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Immune Cell Regulation and Cardiovascular Effects of Sphingosine 1-Phosphate Receptor Agonists in Rodents Are Mediated via Distinct Receptor Subtypes

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ABSTRACT

Sphingosine 1-phosphate (S1P) is a bioactive lysolipid with pleiotropic functions mediated through a family of G protein-coupled receptors, $S1P_{1,2,3,4,5}$. Physiological effects of S1P receptor agonists include regulation of cardiovascular function and immunosuppression via redistribution of lymphocytes from blood to secondary lymphoid organs. The phosphorylated metabolite of the immunosuppressant agent FTY720 (2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol) and other phosphonate analogs with differential receptor selectivity were investigated. No significant species differences in compound potency or rank order of activity on receptors cloned from human, murine, and rat sources were observed. All synthetic analogs were high-affinity agonists on $S1P_1$, with IC_{50} values for ligand binding between 0.3 and 14 nM. The correlation between $S1P_1$ receptor activation and the ED_{50} for lymphocyte reduction was highly significant ($p < 0.001$) and lower for the other receptors. In contrast to $S1P_1$-mediated effects on lymphocyte recirculation, three lines of evidence link $S1P_3$ receptor activity with acute toxicity and cardiovascular regulation: compound potency on $S1P_3$ correlated with toxicity and bradycardia; the shift in potency of phosphorylated-FTY720 for inducing lymphopenia versus bradycardia and hypertension was consistent with affinity for $S1P_1$ relative to $S1P_3$; and toxicity, bradycardia, and hypertension were absent in $S1P_3^{-/-}$ mice. Blood pressure effects of agonists in anesthetized rats were complex, whereas hypertension was the predominant effect in conscious rats and mice. Immunolocalization of $S1P_3$ in rodent heart revealed abundant expression on myocytes and perivascular smooth muscle cells consistent with regulation of bradycardia and hypertension, whereas $S1P_1$ expression was restricted to the vascular endothelium.

Sphingosine 1-phosphate (S1P) is a bioactive lipid derived from metabolism of sphingomyelin (Pyne and Pyne, 2000). S1P has been implicated in the regulation of many cellular functions including proliferation, apoptosis, survival, adhesion, differentiation, and migration (Hla et al., 2001). The diverse signaling has been attributed, in part, to the activation of a family of G protein-coupled receptors called S1P or edg receptors that are differentially expressed and coupled to $G_{i/o}$, G_q, and $G_{12/13}$ proteins (Chun et al., 2002).

Few pharmacological tools with in vivo activity have been described for the S1P receptors, but functions of the individual receptors are beginning to be elucidated. $S1P_1$/edg1 has a widespread distribution and is highly abundant on endothelial cells where it works in concert with $S1P_3$/edg3 to regulate cell migration, differentiation, and barrier function (Lee et al., 1999; Garcia et al., 2001). Although these receptors stimulate some pathways in common, they are not redundant. The $S1P_1$ null embryos are defective in the migration of smooth muscle cell pericytes that are required to support vascular maturation and the embryos die at day 13.5 from hemorrhage (Liu et al., 2000). In contrast, the $S1P_3$ null mouse is phenotypically normal (Ishii et al., 2001). $S1P_2$/edg5 is a potent activator of the Rho pathway and inhibits cell migration, whereas $S1P_1$ and $S1P_3$ stimulate chemotaxis of many cell types (Oka-

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ABBREVIATIONS: S1P, sphingosine 1-phosphate; $I_{K.Ach}$, inwardly rectifying K^+ currents; CHO, Chinese hamster ovary; MAP, mean arterial pressure; DAPI, 4,6-diamidino-2-phenylindole; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; FTY720, 2-amino-2-(2-[4-octylphenyl] ethyl)-1,3-propanediol; PECAM, platelet-endothelial cell adhesion molecule.

moto et al., 2000; Graeler and Goetzl, 2002). Much less is known about the function of $S1P_4$/edg6, which is restricted to hematopoietic and lymphoid tissues (Graeler et al., 1999), and $S1P_5$/edg8, which is predominant in rodent brain (Im et al., 2000) but more broadly expressed in human tissues (Niedernberg et al., 2002).

