Identification of a novel scaffold for a small molecule GPR139 receptor agonist

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GPR139 is an orphan class A G protein-coupled receptor (GPCR) originally identified using bioinformatic searches of the human genome1. In mammals, GPR139 is predominantly expressed in the central nervous system, with the highest expression observed in the striatum, pituitary, habenula, thalamus and hypothalamus2–6. Given its expression profile, GPR139 has been suggested as a therapeutic target for metabolic syndromes6–10 or motor diseases5,6,8,9. Despite its putative value as a drug target, GPR139 has proven challenging to study due to the relative paucity of probe compounds with which to characterize the receptor.

Several endogenous GPR139 ligands have been proposed, including the aromatic amino acids L-tryptophan and L-phenylalanine, the adrenocorticotropic hormone (ACTH), and α- and β-melanocyte-stimulating hormone (EC50 = 220 μM, 320 μM, 2.1 μM, 2.2 μM, and 6.3 μM, respectively)5,6. In addition, multiple surrogate GPR139 agonists have been described by Hu et al.5, Shi et al. from Lundbeck A/S11, Dvorak et al. from Janssen R&D10, Hitchcock et al. from Takeda Pharmaceutical Company Limited12, as well as by Shehata et al.3 (Fig. 1). In contrast, only few GPR139 antagonists, of which none are potent and selective, have been identified to date5,14,15.

Here, we generated a focused compound library, designed to maximize structural diversity while selecting for sensible physicochemical properties, and screened it against GPR139 using a protocol designed to identify both agonist and antagonist hits. We identified 36 novel agonists, including AC4, which exhibits unique structural elements unseen in our recently published pharmacophore based on reported GPR139 ligands3. Moreover, we utilized site-directed mutagenesis to demonstrate that AC4 utilizes the same critical residues for binding at GPR139 as previously reported agonists 1a and 7c. By screening additional analogs, we identified 5 compounds with improved agonist potency that were confirmed in an orthogonal ERK-phosphorylation assay. Together, these ligands represent promising tool compounds and further expand the variety of chemical structures available with which to characterize GPR139 and to guide future campaigns in the development of surrogate ligands.

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Results
Identification of novel GPR139 agonists. To identify novel GPR139 ligand scaffolds, we performed a screening campaign of 4,000 chemically diverse screening compounds in CHO cells stably expressing the GPR139 receptor using a fluorescence-based calcium mobilization assay. Our 2-stage assay protocol enabled us to screen for agonist and antagonists simultaneously. This involved an initial addition of test compounds followed by a second stimulation with an EC80 concentration (800 nM) of the reference GPR139 agonist compound 1a. In this assay format, the addition of agonists led to a Ca2+ response during the initial 10 minute recording, although upon stimulation 1a was not able to activate GPR139 further, due to receptor desensitization in the constant presence of the test agonist (Fig. 2B, example with AC4 an agonist found in this screening campaign). Initial addition of antagonists did not elicit a Ca2+ response during the first compound addition step, but inhibited the response from 1a in the screening step (Fig. 2C, example shown with a published antagonist NCRW0105-E06). By way of comparison, the addition of 1a produced a full Ca2+ response in the wells that were initially tested with buffer or with compounds that had no effect on GPR139 (Fig. 2A, example with buffer). In total 11 hits, all agonists, were identified in the primary screen (Figs 3, S1 and Table S1).

