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Short Title: Screening for novel agonists of GPR35

SYNOPSIS

The poorly characterised 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-β-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 whilst 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 chimeric Gpa1-$G\alpha_{13}$ G protein confirmed that all of the compounds active at human GPR35 in the β-arrestin-2 interaction assay were also able to promote cell growth via $G\alpha_{13}$. Each of these ligands also promoted binding of [^{35}S]GTPγS 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.

Keywords: screening, drug discovery, G protein-coupled receptor

[1]**Abbreviations:** BRET**,** bioluminescence resonance energy transfer; GPCR, G protein-coupled receptor; GTPγS, Guanosine 5'-*O*-(3-thiotriphosphate); PPARγ peroxisome proliferator-activated receptor γ

Chemical names

Cromolyn; 5,5′-(2-hydroxypropane-1,3-diyl)bis(oxy)bis(4-oxo-4*H*-chromene-2-carboxylic acid)
Dicumarol; 3,3'-methylenebis(4-hydroxy-2*H*-chromen-2-one)
Flufenamic acid; 2-{[3-(trifluoromethyl)phenyl]amino}benzoic acid
Luteolin; 2-(3,4-dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone
Niflumic acid; 2-{[3-(trifluoromethyl)phenyl]amino}nicotinic acid.
Oxantel pamoate; (1-methyl-2-(3-hydroxyphenylethenyl)-1,4,5,6-tetrahydropyrimidine) pamoate
Pamoate; 4,4'-methylenebis(3-hydroxy-2-naphthoic acid)
Pyrvinium; (4-[(3-carboxy-2-hydroxynaphthalen-1-yl)methyl]-3-hydroxynaphthalene-2-carboxylic acid; 1-methyl-2-[(*E*)-2-thiophen-2-ylethenyl]-5,6-dihydro-4*H*-pyrimidine) pamoate
Quercetin; -(3,4- dihydroxyphenyl)- 3,5,7- trihydroxy- 4*H*- chromen- 4-one
Zaprinast; 2-(2-Propyloxyphenyl)-8-azapurin-6-one

Introduction:

GPR35 is a poorly characterised G protein-coupled receptor (GPCR[1]) [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 [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 β-arrestin-2 and to cause internalisation of the receptor in transfected cell systems is above 100 μM [8], well above 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 expressing human umbilical vein endothelial cells via a β2-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 PPARγ class [11]. Identification of other ligands and a clear understanding of their relative potency/affinity at species 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 bioluminescence resonance energy transfer (BRET)-based assay [8, 12-13] that reports interactions between GPR35 and β-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 shown recently 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 disodium and the anticoagulant/vitamin K antagonist dicumarol. Significant species selectivity was also observed. For example, the drug congener pamoate was a potent agonist at human GPR35 but almost inactive at rat GPR35 whilst, although less potent, niflumic acid was also highly selective in promoting interactions with β-arrestin-2 via human GPR35. In contrast, both zaprinast and the flavenoid luteolin displayed substantial selectivity for rat GPR35. A number of these compounds may be useful for exploring the physiological functions of GPR35 but, as previously described for zaprinast [7-8], species selectivity needs to be considered before selecting ligands to perform such studies.

Experimental:

Constructs

DNA constructs

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'GCGAAGCTTGCCACCATGGATTACAAGGATGACGACGATAAGAATGGC ACCTACAACACC 3', rat sense, 5'CGCGGATCCGCCACCATGGATTACAAGGAT GACGACGATAAGAACAATACAAATTGTAGCATC 3'; and human antisense: 5'GGCCGCGGCCGCCTCTAGAATTAGGCGAGGG 3', rat antisense: 5'GGCCGCGGCCGCCGGTGAGGCTCAGGCTCTG 3'. The HindIII, BamHI 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 yielding the final N-terminal epitope- and C-terminal fluorescent protein-tagged constructs FLAG-humanGPR35-eYFP-pcDNA5/FRT/TO, and FLAG-ratGPR35-eYFP-pcDNA5/FRT/TO. The integrity of each fusion was confirmed by DNA sequencing.

Cell culture and BRET studies - HEK293T cells were maintained in Dulbecco's modification of Eagle's medium supplemented with 0.292 g/litre L-glutamine and 10% (v/v) newborn calf serum at 37 °C in a 5% CO_2 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 (ratio 1:6 DNA: polyethyleneimine), diluted in 150 mM NaCl, pH 7.4. After incubation at room temperature for 10 min, the mixture was added to HEK293T 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 equalised 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 Hank's Balanced Salt Solution (pH 7.4) (HBSS), and coelentrazine-h (Promega) was added to a final concentration of 5 μM. Cells were incubated in darkness 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 utilised a commercially available 1120 compound Bioactives set of marketed drugs and pharmacologically active agents (Prestwick Chemicals, France). Compounds were formatted into 96-well daughter sets at 1 mM in 100% DMSO (80 compounds per plate) and active working sets stored.

Primary screening

Compounds were assayed in a single point assay at 10 μM with 1% DMSO. Basal wells containing assay buffer with 1% DMSO, stimulation wells containing 10 μM zaprinast and two points containing a reference compound (5 μM zaprinast) were included in all plates. Each plate contained two '*Renilla* luciferase' points with cells

lacking receptor expression and two 'Mock' points with cells transfected with pcDNA3. Cells were washed with HBSS and incubated for 30 min at 37ºC, after this time a fluorescence reading was performed to check the efficiency of transfection with the GRP35-eYFP constructs. Buffer containing 5 µM coelenterazine-h was added to each well and cells were incubated for 10 min at 37ºC. After this time compounds were added and cells were incubated for 5 min at 37ºC and subsequent BRET measurements were carried out using a PHERAstar FS reader (BMG-Labtech, Offenburg, Germany). Compound autofluorescence was assessed for all the hits obtained in the assay in order to detect those compounds that might be false positives simply because they affected BRET measurements

Data analysis and processing

Data from primary screening were analyzed using Microsoft Excel software. The activities of the compounds were calculated following the formula:
Activity (%) = (mBRET compound-mBRET basal)/(mBRET stim-mBRET basal) x100; where mBRET compound was the mBRET value obtained from wells treated with the test compound, mBRET basal was the average of the mBRET values obtained from wells treated with assay buffer and mBRET stim was 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 x SD) 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:

$$Z' = 1-((3x\sigma_{stim}) + (3x\sigma_{basal}))/(\mu_{stim}-\mu_{basal})),$$

where σ_{stim} and σ_{basal} were the standard deviation values of wells containing 10 µM zaprinast and assay buffer, respectively and μ_{stim} and μ_{basal} were the mean of wells containing 10 µM zaprinast and assay buffer, respectively. A Z' value higher than 0.5 was selected as the cutoff in the screen.

