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The Tumor Proteasome Is a Primary Target for the Natural Anticancer Compound Withaferin A Isolated from "Indian Winter Cherry"

Huanjie Yang, Guoqing Shi, and Q. Ping Dou

The Prevention Program, Barbara Ann Karmanos Cancer Institute, and Department of Pathology, School of Medicine, Wayne State University, Detroit, Michigan

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ABSTRACT

Withaferin A (WA) is a steroidal lactone purified from medicinal plant "Indian Winter Cherry" that is widely researched for its variety of properties, including antitumor effects. However, the primary molecular target of WA is unknown. By chemical structure analysis, we hypothesized that Withaferin A might be a natural proteasome inhibitor. Computational modeling studies consistently predict that C_1 and C_{24} of WA are highly susceptible toward a nucleophilic attack by the hydroxyl group of N-terminal threonine of the proteasomal chymotrypsin subunit β5. Furthermore, WA potently inhibits the chymotrypsin-like activity of a purified rabbit 20S proteasome ($IC_{50} = 4.5$ μM) and 26S proteasome in human prostate cancer cultures (at 5–10 μM) and xenografts (4–8 mg/kg/day). Inhibition of prostate tumor cellular proteasome activity in cultures and in vivo by WA results in accumulation of ubiquitinated proteins and three proteasome target proteins (Bax, p27, and IκB-α) accompanied by androgen receptor protein suppression (in androgen-dependent LNCaP cells) and apoptosis induction. Treatment of WA under conditions of the aromatic ketone reduction, or reduced form of Celastrol, had significantly decreased the proteasome-inhibitory and apoptosis-inducing activities. Treatment of human prostate PC-3 xenografts with WA for 24 days resulted in 70% inhibition of tumor growth in nude mice, associated with 56% inhibition of the tumor tissue proteasomal chymotrypsin-like activity. Our results demonstrate that the tumor proteasome β5 subunit is the primary target of WA, and inhibition of the proteasomal chymotrypsin-like activity by WA in vivo is responsible for, or contributes to, the antitumor effect of this ancient medicinal compound.

Natural products have become more and more important in drug discovery for the treatment of human diseases, such as cancer (Newman et al., 2003). However, mechanisms of action of most of the natural medicinal products are unclear. *Withania somnifera* Dunal (ashwagandha, also named "Indian Winter Cherry" or "Indian Ginseng," belongs to the Solanaceae family) has been used for centuries in Ayurvedic medicine, the traditional medical system in India, as a remedy for a variety of musculoskeletal conditions and as a tonic to improve the overall health (Mishra et al., 2000). Its root extract has also recently been accepted as a dietary supplement in the United States (Jayaprakasam et al., 2003). Many reports suggest its broad medical application with anti-inflammation, antitumor, cardioprotection, neuroprotection, and immunomodulation properties (Devi et al., 1992; Agarwal et al., 1999; Gupta et al., 2004; Ahmad et al., 2005; Owais et al., 2005; Rasool and Varalakshmi, 2006). So far, more than 35 chemical constituents have been isolated from this plant, including alkaloids and withanolides, of which Withaferin A (WA), a steroidal lactone, is the most important (Mishra et al., 2000). Several properties of WA have been reported: antitumor effect, radiosensitizing activity (Devi et al., 1995, 1996, 2000; Sharada et al., 1996; Devi and Kamath, 2003), antiangiogenesis through NF-κB inhibition (Mohan et al., 2004; Yokota et al., 2006), cytoskeletal architecture alteration by covalently binding annexin II (Falsey et al., 2006), and apoptosis induction through the protein kinase C pathway in leishmanial cells (Sen et al., 2007).

The proteasome, a highly selective proteinase complex, is

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ABBREVIATIONS: WA, Withaferin A; NF-κB, nuclear factor-κB; N-Thr, amino-terminal threonine; IκB-α, inhibitor of nuclear factor κB-α; DMSO, dimethyl sulfoxide; Suc, *N*-succinyl-; AMC, 7-amino-4-methylcoumarin; PARP, poly(ADP-ribose) polymerase; AR, androgen receptor; TLC, thin-layer chromatography; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; H and E, hematoxylin and eosin; CT, chymotrypsin.

viewed as a possible target for the therapy of cancer (Goldberg, 1995; Dou and Li, 1999). It is composed of two complex components, the cylindric 20S core particle and two 19S cap particles, that dock onto both ends of the 20S proteasome (Goldberg, 1995; Dou and Li, 1999). The barrel-shaped 20S unit consists of two rings of seven α subunits and two rings of seven β subunits, stacked in the order $\alpha\beta\beta\alpha$. The β5, β2, and β1 subunits are responsible for three different catalytic activities of the proteasome: chymotrypsin-like, trypsin-like, and peptidyl glutamyl peptide hydrolyzing, respectively (Goldberg, 1995; Dou and Li, 1999). In all three β-subunits, the amino-terminal threonine (N-Thr) is the catalytically active amino acid. Many endogenous proteins, including cyclins, transcription factors, and tumor suppressors, are degraded by the proteasome (Glotzer et al., 1991; Pagano et al., 1995; Chen et al., 1996). These proteins are marked by ubiquitins for degradation and recognized by the 19S particle of the proteasome (Nandi et al., 2006).

We have reported that some dietary flavonoids containing aromatic ketone structures were able to inhibit proteasomal chymotrypsin-like activity (Chen et al., 2005). We proposed that the aromatic ketone carbon would interact with the hydroxyl group of the N-threonine of the proteasomal β5 subunit, forming a covalent bond and causing inhibition of the proteasomal chymotrypsin-like activity (Chen et al., 2005).

By chemical structure analysis, we noticed that WA contains two conjugated ketone bonds (Fig. 1*A*) and therefore hypothesized that WA might be a proteasome inhibitor. In the current study, we report that the nucleophilic susceptibility and in silico docking studies predict that C_1 and C_{24} of WA are highly susceptible toward a nucleophilic attack by the hydroxyl group of N-terminal threonine of the proteasomal chymotrypsin subunit β5. WA inhibits the chymotrypsin-like activity of a purified 20S proteasome and 26S proteasome in cultured prostate cancer cells and tumors. Inhibition of prostate tumor cellular proteasome in vitro and in vivo by WA was accompanied with the accumulation of the proteasome target proteins Bax, IκB-α, and $p27^{Kip1}$ and induction of apoptosis. Treatment of WA under conditions in