A novel physiological role for S1P in immune regulation has been discovered recently by elucidating the mechanism of FTY720 (Brinkmann et al., 2002; Mandala et al., 2002), an immunosuppressive agent with activity in many models of transplantation and immune-based disease (Dumont, 2000; Brinkmann et al., 2001). FTY720 depletes peripheral blood lymphocytes and sequesters them in secondary lymphoid organs (Chiba et al., 1998). We discovered that FTY720 is phosphorylated in vivo to become a high-affinity ligand (Compound A) for $S1P_{1,3,4,5}$ but not $S1P_2$ (Mandala et al., 2002). A non-hydrolyzable phosphonate analog (Compound B) with similar S1P receptor selectivity was also able to alter lymphocyte recirculation (Mandala et al., 2002). Close analogs of FTY720, such as the (S) enantiomer of 2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol, that were not substrates for phosphorylation did not have immunosuppressive activity, thus providing additional evidence that the phosphorylated metabolite is the active species (Brinkmann et al., 2002; Mandala et al., 2002). The receptor(s) responsible for immune modulation has not been determined, although both $S1P_1$ and $S1P_4$ have been implicated based on their roles in regulating lymphocyte chemotaxis (Brinkmann et al., 2001; Dorsam et al., 2003).

Clinical studies with FTY720 have identified dose-dependent transient asymptomatic bradycardia in stable renal transplant patients (Budde et al., 2002). Although there are no published reports on the effects of FTY720 on heart rate in rodents, S1P decreased heart rate in anesthetized rats (Sugiyama et al., 2000a). Bradycardia is consistent with previous reports of S1P activation of muscarinic receptor-activated inwardly rectifying K^+ currents ($I_{K.Ach}$) and concomitant slowing of sino-atrial node pacemaker activity (Bunemann et al., 1995; Guo et al., 1999). In contrast to observations in rats, S1P administration to a canine isolated heart preparation evoked a sinus tachycardia (Sugiyama et al., 2000b). The effects of S1P receptor agonists on vascular tone and mean arterial pressure (MAP) are similarly complex. FTY720 has not been reported to have any effects on MAP in man. However, FTY720 has been shown to either decrease or increase MAP in anesthetized rats (Tawadrous et al., 2002). On isolated vascular preparations, S1P induced relaxation at low concentrations (10–100 nM) (Dantas et al., 2003) and vasoconstrictor effects at higher concentrations (100 nM to 100 μM) (Bischoff et al., 2000; Tosaka et al., 2001). The complex cardiovascular effects of S1P receptor agonists are likely due to differential effects on S1P receptor subtypes. $S1P_2$ contraction of coronary smooth muscle cells was implicated using a selective antagonist (Ohmori et al., 2003) but antisense to $S1P_3$, not $S1P_2$, inhibited contraction of rat basilar arteries (Salomone et al., 2003). The availability of S1P subtype receptor-selective agonists with in vivo activity that are described in this paper and $S1P_3$ receptor knockout mice allows for a more detailed investigation of the cardiovascular and lymphocyte trafficking effects of S1P agonists.

Materials and Methods

Synthesis of S1P Receptor Agonists. The syntheses of Compounds A and B have been described previously (Mandala et al., 2002). Compound C was prepared in seven steps from 2-acetylamino-2-(2-(4-octylphenyl)ethyl)propane-1,3-dicarboxylic acid, diethyl ester (Durand et al., 2000). Compounds D to F were prepared by reductive amination of the appropriate aryl aldehyde with 3-aminopropylphosphonic acid [$Na(CN)BH_3$, MeOH, 50°C]. All compounds were characterized by ^1H NMR, mass spectroscopy, and high-pressure liquid chromatography and were judged to be >95% pure. Detailed procedures are provided in patents WO 03074008 and WO 03062252.

Mouse Lymphocyte Reduction Assay. Mice (three per group) were dosed intravenously with 0.1 ml of test compound dissolved in vehicle [2% (w/v) hydroxypropyl-β-cyclodextrin (Cerestar, Cedar Rapids, IA) and 0.12 M NaCl], and peripheral blood lymphocyte counts were assessed 3 h later. Mice were euthanized via CO_2 inhalation, the chest was opened, 0.5 ml of blood was withdrawn via direct cardiac puncture into EDTA, and hematology was evaluated using a clinical hematology AutoAnalyzer calibrated for performing murine differential counts (H2000; CARESIDE, Culver City, CA). Toxicity was observed upon administration of some of the test compounds. Severe signs included death, seizure, paralysis, or unconsciousness. Milder signs were also noted and included ataxia, labored breathing, ruffling, or reduced activity relative to normal. To assess lymphopenic activity with these compounds, upon noting symptoms in the first animal, the dosing solution was diluted in the same vehicle and administered to a second mouse for observation. The process was repeated until a dose was reached that produced only brief, mild symptoms. This was considered the maximum tolerated dose. All procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee.

