MLSCN Probe Summary: S1P3 Agonist

The Scripps Research Institute Molecular Libraries Screening Center (SRIMSC)

Grant Number: 1R03MH076534-01
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CRISP Project Title: MLSCN HTS Assays R03 - S1P1

Specific Aim: Identify compounds which provide insight into the molecular mechanism of S1P biological function.

Significance: The biology of S1P receptor subtypes: Sphingosine 1-phosphate (S1P) influences heart rate, coronary artery caliber, endothelial integrity, lung epithelial integrity and lymphocyte recirculation through five related high affinity G-protein coupled receptors. Inhibition of lymphocyte recirculation by nonselective S1P receptor agonists produces clinical immunosuppression preventing transplant rejection, but is associated with transient bradycardia. Understanding the contribution of individual receptors has been limited by the unavailability of selective agonists or antagonists for the 5 receptor subtypes. Separation of receptor subtype usage for control of endothelial integrity will allow the identification of selective S1P receptor agonists and antagonists that could be of use in the control of cardiac function and the prevention of brochospasm. S1P receptor subtype selective agonists and antagonists will be of broad utility in understanding cell functions in vitro, and vascular physiology in vivo, and the success of the chemical approach for S1P1 would suggest that selective tools for the resolution of function across this broad lipid receptor family is now possible.

Rationale: Selective chemical probes of S1P3: S1P3 receptor subtype plays a critical role in cardiac rhythm. S1P1 and S1P3 are coexpressed in some cells, especially endothelium. The association of a dose-dependent bradycardia with administration of the relatively non-selective receptor FTY720 in man led us to study the lymphopenic and heart rate responses that associated with S1P1 and S1P3. Induction of lymphopenia in homozygous S1P3-/- mice was indistinguishable from wild-type mice , with no statistically significant difference in the depth of lymphopenia at 5 hours between the S1P1-selective agonist SEW2871 and the S1P1,3,4 and 5 active prodrug AAL-(R), which is phosphorylated to its active form AFD-(R). Deletion of S1P3

therefore did not affect the S1P receptor agonist-induced inhibition of lymphocyte recirculation. We then tested the ability of the non-selective S1P receptor agonist AFD-(R) for the induction of heart rate changes in conscious mice by ECG analysis. Wild-type mice showed a significant maximal sinus bradycardia (-41.5 + 2.0%; p = 0.0001 by ANOVA) sustained for over 5 hours in response to the administration of AFD(R) or a structurally unrelated non-selective S1P3 agonist. AFD administration in S1P3-deletant mice was statistically equivalent to administration of vehicle alone in wild-type mice, and no bradycardia was seen. We tested the S1P1-selective agonist SEW2871 at a dose of 10 mg/kg that induced full lymphopenia for bradycardia and found no induction of bradycardia in either wild-type or S1P3-/- mice and it was indistinguishable from vehicle alone. Non-selective S1P receptor agonists therefore have effects upon both lymphocyte recirculation and heart rate. The use of SEW2871 together with the S1P3-deletant mice shows that S1P1 and S1P3 appear to have mutually exclusive roles: activation of S1P1 is sufficient to control lymphocyte numbers and plays no discernable role in control of sinus rhythm, whereas S1P3 regulates sinus rhythm and not lymphocyte recirculation. Agonists and antagonists of S1P3 may be useful probes of cardiac function in vivo. Activation of S1P3 also appears to induce significant bronchoconstriction. Antagonism of S1P3 may be useful in pulmonary disease.

PubChem Bioassay Identifier (AID): 373, 439

PubChem Bioassay Name: S1P3 Agonist Primary HTS and Confirmation Assays, S1P3 Agonist Dose-Response Potency Assay

Assay Description
Assay Overview:

Compounds identified from a previously described set of experiments entitled "S1P3 Agonist Primary HTS and Confirmation Assays" were selected for testing in this assay. Further information on the previous set of experiments can be found by searching on this website for PubChem AID = 373.

A cell line containing the human S1P3 receptor as well as the beta-lactamase (BLA) reporter-gene under control of the nuclear factor of activated T-cells (NFAT) promoter was used to measure S1P3 agonism. If the S1P3 receptor was stimulated by agonist, transcription of the NFAT-BLA gene occurred via a G-alpha16 protein coupled signaling cascade. The amount of BLA activity was proportional to the concentration of agonist. BLA activity was measured with a fluorescent BLA substrate.

In this experiment, 69 compounds that passed hit selection criteria were tested to determine individual EC50 values. The compounds were "hit-picked" at a 10 millimolar in DMSO and further serially diluted nine times at three fold-dilutions for a total of 10 different compound concentrations.

