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SPHINGOSINE KINASE TYPE 1 INHIBITION REVEALS RAPID TURNOVER OF CIRCULATING SPHINGOSINE 1-PHOSPHATE

Yugesh Kharel¹, Thomas P. Mathews^{1,2}, Amanda M. Gellett¹, Jose L. Tomsig¹, Perry C. Kennedy¹, Morgan L. Moyer², Timothy L. Macdonald^{1,2}, and Kevin R. Lynch^{1,*†}

¹University of Virginia Department of Pharmacology, 1340 Jefferson Park Avenue, Charlottesville, VA 22908

²University of Virginia Department of Chemistry, McCormick Road, Charlottesville, VA 22904

Abstract

Sphingosine 1-phosphate (S1P) is a signaling molecule involved in a host of cellular and physiological functions – most notably cell survival and migration. S1P, which signals via a set of five G protein coupled receptors (S1P1–5), is formed by the action of two sphingosine kinases (SphKs) from sphingosine. Interfering RNA strategies and SphK1 null mouse studies implicate SphK1 in multiple signaling cascades, yet there is a paucity of potent and selective SphK1 inhibitors necessary to evaluate the effects of rapid onset inhibition of this enzyme. We have identified a set of sub-micromolar, amidine-based SphK1 inhibitors and herein report using a pair of these compounds to probe the cellular and physiological functions of SphK1. In doing so, we demonstrate that our inhibitors effectively lower S1P levels in cell based assays, but we have been unable to correlate SphK1 inhibition with changes in cell survival. However, SphK1 inhibition did diminish epidermal growth factor-driven increases in S1P levels and Akt/ERK phosphorylation. Finally, administration of the SphK1 inhibitor to wild type, but not *Sphk1*^{-/-}, mice resulted in a rapid decrease in blood S1P levels indicating that circulating S1P is rapidly turning over.

Keywords

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

INTRODUCTION

Sphingosine 1-phosphate (S1P) is a bioactive lipid implicated in a host of biologic functions, including cell migration [1–3] and survival [4, 5] as well as angiogenesis [6]. S1P exerts its effects via five G-protein coupled receptors – S1P1–5 [7] and perhaps through less well-defined intracellular targets. Due in part to the remarkable clinical success of the S1P receptor agonist and immunomodulatory pro-drug, fingolimod (FTY720) [8], S1P signaling pathways are currently the subject of many investigations.

[†]To whom correspondence should be addressed: Kevin R. Lynch, Department of Pharmacology, University of Virginia Health System, 1340 Jefferson Park Avenue, Charlottesville, VA 22908-0735, Tele: 434-924-2840, Fax: 434-982-3878, KRLynch@virginia.edu.

^{*}YK and TPM contributed equally to this study

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Conflict of Interest Statement: Y.K., T.P.M., T.L.M. and K.R.L. are inventors on a patent application claiming amidine-based sphingosine kinase inhibitors including **1a** and **1b**. This application has been licensed by the University of Virginia to a commercial entity in which both T.L.M. and K.R.L. have equity positions.

Two sphingosine kinases (SphK1 & 2) are solely responsible for S1P synthesis by catalyzing the phosphorylation of sphingosine (Sph) [9, 10]. Studies with SphK1 null mice reveal that SphK1 is responsible for a substantial fraction of circulating S1P [11]. These kinases have come under increasing scrutiny as drug targets due to their role in the production of S1P and the SphK-S1P axis has been linked to cell growth, survival, angiogenesis and metastasis [12, 13].

SphK1 & 2 play a role in controlling the relative concentrations of S1P and its precursor, sphingosine, and this proposed homeostatic mechanism has been referred to as the 'sphingolipid rheostat' [14]. The implication of this concept is that SphK inhibition would simultaneously decrease S1P levels and increase concentrations of sphingosine and its precursor, ceramide. The concept of a sphingolipid homeostasis, reinforced by numerous studies using interfering RNA strategies, suggests SphKs are important drug targets for the treatment of conditions characterized by hyper-proliferation such as fibrosis and cancer. The relative importance of SphK1 versus SphK2 as a drug target remains a topic of debate with some studies indicating SphK1 [15–18], while other studies point towards SphK2 [19, 20]. Our present work is focused on SphK1.

Previously described SphK inhibitors are relatively low potency (K_i values $> 1 \mu\text{M}$) [21] and for most of these compounds their influence on the Sph : S1P ratio in either cells in culture or in whole animals has not been reported [22–25]. Further, many of the existing inhibitors are long chain bases that, like sphingosine, are toxic to cells at micromolar concentrations [26]. We recently discovered an amidine-based scaffold of SphK inhibitors that has allowed us to generate potent ($K_i < 0.2 \mu\text{M}$) and selective SphK1 inhibitors [27, 28]. In this report, we describe the use of an enantiomeric pair of these inhibitors. We observed that acute SphK1 inhibition results in a pronounced, rapid decrease in S1P levels in both cultured cells and mice. In agreement with the mitogenic effects of S1P, we found that EGF-induced phosphorylation of Akt and ERK in SKOV3 cells was antagonized by our inhibitor. We failed to observe, however, a direct effect of our inhibitor on the survival of U937 and Jurkat T cells.

EXPERIMENTAL PROCEDURES

Materials

Compounds **1a** and **1b** were synthesized as described [28]. *Sphk1*^{-/-} mice were a gift from Dr. R. Proia (NIH/NIDDK, Bethesda, MD). Compound **SKI-II** was purchased from Sigma Aldrich (St Louis, MO). C57BL/6j mice were from Jackson Laboratories (Bar Harbor, ME). Antibodies to ERK, p-ERK, Akt, p-Akt, PARP and caspase 3 were purchased from Cell Signaling Technology (Danvers, MA). Plasmids encoding diacylglycerol kinase alpha and diacylglycerol kinase zeta were gifts from Dr. Kaoru Goto (Yamagata University School of Medicine, Yamagata, Japan) and Dr. Matthew Topham (University of Utah, Salt Lake City, UT), respectively. C17 S1P and C17 sphingosine were purchased from Avanti Polar Lipids (Alabaster, AL).

Kinase assays

SphK activity was measured by a scintillation proximity assay as described by us previously [21]. Briefly, recombinant SphK1 or SphK2 were incubated in 96 well FlashPlates (Perkin-Elmer) with D-erythro-sphingosine and γ -[³³P]ATP and the [³³P]S1P product, which adheres to the plate wall, was quantified by scintillation counting. To assay ceramide kinase and diacylglycerol kinases, the recombinant proteins were incubated with γ -[³²P]ATP and substrate (C6 ceramide and 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 the effect on K_m and V_{max} determined.

Lipid extraction

Extraction protocols and LC/MS procedures were from Shaner *et al.* [29] with minor modifications. Cell pellets (approximately 5 million cells) or whole blood (20 μ L) were mixed with 2 mL of a 3:1 methanol : chloroform mixture and transferred to a capped glass vial. To this suspension was added 10 μ L of internal standard solution containing 1 μ M C17 S1P, 1 μ M C17 sphingosine and 1 μ M of an undecyl analogue of **1a** and **1b** (dodecyl). The mixture was homogenized 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 μ L of 1M KOH in methanol. The samples were again sonicated and incubated at 37°C for 2 hours. After this time, the samples were neutralized through the addition of 20 μ L of glacial acetic acid and transferred to 2 mL microcentrifuge tubes. Samples were then centrifuged at 10,000 \times g for 10 minutes at 4°C. The supernatant fluid was collected in a separate glass vial and the pellets discarded. The resulting solution was evaporated under a stream of nitrogen gas. Immediately prior to LC/MS analysis, the material was dissolved in 300 μ L of methanol and centrifuged at 12,000 \times g for 12 minutes at 4°C. Fifty μ L of the resulting supernatant fluid were analyzed by LC/MS.

LC/MS protocol

Analyses were performed by Liquid Chromatography/ESI Mass Spectrometry (LC/MS) using a triple quadrupole mass spectrometer (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 (50mm \times 2.1 mm, 5 μ m 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) methods previously described [29] as follows: C₁₇S1P (366.4 \rightarrow 250.4); S1P (380.4 \rightarrow 264.4); dihydroS1P (382.4 \rightarrow 266.4); C₁₇sphingosine (286.4 \rightarrow 250.3); sphingosine (300.5 \rightarrow 264.4); sphinganine (302.5 \rightarrow 260.0). Fragmentation of compounds **1a** and **1b** was analyzed by direct infusion of 1 μ M solutions in methanol : formic acid (99:1) and the following transitions were found to produce the most intense product ions in positive mode: C₁₂-proline-amidines **1a**, **1b** (386.3 \rightarrow 273.1); VPC96077 (C₁₁-proline-amidine) (372.5 \rightarrow 259.2). All analytes were analyzed simultaneously using the afore mentioned MRMs. Voltages (DP, EP, CE and CXP) for **1a** and **1b** were: 35, 10, 25, 6; and 156, 10, 25, 14 volts, respectively. Retention times for all analytes under our experimental conditions were between 5.1 and 5.6 min. The following ceramides were measured by monitoring the m/z 264.4 product ion as previously described [29] using a Supelco Supelcosil LC-NH₂ column (50mm \times 2.1 mm, 3 μ m bead size) : 12:0, 16:0, 18:1, and 24:1. Quantification was carried out by measuring peak areas using commercial software (Analyst 1.5.1).

