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Pristimerin Induces Apoptosis by Targeting the Proteasome in Prostate Cancer Cells

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Abstract Pristimerin is a natural product derived from the *Celastraceae* and *Hippocrateaceae* families that were used as folk medicines for antiinflammation in ancient times. Although it has been shown that pristimerin induces apoptosis in breast cancer cells, the involved mechanism of action is unknown. The purpose of the current study is to investigate the primary target of pristimerin in human cancer cells, using prostate cancer cells as a working model. Nucleophilic susceptibility and *in silico* docking studies show that C_6 of pristimerin is highly susceptible towards a nucleophilic attack by the hydroxyl group of N-terminal threonine of the proteasomal chymotrypsin subunit. Consistently, pristimerin potently inhibits the chymotrypsin-like activity of a purified rabbit 20S proteasome (IC_{50} 2.2 μmol/L) and human prostate cancer 26S proteasome (IC_{50} 3.0 μmol/L). The accumulation of ubiquitinated proteins and three proteasome target proteins, Bax, p27 and IκB-α, in androgen receptor (AR)-negative PC-3 prostate cancer cells supports the conclusion that proteasome inhibition by pristimerin is physiologically functional. This observed proteasome inhibition subsequently led to the induction of apoptotic cell death in a dose- and kinetic-dependent manner. Furthermore, in AR-positive, androgen-dependent LNCaP and AR-positive, androgen-independent C4-2B prostate cancer cells, proteasome inhibition by pristimerin results in suppression of AR protein prior to apoptosis. Our data demonstrate, for the first time, that the proteasome is a primary target of pristimerin in prostate cancer cells and inhibition of the proteasomal chymotrypsin-like activity by pristimerin is responsible for its cancer cell death-inducing property.
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Key words: pristimerin; proteasome inhibition; natural compounds; apoptosis; prostate cancer

Apoptosis is an evolutionally conserved cellular suicide program through mitochondira pathway (intrinsic) or the death receptor pathway (extrinsic) [Steller, 1995; Nagata, 1997; Ashkenazi and Dixit, 1998; Green and Reed, 1998; Gross et al., 1999a]. It has been clearly demonstrated that several pro-apoptotic proteins of Bcl-2 family located in the outer membranes of mitochondria control the release of cytochrome *c* into the cytosol [Green and Reed, 1998; Gross et al., 1999b]. The released cytochrome *c* activates caspase-9 that in turn

Abbreviations used: AR, androgen receptor; CT, chymotrypsin; DM, DMSO; IκB, inhiibitor of NF kappa B; OH, hydroxyl group; PARP, poly(ADP-ribose) polymerase; Pri, pristimerin; NT, untreatment; Ub-Prs, polyubiquitinated proteins.

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proteolytically activates downstream caspase-3 that then cleaves a number of important cellular target proteins, including poly(ADP-ribose) polymerase (PARP) [Lazebnik et al., 1994] and retinoblastoma protein (pRB) [An and Dou, 1996], and leads to apoptotic cell death.

Natural compounds have become more important in anticancer drug development since they are more tolerable in human body [Newman et al., 2003]. Pristimerin is a natural compound found in the *Celastraceae* and *Hippocrateaceae* families that are rich in quinone-methide triterpenes [Buffa Filho et al., 2002; Chang et al., 2003; Niampoka et al., 2005]. It has been well established that pristimerin possesses antiinflammatory, antioxidant, antimalarial, and insecticidal activities [Sassa et al., 1994; Dirsch et al., 1997; Figueiredo et al., 1998; Avilla et al., 2000; Luo et al., 2005]. Most recently, it has been shown that pristimerin induces apoptosis in breast cancer MDA-MB-231 cells in a caspase-dependent manner [Wu et al., 2005]. However, the molecular target of apoptosis induction by pristimerin remains unknown.

The ubiquitin-proteasome pathway plays an important role in cell cycle regulation and apoptosis through the degradation of cell cycle or apoptosis related proteins [Goldberg, 1995; Orlowski and Wilk, 2000; Dou et al., 2003; Nandi et al., 2006]. The 26S eukaryotic proteasome is a multicatalytic protease complex consisting of a 20S catalytic particle capped by two 19S regulatory particles. The 20S proteasome is a barrel-shaped complex made up of 28 subunits in 4 rings (7 α or β subunits per ring) stacked in the order of $\alpha\beta\beta\alpha$. The subunits β5, β2, and β1 mediate three main proteolytic activities of the proteasome, chymotrypsin (CT)-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing (PGPH)-like or caspase-like activities, respectively. Furthermore, the β subunits contain a threonine residue at the N terminus (Thr-1), which imparts the catalytic activity of the proteasome [Goldberg, 1995; Orlowski and Wilk, 2000; Dou et al., 2003; Adams, 2004; Nandi et al., 2006]. The 19S proteasome recognizes the target substrates tagged by polyubiquitins and removes them from the targets prior to destruction by the 20S proteasome [Orlowski and Wilk, 2000; Nandi et al., 2006]. Since cancer cells are more sensitive to inhibition of protein degradation by proteasome inhibitors than normal cells [Daniel et al., 2004], it is hypothesized that proteasome inhibitors might act as novel anticancer drugs by killing cancer cells selectively [Orlowski and Wilk, 2000; Dou et al., 2003; Adams, 2004]. Efficacy and tolerance of the first proteasome inhibitor PS-341 (Bortezomib, Velcade) in phase I and II trials further encourage researchers to explore proteasome inhibitors for cancer treatment [Adams and Kauffman, 2004; Papandreou et al., 2004; O'Connor et al., 2005; Orlowski et al., 2005].

