

**JPET # 201798**

## **Title Page**

# **High throughput identification and characterization of novel, species-selective GPR35 agonists**

**Zaynab Neetoo-Isseljee, Amanda E. MacKenzie, Craig Southern, Jeff Jerman, Edward G. McIver, Nicholas Harries, Debra L. Taylor and Graeme Milligan**

MRC Technology Centre for Therapeutics Discovery, 1-3 Burtonhole Lane, Mill Hill, London, NW7 1AD, UK (ZN-I, CS, JJ, EM, DLT), Molecular Pharmacology Group, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, Scotland G12 8QQ, UK (AEM, NH, GM)

**JPET # 201798**

**Running Title Page**

**Running title: HTS identification of GPR35 agonists**

**Address correspondence to:**

**Graeme Milligan, Wolfson Link Building 253, University of Glasgow,  
Glasgow G12 8QQ, Scotland, U.K.**

**Tel +44 141 330 5557**

**FAX +44 141 330 5841**

**e-mail [Graeme.Milligan@glasgow.ac.uk](mailto:Graeme.Milligan@glasgow.ac.uk)**

**manuscript information**

**Text pages 37, Number of Tables 1, Number of Figures 7**

**Number of References 39, Words in: Abstract 247, Introduction 637, Discussion  
1720**

**Non-standard abbreviations**

BRET, bioluminescence resonance energy transfer; CHO-K1, Chinese hamster ovary-K1 cell line; DMEM, Dulbecco's modification of Eagle's medium; GPCR, G protein-coupled receptor; HTRF, homogeneous time-resolved fluorescence; HTS, high-throughput screen; MRCT, Medical Research Council Technology; NINDS, National Institute of Neurological Disorders and Stroke, PEI, polyethylenimine;

**Recommended section**

Cellular and Molecular

## JPET # 201798

### ABSTRACT

Drugs targeting the orphan receptor, GPR35, have potential therapeutic application in a number of disease areas including: inflammation, metabolic disorders, nociception and cardiovascular disease. Currently available surrogate GPR35 agonists identified from pharmacologically relevant compound libraries have limited utility due to the likelihood of off-target effects *in vitro* and *in vivo* and the variable potency that such ligands exhibit across species. We sought to identify and characterize novel GPR35 agonists to facilitate studies aimed at defining the physiological role of GPR35. PathHunter™  $\beta$ -arrestin recruitment technology was validated as a human GPR35 screening assay and a High Throughput Screen of 100,000 diverse low molecular weight compounds conducted. Confirmed GPR35 agonists from five distinct chemotypes were selected for detailed characterization using both  $\beta$ -arrestin recruitment and G protein-dependent assays and each of the human, mouse and rat GPR35 orthologs. These studies identified 4-[(Z)-[(2Z)-2-(2-fluorobenzylidene)-4-oxo-1,3-thiazolidin-5-ylidene]methyl]benzoic acid (compound 1) as the highest potency, full agonist of human GPR35 yet described. As with certain other GPR35 agonists, compound 1 was markedly selective for human GPR35, but displayed elements of signal bias between  $\beta$ -arrestin-2 and G protein-dependent assays. Compound 1 also displayed competitive behavior when assessed against the human GPR35 antagonist, ML-145 (2-hydroxy-4-[4-(5Z)-5-[(E)-2-methyl-3-phenylprop-2-enylidene]-4-oxo-2-sulfanylidene-1,3-thiazolidin-3-yl]butanoylamino]benzoic acid). Of the other chemotypes studied, compounds 2 and 3 were selective for the human receptor, but compounds 4 and 5 demonstrated similar

## **JPET # 201798**

activity at human, rat and mouse GPR35 orthologs. Further characterization of these compounds and related analogues is likely to facilitate a better understanding of GPR35 in health and disease.

## **JPET # 201798**

### **Introduction**

The class A orphan receptor GPR35 has been described as a potentially novel drug target (MacKenzie et al., 2011) based upon reports describing the activation of this receptor in a variety of cell types (Leonard et al., 2005; Wang et al., 2006; Ohshiro et al., 2008; Fallarini et al., 2010), the potential genetic linkage of GPR35 to disease (Shrimpton et al., 2004; Vander Molen et al., 2005; Ellinghaus et al., 2012) and the phenotypic profiling of a GPR35 knockout mouse line (Min et al., 2010). This has resulted in speculation that GPR35 may hold therapeutic potential for inflammatory disorders (Wang et al., 2006; Lattin et al., 2008, Barth et al., 2009), CNS function (Shrimpton et al., 2004; Lein et al., 2007; Guo et al., 2008), nociception (Ohshiro et al., 2008; Zhao et al., 2010; Cosi et al., 2011), metabolic disorders (Leonard et al., 2005, Vander Molen et al., 2005) hypertension and cardiovascular disease (Sun et al., 2008; Min et al., 2010). The physiological and patho-physiological functions of this receptor, however, remain to be fully determined (Milligan, 2011).

The endogenously produced molecules kynurenic acid (Wang et al., 2006) and lysophosphatidic acid (Oka et al., 2010) have been reported as candidate GPR35 agonists, however, the nature of the cognate ligand(s) remains controversial (Jenkins et al., 2011). As a consequence, many studies examining GPR35 activation and intracellular signalling have been performed using the first reported surrogate ligand, zaprinast (Taniguchi et al., 2006) and this has become widely used as a reference compound. Although more widely known as a moderately potent phosphodiesterase type V and VI inhibitor, the GPR35

## JPET # 201798

agonist activity of zaprinast appears to be unrelated to its phosphodiesterase inhibitory activity (Taniguchi et al., 2006).

Work from several laboratories, using a range of screening assays including calcium mobilization,  $\beta$ -arrestin-based receptor interaction and translocation assays and so called 'label free' technologies, have continued to identify an increasing number of structurally diverse surrogate ligands (MacKenzie et al., 2011; Milligan, 2011), not least the potent, but human ortholog selective, agent pamoic acid ((4-[(3-carboxy-2-hydroxynaphthalen-1-yl)methyl]-3-hydroxynaphthalene-2-carboxylic acid), from the Prestwick chemical library of FDA approved drugs (Jenkins et al., 2010, Zhao et al., 2010). Although approaches based on agonist-induced interactions between GPR35 and  $\beta$ -arrestin-2 have been central to these efforts, GPR35 is also able to activate signal transduction through pertussis-toxin-sensitive  $G_i$  family G proteins and, very selectively, via the G protein  $G_{13}$  (MacKenzie et al., 2011; Milligan, 2011). Agonist ligands with the ability to stabilize receptor conformations that selectively promote engagement of one signal transduction pathway in preference to another are generally referred to as biased-ligands (Rajagopal et al., 2011). There is growing interest in the concept that such ligands may offer therapeutic benefit in situations where activation of multiple signals via a receptor can result in both useful and contra-indicated outcomes (Dewire and Violin, 2011). As such, no matter the primary screen used, it is imperative to assess the activity of ligands across the range of signal pathways, both G protein-dependent and – independent, that the receptor is able to modulate.

## JPET # 201798

The availability of structurally diverse, pharmacologically relevant surrogate ligands may facilitate early investigation into receptor pharmacology in multiple cell types, however, many of the current ligands with GPR35 activity also have prominent off-target effects at other pathways, which may diminish their utility for understanding GPR35 signaling and for identifying potential therapeutic uses of GPR35.

To facilitate the investigation of GPR35 biology we now report key outcomes from a High Throughput Screen (HTS) of 100,000 compounds to identify novel agonists, utilizing CHO cells stably expressing human GPR35 and DiscoverX PathHunter™  $\beta$ -arrestin recruitment technology. Candidate hit compounds, including 5 novel ligands that we detail herein, were profiled and characterized in both  $\beta$ -arrestin-2 and G-protein-coupled read-outs to explore potential ligand bias and against rodent orthologs of GPR35 to define potential species selectivity.

## Methods

### *Materials*

Cell culture media were from Life Technologies (Paisley, UK). Cells were routinely passaged in complete growth medium (DMEM/F12 with GlutaMAX + 10% Foetal Bovine Serum). Zaprinast was from Sigma-Aldrich (Gillingham, UK). ML-145 (2-hydroxy-4-[4-(5Z)-5-[(E)-2-methyl-3-phenylprop-2-enylidene]-4-oxo-2-sulfanylidene-1,3-thiazolidin-3-yl]butanoylamino]benzoic acid) was from Tocris Bioscience (Bristol, UK). Commercially available compound libraries were obtained from National Institute

## **JPET # 201798**

of Neurological Disorders and Stroke (NINDS) (National Institutes of Health, USA) and Sequoia Research Products Ltd. (Pangbourne, U.K.).

### ***PathHunter<sup>TM</sup> $\beta$ -arrestin recruitment assay***

PathHunter<sup>TM</sup>  $\beta$ -arrestin recruitment assays were performed using a Chinese hamster ovary (CHO-K1) cell line stably expressing human GPR35 and  $\beta$ -arrestin-2 (DiscoverX, Fremont, CA). In this assay a receptor C-terminally tagged with a short fragment of the enzyme  $\beta$ -galactosidase is co-expressed alongside  $\beta$ -arrestin-2 tagged with the rest of the sequence of  $\beta$ -galactosidase (Eglen et al., 2007). Agonist-induced recruitment of  $\beta$ -arrestin-2 to GPR35 was monitored through  $\beta$ -galactosidase enzyme fragment complementation as this reflects proximity-induced complementation between the fragments of the enzyme. Prior to assay 5,000 cells per well of a 384-well plate were seeded in 10  $\mu$ l Cell Plating Reagent 2 (DiscoverX, Fremont, CA) and incubated overnight at 37°C. For primary screening compounds were tested at a single final assay concentration of 10  $\mu$ M, (1.0 % DMSO). For the determination of EC<sub>50</sub> values compounds were tested across a 10-point concentration-response curve with a highest assay concentration of 100  $\mu$ M. Cells were incubated with compounds at 37°C for 2.5 h to facilitate complementation and enzyme action. This was followed by the addition of 10  $\mu$ l PathHunter<sup>TM</sup> detection reagent (DiscoverX, Fremont, CA) and incubation for 2.5 h at room temperature in the dark. Chemiluminescence was measured on a PHERAstar FS plate reader (BMG Labtech, Germany).



