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SPHINGOSINE KINASE TYPE 2 INHIBITION ELEVATES CIRCULATING SPHINGOSINE 1-PHOSPHATE

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Abstract

Sphingosine 1-phosphate (S1P) is a pleiotropic lipid mediator involved in numerous cellular and physiological functions. Notable among these are cell survival and migration as well as lymphocyte trafficking. S1P, which exerts its effects via five G protein coupled receptors (S1P1-5), is formed by the action of two sphingosine kinases (SphKs). While SphK1 is the more intensively studied isotype, SphK2 is unique in it nuclear localization and has been reported to oppose some of the actions ascribed to SphK1. While several scaffolds of SphK1 inhibitors have been described, there is a scarcity of selective SphK2 inhibitors that are necessary to evaluate the downstream effects of inhibition of this isotype. Herein we report a cationic amphiphilic small molecule that is a selective SphK2 inhibitor. In the course of characterizing this compound in wild type and SphK null mice we discovered that administration of the inhibitor to wild type mice resulted in a rapid increase in blood S1P, which is in contrast to our SphK1 inhibitor that drives circulating S1P levels down. Using a cohort of F2 hybrid mice, we confirmed, compared to wild type mice, that circulating S1P levels were higher in SphK2 null mice and lower in SphK1 null mice. Thus both SphK1 and SphK2 inhibitors recapitulate the blood S1P levels observed in the corresponding null mice. Moreover, circulating S1P levels mirror SphK2 inhibitor levels providing a convenient biomarker of target engagement.

Keywords

Sphingosine Kinase (SphK); Sphingosine 1-phosphate (S1P); SphK inhibitor

Sphingosine 1-phosphate (S1P) is a bioactive lipid involved in a host of cellular functions, including migration, differentiation, survival, angiogenesis and immune cell modulation. Extracellularly, S1P exerts its effects via five G-protein coupled receptors – S1P1-5 and intracellularly, acts to modulate transcription complexes [1]. Due in part to the remarkable clinical success of the S1P receptor agonist and immunomodulatory prodrug, fingolimod (FTY720), S1P signaling is currently the subject of many investigations.

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[*]Yugesh Kharel, Mithun Raje, Kevin Lynch and Webster Santos are co-inventors on a patent application claiming SLR080811. This intellectual property has been licensed to a commercial entity in which Kevin Lynch and Webster Santos have equity.

AUTHOR CONTRIBUTIONS

Mithun Raje, Ming Gao and Webster Santos designed, synthesized and purified **SLR080811**. Yugesh Kharel and Kevin Lynch designed the experiments to characterize **SLR080811**. Yugesh Kharel performed the experiments except that Amanda Gellett did the DAG kinase assays and statistical analyses and Jose Tomsig did the LC/MS sphingolipid analyses. Yugesh Kharel and Kevin Lynch wrote the manuscript, all of the authors participated in its editing.

Two sphingosine kinase isotypes (SphK1 [2] & SphK2 [3]) catalyze the phosphorylation of sphingosine and these enzymes are solely responsible for S1P synthesis [4]. Studies with Sphk1 null mice reveal that SphK1 is responsible for a substantial fraction of S1P production [5]. Not surprisingly, these kinases have come under increasing scrutiny as drug targets due to their role in the production of S1P, which is implicated in a variety of pathological conditions such as cancers, fibrosis, etc.

The relative importance of SphK1 versus SphK2 as potential drug targets remains a topic of debate. While SphK1 is reported to promote growth and survival [6–8], cell-based studies of SphK2 suggest that this enzyme is not protective, rather it opposes proliferation while enhancing apoptosis [9–11]. While several scaffolds of SphK1 inhibitors have been described [12–15], SphK2 inhibitors are less common. One compound, ABC294640, has a K_I of 8 μM at SphK2 [16]. This adamantyl compound has been reported to be efficacious in several disease models including hepatic ischemia-reperfusion injury [17], osteoarthritis [18], Crohn's disease [19], ulcerative colitis [20] and colon cancer [21] among others. However, ABC294640 was recently documented to bind to the estrogen receptor where it has tamoxifen-like properties [22].

In this study, we report the results of our characterization of another SphK2 inhibitor, **SLR080811. SLR080811** is a guanidine-based SphK2 inhibitor with a $T_{1/2}$ of 4–5 hours in mice. While this molecule lowers S1P levels in cultured cells, it drives a SphK1-dependent increase in S1P in mice and thus mimics the elevated S1P levels observed in SphK2 null mice.

MATERIALS AND METHODS

Materials

Sphk1$^{-/-}$ [5] and *Sphk2*$^{-/-}$ [4] mice were gifts from Dr. Richard Proia (NIH/NIDDK). C57BL/6j mice were from Jackson Laboratories (Bar Harbor, ME). The plasmid encoding diacylglycerol kinase alpha and was a gift from Dr. Kaoru Goto (Yamagata University School of Medicine, Yamagata, Japan). Adult mouse kidney fibroblast cultures were a gift from Dr. Amandeep Bajwa (University of Virginia). Deuterated (D7) S1P, S1P, sphingosine, C17 S1P and C17 sphingosine were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Synthesis of SLR080811

The details of the synthesis of **SLR080811** ((*S*)-2-(3-(4-octylphenyl)-1,2,4-oxadiazol-5-yl)pyrrolidine-1-carboximidamide)) and related compounds will be reported elsewhere (Raje, Gao and Santos, in preparation).

Kinase assays

SphK activity was measured by a scintillation proximity assay as previously described [23]. Briefly, recombinant SphK1 or SphK2 were expressed in Sf9 insect cells, crude homogenates were prepared and incubated in 96 well FlashPlates (Perkin-Elmer) in a buffer containing D-*erythro*-sphingosine and γ-[^{33}P]ATP. The [^{33}P]S1P product, which adheres to the plate wall, was quantified by scintillation counting. To assay ceramide kinase or diacylglycerol kinases, the recombinant proteins were incubated with γ-[^{32}P]ATP and substrate (C6 ceramide or 1-O-hexadecyl-2-acetyl-*sn*-glycerol, respectively) and the lipid product, after recovery by organic extraction, was resolved by thin layer chromatography, detected by autoradiography and quantified by liquid scintillation counting. These assays were performed with and without a fixed concentration of inhibitor and its effect on K_M and V_{max} determined.

