

# Aberrant Sphingolipid Signaling Is Involved in the Resistance of Prostate Cancer Cell Lines to Chemotherapy<sup>1</sup>

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## ABSTRACT

Activation of the apoptosis program has been implicated in the response of cancer cells to chemotherapy. Therefore, we postulated that chemotherapy-resistant prostate cancer has developed a lesion in the apoptosis signal transduction cascade. In this study, we investigated the mechanism underlying the resistance of apoptosis-insensitive prostate cancer cells to apoptosis. We approached this by comparing the response of the androgen-sensitive LNCaP cell line and the androgen-insensitive PC3 cell line to treatment with the topoisomerase I inhibitor, camptothecin. We demonstrated that LNCaP cells are susceptible to camptothecin-induced cell death, and PC3 cells are resistant. Additional studies confirmed that the mode of cell death in the LNCaP cells was by apoptosis. We then determined that a component of the resistance to death in the apoptosis-insensitive cells involved a defect in the generation of ceramide, a key lipid mediator of apoptosis. Specifically, we demonstrated that PC3 cells are unable to elevate ceramide in response to treatment with camptothecin. In contrast, elevations in ceramide levels occur in LNCaP cells in response to the same treatment. Significantly, additional studies showed that treatment with exogenous ceramide overcomes the lesion in the PC3 cells and induces apoptosis. In attempting to gain preliminary insight into the nature of the lesion in ceramide formation in the apoptosis-resistant cells, we established that generation of ceramide in LNCaP cells is independent of the *de novo* pathway. These studies present novel insights into the mechanism by which prostate cancer cells may be resistant to induction of apoptosis. The significance of this study lies in the fact that an understanding of the biological and molecular events contributing to the resistance of prostate cancer to therapy is crucial to the development of more effective regimens for advanced disease.

## INTRODUCTION

During the past 50 years, the treatment of cancer has mainly relied on the use of a variety of forms of chemotherapy and radiotherapy. Although these approaches have had positive results on many hematological malignancies and a few solid tumors, many malignancies remain resistant to these interventions (1). One such malignancy is androgen-insensitive prostate cancer, which is recognized as a chemotherapy-resistant disease (2).

Adenocarcinoma of the prostate is the most frequently diagnosed noncutaneous cancer and the second leading cause of cancer-related deaths among men in the United States (3). Approximately 334,000 new cases of prostate cancer were diagnosed in 1997 (4), representing 1 of every 3 newly diagnosed cancers in men (3). More than 42,000 deaths annually are attributed to the disease in this country, reflecting 14% of all current cancer deaths in males (4). In addition, prostate cancer is an age-related disease with over 75% of cases diagnosed in men aged 65 and older. With the continuing shift of the American demographic pattern

toward an older population, an increase of 37% in the number of prostate deaths/year is predicted for the 1985–2000 time period (5).

Despite increased awareness of the disease and improved methods for early detection, a large proportion of prostate cancer patients die of metastatic disease that is resistant to conventional therapies (6). Metastatic disease involves evolution to an androgen-insensitive state (7, 8). Current treatment options for prostate cancer include radiation therapy, chemotherapy, androgen ablation therapy, or combinations of these treatments. One of the challenges in the treatment of metastatic disease is that these conventional therapies exhibit little activity against the androgen-insensitive cell population in the prostate tumor (6).

Recent evidence has established that ionizing radiation and many, if not all, cancer chemotherapeutic agents kill tumor cells *in vitro* and *in vivo* by apoptosis (reviewed in Ref. 1). Apoptosis is an evolutionarily conserved and biochemically driven form of cell death with distinct morphological features that is activated in response to a variety of stimuli (9).

The mechanism underlying the resistance of androgen-insensitive prostate cancer cells to chemotherapy-mediated apoptosis is unknown. Chemotherapy is effective because it activates the cell death machinery; therefore, we postulated that the intrinsic resistance to chemotherapy in androgen-insensitive prostate tumor cells may reflect an inability to successfully activate the apoptosis program. We set out to test the hypothesis that dysregulated apoptosis underlies the resistance of prostate cancer to chemotherapy. We approached this by investigating activation of two families of apoptosis signal-transducing molecules, the sphingolipids and the caspases, in an apoptosis-sensitive and apoptosis-resistant prostate cancer cell line.

Sphingolipids are key apoptotic signal-transducing lipids with a role in various regulatory pathways, including differentiation, cell cycle arrest, senescence, and apoptosis (10, 11). Ceramide, a central molecule in sphingolipid structure and metabolism, appears to be a novel second messenger, mediating the intracellular effects of various inducers to activate the apoptosis program (12, 13). Increases in ceramide levels, followed by induction of apoptosis, are seen in response to serum withdrawal (14), interleukin 1 $\beta$  (15, 16), nerve growth factor (17), dexamethasone (18), activators of Fas (19, 20), ionizing radiation (21), and chemotherapeutic agents (22, 23). Various enzymes appear to contribute to elevation in cellular levels of ceramide. These include: (a) enzymes involved in the *de novo* pathway of ceramide formation; and (b) neutral or acid sphingomyelinases that hydrolyze distinct pools of sphingomyelin (11). In addition, treatment with exogenous short chain homologues of ceramide mimics endogenous generation of ceramide and induces apoptosis (11). We have shown that ceramide activates caspase-3, the protease responsible for cleavage of PARP<sup>3</sup> (24, 25).

