The Selective Sphingosine 1-Phosphate Receptor 1 Agonist Ponesimod Protects against Lymphocyte-Mediated Tissue Inflammation

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ABSTRACT
Lymphocyte exit from lymph nodes and their recirculation into blood is controlled by the sphingolipid sphingosine 1-phosphate (S1P). The cellular receptor mediating lymphocyte exit is S1P1, one of five S1P receptors. Nonselective agonists for S1P receptors lead to blood lymphocyte count reduction. The effects of selective S1P1 agonists on blood lymphocyte count and their impact in models of lymphocyte-mediated tissue inflammation have been less investigated. We describe here the general pharmacology of ponesimod, a new, potent, and orally active selective S1P1 agonist. Ponesimod activated S1P1-mediated signal transduction with high potency (EC50 of 5.7 nM) and selectivity. Oral administration of ponesimod to rats led to a dose-dependent decrease of blood lymphocyte count. After discontinuation of dosing, blood lymphocyte count returned to baseline within 48 h. Ponesimod prevented edema formation, inflammatory cell accumulation, and cytokine release in the skin of mice with delayed-type hypersensitivity. Ponesimod also prevented the increase in paw volume and joint inflammation in rats with adjuvant-induced arthritis. These data show that selective activation of S1P1 using ponesimod leads to blood lymphocyte count reduction and efficacy in models of lymphocyte-mediated tissue inflammation. Immunomodulation with a rapidly reversible S1P1-selective agonist may represent a new therapeutic approach in lymphocyte-mediated autoimmune diseases.

Introduction
The vertebrate immune system uses two major arms to control pathogens: the innate and the adaptive arms. The innate arm consists of cellular and humoral factors prepared to rapidly identify and eliminate pathogens based on the recognition of invariant pathogenic structures (Medzhitov, 2001). The adaptive arm of the immune system, most importantly T and B cells, is able to recognize any given structure, called antigen. In the case of autoimmune diseases recognized by the immune system are antigens of the body itself, referred to as self-antigens. In autoimmune diseases a vicious circle of self-antigen recognition in lymph nodes, followed by T cell expansion, lymph node exit, and migration to damaged tissue expressing the self-antigen perpetuates the lymphocyte-mediated inflammation. T and B cells strive to find their cognate antigen by constantly scrutinizing antigens within secondary lymphoid organs such as lymph nodes or the spleen. T cells that do not encounter their cognate antigen remain naive and recirculate to other lymph nodes. T cells that recognize their antigen, however, become activated, proliferate, differentiate into effector memory T cells, leave the lymph node, and home to the site of inflammation. Both naive and effector memory T cells, but also B cells, rely on a tightly regulated mechanism of exit...
from lymph nodes. A prerequisite for lymph node exit is the passage through the cortico-medullary endothelial barrier. This passage is orchestrated by the chemotactic activity of the sphingolipid sphingosine 1-phosphate (S1P) (Grigorova et al., 2009). It is synthesized by lymphatic endothelium (Pham et al., 2010), leading to a high S1P concentration in lymphatic vessels. Within the lymph node parenchyma, however, S1P concentrations are low, because it is irreversibly degraded by the enzyme S1P lyase (Schwab et al., 2005). This gradient from a low S1P concentration in lymph node parenchyma to a high concentration in lymphatic vessels is a chemotactic gradient that is sensed by the lymphocytes using S1P receptor 1 (S1P1). As effenter lymph vessels transport lymph to larger lymph ducts that drain the lymph into one of the subclavian veins, inhibition of the S1P1-mediated egress of lymphocytes into the lymph vessel leads to a reduction of blood lymphocyte count.

Nonselective S1P receptor agonists are inhibitors of lymphocyte exit from lymph nodes and thereby decrease lymphocyte count in peripheral blood (Chiba et al., 1998; Mandalà et al., 2002). One hypothesis as to why S1P agonists lead to reduced blood lymphocyte count is that the agonistic activity on S1P1 causes receptor internalization. This internalization prevents lymphocytes from sensing the S1P gradient. As a consequence, lymphocytes are blinded to lymph node exit signals, leading to their sequestration within the lymph node parenchyma (Lo et al., 2005). Important immunological functions of the lymphocytes such as antigen recognition, proliferation, formation of immunological memory, and generation of antibodies remain unaffected by exposure to S1P receptor agonists (Pinschewer et al., 2000; Xie et al., 2003). Treatment with S1P receptor agonists is therefore not an immunosuppressive but an immunomodulating approach, owing to the temporary sequestration of otherwise fully competent effector cells within secondary lymphoid organs. One such nonselective S1P receptor agonist is 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol (FTY720), which has been shown to lower blood lymphocyte count (Chiba et al., 1998; Mandalà et al., 2002) and is approved for the treatment of human multiple sclerosis (Kappos et al., 2010).

