

Pristimerin Induces Apoptosis in Prostate Cancer Cells by Down-regulating Bcl-2 through ROS-dependent Ubiquitin-proteasomal Degradation Pathway

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Abstract

Pristimerin is a quinonemethide triterpenoid with the potential of a promising anticancer agent. Pristimerin (PM) has shown anticancer activity against a range of cancer cell lines, but its activity for prostate cancer has not been adequately investigated. In the present study we have examined the underlying mechanisms of the apoptotic response of the hormone-sensitive (LNCaP) and hormone-refractory (PC-3) prostate cancer cell lines to PM. Treatment with PM induced apoptosis in both cell lines as characterized by increased annexin V-binding and cleavage of PARP-1 and procaspases-3 and -9. It also induced mitochondrial depolarization, cytochrome c release from mitochondria and generation of reactive oxygen species (ROS). Response to PM is regulated by Bcl-2 since it down-regulated Bcl-2 expression and overexpression of Bcl-2 rendered prostate cancer cells resistant to PM. ROS plays a role in down-regulation of Bcl-2, since treatment with PM in the presence of various ROS modulators, e.g., n-acetylcysteine (NAC), a general purpose antioxidant; diphenylene iodonium (DPI), a NADPH inhibitor; rotenone (ROT), a mitochondrial electron transport chain interrupter rotenone or MnTBAP, a O₂ scavenger, attenuated the down-regulation of Bcl-2. Furthermore, ROS is also involved in the ubiquitination and proteasomal degradation of Bcl-2 as both of these events were blocked by O₂ scavenger MnTBAP. Thus, pristimerin induces apoptosis in prostate cancer cells predominately through the mitochondrial apoptotic pathway by inhibiting antiapoptotic Bcl-2 through a ROS-dependent ubiquitin-proteasomal degradation pathway.

Keywords: Pristimerin; Apoptosis; ROS; Bcl-2; Ubiquitin; Proteasomes

Abbreviations: PM: Pristimerin; H₂DCF-DA: 6-carboxy-2,7-dichlorodihydrofluorescein diacetate; ROS: reactive Oxygen Species; PARP-1: Polyp-ADP-Ribose Polymerase-1; NAC: N-Acetylcystein; DPI: Diphenylene Iodonium; ROT: Rotenone; MnTBAP: Mn(III) tetrakis(4-benzoic acid)porphyrin; Bcl-2: B-cell lymphoma 2; Bcl-xL: B-cell lymphoma-extra large

Introduction

Carcinoma of the Prostate (CaP) is the most commonly diagnosed cancer and the second leading cause of cancer related death in American males with 238,590 new cases and 29,720 deaths from prostate cancer expected in the United States in 2013. Current therapies (radical prostatectomy, local radiotherapy or brachytherapy) while successful for treating localized prostate cancer are of limited efficacy against metastatic disease [1,2]. Androgen deprivation therapy produces objective responses; however, responses are temporary and the disease eventually progresses to hormone-refractory disease [3]. Therefore, there is an urgent need for novel agents and treatment strategies to combat this malignancy.

Herbal remedies are used in traditional medicine to treat and prevent human diseases including cancer. Numerous plant derived flavonoids and phenolic/polyphenolic compounds with antioxidant and anti-inflammatory activities are currently used by cancer patients as dietary supplements to complement chemotherapy. In fact, isolation and identification of bioactive components from medicinal plants have led to the synthesis and development of several potent anticancer drugs, such as Vinca alkaloids, taxol, camptothecin, etoposide and retinoids. Triterpenoids are members of a larger family of structurally related compounds known as cyclosqualenoids that are widely distributed in nature. Pristimerin is a quinonemethide triterpenoid present in

various plant species in the Celastraceae and Hippocrateaceae families [4,5]. Plant parts containing pristimerin have been used in traditional medicine as anti-inflammatory, antioxidant and antimalarial agents [6-8]. Recent studies have shown potent antiproliferative and apoptosis-inducing activity of pristimerin in diverse types of tumor cell lines, including glioma, leukemia, breast, lung, prostate and pancreatic cancer cell lines [9-12]. Induction of apoptosis by pristimerin involves activation of caspases, mitochondrial dysfunction, inhibition of antiapoptotic nuclear factor kappa B (NF- κ B) and Akt signaling pathways [13-15]. Pristimerin activates c-Jun N-terminal kinase (JNK) and poly (ADP-ribose) polymerase-1 (PARP-1) through the generation of reactive oxygen species [16]. Pristimerin is also capable of inhibiting cell cycle progression, proteasome, tumor cell migration and angiogenesis [11,17-19]. To the best of our knowledge there is only one study in which pristimerin was shown to induce apoptosis in prostate cancer cells through the inhibition of proteasome [11]. Since this report, there has been no other published report on the anticancer activity and mechanism(s) of action of pristimerin in prostate cancer cells. In the present study, we investigated the tumor inhibitory activity of pristimerin using androgen-sensitive and androgen-refractory

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prostate cancer cell lines. The results demonstrated that both hormone-sensitive and hormone refractory cells are equally susceptible to the induction of apoptosis by pristimerin through down-regulation of antiapoptotic Bcl-2 via a ROS-dependent ubiquitin-proteasomal degradation pathway.

Materials and Methods

Materials

Pristimerin (PM) was purchased from Sigma Chemicals (Saint Louis, MO). Anti-caspase-3 and caspase-9 antibodies were purchased from BD Pharmingen (San Diego, CA). Anti-PARP-1, anti-Bcl-2, anti-Bcl-xL and anti-Flag antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 96 Aqueous One Solution Proliferation Assay System was from Promega (Madison, WI). Annexin V-FITC apoptosis detection kit was purchased from BD Pharmingen (San Diego, CA) and mitochondrial potential sensor JC-1 was obtained from Molecular Probes, Invitrogen (San Diego, CA). 100 mM stock solution of PM was prepared in DMSO and all test concentrations were prepared by diluting stock solution in tissue culture medium.

Cell lines

LNCaP and PC-3 human prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). PC-3 cells were grown in F-12K nutrient mixture (Gibco BRL, Rockville, MD) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 25 mM HEPES buffer. LNCaP were grown in RPMI-1640 supplemented with FBS and penicillin/streptomycin. Both cell lines were cultured at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air, and maintained by subculturing cells twice a week.

Measurement of cell viability (MTS assay)

Tumor cells (1×10^4) in 100 μ l of tissue culture medium were seeded into each well of a 96-well plate. After 24 h incubation to allow cells to adhere, cells were treated with PM at concentrations ranging from 0 to 10 μ M. Cultures were incubated for additional 72 h and cell viability was then determined by the colorimetric MTS assay using CellTiter 96 Aqueous One Solution Proliferation Assay System from Promega (Madison, WI). This assay measures the bioreduction by intracellular dehydrogenases of the tetrazolium compound MTS in the presence of electron-coupling reagent phenazine methosulfate. MTS and phenazine methosulfate were added to the culture wells, and cultures were incubated for 2 h at 37°C. The absorbance, which is directly proportional to the number of viable cells in the cultures, was measured at 490 nm using a microplate reader.

