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Discovery of a Potent, $S1P_3$-Sparing Benzothiazole Agonist of Sphingosine-1-Phosphate Receptor 1 ($S1P_1$)

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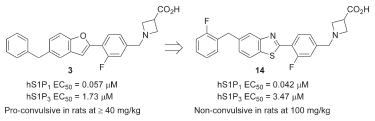
ABSTRACT Optimization of a benzofuranyl $S1P_1$ agonist lead compound (**3**) led to the discovery of 1-(3-fluoro-4-(5-(2-fluorobenzyl)benzo[d]thiazol-2-yl)benzyl)-azetidine-3-carboxylic acid (**14**), a potent $S1P_1$ agonist with minimal activity at $S1P_3$. Dosed orally at 0.3 mg/kg, **14** significantly reduced blood lymphocyte counts 24 h postdose and attenuated a delayed type hypersensitivity (DTH) response to antigen challenge.

KEYWORDS Sphingosine-1-phosphate receptor, $S1P_1$, agonist, inflammation, lymphocyte



Research efforts over the past decade have revealed the lysophospholipid sphingosine-1-phosphate (**1**, S1P; Figure 1) to be a pleiotropic modulator of diverse cellular processes including migration, adhesion, proliferation, and differentiation.[1,2] These effects are mediated, at least in part, by the interaction of S1P with a set of paralogous G protein-coupled receptors ($S1P_{1-5}$), which are widely expressed in the immune, cardiovascular, and central nervous systems.[3−5] The $S1P_1$ receptor, highly expressed in lymphocytes, has recently been the focus of extensive investigation due to its role in the regulation of lymphocyte egress from lymph nodes.[6−9]

Much of this effort has centered on the study of fingolimod (**2**, FTY-720),[10] a synthetic amino diol prodrug, which is stereoselectively phosphorylated in vivo by sphingosine kinase 2 to generate the corresponding (*S*)-phosphate (FTY-720P), which is a potent agonist of $S1P_{1,3-5}$. FTY-720P binding to lymphocyte $S1P_1$ receptors leads to receptor internalization (RI) and proteolysis.[11] Knockout studies in mice have revealed $S1P_1$ receptor expression in T[12,13] and B cells[14] to be required for these lymphocytes to exit secondary lymphoid organs and enter the circulation; FTY-720P-mediated RI thus leads to lymphocyte sequestration in secondary lymphoid organs.[15]

Although FTY-720-mediated lymphocyte sequestration does not impair antiviral and antibacterial immune responses to secondary infections in rodent models,[16,17] decreased lymphocyte recruitment to sites of inflammation has been demonstrated to moderate immune responses in rodent models of multiple sclerosis[18,19] and organ transplantation.[20] Phase III human clinical trials have recently revealed **2** to be efficacious in reducing relapse rates in patients with relapsing−remitting multiple sclerosis.[21,22]

Human clinical trials have additionally shown dosing of **2** to be associated with transient, dose-dependent decreases in mean heart rate and asymptomatic atrioventricular blockade.[23] Studies of **2** in rodents have revealed lymphocyte sequestration and heart rate reduction to be driven by distinct S1P receptor isoforms ($S1P_1$ and $S1P_3$, respectively), however.[24−27] These observations, coupled with the impressive efficacy of **2** emerging from human clinical investigations, have prompted the widespread search for $S1P_1$ agonists with minimal activity at the $S1P_3$ receptor.[28,29] In this communication, we describe the discovery of a potent $S1P_3$-sparing $S1P_1$ agonist, benzothiazole **14**, as well as its in vivo pharmacological and pharmacokinetic characteristics.

In prior work, in silico docking of a library of commercially available molecules with druglike properties into a $S1P_1$ receptor model generated using PREDICT methodology,[30] followed by modeling-driven structure−activity relationship (SAR) studies, led to the identification of a benzofuran-containing hit series, of which **3** was chosen as the lead molecule.[31] Benzofuran **3** demonstrated double-digit nanomolar activity at $hS1P_1$ in an assay measuring RI of an $hS1P_1$-GFP fusion protein in U2OS cells and limited activity at $hS1P_3$ as determined by Ca^{2+} mobilization in $hS1P_3$- and $G_{q/i5}$-transfected CHO-K1 cells (Table 1). Oral dosing (1 mg/kg) of **3** in rats resulted in a statistically significant reduction in circulating lymphocytes 24 h postdose and significantly

