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# Effects of Synthetic Pseudoceramides on Sphingosine Kinase Activity in F9-12 Cells

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## Abstract

Sphingosine kinase (SPHK) has a central role to control cell death and cell proliferation, which is suggested as a sphingolipid rheostat by regulating the levels between ceramide and sphingosine 1-phosphate (S1P). Therefore, physiological regulators of SPHK will be a good candidate to develop a new targeted drug. For this purpose, a series of synthetic pseudoceramides were tested by SPHK assay either cell-based or cell-free system. K10PC-5 strongly inhibited SPHK, while K6PC-5 activated SPHK in cell-free system. Specifically, K6PC-5 activated SPHK under the co-treatment with 50 μM dimethylsphingosine (DMS), a SPHK inhibitor. Collectively, we developed a simple SPHK assay system to find SPHK regulatory pseudoceramide compounds, K10PC-5 and K6PC-5 which may be useful to cancer treatment or immune regulation like FTY720, a synthetic sphingolipid mimetic compound.

**Key Words:** Pseudoceramide, Sphingosine kinase, F9 cells, Assay system

## INTRODUCTION

Sphingosine kinase (SPHK) phosphorylated sphingosine to yield sphingosine 1-phosphate (S1P) which is a bioactive autocrine lipid as it binds to five different G-protein-coupled S1P (S1P1-5) receptors to elicit diverse biological responses (Lee *et al.*, 1996). The produced S1P in mammalian cells is short-lived, due to rapid degradation by S1P lyase and dephosphorylation by S1P phosphohydrolases (Pyne and Pyne, 2000).

However, S1P has emerged as an enigmatic lipid which is implicated in both extracellular and intracellular signaling processes (Hla *et al.*, 2001). S1P has been shown to be secreted into blood stream from platelets, red blood cells (RBCs) or vascular endothelial cells, and binds to S1P receptors on the surface of vascular endothelial cells, triggering such cellular processes as cell differentiation, migration, and mitogenesis (Maceyka *et al.*, 2002). In addition, S1P also worked as a lipid second messenger participating in signaling cascades leading to cytoskeleton changes, motility, release of intracellular calcium stores, and cell protection from apoptosis (Kluk and Hla., 2002; Spiegel *et al.*, 2002; Olivera *et al.*, 2003; Watterson *et al.*, 2003). Whether acting intracellular or extracellular, S1P is generated from phosphorylation of sphingosine by SPHK. Therefore, the enhanced production of S1P in biological systems through an increased SPHK activity has become an interesting research subject.

The ceramide-S1P rheostat theory has been suggested to determine the cell fate such that the intracellular concentration ratio of ceramide to S1P determines whether a cell proliferates or undergoes apoptosis (Spiegel *et al.*, 2002). Ceramide is produced by hydrolysis of sphingomyelin in response to stresses, including exposure to chemotherapeutic drugs and induces apoptosis through multiple cell death signaling pathway (Hannun and Obeid, 1995; Ogretmen and Hamun, 2001; Johnson *et al.*, 2002). Alternatevely, ceramide can be additionally hydrolyzed by ceramidase to produce sphingosine. Sphingosine is then rapidly phosphorylated by SPHK to produce S1P. Ceramidase and SPHK are activated by a number of growth factors and intracellular oncoproteins, leading to rapid increases in the intracellular S1P and concurrent depletion of ceramide. This situation promotes cell proliferation and inhibits apoptosis in tumor cells. Therefore, SPHK enzyme provides a potential target for the development of new anticancer drug (Claus *et al.*, 2000).

There are limited regulators of sphingolipid-metabolizing enzymes, and therefore pharmacological regulation of sphingolipid metabolism remains an untested area toward cancer chemotherapy. Especially, inhibition of SPHK activity and

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subsequent reduction of S1P synthesis should have profound anti-proliferative effect on tumor cells because S1P is the most direct mitogenic messenger. This focus is substantiated by a recent demonstration that SPHK can directly transform the cells (Xia *et al.*, 2000). This paper described the first demonstration that SPHK1 increases V12 RAS-dependent transformation of NIH3T3 fibroblasts to form fibrosarcoma cells when injected into immune compromised mice. Pharmacological studies have used three compounds to inhibit SPHK activity: N,N-dimethylsphingosne (DMS), *D,L-threo*-dihydrosphingosine and *N,N,N*-trimethylsphingosine. However, these compounds are not specific inhibitors of SPHK as they are known to affect other kinases such as protein kinase C (Igarashi *et al.*, 1989), sphingosine-dependent protein kinase (Megidish *et al.*, 1995), 3-phosphoinositide-dependent kinase (King *et al.*, 2000), and casein kinase (McDonald *et al.*, 1991). In this report, we tested a series of synthetic pseudoceramides whether these compounds could regulate SPHK activity and found that K6PC-5, a hydrophobic pseudo-ceramide chemically named N-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide enhanced SPHK activity of F9-12 cell lysates where SPHK was highly expressed.

