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GPR40-Mediated Gα12 Activation by Allosteric Full Agonists Highly Efficacious at Potentiating Glucose-Stimulated Insulin Secretion in Human Islets [S]

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ABSTRACT

GPR40 is a clinically validated molecular target for the treatment of diabetes. Many GPR40 agonists have been identified to date, with the partial agonist fasiglifam (TAK-875) reaching phase III clinical trials before its development was terminated due to off-target liver toxicity. Since then, attention has shifted toward the development of full agonists that exhibit superior efficacy in preclinical models. Full agonists bind to a distinct binding site, suggesting conformational plasticity and a potential for biased agonism. Indeed, it has been suggested that alternative pharmacology may be required for meaningful efficacy. In this study, we described the discovery and characterization of Compound A, a newly identified GPR40 allosteric full agonist highly efficacious in human islets at potentiating glucose-stimulated insulin secretion. We compared Compound A–induced GPR40 activity to that induced by both fasiglifam and AM-1638, another allosteric full agonist previously reported to be highly efficacious in preclinical models, at a panel of G proteins. Compound A was a full agonist at both the Gαq and Gαi2 pathways, and in contrast to fasiglifam Compound A also induced Gα12 coupling. Compound A and AM-1638 displayed similar activity at all pathways tested. The $G\alpha_{12}/G\alpha_{13}$-mediated signaling pathway has been linked to protein kinase D activation as well as actin remodeling, well known to contribute to the release of insulin vesicles. Our data suggest that the pharmacology of GPR40 is complex and that Gα12/Gα13-mediated signaling, which may contribute to GPR40 agonists therapeutic efficacy, is a specific property of GPR40 allosteric full agonists.

Introduction

Activation of the free fatty acid receptor 1, also known as GPR40, potentiates glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells and stimulates the release of incretins, such as glucagon-like peptide 1 (GLP-1), from enteroendocrine cells (Briscoe et al., 2003, 2006; Itoh et al., 2003; Yonezawa et al., 2004; Hardy et al., 2005; Shapiro et al., 2005; Tomita et al., 2005; Latour et al., 2007; Edfalk et al., 2008; Stoddart et al., 2008; Luo et al., 2012; Mancini and Poitout, 2013). GLP-1 further promotes GSIS and also decreases hepatic gluconeogenesis, inhibits glucagon secretion, reduces body weight, and improves insulin sensitivity (Baggio and Drucker, 2007; Holst, 2007; Pocai, 2012; Gorski et al., 2017). Thus, the dual mechanisms of GPR40 in pancreatic β-cells as well as in enteroendocrine cells provide considerable rationale for the development of GPR40 agonists for the treatment of type-2 diabetes mellitus (T2DM), with a potential for weight management.

A number of potent, synthetic GPR40 agonists have been reported and a GPR40 partial agonist, fasiglifam from Takeda, advanced as far as phase III clinical trials (Kaku et al., 2015). In a phase II study in T2DM patients, fasiglifam induced a similar glucose-lowering effect (HbA1c: ca. 1%) to that of glimepiride (Burant et al., 2012; Leifke et al., 2012). In spite of similar promising results in phase III, fasiglifam was withdrawn from development due to drug-induced liver injury (Hedrington and Davis, 2014; Otieno et al., 2017).

Since then, numerous full agonists with superior efficacy both in vitro and in vivo compared with fasiglifam have been

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ABBREVIATIONS: AgoPAM, allosteric agonist with PAM activity; BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CPM, counts per minute; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GLP-1, glucagon-like peptide 1; GPR40, free fatty acid receptor 1, also known as GPR40; GSIS, glucose-stimulated insulin secretion; HbA1c, haemoglobin A1c; HBSS, Hanks' balanced salt solution; HTRF, homogeneous time-resolved fluorescence; IBMX, 3-isobutyl-1-methylxanthine; IP1, inositol-1-phosphate; PAM, positive allosteric modulator; PBS, phosphate-buffered saline; PEI, polyethylenimine; PKD, protein kinase D; PTX, pertussis toxin; T2DM, type-2 diabetes mellitus.

reported (Defossa and Wagner, 2014; Li et al., 2016). Interestingly, these full agonists bind to a recently identified binding site, distinct from previously predicted pockets, and different from that of endogenous fatty acids and fasiglifam or other partial agonists (Lin et al., 2012; Defossa and Wagner, 2014; Hauge et al., 2014; Srivastava et al., 2014; Lu et al., 2017). The presence of multiple binding sites suggests conformational plasticity, highlighting a potential for biased agonism (Kenakin et al., 2012; Kenakin and Christopoulos, 2013; Costa-Neto et al., 2016; Rankovic et al., 2016). GPR40 is mostly known to couple to the heterotrimeric G protein Gαq/11 (Shapiro et al., 2005). However, it has also been shown that GPR40 could couple to other pathways in a ligand-dependent manner and that only allosteric full agonists able to induce the activation of such alternative pathways, such as the Gαs/cAMP pathway, could trigger maximal efficacy in preclinical models (Lin et al., 2012; Defossa and Wagner, 2014; Hauge et al., 2014). GPR40 has also been shown to couple to Gαi/o and arrestin (Schröder et al., 2011; Mancini et al., 2015), and arrestin recruitment has been shown to contribute to GPR40-mediated GSIS (Mancini et al., 2015).

Through a rational design approach, we have identified a new human GPR40 (hGPR40) full agonist at the Gαq/inositol-1-phosphate (IP1)/calcium pathway fully efficacious at enhancing GSIS in human islets. We compared Compound A–induced GPR40 activity at a panel of G proteins and to that induced by both fasiglifam as well as AM-1638, previously reported as a highly efficacious hGPR40 allosteric full agonist (Hauge et al., 2014; Li et al., 2016). Our data indicated that Compound A and AM-1638 were both hGPR40 allosteric full agonists, not only at the Gαq pathway but also at Gαi2, with no to very weak efficacy at the Gαs/cAMP pathway. Interestingly, in contrast to fasiglifam and α-linolenic acid, Compound A and AM-1638 strongly engaged the Gα12 protein. Our data suggest that the pharmacology of GPR40 is complex and that Gα12/Gα13-mediated signaling, which may contribute to the release of vesicles possibly via protein kinase D (PKD) activation and actin remodeling, is a specific property of the GPR40 allosteric full agonists Compound A and AM-1638.

Materials and Methods

Cell Lines and Cell Culture. The hGPR40 low-expressing stable Chinese hamster ovary (CHO)-K1 cell line used in this study was purchased from Multispan, Inc (Hayward, CA). The receptor density in this cell line was evaluated by whole cell radioligand saturation binding at 47,112 ± 5,088 receptors per cell, which was comparable to the GPR40 density in a rat insulinoma β-cell line INS-1 832/13 (41,519 ± 9,516 receptors per cell). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 10 μg/ml puromycin, and then incubated at 37°C with 5% CO_2.

IP1 Homogeneous Time-Resolved Fluorescence (HTRF) Assay. The day before the assay, hGPR40-expressing CHO-K1 cells were plated overnight in 384-well plates (4,000 cells per well) in complete media, with or without 100 ng/ml pertussis toxin (PTX) (Tocris Bioscience, Bristol, United Kingdom). The following day, the culture medium was replaced with assay buffer containing Hanks' balanced salt solution (HBSS) with calcium and magnesium, 20 mM HEPES, and 0.1% fatty acid free bovine serum albumin (BSA), pH 7.4. Compounds were then added and incubated with cells at 37°C for 90 minutes. Analytes were detected according to the manufacturer's protocol (IPone Tb kit; CisBio, Codolet, France). Data presented are representative of at least three independent experiments performed in quadruplicate for each compound. Data are represented as averages ± S.D.

