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Immune Cell Regulation and Cardiovascular Effects of Sphingosine 1-Phosphate Receptor Agonists in Rodents are Mediated via Distinct Receptor Sub-Types.

M. Forrest, S.-Y. Sun, R. Hajdu, J. Bergstrom, D. Card, G. Doherty[1], J. Hale, C. Keohane, C. Meyers, J. Milligan, S. Mills, N. Nomura, H. Rosen[2], M. Rosenbach, G.-J. Shei, I. I. Singer, M. Tian, S. West, V. White, J. Xie, R. Proia[3], S. Mandala*

Departments of Immunology & Rheumatology, Pharmacology, and Medicinal Chemistry, Merck Research Laboratories, Rahway, New Jersey, USA

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Running title: Immune Cell Regulation and Cardiovascular Effects of Sphingosine 1-Phosphate Receptor Agonists

Suzanne Mandala, Ph.D., Merck Research Laboratories, Dept. of Immunology & Rheumatology, PO Box 2000, RY80Y-215, Rahway, NJ, 07065, USA, 732-594-3327 (phone); 732,-594-2254 (fax); suzanne_mandala@merck.com

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ABSTRACT

Sphingosine 1-phosphate 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, 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: 1) compound potency on $S1P_3$ correlated with toxicity and bradycardia; 2) the shift in potency of phosphorylated-FTY720 for inducing lymphopenia vs. bradycardia and hypertension was consistent with affinity for $S1P_1$ relative to $S1P_3$; 3) 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.

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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 d13.5 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 (Okamoto 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).

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A novel physiological role for S1P in immune regulation has been recently discovered 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 $S1P_3$, but 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 7 steps from 2-acetylamino-2-(2-(4-octylphenyl)ethyl)propane-1,3-dicarboxylic acid, diethyl ester (Durand et al., 2000). Compounds D, E and F were prepared by reductive amination of the appropriate aryl aldehyde with 3-aminopropylphosphonic acid ($Na(CN)BH_3$, MeOH, 50 ^oC). All compounds were characterized by ^1H NMR, MS and HPLC and were judged to be >95% pure. Detailed procedures are provided in patents WO 03074008 and WO 03062252.

Mouse Lymphocyte Reduction Assay. Mice (3 per group) were dosed intravenously with 0.1 ml of test compound dissolved in vehicle (2% (w/v) hydroxypropyl-β-cyclodextrin (Cerestar USA), 0.12 M NaCl) and peripheral blood lymphocyte counts were assessed three hours 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. In order 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 PCR 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 $S1P_1$); 5'-GAACCCGGGCATCCACGCATGCGCAG and 5'-GCCGAATTCTCACTTGCAGAGGACCCCATTCTG (rat $S1P_3$). The PCR products were inserted in frame after a FLAG tag using vector pCMV-Tag2 (Stratagene). Stable lines were established by transfecting plasmids into CHO cells using lipofectamine

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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 BSA and added to test compounds diluted in DMSO, 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 % BSA, 50mM HEPES-Na, pH 7.5, 5mM $MgCl_2$, 1mM $CaCl_2$ and 0.3 to 0.7 µg 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 radionuclide 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 protein) were incubated in 96-well plates with test compounds diluted in DMSO, in 100 µl of buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, and 2 – 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; NEN) for an assay concentration of 125 pM. After 60 min incubation at room temperature, membranes were harvested only 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 and 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 hours) 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 to 350 g body weight) with surgically implanted femoral artery and vein catheters were obtained from Charles River Laboratories (Raleigh, NC). Animals were anesthetized with Nembutal (55 mg/kg, ip), a Becton Dickinson DTX pressure transducer (TNF-R, Franklin Lake, NJ) 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 minutes duration) and subsequently compounds were administered intravenously (bolus injection of approximately 10 seconds). Cardiovascular data were recorded continuously and are reported as average values over 1 minute intervals.

Conscious Rat Cardiovascular Assay. For the assessment of cardiovascular function in conscious rats, male Sprague-Dawley rats (300 to 350 g body weight) with surgically implanted femoral artery and vein catheters were obtained from Charles River Laboratories (Raleigh, NC). 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 two day acclimation prior to further experimentation. On the day of study a Becton Dickinson 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 minutes to establish baseline values. Subsequently, compound or vehicle was administered as a continuous intravenous infusion of 25 μL/min for 4 hours. Blood samples, for the evaluation of circulating leukocytes, were obtained from the arterial catheter 30 minutes prior to dosing, and 1 and 4 hours post initiation of the infusion. Cardiovascular data were recorded continuously and are reported as average values over 1 minute intervals.

