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Discovery of a novel series of potent S1P1 agonists

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ABSTRACT

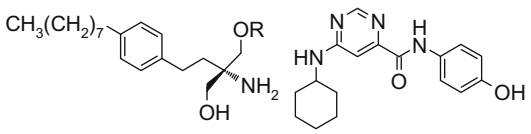
The discovery of a novel series of S1P1 agonists is described. Starting from a micromolar HTS positive, iterative optimization gave rise to several single-digit nanomolar S1P1 agonists. The compounds were able to induce internalization of the S1P1 receptor, and a selected compound was shown to be able to induce lymphopenia in mice after oral dosing.

Sphingosine-1-phosphate is an important chemical mediator, which exerts its effects via five G-protein coupled receptors (S1P1-5). In recent years, S1P1 in particular has attracted wide interest as a therapeutic target, as molecules capable of agonising this receptor demonstrate a potent lymphopenic effect in vivo.[1] In addition, the investigational drug FTY720 (Fingolimod, **1a**, now in Phase 3 clinical trial for relapsing-remitting multiple sclerosis) is believed to provide therapeutic benefit via S1P1: its phosphorylated form **1b** (essentially generated in vivo by sphingosine kinase type 2 activity)[2] is in fact a potent S1P1 agonist and animals treated with FTY720 experience a strong peripheral blood lymphopenia.[3]

Phosphorylated FTY720 is poorly selective against the various S1P receptors, being very active on S1P1, S1P3, S1P4 and S1P5.[3] However, it has been demonstrated that only S1P1 activity is necessary to induce lymphopenia, while it has been shown that bradycardia induced by S1P receptor non-selective agonists in wild type mice is suppressed in S1P3 −/− mice.[4] To attenuate the potential risk of bradycardia more selective compounds are needed. In this Letter, we describe the identification and characterization of new small molecules which are selective and potent S1P1 agonists capable of inducing a lymphopenic effect.

A high-throughput screening run on 100,000 compounds (using a cAMP readout) yielded, among others, compound **2** as positive (Fig. 1). This compound was considered to be a suitable starting point for a medicinal chemistry program.

The general synthetic methods employed are summarized in Scheme 1. The carboxylic acids **5** could be prepared by reaction of an amine with methyl 2,4-dichloropyrimidine-6-carboxylate, followed by reductive dechlorination and hydrolysis of the methyl ester group. These intermediates could be coupled with various amines to explore the SAR of the R^3 substituent, using standard amide coupling reagents. In particular, we routinely used the polymer-supported Mukaiyama reagent for its ease of use.[5] The 2-chloropyrimidine intermediates **4** could also be used to prepare some 2-substituted compounds via either Pd-catalyzed chemistry or S_NAr reactions.



1a FTY720 R=H
1b (*S*)-FTY720-P R=PO_3H_2

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Figure 1. FTY720 (**1a**), (*S*)-FTY720-phosphate (**1b**) and HTS positive **2**.

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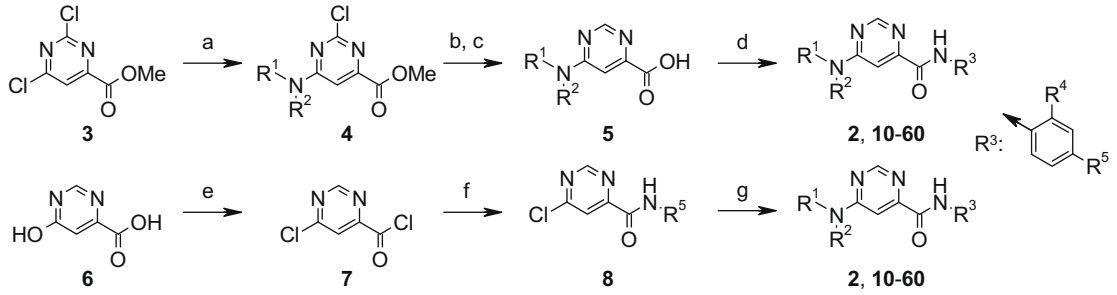

Scheme 1. Reagents and conditions: (a) R^1R^2NH, TEA, THF; (b) $(NH_4)^+HCOO^-$, Pd/C; (c) NaOH, THF/H_2O; (d) R^3NH_2, PS-Mukaiyama reagent, TEA; (e) PCl_5, $POCl_3$; (f) R^3NH_2, TEA, DCM; (g) R^1R^2NH, DIEA, EtOH, 160 °C (microwave irradiation).

