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Discovery of AMG 369, a Thiazolo[5,4-*b*]pyridine Agonist of $S1P_1$ and $S1P_5$

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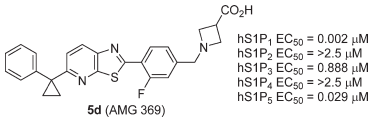
ABSTRACT The optimization of a series of thiazolopyridine $S1P_1$ agonists with limited activity at the $S1P_3$ receptor is reported. These efforts resulted in the discovery of 1-(3-fluoro-4-(5-(1-phenylcyclopropyl)thiazolo-[5,4-*b*]pyridin-2-yl)benzyl)-azetidine-3-carboxylic acid (**5d**, AMG 369), a potent dual $S1P_1$/$S1P_5$ agonist with limited activity at $S1P_3$ and no activity at $S1P_2$/$S1P_4$. Dosed orally at 0.1 mg/kg, **5d** is shown to reduce blood lymphocyte counts 24 h postdose and delay the onset and reduce the severity of experimental autoimmune encephalomyelitis in rat.

KEYWORDS Sphingosine-1-phosphate receptor, $S1P_1$, lymphocyte, inflammation, multiple sclerosis



The lysophospholipid sphingosine-1-phosphate (**1**, S1P) has emerged as a versatile signaling molecule,[1,2] capable of impacting a wide range of cellular functions, including proliferation and apoptosis, differentiation, and migration. The diverse activities of S1P are believed to be mediated through its interaction with the five S1P GPCRs ($S1P_{1-5}$) encoded by the EDG (endothelial differentiation gene) family as well as possible additional intracellular targets.[1−6] The discovery that the potent immunomodulator FTY720 (fingolimod, **2**) is phosphorylated in vivo to the monophosphate ester FTY720P,[7,8] an agonist of all S1P GPCRs except $S1P_2$, sparked intense research into the functions of the individual S1P receptors (see Figure 1 for structures). From these studies, a consensus emerged that the immunomodulatory effects of FTY720 are primarily due to agonism and subsequent downregulation of the $S1P_1$ receptor by FTY720P, which interrupts the normal pattern of lymphocyte migration and leads to a state of peripheral lymphocyte depletion.[3−8] It was also established that, in rodents, agonism of the $S1P_3$ receptor by FTY720P causes a reduction in heart rate[9,10] similar to that observed in human subjects.[11] These findings, combined with the impressive efficacy of FTY720 in clinical trials for the treatment of transplant rejection[12] and multiple sclerosis,[13,14] led to a wide-ranging search for $S1P_1$ agonists with limited activity at the $S1P_3$ receptor.[15,16] Herein we report the discovery of a dual $S1P_1$/$S1P_5$ thiazolo[5,4-*b*]pyridine agonist (**5d**) and describe its in vivo pharmacological and pharmacokinetic characteristics.

Previously, our research team produced a series of potent and selective benzothiazole $S1P_1$ agonists.[17] Compound **3a**

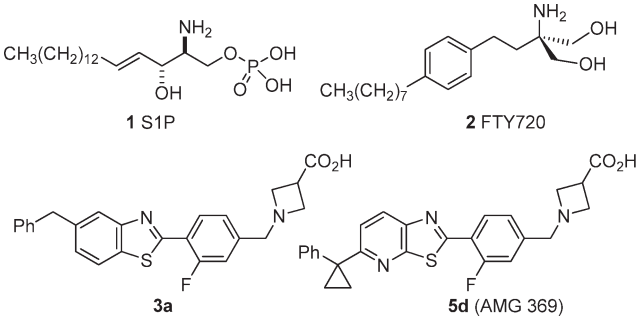


Figure 1. S1P, FTY720, benzothiazole **3a**, and optimized thiazolo-[5,4-*b*]pyridine **5d** (AMG 369).

