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Bioactive Sphingolipids Mediate Apoptosis and Senescence in Human Pancreatic Cancer Cells

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BIOACTIVE SPHINGOLIPIDS MEDIATE APOPTOSIS AND SENESCENCE IN HUMAN PANCREATIC CANCER CELLS

by

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ABSTRACT

Ceramide is a second messenger involved in apoptosis, cell differentiation, and growth arrest. We hypothesize that in tumor cells, insufficient sphingomyelin (SM) limits the amount of ceramide which can be generated for the probagation of the apoptotic signal resulting from treatment with chemotherapy. As a result, we postulated that exogenous SM will facilitate apoptosis and synergize with chemotherapeutic agents.

The current studies examined the response of the Panc1 human pancreatic cancer cell line to gemcitabine, a nucleoside analog, in the presence and absence of sub-toxic doses of SM. Cytotoxic dose response relationships demonstrated an increased chemosensitivity to gemcitabine with SM inclusion which was synergistic. Treatment with gemcitabine and/or SM led to an increased percentage of apoptotic cells with combination treatment as compared to the single agents. Gemcitabine treatment caused a decrease in the expression of anti-apoptotic bcl-2 family proteins in Panc1 cells, irrespective of the inclusion of SM. However, treatment of Panc1 cells with a combination of gemcitabine and exogenous SM resulted in a greater percentage of cells accumulating in the S phase when compared to single agent treatments. Changes in morphology and β-galactosidase activity in cells treated with gemcitabine were consistent with senescence and gemcitabine-induced senescence was abrogated by concomitant SM treatment. Moreover, exposure of Panc1 cells to C_8-ceramide demonstrated ceramide induces both apoptosis and senescence in a concentration dependent manner with senescence occurring at lower concentrations and apoptosis at high concentrations.

These findings support our proposed model which suggests a cell's ability to progress through the cell cycle, undergo apoptosis, or enter into senescence is associated

with intracellular ceramide levels. By supporting ceramide generation via exogenous SM addition, it is possible to increase apoptosis signaling in tumor cells and increase gemcitabine cytotoxicity. However, failure to generate sufficient signaling ceramide will redirect cells to senescence.

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INTRODUCTION

In the United States, pancreatic cancer is currently the fourth leading cause of cancer deaths in the United States, even though it is the eighth most common (http://www.cancer.gov/statistics). Because pancreatic cancer is very aggressive, is usually detected at an advanced stage, and does not respond well to chemotherapy or radiation, treatment options are limited (McKenna and Eatock 2003). Due to the involvement of local anatomical structures (i.e., the aorta), surgery is not an option for a significant number of patients. Even with surgery, less than 5% of patients have a life expectancy of greater than 5 years.

Since surgery has limited utility, greater focus is placed on systemic treatments for this type of cancer. These treatments rarely provide more than relief of some of the symptoms associated with the cancer, such as weight loss, pain, nausea, and anemia (Shirota, Haji et al. 2005). The nucleoside analog gemcitabine [2',2'-difluoro-2'-deoxycytidine], is the front line chemotherapeutic treatment for pancreatic cancer (Lawrence, Davis et al. 2001; Xiong, Rosenberg et al. 2004). It is taken up by the cell, where it is phosphorylated to difluorodeoxycytidine triphosphate (dFdCTP). By competing for DNA polymerase and ribonucleotide reductase, gemcitabine exposure results in decreased intracellular dNTPs, inhibition of DNA synthesis, and subsequent arrest of the cell cycle in S phase (Hui and Reitz 1997; Sun, Urrabaz et al. 2002). The decreased amount of dCTP within the cell can also increase the possibility of gemcitabine-triphosphate becoming incorporated in the DNA strand and, consequently stop the synthesis of DNA. Presently, gemcitabine has proved to be the best option for these patients, whether it is given alone or in combination with other treatments (Berlin,

Catalano et al. 2002; Bold, Chandra et al. 1999; Colucci, Giuliani et al. 2002; Xiong, Rosenberg et al. 2004). Unfortunately, even with gemcitabine treatment, the average survival time for patients with metastatic pancreatic cancer continues to be less than 6 months.

Apoptosis

Apoptosis, or programmed cell death, is a beneficial cellular process that takes place in response to various conditions (Ghobrial, Witzig et al. 2005; Ricci and Zong 2006; Westphal and Kalthoff 2003). These include regulation of normal cell number, some immune responses, and elimination of cells with irreparable DNA damage. When a cell undergoes apoptosis, specific proteases and nucleases are activated, leading to membrane blebbing, cell shrinking, condensation of chromatin, and nuclear fragmentation. As apoptosis progresses, cellular DNA is cleaved and phosphatidylserine moves from the inner to the outer leaflet of the plasma membrane. These changes allow macrophages to identify and remove apoptotic cells without releasing the cellular contents, thus avoiding an inflammatory response.

Apoptosis can occur through one of two pathways: 1) the extrinsic or membrane receptor pathway and 2) the intrinsic or mitochondrial pathway. Both of these pathways are interconnected and the members of one pathway will influence the other. Apoptosis is further regulated through additional protein interactions, including caspases, bcl-2 family proteins, and inhibitor of apoptosis proteins (IAPs).

Whether the signal for a cell to undergo apoptosis is the result of the intrinsic or extrinsic pathway, the pathways converge and result in the activation of a set of

sequence-specific proteases (caspases), which are present within the cell as inactive zymogens (procaspases) (Broker, Kruyt et al. 2005; Chang and Yang 2000; Ghobrial, Witzig et al. 2005). To date, 14 different caspases are known in mammals, and are divided into three groups depending on their function. Caspase 8, caspase 9, and caspase 10 are referred to as initiator or apical caspases and are activated by oligomerization. These caspases initiate the apoptotic pathways by activating downstream caspases (caspase cascades). Caspase 3, caspase 6, and caspase 7 are known as effector or executioner caspases. Once activated, effector caspases are responsible for cleaving cellular proteins irrespective of the apoptotic signal. The third group of caspases is composed of caspase-1, -4, -5, -11, -12, -13, and -14 and is associated with cytokine activity. The activation of caspases stops DNA replication and repair, and inactivates anti-apoptotic proteins. Caspases also act on the cytoskeleton and the nuclear lamina of the cell, resulting in the organized fragmentation of the cell and its organelles.

The extrinsic pathway is initiated as a result of the conditions present outside the cell (Broker, Kruyt et al. 2005; Ghobrial, Witzig et al. 2005; Johnstone, Ruefli et al. 2002; Ricci and Zong 2006). Activation of the extrinsic pathway requires the binding of extracellular ligands to receptors of the tumor necrosis factor family on the plasma membrane. After the ligand binds to the receptor, caspase 8 and caspase 10 bind to form the DISC (death-inducing signaling complex), ultimately resulting in the activation of downstream caspases. The extrinsic pathway is regulated by inhibitors of apoptosis proteins and c-FLIP.

The intrinsic pathway of apoptosis is initiated when damage is present inside of the cell (internal signaling). These stresses include damage to DNA, the presence of

reactive oxygen species (ROS), viral infection, and oncogene activation. Ultimately, this pathway involves the release of pro-apoptotic proteins from the mitochondria which initiate the caspase cascade via caspase 9 activation.

The intrinsic pathway is regulated by the bcl-2 protein family, whose presence or absence regulates mitochondrial membrane permeability and the release of apoptogenic factors from the mitochondria (Adachi and Imai 2002; Ghobrial, Witzig et al. 2005). The members of this protein family are divided into groups based on which of the four bcl-2 homology (BH) domains are present. All four BH domains (BH1-4) are present on the anti-apoptotic members of the bcl-2 family, which include Bcl-2, Bcl-x_L, Mcl-1, Bfl-1/A1, Bcl-W and Bcl-G. The pro-apoptotic members lack the BH4 domain and include Bax, Bak, Bok, Bik, Bim, Bcl-x_s, Krk, Mtd, Nip3, Nix, Noxa, Bcl-B. Members of the bcl-2 family containing only BH3 domains include Bid, Bad, Bim, Puma, Noxa, and Bmf and influence the process of apoptosis by either inhibiting anti-apoptotic or activating pro-apoptotic members (Ricci and Zong 2006; Westphal and Kalthoff 2003).

Bak and Bax are both pro-apoptotic members of the bcl-2 family normally found in the cytosol as monomeric proteins (Jia, Macey et al. 1999). Activation of the intrinsic pathway causes Bax and Bak to translocate to the outer membrane of the mitochondria (Kim, Emi et al. 2006; Lee, Hosotani et al. 1999). At the mitochondria, these proteins will interact with a voltage-dependent anion channel (VDAC)/adenine nucleotide translocator (ANT) complex, thus allowing for the formation of the mitochondrial transition pore (PTP) and resulting in the loss of mitochondrial membrane potential (MMP). This allows for the release of cytochrome c, Smac/DIABLO, and Omi/HrtA2

from the mitochondria (Shimizu, Narita et al. 1999). Once in the cytoplasm, Smac/DIABLO and Omi/HrtA2 will block the function of the inhibitors of apoptosis proteins. Meanwhile, cytochrome c binds to apoptotic protease activating factor 1 (Apaf-1), thus enabling it to engage procaspase-9 to form the protein complex called the apoptosome, activate caspase 9, and propagate the caspase cascade (Broker, Kruyt et al. 2005; Johnstone, Ruefli et al. 2002).

The presence of anti-apoptotic Bcl-2 and Bcl-x_L confer protection to the mitochondria by heterodimerizing with Bak and Bax (Oltvai, Milliman et al. 1993). Bcl-2 is found predominantly on the outer mitochondrial membrane, but also in the endoplasmic reticulum and the nuclear envelop, while Bcl-x_L is normally located in the cytosol (Lee, Hosotani et al. 1999). Although expression levels alone do not determine their function, both Bcl-2 and Bcl-x_L over-expression alter mitochondrial membrane permeability by blocking the formation of the transition pore. This limits the release of pro-apoptotic proteins, such as cytochrome c, from the mitochondria, consequently inhibiting apoptosis (Fleischer, Ghadiri et al. 2006; Yang, Liu et al. 1997). In addition, Bcl-x_L inhibits caspase activation by interacting with Apaf-1, which prevents the formation of the apoptosome (Scorrano and Korsmeyer 2003).

Bad is a pro-apoptotic protein normally found in its inactive form and bound to 14-3-3 scaffold proteins in the cytosol, which prevents Bad from accessing Bcl-2 and Bcl-x_L in the mitochondria (Hashimoto, Hirose et al. 2005; Zha, Harada et al. 1997). Dephosphorylation of the serine residues activates Bad and detaches it from the scaffold proteins (Adachi and Imai 2002; Ayllon, Martinez et al. 2000; Kitada, Krajewska et al.

1998). This enables Bad to move to the mitochondria where it heterodimerizes and inactivates anti-apoptotic proteins, Bcl-2 and Bcl-x_L, thereby promoting apoptosis.

Sphingolipids

Ceramide is an intracellular second messenger involved in apoptosis, senescence, cell cycle, cell differentiation, and growth arrest (Ogretmen 2006; Ruvolo 2003). Cellular stresses, including the presence of cytokines of the TNF family, environmental stress (heat shock), withdrawal of growth factors, radiation (ionizing or UV), and chemotherapeutics, cause ceramide accumulation in the cell. Although the details are not yet clear, it is believed ceramide may function as a "biostat" by measuring and indicating cellular stress. Ceramide affects a number of signaling proteins including cathepsin D, ceramide-activated protein phosphatases (CAPPs), which include PP1 and PP2A, ceramide-activated protein kinase (CAPK, also known as kinase suppressor of Ras), MAPK, RAF-1, PKCζ, and MEKK (Perry and Hannun 1998). Due to the variety of important regulatory proteins ceramide may interact with, it is able to function as a pro-apoptotic signal in both the intrinsic and extrinsic pathways of apoptosis.

Ceramide levels are governed by the rates of formation (*de novo* synthesis and activity of sphingomyelinases) and degradation. *De novo* ceramide synthesis is initiated on the cytosolic face of the endoplasmic reticulum with the condensation of serine and palmitoyl coenzyme A to yield sphinganine (Perry and Hannun 1998; Pettus, Chalfant et al. 2002). This is followed with acylation and dehydrogenation to form ceramide (Figure 1). Once formed, ceramide is transported to the Golgi by the ceramide transfer protein (CERT) where it is used in the synthesis of sphingomyelin and

glycosphingolipids. Through the action of sphingomyelin synthase, phosphatidylcholine (PC) transfers a phosphocholine headgroup to ceramide, forming sphingomyelin (SM) and diacylglycerol (DAG). *De novo* synthesis can be stimulated by Fas and some chemotherapeutics (e.g. Dox).

Sphingomyelinases (SMases) hydrolyze SM to generate ceramide (Perry and Hannun 1998; Ruvolo, Clark et al. 2002). To date, several SMases have been identified and are recognized by their pH optima and location in the cell. Acid sphingomyelinase (aSMase) is located primarily in lysosomes and, to a lesser extent, in caveoli or lipid rafts in the plasma membrane, while neutral sphingomyelinase (nSMase) is found in the plasma membrane, microsomes, Golgi, and mitochondria (Mathias, Pena et al. 1998; Pettus, Chalfant et al. 2002).

