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Regulation of Human Breast Cancer Cell Death by Sphingolipids

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REGULATION OF HUMAN BREAST CANCER CELL DEATH BY SPHINGOLIPIDS

By

Chi Zhang

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ABSTRACT

REGULATION OF HUMAN BREAST CANCER CELL DEATH BY SPHINGOLIPIDS

By

Chi Zhang

Breast cancer is the most common malignant cancer among women and the incidence is increasing in most countries. Improved treatments are needed including new chemotherapeutic agents. This study examined the effects of sphingosine and ceramide, two bioactive metabolites of more complex sphingolipids, on the death of MDA-MB-231 human breast cancer cells which are estrogen receptor-negative and multi-drug resistant. Both the naturally occurring form of sphingosine, D-*erythro*, and a cell-permeable form of ceramide, C_2-ceramide, caused cell death in concentration- and time-dependent manners. Data from terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling, agarose gel electrophoresis of DNA extracts, and flow cytometric analysis suggest that sphingosine induces apoptosis while ceramide causes death via a non-apoptotic pathway. Both exogenously added sphingosine and ceramide increased the cellular level of sphingosine, but the increase caused by sphingosine was much higher than that induced by ceramide. The results suggest that the cellular sphingosine concentration necessary to induce breast cancer cell apoptosis is about 5-10 nmol/mg protein. The unnatural synthetic sphingosine stereoisomers (D-*threo*, L-*threo*, L-*erythro*) also caused death of human breast cancer cells with potencies greater than or equal to D-*erythro*-sphingosine. These studies suggest that D-*erythro*-sphingosine and its unnatural stereoisomers may provide new means to treat patients with advanced breast cancer who are estrogen receptor-negative.

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I. LITERATURE REVIEW

A. Breast cancer is a growing problem

1. Breast cancer epidemiology-The risk of developing breast cancer is about one in eight American women (Claus *et al.*, 1991; Marshall, 1993), and the risk of a woman living in the United States dying of breast cancer is 3.6 percent. Any family history of breast cancer on either the maternal or paternal side increases risk, but the increase is fairly small except in women who have first-degree relatives (mothers or sisters) with breast cancer (Osteen *et al.*, 1986). Some studies indicate that the risk is further increased if a first-degree relative had either premenopausal or bilateral breast cancer (Osteen *et al.*, 1986). The risk of breast cancer decreases as the age of the patient at first full term pregnancy decreases (Osteen *et al.*, 1986). Certain benign, proliferative changes seen in biopsy specimens are associated with an increased risk for subsequent development of breast cancer (Osteen *et al.*, 1986).

2. Causes of breast cancer-Mutations in *BRCA1*, *BRCA2* and potentially a few other genes are responsible for increases in risk of breast cancer incidence on a familial basis. The *BRCA1* gene has been localized to chromosome 17q21 (Miki *et al.*, 1994). Although its normal function is still unknown, it appears to confer tumor suppresser activity. About five percent of female breast cancer patients may have alteration(s) in *BRCA1* (Miki *et al.*, 1994). If their relatives are carriers of the *BRCA1* mutation(s), they have about 85 percent lifetime risk of breast cancer with 50 percent of the breast cancers occurring before age 50 (Miki *et al.*, 1994). Genetic abnormalities of *BRCA2*, which have been localized to chromosome 13q12-13, also confer a high risk of breast cancer (Wooster *et al.*, 1994). Despite inherited defective genes which predispose women to breast cancer,

about 90 percent of breast cancer incidence is believed to be caused by environmental factors such as diet, lifestyle, and exposure to environmental contaminants (Wright, 1990; Lynch *et al.*, 1992).

3. **Breast cancer development and diet**-Certain dietary factors affect the development of breast cancer and prevention of breast cancer through dietary modification is an active area of clinical and epidemiological research. The risk of breast cancer may be related to the total fat content of the diet (Wynder *et al.*, 1997). As women from oriental countries with a low fat content in the diet increase their fat intake or migrate to western countries, the incidence of breast cancer increases, even within the migrating generation and particularly in later generations (Osteen *et al.*, 1986). Although controversial, some studies have suggested that obese, postmenopausal women may have a higher risk of breast cancer than thin women (Cleary and Maihle, 1997). Also overweight or obese breast cancer patients might have a poorer prognosis (Bastarrachea *et al.*, 1994). Some animal studies showed that caloric restriction reduced breast cancer incidences (Klurfeld *et al.*, 1991). High alcohol intake probably increases the risk of breast cancer (Friedenreich *et al.*, 1993). In addition, other studies suggest that dietary carotenoids (Verhoeven *et al.*, 1997) and vitamins A (Sankaranarayanan and Mathew, 1996), C (Verhoeven *et al.*, 1997) and E (Kimmick *et al.*, 1997) might play a preventive role in breast cancer occurrence. Also, many studies have suggested that a lower risk of breast cancer is associated with higher intake of dietary fiber and carbohydrates (Stoll *et al.*, 1996). Taken together, current information suggests that a diet which is low in fat, and high in carbohydrate, fiber, vegetables and fruit may help to reduce the development of breast cancer. Further,

maintenance of body weight within recommended levels by proper food and energy intake is also important.

B. Chemotherapeutic treatments for estrogen receptor-negative breast cancer are needed

1. Breast cancer histology-The majority of breast cancers are poorly differentiated ductal carcinomas (Osteen *et al.*, 1986). Breast cancers often progress from a hormone-dependent, nonmetastatic, antiestrogen-sensitive phenotype to a hormone-independent, antiestrogen- and chemotherapy-resistant phenotype with highly invasive and metastatic growth properties. This progression is usually accompanied by altered function of the estrogen receptor or outgrowth of estrogen receptor-negative cancer cells (Nakshatri *et al.*, 1997).

2. Breast cancer detection and therapy-Mammography and, more recently, breast magnetic resonance imaging are used for breast cancer detection and diagnosis (Orel *et al.*, 1994). Breast cancer is treated by appropriate combinations of surgery, radiation therapy, chemotherapy, and hormonal therapy, and is curable if detected in early stages. The choice of treatment is based on tumor stage, lymph node status, estrogen- and progesterone-receptor levels in the tumor tissue, menopausal status, general health of the patient and patient age (de la Rochefordiere *et al.*, 1993). Normally, primary treatment (mastectomy or lumpectomy with radiation therapy) is followed by hormonal therapy and adjuvant chemotherapy.

3. Estrogen receptor status and breast cancer chemotherapy-Estrogen receptor status is a very important criterion to select appropriate hormonal therapy or adjuvant chemotherapy (*e.g.* cyclophosphamide, doxorubicinn, menthotrexate, 5-

fluorouracil, prednisone, vincristine and tamoxifen) for breast cancer patients at different staging categories (Greenspan, 1996). For patients with positive estrogen receptor status, hormonal therapy (*e.g.* tamoxifen) is the first choice (Fisher *et al.*, 1989). Chemotherapy is usually applied for endocrine-resistant patients, estrogen-receptor negative patients, or patients who have life-threatening metastases (Clavel and Catimel, 1993). Doxorubicin is one of the most effective single cytotoxic agent, giving a 40 percent response rate in previously untreated patients (Ahmann *et al.*, 1974). In most cases, combination chemotherapy regimens are used because they are much more effective than a single agent (Ahmann *et al.*, 1974; Canellos *et al.*, 1976; Jones *et al.*, 1975). Combination chemotherapy regimens generally produce higher response rates, ranging from 50 to 80 percent (Canellos *et al.*, 1976; Jones *et al.*, 1975). However, results are still unsatisfactory because the fraction of complete responders is less that 20 percent, the duration of response is less than 1 year, and the median survival time of these patients is about 2 years (Legha *et al.*, 1979; Eddy, 1992). Therefore, the search for new chemotherapeutic agents and more effective combinations must continue.

C. The sphingolipids, ceramide and sphingosine, inhibit cell growth and induce cell death

1. Sphingolipid metabolism-The influence of specific dietary factors or drugs on the development of breast cancer has received substantial attention in medical research. However, to this point, little has been done to assess the potential of sphingolipids in treating breast cancer. Sphingolipids are a family of bioactive lipids which regulate cell behavior (Bell *et al.*, 1993; Merrill *et al.*, 1997). Specifically, ceramide, sphingosine and sphingosine 1-phosphate are bioactive metabolites of more complex sphingolipids (Figure

1). These bioactive lipids serve as second messengers for the effects of some extracellular agents on cell growth, oncogenesis, differentiation, and cell death. Studies conducted using HL-60 human leukemia cells have shown that the addition of 1α, 25-dihydroxyvitamin D_3, an inducer of differentiation, activates a neutral sphingomyelinase and caused early and reversible hydrolysis of sphingomyelin and the concomitant generation of ceramide (Okazaki *et al.*, 1989) (Figure 1). The effect of 1α, 25-dihydroxyvitamin D_3 on cell differentiation is mimicked by cell-permeable ceramide analogs with shorter N-acyl chains (Okazaki *et al.*, 1994). Tumor necrosis factor-α (TNF-α) and γ-interferon also have been found to induce sphingomyelin turnover and ceramide production (Kim *et al.*, 1991; Mathias *et al.*, 1991; Dressler *et al.*, 1992). In some cases, ceramide can be further metabolized to sphingosine by ceramidase (Nikolova-Karakashian *et al.*, 1997) (Figure 1). Moreover, sphingosine can be phosphorylated by sphingosine kinase to form sphingosine-1-phosphate (Zhang *et al.*, 1991) (Figure 1). Thus, some effects previously attributed to ceramide may be mediated via its conversion to sphingosine or sphingosine-1-phosphate.

