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Discovery, design and synthesis of a selective $S1P_3$ receptor allosteric agonist

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ABSTRACT

Potent and selective $S1P_3$ receptor ($S1P_3$-R) agonists may represent important proof-of-principle tools used to clarify the receptor biological function and assess the therapeutic potential of the $S1P_3$-R in cardiovascular, inflammatory and pulmonary diseases. *N*,*N*-Dicyclohexyl-5-propylisoxazole-3-carboxamide was identified by a high-throughput screening of MLSMR library as a promising $S1P_3$-R agonist. Rational chemical modifications of the hit allowed the identification of *N*,*N*-dicyclohexyl-5-cyclopropylisoxazole-3-carboxamide, a $S1P_3$-R agonist endowed with submicromolar activity and exquisite selectivity over the remaining $S1P_{1,2,4,5}$-R family members. A combination of ligand competition, site-directed mutagenesis and molecular modeling studies showed that the *N*,*N*-dicyclohexyl-5-cyclopropylisoxazole-3-carboxamide is an allosteric agonist and binds to the $S1P_3$-R in a manner that does not disrupt the $S1P_3$-R–S1P binding. The lead molecule herein disclosed constitutes a valuable pharmacological tool to explore the molecular basis of the receptor function, and provides the bases for further rational design of more potent and drug-like $S1P_3$-R allosteric agonists.

Sphingosine-1-phosphate (S1P) is a naturally occurring lysophospholipid produced in most cell types, regulates fundamental biological processes and functions including cell proliferation, cell growth, angiogenesis, and inhibits apoptosis and lymphocyte trafficking.[1] The generation of S1P is mediated by two cytosolic sphingosine kinase isoforms (SPK1 and SPK2) and occurs preferentially in the plasma membrane.[2] S1P has two distinct signaling roles, as an intracellular second messenger and as an extracellular ligand for a specific family of G-protein coupled receptors (GPCRs) named $S1P_{1-5}$-Rs.[3] S1P can also affect cell function by either binding or modifying putative intracellular targets or by affecting the relative levels of other lipid products, particularly sphingomyelin and ceramide whose biological effects oppose those of S1P.[4,2a]

Increasing evidence supports that different subfamilies of ATP-binding cassette (ABC) transporters contribute to, or are involved in, secreting the S1P lipids across the plasma membranes.[5] The differential temporal and spatial pattern and intracellular signaling pathways of each S1P-R enable S1P to exert its diverse biological functions. $S1P_{1-3}$-Rs are widely expressed in almost all organs in mice and humans whereas $S1P_4$-R and $S1P_5$-R expression is restricted to specific organs and cell types.[6] In humans $S1P_3$-R is highly expressed in heart, lungs, spleen, kidney and pancreas. $S1P_3$-R couples with $G_{i/o}$, G_q, and $G_{12/13}$. Interestingly, $S1P_3$-R deficient mice showed no obvious phenotype.[7] However, clear association of $S1P_3$-R has been demonstrated in several cardiovascular functions including regulation of heart rate and blood pressure in rodents,[8] vasorelaxation[9] and cardiac fibrosis.[10] Additionally, regulation of myocardial perfusion and cardiomyocytes protection in ischemia perfusion injury was dependent on the $S1P_3$-R.[11] Furthermore, studies with $S1P_3$-R-null mice and PAR1-deficient dendritic cells (DC) have shown that $S1P_3$-R acts as a downstream component in PAR_1-mediated septic lethality.[12] $S1P_3$-R is also shown to regulate the endothelial cells in splenic marginal sinus organization[13] and the stimulation of endothelial progenitor cells.[14] $S1P_3$-R has also been implicated in mediating and amplifying inflammatory responses in various CNS disorders in autocrine and paracrine fashion.[15] Recently, it has been shown that $S1P_3$-R

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is markedly up-regulated in a subset of adenocarcinoma cells, and knock down of this receptor subtype inhibits proliferation and growth of lung adenocarcinoma cells.[16]