## MATERIALS AND METHODS

### Materials

The five synthetic pseudoceramides, (K-series; K6PC-5, K6PC-7 and K10PC-5) (L-series; L6PC-5 and L6PC-7) were synthesized and were kindly supplied from Neopharm Co. (Daejeon, Korea). Sphingosine 1-phosphate (S1P) and *d-erythro*-sphingosine (So) were purchased from Biomol Research, Inc. (Plymouth Meeting, PA.,USA). $C_{17}$-sphingosine ($C_{17}$-So) and $C_{17}$ sphingosine 1-phosphate ($C_{17}$-So1P) were obtained from Avanti Polar Lipids, Inc. (Alabastar, AL). Alkaline phosphatase (APase) (bovine intestinal mucosca type VII-T), *N,N*-dimethylsphingosine (DMS) and phorbol 12-myristate 13-acetate (PMA) were from Sigma (St. Louis, MO). Serum and culture media were obtained from Life Technologies, Inc. (Gaithersburg, MD). HPLC-grade acetonitrile was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). *o*-phthalaldehyde (OPA) was obtained from Nacalai Tesque (Kyoto, Japan). All organic solvents and chemicals were of analytical grade.

### Cell culture and pseudoceramides treatment

Mouse embryonal carcinoma F9-12 cells (SPHK overexpressed S1P lyase knocked-out) were kindly supplied by Dr. A. Kihara in Hokkaido University, Japan. Cells were grown in Dulbecco modified Eagles medium (DMEM) containing 10% (v/v) FBS and 1% penicillin-streptomycin in 0.1% gelatin-coated dishes. Cells were cultured at 37°C in a humidified 5% $CO_2$ atmosphere and routinely sub-cultured every other day using a solution of trypsin-EDTA from Life Technologies, Inc. (Gaithersburg, MD).

Pseudoceramides were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C in 50 mM stock solution. Ten microliters of stock solution were directly added to 10 ml DMEM media of F9-12 cells growing dish. The cells were further incubated for 24 hr.

### Preparation of cell lysate

Cells were immediately washed twice with ice-cold phosphate-buffered saline and then scraped in 3 ml of lysis buffer (1 mM PMSF, 1 x protease inhibitor cocktail, and 1 mM dithiothreitol) after centrifugation at 10,000 RPM for 3 min. The pellet lysated in 1 ml assay buffer (12 mM β-glycerophosphate, 1 mM sodium pyrophosphate, 5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 x protease inhibitor cocktail, 5 mM sodium orthovanadate, 2 mM dithiothreitol, 0.5 mM 4-deoxypyridoxine) was then sonicated and centrifuged at 1,500 rpm for 3 min at 4°C and the resulting supernatant was determined the protein concentration by performing Bradford assay. The cell lysate was diluted to approximately 1 mg/ml total cell protein.