In this study, we investigate the mechanism underlying the resistance of an androgen-insensitive prostate cancer cell line to apoptosis. We establish the existence of a defect in the apoptosis pathway in the PC3 cell line by demonstrating that these cells are unable to elevate ceramide in response to treatment with camptothecin. In contrast, the

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<sup>3</sup> The abbreviations used are: PARP, poly(ADP-ribose) polymerase; FBS, fetal bovine serum; DAG, diacylglycerol.

androgen-sensitive LNCaP cell line is capable of elevating ceramide levels in response to the same treatment. We provide preliminary insight into the nature of the lesion in ceramide formation in PC3 cells by showing that the mechanism of ceramide generation in the apoptosis-sensitive prostate cancer cells does not require activation of ceramide synthase. These studies present novel insights into the mechanism by which prostate cancer cells may be resistant to induction of apoptosis. This represents a breakthrough in our understanding of the resistance of prostate cancer to therapy and contributes to our understanding of the basic biology of prostate cancer. Such insight into the biological and molecular mechanism contributing to the resistance of prostate cancer to therapy is crucial to the development of more effective regimens for advanced disease.

## MATERIALS AND METHODS

**Cell Lines and Culture Conditions.** The PC3 and LNCaP cell lines were a generous gift from Dr. Cary Robertson (Duke University). These cell lines were routinely maintained in RPMI 1640 with 10% FBS and grown at 37°C in a 5% CO<sub>2</sub> incubator. For general maintenance, PC3 cells were supplemented with 2 mM L-glutamine (Life Technologies, Inc.) and 5 μg/ml insulin and passaged with 0.025% trypsin-EDTA (Life Technologies, Inc.) twice weekly. LNCaP cells were supplemented with 2 mM L-glutamine, 5 μg/ml insulin, and 1% of ITS (insulin, transferrin, and selenium) and passaged by 0.025% trypsin once a week. To ensure that all cell lines had reached the same cell density by the time of treatment, PC3 cells in logarithmic growth were plated at 1.5 × 10<sup>4</sup> cells/ml, and LNCaP cells were plated at 4.5 × 10<sup>4</sup> cells/ml. Cells were plated in complete medium, allowed to grow for 2 days, refed with complete medium, and allowed to grow for an additional 24 h. Prior to treatment, cells were incubated in medium containing 2% FBS for 1 h.

**Chemicals.** D-Erythro-C<sub>6</sub> ceramide [(2S,3R,4E)-N-hexanoyl-sphingosine] was synthesized by the method of Jayadev *et al.* (14). RPMI 1640, FBS, L-glutamine, trypan blue, and trypsin-EDTA were from Life Technologies, Inc. (Gaithersburg, MD). ITS was from Collaborative Biomedical Products, Inc. (Bedford, MA). Proteinase K was from Boehringer-Mannheim (Indianapolis, IN). RNase was from 5 Prime → 3 Prime, Inc. (Boulder, CO). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Camptothecin was dissolved in DMSO at the stock concentration of 10 mg/ml and stored at -20°C.

**Cell Viability Measured by Trypan Blue Dye Exclusion.** Cell viability was determined by the ability of cells to exclude 0.4% trypan blue. An equal volume of trypan blue dye solution (0.1% w/v), PBS, and cell slurry were combined and allowed to sit for a few minutes at room temperature. The sample was loaded onto a hemocytometer, and the cells were scored as living or dead based on uptake of dye.

**Detection of PARP Cleavage by Western Blot.** Cells were harvested by trypsinization. After centrifugation, pellets were washed once in PBS and centrifuged. The pellets (~1.5 × 10<sup>6</sup> cells) were then resuspended in 30 μl of PBS, lysed by addition of 120 μl of ice-cold Laemmli buffer [60 mM Tris (pH 6.8), 2% SDS, 100 mM DTT, and 10% glycerol], and boiled at 100°C for 5 min. The protein content was determined by the method of Bradford (26). Samples containing 25 μg of protein were analyzed by Western blot (27). In brief, protein extracts were subjected to 8% SDS-PAGE, and the separated proteins were electrophoretically transferred to nitrocellulose at 4°C for a minimum of 1 h. The filter was blocked in 5% nonfat dry milk in washing buffer (0.1% Tween 20 in 1× PBS) for 1 h at room temperature, incubated in rabbit polyclonal antiserum raised against a peptide contained in the automodification domain of PARP (28) at a dilution of 1:2000, followed by horseradish peroxidase-conjugated goat antirabbit secondary antibody (Bio-Rad) at a dilution of 1:5000. The signal was detected using the enhanced chemiluminescence (ECL) detection system (Amersham).

**Evaluation of DNA Fragmentation by DNA Gel Electrophoresis.** DNA was isolated according to the method of Preston *et al.* (29). In brief, cells were harvested, washed with ice-cold PBS, and centrifuged. A pellet of ~1.5 × 10<sup>6</sup> cells was resuspended in 50–100 μl (depending on the size of the pellet) of lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.5% N-lauroyl-sarcosine, 1.4 mg/ml proteinase K, and incubated at 50°C for 2 h. Cell lysates were treated with 0.2 mg/ml RNase A at 37°C for 1 h. After extraction with phenol:

chloroform:isoamyl alcohol (25:24:1), the organic phase and interface material were separated from the aqueous phase by centrifugation in Phase Lock Gel (5 Prime → 3 Prime, Inc.). The aqueous phase was then mixed with an equal volume of loading dye (50% Ficoll and Bromphenol) and loaded onto a 1% agarose gel. Electrophoresis was carried out at 100 V for 45 min. Ethidium bromide (0.5 μg/ml) was included in the agarose gel to visualize DNA bands. The migration pattern of the DNA was visualized by a short wave UV transilluminator (Fotodyne, Hartland, WI). Gels were photographed with a Polaroid camera system (model MP-4 land camera with 545 filmholder) using Polaroid high speed film (type 57; Polaroid Corp., Cambridge, MA).

