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Novel Ceramide Analogs as Potential Chemotherapeutic Agents in Breast Cancer

Amanda P. Struckhoff, Robert Bittman, Matthew E. Burow, Sanda Clejan, Steven Elliot, Timothy Hammond, Yan Tang, and Barbara S. Beckman

Departments of Pharmacology (A.P.S., B.S.B.), Pathology (S.C.), and Medicine (T.H., M.E.B), Molecular Cellular Biology Program (M.E.B., S.C., S.E., Y.T., B.S.B.), Center for Bioenvironmental Research (M.E.B., S.C., S.E., Y.T., B.S.B.) Tulane Cancer Center (M.E.B., S.E., Y.T., S.S.B.), and Louisiana General Clinical Research Center (S.C.), Tulane University Health Sciences Center, New Orleans, LA 70112

Department of Chemistry and Biochemistry, Queens College, City University of New York, Flushing, NY 11367 (R.B.)

Veterans Affairs Medical Center, New Orleans, LA 70112 (T.H.)

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Running Title: Ceramide Analogs in Breast Cancer

Address Correspondence to: Barbara S. Beckman

Department of Pharmacology, Tulane Health Sciences Center, 1430 Tulane Ave. SL-83

New Orleans, LA 70112

Phone: (504)-588-5589

Fax: (504)-584-3779

E-mail: bbeckman@tulane.edu

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Abbreviations: Cer, (2*S*,3*R*)-*N*-octanoylsphingosine; DH-Cer, (2*S*,3*R*)-*N*-octanoyl-4,5-dihydrosphingosine; 4,6-diene-Cer, (2*S*,3*R*)-(4*E*,6*E*)-2-octanoylamidooctadecadiene-1,3-diol; 4,6-diene-7-Ph-Cer, (2*S*,3*R*)-(4*E*,6*E*)-2-octanoylamido-7-phenylheptadiene-1,3-diol; 6-ene-Cer, (2*S*,3*R*)-(6*E*)-2-octanoylamidooctadecadiene-1,3-diol; 6-OH-Cer, (2*S*,3*R*,6*S*)-(4*E*)-2-octanoylamidooctadecadiene-1,3,6-triol; 6-OH-4-yne-Cer, (2*S*,3*R*,6*S*)-2-octanoylamido-4-octadecyne-1,3,6-triol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's Modified Eagle Medium; FCS, fetal calf serum; BME, basal medium eagle; MEM, minimum essential medium; TNF-α, tumor necrosis factor α; hTERT, human telomerase; HME, human mammary epithelial; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; PI, propidium iodide; JC-1, 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethyl-benzimidazolylcarbocyanine iodide; DAG, diacylglycerol; D-*e*-C8, D-*erythro*-C8-ceramide, DH-C8, dihydro-C8-ceramide; PTPC, permeabilization transition pore complex; S.E., standard error

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Abstract

Recent evidence suggests a role for aberrant ceramide levels in the pathogenesis of cancer and chemoresistance, and indicates that manipulation of tumor ceramide levels may be a useful strategy in the fight against breast cancer. This study demonstrates that alterations in the degree and position of unsaturation of bonds in the sphingoid backbone of D-*erythro*-*N*-octanoyl-sphingosine (Cer) affect the antiproliferative ability of ceramide analogs in breast cancer cells. The most potent analog of Cer we tested is (2*S*,3*R*)-(4*E*,6*E*)-2-octanoylamidooctadecadiene-1,3-diol (4,6-diene-Cer), which contains an additional trans double bond at C(6)-C(7) of the sphingoid backbone. 4,6-Diene-Cer exhibited higher potency than Cer in TNF-α-resistant (IC_{50} 11.3 μM vs 32.9 μM) and TNF-α-sensitive (IC_{50} 13.7 μM vs 37.7 μM) MCF-7 cells. 4,6-Diene-Cer was also more potent than Cer in inducing cell death in MDA-MB-231 and NCI/ADR-RES breast cancer cell lines (IC_{50} 3.7 μM vs 11.3 μM, and 24.1 μM vs 86.9 μM, respectively). 4,6-Diene-Cer caused a prolonged elevation of intracellular ceramide levels in MCF-7 cells, which may contribute to its enhanced cytotoxicity. Furthermore, treatment of MCF-7 cells with Cer or 4,6-diene-Cer resulted in induction of apoptosis by 8 hr via the mitochondrial pathway, as demonstrated by release of cytochrome c, loss of membrane asymmetry (measured by Annexin V staining), and a decrease in the mitochondrial membrane potential. Importantly, both Cer and 4,6-diene-Cer displayed selectivity toward transformed breast cells over non-transformed breast epithelial cells. These data suggest that these and other novel ceramide analogs represent potential therapeutic agents in breast cancer treatment.

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Breast cancer is the most commonly diagnosed cancer in women, and the American Cancer Society estimates there will be approximately 213,000 new cases diagnosed in 2003. Resistance to therapy is the major reason for failure of cancer treatment. Chemo- and radiotherapies are thought to primarily exert anti-tumor effects through the activation of programmed cell death pathways (Mesner et al., 1997), and resistance to these therapies is often the result of defects in this apoptotic cell death cascade. From a pharmacological perspective, development of new agents that can induce programmed cell death or overcome resistance mechanisms are predicted to improve patient outcomes, prevent relapse, and prolong patient survival.

Ceramide is a sphingolipid signaling molecule that has been shown to mediate a diverse range of biological responses to extracellular stimuli, including proliferation, differentiation, immune responses, senescence, and growth arrest (Hannun and Obeid, 2002; Kolesnick, 2002). The diversity of responses elicited by ceramide suggests the presence of distinct signaling pathways upon which ceramide acts. In the field of cancer biology, ceramide has been intensely studied for its ability to induce both apoptotic and non-apoptotic cell death (Lopez-Marure et al., 2002; Obeid et al., 1993). In tumor cells, chemotherapeutic drugs such as doxorubicin, vincristine, etoposide, and paclitaxel (Senchenkov et al., 2001), as well radiotherapy, increase intracellular ceramide levels following treatment (Haimovitz-Friedman, 1998). In most cases, this treatment-induced rise in ceramide is critical for response to these treatments, and pharmacologic agents that affect production or accumulation of ceramide can alter the response to chemotherapy (Litvak et al., 2003; Olshefski and Ladisch, 2001).

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Consistent with the principle that increasing intracellular ceramide can mediate tumor cell death, aberrant or decreased ceramide signaling has been implicated in contributing to tumor progression and resistance to therapy. Ceramide levels in human specimens of primary and metastatic colon cancer contained approximately half the level of ceramide when compared with respective normal colon mucosa from the same patient (Selzner et al., 2001), and ceramide levels have been inversely correlated with malignant progression in glial specimens (Riboni et al., 2002). In addition, many radiation-resistant cell lines and isolated tumor specimens do not produce ceramide following irradiation (Chmura et al., 1997; Michael et al., 1997), and a number of multi-drug resistant cancer cell lines do not generate, or accumulate, ceramide in response to therapy (Cai et al., 1997; Wang et al., 1999). These data suggest that clinical manipulation of ceramide levels within tumors represents an important mechanism for decreasing both tumor survival and chemotherapeutic resistance mechanisms and makes ceramide signaling an attractive target for chemotherapeutic drug development.

In this study we examine the antiproliferative and proapoptotic activities of a series of novel ceramide analogs in a model of breast cancer resistance. Altering the composition and degree of unsaturation of the sphingoid backbone of ceramide significantly changed the ability of these analogs to decrease viability and proliferation in both chemosensitive and chemoresistant MCF-7 cells. Importantly, the ability of ceramide analogs to induce apoptosis was selective for breast cancer cells compared to normal mammary epithelial cells. Since ceramide has been shown to be involved in tumor sensitivity to apoptosis and chemoresistance, we suggest that development of

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ceramide analogs with increased anti-tumor activity represents a potential new class of chemotherapeutic agents.

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Methods

Reagents. D-*erythro*-C8-Ceramide (Cer) and C8-dihydroceramide (DH-Cer) were purchased from BIOMOL (Plymouth Meeting, PA). In order to achieve cell permeability, all of the ceramide analogs used in the present study have an *N*-octanoyl chain. The syntheses of (2*S*,3*R*)-(4*E*,6*E*)-2-octanoylamidooctadecadiene-1,3-diol (4,6-diene-Cer) and (2*S*,3*R*)-(6*E*)-2-octanoylamidooctadecadiene-1,3-diol (6-ene-Cer) have been described (Chun et al., 2002). The syntheses of (2*S*,3*R*,6*S*)-(4*E*)-2-octanoylamidooctadecadiene-1,3,6-triol (6-OH-Cer) and (2*S*,3*R*,6*S*)-2-octanoylamido-4-octadecyne-1,3,6-triol (6-OH-4-yne-Cer) have also been described (Chun et al., 2003b). (2*S*,3*R*)-(4*E*,6*E*)-2-Octanoylamido-7-phenylheptadiene-1,3-diol (4,6-diene-7-Ph-Cer) was prepared by using a synthetic strategy similar to that employed in the preparation of 4,6-diene-Cer. All ceramide analogs were dissolved in ethanol, and all treatments were adjusted to have identical final ethanol concentrations of less than 0.1%. TNF-α was purchased from R&D Biosystems (Abingdon, UK).

Cell culture. MCF-7N and MCF-7TN-R cells, as well as MDA-MB-231 and NCI/ADR-RES cells, were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, BME amino acids, MEM amino acids, sodium pyruvate, and penicillin-streptomycin (Life Technologies, Gaithersburg, MD). The MCF-7 N cell variant is a subclone of MCF-7 cells from the American Type Culture Collection (Manassas, VA) that was generously provided by Louise Nutter (University of Minnesota, Minneapolis, MN) (Burow et al., 1998).

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Generation of the resistant MCF-7 variant (MCF-7TN-R) was achieved by prolonged exposure of MCF-7N cells to increasing concentrations of TNF-α. Human mammary epithelial (HME) cells transfected with telomerase (hTERT-HME) were purchased from BD Bioscience/Clontech (Palo Alto, CA) and were cultured in Mammary Epithelial Cell Medium (MECM) (Cambrex, San Diego, CA) supplemented with bovine pituitary extract.

MTT viability Assay. MCF-7 cell variants or HME cells were seeded at 7.5 x 10^5 cells per 96-well plate in phenol-free DMEM (or MECM for HME) supplemented with 10% FBS and allowed to adhere overnight. Cells were treated with the indicated concentrations of TNF-α or ceramide analog for 24-48 hr. Twenty-five μL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye (5 mg/ml) were incubated in each well for 4 hr. Cells were lysed with 20% SDS in 50% dimethylformamide. The pH and absorbances were read on an EL_x808 Microtek plate reader (Winooski, VT) at 550 nm, with a reference wavelength of 630 nm. Unless otherwise indicated, viability is expressed as percent of vehicle-treated control. All treatments were carried out in quadruplicate, and all experiments were performed at least three times.