A wealth of S1P-Rs agonists has been described in the literature. However, the development of subtype selective $S1P_3$-R agonists as useful pharmacological tools has been limited as a consequence of targeting the orthosteric binding site for receptor activation. The orthosteric binding site displays a high level sequence homology among the S1P-R family subtypes. $S1P_1$-R and $S1P_3$-R are the most closely related by sequence, particularly at their agonist binding pockets which consist of a lower hydrophobic and an upper polar region where Leu-276 in $S1P_1$-R and Phe-263 in $S1P_3$-R are the main difference. Receptor structure modeling and ligand docking studies revealed that the $S1P_3$-R binding pocket is contracted between the lower lipophilic area and the upper polar section by 1.5–1.8 Å compared to the $S1P_1$-R due to the presence of Phe-263. These differences in steric and space constrains in the $S1P_3$-R orthosteric binding site may explain the difficulty in designing $S1P_3$-R agonists devoid of $S1P_1$-R agonist activity.[17]

Topologically distinct from the conserved orthosteric binding site, an allosteric site provides a means to overcome important selectivity issues associated with the orthosteric ligands, in particular within GPCRs in which the orthosteric site is highly conserved between subtypes. In addition to offering a potential subtype-selectivity, allosteric ligands may stabilize different conformations and functional states, thus activating a distinct repertoire of receptor signaling and regulatory properties that orthosteric ligands are unable to initiate.[18] The S1P binding site is highly conserved among the S1P-R family thus selective allosteric ligands may be useful pharmacological tools to decipher individual receptor biological functions.

A high-throughput screening (HTS) of the Molecular Libraries-Small Molecule Repository (MLSMR) library identified the *N*,*N*-dicyclohexyl-5-propylisoxazole-3-carboxamide **1a** (Fig. 1) as a $S1P_3$-R agonist with acceptable in vitro potency/selectivity profile.[17,19] The structural integrity of the hit was corroborated by the re-synthesis (Scheme 1) of the title compound that showed confirmed EC_{50}'s of 434 nM at $S1P_3$-R, 7.87 μM at $S1P_1$-R and no agonist activity at $S1P_{2,4}$-Rs at concentrations up to 50 μM (Fig. 1).

Our SAR studies commenced varying the amide region C. The synthesis and biological results of **1b–d** are outlined in Scheme 1. The carboxylic acid **2** was transformed into the corresponding acid chloride and coupled with amines **3b–d** to furnish the amide products **1b** and **1d**. Surprisingly, exchanging a cyclohexyl from **1a** for a cyclopentyl group (**1b**) led to loss in potency of 18-fold for the $S1P_3$-R but only of three-fold for the $S1P_1$-R. Furthermore, changing a cyclohexyl for a phenyl (**1d**) or hydrogen (**1c**) led to complete loss of potency at both receptors. These results underscore the key role played by the *N*,*N*-dicyclohexyl amide for binding to the $S1P_3$-R.

Next we explored region A of the HTS-hit while keeping regions B and C constant. Compounds **5a–i** (Table 1) were synthesized from a series of isoxazole carboxylic acids **4a–i** commercially available (**4a–c** and **4f–i**) or readily obtained (**4d** and **4e**) according to literature procedures (Scheme 2).[20]

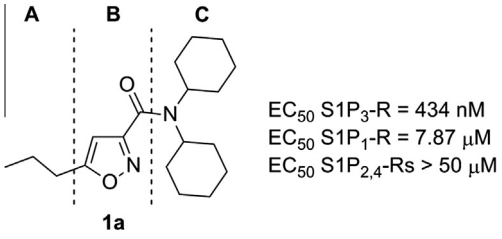
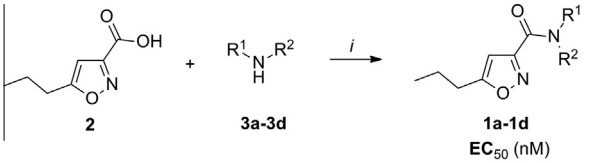


Figure 1. HTS $S1P_3$-R agonist hit **1a**.