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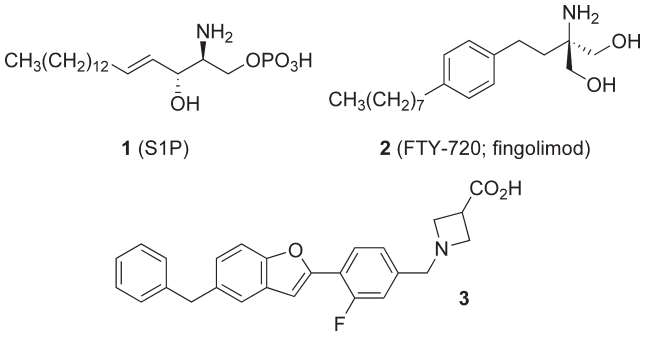
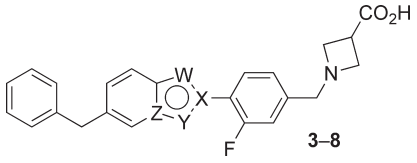


Figure 1. S1P, FTY-720, and benzofuran lead **3**.

Table 1. SAR of 5-Benzyl-Substituted Bicyclic Cores[a]



compd	W	X	Y	Z	hS1P$_1$ RI EC_{50} (μM) (% efficacy)	hS1P$_3$ Ca^{2+} EC_{50} (μM) (% efficacy)
3	O	C	CH	C	0.057 (96)	1.73 (42)
4	O	C	N	C	0.325 (105)	0.354 (77)
5	N	C	S	C	0.221 (84)	3.47 (50)
6	N	C	NH	C	> 6.25	> 25
7	N	C	CH	N	6.72 (115)	> 25
8	N	N	CH	C	2.89 (53)	1.11 (43)

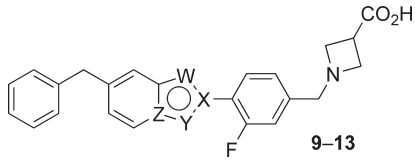
[a] Data represent an average of at least two determinations. Percent efficacy is reported relative to 1.00 μM **9** and 0.200 μM S1P for hS1P$_1$ RI and hS1P$_3$ Ca^{2+} assays, respectively. (S1P demonstrates 91% efficacy relative to 1.00 μM **9** in hS1P$_1$ RI studies.) > [Highest concentration tested] is reported for compounds that do not achieve > 10% of control activity (1.00 μM **9** or 0.200 μM S1P).

attenuated a delayed type hypersensitivity (DTH) response to antigen challenge.[32] One potential liability of **3** identified during preclinical toxicology studies was the observation of proconvulsive activity in rat at oral doses of 40 mg/kg and higher. Herein, we report on a successful effort to identify derivatives of **3** lacking this liability.

As proconvulsive activity had not been reported with prior nonisoform-selective S1P receptor agonists, such activity was tentatively assumed to be peculiar to benzofuran **3**. An effort was therefore undertaken to identify closely structurally related analogues of **3** that possessed similar or improved pharmacological profiles but that were devoid of proconvulsive activity. To this end, a series of compounds was prepared wherein the benzofuran core of **3** was replaced with a range of bioisosteric heterocycles (Tables 1 and 2). Analogues wherein the benzylic substituent of the central 5,6-ring system was located para to the more electronegative substituent ("W") of the six-membered ring were uniformly found to be less potent (4−118-fold) than **3** in the S1P$_1$ RI assay (Table 1). In contrast, several analogues wherein the benzylic substituent was located meta to the more electronegative substituent of the six-membered ring possessed comparable or superior S1P$_1$ activity to **3** (Table 2). Benzothiazole **11** was particularly noteworthy, as it possessed not only comparable in vitro activity to lead **3** but proved similarly active in vivo, bringing about statistically significant lymphocyte depletion upon oral dosing at 1.0 mg/kg.