## JPET # 201798

### *Intracellular calcium assay*

PathHunter™  $\beta$ -arrestin GPR35 CHO–K1 cells were transfected with a chimeric  $G\alpha_{q-15}$  G protein subunit plasmid (Jenkins et al., 2012) using lipofectamine (Life Technologies, Paisley, UK) and incubated overnight at 37°C. Cells were then re-seeded in complete growth medium at 10,000 cells per well in 384-well black, clear bottom microplates and incubated overnight at 37°C. Cells were loaded with Fluo-4 direct calcium assay reagent (Life Technologies, Paisley, UK) for 45 min at 37°C + 15 min at room temperature in the presence of probenecid (5 mM). Compound addition and calcium mobilization were monitored on a Flex Station (Molecular Devices, Sunnyvale, CA).

### *BRET-based $\beta$ -arrestin-2 recruitment assay*

HEK-293T cells, maintained as previously described (Jenkins et al., 2010) were transiently transfected with human, mouse or rat orthologs of GPR35 C-terminally tagged with eYFP and  $\beta$ -arrestin-2-*Renilla* luciferase 6 using polyethylenimine (PEI) (Polysciences, Inc, Warrington, PA) (Jenkins et al., 2010). Transfections with *Renilla* luciferase 6 construct and pcDNA3 expression vector alone were performed as controls. After 24 h, cells were seeded at 50,000 cells per well in poly-D-lysine coated 96-well plates and incubated at 37°C overnight. After 24 h, cells were washed twice with Hank's Balanced Salt Solution (pH 7.4) and incubated in the dark with 5  $\mu$ M coelentrazine-h (Promega, Southampton, UK) for 10 min at 37°C. Following compound addition, the

## JPET # 201798

cells were incubated for a further 5 min at 37°C before sequential reading of emission signals on a PHERAstar FS plate reader (BMG-Labtech, Germany) at 485 nm and 530 nm, which represent the *Renilla* luciferase 6 and eYFP signals respectively. The BRET ratio was calculated as the BRET eYFP divided by *Renilla* luciferase 6. The net BRET ratio was obtained from the BRET ratio minus *Renilla* luciferase 6 control. This value was multiplied by 1000 to obtain mBRET units.

### *Inositol monophosphate accumulation assay*

Utilized a homogeneous time-resolved fluorescence-based (HTRF) assay kit to detect inositol monophosphates derived from the breakdown of inositol 1,4,5, trisphosphate (Trinquet et al. 2011). HEK-293T cells were transiently co-transfected with human, mouse or rat orthologs of FLAG-GPR35-eYFP and a chimeric G protein ( $G\alpha_{q-13}$ ) (Jenkins et al., 2012) using PEI. After 16-24 h incubation at 37°C, cells were resuspended in IP-One stimulation buffer, (10 mM HEPES, 1 mM  $CaCl_2$ , 0.5 mM  $MgCl_2$ , 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH7.4) and seeded at 10,000 cells per well in white, solid bottom 384 well plates. Ligands were diluted in IP-One stimulation buffer according to manufacturer's instructions (Cisbio HTRF IP-One Tb kit, Codolet, France). Cells were incubated with agonist for 2 h at 37°C, before addition of d2-conjugated IP1 and anti-IP1 Lumi4™-Tb cryptate diluted in lysis buffer as per manufacturer's instructions. After incubation at room temperature for 1 h, HTRF was measured using a PHERAstar FS plate reader (BMG-Labtech, Germany).

## JPET # 201798

### *Data analysis*

Curves were fitted by non-linear regression analysis in GraphPad Prism (GraphPad, San Diego, CA).

## RESULTS

### *Screening NINDS and Sequoia libraries using PathHunter™ $\beta$ -arrestin-2 recruitment*

Screening for GPR35 agonists within the Prestwick library of 1200 pharmacologically relevant compounds has previously been reported (Jenkins et al., 2010, Zhao et al., 2010). As an extension to this, initially we developed a 384-well human GPR35 agonist assay using the DiscoverX PathHunter™  $\beta$ -arrestin recruitment technology and performed a pilot screen using NINDS and Sequoia libraries of known drugs and pharmacologically active compounds, which show a degree of compound overlap with the Prestwick library. Zaprinst at an  $EC_{100}$  of 100 $\mu$ M served as a positive control and 1.0 % DMSO as a negative vehicle control on each assay plate. Assay performance was monitored and validated by statistical outcomes; the mean signal:background (S/B) was 2.93, mean Z prime (Z') was 0.66, mean %CV for positive and negative controls were 5.75 and 4.96 respectively and the overall assay hit rate was 2.1% at a 30% activity of zaprinast cut-off. This resulted in selection of 29 compounds encompassing a variety of chemotypes that included both full and partial agonists with a range of potencies and efficacies (**Figure 1**). The assay successfully identified zaprinast

## JPET # 201798

and other previously reported agonists including 5-nitro-2-(3-phenylpropylamino) benzoic acid (Tanaguchi et al., 2008), dicumarol, cromolyn disodium, derivatives of pamoic acid (Zhao et al., 2010, Jenkins et al., 2010), furosemide (Yang et al., 2012), aspirin metabolites (Deng and Fang, 2012) and ellagic acid (Deng et al., 2012b). Novel agonists identified from the NINDS and Sequoia libraries included artemisinin, which behaved as a partial agonist with high potency, amlexanox, tannic acid and 5-fluoroindole-2-carboxylic acid (**Figure 1**). The identification of previously reported as well as novel agonists provided confidence in the validity of the assay and that a robust  $\beta$ -arrestin assay was configured and suitable for HTS.

### *HTS of Medical Research Council Technology diversity compound collection using PathHunter™ $\beta$ -arrestin-2 recruitment*

To gain greater diversity and potentially more chemically tractable ligands we performed a HTS against a diverse collection of 100,000 chemical compounds. This collection was selected to generate a high-quality set of compounds using drug-like filters based on physicochemical properties, together with analysis to maximize template diversity within the library. The compounds from the library were tested in ‘single shot’ format at final assay concentration of 10  $\mu$ M and 753 positives were identified. A further assay run of these 753 positives resulted in a 73% hit confirmation rate. Analysis of the activity distribution profile revealed that only 5% of compounds were as or more active than zaprinast, with approximately 40% of the hits approaching only 20-30% of zaprinast

## JPET # 201798

activity (**Figure 2**). Confirmed hits were profiled in full concentration-response curves and the potency distribution of the pEC<sub>50</sub> values is shown in **Figure 3**. The largest proportion (38.9%) had pEC<sub>50</sub> values within the range 4.5-5.0, whilst few were significantly more potent than zaprinast (pEC<sub>50</sub> ~5.8-6.3), and only a small proportion (3.9%) displayed pEC<sub>50</sub> ≥ 6.

### *Compound profiling in distinct $\beta$ -arrestin-2 and G protein-dependent assay formats*

To extend the initial screening data a chemistry-based triage and assessment process highlighted a number of distinct chemical series among the hits. Seven hit families were prioritized and 86 selected compounds re-purchased. All of the re-supplied compounds were re-assessed for activity and potency in a distinct GPR35- $\beta$ -arrestin-2 interaction assay based on BRET between co-expressed  $\beta$ -arrestin-2-*Renilla* luciferase and C-terminally eYFP-tagged forms of each of human, mouse or rat GPR35 (Jenkins et al., 2012) to explore possible species selectivity. The functionality of 30 compounds, each of which displayed pEC<sub>50</sub> of at least 5.0 at one of the three orthologs is shown (**Supplemental Table 1**). Performed in 96 well plate format these assays produced excellent assay performance with mean Z' values of 0.83 for human, 0.75 for mouse and 0.77 for rat GPR35 and mean signal:background of 8.15 for human, 7.98 for mouse and 4.27 for the rat ortholog. Subsequently, a smaller number of compounds were selected as representatives of 5 different chemotypes and profiled in detail in a range of assays including:  $\beta$ -arrestin-2 interaction and both intracellular calcium mobilization (performed in the PathHunter™ human GPR35 CHO cell line following transient transfection with

## JPET # 201798

the chimeric G protein subunit  $G\alpha_{q-i5}$ ) (Jenkins et al., 2012) and an inositol monophosphate (IP1) accumulation (performed in HEK-293T cells transiently transfected to express human, mouse or rat GPR35 alongside the chimeric G protein subunit  $G\alpha_{q-i3}$ ) assay (Jenkins et al., 2012). These studies were designed to explore if compounds might selectively modulate  $\beta$ -arrestin-2 over G protein-dependent signals. **Figure 4** demonstrates that zaprinast was active in both PathHunter™  $\beta$ -arrestin and  $G\alpha_{q-i5}$   $Ca^{2+}$  elevation assays. A number of other compounds identified in the HTS including 4-[(Z)-[(2Z)-2-(2-fluorobenzylidene)-4-oxo-1,3-thiazolidin-5-ylidene] methyl] benzoic acid (**compound 1**), 2,2'-bithiophene-5,5'-dicarboxylic acid (**compound 2**), 4-chloro-3-(2,5-dimethyl-1H-pyrrol-1-yl)benzoic acid (**compound 3**), 4H-thieno[3,2-b]pyrrole-2-carboxylic acid (**compound 4**) and 9-benzyl-5-methyl-3-(4-methylphenyl)-5,9-dihydro-6H-[1,2,4]triazolo[4,3-e]purine-6,8(7H)-dione (**compound 5**) (see **Table 1** for structures) displayed efficacy equivalent to zaprinast in both these assays (**Figure 4**). Most notably, compound **1** was markedly more potent than zaprinast with  $pEC_{50} = 7.59 \pm 0.05$  in the PathHunter™  $\beta$ -arrestin interaction assay and  $pEC_{50} = 8.36 \pm 0.05$  in the  $G\alpha_{q-i5}$   $Ca^{2+}$  assay (**Figure 4**). It is thus the most potent agonist yet reported for human GPR35.