EC_{50} (nM)

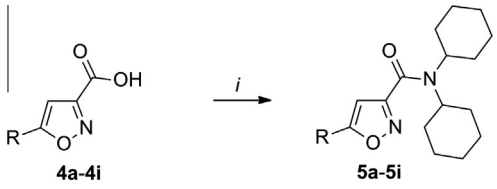
	$S1P_3$-R	$S1P_1$-R
1a, **3a** R^1 = cyclohexyl, R^2 = cyclohexyl	434	7870
1b, **3b** R^1 = cyclopentyl, R^2 = cyclohexyl	7985	23100
1c, **3c** R^1 = H, R^2 = cyclohexyl	> 50000	> 50000
1d, **3d** R^1 = phenyl, R^2 = cyclohexyl	> 50000	> 50000

Scheme 1. Synthesis of **1a–d**. Reagents and conditions: (i) (a) **2** (1 equiv), $SOCl_2$, benzene, reflux, 3 h; (b) **3a–d** (1.5 equiv), DIPEA (1.5 equiv), CH_2Cl_2, 0 °C–rt, 3 h, 70–98% (over two steps).

Table 1
$S1P_3$-R agonist activity of compounds **5a–i**

Compd	Carboxylic acid	R	EC_{50}[α] (nM)	
			$S1P_3$-R	$S1P_1$-R
5a	**4a**	Isobutyl	1375	6420
5b	**4b**	Isopropyl	959	22,250
5c	**4c**	Methyl	6595	>50,000
5d	**4d**	Cyclohexyl	559	>50,000
5e	**4e**	Cyclopentyl	339	>50,000
5f	**4f**	Cyclopropyl	105	33,300
5g	**4g**	Phenyl	103	662
5h	**4h**	4-Methoxyphenyl	323	859
5i	**4i**	3,4-Diethoxyphenyl	8070	1104

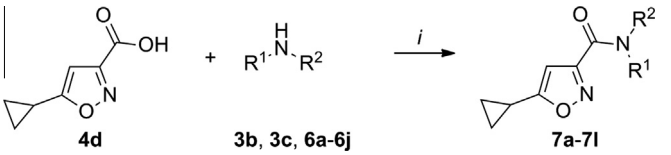
[α] Data are reported as mean of $n = 3$ determinations.



Scheme 2. Synthesis of **5a–i**. Reagents and conditions: (i) (a) **4a–h** (1 equiv), $SOCl_2$, benzene, reflux, 3 h; (b) **3a** (1.5 equiv), DIPEA (1.5 equiv), CH_2Cl_2, 0 °C–rt, 3 h, 85–98% (over two steps).

Interestingly, the isobutyl **5a** was approximately three-fold less potent than the hit at the $S1P_3$-R and four-fold less selective against the $S1P_1$-R. The isopropyl derivative **5b** was approximately two-fold less active than **1a** for the $S1P_3$-R, but the selectivity against the $S1P_1$-R remained similar. The methyl derivative **5c** was 15-fold less potent than **1a**. Remarkably, the cyclohexyl (**5d**) and cyclopentyl (**5e**) analogs were slightly less and more active at the $S1P_3$-R and inactive at the $S1P_1$-R. Of note, the cyclopropyl derivative **5f** (CYM5541) was found four-fold more potent than the hit and nearly 18-fold more selective against the $S1P_1$-R. The phenyl derivative **5g** (CYM5544) was found equipotent to **5f** for the $S1P_3$-R but significantly less selective against the $S1P_1$-R. Interestingly, the 4-methoxyphenyl **5h** was slightly more potent than the hit compound at the $S1P_3$-R, but its selectivity against the $S1P_1$-R was less than three-fold. Intriguingly, 3,4-diethoxyphenyl **5i** was 18-fold less potent than the hit at the $S1P_3$-R but more active (~7-fold) at the $S1P_1$-R. All this information together indicates that an aromatic ring at position five of the isoxazole hit (portion A), although favorable for the $S1P_3$-R activity, is also detrimental for the selectivity against the $S1P_1$-R.

Based on the obtained results, we focused our attention on the SAR studies of **5f**, particularly on the amide region C. The synthesis of **7a–l** is depicted in Scheme 3. The biological results are listed in Table 2.



Scheme 3. Synthesis of **7a–l**. Reagents and conditions: (i) (a) **4d** (1 equiv), $SOCl_2$, benzene, reflux, 3 h; (b) **3a**, **3c**, **6a–j** (1.5 equiv), DIPEA (1.5 equiv), CH_2Cl_2, 0 °C–rt, 3 h, 65–98% (over two steps).