In an effort to further understand the S1P$_1$ and S1P$_3$ SARs of benzothiazole **11**, a series of analogues was prepared wherein the terminal phenyl group of **11** was modified with a range of small lipophilic substituents (Table 3). S1P$_1$ activity was generally maintained across these analogues; however,

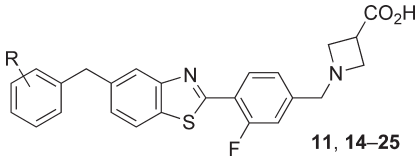
Table 2. SAR of 6-Benzyl-Substituted Bicyclic Cores[a]



compd	W	X	Y	Z	hS1P$_1$ RI EC_{50} (μM) (% efficacy)	hS1P$_3$ Ca^{2+} EC_{50} (μM) (% efficacy)
9	O	C	CH	C	0.013 (110)	0.679 (42)
10	O	C	N	C	0.094 (108)	0.542 (87)
11	N	C	S	C	0.042 (102)	1.21 (24)
12	N	C	CH	N	0.741 (110)	6.09 (50)
13	N	N	CH	C	1.81 (100)	2.71 (48)

[a] Data represent an average of at least two determinations. Percent efficacy is reported as in Table 1.

Table 3. Optimization of Benzothiazole Agonists[a]



compd	R	hS1P$_1$ RI EC_{50} (μM) (% efficacy)	hS1P$_3$ Ca^{2+} EC_{50} (μM) (% efficacy)
11	H	0.042 (102)	1.21 (24)
14	2-F	0.042 (115)	3.47 (14)
15	3-F	0.021 (138)	0.687 (22)
16	4-F	0.033 (129)	1.91 (37)
17	2,4-di-F	0.042 (186)	1.20 (24)
18	2,6-di-F	0.126 (126)	> 25
19	3,4-di-F	0.030 (151)	0.967 (19)
20	2-Me	0.026 (118)	2.44 (40)
21	3-Me	0.054 (92)	> 2.5
22	4-Me	0.077 (132)	1.80 (17)
23	2-Cl	0.042 (121)	4.88 (41)
24	3-Cl	0.049 (141)	3.88 (35)
25	4-Cl	0.159 (117)	> 25

[a] Data represent an average of at least two determinations. Percent efficacy is reported as in Table 1. > [Highest concentration tested] is reported for compounds that do not achieve > 10% of control activity (1.00 μM **9** or 0.200 μM S1P).

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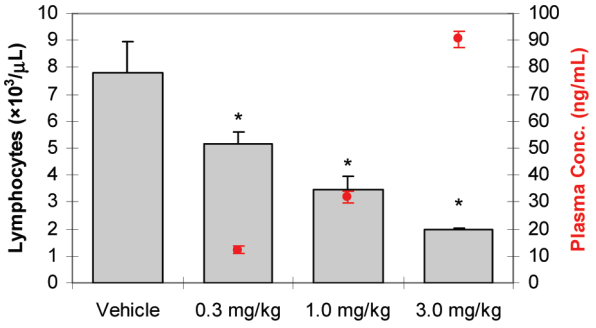

Figure 2. Compound **14** dosed orally reduces blood lymphocyte counts in female Lewis rats 24 h postdose (N = 5/group; bars represent average blood lymphocyte counts + SEs; circles represent average plasma concentration ± SEs; *P < 0.05 vs vehicle by ANOVA/Dunnett's multiple comparison test; vehicle = 20 % captisol, pH 2).

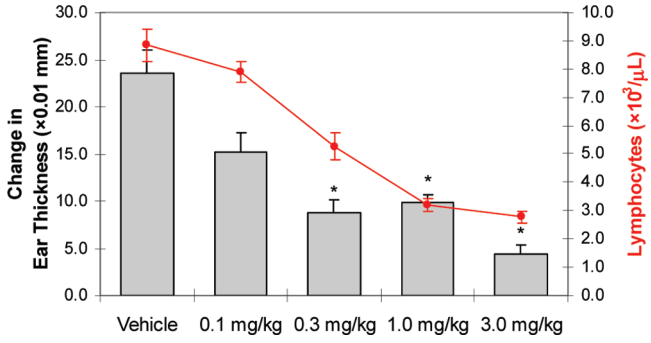

Figure 3. Compound **14** dosed orally (qd for 10 days) reduces ovalbumin (OVA)-induced ear swelling in OVA-immunized female Lewis rats 48 h post-OVA challenge [N = 8/group (vehicle, 0.1, 0.3, and 1.0 mg/kg), N = 4/group (3.0 mg/kg); bars represent average change in ear thickness + SEs; circles represent average blood lymphocyte counts ± SEs; *P < 0.05 vs vehicle by ANOVA/Dunnett's multiple comparison test; vehicle = 20% captisol, pH 2].