Cell culture

U937 and Jurkat T cells were grown as described [22]. Briefly, cells were grown in RPMI 1640 media supplemented with L-glutamate, 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in an atmosphere containing 5% CO₂. SKOV3 cells were grown in MyCoy's 5a media supplemented with 10% FBS and penicillin/streptomycin at 37°C in an

atmosphere containing 5% CO₂. Twenty-four hours before adding inhibitors, the growth media was replaced with media containing 0.5% or 2% FBS.

Western blot analysis

Cells were incubated with various concentrations of inhibitor for the times indicated (usually 2 h). After incubation, cells were washed with phosphate-buffered saline and lysed using a Dounce homogenizer. Equal amounts of protein were resolved by SDS-PAGE analysis using 10% polyacrylamide gels and resolved proteins transferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween 20 for 1 hour at room temperature. After rinsing, membranes were incubated with antibodies (diluted 1:1000 in TBS) against ERK, p-ERK, Akt, p-Akt, caspase-3, PARP or β -actin for 1 hour. After washing three times in TBS buffer, the nitrocellulose membrane was incubated with a 1:2000 dilution (in TBS) of HRP-conjugated anti-IgG antibody. Detection was accomplished by chemiluminescence using a commercial kit (Perkin Elmer Western Lightning).

MTT cell viability assay

U937 and separately Jurkat T cells were plated in a 96 well plate at a density of 50–60,000 cells per well. Cells were grown overnight in 2% FBS and then treated with the indicated concentration of compound overnight. The TACS™ MTT assay was performed according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Briefly, MTT reagent (10 μ L per 100 μ L of cell culture medium) was added to each well and the plate was incubated at 37°C for 4 h, followed by incubation with Detergent Reagent at room temperature for 2 h. Absorbance was measured at a wavelength of 570 nm.

Pharmacokinetic analysis

Groups of 8–12 week old mice (strain: C57BL/6j) were anesthetized with methoxyflurane and injected into the tail vein with either **1a**, **1b** (dose: 10 mg/kg) or an equal volume of vehicle. The vehicle was a 2% solution of hydroxypropyl- β -cyclodextrin (Cargill Cavitrone 82004) in water. After injection, animals were lightly anesthetized and bled from the retro-orbital sinus at the specified time points. (ASAP time points were 1–2 minutes after dosing). Blood was extracted immediately as described above for lipid and drug analysis. 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

We have described previously a set of SphK inhibitors with an amidine 'warhead' [27]. A homology model suggests that the amidine group interacts directly with ATP via a bidentate chelation of the γ -phosphate [28]. In the design of biologically active small molecules, rigid analogues are a structural motif often used to improve selectivity among related targets. For example, we found that restricting the rotatable bonds of FTY720 analogues has a significant impact on their rate of phosphorylation by SphK1 & 2 [30]. To increase selectivity and potency for SphK1, we designed a rigid analogue of our previously reported amidine-based SphK inhibitors. The limited rotational degrees of freedom of rigid analogues were expected to provide greater structural differences between stereoisomers. Indeed, the enantiomers of our proline analogue exaggerated the differences in activity at SphK1 over those described in our initial study [27]. Our structure activity relationship studies also identified the 12 carbon alkyl tail length analogues as the most potent for SphK inhibition

[27, 28]. Taking these structural considerations into account, we selected enantiomers **1a** and **1b** (structures in Table 1) for in depth characterization.

Evaluation of **1a** and **1b** in vitro

We first determined the K_i values of **1a** and **1b** at SphK isotypes by measuring the synthesis of [^{33}P]-S1P catalyzed by recombinant SphK1 and SphK2 [21]. In agreement with our previous findings [27], the *s*-enantiomer (**1a**), derived from L-proline, was significantly more potent at SphK1 than its *r*-counterpart (**1b**). Due to their enantiomeric nature, comparing these compounds in biological systems is useful in establishing the target selectivity of the inhibitors. Importantly, there is a >100-fold difference in potency between **1a** (K_i 0.1 μM) and **1b** (K_i 16 μM) at SphK1, thus differences in biologic responses to these compounds can reasonably be assigned to activity at SphK1. Our amidine-based SphK1 inhibitors, including **1a** and **1b**, are competitive with sphingosine, but not with ATP. Therefore, as a further assessment of selectivity, we tested **1a** at related lipid kinases including ceramide kinase and diacylglycerol kinase isotypes alpha and zeta. At a concentration of 3 μM , there was no inhibition of these lipid kinases (data not shown).

Evaluation of **1a** and **1b** on cultured cells

We next determined whether the inhibitors were capable of penetrating cells and inhibiting SphK1 therein. Due to high levels of SphK1 expression and ease of culture, we chose human monocyte U937 cells as our primary model system for the evaluation of SphK1 inhibitors [31]. Spiegel and co-workers had also evaluated their mono-methylated sphingosine analogue **SKI-1** using U937 cells thus affording comparison of SphK1 inhibitors while recognizing possible differences in strains of U937 cells [22].

We treated U937 cultures with either enantiomer and quantified the accumulation of cell-associated S1P, dhS1P, sphingosine (Sph), sphinganine (dhSph) and **1a** or **1b**. As depicted in Figure 1, treatment of cultures with **1a** or **1b** for 2 hours resulted in diminished S1P (Figure 1a) and dhS1P (Figure 1b) in a drug concentration-dependent manner. As predicted from assessment at recombinant SphK1, enantiomer **1a** was significantly more potent than **1b**; the IC_{50} value for **1a** was about 0.03 μM while the IC_{90} value was about 0.3 μM . Concomitant with the decrease in the phosphorylated species, both sphingosine and sphinganine levels rose on treatment with **1a**, but the extent of the increase was 2-fold or less (Figure 1c). The decrease in S1P was observed as soon as 10 minutes after inhibitor addition (not shown) and persisted for at least 24 hours (Figure 1a). The amount of cell-associated **1a** and **1b** increased proportionate to added compound and significant amounts of compound remain cell-associated after 24 hours (Figure 1d). One abundant ceramide species, C16:0 ceramide, was also slightly increased after 2 hr and significantly elevated after 24 hr of **1a**, but not **1b**, treatment (Figure 1e). A similar increase, albeit to a lesser extent, was also observed with C18:1 and C24:1 ceramides (data not shown). These data are consistent with the observations reported by Paugh *et al.* [22]. Treatment of another cell line, human T cell leukemia Jurkat T cells, for 2 hours with **1a** (but not **1b**) also resulted in decreased S1P and increased sphingosine levels (Figure 1f–1g), although the magnitude of the changes were less than with U937 cells.

To confirm that the observed decrease in S1P accumulation in response to **1a** was the result of decreased synthesis (as opposed to increased degradation / export), we added exogenous sphingosine and measured S1P in U937 cells with or without **1a** in the culture medium. Cells supplemented with sphingosine to 0.3 or 1 μM exhibited pronounced increases in both sphingosine and S1P after two hours (Figure 2). The concomitant addition of **1a** to 0.3 μM largely blocked the appearance of S1P (Figure 2a) while exaggerating the accumulation of sphingosine (Figure 2b). These results indicate that the decrease in S1P levels observed in

U937 cells treated with **1a** is primarily the result of blockade of SphK1 activity. Presumably, the decreased S1P levels observed as a consequence of **1a** treatment (Figure 1a) occur because S1P metabolism by phosphatases and/or S1P lyase, and/or S1P export proceeds unimpeded while synthesis is blocked. These results also document that the inhibitors are readily taken up by U937 and Jurkat T cells.

The ability to block SphK activity in U937 cells enabled an examination of cell signaling and survival by these cells in response to the blockade. A previous report documenting the effects of another SphK1 inhibitor, **SKI-1** (SphK1 K_i 10 μ M), on U937 cells ascribed decreased cell survival to the blockade of S1P biosynthesis [22]. Specifically, treatment with 20 μ M of **SKI-1** blocked the constitutive phosphorylation of ERK and Akt that is characteristic of U937 cells [22]. Therefore, we asked whether treatment with **1a** (K_i 0.1 μ M) might have similar effects on ERK and Akt phosphorylation by U937 cells. As depicted in Figure 3, we did not detect a change in ERK phosphorylation at **1a** applied at 0.3 μ M – a concentration that results in a substantial blockade of S1P synthesis (see Figure 1 and Figure 2). Effects on ERK phosphorylation were observed only at high **1a** concentration (10 μ M) or after prolonged exposure times (> 2h). We observed a similar pattern for Akt phosphorylation, although even longer exposure times (16 hours) were required to observe an effect. Paugh *et al.* [22] also reported activation of PARP cleavage after treating Jurkat T cells with 10 μ M **SKI-1**. We observed this activity after **1a** treatment, but only if the inhibitor was present for 16 hours at a concentration (10 μ M) far in excess of that required to inhibit SphK1 effectively (Figure 3b). Since **SKI-1** was not available to us for direct comparison, we tested the widely used inhibitor, **SKI-II** [32], a low affinity, non-selective SphK inhibitor (K_i 12–40 μ M) [21] that we have found previously lowers S1P levels 4-fold in U937 cells when added at 10 μ M for 2 hours [28]. In the present study, **SKI-II** inhibited ERK phosphorylation but, like **1a**, only when added for > 2 h to U-937 cells. Likewise, cleavage of PARP in response to **SKI-II** treatment required extended (16 hour) treatment of Jurkat T cells.

