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A sphingosine 1-phosphate receptor 2 selective allosteric agonist ☆

Hideo Satsu [a,f], Marie-Therese Schaeffer [b], Miguel Guerrero [c], Adrian Saldana [d], Christina Eberhart [d], Peter Hodder [d], Charmagne Cayanan [b], Stephan Schürer [e], Barun Bhhatarai [e], Ed Roberts [c], Hugh Rosen [b,f], Steven J. Brown [b,*]

[a] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[b] The Scripps Research Institute Molecular Screening Center, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, USA
[c] Department of Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, USA
[d] Scripps Research Institute Molecular Screening Center, Lead Identification Division, Translational Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA
[e] Center for Computational Science, Miller School of Medicine, University of Miami, FL 33136, USA
[f] Department of Chemical Physiology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, USA

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Molecular probe tool compounds for the Sphingosine 1-phosphate receptor 2 (S1PR2) are important for investigating the multiple biological processes in which the S1PR2 receptor has been implicated. Amongst these are NF-κB-mediated tumor cell survival and fibroblast chemotaxis to fibronectin. Here we report our efforts to identify selective chemical probes for S1PR2 and their characterization. We employed high throughput screening to identify two compounds which activate the S1PR2 receptor. SAR optimization led to compounds with high nanomolar potency. These compounds, XAX-162 and CYM-5520, are highly selective and do not activate other S1P receptors. Binding of CYM-5520 is not competitive with the antagonist JTE-013. Mutation of receptor residues responsible for binding to the zwitterionic headgroup of sphingosine 1-phosphate (S1P) abolishes S1P activation of the receptor, but not activation by CYM-5520. Competitive binding experiments with radiolabeled S1P demonstrate that CYM-5520 is an allosteric agonist and does not displace the native ligand. Computational modeling suggests that CYM-5520 binds lower in the orthosteric binding pocket, and that co-binding with S1P is energetically well tolerated. In summary, we have identified an allosteric S1PR2 selective agonist compound.
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1. Introduction

Sphingolipids are an important family of bioactive molecules with cell signaling properties. Sphingosine 1-phosphate (S1P) is a pleiotropic lysophospholipid mediator present in plasma and is released in large amounts from activated platelets. S1P regulates various biological processes such as cell proliferation, migration, survival, and differentiation. The five sphingosine-1-phosphate receptors S1PR1 through S1PR5 mediate cellular functions upon S1P binding.[1] S1PR1, S1PR2 and S1PR3 are widely expressed on various tissues and cell types, whereas the expression of S1PR4 and S1PR5 is prominent in cells of the immune and nervous systems, respectively.

The *S1pr2* gene was cloned from rat smooth muscle cDNA library as an orphan receptor homologous to the *S1pr1* gene.[2] In K562 cells transiently transfected with *S1pr2* cDNA, S1P increased intracelluar calcium levels from intra- and extracellular reserves.[3] S1PR2 is a high affinity subnanomolar receptor for S1P and has been implicated in multiple biological functions, including Rho activation, inhibition of Rac and cell migration, and in 'feed forward' autocrine signaling in NF-κB survival signaling of tumor cells.[4,5] S1PR2 promiscuously couples to the heterotrimeric G proteins Gq, Gs, Gi/o, and $G_{12/13}$.[1] Studies with genetic deletions can provide insights into the physiologic functions of the targeted gene product. Kono et al. reported that S1PR2 expression is essential for proper functioning of the auditory and vestibular system.[6] Skoura et al. reported the essential role of S1PR2 in pathological angiogenesis of the mouse retina.[7] About one half of *S1pr2* gene null mice develop clonal B-cell lymphomas by age 1.5–2 years.[8] In addition to these observations, it is further expected that S1PR2 exerts other unknown physiological functions.

S1P is a unique, amphiphilic GPCR ligand, consisting of a hydrophilic, polar zwitterionic phosphoamine headgroup and a hydrophobic aliphatic straight chain C18 tail (Fig. 1A). The flexibility of the acyl chain may allow binding to many diverse sites. Along with genetic manipulations, chemical approaches provide novel insights into the function of S1P receptors. In the case of S1PR1, SEW-2871

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* Corresponding author.
E-mail address: sbrown@scripps.edu (S.J. Brown).

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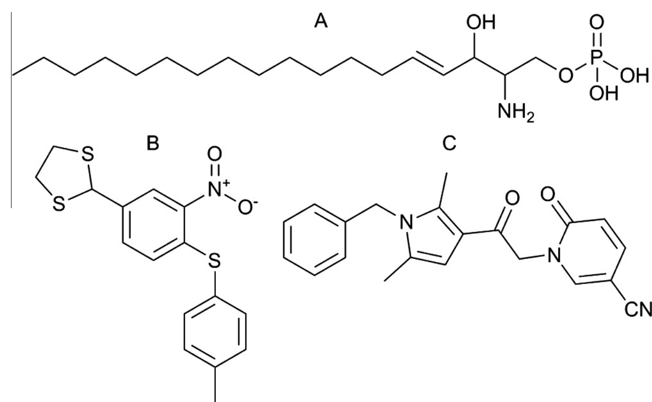

Figure 1. Chemical structure of ligands used in this study. (A) Sphingosine-1-phosphate, (B) XAX-162, and (C) CYM-5520.

is well recognized as an S1PR1-agonist and demonstrates the essential role of S1PR1 in lymphocyte trafficking. Recently a S1PR1 subtype selective agonist provided insights into the pulmonary response to viral infection.[9] Further, Sanna et al. reported that W146, a chiral S1PR1 antagonist, enhances capillary leakage and restores lymphocyte egress in vivo.[10] Therefore S1P receptor subtype selective agonists and antagonists will be of broad utility in understanding the regulatory mechanism of cell functions in vitro and physiological phenomenon in vivo.

