Structural basis for GLP-1 receptor activation by LY3502970, an orally active nonpeptide agonist

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Ly3502970, an orally active nonpeptide agonist

The glucagon-like peptide-1 receptor (GLP-1R) is a nonpeptide agonist that has been shown to activate the GLP-1R without the need for high concentrations of ligand. This activation mechanism is remarkably efficient as it allows for the activation of class B GPCRs by nonpeptide ligands. The discovery of LY3502970, a potent and selective small molecule GLP-1R agonist, has led to the development of a leading class of diabetes medications. However, these peptide-based drugs are administered by subcutaneous injection or, in one case, by a complex oral dosing regimen. We now report the discovery of LY3502970, a potent and selective small molecule GLP-1R agonist. LY3502970 exhibits preclinical pharmacology equivalent to a marketed injectable GLP-1R agonist and possesses pharmacokinetic properties compatible with oral dosing in humans. Cryo-electron microscopy (cryo-EM) studies reveal an ECD-driven receptor binding mode for LY3502970 that provides a favorable pharmacological profile.
the higher drug exposures required to deliver equivalent glucose and body weight–lowering efficacy observed with injectable semaglutide (brand name Ozempic) (14–16). Further, the dosing regimen for oral semaglutide is restrictive for patients since drug absorption is significantly affected by food and fluid in the stomach (17). Specifically, the tablet must be administered after overnight fasting with a prescribed volume of water and at least 30 min before consumption of breakfast or other medicines (14–16). As an alternative approach, nonpeptide agonists could offer more standard drug formulations with simpler dosing practices, which would be especially beneficial for T2DM patients who often require additional daily medications.

Historically, several groups have attempted to discover small-molecule activators of the GLP-1R, including positive allosteric modulators and compounds with agonist properties. Various chemotypes have been reported, such as series of quinoxalines (18, 19), sulfonlythiophenes (20), pyrimidines (21), phenylala-nine derivatives (22), Boc-5 (23), and azoanthracene and oxa-diazoantharcarvines derivatives (24–27). Although this collection of molecules indicated that nonpeptide ligands can modulate GLP-1R activity, these compounds evidently lack potency and pharmacokinetic properties that would be necessary to achieve an efficacy profile similar to that of peptide-based GLP-1R drugs. Therefore, it was hypothesized that small molecules cannot make sufficient contacts throughout the peptide-binding pocket to potently activate the GLP-1R. Here, we challenge this notion by reporting the discovery of a unique ECD-driven binding mechanism for LY3502970, an orally bioavailable nonpeptide agonist of the GLP-1R. This mechanism provides a potent pharmacological profile in vitro and in vivo, supporting the promise of an orally administered GLP-1R agonist drug.