Annexin V-FITC binding

Induction of apoptosis was assessed by the binding of annexin V to phosphatidylserine, which is externalized to the outer leaflet of the plasma membrane early during induction of apoptosis. Briefly, LNCaP and PC-3 cells treated with PM (0 to 5 μ M) for 20 h were resuspended in the binding buffer provided in the annexin V-FITC apoptosis detection kit II (BD Biosciences, Pharmingen). Cells were mixed with 5 μ l of annexin V-FITC reagent, 5 μ l of PI, and incubated for 30 min at room temperature in the dark. Stained cells were analyzed by flow cytometry.

Western blotting

Cell lysates were prepared by detergent lysis (1% Triton-X 100 (v/v), 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 10% glycerol, 2 mM sodium vanadate, 5 μ g/mL leupeptin, 1 μ g/mL aprotinin, 1

μ g/mL pepstatin A, and 10 μ g/mL 4-2-aminoethyl-benzenesulfinyl fluoride). Lysates were clarified by centrifugation at $14,000 \times g$ for 10 min at 4°C, and protein concentrations were determined by Bradford assay. Samples (50 μ g) were boiled in an equal volume of sample buffer (20% glycerol, 4% SDS, 0.2% Bromophenol Blue, 125 mM Tris-HCl (pH 7.5), and 640 mM 2-mercaptoethanol) and separated on 10% SDS-polyacrylamide gels. Proteins resolved on the gels were transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl with 0.05% Tween 20 (TPBS) and probed with protein specific antibodies to procaspase-3 and -9 (1:1000), Bcl-2 (1:500), Bcl-xL (1:500), cytochrome c (1:500) ubiquitin (1:250), Cox IV or β -actin (1:1000) followed by HRP-conjugated secondary antibody. Immune complexes were visualized with enhanced chemiluminescence detection system from Amersham Corp (Arlington Heights, IL). Protein bands were imaged and band densities analyzed using the NIH/Scion image analysis software. The protein band densities were normalized to the corresponding β -actin band densities and percent change in signal strength was calculated.

Measurement of ROS

H₂DCF-DA fluorescent probe was used to measure the intracellular generation of peroxide ROS species. Briefly, 1×10^6 LNCaP or PC-3 cells were plated in 6-well plates and allowed to attach overnight. Cells were treated or not with PM (0 to 10 μ M) for 2.5 h and then reacted with 5 μ M of H₂DCF-DA for 30 min at 37°C. Cells were collected by trypsinization and DCF-DA fluorescence was analyzed by flow cytometry.

Measurement of mitochondrial depolarization and cytochrome c release

The loss of mitochondrial potential by treatment with PM was determined using mitochondrial potential sensor JC-1 (Molecular Probes, Invitrogen, San Diego, CA). Control (untreated) or cells treated with PM (0 to 10 μ M) for 20 h were loaded with mitochondrial sensor by treating 1×10^6 cells in 1 ml culture medium with JC-1 dye (10 μ g/ml) for 10 minutes at 22°C. Cells were analyzed by flow cytometry. In normal cells, dye is aggregated in mitochondria, fluoresces red, and is detected in the FL2 channel. In cells with altered mitochondrial potential, the dye fails to accumulate in the mitochondria, remains as monomers in the cytoplasm, fluoresces green, and is detected in the FL1 channel.

For effect on mitochondrial cytochrome c, LNCaP and PC-3 cells were treated with PM (1.25 to 10 μ M) for 20 h and mitochondrial and cytosolic fractions were prepared using ApoAlert Cell Fractionation Kit (Clontech, Laboratories Inc., CA). Mitochondrial or cytosolic protein (10 μ g) was separated on a 14% SDS-PAGE gel and after transfer of proteins, membrane was probed with cytochrome c antibody.

DNA transfection

For overexpression of Flag-tagged Bcl-2, semi-confluent cultures of LNCaP and PC-3 cells in 60 mm² cell culture dishes were transfected with 10 μ g of empty or Bcl-2 expression plasmid (pCMV-Tag2B) containing Flag-tagged Bcl-2 cDNA under the control of CMV promoter (Addgene, Cambridge, MA) using LipofectAMINE Plus reagent. After incubation for 36 h, cells were analyzed for the expression of Bcl-2 by immunoblotting using anti-Bcl-2 antibody.

Immunoprecipitation

After treatment with PM (0 to 5 μ M) for 20 h cells were washed with cold PBS and lysed in NP 40 cell lysis buffer (Invitrogen, Camarillo, CA,

USA) supplemented with 2 mM sodium vanadate, 5 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/mL pepstatin, and 10 µg/mL 4-2-aminoethylbenzenesulfinyl fluoride for 30 min on ice. Supernatants were collected after centrifugation at 14000g for 10 min and protein concentration was determined. Each sample (400 µg protein) in 200 µl of antibody binding buffer containing anti-Flag antibody (DYKDDDDK epitope) was incubated for 1 h at room temperature followed by 10 µl of protein A agarose beads for 1 h. Immune complexes were washed two times with lysis buffer and analyzed for ubiquitin by western blotting.

Statistical analysis

Data are presented as means ± S.D. The differences between control and treatment groups were analyzed using Dunnett multiple comparison test and differences with $p < 0.05$ were considered statistically significant.

Results

Pristimerin inhibits proliferation of prostate cancer cells

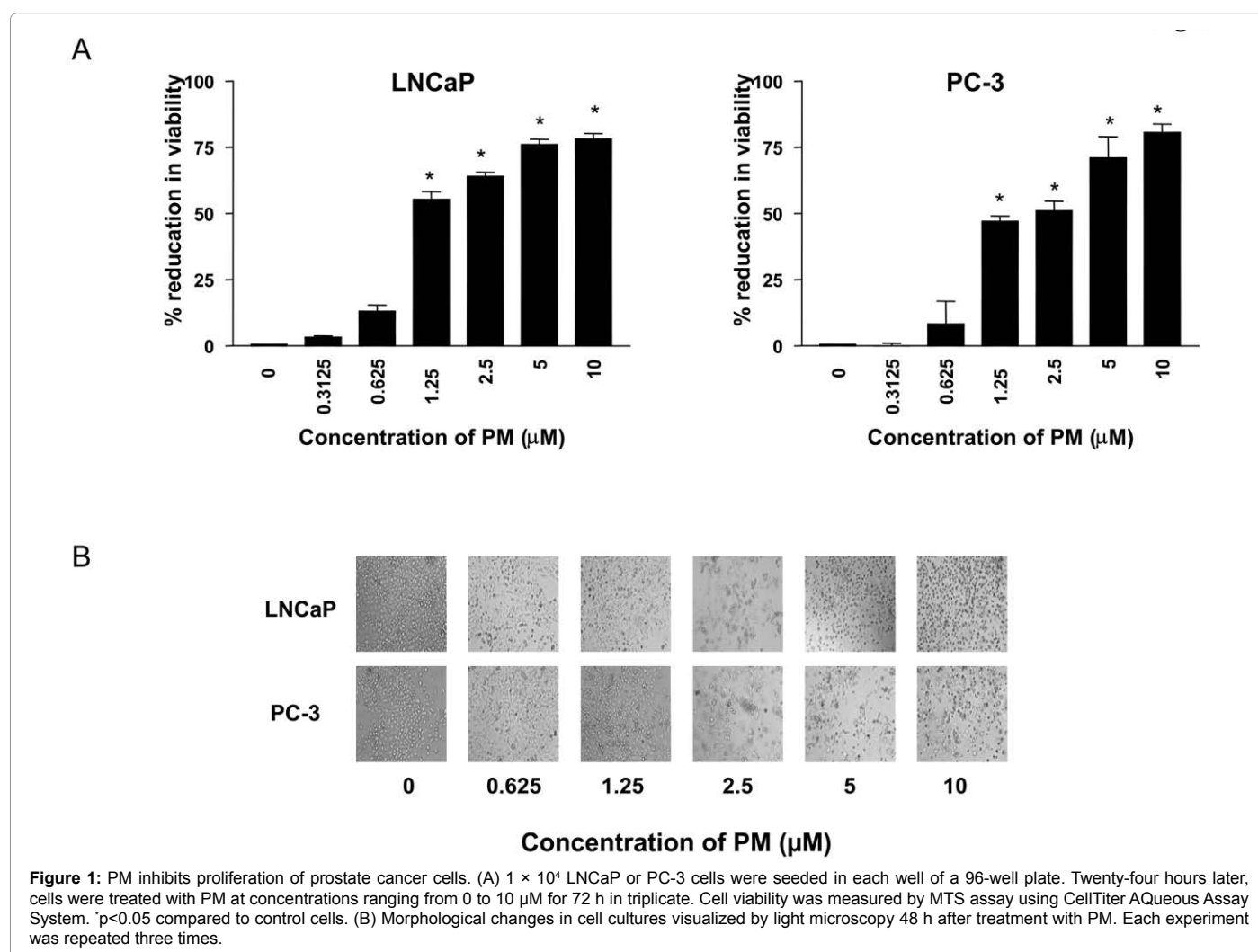
To measure the effect of pristimerin on proliferation of prostate cancer cells, LNCaP and PC-3 cells were treated with PM for 72 h at concentrations ranging from 0.3125 to 10 µM. At the end of the treatment, viability of cultures was determined by MTS assay.

