

Induction of Apoptotic Cell Death by a Ceramide Analog in PC-3 Prostate Cancer Cells

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Ceramide analogs are potential chemotherapeutic agents. We report that a ceramide analog induces apoptosis in human prostate cancer cells. The ceramide analog induced cell death through an apoptotic mechanism, which was demonstrated by DNA fragmentation, the cleavage of poly ADP ribose polymerase (PARP), and a loss of membrane asymmetry. Treating the cells with ceramide analog resulted in the release of various proapoptotic mitochondrial proteins including cytochrome c and Smac/DIBLO into the cytosol, and a decrease in the mitochondrial membrane potential. In addition, the ceramide analog decreased the phospho-Akt and phospho-Bad levels. The expression of the antiapoptotic Bcl-2 decreased slightly with increasing Bax to Bcl-2 ratio. These results suggest that the ceramide analog induces apoptosis by regulating multiple signaling pathways that involve the mitochondrial pathway.

Key words: Ceramide analog, Apoptosis, Akt, Cytochrome c, Smac, Bcl-2

INTRODUCTION

Ceramide is a sphingolipid second messenger that is involved in mediating a variety of cell functions including growth arrest, differentiation and apoptosis (Obeid *et al.*, 1993; Hannun, 1996). A number of diverse apoptosis-inducing agents such as tumor necrosis factor- α , chemotherapeutic agents, Fas ligation, irradiation, and corticosteroids can generate ceramide by the hydrolysis of membrane sphingomyelin through sphingomyelinases or the de novo synthesis via ceramide synthase (Hannun, 1996; Herr *et al.*, 1996; Jaffrezou *et al.*, 1996; Hannun and Obeid, 2002). Exogenous cell permeable ceramide mimics the action of these inducers and induces apoptosis in many different cell types. Therefore, ceramide is considered to be a common mediator of the apoptotic mechanism. Ceramide has several targets in regulating apoptotic cell death. Ceramide activates various proapoptotic mechanisms by acting on the ceramide-activated phosphatases, ceramide-activated kinases, which in turn regulates PKC, Akt, c-Jun, and Bcl-2 (Hannun 2002).

The induction of apoptosis is an effective strategy for cancer chemotherapy. Resistance to apoptosis decreases the sensitivity of cancer cells to various drugs, which can result in the failure of chemotherapy (Wang *et al.*, 1999). For this reason, the search for apoptosis-inducing drugs is an appealing therapeutic approach to the development of novel anticancer agents. Many anticancer drugs such as doxorubicin, vincristine, etoposide, and paclitaxel exert their antitumor effect against cancer cells by inducing apoptosis with an associated increase in cellular ceramide (Cabot *et al.*, 1999; Selzner *et al.*, 2001). It has been suggested that aberrant or decreased ceramide signaling contributes to tumor progression and resistance to therapy (Chimura *et al.*, 1997; Selzner *et al.*, 2001; Struckhoff, *et al.*, 2004). This indicates that various ceramide analogs that mimic the effects of endogenous ceramide might be attractive candidates as cancer chemotherapeutic agents.

This study investigated the antiproliferative effect of a novel ceramide analog on PC-3 human prostate cancer cells. The ceramide analog induced mitochondria-dependent apoptotic cell death by regulating multiple signaling pathways that involve decreasing the antiapoptotic signals in addition to increasing the proapoptotic signals. The ceramide analog may be useful for inducing apoptosis in prostate cancer cells and may be of therapeutic potential for the treatment of cancer.

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MATERIALS AND METHODS

Materials

Fetal bovine serum was purchased from WEL GENE (Daegu, Korea), and the ECL kit was obtained from Amersham Pharmacia. The antibody to cytochrome c was obtained from Cell signaling Technology (Beverly, MA, USA), and the antibody to COX IV was purchased from Molecular Probes (Carlsbad, CA, U.S.A.). The antibodies to Bcl-2, Bcl-xL, Bax, Bad, Bad (Ser136), Akt, pAkt (Ser473), GAPDH and the HRP-conjugated secondary antibody were acquired by Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Ceramide analog

This ceramide analog used in this study is a newly developed conformationally restrained analog of C2-ceramide, which has a diketopiperazine backbone in the place of the polar head of C2-ceramide (Fig. 1).

