Supporting Information

Functional selectivity of a biased cannabinoid-1 receptor (CB$_1$R) antagonist

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**Supplementary Figure 2.** CB₁R inhibition of upper gastrointestinal (GI) motility in lean control mice is mediated via the G-protein pathway. 

**a)** Maximal inhibition of upper GI motility by the CB₁R agonist ACEA is dose-dependently antagonized by MRI-1891 or rimonabant, as quantified using the charcoal test (see Methods). Significant difference from vehicle only controls (*, \(P < 0.05\)) or from the vehicle + ACEA-treated group (#, \(P < 0.05\)) is indicated and determined by 1-way ANOVA followed by Dunnett’s multiple comparisons test. Columns and vertical bars represent means ± SEM from 4-6 experiments; 

**b)** treatment with MRI-1891 or rimonabant alone reveals inverse agonism by these compounds; 

**c)** treatment with ACEA alone induces similar inhibition of upper GI motility in wild-type and βArr2-ko mice. Symbols as in panel a.
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Methods

Chemistry. Reagents available commercially were purchased and used as is. Melting points were determined on a Buchi B-545 instrument and are uncorrected. Proton and carbon nuclear magnetic resonance ($^1$H and $^{13}$C NMR) spectra were recorded on a Varian 400 spectrometer in CDCl$_3$ or DMSO-d$_6$ (unless otherwise noted) with the values given in ppm (TMS as internal standard) and J (Hz) assignments of $^1$H resonance coupling. Mass spectra (HRMS) were recorded on a VG 7070E spectrometer or a JEOL SX102a mass spectrometer. Thin layer chromatography (TLC) analyses were carried out on Analtech silica gel GHLF 0.25 mm plates using various gradients of CHCl$_3$/MeOH containing 1% NH$_4$OH or gradients of EtOAc:n-hexane. Visualization was accomplished under UV light or by staining in an iodine chamber. Flash column chromatography was performed on Teledyne ISCO Combiflash system with Purion mass detector. All derivatives synthesized and tested had > 95% purity. LC/MS detection was carried out on Agilent 1200 system using Luna C18 3 µm column (3 x 75 mm). The mobile phase was 4% to 100% acetonitrile (0.05% TFA) standard gradient. The LC-MS chromatogram showed the correct molecular (MH$^+$) ion as well as a single peak by UV (254 nm). Results of elemental analyses were within 0.4% of the theoretical values. X-ray results were obtained utilizing the X-ray facility at University of California San Diego. Gram-scale chiral separation of (rac)-MRI-1891 was carried out at Regis Technologies Inc.

Synthesis, purification and isolation of compound MRI-1891.
Scheme 1.
Scheme 1: Reagents and conditions: (a) Toluene, reflux; (b) i. PCl₅, chlorobenzene, reflux; ii. N-acetylguanidine, Et₃N, MeOH: CH₂Cl₂, 78°C.

3-(4-chlorophenyl)-4-phenyl-4,5-dihydro-1H-pyrazole (1). Compound 1 was synthesized as described¹.

Methyl (4-(trifluoromethyl)phenyl)sulfonylcarbamate (2). A stirred solution of 4-trifluoromethyl sulfonamide (6.48 g, 28.8 mmol) and Et₃N (12 mL, 86.4 mmol) in acetonitrile (100 ml) was treated with methyl chloroformate (3.34 mL, 43.2 mmol) at 0°C. The reaction was warmed to room temperature over 6 h and the solvent evaporated in vacuo. The residue was dissolved in ethyl acetate and aqueous NaHCO₃ was added. The water layer was extracted and acidified with concentrated HCl in ice to give an oily emulsion, which turned into a white precipitate upon standing. The fluffy precipitate was filtered, washed with water and dried to give compound 2 (5.2 g, 64%). Mp: 85-87°C; ¹H-NMR (400 MHz, CDCl₃): δ 8.19 (d, J = 8.3 Hz, 2H), 7.82 (d, J = 8.4 Hz, 2H), 3.71 (s, 3H), 2.18 (s, NH); ¹³C-NMR (101 MHz, CDCl₃) δ 150.9, 141.8, 136.2-135.2(q), 129.10, 126.4-126.3 (q), 124.5, 121.8, 53.9; LRMS (C₉H₁₀F₃NO₄S) [M+Na]⁺: C₉H₁₀F₃NO₄S 306.0