As shown in Figure 1A, PM significantly reduced the proliferation of both cell lines (measured from the loss of viability of cultures) at a concentration of 1.25 µM (55% and 47% reduction, respectively, $p < 0.05$). The proliferation of both cell lines was further inhibited at higher concentrations of PM in a dose-related manner (LNCaP cells, 64 to 78% inhibition; PC-3 cells, 51 to 81% inhibition at 2.5 to 10 µM PM, $p < 0.05$).

The antiproliferative effect of PM in MTS assay correlated with the morphological changes in cell cultures treated with PM. Microscopic examination of LNCaP and PC-3 cell cultures at 48 h after treatment with PM showed partial rounding of cells at 0.625 µM. However, severe impact of PM on cellular growth was clearly evident at higher concentrations of PM (1.25 to 10 µM) as characterized by cellular detachment, rounding, shrinkage and clumping of cells in a dose-dependent manner (Figure 1B). Together, MTS and morphological data indicated strong antiproliferative effect of PM on both androgen-sensitive (LNCaP) and androgen-resistant (PC-3) prostate cancer cells.

Pristimerin induces apoptosis in prostate cancer cells

Whether PM induces apoptosis in prostate cancer cells was investigated next. We first measured the binding of annexin V-FITC to LNCaP and PC-3 cells treated with PM by flow cytometry. As shown in Figure 2A, only a small percentage of untreated LNCaP or PC-3



cells bound annexin V-FITC (1-4%). In the case of LNCaP cells, after treatment with PM for 20 h the percentage of annexin V-FITC-binding cells increased from 16% at 0.625 μ M to 70% at 5 μ M PM. Similarly, the percentage of annexin V-FITC-binding PC-3 cells also increased from 7% to 38% at 0.625 μ M to 5 μ M PM.

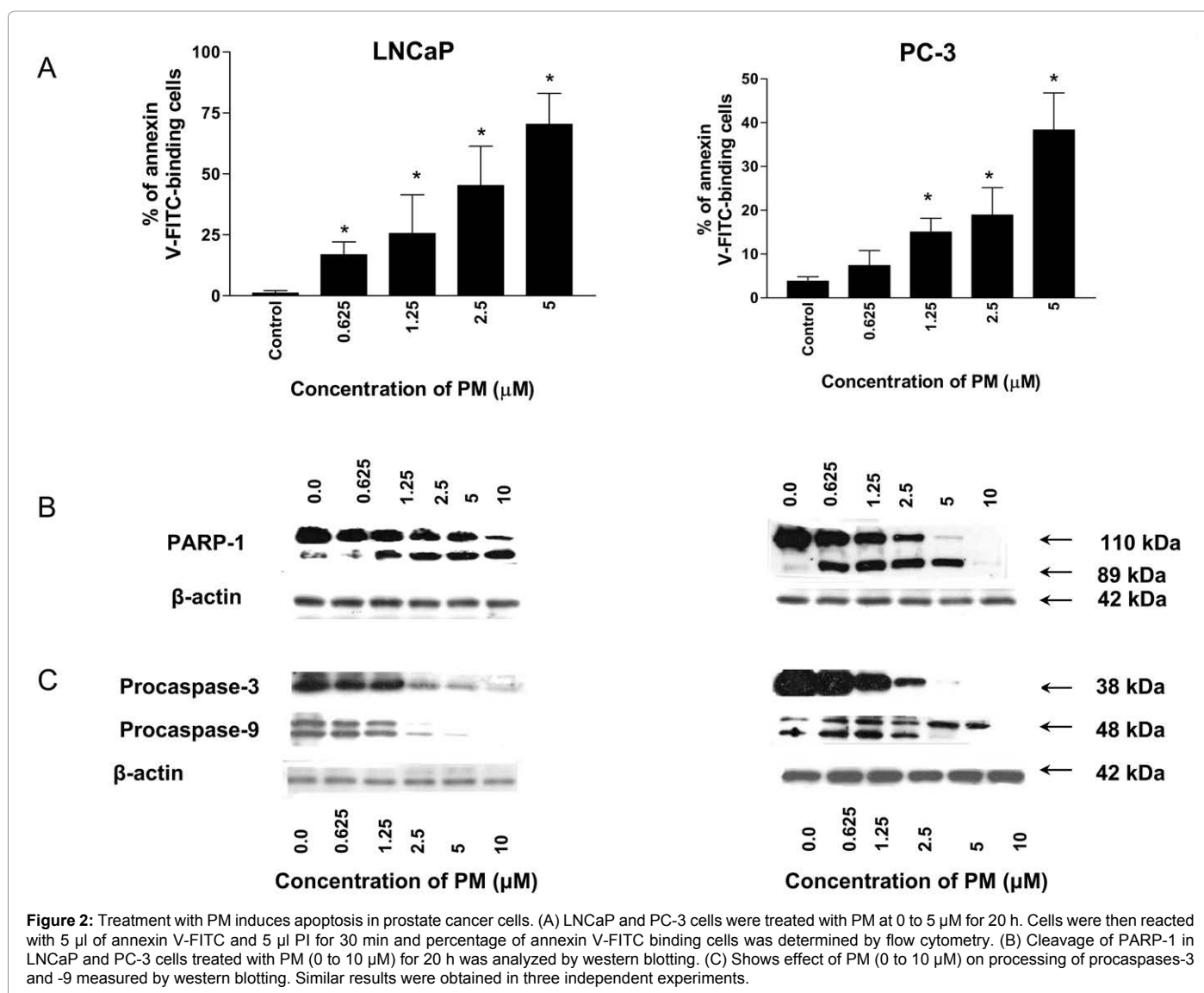
The induction of apoptosis by PM was confirmed by the cleavage of PARP-1 by western blotting. As can be seen in Figure 2B, the cleavage of PARP-1 in LNCaP cells was detectable at 1.25 μ M PM by the appearance of 89 kDa cleavage product. In PC-3 cells the cleavage of PARP-1 was detectable at 0.625 μ M PM. In both cell lines, the cleavage of PARP-1 was more pronounced at 2.5 to 10 μ M PM.

