Supplementary Information for

Discovery of a Functionally Selective Ghrelin Receptor (GHSR₁ₐ) Ligand for Modulating Brain Dopamine

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Chemicals and Compounds. Full length, human acyl-ghrelin 1-28 (Cat. no. 1463), L-692,585 (Cat. no. 2261), YIL781 hydrochloride (Cat. no. 3959), and MK-0677 (Cat. no. 5272) were purchased from Tocris Biosciences (Bristol, United Kingdom). JMV2959 was purchased from Sigma Aldrich (Cat no. 345888). [125I]ghrelin was purchased from Perkin-Elmer (Waltham, MA; Cat no. NEX388010UC). Ghrelin peptide was maintained as a 1 mM stock in 50% glycerol, whereas all other small molecule ligands (including N8279) were maintained as 10 mM stocks in dimethyl sulfoxide (DMSO).

NCGC00538279 (N8279/NCATS-SM8864) Synthesis.

\[
\begin{align*}
1 & \quad \text{Cl} \quad \text{O} \quad \text{OEt} \\
+ & \quad \text{MeO} \quad \text{MeO} \\
+ & \quad \text{H}_2\text{N} \quad \text{N} \\
\rightarrow & \quad \text{Cl} \quad \text{O} \quad \text{N} \quad \text{N} \\
\end{align*}
\]

7-chloro-1-(3,4-dimethoxyphenyl)-2-(3-(dimethylamino)propyl)-1,2-dihydrochromeno[2,3-c]pyrrole-3,9-dione (4). The title compound was prepared according to Vydzhak, R.N.; Panchishin, S.Y. Synthesis of 2-Alkyl-1-aryl-1,2-dihydrochromeno[2,3-c]pyrrole-3,9-dione Derivatives. Russ. J. Gen. Chem. 2008, 78, 2391-2397.)

\(^1\)H NMR (400 MHz, Chloroform-d) \(\delta\) 8.12 (d, \(J = 2.3\) Hz, 1H), 7.71 – 7.61 (m, 2H), 6.92 – 6.81 (m, 2H), 6.65 (d, \(J = 1.9\) Hz, 1H), 5.48 (s, 1H), 3.81 – 3.90 (m, 7H), 2.99 (ddd, \(J = 14.0, 8.2, 5.9\) Hz, 1H), 2.29 – 2.19 (m, 2H), 2.15 (s, 6H), 1.85 – 1.63 (m, 2H); LCMS RT (Method 1) = 4.035 min, \(m/z\) 457.2 [M + H\(^+\)]; HRMS (ESI) \(m/z\) calcd for C\(_{24}\)H\(_{26}\)ClN\(_2\)O\(_5\) [M + H\(^+\)] 457.1530, found 457.1537.

6-chloro-3-(3,4-dimethoxybenzoyl)-N-(3-(dimethylamino)propyl)-4-oxo-4H-chromene-2-carboxamide (5). A solution of 7-chloro-1-(3,4-dimethoxyphenyl)-2-(3-(dimethylamino)propyl)-1,2-dihydrochromeno[2,3-c]pyrrole-3,9-dione (200 mg, 0.438 mmol) was dissolved in DMSO (10
mL). To this solution was bubbled air until complete conversion was observed by LCMS. The reaction mixture was blown dry and purified by column chromatography (Methanol/DCM) to afford the desired product (2:1 ratio 5a:5b) as a light brown solid (86.7 mg, 42%).

5a: 1H NMR (400 MHz, Methylene Chloride-d2) δ 9.48 (s, 1H), 8.12 (d, J = 2.5 Hz, 1H), 7.73 – 7.62 (m, 2H), 7.08 (d, J = 2.2 Hz, 1H), 6.98 (dd, J = 8.4, 2.2 Hz, 1H), 6.84 (d, J = 8.4 Hz, 1H), 3.83 (s, 3H), 3.82 (s, 3H), 3.69 (ddd, J = 14.7, 5.5, 3.9 Hz, 2H), 2.88 (ddd, J = 14.1, 10.4, 3.2 Hz, 2H), 2.33 (m, 2H), 2.22 (s, 6H); 5b: 1H NMR (400 MHz, Methylene Chloride-d2) δ 8.15 (d, J = 2.6 Hz, 1H), 7.77 (dd, J = 8.9, 2.6 Hz, 1H), 7.73 – 7.64 (m, 1H), 7.58 (d, J = 2.0 Hz, 1H), 7.30 (dd, J = 8.4, 2.0 Hz, 1H), 6.84 (d, J = 8.4 Hz, 1H), 3.92 (s, 3H), 3.89 (s, 3H), 3.51 – 3.43 (m, 1H), 2.74 (m, 1H), 2.60 – 2.52 (m, 1H), 2.53 (s, 6H), 2.33 (m, 1H), 1.87 (m, 1H), 1.71 (m, 1H); LCMS RT (Method 1) = 3.921 min, m/z 473.2 [M + H⁺]; HRMS (ESI) m/z calcd for C24H26ClN2O6+ [M + H⁺] 473.1479, found 473.1467.

Analytical analysis was performed on an Agilent LC/MS (Agilent Technologies, Santa Clara, CA). Method 1: A 7-min gradient of 4% to 100% acetonitrile (containing 0.025% trifluoroacetic acid) in water (containing 0.05% trifluoroacetic acid) was used with an 8-min run time at a flow rate of 1.0 mL/min. Method 2: A 3-min gradient of 4% to 100% acetonitrile (containing 0.025% trifluoroacetic acid) in water (containing 0.05% trifluoroacetic acid) was used with a 4.5-min run time at a flow rate of 1.0 mL/min. A Phenomenex Luna C18 column (3 micron, 3 x 75 mm) was used at a temperature of 50 °C. Purity determination was performed using an Agilent diode array detector for both Method 1 and Method 2. Mass determination was performed using an Agilent 6130 mass spectrometer with electrospray ionization in the positive mode.

Plasmids. The 3xHA-hGHSR1aWT plasmid was originally purchased from the cDNA Resource Center (Bloomsberg, PA) and consists of an N-terminally 3xHA-tagged human GHSR1aWT coding sequence cloned into a pcDNA3.1+ backbone. This construct was used to generate all mutant 3xHA-hGHSR1a constructs by QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). The expression vector for the bioluminescent, mitochondria-targeted apoaequorin Ca2+ sensor (miAeq1) was a gift from Dr. Stanley Thayer (University of Minnesota). To generate a C-terminally-tagged 3xHA-hGHSR1aWT plasmid with LgBiT, the coding sequence for 3xHA-hGHSR1aWT was inserted 5′ into a vector containing the coding sequence for the LgBiT NanoLuc fragment (pBiT1.2-C). To generate a complementary N-terminally-tagged β-arrestin2WT plasmid, β-arrestin2WT was inserted 3′ of the coding sequence for the SmBiT NanoLuc fragment (pBiT2.2-N). Both LgBiT and SmBiT vector backbones were purchased from Promega. GoqLgBiT, SmBiT-β1, untagged Gy2, and RIC8A were generous gifts from Dr. Asuka Inoue. The hGHSR1aWT-
The RhoA-dependent serum response element plasmid SRF-RE-Luciferase, cloned into the pGL4.34[luc2P/SRF-RE/Hygro] vector backbone, was purchased from Promega (Cat. no. E1350). pcDNA3.1+ was used as empty vector in all experiments and all constructs were validated by sequencing.

[^125]Ighrelin-GHSR<sub>1a</sub> Radioligand Binding Assays.

**Saturation:**[^125]Ighrelin (Perkin-Elmer, Waltham, MA) saturation binding experiments were performed as described previously with minor modifications. Briefly, hGHSR<sub>1a</sub> WT (1 µg) was transiently transfected into HEK293/T cells cultured on 10cm dishes. After 48 hours, cells were collected, resuspended in hypotonic membrane lysis buffer (50 mM Tris-HCl, pH 7.4), then centrifuged at 21,000xG for 20 minutes to obtain crude membrane pellets. Membranes were resuspended in assay buffer (50 mM Tris-HCl, 5 mM EDTA, 5 mM MgCl₂, 1% BSA), protein content was measured by BCA protein assay, and diluted to 2 µg/mL. Ten µL of GHSR<sub>1a</sub>-expressing membrane (20 µg total protein) was then incubated with increasing concentrations of cold ghrelin (0.8-10 nM) and[^125]Ighrelin with its specific activity diluted 1:100. Non-specific binding was defined in parallel reactions containing 10 µM YIL781. Binding reactions proceeded for 1 hour on ice and were terminated by rapid filtration over 0.3% PEI-soaked GF/B filters with a 96-well Brandel harvester, followed by four washes with ice-cold wash buffer (Tris HCl, pH 7.4).