2. Sphingosine inhibits cell growth and induces apoptosis-Sphingosine is a bioactive compound. The observation that sphingosine is an inhibitor of protein kinase C lead to the initial interest in sphingolipids as possible second messengers (Hannun *et al.*, 1986). Sphingosine added exogenously to tissue culture medium is taken up by the cells and metabolized to form other lipids (Smith and Merrill, 1995). Cellular sphingosine can also activate targets to regulate cell behavior. Some recent research shows that sphingosine plays an important role in apoptosis (Ohta *et al.*, 1994; Ohta *et al.*, 1995; Jarvis *et al.*, 1996; Nakamura *et al.*, 1996). Conflicting observations on the involvement of sphingosine

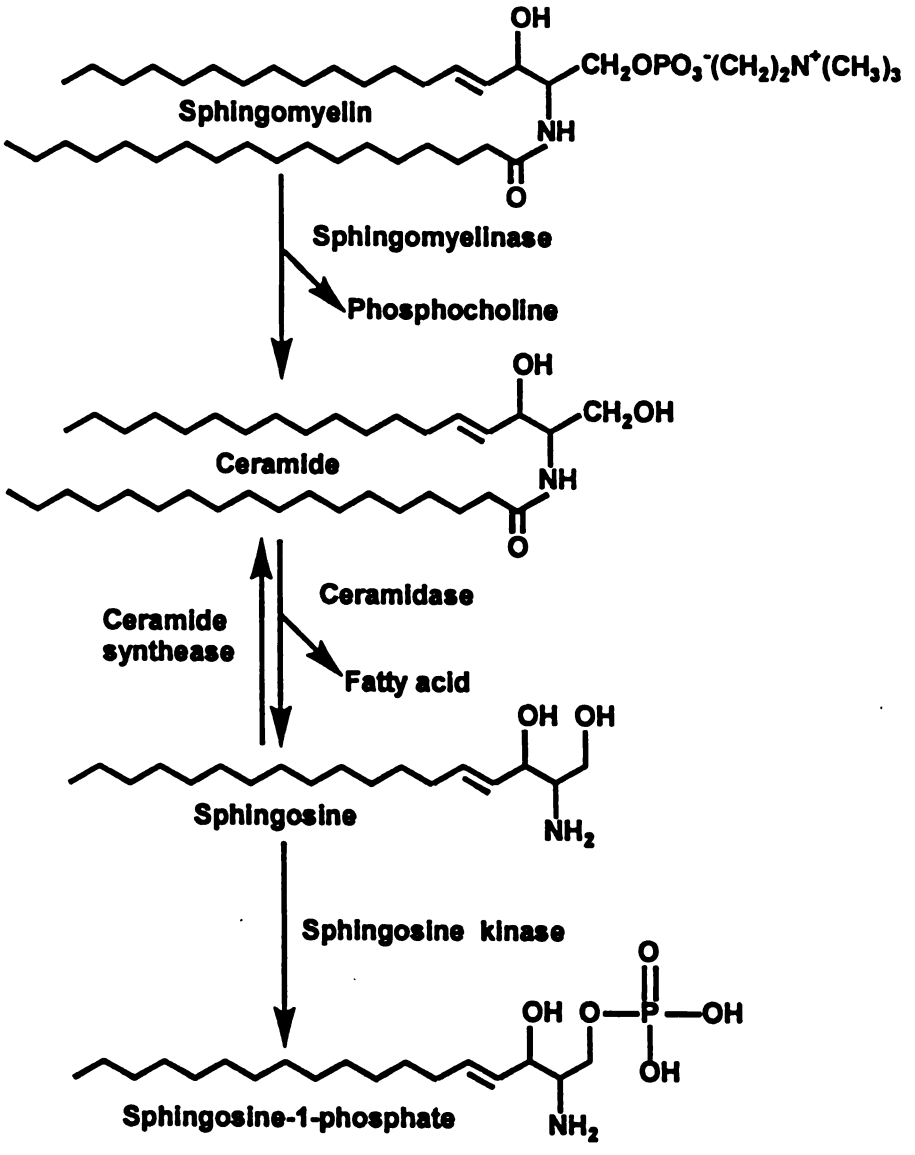


Figure 1. Sphingolipid turnover leads to formation of intracellular second messengers

in apoptosis point to a great variability depending on sphingosine dosage, cell type, phase of cell cycle and intracellular signal transduction pathway.

Exogenous addition of sphingosine was demonstrated to induce apoptosis of human neutrophils (Ohta *et al.*, 1994), human myeloid leukemic HL-60 cells (Ohta *et al.*, 1995; Jarvis *et al.*, 1996) and an interleukin-2-dependent cytotoxic T cell line, CTLL-2 cells (Nakamura *et al.*, 1996). The intracellular level of sphingosine increased in the cases of tumor necrosis factor-α (TNF-α) induced apoptosis of human neutrophils (Ohta *et al.*, 1994) and cardiac myocytes (Ohta *et al.*, 1995) and phorbol ester induced apoptosis of HL-60 cells (Ohta *et al.*, 1995; Jarvis *et al.*, 1996), suggesting that sphingosine may function as an endogenous mediator of apoptosis in these cells. In the experiment conducted with neutrophils, accumulation of both ceramide and sphingosine was observed after exogenous addition of TNF-α, with the ceramide concentration increasing prior to that of sphingosine. Furthermore, exogenous sphingosine (5-15 μM) was capable of inducing apoptosis in neutrophils, but ceramide and sphingosine-1-phosphate at similar concentrations did not induce apoptosis. These findings suggested sphingosine derived from deacylation of ceramide, but not ceramide itself, might mediate cell death in neutrophils after TNF-α treatment (Ohta *et al.*, 1994). Primary cell cultures appear to be less susceptible to sphingosine than cancer cells. Sphingosine did not induce apoptosis in normal epithelial cells such as HUVECs or rat mesangial cells, but did induce apoptosis in their transformed counterparts (Sweeney *et al.*, 1996).

The cellular concentration of sphingosine increased during apoptosis resulting from phorbol ester-induced terminal differentiation of HL-60 cells (Ohta *et al.*, 1995; Jarvis *et al.*, 1996). Apoptosis induced by phorbol ester and sphingosine was accompanied by a

concomitant decrease of expression of *bcl-2* proto-oncogene (a suppresser gene of apoptosis) at both the mRNA and protein levels (Figure 2), while expression of *bcl-X_L* and *bax* mRNA did not change. In contrast, expression of *bcl-2* did not change in apoptosis induced by the pharmaceutical protein kinase C inhibitors 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) or staurosporine. These observation suggested that sphingosine mediates the apoptotic signaling in phorbol ester-induced terminal differentiation of HL-60 cells through down regulation of *bcl-2*, which is probably independent of the protein kinase C inhibition function of sphingosine (Sakakura *et al.*, 1996). Sphingosine also induces apoptosis in androgen-independent human prostatic carcinoma DU-45 cells, which express *bcl-X_L* (another cell death repressor gene) but not *bcl-2* at the protein level, through down regulation of *bcl-X_L* (Figure 2). Again, this probably occurs independently of protein kinase C inhibition by sphingosine. Furthermore, neither ceramide nor sphingosine-1-phosphate induce apoptosis in DU-145 cells (Shirahama *et al.*, 1997). On the other hand, some studies have shown that sublethal concentrations of sphingosine synergistically augment the apoptotic capacity of ceramide in HL-60 and U937 monoblastic leukemia cells (Jarvis *et al.*, 1996). This effect of sphingosine could also be achieved by acute co-exposure to highly selective pharmacological inhibitors of protein kinase C or chronic pre-exposure to the non-tumor-promoting protein kinase C activator, bryostatin 1 (Jarvis *et al.*, 1996). In addition, both sphingosine and H7 induced apoptosis in neutrophils (Ohta *et al.*, 1994). The findings demonstrate a protein kinase C-dependent pathway for sphingosine induced apoptosis. In other words, induction of apoptosis by sphingosine might be related to inhibition of protein kinase C activity (Figure 2).

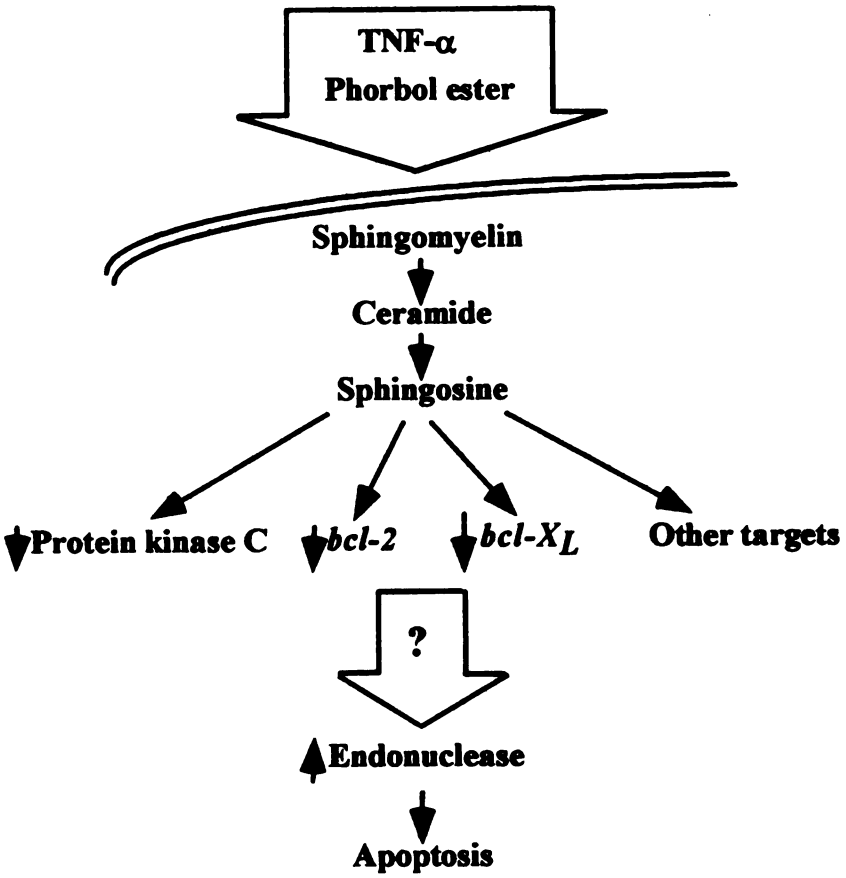


Figure 2. Sphingosine induces apoptosis

3. **Ceramide inhibits cell growth and induces apoptosis-**Ceramide has also been postulated as an intracellular mediator of apoptosis. TNF-α induced apoptosis in U937 monoblastic leukemia cells. Exposure of U937 cells to TNF-α resulted in sphingomyelin hydrolysis and ceramide generation. Treatment of cells with either TNF-α or C_2-ceramide (a short chain, cell-permeable ceramide analog) caused internucleosomal DNA fragmentation characteristic of apoptosis (Obeid *et al.*, 1993). In Molt-4 leukemia cells, serum withdrawal caused a modest apoptotic cell death (Jayadev *et al.*, 1995). Serum deprivation of these cells resulted in significant sphingomyelin hydrolysis and elevation in endogenous levels of ceramide. A distinct, particulate, membrane-associated and magnesium-dependent sphingomyelinase was involved in this process. The addition of exogenous C_6-ceramide resulted in more pronounced apoptosis which occurs much sooner (Jayadev *et al.*, 1995). Moreover, Chouaib's group found that a TNF-resistant variant (R-A1) of MCF-7 human breast carcinoma cells could be induced to undergo cell death after exposure to exogenous sphingomyelinase or cell-permeable C_6-ceramide (Cai *et al.*, 1997). In addition, exogenously added cell-permeable ceramides also induced apoptosis in some other cell lines (Jarvis *et al.*, 1994; Karasavvas *et al.*, 1996; Gill *et al.*, 1997).

