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Discovery and characterization of potent and selective 4-oxo-4-(5-(5-phenyl-1,2,4-oxadiazol-3-yl)indolin-1-yl)butanoic acids as S1P1 agonists

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ABSTRACT

$S1P_1$ receptor driven lymphopenia has proven utility in the treatment of an array of autoimmune disease states. As a part of our efforts to develop potent and selective $S1P_1$ receptor agonists, we have identified a novel chemical series of 4-oxo-4-(5-(5-phenyl-1,2,4-oxadiazol-3-yl)indolin-1-yl)butanoic acid $S1P_1$ receptor agonists.

Introduction

$S1P_1$ receptor mediated immunomodulation has emerged as an intriguing mechanism for the treatment of autoimmune disease.[1] FTY720 (Gilenya®, Fig. 1) recently became the first $S1P_1$ receptor agonist to gain approval in the US for relapsing forms of multiple sclerosis (MS). In phase III clinical trials FTY720 demonstrated efficacy relative to both placebo and Interferon-γ in reducing disease relapse and delaying the progression of disability.[2] This efficacy results from $S1P_1$ dependent sequestration of lymphocytes in the lymphoid tissues following phosphorylation of FTY720 to its active metabolite (FTY720P). Animal studies suggest this mechanism of action may also be useful for the treatment of other autoimmune diseases.[3] The success of FTY720 in MS trials, and the potential for use in other disease areas, has encouraged the development of second generation $S1P_1$ agonists.[4] Achieving increased selectivity against the cardiac expressed $S1P_3$ subtype has been a consistent theme of this effort, as agonism of $S1P_3$ causes bradycardia in rodents. Transient bradycardia has been observed in humans treated with FTY720. Efforts have also been directed at identifying small molecules with improved pharmacokinetic properties. FTY720 has a relatively long half-life in humans (6–9 days), and normalization of peripheral lymphocyte levels upon cessation of drug treatment requires 1–2 months.[2] Other adverse reactions noted in human trials of FTY720 include elevated liver enzymes, macular edema, and respiratory effects. As a part of our efforts to develop potent and selective $S1P_1$ receptor agonists with desirable pharmacokinetic and safety profiles, we have identified a novel chemical series of 4-oxo-4-(5-(5-phenyl-1,2,4-oxadiazol-3-yl)indolin-1-yl)butanoic acid $S1P_1$ receptor agonists (e.g. **1**). The discovery and optimization studies pertaining to this new chemical series are described herein.

An internal HTS campaign identified sulfonamide **2** (Fig. 2) as a potent agonist of the human $S1P_1$ receptor (EC_{50}, Melanophore[5] = 6.7 nM). SAR studies were initiated on the three regions

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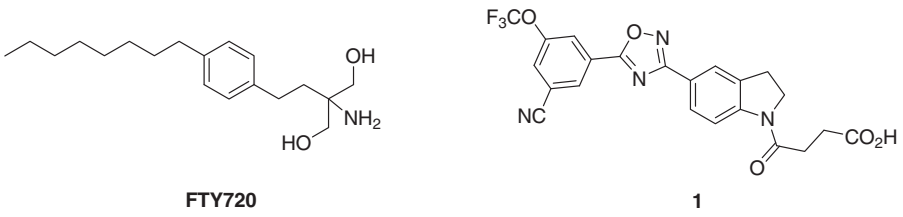


FTY720 1

Figure 1. Structures of FTY720 and Compound **1**.

of the molecule indicated in Figure 2. The sulfonamide linkage allowed for facile variation in each of these areas via alkylation or sulfonylation of the anilinic nitrogen. Replacement of the allyl group with ethyl or n-propyl was tolerated, and small lipophilic phenyl substitution was preferred (Me, Cl, CF_3, etc.). In particular, 3,5-disubstitution on lower phenyl maintained the highest degree of potency. Unfortunately, these early analogs were not effective at lowering lymphocyte count in mice after oral delivery. Only when the SAR diverged from the arylsulfonamide motif did we identify a chemical series that ultimately led to success *in vivo*. Indazole **3** represents a transition point in the SAR. This compound was prepared as a part of a small set of 5,6-fused bicyclic analogs and retained moderate activity for the $S1P_1$ receptor. Subsequent replacement of the sulfonamide group with the 1,2,4-oxadiazole motif, led to a compound of improved *in vitro* potency (Fig. 2, Compound **4**).