Calcium Measurements. The day before the assay, hGPR40-expressing CHO-K1 cells were plated overnight in 384-well plates (20,000 cells per well) in complete media. The following day, the culture medium was replaced with 25 μl of assay buffer containing HBSS with calcium and magnesium, 20 mM HEPES, and 0.1% fatty acid free BSA, pH 7.4, and then starved for 1 hour at 37°C. Calcium-sensitive fluorescent dye (Fluo 6; Molecular Devices, San Jose, CA) was then added in 25 μl assay buffer and the cells were incubated for another hour at 37°C and protected from light. Plates were read on FLIPR Tetra (Molecular Devices) measuring emission at 515–575 nm caused by excitation at 470–495 nm before and up to 8 minutes after addition of 12.5 μl of 5X agonist solution (prepared in assay buffer). The concentration response curves were constructed based on the maximal responses over baseline obtained for different concentrations of each compound. Data presented are representative of three independent experiments performed in quadruplicate for each compound. Data are represented as averages ± S.E.M.

cAMP HTRF Measurements. The day before the assay, hGPR40-expressing CHO-K1 cells were plated overnight in 384-well plates (20,000 cells per well) in complete media. The following day, the culture medium was replaced with assay buffer containing HBSS with calcium and magnesium, 20 mM HEPES, and 0.1% fatty acid free BSA, pH 7.4, and then starved for 1 hour at 37°C. The assay buffer was then replaced with fresh assay buffer containing 500 μM IBMX, and compounds were added in assay buffer (no IBMX) for 30 minutes. Analytes were detected according to the manufacturer's protocol (cAMP Dynamic kit; CisBio). Fluorescence was read with a PHERAstar plate reader (BMG Labtech, Ortenberg, Germany) using an excitation of 337 nm and emissions of 620 and 665 nm. Raw data were converted to nanomolar cAMP values by interpolation from a cAMP standard curve. The maximum effect attributable to the drug (E_{max}) and EC_{50} determinations were made from an agonist-response curve analyzed with a curve fitting program using a four-parameter logistic dose-response equation in GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). Data presented are representative of three independent experiments performed in quadruplicate for each compound. Data are represented as averages ± S.D.

DiscoveRx Arrestin Recruitment. The ability of hGPR40 to recruit β-arrestin-2 was determined using the DiscoveRx PathHunter technology (DiscoveRx, Fremont, CA) that involves enzyme complementation of a fusion-tagged receptor along with an arrestin recruitment modulating sequence and β-arrestin-2 proteins. DLD1 cells expressing hGPR40 (DiscoveRx) were seeded in Cell Plating Media 2 (DiscoveRx) at a density of 15,000 cells/well in 384-well black, clear-bottom plates. The following day, the culture medium was replaced with assay buffer containing HBSS with calcium and magnesium, 20 mM HEPES, and 0.1% fatty acid free BSA, pH 7.4, and then starved for 1 hour at 37°C. The cells were then treated with multiple concentrations of agonists in phosphate-buffered saline (PBS) and incubated at 37°C for 60 minutes. DiscoveRx reagent was then added to the cells according to the manufacturer's recommendations, followed by 1 hour incubation at room temperature, and then luminescence was measured on the PHERAstar reader. Data presented are representative of three independent experiments performed in triplicate for each compound. Data are represented as averages ± S.D.

Bioluminescence Resonance Energy Transfer (BRET)–Based Biosensor Assays (BioSensAll). BioSensAll biosensor assays were conducted at Domain Therapeutics NA Inc. (Montreal, QC, Canada). Assays were performed in HEK-293T cells, cultured in DMEM (Wisent, Quebec, Canada) supplemented with 1% penicillin-streptomycin (Wisent, Quebec, Canada) and 10% FBS (Wisent, Quebec, Canada), and then maintained at 37°C with 5% CO_2. All biosensor-coding plasmids and related information are the property of Domain Therapeutics NA Inc.: GAPL-Gs, GAPL-Gq, GAPL-G11, GAPL-Gi2, GAPL-GoB, GAPL-Gz, and GAPL-G12. Transfections were performed

using 25-kDa linear polyethylenimine (PEI) (Polysciences, Warrington, PA) at a 3:1 μl of PEI/microgram of DNA ratio. Briefly, DNA and PEI were diluted separately in 150 mM NaCl, mixed, and then incubated for at least 20 minutes at room temperature [note: the total amount of DNA transfected was adjusted to a final quantity of 2 μg with salmon sperm DNA (Thermo Fisher Scientific, Waltham, MA)]. During the 20-minute incubation, HEK-293T cells were detached, counted, and resuspended into cell culture medium to a final density of 350,000 cells/ml. At the end of the 20-minute incubation, DNA/PEI complexes were added to the cells followed by gentle mixing. Cells were subsequently distributed in cell culture-treated 96-well plates (White Opaque 96-well Microplates; Greiner Kremsmünster, Austria) at a density of 35,000 cells per well (i.e., 100 μl of cell suspension per well) and incubated at 37°C for 48 hours. For PTX treatment, 24 hours after transfection and the day before the assay the medium was replaced by fresh medium containing 100 ng/ml PTX (Tocris Bioscience). Then, 48 hours after transfection, the transfection medium was removed and cells were washed once with 100 μl of Tyrode-HEPES buffer (Sigma-Aldrich, St. Louis, MO) per well. Wash buffer was then replaced with 100 μl of fresh Tyrode-HEPES buffer per well and plates were incubated for 60 minutes at room temperature. At the end of this equilibration period, 10 μl of 20 μM e-Coelenterazine Prolume Purple (Methoxy e-CTZ; Nanolight, Pinetop, AZ) was added to each well followed immediately by the addition of increasing test compound concentrations. For PAM mode experiments, 10 minutes after the addition of increasing concentrations of test compound, an EC_{20} of α-linolenic acid (1 μM) was added to the cells. Cells were incubated at room temperature for 10 minutes and BRET readings were subsequently collected with a 0.4-second integration time on a Synergy NEO plate reader (BioTek Instruments, Inc., Winooski, VT; filters: 400/70 and 515/20 nm, donor and acceptor filters, respectively). The BRET signal was calculated as the ratio of acceptor emission to donor emission. Data from at least three independent experiments for each compound and performed in duplicates were combined and the symbols presented are the mean ± S.E.M. Note that due to the nature of the sensors, apart from the GAPL-Gs sensor, whose activation leads to a decrease in the BRET signal, activation of the other sensors leads to an increase in the BRET signal.

To calculate the bias factor between some of those pathways for our compounds, we used the Black-Leff operational model to fit the agonist concentration ([A])-response curves as follows (Kenakin et al., 2012):

$$\text{Response} = \frac{E_m[A]^n \tau^n}{[A]^n \tau^n + ([A] + K_A)^n}$$

where the maximal response of the system is given by E_m; n is the transducer slope for the function linking agonist concentration to measured response; and parameters K_A and τ are the equilibrium constants governing the reaction. Log (τ/K_A) is defined as the transduction coefficient, and using α-linolenic acid as a reference we calculated the relative efficiency of our compounds at relevant pathways [$\Delta\log(\tau/K_A)$]. The bias factor [$\Delta\Delta\log(\tau/K_A)$ or log bias] between pathways j_1 and j_2 can be calculated as follows:

$$\text{Bias} = 10^{\Delta\Delta\log(\tau/K_A)_{j_1 - j_2}}$$

where

$$\Delta\Delta\log(\tau/K_A)_{j_1 - j_2} = \log\text{bias} = \Delta\log(\tau/K_A)_{j_1} - \Delta\log(\tau/K_A)_{j_2}$$

Molecular Modeling. Molecular modeling and docking have been performed using the recent co-crystal structure of hGPR40 in complex with MK-8666 and AgoPAM AP8 (PDB code 5TZY) (Lu et al., 2017). Molecular Operating Environment 2015.1001 (Chemical Computing Group Inc., Montreal, QC, Canada) was used for loop modeling, energy minimization (AMBER10:EHT forcefield and Born solvation model), and rescoring of the docking poses. Glide was used for molecular docking of Compound A (Small-Molecule Drug Discovery Suite 2016-3: Glide, version 6.9; Schrödinger, LLC New York, NY). The docking poses generated with Glide-XP were rescored using the GBVI/WSA dG scoring function available in Molecular Operating Environment 2015.1001 (Corbeil et al., 2012). The top ranked pose of Compound A was imported, with the hGPR40 protein structure, into a Pymol session to create all of the pictures (The PyMOL Molecular Graphics System, version 1.8; Schrödinger, LLC).