Proteasome inhibition has been shown to lead to induction of apoptosis [Dou et al., 2003]. In the current study, we applied computer modeling to examine the possible interaction between pristimerin and the β5 subunit of the proteasome. We found that the conjugated ketone carbon, $C_{6,}$ of pristimerin may interact with the N-terminal threonine of the proteasomal β5 subunit. Consistently, pristimerin potently inhibited the chymotrypsin-like activity of a purified 20S proteasome with an IC_{50} value of 2.2 μmol/L. Pristimerin also caused proteasome inhibition in androgen receptor (AR)-negative PC-3 prostate cancer cells, as shown by accumulation of proteasome target proteins Bax, p27, and IκB-α, which was followed by increased caspase-3 activity and PARP cleavage. In AR-positive LNCaP and C4-2B prostate cancer cells, pristimerin caused both proteasome inhibition and AR suppression, again followed by caspase-3 activation and PARP cleavage. Taken together, our results demonstrate that pristimerin targets the proteasome to induce apoptosis in human prostate cancer cells.

MATERIALS AND METHODS

Materials

Pristimerin was a generous gift from $\pi-\pi$ technologies, Inc. (Shenzhen, China). It was dissolved in DMSO at a stock concentration of 50 mmol/L, aliquoted and stored at -20°C. Fetal bovine serum (FBS) was purchased from Tissue Culture Biologicals (Tulare, CA). RPMI 1640 medium, penicillin, and streptomycin were from Invitrogen Co. (Carlsbad, CA). Purified rabbit 20S proteasome, fluorogenic peptide substrates Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activity) and Ac-DEVD-AMC (for caspase-3/-7 activity) were obtained from Calbiochem, Inc. (San Diego, CA). Mouse monoclonal antibody against human PARP was from BIOMOL International LP (Plymouth Meeting, PA). Mouse monoclonal antibodies against Bax

(B-9), p27 (F-8), Ubiquitin (P4D1), and AR (441), rabbit polyclonal antibody against inhibitor of nuclear factor κB-α (IκB-α) (C-15) and goat polyclonal antibody against actin (C-11) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence reagent (ECL) was from Amersham Biosciences (Piscataway, NJ). Bisbenzimide Hoechst No. 33258 stain and 3-4, 5-dimethyltiazol-2-yl-2.5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Whole Cell Extract Preparation

Human prostate cancer PC-3, LNCaP, and C4-2B cells were grown in RPMI 1640 supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cells were maintained at 37°C and 5% CO_2. A whole cell extract was prepared as described previously [An et al., 1998].

Nucleophilic Susceptibility Analysis

The electron density surface colored by nucleophilic susceptibility was created with the use of Quantum CAChe (Fujitsu; Fairfield, NJ) by performing a nuclear susceptibility analysis using the PM5 geometry and PM5 wavefunction in water as described previously [Chen et al., 2005]. The colored "bull's-eye" with a red center denotes atoms that are highly susceptible for nucleophilic attack.

Computational Modeling

The crystal structure of the eukaryotic yeast 20S proteasome used for the docking studies was obtained from the Protein Database [Kazi et al., 2003; Smith et al., 2004]. The yeast 20S proteasome is structurally very similar to the mammalian 20S proteasome, and the chymotrypsin active site between the two species is highly conserved. The AutoDock 3.0 suite of programs and docking parameters were set up as described [Kazi et al., 2003; Smith et al., 2004]. The AutoDock software was run on an i386 architecture computer operating with Redhat Linux 6.0 ^{TM} operating system. The selected docking image for pristimerin was the cluster with the most members and the lowest binding free energy. Structural output from AutoDock was visualized using PyMOL software.

Inhibition of Purified 20S Proteasome or 26S Proteasome in Whole Cell Extract by Pristimerin

A purified rabbit 20S proteasome (35 ng) or PC-3 extract (2 μg) were incubated with 20 μM of fluorogenic peptide substrate (Suc-Leu-Leu-Val-Tyr-AMC for chymotrypsin-like of the proteasome) in 100 μl assay buffer (20 mM Tris-HCl, pH 7.5), in the presence of pristimerin at up to 50 μmol/L or the solvent DMSO for 2 h at 37°C, followed by measurement of inhibition of proteasomal chmotrypsin-like activity as described previously [Nam et al., 2001].

Cell-Free Caspase-3 (or -7) Activity Assay

The prepared whole cell extracts (30 μg per sample) from untreated or treated cells were incubated with 40 μM of the caspase-3/-7 substrate Ac-DEVD-AMC in 100 μl assay buffer at 37°C for at least 2 h. The release of the AMC groups was measured as described above.

Western Blotting Analysis

Whole cell lysates (40 μg) were separated by an SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane. Specific proteins were detected by the ECL, as described previously [Chen et al., 2005].

Hoechst Staining

PC-3 cells were treated with 5 μmol/L of pristimerin for 8 h, followed by 100 μg/ml Hoechst 32258 stain for at least 0.5 h at 4°C. The condensed nucleus was visualized under Zeiss fluorescent microscopy.

MTT Assay

PC-3 cells were grown in a 96-well plate. Triplicate wells of cells were treated with indicated concentrations of pristimerin for 24 h. After aspiration of medium, MTT (1 mg/ml) was then added to the cell cultures, followed by incubation for 3 h at 37°C. After cells were crystallized, MTT was removed and DMSO was added to dissolve the metabolized MTT product. The absorbance was then measured on a Wallac Victor^3 1420 Multilabel counter at 540 nm.