**Measurement of Ceramide Levels.** Approximately 1.5 × 10<sup>6</sup> cells were pelleted by centrifugation (300 × g for 10 min), washed once with ice-cold PBS, and extracted according to the method of Bligh and Dyer (30). Ceramide was then quantified by a modified DAG kinase assay using external ceramide standards (31, 32). In brief, the lipids were dried under N<sub>2</sub>, resuspended in β-octylglucoside:dioleoylphosphatidylglycerol mixed micelles (32, 33), and incubated at 37°C in a water bath for 30 min. The final reaction mixture contained 50 mM imidazole buffer (pH 6.6), 2.0 mM DTT, 1.67 μg DAG kinase (3 units/mg) per sample, and ATP [1 mM final concentration mixed with 0.4 μCi [<sup>32</sup>P]ATP (NEN) per sample]. After 30 min at room temperature, lipids were again extracted by the Bligh and Dyer method (exactly as above, except for the use of 1% HClO<sub>4</sub> in place of H<sub>2</sub>O). An aliquot of the lower organic phase (1.5 ml) was dried under N<sub>2</sub>. The lipids were redissolved in 80 μl of chloroform, and 20 μl of this solution were spotted on silica gel 60A TLC plates (Whatman), separated using a solvent system of chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1), and detected by autoradiography. The radioactive spots corresponding to phosphatidic acid and ceramide-phosphate (the phosphorylated products of DAG and ceramide, respectively) were identified by comparison to known standards. Spots were scraped into a scintillation vial, and the incorporated <sup>32</sup>P was quantified in a liquid scintillation counter. The level of ceramide was determined by comparison with a standard curve generated with known amounts of ceramide. Ceramide content was normalized to total phosphate content as described (31).

## RESULTS

**Camptothecin Induces Death in LNCaP Cells but not in PC3 Cells.** Camptothecin is a topoisomerase I inhibitor that has been shown to induce apoptosis in various cancer cells (34, 35). Cells in log-phase were exposed to 0.5, 1, and 5 μM camptothecin and harvested at 6, 14, 24, and 48 h. The viability of the cells was monitored by the trypan blue exclusion assay. In LNCaP cells treated with the lowest dose of camptothecin (0.5 μM), a 3–4-fold increase in cell death was measured at 48 h after treatment (Fig. 1A). Exposure to increasing concentrations of camptothecin (1–5 μM) resulted in a 7–8-fold increase in cell death by 48 h, as compared with the control cells. In contrast, under the same conditions, there was no measurable increase in cell death in PC3 cells (Fig. 1A).

Although camptothecin does not induce cell death in PC3 cells, it does inhibit cell growth (Fig. 1B), suggesting that the drug is reaching its target in the cell. All three doses of camptothecin were similarly effective in inhibiting cell growth, as determined by cell counts. The reduction in the number of viable cells was as follows: approximately 2-fold at 24 h, 3-fold at 48 h, and 7-fold at 72 h.

**Camptothecin Induces PARP Cleavage in LNCaP but not in PC3 Cells.** Proteolytic cleavage of PARP by one or more members of the ICE/ced-3 family of cysteine protease at a DEVD (Asp-Glu-Val-Asp) motif leads to the generation of an M<sub>r</sub> 85,000 fragment. This fragment is an early and characteristic feature of apoptosis in diverse cellular systems (36, 37). To determine whether the cell death measured in Fig. 1A was occurring by apoptosis, we measured the kinetics of PARP cleavage as a marker of induction of apoptosis. As shown in Fig. 2A, the M<sub>r</sub> 85,000 cleavage product appeared between 6 and 12 h after treatment of LNCaP cells with 1 μM camptothecin. Concurrently, the level of the M<sub>r</sub> 115,000 intact PARP protein was declining. By 36 h, most of the intact PARP protein in the cells had been cleaved to