AC4 binds in the same binding site as the known reference agonists. Our recent mutational study of GPR139 indicates that residues F1093, W2414 and N2717 are required for its activation by the two agonists 1a and 7c. In order to assess if the same residues were important for the recognition of AC4, the most potent agonist discovered in the primary screen (EC50 = 0.22 μM), we tested the ligand on our GPR139 mutants: F109A3, F109L3, W241H6 and N271A7. The expression levels of F109A3, F109L3 and W241H6 were not significantly different from the N-terminally myc-tagged GPR139 wild type (WT) receptor construct, whereas N271A7 was 53% expressed compared to WT. There was no measurable Ca2+ response from AC4 stimulation of mutant F109A3 and N271A7, and only a weak Ca2+ response (17 ± 2% of WT, n = 3) from F109L3 at the highest tested concentration (100 μM) of AC4 (Fig. 4). AC4 had a 12-fold lower EC50 on W241H6 than on WT, with a greatly reduced capacity for receptor activation (Emax = 37 ± 3% of WT, n = 3). This suggests that although the structure of AC4 is unique compared to 1a and 7c, it still occupies, at least partly, the same binding site as these known reference ligands.

Screening and pharmacological characterization of GPR139 compound analogs. To validate these observations and better define the structure-activity relationships of the novel agonists’ pharmacophores, 160 analogs of these initial hits were purchased and tested in Ca2+ mobilization assays. From this new analog library we identified 36 novel GPR139 agonists with potencies in the nanomolar range (Fig. 5, Table 1), 36 weak agonists with potencies between 1–10 μM, and a further 11 very weak agonists with EC50 values between 10–50 μM, respectively (Supplementary Fig. S2, Supplementary Table S2). Interestingly, a close analog of AC4, designated AC170, was completely inactive (Fig. 5, Supplementary Table S2).

The lead compound AC4 and the 5 most potent analogs as well as the inactive analog AC170 and three reference agonists 1a, 7c and L-tryptophan were tested in an orthologous ERK phosphorylation assay to further assess GPR139 activity. All compounds except AC170 led to robust, concentration-dependent ERK activation responses with similar potencies (Fig. 6, Table 2) and potency rank-order to the Ca2+ mobilization assay results (Figs 1 and 5, Table 1).

Figure 1. Chemical structures and EC50 values of GPR139 agonist compounds: AC4 and AC170 (this study), 1a11, 7c10, 3912, DL4313, and L-tryptophan7.
AC4 presents a novel GPCR139 agonist scaffold. Our 36 novel GPR139 agonists were compared to the salient structural elements in our previously published GPR139 pharmacophore model. Only the most potent analog compound, AC224, matched all 6 of our designated pharmacophore elements (Table 1, Supplementary Fig. S3). In contrast to the majority of new agonists that matched at least four of the six pharmacophore elements,  

Figure 2. Representative data of the 2-stage screening protocol which enabled simultaneously screening for agonist and antagonists hits by eliciting different response profiles during the initial addition of (A) buffer, (B) agonist or (C) antagonist and subsequent addition of the reference agonist compounds 1a (cmp1a). Only the second addition of 1a was measured during the screening campaign and the agonist/antagonist activity was then uncovered during subsequent detailed characterization of the hits.
AC4 featured an alternative structure, which only matched three of the elements (Supplementary Fig. S3). Thus, AC4 presents a novel chemical scaffold containing only one terminal aromatic ring, a carboxylic acid at the ligand’s opposite terminal, and an oxadiazole moiety in the linker region (Fig. 1).

**Discussion**

We performed a screening campaign using a diverse chemical library and identified 36 potent GPR139 agonists, among them AC4 which introduces a unique scaffold not shared by any previously described GPR139 agonists. AC4 displays an EC$_{50}$ value of 220 nM and a similar maximal response as the previously published agonist 1a$^{11}$. Similar to the aromatic amino acid agonists (L-tryptophan and L-phenylalanine)$^{5,7}$, the chemical structure of AC4 presents a terminal aromatic ring. This is in contrast to 1a and 7c that feature aromatic systems on each end. Additionally, AC4 contains a carboxylate moiety on its terminal ring system (Fig. 1). Despite the lack of aromaticity at both ends of the molecule, the aliphatic ring in AC4 fits well into the suggested hydrophobic pharmacophore element (Supplementary Fig. S3), previously seen in all reported analogs$^{13}$.