Results

Pristimerin Induces Apoptosis in Prostate Cancer Cells

Pristimerin is a triterpenoid (Fig. 1A) isolated from natural plants. Recently it was reported that pristimerin induced breast cancer cell apoptosis in a caspase-dependent manner [Wu et al., 2005]. We were interested in the involved mechanism of action and, specifically, the cancer-related molecular target of pristimerin,

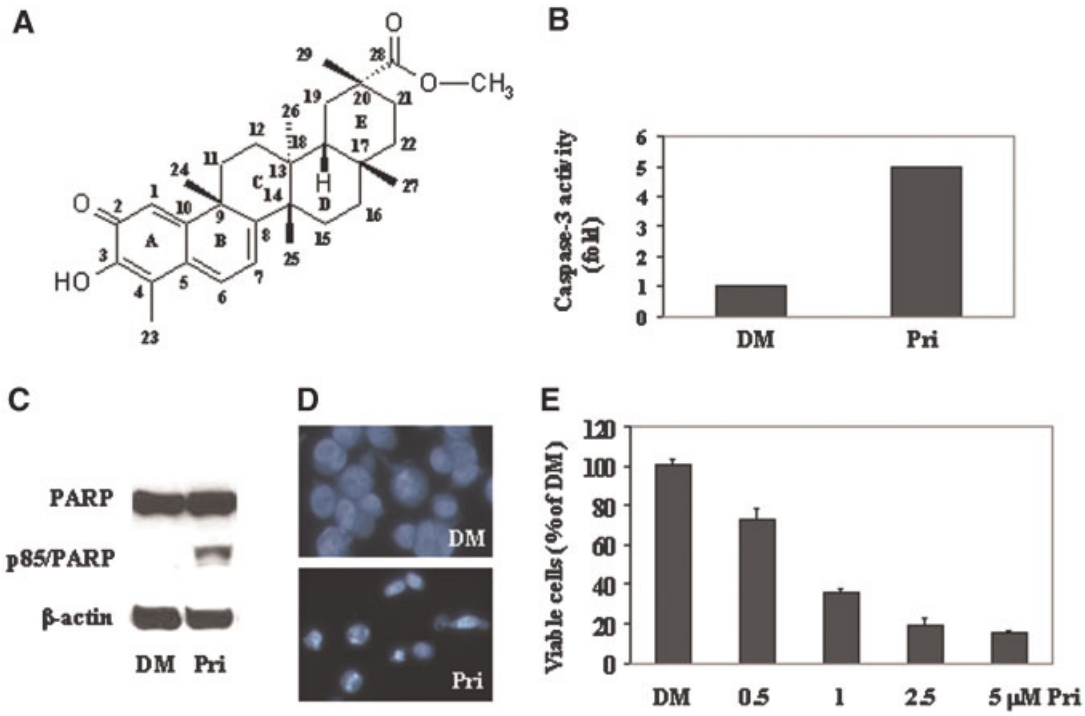

Fig. 1. Pristimerin induces apoptosis in human prostate cancer cells. **A**: Chemical structure of pristimerin. To test the effect of pristimerin on apoptosis in human prostate cancer, PC-3 cells were treated with 5 μmol/L pristimerin (Pri) or solvent DMSO (DM) for 8 h, followed by caspase-3 activity assay (**B**), Western blotting analysis against PARP antibody (**C**), and Hoechst staining (**D**). Molecular weight of intact PARP and cleaved PARP is 116 and 85 kDa, respectively. β-actin was used as a loading control. **E**: Cell viability was decreased by pristimerin in PC-3 cells. 8000 PC-3 cells per well were plated in a 96-well plate and treated with indicated concentrations of Pri or equal volume of DM for 24 h, followed by an MTT assay. All data represent independent triplicate experiments. Bars, SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

if it exists. To start this project, we first investigated whether pristimerin is also able to induce apoptosis in human prostate cancer cells. PC-3 prostate cancer cells were treated with 5 μmol/L of pristimerin for 8 h, followed by detection of apoptosis. Compared to the control treated cells, caspase-3/-7 activity was increased fivefold after pristimerin treatment (Fig. 1B). This finding is similar to what was observed in breast cancer cells [Wu et al., 2005]. Consistent with caspase activation, the p85 kD PARP fragment was detected (Fig. 1C) and condensed nuclei were observed with Hoechst 32258 staining (Fig. 1D) in pristimerin-treated PC-3 cells. As shown in Figure 1E, MTT measurement showed that pristimerin decreased cell growth by 30–85% in PC-3 at 0.5–5μmol/L after 24 h treatment.

Pristimerin Inhibits the Chymotrypsin-Like Activity of a Purified Rabbit 20S Proteasome and 26S Proteasome in Tumor Cell Extract

It has been shown that proteasome inhibition triggers apoptosis [Dou et al., 2003]. If apoptosis induced by pristimerin is related to proteasome inhibition, we expect to see that proteasome activity would be inhibited by pristimerin under the same conditions. In aliquots of the apoptosis experiments, we measured proteassome inhibition by the proteasomal chymotrypsin-like activity assay and polyubiqutinated protein accumulation. Treatment with at 5 μmol/L pristimerin for 8 h caused 30% inhibition of cellular proteasome activity, compared to the control (Fig. 2A). Consistently, pristimerin treatment was able to accumulate polyubiquitinated proteins (Fig. 2B), suggesting that the proteasome inhibition is physiologically functional.

