Dihydromunduletone Is a Small-Molecule Selective Adhesion G Protein–Coupled Receptor Antagonist[S]

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ABSTRACT

Adhesion G protein–coupled receptors (aGPCRs) have emerging roles in development and tissue maintenance and is the most prevalent GPCR subclass mutated in human cancers, but to date, no drugs have been developed to target them in any disease. aGPCR extracellular domains contain a conserved subdomain that mediates self-cleavage proximal to the start of the 7-transmembrane domain (7TM). The two receptor protomers, extracellular domain and amino terminal fragment (NTF), and the 7TM or C-terminal fragment remain noncovalently bound at the plasma membrane in a low-activity state. We recently demonstrated that NTF dissociation liberates the 7TM N-terminal stalk, which acts as a tethered-peptide agonist permitting receptor-dependent heterotrimeric G protein activation. In many cases, natural aGPCR ligands are extracellular matrix proteins that dissociate the NTF to reveal the tethered agonist. Given the perceived difficulty in modifying extracellular matrix proteins to create aGPCR probes, we developed a serum response element (SRE)-luciferase–based screening approach to identify GPR56/ADGRG1 small-molecule inhibitors. A 2000-compound library comprising known drugs and natural products was screened for GPR56-dependent SRE activation inhibitors that did not inhibit constitutively active Gs13-dependent SRE activation. Dihydromunduletone (DHM), a rotenoid derivative, was validated using cell-free aGPCR/heterotrimeric G protein guanosine 5′-3-O-(thio)triphosphate binding reconstitution assays. DHM inhibited GPR56 and GPR114/ADGRG5, which have similar tethered agonists, but not the aGPCR GPR110/ADGRF1, M3 muscarinic acetylcholine, or β2 adrenergic GPCRs. DHM inhibited tethered peptide agonist-stimulated and synthetic peptide agonist-stimulated GPR56 but did not inhibit basal activity, demonstrating that it antagonizes the peptide agonist. DHM is a novel aGPCR antagonist and potentially useful chemical probe that may be developed as a future aGPCR therapeutic.

Introduction

Adhesion G protein–coupled receptors (aGPCRs) are a 33-member subclass of family B G protein–coupled receptors (GPCRs) that have critical roles in tissue specification and replenishment and human cancers (Langenhan et al., 2013; Hamann et al., 2015). It was recently discovered that the extracellular peptide stalks emanating from the first transmembrane-spanning helix of multiple adhesion GPCRs are tethered-peptide agonists that activate aGPCR-mediated heterotrimeric G protein signaling (Liebscher et al., 2014; Demberg et al., 2015; Stoveken et al., 2015; Wilde et al., 2016). Tethered-agonist regulation is intimately linked to the ability of aGPCRs to execute a precise autocatalytic, self-cleavage event in which the P1′ residue becomes the N terminus of the ~17–26 amino acid tethered-agonist stalks (Lin et al., 2004; Arac et al., 2012; Liebscher et al., 2014; Stoveken et al., 2015). Self-cleavage is thought to be constitutive, and the resultant receptor protomers or fragments remain noncovalently bound while in residence at the plasma membrane. In this state, the N terminus of the postcleaved tethered-agonist stalk binds firmly within a hydrophobic β-strand network that would preclude it from engaging the carboxy-terminal fragment (CTF)/seven-transmembrane domain (7TM) orthosteric site.

A current hypothesis which we favor that describes the dynamism of receptor activation consists of two components: 1) anchored protein ligands bind aGPCR N-terminal fragment (NTF) binding determinants, and 2) the action of shear force created by cell movement serves to dissociate the NTF from the CTF to release or decrypt the tethered agonist (Karpus et al., 2013; Langenhan et al., 2013; Scholz et al., 2015; Stoveken et al., 2015). We reconstituted aGPCRs, GPR56 (ADGRG1) and GPR110 (ADGRF1), with purified G protein heterotrimerics and provided a biochemical demonstration that experimentally

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ABBREVIATIONS: aGPCR, adhesion G protein–coupled receptor; CTF, carboxy-terminal fragment; DHM, dihydromunduletone; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethylsulfoxide; ETC, electron transport chain; FBS, fetal bovine serum; GPCR, G protein–coupled receptor; GTPγS, 5′-3-O-(thio)triphosphate; HA, hemagglutinin; HEK293, human embryonic kidney 293; NTF, amino terminal fragment; PAR, protease activated receptors; [35S]GTPγS, 5′-O-3-[35S]thiotriphosphate; SRE, serum response element; 7TM, seven-transmembrane domain.
induced NTF dissociation dramatically enhanced aGPCR-mediated G protein activation (Stoveken et al., 2015). The known protein ligands of aGPCRs are fibrillar extracellular matrix proteins or, in some instances, proteins presented by neighboring cells (Hamann et al., 1996; Sugita et al., 1999; Stacey et al., 2003; Xu et al., 2006; Bolliger et al., 2011; Chiang et al., 2011; Luo et al., 2011; Silva et al., 2011; Boucard et al., 2012; O’Sullivan et al., 2012; Paavola and Hall, 2012; Paavola et al., 2014; Petersen et al., 2015; Scholz et al., 2015). Based on the proposed two-component mode of ligand-mediated aGPCR activation, we anticipate that natural NTF ligands or ligand-based NTF-binding mimetics will be of limited, stand-alone pharmacological use to manipulate receptor activities. Indeed, application of dilute, soluble extracellular matrix protein ligands to aGPCR cell culture–based signaling assays most likely does not recapitulate authentic aGPCR pharmacology. Hence, there is a critical need to develop small-molecule modulators that can bypass the two-component ligand-mediated regulation process and directly modulate receptor activities. A number of adhesion GPCR small-molecule screening efforts are ongoing, but to our knowledge, only a few candidate small-molecule activators were proposed to regulate GPR97 (Gupte et al., 2012; Southern et al., 2013).