There are few existing chemical probes for S1PR2. JTE-013 has been developed as an S1PR2 selective antagonist.[11] However, to our knowledge there is no known S1PR2 selective agonist. An S1P analogue, DS-SG-44, (2*S*,3*R*)-2-amino-3-hydroxy-4-(4-octylphenyl)butyl phosphoric acid), was reported to block isoprenaline-mediated morphological changes in rat C6 glioma cells, and was hypothesized to acted as an S1PR2 receptor agonist.[12] However, no receptor pharmacology for DS-SG-44 is reported, and is likely that it will be problematic for biological studies because of solubility and metabolic liabilities.

We report here HTS-driven identification of novel chemical scaffolds for S1PR2, chemical optimization and characterization of receptor activation via binding to the hydrophobic portion of the S1P biotopic, orthosteric binding site. Receptor binding and activation were delineated by radioligand binding competition, antagonist inhibition of CRE-reporter responses, cAMP biosensor detection of activation of wild type and head group mutant S1PR2 receptors, and molecular modeling studies.

2. Materials and methods

2.1. Chemicals

S1P was purchased from Biomol (Plymouth Meeting, PA) and dissolved in methanol (1 mM) and stored at −80 °C. Forskolin was purchased from Sigma–Aldrich and stored as a 10 mM DMSO solution at −35 °C. JTE-013 was purchased from Calbiochem (San Diego, CA) and Cayman Chemical (Ann Arbor, MI) and stored as a 10 mM DMSO solution at −35 °C.

2.2. S1P reporter and counterscreen assays

S1pr1 CRE-bla CHO, *S1pr2 CRE-bla* CHO, *S1pr3-Gα16 NFAT-bla* CHO, *S1pr4*-TANGO, *S1pr4*-TANGO and the counterscreen *CRE-bla* CHO reporter assays were performed as described.[13] PubChem assays are listed in Table S1.

2.3. Jump-In CHO S1PR2 wild type and head group triple mutant cell lines

Multisite Gateway cloning was utilized to generate in-frame *S1pr2-eGFP* expression constructs from pEnter-15-*S1pr2* and pENTER-52-*eGFP*. The *S1pr2-eGFP* fusion protein expression vector was cloned into pDEST-CMV-JTI (Invitrogen). S1PR2 head group binding side chains were identified by alignment with S1PR1. These mutations were generated by overlapping oligonucleotide PCR.[14] The triple mutant S1PR2 (R108A, E109A and K269A) was generated by overlapping oligonucleotide PCR mutagenesis. All constructs were confirmed by DNA sequencing. These vectors were transfected into CHO JumpIn cells (Invitrogen) and selected with 10 μg/mL blasticidin as described.[15] A homogenous pool of cells was generated by FACS sorting of GFP positive cells.

2.4. Glo-sensor cAMP transient transfection assay

The GloSensor vector (pGLoSensor-20FcAMP, Promega) was transfected using Fugene HD into S1PR2-eGFP or S1PR2-TM-eGFP Jump-In CHO cells. The following day, cells were harvested with 0.05% trypsin EDTA, resuspended to 500,000 cells/mL in CO_2 independent Media (Invitrogen) containing 2% charcol dextran stripped serum (CDS, Invitrogen) and 20 μL of the cell suspension was added to 384 well tissue culture treated white plates (Corning, part number 3570). These plates were incubated overnight at 37 °C, 5% CO_2. 25 μL of CO_2 independent media containing 2%CDS and 4% GloSensor Reagent (Promega) were then added and the plates were incubated for 2 h at room temperature. Antagonist (JTE-013) or vehicle were added and incubated for 20 min followed by agonist compounds or S1P. After 15 min, luminescence was read on a Perkin–Elmer Envision plate reader.

2.5. ^{33}P-S1P radioligand competition binding assay

Sphingosine, D-erythro [^{33}P] 1-phosphate was purchased from American Radiolabeled Chemicals, Inc (St Louis). S1PR2-CRE bla cells were seeded into wells of a 24 well plate at 200,000 cells in 1.0 mL growth media and the plate incubated overnight in an incubator with 100% humidity, 5% CO_2, 37 °C. The media was replaced with 1% CDS serum media for 4 h prior to the assay. At 4 °C, the media was removed and replaced with test compounds or vehicle controls in binding buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 15 mM NaF with freshly added 1 mM Na_3VO_4 and protease inhibitors).

2.6. Compound synthesis and characterization

2.6.1. (CYM- 5482)

1-(1-Benzyl-2,5-dimethyl-1*H*-pyrrol-3-yl)-2-chloroethanone (50 mg, 0.191 mmol) in DMF were added sequentially DIPEA (66 μL, 0.38 mmol) and succinamide (38 mg, 0.38 mmol). The reaction was stirred 40 min in the microwave at 130 °C. The mixture was diluted in water and extracted with Ethyl acetate (4 × 50 mL). The combined organic phase was washed with brine (2 × 50 mL) and concentrated under reduced pressure. The mixture was purified by column chromatography using DCM/MeOH to yield 12 mg (0.036 mmol, 19%) of product as pale yellow powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34–7.25 (m, 3H), 6.87 (d, *J* = 7.68 Hz, 2H), 6.34 (s, 1H), 5.05 (s, 2H), 4.73 (s, 2H), 2.84 (s, 4H), 2.45 (s, 3H), 2.14 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 186.46, 177.91, 137.72, 138.00, 137.20, 129.83, 129.64, 128.42, 126.35, 117.68, 107.64, 47.52, 46.36, 29.20, 13.08, 12.70. IR (cm^{-1}): 1702 s, 1663 s. MS (EI) *m*/*z*: 325 (M+H).

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