To ascertain whether inhibition of SphK correlated with cytotoxicity, we treated cultures of U937 (Figure 4a) and Jurkat T (Figure 4b) cells with 0.3–10 μ M **1a** or **1b** for 24 hours and assessed cell viability with an MTT assay. As documented in Figure 4, both **1a** and **1b** exhibit cytotoxic effects on the cells, but only at concentrations far greater than those required to inhibit S1P synthesis. The threshold for cytotoxicity was about 1 μ M, which is a value 10-fold higher than is required to significantly reduce S1P levels in these cells. Finally, we observed no change in the growth of U937 cells cultured in the presence of **1a** at concentrations of up to 1.0 μ M (not shown).

Our results indicate that inhibition of SphK1 in U937 cells does not correlate with inhibition of Akt or ERK phosphorylation. Because U937 cells are unusual in their constitutively high levels of pAkt and pERK, we considered the possibility that SphK1 may play a role in growth factor-mediated phosphorylation of Akt and ERK, a hypothesis that has been supported in several experimental models [23]. To test this hypothesis we used cultured human ovarian cancer SKOV3 cells. Unlike U937 cells, SKOV3 cells have low basal levels of pAkt and pERK, and pAkt and pERK can be stimulated by epidermal growth factor (EGF) treatment. SKOV3 cells, similar to U937 and Jurkat T cells, are sensitive to inhibitor **1a** as revealed by the reduction of S1P levels observed after treatment with 0.3 μ M **1a** (Figure 5a). We found that S1P levels in SKOV3 cells rose in response to EGF treatment and that this increase in S1P was antagonized by inhibitor **1a**, but not **1b**, treatment (Figure 5b). We observed a similar pattern regarding EGF-evoked increases in pAkt and pERK levels (Figure 5c).

In vivo evaluation of **1a**

Lastly we determined whether **1a** blocked SphK1 in animals. Hla and colleagues reported that intravenously (IV) administered C17-S1P was cleared from mouse circulation with a 15 minute half life [33]. Therefore we reasoned that an effective SphK1 blockade in mice would likewise result in a rapid diminution of endogenous circulating S1P. To this end we determined the levels of sphingosine and S1P in the blood of mice that were injected IV with **1a** or **1b** and the levels of these sphingolipids as well as **1a** or **1b** were measured in blood samples obtained at various times after injection. We found that whole blood S1P levels dropped rapidly to about one-half of control levels shortly after injection of **1a**. In contrast, administration of the same dose (10 mg/kg) of the less potent enantiomer, **1b**, or vehicle, did not change blood S1P (Figure 6a). As an additional assessment of target specificity, we injected **1a** or vehicle into SphK1 null (*Sphk1*^{-/-}) mice. In these mice, the same dose of compound **1a** did not change blood S1P levels (Figure 6a). There was no significant change in blood sphingosine levels in either type of mouse (Figure 6b). Finally, Figure 6c documents that both **1a** and **1b** are rapidly cleared from circulation, *i.e.* > 90% of the compound was cleared from the bloodstream by 60 minutes after injection. We performed a parallel study in rats and observed the same result – a rapid decrease in blood S1P levels and rapid clearance of the test compounds. In the rat study, where blood was drawn over a 4 hr time course, we found that blood S1P level returned to pre-dose levels at 2 hr post injection (not shown).

DISCUSSION

The pro-survival, pro-migratory and mitogenic effects of S1P coupled with the inducibility of SphK1 by a wide variety of stimuli have generated repeated suggestions that SphK1 could be a viable therapeutic target for small molecule inhibition in diseases characterized by hyper-proliferation, particularly cancer [34, 35]. Numerous studies deploying interfering RNA strategies implicate SphK1 in a variety of signaling cascades, which lends credence to SphK1 as a drug target. However, the SphK1 inhibitors necessary to learn whether rapidly inhibiting enzyme activity recapitulates the effects observed after slowly decreasing protein levels are largely lacking. With few exceptions [36], the inhibitors described heretofore are low potency (K_i values are on the order of 10 μ M), non-isotype selective [21] and have not been documented as inhibiting SphK1 enzymatic activity in cells or animals. Further, most of these inhibitors are long chain bases that tend to be cytotoxic to cells, making their use at micromolar concentrations problematic.

In this report, we describe an inhibitor that selectively blocks SphK1. Our claim for selectivity is based on a rank order of IC_{50} values in cells that matches the K_i values (**1a** more potent than its enantiomer, **1b**) and the lack of an effect of **1a** on circulating S1P levels in SphK1 null mice. Although these results build confidence, we cannot rule out off-target effects on, for example, other proteins that bind sphingosine, by these long chain bases particularly at micromolar concentrations. Concern about the non-specific cytotoxicity of long chain bases is addressed to some extent by the use of **1b**. Ultimately, the specificity of SphK1 inhibition by small molecules is best established by comparison of multiple inhibitors with a variety of structures.

Compound **1a** decreased S1P in cultured U937, Jurkat T and SKOV3 cells and prevents the conversion of Sph to S1P in these cells. Further, we observed no evidence of cell toxicity at concentrations (0.3 μ M) of **1a** that effectively inhibit SphK1 for treatment periods up to 24 hours. The cellular toxicity that we did observe occurred at concentrations that far exceeded those required to block S1P production. Moreover, equivalent cytotoxicity was observed at equal concentrations of **1a** and **1b** despite their >100-fold difference in K_i values at SphK1.

Our data do not support the contention of Paugh *et al.* [22] that a SphK1 blockade affects growth, viability or signaling in U937 within a 24 hour time window. Indeed, we were unable to detect any effect on U937 cells despite a nearly complete blockade of the conversion of sphingosine to S1P by SphK1. Only when inhibitor was present at high concentrations (10 μ M) could we evoke a response from these cells, and at these concentrations the enantiomers were equipotent, which suggests to us an off-target mechanism. However, we found that SphK1 blockade with **1a** did inhibit EGF stimulated accumulation of pAkt and pERK in SKOV3 cells. At this juncture, we do not know whether the S1P produced in response to EGF is acting through cell surface S1P receptors in an autocrine fashion or through some other intracellular mechanism that is independent of canonical S1P receptors [37].

The concept of sphingolipid homeostasis – cells balancing the relative levels of sphingosine / ceramide and S1P – is intriguing. An implication of this idea is that blockade of the conversion of sphingosine to S1P will both lower S1P and raise sphingosine and ceramide levels. Reportedly, sphingosine levels are approximately 10-fold higher than S1P in cells and ceramide levels are another log order higher [38]. Given this 100-fold difference between cellular levels of ceramide and S1P, we thought it unlikely that SphK inhibition would increase ceramide substantially *per se*. Indeed, when SphK1 is inhibited by **1a**, S1P levels decrease more than 10-fold, while sphingosine levels rise barely 2-fold in U937 cells while changes in ceramide levels are undetectable (Figure 1).

The rapid decrease in circulating S1P in response to SphK1 inhibition by **1a** observed *in vivo* is intriguing. In addition to providing compelling evidence of the selectivity of **1a** for the SphK1 isotype, this result indicates that blood S1P levels can serve as a biomarker of SphK1 inhibition. We do not know the mechanism(s) whereby S1P is cleared from blood, but an obvious potential route is de-phosphorylation catalyzed by the ubiquitous lipid phosphate phosphohydrolases; another is by S1P lyase. We found that the S1P concentration in whole mouse blood is about 3 μ M (Figure 6a) while that in mouse plasma is about 10-fold lower (not shown). Such a difference is expected in view of the observation that erythrocytes have high S1P levels [39, 40]. It will be interesting to learn whether, following SphK1 inhibition, plasma S1P levels remain constant while whole blood S1P stores are depleted. Regardless of the mechanism(s), our data suggest that the S1P pool in blood is in a state of rapid flux. Interestingly, a structurally similar bioactive lysophospholipid, lysophosphatidic acid, also turns over rapidly in circulation [41, 42].

