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RESEARCH PAPER

Investigating the molecular mechanisms through which FTY720-P causes persistent $S1P_1$ receptor internalization

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BACKGROUND AND PURPOSE

The molecular mechanism underlying the clinical efficacy of FTY720-P is thought to involve persistent internalization and enhanced degradation of the $S1P_1$ receptor subtype (S1P1R). We have investigated whether receptor binding kinetics and β-arrestin recruitment could play a role in the persistent internalization of the S1P1R by FTY720-P.

EXPERIMENTAL APPROACH

[^{3}H]-FTY720-P and [^{33}P]-S1P were used to label CHO-S1P1/3Rs for binding studies. Ligand efficacy was assessed through [^{35}S]-GTPγS binding and β-arrestin recruitment. Metabolic stability was evaluated using a bioassay measuring intracellular Ca^{2+} release. CHO-S1P1/3R numbers were determined, following FTY720-P treatment using flow cytometry.

KEY RESULTS

The kinetic off-rate of [^{3}H]-FTY720-P from the S1P1R was sixfold slower than from the S1P3R, and comparable to [^{33}P]-S1P dissociation from S1P1/3Rs. S1P and FTY720-P stimulated [^{35}S]-GTPγS incorporation to similar degrees, but FTY720-P was over 30-fold less potent at S1P3Rs. FTY720-P stimulated a higher level of β-arrestin recruitment at S1P1Rs, 132% of the total recruited by S1P. In contrast, FTY720-P was a weak partial agonist at S1P3R, stimulating just 29% of the total β-arrestin recruited by S1P. Internalization experiments confirmed that cell surface expression of the S1P1R but not the S1P3R was reduced following a pulse exposure to FTY720-P, which is metabolically stable unlike S1P.

CONCLUSIONS AND IMPLICATIONS

FTY720-P and S1P activation of the S1P1R results in receptor internalization as a consequence of an efficient recruitment of β-arrestin. The combination of slow off-rate, efficacious β-arrestin recruitment and metabolic stability all contribute to FTY720-P's ability to promote prolonged S1P1R internalization and may be critical factors in its efficacy in the clinic.

Abbreviations

FTY720-P, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol phosphate; MEM, minimal essential medium; NSB, non-specific binding; S1P, sphingosine 1-phosphate; SPA, scintillation proximity assay

Introduction

Sphingosine 1-phosphate (S1P) is a blood-borne bioactive sphingolipid secreted by platelets, erythrocytes (Pappu *et al.*, 2007), mast cells and monocytes with diverse functions in regulating cell migration, proliferation, differentiation and survival (Spiegel and Milstien, 2003; Le Stunff *et al.*, 2004). The chemotactic egress of lymphocytes from lymphoid tissue towards the lymph requires signalling through S1P1Rs (Matloubian *et al.*, 2004; Pappu *et al.*, 2007; receptor nomenclature follows Alexander *et al.*, 2013). More recently, it was demonstrated that agonism at endothelial S1P1Rs was also able to prevent egress of lymphocytes from lymphoid tissue (Wei *et al.*, 2005). The balance between these processes potentially plays a key role in the pathophysiology of multiple sclerosis. FTY720 (fingolimod; 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol), a structural analogue of sphingosine, is a novel and potent oral S1P1R modulator which has recently been approved for the treatment of relapsing-remitting multiple sclerosis after completing two pivotal phase 3 trials (Brinkmann *et al.*, 2010; Cohen *et al.*, 2010; Kappos *et al.*, 2010). FTY720 is phosphorylated *in vivo* by sphingosine kinase 2 yielding the potent S1P1/3/4/5R agonist FTY720-P (Lee *et al.*, 2010). The molecular mechanism underlying the influence of FTY720-P on lymphocyte trafficking is only now being fully understood.

FTY720 is thought to act as a functional S1P1R antagonist, causing complete internalization and down-regulation of the S1P1Rs found on lymphocytes (Matloubian *et al.*, 2004; Chiba *et al.*, 2006). Sustained FTY720-P-induced receptor internalization of S1P1Rs has been demonstrated in a number of separate studies (Jo *et al.*, 2005; Oo *et al.*, 2007; Mullershausen *et al.*, 2009). Maximal internalization of S1P1Rs can take up to 30 min (Oo *et al.*, 2007) and is readily reversed in the case of S1P but sustained for FTY720-P following treatment of cells (Jo *et al.*, 2005). In the case of the endogenous ligand S1P, internalized S1P1Rs recycle back to the plasma membrane within hours following treatment of cells (Jo *et al.*, 2005; Oo *et al.*, 2007) whereas FTY720-P-induced receptor internalization does not lead to receptor recycling following ligand removal but results in irreversible degradation and a reduction in S1P1R membrane expression. The mechanism of S1P1R selective internalization and down-regulation following treatment of cells with FTY720-P is not fully understood, but it is thought to be implicated in the immunosuppressive properties of FTY720. It has been postulated that S1P and FTY720-P stabilize different conformational states of the S1P1R leading to so-called functional selectivity or ligand-directed signalling (Oo *et al.*, 2007). These differences in adopted conformations are thought to result in distinct patterns of S1P1R phosphorylation (van Der Lee *et al.*, 2008) and ubiqitinization (Oo *et al.*, 2007) which ultimately leads to differences in the extent of endosomal trafficking to proteosomal/lysosomal degradation pathways. The possibility that differences in the stability of these ligands somehow contribute to the various levels of receptor phosphorylation and ubiqitinization has not been fully explored. In addition, receptor phosphorylation and subsequent β-arrestin recruitment have been shown to be necessary for FTY720-P-induced receptor internalization and subsequent receptor degradation however little is known about the efficiency of β-arrestin recruitment to the receptor following treatment with FTY720-P and S1P (Oo *et al.*, 2007).

Recently, Mullershausen *et al.* (2009) demonstrated clear differences in the pharmacological profile of FTY720-P at human S1P1 and S1P3Rs expressed in CHO cells. From these studies, we surmised that persistent S1P1R internalization could be dependent on the availability of agonist inside the cell following initiation of the internalization process. We hypothesized that trapping of agonist in intracellular vesicles is more likely for agonists that have a slow dissociation rate from the receptor. In order to investigate whether a disparity in receptor dissociation kinetics of FTY720-P and S1P could play a role in their differential effects at S1P1 and S1P3Rs, we have studied the binding of both [^{3}H]-FTY720-P and [^{33}P]-S1P to both human S1P1 and S1P1Rs, individually expressed in CHO cells in order to study each subtype in isolation. Differences in the relative efficacies of S1P and FTY720-P for G protein activation were assessed in a [^{35}S]-GTPγS assay while the PathHunter™ β-arrestin assay was used to quantify β-arrestin recruitment to assess a potential role for intrinsic activity in persistent internalization. In addition, we have compared the stability of both ligands in membrane preparations to determine whether longevity of internalization could be influenced by metabolic stability of the ligand. Finally, we have investigated the relative rates of CHO-S1P1 and S1P3 surface receptor recovery following a pulse treatment with FTY720-P.