Given the high potency of compound **1** at human GPR35 we also examined the activity of a pair of closely related analogs. **Compound 6** (4-[-4-oxo-2-(phenylimino)-1,3-thiazolidin-5-ylidene]methyl]benzoic acid) and **compound 7** (-[2-(4-fluorophenyl)imino]-4-oxo-1,3-thiazolidin-5-ylidene]methyl]benzoic acid) were also both potent agonists at human GPR35 using both PathHunter™ and BRET-based  $\beta$ -

## JPET # 201798

arrestin-2 recruitment assays, but neither displayed higher potency than compound **1** (**Figure 5**).

### *Agonist activity at GPR35 rodent orthologs*

We have previously demonstrated marked differences in potency of certain GPR35 agonists when assessed at species ortholog variants. We next assessed, therefore, this possibility for compounds **1-5**. Using the BRET-based GPR35- $\beta$ -arrestin-2 interaction assay, as reported previously (Jenkins et al., 2010, 2012), in this format zaprinast was substantially more potent at rat than human GPR35 and showed intermediate potency at the mouse ortholog (**Figure 6A**). Although this assay confirmed the ability of compounds **1**, **2** and **3** to recruit  $\beta$ -arrestin-2 via human GPR35, when using either rat or mouse GPR35 both compound **2** and compound **3** were close to inactive at concentrations up to 100  $\mu$ M and, therefore, at least 100 fold less potent in this assay than at the human ortholog (**Figure 6A**). Furthermore, although compound **1** displayed agonist activity at both rodent orthologs at concentrations of 10  $\mu$ M and above, this compound was some 1000 fold more potent at the human receptor (**Figure 6A**). As for compound **1**, the related compounds **6** and **7** also displayed poor potency at the rat and mouse orthologs, being essentially inactive at concentrations up to 100  $\mu$ M (**Figure 5**). By contrast, the potency of compound **4** was similar at all three orthologs in this assay (**Figure 6A**) whilst compound **5** displayed similar potency at the human and rat orthologs but some ten fold lower potency at mouse GPR35 (**Figure 6A**).

## JPET # 201798

The  $G\alpha_{q-15}$   $Ca^{2+}$  elevation assays (**Figure 4**) provide a surrogate endpoint for GPR35-mediated activation of  $G_i$ -family G proteins (Jenkins et al., 2012). However, GPR35 is also known to selectively activate the G protein  $G\alpha_{13}$  (Jenkins et al., 2010). Thus to assess engagement of this pathway we employed IP1 accumulation assays in HEK-293T cells transfected to express an ortholog of GPR35 and the chimeric G protein  $G\alpha_{q-13}$ . This assay again confirmed the rank order of potency of zaprinast as highest at rat and lowest at human GPR35 (**Figure 6B**) and, in general, produced results for the newly identified ligands in agreement with the BRET-based assays. For example, compound **4** was of similar potency at all three orthologs compound **5** somewhat less potent at mouse GPR35 than at either the human or rat orthologs (**Figure 6B**) whilst compound **3** was some 1000 fold more potent at human than at either rodent ortholog (**Figure 6B**).

Despite these similarities some differences were noted. For example, although compound **2** remained substantially less potent at the rodent versions of GPR35 than at human (**Figure 6B**), at the highest concentration tested it did display greater efficacy than zaprinast. It remains unclear if this might be an assay artifact, however, as the production of IP1 did not display signs of saturation. Most interestingly, although a potential partial agonist compared to zaprinast at mouse and rat GPR35 in the BRET-based  $\beta$ -arrestin-2 interaction assay (**Figure 6A**), and remaining less potent than at human GPR35, compound **1** clearly displayed greater efficacy than zaprinast at the rodent orthologs in the IP1 assay (**Figure 6B**).



## JPET # 201798

### *Mode of action of novel GPR35 agonists*

ML-145 is a high affinity, but highly human selective, blocker of GPR35 (Jenkins et al., 2012). In BRET-based  $\beta$ -arrestin-2 interaction assays using human GPR35 increasing concentrations of ML-145 increased the concentration of compound **1** required to achieve half-maximal effect and did so in a fully surmountable fashion (**Figure 7A**). Moreover, ML-145 displayed similar effects when co-incubated with either compound **2** (**Figure 7B**) or compound **3** (**Figure 7C**), also consistent with competitive effects, although the moderate potency of compounds **2** and **3** at human GPR35 made it impossible to demonstrate unambiguously full surmountability of the effect of the antagonist. We also explored the issue of competitiveness with ML-145 in the IP1 accumulation assay. Despite the data analysis being affected by the ligand-independent, constitutive activity of human GPR35 in this assay (Jenkins et al., 2012) and this being reduced in a concentration-dependent fashion by ML-145 due to the inverse agonist nature of ML-145 (Jenkins et al., 2012), similar conclusions as to the competitive nature of interactions between compounds **1-3** and ML-145 were produced (**Figure 7D-F**).

### **Discussion**

Efforts to identify novel chemotypes of ligands at many targets for therapeutic drugs are initiated via HTS campaigns. The poorly characterized GPCR GPR35 has been suggested as a potential target in conditions ranging from cardiovascular disease and

## JPET # 201798

metabolic disorders to inflammation and nociception (Milligan, 2011, MacKenzie et al., 2011). These suggestions have arisen from studies exploring each of tissue distribution, functional studies and genetic associations of GPR35 with disease (Leonard et al., 2005, Vander Molen et al., 2005, Wang et al., 2006, Min et al., 2010, Ellinghaus et al., 2012). However, understanding of the biology of GPR35 has been restricted due to a paucity of high potency/affinity and selective ligands. Furthermore, it remains unclear whether any of the proposed endogenous ligands, including kynurenic acid (Wang et al., 2006) and lysophosphatidic acid (Oka et al., 2010) actually function as the key activator of this receptor *in vivo*. Largely because of this, the surrogate GPR35 agonist zaprinast has become the standard reference ligand, as it is an effective and reasonably potent activator of GPR35 (Tanaguchi et al., 2006, Jenkins et al., 2010, 2011, 2012). However, its use to define the biology of GPR35 is limited by its known action as a PDE V and VI inhibitor (Taniguchi et al., 2006). Moreover, other recently reported GPR35 agonists, although of reasonable potency, have been identified predominantly via screens of small libraries of known, pharmacologically relevant, drugs (Jenkins et al., 2010, Zhao et al., 2010), and/or have known activity at other targets (Yang et al., 2010, 2012; Deng and Fang, 2012; Deng et al., 2012a,b; Hu et al., 2012). Furthermore, certain of these ligands, including the currently described antagonists, display markedly different affinity at human and rodent orthologs of GPR35 (Jenkins et al., 2010, 2012) limiting their potential to define the function of GPR35 in rodent models and in cells derived from these species. To attempt to define novel, distinct chemotypes of GPR35 agonists we therefore conducted a HTS campaign following initial demonstration that the PathHunter™  $\beta$ -arrestin-2 recruitment

## JPET # 201798

assay could be used successfully in 384-well format to identify both previously reported and novel GPR35 agonists from a pair of small scale libraries. A 100,000 compound library produced some 550 confirmed actives and, following triage based on potency, chemical tractability and availability some 86 compounds within seven broad chemotypes were assessed further. Herein we have detailed five distinct exemplars, compounds **1-5**, and compared these to the standard ligand, zaprinast. Zaprinast is used routinely as the standard reference agonist ligand for GPR35 and, therefore herein, akin to the situation with well defined endogenous agonists, efficacy of other ligands has been related to that of zaprinast. However, it should be noted that certain ligands generated greater signal than zaprinast in different assays and, therefore, it should not be assumed that zaprinast is intrinsically a full agonist at GPR35.

$\beta$ -arrestin-2 based cellular translocation and receptor interaction methodologies have become central to many HTS efforts at GPCRs (Hamdan et al., 2005, Verkar et al., 2008), including screens that identified two distinct (but in retrospect highly human selective, (Jenkins et al., 2012)) GPR35 antagonists (Heynen-Genel et al., 2010). This reflects the suitability of GPR35 in such assays and a view that agonist occupancy of most GPCRs will generate a positive readout (Hamdan et al., 2005). Each of the five selected compounds displayed close to full agonism in the PathHunter™  $\beta$ -arrestin-2 recruitment assay, with compound **1** acting as the most potent GPR35 full agonist yet reported. Importantly, each of compounds **1-5** was also able to stimulate intracellular  $\text{Ca}^{2+}$  elevation following transfection of the human GPR35 expressing CHO cell line with

## JPET # 201798

a  $G_{\alpha_{i5}}$  chimeric G protein (Kostenis et al., 2005, Milligan, 2011), indicating the capacity of these ligands to stabilize a conformation of GPR35 that results in G protein activation as well as  $\beta$ -arrestin-2 interactions. Notably, however, in concentration-response studies the Hill slope for each agonist was markedly lower in this assay than in the PathHunter™  $\beta$ -arrestin-2 recruitment assay. Although we have not explored this in detail it may reflect the very different timings of the assays in which  $Ca^{2+}$  elevation is monitored rapidly and dynamically after ligand addition, a situation that is unlikely to allow ligand equilibrium to be achieved. By contrast the PathHunter™  $\beta$ -arrestin-2 assay is based on enzyme-fragment complementation, a relative slow process and measured 2.5 hours after ligand addition. However, it should be noted that although the BRET-based  $\beta$ -arrestin-2 assay also reports on induced-interactions between agonist-occupied GPR35 and  $\beta$ -arrestin-2 and the observed agonist-induced Hill slopes were very similar in the two  $\beta$ -arrestin-2 interaction assays, the BRET-based assay was conducted only 5 minutes after ligand addition and, hence, in a more similar time frame to the  $Ca^{2+}$  elevation studies. As such, the basis for the variation in Hill slope appears to reflect more than aspects of potential non-equilibrium conditions. Understanding of this will require further analysis but is clearly also not related simply to G protein-dependent versus -independent signal pathways as the IP1 accumulations assays, which lie on the same pathway as  $Ca^{2+}$  elevation, also displayed Hill slopes similar to the  $\beta$ -arrestin-2 interaction assays.