**Results and Discussion**

**In Vitro and In Vivo Activity of LY3502970.** Small-molecule activators of the GLP-1R were identified using a screening method that detects compound-induced expression of a urokinase-type plasminogen activator in LLC-PK1 cells (28) expressing the human GLP-1R. Multiple cycles of traditional structure activity relationship work were conducted to optimize affinity and drug-like properties that enabled the discovery of OWL833 (LY3502970) (Fig. 1A) (29). Pharmacological studies using HEK293 cells expressing various densities of the human GLP-1R revealed that LY3502970 is highly potent at stimulating GLP-1R-induced cAMP accumulation with partial agonist activity relative to native GLP-1 (Fig. 1B and C and SI Appendix, Table S1). Further, no detectable recruitment of GLP-1R-mediated β-arrestin was observed for LY3502970 (Fig. 1B and C), a feature that may enhance GLP-1R-induced glucose lowering and body weight reduction (30, 31). Although the biased pharmacology is reminiscent of that observed for the nonpeptide ligand TT-OAD2 (32), the potency and efficacy of LY3502970 to stimulate GLP-1R-mediated cAMP accumulation in cell lines expressing different densities of the receptor are far greater than TT-OAD2 (SI Appendix, Fig. S1A and Table S1). LY3502970 showed no activity on other class B GPCRs (SI Appendix, Fig. S1B) and strikingly, the compound was also inactive on the mouse (Fig. 1D) and other species of the GLP-1R (SI Appendix, Fig. S1C). Therefore, experiments in mice expressing the human GLP-1R were employed to investigate the in vivo efficacy of LY3502970 (33). In these studies, overnight-fasted animals were orally administered various doses of LY3502970 (0.1 to 10 mg/kg body weight), and 5 h later, the mice were challenged with intraperitoneally injected glucose. Exenatide, the active pharmacoequivalent in the registered GLP-1R peptide agonist drugs Byetta and Bydureon, was used as a positive control, but its short half-life required administration (via s.c. injection) 1 h prior to receiving the glucose bolus. LY3502970 demonstrated robust glucose lowering at all doses tested, revealing a potent pharmacodynamic:pharmacokinetic relationship as maximum efficacy was observed with low blood concentrations of the compound (Fig. 1E; 10 mg/kg = [1.257 ± 387 nmol/L]LY3502970, 1 mg/kg = [205 ± 18 nmol/L]LY3502970, 0.1 mg/kg = [24 ± 8 nmol/L]LY3502970; mean ± SEM, n = 5). These head-to-head experiments with exenatide demonstrate that an orally administered nonpeptide agonist of the GLP-1R can reduce hyperglycemia to the same extent as a registered peptide-based GLP-1 drug. Consistent with the in vitro selectivity data, LY3502970 did not lower glucose in Glp1r−/− mice (Fig. 1F). LY3502970 is a low-efficacy, partial agonist for Gαs-cAMP activation, yet it exhibits in vivo glucose lowering efficacy comparable to the full agonist exenatide. We propose for the GLP-1 system that a substantial receptor reserve is present in vivo, in that a full biological response can be achieved with partial receptor occupancy (34). Therefore, a partial agonist can induce cAMP pathway activation equivalent to a full agonist in vivo by achieving a higher relative receptor occupancy. For LY3502970, this level of cAMP modulation is achieved in the absence of β-arrestin recruitment, and this may be therapeutically advantageous given emerging hypotheses that G protein–biased GLP-1R agonists have superior efficacy in preclinical models of diabetes (30, 31, 35).

