Biomedicine & Pharmacotherapy 66 (2012) 103–110

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Original article

Bioactive sphingolipids in docetaxel-induced apoptosis in human prostate cancer cells

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ARTICLE INFO

Article history:
Received 25 August 2011
Accepted 31 October 2011

Keywords:
Prostate cancer
Bioactive sphingolipids
Ceramides
Sphingosine kinase
Glucosyle ceramide synthase
Docetaxel

ABSTRACT

In this study, we examined the possible roles of ceramide/sphingosine-1-phosphate and ceramide/glucosyleceramide signaling in docetaxel-induced apoptosis by examining expression levels of the glucosyleceramide synthase and sphingosine kinase-1 and ceramide synthase gene family. As confirmed by isobologram analysis, docetaxel in combination with agents that increase intracellular ceramide levels increased the cytotoxic and apoptotic effects of docetaxel synergistically. More importantly, RT-PCR results revealed that expression levels of glucosyleceramide synthase and sphingosine kinase-1 were downregulated and ceramide synthase genes were upregulated in response to docetaxel. This study identifies mechanisms underlying the involvement of ceramide metabolizing genes in docetaxel-induced apoptosis in prostate cancer cells.

1. Introduction

Prostate cancer is the second leading cause of cancer-related deaths in men [1,2]. Chemotherapy provides the treatment of choice for the effective management of androgen-independent prostate cancer (AIPC). Docetaxel is a very effective anticancer agent used for the treatment of various types of cancers including prostate cancer [2–4]. Docetaxel inhibits microtubule depolymerization, resulting in cell cycle arrest and apoptosis [3,5,6].

Ceramides are lipid second messengers that mediate various cellular functions including cell growth, proliferation, drug resistance, and apoptosis. Many external factors, such as radiation and chemotherapeutics regulate endogenous ceramide levels [7]. Ceramides can be generated via de novo synthesis by the longevity assurance gene family (LASS) and via hydrolization of sphingomyelin by sphingomiyelinase. Cell-permeable ceramide analogs/mimetics have antiproliferative and apoptotic effects in various types of tumor cells [8,9]. However, aberrations in the generation of ceramides have been linked to resistance to cell death [10]. While increases in endogenous ceramide levels in response to chemotherapy were observed, there were also aberrations in bioactive sphingolipids in chemoresistant cells.

Glucosylceramide synthase (GCS) converts ceramides into antiapoptotic glucosylceramide. Since, GCS is over-expressed in almost all types of multidrug resistant cancer cells, it has been suggested as a potential marker of drug resistance [11,12]. In addition to GCS's roles in regulating proliferation and oncogenic transformation, it was shown that certain anticancer agents induce apoptosis through downregulating expression levels of GCS [13]. Sphingosine-1-phosphate (S1P), the product of SK-1, is shown to be an antiapoptotic lipid, since it has roles in malignant transformation, proliferation, angiogenesis, and resistance to cell death [14]. Inhibition of SK-1 activity was shown to increase cancer cells sensitivity to anticancer agents [15,16]. Up-regulation of SK-1 and S1P levels were determined in various types of cancer cells [17,18].

In the current study, we examined the possibility of increasing sensitivity of prostate cancer cells to docetaxel by increasing intracellular generation and accumulation of apoptotic ceramides. We also examined the roles of bioactive sphingolipids in docetaxel-induced apoptosis.

2. Materials and methods

2.1. Chemicals

Docetaxel was obtained from Sigma (Germany). C8:ceramide, N-(2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl)-decanamide, hydrochloride (PDMP), and SK-1 inhibitor were obtained from Cayman Chemicals (USA), dissolved in DMSO, and stored at −20 °C.

2.2. Cell lines

DU-145 and PC-3 cells were obtained from German Collection of Microorganisms and Cell Cultures (Germany). They were grown

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doi:10.1016/j.biopha.2011.10.003

in RPMI1640 medium (Biological Industries, Israel) containing 10% fetal bovine serum, and 1% penicillin-streptomycin (Invitrogen, USA) at 37 °C in 5% CO_2. DU-145 cells were established from a 69-year-old man with prostate carcinoma in 1975 [19] while PC-3 cells were established from a 62-year-old man with fourth grade prostate cancer [20]. They are both AIPC cells.