Methods

Cell culture and membrane preparation

CHO cells were transfected with the cDNA encoding the human S1P1 and S1P3Rs. Cells were grown in Hams-F12 supplemented with 10% FBS and Glutamax and maintained at 37°C in 5% CO_2/humidified air. Cells were routinely spit 1:10 using trypsin-EDTA to lift cells, and were not used in assays beyond passage 40. CHO cells expressing the human S1P1 and S1P3Rs were grown to 80–90% confluence in 500 cm^2 cell culture plates and cell membranes were subsequently prepared as described by Sykes and Charlton (2012).

[^{35}S]GTPγS binding assay

The [^{35}S]GTPγS binding assays were performed in white 96-well Optiplates in a final volume of 250 μL as follows. In brief, wells were loaded with 150 μL of assay buffer (20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl and 1 mM EDTA), containing 0.1% BSA, 30 μg·mL^{-1} saponin, 50 μg·mL^{-1} CHO-S1P1 or S1P3 membranes, 3.7 μM guanosine 5′-diphosphate sodium salt (GDP) and 2.5 mg·mL^{-1} WGA PVT scintillation proximity assay (SPA) beads. Fifty microlitres of agonist was then added to the plates, which were incubated at room temperature with gentle agitation for 30 min, allowing equilibrium to be reached. Following this, 50 μL of guanosine 5-*O*-(3-[^{35}S]thio)triphosphate (GTPγS) at a final concentration of 300 pM was added to each well, and incubated at room temperature with gentle agitation for 40 min. Following this, the plates were centrifuged (Jouan B4i; Jouan, St. Herblain, France) for 3 min at 1000× *g* before being quantified using single photon counting on a TopCount™ microplate scintillation counter (Perkin Elmer).

PathHunter β-arrestin assay

Human S1P1 and human S1P3Rs were sub-cloned into the DiscoveRx PathHunter ProLink vector (containing Nhe1 and Hind III restriction sites) by restriction digest and ligation. *Escherichia coli* were transformed and positive colonies selected using kanamycin. CHO-human S1P1 and human S1P3 cell lines were grown in Ham-F12 supplemented with glutamax, 10% FBS, 200 μg·mL^{-1} hygromycin B, 500 μg·mL^{-1} geneticin, and incubated at 37°C in 5% CO_2/humidified air.

On the day prior to the experiment, CHO-human S1P cells were harvested with trypsin-EDTA from the cell culture flask, counted and resuspended in cell culture medium. About 20 μL of the cell suspension was transferred to each well of a 384-well white walled, clear bottom plate (ViewPlate Perkin Elmer) at a concentration of 1000 cells/well. Seeded plates were incubated for 24 h at 37°C in 5% CO_2/humidified air. Subsequent agonist stimulation of cells and detection steps were carried out as previously described by Riddy *et al.* (2012).

Common procedures applicable to all radioligand binding experiments

All radioligand experiments were conducted in 96-deep-well plates, in assay binding buffer, HBSS pH 7.4 containing 0.5% BSA and 0.1 mM sodium orthovanadate at room temperature. In all cases, non-specific binding (NSB) was determined in the presence of 0.3 μM FTY720-P or S1P. After the indicated incubation period, bound and free radioligand were separated by rapid vacuum filtration using a FilterMate™ Cell Harvester (Perkin Elmer) onto 96-well GF/C filter plates and rapidly washed three times with ice cold 20 mM HEPES 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 and [^{33}P]-S1P were also quantified accurately to determine how much radioactivity was added to each well using liquid scintillation spectrometry on LS 6500 scintillation counter (Beckman Coulter, High Wycombe, UK).

Equilibrium radioligand binding studies

Binding was performed with a range of concentrations of [^{3}H]-FTY720-P (~7 to 0.003 nM) and [^{33}P]-S1P (~1–0.002 nM) to construct saturation binding curves, as described by Sykes *et al.* (2009). CHO-S1P1 and S1P3 cell membranes (≤10 μg/well) were incubated in 96-deep-well plates at room temperature in assay binding buffer containing radioligand in a total assay volume of 250 μL, with gentle agitation for 2 h to ensure equilibrium was reached.

Determination of the dissociation rate (k_{off}) of [^{3}H]-FTY720-P and [^{33}P]-S1P

The dissociation of [^{3}H]-FTY720-P was determined by allowing approximately 1 nM [^{3}H]-FTY720-P (exact concentration determined by liquid scintillation counting) to reach equilibrium with CHO-S1P1 and S1P3 membranes (10 and 20 μg/well, respectively) in a final volume of 100 μL. Equilibrium was verified by harvesting an identical plate before the start of the experiment and then comparing the total binding on this plate to that on the proceeding (post t = 0) plates. After equilibrium was reached (approximately 1 h), re-association of [^{3}H]-FTY720-P was prevented by the addition of 0.9 mL S1P (0.3 μM final) and GTP (1 mM). Bound [^{3}H]-FTY720-P was measured at multiple points post t = 0. The dissociation of [^{33}P]-S1P was carried out as described above for [^{3}H]-FTY720-P except that CHO-S1P1 and S1P3 membranes were reduced to 5 and 10 μg/well, respectively, and re-association of [^{33}P]-S1P was prevented by the addition of 0.9 mL FTY720-P (0.3 μM final) and GTP (1 mM).

Metabolism of FTY720-P and S1P by CHO cells

CHO-S1P3 cells were seeded at 15 000 cells per well in a 96-well plate and incubated at 37°C for 24 h. On the day of the experiment, the media was removed and replaced with loading buffer (HBSS without Ca^{2+} and Mg^{2+}, 20 mM HEPES, 100 μM sodium orthovanadate, 2.5 mM probenecid, 100 μM brilliant black and 1 μM Fluo-4-AM) and incubated at 37°C for 60 min. This assay was used as a 'bioassay' to indirectly measure the amount of S1P and FTY720-P present following incubation with CHO-S1P3 membranes. Specifically, a range of concentrations of S1P and FTY720-P diluted in assay buffer [HBSS without Ca^{2+} and Mg^{2+}, 20 mM HEPES and 0.1% (w/v) BSA] were incubated in the presence and absence of CHO-S1P3 membranes (2.5 μg/well) for 180 min. A sample of these incubation mixtures was then added to CHO-S1P3 cells, and increases in intracellular Ca^{2+} were measured using a Fluorometric Imaging Plate Reader (FLIPR; Molecular Devices, Sunnyvale, CA, USA).