We conducted a chemical-screening effort to identify small-molecule inhibitors of GPR56, an attractive therapeutic target due to its proposed roles in neurogenesis, neurmaintenance, and cancer progression (Piao et al., 2004; Shashidhar et al., 2005; Xu et al., 2006, 2010; Yang et al., 2011; Saito et al., 2013; Hamann et al., 2015). GPR56 signals through G13 and multiple laboratories showed that GPR56 robustly activates serum response element (SRE) or serum response factor luciferase gene reporters (Iguchi et al., 2008; Kim et al., 2010; Wu et al., 2013; Stoveken et al., 2015; Kishore et al., 2016). We adapted our construct that produces the full tethered-agonist-activated GPR56 CTF for use as an SRE luciferase human embryonic kidney 293 (HEK293) cell high-throughput inhibitor screening platform (Stoveken et al., 2015). A counterscreen was developed in which each library compound was tested for inhibition of constitutively active Ga13-Q226L–activated SRE luciferase. Since G13 activation is the first step downstream of GPR56, all compound hits that inhibited the GPR56 CTF, but not Ga13-Q226L, are likely to act at the receptor level and not at points downstream in the pathway. Sixty-six compounds from the ~2000-compound Spectrum Collection chemical library inhibited GPR56-CTF–dependent luciferase activity, 63 of which also inhibited Ga13-Q226L and were therefore eliminated. The identified compounds were dihydromunduletone (DHM), a derivative of the natural-product mundulone, and two structurally related natural products that we have temporarily termed “H.M.S.1” and “H.M.S.2,” pending chemical syntheses (Burrows et al., 1959; Ollis and Sutherland, 1961).

Here, we present evidence that DHM is a bona fide selective antagonist for a subset of aGPCRs. This is the first identification of a small-molecule aGPCR inhibitor that has potential to uncover novel aGPCR biology and represents a first step in the development of a therapeutic strategy targeting aGPCRs.

**Materials and Methods**

**Reagents and Antibodies.** The GPR56 C-terminal antibody was a gift from Dr. Randy Hall (Emory University, Atlanta, GA) (Paavola et al., 2011). Dihydromunduletone, isorotenone, mundulone, and deguelin were from the Spectrum Collection chemical library and were purchased individually as powders from MicroSource Discovery Systems, Inc. (Gaylordsville, CT). Rotenone and polyclonal hemagglutinin (HA) antibody were purchased from Sigma-Aldrich (St. Louis, MO). Latrunculin B was from Calbiochem (San Diego, CA). Quant-IT PicoGreen double stranded DNA reagent was from Invitrogen (Carlsbad, CA). Streptavidin Sepharose High Performance was from GE Healthcare (Chicago, IL). Sulfo-NHS-Biotin was from Thermo Scientific (Waltham, MA). 5′-O-(3′-P,S-thio)triphostate (dS-TP) and the phLuc-N1 plasmid were from PerkinElmer (Waltham, MA). The pGL4.33 (luc2/SRE/Hygro) plasmid was purchased from Promega (Madison, WI).

**High-Throughput Screen.** HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS). Cells were transiently transfected with GPR56 7TM (Met-Thr383 to Ile693) pcDNA3.1 or GNA13Q226L pcDNA3.1 (cDNA.org) and the SRE-luciferase reporter using a polyethyleneimine transfection method (Wang et al., 2010; Oner et al., 2013; Stoveken et al., 2015). Five hours after transfection, cells were trypsinized, counted, and seeded at 15,000 cells per well in 384-well plates in 20 μl of phenol red–free DMEM buffer with 25 mM Hepes, pH 7.4, and incubated overnight with ~3–5 μM compounds in dimethylsulfoxide (DMSO); Spectrum Collection, at the URMC High Throughput Screening Core (Microsource Discovery Systems, Inc, Gaylordsville, CT). The next morning, 20 μl of StealthyLuciferase reagent (PerkinElmer) was robotically dispensed into each well and incubated for 15 minutes at room temperature. Luminescence was read on a PerkinElmer Envision Plate Reader.