Compound re-supply

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

Yeast $G\alpha_{13}$ assay

Human GPR35 bearing an N-terminal FLAG epitope as previously described [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 [14] to express either wild type yeast Gα protein (Gpa1; strain MMY12) or a chimeric version of Gpa1 in which the last 5 amino acids of the yeast G protein were replaced by the C-terminal 5 amino acids from human $G\alpha_{13}$ (QLMLQ; Gpa1/G_{13}; MMY20) [15-16]. GPR35 coupling through the G_{13} 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, 2% (w/v) glucose) until saturation and diluted to approximately 0.0025 OD_{600}/ml in synthetic drop-out media (SC-ura,-his) supplemented with 10 mM 3-aminotriazole, 10 µM fluorescein di-β-D-galactopyranoside and 0.1 M sodium phosphate pH 7.0, then seeded into 96 well black tissue culture plates (Greiner, Gloucestershire, UK). After

20 hours incubation at 30 ºC with shaking, β-galactosidase-mediated generation of fluorescein was measured using a PHERAstar FS microplate reader (excitation 485 nm, emission 520 nm; BMG Labtech, Offenburg, Germany).

Mammalian Ga_{13} [^{3}5S]GTPγS binding assay

Flp-In™ T-REx™ 293 cells (Invitrogen Ltd, Paisley, UK) 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 $G\alpha_{13}$ construct (Missouri S&T cDNA Resource Center, Rota, Missouri). 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 previously described protocol [8]. Compounds were then tested for their ability to activate $G\alpha_{13}$ using 25 μg of cell membranes per reaction carried out in 1x assay buffer (20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl) containing 1μM GDP, 50nCi [^{35}S]GTPγS, and 1% DMSO. Reactions were incubated for 30 min at 30°C before being terminated by the addition of ice-cold 1x assay buffer. Samples were then centrifuged at 14000 x g for 15 min at 4°C. Supernatants were discarded and the pellets were resuspended in solubilisation buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, and 1.25% Nonidet P-40) containing 0.2% SDS. Samples were pre-cleared with Pansorbin, followed by immunoprecipitation with an anti-EE monoclonal antibody (Covance, Princeton, New Jersey). Immunocomplexes were then washed four times with solubilisation buffer before bound [^{35}S]GTPγS was estimated by liquid scintillation spectrometry.

Results

Human GPR35, modified to incorporate both an N-terminal FLAG epitope tag and C-terminal enhanced yellow fluorescent protein (eYFP), was co-transfected into HEK293T cells along with a form of β-arrestin-2 C-terminally tagged with *Renilla* luciferase. As shown previously [8] this established a bioluminescence resonance energy transfer (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 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 1A**. 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 % 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 percent activation value of > 3 Standard Deviations 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 % 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 2D.**

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, Table 1**). By contrast, both cromolyn disodium and dicumarol were essentially equipotent at the two species orthologues (**Figure 3, Table 1**). Interestingly, two of the hits identified in screening of human GPR35, oxantel pamoate and pyrvinium pamoate, were not identified in the screen of rat GPR35 and concentration-response curves (**Figures 4A, 4B, Table 1**) confirmed 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, 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 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 ($EC_{50} = 1.4 \times 10^{-5}$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 did not display 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 and the closely related anti-oxidant flavenoid, luteolin. These were also purchased and re-tested in concentration-response curves (**Figure 6**). Luteolin was confirmed, like zaprinast, to be substantially more potent at rat than human GPR35 but to be of substantially lower potency than zaprinast (luteolin $EC_{50} = 1 \times 10^{-5}$M, zaprinast $EC_{50} = 6.8 \times 10^{-8}$ M) (**Table 1**). By contrast, although clearly more efficacious at rat than human GPR35 (**Figure 6**), quercetin had similar potency at the two species orthologues ($4.7\text{-}5.6 \times 10^{-6}$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 to zaprinast as the reference agonist. In such studies, when compared to 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**). At rat GPR35 both quercetin and luteolin were partial agonists whilst 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 x 10^{-5}M zaprinast generated a higher signal than the most effective concentration of pamoate (**Figure 8**). However, for both 1 x 10^{-5}M and 1 x 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 data are entirely consistent with pamoate acting as a partial agonist with efficacy of some 50% compared to zaprinast and interacting at an overlapping binding site.

