Hexahydroquinoline Derivatives Are Selective Agonists for the Adhesion G Protein-Coupled Receptor ADGRG1/GPR56

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ABSTRACT

GPR56 is a widely expressed adhesion GPCR (AGPCR) that has pleotropic roles in brain development, platelet function, and cancer. Nearly all AGPCRs possess extracellular regions that bind protein ligands and conceal a cryptic tethered peptide agonist. AGPCR reception of mechanical or shear force is thought to release the tethered agonist permitting its binding to the AGPCR orthosteric site for consequent activation of G protein signaling. This multistep mechanism of AGPCR activation is difficult to target, emphasizing the need for tool compounds and potential therapeutics that modulate AGPCRs directly. We expanded our cell-based pilot screen for GPR56 small molecule activators to screen >200,000 compounds and identified two promising agonists: 2-(furan-2-yl)-1-[(4-phenylphenyl)carbonyl]pyrrolidine, or compound 4, and propan-2-yl-4-(2-bromophenyl)-2,7,8-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate, or compound 36. Both compounds activated GPR56 receptors to have impaired tethered agonists and/or be cleavage deficient. Compound 4 activated a subset of group VIII AGPCRs while compound 36 had exclusive specificity for GPR56 among the GPCRs tested. Compound 36 SAR analysis identified an analog with the isopropyl R group replaced with a cyclopropyl ring and the electrophilic bromine replaced with a CF₃ group. Analog 36.40 had 40% increased potency over compound 36 and was 20-fold more potent than synthetic peptidomimetics designed from the GPR56 tethered agonist. The new GPR56 tool compounds discovered in this screen may be used to further advance understanding of GPR56 function and aid development of AGPCR-targeted therapeutics.

SIGNIFICANCE STATEMENT

Adhesion G protein coupled receptors (AGPCRs) are a large, clinically relevant class of GPCRs with no available therapeutics, in part due to their unique mechanism of activation. GPR56 is a widely expressed model AGPCR involved in cancer metastasis, hemostasis, and neuron myelination. In the present study, we identified novel small molecule agonists for GPR56. These molecules are among the most potent identified thus far and may become useful leads in the development of a GPR56-targeted therapeutic.

Introduction

Adhesion GPCRs (AGPCRs) or family B2 GPCRs consist of 33 members that have variable extracellular regions with adhesive modules that bind a variety of protein ligands. They are important mediators of diverse processes including tissue and organ development, blood cell function, and synaptic regulation (Lin et al., 2005; Luo et al., 2011; Silva et al., 2011; O’Sullivan et al., 2012; Wang et al., 2013; Boucard et al., 2014; Vysokov et al., 2018; Yeung et al., 2020). Common to AGPCRs is the conserved extracellular GPCR autoproteolysis-inducing (GAIN) domain (Arac et al., 2012). The GAIN domain is a constitutive protease that self-cleaves AGPCRs at a conserved loop that links the penultimate and last β-strands of the GAIN domain. The two resulting fragments termed the extracellular N-terminal fragment (NTF) and the C-terminal fragment (CTF) or 7TM domain remain noncovalently bound via the dense network of hydrogen bonds within the GAIN domain. The stalk that emanates from the first transmembrane span of the 7TM lies encrypted within the GAIN domain in the cleaved holoreceptor. Dissociation of the two

ABBREVIATIONS: AGPCRs, adhesion G protein coupled receptors; β2AR, β2 adrenergic receptor; ChemDiv 100K, Chemical Diversity 100K library; CTF, C-terminal fragment; DART 90K, Dart Neurosciences 90K library; 1,4-DHP, 1,4-dihydropyridine; 3-DOG, 3-α-acetoxydihydroxydeoxygedunin; DMEM, Dulbecco’s modified Eagle’s medium; ECL2, extracellular loop 2; GAIN, GPCR autoproteolysis-inducing domain; GPR56-AP, GPR56-activating peptide; HHQ, hexahydroquinoline; MB24K, Maybridge 24K library; M1R, muscarinic acetylcholine receptor 1; NTF, N-terminal fragment; PEI, polyethyleneamine; SRE, serum response element; TA, tethered agonist; 7TM, 7-transmembrane spanning domain.
AGPCR fragments exposes this peptide, and its N-terminus adopts a new conformation when bound to the 7TM domain orthosteric site to act as a tethered peptide agonist (TA) and activate signaling (Liebscher et al., 2014; Stoveken et al., 2015; Vizarraga et al., 2020). The first cryogenic-electron microscopy structures of TA-activated AGPCRs were determined, affirming a unified mechanism of TA-mediated activation; upon decrption, the TA stalk threads into the orthosteric site beneath ECL2 as a partial 3-helical hook-like structure and interacts with the 7TM domain primarily through the P3, P6, and P7 hydrophobic residues of the TA (typically Phe, Leu, and Met, respectively) (Barros-Alvarez et al., 2022; Ping et al., 2022; Qu et al., 2022; Xiao et al., 2022).

GPR56/ADGRG1 is widely expressed in tissues including glial cells, muscle, testis, and platelets (Chen et al., 2010; Wu et al., 2013; White et al., 2014; Mehta and Piao, 2017; Ackerman et al., 2018; Giera et al., 2018; Yeung et al., 2020). GPR56 possesses a Pentraxin/Laminin/neurexin/sex-hormone-binding-globulin-like domain N-terminal to its GAIN domain that binds collagen and transglutaminase-2 (Yang et al., 2014; Salzman et al., 2016; Giera et al., 2018; Salzman et al., 2020). It couples to G protein G12/13 to mediate proliferation of oligodendrocyte precursor cells via RhoA signaling, which supports nerve myelination (Ackerman et al., 2015; Giera et al., 2015, 2018). In platelets, GPR56 interacts with vessel wall-injury exposed collagen via its Pentraxin/Laminin/neurexin/sex-hormone-binding-globulin-like domain to fulfill shear force-dependent platelet shape change via Rho signaling prior to platelet activation (Yeung et al., 2020). GPR56 is also an oncogene in several types of cancer, including colorectal cancer, gliomas, and melanomas (Shashidhar et al., 2005; Ke et al., 2007; Yang et al., 2011; Chiang et al., 2017; Jin et al., 2017; Ji et al., 2018; Zhang et al., 2019). GPR56 upregulation in cancers may provide Rho signaling to drive cancer progression, marking it as a potential biomarker or therapeutic target. Dysregulation of GPR56 also results in the pathogenesis of the recessive human neurodegenerative disease bilateral frontoparietal polymicrogyria, the patients of which suffer severe intellectual deficiencies, epilepsy, and ataxia (Jin et al., 2007; Chiang et al., 2011). Despite a variety of roles in biologic function and disease, no drugs have been designed to target GPR56 or any other adhesion GPCR. Small molecule probes targeting AGPCRs could fill this role or may also serve as useful tools to study AGPCRs in vivo.

