Sphingolipid Metabolic Changes during Chiral C2-Ceramides Induced Apoptosis in Human Leukemia Cells

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N-acetylsphingosine (C2-ceramide) is a synthetic water-soluble ceramide mimicking the activity of natural ceramides. By fixing chiral conformation on carbon numbers 2 and 3 in the ceramide structure, four chiral C2-ceramides naming *d-erythro-*, *l-erythro-*, *d-threo-* and *l-threo* C2-ceramide were synthesized. We have investigated the chiral effects of these C2-ceramides on the sphingolipid metabolism, particularly on both the sphingolipid biosynthetic pathway and on the degradation pathway. In both HL-60 and U937 cells, the chiral C2-ceramide (10 μM) showed sphingosine accumulation monitored fluoromatrically by a high performance liquid chromatographic separation of the sphingoid bases. Most importantly, in HL-60 cells, *l-erythro* C2-ceramide induced a 50 fold increase in sphingosine as compared to the control, while *l-threo* C2-ceramide exhibited a minimal 7-fold increase. In contrast, sphinganine, another sphingoid base, showed less accumulation by any chiral C2-ceramide tested under the same conditions. These results suggested that chiral C2-ceramide primarily acts on the sphingolipid degradation pathway rather than on the sphingolipid biosynthetic route. The strong G_0/G_1 phase arrest in the cell cycle by treatment of *l-erythro* C2-ceramide indicates that the blockade of the sphingolipid degradation pathway might be concomitantly involved in the dysfunction of the cell cycle. On the other hand, the fact that all chiral C2-ceramides tested failed to inhibit the activity of sphingosine kinase acting on the removal of sphingosine by producing sphingosine-1-phosphate demonstrates that chiral C2- ceramides may increase sphingosine by activating various ceramidases by which natural ceramides are divided into sphingosine and free fatty acids. However, the precise steps involved in this interaction are still unknown.

Key words: C2-ceramide, Sphingosine, Apoptosis, Cell cycle, Sphingosine kinase

INTRODUCTION

Recent studies on sphingolipid metabolism suggest that, analogous to glycerophospholipid metabolism, sphingolipid turnover generates intermediates that have important functions (Huwiler *et al.*, 2000). In particular, ceramide, sphingosine, and sphingosine-1-phosphate(SPP) regulate pathophysiological cellular process by growth factors and cytokines (Merrill *et al.*, 1997). Initial study of ceramides has suggested that sphingomyelin hydrolysis and ceramide generation are implicated in a signal transduction pathway that mediates the effects of tumor necrosis factor-α and other agents on cell growth and differentiation (Obeid *et al.*, 1993).

Due to the limited solubility of long-chain ceramides in aqueous environments, the study of ceramide metabolism in cultured cells or in vitro is difficult. This problem can be circumvented by substitution of the N-acyl group with a shorter acyl chain (C2-ceramide). C2-ceramide (*d-erythro* form), a synthetic cell-permeable ceramide analog, has long been used as a valuable tool for investigating the apoptosis mechanism related to the sphingomyelin turnover cycle and cell cycle arrest (Jayadev *et al.*, 1995). An initial study of the influence of stereochemical configuration on metabolism of four C2-ceramides has been conducted (Okazaki *et al.*, 1990).

In the present study, we investigated four short-chain ceramides that affect sphingolipid metabolism and cellular responses in human leukemia cells depending on the stereochemistry of the 2nd and 3rd carbon positions.

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MATERIALS AND METHODS

Chemicals

D-erythro sphingosine, *d-erythro* sphinganine, DAPI (4,6-diamidino-2-phenylindole) and 2-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *d-erythro* C2-ceramide, *l-erythro* C2-ceramide, *d-threo* C2-ceramide, and *l-threo* C2-ceramide were from Matreya Inc.(Pleasant Gap, PA, U.S.A.). OPA was purchased from Nakalai Tesque (Tokyo, Japan). The internal standard, C_{20}-sphinganine, was a kind gift from Dr. Merrill at Emory University (Atlanta, GA, U.S.A.). Other solvents used for extracting sphingolipids and HPLC eluents were used as a HPLC analytical grade. Other materials were commercially available as reagent grade chemicals.