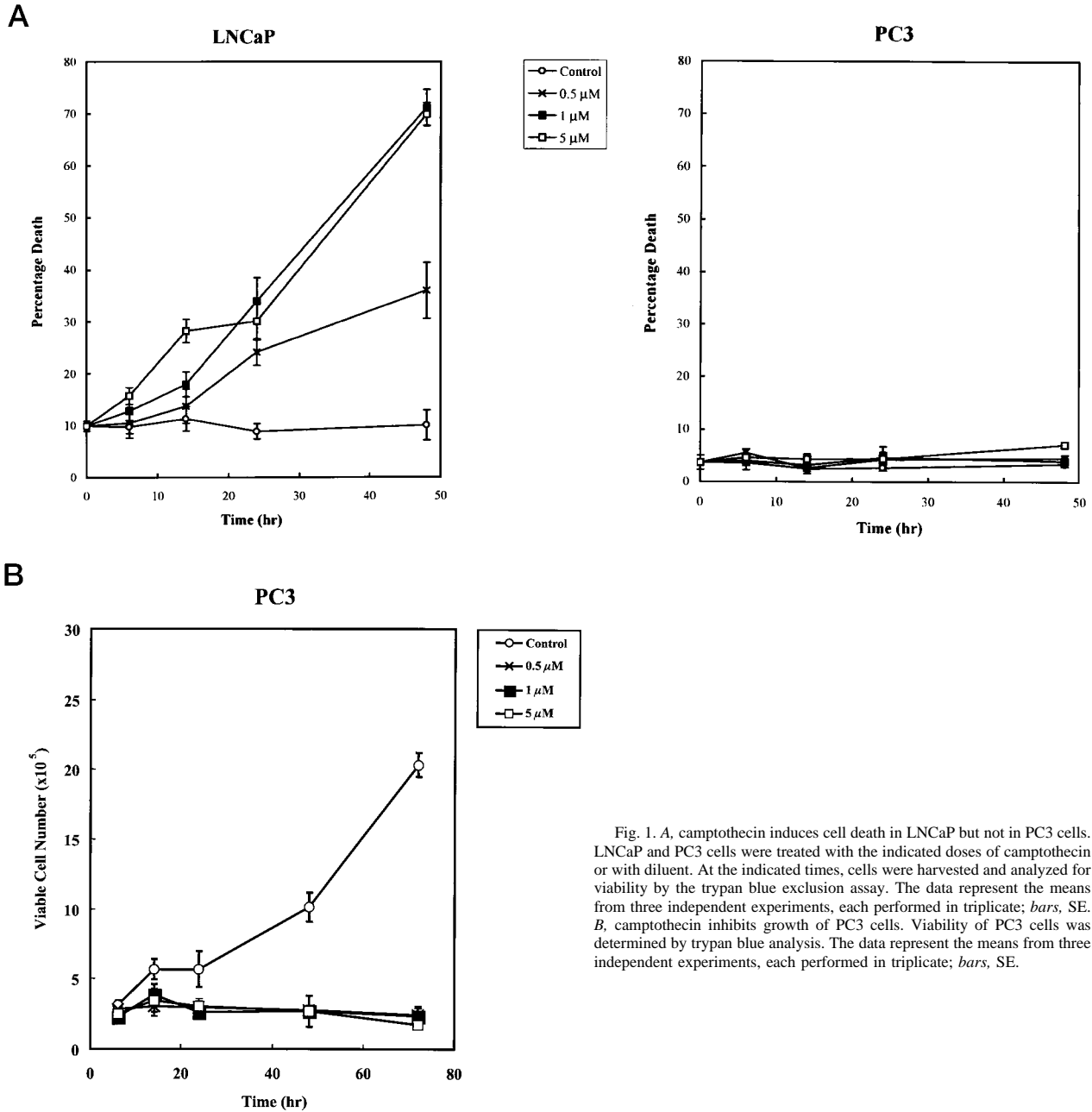


Fig. 1. A, camptothecin induces cell death in LNCaP but not in PC3 cells. LNCaP and PC3 cells were treated with the indicated doses of camptothecin or with diluent. At the indicated times, cells were harvested and analyzed for viability by the trypan blue exclusion assay. The data represent the means from three independent experiments, each performed in triplicate; bars, SE. B, camptothecin inhibits growth of PC3 cells. Viability of PC3 cells was determined by trypan blue analysis. The data represent the means from three independent experiments, each performed in triplicate; bars, SE.

the apoptosis-specific fragment. By 48 h, both fragments were difficult to detect because of the advanced degree of apoptosis. In PC3 cells, there was no PARP cleavage under the same conditions. This result provided support for our hypothesis that aberrations in the mechanism of apoptosis are responsible for the inability of PC3 cells to respond to activators of the death machinery.

**Camptothecin Induces Apoptosis-specific DNA Laddering in LNCaP but not in PC3 Cells.** Treatment of LNCaP cells with 1  $\mu$ M camptothecin resulted in apoptosis-specific DNA degradation by 12 h after treatment, as detected by a DNA ladder (Fig. 2B). However, no DNA laddering was observed in PC3 cells undergoing the same treatment (Fig. 2B). Extended monitoring of the PC3 cells between 48 and 72 h after treatment failed to show any evidence of the apoptosis-specific DNA ladder (data not shown). In addition, flow cytometric analysis determined that a hypodiploid DNA peak was detectable in LNCaP and not in PC3 nuclei in response to treatment with camptothecin (data not

shown). These data are consistent with the trypan blue analyses and PARP data and confirm the resistance of PC3 cells to apoptosis.

**Camptothecin Causes Elevation in Endogenous Ceramide Levels in LNCaP but not in PC3 Cells.** Having established that PC3 cells are insensitive to induction of apoptosis by camptothecin, we next investigated whether the lesion in PC3 cells was upstream or downstream of ceramide generation. We, therefore, measured generation of ceramide in LNCaP and PC3 cells after treatment with camptothecin. As indicated in Fig. 3, camptothecin caused elevation in ceramide levels in LNCaP but not in PC3 cells, as determined by the DAG kinase assay. After treatment with 1  $\mu$ M camptothecin for 24 h, ceramide levels had reached a plateau, culminating in an increase of  $\sim$ 2-fold over control. However, PC3 cells failed to generate ceramide in response to the same treatment. Similar results were obtained after treatment with vincristine (data not shown). These results provide convincing preliminary data indicating that the inability



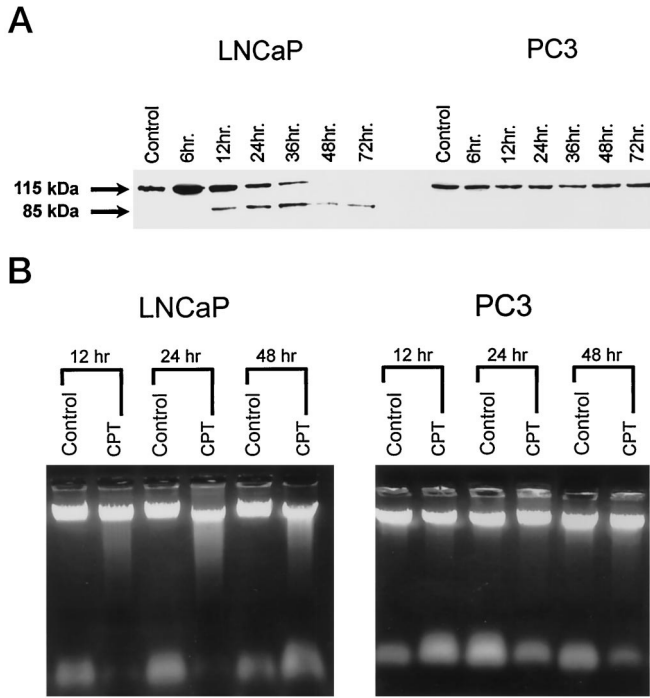


Fig. 2. A, camptothecin induces PARP cleavage in LNCaP but not in PC3 cells. Cells were treated with 1  $\mu$ M camptothecin for the indicated times, and PARP proteolysis to the apoptosis-specific  $M_r$  85,000 fragment was monitored by Western blotting. B, camptothecin (CPT) induces DNA laddering in LNCaP but not in PC3 cells. Cells were treated with 1  $\mu$ M camptothecin. At the indicated time points, genomic DNA was extracted and subjected to agarose gel electrophoresis.