Mouse Cardiovascular Assay. For the assessment of cardiovascular function in conscious mice, the $S1P_3$ receptor ($S1P_3^{-/-}$) was genetically deleted (R.L.P, manuscript submitted) and bred at Taconic Farms Inc. (Germantown, NY). Male (B6.129) $S1P_3^{-/-}$ and $S1P^{+/+}$ mice (20 to 30 g body weight) were anesthetized with ketamine (80-100 mg/kg, IP) and xylazine (10 mg/kg, IP) and catheters 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., Plymouth Meeting, PA) 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 Becton Dickinson 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 minutes to establish baseline values. Subsequently, compound or vehicle was administered intravenously as a bolus of 10 seconds duration. Cardiovascular data were recorded continuously and are reported as average values over 10 second 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 TissueTek cryomolds (Cat# 4557, Torrance, CA) filled with O.C.T. Compound (TissueTek Cat# 4583, Torrance, CA), frozen in liquid nitrogen and stored at -80 ^{o}C. Frozen sections were cut (5 µm thickness) on a Bright Model OTF cryotome (Hacker Instruments, Fairfield, NJ), and mounted on Instrumedics coated slides (Cat# CFSACS, Hackensack, NJ). To block non-specific labeling, sections were treated with 5% donkey serum in PBS for 20 min, then with a clarified solution of 5% non-fat dry milk for 30 min, and finally with Fc blocker (Accurate Chemical, Westbury, NY) for 20 min. Sections were labeled for one hour 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, Cat # 555025). All non-immune IgG controls were obtained from Jackson Laboratories, 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 minutes) from Jackson Laboratories. 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. Non-immune IgG controls were also run in this fashion. Specificity was also demonstrated by preincubating each primary antibody at staining concentrations with its relevant or irrelevant peptide at 5 µg/ml for 1 hour, and centrifugation for 1 hour at 13500 x g at 4^0C (Beckman Microfuge 11, Palo Alto, CA). After staining, the slides were fixed for 30 min. in 4% formaldehyde freshly generated from paraformaldehyde in phosphate

buffer pH 7.4 (Fisher Scientific, Cat# 04042500, Pittsburgh, PA). Coverslips were mounted on the slides with Vectashield plus DAPI nuclear stain (Vector Laboratories, Cat# H1200, 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 fluorochrome conjugated secondary antibodies individually or in combination on the same section in double labeling experiments.

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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 previously found 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 hour 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 two-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 to10-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 were tested against human receptors expressed in CHO cells in competitive ligand binding assays using [^{33}P]-sphingosine 1-phosphate 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 2-3 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 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, E, and 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 previously deduced from the human receptors, the correlation between $S1P_1$ receptor activation and the ED_{50}

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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 x 10^6/ml compared to 5.0 x 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 minutes of compound

administration and returning towards baseline values within 10 minutes. 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 minutes post compound administration before resolving within 20 minutes. 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 $S1P_1$ and $S1P_3$ 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 minutes 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 $\geq$ D > E ~ S1P >> Compound F. The reduced effects of Compounds E and F on heart rate are consistent with their reduced IC_{50} values against $S1P_3$ 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 μg/kg/minute, bradycardia (Fig. 5A) and hypertension (Fig. 5B) were evident within 15 to 30 minutes post initiation 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 minutes. The decrease in

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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 μg/kg/minute, whereas significant changes in heart rate and mean arterial pressure were not evident until doses of 1 μg/kg/minute 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 $S1P_3$ receptor. Accordingly, the cardiovascular effects of S1P receptor agonists were evaluated in $S1P_3^{-/-}$ mice. Administration of either S1P or Compound A to conscious unrestrained mice evoked significant bradycardia compared with pre-dose values in wild type mice (Fig. 7). However, in $S1P_3^{-/-}$ mice administration of either Compound A or S1P did not produce any change in heart rate. Furthermore, as was observed in anesthetized and conscious rats, both compounds produced an increase in MAP in wild type mice. In $S1P_3^{-/-}$ 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 $S1P_3^{-/-}$ mice that received an equivalent volume of vehicle there were no meaningful changes in either heart rate or MAP. $S1P_3^{+/-}$ heterozygote mice had the same response to Compound A as wild type mice (data not shown).

Localization of $S1P_1$ and $S1P_3$ in Rodent Heart. As expected, $S1P_1$ was heavily expressed by the vascular endothelial cells of capillaries (Fig 8A) and larger blood vessels (Fig 8F), but $S1P_1$ expression was not detectable in cardiomyocytes of normal rat heart. In contrast, intense $S1P_3$ labeling was observed along the cell-surface membranes of rat ventricular and atrial cardiomyocytes, and moderate $S1P_3$ expression

was also seen within the myocyte cytoplasm (Fig 8B, E). $S1P_3$ labeling was also heavily localized on the outer cell membranes of wild-type mouse ventricular myocytes (Fig C1), but was not detectable in $S1P_3$ knockout mice (Fig C2), demonstrating the specificity of the $S1P_3$ labeling pattern. In contrast, vascular endothelial cells were similarly labeled by the $S1P_1$ antibody in wild-type and $S1P_3$ knockout mice (data not shown). Further, while $S1P_1$ labeling was readily detected in the vascular endothelium of rat hearts as indicated by double labeling with a PECAM mAb (Fig 8F), no $S1P_3$ expression was observed in the vascular endothelium (Fig 8D & E). However, the subadjacent layer of rat vascular smooth muscle exhibited considerable $S1P_3$-specific staining (Fig 8D). These results were shown to be immuno-specific for both $S1P_1$ and $S1P_3$ expression by the following criteria: a) staining was inhibited by pretreatment of primary antibody with relevant, but not by irrelevant peptide; b) substitution of non-immune rabbit IgG for the primary antibody abolished the labeling; c) similar $S1P_1$ staining patterns occurred using either C-terminal or an N-terminal $S1P_1$ antibodies; d) immunoblotting experiments conducted on crude cellular extracts demonstrated that all S1P antibodies used for immunohistochemistry specifically labeled their respective proteins; e) staining of $S1P_3$ was absent in the $S1P_3^{-/-}$ mouse hearts (Fig 8C) and staining of $S1P_1$ was absent in $S1P_1^{-/-}$ mouse embryos (Allende et al., 2003).