**Competition:** Equilibrium[^125]Ighrelin competition binding assays were performed by incubating 2-20 µg of hGHSR<sub>1a</sub> WT (1 µg transfected, 10cm dish) or hGHSR<sub>1a</sub>L<sup>149G</sup> (0.5 µg transfected, 10cm dish)-expressing HEK293/T cell membranes with a fixed concentration of[^125]Ighrelin (3.2 nM) and increasing concentrations of cold acyl-ghrelin 1-28 or N8279. hGHSR<sub>1a</sub>L<sup>149G</sup> was transfected at half the total amount of cDNA relative to hGHSR<sub>1a</sub> WT because it exhibits enhanced cell surface expression. The data for each ligand was normalized to its respective vehicle condition, representing 100% binding. Equilibrium binding reactions proceeded for 1 hour and were terminated by rapid filtration over 0.3% PEI-soaked GF/B filters using a 96-well Brandel harvester, followed by four washes with ice-cold wash buffer.

**G<sub>αq/11</sub>-Dependent Intracellular Ca<sup>2+</sup> Mobilization.** HEK293/N cells stably expressing 3xHA-hGHSR<sub>1a</sub> WT and the mitochondrial-Aequorin (miAeq) Ca<sup>2+</sup> sensor were grown in standard DMEM supplemented with 10% FBS and antibiotic-antimycotic, as well as selection media (G418 and Puromycin). Cells were split into and seeded into 10cm dishes without antibiotic selection and
grown to confluency overnight. The next morning, cells were serum starved in 10mL of clear optiMEM (supplemented with L-glutamine and HEPES; Gibco, Waltham, MA) for 3-4 hours in a 37°C incubator with 5% CO2. Then, 25µL of coelenterazine H (NanoLight Technology, Pinetop, AZ) was added to each 10mL dish and incubated for another 1-2 hours. For antagonist assays, test compounds were either added directly to the media in 10cm dishes, or cells were collected by gentle washing/trituration and plated onto white, clear-bottomed 96-well plates for 30min - 1hr. Once the coelenterazine H (or antagonist) incubation was complete, cells were harvested from the 10cm dish by gentle washing/trituration and collected in a 15mL conical tube. Cell suspensions were then immediately subjected to assay by injecting cells onto a white, clear-bottomed 96-well plate containing 2x test compounds. Luminescence was read without delay and for 10 seconds per well. To control for cell number variability, cells were lysed in 2x lysis buffer (100mM CaCl₂ + 0.2% Triton-X) immediately following the assay. The max ligand-induced response (Ca²⁺ mobilization: net-miAeq) over 10 seconds was normalized by dividing the ligand-induced luminescence counts (L-miAeq) by the ligand-induced luminescence counts (L-miAeq) plus the luminescence measured upon cell lysis (Lysis-miAeq). Relative, lysis-normalized data were then normalized to the appropriate control/reference condition.

For experiments with WT and Gαq KO HEK293/S cells, 2.5 µg of hGHSR₁αWT, 10 µg of miAEQ, and 2.5 µg of pcDNA3.1 was transiently transfected into 10cm dishes and incubated overnight. For experiments evaluating hGHSR₁α mutant/variant-mediated Ca²⁺ mobilization, HEK293/T (10cm dishes) cells were transiently transfected with hGHSR₁αWT (2.5 µg) or a relative cell surface-expression normalized amount of hGHSR₁α mutant (see Fig. S6 and 6,7), 10 µg of miAEQ, and pcDNA3.1, then incubated overnight. The next morning, transfection media was exchanged with fresh DMEM (supplemented with 10% FBS and antibiotic-antimycotic). Twenty-four hours later, experiments proceeded in accordance with the procedures described above. To control for any possible kinetic differences in genetically-modified cell lines or GHSR₁α variants, the average ligand-induced response over 10 seconds was calculated, then normalized by dividing the L-miAeq by the L-miAeq plus the Lysis-miAeq and normalized to the appropriate control/reference condition.

**βarr₂GFP Translocation**

*Compound screening:* βarr2 translocation was assessed using U2OS cells stably expressing the human hGHSR₁α-vasopressin receptor 2 tail chimera (hGHSR₁αV₂T) and green fluorescent protein (GFP)-tagged βarr2, as described previously 2,5. Replacement of the C terminal sequence of
GHSR\textsubscript{1a} to the C terminal tail of a class B GPCR, vasopressin receptor 2 leads to the formation of stable GHSR\textsubscript{1a}/\beta\text{arrs} complexes in endocytic vesicles. On day 1, stable cells were split into MGB101-1-2-LG glass-bottom 384-well plates (MatriCal, Spokane, WA) using a Multidrop 384 liquid dispenser (Thermo Scientific, Hudson, NH). Each well contained 30 \mu L aliquots of 8,000 cells in Minimum Eagle’s medium (MEM) containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin/ streptomycin (Life Technologies, Grand Island, NY). The plates were incubated overnight at 37°C in 5% CO\textsubscript{2}, and on the following day, media was changed to 30 \mu L clear MEM without serum. Compounds at 50 \mu M in 5% DMSO were added to each well using a MicroLab StarLET liquid handler (Hamilton Robotics, Reno, NV) and diluted 10-fold to 5 \mu M final concentration. The plates were returned to the incubator for 40 min, and the cells were fixed by adding 30 \mu L of 2% paraformaldehyde-phosphate buffered saline (PBS) to each well. Plates were stored at 4°C until analysis on an ImageXpress Ultra (Molecular Devices, Sunnyvale, CA) at 488 nm. Images were analyzed using a wavelet algorithm to measure formation of fluorescence aggregates as described previously \cite{8}. Image results were also confirmed visually.

\textit{Evaluation of ghrelin- and N8279-induced \beta\text{arr2}GFP Translocation:} U2OS cells stably expressing the human GHSR\textsubscript{1a WT} and \beta\text{arr2}GFP were seeded onto 35 mm MatTek (Ashland, MA, USA) glass coverslip dishes in opti-MEM supplemented with 2% FBS and antibiotic-antimycotic and grown overnight in a humified incubator (5% CO\textsubscript{2}, 37°C). The next morning, media was aspirated and replaced with fresh, serum-free opti-MEM. Four hours later, cells were treated with vehicle, ghrelin (100 nM), or N8279 (100 nM), placed back in a humified incubator (5% CO\textsubscript{2}, 37°C) and incubated for 45 minutes. Treatment was terminated by aspiration of media, a PBS wash, and fixation with 4% paraformaldehyde (20 min, RT). Fixed cells were either stored at 4°C or immediately imaged for \beta\text{arr2}GFP translocation with a Zeiss LSM 510 meta confocal microscope.

\textbf{G\alpha\textsubscript{q} \& \beta\text{arr2 NanoBiT Assays}}

\textit{G\alpha\textsubscript{q} dissociation/activation:} G\alpha\textsubscript{q LgBiT} and SmBiT-\beta\textsubscript{1} were transiently co-transfected with human 3xHA-GHSR\textsubscript{1a WT}, untagged G\gamma\textsubscript{2}, and RIC8A\textsuperscript{9} into monolayers of HEK293S-G\alpha\textsubscript{q 11} knockout cells. 3xHA-GHSR\textsubscript{1a WT} and G\alpha\textsubscript{q LgBiT} cDNA was transfected at a 1:1 ratio. The next morning, media (DMEM supplemented with 10% FBS and antibiotic/antimycotic) was exchanged and after 4-6 hours, cells were plated on white 96-well, clear-bottomed assay plates at a density of 45,000 cells/well in opti-MEM supplemented with 2% FBS and antibiotic/antimycotic. The next morning, opti-MEM was removed and cells were incubated with 80 \mu L HBSS supplemented with 20 mM HEPES (pH 7.4) for 4 hours. Cells were then treated with 10 \mu L of the luminescent substrate,
coelenterazine H (5 µM final), for 15 minutes at room temperature prior to the addition of 10 µL of test compound. Approximately two minutes after ligand application, a stable luminescence signal was established and measured by a Mithras 940 plate reader over 20 minutes. The average response was utilized for generating C/R curves.

**βarr2 recruitment:** In agonist and antagonist assays of NanoBiT-based βarr2 recruitment, hGHSR1a<sup>WT</sup>-LgBiT and SmBiT-βarr2<sup>WT</sup> were transiently co-transfected at a 1:1 ratio (250 µg each) into monolayers of HEK293/T cells on 6-well plates along with 2µg of pcDNA3.1. For assays employing the Ala204Glu variant, hGHSR<sub>1α</sub><sup>A204E-LgBiT</sup> was transfected at 2x hGHSR<sub>1α</sub><sup>WT-LgBiT</sup>, as performed for iCa<sup>2+</sup> assays with hGHSR<sub>1α</sub><sup>A204E</sup> described above. For GHSR<sub>1α</sub>-βarr2 saturation assays, a fixed amount of hGHSR<sub>1α</sub><sup>WT-LgBiT</sup> cDNA (250 µg) was transfected with 0, 62.5 (1:0.25), 125 (1:0.5), 250 (1:1), 500 (1:2), or 1000 (1:4) µg of SmBiT-βarr2<sup>WT</sup>. The morning after transfection, media was exchanged and after 4-6 hours, cells were then plated on white 96-well, clear-bottomed assay plates at a density of 45,000 cells/well in opti-MEM supplemented with 2% FBS and antibiotic/antimycotic. The following morning, opti-MEM was removed and cells were incubated with 70-80 µL HBSS supplemented with 20 mM HEPES (pH 7.4) for 4 hours. Cells were then treated with 10 µL of coelenterazine H (5 µM final) for 15-20 minutes at room temperature. Cells were then stimulated with 10 µL of test compounds and 2 min later, luminescence was measured over 5 minutes, which encompassed the peak βarr2 recruitment signal. For antagonist assays, cells were pretreated with the indicated ligand for 5 minutes prior to stimulation with EC<sub>80</sub> ghrelin (40 nM).