4-substitution of the phenyl group with larger substituents led to moderate decreases in $S1P_1$ activity (**22**, EC_{50} = 0.077 μM; **25**, EC_{50} = 0.159 μM), as did 2,6-difluorination (**18**, EC_{50} = 0.126 μM). Substitution of the terminal phenyl group demonstrated a more varied effect on $S1P_3$ activity: Whereas methylation had little impact on $S1P_3$ activity (EC_{50} = 1.80−2.44 μM), chlorination led to moderate to significant decreases in $S1P_3$ activity (EC_{50} = 3.88 to >25 μM), and fluorination resulted both in compounds with increased $S1P_3$ activity (**15**, EC_{50} = 0.687 μM) and in a compound with no detectable activity at $S1P_3$ (**18**, EC_{50} > 25 μM).

One trend to emerge from these studies was that 2-substitution of the terminal phenyl group of **11** uniformly led to moderate, but significant, reductions in $S1P_3$ activity. As a small lipophilic substituent proved nearly equally effective to a larger substituent in reducing $S1P_3$ activity (cf. **14**, CLog P = 4.1, and **23**, CLog P = 4.6),[33] we selected the least lipophilic analogue, 2-fluorobenzyl benzothiazole **14** (RI EC_{50} = 0.042 μM, Ca^{2+} EC_{50} = 3.47 μM), for further in vitro and in vivo profiling.[34]

Dosed orally at 0.3, 1.0, and 3.0 mg/kg in Lewis rats, **14** produced a dose-dependent reduction in circulating blood lymphocytes 24 h postdose, consistent with $S1P_1$ agonism (Figure 2). Statistical significance (P < 0.05) was achieved at a dose of 0.3 mg/kg (34% reduction in lymphocytes vs vehicle), and a 3.0 mg/kg dose resulted in near maximal lymphopenia (74% reduction in lymphocytes vs vehicle).[35]

Compound **14** was subsequently investigated in a DTH antigen challenge model, an in vivo model of cell-mediated immune responses. In this study, Lewis rats were orally dosed with 0.1, 0.3, 1.0, or 3.0 mg/kg **14** or vehicle once daily over the course of 10 days. One day after initiating dosing, rats were immunized with a mixture of ovalbumin and complete Freund's adjuvant. One week later, the immunized rats were rechallenged with ovalbumin by intracutaneous injection into one ear. After an additional 2 days, differential swelling of the inoculated ear (determined by change in ear thickness) and circulating blood lymphocyte counts were measured. As seen in Figure 3, a statistically significant (P < 0.05) reduction in ear swelling was observed at doses of 0.3 mg/kg and higher. Furthermore, reduced ear swelling was found to closely track circulating lymphocyte counts, consistent with the cell-mediated etiology of the DTH immune response.

Table 4. Pharmacokinetic Parameters for **14**

species	Cl (L/h/kg)	V_{ss} (L/kg)	$T_{1/2}$ (h)	MRT (h)	% F
rat[a]	0.33	3.3	7.5	10	68
NHP[b]	0.50	1.6	35.2	3.3	23

[a] Female Sprague−Dawley (iv: 2 mg/kg, DMSO, N = 3; po: 15 mg/kg, 20% captisol, pH 2, N = 3). [b] Male Cynomolgus (iv: 4 mg/kg, 20% captisol, pH 4, N = 3; po: 10 mg/kg, 20% captisol, 1% pluronic F68, 1% HPMC, pH 2, N = 3).

Pharmacokinetic profiling of **14** in rats and nonhuman primates (Table 4) revealed **14** to possess acceptable characteristics for further development: **14** demonstrated low clearance (10−19% of liver blood flow), moderate steady state volumes of distribution (V_{ss} = 1.6−3.3 L/kg), moderate-to-long mean residence times (3.3−10 h), and acceptable oral bioavailability (23−68%). In vitro studies established that **14** neither inhibited nor induced human cytochrome P450 enzymes, was nonmutagenic (Ames and micronucleus negative), and did not significantly inhibit the hERG channel in an electrophysiology assay (PatchXpress;[36] IC_{50} > 10 μM).