Cell proliferation assay

The cell proliferation assays were performed using a Cell counting Kit-8 (Dojindo, Kumamoto, Japan). The PC-3 cells were plated in 96-well plates at 1×10^4 cells per well and cultured in a RPMI growth medium. At the indicated times, the number of cells in triplicate wells were measured at a wavelength of 450 nm, which is the absorbance of the reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt).

Apoptosis assay

The cells were washed with serum-free RPMI. The ceramide analog or vehicle (DMSO) was diluted into serum-free RPMI at the indicated concentrations. The cells were maintained in serum-free RPMI for 2 h before the experiments. The level of apoptosis was analyzed by treating the cells with the ceramide analog for 24 h, resuspending them in a buffer containing 10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl_2 , and pelleting them by centrifugation. The cell pellets were washed in ice-cold PBS, resuspended in a binding buffer, and incubated with FITC-conjugated Annexin V and propidium iodide (Zymed Laboratories, South San Francisco, CA) for 10 min at room temperature in the dark. The stained cells were analyzed on a FACSCaliber (BD Bioscience, Franklin Lakes, NJ) flow cytometer, which

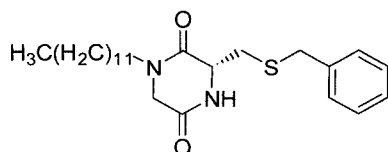


Fig. 1. Structure of the ceramide analog

measured the fluorescence emission at 530 nm for FITC and 638 nm for propidium iodide. Apoptosis was also measured using an enzyme-linked immunosorbent assay (ELISA) based on the detection of the histone-associated DNA fragments in the cytoplasm of the apoptotic cells according to the manufacturer's instructions (Roche, Castle Hill, Australia).

Subcellular fractionation

The basic methodology for the preparation of the mitochondria and cytosol fractions was modified from a previous report (Kim *et al.*, 2001). Briefly, the cells (3×10^6) at the end of the treatment were harvested and washed with ice-cold PBS. The cells were resuspended in 500 μL of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol) containing 250 mM sucrose and a mixture of protease inhibitors (1 mM PMSF, 1% aprotinin, 1 mM leupeptin). The cells were lysed by passing the cell suspension through a 26-gauge needle fitted to a syringe. The unbroken cells, large plasma membrane pieces, and nuclei were removed by centrifuging the homogenates at $1,000 \times g$ at 4°C for 10 min. The resulting supernatant was subjected to centrifugation at $10,000 \times g$ at 4°C for 20 min. The pellet fraction (i.e., mitochondria) was first washed with the abovementioned buffer A containing sucrose and then dissolved in 50 μL of the TNC buffer (10 mM Tris-acetate, pH 8.0, 0.5% NP-40, 5 mM CaCl_2). The supernatant was again centrifuged at $100,000 \times g$ (4°C , 1 h) to generate the cytosol.

Western blot analysis

The proteins (30 μg /well) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on polyacrylamide gels, and transferred electrophoretically onto an Immobilon-P membrane. Blocking was performed in Tris-buffered saline containing 5% skimmed-milk powder and 0.1% Tween-20. The membranes were probed with the antibodies against Smac, cytochrome c, Bcl-2, Bcl-xL, Bax, Bad, pBad, Akt or phospho-Akt. Detection was carried out using an ECL system. The protein content was determined using the BCA method with bovine serum albumin as the standard.

