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TITLE PAGE

$S1P_1$ modulator-induced $G_{\alpha i}$ Signaling and β-Arrestin Recruitment Are Both Necessary to Induce Rapid and Efficient Reduction of Blood Lymphocyte Count *in vivo*

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RUNNING TITLE PAGE

Running title: **$S1P_1$ receptor signaling and lymphopenia**

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Abbreviations: CHO, Chinese hamster ovary; DMR, dynamic mass redistribution; fafBSA, fatty acid-free bovine serum albumin; FBS, fetal bovine serum; $G_{\alpha i}$, G protein alpha i subunit; GPCR, G protein-coupled receptor; HMVEC-LLy, human lung lymphatic microvascular endothelial cells; PBS, phosphate buffered saline; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; $S1P_1$, sphingosine-1-phosphate receptor 1

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ABSTRACT

$S1P_1$ (sphingosine-1-phosphate receptor 1) agonists prevent lymphocyte egress from secondary lymphoid organs and cause a reduction of the number of circulating blood lymphocytes. We hypothesized that $S1P_1$ receptor modulators with pathway-selective signaling properties could help to further elucidate molecular mechanisms involved in lymphocyte trapping. A proprietary $S1P_1$ receptor modulator library was screened for compounds with clear potency differences in β-arrestin recruitment and $G_{\alpha i}$ protein-mediated signaling. We describe here structure-activity relationships of highly potent $S1P_1$ 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 (EC_{50} 0.9 ±1 nM) as compared with the $G_{\alpha i}$-activation assay (167 ± 1.4 nM), whereas ponesimod, a $S1P_1$ modulator that is currently in advanced clinical development in multiple sclerosis, was equipotent in both assays (EC_{50} 1.5 ±1.3 nM and EC_{50} 1.1 ±1.5 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 the $S1P_1$ receptors, the potency in inducing $G_{\alpha i}$ signaling determines the rate of receptor internalization *in vitro*. In contrast to ponesimod, the compound D3-2 did not reduce the number or circulating lymphocytes in rats despite high plasma exposures. Thus, for rapid and maximal $S1P_1$ receptor internalization a high potency in both $G_{\alpha i}$ signaling and β-arrestin recruitment is mandatory and this translates into efficient reduction of the number of circulating lymphocytes *in vivo*.

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INTRODUCTION

Sphingosine-1-phosphate is a bioactive sphingolipid that causes pleiotropic cellular effects, including cell proliferation, migration or survival, by acting as intracellular and extracellular mediator. Extracellular S1P signaling is mediated by five different S1P receptor subtypes, named $S1P_1$ through $S1P_5$ receptors, which show differential expression across a variety of tissues and cell-types. Synthetic $S1P_1$ receptor modulators, like the non-selective $S1P_{1,2,3,4,5}$ receptor modulator FTY720 (fingolimod, Gilenya™) or the selective $S1P_1$ 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 $S1P_1$ 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 $S1P_1$ 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 (Gatfield et al., 2014; Hla and Brinkmann, 2011). This hypothesis is supported by the fact that $S1P_1$ antagonists are also able to induce lymphopenia *in vivo* (Quancard et al., 2012; Tarrason et al., 2011). However, it was recently reported that the synthetic agonist BMS-986104 was able to induce lymphopenia without showing complete internalization of $S1P_1$ receptors (Dhar et al., 2016) and a transgenic mouse, expressing internalization-defective $S1P_1$ receptors, still showed FTY720-induced lymphopenia, albeit with delayed kinetics (Thangada et al., 2010). Previous reports had suggested $S1P_1$ agonism to be a mechanism responsible for lymphocyte egress inhibition induced by synthetic agonists and $S1P_1$ 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.

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Alternatively, synthetic compounds could activate $S1P_1$ receptors on lymphatic endothelial cells, leading to the tightening of cell-cell junctions and thus blocking lymphocyte exit. It has been shown that $S1P_1$ agonist SEW2871, which causes transient $S1P_1$ 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 $S1P_1$ 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 $S1P_1$ receptor engagement and signal transduction, leading to distinct physiological responses. In particular, HDL-S1P was shown to induce association of $S1P_1$ 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 $S1P_1$ receptor - β-arrestin complexes (Galvani et al., 2015). The attractive concept of biased agonism, which posits that ligands can stabilize different GPCR 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 $S1P_1$ receptor, displays biased agonism and induces different pharmacological behaviors, suggested that similar properties might be discovered in synthetic $S1P_1$ receptor modulators. Such compounds could serve as valuable tools to further investigate $S1P_1$ receptor modulator-induced pharmacology at a signaling pathway level.

Here we describe the identification and structure-activity relationship of compounds with novel and unique $S1P_1$ receptor signaling characteristics. We established sensitive *in vitro* assays to quantify kinetic responses that are induced upon $S1P_1$ receptor activation and demonstrate that these novel compounds show an "apparent deficiency" in $G_{\alpha i}$ signaling, whilst retaining β-arrestin recruitment with high potency and efficacy, leading to effective $S1P_1$ receptor internalization and degradation. Using cellular assays in recombinant and primary human lymphatic endothelial cells, we determined

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that $G_{\alpha i}$ signaling influenced the rate of $S1P_1$ receptor internalization, whereas β-arrestin recruitment was necessary for effective internalization. *In vivo*, these novel $S1P_1$ receptor modulators displayed a delayed onset lymphopenia, a striking pharmocokinetic/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 $S1P_1$ receptor internalization, leading to efficient lymphocyte reduction by $S1P_1$ modulators *in vivo*.