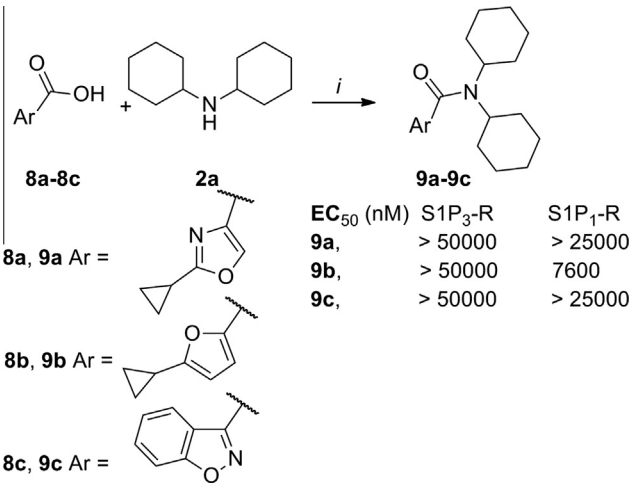
As previously observed, removal of a cyclohexyl group (**7a**) led to complete loss of activity. Similarly, exchanging a cyclohexyl for a cyclopentyl (**7b**), 4-tetrahydropyranyl (**7c**) or cyclohexylmethyl (**7d**) led to 16-, 33- and 32-fold loss of potency, respectively, confirming the importance of the bicyclohexyl amide system. Interestingly, the 2-methylcyclohexyl derivative **7e** (CYM5558) was three-fold less potent and selective than **5f**, probably due to conformational changes caused by the methyl group. Unexpectedly, when both cyclohexyls were substituted with a methyl group (**7f**, CYM5556) the potency decreased by only two-fold compared to **5f** and the selectivity remained nearly similar. The *N*-(2-methylcyclohexyl)-*N*-cycloheptyl derivative **7g** was almost two-fold less potent than **7e** while the 4-tetrahydropyranyl **7h** was slightly more potent than the cyclohexyl derivative **7c**, although the selectivity of both compounds decreased considerably. Surprisingly, the cyclopentyl **7i** was nearly equipotent to **7b**. Furthermore, the *N*-cyclopentyl-*N*-cycloheptyl derivative **7j** was 26-fold less potent than **5f**. Not surprisingly, the installation of an aromatic substituent (e.g., **7k** and **7l**) led to drastic loss in potency.

Next, we explored the middle region B holding constant the bicyclohexyl amide region C as well as the cyclopropyl and phenyl groups from region A. The synthesis and biological activity of representative compounds **9a–c** is depicted in Scheme 4. Interestingly the oxazole **9a** and the furan **9b** were inactive at the $S1P_3$-R; interestingly, **9b** showed micromolar activity for the $S1P_1$-R. Fusing the phenyl ring of **5g** into the isoxazole moiety led to **9c**, which was inactive at the $S1P_3$-R. This modification highlights the isoxazole ring as an important binding motif of this chemotype.

The functional activity of selected compounds was tested against the $S1P_{2,4}$-Rs subtypes (Table 3).[17] Remarkably, all the compounds were highly selective against the $S1P_{2,4}$-Rs. Due to its selectivity profile, **5f** was also tested against the $S1P_5$-R. Remarkably **5f** was found exquisitely selective against $S1P_{1,2,4,5}$-R subtypes.

The selectivity profile of **5f** was further investigated against the Ricerca panel of off target proteins including GPCRs, enzymes and ion channels at a concentration of 30 μM. The compound was found to be highly selective against the pool of therapeutically relevant targets tested (stimulation/inhibition <50% at 30 μM). A borderline inhibition (⩾50%) was found for CYP450 2C19, CYP450 3A4, norepinephrine transporter, cannabinoid CB_1-R, histamine H_1-R, and sodium channel site two. Moderate activation (50%) was found only for nicotinic acetylcholine receptor.

