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Screening for positive allosteric modulators of cholecystikinin type 1 receptor potentially useful for management of obesity

Daniela G. Dengler^{1,*#}, Qing Sun¹, Kaleeckal G. Harikumar², Laurence J. Miller^{2,*#}, Eduard A. Sergienko^{1,*#}

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

²Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, Arizona, USA

Abstract

Obesity has become a prevailing health burden globally and particularly in the US. It is associated with many health problems, including cardiovascular disease, diabetes and poorer mental health. Hence, there is a high demand to find safe and effective therapeutics for sustainable weight loss. Cholecystikinin (CCK) has been implicated as one of the first gastrointestinal hormones to reduce overeating and suppress appetite by activating the type 1 cholecystikinin receptor (CCK1R). Several drug development campaigns have focused on finding CCK1R-specific agonists, which showed promising efficacy for reducing meal size and weight, but fell short on FDA approval, likely due to side effects associated with potent, long-lasting activation of CCK1Rs. Positive allosteric modulators (PAMs) without inherent agonist activity have been proposed to overcome the shortcomings of traditional, orthosteric agonists and restore CCK1R signaling in failing physiologic systems. However, drug discovery campaigns searching for such novel acting CCK1R agents remain limited. Here we report a high-throughput screening effort and the establishment of a testing funnel, which led to the identification of novel CCK1R modulators. We utilized IP-One accumulation to develop robust functional equilibrium assays tailored to either detect PAMs, agonists or non-specific activators. In addition, we established the CCK1R multiplex PAM assay as a novel method to evaluate functional selectivity capable of recording CCK1R-induced cAMP accumulation and β -arrestin recruitment in the same well. This selection and arrangement of methods enabled the discovery of three scaffolds, which we characterized and validated in an array of functional and binding assays. We found two hits incorporating a tetracyclic scaffold that significantly enhanced CCK signaling at CCK1Rs without intrinsically activating CCK1Rs in an overexpressing system. Our results demonstrate that a well-thought-out testing funnel can identify

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*Corresponding authors at: Daniela Dengler and Eduard Sergienko, Conrad Prebys Center for Chemical Genomics, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, 92037. Laurence Miller, Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, AZ, 85259, ddengler@sbsdsccovery.org (D.G. Dengler), miller@mayo.edu (L.J. Miller), esergien@sbsdsccovery.org (E.A. Sergienko).

*Contributed equally to this work.

Declaration of Conflicting Interest

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small molecules with a distinct pharmacological profile and provides an important milestone for the development of novel potential treatments of obesity.

Keywords

Cholecystokinin receptor; Positive allosteric modulator; Obesity treatment; High-throughput screening; G protein-coupled receptor

1. Introduction

Cholecystokinin (CCK) is a gastrointestinal peptide hormone synthesized and secreted from neuroendocrine I cells scattered along the upper small intestine in response to ingestion of various nutrients, particularly fat and protein.¹ It is known to stimulate gallbladder contraction, stimulate pancreatic exocrine secretion, regulate gastric emptying and intestinal transit, and to induce satiety, all involved in nutrient homeostasis. It was the first gastrointestinal hormone described to regulate appetite,² and this function was the major rationale for development of CCK agonists for the treatment of obesity. This activity has been shown to be mediated via activation of the type 1 CCK receptor (CCK1R) present in the periphery on vagal afferent neurons.³

Indeed, several major pharmaceutical companies have developed small molecule full agonists of the CCK1R as potential therapeutics for obesity.⁴⁻¹⁰ While all of these molecules exhibit full agonist activity at this receptor *in vitro* and *in vivo*, none have achieved therapeutic end-points for obesity in clinical trials. The FDA has required such agents to exhibit superiority in weight loss over acute dieting, a very high bar. Behavioral modification with dieting and exercise is well known to be effective acutely, but is a durable modifier of body weight in a small percentage of the population. There has been reticence to develop more potent CCK1R agonists and agonists with long duration of action, due to theoretical concerns about side effects, such as abdominal cramping, nausea, and diarrhea, and potential toxicities, including trophic effects and progression of malignancies.¹¹

We have previously made the case for the development of positive allosteric modulators (PAMs) of CCK action at CCK1R, particularly those with minimal endogenous agonist activity, as a strategy to treat obesity without the side effects and toxicities that could be associated with full agonists.^{12,13} The only such agent reported to date,¹² came from our effort to establish the high-throughput screening strategy we now describe. That preliminary effort involved the screening of 5,000 compounds of the NATx natural product-inspired compound library, where we evaluated potential screening formats, such as calcium flux or IP-One assays. Other agents with a similar or improved activity profile do not yet exist. They would take advantage of the physiologic timing and short duration of action of endogenously released CCK during a meal.¹⁴ They also would likely possess the possible advantages of PAMs, such as increased selectivity and saturability of action adding to safety.¹⁵ It is important to recognize that previous screening strategies for the development of CCK agonists for the treatment of obesity would have missed this type of chemical probe.

This report describes our high throughput screening effort to identify small molecule candidates with PAM activity at CCK1R that possess minimal endogenous agonist activity. We achieved this by screening a 360K compound library using a stable HEK293 cell line expressing high numbers of human CCK1R. The CCK1R is known to be predominantly coupled with Gq, so we utilized IP-One detection as primary screening method. We stimulated the cells with an EC₂₀ concentration of sulfated cholecystokinin octapeptide (CCK) in the assay, providing the opportunity to identify both, endogenous agonist activity and PAM activity, that could be separated and further characterized in subsequent assays.

This screening effort was effective, providing 45 hits with CCK1R PAM activity that exhibited minimal or no intrinsic agonist activity. This provides further evidence for the existence of small molecule probes with the activity profile of interest. These will have to be further validated and optimized for proof-of-concept studies in the future.

2. Materials and methods

2.1. Materials

2.1.1. Peptides and compounds—Sulfated cholecystokinin octapeptide (CCK, #4033010) was obtained from Bachem AG (Bubendorf, Switzerland). HF488-CCK-9s (HiLyteT Fluor 488-RD-Y(SO₃H)-TGW-Nle-DF-NH₂, #SQ-ASPE-74256) was obtained from AnaSpec, Inc. (Fremont, CA, USA). Adenosine 5'-triphosphate disodium salt hydrate (ATP, #A26209) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dry powders for hit validation were purchased through MolPort (Beacon, NY, USA) and were supplied by AK Scientific (Union City, CA, USA), BIONET/Key Organics Ltd. (Camelford, Cornwall, UK), ChemBridge Corporation (San Diego, CA, USA), ChemDiv, Inc. (San Diego, CA, USA), ENAMINE Ltd. (Monmouth Jct., NJ, USA), Life Chemicals Inc. (Niagara-on-the-Lake, ON, Canada), Specs (Zoetermeer, Netherlands), or Vitas-M Laboratory (Champaign, IL, USA) with purity 90%. In preparation for dry-powder dose-response confirmation experiments, compounds were stored in Echo Qualified 384-well low dead volume (384LDV) microplates (Labcyte, San Jose, CA, USA) as 16-point 2-fold dilutions (0 to 10 mM) in DMSO in a desiccator at room temperature.

2.1.2. The NIH Molecular Library Small Molecule Repository (MLSMR)—compound collection is part of NIH Molecular Libraries Program (MLP). The compounds were selected from commercial and academic sources using the following criteria: (a) acceptable physicochemical properties, including DMSO and water solubility, (b) lack of highly-reactive groups, (c) availability of analogs to generate structural clusters of 4-5 compounds, (d) high purity and (e) sufficient quantities for resupply. Majority of compounds in the collection obey Lipinski rule of 5. The collection served as the principal screening library within the MLP Screening Center Network (MLSCN/MLPCN). The data generated within the Network are stored in the NCBI's PubChem database, enabling establishing compound specificity and selectivity profiles from comparison of data in hundreds of assays.

2.1.3. Cells and culture reagents—Human Embryonic Kidney (HEK)-293 cells were received from American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained in growth media consisting of Dulbecco's Modified Eagles Medium (DMEM,

Corning Life Sciences, Cellgro #10-013-CV), 10% FBS (Omega Scientific, Tarzana, CA, USA, #FB-12), 1% penicillin (10,000 units)/ streptomycin (10 mg) (Pen/Strep, Gibco from Thermo Fisher Scientific, Waltham, MA, USA, #15140122), and 1% L-glutamine (200 mM) (Gibco #25030081). Selection antibiotics, Zeocin (Gibco, #R25001), Hygromycin (Hygromycin B, Omega Scientific, #HG-80), G418 (Omega Scientific, #GN-04) and Blastidicin (InvivoGen, #ant-bl-1, San Diego, CA, USA), were added to respective cell lines as specified below. TrypLE Express (Gibco #12605036) was used for cell detachment.