To avoid focusing measures of GPR35 activation only towards recruitment of  $\beta$ -arrestin-2 we deliberately also developed and employed a pair of G protein-dependent

## JPET # 201798

assays. As we have previously shown that GPR35 selectively activates  $G_{13}$  (Jenkins et al., 2011) we wished to monitor downstream effects from this interaction. Direct measures of activation of  $G_{13}$  are both limited and technically challenging (Jenkins et al., 2011). They are also poorly suited to high throughput or the production of concentration-response data. We therefore employed a chimeric G protein methodology. Such assays have been used widely in ligand screens and rely on the selective recognition of the GPCR by the extreme C-terminal  $\alpha$  helix of a G protein  $\alpha$  subunit (Kostenis et al., 2005). Linking this sequence to the body of a distinct  $\alpha$  subunit, in this case  $G\alpha_q$ , that engages an effector enzyme that generates an easy to detect and quantify secondary messenger, allowed receptor activation of  $G_{13}$  to be converted to elevation of IP1 levels. In a similar vein,  $Ca^{2+}$  elevation as a surrogate monitor of GPR35 activation of  $G_i$ -family G proteins was achieved in an equivalent way. By their very nature these are indirect assays and this must be considered in terms of the pharmacology observed. However, despite some clear examples where this has resulted in unexpected, and potentially confusing, pharmacology (see Kostenis et al., 2005 for review) these approaches are widely used and viewed generally as being robust. Although not the primary focus of the current studies it is interesting to note potential variation in activity of certain GPR35 agonists in different functional assays, with compound **1** being a case in point. Although markedly less potent and efficacious at rat and mouse GPR35 than the human ortholog in the BRET-based  $\beta$ -arrestin-2 interaction assay, this compound was more efficacious at rat and mouse GPR35 than at human in the IP1 accumulation assay and the difference in potency between

## JPET # 201798

species was substantially less. Such observations suggest that different conformations of the receptor may engage different signaling modalities and, hence, that ligand bias can be noted between different GPR35 agonist ligands. This idea is also supported by a recent comparison of various natural phenols that act as GPR35 agonists (Deng et al., 2012b). For example, when compared to zaprinast, although ellagic acid displayed as high efficacy in a label-free, dynamic mass redistribution assay it displayed much lower efficacy in a  $\beta$ -arrestin-2 recruitment assay (Deng et al., 2012b). A number of other ligands showed a similar pattern, including niflumic acid, previously identified as a partial agonist at GPR35 in a distinct  $\beta$ -arrestin-2 recruitment assay (Jenkins et al., 2010). Interestingly, although two further previously identified GPR35 partial agonists, luteolin and quercetin (Jenkins et al., 2010) were partial agonists in both dynamic mass redistribution and  $\beta$ -arrestin-2 recruitment assays (Deng et al., 2012b) no ligands were identified with the opposite bias.

As in other detailed studies (Jenkins et al., 2010, 2012), marked variation in agonist potency between species orthologs was observed for a number of the novel agonists identified and, importantly, this was largely conserved between assays. As the HTS was conducted against human GPR35 then, inherently, each of the 5 novel ligands selected for detailed profiling has at least moderate potency at human GPR35. However, the three most potent agonists at human GPR35 from the current series, compounds **1**, **2** and **3**, each displayed very modest if even detectable potency at either rat or mouse GPR35. A challenge in defining the biology of GPR35 has been to identify potent

## JPET # 201798

ligands, either agonists or antagonists, at the rat or mouse orthologs, and despite the inherent limitations of attempting to use zaprinast to interrogate GPR35 function, this remains the most potent ligand currently described for this pair of rodent orthologs. It is likely that more potent ligands at rat and/or mouse GPR35 could be identified by repeating the HTS, but using the rodent orthologs as the target because a number of non-overlapping hits were identified previously when screening the Prestwick library against human and rat GPR35 (Jenkins et al., 2010). This may eventually be required. Although the number of atomic level structures of GPCRs has increased substantially in recent times and may be of considerable use in drug-discovery (Congreve et al., 2011, Salon et al., 2011), none of these receptors is highly related to GPR35 and, therefore, confidence in homology modeling and possible virtual screening at this target remains low.

Overall these studies demonstrate the utility of HTS based on receptor- $\beta$ -arrestin interaction technologies to identify novel and potent GPR35 agonists. However, the identification of potential bias in ligand function at this receptor means that initial identification at this level needs to be assessed further in a series of G protein-dependent assays. Moreover, as noted previously, the marked variation in potency and, indeed, even activity at rodent versus human orthologs means that considerable care must be taken to select ligands suitable for rodent-based studies. However, the growing number of reported ligands derived from distinct chemotypes, and with potency and affinity differences at human and/or rodent orthologs will now provide an opportunity to define

## **JPET # 201798**

the ligand binding sites and to understand the molecular basis of this pharmacological variation.

### **Acknowledgments**

#### **Authorship contributions**

*Participated in research design:* Jerman, Mackenzie, Milligan, Neetoo-Isseljee, Southern, McIver, Taylor

*Conducted experiments:* Harries, Jerman, Mackenzie, Neetoo-Isseljee, Southern,

*Contributed new reagents or analytic tools:* McIver, Southern, Taylor

*Performed data analysis:* Harries, Jerman, McIver, Mackenzie, Milligan, Neetoo-Isseljee, Southern

*Wrote or contributed to the writing of the manuscript:* Mackenzie, Milligan, Neetoo-Isseljee, Southern, with assistance from all other authors.



## JPET # 201798

### References

Barth M, Ahluwalia N, Anderson TJ, Hardy GJ, Sinha S, Alvarez-Cardona JA, Pruitt IE, Rhee EP, Colvin RA, and Gerszten RE (2009) Kynurenic acid triggers firm arrest of leukocytes to vascular endothelium under flow conditions. *J Biol Chem* **284**:19189-19195.

Congreve M, Langmead C, and Marshall FH (2011) The use of GPCR structures in drug design. *Adv Pharmacol* **62**:1-36.

Cosi C, Mannaioni G, Cozzi A, Carla V, Sili M, Cavone L, Maratea D, and Moroni F (2011) G-protein coupled receptor 35 (GPR35) activation and inflammatory pain: Studies on the antinociceptive effects of kynurenic acid and zaprinast. *Neuropharmacol* **60**: 1227-1231.

Deng H, and Fang Y (2012) Aspirin metabolites are GPR35 agonists. *Naunyn Schmiedebergs Arch Pharmacol* **385**: 729-737.

Deng H, Hu H, and Fang Y (2012a) Multiple tyrosine metabolites are GPR35 agonists. *Sci Rep* **2**:373.

Deng H, Hu H, Ling S, Ferrie AM and Fang Y (2012b) Discovery of natural phenols as G protein-coupled receptor-35 (GPR35) agonists. *ACS Med. Chem Lett* **3**: 165-169.

## JPET # 201798

DeWire SM, and Violin JD (2011) Biased ligands for better cardiovascular drugs: dissecting G-protein-coupled receptor pharmacology. *Circ Res* **109**: 205-216.

Eglen RM (2007) Assessing GPCR activation using protein complementation: a novel technique for HTS. *Biochem Soc Trans* **35**: 746-748.

Ellinghaus D, Folseraas T, Holm K, Ellinghaus E, Melum E, Balschun T, Laerdahl JK, Shiryaev A, Gotthardt DN, Weismüller TJ, Schramm C, Wittig M, Bergquist A, Björnsson E, Marschall HU, Vatn M, Teufel A, Rust C, Gieger C, Wichmann HE, Runz H, Sterneck M, Rupp C, Braun F, Weersma RK, Wijmenga C, Ponsioen CY, Mathew CG, Rutgeerts P, Vermeire S, Schrumpf E, Hov JR, Manns MP, Boberg KM, Schreiber S, Franke A, and Karlsen TH (2012) Genome-wide association analysis in sclerosing cholangitis and ulcerative colitis identifies risk loci at GPR35 and TCF4. *Hepatology*. Jul 23. doi: 10.1002/hep.25977. [Epub ahead of print]

Fallarini S, Magliulo L, Paoletti T, de Lalla C, and Lombardi G (2010) Expression of functional GPR35 in human iNKT cells. *Biochem Biophys Res Commun* **398**: 420-425.

Guo J, Williams DJ, Puhl HL 3rd, Ikeda SR.J (2008) Inhibition of N-type calcium channels by activation of GPR35, an orphan receptor, heterologously expressed in rat sympathetic neurons. *J Pharmacol Exp Ther* **324**: 342-351.

Hamdan FF, Audet M, Garneau P, Pelletier J, and Bouvier M (2005). High-throughput screening of G protein-coupled receptor antagonists using a bioluminescence resonance energy transfer 1-based beta-arrestin2 recruitment assay. *J Biomol Screen* **10**: 463-475.

## JPET # 201798

Heynen-Genel S, Dahl R, Shi S, Sauer M, Hariharan S, Sergienko E, Dad S, Chung TDY, Stonich D, Su Y, Caron M, Zhao P, Abood ME, and Barak LS (2010) Selective GPR35 Antagonists - Probes 1 & 2. Probe Reports from the NIH Molecular Libraries Program [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2010-. 2010 Feb 28 [updated 2010 Oct 4]

Hu H, Deng H, and Fang Y (2012) Label-free phenotypic profiling identified D-luciferin as a GPR35 agonist. *PLoS One* **7**: e34934.