Discussion

We have investigated the cardiovascular and lymphopenic effects of differentially selective S1P receptor agonists. $S1P_3$ activity correlated with toxicity and bradycardia, and this association was confirmed using mice genetically deleted for $S1P_3$. In contrast, $S1P_1$ 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 $S1P_1$/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 only recently emerged 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 $CD4^+$, $CD8^+$, 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 $S1P_4$ might be the target for FTY720 action given its expression in leukocytes and effects on actin cytoskeletal rearrangement (Brinkmann et al., 2001). However, $S1P_1$ mRNA is also highly expressed in lymphocytes and retroviral transduction of $S1P_1$ 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 5 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 in spite of a significant loss in potency on the S1P receptors (Mandala et al., 2002). A modest improvement in $S1P_1$ 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 ED_{50} only 2-fold reduced relative to Compound A. None of the synthetic analogs had significant activity on $S1P_2$, but Compounds A, B, C, and D were relatively non-selective on the other receptors. Selectivity against $S1P_3$ 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 $S1P_5$ activity, and only modest effects on $S1P_1$ and $S1P_4$. The correlation between $S1P_1$ 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 $S1P_3$ receptor activity of the phosphonates and toxicity was very high, and compounds did not produce any acute symptoms in the $S1P_3^{-/-}$ mice, although they did induce lymphopenia. However, S1P and Compound A were less toxic than Compound D in spite of enhanced or equivalent $S1P_3$ 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 $S1P_3$ 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 $S1P_3$ than the other S1P receptors. Secondly, in conscious rats the 10-fold shift in potency of Compound A for inducing lymphopenia vs. bradycardia and hypertension is consistent with affinity for $S1P_1$ relative to $S1P_3$. Finally, in conscious $S1P_3$ 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 inwardly rectifying K^+ channel ($I_{K.Ach}$) that is a major contributor to vagally mediated cardiac slowing (Bunemann et al., 1995; Liliom et al., 2001). S1P induced activation of $I_{K.Ach}$ was suggested to be mediated via the $S1P_3$ receptor subtype based on inhibition by the putative $S1P_3$ receptor antagonist suramin (Himmel et al., 2000). However, suramin was only $S1P_3$ selective in oocyte transfectants with heterologous G proteins and required μM levels for inhibition (Ancellin and Hla, 1999), whereas $I_{K.Ach}$ 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 $S1P_3$ activation. The decrease in MAP was followed by an equally rapid restoration of pressure and in many cases a slight overshoot which 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 minutes, whereas a higher dose of FTY720 (5 mg/kg) produces an immediate decline in MAP that reversed within 10 minutes (Tawadrous et al., 2002). However, as these studies did not include

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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 $S1P_3$ receptor knockout and wild type mice are consistent with hypertension mediated via $S1P_3$ receptors. Interestingly, Compound A, but not S1P, produced a modest hypotension in the $S1P_3$ 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 $S1P_3$ 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 >0.1μM (Bischoff et al., 2000; Tosaka et al., 2001; Salomone et al., 2003), whereas vasorelaxation was observed at S1P concentrations <0.1μM (Dantas et al., 2003). The contractile response was attributed to $S1P_3$ activation (Salomone et al., 2003), while 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 vs. those that mediate vasoconstriction.

Immunohistochemical studies on rat and mouse atrial and ventricular heart tissue revealed an intense expression of $S1P_3$ on myocytes and vascular smooth muscle cells that was absent in the $S1P_3^{-/-}$ mice. This report is the first description of $S1P_3$ immunolocalization in heart tissue, although the same distribution pattern for $S1P_3$ mRNA was observed in human heart tissue (Mazurais et al., 2002). In contrast to $S1P_3$,

which was not detectable on endothelium, anti-$S1P_1$ labeled endothelial cells almost exclusively. Intense anti- $S1P_1$ staining of capillaries and endothelium of large blood vessels was observed with significant PECAM co-distribution. In contrast to other reports (Nakajima et al., 2000; Robert et al., 2001) we did not observe any detectable $S1P_1$ expression on cardiomyocytes. Species or antibody specificity may explain the differing results.

In conclusion, we describe novel pharmacological tools that have helped distinguish the sub-type specific roles of S1P receptors in regulating lymphocyte recirculation and cardiovascular function. Further mechanistic insight will be required to confirm the hypothesis that $S1P_1$ is the molecular target for the immunosuppressive effects of FTY720, and to characterize the pathway between $S1P_1$ activation and lymphocyte sequestration. Bradycardia and hypertension were clearly associated with $S1P_3$ 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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FOOTNOTES

[1]Current address: Array BioPharma, Boulder, CO

[2]Current address: The Scripps Research Institute, La Jolla, CA, USA

[3]Address: National Institute of Health, Bethesda, MD

*Corresponding author

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Figure Legends

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

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 hrs later.

