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Sphingosine 1-Phosphate (S1P) Receptor Subtypes $S1P_1$ and $S1P_3$, Respectively, Regulate Lymphocyte Recirculation and Heart Rate*

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Sphingosine 1-phosphate (S1P) influences heart rate, coronary artery caliber, endothelial integrity, and lymphocyte recirculation through five related high affinity G-protein-coupled receptors. Inhibition of lymphocyte recirculation by non-selective S1P receptor agonists produces clinical immunosuppression preventing transplant rejection but is associated with transient bradycardia. Understanding the contribution of individual receptors has been limited by the embryonic lethality of the $S1P_1$ knock-out and the unavailability of selective agonists or antagonists. A potent, $S1P_1$-receptor selective agonist structurally unrelated to S1P was found to activate multiple signals triggered by S1P, including guanosine 5′-3-*O*-(thio)triphosphate binding, calcium flux, Akt and ERK1/2 phosphorylation, and stimulation of migration of $S1P_1$- but not $S1P_3$-expressing cells *in vitro*. The agonist also alters lymphocyte trafficking *in vivo*. Use of selective agonism together with deletant mice lacking $S1P_3$ receptor reveals that agonism of $S1P_1$ receptor alone is sufficient to control lymphocyte recirculation. Moreover, $S1P_1$ receptor agonist plasma levels are causally associated with induction and maintenance of lymphopenia. $S1P_3$, and not $S1P_1$, is directly implicated in sinus bradycardia. The sustained bradycardia induced by S1P receptor non-selective immunosuppressive agonists in wild-type mice is abolished in $S1P_3$−/− mice, whereas $S1P_1$-selective agonist does not produce bradycardia. Separation of receptor subtype usage for control of lymphocyte recirculation and heart rate may allow the identification of selective immunosuppressive $S1P_1$ receptor agonists with an enhanced therapeutic window. $S1P_1$-selective agonists will be of broad utility in understanding cell functions *in vitro*, and vascular physiology *in vivo*, and the success of the chemical approach for $S1P_1$ suggests that selective tools for the resolution of function across this broad lipid receptor family are now possible.

Sphingosine 1-phosphate (S1P),[1] through its high affinity G-protein-coupled receptors, is a physiological mediator with widespread effects upon multiple physiological systems (1). It regulates heart rate (2), coronary artery blood flow (3), blood pressure (4), endothelial integrity in lung (5, 6) and most recently has been shown to regulate the recirculation of lymphocytes (7–11). Many of the physiologically relevant functions occur in the low nanomolar range, including activation of endothelial nitric oxide synthase (12, 13), vasorelaxation (14), and inhibition of thymic egress and lymphocyte recirculation (11). Free plasma levels of S1P are tightly regulated by protein binding to albumin and high density lipoprotein to avoid the deleterious effects of systemic S1P receptor subtype activation at high concentrations of ligand, such as bradycardia and coronary artery vasospasm (3, 15). The choice of S1P, through its receptors, as an acute regulator of the number of blood lymphocytes may represent an interesting evolutionary choice by the immune system, which evolved after the circulatory system. The pleiotropic responses to S1P, mediated by distinct receptor subtypes in a tissue-specific manner, require the definition of the minimal receptor activation and minimal signals necessary and sufficient to regulate these key physiological functions. Advances in understanding the contribution of individual receptors has been limited by the current unavailability of selective agonists or antagonists. Although $S1P_2$ and $S1P_3$ were successfully deleted in mice (16, 17), deletion of the $S1P_1$ gene is embryonic lethal at day 13.5 (18), due to the failure of vascular maturation. New tools that elucidate biological functions of receptor subtypes are needed.

S1P receptor function in the immune system has experimental advantages in approaching selective S1P receptor function at vascular interfaces. The maintenance of efficient immune responses depends upon maximizing the probabilities of T lymphocytes encountering their cognate antigens (7). The dispersed anatomy of the immune system requires recirculation of naive lymphocytes, which emerge from their organs of primary lymphopoiesis (thymus and bone marrow), traversing the blood, entering secondary lymphoid organs across high endothelium, and finally crossing sinus-lining endothelium to return to the blood stream via lymph and thoracic duct (19–22). S1P receptors play a critical role as the molecular gatekeeper regulating lymphocyte recirculation. Relatively non-selective S1P receptor agonists active on $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ but not $S1P_2$, such as the phosphate-ester of the immunomodulatory prodrug FTY720, induce rapid lymphopenia by inhibiting the egress of lymphocytes from lymph nodes into lymph (9), as