Western blot analysis. MCF-7N or MCF-7TN-R cells were plated at 5 x 10^5 cells in 25 cm^2 culture flasks and treated as indicated. Cells were detached using 0.5% EDTA-PBS, pelleted by centrifugation, resuspended in sonication buffer (62.5 mM Tris–HCl, pH 6.8, 4% (w/ v) SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, as well as protease and phosphatase inhibitor cocktails (Sigma, St Louis, MO), and sonicated for

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30 s. After centrifugation at 12,000 x g for 3 min, 50 μg of protein was suspended in sample loading buffer (62.5 mM Tris – HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 3 min, and electrophoresed on 12-15% polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane (Biorad, Hercules, CA) which was blocked with PBS–Tween (0.05%)-5% low-fat dry milk solution at room temperature for 1 hr. The membrane was subsequently probed with polyclonal antibodies raised against cytochrome c (1:500 dilution, Santa Cruz, CA), cytochrome c oxidase (1:1000, Molecular Probes, Eugene, OR). After incubation at 4°C overnight, blots were washed 3x in PBS-Tween (0.05%) solution and incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (1:10,000 dilution; Oxford, Oxford, MI) for 2 hr at room temperature. After four washes with PBS-Tween solution, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham, Arlington Heights, IL) and recorded by fluorography on Hyperfilm (Amersham), according to the manufacturer's instructions.

Annexin V apoptosis assay. FITC Annexin V/ propidium iodide (PI) apoptosis assay kits were purchased from Molecular Probes and assays were performed according to the manufacturer's instructions. Briefly, MCF-7 cells were treated with the indicated ceramide analog for 24 hr, harvested, and pelleted by centrifugation. Cell pellets were washed once in ice-cold PBS, resuspended in Annexin binding buffer (~1 x 10^6 cells/ml), and incubated with FITC-conjugated Annexin V and propidium iodide (1 μg/ml) for 15 min at 37°C in the dark. The stained cells were analyzed on a Becton Dickinson FACStar flow cytometer measuring fluorescence emission at 530 nm for FITC, and 638

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nm for propidium iodide (PI). Cells negative for both Annexin V and PI staining are considered live cells. Annexin V-positive and PI-negative staining cells are undergoing early stages of apoptosis, PI- and Annexin V-positive staining cells are necrotic and/or late apoptotic cells, and PI-positive and Annexin V-negative staining cells are necrotic cells.

Cytochrome c release. Cells (4×10^6) were harvested with 0.5% EDTA-PBS, pooled together with media and washes containing floating cells, and pelleted by centrifugation at 500 × *g* for 3 min at 4°C. Pellets were resuspended with Buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM benzamidine, 1 mM dithiothreitol, 250 mM sucrose, and protease and phosphatase inhibitor cocktails (Sigma), then lysed by passing the suspension through a 25-gauge needle 15 times. Homogenates were centrifuged at 500 × g for 5 min at 4°C. Supernatants were further centrifuged at 10,000 × *g* for 30 min at 4°C, and the resultant mitochondria-rich pellets were resuspended in Buffer A and sonicated. The supernatant from the 10,000 × *g* spin was designated as cytosol. Protein concentrations were determined by the Bradford reagent assay (BioRad). Subcellular fractionations were subjected to immunoblotting as described above. Experiments were performed 3-5 times, and the results of a typical experiment are shown.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$). MCF-7 cells were seeded at 7.5×10^5 cells per 96-well plate in phenol-free DMEM supplemented with 10% fetal calf serum, and allowed to adhere overnight. The cells were treated with 30

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μM of ceramide analog for indicated times. After incubation, the medium was removed, 2.5 μM 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethl-benzimidazolylcarbocyanine iodide (JC-1) (Chemicon International, Temecula, CA) was added to each well, and plates were incubated at 37°C for 20 min. After incubation, JC-1 dye was removed and the cells were carefully washed with 1X incubation buffer, followed by addition of PBS. Emitted fluorescence was measured on a FLx600 fluorometer (Biotek, Winooski, VT) at 530 nm (red) and 590 nm (green) with excitation at 485 nm. The decrease in the mitochondrial membrane potential was estimated by calculating the ratio of fluorescence at 590 nm and 530 nm for each well as described (Stoica et al., 2003). Ratios of treated samples are expressed as the percent of the vehicle-treated control (considered 100%).

Analysis of cellular ceramide levels. Ceramide was quantified by the diacylglycerol (DAG) kinase assay as ^{32}P-incorporated into ceramide 1-phosphate, or by HPLC, as previously described (Clejan, 1998). In the DAG kinase method, MCF-7 cells were treated with TNF-α, Cer, or ceramide analog for the times indicated, washed in PBS, and fixed in ice-cold methanol. The lipids were extracted in chloroform, and the organic phase was dried under a nitrogen stream. The lipids contained in the organic phase extract were resuspended in 20 μl of 7.5% *n*-octyl β-glucopyranoside, 5 mM cardiolipin, 1 mM diethylenetriaminepentaacetic acid (Sigma) and 40 μl of purified DAG kinase in enzyme buffer (20 mM Tris-HCl, 10 mM dithiothreitol, 1.5 M NaCl, 250 mM sucrose, and 15% glycerol, pH 7.4). DAG kinase was added in excess in order to ensure the total conversion of the substrate to the phosphorylated product. Fifty μl of buffer, containing [γ-^{32}P]ATP (10 mM; 1000 dpm/pmol)), was added to start the reaction. After 30 min at 22 C, the reaction was stopped by extraction of lipids with 1 ml of

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chloroform:methanol:hydrochloric acid (100:100:1, v/v). Buffered saline solution (170 µl; 135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 10 mM Hepes, pH 7.2) and 30 µl of 100 mM EDTA were added. The organic phase (lower) was dried under N_2. Ceramide 1-phosphate was resolved by TLC using $CHCl_3$:CH_3OH:acetic acid (65:15:5, v/v/v) as the solvent system and detected by autoradiography (Rf fraction between 0.5 and 0.6), and the incorporated ^{32}P was quantified by phosphorimaging (Fugi BAS1000, Fugi Medical Systems, Stamford, CT). The level of ceramide was determined by comparison with a standard curve composed of a known amount of egg- and brain-derived, mixed *N*-acyl chain ceramides (Avanti Polar Lipids, Alabaster, AL)., with C8-ceramide was used as a positive standard of DAG kinase activity.

Alternatively, ceramide levels were measured by HPLC. A non-enzymatic method based on deacylation of ceramide to the corresponding sphingoid base and derivatization with *o*-phthalaldehyde (OPA) (Merrill et al., 1988) as modified by Yoon (Yoon et al., 1999) was used. Lipid extract aliquots (20 µL) were quantified by reverse-phase HPLC using a Nova Pak C18 column (elution with methanol:6 mM potassium phosphate, pH 7.0 (90:10), flow rate, 0.6 ml/min, detection by spectrofluorometry (excitation, 340 nm; emission, 455 nm), with D-*erythro*-C8-sphingosine as an internal standard Ceramide levels were determined by comparison with a concomitantly run standard curve of known amounts of ceramide (Avanti Polar Lipids) as was done in the DAG kinase method. We compared the quantity of ceramide measured by the DAG kinase assay with that estimated by the HPLC method. The ceramide levels as calculated by the two methods correlated well as determined by linear regression analysis ($r=0.910$, $p<0.004$).

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Results

A Model of MCF-7 Chemoresistance. To investigate the ability of novel ceramide analogs to affect breast cancer chemoresistance and apoptosis, we developed a model of MCF-7 breast cancer resistance based on work previously published by our laboratory (Burow et al., 1998). MCF-7N-TR cells are isogenic variants of MCF-7N cells, and show profound resistance to the apoptosis-inducing effects of TNF-α, even at 100 ng/ml (Fig. 1). This is in contrast to the parental MCF-7N cells where treatment with only 1 ng/ml TNF-α resulted in a 50% reduction in cell number, and 10ng/ml reduced cell viability to less then 25%. Resistance to TNF-induced cell death is not a result of a lack of TNF-α receptors as treatment with TNF-α is still able to activate intracellular signaling pathways, as measured by a NF-κB luciferase reporter gene assay (data not shown). Both the parental and resistant MCF-7 cell variants were used to investigate the ability of ceramide analogs to induce cell death and overcome chemoresistance mechanisms.

TNF-α-resistant MCF-7 cells Do Not Generate Ceramide Following TNF-α Treatment. Many chemoresistant tumor cells exhibit defects in ceramide generation or accumulation. We hypothesized that in MCF-7TN-R cells ceramide generation or accumulation was low, and that this contributed to the observed resistance to TNF-α. Therefore, we investigated the effect of TNF-α treatment ceramide levels in the MCF-7 cell variants. In the sensitive (MCF-7N) cell variant, treatment with 10 ng/ml TNF-α resulted in a rapid rise of intracellular ceramide accumulation which peaks within 15

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min, with a return to basal levels by 2 hr (Fig. 2). In contrast, ceramide levels in the MCF-7N-TR variant did not increase significantly over the course of TNF-α treatment suggesting that defective ceramide accumulation correlates with resistance to the apoptotic effects of TNFα.

Cell Death Resistance Mechanisms Are Overcome by Exogenous Ceramide Treatment. Since the resistant MCF-7TN-R cell line did not generate ceramide in response to TNF-α treatment, we determined the effect of restoring ceramide signaling, via addition of exogenous ceramide, in the resistant and sensitive MCF-7 variants. Synthetic ceramides with a short *N*- acyl chain, such as *N*-octanoyl, are frequently used because they are taken up readily by cultured cells; natural ceramides have a very long *N*-acyl chain are difficult to introduce into cells and are poorly soluble in aqueous medium (Luberto and Hannun, 2000). We used Cer to test whether treatment with exogenous ceramide could restore the ability of the MCF-7TN-R cells to undergo cell death. Treatment of MCF-7 cell variants with Cer resulted in a virtually identical dose-dependent decrease in cell viability over 48 hr (Fig. 3), with an IC_{50} of approximately 30 μM in both the TNF-sensitive and the TNF-resistant cell variants. Ceramide was also able to decrease the long-term survival and colony-forming potential of both cell lines equally, as measured by an 8-day colony assay (data not shown). These findings support the hypothesis that restoration of the ceramide signaling component is able to restore cell death signaling in previously-resistant MCF-7 cells.

