ACS Medicinal Chemistry Letters

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Benzofuran Derivatives as Potent, Orally Active $S1P_1$ Receptor Agonists: A Preclinical Lead Molecule for MS

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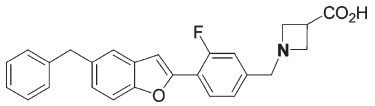
ABSTRACT We have discovered novel benzofuran-based $S1P_1$ agonists with excellent in vitro potency and selectivity. 1-((4-(5-Benzylbenzofuran-2-yl)-3-fluorophenyl)methyl) azetidine-3-carboxylic acid (**18**) is a potent $S1P_1$ agonist with >1000× selectivity over $S1P_3$. It demonstrated a good in vitro ADME profile and excellent oral bioavailability across species. Dosed orally at 0.3 mg/kg, **18** significantly reduced blood lymphocyte counts 24 h postdose and demonstrated efficacy in a mouse EAE model of relapsing MS.

KEYWORDS Sphingosine-1 phosphate receptor, S1P1, S1P3, benzofuran, relapsing MS, immunomodulators, lymphopenia



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Current therapeutic options for multiple sclerosis (MS) are all injectable, and a need for an effective oral agent exists. Myelin reactive T cells (lymphocytes) in the peripheral immune system play a key role in MS.[1−6] Regulation of sphingosine-1 phosphate receptor subtype-1 ($S1P_1$), a G-protein-coupled receptor (GPCR) expressed on T lymphocytes, could produce a new class of immunomodulators with a novel mechanism of action. Lymphocyte egress from lymphoid tissues requires the lipid mediator $S1P_1$ and is required for the emigration of lymphocytes from the thymus and the trafficking of lymphocytes in secondary lymphoid organs.[7−9]

The pro-drug FTY-720 upon phosphorylation activates the $S1P_1$ receptor.[10] It was successful in advanced clinical trials for relapsing−remitting MS and was recently recommended for approval by an FDA advisory committee for relapsing MS.[11,12] It is, however, a nonselective S1P receptor agonist with potent affinity for four of five S1P receptor subtypes. Agonism of $S1P_3$ is believed to cause chronotropic side effects.[13−16] We aimed to develop a novel, selective $S1P_1$ agonist from knowledge-based lead design and computational chemistry approaches.

Findings from Merck[17,18] revealed that unlike FTY-720-P and related compounds requiring intracellular phosphorylation, hydrophobic S1P screening hits such as oxadiazole SEW-2871 when decorated with a polar headgroup not only demonstrate strong binding to $S1P_1$ receptors but are far more selective against $S1P_3$. The structure−activity relationship (SAR) that emerged indicated that minor variation of the core scaffold could influence receptor binding potency as well as selectivity (Figure 1). For example, compounds A, B, and C differ very little (see oxadiazole ring) yet demonstrate >10-fold difference in $S1P_1$ potency between each of them.

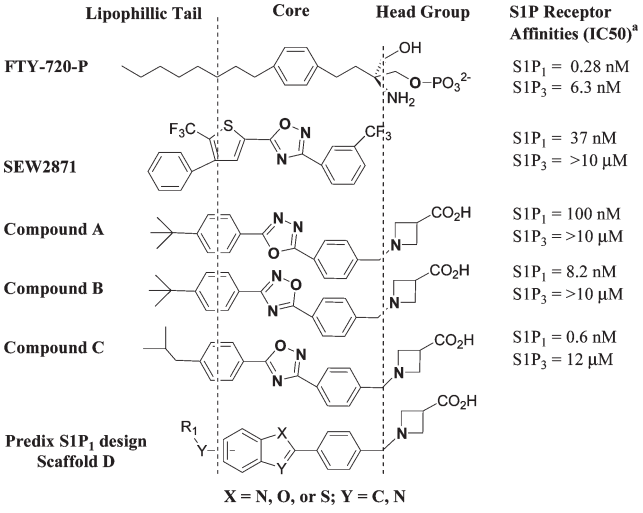

Figure 1. FTY-720-P, early $S1P_1$ agonists, and Predix scaffold. [a]Displacement of [^{33}P]-labeled sphingosine-1-phosphate (S1P) from human S1P receptors expressed on CHO cell membranes.