ity to generate the ceramide signal is, at least, a component of the resistance to death in PC3 cells. These studies indicate that increased ceramide generation occurs early after treatment with camptothecin and peaks by 24 h. In contrast, downstream signals such as PARP cleavage and DNA laddering are still ongoing at later time points (Fig. 2). Increases in permeability to trypan blue (a late event) are measurable at significantly later time points (Figs. 1 and 2).

**LNCaP and PC3 Cells Undergo Cell Death after Treatment with Exogenous Ceramide.** Addition of exogenous short-chain ceramides (D-erythro- $C_2$  and D-erythro- $C_6$ ) has been shown to induce apoptosis in various cell systems (11). The mechanism of this effect is believed to involve elevation of endogenous ceramide levels by the exogenous ceramide. Because PC3 cells are unable to elevate endogenous ceramide in response to treatment with camptothecin, it became important to determine whether we could bypass the failure to transmit the apoptosis signal by addition of exogenous ceramide to PC3 cells. We,

therefore, investigated the response of LNCaP and PC3 cells to treatment with 5, 10, and 15  $\mu$ M  $C_6$ -ceramide. At the indicated times, cells were harvested and analyzed for viability by the trypan blue exclusion assay. As indicated in Fig. 4, measurements of cell viability by trypan blue exclusion established that both cell lines undergo cell death in response to addition of ceramide to the culture medium. Interestingly, both cell lines differ markedly in the kinetics and magnitude of their response. The overall magnitude of the response of the PC3 cells, relative to the internal controls, was considerably higher than that of the LNCaPs. Also, the PC3 cells were significantly more sensitive to the death-inducing effects of the lower doses of ceramide (5 and 10  $\mu$ M). For example, treatment of LNCaP cells with 5 and 10  $\mu$ M  $C_6$ -ceramide resulted in a 2-fold increase in cell death by 48 h after treatment. In contrast, by 24 h after treatment, the level of death in the PC3 line in response to 5 and 10  $\mu$ M  $C_6$ -ceramide was 7- and 12-fold over background, respectively. In LNCaP cells, 15  $\mu$ M  $C_6$ -ceramide was significantly more effective at inducing cell death, resulting in a 4-fold increase in cell death by 24 h and a 5-fold increase by 48 h. Nevertheless, this is a muted response relative to the PC3 cells, where the comparable values are 20- and 23-fold, respectively. In addition, significant increases in cell death occurred early in the PC3 cells. At 6 h, treatment with 15  $\mu$ M  $C_6$ -ceramide resulted in a level of cell death that was 5-fold over background. By 12 h of treatment, even the lowest dose of ceramide (5  $\mu$ M) had caused significant cell death (6-fold over background).

**Treatment with Exogenous  $C_6$ -Ceramide Induces PARP Cleavage and DNA Laddering in PC3 Cells.** Consistent with the induction of cell death in PC3 cells in response to treatment with  $C_6$ -ceramide, these cells undergo PARP cleavage and DNA laddering in response to the same treatment (Fig. 5). This is in contrast to the inability of these cells to undergo apoptosis in response to camptothecin (compare with Fig. 2). PC3 cells were treated with 5  $\mu$ M  $C_6$ -ceramide for 16, 24, and 48 h. Cleavage of PARP was first evident between 16 and 24 h. DNA ladders were also detectable by 24 h after treatment. This is in contrast to the response of PC3 cells to camptothecin, where no evidence of DNA laddering was seen by 72 h.

Although PC3 cells do not generate a ceramide signal in response to camptothecin, they show great sensitivity to ceramide-induced apoptosis. This establishes that the downstream apoptosis machinery is intact in these cells. This lends strong support to our hypothesis that there is a defect in ceramide formation in PC3 cells and that this defect can be overcome by manipulation of key enzymes in the sphingolipid pathways. We suggest that overcoming this defect will be of therapeutic and mechanistic value.

**Fumonisin Does Not Inhibit Camptothecin-induced PARP Cleavage in LNCaP Cells.** On the basis of the foregoing data establishing a lesion in ceramide synthesis in PC3 cells, it became impor-