Internalization assay

The surface expression of S1P1 and S1P3Rs in CHO cells was measured following a 1 h incubation period in the absence or presence of increasing concentrations of FTY720-P, at 37°C, or following a 1 h incubation and 3 h wash period in the absence of compound. Full details of these procedures are outlined below. The vectors encompassing human S1P1 and S1P3Rs contained an N-terminal myc-tag (sequence = EQKLISEEDL). Cells were seeded (10^6 cells) and grown in 6-well plates to 60–80% confluence in αMEM. Following treatment with FTY720-P, the cells were washed with PBS and detached by treatment with PBS/1 mM EDTA. 10^6 cells were incubated either with 4 μg·mL^{-1} of monoclonal mouse anti c-myc IgG1 (Roche Applied Science, Mannheim, Germany) antibody or with isotype control mouse IgG1 (Pharmigen, BD Biosciences, Basel, Switzerland) in balanced salt solution for 60 min at 4°C. The cells were washed once in PBS/5% fetal calf serum, followed by incubation of 60 min at 4°C in the dark with 1 μg·mL^{-1} of Alexa488-labelled goat anti-mouse secondary conjugates (Molecular Probes, Juro Supply, Luzern, Switzerland). Fluorescence-activated cell cytometer measurements were performed with 10 000 viable cells per sample. The viability was tested by the addition of 3 μL of a 1 μM DNA-binding TO-PRO-3 iodide solution (Molecular Probes, Juro Supply) and analysed in flow cytometry. The fraction of receptor expressed at the surface of the cell was calculated as follows: $(F_c - F_b)/(F_0 - F_b)$*100, where F_c is the fluorescence measured at the given concentrations of FTY720-P, F_0 is the fluorescence in untreated cells (maximal expression of S1PR at the surface), and F_b is the fluorescence intensity detected with the isotype control IgG1.

Data analysis

[^{3}H]FTY720-P and [^{33}P]-S1P total and non-specific binding data were analysed by non-linear regression according to a one-site and two-site binding equations, and individual estimates for total receptor number (B_{max}) and radioligand dissociation constant (K_d) were calculated, the best fit was determined using an *F*-test.

The following equation was used, where [*A*] is the concentration of radioligand:

One-site model:

$$\text{Specific} = \frac{B_{max}[A]}{K_d + [A]} \quad \text{NSB} = \text{slope}\,[A] + \text{background}$$

Two-site model:

$$\text{Specific} = \frac{B_{max\,high}[A]}{K_{d\,high} + [A]}$$

$$\text{Specific} = \frac{B_{max\,low}[A]}{K_{d\,low} + [A]}$$

$$\text{NSB} = \text{slope}\,[A] + \text{background}$$

FLIPR, GTPγS, β-arrestin and internalization data were analysed by non-linear regression, sigmoidal dose response (variable slope) according to the following equation:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{bottom})}{1 + 10\,\text{^}\,((\text{LogEC}_{50} - X)\text{Hillslope})},$$

where *Y* is the measurement (e.g. normalized fluorescence). Top denotes maximal asymptotic response and Bottom denotes the minimal asymptotic response.

[^{3}H]FTY720-P and [^{33}P]-S1P dissociation data were fitted to either mono-exponential or bi-exponential dissociation equations and the best fit was determined using an *F*-test. This analysis provided estimates of the dissociation rates and the proportion (population %) of specific binding that dissociates at these rates. Dissociation rate constants were compared using paired Student's *t*-tests. For data best fit to a two-phase exponential, we have termed the half-lives rapid and slow. All data are shown as the mean ± SE mean or range for the indicated number of experiments. All experiments were analysed by either linear or non-regression using Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Materials

[^{3}H]-FTY720-P (specific activity 49 Ci mmol^{-1}) was synthesized by tritiation of the (S,E)-4-(4-(oct-1-en-1-yl)phenethyl)-4-(((3-oxido-1,5-dihydrobenzo[e][1,3,2]dioxaphosphepin-3-yl)oxy)methyl)oxazolidin-2-one dissolved in ethanol in the presence of palladium on charcoal for 2 h followed by lithium hydroxide-mediated hydrolysis of the oxazolidone ring at 80°C for 18 h and HPLC purification. [^{33}P]-S1P (specific activity ~2000 to 3000 Ci mmol^{-1}) was purchased from the American Radiolabeled Chemical Company (ARC, Saint Louis, MO, USA). Guanosine 5-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS; specific activity 1000 Ci mmol^{-1}) and wheat germ agglutinin SPA beads were obtained from Perkin Elmer (Beaconsfield, UK). S1P was purchased from BioMol, FTY720-P was generated in-house. CHO cells stably expressing the human S1P1 and S1P3R were generated in-house. 96-well polypropylene plates, 96-deep-well polypropylene plates, 96-well black plates with clear bottom and lid (tissue culture treated) and 500 cm^2 cell culture plates were purchased from Fisher Scientific (Loughborough, UK). 96-well GF/C filter plates were purchased from Millipore (Watford, UK). HBSS, sodium bicarbonate, EDTA, sodium chloride, HEPES, DMSO, BSA, GTP, saponin and sodium orthovanadate were obtained from Sigma Chemical Co Ltd. (Poole, UK). Cell culture reagents including Fluo-4-AM and pluronic acid were purchased from Gibco (Invitrogen, Paisley, UK).

Results

Equilibrium saturation binding of [^{3}H]-FTY720-P and [^{33}P]-S1P

Specific [^{3}H]-FTY720-P and [^{33}P]-S1P binding to S1P1 and S1P3Rs expressed in CHO cells was in each case saturable and best described by the interaction of the radioligand with a single population of high-affinity binding sites (Figure 1A–D). The equilibrium dissociation constant (K_d) and maximal receptor binding (B_{max}) for each radioligand at both receptors are summarized in Table 1. S1P exhibits a threefold higher affinity for S1P3Rs over S1P1Rs, with K_d values of 0.068 ± 0.005 versus 0.210 ± 0.027 nM respectively ($P < 0.05$). In contrast, the affinity of FTY720-P was threefold higher for S1P1Rs compared with S1P3Rs with K_d values of 0.283 ± 0.055 versus 0.946 ± 0.143 nM respectively.

[^{3}H]-FTY720-P and [^{33}P]-S1P dissociation studies – kinetic binding studies

The kinetics of [^{3}H]-FTY720-P and [^{33}P]-S1P dissociation was studied in the presence of GTP and excess unlabelled competitor. GTP was included to remove the high-agonist affinity complex comprising the guanine nucleotide-free GPCR that is artificially stabilized by removal of guanine nucleotide during the membrane preparation step. In both instances, the observed dissociation was consistent with the presence of two populations of receptor. The population that represents the very rapid dissociation rate is likely to represent the guanine nucleotide-sensitive GPCR that is rapidly removed upon addition of GTP (population 1, see Table 2), whereas the second component (population 2) with slower dissociation kinetics is resistant to GTP so is likely independent of the G protein. [^{3}H]-FTY720-P dissociation from population 2 was significantly slower from S1P1Rs compared with S1P3Rs (Figure 2A, B, 19.0 vs. 3.1 min, $P < 0.05$). In direct contrast, the rates of [^{33}P]-S1P dissociation from the guanine insensitive component of both S1P1 and S1P3Rs were not significantly different from each other ($t½$ of 23.9 vs. 14.0 min, $P > 0.05$), occurring at a similar rate to [^{3}H]-FTY720-P dissociating from the guanine insensitive component of $S1P_1$ receptors ($t½$ of 19.0 min). Full details are presented in Table 2.