Measurement of mitochondrial membrane potential ($\Delta\Psi\text{m}$)

The $\Delta\Psi\text{m}$ of PC-3 cells was measured using the fluorescent probe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanide iodide). JC-1 exists as a monomer at low $\Delta\Psi\text{m}$ values (green fluorescence) while it forms aggregates at a high $\Delta\Psi\text{m}$ (red fluorescence). Therefore, the mitochondria with a normal $\Delta\Psi\text{m}$ concentrate JC-1 forms aggregates. The PC3 cells (6×10^5) were

collected by trypsinisation, washed in warm phosphate buffered saline (PBS, pH 7.4) and incubated for 15 min at 37°C with 2 μ M JC-1. The cells were pelleted at 1,000 rpm for 5 min, and washed in warm PBS. The pellet with resuspended PBS, and analyzed by flow cytometry using a FACScan.

RESULTS

Ceramide analog induces apoptotic cell death in PC-3 prostate cancer cells

The ability of the ceramide analog to induce cell death was determined in the human prostate cancer PC-3 cells.

The cells were treated with different concentrations of the ceramide analog for 24 h, and the cell viability was determined. The ceramide analog exerted a dose-dependent inhibition of cellular proliferation, with a concentration of 10 μ M resulting in the death of 50% of the cell population (Fig. 2A). The induction of apoptosis by the ceramide analog was analyzed by detecting the histone-associated DNA fragments in the cytoplasm of the apoptotic cells (Fig. 2B). The cleavage of PARP into an 89 kDa fragment, which is the main biochemical hallmark of apoptosis (Kaufmann *et al.*, 1993), was observed after treatment (Fig. 2C). The translocation of phosphatidylserine to the outer surface of the cytoplasmic membrane was evaluated