Additional bioactive lipids are generated from the hydrolysis of ceramide by ceramidases (CDases) to yield sphingosine or by ceramide kinase to yield ceramide-1-PO_4 (C1P) (Mathias, Pena et al. 1998; Ruvolo, Clark et al. 2002). In turn, sphingosine can be phosphorylated by sphingosine kinases (SK1 and SK2) to produce sphingosine 1-phosphate (S1P). Furthermore, S1P can be dephosphorylated by S1P phosphatases to regenerate sphingosine or it can be cleaved by S1P lyase to produce ethanolamide-1-phosphate.

Ceramide levels influence the release of pro-apoptotic proteins from the mitochondria (Perry and Hannun 1998; Ruvolo 2003; Siskind 2005). The presence of excess ceramide in the mitochondria causes the activation of PP2A. Activation of PP2A leads to the inactivation of anti-apoptotic Bcl-2 and PKCα, resulting in mitochondrial

depolarization, the release of cytochrome c, and the activation of the apoptosome (Lee, Hannun et al. 2000; Ruvolo, Deng et al. 1999; Zhang, Liu et al. 1997).

The enzymes necessary for ceramide production (*de novo* and via SMases) are formed in different subcellular compartments and may determine the signaling pathway that is activated (Mathias, Pena et al. 1998). For instance, treating cells with exogenous bacterial SMase promotes the cleavage of SM to generate ceramide but does not lead to apoptosis. Thus it can be concluded that SM pools located on the outer leaflet of the plasma membrane are not used as sources of ceramide for apoptosis (Linardic and Hannun 1994). However, targeting bacterial SMase to different cellular compartments, via the introduction of protein localization signals within bacterial SMase, induced apoptosis only when the enzyme was targeted to the mitochondria. In addition, ceramide generated via the *de novo* pathway in the mitochondria has also been associated with apoptosis induction after chemotherapy exposure (Birbes, El Bawab et al. 2001).

Studies have shown the levels of SM in tumor cells differ from those of normal cells (Dyatlovitskaya 1998; Kandyba, Kobliakov et al. 2004). One hypothesis is that tumor cells are unable to initiate apoptosis due to a lack of sufficient SM which limits ceramide levels in the cell. Since ceramide is necessary for apoptosis signaling, tumor cells can bypass the process. Thus, it has been postulated that rational manipulation of ceramide levels will lead to enhanced efficacy.

Senescence

Cellular senescence is one response of cells to the presence of DNA damage (Campisi 2000; Dimri 2005; Kahlem, Dorken et al. 2004; te Poele, Okorokov et al.

2002). Senescent cells are characterized by their flattened appearance, enlarged size and increased cytoplasmic granularity due to an increase in the presence of vacuoles. These changes are accompanied by alterations in gene expression, protein processing, and metabolism. One of the characteristic indicators of senescence is an increase in senescence-associated β-galactosidase activity, which is thought to be the result of greater lysosomal mass in the cells.

Because senescent cells are present in cell cycle arrest (G_0), they are unable to synthesize DNA (Dimri 2005; Kahlem, Dorken et al. 2004; Schmitt 2007; Venable, Lee et al. 1995). These cells remain viable, though mitotically inactive. It is important to note that these cells will continue to be metabolically active and may produce proteins that stimulate tumor activities, such as growth factors, cytokines, and metalloproteases. However, once cells become senescent, they may be unable to enter into apoptosis even if they are exposed to apoptosis-inducing agents.

There are two types of senescence: Replicative and premature. Replicative senescence presumably limits a cell's ability to proliferate and is initiated as a result of telomere shortening due to normal replication (Ben-Porath and Weinberg 2004; d'Adda di Fagagna, Reaper et al. 2003; Kahlem, Dorken et al. 2004; Shay and Wright 2005). Telomeres are the repetitive DNA sequences whose function in the cell is to protect the ends of the chromosomes. Conversely, the induction of premature senescence can come about as a result of DNA damage generated from physiological stresses (Lloyd 2002). Although initially reversible, premature senescence may be irreversible through unknown mechanisms (Beausejour, Krtolica et al. 2003). Irrespective of the stress signal, both the

p53 and *retinoblastoma* (*Rb*) tumor suppressor genes are associated with senescence induction (Shay, Pereira-Smith et al. 1991).

Rb regulates cell proliferation by controlling progression through the G_1 restriction point of the cell cycle. Rb is normally inactive as a result of cyclin dependent kinases (CDKs), which maintain Rb in a phosphorylated state (Campisi 2001). These CDKs are inhibited by p16, p21, and p27, which are often up-regulated in senescent cells (Bringold and Serrano 2000; Roninson 2003). As a result, senescent cells will express active Rb which inhibits the genes involved in cell-cycle progression. However, if Rb is inactivated, cells will re-initiate cell cycle progression and come out of senescence.

Through *in vitro* studies using the Panc1 human pancreatic cancer cell line, the effects of exogenously administered SM in combination with gemcitabine were examined with respect to the enhancement of the apoptotic effects. However, during the course of this work, the focus of the study was expanded to include senescence.

MATERIALS AND METHODS

Cell Culture

The human pancreatic cancer line Panc1 was obtained from Dr. David Gold (Garden State Cancer Center, Belleville, NJ) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Irvine Scientific, Santa Ana, CA) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% Penicillin-Streptomycin-Glutamine (Gibco, Grand Island, NY), 1% non-essential amino acids (Irvine Scientific), and 1% sodium pyruvate (Irvine Scientific). The cells were maintained at 37 °C with 5% CO_2.

Preparation of SM

SM liposomes were generated following published protocols (Modrak, Rodriguez et al. 2002; Modrak, Cardillo et al. 2004). Egg yolk SM (Avanti Polar Lipids, Alabaster, AL) was prepared as a 100 mg/ml suspension in PBS (4.3 mM KH_2PO_4, 1.4 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl) and sonicated for 10 minutes in a warm water bath. The suspension was subsequently passed through 1.0 and 0.1 μm filters with the Avanti Mini-Extruder.

Cell Viability Assay

Cell viability assays were performed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich, St. Louis, MO) quantitative colorimetric assay for mammalian cells (Mosmann 1983). Panc1 cells were harvested using trypsin (Irvine Scientific) and seeded in 96-well plates at 2000 cells/well.

Approximately 24 hours after plating, cells were treated with gemcitabine (Eli Lilly, Indianapolis, IN), SM, or a combination of gemcitabine and SM. Plates were incubated for 4 days (approximately four doublings). Media was removed from wells and replaced with a 0.5 mg/ml MTT in DMEM without additives. After 2-4 hours, the MTT solution was removed and the cells were solubilized with DMSO. Plates were read at A_{570} on a SpectraMax 250 Microplate Reader (Molecular Devices, Sunnyvale, CA). Data was normalized to untreated cells and values were multiplied by 100% to determine the percent of viable cells.

SM Uptake

Panc1 cells were seeded in 6-well plates at 2000 cells/well and allowed to adhere overnight. The media and unattached cells were removed and the cells were treated with 0.5, 0.2, or 0.1 mg/ml [^{3}H-methyl]choline-SM (American Radiolabeled Chemicals, St. Louis, MO) at a specific activity of 1501.6 dpm/nmole SM or 0.5, 0.2, or 0.1 mg/ml unlabeled SM. At 0, 4, 24, 48, 72, and 96 hours post-treatment, media was removed, wells were washed three times with 1X PBS, and cells were solubilized with 1% sodium dodecyl sulfate (Sigma-Aldrich) in water. Samples containing [^{3}H-methyl]choline-SM were transferred to scintillation vials containing 10 ml Ecoscint (National Diagnostics, Atlanta, GA) and radioactivity was determined by using a Liquid Scintillation Analyzer (Packard Instrument Company, Downers Grove, IL). Identically treated, but non-radioactive, samples were analyzed by spectrophotometry at A_{260} and A_{280} to determine protein concentration using the formula: protein (mg/ml) = 1.56 (A_{280}) – 0.7363 (A_{260}). This equation is from the nomograph prepared by E. Adams (distributed by the California

Corporation for Biochemical Research, Los Angeles 63, CA), which was based on the extinction coefficient for enolase and nucleic acid (Warburg and Christian 1942).

Analysis of Apoptosis

Throughout the course of this study, Panc1 cells were treated in the presence of gemcitabine at the IC_{50} and/or SM at the IC_{10} for 4 days before harvesting for subsequent analyses. Panc1 cells to be left untreated or treated with SM were seeded at 0.5×10^6 cells/flask while cells to be treated with gemcitabine or a combination of gemcitabine and SM were seeded at 10^6 cells/flask and allowed to adhere overnight. The media was replaced with treatment media containing 100 nM gemcitabine, 0.2 mg/ml SM, or a combination of the two and the cells were incubated in treatment for 4 days before processing.

Apoptosis was measured with Annexin V-propidium iodide staining and subsequent flow cytometry (Vermes, Haanen et al. 1995). Cells were harvested by scraping and combined with non-adhered cells present in the culture media. The cells were washed with PBS and pellets were resuspended in 250 µl 1X binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Five microliters of Annexin V-FITC (BD Biosciences Pharmigen, San Diego, CA) and 10 µl propidium iodide (Sigma-Aldrich) solution (50 µg/ml stock), were added directly to tubes. Samples were allowed to stand at room temperature, in the dark, for 15 minutes. Immediately before analysis, the samples were diluted with 1.5 times volume PBS. Fluorescence was measured using the Becton-Dickenson FACSCaliber (BD Bioscience Pharmigen). Using

the CellQuest Software, apoptotic cells were identified as being Annexin V-FITC positive while non-viable cells were distinguished by propidium iodide staining.

Preparation of Protein Cell Lysates and Western Blot Analysis

Cells (both adhered and non-adhered) were harvested by scraping, collected at 500 x *g* for 5 minutes, and washed once with PBS. Cell pellets were resuspended in 1 ml PBS and transferred to eppendorf tubes. Samples were centrifuged at 500 x *g* and pellets were resuspended in deionized water. A 10% sodium dodecyl sulfate solution was added to samples for a final concentration of 1% SDS. Each sample was drawn through an insulin syringe to lyse and shear DNA. All cell lysates were stored at -80 °C.

The concentration of total protein was estimated by spectrophotometry at A_{260}/A_{280}. For each sample, 20 µg total protein was heated in sample buffer (final concentration was 70 mM sodium dodecyl sulfate, 60 mM TrisCl, pH 8.0, 100 mM glycerol, 40 mM 2-mercaptoethanol, and trace bromophenol blue) for 5 minutes at 95 °C. The samples were separated on 4-20% SDS gradient gels (Life Therapeutics, Frenchs Forests, Australia) using a Tris-HEPES-SDS running buffer (Life Therapeutics). Proteins were transferred to polyvinylidene fluoride (PVDF) microporous membranes (Millipore, Bedford, MA) for 90 minutes at 40 V. Membranes were blocked overnight at 4 °C with Superblock Blocking Buffer (Pierce, Rockford, IL) containing 0.05% Tween-20 (JT Baker, Phillipsburg, NJ). Antibodies for β-actin (Sigma-Aldrich), Bad, Bax, Bcl-2, Bcl-x_L, p16, p21, and Rb (BD Biosciences Pharmigen) were used to probe blots for 1 hour at room temperature. Blots were washed in Tris buffered saline (20 mM TrisCl, pH 7.5, 100 mM NaCl) four times for 20, 10, 10, and 10 minutes respectively,

followed by a 30 minute incubation with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG antibody (Rockland Immunochemicals, Gilbertsville, PA). Blots were washed a second time, as before. SuperSignal chemoluminescent substrate (Pierce) was used to visualize protein expression. Images were analyzed using the TotalLab software (Nonlinear Dynamics Ltd, Durham, NC) and data was normalized to β-actin.

RNA Isolation

Total RNA was isolated with Trizol (Gibco, BRL) (Ausubel and Seidman 1988). Cells (both adhered and non-adhered) were harvested by scraping, collected at 500 x *g* for 5 minutes, and washed once with PBS. Pellets were resuspended in PBS and transferred to RNase free tubes. Samples were centrifuged and PBS was removed. The cells were disrupted by the addition of 1 ml Trizol and incubated at room temperature for 5 minutes. One fifth volume chloroform was added and the tubes were vigorously shaken by hand, incubated for 3 minutes, and centrifuged at 20,800 x *g* for 15 minutes. The aqueous phase was transferred to new RNase free tubes and isopropanol was added to each sample at 0.5 the original volume of Trizol per tube. Samples were incubated at room temperature for 10 minutes, centrifuged at 20,800 x *g* for 10 minutes and the supernatant removed. Pellets were washed once with 75% ethanol. Excess ethanol was removed by air drying and samples were stored at -80 °C.

RNA pellets were resuspended in DEPC H_2O and re-purified using the RNeasy Mini Kits (Qiagen, Valencia, CA) according to the supplied protocol. Prior to loading the columns, samples were treated with RNase-free-DNase (BD Biosciences Pharmigen)

for 15 minutes to remove DNA contamination. Samples were eluted from the columns with 30 µl RNase-free water.

RNA quantity and quality were determined by measuring optical density at 260 and 280 nm using spectrophotometry. RNA concentration was calculated as 1 OD_{260} is equivalent to 40 µg/ml RNA. The quality of RNA was established by determining the ratio of absorbance at 260 and 280 nm and examined by denaturing (formaldehyde) agarose gel electrophoresis.

