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Letters

Discovery of Potent 3,5-Diphenyl-1,2,4-oxadiazole Sphingosine-1-phosphate-1 (S1P₁) Receptor Agonists with Exceptional Selectivity against S1P₂ and S1P₃

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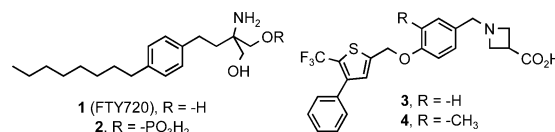
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Abstract: A class of 3,5-diphenyl-1,2,4-oxadiazole based compounds have been identified as potent sphingosine-1-phosphate-1 (S1P₁) receptor agonists with minimal affinity for the S1P₂ and S1P₃ receptor subtypes. Analogue **26** (S1P₁ IC₅₀ = 0.6 nM) has an excellent pharmacokinetics profile in the rat and dog and is efficacious in a rat skin transplant model, indicating that S1P₃ receptor agonism is not a component of immunosuppressive efficacy.

The advent of the novel immunosuppressant 2-amino-2-(4-octylphenyl)ethylpropane-1,3-diol¹ (**1**, FTY720) may represent a new modality of immunosuppression and

has inspired considerable interest in agonists and antagonists of sphingosine-1-phosphate (S1P) receptors.² S1P is a bioactive lysolipid with pleiotropic functions mediated via agonism of a family of G-protein-coupled receptors, S1P_{1–5}.³ It has been demonstrated that **1** is



metabolized across species to a monophosphate ester (**2**), which is a high-affinity ligand for S1P_{1,3–5} but not S1P₂.⁴ Preclinical studies with **1** have revealed that its physiological effects include a redistribution of lymphocytes from blood to secondary lymphoid organs and regulation of cardiovascular function, with the former being a driver of immunosuppressive efficacy.⁵ S1P₁ receptor agonism has been shown to correlate with lymphocyte recirculation,⁶ while S1P₃ receptor agonism has been linked to acute toxicity and bradycardia in rodents.⁷ A dose-dependent transient, asymptomatic bradycardia has been seen in clinical studies with **1**,⁸ suggesting that selecting against S1P₃ may be desirable with second-generation S1P receptor agonist immunosuppressants. A previous report from these laboratories disclosed a series of 1-benzyl-3-carboxyazetidide compounds (exemplified by **3** and **4**) as selective, orally bioavailable S1P receptor agonists.⁹ While **3**, **4**, and many of their analogues were found to be about 500- to 1000-fold selective for S1P₁ over S1P₃, enhanced selectivity in this class of compounds was often accompanied by modest, yet significant, losses of S1P₁ receptor affinity.

Herein, we report the discovery of a class of potency-enhanced S1P₁ receptor agonists based on a 3,5-diphenyl-1,2,4-oxadiazole scaffold that exhibit exceptional selectivity against S1P₂ and S1P₃ receptor subtypes. Their syntheses, structure–activity relationships, and the significance of their enhanced potency and selectivity as it impacts immunosuppressive efficacy and pharmacology attributable to S1P₃ receptor agonism are the subjects of this communication.

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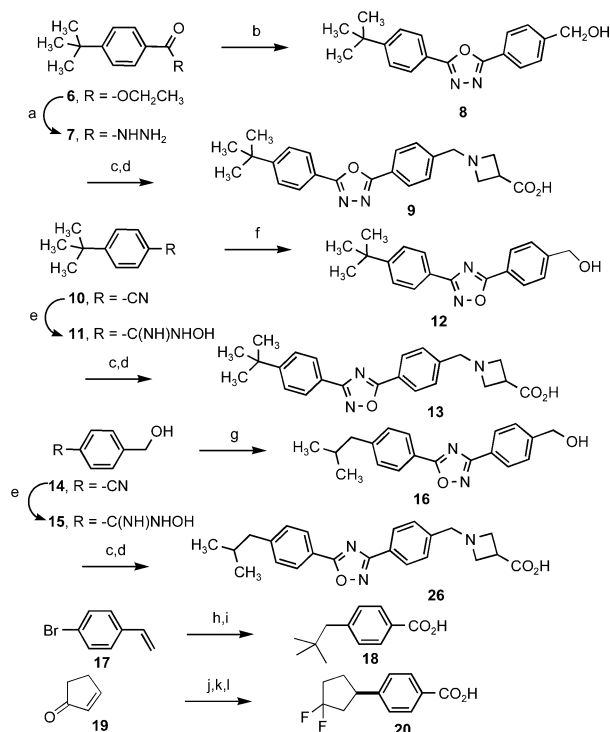
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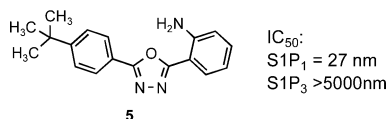
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Scheme 1^a