For further evidence that PM induces apoptosis in prostate cancer cells we also examined the effect of PM on the activation of procaspases-3 and -9. Western blot analysis of cell lysates prepared from cells treated with PM showed partial to complete processing of procaspases 3 and -9 in both cell lines at 1.25 to 10 μ M PM (Figure 2C). Together, increase in annexin V-FITC-binding cells and cleavage of PARP-1 and procaspases-3 and -9 indicated induction of apoptosis in prostate cancer cells by pristimerin.

Pristimerin induces mitochondrial depolarization and release of cytochrome c

We next investigated whether PM utilizes mitochondrial 'intrinsic' pathway in apoptotic death of prostate cancer cells. For this, we evaluated mitochondrial depolarization in cells treated with PM. LNCaP and PC-3 cells treated with PM (0.625-10 μ M) for 20 h were loaded with mitochondrial membrane-potential JC-1 probe and fluorescent shift in cells was measured by flow cytometry. There was a significant change in mitochondrial potential after treatment of both cell lines with PM. The percentage of LNCaP cells with green fluorescence changed from 2% at 0 μ M PM to 7%, 36%, 52%, 63% and 80% at 0.625, 1.25, 2.5, 5 and 10 μ M PM, respectively (Figure 3A). The effect of PM on mitochondrial depolarization in PC-3 was identical to LNCaP cells (e.g., 10%, 23%, 33%, 64%, 71% and 80% of cells with green fluorescence at 0, 0.625, 1.25, 2.5, 5 and 10 μ M PM, respectively).

To further determine the effect of PM on mitochondrial integrity, the release of cytochrome c from mitochondria in LNCaP and PC-3 cells treated with PM was measured. Western blot analysis of mitochondrial and cytosolic fractions of cells treated with PM (0.625-



10 μM) demonstrated concentration-dependent release of cytochrome c from the mitochondria in both cell lines. Mitochondrial cytochrome c levels were more than 90% decreased in LNCaP cells at 2.5 μM and were completely devoid of it at 10 μM PM. Identical effect of PM on the release of cytochrome c from mitochondria was seen in PC-3 cells. As expected, decrease in mitochondrial cytochrome c correlated with corresponding increase in cytosolic cytochrome c in both cell lines in a concentration-related manner (Figure 3B). Thus, loss of mitochondrial membrane potential and release of cytochrome c demonstrated induction of mitochondrial or intrinsic pathway of apoptosis by PM in LNCaP and PC-3 cells.

Pristimerin generates ROS in prostate cancer cells

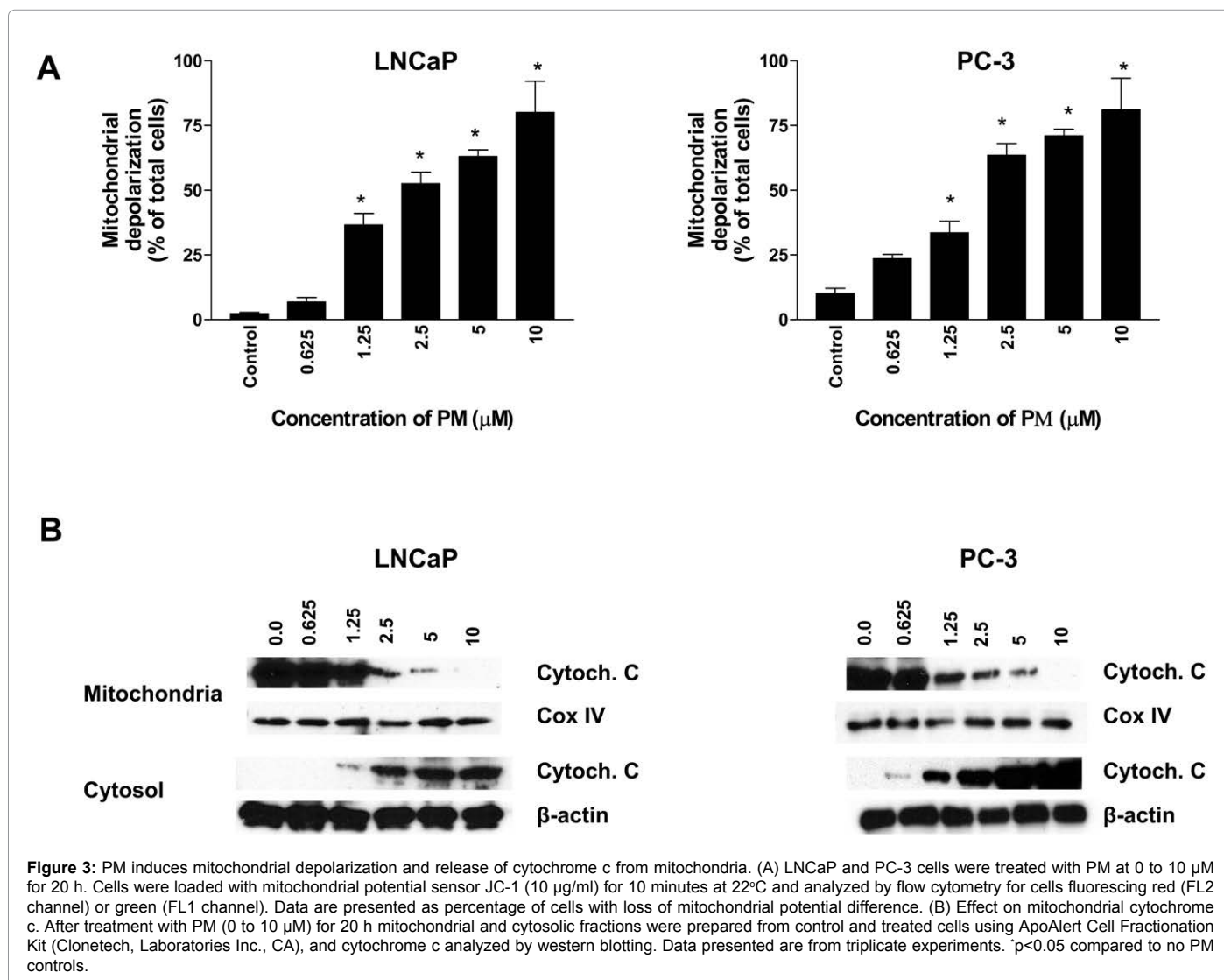
Whether PM causes the generation of intracellular ROS was investigated next. ROS generation was measured by flow cytometry using $\text{H}_2\text{DCF-DA}$ probe, a general purpose oxidative fluorescent probe that can detect multiple ROS. Figure 4 shows flow cytometric analysis of DCF fluorescence intensity in LNCaP and PC-3 cells treated with PM. Treatment of LNCaP with PM at 1.25 to 5 μM for 2.5 hour increased the mean DCF fluorescence intensity from 193% to 274% compared to control cells (100%). In case of PC-3 cells, the mean DCF fluorescence