First-strand cDNA Synthesis

The Brilliant SYBR Green QPCR Core Reagent Kit (Stratagene, La Jolla, CA) was used to generate cDNA from purified RNA as per manufacturer's instructions. For each reaction, 5 µg RNA in DEPC H_2O was primed with oligo$(dT)_{20}$. Samples were heated to 65 °C for 5 minutes and subsequently allowed to slowly cool to 37 °C for 10 minutes. A reaction mix containing dATP, dCTP, dGTP, dTTP, StrataScript buffer, RNase Block Ribonuclease Inhibitor, and StrataScript reverse transcriptase was prepared and added according to the Stratagene first-strand cDNA synthesis protocol supplied with the reverse transcriptase. Samples were incubated at 42 °C for 1 hour and the reaction was stopped by heating to 95 °C for 5 minutes. Samples were stored at -20 °C.

Real-time Quantitative PCR (QPCR)

Human Apoptosis RT^2*Profiler*™ PCR Arrays (SuperArray Bioscience Corporation, Frederick, MD) were used to amplify cDNA with the Brilliant SYBR® Green QPCR Core Reagent Kit (Stratagene). As per manufacturer's instructions, a

master mix consisting of 1X core buffer, 4 mM $MgCl_2$, 0.8 mM dNTP mix, 8% glycerol, 3% DMSO, 30 nM reference dye, 0.167X SYBR Green, and 1.25 Units Taq DNA polymerase was prepared. A total of 5 µg cDNA sample was added to the master mix for a final concentration of 50 ng cDNA per reaction (25 µl final volume per reaction). Equal amounts of the sample mixture were then added to the wells of the microarray which contain the primer sets for specific genes involved in apoptosis. Using the Mx3000P® QPCR System (Stratagene), samples were subjected to an initial denaturation cycle of 10 minutes at 95 °C; 40 cycles of 30 seconds denaturation at 95 °C; 1 minute annealing at 50-60 °C; 1 minute elongation at 72 °C.

The C_t, or threshold cycle, for each reaction was determined by the QPCR software. The average C_t of both GAPDH and β-actin was used to normalize expression. The fold change in gene expression between untreated and treated Panc1 was calculated as $2^{|-\Delta\Delta Ct|}$. A fold change greater than 1 corresponded to a "-fold up-regulation" and a change less than 1 was reported as the negative inverse (i.e. "-fold down-regulation").

Cell Cycle Analysis

Cell cycle distribution was analyzed by propidium iodide staining (Noguchi 1998). Panc1 cells were harvested by scraping, washed, and resuspended in 0.5 ml PBS. The cells were fixed by adding 4.5 ml of 70% EtOH and refrigerated overnight. Cells were collected by centrifugation (200 x *g*, 5 minutes) and washed once with PBS. The cell pellet was resuspended in propidium iodide staining solution (0.1% (v/v)

Triton X-100 in PBS, 0.2 mg/ml DNAse-free RNase A, and 20 μg/ml propidium iodide). Samples were incubated for 15 minutes at 37 °C and DNA content was analyzed by flow cytometry.

Senescence Detection (β-galactosidase Activity)

Senescent cells were identified by examining β-galactosidase activity using C_{12}-FDG (lipophilic fluorescein-di-β-D-galactopyranoside). In senescent cells, the presence of β-galactosidase results in the cleavage of galactose from FDG, leaving the fluorescent molecule associated with the cellular membrane. Panc1 cells were seeded at 10,000 cells/well in 24-well plates and allowed to adhere overnight. The media in each well was replaced with drugs at varying concentrations in the presence of 5 μM C_{12}-FDG. Plates were incubated for 4 days at 37 °C with 5% CO_2. Cells were harvested using 2 mM EDTA in PBS and centrifuged at 500 x *g* for 5 minutes. Pellets were resuspended in 250 μl PBS plus 5 μg/ml propidium iodide and analyzed by flow cytometry.

Statistical Analysis

All data has been expressed as mean ± standard deviation. Student's t-tests were used to determine statistical significance. Only statistically significant *P* values ($P < 0.05$) were reported. Calculation of combination index (CI) was used to determine synergy (Chou and Talalay 1984). The CI values were calculated by determining the percentage of cell growth inhibited by gemcitabine and SM individually and in combination using the formula: $CI = (D_1/Dx_1) + (D_2/Dx_2) + (D_1D_2/Dx_1Dx_2)$, where Dx is the IC_{50} for an individual drug, D is the IC_{50} for an individual drug in the combination

treatment, and the subscripts 1 and 2 refer to gemcitabine and SM, respectively.

Synergism is defined as $CI < 0.9$, additivity as $0.9 < CI < 1.1$ and antagonism as $CI > 1.1$.

RESULTS

Panc1 cells exhibit an increased chemosensitivity to gemcitabine treatment in the presence of SM.

To determine whether exogenous SM can increase chemosensitivity, Panc1 cells were treated with SM alone for 4 days to assess treatment sensitivity. The effect of the treatment on the metabolic viability of the cells was determined using the MTT assay. A dose response profile was generated and the concentrations needed to inhibit cell growth by 50% (IC_{50}) for SM was determined to be 1.2 mg/ml and the IC_{10} (10% growth inhibition) was 0.2 mg/ml (data not shown).

The effects of gemcitabine alone and in combination with 0.2 mg/ml SM on Panc1 were examined by the MTT assay (Figure 2). The IC_{50} for gemcitabine decreased from 78.3 ± 13.7 nM to 13.0 ± 3.0 nM with the inclusion of SM in the treatment ($P < 0.001$). A CI value of 0.36 indicated a synergistic effect between gemcitabine and SM in Panc1 cells. It is also noteworthy that even at the highest doses of gemcitabine approximately 35% of the cells continued to be viable. With the addition of SM, < 10% of the cells remained viable at the highest concentrations.

Uptake of Exogenous SM in Panc1 Cells Increases Over Time.

To determine if Panc1 cells can internalize exogenously administered SM, cells were exposed to 0.5, 0.2, or 0.1 mg/ml [^{3}H-methyl]choline-SM or non-labeled SM and aliquots of cells were analyzed at 0, 24, 48, 72, and 96 hours post-treatment. Samples treated with labeled SM were subjected to liquid scintillation analysis to determine the amount of [^{3}H-methyl]choline-SM present in the cells. Cells treated with non-radioactive

SM were used as a control for normal cell proliferation by determining total protein concentration at the given time points.

The amount of SM taken up by the cells was normalized to total protein levels. Results showed that by 48 hours, > 200 nmole SM (19.0 %) was taken up per mg protein in the presence of 0.5 mg/ml SM, 100 nmole (29.6 %) SM/mg protein with 0.2 mg/ml SM, and 20 nmole (13.8 %) SM/mg protein with 0.1 mg/ml SM (Figure 3). Thus it was concluded that Panc1 cells will incorporate greater amounts of SM with increasing concentrations. At the IC_{10} of SM, a significant increase in SM was taken up by the cells, which peaked at 48 hrs post-treatment.

Panc1 cells treated with a combination of gemcitabine and SM have increased apoptosis.

Apoptosis is a major pathway for the induced death of cells. To determine the percentage of cells undergoing apoptosis in response to treatment, Panc1 cells were treated with 100 nM gemcitabine, 0.2 mg/ml SM, or a combination of gemcitabine and SM for 4 days. The cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry to determine the cytotoxicity of the agents (Figure 4). Since the presence of phosphatidylserine (PS) outside the cell increases during apoptosis, cells stained with Annexin V-FITC, which is specific for PS, were considered to be apoptotic (Vermes, Haanen et al. 1995). Cell death was monitored with propidium iodide, which is actively effluxed in metabolically viable cells. The percentage of apoptotic Panc1 cells present with no treatment, SM alone, gemcitabine alone, and the combination treatments was $3.4 \pm 3.0\%$, $4.4 \pm 3.3\%$, $38.9 \pm 6.8\%$, and $48.4 \pm 6.6\%$, respectively (Figure 5). Both the

untreated and SM treatment groups exhibited a relatively equal percentage of apoptotic cells, which supports the idea that SM alone will not cause the cells to undergo apoptosis. Cells treated with gemcitabine at the IC_{50} show significant apoptosis and the presence of SM enhanced the toxic effect resulting in an increase in apoptotic cells versus gemcitabine alone.

Gemcitabine treatment causes a decrease in the expression of anti-apoptotic bcl-2 family proteins in Panc1 cells.

The effect of SM, gemcitabine, and combination treatment on the expression of pro- and anti-apoptotic proteins in Panc1 cells was examined. Cells were treated as previously described, total cell lysates were prepared, and Bad, Bax, Bcl-2, and Bcl-x_L expression was determined by western blot analysis (Figure 6). These proteins were investigated as a result of their importance in the regulation of apoptosis. β-actin was used as an internal control for each experiment. The relative image density of individual bands was normalized to β-actin and averaged over three experiments (Figure 7). Since untreated Panc1 cells were used as the controls for normal protein expression, its value was set to 1.0.

The results indicated that treating Panc1 cells with SM as a single agent caused a decrease in Bad expression and an increase in both Bcl-2 and Bcl-x_L. In the presence of gemcitabine alone, there was a decrease in the relative levels of Bad, Bcl-2 and Bcl-x_L. Although, combination treatment of gemcitabine and SM decreased levels of both Bcl-2 and Bcl-x_L when compared to the untreated, these changes were not significantly different from those observed with gemcitabine alone.

The current understanding of apoptosis induction suggests that absolute levels of bcl-2 family proteins alone are not sufficient for apoptosis induction, but the ratio of pro- to anti-apoptotic proteins may be more relevant (Breckenridge and Xue 2004; Strobel, Swanson et al. 1996). Although our studies indicated the levels of pro-apoptotic proteins decreased with gemcitabine and combination treatment groups, the levels of anti-apoptotic proteins were significantly lower in comparison. The ratios of total anti- to pro-apoptotic proteins were determined to be 1.47 with SM alone, 0.06 for gemcitabine alone, and 0.04 for combination treatments. Together, our data suggest additional factors are present to promote apoptosis in Panc1 cells.

Treatments alter the expression of genes associated with apoptosis.

To determine if the observed differences in protein expression of Panc1 cells in response to treatment were associated with transcription changes, RT-PCR microarrays were used. Panc1 cells were treated as before, RNA was isolated using Trizol, and gene expression profiles were generated by normalizing individual genes to the *GAPDH* and *β-actin* housekeeping genes for each experiment. Relative changes in gene expression were calculated using the $\Delta\Delta C_t$ method. It should be stressed that these are preliminary studies and that a comprehensive analysis of gene expression changes are outside the scope of this thesis.

These results suggested that changes in expression for a number of bcl-2 protein family genes were dependent on treatment. As shown in Figure 8, a 1.4-, 25.5-, 1.0-, and 13.2-fold increase ($P < 0.5$) in *Bad*, *Bax*, *Bcl-2*, and *Bcl-x_L* gene expression, respectively, was observed in Panc1 cells treated with SM alone. On the other hand, *Bad* and *Bcl-2*

was down-regulated in Panc1 cells treated with gemcitabine alone. Of interest is that the combination of gemcitabine and SM treatment also resulted in the down-regulation of *Bad*, but causes *Bcl-2* levels to return to that of untreated Panc1.

The use of PCR arrays offered the opportunity to obtain additional information for apoptosis-related gene expression (Figure 9). An up-regulation was seen in the expression of *Bak*, *caspase 8*, *caspase 10*, and *p53 binding protein* when Panc1 cells were exposed to SM treatment for 4 days. Gemcitabine alone down-regulated the genes for *caspase 3*, *caspase 8*, *caspase 10*, *FADD*, and *p53 binding protein*. By combining SM with the gemcitabine, an increase in expression of these pro-apoptotic genes similar to that of SM alone was seen. In the case of *caspase 10*, the increase in gene expression was actually greater in the combination treatment than with SM alone. However, *caspase 3* expression decreased with the both single agent gemcitabine and the combination treatment.

Analysis of cell cycle distribution in Panc1 treated cells.

By competing with DNA polymerase and ribonucleotide reductase, gemcitabine serves to inhibit DNA synthesis, subsequently causing an arrest of the cell cycle at the S phase. The cell cycle of Panc1 cells was analyzed to see how the treatment affected cell cycle distribution (Figure 10). Cells were treated with 100 nM gemcitabine, 0.2 mg/ml SM, or a combination of the two agents for a total of 4 days, as before. The cells were then fixed and stained with PI before cytometric analysis. Panc1 cells treated with SM alone did not exhibit significant changes in cell cycle distribution compared to the untreated, which confirmed that SM was non-toxic at the administered concentration.

Treatment with gemcitabine alone resulted in 44.9% of the cells accumulating in sub-G_1 with a concomitant decrease in the number of cells in G_0/G_1 and G_2/M phases. These changes were accented in cells receiving a combination of gemcitabine and SM treatment, where 57.4% of the cells accumulated in sub-G_1. These results indicate that the combination treatment is able to alter the cell cycle in a synergistic manner.

Gemcitabine treatment increases β-galactosidase activity in Panc1 cells.