**Directed Dual SRE-Luciferase Assay.** HEK293T cells maintained in DMEM +10% (v/v) FBS were transiently transfected in 24-well format using the polyethyleneimine reagent with 200 ng of GPR56 7TM (encodes Met-Thr383 to Ile693), 25 ng of GPR56 A386M 7TM (encodes Ala386Met to Ile687), or 200 ng of GNA13Q226L pcDNA3.1 plasmids; 100 ng of the SRE-luciferase reporter, and 1 ng of phFLuc (Stoveken et al., 2015). Total DNA levels were balanced with empty pcDNA3.1. Cells were serum starved for 10 hours the following day; DHM or synthetic-peptide agonist was added to the cell medium at 2 and 4 hours, respectively, during the serum-starving phase. Cells were harvested in culture medium by trituration, washed in Tyrode’s solution (137 mM NaCl, 2.7 mM KC1, 1 mM MgCl2, 1.8 mM CaCl2, 0.2 mM Na2HPO4, 12 mM NaHCO3, and 5.5 mM D-glucose), and lysed in firefly luciferase reagent (Nanolight Technologies, Inc., Pinetop, AZ). Firefly luminescence was quenched using Renilla luciferase buffer containing 3 μM coelenterazine H (Dyer et al., 2000). Luminescence was read using a TriStar* plate reader (Berthold, Wildbad, Germany). All firefly luciferase data were normalized to the Renilla luciferase signal and expressed as fold increase over the signal obtained from cells transfected with SRE-luciferase only.

**Insect Cell Culture, Baculovirus Generation, and Adhesion GPCR Membrane Preparation.** Spodoptera frugiperda 9 and High-Five insect cells were maintained as described (Stoveken et al., 2015). Baculoviruses were generated per the manufacturer’s protocol (Bac-to-Bac manual; Invitrogen). For adhesion GPCR membrane preparations, High-Five insect cells were infected with a 1/50 dilution (1st amplification) or a 1/100 dilution (2nd amplification) of recombinant adhesion GPCR baculovirus stocks for 48 hours prior to cell harvest and preparation of native and urea-treated adhesion GPCR membranes as described (Stoveken et al., 2015).

**Adhesion GPCRα Subunits (Gαq, Gα12, Gα13, Gαi1) were purified from High-Five insect cells using the GST-Ric-8A or B association method (Chan et al., 2011).** Recombinant αα13-GT was purified from High-Five membranes using the Goi-His6 association method (Kozasa and Gilman, 1995). Adhesion GPCR membranes (1–5 μg) were reconstituted with purified G proteins (100 nM Go of interest and 500 nM αα13-GT) in preincubation buffer [50 mM Hepes (pH 7.4), 1 mM dithiothreitol, 1 mM EDTA, and 3 μg/ml bovine serum albumin] with 0 or 20 μM GDP. Compounds (DHM, deguelin, mundulone, and...
isorotenone, rotenone), synthetic-peptide agonist, or DMSO vehicle control (≤5% v/v) was added to the membrane/G protein mixture and incubated for 30 minutes at 25°C. An equal volume of 5'-3-O-(thio)-triphosphate (GTPγS) binding buffer (preincubation buffer containing 10 mM MgCl₂ and 50 mM NaCl) with 2 μM GTPγS, [35S]GTPγS (20,000–50,000 cpm/μM), and the indicated concentrations of compounds or synthetic-peptide agonist was added to initiate the kinetic assay (compounds or peptides were included in both incubation mixtures to maintain the final concentrations). Triplicate samples were removed from the reaction mixtures and quenched at the indicated time points with 20 mM Tris (pH 7.7), 100 mM NaCl, 10 mM MgCl₂, 1 mM GTP, and 0.08% (v/v) deionized polyoxyethylene 10 lauryl ether C12E10. Quenched samples were filtered through BA85 nitrocellulose filters (GE Healthcare Chicago, IL), dried, and counted by liquid scintillation counting as described (Stoveken et al., 2015).

Mitochondrial Complex I (NADH:Ubiquinone Oxidoreductase) Activity Assay. Complex I activity was measured from frozen C57BL/6J mouse heart mitochondria isolated by differential centrifugation (Wojtovich et al., 2011). Mitochondria (25 μg/ml) were suspended in assay buffer (25 mM K₂HPO₄/KH₂PO₄, 10 mM MgCl₂, 2.5 mg/ml bovine serum albumin, 1 mM KCN, 75 μM NADH, pH 7.2, 37°C) in the presence of the indicated isoflavone compounds. After the addition of coenzyme Q₁₀ (0.1 mM), complex I activity was measured as the rotenone-sensitive rate (nmol/min/mg) of NADH oxidation (e = 6180 M⁻¹ cm⁻¹ at 340 nm) in the presence of the indicated isoflavone compounds. The addition of coenzyme Q₁₀ (0.1 mM), complex I activity was measured as the rotenone-sensitive rate (nmol/min/mg) of NADH oxidation (e = 6180 M⁻¹ cm⁻¹ at 340 nm) and expressed as percentage of inhibition (Nadotchy et al., 2007).

