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# Development of a time-resolved fluorescence probe for evaluation of competitive binding to the cholecystokinin 2 receptor

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ABSTRACT

The synthesis, characterization, and use of Eu-DTPA-PEGO-Trp-Nle-Asp-Phe-$NH_2$ (Eu-DTPA-PEGO-CCK4), a luminescent probe targeted to cholecystokinin 2 receptor (CCK2R, aka CCKBR), are described. The probe was prepared by solid phase synthesis. A $K_d$ value of 17 ± 2 nM was determined by means of saturation binding assays using HEK-293 cells that overexpress CCK2R. The probe was then used in competitive binding assays against Ac-CCK4 and three new trivalent CCK4 compounds. Repeatable and reproducible binding assay results were obtained. Given its ease of synthesis, purification, receptor binding properties, and utility in competitive binding assays, Eu-DTPA-PEGO-CCK4 could become a standard tool for high-throughput screening of compounds in development targeted to cholecystokinin receptors.



## 1. Introduction

The cholecystokinin 2 receptor (CCK2R, aka CCKBR) is a member of the GPCR family expressed in several cancers, including medullary thyroid carcinomas, small-cell lung cancers, gastroenteropancreatic neuroendocrine cancers, stromal ovarian cancers, astrocytomas, and gastrointestinal stromal cancers.[1] In many of these cancers, CCK2R is overexpressed on malignant cell surfaces,[2,3] providing an opportunity for targeted imaging and/or therapy. One strategy for detecting overexpression is to employ simultaneous binding of two or more weak ligands presented on a single scaffold. Such multivalent molecules can selectively bind with high avidity to cells overexpressing the targeted receptors.[4,5]

When developing a new series of compounds, rapid screening for binding efficacy using a high-throughput competitive binding assay is a useful strategy for hit selection. By this method, the binding efficacies of a series of unlabeled molecules may be evaluated against a single labeled probe targeted to the same receptor. While many radiolabelled ligands that bind to CCK receptors have been reported,[6–9] luminescent probes targeted to CCKRs were only recently described. Probes bearing lanthanide chelates have become popular due to their sensitivity and the avoidance of radioactive materials.[10–13] They are of special importance in applications where background signal is a significant problem. During the past decade several europium-diethylenetriaminepentaacetic acid (Eu-DTPA) chelates linked to peptide recognition elements have been reported.[10,13–16] These probes were used to evaluate multivalent molecules targeted to melanocortin or cholecystokinin receptors via dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA)-based ligand binding assays.[17] The functional readout of such assays comes from the time-resolved fluorescence (TRF) generated by the europium ions. While TRF probes **1** and **2** based on a CCK8 recognition element[16] have previously been used to characterize ligand binding to CCK2R in competitive binding assays,[18–20] a redesign of these probes could simplify and improve probe preparation and purification, possibly enhance probe solubility and receptor binding, and provide an additional tool for use in bioassays.

*Abbreviations:* CCK4, Trp-Nle-Asp-Phe-$NH_2$; Cl-HOBt, 1-hydroxy-6-chlorobenzotriazole; CuAAC, copper-catalyzed azide–alkyne cyclization; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; DIC, diisopropyl carbodiimide; DMEM, Dulbecco's Modified Eagle Medium; DTPA, diethylenetriaminepentaacetic acid; ESI, electrospray ionization; ICR, ion cyclotron resonance; hCCK2R, human cholecystokinin 2 receptor; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; hMC4R, human melanocortin 4 receptor; HOBt, 1-hydroxybenzotriazole; PEGO, 19-amino-5-oxo-3,10,13,16-tetraoxa-6-azanonadecan-1-oic acid; TBTA, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine; TACP, tetrakis(acetonitrile)copper(I) hexafluorophosphate; TRF, time-resolved fluorescence.

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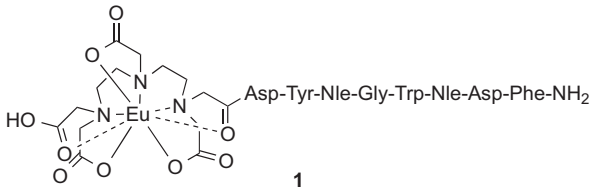


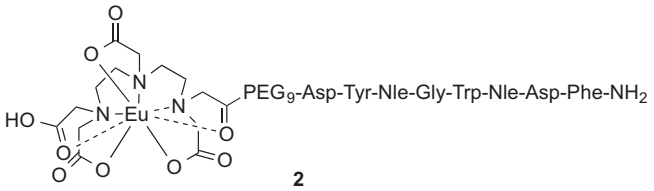


In designing probes for characterization of multivalent binding, we employ the minimum peptide sequence that gives an acceptable level of receptor binding on the theory that a binding competition should involve recognition elements of similar binding affinity. This strategy also minimizes the number of synthetic steps required for probe assembly. In this manner we recently developed a new and useful TRF probe for the evaluation of ligand binding to human melanocortin 4 receptors (hMC4R) and established a robust in vitro binding assay protocol.[15,21] In the present work, we describe a new TRF probe for high-throughput screening of compounds for binding to CCK2R and extend the aforementioned binding assay protocol to bioassays involving this receptor.