Although ligand-promoted interactions between a GPCR and a β-arrestin has 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 $G\alpha_{13}$ compared to either $G\alpha_{12}$ or $G\alpha_{q}$ [8]. We therefore developed and utilised a *Saccharomyces cerevisiae*-based human GPR35 activation assay. This employed a strain expressing a chimeric yeast-mammalian Gpa1-$G\alpha_{13}$ G protein in which the last 5 amino acids of the yeast G protein were replaced by the C-terminal 5 amino acids (QLMLQ) from human $G\alpha_{13}$. 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 (EC_{50} = 1.4 x 10^{-9}M) to niflumic acid (EC_{50} = 4.4 x 10^{-6}M) (**Figure 9A, 9B**). By contrast, in cells expressing full length Gpa1 no activity was recorded for any of these ligands (**Figure 9C**). These data are consistent with each compound promoting interaction and activation of $G\alpha_{13}$ via human GPR35

Despite this conclusion, the yeast-based assay employs a chimeric G protein containing only a fragment of the GPCR recognition domain of $G\alpha_{13}$. To further validate the conclusions we also developed a mammalian $G\alpha_{13}$ [^{35}S]GTPγS binding assay. This was based on the expression and subsequent immunocapture of a form of $G\alpha_{13}$ containing an internal 'EE' epitope-tag to allow efficient immunocapture with an anti-'EE' antibody. Flp-In™ T-REx™ 293 cells induced to express human GPR35-eYFP [8] were transfected with 'EE' $G\alpha_{13}$. Membranes from these cells were incubated with [^{35}S]GTPγS 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 earlier to be active at human GPR35 promoted statistically significant ($p<0.01$) increases in the amount of [^{35}S]GTPγS present in the immunoprecipitates (**Figure 10**), providing direct evidence for the capacity of agonists at human GPR35 to promote activation of $G\alpha_{13}$ whilst flufenamic acid was unable to do so (**Figure 10**). Equally, both luteolin and quercetin produced no significiant stimulation of [^{35}S]GTPγS binding, consistent with their low potency and efficacy at human GPR35 in the GPR35-β-arrestin-2 interaction assay.

Discussion

Although poorly characterised, 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. Firstly, kynurenic acid displays very low potency to activate human GPR35 and hence produce either recruitment of β-arrestin-2 or cause internalisation of the receptor from the surface of transfected cells [8]. Secondly, whilst lysophosphatidic acid has also been suggested to be the endogenous agonist for GPR23 [24] that, based on overall sequence similarity, is the most closely related receptor to GPR35, both these receptors lie well outwith 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 characterised 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 internalisation 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 screen 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 previously demonstrated, zaprinast was some 38 fold more potent at rat than at human GPR35, whilst cromolyn disodium (cromoglicic acid) was an essentially equipotent (EC_{50} = 4.4-7.6 x 10^{-6}M) agonist at both orthologues. This, in part, confirms a very 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 previously reported, dicumarol, was also identified as a non-selective and slightly more potent (EC_{50} = 1.3-2.0 x 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 x 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 to 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 x 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 interation assay neither niflumic acid nor pamoate would, for example, be of use in exploring contributions of activating GPR35 in rat models of disease. This issue of variation in pharmacology at species orthologue drug targets is a vitally important area for pharmaceutical research and possible development.

It was equally clear from the initial screens that certain ligands potentially displayed the reverse species orthologue selectivity. Furthermore the concept that zaprinast is more potent at rat than human GPR35 has already been established [7-8]. Both quercetin and luteolin were active at rat but not human GPR35 in the initial screens. However, although this selectivity was confirmed for luteolin in concentration-response curve studies it was not for quercetin. In the case of quercetin, relatively high efficacy at rat GPR35 resulted in this ligand reporting as a hit in the initial screen, but its low efficacy as an agonist at human GPR35 resulted in this compound being within the defined screen tolerance, although detailed studies indicated it to be equipotent at the two orthologues. Again, this information will provide insight into the mode of binding of ligands and the basis of species orthologue selectivity.

Because ligands may display 'bias' [21] dependent upon the assay used to detect their activity we also wished to demonstrate function of ligands at a G protein-mediated endpoint. We have recently shown both rat and human GPR35 to display marked selectivity to activate $G\alpha_{13}$ compared to either the closely related G protein $G\alpha_{12}$ or the more distantly related $G\alpha_q$ [8]. We therefore established an assay in *Saccharomyces cerevisiae* employing chimeric G proteins that has been used widely in ligand screening studies [15-16]. Herein, with expression of human GPR35 a range of ligands identified in the primary screen were able to promote cell growth via a Gpa1-$G\alpha_{13}$ chimera. We also developed a mammalian $G\alpha_{13}$ activation assay. Although best suited to analysis of the activation of G_i-family G proteins, [^{35}S]GTPγS binding assays have been adapted for other G protein classes [27]. Although this was also able to identify hits from the primary screen, absolute amounts of bound [^{35}S]GTPγS 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 herein add substantially to the available pharmacology of GPR35 and provide tool 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.

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Tables

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

	pEC_{50} (-log (M))	
compound	**human**	**rat**
zaprinast	**5.59 ± 0.04 n=5**	**7.17 ± 0.03 n=5**
cromolyn sodium	**5.12 ± 0.03 n=3**	**5.36 ± 0.03 n=3**
dicumarol	**5.90 ± 0.08 n=3**	**5.70 ± 0.07 n=3**
quercetin	**5.35 ± 0.16 n=3**	**5.20 ± 0.15 n=3**
niflumic acid	**4.84 ± 0.10 n=3**	**>3 n=3**
oxantel pamoate	**7.57 ± 0.44 n=3**	**>3 n=3**
pyrvinium pamoate	**6.17 ± 0.04 n=3**	**>3 n=3**
pamoate	**7.29 ± 0.03 n=3**	**>3 n=3**
luteolin	**4.87 ± 0.3 n=3**	**5.01 ± 0.08 n=3**

Data are presented at means +/ - SEM. Values in parentheses represent the number of experiments. * Significant species variation $p < 0.05$