Cell culture

Human promyelocytic leukemia cell line, HL-60 cells and U937 cells were obtained from the Korea Research Institute of Bioscience and Biotechnology, KIST, Taejon, Korea. The cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin. Cells were subcultured at 37°C in 5% CO_2 atmosphere.

FACS analysis for DNA fragmentation and cell cycle arrest

The harvested cells with density of 1×10^6 cells/ml were washed with phosphate-buffered saline (PBS). Subsequent to centrifugation, the pellets were re-suspended in 1 ml of cold 70% EtOH and were kept for 30 min at 4°C. Following complete removal of the 70% EtOH solution, 1 ml of DNA staining solution (0.1 mM EDTA, pH 7.4, 0.05 mg/ml RNase A and 50 μg/ml propidium iodide) was added. After suspending gently, the sample was kept at room temperature for 1 h and transferred for analysis of apoptotic region and cell cycle arrest in a flow cytometric diagram.

Analysis of viable cells

The cells were maintained at a cell density of 5×10^5 cells/ml. Before the cells were treated with C2-ceramides or with an ethanol vehicle, the cells were re-suspended in serum-free media containing insulin and transferin. The number of viable cells was calculated as the number of exclusion bodies of trypan blue dye by hemocytometer.

OPA reagent preparation and derivatization

The following steps prepared OPA reagent solution. Twenty-five milligrams of OPA and 25 ml of 2-mercaptoethanol were dissolved in 0.5 ml of ethanol. The total volume was adjusted to 50 ml with 3% borate buffer (adjusted pH to 10.5 with KOH) and stored at 4°C under nitrogen in the dark. This reagent solution was prepared weekly or whenever the reactivity with sphingoid base standards decreased.

For the preparation of stock solution of sphingosine, ten milligrams of sphingosine was dissolved in 1 ml of ethanol. The organic phase of the cellular lipid extracts was evaporated in a Speed Vac concentrator (Hanil, Seoul, Korea). The precipitates of the lipid mixture were re-dissolved in 40 μl of ethanol. In order to increase the solubility of the lipid mixture, the ethanol solution was pre-incubated in 1.5 ml PTFE tube at 60°C for 30 min (Yoon *et al.*, 1999). Adding 5 μl of OPA reagent solution to 40 μl of ethanol solution of lipid mixture and incubated 30 min at ambient temperature successfully did the fluorescent OPA derivatization.

The alkaline solution for lipid extraction was prepared daily. Adding 0.1 ml of 2N NH_4OH stock solution to 250 ml of deionized water and adjusting the pH to 9.0 prepared the alkaline solution.

Extraction of cellular sphingoid bases

The total population of 1×10^6 cells was harvested and rapidly precipitated by brief centrifugation at 1,000 rpm for 10 sec. After the supernatant was removed, the pellets were washed twice with 0.5 ml of PBS (pH 7.2) and then precipitated again by the above procedure. In order to extract sphingolipids, 1.5 ml of chloroform-methanol (1:2, v/v) mixture was directly added to the pellets. At the same time, 40 μl (40 pmol) of C_{20}-sphinganine (internal standard) stock solution (1.0 μM) was added and mixed briefly. To transfer the sphingoid bases to the organic layer, 1 ml each of chloroform and alkaline solution (pH 9~10) were added and vortexed vigorously before centrifugal separation. After the lower organic layer was washed twice with 1 ml of alkaline solution, the lower organic phase was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*.