We previously sought small molecule agonists and antagonists for AGPCRs using GPR56 as a model. We identified the steroid-like partial agonist 3-α-acetoxydihydroxycegdunin (3-α-DOG) and the isoflavonoid antagonist dihydromedulone from pilot cell-based high throughput screens. These compounds served as vital probes for identifying the role of GPR56 in platelet shape change (Yeung et al., 2020). Here we expanded upon these pilot screens and conducted a large-scale effort to identify more potent and efficacious activators of GPR56 from three libraries comprising ~200,000 compounds. We developed techniques for large-scale handling and culturing of HEK293T cells to overcome technical challenges that ensured integrity of the screening assay across all 200,000 compounds. From our primary screen we identified 1,327 initial hits, which was narrowed to 155 candidates following counter-screening. Seventy-four of the 155 candidates demonstrated promising concentration-dependent responses. Further vetting and testing in an orthogonal biochemical GPCR reconstitution assay identified 16 final candidate compounds that had equivalent or improved efficacies and potencies over the positive control GPR56-activating peptide (GPR56-AP), a peptidomimetic of the tethered agonist.

One compound, propan-2-yl-4-(2-bromophenyl)-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate, or compound 36, showed substantial activity in the cell-based luciferase assay and GPCR reconstitution assay with potency several fold higher than a GPR56-AP and the previously identified partial agonist 3-α-DOG. Compound 36 and one other compound, 2-((furan-2-yl)-1-(4-phenylphenyl)carbonyl)pyrrolidine, or compound 4, also activated a cleavage-deficient holoreceptor mutant of GPR56. Compounds 36 and 4 selectively activated the G subfamily of AGPCRs. Compound 36 was exclusive for GPR56/ADGRG1, while compound 4 activated both GPR56 and GPR97/ADGHC3. Following structure activity relationship analysis of compound 36 using commercially available analogs, we identified an analog that had ~40% increased potency. Compound 36 and the improved analog were docked in the GPR56 orthosteric site by in silico analysis, which predicted that the R enantiomer interacted more productively than the L enantiomer with features of the binding pocket that are critical for TA engagement. The compounds discovered in our study may be used to advance knowledge of GPR56 function and be tailored to become first-generation AGPCR-targeted therapeutics.

Materials and Methods

Reagents and Antibodies. [35S]-GTP-S was from PerkinElmer (Waltham, MA.). GTP-γ-S was from MilliporeSigma. Rhoetkin-BD beads were from Cytoskeleton, Inc. Steady-Glo Luciferase kits were from Promega. Fresh compound powders were obtained from MolPort, Inc. and reconstituted in anhydro DMSO. Coelenterazine h for dual serum response element (SRE)-Luciferase assays was from NanoLight Technologies and reconstituted in 70% v/v ethanol. The synthetic peptidomimetics or activating peptides GPR56/GPR114 P19 (TYFAVLMLSPALVPAELL-NH2) and GPR56 P7 (TYFAVLML-NH2) were synthesized and HPLC purified by GenScript, dissolved in anhydro DMSO and stored under argon.

Plasmids and Cloning. A list of all plasmids and their sources can be seen in Supplemental Table 1. Human muscarinic acetylcholine receptor 1 (M1R) was purchased from cDNA.org (MAR1000000) and subcloned from pcDNA3.1(+) into pFastBac1 via BamH I and XhoI restriction sites.

Chemical Libraries. The use of all chemical libraries was purchased from the University of Michigan Center for Chemical Genomics. Chemical libraries were cherry-picked collections from larger libraries to select for available dry powders with low molecular weight (< 500 Da), favorable Lipinski drug-like properties (Lipinski et al., 2001), and flexibility for derivatization. The Maybridge 24K library—MB24K—is a library of ~23,500 compounds derived from the Maybridge library with an average molecular weight of 328 Da, Log D of 3.31 and over 99% rate of favorable Lipinski conditions. The Chemical Diversity 100K library—ChemDiv100K—is a library of ~100,000 compounds derived from the public ChemDiv library with an average molecular weight of 364 Da, Log D of 2.73 and 100% rate of favorable Lipinski conditions. The Dart Neurosciences 90K library—DART90K—is a library of ~83,000 compounds cherry-picked from a 264,000-compound library from Dart Neuroscience with an average molecular weight of 334 Da, Log D of 2.86 and 99.99% rate of favorable Lipinski conditions. All libraries are reconstituted in DMSO at stock concentrations of 10 mM.

Preparation of Pretransfected Cells for High Throughput Screening Assays. HEK293T cells were seeded in Corning Hyper-Flasks at 1.72 × 10^5 cells per flask in 550 mL of Dulbecco’s modified Eagle’s medium (DMEM) containing 10% v/v FBS 24 hours prior to transfection. Cells were transfected with 200 mg of SRE-Luc plasmid
and 400 mg of either GPR56 (ΔTA)-pcDNA3.1(+) or pcDNA3.1(+).
Transfections were conducted using polyethylenamine (PEI) as described (Stoevenk et al., 2015). Plasmid DNAs were added to 15 mL of OptiMEM (Gibco), and mixed 1:1 with 15 mL of 100 μg/mL PEI in optinem. Transfection mixtures were incubated at 22°C for 15 minutes and added to 500 mL of fresh DMEM containing 10% v/v FBS. Media in the HyperFlasks were decanted and replaced with the transfection media. The flasks were incubated for 6 hours at 37°C with 5% CO2. Media were removed and cells were trypsinized using 0.05% w/v trypsin in Puck’s G Salt Solution-EDTA (137 mM NaCl, 5.4 mM KCl, 1.1 mM KH2PO4, 1.1 mM NaHPO4, 1 mM EDTA, pH 7.0) for 5 minutes. Cells were diluted in medium, centrifuged at 500 g for 5 minutes, and suspended at 22 x 10^6 cells/mL in DMEM containing 10% v/v FBS and 10% v/v DMSO. The cell suspension (1 mL) was filled per Nunc cryopreservation vial cells and frozen at −80°C overnight in Mr. Frosty freezing containers prior to long-term storage under liquid N2.

**Cell-Based High Throughput SRE-Luciferase Assay.** Cryopreserved cells were thawed at 37°C and diluted to 1.25 x 10^5 cells/mL in Fluorobrite medium (Gibco) supplemented with 0.1% v/v FBS, 20 mM HEPES pH 7.4, and 2 mM L-glutamine. Diluted cells were used to seed 8 x 384-well opaque white plates at 5,000 cells per well in 40 μL using a Thermo Scientific Multidrop combi reagent dispenser. Cells were incubated at 37°C in a 5% CO2 atmosphere for 4 hours. After incubation, 200 nL of 2 mM compounds in DMSO were added to each well using a Scilon ALH 3000 V&P pintool to achieve a final compound concentration of 10 μM and 0.5% v/v DMSO. GPR56/114-AP 19-mer peptide in DMSO was diluted to 90 μM in Fluorobrite medium, and 11.5 μL was added to the final two columns of each 384-well plate using the combi reagent dispenser to achieve a final concentration of 20 μM. Eighteen hours after compound and peptide dispensing, plates were centrifuged at 300 g for 3 minutes and the upper 20 μL of medium was aspirated using a Biotek EL 406 plate washer. Promega Steady-Glo (20 μL) was added to each well using the combi reagent dispenser, and the plates were placed on a plate shaker at 600 rpm for 5 minutes. After shaking, the plates were incubated for 10 minutes in the dark and luminescence was read using a PHERAstar FSX microplate reader. For evaluation of screen quality, the ratios between S.D.s and means of positive and negative controls were used in a Z’ score equation for each plate screened (Zhang et al., 1999):

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Z' = \left( \frac{3σ_{c} + 3σ_{nc}}{|μ_c - μ_{nc}|} \right)
\]

Where σ represents the S.D. and μ represents the mean of the positive (c+) or negative (c−) controls. A score of >0.5 was used as a threshold for an assay that was suitable for screening.

