Novel Ceramide Analogs as Potential Chemotherapeutic Agents in Breast Cancer

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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 ω-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 (2S,3R)-(4E,6E)-2-octanoylamidoctadecadiene-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 tumor necrosis factor (TNF)-α-resistant (IC50 of 11.3 versus 32.9 μM) and TNF-α-sensitive (IC50 of 13.7 versus 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 (IC50 of 3.7 versus 11.3 μM, and 24.1 versus 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 h 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 nontransformed breast epithelial cells. These data suggest that these and other novel ceramide analogs represent potential therapeutic agents in breast cancer treatment.

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 antitumor 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 nonapoptotic cell death (Obeid et al., 1993; Lopez-Marure et al., 2002). In tumor cells, chemotherapeutic drugs such as doxorubicin, vincristine, etoposide, and paclitaxel (Senchenkov et al., 2001), as well radiotherapy, increase intracellular ceramide levels after treatment (Haimovitz-Friedman, 1998). In most cases, this treatment-induced rise in ceramide is critical for response to these treatments, and pharmacological agents that affect production or accumulation of ceramide can alter the response to chemotherapy (Olshefski and Ladisch, 2001; Litvak et al., 2003).

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 one-half the level of ceramide 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 after irradiation (Chmura et al., 1997; Michael et al., 1997), and a number of multidrug 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 tumor cells is not the only 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 with normal mammary epithelial cells. Because ceramide has been shown to be involved in tumor sensitivity to apoptosis and chemoresistance, we suggest that development of ceramide analogs with increased antitumor activity represents a potential new class of chemotherapeutic agents.

Materials and Methods

Reagents. D-erythro-C8-Ceramide (Cer) and C8-dihydroceramide (DH-Cer) were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). To achieve cell permeability, all of the ceramide analogs used in the present study have an N-octanoyl chain. The syntheses of (2S,3R,4E,6E)-2-octanoylamidoctadecane-1,3-diol (4,6-diene-Cer) and (2S,3R,4E,6E)-2-octanoylamidoctadecene-1,3-diol (6-ene-Cer) have been described previously (Chun et al., 2002). The syntheses of (2S,3R,6S)-4E,6E-2-octanoylamidoctadecene-1,3,6-triol (4-OH-Cer) and (2S,3R,6S)-2-octanoylamidoctadecene-1,3,4E,6(E)-2-octanoylamidoctadecene-1,3,6-triol (6-OH-4-yne-Cer) have also been described previously (Chun et al., 2003a). (2S,3R,4E,6E)-2-Octanoylamido-7-phenylheptadiene-1,3-diol (4,6-diene-7-Ph-Cer) was prepared by using a synthetic strategy similar to that used 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’s medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, basal medium Eagle amino acids, minimal essential medium amino acids, sodium pyruvate, and penicillin-streptomycin (Invitrogen). 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). 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 Biosciences Clontech (Palo Alto, CA) and were cultured in mammary epithelial cell medium in Cambrex, San Diego, CA) supplemented with bovine pituitary extract.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide (MTT) Viability Assay. MCF-7 cell variants or HME cells were seeded at 7.5 × 10⁴ cells per 96-well plate in phenol-free DMEM (or mammary epithelial cell medium for HME) supplemented with 10% fetal bovine serum and allowed to adhere overnight. Cells were treated with the indicated concentrations of TNF-α or ceramide analog for 24 to 48 h. Twenty-five microliters of MTT dye (5 mg/ml) was incubated in each well for 4 h. Cells were lysed with 20% SDS in 50% dimethylformamide. The pH and absorbances were read on an ELx808 Microtek plate reader (Bio-Tek Instruments, Winooski, VT) at 550 nm, with a reference wavelength of 630 nm. Unless otherwise indicated, cell viability is expressed as a percentage 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 × 10⁵ cells in 25-cm² 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-Aldrich, St. Louis, MO), and sonicated for 30 s. After centrifugation at 12,000g 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, and 0.01% bromphenol blue), boiled for 3 min, and electrophoresed on 12 to 15% polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), which was blocked with PBS-Tween (0.05%-0.5% low-fat dry milk solution at room temperature for 1 h. The membrane was subsequently probed with polyclonal antibodies raised against cytochrome c (1:500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), cytochrome c oxidase (1:1000 dilution; Molecular Probes, Eugene, OR). After incubation at 4°C overnight, blots were washed three times in PBS-Tween (0.05% solution) and incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (1:10,000 dilution; Oxford, Oxford, MA) for 2 h at room temperature. After four washes with PBS-Tween solution, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham Biosciences Inc., Piscataway, NJ) and recorded by fluorography on Hyperfilm (Amersham Biosciences Inc.,) according to the manufacturer’s instructions.