To provide direct evidence for pristimerin as a proteasome inhibitor, we performed a cell-free proteasome activity assay using a purified rabbit 20S proteasome in the presence of pristimerin at up to 50 μmol/L. The chymotrypsin-like activity of the purified 20S proteasome was significantly inhibited by pristimerin with an IC_{50} value of 2.2 μmol/L (Fig. 2C). Similarly, pristimerin also potently inhibited 26S proteasome chymotrypsin-like activity in an extract

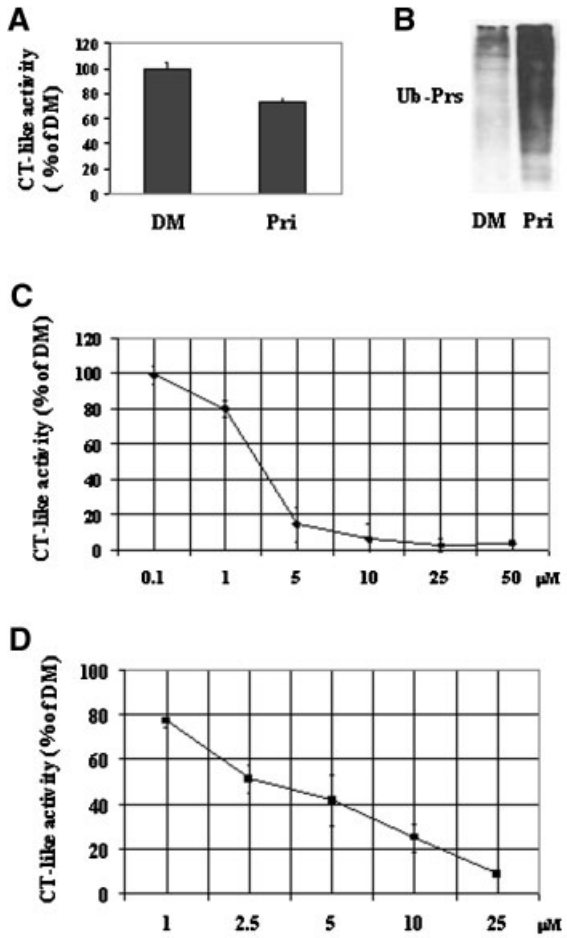

Fig. 2. Proteasome inhibition in tumor cells and purified 20S proteasome. In the same aliquots described above (Fig. 1B–D), proteasome inhibition was measured by chymotrypsin (CT)-like activity assay (**A**) and Western blot against polyubiquitinated proteins (**B**). **C**,**D**: Proteasome inhibition by pristimerin in vitro. Purified 20S rabbit proteasome (35 ng) (C) or PC-3 cell extracts (2 μg) (D) were incubated with indicated concentrations of pristimerin for 2 h, proteasome activity was monitored by calculating the fluorescent group release from substrate for chymotrypsin-like activity. All data represent independent triplicate experiments. Bars, SD.

prepared from exponentially grown PC-3 prostate cancer cells with an IC_{50} value of 3.0 μmol/L (Fig. 2D). Therefore, pristimerin is a natural inhibitor of purified 20S and 26S cellular proteasome.

Computational Electron Density and Docking Studies Verifies the Interaction Between Pristimerin and the Proteasome

To better understand the interaction between the pristimerin molecule and the proteasome, we performed nucleophilic susceptibility analysis. The result showed that pristimerin is highly susceptible to nucleophilic attack at C_2 in the A-ring and C_6 in the B-ring, as denoted by "bull's eyes" with red centers (Figs. 3A vs. 1A). Since the chymotrypsin-like activity is mediated by the β5 subunit of the proteasome, we docked the pristimerin molecule into the proteasomal β5 subunit to investigate their molecular interactions.

Our results demonstrate that pristimerin interacts with the proteasomal β5 subunit in a conformation suitable for proteasome inhibition (Fig. 3B). Out of 100 runs there were only three docking conformations obtained. The selected

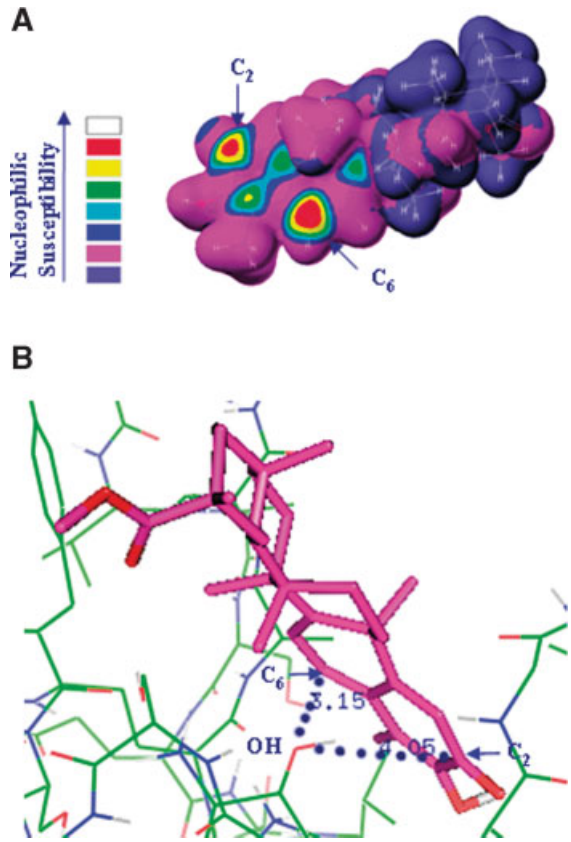

Fig. 3. The interaction between the pristimerin molecule and the proteasome β5 subunit. **A**: Nucleophilic susceptibility analysis of pristimerin. High susceptibility of pristimerin to nuleophilic attack at C_2 and C_6 indicated by ''bull's eyes'' with red center is shown. **B**: Computational modeling of pristimerin interacting with the β5 subunit of the proteasome. Pristimerin is shown in pink while the hydroxyl group (OH) of the N-terminal threonine (Thr) of β5 subunit is labeled. The selected conformation with 78% possibility shows the distances to the OH group of N-Thr from C_6 and C_2 are 3.15 and 4.05 Å, respectively.