Rho GTPase Activation Assay. HEK293T cells were plated at 4 × 10⁵ cells per 10-cm plate. Twenty-four hours later, cells were transfected with 6.5 μg GPR56 A386M 7TM pcDNA3.1 and 1 μg 3XHA-Rh-oPCDNA3.1 (cDNA.org) using the polyethylenimine transfection method (Oner et al., 2013). The next day, cells were trypsinized, pooled, and plated at 4 × 10⁴ cells per 10-cm plate. The seeded transfected cells were allowed to attach for 8 hours and then serum starved overnight. Serum-starved cells were treated with 10 μM DHM or DMSO vehicle for 15 minutes. P7 synthetic peptide agonist or DMSO vehicle for 15 minutes. P7 synthetic peptide agonist or DMSO vehicle was added to the cells and incubated for an additional 5 minutes. Cells were washed once with phosphate-buffered saline + protease inhibitor mixture (23 μg/ml phenylmethylsulfonyl fluoride, 21 μg/ml Na-p-tosyl-L-lysine-chloromethyl ketone, 21 μg/ml L-1-p-tosylamino-2-phenylethyl-chloroketone, 3.3 μg/ml leupeptin, and 3.3 μg/ml lima bean trypsin inhibitor) and lysed in 1 ml of lysis buffer (25 mM Hepes (pH 7.2), 150 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 5% glycerol, protease inhibitor mixture). Lysates were cleared by centrifugation at 21,000 g, and the total protein concentration of each lysate sample was determined by amido black protein assay (Schaffner and Weissmann, 1973). Equal amounts of protein from each lysate were added to tubes containing 60 μg of Rhotekin-RBD agarose (Cytoskeleton, Inc., Denver, CO) and tumbled for 45 minutes. Rhotekin-RBD agarose was washed three times with lysis buffer, and beads were suspended in 50 μl of 2× sample buffer [200 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 200 mM dithiothreitol, 0.02% bromophenol blue] (Laemmli, 1970). Relative levels of active Rho recovered from Rhotekin-RBD agarose was washed three times with lysis buffer, and beads were suspended in 50 μl of 2× sample buffer [200 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 200 mM dithiothreitol, 0.02% bromophenol blue] (Laemmli, 1970). Relative levels of active Rho recovered from Rhotekin-RBD agarose were compared with levels of total Rho obtained from input samples by immunoblotting with the HA antibody.

PicoGreen Cell Detachment Assay. HER293T cells were plated at 2.5 × 10⁴ cells/well in 96-well plates in DMEM + 10% FBS (v/v). Twenty hours later, compounds (DHM, deugulin, isorotenone, maldulone, and rotenone) or DMSO vehicle control was serially diluted in DMEM + 10% FBS from 50 μM to 100 nM, and the cell medium was exchanged. Culture medium was replaced 24 hours later with 100 μl TE buffer [10 mM Tris-HCl (pH 7.7) and 1 mM EDTA] for 1 hour to induce hypotonic cell lysis. Quant-IT PicoGreen double stranded DNA reagent was diluted 1:200 in TE, and 100 μl was added to each well for 15 minutes to allow dye/DNA complex formation. The fluorescent dye was excited at 485 nm using 0.1% lamp energy, and emission was measured at 535 nm using the Berthold TriStar⁴²² Plate Reader. Relative fluorescent units were plotted as functions of compound concentration, and curve fitting was performed using GraphPad Prism (GraphPad Software, La Jolla, CA).

Results
Identification of GPR56 Small-Molecule Inhibitors through High-Throughput Screening. Isolated adhesion GPCR CTFs/7TM domains are highly active due to the engaged state of the tethered-peptide agonist (Paavola et al., 2011, 2014; Liebscher et al., 2014; Stoveken et al., 2015). We exploited this property of the GPR56 7TM to develop a high-throughput inhibitor screening assay using the SRE-luciferase gene reporter. GPR56 activates G13 robustly and thus, Ga13-sensitive SRE- or serum response factor–luciferase reporters (Shashidhar et al., 2005; Iguchi et al., 2008; Stoveken et al., 2015; Kishore et al., 2016). For the high-throughput screen and counterscreen, HEK293 cells were cotransfected with an SRE-luciferase reporter plasmid and GPR56 7TM or Ga13-Q226L pcDNA3.1 plasmids, respectively. Vehicle-treated cells were the positive controls (top limit of the assays), and cells treated with latrunculin B, which disrupts the signaling axis downstream of both GPR56 and Ga13, were the negative controls (bottom limit of the assays) (Evelyn et al., 2007). The Z’ score was 0.67 for the screening assay and 0.8 for the counterscreening assay (Supplemental Fig. 1) (Zhang et al., 1999).

The 2000-compound Spectrum Collection chemical library (estimated ~3–5 μM each) was then screened and counterscreened. In the screen, 66 compounds were identified that inhibited activity >90% (≥3 standard deviations above control), and 63 of these compounds also inhibited activity in the counterscreen, and were thus eliminated (Fig. 1A and B). The three hits were DHM and two compounds we have termed H.M.S.1 and H.M.S.2. Since DHM was highly efficacious and commercially available, we decided to characterize this molecule in detail.

The concentration dependence of DHM inhibition of GPR56 7TM or Ga13-Q226L was evaluated using an independent dual SRE-luciferase assay (Fig. 1D). At concentrations of DHM up to 5 μM, GPR56 7TM activity was inhibited and Ga13-QL activity was not, confirming the results obtained in the screening assay. The library was then scanned for compounds structurally related to DHM to evaluate performance in the screen and counterscreen, even if subpar. Four isoflavonoid compounds were found that are structurally related to DHM, the isoflavone maldulone and the rotenoid isorotenone and deugulin, all of which inhibited GPR56 7TM activity more than the Ga13-QL activity (Fig. 1E), although each result constituted a single trial due to the nature of high throughput screening. The fourth compound, the rotenoid, rotenone inhibited activity in the screen only marginally better than it did in the counterscreen (n = 1) (Fig. 1E).