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 (3 per group). HR and MAP were recorded continuously and are expressed as average values at 1 minute intervals. Error bars have been omitted for the sake of clarity. Compounds were administered (arrow) as an intravenous bolus of approximately 10 seconds duration. Significant differences from baseline control values are shown as * $P<0.05$ for HR and ǂ $P<0.05$ 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 minutes prior to and 30 minutes post compound administration at the doses shown. Decrease in heart rate was calculated as the maximum change in heart rate during the 10 minute period after compound administration in comparison with the average value for 5 minutes prior to compound administration. Each data point represents the mean and vertical lines the standard error of the mean for 3 to 5 animals per group.

Fig. 5. The effects of Compound A, on heart rate (A: HR) and mean arterial pressure (B: MAP) were evaluated in conscious unrestrained rats. Compound A or an equivalent volume of vehicle was infused intravenously for 4 hours during the period as indicated. HR and MAP were recorded continuously and are expressed as average values at 1 minute intervals. Each data point represents the mean and vertical lines the standard error of the mean for 3 to 5 animals per group.

Fig. 6. The effects of Compound A, on heart rate (HR), mean arterial pressure (MAP) and circulating lymphocytes were evaluated in conscious unrestrained rats. Compound A at the doses shown was infused intravenously for 4 hours. Changes in HR and MAP were calculated as the average recorded value for the entire period of infusion as a percentage of the average value for the 20 minute period prior to compound administration. Change in lymphocytes are the number of cells measured in peripheral blood obtained at the conclusion of compound administration as a percentage of the number determined in a blood sample collected 30 minutes prior to compound administration. Each data point represents the mean and vertical lines the standard error of the mean for 3 to 5 animals per group.

Fig. 7. The effects of the S1P receptor agonists, Compound A (A & B) and S1P (C & D) on heart rate (HR: A & C) and mean arterial pressure (MAP: B & D) were evaluated in conscious unrestrained wild type or $S1P_3$ receptor knock out mice (4-7 per group). HR and MAP were recorded continuously and are expressed as average values at 1 minute intervals. Error bars have been omitted for the sake of clarity. Compounds were

administered (arrow) as an intravenous bolus of approximately 10 seconds duration. Significant differences from baseline control values are shown as * $P<0.05$ for wild type and ‡ $P<0.05$ for $S1P_3$ knock out mice.

Fig. 8. Localization of $S1P_1$ and $S1P_3$ expression in rodent heart by immunofluorescence microscopy. A) Anti-$S1P_1$ (red fluorescence) labels the capillary endothelial cells but not the myocytes of the rat heart ventricle. (Bar for A-C & E = 30 μm. B) Anti-$S1P_3$ (red fluorescence) is concentrated on the outer cell membranes of the rat ventricular (B1) and atrial (B2) myocytes. C) $S1P_3$ (red fluorescence) is heavily expressed on the cell membranes of myocytes from wild-type mice (C1) but not in hearts from $S1P_3$ knockout mice (C2). D1) Double labeling of a rat heart atrial blood vessel showing intense PECAM (green fluorescence) staining of the endothelium, and $S1P_3$ expression (red fluorescence) in the surrounding smooth muscle. D2) Same section shown in D1 viewed without green fluorescence. No detectable $S1P_3$ expression is present in the endothelium. Bar for D & F = 15 μm. E) Expression of PECAM (green fluorescence) and $S1P_3$ (red fluorescence) in the rat heart atrium. A small blood vessel exhibits intense endothelial PECAM labeling without co-localized $S1P_3$, which was localized in the surrounding myocytes. F) Double labeling of a rat ventricular blood vessel showing co-distribution of $S1P_1$ (red fluorescence) and PECAM (green fluorescence) staining in the endothelium. The surrounding myocytes do not exhibit $S1P_1$ expression. All sections were counterstained with DAPI nuclear stain (blue fluorescence).

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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 (nM) are the mean of 2 to 6 measurements performed in duplicate. nd, not determined; 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
$S1P_1$ 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	>10000
$S1P_4$ 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

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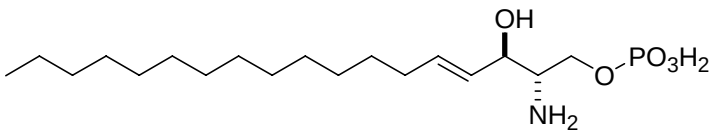
TABLE 2

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

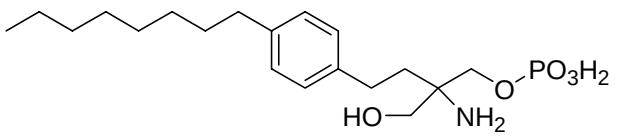
ED_{50} values (+/- standard error) are the dose (in mg/kg) which produces 50% of the maximum reduction in peripheral blood lymphocytes at 3 hours. Toxicity is the maximum tolerated dose (mg/kg) as defined in Materials and Methods. Compounds were tested in vitro in [^{33}P]-S1P binding assays to determine IC_{50} values (nM) using membranes prepared from CHO cells expressing mouse and rat S1P receptors.