The existence of S1P in the bloodstream at low micromolar levels and in plasma at high nanomolar levels suggests a physiologic role for this molecule. Studies with the S1P receptor agonist pro-drug, FTY720 (fingolimod), led to the realization that S1P is involved in egress of lymphocytes from secondary lymphoid tissues and implicated S1P in controlling heart rate [43]. A third role for circulating S1P was suggested by administration of S1P1/3 receptor antagonists, which elicit vascular leakage acutely in mice [44, 45]. The decrease in blood S1P in response to SphK1 inhibition provides, in theory, a method to test whether acute changes in circulating S1P levels influence blood lymphocyte levels, heart rate and/or capillary integrity.

Signaling at the S1P1 receptor is critical for proper lymphocyte egress from lymphoid tissues [46]. Therefore we considered using **1a** to induce lymphopenia in animals. However, the short half-life of **1a** *in vivo* coupled with the observation that normal lymphocyte trafficking is maintained until plasma S1P becomes very low [46] makes detecting changes in circulating lymphocytes problematic with this particular compound. Injected S1P slows heart rate in anesthetized rats [47], but we do not yet know whether the 50% reduction in blood S1P achieved after **1a** injection increases heart rate in rodents. We have measured

pulmonary leakage of Evans blue dye in mice injected with **1a** to assess the effects of a decreased S1P circulatory tone on the endothelial barrier. However, our results to date have been inconsistent. In future studies, we will attempt to magnify the effect of SphK1-selective inhibitors by using SphK2 null (*Sphk2*^{-/-}) mice and analogs that persist longer *in vivo*.

In sum, administration of compound **1a** mounts a sustained, selective blockade of SphK1 in cultured cells and a transient, blockade *in vivo*. This inhibition neither induces a cell stress response nor limits proliferation by U937 cells, but the inhibition does blunt an EGF response in SKOV3 cells. Although too short lived *in vivo* to be useful in animal models of disease, **1a** is adequate in establishing persistent SphK1 inhibition in cultured cells. We will be particularly interested to learn how well the results obtained by the slow onset, partial reduction in SphK1 protein levels correlate with the rapid inhibition of SphK1 catalytic activity achieved by compounds such as **1a**.

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Abbreviations used in this text

S1P	sphingosine 1-phosphate
Sph	sphingosine
SphK1	sphingosine kinase type 1
SphK2	sphingosine kinase type 2
LC/MS	liquid chromatography / mass spectrometry
HPCD	hydroxypropyl-β-cyclodextrin
dhS1P	dihydrosphingosine 1-phosphate
dhSph	dihydrosphingosine (sphinganine)

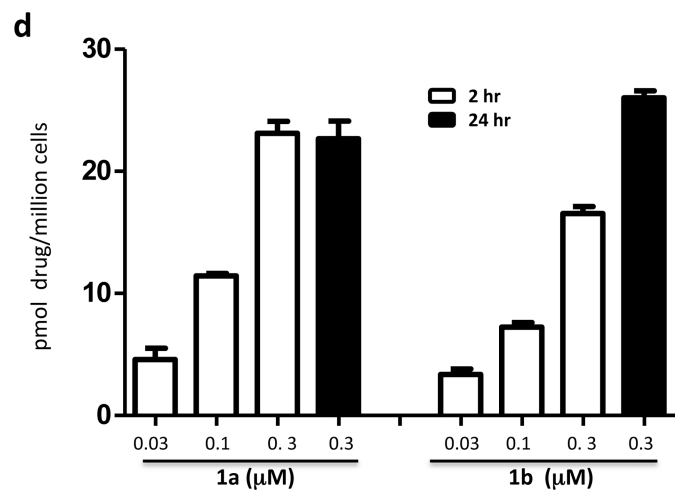
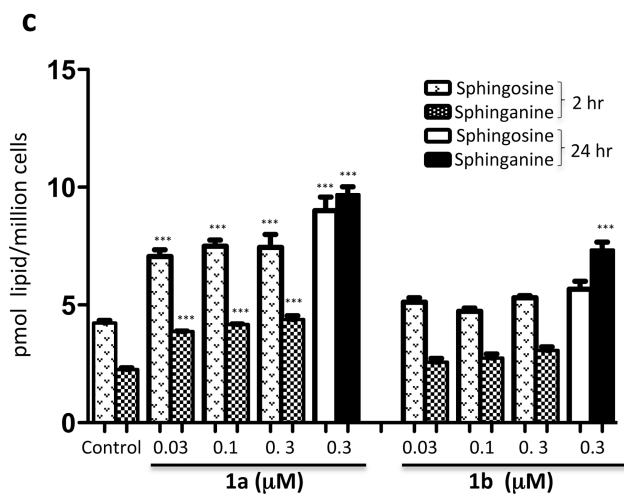
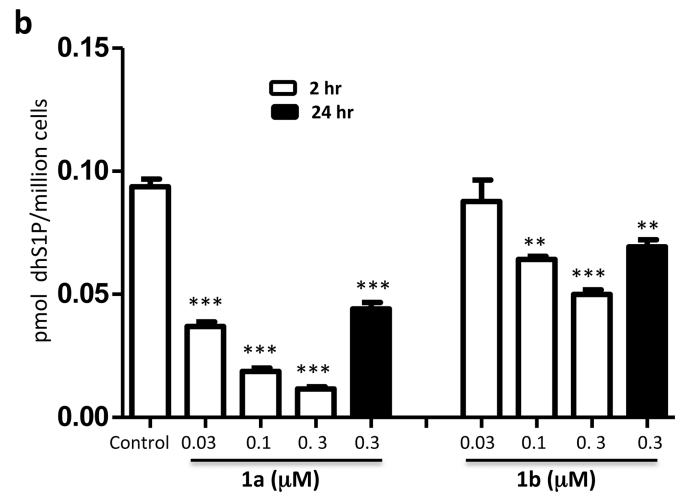
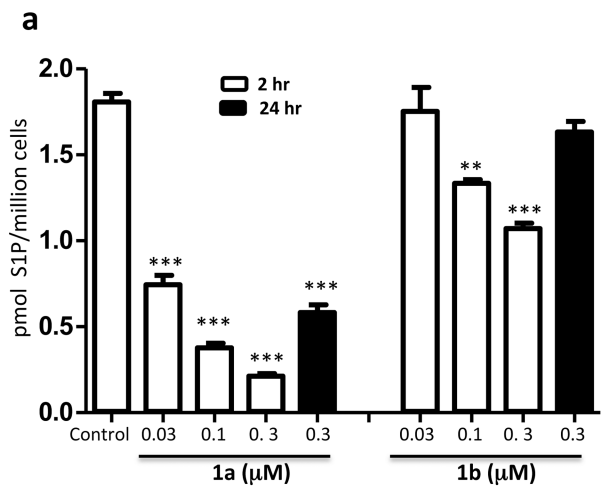
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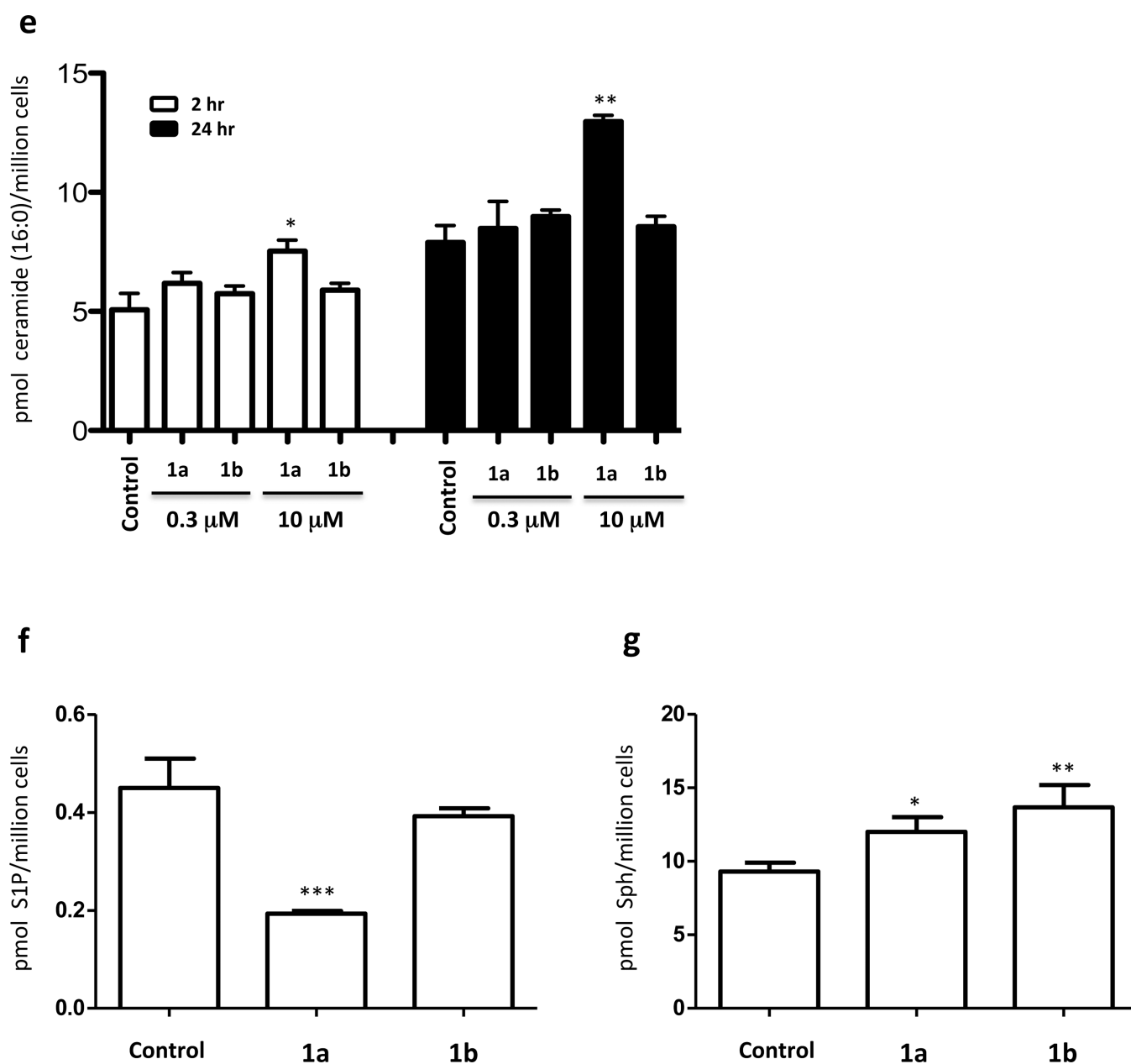