Another unprecedented feature of AC4 is its lack of a hydrogen bond donor in its linker region. Instead, AC4 displays a unique cyclic aromatic 1,3,4-oxadiazole ring. In general, our presented analogues (Fig. S1) demonstrate a new linker cyclization exemplified by an imidazolidine-2,4-dione ring, which can substitute for the polar linkers seen in other GPR139 ligand examples$^{13}$. We previously reported that cyclization in the linker 2 position, as shown in the Hitchcock et al. compound series, is beneficial, whereas cyclization at the linker 5 and 6 positions is unfavorable$^{15}$. 

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**Figure 3.** Calcium mobilization concentration-response curves for 11 initial agonist hits from diverse library in CHO-GPR139 cells. assay. The graphs are mean ± S.D. of representative concentration-response curves out of at least three independent experiments performed in duplicate. Data are normalized to the Ca$^{2+}$ response of buffer (0%) and 8μM 1a (100%).

**Figure 4.** Ca$^{2+}$ mobilization concentration-response curves of AC4 on GPR139 wild-type (WT) and mutants. Data is shown from a representative experiment (mean ± S.D.) out of three independent experiments performed in duplicate. Data is normalized to the Ca$^{2+}$ response of buffer (0%) and 100μM AC4 (100%) on myc-GPR139(WT). Residue numbering in superscript is according to GPCRdb numbering system.
Here, we report 23 nanomolar GPR139 agonists that represent the first compounds with a novel linker cyclization between atoms 3 and 5 (AC123, AC134, AC136, AC137, AC138, AC186, AC187, AC191, AC192, AC197, AC200, AC202, AC205, AC206, AC208, AC209, AC210, AC224, AC225, AC229, AC233, AC234, and AC241), as well as a novel aromatic cyclic linker (AC4) (Supplementary Fig. S3, Supplementary Table S2).

It was interesting to note that a commercially available analog of AC4, designated AC170, did not evoke a GPR139 response in either Ca²⁺ mobilization or ERK phosphorylation assays (Figs 1, 5 and 6, Table 2 and Supplementary Table S2). This could be due to the change in the chemical nature of the substituent on the aromatic ring from being weak electron-withdrawing (halogen on AC4) into a strong electron-withdrawing group (nitrile on AC170). Our previous SAR analysis suggests that the size of both groups can be tolerated in other described ligands. Moreover, due to the difference in electronegativity, the ether oxygen in AC170 could be playing another role in weakening the aromatic ring character more than the thioether sulfur in AC4. Taken together, these features could explain the observed loss of activity.

Based on the available data, we suggested in our previous SAR analysis that a second terminal aromatic group contributes to the high potency of the most potent analogues 1a, 7c, and 39 from Shi et al., Dvorak et al., and Hitchcock et al., respectively. Interestingly, our ligands AC4, AC7, AC76, AC77, AC82, AC177, AC178, AC183, AC186, AC191, AC192, AC194, AC220, AC229, and AC230 all lack a second terminal aromatic ring (Supplementary Fig. S3), yet they all possess EC₅₀ values in the nanomolar range (90–990 nM). Thus, this series of new agonists demonstrate that one aromatic ring can be sufficient for potent GPR139 activity.

Our study also provided the first detailed pharmacological analyses of GPR139-mediated ERK phosphorylation using a state-of-the-art TR-FRET based assay, and show that the receptor is strongly coupled to that pathway (Fig. 6, Table 2). This correlates with and greatly expands a previous report showing that a high concentration of L-tryptophan and L-phenylalanine increased ERK phosphorylation in a Western blot assay. Recent studies have shown that biased agonists can differentially activate intracellular calcium and ERK phosphorylation responses, which potentially can lead to different physiological responses and therapeutic effects. It was thus of interest to determine if the most potent agonists identified in the present study as well as reference agonists 1a, 7c and L-tryptophan displayed such biased signaling or activate both pathways equally well. Our data showed that all compounds shows similar potencies and potency rank-order in both pathways, and thus are not biased with respect to these signaling pathways/endpoints (Figs 1, 5 and 6, Tables 1 and 2).