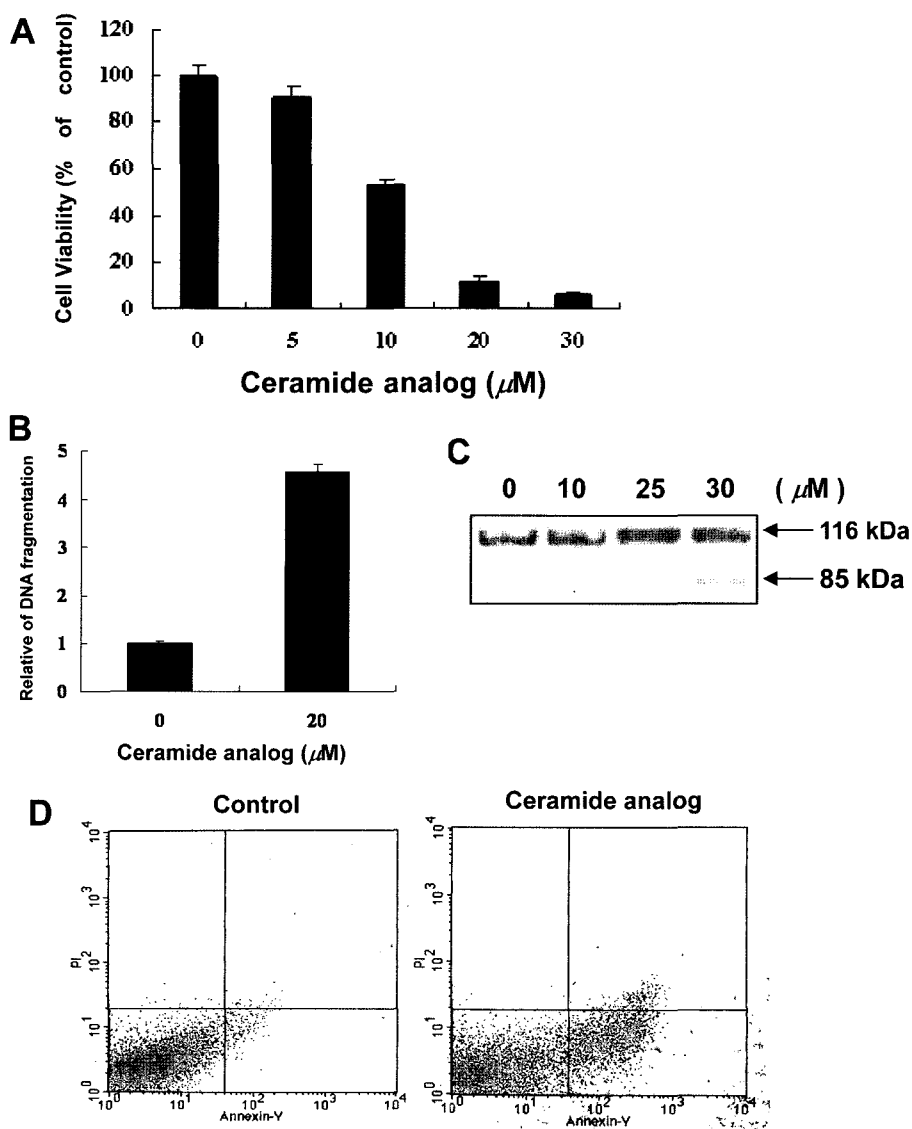


Fig. 2. Ceramide analog induces apoptosis in PC-3 cells. A. The PC-3 cells were treated with the ceramide analog at various concentrations for 24 h, and the cell viability was detected using Cell Counting Kit-8. B. After treatment with the ceramide analog at 20 μ M for 24 h, the level of apoptotic cell death was analyzed using ELISA. C. Extracts from the PC-3 cells treated with the ceramide analog at various concentrations for 24 h were immunoblotted with the anti-PARP antibody. D. The detection of apoptosis by fluorescein isothiocyanate labeled annexin V. Flow cytometric analysis of the apoptotic cells were shown under treatment with the ceramide analog at 25 μ M for 24 h.

using Annexin FITC to determine if the ceramide analog induces apoptosis. The cells were treated with the ceramide analog for 24 h, and the Annexin V-positive cells were determined using flow cytometry. As shown in Fig. 2D, the number of Annexin-positive cells increased after treatment with the ceramide analog, suggesting that a population of cells underwent apoptotic cell death.

Ceramide analog downregulates Akt and Bad phosphorylation

Experiments were carried out to determine if the Akt pathway is involved in the ceramide analog-induced apoptosis. The PI3-kinase/Akt signaling pathway is essential in the inhibition of apoptosis, and molecules that can block the activity of Akt may have significance in cancer therapy (Testa and Bellacosa, 2001). The ceramide analog treatment resulted in the appreciable down-regulation of phospho-Akt and phospho-Bad without having an effect on the total Akt and Bad expression in PC-3 cells (Fig. 3).

Ceramide analog induces release of mitochondrial proteins

The release of various proapoptotic mitochondrial proteins including cytochrome c and Smac/DIABLO into the cytosol is an important process during the apoptosis induced by chemotherapeutic agents (Liu *et al.*, 1996; Green and Reed, 1998; Kuwana and Newmeyer, 2003). In the cytosol, cytochrome c triggers the formation of apoptosome, which then recruits and activates the initiator caspase-9 (Liu *et al.*, 1996). Smac/DIABLO binds in the cytosol to the inhibitor of Apoptosis Proteins (IAPs) and releases caspases from IAPs inhibition (Kuwana and Newmeyer, 2003). Treating the PC3 cells with the ceramide analog caused an increase in the level of cytochrome c and Smac into

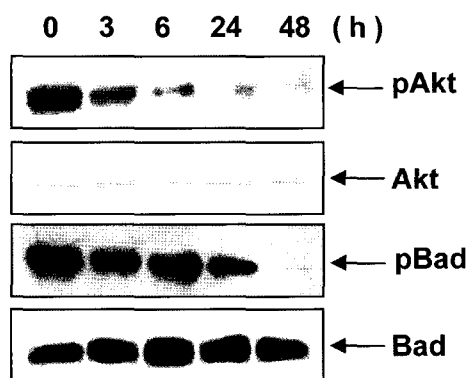


Fig. 3. Ceramide analog inhibits the phosphorylation of Akt and triggers the activation of Bad in PC-3 cells. The PC-3 cells were treated with 25 μ M ceramide analog for the indicated times. The activation of Akt or Bad was analyzed by Western blotting using either the anti-phospho Akt or anti-phospho Bad antibody. The PVDF membranes were stripped and reprobbed with the antibody against total cellular Akt or Bad.

the cytosol, indicating the involvement of the subcellular redistribution of cytochrome c and Smac in the ceramide analog-induced apoptosis (Fig. 4).

