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RESEARCH PAPER

Reassessment of the pharmacology of Sphingosine-1-phosphate $S1P_3$ receptor ligands using the DiscoveRx PathHunter™ and Ca^{2+} release functional assays

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BACKGROUND AND PURPOSE

DiscoverRx's PathHunter™ assay measures GPCR agonist potency, via the recruitment of β-arrestin, independent of the subtype of G_α protein activated. This assay is frequently used in drug discovery although little is known about the agonist pharmacology generated. Here we have compared agonist potency, efficacy and affinity values obtained in PathHunter™ assays with those from more established radioligand binding and functional techniques.

EXPERIMENTAL APPROACH

Using cells expressing the human sphingosine-1-phosphate $S1P_3$ receptor at four different densities, we compared pharmacological affinity and efficacy values of four structurally distinct ligands – FTY720-P, VPC24191, CYM5442 and the endogenous agonist S1P – obtained from competition binding, functional Ca^{2+} release and PathHunter™ assays.

KEY RESULTS

The pK_i values for S1P were significantly different (9.34 ± 0.10 and 8.92 ± 0.15) in clones expressing different receptor levels using the binding assay. In the PathHunter™ and Ca^{2+} assays, S1P and CYM5442 were full agonists, FTY720-P was a partial agonist, while the efficacy of VPC24191 could not be detected in PathHunter™ assays. VPC23019, previously described as a $S1P_{1/3}$ receptor antagonist, behaved as an $S1P_3$ receptor partial agonist in the Ca^{2+} release assay.

CONCLUSIONS AND IMPLICATIONS

Comparison of data from the PathHunter™ assay with binding and functional Ca^{2+} assays suggest that PathHunter™ assays measured a different agonist-bound receptor conformation. While this assay has great utility in drug discovery, care must be taken as high-efficacy, low-affinity agonist compounds would not be detected. Therefore highly amplified, more traditional assays are necessary to identify agonists with low efficacy.

Abbreviations

CYM5442, 2-(4-(5-(3,4-diethoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1H-inden-1-yl amino) ethanol; FTY720-P, 2-amino 2-[2-(4-octylphenyl)ethyl]-1,3-propanediol phosphate; GRK, GPCR kinase; MEM, minimal essential medium; S1P, sphingosine1-phosphate; VPC24191, (S)-phosphoric acid mono-[2-amino-3-(4-octyl-phenylamino)-propyl] ester; VPC23019, (R)-phosphoric acid mono-[2-amino-2-(3-octyl-phenylcarbamoyl)-ethyl] ester

Introduction

Sphingosine-1-phosphate (S1P) is a bioactive lipid that is involved in a wide range of cellular responses including lung epithelial barrier function (Gon *et al.*, 2005), proliferation (An *et al.*, 2000) and cardioprotection (Kennedy *et al.*, 2008). S1P binds to five GPCRs, called $S1P_{1-5}$ receptors, with nanomolar potency (Murata *et al.*, 2000; receptor nomenclature follows Alexander *et al.*, 2011). Each receptor is widely expressed in a variety of tissues including the heart (Spiegel and Milstein, 2003), brain and lungs (Marsolais and Rosen, 2009). The individual $S1P_{1-5}$ receptors differentially signal via numerous G-proteins, including $G\alpha_i$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$, which, in turn, activate different intracellular signalling cascades. This multiple coupling is further complicated by the observation that the host cell employed for recombinant S1P receptor expression can directly affect the repertoire of G_α proteins activated (Holdsworth *et al.*, 2005). This pleiotropic and cell-type dependent coupling behaviour makes the development of a single uniform *in vitro* assay to assess agonist efficacy at all S1P receptor subtypes problematic, as there is no one signalling cascade that is activated by all five of the S1P receptor subtypes.

Recently two G-protein–independent assay technologies, which utilize the recruitment of β-arrestin, have been developed: Invitrogen's Tango™ and DiscoveRx's PathHunter™. Utilization of β-arrestin recruitment allows a direct measurement of agonist binding as activated receptors act as substrates for GPCR kinases (GRKs) that phosphorylate the receptor (Violin and Lefkowitz, 2007). This phosphorylation of serine and threonine residues within the C-terminal tail and the third extracellular loop then function as high-affinity binding sites for β-arrestin (van der Lee *et al.*, 2008). In the case of the DiscoveRx technology, β-arrestin is fused to an N-terminal deletion mutant of β-galactosidase, which when stably expressed alone has no enzymic activity. The GPCR of interest is fused to a smaller (42 amino acids), weakly complementing fragment termed ProLink™. Only in cells that stably express both fusion proteins will ligand stimulation result in the interaction of β-arrestin and the ProLink-tagged GPCR, which in term forces the complementation of the two β-galactosidase fragments. The resulting functional enzyme converts substrate to a detectable signal, which can be used to assess receptor activation by agonists (Figure 1).

The relative ease of these assay formats has led to their increased use in high through-put screening and routine compound profiling. As the technology is independent of the subtype of G_α protein activated, it also permits numerous counterscreen assays to be performed without the consideration of which heterotrimeric G-proteins by each receptor are activated upon agonist binding. Before this technology was available, when different receptor subfamilies coupled to specific G_α proteins, multiple G-protein–dependent assays would have been developed to assess ligand activity across receptor subtypes. For example, there was [^{35}S]-GTPγS binding for G_i coupled receptors, cAMP accumulation for G_s coupled receptors and Ca^{2+} release assays for $G_{q/11}$ coupled receptors. Agonist efficacy can be assessed by two parameters, potency and intrinsic activity (% activity of the system maximum). However, efficacy can be influenced by both receptor expression levels and the point in the G-protein signalling cascade at which the agonist activity is quantified, a concept termed 'receptor reserve' (van Rossum and Ariens, 1962). As β-arrestin binding is the most proximal step to agonist binding, this study aimed to compare agonist pharmacology for both binding and different functional readouts along the $S1P_3$ signalling cascade.

We have used a range of $S1P_3$ receptor agonists (S1P, FTY720-P, VPC24191 and CYM5442), in a cell line expressing different levels of the $S1P_3$ receptor. We compared the affinity

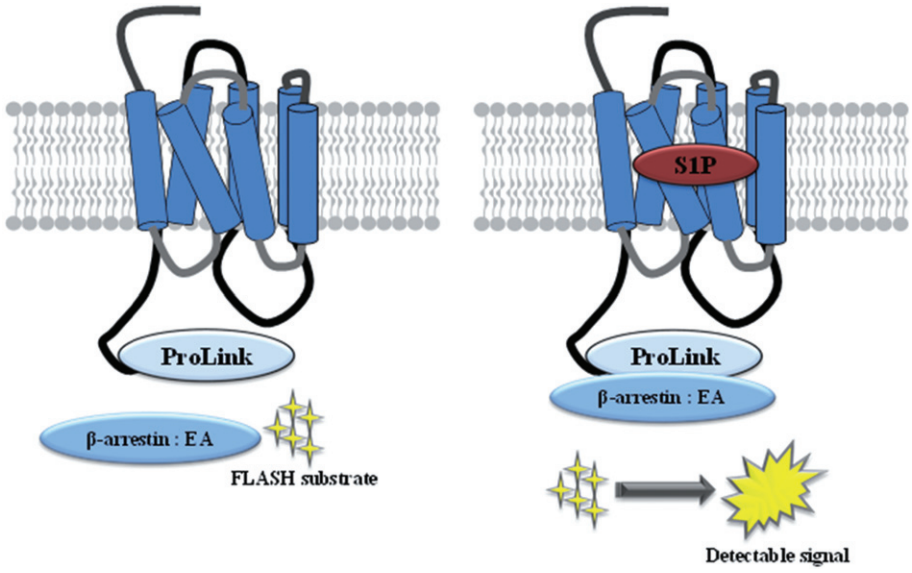

Figure 1

Schematic diagram of DiscoveRx's PathHunter™ assay, which permits the assessment of GPCR agonist potency, independent of the subtype of G_α protein activated.

values obtained from radioligand-binding experiments with relative efficacy measurements from functional assays in order to fully evaluate the pharmacological values obtained from the novel assay technology.