Sample preparation

Sample preparation protocols were from Shaner *et al.* [24] with minor modifications. Cell pellets (2–4 × 10^6 cells), whole blood (20 μl) or plasma (50 μl) was mixed with 2 ml of a methanol : chloroform solution (3:1) and transferred to a capped glass vial. Suspensions were supplemented with 10 μl of internal standard solution containing 10 pmoles each of C17 S1P or deuterated (D7) S1P, C17 sphingosine or deuterated (D7) sphingosine and an undecyl analogue of compound **1a** [25,26]. The mixture was placed in a bath sonicator for 10 minutes and incubated at 48°C for 16 hours. The mixture was then cooled to ambient temperature and mixed with 200 μl of 1M KOH in methanol. The samples were again sonicated and incubated a further 2 hours at 37°C. Samples were then neutralized by the addition of 20 μl of glacial acetic acid and transferred to 2 ml microcentrifuge tubes. Samples were then centrifuged at 12,000 x g for 12 minutes at 4°C. The supernatant fluid was collected in a separate glass vial and evaporated under a stream of nitrogen gas. Immediately prior to LC-MS analysis, the dried material was dissolved in 0.3 ml of methanol and centrifuged at 12,000 x g for 12 minutes at 4°C. Fifty μl of the resulting supernatant fluid was analyzed.

LC/MS protocol

Analyses were performed by Liquid Chromatography-ESI Mass Spectrometry (LC-MS) using a triple quadrupole mass spectrometer (AB-Sciex 4000 Q-Trap) coupled to a Shimadzu LC-20AD LC system. A binary solvent gradient with a flow rate of 1 ml/min was used to separate sphingolipids and drugs by reverse phase chromatography using a Supelco Discovery C18 column (50 mm × 2.1 mm, 5 μm bead size). Mobile phase A consisted of water : methanol : formic acid (79:20:1) while mobile phase B was methanol : formic acid (99:1). The run started with 100% A for 0.5 minutes. Solvent B was then increased linearly to 100% B in 5.1 minutes and held at 100% for 4.3 minutes. The column was finally re-equilibrated to 100% A for 1 min. Natural sphingolipids were detected using multiple reaction monitoring (MRM) protocols previously described [24] as follows: C_{17}S1P (366.4 → 250.4); S1P (380.4 → 264.4); dihydroS1P (382.4 → 266.4); deuterated (D7)C_{18}S1P (387.4 → 271.3);, C_{17}sphingosine (286.4 → 250.3); sphingosine (300.5 → 264.4); sphinganine (302.5 → 260.0), deuterated (D7) sphingosine (307.5 → 271.3). Fragmentation of compound **SLR080811** was analyzed by direct infusion of a 1 μM solution in methanol:formic acid (99:1) and it was found that the transition (371.1 → 140.1) in positive mode provided the most intense signal at the following voltages, DP: 76; EP: 10; CE: 29; CXP: 10. All analytes were analyzed simultaneously using the aforementioned MRMs. Retention times for all analytes under our experimental conditions were between 5.1 and 5.6 min. Ceramide (16:0) was measured in positive mode by monitoring the m/z 264.4 product ion and using a Supelco Supelcosil LC-NH_2 column (50mm × 2.1 mm, 3 μm bead size) as previously described [24].]. Quantification was carried out by measuring peak areas using commercially available software (Analyst 1.5.1).

Cell culture

U937 cells were grown in RPMI 1640 media supplemented with L-glutamate, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in an atmosphere containing 5% CO2 [12]. SKOV3 cells were grown in McCoy's 5a media supplemented with 10% FBS and penicillin/streptomycin at 37°C in an atmosphere containing 5% CO2. Mouse kidney fibroblast were grown in DMEM low glucose media with 10% FBS. Twenty-four hours before adding inhibitors, the growth media was replaced with media containing 0.5% FBS.

Cell viability assay

U937 cells were plated in 96 well plates at a density of 60–80,000 cells per well. Cells were treated with the indicated concentration of compounds for 24 hours. Cell viability was assessed using TACS™ MTT assay according to the manufacturer's protocol (R&D System, Minneapolis, MN). Briefly, 10 μl of MTT reagent was added to 100 μl of cell culture medium and the plate was incubated at 37°C for 4 hours, followed by incubation with 100 μl of Detergent Reagent at room temperature for 2 hours. Absorbance was measured at a wavelength of 570 nm.

Generation of F2 hybrid mice

Sphk1$^{-/-}$ and *Sphk2*$^{-/-}$ mice were mated and 15 (5 male, 10 female) of the resulting F1 heterozygotes were intercrossed to yield a cohort of F2 hybrid mice. Mice were genotyped at weaning (P21) and the various genotypes were found at the expected Mendelian frequencies except for the embryonic lethal double null genotype [4]. After 12–14 hours of fasting, blood was obtained from F2 mice from retro-orbital sinuses under light isofluorane anesthesia. Aliquots of whole blood and plasma were processed for LC-MS analysis of S1P levels as described above.

Pharmacokinetic analysis

Groups of 8–12 week old mice (strain: C57BL/6j) were injected (intraperitoneal route) with either **SLR080811** (dose: 10 mg/kg) or an equal volume of vehicle (2% solution of hydroxypropyl-β-cyclodextrin (Cargill Cavitron 82004)). After injection, animals were bled at the specified time points (ASAP time points were 1–2 minutes after dosing). Whole blood was processed immediately for LC-MS analysis as described above. Animal protocols were approved prior to experimentation by the University of Virginia's School of Medicine Animal Care and Use Committee.

RESULTS

Inhibitor design strategy

In the course of our previous studies of amidine-based SphK1 inhibitors [14,25] to identify compounds with longer half-lives, we reasoned that compounds containing an amidine or an amide might be rapidly hydrolyzed *in vivo*. Therefore, we synthesized compound **SLR080811** (Table 1), which retained the pyrrolidine ring of our SphK1 inhibitor compound **1a** [26] while replacing the amidine with the more stable guanidine isostere. Both compounds feature the carboximidamide 'warhead' [25]. Further, the amide in compound **1a** was replaced by an oxadiazole group. **SLR080811** was evaluated at recombinant SphKs, other lipid kinases, in whole cells and finally, in wild type and SphK null mice.

Evaluation of SLR080811 *in vitro*

SLR080811, prepared as the HCl salt, was tested first at recombinant SphK1 and SphK2 using a broken cell assay (see Methods). In these assays, **SLR080811** was found to have inhibitory constants (K_I) of 1.3 and 12 μM for SphK2 and SphK1, respectively (Table 1). Further, **SLR080811** was found to be competitive with sphingosine, but not with ATP. Because **SLR080811** is a sphingosine analog, we tested **SLR080811** as an inhibitor of related lipid kinases including ceramide kinase and DGKα. At a concentration of 3 μM, no inhibition of either enzyme was observed (data not shown).