**Reconfirmation and Counter Assay of Hits.** A Sample Preparation Technologies Labtech Microplate instrument was programmed to dispense 200 nL of selected activator compounds into 384-well white opaque plates. Cryopreserved HEK293T cells pretransfected with the SRE-luciferase reporter and GPR56 ΔTA or pcDNA3.1 were thawed and seeded into the awaiting compound-seeded plates at 5,000 cells per well. The plates were processed as described previously with Promega Steady-Glo to measure luciferase signals.

**Directed Dual Luciferase Assay.** Early passage HEK293T cells were used to seed 15 cm tissue culture plates at 15 x 10^6 cells per plate and were incubated at 37°C 5% CO2 for 18 hours. Each plate was transfected with 74 μg phBLaC-N1, 7.43 μg SRE-Luc plasmid and 18.57 μg of either receptor plasmid (GPR56 ΔTA, GPR56 7TM, and GPR56 full-length) or empty pcDNA3.1(+) using the PEI method described earlier. After 6 hours, the cells were lifted with 0.05% trypsin in Puck’s G Salt Solution-EDTA, spun at 500 g for 5 minutes and resuspended at 8 x 10^6 cells per mL in DMEM + 10% FBS + 10% v/v DMSO. Cells were aliquoted into cryopreservation vials at 1 mL/2 and frozen at −80°C overnight in Mr. Frosty freezing containers prior to long-term storage under liquid N2.

Prior to each assay, cells were thawed at 37°C, washed in warm DMEM + 10% v/v FBS, and seeded into 96-well plates at 80,000 cells per well in 100 μL of medium. After 16 to 18 hours, cells were serum starved for 4 hours before being treated with 1 mL of compounds or peptides with a final DMSO content of < 1% v/v. After 8 hours, the plates were spun at 300 g for 3 minutes and the top 50 μL from each well was withdrawn and replaced with 50 μL of Promega SteadyGlo reagent. Plates were shaken at 500 rpm for 5 minutes and luminescence was read at 1 second per well.

**Baculovirus Generation and Insect Cell Culture.** *Spodoptera frugiperda* 9 (Sf9) and *Trichoplusia ni* (Tni, High Five) cells were cultured in ESF921 medium (Expression Systems). Recombinant baculoviruses for G proteins and GPCRs were prepared from pFastBac1 donor constructs using the Bac-to-Bac system as per the manufacturer’s instructions. Briefly, pFastBac constructs were transposed into competent DH10Bac cells (Invitrogen), after which cells were plated onto triple antibiotic Luria-Broth agar plates containing 50 μg/mL kanamycin, 10 μg/mL tetracycline HCl, 7 μg/mL gentamicin, 40 μg/mL isopropyl β-D-l-thiogalactopyranoside, and 75 μg/mL halogenated indolyl-β-galactoside (BluoGal) for blue/white colony screening. White colonies were restreaked and used to inoculate triple antibiotic Luria-Broth liquid medium for bacmid DNA preparation. Bacmid DNA (3 mg) was transfected into 9 x 10^6 adherent Sf9 cells in 6-well format using Fugene HD transfection reagent (Promega). After five days, viral supernatants were harvested and amplified twice in Sf9 cells at an infection ratio of 1:100. High titer baculovirus were used to infect 50 to 200 mL cultures of High-Five cells at a ratio of 1:100 for 48 hours, after which cells were centrifuged at 2000 g and frozen for cell membrane preparation.

**G Protein Preparation.** Gz proteins were purified via association with His6G/γ/1/2 as described (Kozasa and Gilman, 1995). Briefly, large-scale membrane homogenates were prepared from pellets of Tni cells overexpressing Gz, Ric-8a, His6G/γ1/2, and Gz2. Membrane homogenates were detergent extracted in 1% w/v sodium cholate with gentle stirring for 1 hour at 4°C. The cholate extract was centrifuged at 100,000 x g for 1 hour, and the clarified supernatant was diluted fourfold with 0.5% w/v decaethylene glycol mono-dodecyl ether (lubrol) and loaded onto Ni-NTA resin in a gravity driven column. The column was washed with a high salt buffer [20 mM Hepes pH 8.0, 300 mM NaCl, 1 mM MgCl2, 10 mM imidazole, 10 mM βME, 10 μM GDP, 0.5% lubrol, and protease inhibitor cocktail (25 μg/mL phenylmethylsulfonyl fluoride, 21 μg/mL N-P-tosyl-L-lysinecholoromethyl ketone, 21 μg/mL L-Tosylamin-2-phenylethylchloroketone, 3.3 μg/mL leupeptin, and 3.3 μg/mL lima bean trypsin inhibitor)] and eluted with an aluminum fluoride buffer (20 mM Hepes pH 8.0, 100 mM NaCl, 50 mM MgCl2, 10 mM βME, 20 μM GDP, 10 mM NaF, 30 μM AlCl3, 1% n-octyl-β-d-glucoside). The aluminum fluoride eluate was resolved over a HiPrep 26/60 Sephacryl S-200 column to isolate monomeric Gz and to buffer exchange the protein into storage buffer (20 mM Hepes pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 2 mM MgCl2, 1 mM dithiothreitol, 10 μM GDP, 11 mM CHAPS). The Gz protein was collected and concentrated in an Amicon ultracentrifugal concentration device with 30,000 MWCO and cryopreserved at −80°C.

**Adhesion GPCR Membrane Homogenate Preparation.** Insect cell pellets overexpressing adhesion GPCRs were thawed at 37°C and resuspended in 20 mL of lysis buffer [10 mM Hepes pH 7.4, 1 mM EDTA, and protease inhibitor cocktail (23 μg/mL phenylmethylsulfonyl fluoride, 21 μg/mL N-P-tosyl-L-lysinecholoromethyl ketone, 21 μg/mL L-Tosylamin-2-phenylethylchloroketone, 3.3 μg/mL leupeptin, and 3.3 μg/mL lima bean trypsin inhibitor)]. The cell suspension was lysed by nitrogen cavitation at 600 PSI. Cell lysates were centrifuged at 100,000 g for 30 minutes. The supernatants were discarded, and membrane pellets were Dounce homogenized into 5 mL of ice-cold lysis buffer with or without 7M urea. The homogenates were recentrifuged at 100,000 g for 30 minutes and washed by Dounce homogenization into ice-cold lysis buffer without urea. Membranes were recollected at 100,000 g
and Dounce homogenized into 2 mL of lysis buffer containing 12% w/v sucrose and cryopreserved into small aliquots.