Domae's group showed that c-*jun*/AP-1 was activated by ceramide early in the process of apoptosis and that the impairment of c-*jun*/AP-1 by curcumin (an inhibitor of AP-1) or antisense oligonucleotides for c-*jun* rescued apoptotic cells (Sawai *et al.*, 1995). This suggested that ceramide was crucially involved in the signal transduction pathway leading to apoptosis through the activation of c-*jun*/AP-1 (Figure 3).

Neither the specificity nor the mechanism of action of ceramide is fully understood. Although C_2-ceramide was able to induce differentiation, neither sphingosine nor N-

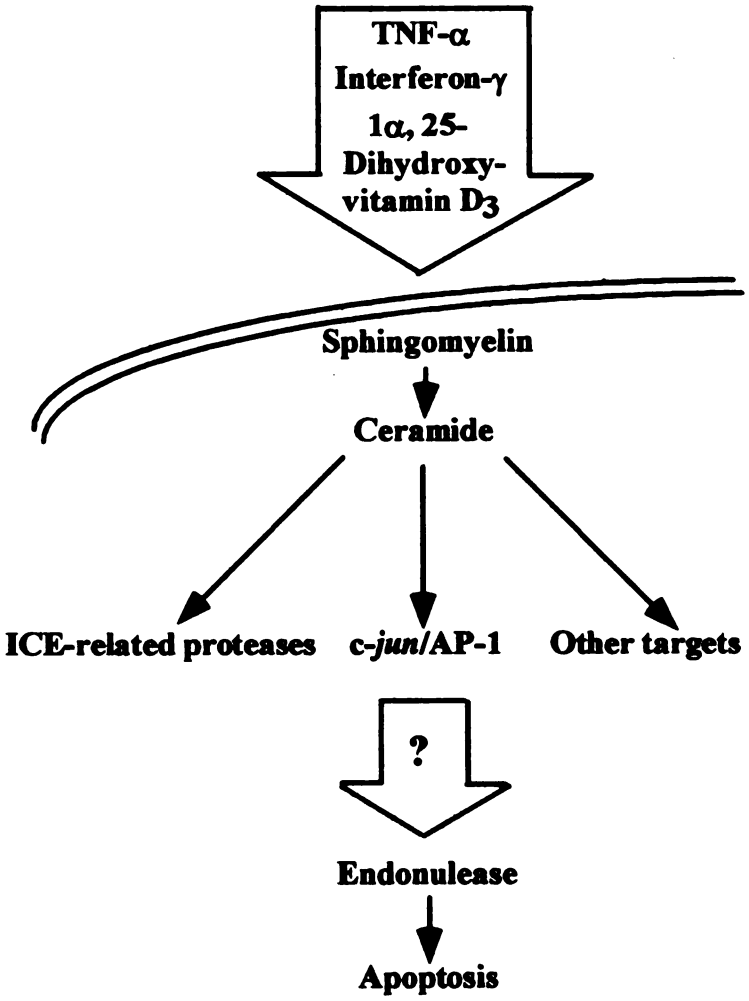


Figure 3. Ceramide induces apoptosis

ethylsphingosine showed any activity (Bielawska *et al.*, 1992); this observation suggested a critical role for the amide-linked fatty acyl group. Furthermore, C_2-dihydroceramide which is identical to C_2-ceramide but lacks the 4,5 *trans* double bond of the sphingoid base backbone failed to induce DNA fragmentation (Obeid *et al.*, 1993), which demonstrated that the double bond was obligatory for ceramide to induce apoptosis.

D. Sphingosine and ceramide may provide a novel means to treat patients with estrogen receptor-negative breast cancer

In preliminary studies, we found that both sphingosine and ceramide inhibited growth and induced death of MDA-MB-231 cells, an estrogen receptor-negative human breast cancer cell line. Both sphingosine and ceramide were more cytotoxic for sub-confluent continuously growing cells than for confluent quiescent cells. This suggests that cancer cells which are more likely to be dividing *in vivo* may be more susceptible to cell death induced by sphingosine and ceramide than neighboring normal tissue that may be less likely to be proliferating. The experiments described herein build upon our preliminary observations.

II. OBJECTIVES

Breast cancer is the most common malignant cancer of women in the western world (Claus *et al.*, 1991; Marshall, 1993). Approximately 910,000 new cases were diagnosed worldwide in 1996 (WHO, 1997), which accounted for nine percent of all new cancers. Many studies are underway to improve human breast cancer prevention, early detection, and treatment. Our preliminary data show that sphingosine and ceramide inhibit growth and induce death of MDA-MB-231 human breast cancer cells which lack an estrogen receptor (Zhang and Schroeder, 1998). The purpose of this project is to study the mechanism by which sphingosine and ceramide induce human breast cancer cell death. The specific aims of this project are to:

A. Determine whether sphingosine and ceramide kill cells by apoptosis or via a non-apoptotic pathway.

B. Determine the structural requirements for the breast cancer cell killing effects of sphingosine and ceramide.

This study may provide insight into the potential of sphingosine and ceramide to serve as chemotherapeutic agents for breast cancer patients who are estrogen receptor-negative.

III. MATERIALS AND METHODS

Cell culture-The MDA-MB-231 human breast cancer cell line was purchased from the American Type Culture Collection. Cells were grown in minimum essential medium (MEM, GIBCO®) supplemented with 10% fetal bovine serum (FBS). Sphingolipids were obtained from Matreya, Inc.. Sphingosine was added as a 1:1 complex with bovine serum albumin (BSA) while C_2-ceramide was dissolved in ethanol and then added directly to the medium.

Nucleic acid assay-Total nucleic acid concentration was measure as described by Li *et al.* (1990). Cells were seeded at 2.5×10^5/mL and incubated with treatments in 1% FBS in MEM for certain times. Then the cells were rinsed twice with phosphate-buffered saline (PBS, pH 7.4) and lysed with 1N NaOH. The absorbance of the clear cell lysate was read at 260 nm by spectrophotometry to measure total nucleic acid content.

Detection of DNA strand breaks by the TUNEL reaction-In Situ Cell Detection Kit-Fluorescein was purchased from Boehringer Mannheim. In this method, terminal deoxynucleotidyl transferase was used for the incorporation of fluorescent labeled nucleotides into DNA strand breaks *in situ* (Gavrieli *et al.*, 1992). Cells were grown and treated in chamber slides (Nunc, Inc.). Air dried cell samples were fixed with freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30 minutes at room temperature. Then cells were incubated in permeablization solution (0.1% Triton® X-100, 0.1% sodium citrate) for 2 minutes on ice (4°C). Each sample was incubated with 50 µL TUNEL reaction mixture in a humidified chamber for 60 minutes at 37°C in the dark. To

identify cells with strand breaks, samples were directly analyzed using a fluorescence microscope.

Qualitative analysis of internucleosomal DNA fragmentation by agarose gel electrophoresis-This method was based on the procedure described by Goruppi *et al.* (1994) by using the Genomix Kit purchased from Tel-Test "B", Inc. Briefly, cells were collected in PBS, and then incubated with a lysing solution and RNase at 65°C for 30 minutes. Chloroform and an acidification solution were then added, and the mixture was separated with a small gel barrier tube. Sample DNA was contained in the upper, aqueous phase. Sample DNA was centrifuged with a precipitate solution at 10,000 rpm for 10 minutes. The pellet was washed with ionic exchange solution and then precipitated with ethanol. The pellet was resuspended in distilled water after a final wash with 70 % ethanol. DNA samples were run on 1.5 % agarose gel at 60 V for 2.5 hours with 0.5× Tris Borate EDTA (TBE) running buffer. The DNA was stained with ethidium bromide, and the gel was photographed under UV light.

Flow cytometric analysis of cell cycle and apoptosis-The cells were prepared based on the procedure described by Telford *et al.* (1994). Briefly, cells were harvested by collecting floating cells in the medium and trypsinization of attached cells. Cells were precipitated and then resuspended in fresh medium (1% fetal bovine serum in minimum essential medium) to obtain single cells suspension. Cells were fixed with 70% ethanol in steps until the final concentration of ethanol was more than 50%. Then cells were stored at 4°C overnight. Cells were pelleted and then resuspended in PBS (pH 7.4) containing 0.1% Triton X-100 and 0.1 mM EDTA (pH 8.0). Cells in single cell suspension were stained by DNA staining solution (PBS containing 0.1% Triton X-100, 0.1 mM EDTA, 0.05 mg/ml

RNase A, 50μg/ml propidium iodide) for at least 1-2 hours before analysis. The cells were analyzed by a state-of-the-art Becton-Dickinson Vantage flow cytometer.