### SPHK activity assay

Cells lysate (30 μg protein) was incubated in total volumes of 160 μl with 10 μl of 40 mM ATP in 200 mM $MgCl_2$ and 10 μl of 100 μM $C_{17}$-sphingosine in 5% Triton X-100. Incubations with cell lysates contained 5 mM NaF and $Na_3VO_4$, included as inhibitors of S1P phosphatase and lyase, respectively, to prevent potential degradation of formed $C_{17}$-S1P. In case of *in vitro* assay, 10 μl from 1 mM K6PC-5 in DMSO was directly added to the cells lysate. The reaction started with $C_{17}$-sphingosine addition to tube and was incubated for 20 min at 37°C for SPHK activation. The enzyme reaction was terminated by adding 20 μl of 1.0 N-HCl and ice-cold 0.8 ml $CHCl_3$/Methanol/HCl (100:200:1, v/v). The tube was gently mixed for 1 min after spiking 200 pmol of $C_{18}$-S1P as an internal standard. Then, 250 μl of $CHCl_3$ and 250 μl of 2M NaCl were added, and the mixture was vigorously vortexed for 10 min. The tube was centrifuged for 4 min at 12,000 rpm at 4°C and the aqueous upper phase was removed. The lower organic phase was transferred to a fresh tube and left on ice for 10 min. Phospholylated $C_{17}$-S1P was extracted by adding 400 μl of $H_2O$ and 40 μl of 3N NaOH. The tube was then vortexed for 10 min and centrifuged at 12,000 rpm for 4 min. The alkaline aqueous phase containing S1P transferred to a fresh tube. The residual S1P in the $CHCl_3$ phase were extracted twice with a 300 μl of $H_2O$ and 10 μl of 3N NaOH. All of the aqueous fractions were then combined. The aqueous fraction of S1P extract was mixed thoroughly with 130 μl of alkaline phosphatase (APase) reaction buffer (200 mM Tris-HCl (pH 7.4), 75 mM $MgCl_2$ in 2M glycine buffer, pH 9.0) and 50 units of APase enzyme for dephosphorylation. The tube was incubated at 37°C for 1 hr. The dephosphorylated sphingosine and $C_{17}$-sphingosine were extracted twice with 500 μl of $CHCl_3$ and 300 μl of $CHCl_3$ then washed 3-times with alkaline water (pH 10.0). The washed $CHCl_3$ phase was transferred to a fresh tube and dried completely under a nitrogen stream in a Pierce heating module.

### HPLC analysis

The dried residues were dissolved again in 120 μl ethanol, and incubated it at 65°C for 25 min and then labeled with 15 μl OPA reagent (50 mg OPA, 1 ml ethanol, 100 μl 2-mercaptoethanol, and 50 ml 3% (w/v) boric acid solution). After incubation of the tubes for 40 min in dark room at room temperature, 100 μl aliquots was injected into the HPLC system which consisted of a Jasco (Tokyo, Japan) Model PU 9850 pump, Cosmosil 5C 18-AR II packed with Nova-Pak C18 (4.6 mm i.d. ×150 mm), Jasco FP-920 fluorescence spectrophotometer (excitation 340 nm and emission 455 nm) and Jasco autosampler. The isocratic mobile phase of 90% acetonitrile was pumped at a flow rate of 1ml/min. The resulting data and chromatographic

profiles were evaluated using the Borwin system manager software (JMBS, France).

### XTT assay

F9-12 cells were harvested, counted, and inoculated at 2.5 ×$10^3$ cells (100 μl) into 96-well plates. After 24 hr, pseudoceramides dissolved in medium were applied (100 μl) to triplicate culture wells, and cultures were incubated for 24 hr at 37°C. XTT (Sigma, St. Louis, MO) was prepared at 5 mg/ml in PBS and stored at 4°C. After 24 hr incubation, XTT was diluted 1 to 5 in medium without serum (containing 100 μl activation solution), and 50 μl were added to microculture wells. After 4 hr incubation at 37°C, the plate was gently shaken to evenly distribute XTT dye in the wells. The absorbance at 540 nm was measured with a Molecular Devices mciroplate reader.

### Statistics

All values were expressed as a mean ± standard deviation (SD). Differences between treatments were analyzed statistically by unpaired Student's t-test. Differences with $p<0.01$ were defined as statistically significant.

## RESULTS

### Design of synthetic pseudoceramides for the regulation of SPHK activity

Five pseudoceramides showing closely related structure to natural ceramides were synthesized by NeoPharm Co.. Three pseudoceramides (K-series) contains a ketone group, two hydroxyl groups, two short alkyl groups, and an amide linkage with a chemical name of N-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide. Other two pseudoceramides (L-series) contains three hydroxyl groups, two short alkyl groups, and an amide linkage with a chemical name of N-(1,3-dihydroxyisopropyl)-2-hexyl-3-hydroxy-decanamide (Fig. 1). Five pseudoceramides were commonly designed to contain a 1,3-dihydroxyisopropyl group, which possibly provides phosphorylation site by SPHK activation.