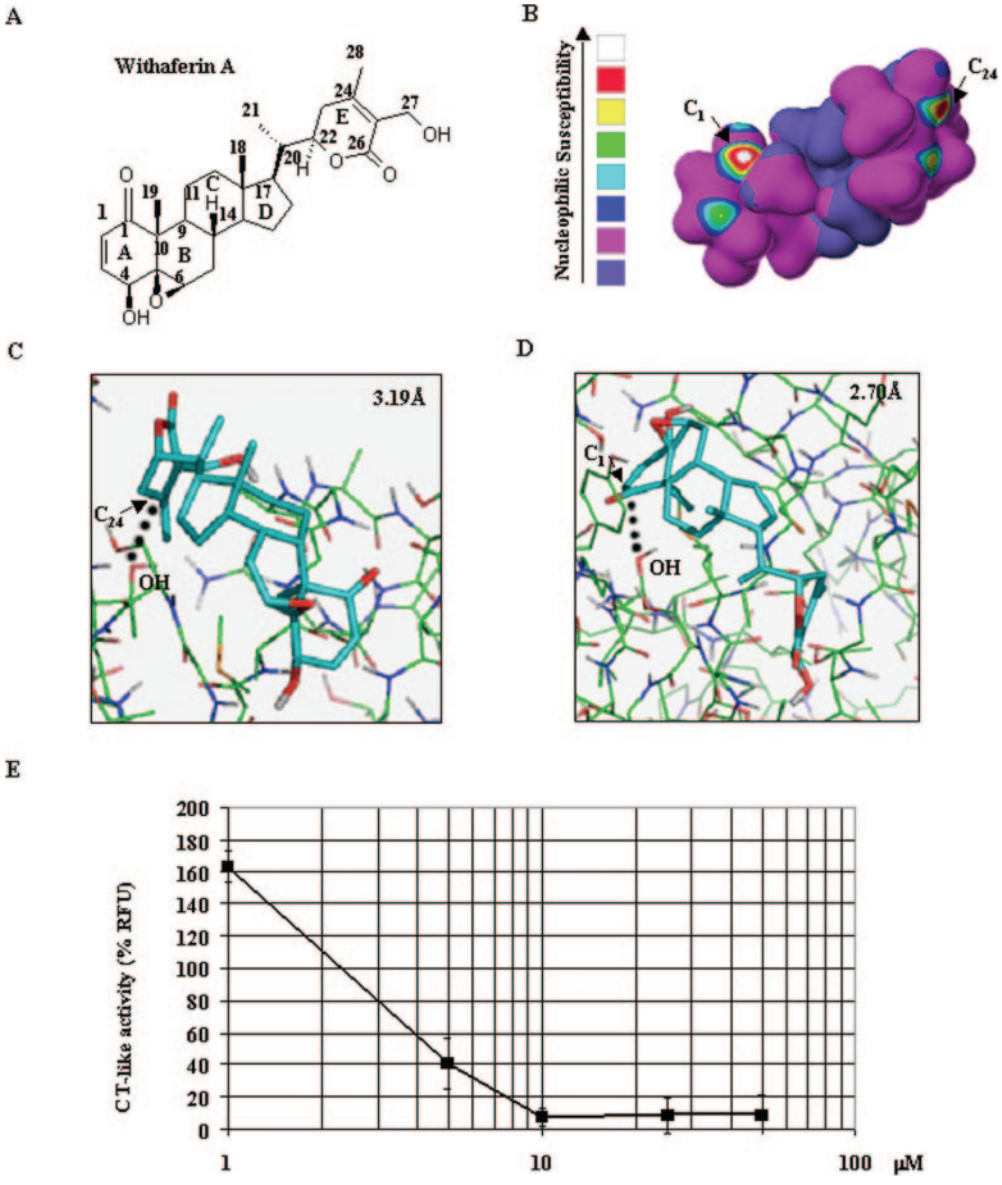

Fig. 1. WA contains two conjugated ketone bonds and can inhibit the chymotrypsin (CT)-like activity of a purified rabbit 20S proteasome. A, the chemical structure of WA was shown. B, nucleophilic susceptibility of WA analyzed using CAChe software. Higher susceptibility was shown at the C_1 and C_{24} positions of WA. C and D, computational modeling of WA interacting with β5 subunit of proteasome. WA is shown in blue, whereas the hydroxyl group (OH) of the N-Thr of β5 subunit is labeled. The distance from C_{24} to the OH of N-Thr was 3.19 Å in one cluster with 42 members (C), whereas the distance from C_1 to the OH of N-Thr was 2.70 Å in another cluster with 26 members (D). To verify computational modeling results, inhibition of CT-like activities of a purified 20S rabbit proteasome (35 ng) by WA was tested (E). The IC_{50} value of WA was 4.5 μM.

which its ketone bond would be reduced, or a reduced form of Celastrol containing a conjugated ketone structure similar to that of WA, results in significant decrease in the abilities to inhibit the proteasome and induce apoptosis. Finally, treatment of human prostate PC-3 xenografts with WA for 24 days caused 70% inhibition of tumor growth that was associated with 56% inhibition of tumor proteasomal chymotrypsin-like activity. Our results suggest that the proteasomal chymotrypsin subunit is a novel molecular target of WA in vitro and in vivo.

Materials and Methods

Materials. Purified WA (>95%) was purchased from Calbiochem Inc. (San Diego, CA) and ChromaDex, Inc. (Santa Ana, CA; used for animal study). WA was dissolved in DMSO (Sigma, St. Louis, MO) at a stock concentration of 20 mM, aliquoted and stored at −80°C. Celastrol and dihydrocelastrol were purchased from Calbiochem Inc. and Gaia Chemical Corporation (Gaylordsville, CT), respectively. Cremophor EL and other chemicals were from Sigma (St. Louis, MO). Fetal bovine serum was from Tissue Culture Biologicals (Tulare, CA). RPMI 1640 medium, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA). Purified rabbit 20S proteasome, fluorogenic peptide substrates Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activity) and *N*-acetyl-DEVD-AMC (for caspase-3/-7 activity) were from Calbiochem Inc. Mouse monoclonal antibody against human poly(ADP-ribose) polymerase (PARP) was from BIOMOL International LP (Plymouth Meeting, PA). Mouse monoclonal antibodies against Bax (B-9), p27 (F-8), ubiquitin (P4D1), NF-κB p65 (F-6), and androgen receptor (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). Mouse monoclonal antibody NCL-p27 was purchased from Novocastra Laboratories Ltd (Newcastle-upon-Tyne, UK). CD31 monoclonal antibody clone JC/70A was purchased from Ventana Medical Systems, Inc. (Tucson, AZ). Enhanced chemiluminescence reagent was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Apoptag Peroxidase In Situ Apoptosis Detection Kit was from Chemicon International, Inc. (Temecula, CA). Hexane, sodium borohydride, iodine, ethyl acetate, and silica gel on thin-layer chromatography (TLC) plates were purchased from Sigma-Aldrich Inc (St. Louis, MO).

Preparation of Reduced Form of WA. WA was dissolved in ethanol at 9.4 mM. Reduction reaction was set up by adding 26 mM $NaBH_4$. The mixture was vortexed for 1 h at 4°C, followed by standing on table for 2 days at 4°C. Reduction product (reduced WA), with WA as a control, was analyzed by TLC and separated by capillary action in a defined solvent [(hexane/methanol/ethyl acetate (5:3:12)].

Cell Cultures. Human prostate cancer PC-3 and LNCaP cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell cultures were maintained at 37°C and 5% CO_2.

Whole-Cell Extract Preparation and Western Blotting Analysis. A whole-cell extract was prepared as described previously (An and Dou, 1996). Western blotting assay using enhanced chemiluminescence reagent was performed as described previously (Li and Dou, 2000).

Nuclear Protein Extraction. The whole-cell pellet was suspended with cell lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.5 mg/ml benzamidine) and incubated on ice, followed by centrifuge. Nuclear pellet was resuspended in nuclear extraction buffer (20 mM

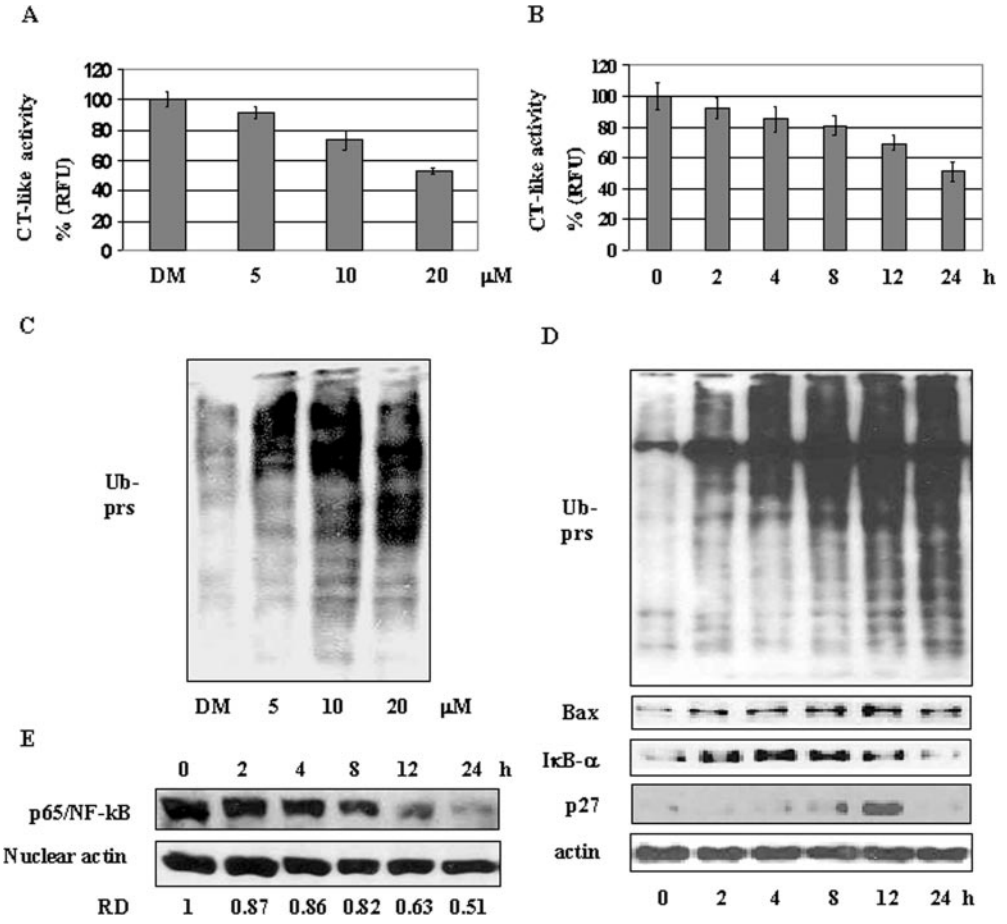

Fig. 2. Proteasome inhibition by WA in PC-3 cells. PC-3 cells were treated with either solvent DMSO (DM) or indicated concentrations of WA for 4 (for dose-dependent ubiquitination) or 16 h (for dose-dependent CT-like activity inhibition), or 10 μM WA for the hours indicated (for kinetics), followed by measuring inhibition of the proteasomal CT-like activity using the fluorescent substrate Suc-LLVY-AMC (A and B) and Western blotting analysis using specific antibodies to ubiquitin (to measure ubiquitinated proteins or Ub-Prs) (C and D), Bax, IκB-α, and p27 (D). The molecular masses of Bax, IκB-α, and p27 were 23, 37, and 27 kDa, respectively. β-Actin was used as loading control. E, inhibition of nuclear translocation of p65/NF-κB by WA. Nuclear proteins from the above kinetic experiment were subject to Western blotting using antibody against NF-κB p65 antibody. Actin extracted from the nuclei was used as loading control. Relative density (RD) values were normalized ratios of the intensities of p65/NF-κB band to the corresponding actin band. Data represent independent triplicate experiments. Bars, SD.

HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.5 mg/ml benzamidine), followed by incubation on ice for 30 min. After centrifugation, nuclear proteins were extracted from the supernatant.

Nucleophilic Susceptibility Analysis. Analysis of electron density surface colored by nucleophilic susceptibility was generated using Quantum CAChe (Fujitsu, Fairfield, NJ). Highly susceptible atoms for nucleophilic attack were showed by two-colored bull's-eyes.

In Silico Docking Study. WA was docked to the 20S proteasome as described previously (Kazi et al., 2003; Smith et al., 2004). The selected dockings for WA were the two clusters with more members and lower binding free energies. Structural output from Autodock was visualized using PyMOL software (http://pymol.sourceforge.net/).

Inhibition of Purified 20S Proteasome Activity by WA. Purified rabbit 20S proteasome (35 ng) was incubated with 40 μM fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activities) in 100 μl of assay buffer (20 mM Tris-HCl, pH 7.5) in the presence of WA, reduced WA, celastrol, or dihydrocelastrol at different concentrations or the solvent DMSO or ethanol for 2 h at 37°C, followed by measurement of hydrolysis of the fluorogenic substrates using a Wallac 1420 Victor^3 (PerkinElmer Life and Analytical Sciences, Boston, MA) multilabel counter with 355-nm excitation and 460-nm emission wavelengths.

Inhibition of the Cellular Proteasome Activity by WA. PC-3 or LNCaP cells were treated as described in the figure legends. Chymotrypsin-like activity was measured in the prepared whole-cell extracts (7.5 μg per sample) as described above. To detect proteasome inhibition by WA in intact cells, 40 μM Suc-LLVY-AMC substrate was incubated in 100 μl of treated or untreated cells (5000–8000 in 96-well plates) for 2 h, followed by measurement of chymotrypsin-like activity.

Caspase-3 (or -7) Activity Assay. PC-3 or LNCaP cells were treated with different concentrations of WA, reduced WA, celastrol, or dihydrocelastrol for indicated hours. The prepared whole-cell extracts (30 μg per sample) were then incubated with a 40 μM concentration of the caspase-3/-7 substrate *N*-acetyl-DEVD-AMC in 100 μl of assay buffer at 37°C for at least 2 h. The release of the AMC groups was measured as described above.

Human Prostate Tumor Xenograft Experiments. Male immunodeficient nude mice aged 5 weeks were purchased from Taconic Research Animal Services (Hudson, NY) and housed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of Wayne State University. Prostate cancer PC-3 cells (8×10^6) suspended in 0.1 ml of serum-free RPMI 1640 medium were inoculated s.c. in the right flank of each nude mouse. When the tumors became palpable (~120 mm^3), mice were randomly divided into one control group and two test groups. The control animals started daily i.p. injection with 100 to 200 μl of the vehicle [10% DMSO, 40% Cremophor/ethanol (3:1), and 50% phosphate-buffered saline], whereas the test animals received WA at 4.0 mg/kg (in 100 μl) or 8.0 mg/kg (in 200 μl). Tumor sizes were measured daily using calipers and their volumes calculated using a standard formula: width^2 $\times$ length/2. Body weight was measured every other day.

Proteasome Inhibition and Apoptosis Assays Using Tumor Tissue Samples. The proteasome or caspase activity assays and Western blotting using animal tumor tissue samples were performed similarly as described above using cultured prostate cancer cells. TUNEL assay, p27^{kip1}, CD31 immunostaining, and hematoxylin and eosin (H and E) staining in tumor tissues were performed according to manufactory protocols (Chen et al., 2006).

Statistical Analysis. One-way analysis of variance was used to evaluate differences between treated and control nude mice with respect to tumor growth, followed by Welch two sample t test to determine the differences between each two groups. $P < 0.05$ was considered statistically significant.

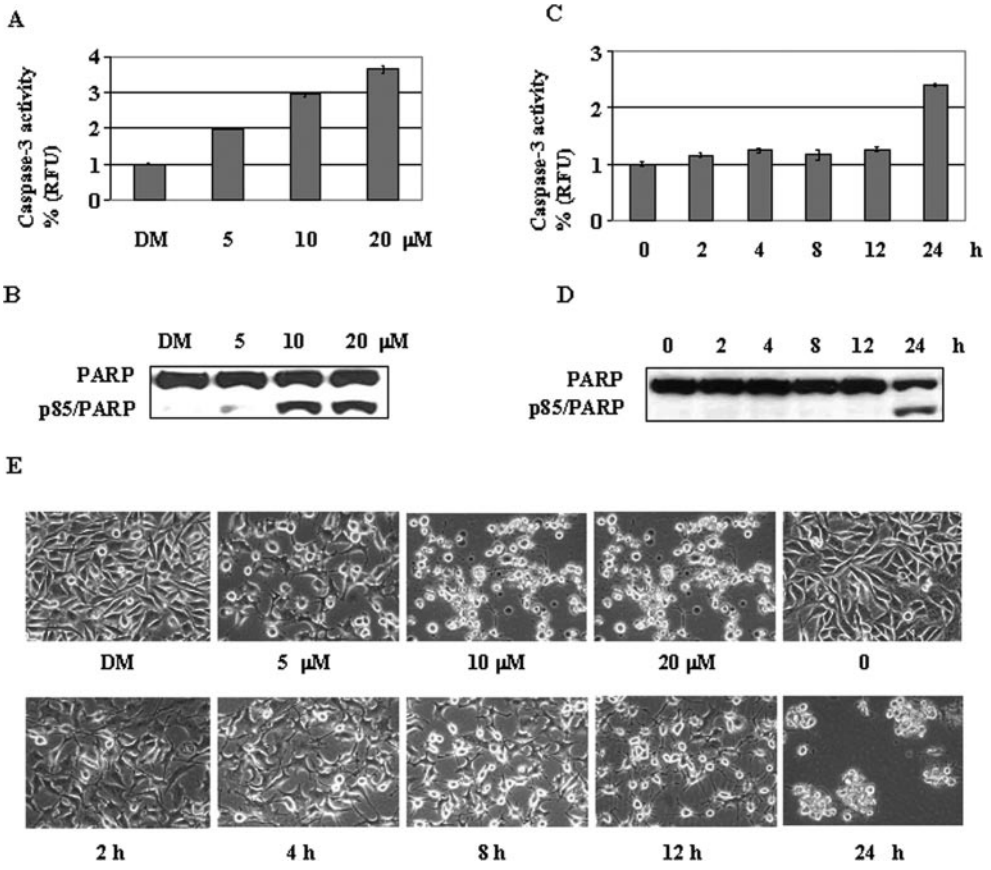

Fig. 3. Induction of apoptosis by WA in PC-3 cells. A and B, dose dependence. PC-3 cells were treated with DMSO (DM) or indicated concentrations of WA for 16 h (for activity assay) or 24 h (for Western blotting), followed by fluorescent detection of Caspase-3 activity (A) or Western blot analysis using PARP antibody (B). C and D, kinetics of apoptosis induction by WA. PC-3 cells were treated with 10 μM WA for the hours indicated, and cell extracts were prepared for the detection of Caspase-3 activity (C) and PARP cleavage (D). E, morphological changes were monitored during each treatment. The molecular masses of intact PARP and cleaved PARP were 116 and 85 kDa, respectively. Data represent independent triplicate experiments. Bars, SD.

Results

Computational Electron Density and Docking Studies Suggest that C_1 and C_{24} in WA Could Interact with the Proteasomal β5 Subunit and Inhibit the Chymotrypsin-like Activity. By chemical structure analysis, we found that WA, isolated from Indian Winter Cherry, has a conjugated ketone structure (Fig. 1A) that might be responsible for interaction with the hydroxyl (OH) group of the N-Thr of the β5 subunit, forming a covalent bond and causing inhibition of the proteasomal chymotrypsin-like activity (Chen et al., 2005; Yang et al., 2006). To test this idea, we first performed computational electron density analysis for the WA molecule. The result (Fig. 1B) predicts that WA is highly susceptible to a nucleophilic attack at C_1 in A-ring and C_{24} in E-ring, denoted by bull's-eyes with white or red center, respectively (Fig. 1B).