Introduction of an acid side chain onto the indazole ring was examined (**5–7**, Fig. 3). It was anticipated that the carboxylic acid group would mimic the phosphate residue found within sphingosine-1-phosphate. Successful phosphate replacement has been demonstrated in several other $S1P_1$ agonists.[4] Introduction of the carboxylic acid was tolerated, producing analogs of comparable potency to Compound **4**. Notably, masking of the carboxylic acid as an ester (**8**, **9**) led to a decrease in *in vitro* potency, supporting the notion of a specific interaction between the carboxylic acid residue and the $S1P_1$ receptor pocket.[6] A small number of alternative ring systems were examined, both with and without an acid side chain (e.g. **10**–**20**). Benzimidazole and indoline were effective replacements for the indazole group (e.g. **14**, **19**, **20**).

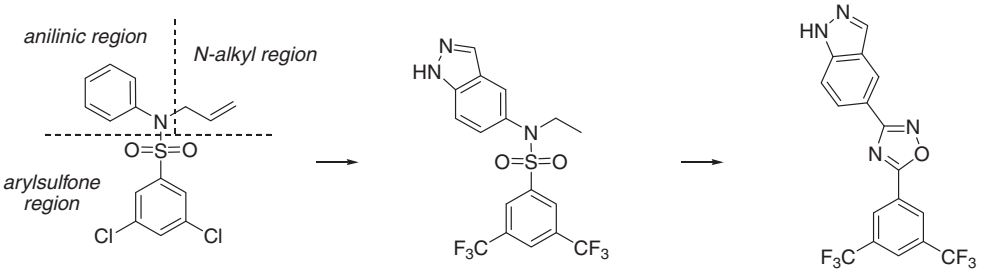
Selected compounds taken from Figure 3 were examined in an abbreviated lymphocyte lowering experiment in BALB/C mice. Lymphocyte levels were measured at a single time point (5 h), following a 1.0 mg/kg oral delivery of test compound. Compounds that failed to cause lymphocyte lowering relative to vehicle at this dose were excluded from further consideration. Often, failure to induce lymphopenia was attributable to low oral bioavailability, as was the case for benzimidazole **19**. Indoline **20** was found to produce a maximal response in the lymphocyte lowering experiment (Fig. 4). Given the robust efficacy of indoline **20** in the acute mPLL experiment, a lead optimization study focused on the 4-oxo-4-(5-(5-phenyl-1,2,4-oxadiazol-3-yl)indolin-1-yl) motif was initiated.

Preparation of Compound **20** and related analogs was performed utilizing commercially available indoline-5-carbonitrile **21** as starting material (Fig. 5). Two synthetic routes were utilized, allowing for late stage variability of either the 5-phenyl-1,2,4-oxadiazole or butyric acid group. Additionally, we found it beneficial to have several methods for unmasking the carboxylic acid, as some functional groups were incompatible under basic saponification conditions. Attempts to prepare Compound **1** via hydrolysis of a methyl ester with KOH were not productive due to competitive reaction at the nitrile. It was possible to introduce the butyric acid group directly upon treatment with succinic anhydride, or in two step sequence utilizing 4-*tert*-butoxy-4-oxobutanoic acid as the coupling reagent.

The 3-(1,2,4-oxadiazol-5-yl)-5-(trifluoromethoxy)benzonitrile group was identified as a suitable replacement for the 5-(3,5-bis(trifluoromethyl)phenyl) group (Fig. 5, Compound **1**). This array conferred a modest improvement in potency ($hS1P_1$ EC_{50} = 1.2 nM) and was thought to offer an advantage in physicochemical properties (Compound **1**, clogP = 3.74; Compound **20**, clogP = 4.68). Notably, the 3-methoxy-5-(1,2,4-oxadiazol-5-yl)benzonitrile variant (Compound **24**, Fig. 5), on the other hand, was less potent ($hS1P_1$ EC_{50} [cAMP] = 10.3 nM).