3-(4-Chlorophenyl)-4-phenyl-N-((4-(trifluoromethyl)phenyl)sulfonyl)-4,5-dihydro-1H-pyrazole-1-carboxamide (3). To a solution of 2 (5 g, 17.7 mmol) in toluene, compound 1 (4.9 mg, 19.4 mmol) was added and the mixture was refluxed for 5 h. The solution was cooled to room temperature and compound 3 crystallized out of the solution. The crystals were collected and washed with cold toluene to yield pure 3 (6.0 g, 67%). Mp: 198-200°C; ¹H-NMR (400 MHz, CDCl₃) δ 8.89 (s, 1H), 8.30 (d, J = 8.1 Hz, 2H), 8.30 (d, J = 8.1 Hz, 2H), 7.81 (d, J = 8.2 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H), 7.25 (d, J = 8.3 Hz, 5H), 7.10 (d, J = 7.0 Hz, 2H), 4.71 (dd, J = 11.6, 5.4 Hz, 1H), 4.31 (t, J = 11.6 Hz, 1H), 3.89 (dd, J = 11.5, 5.5 Hz, 1H). ¹³C-
NMR (101 MHz, CDCl₃): δ 156.7, 147.7, 142.6, 139.1, 136.8, 135.8-134.9 (q), 129.6, 129.2, 129.1, 128.8, 128.3, 128.1, 127.3, 126.2-126.1 (q), 124.6, 121.9, 54.1, 51.6; LRMS (C₂₃H₁₈ClF₃N₃O₃S) [M⁺H]+: 508.1.

N-N'-(3-(4-chlorophenyl)-4-phenyl-4,5-dihydro-1H-pyrazol-1-yl)(((4-(trifluoromethyl)phenyl)sulfonyl)imino)methyl)carbamimidoyl)acetamide

**N-(rac)-MRI-1891** Compound 3 (4.0 g, 7.9 mmol) and PCl₅ (1.9 g, 9.4 mmol) were taken up in chlorobenzene (40 mL) and refluxed for 2 h. After thorough evaporation of the solvent, the formed imidoyl chloride (structure not shown) was dissolved in CH₂Cl₂ and cooled to -78°C. N-acetyl guanidine (1.2 g, 11.9 mmol) premixed with Et₃N (5 mL) in MeOH: CH₂Cl₂ (1:1) (20 ml) was added drop-wise. The reaction was slowly allowed to warm up to room temperature over 8 h. The reaction mixture was washed with water and extracted with CH₂Cl₂ and the solvent evaporated. The residue was subjected to flash chromatography and purified using 60% EtOAc in hexane. The off-white solid was triturated with isopropyl alcohol to give a white powder (2.0 g, 43% yield).

Mp: °C; ¹H-NMR (rac)-MRI-1891 (400 MHz, CDCl₃): δ 8.11 (d, J = 8.2 Hz, 2H), 7.69 (d, J = 8.3 Hz, 2H), 7.38 (d, J = 8.6 Hz, 2H), 7.30 (d, J = 7.4 Hz, 3H), 7.14 (dd, J = 15.9, 7.7 Hz, 4H), 4.53 (dt, J = 17.3, 8.5 Hz, 2H), 4.04 (dd, J = 11.4, 5.0 Hz, 1H), 1.89 (s, 3H). ¹³C-NMR (101 MHz, CDCl₃) δ 172.8, 158.3, 153.7, 139.3, 136.4, 133.2, 132.9, 132.6, 132.3, 129.5, 128.93, 128.79, 128.5, 128.0, 127.6, 127.4, 126.4, 125.74, 125.70, 124.9, 122.2, 119.5, 57.7, 50.6, 24.1. HRMS (C₂₆H₂₃ClF₃N₆O₃S) [M⁺H]+: calc’d: 591.1187 found: 591.1189. Anal. Cal (C₂₆H₂₂ClF₃N₆O₃S) C, 52.84; H, 3.75; N, 14.22; Found: C, 52.70; H, 3.78; N, 13.95.