We then performed an in silico docking study to aid in the understanding of possible binding and inhibition of WA. Results from computational modeling indicate that WA binds the chymotrypsin site in an orientation and conformation that is suitable for a nucleophilic attack by the OH group of N-Thr of β5 subunit. There were only two major docking modes obtained. One with the lowest docked free energy (−9.93 kcal/mol) was repeated for 42 of 100 runs (42% probability), showing that the distance from the electrophilic C_{24} of WA to the OH of β5 N-Thr was 3.19 Å (Fig. 1C). Another docking mode (26% probability; −9.30 kcal/mol) showed 2.70 Å between C_1 and the OH of β5 N-Thr (Fig. 1D). Because nucleophilic attack could occur within 4 Å (Smith et al., 2004), C_{24} and C_1 in WA could interact with the proteasomal β5 and inhibit the chymotrypsin-like activity.

WA Potently Inhibits the Chymotrypsin-like Activity of a Purified Rabbit 20S Proteasome and Cellular Proteasome in Androgen-Independent PC-3 Prostate Cancer Cells. To provide direct evidence for the inhibition of proteasomal chymotrypsin-like activity by WA, we performed a cell-free proteasome activity assay using a purified rabbit 20S proteasome in the presence of WA at up to 50 μM. The chymotrypsin-like activity of the purified 20S proteasome was significantly inhibited by WA with an IC_{50} value of 4.5 μM (Fig. 1E).

To determine in vivo effects of proteasome inhibition by WA, androgen-independent PC-3 prostate cancer cells were treated with WA at 5, 10, or 20 μM for 4 or 16 h (dose-dependent) or 10 μM WA for up to 24 h (kinetics), followed by measuring proteasome inhibition by the cellular proteasomal activity assay. The proteasomal chymotrypsin-like activity was inhibited by WA in both dose- and time-dependent manners (Fig. 2, A and B). Compared with its potency to a purified 20S proteasome (IC_{50} 4.5 μM; Fig. 1E), WA reached 30 to 50% inhibition of the cellular proteasomal chymotrypsin-like activity at 10 to 20 μM (see *Discussion*). The kinetic study also showed that proteasome inhibition occurred at as early as 2 h after addition of WA and increased gradually after 24 h (Fig. 2B). Accompanied by proteasome inhibition, ubiquitinated proteins also accumulated in dose- and time-dependent manners (Fig. 2, C and D). Consistent with kinetic proteasome inhibition, accumulation of ubiquitinated proteins was started as early as 2 h and increased to the highest after 24 h (Fig. 2D). Furthermore, we observed levels of Bax and IκB-α, two well known target proteins of the proteasome (Chen et al., 1996; Li and Dou, 2000), increased from 2 h and peaked at approximately 8 to 12 and 4 to 8 h, respectively (Fig. 2D). Accumulation of another proteasome target protein p27 was peaked at 12 h (Fig. 2D). These results confirm that WA inhibits cellular proteasome activity in PC-3 cells.

It has been shown that WA suppresses angiogenesis through NF-κB inhibition (Mohan et al., 2004) and that proteasome is required by nuclear translocation of p65/NF-κB and therefore activation of NF-κB (Palombella et al., 1994; Panwalkar et al., 2004). We then examined whether WA-induced proteasome inhibition leads to NF-κB inactivation

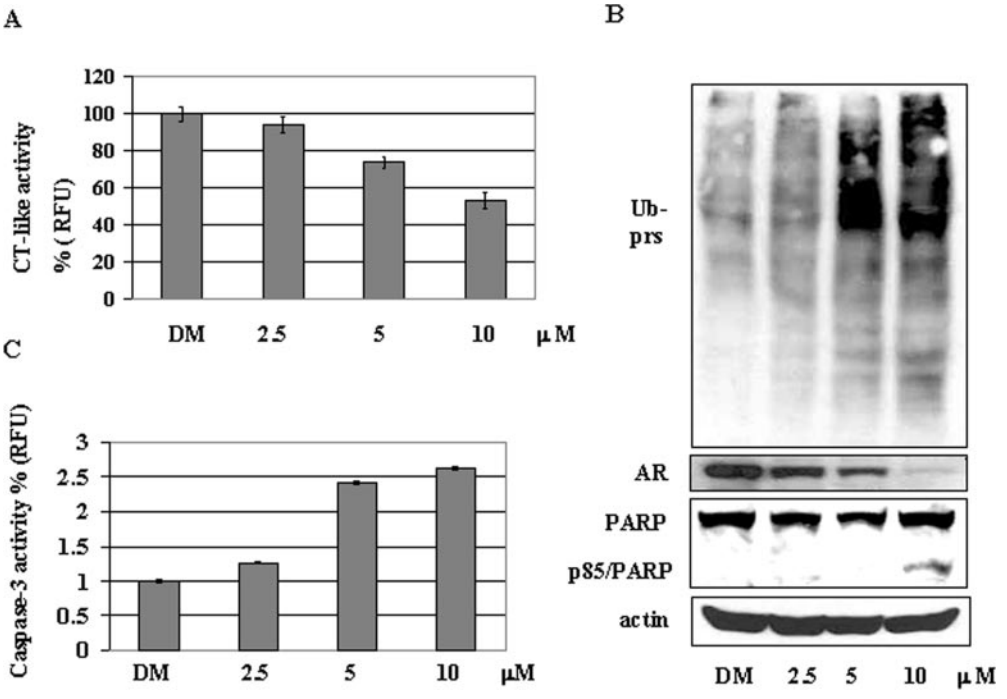

Fig. 4. Dosage effect of WA on proteasome inhibition and apoptosis induction in LNCaP cells. LNCaP cells were treated with either DMSO (DM) or WA at indicated concentrations for 3 h (for ubiquitination) or 6 h, followed by the proteasomal CT-like activity assay (A), Western blotting analysis (B), and caspase-3 activity assay (C). Inhibition of CT-like activity, loss of AR expression, cleavage of PARP, and activation of Caspase-3 were shown. The molecular mass of AR was 110 kDa. Data represent independent triplicate experiments. Bars, SD.

by detecting levels of nuclear p65/NF-κB in the same kinetic study. Figure 2E showed that inhibition of NF-κB nuclear translocation by WA is time-dependent, from 13% at 2 h to ~50% at 24 h. Nuclear actin protein was used to normalize p65/NF-κB levels.

Induction of Apoptosis by WA in Androgen-Independent PC-3 Prostate Cancer Cells. It has been shown that inhibition of the tumor cellular proteasome activity is associated with apoptosis induction (Lopes et al., 1997; An et al., 1998). To determine whether WA could induce apoptotic death in PC-3 cells along with proteasome inhibition and NF-κB inactivation, in the same experiment, we measured apoptotic cell death by caspase-3 activation, PARP cleavage, and cellular apoptotic morphological changes in the aliquots of PC-3 cells treated with WA. Figure 3A shows that WA induces caspase-3 activation in dose-dependent manner, from 2-fold increase by 5 μM WA to 4-fold increase by 20 μM WA after 16 h (Fig. 3A). The cleaved PARP fragment p85/PARP was also detected after treatment of 10 to 20 μM WA for 24 h (Fig. 3B), supporting the activation of caspase-3 by WA. If apoptosis were induced through proteasome inhibition, we would expect that proteasome inhibition should occur before apoptosis. Indeed, in the same kinetic experiment, caspase-3 activation was not detected until after 24 h (Fig. 3C) and PARP cleavage was also detected at 24 h (Fig. 3D), whereas proteasome inhibition was observed at as early as 2 h (Fig. 2, B and D). The morphological changes induced by WA were also consistent with the molecular events, showing that 10 μM WA is enough to induce apoptotic features (rounding and condensation), and treated cells at this concentration started to shrink at 8 h and became round at 24 h (Fig. 3E).

Effects of WA on Androgen-Dependent, AR-Positive LNCaP Prostate Cancer Cells. AR plays a critical role in the development of prostate cancer (Jenster, 1999). Proteasome inhibitors have been shown to reduce levels of AR protein, although the involved molecular mechanisms are unknown (Lin et al., 2002). If WA can inhibit proteasome activity, it should be able to suppress AR expression. To test this possibility, LNCaP cells were treated with different concentrations of WA for 3 or 6 h. We found that ~30% and 50% of proteasomal chymotrypsin-like activity was inhibited by WA at 5 and 10 μM, respectively (Fig. 4A) and that polyubiquitinated proteins were accumulated by WA at 5 μM and further increased at 10 μM (Fig. 4B). Likewise, AR protein expression was decreased in a WA dose-dependent manner (Fig. 4B). In addition, suppression of AR expression is associated with induction of apoptosis. Along with decreased AR protein levels by 5 to 10 μM WA, caspase-3 activity increased by 2.5-fold, and PARP was cleaved by 10 μM WA (Fig. 4, B and C). Levels of apoptotic cells with condensed nucleus were consistently increased after treatment with WA at 10 μM (data not shown; see Fig. 5D).