Measurement of [^{35}S]-GTPγS incorporation

Both S1P and FTY720-P stimulated the incorporation of [^{35}S]-GTPγS into the CHO-S1P1 (Figure 3A) and S1P3 membranes (Figure 3B) and the potency and intrinsic activity for each ligand are detailed in Table 3. FTY720-P was significantly ($P < 0.05$) less potent at S1P3 than S1P1Rs (pEC_{50}s of 8.44 ± 0.08 and 9.32 ± 0.02 respectively) while S1P itself was significantly ($P < 0.05$, Student's *t*-test) more potent at S1P3 than S1P1Rs

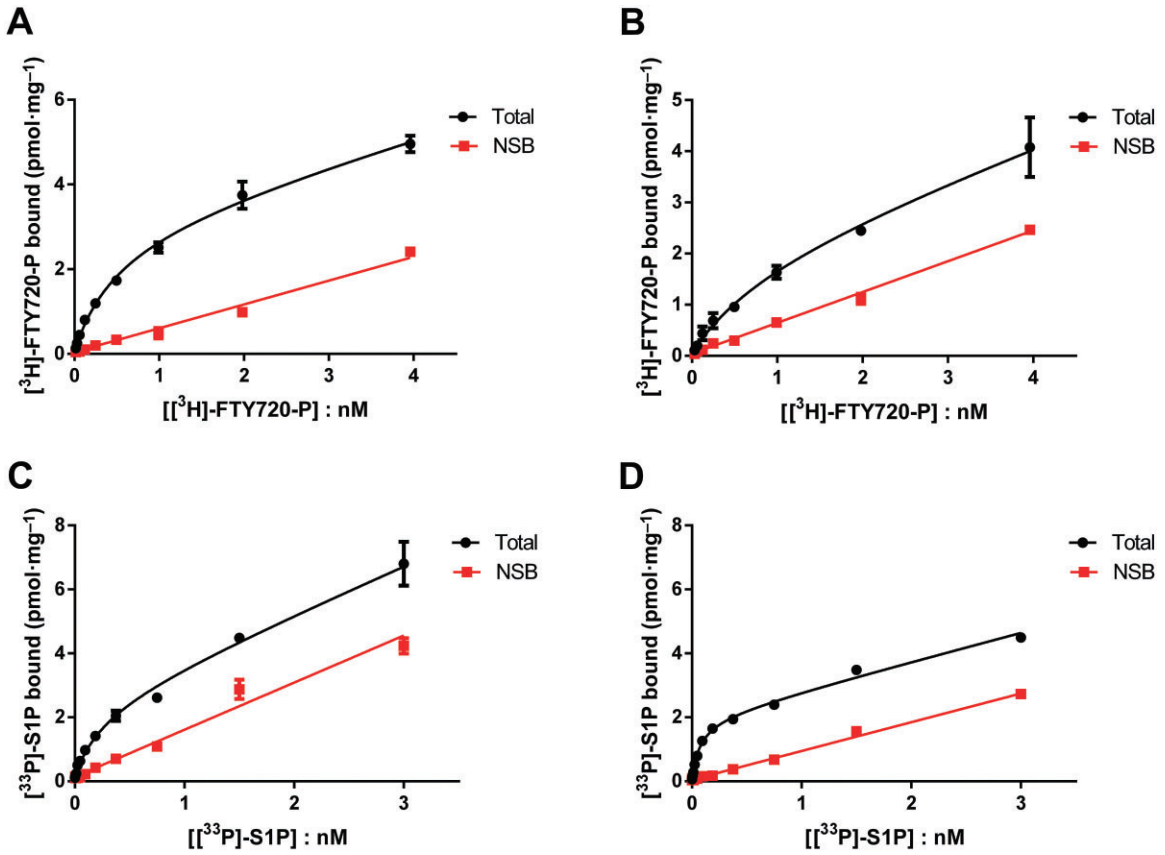

Figure 1

Characterization of [^{3}H]-FTY720-P and [^{33}P]-S1P binding to CHO S1P1 and S1P3Rs. Total and non-specific binding plots for the binding of [^{3}H]-FTY720-P to (A) S1P1 and (B) S1P3Rs and [^{33}P]-S1P to (C) S1P1 and (D) S1P3Rs. Data points shown as pmol·mg^{-1} are the mean ± range of duplicate measurements. Data are from representative experiments repeated three to five times.

Table 1

Saturation binding parameters for [^{3}H]-FTY720-P and [^{33}P]-S1P at S1P1 and S1P3Rs

	S1P1 receptor		S1P3 receptor	
Radioligand	**K_d (nM)**	**B_{max} (pmol·mg^{-1})**	**K_d (nM)**	**B_{max} (pmol·mg^{-1})**
[^{3}H]-FTY720-P	0.283 ± 0.006	2.64 ± 0.26	0.946 ± 0.143	1.62 ± 0.19
[^{33}P]-S1P	0.206 ± 0.028	2.69 ± 0.25	0.067 ± 0.005	1.86 ± 0.03

Data are presented as mean ± SE mean from three to five separate experiments.

(pEC_{50s} of 10.10 ± 0.03 and 9.42 ± 0.02 respectively). Activation of S1P1Rs by S1P resulted in a small but significant difference ($P < 0.05$) in maximal GTPγS incorporation when compared with FTY720-P (4.31 ± 0.03 and 3.90 ± 0.05 respectively). In contrast, maximal GTPγS incorporation in response to both S1P and FTY720-P was not significantly different ($P > 0.05$) at the S1P3R.

β-Arrestin recruitment

GPCR kinase 2 (GRK2)-mediated receptor phosphorylation has been shown to be essential for FTY720-P-induced S1P1R internalization and subsequent receptor degradation (Oo *et al.*, 2007). Phosphorylation leads to the recruitment of β-arrestin to the receptor, which acts as a scaffold for several other proteins required for the internalization process. To investigate the potential differences in β-arrestin recruitment, we have utilized the PathHunter β-arrestin assay that detects recruitment of β-arrestin to the receptor via enzyme complementation (Riddy *et al.*, 2012). Both S1P and FTY720-P recruited β-arrestin in the CHO-S1P1 and CHO-S1P3 cells with S1P being more potent than FTY720-P in both cases, see Figure 4, Table 3. The relative intrinsic activities of the

Table 2

Summary of dissociation rate parameters for [^{3}H]-FTY720-P and [^{33}P]-S1P from S1P1 and S1P3Rs

	S1P1 receptor					
	Rapid phase			Slow phase		
Radioligand	k_{off} (min^{-1})	Dissociation $t_{1/2}$ (min)	% Population 1	k_{off} (min^{-1})	Dissociation $t_{1/2}$ (min)	% Population 2
[^{3}H]-FTY720-P	17.4 ± 3.5	0.04 ± 0.01	78.5 ± 1.5	0.05 ± 0.01	19.0 ± 4.6	21.5 ± 1.5
[^{33}P]-S1P	22.5 ± 2.2	0.03 ± 0.04	79.5 ± 2.1	0.03 ± 0.01	23.9 ± 4.4	20.5 ± 2.1
	S1P3 receptor					
	Rapid phase			Slow phase		
Radioligand	k_{off} (min^{-1})	Dissociation $t_{1/2}$ (min)	% Population 1	k_{off} (min^{-1})	Dissociation $t_{1/2}$ (min)	% Population 2
[^{3}H]-FTY720-P	27.8 ± 3.7	0.026 ± 0.003	79.3 ± 3.5	0.34 ± 0.13[a]	3.1 ± 1.4[a]	20.7 ± 4.7
[^{33}P]-S1P	19.7 ± 2.3	0.037 ± 0.005	76.5 ± 2.2	0.05 ± 0.01	14.0 ± 1.5	23.5 ± 2.2

The dissociation of [^{3}H]-FTY720-P and [^{33}P]-S1P was measured using the procedure described in Figure 2 by allowing ~1 nM of radioligand to reach equilibrium with CHO-S1P1 and S1P3 membranes. Re-association of [^{3}H]-FTY720-P and [^{3}H]-S1P was prevented by the addition of S1P or FTY720-P (0.3 μM final) and GTP (1 mM). Bound radioligand was measured at multiple points post $t = 0$. Normalized data were fitted to mono and bi-exponential dissociation equations, as described in Figure 2, and the best fit was determined using an *F*-test (see *calculations and data analysis*). The analysis provided estimates of the dissociation rates and the proportion (receptor population %) of specific binding that dissociates at these rates. For analysis of % specific bound, the lower plateau was fixed to zero. Data are presented as the mean ± SE mean from three or more experiments. Dissociation rate constants and receptor populations for FTY720-P and S1P at each receptor subtype were compared using paired Student's *t*-tests.
[a] $P < 0.05$.