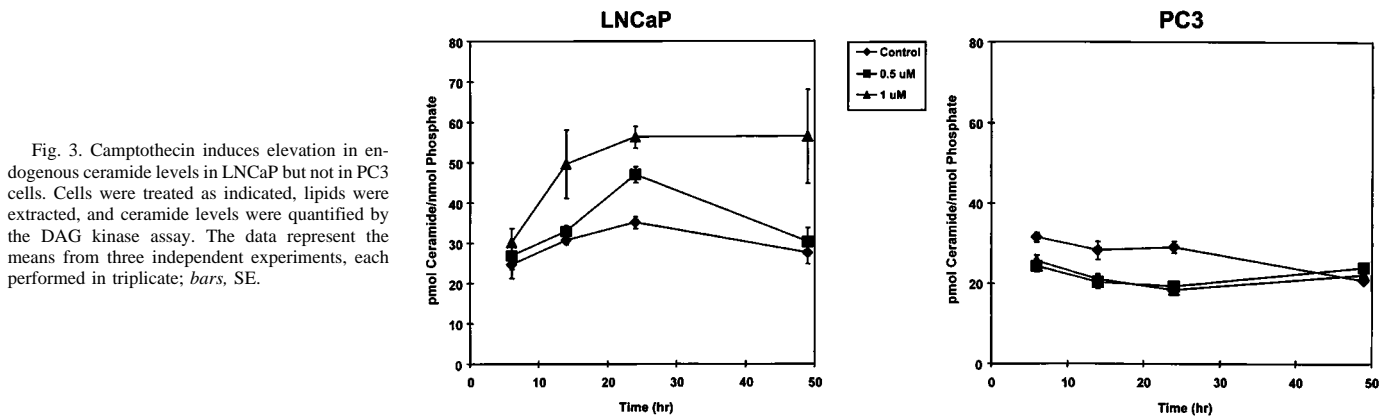


Fig. 3. Camptothecin induces elevation in endogenous ceramide levels in LNCaP but not in PC3 cells. Cells were treated as indicated, lipids were extracted, and ceramide levels were quantified by the DAG kinase assay. The data represent the means from three independent experiments, each performed in triplicate; bars, SE.

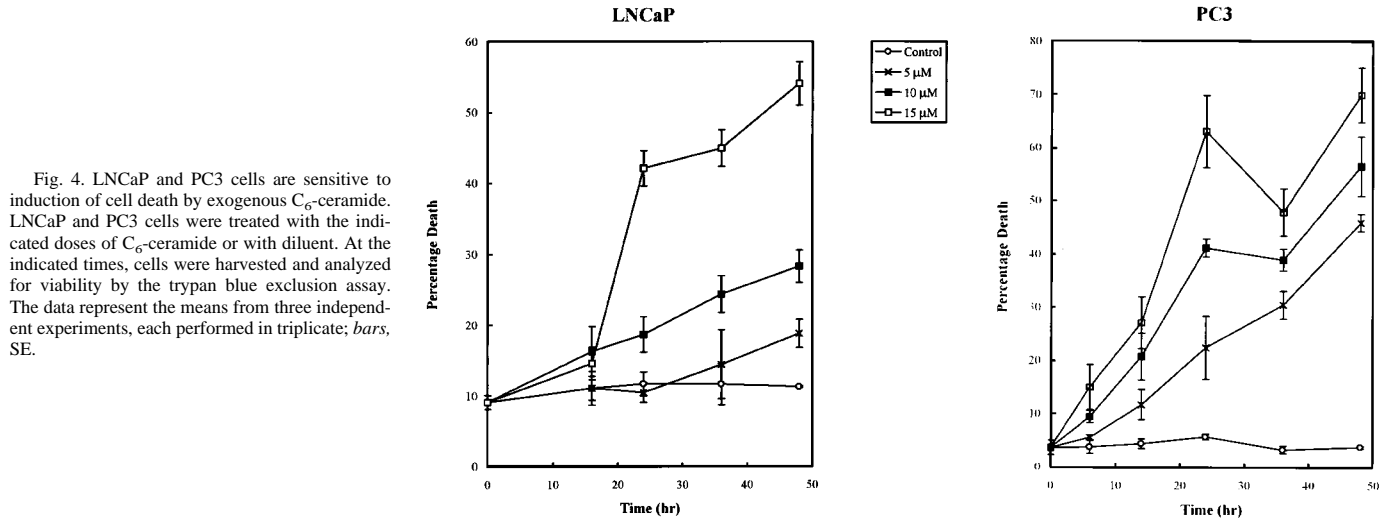


Fig. 4. LNCaP and PC3 cells are sensitive to induction of cell death by exogenous C<sub>6</sub>-ceramide. LNCaP and PC3 cells were treated with the indicated doses of C<sub>6</sub>-ceramide or with diluent. At the indicated times, cells were harvested and analyzed for viability by the trypan blue exclusion assay. The data represent the means from three independent experiments, each performed in triplicate; bars, SE.

tant to determine the mechanism involved in this failure of ceramide generation. To approach this, we decided to first investigate the method of generation of ceramide in the apoptosis-sensitive LNCaP cells. We did this by using fumonisin B1, a natural product of the fungus *Fusarium moniliforme* that is a specific inhibitor of ceramide synthase (38), blocking the conversion of dihydrosphingosine to dihydroceramide in the *de novo* pathway and regeneration of ceramide

from sphingosine in the salvage pathway (see Fig. 6). Fumonisin B1 has also been shown to attenuate apoptosis induced by camptothecin, daunorubicin, tumor necrosis factor- $\alpha$ , and phorbol ester (39–42). LNCaP cells were treated with fumonisin B1 in the presence of an apoptosis-inducing dose of camptothecin. In the experiment presented here, fumonisin B1 (50 and 100  $\mu$ M) was added either concurrently with 1  $\mu$ M camptothecin (cotreatment) or 30 min prior to addition of camptothecin (pretreatment). As shown in Fig. 7, camptothecin induced cleavage of PARP to the apoptosis-specific  $M_r$  85,000 fragment, and this cleavage was not inhibited by fumonisin. Similar results were obtained in experiments where the fumonisin pretreatment was extended by as much as 7 h. These results suggest that camptothecin does not kill LNCaP cells by activating ceramide synthase, either directly or indirectly. This leads us to rule out a role for the *de novo* or the salvage pathway in the generation of ceramide in response to camptothecin in LNCaP cells (Fig. 6).

