Probe Report

Project: High Throughput Screening for S1P Receptor Agonists and Antagonists, S1P2 Agonist

Grant Number: 1R03MH076533-01
Screening Center: The Scripps Research Institute Molecular Screening Center

Principal Investigator of Screening Center: Hugh Rosen, MD PhD

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PubChem Bioassay Identifer (AID): 729, 843,854, 872 and 874

Assay or Pathway Target: S1P2 Receptor

Assay Target: S1P2

Probe PubChem Substance Identifier (SID): 46371210

Specific Aim: The goal of this project is to identify specific agonists of the S1P2 receptor

Significance:

Sphingosine 1-phosphate (S1P) is an important biological mediator which signals via five related high affinity G-protein coupled receptors and modulate a wide range of biological activities including immune response, cardiovascular function and angiogenesis [1, 2]. Subtype-selective modulators of S1P receptors will be of broad utility in understanding cell functions in vitro and vascular physiology in vivo, as well as de-convoluting the role of individual subtypes in cellular processes. The S1P receptor 2 (S1P2), also known as endothelial differentiation sphingolipid G-protein-coupled receptor 5 (EDG5), signals through Gi, Gq and G12/13. A S1P2 selective agonist ligand will enable new studies of the role of this receptor in a variety of biological processes.

Rationale:

As no selective S1P2 agonist exists, the discovery of selective SIP2 agonists should prove useful for uncovering the physiological and pathological roles of this S1P receptor subtype.

Assay DescriptionAssay Overview:
A cell line expressing both the beta-lactamase (BLA) reporter-gene under control of the cAMP Response Element (CRE) promoter and the human S1P2 receptor was used to measure S1P2 agonism. Under normal conditions, S1P2 has low basal activity and therefore cells express low BLA levels.

When the S1P2 receptor is stimulated by an agonist, transcription of the BLA gene occurs proportionally to agonist concentration, and BLA activity in the sample is increased. This increase is monitored by measuring fluorescence resonance energy transfer (FRET) of a cleavable fluorogenic cell permeable BLA substrate.

Protocol Summary:
For this assay, a Chinese Hamster Ovary (CHO) cell line stably transfected with human S1P2 receptor and a cAMP Response Element-beta lactamase (CRE-BLA) reporter construct was used.
Cells were routinely cultured in T-175 sq cm flasks (Corning, part 431080) at 37 deg C and 95% relative humidity (RH). The growth media consisted of Dulbecco's Modified Eagle's Media supplemented with 10% v/v heat inactivated dialyzed fetal bovine serum, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 25 mM HEPES, 5 mM L-Glutamine, 2 mg/mL Geneticin and 1X antibiotic mix (mix of penicillin, streptomycin and neomycin).

Prior to assay, cells were suspended to a concentration of 1.25 million/milliliter in assay media, which consisted of phenol red-free Dulbecco's Modified Eagle's Media supplemented with 2% charcoal/dextran-treated fetal bovine serum, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 25 mM HEPES, 5 mM L-Glutamine and 1X antibiotic mix (mix of penicillin, streptomycin and neomycin).

The assay began by dispensing 4 microliters of cell suspension to each test well of a 1536 well plate (5000 cells/well) followed by incubation at 37 deg C in 5% CO2 for 16 hrs. To the appropriate wells were then added 26 nL of test compound in DMSO (final nominal concentration of 4.5 micromolar, final DMSO concentration of 0.5%) or DMSO only (for negative control wells) followed directly afterwards by 1 microliter of 2% BSA. The S1P positive control was also added to the appropriate control wells to a final concentration of 3.3 micromolar, i.e. a concentration that resulted in 100% activity (EC100). Plates were incubated again at 37 deg C in 5% CO2 for 2 hrs. The fluorogenic LiveBLAzer substrate mixture with 10 mM Probenicid was prepared according to the manufacturer's protocol and this mixture was then added (1 microliter/well).

After a further 2 hours of incubation 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 nm & 460 nm.

Fluorescence values measured for each channel were corrected by subtracting "background" fluorescence, i.e. fluorescence measured in wells containing media and substrate only.