Although the chemotactic activity required for lymph node exit is exclusively mediated by S1P1 (Matloubian et al., 2004), four other S1P receptors (S1P2–5) have been identified. They mediate a wide range of responses to S1P, e.g., effects on smooth muscle cell proliferation and contraction (Kluk and Hla, 2001; Murakami et al., 2010), angiogenesis, vascular permeability, and other functions on a vast array of target cells (Brinkmann, 2007; Rosen et al., 2009). Based on the exclusive role of S1P1 in the process of lymph node exit, we developed a selective S1P1 agonist, ponseimod, as a potential treatment for autoimmune diseases. We hypothesized that this compound would be able to sequester lymphocytes and, therefore, modulate the course of autoimmune disease. In this article, we describe the pharmacological characteristics of ponseimod [l(Z,Z)-5-[3-chloro-4-((2R)-2,3-dihydroxy-propoxy)-benzylidene]-2-propylimino-3-o-tolyl-thiazolidin-4-one] (Fig. 1). We describe its in vitro effects on recombinant receptors, its in vivo effects on peripheral blood lymphocyte count in rat, and its capacity to protect against inflammation in two animal models of lymphocyte-mediated disease.

Materials and Methods

In Vitro Functional Assays

Cell Culture. Recombinant Chinese hamster ovary-K1 cells expressing human S1P1,2,3,4,5, rat S1P1,3, or mouse S1P1,3 receptors were generated, grown, and characterized as described previously (Ziltener et al., 2002).

GTPγS Binding Assay. GTPγS binding assays using membrane preparations of cells expressing recombinant S1P receptors of human, rat, or mouse origin were performed as described previously (Bollì et al., 2010). EC50 values were determined using IC50 witch (Aetelion Pharmaceuticals Ltd., Allschwil, Switzerland). Results were expressed as EC50 using the maximal response generated by S1P (percentage effect of maximal response) as an external maximum and solvent as minimum. Data are expressed as nanomoles of EC50 (geometric mean, geometric standard deviation).

Reduction of Blood Lymphocyte Count

Blood Lymphocyte Count after a Single Oral Dose. The study was performed in male Wistar rats weighing 300 to 330 g (Harlan Laboratories Inc., Füllinsdorf, Switzerland). The rats were housed in climate-controlled conditions with a 12-h light/dark cycle in accordance with the guidelines of the Basel Cantonal Veterinary Office. Rats had free access to normal pelleted chow (Proveni Kliba SA, Kaiseraugst, Switzerland) and water ad libitum. The rats were used for blood sampling after an acclimatization period of at least 7 days. All rats were housed in Makrolon cages with wire mesh tops and standardized softwood bedding and appropriate environmental enrichment. Ponesimod and its vehicle (5% DMSO and 7.5% gelatin) were supplied by Aetelion Pharmaceuticals Ltd. and stored at room temperature (maximum 25°C). Ponesimod was first dissolved in 100% DMSO and then diluted to the appropriate concentration in 7.5% gelatin; solutions were protected from light. The final concentration of DMSO in the formulation was 5%. Peripheral blood lymphocyte count was assessed by hematology using single doses of 0.3, 1, 3, 10, 30, and 100 mg/kg by the oral route (gavage) (n = 6 per dose group). Blood samples from rats were collected before and at 3, 6, 9, 12, 18, and 24 h after oral administration of ponseimod or its vehicle by sublingual puncture. Blood (0.5 ml) was drawn into tubes containing 5% EDTA. For the determination of blood lymphocyte count, undiluted blood was analyzed using a Beckman Coulter 5diffCPC hematology analyzer (Beckman Coulter, Zürich, Switzerland).

Plasma Ponesimod Analysis. After measurement of lymphocyte count, plasma was separated by centrifugation and stored at −20°C. Plasma samples were analyzed for ponseimod concentrations using liquid chromatography coupled to mass spectrometry. The performance of the bioanalytical assay was checked and documented by including quality-control samples.

Blood Lymphocyte Count after Multiple Oral Doses. The study was performed in male Wistar rats weighing 290 to 330 g, with similar housing conditions and adaptation periods as in the single-dose study. Ponesimod and its vehicle were prepared in a similar way.
as in the single-dose study. Repeated doses of 100 mg/kg by the oral route (gavage) (n = 6 per dose group) were given once daily for 7 consecutive days, and peripheral blood lymphocyte count was assessed. Blood samples from rats were collected by sublingual puncture before and at 24 h after the first oral administration and at 24, 36, and 48 h after the seventh oral administration of ponesimod or its vehicle.