Morphological changes consistent with senescence (i.e. large flat cells) were observed in Panc1 cells receiving gemcitabine treatment. With the knowledge that gemcitabine does not cause 100% of cells to undergo apoptosis and that an increase in the size of cells treated with this agent is observed, we examined the possibility of senescence involvement through the measurement of senescence-associated β-galactosidase activity.

By including C_{12}-fluorescein-digalactoside (C_{12}-FDG) in the cell culture media of Panc1 cells treated with gemcitabine and/or SM, senescence-associated β-galactosidase activity was examined. Cells expressing β-galactosidase are able to cleave the C_{12}-FDG, thus liberating the membrane-associated C_{12}-fluorescein component which can be detected by flow cytometry. Cells treated with gemcitabine alone exhibited a dose dependent increase in senescence-associated β-galactosidase activity, which was approximately 4.5 fold greater than the combination treatment (Figure 11). However, Panc1 cells treated with gemcitabine in the presence of SM exhibited nearly identical levels of β-galactosidase activity compared to untreated cells. This indicates that the

inclusion of SM altered the viable-senescent-apoptotic distribution of cells after gemcitabine treatment.

Expression of Rb, p16, and p21 in Panc1 treated cells.

The role of senescence induction in gemcitabine-treated Panc1 cells was examined by determining the expression levels of key cell cycle regulatory proteins. Panc1 cells were treated, and protein cell lysates were prepared 4 days post-treatment, as before, for western blot analysis of Rb, p16, and p21 (Figure 12). The relative density of each band was quantified and results were averaged over three experiments (Figure 13). Panc1 cells treated with SM alone showed increased expression of both Rb and p16 ($P < 0.001$). As a single agent, gemcitabine decreased expression of Rb compared to untreated cells and reduced p16 and p21 expression to undetectable levels. However, cells treated with both agents did not exhibit statistically significant changes in Rb or p21 over gemcitabine alone whereas p16 was detected in this treatment group.

C_8-ceramide induces apoptosis and senescence in Panc1 treated cells

Chemotherapeutic treatments may cause cells to undergo senescence. Our studies have shown that a combination of gemcitabine and SM results in increased apoptosis and reduced senescence. Previous work demonstrated that gemcitabine activated aSMase and increased ceramide formation (Modrak, Cardillo et al. 2004). In order to determine the relation between senescence and apoptosis induction as a result of gemcitabine treatment, Panc1 cells were treated with the water-soluble analog C_8-ceramide (N-octanoyl-D-sphingosine) in the presence of C_{12}-FDG. These cells were analyzed by flow cytometry

for β-galactosidase activity and cell viability using propidium iodide. Our results showed that treatment of Panc1 cells with C_8-ceramide for 4 days resulted in dose dependent cell death with the IC_{50} for C_8-ceramide being 43.2 ± 2.1 μM while concentrations below 30 μM did not affect cell viability (Figure 14). However, changes were also observed in senescence-associated β-galactosidase activity (mean channel fluorescence), which increased with ceramide concentration, reaching a maximum at 21.7 ± 1.5 μM and decreasing at higher ceramide concentrations. These results indicate ceramide induces both apoptosis and senescence in a concentration-dependent manner.

DISCUSSION

Malignant tumors develop when cells undergo uncontrolled proliferation and either lack the ability or are resistant to apoptotic stimuli (Gerhard, Schmid et al. 2002). Numerous mutations in pancreatic cancers, including the presence of cell growth factors, non-functional factors for growth inhibition, and mutated tumor suppressor genes, interfere with the induction of apoptosis, impart generalized drug resistance, and, ultimately, make treatments ineffective. Together, these factors dictate how the cancer will develop, progress, and metastasize.

Sphingolipids have been shown to play a central role in apoptosis regulation (Hannun 1996; Hannun and Obeid 2002; Itoh, Kitano et al. 2003; Ogretmen and Hannun 2001; Ogretmen and Hannun 2004). Ceramide serves as a secondary messenger by interacting with kinases, phosphatases, and proteases. Agents causing cellular stress affect the regulation of sphingolipid metabolism and increase the amount of ceramide present in the cell. Increased basal ceramide levels lead to chemosensitivity, while reduced levels lead to chemoresistance (Hannun and Luberto 2000; Itoh, Kitano et al. 2003; Ogretmen and Hannun 2001). For instance, stimulators of *de novo* ceramide synthesis (e.g., PSC-833), inhibitors of ceramidase [e.g., B13 or (1S, 1R)-D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol] and inhibitors of glycosylceramide synthase (e.g., tamoxifen or DL-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol) increase ceramide and result in chemosensitivity (Samsel, Zaidel et al. 2004; Selzner, Bielawska et al. 2001; Seumois, Fillet et al. 2007; Wang, Giuliano et al. 2002). Generally, it has been observed that chemotherapeutic agents increase ceramide levels

through SM hydrolysis primarily, but examples of chemotherapeutics increasing *de novo* synthesis are known (Ogretmen and Hannun 2001).

Levels of biologically active sphingolipids and enzymes participating in sphingolipid metabolism are altered in cancer cells though not in a predictable or uniform manner (Modrak, Cardillo et al. 2004; Senchenkov, Litvak et al. 2001). We hypothesized that chemoresistance in pancreatic cancer cells may be the result of insufficient levels of the sphingolipid, ceramide, which prevents apoptosis signaling. Furthermore, we hypothesized that this was a result of inadequate intracellular SM substrate available to the drug-activated SMases for conversion to ceramide. Thus, we postulated that concomitant treatment with exogenous SM would allow the cells to enter into apoptosis when treated with chemotherapeutic agents, in line with our previously reported results with colonic and pancreatic cell lines (Modrak, Lew et al. 2000; Modrak 2004).

Pancreatic cancer is a particularly aggressive and drug-resistant disease for which improvements in therapy are much needed. For this reason, we examined the use of exogenous SM to enhance the effect of gemcitabine in pancreatic cancer cells. Previous work demonstrated that gemcitabine activated acidic SMase but that increased ceramide production occurred only in the presence of exogenous SM (Modrak, Cardillo et al. 2004). This study showed no difference in ceramide content in cells that were treated with gemcitabine alone, while cells receiving SM alone had increased ceramide levels in the absence of increased apoptosis. Cells receiving combination treatments had an increase in the levels of ceramide present in the cells. In addition, mitochondrial depolarization, apoptosis, and cell death increased when cells were treated with gemcitabine and SM in combination versus individual agents.

Our initial experiments demonstrated that exogenous SM could enhance gemcitabine chemotherapy in a synergistic manner. Additional results in other pancreatic cell lines and with other drugs demonstrated that this effect was not unique to our Panc1-gemcitabine model (Modrak, personal communication). Similar results were obtained in a study by Tran, et al., where the use of sorafenib and nanoliposomal ceramide inhibited *in vitro* melanoma and breast cancer cell growth in a synergistic manner (Tran, Smith et al. 2008). Flow cytometric analysis confirmed that the inclusion of SM with gemcitabine treatment significantly increased the percentage of apoptotic cells compared to gemcitabine used as a single agent. Together, this evidence supports the idea that the levels of ceramide in gemcitabine treated Panc1 pancreatic cancer cells are not sufficient to signal apoptosis induction and this is likely a result of insufficient SM substrate.

Pancreatic cancer cells have altered expression of bcl-2 family proteins (both pro- and anti-apoptotic), which are crucial in determining chemosensitivity (Lawrence, Davis et al. 2001). Studies to examine the role of Bcl-x_L and Bax in determining apoptosis induction in pancreatic cell lines (Colo357, PancTu1 and Panc1) treated with gemcitabine showed that Bcl-x_L works to stabilize the mitochondrial transmembrane potential while Bax destabilizes it (Schniewind, Christgen et al. 2004). Our studies, showed treatment of Panc1 with gemcitabine as a single agent decreased both Bad and Bcl-x_L expression and reduced Bcl-2 to undetectable levels. Hinz, et al., suppressed Bcl-x_L protein expression with the use of antisense oligonucleotide treatment in pancreatic cancer lines, including Panc1, and demonstrated an increase in apoptosis in response to TRAIL (Hinz, Trauzold et al. 2000). Others have shown that Bcl-2 blocks the release of factors essential for apoptosis preserving mitochondrial membrane potential

through the sequestering of Bax and Bad (Ng, Tsao et al. 2000; Scaffidi, Schmitz et al. 1999). In a study conducted by Bold, et al., over-expression of Bcl-2 in pancreatic cancer cell lines increased gemcitabine chemoresistance (Bold, Chandra et al. 1999). Since untreated and SM treated Panc1 cells express Bcl-2, these cells would be more resistant to chemotherapy. Conversely, the lack of Bcl-2 expression in the cells treated with gemcitabine, both as a single agent and in combination with SM, indicates the cells would be more chemosensitive.

The ratio of pro- to anti-apoptotic proteins is important in determining cell survival and chemoresistance (Bold, Virudachalam et al. 2001; Mirjolet, Barberi-Heyob et al. 2000). When anti-apoptotic proteins such as Bcl-2 and Bcl-x_L are over-expressed, Bax function is suppressed because of the formation of Bcl-2/Bax or Bcl-x_L/Bax heterodimers, thereby inhibiting apoptosis (Hinz, Trauzold et al. 2000; Lee, Hosotani et al. 1999; Oltvai, Milliman et al. 1993). Increasing Bax levels after exposure to drugs (Rampino, Yamamoto et al. 1997) or via transfection-mediated over-expression (Basu and Haldar 1998) facilitates the formation of Bax/Bax homodimers and increases cell death. Zhang, et al., found that, in the absence of an increase in Bax, a decrease in Bcl-2 alone resulted in altered Bax/Bcl-2 ratios and apoptosis (Zhang, Yu et al. 2000). In addition, Adachi showed Bad to have the ability to induce apoptosis without interacting with Bcl-2 (Adachi and Imai 2002). Our results agree with these studies in that we also saw an increase in the pro- to anti-apoptotic protein ratio in the combination treatment group as compared to the untreated cells. However, the lack of difference between the combination and gemcitabine alone group suggests other factors are involved in the initiation of cell death and that protein levels alone are not sufficient to guide the process.

Mammalian cells will respond to cell injury by undergoing cell cycle arrest in an attempt to repair the damage. If the damage is too severe, cells will usually signal for apoptosis, but may undergo senescence (Hannun 1996; Schmitt, Fridman et al. 2002). In the present study, we observed gemcitabine-induced apoptosis in Panc1 cells is enhanced by the addition of SM. Cell cycle analysis of Panc1 cells demonstrated gemcitabine decreased the G_2/M cell population, resulting in an increase in the sub-G_1 (apoptotic) population. Although cell cycle arrest at the S phase is characteristic for gemcitabine (Lawrence, Davis et al. 2001; Ng, Tsao et al. 2000), the inclusion of exogenous SM with the gemcitabine treatment additionally increased the cells in the sub-G_1 phase of the cell cycle compared to single agent gemcitabine. Moreover, cells treated with SM alone showed no significant changes in cell cycle distribution compared to the untreated, thus reinforcing its non-toxic properties at the present concentration. Together, these results support our previous observation that by increasing ceramide levels in the cell through the administration of exogenous SM, there is an increase in the ability of cells to undergo apoptosis.

The complex role of ceramide in the cell includes the regulation of signaling pathways that result in G_0/G_1 cell cycle arrest, which may be followed by apoptosis (Bieberich 2004; Eto, Bennouna et al. 2003) or senescence (Ogretmen and Hannun 2004). Cells that have sustained DNA damage will be stopped at G_1 and G_2 checkpoints of the cell cycle, where lethal damage will result in apoptosis; however, non-lethal damage will result in autophagy or senescence (Kim, Emi et al. 2006). In our study, morphological changes consistent with those observed in senescent cells, such as increased cell size and flattened appearance, were evident in cells receiving gemcitabine

treatment. In addition, we observed that, typically, approximately 35-45% of the cells treated with gemcitabine alone remained metabolically viable. Together these observations suggested that a percentage of pancreatic cancer cells escaped cell death and instead entered into a state of senescence when treated with gemcitabine while the inclusion of SM with gemcitabine treatment allowed the cells to enter into apoptosis.

Increased senescence-associated β-galactosidase activity is the most commonly used marker for identifying senescent cells. Studies have examined β-galactosidase expression *in vivo* in clinical cancers and have shown a percentage of cells became senescent in tumors of treated patients and not in normal cells. One study with breast tumor resections showed a greater percentage of senescence-associated β-galactosidase expression in women who underwent chemotherapy than in resections obtained from untreated patients (about 10% in untreated patients and 42% in treated patients), indicating an increase in senescence cells with treatment (te Poele, Okorokov et al. 2002). Another study examined mice over-expressing the *Bcl-2* gene treated with cyclophosphamide. These results indicated an interruption in DNA replication in the tumors as well as a significant increase in β-galactosidase expression (Schmitt, Fridman et al. 2002).

We explored the possibility of senescence induction in gemcitabine treated Panc1 cells by examining the presence of senescence-associated β-galactosidase activity. Our findings showed a dose dependent increase in senescence-associated β-galactosidase activity in cells treated with gemcitabine alone, reaching a maximum at gemcitabine concentrations above 10 nM and well below the IC_{50} (as determined by the MTT assay). However, in the presence of SM, senescence-associated β-galactosidase activity remained

very low regardless of gemcitabine concentration. Together, this data demonstrated that Panc1 cells treated with gemcitabine entered into senescence, however, by combining gemcitabine and SM, the percentage of cells undergoing senescence was reduced.

