Identification of novel species-selective agonists of the G-protein-coupled receptor GPR35 that promote recruitment of \( \beta \)-arrestin-2 and activate G\( \alpha \)13

Laura JENKINS*, Jose BREA†, Nicola J. SMITH*, Brian D. HUDSON*, Graeme REILLY*, Nia J. BRYANT‡, Marian CASTRO†, María-Isabel LOZA† and Graeme MILLIGAN*†

*Molecular Pharmacology Group, Institute of Neuroscience and Psychology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K., †Departamento de Farmacología, Instituto de Farmacia Industrial, Universidad de Santiago de Compostela, Campus Sur, Santiago de Compostela 15782, Spain, and ‡Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

The poorly characterized G-protein-coupled receptor GPR35 has been suggested as a potential exploratory target for the treatment of both metabolic disorders and hypertension. It has also been indicated to play an important role in immune modulation. A major impediment to validation of these concepts and further study of the role of this receptor has been a paucity of pharmacological tools that interact with GPR35.

Using a receptor-\( \beta \)-arrestin-2 interaction assay with both human and rat orthologues of GPR35, we identified a number of compounds possessing agonist activity. These included the previously described ligand zaprinast. Although a number of active compounds, including cromolyn disodium and dicumarol, displayed similar potency at both orthologues of GPR35, a number of ligands, including pamoate and niflumic acid, had detectable activity only at human GPR35 whereas others, including zaprinast and luteolin, were markedly selective for the rat orthologue.

Previous studies have demonstrated activation of G\( \alpha \)13 by GPR35. A *Saccharomyces cerevisiae*-based assay employing a chimaeric Gpa1–G\( \alpha \)13 G-protein confirmed that all of the compounds active at human GPR35 in the \( \beta \)-arrestin-2 interaction assay were also able to promote cell growth via G\( \alpha \)13. Each of these ligands also promoted binding of \([\text{35S}]\text{GTP}^g\) (guanosine 5′-\( \gamma \)-[\text{35S}]thio\)triphosphate) to an epitope-tagged form of G\( \alpha \)13 in a GPR35-dependent manner. The ligands identified in these studies will be useful in interrogating the biological actions of GPR35, but appreciation of the species selectivity of ligands at this receptor will be vital to correctly attribute function.

Key words: agonist, \( \beta \)-arrestin-2, drug discovery, G-protein-coupled receptor (GPCR), GPR35, screening.

INTRODUCTION

GPR35 is a poorly characterized GPCR (G-protein-coupled receptor) [1] that has been suggested to be a potential exploratory target for the treatment of both metabolic disorders [2] and hypertension [3]. It has also been indicated to play important roles in immune modulation [4] and possibly in the development of gastric cancers [5]. Both the tryptophan metabolite kynurenic acid [6] and the synthetic ligand zaprinast [2-(2-proplyoxyphenyl)-8-azapurin-6-one] [7] have been shown to possess agonist activity at GPR35. The role of kynurenic acid as an endogenous agonist at human GPR35 has, however, been clouded by studies which show that its potency to promote interaction of this receptor with \( \beta \)-arrestin-2 and to cause internalization of the receptor in transfected cell systems is above 100 μM [8], well above the concentrations of kynurenic acid reported to be achieved in bile, even in disease states such as cholecystolithiasis and obstructive jaundice [9]. This contrasts with a recent report of potent effects of this ligand to induce firm arrest of human monocytes on ICAM-1 (intercellular adhesion molecule 1)-expressing human umbilical vein endothelial cells via a \( \beta 2 \)-integrin-mediated process, with action reported at concentrations as low as 300 nM [4]. The use of zaprinast to explore the role of GPR35 in cell systems and in models of disease is also limited because this ligand is also an effective inhibitor of cGMP phosphodiesterases [10]. It is thus challenging to unravel the relative contribution of these two effects. Equally challenging is that rodent forms of GPR35 display significantly higher potency for both zaprinast and kynurenic acid than the human orthologue and, although we have recently shown that two novel thiazolidinediones also display agonism and have similar potency at rat and human GPR35 [8], thiazolidinediones can have a wide range of molecular targets, including nuclear hormone receptors of the PPAR\( \gamma \) (peroxisome-proliferator-activated receptor \( \gamma \)) class [11]. Identification of other ligands and a clear understanding of their relative potency/affinity at orthologues of GPR35 is therefore required to better understand the function and regulation of this receptor. As a step towards this goal, we have screened the Prestwick Chemical Library® for compounds able to activate either or both human and rat GPR35 using a BRET (bioluminescence resonance energy transfer)-based assay [8,12,13] that reports interactions between GPR35 and \( \beta \)-arrestin-2. This library contains 1120 small-molecule marketed drugs with known bioavailability and safety characteristics, thus potentially allowing hits to be used to probe the function of GPR35 in vivo. Following the initial screen and hit re-confirmation we have also assessed whether the ligands are able to promote activation of the heterotrimeric G-protein G\( \alpha \)13, which we have recently shown to be stimulated selectively by GPR35 [8]. Confirmed actives that were essentially equipotent at the human and rat orthologues were the anti-asthma drug cromolyn [5,5′-(2-hydroxypropane-1,3-diy]bis(oxyl)bis(4-oxo-4\( H \)-chromene-2-carboxylic acid)] disodium and the anticoagulant/vitamin K antagonist dicumarol [3,3′-methylenbis(4-hydroxy-2\( H \)-chromen-2-one)]. Significant species selectivity was also observed. For example, the drug congener pamoate [4,4′-methylenbis(3-hydroxy-2-naphthoic...
acid] was a potent agonist at human GPR35 but almost inactive at rat GPR35 whereas, although less potent, niflumic acid (2-[(3-trifluoromethyl)phenyl]amino)nicotinic acid) was also highly selective in promoting interactions with β-arrestin-2 via human GPR35. In contrast, both zaprinast and the flavened luteolin [2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone] displayed substantial selectivity for rat GPR35. A number of these compounds may be useful for exploring the physiological functions of GPR35 but, as described previously for zaprinast [7,8], species selectivity needs to be considered before selecting ligands to perform such studies.