To normalize assay data, values measured from both the probe's fluorescence emission wavelengths were used to calculate a ratio for each well, according to the following mathematical expression:

Ratio = I460 nm/I535 nm

where I represents the measured fluorescence emission intensity at the enumerated wavelength in nanometers.

Percent activation was calculated from the median ratio as follows:
%Activation= ((Ratio_Test_Compound- Median_Ratio_Low_Control)/(Median_Ratio_High_Control - Median_Ratio_ Low_Control))*100

Where:
Low Control: 2% fatty acid free BSA (Calbiochem, part#126609)
High Control: 3.3 micromolar of S1P, corresponding to its EC100 (Biomol, part#SL-140)

A mathematical algorithm was used to determine nominally activating compounds in the primary screen. Two values were calculated: (1) the average percent activation of all compounds tested, and (2) three times their standard deviation. The sum of these two values was used as a cutoff parameter, i.e. any compound that exhibited greater %activation than the cutoff parameter was declared active.

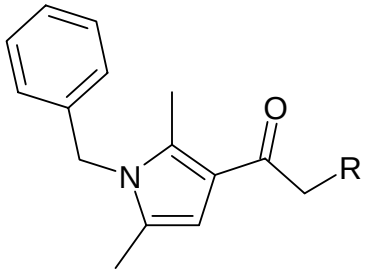
The reported Pubchem_Activity_Score has been normalized to 200% observed primary activation. % Activation values of greater than or equal to 200 are reported as activity score 100. Negative % activation values are reported as activity score zero.

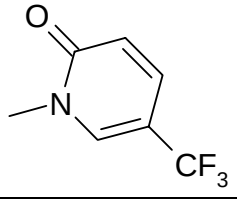
List of reagents:

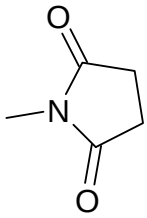
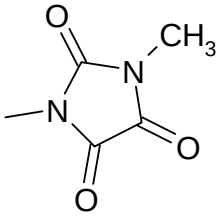
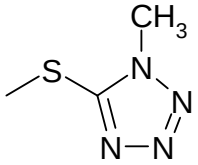
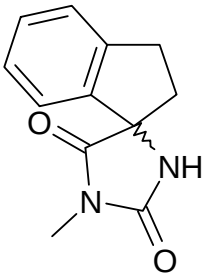
Dulbecco's Modified Eagle's Media with phenol red (Invitrogen, part 11965-092)
Dulbecco's Modified Eagle's Media without phenol red (Invitrogen, part 21063-029)
Fetal Bovine Serum (Invitrogen, part 26400-044)
NEAA (Invitrogen, part 1114-050)
Sodium Pyruvate (Invitrogen, part 11360-070)
HEPES (Invitrogen, part 15630-080)
L-Glutamine (Invitrogen, part 25030-081)
Geneticin (Invitrogen, part 10131-027)
100X Penicillin-Streptomycin-Neomycin mix (Invitrogen, part 15640-055).
Charcoal/dextran treated fetal bovine serum (Hyclone, part SH30068.03)
Probenicid (Sigma, part P8761)
S1P agonist (Biomol, part#SL-140)
LiveBLAzer (Invitrogen, part K1096)

Assay Results:
The MLSCN compound collection, 96,981 compounds, was screened for agonists of the S1P2 receptor and the data are deposited in AID 729. Dose response curves were obtained for 63 available compounds (out of 64 selected with greater than 50% activation in the primary screen in the primary screen and a counter screen for nonspecific activators of the cyclic-AMP response element (CRE). These two AIDS, 854 and 843, identify a lead compound, SID3716068, with significant activity towards the S1P2 receptor. This substance is reported inactive in 144 PubChem assays and active in one additional assay, CYP2C19 inhibition. Note that in this profiling assay, AID 778 from the SDCCG, ~20% of the MLMSR collection was flagged active. Dose response assays on a set purchased of analogues identified a second, related compound, SID 46371153.

SAR: **Table 1. S1P2 Agonists**



PubChem SID	R	EC50 S1P2 (μM)	Max Activity (% of 1 μM S1P)
46371153		0.72	120%

3716068		1.2	104%
46371152		22	68%
46371147		30	55%
46371148		>44	40%

Specificity:
The S1P2 specific antagonist, JTE-013 blocks S1P binding to S1P2 CHO membranes but not S1P1 nor S1P3 –overexpressed CHO membranes[3]. Activity of 46371153 is inhibited by the antagonist, JTE-013 (Figure 1).