To determine the kinetics of proteasome inhibition, AR suppression, and apoptosis induction, LNCaP cells were treated with 10 μM WA for different lengths of time. First,

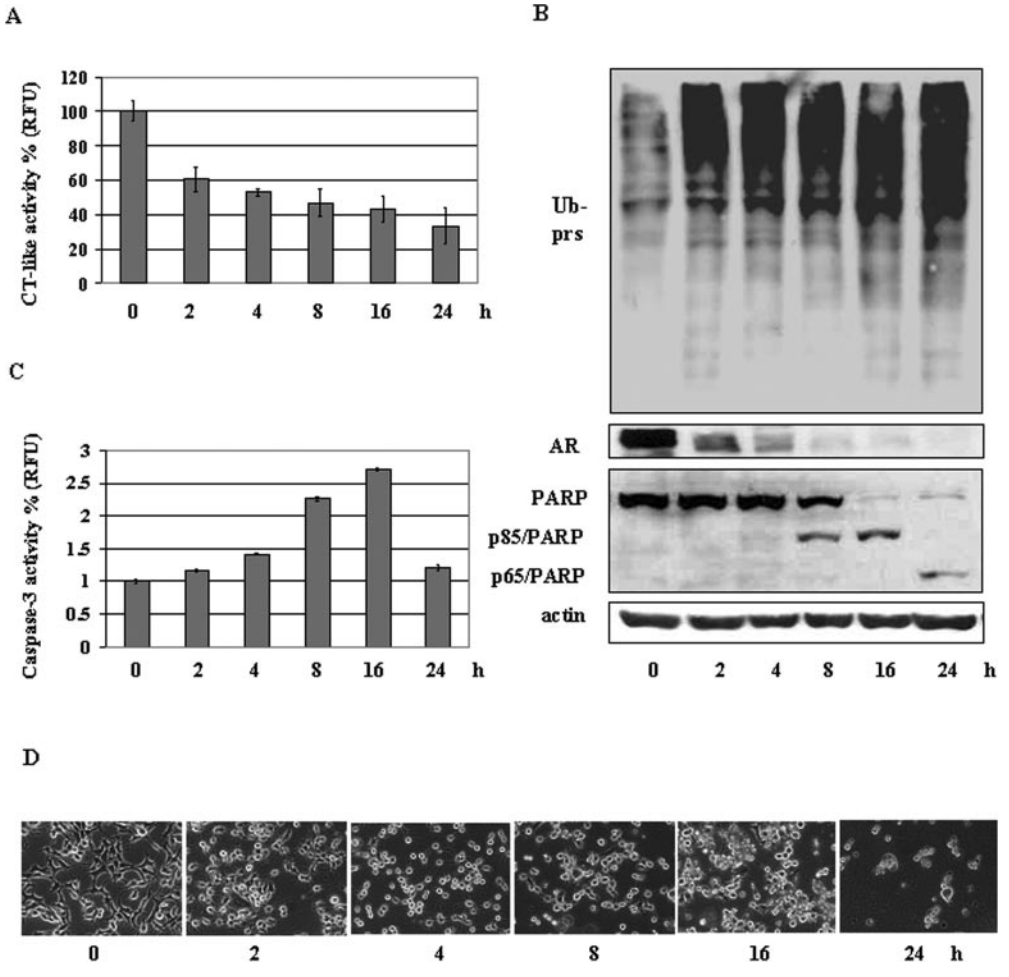

Fig. 5. Kinetic effect of WA on proteasome inhibition and apoptosis in LNCaP cells. Exponentially grown LNCaP cells (0) were treated with 10 μM WA for indicated length of times, followed by the proteasomal CT-like activity assay (A), Western blotting analysis (B), and caspase-3 activity assay (C). Inhibition of CT-like activity, loss of AR expression, cleavage of PARP, activation of Caspase-3 and apoptotic morphological changes (D) are shown. Data represent independent triplicate experiments. Bars, SD.

we found that proteasomal activity was inhibited by 40% at 2 h and by 70% after 24-h treatment (Fig. 5*A*). Consistent with kinetic inhibition of proteasomal activity, polyubiquitinated proteins were accumulated in a time-dependent manner, and this accumulation started as early as 2 h of treatment (Fig. 5B). Associated with the proteasome inhibition, AR protein expression was decreased by WA at 2 h and became almost undetectable after 8 h (Fig. 5B). After proteasome inhibition and AR suppression, PARP cleavage and caspase-3 activation were detected at 8 h after treatment and further increased at 16 h (Fig. 5, B and C). Compared with the proteasome inhibition and AR suppression at 2 h, apoptosis was delayed for at least 6 h. From a morphological standpoint, apoptotic features were found after 8 h treatment (Fig. 5*D*). It is noteworthy that, at 24 h, p65/PARP was the main cleaved product detected (Fig. 5B) that is generated by the active calpain (Tagliarino et al., 2003). At this time point, caspase activity was decreased, associated with loss of p85/PARP (Fig. 5, B and C).

WA Induced Apoptosis Associated with the Inhibition of Proteasomal Chymotrypsin-Like Activity in Human PC-3 Xenograft. To determine whether WA can inhibit the proteasome activity and induce tumor cell apoptosis in vivo, as observed in cultured prostate cancer cells, human PC-3 xenografts were generated s.c. in male nude mice. When the tumors became palpable (~120 mm^3), the mice were i.p. treated with either vehicle control ($n = 8$) or WA at 4.0 mg/kg ($n = 5$) or 8.0 mg/kg ($n = 4$). After 7 days of treatment, one WA-treated tumor (at 4.0 mg/kg) became undetectable, and this mouse remained tumor-free to the end of the experiment. In addition, after 24 days of daily treatment, 8 tumors from control animals grew to an average size of 1406 ± 116 mm^3. In contrast, 4 tumors from WA-treated animals at 4.0 and 8.0 mg/kg grew to an average size of 659 ± 166 and 427 ± 51 mm^3, respectively (Fig. 6A), demonstrating a significant tumor growth inhibition by 54 to 70%. To examine whether the proteasome activity of human tumor tissue in nude mice is inhibited by WA, the tumors were removed, and tissue extracts were prepared for proteasomal chymotrypsin-like activity assay and Western blotting. Figure 6B showed that WA treatment at 4.0 to 8.0 mg/kg caused 28 to 56% inhibition of the proteasomal chymotrypsin-like activity. Accumulation of ubiquitinated proteins and Bax, IκB-α, and p27, the three proteasome targets, was consistently observed in the tissue extracts of WA-treated versus control tumors (Fig. 6C). Thus, proteasome inhibition in vivo by WA was demonstrated by multiple assays. Furthermore, increased caspase-3 activity was found in the tumor extract of WA-treated mouse compared with control mice (data not shown; see Fig. 7), suggesting that apoptosis in PC-3 tumors has been activated by WA treatment.

We next performed immunostaining assays using tumor tissues to further investigate the in situ proteasome inhibition and apoptosis by WA. Compared with vehicle control, increased expression of p27 protein was observed in the tumor tissues from WA-treated mice (Fig. 7A), confirming proteasome inhibition in vivo by WA. In addition, apoptotic cells indicated by TUNEL positivity were observed in tumors from animals treated with WA (Fig. 7B). The apoptosis-inducing ability of WA in tumors was further demonstrated by another apoptotic feature, condensed nuclei, detected in WA-treated tumors by H and E staining (Fig. 7C). It has been shown that WA is an angiogenesis inhibitor (Mohan et al., 2004; Yokota et al., 2006). We consistently found that the expression of the endothelial marker CD31 in tumors was significantly inhibited by WA treatment (Fig. 7D). Finally, as mentioned previously, one WA-treated tumor (at 4.0 mg/kg) became undetectable on day 7 and remained tumor-free to the end of the experiment. The tissues from the tumor location of this mouse were collected and analyzed by anatomy and H and E staining. The results show the presence of normal muscles without any tumorous characteristics (Fig. 7E). Taken together, these data demonstrate that WA targets the tumor proteasome, resulting in apoptosis induction and angiogenesis inhibition, which are responsible for its observed antitumor effect.