Lymphocyte Subset Analysis. The study was performed in male Wistar rats weighing 250 to 300 g, with similar housing conditions and adaptation periods as in the single-dose study. Ponesimod and its vehicle were prepared in a similar way as in the single-dose study. Repeated doses of 3, 10, and 30 mg/kg by the oral route (gavage) (n = 6 per dose group) were given once daily for 7 consecutive days, and peripheral blood lymphocyte subsets and monocytes were assessed by flow cytometry. The degree of lymphocyte subset sequestration was determined relative to vehicle treatment. Blood samples from rats were collected before and at 3, 9, and 24 h after the first dose and before and at 3, 9, 27, 33, 36, and 48 h after the seventh oral administration of ponesimod or its vehicle by sublingual puncture. Whole-blood samples were stained with a total of 10 monoclonal antibodies in different combinations. Dead cells were excluded electronically from analysis by staining with 1 μg/ml propidium iodide. The marker CD45 was used as pan-leukocyte marker, and an intensity of CD45 above a predetermined threshold was used as the criterion for inclusion in the leukocyte analysis. Erythrocytes were excluded by augmenting the threshold level for cell size using the forward scatter parameter. The results obtained in the vehicle-treated group were set at 100%, and values obtained in treated rats were expressed in relation. B cells were identified using an antibody for CD45RA. Other lymphocyte subsets were determined by combining two markers: αβ TCR/CD3+ for αβ T cells, γδ TCR/CD3+ for γδ T cells, CD4+/CD62L+ for naive CD4 T cells, CD8+/CD62L+ for naive CD8 T cells, CD4+/CD8+ for CD4+ T cells, CD8+/CD4+ for CD8+ T cells, NKR-P1A+CD3+ for NK cells, NKR-P1A /CD3+ for NKT cells, and CD4/CD49d+ for monocytes. The monoclonal antibodies and streptavidin used in this study all were titrated for highest fluorescence signal-to-background ratio; anti CD49d antibody was titrated to detect strong expression of CD49d only. The individual antibodies were: anti-rat CD45R AlexaFluor647 (Serotec, Oxford, UK), anti-rat CD3(PE) AO (BD Biosciences, San Jose, CA), anti-rat αβ TCR(PerCP) BD Biosciences), anti-rat γδ TCR(BD Biosciences), anti-rat CD4(BV711) BD Biosciences, anti-rat CD8a(PerCP) BD Biosciences, anti-rat CD45RA(PerCP) BD Biosciences), anti-rat CD49d(PerCP) BD Biosciences), anti-rat NKR-P1A(PerCP) (BD Biosciences). Biotin-labeled antibodies were developed using streptavidin coupled to PE-Cy7 (BD Biosciences). Compensation values for spectral overlap of fluorescence signals were determined using CompBeads (BD Biosciences) and FACSDiva software (BD Biosciences). For each analysis, 30,000 events were registered. The difference between the analysis using conventional hemocytometers and that using specific antibodies followed by flow cytometry was the reference before and at 24 h after the first oral administration and at 24, 36, and 48 h after the seventh oral administration of ponesimod or its vehicle.

Delayed-Type Hypersensitivity Model. Female BALB/c mice weighing 15 to 25 g were used for this study (Harlan Laboratories Inc.). The mice were housed in climate-controlled conditions with a 12-h light/dark cycle in accordance with the guidelines of the Basel Cantonal Veterinary Office. Mice had free access to normal pelleted chow (Provimi Kliba SA) and water ad libitum. All animals (n = 14 per group) were housed in Makrolon cages with wire mesh tops and standardized softwood bedding and appropriate environmental enrichment. The mice were used for the induction of delayed-type hypersensitivity (DTH) after an acclimatization period of at least 7 days. Dinitrofluorobenzene (DNFB) (Sigma Chemicals, Baar, Switzerland) was dissolved shortly before use in acetone/oil (4:1, v/v). Thirty microliters of 0.5% (w/v) DNFB or solvent (control) were painted onto the shaved abdominal skin of BALB/c mice on days 0 and 1 for sensitization. On day 7 under mild isoflurane anesthesia mice were challenged by application of 20 μl of 0.2% (w/v) DNFB onto both sides of the right ear. The same volume of solvent was applied to the left ear as control. Ponesimod and its vehicle (0.25% methylcellulose, 0.05% (v/v) Tween 80) were supplied by Actelion Pharmaceuticals Ltd., stored at room temperature (maximum 25°C), and protected from light. Ponesimod was administered orally, first with a gavage (30 mg/kg) at 19 and 3 h before the first DNFB application on day 1, followed by chronic administration as food admix (175 mg/kg/day) until the day of sacrifice (day 8). Mice were sacrificed by CO2 inhalation 24 h after DNFB challenge (day 8) to assess DTH of the ear skin. A 7-mm-diameter disk was punched out of the ear and weighed. The remaining part of the ear was weighed as well and transferred with the corresponding disk to a 5-ml tube. The complete ear was homogenized for 1 min in 2 ml of Tris buffer (50 mM, pH 8) containing 0.5% hexadecyl-trimethylammonium bromide (Art Miccra D-8; Artmoderne Labortechnik, Müllheim, Germany) or 2 ml of Tris/NaCl buffer (50 mM/0.1 M, pH 8) containing 0.5% hexadecyl-trimethylammonium bromide, 0.1% Triton, and 10 μM protease inhibitor cocktail (Sigma Chemicals). The homogenized samples were centrifuged (1700g, 20 min at room temperature), and the supernatant was collected and filtered through a 0.45-μm polyvinylidene fluoride filter (Roht, Karlsruhe, Germany). Myeloperoxidase (MPO) activity of the ear homogenate was assessed by incubating a 50-μl sample of homogenate with 75 μl of phosphate/citrate buffer (0.05 M, pH 5) containing O-dianisidine (0.1 mg/ml) and hydrogen peroxide (0.0036%) for 15 min in a 96-well plate. The reaction was stopped by the addition of 50 μl of HCl (1 M), and absorbance was read at 405 nm using a microtiter plate reader (Spectramax; Molecular Devices, Sunnyvale, CA). A standard curve was obtained by serial dilutions of horseradish peroxidase (Sigma-Aldrich, St. Louis, MO). In parallel, the protein content of the ear homogenate was measured using a commercial kit (BCA protein assay kit; Thermo Fisher Scientific, Wohlen, Switzerland). For cytokine measurements, the ear tissue was homogenized in Tris/NaCl buffer (50 mM/0.1 M, pH 7.4) containing 0.1% Triton and 10 μl/ml protease inhibitor cocktail (Sigma Chemicals). Concentrations of the cytokines IL-1β, IL-4, IL-6, TNF-α, IFN-γ, MIP-1α, MCP-1, KC, RANTES, interferon γ-induced protein 10 kDa, and granulocyte colony-stimulating factor were determined using a commercial mouse cytokine Luminex kit (MCYTO-70K-PMX; Millipore Corporation, Billerica, MA). For the analysis of inguinal lymph node cells, mice were treated as described above. Inguinal lymph node cells were isolated and single cell suspensions were prepared for lymphocyte subset analysis (described above).