The properties of **5f** were further examined through a competitive binding experiment using [^{33}P]S1P as the orthosteric binding ligand and S1P as the competitive agonist in the control experiment. We found that S1P competes with [^{33}P]S1P for the $S1P_3$-R binding pocket. Remarkably, **5f** does not compete with [^{33}P]S1P



Scheme 4. Synthesis of **9a–c**. Reagents and conditions: (i) (a) **8a–c** (1 equiv), $SOCl_2$, benzene, reflux, 3 h; (b) **2a** (1.5 equiv), DIPEA (1.5 equiv), CH_2Cl_2, 0 °C–rt, 3 h, 70–98% (over two steps).

Table 3
$S1P_{2,4,5}$-Rs selectivity counter screen of selected compounds

Compd	EC_{50}[α] (nM)				
	$S1P_3$-R	$S1P_1$-R	$S1P_2$-R	$S1P_4$-R	$S1P_5$-R
3a	434	7870	>50,000	>50,000	NT
5b	959	22,250	>50,000	>50,000	NT
5f	105	33,300	>50,000	>50,000	>25,000
5g	103	662	>50,000	>50,000	NT
5h	323	859	>50,000	>50,000	NT
7f	202	>50,000	>50,000	>50,000	NT
7e	337	33,100	>50,000	>50,000	NT
7g	535	4703	>50,000	>50,000	NT

NT = not tested.
[α] Data are reported as mean of $n = 3$ determinations.

Table 2
$S1P_3$-R agonist activity of compounds **7a–l**

Compd	Amine	R^1	R^2	EC_{50}[α] (nM)	
				$S1P_3$-R	$S1P_1$-R
7a	**3c**	H	Cyclohexyl	>50,000	>50,000
7b	**3b**	Cyclopentyl	Cyclohexyl	1683	>50,000
7c	**6a**	4-Tetrahydropyranyl	Cyclohexyl	3536	>50,000
7d	**6b**	Cyclohexylmethyl	Cyclohexyl	3430	>50,000
7e	**6c**	2-Methylcyclohexyl	Cyclohexyl	337	33,100
7f	**6d**	2-Methylcyclohexyl	2-Methylcyclohexyl	202	>50,000
7g	**6e**	2-Methylcyclohexyl	Cycloheptyl	535	4700
7h	**6f**	2-Methylcyclohexyl	4-Tetrahydropyranyl	2820	3260
7i	**6g**	2-Methylcyclohexyl	Cyclopentyl	1740	>50,000
7j	**6h**	Cyclopentyl	Cycloheptyl	2753	34,150
7k	**6i**	Phenyl	Benzyl	7000	44,900
7l	**6j**	2-Pyridinyl	Benzyl	6563	>50,000

[α] Data are reported as mean of $n = 3$ determinations.

for the binding pocket of the $S1P_3$-R and binds in a manner that does not disturb the [^{33}P]S1P–$S1P_3$-R binding.[21] Site-directed mutagenesis of $S1P_1$-R/$S1P_3$-R and docking studies performed on an $S1P_3$-homology model showed that **5f** binds in an allosteric binding pocket.

Even though **5f** is a highly selective $S1P_3$-R agonist with appropriate potency for further biological studies, its physicochemical properties remain suboptimal (tPSA = 41.9, *C*log*P* = 3.9, solubility in PBS = 1.24 μM).

Based on the characterized ligand/binding pocket interactions, our research group will embark on a medicinal chemistry program aiming to further improve the potency and physicochemical properties while maintaining selectivity of **5f**.

In summary, rational chemical modifications of the MLSMR hit **1a** led to the development of $S1P_3$-R agonists **5f**, **7e** and **7f** (CYM5541, CYM5558, CYM5556) exquisitely selective against the remaining $S1P_{1,2,4,5}$-R subtypes. Noteworthy, we have reported the discovery, design and synthesis of the first small molecule $S1P_3$-R allosteric agonist based on a *N*,*N*-dicyclohexyl-5-alkylisoxazole-3-carboxamide chemotype, **5f**, that is highly selective against a panel of therapeutically relevant targets. The studies herein described provide novel pharmacological tools to decipher the biological function and assess the therapeutic utility of the $S1P_3$-R. Further studies of our research program aiming to improve physicochemical properties and potency while maintaining selectivity will be communicated in due course.