Methods

Lipofectamine™2000 transfection

PathHunter™ CHO-K1 EA-Arrestin2 Parental cells (DiscoveRx, Freemont, CA, USA; 93–0164) were plated out in 6-well plates at a concentration of 3 x 10^5 cells per·well, in 2 mL F12 medium (Invitrogen, Carlsbad, CA, USA; 31765-027) supplemented with glutamax and 10% foetal bovine serum (FBS) (Invitrogen 26010-074), and incubated at 37°C and in 5% CO_2. Once confluent, the cells were transfected with human $S1P_3$ receptor plasmid DNA (pCMV-ProLink $S1P_3$) according to the manufacturer's instructions. In short, 4 µg of plasmid DNA was added to 250 µL Opti-MEM® I Medium (Invitrogen 11058-021) and mixed gently. To 250 µL of Opti-MEM® I Medium, 10 µL of Lipofectamine™2000 (Invitrogen 11668-019) was added and incubated for 5 min at room temperature. Following this incubation, the two 250 µL samples were combined, mixed and incubated for 20 min at room temperature. Following this incubation, the mixture was added to one well containing cells and medium and incubated at 37°C/5% CO_2. Medium was changed 6 h after transfection. The following day, the medium was changed for growth medium, F12 medium supplemented with glutamax, 10% FBS 200 µg·mL^{-1} hygromycin B (Invitrogen 10687-010), 500 µg·mL^{-1} geneticin (Invitrogen 10131-019). The cells were grown to a suitable level and then single-cell sorted into a 96-well tissue culture plate using a BD FACSCalibur™ Flow Cytometer (Becton-Dickinson Diagnostic Instrument Systems, Oxford, UK). The cells were grown until confluent and then tested for their ability to elicit a response to a full concentration range of S1P (Biomol SL140-0001) using the PathHunter™ assay. Four clones were chosen for further profiling, denoted B3, B6, E5 and F6.

PathHunter™ assay

CHO-K1 human $S1P_3$ cells were grown in F12 medium supplemented with glutamax, 10% FBS, 200 µg·mL^{-1} hygromycin B, 500 µg·mL^{-1} geneticin and incubated at 37°C and in 5% CO_2. On the day before the experiment, the cells were harvested with trypsin-EDTA (Invitrogen 15400-054) from the cell-culture flask, counted and resuspended in 20 µL medium and plated into a 384-well white-walled, clear bottom plate (ViewPlate Perkin Elmer, Seer Green, UK; 6005262) at a concentration of 1000 cells per well. Seeded plates were incubated for 24 h at 37°C and in 5% CO_2. Ten-point concentration response curves to the four agonists were prepared using the Biomek FX^P (Becton-Dickinson Diagnostic Instrument Systems) at 100× concentration in 100% DMSO. This plate was diluted 1:20 using assay buffer, HBSS with calcium and magnesium (Invitrogen 14025-050), 40 mM HEPES (Invitrogen 15630-056), 0.1% BSA essentially fatty acid free (Sigma, St Louis, MO, USA; A7030) and 100 µM of freshly prepared Na_3VO_4 (Sigma S6508). After 24 h, the medium was removed from the cell plates and replaced with 20 µL per well of assay buffer and incubated for 30 min at 37°C/5% CO_2. Following this incubation, 5 µL·per well of compound were added to the cell plate using the Biomek FX^P and incubated for 90 min at 37°C and in 5% CO_2. The experiment was concluded upon the addition of 25 µL per well of Flash reagent (detectable solution and lysis buffer). Flash was added to each plate using the Biomek FX^P and placed in a shaking incubator for 30 min at 37°C and in 5% CO_2. To each plate, a bottom seal and a black solid lid was added. The plates were read using a GE Healthcare LEADseeker™ imaging system. The exposure time was determined for each clone independently on the day of experimentation.

Membrane preparation

CHO-K1 human $S1P_3$ cells were grown to 80–90% confluency in 500 cm^2 cell-culture trays at 37°C/5% CO_2. All subsequent steps were conducted at 4°C to avoid receptor degradation. The cell-culture medium was removed and ice cold buffer (10 mL^{-1} tray; 10 mM HEPES, 0.9% w/v NaCl, 0.2% w/v EDTA, pH 7.4) was added to the cells. The cells were scraped from the trays into a 50 mL Corning tube and subsequently centrifuged at 250× *g* for 5 min. The supernatant fraction was aspirated and 10 mL^{-1} 500 cm^2 tray of wash buffer (10 mM HEPES, 10 mM EDTA, pH 7.4) was added to the pellet. This was homogenized using an electrical homogenizer 'Werker, Ultra-Turrax' (position 6, 4 × 5 s bursts) and subsequently centrifuged at 48 000× *g* at 4°C (Beckman Avanti J-251 Ultracentrifuge) for 30 min. The supernatant was discarded and the pellet re-homogenized and centrifuged as described above, in wash buffer. The final pellet was suspended in ice cold assay buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4) at a concentration of 1–2 mg·mL^{-1}. Protein concentration was determined by the bicinchoninic acid assay based on the method of Smith *et al*. (1985), using BSA as a standard and aliquots maintained at –80°C until required.

Common procedures applicable to all radioligand-binding experiments

All radioligand experiments were conducted in 96 deep well plates, in assay-binding buffer (HBSS, 0.5% BSA, 40 mM HEPES, 0.1 mM Na_3VO_4, pH 7.4) at room temperature. In all cases, non-specific binding was determined in the presence of 1 µM S1P. After the indicated incubation period, bound and free [^{3}H]-FTY720-P were separated by rapid vacuum filtration using a FilterMate™ Cell Harvester (Perkin Elmer, Beaconsfield, UK) onto 96-well GF/C filter plates and rapidly washed three times with ice cold wash buffer (10 mM HBSS, 20 mM HEPES, 4.2 mM sodium bicarbonate, pH 7.4). After drying (>4 h), 40 µL of Microscint™ 20 (Perkin Elmer) was added to each well and radioactivity quantified using single photon counting on a TopCount™ microplate scintillation counter (Perkin Elmer). Aliquots of [^{3}H]-FTY720-P were also quantified accurately to determine how much radioactivity was added to each well using liquid scintillation spectrometry and a LS 6500 scintillation counter (Beckman Coulter, High Wycombe, UK). In all experiments, total binding never exceeded more than 10% of that added, limiting complications associated with depletion of the free radioligand concentration (Carter *et al*., 2007).

Equilibrium radioligand-binding studies

Binding was performed with a range of concentrations of [^{3}H]-FTY720-P (30–0.01 nM**)** to construct saturation-binding curves. CHO-K1 human $S1P_3$ cell membranes (10 μg per well) were incubated in 96 deep well plates at room temperature in assay-binding buffer containing radioligand and Na_3VO_4 in a total assay volume of 250 μL, with gentle agitation for 2 h to ensure equilibrium was reached. For equilibrium dissociation studies a mean pK_d of 8.5 was used.

Ca^{2+} release assay

CHO-K1 human $S1P_3$ cells were grown in F12 medium supplemented with glutamax, 10% FBS, 200 μg·mL^{-1} hygromycin B, 500 μg·mL^{-1} geneticin and incubated at 37°C and in 5% CO_2. On the day prior to the experiment, the cells were harvested with trypsin-EDTA from the cell-culture flask, counted and resuspended in 20 μL per well medium and plated into a 384-well black-walled, clear bottom plate (Corning Costar, 3712) at a concentration of 20 000 cells per well. Seeded plates were incubated for 24 h at 37°C/5% CO_2. Compound plates were prepared as described earlier. After 24 h, the medium was removed from the cell plates and replaced with 20 μL per well Fluo-4 calcium dye (Molecular Devices Co., Menlo Park, CA, USA; R8141) and incubated for 45 min at 37°C and in 5% CO_2. Following this incubation, 5 μL per well of the compound was added to the cell plate using the Hamamatsu FDSS7000 (Hamamatsu, Herts, UK) and read for 2 min with 1 s increments.