N-N'-(S)-3-(4-chlorophenyl)-4-phenyl-4,5-dihydro-1H-pyrazol-1-yl)(((4-(trifluoromethyl)phenyl)sulfonyl)imino)methyl)carbamimidoyl)acetamide

**(-)-MRI-1891** Chiral preparative HPLC separation on racemic compound (50 mg) using a (R,R)-Whelk-O1 as the chiral stationary phase (25 cm x 21mm) yielded (-)-MRI-1891 (17 mg) and (+)-MRI-1891 (19 mg), respectively. The mobile phase was 100% ethanol. Gram-scale chiral separation was carried out using (S,S)-Whelk-O1and Super critical fluid chromatography (SFC). [α]25° = -13.0°, c = 0.23, CHCl₃; Mp 144-146 °C; ¹H-NMR (-)-MRI-1891 (400 MHz, CDCl₃): δ 8.13 (d, J = 8.0 Hz, 2H), 7.72 (d, J = 8.1 Hz, 2H), 7.38 (d, J = 8.2 Hz, 2H), 7.31 (d, J = 7.3 Hz, 3H), 7.17 (d, J = 8.0 Hz, 4H), 4.63 (dd, J = 11.1, 4.6 Hz, 1H), 4.52 (t, J = 11.7 Hz, 1H), 4.08 (dd, J = 12.1, 4.9 Hz, 1H), 1.98 (s, 3H). LCMS (C₂₆H₂₃ClF₃N₆O₃S) [M⁺H]+: found: 591.1.
N-N’-((R)-3-(4-chlorophenyl)-4-phenyl-4,5-dihydro-1H-pyrazol-1-yl)((4-(trifluoromethyl)phenyl)sulfonylimino)methyl)carbamimidoyl)acetamide ( (+)-MRI-1891) 

$[\alpha]_D^{25} = +12.6^\circ \text{ c } = 0.23$, CHCl$_3$; Mp 144-146 °C; $^1$H-NMR (400 MHz, CDCl$_3$): δ 8.10 (d, $J = 7.9$ Hz, 2H), 7.67 (d, $J = 7.7$ Hz, 2H), 7.40 (d, $J = 8.2$ Hz, 2H), 7.30 (dd, $J = 16.1$, 9.0 Hz, 3H), 7.15 (t, $J = 9.4$ Hz, 4H), 4.64-4.52 (m, 2H), 4.06-4.03 (m, 1H), 1.98 (s, 3H). LCMS (C$_{26}$H$_{23}$ClF$_3$N$_6$O$_3$S) [M$^+$H$^+$]: found: 591.1.
**X-Ray Diffraction.**

1. (-)-MRI-1891.

The single crystal X-ray diffraction studies were carried out on a Bruker Kappa APEX-II CCD diffractometer equipped with Cu K$_\alpha$ radiation ($\lambda = 1.5478$). Crystals of the subject compound were grown by dissolving approximately 1mg of sample in 350µL of Dichloromethane, which was then vapor diffused with Pentane over 2 days. A 0.115 x 0.091 x 0.053 mm colorless block was mounted on a Cryoloop with Paratone oil. Data were collected in a nitrogen gas stream at 100(2) K using $\phi$ and $\omega$ scans. Crystal-to-detector distance was 45 mm using variable exposure time (1s-5s) depending on $\theta$ with a scan width of 1.0°. Data collection was 99.4% complete to 68.00° in $\theta$. A total of 15223 reflections were collected covering the indices, -9<=h<=10, -10<=k<=10, -19<=l<=18. 4110 reflections were found to be symmetry independent, with a R$_{int}$ of 0.0267. Indexing and unit cell refinement indicated a primitive, monoclinic lattice. The space group was found to be P2$_1$. The data were integrated using the Bruker SAINT software program and scaled using the SADABS software program. Solution by direct methods (SHELXT) produced a complete phasing model consistent with the proposed structure.