The Conjugated Ketone Structure of WA Is Required for Its Proteasome-Inhibitory Activity. To provide direct

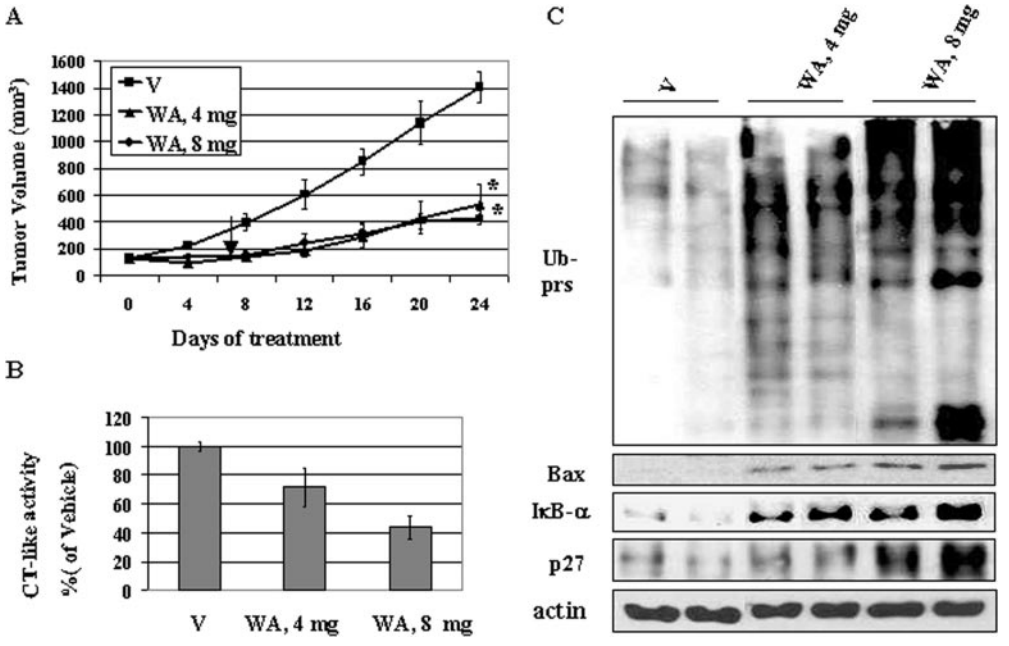

Fig. 6. Antitumor effects of WA associated with tumor proteasome inhibition in vivo. Male nude mice bearing PC-3 tumors were treated with either vehicle (V) or WA at 4.0 or 8.0 mg/kg/day for 24 days. A, PC-3 tumor growth inhibition by WA. Points, mean tumor volume; bars, SE. *, $p < 0.01$ After 7 days of treatment, one tumor treated with WA at 4.0 mg/kg became undetectable (arrow). By the end of treatment, tumors were removed and tissue extracts were prepared for the activity and Western blotting assays. B, proteasome inhibition by WA in vivo. C, Western blotting showed increased levels of ubiquitinated proteins Bax, IκB-α and p27.

evidence for supporting our hypothesis that the conjugated ketone carbons are required for the proteasome inhibition, we tried to generate the reduced form of WA by reduction reaction because it is not commercial available. TLC analysis of the production after reduction reaction is consistent with formation of a reduced form of WA, because 1) the new product has different mobility compared with WA and 2) the new product has increased polarity (data not shown).

We first measured the effect of the reduced WA on the chymotrypsin-like activity of purified rabbit 20S proteasome using WA as a positive control. WA at 10 μM caused proteasome inhibition by 90%, whereas the reduced WA at 10 μM caused inhibition by only 30% (Fig. 8A). Even at 25 μM, the reduced WA did not inhibit more than 35% of the proteasomal activity (data not shown). These data suggest that reduction of WA caused a 3-fold decrease in its proteasome-inhibitory activity (Fig. 8A). We then examined whether WA in reduced form was less able to inhibit cellular proteasome activity. LNCaP cells were treated with 10 μM WA or reduced WA for up to 16 h, followed by proteasomal chymotrypsin-like activity assay. WA inhibited the cellular proteasome by 40% after 2 h treatment, whereas reduced WA caused only 20% proteasome inhibition (Fig. 8B). After 16-h treatment, WA-mediated proteasome inhibition increased to ~60%, whereas reduced WA retained 20% inhibition (Fig. 8B). Furthermore, WA caused dramatic decrease of AR protein expression at 2 h, similar to what we observed previously (Figs. 8C versus 5B). In sharp contrast, the reduced WA had little effect after even 16 h of treatment (Fig. 8*C*).

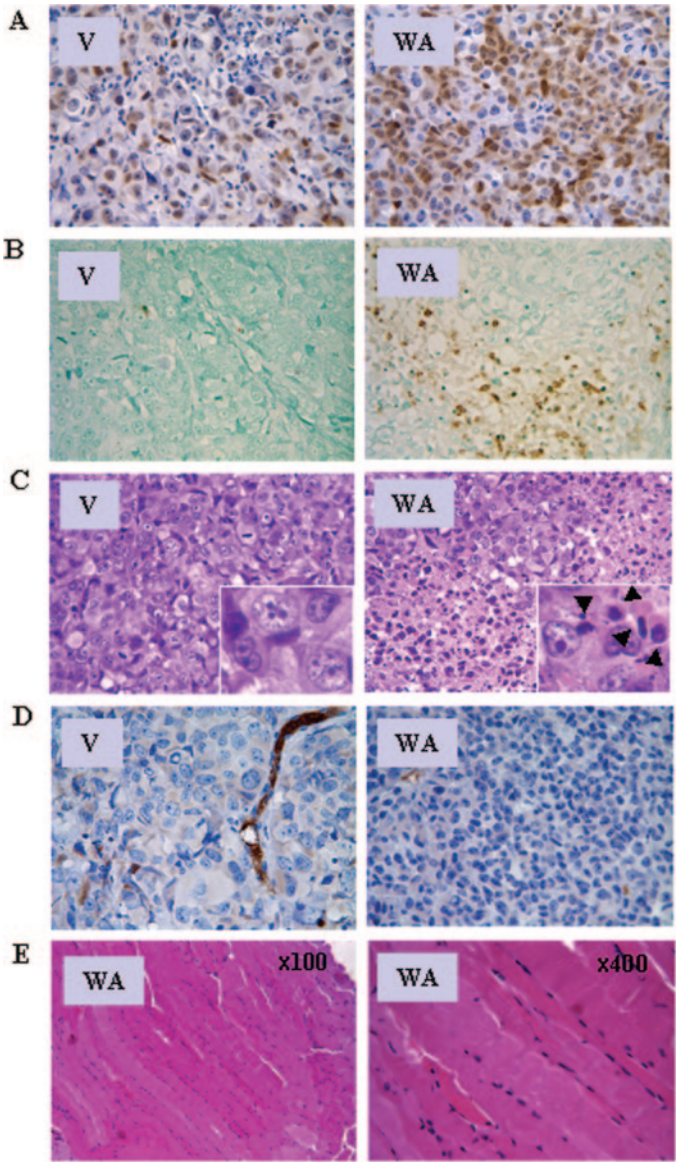

Fig. 7. Immunohistochemistry and H and E staining for tumor tissues. To verify the in situ proteasome inhibition and apoptosis induction by WA, tissue slides, prepared from tumors treated with vehicle (V) or WA, were used for immunostaining for p27 (A), TUNEL (B), H and E staining (C; arrows indicate apoptosis-specific condensed nuclei), and CD31 (D). E, To further detect existence of tumor cells in one tumor-free sample, tissues from the site where tumor was inoculated were prepared for H and E staining and only normal muscle tissue was detected. Original magnification, 100× (E, left) or 400× (others).

To investigate whether the decreased proteasome-inhibitory and AR-suppressing activities of reduced WA was associated with decreased apoptosis induction, LNCaP cells treated with WA or reduced WA in the same experiment were analyzed for apoptosis. We found that WA, but not the reduced WA, caused time-dependent caspase-3 activation and PARP cleavage (Fig. 8, C and D).

The conjugated ketone structure of WA is very similar to that present in celastrol (Figs. 1*A versus. 9A*). Because dihydrocelastrol (Fig. 9*B*), the reduced form of celastrol, is commercially available, we used the pair (celastrol and dihydrocelastrol) to further investigate the requirement of the conjugated ketone carbons for proteasome inhibition. As we reported previously, celastrol inhibited the chymotrypsin-like activity of the purified 20S proteasome with an IC_{50} of 2.5 μM (Fig. 9C) (Yang et al., 2006). In comparison, the IC_{50} value of dihydrocelastrol to the purified proteasome was determined to be 10 μM (Fig. 9C), suggesting that reduction of the ketone in celastrol caused a 4-fold decrease in its proteasome-inhibitory activity. Likewise, treatment of LNCaP cells with 5 μM Celastrol for 16 h results in greater proteasome inhibition than with dihydrocelastrol under the same conditions (48% versus 4%; Fig. 9D). In the kinetic experiment, celastrol, but not dihydrocelastrol, caused significant reduction of AR protein expression (Fig. 9*E*). Finally, celastrol has greater apoptosis-inducing ability than dihydrocelastrol, as shown by caspase activation (Fig. 9F) and PARP cleavage (Fig. 9E) at both earlier time points and higher levels. Thus the comparison of highly oxidized WA or celastrol at ketone structure with their reduced forms supports the idea that the conjugated ketone structure is critical for proteasome inhibition and apoptosis induction.