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MATERIALS AND METHODS

Materials. S1P was purchased from Avanti Polar Lipids, Inc. 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. SEW2871 (5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole) was purchased from Cayman Chemical. All other $S1P_1$ receptor modulators described herein were synthesized by Actelion Pharmaceuticals Ltd. HMVEC-LLy, human lung lymphatic microvascular endothelial cells, were purchased from Lonza. Tango™ EDG1-*bla* U2OS cells were obtained from Invitrogen.

EPIC assay. 10'000 CHO cells stably expressing $hS1P_1$ receptor (CHO-$hS1P_1$) were seeded in 384-well EPIC® sensor microplates (Corning) and grown for 24 h in Ham's F12 medium supplemented with 10 % charcoal-treated FBS, 300 µg/ml geneticin, 100 U penicillin and 1 µg/ml streptomycin. The medium was removed, the wells filled with 40 µl assay buffer (HBSS with 20 mM Hepes, 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 µl of test compounds in assay buffer were added and a real-time DMR changes recorded for 30 minutes. The end data from EPIC® Analyser software (Corning) were exported to Microsoft Excel and used to determine compound potency with the IC_{50} 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. 5'000 Tango™ EDG1-*bla* U2OS cells were plated in 30 µl FreeStyle Expression Medium (Life Technologies) in black, clear-bottom 384-well plates and stimulated with 10 µl of test compounds for 16 h at 37°C in a humidified CO_2 incubator. Cells were then loaded with LiveBLAzer-FRET B/G substrate (Life Technologies) for 2 h at room temperature in the dark and the fluorescence emissions at 460 nm end 530 nm were measured with SynergyMX reader (BioTek)

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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 IC_{50} 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-$S1P_1$ receptor internalization assay. 30'000 HeLa cells stably expressing $hS1P_1$ receptor with the N-terminal SNAP-tag (HeLa-SNAP-$hS1P_1$) were seeded in gelatin-coated, black, clear-bottom 384-well plates and grown for 24 h in RPMI 1640 medium supplemented with 10% FBS, 1 mg/ml geneticin, 100 U penicillin and 1 µg/ml streptomycin. The medium was changed to 60 µl starvation medium (RPMI 1640 with 0.1 % fafBSA, 100 U penicillin and 1 µg/ml streptomycin) and incubated at 37°C in a humidified CO_2 incubator before addition of 20 µl of test compounds. After incubation with compounds for the indicated times, the medium was removed and the SNAP-tagged surface $S1P_1$ receptors labeled with SNAP-surface Alexa Fluor 488 substrate (1 µM; New England Biolabs) 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 µl ice-cold starvation medium and the fluorescence of the SNAP-labeled surface $S1P_1$ 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 $S1P_1$ receptor internalization or to determine compounds' potency at 16 h with the IC_{50} 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. 50'000 HMVEC-Lly were seeded into gelatin-coated 8-chamber slides (BD Falcon) and grown for 48 h in EGM-2MV medium (Lonza). After serum-starvation for 4 h, 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 PBS, 1 % BSA (bovine-

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serum albumin) using rabbit anti-h$S1P_1$ receptor antibodies (Santa Cruz Biotechnology) 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).

ERK and Akt activation assay. 10'000 HMVEC-Lly were seeded in gelatin-coated 12-well plates and grown for 48 h in EGM-2MV medium (Lonza). After serum-starvation for 4 h, the test compounds were added and incubated for the indicated times. Cells were washes with ice-cold PBS and lysed in RIPA lysis buffer (Sigma) supplemented with 10 mM NaF, 4 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT and 100 U/ml benzonaze. Equal protein amounts were separated on 4-12 % Bis-Tris polyacrylamide gel (Life Technologies) and transferred to nitrocellulose membranes for immunobloting. Phosphorylated ERK1/2, total ERK1/2, phosphorylated Akt and total Akt were detected with rabbit anti-phospho-p44/42 MAPK (Cell Signaling Technology), anti-MAP kinase 1/2 (Merck Millipore), anti-phospho-Akt (Ser473) (Cell Signaling Technology), and anti-Akt (Cell Signaling Technology) antibodies, respectively. As a secondary reagent, HRP-linked donkey anti-rabbit antibodies (Amersham Biosciences) were used. The Plus-ECL chemiluminescence detection substrate (Perkin Elmer) was used for detection. Comparative densitometry of immunoblots was performed on LAS400 imager (Fujifilm) using the Multi Gauge software (Fujifilm).

HMVEC-LLy chemotaxis assay. HMVEC-Lly were serum-starved for 2 h and then labeled with Calcein-AM (5 µg/ml; Life Technologies) for 30 minutes. The labeled cells were washed and resuspended in EBM-2 medium (Lonza) supplemented with 0.1 % fafBSA (Sigma) at 400'000 cells/ml. 50 µl cell suspensions were added to the top chamber of 96-well 8 µm FluoroBlok inserts (Corning) and combined with the bottom chambers containing 220 µl test compounds in EBM-2 medium supplemented with 0.1 % fafBSA. The plate was placed at 37°C in a humidified CO_2 incubator and the fluorescence of the migrated cells in the lower wells was measured after a given time (45 minutes, 2 h, 3 h and 4 h) at 485 nm excitation and 530 nm emission on a Cytofluor 4000 (Perseptive Biosystems).

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Measurement of lymphocyte count. Lymphocyte count was performed in male Wistar rats weighing 294 to 510 g (Harlan Laboratories Inc., Switzerland). The rats were housed in climate-controlled conditions with a 12 h light/dark cycle with free access to normal pelleted chow (Provimi Kliba SA, 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, 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 per group) or vehicle (n = 6 per group). Blood samples from rats were collected before and at 3, 6 and 24 h 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 Beckman Coulter 5diffCP hematology analyzer (Beckman Coulter).