Jenkins L, Alvarez-Curto E, Campbell K, de Munnik S, Canals M, Schlyer S, and Milligan G (2011) Agonist activation of the G protein-coupled receptor GPR35 involves transmembrane domain III and is transduced via Galpha and beta-arrestin-2. *Br J Pharmacol* **162**: 733-748.

Jenkins L, Brea J, Smith NJ, Hudson BD, Reilly G, Bryant NJ, Castro M, Loza MI, and Milligan G (2010) Identification of novel species-selective agonists of the G-protein-coupled receptor GPR35 that promote recruitment of beta-arrestin-2 and activate Galpha13. *Biochem J* **432**: 451-459.

Jenkins, L., Harries, N., Lappin, J.E., MacKenzie, A.E., Neetoo-Isseljee, Z., Southern, C., McIver, E.G., Nicklin, S.A., Taylor, D.L., Milligan, G. (2012) Antagonists of GPR35

## JPET # 201798

display high species ortholog selectivity and varying modes of action. *J Pharmacol Exp Ther* **343**: 683-695.

Kostenis E, Waelbroeck M, and Milligan G (2005) Techniques: promiscuous Galpha proteins in basic research and drug discovery. *Trends Pharmacol Sci* **26**: 595-602.

Lattin JE, Schroder K, Su AI, Walker JR, Zhang J, Wiltshire T, Saijo K, Glass CK, Hume DA, Kellie S, and Sweet MJ (2008) Expression analysis of G Protein-Coupled Receptors in mouse macrophages. *Immunome Res* **4**:5.

Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski MS, Brockway KS, Byrnes EJ, Chen L, Chen L, Chen TM, Chin MC, Chong J, Crook BE, Czaplinska A, Dang CN, Datta S, Dee NR, Desaki AL, Desta T, Diep E, Dolbeare TA, Donelan MJ, Dong HW, Dougherty JG, Duncan BJ, Ebbert AJ, Eichele G, Estin LK, Faber C, Facer BA, Fields R, Fischer SR, Fliss TP, Frensley C, Gates SN, Glattfelder KJ, Halverson KR, Hart MR, Hohmann JG, Howell MP, Jeung DP, Johnson RA, Karr PT, Kawal R, Kidney JM, Knapik RH, Kuan CL, Lake JH, Laramée AR, Larsen KD, Lau C, Lemon TA, Liang AJ, Liu Y, Luong LT, Michaels J, Morgan JJ, Morgan RJ, Mortrud MT, Mosqueda NF, Ng LL, Ng R, Orta GJ, Overly CC, Pak TH, Parry SE, Pathak SD, Pearson OC, Puchalski RB, Riley ZL, Rockett HR, Rowland SA, Royall JJ, Ruiz MJ, Sarno NR, Schaffnit K, Shapovalova NV, Sivasay T, Slaughterbeck CR, Smith SC, Smith KA, Smith BI, Sodt AJ, Stewart NN, Stumpf KR, Sunkin SM, Sutram M, Tam A, Teemer CD, Thaller C, Thompson CL, Varnam LR, Visel A, Whitlock RM, Wohnoutka

## JPET # 201798

PE, Wolkey CK, Wong VY, Wood M, Yaylaoglu MB, Young RC, Youngstrom BL, Yuan XF, Zhang B, Zwingman TA, Jones AR. (2007) Genome-wide atlas of gene expression in the adult mouse brain. *Nature* **445**: 168-176.

Leonard JN, Chu ZL, Unett DJ, Gatlin JE, Gaidarov I, Qiu J, Skinner PJ, and Boatman .D (2005) GPR35 and modulators thereof for the treatment of metabolic-related disorders. Patent Application # G01N33/50

MacKenzie AE, Lappin JE, Taylor DL, Nicklin SA and Milligan G (2011) GPR35 as a novel therapeutic target. *Front Endocrin Molecular and Structural Endocrinology* **2**: 68.

Milligan G (2011). Orthologue selectivity and ligand bias: translating the pharmacology of GPR35. *Trends Pharmacol Sci* **32**: 317-325.

Min KD, Asakura M, Liao Y, Nakamaru K, Okazaki H, Takahashi T, Fujimoto K, Ito S, Takahashi A, Asanuma H, Yamazaki S, Minamino T, Sanada S, Seguchi O, Nakano A, Ando Y, Otsuka T, Furukawa H, Isomura T, Takashima S, Mochizuki N, and Kitakaze M (2010) Identification of genes related to heart failure using global gene expression profiling of human failing myocardium. *Biochem Biophys Res Commun* **393**: 55-60.

Ohshiro H, Tonai-Kachi H, and Ichikawa K (2008) GPR35 is a functional receptor in rat dorsal root ganglion neurons. *Biochem Biophys Res Commun* **365**: 344-348.

## JPET # 201798

Oka S, Ota R, Shima M, Yamashita A, and Sugiura T (2010) GPR35 is a novel lysophosphatidic acid receptor. *Biochem Biophys Res Commun* **395**: 232-237.

Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, Dewire SM, Violin JD, and Lefkowitz RJ. (2011) Quantifying ligand bias at seven-transmembrane receptors. *Mol Pharmacol* **80**: 367-377.

Salon JA, Lodowski DT, and Palczewski K (2011) The significance of G protein-coupled receptor crystallography for drug discovery. *Pharmacol Rev* **63**: 901-937.

Shrimpton AE, Braddock BR, Thomson LL, Stein CK, and Hoo JJ (2004) Molecular delineation of deletions on 2q37.3 in three cases with an Albright hereditary osteodystrophy-like phenotype. *J Clin Genet* **66**: 537-544.

Sun YV, Bielak LF, Peyser PA, Turner ST, Sheedy PF 2nd, Boerwinkle E and Kardia S L (2008). Application of machine learning algorithms to predict coronary artery calcification with a sibship-based design. *Genet Epidemiol* **32**: 350-60.

Taniguchi Y, Tonai-Kachi H, and Shinjo, K (2006) Zaprinast, a well-known cyclic guanosine monophosphate-specific phosphodiesterase inhibitor, is an agonist for GPR35. *FEBS Lett* **580**: 5003-5008.

**JPET # 201798**

Taniguchi Y, Tonai-Kachi H, Shinjo K. (2008) 5-Nitro-2-(3-phenylpropylamino)benzoic acid is a GPR35 agonist. *Pharmacology* **82**: 245-249.

Trinquet E, Bouhelal R, and Dietz M (2011) Monitoring Gq-coupled receptor response through inositol phosphate quantification with the IP-One assay. *Expert Opin Drug Discov* **6**: 981-994.

Vander Molen J, Frisse LM, Fullerton SM, Qian Y, Del Bosque-Plata L, Hudson RR, and Di Rienzo A. (2005) Population genetics of CAPN10 and GPR35: implications for the evolution of type 2 diabetes variants. *Am J Hum Genet* **76**: 548-60.

Verkaar F, van Rosmalen JW, Blumenröhr M, van Koppen CJ, Blankesteyn WM, Smits JF, and Zaman GJ (2008) G protein-independent cell-based assays for drug discovery on seven-transmembrane receptors. *Biotechnol Annu Rev* **14**:253-274.

Wang J, Simonavicius N, Wu X, Swaminath G, Reagan J, Tian H, and Ling L (2006) Kynurenic acid as a ligand for orphan G protein-coupled receptor GPR35. *J Biol Chem* **281**: 22021-22028.

Yang Y, Lu JY, Wu X, Summer S, Whoriskey J, Saris C, and Reagan JD (2010) G-protein-coupled receptor 35 is a target of the asthma drugs cromolyn disodium and nedocromil sodium. *Pharmacology* **86**:1-5.

**JPET # 201798**

Yang Y, Fu A, Wu X, and Reagan JD (2012) GPR35 is a target of the loop diuretic drugs bumetanide and furosemide. *Pharmacology* **89**:13-17.

Zhao P, Sharir H, Kapur A, Cowan A, Geller EB, Adler MW, Seltzman HH, Reggio PH, Heynen-Genel S, Sauer M, Chung TD, Bai Y, Chen W, Caron MG, Barak LS, and Abood ME (2010). Targeting of the orphan receptor GPR35 by pamoic acid: a potent activator of extracellular signal-regulated kinase and beta-arrestin-2 with antinociceptive activity. *Mol Pharmacol* **78**: 560-568.



## **JPET # 201798**

### **Footnotes**

AEM thanks the Biotechnology and Biosciences Research Council for studentship support.

## **JPET # 201798**

### **Legends for Figures**

#### **Figure 1 Development of a HTS compatible $\beta$ -arrestin recruitment assay for human GPR35**

Concentration-response curves for agonist hits identified from a pilot screen of NINDS/Sequoia compound libraries were performed using the human GPR35 PathHunter™  $\beta$ -arrestin recruitment assay. Data are expressed as % of the response to 100  $\mu$ M zaprinast.  $pEC_{50}$  values for each compound are provided in parentheses. Data are means  $\pm$  SEM from two separate experiments performed in duplicate.

#### **Figure 2 Analysis of HTS data: efficacy of hits at human GPR35**

A distribution of agonist activity recorded in the 100,000 compound library HTS is shown. Compound activity at 10  $\mu$ M using a human GPR35 PathHunter™  $\beta$ -arrestin recruitment assay is shown both as hit frequency, binned as % agonist activity versus zaprinast, with number of hits for each % activity indicated above each bar. The pie chart shows the percentage of hits within each % activity group.

#### **Figure 3 Analysis of HTS data: potency of hits at human GPR35**

Distribution of  $pEC_{50}$  values determined for 348 agonist compounds identified from a 100,000 compound library HTS performed using a human GPR35 PathHunter™  $\beta$ -arrestin recruitment assay. Data are displayed as the percentage of these compounds within distinct  $pEC_{50}$  ranges.