Ceramide Analogs Induce Cell Death in MCF-7 Variants. Since exogenous ceramide addition bypassed chemoresistance mechanisms in the MCF-7 breast cancer variants, we next determined whether the ceramide structure could be altered to

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increase the ability of ceramide to reduce breast cancer cell viability. Five novel ceramide analogs (Fig. 4)) were tested for their ability to induce changes in MCF-7 cell viability. The analogs varied in the structure and composition of the ceramide sphingoid backbone. Their dose-response curves are shown in Fig. 5A, along with those for Cer and DH-C8; the latter compound differs from ceramide by its lack of the 4,5 double bond and is the commonly used negative control for ceramide treatment. Moving the position of the C(4)-C(5) double bond on the sphingoid backbone of ceramide to the C(6)-C(7) position (6-ene-Cer) decreased the ability of this compound to induce cell death compared with Cer, except at doses that exceeded 50 μM. However, the addition of a C(6)-C(7) double bond with retention of the original C(4)–C(5) – trans double bond resulted in a marked improvement in potency; the IC_{50} of 4,6-diene-Cer was 11.3 μM, whereas that of Cer was 33.9 μM. The same C(4)–C(5)/C(6)-C(7)– trans double bond system was also tested with a phenyl ring at C(7) in place of the long, sphingoid hydrocarbon chain (4,6-diene-7-Ph-Cer). This change, however, reduced the efficacy of this compound at all concentrations tested. Finally, novel analogs were synthesized with the addition of a hydroxyl group at C(6), with either the original double bond of ceramide (6-OH-C8) or a triple bond at C(4)-C(5) (6-OH-4-yne-C8). Interestingly, both compounds were more potent than Cer in reducing MCF-7TN-R viability, with the 6-OH-4-yne-C8 being the more cytotoxic of the two. All analogs were also tested in the MCF-7N variant with similar results (IC_{50} 37.7 μM and 13.7 μM, for Cer and 4,6-diene-Cer, respectively) (data not shown). To more easily evaluate the relative potencies of the ceramide analogs, we present the MCF-7 cell viability after 48-hr treatment with 30 μM

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of the drug in Fig. 5B. As shown in Figure 5B, 4,6-diene-Cer was the most potent at decreasing MCF-7 cell number.

Cer and 4,6-diene-Cer Induce Apoptosis in MCF-7 Cells. Ceramide has been shown to reduce cell number by several mechanisms, including induction of cell death by both apoptotic and non-apoptotic mechanisms (Lopez-Marure et al., 2002; Obeid et al., 1993). We tested the hypothesis that treatment of MCF-7 cells with Cer induced apoptosis in our model system, and that 4,6-diene-Cer initiated cell death in a similar manner. An early event in apoptosis is a loss of membrane asymmetry that results in phosphatidylserine (PS) "flipping" from the inner to the outer leaflet of the cell membrane, an event that may signal to phagocytes to clear dying cells. PS is bound by Annexin V, and therefore recognition of Annexin V binding via a FITC-labeled anti-Annexin V antibody serves as a useful marker of early apoptotic events. Treatment of MCF-7N cells with 30 μM Cer for 24 hr resulted in a 2.9-fold increase in the percentage of Annexin-(+) cells (from 7.1% for the untreated control ((Fig. 6A, B) to 27.8%), while treatment with DH-Cer resulted in no increase in Annexin binding under the same conditions. The treatment with the same concentration of 4,6-diene-Cer resulted in a greater increase (7.6-fold) in cells undergoing early apoptosis (61.2%) over the vehicle-treated control (Fig. 6C). These data suggest that the reductions in MCF-7 cell number shown in Fig. 5 are the result of greater induction of apoptosis by 4,6-diene-Cer. Results with the resistant MCF-7TN-R cell variant were similar (data not shown).

Ceramide Analogs-Induced Apoptosis Occurs Through the Activation of the Mitochondria. Ceramide is reported to utilize the mitochondrial pathway of apoptosis (Birbes et al., 2002) and to depend on release of cytochrome c from the

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mitochondria for the induction of apoptosis. To confirm that Cer-induced apoptosis occurs through mitochondrial activation and to test whether 4,6-diene-Cer induces cell death via this pathway, we used immunoblotting to detect release of cytochrome c from the mitochondria. Treatment of MCF-7N cells with Cer resulted in release of cytochrome c into the cytosol by 8 hr compared with vehicle-treated control (Fig. 7A). Fig. 7B shows that treatment with 4,6-diene-Cer caused similar changes in the mitochondrial permeability of cytochrome c at 8 hr, which continued to increase to 24 hr. Cytosolic fractions from Cer (data not shown) or 4,6-diene-Cer showed no immunoreactivity to anti-cytochrome oxidase II, demonstrating that fractions were free of mitochondrial contamination.

The opening of the permeabilization transition pore complex (PTPC) is thought to mediate release of cytochrome c from the mitochondria. One of the markers of the opening of PTPC is a decrease in mitochondrial membrane potential ($\Delta\Psi_m$). We used the cell-permeant JC-1 dye to monitor changes in $\Delta\Psi_m$ following Cer or 4,6-diene-Cer treatment. In healthy mitochondria with an intact $\Delta\Psi_m$, the JC-1 dye accumulates and forms aggregates with a red fluorescence. As the $\Delta\Psi_m$ is lost, the dye is not actively taken up into the mitochondria and remains as a monomer in the cytosol. This cytosolic, monomeric form is distinguishable from the aggregate form by its green fluorescence. As shown in Fig. 7C, treatment with either 30 μM Cer or 4,6-diene-Cer led to a significant decrease in the mitochondria membrane potential within 8 hr and continued to decrease to 24 hr, demonstrating the involvement of the mitochondria in Cer- and 4,6-diene-Cer-induced apoptosis.

4,6-Diene-Cer Treatment Results in Prolonged Ceramide Generation.

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Exogenous ceramide has been shown to generate endogenous ceramide, leading to speculation that this endogenous ceramide may be responsible for the cellular effects of ceramide treatment (Ogretmen et al., 2001). To determine whether addition of exogenous Cer or 4,6-diene-Cer affected ceramide accumulation, we treated MCF-7 cells with either 30 μM Cer or 4,6-diene-Cer. While treatment with Cer resulted in a pattern of ceramide accumulation that resembled that of TNF-α, ceramide accumulation in response to 4,6-diene-Cer was markedly different (Fig. 8). Cer treatment led to a 4.2-fold increase in ceramide and peaked within 15 min and returned to baseline by 1 hr. After the addition of 4,6-diene-Cer, the ceramide level rose by 3.6-fold over the untreated levels within 15 min. However, unlike TNF-α or Cer, 4,6-diene-Cer caused ceramide levels to continue to rise up to 1 hr and remained significantly elevated even to 24 hr. In comparison, treatment of MCF-7 cells with either 4,6-diene-7-Ph-Cer or 6-OH-Cer did not increase ceramide levels significantly. HPLC was used to confirm the results of the DAG kinase assay. The results correlated well, with continuous elevation of ceramide at times up to 12h (24h was not measured by the HPLC method) (data not shown).

4,6-Diene-Cer Reduces Viability in Other Breast Cancer Cell lines. In order to determine whether the ability of 4,6-diene-Cer to decrease cell proliferation and viability was restricted to the MCF-7 breast cancer cell line, we investigated the effect of 4,6-diene-Cer treatment in two other well-studied breast cancer cell lines with varying phenotypes. The NCI/ADR-RES (formerly MCF-7 ADR (Scudiero et al., 1998)) cell line is a commonly used model of chemoresistant, Pgp-positive, ER-negative breast cancer, while MDA-MB-231 cells are a model of invasive, ER-negative breast cancer. As

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shown in Fig. 9, treatment of either MDA-MB-231 (A) or NCI/ADR-RES (B) cells with Cer or 4,6-diene-Cer resulted in a dose-dependent decrease in viability. Importantly, 4,6-diene-Cer was more potent than Cer in both the MDA-MB-231 and NCI/ADR-RES cell lines at all doses we used, demonstrating that the increased potency of 4,6-diene-Cer is not restricted to the MCF-7 cell model. The IC_{50} values of 4,6-diene-Cer were 3.7 μM and 24.1 μM for the MDA-MB-231 and NCI/ADR-RES cell lines, respectively, compared with 11.3 μM and 86.9 μM, respectively, for Cer.

Ceramide and Ceramide Analogs Are Less Toxic to Normal Breast Epithelial Cells. Ideally, a chemotherapeutic agent should possess a level of selectivity toward tumor cells over their normal, healthy counterparts. Since ceramide and novel ceramide analogs induce apoptosis in MCF-7 breast carcinoma cells, we sought to determine the effect of these compounds in a cell line of breast epithelial cells (hTERT-HME) that are immortalized with the human telomerase gene while retaining a normal epithelial phenotype (Jiang et al., 1999). Treatment of these cells with either Cer or 4,6-diene-Cer did not result in a similar loss of viability as seen in the MCF-7 cell variants (Fig. 10). These cells were more refractory to treatment with either Cer or 4,6-diene-Cer even at concentrations that were quite toxic to MCF-7 cell variants.

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Discussion

This study demonstrates that alterations in the degree and position of unsaturation of bonds in the sphingoid backbone of Cer affect the antiproliferative activity of ceramide analogs in breast cancer cells. The most efficacious analog of Cer we tested is 4,6-diene-Cer, which contains an additional trans double bond at C(6)-C(7) of the sphingoid backbone. 4,6-Diene-Cer exhibited higher cytotoxicity than Cer in all of the breast cancer cell lines we tested (Figs. 5 and 9) and displayed selectivity toward transformed breast cells over non-transformed breast epithelial cells (Fig. 10). Treatment of MCF-7 cells with Cer or 4,6-diene-Cer resulted in induction of apoptosis via the mitochondrial pathway by 8 hr, as demonstrated by the release of cytochrome c, loss of membrane asymmetry (as measured by Annexin V staining), and decrease in the mitochondrial membrane potential (Figs. 6 and 7). Furthermore, 4,6-diene-Cer caused a prolonged elevation of intracellular ceramide levels in MCF-7 cells (Fig. 8), which may contribute to its enhanced cytotoxicity. These data suggest that these and other novel ceramide analogs represent potential therapeutic agents in breast cancer treatment.

We and others have shown that defective ceramide generation following cytotoxic stress leads to increased cellular resistance to such agents; tumor cells are often defective in apoptotic signaling as a result of alterations in the ceramide pathway (Senchenkov et al., 2001). In this study, our TNF-resistant MCF-7TN-R cell variant demonstrated no significant generation of ceramide following TNF-α exposure (Fig. 2), a finding that correlates with other reports regarding ceramide generation and resistance to apoptosis (Cai et al., 1997; Wang et al., 1999). Restoration of ceramide

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signaling (via exogenous ceramide) decreased MCF-7TN-R cell viability in a manner that was nearly identical to its TNF-sensitive counterpart (Fig. 2), demonstrating that the ceramide signaling pathway plays an important role in the apoptotic sensitivity of these cells. This observation supports the hypothesis that ceramide levels are low in resistant tumor cells, and in these types of cells ceramide replacement therapy would provide a significant benefit. This hypothesis is further supported by our finding that the telomerase-transfected breast epithelial cells (hTERT-HME), which retain a normal epithelial phenotype, are not greatly affected by Cer or 4,6-diene-Cer treatment (Fig. 10). This correlates with findings by others that many non-transformed cells are less sensitive to ceramide-induced cell death (Lopez-Marure et al., 2002; Selzner et al., 2001), and suggests that ceramide-based chemotherapy may be selective for tumor cells *in vivo*.