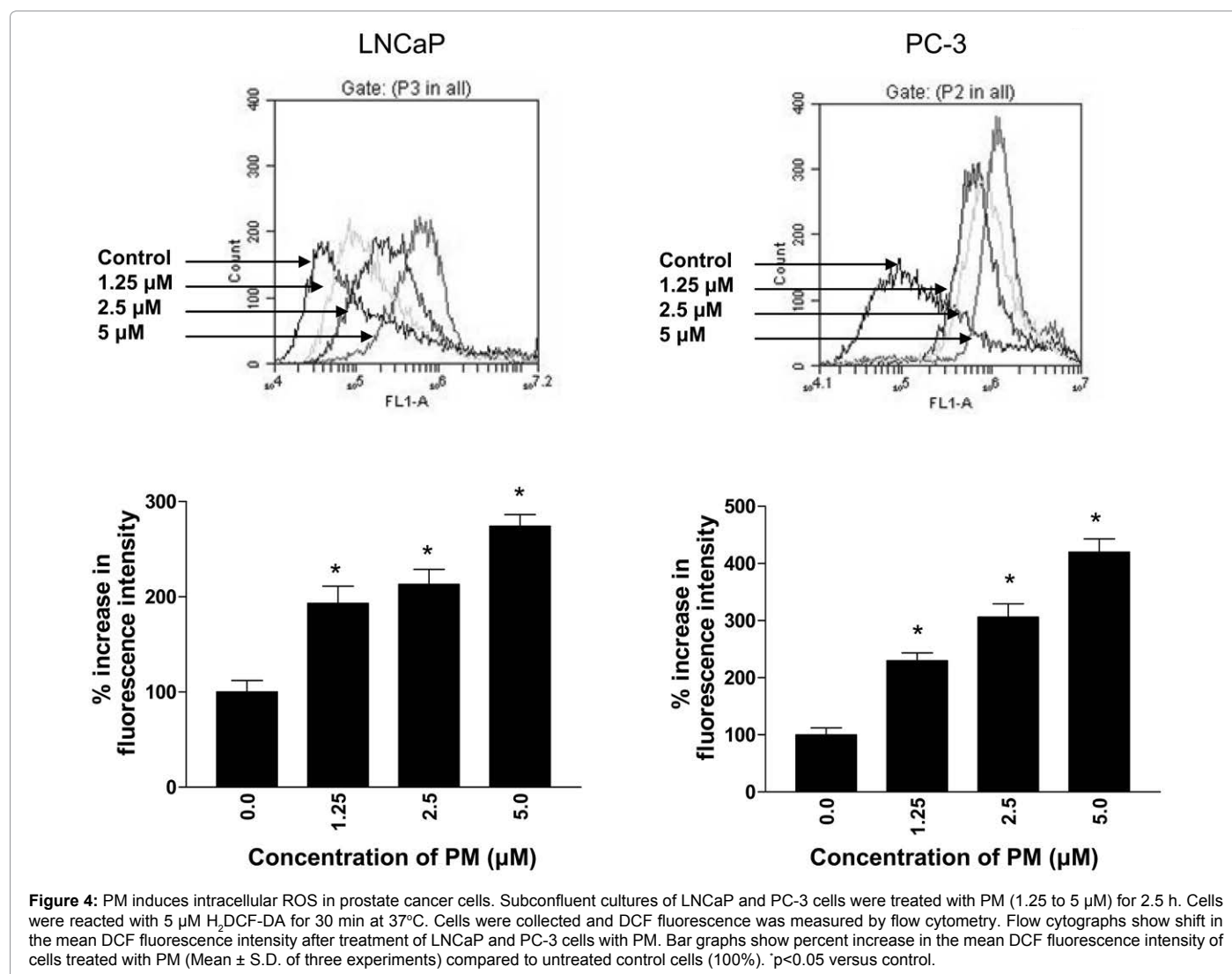
intensity increased from 230% to 420% at similar concentrations of PM. These data demonstrated generation of ROS by PM in prostate cancer cells.

Pristimerin inhibits Bcl-2 and Bcl-xL expression in prostate cancer cell

Bcl-2 and Bcl-xL are antiapoptotic proteins located in mitochondrial wall that regulate mitochondrial or “intrinsic” apoptosis pathway by controlling mitochondrial permeability. We determined the effect of PM on expression of these proteins in prostate cancer cells. Lysates of cells treated with PM (0.625 to 10 μM) for 20 h were analyzed by western blotting for the levels of Bcl-2 and Bcl-xL. As shown in Figure 5A, PM partially to completely inhibited Bcl-2 in LNCaP cells at 1.25 to 10 μM PM. PC-3 cells appear to be more sensitive with respect to the effect of PM on Bcl-2 expression, since significant reduction of Bcl-2 was seen in these cells even at 0.625 μM PM. Bcl-xL levels were also significantly to completely reduced in both cell lines at 0.625 to 10 μM PM.

To further determine the significance of Bcl-2 in response of tumor cells to PM, we overexpressed Bcl-2 in LNCaP and PC-3 cells





and measured their response to PM in MTS assay. As shown in Figure 5B, overexpression of Bcl-2 significantly reduced the susceptibility of both cell lines to PM at concentrations of 0.625 to 10 μM. Transfection with empty vector did not affect response to PM in either cell line (not shown).

