

Synthesis and evaluation of sphingoid analogs as inhibitors of sphingosine kinases

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Abstract—Sphingosine 1-phosphate (S1P), a product of sphingosine kinases (SphK), mediates diverse biological processes such as cell differentiation, proliferation, motility, and apoptosis. In an effort to search and identify specific inhibitors of human SphK, the inhibitory effects of synthetic sphingoid analogs on kinase activity were examined. Among the analogs tested, we found two, SG12 and SG14, that have specific inhibitory effects on hSphK2. *N,N*-Dimethylsphingosine (DMS), a well-known SphK inhibitor, displayed inhibitory effects for both SphK1 and SphK2, as well as protein kinase C. In contrast, SG12 and SG14 exhibited selective inhibitory effects on hSphK2. Furthermore, SG14 did not affect PKC. In isolated platelets, SG14 blocked the conversion of sphingosine into sphingosine 1-phosphate significantly. This is the first report on the identification of a hSphK2-specific inhibitor, which may provide a useful tool for studying the biological functions of hSphK2.

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1. Introduction

The sphingolipid metabolites ceramide (CER), sphingosine (SPH), and sphingosine 1-phosphate (S1P) play an important role in the regulation of cell proliferation, survival, and apoptosis.^{1,2} S1P mediates diverse biological processes such as cell differentiation, proliferation, motility, and apoptosis, both extracellularly by binding to members of the S1P receptor and intracellularly.^{3–5} S1P usually stimulates proliferation and suppresses apoptosis, while CER and SPH inhibit proliferation and promote apoptosis. Therefore, it has been proposed that the balance between S1P and SPH/CER may determine cell fate. One sphingolipid metabolizing enzyme, sphingosine kinase (SphK), which phosphorylates SPH

to form S1P,⁶ has been implicated as playing a role in determining cell fate because it not only produces anti-apoptotic S1P but also decreases pro-apoptotic CER and SPH.

SphK is a highly conserved lipid kinase, and the distribution of its activity varies among cell types and tissues.^{7,8} SphK activity is increased by many growth and survival factors, including platelet-derived growth factor,⁹ nerve growth factor,¹⁰ epidermal growth factor,¹¹ and vascular endothelial growth factor.¹² To date, two mammalian SphKs, SphK1 and SphK2, have been identified,^{13,14} and differences between the two have been recognized. While SphK1 is predominantly localized in the cytoplasm, SphK2 is localized in the nucleus as well as in the cytosol.^{15,16} SphK1 stimulates cell growth and survival, but SphK2 enhances apoptosis and suppresses cellular growth in various cell types.¹⁷ Additionally, each has its own substrate specificity. SphK1 phosphorylates *D-erythro*-sphingosine (SPH) and, to a lesser extent, dihydrosphingosine (DHS), but

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acts very poorly on phytosphingosine (PHS). On the other hand, SphK2 phosphorylates both *D-erythro*-sphingosine and dihydrosphingosine, with nearly the same efficiency, and phytosphingosine, though to a lesser extent. Furthermore, the immunomodulatory pro-drug FTY-720 is phosphorylated 30-fold more efficiently by SphK2 than SphK1.^{18,19}

SPH, DHS, and PHS, which can serve as precursors of many biologically important compounds such as cerebrosides, gangliosides, and sphingomyelin, are composed of three structural units: a long-chain aliphatic 2-amino-1,3-diol, a fatty acid, and a polar head group.²⁰ The inherent structural variation of sphingosine and its derivatives attracts great interest, because of the diverse biological activities of these lipids including the cell regulation and signal transduction.^{21–23} We recently reported that the key structural unit of sphingolipids, enantiomerically pure 2-amino-1,3-propanediols, could be synthesized from suitably substituted chiral aziridine.²⁴ Various enantiomerically pure 2-acylaziridines were prepared efficiently from the corresponding aziridine-2-carboxylate via Weinreb's amide^{25–28} and organometallic compounds. The carbonyl group was reduced stereoselectively by a suitable reducing agent, and then the aziridine ring was regioselectively opened with AcOH, pyrrolidine, and morpholine. Using this methodology, we have prepared various sphingosine analogues, with high yields.

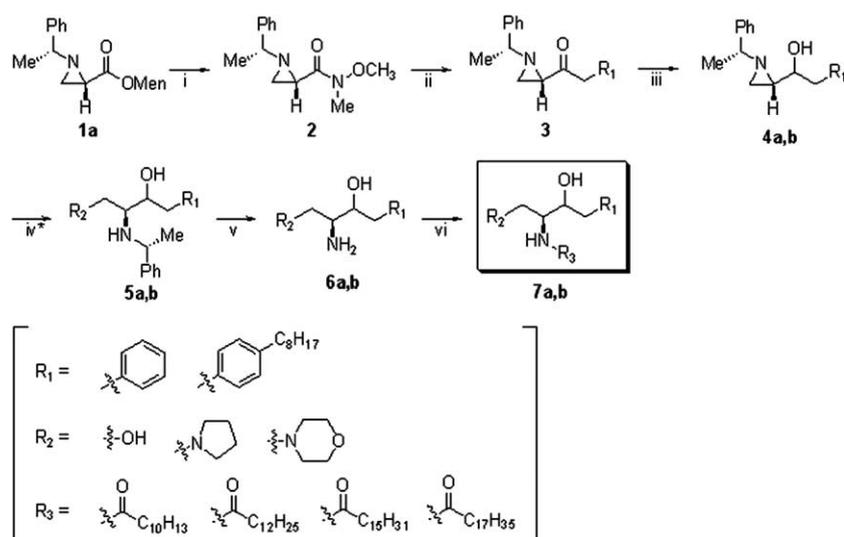
Sphingolipid metabolism is often found to be abnormal in a number of apoptosis-related pathological conditions. In many cancer types, SphK inhibitors strongly induce apoptosis and enhance sensitivity to gamma irradiation and anti-cancer drugs.²⁹ Thus, SphK inhibitors have been implicated as a novel sensitizer for cancer therapeutics. Furthermore, understanding SphK functions could uncover new targets for therapeutic applications.

In this study, we prepared various novel sphingoid analogs to find specific inhibitors of SphK1 and/or SphK2. We found SphK2-specific inhibitor that did not affect SphK1 or protein kinase C (PKC) activity. These results provide the first SphK2-specific inhibitor. Such an inhibitor will be useful not only in understanding the SphK functions but also in designing therapeutics based on the sphingoid structure.

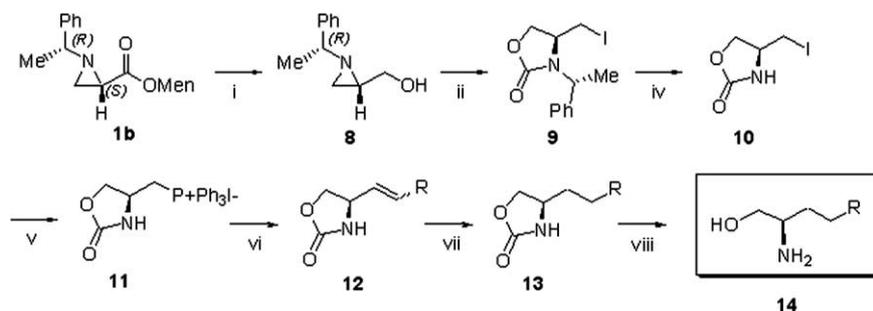
2. Results

2.1. Synthesis of sphingoid analogs

The reaction of *N,O*-dimethyl hydroxylamine hydrochloride and a commercially available *N*-[(*R*)-(+)- α -methylbenzyl]-2(*S*)-carboxylic acid menthol ester **1a** (Scheme 1), in the presence of *i*-PrMgCl in THF provided the Weinreb's amide **2**, with a 92% yield. The Weinreb's amide **2** was reacted with various organometallic compounds to provide the corresponding ketones **3** in high yields. Reduction of the ketones **3** with LAH resulted in a diastereomeric mixture of hydroxy aziridines **4a,b** (approximately R/S = 1/1 to 2/1), which were readily separable by flash chromatography.^{30,31} A regioselective ring opening reaction of aziridine-2-methanols **4a,b** by AcOH and various amine nucleophiles provided **5a,b**. TMS-I was used for the activation of the basic ring nitrogen of the aziridine-2-methanols and was also used as the source of the iodide nucleophiles to attack the less substituted C(3) of the aziridine.³² Aziridine-2-methanol readily opens up regioselectively, and these results prompted us to investigate the preparation of enantiomerically pure **7a,b** by debenzoylation and amide bond formation. The debenzoylation reaction did not proceed via the usual procedure with Pearlman's catalyst, and the reactivity was influenced by solvent, temperature and pressure. *N*-acylation provided various ceramide analogues, and the results are shown in Scheme 1.