2.1.4. Cell line development and membrane preparation—Monoclonal cell lines (HEK-293 CCK1R, HEK-293 SNAP-CCK1R, HEK-293 CCK1R-SmBiT LgBiT-ARRB2) and membranes (HEK-293 SNAP-CCK1R) for primary and secondary assays were generated as described previously,¹⁶ but utilizing constructs carrying the coding sequence of human CCK1R (NM_000730.3). The pcDNA3.1/Zeo(+)-CCK1R construct was transfected to develop a monoclonal HEK-293 CCK1R cell line with selection induced by Zeocin, whereas HEK-293 SNAP-CCK1R cells were selected by addition of G418 after transfection with pcDNA3.1(+) SNAP-CCK1R. Fusion vectors for generating HEK-293 CCK1R-SmBiT LgBiT-ARRB2 cells were obtained from Promega (Promega Corp., Madison, WI, USA) and prepared as described in a previous report.¹⁶ The monoclonal cell line was obtained via limiting dilution of Hygromycin- and Blastidicin-resistant cells. The HEK-293 CCK1R cells expressed $57,000 \pm 3,000$ copies of this receptor on their surface, as determined by direct CCK radioligand binding analysis.

For preparing cryopreserved high-density cell stocks, which were deployed in IP-One screening assays, cells were re-suspended in freeze media (10% DMSO in growth media) at 20 million cells/mL after reaching 80-90% confluency. For each cryopreserved batch of cells, respective ligand dose-response curves were measured to determine control concentrations (EC_0 , EC_{20} , EC_{95}).

2.2. Methods

All assays were performed at the Conrad Prebys Center for Chemical Genomics (CPCCG).

2.2.1. IP-One Gq assay—TR-FRET based myo-inositol 1 phosphate (IP-One) accumulation assays were conducted using cryopreserved stocks of the clonal HEK-293 CCK1R cell line overexpressing CCK1Rs, or parental HEK-293 cells.

2.2.1.1. General procedure.: IP-One assays were performed utilizing Cisbio's IP-One Gq detection kit (Cisbio US Inc., Bedford, MA, USA, #62IPAPEJ) and following manufacturer's instructions with modifications.

In brief, cryopreserved cell stocks were thawed in a 37°C water bath and immediately re-suspended in IP-One assay media (phenol-red free DMEM (Corning, #17-205-CV) with 10% FBS, 1% Pen/Strep and 1% L-glutamine) to obtain desired cell densities. The cell suspension was dispensed with a Multidrop Combi dispenser (Thermo Fisher Scientific) at 4 μ L/well into a 1536-well TC-treated microplate (Corning, #3727). The plate was sealed with a MicroClime lid (Labcyte), which had been filled with autoclaved water, and incubated overnight at 37°C in a SteriStore incubator (HighRes Biosolutions, Beverly, MA, USA). The

next day, DMSO or compounds were added onto microplates containing adherent cells using Echo liquid handler (Labcyte), resulting in final assay compound concentrations of 6.25 μM for the primary screening, 5.8 μM for hit confirmation, up to 25 μM for hit prioritization, and up to 50 μM for dry powder validation. Orthosteric stimulator dilutions for control wells were prepared in assay media containing 150 mM lithium chloride (LiCl, 50 mM final, Sigma-Aldrich, #L7026). After incubation of plates containing cells in the presence of tested compounds for 30 min at 37°C, 2 μL of orthosteric stimulator dilutions were added to designated wells using BioRaptor (Beckman Coulter, Brea, CA, USA). The plate was centrifuged for 1 min at 1000 rpm, followed by an incubation period of 1 h at 37°C. After the plate was equilibrated to room temperature (RT) for 10-15 min, IP-One detection reagent was added at 2 $\mu\text{L}/\text{well}$ using Multidrop Combi. The microplate was centrifuged at 1000 rpm for 1 min, covered with MicroClime lid, incubated for 1 h at RT, and read with the HTRF (homogeneous time resolved fluorescence) module on a Pherastar FS microplate reader (BMG Labtech, Ortenberg, Germany). Raw data of the primary screening was uploaded to GeneData Screener (Genedata, Basel, Switzerland) and processed to remove plate patterns. Dose-response dilutions of the positive control ligand were included on each plate, and corresponding response curves of 4-6 plates per run were plotted using GraphPad Prism 9.3.1 (San Diego, CA, USA) to validate adequate control and test well concentrations of the orthosteric stimulator. Data of hit confirmation, prioritization and validation were uploaded and analyzed with CBIS (Chemical and Biology Information System software, ChemInnovation Software, Inc., San Diego, CA, USA). Further data analysis was conducted using the TIBCO Spotfire software (PerkinElmer, Waltham, MA, USA). Select compound dose-responses were analyzed by interpolating the data using IP-One standard curves to convert HTRF ratios to IP-One formed (nM) and subjected to nonlinear regression curve fitting of GraphPad Prism 9.3.1.

2.2.1.2. HEK-293 CCK1R, PAM format.: For IP-One PAM assays on HEK-293 CCK1R cells, the positive control wells contained EC₉₅ concentration of CCK. The negative control and test wells contained an EC₂₀ concentration of orthosteric stimulator CCK.

2.2.1.3. HEK-293 CCK1R, agonist format.: For IP-One agonist assays on HEK-293 CCK1R cells, the positive control wells contained EC₉₅ concentration of CCK. The negative control and test wells contained equivalent volumes of DMSO.

2.2.1.4. HEK-293 parental, PAM counter-screen.: For IP-One PAM assays on HEK-293 parental cells used as counter-screen, the positive control wells contained EC₉₅ concentration of ATP. The negative control and test wells contained an EC₃₀ concentration of orthosteric stimulator ATP. 200 mM ATP stock solution in assay media for preparation of the ligand dilutions was made freshly on day of the experiment.

2.2.2. TR-FRET SNAP-CCK1R competition binding—HEK-293 SNAP-CCK1R membranes labeled with Tag-lite SNAP-Lumi-4-Terbium (Cisbio US, Inc., Bedford, MA, USA, #SSNPTBD, TR-FRET donor) were prepared as described previously.¹⁶ HF488-CCK-9s, a CCK peptide where the free amino-group of sulfated (Thr²⁸,Nle³¹)-Cholecystokinin (25-33) had been ligated with HiLyte Fluor 488 (HF488), was utilized as

the orthosteric ligand carrying the TR-FRET acceptor fluorophore. The binding experiments were performed as reported in our previous study¹⁶ with modifications: Saturation binding experiments afforded the equilibrium dissociation constant K_D of HF488-CCK-9s, which guided the HF488-CCK-9s concentration used for competition binding assays. Ligands and test compounds were diluted in DMSO and dispensed onto a 1536-well microplate (Corning, #3725) using Echo liquid handler, whereby positive control wells contained HF488-CCK-9s and DMSO, negative control wells contained HF488-CCK-9s and Devazepide, and test wells contained HF488-CCK-9s and test compounds. HEK-293 SNAP-CCK1R membranes were thawed in a RT water bath and subsequently diluted in binding buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 1 mM ascorbic acid, 0.2% BSA (7.5% DTPA-purified Bovine Serum Albumin, PerkinElmer, #CR84-100)) using a dounce homogenizer. The resulting suspension was added to the microplate containing compounds and ligands using Multidrop Combi at 5 μ L per well and 10 μ g/mL. After a centrifugation step at 1000 rpm for 1 min, the plate was incubated for 2 h at RT. Remaining HF488-CCK-9s bound to SNAP-CCK1Rs was determined using the LanthaScreen module of a Pherastar FSX reader. Results were uploaded and analyzed with CBIS (Chemical and Biology Information System software, ChemInnovation Software, Inc., San Diego, CA, USA) to assess compound effects on CCK binding. Select compounds were evaluated using GraphPad Prism 9.3.1. with statistical significance determined using ordinary one-way ANOVA and Dunnett's multiple comparisons test ($\alpha = 0.05$).

