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Synthetic Phytoceramides Induce Apoptosis with Higher Potency than Ceramides

ONYOU HWANG, GUNCHEOL KIM, YEON JOO JANG, SEONG WHO KIM, GUIYONG CHOI, HYUN JIN CHOI, SEON YOUNG JEON, DON GYU LEE, and JAE DAM LEE

Department of Biochemistry, University of Ulsan College of Medicine, Seoul, Korea (O.H., Y.J.J., S.W.K., H.J.C., J.D.L.); Department of Chemistry, College of Natural Sciences, Chungnam National University, Taejon, Korea (G.K., S.Y.J., D.G.L.); and The SphingoBiolipid Corp, Yongin, Korea (G.C.)

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ABSTRACT

Ceramides are naturally occurring compounds recognized to mediate apoptosis. *N*-acylsphingosines, containing a double bond at carbons 4 and 5 of their sphingoid backbone, are thought to be the active form, because *N*-acylsphinganines with completely saturated sphingoid are inactive. In the present study, we synthesized a series of *N*-acyl-4D-ribo-phytosphingosines (phytoceramides) that contain a hydroxyl group at carbon 4 and investigated structure-cytotoxicity relationship of the presumed functional groups in ceramides. *N*-Acetylphytoceramide (PCer2) and *N*-hexanoylphytoceramide (PCer6) were found to be more cytotoxic than ceramides as determined by released lactate dehydrogenase activity and morphological criteria. This was not caused by intracellular conversion of phytoceramides to ceramides, because no *N*-hexanoylsphingosine was formed after incubation of cell lysate with PCer6. Among phytoceramides having acyl chains two to eight carbons long, the cytotoxicity was highest with five or six carbons. The carbonyl group of the amide bond did not seem to be critical, because substitution of the oxygen with sulfur did not influence the cytotoxicity. The phytoceramide-induced cell death was observed to be apoptotic in nature with the use of terminal deoxynucleotidyl transferase dUTP nick-end labeling and propidium iodide staining. Because phytoceramides can be readily synthesized from yeast sources, they may present a potential and economical alternative to ceramide in future studies and therapies.

Ceramides, products of sphingolipid metabolism, have been recently recognized as second messengers mediating apoptosis in the mammalian system. Increased ceramide productions have been demonstrated in response to a variety of extracellular death signals, such as tumor necrosis factor-α (Dressler et al., 1992; Wiegmann et al., 1994), interleukin-Iβ (Mathias et al., 1993), γ-interferon (Kim et al., 1991; Agresti et al., 1996), and Fas-induced toxicity (Tepper et al., 1995), as well as by physical stresses such as radiation, heat shock, and chemotherapeutic drugs (Santana et al., 1996; Hartfield et al., 1997).

Naturally occurring ceramides consist of a long-chain sphingoid base with an amide-linked fatty acid substituent (typically with acyl chain lengths of 16–24 carbon atoms). Among these, *N*-acylsphingosines (ceramides), possessing a double bond between carbons 4 and 5 of the sphingoid, are thought to be the ceramide form mediating the apoptotic response. In comparison, *N*-acylsphinganines (dihydroceramides), the ceramides with completely saturated sphingoid chains, are reported to exhibit no apoptosis-inducing activity (Bielawska et al., 1993; Obeid et al., 1993; Jayadev et al., 1995; Jarvis et al., 1996; Kaipia et al., 1996; Hartfield et al., 1997). Based on these findings, the double bond between carbons 4 and 5 has been believed to be critical for the ceramides' apoptotic activity and the dihydroceramides are considered inert compounds that serve as intermediates of the ceramide metabolism.

N-acyl-4D-ribo-phytosphingosines (phytoceramides), another form of ceramides, differ from the dihydroceramides by having a hydroxyl group instead of the hydrogen at carbon 4 of the sphingoid. The phytoceramides are recently recognized to mediate regulation of cell growth and stress responses in yeast (Saba et al., 1997; Mao et al., 1999; Skrzypek et al., 1999) and are thought to be the yeast counterpart of the mammalian ceramide. Because the phytoceramides do not possess the double bond between carbons 4 and 5, whether they also exert cytotoxicity would definitively resolve whether the double bond is indeed critical for the biological activity.

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ABBREVIATIONS: HPLC, high-performance liquid chromatography; Cer2, *N*-acetylsphingosine; DHCer2, *N*-acetylsphinganine; Cer6, *N*-hexanoylsphingosine; DHCer6, *N*-hexanoylsphinganine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; DMSO, dimethyl sulfoxide; MS, mass spectrometry; ESI, electrospray ionization; PCer2, *N*-acetyl-4D-ribo-phytosphingosine; PCer6, *N*-hexanoyl-4D-ribo-phytosphingosine; Pcer6S, *N*-thio-hexanoylphytosphingosine; LDH, lactate dehydrogenase; ANOVA, analysis of variance.

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In addition to the double bond, other functional groups of ceramides presumed to be important for activity include the hydroxyl groups at carbons 1 and 3 of the sphingoid backbone (Lister et al., 1995; reviewed by Dyatlovitskaya, 1998) and the carbonyl group (—C═O) of the amide bond (reviewed by Dyatlovitskaya, 1998). Furthermore, because the hydrocarbon chains may facilitate the hydrophobic interaction between ceramides and their cell targets, the acyl chain length may play a role as well.

Because of the insolubility of the natural ceramides in water, most studies on their biological activity have been performed with synthetic ceramides with short acyl chains of two to eight carbon atoms. These analogs have been reported to induce apoptosis in various culture systems, including the catecholaminergic mesencephalon neurons (Brugg et al., 1996) and PC 12 cells (Hartfield et al., 1997).

Because the chemical structure of ceramides would be important for their biological efficiency, modification of different parts of the molecules should have different biological effects. In the present study, we synthesized a series of phytoceramides and sought to determine whether they, too, exhibit cytotoxic activity and, if so, to understand the structure-function relationship of the presumed functional groups using various synthetic phytoceramides in catecholaminergic neuroblastoma cell lines.