Receptors and Cell Lines. CHO cells stably expressing human $S1P_{1,2,3,4,5}$ were as previously described (Mandala et al., 2002). cDNA sequences encoding rodent S1P receptors were cloned from genomic DNA by polymerase chain reaction using the following primers for each respective receptor: 5′-GAACCCGGGTGTCCACTAGCATCCCGG and 5′-CCCGAATTCTTAGGAAGAAGAATTGACGTTTCC (mouse $S1P_1$), 5′-GAACCCGGGCGGCTTATACTCAGAGTACC and 5′-GGCGAATTCTCAGACCACTGTGTTACCCTC (mouse $S1P_2$), 5′-GAACCCGGGCAACCACGCATGCGCAGG and 5′-GTCGAATTCTCACTTGCAGAGGACCCCG (mouse $S1P_3$), 5′-GAACCCGGGAACATCAGTACCTGGTCCACGC and GCGGAATTCTAGGTGCTGCGGACGCTGG (mouse $S1P_4$), 5′-GAACCCGGGCTGCTGCGGCCGG and 5′-CGCGAATTCAGTCTGTAGCAGTAGGCACC (mouse $S1P_5$), 5′- GTAGGATCCGTGTCCTCCACCAGCATC and 5′-GGCCGAATTCTTAAGAAGAAGAATTGACGTTTC (rat $S1P_1$), and 5′- GAACCCGGGCATCCACGCATGCGCAG and 5′-GCCGAATTCTCACTTGCAGAGGACCCCATTCTG (rat $S1P_3$). The polymerase chain reaction products were inserted in frame after a FLAG tag using vector pCMV-Tag2 (Stratagene, La Jolla, CA). Stable lines were established by transfecting plasmids into CHO cells using lipofectamine reagent, selecting for neomycin resistance, and screening single cell cultures for increased [^{33}P]S1P-specific binding. Membranes were prepared from positive clones and confirmed in [^{33}P]S1P and [^{35}S]GTPγS binding assays.

S1P Receptor Assays. Binding assays were conducted as previously described (Mandala et al., 2002). In brief, [^{33}P]S1P was sonicated with fatty acid-free bovine serum albumin, added to test compounds diluted in dimethyl sulfoxide, and mixed with membranes in 200 μl in 96-well plates with assay concentrations of 0.1 nM [^{33}P]S1P (22,000 dpm), 0.5% bovine serum albumin, 50 mM HEPES-Na (pH 7.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.3 to 0.7 μg of membrane protein. Binding was performed for 60 min at room temperature and terminated by collecting the membranes onto GF/B filter plates with a Packard Filtermate Universal Harvester. Filter bound radionu-

clide was measured on a PerkinElmer 1450 MicroBeta. Specific binding was calculated by subtracting radioactivity that remained in the presence of 1000-fold excess of unlabeled S1P.

To measure functional activation of the S1P receptors, [^{35}S]GTPγS binding was measured. Membranes (1–4 μg of protein) were incubated in 96-well plates with test compounds diluted in dimethyl sulfoxide in 100 μl of buffer containing 20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, and 2 to 10 μM GDP, depending on the expressed receptor. The assay was initiated with the addition of 100 μl of [^{35}S]GTPγS (1200 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) for an assay concentration of 125 pM. After 60 min of incubation at room temperature, membranes were harvested, onto GF/B filter plates and bound radionuclides were measured as described for ligand binding. S1P was subject to significant dephosphorylation in the [^{35}S]GTPγS binding assay as measured with [^{33}P]S1P or [^{3}H]S1P. EC_{50} values are not reported for S1P. Degradation of S1P was less than 10% in the [^{33}P]S1P binding assay, and [^{3}H]-Compound A was not metabolized under either assay condition.

Assessment of Cardiovascular Function. Cardiovascular function was assessed in anesthetized and conscious rats and in conscious mice. Anesthetized rats were used to evaluate the dose-dependent profile of cardiovascular responses to S1P receptor agonists of differing S1P receptor selectivity. Conscious rats were used to verify data from anesthetized animals and to allow a longer period of observation (4 h) that included a concurrent assessment of the induction of lymphopenia. The use of conscious mice allowed a comparison of compound effects in wild-type animals and mice with genetic deletion of the $S1P_3$ receptor.