Radioligand Binding Experiment. Membranes were prepared as follows. Cells stably expressing hGPR40 were harvested by centrifugation (10 minutes at 5,000*g*). The pellet was resuspended in lysis buffer [10 mM Tris-HCl, pH 7.4, 137 mM NaCl, and complete protease inhibitor cocktail (1 tablet per 40 ml; Roche, Basel, Switzerland)], and lysed using 30 strokes with a dounce homogenizer on ice. The homogenate was centrifuged at 4°C (10 minutes at 900*g*). The supernatant was centrifuged at 4°C for 60 minutes at 100,000*g*. The resulting pellet was resuspended in wash buffer [10 mM Tris-HCl, pH 7.4, 1 M NaCl, and complete protease inhibitor cocktail (1 tablet per 40 ml)]. The homogenate was centrifuged at 4°C for 30 minutes at 100,000*g*. Membranes were resuspended at 10 mg/ml protein in 10 mM Tris-HCl, pH 7.4, and 137 mM NaCl.

Test compounds were serially diluted in binding buffer (PBS + 0.1% fatty acid-free BSA). Each well of the 96-well assay plate contained diluted test compounds, 50 nM [^{3}H]-Compound A or 10 nM [^{3}H]-AM-1638, and 10 μg/well hGPR40 membrane suspension in a total volume of 100 μl. The binding reaction was allowed to equilibrate for 60 minutes at room temperature with shaking. Binding assays were terminated using a Harvester Filtermate 96 (PerkinElmer, Waltham, MA). Bound and free radioligands were separated by collecting the membrane-bound fraction onto GF/B filter plates impregnated with PEI 0.5% and prewetted with binding buffer. Filter plates were washed four times with ice-cold binding buffer and dried for 2 hours. Microscint O (50 μl) was added to each well and radioactivity was counted using Topcount (PerkinElmer). Nonspecific binding was determined using 10 μM cold Compound A or AM-1638. Data analysis was performed using GraphPad Prism 7.0 (GraphPad Software, Inc.). Data presented are representative of three independent experiments performed in triplicate for each compound. Data are represented as averages ± S.D.

For receptor densities evaluation, whole cell saturation binding experiments were performed according to what has been previously described (Jin et al., 2009), using a binding buffer composed of DMEM, 25 mM HEPES, and 0.1% fatty acid-free BSA (pH 7.4). For receptor number determination, a normalization sample (NORM) was used and the receptor density was calculated as follows:

$$\text{Number of receptors per cell} = \frac{6.022 \times 10^{23} \times B_{max} \times \text{number of mol radioligand used for NORM}}{\text{Number of cells} \times \text{CPM of NORM}}$$

Insulin Secretion in Human Islets. Human islets were dispersed with Accutase (Thermo Fisher Scientific, Waltham, MA) for 10 minutes at 37°C. Twenty thousand cells per well were plated in V-bottom 96-well plates and cultured overnight in complete medium containing: CMRL Media (Thermo Fisher Scientific), 10 mM Niacinamide, (1 mg/ml, 0.55 mg/ml, 0.67 μg/ml insulin-transferrin-selenium) ITS (Thermo Fisher Scientific), 16.7 mM zinc sulfate, 5 mM sodium pyruvate, 2 mM glutamax (Thermo Fisher Scientific), 25 mM HEPES, and 10% FBS. The next day, medium was replaced with assay buffer (Krebs Ringer) and cells were preincubated in 2 mM glucose for 1 hour. Next, the indicated concentrations of compounds were added in either 2 or 12 mM glucose and the cells were incubated at 37°C for 1 hour. The supernatant was then collected and tested for insulin using the CisBio HTRF insulin assay kit. Data are represented as averages ± S.E.M. from three different islets. Donors are different between Figures 2A and 2B. Statistical significance was determined by one-way analysis of variance with Dunnett's post hoc analysis using GraphPad Prism 7.0 (GraphPad Software, Inc.).

Statistics. All data are expressed as the mean ± S.E.M. or S.D. as indicated in *Materials and Methods* and the figure legends of the

indicated number of experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) with Dunnett's post hoc analysis using GraphPad Prism 7.0 (GraphPad Software, Inc.).

Materials. The synthesis of Compound A is summarized in the Supplemental Methods. Forskolin and IBMX were obtained from Tocris Bioscience. DMEM/F12 and DMEM/high glucose media, penicillin/streptomycin, L-glutamine, G418, and hygromycin were purchased from Thermo Fisher Scientific. FBS was purchased from Hyclone, GE Healthcare Life Sciences (Logan, UT). HBSS and HEPES were purchased from CellGro, Corning Inc. (Corning, NY). Fatty acid free BSA was purchased from Sigma-Aldrich (St. Louis, MO). Human islets were obtained from healthy donors through Prodo Laboratories Inc. (Aliso Viejo, CA).

Results

Identification and Characterization of a New hGPR40 Full Agonist

Compound A Is a Full Agonist at the IP1/Calcium Pathway. Multiple series of hGPR40 agonists were rationally designed based on existing structures and evaluated in a calcium assay using a hGPR40 low-expressing CHO-K1 stable cell line to allow differentiation between partial and full agonists. In this assay, Compound A (Fig. 1A; Supplemental Methods) showed similar efficacy to AM-1638, previously reported as a highly efficacious hGPR40 full agonist (Hauge et al., 2014; Li et al., 2016), while fasiglifam was only weakly efficacious (Fig. 1B). To confirm the activity of Compound A, we used an IP1 HTRF assay, which detects the accumulation of IP1 inside the cells that follows the rapid degradation of IP3 (Fig. 1C). The IP1 HTRF assay has indeed been shown to generate less false positive results (Cassutt et al., 2007). Compound A was as efficacious as AM-1638 and showed superior efficacy compared with fasiglifam (E_{max} fasiglifam = 50.9% ± 1.2% compared to that of Compound A; ****P < 0.0001) (Fig. 1C). However, compared to Compound A and AM-1638 (EC_{50} = 225 ± 80 and 158 ± 27 nM, respectively) (Fig. 1C), fasiglifam (EC_{50} = 78 ± 30 nM) was about two to three times more potent (*P < 0.05).

We also profiled the activity of Compound A at the β-arrestin2 pathway and obtained similar results (Supplemental Fig. 1), with fasiglifam inducing about 50% efficacy compared with the full agonists (51.2% ± 8.3%; ****P < 0.0001). No significant bias was observed for any of the compounds between Gαq/11 and β-arrestin2.