^a Reagents: (a) H_2NNH_2 , EtOH, reflux (99%); (b) 4-(hydroxymethyl)benzoic acid, 2-chloro-1,3-dimethylimidazolium chloride, TEA, CH_2Cl_2 (50%); (c) $(\text{COCl})_2$, DMSO, DIEA, CH_2Cl_2 , -78°C to room temp; (d) 3-carboxyazetidine, $\text{Na}(\text{CN})\text{BH}_3$, HOAc, MeOH (yields, two steps: **8**, **9**, 50%; **12**, **13**, 50%; **16**–**26**, 53%); (e) $\text{HONH}_2\cdot\text{HCl}$ NaHCO_3 , MeOH, reflux (**10**, **11**, 95%; **14**, **15**, 99%); (f) 4-(hydroxymethyl)benzoic acid, EDC, HOBT, CH_3CN , reflux (85%); (g) 4-(2-methylpropyl)benzoic acid, EDC, HOBT, CH_3CN , reflux (85%); (h) 2,2-dimethylpropylmagnesium bromide, $\text{Ni}(\text{dppe})\text{Cl}_2$, ether, reflux; (i) cat. RuO_2 , NaIO_4 , $\text{EtOAc}/\text{H}_2\text{O}$ (52%, two steps); (j) 4-(bromo)phenylboronic acid, cat. (*R*)-BINAP, cat. $\text{Rh}(\text{acac})(\text{C}_2\text{H}_4)_2$, 9:1 v/v dioxane/ H_2O (54%, 91% ee); (k) [bis(2-methoxyethyl)amino]sulfur trifluoride, $\text{BF}_3\cdot\text{Et}_2\text{O}$, toluene (65%); (l) *n*-BuLi, CO_2 , THF/ether, -78°C (75%).

After in-house leads identified in a high-throughput screen for S1P₁ agonists¹⁰ led to the rational design of **3** and **4**, approximately 1000 commercially available compounds related to those leads were purchased and tested for S1P receptor agonism activity. Compound **5**,



with a 3,5-diphenyl substituted oxadiazole moiety, was identified in this set of analogues. Employing **5** as we had our previous leads, we started the investigation of 3,5-diphenyl substituted oxadiazole based compounds, which has led to the discovery of compound **26**.

Representative syntheses are outlined in Scheme 1. The key step in obtaining **9**, **13**, and **26** was the formation of their respective appropriately substituted oxadiazole heterocycles; this was efficiently realized using chemistry based on established literature procedures.¹¹ Syntheses of 4-(cyclopropyl)-,^{12a} 4-(cyclobutyl)-,^{12a} 4-(cyclopentyl)-,^{12a} and 4-(4,4-difluorocyclohexyl)benzoic acid^{12b} have appeared in the literature. The preparation of benzoic acid **18**, which featured a nickel-catalyzed cross-coupling,¹³ is representative of the

