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


Departments of Immunology and Rheumatology, Pharmacology, and Medicinal Chemistry, Merck Research Laboratories, Rahway, New Jersey; and National Institutes of Health, Bethesda, Maryland (R.L.P.)

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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, S1P1,2,3,4,5. 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 S1P1, with IC50 values for ligand binding between 0.3 and 14 nM. The correlation between S1P1 receptor activation and the ED50 for lymphocyte reduction was highly significant (p < 0.001) and lower for the other receptors. In contrast to S1P1-mediated effects on lymphocyte recirculation, three lines of evidence link S1P3 receptor activity with acute toxicity and cardiovascular regulation: compound potency on S1P3 correlated with toxicity and bradycardia; the shift in potency of phosphorylated-FTY720 for inducing lymphopenia versus bradycardia and hypertension was consistent with affinity for S1P1 relative to S1P3; and toxicity, bradycardia, and hypertension were absent in S1P3−/− mice. Blood pressure effects of agonists in anesthetized rats were complex, whereas hypertension was the predominant effect in conscious rats and mice. Immunolocalization of S1P3 in rodent heart revealed abundant expression on myocytes and perivascular smooth muscle cells consistent with regulation of bradycardia and hypertension, whereas S1P1 expression was restricted to the vascular endothelium.
moto et al., 2000; Graeler and Goetzl, 2002). Much less is known about the function of S1P_{1,3,4,5}, which is restricted to hematopoietic and lymphoid tissues (Graeler et al., 1999), and S1P_{6,edg6}, 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 phosphate 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-heptyloxypbenyl)-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_{3} 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 decreases 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 was implicated using a single cell cultures for increased [33P]S1P-specific binding. The process was repeated until a dose was reached that produced only brief, mild symptoms. This was considered the maximum tolerable 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.

**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-aminomorpholinosulfonic acid [NaCN\(\text{BH}_3\), 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; CARESIEIDE, 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′-GAACCCGGGTGTCCACTAGCCTC-CGGG and 5′-CCCCAATTTACTAGAGAATTGGCCTTTT (mouse S1P_{1}), 5′-GAACCCGGGTGTCCACTAGCCTC-CGGG and 5′-CCCCAATTTACTAGAGAATTGGCCTTTT (mouse S1P_{2}), 5′-GAACCCGGGTGTCCACTAGCCTC-CGGG and 5′-CCCCAATTTACTAGAGAATTGGCCTTTT (mouse S1P_{3}), 5′-GAACCCGGGTGTCCACTAGCCTC-CGGG and 5′-CCCCAATTTACTAGAGAATTGGCCTTTT (mouse S1P_{4}), 5′-GAACCCGGGTGTCCACTAGCCTC-CGGG and 5′-CCCCAATTTACTAGAGAATTGGCCTTTT (mouse S1P_{5}). The polymerase chain reaction products were inserted in frame after a FLAG tag using vector pCMV-Tag2 (Strategene, 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 [35S]GTPgammaS-binding. Membranes were prepared from positive clones and confirmed in [32P]SIP and [35S]GTPgammaS binding assays.

**S1P Receptor Assays.** Binding assays were conducted as previously described (Mandala et al., 2002). In brief, [32P]SIP 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 [32P]SIP (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 radiolu-
binding was measured on a Perkin-Elmer 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, [35S]GTP-S 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 MgCl2, and 2 to 10 μM GDP, depending on the expressed receptor. The assay was initiated with the addition of 100 μl of [35S]GTP-S (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 filters plates and bound radioligand were measured as described for ligand binding. S1P was subject to significant dephosphorylation in the [35S]GTP-S binding assay as measured with [35S]S1P or [3H]S1P. EC50 values are not reported for S1P. Degradation of S1P was less than 10% in the [35S]S1P binding assay, and [3H]-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 S1P3 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). Arterial blood pressure was 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 S1P3 receptor was genetically deleted (R. L. Proia, manuscript submitted for publication) and bred at Taconic Farms Inc. (Germantown, NY). Male (B6.129) S1P3−/− and S1P3−/− 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 (CHI95) and swivel (375/225P; 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 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.

