Title

Assay of Sphingosine 1-phosphate Transporter Spinster Homolog 2 (Spns2) Inhibitors

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Introduction

Sphingosine 1-phosphate (S1P) was discovered as the final metabolite in the catabolism of sphingolipids1. While this remains the only recognized function of S1P in most eukaryotes, in vertebrates S1P is also an extra-cellular signaling molecule2. The presence of extra-cellular S1P in, for example, the bloodstream and lymph as well as cell surface S1P receptors indicates that there exists an S1P export mechanism. The expected S1P transporter was discovered when screening chemically mutagenized zebrafish embryos revealed a phenotype (twin hearts with tail blisters – previously associated with an S1P receptor3) that mapped onto the spinster homolog 2 (zSpns2) gene4,5. The phenotype was rescued by both wildtype zebrafish Spns2 and human SPNS2, but not by zSpns1, zSpns3 or the namesake gene from Drosophila, Spinster4,5. However, Spns2 is not present in red blood cells (RBCs), which are the main source of plasma S1P. Germ line deletion of an ‘orphan’ transporter-encoding gene (Mfsd2b) revealed that MFSd2b is required for RBC S1P release in the mouse6. While Spns2 and MFSd2b are both Major Facilitator Superfamily proteins that transport S1P, their amino acid sequences are dissimilar. Insofar as is known, Spns2 is an exporter – there appears to be little or no import of S1P from the extracellular environment.

S1P signaling was recognized as a bona fide drug target when an experimental drug, FTY720 (later known as fingolimod), was found to be a prodrug and its active metabolite, phospho-FTY720, was discovered to be a high affinity S1P receptor agonist, most prominently at the S1P1 receptor7,8. Investigation of the mechanism of action of fingolimod and other S1P1 agonists revealed that S1P
signaling at the lymph – lymph node interface is required for normal lymphocyte trafficking, specifically the migration of T-lymphocytes from secondary lymphoid tissues to efferent lymph\textsuperscript{9}. Agonist stimulation of lymphocyte S1P\textsubscript{1} receptors results in their desensitization and thereby disrupts the S1P signaling that is necessary for proper lymphocyte positioning\textsuperscript{10}. A suite of S1P receptor agonist drugs (fingolimod, siponimod, ozanimod, ponesimod) were eventually approved as medicines for treatment of multiple sclerosis and, in the case of ozanimod, ulcerative colitis. The adverse events associated with S1P\textsubscript{1} receptor agonist drugs in humans are initial dose bradycardia and immunosuppression\textsuperscript{11}. There is also concern that such drugs might compromise endothelial barrier function\textsuperscript{12}. Our laboratories are exploring the alternative approach of inhibiting targets that lie ‘upstream’ of S1P receptors, \textit{e.g.}, blocking S1P release via Spns2 into lymph. Spns2 inhibitors are needed to enable testing of the hypothesis that such compounds will capture the efficacy of S1P receptor modulators with lessened adverse events. To conduct a medicinal chemistry campaign, a robust assay to interrogate numerous new chemical entities is necessary.

Spns2 only exports S1P from cells, therefore we first generated a cell line that would release detectable amounts of S1P in the extracellular media in a Spns2-dependent manner. We chose HeLa cells, which are widely available, easily cultured and only release detectable S1P into their culture media when transfected with a plasmid encoding Spns2 (we have tested zebrafish, mouse and human Spns2). To maximize S1P release from Spns2-expressing HeLa cells, cells are treated with phosphatase and S1P lyase inhibitors to retard S1P catabolism. Further, fatty acid free bovine serum albumin (BSA) is added to the release media as a chaperone for S1P, which is sparingly soluble in aqueous media. Under these conditions, sufficient S1P is released over an 18-hour period to be readily detectable by tandem liquid chromatography mass spectrometry (LC-MS). The assay can be performed with transiently transfected HeLa cells or cell pools generated by culturing transfected cells in the presence of a positive selection marker (geneticin, G418). The cells are grown in standard 12 well culture dishes wherefrom 1.8 mL of culture media is collected for analysis. When the concentration of an inhibitor is varied, the assay yields inhibition curves and therefore IC\textsubscript{50} values. Three of our recent publications feature this assay\textsuperscript{13-15}. 