EXPERIMENTAL

Epitope tagging of GPR35

A FLAG epitope (amino acid sequence DYKDDDDK) was introduced in the N-terminal end of human and rat GPR35 cDNA by PCR using the following primers: human sense, 5′-GGGAAGCTTCCACCATGGATTACAAGGATGACGACGAGACTAAGAATGGCACCTACAACCCAACC-3′; rat sense, 5′-CGCGGATCCGCCACCATGGATTACAAGGATGACGACGAGATAAGAATGGCACCTACAACCCAACC-3′; rat anti-sense, 5′-GGCGGCCGCAGGCGATATAgGATGACGACGAGGCCTAGAGTTGACCAAC-3′; and human antisense, 5′-GGCGGCCGCAGGCGATATAgGATGACGACGAGGCCTAGAGTTGACCAAC-3′. The HindIII and NotI restriction sites used for cloning are underlined. The resulting cDNA was subsequently cloned in-frame into the HindIII/BamHI and NotI sites of an EYFP-pcDNA5/FRT/TO plasmid (where EYFP is enhanced yellow fluorescent protein) yielding the final N-terminal epitope- and C-terminal fluorescent-protein-tagged constructs FLAG–human GPR35–EYFP constructs. Buffer containing 5 M zaprinast and assay buffer (1% DMSO) were included in all wells. Each plate contained two control wells containing 0.5% DMSO, and two ‘Mock’ points with cells transfected with pcDNA3. Cells were washed with HBSS and incubated for 30 min at 37 °C, after this time a fluorescence reading was performed to check the efficiency of transfection with the GPR35–EYFP constructs. Buffer containing 5 M coelenterazine-h was added to all wells and cells were incubated for 10 min at 37 °C. After this time, compounds were added and cells were incubated for 5 min at 37 °C and subsequent BRET measurements were carried out using a PHERAstar FS reader (BMG-Labtech). Compound autofluorescence was assessed for all of the hits obtained in the assay in order to detect those compounds that might be false positives simply because they affected BRET measurements.