Compound A Is Fully Efficacious at Potentiating GSIS in Human Islets. We then tested the ability of Compound A to potentiate GSIS in human islets from healthy donors. All donors tested were responsive to 12 mM glucose and non glucose-dependent insulin secretagogues, KCl, and glibenclamide. In the presence of 12 mM glucose, Compound A (3 and 10 μM) significantly potentiated insulin secretion (79.6 ± 18.5 and 78.7 ± 10.2 ng/ml, respectively; P < 0.0001) compared with islets treated with glucose alone (11.6 ± 6.2 ng/ml) (Fig. 2A). Fasiglifam potentiated 12 mM glucose-induced insulin secretion (26.7 ± 15.9 ng/ml; P = 0.0008) but to a lesser extent than Compound A (22.4% ± 6% of the potentiation induced by Compound A; P < 0.0001). The effects observed with Compound A and fasiglifam were consistent with the potentiation induced by AM-1638 compared with other partial agonists (Luo et al., 2012). Interestingly, in the presence of 2 mM glucose fasiglifam was inactive, whereas Compound A (3 and 10 μM) significantly potentiated insulin secretion (16.6 ± 4.7 and 41.2 ± 12.1 ng/ml, respectively; P < 0.0001) compared with islets treated with glucose alone (3.9 ± 1.2 ng/ml) (Fig. 2B).

Compound A Is an Allosteric Full Agonist at Gαq, Gαi2, and Gα12

Compound A Is a Full Agonist at Gαq and Gαi2, and Engages the Gα12 Pathway. In addition to the Gαq/IP1/calcium pathway, some GPR40 agonists have been shown to activate alternative pathways (Schröder et al., 2011; Lin et al., 2012; Defossa and Wagner, 2014; Hauge et al., 2014; Mancini et al., 2015). Thus, we used BRET-based biosensors to fully characterize other G proteins downstream from hGPR40 activation. Resonance energy transfer between a luminescent enzymatic donor and a fluorescent protein acceptor typically occurs in the 1–10 nm range, which makes BRET an ideal platform to study protein-protein interactions in living cells. BRET has indeed been extensively used to study G protein activation by multiple GPCRs (Denis et al., 2012; Salahpour et al., 2012; Namkung et al., 2016) and allows monitoring in real time the activation of G proteins of interest following GPR40 agonist treatment. We first used Gαq and Gα11 sensors (Fig. 3A; Supplemental Fig. 2A) to confirm the engagement of those pathways. IP1 production can indeed originate from other G protein couplings (Rives et al., 2009)

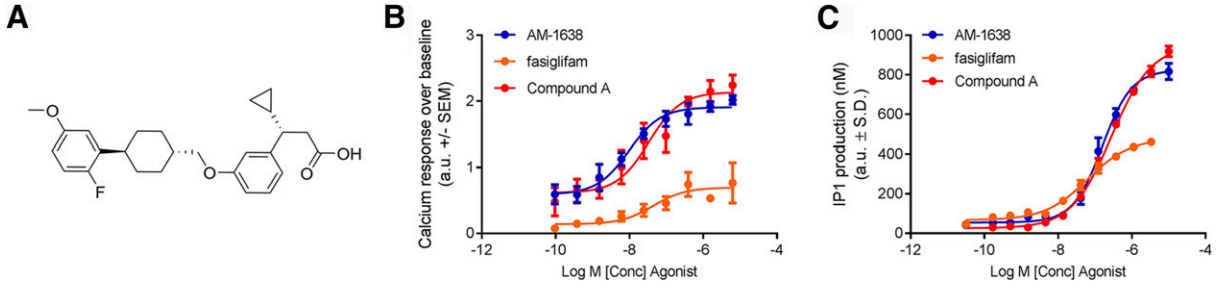

Fig. 1. Identification of a new full hGPR40 agonist, Compound A, at the Gαq/IP1/calcium pathway. (A) Structure of Compound A. (B) Calcium signaling in a CHO-K1 cell line stably expressing hGPR40. Compound A showed similar efficacy to AM-1638, previously reported as a highly efficacious hGPR40 full agonist and fasiglifam was only partially efficacious. Data presented are representative of three independent experiments performed in quadruplicate for each compound. Data are represented as averages ± S.E.M. (C) In a CHO-K1 cell line stably expressing hGPR40, Compound A was a full agonist at the IP1 pathway, with similar efficacy as AM-1638. Fasiglifam was a partial agonist with about 50% efficacy (50.9% ± 1.2%; P < 0.0001) compared with Compound A and AM-1638. Data presented are representative of three independent experiments performed in quadruplicate for each compound. Data are represented as averages ± S.D. Statistical significance was determined by one-way ANOVA with Dunnett's post hoc analysis using GraphPad Prism 7.0 (GraphPad Software, Inc.).

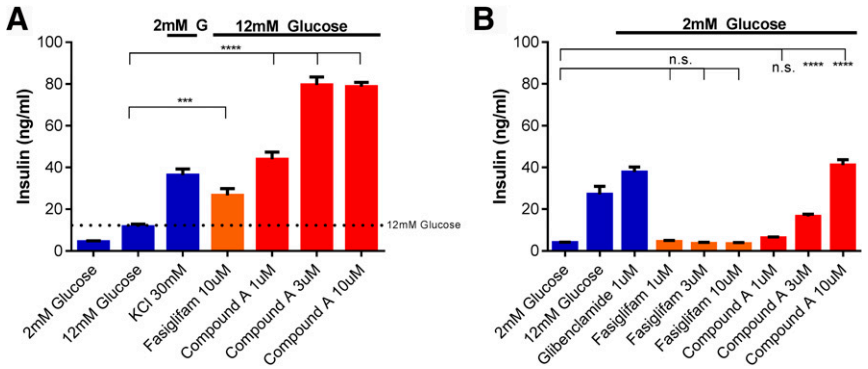

Fig. 2. Compound A is fully efficacious at potentiating GSIS in human islets. All donors tested were responsive to 12 mM glucose and non glucose-dependent insulin secretagogues, KCl or glibenclamide. (A) In the presence of 12 mM glucose, Compound A significantly potentiated insulin secretion compared with islets treated with glucose alone. The potentiation observed with fasiglifam was 22.4% ± 6% ($P < 0.0001$) of the potentiation induced by Compound A. (B) In the presence of low glucose (2 mM), fasiglifam was not able to potentiate GSIS. Stimulation with Compound A led to significant potentiation of insulin secretion but at higher concentrations than in the presence of high glucose. Data are represented as averages ± S.E.M. from three different islets. Donors are different between graphs (A and B). Statistical significance was determined by one-way ANOVA with Dunnett's post hoc analysis using GraphPad Prism 7.0 (GraphPad Software, Inc.).

and BRET sensors provide a straightforward approach to directly assess G protein activation, independently of downstream effectors and potential cross regulation between pathways. Compared with the IP1 assay, we obtained similar results with the Gαq and Gα11 sensors (Fig. 3A; Supplemental Fig. 2A). Compound A was a full agonist at the Gαq and Gα11 pathways with similar efficacy as α-linolenic acid, an endogenous GPR40 agonist, as well as AM-1638, and fasiglifam was a partial agonist with about 40% efficacy ($P < 0.0001$) (Fig. 3A; Table 1). However, in contrast to the IP1 assay, fasiglifam was less potent than Compound A and AM-1638 at recruiting Gαq (Table 1), suggesting fasiglifam might trigger the activation of other pathways leading to IP1 accumulation. Additionally, at both the Gαq and Gα11 pathways, Compound A- and AM-1638-induced responses, but not that induced by fasiglifam, appeared highly cooperative (Hill slope > 1) (Table 1).

We then measured the ability of Compound A to activate the Gαi/o pathway in hGPR40 expressing cells using Gαi2 (Fig. 3B), GαoB, and Gαz sensors (Table 1). Both Compound A and AM-1638 were full agonists at the Gαi/o pathway (Fig. 3B; Table 1) compared to α-linolenic acid, with Compound A being slightly more potent (Table 1). Interestingly, fasiglifam displayed intra-Gαi/o family bias by promoting partial activation of Gαi2 (~50% efficacy compared with Compound A and AM-1638), while being completely inactive on GαoB and Gαz (Fig. 3B; Table 1).

