A Rational Utilization of High-Throughput Screening Affords Selective, Orally Bioavailable 1-Benzyl-3-carboxyazetidine Sphingosine-1-phosphate-1 Receptor Agonists

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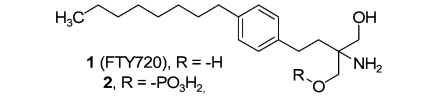
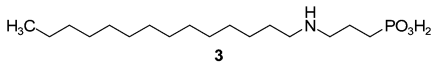
Abstract: Moderately potent, selective $S1P_1$ receptor agonists identified from high-throughput screening have been adapted into lipophilic tails for a class of orally bioavailable amino acid-based $S1P_1$ agonists represented by **7**. Many of the new compounds are potent $S1P_1$ agonists that select against the $S1P_2$, $S1P_3$, and $S1P_4$ (although not $S1P_5$) receptor subtypes. Analogues **18** and **24** are highly orally bioavailable and possess excellent pharmacokinetic profiles in the rat, dog, and rhesus monkey.

A rationale has recently emerged for the investigation of sphingosine-1-phosphate-1 ($S1P_1$) receptor agonists as immunomodulatory agents. The novel immunosuppressant and clinical development candidate 2-amino-2-(4-octylphenyl)ethylpropane-1,3-diol (**1**, FTY720) has been demonstrated to be metabolized across species to a monophosphate ester (**2**), which is a potent agonist of four of the five known S1P receptors ($S1P_{1,2,3,4,5}$).[1] The systemic administration of either **1** or **2** induces a dose-responsive lowering of circulating T and B lymphocytes; this phenomenon has been replicated with structurally distinct phosphonate-based S1P receptor agonists which would indicate that it is regulated by S1P receptor agonism.[2] The observed alterations in lymphocyte trafficking have been proposed to be responsible for the efficacy of **1** in the prevention of organ allograft rejection and in models of autoimmune disorders.[3] The distinct tissue distributions of the S1P receptor subtypes[4] suggest that nonselective agonists would not be required for immunosuppression. The notion that an agonist-driven $S1P_1$ antagonism is the key component in the immunosuppressive activity of **1** is strongly supported by reports detailing the similarities observed in the phenotypes of the lymphocyte cell-specific $S1P_1$ knockout mice and the changes in thymic emigration and lymphocyte circulation in wild-type mice that have been treated with **1**.[5]



1 (FTY720), R = -H
2, R = -PO_3H_2,



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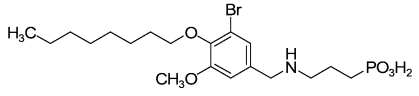

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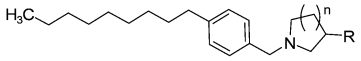
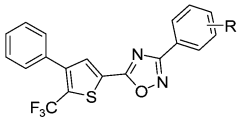


5, R = -PO_3H_2, n = 1
6, R = -CO_2H, n = 1
7, R = -CO_2H, n = 0



8, R = -H
9, R = 2,4-di-Cl
10, R = 3-CF_3

The primary clinical adverse effect that has been reported for **1** is a transient, asymptomatic bradycardia;[6] bradycardia is driven by $S1P_3$ agonism in rodents.[7] We recently disclosed that specific modifications of a nonselective S1P agonist (**3**) resulted in new compounds (e.g., **4**) that select against the $S1P_3$ subtype (Table 1).[2c] These more selective compounds were fully efficacious in their ability to alter lymphocyte trafficking, yet had an attenuated potential to cause the acute bradycardia and hypertension that had been observed in rodents challenged with more promiscuous S1P receptor agonists. While the clinical experience with **1** would indicate that selecting against $S1P_3$ may be desirable, our work affording analogues such as **4** demonstrated that it could be realized in a practical manner.

Another extension of our work with **3** involved an empirical investigation of constrained analogues with the goal of identifying new classes of leads possessing enhanced selectivity or pharmacokinetic properties.[8] While several novel scaffolds were identified, none of these afforded advantages in S1P receptor subtype selectivity. A possible explanation for this may be that these compounds interact with the Arg120, Glu121, and Arg292 of S1P receptors similarly to S1P,[9] but the environment around these conserved residues in $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ provides no basis for influencing selectivity. While the iv pharmacokinetic profiles of a number of our earlier acyclic phosphonate-based S1P receptor agonists as well as those of their conformationally constrained counterparts made them suitable for use in investigating S1P receptor pharmacology, all were found to have negligible oral bioavailability in rodents.

