

S1P₁ Modulator-Induced G_{αi} Signaling and β-Arrestin Recruitment Are Both Necessary to Induce Rapid and Efficient Reduction of Blood Lymphocyte Count In Vivo[§]

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ABSTRACT

S1P₁ (sphingosine-1-phosphate receptor 1) agonists prevent lymphocyte egress from secondary lymphoid organs and cause a reduction in the number of circulating blood lymphocytes. We hypothesized that S1P₁ receptor modulators with pathway-selective signaling properties could help to further elucidate the molecular mechanisms involved in lymphocyte trapping. A proprietary S1P₁ receptor modulator library was screened for compounds with clear potency differences in β-arrestin recruitment and G protein α i subunit (G_{αi}) protein-mediated signaling. We describe here the structure-activity relationships of highly potent S1P₁ modulators with apparent pathway selectivity for β-arrestin recruitment. The most differentiated compound, D3-2, displayed a 180-fold higher potency in the β-arrestin recruitment assay (EC₅₀

0.9 nM) compared with the G_{αi}-activation assay (167 nM), whereas ponesimod, a S1P₁ modulator that is currently in advanced clinical development in multiple sclerosis, was equipotent in both assays (EC₅₀ 1.5 and 1.1 nM, respectively). Using these novel compounds as pharmacological tools, we showed that although a high potency in β-arrestin recruitment is required to fully internalize S1P₁ receptors, the potency in inducing G_{αi} signaling determines the rate of receptor internalization in vitro. In contrast to ponesimod, the compound D3-2 did not reduce the number of circulating lymphocytes in rats despite high plasma exposures. Thus, for rapid and maximal S1P₁ receptor internalization a high potency in both G_{αi} signaling and β-arrestin recruitment is mandatory and this translates into efficient reduction of the number of circulating lymphocytes in vivo.

Introduction

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that causes pleiotropic cellular effects, including cell proliferation, migration, and survival, by acting as an intracellular or extracellular mediator. Extracellular S1P signaling is mediated by five different S1P receptor subtypes, named S1P₁ through S1P₅ receptors, which show differential expression across a variety of tissues and cell types. Synthetic S1P₁ receptor modulators, such as the nonselective S1P_{1,2,3,4,5} receptor modulator fingolimod (FTY720/Gilenya) or the selective S1P₁ receptor modulator ponesimod block lymphocyte egress, leading to rapid and reversible lymphopenia, a property that is used as a mechanistic basis to treat autoimmune conditions

(Hla and Brinkmann, 2011; Piali et al., 2011). Lymphocyte egress from secondary lymphoid organs into lymph is dependent on their expression of S1P₁ receptor that allows them to chemotax along the S1P gradient (Allende et al., 2004; Matloubian et al., 2004), which is formed by high S1P concentrations in blood and lymph and generally low concentrations in tissues (Spiegel and Milstien, 2003). Even though synthetic compounds activate S1P₁ receptors, it has been suggested that they act in fact as functional antagonists, causing rapid and sustained receptor internalization and degradation, thus abolishing the lymphocytes' ability to sense and migrate along the S1P gradient (Hla and Brinkmann, 2011; Gatfield et al., 2014). This hypothesis is supported by the fact that S1P₁ antagonists are also able to induce lymphopenia in vivo (Tarrasón et al., 2011; Quancard et al., 2012). However, it was recently reported that the synthetic agonist BMS-986104 was able to induce lymphopenia without showing complete internalization of S1P₁ receptors (Dhar et al., 2016) and a transgenic mouse, expressing internalization-defective S1P₁ receptors, still showed FTY720-induced lymphopenia, albeit with delayed kinetics (Thangada et al., 2010). Previous reports had suggested S1P₁ agonism to be a mechanism responsible for lymphocyte egress inhibition induced by synthetic agonists

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ABBREVIATIONS: DMSO, dimethylsulfoxide; fafBSA, fatty acid-free bovine serum albumin; FTY720, fingolimod; G_{αi}, G protein α i subunit; HMVEC-LLy, human lung lymphatic microvascular endothelial cells; SEW2871, 5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole; S1P, sphingosine-1-phosphate.

and S1P₁ agonism could in fact disrupt the S1P gradient, thus blinding the lymphocytes to the exit. The fact that the blockade of S1P lyase activity, the enzyme responsible for S1P gradient formation, leads to lymphocyte sequestration (Weiler et al., 2014) supports this hypothesis. Alternatively, synthetic compounds could activate S1P₁ receptors on lymphatic endothelial cells, leading to the tightening of cell-cell junctions and thus blocking lymphocyte exit. It has been shown that S1P₁ agonist 5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)-phenyl]-1,2,4-oxadiazole (SEW2871), which causes transient S1P₁ internalization and recycling, reduced T cell egress in lymph node explants within 5 minutes, suggesting it closed normally open stromal gates (Sanna et al., 2006). Therefore, the individual contribution of functional antagonism or agonism leading to synthetic S1P₁ receptor modulator-induced lymphopenia is far from being fully understood.

Recently, it was shown that chaperone-dependent presentation of the natural ligand S1P shows differences in S1P₁ receptor engagement and signal transduction, leading to distinct physiologic responses. In particular, high-density lipoprotein-S1P was shown to induce association of S1P₁ receptors with β -arrestin 2, which has an anti-inflammatory activity, whereas albumin-S1P led to suppression of forskolin-induced cAMP but did not induce formation of S1P₁ receptor/ β -arrestin complexes (Galvani et al., 2015). The attractive concept of biased agonism, which posits that ligands can stabilize different G protein-coupled receptor conformation states to induce individual intracellular signaling pathways (Luttrell et al., 2015), may offer a theoretical framework for the observed effects. In addition, considering that the natural ligand, depending on the way it is presented to the S1P₁ receptor, displays biased agonism and induces different pharmacological behaviors, suggested that similar properties might be discovered in synthetic S1P₁ receptor modulators. Such compounds could serve as valuable tools to further investigate S1P₁ receptor modulator-induced pharmacology at a signaling pathway level.

Here, we describe the identification and structure-activity relationship of compounds with novel and unique S1P₁ receptor signaling characteristics. We established sensitive *in vitro* assays to quantify kinetic responses that are induced upon S1P₁ receptor activation and demonstrated that these novel compounds show an apparent deficiency in G protein α i subunit ($G_{\alpha i}$) signaling, while retaining β -arrestin recruitment with high potency and efficacy, leading to effective S1P₁ receptor internalization and degradation. Using cellular assays in recombinant and primary human lymphatic endothelial cells, we determined that $G_{\alpha i}$ signaling influenced the rate of S1P₁ receptor internalization, whereas β -arrestin recruitment was necessary for effective internalization. *In vivo*, these novel S1P₁ receptor modulators displayed a delayed onset lymphopenia, a striking pharmacokinetic/pharmacodynamics disconnect, and surprisingly they were no longer able to induce fully efficient lymphopenia. These results suggest that both $G_{\alpha i}$ protein signaling and β -arrestin recruitment are necessary to induce rapid S1P₁ receptor internalization, leading to efficient lymphocyte reduction by S1P₁ modulators *in vivo*.

Materials and Methods

Materials. S1P was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Ponesimod [(Z,Z)-5-[3-chloro-4-(2R)-2,3-dihydroxy-propoxy]-benzylidene]-2-propylimino-3-o-tolylthiazolidin-4-one] was synthesized by Actelion Pharmaceuticals Ltd (Allschwil, Switzerland). SEW2871 was purchased from Cayman Chemical. All other S1P₁

receptor modulators described herein were synthesized by Actelion Pharmaceuticals Ltd. Human lung lymphatic microvascular endothelial cells (HMVEC-LLy) were purchased from Lonza (Verviers, Belgium). Tango EDG1-*bla* U2OS cells were obtained from Invitrogen (Carlsbad, CA).

EPIC Assay. In this study, 10,000 Chinese hamster ovary cells stably expressing hS1P₁ receptor were seeded in 384-well EPIC sensor microplates (Corning; Corning, NY) and grown for 24 hours in Ham's F12 medium supplemented with 10% charcoal-treated fetal bovine serum, 300 μ g/ml geneticin, 100 U penicillin, and 1 μ g/ml streptomycin. The medium was removed, the wells were filled with 40 μ l assay buffer [Hanks' balanced salt solution with 20 mM Hepes, 0.05% fatty acid-free bovine serum albumin (fattyBSA) and 2% dimethylsulfoxide (DMSO)], and the plate was loaded in the EPIC reader (Corning) for 2-hour equilibration. After the baseline dynamic mass redistribution measurement, 10 μ l of test compounds in assay buffer were added and real-time dynamic mass redistribution changes were recorded for 30 minutes. The end data from EPIC Analyzer software (Corning) were exported to Microsoft Excel and used to determine compound potency with the IC₅₀ Witch software (Actelion Pharmaceuticals Ltd.) using the following settings: minimum defined by the vehicle control (DMSO), maximum defined by the maximal response of the compound (smart max), no weighting, and variable slope.

β -Arresting Recruitment Assay. In this study, 5000 Tango EDG1-*bla* U2OS cells were plated in 30 μ l FreeStyle Expression Medium (Life Technologies, Carlsbad, CA) in black, clear-bottom 384-well plates and stimulated with 10 μ l of test compounds for 16 hours at 37°C in a humidified CO₂ incubator. Cells were then loaded with LiveBLazer-FRET B/G substrate (Life Technologies) for 2 hours at room temperature in the dark and the fluorescence emissions at 460 and 530 nm were measured with SynergyMX reader (BioTek, Winooski, VT) using 409 nm excitation filter. Following background subtraction from both channels, the 460/530 nm emission ratio for each well was calculated and used to determine compound potency with the IC₅₀ Witch software (Actelion Pharmaceuticals Ltd.) using the following settings: minimum defined by the vehicle control (DMSO), maximum defined by the maximal response of the compound (smart max), no weighting, and variable slope.

SNAP-S1P₁ Receptor Internalization Assay. In this study, 30,000 HeLa cells stably expressing hS1P₁ receptor with the N-terminal SNAP-tag (HeLa-SNAP-hS1P₁) were seeded in gelatin-coated, black, clear-bottom 384-well plates and grown for 24 hours in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mg/ml geneticin, 100 U penicillin, and 1 μ g/ml streptomycin. The medium was changed to 60 μ l starvation medium (RPMI 1640 with 0.1% fattyBSA, 100 U penicillin, and 1 μ g/ml streptomycin) and incubated at 37°C in a humidified CO₂ incubator before addition of 20 μ l of test compounds. After incubation with compounds for the indicated times, the medium was removed and the SNAP-tagged surface S1P₁ receptors labeled with SNAP-surface Alexa Fluor 488 substrate (1 μ M; New England Biolabs, Ipswich, MA) in starvation medium for 20 minutes at 37°C. The cells were washed with ice-cold starvation medium and incubated for 30 minutes at 4°C in the dark. The medium was changed again to 20 μ l ice-cold starvation medium, and the fluorescence of the SNAP-labeled surface S1P₁ receptors was measured at 506 nm excitation and 526 nm emission with SynergyMX reader (BioTek). The data were used either to calculate the percentage of S1P₁ receptor internalization or to determine compounds' potency at 16 hours with the IC₅₀ Witch software (Actelion Pharmaceuticals Ltd.) using the following settings: maximum defined by the vehicle controls (DMSO, 0% internalization), minimum defined by the negative controls (empty HeLa cells, 100% internalization), no weighting, and variable slope.