Compared to Compound A and AM-1638, fasiglifam was more potent at recruiting Gαi2 than Gαq/11 (Fig. 3, A and B; Table 1). Using the Black-Leff operational model and α-linolenic acid as a reference compound, we evaluated that fasiglifam was biased toward Gαi2 versus Gαq (bias factor = 5.86 compared to 0.19 for both Compound A and AM-1638), while Compound A and AM-1638 were slightly biased toward Gαq versus Gαi2 (bias factor = 5.2 compared to 0.17 for fasiglifam). These data could explain why fasiglifam was more potent than Compound A and AM-1638 at the IP1 pathway compared with the Gαq activation assay. It is, in fact, well known that Gαi/o coupling can lead to IP production and calcium signaling (Rives et al., 2009). To confirm the involvement of the Gαi/o pathway in fasiglifam-induced IP1 responses, we measured GPR40-mediated IP1 production following treatment with Compound A, AM-1638, and fasiglifam in the presence of PTX. PTX activity was first validated using the BRET Gαq and Gαi2 sensors. While PTX had no significant effect on Gαq activation (Supplemental Fig. 3A), it completely abolished Gαi2 coupling (Supplemental Fig. 3B).

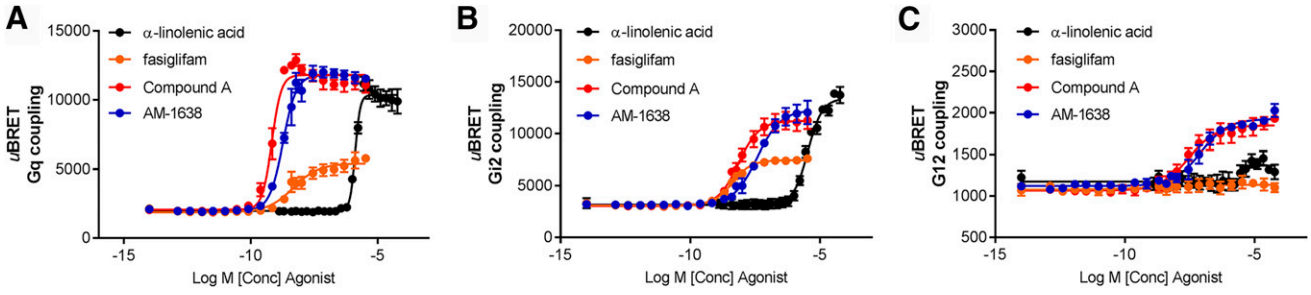

Fig. 3. Compound A is a full agonist at Gαq and Gαi2 and engages the Gα12 pathway. (A–C) BRET-based biosensor assays were used to directly monitor G protein activation following GPR40 agonist treatment. (A) Gαq sensor. Compound A and AM-1638 were full agonists at the Gαq pathway with similar efficacy as α-linolenic acid. Fasiglifam was a partial agonist with about 40% efficacy compared with Compound A. (B) Gαi2 sensor. Compound A and AM-1638 were highly efficacious agonists at the Gαi2 pathway with 82.5% ± 4.6% and 91.6% ± 4.5% efficacy, respectively, compared with α-linolenic acid. Fasiglifam was a partial agonist at Gαi2 with about 40% efficacy (43.5% ± 2.0%; $P < 0.0001$) compared with Compound A. (C) Gα12 sensor. While fasiglifam was inactive at Gα12, Compound A and AM-1638 induced activation of the Gα12 protein similarly to the ghrelin receptor (Supplemental Fig. 2B) (Sivertsen et al., 2011; Evron et al., 2014). Symbols represent the mean ± S.E.M. from at least three independent experiments performed in duplicates.

TABLE 1
Potency (EC_{50}) and efficacy relative to α-linolenic acid (E_{max}, percentage of α-linolenic acid ± S.D.) of fasiglifam, Compound A, and AM-1638 at multiple G proteins using BRET-based sensors

G Protein and Parameters	Fasiglifam	Compound A	AM-1638
Gq			
E_{max} (% α-linolenic acid)	40.9 ± 4.4[a]	112.8 ± 5.6	115.2 ± 2.9
EC_{50} (nM) (average ± S.D.)	8.3 ± 2.2[a]	0.55 ± 0.03	1.9 ± 0.3
Hill Slope, average (95% CI)	0.9 (0.5–1.5)	2.3 (1.7–4.4)	1.6 (1.3–2.1)
G11			
E_{max} (% α-linolenic acid)	35.8 ± 4.4[a]	122.7 ± 10.0	111.9 ± 9.4
EC_{50} (nM) (average ± S.D.)	10.1 ± 4.9[a]	0.3 ± 0.3	1.1 ± 0.3
Hill Slope, average (95% CI)	0.5 (0.3–0.8)	2.8 (2.0–6.5)	2.5 (1.7–∞)
G12			
E_{max} (% α-linolenic acid)	N.A.[b]	>300	>300
EC_{50} (nM) (average ± S.D.)	N.A.[b]	28.7 ± 12.5	83.9 ± 26.5
Hill Slope, average (95% CI)	N.A.[b]	0.8 (0.5–1.3)	0.8 (0.6–1.1)
G13			
E_{max} (% α-linolenic acid)	N.A.[b]	>200	>200
EC_{50} (nM) (average ± S.D.)	N.A.[b]	209 ± 105	154 ± 96
Hill Slope, average (95% CI)	N.A.[b]	1.0 (0.6–2.0)	1.0 (0.6–2.0)
Gs			
E_{max} (% α-linolenic acid)	N.A.[b]	N.D.	N.D.
EC_{50} (nM) (average ± S.D.)	N.A.[b]	N.D.	N.D.
Hill Slope, average (95% CI)	N.A.[b]	N.D.	N.D.
Gi2			
E_{max} (% α-linolenic acid)	43.5 ± 2.0[a]	82.5 ± 4.6	91.6 ± 4.5
EC_{50} (nM) (average ± S.D.)	4.2 ± 1.0	7.2 ± 1.8	38.0 ± 9.3
Hill Slope, average (95% CI)	1.2 (0.9–1.6)	1.1 (0.8–1.5)	1.0 (0.8–1.2)
GoB			
E_{max} (% α-linolenic acid)	N.A.[b]	72.9 ± 4.5	81.7 ± 5.9
EC_{50} (nM) (average ± S.D.)	N.A.[b]	69.9 ± 84.1	277 ± 122
Hill Slope, average (95% CI)	N.A.[b]	0.7 (0.5–1.0)	0.6 (0.5–0.9)
Gz			
E_{max} (% α-linolenic acid)	N.A.[b]	151.5 ± 10.0	148.4 ± 25.6
EC_{50} (nM) (average ± S.D.)	N.A.[b]	11.5 ± 4.4	114 ± 58
Hill Slope, average (95% CI)	N.A.[b]	0.8 (0.7–0.9)	0.7 (0.6–0.9)

CI, confidence interval; N.A., not applicable; N.D., not determined.
[a]Significantly different from that of Compound A and AM-1638 ($P < 0.05$).
[b]$EC_{50} > 50$ μM and/or $E_{max} < 10$.

The efficacy of Compound A and AM-1638 at inducing IP1 production was not significantly affected by PTX treatment but the potency of both compounds was slightly reduced (3.5 ± 0.4-fold and 2.4 ± 0.1-fold, respectively) (Supplemental Fig. 3, C and D). However, fasiglifam-induced IP1 response was almost completely abolished by PTX treatment (Supplemental Fig. 3E), suggesting that in contrast to Compound A and AM-1638, fasiglifam-induced IP1 production was mostly driven by Gαi/o coupling.