We characterized **SLR080811** in detail because of its SphK2-selectivity that, although modest (10-fold), is unusual in our carboximidamide series. We chose human leukemia U937 cells for the evaluation of SphK2 inhibitors because they exhibit high SphK1 and

SphK2 activities, can be cultured with ease, and more importantly, these cells have been used in the past by us and other authors [12] to test SphK1 inhibitors enabling comparisons of the effects of inhibitors.

We first treated U937 cultures with either vehicle or **SLR080811** and measured the intracellular levels of S1P, sphinganine 1-phosphate (dhS1P), sphingosine (Sph), sphinganine (dhSph) and **SLR080811**. We observed that treatment of U937 cells with **SLR080811**, but not vehicle, resulted in decreased amounts of phosphorylated sphingolipids S1P and dhS1P (Figures 1a and 1c) and the concomitant increase of the corresponding non-phosphorylated precursors sphingosine and sphinganine (Figures 1b and 1d). The data in Figures 1a and 1c indicate that the IC_{50} values of **SLR080811** are less than the its K_I (1.3 μM) determined at recombinant SphK2. In addition to sphingolipids, we also measured the intracellular concentration of SLR080811. As shown in Figure 1e, **SLR080811** accumulates inside U937 cells in a concentration dependent manner, which might explain its potent effect on the levels of intracellular sphingolipids shown in Figure 1a–1d.

Treatment of human Jurkat T leukemia cells or human SKOV3 ovarian cancer cells with S**LR080811** for 2 hours also resulted in decreased S1P (data not shown). In U937 cells, the effect of **SLR080811** on intracellular sphingolipids was observed as early as 20 minutes after **SLR080811** exposure (data not shown) and persisted for at least 72 hours as documented in Figures 2a and 2b. We also quantified one of the prominent ceramide species (C16:0) in these cells and found that this ceramide was significantly elevated, but only at the 48 and 72 hour time points (Figure 2c).

The most obvious explanation for the decline in U937 cell-associated S1P and dhS1P in response to **SLR080811** is decreased synthesis, but it is conceivable that the decline was somehow the result of increased metabolism via, for example, S1P phosphatase or S1P lyase, or increased S1P export. To discriminate between these possibilities, we used FTY720, an SphK2-selective substrate [27,28]. We observed that treatment of U937 cells with **SLR080811** impaired their ability to convert FTY720 into FTY720-phosphate. As shown in Figure 3, we observed much lower levels of intracellular FTY720-phosphate in **SLR080811**-treated cells that, as expected, correlated with correspondingly higher levels of FTY720. This suggests that the reduction of intracellular S1P levels in U937 cells produced by **SLR080811** is due to SphK2 inhibition rather than the alternative mechanisms mentioned above.

To evaluate further the selectivity of **SLR080811** *in vitro*, we used SphK1 null and SphK2 null mouse kidney fibroblasts. Because the wild type fibroblasts derived from adult mouse kidney have both SphK1 and SphK2 activity (not shown), null cells are a useful model for testing compound selectivity. We found that **SLR080811** reduces the levels of intracellular S1P in both wild type and SphK1 null cells but not in SphK2 null cells (Figure 4a). This suggests SphK2, but not SphK1, is a target for **SLR080811**. Contrary to our expectations however, the effect of **SLR080811** on sphingosine levels was not selective for SphK2. As shown in Figure 4b, both SphK1 null and SphK2 null fibroblasts exhibited increased concentrations of sphingosine on treatment with **SLR080811** suggesting that other mechanisms may be at play in the regulation of these sphingolipids.

Finally, we tested whether the effects of **SLR080811** on U937 cells included cell toxicity. In general, we found that **SLR080811** has no obvious cytotoxic effects on U397 cells. For example, cultures grew normally in medium containing up to 3 μM **SLR080811** and there were no signs of cell growth inhibition (data not shown). Further, we investigated the effect of **SLR080811** on U397 cells using a standard assay that correlates cell viability with their redox potential (MTT assay, see Methods section). We found that **SLR080811** had a slight

cytotoxic effect that is apparent even at the lowest concentration tested but was not concentration dependent (Figure 5).

Evaluation of SLR080811 *in vivo:* Isotype selectivity and pharmacokinetics

As an extension of our experiments *in vitro*, we sought to evaluate the sphingosine kinase isotype selectivity of **SLR080811** *in vivo*. To this end, we injected groups of SphK1 null, SphK2 null, and wild type mice with a single intraperitoneal dose of **SLR080811** and analyzed the blood levels of S1P. We observed (Figure 6a) that SphK1 null mice exhibit reduced levels of blood S1P after injection whereas in SphK2 null animals the blood levels of S1P did not change. This result once again suggests that **SLR080811** is a selective inhibitor of SphK2. Levels of S1P in SphK1-null mice reached a nadir between 2 and 4 hours after injection and slowly returned to pre-treatment levels at approximately 24 hours. In addition to S1P, we also measured the blood levels of **SLR080811**. The kinetics of **SLR080811** approximate that of S1P in the sense that we observed an early **SLR080811** peak at 1 h, rather than 2 h (Figure 6b) as in the case of S1P, and then a slow disappearance of the compound over the subsequent 24 h.

The effect of a single dose of **SLR080811** in wild type mice was surprising, in that S1P levels increased, rather than decreased (Figure 6a). To investigate this seemingly paradoxical result, we generated a cohort of age-matched F2 hybrid *Sphk1 Sphk2* mice (see Methods) and measured their blood and plasma S1P levels. These mice constitute a genetically homogeneous population whose levels of blood S1P that should be less influenced by genetic variation than the parent strains. In this population we observed, as shown in Figure 7, the same pattern of blood S1P levels: high circulating S1P in SphK2 null animals, lower S1P levels in wild type animals and low levels of S1P in SphK1 null animals. Thus it appears that **SLR080811**, by inhibiting SphK2, mimics the effect of lack of functional *Sphk2* alleles. However, we note that a different SphK2 inhibitor, ABC294640, was reported to lower circulating S1P, albeit after five weeks of daily dosing into xenograft-bearing SCID mice [29].