We have also investigated replacing the phosphonic acid in analogues of **3** with other acidic functional

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Table 1. S1P Receptor Affinities (IC_{50}, nM)[a]

compd	$S1P_1$	$S1P_2$	$S1P_3$	$S1P_4$	$S1P_5$
S1P	0.67	0.35	0.26	34	0.55
1	840	>10000	>10000	>10000	2100
2	0.28	1100	6.3	15	0.77
3	2.3	580	3.6	140	13
4	4.1	>10000	2100	80	10
5	6.9	>10000	220	45	6.3
6	20	>10000	2100	2500	29
7	18	>10000	4900	5400	11
8	25	>10000	>10000	>10000	>10000
9	78	>10000	>10000	>10000	>10000
10	37	>10000	>10000	>10000	4600

[a] Displacement of [^{33}P]-labeled sphingosine-1-phosphate (S1P) by test compounds from human S1P receptors expressed on CHO cell membranes. Data are reported as mean for $n = 3$ determinations. SD were generally ± 20% of the average. See ref 7b for assay protocol.

groups that would be expected to enhance the potential for oral absorption.[2b] This generally afforded analogues with 50- to 100-fold lower affinity for $S1P_1$, making them moderately interesting; similar results were seen when this was attempted with many of the constrained versions of **3**. When this strategy was extended to pyrrolidine-3-phosphonic acid **5**; however, a more modest 3-fold loss in $S1P_1$ affinity was observed with the pyrrolidine carboxylate **6** (Table 1). While ring-expanded or chain-extended analogues of **6** were found to lose significant S1P receptor affinity, ring contraction giving azetidine carboxylate **7** was found to be tolerated. Both **6** and **7** were found to lower circulating lymphocytes in mice 3 h after oral administration with pharmacodynamic ED_{50} values determined to be 23 mpk and 5.2 mpk, respectively. Pharmacokinetic experiments with these compounds showed that they both had high oral bioavailability (*%F* > 70) in the rat with moderate plasma clearance rates but relatively short half-lives (**6**, $t_{1/2} = 0.9$ h; **7**, $t_{1/2} = 1.4$ h).

A method to integrate [^{35}S]-GTPγS scintillation proximity bead-based binding assays with a fully automated robotics platform amenable to the high-throughput screening (HTS) of G_i-coupled $S1P_1$ receptor agonists has been reported.[10] Such screening of the Merck sample collection was conducted, and 90 compounds were identified as genuine leads after counterscreening for the nonspecific induction of [^{35}S]-GTPγS binding. A subset of the actives was commercially available 3,5-diaryl-1,2,4-oxadiazole analogues exemplified by **8**−**10** which after further evaluation were found to be moderately potent, but highly selective $S1P_1$ receptor agonists (Table 1).

The structure−activity relationships that had been developed for analogues of **4**[2c] suggested to us that **8**−**10** could be incorporated into our existing lead series with the idea that they be adapted to occupy the same molecular space as the lipophilic side chains of our earlier compounds (Figure 1). While either phenyl ring of **8**−**10** could be envisioned to overlap with the benzylic phenyl ring of **4**, the fact that moderately polar groups (ether, ketone, ester, small heterocycle) directly adjacent to the benzylic phenyl ring were tolerated in these analogues indicated that an alignment as shown in Figure 1 was perhaps the more reasonable as a first approach. Analogues with hybrid lipophilic tails (i.e., those in which the 1,2,4-oxadiazole was replaced with -CH_2O-) also seemed to be rational in their design. Since