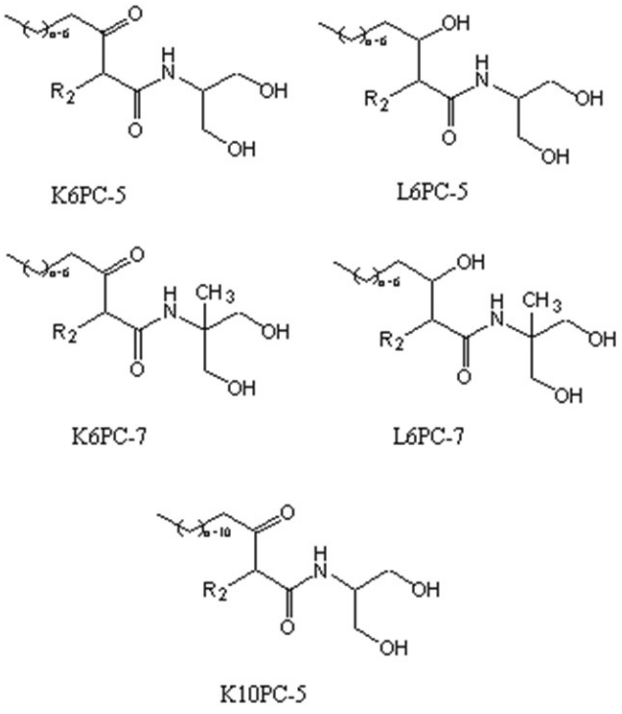


**Fig. 1.** Chemical structure of pseudoceramides. R2 contains hexyl group. K-series contains a ketone group while L-series contains a hydroxyl group.

### Effect of synthetic pseudoceramides on SPHK activity in F9-12 cells

Previously, PMA has been well used as a typical SPHK activation marker in a cell-based assay system. SPHK activity in F9-12 cells was 1.5-fold activated in 1 hr treatment, indicating that F9-12 cell-based system is a reliable tool to search for SPHK regulators. The internal SPHK activities in F9-12 cells were relatively high because F9-12 cells were SPHK over-expressed mutant selected from a colony resistant to G418 (Geneticin®) treatment. Therefore, SPHK activity with 10 ul of DMSO treatment (control) was measured 780 pmol/min/mg protein. Pseudoceramide K10PC-5 greatly inhibited SPHK activity compared to other pseudoceramides (Fig. 2). This unusual inhibition by K10PC-5 might be contributed to the existence of relatively long chain of 3-oxo-tetradecanamide. L6PC-7 which containing 2-methyl group on N-(1,3-dihydroxyisopropyl) structure also mildly inhibited SPHK activity. Interestingly, K6PC-5 was seem to activate SPHK activity, suggesting that two hydroxyl sites in N-(1,3-dihydroxyisopropyl) structure may activate SPHK through providing phosphorylation site like as a major substrate, sphingosine.

### Inhibition of SPHK activity by K10PC-5

K10PC-5 strongly inhibited SPHK activity from the concentration above 20 μM, while 10 uM did not show any inhibition on SPHK activity (Fig. 3A). The 88% of SPHK activity was reduced by 20 μM K10PC-5, suggesting that K10PC-5 will be a pharmacological candidate for SPHK enzyme inhibitor which may induce cancer cells death. Next, inhibition of SPHK activ-

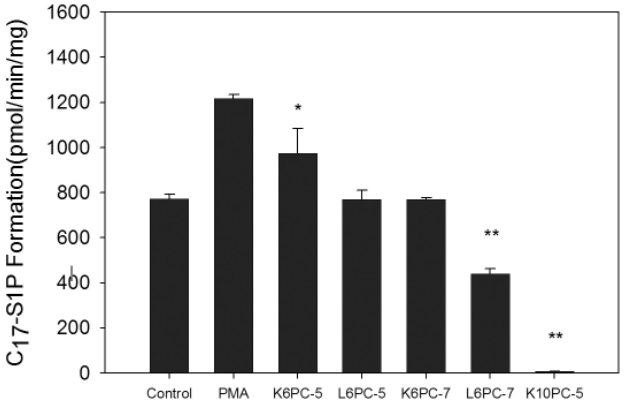


**Fig. 2.** Effect of pseudo-ceramides on SPHK activity in F9-12 cells. F9-12 cells were treated with 300 nM of PMA and incubated for 1 hr. The C17-S1P formation by PMA treatment used as a positive SPHK activation biomarker. Five pseudo-ceramides, K6PC-5, L6PC-5, K6PC-7, L6PC-7 and K10PC-5 were treated with the concentration of 50 μM in F9-12 cells and further incubated for 24 hr. Control means 10 μl DMSO treatment. Data are shown as mean±S.D. *$p<0.05$, **$p<0.01$ compare to control group.