We employed novel ceramide analogs to directly target the cell death machinery in breast cancer cells. Specifically, we have used novel ceramide analogs that are altered with respect to the degree and position of unsaturation of bonds in the sphingoid backbone of D-*erythro*-*N*-octanoyl-ceramide. Others have demonstrated that changes in ceramide's structure can result in increased efficacy of these compounds (Chun et al., 2003; Macchia et al., 2001; Van Overmeire et al., 2000; Wieder et al., 1997). For example, Macchia et al. used DNA fragmentation and release of cytochrome c to analyze the effect of replacing the polar portion of the ceramide molecule with uracil or thiouracil. The compounds produced potent *in vivo* antitumor activity in a model of mouse colon cancer with few systemic side effects, demonstrating the potential of ceramide analogs as a treatment strategy. Here we demonstrate that the double bond

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character of the sphingoid backbone of ceramide plays a key role in determining the ability of our ceramide analogs to induce cell death effectively. We show that 4,6-diene-Cer exhibited higher cytotoxicity than Cer in TNF-α-resistant (IC_{50} 11.3 μM vs 32.9 μM) and TNF-α-sensitive (IC_{50} 13.7 μM vs 37.7 μM) MCF-7 cells treated with the sphingolipids for 48 hr. 4,6-Diene-Cer was also more effective than Cer in inducing cell death in MDA-MB-231 and NCI/ADR-RES breast cancer cell lines (IC_{50} 3.7 μM vs 11.3 μM, and 24.1 μM vs 86.9 μM, respectively).

The double bond at C(4)–C(5) has previously been shown to be important in mediating some of the biological and apoptotic effects of ceramide (Bielawska et al., 1993; He et al., 1999; Karasavvas et al., 1996). This selectivity of location of unsaturation in the sphingoid backbone is supported by our data, since 6-ene-Cer, which has the unsaturation only at C(6)–C(7), was considerable less efficacious than analogs that contained a C(4)–C(5) trans double bond (with the exception of 4,6-diene-7-Ph-Cer), with an IC_{50} value of 43.2 μM. However, the presence of the C(4)–C(5) and C(6)–(C)7 double bonds alone is not sufficient to confer increased efficacy as 4,6-diene-7-Ph-Cer did not induce greater than 55% cell death at concentrations up to 100 μM. The replacement of the long-chain base with a phenyl group may alter the lipophilicity of the compound such that it is not taken up efficiently or inserted into cellular membranes. Van Overmeire et al. (2000) also investigated ceramide analogs with an aromatic ring similar to 4,6-diene-7-Ph-Cer, and found these compounds to be quite effective. However, it is difficult to correlate these results with those in the present study since the biological response measured in that study dealt with overcoming axonal growth inhibition in hippocampal neurons. Interestingly, treatment of MCF-7 cells with

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ceramide analogs containing an additional hydroxyl group at C-6 resulted in a higher cytotoxicity than that found with Cer; the IC_{50} value of 6-OH-Cer was reduced by 27% to 23.7 μM, and introduction of further unsaturation at C(4)–C(5), as in the triple-bond analog 6-OH-4-yne-Cer, yielded a further increase in cytotoxicity (IC_{50} = 13.9 μM, i.e., a 58% decrease vs Cer).

Mitochondria play a central role in the intrinsic pathway of apoptosis. Stimuli that activate this pathway lead to increased permeability of the outer mitochondrial membrane and decreased transmembrane potential, release of cytochrome c, and production of reactive oxygen species (ROS), which ultimately lead to activation of downstream effector caspases (Kaufmann and Earnshaw, 2000). Both endogenously derived or exogenously added ceramide can cause generation of free radicals and hydrogen peroxide at the mitochondria, disruption of mitochondrial membrane potential (Garcia-Ruiz et al., 1997; Gudz et al., 1997; Quillet-Mary et al., 1997), and release of cytochrome c. Here we demonstrate that treatment with both Cer and 4,6-diene-Cer results in a decrease mitochondrial membrane potential ($\Delta\Psi_m$) by 2 hr, which is significant by 8 hr, and continues to decrease to 24 hr (Fig. 7C). The timing of the significant decrease in $\Delta\Psi_m$ at 8 hr coincides with the first observable release of cytochrome c from the mitochondria into the cytosol and suggests that dissipation of $\Delta\Psi_m$ promotes the release of cytochrome c. Release of cytochrome c was followed by further signs of apoptosis execution as measured by loss of membrane symmetry (Annexin V staining). Treatment of MCF-7 cells with 4,6-diene-Cer resulted in more than twice the level of Annexin V staining (Fig. 6) compared with that found with Cer, which correlates with the increased loss of cell viability.

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An interesting observation that also requires further investigation is the pronounced ability of 4,6-diene-Cer to increase intracellular ceramide levels for up to 24 hr. We hypothesize that this leads to a continuous stimulation of ceramide-mediated death signaling, rather than the short burst seen with Cer treatment. The altered structure of 4,6-diene-Cer may allow it to differentially activate or inhibit enzymes in the ceramide metabolic pathway (Bielawska et al., 1996; Selzner et al., 2001).

In conclusion, our results demonstrate that novel ceramide analogs may represent chemotherapeutic agents capable of overcoming apoptotic-resistance mechanisms in breast cancer. We demonstrate that manipulation of the ceramide structure can increase the ability of these agents to target chemoresistance and apoptosis machinery in tumor cells. While the use of any chemotherapeutic agent can be limited when used alone, deliberate pharmacological manipulation of ceramide levels via ceramide analogs such as those described here may lead to an effective therapeutic approach to treating breast cancer.

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Please send reprint requests to: Barbara S. Beckman, Ph.D.

Professor

Department of Pharmacology

Tulane University Health Science Center

1430 Tulane Avenue, SL-80

New Orleans, LA 70112

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Legends for Figures

Fig. 1. The MCF-7TN-R breast cancer cell variant is resistant to the cytotoxic effects of TNF-α. Cultured MCF-7TN-R (closed circles, ●) or MCF-7N (open circles, ○) breast cancer cell variants were treated with increasing doses of TNF. After 48 hr, cell viability was estimated using the MTT assay. Data are presented as percent viability of vehicle-treated control cells. Mean values ± S.E. of four different experiments in triplicate are reported.

Fig. 2. Resistant MCF-7 cells do not accumulate significant levels of ceramide following TNF-α treatment. MCF-7TN-R or MCF-7N cell variants were exposed to 10 ng/ml TNF-α for the indicated times. Cells were fixed in ice-cold methanol, harvested, and ceramide levels were measured. Changes in ceramide levels are expressed as fold change over vehicle-treated control (normalized to one). ●, MCF-7TN-R; ○, MCF-7N. Mean values ± S.E. are reported.

Fig 3. Effect of Cer treatment is similar in MCF-7TN-R and MCF-7N cells. MCF-7 cells variants were incubated with the indicated doses of Cer for 48 hr. Following incubation, cell viability was estimated using the MTT assay. Data are presented as percent viability of vehicle-treated control cells. ●, MCF-7TN-R; ○, MCF-7N. Mean values ± S.E. of four different experiments in triplicate are reported.

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Fig 4. Structures of D-*erythro*-C8-ceramide (Cer), D-*erythro*-4,5-dihydro-C8-ceramide (DH-Cer), and the D-*erythro*-C8 ceramides in which the degree of saturation in the long-chain base was modified.

Fig 5. 4,6-Diene-Cer is highly effective at inducing MCF-7 cell death. (A) Effect of novel analogs on cell viability. MCF-7TN-R variant was treated with Cer, DH-Cer, or ceramide analogs for 48 hr. Following incubation, cell viability was estimated using the MTT assay. Data are presented as mean from at least four experiments and are expressed as percent viability of vehicle-treated control cells. (B) Comparison of novel analogs at the IC_{50} of Cer (30 μM). The percent viability of each analog at 30 μM after 48 hr., the IC_{50} of Cer, is shown. Data are presented as mean ± SEM of at least four experiments performed in triplicate. *, $p < 0.05$ compared with Cer-treated MCF-7 cells. Note: S.E. is not shown in (A) for simplicity, but is shown for (B).

Fig 6. 4,6-Diene-Cer induces apoptosis in MCF-7 cells. MCF-7N cells were treated with either vehicle (A), Cer (B), or 4,6-diene-Cer (C) for 24 hr at 30 μM. Cells were analyzed by flow cytometry with Annexin V and propidium iodide to determine the percent apoptosis. Similar results were obtained for three experiments. (D) Average percentage of cells in viable and early apoptotic populations. Data are presented as mean ± S.E. from three independent experiments. *, $p < 0.05$; **, $p < 0.01$

Fig 7. 4,6-Diene-Cer causes mitochondrial depolarization and release of cytochrome c. (A) MCF-7 cells were treated with Cer or (B) 4,6-diene-Cer (30 μM) for 8,16,or 24 hr.

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Cells were fractionated using centrifugation and cytosolic proteins were analyzed by immunoblotting with anti-cytochrome c, anti-cytochrome c oxidase II, or β-actin. Cytochrome c oxidase serves as a marker for mitochondrial contamination of cytosolic fractions. Purified cytochrome c was used as a positive control for cytochrome c staining, a whole cell lysate was used as a (+) control for cytochrome oxidase, and anti-β-actin was used as a control for protein loading. Data shown are representative results from three separate experiments. (C) MCF-7 cells were treated with DH-Cer, Cer, 4,6-diene-Cer (30 μM) for the indicated times. Change in mitochondrial membrane potential was estimated using a ratio of JC-1 fluorescence at 590 nm (green) and 530 nm (red) as described in Methods. Data are presented as mean ± S.E. of three experiments performed in quadruplicate.

Fig. 8. 4,6-Diene-Cer treatment results in prolonged elevation of intracellular ceramide. MCF-7 cells were treated with ceramide analogs (30 μM) for the indicated times. Cells were fixed in ice-cold methanol, harvested, and ceramide levels were measured. Changes in ceramide levels are expressed as fold change over vehicle-treated control. Mean values ± S.E. are reported. *, $p < 0.05$; **, $p < 0.01$; #, $p < 0.001$

Fig 9. 4,6-Diene-Cer is effective in inducing apoptosis in other breast cancer cell lines. (A) MDA-MB-231 or (B) NCI/ADR-RES breast cancer cells (formerly known as MCF-7/ADR) were treated with Cer or 4,6-diene-Cer for 24 hr. Following incubation, cell viability was estimated using the MTT assay. Data are presented as percent viability of

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vehicle-treated control cells. □, DH-Cer; ●, Cer; ○, 4,6-diene-Cer. Mean values ± S.E. of three different experiments in triplicate are reported.

Fig 10. Normal breast epithelial cells are more refractory to ceramide analog treatment. Human mammary epithelial cells were treated with Cer or 4,6-diene-Cer for 24 hr. Following incubation, cell viability was estimated using the MTT assay. Data are presented as percent viability of vehicle-treated control cells. Mean values ± S.E. of three different experiments in triplicate are reported. (Note: The dose-response curves of Cer and 4,6-diene-Cer in MCF-7 cells are shown for comparison.)