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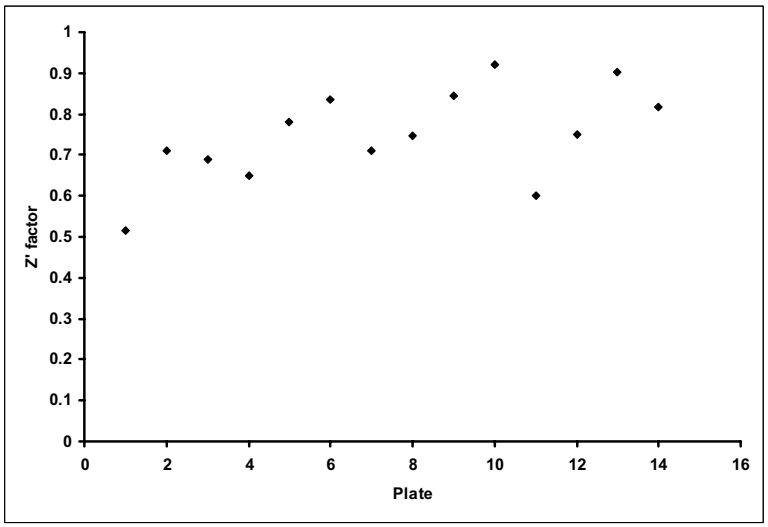
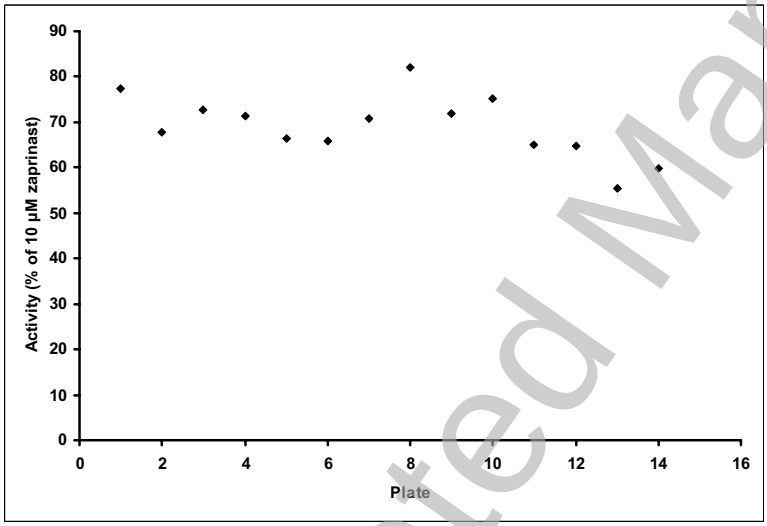
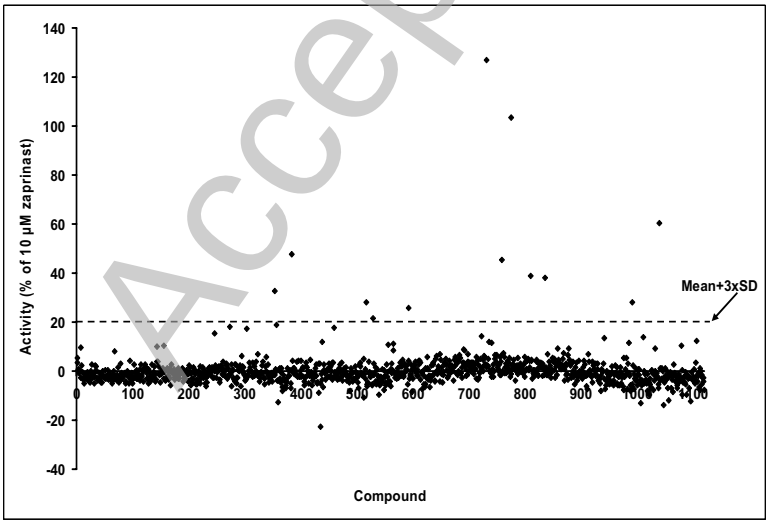
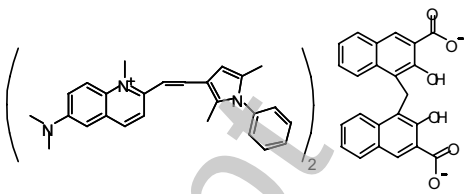
Figure 1 Jenkins et al.,

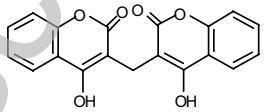




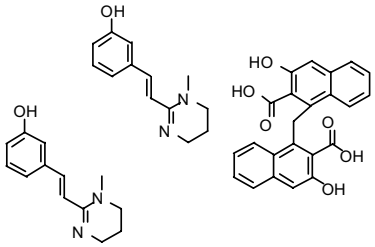




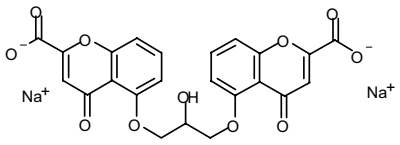
Pyrvinium pamoate
(61.4 ± 1.5)



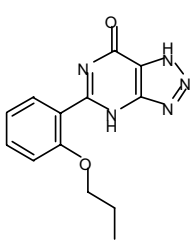
Dicumarol
(56.7 ± 15.8)



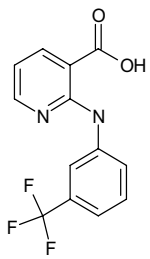
Oxantel pamoate
(51.0 ± 4.7)



Cromolyn disodium
(39.2 ± 0.6)



Zaprinast
(38.3 ± 7.7)



Niflumic acid
(20.0 ± 2.8)