Table 1. S1P Receptor Affinities (IC_{50} , nM)^a and Rat Pharmacokinetic Data^b

cpd	R	S1P ₁	SP ₃	S1P ₄	S1P ₅	rat PK
9	--	100	>10000	4300	1000	nd
13	--	8.2	>10000	640	20	nd
21	(CH_3) ₃ C-	3.8	12000	370	5.2	nd
22	$\text{CH}_3\text{CH}_2(\text{CH}_3)_2\text{C}-$	1.3	3086	75	1.8	nd
23	$\text{CH}_3\text{CH}_2\text{CH}_2-$	1.3	58000	150	1.6	$\text{Cl}_p = 32$ $\text{Vd}_{ss} = 1.7$ $t_{1/2} = 0.8$
24	$\text{CH}_3(\text{CH}_2)_2\text{CH}_2-$	2.9	>10000	350	2.4	nd
25	$\text{CH}_3(\text{CH}_2)_4\text{CH}_2-$	29	>10000	>10000	6.4	nd
26	(CH_3) ₂ CHCH ₂ -	0.6	12000	70	1.0	$\text{Cl}_p = 4.1$ $\text{Vd}_{ss} = 2.8$ $t_{1/2} = 8.5$
27	(CH_3) ₃ CCH ₂ -	3.8	~10000	90	6.6	nd
28	$\text{CH}_3\text{CH}_2\text{O}-$	5.1	>1000	>1000	48	nd
29	(CH_3) ₂ CHO-	1.8	>10000	370	12	$\text{Cl}_p = 4.1$ $\text{Vd}_{ss} = 2.2$ $t_{1/2} = 7.1$
30		4.5	>10000	>1000	12	nd
31		2.2	>10000	520	4.0	nd
32		0.4	14000	49	0.5	$\text{Cl}_p = 70$ $\text{Vd}_{ss} = 5.6$ $t_{1/2} = 1.2$
33		1.4	12000	40	0.8	$\text{Cl}_p = 51$ $\text{Vd}_{ss} = 4.6$ $t_{1/2} = 1.2$
34		1.7	>10000	240	11	$\text{Cl}_p = 2.9$ $\text{Vd}_{ss} = 2.8$ $t_{1/2} = 11$
35	$\text{CF}_3\text{CH}_2\text{CH}_2-$	0.4	13000	430	0.3	$\text{Cl}_p = 5.3$ $\text{Vd}_{ss} = 2.3$ $t_{1/2} = 5.8$
36		0.9	4282	68	0.5	$\text{Cl}_p = 10$ $\text{Vd}_{ss} = 3.0$ $t_{1/2} = 4.1$
37		0.8	4018	89	0.4	$\text{Cl}_p = 5.8$ $\text{Vd}_{ss} = 3.0$ $t_{1/2} = 6.6$

^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 the mean of $n = 3$ determinations. All compounds had S1P₂ $\text{IC}_{50} > 10\,000$ nM. SD were generally $\pm 20\%$ of the average. See ref 7a for assay protocol. ^b 1 mg/kg iv. Units: Cl_p , $\text{mL min}^{-1} \text{kg}^{-1}$, Vd_{ss} , L/kg, $t_{1/2}$, h. Data are reported as the average for $n = 2$ iv. Compound plasma levels for individual animals used to calculate PK parameters were with $\pm 25\%$ of average. See ref 19.

chemistry used to prepare the benzoic acids needed for targets **27** and **35**. The synthesis of benzoic acid **20** (required for target **36**) featured an asymmetric conjugate addition of 4-(bromo)phenylboronic acid to cyclopentenone;¹⁴ analogous chemistry was used to prepare the benzoic acids needed for **37**.

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.^{4a} S1P receptor agonism by the test compounds was determined by measurement of ligand-induced [^{35}S]-5'-O-3-thiotriphosphate (GTP γ S) binding.¹⁵ Some general features about the data generated for the new compounds are noteworthy (Table 1). While the S1P₁ IC_{50} values for the new compounds (**9**, **13**, **21**–**37**) spanned almost 100-fold, all of them had minimal affinity for S1P₂ and S1P₃ receptors. This absolute disconnection of S1P₁ and S1P₃ SARs is in sharp contrast to what was seen previously for other classes of S1P receptor agonists where these receptor affinities were separable but effected by changes in structure in similar ways.^{9,16} Most of the new

compounds showed a more modest 100-fold selectivity against S1P₄ while being nonselective in regard to S1P₅. Interestingly, compounds such as **26** were found to be inactive in the GTP γ S binding and ERK activation¹⁷ assays used to assess S1P₄ functional activity which indicates that the new compounds differ from compounds such as **3** and **4** in that they are weak antagonists of the S1P₄ receptor.