**Structural Basis of GLP-1R Activation by LY3502970.** To investigate the receptor-binding mechanism of LY3502970, cryo-EM was used to determine the structure of the GLP-1R bound to the compound in complex with heterotrimeric Gqα16, camelid antibody Nb35, and single-chain variable fragment scFv16 at a global nominal resolution of 3.1 to 3.2 Å (Fig. 1G and SI Appendix, Fig. S2 and Table S2), with well-defined density for each transmembrane (TM) helix, LY3502970, and the majority of the ECD (SI Appendix, Figs. S2 and S3). Local resolution near the binding site for LY3502970 is comparable to the overall resolution (2.8 to 3.4 Å; SI Appendix, Fig. S2E), and the well-defined density for the compound allowed confident modeling of the ligand and residues of the pocket (SI Appendix, Fig. S3). A positive allosteric modulator was included in the sample preparation to further stabilize the complex; its density was observed near the kinked region of TM6, but the disposition of the potentiatior could not be unequivocally determined and was therefore not included in the final model (SI Appendix, Fig. S2E). We hypothesize that the modulator stabilizes the conformation of TM6, thereby enabling a high-affinity complex for structural studies, but the compound was not included in the in vivo studies that show full glucose lowering by treatment with LY3502970. In the cryo-EM structure, the receptor is in an active conformation, exhibiting typical active-state class B GPCR structure features on the intracellular side, similar to other GLP-1R/G protein complex structures (2, 32, 36) (SI Appendix, Fig. S4 A and B). However, the binding mode of LY3502970 is unique, resulting in a distinct conformation of the ECD and the extracellular portion of the 7TM segments. The compound binds high in the helical bundle, interacting with residues within the ECD, TM1, TM2, TM3, ECL2, and TM7 (Fig. 1H and I) but having no interactions with TM4, TM5, and TM6. This contrasts with GLP-1, which interacts with all TM segments except TM4. The binding site of LY3502970 partially overlaps with the area where TT-OAD2 binds (32), although the overall binding modes of these ligands are different (SI Appendix, Fig. S5). TT-OAD2 adopts a U-shaped orientation with both ends of its backbone sitting between TM2, TM3, and ECL1 (32). While the 2,2-dimethyl-tetrahydropryamo moiety (Fig. 1J, green shading) of LY3502970 and the 2,3-dime-thylpyridine ring of TT-OAD2 occupy similar positions, the 4-fluoro-1-methyl-indazole moiety (Fig. 1J, blue shading) at the other end of LY3502970 extends into the space between TM1 and TM2. Further, the 3.5-dimethyl-4-fluoro-phenyl ring (Fig. 1L, orange shading) interacts with TM1 and TM7 (Fig. 1F and SI Appendix, Fig. S5). In addition to the extensive hydrophobic and
Fig. 1. Pharmacology and structural analysis of LY3502970 in complex with GLP-1R/GsiN18/Nb35/scFv16. (A) Chemical structure of LY3502970 (molecular weight: 882.96, formula: C48H48F2N10O5). Moieties that are discussed in the cryo-EM structural analysis are highlighted in colored boxes. (B and C) The signal transduction pharmacology of LY3502970 and GLP-1(7-36) was determined. Human GLP-1R density-dependent pharmacology of ligands was quantified by measuring the potency and efficacy for cAMP accumulation at increasing levels of receptor density (high, medium, low). Functional potency and efficacy for β-arrestin recruitment using enzyme fragment complementation was determined. Representative concentration response curves are presented. Summarized data with statistics are presented in SI Appendix, Table S1. (D) LY3502970 does not stimulate cAMP accumulation in HEK293 cells expressing the mouse GLP-1R. Data are presented as the mean ± SD of three independent experiments. (E) Mice expressing the human GLP-1R (n = 5 mice/group) were fasted overnight and orally administered vehicle or LY3502970 (0.1 to 10 mg/kg). Five hours later, animals received an intraperitoneal (i.p.) injection of glucose (2 g/kg). As a control group, one cohort was dosed with a s.c. injection of exenatide (1 nmol/kg) 1 h prior to receiving the i.p. glucose. For all mice, the circulating concentration of glucose over various time points was measured using glucometers. Each dose of LY3502970 reduced the glucose excursion AUC versus vehicle (P < 0.05; one-way ANOVA followed by the Dunnett's test). (F) Similar studies were performed in Glp1r null mice. In these experiments, vehicle or LY3502970 (10 mg/kg) was administered orally and exenatide (1 nmol/kg) or gastric inhibitory polypeptide, (GIP) (30 nmol/kg) was dosed via s.c. injection. Data are mean ± SEM (n = 4 to 5 mice/group). (G) Overall structure of GLP-1R/LY3502970/GsiN18/Nb35/scFv16. Each subunit or ligand is shown with a different color (GLP-1R: green for 7TM and blue green for ECD; LY3502970: magenta; GsαiN18: bright blue; Gβ: light orange; Gγ: green cyan; scFv16: salmon; Nb35: violet). (H) Surface representation of LY3502970 binding pocket. The 7TM domain of GLP-1R is colored in green, while ECD is colored blue green. LY3502970 is shown by spheres. (I) LY3502970 interacts with residues from both the ECD and 7TM of GLP-1R. LY3502970, and its interaction residues are shown by sticks. Hydrogen bonds are indicated by dashed lines.
aromatic interactions, 4H-1,2,4-oxadiazol-5-one (Fig. 1A, yellow shading) of LY3502970 forms strong hydrogen bonds with Lys1972.67 (Fig. 1I). Together, the conformation of the three branches of LY3502970 establishes a unique binding mode for this molecule.

Domain-swapping experiments investigating the molecular basis for the specificity of LY3502970 activating the human but not the mouse GLP-1R provided functional insight into the receptor activation mechanism of LY3502970. Exchange of the mouse ECD with the corresponding sequence of the human receptor enabled LY3502970 to activate the mouse GLP-1R (Fig. 2B). Comparison of the amino acids of the ECD across species shows tryptophan at position 33 (Trp33ECD) in primates, while other species have serine at this location (Fig. 2A). Site-directed mutagenesis of this residue revealed gain and loss of function in the ability of LY3502970 to activate the mouse and human GLP-1Rs, demonstrating an essential role of Trp33ECD in the receptor activation mechanism of the compound (Fig. 2 C and D). Indeed, the cyro-EM structure reveals aromatic and hydrophobic interactions between the indole-tetrahydropyran branch (Fig. 1A, green shading and the connecting moiety) of LY3502970 and Trp33ECD, which forms a lid over this branch of the compound (Figs. 1I and 2E). The Trp33ECD lid is further stabilized through hydrogen bonding with Thr298ECL2 of ECL2 and van der Waals interactions with Ser222ECL1 of ECL1 and the disulfide bonding between Cys226ECL1 and Cys296ECL2 (Fig. 2F).