Immuno histochemical Localization of S1P1 and S1P3 in Rat Heart. Peptides to the N-terminus of mouse S1P1 (ATTAHG HQPV-LGNDYLREHYDYYKVLGLRRLDPPEGTL) and mouse S1P3 (VSTPIEVRKARSSVSVDNYDDIIVRHYNTGKLNIGAEKDHGIRK) and the C-terminus of S1P3 (EGDNPETMSSSGNVNSSS) 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 S1P1 was specific for the rodent S1P3 receptors. For the S1P3 antisera, MS2029 to the N terminus was specific for rodent S1P1, whereas the C-terminal antisera, MS1766, recognized human and rodent S1P1. 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 pentobarbita!i were rapidly dissected out and immediately placed in cryomolds (catalog no. 4557; TissueTek, Torrance, CA) filled with OCT 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; Instrumented, 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 with 1 μg/ml of affinity-purified primary antibodies or appropriate IgG controls (5 μg/ml): rabbit anti-mouse S1P1, rabbit anti-human S1P1, rabbit anti-mouse S1P3, rabbit anti-rat S1P3, and rabbit 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 (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 fluoros.
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 ED₅₀ of 2.2 mg/kg. No toxicity was observed with Compounds E and F.

To investigate S1P receptor selectivity of the analogs, they

![Chemical structures of S1P and synthetic S1P receptor agonists.](image1)

![Dose response of S1P receptor agonists in peripheral blood lymphocyte depletion.](image2)
were tested against human receptors expressed in CHO cells in competitive ligand binding assays using \(^{35}\text{S}\)GTP \(\gamma\)S 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 Compounds A and B, and maximal efficacy in \(^{35}\text{S}\)GTP \(\gamma\)S 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 94%, 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.

### TABLE 1

Activity of compounds on human S1P receptors

<table>
<thead>
<tr>
<th>S1P</th>
<th>A</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC(_{50})</td>
<td>EC(_{50})</td>
<td>IC(_{50})</td>
<td>EC(_{50})</td>
<td>IC(_{50})</td>
</tr>
<tr>
<td>S1P(_1)</td>
<td>0.5</td>
<td>0.3</td>
<td>3.2</td>
<td>0.9</td>
<td>2.4</td>
</tr>
<tr>
<td>S1P(_2)</td>
<td>0.3</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>S1P(_3)</td>
<td>0.3</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>S1P(_4)</td>
<td>0.2</td>
<td>5.0</td>
<td>125</td>
<td>7.9</td>
<td>1424</td>
</tr>
<tr>
<td>S1P(_5)</td>
<td>3.0</td>
<td>109</td>
<td>7.3</td>
<td>1145</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

ND, not determined.
This late hypertension attained statistical significance in animals administered S1P but not in those administered Compound A.

The effects of S1P receptor agonists with varying in vitro potency for activation of S1P1 and S1P3 receptors were evaluated for their ability to evoke bradycardia in the anesthetized rat. The extent of bradycardia, expressed as the peak decrease in heart rate as a percentage change from the average baseline value recorded for 20 min prior to compound administration, is shown in Fig. 4. The rank order of potency of S1P agonists for producing bradycardia in the anesthetized rat was Compound A \textgreater{} D \textgreater{} E \textgreater{} S1P \textgreater{} F. The reduced effects of Compounds E and F on heart rate are consistent with their reduced IC50 values against S1P3 in vitro.

Effects of S1P Receptor Agonists in Conscious Rats. Administration of Compound A by continuous intravenous infusion to conscious rats produced time- and dose-dependent lymphopenia, bradycardia, and hypertension. At a dose of 10 \(\mu\)g/kg/min, bradycardia (Fig. 5A) and hypertension (Fig. 5B) were evident within 15 to 30 min postinitiation of infusion, with slower onset of effects at lower doses of compound (data not shown). Similarly, upon cessation of infusion, cardiovascular parameters returned to baseline values within 15 to 30 min. The decrease in circulating leukocytes was also dose dependent (Fig. 6). The dose dependence for the pharmacological effects of Compound A in conscious rats (Fig. 6) indicates that maximal lymphopenia was obtained at an infusion dose of less than 0.1 \(\mu\)g/kg/min, whereas significant changes in heart rate and mean arterial pressure were not evident until doses of 1 \(\mu\)g/kg/min or greater were administered.

Effects of S1P Receptor Agonists in Conscious Mice. Toxicity of S1P and Compound D were abrogated in mice with a genetic deletion of the S1P3 receptor. In accordance, the cardiovascular effects of S1P receptor agonists were evaluated in mice compared with binding activity on rodent S1P receptors.

**TABLE 2**

Lymphopenic and toxic effects of compounds in mice compared with binding activity on rodent S1P receptors.