Scheme 1. Reagents and conditions: (i) Weinreb's amine, *i*-PrMgCl, THF, 0 °C; (ii) benzyl bromide, Mg, THF, 50 °C; (iii) LAH, THF, 0 °C, 85%; (iv)* (a) AcOH, CH₂Cl₂, rt, (b) KOH, EtOH, 90% (two steps) or NaI, TMSI, pyrrolidine, MeCN, 90%; (v) Pd(OH)₂, H₂, (100 psi), MeOH; (vi) Acyl chloride, NaOH, THF, 76% (two steps).



Scheme 2. Reagents and conditions: (i) LAH, THF, 0 °C; (ii) carbonyldiimidazole, CH₂Cl₂, rt; (iii) NaI, TMSCl, MeCN, rt, 75% (two steps); (iv) anisole, MsOH, toluene, 60 °C, 95%; (v) PPh₃, DMF, 100 °C, 90%; (vi) 4-octylbenzaldehyde, LHMDS, THF, 0 °C, 80%; (vii) Pd/C, H₂, MeOH, rt, 90%; (viii) LiOH·H₂O, EtOH, 70 °C, 90%.

The reduction of the aziridine-2-carboxylate **1b** to the corresponding aziridine-2-methanol was fairly straightforward using LAH in THF. The enantiomerically pure phenylethyl-protected 4(*S*)-iodomethyl-2-oxazolidinone was prepared from the aziridine 2(*S*)-methanol **8** through regiospecific C(3)–N bond cleavage by iodotrimethylsilane, followed by intramolecular cyclization with carbonyldiimidazole (CDI).³³ The phenylethyl group on the nitrogen was successfully removed by heating compound **9** in the presence of anisole and methanesulfonic acid³⁴ in toluene, to provide the (4*S*)-iodomethyl-2-oxazolidinone **10** in an almost quantitative yield. The iodide **10** was converted to the corresponding phosphonium salt **11** in a 90% yield, by stirring with triphenylphosphine in toluene at 100 °C for 24 h.³⁵ Treatment of the phosphonium salt **11** with LiHMDS at –78 °C generated the ylide, which was then reacted with various benzaldehyde analogues to provide alkenes in high yield. The utility of the olefination procedure can be further demonstrated by the ready conversion of the oxazolidinone to the corresponding aryl-substituted amino alcohol by hydrogenation (5% Pd/C, H₂) to give the 4-substituted (4*R*)-phenylethyl-2-oxazolidinone **13**. This was then hydrolyzed in aqueous EtOH by LiOH to provide (*R*)-2-amino-4-(4-octylphenyl)butan-1-ol **14**, in a 90% yield (Scheme 2).

3. Expression of hSphK1 and hSphK2

To examine the inhibitory effect of the synthetic sphingoid analogs on the hSphK1 and SphK2, Chinese hamster ovary cells (CHO-K1) were transfected with pcDNA3-HA, pcDNA3-HA-hSphK1, or pcDNA3-HA-hSphK2. Twenty-four hours after transfection, the expression levels of the hSphK1 and hSphK2 were determined by Western blotting (Fig. 1A). Quantification using a densitometer (NIH image analyzer, 1.62) revealed an expression level of the hSphK1 protein 20-fold higher than that of hSphK2. Next, we examined the sphingosine kinase activities of SphK1 and SphK2 with pcDNA3-HA-hSphK1 and pcDNA3-HA-hSphK2-transfected cells (Fig. 1B). Despite the much higher expression level of the protein, the kinase activity of the hSphK1 transfectant was only about 4-fold higher than that of the hSphK2. Taken together, these results imply that hSphK2 has comparable sphingosine kinase activity to that of hSphK1.

3.1. Inhibitory effect of sphingoid analogs on in vitro SphK activity

To evaluate the SphK inhibitory effect of sphingoid analogs, in vitro SphK inhibition assays were performed.

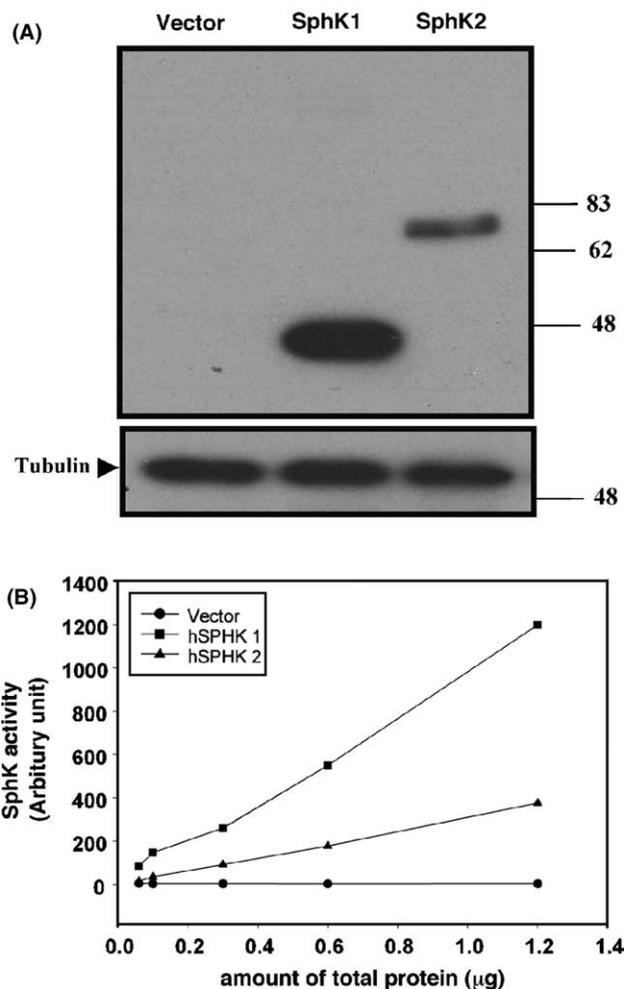


Figure 1. Expression and enzymatic activities of hSphK1 and hSphK2. CHO-K1 cells were transiently transfected with pcDNA3-HA, pcDNA3-HA-hSphK1, or pcDNA3-HA-hSphK2. A. HA-hSphK1 and HA-hSphK2 proteins were separated by SDS-PAGE and analyzed by Western blotting with an anti-HA antibody. An anti-tubulin blot was performed as a loading control. B. Cell lysates were prepared and SphKs activities were measured with 4 μM D-e-sphingosine, 2 μCi[γ-³²P] ATP, and 0.5 mM cold ATP, at 37 °C for 30 min.

N,N-Dimethylsphingosine (DMS), a well known SphK inhibitor, was used as a positive control and exhibited very strong inhibitory effect on both hSphK1 and hSphK2 (Table 1). More than half of the sphingoid analogs tested displayed inhibitory effects, and in general, the sphingoid analogs showed a more inhibitory effect on hSphK2 than hSphK1. From these data, together with reported results of hSphK2 phosphorylating phytosphingosine and FTY-720 more efficiently than hSphK1,^{18,19} we can infer that hSphK2 may have broader substrate availability than hSphK1. Two of the tested sphingoid analogs, SG12 and SG14, exhibited strong inhibitory effects against hSphK2 with no effect on hSphK1 (Fig. 2A and B). The IC₅₀ values of SG12 and SG14 on SphK2 were 22 and 4 μM, respectively, while the IC₅₀ of the positive control DMS was 16 μM. These results indicate that SG14 has a stronger inhibitory effect than DMS on hSphK2.