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, the one-way analysis of variance (Anova) with Dunnett's post hoc test was performed. When the p value was < 0.05, the results were considered significant (GraphPad Prism 7 Software, San Diego, CA, USA).

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RESULTS

Synthetic $S1P_1$ receptor agonists with different potencies in $G_{\alpha i}$ signaling and β-arrestin recruitment.

To reveal potential differences in synthetic $S1P_1$ 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 $G_{\alpha i}$ protein-mediated and β-arrestin-mediated $S1P_1$ receptor signaling. A label-free resonant waveguide grating assay (EPIC®) was developed to measure in real-time $G_{\alpha i}$ protein-mediated responses in recombinant CHO-h$S1P_1$ cells (Nayler et al., 2010). The S1P-induced EPIC® signal increased rapidly and peaked 6 to 12 minutes after ligand addition. The signal subsequently decreased and for lower S1P concentrations reached baseline within 25 minutes after addition (Figure 1A). The S1P-induced EPIC® response was efficiently blocked with the $G_{\alpha i}$ protein inhibitor pertussis toxin (PTX) or the selective $S1P_1$ receptor antagonist TASP0277308 (Fujii et al., 2012) (Supplemental Figure 1), demonstrating that the observed responses in this assay were $S1P_1$ receptor specific and $G_{\alpha i}$ protein-mediated.

To assess $S1P_1$ receptor internalization kinetics, recombinant HeLa cells with stable expression of a SNAP-tagged-h$S1P_1$ receptor construct were employed using a fluorescence-based read-out. In this assay, ponesimod (Bolli et al., 2010) led to a potent and sustained 80-90 % reduction of cell surface residing $S1P_1$ receptors after 1 hour (EC_{50} ~8 nM) (Figure 1B), which confirmed our previous results using FACS analyses (Gatfield et al., 2014). β-arrestin recruitment to the $S1P_1$ 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 $S1P_1$ receptor agonists correlated very well with the potencies that were observed with the SNAP-tagged-h$S1P_1$ receptor internalization assay 16 hours after ligand addition (Supplemental Figure 2A), supporting that β-arrestin recruitment and receptor internalization were functionally linked. As expected, the $S1P_1$ agonist-induced responses at 16 hours in both assays, i.e. Tango™ and

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SNAP-tag, were insensitive to PTX treatment (Supplemental Figure 2B,C,D,E), suggesting that receptor internalization and β-arrestin recruitment could be uncoupled from $G_{\alpha i}$ protein signaling. The EPIC® and Tango™-β-arrestin assays were then used in parallel to screen a collection of proprietary synthetic $S1P_1$ receptor modulators. The prototypic synthetic $S1P_1$ 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 $G_{\alpha i}$ protein-mediated signaling within 30 minutes in the EPIC® assay. While ponesimod and SEW2871 at high concentrations (≥80 nM) reached their half maximal signal within 7-9 minutes (Figure 2A,B), the compound D3-2 required 14-15 minutes and after 30 minutes it remained considerably less potent (Figure 2C,D). Interestingly, a consistent structure-activity relationship could be established around these compounds (Table 1). The residue attached to position 5 of the thiophene moiety (R_1) 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 $G_{\alpha i}$ 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 R_2 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 $G_{\alpha i}$ protein-signaling (EPIC® assay) and β-arrestin recruitment (Tango™ assay), like compounds C2-2, C3-2 or D3-2 (Table 1). The apparent lower potency in $G_{\alpha i}$ protein-mediated signaling by these compounds was confirmed in classical GTPγS assay (Supplemental Table 1). This apparent deficiency in $G_{\alpha i}$ signaling neither compromised the activity of the compounds to internalize the $S1P_1$ receptor within 16 hours (Table 2) nor did it prevent $S1P_1$ receptor degradation (Supplemental Figure 3). These uniquely differentiated compounds were in terms of their functional profiles more similar to

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ponesimod, which potently activated $G_{\alpha i}$ signaling, β-arrestin recruitment, internalization and degradation (Table 2; Supplemental Figure 3) (Bolli et al., 2010; Gatfield et al., 2014) than to SEW2871, which potently activated $G_{\alpha i}$ signaling, but was >20-fold less potent than ponesimod in β-arrestin recruitment and in $S1P_1$ internalization (Table 2) and induced only limited degradation of $S1P_1$ (Gonzalez 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.

$G_{\alpha i}$ protein-coupling deficient $S1P_1$ agonists can maximally internalize $S1P_1$ receptors but at a slower rate.

We next studied the kinetics of $S1P_1$ receptor internalization and investigated representative compounds with different $G_{\alpha i}$ protein signaling and β-arrestin recruitment potencies (ponesimod, D2, D3, D3i, D3-2 and SEW2871) at different concentrations using the SNAP-tagged-h$S1P_1$ receptor assay. The amount of internalized $S1P_1$ receptor was measured at different times for up to 20 hours after compound addition (Figure 3). At the highest concentration (1 μM), which was well above the EC_{50} values of all compounds in $G_{\alpha i}$ protein signaling and β-arrestin assays, there was no obvious difference in the rate or magnitude of $S1P_1$ receptor internalization and all compounds induced almost 100 % internalization within 4 to 6 hours after compound addition (Figure 3A,D). However, at lower concentrations (i.e. 100 nM and 30 nM) different internalization rates were apparent (Figure 3B,C,D). Compounds D3i and D3-2 with the lowest potencies in $G_{\alpha i}$ protein signaling (EC_{50} 63.3 and 167.9 nM, respectively) and similar β-arrestin potencies (EC_{50} 0.7 and 0.9 nM, respectively) displayed the slowest rate of $S1P_1$ receptor internalization among these compounds (Figure 3D). Eventually, maximal $S1P_1$ receptor internalization was reached at all investigated concentrations, but only after prolonged incubation times. In contrast, SEW2871 always reached a plateau after 4 to 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 $S1P_1$ receptors, the potency in the $G_{\alpha i}$ signaling assay actually determines the rate of

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$S1P_1$ receptor internalization. Thus, for rapid and maximal $S1P_1$ receptor internalization a high potency in the $G_{\alpha i}$ signaling and in the β-arrestin assay is mandatory.