A potential indirect mode of DHM action would be to negatively regulate GPR56 biosynthesis or trafficking or both. To rule out this possibility, GPR56 7TM High-Five insect cells were cultured in the presence or absence of 5 μM DHM for 24 hours. The cells were collected and the relative amounts of cell-surface receptor levels were found to be identical (Supplemental Fig. 2; Supplemental Method 1). However, when rotenone, isorotenone, deugulin, or maldulone (5 μM) was included in an overnight High-Five insect cell culture, the cells appeared to die or arrest growth, and thus relative GPR56 cell
surface levels were not measured. This is not a surprising outcome given that rotenoids, predominantly rotenone, are used commercially as piscicides and insecticides. Upon application to a natural water source, rotenoids efficiently enter the bloodstream through fish gills, potently inhibit complex I of the electron transport chain (ETC), and cause death (Chance et al., 1963; Li et al., 2003; Ling, 2003; Tomlin, 2009).

We were concerned that this known property of rotenoids to induce cell/organism death through ETC inhibition might prevent the usefulness of DHM as an adhesion GPCR inhibitor. A comparative analysis was conducted on DHM, mundulone, and rotenoid inhibition of ETC complex I versus the ability to induce death of cultured cells. HEK293 cells were treated with increasing concentrations of compounds, and 24 hours later, the cell medium and detached cells were removed. The adherent cells that remained were measured by a proxy assay in which the relative amounts of total DNA were quantified by PicoGreen assay. All of the compounds induced some degree of HEK293 cell detachment, but DHM was the least potent and could safely be used in cultured HEK293 cell assays at concentrations up to $10^{-6}$ M (Fig. 2A). Similarly, we tested each compound to determine potency of ETC complex I inhibition in an isolated mitochondria assay. Again, DHM was the least-potent complex I inhibitor, which correlated with its property as the least-potent mediator of HEK293 cell detachment (Fig. 2B). It is important to note that the apparently
compound and reduced the rate at which GPR56 7TM activated G13 >75% (from 0.18 to 0.04 minute⁻¹) (Fig. 3B).

The potency of DHM inhibition was determined by measuring the initial linear rates (0– to 3-minute time courses) of GPR56 7TM–stimulated G13 activation in response to increasing DHM concentrations. The IC₅₀ of DHM inhibition was ~21 μM, which is quite potent considering that DHM may interfere with the first-order action of a tethered-peptide agonist (Fig. 3C). DHM (50 μM) also inhibited intact full-length GPR56 activation of G13 4-fold (from 0.16 to 0.04 minute⁻¹) and full-length GPR56, in which we chemically dissociated the NTF with 7 M urea to induce the tethered-agonist-activated state (from 0.70 to 0.18 minute⁻¹) (Fig. 3D). The high constitutive activity (0.16 minute⁻¹) of the “intact” receptor may represent two populations: one that is truly intact and has the NTF bound to the CTF, and a second population in which the NTF has been shed or dissociated from the CTF either spontaneously during live cell culture or procedurally during preparation of native receptor membranes, which involves multiple washing and Dounce homogenization steps. The latter population would be active with a decrypted tethered-peptide agonist. These points are important when considering the potential mechanisms of DHM inhibition as a competitive antagonist to the tethered agonist or as a negative allosteric modulator.

**DHM Inhibits GPR56-Related GPR114, but Not GPR110 or Class A GPCRs.** GPR114/ADGRG5 is an adhesion GPCR that promotes cyclic AMP production in cells and shares an identical amino acid sequence with GPR56 at the N-terminal tip of its tethered agonist (Gupte et al., 2012; Wilde et al., 2016) (Fig. 4A). We reconstituted the GPR114 7TM domain with purified Gα₉ and an electron acceptor (Q1), and the loss of NADH over 5 minutes at 340 nM was measured in the presence of the indicated amount of each flavonoid. IC₅₀ values were calculated from monoeponential association curves fitted using GraphPad Prism. R.F.U., relative fluorescent units. Error bars are the average ± the s.d. of three experimental replicates.

**Secondary Assay Validation of DHM Inhibition of GPR56 Activity.** Our measurements of adhesion GPCR activation of reconstituted G proteins provide an entirely independent, cell-free assay system to evaluate small-molecule modulators obtained from cell-based screens (Stoveken et al., 2015). Insect cell membranes expressing the recombinant adhesion GPCR of interest are prepared and reconstituted with purified, recombinant Gα and Gβ₁γ₂. Assays are initiated by the addition of [³⁵S]GTPγS, and the rates of aGPCR-stimulated G protein activation ([³⁵S]GTPγS binding to Gα) are measured with or without the influence of added compounds. DHM and related compounds (deguelin, mundulone, isorotenone, and rotenone; 50 μM each) inhibited the kinetics of GPR56 7TM–stimulated G13 GTPγS binding to varying degrees (Fig. 3A). DHM was the best inhibitory

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**Fig. 2.** The rank potency of flavonoid derivative–induced HEK293 cell detachment correlates with electron transport chain complex I inhibition. (A) Adherent HEK293 cells were grown for 24 hours in the presence of the indicated concentrations of the five flavonoids (DHM, mundulone, rotenone, deguelin, isorotenone). Detached cells were removed and the relative levels of adhered cells remaining were measured by proxy assay of PicoGreen quantification of total DNA. (B) Isolated mitochondria were supplied with NADH and an electron acceptor (Q1), and the loss of NADH over 5 minutes at 340 nM was measured in the presence of the indicated amounts of each flavonoid. IC₅₀ values were calculated from monoeponential association curves fitted using GraphPad Prism. R.F.U., relative fluorescent units. Error bars are the average ± the s.d. of three experimental replicates.