Interestingly, SG7, in which the R4 –OH of SG12 is substituted with –H, lost its hSphK2 selectivity, suggesting the importance of the –OH substitution in its specificity. In addition, SG9 and SG15, stereoisomers of SG12 and SG14, respectively, displayed weak inhibitory effects on hSphK2. These results indicate that the stereochemistry of SG12 and SG14 is essential for the inhibitory effect.

3.2. Cytotoxic effects of a hSphK2-specific inhibitor in CHO-K1 cells

It is well understood that SphK inhibitors cause cell death by inducing the accumulation of ceramide. In many studies using various cell types, DMS induces cell death very strongly. To investigate the influence of hSphK2-specific inhibitors on cell growth, their cytotoxic effects were measured using 2-(2-methoxy-4-

nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrasolium (Fig. 3). After twenty-four hours, SG12 and SG14 treatments induced dose-dependent cell death in CHO-K1 cells. Interestingly, SG15, which is a stereoisomer of SG14 and a weak SphK2 inhibitor, had no cytotoxic effect. Though we do not yet know if this cell death was induced by SphK2 inhibition, these results indicate that SG12 and SG14 have cytotoxic effects comparable to those of DMS.

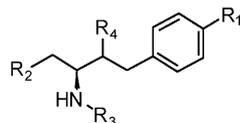
3.3. Inhibition of in vitro protein kinase C activity by SphK inhibitors

In many studies, DMS is the inhibitor most widely used. It inhibits SphK1 and SphK2 as well as protein kinase C³⁶ and induces cell death in various cell types. It is difficult to say, however that DMS induces cell death via SphK inhibition, since it also inhibits PKC. Thus, DMS is imperfect as an experimental and/or therapeutic tool. With this in mind, we performed in vitro PKC inhibition assays to determine whether the sphingoid analogs specifically inhibit SphK2. Our results demonstrated that SG14 did not inhibit PKC, while DMS inhibited PKC very strongly (Fig. 4). On the other hand, SG12 exhibited moderate inhibitory effects on PKC. In summary, SG14 can be used as a SphK2-specific inhibitor which does not affect PKC or SphK1. Thus, SG14 can overcome the imperfections of DMS and has great advantages as an experimental and/or therapeutic tool.

3.4. Inhibition of the phosphorylation [³H] sphingosine into [³H] sphingosine 1-phosphate in platelets

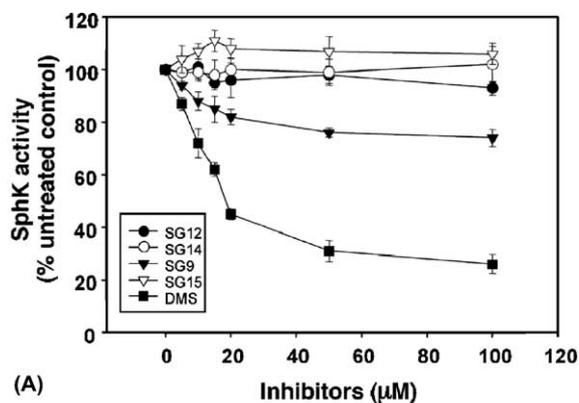
To determine whether SG12 and SG14 can inhibit endogenous SphK activity, we studied the effect of these SphK inhibitors on [³H] sphingosine metabolism using platelets. Blood platelets store S1P abundantly, possibly

Table 1. Inhibitory effects of sphingoid analogues on human sphingosine kinase 1, 2

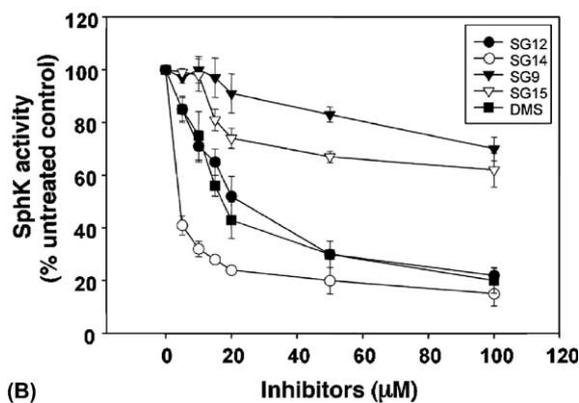


ID	R1	R2	R3	R4	hSphK1 (%)	hSphK2 (%)
DMS					24	23
SG-1	H	OH	Tri-decanoyl	OH (S)	98	56
SG-2	H	OH	Tridecanoyl	OH (R)	97	104
SG-3	H	OH	Palmitoyl	OH (S)	149	91
SG-4	H	OH	Palmitoyl	OH (R)	98	71
SG-5	H	OH	Tridecanoyl	H	66	49
SG-6	H	H	Palmitoyl	H	71	85
SG-7	Octyl	OH	H	H	54	44
SG-9	Octyl	OH	H	OH (S)	70	83
SG-10	H	Morpholine	H	OH (R)	95	91
SG-11	H	Pyrrolidine	Decanoyl	H	96	46
SG-12	Octyl	OH	H	OH (R)	97	25
SG-13	H	Pyrrolidine	Decanoyl	OH (S)	72	96
SG-14	H	Pyrrolidine	Stearoyl	OH (S)	98	21
SG-15	H	Pyrrolidine	Stearoyl	OH (R)	98	65
SG-16	H	Morpholine	Decanoyl	OH (S)	95	60

Inhibitory effects toward hSphK1 and hSphK2 activities were compared at inhibitor concentrations of 50 μM (% untreated control); These data represent an average of three different experiments; DMS was used as a positive control.



(A)



(B)

Figure 2. In vitro sphingosine kinase inhibition by sphingoid analogs. (A) Inhibition assays of sphingoid analogs on hSphK1. 1 µg protein from lysates of hSphK1-overexpressing CHO-K1 cells were incubated with 4 µM D-e-sphingosine, 2 µCi[γ - 32 P] ATP, and 0.5 mM cold ATP at 37 °C for 30 min. (B) Inhibition assays of sphingoid analogs on hSphK2. Assays were performed as in A but in the presence of 200 mM KCl and using lysates from the hSphK2-overexpressing CHO-K1 cells. Data are shown as the percentage of SphK activity relative to untreated controls from three independent experiments (mean \pm S.D.).

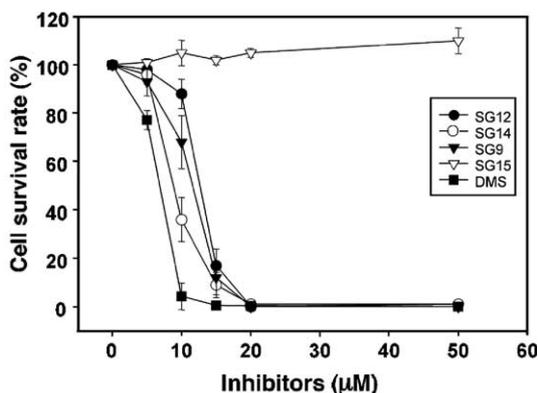


Figure 3. Cytotoxic effects of hSphK inhibitors. The cytotoxic effect was measured using tetrasolium salt. CHO cells (5×10^3 cell/100 µL) were incubated with the indicated inhibitors at 37 °C for 24 h, then the absorbance of the culture supernatant was measured by spectrophotometry at a wavelength of 450 nm. Data are shown as the percentage of live cells relative to untreated controls from three independent experiments (mean \pm S.D.).