To explore the SAR of the R^1 and R^2 substituents, a different strategy was employed: 6-hydroxypyrimidine-4-carboxylic acid was first converted to 6-chloropyrimidine-4-carbonyl chloride **7**, which was reacted with the desired aniline compounds. The intermediates **8** could then be reacted with a variety of amines under microwave irradiation to give the desired compounds.

At first, the SAR of the R^1 substituent was explored (Table 1). Of the compounds present in the original screening library, only those possessing a cycloalkyl substituent were found active. The crucial importance of this substituent was confirmed during our investigation: smaller substituents lead to a drastic loss of potency (e.g., **9** and **10**). Replacing the cycloalkyl substituent with variously substituted aromatic groups led to poorly active compounds, with the only exception being the *o*-tolyl derivative **13**, which kept a similar level of activity. Replacing the cycloalkyl amine moiety with a number of cyclized substituents, such as variously substituted piperidines, morpholines or piperazines led to inactive compounds (results not shown).

Table 1

Compds	R^1	R^2	R^4	R^5	S1P1 GTPγS EC_{50}[a] (nM)
2	*c*-Hexyl	H	H	OH	1780
9	*c*-Propyl	H	Me	OH	>20,000
10	*i*-Butyl	H	Me	OH	>20,000
11	*c*-Pentyl	H	H	OH	16,700
12	Ph	Me	H	OH	>20,000
13	*o*-Tolyl	H	H	OH	2100
14	*c*-Heptyl	H	Me	OH	973
15	*c*-Hexyl	Me	H	OH	700
16	*c*-Hexyl	Me	Me	OH	655
17	*c*-Hexyl	Et	Me	OH	405
18	*c*-Hexyl	*c*-$PrCH_2$	Me	OH	25
19	Pr	Pr	H	OH	375
20	*c*-Hexyl	H	Me	OH	1160
21	*c*-Hexyl	H	F	OH	440
22	*c*-Hexyl	H	Cl	OH	390
23	*c*-Hexyl	H	CF_3	OH	382
24	*c*-Hexyl	H	H	CH_2OH	>20,000
25	*c*-Hexyl	H	H	CH_2CH_2OH	>20,000
26	*c*-Hexyl	H	F	F	>20,000
27	*c*-Hexyl	H	H	OMe	>20,000
28	*c*-Hexyl	H	H	$NHSO_2Me$	960
29	*c*-Hexyl	H	H	NHCOMe	>20,000
30	*c*-Hexyl	H	H	SO_2NH_2	1990
31	*c*-Hexyl	H	Me	SO_2NH_2	440
32	*c*-Hexyl	Me	Me	SO_2NH_2	65
33	*c*-Hexyl	*c*-$PrCH_2$	Me	SO_2NH_2	1.6
34	*c*-Hexyl	Pr	Me	SO_2NH_2	2.9
35	Pr	Pr	Me	SO_2NH_2	36
36	*c*-Hexyl	*c*-$PrCH_2$	H	SO_2Me	193
37	*c*-Hexyl	*c*-$PrCH_2$	H	$CONH_2$	73
38	*c*-Hexyl	*c*-$PrCH_2$	Me	SO_2NHMe	6
39	*c*-Hexyl	*c*-$PrCH_2$	H	CH_2NH_2	39
40	*c*-Hexyl	*c*-$PrCH_2$	H	CH_2NMe_2	104

[a] Average of two experiments. SD were generally within 50% of average.