Fig. 1. Levels of sphingolipids and compounds **1a** and **1b** in cell cultures treated with various concentrations of compounds **1a** and **1b**. Cultured U937 and Jurkat T cells were exposed to different concentrations of compounds **1a** and **1b** as indicated in the figure. After a 2 or 24 h period of exposure, cells were harvested, lysed and the amounts of sphingolipids and compounds **1a** and **1b** in the lysates were measured by LC/MS as described in the Methods section. **a**: S1P in U937 cells; **b**: dihydroS1P in U937 cells; **c**: sphingosine and sphinganine in U937 cells; **d**: **1a** and **1b** in U937 cells; **e**: C16:0 ceramide level in U937 cells; **f**: S1P in Jurkat T cells; **g**: sphingosine in Jurkat T cells. Amounts in cells are expressed as the number of pmoles *per* million cells. Drug concentrations refer to the concentration of drugs in the culture medium. Data are means \pm SD of three independent experiments. * $p < 0.05$, ** $p <$

0.01, *** $p < 0.001$ (1 way ANOVA, and Dunnett's Multiple Comparison Post Test, compared to Control).

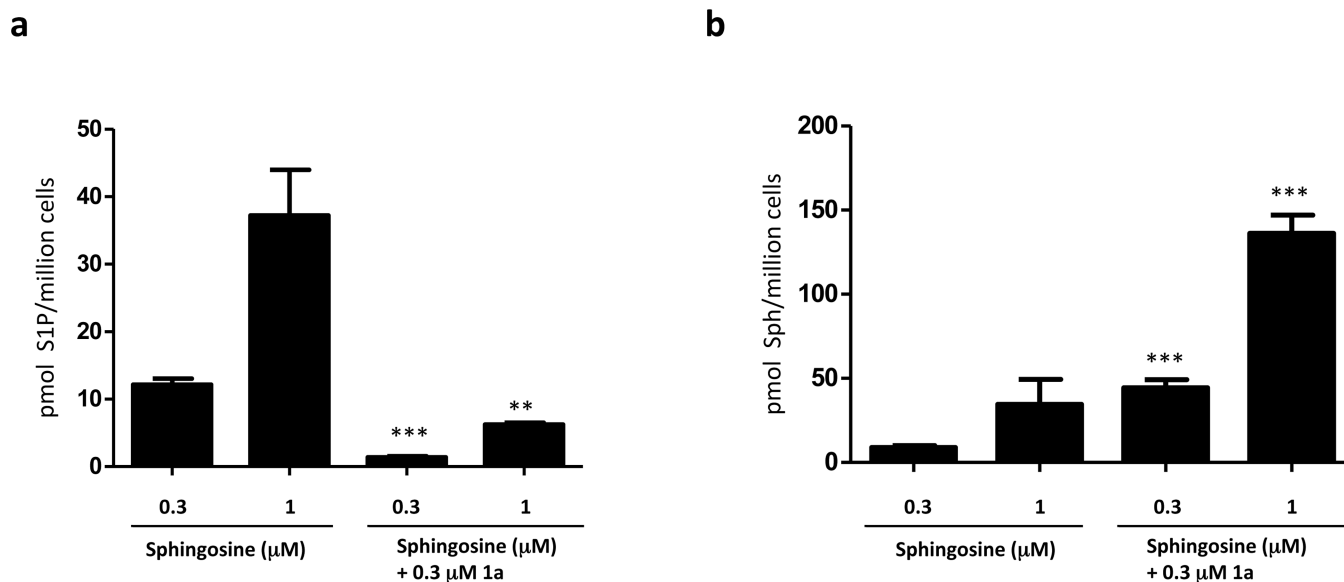
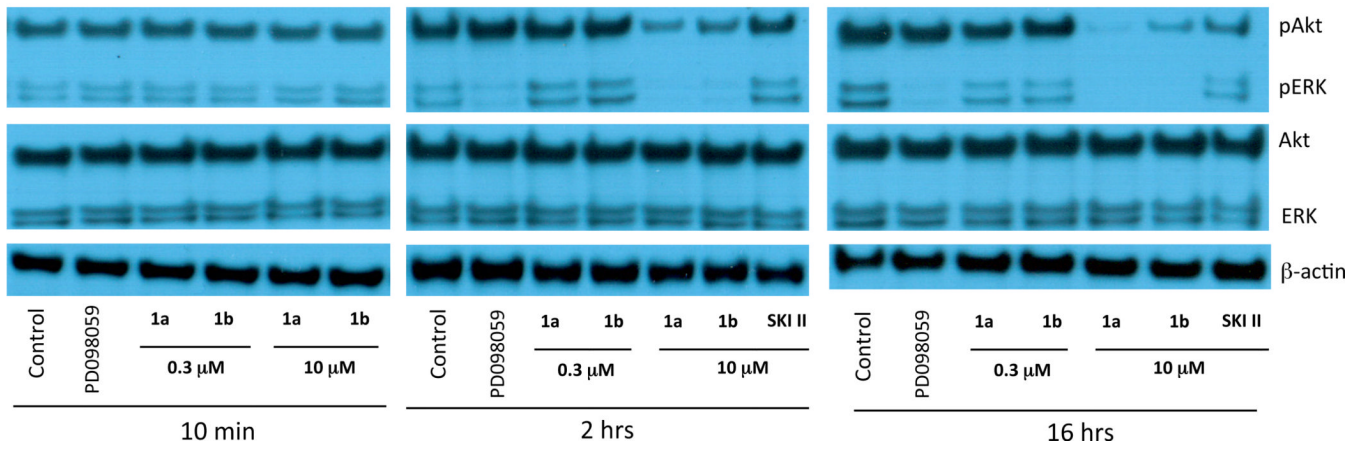
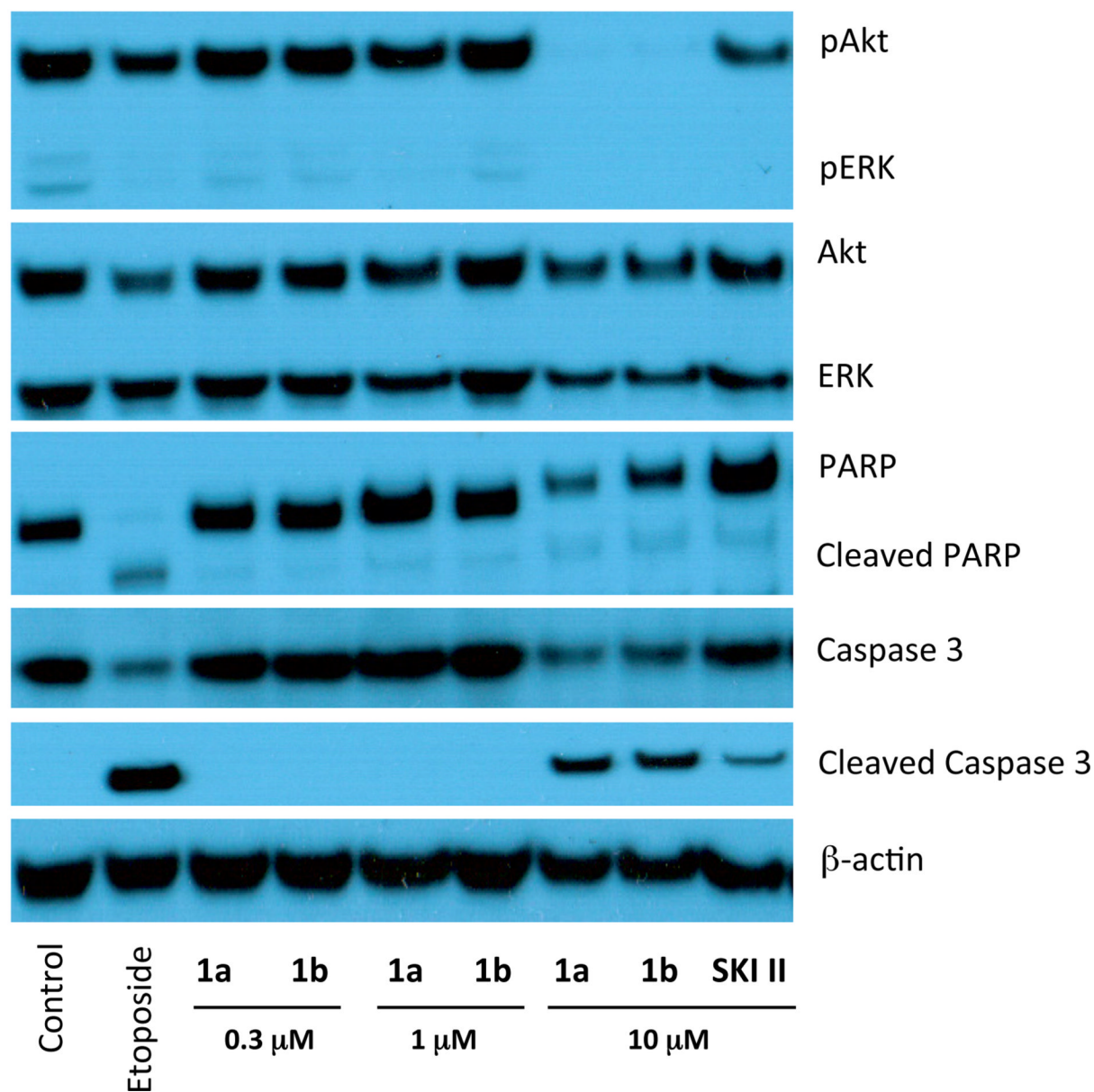