<table>
<thead>
<tr>
<th>S1P</th>
<th>ED50 (mg/kg)</th>
<th>Toxicity (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL</td>
<td>ND</td>
<td>0.03 (0.01)</td>
</tr>
<tr>
<td>mS1P1</td>
<td>0.03 (0.08)</td>
<td>0.06 (0.007)</td>
</tr>
<tr>
<td>rS1P1</td>
<td>0.05</td>
<td>&gt;10</td>
</tr>
<tr>
<td>mS1P2</td>
<td>0.3</td>
<td>&gt;10</td>
</tr>
<tr>
<td>rS1P2</td>
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</tr>
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</tr>
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<td>6.4</td>
</tr>
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<td>4.2</td>
</tr>
<tr>
<td>mS1P5</td>
<td>2.2</td>
<td>4.2</td>
</tr>
</tbody>
</table>

ND, not determined.

**Fig. 3.** The effects of S1P (A) and Compound A (B) on mean arterial pressure (MAP) and heart rate (HR) were evaluated in barbiturate-anesthetized rats (three per group). HR and MAP were recorded continuously and are expressed as average values at 1-min intervals. Error bars have been omitted for the sake of clarity. Compounds were administered (arrow) as an intravenous bolus of approximately 10-s duration at 0.2 (A) or 0.05 (B) mg/kg. Significant differences from baseline control values are shown as: *p < 0.05 for HR; and †p < 0.05 for MAP.

**Fig. 4.** The effects of S1P receptor agonists on heart rate (HR) were evaluated in barbiturate-anesthetized rats. HR was determined for at least 15 min prior to and 30 min post-compound administration at the doses shown. Decrease in heart rate was calculated as the maximum change in heart rate during the 10-min period after compound administration in comparison with the average value for 5 min prior to compound administration. Each data point represents the mean, and vertical lines represent the S.E.M. for three to five animals per group.
Administration of either S1P or Compound A to conscious unrestrained mice evoked significant bradycardia compared with predose values in wild-type mice (Fig. 7). However, in S1P3−/− mice, administration of either Compound A or S1P did not produce any change in heart rate. Furthermore, both compounds produced an increase in MAP in wild-type mice. In S1P3−/− mice, administration of Compound A evoked a small but significant decrease in MAP, whereas S1P administration was without effect on blood pressure. In wild-type or S1P3−/− mice that received an equivalent volume of vehicle, there were no meaningful changes in either heart rate or MAP. S1P3−/− heterozygote mice had the same response to Compound A as wild-type mice (data not shown).

Localization of S1P1 and S1P3 in Rodent Heart. As expected, S1P1 was heavily expressed by the vascular endothelial cells of capillaries (Fig. 8A) and larger blood vessels (Fig. 8F), but S1P1 expression was not detectable in cardiomyocytes of normal rat heart. In contrast, intense S1P3 labeling was observed along the cell surface membranes of rat ventricular and atrial cardiomyocytes, and moderate S1P3 expression was also seen within the myocyte cytoplasm (Fig. 8, B and E). S1P3 labeling was also heavily localized on the outer cell membranes of wild-type mouse ventricular cardiomyocytes (Fig. 8C1) but was not detectable in S1P3 knockout mice (Fig. 8C2), demonstrating the specificity of the S1P3 labeling pattern. In contrast, vascular endothelial cells were similarly labeled by the S1P1 antibody in wild-type and S1P3 knockout mice (data not shown). Furthermore, while S1P1 labeling was readily detected in the vascular endothelium of rat hearts as indicated by double labeling with a PECAM mAb (Fig. 8F), No S1P3 expression was observed in the vascular endothelium (Fig. 8, D and E). However, the subadjacent layer of rat vascular smooth muscle exhibited considerable S1P3-specific staining (Fig. 8D). These results were shown to be immunospecific for both S1P1 and S1P3 expression by the following criteria: staining was inhibited by pretreatment of primary antibody with relevant, but not by irrelevant peptide; substitution of nonimmune rabbit IgG for the primary antibody abolished the labeling; similar S1P1 staining patterns occurred using either C- or N-terminal S1P1 antibodies; immunoblotting experiments conducted on crude cellular extracts demonstrated that all S1P antibodies used for immunohistochemistry specifically labeled their respective proteins; and staining of S1P3 was absent in the S1P3−/− mouse hearts (Fig. 8C), and staining of S1P1 was absent in S1P3−/− mouse embryos (Allende et al., 2003).
We have investigated the cardiovascular and lymphopenic effects of differentially selective S1P receptor agonists. S1P3 activity correlated with toxicity and bradycardia, and this association was confirmed using mice genetically deleted for S1P3. In contrast, S1P1 activity correlated significantly with depletion of circulating lymphocytes. S1P has been known to have essential functions on endothelial cells in regulating migration, angiogenesis, adherens junctions, and vascular permeability (Lee et al., 1999; Garcia et al., 2001), and S1P1/edg1 is required for vascular maturation (Liu et al., 2000; Allende et al., 2003). The physiological role for S1P receptors in regulating immune cell function has emerged only recently with the discovery that FTY720, a compound in clinical trials for transplantation, is a prodrug and that its phosphorylated active metabolite is an agonist on four of the five S1P receptors (Brinkmann et al., 2002; Mandala et al., 2002).

