Supplementary Information

Development of a Testing Funnel for Identification of Small Molecule Modulators Targeting Secretin Receptors

Daniela G. Dengler[1, *], Qing Sun[1], John Holleran[1], Sirkku Pollari[1], Jannis Beutel[2], Brock T. Brown[1], Aki Shinoki Iwaya[1], Robert Ardecky[1], Kaleeckal G. Harikumar[3], Laurence J. Miller[3] and Eduard A. Sergienko[1, *]

[1]Conrad Prebys Center for Chemical Genomics, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California, USA.

[2]Department of Chemistry and Pharmacy, Chemikum, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany.

[3]Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, Arizona, USA.

*Correspondence to: ddengler@sbpdiscovery.org; esergien@sbpdiscovery.org.

Methods

Plasmids and construct generation by Flexi-Cloning (Promega)

Fusion vectors ARRB2-LgBiT (CS1603B121, CMV/HygR/KanR) and LgBiT-ARRB2 (CS1603B121, CMV/HygR/KanR) containing human arrestin beta 2 (ARRB2) coding sequence (NM_004313) as well as Flexi vectors pF4A CMV Flexi (AY753580, CMV/AmpR) and pFC220K SmBiT Flexi Vector (CS1603B33C, CMV/BlastR/KanR) were obtained from Promega (Promega Corp., Madison, WI, USA).

In general, Promega's technical manual "Flexi Vector Systems" was followed to generate pFC220K-SCTR-SmBiT and pFC220K-AVP2R-SmBiT fusion vectors. Exemplary for pFC220K-SCTR-SmBiT in brief, forward primer (ttccGCGATCGCCACCATGCGTCCCCACCTGT) and reverse primer (ttccGTTTAAACGATGATGCTGGTCCTGCAGG) were used to PCR-amplify the coding sequence of human SCTR between Flexi restriction sites SgfI and PmeI. PCR was performed on 10ng/µL pcDNA3-SCTR (NM_002980.2) using Q5 High Fidelity DNA Polymerase (NEB, M0491S) according to manufacturer's instructions. PCR reaction was performed in a Thermal Cycler (Eppendorf), whereby initial denaturation was at 98 °C for 30 sec, followed by 30 cycles of 98°C for 10 sec (denaturation), 72°C for 30 sec (annealing), and 72°C for 1 min 15 sec (elongation) and at the end a final extension at 72°C for 2 min. PCR product was purified by gel and diluted in 20 µL nuclease-free water. Digestion of SCTR-Flexi PCR product and pF4A CMV acceptor Flexi vector was performed using Flexi Enzyme Blend (SgfI & PmeI, Promega, R1851)

according to Promega's technical manual. After 30 minutes at 37°C, SCTR-product digestion reaction was column purified using Zymo PrepKit DNA Clean & Concentrator and pF4A digestion reaction was heat inactivated at 65°C for 20 min. Ligation reaction was carried out using T4 DNA Ligase (NEB, M0202) on around 50 ng of acceptor Flexi vector pF4A and 100 ng SCTR-PCR product at RT for 1 h. After transformation reaction in NEB 5-alpha competent E. coli (High Efficiency) cells (NEB, C2987H) according to manufacturer's protocol, cells were plated on LB Agar Plates Ampicillin-100 (Teknova, L1004) and incubated overnight at 37°C. Several clones were picked and sent to direct colony sequencing at Eton Bioscience (San Diego, CA). Clones containing SCTR sequence were grown in LB Broth with Ampicillin and used for plasmid isolation applying NucleoSpin Plasmid Mini kit for plasmid DNA (Macherey-Nagel GmbH & Co. KG, l, Düren, Germany, #740588.50) yielding pF4A-SCTR with a final concentration of 0.24 µg/µL in nuclease-free water. According to technical manual, 200 ng pF4A-SCTR and 400 ng pFC220K SmBiT Flexi vector were digested using Flexi Enzyme Blend (SgfI & PmeI, Promega, #R1851) or Carboxy Flexi Enzyme Blend (SgfI & EcoICRI, Promega, #R1901), respectively. Heat inactivation was performed at 65°C for 20 min. Ligation reaction was carried out using T4 DNA Ligase (NEB, #M0202) on around 50 ng of both digestion products at RT for 1 h. After transformation reaction in 5-alpha competent E. coli (High Efficiency) cells (NEB, #C2987H) according to manufacturer's protocol, cells were plated on LB Agar Plates Kanamycin-50 (Teknova, #L1025) and incubated overnight at 37°C. Several clones were picked and sent to direct colony sequencing at Eton Bioscience (San Diego, CA). Clones containing SCTR sequence were grown in LB Broth with Kanamycin and used for plasmid isolation employing NucleoSpin Plasmid Mini kit for plasmid DNA yielding fusion vector pFC220K-SCTR-SmBiT in a final concentration of 0.28 µg/µL in nuclease-free water. pFC220K-AVP2R-SmBiT was prepared following protocol for pFC220K-SCTR-SmBiT employing forward primer (ttccGCGATCGCCACCATGCTCATGGCGTC) and reverse primer (ttccGTTTAAACCGATGAAGTGTCCTTGGCCA) on pcDNA3.1 vector containing coding sequence for human AVP2R (NM_000054.6) for PCR amplification.