Speculation exists as to the ability of senescent cell(s) to participate in tumor growth, either by re-entering cell cycling or by providing growth factors that support tumor growth (eg, VEGF). Both normal and treated cells undergo senescence under the influence of the *p53*, *p21*, and *p16* genes, all of which are often mutated or inactive in pancreatic cancer cells (Roninson 2003). Mice that have *p53*, *Rb*, or *p16* mutations are more likely to develop tumors, suggesting one role of senescence is to prevent the formation of tumors (Ben-Porath and Weinberg 2004). Since the *p16* gene is involved in the inhibition of several cell cycle regulators, mutations of this gene have been association with aggressive cell growth and up-regulation of tumor promoting factors (Roninson 2003; Talar-Wojnarowska and Malecka-Panas 2006). Numerous studies have provided evidence linking *p16* mutations in pancreatic cancer to negative treatment outcomes (Talar-Wojnarowska and Malecka-Panas 2006; Watanabe, Lee et al. 1997). An *in vivo* study demonstrated that mice implanted with lymphomas and administered several cycles of chemotherapy over time resulted in selection of mutant *p16* and *p53* genes, leading to tumors that not only re-grew, but were more aggressive in growth (Kahlem, Dorken et al. 2004). In addition, the presence of non-functional p16 not only prevents apoptosis but also causes cells to lose contact with the extracellular matrix, which may increase the possibility of metastasis (Plath, Detjen et al. 2000).

The presence of β-galactosidase in cells treated with gemcitabine in our findings, made us examine other senescence-associated proteins. We observed an increase in Rb

and p16 expression in Panc1 cells treated with SM treatment alone. However, compared to the untreated cells, single agent gemcitabine treatment decreased expression of Rb and reduced p16, and p21 expression to an undetectable level. Although the levels of the senescence related proteins we examined are not indicative of senescent cells, these observations also fail to exhibit a pattern of protein expression to support apoptosis induction in gemcitabine treated cells. In a study conducted by te Poele, et al., various p53 wild-type cells lines treated with topoisomerase inhibitors exhibited characteristics of senescence while in the presence of treatment and had constant levels of p16 which increased only upon treatment removal (te Poele, Okorokov et al. 2002).

Cellular stress associated ceramide increases PP1 (protein phosphatase 1) and PP2A activity which results in the dephosphorylation of the retinoblastoma protein (Rb) and subsequent growth arrest (Hannun 1996; Perry and Hannun 1998; Pettus, Chalfant et al. 2002; Ruvolo 2003). Senescence may be reversed if ceramide levels are decreased in the cell (Venable, Lee et al. 1995). In a study conducted by Zhang, et al., Molt-4 T-leukemia cells were used to show the ability of ceramide to activate Rb, thus resulting in cell cycle arrest (Zhang, Alter et al. 1996). Another study showed that short chain ceramide will lead to dephosphorylation of Rb, which leads to cell death (Perry and Hannun 1998). In another study using hepatocarcinoma cells, ceramide-mediated G_1 arrest occurred in a p53 independent manner through p21-mediated inhibition of cdk2 activity and dephosphorylation of Rb (Kim, Emi et al. 2006).

The results of our study provide evidence for the role of ceramide in both apoptosis and senescence. Incubating Panc1 cells with water soluble C_8-ceramide led to an induction of both apoptosis and senescence in a dose dependent manner with

senescence occurring at lower concentrations and apoptosis at higher concentrations. Our results showed that β-galactosidase reached a maximum at 21.7 ± 1.5 μM, declining thereafter. Cell death was not achieved at concentrations lower than 35 μM C_8-ceramide, with the IC_{50} of 43.6 ± 2.5 μM. Together this data supports the idea that ceramide levels are important in determining both apoptosis and senescence. Thus, by manipulating the availability of ceramide, cells can be directed to undergo apoptosis.

Ceramide levels have been shown to quickly increase in the mitochondria immediately before the activation of caspases that are associated with apoptosis induction (Hannun 1996). However, ceramide levels have also been shown to remain elevated throughout the process of apoptosis, which would indicate that ceramide is further involved in the execution phase of apoptosis (Siskind 2005). Nevertheless, cells that have become senescent will also have increased levels of intracellular ceramide (Venable, Lee et al. 1995). Combining this data with the results of our studies, we propose ceramide levels determine a cell's ability to progress through the cell cycle, undergo apoptosis, or enter into senescence (Figure 15). In normal cell proliferation, ceramide levels increase during the G_1 phase of the cell cycle and decrease after the G_1/S restriction point. However, in response to cellular stress, the levels of ceramide increase and lead to cell cycle arrest, where high concentrations of intracellular ceramide will induce apoptosis. We postulate cellular senescence occurs with intermediary ceramide levels, which maintain the cell in growth arrest but are insufficient for apoptosis.

Our proposed model for ceramide involvement in cell cycle control supports both our hypothesis and the results obtained throughout the course of our study. Treating cancer cells with gemcitabine activates aSMase to convert SM to ceramide (Modrak,

Cardillo et al. 2004). Since tumor cells lack sufficient substrate for ceramide generation, the addition of exogenous SM augments cellular SM signaling pools and allows increased ceramide-mediated apoptosis signaling resulting from gemcitabine exposure. However, if a sufficient amount of signaling ceramide fails to be generated, the cells cannot enter apoptosis and will, instead, enter into a state of senescence.

Cancerous tumors are composed cells at different stages of division and with mutations which impart chemoresistance. As studies continue to elucidate the complex involvement of sphingolipids in cell signaling, it is becoming evident that ceramide plays a critical role in a number of cell processes including the determination of cell survival. Understanding the cellular origins and the key biochemical pathways that participate in pancreatic cancer will offer new opportunities for the treatment of the disease. In so doing, we may not only increase patient survival time but possibly find a cure for the devastating illness that is pancreatic cancer.

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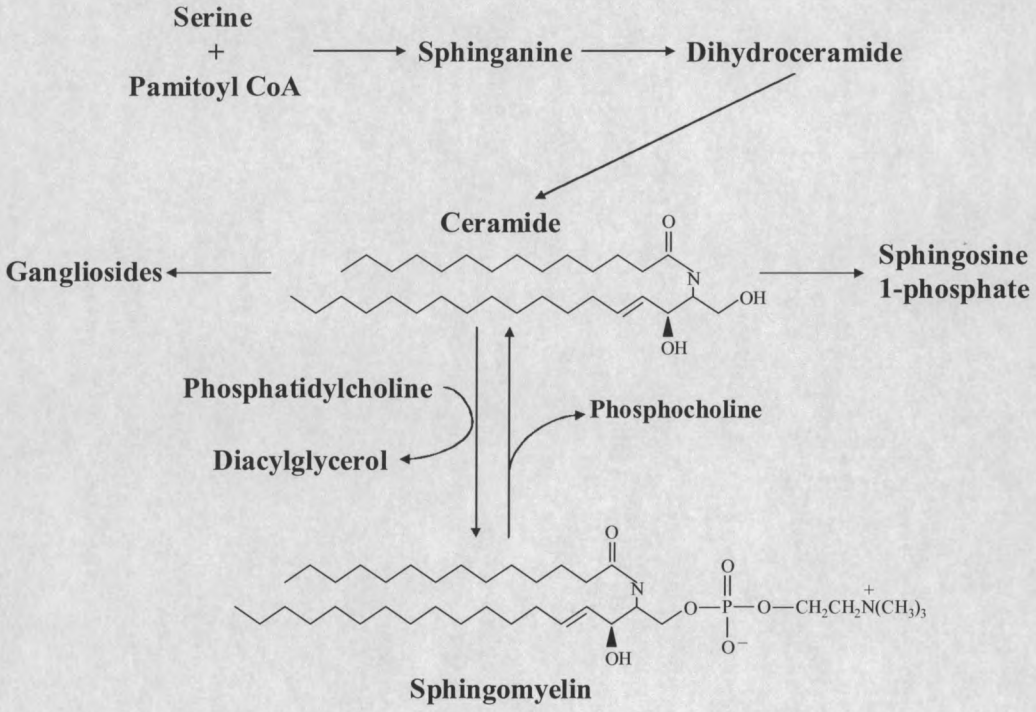


Figure 1. Sphingolipid metabolism. *De novo* synthesis of ceramide begins with the condensation of serine and palmitoyl CoA to yield sphinganine, followed by acylation to dihydroceramide and dehydrogenation to ceramide. Ceramide is then the root of all other sphingolipids. The addition of glucose or galactose is the next step in the formation of higher order gangliosides and is the major use of ceramide. The other major use of ceramide is the production of SM via the transfer of a phosphocholine headgroup from phosphatidylcholine by SM synthase. SM may be degraded to ceramide by the action of SMases. Ceramide may be degraded to sphingosine by the action of ceramidase.

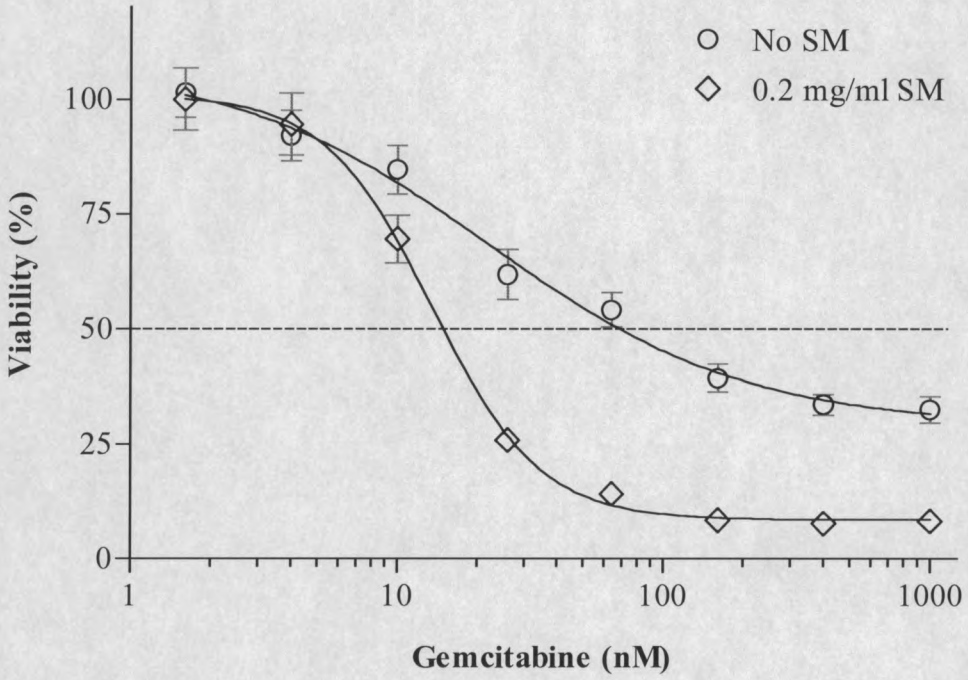


Figure 2. Dose dependent response of Panc1 cells to gemcitabine in the presence of SM. Panc1 cells were treated for 4 days with different concentrations of gemcitabine as a single agent and in combination with 0.2 mg/ml (IC_{10}) SM before assessment of viability with MTT reagent. Typical results are shown. The IC_{50} values were determined to be 78.3 ± 13.7 nM for gemcitabine alone and 13.0 ± 3.0 nM for the combined treatment ($P < 0.001$). These values represent the mean ± standard error from > 20 independent experiments.