Cardiovascular safety studies of **14** in telemetered rats established a no-effect level for heart rate and mean arterial pressure changes of 30 mg/kg (po), indicating a wide margin for $S1P_3$-associated cardiovascular toxicity. Most significantly, **14** did not exhibit proconvulsive activity in Sprague−Dawley rats (N = 3) at a dose of 100 mg/kg when administered orally once daily for 4 days. The exposure of **14** at the end of the fourth day (C_{max} = 49 ± 10 μg/mL, AUC_{0-24} = 987 ± 185 μg·h/mL) was significantly higher (3-fold C_{max}, 40-fold AUC_{0-24}) than the exposure at which proconvulsive activity was observed with benzofuran **3**. As the plasma free fractions[37] of **14** (2.1%) and **3** (1.5%) in rat were similar, the lack of proconvulsive activity of **14** relative to **3** is currently not well understood.

The synthesis of benzothiazole **14** is described in Scheme 1. Aryl bromide **26**, prepared in one step from commercially available 3-fluoro-4-bromobenzaldehyde, was treated with

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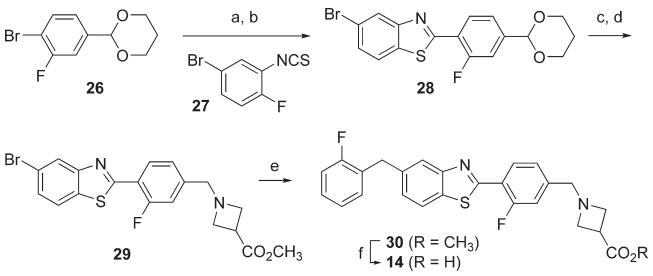
Scheme 1. Synthesis of **14**[a]



[a] Reagents and conditions: (a) *n*-BuLi, **27**, THF, −78 °C, 63%. (b) Na_2CO_3, DMF, 110 °C, 93%. (c) 5 N HCl, THF, 55 °C, 100%. (d) Methyl azetidine-3-carboxylate hydrochloride, AcOH, DIPEA, $NaBH_3CN$, MeOH/CH_2Cl_2, 71%. (e) Bis(di-*tert*-butyl(phenyl)phosphine) palladium dichloride (5 mol %), (2-fluorobenzyl)zinc chloride, THF, 85%. (f) LiOH, THF/H_2O, then HCl and pH 6 sodium phosphate buffer, 99%.

n-butyllithium at −78 °C, and 2-fluoro-5-bromophenyl isothiocyanate **27** was then added to the resulting aryl lithium species, generating the corresponding thioamide. Incubation of the thioamide with a suspension of sodium carbonate in hot DMF resulted in clean cyclization to benzothiazole **28**. Exposure of **28** to HCl in warm THF resulted in cleavage of the acetal protecting group; reductive amination of the resulting aldehyde subsequently provided methyl azetidine carboxylate **29**. Negishi coupling of **29** with (2-fluorobenzyl)zinc chloride delivered 5-benzylbenzothiazole **30**, and saponification of **30** followed by pH adjustment of the resulting reaction mixture resulted in the precipitation of benzothiazole **14** as its zwitterion.

In summary, replacement of the benzofuranyl core of a novel $S1P_1$ agonist (**3**) with a series of bioisosteric heterocycles led to the identification of benzothiazole **11**, which retained both the $S1P_1$ activity and the $S1P_3$ selectivity of **3**. Subsequent substitution of the terminal phenyl group of **11** with a range of small lipophilic substituents led to the discovery of 2-fluorobenzyl benzothiazole **14**, which was found to be a potent $S1P_1$ agonist (EC_{50} = 0.042 μM) with reduced activity at $S1P_3$ (EC_{50} = 3.47 μM). Oral dosing of **14** in rats (0.3 mg/kg) resulted in a statistically significant reduction in circulating lymphocytes 24 h postdose, as well as a statistically significant attenuation of a DTH response to antigen challenge. Furthermore, benzothiazole **14** was devoid of the proconvulsive activity of **3** at doses of up to 100 mg/kg. In vitro and in vivo pharmacokinetic studies and safety studies have established that **14** possesses acceptable properties for further development.