#### **Figure 4 Comparison of agonist activity in $\beta$ -arrestin and $Ca^{2+}$ mobilization assays**

## **JPET # 201798**

Five selected compounds (**1-5**) re-supplied following hit identification were compared to zaprinast for activity and potency in the human GPR35 PathHunter™  $\beta$ -arrestin recruitment assay (**A**) and in intracellular calcium mobilization studies (**B**). Data in **A** are expressed as % of the maximum response to zaprinast and in **B** as Fluorescence Intensity Units (FIU). Means  $\pm$  SEM from two separate experiments performed in duplicate.

### **Figure 5 Analogs of compound 1 are also potent agonists of human GPR35**

The potency of compound **1** (circles) was compared to that of the related analogs, compounds **6** (squares) and **7** (triangles) as well as zaprinast (diamonds) in the human GPR35 PathHunter™  $\beta$ -arrestin recruitment assay (**A**). (**B**). Using the BRET-based  $\beta$ -arrestin-2 interaction assay the potency of these ligands were compared at human, rat and mouse GPR35. Data are means  $\pm$  SEM, n = 3.

### **Figure 6 Comparison of agonist activity at GPR35 orthologs**

Compounds **1-5** as well as zaprinast were employed in both BRET-based  $\beta$ -arrestin-2 recruitment assays (**A**) and in IP1 accumulation assays (**B**) conducted in HEK-293T cells transfected to transiently express human, mouse or rat GPR35. Data are expressed as % of the maximum response to zaprinast. Data represent means  $\pm$  SEM from three separate experiments performed in duplicate.

### **Figure 7 ML-145 is a competitive antagonist of compounds 1-3 at human GPR35**

## **JPET # 201798**

The ability of varying concentrations of ML-145 to alter concentration-response curves to compound **1 (A, D)**, compound **2 (B, E)** and compound **3 (C, F)** at human GPR35 was assessed in either BRET-based  $\beta$ -arrestin-2 recruitment (**A-C**) or IP1 accumulation (**D-F**) assays. In each case fitting of the data was consistent with competitive interactions. Data represent means  $\pm$  SEM,  $n = 3$  in each case.

JPET # 201798

## Tables

**Table 1 Characterization of novel GPR35 agonists**

The chemical structures of compounds 1-5 are shown as are pEC<sub>50</sub> and E<sub>max</sub> values obtained in the various assays employed. Hu = human, Mu = mouse.

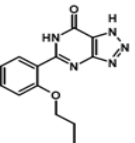
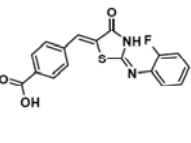
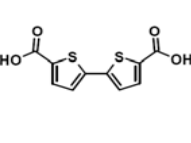
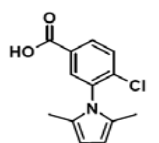
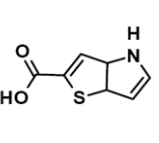
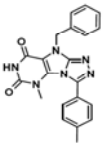
			Zaprinast	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5
								
PathHunter β-arrestin	Hu GPR35	pEC <sub>50</sub>	5.1+/-0.01	7.6+/-0.05	6.3+/-0.05	5.4+/-0.11	5.6+/-0.01	4.9+/-0.07
		Max	100.0+/-0.00	95.3+/-2.24	98.3+/-5.22	105.0+/-9.95	101.8+/-8.75	72.0+/-5.58
PathHunter (Gqi5)	Hu GPR35	pEC <sub>50</sub>	6.0+/-0.34	8.5+/-0.05	6.0+/-0.04	5.5+/-0.10	4.9+/-0.03	6.2+/-0.09
		Max	1.7+/-0.05	1.6+/-0.05	1.7+/-0.02	1.8+/-0.06	1.5+/-0.03	1.6+/-0.01
BRET β-arrestin	Hu GPR35	pEC <sub>50</sub>	5.6+/-0.03	7.6+/-0.03	5.5+/-0.04	5.5+/-0.03	4.9+/-0.06	4.7+/-0.04
		Max	100+/-1.35	100.2+/-1.32	96.1+/-1.81	123.0+/-1.98	104.3+/-4.25	134.4+/-4.10
	Mu GPR35	pEC <sub>50</sub>	6.6+/-0.04	4.78+/-0.05	<4	<4	4.7+/-0.16	4.0+/-0.24
		Max	101.1+/-1.53	61.7+/-2.52	-	-	68.1+/-7.97	109.4/-32.7
	Rat GPR35	pEC <sub>50</sub>	7.1+/-0.04	5.1+/-0.08	<4	<4	4.3+/-0.09	4.9+/-0.05
		Max	100.0+/-1.67	36.0+/-1.53	-	-	88.4+/-8.00	116.9+/-3.8
Inositol phosphate (Gaqa13)	Hu GPR35	pEC <sub>50</sub>	6.9+/-0.06	8.1+/-0.09	6.5+/-0.06	6.8+/-0.07	5.6+/-0.10	6.0+/-0.10
		Max	99.0+/-2.87	86.0+/-3.53	100.9+/-3.1	84.1+/-4.63	96.8+/-5.60	92.5+/-5.18
	Mu GPR35	pEC <sub>50</sub>	7.9+/-0.60	5.6+/-0.09	4.5+/-0.14	<4	6.0+/-0.08	5.0+/-0.16
		Max	99.3+/-5.49	133.7+/-6.08	197.0+/-20.7	-	107.3+/-3.80	101.4+/-11.02
	Rat GPR35	pEC <sub>50</sub>	8.3+/-0.42	5.6+/-0.10	4.5+/-0.14	<4	5.8+/-0.11	5.8+/-0.93
		Max	97.9+/-6.36	110.6+/-6.29	197.2+/-21.3	-	92.9+/-5.81	105.5+/-4.96