was representative of this series and exhibited double-digit nanomolar potency in an assay measuring receptor internalization (RI) of an $hS1P_1$-GFP fusion protein in U2OS cells, and it exhibited limited activity at $hS1P_3$ as determined by Ca^{2+} mobilization in $hS1P_3$- and $G_{q/i5}$-transfected CHO-K1 cells (Table 1).[18] Compound **3a** also demonstrated on-mechanism activity in rat, with a single oral dose of 1 mg/kg producing a significant reduction of circulating blood lymphocytes 24 h postdose. However, **3a** is characterized by both relatively high lipophilicity (CLogP = 3.9) and low topological polar surface area (tPSA = 53 Å^2),[19] and therefore, it

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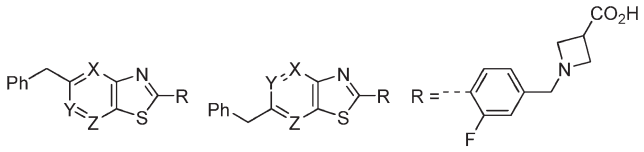
Table 1. SAR of Thiazolopyridine Analogues[a]



compd	X	Y	Z	hS1P$_1$ RI EC$_{50}$, μM (% efficacy)	hS1P$_3$ Ca^{2+} EC$_{50}$, μM (% efficacy)
3a	CH	CH	CH	0.041 (102)	1.21 (24)
3b	N	CH	CH	0.144 (106)	10.1 (63)
3c	CH	N	CH	0.429 (115)	> 25
3d	CH	CH	N	0.138 (125)	2.89 (36)
4a	CH	CH	CH	0.221 (84)	3.47 (50)
4b	N	CH	CH	1.60 (75)	> 25
4c	CH	N	CH	3.98 (77)	5.46 (64)
4d	CH	CH	N	0.199 (92)	0.478 (94)

[a] Data represents an average of at least two determinations. % efficacy is reported relative to 1.00 μM 1-(4-(6-benzylbenzofuran-2-yl)-3-fluorobenzyl)azetidine-3-carboxylic acid and 0.200 μM S1P for hS1P$_1$ RI and hS1P$_3$ Ca^{2+} assays, respectively; > [highest concentration tested] is reported for compounds that do not achieve > 10% of control activity. For statistical analysis, see the Supporting Information.

resides in a region of physicochemical space with a higher probability of off-target toxicity (CLogP > 3, tPSA < 75 Å^2).[20] Therefore, modifications of **3a** were undertaken with the goal of identifying S1P$_1$ agonists with lower CLogP and higher tPSA. Single nitrogen for CH substitutions were identified as a means of lowering CLogP (−1.1 units) and raising tPSA (+12 Å^2). The three possible aza-analogues of **3a** (**3b**−**3d**) arising from nitrogen substitution at each benzothiazole C−H position were investigated (Table 1) and found to exhibit reduced potency (3−10-fold loss vs **3a**) in the S1P$_1$ receptor internalization assay. Aza-derivatives (**4b**−**4d**) of the isomeric 6-benzyl-benzothiazole **4a** were also investigated, and in one case, the substitution of nitrogen for CH resulted in no loss in potency (**4d**, S1P$_1$ RI EC$_{50}$ = 0.199 μM) relative to the parent (**4a**, hS1P$_1$ RI EC$_{50}$ = 0.221 μM). Of the six aza-analogues, **3b**, **3d**, and **4d** exhibited reasonable S1P$_1$ potency (EC$_{50}$ = 0.138−0.199 μM) and were selected for further optimization.