Cell line generation and clonal selection

HEK-293 SCTR-SmBiT LgBiT-ARRB2

To generate a stably expressing cell line, Hygromycin and Blasticidin were added to growth media 24 h post-transfection for a time period of over two weeks. Once Hygromycin/Blasticidin resistant cells were sufficiently proliferating, cells were harvested, re-suspended in growth media to a cell density of 10 cells/mL and dispensed into a 384-well microplate (Greiner #781098) using a 16-channel Finnpipette (Thermo Fisher Scientific), 50 µL/well. Plate was incubated at 37 °C and 5% CO_2 for approximately two weeks. After the average of cell clones reached confluency, plate was duplicated and after incubation overnight, Sec-FL and NanoBiT detection reagent were added to each well using Echo liquid dispenser. Several clones were selected in accordance with

luminescence detected by Pherastar FSX. Clones were expanded in growth media and further characterized by β-arrestin-2 recruitment assay.

HEK-293 SCTR CRELuc

HEK-293 SCTR CRELuc cells were obtained by transient transfection of HEK-293 cells with pcDNA3(+) SCTR (NM_002980.2) and pGL4.29 pGL4.29[luc2P/CRE/Hygro] Vector (Promega part number E8471). HEK-293 cells were seeded in a 6 well plate at 600,000 cells/well in 2mL total volume in HEK cell growth media (DMEM, 10% FBS, 1% Pen/Strep, 1% L-Glutamine). After 3h at 37°C and 5% CO2 incubation transfection complexes were prepared using 8µL of TransIT-LT1 transfection Reagent (Mirusbio, Madison, WI, USA, #MIR2300), 100µL Opti-MEM, and 1µg DNA of each construct per well. Complexes were formed at room temperature for 10 minutes then added drop wise to the 6 well plate. To generate a stably expressing cell line, Hygromycin and G418 antibiotics were added to the growth media 24h after transfection for a time period of over two weeks. After Hygromycin/G418 resistant cells were sufficiently proliferating, cells were harvested, re-suspended in growth media to a cell density of 10 cells/mL and dispensed into a 384-well microplate (Greiner #781098) using a 16-channel Finnpipette, 50 µL/well. Plate was incubated at 37 °C and 5% CO_2 for approximately two weeks. Once the average of cell clones reached confluency, clones were expanded in growth media and further characterized by CRE-Luc2P luciferase activity assay using either forskolin or Sec-FL.

HEK-293 SNAP-SCTR

Coding sequence of human SCTR (NM_002980.2) with N-terminal SNAP-tag but eliminated signal peptide was cloned into pcDNA3.1(+) vector by GenScript (Piscataway, NJ, USA, customized order) using 5'BamHI-3'XbaI restriction sites resulting in pcDNA3.1(+) SNAP-SCTR.

HEK-293 cells in HEK cell growth media were seeded into a 6-well plate at a cell density of 0.3M/well and were incubated overnight at 37 °C and 5% CO_2. Following manufacturer's manual pcDNA3.1(+) SNAP-SCTR was transfected using TransIT®-LT1 transfection reagent (Mirusbio) delivering 1 µg DNA per well. After 24 h, surface expression was determined by labeling with SNAP-Surface Alexa Fluor 488 (New England Biolabs (NEB), Ipswich, MA, USA, # S9129S) and subsequent microscopic analysis. To generate a stably expressing cell line, G418 (Geneticin) was added to growth media for a time period of over two weeks. Once G418 resistant cells were sufficiently proliferating, cells were harvested, re-suspended in growth media to a cell density of 10 cells/mL and dispensed into a 384-well microplate (Greiner 781098) using a 16-channel Finnpipette (Thermo Fisher Scientific), 50 µL/well. Plate was incubated at 37 °C and 5% CO_2 for approximately two weeks. After the average of cell clones reached confluency, plate was duplicated and after incubation overnight, SNAP-tag labeling using SNAP-Surface Alexa Fluor 488 and microscopic analysis revealed cell clones expressing construct of interest. Clones were selected and further investigated regarding their ability to accumulate cAMP.

Membrane preparation of HEK-293 SNAP-SCTR cells

HEK-293 SNAP-SCTR Clone 1 with the closest profile to wildtype SCTR was selected and grown to confluency. Cells were labeled with Tag-lite SNAP-Lumi4-Tb (Cisbio US, Inc., Bedford, MA, USA, #SSNPTBD) based on manufacturer's manual in HEK cell growth media and incubated at 37 °C and 5% CO2. After three wash steps with PBS, cells were detached, re-suspended in growth media and centrifuged at 300g for 5-10 min. The cell pellet was diluted in lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 0.5 mM MgCl2, 1 mM EGTA (ethylene glycol tetraacetic acid) plus protease inhibitor cocktail (Sigma Aldrich, #P8340)) and homogenized on ice using Tissue Tearor (Biospec products, Inc., Bartlesville, OK, USA, #985370). After centrifugation at 19,000 rpm for 30 min at 4 °C, the supernatant was discarded, the pellet re-suspended in lysis buffer and homogenized on ice. Centrifugation was repeated and remaining cell pellet homogenized in pre-binding buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$) plus protease inhibitor cocktail. Protein concentration was determined via Bradford assay and membranes at 1.2 mg/mL were stored in aliquots at -80 °C.