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[1] The abbreviations used are: S1P, sphingosine 1-phosphate; hS1P, human S1P; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; SEW2871, 5-(4-phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-(1,2,4)-oxadiazole; CHO, Chinese hamster ovary; BSA, bovine serum albumin; FLIPR, Fluorescence Imaging Plate Reader; FACS, fluorescence-activated cell sorting; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; GC-MS, gas chromatography-mass spectrometry; AFD-(R), phosphate ester of AAL; AAL-(R), 2-amino-4-(4-heptyloxyphenyl)-2-methyl butanol.

well as from thymus into blood (11, 23). Inhibition of lymphocyte egress is associated with clinically useful immunosuppression in both transplantation and autoimmune disease models (24–26). Pleiotropic responses at low nanomolar plasma concentrations is seen in this system, because FTY720 mediates both lymphopenia and a transient dose-dependent bradycardia on initial dosing in humans (27). The therapeutic window for S1P receptor agonists may therefore depend on the association of single receptors with critical functions.

We have combined a chemical approach with the use of S1P receptor null mice to help define receptor selectivity. We chose a chemical approach for S1P$_1$, because of the absence of the knock-out. Published data on FTY720 phosphonate (respective IC_{50} values for human S1P$_1$ (8.2 nM), S1P$_2$ (>10,000 nM), S1P$_3$ (151 nM), S1P$_4$ (33 nM), and S1P$_5$ (178 nM)) suggested that S1P$_1$ is responsible for inhibition of lymphocyte egress (9), a fact that was subsequently strengthened by structure-activity correlations among a collection of semi-selective S1PR agonists (43, 44).[2] We now show that the discovery of selective S1P receptor agonists is useful in demonstrating that selective biochemical signals can regulate complex *in vivo* biology.

EXPERIMENTAL PROCEDURES

S1P Receptor Agonists

AFD-(R) was the kind gift of Novartis Pharma (Basel, Switzerland, Volker Brinkmann). 5-(4-Phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-(1,2,4)-oxadiazole was purchased from Maybridge (Tintagel, Cornwall).

Cells and Plasmids

CHO cells stably expressing human S1P receptors (hS1P) hS1P$_1$, hS1P$_2$, hS1P$_3$, hS1P$_4$, and hS1P$_5$, were kindly provided by Danilo Guerini (Novartis Pharma).

Membrane Preparations

Membranes were prepared from CHO cells expressing human or murine S1P$_1$, S1P$_2$, S1P$_3$, S1P$_4$, and S1P$_5$, for use in ligand and [^{35}S]GTPγS binding studies as described previously (9) and suspended in Buffer B with 15% glycerol and stored at −80 °C.

Agonist Assays

Measurements of [^{35}S]GTPγS Binding—Serial dilutions of S1P (diluted in 4% BSA) or SEW2871 (diluted in Me_2SO) were added to membranes (1–10 μg of protein/well) and assayed as described (9).

Measurements of Ca^+ Flux—Calcium flux assays in the Fluorescence Imaging Plate Reader (FLIPR; Molecular Devices) format were performed as described (9). The assay was initiated by transferring an equal volume of ligand to the cell plate, and calcium flux was recorded over a 3-min interval. Cellular response was quantitated as maximal peak height by averaging triplicate wells and expressing as percent response relative to S1P activation without pretreatment. Spleen cells, after lysis of erythrocytes with 0.17 M NH_4Cl, were separated by adherence to tissue-culture plastic and adherent (stromal cells, macrophages, and neutrophils), and non-adherent (lymphocytes) were assayed for calcium flux in response to SEW2871 or S1P or ionomycin separately. Fluorescence intensity is an absolute measure of fluorescence emission upon laser excitation. Flow cytometric measurement of calcium flux was performed on cells isolated by teasing apart lymph nodes to single cell suspensions, followed by loading with Fluo-3 (Molecular Probes, Cell-permeant fluorescence dye 3, calcium binding, 1 μM at 4×10^8 viable cells/ml). Cells were then labeled with non-activating antibodies to CD4 (CD4-PE, BD Pharmingen) and CD8 (CD8-PE), respectively, in the presence of propidium iodide. Absolute fluorescence intensity over a four-log scale is the standard method for comparisons of fluorescence intensity by FACS. Agonist challenge with 1 μM SEW2871, S1P, or ionomycin (Sigma) at 37 °C was performed in a temperature-controlled FACSCalibur flow cytometer (BD Bioscience, Mountain View, CA). FACS events were collected using CELLQUEST software (BD Bioscience), and then analyzed with FLOWJO (Treestar, San Carlos, CA). FACS events were collected for 30 s, and then ionomycin, S1P, or SEW2871 were added and events were collected for an additional 10 min. The ratio fluorescence at 420 nm to that at 510 nm was used to measure calcium flux of propidium iodide-negative cells.