Experimental Procedures

Materials. 4D-Ribo-phytosphingosine [(2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecanetriol], isolated in the form of tetra-acetylphytosphingosine from the yeast *Hansenula ciferrii* and subsequently produced by hydrolysis, was obtained from SphingoBiolipid Corp. (Yongin, Korea). Purity of the phytosphingosine was >90% by HPLC. *N*-acetylsphingosine (Cer2), *N*-acetylsphinganine (DHCer2), *N*-hexanoylsphingosine (Cer6), and *N*-hexanoylsphinganine (DHCer6) were purchased from Biomol (Plymouth Meeting, PA). SK-N-BE(2)C and N1E-115 cells were obtained from American Type Culture Collection (Manassas, VA). All culture media, fetal bovine serum, L-glutamine, trypsin/EDTA, and penicillin-streptomycin were from Life Technologies (Gaithersburg, MD). TUNEL staining kit (In Situ Cell Death Detection Kit POD) was obtained from Roche Molecular Biochemicals (Indianapolis, IN) and propidium iodide from Sigma Chemical (St. Louis, MO). All other chemicals were reagent grades and were from Sigma or Merck (Rahway, NJ).

Syntheses of Phytoceramides. Phytoceramides were synthesized by the conventional method of peptide preparation, coupling 4D-ribo-phytosphingosine with 1.2 Eq of corresponding acid halides or acid anhydrides in the presence of a base catalyst. Before aqueous work-up of the reaction, hydrolysis of the crude product was performed using 10% NaOH solution in methanol to remove minor acyl groups formed on the hydroxyl group, although the major product was found to be the ceramide with free alcohols. Each of the compounds was then purified by silica-gel column chromatography and proved pure on the basis of thin-layer chromatography on silica gel H plates developed with CH_2Cl_2 and methanol (9:1, v/v) and visualized with phosphomolybdic acid. The compounds were identified by IR and H^1- and C^{13}-NMR spectra, including ^1H-^{13}C-correlation spectroscopy, and mass spectra, and also confirmed to be >95% pure by HPLC. The chemical homogeneity of samples was compared with what is reported in the literature for synthetic standards (Shirota et al., 1999).

For example, *N*-hexanoyl-4-D-ribo-phytosphingosine (PCer6) was prepared as follows: the phytosphingosine (2.0 g, 6.3 mmol) was dissolved in 20 ml of anhydrous chloroform containing triethylamine (2.6 ml, 18.9 mmol). Hexanoic anhydride (1.8 ml, 7.6 mmol) was added to the solution and the mixture was stirred under N_2 overnight at room temperature. To the resulting solution was added 10 ml of 10% NaOH solution in methanol. The mixture was stirred vigorously for 2 h and the organic layer was diluted with methylene chloride and washed with saturated aqueous ammonium chloride solution and brine. After filtration and concentration, the crude product was purified by silica gel column chromatography eluting ethyl acetate first and methanol/methylene chloride (5:95, v/v) to afford 876 mg of the PCer6 product (31% yield).

All other phytoceramides varying in acyl chain length were prepared by the same procedure except that the corresponding acid halides or anhydrides were used instead of hexanoic anhydride. PCer6S was readily obtained by treatment of PCer6 with Lawesson's reagent in toluene.