$S1P_1$ receptor signaling in lymphatic endothelial cells.

$S1P_1$ receptor activation is known to induce ERK1/2 and Akt phosphorylation (Igarashi et al., 2001), as well as induce cell chemotaxis through activation of $G_{\alpha i}$ protein signaling (Wang et al., 1999). We thus investigated these 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 non-recombinant cell system.

HMVEC-Lly were treated with 1 μM 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 (Figure 4). The phosphorylation induced by D3i was delayed and reached a maximum at 15 minutes after stimulation (Figure 4C and 4F), 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 (Figure 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 μM 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 $S1P_1$ receptors in HMVEC-Lly cells. To this end, HMVEC-Lly were incubated for different times with these compounds (1 μM), followed by immunofluorescence staining of $S1P_1$ receptors. As shown in Figure 6, ponesimod induced strong $S1P_1$ receptor translocation from the cell surface into intracellular compartments already after 15 minutes of incubation. $S1P_1$ internalization induced with compounds

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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 $S1P_1$ receptor-expressing HeLa cells. Altogether, these results show that also in native human cells $S1P_1$ 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 $S1P_1$ 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 $S1P_1$ modulators *in vivo*. As we could not observe major differences in the magnitude of $S1P_1$ 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 post-dosing (Figure 7B). The effect remained for at least 3 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 (Figure 7A,B). Surprisingly, we observed that compounds with attenuated $G_{\alpha i}$ signaling but with a similarly high potency in β-arrestin recruitment and $S1P_1$ receptor internalization assays as ponesimod, displayed an obvious disconnect between the pharmacodynamic (Figure 7B) and pharmacokinetic effects (Figure 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

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maximal effect on the lymphocyte count was reached later. Furthermore, a clear trend to lower maximal lymphocyte count reduction was seen with those compounds as 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 versus 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 $G_{\alpha i}$ protein signaling deficit, reached very high plasma concentrations already after 3 and 6 hours without any obvious signs of lymphocyte count reduction. Moderate lymphocyte count reduction (approx. 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 were similar. Furthermore, the compounds activated and desensitized rat $S1P_1$ receptors with similar potencies as the human $S1P_1$ receptors, as exemplified by the compound D3-2 (Supplemental Figure 4). These results suggest that for rapid onset and substantial blood lymphocyte count reduction *in vivo*, potent $G_{\alpha i}$ protein signaling and β-arrestin recruitment are both necessary.

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DISCUSSION

To current knowledge, $S1P_1$ 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 $S1P_1$ receptors mediating lymphocyte exit from lymph nodes has been demonstrated using different genetic and pharmacological approaches. It has been shown that $S1P_1$ deficient lymphocytes cannot egress from thymus or secondary lymphoid organs (Matloubian et al., 2004) and $S1P_1$ heterozygous lymphocytes egress more slowly than wild type lymphocytes (Lo et al., 2005). Pharmacological blockade of $S1P_1$ receptors with specific antagonists also induces lymphopenia *in vivo* (Quancard et al., 2012; Tarrason et al., 2011). Together, this led to the current thinking that $S1P_1$ receptor modulators induce lymphopenia through functional antagonism, following sustained $S1P_1$ 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 $S1P_1$ receptor modulators, since SEW2871 causes only transient $S1P_1$ receptor internalization and recycling, but is nevertheless lymphopenic *in vivo* (Jo et al., 2005). For example, lymphatic endothelial cells express high levels of $S1P_1$ 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 $S1P_1$ receptor modulator-induced lymphopenia remain to be fully elucidated.

We describe here the discovery of novel synthetic $S1P_1$ 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 it specifically aimed at the identification of compounds with

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deficiency in $G_{\alpha i}$ protein signaling and preserved potency and efficacy in β-arrestin recruitment and $S1P_1$ receptor internalization. We thus identified novel structure-activity relationships of synthetic $S1P_1$ receptor modulators with significantly reduced potency in $G_{\alpha i}$ 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 to 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 to non-biased agonists in models of acute cardiac injury (Kim et al., 2012). Together, such examples appear to support the hypothesis that G_{α} protein-mediated and β-arrestin-mediated signaling are dissociable and trigger distinct 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 GPCR conformations (Pupo et al., 2016), they may influence G_{α} protein dissociation rates (Furness et al., 2016), or show different thermodynamic properties in GPCR binding (Klein Herenbrink et al., 2016).

Nevertheless, using our novel $S1P_1$ 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 $S1P_1$ receptor internalization rate, without compromising $S1P_1$ receptor internalizing and degrading efficacy. With increasing compound concentration and consequently more $G_{\alpha i}$ protein signaling engagement, $S1P_1$ receptor internalization rate could be accelerated and the pharmacology became closer to the "classical" $S1P_1$ receptor modulators, such as ponesimod. Based on the *in vitro* data and adhering to the idea of functional $S1P_1$ receptor antagonism being responsible for lymphopenia, we thus anticipated a delayed onset of lymphopenia, without major consequences on maximal lymphocyte reduction *in vivo*. However, $S1P_1$ modulators with increasingly lower $G_{\alpha i}$