image (Fig. 3B) was from the cluster with the most members (78%) and the lowest docked free energy (−10.31 kcal/mol), showing that pristimerin can be oriented within the active site of the proteasomal β5 subunit (Fig. 3B). The docking results also indicated that pristimerin binds in an orientation and conformation that is suitable for a nucleophilic attack by the OH group of the N-Thr within the β5 subunit (Fig. 3B). The distances from the electrophilic C_6 and C_2 of pristimerin to the OH of β5 N-Thr were 3.15 and 4.05 Å, respectively (Fig. 3B). Since nucleophilic attack could occur within 4 Å [Smith et al., 2004], these computational docking results suggest that C_6 of pristimerin more likely interacts with the N-Thr of the β5 subunit and potentially inhibits the proteasomal chymotrypsin-like activity.

Kinetics of Pristimerin-Induced Proteasome Inhibition and Apoptosis in AR-Negative PC-3 Prostate Cancer Cells

The results described above have demonstrated that pristimerin is capable of inhibiting the proteasome and inducing apoptosis. Whether proteasome inhibition by pristimerin is the cause of or the subsequent event of apoptosis needs to be further determined. To do so, PC-3 cells were treated with 2.5 μmol/L of pristimerin for up to 16 h, followed by measurement of proteasome inhibition and apoptosis. We found that accumulation of IκB-α and Bax, two well-known target proteins of the proteasome [Chen et al., 1995; Li and Dou, 2000], occurred at as early as 1 h after addition of pristimerin and lasted for 8 and 16 h, respectively, while accumulation of another proteasome target protein p27 [Pagano et al., 1995], occurred at 8 h and increased further at 16 h (Fig. 4A). Also, polyubiquitinated proteins were accumulated as early as 1 h and increased to the peak after 8 h (Fig. 4A). These results indicate that the functional proteasome inhibition occurs early-on after pristimerin treatment. In the same kinetic experiment, apoptosis was detected at later time-points as shown by six- to eight-fold increased levels of caspase-3 activity after 8 h (Fig. 4B) and PARP cleavage after 16 h treatment compared to the control (Fig. 4A). Our studies indicate that apoptosis was delayed for at least 7 h compared to the onset of proteasome inhibition in PC-3 cells treated with pristimerin (Fig. 4).

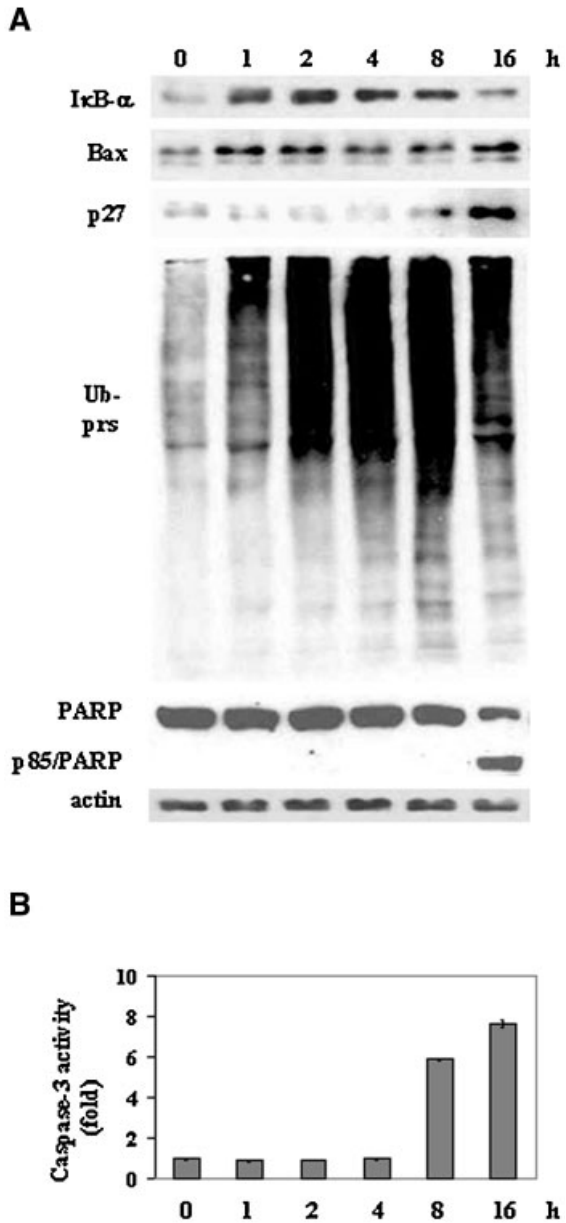

Fig. 4. Kinetic study on proteasome inhibition and apoptosis induction in PC-3 cells. PC-3 cells were treated with 2.5 μmol/L pristimerin for up to 16 h, followed by Western blotting using Bax, IκB-α, polyubiquitinated proteins and PARP antibodies (**A**) and caspase-3 activity detection (**B**). Molecular weight of IκB-α, Bax and p27 are 37, 23, and 27 kDa, respectively. β-actin was used as a loading control. All data represent independent triplicate experiments. Bars, SD.