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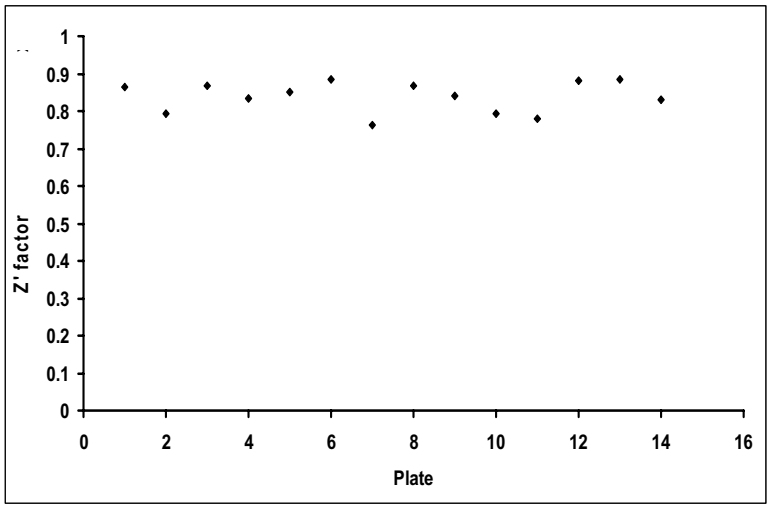
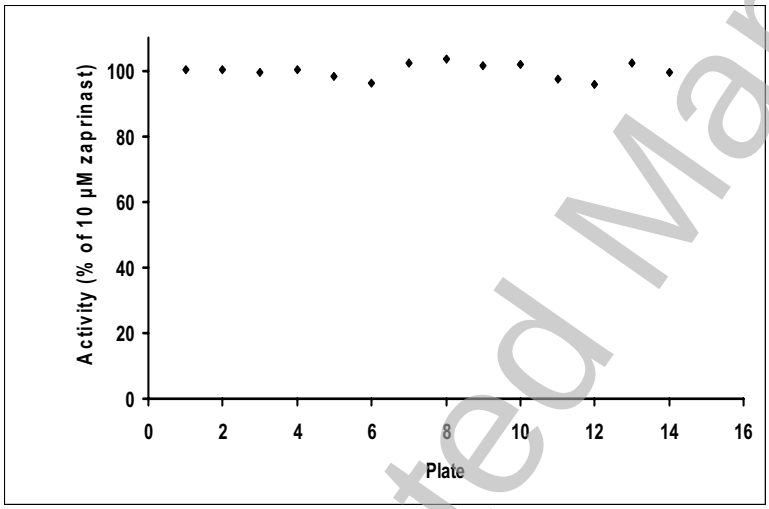
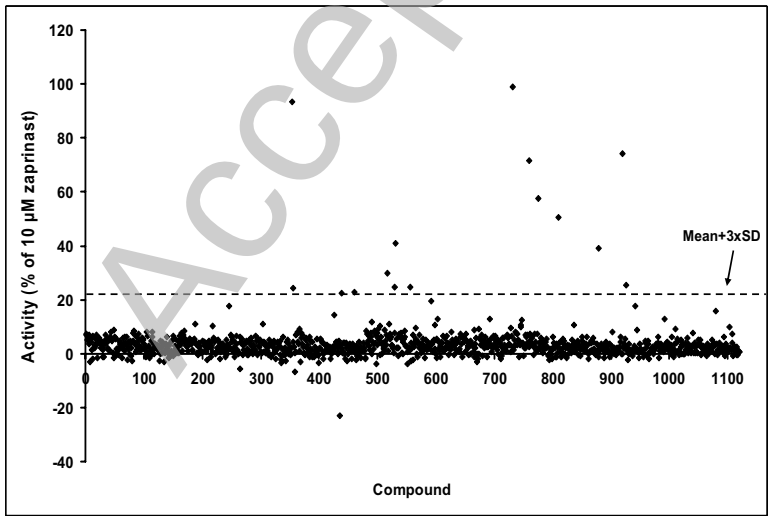
Figure 2 Jenkins et al.,







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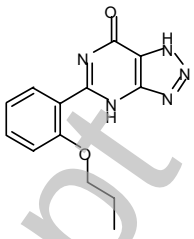
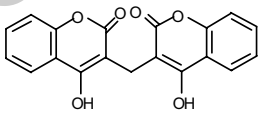
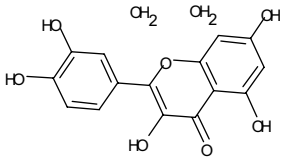


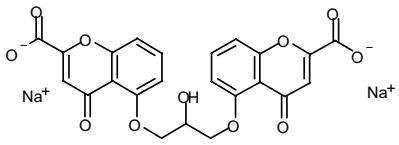
Zaprinast
(97.4 ± 5.8)



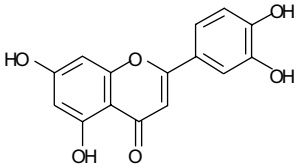
Dicumarol
(62.1 ± 13.6)



Quercetin dihydrate
(52.0 ± 15.9)



Cromolyn disodium
(50.2 ± 0.6)



Luteolin
(37.9±1.7)

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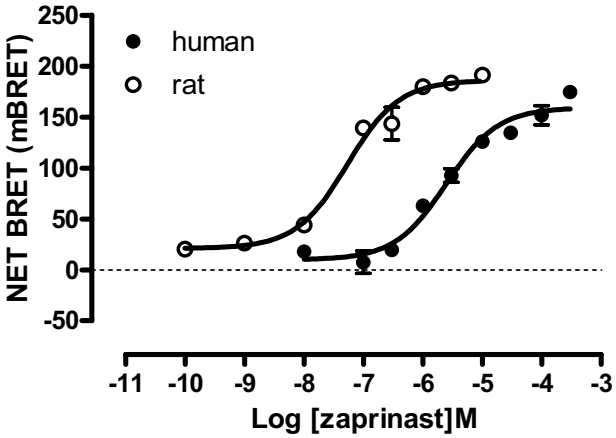
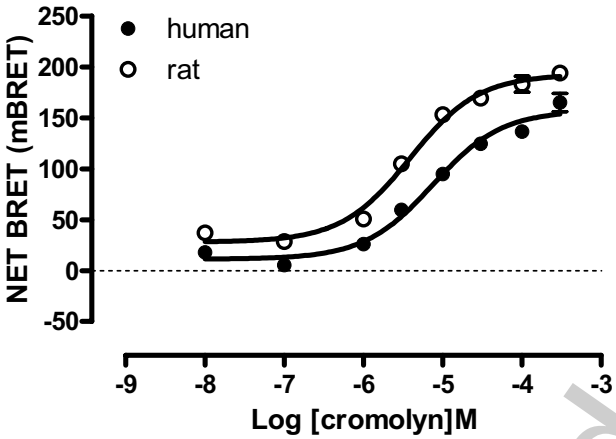
Figure 3 Jenkins et al.,