The DNFB solvent was slightly irritating to the skin and induced an acute inflammatory response in the absence of sensitization, i.e., independent of T cells. To subsequently correct for this acute inflammation, we included groups of mice sensitized with the DNFB solvent (T cell-independent inflammation). Each experiment consisted of four groups: group 1, sensitization with solvent, treatment with vehicle; group 2, sensitization with solvent, treatment with ponesimod; group 3, sensitization with ponesimod, treatment with vehicle; group 4, sensitization with ponesimod, treatment with ponesimod.
mod; group 3, sensitization with DNFb, treatment with vehicle; and group 4, sensitization with DNFb, treatment with ponesimod.

The mean values obtained in groups 1 and 2 were subtracted from values obtained in groups 3 and 4, respectively.

**Adjuvant-Induced Arthritis Model**

Male Lewis rats weighing 200 to 250 g (Harlan Laboratories Inc.) were used. The rats (n = 5 per treatment group) were housed in similar conditions as described for the reduction of blood lymphocyte count. Arthritis was induced on day 0 by a single intradermal injection of 0.1 ml of complete Freund’s adjuvant into the base of the tail. Complete Freund’s adjuvant was prepared by suspending 6 mg of heat-inactivated *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI) in 1 ml of liquid paraffin, also known as incomplete Freund’s adjuvant (Sigma Chemicals). Control rats received 0.1 ml of incomplete Freund’s adjuvant. Ponesimod and its vehicle [0.25% methylcellulose and 0.05% (v/v) Tween 80] were supplied by Actelion Pharmaceuticals Ltd., stored at room temperature (maximum 25°C), and protected from light. Ponesimod was administered orally, first with a gavage (30 mg/kg) at 3 h before arthritis induction and again 6 h after induction of arthritis on day 0, followed by chronic administration as food admix (100 mg/kg/day) until the day of sacrifice (day 18). Onset and progression of AIA was measured by hind paw edema, body weight, and blood inflammation markers at different time points after AIA induction. The hind paw volume was measured with an electronic water plethysmograph (Ugo Basile, Comerio, Italy) by immersing the paw vertically into a calibrated plethysmography chamber filled with water, and the displaced volume was recorded. Results were expressed as changes in hind paw volume relative to the volume on day 0. Erythrocyte sedimentation rate (ESR) (BD vacutainer kit; BD Biosciences) and plasma C-reactive protein (CRP) (rat CRP kit; Dunn Labortecnich, Asbach, Germany) were assessed in blood samples collected by sublingual puncture. Rats were sacrificed by intraperitoneal injection of 150 mg/kg pentobarbital (Vetanarcol; Veterinaria AG, Zürich, Switzerland). After dissection, the two hind paws were frozen in liquid nitrogen and pulverized in a tissue pulverizer (Thermo Fisher Scientific). Thereafter protein was extracted with a digestion buffer consisting of 50 mM Tris buffer, pH 7.4 containing 0.1 M NaCl and 0.1% Triton X-100 and filtered. The cytokines and chemokines GRO/KC, IL-1β, IL-6, IL-12(p70), IL-17, IL-18, MCP-1, MIP-1α, and RANTES were measured in the paw homogenates using a commercial rat cytokine Luminex kit (RCYTOSK-PMX; Millipore Corporation).

**Statistical Analysis**

The results of blood lymphocyte counts after oral administration (gavage) of ponesimod are presented as mean ± S.E.M. Student’s t test (unpaired, two-tailed) was used to examine the null hypothesis. In the DTH and AIA models, data are expressed as mean ± S.E.M. Student’s t test (unpaired, two-tailed) or a one-way analysis of variance followed by Dunnett’s test was used to examine the null hypothesis. A p value < 0.05 was considered significant.

**Drugs**

The chemical synthesis and initial characterization of ponesimod has been described elsewhere (Bolli et al., 2010). Ponesimod was synthesized by Actelion Pharmaceuticals Ltd. and dissolved in 100% DMSO. For oral administration (gavage) to animals ponesimod was freshly prepared every day. Two formulations were used to prepare ponesimod for oral administration: either 7.5% gelatin/5% DMSO or 0.25% methylcellulose (w/v)/0.05% Tween 80 (v/v). Vehicle-treated animals received the same volume of the excipients by oral gavage without the test compound. Compounds and vehicle were given at a volume of 5 to 10 ml/kg to rats and 5 ml/kg to mice. For chronic studies, pelleted chow containing ponesimod at 1 g/kg food as well as control chow were prepared by Provimi Kliba SA.