**GPCR/G Protein Reconstitution Assays.** For all assays, adhesion GPCR membrane homogenates (5 μg nontreated homogenates/assay time point or equivalent volume of urea-treated homogenates) were reconstituted with 200 nM purified Gα13, Gαq, or Gαs,short and 500 nM purified Gβγ in binding buffer (50 mM Hepes pH 7.4, 1 mM diithiothreitol, 1 mM EDTA, and 3 μg/mL purified BSA). To initiate GTPγS binding, the reconstituted membrane homogenates were combined 1:1 with binding buffer containing 50 mM NaCl, 10 mM MgCl2, 20 μM GDP, and 4 μM [35S]GTP-S (25-50,000 cpm/pmol). Endpoint assays were quenched with 20 mM Tris pH 7.7, 100 mM NaCl, 10 mM MgCl2, 1 mM GTP, 0.08% w/v lubrol, C12E10 and filtered through Whatman GF/C filters using a Brandel vacuum. The filters were washed, dried, and subjected to liquid scintillation counting. For compound activation experiments, reconstituted membranes were preincubated with DMSO, GPR56-AP P7, or activator compounds for 10 minutes at 22 °C prior to the start of reactions with DMSO content of ≤ 1.0% v/v.

**In Silico Docking.** The cryo-EM structure of TA-activated GPR56 7TM (PDB: 7SF8) (Barros-Alvarez et al., 2022) with 13 N-terminal residues (T383-V395) removed was prepared in Maestro 2022-3 with the OPLS force field. R and L enantiomers of both compound 36 and 36.40 were docked within a 25 Å × 25 Å × 25 Å grid centered around W6.53 using Glide XP Docking with sampling of hydrogen rotamers for residues within 10 Å of W6.53 (Friesner et al., 2006), outputting the five docked poses for each ligand. Pairs for each ligand were selected based on top score.

**Statistical Analysis.** All data are presented as the mean ± S.D. of three independent reactions or experiments. GraphPad Prism 8.0 software was used to conduct all data and significance analyses. Unless otherwise stated, significance was evaluated using repeated measures of one-way analysis of variance using Dunnett’s multiple comparisons test. The threshold for statistical significance was P < 0.05.

**Results**

**Cell-Based High Throughput Screening for GPR56 Activators.** GPR56/ADGRG1 activates G12/13 and the human homolog modestly activates G1q (Iguchi et al., 2008; Paavola et al., 2011; Ackerman et al., 2015; Stoveken et al., 2015; Yeung et al., 2020). Rho signaling through G12/13 can be monitored in HEK293T cells via a firefly luciferase reporter driven by the SRE promoter (Fig. 1A). We used this previously to conduct pilot screens of a small chemical library and identified a corticoid-like partial agonist and isoflavonoid antagonist for GPR56 (Stoveken et al., 2016, 2018). Engineered GPR56 receptors lacking the NTF were used to selectively screen for molecules that target the 7TM. GPR56 7TM with an intact TA exhibited maximal SRE-Luc activity that was several-fold higher than the GPR56 holoreceptor and was used to screen for inhibitory compounds (Stoveken et al., 2016), while a GPR56 7TM construct encoding a four-residue truncation of its TA (GPR56 ΔTA) exhibited low activity and was used to screen for agonists (Fig. 1, B–D). A synthetic peptide modeled from GPR114 that mimics the identical TA sequences of GPR56 and GPR114 was used as a positive control for the activator screen assay (GPR56/114-AP, Fig. 1C) (Wilde et al., 2016; Stoveken et al., 2018). This 19-mer peptide agonist (20 μM) provided strong activation of GPR56 ΔTA (Z = 0.65, Fig. 1D). As a positive control for the GPR56 7TM inhibitory screen, the ac-tin polymerization inhibitor Latrunculin B (1 μM) provided complete inhibition (Z = 0.51, Fig. 1D). A receptor-minus counter-screen assay was developed to vet GPR56 activating compounds obtained in the primary screen. Cells were transfected with the luciferase reporter only and treated with FBS or GPR56/114-AP (Fig. 1D). These SRE reporter-only cells were activated by FBS (Z = 0.57) but not by the GPR56/114-AP (Fig. 1E).

We expanded our previous pilot activator screen of 2,000 compounds to conduct a large screen of more than 200,000 compounds (Fig. 2) (Stoveken et al., 2018). It was not logistically possible to freshly transfect a sufficient amount of HEK293T cells to screen all compounds at once given our equipment, so we developed a cell transfection/cryopreservation regimen that ensured plate-to-plate reproducibility over an extended time. Corning HYPERFlasks were used to culture 2 x 10⁶ HEK293T cells for transfection en masse with the SRE-luciferase gene reporter and GPR56 ΔTA receptor. After transfection, cells were pooled and cryopreserved in assay-ready aliquots that were thawed and seeded in increments of eight 384-well plates for stimulation with robotically dispersed compounds (Fig. 2A). Small molecules were screened from three commercial libraries: a MB24K, a ChemDiv100K, and a DART90K. For the screens, luminescence signals were normalized to DMSO as the negative control and 20 μM GPR56/114-AP as positive control. A threshold of 35% activity was used to define hits. We established this threshold as it closely matches the high throughput screening standard of 3 S.D. above the negative control on plates with few hits, while not excessively excluding compounds from plates with many hits. From the screen we identified 1,327 primary hits as candidate GPR56 activators, 801 of which were from the MB24K and ChemDiv libraries and 526 that were from the DART90K library (Fig. 2, B and C). The overall hit rate of the screen was 0.64%.

A limited confirmatory check and counter-screen were conducted to eliminate false positives or pathway activators downstream of GPR56 in the serum response. Compounds were robotically cherry-picked via an STP Mosquito instrument and consolidated into 384-well plates in triplicate. HEK293T cells pretransfected with GPR56 ΔTA, and the SRE-Luc reporter or reporter alone were cultured atop the compounds before measurement of luciferase activities. We found that 796 of the 1,327 initial hits (516 from the ChemDiv library + 280 from the DART library) stimulated activity >35% of the positive control, thereby reconfirming the original activities (Fig. 3, A and B). Counter-screening found that 891 of the 1,327 initial hits (533 from the ChemDiv library + 358 from the DART library) elicited >35% of the serum response and were thus eliminated as off-target or pathway activators (Fig. 3, C and D). A lower hit rate was obtained using the Maybridge library screen with only eight candidate hits, of which two were eliminated as pathway activators (Supplemental Fig. 1). In total, 155 hits across all three libraries (96 from ChemDiv, 6 from Maybridge, and 53 from DART) survived confirmation and counter-screening, giving a final hit rate of 0.076% for the entire screen. To further hone the list of GPR56 agonist candidates, we tested compounds in concentration response assays between 2.8 and 100 μM to acquire initial measurements of efficacy and potency. Seventy three of the 155 candidate activators were eliminated after demonstrating peak efficacies or potencies substantially below that of GPR56/114-AP (Supplemental Fig. 2). Eight additional compounds were eliminated for eliciting concentration responses with poor hill slopes, which we attributed to compound cytotoxicity, instability, and/or insolubility. From this analysis 74 compounds with favorable concentration response profiles were purchased as fresh powders.
Structurally related analogs that each had efficacy equivalent to GPR56-AP (Supplemental Fig. 3D). The remaining 58 compounds had reduced efficacy of the top 12 compounds plus variants lacking the NTF used for screening. Constitutively-active GPR56 7TM has an intact tethered agonist (TA, red) and GPR56 ΔTA (green) has a compromised tethered agonist resulting from a four-residue truncation. (C) TA (yellow text) and stalk sequences (white text) of the GPR56 7TM constructs used for high throughput screening aligned with a synthetic peptidomimetic agonist that activates GPR56 or GPR114 (GPR56/114-AP, purple) and was used to calibrate the screening assays. (D) Calibration of HEK293T cell-based high throughput SRE-luciferase screening assays. The GPR56 7TM assay exhibits high activity that was blunted with Latrunculin B (LatB) and used to screen for inhibitors. The GPR56 ΔTA assay exhibits low activity that could be surmounted with GPR56/114-AP and was used to screen for activators. (E) Calibration of the HEK293T cell target-minus high throughput counter assay, which uses cells that only express the SRE-luciferase reporter. FBS, but not GPR56/114-AP, activated the SRE-luciferase reporter in the absence of GPR56. Z-scores are of assay quality, with 0.5 being the threshold suitable for high throughput screening. Error bars are the mean ± SD of three biologic replicates.