Automated Peptide Synthesis of Fluo-Sec

Peptide synthesis was performed in 25 µmol scale on 0.24 mmol/g TentaGel S-RAM resin (Rapp Polymere GmbH, Tuebingen, Germany, #S30023). The Fmoc group was removed by treatment with 20% piperidine–DMF (2 x 5 min). Subsequently a standard double coupling procedure (2 x 60 min) is employed, using two times 5 equivalents of the Fmoc amino acid (0.4 M) in Oxyma pure/DMF (0.5 M), activated by addition of DIC/DMF (20 %, 10 equivalent). Formation of deletion sequences is prevented by a subsequent capping step using a mixture of acetic anhydride–pyridine–DMF (1:2:3, 500 µL, 5 min). Deprotection of Lys(Alloc): 1,3-Dimethylbarbituric acid (4.5 equiv., 115 µmol, 18 mg) and Pd(PPh3) (0.5 equiv., 12.5 µmol, 12 mg) are dispensed in DMF (1 mL). The mixture is degassed via nitrogen bubbling (5 min) and subsequently incubated with the resin for 3 hours. After incubation the resin is washed with DCM (2 x, 0.8 mL), DMF (2 x, 0.8 ml), AllocWash (Diethyldithiocarbamic acid (20 mg) in DMF (5 mL)) (6 x, 0.8 mL) and DCM (4 x, 0.8 mL). Manual Coupling Procedure: The coupling of 5(6)-Carboxyfluorescein to the deprotected ε-amino group of the lysine side chain was performed by dissolving 5(6)-Carboxyfluorescein (5 equiv., 125 µmol, 47 mg) in 0.5 M Oxyma pure (35.5 mg in 0.5 mL DMF) and addition of DIC (10 equiv., 250 µmol, 38.7 µL) (o.n.). Preparative Cleavage: Peptides were cleaved from the resin using Reagent B (88% TFA [880 µl], 5% [50 mg] Phenol, 5% [50 µL] HPW, 2% [20 µL] TIPS, 2x 1 mL: 3 h, 1.5 h). The cleaved peptide is precipitated in cold methyl t-butyl ether (MTBE) (15 mL), extracted with water (5 mL) and lyophilized. Subsequently the crude peptide is treated with Reagent B again (2 h), precipitated by addition of cold MTBE (15 mL), extracted with water (5 mL), lyophilized twice, purified by preparative HPLC and characterized by LC-MS. LC-MS: Analytical LC-MS was performed using ESI mass spectrometry detection combined with an Agilent HP 1100 HPLC system. Column: Kinetex C18, 2.6 µm, 100 Å, 50 × 2.1 mm, flow rate: 0.4 mL/min, gradient: 5–95% ACN–water (both containing 0.1% TFA)

in 15 min. Mass spectra were measured on an AB SCIEX API 2000. The recorded spectra were analyzed using the Analyst software (1.4.2) (AB SCIEX). Preparative HPLC:Column: Reprosil 100 C18, 5 µm, 250 × 10 mm, flow rate: 9 mL/min, gradient: 20-60% ACN–water (both containing 0.1% TFA) in 80 min; UV detection at 220 and 280 nm.

TR-FRET SNAP-SCTR binding assay

Binding experiments were performed as reported previously[1-4] with following modifications:

Saturation binding

Fluo-Sec (12-point (1:1), 0-200 µM) was diluted in DMSO in 384LDV plates. DMSO and Sec-FL diluted to a final concentration of 1 mM were transferred into adjacent wells. Fluo-Sec titration (25 nL) was dispensed by Echo liquid handler into all test wells of a 384-well plate (Greiner Bio-One small volume 784075). DMSO (25 nL) or Sec-FL (25 nL) were added on top. Using a dounce homogenizer thawed HEK-293 SNAP-SCTR membranes were diluted in binding buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM ascorbic acid, 0.2% BSA) to a final concentration of 5 µg/mL. Membrane solution was added via a Viaflo multichannel pipette (Integra Biosciences Corp., Hudson, NH, USA) at 10 µL/well. The plate was centrifuged 1000 rpm for 1 min and incubated at RT for 2 h. Saturation binding curve was recorded by Pherastar FSX microplate reader (BMG Labtech, Ortenberg, Germany) using the LanthaScreen module ((520nm/490nm) x 10,000) and data was analyzed via GraphPad Prism 8.4.0 using the equation "One site - Fit total and nonspecific binding" to determine dissociation constant K_d and maximum specific binding B_{max}.