Fig. 2. Levels of S1P and sphingosine in U937 cells treated with sphingosine and compound **1a**. Cultured U937 cells were exposed to different concentrations of sphingosine and 0.3 μM **1a** as indicated in the figure. After 2 hours, cells were harvested by centrifugation and the amounts of S1P and sphingosine associated with the cell pellet were measured by LC/MS. a: S1P; b: sphingosine. Amounts are expressed as the number of pmoles *per* million cells. Drug and sphingosine concentrations refer to the concentration of these molecules in the culture medium. Data are means ± SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ (Unpaired two-tailed T-test, comparing 0.3 μM sphingosine treatment to 0.3 μM sphingosine + 0.3 μM **1a** treatment; separately comparing 1 μM sphingosine treatment to 1 μM sphingosine + 0.3 μM **1a** treatment.)

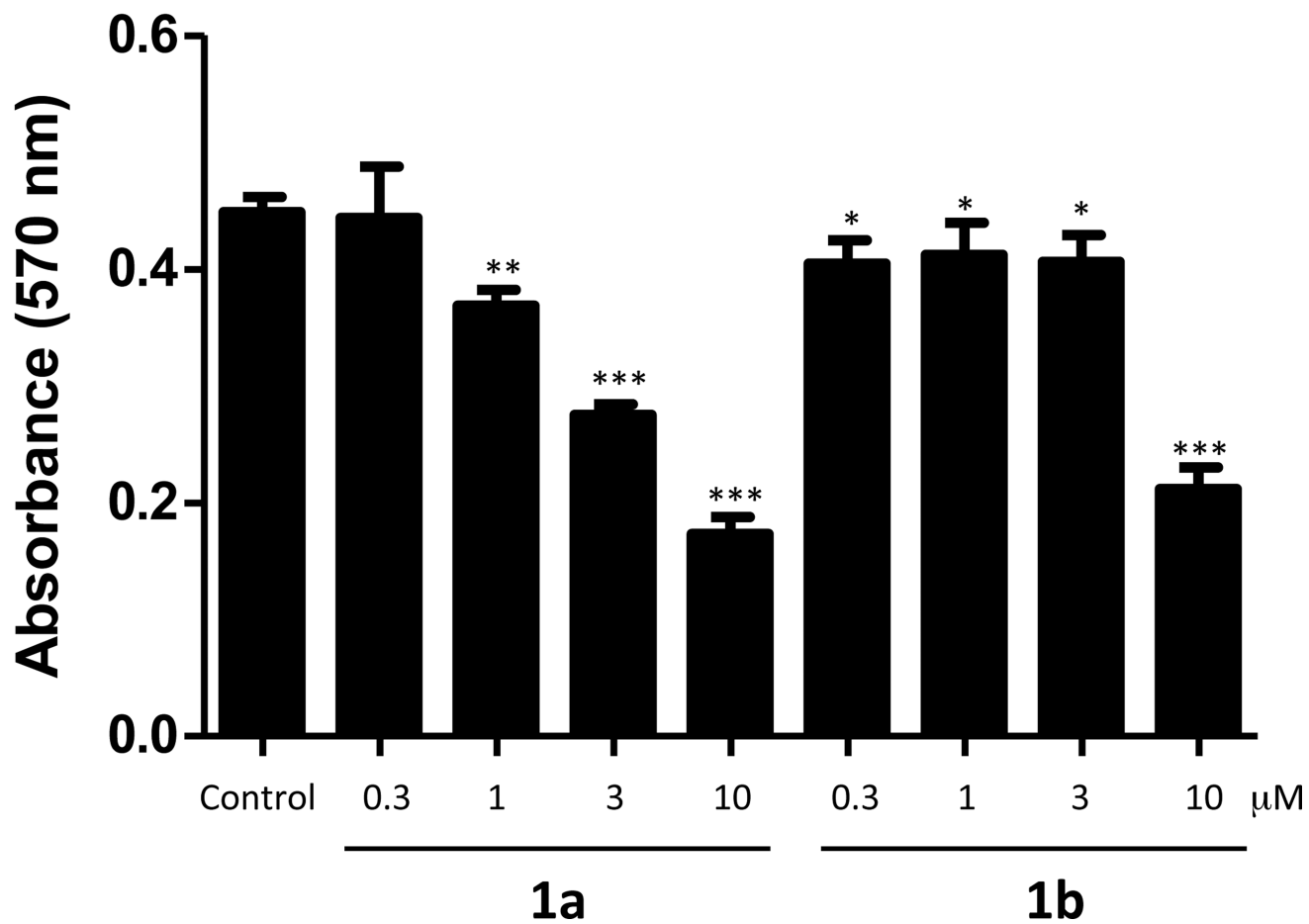
a



b**Fig. 3.**

Expression of proteins related to cell viability in U937 and Jurkat T cells treated with compounds **1a** and **1b**. a: Cultured U937 cells were treated with the indicated concentrations of compounds **1a** and **1b** (or 10 μM **SKI-II**) for the indicated times. Cells were then lysed and samples containing the same amount of protein (40 μg) were analyzed by SDS PAGE/ Western blotting using primary antibodies against the phosphorylated (pERK and pAkt) and de-phosphorylated forms of ERK and Akt. PD098059 (10 μM) was used as a positive control for inhibition of ERK phosphorylation. b: Cultured Jurkat T cells were treated with the indicated concentrations of compounds **1a** and **1b**, or 10 μM **SKI-II**, for 16 h. Cells were

then lysed and analyzed as described for panel using primary antibodies against the phosphorylated (pAkt/pERK) and de-phosphorylated forms of Akt/ERK, and the cleaved and un-cleaved forms of Caspase 3 and PARP. Etoposide (5 μ M) was used as a positive control for caspase activation. In both panels β -actin was used as a loading control.