**Gα<sub>q</sub> & βarr2 BRET Assays**

**TRUPATH Gα disassociation:** G<sub>αq</sub>(1/1/2/3/13/13/8<sup>RLuc8</sup>) and G<sub>γ</sub>8<sup>GFP2</sup> or G<sub>γ</sub>9<sup>GFP2</sup> were transiently co-transfected with human 3xHA-GHSR<sub>1α</sub><sup>WT</sup> and untagged Gβ3 at a 1:1:1:1 ratio (100 µg each) into monolayers of HEK293/T cells along with 1.6 µg pcDNA3.1. The next morning, media (DMEM supplemented with 10% FBS and antibiotic/antimycotic) was exchanged and after 4-6 hours, cells were plated on white 96-well, clear-bottomed assay plates at a density of 45,000 cells/well in opti-MEM supplemented with 2% FBS and antibiotic/antimycotic. The next morning, opti-MEM was removed and cells were incubated with 80 µL HBSS supplemented with 20 mM HEPES (pH 7.4) for 4 hours. Cells were then treated with 10 µL of the luminescent substrate, coelenterazine 400a (5 µM final), for 5 minutes at room temperature prior to the addition of 10 µL of test compound.
Five minutes after ligand application, luminescence was measured by a Mithras 940 plate reader over 30 minutes and the maximal response was utilized for generating all C/R curves.

**βarr2 recruitment:** 3xHA-hGHSR1aWT-RLucII and mVenus-βarr2WT were transiently co-transfected at a 1:15 ratio (100 ng:1.5 μg) into monolayers of HEK293/T cells on 6-well plates along with 0.9μg of pcDNA3.1. Due to increased surface expression of hGHSR1aL149G, 50 ng of 3xHA-hGHSR1aL149G-RLucII was transfected with 750 ng of mVenus-βarr2WT (1:15 ratio). The morning after transfection, media was exchanged and after 4-6 hours, cells were then plated on white 96-well, clear-bottomed assay plates at a density of 45,000 cells/well in opti-MEM supplemented with 2% FBS and antibiotic/antimycotic. The following morning, opti-MEM was removed and cells were incubated with 70-80 μL HBSS supplemented with 20 mM HEPES (pH 7.4) for 4 hours. Cells were then treated with 10 μL of coelenterazine H (5 μM final) for 5-10 minutes at room temperature. Cells were then stimulated with 10 μL of test compounds and 2 min later, luminescence was measured over 60 minutes and the maximal response was utilized for generating C/R curves.

**Quantitative Fixed-Cell ELISA.** 3xHA-hGHSR1aWT or pcDNA3.1 was transiently transfected into monolayers of HEK293/T cells on 6-well plates. The next morning, media (DMEM supplemented with 10% FBS and antibiotic/antimycotic) was exchanged. Later that afternoon, cells were plated on white 96-well, clear-bottomed assay plates at a density of 40,000 cells/well in opti-MEM (supplemented with 2% FBS and antibiotic/antimycotic). The next morning, media was removed and cells were serum starved with 90 μL of opti-MEM (containing no serum) for 2-3 hours at 37°C. Cells were then stimulated with 10x test compounds (diluted in serum-free opti-MEM) for 45 min at 37°C in a humidified incubator. After 45 min, cells were fixed with 4% paraformaldehyde for 15 min (RT). Fixed cells were then washed three times with PBS (pH 7.4, Gibco) and blocked with fish gelatin in TBS (Rockland, Gilbertsville, PA) for 45 min. Cells were then incubated with a rabbit, horseradish peroxidase (HRP)-conjugated anti-HA antibody (1:2,500; Novus Biologicals, Littleton, CO) for 1 hour (RT). After three washes with PBS, cells were treated with 50 μL of SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, Waltham, MA) and luminescence was measured by a Mithras 940 plate reader over ~12 minutes. The average luminescence was used and the data for each ligand were normalized its corresponding vehicle treatment conditions.
**Bystander BRET (bBRET).** hGHSR$_{1a}^{\text{WT-RLucII}}$ and plasma membrane marker, MyrPalm$m\text{Venus}$, or the early endosomal marker, 2x-FYVE$m\text{Venus}$, was transiently co-transfected in HEK293/T cells at a ratio of 1:5 or 1:12.5, respectively. The next morning, media (DMEM supplemented with 10% FBS and antibiotic/antimycotic) was exchanged. Later that afternoon, cells were plated on white 96-well, clear-bottomed assay plates at a density of 45,000 cells/well in opti-MEM (supplemented with 2% FBS and antibiotic/antimycotic). The next morning, opti-MEM was removed and cells were incubated with 70-80 µL HBSS + 20 mM HEPES for 4 hours at 37°C. Cells were treated with 10 µL of coelenterazine H (5 µM final) for 5-15 minutes at 37°C, then stimulated with 10 µL of 10x test compound for 5 min at 37°C. Plates were then read by a Mithras 940 plate reader every 5 min over 1 hour (MyrPalm$m\text{Venus}$) or 2 hours (2xFYVE$m\text{Venus}$) to determine a BRET ratios. To generate C/R curves, the average net BRET over 60 minutes post-treatment was used.