Our mutagenesis data, suggest that F109, W241, and N271 are important for GPR139 activation by AC4, as previously shown for 1a and 7c. This provides strong evidence that the novel scaffold of AC4 is equipotent, and it also shares the same binding site as the other GPR139 agonists, despite the differences between their chemical structures.

Given our successful identification of a new agonist scaffold, it was striking that we did not identify any GPR139 antagonists in our screen. It appears that these have been challenging given the discrepancy between published findings on small molecule agonists versus antagonists that target GPR139. The development of more potent and selective antagonists remains an area of great interest, and would be of particular value for in vivo studies to delineate the biological function of the receptor.

In summary, we have identified 36 novel GPR139 agonists with nanomolar potency. Interestingly, we discovered AC4, a compound with novel structural features that distinguish it from other known GPR139 agonists. We showed via mutagenesis that AC4 activates GPR139 in a similar manner as previously published agonists despite
Table 1. Potency and maximal response of the 36 identified GPR139 agonists in calcium mobilization assay in CHO-GPR139 cells. The data is based on at least three independent full concentration-response curves in duplicate, normalized to buffer (0%) and 8 μM 1a (100%). *Number of matching pharmacophore elements from Supplementary Fig. S3.

| ID   | Enamine ID | EC50 μM | pEC50 ± S.E.M. | Emax ± S.E.M. | n | Matches* |
|------|------------|---------|----------------|--------------|---|----------|
| AC224| Z30029744  | 0.09    | 7.03 ± 0.02    | 106 ± 1      | 3 | 6        |
| AC177| Z1618891443| 0.09    | 7.02 ± 0.11    | 103 ± 1      | 4 | 5        |
| AC183| Z352587398 | 0.18    | 6.74 ± 0.19    | 110 ± 10     | 3 | 5        |
| AC197| Z16669083  | 0.19    | 6.71 ± 0.14    | 103 ± 1      | 3 | 5        |
| AC194| Z18052622  | 0.21    | 6.68 ± 0.02    | 106 ± 4      | 3 | 5        |
| AC4  | Z446416294 | 0.22    | 6.66 ± 0.06    | 116 ± 3      | 5 | 3        |
| AC225| Z13589494  | 0.28    | 6.56 ± 0.05    | 120 ± 5      | 3 | 5        |
| AC178| Z1618764723| 0.28    | 6.55 ± 0.07    | 97 ± 5       | 3 | 5        |
| AC209| Z18053107  | 0.29    | 6.54 ± 0.17    | 107 ± 3      | 4 | 5        |
| AC82 | Z352587564 | 0.29    | 6.54 ± 0.08    | 102 ± 6      | 3 | 4        |
| AC202| Z16669106  | 0.34    | 6.47 ± 0.09    | 102 ± 2      | 3 | 4        |
| AC233| Z14078826  | 0.34    | 6.47 ± 0.19    | 80 ± 6       | 3 | 5        |
| AC191| Z18052722  | 0.35    | 6.46 ± 0.16    | 99 ± 2       | 3 | 5        |
| AC243| Z14078663  | 0.35    | 6.45 ± 0.11    | 86 ± 5       | 3 | 4        |
| AC206| Z16668711  | 0.38    | 6.42 ± 0.06    | 94 ± 3       | 3 | 5        |
| AC234| Z14075523  | 0.38    | 6.42 ± 0.07    | 99 ± 3       | 3 | 5        |
| AC7  | Z16668610  | 0.39    | 6.41 ± 0.12    | 112 ± 5      | 4 | 4        |
| AC186| Z14077796  | 0.41    | 6.39 ± 0.12    | 89 ± 5       | 3 | 5        |
| AC241| Z165543100 | 0.42    | 6.38 ± 0.06    | 88 ± 3       | 3 | 5        |
| AC229| Z14075450  | 0.43    | 6.36 ± 0.16    | 95 ± 2       | 4 | 4        |
| AC205| Z18052636  | 0.44    | 6.36 ± 0.14    | 100 ± 3      | 3 | 5        |
| AC200| Z18052594  | 0.46    | 6.34 ± 0.11    | 111 ± 5      | 3 | 5        |
| AC220| Z16668660  | 0.49    | 6.31 ± 0.01    | 103 ± 3      | 3 | 5        |
| AC230| Z14075785  | 0.49    | 6.31 ± 0.27    | 89 ± 1       | 3 | 4        |
| AC137| Z16669312  | 0.54    | 6.27 ± 0.08    | 92 ± 3       | 3 | 5        |
| AC77 | Z336570670 | 0.60    | 6.22 ± 0.17    | 102 ± 4      | 3 | 5        |
| AC210| Z18052723  | 0.63    | 6.20 ± 0.24    | 115 ± 8      | 4 | 5        |
| AC123| Z54783382  | 0.74    | 6.13 ± 0.14    | 104 ± 1      | 3 | 5        |
| AC187| Z14077801  | 0.74    | 6.13 ± 0.22    | 102 ± 5      | 4 | 4        |
| AC192| Z16669294  | 0.75    | 6.12 ± 0.13    | 95 ± 1       | 3 | 4        |
| AC14 | Z336589360 | 0.78    | 6.11 ± 0.12    | 93 ± 3       | 3 | 5        |
| AC138| Z16688376  | 0.79    | 6.10 ± 0.14    | 100 ± 5      | 4 | 5        |
| AC134| Z16669311  | 0.80    | 6.09 ± 0.15    | 103 ± 5      | 4 | 5        |
| AC208| Z18053097  | 0.88    | 6.05 ± 0.60    | 134 ± 26     | 3 | 5        |
| AC76 | Z336570672 | 0.94    | 6.03 ± 0.10    | 97 ± 4       | 3 | 5        |
| AC136| Z16669296  | 0.99    | 6.01 ± 0.03    | 67 ± 6       | 3 | 5        |