Spectral Analyses. *N*-acetyl-4D-ribo-phytosphingosine [PCer2; R_f = 0.25 in methanol/methylene chloride (10:90, v/v)]: ^{1}H-NMR (400 MHz, $CDCl_3$ + DMSO-*d6*) d6.79 (d, *J* = 8.2Hz, 1H), 4.60 (broad s, 1H), 4.28 (broad s, 1H), 4.10 (broad s, 1H), 3.95 (broad s, 1H), 3.77 (dd, *J* = 11.3 and 3.1 Hz, 1H), 3.63 (dd, *J* = 11.3 and 5.2 Hz, 1H), 3.50–3.51 (m, 2H), 1.90 (S, 3H), 1.65 (m, 1H), 1.50–1.10 (m, 25H), 0.80 (t, *J* = 6.6 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$ + DMSO-*d6*) d170.9, 76.3, 72.7, 61.7, 52.7, 33.5, 32.2, 30.1 × 2, 30.0 × 3, 29.9 × 3, 29.6, 26.2, 23.5, 22.9, 14.4; MS (ESI) *m/z* 360 $[M+H]^+$, 382 $[M+Na]^+$. *N*-propionyl-4D-ribo-phytosphingosine, R_f = 0.30 in methanol/methylene chloride (10:90, v/v): ^{1}H-NMR (400 MHz, $CDCl_3$ + DMSO-*d6*) d6.64 (d, *J* = 8.2Hz, 1H), 4.52 (broad s, 1H), 4.28 (broad s, 1H), 4.10 (broad s, 1H), 3.95 (broad s, 1H), 3.72 (dd, *J* = 11.5 and 3.4 Hz, 1H), 3.56 (dd, *J* = 11.5 and 4.3 Hz, 1H), 3.41–3.47 (m, 2H), 2.10 (q, *J* = 7.6 Hz, 3H), 1.53 (m, 1H), 1.50–1.10 (m, 25H), 0.98 (t, *J* = 7.5 Hz, 3H), 0.80 (t, *J* = 6.6 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$ + DMSO-*d6*) d174.7, 76.3, 72.8, 61.8, 52.7, 33.5, 32.2, 30.1 × 2, 30.0 × 2, 29.9 × 4, 29.2 × 2, 26.2, 23.0, 22.9, 14.5, 10.2; MS (ESI) *m/z* 374 $[M+H]^+$, 396 $[M+Na]^+$; *N*-butyryl-4D-ribo-phytospingosine, R_f = 0.40 in methanol/methylene chloride (10:90, v/v): ^{1}H-NMR (400 MHz, $CDCl_3$ + DMSO-*d6*) d6.66 (d, *J* = 8.2Hz, 1H), 4.54 (broad s, 1H), 4.29 (broad s, 1H), 4.10 (broad s, 1H), 3.95 (broad s, 1H), 3.78 (dd, *J* = 11.4 and 2.8 Hz, 1H), 3.63 (dd, *J* = 11.4 and 5.3 Hz, 1H), 3.52–3.54 (m, 2H), 2.15 (t, *J* = 7.6 Hz, 2H), 1.60 (m, 1H), 1.53 (m, 2H), 1.50–1.10 (m, 25H), 0.86 (t, *J* = 7.5 Hz, 3H), 0.82 (t, *J* = 7.0 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$ + DMSO-*d6*) d174.4, 76.5, 72.8, 61.9, 52.8, 36.7, 33.5, 32.3, 30.1 × 5, 30.0 × 2, 29.7, 28.2, 26.3, 23.0, 22.7, 14.9, 14.2; MS (ESI) *m/z* 388 $[M+H]^+$, 410 $[M+Na]^+$; *N*-valeryl-4D-ribo-phytosphingosine, R_f = 0.35 in in methanol/methylene chloride (10:90, v/v): ^{1}H-NMR (400 MHz, $CDCl_3$ + DMSO-*d6*) d6.68 (d, *J* = 8.2Hz, 1H), 4.54–3.95 (broad s, 2H), 4.10 (broad s, 1H), 3.85 (dd, *J* = 11.5 and 3.0 Hz, 1H), 3.63 (dd, *J* = 11.5 and 5.5 Hz, 1H), 3.60–3.64 (m, 2H), 2.22 (t, *J* = 7.8 Hz, 2H), 1.63 (m, 1H), 1.59 (m, 2H), 1.50–1.10 (m, 27H), 0.92 (t, *J* = 7.3 Hz, 3H), 0.88 (t, *J* = 7.0 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$ + DMSO-*d6*) d174.9, 76.4, 73.1, 61.8, 52.9, 36.8, 33.6, 32.3, 30.1 × 8, 29.8, 28.2, 26.3, 23.1, 22.8, 14.5, 14.2; MS (ESI) *m/z* 402 $[M+H]^+$, 424 $[M+Na]^+$; *N*-hexanoyl-4D-ribo-phytosphingosine (PCer6, R_f = 0.40 in methanol/methylene chloride (10:90 v/v)): ^{1}H-NMR (400 MHz, $CDCl_3$ + DMSO-*d6*) d6.62 (d, *J* = 8.1Hz, 1H), 4.54–3.95 (broad s, 3H), 4.10 (broad s, 1H), 3.78 (dd, *J* = 11.3 and 2.9 Hz, 1H), 3.63 (dd, *J* = 11.3 and 5.4 Hz, 1H), 3.50–3.54 (m, 2H), 2.14 (t, *J* = 7.4 Hz, 2H), 1.60 (m, 1H), 1.56 (m, 2H), 1.50–1.10 (m, 29H), 0.83 (t, *J* = 6.7 Hz, 3H), 0.82 (t, *J* = 6.9 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$ + DMSO-*d6*) d174.3, 76.6, 72.9, 61.9, 52.9, 37.0, 33.5, 32.3, 31.8, 30.1 × 6, 30.0 × 2, 29.7, 26.3, 25.8, 23.0, 22.8, 14.5, 14.3; MS (ESI) *m/z* 416 $[M+H]^+$, 438 $[M+Na]^+$; *N*-heptanoyl-4D-ribo-phytosphingosine, R_f = 0.42 in methanol/methylene chloride (10:90, v/v): ^{1}H-NMR (400 MHz, $CDCl_3$ + DMSO-*d6*) d6.70 (d, *J* = 7.9 Hz, 1H), 4.64 (broad s, 2H), 4.13 (broad s, 1H), 3.60 (dd, *J* = 11.5 and 3.0 Hz, 1H), 3.71 (dd, *J* = 11.5 and 5.6 Hz, 1H), 3.60–3.66 (m, 2H), 2.22 (t, *J* = 7.6 Hz, 2H), 1.65 (m, 1H), 1.61 (m, 2H), 1.50–1.10 (m, 29H), 0.88 (t, *J* = 5.2 Hz, 3H), 0.87 (m, 2H), 0.86 (t, *J* = 6.10 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$ + DMSO-*d6*) d175.0, 76.4, 73.2, 61.8, 52.9, 37.0, 33.6, 32.3, 31.9 × 2, 30.1 × 5, 29.8 × 2, 29.3, 29.2, 26.3, 26.1, 23.1,

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22.9, 14.5, 14.4; MS (ESI) *m/z* 430 $[M+H]^+$, 452 $[M+Na]^+$; *N*-octanoyl-4D-ribo-phytosphingosine, R_f = 0.50 in methanol/methylene chloride (10:90, v/v): ^{1}H-NMR (400 MHz, $CDCl_3$ + DMSO-*d6*) d6.62 (d, *J* = 8.1 Hz, 1H), 4.08–4.20 (broad s, 3H), 4.10 (broad s, 1H), 3.78 (dd, *J* = 11.4 and 3.1 Hz, 1H), 3.61 (dd, *J* = 11.4 and 5.4 Hz, 1H), 3.49–3.55 (m, 2H), 2.14 (t, *J* = 7.9 Hz, 2H), 1.63 (m, 1H), 1.59 (m, 2H), 1.50–1.10 (m, 31H), 0.81 (t, *J* = 6.4 Hz, 3H), 0.80 (m, 2H), 0.79 (t, *J* = 6.4 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$ + DMSO-*d6*) d174.3, 76.6, 72.9, 61.9, 52.9, 37.0, 33.5, 32.3, 32.0, 30.1 × 6, 30.0 × 3, 29.7, 29.6, 26.3, 26.1, 23.0, 22.9, 14.5, 14.4; MS (ESI) *m/z* 444 $[M+H]^+$, 466 $[M+Na]^+$.

Cell Cultures. SK-N-BE(2)C and N1E-115 cells were grown in RPMI 1640 and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% fetal bovine serum. The cells were grown as monolayers in the presence of 100 IU/l penicillin and 10 μg/ml streptomycin; when confluent, they were subcultured 1:3 to 1:5 after dispersing adherent cells using trypsin/EDTA. Cultures were maintained at 37°C in 95% air/5% CO_2 and humidified atmosphere. For experiments, cells were plated on polystyrene tissue culture dishes at a density of 2×10^5 cells/well in 24-well culture plates. After 24 h, cells were fed with fresh media, at which time the ceramides were added.

Determination of Lactate Dehydrogenase Activity. Extent of cell death was assessed by lactate dehydrogenase (LDH) released into the culture medium. LDH activity was measured by monitoring the production of NAD^+ from NADH during the conversion of pyruvate to lactate. Aliquots (50 μl) of cell culture medium were incubated at room temperature in the presence of 0.26 mM NADH, 2.87 mM sodium pyruvate, and 100 mM potassium phosphate buffer, pH 7.4, in total volume of 200 μl. The rate of NAD^+ formation was monitored for 5 min at 2-s intervals at 340 nm using a microplate spectrophotometer (SPECTRA MAX 340 pc; Molecular Devices, Menlo Park, CA).