While commonly used immortal mammalian cell lines generally do not express Spns2, an exception is U-937 cells. Activated monocytes are atypical among white blood cells in that they express Spns2, thus, it is not unexpected that the human histiocytic leukemia U-937 cells express Spns2, a property shared by a similar cell line, THP-1. In our characterization of SphK inhibitors, we often used U-937 cells both because of their strong S1P synthetic capacity and relatively high levels of intracellular S1P as well as ease of culture. We format the U-937 cell-based Spns2 assay like our HeLa assay except no transfection of the U-937 cells is necessary.

Because functional transporter assays are vectorial, cell-free assays are problematic, particularly when an assay that would support a stream of new chemical entities generated in a medicinal chemistry campaign is desired. With S1P transporters, assays are complicated by the impermeability of cells to S1P and, as mentioned previously, Spns2 only extrudes S1P from cells. Thus, only S1P (or close structural analogs) that are synthesized intracellularly are transported. An assay similar to ours used hamster CHO cells expressing human SPNS2 and human sphingosine kinase type 1 (SPHK1). The Hisano et al. assay was formatted similar to ours, for example, 1% BSA and phosphatase inhibitors were included in the release medium. In addition to detecting released S1P by LC-MS, another version of the assay involved adding [3H]sphingosine (Sph) to the medium to enable the detection of released [3H]S1P. Although highly sensitive, this assay format is cumbersome as [3H]S1P was separated from [3H]Sph by thin layer chromatography and the cost of [3H]Sph is prohibitive to most laboratories.

The requisite control is to determine that S1P release is dependent on the expression of Spns2. HeLa cells release only a small amount of detectable S1P without the forced expression of Spns2. We have ascertained that transfection of HeLa cells with a transport inactive form of Spns2 (Spns2Arg200Ser) results in cell cultures that release little detectable S1P (Figure 1). The only specialized expertise involved in our protocol is the use of an LC-MS instrument to quantify S1P. The salient limitation of our protocol is that of any cell-based assay. That is, the assay will not work with compounds applied at cytotoxic concentrations or, presumably, with compounds that do not penetrate the cell. Further, the assay is not a direct measure of the affinity of inhibitors for Spns2 and yields an IC50 value, which is assay dependent. A specific limitation of the assay is that a cell line used must have sufficient S1P.
synthetic capacity (as do HeLa and U-937 cells). However, there is the option of forcing the expression of a sphingosine kinase to increase intracellular S1P production (and subsequent release) as reported by Hisano et al.\textsuperscript{19}

We have used this protocol with HeLa cells, U-937 cells and a mouse pericyte cells in three publications.\textsuperscript{13-15}

Materials

Reagents

<table>
<thead>
<tr>
<th>Tissue culture media</th>
<th>Final concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640 (U-937 cells)</td>
<td></td>
<td>500 mL</td>
</tr>
<tr>
<td>DMEM (HeLa cells)</td>
<td></td>
<td>500 mL</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>1%</td>
<td>5 mL</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>10%</td>
<td>50 mL</td>
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</table>

<table>
<thead>
<tr>
<th>Release assay media</th>
<th>Final concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI or DMEM without serum</td>
<td></td>
<td>500 mL</td>
</tr>
<tr>
<td>BSA (fatty acid free)</td>
<td>0.2% (w/v)</td>
<td>1 g</td>
</tr>
<tr>
<td>4-deoxypyridoxine</td>
<td>1 mM</td>
<td>0.5 mL of 1 M stock solution</td>
</tr>
<tr>
<td>NaF</td>
<td>2 mM</td>
<td>1.0 mL of 1 M stock solution</td>
</tr>
<tr>
<td>Na\textsubscript{3}VO\textsubscript{4}</td>
<td>0.2 mM</td>
<td>0.5 mL of 200 mM stock solution</td>
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</table>

<table>
<thead>
<tr>
<th>S1P recovery</th>
<th>Final concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>d7-S1P (Avanti Polar Lipids)</td>
<td>1.25 nM</td>
<td>2.5 pmole (5 µL of 0.5 µM solution in LC-MS grade methanol), store at -20°C</td>
</tr>
<tr>
<td>trichloroacetic acid (TCA)</td>
<td>10%</td>
<td>100% (w/w) in water, store at 4°C</td>
</tr>
<tr>
<td>caution: caustic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>methanol (LC-MS grade)</td>
<td>100%</td>
<td>Fisher Optima grade</td>
</tr>
</tbody>
</table>
Equipment

A tandem quadrupole mass spectrometer with a UPLC inlet is necessary to quantify S1P levels. We use a Waters Xevo TQ-S micro MS with a Waters Acquity h-class + UPLC.