Finally, in the course of the investigations described in this paper we noticed that the recovery of the C17-S1P internal standard was greater than 100% when samples from *SphK2* $^{-/-}$ mice were analyzed. This prompted us to investigate the possible presence of endogenous C17-S1P or an interfering analyte in those samples. We found that *Sphk2* $^{-/-}$ samples contain an analyte that is indistinguishable from authentic C17-S1P by LC/MS, *i.e.* their chromatographic retention times and transitions are indistinguishable. Because of these striking similarities we refer to this analyte as C17-S1P with the caveat that a definitive identification will require confirmation by additional analysis. Moreover we found that wild type and *Sphk1* $^{-/-}$ mice samples also contain C17-S1P, albeit to a lesser extent. From the practical point of view, and regardless of the identity of this analyte, it was obvious to us that C17-S1P should not be used as an internal standard for our samples rather we used deuterated (D7) S1P. A representative experiment illustrating this technical issue can be seen in Figure 8.

DISCUSSION

In this paper we disclose, **SLR080811**, a novel SphK2-selective inhibitor. **SLR080811** was synthesized by modifying the chemical structure of compound 1a, a SphK1-selective inhibitor that we have reported previously [26]. The amidine and the amide group in compound 1a were replaced by guanidine and oxadiazole groups respectively in **SLR080811**. Although our original intention was to obtain a longer-lived version of compound 1a by switching to the more stable guanidine and oxadiazole groups, we unexpectedly found that these changes also brought about a reversal of selectivity for SphK

isotypes. In the context of this report, “selectivity” refers not to absolute selectivity for SphK2, *i.e.* no effect on SphK1, but rather to relative selectivity. As shown in Table I, **SLR080811** is about one order of magnitude more potent inhibitor of SphK2 as compared to SphK1 based on the K_I values obtained *in vitro* with recombinant enzymes. In addition to the selectivity for SphK2, we found that **SLR080811** does indeed have a longer half-life than compound 1a according to our original expectations. As shown in Figure 6 we estimate the half-life of **SLR080811** to be 4–5 hours whereas the half-life for compound 1a is less than one hour [26]. We are currently testing **SLR080811** analogs to identify compounds with enhanced potency, half-life and/or isotype selectivity.

Our contention that **SLR080811** is a SphK2-selective inhibitor is based on observations carried out in different biological models both *in vivo* and *in vitro*. In agreement with our results with recombinant enzymes, the effects of **SLR080811** in cultured cell lines such as U937 correspond to inhibition of S1P synthesis, namely reduction of S1P and dhS1P levels and concomitant elevation of their aminoalcohol precursors sphingosine and sphinganine (Figure 1). That these effects are mediated at least in part by SphK2 blockade is demonstrated by our experiments with FTY720. This drug is a selective SphK2 substrate and, as shown in Figure 3, its phosphorylation is greatly diminished in presence of **SLR080811**.

Consistent with our observations with U937 cells, **SLR080811** had no effect on S1P levels in SphK2 null fibroblasts while it reduced S1P levels in wild type and SphK1 null fibroblasts as shown in Figure 4a. Taken together these results corroborate the SphK inhibition by **SLR080811** and its SphK2 selectivity. Our observations on sphingosine levels in these cells however were not completely consistent with this picture. Whereas we were able to detect the anticipated accumulation of sphingosine in both wild type and SphK1 null fibroblasts, we unexpectedly observed that sphingosine levels were higher in SphK2 null fibroblasts as well (Figure 4b). There is no simple explanation for this observation other than to suggest that some other mechanism(s) not presently known may be involved in the regulation of the metabolism of sphingolipids. One possibility is that **SLR080811** might have some inhibitory effect on ceramide synthase.

Our *in vivo* observations support the notion that **SLR080811** is a SphK2 selective inhibitor in the sense that it lowers the levels of blood S1P in of SphK1 null mice but does not have an effect on blood S1P when administered to mice lacking SphK2 (Figure 6). It is noteworthy that the basal levels of blood S1P in SphK2 null animals are much higher than those in SphK1 null or wild type mice. This was reported previously by Zemann *et al.* [30] with their SphK2 null mice and then by Olivera *et al.* [31] and Sensken *et al.* [32]. It may be hypothesized therefore that SphK1 is upregulated when SphK2 activity is reduced. However, increased SphK1 activity by itself seems to be insufficient to raise S1P levels as was demonstrated in transgenic mice with forced global expression of SphK1 [33]. These animals have high tissue SphK1 activity but have levels of plasma S1P that are not different from wild type animals. It appears that SphK2 suppression may have more complex consequences than just increasing SphK1 activity. A recent report showing that SphK2 may function as a transcriptional modulator [1] supports this contention, although this does not seem to be the case for the SphK2 null mice whose SphK1 mRNA levels were not different from wild type mice [30]. According to all these observations, it appears that there are factors at play in the regulation of S1P metabolism that we do not fully understand.

Our previous work with compound 1a, our SphK1-selective inhibitor, included the observation that this compound reduces the levels of blood S1P in wild type mice [26]. This effect appears to be the logical consequence of SphK inhibition in the context of a simple model of S1P metabolism whereby reducing the synthesis of S1P results in a drop in S1P

levels. We expected **SLR080811** to exhibit a qualitatively similar effect because both compounds are SphK inhibitors and because they exhibit other similarities. For example, both compounds accumulate to high levels in U937 cells possibly because they are recognized by the uptake system(s) that take up long chain bases such as sphingosine and sphinganine from the extracellular environment [34,35]. In addition, neither compound exerts major effects on cell viability at concentrations that result in extensive SphK blockade. However, we observed - contrary to our expectations - that **SLR080811** increases the levels of blood S1P in wild type mice, *i.e.* the opposite effect of compound 1a. Obviously, this observation cannot be accommodated in a simple model of S1P metabolism. It seems that the most likely hypothesis that can be advanced to explain this effect is to postulate that SphK2 inactivation, whether genetic or pharmacological, leads to increased S1P levels. In this context, the effect of **SLR080811** on wild type animals can be related to the increased levels of S1P in SphK2 null mice. In any event, if the effects of compounds 1a and **SLR080811** prove to be a general property of SphK inhibitors and persist with chronic dosing then it should be possible to use SphK inhibitors to adjust the levels of S1P both positively and negatively. This may be useful not only in the investigation of S1P metabolism but in a clinical setting as well.