Anesthetized Rat Cardiovascular Assay. For the assessment of cardiovascular function in anesthetized rats, male Sprague-Dawley rats (300–350 g. b.wt.) with surgically implanted femoral artery and vein catheters were obtained from Charles River Laboratories (Raleigh, NC). Animals were anesthetized with Nembutal (55 mg/kg, i.p.), and a DTX pressure transducer (TNF-R; BD Biosciences, San Jose, CA) was attached to the arterial catheter and subsequently to a Gould ACQ-7700 data acquisition system using Po-Ne-Mah software (PNM-P3P) (Gould Instrument Systems, Valley View, OH). Heart rate was derived from the arterial pulse wave. Following an acclimation period, baseline values were determined (approximately 20-min duration); subsequently, compounds were administered intravenously (bolus injection of approximately 10 s). Cardiovascular data were recorded continuously and are reported as average values over 1-min intervals.

Conscious Rat Cardiovascular Assay. For the assessment of cardiovascular function in conscious rats, male Sprague-Dawley rats (300–350 g. b.wt.) with surgically implanted femoral artery and vein catheters were obtained from Charles River Laboratories. The catheters were connected to a tether (CIH95) and swivel (375/20; Instech Laboratories, Inc., Plymouth Meeting, PA), allowing the animal to move freely around the cage. Animals were allowed a minimum 2-day acclimation prior to further experimentation. On the day of study, a BD Biosciences DTX pressure transducer (TNF-R) was attached to the arterial catheter and subsequently to a Gould ACQ-7700 data acquisition system using Po-Ne-Mah software. Heart rate was derived from the arterial pulse wave. Heart rate and arterial pressure were measured for between 30 and 60 min to establish baseline values. Subsequently, compound or vehicle was administered as a continuous intravenous infusion of 25 μl/min for 4 h. Blood samples, for the evaluation of circulating leukocytes, were obtained from the arterial catheter 30 min prior to dosing and 1 and 4 h post initiation of the infusion. Cardiovascular data were recorded continuously and are reported as average values over 1-min intervals.

Mouse Cardiovascular Assay. For the assessment of cardiovascular function in conscious mice, the $S1P_3$ receptor was genetically deleted (R. L. Proia, manuscript submitted for publication) and bred at Taconic Farms Inc. (Germantown, NY). Male (B6.129) $S1P_3^{-/-}$ and $S1P_3^{+/+}$ mice (20–30 g b.wt.) were anesthetized with ketamine (80–100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and catheters were placed in a carotid artery (PE-50, with tip modified) and Jugular vein (PE-10). The catheters were tunneled subcutaneously to the nape and exteriorized. The catheters were connected to a tether (CIH62) and swivel (375/25P; Instech Laboratories, Inc.), allowing the animal to move freely around the cage. Following surgery, animals were allowed an overnight recovery period prior to further experimentation. On the day of study, a BD Biosciences DTX pressure transducer was attached to the arterial catheter and subsequently to a Gould ACQ-7700 data acquisition system using Po-Ne-Mah software. Heart rate was derived from the arterial pulse wave. Heart rate and arterial pressure were measured for between 30 and 60 min to establish baseline values. Subsequently, compound or vehicle was administered intravenously as a bolus of 10-s duration. Cardiovascular data were recorded continuously and are reported as average values over 10-s intervals.

Immunohistochemical Localization of $S1P_1$ and $S1P_3$ in Rat Heart. Peptides to the N terminus of mouse $S1P_3$ (ATTHAQGHQPVLGNDTLREHYDYVGKLAGRLRDPPEGGTL) and mouse $S1P_1$ (VSTSIPEVKALRSSVSDYGNYDIIVRHYNYTGKLNIGAEKDHGIK) and the C terminus of $S1P_1$ (EGDNPETIMSSGNVNSSS) were synthesized (SynPep Corp., Dublin, CA), conjugated to KLH, and used to immunize rabbits (Covance Research Products, Denver, PA). Specific IgG fractions were affinity purified using the immunizing peptides. The resulting antisera were tested by Western blot across a panel of human, mouse, and rat S1P receptors. MS2031 to $S1P_3$ was specific for the rodent $S1P_3$ receptors. For the $S1P_1$ antisera, MS2029 to the N terminus was specific for rodent $S1P_1$, whereas the C-terminal antisera, MS1766, recognized human and rodent $S1P_1$. Staining by Western and immunohistochemistry was blocked by incubation with the relevant but not the irrelevant peptide.