**SRF-RE Transcriptional Activity.** hGHSR$_{1a}^{\text{WT}}$ and SRF-RE (Promega) were transiently co-transfected at a 1:40 ratio into monolayers of HEK293/T cells on 6-well plates. The next morning, standard media was exchanged and later that afternoon, cells were plated on white 96-well, clear-bottomed assay plates at a density of 45,000 cells/well in opti-MEM supplemented with 2% FBS and antibiotic/antimycotic. The next morning, media was removed and cells were serum starved with 90 µL of opti-MEM (containing no serum) for 2 hours at 37°C. Cells were then stimulated with 10x test compounds (diluted in serum-free opti-MEM) for 5 hours at 37°C in a humidified incubator. Cells were then lysed in 20µL of 1x passive lysis buffer (Promega) for 10min on a shaker (RT). To measure luminescence, 40 µL of luciferin (in HBSS supplemented with 20 mM HEPES) was injected into in each well and immediately read by a Mithras 940 plate reader for 10 seconds. To generate C/R curves, the average luminescence produced over the 10 seconds was was used.
Figure S1. Summary of high-throughput screening (HTS) and identification of a novel lead compound. (A) Study flow chart of HTS compound screening, the discovery of N8279, and the chemical structures of the (B) inactive parental scaffold N1956 and (C) active, lead compound N8279 (NCATS-SM8864).
Figure S2. Hit compounds from a high-throughput screen of GHSR1α-βarr activation. The initial screening of ~47,000 compounds in a βarr1 DiscoverX Path Hunter assay resulted in 36 hits. The hits were further analyzed by Ca²⁺ mobilization assays using the Ca²⁺ sensor mitochondrial Aequorin (Aeq Max) for Gαq/11-dependent signaling and βarr2 translocation imaging assay (βarrestin agonist) for βarr signaling. The maximum responses (at 30µM) of the compounds are ordered by Gαq/11/Ca²⁺ response. Data in each assay were normalized to activity of the unbiased, full agonist L585.
Figure S3. N8279 treatment activates receptor-dependent, Gαq/11-mediated signaling at the apo- and orthosteric agonist-bound GHSR1a. (A) hGHSR1aWT saturation binding with [125I]ghrelin. Hyperbola were fit by one-site, non-linear regression (Bmax = 755.7 CPM ± 112.7; Kd = 4.2 nM ± 1.2). HEK293/N cells stably expressing hGHSR1aWT and miAEQ were stimulated with EC80 (B) N8279, (C) ghrelin, or (D) L585 following 30 minute pretreatment with increasing concentrations of the antagonist YIL781 or JMV2959. The data in each panel are normalized to their respective baseline (100%) and maximal inhibitory effect (0%). Data were fit by three- or four-parameter non-linear regression and represent the mean ± SEM from 3 independent experiments. (E) 10μM ghrelin (black), N8279 (red), and L585 (blue)-induced iCa2+ in WT or Gαq/11 KO HEK293/S cells. Data were normalized to ghrelin (% reference; N=2 independent experiments). Two-way ANOVA analysis revealed a significant effect of cell line (F(1,18) = 972.8, p < 0.0001)) and Sidak’s multiple comparisons analyses showed a significant effect between WT and Gαq/11 KO cells for each ligand (****, p < 0.0001). (F) N8279-induced iCa2+ with (+vehicle, red) or without ~EC25 MK-0677 (orange) or ~EC50 ghrelin (purple). Data are normalized to the N8279 + vehicle Emax (% N8279 Emax). Curves with h ≠ 1 were fit by four-parameter nonlinear regression (N8279 + vehicle: * = p < 0.05), whereas curves h = 1 were fit by three-parameter regression (N8279 + MK0677EC25 and N8279 + ghrelinEC50: p > 0.05 = #), as determined
by extra sum-of-squares ANOVA. ANOVA analysis across conditions revealed a significant effect of *Hill Slope*: \( F(2,288) = 15.23, p < 0.0001 \), *Top*: \( F(2,288) = 3.2, p = 0.041 \), and *Bottom*: \( F(2,288) = 75.2, p < 0.0001 \), but not *logEC_{50}*: \( F(2,288) = 1.2, p = 0.290 \). (G) *In silico* model of N8279 (green) docked concomitantly into a NMR-based homology model of the GHSR\(_{1a}\) with ghrelin 1-17 bound (purple). N8279 sits atop ghrelin, interacting with Asp191 in ECL2 (docking score = -4.871). (H) hGHSR\(_{1a}\)WT-mediated G\(_{\alpha q}\) activation using NanoBiT. G\(_{\alpha q}\) activation was quantified as the average % decrease from baseline (0%) after ligand treatment (over 20 min). N8279 and ghrelin were potent (N8279 pEC\(_{50}\) = 7.60 ± 0.19; ghrelin pEC\(_{50}\) = 7.99 ± 0.17), full agonists (N8279 E\(_{max}\) = 118.3 ± 9.8; ghrelin E\(_{max}\) = 100.0 ± 7.35) and potencies did not significantly differ (N8279 vs ghrelin logEC\(_{50}\): \( F(1,150) = 1.51, p = 0.22 \)).
Figure S4. Ghrelin-, N8279-, and L585-induced G\(_{\alpha}\) dissociation from the GHSR\(_{1a}^{WT}\) using the BRET-based TRUPATH assay. Ghrelin (black, solid)-, N8279 (red, dotted)-, and L585 (blue, solid)-induced G protein activation at the hGHSR\(_{1a}^{WT}\) with (A) G\(_{\alpha_q}\) (B) G\(_{\alpha_1}\) (C) G\(_{\alpha_2}\) (D) G\(_{\alpha_oA}\) (E) G\(_{\alpha_{12}}\) (F) G\(_{\alpha_{13}}\) and (G) G\(_{\alpha_{SS}}\). Data are plotted as the minimum \(\Delta\) net BRET (maximal response) over 30 min post-treatment and fit by three- or four-parameter non-linear regression. All data represent the mean ± SEM from >3 independent experiments.
Figure S5. N8279 bias factor using an assay-standardized calculation. N8279 bias factor ($\beta$) relative to ghrelin, calculated using the RA$_i$ method as in Fig. 2L. C/R BRET data were derived from Fig. 1H (GHSR$_{1a}^{WT}$-mediated $G_\alpha_q$ dissociation, TRUPATH) and Fig. 2D (GHSR$_{1a}^{WT}$-RLucII-Venus$\beta$arr2 BRET), whereas C/R NanoBiT data were derived from Fig. S3H (GHSR$_{1a}^{WT}$-mediated $G_\alpha_q$ dissociation, NanoBiT) and Fig. 2A (GHSR$_{1a}^{WT}$-LgBiT-SmBiT$\beta$arr2 NanoBiT). N8279-BRET $\beta = 1.16 \pm 0.11$ (~14-fold) and N8279-NanoBiT $\beta = 1.36 \pm 0.33$ (~23-fold) relative to ghrelin.
Figure S6: Cell surface expression, G\(\alpha_q\) dissociation, and iCa\(^{2+}\) of GHSR\(_{1a}\) mutants. (A) Surface expression of hGHSR\(_{1a}\) variants determined by on-cell ELISA. The expression of hGHSR\(_{1a}\)^{D99A} (D99A, blue), hGHSR\(_{1a}\)^{E197A} (green), hGHSR\(_{1a}\)^{R199A} (R199A, orange), hGHSR\(_{1a}\)^{P200A} (P200A, purple), hGHSR\(_{1a}\)^{A204E} (A204E, yellow), and hGHSR\(_{1a}\)^{N305A} (N305A, turquoise) was assessed relative to hGHSR\(_{1a}\)^{WT} (100%; WT, black) and data were analyzed by one-way ANOVA. **, p < 0.01, ****, p < 0.0001. Mean expression (%) ± SEM: WT = 100 ± 1.2; D99A = 37.6 ± 2.5; E197A = 67.0 ± 2.6; R199A = 114.4 ± 11.9; P200A = 128.7 ± 13.0; A204E = 55.5 ± 8.9; N305A = 89.5 ± 10.1. Data represent the pooled results from ≥2 independent experiments with at least 2 technical replicates per experiment. C/R curves produced by ghrelin- and N8279-induced G\(\alpha_q\) dissociation (TRUPATH) (B, C) and iCa\(^{2+}\) (D, E) assays in HEK293/T cells transiently expressing human GHSR\(_{1a}\) WT (red), D99A (blue), E197A (green), R199A (orange), P200A (purple), or N305A (turquoise). Mutants with ~50% the surface expression of the WT GHSR\(_{1a}\) were transfected at 2x the amount of the GHSR\(_{1a}\)^{WT}. iCa\(^{2+}\) and G\(\alpha_q\) dissociation data at the human GHSR\(_{1a}\)^{A204E} mutant were omitted from panels B-E because the data are shown in Figs. 3B,C (ghrelin) and 3E,F (N8279). For all iCa\(^{2+}\) assays with GHSR\(_{1a}\) variants, the average N8279- or ghrelin-stimulated response over 10 sec (see SI Methods and Materials) was calculated and the data were normalized to the GHSR\(_{1a}\)^{WT} E\(_{max}\) for each ligand (100%); whereas, the max effect (\(\Delta\) net BRET) over 30 min was used for all G\(\alpha_q\) dissociation BRET assays. Dashed lines indicate a significant difference in potency or efficacy from the GHSR\(_{1a}\)^{WT} control, and dotted lines indicate no measurable agonist-stimulated
response. Data represent the pooled results from ≥3 independent experiments (N = 2 independent experiments for panel E, D99A-ghrelin) with at least 2 technical replicates per experiment.
Figure S7: Docking of N8279 into an antagonist-bound GHSR$_{1a}$ crystal structure (6KO5). (A) Differences in the side chain conformations of Asp99, Gln120, Glu124, Phe279, Arg283 and Phe286 between the ghrelin-bound model structure (blue) and the antagonist-bound x-ray crystal structure (6KO5, green). (B) Best docking pose of N8279 docked to the antagonist-bound x-ray crystal structure (6KO5), with a docking score of -5.040. Dash lines indicate hydrogen bonds (yellow), π-π stacking interactions (turquoise), and halogen bonds (purple).
Figure S8. Mean concentration (±SD) time profiles of N8279 in C57BL/6 mice after single dose treatment of 1 mg/kg (IV), 5 mg/kg (PO) and 5 mg/kg (IP).
Figure S9. N8279-induced open field locomotor activity in WT C57BL/6J mice. (A) Novelty-induced locomotor activity in N8279-treated, male and female inbred C57BL/6J mice. Treatment and behaviorally naive mice were administered (IP) vehicle (5% DMSO and saline, black), 2.5 mg/kg N8279 (orange), 5 mg/kg N8279 (red), or 10 mg/kg N8279 (purple) and immediately allowed to explore the open field chamber. N = 14-17 mice per treatment group. No statistically significant differences were identified between any treatment groups (p > 0.05).
### Table S1. Structure-Activity Relationship (SAR) around candidate scaffold N1956