2.2.3. NanoBiT (NanoLuc binary technology) β -arrestin-2 recruitment and cAMP accumulation PAM multiplex assay—Cisbio Gs dynamic cAMP assays (Cisbio US, Inc., Bedford, MA, USA) and β -Arrestin-2 recruitment assays were developed and performed as reported previously,^{16,17} with the following modifications to enable multiplex analysis of both signaling pathways: The monoclonal cell line HEK-293 CCK1R-SmBiT LgBiT-ARRB2 was maintained in culture and was harvested at 80-90% confluency at passage numbers below 35. After centrifugation at 300 x g for 3 min, the cell pellet was re-suspended in assay buffer (HBSS with Mg²⁺ and Ca²⁺ (Hank's Balanced Salt Solution, Gibco, #24020117, 5 mM HEPES (hydroxyethyl piperazineethanesulfonic acid), 0.1% BSA) containing NanoBiT substrate and diluted to target cell density. The cell suspension was incubated at RT for 2 h, whereby the tube was gently inverted occasionally to ensure a homogeneous mixture. IBMX (3-isobutyl-1-methylxanthine, Sigma-Aldrich, #I5879) and CCK dilutions were prepared in DMSO in 384LDV plates. Compounds and ligands were then transferred into a 1536-well microplate (Corning, #3725) using Echo liquid handler. An EC₂₀₋₃₀ concentration of CCK peptide was dispensed into sample and negative control wells, and an EC₉₅ concentration of CCK was added to positive control wells. IBMX was transferred on top of all wells (0.5 mM final). Using a Multidrop Combi the cell suspension was added to the microplate at 4 μ L/well, followed by a 1 min spin at 1000 rpm. Peak luminescence quantifying β -arrestin-2 recruitment was determined after 5 min using a Pherastar reader. After a 30 min incubation at RT, detection reagents of the Cisbio cAMP Gs Dynamic HTRF kit (Cisbio GsD, Cisbio US, Inc., #62AM4PEC) were added at 4 μ L per well with Multidrop Combi. The microplate was centrifuged at 1000 rpm for 1 min and cAMP accumulation was recorded after 30 min at RT utilizing the HTRF module of a Pherastar reader. Raw data was analyzed with CBIS and Graphpad Prism.

3. Results

3.1. Development of the CCK1R IP-One PAM assay as primary screening method

To identify PAMs without intrinsic agonist activity at CCK1Rs, we developed an assay monitoring IP-One accumulation in HEK-293 cells overexpressing CCK1Rs. CCK1Rs preferably couple to Gq proteins leading downstream to increased levels of inositol monophosphate (IP-One), a metabolite of inositol triphosphate. Therefore, the sought-after CCK1R PAM might silently bind to the receptor but only enhance the accumulation of IP-One in CCK1R-bearing cells while an orthosteric, endogenous stimulator is present, such as CCK. We used this concept in the design of our primary screening assay. After letting HEK-293 CCK1R cells attach overnight, we first pre-incubated the compounds with the cells for 30 min for equilibration with the receptor. Then, we added simultaneously an EC₂₀ concentration of CCK as orthosteric stimulator probe and lithium chloride. PAMs would enhance the effect of CCK, while lithium chloride inhibits the degradation of IP-One to myo-inositol. We detected the resulting accumulation of IP-One after 1 hour at 37°C using the TR-FRET-based IP-One kit from Cisbio. To avoid any potential fluorescence interference, we chose growth media without phenol red for cell seeding and as assay buffer.

As mentioned in the introduction, we compared IP-One to calcium flux assays in a pilot effort by screening a 5,000-compound library from the NATx collection. We recognized that the ability to screen robustly in a 1536-well format greatly enhanced the high-throughput feasibility for IP-One assays. Therefore, we decided in favor of the IP-One format.

3.2. Performance of HTS and hit confirmation

Using the established CCK1R PAM IP-One assay, we screened 362,388 compounds part of a library from the NIH Molecular Libraries Small Molecules Repository at a final concentration of 6.25 µM. We completed 19 to 22 plates per day and were able to finish the primary screening effort after fourteen working days.

Since we found it crucial for reproducibility, assay window and comparability that the concentration of the orthosteric stimulator probe is within a range of EC₁₀ to EC₃₀ of the maximal response, we included a 6-pt CCK dose-response curve in quintuplicate to each plate. This way, we established an internal control capable of identifying detrimental variations in the orthosteric stimulator probe and were able to intervene in a timely manner. Every screening day, we plotted the curves of six plates, which were selected to capture the performance during the day. In Fig. 1A, we depict an example graph for day 14 of the primary screening effort. We found a great overlap of the CCK standard curves. The orthosteric stimulator probe concentrations ranged from EC₁₈ to EC₂₀ on that day, indicating a very successful screening performance. Even though the signal-to-background ratio (S/B) varied from 2.3 to 3, the assay performed very robustly resulting in an average Z²-factor of 0.79.

Encouraged by the great performance of the primary assay, we expanded our range for hit selection to be able to confirm a larger set of potential hits. Compounds with an activity over 15% or a Z-Score below -4 were selected for hit confirmation. The Z-Score was determined utilizing the mean of negative control (EC₂₀ CCK) wells. Since IP-One

accumulation results in a loss of signal, negative Z-Score values indicated the desired response. Occurrence histograms indicated a normal distribution of activity (Fig. 1B), while the Z-Score occurrence was slightly negatively skewed (Fig. 1C). We identified 4,920 compounds that met the selection criteria resulting in a 1.36% hit rate. Following our standard practice, we evaluated compounds using a parameter called F-ratio, which monitors fluorescence interference of compounds through comparison of the TR-FRET donor channel of compound well with the average value of the donor channel negative control wells. We depicted compound activity against F-ratio in a scatterplot (Fig. 1C) and noted a major sub-group of active compounds that had significantly higher F-ratios than neutral (negative) or stimulator (positive) control wells. We decided to proceed with the initial selection of hits to avoid accidentally excluding any promising candidate at this early stage yet employed the F-ratio criterion-based selection at a later stage. Hence, we conducted hit confirmation testing 4,920 compounds in triplicates at a final concentration of 5.8 μM in our primary screening assay. As in the previous step, we decided to use lenient selection criteria (activity > 6% or Z-Score < -1), which resulted in 646 hits and a hit confirmation rate of 13.1% (Fig. 1D).

3.3. Evaluation of non-specific modulators and assay artifacts

We developed an IP-One assay to determine non-specific activators of the IP-One signaling pathway and artifacts of the detection kit, which we describe as counter-screen assay. PAM assays rely on a significant basal stimulation induced by the orthosteric stimulator probe. Based on our experience with PAM screening efforts, we found it crucial to imitate similar basal IP-One levels in order to successfully identify non-specific activators. As host cell line for our counter-screen we selected HEK-293 parental cells, which do not endogenously express CCK1Rs but possess inherent levels of Gq-coupled purinergic receptors, such as P2Y1R, P2Y2R and particularly P2Y11R.¹⁸ Hence, we were able to use adenosine triphosphate (ATP) as orthosteric stimulator probe as it had been shown to activate P2Y2 and P2Y11 receptors.^{19,20} We tested 646 confirmed hits at 5.8 μM in triplicates in the P2YR PAM IP-One assay, thereby using same compound conditions of the hit confirmation stage to ensure direct comparability of results. We plotted ATP dose-response curves, which were included in the plates, to validate adequate basal IP-One levels were achieved by the ATP concentration added to negative and compound wells (Fig. 2A). Indeed, the orthosteric basal stimulation of the counter-screen was not only in the desired EC₁₀-EC₃₀ range of the maximal response, but also resulted in similar TR-FRET ratios seen in the primary screening assay indicating comparable basal IP-One stimulation in both formats. Overall, the counter-screen performed exceptionally well with a S/B ratio of 3.8 and an average Z'-factor of 0.89 (Fig. 2B). We further evaluated whether there was a correlation of activities in hit confirmation and counter-screen assays and F-ratios of tested compounds (Fig. 2C, D). Interestingly, we found that there was a great overlap of active hits in the counter-screen assay and compounds that displayed high F-ratios in both, the CCK1R PAM and P2YRs PAM IP-One assays. Hence, this pattern supported our initial hypothesis that these compounds were likely assay artifacts interfering with the TR-FRET donor channel. We further excluded compounds with activity lower than 7.8% in hit confirmation CCK1R assays and other non-specific activators, which we characterized as having an activity in the counter-screen assay higher than 15%. The latter represented roughly the 95% confidence

interval of the assay. To account for the lenient selection criteria chosen for the primary assay, we only included hits with counter-screen activity between 4 and 15% that showed a 1.8-fold selectivity toward CCK1R. Thus, we conducted further tests for hit prioritization on a set of 296 hits.