Determination of Lipids. SK-N-BE(2)C cells grown on 60-mm tissue culture dishes were harvested and lysed in 800 μl of 0.1% Triton X-100 in 50 mM phosphate buffer, pH 7.0. The cell lysate (315 μg each) was incubated in the presence of various concentrations of PCer6 in total volume of 400 μl for 2 h at 37°C. The reaction was stopped by addition of 400 μl of chloroform/methanol (1:2, v/v). After two rounds of extraction with chloroform/methanol, the organic phase was vacuum-dried, dissolved in 20 μl of chloroform, and subjected to lipid analysis by HPLC-evaporated light scattering detector system. The Alliance HPLC System with Waters 2690 Separation Module and Autosampler (Waters, Milford, MA), Alltima silica column (250-mm × 4.6-mm i.d., 5-μm particle size; Alltech Associates, Deerfield, IL), and evaporated light scattering detector Model ELSA 2000 (Alltech Associates) were used. Program control, data acquisition, and analysis were carried out using Millennium 32 software. Nebulization temperature for the detector was 85.0°C and the flow rate for the nebulizer gas (nitrogen) was 2.5 ml/min. The mobile phase was a mixture of hexane/acetone/chloroform (25:35:65, v/v) and the flow rate was 1.3 ml/min. Cer6 and PCer6 (10 nmol each) were injected as standards.

Morphological Studies. After the cells were exposed to various ceramides for 24 h, their morphological changes were examined and photomicrographs were taken using a Leica DM IRB inverted microscope (Leica, Wetzlar, Germany).

TUNEL Staining. Cells were treated with or without PCer6 for 6 h, washed with Dulbecco's phosphate-buffered saline, and fixed in 4% paraformaldehyde for 15 min. TUNEL assay was performed according to the instructions provided by the manufacturer. Fluorescence photographs were taken using Leica Leitz DM RBE fluorescence microscope.

Propidium Iodide Staining. Cells were treated with PCer6 for 4 h, washed with Dulbecco's phosphate-buffered saline, and fixed in 100% ethanol for 10 min at 4°C. The cells were then incubated in the presence of propidium iodide (50 μg/ml) for 30 min at room temperature with mild shaking. After three washes, fluorescence and phase contrast photographs were taken using an Olympus IX70 fluorescence microscope (Olympus, Tokyo, Japan).

Data Analyses. All data are reported as mean ± S.D. Comparisons were made using ANOVA and Newman-Keuls multiple comparison tests.

Results

Comparison of Cytotoxicity of Phytoceramides with Ceramides and Dihydroceramides. To test whether phytoceramides exhibit cytotoxic activity, we compared the three ceramide derivatives Cer6, DHCer6, and PCer6. For this, PCer6 was first produced by organic synthesis using phytosphingosine derived from yeast as the starting material, and its identity and purity were confirmed by IR HPLC (not shown) and ^{1}H-NMR analyses as described under *Experimental Procedures*. The human neuroblastoma SK-N-BE(2)C cells were exposed to various concentrations of each compound for 24 h and extents of cell death were assessed by measuring the activity of LDH released into the medium.

As shown in Fig. 1, Cer6 was dose dependently toxic, whereas DHCer6 showed no cytotoxicity at all concentrations tested, confirming the previous report that the removal of the double bond from ceramide by addition of hydrogen atoms at carbons 4 and 5 resulted in a loss of the activity. On the other hand, PCer6 exhibited enhanced cytotoxicity compared with Cer6. Whereas Cer6 began to be effectively toxic at 10 μM with an increase in LDH to 128 ± 10% of untreated control, PCer6 toxicity was observed at concentrations as low as 5 μM (136 ± 17%) and was significantly higher than Cer6 at 10 μM (157 ± 15%). Overall, PCer6 was 20–30% more effective compared with Cer6 at all concentrations tested. Thus, the phytoceramide lacking the double bond was found to be more cytotoxic than the ceramide. To further confirm this finding, PCer2 was synthesized and similar experiments were performed. As shown in Fig. 2, again, PCer2 was more cytotoxic than Cer2 and DHCer2 was inactive. At 70 μM, PCer2 caused an increase in LDH to 186 ± 11%, whereas Cer2 and

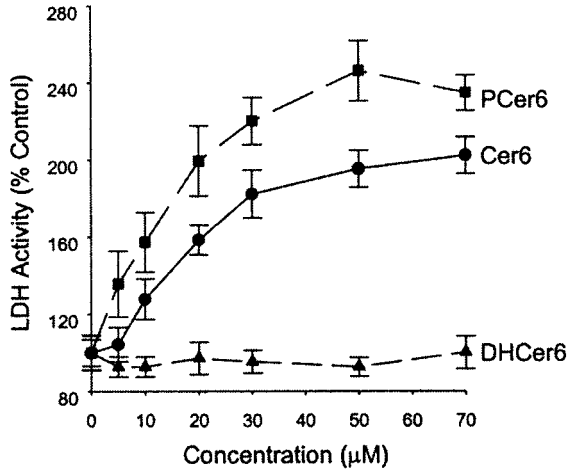

Fig. 1. Comparison of cytotoxic activities of DHCer6, PCer6, and Cer6. SK-N-BE(2)C cells were incubated with 0 to 70 μM DHCer6, PCer6, or Cer6 for 24 h and cell death was assessed by LDH activity released into the medium. Data were obtained from two independent experiments performed in quadruplicate and expressed as mean ± S.D. of LDH activity in percentage of untreated control cells. ANOVA, Newman-Keuls multiple comparisons test. PCer6 values were significantly different from Cer6 and DHCer6 at all concentrations ($p < 0.005$).

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DHCer2 yielded 144 ± 13 and 102 ± 7% of untreated control, respectively.

Morphological observation also corresponded well with the LDH data (Fig. 3). Cells treated with 10 μM DHCer6 did not seem to be different from the untreated control cells. Cer6 caused significant cell death, and the cells have become characteristically shrunken, granular, and round. More extensive cell death was observed in the cells treated with PCer6, most of which have detached from the bottom of the culture plate. PCer2 was also highly toxic at 60 μM, whereas Cer2 was only mildly toxic. Hence, the phytoceramides were more cytotoxic compared with their corresponding ceramides and dihydroceramides by both biochemical and morphological criteria. The results also showed that the double bond was not necessary for the ceramides' cytotoxic activity and that a hydroxyl group at carbon 4 augmented the toxicity.