Annexin V 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 h, harvested, and pelleted by centrifugation. Cell pellets were washed once in ice-cold PBS, resuspended in Annexin binding buffer (~1 × 10⁶ 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 BD Biosciences FACStar flow cy-
in phenol-free DMEM supplemented with 10% fetal calf serum and pelleted by centrifugation at 500g 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 MgCl₂, 1 mM EDTA, 1 mM EGTA, 10 mM benzamidine, 1 mM dithiothreitol, 250 mM sucrose, and protease and phosphatase inhibitor cocktails; Sigma-Aldrich), and then lysed by passing the suspension through a 25-gauge needle 15 times. Homogenates were centrifuged at 500g for 5 min at 4°C. Supernatants were further centrifuged at 10,000g for 30 min at 4°C, and the resultant mitochondria-rich pellets were resuspended in buffer A and sonicated. The supernatant from the 10,000g spin was designated as cytosol. Protein concentrations were determined by the Bradford reagent assay (Bio-Rad). Subcellular fractionations were subjected to immunoblots as described above. Experiments were performed three to five times, and the results of a typical experiment are shown.

Measurement of Mitochondrial Membrane Potential (ΔΨm). MCF-7 cells were seeded at 7.5 × 10⁴ 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 μM of ceramide analog for indicated times. After incubation, the medium was removed, 2.5 μM 5,5′,6,6′-tetracloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Chemicon International, Temecula, CA) was added to each well, and plates were incubated at 37°C for 10 min. After incubation, JC-1 dye was removed and the cells were carefully washed with 1× incubation buffer, followed by addition of PBS. Emitted fluorescence was measured on an FLA6000 fluorometer (Bio-Tek Instruments) 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 percentage of the vehicle-treated control (considered 100%).

Analysis of Cellular Ceramide Levels. Ceramide was quantified by the dicycglycerol (DAG) kinase assay as [32P]incorporated into ceramide 1-phosphate, or by HPLC, as described previously (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 nitrogen stream. The lipids contained in the organic phase were re-suspended in 20 μl of 7.5% n-octyl β-glucopyranoside, 5 mM calcium, 1 mM diethylenetriaminepentaacetic acid (Sigma-Aldrich), 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 to ensure the total conversion of the substrate to the phosphorylated product. Fifty microliters of buffer containing [γ-32P]ATP (30 mM; 1000 dcpm/μmol) were added to start the reaction. After 30 min at 22°C, the reaction was stopped by extraction of lipids with 1 ml of chloroform/methanol/hydrochloric acid (100:100:1, v/v/v) and washed with 135 mM NaCl, 1.5 mM MgCl₂, 0.5 mM CaCl₂, 5.6 mM glucose, and 10 mM HEPES, pH 7.2). The pellet was washed with methylene chloride (100:100:1, v/v/v) and detected by autoradiography (Rf fraction between 0.5 and 0.6), and the incorporated 32P 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), and Cer was used as a positive standard of DAG kinase activity.

Alternatively, ceramide levels were measured by HPLC. A nonenzymatic method based on deacylation of ceramide to the corresponding sphingoid base and derivatization with o-phthalaldehyde (Merill et al., 1988) as modified by 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 Cer 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).

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 published previously by our laboratory (Burrow et al., 1998). MCF-7-TN-TR cells are isogenic variants of MCF-7 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-7 cells where treatment with only 1 ng/ml TNF-α resulted in a 50% reduction in cell number, and 10 ng/ml reduced cell viability to less than 25%. Resistance to TNF-induced cell death is not a result of a lack of TNF-α receptors because treatment with TNF-α is still able to activate intracellular signaling pathways, as measured by a nuclear factor-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.

Fig. 1. The MCF-7TN-R breast cancer cell variant is resistant to the cytotoxic effects of TNF-α. Cultured MCF-7TN-R (○) or MCF-7N (□) breast cancer cell variants were treated with increasing doses of TNF. After 48 h, cell viability was estimated using the MTT assay. Data are presented as percentage of viability of vehicle-treated control cells. Mean values ± S.E. of four different experiments in triplicate are reported.
TNF-α-Resistant MCF-7 Cells Do Not Generate Ceramide after 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 on 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 min, with a return to basal levels by 2 h (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 TNF-α.