Received Date: September 29, 2010
Accepted Date: November 2, 2010
Published on Web Date: November 09, 2010

DOI: 10.1021/ml100227q | *ACS Med. Chem. Lett.* **2011**, *2*, 97–101

ACS Medicinal Chemistry Letters
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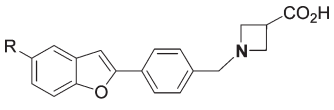
Table 1. SAR of 5-Substituted Benzofuran Core[a]



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no.	R	GTPγS[b] hS1P$_1$ EC$_{50}$ (μM)	hS1P$_3$ Ca^{2+} EC$_{50}$ (μM)	hS1P$_1$ RI EC$_{50}$ (μM)
1	*n*-Bu	0.35	> 25	2.48
2	O−*n*-Bu	0.52	> 7.2	NA
3	CH_2Ph	0.44	> 4.3	2.07
4	cHex	0.6	> 25	0.46
5	$CH_2CH(CH_3)_2$	0.38	1.9	0.15
6	CH_2CH_2Ph	0.58	> 25	0.354
7	3-Pyr	> 1	> 25	5.03
8	O−Ph	0.51	> 25	NA
9	$N(CH_2)_5$	1.5	1.44	0.62

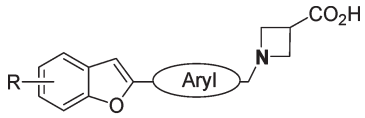
[a] Data represents an average of at least two determinations. [b] Compound induced binding of [^{35}S]-GTPγS to human S1P-1 receptors expressed on CHO cell membranes (EC$_{50}$). NA, not available.

Selectivity against S1P$_3$ receptor of course improves significantly as a consequence.

Our in silico group developed models of the S1P$_1$ receptor using PREDICT methodology.[19] Docking of the Merck inhibitors provided insight and supported the medicinal chemistry observation that small changes to the core five-member ring structures could lead to potent, selective, and novel new S1P$_1$ agonists. Utilizing this SAR insight, we designed several novel scaffolds each consisting of the azetidine carboxylate, a moderately polar core that lacks an H-bond donor and hydrophobic tail group. The scaffolds were designed with the additional consideration that the initial hit exploration library must be synthesizable using cluster synthesis approach. Mol wt, CLog *P*, and calculated ADME properties were evaluated prior to synthesis decisions. Among conceived designs, scaffold D (Figure 1), with its fused 5,6-heterocycle core with several combinations of the X and Y atoms, was favored due to overall superior match with our pharmacophore hypothesis. In this paper, we describe the successful discovery of benzofuran (X = O; Y = C)-based potent, selective S1P$_1$ agonists.

Benzofuran **1** with the tail 5-*n*-butyl group, one of the first members of initial benzofuran lead-seeking compounds, demonstrated submicromolar potency in the GTPγS assay for the S1P$_1$ receptor (Table 1). It had no activity at hS1P$_3$ as determined by Ca^{2+} mobilization in hS1P$_3$- in G$_{qi5}$- transfected CHO-K1 cells. In an assay measuring receptor internalization (RI) of an hS1P$_1$-GFP fusion protein in U2Os cells, compound **1** demonstrated micromolar potency. The RI data were deemed to be functionally more relevant and were run in parallel whenever feasible. The two assays generally correlated with each other. For SAR purposes, however, the GTPγS assay for the S1P$_1$ receptor remained a primary screen. An effort was undertaken to investigate the benzofuran motif over other exploratory scaffolds in view of its novelty, initial potency, and generally good early ADME properties. Initial changes focused on the tail region. Analogue **2** with a 5-*n*-butyloxy

Table 2. Lead Optimization of Benzofuran Core[a]