**Results**

**Chemical Structure of Ponesimod.** The chemical structure of ponesimod ([Z,Z]-5-[3-chloro-4-((2R,2S)-3,3-dihydroxypropoxy)-benzylidene]-2-propylaminio-3-o-tolyl-thiazolidin-4-one) is shown in Fig. 1.

**In Vitro Receptor Potency and Selectivity of Ponesimod.** The potency of sphingosine 1-phosphate and ponesimod on human S1P1, S1P2, S1P3, S1P4, and S1P5 receptors, measured as G protein activation in recombinant Chinese hamster ovary cells, is shown in Table 1. Relative to the potency of S1P, the potency of ponesimod at human recombinant receptors was 4.4-fold higher for S1P1 and 150-fold lower for human S1P3. Therefore, ponesimod was ~650-fold more selective for human S1P1 over S1P3 than the natural ligand. The rat and mouse S1P1 and S1P3 receptors were activated by ponesimod with EC50 values comparable with those measured at the human receptors (Table 1). Determination of the selectivity ratio for activation of mouse and rat S1P1 versus S1P3 was not straightforward, because ponesimod acted as a partial agonist on both rat and mouse S1P3 receptors (maximal effect). Nevertheless, ponesimod activated rat and mouse S1P1 with lower EC50 values than were measured at the respective S1P3 receptors (Table 1).

**Kinetics and Dose Dependence of Blood Lymphocyte Count Reduction after a Single Ponesimod Dose.** Ponesimod caused a dose-dependent reduction in lymphocyte count, with a plateau effect being reached at 3 mg/kg. This maximal effect represented a reduction of lymphocyte count from 6900 to 2400 lymphocytes/μl of blood (corresponding to a decrease of 64 ± 4%; p < 0.001) (Fig. 2A). The duration of lymphocyte count reduction was also dose-dependent. At 3 mg/kg ponesimod the lymphocyte count returned to predose levels within 12 h, whereas at 10 mg/kg the blood lymphocyte count returned within 18 h. At doses of 30 mg/kg and above, the peripheral lymphocyte count was reduced by more than 50% for 24 h (Fig. 2A). The plasma concentration of ponesimod increased in a dose-dependent manner and reached a maximum after 0.5 to 2 h. At the time of maximal reduction of lymphocyte count (3 h), plasma concentrations ranged from 18 ± 1 ng/ml (0.3 mg/kg) to 3966 ± 595 ng/ml (100 mg/kg) (Table 2). Maximum lymphocyte count reduction was reached at plasma concentrations of approximately 70 ng/ml (152 nM) or higher of ponesimod (Fig. 2B).

**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>EC50 (nM)</th>
<th>Maximal Effect (%)</th>
</tr>
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<tbody>
<tr>
<td>Human S1P1</td>
<td>5.7 (1.2)</td>
<td>25.3 (1.1)</td>
</tr>
<tr>
<td>Human S1P3</td>
<td>&gt; 10,000</td>
<td>43.9 (1.2)</td>
</tr>
<tr>
<td>Human S1P4</td>
<td>105 (1.3)</td>
<td>0.7 (1.3)</td>
</tr>
<tr>
<td>Human S1P5</td>
<td>1108 (1.2)</td>
<td>164 (1.2)</td>
</tr>
<tr>
<td>Human S1P5</td>
<td>59.1 (1.9)</td>
<td>121 (1.2)</td>
</tr>
<tr>
<td>Rat S1P1</td>
<td>1.9 (2.9)</td>
<td>31.2 (1.9)</td>
</tr>
<tr>
<td>Rat S1P3</td>
<td>1496.4 (1.1)</td>
<td>3.8 (1.4)</td>
</tr>
<tr>
<td>Mice S1P1</td>
<td>1.4 (1.4)</td>
<td>10.1 (1.2)</td>
</tr>
<tr>
<td>Mice S1P3</td>
<td>585.7 (1.1)</td>
<td>0.2 (2.7)</td>
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N.A., not available.
subsets, NKT cells were more susceptible than γδ T cells to ponesimod-mediated sequestration. The degree of reduction at nadir for conventional T cell subsets (αβ T cells) was 95%, whereas the maximal degree of NKT and γδ T cell reduction reached 60% (at 3 h; not shown) and 90% (at 9 h; Fig. 4).

Prevention of Delayed-Type Hypersensitivity by Ponesimod in the Mouse. All mice sensitized with DNFB showed similarly enlarged inguinal lymph nodes at sacrifice, suggesting that an immune response against DNFB occurred after the initial application of DNFB onto the abdominal skin. No enlargement of inguinal lymph nodes was seen in control-sensitized mice. Within enlarged inguinal lymph nodes the number of T cells increased significantly. The absolute number of T cells (total and activated) did not differ in vehicle- and ponesimod-treated mice after DNFB sensitization (Table 3). In control-sensitized mice, the DNFB challenge at day 7 evoked a weak inflammatory response in the ear skin. In mice sensitized and challenged with DNFB, ponesimod caused a 60 to 90% reduction of DTH parameters of the skin, such as edema, protein extravasation, and neutrophil MPO activity (Fig. 5A). In addition, ponesimod significantly reduced skin levels of proinflammatory cytokines, such as IL-1β, IL-6, IFN-γ, TNFα, and several chemokines to different degrees ranging from −27 to −100% (Fig. 5B).