To reduce byproducts in OPA reaction, the alkaline hydrolysis with 1 ml of 0.1 M KOH in chloroform-methanol (1:2, v/v) underwent for 60 min at 37°C to break down cellular acylglycerolipids and phospholipids. After hydrolysis, the same extraction procedure as outlined above was applied to the hydrolysates to extract free sphingoid bases.

Chromatography for sphingoid bases analysis

The analytical HPLC system was equipped with an L-6200 pump (Hitachi, Tokyo, Japan), a Rheodyne 7125 injector, a 655A-52 column oven (Hitachi, Tokyo, Japan) and a FP-720 fluorescent detector (Jasco, Tokyo, Japan). The OPA derivatives of the sphingoid bases were separated by using 82% acetonitrile at a flow rate of 1.0 ml/

min on a reversed-phase column (Cosmosil 5C18-AR, 4.6 mm i.d. × 150 mm) at 40°C. The OPA derivatives of the sphingoid bases were monitored fluoromatrically at 340 nm for excitation wavelength and 455 nm for emission wavelength.

Preparation of sphingosine kinase

The LLC-PK_1 cells were washed twice with cold PBS and scraped in a 0.1 M phosphate buffer (pH7.4) containing 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, phophatase inhibitors (20 mM $ZnCl_2$, 1 mM sodium orthovanadate and 14 mM NaF), protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF) and the pyridoxal phosphate analog, 4-deoxy-pyridoxine (0.5 mM) to inhibit the pyridoxal-dependent sphingosine-1-phosphate lyase (buffer A). Cells were then disrupted by freeze thawing and centrifuged at 105,000 × *g* for 90 min and the supernatants were stored at -70°C. The protein concentration of the supernatants was adjusted to 0.8~1.0 mg/ml. Sphingosine kinase activity was stable for several months.

Sphingosine kinase activity measurement

The cytosolic fraction containing sphingosine kinase activity, prepared as described above, was added to the solubilized lipids. The samples were gently mixed and incubated at room temperature for 5 min. Reactions were initiated by addition of 5 μl of [γ-^{32}P] ATP (2 mCi/ml) containing 10 mM $MgCl_2$, and incubated for 20 min 30°C. The reaction was stopped by the addition of 20 μl of 1 N HCl followed by 0.8 ml of chloroform : methanol : concentrated HCl (100:200:1, v/v). After vigorous vortexing, 240 μl of chloroform and 240 μl of 1 M KCl were added for phase separation. The labeled lipids in the organic phase were separated by TLC on silica gel G60 using chloroform:methanol:acetic acid:water (90:90:15:6, v/v) and visualized by autoradiography. Alternatively, unlabeled sphingosine-1-phosphate can be added to each sample to allow visualization of this compound on the TLC plates

Statistical analysis

Data is from experiments repeated two or four times, and is expressed as the mean ± SD. The Student's *t*-test was used for comparison of the paired and unpaired measurements. The *p* values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Apoptosis induction by *d-erythro* C2-ceramide

The enhanced solubility and uptake of short-chain ceramides by cultured cells make them useful substrates in which to identify the intracellular fate of ceramide and the specificity of enzymes involved in ceramide metabolism. *D-erythro* C2-ceramide (N-acetylated *d-erythro* sphingosine) effectively induced internucleosomal DNA fragmentation, which was inhibited by zinc ion in the range of 0.6 to 5 μM (Obeid *et al.*, 1993). Similar findings are shown here; however, C2-ceramide concentration above 5 μM was minimally required to find the apoptotic region in the diagram of flow cytometric analysis (FACS) for 24 h. This effective dose difference may be originated from culture media conditions; i.e., Obeid *et al.* incubated HL-60 cells in serum-free media because the tested period was a very short 3 h. However, in our study, instead of a serum supplement, a media condition with basic growth factors including insulin and transferin was added for the prevention of sphingolipid "burst" due to shock when the media is changed (Smith *et al.*, 1995). These growth factors may activate the degradation process of natural ceramides (Perry *et al.*, 1998) and therefore suppress the apoptosis process triggered by C2-ceramide. A dose above 10 μM of *d-erythro* C2-ceramide began to increase the apoptotic cell population from 6 h after treatment and raised the ratio in a time dependent manner until 24 h (Fig. 1). Apoptosis induction by other chiral C2-ceramide showed similar patterns above a 10 μM dose (data not shown). Under our conditions, at least a 10 μM concentration of chiral C2-ceramides was required to achieve DNA fragmentation in 24 h.