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Fig. 1

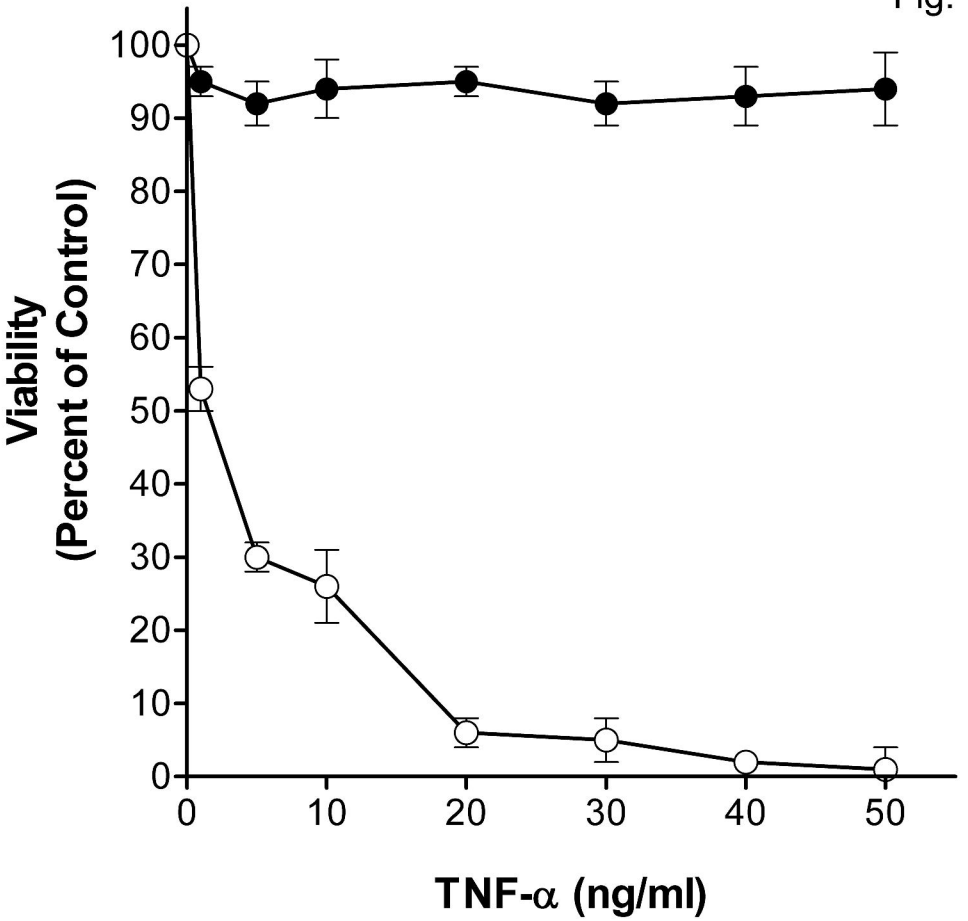

Viability
(Percent of Control)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
TNF-α (ng/ml)

Fig. 2

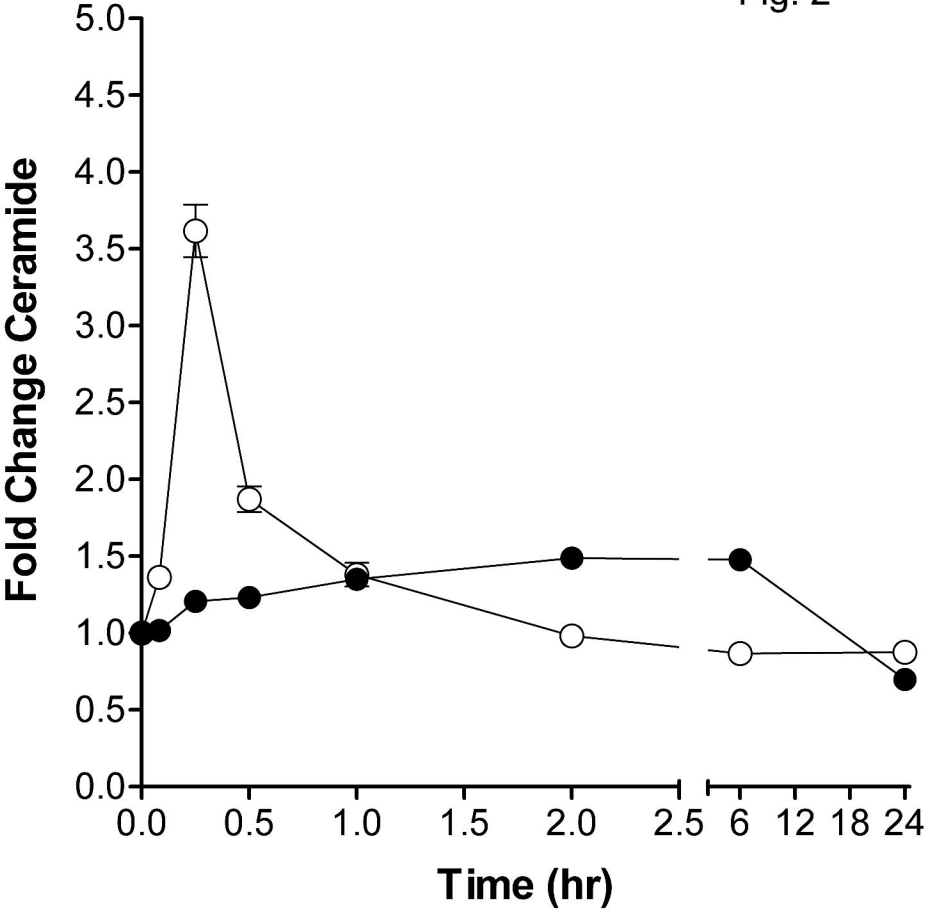

Fold Change Ceramide
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
2.5
6
12
18
24
Time (hr)

Fig. 3

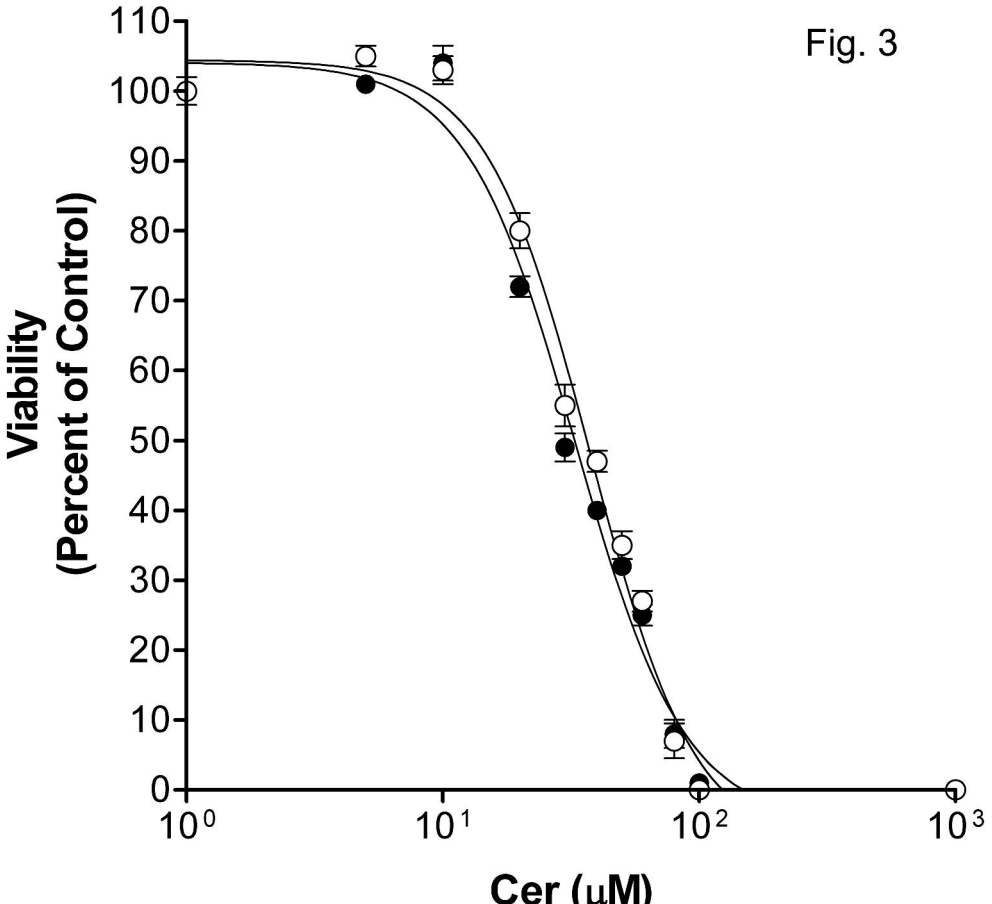
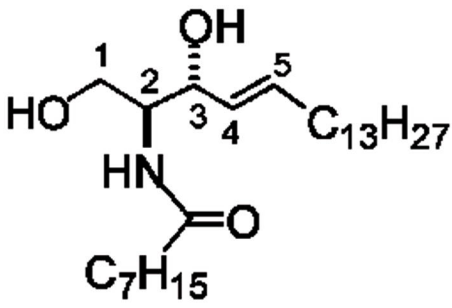
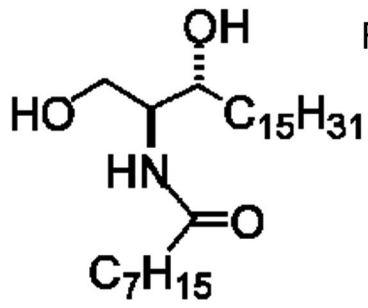

Viability
(Percent of Control)
110
100
90
80
70
60
50
40
30
20
10
0
10^0
10^1
10^2
10^3
Cer (μM)

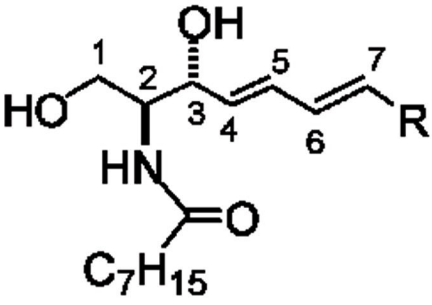
Fig. 4



Cer

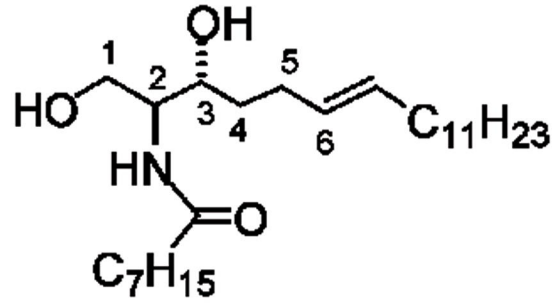


DH-Cer

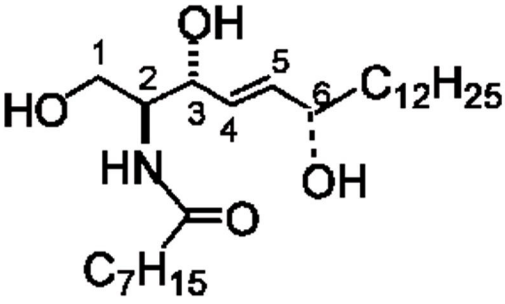


4,6-diene-Cer (R = $C_{11}H_{23}$)