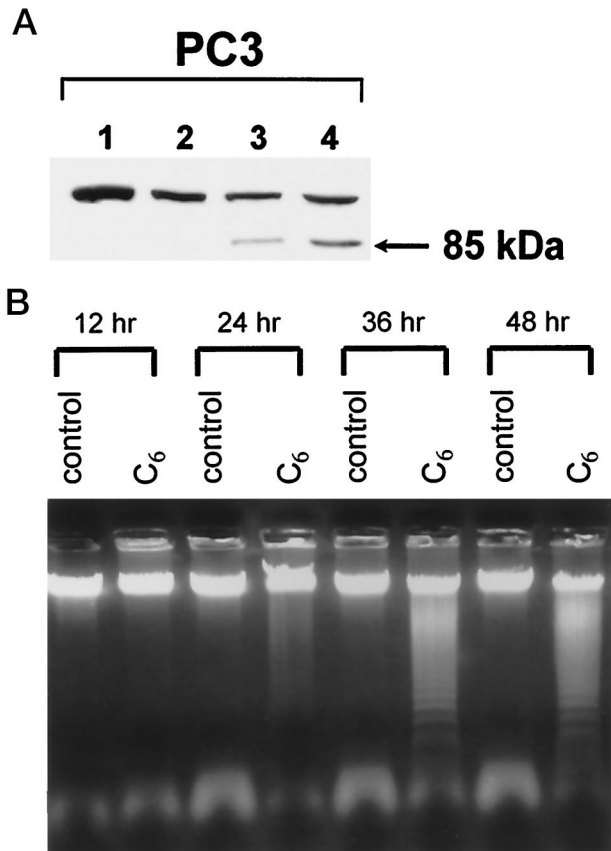


Fig. 5. A, induction of PARP cleavage by exogenous C<sub>6</sub>-ceramide in PC3 cells. PC3 cells were treated with 5  $\mu$ M C<sub>6</sub>-ceramide for 16 h (Lane 2), 24 h (Lane 3), and 48 h (Lane 4). Lane 1, diluent control. PARP proteolysis to the apoptosis-specific  $M_r$  85,000 (85 kDa) fragment was monitored by Western blotting. B, induction of DNA ladders by exogenous C<sub>6</sub>-ceramide (C<sub>6</sub>) in PC3 cells. Cells were treated with 5  $\mu$ M C<sub>6</sub>-ceramide. At the indicated time points, genomic DNA was extracted and subjected to agarose gel electrophoresis.

DISCUSSION

The studies presented here investigate activation of the apoptosis machinery in camptothecin-sensitive and camptothecin-resistant prostate cancer cells. Our studies have demonstrated that camptothecin induces apoptosis in the androgen-sensitive LNCaP cell line but not in the androgen-insensitive PC3 cell line. We defined the existence of a defect in the apoptosis pathway in PC3 cells by demonstrating that these cells are unable to elevate ceramide after treatment with camptothecin. In contrast, LNCaP cells are capable of elevating ceramide levels in response to the same treatment. Significantly, treatment with exogenous ceramide overcomes the lesion in the PC3 cells and induces death. Preliminary investigations of the mechanism of ceramide generation in LNCaP cells suggest that the mechanism of ceramide generation is independent of the *de novo* and salvage pathways. In summary, these studies provide important and fundamental insights into signal transduction during activation of the apoptosis cascade in prostate cancer cell lines.

There is now abundant evidence that chemotherapeutics and other cancer therapies induce apoptosis in tumor cells *in vivo* and *in vitro* (43), raising the possibility that resistance to therapy may involve defects in the regulation of apoptosis (44). This led us to postulate that hormone refractory prostate cancer has an inherent defect in apoptotic signaling that is the cause of the failure of these cells to die in response to antineoplastic drug treatment. The data in this report provide the first evidence for involvement of aberrations in sphingo-

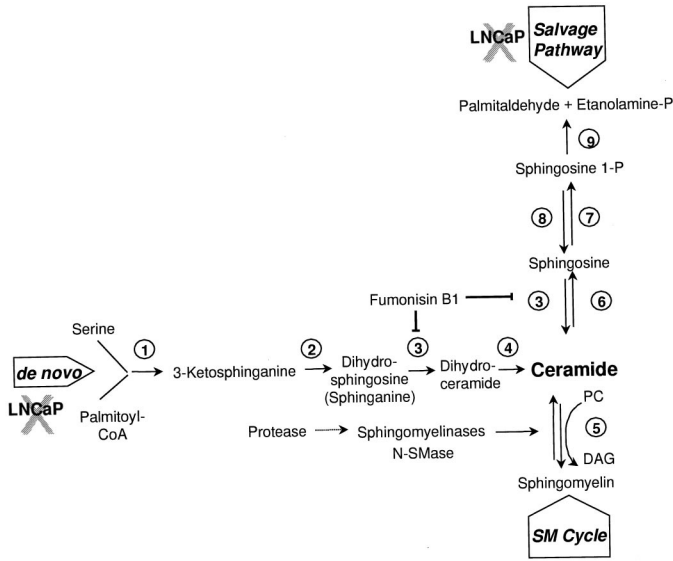


Fig. 6. Scheme of ceramide generation in LNCaP cells in response to camptothecin. As discussed in the text, the data presented here suggest that ceramide synthase is not involved in the generation of ceramide in LNCaP cells in response to treatment with camptothecin. The enzymes indicated are: 1, serine palmitoyltransferase; 2, NADPH-dependent reductase; 3, ceramide synthase (dihydroceramide synthase); 4, dehydrogenase (desaturase); 5, sphingomyelin synthase; 6, ceramidase; 7, sphingosine kinase; 8, sphingosine 1-P phosphatase; and 9, lyase. SM, sphingomyelin.

lipid accumulation in the resistance of an androgen-insensitive prostate cancer cell line to chemotherapy. This contributes novel insight into the mechanism by which androgen-insensitive prostate cancer cells may be resistant to induction of apoptosis.