Mass measurements of sphingoid bases-Cells were washed with 3 mL of PBS (pH 7.4) and harvested in 0.5 mL PBS. Mass measurements of the long-chain bases were conducted using a sensitive and reproducible HPLC method (Merrill *et al.*, 1988). Briefly, the unnatural sphingoid bases, C_{20}-sphinganine and C_{20}-sphingosine, were used as internal standards and the long-chain bases were extracted with chloroform and methanol and then treated with base to remove interfering glycerolipids. After preparation of the o-phthalaldehyde (OPA) derivatives, the long-chain bases were separated by reverse-phase HPLC using a C18-column eluted isocratically with methanol:5 mM potassium phosphate (pH 7.0), 90:10 (v/v).

Measurement of cell protein-Total cell protein was determined by the method described by Lowry *et al.* (1951). The cells were collected in PBS (pH 7.4). Then 1.0 mL of Lowry reagent (25 mg $Na_2CuEDTA$, 2 g Na_2CO_3, 0.4 g NaOH in 100 mL water) was added to 100 μL of sample or BSA standard. After 10 minutes, the samples were incubated with 100 μL of phenol-(FOLIN)-reagent:water (1:1) for 30 minutes at room temperature. Absorbance was determined at 500 nm.

Statistical Analyses-Two-way factorial analysis of variance (ANOVA) was applied to the data. Also, two-sample t-test (two tailed, assuming equal variance) was used to identify significant difference between control and treatment groups at specific time points. The significance level was adjusted by Bonferroni method. Significant effects were at $P<0.05$.

IV. RESULTS

A. Sphingosine and ceramide differentially affect death of estrogen receptor-negative MDA-MB-231 human breast cancer cells

Sphingosine and ceramide cause death of human breast cancer cells-To assess whether sphingosine and ceramide inhibit growth and cause death of human breast cancer cells, subconfluent MDA-MB-231 cells were cultured with either D-*erythro*-sphingosine or C_2-ceramide (a cell permeable, short chain analog of naturally occurring ceramide). At various times, dead cells were rinsed away and live cells were harvested and nucleic acid content was measured as an index of cell number (Figure 4). The nucleic acid concentration in control cultures decreased ~20-25% within the first 3-6 hours (Figure 4A & 4B). Over the subsequent 18-21 hours, the nucleic acid content doubled. The addition of D-*erythro*-sphingosine caused concentration- and time-dependent decreases in total nucleic acid concentration (Figure 4A). D-*erythro*-sphingosine at 5 μM significantly reduced nucleic acid concentration within 24 hours compared to the corresponding control. In comparison, D-*erythro*-sphingosine at 10 μM reduced nucleic acid concentration to ~40% of the corresponding control within 3 hours. Total nucleic acid was still ~40% of control at 24 hours.

Like sphingosine, C_2-ceramide also inhibited growth and killed MDA-MB-231 cells in a concentration- and time-dependent manner (Figure 4B). C_2-ceramide at 5 μM significantly reduced nucleic acid concentration with 18 hours compared to the corresponding control. At 10 μM, C_2-ceramide reduced the nucleic acid content of cells to about 75% of the corresponding control within 3 hours. By 24 hours, the nucleic acid

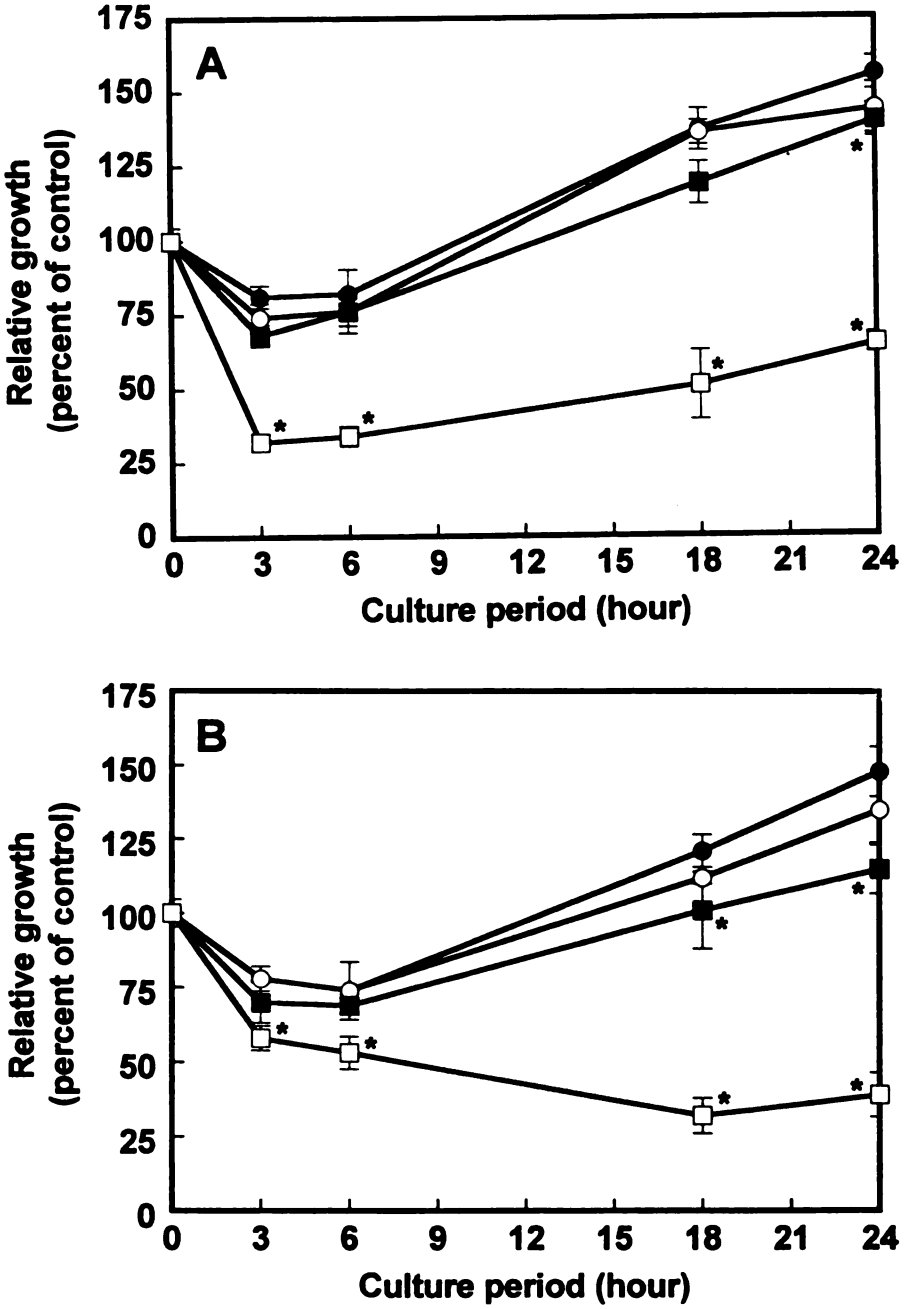


Figure 4. Sphingosine and ceramide inhibit growth and induce death of human breast cancer cells. Subconfluent MDA-MB-231 cells were cultured with D-*erythro*-sphingosine (A) or C_2-ceramide (B) at 0 (●), 2 (○), 5 (■), or 10 (□) μM. At various times, cells were harvested and nucleic acid content was measured by spectrophotometry (λ=260 nm). Each point is the mean ± SD (n=3). At each time, means with an asterisk (*) are significantly different ($P < 0.05$) than the corresponding control.

concentration for cells cultured with 10 μM C_2-ceramide was about 30% of the corresponding control.

Sphingosine and ceramide differentially affect death of human breast cancer cells- To determine whether sphingosine and ceramide kill breast cancer cells by inducing apoptosis, subconfluent cultures of MDA-MB-231 cells were cultured with cytotoxic concentrations of the sphingolipids. Then three techniques were utilized to assess DNA fragmentation, a hallmark of apoptosis. For the TUNEL reaction, cells were cultured with or without the sphingolipid at 5 μM for 4 hours. Then DNA strand breaks were labeled with fluorescein using terminal deoxynucleotidyl transferase and cells were examined via microscopy under both phase contrast (Figure 5, left panels) and under fluorescent light (Figure 5, right panels). Cells with DNA strand breaks fluoresce and are considered TUNEL positive. Control cells were TUNEL negative indicating they had intact DNA (Figure 5A, right panel). Cells cultured with either 5 μM sphingosine (Figure 5B) or C_2-ceramide (Figure 5C) were TUNEL positive (right panels). These studies indicate that both sphingosine and ceramide cause DNA strand breaks.

To further investigate the mechanism of cell death, agarose gel electrophoresis was used to detect DNA fragmentation in MDA-MB-231 cells cultured with or without sphingolipids. Control cultures had intact, high molecular weight DNA which appeared at the top of the agarose gels (Figure 6A and 6B, lane 2). Like DNA from control cultures, DNA from cells cultured with C_2-ceramide at 2-10 μM for either 6 hours (Figure 6A) or 24 hours (Figure 6B) was not fragmented. In contrast, DNA extracted from cells cultured for 6 hours with 5 μM D-*erythro*-sphingosine was fragmented and showed a characteristic

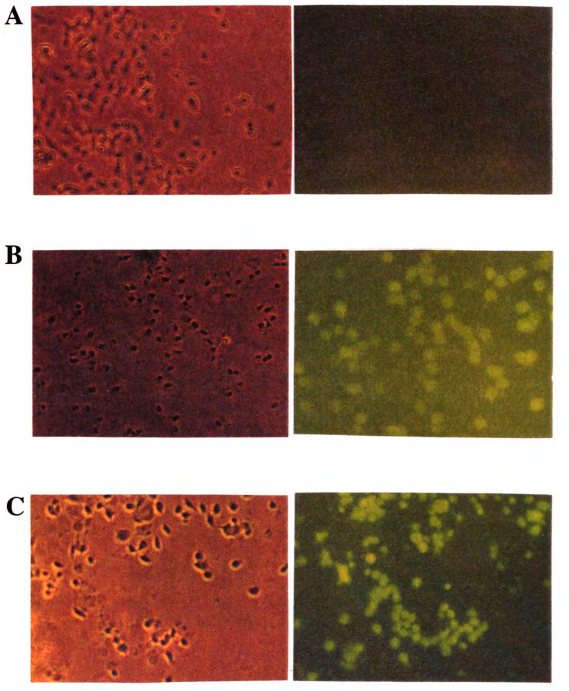


Figure 5. Sphingosine and ceramide cause DNA strand breaks in human breast cancer cells. Subconfluent MDA-MB-231 cells were cultured either without sphingolipid (A), or with 5 μM D-*erythro*-sphingosine (B) or 5 μM C_2-ceramide (C) for 4 hours and DNA strand breaks were labeled with fluorescein *in situ* via the TUNEL reaction. For each pair of photographs, the left panel represents cells under phase contrast and the right shows cells under fluorescent light.