The conformation of Trp33ECD is also likely a driving force behind the unique ECD conformation. Other reports have shown that the ECD exhibits a high degree of flexibility and can adopt multiple open or closed conformations (37). In the structure presented here, the ECD is oriented toward ECL1 (Fig. 2F and G). An aromatic patch of the ECD consisting of Trp39ECD, Tyr69ECD, and Tyr88ECD is packed against His212ECL1 and Trp214ECL1 of the ECL1 (Fig. 2F and SI Appendix, Fig. S6). In the native GLP-1 and exendin-5 (ExP5) bound GLP-1R structures, these aromatic patches on the ECD and ECL1 are physically separated by the peptide, engaging in aromatic and hydrophobic interactions with Phe28GLP1 (or Phe23ExP5) (Fig. 3A) and Ile29GLP1 (or Ile24ExP5) (Fig. 3B) and Trp31GLP1 (or Trp29ExP5) and Leu32 (or Leu27) spanning across the C-terminal region of the peptide (SI Appendix, Fig. S6) (2, 36).

Taken together, the functional importance of Trp33ECD revealed by the mutagenesis experiments is fully supported by the cryo-EM data. This finding suggests that stabilizing the binding pocket and consistent with an inability of LY3502970 to activate receptors containing Ser33ECD. Competition binding experiments further substantiated this mechanism as Trp33ECD was determined to be necessary for GLP-1R binding of LY3502970 (Fig. 2H and I). More broadly, although Trp33ECD is not critical for receptor activation by native GLP-1, additional experiments revealed that another nonpeptide agonist PF-06882961 (38, 39) also requires Trp33ECD for GLP-1R activity (SI Appendix, Fig. S7 A and B).

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Fig. 2. Trp33ECD mediates key interaction with LY3502970 and the surrounding region. (A) Amino acid sequence comparison of the ECD regions for GLP-1Rs of different species. (B) LY3502970 increases cAMP accumulation in HEK293 cells expressing a chimeric form of the mouse GLP-1R (domains shown in green) where the N-terminal 142 residues have been replaced by the corresponding region of the human GLP-1R (domains shown in blue). (C) Mutation of Ser33ECD to Trp33ECD in the mouse GLP-1R enables LY3502970 to stimulate receptor-induced cAMP accumulation, while the reciprocal mutation in the human GLP-1R abolishes compound function. The chimera and mutant data are presented as the mean ± SD of three independent experiments. (F) Trp33ECD interacts with LY3502970 and residues on TM2, ECL1, and ECL2. Residues and ligands are shown by sticks except Trp33ECD, which is also shown by spheres. Hydrogen bonds are indicated by dashed lines. (G) GLP-1R ECD orientation in the LY3502970 bound structure stabilized by aromatic interactions with ECL1. (H) The unique ECD orientation of ECD in the LY3502970 bound structure as a result of the unique Trp33ECD position. The structures of GLP-1R bound to LY3502970 (7TM in green, ECD in blue green, LY3502970 in magenta), GLP-1 (yellow, PDB ID code 6VCB), and peptide 5 (gray, PDB ID code 5NX2) are aligned. Trp33ECD is shown by a stick. Other peptide bound GLP-1R structures (GLP-1, PDB ID code 5VAI; ExP5, PDB ID code 6B3J) are not shown here, but their ECD orientations are very similar to GLP-1 bound structure 6VCB in yellow. (H and I) Competitive inhibition of [125I]GLP-1(7-36) binding to membranes isolated from cells expressing the human GLP-1R (H) or human W33S GLP-1R (I). Data are represented as the mean ± SD of three independent experiments.
tested in cynomolgus monkeys to evaluate the ability of the compound to enhance glucose-stimulated insulin secretion and reduce food intake, both therapeutic hallmarks of GLP-1R agonism. Intravenous glucose tolerance tests (IVGTTs) were conducted to assess the ability of LY3502970 to enhance insulin secretion. The compound or exenatide was i.v. administered, followed by continuous infusion to maintain steady-state drug concentrations during the test. Glucose was administered 40 min after the infusion of LY3502970 or exenatide (Fig. 5A). Prior to the glucose administration, neither LY3502970 nor exenatide stimulated insulin secretion. After the glucose infusion, blood glucose concentrations in the vehicle-treated control were measured.