Cell culture and BRET studies

HEK-293T cells [human embryonic kidney-293 cells expressing the large T-antigen of SV40 (simian virus 40)] were maintained in Dulbecco’s modified Eagle’s medium supplemented with 0.292 g/L-glutamine and 10% (v/v) newborn calf serum at 37 °C in a 5% CO2 humidified atmosphere. Cells were transfected with the required receptor species orthologue of GPR35 tagged with EYFP and with β-arrestin-2–Renilla luciferase 6 (ratio 4:1), using 1 mg/ml polyethyleneimine, linear MW-25000 (molecular mass 25000 Da) (ratio 1:6 DNA/polyethyleneimine), diluted in 150 mM NaCl, pH 7.4. After incubation at room temperature (22 °C) for 10 min, the mixture was added to HEK-293T cells. Cells were incubated for 24 h then transferred (50 000 cells per well) to 96-well plates coated with poly-D-lysine. In all experiments, the total amount of DNA transfected was equalized between constructs by the addition of the empty expression vector. For the BRET studies, an additional transfection was performed with only the Renilla luciferase construct and empty expression vector. After 24 h, cells were washed twice with HBSS (Hanks balanced salt solution) (pH 7.4), and coelenterazine-h (Promega) was added to a final concentration of 5 μM. Cells were incubated in the dark for 10 min at 37 °C before addition of ligands. Cells were incubated for a further 5 min at 37 °C before BRET measurements were performed using a PHERAstar FS reader (BMG-Labtech, Offenburg, Germany). The BRET ratio was calculated as emission at 530 nm/emission at 485 nm. Net BRET was defined as the 530 nm/485 nm ratio of cells co-expressing Renilla luciferase and EYFP minus the BRET ratio of cells expressing only the Renilla luciferase construct in the same experiment. This value was multiplied by 1000 to obtain mBRET units.

Compound collection and storage

For the screen, we utilized a commercially available 1120 compound bioactives set of marketed drugs and pharmacologically active agents (Prestwick Chemicals). Compounds were formatted into 96-well daughter sets at 1 mM in 100% DMSO (80 compounds per plate) and active working sets stored at −20 °C until required.

Data analysis and processing

Data from primary screenings were analysed using Microsoft Excel software. The activities of the compounds were calculated following the formula:

\[ \text{Activity (\%) = (mBRET compound - mBRET basal)/} \]
\[ \text{(mBRET stim - mBRET basal) \times 100} \]

where mBRET compound is the mBRET value obtained from wells treated with the test compound, mBRET basal is the average of the mBRET values obtained from wells treated with assay buffer and mBRET stim is the average of the mBRET values obtained from cells treated with 10 μM zaprinast. Compounds were considered to be possible hits if the activity was higher than the mean + (3 × S.D.) of the overall activity in the whole assay. Hits were considered to be confirmed if the activity remained over this threshold in a second independent assay.

Reliability of the assay was estimated by calculating Z’ values for each plate, using the formula:

\[ Z' = 1 - \left[ \frac{(3 \times \sigma_{\text{stim}}) + (3 \times \sigma_{\text{basal}})}{(\mu_{\text{stim}} - \mu_{\text{basal}})} \right] \]

where σ_{stim} and σ_{basal} are the S.D. values of wells containing 10 μM zaprinast and assay buffer respectively and μ_{stim} and μ_{basal} are the means for wells containing 10 μM zaprinast and assay buffer respectively. A Z’ value higher than 0.5 was selected as the cut-off in the screen.
Compound re-supply

Compounds that were active by the definition above were re-ordered from Sigma.

Yeast Gα13 assay

Human GPR35 bearing an N-terminal FLAG epitope as described previously [8] was subcloned from pcDNA5/FRT/TO into a yeast high-copy number 2μ plasmid containing a strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (p426GPD) using BamHI and PaeR7I and verified by DNA sequencing. FLAG–GPR35 or empty vector was transformed into Saccharomyces cerevisiae strains modified as described previously [14] to express either wild-type yeast Gα protein (Gpa1; strain MMY12) or a chimaeric version of Gpa1 in which the last five amino acids of the yeast G-protein were replaced by the C-terminal five amino acids from human Gα13 (QLMLQ; Gpa1–Gα13; MMY20) [15,16]. GPR35 coupling through the Gα13 pathway was assessed using HIS3 and lacZ reporters as described previously [15], but with the following variations: individual transformants (minimum of four per construct) were grown overnight in YPD [1% (w/v) yeast extract, 1% (w/v) bacto-peptone and 2% (w/v) glucose] until saturation and diluted to approx. 0.0025 M fluorescein di-β-D-galactopyranoside and 0.1 M sodium phosphate, pH 7.0, then seeded into 96-well black tissue culture plates (Greiner). After 20 h of incubation at 30°C with shaking, β-galactosidase-mediated generation of fluorescein was measured using a PHERAstar FS microplate reader (excitation 485 nm, emission 520 nm; BMG-Labtech).