Table 3

Comparison of S1P and FTY720-P stimulated [^{35}S]GTPγS binding and β-arrestin recruitment at S1P1 and S1P3Rs

	S1P1 receptor				S1P3 receptor			
	[^{35}S]GTPγS binding		β-arrestin binding		[^{35}S]GTPγS binding		β-arrestin binding	
Agonist	pEC_{50}	Maximal response (fold over basal)	pEC_{50}	Maximal response (fold over basal)	pEC_{50}	Maximal response (fold over basal)	pEC_{50}	Maximal response (fold over basal)
S1P	9.42 ± 0.02	4.31 ± 0.03[a]	9.07 ± 0.05	2.97 ± 0.10[a]	10.10 ± 0.03	2.28 ± 0.02	8.48 ± 0.08	9.37 ± 1.09[a]
FTY720-P	9.32 ± 0.02	3.90 ± 0.05	8.44 ± 0.06	3.92 ± 0.17	8.44 ± 0.08	2.14 ± 0.08	7.86 ± 0.10	2.74 ± 0.42

Data are presented as mean ± SE mean from three separate experiments. Maximal responses to S1P and FTY720-P at each receptor subtype were compared using paired Student's *t*-tests.
[a] $P < 0.05$.

compounds were strikingly different at the two receptors. At S1P1, FTY720-P stimulated a higher maximal level of β-arrestin recruitment than S1P, being 132% of that to S1P, see Figure 4A. In contrast, FTY720-P was a low partial agonist at S1P3Rs, stimulating just 29% of the total β-arrestin recruited by S1P, see Figure 4B.

Metabolism of FTY720-P and S1P by CHO cells

S1P is known to be metabolically unstable *in vitro* in the presence of biological membranes (Bradley *et al.*, 2011); however, less is known about the relative stability of FTY720-P. The levels of S1P and FTY720-P remaining after incubation with CHO cell membranes were tested using S1P3-mediated calcium signalling as a bioassay (described in detail by Bradley *et al.*, 2011), data are shown in Figure 5A, B. Freshly prepared S1P had a pEC_{50} of 9.09 ± 0.04 at the S1P3 receptor expressed in CHO cells. In contrast, S1P that had been pre-incubated with CHO cell membranes for 3 h had a reduced potency of 7.43 ± 0.03, indicating that the concentration of S1P was reduced by approximately 30-fold, see Figure 5A. The addition of sodium orthovanadate during the

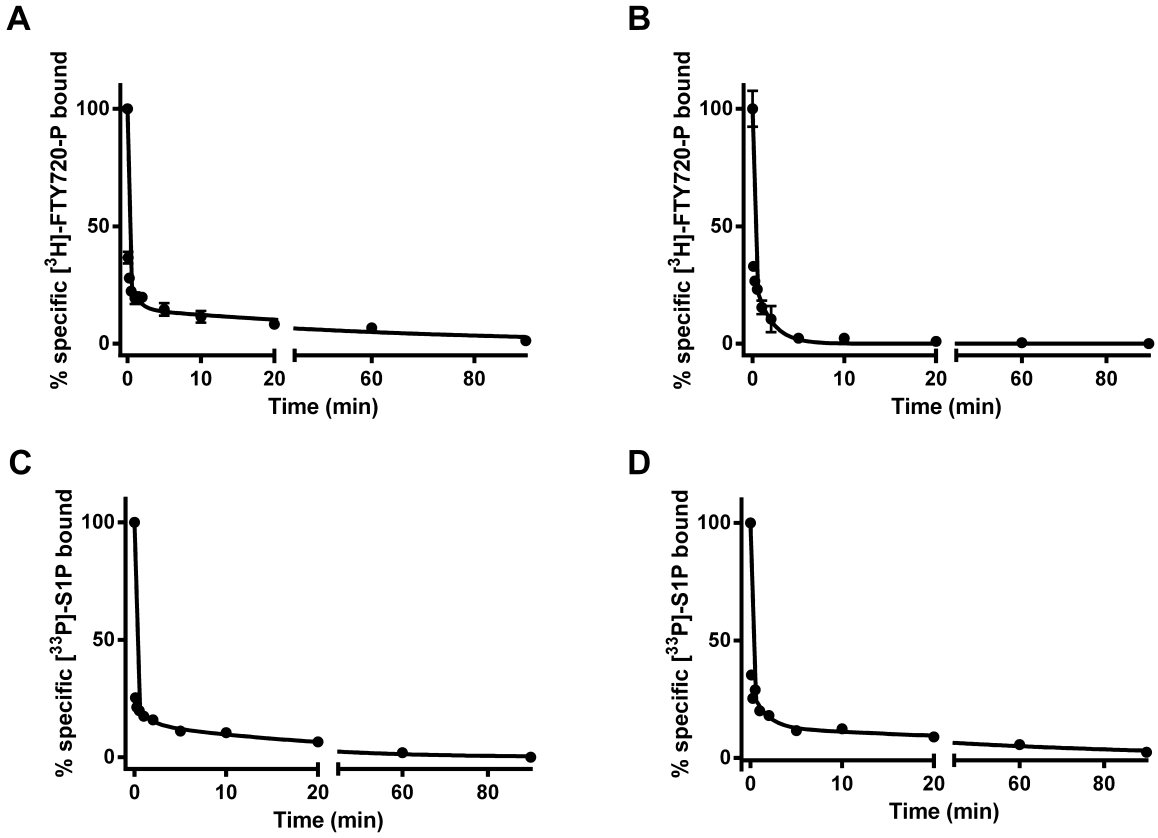

Figure 2

Dissociation of [^{3}H]-FTY720-P and [^{33}P]-S1P from CHO S1P1 and S1P3Rs. (A) [^{3}H]-FTY720-P dissociation in the presence of S1P (300 nM) and GTP (1 mM) from S1P1Rs. (B) [^{3}H]-FTY720-P dissociation in the presence of S1P (300 nM) and GTP (1 mM) from S1P3Rs. (C) [^{33}P]-S1P dissociation in the presence of FTY720-P (300 nM) and GTP (1 mM) from S1P1Rs. (D) [^{33}P]-S1P dissociation in the presence of FTY720-P (300 nM) and GTP (1 mM) from S1P3Rs. Radioligand concentration was typically ~1 nM. Dissociation data obtained in the presence of GTP were fitted to a bi-exponential which provided a better fit to the data ($P < 0.05$) than a single-rate function in all cases (F-test). For normalization of presented data, specific binding at time 0 was calculated by subtracting non-specific binding (300 nM S1P or FTY720-P plus GTP 1 mM included in the equilibration reaction) from total binding (harvested without the addition of unlabeled ligand, following the equilibration reaction). Values are shown as % specific binding. Data are from representative experiments that were repeated three or more times. Data points are the mean ± SE mean of triplicate measurements, and in some cases error bars are enclosed by the symbols. Parameter estimates from these experiments are summarized in Table 2.