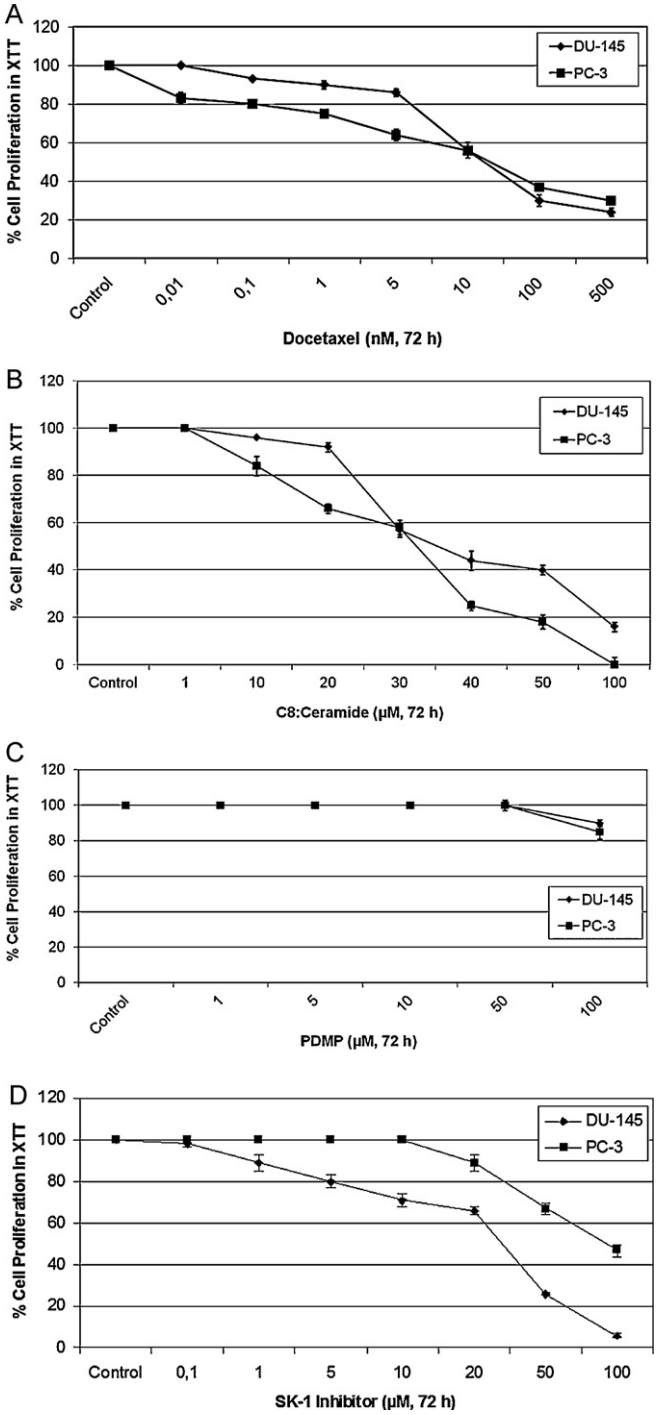

Fig. 1. Antiproliferative effects of docetaxel (A), C8:ceramide (B), PDMP (C), and SK-1 Inhibitor (D) on DU-145 and PC-3 cells. The IC50 concentration of docetaxel, C8:ceramide, IC10 values of PDMP, and SK-1 inhibitor were calculated from cell proliferation plots. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviations. Statistical significance was determined using two-way analysis of variance, and $P < 0.05$ was considered significant.

2.3. Measurement of growth by XTT

IC50 values (drug concentration inhibiting proliferation of 50% of cell population) of docetaxel and C8:ceramide, and IC10 values of SK-1 inhibitor, and GCS inhibitor, PDMP, were determined by XTT assay [21]. In short, 1×10^4 cells/well were seeded into 96-well plates containing 100 μl of the growth medium in the absence or presence of increasing concentrations of agents and then incubated at 37 °C in 5% CO_2. After 72 h, cells were treated with 50 μl XTT for 4 h. Then, the plates were read at 492 nm by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland). Finally, IC values were calculated from the cell proliferation plots. In order to determine possible synergism, docetaxel in combination with C8:ceramide, PDMP, or SK-1 inhibitor were applied to the cells and the same XTT procedure was applied.

2.4. Isobologram analysis for median dose effect

The CalcuSyn for Windows® computer program (CalcuSyn software, Biosoft, UK) was used to perform isobologram analysis of combinations [22]. Experimental data points, represented by dots located below, on, or above the line, indicate synergism, additivity,