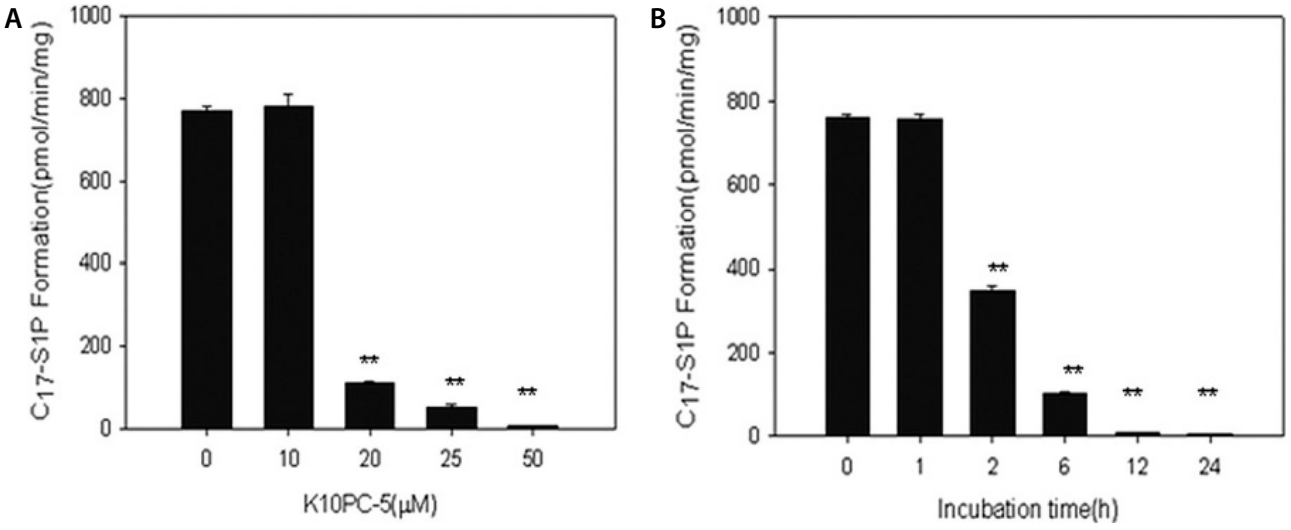


**Fig. 3.** Inhibition of SPHK activity by K10PC-5. A) F9-12 cells were treated with indicated concentrations (0, 10, 20, 25, 50 μM) of K10PC-5 and then incubated for 24 hr. B) F9-12 cells was treated with 50 μM K10PC-5 and incubated with indicated hours. The SPHK enzyme activity was measured. ***p*<0.01 compare to control group.

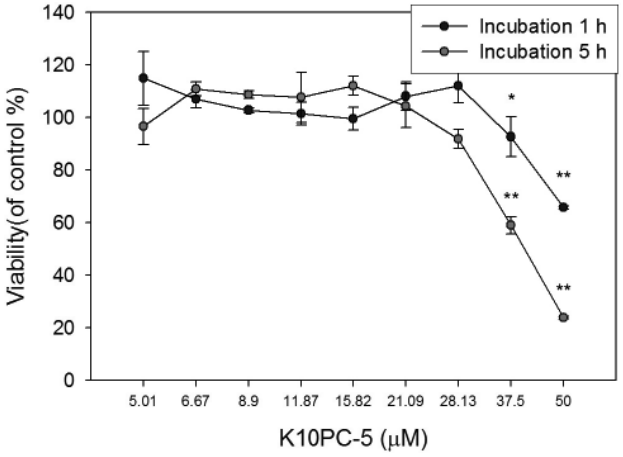


**Fig. 4.** Effect of K10PC-5 on F9-12 cells viability. F9-12 cells in $2.5\times10^4$ cells/ml were seeded. After 24 hr incubation, F9-12 cells were treated with the indicated concentrations (5.01, 6.67, 8.9, 11.87, 15.82, 21.09, 28.13, 37.5, 50 μM) of K10PC-5 and then incubated for 1 hr or 5 hr. XTT assay were applied for measuring the cell viability. Black circle represents the 1 hr incubation at the given concentrations of K10PC-5, and grey circle represents the 5 hr incubation. Data are shown as mean ± S.D. **p*<0.05, ***p*<0.01 compare to control group.