Immunofluorescence Microscopy. In this study, 50,000 HMVEC-LLy were seeded into gelatin-coated eight-chamber slides (BD Falcon, Bedford, MA) and grown for 48 hours in EGM-2MV medium (Lonza). After serum starvation for 4 hours, the test compounds were added and incubated for the indicated times. Cells

were fixed (4% paraformaldehyde), permeabilized with 0.1% Triton X-100, and stained in phosphate-buffered saline, 1% bovine serum albumin using rabbit anti-hS1P₁ receptor antibodies (Santa Cruz Biotechnology, Dallas, TX) and Alexa Fluor 546 goat anti-rabbit antibodies (Life Technologies). Nuclei were stained with Hoechst 33342 (Life Technologies) and F-actin with Alexa Fluor 488 phalloidin (Life Technologies). Images were acquired with the Axiovert 200 fluorescence microscope (Zeiss, Jena, Germany).

ERK and Akt Activation Assay. In this study, 10,000 HMVEC-Ly were seeded in gelatin-coated 12-well plates and grown for 48 hours in EGM-2MV medium (Lonza). After serum starvation for 4 hours, the test compounds were added and incubated for the indicated times. Cells were washed with ice-cold phosphate-buffered saline and lysed in radioimmunoprecipitation assay lysis buffer (Sigma, St. Louis, MI) supplemented with 10 mM NaF, 4 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, and 100 U/ml benzamide. Equal protein amounts were separated on 4%–12% Bis-Tris polyacrylamide gel (Life Technologies) and transferred to nitrocellulose membranes for immunoblotting. Phosphorylated ERK1/2, total ERK1/2, phosphorylated Akt, and total Akt were detected with rabbit anti-phospho-p44/42 mitogen-activated protein kinase (Cell Signaling Technology, Danvers, MA), anti-mitogen-activated protein kinase 1/2 (Merck Millipore, Temecula, CA), anti-phospho-Akt (Ser473) (Cell Signaling Technology), and anti-Akt (Cell Signaling Technology) antibodies, respectively. As a secondary reagent, horseradish peroxidase-linked donkey anti-rabbit antibodies (Amersham Biosciences, Little Chalfont, UK) were used. The Plus-ECL chemiluminescence detection substrate (Perkin Elmer, Groningen, The Netherlands) was used for detection. Comparative densitometry of immunoblots was performed on LAS400 imager (Fujifilm) using the Multi Gauge software (Fujifilm, Dusseldorf, Germany).

HMVEC-Ly Chemotaxis Assay. HMVEC-Ly specimens were serum starved for 2 hours and then labeled with Calcein-AM (5 μ g/ml; Life Technologies) for 30 minutes. The labeled cells were washed and resuspended in EBM-2 medium (Lonza) supplemented with 0.1% fBSA (Sigma) at 400,000 cells/ml. 50 μ l cell suspensions were added to the top chamber of 96-well 8 μ m FluoroBlok inserts (Corning) and combined with the bottom chambers containing 220 μ l test compounds in EBM-2 medium supplemented with 0.1% fBSA. The plate was placed at 37°C in a humidified CO₂ incubator and the fluorescence of the migrated cells in the lower wells was measured after a given time (45 minutes and 2, 3, and 4 hours) at 485 nm excitation and 530 nm emission on a Cytofluor 4000 (Perseptive Biosystems, Framingham, MA).

Measurement of Lymphocyte Count. Lymphocyte count was performed in male Wistar rats weighing 294–510 g (Harlan Laboratories Inc., Itingen, Switzerland). The rats were housed in climate-controlled conditions with a 12-hour light/dark cycle with free access to normal pelleted chow (Provimi Kliba SA, Kaiseraugst, Switzerland) and water ad libitum. The rats were used for blood sampling after an acclimatization period of at least 7 days. All rats were housed in Makrolon cages (Indulab, Gams, Switzerland) with wire mesh tops and standardized softwood bedding and appropriate environmental enrichment. The compounds were first dissolved in 100% DMSO and then diluted to the appropriate concentration in 7.5% gelatin; solutions were protected from light. The final concentration of DMSO in the formulation was 5%. Peripheral blood lymphocyte count was assessed by hematology following single oral (gavage) dose of 10 mg/kg compound (n = 6/group) or vehicle (n = 6/group). Blood samples from rats were collected before and at 3, 6, and 24 hours after oral administration of compound or its vehicle by sublingual puncture. Blood (0.5 ml) was drawn into tubes containing 5% EDTA. For the determination of blood lymphocyte count, undiluted blood was analyzed using a 5diffCP Hematology Analyzer (Beckman Coulter, Brea, CA).

Measurement of Compound Concentrations in Plasma. Plasma was separated by centrifugation and stored at –20°C. Compound concentrations in plasma samples were determined using liquid chromatography coupled to mass spectrometry.

Ethics Statement. All animal experimental procedures were conducted in accordance with the Swiss animal welfare ordinance and Actelion Animal Welfare policy on the use of experimental animals. The studies were approved by the Baselland Cantonal Veterinary Home Office.

Statistical Analysis. For statistical analysis, one-way analysis of variance with Dunnett's post hoc test was performed. When the P value was <0.05, the results were considered significant (GraphPad Prism 7 Software; GraphPad, San Diego, CA).

Results

Synthetic S1P₁ Receptor Agonists with Different Potencies in G_{αi} Signaling and β -Arrestin Recruitment. To reveal potential differences in synthetic S1P₁ receptor agonist-induced signaling and to explore the possible consequences on lymphocyte trafficking in vivo, we first established sensitive in vitro assays to quantify kinetic responses of G_{αi} protein-mediated and β -arrestin-mediated S1P₁ receptor signaling. A label-free resonant waveguide grating assay (EPIC) was developed to measure in real-time G_{αi} protein-mediated responses in recombinant Chinese hamster ovary hS1P₁ cells (Nayler et al., 2010). The S1P-induced EPIC signal increased rapidly and peaked 6–12 minutes after ligand addition. The signal subsequently decreased, and for lower S1P concentrations reached baseline within 25 minutes after addition (Fig. 1A). The S1P-induced EPIC response was efficiently blocked with the G_{αi} protein inhibitor pertussis toxin or the selective S1P₁ receptor antagonist TASP0277308 (Fujii et al., 2012) (Supplemental Fig. 1), demonstrating that the observed responses in this assay were S1P₁ receptor specific and G_{αi} protein-mediated.

To assess S1P₁ receptor internalization kinetics, recombinant HeLa cells with stable expression of a SNAP-tagged-hS1P₁ receptor construct were employed using a fluorescence-based readout. In this assay, ponesimod (Bolli et al., 2010) led to a potent and sustained 80%–90% reduction of cell surface residing S1P₁ receptors after 1 hour (EC₅₀ ~8 nM) (Fig. 1B), which confirmed our previous results using fluorescence-activated cell sorting (Gatfield et al., 2014). β -Arrestin recruitment to the S1P₁ receptor was assessed using the Tango EDG1-*bla*-U2OS cell reporter gene assay and was quantified 16 hours after compound addition (Wetter et al., 2009). The Tango-based assay potencies of a selection of synthetic S1P₁ receptor agonists correlated very well with the potencies that were observed with the SNAP-tagged-hS1P₁ receptor internalization assay 16 hours after ligand addition (Supplemental Fig. 2A), supporting that β -arrestin recruitment and receptor internalization were functionally linked. As expected, the S1P₁ agonist-induced responses at 16 hours in both assays, i.e., Tango and SNAP-tag, were insensitive to pertussis toxin treatment (Supplemental Fig. 2, B–E), suggesting that receptor internalization and β -arrestin recruitment could be uncoupled from G_{αi} protein signaling.

The EPIC and Tango- β -arrestin assays were then used in parallel to screen a collection of proprietary synthetic S1P₁ receptor modulators. The prototypic synthetic S1P₁ receptor agonists ponesimod (Bolli et al., 2010) and SEW2871 (Sanna et al., 2004) were also included in this screen. Interestingly, we identified a small subset of compounds that induced potent and efficient β -arrestin recruitment, but in contrast to the majority of compounds, including ponesimod and SEW2871, displayed clearly slower kinetics and lower potencies in inducing G_{αi} protein-mediated signaling within 30 minutes

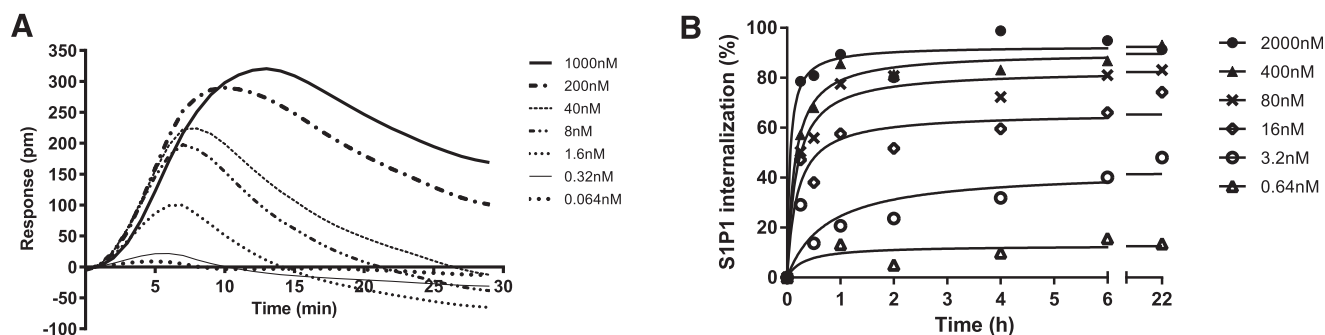


Fig. 1. Assays used to measure S1P₁ agonist-induced signaling. Real-time EPIC traces of Chinese hamster ovary cells stably expressing S1P₁ receptor and stimulated with increasing concentrations of S1P (A). SNAP surface staining of HeLa-SNAP-hS1P₁ cells treated with increasing concentrations of ponesimod (B).

in the EPIC assay. While ponesimod and SEW2871 at high concentrations (≥ 80 nM) reached their half-maximal signal within 7–9 minutes (Fig. 2, A and B), the compound D3-2 required 14–15 minutes, and after 30 minutes it remained considerably less potent (Fig. 2, C and D).

Interestingly, a consistent structure-activity relationship could be established around these compounds (Table 1). The residue attached to position five of the thiophene moiety (R₁) differentially influenced the compound's potency in the two assays. In general, a small substituent-like hydrogen or methyl led to compounds with higher potency in the G_{αi}

signaling assay, while larger groups such as an iso-butyl chain or a cyclopentane ring gave compounds with a preference for β -arrestin recruitment. This preference for β -arrestin recruitment was further enhanced when the two methyl groups in R₂ were replaced by two ethyl groups. In contrast, the nature of the side chain attached to the phenyl ring of the tri-aryl scaffold appeared to have little impact on the compound's signaling preference.