Ceramide analog reduces mitochondrial membrane potential ($\Delta\Psi_m$)

The opening of the permeabilization transition (PT) pore complex is believed to mediate the release of the proapoptotic proteins from the mitochondria (Green and Reed, 1999; Kuwana and Newmeyer, 2003). One of the markers of the opening of the PT pore is a decrease in the mitochondrial membrane potential ($\Delta\Psi_m$). Therefore, the effect of the ceramide analog on the $\Delta\Psi_m$ was measured using a fluorescent cationic dye. The cell-permeable JC-1 dye was used to monitor the changes in the mitochondrial membrane potential after the ceramide analog treatment. In non-apoptotic cells with an intact membrane potential, the JC-1 dye accumulates and forms aggregates with a red fluorescence. On the other hand, in apoptotic and dead cells, the dye is not actively taken up into the mitochondria and remains in its monomeric form, which appears green. Therefore, as shown in Fig. 5, treatment with the ceramide analog caused a significant decrease in the mitochondrial membrane potential compared with the control. This indicates that the mitochondria are important targets for ceramide analog-induced apoptosis.

Ceramide analog regulates the expression of Bcl-2 family proteins

The expression of the antiapoptotic and proapoptotic Bcl-2 proteins in the ceramide analog-treated PC-3 cells was measured to determine if the Bcl-2 family proteins are involved in the apoptosis induced by the ceramide analog. As shown in Fig. 6, the expression of the antiapoptotic Bcl-2 and Bcl-xL proteins was slightly reduced

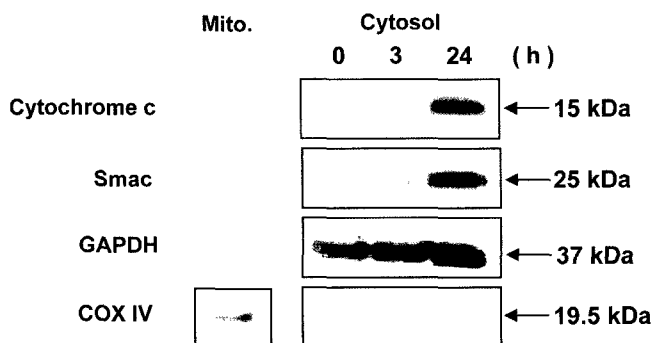


Fig. 4. The ceramide analog induces the release of mitochondrial cytochrome c and Smac into the cytosol of the PC-3 cells. The cytosolic extracts from the PC-3 cells were treated for the indicated times with 25 μ M of the ceramide analog and were immunoblotted with anti-cytochrome c, anti-Smac, anti-GAPDH or anti-COX IV. A mitochondrial extract from the untreated cells (Mito) was used as the mitochondrial marker.