3.4. Hit prioritization with further functional and binding formats

To further prioritize hits, we performed the primary CCK1R PAM IP-One assay and our counter-screen with the remaining 296 hits in a 6-point dose-response format (0 - 25 μ M in duplicates). Increasing the concentration further revealed non-specific activators and assay artifacts that were therefore excluded from dry powder validation. Being aware that our primary PAM assay can detect both, modulators with and without intrinsic agonist activity, we tested the hits in a HEK-293 CCK1R IP-One assay without having the orthosteric stimulator present in sample wells, and compared the maximal efficacy (E_{max}) of compounds in this IP-One CCK1R Agonist assay to their maximal effect in the IP-One CCK1R PAM assay (Fig. 3A). As depicted in the scatterplot, a majority of compounds displayed very similar activities in Agonist and PAM mode indicating intrinsic agonist activity. However, a subset of hits showed no or minimal activity in the Agonist format, while having significant activity in the CCK1R PAM IP-One assay. Thus, these hits were promising candidates to exert the desired pharmacological profile.

Based on a previous report,¹⁶ we developed a TR-FRET-based homogeneous binding assay. We prepared HEK-293 cell membranes over-expressing CCK1R with a SNAP-tag that was labeled with Lumi-4-Terbium cryptate serving as donor fluorophore. Sulfated (Thr²⁸,Nle³¹)-Cholecystokinin (25-33), a stabilized CCK analog,²¹ was ligated to the acceptor fluorophore HiLyte Fluor 488, resulting in the fluorophore-labeled CCK analog HF488-CCK-9s. Since we were interested in allosteric modulators, which do not displace endogenous CCK nor reduce its binding affinity, we screened the 296 hits in competition binding mode (5 μ M, in triplicate). We found that compounds that displayed higher agonist activity were more likely to also show a significant displacement of HF488-CCK-9s (Fig. 3B, C). The subset of hits with no or minimal agonist activity did not show a significant effect on HF488-CCK-9s binding to CCK1R. In contrast, the great majority of compounds that were intrinsically active displayed reduced levels of bound HF488-CCK-9s. We hypothesized that this group of hits were likely orthosteric agonists displacing the fluorophore-labeled CCK analog from its binding site. Hence, we excluded those hits from further studies. We further prioritized hits based on Pan Assay Interference Compounds (PAINS) filter and F-ratios, which largely overlapped with activity in the counter-screen (Fig. 3A). Altogether, we selected 45 compounds with significant PAM effect in HEK-293 CCK1R cells, but no or minimal intrinsic agonist activity and lack of HF488-CCK-9s displacement. 36 of these compounds were commercially available and purchased as dry powders for validation and further characterization.

3.5. Development of a CCK1R PAM multiplex assay recording well-specific β -arrestin recruitment and cAMP production

Even though CCK1Rs preferably signal through Gq proteins, they can stimulate Gs-mediated cAMP production and recruitment of β -arrestins when exposed to higher

concentrations of some agonist ligands. PAMs might enhance one downstream pathway over another, thereby exacerbating or alleviating functional selectivity. To characterize potential candidates regarding their signaling profile and to have an additional tool for validating selected hits, we developed a β -arrestin recruitment assay based on Promega's NanoBiT technology.^{22,23} Preliminary kinetic studies in transiently transfected HEK-293 cells demonstrated that the luminescence resembling β -arrestin recruitment to CCK1R peaked around 5 minutes (Fig. 4A). We also developed a cAMP accumulation assay using Cisbio's Gs dynamic cAMP detection kit. Since assay conditions and timing of both functional assays appeared compatible, we established a protocol allowing the detection of CCK1R-mediated β -arrestin recruitment and cAMP production in the same well. We equilibrated the established monoclonal cell line HEK-293 CCK1R-SmBiT LgBiT-ARRB2 with the NanoBiT substrate for 2 hours. We dispensed CCK ligand dilutions and IBMX onto a dry plate and subsequently added the cell suspension with the NanoBiT substrate. After 5 minutes, we recorded the luminescence to determine levels of β -arrestin recruitment. 30 minutes after the addition of cell suspension, we dispensed the cAMP detection reagents in lysis buffer. Following another 30-minute incubation at room temperature, we measured TR-FRET ratios to evaluate cAMP accumulation.

Compared to cAMP production induced by CCK in HEK-293 CCK1R cells, we determined an approximate 4.5-fold rightward shift in potency in HEK-293 CCK1R-SmBiT LgBiT-ARRB2 cells (Fig. 4B). However, we found this to be within an acceptable range and hypothesized that this is likely the result of slightly different receptor expression levels. Interestingly, CCK potencies for both, β -arrestin recruitment and cAMP accumulation of the multiplex format, were in a similar range at 1.4 nM and 0.9 nM, respectively (Fig. 4C). This represented a 10-15-fold rightward-shift compared to Gq-mediated IP-One signaling. We determined that our newly established multiplex assay is suitable for characterization of CCK1R PAMs, since the CCK concentration used as orthosteric stimulator probe fell in the EC₁₀-EC₃₀ range of the maximal response for both downstream pathways.

3.6. Validation of PAM scaffolds without intrinsic agonist activity

We tested the 36 dry powders in a 16-point dose-response format up to 50 μ M in duplicates in CCK1R IP-One PAM and Agonist assays, in the IP-One counter-screen in HEK-293 parental cells, in our newly developed CCK1R PAM cAMP and β -arrestin recruitment multiplex assay, and in the TR-FRET based binding assay. Overall, we identified 14 CCK1R-specific hits, which demonstrated greater PAM than agonist activity. We focused on 4 hits that we found to display the most significant PAM effects and thereby showed the highest potential for further development. We depict the structures of these 4 hits in Fig. 5A. Of note, hit 1 and 6 were based on a tetracyclic scaffold. In addition, they shared the incorporation of a tertiary basic amine function. Hit 3 was structurally unrelated to hit 1 and 6, however, it contained a tertiary amine as well. We think that this might be an indicator of a common interaction site with the receptor. Hit 7 did not share any structural functions with the other hits.

There are multiple approaches to analyze data from immune-competitive formats, such as the IP-One assay from Cisbio. The choice of which one to use depends on the application

and purpose of the study. It has been found that for improved characterization of compound pharmacology, it is recommended to convert TR-FRET ratios to analyte formed using analyte standard curves.²⁴ To identify the true nature of our novel PAMs, we analyzed the results by assessing IP-One formed.

None of the 4 hits showed any effect in the CCK1R IP-One Agonist format (Fig. 5C), which supported their lack of intrinsic activity at CCK1Rs. In the CCK1R IP-One PAM assay (Fig. 5B), hit 1 achieved the highest IP-One levels (EC_{50} 19.4 μ M, Span 356 nM), followed by hit 3 (EC_{50} n.d., Span 287 nM) and hit 6 (EC_{50} 8.4 μ M, Span 144 nM). Even though hit 7 was the most potent agent among the 4 hits (EC_{50} 6.5 μ M, Span 119 nM), it also demonstrated some non-specific effects in the counter-screen format (EC_{50} 6.0 μ M, Span 43 nM) in the same potency range (Fig. 5D). Since it also did not enhance CCK stimulation in cAMP and β -arrestin recruitment formats (Fig. 5E, F), we thought it to be the least interesting scaffold of the four hits. In contrast, we found significant potentiation of CCK-induced cAMP production for the three hits containing a tertiary amine showing potencies and efficacies in a similar range; potencies were between 8 and 18 μ M and efficacies ranged from 34 to 45% (Fig. 5D). Interestingly, hit 6 also slightly enhanced the effect of CCK on μ -arrestin recruitment to CCK1Rs (EC_{50} 3.6 μ M, E_{max} 14%, Fig. 5E). None of the four compounds reduced the fraction of bound HF488-CCK-9s in a competition binding experiment (Fig. 5F), which is likely an indication for acting via an allosteric binding site. Altogether, we found that the tetracyclic scaffold, represented by hits 1 and 6, displayed the desired pharmacological profile of a CCK1R-specific PAM without intrinsic activity and possessed chemical accessibility. Further pharmacological characterization and chemical optimization studies for this scaffold are in progress.

4. Discussion

Obesity is a major public health problem, in the United States and throughout the developed world. It is a prominent cause of morbidity and mortality, both directly and secondary to comorbidities such as diabetes mellitus and cardiovascular disease. There have been numerous efforts to prevent and manage obesity, including lifestyle modifications such as diet and exercise, medications, and bariatric surgery. Lifestyle modifications are highly successful in the short term, but have a very low durability, as people tend to return to their normal habits, often regaining their lost weight or even ending up at a higher weight. Bariatric surgery has been extremely successful in morbidly obese patients, but it is not adequately scalable to include the large numbers of patients in need of weight loss. Relatively few medications have been approved for weight reduction, with several carrying warnings about serious side effects. Most recently, GLP-1 agonists have been approved, representing a class of drugs first recognized to manage type 2 diabetes and reflecting the use of a signaling pathway engaged by a gastrointestinal hormone.