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Abbreviations used in the text

SphK	sphingosine kinase
S1P	sphingosine 1-phosphate
LC/MS	liquid chromatography/mass spectrometry

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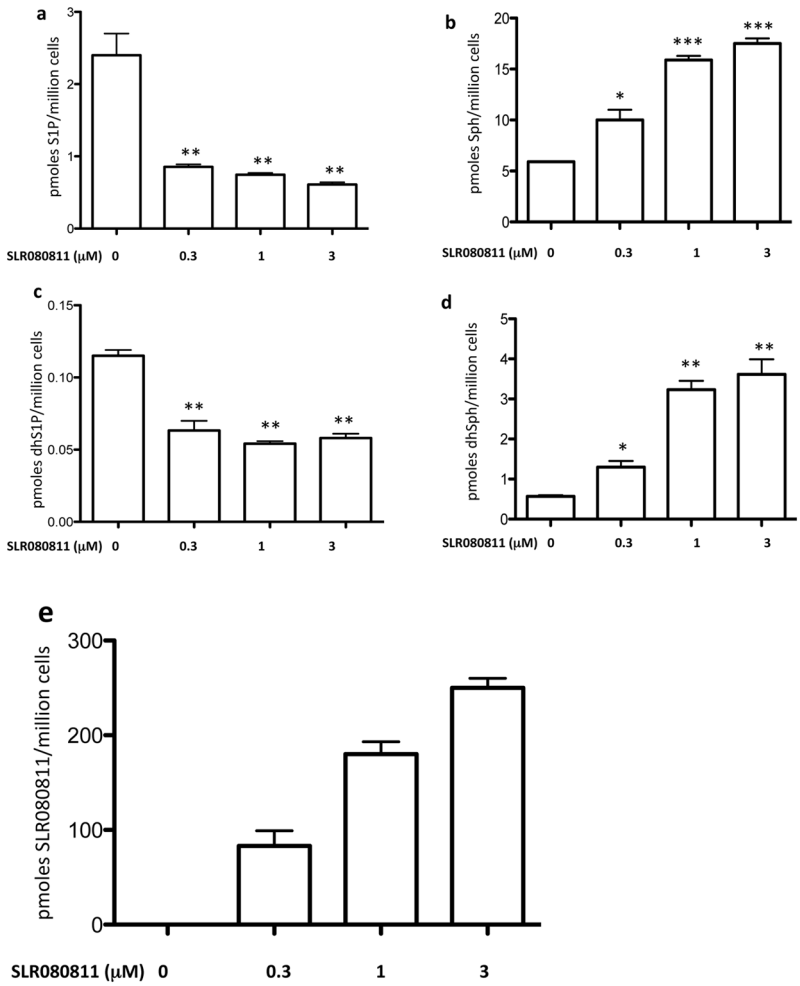


Figure 1.

Levels of sphingolipids and compound **SLR080811** in U937 treated with various concentrations of compound **SLR080811** as indicated. After a 2 hour period of exposure, cells were harvested by centrifugation, lysed and the amounts of sphingolipids and **SLR080811** in the lysates were measured by LC-MS as described in the Methods section. Amounts associated with cells are expressed as the number of pmoles *per* million cells. a: S1P; b: sphingosine; c: dihydroS1P; d: sphinganine: e: **SLR080811**. Data are presented as means ±SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (one way ANOVA, and Bonferroni's Multiple Comparison Test, compared to vehicle alone).

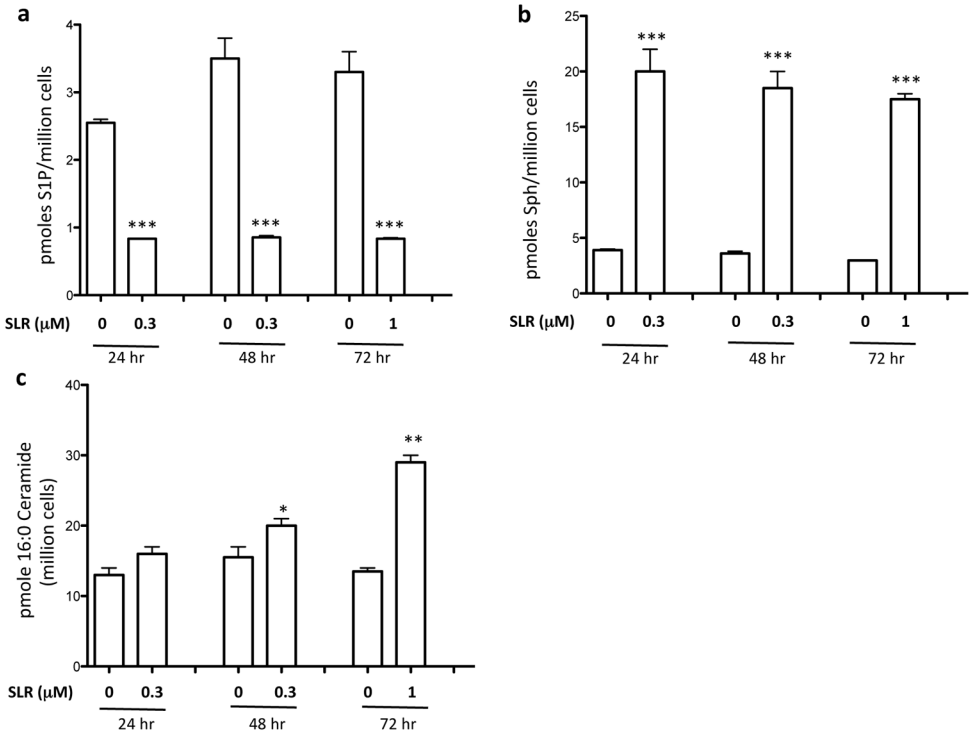


Figure 2.

Cultured U937 cells were treated with vehicle or **SLR080811** (1 μM) for different times as indicated. Cells were harvested by centrifugation, lysed and the amounts of sphingolipids and **SLR080811** in the lysates were measured by LC-MS as described in the Methods section. Amounts are expressed as the number of pmoles *per* million cells. a: S1P b: sphingosine; c: C16:0-ceramide. Data are presented as means ±SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student *t*-test, compared with the control (no compound treatment)).