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#	R	Aryl	GTPγS[b] hS1P$_1$ EC50, μM	hS1P$_3$ Ca^{2+} EC50, μM	hS1P$_1$ RI EC50, μM (% eff.)[c]
10	6-O-nBu	Ph	2	>25	2.29 (89)
11	5-O-nBu		3.2	>25	>1
12	5-O-nBu		1.6	na	na
13	5-O-nBu		0.52	na	na
14	5-O-nBu		0.42	>25	0.653 (117)
15	5-O-nBu		na	10.3	0.158 (104)
16	5-cyHex		0.72	>25	0.424 (115)
17	5-CH2-Ph		0.62	3.43	0.253 (100)
18	5-CH2-Ph		0.16	27	0.057 (96)
19	5-cyHex		0.16	>25	0.048 (100)
20	5-O-CH2-cyPentyl		na	>25	0.454 (71)
21	5-O-CH2-cypropyl		na	4.8	0.29 (156)

[a] Data represents an average of at least two determinations. [b] Compound induced binding of [^{35}S]-GTPγS to human S1P-1 receptors expressed on CHO cell membranes. [c] Percent efficacy is reported relative to 1.0 μM 1-(4-(6-benzylbenzofuran-2-yl)-3-fluorobenzyl)azetidine-3-carboxylic acid **18** (for hS1P1 RI); NA, not available.

group retained submicromolar S1P$_1$ potency. Compounds **3** with 5-benzyl group, **4** with 5-cyclohexyl, and **5** with 5-isobutyl all demonstrated submicromolar activities in the GTPγS assay. Compounds **3** and **4** were full agonists in the RI screen (data not shown). Substitution with a 5-phenethyl group (**6**) or a phenoxy (**8**) was tolerated. Introduction of more polar groups such as a

DOI: 10.1021/ml100227q | ACS Med. Chem. Lett. 2011, 2, 97–101

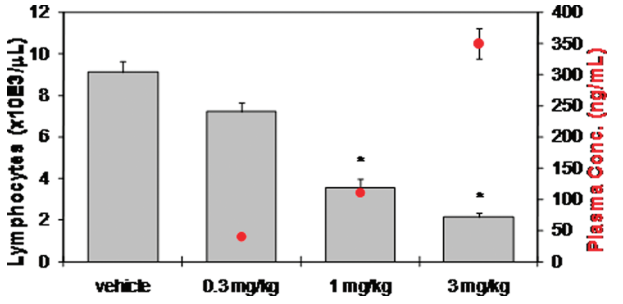


Figure 2. Compound **18** dosed orally reduces blood lymphocyte counts in female Lewis rats 24 h postdose (N = 5/group; bars represent average blood lymphocyte counts + SE; circles represent average plasma concentration ± SE; *P < 0.05 vs vehicle by ANOVA/Dunnett's multiple comparison test; vehicle = 20% captisol, pH 2).

pyridyl (**7**) led to substantial loss of $S1P_1$ potency. Interestingly, the piperidine derivative **9** retained micromolar $S1P_1$ potency; it, however, was poorly selective (~2×) over $S1P_3$ receptor. This initial data demonstrated that hydrophobic groups were preferred in the tail region of the benzofuran core.

Data from the lead optimization phase of benzofuran S1P modulators are shown in Table 2. Moving the tail substituent from the 5-position to the benzofuran-6-position led to loss in $S1P_1$ receptor potency (**10**). Replacement of the core phenyl ring with 5-atom heteroaromatic moieties such as thiazole (**11**) also led to loss of potency. The thiophene (**12**) retained potency. We next studied substituents in the core phenyl ring. Small groups such as fluoro (**13**), chloro (**15**), and methoxy (**14**) at the 3-position were tolerated. Interestingly, a 3-pyridyl replacement (**16**) of phenyl demonstrated retention of in vitro potency and selectivity. Combining the 5-benzyl tail substituent with the core 3-fluoro-phenyl ring produced compound **18** with less than 200 nM potency in the GTPγS screen. Furthermore, it demonstrated less than 100 nM potency in the hS1P1 RI assay. The 2-fluorophenyl analogue **17** showed a 4−5-fold reduced $hS1P_1$ potency relative to 3-fluorophenyl analogue **18**. The 3-fluorophenyl group when combined with 5-cyclo-hexyl tail moiety demonstrated potency similar to analogue **18**. Retaining the optimal 3-fluorophenyl ring but combining with somewhat more polar tail groups (**20** and **21**) led to loss in potency.