Data analysis

Due to the amount of radioactivity varying slightly for each experiment (<5%), data are shown graphically as the mean ± range for individual representative experiments, whereas all values reported in the text and tables are mean ± SEM for the indicated number of experiments. All experiments were analysed by either linear or non-linear regression using Prism 5.04 (GraphPad Software Inc., San Diego, CA, USA). Competition displacement binding data were fitted to sigmoidal (variable slope) curves using a four-parameter logistic equation:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10\text{^}[(\log IC_{50} - X)\text{Hillslope}]} \quad (1)$$

Where Y is the % bound of radioligand or % response. Top denotes maximal asymptotic binding and bottom denotes the minimal asymptotic binding.

Agonist concentration response curves were fitted to sigmoidal (variable slope) using a four-parameter logisitic equation:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10\text{^}[(\log EC_{50} - X)\text{Hillslope}]} \quad (2)$$

Where Y is the response measured in relative fluorescent units or relative luminescence units. Top denotes maximal asymptotic response and bottom denotes minimal asymptotic response.

Saturation-binding isotherms were analysed by non-linear regression according to a hyperbolic, one-site binding model, and individual estimates for total receptor number (B_{max}) and radioligand dissociation constant (K_d) were calculated. The following equation was used, where [A] is the concentration of radioligand:

$$Y = \frac{B_{\max}[A]}{K_d + [A]} \quad (3)$$

Statistical analysis was performed using one-way ANOVA with a Bonferroni post-test versus the highest expressing clone, F6 *P < 0.05, **P < 0.01 and ***P < 0.001 deemed significant.

Materials

FTY720-P was synthesized in house, VPC24191 and VPC23019 were supplied by Avanti Polar Lipids Inc (Alabaster, AL, USA), CYM5442 was supplied by Sigma-Aldrich Co (Exeter, UK) and S1P was supplied by Enzo Life Sciences Ltd (Exeter, UK). [^{3}H]-FTY720-P (specific activity, 1734GBq mmol^{-1}) was synthesized by DMPK Isotope Laboratories, Basel.

Results

Stable cell line generation and S1P pharmacology

In order to examine agonist affinity and efficacy in the PathHunter™ assay, cell lines stably expressing different levels of the $S1P_3$ receptor were generated. From a large panel of potential transfects, four clones were selected (B3, B6, E5 and F6) based on their response profile to S1P using the PathHunter™ assay format. The pEC_{50} and E_{max} values for S1P obtained for B3, B6, E5 and F6 clones are shown in Figure 2 and Table 2, with a rank order of F6 > B3 > E5 > B6. All data were obtained using an equivalent exposure time of 0.3 s. As these E_{max} values could be dependent on other factors, such as differences in clonal growth rates, we utilized radioligand binding to confirm that E_{max} values were dependent on receptor density.

Characterization of [^{3}H]-FTY720-P binding and receptor expression levels

Specific [^{3}H]-FTY720-P binding to $S1P_3$ receptors in CHO membranes was saturable and best described by a single-site binding equation (Figure 3). The rank order for the calculated B_{max} values (Table 1) was similar to that described for the E_{max} in the PathHunter™ assay (F6 = B3 > B6 = E5). Interestingly, there was no statistically significant change in pK_d with a mean value of 8.48 ± 0.26. In all clones, the specific binding of [^{3}H]-FTY720-P was abolished in the presence of 100 μM GTP, indicating binding to high-affinity G-protein–sensitive receptor conformation (Figure 4). In order to compare agonist binding affinity with functional potency, competition binding experiments with [^{3}H]-FTY720-P was performed to obtain affinity estimates.

[^{3}H]-FTY720-P competition binding studies

The [^{3}H]-FTY720-P competition binding profile was determined for each of the S1P agonists in the four different $S1P_3$

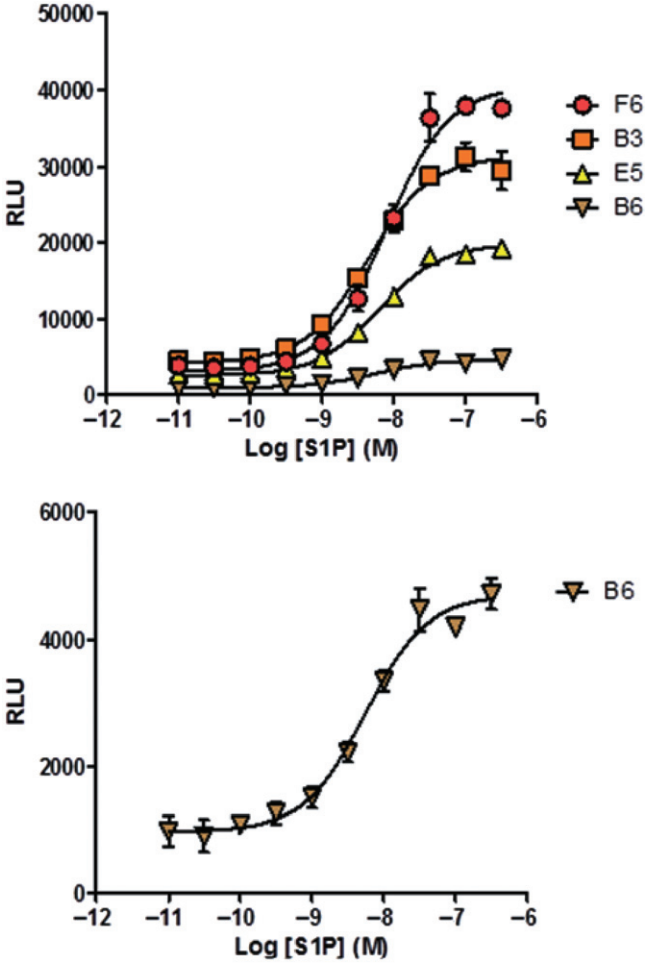

Figure 2

Concentration response curves to S1P in four clonal cell lines, F6, B3, E5 and B6, expressing the human $S1P_3$ receptor in the PathHunter™ assay. The lower graph shows data from clone B6 on a larger scale; note expanded Y-axis. S1P was incubated for 90 min at 37°C and in 5% CO_2 and the experiment concluded upon the addition of 25 μL per well of Flash reagent (detectable solution and lysis buffer) and the exposure time determined for each clone independently on the day of experimentation. Chemiluminescence is indicated as relative luminescence units (RLU). A representative graph from an independent experiment is presented. Data shown are means ± SEM (n = 3).

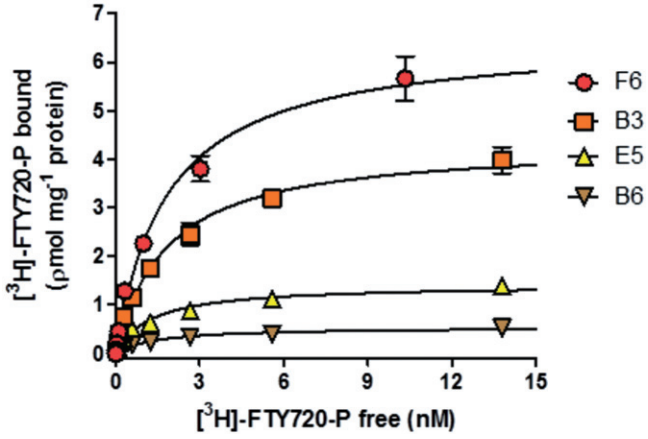

Figure 3

Saturation binding of [^{3}H]-FTY720-P in four clonal cell lines expressing the human $S1P_3$ receptor, F6, B3, E5 and B6. Non-specific binding values were determined in the presence of 1 μM S1P. Plates were harvested following 2 h incubation at room temperature and data were fitted to a one-site specific binding equation. A representative graph from an independent experiment is presented. Data shown are means ± SEM (n = 3).