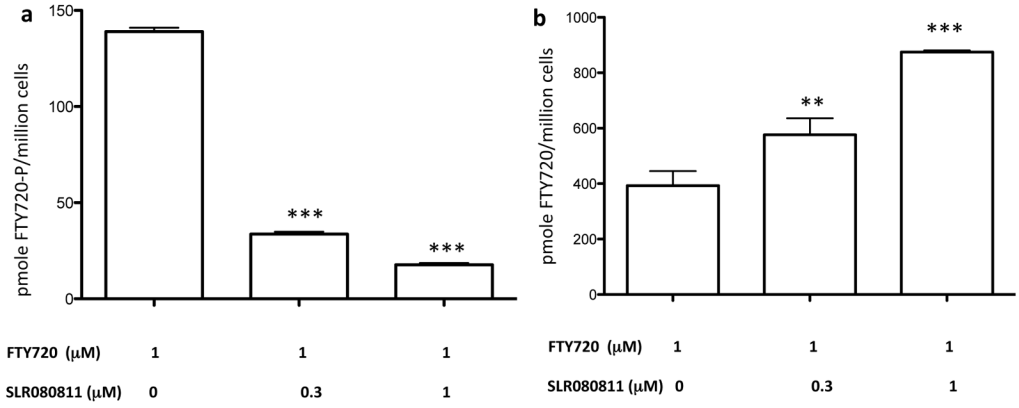


Figure 3.

Levels of FTY720-P and FTY720 in U397 cells treated with FTY720 and **SLR080811**. Cultured U937 cells were exposed to 1 μM of FTY720 and two concentrations of **SLR080811** as indicated in the figure. After 2 hours of exposure, cells were harvested by centrifugation, lysed and the amounts of FTY720 and phospho-FTY720 were measured by LC-MS as described in the Methods section. a: accumulation of FTY720-P; b: accumulation of FTY720. Amounts are expressed as the number of pmoles *per* million cells. Drug and FTY720 concentrations on the x axis refer to the concentration of these molecules in the culture medium. Data are presente as means ±SD of three independent experiments. *p < 0.05, **p <0.01, ***p< 0.001 (one-way ANOVA, and Bonferroni's multiple comparison test, compared with the control (no compound treatment)).

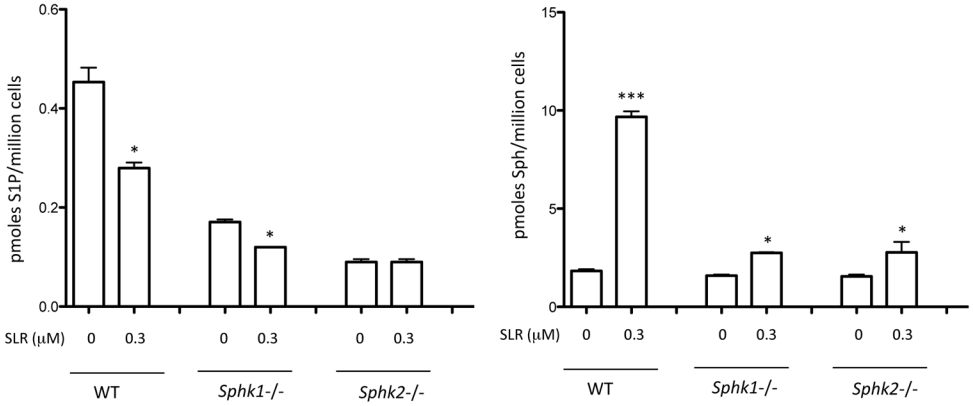


Figure 4.

Selectivity of **SLR080811** inhibition: WT, *Sphk1−/−* and *Sphk2−/−* adult mouse kidney fibroblast were exposed to 1 μM **SLR080811** for 2 hours. Cells were harvested by centrifugation, lysed and sphingolipids were measured by LC-MS as described in the Methods section. a: S1P; b: sphingosine. Data are presented as means ±SD of three independent experiments. *$p < 0.05$, ***$p < 0.001$ (Student *t*-test, compared with the control (no inhibitor)).

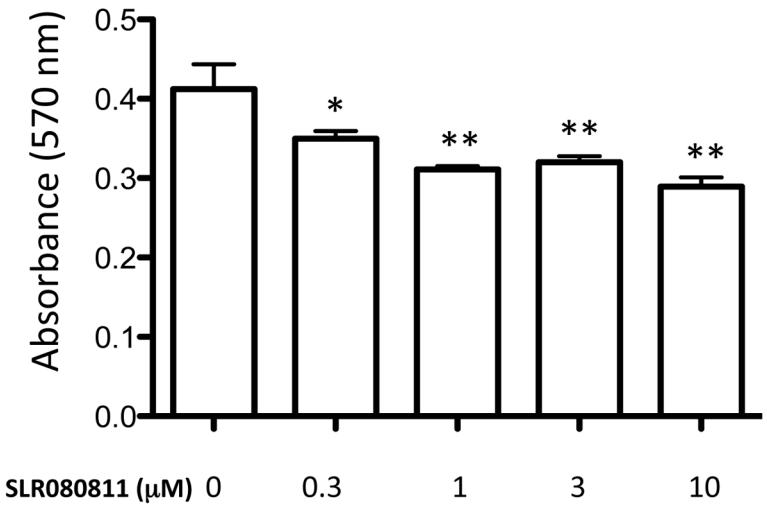


Figure 5.

Viability of U937 cells treated with **SLR080811** (a) or both **SLR080811** and compound **1a** (b). U937 cells were exposed to various concentrations of compounds for 24 hours as indicated. The viability of the cells was measured by MTT assay as described in the Methods section. Viability is directly proportional to the amount of formazan dye produced by live cells as measured by absorbance at 570 nm. Data are presented as means ±SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ (one-way ANOVA, and Bonferroni's multiple comparison post test, compared with the control (no inhibitor)).