pre-incubation stage prevented the degradation of S1P by CHO membranes (data not shown), suggesting the metabolism is via a phosphatase enzyme. Unlike S1P, the potency of FTY720-P was not affected by pre-incubation with CHO cell membranes, with pEC_{50}s of 8.26 ± 0.03 with freshly prepared compound and 8.22 ± 0.05 for pre-incubated material, see Figure 5B. This suggests that FTY720-P is metabolically stable in the presence of biological membrane while S1P is rapidly degraded.

Receptor internalization

S1P and FTY720-P-induced receptor internalization and subsequent degradation of S1P1Rs have been studied previously (Oo *et al.*, 2007); however, a comparison of the relative levels of FTY720-P-induced S1P1 and S1P3Rs internalization has not been examined in the same study. FTY720-P-induced internalization of S1P1 and S1P3Rs was determined using fluorescent antibody staining with flow cytometry following a 60 min incubation period. FTY720-P produced a persistent internalization of the S1P1R in a dose-dependent manner; the pEC_{50} values for internalization of S1P1 and S1P3R by FTY720-P were 9.15 ± 0.10 and 8.42 ± 0.26, respectively, as shown in Figure 6A, B. This difference in potency is likely to be related to the lower receptor affinity, combined with the reduced potency and efficacy of FTY720-P at recruiting β-arrestin to the S1P3R compared with the S1P1R. This persistent internalization was only partially reversed when the cells were washed to remove the exogenously applied FTY720-P. When applied to CHO-S1P3 cells, FTY720-P produced internalization of the S1P3R that was more easily reversed by washout than that seen for the S1P1R, suggesting that although FTY720-P is able to promote receptor internalization, it is less able to prevent S1P3R recycling to the cell surface, as shown in Figure 6B.

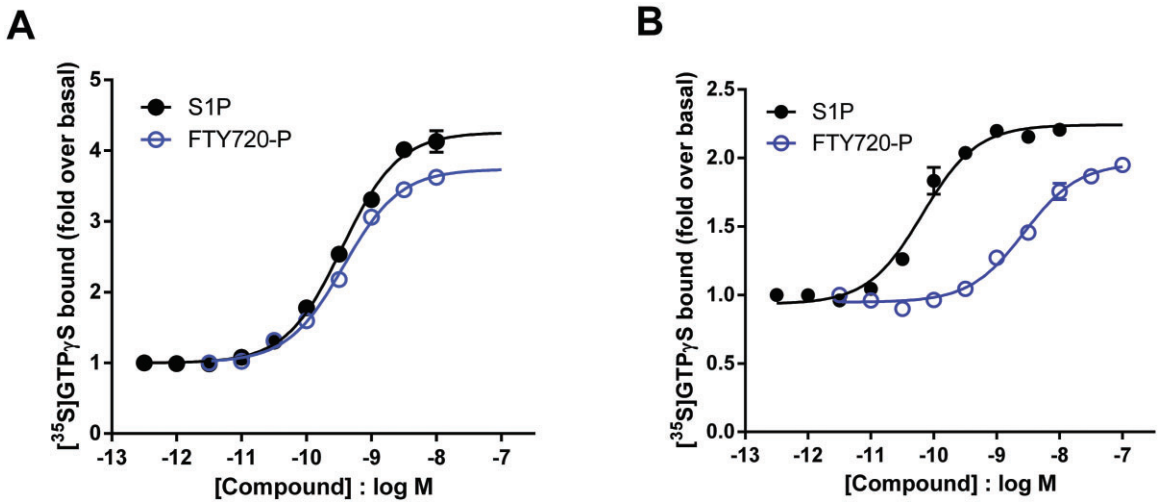

Figure 3

Effect of S1P and FTY720-P on [^{35}S]-GTPγS binding to G proteins in (A) CHO-S1P1 and (B) CHO-S1P3 cell membranes. [^{35}S]-GTPγS binding assays performed as described in the methods data points are the means of duplicate determinations of a single representative experiment. Parameter estimates from three separate experiments are summarized in Table 3.

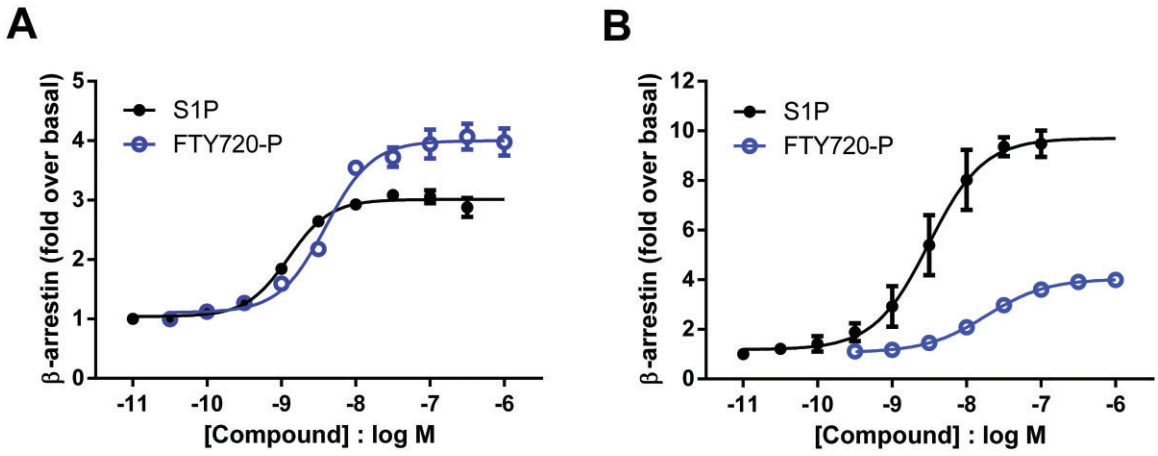

Figure 4

Effect of S1P and FTY720-P on β-arrestin recruitment in (A) CHO-S1P1 and (B) CHO-S1P3 cells. β-arrestin assays performed as described in the methods data points are the means of quadruplicate determinations of a single representative experiment. Parameter estimates from three separate experiments are summarized in Table 3.