| Molecule ID | Calcium signaling | βarrestin translocation |
|-------------|-------------------|-------------------------|
|             | EC50 [M] | 95% CI | ECmax (%) | EC50 [M] | 95% CI | ECmax (%) |
| L626.585    | 1.46E-08 |          |           | 2.40E-08 |          |           |
| NCGC00141956 | 3.32E-07 |          |           | 2.31E-06 |          |           |
| NCGC00136164 | 6.17E-08 |          |           | 2.53E-07 |          |           |
| ML000103531 | 3.20E-10 |          |           | 8.30E-06 |          |           |
| MLS00115599 | 2.04E-05 | 1.28E-09 to 0.3396 | 71 | 2.86E+00 | N/A |         |
| MLS00115606 | 2.29E-01 |          |           | N/A | 3.12E-08 | 7.451E-02 to 0.000136 | 24 |
| MLS002585710 | 1.76E-08 |          |           | 56 | 1.85E+00 | N/A |         |
| MLS00115604 | 8.04E-09 | 1.598E-010 to 0.044E-007 | 51 | 2.98E-09 | 4.727E-016 to 0.01877 | 38 |
| MLS00115603 | 6.81E-09 | 3.904E-010 to 1.186E-007 | 48 | ~ 0.004690 | (Very wide) | N/A |         |
| MLS00036469 | 3.08E-09 | 3.620E-010 to 2.456E-008 | 50 | 1.47E-04 | 1.275E-016 to 1.704E+00 | N/A |         |
| MLS00115621 | 2.38E-06 | 1.165E-006 to 4.875E-006 | 128 | N/A | N/A | 0 |
| NCGC00101911 | 2.26E-09 | 1.422E-010 to 3.585E-008 | 44 | 3.59E-01 | N/A | N/A |
| NCGC00101920 | ~ 0.01848 | (Very wide) | N/A | 1.45E-08 | 6.914E-012 to 3.957E-005 | 19 |
| NCGC00136166 | ~ 0.002955 | (Very wide) | N/A | 7.16E-08 | 2.273E-0120 to 2.411E+012 | 11 |
| NCGC00136184 | 1.31E-05 | 2.768E-006 to 6.231E-005 | 129 | 2.33E-06 | 8.463E-011 to 0.06430 | 24 |
| NCGC00101634 | 7.71E-06 | 3.90E-007 to 0.0001525 | 61 | 4.26E-07 | 1.016E-019 to 1.787E+006 | 10 |
| NCGC00101917 | 4.30E-09 | 7.476E-011 to 2.478E-007 | 39 | 4.24E-09 | 1.341E-014 to 0.001341 | 20 |
| NCGC00101942 | 8.22E-15 | N/A | 57 | 5.28E-07 | 6.135E-016 to 454.2 | 12 |
| NCGC00101945 | 1.20E-06 | 6.134E-008 to 2.342E-005 | 63 | 2.00E-06 | 1.223E-012 to 3.267 | 7 |
| NCGC00101939 | 4.90E-06 | 2.430E-006 to 8.960E-006 | 121 | 1.16E-08 | 4.039E-012 to 3.314E-005 | 20 |
| NCGC00101941 | ~ 0.02693 | (Very wide) | N/A | 9.50E-08 | 6.833E-009 to 1.321E-006 | 83 |
| NCGC00140156 | 9.38E-07 | 7.854E-008 to 1.119E-005 | 68 | 2.19E-09 | 8.034E-016 to 0.005953 | 19 |
| NCGC00141954 | 7.14E-07 | 2.775E-007 to 1.835E-006 | 105 | 5.33E-06 | 2.970E-007 to 9.575E-005 | 196 |
| NCGC00119774 | 5.62E-06 | 3.974E-007 to 7.948E-005 | 75 | 3.05E-06 | 9.342E-008 to 9.943E-005 | 163 |
| NCGC00101673 | 3.24E-05 | 8.270E-011 to 12.68 | 115 | 2.40E-06 | 5.250E-009 to 0.001097 | 49 |
| NCGC00119776 | 1.20E-05 | 4.349E-006 to 3.302E-005 | 125 | 4.99E-08 | 3.464E-010 to 7.181E-006 | 26 |
| NCGC00119778 | ~ 0.06196 | (Very wide) | N/A | 2.97E+00 | N/A | N/A |
| NCGC00116418 | ~ 0.003007 | (Very wide) | N/A | N/A | N/A | N/A |
| NCGC00101907 | 3.35E-06 | 1.355E-007 to 8.282E-005 | 40 | 5.58E-07 | 2.145E-011 to 0.01451 | 39 |
| NCGC00101948 | ~ 0.01115 | (Very wide) | N/A | N/A | N/A | N/A |
| NCGC00101937 | 2.78E-06 | 1.819E-006 to 4.243E-006 | 112 | 8.55E-07 | 4.555E-008 to 1.603E-005 | 49 |
| NCGC00101919 | ~ 0.00517 | (Very wide) | N/A | 1.74E-06 | 1.186E-007 to 2.545E-005 | 99 |
| NCGC00101915 | ~ 0.01916 | (Very wide) | N/A | 1.36E-05 | 2.339E-007 to 0.0007879 | 368 |
| NCGC00101909 | ~ 0.003015 | (Very wide) | N/A | 2.54E-07 | 6.660E-010 to 9.708E-005 | 64 |
| NCGC00140108 | 1.29E-06 | 5.769E-007 to 2.878E-006 | 97 | 2.03E-06 | 2.389E-007 to 1.720E-005 | 142 |
| NCGC00101913 | ~ 0.01803 | (Very wide) | N/A | 1.55E-06 | 1.386E-007 to 1.739E-005 | 153 |

A small library of commercially available analogs of the candidate scaffold NGC141956 was screened in assays of G protein (iCa\(^{2+}\)) and βarr (βarr\(^{2GFP}\) translocation) signaling along with the unbiased, small molecule GHSR\(_{1a}\) agonist L585. The lead compound NCGC1136164 was identified as a potent full agonist with >1 log order (>10-fold) G protein-bias. Results display EC\(_{50}\) and EC\(_{max}\) values for iCa\(^{2+}\) and βarr translocation assays, respectively. EC\(_{max}\) values are presented as percentage of the non-biased, full GHSR\(_{1a}\) agonist L585-induced maximal response.
| Property                        | Value                        |
|--------------------------------|------------------------------|
| Report date                    | 2019-12-09                   |
| Identification code            | N8279                        |
| Empirical formula              | C24 H25 Cl N2 O6             |
| Molecular formula              | C24 H25 Cl N2 O6             |
| Formula weight                 | 472.91                       |
| Temperature                    | 285 K                        |
| Wavelength                     | 1.54178 Å                    |
| Crystal system                 | Monoclinic                   |
| Space group                    | P 1 21/c 1                   |
| Unit cell dimensions           | a = 15.5969(5) Å             |
|                                | b = 14.5091(6) Å             |
|                                | c = 21.7738(9) Å             |
| Volume                         | 4845.6(3) Å³                 |
| Z                              | 8                            |
| Density (calculated)           | 1.296 Mg/m³                  |
| Absorption coefficient         | 1.747 mm⁻¹                   |
| F(000)                         | 1984                         |
| Crystal size                   | 0.267 x 0.243 x 0.228 mm³    |
| Crystal color, habit           | Colorless Block              |
| Theta range for data collection| 2.881 to 59.009°              |
| **Index ranges** | -17≤h≤17, -16≤k≤16, -24≤l≤24 |
|-----------------|---------------------------------|
| **Reflections collected** | 58959 |
| **Independent reflections** | 6947 [R(int) = 0.0596, R(sigma) = 0.0335] |
| **Completeness to theta = 59.009°** | 99.5% |
| **Absorption correction** | Semi-empirical from equivalents |
| **Max. and min. transmission** | 0.3085 and 0.1652 |
| **Refinement method** | Full-matrix least-squares on F² |
| **Data / restraints / parameters** | 6947 / 2 / 611 |
| **Goodness-of-fit on F²** | 1.049 |
| **Final R indices [I>2sigma(I)]** | R₁ = 0.0641, wR₂ = 0.1578 |
| **R indices (all data)** | R₁ = 0.0848, wR₂ = 0.1705 |
| **Extinction coefficient** | NA |
| **Largest diff. peak and hole** | 0.266 and -0.271 e.Å⁻³ |

**Table S2. N8279 NMR, crystal, and structure refinement data.** 1H NMR of N8279 in CD₂Cl₂. An equilibrium exists between the two different isomers whereby it is dependent on solvent, temperature, and concentration. Ratio of 5a:5b (1:2). Single X-ray diffraction derived structural data for N8279.
Table S3. Ligand C/R curve parameters and statistical comparisons presented in main text Fig. 1.