Dissociation binding

Using Echo liquid dispenser, Fluo-Sec (25 nL, 10 nM final) was dispensed into a 1536-well plate (Corning #3725). For pos. ctrl wells and sample wells DMSO (12.5 nL) and for neg. ctrl wells Sec-FL (12.5 nL, 5 µM final) were echoed on top. Using a dounce homogenizer thawed HEK-293 SNAP-SCTR membranes were diluted in binding buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM ascorbic acid, 0.2% BSA) to a final concentration of 5 µg/mL. Membrane solution was added via a Multidrop Combi dispenser at 5 µL/well and the plate was centrifuged at 1000 rpm for 1 min. After a 2 h incubation at RT, the plate was read with Pherastar FSX (Lanthascreen, kinetic mode, 1 cylce). DMSO (12.5 nL) or CMPD A1/B1 (12.5 nL, 12.5 µM final) were dispensed via Echo into sample wells and after centrifugation at 1000 rpm for 1 min, the plate was read with Pherastar FSX (Lanthascreen, kinetic mode, 1 cycle). Immediately thereafter, dissociation buffer (binding buffer, 5 µM Sec-FL ± 100 µM GTPγS) was added to sample wells and binding buffer was added to control wells using Multidrop Combi. Dissociation binding was measured via Pherastar FSX (LanthaScreen, kinetic mode, 10 cycles, 45 min). Dissociation half-lives were determined using GraphPad Prism 8.4.0 applying equation "Dissociation – One phase

exponential decay". Experiments were performed in triplicates in at least three independent experiments

cAMP accumulation assays – specific procedures

Assays were performed according to the general procedure described in the main manuscript with following modifications:

LANCE Ultra cAMP detection (PerkinElmer) screening assay, Sec-FL & SCTR-CHO cells

LANCE Ultra PAM screening assay was performed in 1536-well microplates (Corning #3725). The assay wells contained 10 nL compound, 2.5 nL ligand (positive control (pos. ctrl.): Sec-FL 4 pM, negative control (neg. ctrl.) and compound wells: Sec-FL 0.1 pM) and 2 µL of cell suspension (112,500 cells/mL). For detection, 2 µL 100-fold-diluted Eu-cAMP stock and 2 µL 300-fold-diluted Ulight Ab stock in detection buffer were added using Multidrop Combi dispenser. HTRF (homogeneous time resolved fluorescence) was measured after incubation at RT for 60 min.

LANCE Ultra cAMP detection screening assay, Forskolin & parental CHO-K1 cells

LANCE Ultra screening assay in parental cells was performed in 1536-well microplates (Corning #3725). The assay wells contained 10 nL compound, 2.5 nL ligand (pos. ctrl: Forskolin 7 µM , neg. ctrl. and compound wells: DMSO) and 2 µL of cell suspension (112,500 cells/mL). For detection, 2 µL 100-fold-diluted Eu-cAMP stock and 2 µL 300-fold-diluted Ulight Ab stock in detection buffer were added using Multidrop Combi dispenser. HTRF was measured after incubation at RT for 60 min.

Cisbio cAMP Gs Dynamic HTRF (Cisbio GsD, Cisbio US, Inc., Bedford, MA, USA, #62AM4PEC) screening assay, Sec-FL & SCTR-CHO cells

Cisbio GsD cAMP Sec-FL PAM screening assay was performed in 384-well microplates (Greiner Bio-One small volume #784075). The assay wells contained 25 nL compound, 5 nL ligand (pos. ctrl.: Sec-FL 20 pM , neg. ctrl. and compound wells: Sec-FL 0.4 pM) and 5 µL of cell suspension (250,000 cells/mL). For detection, 2.5 µL 20-fold-diluted cAMP-d2 and 2.5 µL 20-fold-diluted anti-cAMP cryptate in detection buffer were added using Multidrop Combi dispenser. HTRF was measured after incubation at RT for 40-60 min.

Cisbio cAMP Gs Dynamic HTRF screening assay, 3 peptide mix & SCTR-CHO cells

Cisbio GsD cAMP 3-peptide mix PAM screening assay was performed in 384-well microplates (Greiner Bio-One small volume #784075). The assay wells contained 25 nL compound, 7.5 nL ligand (pos. ctrl: 5 pM Sec-FL + 1 nM Sec 1-23 + 14 nM Sec 3-27 , neg. ctrl. and compound wells: 0.1 pM Sec-FL + 28 pM Sec(1-23) + 530 pM Sec(3-27)) and 5 µL of cell suspension (250,000 cells/mL). For detection, 2.5 µL 20-fold-diluted cAMP-d2 and 2.5 µL 20-fold-diluted anti-cAMP

cryptate in detection buffer were added using Multidrop Combi dispenser. HTRF was measured after incubation at RT for 30-60 min.