Keeping the cyclohexyl group fixed, we then explored the influence of a second substituent R^2 on the nitrogen. Already, a simple methyl group led to a twofold increase in potency. Bigger increases in potency could be obtained with longer substituents (ethyl, propyl, cyclopropylmethylene) giving rise to very potent compounds (e.g., **18**, EC_{50} 25 nM). When in conjunction with second substituent of sufficient length (e.g., a propyl group), the cycloalkyl group could be substituted with a non-cyclic group (e.g., a second propyl group, **19**) with an approximately 10-fold drop in potency.

A few modifications of the central core of the molecule were attempted, all leading to dramatic reduction in potency. Even small substituents on the C2 position of the pyrimidine ring (Me, OMe, Cl) lead to significant loss of potency (data not shown). The same was observed after methylation of the amide nitrogen.

At the same time, a preliminary SAR was conducted on R^3 (Table 1). We quickly discovered that a small lipophilic substituent on the position *meta* to the phenol (R^4) was advantageous, leading to a 2–4-fold increase in potency (**20–23**). On the other hand, substitution on the position *ortho* to the phenol had a markedly inferior effect (data not shown).

However, our main concern was the presence of the *p*-anilinophenol moiety, both for the possibility of formation of toxic metabolites as well as for the possibility of Phase 2 metabolism on the phenolic group. In fact, the pharmacokinetic (PK) profile of compound **20** was found to be a parameter to be optimized: despite a medium to low clearance in microsomes (2 and 18 μL/min/mg respectively in human and rat liver microsomes), the compound exhibited very high clearance (4.85 L/h/kg) in mice, and significant amounts of the glucuronide adduct could be found in the PK samples, confirming that the phenolic group had to be replaced.

A number of substituents (fluorine, methoxy, carboxy, hydroxymethyl or hydroxyethyl, see Table 1, compds **24**–**27**) were tested to replace the phenol as R^5, without much success. Also, moving the phenol substituent to the *meta* position of the aniline led to less potent compounds (data not shown). The data clearly indicated the preference for an H-bond donor (HBD) as R^5.

The first alternative R^5 substituents bearing an HBD that were investigated were *N*-arylsulfonamide **28** and *N*-arylacetamide **29**. Interestingly, the activity of these two compounds seemed to be dependent on the acidity of the compound, with a pK_a similar to that of the phenol being favoured. So the sulfonamide **28** (EC_{50} 960 nM, calcd pK_a 9.9) was found to be significantly more potent than the corresponding acetamide **29** (EC_{50} >20 μM, calcd pK_a 13.6). However, the presence of a 1,4-diamino phenyl unit was not considered as being of particular advantage in terms of potentially toxic metabolites. In addition, the reversed sulfonamide **30** was also found to possess a similar potency, indicating that there

was some scope for changing the position of the HBD. Quite satisfyingly, the pharmacokinetic profile of **30** proved to be markedly superior to the phenol **20**, with a low clearance of 0.3 L/h/kg.

This new R^5 group was then combined with some of the best substituents for the lipophilic tail. The SAR was found to be very similar to that for the phenol subseries, and the compounds bearing a cyclohexyl substituent and a propyl or cyclopropylmethylene group (**33**, **34**) proved to be very potent, with EC_{50} of 1.6 and 2.9 nM, respectively. As in the phenol subseries, the derivative bearing two smaller chains (**35**) were less active by approximately one order of magnitude. The fact that this reversed sulfonamide group was still mainly behaving as an HBD was proved by the fact that methyl sulfone **36** was less active by ca. 2 orders of magnitude than the corresponding sulfonamide **33**. The primary amide **37**, possessing less acidic protons, also showed an inferior activity.

We also tested some compounds bearing a chargeable nitrogen to act as HBD (compds **39**–**40**). These compounds retained potency in the same range as for the corresponding phenol compounds, although not as good as for the sulfonamide derivatives.