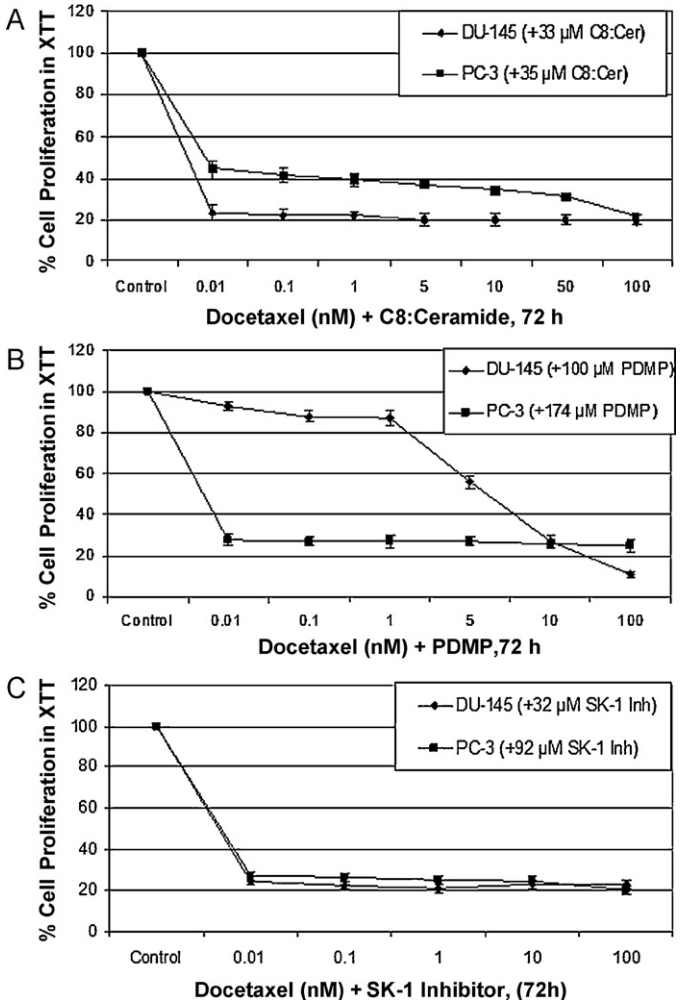

Fig. 2. Synergistic effects of docetaxel in combination with C8:ceramide, PDMP, and SK-1 inhibitor on proliferation of DU-145 and PC-3 cells. Antiproliferative effects of combinations were determined by the XTT cell proliferation test in a 72 h culture. The XTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviation. Statistical significance was determined using two-way analysis of variance, and $P < 0.05$ was considered significant.

and antagonism, respectively. The CI is an analysis of the combined effects of two agents, using a median effect plot analysis. A CI value < 1 indicates synergistic effect (0.1–0.5 strong synergism; < 0.1 very strong synergism); CI value of 1 indicates additive effect; and CI value > 1 indicates antagonistic effect (3.3–10 strong antagonism, > 10 very strong antagonism).

2.5. Measurement of caspase-3 enzyme activity

Changes in caspase-3 activity were determined using the caspase-3 colorimetric assay (R&D Systems, USA) [17]. The cells that had been induced to apoptosis were collected by centrifugation at 1000 rpm for 10 min. Then, the collected cells were