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protein signaling potency displayed a clear tendency for delayed lymphopenia, but unexpectedly, 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 $S1P_1$ receptors. Our data do not exclude that functional antagonism is necessary to induce fully efficacious lymphopenia by $S1P_1$ 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.* (Xu et al., 2013) who analyzed the correlation between *in vitro* potency testing of $S1P_1$ receptor modulators and peripheral lymphocyte count reduction. Interestingly, cAMP signaling, β-arrestin recruitment and $S1P_1$ 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 $S1P_1$ 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 $S1P_1$ receptors were replaced by internalization-deficient mutants (S5A-$S1P_1$), was also used to dissect the effects of $S1P_1$ receptor signaling and $S1P_1$ internalization in the context of lymphocyte homing (Thangada et al., 2010). In this system, mice with T cells expressing mutant S5A-$S1P_1$ 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

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the effects of mutant $S1P_1$ receptors were solely T cell driven. The genetic work shows that $S1P_1$ receptor internalization rates influence lymphocyte egress kinetics and that mutant-induced changes in $S1P_1$ receptor internalization alter lymphocyte egress kinetics in response to FTY720. Our findings using $S1P_1$ 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 to 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 $S1P_1$ receptor internalization *in vitro* and the notion that *in vivo* lymphopenia was observed to occur late, for example for D3-2 only after 24 h, when plasma concentrations were already low. This would allow lymphocytes to exit secondary lymph nodes before a substantial amount of $S1P_1$ 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 bigger reduction of blood lymphocyte count. Alternatively, other cell types, such as endothelial cells, expressing $S1P_1$ receptors could contribute in the exit process through $S1P_1$ receptor-induced and $G_{\alpha i}$-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 $S1P_1$ receptor modulators.

In summary, our study demonstrates that $S1P_1$ receptor modulator-induced $G_{\alpha i}$-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 $S1P_1$ receptor

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modulators induce lymphopenia. Unraveling those mechanisms might facilitate the discovery of novel $S1P_1$ receptor modulators with unique signaling properties to treat human disease.

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FOOTNOTES

The authors declare that they are all employees of Idorsia Pharmaceuticals Ltd.

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Disclosures: All experiments described in this report have been conducted in the research facilities of Actelion Pharmaceuticals Ltd. and all authors acknowledge that the work was performed as employees of Actelion Pharmaceuticals Ltd. During manuscript review, Actelion Pharmaceuticals Ltd. was acquired by Johnson & Johnson, and its drug discovery and early development activities subsequently transferred into a newly created company, Idorsia Pharmaceuticals Ltd.

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FIGURE LEGENDS

Figure 1. Assays used to measure $S1P_1$ agonist-induced signaling

Real-time EPIC traces of CHO cells stably expressing $S1P_1$ receptor and stimulated with increasing concentrations of S1P (A). SNAP surface staining of Hela-SNAP-h$S1P_1$ cells treated with increasing concentrations of ponesimod (B).

Figure 2. Real-time EPIC traces induced by synthetic $S1P_1$ receptor agonists.

CHO-h$S1P_1$ 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).

Figure 3. $S1P_1$ receptor internalization kinetics.

SNAP surface staining of Hela-SNAP-h$S1P_1$ cells treated with 1 µM (A), 100 nM (B) and 30 nM (C) of $S1P_1$ agonists. Shown is one representative experiment done in triplicates. % internalization after 2 h for each $S1P_1$ agonist was plotted as mean ± sdev (D); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to internalization induced by ponesimod.

Figure 4. ERK1/2 and Akt phosphorylation upon stimulation with $S1P_1$ agonists in HMVEC-Lly.

HMVEC-Lly were stimulated with 1 µM ponesimod (pon), compound D3i, compound D3-2 or vehicle (VH) for the indicated times and cell extracts 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 to vehicle-treated cells (C and F).

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Figure 5. HMVEC-Lly chemotaxis upon stimulation with $S1P_1$ agonists.

Chemotactic responses induced by the indicated nM concentrations of $S1P_1$ agonists or vehicle (VH) were measured after 45 minutes by transwell migration assays. Shown is one representative experiment done in duplicates. Mean ± sdev; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Figure 6. $S1P_1$ internalization upon stimulation with $S1P_1$ agonists in HMVEC-Lly.

HMVEC-Lly were stimulated with 1µM ponesimod, compound D3i, compound D3-2 or vehicle (VH) for the indicated times and immunofluorescence of $S1P_1$ receptors (red), F-actin (Phalloidin, green) and nuclei (Hoechst 33342, blue) performed.

Figure 7. Disconnection of pharmacokinetics and pharmacodynamics of $G_{\alpha i}$ protein-coupling deficient $S1P_1$ agonists in rats.

Plasma concentrations of ponesimod (pon), compound D2, compound D3, compound D3i and compound D3-2 at 3, 6 and 24 h after single oral administration of 10 mg/kg of each compound to rats (A) and reduction of the peripheral lymphocyte numbers in % (B). Values are means (*n=6*); 6 rats were dosed with each compound.

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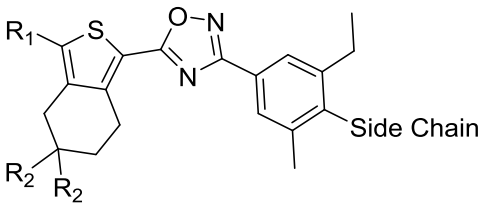
TABLES

Table 1. Structure-activity relationship of Side Chain and Alkyl Chain (R)



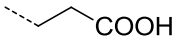
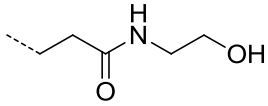
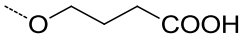
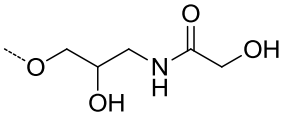
		EPIC EC_{50} (nM)								
		Tango EC_{50} (nM)								
		R_2 = Methyl							R_2 = Ethyl	
		R_1							R_1	
Side Chain	H	Methyl	Ethyl	n-Propyl	iso-Propyl	n-Butyl	iso-Butyl	Cyclo pentyl	Ethyl	n-Propyl
	**A0**	**A1**	**A2**	**A3**	**A3i**	**A4**	**A4i**	**A5c**	**A2-2**	**A3-2**
	30	6	10	21	38	41	74	113	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