SUPPORTING INFORMATION AVAILABLE Statistical analysis of data presented in Tables 1−3, experimental procedures and characterization data for **3**−**25**, and details of in vitro and in vivo assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Hannun, Y. A.; Obeid, L. M. Principles of Bioactive Lipid Signaling: Lessons from Sphingolipids. *Nature Rev. Mol. Cell. Biol.* **2008,** *9*, 139–150.
(2) Kihara, A.; Mitsutake, S.; Mizutani, Y.; Igarashi, Y. Metabolism and Biological Functions of Two Phosphorylated Sphingolipids, Sphingosine-1-Phosphate and Ceramide 1-Phosphate. *Prog. Lipid Res.* **2007,** *46*, 126–144.
(3) Rosen, H.; Gonzalez-Cabrera, P. J.; Sanna, M. G.; Brown, S. Sphingosine 1-Phosphate Receptor Signaling. *Annu. Rev. Biochem.* **2009,** *78*, 743–768.
(4) Cyster, J. G. Chemokines, Spingosine-1-Phosphate, and Cell Migration in Secondary Lymphoid Organs. *Annu. Rev. Immunol.* **2005,** *23*, 127–159.
(5) Rosen, H.; Goetzl, E. J. Sphingosine 1-Phosphate and its Receptors: An Autocrine and Paracrine Network. *Nature Rev. Immunol.* **2005,** *5*, 560–570.
(6) Marsolais, D.; Rosen, H. Chemical Modulators of Sphingosine-1-Phosphate Receptors as Barrier-Oriented Therapeutic Molecules. *Nature Rev. Drug Discovery* **2009,** *8*, 297–307.
(7) Brinkmann, V. Sphingosine 1-Phosphate Receptors in Health and Disease: Mechanistic Insights from Gene Deletion Studies and Reverse Pharmacology. *Pharmacol. Ther.* **2007,** *115*, 84–105.
(8) Gardell, S. E.; Dubin, A. E.; Chun, J. Emerging Medicinal Roles for Lysophospholipid Signaling. *Trends Mol. Med.* **2006,** *12*, 65–75.
(9) Mandala, S.; Hajdu, R.; Bergstrom, J.; Quackenbush, E.; Xie, J.; Milligan, J.; Thornton, R.; Shei, G.-J.; Card, D.; Keohane, C.; Rosenbach, M.; Hale, J.; Lynch, C. L.; Rupprecht, K.; Parsons, W.; Rosen, H. Alteration of Lymphocyte Trafficking by Sphingosine-1-Phosphate Receptor Agonists. *Science* **2002,** *296*, 346–349.
(10) Adachi, K.; Chiba, K. FTY720 Story. Its Discovery and the Following Accelerated Development of Sphingosine 1-Phosphate Receptor Agonists as Immunomodulators Based on Reverse Pharmacology. *Perspect. Med. Chem.* **2007,** *1*, 11–23.
(11) Oo, M. L.; Thangada, S.; Wu, M.-T.; Liu, C. H.; Macdonald, T. L.; Lynch, K. R.; Lin, C.-Y.; Hla, T. Immunosuppressive and Anti-angiogenic Sphingosine 1-Phosphate Receptor-1 Agonists Induce Ubiquitinylation and Proteasomal Degradation of the Receptor. *J. Biol. Chem.* **2007,** *282*, 9082–9089.
(12) Matloubian, M.; Lo, C. G.; Cinamon, G.; Lesneski, M. J.; Xu, Y.; Brinkmann, V.; Allende, M. L.; Proia, R. L.; Cyster, J. G. Lymphocyte Egress from Thymus and Peripheral Lymphoid Organs is Dependent on S1P Receptor 1. *Nature* **2004,** *427*, 355–360.
(13) Allende, M. L.; Dreier, J. L.; Mandala, S.; Proia, R. L. Expression of the Sphingosine 1-Phosphate Receptor, S1P1, on T-cells Controls Thymic Emigration. *J. Biol. Chem.* **2004,** *279*, 15396–15401.
(14) Kabashima, K.; Haynes, N. M.; Xu, Y.; Nutt, S. L.; Allende, M. L.; Proia, R. L.; Cyster, J. G. Plasma Cell S1P1 Expression Determines Secondary Lymphoid Organ Retention Versus Bone Marrow Tropism. *J. Exp. Med.* **2006,** *203*, 2683–2690.
(15) Chiba, K.; Yanagawa, Y.; Masubuchi, Y.; Kataoka, H.; Kawaguchi, T.; Ohtsuki, M.; Hoshino, Y. FTY720, a Novel Immunosuppressant, Induces Sequestration of Circulating Mature Lymphocytes

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by Acceleration of Lymphocyte Homing in Rats. I. FTY720 Selectively Decreases the Number of Circulating Mature Lymphocytes by Acceleration of Lymphocyte Homing. *J. Immunol.* **1998,** *160*, 5037–5044.