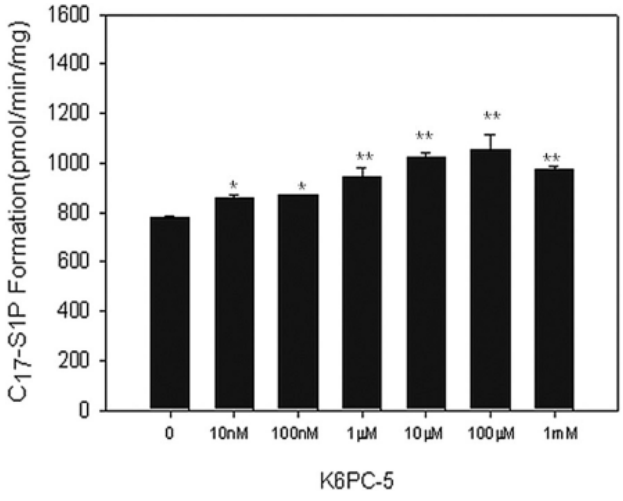


**Fig. 5.** Activation of SPHK activity in cell lysate by K6PC-5. Cell lysate was treated with the indicated concentrations (0, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM and 1 mM) of K6PC-5, then incubated for 20 min at 37°C.Data are shown as mean ± S.D. **p*<0.05, ***p*<0.01 compare to control group.

ity by K10PC-5 was measured to find the minimal time point by varying the incubation time. As shown in Fig. 3B, K10PC-5 (50 μM) started to inhibit 50% of C17-S1P formation within 2 hr. These results indicated that K10PC-5 has a strong potency to inhibit SPHK activity.

In short hour treatment of K10PC-5 from 1 hr to 5 hr, cell viability was not changed below the concentration of 30 μM (Fig. 4). However, in high concentration of K19PC-5 cell viability was abruptly reduced in a dose-dependent manner, suggesting that the inhibition of SPHK activity might trigger a variety of death signals.

### Specific activation of SPHK activity by K6PC-5

Meanwhile, K6PC-5 compound which has a relatively short chain 3-oxo-decanamide moiety was well dissolved in culture medium. As shown in Fig. 1, K6PC-5 showed a unique character to enhance SPHK activity in cell-based assay. To clarify SPHK regulation specificity of K6PC-5 in cell-free system, cell lysate was incubated with various concentrations of K6PC-5 for 20 min at 37°C. In cell-free system, K6PC-5 also activated SPHK activity in a dose dependent manner, although a high concentration of 1.0 mM K6PC-5 mildly activated SPHK activity (Fig. 5).

DMS has been well known as a strong SPHK inhibitor *in*

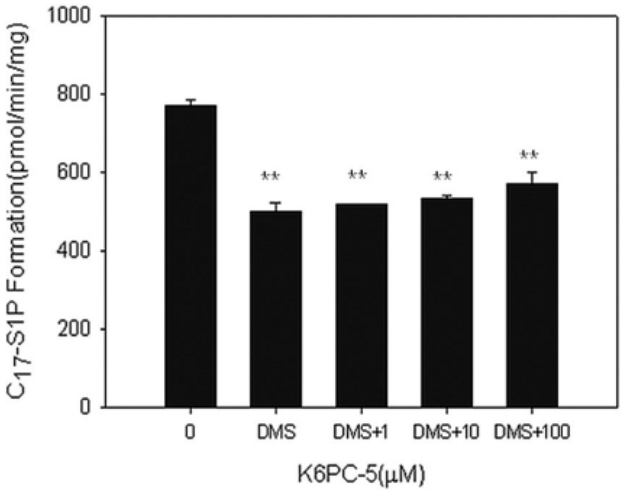


**Fig. 6.** Effect of K6PC-5 on SPHK activity during SPHK inhibition by 50 μM DMS. Cell lysate was co-treated with 50 μM DMS and three different concentrations (1, 10 and 100 μM) of K6PC-5 for 20 min. Data are shown as mean ± S.D. ***p*<0.01 compare to control group.