Prevention of Adjuvant-Induced Arthritis by Ponesimod in the Rat. In rats injected with adjuvant, the incidence of arthritis reached 100% (5/5 rats) in the vehicle-treated group, whereas all rats treated with ponesimod remained arthritis-free for the duration of the experiment (18 days), similar to the control rats (Fig. 6A). The blood markers of inflammation CRP and ESR continuously increased from baseline (day 1) to day 18 in vehicle-treated rats. At day 7, no significant differences between vehicle- and ponesimod-treated rats were observed. At days 14 and 18, however, both CRP and ESR were significantly lower in the rats receiving ponesimod compared with rats receiving vehicle. In control rats, CRP levels did not change throughout the course of the experiment (Fig. 6B). ESR increased in both vehicle- and ponesimod-treated rats until day 7 to a similar level. At days 14 and 18 the ESR continued to increase in vehicle-treated rats, whereas the ESR returned to baseline levels in ponesimod-treated rats (Fig. 6B). The cytokines IL-1β, IL-6, IL-12, IL-17, and IL-18 and the chemokines GRO/KC, MCP-1, MIP-1α, and RANTES, measured in hind paw homogenates at sacrifice, were increased in the vehicle-treated rats compared with control rats without arthritis. Ponesimod significantly decreased the concentrations of these cytokines and chemokines to levels similar to those in the control group (Fig. 6C).

Discussion

Since the discovery of the unique role played by S1P1 in lymphocyte exit from thymus and lymph node (Matloubian et al., 2004), much research has focused on the S1P/S1P1 system as a potential therapeutic target for the treatment of lymphocyte-mediated diseases. We have developed a selective S1P1 agonist, ponesimod, and studied its pharmacological properties in vitro and in vivo. The compound has high oral availability and induces blood lymphocyte count reduction within a few hours. Ponesimod was able to prevent inflammation at doses leading to a 24-h reduction of lymphocyte count when administered to animals developing de-
the agonist, but are not readily available to the peripheral
cellular T and B cells are formed even in the continuous presence
FTY720, a nonselective S1P receptor agonist, antigen-spe-
cifically recirculate via the blood. As has been shown for
important first-line defense cells for infections and continu-
NK cells, monocytes, and polymorphonuclear leukocytes are
leaving NK cells, monocytes, and polymorphonuclear leuko-
selective modulation of the adaptive immune system while
layed-type hypersensitivity of the skin and adjuvant-induced

TABLe 2

Plasma concentrations of ponesimod after single oral dose in Wistar rats

<table>
<thead>
<tr>
<th>Dose</th>
<th>3 h</th>
<th>6 h</th>
<th>9 h</th>
<th>12 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td>0.3 mg/kg</td>
<td>17.8 ± 1.3</td>
<td>7.2 ± 0.4</td>
<td>3.1 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>58.9 ± 3.0</td>
<td>24.8 ± 2.9</td>
<td>8.0 ± 1.2</td>
<td>2.0 ± 1.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>150.5 ± 12.7</td>
<td>69.5 ± 10.9</td>
<td>31.8 ± 6.1</td>
<td>14.8 ± 2.6</td>
<td>5.7 ± 0.0</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>683.2 ± 56.2</td>
<td>322.8 ± 34.4</td>
<td>133.8 ± 27.3</td>
<td>47.0 ± 8.0</td>
<td>17.8 ± 4.0</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>2005 ± 154</td>
<td>1016 ± 110</td>
<td>482 ± 64</td>
<td>255 ± 33</td>
<td>122 ± 61</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>3966 ± 595</td>
<td>2004 ± 355</td>
<td>1092 ± 230</td>
<td>1090 ± 201</td>
<td>1077 ± 374</td>
</tr>
</tbody>
</table>

N.D., not determined.

Fig. 3. Effect of discontinuation after repeated oral administration of ponesimod (100 mg/kg/day once a day per gavage, days 1–7; gray box) on circulating lymphocyte (LY) count in Wistar rats. Lymphocyte count was measured before administration of ponesimod, 24 h after the first admin-
istration, and 24, 36, and 48 h after the last administration. Data are presented as mean ± S.E.M. ***p < 0.001 versus vehicle.

Fig. 4. Effect of multiple oral administration of ponesimod (30 mg/kg/day once a day per gavage) on circulating lymphocyte subsets and monocytes in Wistar rats. Blood lymphocyte subsets and monocytes were determined using a flow cytometer at 9 h after the last dose (day 7). T cells expressing the αβ T cell receptor (αβ T), B cells (B), T cells expressing the γδ T cell receptor (γδ T), NKT cells (NKT), NK cells (NK), and monocytes (Mono) were quantified. Results are expressed as mean ± S.E.M. percentage versus vehicle (vehicle set at 100%).

