15-MSH7 ligand competed with known amounts of Eu-labeled ligand (10 nmol/L Eu-NDP-α-MSH or 10 nmol/L Eu-DPLCE), or the MSH7-Pego-[PG]6-Pego-CCK6 ligand competed with 10 nmol/L Eu-NDP-α-MSH or 0.1 nmol/L Eu-CCK8.

For screening, one-point ligand binding assays were done for each clone. Respectively, MC4R, δOR, or CCK2R expression was determined by incubation with 10 nmol/L Eu-NDP-α-MSH, 10 nmol/L Eu-DPLCE, or 0.1 nmol/L Eu-CCK8 in the presence or absence of unlabeled 10 μmol/L NDP-α-MSH, 10 μmol/L Naloxone, or 1 μmol/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, RTMC4Rab) recognizes an epitope on the cell exterior. Hence, live cells were incubated with this antibody (10 μg/mL) for 5 min, washed in antibody-free buffer (2 × 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 nmol/L glycine and permeabilized with 0.1% TritonX-100 (22). Antibodies for δOR (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 δOR, MC4R-labeled coverslips were incubated sequentially with the primary antibody to δOR, 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× concentration of nonlabeled anti-rabbit IgG to block free anti-rabbit IgG sites remaining on the MC4R-specific polyclonal antibody, washed (3 × 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 × 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 μmol/L of NDP-α-MSH, or CCK8 or MSH7-Pego-[PG]6-Pego-CCK6 for 24 or 48 h. Visible 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, Kd...
values were determined after correction for nonspecific binding as the concentration that yielded half-maximal binding. For competitive binding assays, the IC50 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 δOR**

Although tremendous efforts were made to construct a cell line stably expressing both MC4R and δOR, this was not achieved (data not shown). However, the MC4R and δOR were observed to coexpress well in CHO cells for a short period of time using the pBudCE-MC4R-δOR 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 δOR with a KD of 10.5 ± 2.6 nmol/L and Bmax of 24,000 ± 2,000 AFU (R² = 0.91). Points, average of four samples; bars, SE. B, saturation binding assay of Eu-NDP-α-MSH ligand to MC4R in CHO/MC4R/δOR cells. The curve shows MC4R-specific binding only. From these data, the KD of 5.6 ± 2.7 nmol/L and Bmax of 7,700 ± 1,400 AFU (R² = 0.83). C and D, competitive binding to CHO cells coexpressing MC4R and δOR. C, increasing concentrations of Naloxone were added in the presence of 10 nmol/L Eu-DPLCE. From these data, the IC50 was 65 nmol/L with R² = 0.90. D, increasing concentration of NDP-α-MSH were added to cells in the presence of 10 nmol/L Eu-NDP-α-MSH. From these data, IC50 was 0.77 nmol/L with R² of 0.89.

Figure 2. MC4R and δOR saturation binding and competitive binding analysis. A, saturation binding of Eu-DPLCE ligand to δOR in CHO/MC4R/δOR cells. The curve shows δOR specific binding only (total-nonspecific). From these data, the KD of 10.5 ± 2.6 nmol/L and Bmax of 24,000 ± 2,000 AFU (R² = 0.91). Points, average of four samples; bars, SE. B, saturation binding assay of Eu-NDP-α-MSH ligand to MC4R in CHO/MC4R/δOR cells. The curve shows MC4R-specific binding only. From these data, the KD of 5.6 ± 2.7 nmol/L and Bmax of 7,700 ± 1,400 AFU (R² = 0.83). C and D, competitive binding to CHO cells coexpressing MC4R and δOR. C, increasing concentrations of Naloxone were added in the presence of 10 nmol/L Eu-DPLCE. From these data, the IC50 was 65 nmol/L with R² = 0.90. D, increasing concentration of NDP-α-MSH were added to cells in the presence of 10 nmol/L Eu-NDP-α-MSH. From these data, IC50 was 0.77 nmol/L with R² of 0.89.

To further investigate these dual expressing cells, competitive binding assays were done (Fig. 2C and D). Naloxone or NDP-α-MSH effectively displaced Eu-DPLCE or Eu-NDP-α-MSH with IC50 values of 65 nmol/L (R² = 0.90) or 0.77 nmol/L (R² = 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 IC50 value for NDP-α-MSH to displace Eu-DPLCE or Eu-NDP-α-MSH with IC50 values of 65 nmol/L (R² = 0.90) or 0.77 nmol/L (R² = 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 IC50 value for NDP-α-MSH to displacement Eu-NDP-α-MSH was 0.65 nmol/L when δOR was blocked with Naloxone, which is similar to the IC50 value of 0.77 nmol/L measured in the absence of naloxone (as shown in Fig. 2D). Similarly, the IC50 of Naloxone to displace Eu-DPLCE from the δOR was 55 nmol/L in the presence of excess NDP-α-MSH, compared with an IC50 value of 65 nmol/L in the absence of NDP-α-MSH (Fig. 2C).
Hence, blocking the δOR 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-δOR plasmid and the percentage of cells expressing GFP was measured 48 hours after transfection. Transfection efficiency was 33 ± 4% (n = 9), which is similar to that typically achieved using lipid-based transfection reagents.