Compound dose response studies

The selected compounds A1, A9, B1, C1 and D1 were stored in 384LDV microplates in a desiccator as 16-point 2-fold dilutions in DMSO. All the Cisbio cAMP assays were run on 1536-well plates (Corning #3725). For detection, cAMP-d2 and anti-cAMP cryptate, diluted in Detection Buffer, were added to the wells to achieve the final dilution of 1:90 each. HTRF was measured 30 min after adding the detection reagents. All experiments were performed in duplicate in at least three independent experiments.

In the SCTR agonist assay, the assay wells contained 25 nL compound, 22.5 nL ligand and 500 cells/5 µL. The positive control wells contained 25 pM Sec-FL (final concentration) and negative control and compound wells DMSO only instead.

In the SCTR/Sec-FL PAM assay, the assay wells contained 25 nL compound, 22.5 nL ligand and 500–1000 cells/5 µL. The positive control wells contained 25–60 pM Sec-FL (final concentration) and negative control and compound wells 0.1–0.3 pM Sec-FL (final concentration).

In the SCTR/3-peptide mix PAM assay, the assay wells contained 25 nL compound, 22.5 nL ligand and 500–1000 cells/5 µL. The positive control wells contained 2 pM Sec-FL + 3 nM Sec (1-23) + 260 nM Sec (3-27) (final concentration) and negative control and compound wells 0.04 pM Sec-FL + 170 pM Sec (1-23) + 23 nM Sec (3-27) (final concentration).

In the AVP2R/AVP PAM assay, the assay wells contained 25 nL compound, 22.5 nL ligand and 1000 cells/5 µL. The positive control wells contained 14 pM AVP (final concentration) and negative control and compound wells 0.3 pM AVP (final concentration).

In the GLP-1R/BETP assay, the assay wells contained 12.5 nL BETP or DMSO and 22.5 nL ligand in 16-point dose response (GLP-1: 0-250 pM final; GLP-1 (9-36): 0-0.5 µM final) and 800 cells/5 µL. All experiments were performed in duplicate in at least three independent experiments.

References

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4. Sykes, D. A.; Stoddart, L. A.; Kilpatrick, L. E.; et al. Binding kinetics of ligands acting at GPCRs. *Mol Cell Endocrinol* **2019**.