Orthogonal Assay Validation of GPR56 Activators. The activities of GPR56 agonists were evaluated using an orthogonal biochemical GPCR reconstitution assay that directly measures GPR56-stimulated G protein activation. Agonists were preincubated with membrane homogenates prepared from cells overproducing GPR56 7TM and reconstituted with purified heterotrimeric G proteins (Gz13 and Gβ5(Gαq). Kinetic reactions were initiated and the accumulation of Gz13-bound [35S]-GTPγS was measured. Sixteen of the 74 compounds exhibited efficacy ≥75% of GPR56-AP (Supplemental Fig. 3, A–C). Four of these compounds, compounds 32, 33, 36, and 41, comprise a cluster of structurally related analogs that each had efficacy exceeding GPR56-AP (Supplemental Fig. 3D). The remaining 58 compounds had reduced efficacies or apparent solubility issues. Notably, none of the compounds discovered in the screen were analogs of the previously identified GPR56 partial agonist 3-z-DOG, as steroid-like compounds were not present in the screened libraries (Stoveken et al., 2018). We compared the efficacies of the top 12 compounds plus two compounds from the structural cluster, compounds 36 and 32, to the activity of constitutively active GPR56 7TM that contains an intact TA (Fig. 4, A–C). Compound 36 was the only one of the 14 compounds capable of activating GPR56 ΔTA with efficacy equivalent to GPR56 7TM. Compound 36, or propan-2-yl-4-(2-bromophenyl)-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate, is a hexahydroquinoline (HHQ) derivative that contains a central 1,4-dihydropyridine ring (1,4-DHP). Another interesting compound identified was 2-(furan-2-yl)-1-[(4-phenylphenyl)carbonyl]pyrrolidine, or compound 4. Compound 4 has a simple structure consisting of two benzene rings connected to a 2-(furan-2-yl) pyrrolidine group by a carbonyl group. We compare compound 36 and compound 4 in a concentration response format for activation of GPR56 ΔTA (Fig. 4D). Compound 36 had an EC50 of 2.95 ± 0.41 μM and an efficacy matching that of GPR56 7TM. This is more than 10-fold more potent than GPR56-AP [EC50 = 35 μM (Stoveken et al., 2015)] and was ~40% more potent than the previously identified partial agonist 3-z-DOG [EC50 = 4.8 μM (Stoveken et al., 2018)]. By contrast, compound 4 has a maximum efficacy ~50% of GPR56 7TM. The potency of compound 4 was too low to accurately determine an EC50.

Compound Activation of the GPR56 Holoreceptor. Following validation of the 14 activators in the GPR56 ΔTA/G13 reconstitution assay, we evaluated whether the compounds could activate the GPR56 holoreceptor. Measuring small molecule agonist stimulation of the GPR56 holoreceptor is not straightforward because GPR56 is efficiently self-cleaved and incidental dissociation or shedding of its NTF results in substantial TA-dependent background G13 signaling that confounds measurement of compound agonist activities (Stoveken et al., 2015, 2018; Barros-Alvarez et al., 2022). We used three full-length GPR56 mutants that have impaired autoproteolytic activity and/or an impaired TA to minimize TA-dependent background signaling. GPR56 H381S
is a point mutant of the GPCR proteolysis site that abrogates autoproteolysis; GPR56 F385A/M389A contains two point mutations within the TA that are vital for its ability to engage the 7TM orthosteric site; and GPR56 L388A/M389A contains a different set of TA point mutations that are more distal to the cleavage site and retain full autoproteolytic activity (Fig. 5, A and B) (Barros-Alvarez et al., 2022; Perry-Hauser et al., 2022; Ping et al., 2022; Qu et al., 2022; Xiao et al., 2022).

GPR56 holoreceptor membrane homogenates were treated with urea to dissociate the NTFs from membrane intercalated CTFs/7TMs. Both GPR56 H381S and F385A/M389A mutants were predominantly uncleaved, whereas urea treatment markedly reduced the amount of NTF in the membrane fraction of wild-type GPR56 and GPR56 L388A/M389A (Fig. 5B). However, only the wild-type GPR56 holoreceptor exhibited appreciable urea-dependent (i.e., TA-dependent) activation (Fig. 5B). GPR56 H381S, GPR56 F385A/M389A, and GPR56 L388A/M389A had low basal signaling that were not enhanced by urea treatment. For the GPR56 L388A/M389A double mutant, this demonstrates that TA impairment fully blocks the ability of this efficiently cleaved receptor to activate G proteins (Perry-Hauser et al., 2022).

The GPR56 H381S mutant was used to assess whether the 14 GPR56 agonists could activate the holoreceptor. GPR56 H381S membrane homogenates were preincubated with each compound (20 μM) prior to measurement of G13 GTP binding. Compounds 4, 29, 32, 36, 37, 59, and 73 stimulated GPR56 H381S significantly above basal signaling, with compounds 4 and 36 being the most effective agonists that exceeded greater than fourfold signaling above basal activity (Fig. 5D). GPR56-AP was incapable of activating the GPR56 H381S holoreceptor, as reported, with speculation that the NTF hinders its access to the orthosteric site (Stoveken et al., 2018). Compounds 4 and 36 were tested in GPR56 H381S concentration response assays. Both compounds substantially increased receptor-mediated G protein activation (Fig. 5E). Compound 36 had an EC_{50} of 6.69 ± 0.56 μM, while the right shifted curve of compound 4 did not reach maximal efficacy and could not provide an accurate estimation of EC_{50}. Interestingly, compound 36 provided near full activation of the GPR56 F385A/M389A and GPR56 L388A/M389A receptors, but compound 4 had negligible activation (Fig. 5, F and G). We do not have a full explanation for this but speculate that both double mutants possess some population of freed CTF receptor.