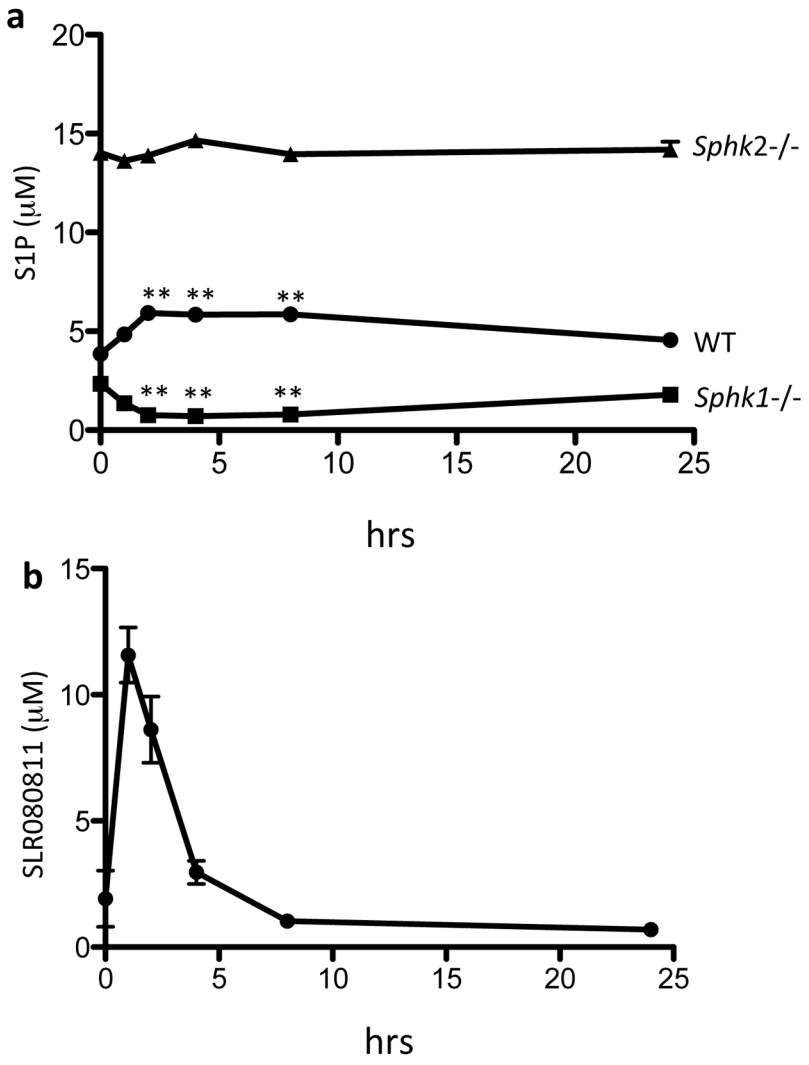


Figure 6.

S1P and **SLR080811** levels in the blood of mice injected with **SLR080811**. Wild type or Sphk1 or Sphk2 null mice were administered **SLR080811** (dose: 10 mg/kg, intraperitoneal route). Blood samples were drawn at times 0, 1, 2, 4, 8 and 24 hours post injection. Levels of S1P (a) and **SLR080811** in WT mice (b) in blood samples were measured by LC-MS. Data are presented as means ±SD of 3–5 mice per group. *$p < 0.05$, **$p < 0.01$ (repeated measures two way ANOVA, and Bonferroni's multiple comparison test compared to ASAP time point after injection of the compound (time 0)).

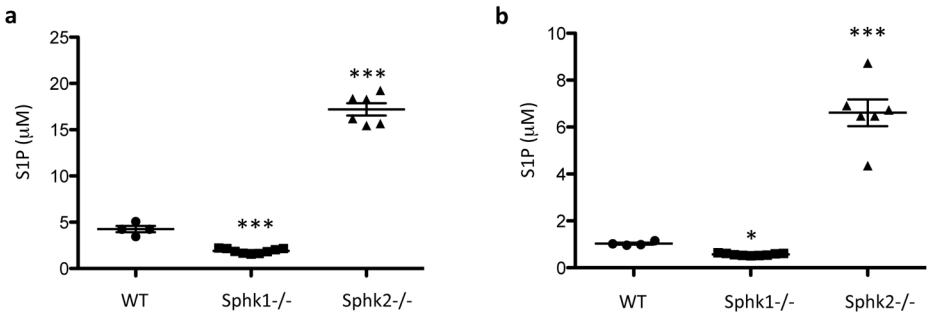
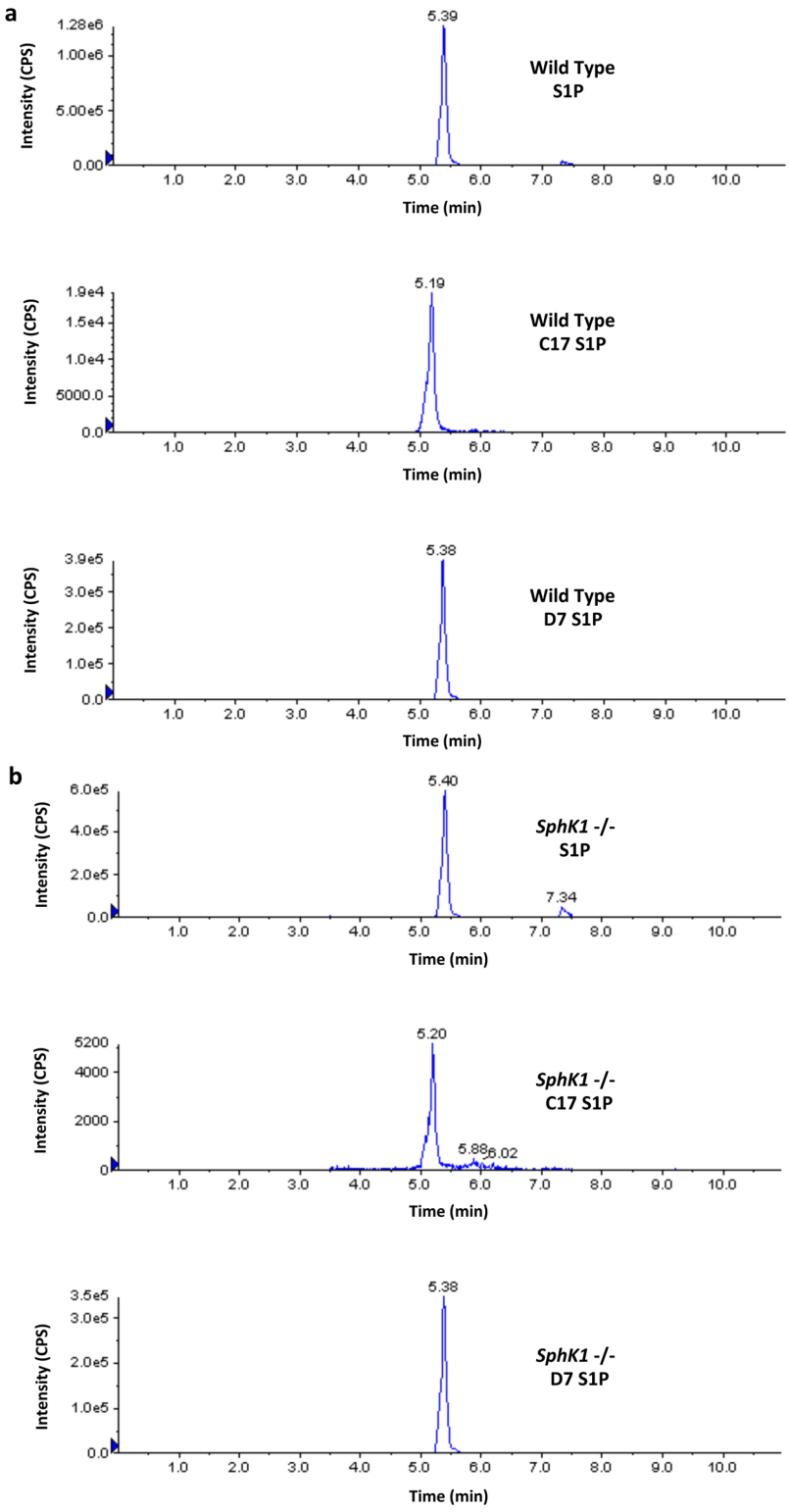


Fig. 7.