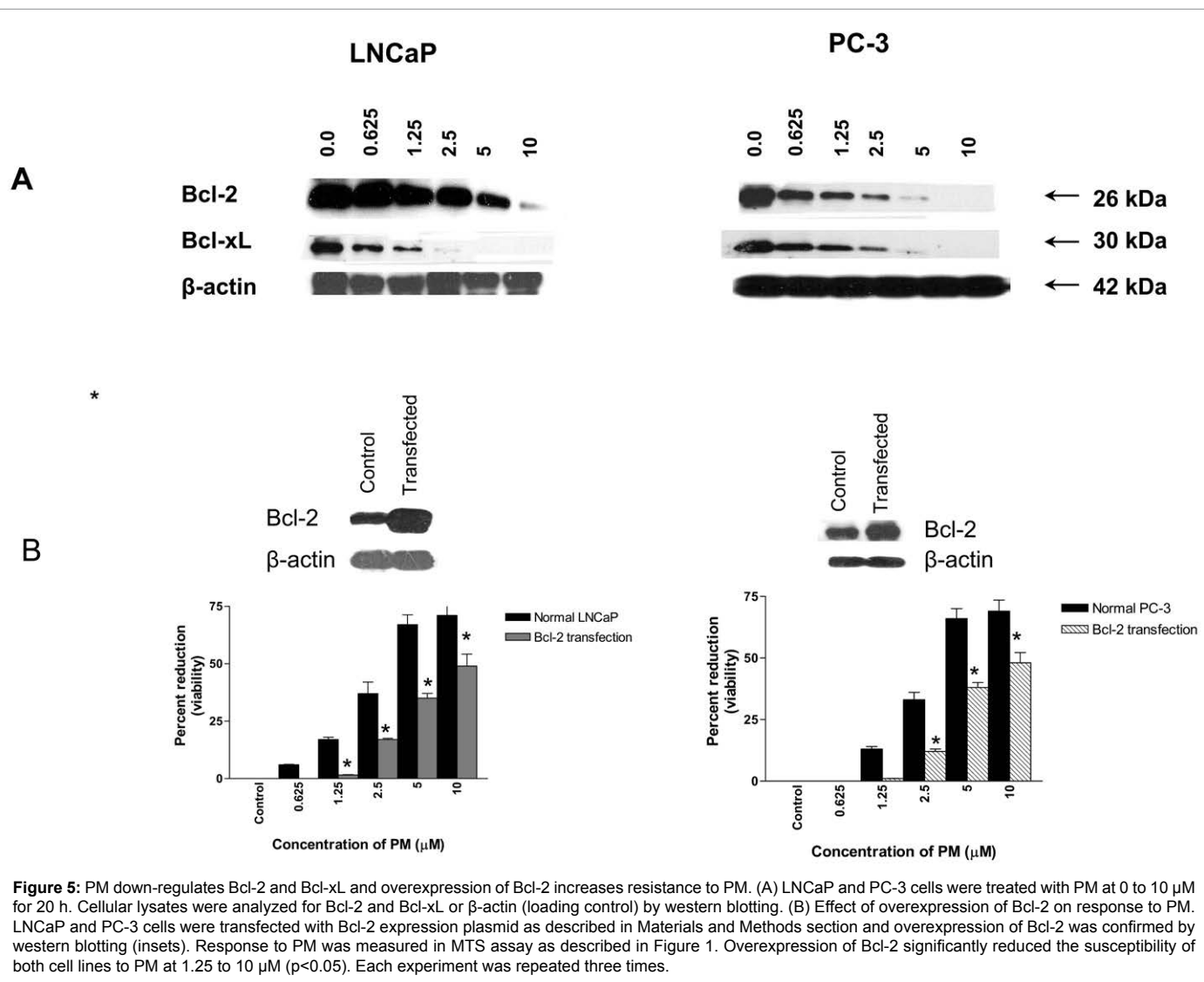
ROS plays a role in down-regulation of Bcl-2 by pristimerin

ROS can mediate apoptosis by chemical agents by facilitating down-regulation of antiapoptotic proteins including Bcl-2. To determine the role of ROS in inhibition of Bcl-2 by PM, LNCaP and PC-3 cells were treated with PM in the presence or absence of ROS modulators, such as general purpose antioxidant NAC or NADPH inhibitor (DPI) or mitochondrial electron transport chain interrupter (rotenone) or O₂ scavenger (MnTBAP) and Bcl-2 expression was analyzed by western blotting. As shown in Figure 6, PM-induced down-regulation of Bcl-2 was strongly reversed by NAC, DPI, ROT and MnTBAP in both cell lines. In addition, treatment with LY83583, a O₂ donor, also down-regulated Bcl-2 and this was reversed by MnTBAP. Together, these data indicated the involvement of ROS in down-regulation of Bcl-2 by PM.

Role of ROS in proteasomal degradation of Bcl-2

Since Bcl-2 is primarily down-regulated through the proteasomal degradation pathway, we next examined whether ROS plays a role in PM-induced ubiquitination and proteasomal degradation of Bcl-2. First, we established the involvement of proteasome in PM-induced down-regulation of Bcl-2. For this, PC-3 cells overexpressing Bcl-2 were treated with PM for 20 h in the presence or absence of proteasome inhibitors lactacystin (LAC) or MG132 and Bcl-2 was analyzed by western blotting. The down-regulation of Bcl-2 by PM was abolished in the presence of both proteasome inhibitors (Figure 7A), indicating that decrease in level of Bcl-2 protein by PM is mediated through the proteasomal pathway.

To analyze the role of ROS in PM-induced proteasomal degradation of Bcl-2, the effect of O₂ scavenger MnTBAP on ubiquitination of Bcl-2 by PM was examined. PC-3 cells transfected with pCMV-Tag-2B plasmid containing Flag-tagged Bcl-2 cDNA were treated with PM for 6 hours in the presence or absence of MnTBAP or proteasomal inhibitors LAC or MG132. In these experiments cells were treated with PM for 6 hours because treatment with PM for 6 hours was found to induce maximal ubiquitination of Bcl-2. Cell



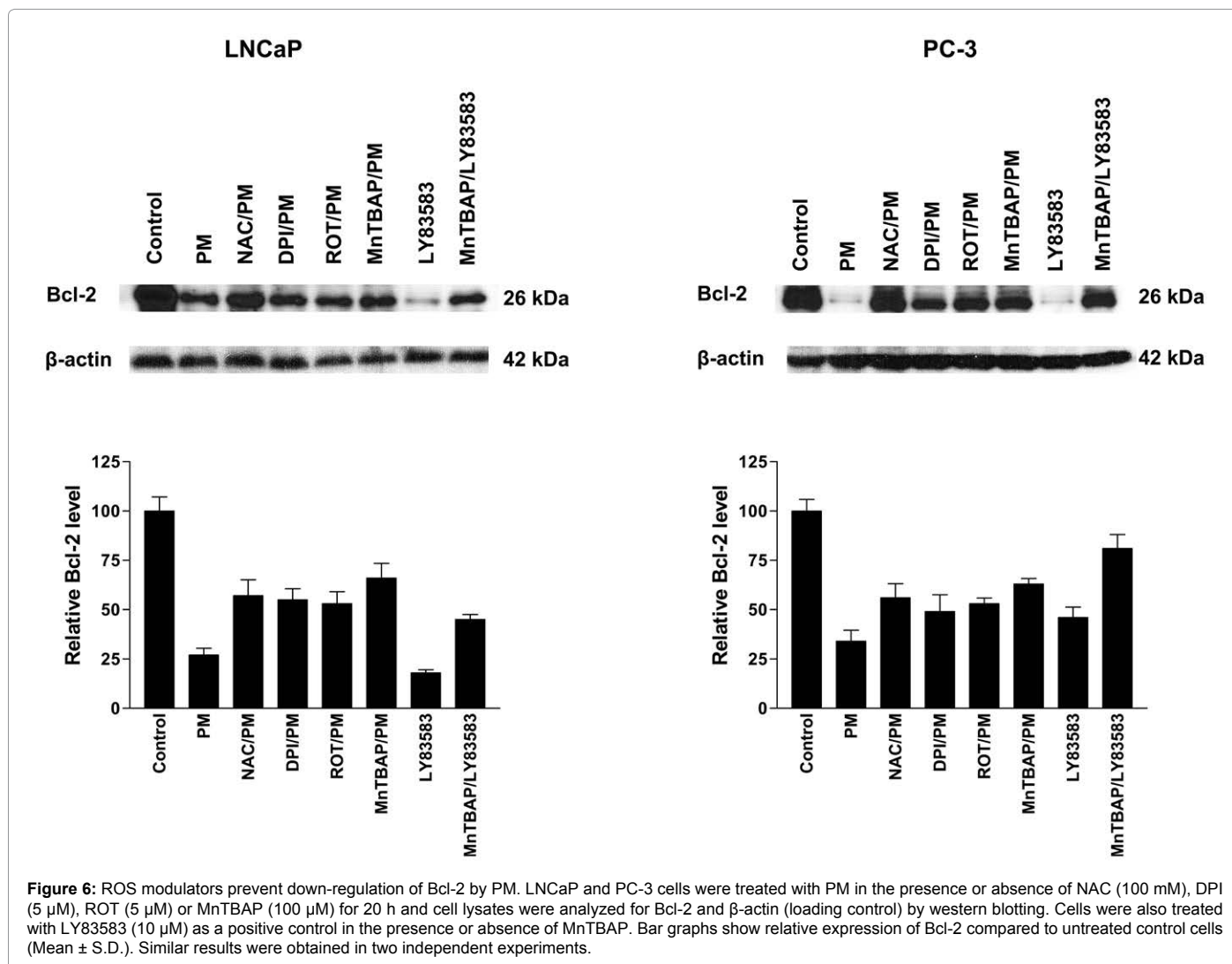
lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblotting with anti-ubiquitin antibody to reveal ubiquitinated Bcl-2 products. As shown in Figure 7B, treatment of cells in the presence of MnTBAP inhibited PM-induced ubiquitination of Bcl-2. On the other hand, treatment with PM in the presence of proteasome inhibitors LAC or MG132 resulted in accumulation of the polyubiquitinated Bcl-2 products. Treatment with LY83583 (O_2 donor) also independently induced ubiquitination of Bcl-2. Together, these data indicated that Bcl-2 down-regulation by PM is mediated through a ROS-dependent ubiquitin-proteasomal degradation pathway.