One of our key preclinical lead criteria was >1000-fold $S1P_1$ selectivity over $S1P_3$. Benzofuran analogue **18** achieved highest selectivity with demonstrated EC_{50} values of 16 nM for $hS1P_1$ in the GTPγS binding assay and 27 μM for $hS1P_3$ in the Ca^{2+}-flux assay, respectively. The ADME profile was favorable with a half-life in human liver microsomes of over 90 min. Clearance in the rat appeared to be primarily metabolic. Permeability across Caco-2 monolayers was moderate to good with low efflux characteristics.

Compound **18** produced comparable lymphopenia activity to FTY720 in Lewis rats with dose-dependent reduction in circulating blood lymphocytes 24 h postdose. Near maximal lymphopenia (75% reduction in lymphocytes vs vehicle) was achieved at doses of 1 and 3 mg/kg (Figure 2). Onset of lymphopenia was rapid across species in mice, rat, and nonhuman primates, with short recovery times in lymphocytes counts (less than 48 h).

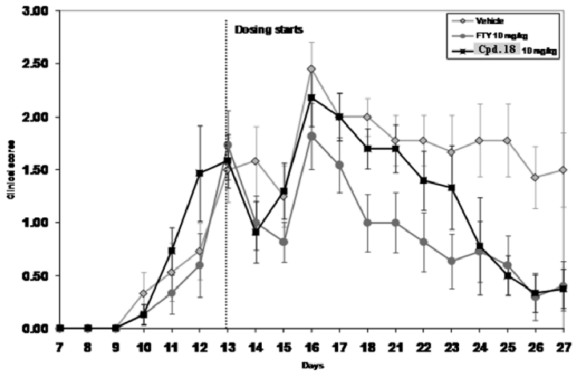


Figure 3. Compound **18** data in experimental autoimmune encephalitis (EAE) SJL mice model of MS.

Table 3. Mean Compound **18** PK Parameters in Pie Clinical Species

species	CL (L/h/kg)	Vss (L/kg)	$T_{1/2,Z}$ (h)	MRT (h)	F %
rat[a]	0.138	1.7	12.1	12.7	80
NHP[b]	0.309	1	4.7	3.4	88
canine[c]	0.266	2.2	21.1	9	51

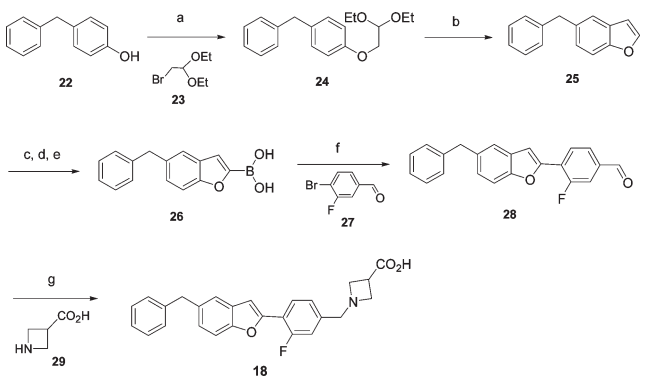
[a] Male Sprague−Dawley (iv: 1 mg/kg, DMSO, N = 3; po: 3 mg/kg, 20% captisol, pH 4.0, N = 3). [b] NHP = nonhuman primate, male cynomolgus (iv: 1 mg/kg, 5% hydroxypropyl-β-cyclodextrin, N = 3; po: 5 mg/kg, 5% hydroxypropyl-β-cyclodextrin, N = 3.). [c] Male beagle (iv: 2 mg/kg, 20% captisol, pH 4.0, N = 3; po: 3 mg/kg, 20% captisol, pH 4.0, N = 3).

Compound **18** demonstrated efficacy comparable to FTY-720 at (10 mg/kg po) nearly completely reversing the course of disease in Experimental Autoimmune Encephalitis (EAE) SJL mice model of multiple sclerosis (Figure 3). Preclinical pharmacokinetic (PK) in rat, nonhuman primate, and canine indicated that compound **18** had low clearance (corresponding to 4−15% of liver blood flow), a moderate steady state volume of distribution (1.0−2.2 L/kg), a moderate to long half-life (5−21 h), and good oral bioavailability (51−88%; Table 3). In vitro studies established that compound **18** neither inhibited nor induced human cytochrome P450 enzymes. Compound **18** was nonmutagenic (Ames and micronucleus negative) and did not significantly inhibit the hERG channel (patch clamp: IC_{50}, 17 μM). In humans, it was predicted to have low clearance and a 5−10 h half-life, which would make it suitable for once a day dosing.