Western Blotting of S1P-activated Kinases

Control CHO cells and the CHO cells stably transfected with human S1P$_1$ or S1P$_3$ were cultured to 50% confluence on 6-well plate in complete RPMI 1640 supplemented with 10% fetal bovine serum. Cells were serum-starved for 16 h and stimulated with SEW2871 diluted to various concentrations in the serum-free medium with 0.1% fatty acid-free BSA. At 5 min, cells were lysed in 50 mM Tris, pH 8.0, 125 mM NaCl, 20 mM CHAPS, 2 mM dithiothreitol, 1 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture. Cell lysates were analyzed by Western blotting after separation on 10% SDS-PAGE using mouse monoclonal anti-phospho-ERK1/2 antibody (sc-7383; Santa Cruz Biotechnology) and rabbit polyclonal anti-phospho-Akt antibody (BD Biosciences). Total ERK1 and ERK2 were detected using a rabbit affinity-purified polyclonal anti-ERK antibody (sc-94; Santa Cruz Biotechnology), and total Akt was detected using a rabbit affinity-purified polyclonal anti-Akt1 antibody (BD Biosciences). Band intensities corresponding to pERK1, pERK2, and pAkt were quantitated by imaging (Kodak 1D Scientific Imaging Systems). Amounts of pERK1/2 and pAkt were normalized for the total amounts of ERK1/2 and Akt. Primary lymph node lymphocytes were teased from the peripheral and mesenteric nodes of C57 BL6 mice kept rigorously at 4 °C. Cells were warmed to 37 °C, stimulated for 5 min with S1P or SEW2871 (50 nM and 500 nM), or 50 ng/ml phorbol myristate acetate or vehicle, and Akt and ERK phosphorylation was determined as above.

Assay of S1P Receptors-dependent Cell Migration

Cell adhesion and migration assays were performed as follows. Cells expressing CHO, CHO-S1P$_1$, or CHO-S1P$_3$ were starved overnight in regular medium without fetal bovine serum prior to migration assay. Cell migration assays were performed using modified Boyden chambers (tissue culture-treated, 6.5-mm diameter, 10-μm thickness, 8-μm pores, Transwell®; Costar Corp., Cambridge, MA) containing polycarbonate membranes coated on the underside of the membrane with 5 μg/ml fibronectin in PBS for 2 h at 37 °C, rinsed once with PBS, and then placed into the lower chamber containing 500 μl of migration buffer (RPMI with 0.5% BSA; Invitrogen, San Diego, CA). Serum-starved cells were removed from culture dishes with Hanks' balanced salt solution containing 5 mM EDTA and 25 mM Hepes, pH 7.2, and 0.01% trypsin, washed twice with migration buffer, and then resuspended in Migration buffer (10^6 cells/ml). 75,000 cells were then added to the top of each migration chamber and allowed to migrate to the underside of the top chamber for 3 h in the presence or absence of either S1P or SEW2871 (100 nm or 1 μM), which had been added to the lower chamber. The non-migratory cells on the upper membrane surface were removed with a cotton swab, and the migratory cells attached to the bottom surface of the membrane were fixed with 4% paraformaldehyde and stained with propidium iodide (1 μg/ml) in PBS for 20 min at room temperature. The number of migratory cells per membrane was evaluated by looking at five different fields with an inverted microscope using a 40× objective. Each determination represents the average of three individual wells. In control experiments, cell migration on vehicle control was less than 0.01% of the total cell population.

Pharmacokinetic Analysis

All samples were analyzed after $CHCl_3$ extraction, evaporation to dryness, and redissolution in 0.1 ml of $CHCl_3$, followed by splitless injection on an Agilent 6890N gas chromatograph. Sample detection was carried out by using a 5973 mass-selective detector with single ion monitoring at 440 *m/z* for SEW2871 and 372 *m/z* for a spiked and structurally related internal standard, SEW2898. Limit of quantitation was 0.4 ng/μl in plasma, based on spikes into human serum. Sample amounts were determined by comparison to a standard curve, R^2 = 0.99. Non-compartmental pharmacokinetic analysis of plasma levels was performed by using PK Solutions 2.0 software (Summit Research Services, Montrose, CO).