Hence, chemical optimization led to compounds with near 100-fold potency difference between G_{αi} protein-signaling (EPIC assay) and β -arrestin recruitment (Tango assay), like

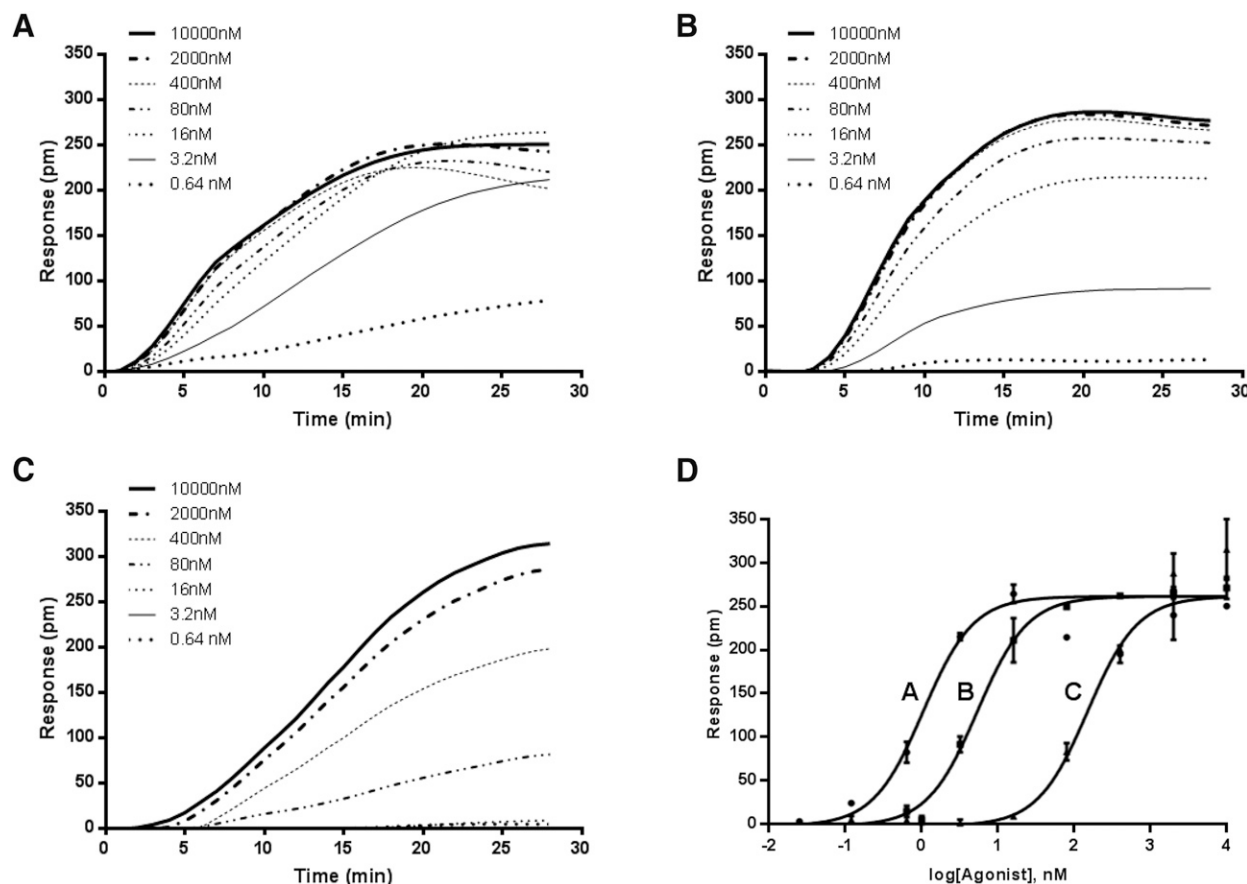
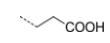
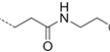
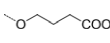
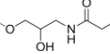


Fig. 2. Real-time EPIC traces induced by synthetic S1P₁ receptor agonists. Chinese hamster ovary hS1P₁ cells were stimulated with increasing concentrations of ponesimod (A), SEW2871 (B), and compound D3-2 (C), and dynamic mass redistribution changes were recorded for 30 minutes. The end data were used to plot the dose response and determine compound potency (D).

TABLE 1

Structure-activity relationship of side chain and alkyl chain (R)

Side Chain	EPIC EC ₅₀ (nM)/Tango EC ₅₀ (nM)									
	R ₂ = Methyl/R ₁								R ₂ = Ethyl/R ₁	
	H	Methyl	Ethyl	<i>n</i> -Propyl	iso-Propyl	<i>n</i> -Butyl	iso-Butyl	Cyclopentyl	Ethyl	<i>n</i> -Propyl
	A0	A1	A2	A3	A3i	A4	A4i	A5c	A2-2	A3-2
	30	6	10	21	38	41	74	113	15	44
	50	6	0.4	0.6	0.5	2	1	3	0.2	0.6
	B0	B1	B2	B3	B3i	B4	B4i	B5c	B2-2	B3-2
	13	15	22	59	37	34	151	141	38	152
	211	17	0.8	1	1	3	4	12	1.1	2
	C0	C1	C2	C3	C3i	C4	C4i	C5c	C2-2	C3-2
	7	2	9	30	32	30	34	75	16	38
	40	3	0.2	0.3	0.4	2	2	3	0.1	0.4
	D0	D1	D2	D3	D3i	D4	D4i	D5c	D2-2	D3-2
	3	5	14	29	63	63	61	160	29	168
	5	0.5	0.2	0.6	0.7	2	3	2	0.7	0.9

compounds C2-2, C3-2, or D3-2 (Table 1). The apparent lower potency in G_{αi} protein-mediated signaling by these compounds was confirmed in classic GTPγS assay (Supplemental Table 1). This apparent deficiency in G_{αi} signaling did not compromise the activity of the compounds to internalize the S1P₁ receptor within 16 hours (Table 2) nor did it prevent S1P₁ receptor degradation (Supplemental Fig. 3). These uniquely differentiated compounds were in terms of their functional profiles more similar to ponesimod, which potently activated G_{αi} signaling, β-arrestin recruitment, internalization, and degradation (Supplemental Fig. 3; Table 2) (Bolli et al., 2010; Gatfield et al., 2014) than to SEW2871, which potently activated G_{αi} signaling, but was >20-fold less potent than ponesimod in β-arrestin recruitment and S1P₁ internalization (Table 2) and induced only limited degradation of S1P₁ (González Cabrera et al., 2007). Thus, based on these data it appears that a high potency in β-arrestin recruitment is mandatory to achieve substantial receptor internalization and degradation.

G_{αi} Protein-Coupling Deficient S1P₁ Agonists Can Maximally Internalize S1P₁ Receptors but at a lower Rate. We next studied the kinetics of S1P₁ receptor internalization and investigated representative compounds with different G_{αi} protein signaling and β-arrestin recruitment potencies (ponesimod, D2, D3, D3i, D3-2, and SEW2871) at different concentrations using the SNAP-tagged-hS1P₁ receptor assay. The amount of internalized S1P₁ receptor was measured at different times for up to 20 hours after compound addition (Fig. 3). At the highest concentration (1 μM), which

was well above the EC₅₀ values of all compounds in G_{αi} protein signaling and β-arrestin assays, there was no obvious difference in the rate or magnitude of S1P₁ receptor internalization and all compounds induced almost 100% internalization within 4–6 hours after compound addition (Fig. 3, A and D). However, at lower concentrations (i.e., 100 and 30 nM) different internalization rates were apparent (Fig. 3, B–D). Compounds D3i and D3-2 with the lowest potencies in G_{αi} protein signaling (EC₅₀ 63.3 and 167.9 nM, respectively), and similar β-arrestin potencies (EC₅₀ 0.7 and 0.9 nM, respectively) displayed the slowest rate of S1P₁ receptor internalization among these compounds (Fig. 3D). Eventually, maximal S1P₁ receptor internalization was reached at all investigated concentrations, but only after prolonged incubation times. In contrast, SEW2871 always reached a plateau after 4–6 hours; the magnitude of effect was dose dependent and close to maximal internalization was reached at 1 μM. These experiments demonstrate that although a high potency in the β-arrestin assay is required to fully internalize the S1P₁ receptors, the potency in the G_{αi} signaling assay actually determines the rate of S1P₁ receptor internalization. Thus, for rapid and maximal S1P₁ receptor internalization a high potency in the G_{αi} signaling and in the β-arrestin assay is mandatory.

S1P₁ Receptor Signaling in Lymphatic Endothelial Cells. S1P₁ receptor activation is known to induce ERK1/2 and Akt phosphorylation (Igarashi et al., 2001), as well as induce cell chemotaxis through activation of G_{αi} protein signaling (Wang et al., 1999). We thus investigated these

TABLE 2

Representative compounds with potency differences in G_{αi} and β-arrestin signalingThe EC₅₀ values are in nanomolars

Compound	β-arrestin Tango			G _{αi} EPIC			SNAP-S1P ₁ Internalization		
	Geometric Mean	Geometric S.D.	<i>n</i>	Geometric Mean	Geometric S.D.	<i>n</i>	Geometric Mean	Geometric S.D.	<i>n</i>
Ponesimod	1.5	1.31	11	1.1	1.55	4	5.0	1.86	8
SEW2871	39.5	1.59	4	3.5	1.46	3	103.9	1.10	3
D2	0.2	1.44	6	13.9	1.79	6	3.6	1.31	5
D3	0.6	1.52	5	29.1	1.47	5	7.6	1.84	8
D3i	0.7	1.90	3	63.3	1.51	4	14.0	1.53	9
D3-2	0.9	1.02	2	167.9	1.40	2	10.6	1.34	5
D4	1.9	1.01	2	62.7	1.10	2	28.1	1.67	6
D4i	2.8	1.04	2	61.1	1.22	2	18.2	1.54	6
D5c	2.1	1.05	2	159.8	1.06	2	32.8	1.95	6