Given the high lipophilicity of the endogenous S1P$_1$ ligand, S1P (**1**, CLogP = 5.5), we hypothesized that the reduced potency of the aza-analogues could be remedied by addition of small lipophilic groups, with the goal of not exceeding the lipophilicity of **3a** (CLogP = 3.9). After extensive exploration of more than seventy analogues of **3b**, **3d**, and **4d** bearing small lipophilic groups, it was found that the α-methylbenzyl analogues of **4d**, the thiazolo[5,4-*b*]pyridines **5a**−**b** (Table 2), showed a 5-fold improvement in S1P$_1$ potency and a 2-fold loss of S1P$_3$ activity relative to the parent **4d**. Geminal dimethyl substitution (**5c**) resulted in comparable S1P$_1$ potency and reduced S1P$_3$ activity. Encouraged by these results, benzylic 1,1-disubstituted carbocycles were investigated (**5d**−**g**). Of this series, cyclopropane-containing **5d** was found to uniquely exhibit single-digit nanomolar potency at the S1P$_1$ receptor (EC$_{50}$ = 0.002 μM), with much weaker activity at the S1P$_3$ receptor (EC$_{50}$ = 0.888 μM). The calculated physicochemical properties of **5d** (ClogP = 3.2, tPSA = 65 Å^2) were also favorable relative to **3a** (ClogP = 3.9, tPSA = 53 Å^2). Further testing of **5d** in a Ca^{2+} mobilization format revealed potent agonism of the hS1P$_5$ receptor (EC$_{50}$ = 0.029 μM, 50% efficacy) and a lack of activity at hS1P$_2$ and hS1P$_4$ receptors at concentrations up to 2.5 μM. The S1P$_5$ activity of **5d** was not considered a liability, as it has been suggested that agonism of oligodendrocyte S1P$_5$ receptors by FTY720P may contribute to the efficacy of FTY720 (**2**) in the treatment of multiple sclerosis.[21]

The greater than 10-fold difference in S1P$_1$ potency between **5d** and both the *gem*-dimethyl analogue **5c** and *des*-aza cyclopropane analogue **6** (Table 2) suggests that the presence of both cyclopropane and pyridine endow **5d** with either a unique conformation or unique interactions with the S1P$_1$ receptor, or both. To further understand the conformational preferences of **5c**, **5d**, and **6**, quantum mechanical calculations[22] were performed on fragments corresponding to **5c**, **5d**, and **6** (Figure 2, A, B, and C, respectively) to characterize the rotational energy profile about the indicated NCCC or CCCC bonds. The profiles for the *gem*-dimethyl fragment **A** and cyclopropyl fragment **B** show striking differences. Fragment **A** exhibits a fairly shallow profile and global minima at 120 and 240° angles, whereas **B** exhibits a steep profile greatly favoring a 180° dihedral angle. The *des*-aza fragment **C** does not share the profile of **B**; rather, a slight preference for a 0° dihedral angle is observed. It was further established that, like **B**, the simple fragment 2-(1-phenylcyclopropyl)pyridine also shows a strong preference for a 180° NCCC dihedral angle (data not shown, see Supporting Information). The unique conformational profile of 2-(1-phenylcyclopropyl)pyridines has, to the best of our knowledge, not been previously described. It may be that the **5d**-S1P$_1$ complex that is conducive to efficient receptor internalization contains **5d** with a 180° NCCC dihedral angle and, therefore, benefits entropically from ligand preorganization.

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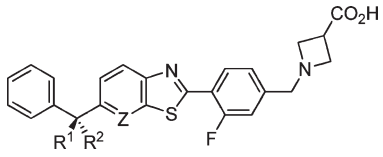
Table 2. Optimization of Thiazolo[5,4-*b*]pyridine Agonists[a]



compd	Z	R^1	R^2	hS1P$_1$ RI EC_{50}, μM (% efficacy)	hS1P$_3$ Ca^{2+} EC_{50}, μM (% efficacy)
4d	N	H	H	0.199 (92)	0.478 (94)
5a	N	Me	H	0.035 (99)	0.877 (44)
5b	N	H	Me	0.038 (87)	0.996 (45)
5c	N	Me	Me	0.033 (103)	2.51 (64)
5d	N	-$(CH_2)_2$-		0.002 (98)	0.888 (26)
5e	N	-$(CH_2)_3$-		0.119 (124)	1.31 (68)
5f	N	-$(CH_2)_4$-		0.026 (76)	12% @ 2.5 μM
5g	N	-$(CH_2)_5$-		0.033 (108)	0.836 (41)
6	CH	-$(CH_2)_2$-		0.038 (93)	4.67 (56)