Evaluation of Phytoceramide Conversion to Ceramide. It was possible that the mechanism by which the phytoceramides led to cellular demise was their intracellular conversion to respective ceramides, although no enzyme catalyzing such conversion has been identified thus far. To test this possibility, we determined whether Cer6 might be produced after incubation of cell lysate with PCer6 after separation of lipids and detection by HPLC-evaporated light scattering detector system. As shown in Fig. 4, the Cer6 and PCer6 standards were separated with retention times of 10.1 min and 18.6 min, respectively. In the cell lysate incubated with PCer6, no Cer6 was detected. In comparison, the PCer6 peak was increased proportionally with the added amount of PCer6. Thus, at least to the limit of the sensitivity of our detection method, phytoceramide did not seem to be converted to ceramide by the cell components.

Role of Chain Length of Ceramides in Cytotoxic Activity. Comparison of Figs. 1 and 2 reveals that Cer6 and PCer6 were more cytotoxic than Cer2 and PCer2, respectively, suggesting a relationship between the acyl chain length and the extent of toxicity. A series of ceramides with varying chain lengths was required for a more systematic test of this hypothesis. Because the phytoceramides were found to be more effective than ceramides and we were able to obtain phytosphingosine in large amounts, we synthesized phytoceramides having two to eight carbons in their acyl chain. Cells were exposed to each phytoceramide for 24 h and extent of cell death was assessed by LDH assay in two different catecholaminergic cell lines, SK-N-BE(2)C and the mouse neuroblastoma N1E-115. As shown in Fig. 5, for both cells, cytotoxicity was elevated when the acyl chain was increased from two to five. The highest activities were observed

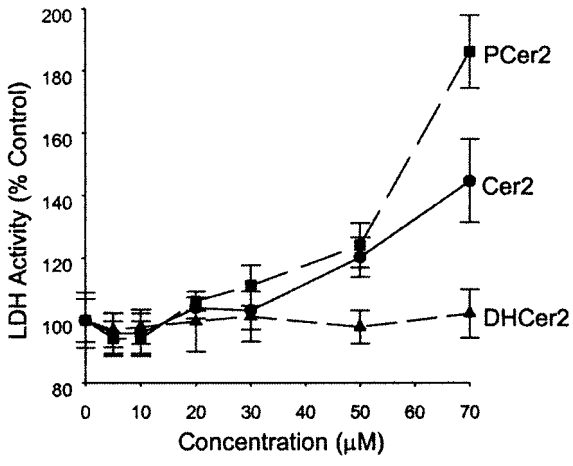

Fig. 2. Comparison of cytotoxic activities of DHCer2, PCer2, and Cer2. SK-N-BE(2)C cells were incubated with 0 to 70 μM DHCer2, PCer2, or Cer2 for 24 h and the degrees of cell death were assessed by LDH activity released into the medium. Data were obtained from two independent experiments performed in quadruplicate and expressed as mean ± S.D. of LDH activity in percentage of untreated control cells. ANOVA, Newman-Keuls multiple comparisons test. PCer2 values were significantly different ($p < 0.005$) from Cer2 at 70 μM and from DHCer2 at 50 and 70 μM.

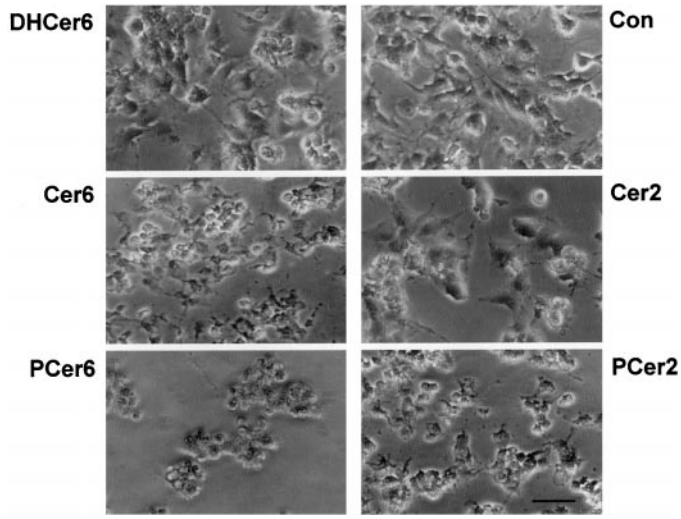

Fig. 3. Representative photographs of morphological analysis. SK-N-BE(2)C cells were incubated with 10 μM DHCer6, Cer6, and PCer6 or 60 μM Cer2 and PCer2 for 24 h and their morphological changes were examined under inverted microscope. Bar, 50 μm.

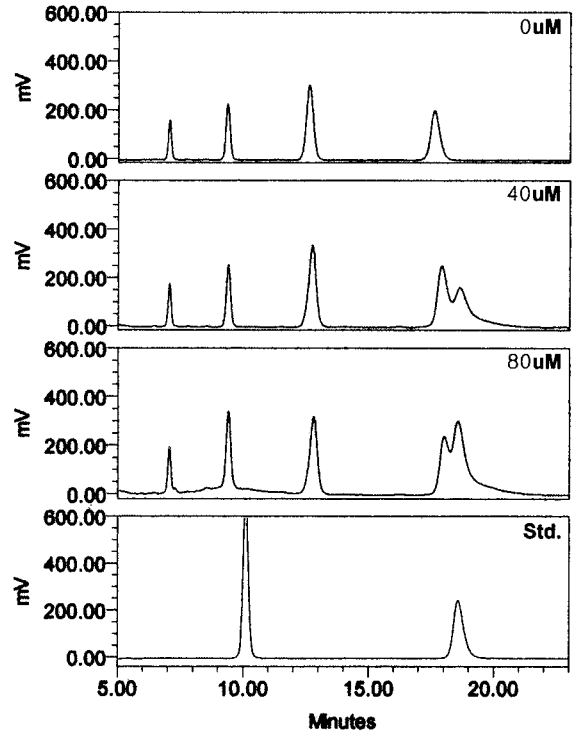

Fig. 4. Evaluation of phytoceramide conversion to ceramide. A typical HPLC-evaporated light scattering detection chromatogram of lipid extracted from SK-N-BE(2)C cell lysate incubated in the presence of 0, 40, or 80 μM PCer6 for 2 h at 37°C. Retention times for the Cer6 and PCer6 standards were determined to be 10.1 and 18.6 min, respectively. No production of Cer6 was detected after the incubation with PCer6.