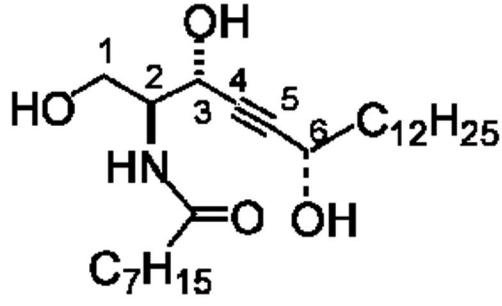
4,6-diene-7-Ph-Cer (R = Ph)



6-ene-Cer



6-OH-Cer



6-OH-4-yne-Cer

Fig. 5

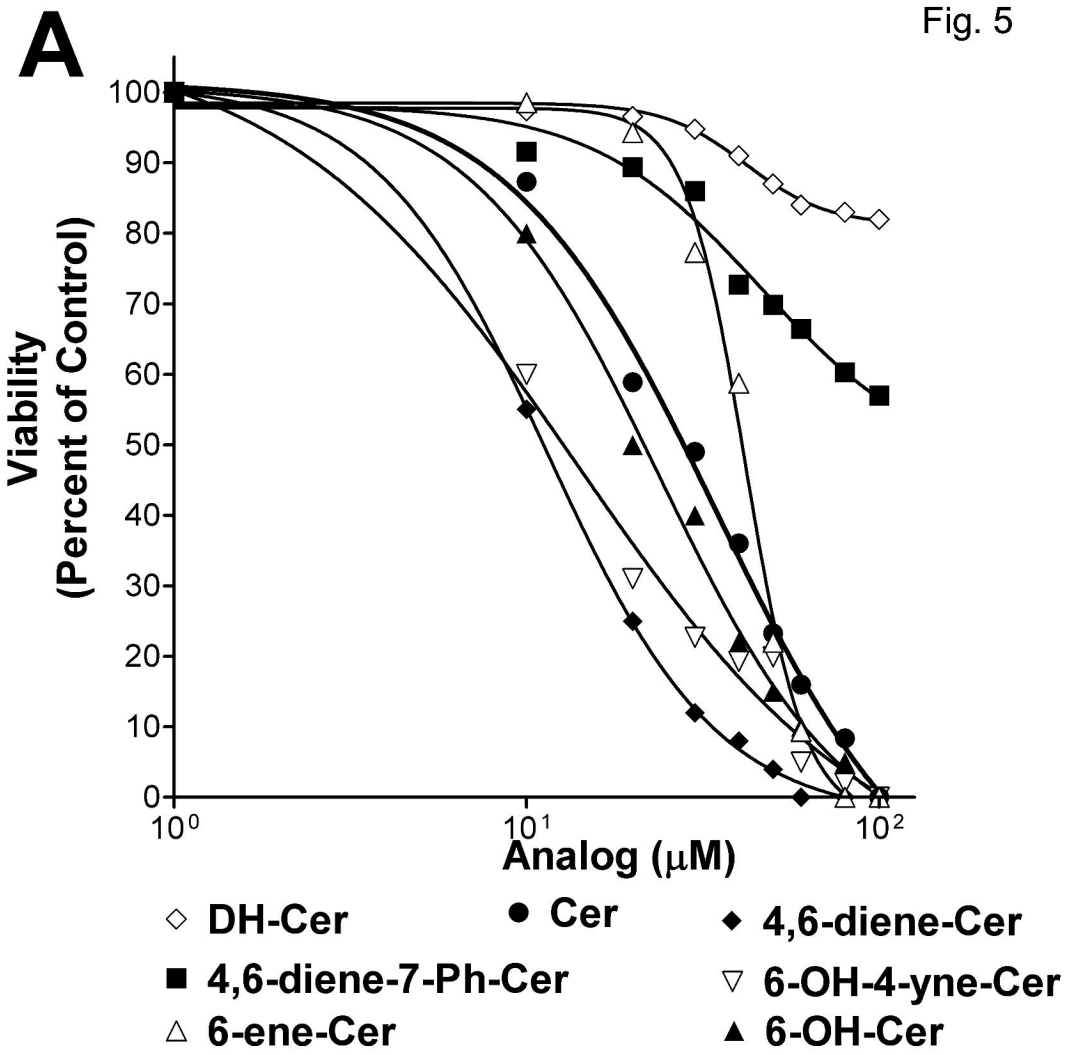

A
Viability
(Percent of Control)
100
90
80
70
60
50
40
30
20
10
0
10^0
10^1
10^2
Analog (μM)
DH-Cer
Cer
4,6-diene-Cer
4,6-diene-7-Ph-Cer
6-OH-4-yne-Cer
6-ene-Cer
6-OH-Cer

Fig. 5B

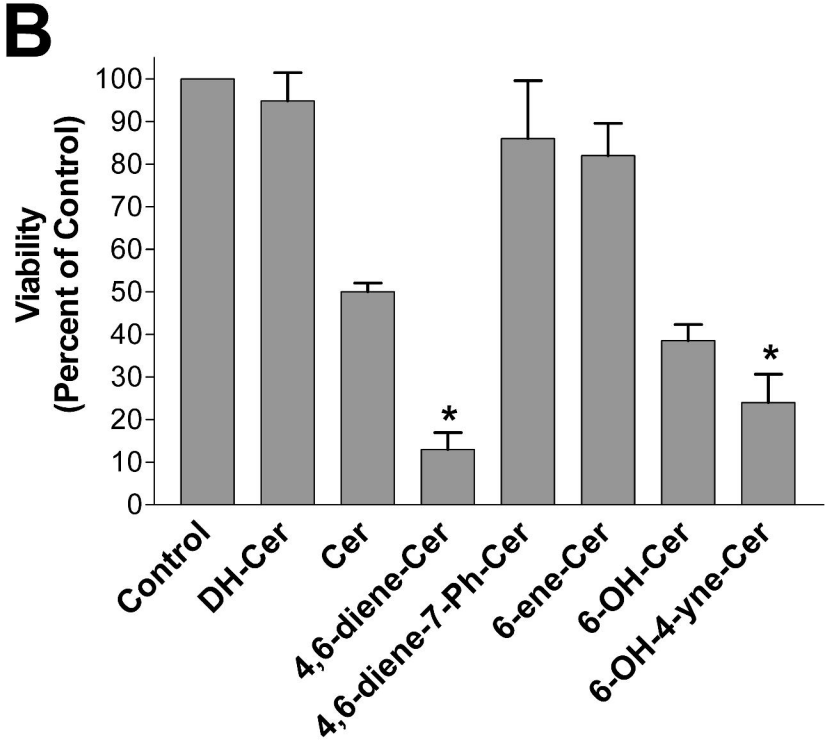

B
Viability
(Percent of Control)
100
90
80
70
60
50
40
30
20
10
0
*
*
Control
DH-Cer
Cer
4,6-diene-Cer
4,6-diene-7-Ph-Cer
6-ene-Cer
6-OH-Cer
6-OH-4-yne-Cer

Fig. 6A-D

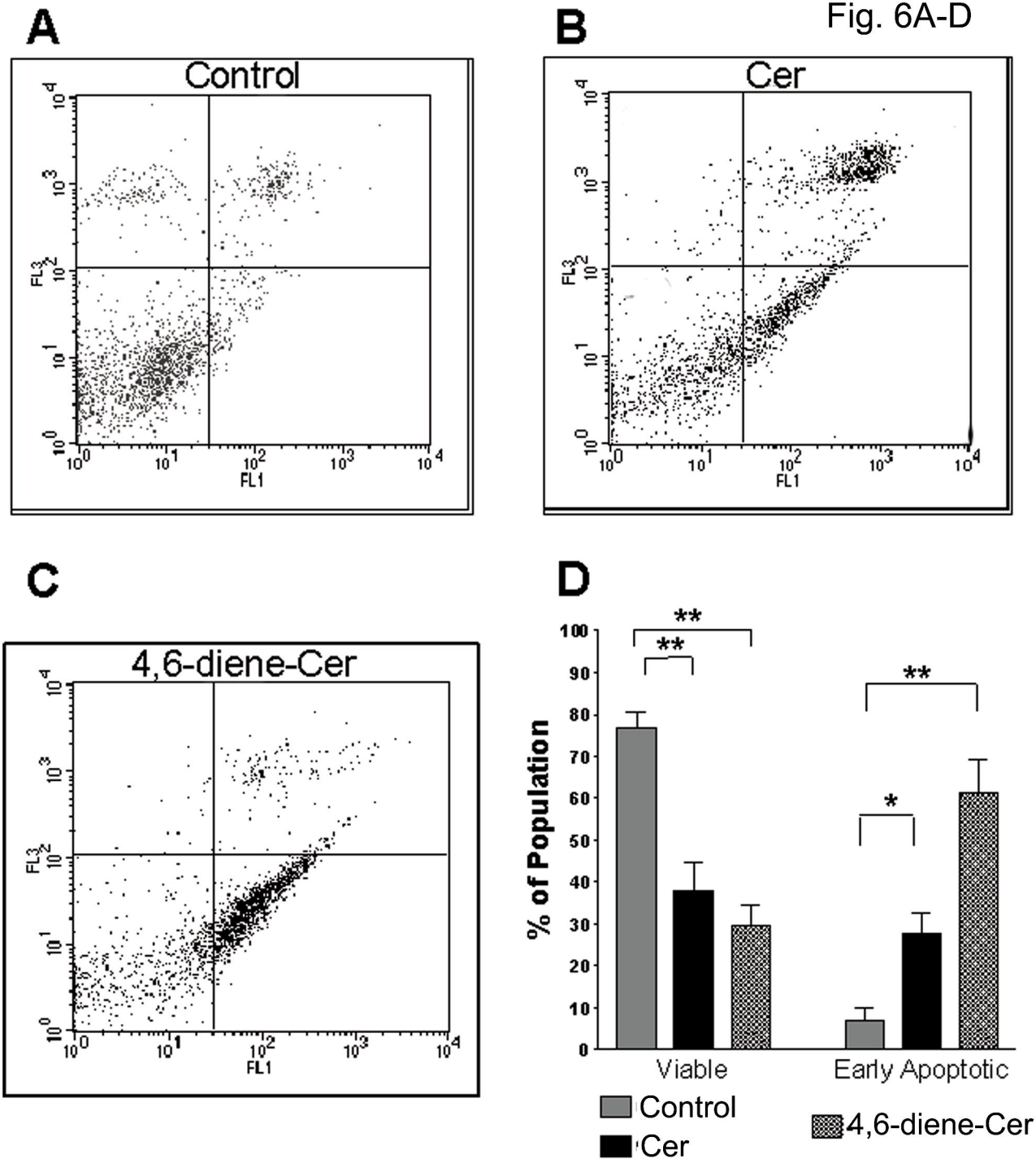

A
Control
B
Cer
C
4,6-diene-Cer
D
% of Population
Viable
Early Apoptotic
Control
Cer
4,6-diene-Cer
FL1
FL3