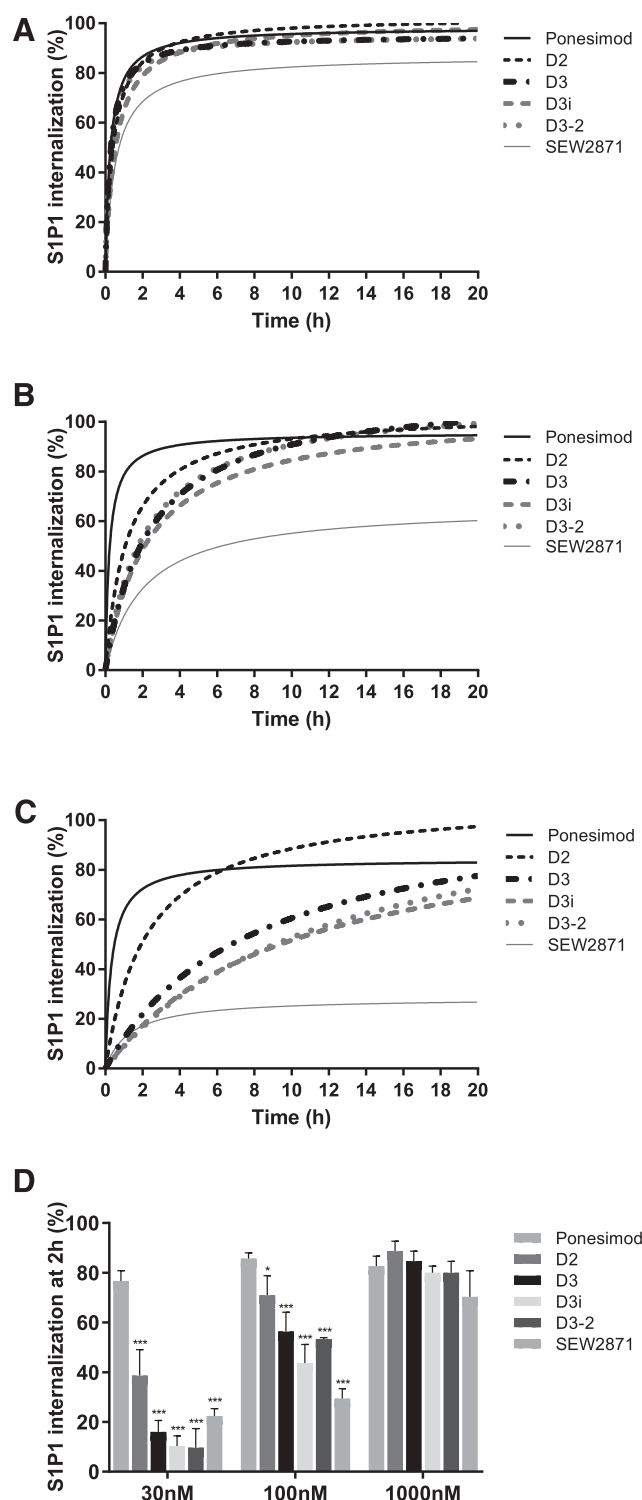


Fig. 3. S1P₁ receptor internalization kinetics. SNAP surface staining of HeLa-SNAP-hS1P₁ cells treated with 1 μ M (A), 100 (B), and 30 nM (C) of S1P₁ agonists. Shown is one representative experiment done in triplicates. Percentage of internalization after 2 hours for each S1P₁ agonist is plotted as mean \pm S.D. (D); * P < 0.05, ** P < 0.01, and *** P < 0.001, compared with internalization induced by ponesimod.

effects in human lymphatic endothelial cells (HMVEC-LLy) to further characterize the signaling properties of the newly identified compounds in a physiologically relevant, human, and nonrecombinant cell system.

HMVEC-LLy were treated with 1 μ M of two of the most differentiated agonists D3i and D3-2, or ponesimod, and phosphorylated ERK and Akt were measured in cell extracts. Phosphorylation of ERK1/2 and Akt induced by ponesimod was maximal at 5 minutes after stimulation and decreased to basal levels within 30 minutes (Fig. 4). The phosphorylation induced by D3i was delayed and reached a maximum at 15 minutes after stimulation (Fig. 4, C and F), while the compound D3-2, which was 180-fold more potent in the β -arrestin than in the $G_{\alpha i}$ signaling assay, was highly inefficient in inducing ERK1/2 and Akt phosphorylation for up to 30 minutes. Furthermore, ponesimod potently induced HMVEC-LLy chemotaxis in a dose-dependent manner (Fig. 5), whereas the compounds D2, D3, and D3i were clearly less potent, which was in line with their loss of potency in the $G_{\alpha i}$ protein signaling assay. Compound D3-2 was only able to induce HMVEC-LLy chemotaxis at 10 μ M concentration, confirming its inefficient $G_{\alpha i}$ signaling. This compound did not significantly increase chemotaxis of HMVEC-LLy for up to 4 hours (data not shown).

Next, ponesimod, D3i, and D3-2 were compared in regard of their ability to internalize S1P₁ receptors in HMVEC-LLy cells. To this end, HMVEC-LLy were incubated for different times with these compounds (1 μ M), followed by immunofluorescence staining of S1P₁ receptors. As shown in Fig. 6, ponesimod already induced strong S1P₁ receptor translocation from the cell surface into intracellular compartments after 15 minutes of incubation. S1P₁ internalization induced with compounds D3i and D3-2 was clearly slower compared with ponesimod, but after 2 hours there was a much smaller difference observed between the three agonists, only compound D3-2 seemed to be slightly less efficient at this time point. No compound differences were apparent after 24 hours of incubation, corroborating the observations in recombinant S1P₁ receptor-expressing HeLa cells. Altogether, these results show that also in native human cells S1P₁ receptor internalization can be induced by synthetic agonists, even by those with $G_{\alpha i}$ signaling deficiencies, albeit at a clearly lower rate. However, high potency in $G_{\alpha i}$ protein signaling is required to strongly activate additional (intra)cellular responses, such as ERK and Akt phosphorylation or cell chemotaxis.

Reduction of Peripheral Lymphocyte Counts In Vivo. Efficient S1P₁ receptor internalization and consequently functional antagonism has been proposed as a mechanistic basis for the observed reduction of the number of circulating blood lymphocytes upon administration of synthetic S1P₁ modulators in vivo. Since we could not observe major differences in the magnitude of S1P₁ internalization in vitro between ponesimod and compounds with attenuated $G_{\alpha i}$ protein signaling, we tested the ability of these novel compounds to induce lymphopenia in vivo. To this end, we used single oral administration (10 mg/kg) of each compound in rats and measured blood lymphocyte counts as well as compound plasma concentration 3, 6, and 24 hours later. As expected (Bolli et al., 2010; Piali et al., 2011), ponesimod induced a rapid and significant reduction of circulating lymphocyte counts that reached a plateau 3 hours postdosing (Fig. 7B). The effect remained for at least three additional hours and then almost fully returned to baseline after 24 hours. For ponesimod a good correlation of lymphocyte count reduction and plasma concentration was observed (Fig. 7, A and B). Surprisingly, we observed that compounds with attenuated $G_{\alpha i}$

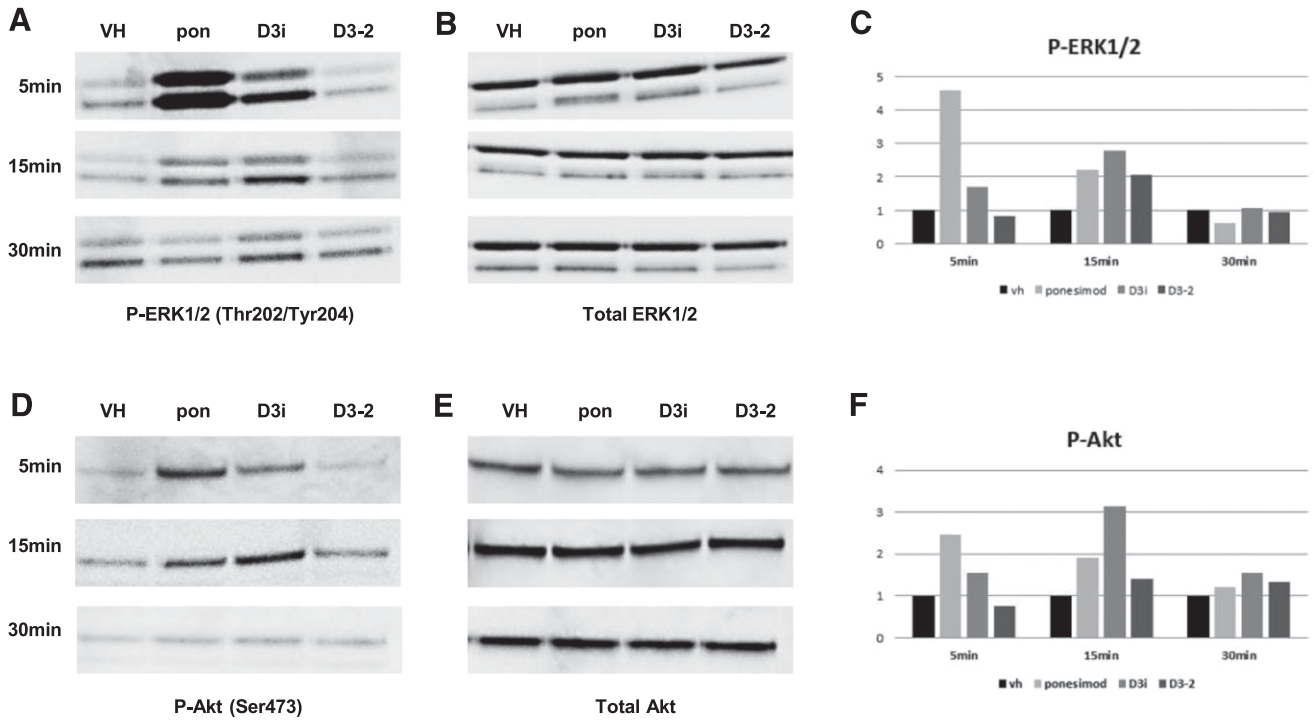


Fig. 4. ERK1/2 and Akt phosphorylation upon stimulation with S1P₁ agonists in HMVEC-LLy. HMVEC-LLy cells were stimulated with 1 μ M ponesimod (pon), compound D3i, compound D3-2, or vehicle (VH) for the indicated times and cell extracts were immunoblotted for phosphorylated ERK (A) and Akt (D). The band intensities were integrated, normalized against total ERK (B) and Akt (E), and expressed as fold phosphorylation compared with vehicle-treated cells (C and F).

signaling but with a similarly high potency in β -arrestin recruitment and S1P₁ receptor internalization assays as ponesimod, displayed an obvious disconnect between the pharmacodynamic (Fig. 7B) and pharmacokinetic effects (Fig. 7A). Although the compounds reached similar or even higher plasma exposures compared with ponesimod, the onset of blood lymphocyte count reduction was slower and the maximal effect on the lymphocyte count was reached later. Furthermore, a clear trend to lower maximal lymphocyte count reduction was seen with those compounds compared with ponesimod. A slight trend was already observed for compound D2, but a more pronounced effect was seen with compounds D3 and D3i, which showed a clear delay in the onset of lymphocyte count reduction (6 hours vs. 3 hours) and the plateau was only reached after 24 hours instead of 3 hours, although the plasma concentrations already

peaked at 3 hours and were clearly lower after 24 hours. Remarkably, compound D3-2, which is the compound with the most pronounced G α_i protein signaling deficit, already reached very high plasma concentrations after 3 and 6 hours without any obvious signs of lymphocyte count reduction. Moderate lymphocyte count reduction (approximately 20%) was observed only after 24 hours when the plasma concentration was already much lower again. Of note, rat plasma protein binding of compounds D2 to D3-2 was similar. Furthermore, the compounds activated and desensitized rat S1P₁ receptors with similar potencies as the human S1P₁ receptors, as exemplified by the compound D3-2 (Supplemental Fig. 4). These results suggest that for rapid onset and substantial blood lymphocyte count reduction in vivo, potent G α_i protein signaling and β -arrestin recruitment are both necessary.