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**Fig. 3.** LY3502970 binding and the conformation of TM1, TM2, and TM7 of the GLP-1R. (A) LY3502970 (magenta) interactions with TM1, TM2, and TM7 of GLP-1R (green). Residues and ligands are shown by sticks. (B) TM1, TM7, and ECL3 of the inactive state structure of GLP-1R (salmon, PDB ID code 6LN2) need to shift to interact with LY3502970 (magenta sphere). (C) Unique conformation of TM1 and TM2 to accommodate LY3502970. The structures of GLP-1R bound to LY3502970, GLP-1 (yellow, PDB ID code 6VCB), and TT-OAD2 (cyan, PDB ID code 6ORV) are aligned. Other peptide bound GLP-1R structures (GLP-1, PDB ID code 5VAI; ExP5, PDB ID code 6B3J; peptide 5, PDB ID code 5NX2) are not shown here, but their TM1, TM2, and TM3 conformations are very similar to GLP-1 bound structure 6VCB in yellow.

**Fig. 4.** Structures of GLP-1R bound to agonists with different signaling profiles shed light on the structural basis for partial agonism and biased signaling. (A) Structures of the GLP-1R bound to LY3502970 (TTM in green, LY3502970 shown by magenta spheres), native GLP-1 (receptor in yellow, GLP-1 in beige, PDB ID code 6VCB), ExP5 (receptor in orange, ExP5 in pink, PDB ID code 6B3J), or TT-OAD2 (receptor in cyan, TT-OAD2 in blue, PDB ID code 6ORV) are aligned and shown from an identical view. Critical residues are shown by sticks. Hydrogen bonds are indicated by dashed lines. (B) The structure of GLP-1R bound to LY3502970 is aligned with GLP-1R bound to ExP5 and TT-OAD2 (Upper) and GLP-1 (Lower).
elevated and thereafter declined gradually over time. Serum insulin levels were slightly increased and remained elevated for 40 min. Treatment with LY3502970 or exenatide significantly increased the insulin concentrations and lowered blood glucose during the experiment (Fig. 5 B–E). Insulin secretion effected by the high dose of LY3502970 (steady-state concentration: 9.1 ± 0.8 nmol/L; mean ± SEM, n = 7) was comparable to that stimulated by high-dose exenatide (43.0 ± 4.1 pmol/L; mean ± SEM, n = 7). These results indicate that LY3502970 can reduce hyperglycemia via an insulinotropic mechanism to an extent similar to exenatide.

Since peptide-based GLP-1R agonists exhibit an anorexigenic effect as part of their overall ability to improve metabolic control, LY3502970 was orally administered to monkeys to examine the ability of the compound to reduce feeding. Following LY3502970 or exenatide treatment, food consumption was measured for 90 min. For these studies, LY3502970 was administered orally 180 min before feeding, and exenatide was s.c. injected 30 min prior to food availability, in line with the time of maximum concentration observed (Tmax) in monkey pharmacokinetic studies. Dosing was conducted once daily for 5 d with a 2 d recovery period. LY3502970 at 0.05 and 0.1 mg/kg decreased food consumption from the first to the fifth days of dosing in a dose-dependent manner (Fig. 5F), similar to that observed for 0.3 and 0.6 μg/kg of exenatide (Fig. 5G). The mean LY3502970 and exenatide concentrations which decreased food consumption were 8.3 ± 0.8 nmol/L and 83.1 ± 4.5 pmol/L, respectively (mean ± SEM, n = 8). These results indicate that orally dosed LY3502970 can achieve a reduction of food intake similar to the injectable GLP-1R agonist, exenatide. Taken together, LY3502970 displays a preclinical pharmacodynamic profile similar to marketed peptide-based GLP-1R agonists and possesses pharmacokinetic properties compatible with oral dosing in humans. Consequently, LY3502970 is currently being evaluated in early stage clinical trials for its potential as an antidiabetic agent (identifier, NCT04432647).

Methods

**In Vitro Pharmacology.** cAMP accumulation, β-arrestin recruitment, and receptor binding assays were performed as previously described (28, 40).

**Mutant Receptor Construction.** A plasmid expression vector containing a chimeric sequence where the ECD of the human GLP-1R replaced the mouse ECD was generated using the PCR products (PrimeSTAR Max, Takara) introduced to the multicloning site of pCMV6 entry using an In-Fusion HD cloning kit (Takara). The PCR primers used to create these constructs are as follows: for hmGLP-1R, 5’-CCGAGGAGACGAGCTCGTCCCTTGACATTATCTAGCAAGGG-3’ and 3’-AGGGGTCCTCTGTGAAGGAC5’ and, for mhGLP-1R, 5’-TTCCCTACATCATCCAGGGTCGGC3’ and 3’-GAAAGACCCTTTGTCGAGGACAAGAGATGTAGTAGATG-5’.