Discussion

Pristimerin is a quinonemethide triterpenoid and pristimerin containing plant products have been used in traditional medicine to treat a variety of diseases including inflammation and cancer. The purported medicinal effects of pristimerin have only recently come under scientific validation. The anti tumor activity of pristimerin has been reported in a small number of studies showing the antiproliferative and apoptosis-inducing activity of pristimerin in diverse types of tumor cell lines, including glioma, leukemia, breast and pancreatic cancer

cell lines [9-17]. These studies have provided some insights into the mode of cell death by pristimerin; however, molecular mechanism of the proapoptotic activity of pristimerin has remained unknown. In the present study, we investigated the mechanism of the antitumor activity of pristimerin in androgen-sensitive (LNCaP) and androgen-refractory (PC-3) prostate cancer cell lines with a focus on the effect of pristimerin on antiapoptotic Bcl-2 protein expression. PM significantly inhibited the proliferation of both cell lines at 1.25 to 2.5 μM with antiproliferative effect plateauing at 5 to 10 μM PM. Pristimerin was reported to inhibit the proliferation of pancreatic cancer cells by causing cell cycle arrest in G1 phase [17]. Whether antiproliferative activity of PM in prostate cancer cells also involves cell cycle arrest remains to be investigated.

The inhibition of cell proliferation invariably leads to the induction of apoptosis in tumor cells. Indeed, PM induced apoptosis both in LNCaP and PC-3 cells as demonstrated by the increased binding of annexin V due to externalization of the phosphatidylserine to the outer leaflet of the cell membrane and cleavage of PARP-1, both well recognized markers of apoptosis. These result indicated that induction of apoptosis is part of the mechanism by which PM destroys prostate cancer cells and corroborates the result of a previous study that also



showed induction of apoptosis in prostate cancer cells by PM [11]. Two major pathways of apoptotic cell death program have been identified, namely receptor-mediated (extrinsic) and mitochondrial (intrinsic) apoptotic cell death pathway [20]. In both cases, caspases, a family of cysteine proteases, play an important role in the apoptotic cell death. In the extrinsic pathway, binding of the death ligands (e.g., TNF- α , FasL, TRAIL) with their cognate receptors activates initiator caspase-8 which then cleaves and activates effector caspases-3, -6, and -7 leading to apoptosis [21]. In mitochondrial or intrinsic pathway, undefined signals induce release of cytochrome c from mitochondria, which in conjunction with Apaf-1 causes activation of initiator caspase-9. Activated caspase-9, in turn, activates effector caspases-3, -6, and -7 [20]. Pristimerin induced the cleavage of procaspases-9 and -3. The cleavage of procaspase-9 indicated that PM activates the mitochondrial (intrinsic) pathway of apoptosis in prostate cancer cells. The activation of the mitochondrial pathway by PM is also supported by the loss of mitochondrial membrane potential and release of cytochrome c from mitochondria in prostate cancer cells treated with PM (Figure 3). Thus, the cleavage of procaspase-9 and release of cytochrome c from mitochondria leading to the activation of effector caspase-3 indicated that the mitochondrial apoptosis pathway plays a critical role in the apoptotic cell death of prostate cancer cells by PM. Whether PM

induces the activation of extrinsic pathway of apoptosis in prostate cancer cells remains to be determined.

Intracellular generation of ROS is part of the mechanism by which most chemotherapeutic agents kill tumor cells [22-24]. In normal cells ROS are generated as byproducts of normal aerobic metabolism or as second messengers in various signal transduction pathways or in response to environmental stress [25]. Depending upon the concentration, ROS elicit a wide spectrum of biological responses ranging from mitogenic to proliferative effects at low concentration to macromolecular damage and apoptosis at high concentrations [26-28]. ROS can also activate mitochondrial permeability transition and mitochondrial depolarization [29]. Treatment with PM generated ROS in prostate cancer cells (Figure 4), suggesting that loss of mitochondrial potential and release of cytochrome c from mitochondria by PM may be, at least in part, mediated through ROS. This result corroborates previous reports in which ROS was also implicated in induction of apoptosis and mitochondrial dysfunction in glioma and cervical cancers by PM [12,16], but the precise mechanism by which PM increases ROS levels and activates mitochondrial pathway of apoptosis in cancer cells remains undetermined. In addition, it seems counterintuitive to consider that PM which has been shown to have antioxidant activity