(16) Pinschewer, D. D.; Ochsenbein, A. F.; Odermatt, B.; Brinkmann, V.; Hengartner, H.; Zinkernagel, R. M. FTY720 Immunosuppression Impairs Effector T Cell Peripheral Homing Without Affecting Induction, Expansion, and Memory. *J. Immunol.* **2000,** *164*, 5761–5770.

(17) Kursar, M.; Jänner, N.; Pfeffer, K.; Brinkmann, V.; Kaufmann, S. H. E.; Mittrücker, H.-W. Requirement of Secondary Lymphoid Tissues for the Induction of Primary and Secondary T cell Responses Against *Listeria monocytogenes*. *Eur. J. Immunol.* **2008,** *38*, 127–138.

(18) Kataoka, H.; Sugahara, K.; Shimano, K.; Teshima, K.; Koyama, M.; Fukunari, A.; Chiba, K. FTY720, Sphingosine 1-Phosphate Receptor Modulator, Ameliorates Experimental Autoimmune Encephalomyelitis by Inhibition of T Cell Infiltration. *Cell. Mol. Immunol.* **2005,** *2*, 439–448.

(19) Fujino, M.; Funeshima, N.; Kitazawa, Y.; Kimura, H.; Amemiya, H.; Suzuki, S.; Li, X.-K. Amelioration of Experimental Autoimmune Encephalomyelitis in Lewis Rats by FTY720 Treatment. *J. Pharmacol. Exp. Ther.* **2003,** *305*, 70–77.

(20) Chiba, K.; Hoshino, Y.; Suzuki, C.; Masubuchi, Y.; Yanagawa, Y.; Ohtsuki, M.; Sasaki, S.; Fujita, T. FTY720, A Novel Immunosuppressant Possessing Unique Mechanisms. I. Prolongation of Skin Allograft Survival and Synergistic Effect in Combination with Cyclosporine in Rats. *Transplant. Proc.* **1996,** *28*, 1056–1059.

(21) Kappos, L.; Radue, E.-W.; O'Connor, P.; Polman, C.; Hohlfeld, R.; Calabresi, P; Selmaj, K.; Agoropoulou, C.; Leyk, M.; Zhang-Auberson, L.; Burtin, P. A Placebo-Controlled Trial of Oral Fingolimod in Relapsing Multiple Sclerosis. *N. Engl. J. Med.* **2010,** *362*, 387–401.

(22) Cohen, J. A.; Barkhof, F.; Comi, G.; Hartung, H.-P.; Khatri, B. O.; Montalban, X.; Pelletier, J.; Capra, R.; Gallo, P.; Izquierdo, G.; Tiel-Wilck, K.; de Vera, A.; Jin, J.; Stites, T.; Wu, S.; Aradhye, S.; Kappos, L. Oral Fingolimod or Intramuscular Interferon for Relapsing Multiple Sclerosis. *N. Engl. J. Med.* **2010,** *362*, 402–415.

(23) See refs 21, 22, and Schmouder, R.; Serra, D.; Wang, Y.; Kovarik, J. M.; DiMarco, J.; Hunt, T. L.; Bastien, M.-C. FTY720: Placebo-Controlled Study of the Effect on Cardiac Rate and Rhythm in Healthy Subjects. *J. Clin. Pharmacol.* **2006,** *46*, 895–904.

(24) Forrest, M.; Sun, S.-Y.; Hajdu, R.; Bergstrom, J.; Card, D.; Doherty, G.; Hale, J.; Keohane, C.; Meyers, C.; Milligan, J.; Mills, S.; Nomura, N.; Rosen, H.; Rosenbach, M.; Shei, G.-J.; Singer, I. I.; Tian, M.; West, S.; White, V.; Xie, J.; Proia, R. L.; Mandala, S. Immune Cell Regulation and Cardiovascular Effects of Sphingosine-1-Phosphate Receptor Agonists in Rodents are Mediated via Distinct Receptor Subtypes. *J. Pharmacol. Exp. Ther.* **2004,** *309*, 758–768.

(25) Sanna, M. G.; Liao, J.; Jo, E.; Alfonso, C.; Ahn, M.-Y.; Peterson, M. S.; Webb, B.; Lefebvre, S.; Chun, J.; Gray, N.; Rosen, H. Sphingosine 1-Phosphate (S1P) Receptor Subtypes $S1P_1$ and $S1P_3$, Respectively, Regulate Lymphocyte Recirculation and Heart Rate. *J. Biol. Chem.* **2004,** *279*, 13839–13848.