*vivo* or *in vitro* system and still have used to be a control compound. In this experiment, K6PC-5 were treated with 50 μM DMS in the indicated different concentrations for measuring the SPHK activating ability by K6PC-5. DMS only treatment inhibited ca. 40% of total SPHK activity. However, by co-treatment of 100 μM K6PC-5 with 50 μM DMS, reduced-SPHK activity was slightly recovered in a dose dependent manner (Fig. 6).

## DISCUSSION

We newly synthesized five chemically related compounds which their representative compound, K6PC-5 containing a ketone group, two hydroxyl groups, two short alkyl groups, and an amide linkage, characterized as a "pseudo-ceramide" backbone with a chemical name of N-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide. K6PC-5 was originally reported to induce the keratinocyte differentiation (Kwon *et al.*, 2007).

The synthesis of pseudoceramides was basically designed on the fact that the reduction of carbon chain length may reduce the molecular size which gives similar hydrophobicity to sphingosine, a SPHK substrate. In addition, 1,3-dihydroxyisopropyl structure may provide the double phosphorylation sites to SPHK enzyme. Indeed, FTY720, a synthetic sphingosine-like compound containing a 1,3-dihydroxyisopropyl group was well phosphorylated by SPHK activation (Billich *et al.*, 2003). The methyl moiety in 1,3-dihydroxyisopropyl group of K6PC-7 and L6PC-7 may not be a significant factor for phosphorylation, rather be a disturbance factor for phosphorylation (Fig. 2).

In the presence of increased hydrophobicity by the extension of chain length from 3-oxo-decanamide to 3-oxo-tetradecanamide, K10PC-5 greatly inhibited SPHK (Fig. 3). This inhibition also influenced on cell viability after the treatment of 50 μM K10PC-5 in 1 hr (Fig. 4). However, this cell-based assay did not exclude the possibility that K10PC-5 possibly triggers the multiple set of cell death signaling pathway. Recently, SPHK was suggested as a new drug target for the treatment of cancer and many chronic diseases which is related to inflammatory responses and already some of SPHK specific inhibitors were developing for chemotherapy resistant cancer therapy (Hengst *et al.*, 2010; Sharma *et al.*, 2010). In this initial study, we developed a lipid drug candidate for cancer treatment showing an enhanced solubility which may be a critical factor for toxicological evaluation and further drug development process.

K6PC-5 was found to activate SPHK in cell-based assay (Fig. 1). In general, SPHK activation is triggered by various growth factors, for example, VEGF, PDGF and EGF or TNF-a signals (Pyne and Pyne, 2008). Therefore, K6PC-5 may also trigger the multiple set of cell growth signaling pathway. To exclude this possibility, we measured SPHK activation by K6PC-5 in cell-free assay system. Interestingly, 10 μM K6PC-5 showed 1.3-fold increase of SPHK activity which is comparable to the SPHK activation by 300 nM TNF-a treatment (Xia *et al.*, 1999). Although a high concentration of K6PC-5 was required to overcome the inhibition by a competitive SPHK inhibitor DMS, K6PC-5 might be a direct SPHK activator. These data suggested that a synthetic pseudo-ceramide mimics the physiological role of S1P, which produced by SPHK activation and worked as either a second messenger or a ligand of S1P receptors.

FTY720 (fingolimid) having 1,3-dihydroxyisopropyl structure was synthesized and has been developed as an immunosuppressant, which was known to inhibit lymphocyte trafficking from lymph node to blood stream (Brinkmann, 2009). FTY720 has also inhibited SPHK and specifically phosphorylated by SPHK2 to bind to S1P receptors (Paugh *et al.*, 2003). Recently, FTY720 was developed as a drug for multiple sclerosis (Walter and Fassbender, 2010). Therefore, synthetic strategy to develop SPHK inhibitor or activator will derive a new therapeutic candidate which controls cell death and cell proliferation. The finding the success of SPHK inhibition in cancer and a range of other disease models were demonstrated that the inhibitors of the SPHK pathway promotes the continued interest in targeting the SPHK for therapeutic benefit (Pitman and Pitson, 2010).

Collectively, K10PC-5 and K6PC-5 regulated SPHK activity in cell-based assay and particularly SPHK was activated by K6PC-5, which suggesting the possibility of K6PC-5 working as a S1P mimicking second messenger or a ligand agonist for S1P receptors as like FTY720.

## ACKNOWLEDGMENTS

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