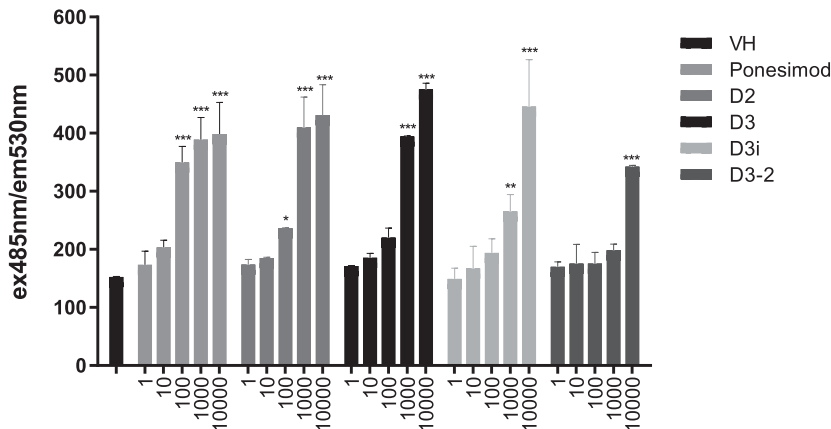


Fig. 5. HMVEC-LLy chemotaxis upon stimulation with S1P₁ agonists. Chemotactic responses induced by the indicated nanomolar concentrations of S1P₁ agonists or vehicle (VH) were measured after 45 minutes by transwell migration assays. Shown is one representative experiment done in duplicates. Mean \pm S.D.; * P < 0.05; ** P < 0.01; *** P < 0.001.

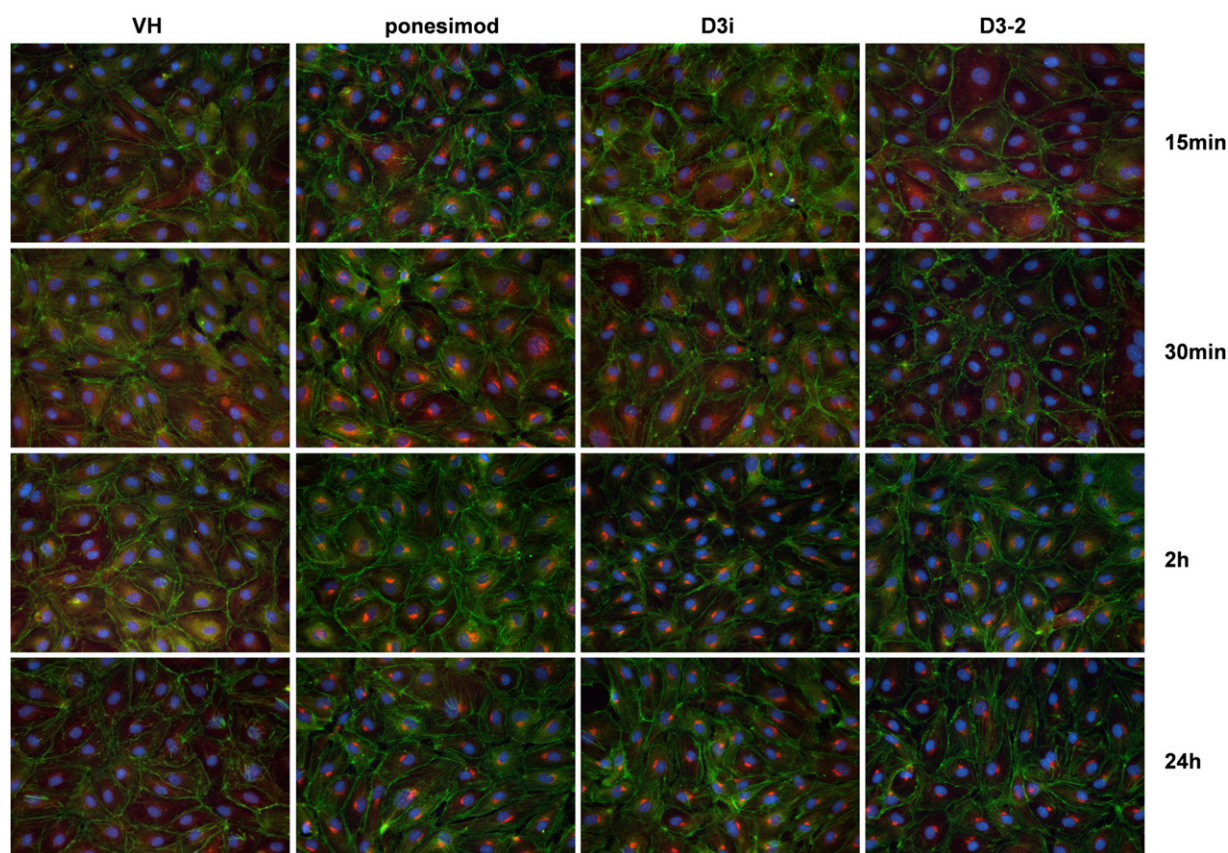


Fig. 6. S1P₁ internalization upon stimulation with S1P₁ agonists in HMVEC-LLy. HMVEC-LLy cells were stimulated with 1 μ M ponesimod, compound D3i, compound D3-2, or vehicle (VH) for the indicated times and immunofluorescence of S1P₁ receptors (red), F-actin (Phalloidin, green), and nuclei (Hoechst 33342, blue) was performed.

Discussion

To current knowledge, S1P₁ receptor modulators cause lymphocyte trapping in secondary lymphoid organs, reduce circulating lymphocytes, and attenuate autoimmune disease symptoms in various animal models and humans (Urbano et al., 2013). The absolute requirement for S1P₁ receptors mediating lymphocyte exit from lymph nodes has been demonstrated using different genetic and pharmacological approaches. It has been shown that S1P₁-deficient lymphocytes cannot egress from thymus or secondary lymphoid organs (Matloubian et al., 2004) and S1P₁ heterozygous lymphocytes egress more slowly than wild-type lymphocytes (Lo et al., 2005). Pharmacological blockade of S1P₁ receptors with specific antagonists also induces lymphopenia in vivo (Tarrasón et al., 2011; Quancard et al., 2012). Together, this led to the current thinking that S1P₁ receptor modulators induce lymphopenia through functional antagonism, following sustained S1P₁ receptor internalization and proteasomal degradation (Hla and Brinkmann, 2011). However, with the discovery of SEW2871 it appeared that other mechanisms might also contribute to the induction of lymphopenia, which is so effectively induced by FTY20/fingolimod/Gilenya and other S1P₁ receptor modulators, since SEW2871 causes only transient S1P₁ receptor internalization and recycling, but is nevertheless lymphopenic in vivo (Jo et al., 2005). For example, lymphatic endothelial cells express high levels of S1P₁ receptor and SEW2871 has been shown to rapidly reduce T cell egress in lymph node explants, suggesting its action on stromal gates rather than lymphocytes

(Sanna et al., 2006). Therefore, the exact molecular events leading to S1P₁ receptor modulator-induced lymphopenia remain to be fully elucidated.

We describe here the discovery of novel synthetic S1P₁ receptor modulators with unique pharmacological profiles. We used these compounds as pharmacological tools to better understand the molecular mechanisms leading to lymphopenia. Our screening strategy was built on the recently emerging concept of biased agonism and was specifically aimed at the identification of compounds with deficiency in G_{ai} protein signaling and preserved potency and efficacy in β -arrestin recruitment and S1P₁ receptor internalization. We thus identified novel structure-activity relationships of synthetic S1P₁ receptor modulators with significantly reduced potency in G_{ai} protein signaling and downstream activation of ERK1/2 and Akt, but with fully retained β -arrestin recruitment capacity. Compound D3-2 displayed the most pronounced differential (180-fold potency shift) pathway activation. Compounds with an apparent β -arrestin bias had been previously described in the case of type 1 parathyroid hormone receptors and they demonstrated clearly different pharmacological effects compared with the non-biased counterparts (Gesty-Palmer et al., 2006). Another well-known example is the angiotensin 1 receptor agonist, TRV120023, which was shown to have different and potentially advantageous effects compared with nonbiased agonists in models of acute cardiac injury (Kim et al., 2012). Together, such examples appear to support the hypothesis that G_{ai} protein-mediated and β -arrestin-mediated signaling is dissociable and triggers distinct

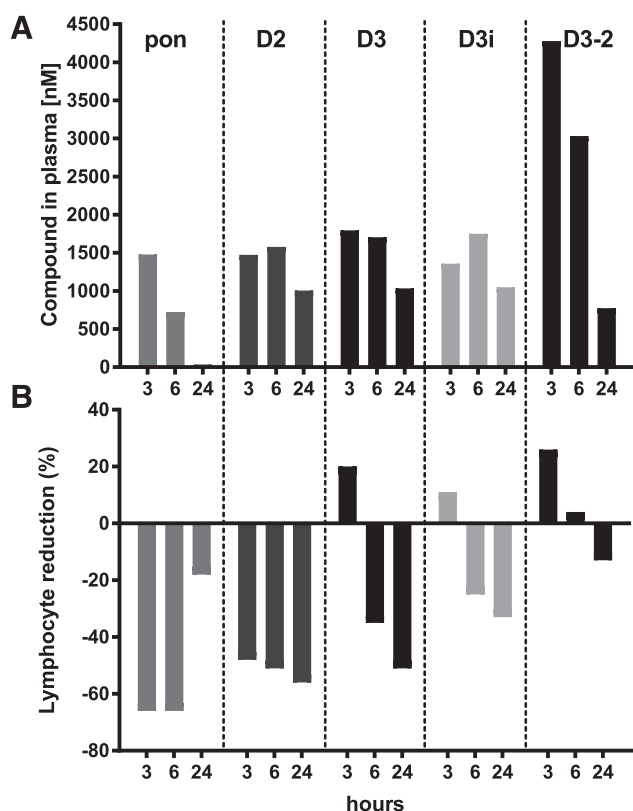


Fig. 7. Disconnection of pharmacokinetics and pharmacodynamics of $G_{\alpha i}$ protein-coupling deficient S1P₁ agonists in rats. Plasma concentrations of ponesimod (pon), compound D2, compound D3, compound D3i, and compound D3-2 at 3, 6, and 24 hours after single oral administration of 10 mg/kg of each compound to rats (A) and reduction in the percentage of peripheral lymphocyte numbers (B). Values are mean ($n = 6$); six rats were dosed with each compound.

pharmacological effects. Similar to most known examples, we have not fully elucidated the exact molecular mechanism, leading to the apparent difference in activities between these assays. The compounds may show preference to stabilize certain G protein-coupled receptor conformations (Pupo et al., 2016), they may influence $G_{\alpha i}$ protein dissociation rates (Furness et al., 2016), or show different thermodynamic properties in G protein-coupled receptor binding (Klein Herenbrink et al., 2016).