A brief SAR study focused on the butyric acid group was performed (Table 1). An amino acid motif was found to be a potency driver (Compound **28**); however, this compound did not produce a substantial reduction in circulating lymphocytes in the mPLL assay. We attribute the lack of *in vivo* efficacy to low oral bioavailability, as this compound is zwitterionic and poorly soluble in water (<0.01 mg/mL). Other analogs prepared in this exercise (**25**–**27**) exhibited equivalent or reduced potency compared to Compound **20**, thus we retained the butyric acid group as our preferred acid fragment.

An evaluation of compounds **20** and **1** across species and in a human S1P subtype selectivity *in vitro* panel was undertaken. $S1P_1$ functional data for mouse, rat, dog, and monkey was



2 hS1P1 EC_{50} = 6.7 nM

3 hS1P1 EC_{50} = 372 nM

4 hS1P1 EC_{50} = 9.4 nM

Figure 2. Identification the 5-(phenyl)-3-(1H-indazol-5-yl)-1,2,4-oxadiazole motif (**4**). Human $S1P_1$ EC_{50} values were determined in Melanophore[5] and are the mean of three or more replicates.

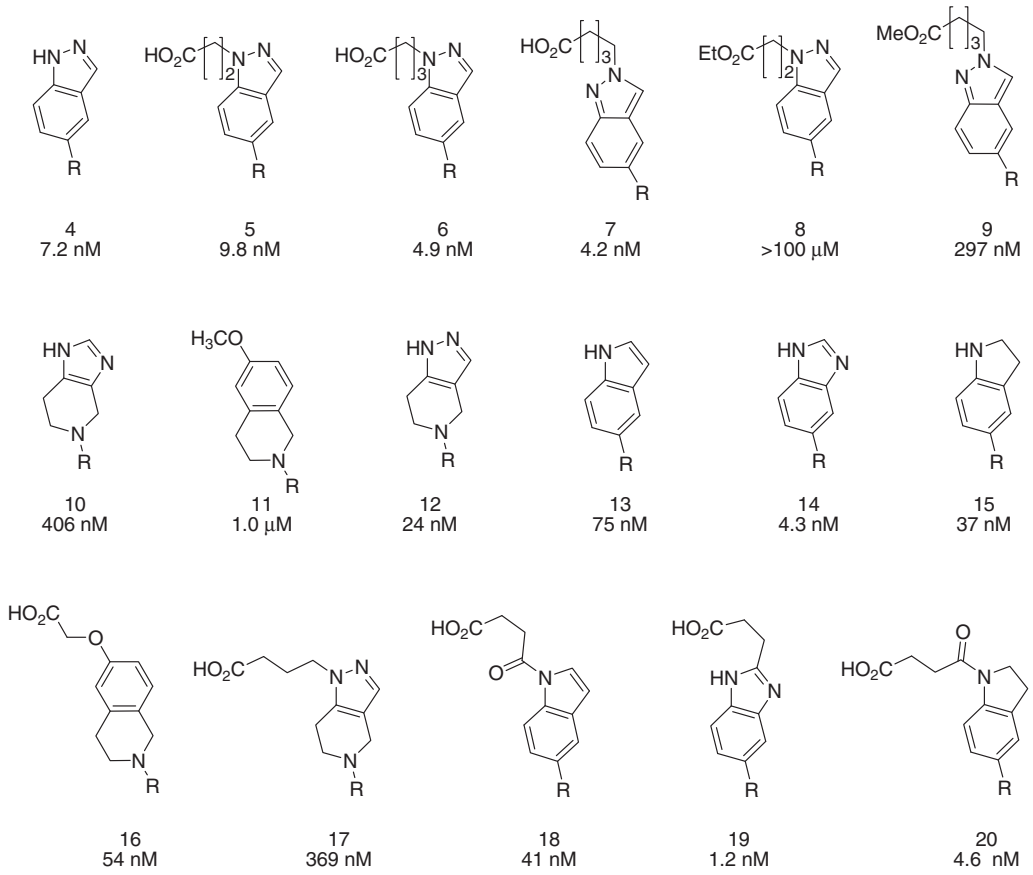


Figure 3. Compound 4 analogs, R = 3-(3,5-bis(trifluoromethyl)phenyl)-1,2,4-oxadiazole. Human $S1P_1$ HTRF cAMP EC_{50} values are reported as the mean of three or more replicates.