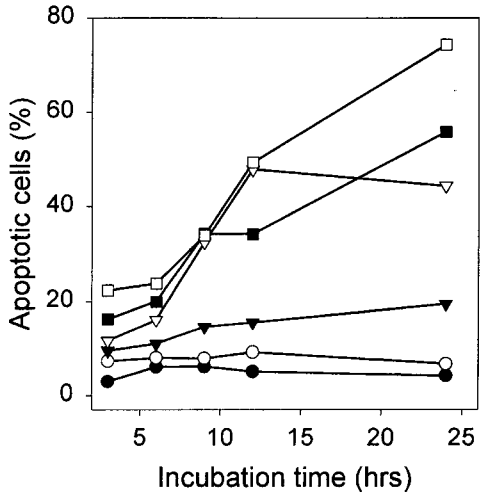

Fig. 1. Flow cytometric analysis of apoptosis induction by *d-erythro* C2-ceramide in serum-free medium containing insulin and transferrin. Concentration and time dependent increases of apoptotic cell populations by *d-erythro* C2-ceramide treatment were measured and expressed as the percentage of hypodiploid cells during 24hrs incubation in HL-60 cells. The treated *d-erythro* C2-ceramide concentration is expressed as symbols: ●, control; ○, 1 μM; ▼, 5 μM; ▽, 10 μM; ■, 15 μM; □ 20 μM, respectively. Data is expressed as the mean value obtained in duplicated experiments.

Sphingoid bases accumulation

The HL-60 cell suspension was incubated with chiral C2-ceramides (each 10 μM) for 24 h (Fig. 2). All four chiral C2-ceramide treatments increased sphingosine concentration without any change in another sphingoid base metabolite, sphinganine. In general, the free sphingoid base concentration in intact cells was below 5 pmol/ 1×10^6 cells. Differences in the specificity of sphingosine accumulation were observed for chiral C2-ceramide isomers. *L-erythro* C2-ceramide treatment significantly raised intracellular sphingosine concentrations 50-fold greater than the control. However, *l-threo* C2-ceramide treatment increased sphingosine by only 7-fold (Fig. 3a). A similar pattern was also observed in U937 cells (Fig. 3b). Results from these experiments suggest that chiral C2-ceramides isomers were quite distinctive from each other according to sphingosine accumulation, which may explain the relationship between stereospecific configuration and different activities.

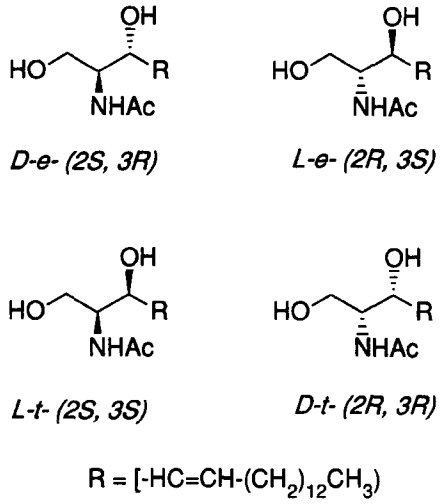

Fig. 2. Structures of chiral C2-ceramides. Shown are the structures for the four stereoisomers of C2-ceramide where R is part of the sphingoid backbone. Ceramide stereoisomers, sphingosine constitutes the sphingoid backbone that contains a C4-C5 double bond. The R/S nomenclature is indicated which is the preferred method of describing the stereochemical configuration of carbons 2 and 3 due to the fact that the *erythro/threo* designator can sometimes result in ambiguous assignment.