All nonhydrogen atoms were refined anisotropically by full-matrix least-squares (SHELXL-2014). All hydrogen atoms were placed using a riding model and their positions were constrained relative to their parent atom using the appropriate HFIX command in SHELXL-2014. The absolute stereochemistry of the molecule was established by anomalous dispersion using the Parson’s method with a Flack parameter of 0.011(4). Atomic coordinates for (-)-MRI-1891 have been deposited with the Cambridge Crystallographic Data Centre (deposition number: 1817722-1817723). Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [fax: +44-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk]

2. (+)-MRI-1891.

The single crystal X-ray diffraction studies were carried out on a Bruker Kappa APEX-II CCD diffractometer equipped with Cu K$_\alpha$ radiation
(λ = 1.5478). A 0.053 x 0.047 x 0.005 mm piece of a colorless plate was mounted on a Cryoloop with Paratone oil. Data were collected in a nitrogen gas stream at 100(2) K using φ and θ scans. Crystal-to-detector distance was 40 mm using variable exposure time (5s-30s) depending on θ with a scan width of 1.0°. Data collection was 99.8% complete to 68.00° in θ. A total of 73901 reflections were collected covering the indices, -14<=h<=14, -22<=k<=23, -31<=l<=31. 11342 reflections were found to be symmetry independent, with a Rint of 0.0818. Indexing and unit cell refinement indicated a primitive, orthorhombic lattice. The space group was found to be P2₁2₁2₁. The data were integrated using the Bruker SAINT software program and scaled using the SADABS software program. Solution by direct methods (SHELXT) produced a complete phasing model consistent with the proposed structure. All nonhydrogen atoms were refined anisotropically by full-matrix least-squares (SHELXL-2014). All hydrogen atoms were placed using a riding model. Their positions were constrained relative to their parent atom using the appropriate HFIX command in SHELXL-2014. Atomic coordinates for (+)-MRI-1891 have been deposited with the Cambridge Crystallographic Data Centre (deposition number: 1817722-1817723). Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [fax: +44-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk]
**Materials.** Rimonabant and CP-55,940 were obtained from the National Institute of Drug Abuse Drug Supply Program (Research Triangle Park, NC). [3H]AEA was synthesized by the reaction of arachidonoyl chloride with [3H]ethanolamine. [3H]CP55,940, [35S]GTPγS, [3-3H]glucose, 2-deoxy-D-[1-14C]glucose were from Perkin-Elmer (Waltham, MA). Arachidonoyl-2’-chlooroethylamine (ACEA) was from Cayman (Ann Arbor, MI). All other chemicals were from Sigma–Aldrich (St. Louis, MO).

**Animals.** Male 8- to 10-week old C57BL/6J wild-type mice and β-arrestin-1−/− (βarr1-KO) and β-arrestin-2−/− (βarr2-KO) mice on a C57Bl/6J background were purchased from The Jackson Laboratory. Mice were maintained under a 12-h light/12-h dark cycle and fed *ad libitum*. To generate diet-induced obesity (DIO), mice were maintained on a high-fat diet (D12492, Research Diets, Inc., New Brunswick, NJ, 60% of calories as fat), whereas lean controls were kept on a standard laboratory diet (NIH-31 rodent diet) for 14 weeks.

**CB,R binding assays.** The assay was performed as described. Briefly, binding affinity of the compounds to CB,R and CB,R was determined by radioligand displacement assays using 1 and 0.6 nM of [3H]CP55,940 as the radioligand, respectively. Plasma membranes were prepared from mouse and rat brain or from cultured CHO-K1 cells stably transfected with human CB,R or CB,R (Perkin Elmer, MA). Twenty μg or 4 μg plasma membrane protein were used in 1 mL reaction mixture for rodent and human sources, respectively. Ki values were derived by computerized curve fitting and using the Cheng-Prusoff equation to account for the affinity of the radioligand, using the GraphPad Prism 6 program (GraphPad Prism Software Inc., San Diego, CA).