Induction of Lymphopenia in Mice

C57BL6 or S1P$_3$−/− mice (16) or their S1P$_3$+/+ litter mate controls were gavaged with increasing doses of SEW2871 or vehicle (10% Me_2SO/25% Tween 20 v/v), and blood collected into EDTA tubes (BD Biosciences). Full blood counts were determined by veterinary autoanalyzer calibrated for mouse blood (H2000, CARESIDE, Culver City, CA) at times stated as described previously (9). All animal studies were approved by the Institutional Animal Care and Use Committee.

[2] S. Mandala, J. Hale, R. Hajdu, and H. Rosen, unpublished results.

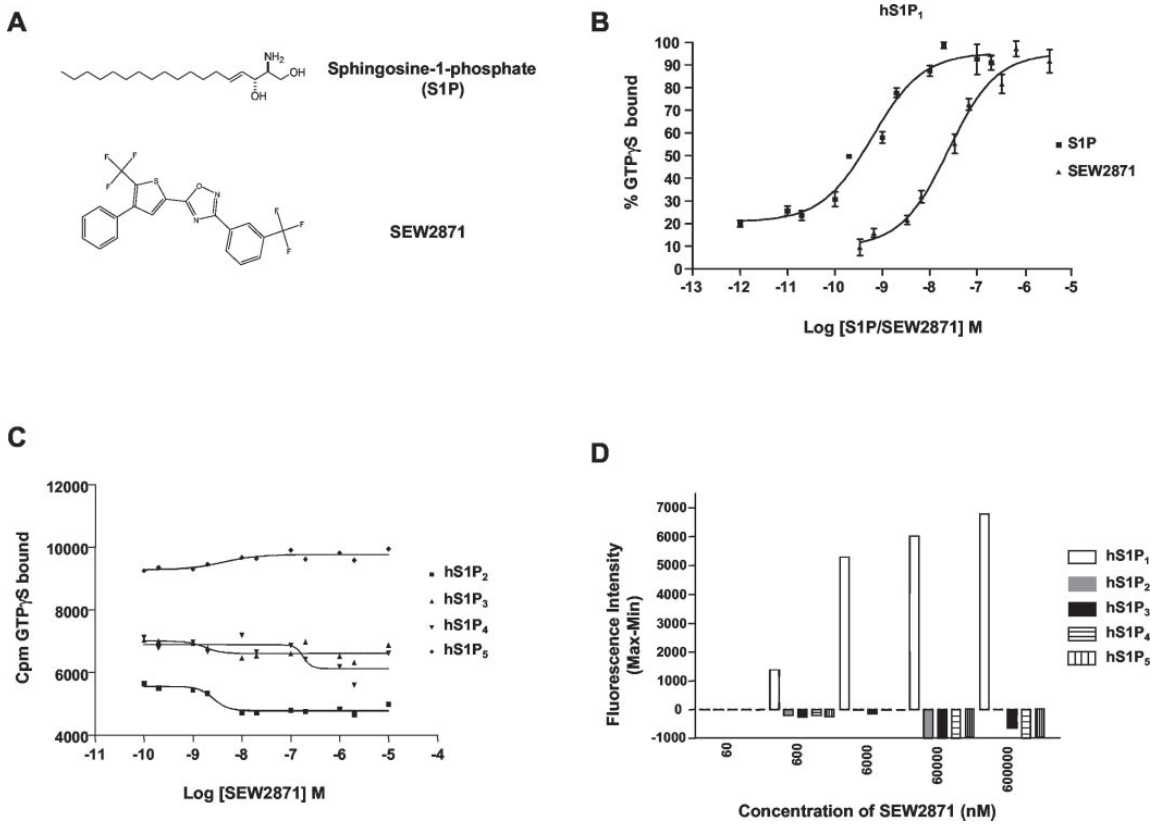

FIG. 1. **SEW2871 is a selective agonist for hS1P$_1$ receptor.** *A*, structures of S1P and SEW2871. *B*, S1P and SEW2871 activation of hS1P$_1$ receptor. CHO cell membranes expressing stably transfected hS1P$_1$ receptor were tested for agonism in a GTPγS binding assay. SEW2871 (*upright triangles*, ▲) was compared with the physiological ligand S1P (*closed squares*, ■) and results normalized to percentage of GTPγS induced at maximal S1P concentrations. EC$_{50}$ values (mean ± S.D.; *n*) were 13.8 ± 8.3 nM (*n* = 3) for SEW2871 and 0.4 ± 0.24 nM (*n* = 6) for S1P. *C*, SEW2871 does not agonize hS1P$_{2-5}$. CHO cell membranes stably expressing one of hS1P$_{2-5}$ were assayed for ligand-induced GTPγS binding. All receptor EC$_{50}$ values for S1P are shown in the text. SEW2871 had no effect on hS1P$_3$ (*closed squares*, ■); hS1P$_2$ (*inverted triangles*, ▼); hS1P$_4$ (*upright triangles*, ▲); hS1P$_5$ (*diamonds*, ♦) at concentrations up to 10 μM. *D*, SEW2871 induced a concentration-dependent ligand-dependent calcium flux on hS1P$_1$ (*clear bars*, □) but not hS1P$_{2-5}$ in a FLIPR format assay.

Histology

C57Bl/6 mice were gavaged with 0.1 ml of vehicle or SEW 2871 (10 mg/kg). Sixteen hours later, mesenteric and inguinal lymph nodes were fixed in 10% formalin in PBS and paraffin-embedded, and 5-μm sections were stained with hematoxylin and eosin. Images were acquired by Metamorph software on an Olympus AX70 microscope.

Measurement of Heart Rate in Conscious Mice