Supplementary Figures and Tables

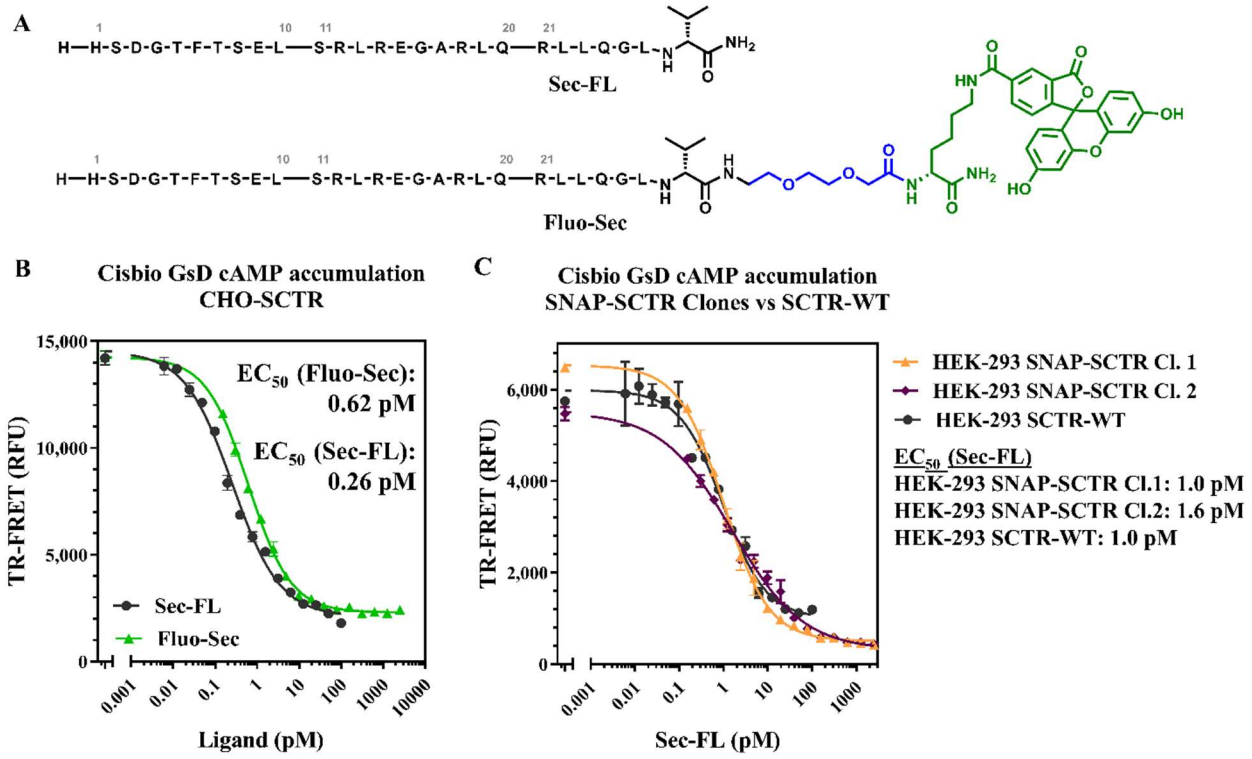

Figure S1: Development of a radiolabel-free SCTR binding assay: (A) Structure of Sec-FL (top) and Fluo-Sec (bottom), a fluorescein-labeled Sec-FL analog. (B) Functional characterization of Fluo-Sec (green) in SCTR-WT expressing CHO-K1 cells in comparison to endogenous ligand Sec-FL (dark grey) regarding their effect on cAMP accumulation. EC_{50} values were determined by curve fitting analysis using GraphPad Prism 8.4.0 and were ranging from 0.26 to 0.62 pM. (C) Functional characterization of HEK-293 SNAP-SCTR single cell clones 1 (Cl. 1, orange) and 2 (Cl. 2, purple) in Sec-FL stimulated cAMP accumulation studies compared to SCTR-WT overexpressing HEK-293 cells (grey). EC_{50} of Sec-FL was determined by curve fitting analysis using GraphPad Prism 8.4.0 and were ranging from 1.0 to 1.6 pM. (B, C) Experiments were performed in duplicate.