CCK was the first gastrointestinal hormone identified to induce satiety after eating,²⁵ and recognized as a possible strategy to reduce caloric intake and appetite.² It achieves this through activation of type 1 CCK receptors on vagal afferent neurons.²⁶ Several pharmaceutical companies took advantage of this observation to develop full agonists of CCK1R,²⁶ with some of these progressing to clinical trials. Unfortunately, none of these

compounds achieved superiority over acute dieting for weight loss potentially suggesting the high bar the weight loss therapies are facing on the path for FDA approval. Further, there has been theoretical concern about increasing potency and/or efficacy or duration of action of CCK1R agonists, due to side effects or possible toxicity. Our hypothesis from the start was that PAMs acting at CCK1R that do not possess substantial intrinsic agonist activity would have much more desirable activity and safety profiles.^{12,26} Compounds with this type of activity profile have the potential to enhance the signaling profile of endogenous CCK released after a meal, providing temporal control and short duration of action when physiologically appropriate for affecting appetite.

Another potential advantage of a CCK1R PAM is its ability to correct the defect that has been described in the setting of excess membrane cholesterol that causes aberrant stimulus-activity coupling.^{27–29} This is proposed to be active in some obese patients and to help explain previous negative clinical trials of CCK agonists.²⁹ A corrective PAM could help make such people more responsive to this hormonal regulator of appetite. Cooperative effects of PAMs on endogenous, orthosteric agonists may be manifested as augmentation of orthosteric agonist affinity or agonist efficacy, also known as α or β effect, respectively, based on the operational model of allosterism.¹⁵ There have been indications that β -PAMs could be the key to rescue attenuated or defective physiologic conditions.³⁰ For example, Leach et al. demonstrated that the β -PAM LY2033298 was able to restore signaling of endogenous agonist acetylcholine at functionally impaired muscarinic M4 receptors.³¹ Therefore, we hypothesized that a CCK1R-specific PAM with β -action would be an effective agent to rejuvenate CCK1R activation in a high-cholesterol environment. Another feature of the desired PAM would be the silent occupation of CCK1Rs prior to release and binding of endogenous CCKs.

Having the preferred compound characteristics in mind, we searched for a suitable primary screening assay, which would allow preincubation of test substances prior to CCK addition and likely recognizes β -PAMs. We found that calcium release and IP-One accumulation represented the two most common functional assays for screening of Gq-coupled receptors.³² Pilot screens of a 5,000-compound library in both formats were successful, leading to a CCK1R PAM with minimal intrinsic agonist activity.¹² However, we found the IP-One assay more suitable for a large-scale screening due to its homogeneous 1536-well format. Moreover, a previous study suggested that nonequilibrium formats, such as the calcium assay, are inferior to IP-One equilibrium assays with respect to classification and determination of pharmacological profiles.³⁰ In contrast to the IP-One format, the calcium assay was suboptimal in establishing agonist or β activity of a PAM and resulted regularly in misleading classifications of mechanisms when compounds needed to be pre-equilibrated with the receptor. Since these two factors were crucial for our experimental set-up in order to identify PAMs without intrinsic agonist activity, we decided to use the IP-One assay in our primary screening effort.

We followed a testing funnel with three stages (Fig. 6): primary screening, hit prioritization and hit validation. In the first stage, we screened 360K compounds from the MLSMR collection. The IP-One primary assay performed very robustly with an average Z' -factor of 0.79 and yielded 4920 hits, of which 646 hits we were able to confirm. After a PAM

counter-screen using parental HEK-293 cells targeting P2YRs to identify non-specific hits, we selected 296 hits for prioritization studies. We found that compounds with a high F-ratio and thus, likely resulting in false positives due to interference with the TR-FRET detection in the assay, greatly overlapped with active hits in the counter-screen. This confirmed that the F-ratio is a great parameter to pinpoint the potential assay artifacts in TR-FRET based assay systems.

In the hit prioritization stage, we tested liquid stocks of the 296 compounds up to a concentration of 25 μM in 6-point dose response format in three assays. The primary IP-One CCK1R PAM assay relied on an addition of an EC_{20} concentration of CCK as orthosteric stimulator probe after a 30-minute pre-equilibration period with test compounds. The submaximal CCK concentration was crucial to identify PAMs without intrinsic agonist activity, however, this format is not able to distinguish between PAM and agonist effects. Therefore, we screened the hits in CCK1R agonist format without CCK present, which detected intrinsic agonist activity in a majority of hits. In our efforts, we utilized a cell line that overexpressed CCK1Rs, since it would be expected to increase the sensitivity of the assay, amplifying the biological response to CCK and endogenous agonists. This is also known to enhance the response to weak agonists and may detect agonist activity when little or no such activity may be present at physiological levels of receptor expression.³³ Thus, we hypothesized that compounds with minimal agonist activity in this cell line might act as pure PAMs in physiologic systems. A subset of hits displayed significant PAM activity in the absence of detectable agonist activity, even in the cell line with high expression levels of CCK1Rs.

We further developed a TR-FRET based homogeneous binding assay capable of identifying compounds that would decrease the binding of a fluorophore-labeled CCK analog, HF488-CCK-9s. The reduced fraction of HF488-CCK-9s bound would either indicate displacement of the orthosteric ligand or negative cooperative effects on the ligand's binding affinity. Our desired PAM should not exhibit either of those properties, therefore, we sought to exclude hits decreasing the binding of HF488-CCK-9s to the receptor. We found a great overlap of hits with intrinsic agonist activity and reduced binding of HF488-CCK-9s, which is likely an indication for competitive behavior for the orthosteric binding site of CCK. We further eliminated hits based on activity in the counter-screen or PAINS filter leading to 45 compounds that displayed the desired activity profile.

For the third stage, hit validation, we purchased dry powders of 36 commercially available hits and screened them as 16-point dose responses up to a concentration of 50 μM in six assay formats. Next to the three IP-One assays, CCK1R PAM, CCK1R agonist and P2YRs PAM, we established a multiplex assay recording the effects of CCK and PAMs on secondary effector proteins Gs and β -arrestin through detection of cAMP accumulation and NanoBiT complementation of CCK1R and β -arrestin-2 tagged with split-luciferase fragments. We believe that this multiplex PAM assay will be a great tool to screen for functional selectivity of scaffolds through our compound development campaign. We highlighted four of the most promising hits from this high throughput screen in the six assay formats. We found that two of the hits, hit 1 and 6, shared a tetracyclic scaffold, while one further hit, hit 3, also had a tertiary amine in common. These exhibited the activity profile

we had sought, demonstrating positive allosteric modulation of the action of CCK, while exhibiting minimal or no intrinsic agonist activity. Our preliminary studies suggested that hit 6 might display a balanced activity profile, while hit 1 and 3 appeared to be biased toward G protein signaling of CCK1Rs. However, the activity of hit 6 for β -arrestin-2 recruitment was low and further optimization of this candidate might display a clearer profile. Furthermore, we characterized the 36 hits regarding their effect on CCK – receptor ligand binding. We could not detect significant effects of the most promising candidates in this binding assay. This might indicate that the identified PAMs base their activity on β -effects rather than on α -effects impacting CCK binding affinity. However, the binding assay was conducted in a co-addition format, and we hypothesize that preincubation with the putative PAMs might induce an effect on receptor conformation and stabilize CCK-receptor complexes. This and further pharmacological characterization and chemical optimization studies for the compounds with the tetracyclic scaffold are currently being pursued. Our aim is to improve the small molecules for enhanced CCK1R PAM activity in normal and high-cholesterol environments, while maintaining minimal or no intrinsic agonist activity at CCK1Rs. In addition, we are currently evaluating pharmacokinetic properties and extended selectivity profiles to ensure suitability of our novel PAMs for in vivo studies.

Altogether, we were able to identify small molecule compounds displaying the desired pharmacological profile, a CCK1R PAM without any apparent intrinsic agonist activity, by establishing and following a testing funnel with a robust equilibrium primary screening assay and novel secondary formats, such as the CCK1R multiplex PAM assay. We identified four promising hits incorporating three scaffolds. Particularly intrigued by the chemical and pharmacological properties of the tetracyclic scaffold, we focus current and future studies on these molecules. In a previous study, we had identified a CCK1R PAM using the hemi-equilibrium calcium release assay. Further characterization revealed around 25% intrinsic agonist activity at a concentration of 32 μ M.¹² Our newly identified tetracyclic analogs did not display intrinsic agonist activity up to a concentration of 50 μ M in the CCK1R overexpressing cell line, thereby representing an improvement over the previously identified small molecule. We hypothesize that the use of the equilibrium IP-One format might have supported our endeavor to discover a pure CCK1R PAM.