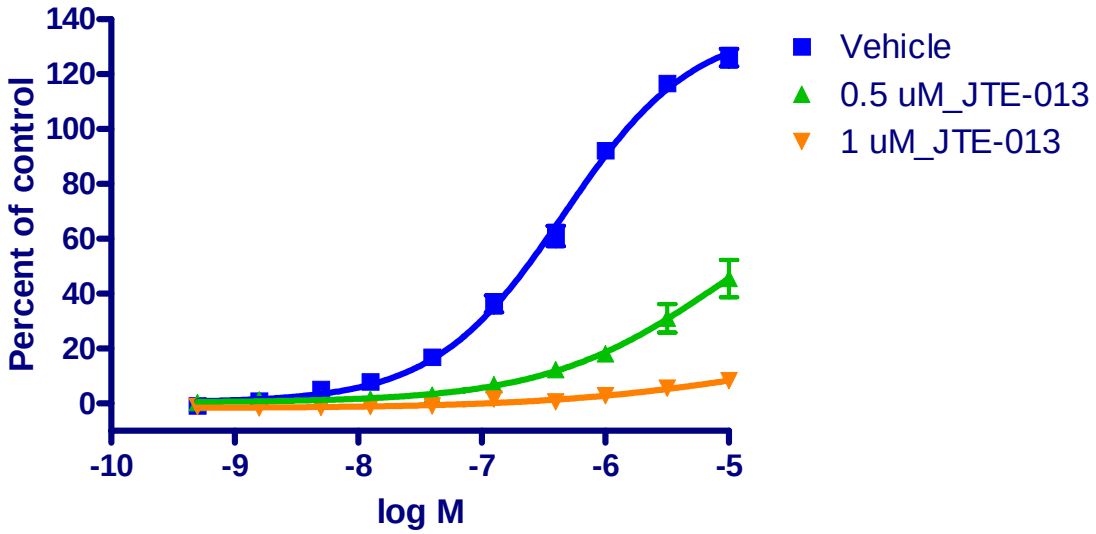


Figure 1

SID 46371153 is inactive towards the S1P1 and S1P3 receptors and the CRE-bla parental cell line. Activity towards the S1P1 receptor is modest; with ~70% of control at 10 uM The apparent S1P1 activity may be due to nonspecific activity, which prevents the S1P1-CRE-bla/CHO cells from making b-lactamase protein. Evaluation in the CRE-bla/CHO parental assay is planned.

Prior Probes:
DS-SG-44, (2S,3R)-2-amino-3-hydroxy-4-(4-octylphenyl)butyl phosphoric acid)(10 uM) is reported to induce reversion of isoprenaline induced morphology in rat C6 glioma cells as does S1P [4]. While DS-SG-44 appears to be acting on the S1P2 receptor, the effects were also through the S1P1/3 receptors. Additionally, this ligand is a phospho-aminoacohol, and as such should prove to be an intrinsically difficult molecule to use in biological studies, due to issues with solubility, metabolism (e.g. lipid phosphatases) and activity on multiple cellular targets.

Probe Summary
SID 46371153 is available from Enamine (T5433485). It is the first small molecule, S1P2 selective agonist reported. As such it should be useful in investigating the role of S1P2 in multiple biological model systems.

References

1. Peters, S.L.S.L.M. and A.E.A.E. Alewijnse, *Sphingosine-1-phosphate signaling in the cardiovascular system.* Current opinion in pharmacology, 2007. **7**(2): p. 186-92.
2. Rosen, H.H. and E.J.E.J. Goetzl, *Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network.* Nature reviews. Immunology, 2005. **5**(7): p. 560-70.
3. Osada, M.M., et al., *Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist.* Biochemical and biophysical research communications, 2002. **299**(3): p. 483-7.
4. Kim, K.K., et al., *Sphingosine 1-phosphate (S1P) induces shape change in rat C6 glioma cells through the S1P2 receptor: development of an agonist for S1P receptors.* Journal of Pharmacy and Pharmacology, 2007. **59**(7): p. 1035-41.