Table 2 shows plasma concentrations of ponesimod after single oral dose in Wistar rats. The table presents the concentrations at different time points after administration. The data are presented as mean ± S.E.M. for each dose level.

The mode of action of S1P receptor agonists allows for the selective modulation of the adaptive immune system while leaving NK cells, monocytes, and polymorphonuclear leukocytes unaffected. NK cells, monocytes, and polymorphonuclear leukocytes are important first-line defense cells for infections and continuously recirculate via the blood. As has been shown for FTY720, a nonselective S1P receptor agonist, antigen-specific T and B cells are formed even in the continuous presence of the agonist, but are not readily available to the peripheral pool.
tion of γδ T cells and NKT cells. Both of these cell types, γδ and NKT cells, have been reported to have both protective and detrimental properties in inflammatory diseases (Gandhi et al., 2010). Currently, it is difficult to predict the importance of sequestration of these two cell types in specific indications, but there is evidence that γδ T cells are important contributors to the pathogenesis of multiple sclerosis (Wohler et al., 2009).

Ponesimod was tested in mouse DTH of the skin to demonstrate the prevention of lymphocyte-mediated tissue inflammation in vivo. Ponesimod significantly prevents inflammation of the skin when given before sensitization and throughout the study period. In the sensitization phase, dendritic cells of the skin (Langerhans cells) capture and process the antigen and migrate to the draining lymph nodes, where they activate naive T cells to differentiate into antigen-specific effector memory T cells (Gollmann et al., 2008). These effector memory T cells later leave the lymph nodes, re-enter the blood circulation, and move into peripheral tissues, where they will be reactivated locally and cause tissue damage. To be fully efficacious, ponesimod had to be dosed before the sensitization phase. Treatment with ponesimod immediately before antigen challenge to the ear did not reduce inflammation (data not shown). These results are in agreement with a previous report showing that FTY720 was efficacious in a DTH model only when given during the sensitization but not during the challenge phase (Nakashima et al., 2008). Why ponesimod has to be present during the sensiti-

### Table 3

<table>
<thead>
<tr>
<th>T Cell Number</th>
<th>CD3⁺T</th>
<th>CD4⁺T</th>
<th>CD8⁺T</th>
<th>CD4⁺TCM</th>
<th>CD8⁺TCM</th>
<th>CD4⁺TEM</th>
<th>CD8⁺TEM</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>3.1 ± 0.9</td>
<td>1.8 ± 0.5</td>
<td>0.6 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Ponesimod</td>
<td>3.8 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>0.8 ± 0.5</td>
<td>0.5 ± 0.0</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td><strong>DNFB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>7.5 ± 1.0**</td>
<td>4.5 ± 0.6**</td>
<td>1.5 ± 0.2*</td>
<td>2.0 ± 0.6*</td>
<td>0.6 ± 0.1*</td>
<td>1.2 ± 0.1**</td>
<td>0.4 ± 0.1*</td>
</tr>
<tr>
<td>Ponesimod</td>
<td>6.5 ± 0.4**</td>
<td>3.9 ± 0.2***</td>
<td>1.4 ± 0.1**</td>
<td>0.9 ± 0.1**</td>
<td>0.5 ± 0.6**</td>
<td>1.0 ± 0.7***</td>
<td>0.3 ± 0.0**</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, ***p < 0.001, DNFB group compared with corresponding solvent group.

### Fig. 5

**Effect of ponesimod on skin inflammation. A**, effect of ponesimod (30 mg/kg by gavage 19 and 3 h before sensitization followed by 175 mg/kg/day by administration as food admix for 8 days) on change of ear punch weight increase, protein content increase, and MPO activity increase in BALB/c mice treated with vehicle or ponesimod 24 h after DNFB challenge. The values were corrected for the T cell-independent inflammatory response. **B**, effect of ponesimod on skin cytokine levels. Results are expressed as mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001 versus vehicle. G-CSF, granulocyte colony-stimulating factor. IP10, interferon-γ-induced protein 10 kDa.
zation phase in this model is not known. One possibility is that the compound inhibits Langerhans cell or cutaneous dendritic cell migration and that prevention of antigen presentation during the sensitization phase is crucial for efficacy in this model (Gollmann et al., 2008; Reines et al., 2009). Another possibility is that the compound reduced the rate of interaction of naive DNFB-specific T cells with antigen-presenting cells (Xie et al., 2003; Nakashima et al., 2008), because the antigen-specific naive T cells could be sequestered in a nondraining lymph node by treatment with S1P1 agonists. Alternatively, these findings may imply that the first wave of antigen-specific effector memory T cells are formed in sufficient amounts to effect skin inflammation independently of further proliferation and supply from skin-draining lymph nodes. We have shown that effector and memory T cells are induced within the draining lymph node within 7 days after sensitization. This increase in cell number was not different in vehicle- and ponesimod-treated groups (see Table 3). In the mouse, CD44 is used as a reliable activation marker for T cells (Budd et al., 1987). In the lymph nodes of DNFB-sensitized animals CD44 was significantly up-regulated on T cells. The total number of CD4+ T cells expressing this activation marker was not different in vehicle- and ponesimod-treated mice. These latter findings indicate that the primary response to DNFB is not significantly inhibited. Because effector memory cells have been elicited by percutaneous immu-