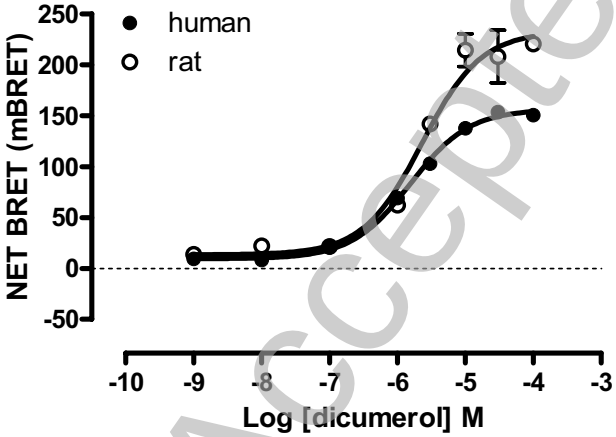


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Figure 4 Jenkins et al.,

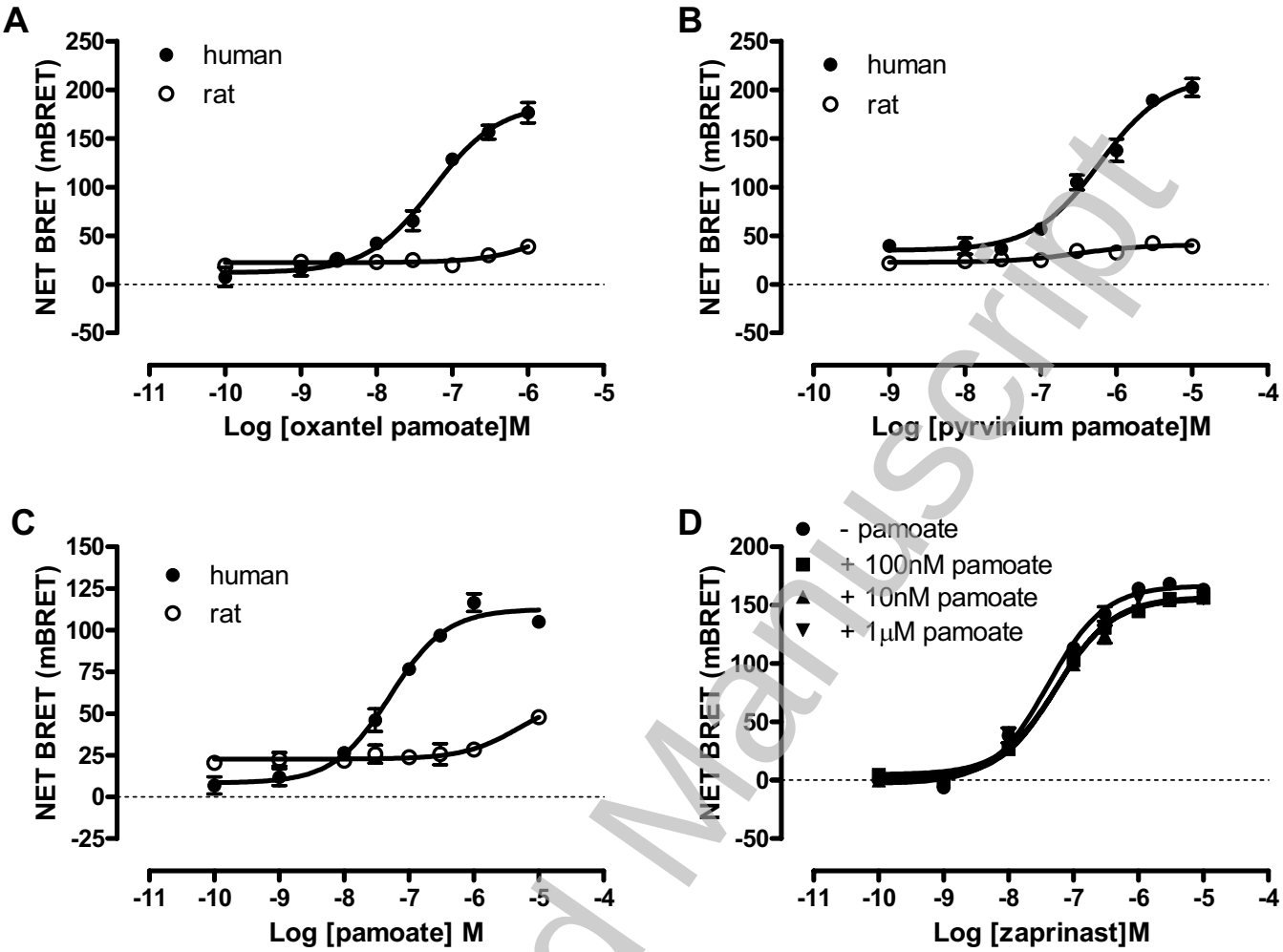

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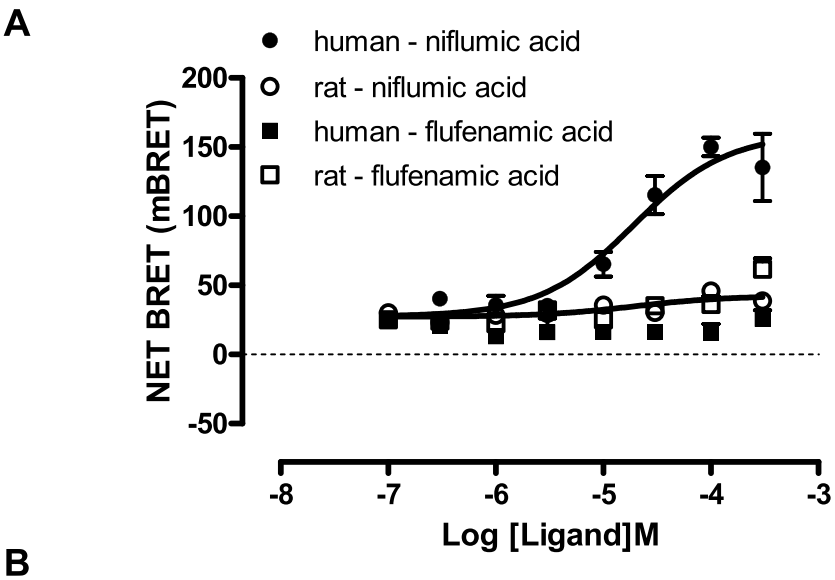
Figure 5 Jenkins et al.,