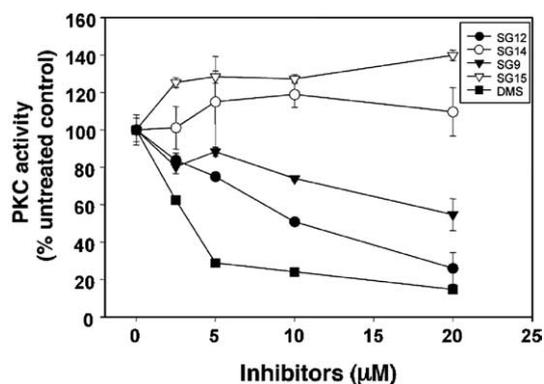


Figure 4. Effect of hSphK2-specific inhibitors on in vitro protein kinase C activity. To monitor the influence of the hSphK2-specific inhibitors on PKC activity, the indicated quantities of inhibitors were supplemented into a PKC assay system containing phosphatidylserine, diacylglycerol, [γ - 32 P]ATP and the biotinylated peptide Neurogranin (AAKIQAS*FRGHMARKK). The reaction was initiated by adding 5 ng PKC (an enzyme mixture which consisting of the α , β and γ isoforms with lesser amounts of the δ and ζ isoforms). Data are shown as the percentage of live cells relative to untreated controls from three independent experiments (mean \pm S.D.).

the result of an SphK activity and a lack of S1P lyase activity.⁴ In fact, S1P is produced very rapidly from exogenously added SPH. Fifteen minutes after adding [3 H] SPH to platelets, about 80% was converted into

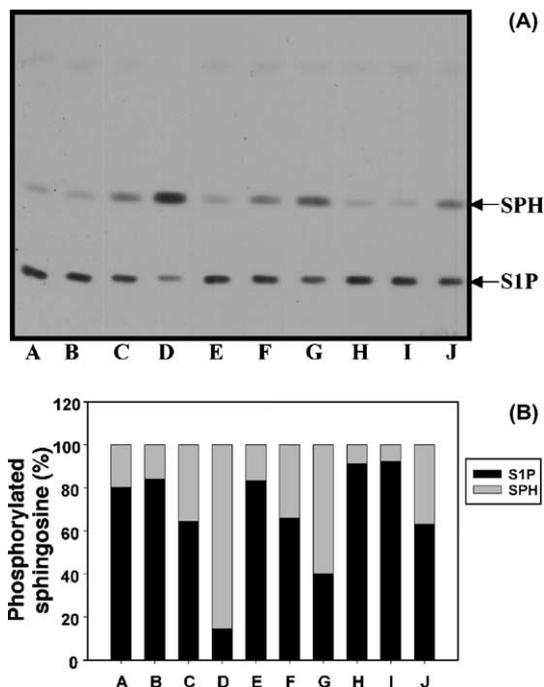


Figure 5. Effect of SphK inhibitors on [3 H] Sphingosine metabolism. Platelet suspensions were pretreated for 5 min with increasing concentrations of SphK inhibitors and then incubated with [3 H] Sphingosine for 15 min. (A) Autoradiography. (B) Quantification of the autoradiography was performed. Data are shown as the percentage of phosphorylated [3 H] sphingosine (S1P) in the total of phosphorylated and unphosphorylated forms of [3 H] Sphingosine. Lane A: negative control, B–D: DMS 5 µM, 20 µM, 50 µM; E–G: SG12 5 µM, 20 µM, 50 µM; H–J: SG14 5 µM, 20 µM, 50 µM.

[³H] S1P (Fig. 5A and B). In the presence of the SphK inhibitors, however, this conversion was inhibited in a dose-dependent manner. In the presence of 50 μM DMS, [³H] SPH conversion into [³H] S1P was reduced to 15%. In the same concentration of SG12 or SG14, the conversion was reduced to 40% or 60%, respectively (Fig. 5B). The more significant inhibitory effect of the non-selective DMS, in contrast to the more moderate effects of SphK2-specific SG12 and SG14, is probably due to the considerable contribution in platelets of SphK1 activity.⁸ These results imply that SG12 and SG14 have selective inhibitory activities on endogenous SphKs in platelets. Although it is still unknown exactly what kind of SphKs exist in platelets,^{7,8} these data suggests the possibility that considerable SphK2 activity is present.

3.5. Inhibitory effects of SG14 on SphK2 activity toward various substrate

SphK2 can phosphorylate phytosphingosine and the prodrug FTY720 very efficiently compared to SphK1.^{18,19} To verify whether SG12 and SG14 can spe-

cifically inhibit SphK2 activity on these substrates, in vitro SphK inhibition assays were performed. First, we examined the relative phosphorylation efficiency for each substrate (Fig. 6B). The phosphorylation activity of SphK1 on sphingosine was 7-fold and 80-fold higher compared to its activity on phytosphingosine and FTY720, respectively (Fig. 6B). In contrast, the phosphorylation activity of SphK2 on sphingosine was 0.5-fold less compared to that on phytosphingosine and 3-fold higher compared to that on FTY720. As expression level of the hSphK1 protein is 20-fold higher than that of hSphK 2 (Fig. 1A), SphK2 phosphorylated phytosphingosine more efficiently than SphK1, by a 20-fold margin. In addition, SphK2 phosphorylated FTY720 more efficiently than SphK1, by 37-fold. These results are consistent with previous reports.^{18,19}

Next, we examined the inhibitory effect of SG14 on the phosphorylation of sphingosine (SPH), phytosphingosine (PHS), and FTY720. DMS inhibited the phosphorylation of all three substrates by either SphK1 or SphK2. However SG14 inhibited only the SphK2 phosphorylation of the three different substrates (Fig. 6A). Through this result, we can verify that SG14 specifically inhibits SphK2, regardless of the substrate involved.

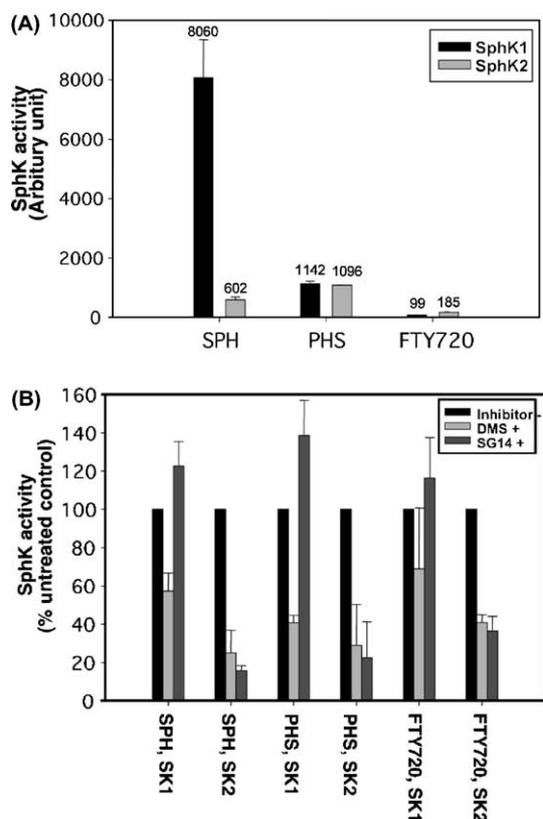


Figure 6. Effects of SphK inhibitors on phosphorylation of various substrates. In vitro sphingosine kinase inhibition assays were performed on a variety of substrates, including sphingosine (SPH), phytosphingosine (PHS) and immunosuppressive prodrug FTY720. (A) Comparative SphK activity against various substrates. Data are shown as the percentage of SphK activity relative to the SphK1 activity on sphingosine. (B) Inhibition activity of DMS and SG14 against SphK activity toward various substrates. Inhibitors were used at the concentration of 20 μM. Data are shown as the percentage of SphK activity relative to untreated controls from three independent experiments (mean ± S.D.).

4. Discussion

In this study, we prepared various sphingoid analogs using suitably substituted chiral aziridine. Various enantiomerically pure 2-acylaziridines were produced from the corresponding aziridine-2-carboxylate via Weinreb's amide and organometallic compounds. Reduction of the carbonyl group and aziridine ring opening allows for the synthesis of a variety of sphingosine analogues, with high yields.

Among the analogs tested in this study, we found specific inhibitors for SphK2. Until now, the well known SphKs inhibitor DMS was used most widely in studies even though it lacks subtype selectivity and also inhibits PKC. In contrast, our novel sphingoid analog SG14 specifically inhibited SphK2 and did not affect SphK1 or PKC. Interestingly, SG15, a stereoisomer of SG14, lacked most of the inhibitory activity, indicating that the stereochemistry may be essential for the inhibitory effect and its selectivity.