Nevertheless, using our novel S1P₁ receptor modulators, we were able to demonstrate in vitro that reduced $G_{\alpha i}$ protein signaling potency with preserved β -arrestin recruitment potency led to a clearly slower S1P₁ receptor internalization rate, without compromising S1P₁ receptor internalizing and degrading efficacy. With increasing compound concentration and consequently more $G_{\alpha i}$ protein signaling engagement, the S1P₁ receptor internalization rate could be accelerated and the pharmacology became closer to the classical S1P₁ receptor modulators, such as ponesimod. Based on the in vitro data and adhering to the idea of functional S1P₁ receptor antagonism being responsible for lymphopenia, we thus anticipated a delayed onset of lymphopenia, without major consequences on maximal lymphocyte reduction in vivo. However, S1P₁ modulators with increasingly lower $G_{\alpha i}$ protein signaling potency displayed a clear tendency for delayed lymphopenia, but unexpectedly the maximal lymphocyte count reduction was also significantly compromised. In extremis, D3-2, the compound with the most pronounced potency differences in $G_{\alpha i}$

protein versus β -arrestin signaling assays barely reduced circulating lymphocytes, despite having plasma exposures exceeding those of all other tested compounds, suggesting a complete pharmacokinetic/pharmacodynamic disconnect. Furthermore, the lack of lymphocyte count reduction cannot be explained by the difference in plasma protein binding or in potency on rat S1P₁ receptors. Our data do not exclude that functional antagonism is necessary to induce fully efficacious lymphopenia by S1P₁ receptor modulators, but there might be additional mechanisms in vivo that depend on efficient $G_{\alpha i}$ protein signaling. This was previously proposed to explain SEW2871- and S1P-induced lymphopenia (Jo et al., 2005). The requirement for potent and effective $G_{\alpha i}$ protein signaling has been also indirectly suggested by Xu et al. (2013), who analyzed the correlation between in vitro potency testing of S1P₁ receptor modulators and peripheral lymphocyte count reduction. Interestingly, cAMP signaling, β -arrestin recruitment, and S1P₁ receptor internalization were all found to be good predictors of in vivo lymphopenia, but inhibition of cAMP accumulation, which is caused by $G_{\alpha i}$ protein signaling, clearly showed the highest correlation (Xu et al., 2013). Similarly, BMS-986104, a S1P₁ receptor modulator that is potent and fully efficacious in $G_{\alpha i}$ protein signaling, but which induces only partial receptor internalization, was recently reported to be fully efficacious in inhibiting lymphocyte egress in mice (Dhar et al., 2016).

A genetic mouse model, in which wild-type S1P₁ receptors were replaced by internalization-deficient mutants (S5A-S1P₁), was also used to dissect the effects of S1P₁ receptor signaling and S1P₁ internalization in the context of lymphocyte homing (Thangada et al., 2010). In this system, mice with T cells expressing mutant S5A-S1P₁ receptors exhibited delayed onset lymphopenia upon FTY720 treatment and these mice were refractory to S1P lyase inhibition. Adoptive transfer experiments then demonstrated that the vascular compartment did not contribute to the observed pharmacology and that the effects of mutant S1P₁ receptors were solely T cell driven. The genetic work shows that S1P₁ receptor internalization rates influence lymphocyte egress kinetics and that mutant-induced changes in S1P₁ receptor internalization alter lymphocyte egress kinetics in response to FTY720. Our findings using S1P₁ receptor modulators with reduced $G_{\alpha i}$ protein signaling and lower receptor internalization rates are in agreement with these findings.

However, in contrast to the genetic model, where $G_{\alpha i}$ signaling was still fully maintained and FTY720 induced delayed lymphopenia, we now observe an almost complete loss of lymphopenia by using compound D3-2 that displayed the largest deficit in $G_{\alpha i}$ signaling compared with β -arrestin recruitment. This suggests that the apparent compound-intrinsic lack of potent $G_{\alpha i}$ protein signaling significantly impacts the ability to induce lymphopenia. This might indicate that lymphocyte egress kinetics are faster than compound-induced internalization, in agreement with the observed slow S1P₁ receptor internalization in vitro and the notion that in vivo lymphopenia was observed to occur late, for example, for D3-2 only after 24 hours when plasma concentrations were already low. This would allow lymphocytes to exit secondary lymph nodes before a substantial amount of S1P₁ receptor is internalized and made refractive from sensing the S1P gradient. However, repetitive administration over 3 days of compounds with attenuated $G_{\alpha i}$ protein signaling did not result in a bigger reduction of blood lymphocyte

count. Alternatively, other cell types, such as endothelial cells, expressing S1P₁ receptors could contribute in the exit process through S1P₁ receptor-induced and G_{αi}-mediated processes. It is thus conceivable that different mechanisms such as S1P gradient obliteration, lymphatic endothelial barrier function increase, or modulation of T cell sensing of S1P gradients influence the lymphopenic response to S1P₁ receptor modulators.

In summary, our study demonstrates that S1P₁ receptor modulator-induced G_{αi}-mediated signaling and β-arrestin recruitment are both necessary to induce efficient and rapid lymphopenia in vivo. Future studies using these compounds as pharmacological tools, possibly in combination with additional genetic models, might help to elucidate the exact mechanism by which the synthetic S1P₁ receptor modulators induce lymphopenia. Unraveling those mechanisms might facilitate the discovery of novel S1P₁ receptor modulators with unique signaling properties to treat human disease.

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Authorship Contributions

Participated in research design: Birker-Robaczewska, Bolli, de Kanter, Kohl, Lescop, Steiner, Nayler.

Conducted experiments: Birker-Robaczewska, Poirey, Boucher, Rey.

Performed data analysis: Birker-Robaczewska, Bolli, de Kanter, Lescop, Steiner, Nayler.

Wrote or contributed to the writing of the manuscript: Birker-Robaczewska, Nayler.

References

- Allende ML, Dreier JL, Mandala S, and Proia RL (2004) Expression of the sphingosine 1-phosphate receptor, S1P₁, on T-cells controls thymic emigration. *J Biol Chem* **279**:15396–15401.
- Bolli MH, Abele S, Binkert C, Bravo R, Buchmann S, Bur D, Gatfield J, Hess P, Kohl C, Mangold C, et al. (2010) 2-imino-thiazolidin-4-one derivatives as potent, orally active S1P₁ receptor agonists. *J Med Chem* **53**:4198–4211.
- Dhar TG, Xiao HY, Xie J, Lehman-McKeeman LD, Wu DR, Dabros M, Yang X, Taylor TL, Zhou XD, Heimrich EM, et al. (2016) Identification and preclinical pharmacology of BMS-986104: a differentiated S1P₁ receptor modulator in clinical trials. *ACS Med Chem Lett* **7**:283–288.
- Fujii Y, Hirayama T, Ohtake H, Ono N, Inoue T, Sakurai T, Takayama T, Matsumoto K, Tsukahara N, Hidano S, et al. (2012) Amelioration of collagen-induced arthritis by a novel S1P₁ antagonist with immunomodulatory activities. *J Immunol* **188**:206–215.
- Furness SG, Liang YL, Nowell CJ, Halls ML, Wookey PJ, Dal Maso E, Inoue A, Christopoulos A, Wootten D, and Sexton PM (2016) Ligand-dependent modulation of G protein conformation alters drug efficacy. *Cell* **167**:739–749 e711.
- Galvani S, Sanson M, Blaho VA, Swendeman SL, Obinata H, Conger H, Dahlbäck B, Kono M, Proia RL, Smith JD, et al. (2015) HDL-bound sphingosine 1-phosphate acts as a biased agonist for the endothelial cell receptor S1P₁ to limit vascular inflammation. *Sci Signal* **8**:ra79.
- Gatfield J, Monnier L, Studer R, Bolli MH, Steiner B, and Nayler O (2014) Sphingosine-1-phosphate (S1P) displays sustained S1P₁ receptor agonism and signaling through S1P lyase-dependent receptor recycling. *Cell Signal* **26**:1576–1588.
- Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Wang S, Eckhardt AE, Cowan CL, Spurney RF, Luttrell LM, et al. (2006) Distinct β-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J Biol Chem* **281**:10856–10864.
- González Cabrera D, Koivisto BD, and Leigh DA (2007) A metal-complex-tolerant CuAAC 'click' protocol exemplified through the preparation of homo- and mixed-metal-coordinated [2]rotaxanes. *Chem Commun (Camb)* **41**:4218–4220.