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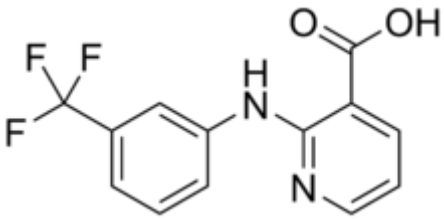
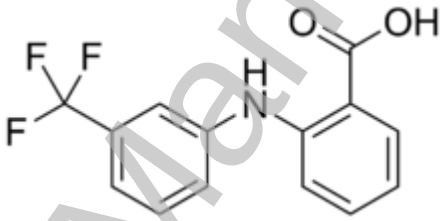


Niflumic acid



Flufenamic acid

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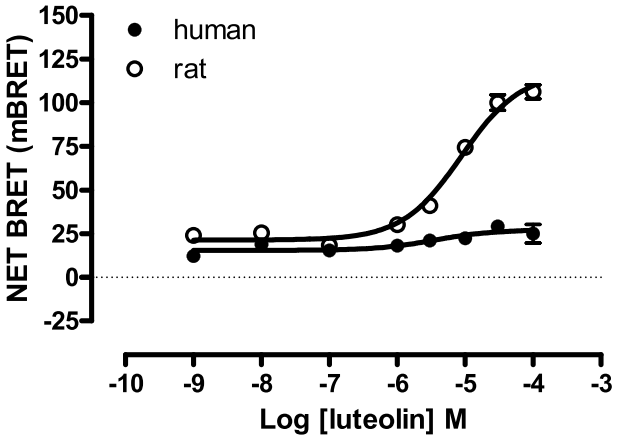
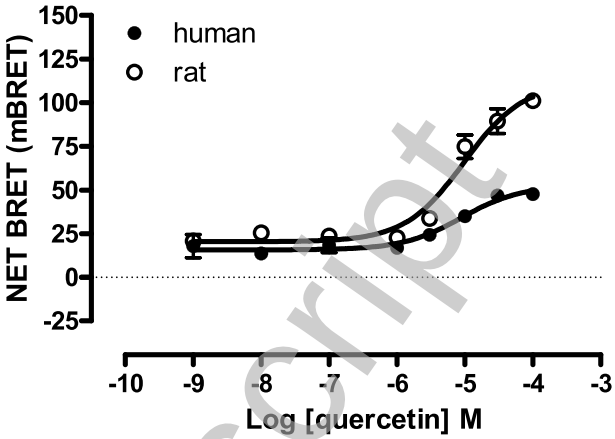
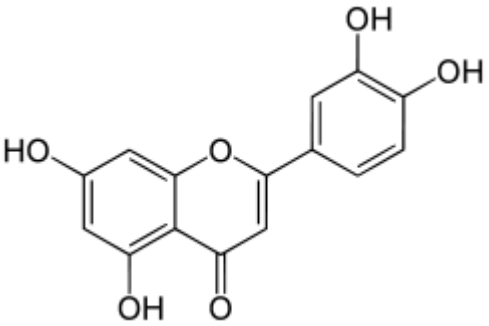
Figure 6 Jenkins et al.,