Fig. 7A,B

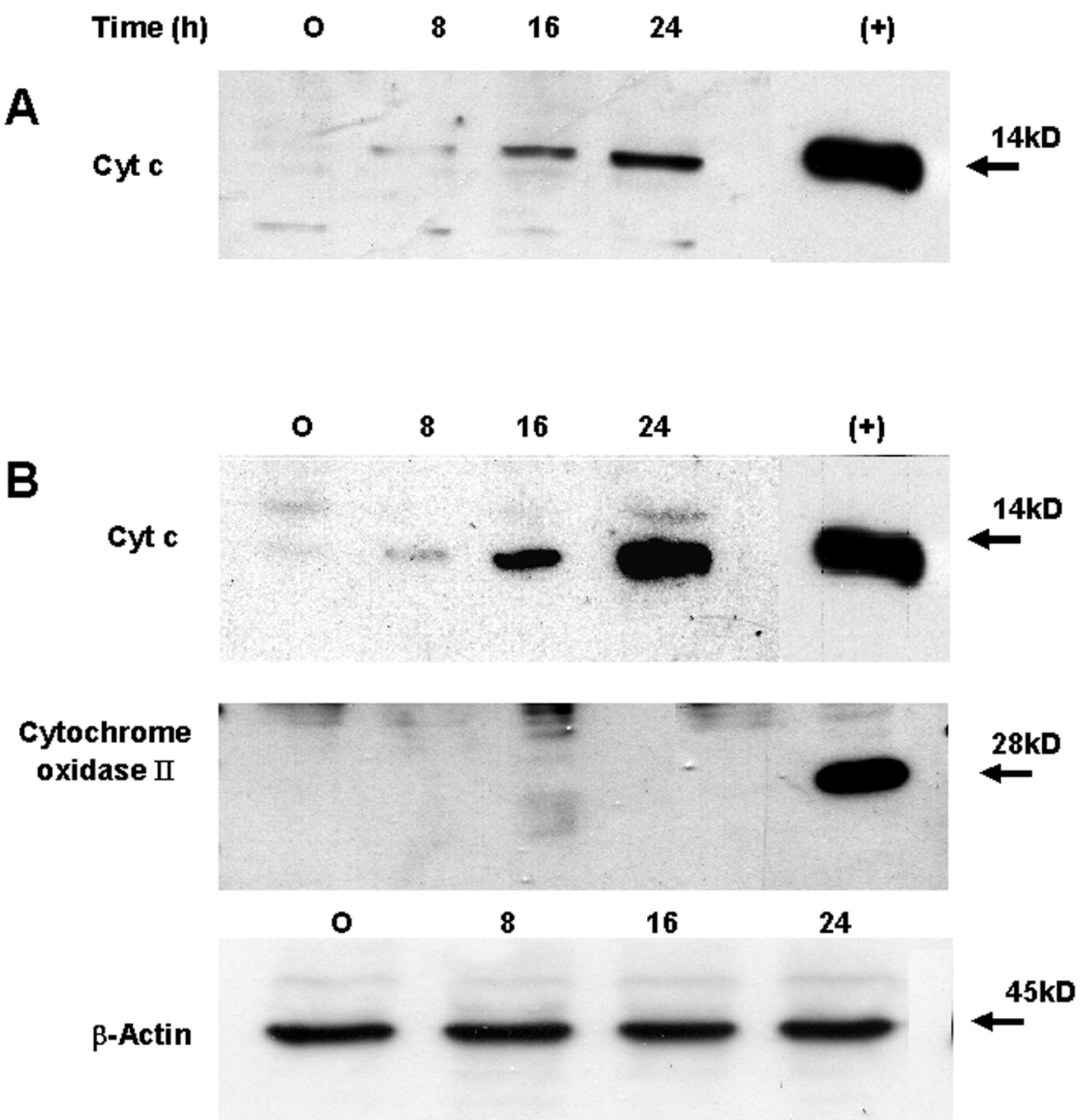

Time (h)
O
8
16
24
(+)
A
Cyt c
14kD
O
8
16
24
(+)
B
Cyt c
14kD
Cytochrome oxidase Ⅱ
28kD
O
8
16
24
β-Actin
45kD

Fig. 7C

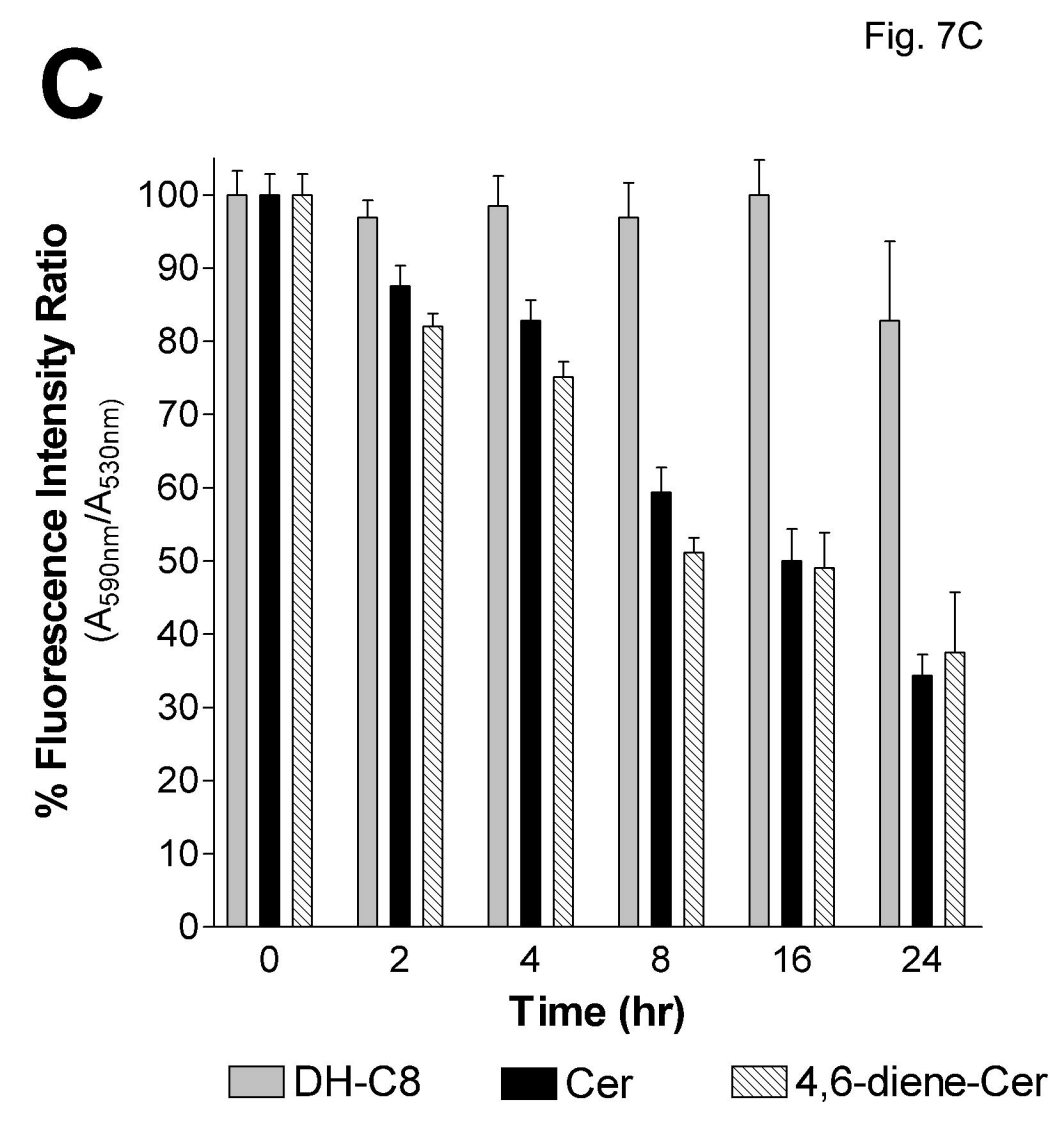

C
% Fluorescence Intensity Ratio
(A_{590nm}/A_{530nm})
100
90
80
70
60
50
40
30
20
10
0
0
2
4
8
16
24
Time (hr)
DH-C8
Cer
4,6-diene-Cer

Fig. 8

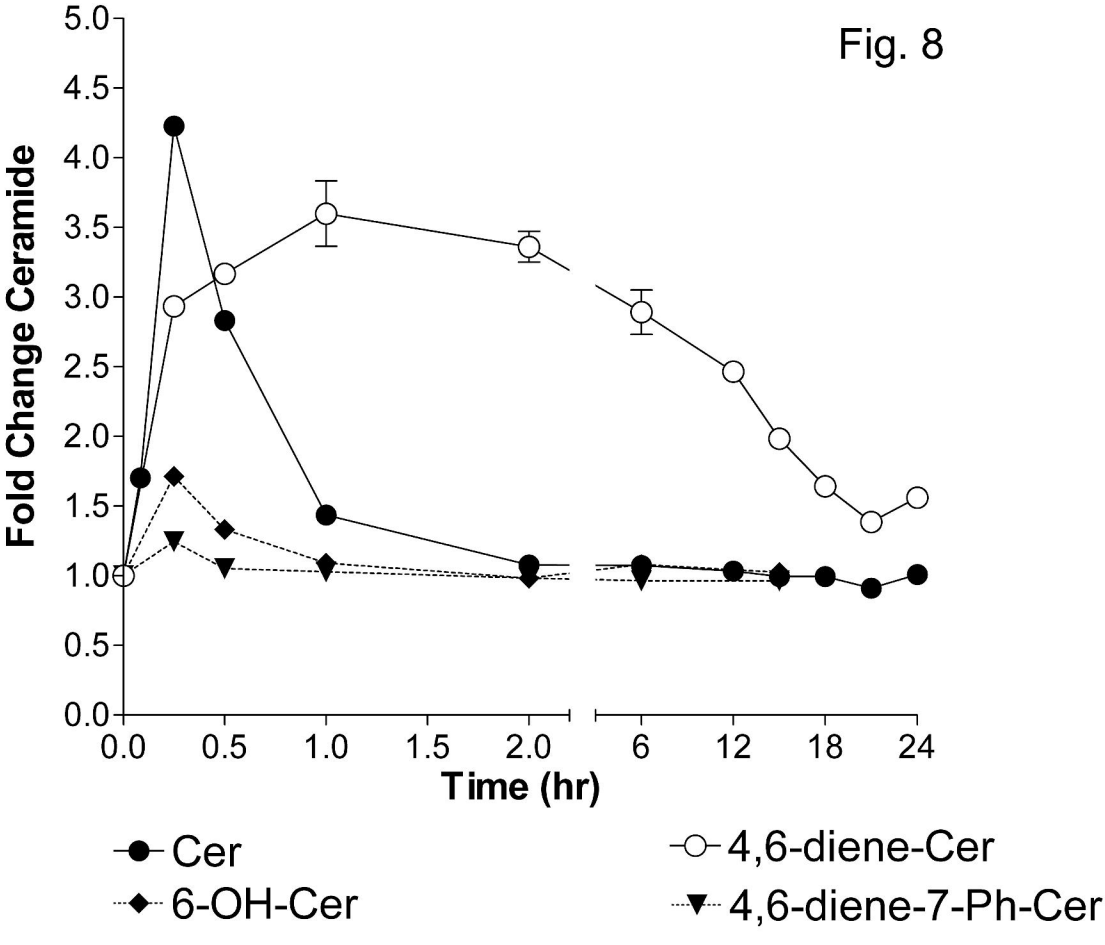

Fold Change Ceramide
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
6
12
18
24
Time (hr)
Cer
4,6-diene-Cer
6-OH-Cer
4,6-diene-7-Ph-Cer