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Table 2. Representative compounds with potency differences in $G_{\alpha i}$ and β-arrestin signaling

	β-arrestin Tango			**$G_{\alpha i}$ EPIC**			**SNAP-S1P$_1$ internalization**		
	geomean	*geosdev*	*n*	geomean	*geosdev*	*n*	geomean	*geosdev*	*n*
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*

EC_{50} (nM)

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FIGURES

Figure 1

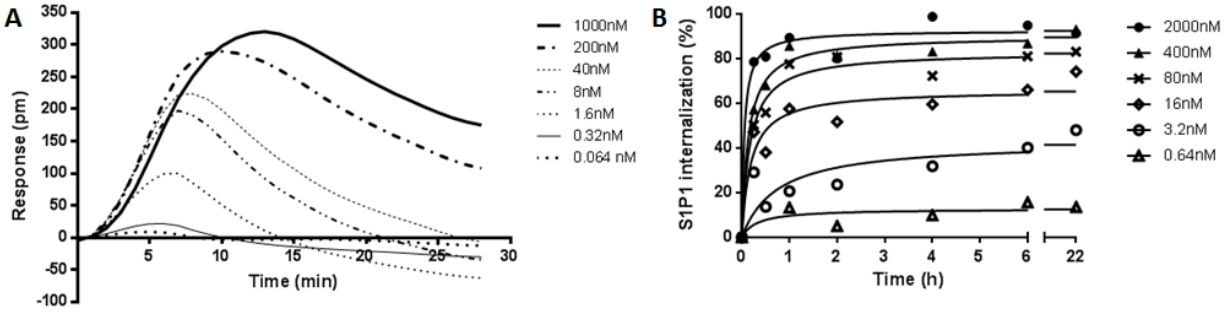

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Figure 2

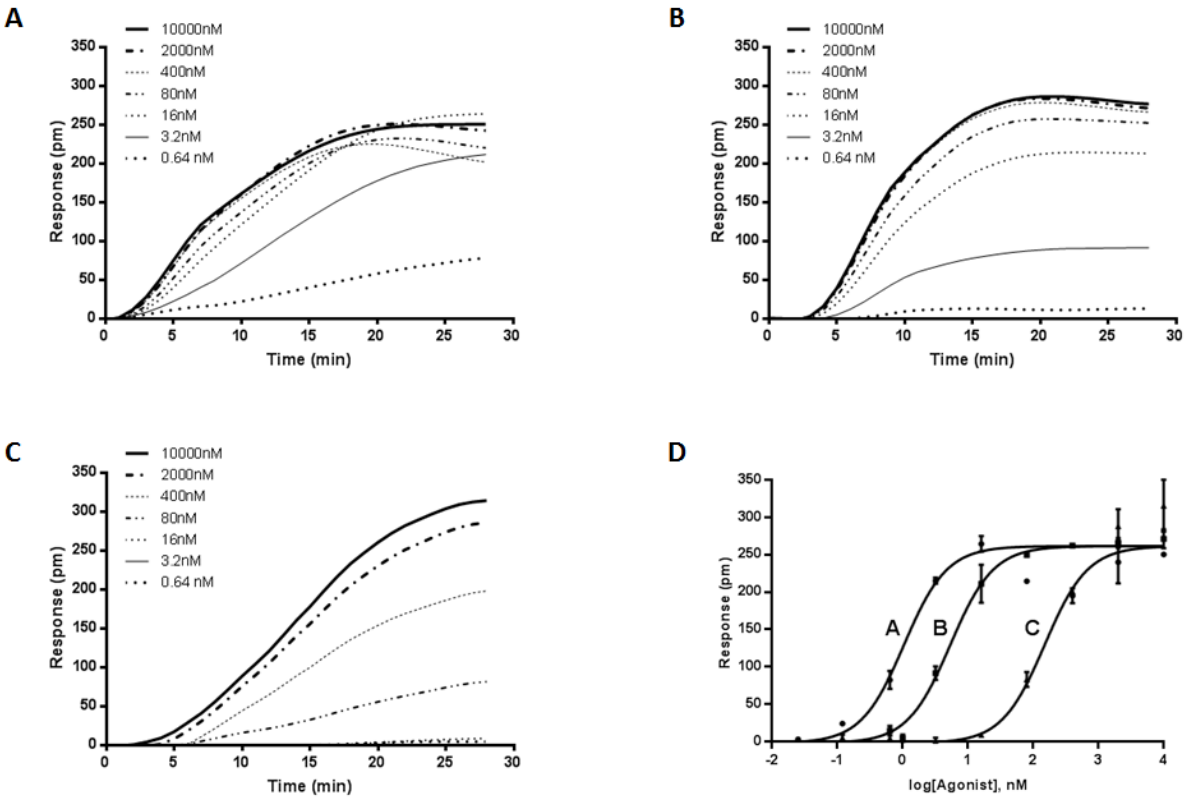

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Figure 3

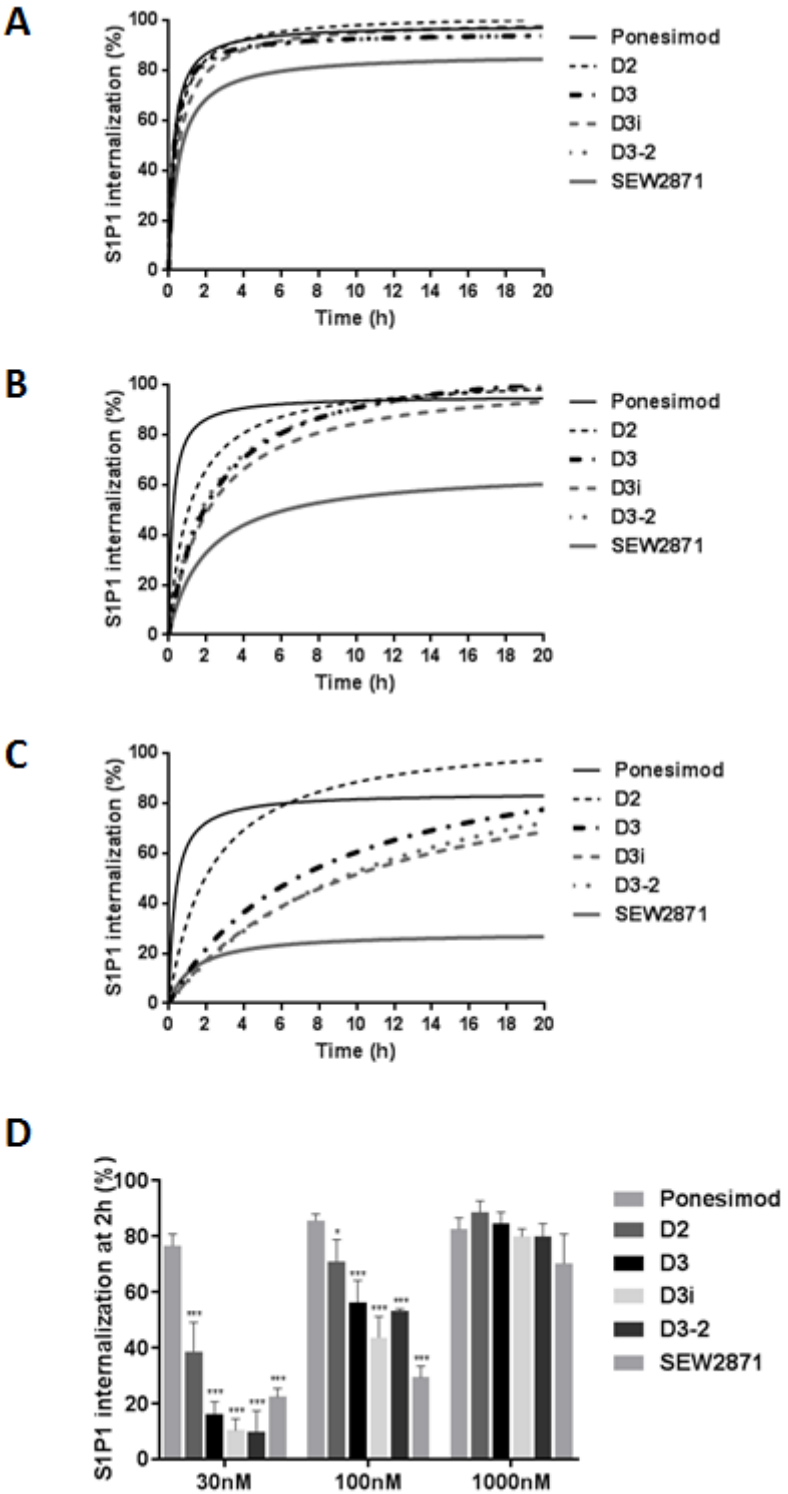