Table 1

Saturation binding data using [^{3}H]-FTY720-P, determining B_{max} and K_d in clonal cell lines expressing the human $S1P_3$ receptor

	B_{max} ρmol mg^{-1}	pK_d pK_d
F6	14.02 ± 2.65 (n = 3)	8.67 ± 0.23 (n = 3)
B3	13.99 ± 2.17 (n = 3)	8.38 ± 0.15 (n = 3)
E5	2.29 ± 0.32*** (n = 3)	8.58 ± 0.20 (n = 3)
B6	3.93 ± 1.89** (n = 3)	8.27 ± 0.44 (n = 3)

Data are expressed as ρmol·mg^{-1} and pK_d ± SEM. **P < 0.01, ***P < 0.001, significantly different from values with F6; one-way ANOVA with a Bonferroni post-test.

expressing clones. For each clone, the concentration of [^{3}H]-FTY720-P employed in competition binding experiments was chosen as the mean pK_d value described in the preceding section. All agonists, in each clone, were able to concentration-dependently inhibit [^{3}H]-FTY720-P binding, with slope factors not different from unity, as shown in Figure 5a–d and Tables 2–5, indicating that all four compounds can bind to the $S1P_3$ receptor. A comparison between the different expression clones showed there was no difference in calculated pK_i values for FTY720-P, VPC24191 and CYM5442 (Tables 3–5). However, there was a significant change in calculated pK_i between the two highest expressing clones, F6 and B3 and the lowest expressing clone, B6, when using S1P (*P < 0.05), indicating that the affinity measurements for the endogenous agonist may be dependent on the receptor expression levels (Table 2).

Characterization of agonist potency using the PathHunter™ assay

To ensure that any differences identified were due to changes in receptor pharmacology and not to variation in experimental conditions, we performed all assays using either whole cells or membrane preparations from the same batch and used identical buffers for all experiments. Only three of the four agonists tested were able to cause a concentration-dependent increase in β-arrestin binding as measured by an increase in β-galactosidase enzymic activity (Figure 6a–d, Tables 2–5). VPC24191 failed to elicit any significant response in this assay regardless of receptor expression levels (Figure 6c), suggesting that either the compound had poor efficacy or, more intriguingly, did not recruit β-arrestin. S1P

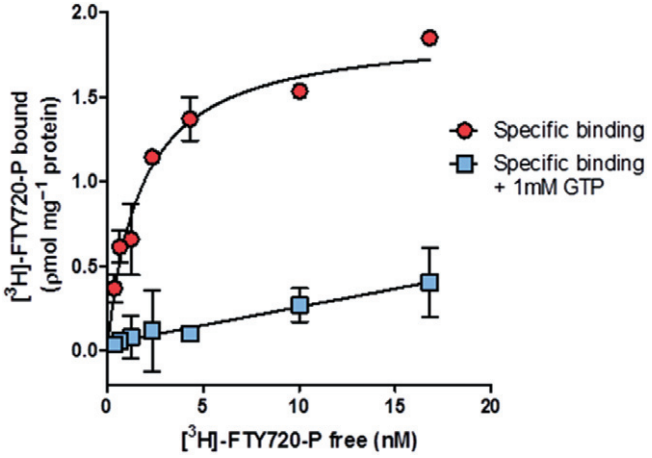

Figure 4

Saturation binding of [^{3}H]-FTY720-P in the highest expressing cell line, F6, in the presence and absence of 1 mM GTP. Membranes were incubated in the presence of 1 mM GTP for 1 h before addition to the assay and non-specific binding values were determined in the presence of 1 μM S1P. Plates were harvested following 2 h incubation at room temperature and data were fitted to a one-site specific binding equation. A representative graph from an independent experiment is presented. Data are means ± SD (n = 2).

and CYM5442 appeared to be full agonists in this functional assay (Figure 6a,d, Tables 2 and 5), with FTY720-P being a partial agonist, (Figure 6b, Table 3) when compared with S1P. This data correlates with that already published by Brinkmann *et al*. (2002) where they demonstrated, using a [^{35}S]-GTPγS binding assay, that FTY720-P produces a 34% response when compared with S1P. There was no statistically significant change in the pEC_{50} values for any of the compounds tested, S1P, FTY720-P or CYM5442.

When the potency of the agonists were compared with the determined pK_i values some interesting differences were noticed. For agonists the functional potency measurements are frequently higher than binding estimates due to non-linear stimulus coupling. However this was not the case for all agonists. In this study, for example, the potency of S1P in the PathHunter™ assay was statistically significantly lower than the binding value obtained (8.28 ± 0.10 compared with 9.34 ± 0.10, Table 2) in all clones. To investigate if this effect was specific to the β-arrestin assay, we next profiled the same agonist in a more distal functional readout for all clones.

EC_{50} determination using a Ca^{2+} Fluo-4 assay

In order to compare the pharmacological values obtained for the agonists in the PathHunter™ assay with another functional assay we next profiled the compounds for their ability to modulate Ca^{2+} release via the $S1P_3$ receptor. The intrinsic efficacy and potency values for all agonists in both the Ca^{2+} and PathHunter™ assay were unaffected by pre-incubation with *Pertussis* toxin (data not shown) indicating that neither response was dependent on G_i coupling. All four agonists caused a concentration-dependent increase in intracellular Ca^{2+}concentration in all four $S1P_3$ expressing clones (Figure 7a–d). S1P clearly showed increases in E_{max} with increasing receptor expression levels. The pEC_{50} values (Table 2) also increased, demonstrating that, with increasing expression level, S1P became more potent. CYM5442 also showed increasing E_{max} values with increasing receptor expression levels, although with only a small change in pEC_{50} values (Table 5). In contrast to the β-arrestin assay, VPC24191 appeared to be a full agonist in the Fluo-4 Ca^{2+} assay (Figure 7c). With the highest expressing clone (F6), both VPC24191 and CYM5442 appeared to be as efficacious as S1P (Figure 7a and d, Tables 4 and 5), although data for CYM5442 did show increased variability. One reason for this may be because CYM5442 is structurally different from the other ligands with increased acidic physicochemical properties which may cause a reduction in the dissolution rate. Finally FTY720-P only showed a response in the two highest expressing cell lines (F6 and B3), with partial responses compared with S1P (Figure 7b). This confirms that FTY720-P is a partial agonist at the human $S1P_3$ receptor, but requires a certain level of receptor reserve to elicit a response. Furthermore, a known antagonist of human $S1P_{1/3}$ receptors VPC23019 (Figure 7e and Table 6), showed partial agonist activity, confirming data published by Jongsma *et al*. (2009), when profiled in an amplified system with a high receptor-expressing cell line, highlighting the need of caution for interpreting neutral antagonists in an assay format which allows for signal amplification.

Discussion

The G-protein–independent, DiscoveRx PathHunter™ assay utilizes the recruitment of β-arrestin upon receptor activation and is becoming more popular for high through-put screening high through-put screening. The G-protein–independent approach also allows numerous counter screen assays to be performed without the complexities of which heterotrimeric G-proteins couple to each receptor. The PathHunter™ assay utilizes the endogenous recruitment of β-arrestin upon agonist binding and, through complementation, a detectable signal is produced. This is achieved by using an engineered cell line that contains a 42 amino acid C-terminal tagged ProLink and β-arrestin fusion protein. With the C-terminal tag being part of the receptor this limits any off-target effects seen with test compounds. As very little investigation into agonist pharmacology using this assay format had been performed; we compared the affinity values obtained from radioligand-binding experiments with relative efficacy measurements from functional assays in order to fully evaluate the pharmacological values obtained from the novel assay technology.