For histochemical studies, blocks of atrium or ventricle from rats or mice treated with a lethal dose of sodium pentobarbital were rapidly dissected out and immediately placed in cryomolds (catalog no. 4557; TissueTek, Torrance, CA) filled with O.C.T. Compound (TissueTek catalog no. 4583), frozen in liquid nitrogen, and stored at −80°C. Frozen sections were cut (5-μm thickness) on a Bright Model OTF cryotome (Hacker Instruments, Fairfield, NJ) and mounted on coated slides (catalog no. CFSACS; Instrumedics, Inc., Hackensack, NJ). To block nonspecific labeling, sections were treated with 5% donkey serum in PBS for 20 min, then with a clarified solution of 5% nonfat dry milk for 30 min, and finally with Fc blocker (Accurate Chemical, Westbury, NY) for 20 min. Sections were labeled for 1 h with affinity-purified primary antibodies or appropriate IgG controls (5 μg/ml): rabbit anti-mouse $S1P_1$, rabbit anti-human $S1P_1$, rabbit anti-mouse $S1P_3$, and mab anti-rat PECAM (CD31 Pharmingen, catalog no. 555025; BD PharMingen, San Diego, CA). All nonimmune IgG controls were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Slides were washed and incubated with affinity-purified F(ab′)$_2$ donkey anti-rabbit or rat Cy2 (green fluorescence) or Cy3 (red fluorescence) conjugated secondary antibodies (5 μg/ml, 30 min) from Jackson ImmunoResearch Laboratories Inc. For double labeling studies, two primary antibodies raised in different species or corresponding species-specific fluorescent secondary antibodies were mixed together and incubated simultaneously on each slide. Nonimmune IgG controls also were run in this fashion. Specificity also was demonstrated by pre-incubating each primary antibody at staining concentrations with its relevant or irrelevant peptide at 5 μg/ml for 1 h and centrifugation for 1 h at 13,500g at 4°C (Beckman Microfuge 11; Beckman Coulter, Fullerton, CA). After staining, the slides were fixed for 30 min in 4% formaldehyde freshly generated from paraformaldehyde in phosphate buffer (pH 7.4; catalog no. 04042500; Fisher Scientific Co., Pittsburgh, PA). Coverslips were mounted on the slides with Vectashield plus DAPI nuclear stain (catalog no. H1200, Vector Laboratories, Burlingame, CA). Sections were photographed and analyzed with an Everest imaging system from Intelligent Imaging Innovations (Denver, CO) equipped with an Axioplan 2 microscope (Carl Zeiss, Göttingen, Germany). This system allows the viewer to visualize two different fluoro-

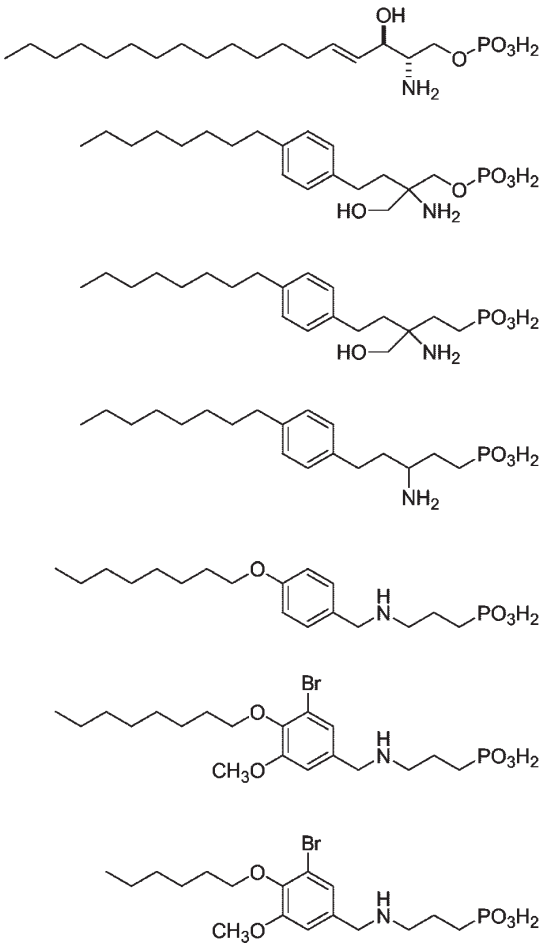

Fig. 1. Chemical structures of S1P and synthetic S1P receptor agonists.

chrome conjugated secondary antibodies individually or in combination on the same section in double labeling experiments.