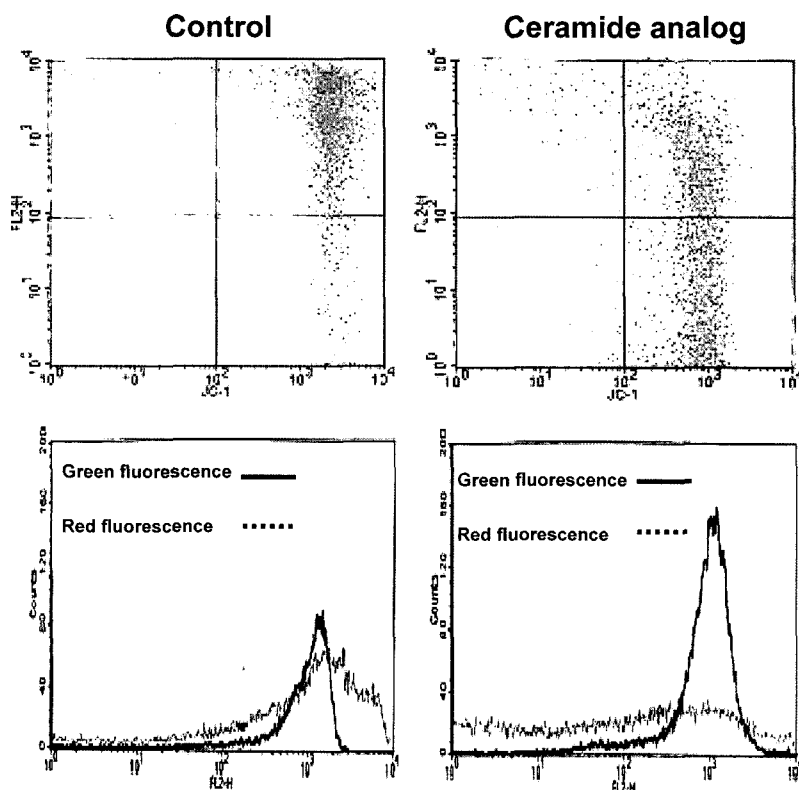


Fig. 5. Ceramide analog induces the loss of the mitochondrial transmembrane potential. The detection of the mitochondrial transmembrane potential by 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazole carbocyanide iodide (JC-1) was analyzed by flow cytometry. The photomultiplier settings were adjusted to detect the JC-1 monomer fluorescence signals on the filter 1 (JC-1) detector (green fluorescence) and JC-1 aggregate fluorescence signals on the FL2 detector (red fluorescence). The histograms represent the JC-1 fluorescence of normal cells (left) and those treated with 25 μ M of the ceramide analog for 24 h (right). Green fluorescence (solid line) increased in the ceramide analog treated cells while the red fluorescence (dotted line) decreased, suggesting a decrease in the mitochondrial membrane potential.

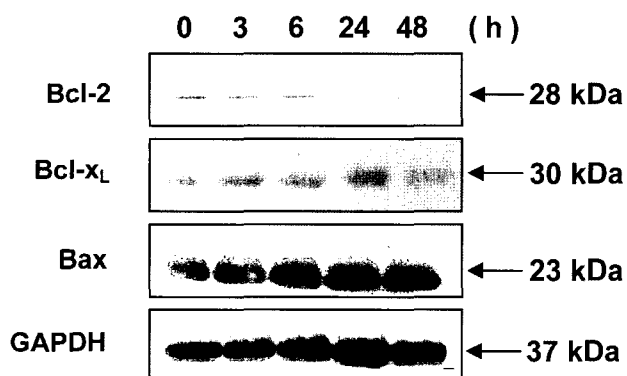


Fig. 6. Ceramide analog treatment regulates the expression of the Bcl-2 family proteins in PC-3 cells. Extracts from the PC-3 cells treated with the 25 μ M ceramide analog for the indicated times were immunoblotted with anti-Bcl-2, anti-Bax, anti-BCL-xL, or anti-GAPDH.

by the treatment with ceramide analog whereas the level of the proapoptotic Bax was increased.

DISCUSSION

Apoptosis is an important regulatory mechanism that removes unwanted cells during the development and maintenance of cell homeostasis. The failure of apoptotic cell death has been implicated in various cancers. The molecular mechanisms by which anticancer drugs induce apoptosis involve the activation of proapoptotic signaling or the inhibition of survival signaling. Therefore, the balance between the survival and death signal transduction pathways is important in controlling apoptosis. Ceramide is a lipid mediator that has proapoptotic activity in cancer cells. The effect of several chemotherapeutic agents is linked to the induction of apoptosis and is associated with an increase in the level of cellular ceramide (Bieberich *et al.*, 2000; Macchia *et al.*, 2001). Therefore, the development of ceramide analogs with increased antitumor activity represents a potential new class of chemotherapeutic agents.