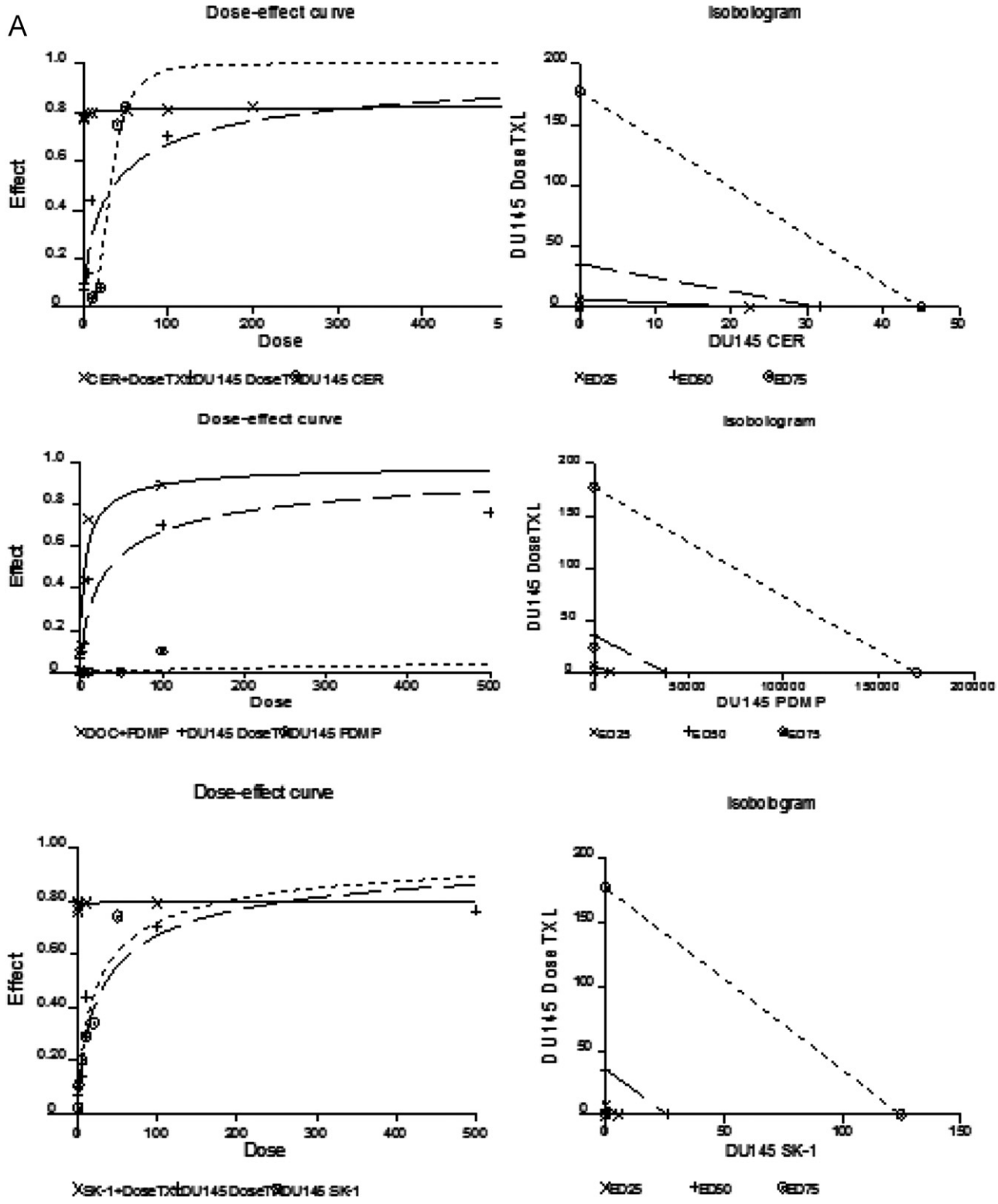

Fig. 3. Isobologram analysis of combination of docetaxel with C8:ceramide, PDMP and SK-1 inhibitor in DU-145 (A) and PC-3 (B) cells.