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**Fig. 4.** The rank potency of flavonoid derivative–induced HEK293 cell detachment correlates with electron transport chain complex I inhibition. (A) Adherent HEK293 cells were grown for 24 hours in the presence of the indicated concentrations of the five flavonoids (DHM, mundulone, rotenone, deguelin, isorotenone). Detached cells were removed and the relative levels of adhered cells remaining were measured by proxy assay of PicoGreen quantification of total DNA. (B) Isolated mitochondria were supplied with NADH and an electron acceptor (Q1), and the loss of NADH over 5 minutes at 340 nM was measured in the presence of the indicated amounts of each flavonoid. IC₅₀ values were calculated from monoeponential association curves fitted using GraphPad Prism. R.F.U., relative fluorescent units. Error bars are the average ± the s.d. of three experimental replicates.

**Conclusion:**
measure DHM/P7 competitive action/binding (Schild, 1949; Arunlakshana and Schild, 1959; Bindseil, 2008). As the peptide-agonist concentration response was performed in response to increasing concentrations of DHM, the sigmoidal curves exhibited clear rightward shifts. However, sufficient synthetic peptide agonist could not be supplied to achieve maximal efficacies, as concentrations above 100–150 μM typically decreased activity by an unknown means (Fig. 5B). These results clearly demonstrate the effectiveness of DHM as an aGPCR inhibitor and leave open the possibility that DHM is an orthosteric antagonist.

To test antagonism in another manner, we used the GPR56 A386M 7TM receptor, which has a more severely compromised tethered agonist that essentially renders the receptor a model of basal activity (Fig. 5A) (Stoveken et al., 2015). The sensitivity of our G protein reconstitution assay was increased by the use of five times more GPR56 A386M 7TM receptor and by not including 20 μM GDP in the assay to promote the most efficient G13:receptor precoupling. Under these conditions, GPR56 A386M 7TM provided modest G13 activation. However, DHM (50 μM) failed to inhibit this basal, tethered-agonist-independent activity (Fig. 5B). DHM did not inhibit basal signaling of GPR56 A386M 7TM when standard assay conditions were used (Fig. 5D). The synthetic P7 peptide agonist TYFAVLM (100 μM) was then used as a surrogate to the tethered agonist to markedly stimulate GPR56 A386M 7TM. However, DHM (50 μM) and P7 (100 μM) were coapplied to GPR56 A386M 7TM, DHM provided nearly complete inhibition of the synthetic peptide-agonist-induced receptor activation (Fig. 5D).

To verify GPR56 inhibition by DHM in cells, Rho GTPase activation assays were performed to test acute receptor inhibition. GPR56 activates G13 to stimulate Rho guanine nucleotide exchange factor-mediated Rho-GTP production (Iguchi et al., 2008; Luo et al., 2011; Paavola et al., 2011). HEK293T cells transfected with HA-tagged RhoA and GPR56 A386M 7TM were treated with DHM (10 μM) or vehicle. RhoA-GTP production was stimulated by the application of P7 peptide agonist (100 μM) or vehicle to the cell culture medium for 5 minutes. RhoA-GTP was isolated from each treated cell group by Rhotekin pull-down assay and visualized by anti-HA immunoblotting (Fig. 5E). Treatment with DHM alone did not affect basal RhoA-GTP levels. P7 treatment alone provided an increase in the amount of RhoA-GTP produced, but P7 peptide activation was clearly blocked by DHM.

DHM inhibition of the P7 peptide response was then recapitulated using GPR56 SRE-luciferase assays. Cells transfected with GPR56 A386M 7TM were untreated or treated with ice-cold 7 M urea to mimic the proposed process of ligand-mediated receptor activation via N-terminal extracellular fragment dissociation. The kinetics of receptor-activated G13 GTP binding were measured in the presence of 50 μM DHM or DMSO control. Error bars were frequently smaller than the plotted symbols and are the average 

\[ \text{mol GTP·S} \cdot \text{mol G13}^{-1} \cdot \text{min}^{-1} \]

of three experimental replicates. (D) Membranes containing full-length (Karpus et al., 2013) GPR56 receptor were untreated or treated with ice-cold 7 M urea to mimic the proposed process of ligand-mediated receptor activation via N-terminal extracellular fragment dissociation. The kinetics of receptor-activated G13 GTP-S binding were measured in the presence of 50 μM DHM or DMSO control. Error bars were frequently smaller than the plotted symbols and are the average ± the s.d. of three experimental replicates. Full-length FL, full-length.
treated with a fixed concentration of 3 \( \mu M \) DHM or vehicle and then stimulated with an increasing concentration of P7 peptide agonist. DHM treatment blunted P7 peptide activation at each concentration (Fig. 5G). In conclusion, DHM antagonizes synthetic-peptide agonist and tethered-peptide agonist–mediated aGPCR activation in isolated membranes and HEK293T cell–based assays, but it does not inhibit basal receptor signaling. This strongly indicates that the mode of DHM inhibition is to act as an antagonist that interferes with peptide agonist binding, but does not preclude the possibility that DHM may act as a negative allosteric modulator.