In an experiment using platelets, DMS, SG12, and SG14 caused the accumulation of SPH by blocking the conversion of SPH into S1P. The inhibitory effect of the non-selective DMS was the strongest, while that of SG12 or SG14 was more moderate. There is considerable SphK1 activity in platelets,⁸ so this lower inhibition likely reflects the SphK2-selectivity of the analogs. In addition, this result provided evidence that SG12 and SG14 are able to inhibit endogenous SphK2 although the presence of other kinases, which might also be inhibited by these analogs, cannot be ruled out.

The exact mechanism and biological functions of SphK regulation are not clear, but sphingolipid metabolism is

often found to be unusual in diseases like cancer. Significantly elevated SphK expression has been observed in solid tumors, as compared with normal tissue from same patients.³⁷ Also, cells overexpressing SphK are transforming and tumorigenic.³⁸ Conversely, inhibition of SphK has been shown to suppress tumor growth.³⁹ Moreover, S1P regulates angiogenesis during tumor progression in an autocrine or paracrine manner.^{40,41} Thus, it is considered that SphK regulates tumor growth and can act as an oncogene. Anti-cancer therapeutics targeting SphK are very plausible. Indeed, in many studies the SphK inhibitors, *N,N*-dimethylsphingosine (DMS) and *D,L*-*threo*-dihydrosphingosine (Safingol) have been shown to induce growth arrest or cell death, although whether this occurs through the inhibition of SphK or PKC has not been established.^{42–45} In preliminary data from a phase I trial, Safingol, when combined with doxorubicin and fenretinide, appeared to increase the anti-cancer efficacy.^{46–48} As the cytotoxicity of SG12 and SG14 were comparable to DMS, examining their anti-cancer efficacy would be a reasonable next step in our investigations.

In a recent study, SphK1 null mice were found to be viable, fertile and without obvious abnormalities.⁴⁹ In that paper, the total SphK activity was reduced in most tissue, but S1P levels were not markedly decreased except in serum. These results indicate the presence of multiple SphKs or the ability of SphK2 to considerably compensate for the loss of SphK1 activity. Additionally, the function of the prodrug FTY-720 regulating lymphocyte trafficking and distribution was not affected in the SphK1 *-/-* mice. This may suggest the possibility that SphK2 can affect the functional activation of the prodrug FTY-720. In this regard, SG14 inhibited the phosphorylation of FTY-720 by hSphK2 (Fig. 6B). Therefore, SG14 might be a potent tool for regulating the functional activation of FTY720. Additionally, SG12 was apparently phosphorylated by SphK2. This provides an interesting basis for further investigation of possible therapeutic properties of this compound.

Many studies have established a relationship between SphK1 and pathological conditions like cancer and inflammation. However, until now, the participation of SphK2 has not been well understood, despite significant research recently to identify its function. A SphK2-specific inhibitor will be useful, then, not only in understanding the function of SphK2 but also in the design of novel therapeutics.

5. Experimental

5.1. Synthesis of sphingoid analogs

¹H NMR spectra were recorded at 300 MHz and ¹³C NMR spectra at 75 MHz, with either TMS ($\delta = 0$) or the signal for residual CHCl₃ in the CDCl₃ solvent ($\delta = 7.27$) as internal standards. *J* values are reported in Hertz. Flash column chromatography was performed with Kieselgel 60 Art 9385 (230–400 mesh). Organic

extracts were dried over a drying agent, MgSO₄, and Na₂SO₄, and concentrated under reduced pressure with the aid of a rotary evaporator. Analytical TLC (thin layer chromatography) was performed with 0.2 mm coated commercial silica gel, and the detection was carried out using a UV lamp and PMA solution, *p*-anisaldehyde solution, or ninhydrine solution.

5.2. Procedure (SG-12)

5.2.1. (*S*)-*N*-Methoxy-*N*-methyl-1-((*R*)-1-phenylethyl)aziridine-2-carboxamide.² To a flask charged with 100 mg of aziridine menthol ester (**1**) in THF (0.3 M) and 45 mg of Weinreb's amine was slowly added 0.46 mL of isopropyl magnesium chloride (2.0 M solution in THF), at 0 °C under N₂ atmosphere. The resulting mixture was warmed to room temperature for 10 min. After disappearance of the starting aziridine menthol ester (as monitored by TLC), the reaction mixture was partitioned into CH₂Cl₂ and H₂O. The organic layer was dried over MgSO₄, concentrated in vacuo, and purified by column chromatography with 50% EtOAc in hexane to give 67 mg of product in a 94% yield.

5.2.2. 2-(4-Octylphenyl)-1-((*S*)-1-((*R*)-1-phenylethyl)aziridin-2-yl)ethanone. The solution of (*S*)-*N*-methoxy-*N*-methyl-1-((*R*)-1-phenylethyl)aziridine-2-carboxamide (450 mg, 1.92 mmol) in THF (0.3 M) was added to 180 mg of Mg, while mixing, and 660 mg of 1-(bromomethyl)-4-octylbenzene at room temperature under N₂ atmosphere. The resulting mixture was heated to reflux at 50 °C for 2–6 h. After completion, the reaction mixture was extracted with EtOAc and H₂O, dried over MgSO₄, and purified by column chromatography to give 580 mg of product in 80% yield.

5.2.3. (*R*)-2-(4-Octylphenyl)-1-((*S*)-1-((*R*)-1-phenylethyl)aziridin-2-yl)ethanol. To a stirred solution of 2-(4-octylphenyl)-1-((*S*)-1-((*R*)-1-phenylethyl)aziridin-2-yl)ethanone (1 g, 2.64 mmol) in THF (0.3 M) was slowly added 120 mg of LAH at 0 °C for 1 h. The mixture was quenched by saturated aqueous KHSO₄ and extracted with EtOAc and H₂O. The organic layer was concentrated and dried over MgSO₄ and purified by chromatography with 20% EtOAc in hexanes to give (*S*)-2-(4-octylphenyl)-1-((*S*)-1-((*R*)-1-phenylethyl)aziridin-2-yl)ethanol (*R_f*: 0.5, 20% EtOAc in hexanes) in a 35–50% yield and (*R*)-2-(4-octylphenyl)-1-((*S*)-1-((*R*)-1-phenylethyl)aziridin-2-yl)ethanol (*R_f*: 0.3, 20% EtOAc in hexanes) in a 50–65% yield.

5.2.4. (2*S*,3*R*)-2-((*R*)-1-Phenylethylamino)-3-hydroxy-4-(4-octylphenyl)butyl acetate. A round flask was charged with (*R*)-2-(4-octylphenyl)-1-((*S*)-1-((*R*)-1-phenylethyl)aziridin-2-yl)ethanol in CH₂Cl₂ (0.5 M). To the solution was added AcOH (10 equiv of the substrate) at room temperature, and the mixture was stirred for 12–18 h. The reaction mixture was quenched with aqueous NaHCO₃ and extracted with CH₂Cl₂. The organic solution was dried over MgSO₄ and purified by column chromatography to give the ring opening product in a 90% yield.

5.2.5. (2*S*,3*R*)-2-((*R*)-1-Phenylethylamino)-4-(4-octylphenyl)butane-1,3-diol. A flask was charged with 100 mg of (2*S*,3*R*)-2-((*R*)-1-phenylethylamino)-3-hydroxy-4-(4-octylphenyl)butyl acetate in MeOH (0.5 M). To the solution was added 26 mg of KOH at room temperature. After 2 h, the reaction mixture was extracted with CH₂Cl₂, dried over MgSO₄, and concentrated to give free alcohol in a quantitative yield.

5.2.6. (2*S*,3*R*)-2-Amino-4-(4-octylphenyl)butane-1,3-diol. A mixture of (2*S*,3*R*)-2-((*R*)-1-phenylethylamino)-4-(4-octylphenyl)butane-1,3-diol and Pd(OH)₂ (20 wt %) in EtOH (0.5 M) was stirred at room temperature under H₂ (100 psi). The reaction mixture was monitored by TLC through the completion of the reaction. The mixture was filtered using Celite. After evaporation of the solvent, the crude product was purified by column chromatography to give the debenzoylation product in an 80% yield.