[a] Data represents an average of at least two determinations. % efficacy is reported as in Table 1. For statistical analysis, see the Supporting Information

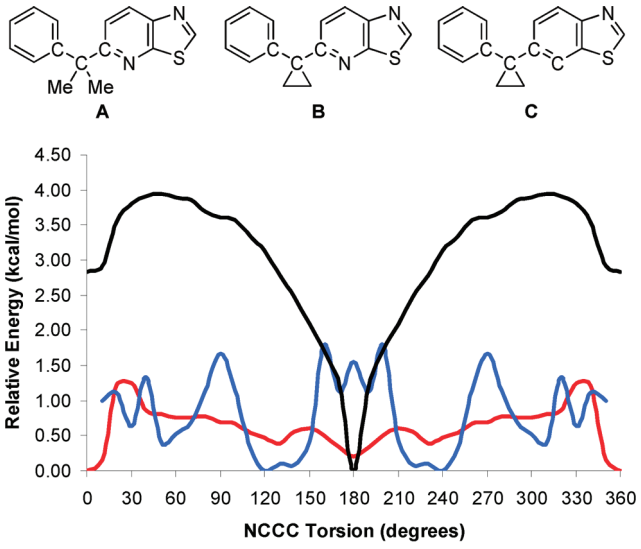


Figure 2. Rotational energy profile of fragments **A** (blue), **B** (black), and **C** (red) corresponding to **5c**, **5d**, and **6**, respectively (B3LYP/6-31G* with Polarizable Continuum Model to simulate aqueous media). Minimization of fragment **A** at a 0° NCCC dihedral was not possible.

The remarkable in vitro potency of **5d** translated to in vivo activity at doses that were more than an order of magnitude lower than had previously been achieved in our program (Figure 3a). Dosed orally at 0.01, 0.03, and 0.1 mg/kg to Lewis rats, **5d** produced a dose-dependent reduction in circulating blood lymphocyte counts consistent with S1P$_1$ agonism[2] 24 h postdose.[23] Statistical significance ($P < 0.05$) was achieved at the 0.03 mg/kg (45% reduction in lymphocytes vs vehicle) and 0.1 mg/kg doses (65% reduction in lymphocytes vs vehicle). Since S1P$_1$ agonists typically produce a maximal 70% reduction in lymphocyte counts,[24] the 0.1 mg/kg dose of **5d** in the rat provides a nearly maximal