It was previously reported that four chiral C2-[3-^{3}H] ceramides were incorporated over approximately 95% into cells and were stable without degradation for 24 h (Bielawska *et al.*, 1993). In a recent advanced investigation, depending on the stereospecific configuration of the 2nd and 3rd carbon positions and acyl chain length in the ceramide structure, short-chain ceramides undergo metabolism to short-chain sphingolipids and long-chain bases, which are then utilized for endogenous ceramide and sphingolipid synthesis; treated *d-erythro* C2-[3-^{3}H] ceramide converted into sphingomyelin (1%), glucosylceramide (1%), and sphingoid bases (0.1%) however *l-threo* C2-[3-^{3}H]ceramide less converted into sphingomyelin (0.1%), glucosylceramide (0.1%), and sphingoid bases (0.05%) (Ridgway *et al.*, 1995). Although sphingoid bases were derived from the metabolism of treated 10 μM (10,000 pmol/ml media) of C2-ceramide, their cellular accumulation was expected to reach approximately 10 pmol as theoretically calculated from a reference (Ridgway *et al.*, 1995). Based on the higher increase of sphingosine to over 200 pmol in cells, our data indicated that chiral C2-ceramide endogenously accumulated sphingosine by interrupting the sphingosine metabolic pathway but not

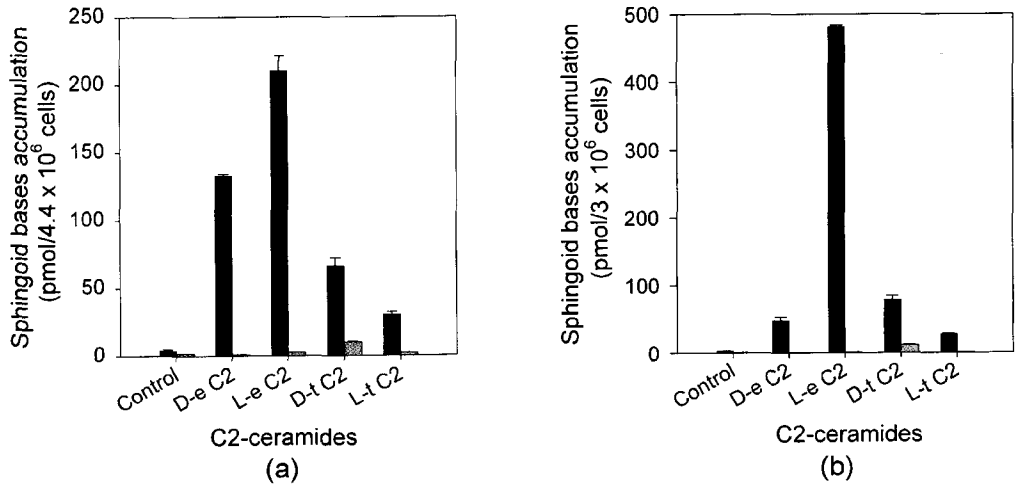

Fig. 3. Sphingoid bases accumulation profile by chiral C2-ceramides in HL-60 (a) and U937 cells (b). Cells were incubated with the indicated chiral C2-ceramide for 24 h and were extracted as described in "materials and methods", and the OPA-derivatized sphingoid bases were analyzed by HPLC. Results represent the mean ± S.D. of three experiments.; black bar, sphingosine; gray bar, sphinganine.

by producing its metabolite, sphingosine.