Thus, we believe the discovery of the novel CCK1R PAM scaffold is an important milestone on our way to develop agents to restore impaired CCK1R signaling in high-cholesterol environments, which could provide a novel and safe treatment for obesity.

Acknowledgements

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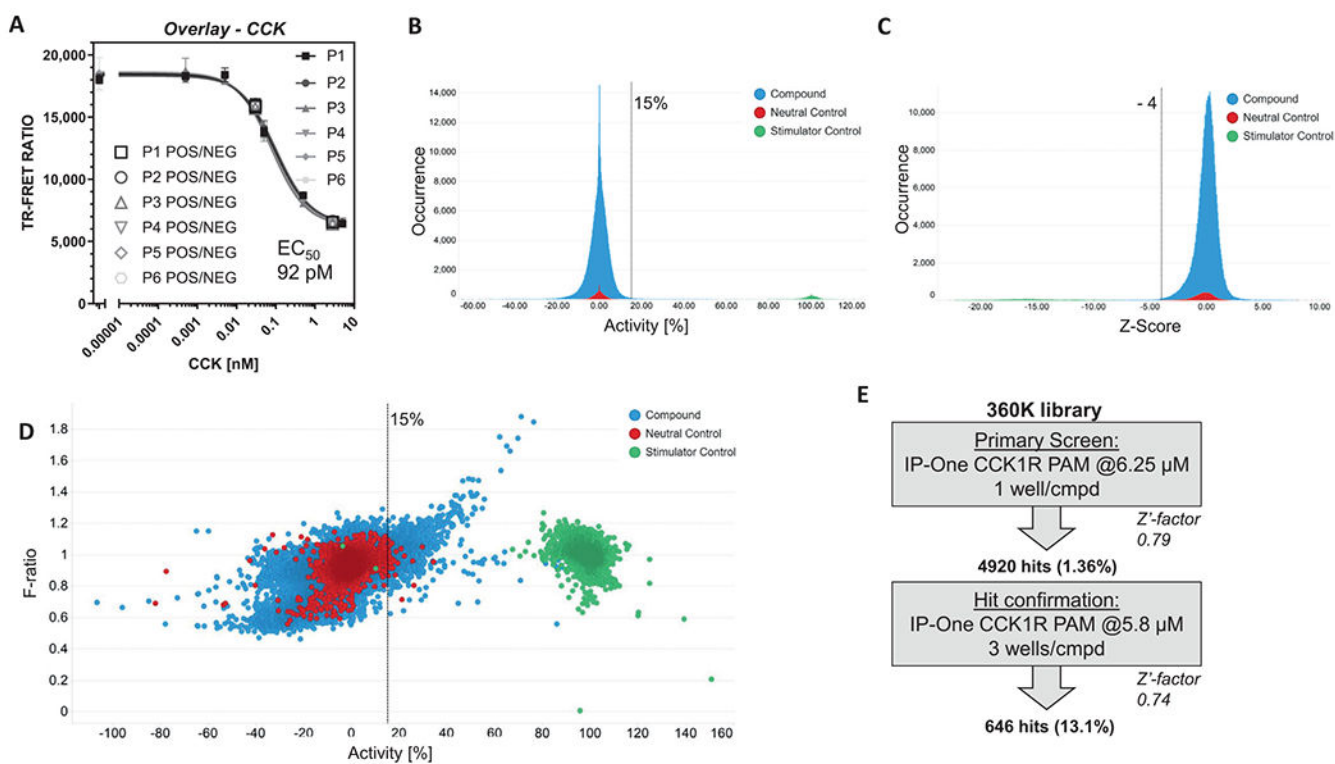


Fig. 1. Performance of primary screen:

(A) Overlay of CCK dose-response curves of one screening day (plates P1-6) with positive and negative control ratios in open symbols; graphs plotted using GraphPad Prism; TR-FRET ratios shown as mean \pm SD. Primary screening occurrence histograms displaying distribution of (B) activity [%] or (C) Z-score with hit selection cut-off line; (D) scatterplot showing % activity in primary screen (x-axis) in correlation to F-ratio (y-axis) with hit selection cut-off line; histograms (B, C) and scatterplot (D) created using TIBCO Spotfire. (E) Testing funnel describing conditions and performance of primary screen and hit confirmation.

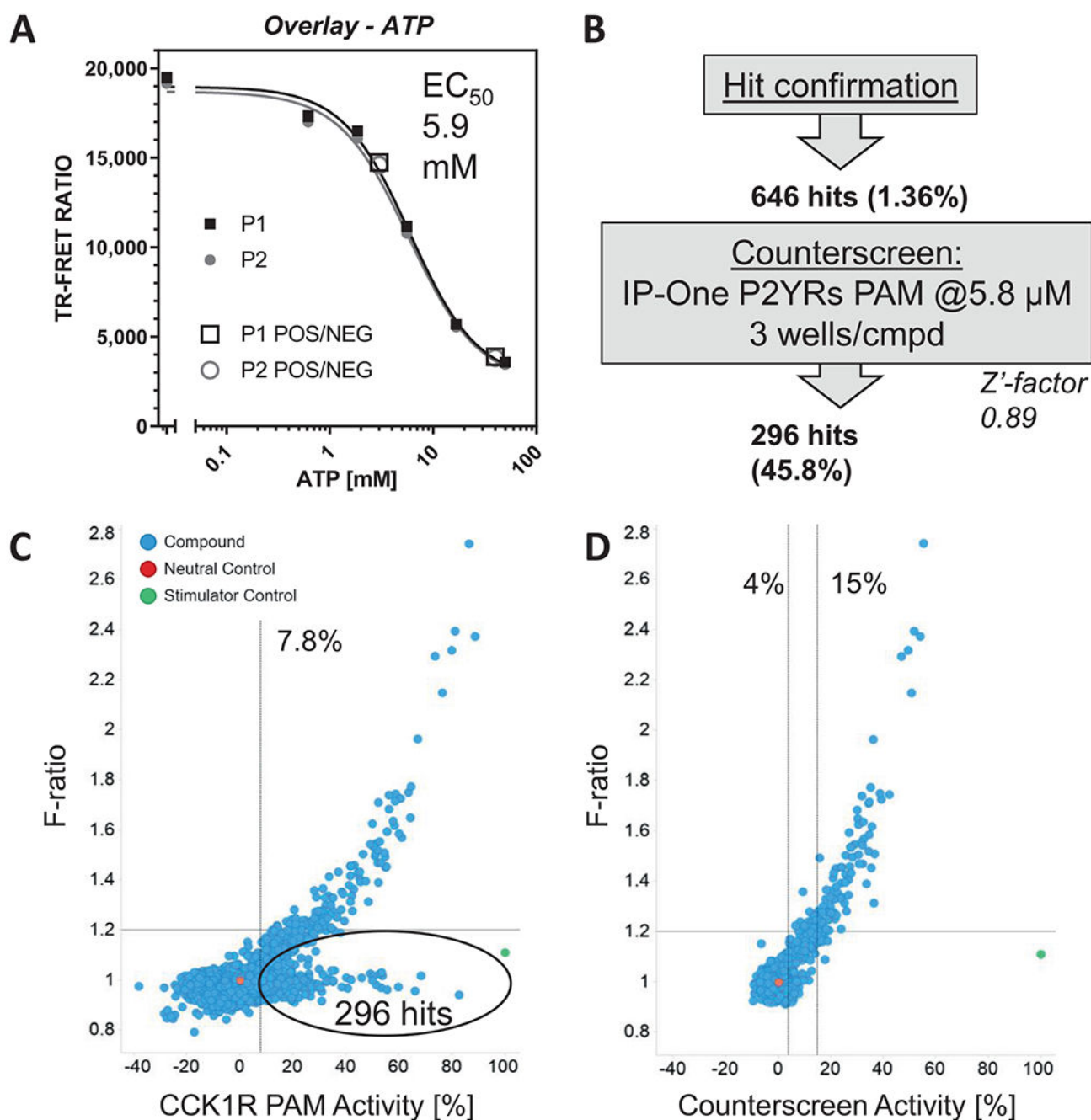


Fig. 2. Hit confirmation and P2YR counter-screen yielded 296 compounds for hit prioritization: (A) Overlay of ATP dose-response curves of P2YR counter-screen (plates P1-2) in HEK-293 parental cells, with positive and negative control ratios in open symbols; graphs plotted using GraphPad Prism; TR-FRET ratios shown as mean \pm SD. (B) Testing funnel stages describing conditions and performance of IP-One P2YRs PAM counter-screen resulting in 296 hits for prioritization assays. Scatterplots depicting % activity in (C) IP-One CCK1R PAM or (D) IP-One P2YRs PAM counter-screen assays (x-axis) in correlation to F-ratio

(y-axis) with hit selection cut-off lines and (C) visualization of 296 compounds; scatterplots were created using TIBCO Spotfire.