5.3. Compound characterization data

5.3.1. *N*-((2*S*,3*S*)-1,3-Dihydroxy-4-phenylbutan-2-yl)tridecanamide (SG-1). ¹H NMR (300 MHz, CDCl₃) δ 7.13–7.22 (m, 5H), δ 6.42 (d, *J* = 8.7 Hz, 1H), δ 4.12 (td, *J* = 6.3, 1.5 Hz, 1H), δ 3.95–3.85 (m, 1H), δ 3.65 (d, *J* = 4.8, 2H), δ 2.74 (d, *J* = 1.5 Hz, 1H), δ 2.71 (d, *J* = 3.6 Hz, 1H), δ 1.69–1.58 (m, 2H), δ 1.38–1.20 (m, 20H), δ 0.87 (t, *J* = 6.9 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 174.3, 137.7, 129.3, 128.3, 126.6, 72.7, 64.2, 52.8, 40.7, 36.8, 31.854, 29.6, 29.5, 29.3, 29.2, 25.9, 22.6, 14.0; Anal. Calcd for C₂₃H₃₉NO₃: C, 73.17; H, 10.41; N, 3.71. Found: C, 73.32; H, 10.32; N, 3.69.

5.3.2. *N*-((2*S*,3*R*)-1,3-Dihydroxy-4-phenylbutan-2-yl)tridecanamide (SG-2). ¹H NMR (300 MHz, CDCl₃) δ 7.2–7.36 (m, 5H), δ 6.38 (d, *J* = 7.2 Hz, 1H), δ 3.9–4.15 (m, 2H), δ 3.76 (dd, *J* = 11.1, 2.7 Hz, 1H), δ 2.98–2.75 (m, 2H), δ 2.21 (t, *J* = 7.2 Hz, 2H), δ 1.9–1.54 (m, 2H), δ 1.4–1.25 (m, 20H), δ 0.88 (t, *J* = 5.7 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 173.7, 137.6, 129.0, 128.5, 126.7, 74.5, 62.2, 53.8, 40.8, 36.8, 34.0, 31.9, 29.7, 29.5, 29.4, 29.4, 29.3, 29.2, 25.6, 24.8, 22.6, 14.0; Anal. Calcd for C₂₃H₃₉NO₃: C, 73.17; H, 10.41; N, 3.71. Found: C, 73.02; H, 10.42; N, 3.65.

5.3.3. *N*-((2*S*,3*S*)-1,3-Dihydroxy-4-phenylbutan-2-yl)palmitamide (SG-3). ¹H NMR (300 MHz, CDCl₃) δ 7.34–7.15 (m, 5H), δ 6.44 (d, *J* = 8.7 Hz, 1H), δ 4.23 (br s, 1H), δ 4.14 (t, *J* = 6.3 Hz, 1H), δ 4.0–3.9 (m, 2H), δ 3.69 (d, *J* = 4.2 Hz, 2H), δ 2.8–2.68 (m, 2H), δ 2.35–2.2 (m, 2H), δ 1.7–1.5 (m, 2H), δ 1.4–1.24 (m, 26H), δ 0.88 (t, *J* = 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 174.4, 137.6, 129.3, 128.6, 126.6, 73.0, 64.4, 52.8, 40.7, 36.8, 34.0, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 25.9, 24.8, 22.6, 14.1; Anal. Calcd for C₂₆H₄₅NO₃: C, 74.42; H, 10.81; N, 3.34. Found: C, 74.52; H, 10.92; N, 3.29.

5.3.4. *N*-((2*S*,3*R*)-1,3-Dihydroxy-4-phenylbutan-2-yl)palmitamide (SG-4). ¹H NMR (300 MHz, CDCl₃) δ 7.35–7.2 (m, 5H), δ 6.47 (d, *J* = 7.8 Hz, 1H), δ 4.1–3.98 (m, 2H), δ 3.96–3.9 (m, 1H), δ 3.75 (dd, *J* = 11.4, 3.0 Hz,

1H), δ 2.96–2.76 (m, 2H), δ 2.2 (t, *J* = 7.8 Hz, 2H), δ 1.7–1.5 (m, 2H), δ 1.35–1.2 (m, 26H), δ 0.88 (t, *J* = 6.9 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 173.8, 137.7, 129.2, 128.7, 126.7, 74.6, 62.2, 53.8, 40.8, 36.8, 34.0, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 25.7, 24.8, 22.7, 14.1; Anal. Calcd for C₂₆H₄₅NO₃: C, 74.42; H, 10.81; N, 3.34. Found: C, 74.57; H, 10.76; N, 3.23.

5.3.5. *N*-((*R*)-1-Hydroxy-4-phenylbutan-2-yl)tridecanamide (SG-5). ¹H NMR (300 MHz, CDCl₃) δ 7.3–7.15 (m, 5H), δ 5.65 (d, *J* = 7.8 Hz, 1H), δ 4.05–3.95 (m, 1H), δ 3.75–3.55 (m, 2H), δ 2.83 (br s, 1H), δ 2.68 (t, *J* = 7.8 Hz, 2H), δ 2.15 (t, *J* = 7.8 Hz, 2H), δ 1.95–1.7 (m, 2H), δ 1.65–1.55 (m, 2H), δ 1.25 (br s, 20H), δ 0.88 (t, *J* = 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 174.3, 141.5, 128.6, 128.3, 126.3, 65.6, 51.5, 36.9, 32.8, 32.5, 31.9, 29.7, 29.6, 29.4, 29.2, 29.3, 25.7, 22.6, 14.3; Anal. Calcd for C₂₃H₃₉NO₂: C, 76.40; H, 10.87; N, 3.87. Found: C, 76.53; H, 10.95; N, 3.79.

5.3.6. *N*-((*R*)-1-Hydroxy-4-phenylbutan-2-yl)palmitamide (SG-6). ¹H NMR (300 MHz, CDCl₃) δ 7.3–7.1 (m, 5H), δ 5.7 (d, *J* = 7.8 Hz, 1H), δ 4.05–3.92 (m, 1H), δ 3.68 (dd, *J* = 11.1, 3.6 Hz, 1H), δ 3.59 (dd, *J* = 11.1, 5.4 Hz, 1H), δ 2.94 (br s, 1H), δ 2.68 (t, *J* = 7.8 Hz, 2H), δ 2.15 (t, *J* = 7.8 Hz, 2H), δ 1.96–1.74 (m, 2H), δ 1.65–1.45 (m, 2H), δ 1.25 (br s, 26H), δ 0.88 (t, *J* = 6.6 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 174.1, 141.4, 128.5, 128.3, 126.1, 65.7, 51.6, 36.9, 32.8, 32.5, 31.9, 29.7, 29.6, 29.5, 29.3, 29.3, 25.8, 22.7, 14.1; Anal. Calcd for C₂₆H₄₅NO₂: C, 77.37; H, 11.24; N, 3.47. Found: C, 77.42; H, 11.38; N, 3.39.

5.3.7. (*R*)-2-Amino-4-(4-octylphenyl)butan-1-ol (SG-7). ¹H NMR (300 MHz, CDCl₃) δ 7.1–6.95 (m, 4H), δ 3.59 (dd, *J* = 10.5, 3.6 Hz, 1H), δ 3.32 (dd, *J* = 10.5, 7.8 Hz, 1H), δ 2.9–2.8 (m, 1H), δ 2.7–2.6 (m, 2H), δ 2.58 (t, *J* = 7.8 Hz, 2H), δ 1.8–1.65 (m, 1H), δ 1.6–1.5 (m, 2H), δ 1.38–1.15 (m, 12H), δ 0.88 (t, *J* = 6.0 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 140.5, 138.7, 128.4, 128.1, 66.4, 52.4, 35.9, 35.5, 31.9, 31.9, 31.5, 29.4, 29.3, 29.2, 22.6, 14.0; Anal. Calcd for C₁₈H₃₁NO: C, 77.92; H, 11.26; N, 5.05. Found: C, 77.84; H, 11.31; N, 5.12.