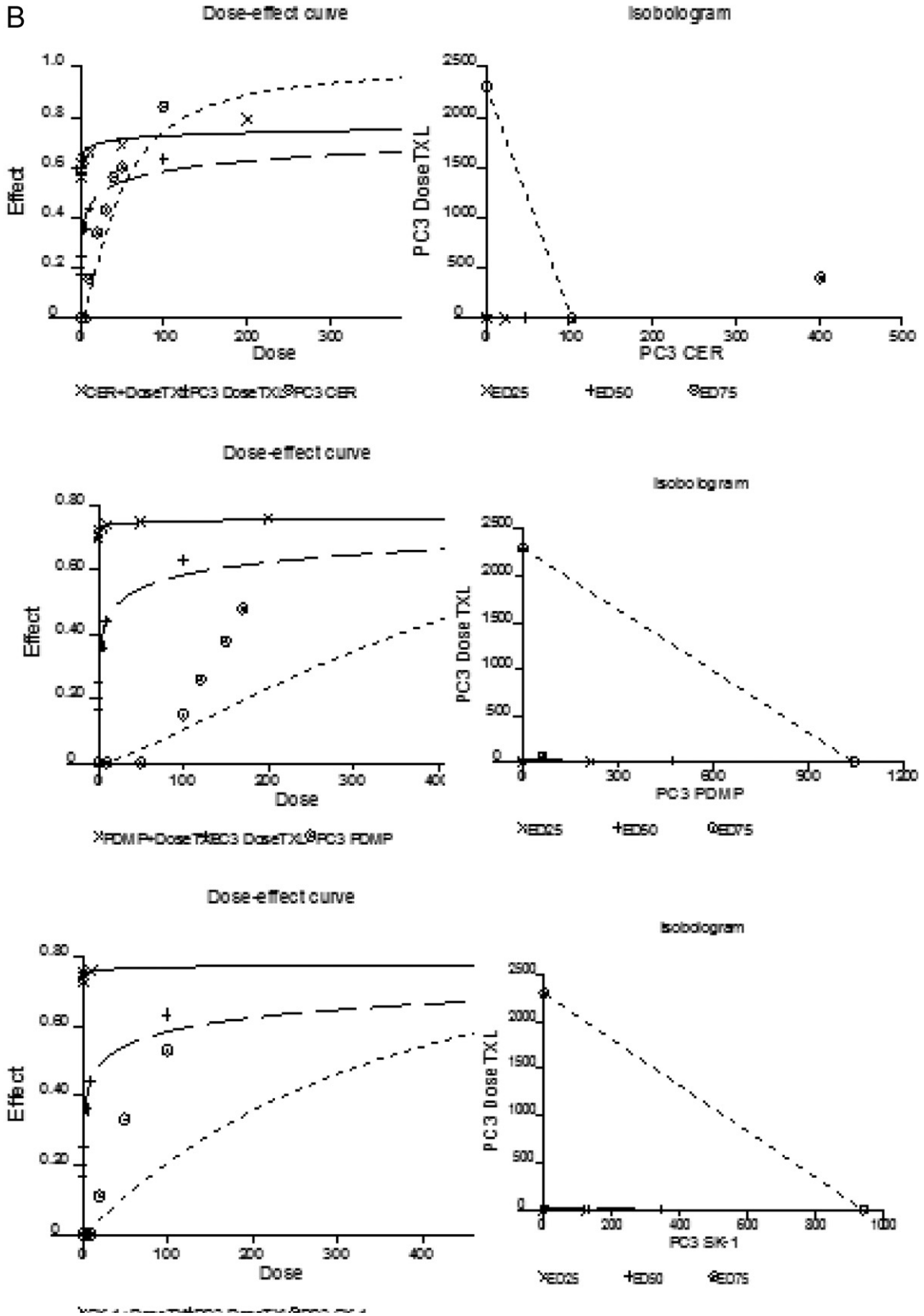

Fig. 3. (*Continued*).

lysed by addition of 100 μl of cold lysis buffer (1 ×). After incubation of the cells on ice for 10 min, they were centrifuged at 14000 rpm for 1 min. Supernatants were taken to new eppendorf tubes and the reaction mixture was prepared in 96-well plates adding 20 μl of assay buffer (5 ×), 25 μl of sample, 50 μl of sterilized water, and 5 μl of caspase-3 colorimetric substrate. After 2 hours of incubation at 37 °C in CO_2 incubator, the plate was read at 405 nm.

2.6. Detection of the loss of mitochondrial membrane potential (MMP)

APO LOGIX JC-1 Assay Kit (Cell Technology, USA) was used to measure the MMP in DU-145 and PC-3 cells [17]. At the beginning, the cells that had been induced to apoptosis were collected by centrifugation at 1000 rpm for 10 min. Supernatants were removed and 500 μl of JC-1 dye (1%) was added onto the pellets. After incubation of cells for 15 min at 37 °C in CO_2 incubator, they were centrifuged at 1000 rpm for 5 min. Then, 2 ml of assay buffer was added onto the pellets and they were centrifuged for 5 min at 1000 rpm again. All pellets were resuspended with 500 μl assay buffer and 150 μl from each of them was added into the 96-well plate in triplicate. The aggregate red form which remains within the intact mitochondria has absorption/emission maxima of 585/590 nm and the green monomeric form which is released to the cytoplasm due to the loss of MMP has absorption/emission maxima of 510/527 nm.

2.7. Total RNA isolation from cells and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The cells were incubated in the absence and presence of increasing concentrations of docetaxel, and total RNAs were isolated by using RNA Isolation Kit (Macherey-Nagel, USA). mRNAs from the total RNA population were reverse-transcribed into cDNA by using reverse transcriptase enzyme (Moroney Murine Leukemia Virus Reverse Transcriptase, Fermentas, USA). The resulting total cDNA was used in PCR to measure the mRNA levels of LASS1-6, SK-1, and GCS. mRNA levels of β actin were used as internal positive control.