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with *N*-valeryl-4D-ribo-phytosphingosine (223 ± 8% and 236 ± 14 for SK-N-BE(2)C and N1E-115, respectively) and PCer6 (222 ± 8% and 219 ± 17%, respectively). Interestingly, however, *N*-heptanoyl-4D-ribo-phytosphingosine exhibited lower toxicity and *N*-octanoyl-4D-ribo-phytosphingosine showed no activity.

Role of Carbonyl Group of the Amide Bond in Ceramide Cytotoxicity. Previous studies have indicated that induction of differentiation and inhibition of cell growth by ceramide required the presence of an amide-linked acyl chain (Okazaki et al., 1990). On the other hand, *N*-octylsphingosine is reported to stimulate apoptosis more effectively than *N*-octanoylsphingosine (Karasavvas et al., 1996), suggesting that the amide group is not necessary. This prompted us to determine whether the oxygen in the amide group might be required for the cytotoxic activity. For this, we synthesized PCer6S, a PCer6 analog with the amidyl oxygen substituted with a sulfur, and compared its cytotoxicity with PCer6, DHCer6, and Cer6 in SK-N-BE(2)C cells. As shown in Table 1, PCer6S exhibited toxicity comparable with PCer6 and higher than Cer6 and DHCer6. Thus, the presence of sulfur in place of oxygen in the amide group did not affect the phytoceramide's cytotoxic activity.

Characterization of the Phytoceramide-Induced Cell Death. Time course of LDH activity in the medium after the PCer6 treatment showed that cell death began to be observed after 4 h (130 ± 21% of untreated control) and steadily increased up to 24 h (Fig. 6A). Most of the PCer6 cytotoxicity seemed to occur during the initial 4 to 6 h.

Because ceramides were known to cause apoptotic cell death, we determined whether the cell death by phytoceramides was also apoptotic. As detection of internucleosomal DNA fragmentation provides a sensitive means to monitor apoptosis, TUNEL staining assay was performed on SK-N-BE(2)C cells exposed to PCer6. As shown in Fig. 6B, after 6 h, virtually all cells in the PCer6-treated group was TUNEL-staining positive, whereas very few cells (less than 1%) were positive in the untreated control group. Propidium iodide staining (Fig. 6C) showed that within 4 h, the nuclei of the PCer6-treated cells exhibited fragmentation. Taken together, the phytoceramide-induced cell death was likely apoptotic in nature.

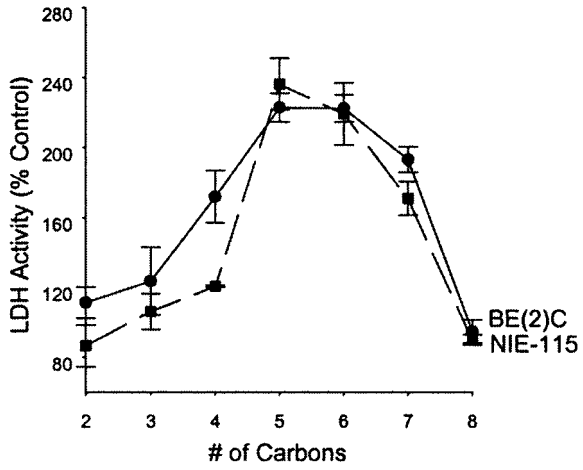

Fig. 5. Comparison of cytotoxic activities of phytoceramides with varying acyl chain lengths. SK-N-BE(2)C and N1E-115 cells were incubated with 30 μM each phytoceramide for 24 h and the degrees of cell death were assessed by LDH activity released into the medium. Data were obtained from two independent experiments performed in quadruplicate and expressed as mean ± S.D. of LDH activity in percentage of untreated control cells. ANOVA, Newman-Keuls multiple comparisons test, $p < 0.005$ versus untreated control values for four, five, six, and seven carbons for both SK-N-BE(2)C and N1E-115.

Discussion

Although it has been generally believed that the *trans* double bond between carbons 4 and 5 of the sphingoid backbone is essential for ceramide's cytotoxicity (Bielawska et al., 1993; Obeid et al., 1993), we show for the first time that the double bond is not an absolute requirement. Whereas its removal by the addition of hydrogen atoms (producing a dihydroceramide) indeed abolishes the cytotoxic activity, addition of hydroxyl group at position 4 (producing a phytoceramide) not only maintains but also enhances the activity. Thus, the cytotoxic activity seems to be rendered by the presence of the double bond as well as the presence of a hydroxyl group at position 4. Although the reason for the enhanced cytotoxicity by the hydroxyl group remains obscure, it is conceivable that changes in hydrogen bonding ability and/or electrophilicity may have occurred, facilitating the molecule's interaction with its targets.

We show that the substitution of sulfur in place of oxygen in the amide-acyl link does not influence the cytotoxicity. In this molecule, hydrogen-bonding ability of the thio-amide group is expected to be reduced compared with the amide, and the sulfur atom, which is larger and more polarizable than oxygen, would exhibit stronger binding ability toward electrophilic sites. That this substitution does not influence the cytotoxic activity suggests that such changes in hydrogen bonding ability are not critical for ceramides interaction with their targets. This is also in agreement with the previous reports that *N*-acetylsphingosine, whose amidyl oxygen is replaced with sulfur, exhibits cytotoxicity (Wieder et al., 1997).

The extent of cell death was augmented with increasing acyl chain length up to five or six, but was reduced with longer chains. Because molecular conformation of ceramides should depend on the hydrophobic interaction between the sphingoid backbone and the acyl chain, which in turn would influence the interaction with their cellular targets, it is possible that phytoceramides that have five or six acyl chains yield the most effective conformation for such interaction. One can speculate that the phytoceramides with longer acyl chains cannot easily penetrate the cell membrane, as demonstrated by the fact that the natural ceramides containing long acyl chains do not freely enter the cells.

TABLE 1

Comparison of cytotoxic activities of PCer6S with other ceramide derivatives.

SK-N-BE(2)C cells were incubated with 20 μM of each compound for 24 h and the degrees of cell death were assessed by LDH activity released into the medium. Data were obtained from two independent experiments performed in quadruplicate and expressed as percentage of untreated control cells ± S.D. ANOVA, Newman-Keuls multiple comparison test, *$p < 0.005$ versus untreated control; PCer6S was not significantly different from PCer6 ($p > 0.5$).