Pristimerin Induces Proteasome Inhibition and Apoptosis Induction in Androgen-Independent, AR-Positive C4-2B Prostate Cancer Cells

AR plays a critical role in the development of prostate cancer [Jenster, 1999]. It has been

shown that proteasome inhibitors can reduce levels of AR expression even though the involved mechanism is unknown [Lin et al., 2002; Yang et al., 2006]. If pristimerin can inhibit the proteasome activity, it should be able to suppress AR expression. To test this possibility, androgen-independent, AR-positive C4-2B cells were treated with pristimerin at 1–5 μmol/L for 24 h. First we measured proteasome inhibition using the proteasomal chymotrypsin-like activity assay and Western blotting. The chymotrypsin-like activity of the proteasome was inhibited by 10, 20, and 40% by pristimerin at 1, 2.5, and 5 μmol/L, respectively (Fig. 5A). Consistent with proteasome inhibition, polyubiquitinated proteins were also accumulated at 2.5 and 5 μmol/L (Fig. 5B). Then we measured AR protein level in the same aliquots. Expression of AR proteins was decreased after pristimerin treatment in a dose-dependent manner and complete suppression of AR was found after pristimerin treatment at 5 μmol/L (Fig. 5B). Associated with AR protein decrease (2.5–5 μmol/L), a cleaved PARP fragment was detected after treatment with 2.5 μmol/L pristimerin and a very strong cleaved PARP band was found in cells treated with 5 μmol/L pristimerin (Fig. 5B). Under the same conditions for PARP cleavage, caspase-3 activity was increased by four- to fivefold (Fig. 5C vs. 5B). Consistent with these molecular changes, apoptotic morphological changes (rounded-up and

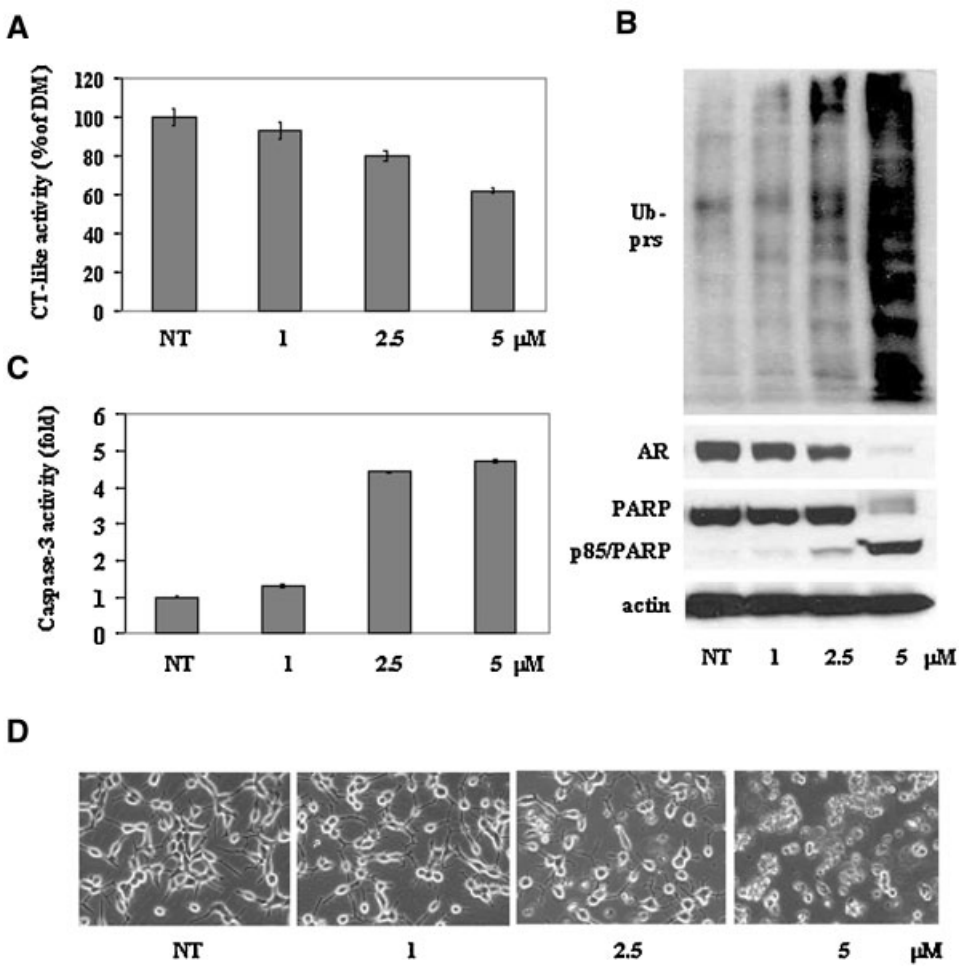

Fig. 5. Dosage effect on proteasome inhibition and apoptosis induction by pristimerin in C4-2B cells. C4-2B cells (untreated or NT) were treated with pristimerin at indicated concentrations for 24 h, followed by the proteasomal CT-like activity assay (**A**), Western blotting analysis (**B**), and caspase-3 activity assay (**C**). Inhibition of CT-like activity, accumulation of polyubquitinated proteins, loss of AR expression, cleavage of PARP, activation of caspase-3, and apoptotic morphological changes (**D**) are shown. Molecular weight of AR is 110 kDa. β-actin was used as a loading control. All data represent independent triplicate experiments. Bars, SD.

condensed nucleus) were found mainly after the 5 μmol/L pristimerin treatment (Fig. 5D).