Results

Lymphopenia, Toxicity, and in Vitro Activity of S1P Receptor Agonists. The phosphonate analog (Compound B) of the phosphate-ester metabolite of FTY720 (Compound A) was found previously to deplete peripheral blood lymphocytes with a 15-fold right shift in potency relative to FTY720 (Mandala et al., 2002). The shift in potency was consistent with its reduced affinity for S1P receptors. In an effort to identify more potent immunosuppressive compounds and explore structure activity relationships, additional analogs were synthesized (Fig. 1). Using a 3-h murine assay, Compound C and several analogs in the secondary amine phosphonate series were found to reduce circulating blood lymphocytes (Fig. 2). Compound D was 2-fold less active than Compound A and was the most potent phosphonate analog tested. However, efficacy could only be assessed within a narrow concentration range due to toxicity. Doses higher than 0.05 mg/kg of Compound D induced symptoms of ataxia and paralysis, and 0.25 mg/kg was lethal. Compounds C and E were 5- to 10-fold less active than D in reducing peripheral blood lymphocytes, and compound F had the weakest activity with an ED_{50} of 2.2 mg/kg. No toxicity was observed with Compounds E and F.

To investigate S1P receptor selectivity of the analogs, they

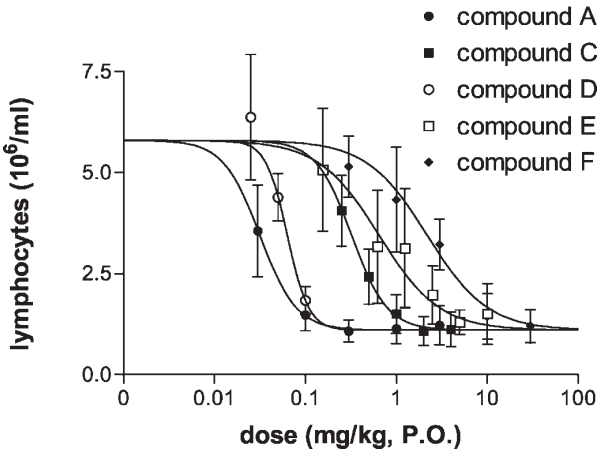

Fig. 2. Dose response of S1P receptor agonists in peripheral blood lymphocyte depletion. Compounds were administered as an intravenous bolus to mice (n = 3), and total blood lymphocytes were determined 3 h later.

were tested against human receptors expressed in CHO cells in competitive ligand binding assays using [^{33}P]S1P as the ligand and functional assays of G protein coupling using [^{35}S]GTPγS binding (Table 1). As previously determined for Compounds A and B, none of the phosphonates had significant activity against $S1P_2$. All compounds were agonists on the other four S1P receptors, and maximal efficacy in [^{35}S]GTPγS binding was similar to that observed with Compound A. Compound D was the most potent phosphonate on $S1P_1$ with an IC_{50} of 0.9 nM that was only 2- to 3-fold less active than S1P or Compound A. Compound D was also the most active synthetic analog on $S1P_3$, with an IC_{50} of 7.9 nM that was equivalent to Compound A but less potent relative to S1P. The 2-bromo and 5-methoxy substitutions to the phenyl ring found in Compounds E and F resulted in modest decreases in affinity for $S1P_1$ and a substantial loss in activity on $S1P_3$, which was further reduced by shortening the alkyl chain length in Compound F. All of the phosphonates had similar activity on $S1P_4$, making this receptor unlikely to account for the differential effects of the analogs on lymphopenia or toxicity. Likewise, $S1P_5$ did not appear to be a probable candidate for either in vivo effect given the equivalent potency of all three of the secondary amine compounds and the 20-fold reduced activity of Compound C on $S1P_5$ relative to Compounds D to F.