	S1P	A	C	D	E	F
PBL ED_{50}	nd	0.03 (0.01)	0.32 (0.08)	0.063 (0.007)	0.7 (0.4)	2.2 (0.8)
Toxicity	0.4	1.0	4.0	0.05	>10	>30
m$S1P_1$ IC_{50}	0.3	0.3	2.5	0.9	4.1	14.6
r$S1P_1$ IC_{50}	0.3	0.3	2.1	0.8	4.2	6.4
m$S1P_3$ IC_{50}	0.3	2.7	71	1.8	605	6576
r$S1P_3$ IC_{50}	0.3	3.1	74	2.6	825	7172
m$S1P_4$ IC_{50}	40	28	99	130	109	200
m$S1P_5$ IC_{50}	0.3	0.5	29	2.8	7.1	4.2

Fig. 1



Sphingosine-1-phosphate (S1P)



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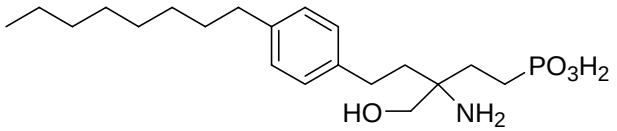

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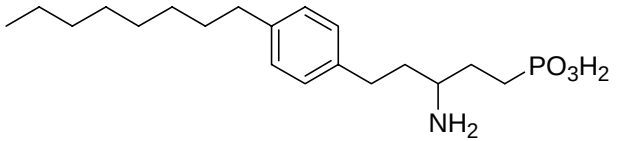

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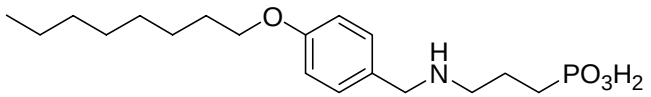
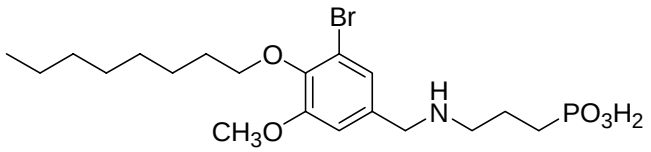
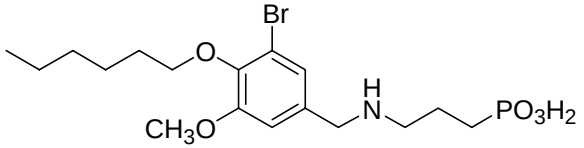