Discussion

We support a hypothesis where adhesion GPCR activation requires at least two separable events to occur in a prescribed order: 1) adhesion GPCR NTFs bind to anchored protein ligands, and 2) shear force generated by cell movement in relation to the anchored-ligand:NTF complex overcomes large energetic and entropic barriers that are required to dissociate the NTF from the CTF (Yona et al., 2008; Karpus et al., 2013; Langenhan et al., 2013; Scholz et al., 2015; Stoveken et al., 2015). NTF dissociation results in decryption of the adhesion GPCR tethered-peptide agonist, which rapidly and perhaps irreversibly binds its orthosteric site on the 7TM domain (CTF). This model nearly mandates that adhesion GPCR signaling deactivation follows an internalization/desensitization mechanism. Based on this model, the development of natural adhesion GPCR ligand mimetics seems to be a very unlikely means to create receptor modulators. It may be far better to develop synthetic-peptide modulators based on the tethered-peptide agonists that activate multiple adhesion GPCRs (Liebscher et al., 2014; Demberg et al., 2015; Stoveken et al., 2015; Wilde et al., 2016). Such peptides would act akin to protease activated receptors (PAR) agonist peptides that modulate protease-activated receptors (PAR GPCRs) (Vu et al., 1991; Scarborough et al., 1992). Alternatively, and perhaps even more effectively, small-molecule modulators could be identified that would bypass the two event-mediated activation processes and regulate adhesion GPCR activities directly.

Using a cell-based high-throughput screening assay to uncover small-molecule inhibitors of tethered-agonist-activated GPR56, we identified DHM as a novel adhesion GPCR antagonist. In direct receptor-stimulated G protein–activation assays, DHM inhibited two of three adhesion GPCRs tested, but not two class A GPCRs, indicating that DHM has selective action for a subset of adhesion GPCRs. DHM inhibition of GPR56 was verified using a cell-based RhoA GTPase effector enzyme assay and by an independent, dual luciferase-gene-reporter assay. Mechanistically, DHM did not inhibit basal GPR56 activity, but did inhibit GPR56 activity stimulated by its tethered-peptide agonist or by a synthetic-peptide agonist. These data establish that the mode
Fig. 5. DHM inhibits tethered- and synthetic-peptide agonist–stimulated GPR56, but does not inhibit basal receptor activity, a mode of action consistent with that of a neutral antagonist. (A) Alignment of stalk regions of intact tethered agonist and compromised tethered agonist GPR56 7TM domain receptors. The seven amino acid synthetic GPR56 agonist peptide is aligned underneath. (B) Partially compromised tethered agonist GPR56 F385M 7TM receptor membranes were reconstituted with G13, and P7 peptide concentration–dependent activation of G13 GTP\(_{\gamma}S\) binding was measured in response to increasing concentrations of DHM. Initial rates are plotted and were determined from four point (t = 0, 1, 2, 3 min.) linear functions. Error bars are the average ± the s.d. of three technical replicates. (C) The ability to measure near-basal activity of the GPR56 A386M 7TM receptor with a negligibly active tethered agonist was enhanced by use of five times more receptor membranes and excluding GDP from the reconstitution assay. GPR56 A386M 7TM basal activation of G13 GTP\(_{\gamma}S\) binding was measured in the presence of 50 μM DHM or the DMSO vehicle control. Error bars are the average ± the s.d. of three experimental replicates. (D) DHM inhibits P7 synthetic-peptide agonist stimulation of the compromised tethered agonist GPR56 A386M 7TM receptor. GPR56 A386M 7TM membranes were reconstituted with G13, and receptor-stimulated G13 GTP\(_{\gamma}S\) binding kinetics were measured in response to 100 μM P7 agonist peptide and/or 50 μM DHM in the presence of 20 μM GDP. Error bars are the average ± the s.d. of three experimental replicates. (E) P7 agonist-peptide (10 μM) stimulation of the GPR56 A386M 7TM receptor in intact HEK293 cells activated RhoA.
of DHM inhibition may be as a neutral antagonist that competes with the tethered-peptide agonist for binding to its orthosteric site. If DHM indeed acts as an orthosteric antagonist, this provides a solidifying pharmacological argument supporting the emerging hypothesis that adhesion GPCRs are activated by tethered-peptide agonists (Liebscher et al., 2014; Stoveken et al., 2015).

Four additional compounds in the Spectrum Collection that are structurally related to DHM were assayed by SRE-luciferase and GPR56 reconstitution assays. Mundulone, rotenone, isorotenone, and deguelin all inhibited GPR56-mediated G13 activation, but with lower efficacies than DHM. Rotenone has a well established cellular target; it directly inhibits complex I of the mitochondrial ETC (Chance et al., 1963). Complex I inhibition explains the toxic effects of rotenone as an insecticide and potent piscicide (Chance et al., 1963; http://www.doc.govt.nz/documents/science-and-technical/SFC211.pdf). Rotenone is less toxic to humans but can cause death if a large quantity is ingested (Wood et al., 2005). We directly compared the ability of all five compounds to inhibit ETC complex I versus toxicity toward cultured HEK293 cells using a proxy assay that measured compound ability to induce cell detachment over 24 hours (Fig. 2). DHM was clearly the least-potent inhibitor of complex I, which correlated with it being the poorest mediator of cell detachment. Importantly, DHM was the most efficacious inhibitor of GPR56 and GPR114.