Effects on heart rate in S1P$_3$−/− or wild type littermates or C57BL6 controls were measured by ECG analysis in conscious mice using the ANONYmouse ECG screening system (MouseSpecifics, Boston, MA), before and after injection of the non-selective S1P receptor agonist AFD-(R) or vehicle control. No difference between WT littermates and C57BL6 mice were seen.

RESULTS

High Throughput Screening Identifies S1P$_1$-selective Agonists—Published binding studies on hS1P$_1$ with FTY720 and FTY-P (9), as well as mutagenesis and modeling with natural ligand S1P (28, 29), suggested a two-site binding model. The hydrophobic-aromatic residues bind within receptor transmembrane domains and the ligand headgroups form salt bridges with glutamate and arginine side chains. Specifically, FTY720 has a measurable IC$_{50}$ (300 nM) for S1P$_1$ that is enhanced 1000-fold by the enantioselective addition of the phosphate ester (9). FTY720 binding implies that G-protein-coupled receptor privileged structures, structurally unrelated to S1P, could likely access the transmembrane site as agonists, with sequence differences between receptor subtypes making the discovery of selective agonists probable (30). Indeed, such agents, including the featured compound in this report, have previously been identified and characterized (43).[2]

SEW2871 Activates Signals and Responses through S1P$_1$ Alone Comparable to S1P in GTPγS Activation, Calcium Flux, Kinase Phosphorylation, and Cell Migration—5-(4-Phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-(1,2,4)-oxadiazole (SEW2871) (Fig. 1*A*) is a novel selective agonist for hS1P$_1$ structurally unrelated to S1P. Unlike S1P, it has no solubilizing or charged headgroups. S1P showed 50% maximal receptor activation in the GTPγS binding assays (EC$_{50}$) of 0.4 ± 0.24 nM (mean ± S.D.; *n* = 6) on human S1P$_1$ (hS1P$_1$), whereas EC$_{50}$ values for SEW2871 on hS1P$_1$ were 13 ± 8.58 nM (mean ± S.D.; *n* = 3) (Fig. 1*B*). Like S1P, SEW2871 was a full agonist with levels of receptor activation comparable to S1P (Fig. 1*B*). Although S1P is a non-selective agonist with EC$_{50}$ values (mean ± S.D.) of 3.8 ± 3.5 nM (hS1P$_2$; *n* = 4), 0.6 ± 0.35 nM (hS1P$_3$; *n* = 6), 67 ± 13 nM (hS1P$_4$; *n* = 4), 0.5 ± 0.39 nM (hS1P$_5$; *n* = 3) on the respective human receptors, SEW2871 was inactive at 10,000 nM on hS1P$_2$, hS1P$_3$, hS1P$_4$, and hS1P$_5$ (Fig. 1*C*).