Mammalian Gα13 [35S]GTP[S] (guanosine S-1y-β(S)thio)triphosphate)-binding assay

Flp-InTm T-RExtm 293 cells (Invitrogen, Paisley, U.K.) able to express C-terminally EYFP-tagged human GPR35 in an inducible tetracycline/doxycycline-dependent manner [8] were transiently transfected with an internally ‘EE’ (EYMPME) epitope-tagged Gα13 construct (Missouri S&T cDNA Resource Center). After culturing the cells for 24 h in the presence of 100 ng/ml doxycycline to induce GPR35–EYFP expression, membranes were prepared according to a protocol described previously [8]. Compounds were then tested for their ability to activate Gα13 using 25 μg of cell membranes per reaction carried out in 1× assay buffer (20 mM Heps, pH 7.4, 3 mM MgCl2 and 100 mM NaCl) containing 1 μM GDP, 50 nCi [35S]GTP[S] and 1% DMSO. Reactions were incubated for 30 min at 30°C before being terminated by the addition of ice-cold 1× assay buffer. Samples were then centrifuged at 14,000 g for 15 min at 4°C. Supernatants were discarded and the pellets were resuspended in solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA and 1.25% Nonidet P40, pH 7.4) containing 0.2% SDS. Samples were pre-cleared with Pansorbin, followed by immunoprecipitation with an anti-EE monoclonal antibody (Covance). Immunocomplexes were then washed four times with solubilization buffer before bound [35S]GTP[S] was estimated by liquid scintillation spectrometry.

RESULTS

Human GPR35, modified to incorporate both an N-terminal FLAG epitope tag and a C-terminal EYFP, was co-transfected into HEK-293T cells along with a form of β-arrestin-2 C-terminally tagged with Renilla luciferase. As shown previously [8], this established a BRET-based human GPR35–β-arrestin-2 interaction assay [12,13]. Using this assay, we screened the entire Prestwick Chemical Library® of 1120 small-molecule marketed drugs at 10 μM in a 96-well plate format for compounds potentially able to promote this interaction. The previously described GPR35 agonist zaprinast [7] is present within the Prestwick Chemical Library® and, indeed, was detected as a hit, but this compound was also included in every plate as a reference standard. The performance of the screen, measured by the Z’ value [17] for each plate, is shown in Figure 1(A). The assay was robust and achieved a signal to background of >11:1 with an average Z’ value for the screen of 0.75 ± 0.11, and a mean percentage for the reference compound of 69.0 ± 6.9% (Figure 1B). Statistical analysis of the hit data relative to the error around the mean of the overall activity indicated that a percentage activation value of >3 S.D. above the mean would constitute a putative positive hit. In this case, we considered as possible hits compounds showing activity over 21.5% (Figure 1C). A number of potential hits were detected and these were cherry-picked and re-tested. Compounds re-confirmed in this manner and without colour or autofluorescence that would be expected to interfere with the assay (Figure 1D) were re-purchased from commercial sources.

Equivalent studies were then performed but replacing human GPR35 with the rat orthologue. Again, the performance of the
screen for each plate was found to be robust and achieved a signal to background of >12:1 with a Z′ value for the screen of 0.84 ± 0.04, (Figure 2A) and a mean percentage for the reference compound of 99.9 ± 2.3 % (Figure 2B). With the same criteria of hit definition as for human GPR35, a number of putative hits, showing activity values higher than 22.9 %, were recorded (Figure 2C). These were also cherry-picked and re-tested. Compounds confirmed as putative hits and lacking colour or autofluorescence that would interfere with the assay are shown in Figure 2(D).