Methods

Diverse compound library generation. Compound 1a was kindly provided by H. Lundbeck A/S, Denmark, and carbamoylcholine chloride (carbachol) was obtained from Sigma-Aldrich (C4382). The screening compound library (Enamine, Kiev, Ukraine) consisted of 4,000 compounds. The compounds were selected from a pool of 30,300 compounds from the Enamine screening compound catalogue based on the four following filters: (1) Molecular weight (g/mol) 200–500, (2) Hydrogen bond donors 1–5, (3) Hydrogen bond acceptors 2–10, (4) Maximum calculated logP = 4.5. These criteria yielded 7,339 unique structures. Similar compounds were removed based on their Tanimoto similarity score, in order to obtain a structurally diverse library with 4,000 compounds.

Cells and cell culture. All compounds were tested on a CHO-k1 cell line stably expressing GPR139 (CHO-GPR139) kindly provided by H. Lundbeck A/S, Denmark. GPR139-specific responses were assessed using a CHO-k1 cell line stably expressing the muscarinic acetylcholine receptor M1 (CHO-M1) (The Missouri

its different chemical structure. We also identified 5 more potent analogs of the original hits that share a similar rank order of potency in both Ca2+ mobilization and ERK assays. All together this data adds to our knowledge about surrogate agonists for GPR139.
S&T cDNA Resource Center, # CEM100TN00). The CHO-GPR139 cells were grown in Dulbecco's modified Eagle's medium (DMEM) F12-Kaighn’s (ThermoFisher Scientific # 21127) supplemented with 10% dialyzed fetal bovine serum (United States origin; ThermoFisher Scientific # 26400), 1% GlutaMAX-I (100X) (ThermoFisher Scientific # 35050061), and 100 units/mL penicillin and 100 µg/mL streptomycin (ThermoFisher Scientific # 15140122) and 1 mg/mL geneticin (ThermoFisher Scientific # 11811031). The CHO-M1 cells were grown in Ham's F12 (ThermoFisher Scientific # 21765) supplemented with 10% fetal bovine serum (South American origin; ThermoFisher Scientific # 10270) + 100 units/mL penicillin and streptomycin and 0.25 mg/mL geneticin.