**[35S]GTPγS binding assay.** The assay was performed as described. Inverse agonism by MRI-1891 and rimonabant was determined in the absence of agonist, whereas their potency as antagonist (IC50) was determined in the presence of the agonist CP55,940 (300 nM), which generated CB,R-mediated increases in GTPγS binding at the ~EC80 level. Ten μg plasma membrane protein was used in a 1.0 mL reaction mixture.

**PathHunter eXpress CNR1 CHO-K1 β-Arrestin GPCR Assay.** β-arrestin-2 recruitment assay for CB,R signaling for both hCNR1 (cat# 93-0959E2CP) and mCnr1 (cat#93-0471E2MCP) were purchased from DiscoveRX (Fremont, CA). The assay was performed following supplier’s instructions. CB,R antagonistic potency (IC50) of MRI-1891 and rimonabant was determined in the presence of the CB,R agonist CP55,940 (30 nM), which increased β-arrestin-2 recruitment at the ~EC80 level. The presence of a
full length, unmodified hCNR1 in the plasmid construct used in this assay was verified by direct sequencing.

**Site-directed mutagenesis.** Point mutations were made in human CB₁ receptor (hCNR₁, NM_016083) in the pCI vector (Promega) for standard GPCR signaling assays, and in the pCMV-hCNR1-PK vector (Eurofins/DiscoverX) for β-arrestin recruitment assays via the PathHunter system. All mutagenesis reactions were performed using the QuikChange II site directed mutagenesis kit from Agilent. Custom oligonucleotide primer sets were from ThermoFisher Scientific.

The following single mutations were made:

I105A, M109A, S123A, M384S

The primer list below includes forward primers only. Reverse primers were the reverse complement of the forward primers. Mutated residues are bold and underlined.

A105: GGGAGAACTTCATGGACGCCGAGTGTTTCATGGTCCTG
A109: TTCATGGACATAGAGTGTTTGCCGTCCTGAACCCCAGCCAG
A123: CTGGCCATTGCAGTCCTG GCCCTCACGCTGGGCACCTTC
S384: ACGGTGTGATCTCAGCAGTACGTCTCTGCTGAACCTCCACC

**Drug treatment.** The compounds were administered by daily oral gavage of the indicated doses, dissolved in a vehicle of DMSO:Tween 80:saline (1:1:18). Oral formulations were applied at 3, 1, 0.3, 0.1, 0.03 or 0.01 mg/mL concentrations to achieve doses of 30, 10, 3, 1, 0.3 or 0.1mg/kg, respectively.

**Nonspecific binding in brain.** Non-specific binding of MRI-1891 and rimonabant to brain tissue was determined by equilibrium dialysis as described⁵, using crude brain membrane preparations from CB₁R⁻/⁻ mice. The membranes were incubated with 2 μM MRI-1891 and rimonabant in Rapid equilibrium dialysis (RED) device (Thermo Scientific, Rockford, IL). The levels of free (unbound) MRI-1891 and rimonabant were determined by LC-MS/MS.

**Tissue levels of drugs.** Tissues and plasma were extracted as described⁶, and MRI-1891 levels were determined by LC-MS/MS using an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies) coupled to an Agilent 1200 LC system (Agilent Technologies). Chromatographic and mass spectrometer conditions were set as described⁷. Levels of each compound were analyzed by multiple reactions monitoring (MRM). The molecular ion and fragments for each compound were measured as follows: m/z 591.1→293 and 591.1→145 for MRI-1891 (CID-energy: 28 V and 72 V, respectively), m/z 463.1→363 and 463.1→84.1 for rimonabant (CID-energy: 28 V and 24 V, respectively). The amounts of
MRI-1891 and rimonabant in the samples were determined against standard curves. Values are expressed as ng/g or ng/mL in wet tissue weight or plasma volume, respectively.

**Upper GI motility assay.** The assay was performed as described\(^4\). To determine inverse agonism, MRI-1891 or rimonabant was administered orally by gavage to male, 8-10-week-old mice 90 min prior to an oral bolus of 10% charcoal suspension in 5% gum arabic. Thirty min later, the mice were sacrificed, and the distance travelled by the head of the marker between the pylorus and the caecum was measured and expressed as percent of the total length of the small intestine. To assess *in vivo* CB\(_1\)R antagonism, MRI-1891 or rimonabant was administered orally 30 min before an intraperitoneal dose of the CB\(_1\)R agonist ACEA (5 mg/kg), with the rest of the procedure as described above.