To investigate the PathHunter™ assay, we first determined whether changes in receptor expression levels could influence agonist affinity values estimated from radioligand-binding studies. There was no statistically significant change in the pK_i values for the synthetic $S1P_3$ receptor agonists, FTY720-P or CYM5442; however, the affinity values for the most efficacious agonist, S1P, increased with receptor expression. Specific binding of [^{3}H]-FTY720-P was sensitive to the inclusion of GTP, indicating binding to a single high-affinity receptor conformation. The simplest model to explain high- and low-agonist affinity values is the two-state model of

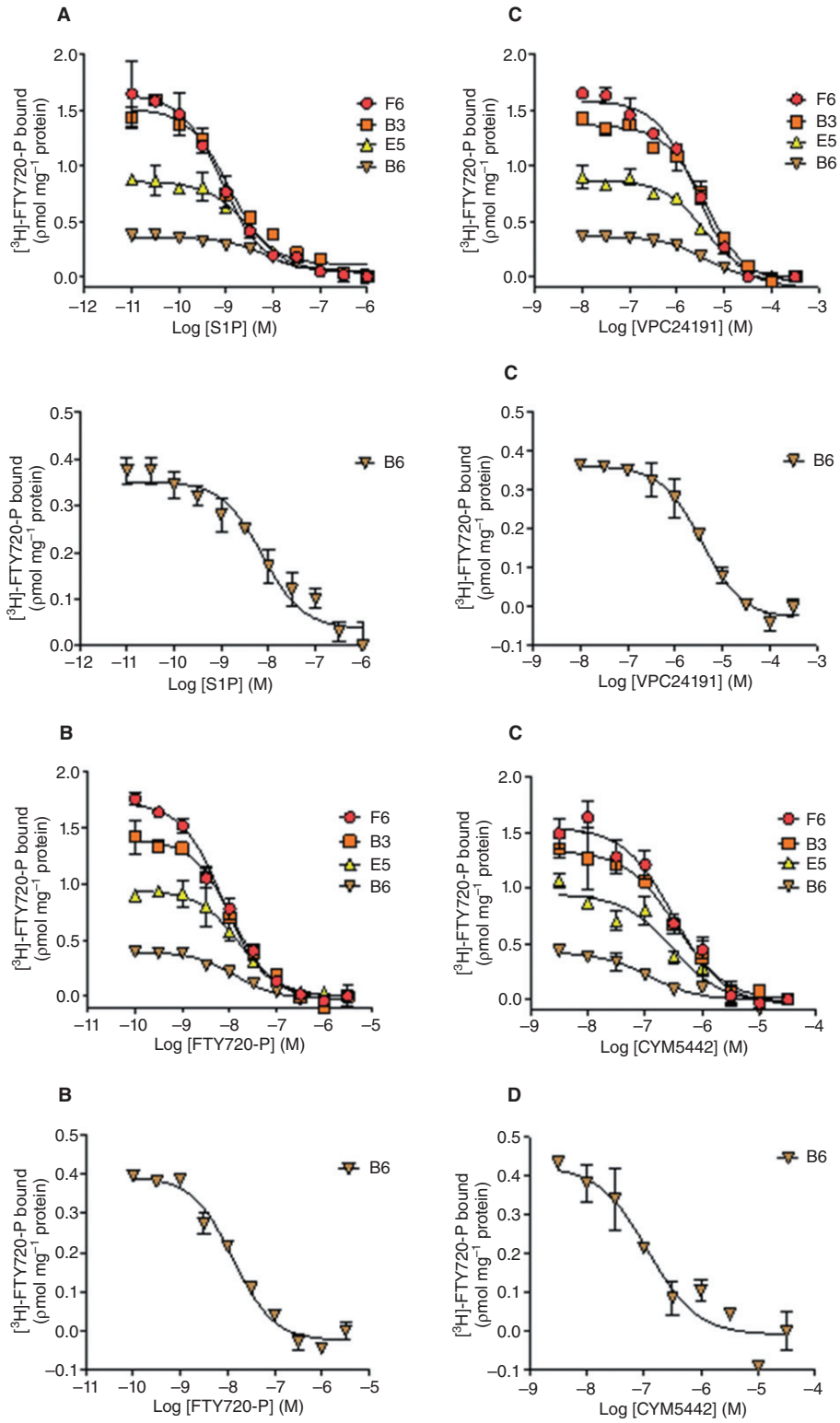

Figure 5

Displacement of [^{3}H]-FTY720-P by (A) S1P, (B) FTY720-P, (C) VPC24191 and (D) CYM5442 in four clonal cell lines expressing the human $S1P_3$ receptor, F6, B3, E5 and B6. The lower graphs in A, B, C and D show data from clone B6 on a larger scale; note expanded Y-axis. Non-specific binding values were determined in the presence of 1 μM S1P. Plates were harvested following 2 h incubation at room temperature and data were fitted to sigmoidal (variable slope) curves using a four-parameter logistic equation. A representative graph from an independent experiment is presented. Data shown are means ± SEM (n = 3).

Table 2

Affinity, potency and efficacy determinations in response to S1P in three-assay formats, radioligand binding, PathHunter™ and Fluo-4 Ca^{2+} using clonal cell lines expressing the human $S1P_3$ receptor

	S1P				
	Binding	PathHunter™		Fluo-4 Ca^{2+}	
	pK_i	pEC_{50}	Efficacy %	pEC_{50}	Efficacy %
F6	9.34 ± 0.10 ($n = 3$)	8.28 ± 0.10 ($n = 7$)	100 ($n = 5$)	9.81 ± 0.06 ($n = 5$)	100 ($n = 5$)
B3	9.69 ± 0.06 ($n = 3$)	8.29 ± 0.09 ($n = 7$)	100 ($n = 5$)	9.80 ± 0.18 ($n = 5$)	100 ($n = 5$)
E5	9.00 ± 0.15 ($n = 3$)	8.46 ± 0.04 ($n = 7$)	100 ($n = 5$)	9.56 ± 0.12 ($n = 5$)	100 ($n = 5$)
B6	8.92 ± 0.15* ($n = 3$)	8.37 ± 0.20 ($n = 7$)	100 ($n = 5$)	9.35 ± 0.15 ($n = 5$)	100 ($n = 5$)

Efficacy is expressed as % response compared with S1P. Data are expressed as means ± SEM. *$P < 0.05$, significantly different from values with F6; one-way ANOVA with a Bonferroni post-test.

Table 3

Affinity, potency and efficacy determinations in response to FTY720-P in three-assay formats, radioligand binding, PathHunter™ and Fluo-4 Ca^{2+} using clonal cell lines expressing the human $S1P_3$ receptor

	FTY720-P				
	Binding	PathHunter™		Fluo-4 Ca^{2+}	
	pK_i	pEC_{50}	Efficacy %	pEC_{50}	Efficacy %
F6	8.15 ± 0.10 ($n = 3$)	7.86 ± 0.10 ($n = 7$)	42.3 ± 4.4 ($n = 5$)	8.25 ± 0.17 ($n = 5$)	88.7 ± 1.0 ($n = 5$)
B3	7.93 ± 0.11 ($n = 3$)	7.67 ± 0.19 ($n = 7$)	50.6 ± 8.2 ($n = 5$)	8.30 ± 0.28 ($n = 4$)	60.7 ± 5.0 ($n = 5$)
E5	7.80 ± 0.14 ($n = 3$)	8.10 ± 0.19 ($n = 6$)	53.4 ± 7.6 ($n = 5$)	n/d	n/d
B6	7.88 ± 0.19 ($n = 3$)	7.68 ± 0.09 ($n = 4$)	54.0 ± 5.8 ($n = 5$)	n/d	n/d

n/d, no data. Efficacy is expressed as % response compared with S1P. Data are expressed as means ± SEM.