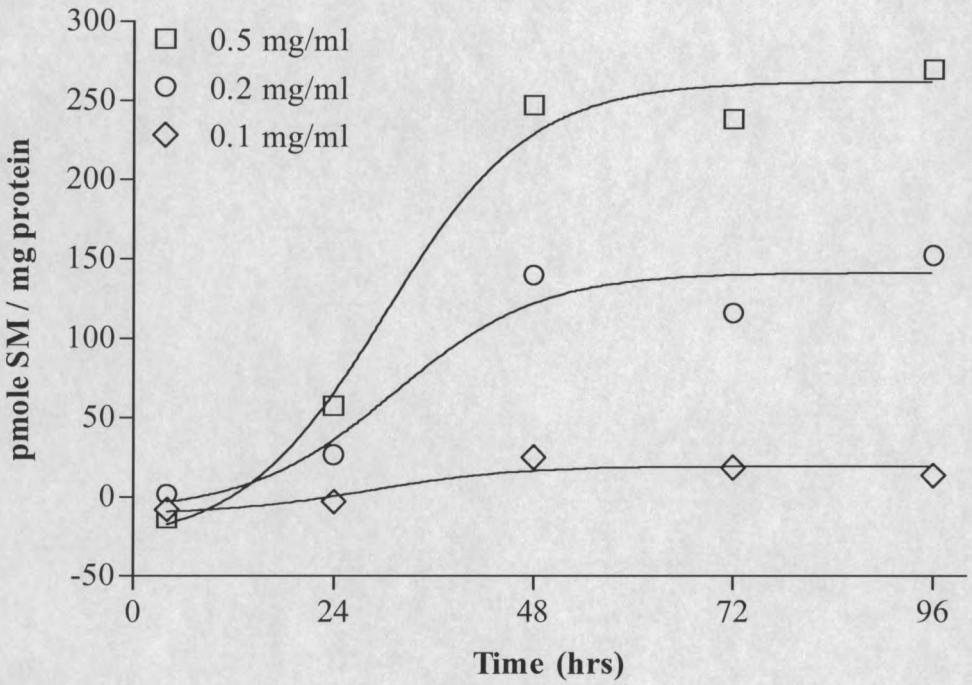
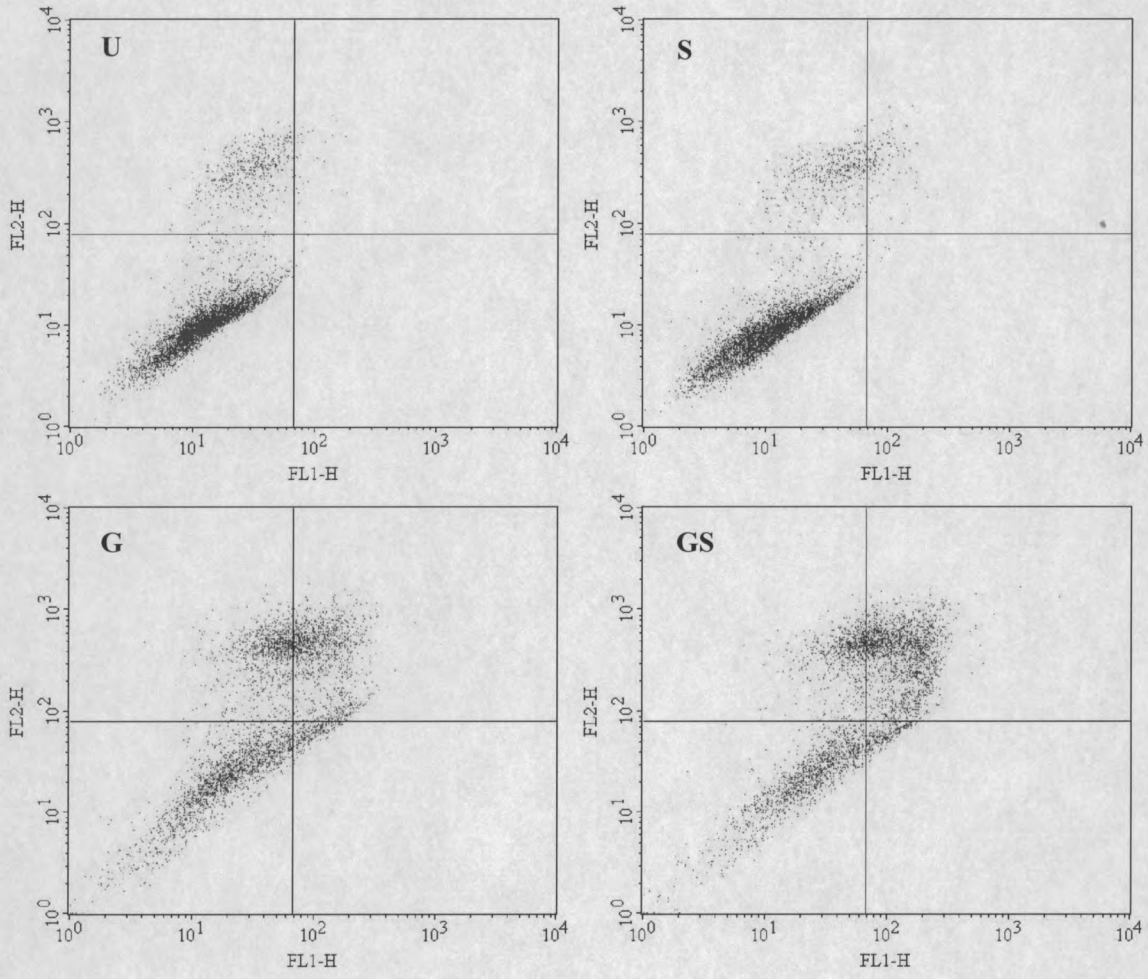


Figure 3. Exogenous sphingomyelin uptake in Panc1 over time. Panc1 cells were treated with [^{3}H-methyl]choline-SM and solubilized at 0, 24, 48, 72, and 96 hours. The data were normalized to total protein levels determined from cells treated with non-radioactive SM. The figure is representative of data obtained from > 3 experiments.

A

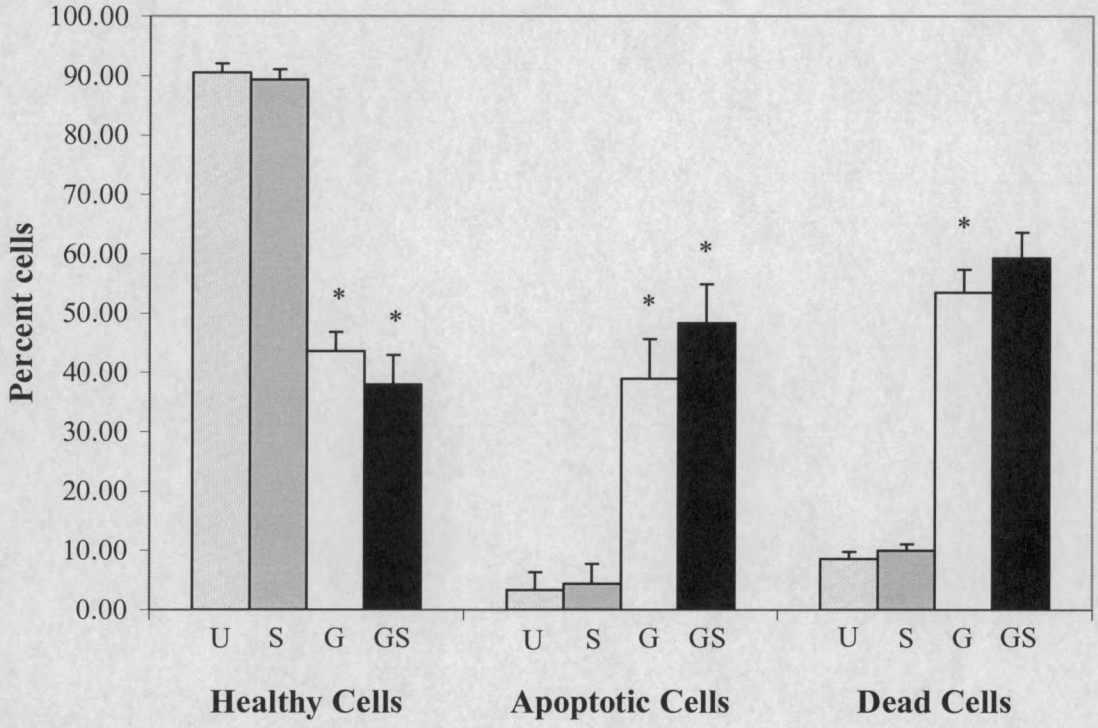


B

	Treatment	Healthy Cells	Apoptotic Cells	Dead Cells
U	Untreated	90.6	0.7	9.3
S	Sphingomyelin	90.1	2.2	9.8
G	Gemcitabine	44.7	33.0	50.4
GS	Combination	30.9	52.8	63.1

Figure 4. Flow cytometric analysis of Panc1 cells after gemcitabine ± SM treatment. Panc1 cells were treated with 100 nM gemcitabine in the presence or absence of 0.2 mg/ml SM for 4 days. Apoptotic cells were detected by Annexin V-FITC and propidium iodide staining. (A) Cells in the lower left quadrant are viable, in the upper and lower right quadrants are apoptotic, and the left and right upper quadrants are dead. (B) Values correspond with the percentage of healthy (viable, non-apoptotic), apoptotic, and dead cells in the above experiment. The figure is representative of data obtained from separate experiments with similar results.

A



B

	Treatment	Healthy Cells	Apoptotic Cells	Dead Cells
U	Untreated	90.5 ± 1.5	3.4 ± 3.0	8.6 ± 1.1
S	Sphingomyelin	89.3 ± 1.8	4.4 ± 3.3	10.0 ± 1.2
G	Gemcitabine	43.6 ± 3.3	38.9 ± 6.8	53.5 ± 3.9
GS	Combination	37.9 ± 5.0	48.4 ± 6.6	59.3 ± 4.4

Figure 5. Aggregated data for apoptotic Panc1 cells. Panc1 cells were treated with 100 nM gemcitabine in the presence or absence of 0.2 mg/ml SM and stained with Annexin V-FITC and propidium iodide. Cells positive for Annexin V-FITC were considered apoptotic and dead cells were positive for propidium iodide staining. (A) The percentage of healthy (viable, non-apoptotic), apoptotic, and dead cells are represented as the mean ± standard error of six separate experiments. (B) The table lists the values that correspond with the graph above. *P* values < 0.05 are indicated by an asterisk (*).

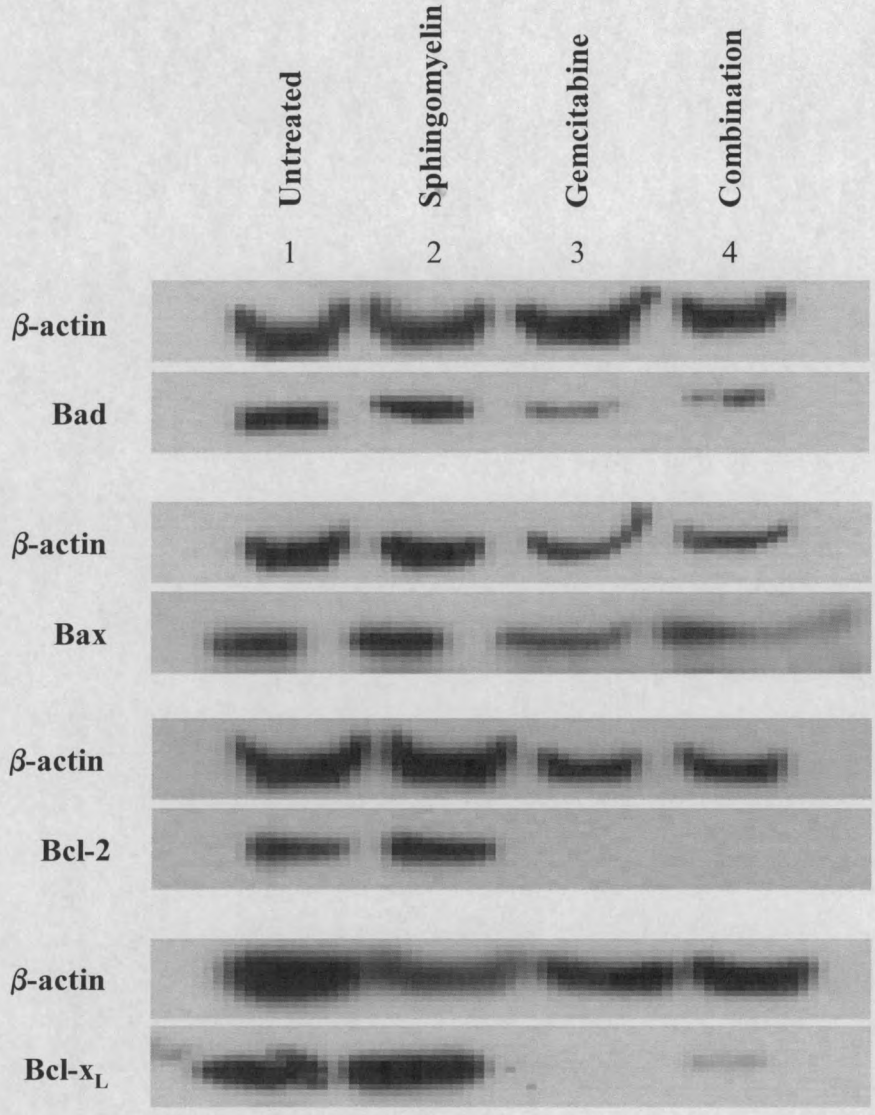
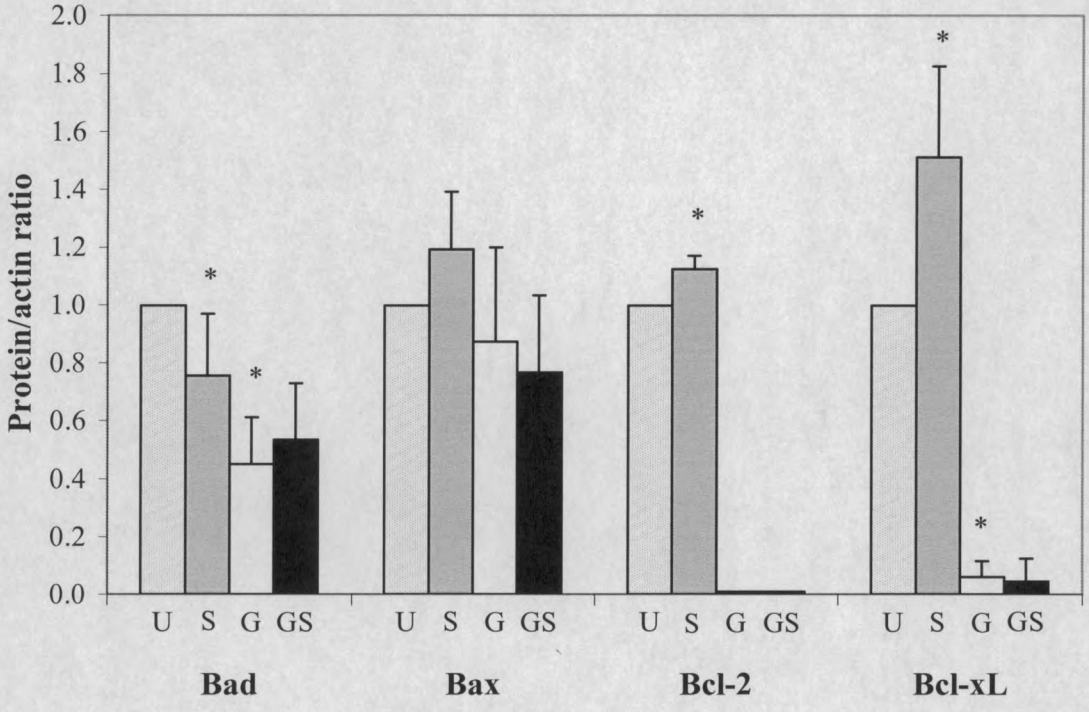


Figure 6. Expression levels of select bcl-2 family proteins in Panc1 cells. Western blot analysis of Bad, Bax, Bcl-2, and Bcl-x_L expression from total cell lysates in Panc1 cells following treatment with 100 nM gemcitabine and/or 0.2 mg/ml SM. The presence of β-actin was used for normalization of results. The figures are representative of three independent experiments with similar results.