**Hyperambulatory activity.** The assay was performed as described\(^4\). Locomotor activity of drug-naïve mice treated with an oral bolus dose of a CB\(_1\)R antagonist or vehicle was quantified by the number of disruptions of infrared beams in two dimensions in an activity chamber. The drugs were administered 3 h after the start of the light cycle.

**Elevated plus-maze (EPM) test.** Anxiety-related behavior was assessed using the EPM test as described\(^8\). Mice were tested 1 h after either acute or 28-day chronic oral administration of rimonabant or MRI-1891.

**Positron emission tomography (PET).** CNS CB\(_1\)R occupancy by MRI-1891 or rimonabant was assessed *in vivo* by their ability to displace the CB\(_1\)R PET radiotracer \(^{[18]}\text{F}\)FMPEP-d2, as described\(^5\). The specific activity of the PET radiotracer at the time of injection was 946.1 ± 243.2 mCi/µmol (mean±SD). Lean control mice (27.1 ± 1.8 g) were treated orally with MRI-1891 (1, 10 mg/kg), rimonabant (3 mg/kg) or vehicle 1h prior to radioligand injection. Concurrent to injection of 122.4 ± 34.3 µCi of \(^{[18]}\text{F}\)FMPEP-d2, PET scans began and continued for 120 min on a Siemens microPET Focus 120 camera (Siemens Medical Solutions, Knoxville, TN). Images were reconstructed without attenuation and scatter correction. For each animal, brain time-activity curve was constructed and expressed in standardized uptake value, which compensates for the injected activity and body weight. Whole brain uptake was determined by area under the time activity curve from 60 to 120 min post radioligand injection.

**Food intake and body weight** The amount of food intake and body weight were recorded daily around 5-6 pm before gavage.
**Glucose tolerance test (ipGTT) and insulin sensitivity test (ipIST).** The assays were performed following one week of daily oral administration of MRI-1891, as described. ipGTT was performed after overnight fasting by intraperitoneal administration of glucose at 1.5 mg/kg and subsequent sampling of tail blood at 15 min intervals. ipIST was performed on the next day after a 6 h fast by treating mice with a bolus dose of insulin (0.75 U/kg). Blood glucose levels were measured from tail blood.

**Blood glucose.** Blood glucose was determined using the Elite glucometer (Bayer).

**Plasma insulin.** Plasma insulin was determined using the Ultrasensitive Mouse Insulin EIISA kit (ALPCO Diagnostics).

**Statistical analyses.** Values shown are expressed as means ± SEM. Statistical analysis was performed by 1-way ANOVA or 2-way ANOVA followed by Dunnett’s or Sidaks’s multiple comparisons test, respectively, as appropriate. \( P < 0.05 \) was considered significant.

**Study approval.** Animal experiments and protocols were approved by the Institutional Animal Care and Use Committee of the NIAAA, NIH, Rockville, MD 20852.

**Cell culture and gene transfection of C2C12 cells.** C2C12 myoblasts were purchased from ATCC (Manassas, VA, USA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/L glucose with 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% \( \text{CO}_2 \) in air. To induce differentiation, the medium was replaced by 2% horse serum when the cells reached confluence. For knockdown of \( \beta \)-arrestin-2 gene expression, C2C12 cells at 4 days of differentiation were seeded into 6-well plates at ~70% confluency and transfected with \( \beta \)-arrestin-2 siRNA or scrambled siRNA (GE Dharmaco, Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen, Gaithersburg, MD) for 48 h as described. Cells were then incubated with 100 nM MRI-1891 or vehicle (DMSO) overnight in serum-starved medium in the absence or presence of 1µM CP55,940 for 2 h and 100 nM insulin for 30 min. Over-expression of mouse \( Cnrip1 \) gene carried by pcDNA3.1 vector in C2C12 cells was performed with Lipofectamine 2000 (Invitrogen, Gaithersburg, MD). Experiments were repeated at least 3 times, using different passages of C2C12 cells. Gene knock-down efficiency and over-expression levels were monitored by qRT-PCR with pre-designed primers from QIAGEN (Valencia, CA).
**Western blot.** Protein was extracted from cultured C2C12 cells. Western blot was performed as described\(^1\). Akt and phospho-AktS473 antibodies were from Cell Signaling Technology (Danvers, MA). β-actin antibody was from Abcam (Cambridge, MA). Western blot bands were quantified and analyzed by densitometry using ImageJ software.