Compounds that passed all of these preliminary checks at both human and rat GPR35 included zaprinast, cromolyn disodium and dicumarol. Following re-supply, these ligands were subjected to concentration–response studies. As demonstrated previously [7,8] zaprinast was substantially (38-fold) more potent at rat GPR35 than human GPR35 (Figure 3 and Table 1). In contrast, both cromolyn disodium and dicumarol were essentially equipotent at the two species orthologues (Figure 3 and Table 1). Interestingly, two of the hits identified in the screen of human GPR35, oxantel pamoate {{1-methyl-2-(3-hydroxyphenylethenyl)-1,4,5,6-tetrahydropyrimidine} pamoate} and pyrvinium pamoate {{4-[(3-carboxy-2-hydroxynaphthalen-1-yl)methyl]-3-hydroxyanthracene-2-carboxylic acid; 1-methyl-2-[(E)-2-thiophen-2-ylethenyl]-5,6-dihydro-4H-pyrimidine} pamoate}, were not identified in the screen of rat GPR35 and concentration–response curves (Figures 4A and 4B, and Table 1), confirming the lack of activity at rat GPR35 but activity at human GPR35. We also noted that both these ligands are provided in the Prestwick Chemical Library® as a combination of the purported active drug along with pamoate. We thus considered whether pamoate might be the active GPR35 ligand, not least because other reported GPR35 agonists are either carboxylic acids or at least contain an acid bioisostere [8] and we have shown previously that an arginine to alanine mutation in transmembrane domain III in both rat and human GPR35 essentially abolishes responses to both kynurenic acid and zaprinast [8]. Indeed, when purchased separately, pamoate was a potent agonist (5.1 × 10−8 M) of human GPR35 but essentially inactive at rat GPR35 (Figure 4C and Table 1). Furthermore, at concentrations up to 1 × 10−6 M, pamoate did not alter the concentration–response curve to zaprinast at rat GPR35 (Figure 4D) and hence was, indeed, inactive at rat GPR35 rather
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Figure 4 Pamoate is a high potency human-specific GPR35 agonist

Concentration–response curves to oxantel pamoate (A) and pyrvinium pamoate (B) in GPR35–β-arrestin-2 interaction assays are shown. On the basis of the presence of pamoate in both of the samples above, pamoate was assessed in isolation (C). Because of the lack of agonist activity of pamoate at rat GPR35, this compound was tested as a potential antagonist at rat GPR35 by examining its ability to modify the concentration–response curve to zaprinast (D). Results represent means ± S.E.M., n = 3.

Table 1 Potency values for agonist ligands at human and rat GPR35 in the β-arrestin-2 interaction assays

Results are presented as means ± S.E.M. *Significant species variation P < 0.05. n = 3, except for zaprinast, where n = 5.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human EC50 ([-\log (M)])</th>
<th>Rat EC50 ([-\log (M)])</th>
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<tbody>
<tr>
<td>Zaprinast</td>
<td>5.59 ± 0.04</td>
<td>7.17 ± 0.03*</td>
</tr>
<tr>
<td>Cromolyn sodium</td>
<td>5.12 ± 0.03</td>
<td>5.36 ± 0.03</td>
</tr>
<tr>
<td>Dicumarol</td>
<td>5.90 ± 0.08</td>
<td>5.70 ± 0.07</td>
</tr>
<tr>
<td>Quercetin</td>
<td>5.35 ± 0.16</td>
<td>5.20 ± 0.15</td>
</tr>
<tr>
<td>Niflumic acid</td>
<td>4.84 ± 0.10</td>
<td>&gt;3*</td>
</tr>
<tr>
<td>Oxantel pamoate</td>
<td>7.57 ± 0.44</td>
<td>&gt;3*</td>
</tr>
<tr>
<td>Pyrvinium pamoate</td>
<td>6.17 ± 0.04</td>
<td>&gt;3*</td>
</tr>
<tr>
<td>Pamoate</td>
<td>7.29 ± 0.03</td>
<td>&gt;3*</td>
</tr>
<tr>
<td>Luteolin</td>
<td>4.87 ± 0.03</td>
<td>5.01 ± 0.08</td>
</tr>
</tbody>
</table>

than acting as an antagonist. A further ligand identified in the human GPR35 screen but not in the screen using rat GPR35 was niflumic acid. Although not particularly potent (EC50 = 1.4 × 10⁻⁵ M) at human GPR35, detailed studies also confirmed an apparent lack of activity of this ligand at rat GPR35 (Figure 5). Interestingly, although differing only in a single atom, flufenamic acid ([2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one]) did not display any activity at either rat or human GPR35 at concentrations up to 1 × 10⁻⁴ M (Figure 5).

In the initial screens, we also noted compounds as hits at rat GPR35 but not at the human orthologue. These included quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one] and the closely related antioxidant flavenoid luteolin. These were also purchased and re-tested in concentration–response curves (Figure 6). Luteolin was confirmed, similar to zaprinast, to be substantially more potent at rat than human GPR35, but to be of substantially lower potency than zaprinast (luteolin EC50 = 1 × 10⁻⁵ M, zaprinast EC50 = 6.8 × 10⁻⁸ M) (Table 1). In contrast, although clearly more efficacious at rat than human GPR35 (Figure 6), quercetin had similar potency at the two species orthologues (4.7–5.6 × 10⁻⁶ M) (Table 1). Hence the apparent selectivity of quercetin in the initial screen reflected variation in ligand efficacy rather than potency.