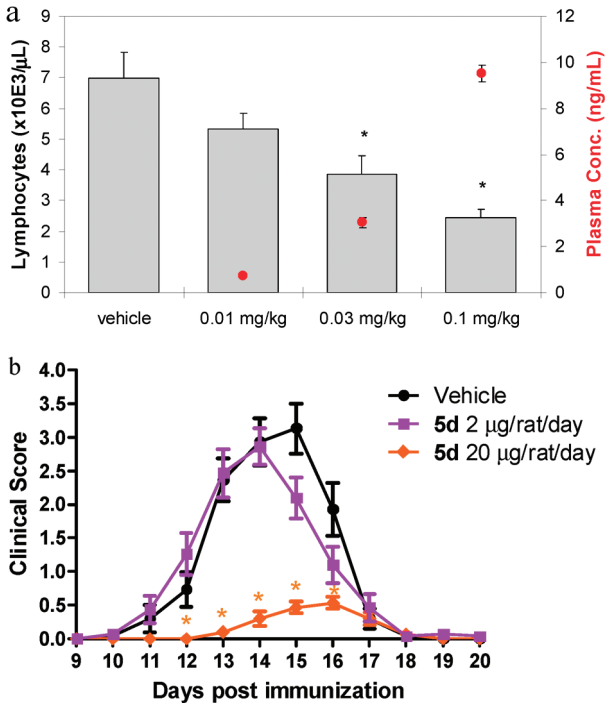


Figure 3. In vivo characterization of **5d**. (a) **5d** dosed orally reduces blood lymphocyte counts in female Lewis rats 24 h postdose (N = 5/group; bars represent average blood lymphocyte counts + SE; circles represent average plasma concentration ± SE; *$P < 0.05$ vs vehicle by ANOVA/Dunnett's Multiple Comparison Test). (b) Efficacy of **5d** dosed orally at 2 and 20 μg/rat/day (approximately 0.01 and 0.1 mg/kg, respectively) in delaying onset and reducing disease score in an experimental autoimmune encephalomyelitis (EAE) model in female Lewis rats (N = 15/group; *$P < 0.05$ vs vehicle by Wilcoxon exact 2-sided test). Vehicle = 20% captisol, pH 2.

pharmacologic effect.[25] Compound **5d** was further profiled in a rat experimental autoimmune encephalomyelitis (EAE)

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Table 3. Pharmacokinetic Parameters for **5d**

species	Cl (L/(h kg))	V_{ss} (L/kg)	$T_{1/2}$ (h)	MRT (h)	% F
rat[a]	0.18	1.8	5.9	10	64
canine[b]	0.071	1.2	38	17	41
NHP[c]	0.50	2.3	24	4.7	34

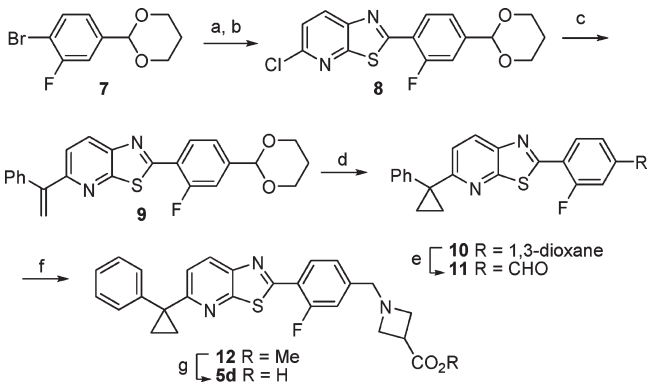
[a] Male Sprague−Dawley (iv: 1 mg/kg, DMSO, N = 3; po: 2 mg/kg, 20% captisol pH 4.0, N = 2). [b] Male beagle (iv: 1 mg/kg, 20% captisol pH 4.0, N = 3; po: 1 mg/kg, 20% captisol/1% pluronic F68/1% HPMC pH 2.1, N = 3). [c] NHP = nonhuman primate, male cynomolgus (iv: 1 mg/kg, 20% captisol pH 4.0, N = 3; po: 1 mg/kg, 20% captisol/1% pluronic F68/1% HPMC pH 2.1, N = 3).

model which has characteristics of the human disease multiple sclerosis (Figure 3b).[26] In this EAE model, Lewis rats are immunized with guinea pig myelin basic protein and scored daily on a 0−6 scale reflecting the degree of disability observed (0 = no sign of disability), with the investigator assessing the clinical score blinded to the study group of the individual rats. The onset of disease typically occurs 10 days postimmunization, with complete resolution of disease at 20 days postimmunization. **5d** dosed orally once daily at 20 μg per day (approximately 0.1 mg/kg) at the time of immunization resulted in delayed onset and significant reduction of disease score (Figure 3). A 10-fold lower dose of **5d** (2 μg, approximately 0.01 mg/kg) did not significantly impact disease score in this model.