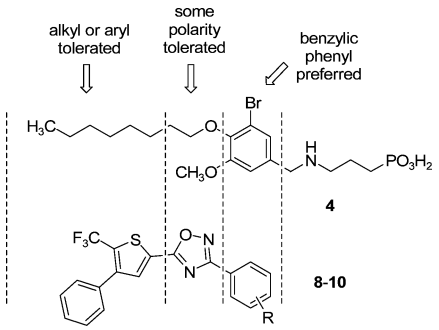
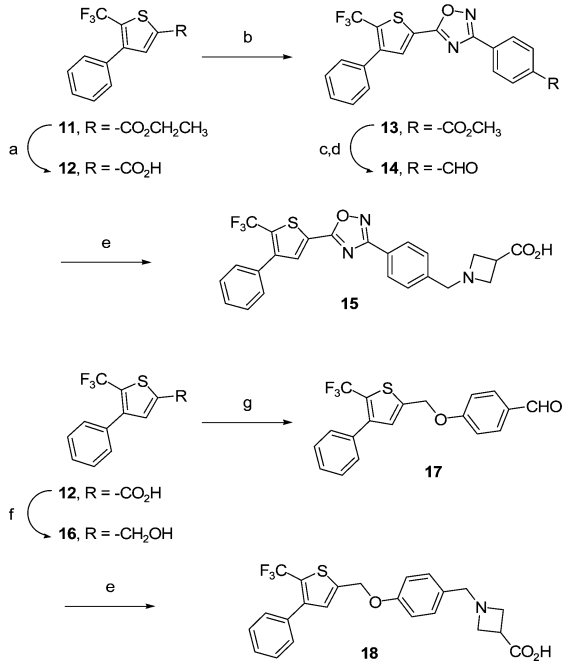


Figure 1. SAR summary and rationale for HTS lead utilization.

Scheme 1[a]



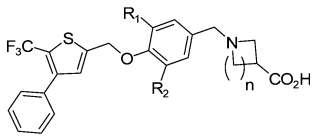
[a] Reagents: (a) NaOH, aq EtOH, rt (93%); (b) $(COCl_2)_2$, cat. DMF, CH_2Cl_2, then 4-(carbomethoxy)benzamidoxime, xylenes/pyridine, reflux (65%); (c) DIBALH, CH_2Cl_2, −78 °C (89%); (d) cat. TPAP, NMMO, 4 Å mol sieves, CH_3CN (66%); (e) azetidine-3-carboxylic acid, $NaB(CN)BH_3$, MeOH (**15**, 70%; **18**, 48%); (f) $BH_3{\cdot}S(CH_3)_2$, THF, reflux (98%); (g) 4-hydroxybenzaldehyde, DEAD, Ph_3P, THF (60%).

amino acid compounds such as **6** and **7** had demonstrated oral bioavailability in rodents, the first "HTS side chain" analogues were prepared in these series. The syntheses of **15** and **18** (Scheme 1) are representative of those used to prepare the new compounds.

Ligand competition studies between [^{33}P]-S1P and all new compounds were carried out for each of the five human S1P receptors stably expressed in Chinese Hamster Ovary (CHO) cell membranes.[7b] S1P receptor agonism by the test compounds was determined by measurement of ligand-induced [^{35}S]-5′-*O*-3-thiotriphosphate (GTPγS) binding. In general, all new compounds were found to be agonists of S1P receptors with calculated EC_{50} values 2- to 3-fold lower than IC_{50} values. Analogue **15** was the outlier in this respect, showing much more pronounced $S1P_1$ and $S1P_3$ EC_{50} shifts of 40-fold and 60-fold, respectively. Reasons for this are not fully understood at this time.

The S1P receptor data generated for the new compounds (Table 2) is noteworthy for several reasons. All

Table 2. S1P Receptor Affinities (IC_{50}, nM)[a] for "HTS Side Chain" Analogues



compd	*n*	R_1	R_2	$S1P_1$	$S1P_3$	$S1P_4$	$S1P_5$
S1P				0.67	0.26	34	0.55
2				0.28	6.3	15	0.77
15				8.3[b]	1000[c]	10000	16
18	1	H	H	1.2	530	1600	23
19[d]	2	H	H	4.3	1000	790	120
20	1	CF_3	H	7.7	>10000	340	21
21	1	Br	H	13	>10000	2400	53
22	1	Cl	H	3.2	2000	1300	18
23	1	F	H	2.7	2100	2700	41
24	1	CH_3	H	1.8	7500	1500	13
25	1	CH_2CH_3	H	3.5	4300	>10000	10
26	1	OCH_3	H	33	>10000	2800	77
27	1	CH_3	CH_3	4.0	10000	120	12
28	1	Cl	Cl	7.5	>10000	640	33