The assay was conducted in 1536-well format. Each compound dilution series was assayed in triplicate. S1P was used as the positive control. All data reported was normalized on a per-plate basis to wells that contained cells in the presence of 1

micromolar S1P (i.e. 100% activation). In this assay, S1P had a 50% effective concentration (EC50) of approximately 200 nM.

Dose-response curves were plotted and fitted to a four parameter equation describing a sigmoidal concentration-response curve with adjustable baseline using Assay Explorer software by MDL. The reported EC50 values are generated from fitted curves by solving for x-intercept at the 50% activity level of Y-intercept. In cases where the highest concentration tested (45 micromolar) did not result in > 50% inhibition, the EC50 was determined manually as > 45 micromolar. Compounds with EC50 values of greater than 10 micromolar were considered inactive, compounds with EC50 equal to less than 10 micromolar are considered active. The activity score is reported as normalized EC50 for samples with EC50 of less than 45 micromolar and as zero for samples with EC50 greater than or equal to 45 micromolar.

Protocol Summary:

For all assays, a Chinese Hamster Ovary (CHO) cell line stably transfected with human S1P3 receptor, nuclear factor of activated T-cell-beta lactamase (NFAT-BLA) reporter construct and the G-alpha-16 pathway coupling protein was used.

Cells were cultured in T-175 sq cm Corning flasks (part 431080) at 37 deg C and 95% RH. The growth media consisted of Dulbecco's Modified Eagle's Media (Invitrogen, part 11965-092) containing 10% v/v heat inactivated bovine growth serum (Hyclone, part SH30541.03), 0.1 mM NEAA (Invitrogen, part 1114-050), 1 mM Sodium Pyruvate (Invitrogen, part 11360-070), 25 mM HEPES (Invitrogen, part 15630-080), 5 mM L-Glutamine (Invitrogen, part 25030-081), 2 mg/mL Geneticin (Invitrogen, part 10131-027), 0.2 mg/mL Hygromycin B (Invitrogen, part 10687-010) and 1x penicillin-streptomycin (Invitrogen, part 15140-122).

Prior to the start of the assay, cells were suspended to a concentration of 1 million/milliliter in phenol red free Dulbecco's Modified Eagle's Media (Invitrogen, part 21063-029) containing 0.5% charcoal/dextran treated fetal bovine serum (Hyclone, part SH30068.03), 0.1 mM NEAA, 1 mM Sodium Pyruvate, 25 mM HEPES, and 5 mM L-Glutamine.

The assay began by dispensing 5 microliters of cell suspension to each test well of a 1536 well plate. The cells were then allowed to incubate in the plates overnight at 37 deg C in 5% CO2. The next day, 25 nL of test compound or DMSO control was added. The S1P positive control was also added to the appropriate control wells to a final concentration of 1 micromolar. Plates were then incubated at 37 deg C in 5% CO2 for 4 hrs. After the incubation, 1 microliter/well of the GeneBLAzer's fluorescent substrate mixture (Invitrogen, LiveBLAzer, part K1085), prepared according to the manufacturer's protocol and containing 200 mM probenicid (Sigma, part P8761) was added. After 2 hours of incubation at room temperature, plates were read on the EnVision plate reader (PerkinElmer Lifesciences, Turku, Finland) at an excitation wavelength 405 nm and

emission wavelengths of 535 nm & 460 nm. Each channel of raw data was corrected by subtracting "background" (i.e. wells containing media and substrate only) before the ratio of 460 nm/535 nm fluorescence emission was calculated. Percent activation was calculated from the median ratio of the positive control after subtracting the basal signal ratio from the sample well and the positive control.

The Primary HTS and Dose Response assays were conducted in 1536-well format. All compounds were tested once at a 10 micromolar final concentration.

Center Summary of Screen and Followup:
Screening, Dose Response and Parental Cell line Counterscreen: We screened 59,805 compounds and 62 were identified as actives. A dose response confirmation assay of the primary screening hits identified 13 repeatedly active compounds. Based upon potency, 4 were selected for further efforts. These were tested against the parental cell line and for their ability to induce calcium flux in the parental cell line.

Center Comments. See below.
65 structures were identified as active in the primary assay and EC50 values were determined in the antagonist assay for S1P3. The 65 structures were clustered using a 0.7 similarity threshold and Leadscope fingerprints to identify 13 clusters and 19 singletons. The top 10 clusters and singletons were identified based on their best activity. Structural classes that show activity against S1P1 were removed and the best 4 clusters including 1 singleton are shown in Table 1.

Table 1. Activity of the top 4 S1P3 agonist structural representatives.