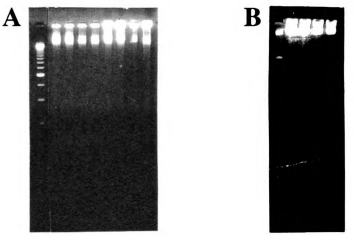


Figure 6. Ceramide does not cause internucleosomal DNA fragmentation in human breast cancer cells. Subconfluent MDA-MB-231 cells were cultured with various concentrations of C_2-ceramide for 6 hours (A) and 24 hours (B). Then DNA was extracted, stained with ethidium bromide, and analyzed for fragmentation by agarose gel electrophoresis (1.5% agarose gel, 60V). Figure 6A lane 1, DNA 250 base pair molecular weight markers; lanes 2 and 3, control; lanes 4 and 5, 2 μM C_2-ceramide; lanes 6 and 7, 5μM C_2-ceramide; lanes 8 and 9, 10 μM C_2-ceramide. Figure 6B lane 1, DNA 250 base pair molecular weight markers; lanes 2 and 3, control; lanes 4 and 5, 5μM C_2-ceramide.

ladder pattern indicative of apoptosis (Figure 7-lanes 2 and 3). These results provide strong evidence that sphingosine kills MDA-MB-231 cells by inducing apoptosis; whereas, ceramide causes death of these cells via a non-apoptotic pathway.

The differential effects of sphingosine and ceramide on human breast cancer cells were further examined via flow cytometric analysis. For these studies, MDA-MB-231 cells were cultured with cytotoxic concentrations of the sphingolipids and then DNA was stained with propidium iodide. Upon flow cytometric analysis, necrotic cells appear to have reduced DNA content and form a plateau in the hypodiploid pre-G_0/G_1 region; whereas, apoptotic cells appear to have reduced DNA content and form a sharp hypodiploid pre-G_0/G_1 peak. Flow cytometry showed that control cultures had subpopulations of cells in each phase of the cell cycle (G_0/G_1, S, G_2/M) indicative of subconfluent, proliferating cells (Figures 8A and 9A). In comparison, C_2-ceramide (2, 5, 10 μM) caused concentration-dependent increases in the number of cells in the G_0/G_1 phase and in the pre-G_0/G_1 region as a plateau indicative of necrosis (Figure 8B-D). In contrast, D-*erythro*-sphingosine (5, 10, 15 μM) caused concentration-dependent increases in the number of cells in the pre-G_0/G_1 region as a peak indicative of apoptosis (Figure 9B-D). The percentage of apoptotic cells in cultures treated with 10 μM and 15 μM D-*erythro*-sphingosine were ~65% (Figure 9C) and ~85% (Figure 9D), respectively.

Exogenous sphingosine and ceramide increase cellular sphingosine in human breast cancer cells-To this point, the results indicate that both D-*erythro*-sphingosine and C_2-ceramide kill human breast cancer cells; however, only D-*erythro*-sphingosine kills cells by inducing apoptosis, while C_2-ceramide kills cells via a non-apoptotic mechanism. The

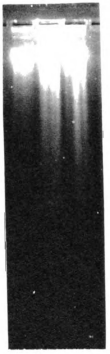


Figure 7. Sphingosine induces internucleosomal DNA fragmentation in human breast cancer cells. Subconfluent MDA-MB-231 cells were cultured without sphingolipid (lane 1) or with 5 μM D-*erythro*-sphingosine (lanes 2 and 3) for 6 hours. Then DNA was extracted, stained with ethidium bromide, and analyzed for fragmentation by agarose gel electrophoresis (1.5% agarose gel, 60V).

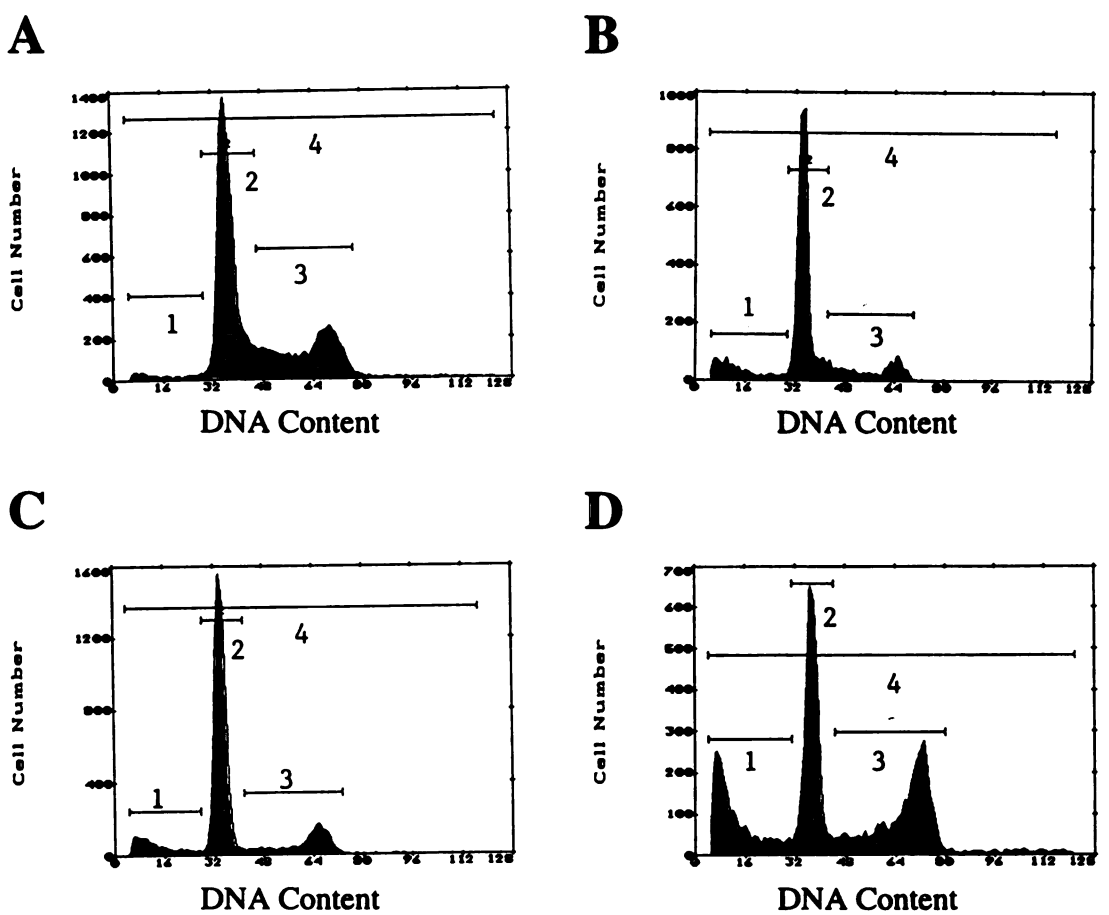


Figure 8. Flow cytometric analysis of human breast cancer cells cultured with ceramide. Subconfluent MDA-MB-231 cells were cultured with 0 (A), 2 (B), 5 (C), or 10 μM (D) D- C_2-ceramide for 24 hours. Subsequently, the cells were fixed with ethanol and DNA was stained with propidium iodide. Pre-G_0/G_1 cells, *bar 1*; G_0/G_1 cells, *bar 2*; S and G_2/M cells, *bar 3*; total cells, *bar 4*.