The S1P₁ receptor data (Table 1) for the three 4-(*tert*-butyl)phenyloxadiazole analogues **9**, **13**, and **21** indicated a preference for the heterocycle of the last isomer. There appears to be an optimal length and/or size of the substituent of analogues of **21** at which S1P₁ affinity is maximized. Extending the carbon chain on the pendent phenyl ring of analogues of **21** was found to be S1P₁ affinity-enhancing with isobutyl analogue (**26**) having subnanomolar S1P₁ IC₅₀ values, but this had limits because *n*-hexyl analogue **25** was significantly less potent. The S1P₁ data for cycloalkyl analogues **30**–**33** and phenyl analogue **34** also support the notion that an appropriately sized alkyl substituent affords maximal binding to S1P₁. Fluorination of some selected alkyl groups was found to be tolerated and in one case S1P₁-potency-enhancing (compare **35** vs **23**). As previously noted, none of these structural changes significantly altered selection against S1P₄ or S1P₅ compared to that seen for lead analogue **21**.

It was possible to differentiate among the new compounds based on their rat pharmacokinetics (PK) and their ability to drive a pharmacodynamic (PD) response characteristic of S1P₁ receptor agonists, i.e., the dose response of lowering peripheral blood lymphocyte counts.^{4a,6,18} While all of the compounds for which rat pharmacokinetics were determined¹⁹ were highly orally bioavailable (>50%), many of the more interesting simple alkyl and cycloalkyl analogues (e.g., **23**, **32**, **33**) were found to have high rates of plasma clearance in relation to relatively modest steady-state volumes of distribution. Isobutyl analogue **26** and phenyl analogue **34** were outliers in this respect with significantly longer half-lives in the rat (**26** *t*_{1/2} = 8.5 h, **34** *t*_{1/2} = 11 h). It was proposed that metabolism of the terminal alkyl groups of the higher clearance compounds might be responsible in part for their poorer pharmacokinetics; a subsequent metabolism and disposition experiment in bile duct cannulated Sprague-Dawley rats with [¹⁴C]-**26** revealed that oxidation of the isobutyl group, the phenyl ring to which it is attached, and the azetidine nitrogen were major phase I metabolic processes for that compound.²⁰ The problem of high clearance could be remedied with fluorination of the pendent alkyl groups; analogues **35**–**37** were all found to have extended half-lives compared to their *des*-fluoro counterparts. While it is not clear why isobutyl analogue **26** is a better pharmacokinetics player than many of its close analogues, it is interesting to note that isopropoxy analogue **29** (which is isosteric to **26**) also had an extended rat half-life (*t*_{1/2} = 7.1 h). Beagle dog pharmacokinetics⁹ for selected compounds (**23**, **26**, **29**, **32**, **33**) were found to parallel those in the rat, with **26** having a superior profile (Cl_p = 3.3 mL min⁻¹ kg⁻¹, Vd_{ss} = 2.5 L/kg, *t*_{1/2} = 10 h, % *F* = 66 after 1.0 mpk po and 0.5 mpk iv doses).

Since the immunosuppressive efficacy of **1** has been proposed to arise from its ability to promote the seques-

Table 2. Minimum Single Dose (mg/kg, po) Required for Maximal PBL Lowering Response 24 h Postdose

compd	mouse	rat	dog
1	1.0 ^a	0.2 ^a	0.1 ^c
3	10 ^{a,b}	3.0 ^c	1.0 ^c
26	10 ^a	0.5 ^a	0.5 ^c

^a Data generated 24 h after compound challenge in *n* = 3 animals according to protocol described in ref 4a. ^b b.i.d. ^c Data generated from PK/PD time-course studies in *n* = 3 animals as described in ref 9.

tration of peripheral blood lymphocytes (PBLs), measurement of PBL levels can be used as a surrogate marker for efficacy amenable to the screening of multiple analogues in vivo.¹⁸ The ability of these new compounds to lower PBL counts after oral administration⁹ provided another basis for analogue differentiation. While many of the compounds in Table 1 were capable of eliciting a maximal PBL lowering response 3 h after a sufficient oral dose, only **26**, **29**, **32**, **36**, and **37** were capable of doing so after a dose as low as 0.3 mpk po. While murine pharmacokinetics were not determined as part of these screening experiments, it is noteworthy that the most potent of the new compounds in this assay were also the ones with the best rat pharmacokinetics profiles. The pharmacodynamic ED₅₀ for **26** in the PBL lowering screening assay was determined to be 0.03 mpk po, making it comparable to **1** and approximately 10-fold more potent than **3** or **4**. The oral doses of **1**, **3**, and **26** required to elicit a maximum PBL lowering response 24 h postdose in the mouse, rat, and dog were also determined (Table 2); PBL counts appeared to rebound in all three species when plasma concentrations of **26** reached 25–50 nM, indicating that this compound was at least 3- to 4-fold more potent than **3** in its ability to drive the lowering of PBLs.²¹

On the basis of its S1P₁ receptor agonist potency, selectivity, pharmacokinetics/pharmacodynamic properties, and the ready availability of the intermediates, **26** was subjected to more in-depth characterizations.