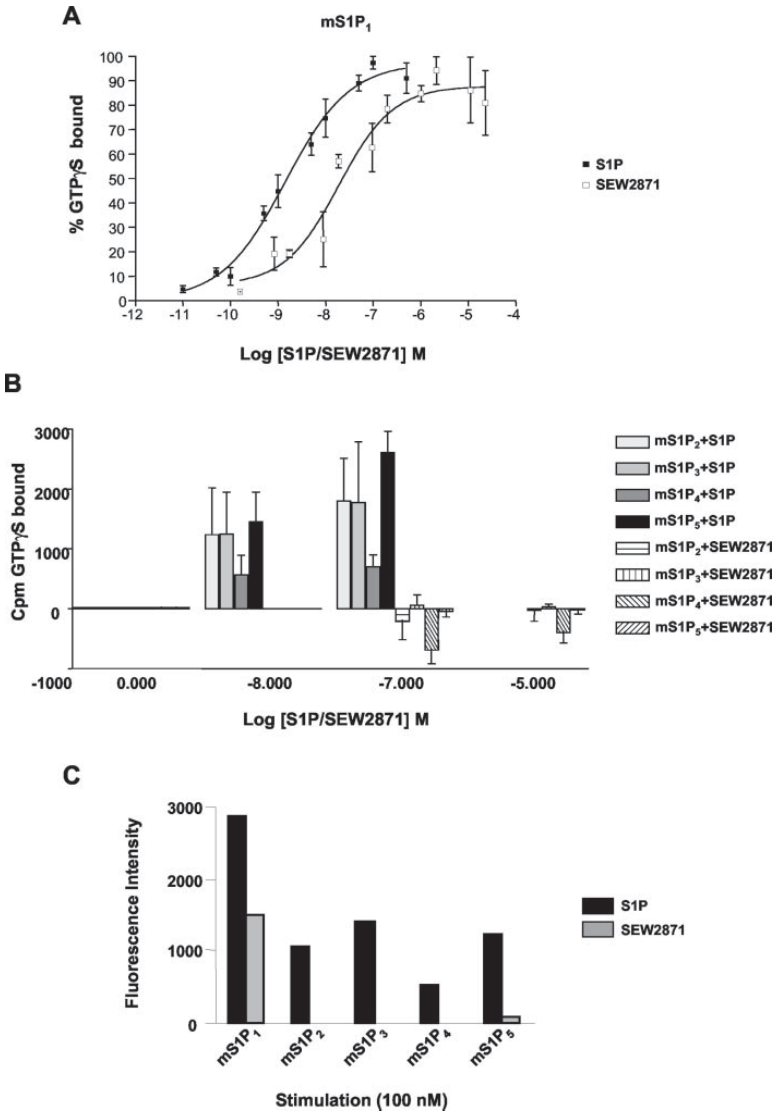

FIG. 2. **SEW2871 is a selective agonist for mS1P$_1$ receptor.** *A*, S1P and SEW2871 activation of mS1P$_1$ receptor. CHO cell membranes expressing transiently transfected mS1P$_1$ receptor were tested for agonism in a GTPγS binding assay. SEW2871 (*open squares*) was compared with the physiological ligand S1P (*closed squares*) and results normalized to percentage of GTPγS induced at maximal S1P concentrations. EC$_{50}$ values were 20.7 nM for SEW2871 and 1.4 nM for S1P. *B*, SEW2871 is not an agonist on mS1P$_{2-5}$. CHO cell membranes transiently expressing one of mS1P$_{2-5}$ were assayed for ligand-induced GTPγS binding. Responses to S1P (see figure for *symbol key*) are shown at 10 and 100 nM for: mS1P$_2$, mS1P$_3$, mS1P$_4$, and mS1P$_5$, respectively. SEW2871 had no effect on mS1P$_2$, mS1P$_3$, mS1P$_4$, and mS1P$_5$ at concentrations up to 10 μM. *C*, comparison of calcium flux stimulation on the murine S1P receptors by 100 nM S1P (*black columns*) or SEW2871 (*gray column*) by FLIPR. (Only mS1P$_1$ showed a significant calcium flux to SEW2871.) Fluorescence intensity is intended as the absolute measure of fluorescence emission upon laser excitation.

We confirmed full selective agonism for hS1P$_1$ alone in the ligand-dependent calcium flux assay (Fig. 1*D*) for SEW2871 in stably transfected CHO cell lines, with no significant activation of hS1P$_{2-5}$ (Fig. 1*D*) up to ≥ 10 μM. We found evidence for selective but full agonism of murine S1P$_1$ (mS1P$_1$), EC$_{50}$ = 20.7 nM (Fig. 2*A*), with no activity at 10 μM on mS1P$_{2-5}$ (Fig. 2*B*). EC$_{50}$ values for S1P on the transiently transfected murine receptors S1P$_{1-5}$ were 1.4 nM (mS1P$_1$), 2.0 nM (mS1P$_2$), 2.3 nM (mS1P$_3$), 75 nM (mS1P$_4$), and 16 nM (mS1P$_5$), respectively. Identical selectivity was seen both in membranes of CHO cells transiently transfected with the respective murine receptors and assayed for agonism in GTPγS binding assays, as well as in intact cells in calcium flux assays (Fig. 2*C*). Pretreatment with 30 ng/ml pertussis toxin in the GTPγS assay fully inhibited GTPγS binding induced by either S1P or SEW2871, confirming that SEW2871 is also acting through the G$_i$-coupled receptor.