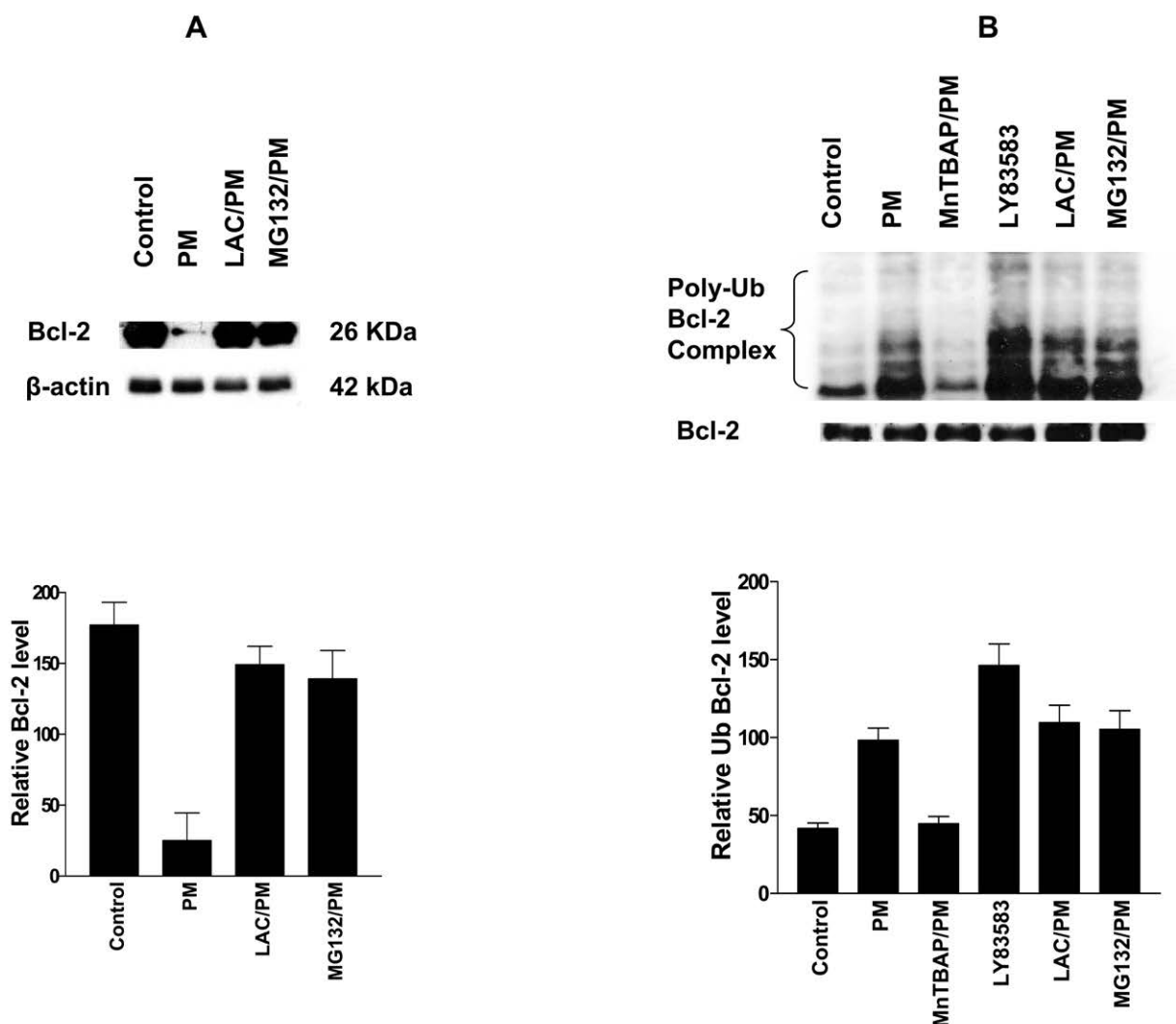


Figure 7: Involvement of ROS in ubiquitination and proteasomal degradation of Bcl-2. (A) PC-3 cells overexpressing Bcl-2 were treated PM (5 μM) in the presence or absence of proteasome inhibitors LAC (10 μM) or MG132 (20 μM) for 20 h. Cell lysates were analyzed for Bcl-2 expression by western blotting. (B) PC-3 cells were treated with PM (5 μM) in the presence or absence of MnTBAP (100 μM), LAC or MG132. Cells were also treated with LY83583 alone (10 μM) for 6 h. Cell lysates were immunoprecipitated with anti-Flag antibody and immune complexes were analyzed for ubiquitin by western blotting. Ubiquitin was analyzed after treatment with PM for 6 h because ubiquitination was found to be maximal at this time point. Bar graphs show relative levels of Bcl-2 (A) and poly-ubiquitinated Bcl-2 (B). Similar results were obtained in two independent experiments.

is also able to induce intracellular ROS generation. In fact, this dual functionality (antioxidant/prooxidant activity) is not unique to pristimerin, since a number of cancer chemopreventive compounds, including but not limited to sulforaphane, diallyl trisulfide, lipoic acid, oleanane triterpenoids have been shown to exhibit both antioxidant and prooxidant activity [30-32]. Experimental variables such as concentration of the compound, cellular context and redox status of the cells may determine the oxidative response of tumor cells to these compounds.

The intrinsic (mitochondrial) pathway of apoptosis is regulated by members of the Bcl-2 family of proteins that includes both pro- as well as anti-apoptotic molecules. Bcl-2 and Bcl-xL are two major antiapoptotic members of Bcl-2 family that reside in the mitochondrial membrane and inhibit apoptosis by preventing the activation of inner mitochondrial permeability transition pore and release of

proapoptogenic mitochondrial contents including cytochrome c [33]. PM inhibited both Bcl-2 and Bcl-xL in concentration-related manner (Figure 5). It is perhaps due to the inhibition of Bcl-2 and Bcl-xL that PM facilitates Bax and Bak mediated mitochondrial permeability transition and cytochrome c release.

Different ROS species have been shown to play a role in the degradation of Bcl-2 [34,35]. Our data showed that PM-induced down-regulation of Bcl-2 was reversed in the presence of ROS modulators, such as NAC, DPI, rotenone or O₂ scavenger MnTBAP (Figure 6). The finding that O₂ donor LY83583 also down-regulated Bcl-2 further supports the role of ROS in PM-induced down-regulation of Bcl-2. Although no attempt was made to identify specific ROS species involved in inhibition of Bcl-2 by PM, pharmacological inhibitors used in these experiments collectively suggest that both cytosolic and mitochondrial ROS are involved with mitochondrial superoxide anion (O₂⁻) playing a significant role in down-regulation of Bcl-2 by PM.

The stability and levels of Bcl-2 are regulated through post-translational modifications, such as dephosphorylation, ubiquitination and proteasomal degradation [36,37]. PM-induced down-regulation of Bcl-2 is also mediated through proteasomal degradation, since treatment with PM in the presence of proteasome inhibitors lactacystin (a 20S proteasome inhibitor) and MG132 stabilized Bcl-2 (Figure 7A). To explore the role of ROS in ubiquitination of Bcl-2 by PM, which predisposes it for degradation by ubiquitin-proteasome pathway; we examined the effect of O₂ scavenger MnTBAP on ubiquitination of Bcl-2 by PM. MnTBAP completely inhibited ubiquitination of Bcl-2 by PM (Figure 7B), whereas treatment with PM in the presence of proteasome inhibitors LAC or MG132 resulted in the accumulation of polyubiquitinated Bcl-2 products. We also observed ubiquitination of Bcl-2 when cells were treated with O₂ donor LY83583 alone. Taken together, these results indicated the important role O₂ plays in ubiquitination and proteasomal degradation of Bcl-2 by PM. These results are in agreement with previous reports showing involvement of ROS in ubiquitination and proteasomal degradation of Bcl-2 by other compounds [34-37]. In addition to delineating the role of individual ROS species in Bcl-2 ubiquitination and proteasomal degradation, finding the chemopreventive/therapeutic efficacy of pristimerin in an experimental model of prostate tumorigenesis is a major focus of our continuing studies on pristimerin.

Conclusion

The results of the present study provide an insight into the mechanism of the apoptotic cell death induced by pristimerin in prostate cancer cells. Pristimerin activates the mitochondrial apoptotic pathway through the generation of intracellular ROS. The antiproliferative/apoptotic effect of pristimerin is regulated by Bcl-2 and it mediates down-regulation of Bcl-2 through ROS-dependent ubiquitin-proteasomal degradation pathway. Thus, better understanding of the mechanism of the antitumor activity will facilitate designing of the in vivo efficacy study of pristimerin in a preclinical model of prostate tumorigenesis.

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