Table 4

Affinity, potency and efficacy determinations in response to VPC24191 in three-assay formats, radioligand binding, PathHunter™ and Fluo-4 Ca^{2+} using clonal cell lines expressing the human $S1P_3$ receptor

	VPC24191				
	Binding	PathHunter™		Fluo-4 Ca^{2+}	
	pK_i	pEC_{50}	Efficacy %	pEC_{50}	Efficacy %
F6	5.40 ± 0.14 ($n = 3$)	n/d	n/d	7.81 ± 0.19 ($n = 5$)	87.5 ± 3.4 ($n = 5$)
B3	5.47 ± 0.14 ($n = 3$)	n/d	n/d	8.09 ± 0.32 ($n = 2$)	72.7 ± 5.6 ($n = 5$)
E5	5.35 ± 0.08 ($n = 3$)	n/d	n/d	7.67 ± 0.06 ($n = 2$)	73.9 ± 10.3 ($n = 5$)
B6	5.43 ± 0.08 ($n = 3$)	n/d	n/d	7.51 ± 0.10 ($n = 5$)	58.6 ± 9.2 ($n = 5$)

n/d, no data. Efficacy is expressed as % response compared with S1P. Data are expressed as means ± SEM.

receptor activation (Leff, 1995). Central to this model is the predicted existence of two receptor conformations: *R* and *R**. *R* represents the receptor in its inactive or resting conformation, and *R** represents the same receptor in a conformation that is active, i.e., able to couple productively to G-protein(s) to initiate a measurable response while stabilizing the receptor. Using this model, it would be predicted that in our system, we could only detect $S1P_3$ receptor agonists binding with high affinity to the *R** conformation, and that this affinity value should be independent of receptor density. Moreover this model actually predicts a decrease in agonist affinity with increasing receptor expression, probably due to depletion of available G-proteins required to form the high-affinity complex. As stated earlier, in our assay, S1P affinity increased with receptor expression. One suggestion for this may be that during our clonal selection, we inadvertently identified clones with different cellular background conditions, i.e., changes in the levels of accessory proteins, therefore altering the affinities for the endogenous agonist. However, in this scenario, we would then expect an increase

Table 5

Affinity, potency and efficacy determinations in response to CYM5442 in three-assay formats, radioligand binding, PathHunter™ and Fluo-4 Ca^{2+} using clonal cell lines expressing the human $S1P_3$ receptor

	CYM5442				
	Binding	PathHunter™		Fluo-4 Ca^{2+}	
	pK_i	pEC_{50}	Efficacy %	pEC_{50}	Efficacy %
F6	6.50 ± 0.01 (*n* = 2)	5.53 ± 0.08 (*n* = 6)	126.0 ± 13.8 (*n* = 5)	6.83 ± 0.26 (*n* = 5)	107.1 ± 7.6 (*n* = 5)
B3	6.46 ± 0.00 (*n* = 2)	5.81 ± 0.09 (*n* = 6)	110.3 ± 8.7 (*n* = 5)	6.65 ± 0.21 (*n* = 2)	103.4 ± 10.4 (*n* = 5)
E5	6.51 ± 0.02 (*n* = 2)	5.71 ± 0.04 (*n* = 5)	142.6 ± 24.3 (*n* = 5)	6.56 ± 0.23 (*n* = 2)	115.7 ± 20.6 (*n* = 5)
B6	6.74 ± 0.03 (*n* = 2)	5.66 ± 0.04 (*n* = 6)	120.6 ± 16.8 (*n* = 5)	6.01 ± 0.21 (*n* = 5)	94.8 ± 16.1 (*n* = 5)

Efficacy is expressed as % response compared with S1P. Data are expressed as means ± SEM or SD.

in affinity of all agonists tested. Another explanation is that when agonist binding occurs, it stabilizes the receptor in a conformation that partly resembles *R**, even in the presence of GTP; therefore, agonist affinity contains a composite of both agonist binding and efficacy (Strange, 1999), explaining the increase in agonist affinity observed with the most efficacious agonist, S1P.

Next, using S1P, FTY720-P and CYM5442 we have demonstrated that these compounds were able to recruit β-arrestin with varying potency and efficacies. Our data also confirmed that FTY720-P was a partial agonist in the PathHunter™ assay, supporting data previously published by Brinkmann *et al*. (2002) and Jongsma *et al*. (2009). However, unlike other functional assays, where increases in receptor expression have been demonstrated to exhibit concomitant increases in agonist potency, no changes were observed, suggesting little or no receptor reserve present in the PathHunter™ assays. This would be expected as it is assumed there is a simple 1:1 stoichiometry between the activation of the receptor, and the recruitment of β-arrestin. Another $S1P_3$ receptor agonist, VPC24191, is an agonist in two different functional assays, inhibition of cAMP accumulation and elevation of intracellular calcium concentrations (Jongsma *et al*., 2009). Interestingly VPC24191 failed to elicit any response in the PathHunter™ assays, potentially indicating that the compound may not recruit β-arrestin. While our data cannot prove or disprove that VPC24191 is a biased ligand, we suggest that the compound is more likely to be driven by efficacy. Moreover, Jongsma *et al*. (2009) have shown internalization data of $S1P_3$ receptors using VPC24191, suggesting that the compound was able to recruit β-arrestin during the process of receptor internalization. Furthermore, when we profiled this compound in the Ca^{2+} release assay, we found that the compound was an $S1P_3$ receptor agonist but with very low efficacy, when compared with the full agonist S1P and therefore only detectable in a highly amplified system. This highlights a potential issue with using the PathHunter™ assay for identifying agonists. As there is little or no receptor reserve, low efficacy agonists may not be detected and could appear to be inactive. This issue is also demonstrated with the data obtained for the $S1P_{1/3}$ receptor antagonist VPC23019. Here, it behaved as a partial agonist with low efficacy in the Ca^{2+} release assay rather than a neutral antagonist as claimed (Davis *et al*., 2005). An alternative explanation for the agonist activity of VPC23019 was proposed by Pyne and Pyne (2011). These authors suggested that VPC23019 bound and stabilized an $S1P_3/G_i$ conformation which then induced Ca^{2+} mobilization. The antagonist activity observed by Davis *et al*. (2005) could be due to stabilization of a $S1P_3/G_q$ conformation, with the outcome being dependent on which conformation exists at equilibrium. This mechanism supports data by Jongsma *et al*. (2009) who demonstrated that VPC23019 induced a *Pertussis* toxin-sensitive mobilization of Ca^{2+}. These findings illustrate the importance of using multiple functional assays when defining agonist pharmacology.

Data from the Ca^{2+} release assay are what would be expected. The full agonists, S1P and CYM5442, show increased potencies while the partial agonists FTY720-P and VPC24191 show increased efficacies in clones with higher expression levels. These data are consistent with there being a hyperbolic relationship between conversion of an agonist–receptor complex to response and efficacy of the agonist, termed the operational model of receptor function described by Black and Leff (1983),

When comparing the affinity values (pK_i) with the pEC_{50} values, our results show that the PathHunter™ assays lie to the right of both the binding and Ca^{2+} data (Figure 8), thus supporting the hypothesis that the PathHunter™ assay was detecting a different agonist-bound receptor conformation. However, for the partial agonist, FTY720-P, the pEC_{50} calculated in the PathHunter™ assay approximates to the pK_i. This may be due to its partial agonism; however, further work is required to fully understand this finding. For the full agonists, S1P and CYM5442, the pEC_{50} values determined in the PathHunter™ assay are significantly lower than their pK_i. One explanation for this finding could be that the PathHunter™ assay measures a different agonist-bound receptor conformation. Upon agonist binding and the association of different effector proteins, a change in the receptor conformation occurs. With the recruitment of β-arrestin, the receptor may be altered into a more restricted conformation and therefore comprises a different population from that being measured in both the binding and Ca^{2+} release assays, albeit still in an agonist-bound receptor conformation. It has also been suggested that formation of homodimers is required before binding of β-arrestin can occur (Lagane *et al*., 2008). However, this contradicts the suggestion that there is a simple 1:1 stoichiometry between receptor activation and β-arrestin