| Figure | Panel | Experiment Description | F Statistics | Multiple Comparisons | N | Condition/Treatment | Parameter | Mean ± SEM | Units |
|--------|-------|------------------------|--------------|----------------------|---|---------------------|-----------|------------|-------|
| E      |       | [125]Ighrelin competition binding at the hGHSR<sub>1a</sub><sup>WT</sup> | logEC<sub>50</sub> & logK<sub>i</sub>: F(1,190) = 77.8, p < 0.0001 | NA | 5-6 | ghrelin | pIC<sub>50</sub> K | 8.5 ± 0.1 | 1.4 nM |
|        |       |                         |              |                      |    | N8279               | pIC<sub>50</sub> K | 5.8 ± 0.1 | 761.9 nM |
| F      |       | iCa<sup>2+</sup> in HEK293/N cells expressing hGHSR<sub>1a</sub><sup>WT</sup> & mitochondrial Aequorin (miAeq) | logEC<sub>50</sub>: F(3,365) = 1009.0, p < 0.0001 | Hill slope (h): F(3,365) = 10.1, p < 0.0001 | 3-9 | ghrelin | E<sub>max</sub> pEC<sub>50</sub> h | 100<sup>±</sup> | 6.5 ± 0.1 | RLU, % reference (ghrelin) |
|        |       |                         |              |                      |    | MK-0677            | E<sub>max</sub> pEC<sub>50</sub> h | 100<sup>±</sup> | 8.2 ± 0.06 | |
|        |       |                         |              |                      |    | L585               | E<sub>max</sub> pEC<sub>50</sub> h | 100<sup>±</sup> | 8.0 ± 0.05 | |
|        |       |                         |              |                      |    | N8279              | E<sub>max</sub> pEC<sub>50</sub> h | 100<sup>±</sup> | 7.4 ± 0.01 | |
| G      |       | Ago-allosteric agonism of N8279 with ghrelin-induced iCa<sup>2+</sup> | Omnibus ANOVA (three parameter) |              | 4-7 | ghrelin + [N8279] μM | 0 | 2.8 ± 1.2 | RLU, % reference (ghrelin + vehicle) |
|        |       |                         |              | Bottom: F(6,403) = 86.68, p < 0.0001 |    | Bottom | E<sub>max</sub> pEC<sub>50</sub> h | 100 ± 2.2 | 6.6 ± 0.06 | |
|        |       |                         |              | E<sub>max</sub>: F(6,403) = 2.28, p = 0.034 |    | 0.001 | Bottom | E<sub>max</sub> pEC<sub>50</sub> h | 107.3 ± 2.4 | 6.6 ± 0.05 | |
|        |       |                         |              | 0.01 | Bottom | E<sub>max</sub> pEC<sub>50</sub> h | 28.7 ± 1.8 | 107.2 ± 3.7 | |
|        |       |                         |              | 0.03 | Bottom | E<sub>max</sub> pEC<sub>50</sub> h | 54.2 ± 1.6 | 108.3 ± 2.8 | |
|        |       |                         |              | 0.1 | Bottom | E<sub>max</sub> pEC<sub>50</sub> h | 79.3 ± 1.0 | 107.2 ± 2.2 | |
|        |       |                         |              | 1 | Bottom | E<sub>max</sub> pEC<sub>50</sub> h | 92.3 ± 0.7 | 104.0 ± 1.1 | |
|        |       |                         |              | 10 | Bottom | E<sub>max</sub> pEC<sub>50</sub> h | 94.6 ± 0.7 | 103.7 ± 0.9 | |
| H      |       | Gq dissociation (TRUPATH) in hGHSR<sub>1a</sub><sup>WT</sup>-expressing HEK293/T cells. | logEC<sub>50</sub>: F(2,222) = 73.5, p < 0.0001 |              | 3-5 | ghrelin | E<sub>max</sub> pEC<sub>50</sub> h | 100<sup>±</sup> | 8.2 ± 0.04 | ∆ net BRET, % reference (ghrelin) |
|        |       |                         |              | L585 | E<sub>max</sub> pEC<sub>50</sub> h | 100<sup>±</sup> | 7.6 ± 0.06 | |

Notes:
- <sup>±</sup> indicates ± SEM.
|   | Heat map of ghrelin, N8279, and L585 potencies (pEC50) at seven different G proteins |   |   |
|---|---|---|---|
| I | NA | NA | 2-6 |

**J**

|   | Ga TRUPATH potency (pEC<sub>50</sub>) |
|---|---|
| Ga<sub>a</sub>: F(6,148) = 51.8, p < 0.0001 | ghrelin vs N8279, p = 0.167 |
| ligand: F(2,148) = 52.5, p < 0.0001 | ghrelin vs L585, p = 0.819 |
| interaction: F(12,148) = 43.1, p < 0.0001 | N8279 vs L585, p = 0.161 |
|   | 2-6 |

**K**

|   | Ga<sub>a</sub> TRUPATH max efficacy (10 µM ligand) |
|---|---|
| Ga<sub>a</sub>: F(6,144) = 139.1, p < 0.0001 | ghrelin vs N8279, p = 0.997 |
| ligand: F(2,144) = 14.5, p < 0.0001 | ghrelin vs L585, p = 0.686 |
| interaction: F(12,144) = 5.9, p < 0.0001 | N8279 vs L585, p = 0.850 |
|   | 2-6 |

### Results

|   | N8279 | E<sub>max</sub> | 100<sup>y</sup> |
|---|---|---|---|
| ghrelin | pEC<sub>50</sub> | 7.4 ± 0.04 | 0.66<sup>y</sup> |
| L585 | NA | NA | NA |
| N8279 | NA | NA | NA |

**Note:**

- pEC<sub>50</sub> values are reported for each ligand's potency.
- E<sub>max</sub> values indicate the maximum efficacy achieved.
- 100% effect is noted in the last column for each respective condition.

**References:**

- Ga<sub>a</sub> potency and efficacy values are derived from statistical analyses with corresponding p-values.
- L585 and N8279 potencies are compared for their max efficacy and significance levels.

**Additional Notes:**

- The table includes a summary of the observed interactions and their respective p-values, highlighting the significance of ghrelin and other ligands on Ga<sub>a</sub> potency and efficacy.
- The table concludes with a reference to the percentage of the expected effect, indicating how close the observed results are to the theoretical max efficacy.
|                      | N8279 vs L585, p = 0.0004 |
|----------------------|---------------------------|
|                      | G13                       |
| ghrelin vs N8279,    | p < 0.0001                |
| ghrelin vs L585,     | p = 0.738                 |
| N8279 vs L585,       | p < 0.0001                |
|                      | Gs                        |
| ghrelin vs N8279,    | p = 0.995                 |
| ghrelin vs L585,     | p = 0.923                 |
| N8279 vs L585,       | p = 0.897                 |

N, number of independent experiments with >2 technical replicates per condition; pIC$_{50}$, absolute value of half-maximal inhibitory concentration; K$_i$, inhibition constant/binding affinity; pEC$_{50}$, absolute value of half-maximal effective concentration; E$_{max}$, maximal response; h, Hill slope; # parameter constrained or shared; max efficacy, response at the highest single concentration of drug; cpm, counts per million; RLU, relative luminescence units; NA, not applicable; UD, undeterminable; NC, not calculated
Table S4. Ligand C/R curve parameters and statistical comparisons presented in main text Fig. 2.

| Figure | Panel | Experiment Description | F Statistics | Multiple Comparisons | N | Condition/Treatment | Parameter | Mean ± SEM | Units |
|--------|-------|------------------------|--------------|----------------------|---|---------------------|-----------|------------|-------|
| A      |       | NanoBit-based SmbIT
SmBiTArr2 recruitment to apo-hGHSR<sub>1α</sub>WT-L<sub>LgBiT</sub> | $E_{max}$: $F(2,459) = 4.7$, $p = 0.008$
$\log EC_{50}$: $F(2,459) = 34.7$, $p < 0.0001$ | NC | 6-10 | ghrelin | $E_{max}$ pEC<sub>50</sub> | 100 ± 3.3 8.02 ± 0.09 | RLU, % reference (ghrelin) |

B, inset

|       |       | Agonist-induced hGHSR<sub>1α</sub>WT-L<sub>Ghrelin</sub> recruitment to hGHSR<sub>1α</sub>SmBiT
SmBiTArr2 saturation | $BT_{max}$: $F(2,167) = 5.0$, $p = 0.0072$
$BT_{50}$: $F(2,167) = 46.6$, $p < 0.0001$
Inset $BT_{50}$: $F(2,167) = 31.6$, $p = 0.0003$
Inset $ghrelin$ vs $N8279^{100nM}$ $p = 0.0003$
$ghrelin$ vs $N8279^{200nM}$ $p = 0.0002$
$N8279^{100nM}$ vs $N8279^{200nM}$ $p = 0.073$ | 3-4 | ghrelin (100 nM) | $BT_{max}$ BiT<sub>d</sub> | 100.0 ± 2.4 0.97 ± 0.06 | RLU, % reference Bi<sub>max</sub> (ghrelin) |

C

|       |       | [<sup>125I</sup>]ghrelin competition binding at the hGHSR<sub>1α</sub>L<sub>149G</sub> | $logIC_{50}$: $F(1,143) = 19.8$, $p < 0.0001$ | NA | 4 | ghrelin | pIC<sub>50</sub> K<sub>i</sub> | 8.23 ± 0.18 NC | cpm, % reference (ghrelin) |