(26) Recent research has suggested that $S1P_1$ rather than $S1P_3$ mediates heart rate reduction in humans: Gergely, P.; Wallström, E.; Nuesslein-Hildesheim, B.; Bruns, C.; Zécri, F; Cooke, N.; Traebert, M.; Tuntland, T.; Rosenberg, M.; Saltzman, M. Phase I study with the selective $S1P_1/S1P_5$ receptor modulator BAF312 indicates that $S1P_1$ rather than $S1P_3$ mediates transient heart rate reduction in humans. *Mult. Scler.* **2009,** *15*, S125–S126. See also ref 27.

(27) Brossard, P.; Hofmann, S.; Cavallaro, M.; Halabi, A.; Dingemanse, J. Entry-into-Humans Study with ACT-128800, A Selective $S1P_1$ Receptor Agonist: Tolerability, Safety, Pharmacokinetics, and Pharmacodynamics. *Clin. Pharmacol. Ther.* **2009,** *85*, S63–S64.

(28) Buzard, D. J.; Thatte, J.; Lerner, M.; Edwards, J.; Jones, R. M. Recent Progress in the Development of Selective S1P1 Receptor Agonists for the Treatment of Inflammatory and Autoimmune Disorders. *Expert Opin. Ther. Patents* **2008,** *18*, 1141–1159.

(29) Cooke, N.; Zécri, F. Sphingosine 1-Phosphate Type 1 Receptor Modulators: Recent Advances and Therapeutic Potential. *Annu. Rep. Med. Chem.* **2007,** *42*, 245–263. For reports from 2008 to the present and modeling-based approaches to rationalizing isoform selectivity, see the Supporting Information.

(30) Shacham, S.; Marantz, Y.; Bar-Haim, S.; Kalid, O.; Warshaviak, D.; Avisar, N.; Inbal, B.; Heifetz, A.; Fichman, M.; Topf, M.; Naor, Z.; Noiman, S.; Becker, O. M. PREDICT Modeling and In-Silico Screening for G-Protein Coupled Receptors. *Proteins* **2004,** *57*, 51–86.

(31) Saha, A. K.; Yu, X.; Lin, J.; Lobera, M.; Sharadendu, A.; Cheruku, S.; Schutz, N.; Segal, D.; Marantz, Y.; McCauley, D.; Buys, J.; Horner, M.; Salyers, K.; Schrag, M.; Vargas, H. M.; Xu, Y.; McElvain, M.; Xu, H. Benzofuran-based Sphingosine-1 Phosphate Receptor-1 (S1P1) Agonists: Discovery of a Potent, Selective and Orally Bioavailable Preclinical Lead Molecule. *ACS Med. Chem. Lett.*, in press.

(32) Ovalbumin/complete Freund's adjuvant. For experimental protocol, see the Supporting Information.

(33) ChemBioDraw Ultra, version 11.0, Cambridgesoft, 100 CambridgePark Drive, Cambridge, MA 02140.

(34) Dosed orally (1 mg/kg) in female Lewis rats, **14**, **20**, and **23** were similarly efficacious in reducing blood lymphocyte counts 24 h postdose (63−73% reduction).

(35) $S1P_1$ agonists have previously been reported to produce a maximal ~70% reduction in lymphocyte count: Hale, J. J.; Lynch, C. L.; Neway, W.; Mills, S. G.; Hajdu, R.; Keohane, C. A.; Rosenbach, M. J.; Milligan, J. A.; Shei, G.-J.; Parent, S. A.; Chrebet, G.; Bergstrom, J.; Card, D.; Ferrer, M.; Hodder, P.; Strulovici, B.; Rosen, H.; Mandala, S. A Rational Utilization of High-Throughput Screening Affords Selective, Orally Bioavailable 1-Benzyl-3-carboxyazetidine Sphingosine-1-phosphate-1 Receptor Agonists. *J. Med. Chem.* **2004,** *47*, 6662–6665.

(36) PatchXpress 7000A Automated Parallel Patch-Clamp System, Molecular Devices, 1311 Orleans Drive, Sunnyvale, CA 94089.

(37) Determined by ultracentrifugation at 5.0 μg/mL test compound concentration.

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