[a] Displacement of [^{33}P]-labeled sphingosine-1-phosphate (S1P) by test compounds from human S1P receptors expressed on CHO cell membranes. Data are reported as mean for $n = 3$ determinations. SD were generally ± 20% of the average. All new compounds had $S1P_2$ IC_{50} >10000 nM. See ref 7b for assay protocol. [b] EC_{50} = 0.2 nM. [c] EC_{50} = 17 nM. [d] Racemate.

Table 3. Murine Peripheral Lymphocyte Lowering[a](PLL) and Pharmacokinetic (PK) Data for Selected Compounds

	compound			
	15	**18**	**22**	**24**
PLL ED_{50} (mpk, po)	0.07	0.44	0.54	0.18
	Rat PK[b]			
Cl_p (mL/min/kg)	2.2	12.3	8.2	8.2
Vd_{ss} (L/kg)	1.9	10.0	3.8	5.7
C_{max} (μM)	1.3	0.3	0.5	0.5
$t_{1/2}$	10.5	6.7	7.0	8.9
%F	64	79	62	88
	Dog PK[c]			
Cl_p (mL/min/kg)	nd[d]	2.2	nd[d]	0.5
Vd_{ss} (L/kg)		1.6		0.9
C_{max} (μM· h)		1.3		3.2
$t_{1/2}$ (h)		9.7		22.3
%F		66		100
	Rhesus PK[c]			
Cl_p (mL/min/kg)	nd[d]	1.5	nd[d]	1.3
Vd_{ss} (L/kg)		1.4		1.2
C_{max} (μM)		1.8		1.8
$t_{1/2}$ (h)		12.5		12.6
%F		76		96

[a] Individual data points for dose-titrations were the average percentage decrease of PBL counts in $n = 3$ animals vs control ($n = 3$) 3 h after po administration of the test compound. SD were generally ±20% of the average. See ref 2a for assay protocol. [b] 2.0 mpk po, 1.0 mpk iv. [c] 1.0 mpk po, 0.5 mpk iv. [d] Not determined.

of the new compounds have enhanced affinity for $S1P_1$ as compared to amino acid analogues **6** and **7** or the HTS leads **8**−**10**. Ether-linked analogues such as **18** and **24** are 3- to 20-fold more selective against $S1P_3$, $S1P_4$, and $S1P_5$ than their 4-(nonyl)benzyl counterpart **7**. Compounds such as **15** ($S1P_1$ EC_{50} = 0.2 nM), **18**, and **24** were less selective than the HTS leads (especially against $S1P_5$), but the data for them clearly demonstrate that side chain modifications can lead to selection against the $S1P_2$, $S1P_3$, and $S1P_4$ subtypes in compounds with $S1P_1$ affinity approaching that of **2**. The facts that compound **15** is a ligand for S1P receptors and enhanced selectivity against $S1P_3$ is obtained in analogues of **18** when R_1 is larger than a hydrogen or fluorine atom support our hypothesis regarding the overlap of the HTS leads **8**−**10** and the side chains of our earlier phosphonate-based compounds.

The immunosuppressive efficacy of **1** has been proposed to arise from its ability to promote the sequestration of $CD4^+$ and $CD8^+$ T cells and B cells in secondary lymphoid organs which prevents their infiltration into transplanted or antigen-bearing nonlymphoid tissues.[3] Measurement of peripheral blood lymphocyte (PBL) counts in rodents after the administration of test compounds provides a convenient surrogate marker for efficacy that is amenable to the screening of multiple analogues in vivo.[7b] The ability of all of the new compounds to lower PBLs in mice after oral administration was the first indication that they were analogous to **6** and **7** in regard to oral bioavailability. Dose−titration experiments in the mouse with **15**, **18**, **22**, and **24** (Table 3) indicated that they were all at least 10-fold more potent than **7** with pharmacodynamic ED_{50} values in this assay comparable to that of **1** (ED_{50} = 0.15 mpk po). Compounds **15**, **18**, **22**, and **24** were all found to be orally bioavailable in the rat with extended half-lives as compared to **6** and **7**; further evaluation of **18** and **24** established that they had favorable pharmacokinetic profiles in the beagle dog and rhesus monkey. Complete blood counts were determined in parallel to compound plasma concentrations as part of the dog and rhesus pharmacokinetic experiments; a maximal lowering of PBLs was observed 24 h after 1.0 mpk single oral doses of **18** and **24** in both species with the lymphocyte nadir being initially reached 8 h postdose in the beagle dog and 4 h postdose in the rhesus.