In a 4 day repeated dose toxicity study in rats, exposure was dose proportional up to 60 mg/kg. The brain/serum ratio was consistent across doses (10−16-fold), while the volume of distribution was 10-fold lower than FTY720. The NOAEL was 20 mg/kg. To examine if lack of $S1P_3$ potency corresponded to an absence of chronotropic effect, compound **18** was dosed in telemetered female SD rats as an oral bolus in a volume of 5 mg/mL in 20% captisol. The mean arterial pressure (MAP) was moderately elevated at 20 and 40 mg/kg from 4 to 6 h after dosing, with peak effects corresponding to C_{max}. There was no effect on heart rate at all doses, confirming a no-effect level for HR changes at 40 mg/kg po, indicating a wide margin for $S1P_3$-mediated cardiovascular toxicity. The mechanism underlying the elevated MAP is uncertain.

DOI: 10.1021/ml100227q | ACS Med. Chem. Lett. 2011, 2, 97–101

Scheme 1. Synthesis of **18**[a]



[a] (a) KOH, DMSO, 20 h, 84%. (b) PPA, benzene, 89%. (c) *n*-BuLi, THF, −78 °C. (d) $B(iPrO)_3$. (e) 2 N HCl or NH_4Cl (66% yield after steps c−e). (f) $PdCl_2(Ph_3P)_2$ (5 mol %) Et_3N, EtOH, 100 °C, microwave, 65%. (g) AcOH, $NaCNBH_3$, DCM/MeOH, 56%.

The synthesis of compound **18** is described in Scheme 1. The 5-benzyl benzofuran **25** was prepared via a two-step reaction, starting from the alkylation of phenol **22** with 2-bromo-1,1- diethoxyethane **23**, followed by cyclization with polyphosphoric acid (PPA) in benzene at reflux temperature. The boronic acid functionality was selectively incorporated at the 2-position of the benzofuran ring of **26** under our modified conditions using *n*-BuLi and $B(^iPrO)_3$ at low temperature. Suzuki C−C coupling of boronic acid **26** and aryl halide **27** was carried out in a microwave apparatus using 5 mol % palladium(II) catalyst, triethyl amine, and ethanol as the solvent at 100 °C, cleanly yielding 4-(5-benzylbenzofuran-2-yl)-3-fluorobenzaldehyde **28**. Reductive amination of the aldehyde with azetidine-3-carboxylic acid **29** provided the desired compound **18** as its zwitterion.

In summary, knowledge-based scaffold hopping combined with computation chemistry tools led to identification of the benzofuranyl core as a novel new $S1P_1$ lead series. Rapid optimization efforts designed to enhance potency, while balancing lipophilicity led to 3-fluorophenyl benzofuran **18**, which was found to have potent $S1P_1$ activity and excellent $S1P_3$ selectivity. Oral dosing of **18** in rats (1 mg/kg) resulted in a statistically significant reduction in circulating lymphocytes 24 h postdose, as well as efficacy in the mice EAE model of MS. The overall profile of compound **18** led to nomination for preclinical development including follow-on GLP toxicology studies. Such studies revealed pro-convulsive activity at doses of 40 mg/kg and higher. Optimization efforts toward scaffolds that avoid such toxic properties will be the subject of upcoming manuscripts.

SUPPORTING INFORMATION AVAILABLE

Experimental procedures and characterization data for **1**−**21** 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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ACKNOWLEDGMENT

We thank Sheila Dewitt, Epix, and Sharon Pick and Victor Cee, Amgen, for assistance in coordinating completion of this manuscript. We also thank Pini Orbach, Sharon Shacham, and Oren Becker for their contributions to the S1P program at Epix.

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DOI: 10.1021/ml100227q | *ACS Med. Chem. Lett.* **2011**, *2*, 97–101