a

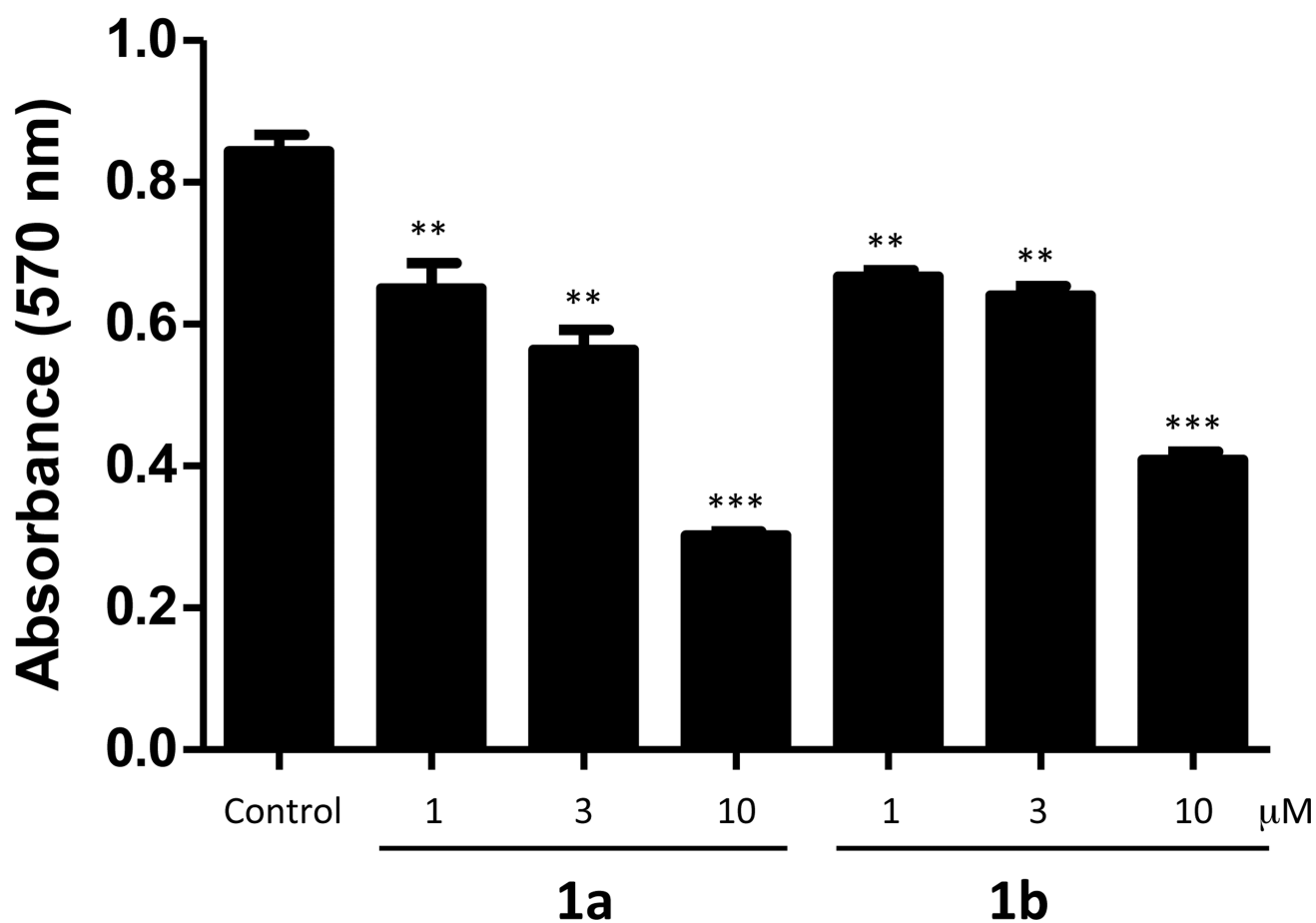
b

Fig. 4. Viability of U937 and Jurkat T cells treated with compounds **1a** and **1b**. Cultured U937 and Jurkat T cells were exposed to different concentrations of compounds **1a** and **1b** as indicated in the figure. After a 24 h period of exposure, the viability of cells was measured as their ability to reduce a tetrazole compound to the corresponding purple formazan dye (MTT assay). Viability is directly proportional to the amount of formazan dye produced as measured by light absorbance at 570 nm. a: U937 cells, b: Jurkat T cells. Data are means \pm SD of six independent experiments. * $p < 0.05$, *** $p < 0.001$ (1 way ANOVA, and Dunnett's Multiple Comparison Post Test, compared to Control).

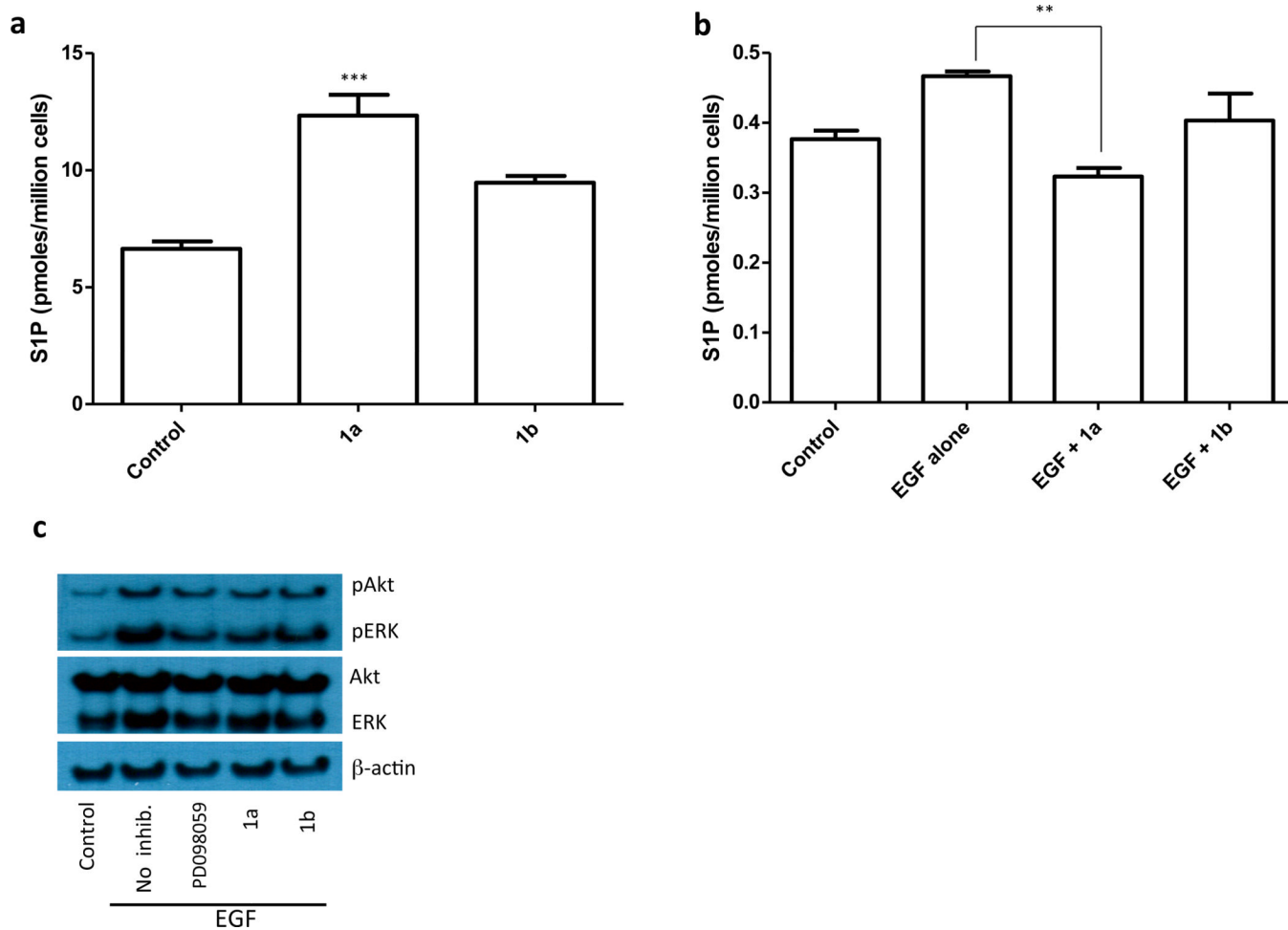
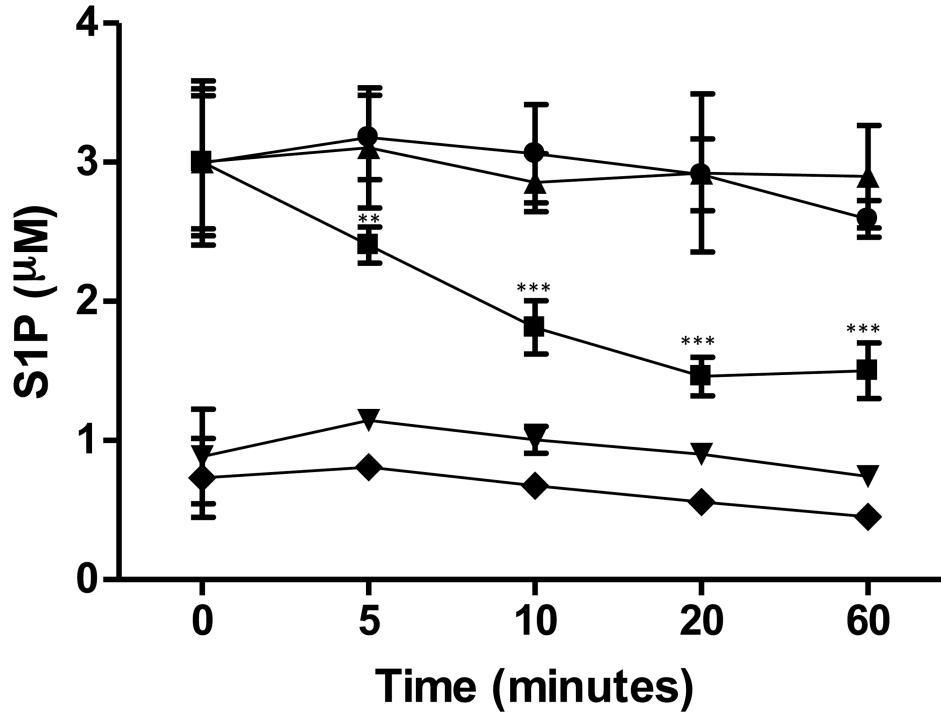