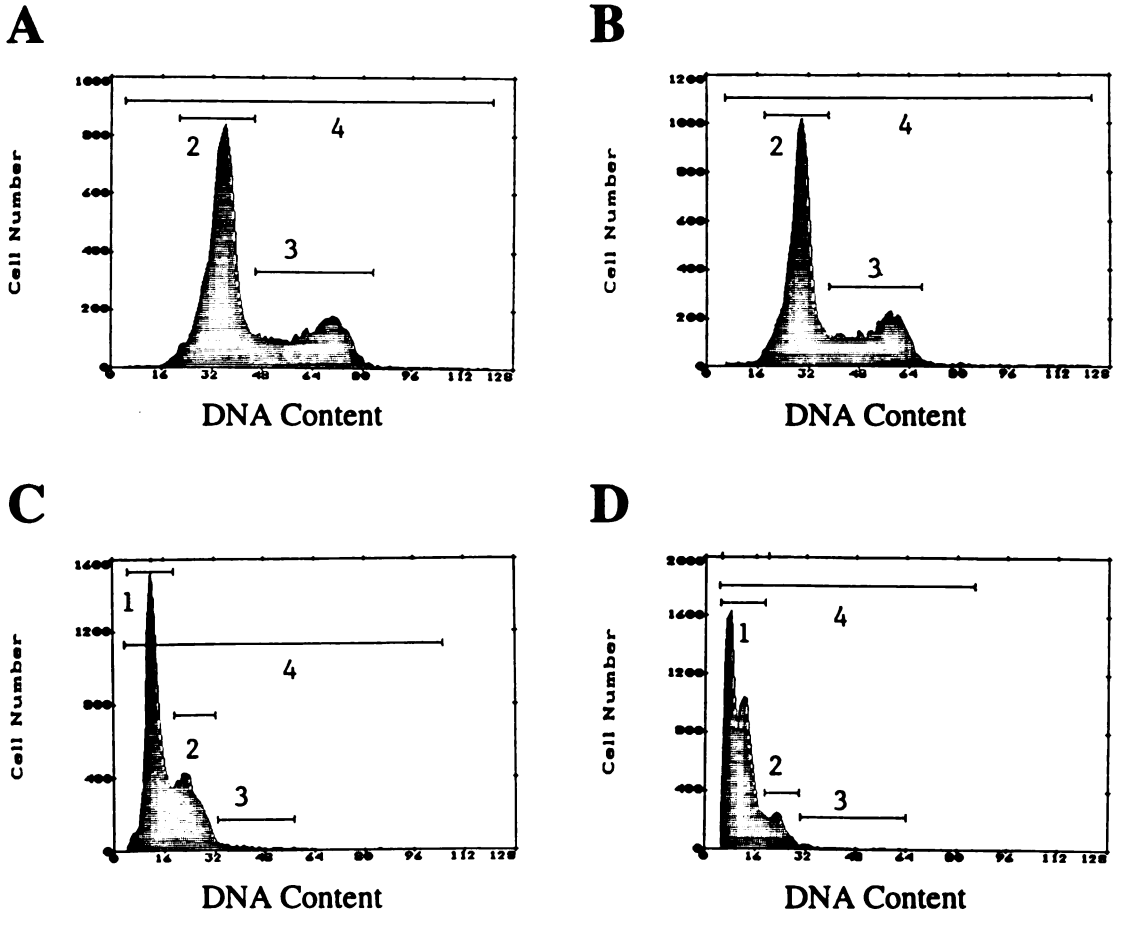


Figure 9. Flow cytometric analysis of human breast cancer cells cultured with sphingosine. Subconfluent MDA-MB-231 cells were cultured with 0 (A), 5 (B), 10 (C), or 15 μM (D) D-*erythro*-sphingosine for 6 hours. Subsequently, the cells were fixed with ethanol and DNA was stained with propidium iodide. Pre-G_0/G_1 cells, *bar 1*; G_0/G_1 cells, *bar 2*; S and G_2/M cells, *bar 3*; total cells, *bar 4*.

ability of cells to metabolize ceramide to sphingosine raised the question as to why ceramide did not also induce apoptosis similar to sphingosine. To address this question, we examined the effects of exogenously added cytotoxic concentrations of sphingolipids on the cellular concentration of sphingosine. In control cells, cellular sphingosine remained at ~250 pmol/mg protein over 60 minutes of culture (Figures 10 and 11). In comparison, the addition of exogenous D-*erythro*-sphingosine (2, 5, 10 μM) caused concentration- and time-dependent increases in cellular sphingosine (Figure 10). Upon addition of D-*erythro*-sphingosine at 2, 5, and 10 μM, cellular sphingosine increased to ~1.5, 5.5, and 10 nmol/mg cell protein, respectively, after 30 minutes. After 60 minutes of culture, the concentrations were lower indicating that cellular sphingosine was metabolized. C_2-ceramide (2, 5, and 10 μM) also caused concentration- and time-dependent increases in cellular sphingosine (Figure 11); however, the cellular sphingosine concentrations which were achieved were lower than that for exogenously added D-*erythro*-sphingosine. With 10 μM C_2-ceramide treatment, cellular sphingosine only increased to ~1 nmol/mg protein after 30 minutes and ~1.5 nmol/mg protein after 60 minutes (Figure 11).

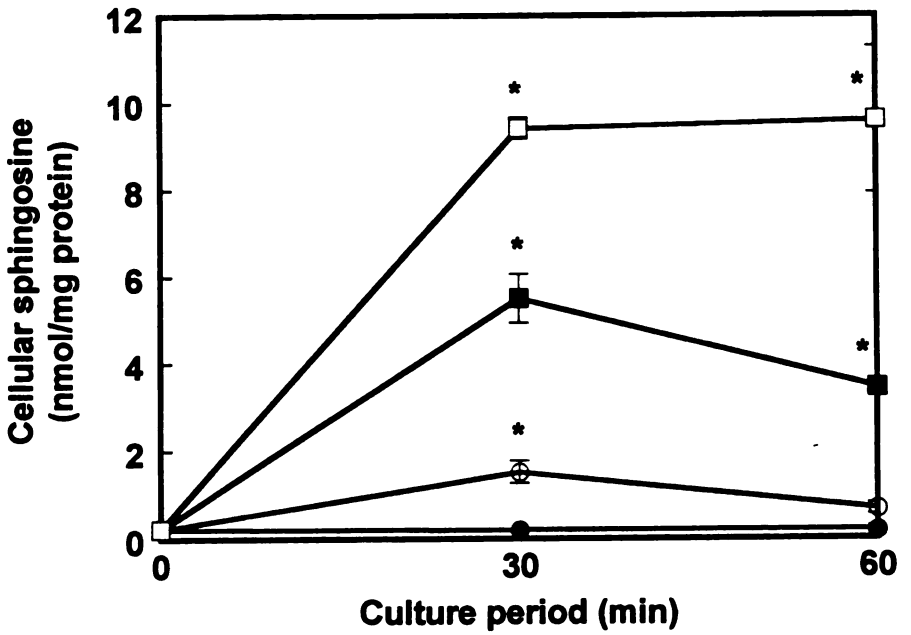


Figure 10. Exogenous sphingosine increases cellular sphingosine concentration. Subconfluent MDA-MB-231 cells were cultured with 0 (●), 2 (○), 5 (■), or 10 μM (□) D-*erythro*-sphingosine. Subsequently, cellular sphingosine concentrations were measured at 0, 30, and 60 minutes via HPLC. Each point is the mean ± SD (n=3). At each time, means with an asterisk (*) are significantly different ($P < 0.05$) than the corresponding control.

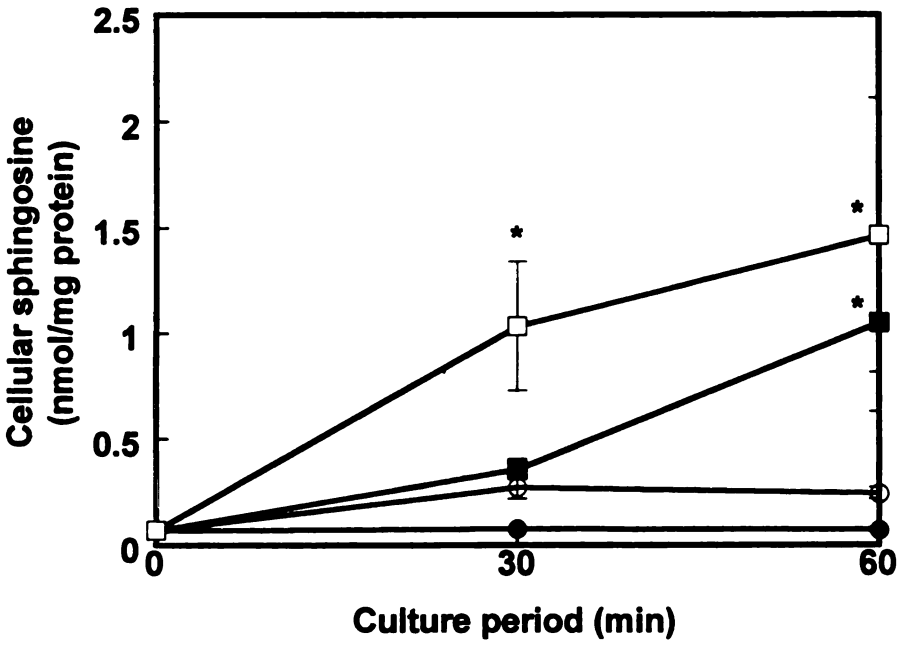


Figure 11. Exogenous ceramide increases cellular sphingosine concentration. Subconfluent MDA-MB-231 cells were cultured with 0 (●), 2 (○), 5 (■), or 10 μM (□) C_2-ceramide. Subsequently, cellular sphingosine concentrations were measured at 0, 30, and 60 minutes via HPLC. Each point is the mean ± SD (n=3). At each time, means with an asterisk (*) are significantly different ($P < 0.05$) than the corresponding control.

B. Structural requirements for sphingosine and ceramide induced death

The results of the first specific aim indicate that the naturally occurring stereoisomer of sphingosine, D-*erythro*-sphingosine, kills MDA-MB-231 human breast cancer cells by inducing apoptosis. To gain additional information about the molecular mechanism, we also examined the effects of the three unnatural stereoisomers (D-*threo*, L-*threo* and L-*erythro*) (Figure 12).

Like D-*erythro*-sphingosine (Figure 4A), the unnatural sphingosine stereoisomers inhibited growth and caused death of MDA-MB-231 human breast cancer cells in concentration- and time-dependent manners (Figure 13 and 14). All of the unnatural stereoisomers at 5 μM caused significant reductions in cell number within 3 hours of culture compared to the corresponding controls (Figure 13). L-*erythro*-sphingosine had the most potent cytotoxic effect, with an LD_{50} of ~2-3 μM at 3 hours (Figure 13B). In comparison, the LD_{50}s of other sphingosine stereoisomers (D-*erythro*-, D-*threo*-, L-*threo*) at 3 hours of culture were ~5 μM (Figures 4 and 13)

The necessity of the 4,5 *trans* double bond in the sphingoid base backbone for the cytotoxic effect of C_2-ceramide was also examined using C_2-dihydroceramide which lacks the double bond (Figure 15). At 5 μM, C_2-dihydroceramide did not affect growth of MDA-MB-231 human breast cancer cells (data not shown) and did not cause DNA strand breaks (Figure 16). These data indicate that the 4,5 *trans* double bond of the sphingoid base backbone of ceramide is obligatory for the cytotoxic action of ceramide in human breast cancer cells.