Discussion

WA is a natural product isolated from *Withania somnifera* Dunal (Solanaceae family) that has been used in traditional Indian medicine (Mishra et al., 2000). WA has been shown to exert antitumor properties (Devi et al., 1995; Sharada et al., 1996). However, the involved direct molecular target has never been identified. Here, we report that the proteasomal chymotrypsin subunit (β5) is a primary target of WA in vitro and in vivo. WA inhibits the chymotrypsin-like activity of purified 20S proteasome (IC_{50} = 4.5 μM) and 26S proteasome in human prostate cancer cells (5–10 μM) and tumors (4–8 mg/kg), leading to apoptosis induction, angiogenesis suppression, and tumor growth inhibition.

By analysis of the chemical structure (Fig. 1A), we proposed that the conjugated ketone carbon of WA should be able to inhibit the proteasomal activity (Chen et al., 2005; Yang et al., 2006). The results of computational modeling further predicted that both C_1 and C_{24} should be critical for this proteasome-inhibitory function: 1) these two carbons show high susceptibility toward nucleophilic attack (Fig. 1B); 2) both of them can be placed into S_1 pocket (data not shown), the active site of β5 subunit of the proteasome; 3) the distances from these two carbons to the β5 N-terminal threonine are within the range for them to interact with the N-terminal threonine, the catalytically active amino acid of β5 subunit (Fig. 1, C and D); and 4) the lowest docking free energies of the two major docking modes (Fig. 1, C and D) support the existence of the WA-proteasome complexes.

Consistent with the results from the nucleophilic susceptibility and in silico docking studies, WA directly and potently inhibited the chymotrypsin-like activity of the purified 20S proteasome with an IC_{50} value of 4.5 μM (Fig. 1E). These results also further support that conjugated ketone carbon contributed to the proteasome-inhibitory potency (Chen et al., 2005). It has been suggested that a double bond at position C_{2-3} of WA was responsible for the cytotoxicity (Fuska et al., 1984). Because removal of the C_{2-3} double bond would affect the electron density of C_1, our results are consistent with those of the previous study. The same report also suggested that dissociation of the double bond at C_{24-25} caused no significant biological effects (Fuska et al., 1984). Whether such a change would affect the proteasome-inhibitory activity of WA needs to be determined.

To provide direct evidence for the requirement of the conjugate ketone structure for the proteasome inhibition and the subsequent apoptosis induction, we prepared the reduced form of WA by reduction reaction and tested its effects using WA as a comparison. The obtained results of the proteasome activity assay in vitro and in cells demonstrated that reduction at the ketone structure of WA caused decreased proteasome-inhibitory activity (Fig. 8, A and B), leading to failure of AR suppression and apoptosis induction, as shown by lack of caspase-3 activation and PARP cleavage (Fig. 8, C and D). The known natural proteasome inhibitor celastrol has an identical ketone structure to that of WA (Figs. 9A versus 1A). We then tested the effect of dihydrocelastrol, the reduced analog of celastrol. The IC_{50} of dihydrocelastrol to the purified proteasome was decreased by 4-fold compared with celastrol (Fig. 9C). Deihydrocelastrol also has decreased potency to inhibit the cellular proteasome activity, decrease AR expression, and induce apoptosis (Fig. 9, C–F), further confirming that the conjugated ketone structure is critical for the proteasome-inhibitory and apoptosis-inducing function.

Compared with inhibition of the purified proteasome (IC_{50}, 4.5 μM), we found that more WA (10–20 μM) is needed to

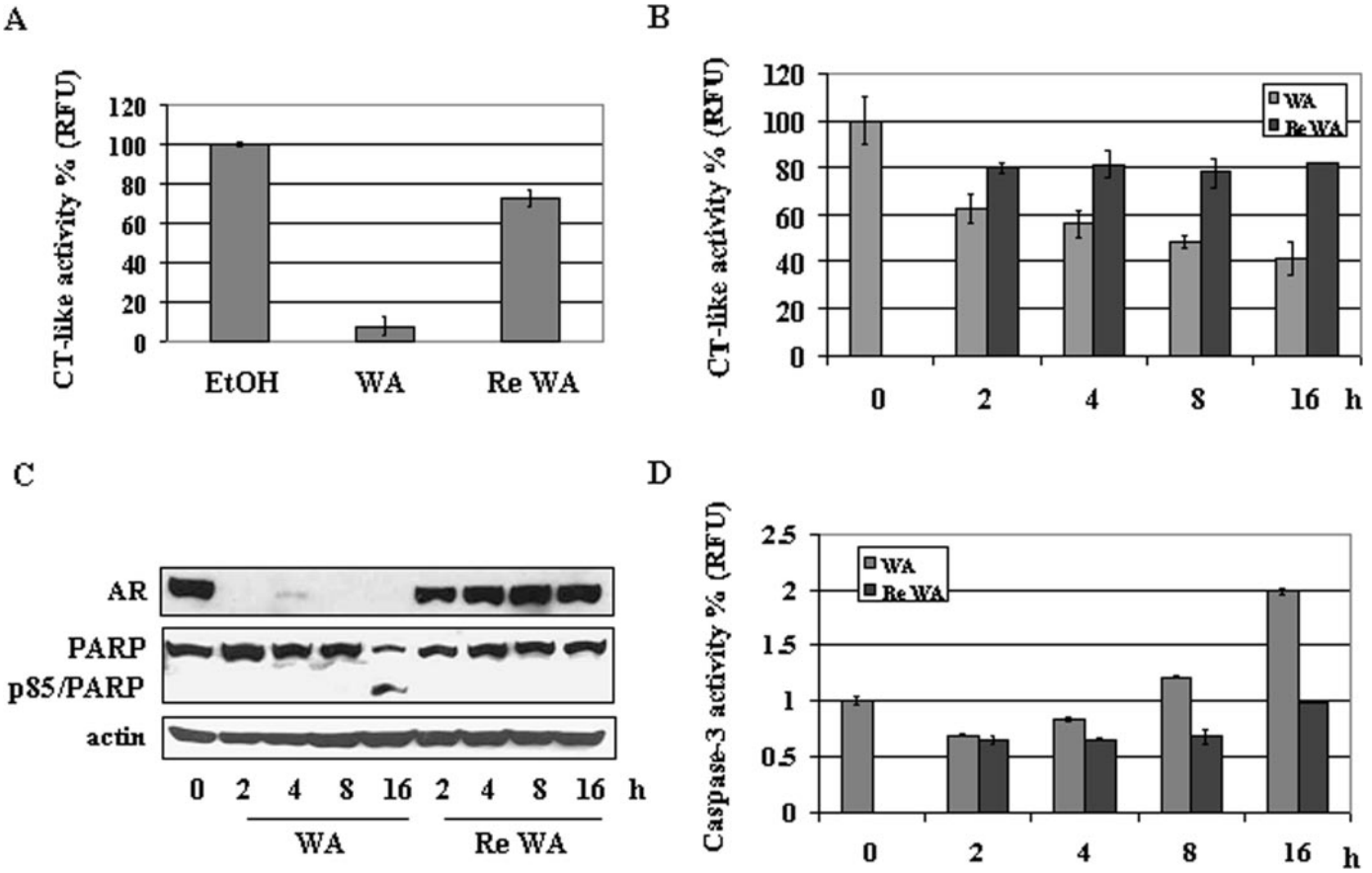

Fig. 8. Decreased proteasome-inhibitory activity and apoptosis-inducing effect of reduced form of WA. To investigate whether the conjugated ketone structure meet the requirement for proteasome inhibition, we generated reduced form of WA. Rabbit purified proteasome (35 ng) was incubated with WA or its reduced form (Re WA) at 10 μM, followed by CT-like activity assay (A). B to D, kinetic effects on cellular proteasome inhibition and apoptosis induction. LNCaP cells were treated with 10 μM WA or reduced WA for up to 16 h, followed by CT-like activity assay (B), Western blotting analysis using antibodies against AR, PARP (C), and caspase-3 detection (D). Data represent independent triplicate experiments. Bars, SD.

reach 50% proteasomal inhibition in prostate cancer cells (Figs. 2, A and B, 4A, and 5A). This difference could be due to instability of WA in cells and/or existence of other WA-binding proteins. In androgen-independent PC-3 cells, inhibition of cellular proteasome by WA was further confirmed by the time-dependent accumulation of proteasomal substrates Bax, IκB-α, and p27 as well as ubiquitinated proteins (Fig. 2C). In androgen-dependent LNCaP cells, we observed that WA induced AR suppression in company with the inhibition of proteasomal chymotrypsin-like activity (Figs. 4 and 5). Our finding was consistent with those of previous reports (Lin et al., 2002; Ikezoe et al., 2004; Pajonk et al., 2005). MG132, a well known proteasome inhibitor, has been shown to block the transactivation of AR (Lin et al., 2002). Whether WA down-regulates AR expression on the level of activity, protein, or mRNA needs to be further investigated. Taken together, these data suggest that WA potently inhibits the proteasome activity and inhibits AR protein expression in cultured prostate cancer cells.