Discussion and conclusions

The aim of this study was to investigate the mechanism of persistent internalization of FTY720-P at S1P1Rs. The persistency of receptor internalization following a short exposure to FTY720-P represents a unique property of this agonist/receptor pair (Jo *et al.*, 2005; Oo *et al.*, 2007) and does not occur in the case of FTY720-P binding to S1P3Rs (Sensken *et al.*, 2008) or S1P binding to S1P receptor subtypes (Oo *et al.*, 2007). This persistent S1P1R internalization upon ligand removal is partly due to reduced recycling of the receptor back to the plasma membrane, a feature of FTY720-P-treated cells not shared by S1P itself (Jo *et al.*, 2005; LaMontagne *et al.*, 2006). As a consequence of persistent internalization, FTY720-P effectively renders the cells unresponsive to any subsequent application of S1P, a factor important in its overall mechanism of action. For the first time in a single study, we demonstrate the potential for slow receptor dissociation and efficacious β-arrestin recruitment in combination with high metabolic stability as contributing to FTY720-P's ability to promote prolonged selective S1P1R internalization. This study highlights the value of investigating drug-receptor binding kinetics and exploring both G protein-dependent and independent signalling pathways when selecting compounds to take forward into development.

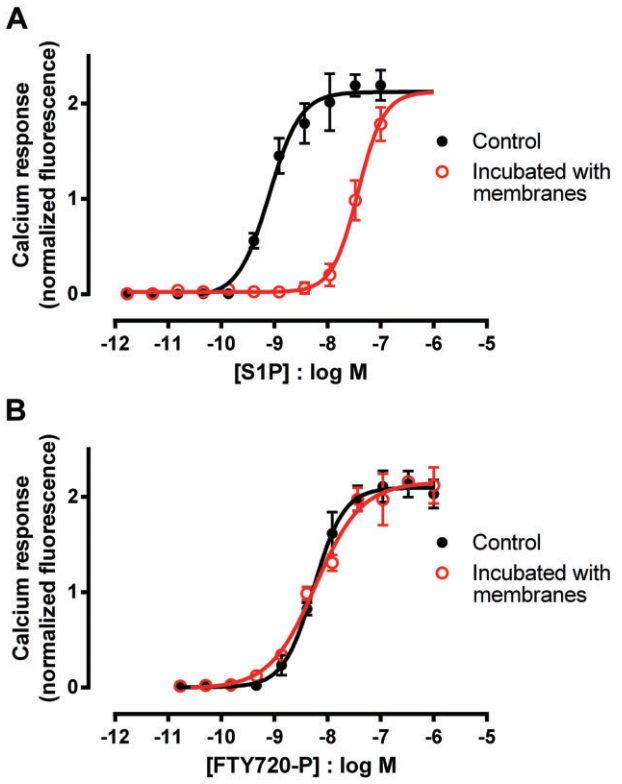

Figure 5

Metabolism of S1P by CHO membranes. CHO-S1P3 cell calcium responses following incubation of (A) S1P with S1P3 membranes. S1P is readily degraded following a 3 h incubation with membranes resulting in a reduction in potency. In contrast, (B) FTY720 incubated with S1P3 membranes has no effect on the potency of FTY720-P demonstrating its inherent stability. Data points are the means of triplicate determinations of a single representative experiment.

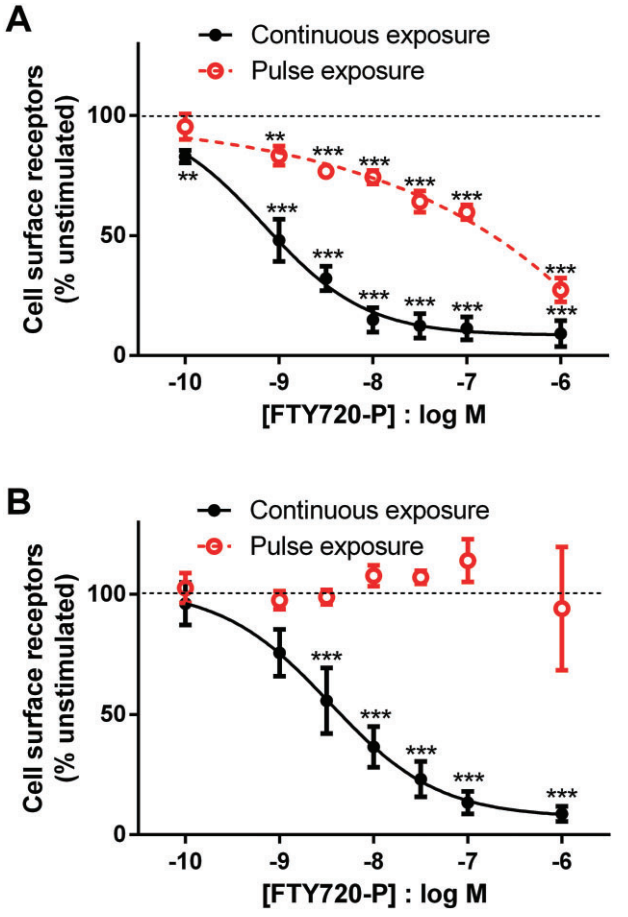

Figure 6

Internalization of (A) CHO-S1P1 and (B) CHO-S1P3 cells in response to FTY720-P following a 60 min incubation period and recovery of cell surface receptors following a subsequent 3 h washout. Internalization assays were performed as described in the methods, data points are the means of at least three determinations from multiple experiments. Statistical analysis by two-way ANOVA and Dunnett's post-test versus unstimulated control. Significance: $^{***}P < 0.001$, $^{**}P < 0.01$ within each group is displayed.

Initially, we postulated that differences in the dissociation rate of FTY720-P from S1P1 and S1P3Rs could play a role in the persistent internalization of FTY720-P at S1P1Rs. To determine the dissociation kinetics of FTY720-P and S1P, we used a dilution method in the presence of an excess of competitor and high (mM) GTP concentrations to recreate the intracellular environment.

The addition of GTP caused a rapid dissociation of FTY720-P and S1P from approximately 80% of the S1P1 and S1P3R sites (population 1), with the remaining 20% of receptor sites seemingly resistant to the effect of GTP (population 2), with dissociation of both ligands being significantly slower at this form of the receptor. It is possible that these G protein-independent receptor sites are stabilized by interaction with an alternative binding partner, such as β-arrestin. Support for this comes from binding studies demonstrating that overexpression of β-arrestin readily increases agonist affinities (Gurevich *et al.*, 1997). Interestingly, dissociation of FTY720-P from population 2 of the S1P1R was six times slower compared with the $S1P_3$ receptor (19.0 vs. 3.1 min), suggesting FTY720-P has a higher affinity for the G protein-independent form of the S1P1R than the S1P3R consistent with FTY720-P being more effective at recruiting arrestin via S1P1R than S1P3Rs. This may explain why persistent abrogation of S1P3 signalling is not observed following treatment of cells with FTY720-P since the majority of FTY720-P will dissociate before a significant proportion of receptors are internalized into the cell. As a consequence, S1P3R trafficking is not delayed by FTY720-P as the agonist-free receptors are readily recycled from vesicular compartments to the cell surface. The slower dissociation of FTY720-P from the S1P1R may result in FTY720-P remaining bound to the receptor long enough for it to be trapped within the internalized receptor vesicle, being available to rebind receptor. This persistent occupancy by the agonist may maintain an active, phosphorylated and arrestin-bound conformation, preventing receptor recycling. It has been shown in a previous study that FTY720-P does not readily accumulate inside the cell, remaining almost exclusively in the supernatant of the cells to which it has been applied (Sensken *et al.*, 2009), so endocytosis of receptor-bound FTY720-P may be the only route by

which the ligand can access this intracellular receptor compartment. Thus, receptor kinetics may contribute to the observation that FTY720 preferentially internalizes S1P1 over S1P3Rs, but this does not explain why only FTY720-P and not S1P causes persistent internalization of S1P1Rs, as the kinetics of FTY720-P and S1P are very similar at the S1P1R.