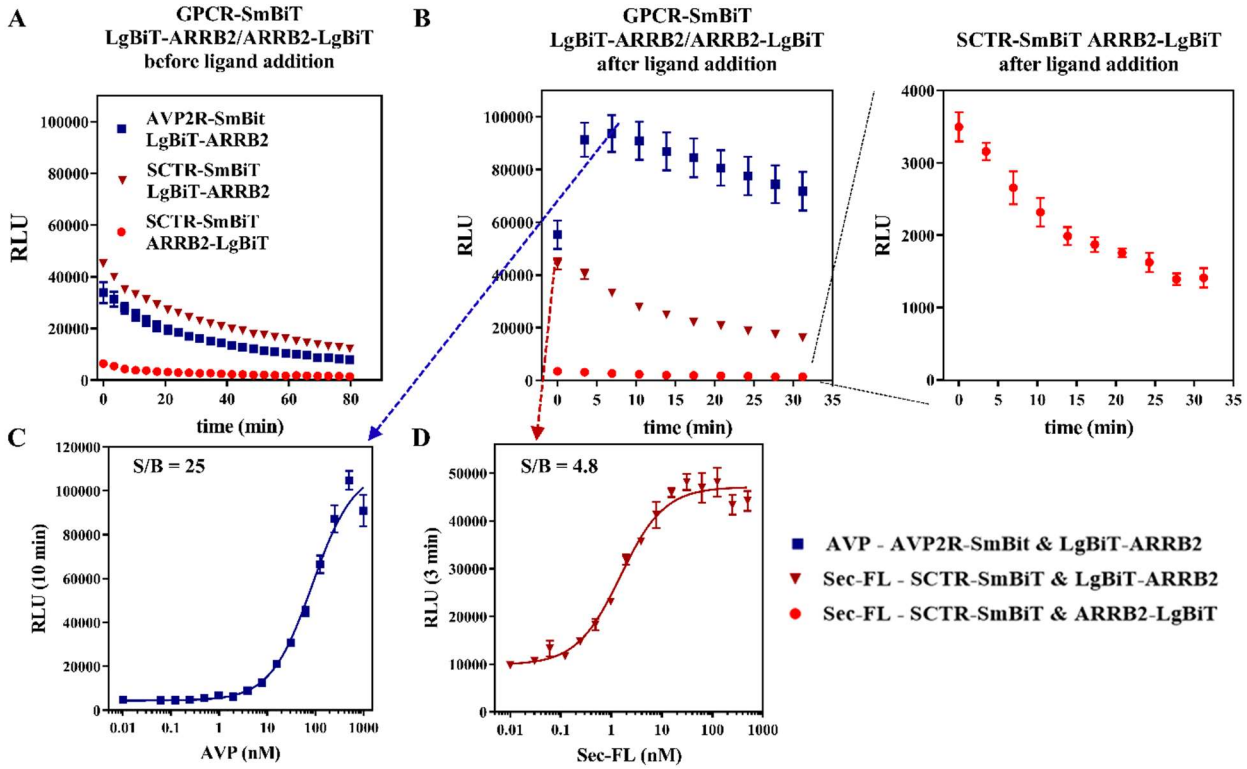

Figure S2: Development of split luciferase complementation-based β-arrestin-2 recruitment assay employing NanoBiT technology: (A) GPCR-SmBiT (Small BiT) & N-terminal LgBiT (Large BiT)-ARRB2 (β-arrestin-2) or ARRB2-LgBiT (with C-terminal LgBiT tag) transiently expressed in HEK-293 cells; kinetic luminescence signal recorded before ligand addition; model constructs (according to Promega's technical manual) AVP2R-SmBiT & LgBiT-ARRB2 (blue); novel constructs: SCTR-SmBiT & LgBiT-ARRB2 (dark red) and SCTR-SmBiT & ARRB2-LgBiT (light red). (B, left panel) GPCR-SmBiT & LgBiT-ARRB2 or ARRB2-LgBiT transiently expressed in HEK-293 cells; kinetic luminescence signal recorded after ligand addition; AVP2R-SmBiT & LgBiT-ARRB2 stimulated with AVP (arginine vasopressin, blue); novel constructs: SCTR-SmBiT & LgBiT-ARRB2 (dark red) and SCTR-SmBiT & ARRB2-LgBiT (light red) stimulated with Sec-FL. (B, right panel) Magnified illustration of SCTR-SmBiT & ARRB2-LgBiT (light red) stimulated with Sec-FL. (C) AVP dose-response curve measured at 10 min after AVP addition (S/B ~ 25). (D) Sec-FL dose-response curve measured at 3 min after Sec-FL addition (S/B ~ 4.8). (A-D) Experiments performed in triplicate and results representative of 1-2 independent experiments. Data analysis and curve fitting performed via GraphPad Prism 8.4.0.

Table S1: Summary of assay performance in primary screen of four different methods. Z′ factor, signal-to-background (S/B), signal-to-noise (S/N) and signal window presented as mean ± standard deviation:

Screening assay	Z′ factor	S/B	S/N	Signal Window
LANCE Ultra Sec-FL	0.60 ± 0.02	3.6 ± 0.19	18.5 ± 1.71	7.6 ± 0.65
Cisbio GsD Sec-FL	0.61 ± 0.06	3.4 ± 0.20	31.2 ± 5.51	6.7 ± 1.70
Cisbio GsD 3-pep mix	0.59 ± 0.07	3.0 ± 0.18	29.6 ± 6.10	6.0 ± 1.84
CRELuc Sec-FL	0.54 ± 0.08	4.7 ± 0.77	37.8 ± 10.1	4.8 ± 1.68

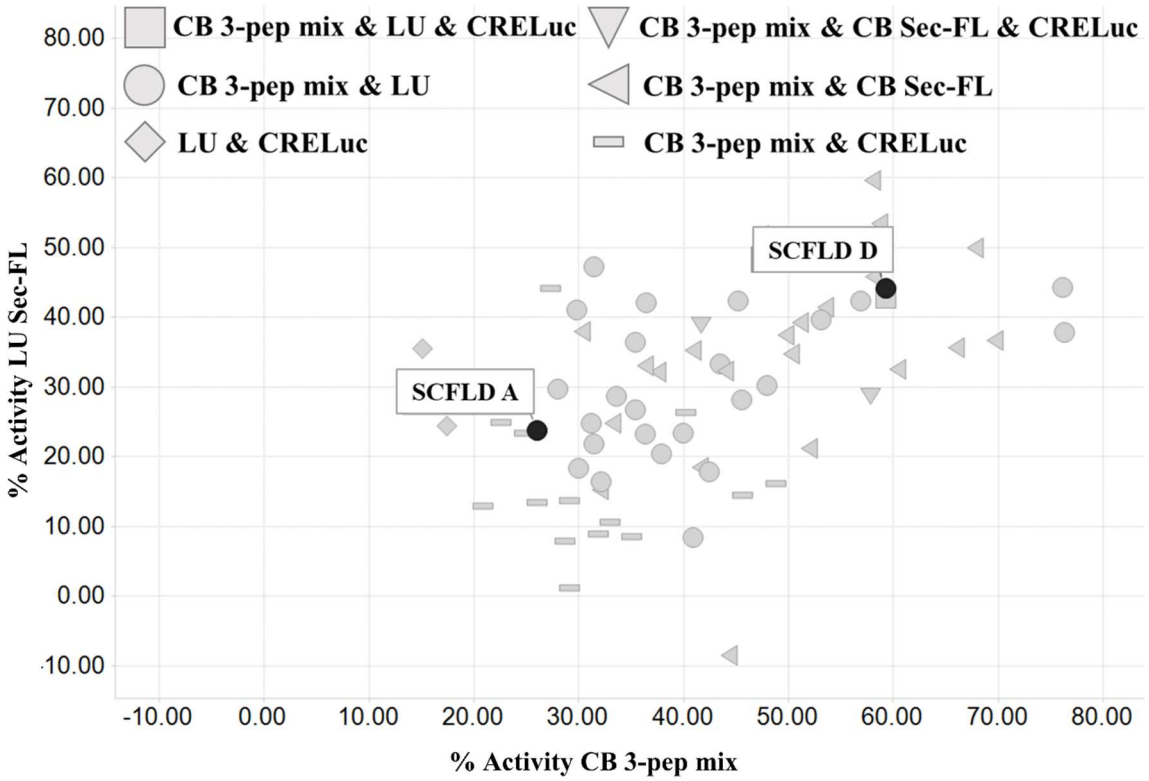

Figure S3: Pilot screens comparing three different detection methods and two sets of ligand probes elucidate strength of combining Sec-FL, Sec (1-23) and Sec (3-27), i.e. 3-peptide mix as orthosteric stimulator: Scatterplot demonstrating % activity in Cisbio GsD 3-pep mix (x-axis) versus % activity in LANCE Ultra Sec-FL (y-axis) of 70 hits confirmed in multiple assays including scaffold A and D; shapes indicate method(s) leading to hit confirmation; Scatterplot generated via TIBCO Spotfire.