Acknowledgments

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19. Chinese Hamster Ovary (CHO) cell line stably transfected with human S1P3-R, nuclear factor of activated T-cell-beta lactamase (NFAT-BLA) reporter construct and the Gα16 pathway coupling protein was used. Cells were cultured in T-175 sq cm flasks at 37 °C and 95% relative humidity (RH). The growth medium consisted of Dulbecco's Modified Eagle's Media containing 10% v/v heat inactivated bovine growth serum, 0.1 mM NEAA, 1 mM sodium pyruvate, 25 mM HEPES, 2 mg/mL 5 mM L-glutamine, 0.2 mg/mL hygromycin B and 1× penicillin-streptomycin. Prior to the start of the assay, cells were suspended to a concentration of 1.25×10^6/mL in phenol red free Dulbecco's Modified Eagle's Media containing 0.5% charcoal/dextran treated fetal bovine serum, 0.1 mM NEAA, 1 mM sodium pyruvate, 25 mM HEPES, and 5 mM L-glutamine. The assay began by dispensing 10 mL of cell suspension to each test well of a 384 well plate. The cells were then allowed to incubate in the plates overnight at 37 °C in 5% CO_2 and 95% RH. The next day, 50 nL of test compound (50 μM final concentration) in DMSO was added to sample wells, and DMSO alone (0.5 final concentration) was added to high control wells. Next, S1P prepared in 2% BSA (0.7 micromolar final nominal concentration, corresponding to the EC_{80} of S1P) was added to the appropriate wells. After 4 h of incubation, 2.2 μL/well of the GeneBLAzer fluorescent substrate mixture, prepared according to the manufacturer's protocol and containing 10 mM probenicid, was added to all wells. The plates were then incubated for 2 h at room temperature. Plates were read on the EnVision plate reader (PerkinElmer Lifesciences, Turku, Finland) at an excitation wavelength of 405 nm and emission wavelengths of 535 and 460 nm.
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21. $S1P_3$ jump-in CHO cells were cultured in T-175 sq cm flasks at 37 °C and 95% RH. The growth media consisted of Dulbecco's Modified Eagle's Media (DMEM) (with GlutaMAX) containing 10% v/v heat inactivated fetal bovine serum (dialyzed), 0.1 mM NEAA, 1 mM sodium pyruvate, 25 mM HEPES, and 1× penicillin–streptomycin–neomycin. On the day before the assay, cells were suspended at a concentration of 0.2×10^6/mL in the growth media and plated at 0.1×10^6/well in a 24-well plate. On the day of the assay, the growth medium was replaced with serum-starvation medium consisted of DMEM (with GlutaMAX) containing 0.1 mM NEAA, 1 mM sodium pyruvate, and no antibiotics. The cells were incubated at 37 °C for 4 h. After 4 h of serum starvation, the medium was removed and the cells were rinsed with 200 μL of ice-cold binding buffer. The binding buffer consisted of 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 15 mM NaF, 0.5 mM EDTA, 1 mM Na_3VO_4, 0.5% fatty acid-free BSA, and 1× protease inhibitor cocktail. Experiment 1: for the $S1P_3$ agonist competition assay, 30 μL of increasing concentrations of **5f** (final **5f** concentrations of 0.001 nM to 10 μM) and 270 μL ice-cold binding buffer containing [^{33}P]S1P (final concentration of 0.1 nM) were added to the cells in each well. Experiment 2: for the control assay, 30 μL of increasing concentrations of cold S1P (final S1P concentrations of 0.001 nM to 10 μM) and 270 μL ice-cold binding buffer containing [^{33}P]S1P (final concentration of 0.1 nM) were added to the cells in each well. For both experiments, the cells were incubated at 4 °C for 30 min. The cells were then washed three times with 500 μL ice-cold binding buffer. The cells were lysed with 300 μL 0.5% SDS and transferred to scintillation vials. 5 mL of scintillation cocktail was dispensed into each vial and the vial vortexed. The ^{33}P radioactivity (cpm) was counted for 5 min/vial in a Beckman LS 6000SC counter.