- Hla T and Brinkmann V (2011) Sphingosine 1-phosphate (S1P): physiology and the effects of S1P receptor modulation. *Neurology* **76** (Suppl 3):S3–S8.
- Igarashi J, Bernier SG, and Michel T (2001) Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase. Differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. *J Biol Chem* **276**:12420–12426.
- Jo E, Sanna MG, Gonzalez-Cabrera PJ, Thangada S, Tigyi G, Osborne DA, Hla T, Parrill AL, and Rosen H (2005) S1P₁-selective in vivo-active agonists from high-throughput screening: off-the-shelf chemical probes of receptor interactions, signaling, and fate. *Chem Biol* **12**:703–715.
- Kim KS, Abraham D, Williams B, Violin JD, Mao L, and Rockman HA (2012) β-Arrestin-biased AT1R stimulation promotes cell survival during acute cardiac injury. *Am J Physiol Heart Circ Physiol* **303**:H1001–H1010.
- Klein Herenbrink C, Sykes DA, Donhamsetti P, Canals M, Coudrat T, Shonberg J, Scammells PJ, Capuano B, Sexton PM, Charlton SJ, et al. (2016) The role of kinetic context in apparent biased agonism at GPCRs. *Nat Commun* **7**:10842.
- Lo CG, Xu Y, Proia RL, and Cyster JG (2005) Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. *J Exp Med* **201**:291–301.
- Luttrell LM, Maudsley S, and Bohn LM (2015) Fulfilling the promise of "biased" G protein-coupled receptor agonism. *Mol Pharmacol* **88**:579–588.
- Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, and Cyster JG (2004) Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* **427**:355–360.
- Nayler O, Birker-Robaczewska M, and Gatfield J (2010) Integration of label-free detection methods in GPCR drug discovery, in *GPCR Molecular Pharmacology and Drug Targeting* (Gilchrist M ed) pp 252–275, Wiley, Hoboken.
- Piali L, Froidevaux S, Hess P, Nayler O, Bolli MH, Schlosser E, Kohl C, Steiner B, and Clozel M (2011) The selective sphingosine 1-phosphate receptor 1 agonist ponesimod protects against lymphocyte-mediated tissue inflammation. *J Pharmacol Exp Ther* **337**:547–556.
- Pupo AS, Duarte DA, Lima V, Teixeira LB, Parreiras-E-Silva LT, and Costa-Neto CM (2016) Recent updates on GPCR biased agonism. *Pharmacol Res* **112**:49–57.
- Quancard J, Bollbuck B, Jansen P, Angst D, Berst F, Buehlmeier P, Streiff M, Beerli C, Brinkmann V, Guerini D, et al. (2012) A potent and selective S1P₁ antagonist with efficacy in experimental autoimmune encephalomyelitis. *Chem Biol* **19**:1142–1151.
- Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS, Webb B, Lefebvre S, Chun J, Gray N, et al. (2004) Sphingosine 1-phosphate (S1P) receptor subtypes S1P₁ and S1P₃, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem* **279**:13839–13848.
- Sanna MG, Wang SK, Gonzalez-Cabrera PJ, Don A, Marsolais D, Matheu MP, Wei SH, Parker I, Jo E, Cheng WC, et al. (2006) Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P₁ antagonist in vivo. *Nat Chem Biol* **2**:434–441.
- Spiegel S and Milstien S (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* **4**:397–407.
- Tarrasón G, Aulí M, Mustafa S, Dolgachev V, Domènech MT, Prats N, Domínguez M, López R, Aguilar N, Calbet M, et al. (2011) The sphingosine 1-phosphate receptor-1 antagonist, W146, causes early and short-lasting peripheral blood lymphopenia in mice. *Int Immunopharmacol* **11**:1773–1779.
- Thangada S, Khanna KM, Blaho VA, Oo ML, Im DS, Guo C, Lefrançois L, and Hla T (2010) Cell-surface residence of sphingosine 1-phosphate receptor 1 on lymphocytes determines lymphocyte egress kinetics. *J Exp Med* **207**:1475–1483.
- Urbano M, Guerrero M, Rosen H, and Roberts E (2013) Modulators of the sphingosine 1-phosphate receptor 1. *Bioorg Med Chem Lett* **23**:6377–6389.
- Wang F, Van Brocklyn JR, Hobson JP, Movafagh S, Zukowska-Grojec Z, Milstien S, and Spiegel S (1999) Sphingosine 1-phosphate stimulates cell migration through a G_i-coupled cell surface receptor. Potential involvement in angiogenesis. *J Biol Chem* **274**:35343–35350.
- Weiler S, Braendlin N, Beerli C, Bergsdorf C, Schubart A, Srinivas H, Oberhauser B, and Billich A (2014) Orally active 7-substituted (4-benzylphthalazin-1-yl)-2-methylpiperazin-1-yl-nicotinonitriles as active-site inhibitors of sphingosine 1-phosphate lyase for the treatment of multiple sclerosis. *J Med Chem* **57**:5074–5084.
- Wetter JA, Revankar C, and Hanson BJ (2009) Utilization of the Tango β-arrestin recruitment technology for cell-based EDG receptor assay development and interrogation. *J Biomol Screen* **14**:1134–1141.
- Xu H, McElvain M, Fiorino M, Henkle B, Sherman L, Xu Y, Tominey E, Kelley K, Adam M, Bürlí R, et al. (2013) Predictability of peripheral lymphocyte reduction of novel S1P₁ agonists by in vitro GPCR signaling profile. *J Biomol Screen* **18**:997–1007.

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SUPPLEMENTAL DATA

S1P₁ modulator-induced G_{ai} Signaling and β -Arrestin Recruitment Are Both Necessary to Induce Rapid and Efficient Reduction of Blood Lymphocyte Count *in vivo*

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MOLECULAR PHARMACOLOGY

SUPPLEMENTAL MATERIALS AND METHODS

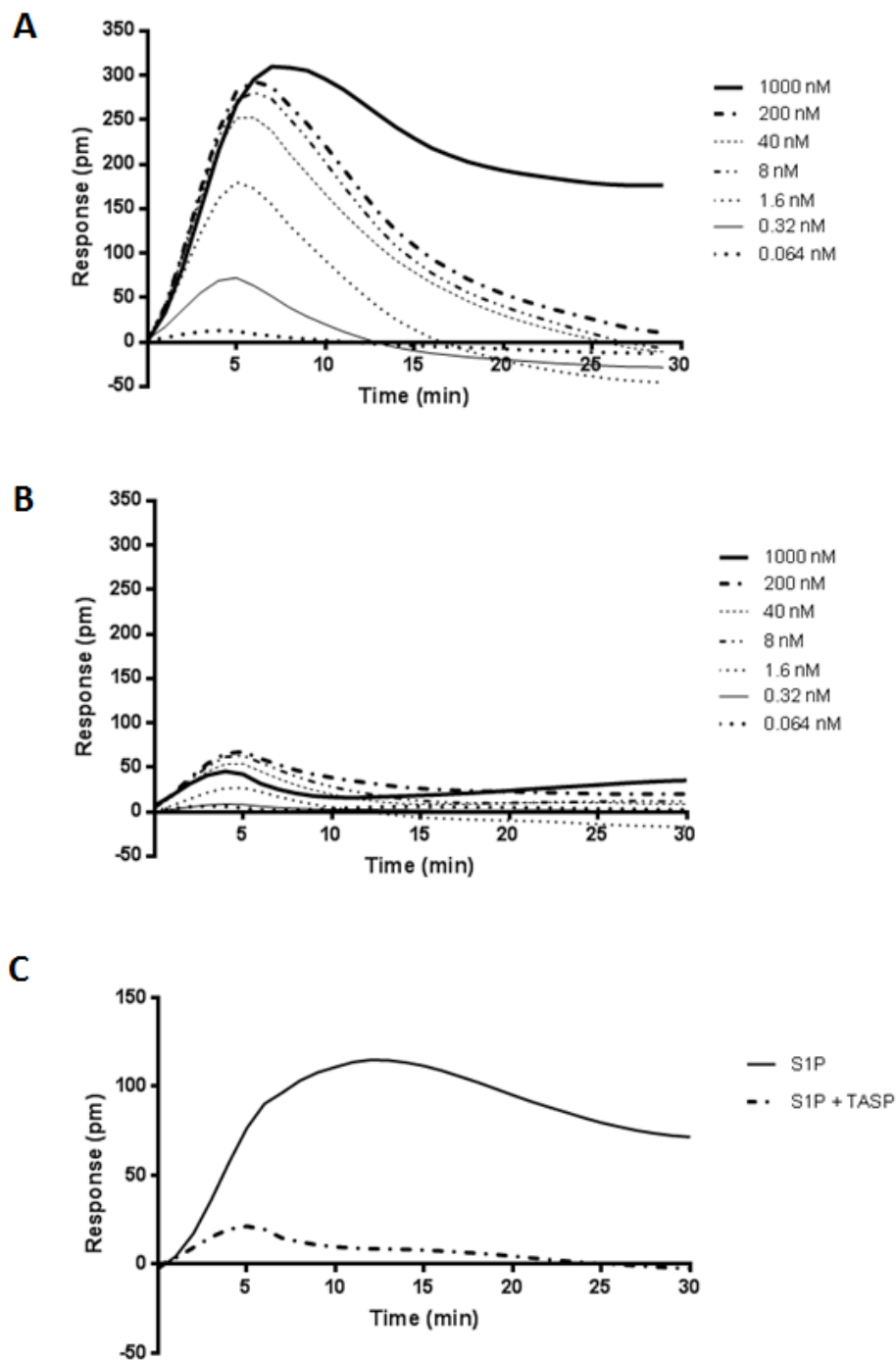
GTP γ S assay. Membrane preparations of CHO cells expressing recombinant hS1P₁ receptors were used for (³⁵S)-GTP γ S binding assay to measure intracellular G_{ai} protein activation following agonist binding. The assays were essentially performed as described previously (Bolli et al., 2010). EC₅₀ values were calculated with the IC₅₀ Witch software (Actelion Pharmaceuticals Ltd.) using the following settings: minimum defined by the no-effect level of the lowest compound concentration, maximum defined by the maximal response of the compound (smart max), no weighting and variable slope.

EPIC desensitization assay. 10'000 CHO cells stably expressing human S1P₁ receptor (CHO-hS1P₁) or rat S1P₁ receptor (CHO-rS1P₁) were seeded in uncoated (CHO-hS1P₁) or fibronectin-coated (CHO-rS1P₁) 384-well EPIC[®] sensor microplates (Corning) and grown for 24 h in Ham's F12 medium supplemented with 10 % charcoal-treated FBS, 300 μ g/ml (CHO-hS1P₁) or 1000 μ g/ml (CHO-rS1P₁) geneticin, 100 U penicillin and 1 μ g/ml streptomycin. The medium was removed, the wells filled with 40 μ l starvation medium (Ham's F12 with 0.05 % fafBSA and 2 % DMSO) and the plate loaded in the EPIC[®] reader (Corning) for 2 h equilibration. After the baseline dynamic mass redistribution (DMR) measurement, 10 μ l of test compounds in starvation medium were added and a real-time DMR changes recorded for 30 minutes. Next, the plate was placed at 37°C in a humidified CO₂ incubator. After 16 h incubation, the plate was washed twice with assay buffer (HBSS with 20 mM Hepes, 0.05 % fafBSA and 2 % DMSO), the wells filled with 40 μ l assay buffer and the plate loaded in the EPIC[®] reader (Corning) for 2 h equilibration. After the baseline dynamic mass redistribution (DMR) measurement, 50 nM of S1P in assay buffer were added and a real-time DMR changes recorded for 30 minutes. The data from EPIC[®] Analyser software (Corning) were exported to Microsoft Excel and used to determine compound potency with the IC₅₀ Witch software (Actelion Pharmaceuticals Ltd.). For determination of compounds EC₅₀ the following settings were used: minimum defined by the vehicle control (DMSO), maximum defined by the maximal response of the

compound (smart max), no weighting and variable slope. For determination of desensitization IC_{50} the following settings were used: minimum defined by the maximal response of the compound (smart min), maximum defined by the no-effect level of the compound (smart max), no weighting and variable slope.