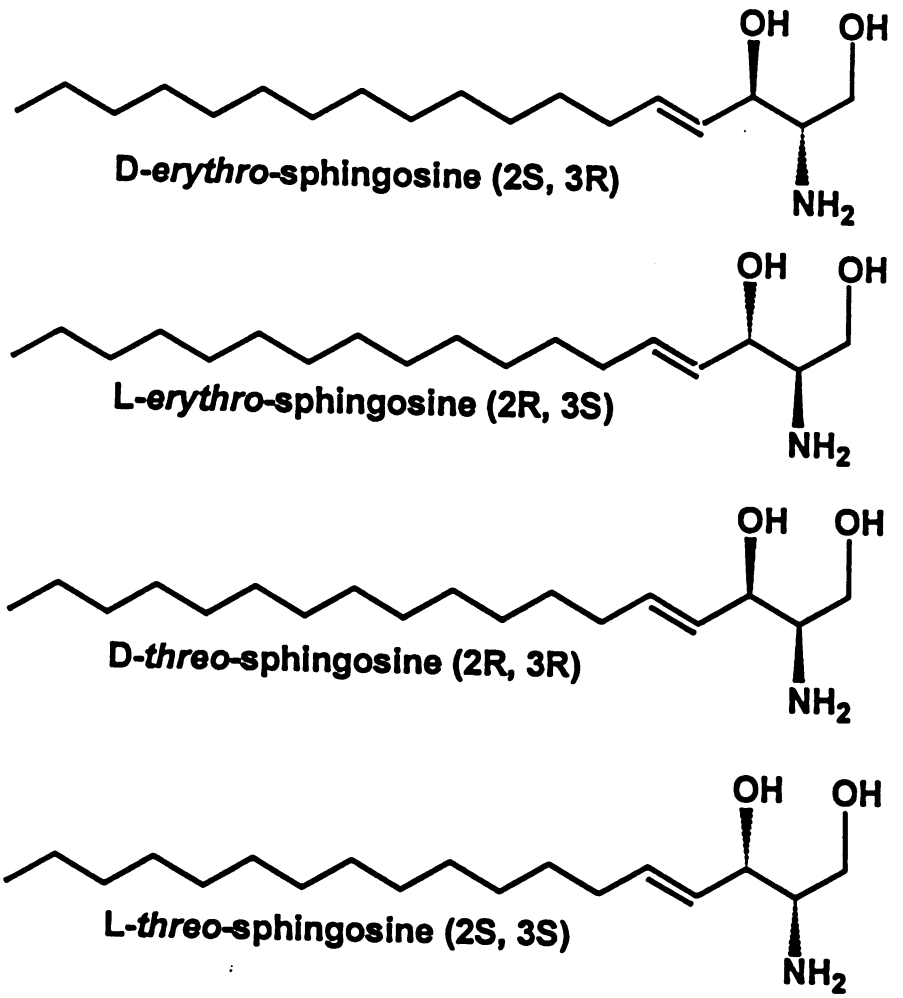


Figure 12. Structures of sphingosine stereoisomers

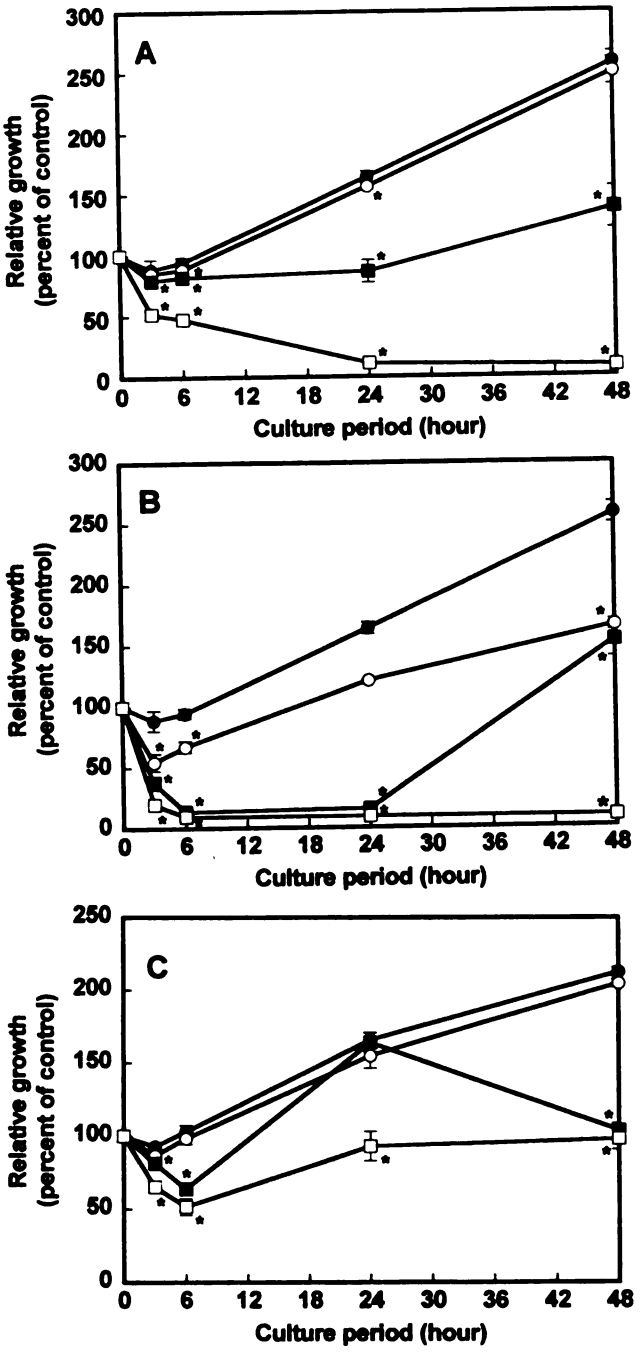


Figure 13. Sphingosine stereoisomers inhibit growth and induce death of human breast cancer cells. Subconfluent MDA-MB-231 cells were cultured with D-*threo*-sphingosine (A), L-*erythro*-sphingosine (B), and L-*threo*-sphingosine (C) at 0 (●), 2 (○), 5 (■), or 10 (□) μM. At various times, cells were harvested and nucleic acid content was measured by spectrophotometry (λ=260 nm). Each point is the mean ± SD (n=3). At each time, means with an asterisk (*) are significantly different ($P < 0.05$) than the corresponding control.

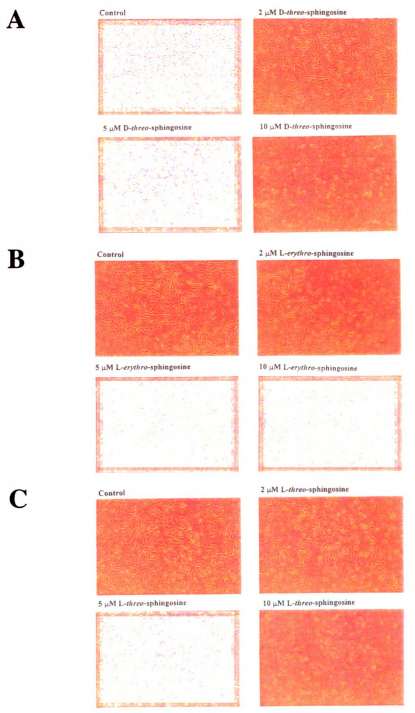
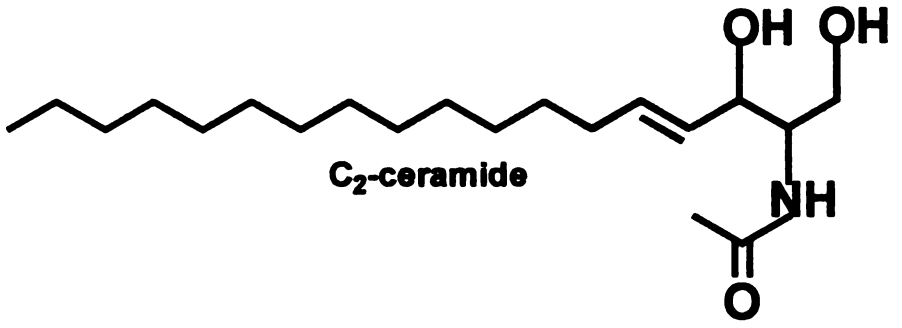


Figure 14. Sphingosine stereoisomers inhibit growth and induce death of human breast cancer cells (photographs). Subconfluent MDA-MB-231 cells were cultured with D-*threo*-sphingosine (A), L-*erythro*-sphingosine (B), and L-*threo*-sphingosine (C) at 0, 2, 5, or 10 μM for 24 hours.



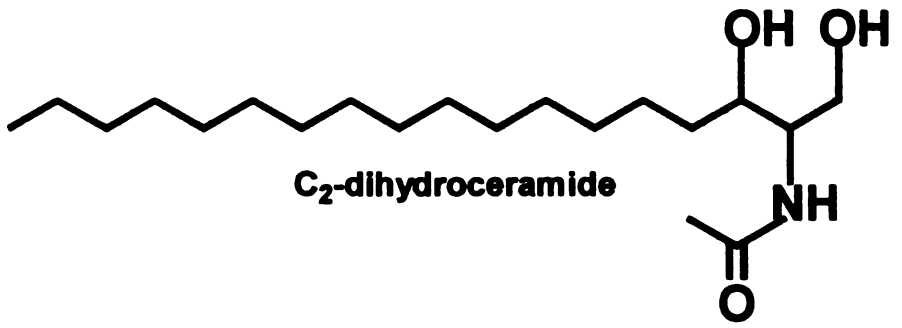


Figure 15. Structures of C_2-ceramide and C_2-dihydroceramide

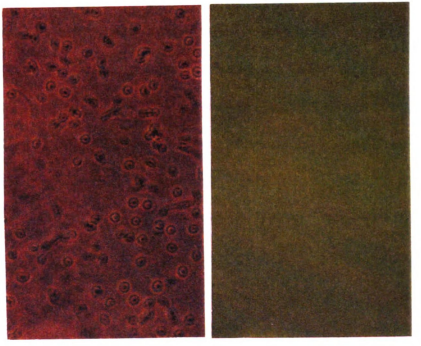


Figure 16. C_2-dihydroceramide does not cause DNA strand breaks in human breast cancer cells. Subconfluent MDA-MB-231 cells were cultured with C_2-dihydroceramide for 4 hours and DNA strand breaks were labeled with fluorescein *in situ* via the TUNEL reaction. The left panel represents cells under phase contrast and the right shows cells under fluorescent light.