It was obvious in the analysis of hits from the library screen that a number of the identified agonists were not equi-efficacious. We, therefore, tested in parallel compounds at maximally effective concentrations and compared these with zaprinast as the reference agonist. In such studies, when compared with zaprinast, only cromolyn disodium was a full agonist at human GPR35, with other compounds acting as partial agonists with efficacy between 50–70% (Figure 7A). For rat GPR35, both quercetin and luteolin were partial agonists, whereas both dicumarol and cromolyn disodium were close to full agonists (Figure 7B). More detailed studies were performed with pamoate at human GPR35. Concentration–response curves to pamoate were performed in the presence of different fixed concentrations of zaprinast. As a higher efficacy agonist, at concentrations at and above 1 × 10⁻⁵ M zaprinast generated a higher signal than the most effective concentration of pamoate (Figure 8). However, for both 1 × 10⁻⁵ M and 1 × 10⁻⁴ M zaprinast, high concentrations of pamoate reduced the measured response in a concentration-dependent manner, and the asymptote of these curves reached the same level as the highest concentrations of pamoate in the absence of zaprinast (Figure 8). These results are entirely consistent with pamoate acting as a partial agonist with efficacy of some 50% compared with zaprinast and interacting at an overlapping binding site.

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Figure 5 Niflumic acid but not the closely related flufenamic acid is a human-selective GPR35 agonist

(A) Concentration–response curves to niflumic acid (circles) and flufenamic acid (squares) at both human (filled symbols) and rat (open symbols) GPR35–β-arrestin-2 interaction assays are shown. Results represent means ± S.E.M., n = 3. (B) The chemical structures of niflumic acid and flufenamic acid.

Although ligand-promoted interactions between a GPCR and a β-arrestin have become a popular screening paradigm [12,13,18,19], this reports on an effect kinetically delayed from, and independent of, G-protein activation. Furthermore, it has been shown that ‘biased’ ligands may differentially promote G-protein activation or interactions with β-arrestins [20,21]. We have recently shown a selective capacity of GPR35 to activate Gα13 compared with either Gα12 or Gαq [8]. We therefore developed and utilized a S. cerevisiae-based human GPR35 activation assay. This employed a strain expressing a chimaeric yeast–mammalian Gpa1–Gα13 G-protein in which the last five amino acids of the yeast G-protein were replaced by the C-terminal five amino acids (QLMLQ) from human Gα13. Following introduction of human GPR35 into these cells, compounds including pamoate, cromolyn disodium, oxantel pamoate, pyrvinium pamoate, dicumarol, niflumic acid and zaprinast were assessed for their capacity to support cell growth. All did so, although with potencies ranging from pamoate (EC50 = 1.4 × 10⁻⁹ M) to niflumic acid (EC50 = 4.4 × 10⁻⁶ M) (Figures 9A and 9B). In contrast, in cells expressing full length Gpa1, no activity was recorded for any of these ligands (Figure 9C). These results are consistent with each compound promoting interaction and activation of Gα13 via human GPR35.

Despite this conclusion, the yeast-based assay employs a chimaeric G-protein containing only a fragment of the GPCR-recognition domain of Gα13. To further validate the conclusions, we also developed a mammalian Gα13 [35S]GTP[S]-binding assay. This was based on the expression and subsequent immunocapture of a form of Gα13 containing an internal ‘EE’ epitope-tag to allow efficient immunocapture with an anti-‘EE’ antibody. Flp-InTM T-RExTM 293 cells induced to express human GPR35–EYFP [8] were transfected with ‘EE’ Gα13. Membranes from these cells were incubated with [35S]GTP[S] in the absence and presence of ligands identified in the screen, including zaprinast. Subsequently, samples were immunoprecipitated with anti-‘EE’, washed and counted. Each of the ligands shown above to be active at human GPR35 promoted statistically significant (P < 0.01) increases in the amount of [35S]GTP[S] present in the immunoprecipitates (Figure 10), providing direct evidence for the capacity of agonists at human GPR35 to promote activation of Gα13, whereas flufenamic acid was unable to do so (Figure 10). Equally, both luteolin and quercetin produced no significant stimulation of [35S]GTP[S] binding, consistent with their low potency and efficacy at human GPR35 in the GPR35–β-arrestin-2 interaction assay.