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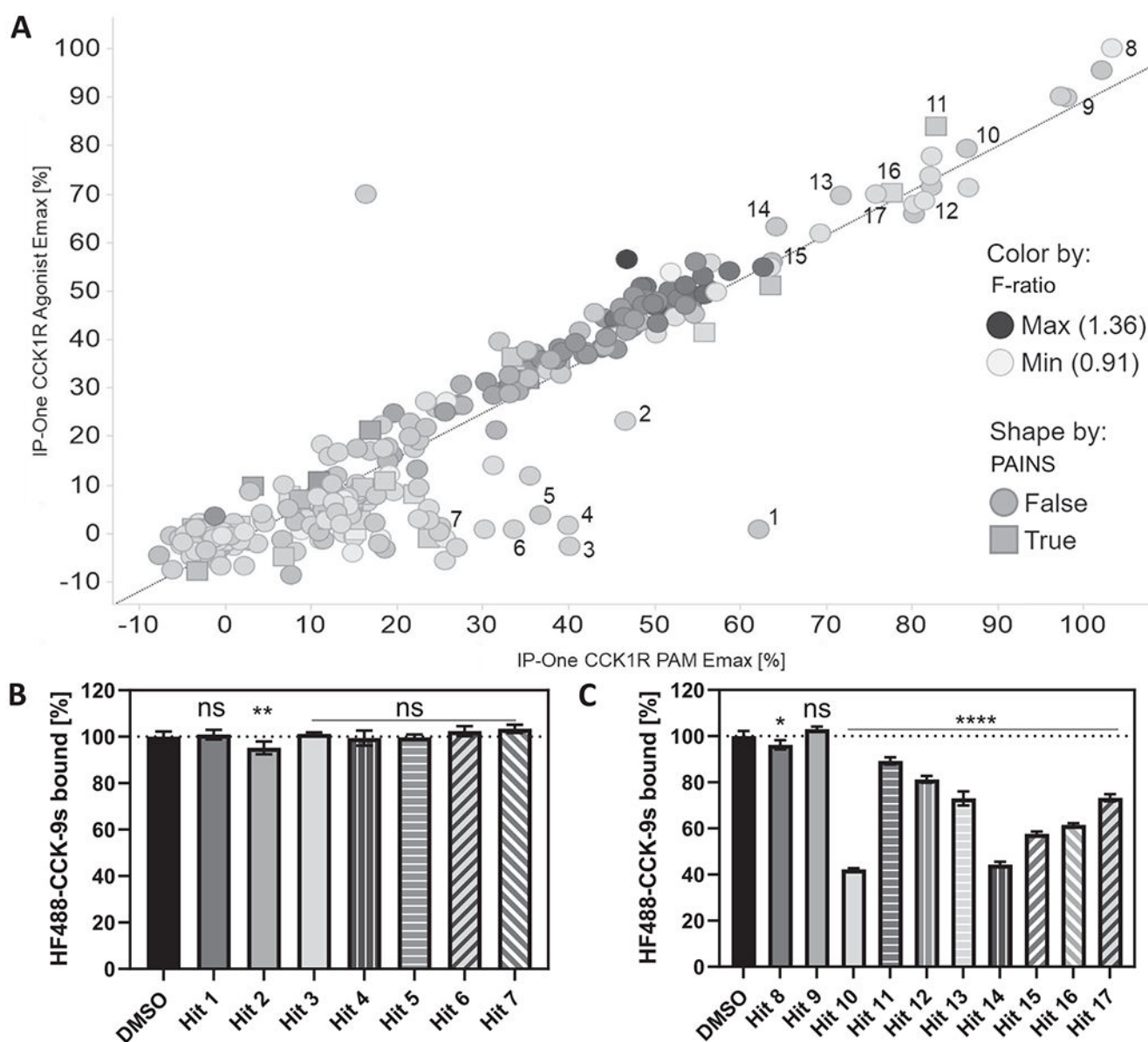


Fig. 3. Hit prioritization distinguished modulators with and without intrinsic agonist activity: (A) Scatterplot illustrating % maximal efficacy (E_{max}) in IP-One CCK1R PAM format (x-axis) in correlation to % E_{max} in IP-One CCK1R agonist mode; hits identified through Pan Assay Interference Compounds (PAINS) filter depicted in squares; higher color intensity indicates higher F-ratios; numbers refer to hits depicted in (B) and (C); scatterplot was generated in TIBCO Spotfire. Bar graphs showing (B) hits with minimal or no intrinsic agonist activity and (C) hits with intrinsic agonist activity tested in TR-FRET competition binding assays in HEK-293 SNAP-CCK1R membranes; TR-FRET ratios normalized to acceptor ligand HF488-CCK-9s bound [%] using GraphPad Prism; statistical significance determined using GraphPad Prism's ordinary one-way ANOVA and Dunnett's multiple comparisons test ($\alpha = 0.05$); P values depicted according to the following classification: (* $p = 0.01-0.05$), (** $p = 0.001-0.01$), (***) $p = 0.0001-0.001$), (**** $p < 0.0001$).

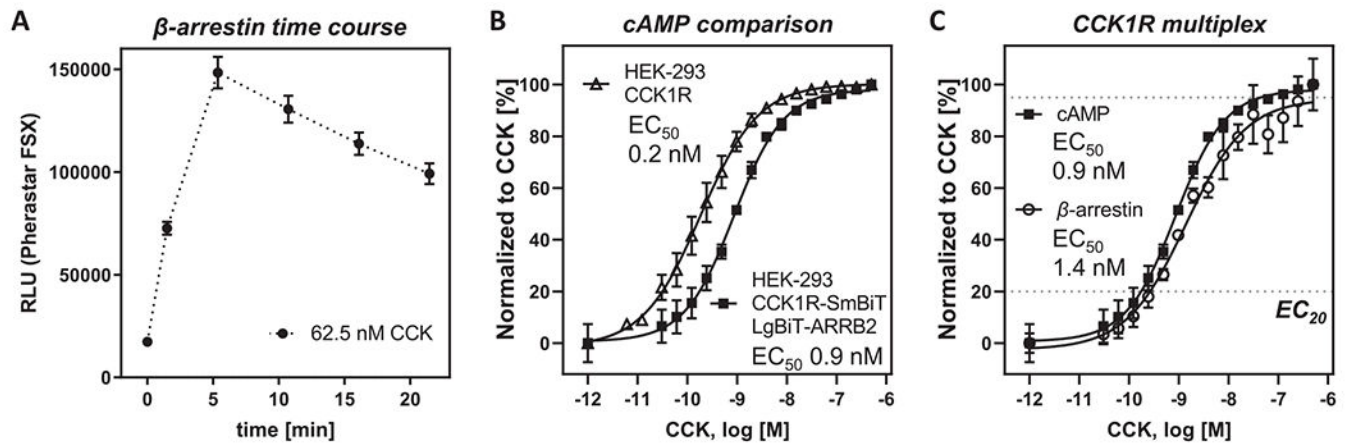


Fig. 4. Development of CCK1R multiplex assay monitoring β -arrestin-2 recruitment and cAMP accumulation:

(A) Development of β -arrestin recruitment assay based on Promega's NanoBiT technology; luminescence time course recorded after addition of 62.5 nM CCK to HEK-293 cells transiently expressing CCK1R-SmBiT and LgBiT-ARRB2; relative luminescence units (RLU) measured using Pherastar FSX plate reader; experiment performed in triplicate and results representative of independent experiments. (B) Comparison of CCK induced cAMP accumulation in HEK-293 CCK1R and HEK-293 CCK1R-SmBiT LgBiT-ARRB2 monoclonal, stable cell lines; TR-FRET ratios normalized to CCK. (C) CCK dose-response for cAMP production and β -arrestin recruitment determined in multiplex format in HEK-293 CCK1R-SmBiT LgBiT-ARRB2 cells; dotted lines indicate assay window for both signaling pathways; TR-FRET ratios and RLUs normalized to CCK. (B, C) Experiments performed in duplicate in at least three independent experiments. (A-C) Data shown as mean \pm SEM; analysis and curve fitting conducted using GraphPad Prism 9.3.1.