2

D

|       |       | BRET-based SmBit
SmBitArr2 recruitment to hGHSR<sub>1α</sub>L<sub>WT-LgBiT</sub> or hGHSR<sub>1α</sub>L<sub>149G-Rluc</sub> | $E_{max}$: $F(3,188) = 36.5$, $p < 0.0001$
$log EC_{50}$: $F(3,188) = 142.8$, $p < 0.0001$ | $E_{max}$ WT-ghrelin vs L149-Ghrelin: $F(1,40) = 19.2$, $p < 0.0001$
$E_{max}$ WT-N8279: $F(1,54) = 350.7$, $p < 0.0001$
$E_{max}$ L149-G-N8279: $F(1,40) = 1584.0$, $p < 0.0001$
$log EC_{50}$ WT-ghrelin vs L149-Ghrelin: $F(1,40) = 303.6$, $p < 0.0001$
$log EC_{50}$ WT-N8279: $F(1,54) = 0.2$, $p = 0.630$
$log EC_{50}$ L149-G-N8279: $F(1,40) = 291.5$, $p < 0.0001$ | 3-4 | WT: ghrelin | $E_{max}$ EG<sub>50</sub> | 100.0 ± 3.3 8.93 ± 0.09 | Δ net BRET, % reference (ghrelin) |

|       |       | E<sub>Ce</sub> ghrelin-induced SmBit
SmBitArr2 recruitment to the hGHSR<sub>1α</sub>L<sub>WT-LgBiT</sub> after N8279 or antagonist pretreatment | $E_{max}$/Bottom: $F(2,266) = 17.4$, $p < 0.0001$
$log EC_{50}$: $F(2,266) = 3.2$, $p = 0.041$ | NC | 2-5 | YIL781 | $E_{max}$/Bottom pIC<sub>50</sub> | -8.4 ± 4.9 7.30 ± 0.11 | RLU, % baseline |

|       |       | Representative confocal images of agonist-induced Arr2<sup>DP</sup>
translocation in hGHSR<sub>1α</sub>L<sub>WT</sub> | NA | 3 | vehicle | NA | NA | NA |

|       |       | ghrelin (100 nM) | NA | NA | NA |

|       |       | N8279 (100 nM) | NA | NA | NA |
|   | expressing U2OS cells | G | On-cell ELISA-based hGHSR<sub>1a</sub><sup>WT</sup> internalization | NA | 4 | 0.59 ± 0.18 | 0.59 ± 0.18 | 2.63 ± 0.19 | 2.63 ± 0.19 | β (log<sub>10</sub>) | NA |
|---|-------------------|---|---------------------------------|----|----|----------------|----------------|----------------|----------------|----------------|----|
|   |  | G | F(1,95) = 11.7, p = 0.0009 | logIC<sub>50</sub>: F(1,95) = 24.3, p < 0.0001 |  |  |  |  |  |  |
|   |  | H | F(1,191) = 4.8, p = 0.028 | logIC<sub>50</sub>: F(1,191) = 25.3, p < 0.0001 |  |  |  |  |  |  |
|   |  | I | F(1,114) = 9.4, p = 0.0026 | logIC<sub>50</sub>: F(1,114) = 11.2, p = 0.0011 |  |  |  |  |  |  |
|   |  | J | F(7,104) = 12.7, p < 0.0001 | logIC<sub>50</sub>: F(7,104) = 1.2, p = 0.300 |  |  |  |  |  |  |
|   |  | K | F(2,153) = 9.9, p < 0.0001 | logIC<sub>50</sub>: F(2,153) = 163.6, p < 0.0001 |  |  |  |  |  |  |
|   |  | K | F(1,57) = 615.3, p < 0.0001 | L585: F(1,41) = 9.9, p = 0.003 |  |  |  |  |  |  |
|   |  | K | F(1,57) = 12.2, p = 0.0009 | L585: F(1,41) = 27.6, p < 0.0001 |  |  |  |  |  |  |
|   |  | K | E<sub>max</sub>Bot: F(1,57) = 100 ± 1.6 | E<sub>max</sub>Bot pEC<sub>50</sub> |  |  |  |  |  |  |
|   |  | K | E<sub>max</sub>Bot: F(1,57) = 101.8 ± 1.6 | E<sub>max</sub>Bot pIC<sub>50</sub> |  |  |  |  |  |  |
|   |  | K | N8279: F(1,57) = 7.26 ± 0.04 | L585 (ref.) proximal downstream |  |  |  |  |  |  |
|   |  | K | N8279: F(1,57) = 105.8 ± 1.6 | L585 (ref.) proximal downstream |  |  |  |  |  |  |
N, number of independent experiments with >2 technical replicates per condition; pIC$_{50}$, absolute value of half-maximal inhibitory concentration; K$_i$, inhibition constant/binding affinity; pEC$_{50}$, absolute value of half-maximal effective concentration; E$_{max}$, maximal response; BiT$_{max}$, maximal NanoBiT response; BiT$_d$, half-maximal NanoBiT response; max efficacy, response at the highest single concentration of drug; cpm, counts per million; RLU, relative luminescence units; NA, not applicable; UD, undeterminable; NC, not calculated
Table S5. Ligand C/R curve parameters and statistical comparisons presented in main text Fig. 3.

| Figure | Panel | Experiment Description | F Statistics | Multiple Comparisons | N | Condition/Treatment | Parameter | Mean ± SEM | Units |
|--------|-------|------------------------|--------------|----------------------|---|---------------------|-----------|------------|-------|
| B      |      | ghrelin-induced iCa²⁺ at the hGHSR₁α <sub>WT</sub> or hGHSR₁α<sub>A204E</sub> | Basal activity/Bottom: F(1,82) = 94.1, p < 0.0001  
  \( E_{\text{max}} \): F(1,82) = 0.1, p = 0.680  
  \( \log E_{\text{EC}_{50}} \): F(1,82) = 0.1, p = 0.679 | NA | 3 | WT | Bottom \( E_{\text{max}} \) \( pE_{\text{C50}} \) | 16.6 ± 1.4  
  101.6 ± 2.6  
  6.50 ± 0.1 | RLU, % reference (WT) |
| C      |      | ghrelin-induced Go₄ dissociation (TRUPATH) at the hGHSR₁α<sub>WT</sub> or hGHSR₁α<sub>A204E-LgBiT</sub> | \( E_{\text{max}} \): F(1,85) = 3.7, p = 0.056  
  \( \log E_{\text{EC}_{50}} \): F(1,85) = 3.7, p = 0.057 | NA | 3 | WT | \( E_{\text{max}} \) \( EC_{50} \) | 100.6 ± 2.7  
  6.31 ± 0.10 | Δ net BRET, % reference (WT) |
| D      |      | ghrelin-induced SmBiT arr2 recruitment at the hGHSR₁α<sub>WT-LgBiT</sub> or hGHSR₁α<sub>A204E-LgBiT</sub> | Basal activity/Bottom: F(1,82) = 132.7, p < 0.0001  
  \( E_{\text{max}} \): F(1,82) = 10.32, p = 0.002  
  \( \log E_{\text{EC}_{50}} \): F(1,82) = 75.98, p < 0.0001 | NA | 3 | WT | \( E_{\text{max}} \) \( EC_{50} \) | 100.0 ± 4.0  
  7.58 ± 0.1 | RLU, % reference (WT) |
| 3      |      | N8279-induced iCa²⁺ at the hGHSR₁α<sub>WT</sub> or hGHSR₁α<sub>A204E</sub> | Basal activity/Bottom: F(1,82) = 34.0, p < 0.0001  
  \( E_{\text{max}} \): F(1,82) = 10.32, p = 0.002  
  \( \log E_{\text{EC}_{50}} \): F(1,82) = 75.98, p < 0.0001 | NA | 3 | WT | \( E_{\text{max}} \) \( EC_{50} \) | 12.4 ± 2.1  
  100.0 ± 2.4  
  7.20 ± 0.06 | Δ net BRET, % reference (WT) |
| F      |      | N8279-induced Go₄ dissociation (TRUPATH) at the hGHSR₁α<sub>WT</sub> or hGHSR₁α<sub>A204E</sub> | \( E_{\text{max}} \): ND  
  \( \log E_{\text{EC}_{50}} \): F(1,84) = 30.3, p < 0.0001 | NA | 3 | WT | \( E_{\text{max}} \) \( EC_{50} \) | 100.2 ± 3.4  
  7.30 ± 0.08 | Δ net BRET, % reference (WT) |
| G      |      | N8279-induced SmBiT arr2 recruitment at the hGHSR₁α<sub>WT-LgBiT</sub> or hGHSR₁α<sub>A204E-LgBiT</sub> | \( E_{\text{max}} \): ND  
  \( \log E_{\text{EC}_{50}} \): F(1,77) = 10.6, p = 0.001 | NA | 2-3 | WT | \( E_{\text{max}} \) \( EC_{50} \) | 100.0 ± 3.7  
  6.85 ± 0.09 | RLU, % reference (WT) |
| M      |      | Ghrelin and N8279 Go₄ dissociation (TRUPATH) potency (pEC₅₀) and maximal efficacy (Eₘₐₓ) at | \( pEC_{50} \): ligand: F(1,116) = 310.4, p < 0.0001  
  ghrelin (pEC₅₀): WT vs D99A: p < 0.0001  
  E197A: p = 0.145  
  R199A: p = 0.051  
  P200A: p = 0.681  
  A204E: p = 0.067  
  \( ghrelin \) WT  
  \( pE_{C50} \):  
  D99A  
  E197A | 8.11 ± 0.03  
  100.0 ± 1.0  
  7.14 ± 0.23  
  7.89 ± 0.07  
  89.5 ± 2.5 | Δ net BRET, % reference (WT) |
|     | hGHSR<sub>a</sub> mutants                                      | N     | Ghrelin and N8279 iCa<sup>2+</sup> potency (pEC<sub>50</sub>) and maximal efficacy (E<sub>max</sub>) at hGHSR<sub>a</sub> mutants |
|-----|----------------------------------------------------------------|-------|---------------------------------------------------------------------------------------------------------------------------------|
|     | mutation: F(6,116) = 472.8, p < 0.0001                           |       |                                                                                                                              |
|     | interaction: F(6,116) = 310.4, p < 0.0001                         |       |                                                                                                                              |
|     | E<sub>max</sub>: ligand: F(1,116) = 364.8, p < 0.0001             |       |                                                                                                                              |
|     | mutation: F(6,116) = 75.7, p < 0.0001                             |       |                                                                                                                              |
|     | interaction: F(6,116) = 84.1, p < 0.0001                          |       |                                                                                                                              |
|     | pEC<sub>50</sub>: ligand: F(1,142) = 70.5, p < 0.0001             |       |                                                                                                                              |
|     | mutation: F(6,142) = 405.4, p < 0.0001                            |       |                                                                                                                              |
|     | interaction: F(6,142) = 86.0, p < 0.0001                          |       |                                                                                                                              |
|     | E<sub>max</sub>: ligand: F(1,142) = 16.0, p < 0.0001              |       |                                                                                                                              |
|     | mutation: F(6,142) = 142.8, p < 0.0001                            |       |                                                                                                                              |
|     | interaction: F(6,142) = 20.4, p < 0.0001                          |       |                                                                                                                              |
| N   | hGHSR<sub>a</sub> mutants                                      |       |                                                                                                                              |
|     | mutation: F(6,116) = 472.8, p < 0.0001                           |       |                                                                                                                              |
|     | interaction: F(6,116) = 310.4, p < 0.0001                         |       |                                                                                                                              |
|     | E<sub>max</sub>: ligand: F(1,116) = 364.8, p < 0.0001             |       |                                                                                                                              |
|     | mutation: F(6,116) = 75.7, p < 0.0001                             |       |                                                                                                                              |
|     | interaction: F(6,116) = 84.1, p < 0.0001                          |       |                                                                                                                              |
|     | pEC<sub>50</sub>: ligand: F(1,142) = 70.5, p < 0.0001             |       |                                                                                                                              |
|     | mutation: F(6,142) = 405.4, p < 0.0001                            |       |                                                                                                                              |
|     | interaction: F(6,142) = 86.0, p < 0.0001                          |       |                                                                                                                              |
|     | E<sub>max</sub>: ligand: F(1,142) = 16.0, p < 0.0001              |       |                                                                                                                              |
|     | mutation: F(6,142) = 142.8, p < 0.0001                            |       |                                                                                                                              |
|     | interaction: F(6,142) = 20.4, p < 0.0001                          |       |                                                                                                                              |