Because proteasome inhibition by WA caused accumula-

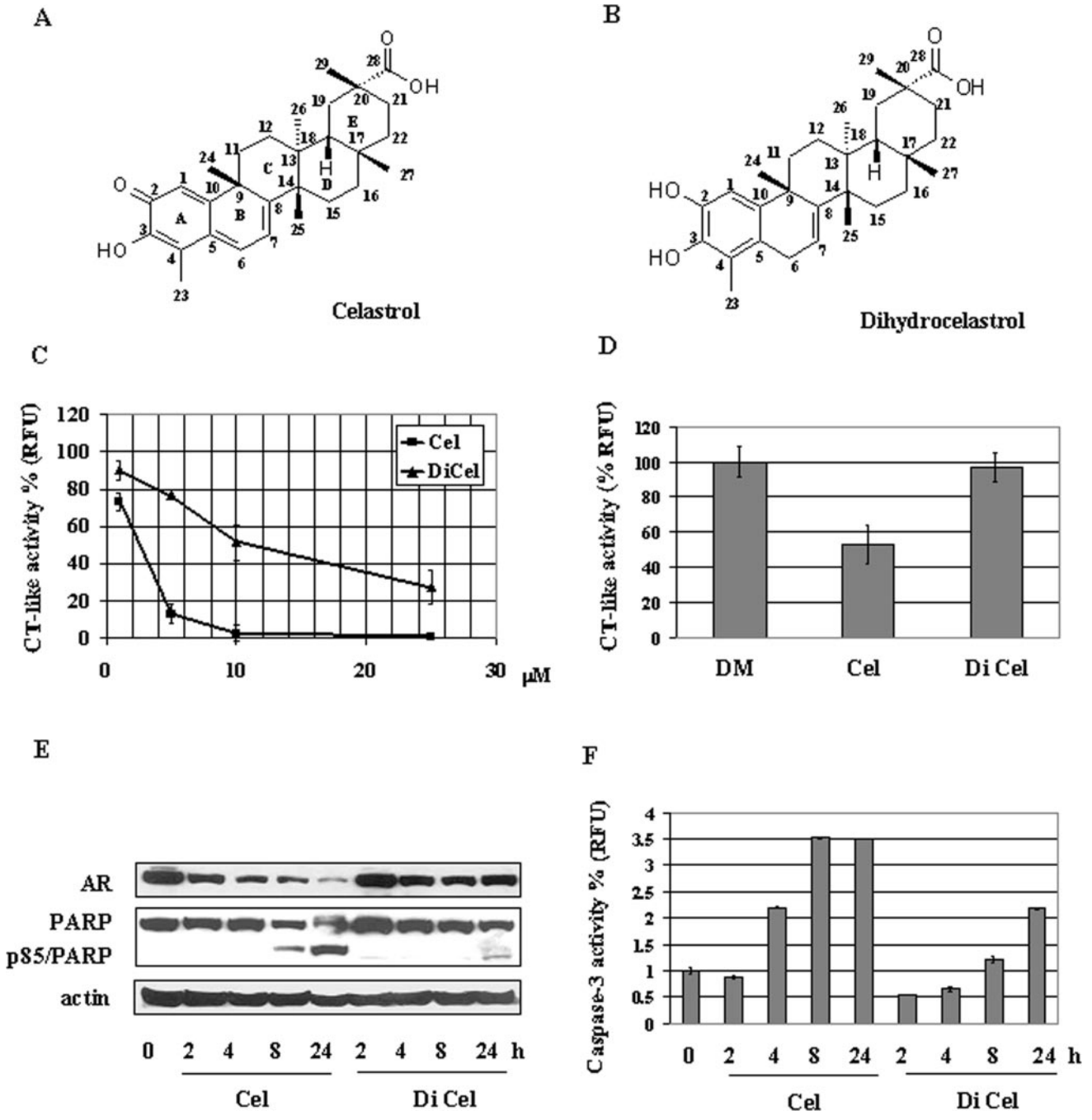

Fig. 9. Comparison between celastrol and its reduced form dihydrocelastrol on proteasome inhibition and apoptosis induction in LNCaP cells. Celastrol (Cel) that has identical ketone to WA and its reduced form dihydrocelastrol (Di Cel) were selected to confirm that the conjugated ketone structure is critical for the proteasome inhibition. The chemical structures of celastrol and dihydrocelastrol are shown (A and B), and proteasome inhibition in vitro (C) and in intact cells (D) were measured as described in *Materials and Methods*. E and F, kinetic effects of celastrol and dihydrocelastrol on AR suppression and apoptosis induction. LNCaP cells were treated with 5 μM celastrol (Cel) or dihydrocelastrol (Di Cel) for different times, followed by Western blotting analysis using antibodies against AR, PARP, and actin (E) and caspase-3 activity (F).

tion of apoptosis-related proteins Bax, p27, and IκB-α, we then investigated whether WA-induced proteasome inhibition triggers further apoptosis in the cultured prostate cancer cells. Indeed, we found that the proteasome inhibition by WA was associated with apoptosis induction, as shown by caspase-3 activation, PARP cleavage, and morphological changes (condensed nucleus) in both dose- and time-dependent manners (Figs. 3–5). More importantly, we noticed that the proteasomal inhibition occurred much earlier than apoptosis. The proteasomal inhibition was observed at as early as 2 h after treatment with WA whereas caspase-3 activation and cleaved PARP were detected after 16 to 24 h of treatment in androgen-independent PC-3 cancer cells (Fig. 3). In androgen-dependent LNCaP cells, we observed that WA induced apoptosis with a 6-h delay after proteasome inhibition and AR suppression (Fig. 5). From a morphological standpoint, WA-induced apoptotic features (i.e., rounding and condensed nuclei) were also detected after proteasome inhibition (Figs. 2–5). Taken together, these results demonstrate that proteasomal inhibition by WA triggers tumor cell apoptosis.

Next, we determined whether the proteasome is an in vivo target of WA in PC-3 xenografts. We found that treatment of tumors with WA, but not vehicle, caused inhibition of proteasomal chemotrypsin-like activity by 28 to 56% and accumulation of its substrates IκBα, Bax, and p27 as well as ubiquitinated proteins (Fig. 6, B and C). The proteasome inhibition was also visualized by the extensive accumulation of p27 in situ in WA-treated tumor tissues (Fig. 7A). The same WA-treated tumor samples also showed TUNEL positivity and apoptotic morphological features, demonstrating that proteasome inhibition in vivo by WA also triggers apoptosis (Fig. 7). Finally, WA at 4.0 to 8.0 mg/kg daily treatment caused 54 to 70% tumor growth inhibition (Fig. 6A), and complete tumor growth regression was found in one WA-treated tumor (Fig. 7E). All these results suggest that WA can reach a therapeutic concentration in vivo that can directly target and inhibit the tumor cellular proteasome, resulting in apoptosis induction, angiogenesis suppression, and tumor growth inhibition.

Natural medicines have been used for many years for cancer treatment, although the mechanisms of action of most of them remain unknown. WA has been shown to exhibit angiogenesis inhibition through NF-κB inhibition, and the ubiquitin-proteasome pathway was thought to be involved (Mohan et al., 2004). However, the direct target molecule of WA involved in the multiple steps of the proteasome-ubiquitin system has never been identified. Results of the present study show, for the first time, that the β5 subunit of the proteasome is the primary target of WA, inhibition of which leads to prostate cancer cell apoptosis. More importantly, our work also demonstrates that the tumor proteasomal chymotrypsin subunit is also an in vivo biological target of WA in human cancer, inhibition of which is associated with tumor growth inhibition. The present study also supports the usefulness of computational modeling for identifying natural proteasome inhibitors and understanding their molecular mechanisms of action.

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Address correspondence to: Q. Ping Dou, The Prevention Program, Barbara Ann Karmanos Cancer Institute, and Department of Pathology, School of Medicine, Wayne State University, 640.1 HWCRC, 4100 John R Road, Detroit, MI 48201. E-mail: doup@karmanos.org