Figure 1

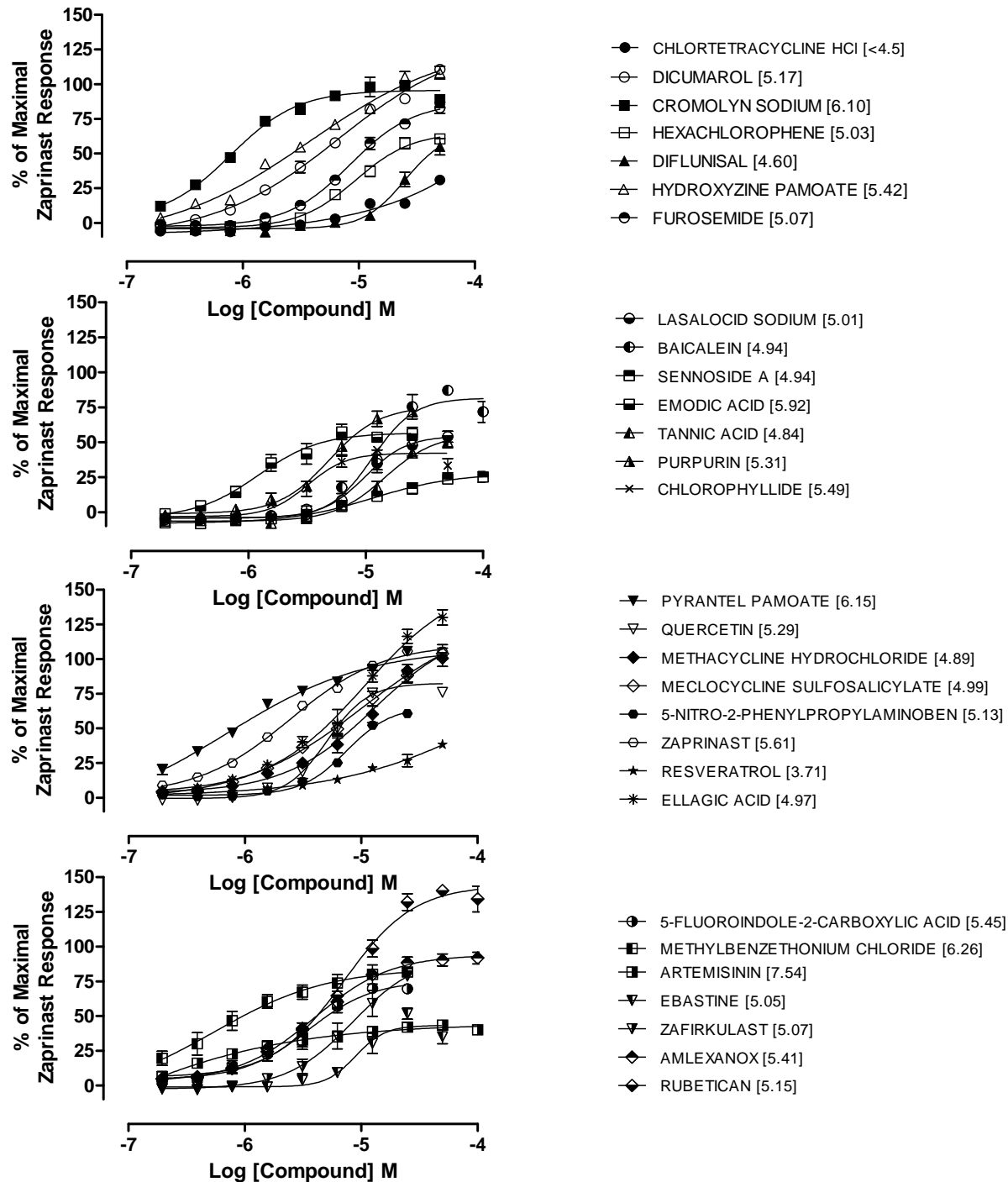


Figure 2

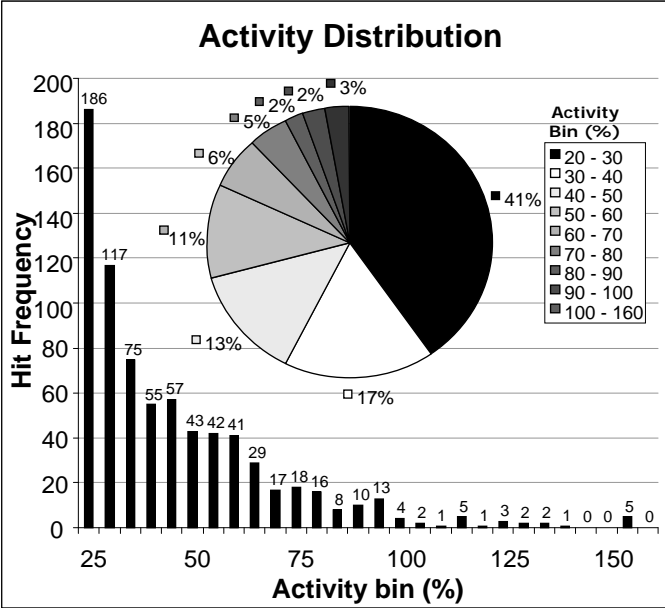


Figure 3

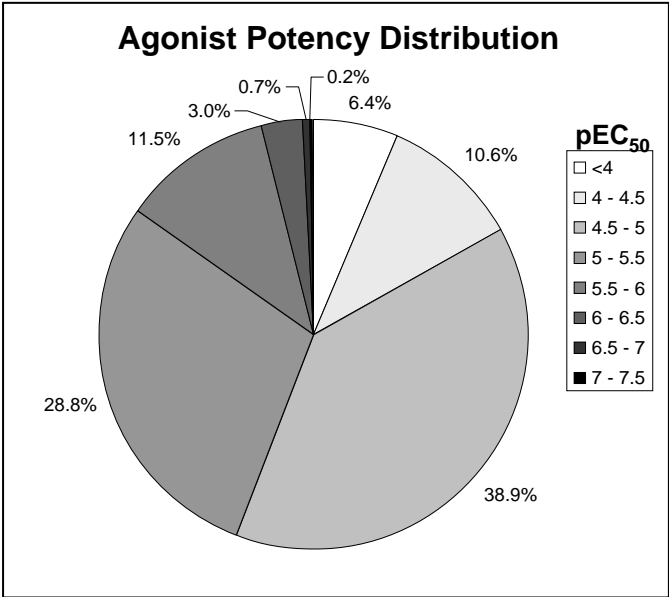
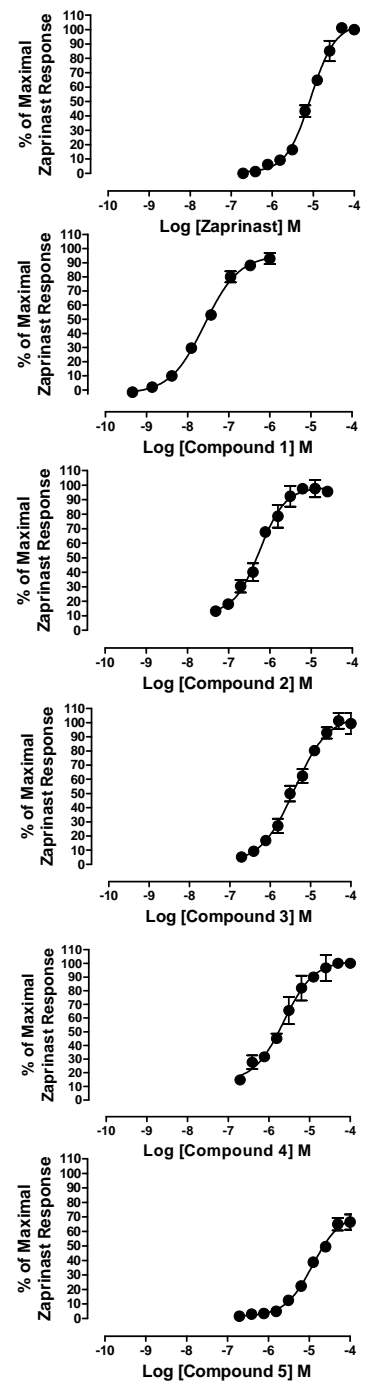




Figure 4

A PathHunter  $\beta$ -arrestin



B Calcium mobilization

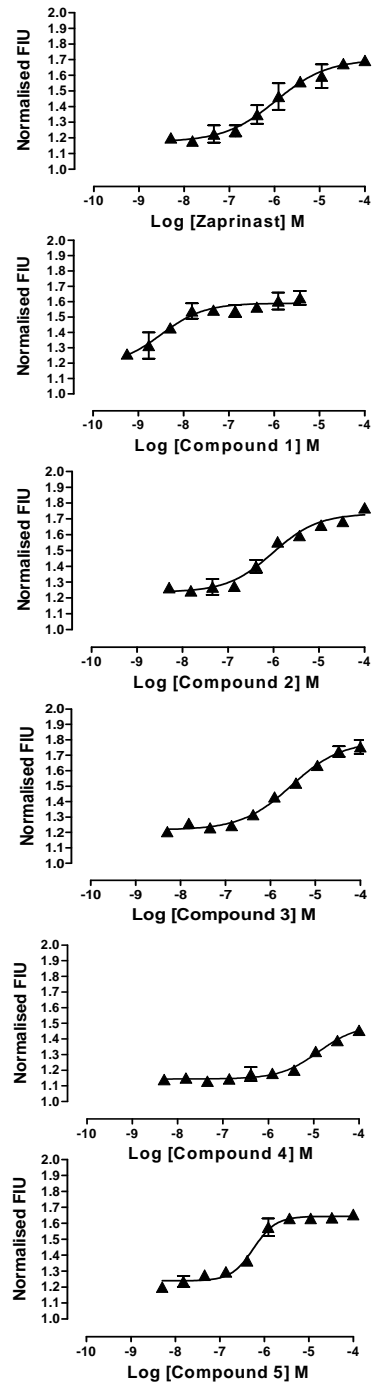


Figure 5

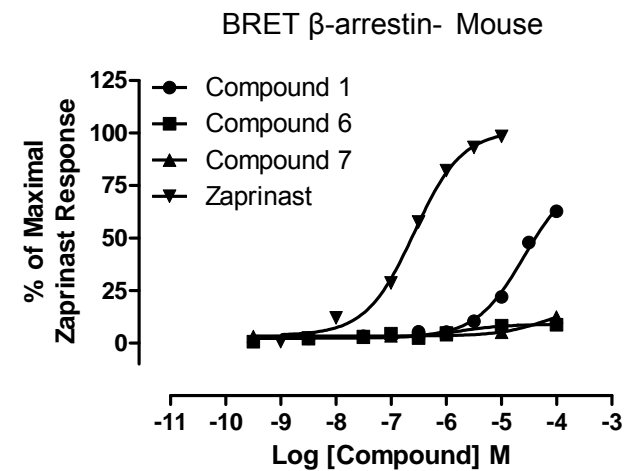
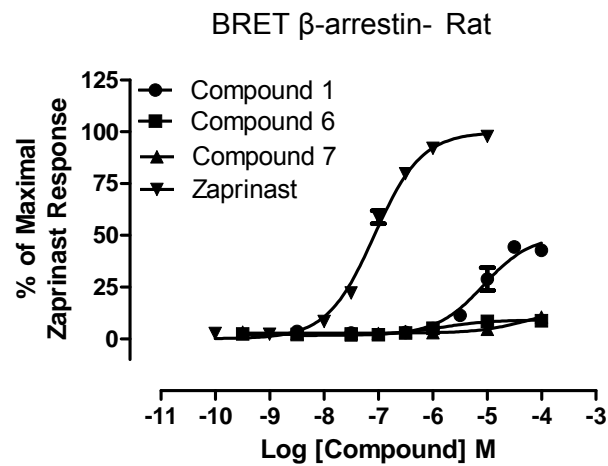
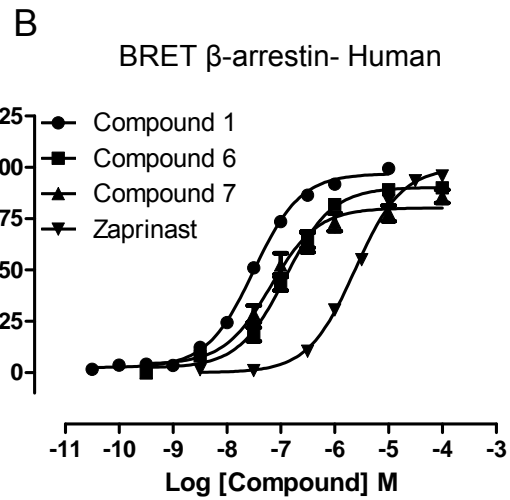
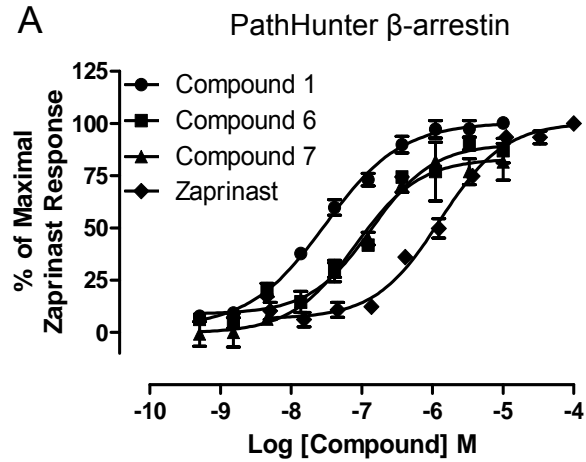