Human W33S GLP-1R and mouse S33W GLP-1R plasmids were generated by PCR-based mutagenesis; then the PCR products were introduced to pCMV6 entry described above. The PCR primers used to create these
constructs are as follows: for hW33S GLP-1R, 5'-TGGCCTTCTGAGACGCTGGACG-TACAGGCGAACT-3' and 3'-CCACGGGCAAGGGAGACCTCTTGCC-5' and for m333W GLP-1R, 5'-TCCCTGTGGAGAGCGCTGAAATGAGTGGTGG-3' and 3'-CATGGTTGGCAGGGGAGACCTCTTGCC-5'. DNA sequencing confirmed the chimeras and single-point mutant constructs.

Cryo-EM Method.

GLP-1R/LY3502970/GsiN18 complex formation and purification. The GLP-1R/LY3502970/GsiN18 complex (referred to as “the complex”) in the following text was formed on membrane as previously described (2, 40) with slight modification. The main difference was that we used a modified version of the alpha subunit of the stimulatory G protein, namely, GsiN18, which has the 1 to 18 residues of Ga1i at its N terminus, replacing the original 1 to 25 residues of Ga1i. This modification does not change any regions on Ga1i that are involved in receptor coupling or nucleotide binding but allows the coupling to sGv16 to aid stabilization of the complex and cryo-EM structure determination (42). To prepare the complex sample, SYF cells infected with virus containing human GLP-1R (recessive 24 to 422) were lysed, and a membrane sample was collected and washed. The complex was formed by incubating the purified molar excess of GsiN18, NB35, sGv16, 10 μM LY3502970, and 2 μM LSN3451217 with membranes and then solubilized in buffer composed of 1% n-dodecyl β-β-maltoside (DDM), 0.5% 3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS), 0.04% cholesterol hemisuccinate, 30 mM Hepes (pH 7.8), 150 mM NaCl, 30% glycerol, 25 μM TCEP (Tetraethylthiuram disulfide), 10 μM MgCl2, 10 μM MnCl2, and 10 μM ZnCl2. The complex was then purified by affinity chromatography using anti-FLAG M1 resin and exchanged for buffer composed of 30 mM Hepes (pH 7.5), 150 mM NaCl, 25 μM CaCl2, 10 μM LY3502970 and 2 μM LSN3451217, 25 μM TCEP, 0.25% lauryl maltose neopentyl glycol (MNG, NG310 Anatrace), 0.05% GDN101 (Anatrace), 0.048% 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-inositol (POPI, Avanti), and 0.03% cholesterol (Sigma-Aldrich). The complex was eluted and further purified by size exclusion chromatography using a Superdex S200 10/300 GL column with a running buffer of 30 mM Hepes (pH 7.5), 150 mM NaCl, 10 μM LY3502970 and 2 μM LSN3451217, 100 μM TCEP, 0.015% MNG, 0.005% GDN101, 0.00192% POPG, and 0.0012% cholesterol. The fractions for the monomeric complex were collected and concentrated individually for electron microscopy experiments. Crystal growth of the complex above was carried out as previously described (40). A sample of 3.5 μL of purified GLP-1R/LY3502970/GsiN18 complex at a concentration of ~10 mg/mL was applied to glow-discharged holey carbon grids (Quantifoil R1.2/1.3, 200 mesh) and subsequently vitrified using a Vitrobot Mark IV (Thermo Fisher Scientific). The discharged holey carbon grids (Quantifoil R1.2/1.3, 200 mesh) and subsequently vitrified using a Vitrobot Mark IV (Thermo Fisher Scientific). The complex was then formed on membrane as previously described (2, 40) with slight modification. The main difference was that we used a modified version of the alpha subunit of the stimulatory G protein, namely, GsiN18, which has the 1 to 18 residues of Ga1i at its N terminus, replacing the original 1 to 25 residues of Ga1i. This modification does not change any regions on Ga1i that are involved in receptor coupling or nucleotide binding but allows the coupling to sGv16 to aid stabilization of the complex and cryo-EM structure determination (42). To prepare the complex sample, SYF cells infected with virus containing human GLP-1R (recessive 24 to 422) were lysed, and a membrane sample was collected and washed. 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The complex was eluted and further purified by size exclusion chromatography using a Superdex S200 10/300 GL column with a running buffer of 30 mM Hepes (pH 7.5), 150 mM NaCl, 10 μM LY3502970 and 2 μM LSN3451217, 100 μM TCEP, 0.015% MNG, 0.005% GDN101, 0.00192% POPG, and 0.0012% cholesterol. The fractions for the monomeric complex were collected and concentrated individually for electron microscopy experiments. Crystal growth of the complex above was carried out as previously described (40). A sample of 3.5 μL of purified GLP-1R/LY3502970/GsiN18 complex at a concentration of ~10 mg/mL was applied to glow-discharged holey carbon grids (Quantifoil R1.2/1.3, 200 mesh) and subsequently vitrified using a Vitrobot Mark IV (Thermo Fisher Scientific). The specimen was visualized with a Titan Krios electron microscope (Thermo Fisher Scientific) and an energy filter operating at 300 kV accelerating voltage at a nominal magnification of 130,000× using a K2 Summit direct electron detector (Gatan, Inc.) in counting mode, corresponding to a pixel size of 1.04 Å on the specimen level. A total of 5,427 images was collected, and the statistics of data collection are listed in SI Appendix, Table S2.