Primary screening using calcium mobilization. The Fluo-4 Ca²⁺-assay was performed as described previously, with minor modifications. After 1 hour incubation with the Fluo-4 dye, the CHO-GPR139 cells were washed with 100 µL HEPES buffer (Hank's Balanced Salt Solution (HBBS, ThermoFisher Scientific # 14175053) supplemented with 20 mM HEPES, 1 mM MgCl₂, 1 mM CaCl₂, pH = 7.4) and the compound library was then added in concentrations between 25–63 µM dissolved in HEPES buffer (supplemented with 2.5 mM probenecid and 1% DMSO) and pre-incubated for 10 min at 37 °C. 33 µL of 3.2 µM 1a were added automatically after baseline measurements. Intracellular Ca²⁺ changes were recorded on a NOVOstar instrument (BMG Labtech) at 37 °C with an excitation filter of 485 nm and an emission filters 520 nm. This set-up allowed us to identify both antagonists and agonists (Fig. 2).

Hit validation and analog purchases. The primary screening of 4,000 diverse screening compounds on GPR139, led us to identify 108 initial actives in GPR139-expressing cells. These 108 compounds inhibited the

| ID      | Enamine ID      | EC₅₀ µM | pEC₅₀ ± SEM | Emax ± SEM | n |
|---------|-----------------|--------|-------------|------------|---|
| Compound 7c | —               | 0.013  | 7.89 ± 0.15 | 89.08 ± 4.38 | 3 |
| Compound 1a | —               | 0.028  | 7.55 ± 0.14 | 81.55 ± 4.12 | 6 |
| L-tryptophan | —               | 0.77   | 3.12 ± 0.26 | 76.62 ± 8.59 | 2 |
| AC224   | Z30029744       | 0.031  | 7.51 ± 0.28 | 69.37 ± 8.01 | 3 |
| AC177   | Z1618891443     | 0.022  | 7.65 ± 0.13 | 78.10 ± 3.19 | 5 |
| AC183   | Z352587398      | 0.039  | 7.41 ± 0.25 | 64.38 ± 5.28 | 5 |
| AC197   | Z16669083       | 0.94   | 7.03 ± 0.14 | 78.70 ± 4.05 | 5 |
| AC194   | Z18052622       | 0.039  | 7.41 ± 0.30 | 62.32 ± 6.66 | 3 |
| AC4     | Z446416294      | 0.18   | 6.75 ± 0.21 | 77.22 ± 6.53 | 3 |
| AC170   | Z838953070      | n.d.   | —           | —           | 3 |

Table 2. New GPR139 agonists activate ERK with similar rank order of potency. Agonist potency (EC₅₀ and pEC₅₀) and efficacy (Emax, expressed relative to FBS positive control) of the 6 most potent new GPR139 agonists and structurally similar inactive analogue AC170. Previously reported GPR139 agonists (compound 1a, compound 7c and L-tryptophan) are reported for comparison. n.d. (not determined, as concentration-response curves could not be generated).
Ca²⁺ response induced by 800 nM 1a (≈EC₉₀) by more than 75%. We excluded compounds that had (1) an effect on another simultaneously tested GPCR (data not shown), (2) reactive groups, or (3) poor predicted solubility. 52 of the active compounds were re-purchased for validation, which were tested at three concentrations (100, 50, and 25 μM) using the same methodology as in the screening campaign. Out of the 52 compounds, 13 compounds had no effect (excluded as false positives), 28 compounds elicited an equal response in GPR139 and muscarinic acetylcholine receptor M1 cells and were designated as not selective, and 11 compounds were identified as viable candidates (AC4, AC7, AC8, AC9, AC10, AC11, AC12, AC13, AC14, AC16, and AC37) (Supplementary Fig. S1). None of the 11 hits promoted calcium mobilization in the M1 receptor cells (data not shown). The 11 hits were then screened in pure agonist mode (i.e., without the presence of 1a) to evaluate if they were agonists or antagonists (Fig. 3 and Supplementary Table S1). All 11 hits were agonists.