Fig. 6. Effect of ponesimod on joint inflammation. A, effect of ponesimod (30 mg/kg by gavage 3 h before and 6 h after adjuvant injection followed by 100 mg/kg/day by administration as food admix for 18 days) on body weight and change in hind paw volume in Lewis rats without adjuvant (Control), with adjuvant treated with vehicle (Vehicle), or with adjuvant treated with ponesimod (Ponesimod) at 3, 4, 6, 7, 10, 11, 12, 13, 14, 17, and 18 days after adjuvant injection. B, effect of ponesimod on CRP and ESR measured at 7, 14, and 18 days after adjuvant injection. C, effect of ponesimod on hind paw cytokine/chemokine levels at day of sacrifice (day 18). Results are expressed as mean ± S.E.M. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus vehicle.
nization in this model, the probability that the effector memory cells preferentially home back to the skin is increased (Butcher and Picker, 1996; Campbell and Butcher, 2002). Full efficacy in this particular model probably requires the sequestration of the very first effector memory cells that are formed during the sensitization. Further studies are required to delineate the relative contributions of the different cell types in the pathology of DTH.

In the AIA model of inflammatory joint damage in the rat, clinical and biochemical parameters were measured. Clinical improvement was observed alongside reductions in C-reactive protein and erythrocyte sedimentation rate. The results observed here are in line with and extend observations made in models of arthritis using FTY720 (Matsuura et al., 2000a,b). The improvements on blood variables, together with the prevention of cytokine expression within the lesion, indicate that ponesimod has the capacity to modulate diseases with complex expression patterns. Some of the cytokines measured in this model are not secreted by T cells but rather by cells of the myeloid lineage, for example, IL-12 by macrophages and dendritic cells (Zhang and Wang, 2008) and IL-1β by activated macrophages (Dinarello, 1991). The pool of peripheral blood lymphocytes represents 1 to 2% of the total body lymphocyte number (Morris et al., 2005). Therefore, by virtue of sequestering a small proportion of the overall circulating lymphocyte population to locally draining lymph nodes, major effects on tissue-resident cell types can be achieved; this indicates that the function of nonlymphocytes, such as macrophages and/or dendritic cells, is controlled by lymphocytes reaching the peripheral tissue via the blood.

The pharmacokinetic and therefore pharmacodynamic effects of oral administration of ponesimod are different in rat and mouse. In the mouse ponesimod is more rapidly metabolized and therefore a higher dose is required for full efficacy over 24 h on lymphocyte numbers in blood (not shown). The oral administration of ponesimod (by gavage) at 30 mg/kg twice at the beginning of each in vivo experiment was done to make sure the animals were maximally exposed to the compound from the beginning. The dosing regimen used allowed for the full coverage with ponesimod throughout the duration of the experiment starting from day 0.

Lower doses were used previously in a similar adjuvant-induced arthritis model in the rat (1 and 6 mg/kg b.i.d., oral gavage). At 6 mg/kg b.i.d. the rats showed no increase in clinical score or paw edema over the entire observation period, whereas at 1 mg/kg b.i.d. four of eight animals developed arthritis and showed significant hind paw edema (not shown). In the skin DTH experiment lower doses have not been assessed.

The lymphocyte count reduction and the efficacy of ponesimod in models of lymphocyte-mediated disease confirm that selective activity on S1P1 is sufficient for immunomodulatory activity. Other selective S1P1 receptor agonists have been described previously (Hale et al., 2004; Jo et al., 2005). Similar to ponesimod, the compound CYM-5442 [2-[4-(5-(3,4-diethoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1H-inden-1-yl amino) ethanol] induces lymphocyte count reduction at plasma concentrations in the range of 50 nM (Gonzalez-Cabrer et al., 2008). The S1P-selective compounds SEW-2871 [5-[4-phenyl-5-(trifluoromethyl)-thiophen-2-yl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole], AUY954 [3-((2-(2-(trifluoromethyl))))

[1,1’-biphenyl]-4-yl)benz[o][thiophen-5-yl](methylamino) propanoic acid], and KRP-203 [2-amino-2-[2-[4-(3-benzoxyl-phenylthio)]-2-chlorophenyl]ethyl]-1,3-propanediol hydrochloride] were characterized in animal models of kidney injury after ischemia-reperfusion (Awad et al., 2006), transplantation (Pan et al., 2006; Yan et al., 2006), and colitis (Song et al., 2008), respectively.

The ability to sequester peripheral blood lymphocytes represents a new paradigm for the treatment of lymphocyte-mediated tissue inflammation, such as in autoimmune diseases. Ponesimod is a new, potent, and selective S1P1 receptor agonist with pharmacokinetic properties allowing rapid restoration of lymphocyte count in peripheral blood upon discontinuation. It can be reasonably assumed that ponesimod has the potential to be as effective in animal models of autoimmunity and human autoimmune disease via its effect on T and B cell blood count. As a result, ponesimod may represent a new therapeutic option for the treatment of autoimmune diseases.

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References


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