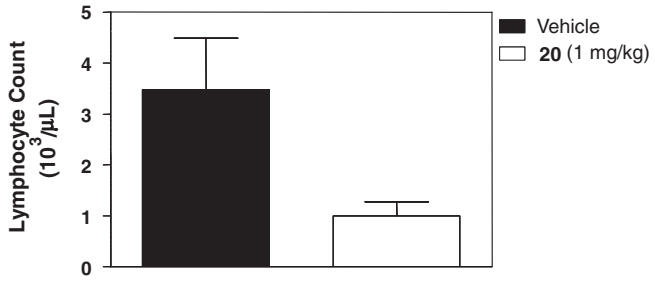


Figure 4. Blood lymphocyte count at 5 h after a 0.00 (vehicle, 0.5% methylcellulose) or 1.00 mg/kg oral dose of Compound **20** in male BALB/c mice (mean ± SD; $n = 6$ per dose group).

determined in the HTRF cAMP assay, and both compounds maintained similar cross species activity (Table 2). Selectivity for the human $S1P_1$ receptor versus the other high affinity subtypes ($S1P_{2-5}$) was performed in the β-arrestin platform. Both compounds appeared to be less potent $S1P_1$ agonists in β-arrestin versus cAMP. Compounds **20** and **1** were selective against human $S1P_2$ and $S1P_3$,[7] while each maintained some activity at the human $S1P_4$ and $S1P_5$ receptors. Avoiding $S1P_3$ agonist activity had been a criteria of ours given the associated bradycardia observed upon activation of this subtype.[8] Compounds **20** and **1** were also examined in a Cerep diversity panel comprised of ninety-seven binding assays and thirteen enzymatic assays. Both compounds showed no effect, or weak effects (0–50% inhibition or stimulation) at a 10 μM test concentration against this panel of receptors.

Further characterization of Compounds **20** and **1** revealed a favorable ADME profile (Table 3). Both compounds were stable in both human and rat liver microsomes ($t_{1/2} > 60$ min.). Not unexpectedly, **20** and **1** were found to be highly protein bound in both human and rat. Pharmacokinetic analysis performed in male Sprague Dawley rats revealed a low systemic clearance, low volume of distribution, and high C_{max}. Consistent with the low volume of distribution and systemic clearance, these compounds had good oral bioavailability (30–50%) and a moderate terminal half-life (3–8 h). As mouse was our pharmacologically relevant species, mouse PK was obtained for Compound **1** and was found to be similar to rat, with Compound **1** having a terminal half-life of approximately 3 h.

Subsequent *in vitro* toxicity profiling (Table 4) demonstrated that neither compound were inhibitors of the major CYP P450 isoforms, and no hERG interaction was observed in ^{3}H-astemizole binding ($IC_{50} > 100$ μM) or patch clamp (<10% at 3.0 μM). We observed no liabilities in our Essential Cell Function (ECF) panel which measures several markers of cellular toxicity such as changes in nuclear size (NI), membrane integrity (MI), intracellular calcium release (IC), mitochondrial membrane potential (MMP). Ames testing showed no frame-shift or base-pair mutations relative to vehicle in the five strains examined in the presence and absence of S9 fraction (TA97a, TA98, TA100, TA1535, and WP2*uvr*ApKM101).