**Calcium mobilization assay for concentration-response curves of hits and analogs.** Agonist mode. Each compound, for which a concentration response curve was made, was tested at least three times in duplicate measurements. After the incubation with the Fluor-4 dye and a wash, 100 μL HEPES buffer (supplemented with 2.5 mM probenecid) was added to each well and incubated for 10 minutes at 37 °C before recording. 33 μL of compounds (4× concentrated) were added automatically after baseline measurements. All concentration response curves of the 11 hits and 160 analogs were measured on a FlexStation 3 Benchtop Multi-Mode Microplate Reader (Molecular Devices) at 37 °C with an excitation filter of 485 nm and emission at 525 nm.

Antagonist mode. When screening the compounds as antagonists they were pre-incubated for 10 min as in the HTS. 33 μL of 3.2 μM 1a on the CHO-GPR139 cells or 20 μM carbachol on the CHO-M1 cells were added automatically after baseline measurements. These data were measured on the FlexStation.

**Concentration-response curves of analogs in phospho-ERK assay.** Phospho-ERK measurements were made using the Advanced Phospho-ERK1/2 (Thr202/Tyr204) HTRF kit by Cisbio (Codolet, France). Approximately 24 hours prior to the experiment, CHO-GPR139 cells were plated at 50,000 cells/well onto a 96-well plate and cultured as detailed above. Approximately 6 hours later, the media was aspirated, replaced with 50 μL serum-free media, and the cells were allowed to starve overnight. The day of the experiment, serial dilutions of the test compounds and controls were made in serum-free media at 2x final concentration. 50 μL of test compounds were added directly to the appropriate wells for 5 minute stimulation at 37 °C. At this point, the media and compounds were rapidly aspirated, lysis/blocking buffer was added, and cells were lysed on a shaker for 30 minutes at room temperature. Lysates were combined with detection solution, incubated for 4 hours, and read on an Envision plate reader measuring emission at 665 and 620 nm.

**Mutational studies.** The mutational studies were performed as previously described18.

**Data analysis.** The Ca²⁺ and ERK responses were normalized as indicated and the concentration response curves were fitted by Prism 6 software (GraphPad) using nonlinear regression in a sigmoidal model with variable slope, as has been previously described19.

**Ligand preparation and pharmacophore matching.** LigPrep was used for ligand preparation19. Macromodel was employed to do the conformational analysis on ligands using default settings20. This includes using the OPLS_3 force field21, and the Monte Carlo approach for sampling the different conformations. The global energy non-collapsed conformation of the ligands was picked for further analysis and superposition. Phase was used to match the pharmacophore to the herein presented analogues with default settings22–24.

**Data Availability**

The authors declare that all data supporting the findings of this study are available within the article and Supplementary Information, or are available from corresponding authors upon request.

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Author Contributions
D.E.G. and H.B.-O. designed the study. A.C.N. and D.P. conducted the compound library screening and performed this data analysis. A.C.N. and M.V. conducted the pharmacological characterization of screening hits and analogs hereof in the calcium mobilization assay and performed this data analysis. A.C.N. conducted the pharmacological characterization of the receptor mutants and performed this data analysis. R.P., S.R.F. and P.R.G. conducted the pharmacological characterization of the most active compounds in the ERK phosphorylation assay and performed this data analysis. M.A.S. and D.E.G. conducted the computational modeling and designed the compound library. All authors wrote or contributed to the writing of the manuscript. D.E.G. and H.B.-O. supervised the computational and pharmacological work, respectively.

Additional Information

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