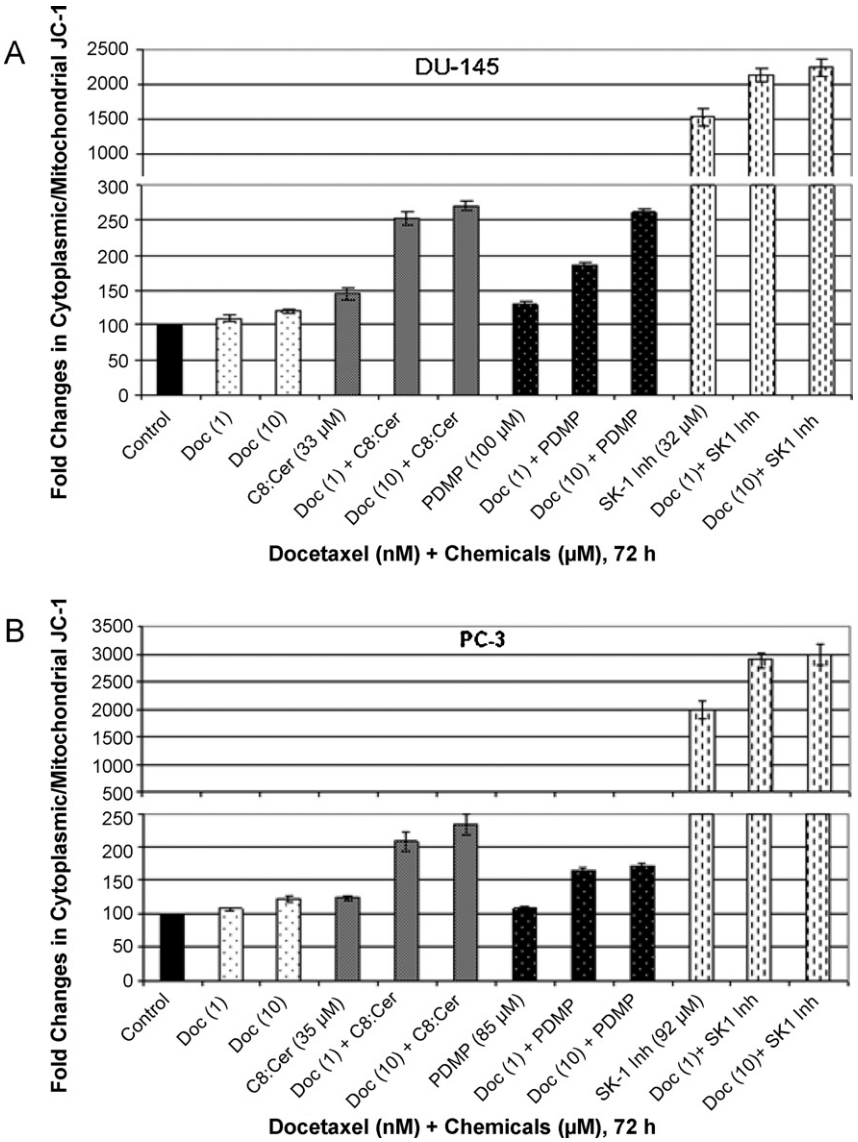

Fig. 4. Fold changes in cytoplasmic/mitochondrial JC-1 in DU-145 and PC-3 cells exposed to combinations of docetaxel with C8:ceramide, PDMP, and SK-1 inhibitor. The results are the means of two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $P < 0.05$ was considered significant.

3. Results

3.1. Antiproliferative effects of docetaxel, C8:ceramide, PDMP and SK-1 inhibitor on prostate cancer cells

IC50 values of docetaxel for DU-145 and PC-3 were found to be 30 and 38,4 nM (Fig. 1A), respectively. Dose-dependent decreases in proliferation of DU-145 and PC-3 cells were detected in response to C8:ceramide (IC50: 33 and 35 μM, respectively, Fig. 1B), PDMP (IC10: 100 and 85 μM, respectively, Fig. 1 C) and SK-1 inhibitor (IC10:32 and 92 μM, respectively, Fig. 1D).

3.2. Increasing intracellular concentrations of ceramides sensitized prostate cancer cells to docetaxel

Increasing concentrations of docetaxel with the IC50 value of C8:ceramide or IC10 values of PDMP or SK-1 inhibitor were applied to DU-145 and PC-3 cells to determine possible synergistic cytotoxicity. The data revealed that docetaxel in combination with C8:ceramide (Fig. 2A), PDMP (Fig. 2B) and SK-1 inhibitor (Fig. 2C) improved growth-inhibitory effects of docetaxel in both cells significantly. The CI values obtained by Calcusyn Programme for combination of docetaxel with C8:ceramide, PDMP and SK-1 inhibitor in DU-145 cells were 5.256E-021, 0.14462, and 1.4137E-033, respectively (Fig. 3A) while the values were 0.00018, 1.3924E-015, and 5.9743E-023 in PC-3 cells, respectively (Fig. 3B). All CI values showed very strong synergism for combination of docetaxel with the chemicals targeting bioactive sphingolipids.

3.3. Apoptotic effects of docetaxel alone or in combination with ceramide metabolism targeting agents on prostate cancer cells

It has been shown that docetaxel induces apoptosis in a dose-dependent manner through loss of MMP and increase of capase-3 enzyme activity in both DU-145 and PC-3 cells. While application of C8:ceramide, PDMP, or SK-1 inhibitors alone induced apoptosis, docetaxel in combination with C8:ceramide, PDMP or SK-1 inhibitor resulted in apoptosis synergistically. Apoptotic synergism was detected by increases in loss of MMP as compared to any agent alone or untreated controls in DU-145 (Fig. 4A) and PC-3 (Fig. 4B) cells. In order to confirm MMP and XTT data, we monitored the changes in caspase-3 enzyme activity in both DU-145 (Fig. 5A) and PC-3 (Fig. 5B) cells. Changes in caspase-3 enzyme activity in DU-145 and PC-3 cells confirmed previous data indicating synergistic apoptotic effects of docetaxel with sphingolipids targeting agents.