5.3.8. (2*S*,3*S*)-2-Amino-4-(4-octylphenyl)butane-1,3-diol (SG-9). ¹H NMR (300 MHz, CD₃OD) δ 7.16 (d, *J* = 7.8 Hz, 2H), δ 7.07 (d, *J* = 7.8 Hz, 2H), δ 3.85–3.75 (m, 1H), δ 3.69 (dd, *J* = 10.8, 5.4 Hz, 1H), δ 3.5 (dd, *J* = 10.8, 6.6 Hz, 1H), δ 2.84 (dd, *J* = 13.8, 5.4 Hz, 1H), δ 2.75–2.62 (m, 2H), δ 2.55 (dd, *J* = 7.8, 7.2 Hz, 2H), δ 1.64–1.5 (m, 2H), δ 1.28 (br s, 12H), δ 0.89 (t, *J* = 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 140.1, 138.2, 128.6, 128.5, 52.1, 35.4, 35.2, 31.6, 31.1, 31.0, 29.7, 29.6, 29.5, 22.6, 14.0; Anal. Calcd for C₁₈H₃₁NO₂: C, 73.67; H, 10.65; N, 4.77. Found: C, 73.68; H, 10.71; N, 4.67.

5.3.9. (2*R*,3*S*)-3-Amino-4-morpholino-1-phenylbutan-2-ol (SG-10). ¹H NMR (300 MHz, CDCl₃) δ 7.35–7.2 (m, 5H), δ 3.8–3.6 (m, 5H), δ 2.96 (dd, *J* = 13.2, 2.4 Hz, 1H), δ 2.92 (dd, *J* = 13.2, 6.6 Hz, 1H), δ 2.68 (dd, *J* = 13.5, 8.7 Hz, 1H), δ 2.6–2.4 (m, 6H); ¹³C NMR

(75 MHz, CDCl₃) δ 138.9, 129.2, 128.3, 126.2, 77.8, 66.7, 63.9, 54.0, 49.9, 40.1; Anal. Calcd for C₃₂H₅₆N₂O₂: C, 67.17; H, 8.86; N, 11.19. Found: C, 67.22; H, 8.92; N, 11.09.

5.3.10. *N*-((*R*)-4-Phenyl-1-(pyrrolidin-1-yl)butan-2-yl)decanamide (SG-11). ¹H NMR (300 MHz, CDCl₃) δ 7.4–7.1 (m, 5H), δ 5.7 (d, *J* = 7.5 Hz, 1H), δ 4.1–4.0 (m, 1H), δ 2.7–2.55 (m, 2H), δ 2.54–2.4 (m, 6H), δ 2.16 (t, *J* = 7.2 Hz, 2H), δ 2.0–1.85 (m, 1H), δ 1.8–1.7 (m, 4H), δ 1.68–1.55 (m, 2H), δ 1.38–1.25 (m, 14H), δ 0.87 (t, *J* = 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 173.1, 142.0, 128.3, 125.7, 59.5, 54.5, 48.4, 36.9, 35.5, 32.2, 31.8, 29.4, 29.3, 29.2, 25.8, 23.6, 22.6, 14.0; Anal. Calcd for C₂₄H₄₀N₂O: C, 77.37; H, 10.82; N, 7.52. Found: C, 77.45; H, 10.94; N, 7.64.

5.3.11. (2*S*,3*R*)-2-Amino-4-(4-octylphenyl)butane-1,3-diol (SG-12). ¹H NMR (300 MHz, CDCl₃) δ 7.16 (d, *J* = 7.8 Hz, 2H), δ 7.08 (d, *J* = 7.8 Hz, 2H), δ 3.85–3.7 (m, 2H), δ 3.54 (dd, *J* = 10.8, 7.8 Hz, 1H), δ 1.64–1.5 (m, 2H), δ 1.28 (br s, 12H), δ 0.89 (t, *J* = 6.0 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 139.8, 137.9, 128.3, 128.2, 51.8, 35.1, 34.9, 31.3, 29.9, 29.7, 29.4, 29.3, 29.2, 22.3, 13.9; Anal. Calcd for C₁₈H₃₁NO₂: C, 73.67; H, 10.65; N, 4.77. Found: C, 73.75; H, 10.72; N, 4.69.

5.3.12. *N*-((2*S*,3*S*)-3-Hydroxy-4-phenyl-1-(pyrrolidin-1-yl)butan-2-yl)decanamide (SG-13). ¹H NMR (300 MHz, CDCl₃) δ 7.3–7.16 (m, 5H), δ 6.38 (d, *J* = 9.0 Hz, 1H), δ 5.3–5.2 (m, 1H), δ 4.44–4.32 (m, 1H), δ 2.94–2.8 (m, 2H), δ 2.72 (dd, *J* = 12.0, 9.0 Hz, 1H), δ 2.6–2.4 (m, 5H), δ 2.3–2.2 (m, 4H), δ 1.8–1.6 (m, 6H), δ 1.58–1.42 (t, *J* = 6.9 Hz, 2H), δ 1.36–1.2 (m, 14H), δ 0.88 (t, *J* = 6.6 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 173.2, 136.8, 129.3, 128.1, 126.3, 74.3, 56.3, 53.6, 48.9, 37.7, 36.6, 34.1, 31.7, 31.7, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 28.9, 25.6, 24.7, 23.2, 22.5, 13.9; Anal. Calcd for C₂₄H₄₀N₂O₂: C, 74.18; H, 10.38; N, 7.21. Found: C, 74.32; H, 10.42; N, 7.19.

5.3.13. *N*-((2*S*,3*S*)-3-Hydroxy-4-phenyl-1-(pyrrolidin-1-yl)butan-2-yl)stearamide (SG14). ¹H NMR (300 MHz, CDCl₃) δ 7.4–7.2 (m, 5H), δ 6.16 (d, *J* = 8.1 Hz, 1H), δ 4.18 (t, *J* = 6.3 Hz, 1H), δ 4.0–3.9 (m, 1H), δ 2.87 (dd, *J* = 7.2, 3.6 Hz, 2H), δ 2.85–2.75 (m, 2H), δ 2.74–2.68 (m, 2H), δ 2.62–2.54 (m, 2H), δ 2.27 (dd, *J* = 7.8, 6.9 Hz, 2H), δ 1.8–1.65 (m, 6H), δ 1.4–1.26 (m, 30H), δ 0.91 (t, *J* = 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 173.0, 138.0, 129.3, 128.5, 126.4, 75.2, 59.4, 55.9, 50.4, 40.4, 36.9, 31.9, 29.7, 29.6, 29.5, 29.3, 25.8, 23.7, 22.6, 14.1; Anal. Calcd for C₃₂H₅₆N₂O₂: C, 76.75; H, 11.27; N, 5.59. Found: C, 76.81; H, 11.32; N, 5.48.

5.3.14. *N*-((2*S*,3*R*)-3-Hydroxy-4-phenyl-1-(pyrrolidin-1-yl)butan-2-yl)stearamide (SG-15). ¹H NMR (300 MHz, CDCl₃) δ 7.3–7.1 (m, 5H), δ 5.84 (d, *J* = 7.5 Hz, 1H), δ 5.35–5.27 (m, 1H), δ 4.3–4.2 (m, 1H), δ 2.95–2.85 (m, 2H), δ 2.71 (dd, *J* = 12.3, 9.3 Hz, 1H), δ 2.58–2.42 (m, 4H), δ 2.25–2.8 (m, 4H), δ 1.74 (br s, 4H), δ 1.65–1.55 (m, 2H), δ 1.5–1.4 (m, 2H), δ 1.25 (br s, 30H), δ 0.88 (t, *J* = 6.0 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ

173.2, 137.3, 129.2, 128.3, 126.4, 74.7, 54.8, 54.2, 50.1, 37.1, 36.8, 34.3, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 25.7, 24.8, 23.5, 22.6, 14.1; Anal. Calcd for C₃₂H₅₆N₂O₂: C, 76.75; H, 11.27; N, 5.59. Found: C, 76.79; H, 11.32; N, 5.45.