Ceramides	Cytotoxic Activity (% Untreated control)
PCer6S	200.13 ± 8.14*
PCer6	199.50 ± 18.15*
Cer6	158.53 ± 7.69*
DHCer6	97.01 ± 8.30

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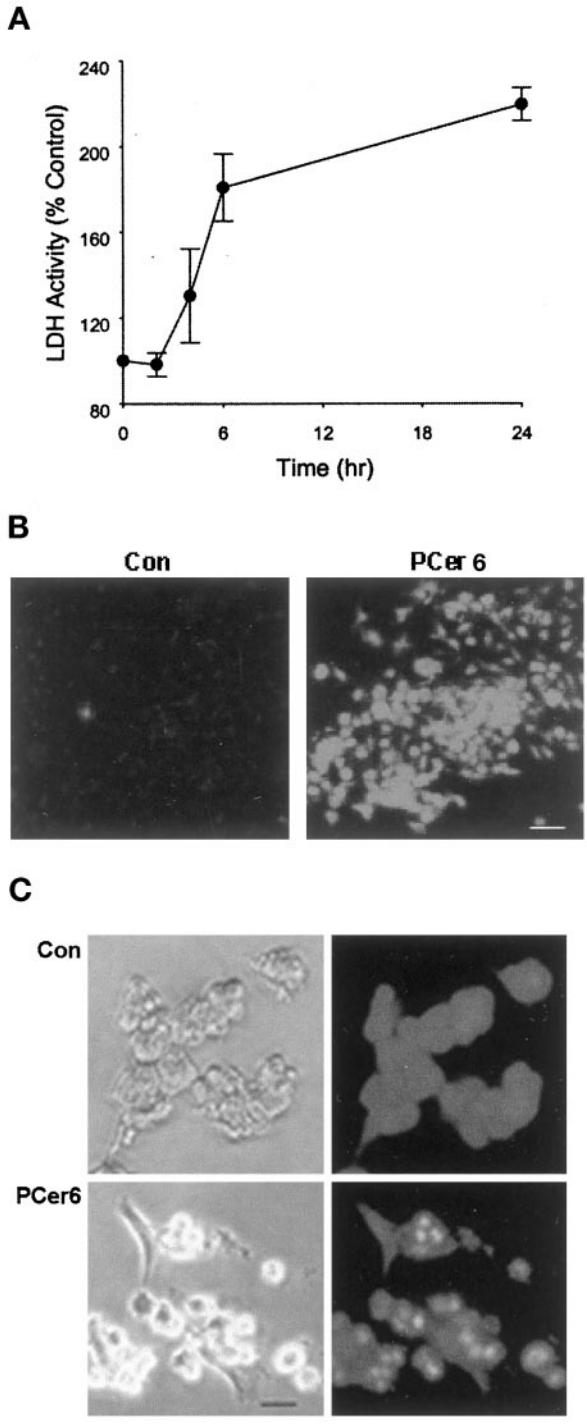

Fig. 6. Characterization of the phytoceramide-induced cell death. A, time course of cell death by PCer6. SK-N-BE(2)C cells were incubated with 30 μM PCer6 for 0 to 24 h and the degrees of cell death were assessed by LDH activity released into the medium. Data were obtained from two independent experiments performed in quadruplicate and expressed as mean ± S.D. of LDH activity in percentage of untreated control cells. ANOVA, Newman-Keuls multiple comparisons test, $p < 0.005$ versus untreated control values for 4-, 6-, and 24-h treatments. B, a representative photograph of TUNEL staining. SK-N-BE(2)C cells were exposed to 20 μM PCer6 for 6 h and TUNEL staining was performed as described under *Experimental Procedures*. The PCer6 treatment caused essentially every cell TUNEL-positive. Bar, 50 μm. C, a representative photograph of propidium iodide staining. SK-N-BE(2)C cells were exposed to 20 μM PCer6 for 4 h and stained with propidium iodide; left , phase contrast microscopy; right, fluorescence microscopy. Bar, 15 μm.

Activation of a variety of intracellular signaling systems by ceramide is believed to lead to activation of caspases, endonucleases, DNA fragmentation and, consequently, apoptosis. Our data suggest that the cell death induced by phytoceramide is probably apoptotic as well, as evidenced by DNA fragmentation. Thus, it is conceivable that phytoceramides make use of the intracellular pathway normally used by ceramides in mediation of the cell demise. Although one can also imagine that the phytoceramide is first converted to ceramides, which in turn mediate the signaling, the present findings that the phytoceramides were more cytotoxic than their respective ceramide counterparts and that the phytoceramide was not converted to ceramide would argue against such a notion. In addition, thus far, no enzyme responsible for the conversion of phytoceramide to ceramide has been identified.

Phytoceramide serves as a building block of complex sphingolipids in yeast and is thought to be the yeast counterpart of the mammalian ceramide. Enzymes responsible for its synthesis (Haak et al., 1997; Grilley et al., 1998; Mao et al., 2000) and degradation (Mao et al., 2000) have been identified in yeast. The availability of the synthetic, short-chain phytoceramides should facilitate elucidation of the mechanisms by which phytoceramide mediates various cellular responses in yeast. In addition, because the yeast and mammalian systems seem to share similarities in lipid metabolism (Mao et al., 2000), gaining knowledge in intracellular signaling and metabolism of phytoceramide should shed insight into understanding of the mammalian system.

Because of their cytotoxic effects, the use of ceramides in skin therapy (Rodrigues et al., 1998) and as antitumor drugs (Kishida et al., 1997) has been suggested. The fact that phytoceramides are more effective than ceramide in cytotoxic activity and can be easily synthesized from phytosphingosine abundantly obtained from yeast sources makes phytoceramides a more effective and economical alternative. Thus, phytoceramides might be useful in development of a new type of antitumor drug and skin therapy. In addition, this availability of low cost and more effective ceramide analogs should facilitate studies requiring large amounts of ceramides, such as in vivo experiments.

References

Agresti C, D'Urso D and Levi G (1996) Reversible inhibitory effects of interferon-γ and tumour necrosis factor-α on oligodendroglial lineage cell proliferation and differentiation in vitro. *Eur J Neurosci* **8:**1106–1116.

Bielawska A, Crane HM, Liotta D, Obeid LM and Hannun YA (1993) Selectivity of ceramide-mediated biology. Lack of activity of erythro-dihydroceramide. *J Biol Chem* **268:**26226–26232.