A

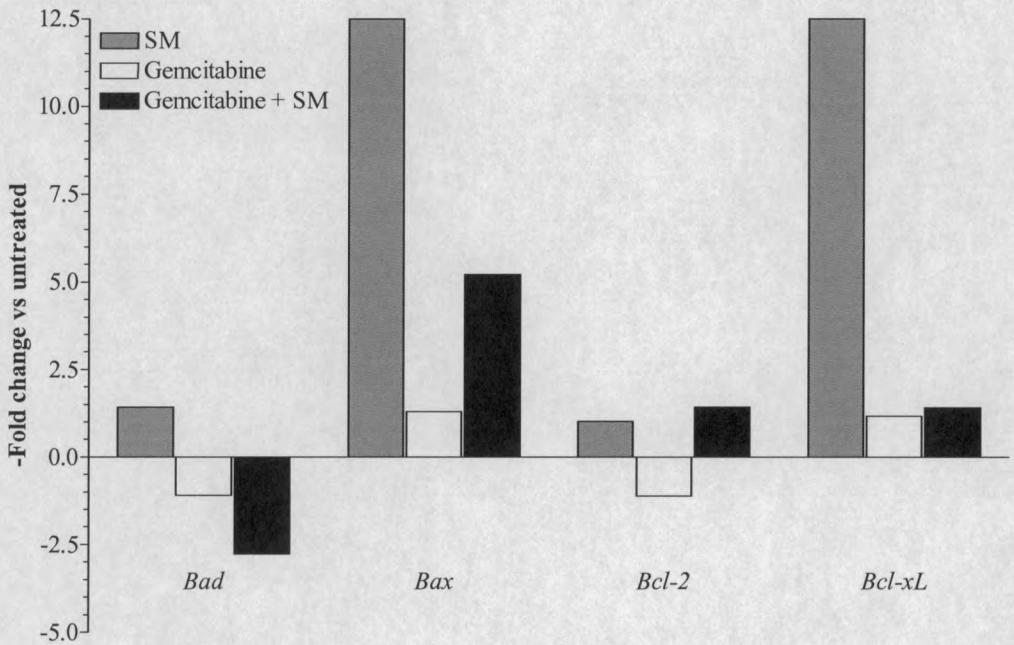


B

	Treatment	Bad	Bax	Bcl-2	Bcl-x_L
U	Untreated	1.0	1.0	1.0	1.0
S	Sphingomyelin	0.8 ± 0.2	1.2 ± 0.2	1.1 ± 0.0	1.5 ± 0.3
G	Gemcitabine	0.4 ± 0.2	0.9 ± 0.3	ND	0.1 ± 0.1
GS	Combination	0.5 ± 0.2	0.8 ± 0.3	ND	0.1 ± 0.1

Figure 7. Expression levels of select bcl-2 family proteins in treated Panc1 cells. Levels of Bax, Bad, Bcl-2, and Bcl-x_L in Panc1 cells were determined by western blot analysis. Bands were quantified using the TotalLab software, normalized to β-actin, and expressed as values relative to protein levels in the untreated cells. Changes in expression in SM and gemcitabine treatments as individual agents were evaluated against the untreated, and the combination treatment was compared to gemcitabine. *P* values < 0.05 are noted with an asterisk (*). (A) The ratio of protein to β-actin expression is representative of the mean ± standard error from > 3 independent experiments. (B) The table lists the values that correspond with the graph. ND = not detected.

A



B

Treatment	Bad	Bax	Bcl-2	Bcl-x_L
Untreated	7.25 ± 1.30	2.54 ± 1.76	3.28 ± 1.37	3.33 ± 1.56
Sphingomyelin	6.74 ± 2.61	-2.30 ± 0.21*	3.24 ± 0.11	-0.39 ± 1.87*
Gemcitabine	7.38 ± 1.37	2.15 ± 0.94	3.44 ± 0.31	3.11 ± 0.72
Combination	8.72 ± 0.44	0.15 ± 0.21*	2.76 ± 0.40*	2.83 ± 0.78

Figure 8. Analysis of selected bcl-2 family gene expression in Panc1 cells. RNA extracted from Panc1 cells treated with 100 nM gemcitabine in the absence and presence of 0.2 mg/ml SM was examined by RT-PCR arrays. (A) The graph shows the fold change in *Bad*, *Bax*, *Bcl-2*, and *Bcl-x_L* gene expression normalized to untreated Panc1 cells in three separate experiments. Values greater than 2 require further investigation. (B) The table summarizes the average ΔC_t values from separate experiments. *P* values < 0.05 are indicated by an asterisk (*).

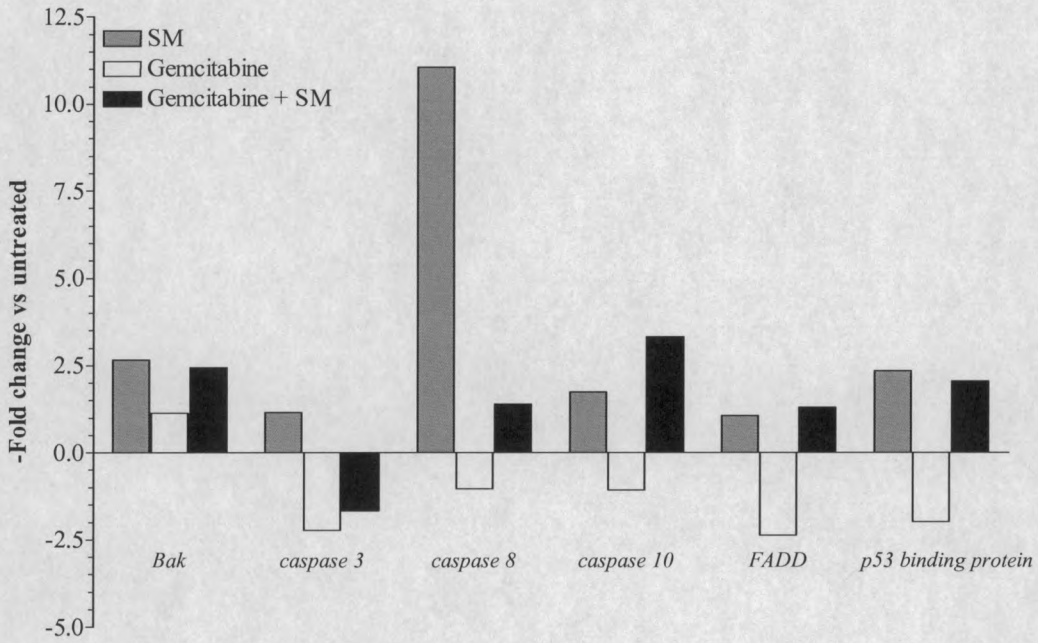
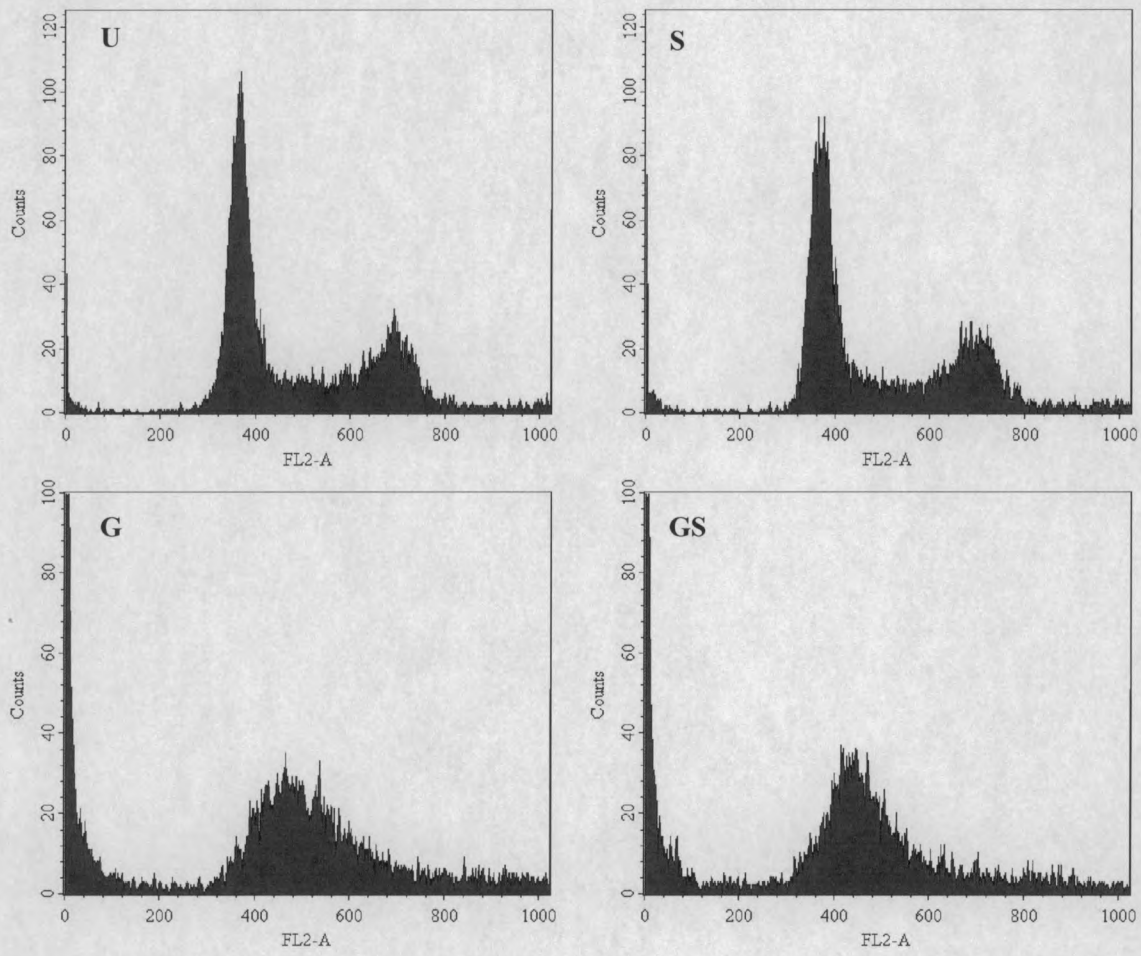


Figure 9. Analysis of apoptosis related gene expression in Panc1 cells. Panc1 cells were treated with 100 nM gemcitabine and/or 0.2 mg/ml SM for 4 days. RNA was isolated and apoptosis related genes were analyzed with RT-PCR arrays. The figure summarizes the fold change in gene expression normalized to untreated Panc1 cells in three separate experiments.

A



B

	Treatment	G_0/G_1	S	G_2/M
U	Untreated	57.1 ± 2.7	19.6 ± 2.1	23.2 ± 3.9
S	Sphingomyelin	58.0 ± 3.4	20.3 ± 2.7	21.7 ± 6.0
G	Gemcitabine	17.5 ± 4.7*	59.7 ± 6.4*	21.0 ± 1.8
GS	Combination	23.7 ± 7.2	55.6 ± 9.2	16.9 ± 3.6

Figure 10. Cell cycle distribution of Panc1 cells. Panc1 cells were treated with 100 nM gemcitabine alone and in combination with 0.2 mg/ml SM for 4 days. Cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. (A) The figure is representative of data obtained from > 3 independent experiments with similar results. (B) The table expresses values characteristic of Panc1 cell cycle as observed in separate experiments. *P* values < 0.05 are indicated by an asterisk (*).