Alternatives: Other models of tandem quadrupole mass spectrometers coupled with a HPLC or UPLC inlet would no doubt suffice.

Software

We use Waters MassLynx ver. 4.2 software for data acquisition and TargetLynx ver. 1.4 software for peak analysis. GraphPad Prism 9 was used for statistical analysis. Analogous software packages would suffice.

Procedure

Step 1

Timing: 2 days

Overnight incubation time is required to allow sufficient S1P to be released into media.

1. Transfect HeLa cells adhered in a 100 mm culture dish with pcDNA3.1 plasmid encoding Spns2 using lipofectamine.
2. Select a pool of cells that survive culturing with the neomycin analog, geneticin (G418).
3. Seed wells of a standard 12 well tissue culture dish with about 2 x 10^5 G418 resistant HeLa cells expressing Spns2 in standard growth media (DMEM with 10% FBS, 1% pen/strep).
   a. Allow to grow to near confluence (1-2 days).
   b. Aspirate off growth media, wash 1X with warm PBS, add 2 mL of release medium containing desired concentration of test article.
   c. Incubate in tissue culture incubator for 18 ± 1 hours.

CRITICAL:
1. In the case of transient transfections, it is advisable to trypsinize cells 24 hours after transfection and seed cells onto 12 well plates, performing the assay the next day (48 hours...
Although we do not know the efficiency of transfection, we observe sufficient signal after transient transfection.

2. Cells will not release S1P unless a chaperone protein such as albumin is in the media. We use fatty acid free BSA because of low endogenous S1P levels.

Optional: In our experience, 24 well culture dishes do not result in sufficient S1P release. HeLa cells are used as a convenience. Although we lack direct experience, we presume that a variety of other cell lines, e.g., HEK293 cells could be substituted. Insofar as we are aware, Spns2 from any species could be used.

Note: We select pools of Spns2-expressing HeLa cells with geneticin (G418). Cells with endogenous expression of Spns2 (U-937, THP-1, mouse pericytes) do not require transfection. Conversely, cell types that release too little S1P after transfection with Spns2 might benefit from co-transfection of a sphingosine kinase. Further, cells that release relatively large amounts of S1P (e.g. U-937 cells) can be incubated for shorter time periods (6 vs. 18 hours). Likewise, co-transfection with a sphingosine kinase expressing plasmid might also shorten the needed incubation time.

Step 2

Timing: 3 hours

Collecting released S1P (bound to albumin)

1. Remove 1.8 mL of culture media from each well and place in a 2.0 mL microcentrifuge tube on ice.
2. Add 5 µL of 0.5 µM d7-S1P (internal standard) to each tube, mix by vortexing.
3. Add 0.2 mL of 100% TCA, mix by vortexing, hold on ice for about 45 minutes.
4. Centrifuge at 10,000 x g for 10 minutes in cold room.
5. Wash pellet (vigorous vortexing) with 1 mL cold LC-MS grade water.
6. Centrifuge at 10,000 x g for 10 minutes.
7. Aspirate off supernatant fluid.
8. Add 0.3 LC-MS grade methanol to pellet, vortex, let sit at room temperature for 15 minutes.
9. Centrifuge 10,000 x g for 5 minutes.
10. Transfer supernatant fluid to LC-MS vial.

Optional: Released S1P will be bound to albumin, which is readily concentrated by TCA precipitation. We have tried precipitation with ammonium sulfate or cold acetone, both are inferior to TCA in our experience. A commonly reported method to recover S1P from cell culture media, plasma, etc. uses a modified Bligh & Dyer protocol (acidification, followed by extraction into chloroform : methanol with phase separation). However, this procedure is too laborious to support a medicinal chemistry campaign and would generate large amounts of halogenated solvent waste. A recent report used a mixture of methanol and acetonitrile to precipitate S1P from conditioned media overnight at -20°C. However, this maneuver dilutes released S1P while our protocol (TCA precipitation) concentrations
the analyte. Various solid phase extraction materials are available, but we are without experience with these.