As Compound **1** satisfied our selectivity and safety requirements, this compound was advanced into a mouse lymphocyte lowering dose response experiment. Mice received an oral dose of Compound **1** at 0 (vehicle), 1, 3, or 10 mg/kg, after which blood samples were taken up to 24 h post-dose for lymphocyte and drug

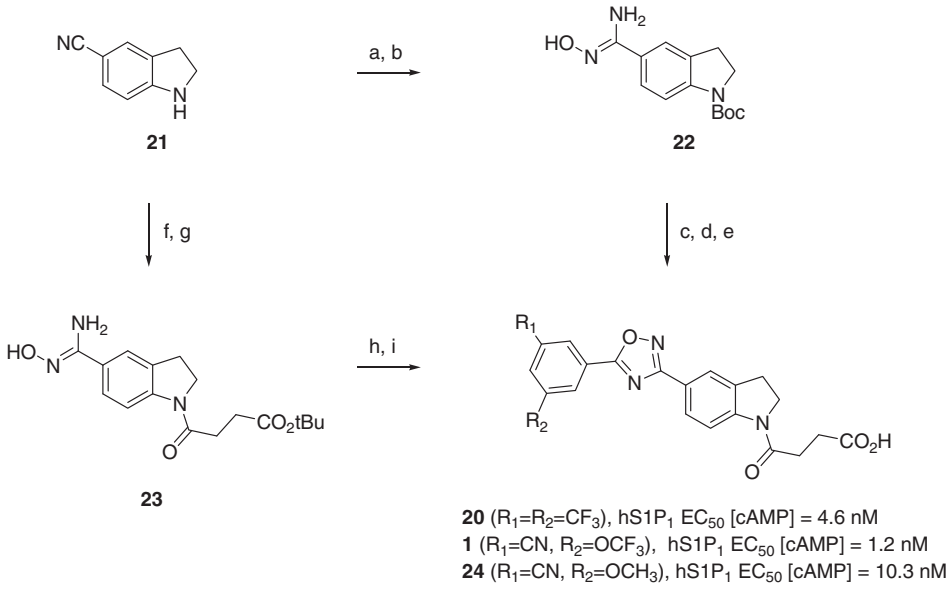
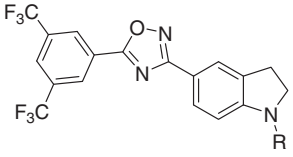


Figure 5. Synthetic methods. (a) Boc_2O, THF (b) 50% aq. hydroxylamine, EtOH (c) ArCOCl, Et_3N or $ArCO_2H$, CDI (d) TFA (e) methyl 4-chloro-4-oxobutanoate then KOH, or succinic anhydride (f) 4-*tert*-butoxy-4-oxobutanoic acid, EDC (g) 50% aq. hydroxylamine, EtOH (h) ArCOCl, Et_3N or $ArCO_2H$, CDI (i) TFA.

Table 1
Acid side chain SAR. Human $S1P_1$ HTRF cAMP EC_{50} values are reported as the mean of three or more replicates



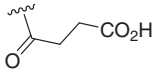
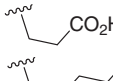
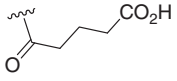
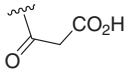
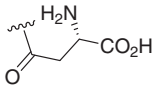
Compound	R	hS1P$_1$ EC$_{50}$ [nM]
20		4.6
25		6.1
26		6.6
27		102
28		1.63

Table 2
In vitro S1P receptor functional data. EC_{50} values shown in this table are the mean of three or more replicates

Assay/parameter	**20** (nM)	**1** (nM)
S1P$_1$ cross species [cAMP]		
Human	4.6	1.2
Mouse	1.4	1.1
Rat	1.3	1.0
Dog	1.3	0.70
Monkey	1.1	0.80
Human S1P subtypes [β-arrestin]		
S1P$_1$	39	31
S1P$_2$	>100,000	>100,000
S1P$_3$	3,100	>100,000
S1P$_4$	160	1,330
S1P$_5$	88	109

Table 3
Compounds **20** and **1** ADME profile

Assay/parameter	**20**	**1**
Microsomal stability $T_{1/2}$[a]	>60 min (HLM, RLM)	>60 min (HLM, RLM)
% Protein binding (species)[b]	99.4% (h), 100% (r)	99.7% (h), 99.3% (r)
SD Rat PK[c]		
Cl (L/h/kg)	0.038	0.045
V_{ss} (L/kg)	0.564	0.275
C_{max} (μg/mL)	1.74	3.82
T_{max} (h)	3.0	2.0
AUC_{last} (h ∗ μg/mL)	16.02	20.2
$T_{1/2}$ (h)	8.5	2.9
%*F*	50.2	32.8