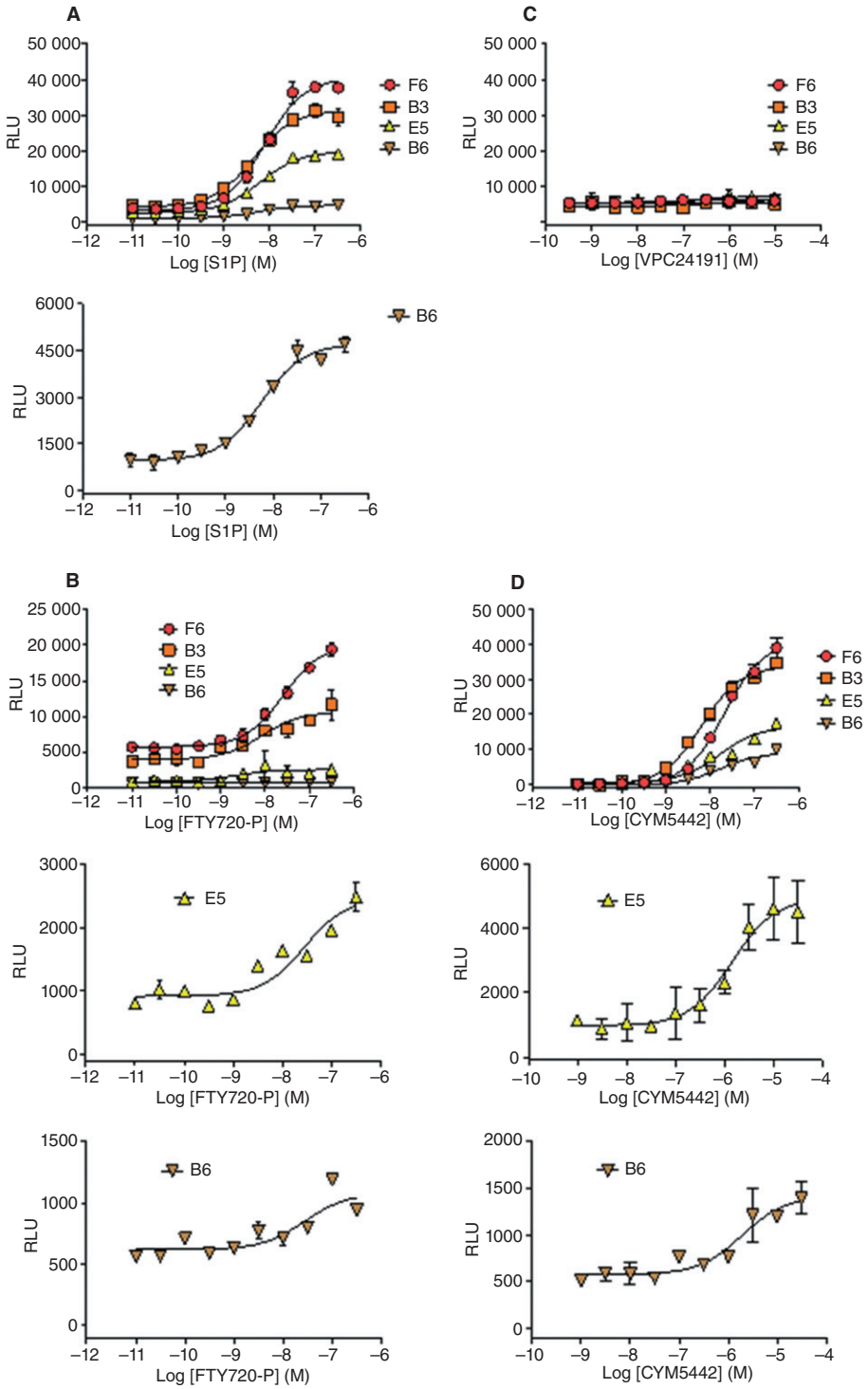

Figure 6

Concentration response curves to (A) S1P; (B) FTY720-P; (C) VPC24191; and (D) CYM5442 in four clonal cell lines expressing the human $S1P_3$ receptor, F6, B3, E5 and B6, in the PathHunter™ assay. The lower graph in A shows data from clone B6 on a larger scale (note expanded Y-axis) and the lower graphs in B and D show similarly expanded results from clones E5 and B6.:The agonists were incubated for 90 min at 37°C/5% CO_2 and the experiment concluded upon the addition of 25 $\mu L \cdot well^{-1}$ of Flash reagent (detectable solution and lysis buffer). The exposure time was determined for each clone independently on the day of experimentation. Chemiluminescence is indicated as relative luminescence units (RLU). A representative graph from an independent experiment is presented. Data shown are means ± SEM (n = 1–7).

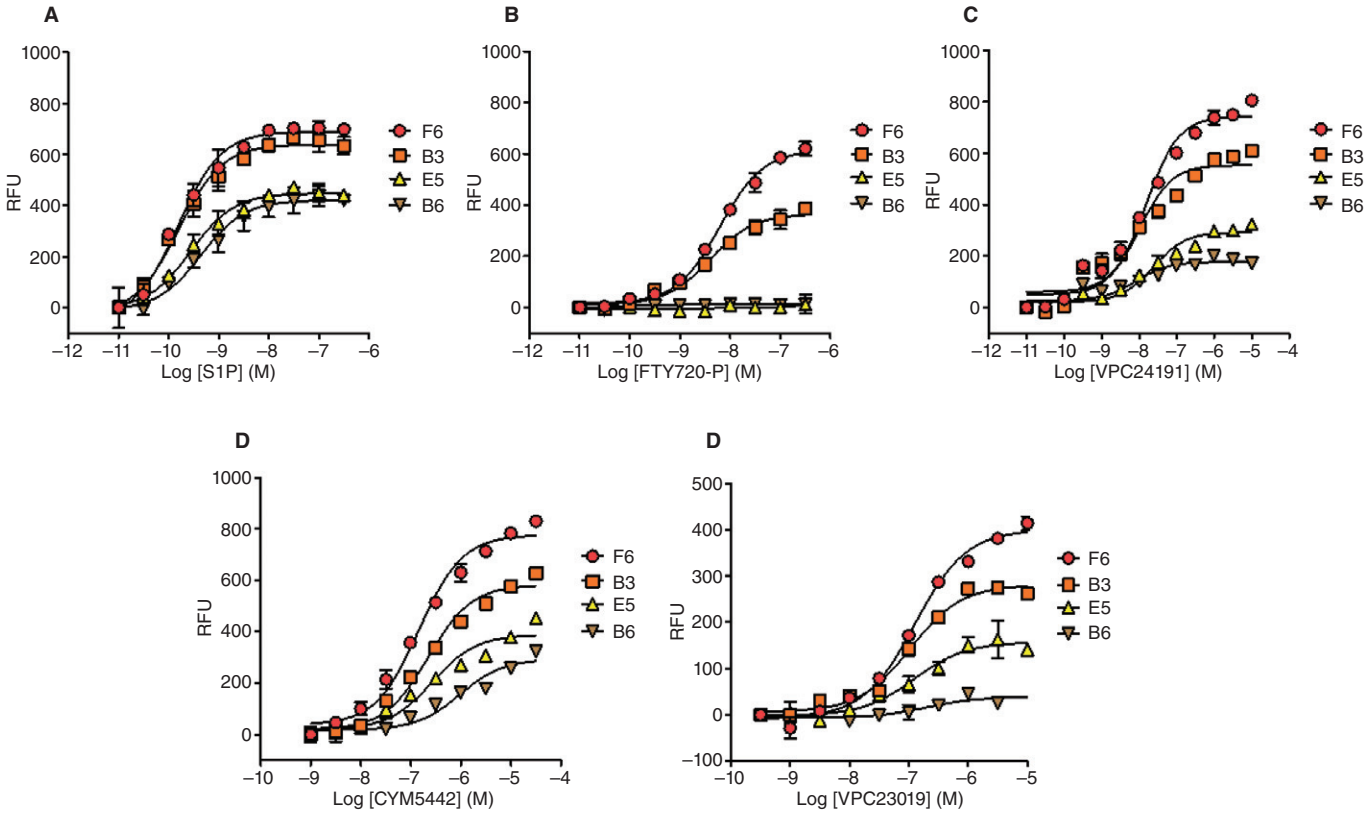

Figure 7

Concentration response curves to (A) S1P; (B) FTY720-P; (C) VPC24191; (D) CYM5442; and (E) VPC23019 in the four clonal cell lines expressing the human $S1P_3$ receptor, F6, B3, E5 and B6, in the Fluo-4 calcium assay. The cells were loaded with Fluo-4 calcium dye and incubated for 45 min at 37°C/5% CO_2. Agonists were added and the release of intracellular calcium measured for 2 min. Basal values were determined for 10 s prior to addition of agonist. Fluorescence is indicated as relative fluorescence units (RFU). A representative graph from an independent experiment is presented. Data are expressed in mean ± SEM (n = 2–5).