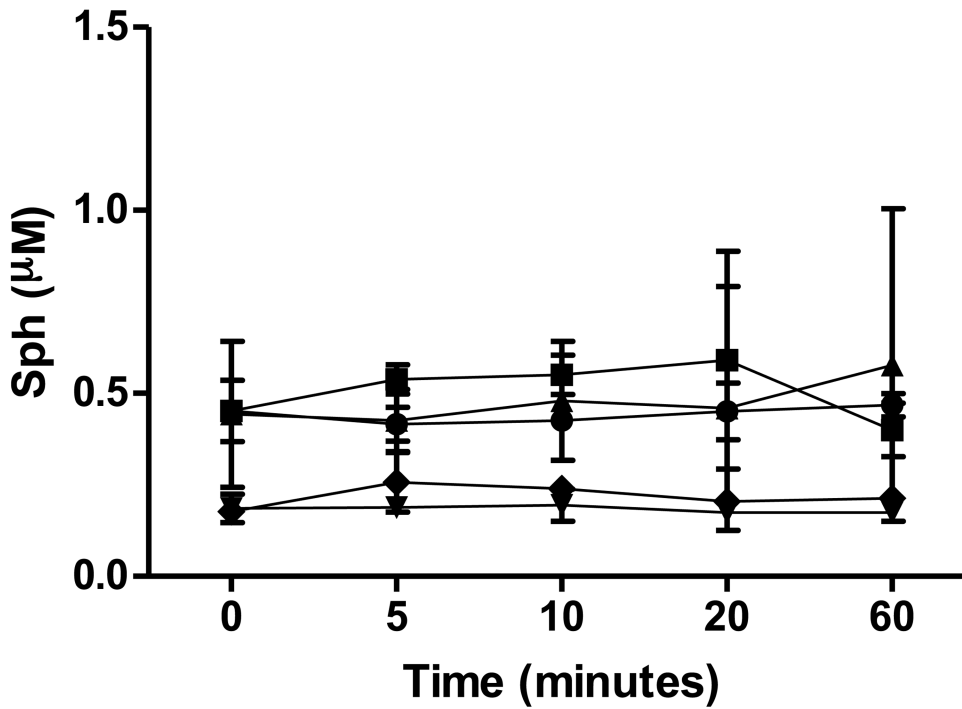
Fig. 5. Effect of SphK1 inhibition on S1P levels and Akt/ERK phosphorylation by SKOV3 cells. **a:** Cultured SKOV3 cells were treated with 0.3 μ M **1a** or **1b** for 1 hour. Cells were then, harvested and the amount of cell-associated S1P was measured by LC/MS. **b:** SKOV3 cells were treated for 1 hour with 0.3 μ M **1a** or **1b** and then stimulated with EGF (10 ng/ml) for 10 min. Cells were then harvested, lysed and the amount S1P was measured by LC/MS. **c:** Cultured SKOV3 cells treated as described in panel b were analyzed by Western blotting using primary antibodies against the phosphorylated Akt/ERK, total Akt/ERK and β -actin (loading control). The MAP kinase inhibitor PD098059 was used as a positive control.

a



- Vehicle
- 1a
- ▲ 1b
- ▼ SK1-/- Veh
- ◆ SK1-/- 1a

b



- Vehicle
- 1a
- ▲ 1b
- ▼ SK1-/- Veh
- ◆ SK1-/- 1a

c

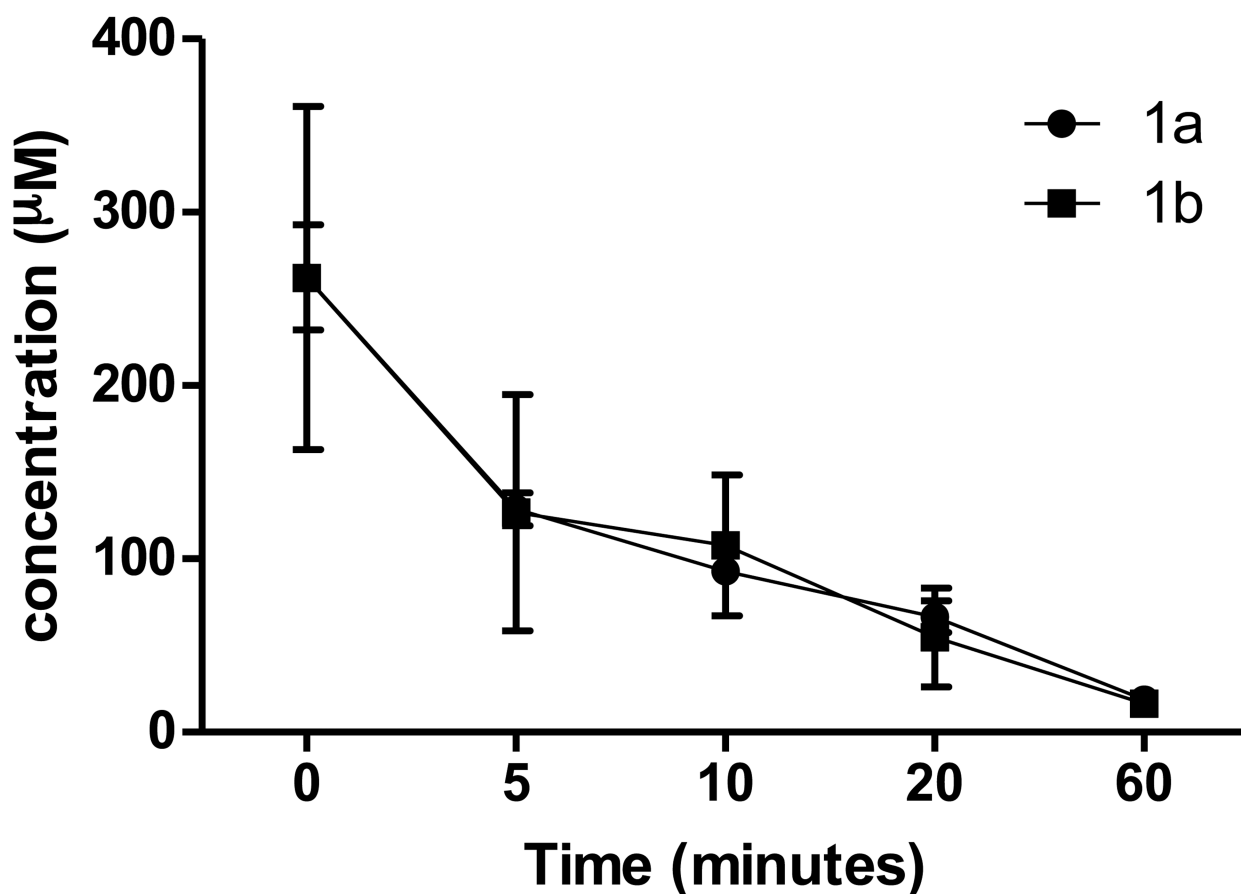
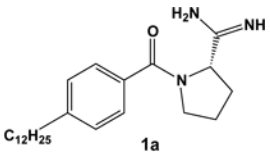
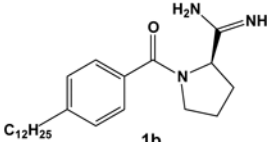


Fig. 6. S1P, sphingosine and compounds **1a** and **1b** levels in the blood of mice injected with compounds **1a** and **1b**. Wild type or Sphk1 null mice were dosed with 10 mg/kg of compounds **1a** or **1b** or vehicle via the tail vein. Blood samples were obtained after injection at the times indicated in the figure. Levels of S1P, sphingosine and compounds **1a** or **1b** in blood samples were measured by LC/MS. a: S1P; b: sphingosine, c: compounds **1a** and **1b**. The lower blood levels of S1P observed in SphK1 null mice are consistent with literature reports [47, 48]. Data are means \pm SD of three measurements in different animals. Sphk1 null mice are referred to in the figure as 'SK1 $-/-$ '. ** $p < 0.01$, *** $p < 0.001$ (repeated measures two way ANOVA, and Bonferroni's Multiple Comparison Post Test).

Table 1

Chemical structure and inhibitory properties of compounds **1a** and **1b**. The chemical structures of compounds **1a** and **1b** along with their inhibitory constants (K_i) for recombinant SphK1 1 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 **1a** and **1b**. 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 K_m' and K_m 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 [21].

Compound	SphK1 K_i	SphK2 K_i
 <p>1a</p>	0.1 μM	1.5 μM
 <p>1b</p>	16 μM	5 μM