3.4. Expression levels of ceramide metabolizing genes in response to docetaxel

The roles of ceramide metabolising genes in docetaxel-induced apoptosis were investigated by examining mRNA levels of *LASS1-6*, *SK-1*, and *GCS* genes in human prostate cancer cells exposed to increasing concentrations of docetaxel for 72 h. Significant decreases in expression levels of *SK-1* and *GCS* genes were detected in both cells in response to docetaxel as compared to untreated controls and normalized to β actin levels (Fig. 4). There were no

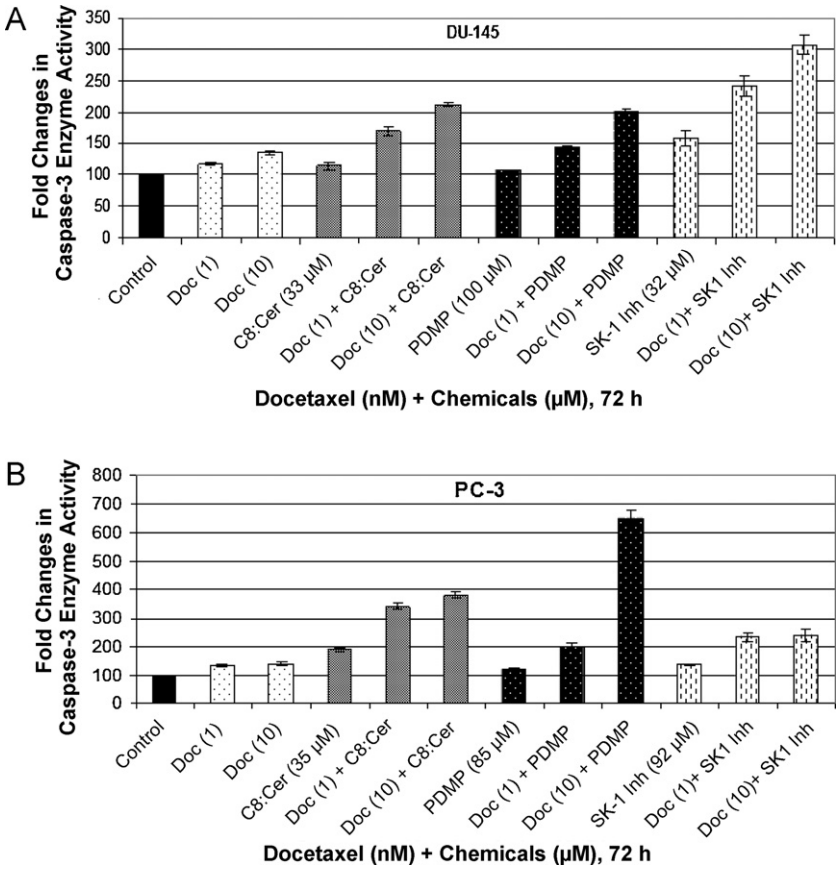

Fig. 5. Fold changes in caspase-3 enzyme activity in response to coadministration of docetaxel with C8:ceramide, PDMP, and SK-1 inhibitor. The results are the means of two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $P < 0.05$ was considered significant.