Figure 6

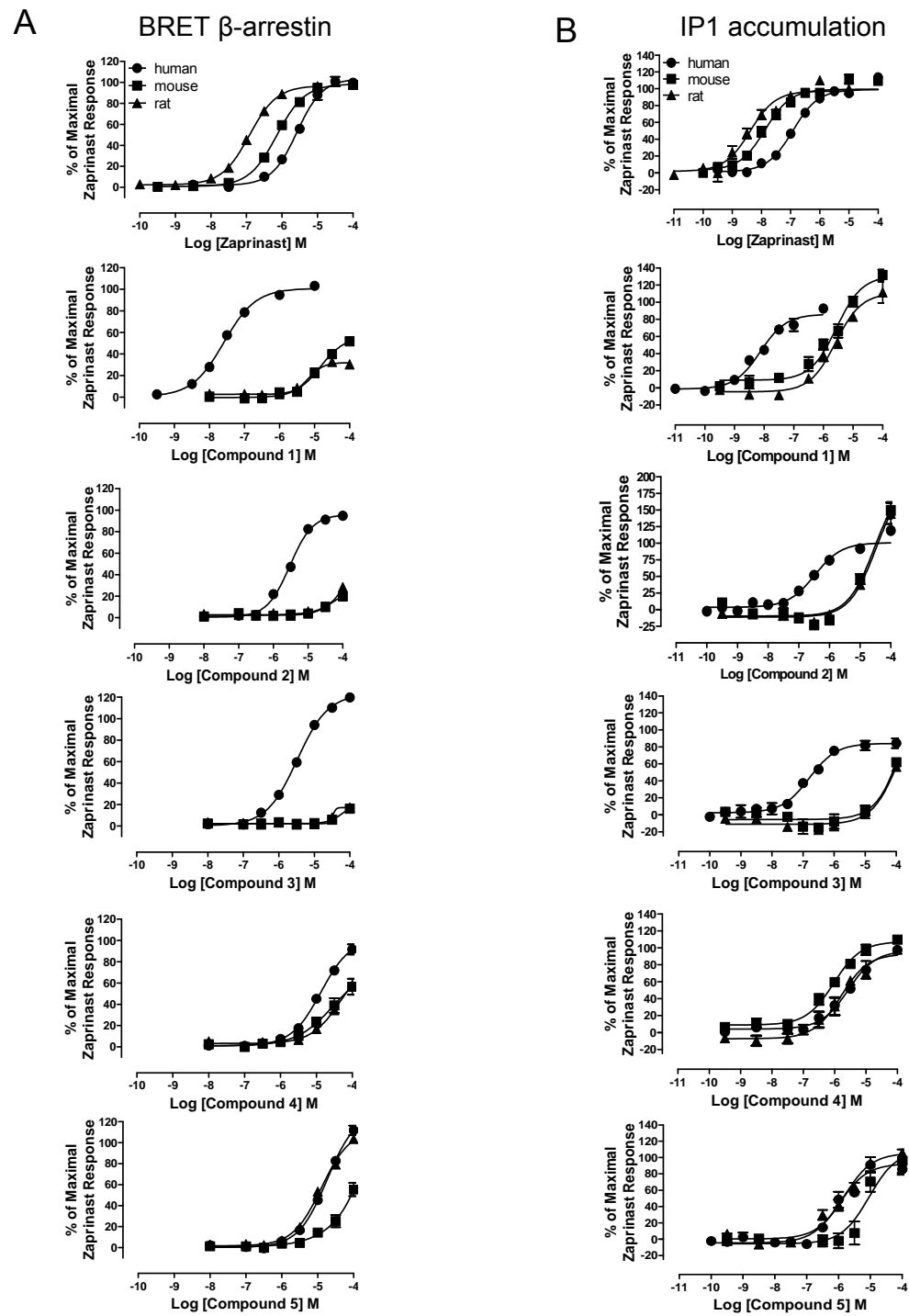
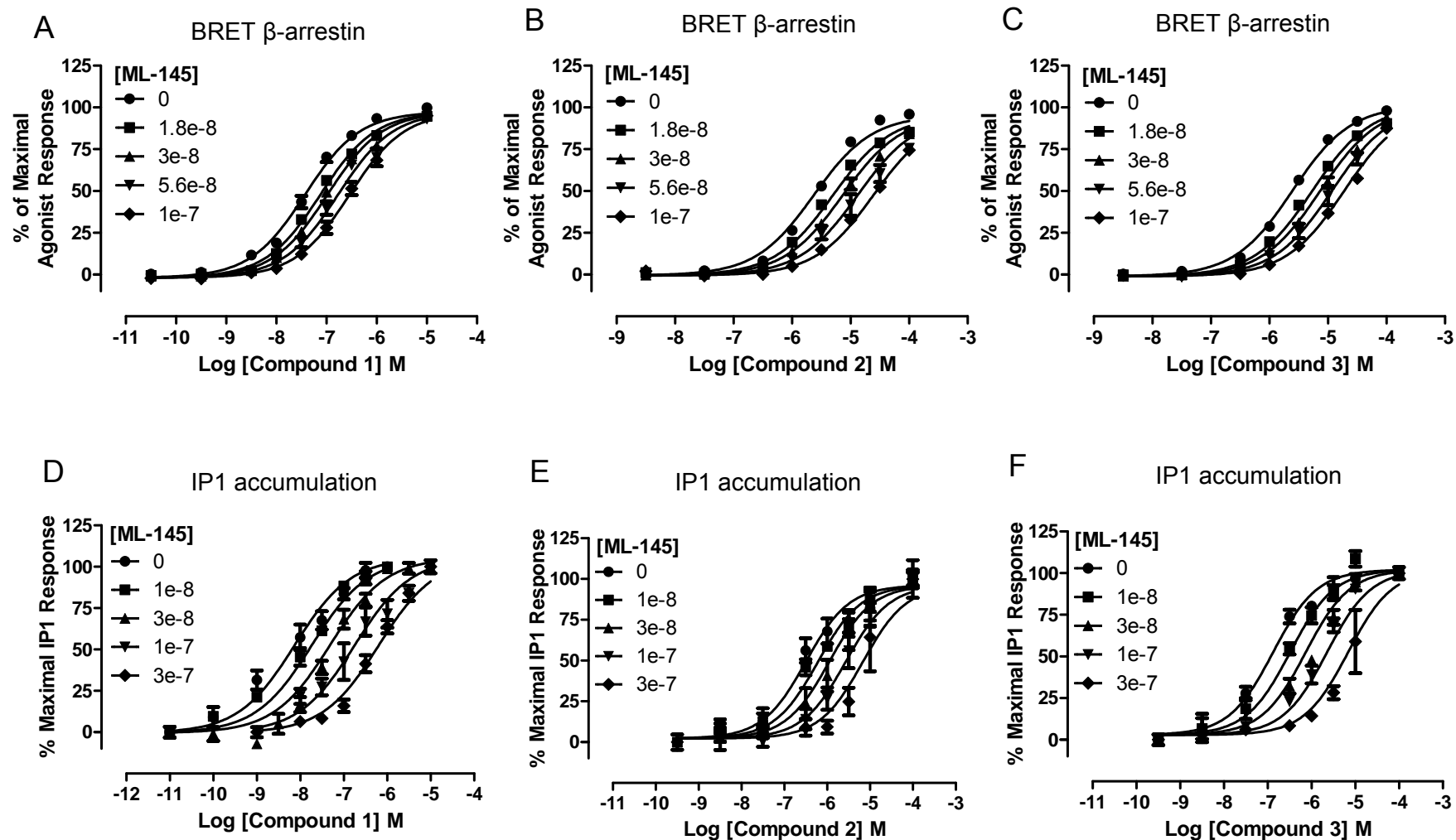


Figure 7



## Supplementary Table 1

### High throughput identification and characterization of novel, species-selective

#### GPR35 agonists

Zaynab Neetoo-Isseljee, Amanda E. MacKenzie, Craig Southern, Jeff Jerman,

Edward G. McIver, Nicholas Harries, Debra L. Taylor and Graeme Milligan.

JPET201798

	pEC <sub>50</sub>			E <sub>max</sub> (100µM)		
MRCT code	Human	Mouse	Rat	Human	Mouse	Rat
1007	4.3	<4	5.5	58	42	34
18030	4.8	5.2	6.1	189	135	164
18036	5.9	6.8	6.4	79	82	105
26822	5	4.9	4.8	75	94	41
39029	<4	4.9	5.2	5	67	34
41311	5.2	<4	<4	25	0	1
42475	5.3	4.6	5.1	40	60	60
42699	<4	4.2	5.1	29	103	85
50003	5	<4	<4	19	0	3
50351	4.7	5	<4	71	40	50
54454	7	4.9	5.5	94	62	41
59297	4.8	5.7	6.1	54	66	51
59416	5.5	5.1	4.3	92	74	51
59439	5.1	4.2	5	87	47	86
59521	5.1	<4	<4	61	18	23
59872*	6.8	6.5	5.3	67	60	56
60030	5	<4	<4	68	50	44
60211	5.5	<4	4.2	99	38	46
104124	5.6	<4	<4	98	9	14
121525	5.1	<4	<4	83	6	4
122758	5.3	<4	<4	80	15	29
126907	<4	5.2	5.2	9	39	30
127254	5.3	4.5	<4	44	46	38
132295	5.8	4.8	5	21	29	42
141051	5.1	4.6	4.9	92	82	62
149265	<4	5.4	4	38	23	70
177540	4.5	5.8	4.4	87	76	60
179831	4.6	<4	5.3	103	23	127
192086	<4	5.5	5.8	9	48	10
192973	5.5	5.2	5.2	23	33	44

**Supplementary Table 1** The potency and efficacy of a series of compounds repurchased following initial screening of the diversity library against human GPR35 was assessed in BRET-based GPR35- $\beta$ -arrestin-2 interactions assays using each of the human, rat and mouse orthologs of the receptor. Efficacy values are compared to zaprinast. \* One compound was insufficiently soluble to be assayed at 100  $\mu$ M and therefore efficacy values reflect function at 10  $\mu$ M. The chemical identity of any of these ligands is available on request.