V. DISCUSSION

Breast cancer is one of the most prevalent malignant cancers encountered in the western world (Marshall, 1993) and currently a great deal of interest is focused on ways to prevent and treat this disease. Combinations of surgery, radiation therapy, hormone therapy and chemotherapy are used to treat breast cancer patients. Although chemotherapy helps to improve survival of patients, the prognoses are far from satisfying, especially for advanced breast cancer patients who are endocrine resistant, estrogen receptor-negative, or who have life-threatening metastasis. The present study has utilized MDA-MB-231 human breast cancer cells as a model system to evaluate the chemotherapeutic potential of several sphingolipids. MDA-MB-231 cells were derived from a breast adenocarcinoma (Cailleau *et al.*, 1974). These cells are estrogen receptor-negative and resistant to a variety of drugs. For example, they are insensitive to antiestrogens (*e.g.* estradiol, tamoxifen, benzothiophene) (Thompson *et al.*, 1988; Lippman *et al.*, 1989), DNA-damaging agents (*e.g.* preactivated merocyanine 540, merodantoin) (Sharma *et al.*, 1995), anti-polyamine (*e.g.* alpha-difluoromethyl-ornithin) (Manni *et al.*, 1992), retinoic acid (Yu *et al.*, 1996) and some apoptotic inducing agents (Nakshatri *et al.*, 1997). Thus, the MDA-MB-231 cell-line provides an excellent model to study chemotherapy *in vitro* for those advanced breast cancer patients who have negative estrogen receptor status. This study demonstrates that both D-*erythro*-sphingosine and ceramide inhibit growth and cause death of MDA-MB-231 human breast cancer cells. Although a number of other studies have shown that both sphingosine and ceramide can kill cancer cells (Ohta *et al.*, 1995; Jayadev *et al.*, 1995; Jarvis *et al.*, 1996; Cai *et al.*, 1997), we believe our study is the first to show that these sphingolipids are cytotoxic for estrogen receptor-negative human breast cancer cells.

There are several possible modes by which D-*erythro*-sphingosine and ceramide might kill human breast cancer cells. For example, these sphingolipids might induce apoptosis, a highly-regulated form of physiological cell death which occurs only in targeted cells. Cellular shrinkage, chromatin condensation, activation of an endogenous endonuclease which causes extensive DNA fragmentation, and formation of apoptic bodies are key events in the process of apoptosis (Kerr and Harmon; 1991, Wyllie *et al.*, 1992). Alternatively, sphingosine and ceramide could cause death of human breast cancer cells via a non-apoptotic mechanism such as necrosis which is highly unregulated and not targeted to specific cells. In the present study, though ceramide did induce DNA strand breaks indicative of DNA damage, it did not cause DNA fragmentation that was detectable via agarose gel electrophoresis. Moreover, flow cytometric analysis revealed the presence of a subpopulation of cells in the pre-G_0/G_1 region in the form of a plateau indicative of necrosis. In contrast, D-*erythro*-sphingosine caused DNA strand breaks, DNA fragmentation on agarose gel electrophoresis, and a pre-G_0/G_1 peak indicative of apoptosis.

Currently, many successful chemotherapeutic treatments involve induction of apoptosis in the target cancer cells (Kaufmann, 1989; Walker *et al.*, 1991; Huschtscha *et al.*, 1996). Apoptotic inducing agents are appealing in cancer chemotherapy because they have two significant advantages: 1) Apoptotic agents eliminate cancer cells from the human body instead of just inhibiting the growth of cancer cells. Clinically, this is considered a "true" cure; and 2) Apoptotic agents have low side effects in cancer patients because they selectively kill cancer cells without having cytotoxic effects on neighboring normal tissue. The finding that ceramide kills breast cancer cells via a non-apoptotic

mechanism suggests that it may not be very effective as a chemotherapeutic agent because of its potential lack of specificity of action. In contrast, the ability of D-*erythro*-sphingosine to induce apoptosis in MDA-MB-231 cells strongly suggests that this compound has potential as a chemotherapeutic agent either alone or in a combination regimen. In chemotherapy, combination regimens (*e.g.* CMFVP, cytoxan, methotrexate, fluorouracil, vincristine, prednisone) are more commonly used than single cytotoxic agents (*e.g.* doxorubicin) because it is often possible to lower the dosage of the individual agents (and minimize side effects) while still generating higher response rates (Clavel and Catimel, 1993).

Our discovery that D-*erythro*-sphingosine kills human breast cancer cells by inducing apoptosis; whereas, ceramide kills the cells via a non-apoptotic pathway was puzzling in light of the ability of cells to metabolize ceramide to sphingosine. Our subsequent results suggest that this may be due to the inability of ceramide to raise cellular sphingosine high enough to induce apoptosis. C_2-ceramide at 10 μM only increased the cellular sphingosine concentration to about 1 nmol/mg protein; whereas, exogenous D-*erythro*-sphingosine at 5-10 μM (which was apoptotic) increased the cellular sphingosine concentration to about 5-10 nmol/mg protein. These findings suggest that the critical concentration of cellular sphingosine necessary to induce apoptosis is 5-10 nmol/mg protein.

The mechanism by which sphingosine induces apoptosis of human breast cancer cells is not clear. Apoptosis could be due to a direct action of sphingosine on a variety of systems they are known to affect (Merrill *et al.*, 1997). For example, sphingosine has been shown to inhibit protein kinase C (Hannun *et al.*, 1986), phosphatidic-acid phosphohydrolase (Lavie and Liscovitch, 1990; Jamal *et al.*, 1991; Mullmann *et al.*, 1991),

and the Na^+/K^+-ATPase (Oishi *et al.*, 1990) and to activate the epidermal growth factor receptor kinase (Faucher *et al.*, 1988; Wedegaertner and Gill, 1989) and other sphingosine-specific protein kinases (Pushkareva *et al.*, 1992). In addition, D-*erythro*-sphingosine has been shown to induce apoptosis via suppression of *bcl-2* in HL-60 cells (Sakakura *et al.*, 1996) and via suppression of *bcl-x_L* in androgen-independent human prostatic carcinoma DU-145 cells (Shirahama *et al.*, 1997). A recent study has demonstrated that MDA-MB-231 human breast cancer cells have constitutively active nuclear factor-κB (*NF-κB*) which protects the cells from apoptotic agents (Nakshatri *et al.*, 1997). Therefore, sphingosine probably induces apoptosis by overcoming or bypassing this protective mechanism.

Our findings that both D-*erythro*-sphingosine and C_2-ceramide kill estrogen receptor-negative breast cancer cells raises the possibility that close structural analogs may be even more effective as chemotherapeutic agents. Our observation that C_2-dihyroceramide does not induce DNA strand breaks or kill cells strongly indicates that the 4-5 *trans* double bond of the sphingoid base backbone is necessary for the cytotoxic effect of ceramide. Further, our discovery that the unnatural L-*threo* and D-*threo* stereoisomers of sphingosine are equally as potent as D-*erythro*-sphingosine and that L-*erythro*-sphingosine is more potent indicate that these compounds may also find utility as breast cancer chemotherapeutic agents. The more potent effect of L-*erythro*-sphingosine may be due to poorer cellular metabolism compared to the other sphingosine stereoisomers (Schroeder *et al.*, 1994).

The results of these studies raise several important new questions: Does sphingosine bypass a key protection step to cause the cells to undergo apoptosis? What are the downstream targets of sphingosine in the cell death signal transduction pathway? How is

the endonuclease activated during this process? The answers to these questions may lead to new means to treat estrogen receptor-negative breast cancer.

VI. SUMMARY AND FUTURE RESEARCH

The naturally occurring form of sphingosine, D-*erythro*, caused MDA-MB-231 estrogen receptor-negative human breast cancer cells to undergo apoptosis with an LD_{50} of ~5-10 μM. The concentration of cellular sphingosine necessary to induce apoptosis is ~5-10 nmol/mg protein. Like D-*erythro*-sphingosine, all of the synthetic, unnatural stereoisomers, L-*erythro*, L-*threo*, and D-*threo*, also caused death of MDA-MB-231 human breast cancer cells, though the mechanism of cell death is not yet known. Among the four stereoisomers, L-*erythro*-sphingosine was the most potent with an LD_{50} of ~2-3 μM; whereas, the potencies of L-*threo*- and D-*threo*-sphingosine were similar to that of D-*erythro*-sphingosine. C_2-ceramide also induced death of MDA-MB-231 human breast cancer cells, but via a non-apoptotic pathway. In contrast, C_2-dihydroceramide did not cause DNA strand breaks or cell death in breast cancer cells, indicating that the 4,5 *trans* double bond of the sphingoid base backbone is critical for ceramide to cause cell death.

The ability of D-*erythro*-sphingosine to induce apoptosis in estrogen receptor-negative human breast cancer cells suggests that this molecule has potential as a chemotherapeutic agent for postmenopausal women who are estrogen receptor-negative. Moreover, the finding that L-*erythro*-sphingosine is even more potent than D-*erythro*-sphingosine at inducing cell death suggests that L-*erythro*-sphingosine may be even more effective as a chemotherapeutic agent. Sphingosine stereoisomers could find roles in chemotherapy used either alone or in combination with other drugs.

Additional studies are necessary to further evaluate the potential of sphingosine stereoisomers as chemotherapeutic agents. Future studies should: 1) Determine whether

the unnatural stereoisomers (L-*erythro*, L-*threo*, and D-*threo*) cause death by inducing apoptosis; 2) Determine the molecular mechanism by which sphingosine stereoisomers induce apoptosis; and 3) Determine the effect of sphingosine stereoisomers on normal human breast epithelial cells. Studies which take the approach outlined above have the potential not only to further evaluate the potential of sphingosine stereoisomers as chemotherapeutic agents, but also to identify targets which are amenable to therapy and to assess the chemopreventive potential of sphingosine stereoisomers.

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