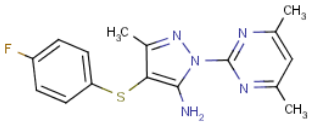
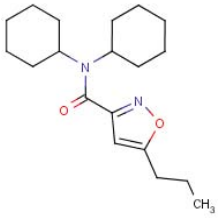
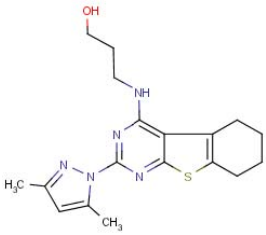
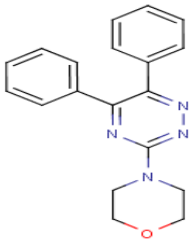
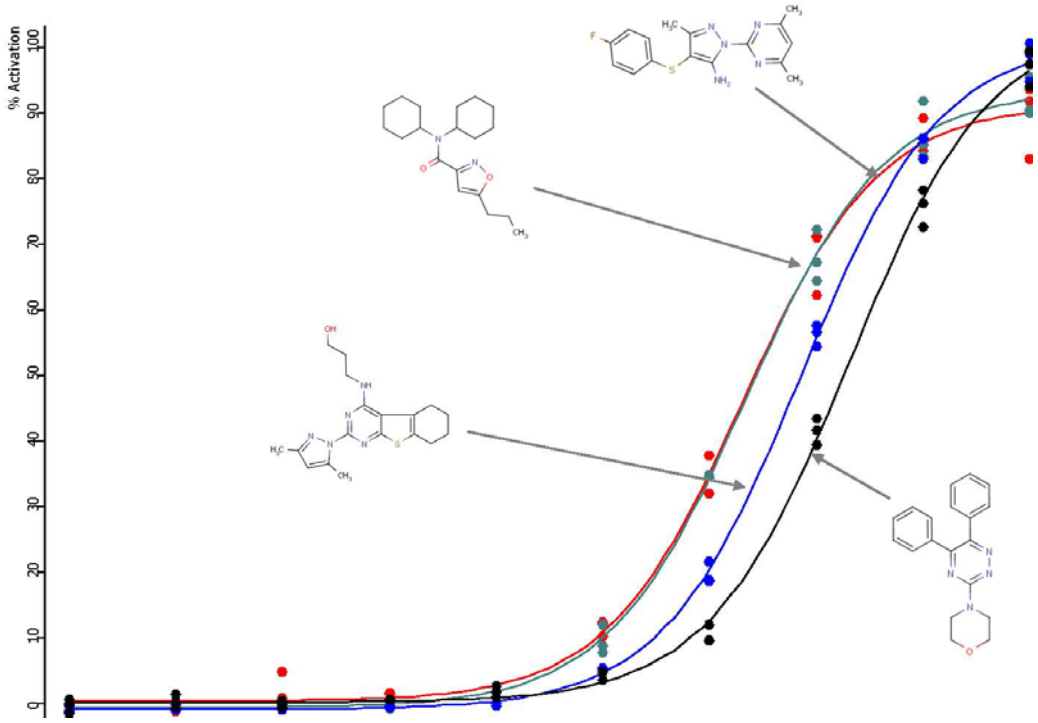
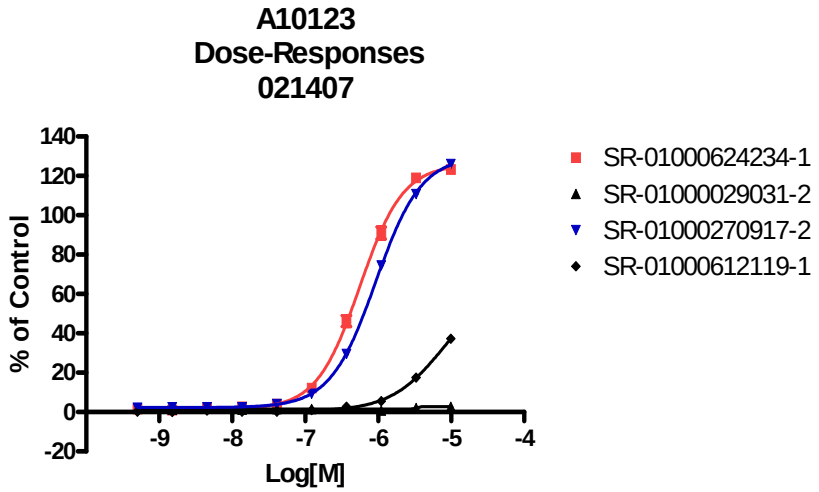
Structure	SID	PUR.	Cluster ID Num	S1P3 AG EC50 Inhib Prim	ANT Inhib Prim	S1P1 PTR / AG Inhib
	3714904	100 %	32 1	2.34 µM 60.1 %	8 %	-74.5 %
	7967985	100 %	13 2	2.7 µM 58 %	30 %	13.1 %
	864271	100 %	9 5	4.28 µM 41.1 %	3.4 %	-83.7 %
	7977380	100 %	5 2	6.63 µM 31.2 %	2.6 %	8.1 %