Sphingosine kinase activity

Sphingosine kinase is an enzyme to catalyze the phosphorylation of sphingosine in order to produce sphingosine-1-phosphate, which in turn blocks the apoptosis process induced by ceramides or other anti-carcinogenic drugs. Primarily, we supposed that sphingosine accumulation was attributed to the inhibition of sphingosine kinase activity by chiral C2-ceramide treatment. Four chiral C2-ceramides were less potent for blocking sphingosine phosphorylation with ^{32}P-γ-ATP than N,N-dimethylsphingosine (DMS) which is a strong inhibitor of this enzyme (Yang *et al.*, 1999). Furthermore, no significant difference in activity was found in regard to the stereo-specific configuration of the 2nd and 3rd carbon positions of chiral C2-ceramide (Fig. 4).

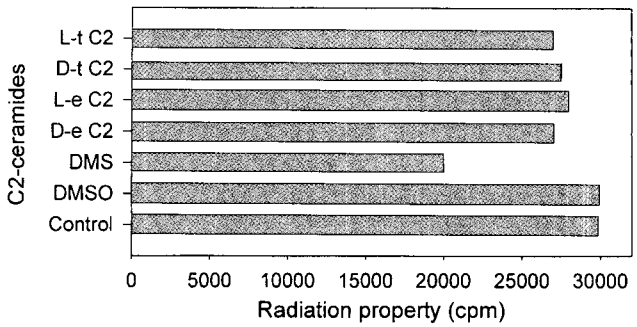

Fig. 4. Effects of chiral C2-ceramides on sphingosine kinase activity in HL-60 cells. Sphingosine kinase activity was expressed as the degree of radiation of a newly synthesized sphingosine-1-[^{32}P]phosphate spot on a TLC plate in sphingosine kinase preparation. Dimethylsphingosine (DMS), a well-known sphingosine kinase inhibitor, was used as a positive control. Data is expressed as the mean value of two independent experiments.

Cell cycle arrest

Previous study has revealed that *d-erythro* sphingosine induces rapid and potent Rb dephosphorylation (Chao *et al.*, 1992). The exogenous addition of C6-ceramide to cells resulted in the dephosphorylation of retinoblastoma (Rb), correlating with the induction of G_0/G_1 cell cycle arrest. The effects of C6-ceramide on the dephosphorylation state of Rb were independent of the conversion to sphingosine (Dbaibo *et al.*, 1995).

In our data, interestingly, the sphingosine concentration was concomitantly increased by chiral C2-ceramides when G_0/G_1 cell cycle arrest by chiral C2-ceramides was observed (Fig. 5).

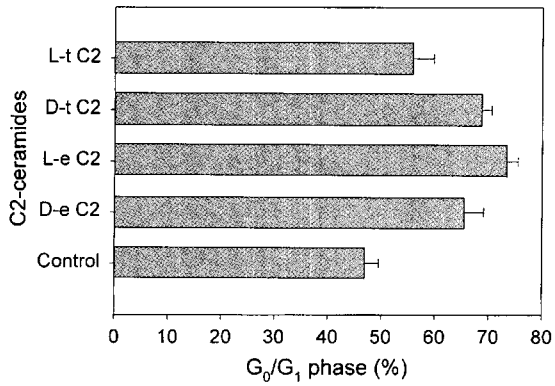

Fig. 5. Cell cycle arrest in G_0/G_1 phase by chiral C2-ceramides in HL-60 cells. Cells were incubated with chiral C2-ceramides for 24 h and were stained with DNA staining solution before FACS analysis. Results represent the mean ± S.D. of three experiments.

In conclusion, the stereo-chemical configuration of C2-ceramides may constitute an important component of the apoptosis induction mechanisms operating in the regulation of cell cycle arrest. More interestingly, the differences of endogenous sphingosine accumulation among the four chiral C2-ceramides suggested that sphingosine might not accumulate by inhibiting sphingosine kinase or by metabolizing chiral C2-ceramide. Recently, sphingosine has been proposed to be involved in mitochondria-dependent Fas-induced apoptosis and the acid ceramidase by which natural ceramides are disintegrated to produce sphingosine and free fatty acids has been proposed as a crucial modulator in regulating cellular levels of ceramides and sphingosine (Cuvillier *et al.*, 2000). In regard to these observations, our finding suggests that the factor increasing sphingosine in cells may be regulated stereo-specifically by the configuration of the 2nd and 3rd carbon positions of chiral C2-cera-mide during G_0/G_1 cell cycle arrest and the apoptosis process.