**Fig. 2.** Primary screen for GPR56 small molecule activators. (A) Workflow of high throughput screening procedure. HEK293T cells were transfected en masse with GPR56 ΔTA and the SRE-Luciferase reporter 24h prior to being harvested, pooled, and cryopreserved in assay-ready aliquots. thawed cells were dispensed into 384-well plates and small molecules were pin-tooled into the wells at 10 μM for 18 hours prior to measurement of SRE-luciferase activity. (B) and (C) Primary results of GPR56 activator screens using the MayBridge and ChemDiv or DART90K small molecule libraries. ~123,000 compounds were screened from the MayBridge (left) and ChemDiv (right) libraries, and 82,000 compounds were screened from the DART90K library. Each dot represents the luciferase activity of a single compound, with luminescence normalized to 20 μM GPR56/114-AP (positive control, red). DMSO (vehicle) was the negative control and established as 0% activity (blue). Compounds that elicited activity ≥ 35% of GPR56/114-AP activity were considered primary hits (green).
that may have the defunct TA embedded within the orthosteric site. It is possible compound 4 is not potent enough to compete with the mutated tethered agonist and activate the receptor, whereas compound 36 is sufficient. GPR56 H381S is strongly cleavage deficient with no or little population of receptor engaged by its TA, which may account for its ability to be activated by compound 4.

**GPCR Selectivity of Compounds 4 and 36.** Compounds 4 and 36 were tested for the abilities to modulate a small panel of adhesion GPCRs and two class A GPCRs. ADGRG5/GPR114, ADGRG3/GPR97, and ADGRG1/GPR56 are from the same adhesion GPCR subfamily. GPR114 and GPR56 have identical tethered agonists and can be activated by 3-α-DOG and the same TA-peptidomimetics (Stoveken et al., 2015, 2018; Wilde et al., 2016). ADGRL3/Latrophilin-3 and ADGRF1/GPR110 are representatives of two distinct adhesion GPCR subfamilies. Compounds 4 and 36 (20 μM) were tested for their abilities to activate TA-impaired 7TM versions of each adhesion GPCR and compared with the activity of intact TA 7TM receptors. Compounds 4 and 36 were investigated to identify critical functional groups and to identify derivatives with improved potency and efficacy. A first set of 22 analogs were obtained that had functional group deletions or functional groups with altered positions. Supplemental Fig. 5 shows the structures of all compound 36 analogs that were evaluated for the ability to activate GPR56 DTA via GPCR reconstitution assay (Fig. 7). All functional groups were essential for full compound 36 activity, but two regions of the structure seemed suitable for optimization: the bromine and isopropyl groups (Fig. 7A). Analog 36.4, which lacks a bromine on the bromobenzene group and contains a tert-butyl group instead of an isopropyl group, activated GPR56 at an efficacy approximately 60% that of the original compound. Analog 36.18 is identical to compound 36 but has a truncation of the isopropyl to a methyl group, which resulted in almost complete abrogation of GPR56 activity. These results suggested that both an electrophilic ortho group on the benzene ring and a hydrophobic group adjacent to the ester are necessary for full activity and might be positions to place alternative functional groups to optimize compound potency. This may explain how compound 32, which contains large differences in the positioning of its rings, was still capable of activating GPR56 to the level of GPR56-AP (Supplementary Fig. 3). Compound 32 has an additional benzene ring that may fill a hydrophobic pocket normally occupied by the hydrophobic isopropyl group of compound 36, giving it some capacity to activate GPR56. Additionally, we noticed the positioning of the bromine on compound 36 could not be altered without abrogating activity, as analog 36.15 has a shift of the bromine to the meta position and could only

**Structure-Activity Relationship of Compound 36 Analogs.** Commercially available analogs of compound 36 were investigated to identify critical functional groups and to identify derivatives with improved potency and efficacy. A first set of 22 analogs were obtained that had functional group deletions or functional groups with altered positions. Supplemental Fig. 5 shows the structures of all compound 36 analogs that were evaluated for the ability to activate GPR56 DTA via GPCR reconstitution assay (Fig. 7). All functional groups were essential for full compound 36 activity, but two regions of the structure seemed suitable for optimization: the bromine and isopropyl groups (Fig. 7A). Analog 36.4, which lacks a bromine on the bromobenzene group and contains a tert-butyl group instead of an isopropyl group, activated GPR56 at an efficacy approximately 60% that of the original compound. Analog 36.18 is identical to compound 36 but has a truncation of the isopropyl to a methyl group, which resulted in almost complete abrogation of GPR56 activity. These results suggested that both an electrophilic ortho group on the benzene ring and a hydrophobic group adjacent to the ester are necessary for full activity and might be positions to place alternative functional groups to optimize compound potency. This may explain how compound 32, which contains large differences in the positioning of its rings, was still capable of activating GPR56 to the level of GPR56-AP (Supplementary Fig. 3). Compound 32 has an additional benzene ring that may fill a hydrophobic pocket normally occupied by the hydrophobic isopropyl group of compound 36, giving it some capacity to activate GPR56. Additionally, we noticed the positioning of the bromine on compound 36 could not be altered without abrogating activity, as analog 36.15 has a shift of the bromine to the meta position and could only
activate GPR56 with ~35% efficacy. Given the importance of the electrophilic bromine group and the aliphatic isopropyl group, we designated them as the X and R functional groups, respectively.

A second set of analogs that consisted of modifications to the X and R functional groups were tested. Most of these analogs retained some ability to activate GPR56 but with variable efficacies (Fig. 7B). We again observed some malleability of the X group, as replacing the bromine with various functional groups caused reductions in efficacy but not complete loss of activity. These substitutions included chlorine (36.30), iodine (36.37), CH₃ (36.32), CH₂O (36.31), and CF₃ (36.33, 36.35, 36.40, and 36.42 through 36.46). The degree of hydrophobicity of the R group was also observed to impact activity. Analogs 36.28, 36.35, and 36.36 had truncated R groups and exhibited reduced activity. A one-carbon extension of the ethyl group of analog 36.28 to the propyl group of analog 36.30 strongly accentuated activity to ~80% efficacy. These data suggest that larger and bulkier R groups correlate with higher activity. This was supported by analogs 36.25 and 36.40, which have large cyclopentane rings and activate GPR56 to near full efficacy. Compounds 36.32 and 36.41 also contain this cyclopentane ring, but alterations of its benzene ring reduced efficacy. A lack of an electrophilic group in analog 36.32 slightly reduced efficacy, while addition of a para chlorine atom completely abrogated activity. In sum, compounds with large aliphatic R groups and/or additional functional groups substituted for the X group activated GPR56 most effectively.