Unlike other immunosuppressants, FTY720 depletes peripheral blood CD4+ and CD8+ T cells and B lymphocytes and sequesters them in secondary lymphoid organs (Chiba et al., 1998). Within the lymph node, antigen presentation, activation, and proliferation of T cells are normal, memory and humoral responses are intact, but effector cells are unable to egress to mount a local immune response (Pinschewer et al., 2000; Xie et al., 2003). The mechanism of PBL depletion by FTY720 was first attributed to accelerated homing (Chiba et al., 1998), and early reports suggested that S1P4 might be the target for FTY720 action given its expression in leukocytes and effects on actin cytoskeletal rearrangement (Brinkmann et al., 2001). However, S1P1 mRNA is also highly expressed in lymphocytes, and retroviral transduction of S1P1 into a Th1 line induced migratory responses to S1P (Dorsam et al., 2003). FTY720 was proposed to antagonize S1P-induced migration (Graeler and Goetzl, 2002), whereas we find that S1P acts similarly to FTY720 in redistributing blood lymphocytes to secondary lymphoid organs (Mandala et al., 2002).

Studies with analogs of FTY720 revealed that depletion of circulating lymphocytes correlated well with efficacy in promoting rat skin allograft survival (Kiuchi et al., 2000). Therefore, we used lymphopenia as a pharmacodynamic assay to analyze structure activity relationships of S1P receptor agonists. Ligand binding and G protein coupling assays with
human, mouse, and rat receptors indicated that S1P and the five synthetic analogs described here have similar potencies and the same rank order of activity on the receptors from different species. Previous characterization of Compound B, the phosphonate analog of Compound A, revealed that the metabolically stabilized analog was able to induce lymphopenia despite a significant loss in potency on the S1P receptors (Mandala et al., 2002). A modest improvement in S1P₁ potency and in vivo activity was achieved with Compound C, the deshydroxy analog of B. We discovered that the primary amine was not essential for receptor activity and identified Compound D as one of the most potent phosphonate analogs. Compound D induced lymphopenia with an ED₅₀ only 2-fold reduced relative to Compound A. None of the synthetic ana-
logs had significant activity on S1P₃, but Compounds A to D were relatively nonselective on the other receptors. Selectivity against S1P₄ was achieved in the secondary amine series (Compounds E and F) by substitutions to the phenyl ring and alkyl chain shortening, with no change on S1P₂ activity and only modest effects on S1P₁ and S1P₄. The correlation between S1P₁ receptor activity and lymphopenia is highly significant, whereas none of the other receptor activities are consistent with efficacy.

Intravenous administration of compounds evoked toxicity that ranged from lethality (S1P and Compound D), transient symptoms of ruffling and paralysis (Compound A and C), to no adverse symptoms (Compounds E and F). The correlation between S1P₃ receptor activity of the phosphonates and toxicity was very high, and compounds did not produce any acute symptoms in the S1P₃⁻/⁻ mice, although they did induce lymphopenia. However, S1P and Compound A were less toxic than Compound D despite enhanced or equivalent S1P₃ potency, which we attribute to the metabolic lability of the phosphate ester bond. Compound A is relatively stable in blood but rapidly reaches equilibrium with FTY720 in vivo (Mandala et al., 2002), suggesting that the high kinase activity in blood is balanced by phosphatases.

The underlying mechanism of toxicity may be the cardiovascular effects of S1P receptor agonists mediated through activation of S1P₃ receptors. This conclusion is derived from three observations. The rank order of compound potency for bradycardia is more closely aligned with the potency for activation of S1P₃ than the other S1P receptors. Second, in conscious rats, the 10-fold shift in potency of Compound A for inducing lymphopenia versus bradycardia and hypertension is consistent with affinity for S1P₁ relative to S1P₃. Finally, in conscious S1P₃ receptor knockout mice, S1P and Compound A did not evoke bradycardia, whereas significant decreases in heart rate were observed in control wild-type mice.