Several investigators have previously examined induction of apoptosis in prostate cancer cell lines (42, 45–50). For example, LNCaP cells have been shown to undergo apoptosis in response to activation of protein kinase C $\alpha$  after treatment with 12-*O*-tetradecanoyl-phorbol 13-acetate (50). In contrast to the results presented here after treatment with camptothecin, the 12-*O*-tetradecanoyl-phorbol 13-acetate-induced apoptosis occurred by *de novo* generation of ceramide, after activation of ceramide synthase (42). Our ongoing studies investigating the mechanism of ceramide generation in apoptosis-sensitive prostate cancer cells may provide insight into the basis for these different mechanisms of ceramide generation in LNCaP cells. On the basis of the data in Fig. 7, the sphingomyelin cycle is now the main focus for our ongoing studies.

The contrast in the kinetics and magnitude of the response of PC3 and LNCaP cells to treatment with the short-chain ceramide analogues also merits further investigation.

Treatment with exogenous short chain membrane-permeable ceramides has been shown to reproduce the antiproliferative and differentiative effects of these agonists in myeloid and lymphoid cells (reviewed in Refs. 51 and 52). In addition, LNCaP cells have been shown previously to undergo apoptosis in response to treatment with ceramide homologues (47). Our data showing the effects of exogenous ceramide on apoptosis in prostate cancer cells provide the exciting insight that the lesion in PC3 cells can be overcome by use of ceramide homologues to manipulate the signal transduction machinery. This suggests that overexpression of enzymes of ceramide synthesis by gene therapy may provide a means for activating the apoptosis machinery to eliminate apoptosis-resistant prostate cancer cells. As normal cells undergo cell cycle arrest in response to elevations in ceramide levels and cancer cells undergo apoptosis (11), this selective response may provide a powerful therapeutic window of opportunity.

Camptothecin is an inhibitor of DNA topoisomerase I, which is a

nuclear enzyme involved in the metabolism of DNA. Because topoisomerase I plays a critical role in normal cellular physiology, inhibitors of topoisomerase I (such as the camptothecins) are effective drugs for treatment of human cancers (53, 54). Previous studies have investigated the effect of camptothecin on PC3 cells. For example, it has been suggested that the resistance of PC3 cells to anticancer drugs may be related to deletion of the *p53* gene (48). These authors have enhanced the cytotoxicity of camptothecin in PC3 cells by infecting these cells with adenovirus expressing the human wild-type *p53* gene. Studies investigating the relationship between the ceramide and *p53* pathways of growth inhibition have suggested that, in situations where *p53* performs a critical regulatory role (such as the response to genotoxic stress), it functions upstream of ceramide (11). We have demonstrated *p53*-independent ceramide accumulation in response to tumor necrosis factor- $\alpha$  in human leukemia cells and *p53*-dependent ceramide accumulation in response to genotoxic damage induced by actinomycin D or  $\gamma$ -irradiation in human leukemia cells (55). The relationship between *p53* and ceramide requires further investigation in prostate cancer cell lines that differ in their *p53* status.

The caspases are essential mediators of mammalian apoptosis (56). Activation of the caspases has been investigated previously in LNCaP cells, where caspase-7 has been identified as a potential mediator of lovastatin-induced apoptosis (46). We have shown previously that ceramide mediates the proteolytic cleavage of PARP by establishing that caspase-3 is a downstream target for ceramide-induced apoptosis (24, 25). The data in this present study documenting the inability of PC3 cells to elevate ceramide and to cleave PARP in response to treatment with camptothecin are consistent with these previous results.

Although the focus in the present study has been on downstream events in apoptosis signaling, it is also important to consider proximal mechanisms of resistance that could be operative in hormone-resistant prostate cancer. Because treatment of the apoptosis-resistant PC3 cells with camptothecin resulted in a significant inhibition of cell growth, this suggests that the drug is reaching its target in this cell line. To confirm this, we are engaged in ongoing approaches to evaluate drug accumulation and effect on target in the apoptosis-resistant and apoptosis-sensitive cell lines. The inhibition of cell growth seen here is consistent with data showing that a new derivative of camptothecin, CPT-11, inhibits the growth of PC3 cells (57).

Metastatic prostate cancer is a fatal disease because there is no effective systemic therapy for the control of the androgen-independent prostate cancer cells that develop during tumor progression (6, 8, 58). To increase survival of men with metastatic prostate cancer, a modality that can eliminate these apoptosis-resistant prostate cancer cells is urgently needed (8). Ultimately, it is anticipated that insights from studies of apoptosis signal transduction in prostate cancer will enable us to alter the apoptosis threshold of apoptosis-resistant prostate cancer cells and translate these insights into improved outcomes for patients with prostate cancer.

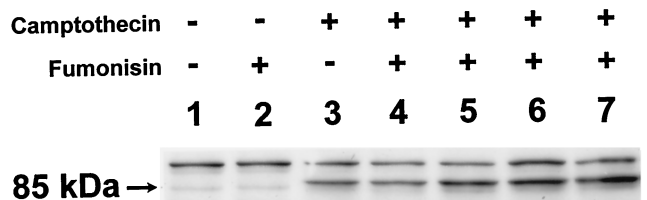


Fig. 7. Fumonisin does not inhibit camptothecin-induced PARP cleavage in LNCaP cells. Cells were treated with diluent alone (Lane 1), 100  $\mu$ M fumonisin alone (Lane 2), 1  $\mu$ M camptothecin (Lane 3), 1  $\mu$ M camptothecin + 50  $\mu$ M fumonisin (cotreatment; Lane 4), 1  $\mu$ M camptothecin + 100  $\mu$ M fumonisin (cotreatment; Lane 5), 1  $\mu$ M camptothecin + 50  $\mu$ M fumonisin (30-min pretreatment with fumonisin; Lane 6), or 1  $\mu$ M camptothecin + 100  $\mu$ M fumonisin (30-min pretreatment with fumonisin; Lane 7). Samples were harvested after 24 h of camptothecin treatment.



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