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REFERENCES

Bielawska, A., Crane, H. M., Liotta, D., Obeid, L. M., and Hannun, Y. A., Selectivity of ceramide-mediated biology. Lack of activity of erythro-dihydroceramide. *J. Biol. Chem.*, 268, 26226 (1993).

Chao, R., Khan, W., and Hannun, Y. A., Retinoblastoma protein dephosphorylation induced by D-erythro sphingosine. *J. Biol. Chem.*, 267, 23459 (1992).

Cuvillier, O., Edsall, L., and Spiegel, S., Involvement of sphingosine in mitochondria-dependent Fas-induced apoptosis of type II Jurkat T cells. *J. Biol. Chem.*, 275,

15691 (2000).
Dbaibo, G. S., Pushkareva, M. Y., Jayadev, S., Schwarz, J.K., Horowitz, J. M., Obeid, L. M., and Hannun, Y. A., Retinoblastoma gene product as a downstream target for a ceramide-dependent pathway of growth arrest. *Proc. Natl. Acad. Sci. USA,* 92, 1347 (1995).
Huwiler, A., Kolter, T., Pfeilschifter, J. and Sandhoff, K., Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochim. Biophys. Acta,* 1485, 63 (2000).
Jayadev, S., Liu, B., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M. Y., Obeid, L. M. and Hannun, Y. A., Role for ceramide in cell cycle arrest. *J. Biol. Chem.,* 270, 2047 (1995).
Merrill, A. H., Schmelz, E. M., Dillehay, D. L., Spiegel, S., Shayman, J. A., Schroeder, J. J., Riley, R. T., Voss, K. A. and Wang, E., Sphingolipids-the enigmatic lipid class: biochemistry, physiology, and pathophysiology. *Toxicol. Appl. Pharmacol.,* 142, 208 (1997).
Obeid, L. M., Linardic, C. M., Karolak, L. A. and Hannun Y.A., Programmed cell death induced by ceramide. *Science,* 259, 1769 (1993).
Okazaki, T., Bielawski, A., Bell, R. M., and Hannun, Y. A., Role of ceramide as a lipid mediator of 1α, 25-dihydroxyvitamin D3-induced HL-60 cell differentiation. *J. Biol. Chem.,* 265, 15823 (1990).
Perry, D. K., and Hannun, Y. H., The role of ceramide in cell signaling. *Biochim. Biophys. Acta,* 1436, 233 (1998).
Ridgway, N. D. and Merriam, D. L., Metabolism of short-chain ceramide and dihydroceramide analogues in Chinese hamster ovary (CHO) cells. *Biochim. Biophys. Acta,* 1256, 57 (1995).
Smith, E. R. and Merrill, A. H., Differential roles of *de novo* sphingolipid biosynthesis and turnover in the "burst" of free sphingosine and sphinganine, and their 1-phosphates and *N*-acyl-derivatives, that occurs upon changing the medium of cells in culture. *J. Biol. Chem.,* 270, 18749 (1995).
Yang, L., Yatomi, Y., Satoh, K., Igarashi, Y., and Ozaki, Y., Sphingosine-1-phosphate formation and intracellular Ca^{++} mobilization in human platelets: Evaluation with sphingosine kinase inhibitors. *J. Biochem. (Japan),* 126, 84 (1999)
Yoon, H. T., Yoo, H. S., Shin, B. K., Lee, W. J., Kim, H. M., Hong, S. P., Moon, D. C., and Lee, Y. M., Improved fluorescent determination method of cellular sphingoid bases in high-performance liquid chromatography. *Arch. Pharm. Res.,* 22, 294-299 (1999).