We then explored the possibility of benzofused heterocycles (Fig. 2) as bioisosteres for the phenolic moiety (Table 2). The correlation between the acidity of the proton and the affinity of the compounds for the S1P1 receptor was confirmed: indazoles **41** and **42** (calcd pK_a 13.6) turned out to be more active than indole **43** (calcd pK_a 16.8). Benzotriazole **44** (calcd pK_a 8.6) was also less active, although this could be due to the existence of a second tautomer. Contrary to what was observed before for the indazole-containing compounds, no significant difference of activity was found between the cyclohexyl-containing compound **45** and its propyl analogues **46** and **47**, with di-propyl analogue **47** being actually the most potent.

Pursuing our exploration of R^3 groups (Fig. 2), we shifted our attention to the possibility of integrating a carboxylic acid moiety in our compounds, to improve their solubility and decrease their Log $D_{7.4}$. We chose to integrate this new feature in both our sulfonamide and amine-based R^3 groups, using spacers of differing length (**51**–**60**) and in both cases we could identify very potent compounds, with a preference being for the compounds with a 2-carbon spacer (**53**, **54**, **59**). Of particular advantage was, in the case of the amino-acid spacer, the use of the azetidine linker (**60**, EC_{50} 3 nM). In this case, the potency of the azetidine compound is remarkably better than the corresponding dimethylamino compound (**39**), clearly indicating a significant positive contribution of the carboxylic acid group to the binding to the S1P1 receptor. To verify that the effect could be exclusively attributed to the carboxylic acid, we also prepared the corresponding azetidine derivative without the carboxylic acid group, which turned out to be significantly less active. Inclusion of other solubility-enhancing substituents on our sulfonamide or amino compounds, such as hydroxylated alkyl chains, had a detrimental effect on the potency, as well as a very limited impact on the solubility of the compounds (data not shown).

Selectivity data for some derivatives can be found in Table 3. Selectivities versus S1P3 were generally moderate to good, with the compounds containing both a sulfonamide and acid groups

Table 2

Compds	R^1	R^2	R^3	S1P1 GTPγS EC_{50}[a] (nM)
41	*c*-Hexyl	H	A[b]	2710
42	*c*-Hexyl	Me	A	418
43	*c*-Hexyl	H	C	12,100
44	*c*-Hexyl	Me	D	7300
45	*c*-Hexyl	*c*-PrCH$_2$	A	9
46	Pr	*c*-PrCH$_2$	A	13
47	Pr	Pr	A	7
48	*c*-Hexyl	Me	B	211
49	*c*-Hexyl	*c*-PrCH$_2$	B	6
50	Pr	*c*-PrCH$_2$	B	12
51	*c*-Hexyl	*c*-PrCH$_2$	E	12
52	Pr	*c*-PrCH$_2$	E	208
53	*c*-Hexyl	*c*-PrCH$_2$	G	3
54	Pr	*c*-PrCH$_2$	G	33
55	*c*-Hexyl	*c*-PrCH$_2$	L	20
56	Pr	*c*-PrCH$_2$	L	238
57	*c*-Hexyl	*c*-PrCH$_2$	M	35
58	*c*-Hexyl	*c*-PrCH$_2$	N	94
59	*c*-Hexyl	*c*-PrCH$_2$	Q	19
60	*c*-Hexyl	*c*-PrCH$_2$	R	3

[a] Average of two experiments. SD were generally within 50% of average.
[b] See Figure 2.

Table 3
Selectivity of selected compounds

Compds	S1P1 GTPγS EC_{50}[a] (nM)	S1P3 GTPγS EC_{50}[a] (nM)	S1P5 GTPγS EC_{50}[a] (nM)
2	1780	>30,000	ND[b]
18	25	>30,000	191
19	375	2320	ND[b]
20	1160	>30,000	258
31	440	>30,000	ND[b]
33	1.6	>30,000	96
46	13	416	142
49	6	3270	ND[b]
53	3	62	114
59	19	1094	6.3
60	3	236	0.6

[a] Average of two experiments. SD were generally within 50% of average.
[b] Not determined.

(e.g., **53**) being the least selective. Sulfonamide **33** showed the best selectivity, with more than four orders of magnitude. Compounds bearing the cyclohexyl substituent tended to give significantly better selectivity versus S1P3 (cf., e.g., **19** vs **18** and **49** vs **46**). In general, the compounds were not selective versus S1P5, with the exception of compds **33** and **53** showing a moderate selectivity.