Compound D

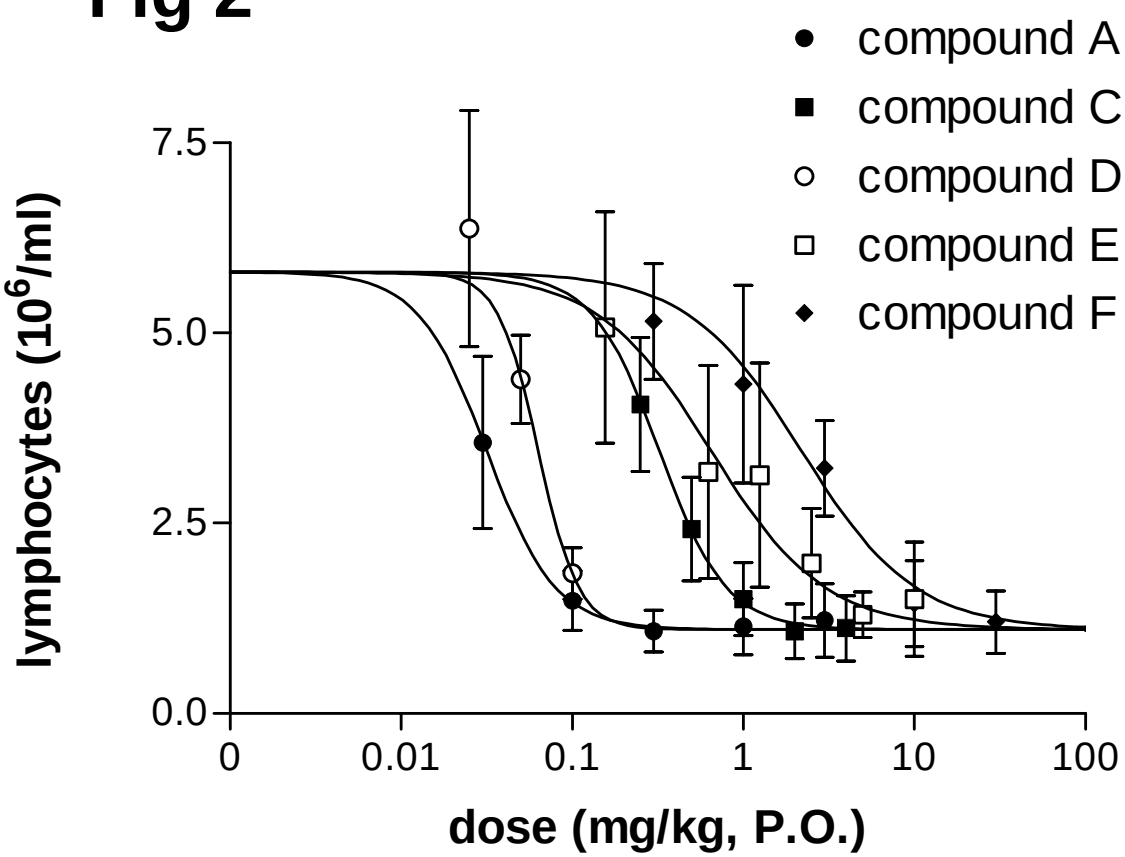


Compound E



Compound F

Fig 2



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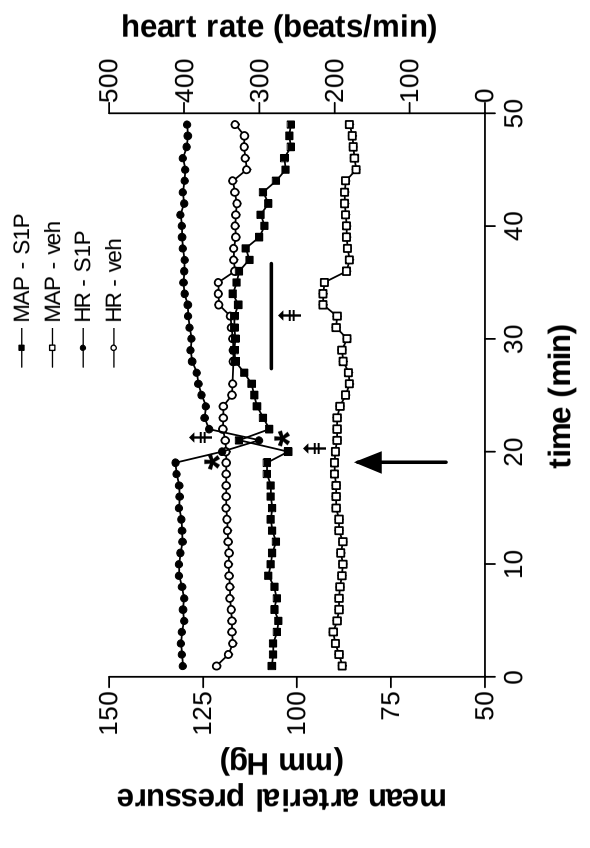