The most efficacious compound 36 analogs from both rounds of SAR were tested in concentration response assays (Fig. 7C). Included were the three compound 36 analogs identified in the primary screens, compounds 32, 33, and 41. Compound 33 has a CH₃O X group and a cyclopentane ring as its R group, and compound 41 has a CH₃ X group with an unchanged R group. Compound 32 is much more different in structure, with a central seven-membered ring replacing the 1,4-DHP ring of compound 36 and an additional benzene ring adjacent to it. All three compounds activated GPR56 with efficacies greater than GPR56-AP peptide (Supplemental Fig. 3). In concentration response assays, three analogs were found to have modestly increased potencies over compound 36 (EC₅₀ of 2.95 ± 0.41 µM). Analogs 36.40, 36.25, and 36.32 activated GPR56 with EC₅₀ of 1.75 ± 0.13 µM, 1.89 ± 0.22 µM, and 2.15 ± 0.033 µM, respectively. All three analogs contained the aforementioned cyclopentyl group in place of the isopropyl R group but had different X groups in place of the bromine (Fig. 7D). Analogs 36.40 had a CF₃ group, analog 36.25 had a chlorine group, and analog 36.32 had a CH₃ group in the X position.
compound 36 that approached GPR56 7TM full agonism. Overall, the combination of the cyclopentyl R group and trifluoromethyl X group of 36.40 contributed to its 40% increased potency over compound 36. Future efforts of compound 36.40 derivatization may be taken to enhance its activity toward GPR56.

In Silico Docking of Compound 36 and 36.40 in the GPR56 Orthosteric Site. Compound 36 and its higher potency analog 36.40 have a chiral center in which the bromo- or trifluoromethyl-benzene ring may rotate. The syntheses of these compounds are most likely racemic mixtures. To model the binding sites of the compound 36 pharmacophore, we conducted in silico docking of the R and L enantiomers to active-state GPR56 7TM (PDB: 7SF8) (Barros-Alvarez et al., 2022) with its TA removed (i.e., residues T383 through V395). Both compound 36 enantiomers docked within the orthosteric site at a region normally occupied by the intact TA and was proximal to several residues that are critical for TA interactions, notably F2.64, W45.51, W6.53, and F7.42 (Fig. 8, A and B) (Barros-Alvarez et al., 2022). The bromobenzene ring of both enantiomers was locked in a fixed position proximal to W45.51 of ECL2, and the poses of the other rings were nearly mirror images along a vertical axis about the central pyridine ring. The isopropyl group of the L enantiomer of compound 36 was facing TM5, close to N5.39, whereas the R enantiomer was docked such that the isopropyl group occupied a larger hydrophobic pocket next to F2.64. In both poses the pyridine ring was positioned deep within the orthosteric site in a hydrophobic region proximal to both W6.53 and P7.42. When analog 36.40 was docked, the CF3 group of the R enantiomer...
appeared to fit readily into the pocket adjacent to ECL2 and the cyclopentyl group occupied the additional available hydrophobic space proximal to F2.64 (Fig. 8C). In active-state GPR56 7TM, these pockets are occupied by residues L388 and F385 of the TA, respectively (Fig. 8D). When the compound 36.40 R enantiomer pose was overlaid with the TA, the cyclopentyl ring overlapped almost entirely with the aromatic ring of TA residue F385 (Fig. 8E).

These models suggest that the R enantiomer of the compound 36 pharmacophore may be the active compound, thereby explaining why substituting the isopropyl group of compound 36 with larger hydrophobic groups improved its activity. Overall, these models predict that compound 36 R enantiomer analogs interact with the critical residues that also interact with the tethered agonist. To assess the importance of these TA-interacting residues for compound 36 activation, we tested compound 36 or compound 4 with GPR56 7TM point mutants F2.64A, W45.51A, W6.53A, or F7.42A for G protein activation (Barros-Alvarez et al., 2022; Gupta et al., 2022). All four mutations abrogated activity when compared with TA-activated GPR56 7TM (Fig. 8D). Compounds 36 and 4 had minimal abilities to activate the four-point mutant GPR56 ΔTA receptors, with compound 36 providing modest activation of the F2.64A mutant and compound 4 appearing to weakly inhibit the W2.54A and F7.24A mutants.

We next attempted to analyze binding pocket residues from the compound 36 R enantiomer docking simulation that may participate in compound binding and not TA binding (Supplemental Fig. 6). The GPR56 7TM substitution mutants L.336F, N.39V, and S.35H had dramatically reduced abilities to respond to the TA, with only L.336F retaining a significant TA-mediated response. Interestingly, compound 36.40 did not stimulate GPR56 ΔTA L.336F. The bulky F residue was chosen for mutagenesis because it was predicted to sterically occlude compound binding but not TA binding, which coincides with the observed results. GPR56 S.35H exhibited the opposite pattern and was partially responsive to compound 36.40 but not its TA. This mutant was chosen to potentially interfere with compound hydrogen bonding to GPR56, but we could not support this conclusion from the results. In sum, the docking and mutagenesis results suggest that there is substantial overlap of the tethered agonist and R-enantiomer compound binding pockets.

Discussion

Knowledge of adhesion GPCR function and therapeutic potential is emerging rapidly. The recent solution of active-state structures of seven adhesion GPCRs including GPR56 (Barros-Alvarez et al., 2022; Ping et al., 2022; Qu et al., 2022; Xiao et al., 2022) affirmed a common mechanism whereby the tethered agonist adopts a partial α-helical hook-like conformation that binds its orthosteric site within the 7TM core beneath ECL2. The P3, P6, and P7 residues of the TA are typically
phenylalanine, leucine, and methionine, respectively, and form critical hydrophobic interactions with residues of the TM spans and ECL2. Despite this new understanding, most AGPCRs are orphans with few molecular tools to study them. Synthetic peptides that mimic the tethered agonists have some utility but often suffer from poor solubility, low potency, and receptor inaccessibility (Liebscher et al., 2014; Demberg et al., 2015, 2017; Stoveken et al., 2015; Wilde et al., 2016; Brown et al., 2017). This calls for a need for small molecule AGPCR agonists and antagonists with improved characteristics. Here we expanded on our previous small molecule pilot screens to identify a potent full agonist that has apparent exclusive specificity for GPR56. The compound has improved characteristics and may serve as a future lead in the development of a first-in-class AGPCR therapeutic.

The activator screen for GPR56 agonists comprised more than 200,000 compounds and was 100-fold larger than our previous pilot screen that revealed 3-α-DOG as a partial agonist for GPR56 (Stoveken et al., 2018). After secondary vetting and counter assay testing in cell-based assays, 74 hits were identified, which was then narrowed to 16 hits following orthogonal assay testing. Within these final hits, compounds 32, 33, 36, and 41 are close structural analogs. The remaining 12 hits are structurally distinct but shared common moieties, including a sulfonamide linker (compounds 7, 12, 29, and 37), a carboxamide linker (compounds 67, 51, 73, 56, and 74), or a terminal 2,5-dimethoxybenzene ring (compounds 7, 12, and 48). These common structural features may indicate similar binding modalities for GPR56. Additionally, the sulfonamide compounds may serve as therapeutically viable leads, given that sulfonamide analogs are noteworthy for their ability to improve therapeutic properties of drugs (Zhao et al., 2019).