Additional profiling established that **5d** possesses acceptable characteristics for further development. In rat, nonhuman primate, and dog (Table 3), the compound had low clearance (4−21% of liver blood flow), a moderate steady state volume of distribution of total drug (V_{ss} = 1.2−2.3 L/kg), a moderate to long mean residence time (5−17 h), and acceptable oral bioavailability (34−64%). Cardiovascular safety studies in telemetered rats established a no-effect level for heart rate and mean arterial pressure changes of 10 mg/kg (po), indicating a wide margin for $S1P_3$-associated cardiovascular toxicity. In vitro studies established that **5d** was not an inhibitor or inducer of human cytochrome P450 enzymes, was nonmutagenic (Ames and micronucleus negative), and did not significantly inhibit the hERG channel in an electrophysiology assay at the highest concentration tested (2.3 μM).[27]

The synthesis of **5d** is detailed in Scheme 1. Aryl bromide **7**, synthesized in one step from the readily available 4-bromo-3-fluorobenzaldehyde, was treated with *n*-BuLi to effect lithium-halogen exchange, and the resulting anion was allowed to react with 2,6-dichloro-3-isothiocyanatopyridine to give an intermediate thioamide. Treatment of the thioamide with sodium carbonate in DMF at elevated temperature effected cyclization to the thiazolo[5,4-*b*]pyridine **8**. A Suzuki reaction between **8** and 1-phenylvinylboronic acid catalyzed by $PdCl_2\{P^tBu_2\text{-}p\text{-}NMe_2\text{-}Ph\}_2$[28] provided **9**. The cyclopropane was installed with dimethyloxosulfonium methylide under Corey−Chaykovsky conditions[29] to give **10**, and deprotection of the acetal revealed aldehyde **11**. Reductive amination of **11** with methyl azetidine-3-carboxylate hydrochloride provided the penultimate intermediate **12**. Saponification of **12** afforded **5d**, which was isolated in high yield as its zwitterion.

Scheme 1. Synthesis of **5d**[a]



[a] (a) *n*-BuLi, 2,6-dichloro-3-isothiocyanatopyridine, THF, −78 °C; (b) Na_2CO_3, DMF, 90 °C, 83% (two steps); (c) bis(4-(di-*tert*-butylphosphino)-*N*,*N*-dimethylbenzenamine) palladium dichloride (2.5 mol %), 1-phenylvinylboronic acid, K_2CO_3, dioxane/water, 80 °C, 74%; (d) Me_3SOI, *t*BuOK, DMSO/THF, 75%; (e) 5 N HCl, THF, 65 °C, 93%; (f) methyl azetidine-3-carboxylate hydrochloride, AcOH, DIPEA, $NaBH_3CN$, MeOH/CH_2Cl_2, 75%; (g) NaOH/THF, then HCl and pH 6 sodium phosphate buffer, 86%.

In conclusion, a thiazolopyridine series of $S1P_1$ agonists was developed with the goal of reducing the lipophilicity and increasing the topological polar surface area of an existing benzothiazole series. The moderate potency of the thiazolo[5,4-*b*]pyridine series was improved by addition of small lipophilic groups at the benzylic position. The cyclopropyl-containing derivative **5d** had improved calculated physicochemical properties, exhibited single-digit nanomolar $S1P_1$ potency with selectivity against $S1P_2$, $S1P_3$, and $S1P_4$, and was capable of reducing blood lymphocyte counts and the severity of experimental autoimmune encephalomyelitis in rat at a dose of 0.1 mg/kg. Molecular modeling experiments revealed an unanticipated preference of the 5-(1-phenylcyclopropyl)thiazolo[5,4-*b*]pyridine fragment for a rotamer with an NCCC dihedral angle of 180°, which differentiates **5d** from related less potent analogues. Additional in vitro and in vivo experiments established that **5d** possesses acceptable properties for further development.

SUPPORTING INFORMATION AVAILABLE

Statistics for in vitro data presented in Tables 1 and 2. Tabulated data for quantum mechanical calculations for fragments **A**−**C** and 2-(1-phenylcyclopropyl)pyridine, as well as a complete ref 22. Experimental procedures and characterization data for **3a**−**d**, **4a**−**d**, **5a**−**g**, and **6**. 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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