Having discovered a series of very potent analogues, these were profiled in a battery of in vitro assays to determine their suitability for in vivo testing. The results of some representative compounds are summarized in Table 4. As expected, all compounds were lipophilic, with Log *P* values above four for almost all compounds tested. All compounds lacking an ionizable group showed poor solubility in aqueous solutions. Predictably, compounds bearing the

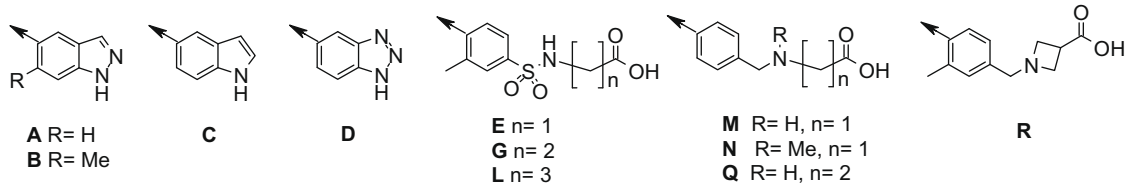

Figure 2. Alternative R^3 groups (see Table 2).

Table 4
Summary of in vitro profiling data

Compds	Log *P*	Solubility[a] (μg/mL)	Microsome Clint[b] (human/rat) (μL/min/mg)	Caco-2 permeability (Papp,[c] 10^{-6} cm/s)
18	7.4	8	22/52	ND[d]
19	ND[d]	16	4/7	21.4
20	4.1	>32	2/18	21.8
31	3.6	5	43/15	12.5
33	5.7	1	130/14	ND[d]
46	4.6	11	17/57	8.5
49	6.1	5	<10/26	0.1
53	ND[d]	>51	25 / 25	2.0
59	ND[d]	>45	<10/<10	0.8
60	>2.7[e]	22	<10/<10	2.4

[a] Nephelometric kinetic solubility in PBS (1% DMSO).
[b] Intrinsic clearance (see Supplementary data).
[c] Apparent permeability (see Supplementary data).
[d] Not determined.
[e] Log $D_{7.4}$.

carboxylic acid groups were considerably more soluble; however, they suffered from a reduced permeability in the Caco-2 assay compared to the neutral compounds. In most cases, the compounds were sufficiently stable in the presence of human or rat microsomes, the only exception being compound **33** which showed instability only in the human system.

In parallel, we verified if the agonistic effect of our compounds on the S1P1 receptor lead to its internalization, which is believed to be the cause of the lymphopenic effect in vivo. This was tested using human osteosarcoma U2OS cells expressing the S1P1 receptor fused to Green Fluorescent Protein (GFP).[6] Internalization of the receptor could be verified by imaging, and the relative EC_{50}s could be calculated for some compounds, with results in the same range as those obtained in the GTPγS binding assay. For example, compds **46** and **60** had EC_{50}s of 12 and 3 nM, respectively, in the internalization assay (13 and 3 nM, respectively, in the GTPγS assay).

Finally, compound **46** was used to test if the effect on S1P1 receptor internalization could translate into significant lymphopenia in vivo. When dosed at 30 mg/kg po, **46** caused a 72% decrease of blood lymphocytes in mice at 4 h after dosing (without any effect on erythrocytes and platelets).

In summary, starting from a weakly potent S1P1 agonist, we have discovered several single-digit nanomolar agonists. We have successfully replaced the unwanted phenol group of the original hit with different groups, which are more acceptable from a toxicity and pharmacokinetic point of view. We have verified that the agonistic behaviour of our compounds towards the S1P1 receptor translated as well in the desired internalization of the receptor. For a selected compound, we confirmed that this functional antagonism could induce a significant lymphopenia in mice.

Supplementary data

Typical conditions for the synthesis of compounds, as well as experimental details on the in vitro assays. Full experimental conditions and characterization of all compounds can be found in patent WO2009019167. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.102.

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