S1P levels in 8 weeks old WT, *Sphk1−/−* and *Sphk2−/−* littermates. Blood were drawn from wild type, Sphk1 and SphK2 null mice and the S1P levels were measured by LC-MS as described in the Methods section. Data shown are independent measurements of whole blood (A) or plasma (B) from 4 WT, 9 *Sphk1−/−* and 6 *Sphk2−/−* mice. The null mice were either heterozygous or wild type at the other SphK locus. *$p < 0.05$; ***$p < 0.001$ (one-way ANOVA, and Bonferroni's multiple comparison test, compared with WT).



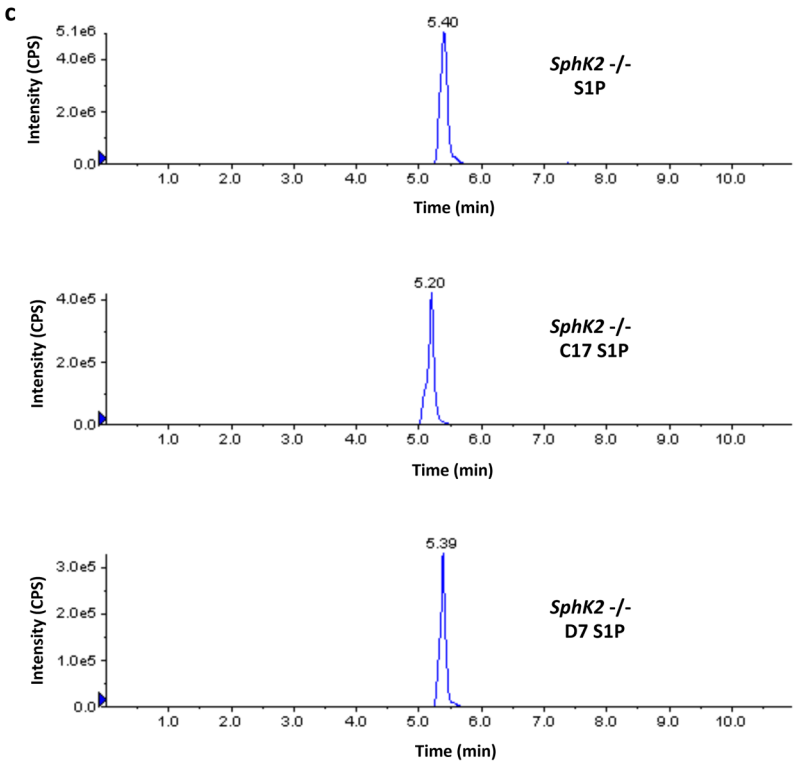
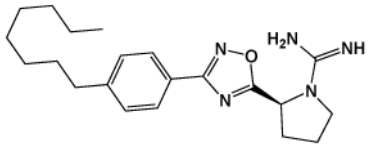
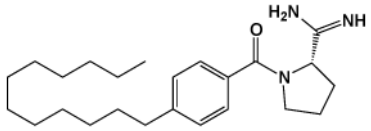


Fig. 8.

Levels of S1P and C17-S1P in whole blood of wild type, SphK1 *null* and SphK2 *null* mice. Blood samples (20 μl) from a wild type (panel A), a *Sphk1* $^{-/-}$ (panel B) and a *Sphk2* $^{-/-}$ (panel C) mouse (all F2 hybrids) were analyzed by LC/MS for the presence of S1P and C17-S1P. Recovery was evaluated using deuterated (D7) S1P as an Internal Standard. The figure shows the chromatograms produced by the Analyst software for these three analytes, which were obtained using the following transitions: S1P (380.4→264.4), C17-S1P (366.4→250.4), and D7-S1P (387.4→271.4). The intensity of the signals corresponds to the number of counts per second (cps) recorded by the mass spectrometer's ion detector. Because the Analyst software uses the height of the tallest peak as a full scale, the y-axis scale (cps) is different for each chromatogram. The numbers above the peaks correspond to the retention times in minutes. Retention time for authentic C17-S1P was 5.23 ± 0.01 min, n=3 (not shown). Amounts of analytes were calculated using the areas under the peaks and a standard curve. Areas in cps x min for the S1P, C17-S1P and D7-S1P peaks shown in the figure were as follows: wild type (7.93E+06, 1.37E+05, 2.36E+06), *Sphk1* $^{-/-}$ (3.67E+06, 3.43E+04, 2.20E+06), *Sphk2* $^{-/-}$ (4.23E+07, 2.95E+06, 2.07E+06). In these mice, which are representative of our F2 dihybrid cohort, C17-S1P is less than 2% of S1P in wild type mice and less than 1% in SphK1 null mice but is 7% in SphK2 null mice. In addition to revealing that the mole fraction of C17-S1P is higher in SphK2 null mouse blood, these chromatograms reveal also that the absolute concentration of S1P is 4–5-fold higher in SphK2 null mice than in wild type mice.

Table 1

Chemical structure and inhibitory constants of compounds **SLR080811** and **1a** [26]. The chemical structure of compounds **SLR080811** and **1a** along with their inhibitory constants (K_I) for recombinant SphK1 and SphK2 are shown. Inhibitory constants were obtained by kinetic analysis of S1P production using variable concentrations of sphingosine and a fixed concentration of ATP in presence and absence of compounds. These compounds exhibit a pattern of competitive inhibition therefore K_I's were calculated as $K_I = [I]/(K_M'/K_M - 1)$, where [I] is the concentration of inhibitor, and Km' and Km are the Michaelis constants obtained in presence and absence of inhibitor. Measurements were carried out using [^{33}P]-ATP as a tracer and a microplate-based scintillation proximity assay for the detection of [^{33}P]-S1P as previously described [23].

Compound	SphK1 K_I (uM)	SphK2 K_I (uM)
 SLR080811	12	1.3
 1a	0.1	1.5