We also profiled the activity of Compound A at the Gα12/13 pathway. Interestingly, while fasiglifam failed to recruit Gα12, Compound A and AM-1638 strongly activated the Gα12 protein in hGPR40-expressing cells (Fig. 3C; Table 1). The magnitude of the Gα12 response following activation of hGPR40 by Compound A and AM-1638 was substantial and similar to that induced by ghrelin in cells expressing the ghrelin receptor (Supplemental Fig. 2B) (Sivertsen et al., 2011; Evron et al., 2014). Interestingly, α-linolenic acid only very weakly activated the pathway, suggesting that the ability to activate Gα12 is a unique property of the synthetic full agonists AM-1638 and Compound A. We obtained similar results at Gα13 (Supplemental Fig. 2C). Although the Gα12/G13-mediated signaling pathway is poorly understood, it has been linked to PKD activation as well as actin remodeling (Yuan et al., 2001; Siehler, 2007), which are well known to contribute to the release of vesicles.

Compound A Only Weakly Triggers Gαs Activation/cAMP Production. As mentioned previously, it has been shown that in addition to the Gαq/IP1/calcium pathway, some GPR40 agonists could induce coupling to other pathways (Schröder et al., 2011; Mancini and Poitout, 2013; Hauge et al., 2014, 2017; Mancini et al., 2015). More specifically, it has been shown that allosteric full agonists such as AM-1638, but not partial agonists, induced coupling to the Gαs/cAMP pathway and that only agonists at both Gαq and Gαs could trigger maximal efficacy in relevant preclinical models, such as GLP-1 secretion in mice (Luo et al., 2012; Hauge et al., 2014). To assess the ability of Compound A to induce signaling through the Gαs/cAMP pathway, we also used a BRET-based Gαs sensor (Fig. 4A). In cells transfected with the GLP-1 receptor, a well-known Gαs-coupled receptor, GLP-1[7–36] induced a strong Gαs response, confirming the functionality of the Gαs biosensor. However, in hGPR40-transfected cells, only a very weak response could be measured after stimulation with either Compound A or α-linolenic acid, about 10%–20% of the GLP-1 response. Fasiglifam was inactive (Fig. 4A). Surprisingly, AM-1638 also only weakly induced Gαs activation (Fig. 4A).

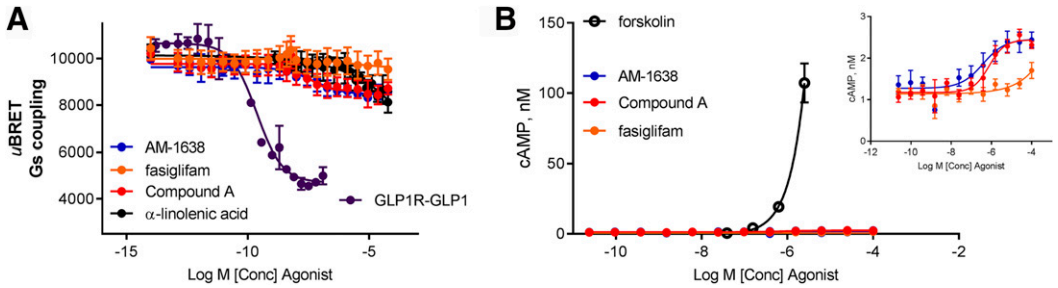

Fig. 4. Compound A and AM-1638 only weakly activate the Gαs/cAMP pathway. (A) BRET-based Gαs biosensor was used to directly monitor Gαs protein activation following GPR40 agonist treatment. In cells transfected with the GLP-1 receptor, a well-known Gαs-coupled receptor, GLP-1[7–36] induced a strong Gαs response. In hGPR40-transfected cells, Compound A, AM-1638, and α-linolenic acid only induced a very weak response and fasiglifam was inactive. (B) In hGPR40-expressing cells, fasiglifam was inactive at inducing increases in cAMP production and Compound A and AM-1638 only weakly activated the cAMP pathway compared with the forskolin control performed in the same cells. Data presented are representative of three independent experiments performed in quadruplicate for each compound. Data are represented as averages ± S.D.

To confirm those findings, we also measured cAMP accumulation in the hGPR40 stable CHO-K1 cell line mentioned previously. As previously described (Hauge et al., 2014), fasiglifam was inactive and did not induce any significant increases in cAMP accumulation. Interestingly, although Compound A and AM-1638 induced some cAMP accumulation, the magnitude of the cAMP response was very weak compared with the forskolin control performed in the same cells (Fig. 4B).

Compound A Is an Allosteric Full Agonist. Three distinct binding sites have been described for GPR40, one that binds endogenous fatty acids such as α-linolenic acid, one that binds partial agonists such as fasiglifam, and one that binds allosteric full agonists, such as AM-1638 and the recently reported AP8 (Lin et al., 2012; Defossa and Wagner, 2014; Hauge et al., 2014; Srivastava et al., 2014; Lu et al., 2017). The resolution of the crystal structure of hGPR40 in complex with both a partial agonist and the full agonist AP8 recently identified the allosteric full agonists' binding site as a lipid-facing pocket outside the transmembrane helical bundle (Lu et al., 2017), between TM4 and TM5.

To assess the orthosteric or allosteric nature of Compound A, we analyzed the functional cooperativity between α-linolenic acid and Compound A using BRET-based Gαq (Fig. 5A) and Gαi2 sensors (Fig. 5B) compared with that of fasiglifam and AM-1638. All three compounds could potentiate an EC_{20} of α-linolenic acid at inducing Gαq and Gαi2 coupling. The relative potencies and efficacies of Compound A, AM-1638, and fasiglifam in (PAM) mode (Fig. 5) were consistent with those previously observed in agonist mode (Fig. 3). Compound A (0.17 ± 0.04 and 2.0 ± 0.4 nM at Gαq and Gαi2, respectively) was slightly more potent than AM-1638 (0.6 ± 0.3 and 5.0 ± 1.5 nM at Gαq and Gαi2, respectively) at potentiating α-linolenic acid–induced Gαq and Gαi2 coupling. Moreover, compared with Compound A and AM-1638, fasiglifam only induced a partial potentiation of α-linolenic acid responses (60% ± 6% and 53% ± 3% at Gαq and Gαi2, respectively) (Fig. 5). These data confirm the allosteric nature of Compound A, potentiating α-linolenic acid–induced responses.

We then used a computational approach to assess whether Compound A could bind to the same binding site as other reported allosteric full agonists. Compound A was docked in the lipid-facing pocket identified by Lu et al. (2017) between TM4 and TM5. The best docking pose of Compound A revealed a similar binding mode as AP8 (Fig. 6A). Among the interactions between Compound A and the protein, the carboxylate group anchored the compound between TM4 and TM5 via a complex H-bond network with Ser123 and Tyr44, and probably with Tyr114 from intracellular loop 2, folded in an alpha helix in the presence of the full agonists (Fig. 6B). The 5-fluoro-2-methoxy phenyl ring formed a CH:π interaction with the side chain of Pro194. The rest of Compound A made numerous Van der Walls contacts with the hydrophobic residues forming the binding grove (Ala98, Ala99, Ala102, Val126, Ile130,