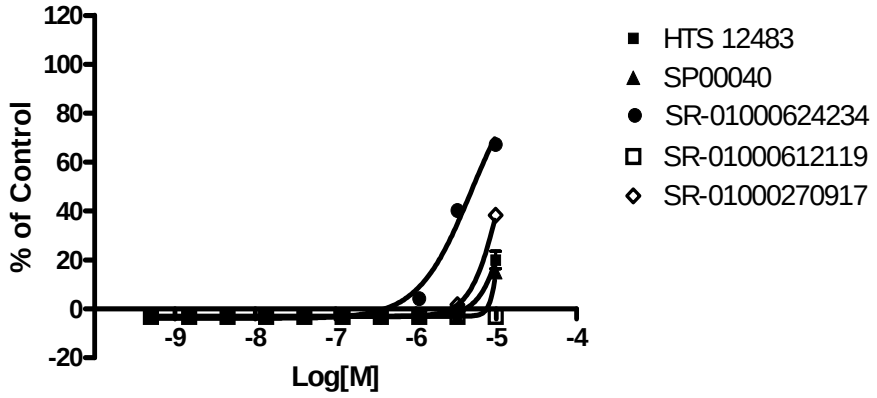
Figure 1. Dose response curves of the top4 structural representatives.





	SR-01000624234-1	SR-01000029031-2	SR-01000270917-2	SR-01000612119-1
HILLSLOPE	1.471	18.56	1.397	1.188
EC50	5.587e-007	3.388e-006	9.250e-007	7.993e-006

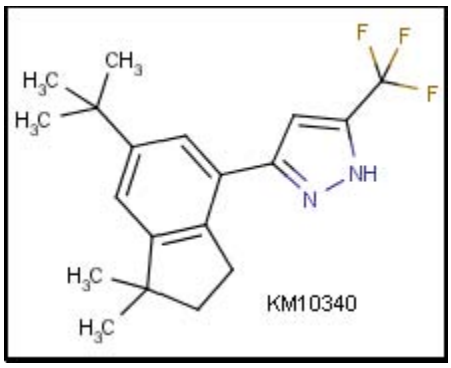
Figure 2. Dose response curves for compounds identified as S1P3 agonist probes.



	HTS 12483	SP00040	SR-01000624234	SR-01000612119	SR-01000270917
HILLSLOPE	2.558	7.497	1.312		2.364
EC50	1.614e-005	1.219e-005	4.900e-006		1.173e-005

Figure 3. FLPR Responses

Based on our screening results we searched commercial databases for further analogs based in structural similarity and scaffolds. The identified commercially available derivatives were prioritized based on structural features of the actives using extended connectivity fingerprints and by chemical intuition. The best commercial analog is shown below and has been further investigated in DMPK studies.



S1P1/S1P3 Agonist Activity:

S1P3: EC_{50} = 189 nM

S1P1 EC_{50} = > 10 µM

Pharmacokinetics

Table 2. Pharmacokinetics results of KM10340. Formulation : 1 mg/mL solution in 10% DMSO/20% w:v cyclodextrin

IV 1 mg/kg	Avg[1)]	%RSD	PO 2 mg/kg	Avg[1)]	%RSD
t 1/2 (hr) [2)]	3.1	5.0	AUC (∞, ng.min/mL)	1959872.5	22.0
Cl (ml/min/kg)	6.2	19.5	AUC (∞, µM.hr)	97.2	22.0
Vss (L) [3)]	1.5	10.4	Cmax (µM)	16.3	36.7
AUC∞ (ng.min/ml)	166301.8	20.1	Tmax (hr)	2.0	0.0
AUC∞ (µM.hr)	8.22	20.2			
			F%	589.3%	22.0

[1)] Average over 3 rats [2)] Based on: 60-480 min [3)] =Vdss(L/Kg) based on 1kg rat

Compound (concentration)	% plasma bound
Ranitidine	37 %
Midazolan	99 %
Warfarin	99.5 %
KM10340 (10 µM) KM10340 (1 µM) KM10340 (0.1 µM)	100 % 100 % 100 %