**Hyperinsulinemic/euglycemic insulin clamp.** Experiments were performed as described previously\(^{12,13}\), with modifications. Briefly, 5 days prior to the experiment, the left common carotid artery and the right jugular vein of mice were catheterized under isofluorane anesthesia. Animals were allowed to recover for 5-7 days before the experiment. Following a 5-h fast, clamps were performed using unrestrained, conscious mice. The clamp procedure consisted of a 120-min tracer equilibration period (−120 to 0 min), followed by a 120-min clamp period (0 to 120 min). A 1.2-μCi bolus of [3-\(^3\)H]glucose tracer (PerkinElmer, USA) was given at the onset of the equilibration period (t = −120 min), followed by a 0.04 μCi/min continuous infusion for 2 h at a pump rate of 1 μL/min (CMA Microdialysis, USA). The insulin clamp started at 0 min with an infusion of human insulin (4 mU·kg\(^{-1}\)·min\(^{-1}\); Humulin R; Eli Lilly, USA), delivered at a pump rate of 0.4 μL/min. Euglycemia (blood glucose: \(\sim\)120–160 mg/dL) was maintained during clamps by measuring blood glucose in arterial blood every 10 min and adjusting as necessary the infusion rate of the mix of 40 μCi [\(^3\)H]-glucose in 600 μL of 45% glucose (hot glucose infusion or HOT GINF). The clamp steady state was achieved within 60–70 min. Blood samples were collected at −15 min and −5 min of the equilibration period and every 10 min during the steady-state clamp period (from 80 min to 120 min) and processed to determine glucose-specific activity. Mice also received an infusion of saline-washed erythrocytes from donors throughout the clamp period (3.5 μL/min) to compensate for a loss of blood during the clamp. To estimate insulin-stimulated glucose fluxes in tissues, 2-deoxy-d-[\(^{14}\)C]glucose (Perkin Elmer) was administered as a bolus (10 μCi) at the end of the steady-state (120 min). Blood samples were collected 2, 5, 10, 15, 25 min after the bolus injection. At the end of the clamp, animals were anesthetized with pentobarbital sodium...
(50 mg/kg i.v.), and tissues were collected immediately and frozen in liquid nitrogen for analysis.

To determine [3-3H]glucose-dependent variables, plasma samples were deproteinized using barium hydroxide and zinc sulfate. Rates of glucose production and disappearance (total glucose turnover; Rd) were determined using Steele's non-steady-state equations\textsuperscript{14}. Hepatic endogenous glucose production rate during clamp was determined by subtracting the glucose infusion rate (GIR) from Rd. The glucose uptake by tissues and glycogen synthesis rates were calculated as described previously\textsuperscript{15}.

\textbf{2-Deoxyglucose uptake in skeletal muscle.} 2-Deoxy-d-glucose uptake into soleus muscle was analyzed as described\textsuperscript{16} with modifications. Briefly, following a 5-h fast, blood was collected from mice for determining baseline glycemic parameters. The animals then received a bolus injection through the tail vein of insulin (0.75U/kg body weight) and 10 µCi 2-deoxy-d-[\textsuperscript{1-14}C] glucose (PerkinElmer) dissolved in 150 µL of 0.9% saline. Thirty minutes later, the mice were sacrificed, trunk blood and soleus muscle tissue were collected and glucose uptake into solus muscle was calculated as described\textsuperscript{15}.