Correlations between rodent in vivo pharmacological effects and human in vitro receptor assays can be misleading unless there is a high degree of conservation in the ligand binding pocket of the receptors. To address this issue, we cloned mouse $S1P_{1,3,4,5}$ and rat $S1P_{1,3}$ receptors and established stable CHO lines and assays that were comparable to those used to assess the human receptors. The overall homology between rodent and human S1P receptors ranged from 80% to 93%, with $S1P_1$ being the most conserved protein. Ligand binding assays using the rodent receptors (Table 2) revealed that the natural ligand and the synthetic compounds maintained similar potency and the same rank order of activity, thus indicating that there were no significant differences between rodent and human structure activity relationships. As was deduced previously from the human receptors, the correlation between $S1P_1$ receptor activation and the ED_{50} for lymphopenia was highly significant ($p < 0.001$) and much lower for the other receptors. All of the compounds had low or sub-nanomolar potency on $S1P_1$, and the rank order of activity was Compound A > D > C > E > F.

Intravenous administration of some of the compounds evoked toxicity with symptoms that ranged from transient ruffling and paralysis to lethality. Compound D was the least tolerable compound tested and the most potent synthetic analog on mouse $S1P_3$ with an IC_{50} of 1.8 nM. The other two compounds in the secondary amine series (E and F) had considerably reduced potency at mouse $S1P_3$ with IC_{50} values of 0.6 and 6.6 μM, respectively, and did not induce any adverse symptoms in mice. Only mild, transient toxicity was observed with Compound C, which had intermediate activity on $S1P_3$. Compound A induced transient signs of toxicity in mice but was not lethal up to 10 mg/kg, whereas S1P was highly toxic and was the most potent ligand for $S1P_3$. To test whether toxicity was mediated via $S1P_3$, as suggested by the correlation of in vitro receptor potency with the maximal tolerated dose (Table 2), the severely toxic compounds were studied in $S1P_3$ null mice. In $S1P_3^{-/-}$ mice, S1P and Compound D did not induce any toxic symptoms at 25 and 5 mg/kg, respectively, whereas they were rapidly lethal in their wild-type litter mate controls at doses of 5 and 0.25 mg/kg, respectively. $S1P_3^{-/-}$ mice were fully susceptible to agonist-mediated lymphocyte depletion; peripheral blood lymphocyte counts from Compound D treated mice were 1.6×10^6/ml compared with 5.0×10^6/ml for vehicle-treated mice ($p < 0.01$).

Effects of S1P Receptor Agonists in Anesthetized Rats. Administration of either S1P (Fig. 3A) or the $S1P_{1,3,4,5}$ agonist, Compound A (Fig. 3B), to anesthetized rats evoked an immediate decrease in heart rate, reaching a nadir within 1 to 2 min of compound administration and returning toward baseline values within 10 min. The effects on mean arterial pressure were more complex. Initially, a rapid hypotension was observed that reversed concomitantly with an increase in heart rate. In some instances, an overshoot to a transient hypertension was observed before returning to baseline. Subsequently, a gradually developing somewhat variable hypertension was seen that peaked approximately 10 min post-compound administration before resolving within 20 min.

TABLE 1
Activity of compounds on human S1P receptors
Compounds were tested in [^{33}P]S1P binding assays to determine IC_{50} values and [^{35}S]GTPγS binding assays to determine EC_{50} values using membranes prepared from CHO cells expressing human S1P receptors. Values are the mean of two to six measurements performed in duplicate. S1P is subject to extensive metabolism under standard [^{35}S]GTPγS binding assay conditions, and EC_{50} values are not shown.

	S1P	A	C	D	E	F
			nM			
$S1P_1$						
IC_{50}	0.5	0.3	3.2	0.9	2.4	12.0
EC_{50}	ND	0.3	2.9	1.2	1.9	12.8
$S1P_2$						
IC_{50}	0.3	>1000	>1000	>1000	>1000	>1000
EC_{50}	ND	>1000	>1000	>1000	>1000	>1000
$S1P_3$						
IC_{50}	0.2	5.0	125	7.9	1424	9265
EC_{50}	ND	3.0	109	7.3	1145	>10,000
$S1P_4$						
IC_{50}	55	5.9	160	114	77	380
EC_{50}	ND	4.3	45	ND	83	ND
$S1P_5$						
IC_{50}	0.5	0.6	108	5.0	7.5	6.8
EC_{50}	ND	1.0	171	1.3	4.4	5.6

ND, not determined.

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