Table S2: Potencies (EC_{50} [µM]) and gain of orthosteric stimulus efficacy (ΔEfficacy [%]) of compound dose response studies in Cisbio GsD cAMP accumulation assays; 3-PM = 3-peptide mix; data analysis via GraphPad Prism 8.4.0; experiments were performed in duplicate in at least three independent experiments:

Assay format / CMPD	SCTR agonist		SCTR PAM Sec-FL		SCTR PAM 3-PM		AVP2R PAM AVP	
	EC_{50} [µM]	ΔEfficacy [%]	EC_{50} [µM]	ΔEfficacy [%]	EC_{50} [µM]	ΔEfficacy [%]	EC_{50} [µM]	ΔEfficacy [%]
A1	162	22	4.6	39	5.4	44	4.7	18
A9	8.4	<10	1.1	28	2.3	34	n.d.	<10
B1	4.7	12	1.2	25	2.9	30	22	15
C1	30	17	2.3	35	5.8	43	n.d.	<10
D1	0.8	<10	0.6	20	4.1	53	n.d.	<10
Sec-FL	$0.5x10^{-6}$	95	$0.3x10^{-6}$	99	$0.5x10^{-6}$	100	-	-
AVP	-	-	-	-	-	-	$1.4x10^{-6}$	104

Table S3: Average potencies (EC_{50} [µM] ± standard deviation (SD)) and average compound efficacy (E_{max} [%] ± SD) of compound dose response studies in screening mode. Compounds were tested in Cisbio GsD cAMP accumulation assays and SNAP-SCTR binding experiments (data analysis via CBIS and Microsoft Excel; experiments performed in duplicate in 2-4 independent experiments):

Assay format	Cisbio cAMP SCTR Sec-FL PAM		Cisbio cAMP SCTR 3-pep mix PAM		Cisbio cAMP SCTR agonist		SNAP-SCTR Binding		Cisbio cAMP AVP2R AVP PAM	
CMPD	EC_{50} [µM]	E_{max} [%]	EC_{50} [µM]	E_{max} [%]	EC_{50} [µM]	E_{max} [%]	EC_{50} [µM]	E_{max} [%]	EC_{50} [µM]	E_{max} [%]
A1	**5.2** ± 1.4	**49.3** ± 13.1	**6.1** ± 2.4	**62.3** ± 16.4	**37.9** ± 8.8	**34.0** ± 3.9	**4.9** ± 2.8	**141.0** ± 7.3	**19.0** ± 17.5	**26.3** ± 11.1
A9	**1.5** ± 0.8	**37.9** ± 14.8	**2.6** ± 0.4	**46.9** ± 7.1	**n.d.** ± n.d.	**n.d.** ± n.d.	**7.5** ± 11.2	**154.0** ± 30.2	**n.d.** ± n.d.	**n.d.** ± n.d.
B1	**1.3** ± 0.3	**33.4** ± 11.6	**3.6** ± 0.6	**46.9** ± 16.1	**21.8** ± 19.0	**15.7** ± 6.5	**4.2** ± 2.8	**159.8** ± 24.3	**43.9** ± 8.6	**30.6** ± 0.0
C1	**2.7** ± 2.2	**38.1** ± 12.4	**6.1** ± 1.0	**59.2** ± 11.7	**42.0** ± 13.8	**25.8** ± 0.0	**2.5** ± 2.8	**135.8** ± 11.1	**n.d.** ± n.d.	**n.d.** ± n.d.
D1	**0.6** ± 0.1	**21.3** ± 0.8	**3.8** ± 0.1	**80.8** ± 0.8	**n.d.** ± n.d.	**n.d.** ± n.d.	**2.4** ± 0.5	**154.5** ± 5.5	**n.d.** ± n.d.	**n.d.** ± n.d.
Sec-FL	**4.6E-07** ± 3.2E-07	**103.0** ± 4.6	**5.6E-07** ± 1.5E-07	**119.3** ± 10.9	**6.1E-07** ± 1.7E-07	**111.5** ± 8.4	**4.7E-03** ± 1.5E-03	**105.3** ± 9.6	**n.d.** ± n.d.	**n.d.** ± n.d.
AVP	**n.d.** ± n.d.	**n.d.** ± n.d.	**n.d.** ± n.d.	**n.d.** ± n.d.	**n.d.** ± n.d.	**n.d.** ± n.d.	**n.d.** ± n.d.	**n.d.** ± n.d.	**1.3E-06** ± 3.2E-07	**108.6** ± 2.5