In clinical trials with FTY720, a dose-associated bradycardia has been reported (Budde et al., 2002). Furthermore, a negative chronotropic effect of S1P receptor agonists was observed in isolated perfused guinea pig hearts (Liliom et al., 2001) and in rabbit sinoatrial node cells (Guo et al., 1999). In contrast, S1P increases sinoatrial rate in a canine preparation (Sugiyama et al., 2000b). The discrepancy between these observations is not clear but could be a species-dependent difference. S1P and sphingosine phosphorylcholine activate an IᵥᵥCa₉ channel that is a major contributor to vagally mediated cardiac slowing (Bunemann et al., 1995; Liliom et al., 2001). S1P-induced activation of IᵥᵥCa₉ was suggested to be mediated via the putative S1P₃ receptor antagonist suramin (Himmel et al., 2000). However, suramin was only S1P₃ selective in oocyte transfectants with heterologous G proteins and required micromolar levels for inhibition (Ancellin and Hla, 1999), whereas IᵥᵥCa₉ channel activity was inhibited at nM concentrations of suramin (Himmel et al., 2000).

The effects of S1P receptor agonists on MAP are complex. Intravenous administration of either S1P or Compound A to anesthetized rats evoked an initial decline in blood pressure contemporaneous with a rapid decline in heart rate. We surmise that the initial rapid decline in MAP may be secondary to reduced heart rate and cardiac output from S1P₃ activation. The decrease in MAP was followed by an equally rapid restoration of pressure and, in many cases, a slight overshoot that may be a baroreflex response. Published studies on the effects of S1P agonists in anesthetized rats also describe a rapid decrease in MAP of short duration in response to intravenous administration of S1P (Sugiyama et al., 2000a). Intravenous administration of FTY720 (1 mg/kg) produces an increase in MAP that normalized within 30 min, whereas a higher dose of FTY720 (5 mg/kg) produces an immediate decline in MAP that reversed within 10 min (Tawadrous et al., 2002). However, because these studies did not include information on changes in heart rate, it is difficult to provide a meaningful comparison with our own data.

Continuous intravenous infusion of S1P receptor agonists in conscious animals induced hypertension. The data obtained in S1P₃ receptor knockout and wild-type mice are consistent with hypertension mediated via S1P₃ receptors. Interestingly, Compound A, but not S1P, produced a modest hypotension in the S1P₃ knockout mice. It is conceivable that the hypotensive response evoked with Compound A may not be mediated via S1P receptors. Alternatively, S1P receptor agonists may have opposing effects on MAP, a hypertensive response mediated via S1P₃ receptors and a hypotensive response mediated via other S1P receptor subtype(s). Indeed, S1P has been reported to evoke vasoconstriction in isolated vascular preparations at concentrations >0.1 μM (Bischoff et al., 2000; Tosaka et al., 2001; Salomone et al., 2003), whereas vasorelaxation was observed at S1P concentrations <0.1 μM (Dantas et al., 2003). The contractile response was attributed to S1P₃ activation (Salomone et al., 2003), whereas relaxation was attributed to S1P receptor-mediated activation of endothelial nitric oxide synthase and NO-dependent vasodilation (Dantas et al., 2003). Thus, the net effect of S1P on MAP will depend on the relative affinities for S1P receptors that mediate vasorelaxation versus those that mediate vasoconstriction.

Immunohistochemical studies on rat and mouse atrial and ventricular heart tissue revealed an intense expression of S1P₃ on myocytes and vascular smooth muscle cells that was absent in the S1P₃⁻/⁻ mice. This report is the first description of S1P₃ immunolocalization in heart tissue, although the same distribution pattern for S1P₃ mRNA was observed in human heart tissue (Mazurais et al., 2002). In contrast to S1P₃, which was not detectable on endothelium, anti-S1P₁ labeled endothelial cells almost exclusively. Intense anti-S1P₁ staining of capillaries and endothelium of large blood vessels was observed with significant PECAM codistribution. In contrast to other reports (Nakajima et al., 2000; Robert et al., 2001), we did not observe any detectable S1P₁ expression on cardiomyocytes. Species or antibody specificity may explain the differing results.

In conclusion, we describe novel pharmacological tools that have helped distinguish the subtype-specific roles of S1P receptors in regulating lymphocyte recirculation and cardiovascular function. Further mechanistic insight will be required to confirm the hypothesis that S1P₁ is the molecular target for the immunosuppressive effects of FTY720 and to characterize the pathway between S1P₁ activation and lymphocyte sequestration. Bradycardia and hypertension were clearly associated with S1P₁ activation and its expression patterns in cardiac tissue; these reagents should be useful in further dissecting the complexities of S1P mediated regulation of blood pressure.
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Bischoff A, Czyborra P, Fetscher C, zu Heringdorf DM, Jakobs KH, and Michel MC
Address correspondence to: Dr. Suzanne Mandala, Merck Research Laboratories, Department of Immunology and Rheumatology, P.O. Box 2000, RY80Y-215, Rahway, NJ 07065. E-mail: Suzanne_mandala@merck.com
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