N, number of independent experiments with ≥2 technical replicates per condition; EC<sub>50</sub>, half-maximal effective concentration; E<sub>max</sub>, maximal response; max efficacy, response at the highest single concentration of drug; RLU, relative luminescence units; NA, not applicable; UD, undeterminable.
### Table 6. Pharmacokinetic parameters of N8279 in C57BL6 mice

| Parameter                  | Units   | Plasma  | Brain  | Liver  | Plasma  | Brain  | Liver  | Plasma  | Brain  | Liver  |
|----------------------------|---------|---------|--------|--------|---------|--------|--------|---------|--------|--------|
| **Route**                  |         | IV      | PO     | IP     |         |        |        |         |        |        |
| **Dose**                   | mg/kg   | 1       | 5      | 5      |         |        |        |         |        |        |
| **C<sub>max</sub>**        | ng/mL   | 310     | 109    | 11000  | 126     | 37.7   | 13200  | 1030    | 123    | 22300  |
| **T<sub>max</sub>**        | hr      | 0.083*  | 2      | 1      | 2       | 1      | 1      | 0.167   | 2      | 0.5    |
| **AUC<sub>0-t</sub>**     | ng.hr/mL| 1270    | 933    | 52100  | 418     | 333    | 36200  | 1750    | 1050   | 86700  |
| **AUC<sub>0-inf</sub>**   | ng.hr/mL| 1300    | 1040   | 52200  | 470     | 436    | 36200  | 1760    | 1130   | 86800  |
| **AUC<sub>extrap</sub>**  | %       | 2.4     | 10     | 0.2    | 11      | 24     | 0.1    | 0.6     | 7.1    | 0.1    |
| **t<sub>1/2</sub>**       | hr      | 4.9     | 7.5    | 2.8    | 1.9     | 11     | 2.4    | 3.8     | 6.6    | 2.7    |
| **CL**                     | mL/min/kg| 13     |        |        |         |        |        |         |        |        |
| **V<sub>dss</sub>**       | L/kg    | 3.9     |        |        |         |        |        |         |        |        |
| **Bioavailability (F)**    | %       | 7.2     |        |        | 27      |        |        |         |        |        |
| **AUC ratio (tissue/plasma)** |        | 0.8 | 40 | 0.9 | 77 | 0.6 | 49 |

* First sampling time point after IV administration

**CL<sub>p</sub>: Plasma clearance

***Bioavailability was calculated by the AUC ratio between PO and IV administration or IP and IV administration

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Table S6. Pharmacokinetic parameters of N8279 in C57BL/6 mice after IV, PO, and IP administration.
SI REFERENCES

1. Rizzuto R, Simpson AW, Brini M, Pozzan T. Rapid changes of mitochondrial Ca2+ revealed by specifically targeted recombinant aequorin. Nature. 1992;358(6384):325-7.

2. Toth K, Nagi K, Slosky LM, Rochelle L, Ray C, Kaur S, Shenoy SK, Caron MG, Barak LS. Encoding the beta-Arrestin Trafficking Fate of Ghrelin Receptor GHSR1a: C-Tail-Independent Molecular Determinants in GPCRs. ACS Pharmacol Transl Sci. 2019;2(4):230-46. PMC7088988.

3. Snyder JC, Rochelle LK, Ray C, Pack TF, Bock CB, Lubkov V, Lyerly HK, Waggoner AS, Barak LS, Caron MG. Inhibiting clathrin-mediated endocytosis of the leucine-rich G protein-coupled receptor-5 diminishes cell fitness. J Biol Chem. 2017;292(17):7208-22. PMC5409487.

4. Shiimura Y, Horita S, Hamamoto A, Asada H, Hirata K, Tanaka M, Mori K, Uemura T, Kobayashi T, Iwata S, Kojima M. Structure of an antagonist-bound ghrelin receptor reveals possible ghrelin recognition mode. Nat Commun. 2020;11(1):4160. PMC7438500.

5. Evron T, Peterson SM, Urs NM, Bai Y, Rochelle LK, Caron MG, Barak LS. G Protein and beta-arrestin signaling bias at the ghrelin receptor. J Biol Chem. 2014;289(48):33442-55. PMC4246099.

6. Pantel J, Legendre M, Cabrol S, Hilal L, Hajaji Y, Morisset S, Nivot S, Vie-Luton MP, Grouselle D, de Kerdanet M, Kadiri A, Epelbaum J, Le Bouc Y, Amselem S. Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. J Clin Invest. 2006;116(3):760-8. PMC1386106.

7. Liu G, Fortin JP, Beinborn M, Kopin AS. Four missense mutations in the ghrelin receptor result in distinct pharmacological abnormalities. J Pharmacol Exp Ther. 2007;322(3):1036-43.

8. Kapur A, Zhao P, Sharir H, Bai Y, Caron MG, Barak LS, Abood ME. Atypical responsiveness of the orphan receptor GPR55 to cannabinoid ligands. J Biol Chem. 2009;284(38):29817-27. PMC2785612.

9. Inoue A, Raimondi F, Kadji FMN, Singh G, Kishi T, Uwamizu A, Ono Y, Shinjo Y, Ishida S, Arang N, Kawakami K, Gutkind JS, Aoki J, Russell RB. Illuminating G-Protein-Coupling Selectivity of GPCRs. Cell. 2019;177(7):1933-47 e25. PMC6773469.

10. Olsen RHJ, DiBerto JF, English JG, Glaudin AM, Krumm BE, Slocum ST, Che T, Gavin AC, McCorvy JD, Roth BL, Strachan RT. TRUPATH, an open-source biosensor platform for interrogating the GPCR transducerome. Nat Chem Biol. 2020;16(6):841-9. PMC7648517.