[a] Human and rat liver microsomes.
[b] Human and rat.
[c] Male Sprague Dawley rat, vehicle = PEG400, Compound **20** 1/1.5 mpk (IV/PO), Compound **1** 2/3 mpk (IV/PO).

plasma concentration measurements (Fig. 6). Compound **1** produced a dose dependent reduction in circulating lymphocytes up to 16 h post-dose with lymphocyte levels returning to vehicle levels by 24 h post-dose. An indirect pharmacokinetic/pharmacodynamic (PK/PD) model was created to estimate the blood concentration of the drug needed to suppress lymphocyte levels by 50% (IC_{50}). Compound **1** was determined to have an *in vivo* IC_{50} value of 431 ng/mL, which is in good agreement with the β-arrestin data after correcting for plasma protein binding.

The dose response experiment provided us with benchmark for further optimization of this series. We sought to identify a molecule with improved potency in the lymphocyte lowering experiment. Toward this end, we initiated an SAR campaign focused on 3,4-disubstituted phenyl analogs (Table 5). Groups in the 4-position consisted of alkyl, alkoxy, aryl, and cycloalkyl, and were paired with small lipophilic groups occupying the adjacent position. Generally, small lipophilic groups in the 4-position compared favorably with the 5-position although only one direct comparison was possible from this subset of compounds (**39** and **24**). Larger groups in the 4-position were tolerated, and afforded several analogs of

Table 4
In vitro cytotoxicity profiling

Assay/parameter	**20**	**1**
CYP P450 inhibition (IC_{50}, μM)	1A2 > 50, 2D6 > 50 2C9 = 34.8, 2C19 > 50 3A4 > 50	1A2 > 50, 2D6 > 50 2C9 > 50, 2C19 > 50 3A4 > 50
^{3}H-Astemizole binding (IC_{50}, μM)	>100	>100
Patch clamp (% inhib. at 3.0 μM)	<10	13
ECF panel (IC_{50}, nM)[a]	MI, NS, MMP, IC > 100	MI, NS, MMP, IC > 100
Micro Ames (±S9 fraction)	Negative: TA97a, TA98, TA100 TA1535, WP2uvrApKM101	Negative: TA97a, TA98, TA100 TA1535, WP2uvrApKM101

[a] Essential Cell Function (ECF): membrane integrity (MI), nuclear size (NS), mitochondrial membrane potential (MMP), intracellular calcium (IC).

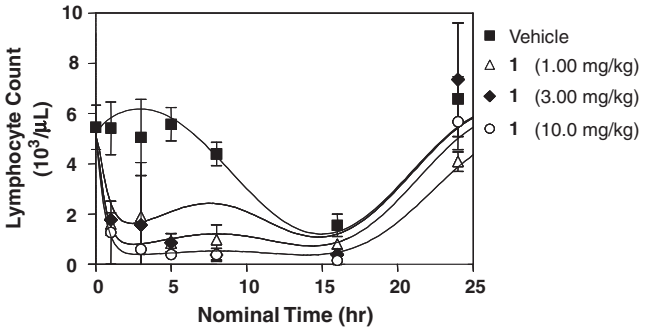


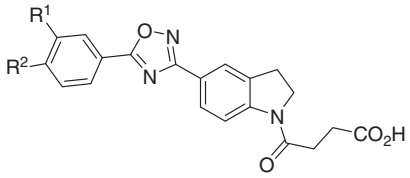
Figure 6. Blood lymphocyte time-profile after a 0.00 (vehicle), 1.00, 3.00 or 10.0 mg/kg oral dose of Compound **1** in male BALB/c mice. Symbols represent observed values (mean ± SD) and the lines represent predicted values from the pharmacokinetic/pharmacodynamic model. Vehicle = 0.5% methylcellulose in water.