Table 6

Affinity, potency and efficacy determinations in response to VPC23019 in three-assay formats, radioligand binding, PathHunter™ and Fluo-4 Ca^{2+} using clonal cell lines expressing the human $S1P_3$ receptor

	VPC23019				
	Binding	PathHunter™		Fluo-4 Ca^{2+}	
	pK_i	pEC_{50}	Efficacy %	pEC_{50}	Efficacy %
F6	4.89 ± 0.14 (n = 3)	n/d	n/d	6.82 ± 0.21 (n = 5)	59.2 ± 3.6 (n = 5)
B3	4.88 ± 0.15 (n = 3)	n/d	n/d	7.22 ± 0.32 (n = 1)	41.2 ± 5.1 (n = 5)
E5	4.94 ± 0.05 (n = 3)	n/d	n/d	6.87 ± 0.31 (n = 2)	31.8 ± 13.4 (n = 5)
B6	5.07 ± 0.13 (n = 3)	n/d	n/d	6.53 ± 0.23 (n = 5)	6.1 ± 14.4 (n = 5)

n/d, no data. Efficacy is expressed as % response compared with S1P. Data are expressed as means ± SEM or SD.

recruitment, highlighting the importance of further investigation into this mechanism. The lack of statistical significance between the pK_i and the pEC_{50} values determined in the Ca^{2+} release assay could be attributed to the binding only measuring a high-affinity complex, or a different population of activated receptors, therefore overestimating the true ground state affinity. Finally, VPC24191 could not be analysed in this manner because it did not produce a suitable response in the PathHunter™ assay.

In summary, we have investigated the pharmacology of S1P receptor agonists in the G-protein–independent DiscoveRx PathHunter™ assay using cell lines over expressing the human $S1P_3$ receptor at different expression levels. We have identified that the apparent pEC_{50} values correlate with

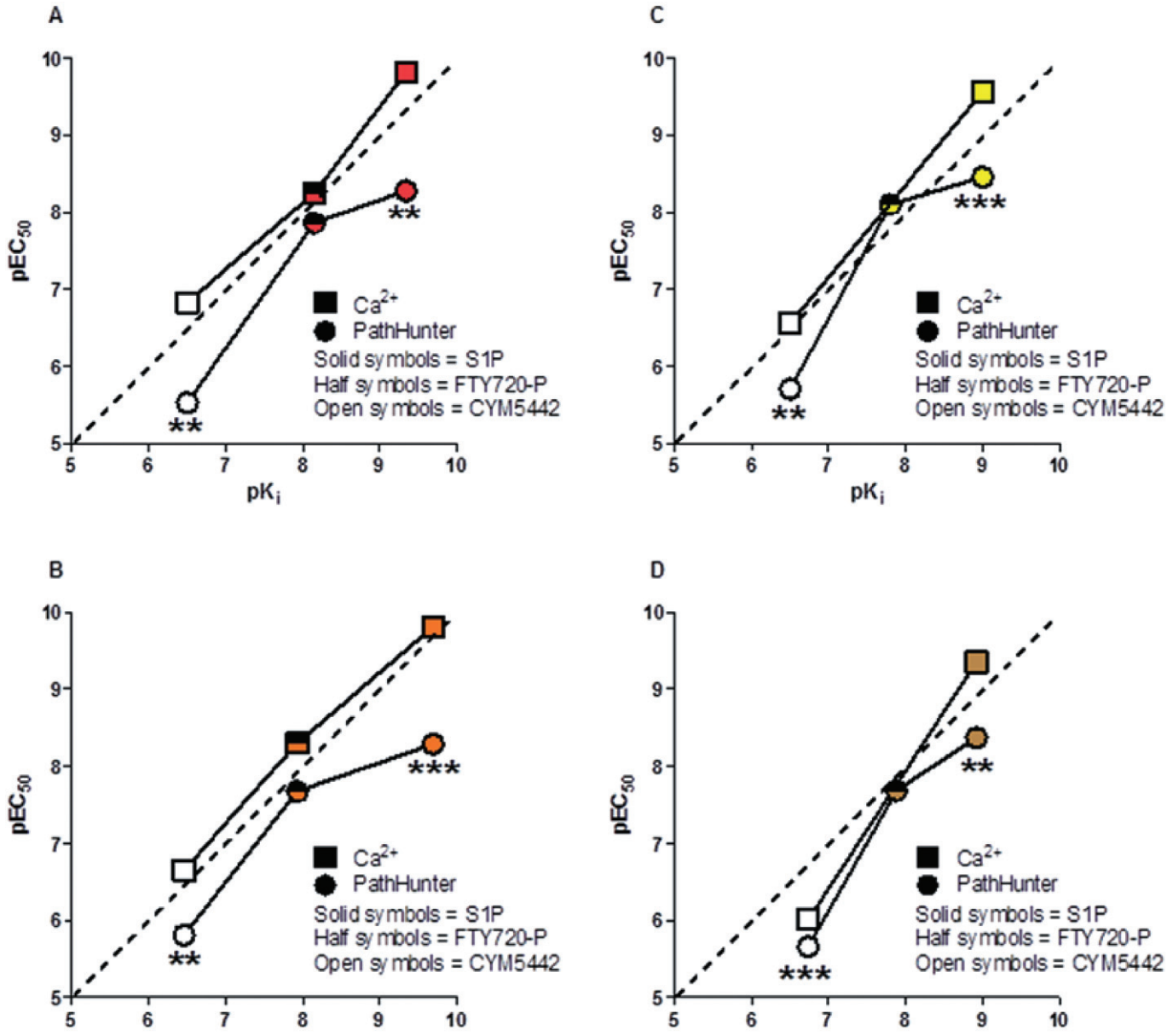

Figure 8

Correlation data plots of the PathHunter™ and Ca^{2+} assays for each clone, (A) F6; (B) B3; (C) E5; and (D) B6 against three S1P agonists, S1P, FTY720-P and CYM5442. $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from 1:1 correlation (dashed line); one-way ANOVA with Bonferroni post-test.

the compound affinity for the partial agonist, FTY720-P. However, the differences between the pK_i and pEC_{50} values of the highly efficacious agonists in the PathHunter™ assay indicate that the binding may be overestimating the apparent affinities. We have demonstrated that the lack of efficacy for VPC24191 in the PathHunter™ assay can be explained by differences in intrinsic efficacy, highlighting potential limitations in screening for agonists, where partial- or low-efficacy compounds can appear inactive. We have also shown that in the highly amplified Ca^{2+} release assay, VPC23019 is a partial agonist. Therefore relying on a single functional assay, especially one with low receptor reserve, can potentially lead to inappropriate agonist/antagonist classification. This is of critical importance as some of the compounds have been used both *in vitro* and *in vivo* to dissect the involvement of a particular S1P receptor subtype (Lichte *et al.*, 2008; Salomone *et al.*, 2008). Therefore, these conclusions are potentially misleading if the pharmacology of the ligand is not truly defined.

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Conflicts of interest

All authors were employees of Novartis while these experiments were carried out.

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