The ability of **1** to prolong allograft survival in a variety of preclinical species and to synergize with agents that effect lymphocyte proliferation and IL-2 production (such as CsA and FK-506) is well-established.²² To demonstrate that an S1P₁ agonist with attenuated affinity for S1P₃ receptors could be expected to maintain those properties of **1**, a rat skin allograft experiment based on a previously published protocol²³ was conducted. Compounds **26** (5.0 mpk po/day, 28 days, *n* = 14 rats) and **1** (1 mpk po/day, 28 days, *n* = 14 rats) were independently combined with a subtherapeutic dose of CsA (1 mpk/day, delivered intraperitoneally till graft death) and tested in Lewis rats that received skin grafts from DA rats. The median graft survival times were 22 days in the **26** + CsA arm (100% of grafts were rejected by day 43) and 13 days in the **1** + CsA arm (100% of grafts were rejected by day 34). Rats treated with vehicle or CsA alone had 100% graft death between days 10 and 12, respectively. Exposure to CsA was comparable in all of the cohorts to which it was administered. These results indicate that S1P receptor agonists that select against the S1P₃ subtype should be expected to be efficacious as immunosuppressive agents. The consequences for efficacy of selecting against the S1P₄ and/or S1P₅ receptor subtypes remain to be established.

Cardiovascular evaluations of the new compounds in the previously described^{7a} conscious rat model were hindered by our inability to administer compounds at doses sufficient to elicit responses attributable S1P₃ agonism.²⁴ Fortunately, a determination of compound effects on airways resistance in the rat appears to provide an attractive means for demonstrating the undesirability of S1P₃ agonism. In addition to modulating lymphocyte trafficking, it was recently demonstrated that S1P can stimulate the contraction of human airway smooth muscle in vitro.²⁵ While this finding implies that S1P has a role in airway tone, it was uncertain whether S1P receptor activation would augment airway resistance in a whole animal model. To assess whether S1P receptors can alter airway resistance, the respiratory effects of **1**, **3**, and **26** were evaluated in anesthetized rats. In this study, airway resistance was measured directly using a computer-controlled small animal ventilator to measure drug-induced changes in respiratory mechanics.²⁶ In this model, intravenous infusion of the muscarinic agonist methacholine ($30 \mu\text{g kg}^{-1} \text{ min}^{-1} \times 30 \text{ min}$), a known bronchoconstrictor, caused a significant increase in airway resistance ($152 \pm 6\%$) in anesthetized rats. Administration of **1** and **3** at 10 mpk iv infused over 30 min induced bronchoconstriction and elevated airway resistance by $344 \pm 33\%$ and $140 \pm 5\%$ compared to baseline. Infusion of **26** was devoid of any effect on airway resistance and was similar to vehicle treatment ($104 \pm 1\%$). These findings suggest that agonists with high to moderate affinity for S1P₃ receptors, e.g. **1** and **3**, can produce bronchoconstriction in the rat and suggest that airways smooth muscle contraction can be mediated by S1P₃ receptors. This is supported by the observation that a highly selective S1P₁ agonist with minimal affinity for S1P₃ receptors, e.g. **26**, lacked the ability to stimulate airway smooth muscle contraction in vivo.

In conclusion, a series of potent S1P₁ receptor agonists exemplified by **26** with high selectivity against the S1P₂ and S1P₃ receptor subtypes have been discovered. While it has been previously shown that lymphocyte trafficking is not influenced by S1P₃ agonism, the ability of **26** to prolong allograft survival in a rat skin transplant model serves as an indication that S1P₃ agonism is not required for immunosuppressive efficacy. Moreover, our initial findings regarding the relative respiratory effects of **1**, **3**, and **26** further support the notion that agonism of S1P₃ may be associated with undesirable pharmacologies.⁷ Details of the further evaluation of **26** and our investigations of other S1P receptor agonists as a novel immunosuppressive agents will be reported in due course.

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Supporting Information Available: Experimental details and characterization data for final in vivo tested compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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