Fig 3A

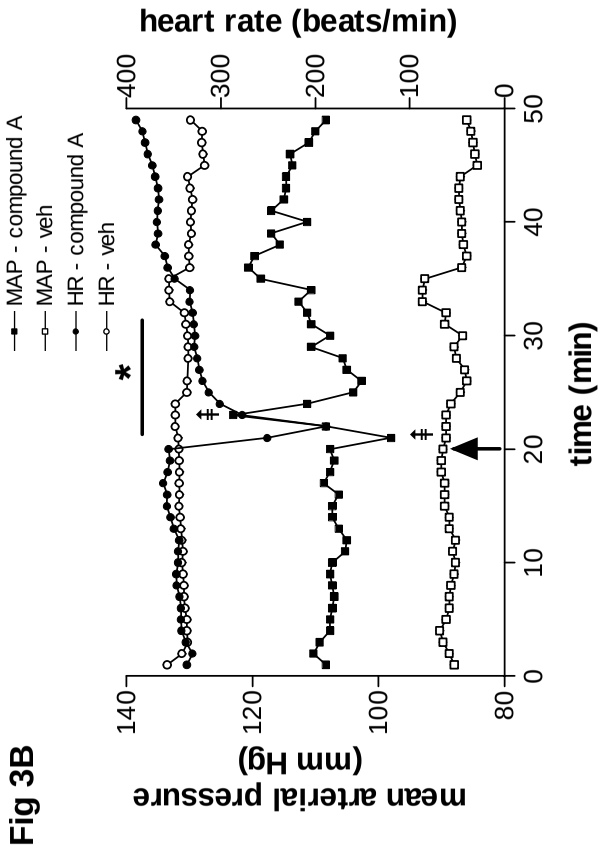


Fig 3B

Fig 4

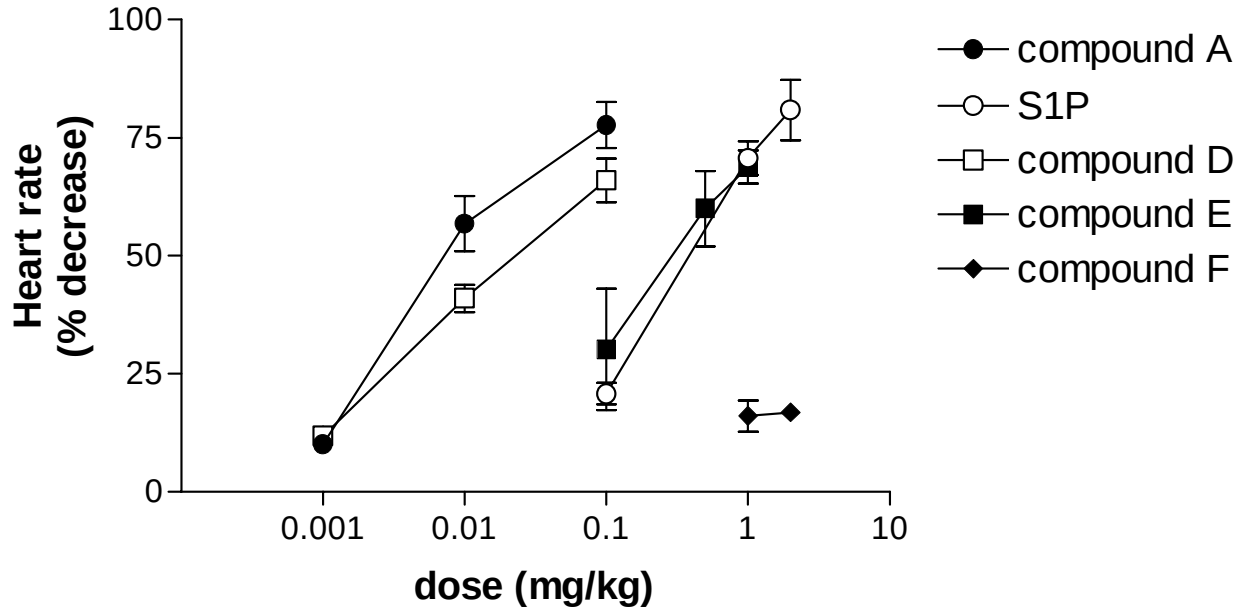


Fig 5A

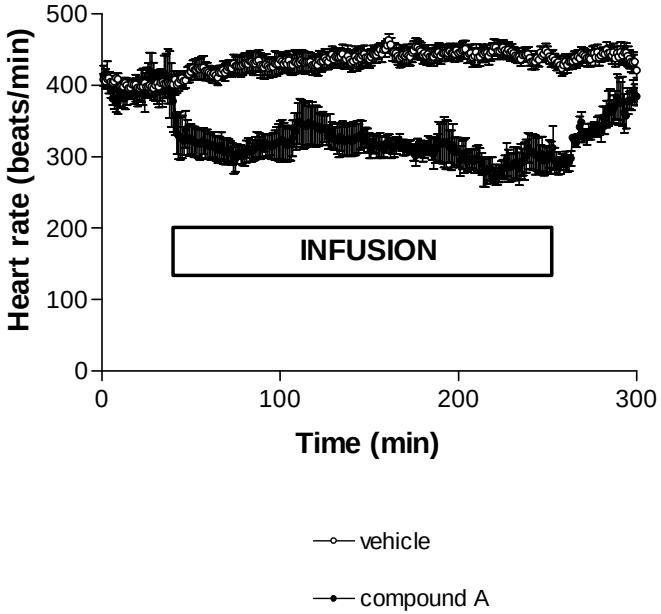
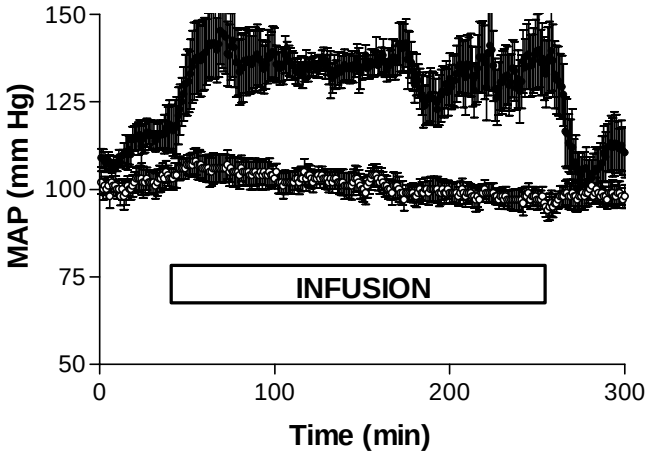


Fig 5B



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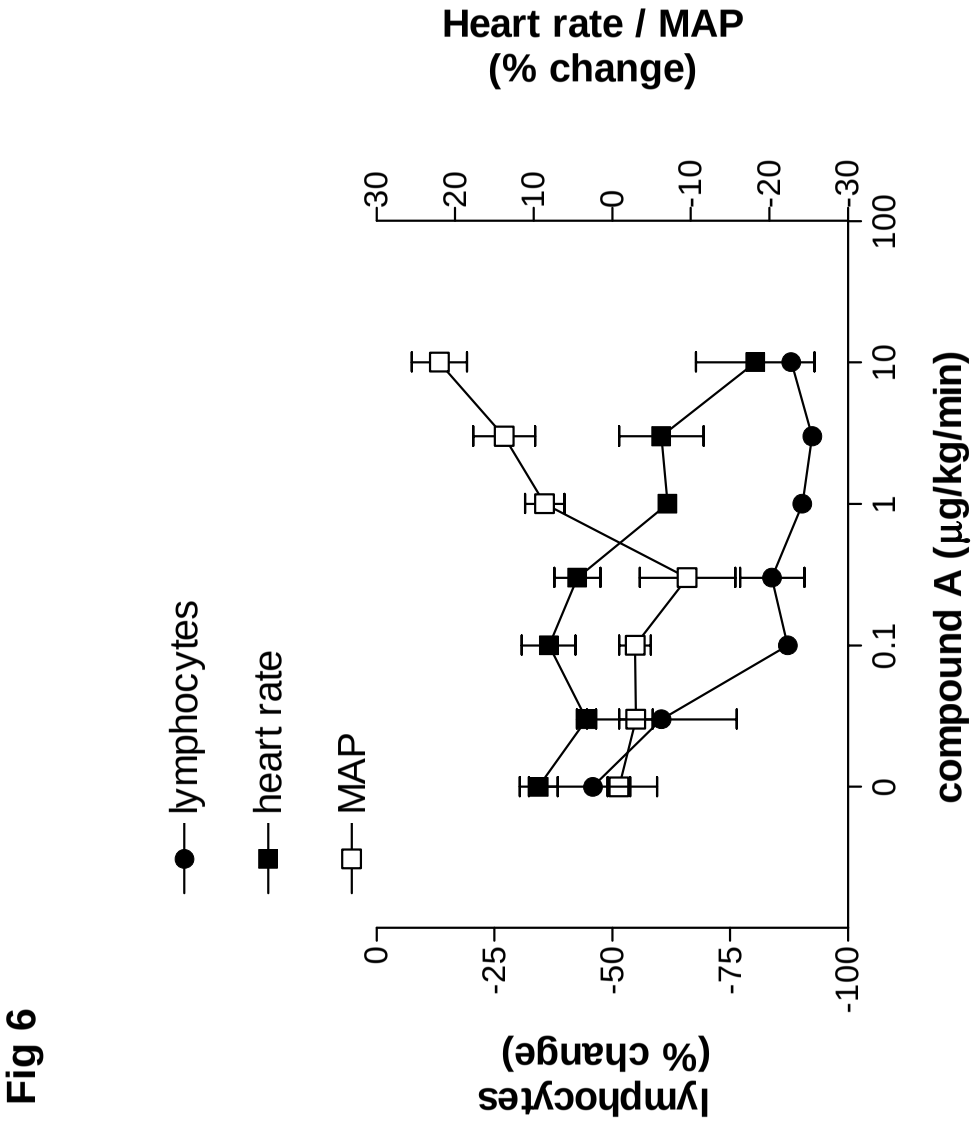