5.3.15. *N*-((2*S*,3*S*)-3-Hydroxy-1-morpholino-4-phenylbutan-2-yl)decanamide (SG-16). ¹H NMR (300 MHz, CDCl₃) δ 7.35–7.15 (m, 5H), δ 6.17 (d, *J* = 8.4 Hz, 1H), δ 5.09 (br s, 1H), δ 4.1–3.9 (m, 2H), δ 3.65 (t, *J* = 4.5 Hz, 4H), δ 2.76 (d, *J* = 2.7 Hz, 1H), δ 2.74 (br s, 1H), δ 2.69 (d, *J* = 5.4 Hz, 1H), δ 2.6–2.4 (m, 5H), δ 2.22 (t, *J* = 6.9 Hz, 2H), δ 1.7–1.6 (m, 2H), δ 1.4–1.2 (m, 14H), δ 0.88 (t, *J* = 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 173.2, 137.8, 129.2, 128.5, 126.5, 73.5, 66.8, 60.8, 54.6, 49.3, 40.5, 36.8, 31.8, 29.4, 29.3, 29.2, 29.1, 25.8, 22.6, 14.0; Anal. Calcd for C₂₄H₄₀N₂O₃: C, 71.25; H, 9.97; N, 6.92. Found: C, 71.33; H, 10.04; N, 6.83.

5.4. DNA construction

HA-tagged hSphK1 and HA-tagged hSphK2 were generous gifts from Dr. Wada (this laboratory). All nucleotides were synthesized with BamHI and EcoRI ends. The antisense primers were treated with T4 polynucleotide kinase (Takara, Ohtsu, Japan). The PCR was carried out with KOD plus (Toyobo, Osaka, Japan) in the presence of 0.2 mM dNTPs, 1 mM MgCl₂ and 0.3 μ M polynucleotide kinase-treated primers, for 25 cycles. The PCR products were ligated using Ligation High (Toyobo, Osaka, Japan). After a BamHI-EcoRI digestion, the fragment was ligated into a BamHI-EcoRI-digested pcDNA3-HA vector.

5.5. Cell culture and DNA transfection

Chinese hamster ovary (CHO-K1) cells were grown in Ham's F-12 medium (Sigma, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (Iwaki, Japan), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Sigma) at 37 °C in a humidified atmosphere of 5% carbon dioxide. Cells were transfected with the N-terminal hamagglutinin(HA)-tagged plasmids pcDNA3-HA-hSphK1 and pcDNA3-HA-hSphK2 using LIPOFECTAMINE-PLUS (Invitrogen) according to the manufacturer's instructions. Plasmids used in the transfection were prepared with a Quantum Prep Plasmid Miniprep Kit (BIO-RAD).

5.6. Immunoblotting

Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking with 5% skimmed milk, the membrane was incubated for 1 h with a 1:1000 dilution of HA-Y11 antibodies (Santa Cruz, CA). The blot was washed with TBS-T (137 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 0.05% Tween 20) and then incubated with a 1:10,000 dilution of anti-rabbit-IgG-HRT (Amersham Pharmacia Biotech.). The blot was washed again with TBS-T and detection was performed with an Enhanced Chemiluminescence Detection Kit (ECL, Amersham

Pharmacia Biotech.). An anti-tubulin (Sigma) blot was performed as a loading control.

5.7. In vitro sphingosine kinase assay

Protein (1 μg) from each transfected cell lysate was incubated with 2 μCi [γ - ^{32}P]ATP (10 mCi/ μmol , Perkin–Elmer Life Science, Boston, MA), 0.5 mM cold ATP, and 40 μM substrate (D-erythro-sphingosine (Sigma), phytosphingosine (BIOMOL, Plymouth, PA), or FTY-720 (generous gift from Sankyo, Co., Ltd., Japan)) at 37 °C for 30 min in assay buffer (20 mM Tris–HCl (pH 7.5), 0.25 mM EDTA, 1 mM magnesium chloride, 12 mM β -glycerophosphate, 1 mM sodium pyrophosphate, 5 mM sodium fluoride, 5 mM sodium orthovanadate, 2 mM dithiothreitol, and 1 \times protease inhibitor cocktail (Roche)). For SphK2 assay, 200 mM KCl was added in assay buffer. Lipids were extracted by adding 200 μL chloroform/methanol/HCl (100:200:1) and vortexing for 1 min. Consequently, 65 μL of chloroform and 65 μL of 1 M KCl were added and the mixture was vortexed vigorously for 5 min. The mixtures were centrifuged for 2 min at 5000 $\times g$ and the upper phase was removed. The samples were evaporated and resuspended in chloroform/methanol (2:1). Lipids were separated by TLC with 1-butanol/acetic acid/water (3:1:1). A sphingosine 1-phosphate band was visualized and quantified using a Fujix Bio-Imaging Analyzer, BAS2500 (Fuji Photo Film, Japan).

5.8. Cytotoxicity assay

The cytotoxic effect was measured using 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrasolium (Cell counting Kit-8, Dojindo, Japan). CHO-K1 cells (5×10^3 cell/100 μL) were plated in 96-well dishes and incubated at 37 °C for 24 h in a humidified atmosphere of 5% CO_2 . Then, cells were incubated with 10 μL of Sphingoid analogs for an additional 24–48 h. Next, 10 μL of Cell counting kit-8 were added in each well, and the samples were incubated for another 4 h. The absorbance at a wavelength of 450 nm was measured to detect mitochondrial dehydrogenase enzymatic activity.

5.9. In vitro protein kinase C assay

PKC activity was measured by using a SignaTech™ PKC assay system (Promega, Madison, WI) according to the manufacturer's instructions. This assay depends on the transfer of ^{32}P from [γ - ^{32}P]ATP to the biotinylated peptide, Neurogranin (AAKIQAS*FRGHMARKK), the most specific substrate commercially available for PKC activity. To monitor the influence of various synthetic compounds on PKC activity, the indicated quantities of the SG samples were supplemented into a PKC assay buffer mixture [1.6 mg/mL phosphatidylserine, 0.16 mg/mL diacylglycerol, 100 mM Tris–HCl (pH 7.5), 50 mM MgCl_2 , 1.25 mM EGTA, 2 mM CaCl_2 , 0.5 mg/mL BSA and [γ - ^{32}P]ATP] to a final volume of 20 mL. The reaction was initiated by adding 5 mL of PKC (5 ng PKC enzyme, purified from rat brain) then was incubated for 5 min at 30 °C. The biotinylated,

^{32}P -labeled substrate was recovered from the reaction mix with a biotin capture membrane, and placed in scintillation vials.

5.10. Platelet preparation

Preparation of washed platelets was performed as previously described.⁵⁰ The platelets were finally resuspended in a pH 7.4 buffer (138 mM NaCl, 3.3 mM NaH_2PO_4 , 2.9 mM KCl, 1 mM MgSO_4 , 1 mg/mL glucose, and 20 mM Hepes), and 1% bovine serum albumin (fatty acid free) was added. All experiments using intact platelet suspensions were performed at 37 °C.

5.11. Metabolism of [3H] sphingosine in platelets

100 μL (3×10^7 platelets/100 μL) platelets suspensions were pre-incubated with inhibitors for 5 min, then 0.2 μCi [^3H] sphingosine (Perkin–Elmer Life Science, Boston, MA) were added and the mixture was incubated for 15 min. The reaction was terminated by adding 400 μL of chloroform/methanol/HCl (100:200:1), and 130 μL of chloroform and 130 μL of 1 M KCl were added, and the mixture was vortexed vigorously for 5 min. The mixtures were centrifuged for 2 min at 5000 $\times g$ and the upper phase was removed. The samples were evaporated and resuspended in chloroform/methanol (2:1). Lipids were separated by TLC with 1-butanol/acetic acid/water (3:1:1). The sphingosine 1-phosphate band was visualized by autoradiography.

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