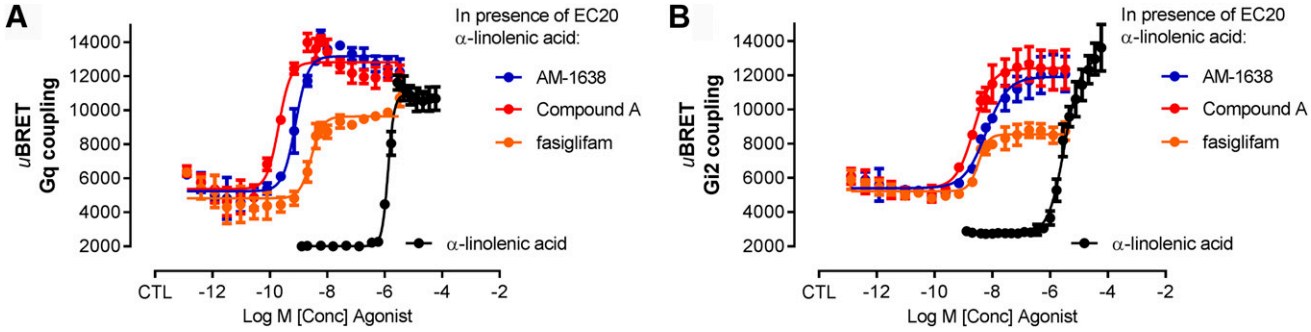

Fig. 5. Compound A, AM-1638, and fasiglifam potentiate α-linolenic acid–induced coupling to Gαq and Gαi2. (A and B) BRET-based biosensor assays were used to directly monitor G protein activation following GPR40 agonist treatment. (A) Gαq sensor. Compound A, AM-1638, and fasiglifam potentiated an EC_{20} of α-linolenic acid at inducing Gαq coupling to hGPR40. (B) Gαi2 sensor. Compound A, AM-1638, and fasiglifam potentiated an EC_{20} of α-linolenic acid at inducing Gαi2 coupling to hGPR40. For each compound, data from two independent experiments performed in duplicates were combined and symbols presented are the mean ± S.E.M.

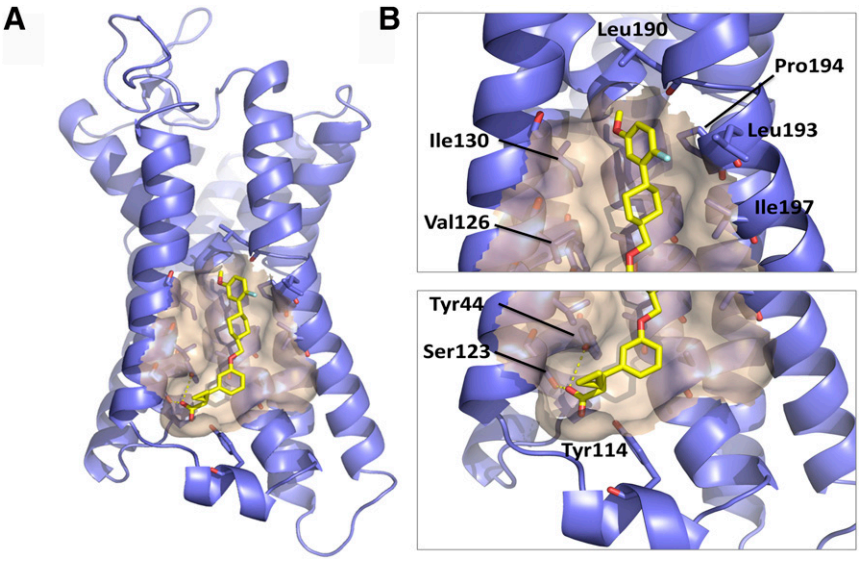

Fig. 6. Docking of Compound A in an allosteric pocket. (A) Docking of Compound A in the lipid-facing pocket between TM4 and TM5 identified by Lu et al. (2017) as the allosteric full agonists' binding site. (B) Ligand: receptor interactions between Compound A and hGPR40 predicted from the molecular docking of Compound A into in the lipid-facing pocket identified by Lu et al. (2017) between TM4 and TM5.

Leu193, and Ile197) (Fig. 6B). While it is clear that multiple ligand:protein interactions contribute to the potency of Compound A, the physicochemical properties of the compound suggest it could also make numerous contacts with surrounding membrane lipids (missing in the X-ray structure).

Furthermore, we also performed radioligand binding experiments using both [^{3}H]-Compound A and [^{3}H]-AM-1638, providing additional evidence that Compound A could bind to the same site as AM-1638. Competition binding experiments showed that Compound A, as well as AM-1638, completely displaced the binding of both [^{3}H]-Compound A (Supplemental Fig. 4A) and [^{3}H]-AM-1638 (Supplemental Fig. 4B). Data were fitted quite well by a one-site competition binding model (Supplemental Table 1), providing additional evidence that both compounds bind to an identical unique binding site. Additionally, fasiglifam had a positive cooperative effect on the binding of [^{3}H]-Compound A (Supplemental Fig. 4A). The effects observed with fasiglifam are similar to those previously reported in the literature (Yabuki et al., 2013; Lu et al., 2017; Plummer et al., 2017) and are consistent with the allosteric nature of this compound.

Discussion

GPR40 is a clinically validated molecular target for the treatment of diabetes. Although the partial agonist fasiglifam (TAK-875) showed efficacy in phase III clinical trials, its efficacy did not significantly differentiate from glimepiride and attention has shifted toward the development of full agonists that exhibit superior efficacy in preclinical models (Schröder et al., 2011; Luo et al., 2012; Hauge et al., 2014, 2017; Mancini et al., 2015). In the present study, we described the pharmacology of Compound A, a newly identified GPR40 allosteric full agonist at the Gαq/IP1/calcium pathway fully efficacious at enhancing GSIS in human islets. We compared Compound A–induced GPR40 activity at a panel of G proteins and to that of both fasiglifam and AM-1638, another allosteric full agonist previously reported to be highly efficacious in preclinical models (Luo et al., 2012; Hauge et al., 2014).

In human islets, in the presence of high glucose, Compound A was highly efficacious at potentiating insulin secretion and data were consistent with those reported for AM-1638 (Luo et al., 2012). Despite 40%–50% efficacy compared to Compound A and AM-1638 at the Gαq/IP1/calcium pathway, in human islets and in presence of high glucose, fasiglifam efficacy was only about 22.4% of that of Compound A at potentiating insulin secretion. Moreover, Compound A, but not fasiglifam, could potentiate insulin secretion in low glucose conditions. These data suggest that the pharmacology of GPR40 is complex and that the activation of additional pathways might be responsible for the superior efficacy of Compound A in human islets.

Previous studies have suggested that activation of alternative pathways in addition to the Gαq/calcium pathway was required for maximal efficacy in preclinical models (Lin et al., 2012; Defossa and Wagner, 2014; Hauge et al., 2014). Thus, only allosteric full agonists, such as AM-1638, which in

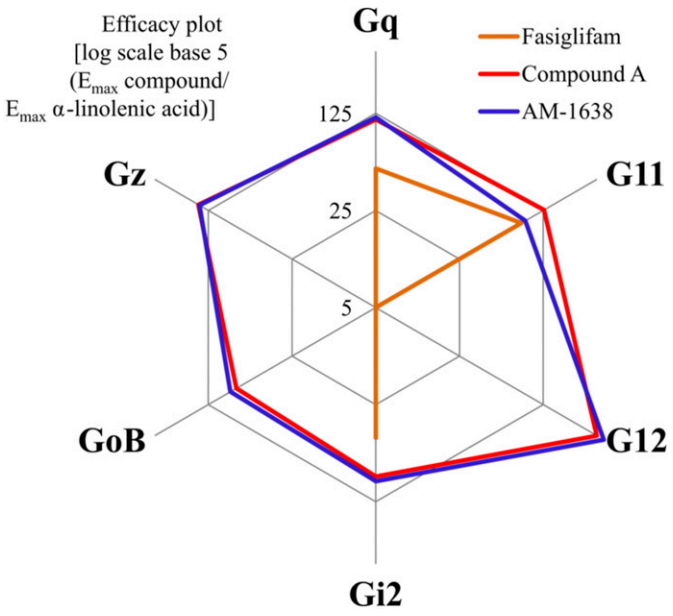

Fig. 7. Efficacy plot of Compound A, AM-1638, and fasiglifam at multiple G proteins compared to α-linolenic acid: [log scale base 5(E_{max} compound/E_{max} α-linolenic acid)].