Fig 6

Fig 7

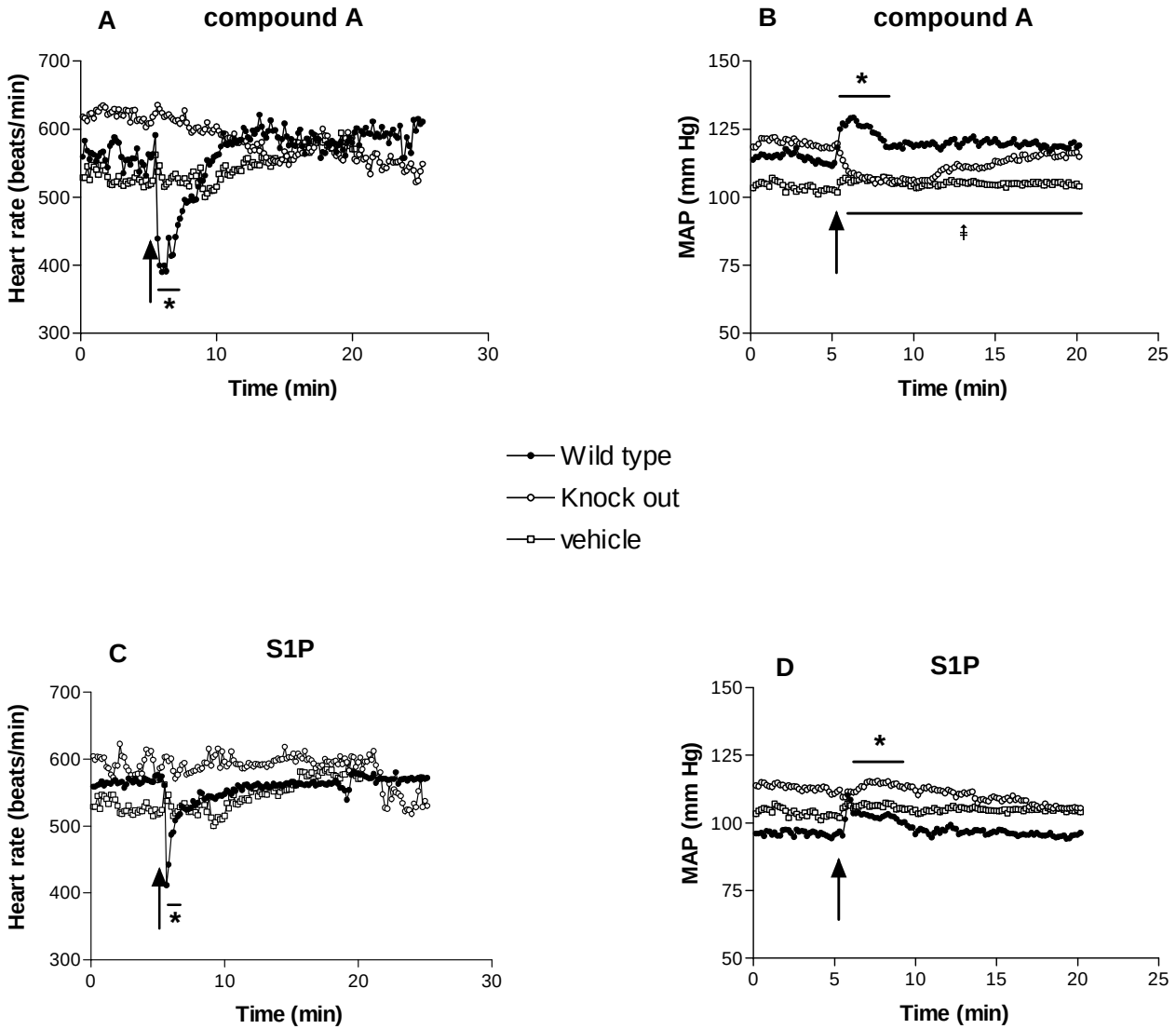

Figure 8