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Figure 4

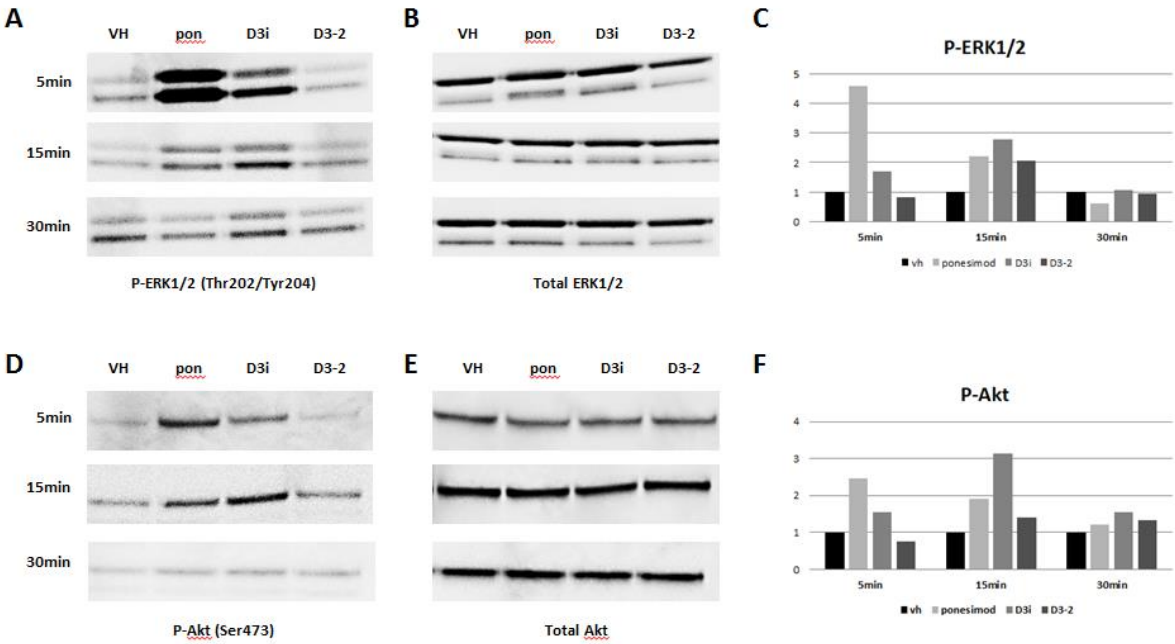

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Figure 5

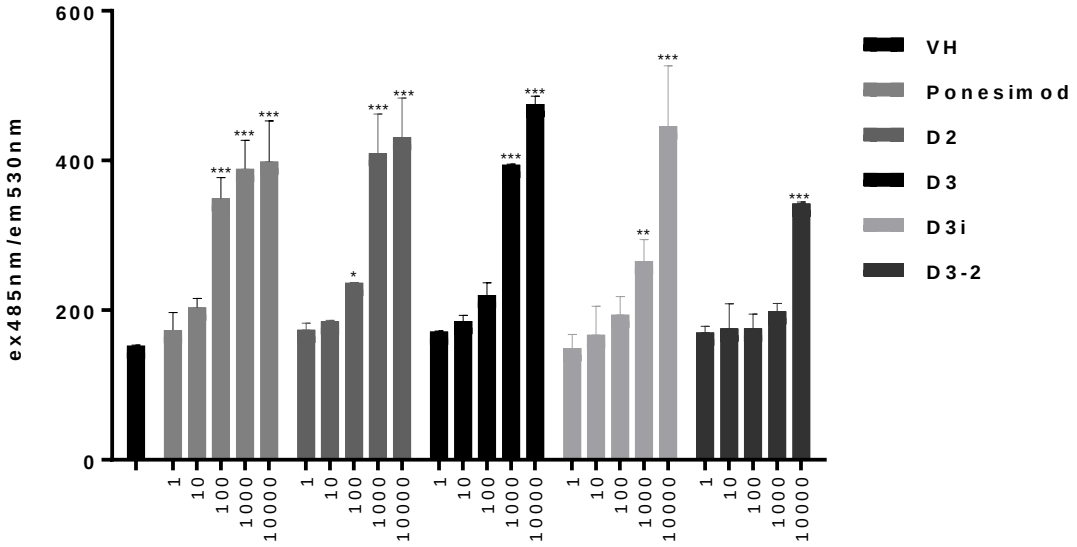

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Figure 6

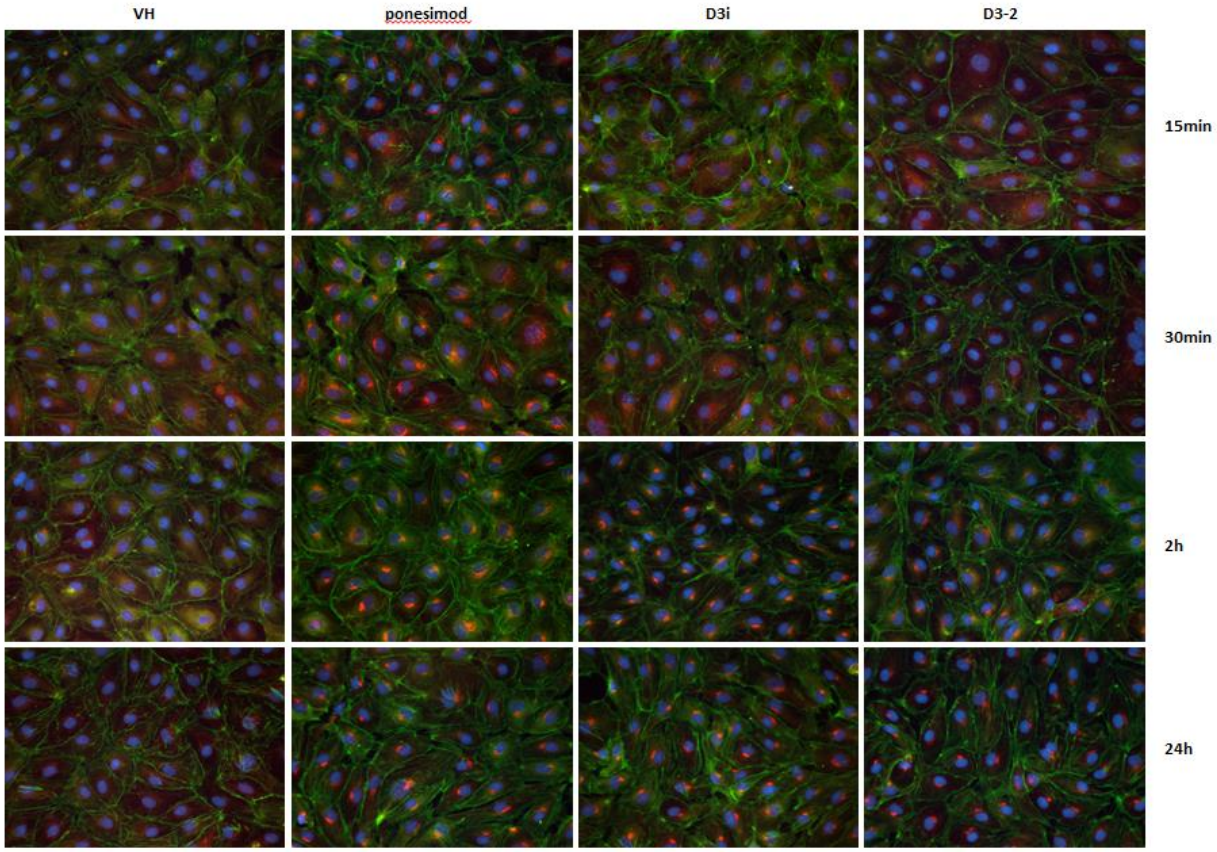

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Figure 7