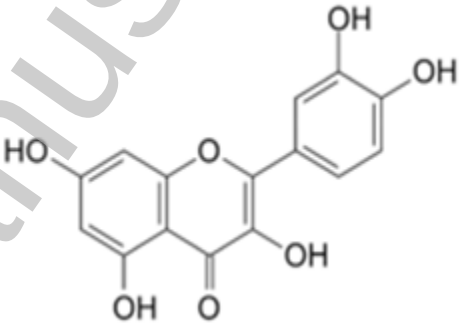


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Figure 7 Jenkins et al.,

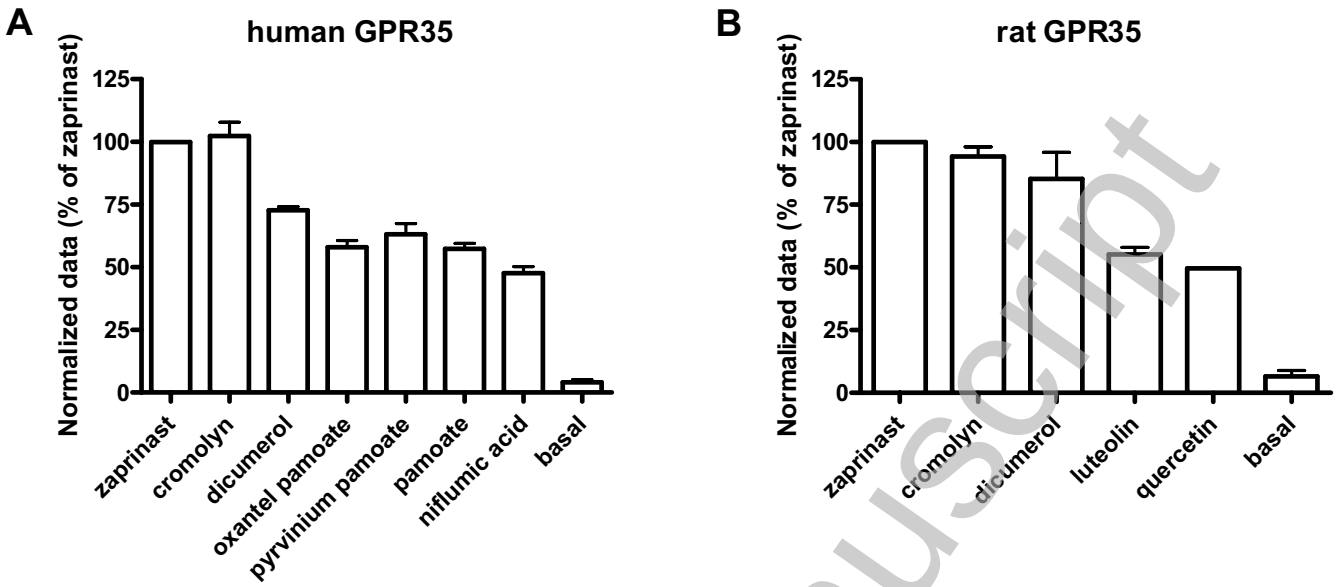

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Figure 8 Jenkins et al.,

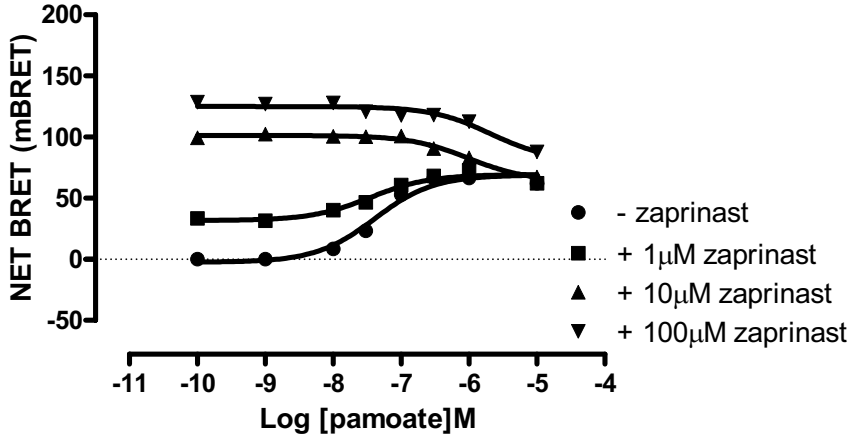

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Figure 9 Jenkins et al.,

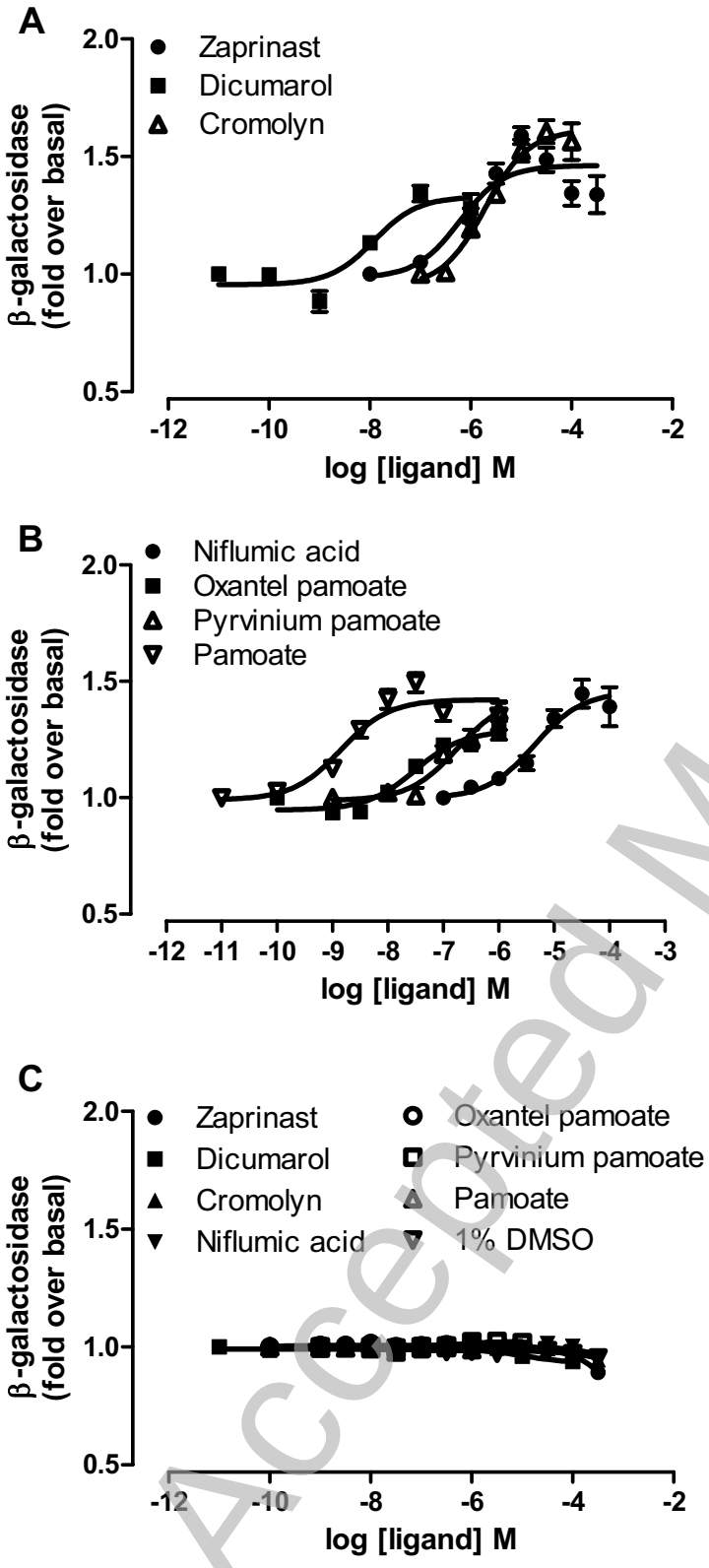

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Figure 10 Jenkins et al.,