\textit{Quantum chemistry and Molecular Dynamics simulations.} The initial coordinates of human CB\textsubscript{1}R were taken from the co-crystal with the antagonist taranabant (PDB: 5TJV, superseded by 5U09). The PGS protein replacing ICL 3 in the crystal was removed; ICL 3 was not modeled, so the intracellular portions of TMHs 5 and 6 were distance-constrained to prevent distortions during the simulation. The receptor was then embedded in a membrane-like environment composed of zwitterionic 1-palmitoyl-2-oleoyl-phosphatidylycholine lipid molecules, the orientation of which was obtained from the OPM database Fig. 8a, left). The system was hydrated with TIP3P water, and simulations were conducted at constant temperature (37°C) and pressure (1 atm), using PBC and PME, with the all-atom representation of the all-atom CHARMM force field. All residues were assumed to be neutral at pH 7 except D\textsuperscript{−}, E\textsuperscript{−}, K\textsuperscript{+} and R\textsuperscript{+}; Cl\textsuperscript{−} ions were placed randomly in the water phase to neutralize the systems and 120 mM concentrations of Na\textsuperscript{+} and Cl\textsuperscript{−} ions were distributed randomly in the extracellular (EC) and intracellular (IC) regions. After removal of the co-crystallized ligand, simulations were performed to relax the cavity and obtain an equilibrated CB\textsubscript{1}R structure, which was taken as the initial coordinates for all the simulations. The initial coordinates of S-MRI-1891 and rimonabant were obtained from their respective crystals (cf. Fig.
S4), and conformers were generated upon rotations of the four dihedral angles $\phi_i$ (Fig. 8a). Nine low-energy conformers were found upon optimization in the gaseous phase with density functional theory (DFT) at the B3LYP/6-31G* level as implemented in Gaussian 09 software\textsuperscript{17}. Four conformers, which includes the optimized X-ray structure, are very similar in energy (within a Gibbs free energy of 0.2 kcal/mol at 298.15 K), with conformer conf. 1 (Fig. 8a), obtained by rotation of $\phi_2$, having the lowest energy. The other four conformers, obtained by a 180° rotation of $\phi_1$ relative to the first set, are $\sim 3kT$ higher in energy. One conformer, obtained upon rotation of $\phi_3$ (double bond), resulted in the lowest energy, about $4kT$ lower than conf. 1. Because this energy can be conceivably overcome by favorable interactions with the receptor, all eight conformers were considered in the simulations. Each conformer was initially docked into the receptor's pocket by overlaying it onto AM6538 as co-crystallized with CB$_1$R, using the five-member ring's heavy atoms as a common docking point. This initial docking was followed by a short rigid-body Monte Carlo simulation to obtain a relaxed initial configuration of the ligand from which the dynamics simulation was started. All the MD simulations followed the standard protocol of minimization, heating, equilibration, and productive phase, where data were collected for analysis. Given that ICL 3 was not modeled, the length of the productive phase of each simulation was restricted to 20-40 ns, which provided enough sampling for statistical analysis of the interactions (CB$_1$R/MRI-1891, CB$_1$R/water, and MRI-1891/water H-bonds, electrostatics, hydrophobic/nonpolar contacts; halogen bonding may play a role but were not modeled in this study, thus not analyzed).

While all the conformers were well stabilized throughout the simulation, as were rimonabant and ibipinabant, only conf. 1 in the relative orientation of Fig. 7 showed optimal and persistent interactions with the receptor, mainly through hydrogen bonding and nonpolar/hydrophobic contacts, and was compatible with all the mutation studies performed, including S123. Two other modes are noted, as they provide clues for further chemical modifications of MRI-1891 or related compounds: conf. 1 rotated as a rigid body and conf. 0 with the same orientation of Arm 1 and 2 (not shown). The former appears to be less stable than the mode in Fig. 7 because the Cl atom of Arm 1 interacts less efficiently with the receptor, and some of the acceptor and donor groups in Arms 3 and 4 are not H-bonded to either water or the receptor; the latter adopts a pose such that Arm 3 does not interact with S123, thus not compatible with the mutation data. Both the QM calculations and the MD simulations thus suggest that MRI-1891 reaches the receptor as conf. 0 (over 90% of the population) and then switches to conf. 1 in the mode of Fig. 7, which is stabilized by multiple interactions.

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