We also compared kinase phosphorylation in response to S1P and SEW2871 stimulation in both S1P$_1$ and S1P$_3$ CHO cell lines (Table I). Substantial ligand concentration-dependent pAKT and pERK1 signals were induced by SEW2871 in S1P$_1$ but not S1P$_3$ CHO cells, whereas modest phosphorylation of pERK2 was also seen. In contrast, S1P activated kinases in both cell lines equally (not shown).

The multiple signals induced by SEW2871 are sufficient to replicate complex functional responses of S1P through S1P$_1$. In a Transwell migration assay, SEW2871 (Fig. 3*B*) and S1P (Fig. 3*E*) induced equivalent cell migration *in vivo* in S1P$_1$-CHO cells with obvious morphology for stimulation of cytoskeletal rearrangements. Minimal cell migration or cytoskeletal reorganization occurred in response to either S1P or SEW2871 in untransfected CHO cells (<0.01% of cell migrated) (Fig. 3, *A* and *D*), whereas S1P$_3$ CHO cells migrated and changed shape in response to S1P (Fig. 3*C*) but not SEW2871 (Fig. 3*F*), confirming the selectivity of SEW2871. Despite its structural dissimilarities to S1P, and lack of headgroups, SEW2871 is a selective low nanomolar full agonist of S1P$_1$ in all biochemical parameters and one complex cellular behavior tested, and could potentially be usefully studied *in vivo*.

TABLE I
S1P$_1$-mediated Akt and ERK1/2 phosphorylation
The -fold increases from the control Me_2SO-treated cells are shown.

SEW2871	pAkt		pERK2		pERK1	
	S1P$_1$-CHO	S1P$_3$-CHO	S1P$_1$-CHO	S1P$_3$-CHO	S1P$_1$-CHO	S1P$_3$-CHO
nM						
5	4.7×	1.9×	1.5×	1.1×	4.2×	ND[a]
50	12.1×	1.7×	1.8×	0.6×	4.7×	ND
500	13.8×	1.7×	1.8×	1.1×	5.8×	ND

[a] ND, not detected.

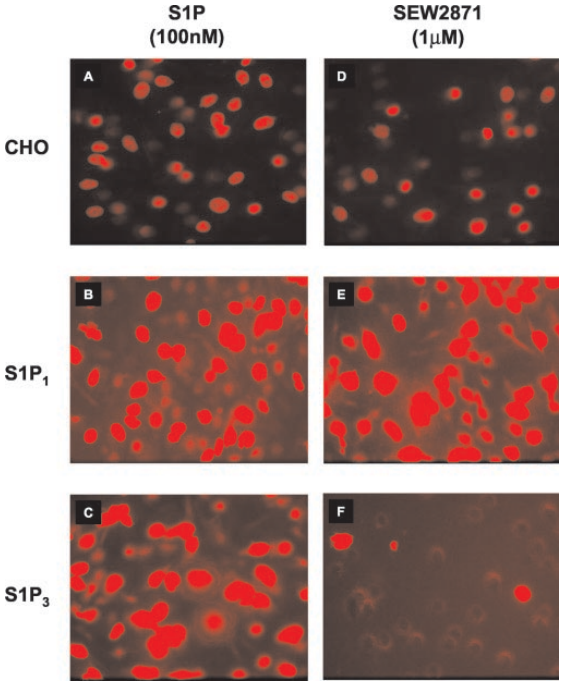

FIG. 3. **Migration assay on S1P$_1$- and S1P$_3$-expressing cells in response to S1P and SEW2871.** CHO, CHO-S1P$_1$, and CHO-S1P$_3$ cells were assayed in a cell migration assay on Transwell membranes upon stimulation with 100 nM S1P (*A–C*) or 1 μM SEW2871 (*D–F*). Only cells passing through the membranes are stained with propidium iodide and appeared *red*. Although SEW2871 (*B*) and S1P (*E*) induced equivalent cell migration *in vivo* in S1P$_1$-CHO cells, S1P$_3$-CHO cells migrated only in response to S1P (*C*) but not SEW2871 (*F*), confirming the selectivity of SEW2871. Minimal cell migration or cytoskeletal reorganization occurred in response to either S1P or SEW2871 in untransfected CHO cells (<0.01% of cell migrated) (*A* and *D*).