Fig. 9A

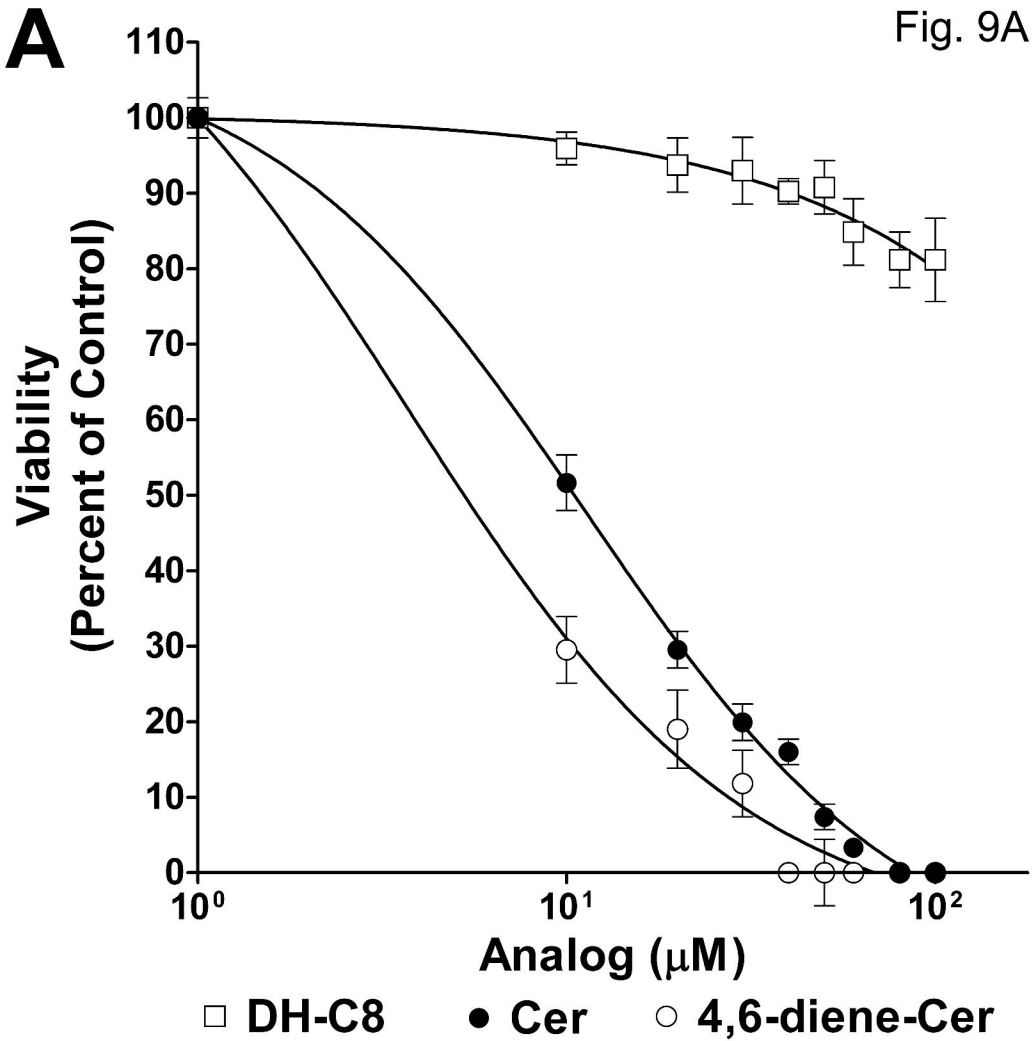

A
110
100
90
80
70
60
50
40
30
20
10
0
Viability
(Percent of Control)
10⁰
10¹
10²
Analog (μM)
□ DH-C8
● Cer
○ 4,6-diene-Cer

Fig. 9B

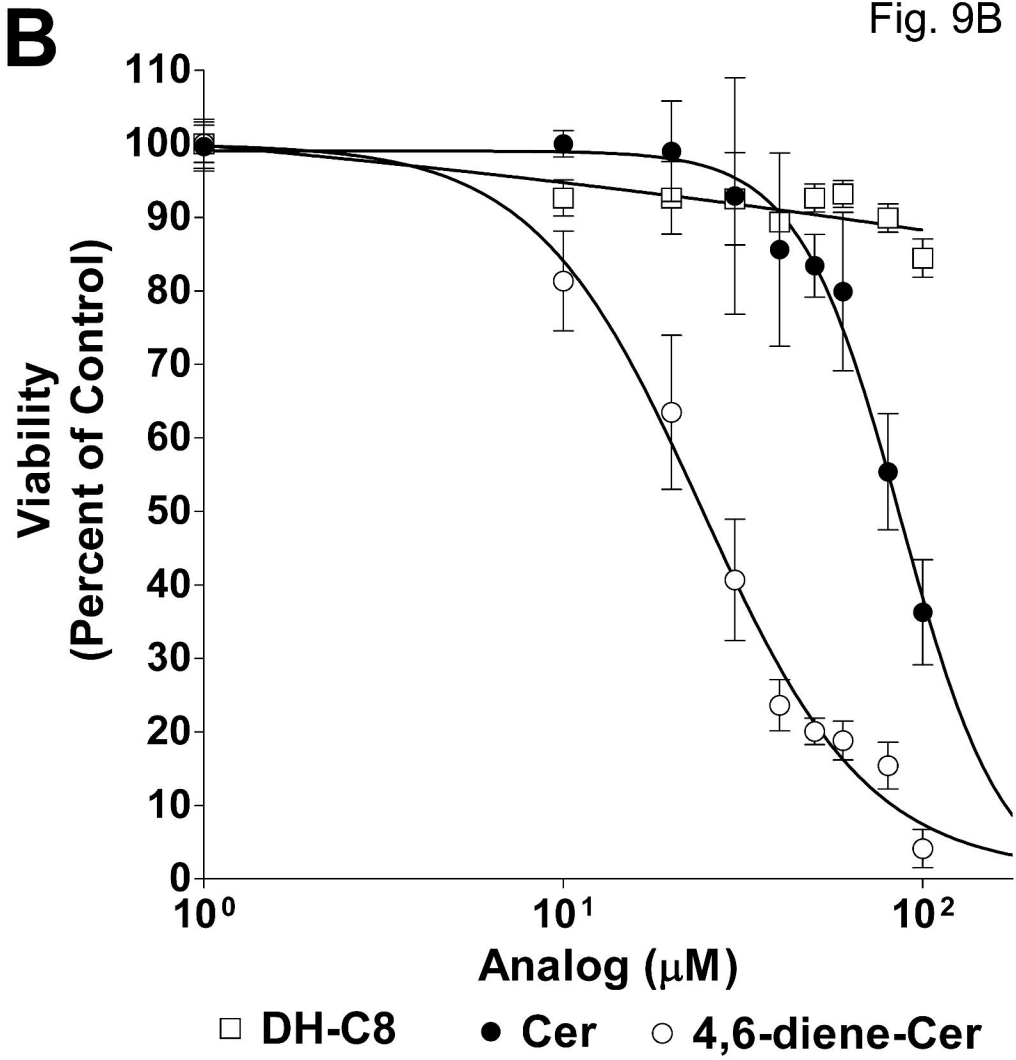
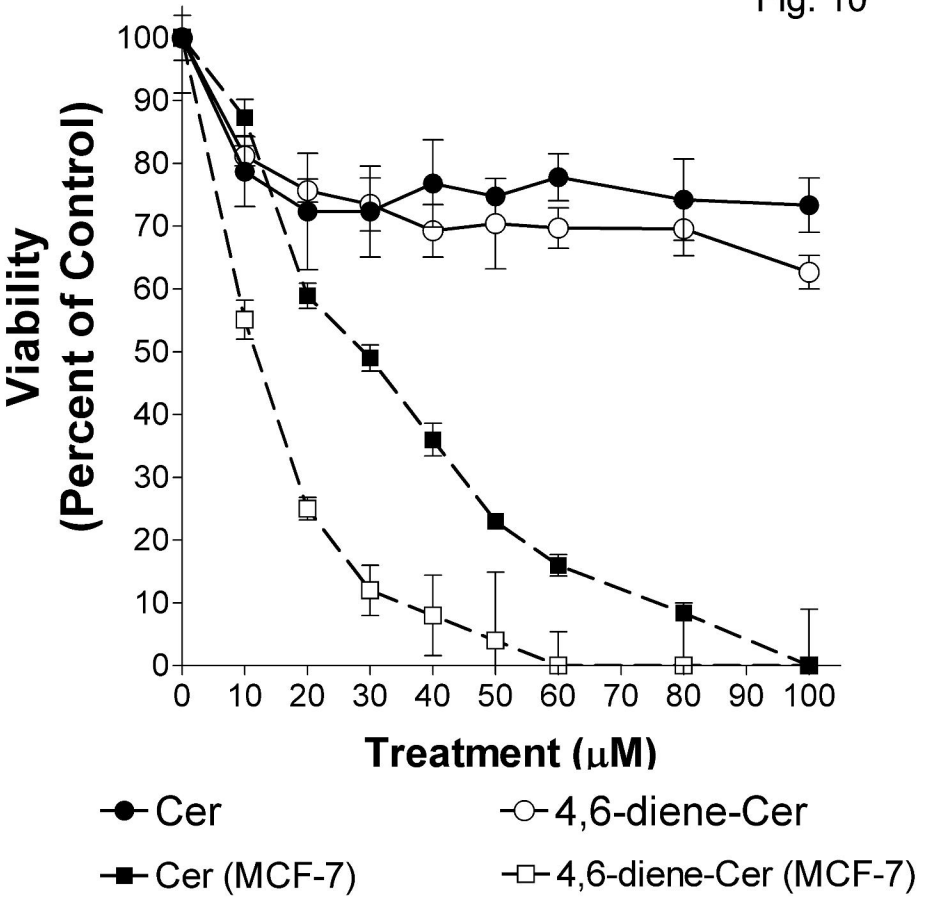

B
Viability
(Percent of Control)
110
100
90
80
70
60
50
40
30
20
10
0
10⁰
10¹
10²
Analog (μM)
□ DH-C8
● Cer
○ 4,6-diene-Cer

Fig. 10


Viability
(Percent of Control)
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
Treatment (μM)
Cer
4,6-diene-Cer
Cer (MCF-7)
4,6-diene-Cer (MCF-7)