The two most promising compounds identified were propan-2-yl-4-(2-bromophenyl)-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate, or compound 36, and 2-(furan-2-yl)-1-[(4-phenylphenyl)carbonyl]pyrrolidine, or compound 4. Compound 36 was the most efficacious of its structural cluster and is a 1,4-DHP derivative of hexahydroquinoline. 1,4-DHP compounds, including those that are HHQ derivatives,
were identified as L-type calcium channel blockers and marketed as drugs (e.g., nifedipine) to treat hypertension (Murakami et al., 1972; Epstein et al., 2007; Bladen et al., 2014; Liu et al., 2015). It is possible that compound 36 and its analogs could have off-target hypotensive effects, as previous SAR work showed that closely-related HHQ derivatives inhibited Cav1.1 channel activation (Takahashi et al., 2008). However, compounds with larger hydrophobic R groups were less efficacious at inhibiting Cav1.1. The inverse is true for GPR56 activation; compounds with larger R groups were most effective. Consequently, we expect that the 36.40 derivative with its bulky cyclopentyl R group would have limited activity for L-type calcium channels. We also predict that L-type calcium channel blockers would be poor GPR56 agonists. Many 1,4-DHP-based calcium channel blockers possess functional groups at the third and fourth carbons of the aryl group. We observed in our SAR analysis that electron withdrawing groups at these positions render the compounds incapable of activating GPR56. Included in our SAR analysis were compounds with symmetric 1,4-DHP rings that closely match structures of calcium channel blockers: compounds 36.11, 36.19, 36.20, 36.21, and 36.23. Each of these compounds had little effect on GPR56.

Compounds 36 and 36.40 are full agonists that stimulated GPR56 7TM to the maximal efficacy of GPR56 7TM that has an intact, endogenous tethered agonist. Compound 36 did not synergize with GPR56-AP, as GPR56 ΔTA coactivation by peptide and compound 36 showed no enhanced potency or efficacy (Supplemental Fig. 4B). In fact, GPR56-AP diminished the maximal efficacy imparted by compound 36, suggesting that the TA peptidomimetic is a partial agonist that may compete with compound 36. Compound 4 was less potent than compound 36 and only activated GPR56 7TM to ~50% maximal efficacy of GPR56 7TM. Compounds 4 and 36 were the only hits that activated the cleavage-deficient GPR56 holoreceptor. GPR56-AP and the partial agonist 3-α-DOG are incapable of activating the GPR56 holoreceptor in vitro (Stoveken et al., 2015, 2018). It is possible that these agonists do not activate GPR56 due to NTF-mediated occlusion of the 7TM orthosteric site. However, low-resolution structural models of AGPCR holoreceptors demonstrated NTF flexibility in relation to the 7TM. A full understanding of the basis of agonist entry to AGPCR orthosteric sites awaits further investigation (Barros-Alvarez et al., 2022).

Interestingly, compound 36 but not compound 4 activated GPR56 F385A/M389A and GPR56 L388A/M389A double TA mutant holoreceptors, the former being partially cleavage-deficient and the latter cleaved efficiently. We do not fully understand the basis of why compound 4 activated the severely cleavage-defective H381S holoreceptor but not the compromised tethered agonist holoreceptors. We speculate this may be due to a combination of factors including differential access to the orthosteric site and the prospect that the TA-impaired mutants may experience some spontaneous NTF shedding followed by mutant tethered agonist nonproductive

Fig. 8. In silico docking prediction of compound 36 and 36.40 enantiomers. (A) and (B) Compound 36 (red) docked within the orthosteric site of GPR56 7TM (teal, PDB: 7SF8) with its tethered agonist removed. The L (A) or R (B) enantiomers docked in a similar position within the orthosteric site. Prominent residues that interact with the GPR56 TA are denoted (dark green). (C) Compound 36.40 (R enantiomer) was docked into the same pocket in the same manner as compound 36. (D) The structure of the tethered agonist (blue) engaged within the orthosteric pocket. The three residues vital for TA engagement are highlighted. (E) The structure of the tethered agonist overlaid with docked compound 36.40. (F) Activation of GPR56 7TM with various point mutations that abrogate TA binding in the GPCR reconstitution assay. Either DMSO or 20 μM of compound 36 or compound 4 was preincubated with membrane homogenates prior to the start of assay. Data points are the mean ± S.D. of three independent reactions. Statistical significance was determined by repeated measures of two-way analysis of variance. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
engagement of the orthosteric site but in a manner that blocks compound 4 binding.

An in silico approach predicted that compounds 36 and 36.40 dock in the GPR56 orthosteric site through a binding mechanism that has parallels to tethered agonist binding. The positioning of the 2-bromophenyl or 2-trifluoromethylphenyl groups of the compounds near W45.51 may explain why addition of functional groups to the third or fourth carbons of the aryl group dramatically decrease compound activity in our SAR analysis. Electron withdrawing groups like bromine or chlorine in these positions would be positioned in unfavorable proximity to W45.51. The R and L enantiomers of compounds 36 and 36.40 docked as mirror images within the orthosteric site. Our analysis suggests that the R enantiomer is the active species, as the cyclopropyl R group of compound 36.40 closely overlaps with the position of TA residue F385 and may likewise engage TM2 residue F2.64. This provides a plausible explanation of why substituting the isopropyl R group of compound 36 with larger hydrophobic groups increased activity. Bulkiier hydrophobic groups at this position may allow the compound to interact more favorably with F2.64. Notably, we included in our SAR analysis an analog that contained a benzene ring as its R group (analog 36.45), which could potentially form favorable pi-pi interactions with F2.64. This compound exhibited only ~70% efficacy compared with the base compound. The reduction in efficacy may be explained by the presence of a one-carbon extension prior to the benzene compared with analog 36.40. With these models in mind, we plan to conduct future derivatization of compound 36.40 by targeting its hydrophobic R group. We anticipate variations of a benzene ring will increase activity toward both GPR56 ΔTA and the holoreceptor. Substitutions of the electron withdrawing X group are unlikely to provide enhanced activity, as steric hindrance within the pocket beneath ECL2 is a concern.

Interestingly, compound 36 was selective for GPR56 and did not activate other AGPCRs, even within the same G subfamily. This includes GPR114, which contains an identical TA to GPR56 and consequently a similar orthosteric binding site (Ping et al., 2022). However similar, GPR114 has slightly altered positions for F2.64, F7.42, W45.52, and W6.53 within its orthosteric site, enough so that it may not bind productively to compound 36 (Barros-Alvarez et al., 2022; Ping et al., 2022). In sum, compound 36 and its analog 36.40 are the most potent activators (Barros-Alvarez et al., 2022). Notably, we included in our SAR analysis enantiomers of compounds 36 and 36.40. This compound exhibited only ~70% efficacy compared with the base compound. The reduction in efficacy may be explained by the presence of a one-carbon extension prior to the benzene compared with analog 36.40. With these models in mind, we plan to conduct future derivatization of compound 36.40 by targeting its hydrophobic R group. We anticipate variations of a benzene ring will increase activity toward both GPR56 ΔTA and the holoreceptor. Substitutions of the electron withdrawing X group are unlikely to provide enhanced activity, as steric hindrance within the pocket beneath ECL2 is a concern.

Data Availability

The data generated for this study are available within this article and its supplementary files.

Authorship Contributions

Participated in research design: Vizurraga, Robertson, Tall.
Conducted experiments: Vizurraga, Robertson.

Cloning and molecular biology: Vizurraga, Yu, Tall.
Performed data analysis: Vizurraga, Robertson, Tall.
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Hexahydropyrindine Derivatives Activate ADGRG1/GPR56


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