Brugg B, Michel PP, Agid Y and Ruberg M (1996) Ceramide induces apoptosis in cultured mesencephalic neurons. *J Neurochem* **66:**733–739.

Dressler KA, Mathias S and Kolesnick RN (1992) Tumor necrosis factor-alpha activates the sphingomyelin signal transduction pathway in a cell-free system. *Science (Wash DC)* **255:**1715–1718.

Dyatlovitskaya EV (1998) Correlation between bioeffector characteristics of sphingolipids and the structure of their hydrophobic fragment. *Biochemistry (Mosc)* **63:**55–61.

Grilley MM, Stock SD, Dickson RC, Lester RL and Takemoto JY (1998) Syringomycin action gene SYR2 is essential for sphingolipid 4-hydroxylation in Saccharomyces cerevisiae. *J Biol Chem* **273:**11062–11068.

Haak D, Gable K, Beeler T and Dunn T (1997) Hydroxylation of Saccharomyces cerevisiae ceramides requires Sur2p and Scs7p. *J Biol Chem* **272:**29704–29710.

Hartfield PJ, Mayne GC and Murray AW (1997) Ceramide induces apoptosis in PC12 cells. *FEBS Lett* **401:**148–152.

Jarvis WD, Fornari FA, Traylor RS, Martin HA, Kramer LB, Erukulla RK, Bittman R and Grant S (1996) Induction of apoptosis and potentiation of ceramide-mediated cytotoxicity by sphingoid bases in human myeloid leukemia cells. *J Biol Chem* **271:**8275–8284.

Jayadev S, Liu B, Bielawska AE, Lee JY, Nazaire F, Pushkareva MY, Obeid LM and Hannun YA (1995) Role for ceramide in cell cycle arrest. *J Biol Chem* **270:**2047–2052.

Kaipia A, Chun SY, Eisenhauer K and Hsueh AJ (1996) Tumor necrosis factor-alpha

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and its second messenger, ceramide, stimulate apoptosis in cultured ovarian follicles. *Endocrinology* **137:**4864–4870.
Karasavvas N, Erukulla RK, Bittman R, Lockshin R and Zakeri Z (1996) Stereospecific induction of apoptosis in U937 cells by N-octanoyl-sphingosine stereoisomers and *N*-octyl-sphingosine. The ceramide amide group is not required for apoptosis. *Eur J Biochem* **236:**729–737.
Kim MY, Linardic C, Obeid L and Hannun Y (1991) Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor α and γ-interferon. Specific role in cell differentiation. *J Biol Chem* **266:**484–489.
Kishida E, Kasahara M, Takagi Y, Matsumura M, Hayashi T, Kobayashi S and Masuzawa Y (1997) Evaluation of a trans configuration for the apoptosis-inducing activity of ceramide. *J Lipid Mediat Cell Signal* **16:**127–137.
Lister MD, Ruan ZS and Bittman R (1995) Interaction of sphingomyelinase with sphingomyelin analogs modified at the C-1 and C-3 positions of the sphingosine backbone. *Biochim Biophys Acta* **1256:**25–30.
Mao C, Saba JD and Obeid LM (1999) The dihydrosphingosine-1-phosphate phosphatases of *Saccharomyces cerevisiae* are important regulators of cell proliferation and heat stress responses. *Biochem J* **342:**667–675.
Mao C, Xu R, Bielawska A and Obeid LM (2000) Cloning of an alkaline ceramidase from Saccharomyces cerevisiae. An enzyme with reverse (CoA-independent) ceramide synthase activity. *J Biol Chem* **275:**6876–6884.
Mathias S, Younes A, Kan CC, Orlow I, Joseph C and Kolesnick RN (1993) Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1 beta. *Science (Wash DC)* **259:**519–522.
Obeid LM, Linardic CM, Karolak LA and Hannun YA (1993) Programmed cell death induced by ceramide. *Science (Wash DC)* **259:**1769–1771.
Okazaki T, Bielawska A, Bell RM and Hannun YA (1990) Role of ceramide as a lipid mediator of 1α,25-dihydroxyvitamin D3-induced HL-60 cell differentiation. *J Biol Chem* **265:**15823–15831.
Rodrigues L, da Silva PA, Pinto P, Galego N, Silva N and Pereira LM (1998) Identification of the in vivo topically applied "human-identical" ceramides included in negatively charged liposomes. *Boll Chim Farm* **137:**395–402.
Saba JD, Nara F, Bielawska A, Garrett S and Hannun YA (1997) The BST1 gene of Saccharomyces cerevisiae is the sphingosine-1-phosphate lyase. *J Biol Chem* **272:**26087–26090.
Santana P, Llanes L, Hernandez I, Gonzalez-Robayna I, Tabraue C, Gonzalez-Reyes J, Quintana J, Estevez F, Ruiz de Galarreta CM and Fanjul LF (1996) Interleukin-1 beta stimulates sphingomyelin hydrolysis in cultured granulosa cells: evidence for a regulatory role of ceramide on progesterone and prostaglandin biosynthesis. *Endocrinology* **137:**2480–2489.
Shirota O, Nakanishi K and Berova N (1999) Phytosphingosines—a facile synthesis and spectroscopic protocol for configurational assignment. *Tetrahedron* **55:**13643–13658.
Skrzypek MS, Nagiec MM, Lester RL and Dickson RC (1999) Analysis of phosphorylated sphingolipid long-chain bases reveals potential roles in heat stress and growth control in *Saccharomyces*. *J Bacteriol* **181:**1134–1140.
Tepper CG, Jayadev S, Liu B, Bielawska A, Wolff R, Yonehara S, Hannun YA and Seldin MF (1995) Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. *Proc Natl Acad Sci USA* **92:**8443–8447.
Wieder T, Geilen CC, Kolter T, Sadeghlar F, Sandhoff K, Brossmer R, Ihrig P, Perry D, Orfanos CE and Hannun YA (1997) Bcl-2 antagonizes apoptotic cell death induced by two new ceramide analogues. *FEBS Lett* **411:**260–264.
Wiegmann K, Schutze S, Machleidt T, Witte D and Kronke M (1994) Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell* **78:**1005–1015.

Send reprint requests to: Jae Dam Lee M.D., Ph.D., Department of Biochemistry, University of Ulsan, College of Medicine 388–1 Poongnap-dong, Songpa-ku Seoul 138–736, Korea. E-mail: jdlee@www.amc.seoul.kr

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