Data processing was performed in Relion3.0 (43). Dose-fractionated image stacks were subjected to beam-induced motion correction using MotionCor2 (44). Contrast transfer function (CTF) parameters for each micrograph were determined using Gctf (45). Manual selection of micrographs based on the presence of the test article, dosing of LY3502970 or exenatide, and were 6.5 and 21.8 ng kg−1·h−1 for low- and high-dose exenatide, respectively, for LY3502970 and 4.2 and 13.4 ng kg−1·h−1 for exenatide. Dosing volumes were 2 mL/kg for the bolus administration, and the infusion rates for low- and high-dose LY3502970 were 1,280 and 3,840 ng kg−1·h−1 and were 6.5 and 21.8 ng kg−1·h−1 for low- and high-dose exenatide. Infusion volume was 2.7 mL/kg at a speed of 2 mL/kg·h−1. Forty minutes after initiation of dosing, 40% glucose (Otsuka Pharmaceutical Factory) was administered at 1.25 mL·kg−1·min−1 via the cephalic or saphenous vein. Blood was collected from the femoral vein 5 min before and after dosing and then at S, 10, 15, 20, 30, and 40 min following administration of 40% glucose. The studies were conducted at intervals of 7 or 24 d (days 8, 15, 22, 29, 36, and 60) using a 7 × 6 cross-over design.

Food Consumption Studies.

Eight male cynomolgus monkeys (7.5 to 9.3 kg, Shin Nippon Biomedical Laboratories, Ltd.) were administered LY3502970, exenatide, or vehicle once daily for 5 d with a 2 d recovery period using an

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**Appendix, Fig. S2C.** The final refinement statistics for the model are summarized in **SI Appendix, Table S2**. Atomic coordinates and the cryo-EM density map have been deposited in the PDB under PDB ID code6XOX and the Electron Microscopy Data Bank (EMDB) entry ID EMD-22283.
8 × 5 cross-over design. Food consumption during the 90 min period following presentation of food was measured in animals previously administered LY5302970 or exenatide, or vehicle as follows: 1) LY5302970 at 0.05 or 0.1 mg/kg by oral administration 180 min before feeding, 2) exenatide at 0.3 or 0.6 μg/kg by s.c. injection 30 min before feeding, or 3) the matched vehicle administered at the appropriate time.

Statistical Analysis. Statistical analyses of circulating glucose, insulin, and food consumption were performed by linear mixed model ANOVA with animal as a random effect and dose level as a fixed effect for comparisons by Dunnett's test with adjustment for multiplicity and by one-sided significance level of 2.5% for the order of increase (insulin) or decrease (glucose, mean food consumption) using SAS System for Windows, Release 9.2 (SAS Institute Inc.). Quantitative results are represented as the mean ± SEM.

The chemical structure of LY5302970 (OWL833) and some of the pharmacology data are shown in WO2018056453.

Data Availability. Atomic coordinates and the cryo-EM density map have been deposited in the Protein Data Bank (PDB ID code 6XOX) and EMDB (entry ID EMD-22283).

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