Some of the relationships between pharmacokinetics and the PBL lowering response have been characterized in the rat. A dose−titration experiment with **18** indicated that a 3 mpk po dose was minimally required to sustain maximal PBL lowering at 24 h postdose. The pharmacokinetics of **18** appeared to be linear over a dose range of 1 to 30 mpk po in the rat and PBL counts 24 h after doses of 10 and 30 mpk po were not significantly different than those seen after the 3 mpk po dose. A pharmacokinetic/pharmacodynamic time course experiment with 3 mpk po of **18** (Figure 2) demonstrated that the kinetics for the onset of PBL lowering in the rat appear to be analogous to those observed after oral administration of **1**[1a] with the lowest PBL counts initially observed 3 to 4 h postdose. In this experiment, the rebound of PBLs started to occur after 24−32 h with a return to predose levels being reached by 72 h. A second challenge of the same animals with 3 mpk po of **18** replicated the PBL response seen after the first dose.

A dose-responsive lowering of PBLs in the rat was also observed when **18** was delivered via continuous infusion. Administering compound at a rate of at least 24 μg/kg/h was required to maximize the PBL response and found to maintain a 100−200 nM plasma concentration of **18**. Interestingly, PBL rebound in the rat pharmacokinetic/pharmacodynamic experiment described in the previous paragraph occurred when plasma concentration of **18** had reached approximately 100 nM; taken together these data indicate that there is a strong

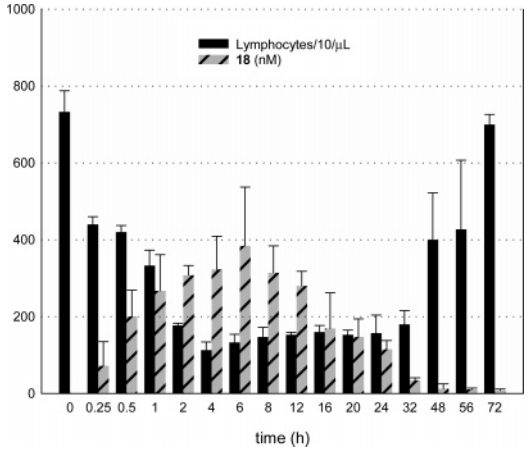


Figure 2. PBL counts vs compound plasma concentration (nM) in femoral artery cannulated Lewis rats ($n = 3$) after a single 3 mpk po dose of **18**.

likelihood that $S1P_1$ agonist-induced alterations to lymphocyte trafficking are driven and maintained by trough, not peak, plasma compound concentrations.

In conclusion, the incorporation of the structures of moderately potent, selective $S1P_1$ receptor agonists identified from high-throughput screening into an existing lead series $S1P_1$ agonists has been found to provide potent $S1P_1$ agonists that select against the $S1P_2$, $S1P_3$, and $S1P_4$ receptor subtypes. Initial investigations have shown that the systemic administration of 1-benzyl-3-carboxyazetidine analogues such as **18** and **24** induce a lowering of circulating lymphocytes in rodents similarly to **1**, and that these new analogues have excellent cross-species pharmacokinetic profiles. The further optimization of the $S1P_1$ receptor agonists described herein as well as details of the pharmacology and immunosuppressive efficacy of these compounds will be the subject of future reports.

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Supporting Information Available: Experimental procedures and characterization data for **15**, **18**, **22**, and **24**, ligand-induced [^{35}S]-GTPγS $S1P_1$ binding for the compounds in Table 2, 24 h PBL lowering dose−response data for **18** in the rat, and data used to generate Figure 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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