comparable potency to Compound **1** (e.g. **34** EC_{50} [cAMP] ~ 1 nM). EC_{50} determination performed in β-arrestin; however, revealed that Compound **34** was 4.5 fold more potent than Compound **1** in this assay (**34**, EC_{50} = 6.8 nM vs. **1**, EC_{50} = 31 nM). It was hypothesized that β-arrestin may be a more accurate predictor of *in vivo* activity than the cyclase data, due to the high levels of $S1P_1$ receptor expression in the cyclase assay. Furthermore, the *in vivo* IC_{50} value for Compound **1** was in better agreement with the β-arrestin data than cyclase.

To determine if an increase in β-arrestin potency coincided with an improvement in *in vivo* potency, Compound **34** was selected for IC_{50} determination in the mouse lymphocyte lowering experiment. Given the improved potency associated with this compound, the three dose groups were reduced to 0.3, 1.0, and 3.0 mg/kg, respectively. A plot of the lymphocyte levels over time is depicted in Figure 7. Complete suppression of circulating lymphocytes was observed very quickly with levels returning to normal by approximately 8 h. Consistent with the β-arrestin data, Compound **34** was determined to be more potent *in vivo* than Compound **1**. The IC_{50} value for Compound **34** was estimated to be 22.6 ng/mL, which translates into an approximate 19-fold improvement in *in vivo* potency when compared to Compound **1**.

In conclusion, a hit to lead optimization study was carried out which resulted in the discovery of a new chemical series of 4-oxo-4-(5-(5-phenyl-1,2,4-oxadiazol-3-yl)indolin-1-yl)butanoic acid $S1P_1$ agonists. Analogs from within this series were

Table 5
Phenyl SAR. Human $S1P_1$ HTRF cAMP EC_{50} values are reported as the mean of three or more replicates



Compound	R^1	R^2	$hS1P_1$ EC_{50} [nM]
29	Cl^-		0.82
30	CF_3^-		1.3
31	CN^-		18
32	Cl^-		45
33	MeO^-	CHF_2O^-	1.5
34	CN^-	$(CH_3)_2CHO^-$	1.1 (6.8)[a]
35	CF_3O^-	MeO^-	1.0
36	Br^-	$(CH_3)_2CHO^-$	4.0
37	CF_3^-	$CH_3CH_2^-$	2.7
38	CF_3O^-	Cl^-	5.3
39	CN^-	MeO^-	6.3
40	CF_3^-	MeO^-	11
41	CH_3^-	MeO^-	18
42	Cl^-	MeO^-	11
43	$CH_3SO_2^-$	$CH_3CH_2^-$	38
44	H^-	$CF3O^-$	68

[a] value in parentheses is the EC_{50} determined in β-arrestin.

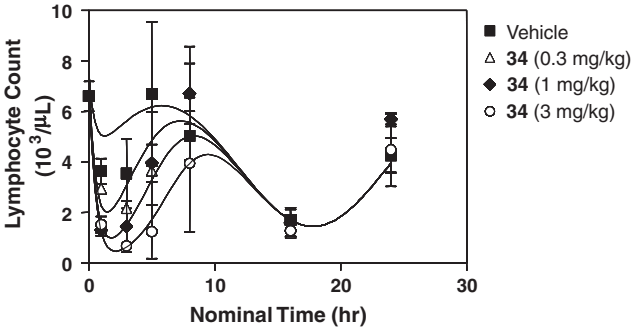


Figure 7. Blood lymphocyte-time profile after a 0.00 (Vehicle), 0.300, 1.00 and 3.00 mg/kg oral dose of Compound **34** in Male BALB/c Mice (mean ± SD). Symbols represent observed values and the lines represent predicted values from the pharmacokinetic/pharmacodynamic model.

demonstrated to possess desirable safety and ADME profiles, and were shown to be efficacious at reducing circulating lymphocytes in mice after a low oral dose. Further optimization of this chemical series resulted in the identification of a new lead molecule possessing significantly improved potency in the mouse lymphocyte lowering experiment.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.05.110.

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