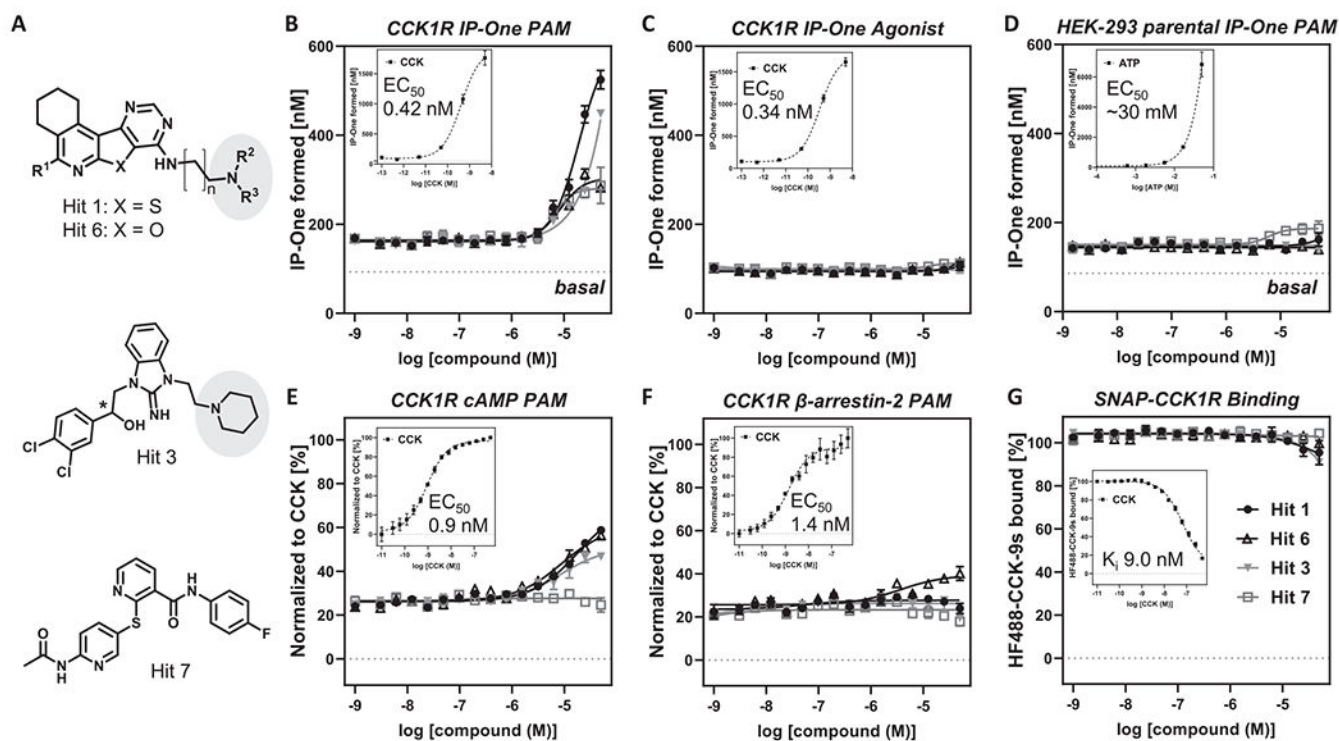


Fig. 5. Validation and pharmacological characterization of promising scaffolds:

(A) Top down: chemical structures of hits 1, 6 (tetracyclic scaffold) and hits 3, 7 (exact structures); tertiary amine highlighted in hit 1, 6 and 3. Ligand and hit titration on (B, C) HEK-293 CCK1R cells in CCK PAM (B) and agonist (C) format or on (D) HEK-293 parental cells in ATP PAM mode; (B-D) IP-One accumulation determined as TR-FRET ratios and converted to IP-One concentrations formed in nM by interpolating the results using IP-One standard curves. CCK1R multiplex PAM assay in HEK-293 CCK1R-SmBiT LgBit-ARRB2 recording compound effect on CCK induced (E) cAMP accumulation or (F) β -arrestin-2 recruitment; TR-FRET ratios (E) or RLU's (F) normalized to CCK response. (G) Competition binding studies in HEK-293 SNAP-CCK1R membranes; fraction of HF488-CCK-9s bound obtained as TR-FRET ratios and normalized to HF488-CCK-9s and DMSO-treated control wells. (B-G) Hit 1 (filled circle), hit 6 (open triangle), hit 3 (filled triangle) and hit 7 (open square) tested at up to 50 μ M as 2-fold dilutions; experiments performed in duplicate in at least three independent experiments; data shown as mean \pm SEM and analyzed with GraphPad Prism 9.3.1.

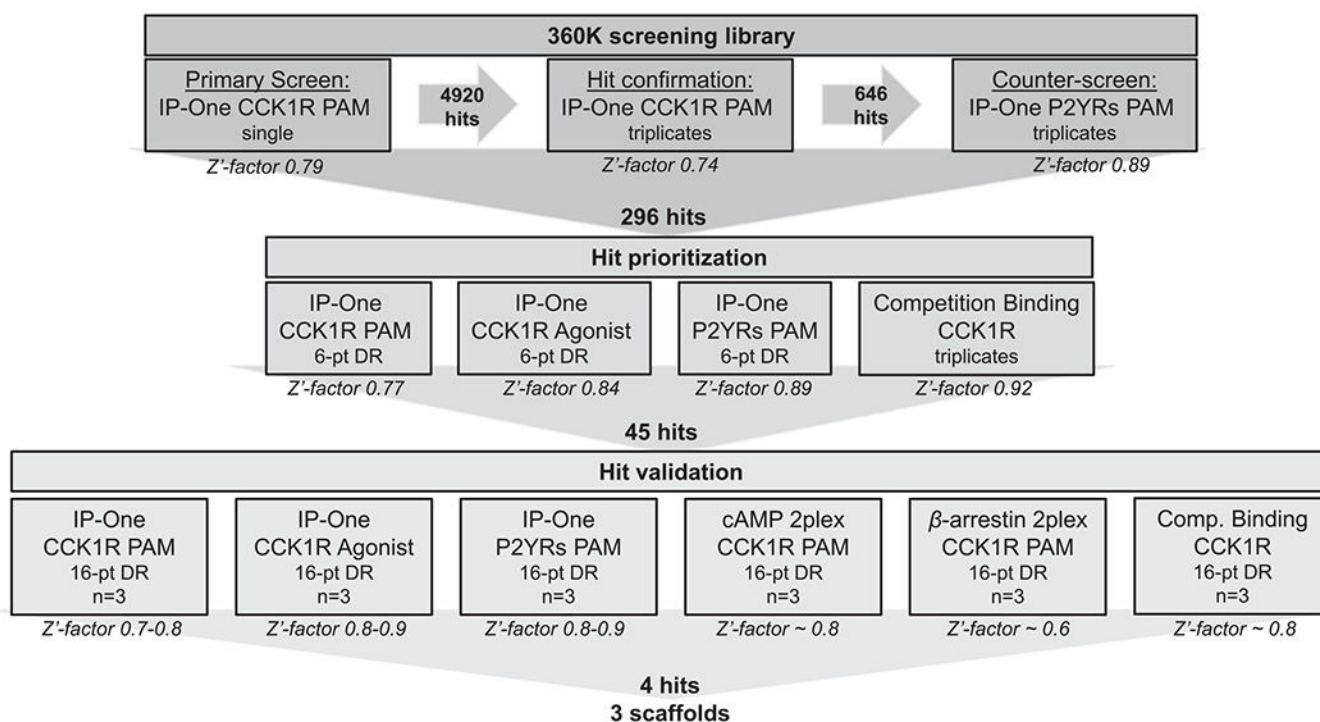


Fig. 6. Testing funnel to identify CCK1R PAMs without intrinsic agonist activity:

Complete testing funnel describing conditions and performance of different stages: Stage 1 comprised primary screening of 360K compound library, hit confirmation and counter-screen to exclude non-specific hits; followed by stage 2, which included hit prioritization of 296 identified hits tested as 6-point dose response in IP-One CCK1R PAM and agonist, and P2YR counter-screen formats, as well as single concentration competition binding studies in triplicates resulting in 45 hits displaying desired profile. Stage 3 referred to dry powder hit validation comprising CCK1R PAM, agonist and P2YRs PAM IP-One assays, multiplex screening for cAMP accumulation and β -arrestin recruitment and CCK1R competition binding experiments in 16-point dose-response format leading to 4 hits and three scaffolds exerting specific CCK1R PAM but no intrinsic agonist activity.