Figure 6 Identification of luteolin, but not the closely related flavenoid quercetin, as a rat GPR35-selective agonist

In the primary screens, both luteolin and quercetin were identified as hits at rat but not human GPR35. Concentration–response curves to each ligand at both orthologues in β-arrestin-2 interaction assays are shown. Luteolin displayed minimal activity at human GPR35 and, hence, is selective for rat GPR35, whereas quercetin had a similar potency at each orthologue but substantially lower efficacy at human GPR35, explaining why it was not detected in the primary screen using human GPR35. Results represent means ± S.E.M., n = 3.
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Figure 7 Relative efficacies of GPR35 agonists

The relative efficacy of various ligands in the β-arrestin-2 interaction assay compared with zaprinast at maximally effective concentrations at either (A) human or (B) rat GPR35. Results represent means ± SEM, n = 3.

Figure 8 Pamoate is a partial agonist at human GPR35

Concentration–response curves to pamoate were performed in the human GPR35–β-arrestin-2 interaction assay in the absence or presence of various concentrations of zaprinast.

DISCUSSION

Although poorly characterized, GPR35 has been suggested to be a potential exploratory target for conditions ranging from diabetes [2] to inflammation [4], asthma [22] and hypertension [3]. A major impediment to validation of these ideas and further study of the role of the receptor has been a paucity of pharmacological tools to study GPR35. GPR35 also remains potentially an orphan receptor, although both the tryptophan metabolite kynurenic acid [6] and lysophosphatidic acid [23] are endogenously produced ligands reported to activate GPR35. The suggestion that it may remain an orphan, despite these reports, is based on two factors. First, kynurenic acid displays very low potency to activate human GPR35 and hence produces either recruitment of β-arrestin-2 or causes internalization of the receptor from the surface of transfected cells [8]. Secondly, although lysophosphatidic acid has also been suggested to be the endogenous agonist for GPR23 [24], which, based on overall sequence similarity, is the most closely related receptor to GPR35, both of these receptors lie well outside the widely accepted lysophosphatidic acid receptor group of lipid receptors and recent efforts to re-confirm GPR23 as a receptor for lysophosphatidic acid have been successful in some [18] but not other [19] reports.

Notwithstanding these issues, neither kynurenic acid nor lysophosphatidic acid are appropriate to explore the functional biology of GPR35 and the best characterized synthetic GPR35 agonist zaprinast both displays considerable species orthologue selectivity [7,8] and is also a cGMP phosphodiesterase inhibitor [10]. As such, novel ligands are required, along with an understanding of their potential species selectivity.

To initiate such a programme, we screened the Prestwick Chemical Library® of 1120 small-molecule marketed drugs for compounds able to activate either or both human and rat GPR35. GPR35 couples selectivity to G\(_{\alpha 13}\) [8], a G-protein not linked to the direct regulation of conventional secondary messengers for which robust assay screens are available [25,26]. However, agonists at GPR35 cause marked translocation of β-arrestin-2 and internalization of the receptor [8]. As a primary screen, we therefore employed a BRET-based assay that reports interactions between GPR35 and β-arrestin-2. For both human and rat GPR35, the assay was robust, providing high confidence in potential hits that were not coloured or fluorescent. Following re-confirmation, a number of potential GPR35 agonists were purchased from commercial vendors and re-tested in concentration–response mode, again using the GPR35 and β-arrestin-2 BRET interaction assay. As demonstrated previously [8], zaprinast was some 38-fold more potent at rat than at human GPR35, whereas cromolyn disodium (cromoglicic acid) was an essentially equipotent (EC\(_{50}\) = 4.4–7.6 × 10\(^{-6}\) M) agonist at both orthologues. This, in part, confirms a recent study demonstrating cromolyn to be a GPR35 agonist, although these workers suggested cromolyn to be selective for human GPR35 over the rat orthologue [22]. Although not reported previously, dicumarol was also identified as a non-selective and slightly more potent (EC\(_{50}\) = 1.3–2.0 × 10\(^{-6}\) M) agonist. At least as interesting as these observations, a number of ligands were markedly selective for human over rat GPR35. Although not particularly potent, niflumic acid (EC\(_{50}\) = 1.4 × 10\(^{-5}\) M) was an agonist at human GPR35 but was without significant potency at rat GPR35. Furthermore, although flufenamic acid differs in a single atom from niflumic acid, no detectable agonist activity was recorded in the GPR35–β-arrestin-2 BRET interaction assay for either species orthologue. Even more interestingly, both oxantel pamoate and pyrvinium pamoate were apparent highly selective agonists at human compared with rat GPR35. However, given that the drug congener pamoate was present in both these ligand samples, we tested whether pamoate might be the active agent and common link. Indeed pamoate was a potent (EC\(_{50}\) = 5.1 × 10\(^{-8}\) M) agonist.
at human GPR35 but essentially inactive at rat GPR35. These variations in activity and potency should be useful in helping to determine the mode of binding of ligands to species orthologues of GPR35. An understanding of this will be vital to the possible validation of GPR35 as a therapeutic target. Clearly, based on the GPR35–β-arrestin-2 interaction assay, neither niflumic acid nor pamoate would, for example, be of use in exploring contributions of activating GPR35 in rat models of disease.