addition to the Gαq/calcium pathway were shown to induce cAMP production, could trigger maximal efficacy in preclinical models, such as GLP-1 secretion in mice (Luo et al., 2012; Hauge et al., 2014, 2017). Interestingly, even though Compound A binds to the same site as AM-1638 (Fig. 6; Supplemental Fig. 4), it showed no to very little efficacy at the Gαs/cAMP pathway. The magnitude of the cAMP response produced after stimulation with Compound A was very low and hGPR40 only very weakly coupled to Gαs after stimulation with Compound A (Fig. 4). Surprisingly, AM-1638 also only weakly induced Gαs activation and it is noteworthy that the magnitude of cAMP accumulation observed was similar to that previously reported (Hauge et al., 2014). Thus, although we cannot exclude the possibility that weak GPR40-mediated cAMP accumulation is enough to potentiate GLP-1 secretion or that mouse GPR40 coupling properties might significantly differ from hGPR40, our data suggest that hGPR40 does not efficiently couple to the Gαs/cAMP pathway and that pathways other than Gαs might be involved in GPR40 agonists in vivo efficacy. Moreover, our findings suggest that cAMP production measured in vitro may originate from other non-Gαs-mediated couplings. It has indeed been shown that some adenylyl cyclase isoforms are calcium sensitive (Halls and Cooper, 2011), raising the possibility that the weak cAMP responses observed after GPR40 stimulation could come from cross regulation between pathways.

We, therefore, assessed the ability of our compound to induce the activation of other G proteins. Figure 7 shows an efficacy plot representing the relative efficacy of Compound A and AM-1638, as well as fasiglifam, relative to α-linolenic acid at multiple G proteins. The graph highlights the ability of Compound A and AM-1638 to activate the Gαq/11 and Gαi/o protein families, while fasiglifam was only a partial agonist at some of those pathways. The poor efficacy of fasiglifam is consistent with recent crystallography studies, showing that in complex with fasiglifam the intracellular portion of the receptors was in an inactive-like state (Srivastava et al., 2014; Lu et al., 2017). Moreover, in contrast to fasiglifam, at both Gαq and Gα11, Compound A- and AM-1638-induced responses appeared highly cooperative (Hill slope > 1) (Table 1). Since the binding data (Fig. 6; Supplemental Fig. 4; Supplemental Table 1) suggest the existence of only one binding site for these compounds, it is likely that Compound A and AM-1638 stabilize a unique conformation of the receptor, distinct from that stabilized by fasiglifam, and that this conformation is further stabilized by Gαq, but not other G proteins. It is indeed now well known that downstream effectors such as G proteins can allosterically modulate the receptor and stabilize active or inactive conformations (Rasmussen et al., 2011).

Although activation of the Gαq/IP1/Ca^{2+} pathway was shown to lead to insulin secretion and Gαi/o coupling is known to potentiate Gαq-mediated IP1 and calcium responses (Rives et al., 2009), the activation of Gαi/o-coupled receptors is usually associated with a decrease in GSIS, through the inhibition of adenylyl cyclases (Fridlyand and Philipson, 2016). In contrast to Compound A and AM-1638, fasiglifam appeared slightly biased toward Gαi2 versus Gαq and fasiglifam-induced IP1 production was more sensitive to PTX treatment than that of Compound A and AM-1638. The extent to which these differences contribute to differences in efficacy and/or safety is not clear but it could explain the weak efficacy of fasiglifam at potentiating GSIS (<25%) despite 40%–50% efficacy compared to Compound A and AM-1638 at the Gαq/IP1 pathway.

Compound A and AM-1638 also induced hGPR40 coupling to Gα12 (Fig. 3C), while fasiglifam was inactive and α-linolenic acid only weakly activated the pathway. Although other agonists should be evaluated in this assay, these data suggest that the ability to activate Gα12 is a unique property of synthetic allosteric full agonists. The role of the Gα12/13 pathway in insulin and incretin secretion is poorly understood but it has been linked to PKD activation as well as actin remodeling, well known to contribute to vesicle release (Siehler, 2007; Ferdaoussi et al., 2012; Kalwat and Thurmond, 2013; Arous and Halban, 2015). Insulin secretion in response to glucose is biphasic, with a rapid and transient first phase followed by a slower but prolonged second phase. It is believed that first-phase insulin secretion corresponds to the exocytosis of a readily releasable pool of insulin granules predocked at the plasma membrane, whereas the second phase relies on the mobilization of an intracellular granule pool to the plasma membrane via a process that requires cytoskeletal remodeling. PKD activation has also been linked to the second phase of insulin release (Ferdaoussi et al., 2012; Kalwat and Thurmond., 2013).

The $G\alpha_{12}/G_{13}$ proteins activate the monomeric GTPases RhoA. RhoA effectors include Rho kinase, which leads to Jun kinase activation and the induction of actin stress fiber formation (Siehler, 2007). The involvement of the cytoskeleton in secretion mechanisms was proposed almost 50 years ago, and although the precise mechanisms are not yet fully understood, it is now well accepted that actin regulates insulin granule trafficking and exocytosis (Arous and Halban, 2015). Constitutively active Gα12/13 was found to induce stress fiber formation and focal adhesion assembly in fibroblasts, similarly to activated Gα12/13-linked lysophosphatidic acid receptors and constitutively active RhoAQ63L (Siehler, 2007). This suggests that Gα12 activation might play a critical role in secretion mechanisms, and our data raise the intriguing possibility that despite weak Gαs signaling, the ability of Compound A and AM-1638 to signal through the $G\alpha_{12}$ pathway may contribute to the release of vesicles and be an important determinant of GPR40 agonist efficacy. It would be interesting to assess the efficacy of Compound A in mice at inducing GLP-1 secretion, as well as in type-2 diabetes human islets where actin remodeling has been shown to be altered (Arous and Halban, 2015). Although the role of Gα12/13 downstream of GPR40 in insulin and incretin secretion needs to be validated both ex vivo and in vivo, while Compound A was more potent at Gαq and Gαi2 compared to Gα12 (Table 1), in human islets, it is noteworthy that Compound A showed maximal efficacy only at concentrations greater than 1 μM (Fig. 2).

Nevertheless, the superior efficacy of Compound A in human islets in low glucose conditions suggests that Compound A administration might lead to hypoglycemia and activation of Gα12/13 could be contraindicated to avoid insulin secretion in low glucose conditions. Moreover, in addition to its role in insulin secretion, PKD activation has been linked to NF-kB activation, the development of inflammation, and pancreatitis (Yuan and Pandol, 2016). Although GPR40 does not seem expressed in the exocrine pancreas, it would be interesting to assess whether Compound A could yield inflammatory responses after either acute or chronic treatment.

In conclusion, we have identified Compound A, a new GPR40 allosteric full agonist fully efficacious at enhancing GSIS in human islets. Compound A was a full agonist at Gαq, Gαi2, and Gα12, with no to very weak efficacy at the Gαs/cAMP pathway. Although more work is needed to validate the role of the GPR40-mediated Gα12 pathway in secretion mechanisms, our data suggest that the pharmacology of GPR40 is complex and that engagement of multiple signaling pathways may be critical to achieve sufficient therapeutic efficacy.

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Authorship Contributions

Participated in research design: Rives, Rady, Zhao, Bakaj, Lee, Player, Pocai.

Conducted experiments: Rives, Rady, Swanson, Zhao, Qi, Bakaj, Mancini.

Contributed new reagents or analytic tools: Player.

Performed data analysis: Rives, Rady, Zhao, Arnoult, Bakaj, Mancini, Breton, Lee, Player, Pocai.

Wrote or contributed to the writing of the manuscript: Rives, Rady, Arnoult, Mancini, Breton, Player.

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