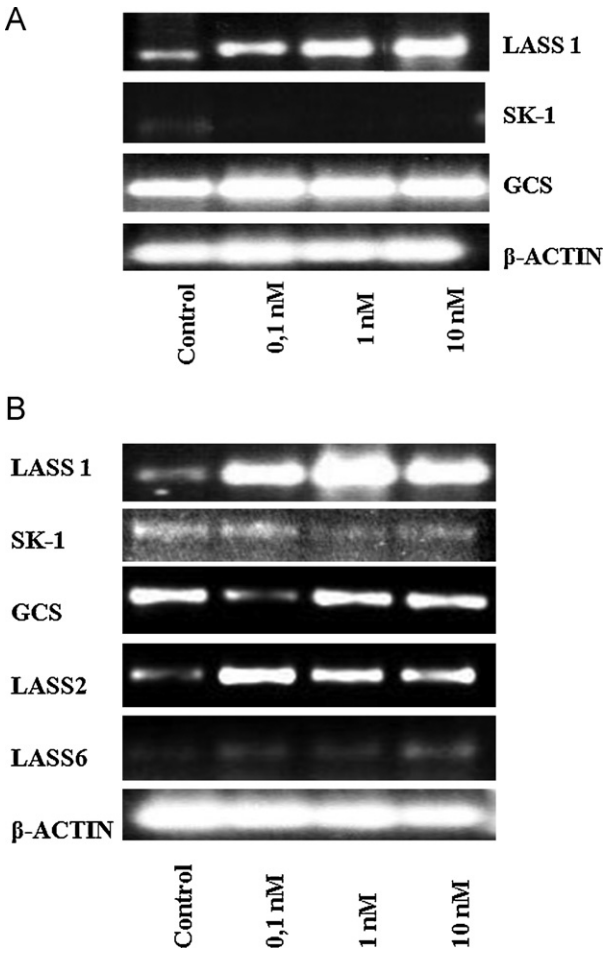

Fig. 6. mRNA levels of ceramide metabolising genes in response to docetaxel in DU-145 (A) and PC-3 (B) cells. 0,1-, 1-, and 10 nM docetaxel were applied to DU-145 and PC-3 cells for 72 h and expression levels of ceramide metabolising genes were determined by RT-PCR. Expression levels of β actin was detected as an internal positive control.

significant changes in expression levels of LASS2, LASS4, LASS5, and LASS6 in response to docetaxel in DU-145 cells. Increases in expression levels of LASS2 and LASS6 but not LASS4 and LASS5 were observed in PC-3 cells. The *LASS1* gene, responsible for C18:ceramide generation, was upregulated in both DU-145 and PC-3 cells (Fig. 6).

4. Discussion and conclusion

In this study, the roles and mechanisms of action of ceramide metabolism in the regulation of docetaxel-induced cell death were examined. The data obtained from this study suggest a novel mechanism of docetaxel-triggered apoptosis in prostate cancer cells. The results showed using ceramide analogs/mimetics or inhibition of GCS and SK-1 enzymes resulted in the increasing intracellular generation and accumulation of ceramides which decreased proliferation of prostate cancer cells and induced apoptosis through loss of MMP and increased caspase-3 enzyme activity. Above all, the results revealed that the addition of short chain ceramide, C8:ceramide or PDMP, or SK-1 inhibitor to docetaxel synergistically increases the sensitivity of prostate cancer cells, as compared to any agent alone. This study demonstrated that modulation of bioactive sphingolipids can provide a promising alternative approach for the treatment of AIPC.

Strategies that either mimic/antagonize bioactive sphingolipids or modulate their levels could provide a new way for treatment of cancer. Accumulating ceramide levels by molecular and/or biochemical methods has proved to increase apoptotic effects of different chemotherapeutic agents in various types of cancers [17,23]. Combination of short chain ceramide with paclitaxel increased therapeutic efficiency in both sensitive and multidrug resistant ovarian cancer cells [9,23]. Application of cell-permeable exogenous C6-ceramide sensitized different types of cancer cells to doxorubicin [24]. C(2)-ceramide induced apoptosis in human colon cancer cells [25] and increased the sensitivity of human NSCLC H1299 non-small cell lung cancer cells to paclitaxel-induced apoptosis [26]. A novel ceramide analog AL6 together with gemcitabine resulted in synergistic cytotoxicity and increased apoptosis in pancreatic cancer cells [27]. In parallel with these studies, we have shown that a combination of short chain C8:ceramide with docetaxel inhibited cell proliferation and induced apoptosis in prostate cancer cells, synergistically. In addition, we have shown for the first time that while docetaxel upregulates expression levels of LASS1 in both PC-3 and DU-145 cells, it up-regulates LASS2 and LASS6 only in PC-3 cells.

An inhibition of GCS and SK-1 provides a novel therapeutic option for the treatment of various types of cancers. Likewise, it has been shown that a combination of docetaxel with GCS or SK-1 inhibitors suppressed proliferation of prostate cancer cells and induced apoptosis synergistically. Dose-dependent decreases in expression levels of GCS and SK-1 in response to docetaxel in both cells were also observed. Dijkhuis et al. showed that inhibition of GCS by PDMP increased sensitivity of neuroblastoma cells to paclitaxel through inhibition of cell cycle progression [28]. It was also demonstrated that increasing accumulation of ceramides by inhibition of GCS increased sensitivity of p53 mutant human ovarian cancer cells to doxorubicine [29].

In conclusion, these results show that targeting ceramide metabolism by increasing its generation and/or accumulation might provide improved strategies for the treatment of prostate cancer. More importantly, the data presented here also show for the first time that docetaxel induces apoptosis in prostate cancer cells through increasing intracellular generation and accumulation of ceramides.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article. They do not have any financial and personal relationships with other people or organisations that could inappropriately influence (bias) our work.

Original publication: they confirm that this manuscript contains original unpublished work and is not being submitted for publication elsewhere at the same time.

Acknowledgment

They thank Prof Dr Anne Frary for critically reviewing the manuscript. This study was supported by TUBITAK project number 109S215 to Y.B and by the Turkish Academy of Sciences Outstanding Young Investigator Programme to Y.B.

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