SEW2871 Induces and Maintains Lymphopenia in Mice, Which Correlates with Plasma Agonist Levels—Relatively non-selective S1P receptor agonists, such as FTY720-P and its phosphonate, produce rapid lymphopenia in peripheral blood that is the basis of their immunosuppression. Because SEW2871 was a selective agonist of S1P$_1$ alone, we tested its efficacy for the induction and maintenance of lymphopenia *in vivo*. We gavaged mice with SEW2871 (1.25 to 20 mg/kg) and measured compound plasma levels and circulating blood lymphocyte numbers at 5 h.

Plasma levels of SEW2871, measured at 5 h by GC-MS were linear with oral dose in the range of 0–30 mg/kg (Fig. 4*A*) in mice. SEW2871 produced a rapid and dose-dependent peripheral blood lymphopenia after 5 h (ED_{50} = 5.5 ± 1.04 mg/kg (mean ± S.E.; n = 4; Fig. 4*B*) when tested at doses up to 150 mg/kg. Because S1P agonists induce lymphopenia without affecting peripheral blood myelomonocytic cells, there was a decline in lymphocyte numbers with a correlative decline in the percentage of lymphocytes within the leukocyte differential count (Fig. 4*C*).

The plasma EC_{50} of SEW2871 for lymphopenia was ~2 μM (Fig. 4*B*). There was a dose-response relationship between plasma SEW2871 levels and the number of blood lymphocytes. We showed the relationship between plasma levels of S1P$_1$ receptor agonist and the maintenance of circulating blood lymphocyte numbers in the duration of action study (Fig. 4*D*), where 20 mg/kg SEW2871 was gavaged and blood lymphocyte numbers and compound plasma concentrations were measured for the first 42 h. The curves for plasma levels of SEW2871 and the induction and maintenance of lymphopenia were mirror images of each other. Induction of lymphopenia was as rapid for SEW2871, as for non-selective S1P receptor agonists such as AAL(R) (9–11), and full lymphopenia was maintained for more than the first 12 h. As SEW2871 concentrations in plasma decline, the lymphopenia reverses, suggesting that the continuous presence of S1P$_1$ receptor agonist is necessary for the maintenance of lymphopenia.

SEW2871 Inhibits Lymphocyte Migration into Murine Lymphatic Sinuses—S1P receptor agonists inhibit egress of lymphocytes into lymphatic sinus in peripheral and mesenteric lymph nodes and Peyer's patch but not spleen (7, 9). Effects are easily seen histologically within 6–15 h of a single dose of agonist. SEW2871 (Fig. 5*B*) but not vehicle (Fig. 5*A*) induced clearing of lymphatic sinuses (*arrows*) and the log-jamming of lymphocytes immediately subjacent to sinus-lining endothelium in lymph nodes. SEW2871 histological changes were indistinguishable from those seen with the non-selective S1P receptor agonist control AAL-(R) (not shown) and those published for FTY720 (9). Both SEW2871 and the non-selective S1P agonists inhibit the egress of lymphocytes across sinus lining endothelium supporting the conclusion that activation of S1P$_1$ alone is sufficient to shut down entry of lymphocytes into lymph.

Freshly Isolated Lymphocytes from Spleen or Lymph Node Do Not Respond to SEW2871 with a Ligand-evoked Calcium Flux—SEW2871 has rapid effects upon the bulk trafficking of lymphocytes *in vivo*, although these effects are confined to lymph node and thymus but not spleen, despite the facts that naïve lymphocyte populations in lymph node and spleen show no distinguishing characteristics. Inhibition of lymphocyte egress from lymph node and thymus but not spleen suggests that this mechanism may therefore depend upon non-lymphocytic stromal cell effects in addition perhaps to direct effects upon lymphocytes. To assess whether SEW2871 mediated its effects upon lymphocytes directly or indirectly, we looked for evidence of S1P$_1$ activation and expression on freshly isolated murine lymphocytes that had not been cultured at all.

Spleen adherent cells, but not lymphocytes freshly isolated from spleen, lymph node, or thymus responded to SEW2871 with a ligand-induced calcium flux in FLIPR format assays (Fig. 6*A*). These data were confirmed by flow cytometry, where freshly isolated CD4+ (shown) or CD8+ (data not shown) T

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