Expression of δOR 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 δOR allowed access to intracellular compartments, a significant amount of δOR 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-α-MSH bound to Hek293/MC4R/CCK2R cells with a Kd of 8.3 ± 1.9 nmol/L and a Bmax of 732,000 ± 59,000 AFU, as shown in Fig. 4A. This Kd 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 Kd of 34.6 ± 3.9 nmol/L, and a Bmax of 1,600,000 ± 83,000 AFU (Fig. 4B). This Kd value was consistent with that observed in the cell line expressing the CCK2R alone, e.g., Kd of 38.6 ± 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 μmol/L for both images pairs.
Determination of Receptor Numbers on Cells

Standard curves for Eu-labeled ligands were generated by adding increasing amounts of Eu-NDP-α-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 Bmax 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/δOR cells, the Bmax was 7,700 ± 1,400 AFU, which corresponds to 0.76 ± 0.14 fmol per well. For δOR binding, the Bmax was 24,000 ± 2,000 AFU which corresponds to 4.30 ± 0.36 fmol per well. The average cell number per well was 75,000 ± 2,300 cells (n = 4). After correcting for transfection efficiency (33 ± 4%), it was calculated that there were 18,000 ± 3,300 MC4R/cell and 100,000 ± 8,600 δOR/cell. Thus, δOR outnumbered MC4R by a ratio of ~6:1.

Taking a similar approach, receptor numbers were determined in Hek293/MC4R/CCK2R cells. It was calculated that there were 1,100,000 ± 56,000 CCK2 receptors/cell and 640,000 ± 52,000 MC4R receptors/cell in this stably expressing line. Hence, the CCK2 receptors outnumbered MC4R by a ratio of ~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 blockade using excess unlabeled ligand. This system was used to test the binding of the heterobivalent MSH-δOR ligand.

Heterobivalent ligands were prepared to target the MC4R-δOR and MC4R-CCK2R combinations. DeltII-[PG]15-MSH7 was constructed and tested for binding to the MC4R and δOR receptors as described above. This ligand competed Eu-NDP-α-MSH with an IC50 value of 3.3 ± 1.8 nmol/L and 159.6 ± 46.3 nmol/L in the absence and presence of naloxone (an δOR antagonist), respectively (number of replicates, n = 5, P < 0.01). Figure 6A shows a representative binding curve in competition with Eu-NDP-α-MSH in the absence (dimer) and presence (monomer) of naloxone. The DeltII-[PG]15-MSH7 ligand competed with Eu-DPLCE with IC50 values of 230 ± 74 nmol/L and 500 ± 90 nmol/L in the absence and presence of excess NDP-a-MSH (n = 5, P > 0.05), respectively. From the hMC4R data, the heterodimer bound with much higher affinity when both complimentary receptors are available, compared with its binding when the δOR was blocked.

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 complimentary receptor and another that expresses both of the complimentary receptors, whereas another approach uses the same cell type but blocks binding at one receptor by addition of an agent (B1 and B2).

Dual Expression GPCRs for Heterobivalent Ligands

Figure 4. MC4R and CCK2R saturation binding analysis. A, saturation curve of Eu-NDP-α-MSH obtained from the MC4R and CCK2 dual expression cell line. The figure shows total binding (●) and binding in the presence of 10 μmol/L NDP-α-MSH (▲). From these data, the Kd of 8.3 ± 1.9 nmol/L and Bmax of 732,000 ± 59,000 AFU. Lines, the computer modeled best fit of the data using GraphPad Prism software using the nonlinear regression, one-site-binding equation, with a R2 value of 0.81. Points, the average of four samples; bars, SE. B, saturation curve of Eu-CCK obtained from the MC4R and CCK2 dual expression cell line. The figure shows total binding (●) and binding in the presence of 1 μmol/L CCK8 (▲). From these data, the Kd of 34.8 ± 3.9 nmol/L, and Bmax of 1,600,000 ± 83,000 AFU, with a R2 value of 0.96.
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 contrast, binding to the δOR 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 δOR can be thus occupied. The remaining 5 of 6 of the δOR receptors thus must bind monovalyent. In all, these data are consistent with receptor cross-linking and heterobivalent interactions (Supplementary Fig. S4). Notably, a short linker ligand, DelIII-[PG]3-MSH7 (18-atom long linker) showed no significant increase of binding affinity between MC4R monovalent (IC50, 110 ± 9 nmol/L) and bivalent (IC50, 180 ± 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]6-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-α-

MSH as a monomer with an IC50 value of 251.4 ± 38.8 nmol/L (n = 5) and as a dimer with an IC50 value of 3.1 ± 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 IC50 value of 190.0 ± 51.3 nmol/L and 167.5 ± 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, receptor distribution was followed (Fig. 3C). By 10 minutes, all ligand was observed to be in punctate structures assumed to be receptor capturing zones (Fig. 3D). To determine if the receptor/ligand complex was significantly internalized, CCK ligand (50 μmol/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 μmol/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 cooverexpressed in the same cell, such as the β2- and β2-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 δOR 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 δOR 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 δ-opiod, 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-δOR), 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 δOR as described in this study (41). A construct with the optimal linker length for binding enhancement, i.e., DelIII-[PG]15-MSH 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]22-Pego-CCK6 (76 atoms), was used herein and exhibited potent binding compared with compounds with shorter linkers, i.e., MSH-Pego-[PG]6-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 μmol/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.

References

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