This study found that the ceramide analog produced the dose-dependent inhibition of PC-3 cell proliferation. The ceramide analog induced apoptotic cell death in PC-3

cells, which was detected by DNA fragmentation or phosphatidylserine exposure, as measured by the level of Annexin V-binding. In an effort to characterize the molecular mechanisms by which the ceramide analog induces apoptosis, this study evaluated whether or not apoptosis involves the inhibition of the cell survival signals or the activation of the cell death signals. It was found that the ceramide analog induced the downregulation of Akt phosphorylation and Bcl-2 protein expression. Akt is an important survival factor in the signal transduction pathways involved in cell growth, and is considered a possible target in cancer therapy (Zhou *et al.*, 2000; Osaki *et al.*, 2004). Akt inhibits apoptosis by inactivating various proapoptotic proteins such as Bad. The PI3K/Akt pathway is constitutively active in prostate cancer cells (Van Duijn and Trapman, 2006). These results showed that the ceramide analog inhibits the activation of Akt detected by decreasing levels of phosphorylation, leading to Bad dephosphorylation. Hence, the inhibition of the Akt pathway may be an important mechanism for the apoptosis induced by the ceramide analog in PC3 cells.

The Bcl-2 family proteins are critical regulators of the apoptotic pathways (Gross *et al.*, 1999; Zang *et al.*, 2000). The over-expression of Bcl-2 is associated with many types of cancer including prostate carcinomas (DiPaola and Aisner, 1999). The antiapoptotic Bcl-2 and Bcl-xL proteins reside on the outer membrane of the mitochondria and can inhibit apoptosis in the presence of various apoptotic stimuli and promote cell survival (Finucane *et al.*, 1999). Bax is a proapoptotic member of the Bcl-2 family that resides in the cytosol and translocates to the mitochondria upon the induction of apoptosis (Gross *et al.*, 1999; Zhang *et al.*, 2000). It was reported that changes in the ratio between the proapoptotic and antiapoptotic members of the Bcl-2 family proteins, rather than the absolute expression level of any single Bcl-2 member protein, could determine the apoptotic sensitivity (Zang *et al.*, 2000). These results showed that the ceramide analog downregulates antiapoptotic Bcl-2 and Bcl-xL but upregulates the level of the proapoptotic Bax protein, thereby increasing the Bax/Bcl-2 and Bax/Bcl-xL ratios.

The mitochondria are central to many forms of cell death, usually through the release of pro-apoptotic proteins, including cytochrome c and Smac/DIABLO, from the mitochondrial intermembrane space (Kuwana and Newmeyer, 2003). The release of protein from the intermembrane space can occur as a result of mitochondrial outer membrane permeabilization, which is believed to be regulated by the proteins of the Bcl-2 family (Gross *et al.*, 1999; Kuwana and Newmeyer, 2003). These results show that a treatment with the ceramide analog caused a loss of the mitochondrial membrane potential, and the release of cytochrome c and Smac into the cytosol. It was reported

that the activation of the PI3K/Akt inhibits the release of cytochrome c (Kennedy *et al.*, 1999; Kim *et al.*, 2003). Since the ceramide analog inhibited Akt phosphorylation, it is believed that the inhibition of Akt might be a signaling event upstream of the mitochondria in ceramide analog-induced cell death.

Overall, these results indicate the presence of multiple pathways for the ceramide analog-induced apoptosis of cancer cells. The ceramide analog-induced apoptosis in prostate cancer cells is associated with mitochondrial signaling, which involves a decrease in the mitochondrial membrane potential, followed by the release of various proapoptotic mitochondrial proteins, cytochrome c and Smac. In addition, the ceramide analog reduces anti-apoptotic phosphorylation of Akt and the expression of Bcl-2, leading to the inhibition of various survival signals as well as increasing the death signals. However, more study of the ceramide analog will be needed to confirm its potential as a candidate for cancer chemotherapy.

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