Our future and ongoing optimization of DHM will use a dual structure-activity relationship approach to seek derivatives that widen the gap between higher-potency adhesion GPCR inhibition and lower-potency complex I inhibition (and associated cytotoxicity). A comparison of the chemical structures of the five isoflavonoid compounds provides insight into the key structural features to focus on and may explain the different potencies of adhesion GPCR inhibition (Fig. 6). Rotenone, isorotenone, and deguelin consist of five joined hydrocarbon rings that share an isoflavone-like core. The contiguous nature of these rings imparts a rigid, planar structural orientation. DHM and mundulone each possess a moiety that disrupts the contiguousness of the five hydrocarbon rings to confer increased flexibility. DHM and mundulone differ in that the former has a 2-phenylacetophenone core, and the latter has an isoflavone core. The 2-phenylacetophenone core in DHM would confer additional flexibility over mundulone, and thus, DHM has the most flexibility of the five studied isoflavonoids. Examples of potential DHM conformers are shown in Fig. 6. Additionally, the phenolic hydroxyl of DHM, which is blocked in mundulone, could be important for adhesion GPCR target interaction.

The mode of DHM inhibitory action remains a partially open question, although our data are consistent with those of a neutral antagonist that competes with a tethered-peptide agonist for binding to its orthosteric site. Still, the data do not eliminate the possibility that DHM may serve as a negative allosteric modulator. A Schild analysis was attempted to directly measure DHM competitive action to the GPR56 synthetic peptide agonist P7 (Fig. 4B) (Schild, 1949; Arunlakshana and Schild, 1959; Bindslev, 2008). When the P7 peptide agonist concentration response was performed with increasing DHM concentrations, the response curves exhibited clear rightward shifts, but could not be fit to full sigmoidal functions because maximum efficacy of P7-mediated activation (rate of G protein GTPγS binding) was unachievable. Activation of GPR56 with P7 peptide agonist concentrations >150 μM results in diminishing efficacy. The reason for this is unknown, although we speculate that use of...
very high adhesion GPCR synthetic peptide agonist concentrations results in nonproductive aggregation of an essential assay component. Since sufficient peptide agonist cannot be used to overcome DHM antagonism, the observed curve flattening might be construed to preclude DHM action as an orthosteric antagonist. However, it could also indicate that DHM has greater affinity for the orthosteric site than the peptide agonist (Arulkumaran and Schild, 1959). Our future work in this area will include development of labeled synthetic peptide agonist or labeled DHM probes that will be used to perform direct adhesion GPCR competition binding analyses.

An important prospective use of a labeled DHM probe will be to determine the active fraction of adhesion GPCR present at the cell surface in response to perturbations intended to alter receptor activation (e.g., peptide agonists, small-molecule modulators, or natural ligands). Our previous work showed that the F7 synthetic-peptide agonist failed to activate an intact (full-length) GPR56 receptor with its NTF bound to the CTF (Stoveken et al., 2015). We proposed that the synthetic-peptide agonist was sterically blocked by the NTF from accessing its CTF orthosteric binding site. If the same holds true for a DHM probe, then quantitative measurement of its binding to cell surfaces would provide a measurement of the population of active adhesion GPCR CTF that has had its NTF dissociated. DHM inhibits the GPR56 7TM and urea-activated full-length GPR56 (NTF dissociated), which are both tethered-agonist-activated receptors. However, DHM also provided very good inhibition of intact, full-length GPR56 that did not have its NTF dissociated via the urea-treatment activation mimetic (Fig. 3D). Intact or holoreceptor GPR56 has substantial baseline activity, and we speculate that, in our assays, “intact” GPR56 is actually a mixed population of NTF bound to CTF (a low-activity-state holoreceptor) and free CTF (tethered-agonist-activated), which was generated by spontaneous NTF shedding during cell culture and/or by the mechanical treatments used during receptor membrane preparation. If a labeled DHM probe can only bind with high affinity to the free GPR56 CTF, it could be used as a means to determine whether intact GPR56 is a mixed population of free CTF and holoreceptor or mostly holoreceptor. If the receptor proves to be mainly intact, then this corroborate recent findings that intact GPR56 has high basal signaling activity (Kishore et al., 2016).

Here, we have identified and validated DHM as a new inhibitor of select adhesion GPCRs (GPR56/GPR114). Work with additional receptors is underway to determine a more complete profile of adhesion GPCR substrate specificity. To our knowledge, DHM is the first reported small-molecule adhesion GPCR antagonist, and its development as a probe compound will meet a critical need for a larger pharmacological toolbox to study adhesion GPCRs. DHM also represents a lead compound that may be developed for increased selectivity and potency toward specific GPCRs to treat adhesion GPCR–directed disease.

**References**