Figure 9  Agonists at human GPR35 in the β-arrestin-2-interaction assay all promote yeast growth via activation of a Gpa1–Gα13 chimaera but not via Gpa1

Membranes from Flp-In™ T-REX™ 293 cells induced to express human GPR35–EYFP that were transfected to transiently express EE epitope-tagged Gα13 were used in [35S]GTP[S]-binding studies. Ligands identified as agonists at human GPR35 in the GPR35–β-arrestin-2 interaction assay were used at maximally effective concentrations and EE epitope-tagged Gα13 was subsequently immunoprecipitated and incorporation of [35S]GTP[S] was assessed. Flufenamic acid, luteolin and quercetin were also tested at 1 × 10^{-4} M. Results represent means ± S.E.M., n = 3.

Figure 10  GPR35 agonists promote binding of [35S]GTP[S] to Gα13

Membranes from Flp-In™ T-REX™ 293 cells induced to express human GPR35–EYFP that were transfected to transiently express EE epitope-tagged Gα13 were used in [35S]GTP[S]-binding studies. Ligands identified as agonists at human GPR35 in the GPR35–β-arrestin-2 interaction assay were used at maximally effective concentrations and EE epitope-tagged Gα13 was subsequently immunoprecipitated and incorporation of [35S]GTP[S] was assessed. Flufenamic acid, luteolin and quercetin were also tested at 1 × 10^{-4} M. Results represent means ± S.E.M., n = 4 individual transformants.

Because ligands may display ‘bias’ [21] dependent upon the assay used to detect their activity, we also wished to demonstrate the function of ligands at a G-protein-mediated end point. We have recently shown both rat and human GPR35 to display marked selectivity to activate Gα13 compared with either the closely related G-protein Gα12 or the more distantly related Gαq [8]. We therefore established an assay in S. cerevisiae employing chimaeric G-proteins that has been used widely in ligand screening studies [15,16]. In the present study, with expression of human GPR35 a range of ligands identified in the primary screen were able to promote cell growth via a Gpa1–Gα13 chimaera. We also developed a mammalian Gα13 activation assay. Although best suited to analysis of the activation of G_q family of G-proteins, [35S]GTP[S]-binding assays have been adapted for other G-protein classes [28]. Although this was also able to identify hits from the primary screen, absolute amounts of bound [35S]GTP[S] recovered in the
assay were low and the signal to background substantially smaller than for either the yeast-based assay or, particularly, the GPR35-β-arrestin-2 interaction assay. Thus, although useful in confirming hits, this assay would not have been appropriate, either in terms of screening statistics or time- and cost-effectiveness, to be used as the primary screen for novel agonists.

The ligands reported in the present study add substantially to the available pharmacology of GPR35 and provide reagents to further probe its function. The results also underline the importance of understanding the extent and basis of species orthologue selectivity before using cellular and, potentially, animal models of receptor function and disease.

AUTHOR CONTRIBUTION
Graeme Milligan and María Isabel Loza conceived the studies; Laura Jenkins, Jose Brea, Nicola Smith, Brian Hudson, Graeme Reilly and Marian Castro performed the experiments; Nia Bryant oversaw the yeast-based work; and Graeme Milligan wrote the paper with input from all of the other authors.

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