## 2. Materials and methods

### 2.1. General

Commercial reagents were used as supplied unless otherwise noted. Dichloromethane (DCM) and tetrahydrofuran (THF) were dried by passage through activated alumina. Dimethylsulfoxide (DMSO) and *N*,*N*-dimethylformamide (DMF) were dried by contact with activated 4 Å molecular sieves, followed by distillation under reduced pressure. Analytical thin-layer chromatography (TLC) was carried out on pre-coated silica gel 60 F-254 plates with staining by 10% phosphomolybdic acid solution in ethanol or aqueous potassium permanganate solution and heat. Column chromatography was performed using silica gel 60 (200–400 mesh). Melting points were recorded on an Electrothermal® Mel-Temp® apparatus (Model 1001) and are uncorrected. IR spectra were recorded on a Thermo Nicolet iS5 FT-IR Spectrophotometer using NaCl plates. NMR spectra were recorded at 500 MHz for $^{1}$H NMR and at 125 MHz for $^{13}$C NMR on a Bruker DRX-500 NMR instrument. Chemical shifts ($\delta$) are expressed in ppm and are internally referenced to chloroform (7.26 ppm and 77.16 ppm for $^{1}$H and $^{13}$C NMR, respectively). Reactions under microwave irradiation utilized a Biotage Initiator 2.0 microwave reactor. Preparative scale reversed-phase HPLC was performed using a 19 × 250 mm Waters XBridge™ 10 μm OBD™ $C_{18}$ preparative HPLC column. The flow rate was 10 mL/min and a dual channel UV detector was used at 230 and 280 nm. Analytical HPLC was performed on a 3.0 × 150 mm Waters XBridge™ 3.5 μm $C_{18}$ analytical HPLC column. For the analysis of compound **3**, a linear gradient of mobile phase from 10% to 90% MeCN/triethylammonium acetate buffer (0.1% v/v of triethylamine in HPLC grade water adjusted to pH = 6.0 by the addition of acetic acid) was used over 30 min. For all other compounds, a linear gradient of mobile phase from 10% to 90% MeCN/water containing 0.1% TFA was used over 30 min. The flow rate was 0.3 mL/min and a dual channel UV detector was used at 220 and 280 nm. A VWR SympHony™ pH meter (Model SB20) equipped with a Ag/AgCl electrode was used for pH measurements. ESI experiments were performed on a Bruker 9.4 T Apex-Qh hybrid Fourier transform ion-cyclotron resonance (FT-ICR) instrument using standard ESI conditions. Samples were dissolved in MeCN/water (1:1) or MeCN/MeOH (1:1) containing 0.1% formic acid in a concentration range of 1–30 μM. HEK-293 cells engineered[18] to overexpress both hCCK2R and hMC4R were used to measure the affinity of the probe for binding to hCCK2R by means of saturation binding assays. All compounds evaluated in bioassays had purities of ⩾95% as determined by HPLC. Unless otherwise specified, all cell incubations were done in a Fisher Scientific™ Isotemp™ $CO_2$ incubator (Model 3530) maintained at 37 °C and 5% $CO_2$ atmosphere. Europium-based TRF competitive binding assays were employed to study the binding of all multivalent constructs and controls. Centrifugations were performed using a VWR Galaxy 7 microcentrifuge or a Fischer Scientific Model 59A microcentrifuge. TRF was measured using a VICTOR™ X4 2030 Multilabel Reader (PerkinElmer) employing the standard Eu TRF measurement settings (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm).

### 2.2. Solid-phase synthesis

#### 2.2.1. Resin-bound protected CCK4 peptide 4

For the synthesis of probe **3** (see Scheme 1) and compounds **6**, **10**, **12**, and **14** (see Schemes 2 and 3), resin-bound protected CCK4 peptide **4** was synthesized manually via an $N^{\alpha}$-Fmoc solid-phase peptide synthesis strategy on Rink amide AM resin (200–400 mesh, 0.68 mmol/g loading). Resin (1 g) was allowed to swell in THF for 1 h in a polypropylene syringe equipped with a polypropylene frit. THF was removed, a solution of 20% piperidine in DMF (15 mL) was added, and the tube was shaken for 2 min. This solution was removed, 20% piperidine in DMF (15 mL) was again added, and the mixture was shaken for 18 min. After removal of the solution, the resin was washed sequentially with DMF (3 × 15 mL), DCM (3 × 15 mL), DMF (3 × 15 mL), 0.5 M HOBt in DMF (15 mL), 0.5 M HOBt in DMF + one drop of 0.01 M bromophenol blue solution in DMF (15 mL), DMF (2 × 15 mL), and DCM (15 mL) in that order. Unless otherwise specified, all resin wash steps in the solid phase peptide synthesis were done by shaking the resin in contact with the wash solvent for 1 min. The amino acid (3 equiv) to be coupled was activated by reaction in a glass vial with 1-hydroxy-6-chlorobenzotriazole (Cl-HOBt, 3 equiv) and diisopropyl carbodiimide (DIC, 6 equiv) in DMF (15 mL) over two min. This solution was then added to the resin and the syringe shaken for 1 h. The coupling solution was removed and the resin was washed with DMF (3 × 15 mL), DCM (3 × 15 mL), and DMF (3 × 15 mL). Free amine groups were capped by shaking the resin with acetic anhydride/pyridine (1:1, 6 mL) for 20 min. The resin was washed with DMF (3 × 15 mL), DCM (3 × 15 mL), and DMF (3 × 15 mL). The coupling cycle was then repeated for each of the remaining amino acids in the sequence. The Kaiser test[22] was used to determine coupling completion at each attachment step. Following the four coupling cycles, resin-bound **4** was obtained in its protected form.

#### 2.2.2. DTPA-PEGO-CCK4 (5)

The PEGO linker was attached to a portion (~0.34 mmol) of resin-bound peptide **4** by a conventional coupling cycle following activation of Fmoc-PEGO-OH (Novabiochem 8510310001, 1.02 mmol in 2.04 mL of DCM) with Cl-HOBt (173 mg, 1.02 mmol) and DIC (256 mg, 2.04 mmol) in DMF (5 mL). After 2 h, coupling completion was ascertained by the Kaiser test, and the resin was washed sequentially with DMF (3 × 8 mL), DCM (3 × 8 mL), and DMF (3 × 8 mL).