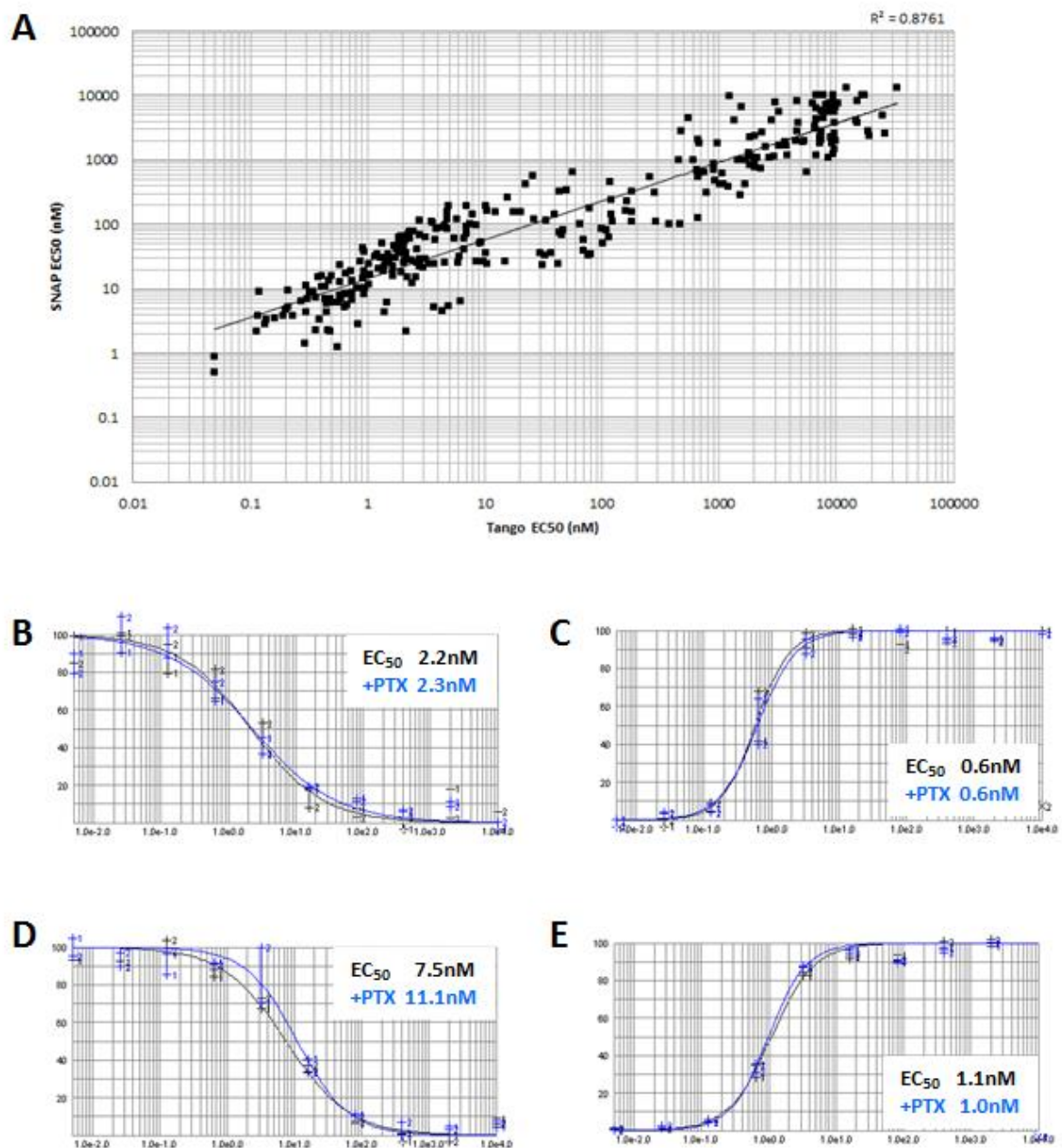
	EPIC			GTP γ S		
	geomean	<i>geosdev</i>	<i>n</i>	geomean	<i>geosdev</i>	<i>n</i>
Ponesimod	1.1	<i>1.55</i>	<i>4</i>	7.1	<i>1.17</i>	<i>2</i>
D2	13.9	<i>1.79</i>	<i>6</i>	9.1	<i>1.28</i>	<i>2</i>
D3	29.1	<i>1.47</i>	<i>5</i>	41.7	<i>1.34</i>	<i>2</i>
D3i	63.3	<i>1.51</i>	<i>4</i>	54.9	<i>1.65</i>	<i>2</i>
D3-2	167.9	<i>1.40</i>	<i>2</i>	215.4	<i>1.04</i>	<i>2</i>
D4	62.7	<i>1.10</i>	<i>2</i>	108.4	<i>1.13</i>	<i>2</i>
D4i	61.1	<i>1.22</i>	<i>2</i>	87.2	<i>1.64</i>	<i>2</i>
D5c	159.8	<i>1.06</i>	<i>2</i>	186.4	<i>1.08</i>	<i>2</i>
EC ₅₀ (nM)						

Supplemental Table 1. Comparison of compound potencies in different assays measuring G_{αi} activity of S1P₁ receptors.



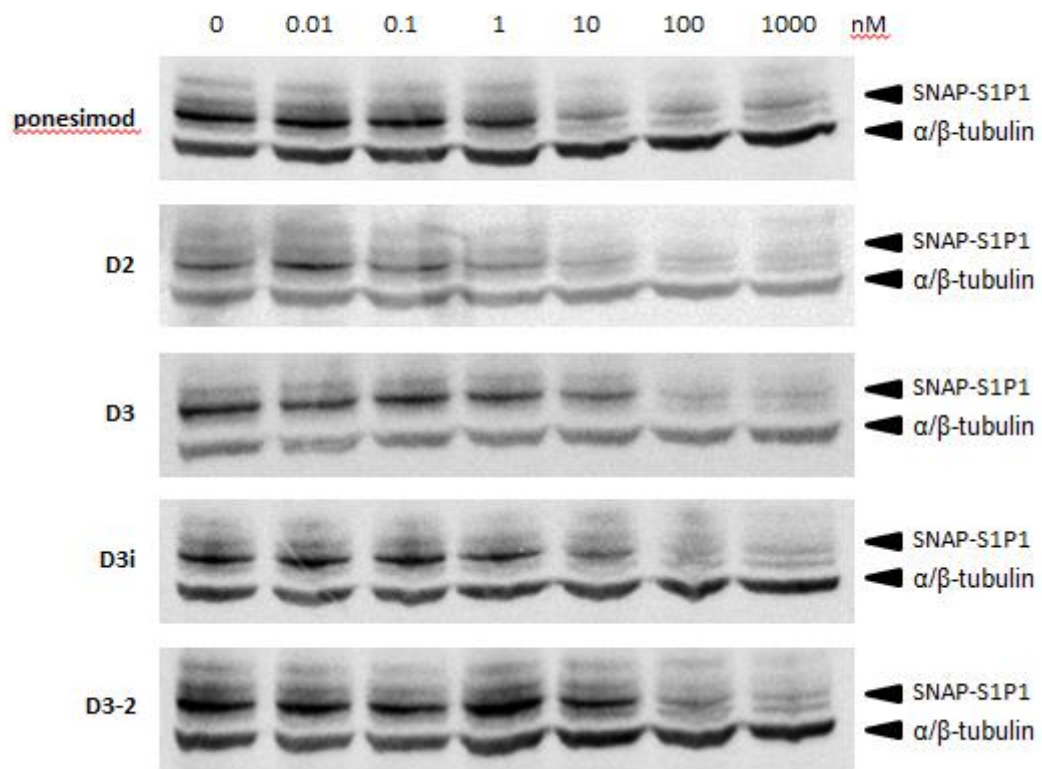
Supplemental Figure 1. Real-time EPIC traces in CHO-S1P₁ cells.

CHO cells stably expressing S1P₁ receptor were stimulated with a dose response of S1P in absence (A) or presence of PTX (300 ng/ml O/N) (B). (C) Response to 50 nM S1P in presence of 10'000 nM TASP0277308.



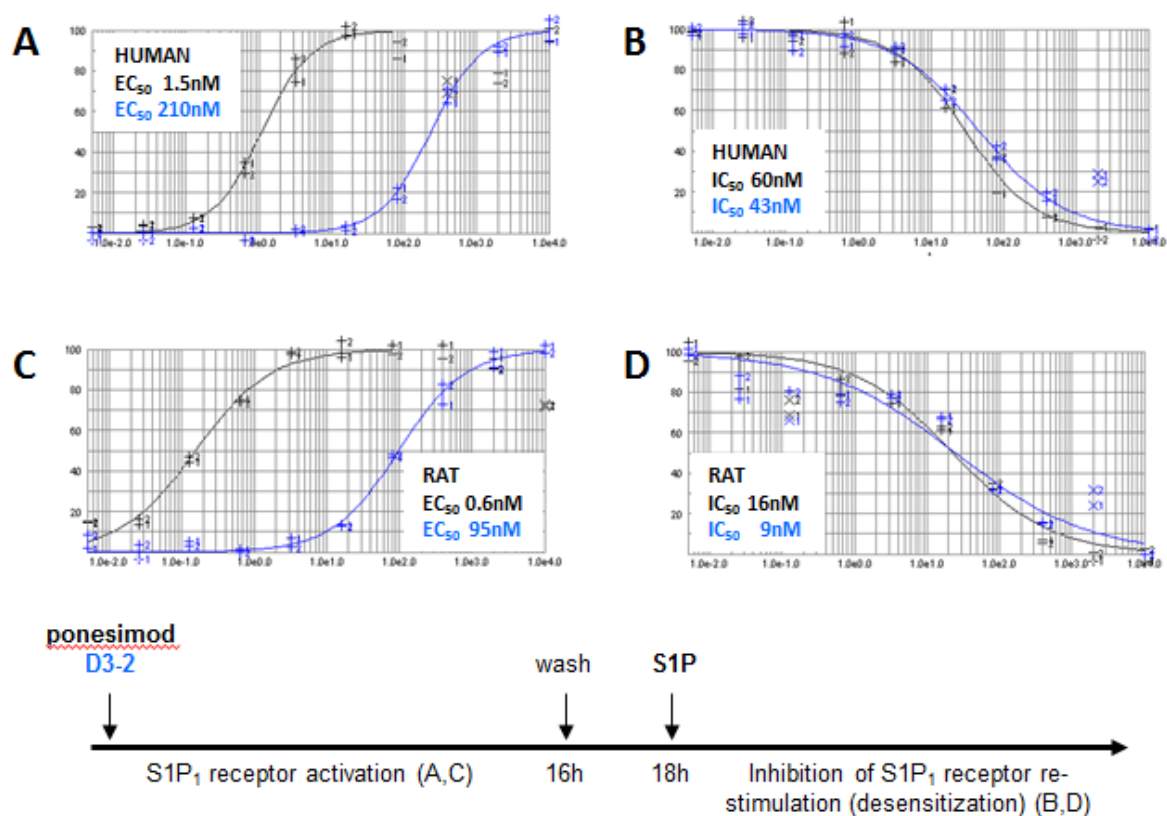
Supplemental Figure 2. β -arrestin recruitment and receptor internalization are functionally linked.

Correlation between EC₅₀ determined in S1P₁ internalization (SNAP) and β -arrestin recruitment (Tango) assays (A). EC₅₀ of ponesimod (B,C) and compound D3-2 (D,E) in SNAP-S1P₁ internalization (B,D) and β -arrestin Tango (C,E) in absence (black curves) and presence of PTX (blue curves).



Supplemental Figure 3. S1P₁ receptor degradation.

Immunoblotting with anti-S1P₁ and α/β -tubulin antibodies of lysates from HeLa-SNAP-hS1P₁ cells treated for 16h with the indicated concentrations of S1P₁ agonists.



Supplemental Figure 4. Activation and desensitization of human and rat S1P₁ receptors. CHO-hS1P₁ cells were stimulated with increasing concentrations of ponesimod (black curves) and compound D3-2 (blue curves) and EC₅₀ determined in EPIC assay (A). After 16 h incubation the compounds were removed, the cells washed and 2 h later re-stimulated with 50 nM S1P. Inhibition of S1P-induced response was expressed as desensitization IC₅₀ (B). The same experiment performed on CHO-rS1P₁ cells with corresponding EC₅₀ (C) and desensitization IC₅₀ (D). Shown is one representative experiment. Values are geomeans ($n=6-12$)