**Note:** The literature describes a number of LC-MS methods to quantify S1P. Our preferred method is as follows: S1P, internal standard (d7-S1P,) and sphingosine quantification were performed using a tandem quadrupole mass spectrometer (Waters Xevo TQ-S micro) with a UPLC (Waters Acquity h-class+) inlet equipped with a reverse phase C-18 UPLC column (Waters BEH C-18 1.7 µm bead size, 2.1mm x 50mm). Our modified chromatography protocol used a binary solvent gradient with a flow rate of 0.4 mL/min and the column temperature of 60 °C. Mobile phase A (MPA) consisted of water/methanol/formic acid (79:20:1) while Mobile phase B (MPB) was methanol/acetone/water/formic acid (68:29:2:1). The run begins with 50:50 MPA:MPB for 0.5 min. MPB is then increased linearly to 100% within 3.5 min and held at 100% MPB for another 3 min. The column is re-equilibrated to 50:50 MPA:MPB for 1.5 min. Three µL is injected in the column. Analytes were detected using MRM protocols as follows: S1P (380.1>264.4, voltages: cone 18, collision 16), deuterated d7-S1P (387.2>271.4, voltages: cone 24, collision 16) and sphingosine (300.3>252.3, voltages: cone 30, collision 18), all in ESI positive mode. Peak analysis was accomplished using Waters TargetLynx software ver. 1.4.

**Data Analysis**

The integration of S1P and d7-S1P peaks were achieved by Waters TargetLynx ver. 1.4 followed by manual verification. For routine compound screening, quantification was accomplished by taking the ratio of S1P to d7-S1P. Statistical analyses were performed using GraphPad Prism 9.

**Anticipated Results**

As depicted in Figure 1, HeLa cell over-expressing wild-type Spns2 release S1P in the media while a transport dead mutant (arginine 200 to serine mutation results in significant attenuation of S1P transport. The addition of Spns2 inhibitor SLF1081851 inhibits the release of S1P into the media in a dose-dependent manner affording similar IC50 values in various cell lines (Table 1).13,14

**Troubleshooting**
Problem 1: No S1P detected.
Potential solution:
1. Ascertain that internal standard (d7-S1P) is detected.

Problem 2: Very low levels of S1P detected.
Potential solution:
1. Co-transfect with a plasmid encoding SphK1.
2. Co-transfect with a plasmid encoding SphK2.
3. Use U-937 cells instead of HeLa cells.

Problem 3: Test article does not show inhibition of S1P release.
Potential solution:
Use SLF1081851 (commercially available) as a control – do not exceed final concentration of 10 µM (cytotoxic).

Limitations
The mammalian cell assays described, while neither high throughput nor rapid, have proved sufficient to support a medicinal chemistry campaign that has yielded low nanomolar inhibitors of Spns2-dependent S1P export. There are two drawbacks inherent to our assay. First, the output is IC$_{50}$ values that, unlike K$_{i}$ values, are assay dependent. Nevertheless, IC$_{50}$ measurements are useful in assessing rank order of potency among compounds assessed on the same platform. Second, ours is a phenotypic assay, i.e., we are measuring the effect of S1P release from HeLa cells that is dependent on forced expression of Spns2, rather than direct interaction with a target protein.

Acknowledgments
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Author contributions
Y.K. and T.H. performed the experiments, collected, and analyzed data. W.L.S. and K.R.L conceived the project, and K.R.L. oversaw the experiments and wrote the manuscript. All of the authors edited the manuscript.

Declaration of Competing Interest
Two authors (WLS & KRL) are among the co-founders of S1P Therapeutics Inc, which was created to commercialize S1P-related discoveries (including S1P transport inhibitors) from their laboratories.

References


Table 1. IC₅₀ (µM) values of SLF1081851 in different cell lines¹³,¹⁴

<table>
<thead>
<tr>
<th>Compound</th>
<th>U937</th>
<th>THP-1</th>
<th>mSpns2/Hela cells</th>
<th>mouse kidney pericytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLF1081851</td>
<td>1.67±0.27</td>
<td>1.78±0.23</td>
<td>1.92±0.33</td>
<td>1.45±0.39</td>
</tr>
</tbody>
</table>

Figure 1. S1P release in Spns2 (wild-type) and Spns2R200S (transport dead mutant) plasmid DNA transfected Hela cells media. IS = internal standard.

Conflict of interest

W.L.S. and K.R.L. are among the co-founders of S1P Therapeutics Inc, which was created to commercialize S1P-related discoveries, including Spns2 inhibitors, discovered and characterized in their laboratories.