To investigate the kinetic relationship between proteasome inhibition, AR suppression, and apoptosis, C4-2B cells were treated with 5 μmol/L of pristimerin for up to 24 h, followed by the measurement of proteasome inhibition and apoptosis. Proteasome activity was inhibited by 15% after 1 h treatment and was further inhibited to 40% by 2 h (Fig. 6A). This inhibition was maintained for at least 24 h. Consistently, increased polyubiquitinated proteins were accumulated as early as 1 h and lasted for 24 h (Fig. 5B). Accompanying proteasome inhibition, AR protein expression was suppressed as early as 1 h and decreased further at 2–24 h (Fig. 6B). Following proteasome inhibition and AR suppression, caspase-3 was activated after 4 h (more than threefold increase) and peaked at 8 h (sixfold increase) during the treatment and then slightly decreased (to fivefold) at 24 h (Fig. 6C). After caspase-3 activation, cleaved PARP was detected at 24 h (Fig. 6B). Compared to proteasome inhibition and AR suppression starting at 1 h after treatment, apoptosis was delayed for at least 3 h in C4-2B cells treated with pristimerin. Morphologically, apoptotic features were found after 8 h treatment (Fig. 6D).

Pristimerin Induces Proteasome Inhibition and Apoptosis Induction in Androgen-Dependent, AR-Positive LNCaP Prostate Cancer Cells

To further confirm the association of pristimerin-induced AR suppression with apoptosis, another AR-positive cell line LNCaP was treated with 5 μmol/L pristimerin for up to 8 h. Again, we found that AR protein was decreased dramatically after 1 h and further decreased gradually to complete ablation after 8 h treatment (Fig. 6A). Following the AR decrease, caspase-3 activation was increased fivefold compared to the control and PARP cleavage was detected after 8 h treatment (Fig. 6A, B). Taken together, we conclude that potent inhibition of prostate cancer proteasome activity by

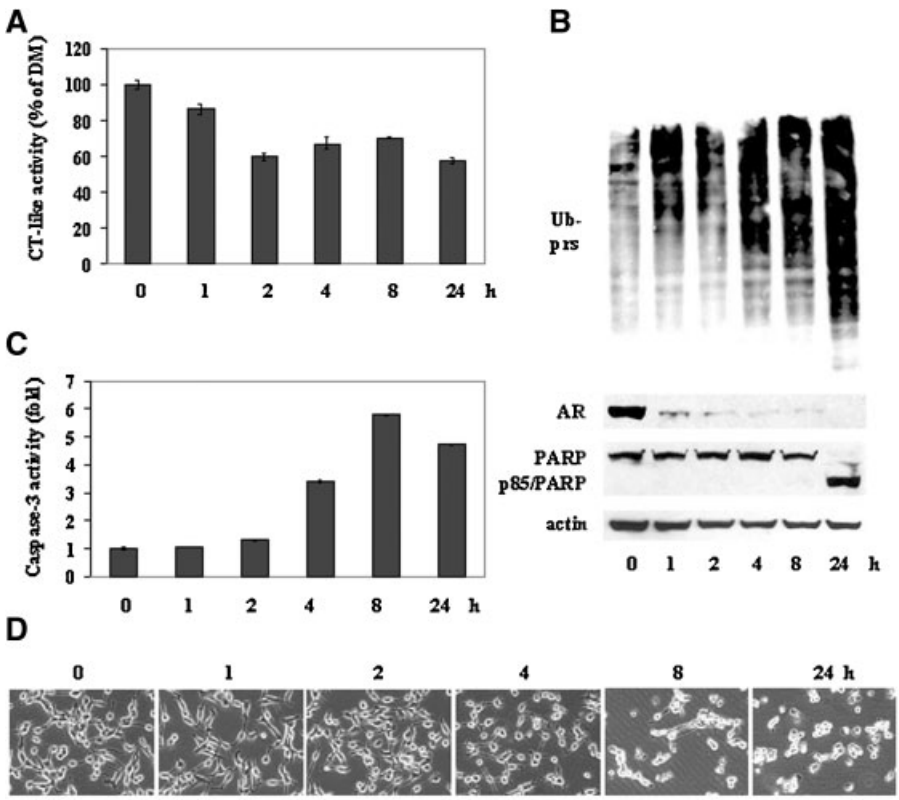

Fig. 6. Kinetic effect on proteasome inhibition and apoptosis induction by pristimerin in C4-2B cells. Exponentially growing C4-2B cells (0 h) were treated with 5 (mol/L pristimerin for indicated times, followed by the proteasomal CT-like activity assay (**A**), Western blotting analysis against polyubquitinated proteins, AR, PARP (**B**), caspase-3 activity assay (**C**), and visualization of apoptotic morphological changes (**D**). β-actin was used as a loading control (B). All data represent independent triplicate experiments. Bars, SD.

pristimerin leads to AR suppression and apoptosis induction.

DISCUSSION

Novel anticancer drug discovery will depend on understanding the mechanisms of drug actions and targets. Pristimerin, a natural product that is derived from several species belonging to the *Celastraceae* and *Hippocrateaceae* families has been shown to induce apoptosis in human breast cancer cells [Wu et al., 2005]. In the current study, we found that pristimerin directly targets the proteasome in human prostate cancer cells, an event that is responsible for the subsequent apoptosis induction.

Pristimerin-induced apoptosis in human prostate cancer was detected by decreased cell viability, caspase-3 activation and PARP cleavage (Figs. 1, 4–7). Since the ubiquitin-proteasome pathway regulates apoptosis-related proteins [Li and Dou, 2000], we focused on the interaction between pristimerin and the proteasome, and the biological effect of this interaction on apoptosis induction.