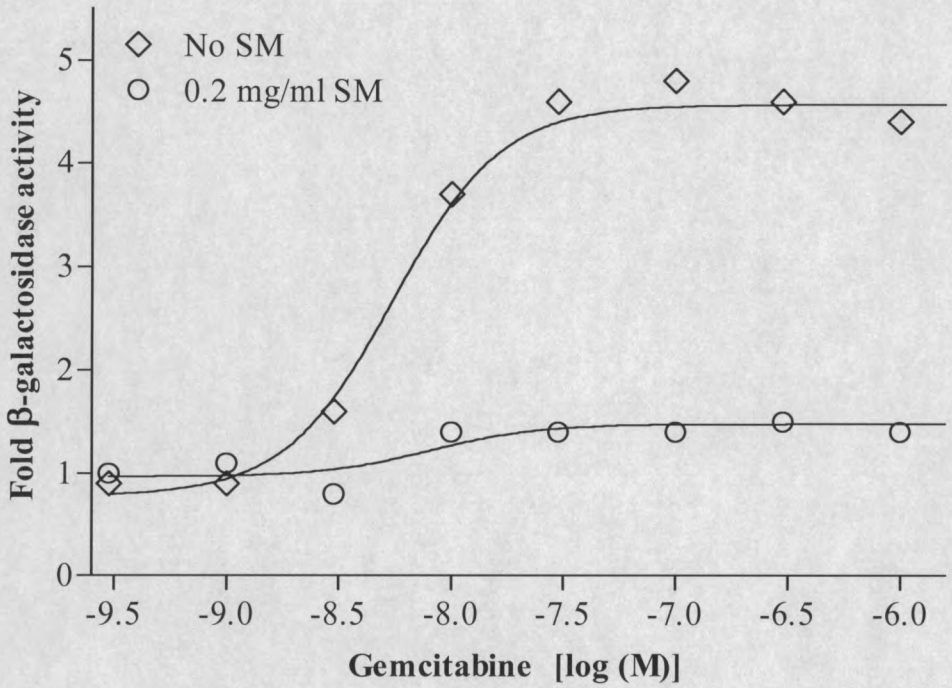


Figure 11. Comparison of β-galactosidase activity in Panc1 cells treated with gemcitabine in the presence and absence of SM. Panc1 cells were incubated in the presence of C_{12}-FDG, gemcitabine and/or SM for 4 days. Cells were analyzed by flow cytometry and mean channel fluorescence was determined. Treated cells were normalized to the untreated. The figure is representative of three independent experiments with similar results.

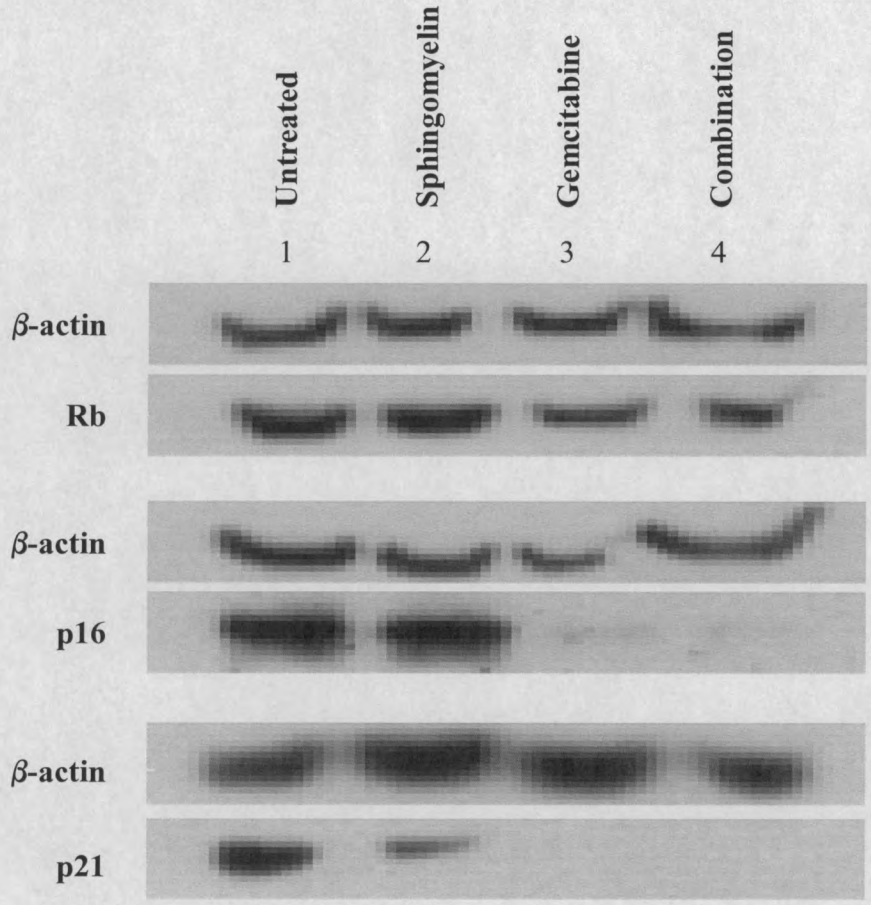
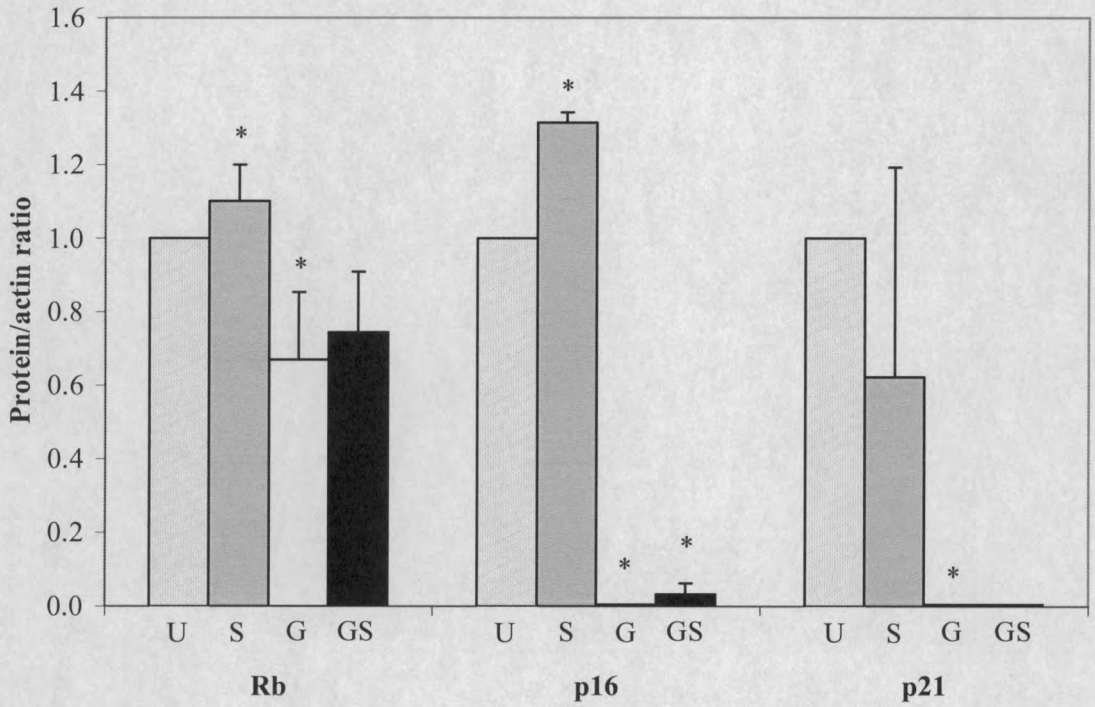


Figure 12. Western blot analysis of proteins associated with senescence in Panc1 cells. Expression of Rb, p16, and p21 in Panc1 cells in total cell lysates was determined by western blot analysis. β-actin was used as an internal control. The figures are representative of triplicate experiments with similar results.

A



B

	Treatment	Rb	p16	p21
U	Untreated	1.0	1.0	1.0
S	Sphingomyelin	1.1 ± 0.1	1.3 ± 0.03	0.6 ± 0.5
G	Gemcitabine	0.7 ± 0.2	ND	ND
GS	Combination	0.7 ± 0.2	0.03 ± 0.03	ND

Figure 13. Quantification of senescence related proteins in treated Panc1 cells. Total cellular levels of Rb, p16, and p21in Panc1 cells treated with 100 nM gemcitabine and/or 0.2 mg/ml SM were determined by western blot analysis. Bands were quantified by the TotalLab software and were normalized to β-actin. P values < 0.05 are noted with an asterisk (*). (A) The ratio of protein to β-actin expression was determined and normalized to the untreated from three independent experiments. (B) The table lists the values that correspond with the graph. ND = not detected.

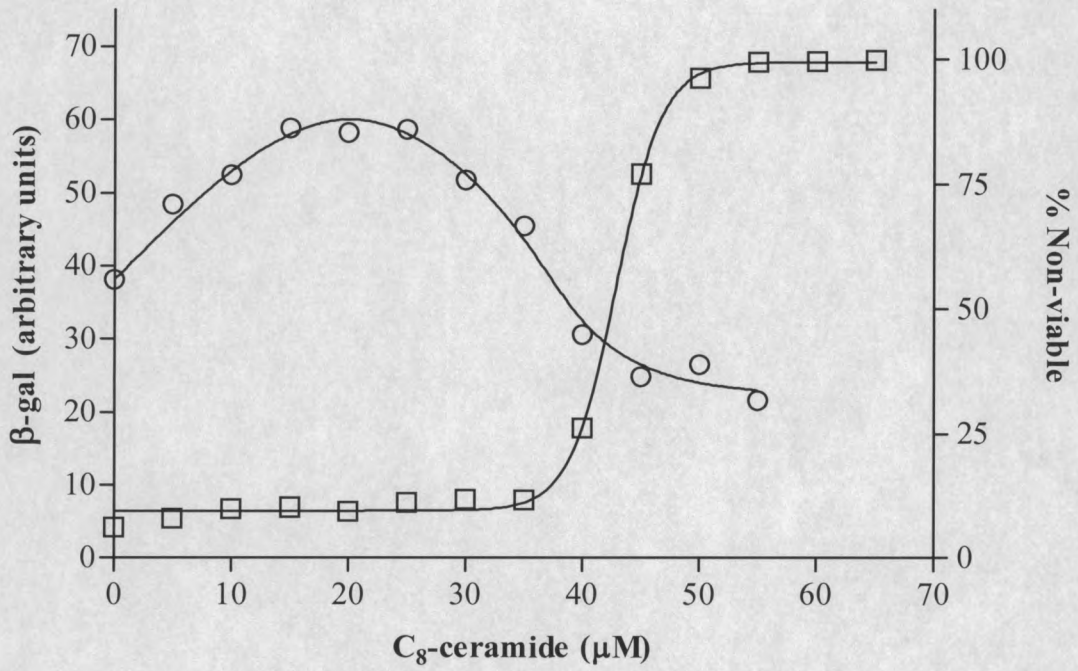


Figure 14. Flow cytometric analysis of viability and β-galactosidase activity in Panc1 cells following C_8-ceramide treatment. Cells were treated with C_8-ceramide for 4 days in the presence of C_{12}-FDG for the identification of senescent cells. Viability was determined by propidium iodide staining (□) and senescence by β-galactosidase activity (○). The figure is representative of three independent experiments with similar results.

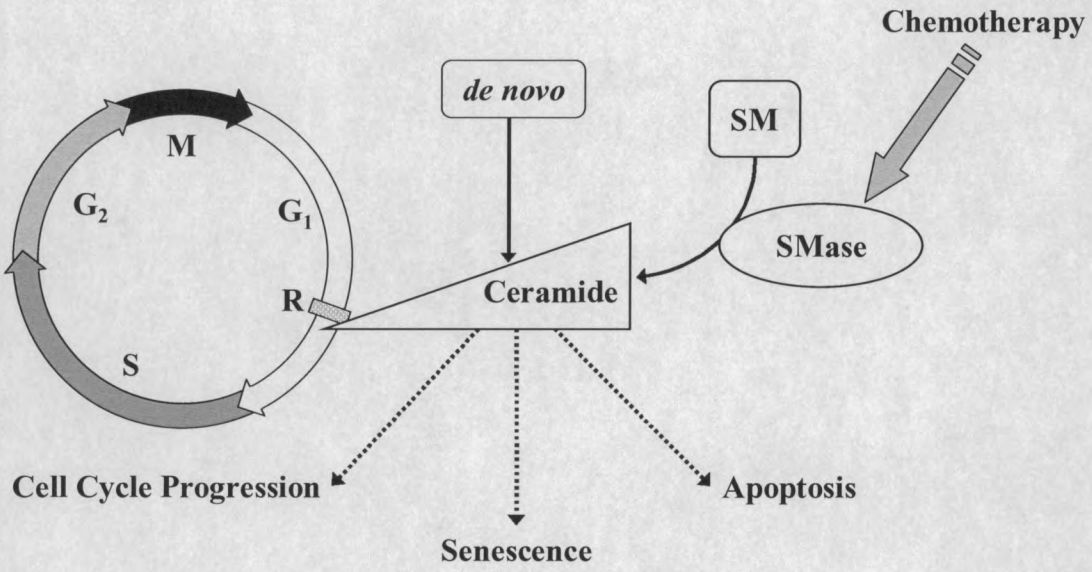


Figure 15. Proposed model for the involvement of ceramide in cell cycle control. The level of ceramide in the cell is proposed to determine whether a cell progresses through the cell cycle, enters senescence, or initiates apoptosis. During the cell cycle, ceramide levels increase until the restriction point in G_1. Cell cycle progression occurs when ceramide levels are low, senescence when levels are moderate, and apoptosis when levels are high.