We have previously shown that S1P is rapidly metabolized by membrane preparations from CHO cells (Bradley *et al.*, 2011), meaning that even if S1P internalizes with the receptor it is likely to be degraded rapidly within the sorting vesicles, allowing receptor recycling. If FTY720-P proved to be more stable, it would be retained in the vesicles for longer periods at concentrations sufficiently high to allow for repeated dissociation-association cycles of the ligand receptor pair. To investigate this, we measured the relative stability of both ligands in our CHO cell system. As receptors are internalized, they are engulfed by plasma membrane and the external face is not exposed to the cytosol of the cell. As several enzymes present in the cytosol degrade S1P, we chose to investigate the stability of the ligands in membrane preparations rather than whole cells as this represents a more relevant environment.

Using a method previously described by Bradley *et al.* (2011), we have shown that after 3 h, S1P concentrations are reduced by approximately 30-fold in this preparation. FTY720-P levels, in contrast, were stable over the 3 h time period, suggesting it is far more resistant to metabolism. This is entirely consistent with previous studies in HEK293 cells overexpressing sphingosine phosphate lyase where it was shown that FTY720-P, unlike S1P, is not degraded by S1P lyases and therefore has a comparatively high metabolic stability (Bandhuvula *et al.*, 2005). These studies highlight the importance of studying compound stability during long-term incubations.

In addition to receptor kinetics, we examined whether differences in intrinsic efficacy measured at the level of GTPγS accumulation could play a role in the differential internalization properties of the two ligands. The [^{35}S]-GTPγS binding assay measures the level of G protein activation or more precisely guanine nucleotide exchange following agonist occupation of a GPCR. The advantage of this assay format is that it measures a functional consequence of receptor occupancy at one of the earliest receptor-mediated events, with the advantage that agonist measures are not subjected to a large degree of amplification or modulation that may occur when analysing parameters further downstream of the receptor. There was no difference in the potency of S1P and FTY720-P at the $S1P_1$ receptors; however, there was a small but significant difference in the intrinsic activity of S1P and FTY720-P, with S1P displaying a higher intrinsic activity. At the S1P3R, however, FTY720-P was more than 30-fold less potent than S1P, despite the fact that they have very similar affinities for the receptor. This could potentially be a consequence of a lower intrinsic efficacy for FTY720-P, which may contribute to the reduced tendency to internalize receptors.

In order to further explore the influence of downstream efficacy on persistent internalization, we examined the ability of the two ligands to stimulate β-arrestin recruitment to the receptors. β-arrestin plays an important role in the initiation of receptor internalization and has also been shown to possess signalling properties in its own right (Shenoy and Lefkowitz, 2011). Previous research has suggested that FTY720-P selectively activates only G_i protein-mediated receptor signalling through the S1P3R without initiating β-arrestin recruitment and receptor internalization (Sensken *et al.*, 2008; Wetter *et al.*, 2009). S1P, on the other hand, activates both G_i and G_q signalling and initiates receptor internalization (Sensken *et al.*, 2008). We have shown that both FTY720-P and S1P are able to induce recruitment of β-arrestin to S1P1 and S1P3Rs, but that the overall magnitude of this response is very different. At the S1P1R, FTY720-P is able to stimulate a higher level of β-arrestin recruitment than S1P, whereas at the S1P3R, FTY720-P is a low partial agonist when compared with S1P, maximally recruiting only 29% of the total levels of β-arrestin achieved by S1P. These differences in efficacy of β-arrestin recruitment may contribute to the improved ability of FTY720-P to internalize the S1P1R over the S1P3R. Importantly, these differences suggest that if FTY720-P were to bind the S1P3R, its lower efficacy at this receptor will mean the functional consequences of binding will be reduced. Interestingly, the reversal in intrinsic activity of S1P and FTY720-P for GTPγS binding and β-arrestin recruitment suggests that FTY720-P has the potential to behave as a biased agonist at the S1P1R, favouring β-arrestin recruitment and therefore receptor internalization over G protein-mediated signalling.

We have speculated that a longer residency time of FTY720-P at the $S1P_1$ receptor leads to trapping of the ligand in intracellular vesicles as the receptor is internalized. This ligand is then free to bind and rebind to the receptors inside the cell, preventing recycling irrespective of the concentration of drug outside the cell. We therefore tested the effect of washing extracellular ligand away once receptor internalization had been induced in order to examine the relative degree of reversibility at the S1P1 and S1P3Rs. FTY720-P was effective at internalizing both S1P1 and S1P3Rs to a similar magnitude and potency. After washing free ligand from the extracellular environment, S1P3R expression at the cell surface was fully recovered. In contrast, the surface expression of the S1P1R was not fully recovered, even after 3 h post washing, supporting the notion that that FTY720-P is trapped within the vesicle, preventing recycling.

In conclusion, we suggest that multiple factors are involved in the persistent internalization of the S1P1R by FTY720-P. Firstly, FTY720-P activation of the S1P1R results in a strong recruitment of β-arrestin and rapid receptor internalization. FTY720-P remains bound to the S1P1R due to its slow off-rate from the receptor ($t½$ of 19.0 min) and is therefore readily internalized with the receptor. Once internalized, the metabolically stable ligand is free to bind and rebind internalized receptor, maintaining β-arrestin recruitment and preventing receptor recycling. Although FTY720-P is able to effectively internalize the S1P3R following a prolonged treatment with FTY720-P, its rapid ($t½$ of 3.1 min) dissociation rate from this uncoupled form of the receptor means that there is less chance that FTY720-P will be internalized with the receptor. Indeed, this rapid off-rate from the S1P3R may be in part responsible for the significantly lower levels of β-arrestin recruited following FTY720-P-stimulated S1P3R activation. As a consequence, less FTY720-P becomes trapped in the internalized vesicles and receptors are readily reinserted at the cell surface following washing of the cells. S1P has a long residency time at both receptors ($t½$ of 23.9 and

14.0 min respectively), so is likely to be internalized with the receptors in both cases. However, due to its inherent metabolic instability, S1P is readily degraded in this environment, meaning that β-arrestin recruitment will be shorter lived, allowing the internalized receptors to recycle to the cell surface. Thus, the reduced metabolic stability of S1P is likely to be the most important distinguishing feature between FTY720-P and S1P acting on S1P1Rs.

This specific, prolonged internalization achieved with FTY720-P at the S1P1R likely plays an important role in its clinical efficacy. These data suggest that the future development of compounds with strong β-arrestin recruitment, slow receptor dissociation rate and high metabolic stability within intracellular vesicles could potentially result in new drugs that persistently internalize receptors to achieve clinical efficacy.

Conflict of interest

None.

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