First, computational modeling results revealed the interaction between pristimerin and the proteasome β5 subunit. Nucleophilic susceptibility analysis showed that both ketone C_2 and C_6 of pristimerin were involved in the nucleophilic attack (Fig. 3A). Docking solutions further verified pristimerin binding to the β5 subunit of the proteasome, as shown by 78% binding probability to N-Thr, and a low docked free energy (−10.31 kcal/mol). The distance from the ketone carbon C_6 to N-Thr was within 4 Å (3.15 Å), while the ketone carbon C_2 was found to be slightly greater than 4 Å, suggesting that C_6 was more suitable than C_2 for interaction with the β5 subunit (Fig. 3B). These results suggest that pristimerin interacts with the catalytically active amino acid (N-Thr) of the proteasome β5 subunit in a manner suitable for proteasome inhibition.

Next, proteasome activity in vitro and in cells supported proteasome inhibition by pristimerin, as shown: (i) pristimerin inhibited the purified proteasome directly, with an IC_{50} value of 2.2 μmol/L (Fig. 2C); (ii) pristimerin inhibited 26S proteasome from tumor cell extract with an IC_{50} value of 3.0 μmol/L (Fig. 2D), and (iii) pristimerin caused ~30% cellular proteasome inhibition at 5 μmol/L in AR-negative PC-3 cells and ~40% inhibition at 5 μmol/L in AR-positive C4-2B cells (Figs. 2, 5, 6). More importantly, proteasome inhibition by pristimerin was physiologically functional, as shown by polyubiquitinated protein accumulation (Figs. 2, 4–7).

To investigate whether apoptosis induced by pristimerin was associated with proteasome inhibition, we performed both dose- and time-dependent experiments. Pristimerin caused proteasome inhibition, as shown by decreased proteasomal chymotrypsin-like activity and decreased AR protein level as well as accumulation of poyubiquitinated proteins in a dose-dependent manner. Also apoptosis as shown by caspase-3 activation, PARP cleavage and apoptotic features (shrinkage, round-up) occurred in the same manner (Fig. 5). One previous report showed that pristimerin induced caspase-dependent apoptosis in breast cancer cells [Wu et al., 2005]. Our results suggest that in prostate cancer cells, pristimerin-induced apoptosis is also caspase-dependent, evidenced by caspase-3 activation and PARP cleavage (Fig. 4B vs. A; Fig. 6C vs. B, and Fig. 7B vs. A).

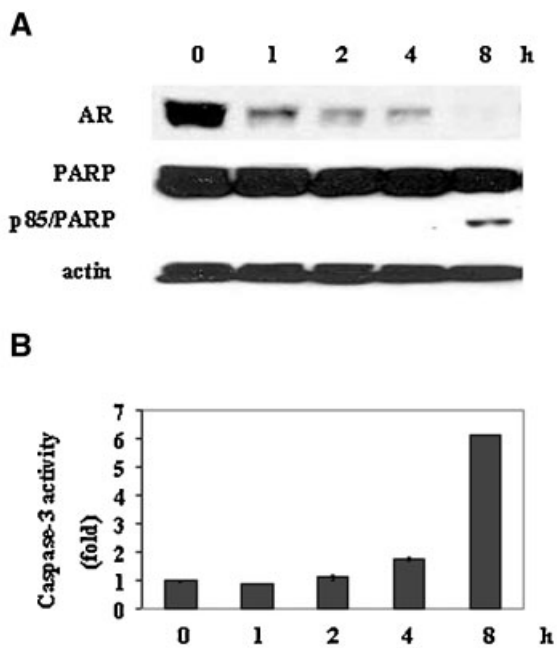

Fig. 7. Proteasome inhibition and apoptosis induction by pristimerin in LNCaP cells. LNCaP cells were treated with 5 μmol/L pristimerin up to 8 h, followed by Western blotting analysis (**A**) and caspase-3 activity assay (**B**). Loss of AR expression, cleavage of PARP, and activation of caspase-3 are shown. β-actin was used as a loading control (A). All data represent independent triplicate experiments. Bars, SD.

The sequence of biological events leading up to apoptosis induced by pristimerin was also examined in kinetic studies. We found that proteasome inhibition by pristimerin was initiated much earlier (at 1 h), as shown by the accumulation of IκB-α, Bax, and p27, three well-known substrates of the proteasome. Apoptosis induction was followed (at 8–16 h), as shown by caspase-3 activation and PARP cleavage in AR-negative PC-3 cells (Fig. 4). Compared to proteasome inhibition starting at 1 h, apoptosis induction delayed for 7 h (Fig. 4B vs. A). In AR-positive cancer cells, proteasome inhibition started as early as the first hour of pristimerin treatment and was accompanied by AR suppression, which was followed by apoptosis induction (Figs. 6–7). These data suggest that AR suppression caused by proteasome inhibition was required for apoptosis induction even though the mechanism for AR decrease remains unknown.

We notice that the IC_{50} value for proteasome inhibition by pristimerin is 2.2–3.0 μmol/L in purified 20S proteasome or tumor cell extract, however it caused 30–40% proteasome inhibition at 5 μmol/L in tumor cells. This discrepancy is in agreement with other reported specific proteasome inhibitors [Nam et al., 2001], indicating that these natural compounds might have more than one cellular targets and/or might not be very stable in cells. We also found that even though the proteasome activity was not completely inhibited (30–40% inhibition) in cultured tumor cells, apoptosis was still induced by pristimerin, suggesting that the proteasome is probably one of major cellular targets of pritimerin to induce apoptosis, but other pathways might also be involved in the same action.

Taken together, our results clearly demonstrate that the proteasome is one important cellular target of pristimerin in human prostate cancer cells, inhibition of which is associated with apoptosis induction. The potency of pristimerin on proteasome inhibition and apoptosis induction suggests its potential use for cancer prevention and treatment.

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