Cell Death Resistance Mechanisms Are Overcome by Exogenous Ceramide Treatment. Because 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 and 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 h (Fig. 3), with an IC50 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. Because exogenous ceramide addition bypassed chemoresistance mechanisms in the MCF-7 breast cancer variants, we next determined whether the ceramide structure could be altered to 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 dihydrosphingosine-ceramide; 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 of the ceramide sphingoid backbone 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 IC50 of 4,6-diene-Cer was 11.3 μM, whereas that of Cer was 32.9 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 (IC50 of 37.7 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-h treatment with 30 \( \mu \text{M} \) of the drug in Fig. 5B. As shown in Fig. 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 nonapoptotic mechanisms (Obeid et al., 1993; Lopez-Marure et al., 2002). 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 “flipping” from the inner to the outer leaflet of the cell membrane, an event that may signal to phagocytes to clear dying cells. Phosphatidylserine is bound by Annexin V, and therefore recognition of Annexin V binding via a FITC-labeled Annexin V serves as a useful marker of early apoptotic events. Treatment of MCF-7N cells with 30 \( \mu \text{M} \) Cer for 24 h resulted in a 2.9-fold increase in the percentage of Annexin- (+) cells (from 7.1% for the untreated control; Fig. 6, A and B) to 27.8%, whereas 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 Analog-Induced Apoptosis Occurs through the Activation of the Mitochondria. Ceramide is reported to use the mitochondrial pathway of apoptosis (Birbes et al., 2002) and to depend on release of cytochrome c from the 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 cyto-
into the cytosol by 8 h compared with vehicle-treated control (Fig. 7A). Figure 7B shows that treatment with 4,6-diene-Cer caused similar changes in the mitochondrial permeability of cytochrome c at 8 h, which continued to increase to 24 h. 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 is thought to mediate release of cytochrome c from the mitochondria. One of the markers of the opening of permeabilization transition pore complex is a decrease in ΔΨ<sub>m</sub>. We used the cell-permeant JC-1 dye to monitor changes in ΔΨ<sub>m</sub> after Cer or 4,6-diene-Cer treatment. In healthy mitochondria with an intact ΔΨ<sub>m</sub>, the JC-1 dye accumulates and forms aggregates with a red fluorescence. As the ΔΨ<sub>m</sub> 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 h and continued to decrease to 24 h, 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. Exogenous ceramide has been shown to

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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 h at 30 μM. Cells were analyzed by flow cytometry with Annexin V and propidium iodide to determine the percentage of 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.
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. Although 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 h. 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 h and remain significantly elevated even to 24 h. 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 12 h (24 h was not measured by the HPLC method) (data not shown).

4,6-Diene-Cer Reduces Viability in Other Breast Cancer Cell Lines. 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, P-glycoprotein-positive, estrogen receptor-negative breast cancer, whereas MDA-MB-231 cells are a model of invasive, estrogen receptor-negative breast cancer. As 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 IC50 values of 4,6-diene-Cer were 3.7 and 24.1 μM for the MDA-MB-231 and NCI/ADR-RES cell lines, respectively, compared with 11.3 and 86.9 μM, respectively, for Cer.

Ceramide and Ceramide Analogs Are Less Toxic to Normal Breast Epithelial Cells. Ideally, a chemothera-
A therapeutic agent should possess a level of selectivity toward tumor cells over their normal, healthy counterparts. Because 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 (formerly known as MCF-7/ADR) 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 very toxic to MCF-7 cell variants.

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 h, 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 after 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-7/TN-R cell variant demonstrated no significant generation of ceramide after 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 signaling (via exogenous ceramide) decreased MCF-7/TN-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 nontransformed cells are less sensitive to ceramide-induced cell death (Selzner et al., 2001; Lopez-Marure et al., 2002) and suggests that ceramide-based chemotherapy may be selective for tumor cells in vivo.

We used 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 n-erythro-N-octanoyl-ceramide. Others have demonstrated that changes in ceramide's structure can result in increased efficacy of these compounds (Wieder et al., 1997; Van Overmeire et al., 2000; Macchia et al., 2001; Chun et al., 2003b). For example, Macchia et al. (2001) 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 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 (IC50 of 11.3 versus 32.9 μM) and TNF-α-sensitive (IC50 of 13.7 versus 37.7 μM) MCF-7 cells treated with the sphingolipids for 48 h. 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 (IC50 of 3.7 versus 11.3 μM, and 24.1 versus 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; Karasavvas et al., 1996; He et al., 1999). This selectivity of location of unsaturation in the sphingoid backbone is supported by our data, because 6-ene-Cer, which has the unsaturation only at C(6)-C(7), was considerably less potent than analogs that contained a C(4)-C(5) trans double bond (with the exception of 4,6-diene-7-Ph-Cer), with an IC50 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 very effective. However, it is difficult to correlate these results with those in the present study because the biological response measured in that study dealt with overcoming axonal growth inhibition in hippocampal neurons. Interestingly, treatment of MCF-7 cells with ceramide analogs containing an additional hydroxyl group at C-6 resulted in a higher cytotoxicity than that found with Cer; the IC50 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 (IC50 of 13.9 μM, i.e., a 58% decrease versus 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, 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; Guzd 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 in ΔΨm by 2 h, which is significant by 8 h, and continues to decrease to 24 h (Fig. 7C). The timing of the significant decrease in ΔΨm at 8 h coincides with the first observable release of cytochrome c from the mitochondria into the cytosol and suggests that dissociation of ΔΨm 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.

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 h. 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. Although 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.

References


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