Title Page

(Revised)

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

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Running Title Page

Running Title: Withaferin A targets prostate tumor proteasome *in vitro* & *in vivo*

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Abbreviation: Withaferin A, WA; IκB-α, inhibitor of nuclear factor κB-α; AR, androgen receptor; N-Thr, amino terminal threonine; PARP, poly (ADP-ribose) polymerase; DMSO, dimethyl sulphoxide; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyltransferase enzyme-mediated dUTP end labeling; H & E, Hematoxylin and Erosin; TLC, thin layer chromatography.

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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 anti-tumor effect. However, the primary molecular target of WA is unknown. By chemical structure analysis, we hypothesized that Withaferin A might be a natural proteasome inhibitor. Consistently, computational modeling studies predict that C_1 and C_{24} of WA are highly susceptible towards 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 μmol/L) and 26S proteasome in human prostate cancer cultures (at 5-10 μmol/L) 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 anti-tumor effect of this ancient medicinal compound.

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Introduction

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", belonged to Solanaceae family) has been used for over 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 been accepted as a dietary supplement in the United States recently (Jayaprakasam et al., 2003). Many reports suggest its broad medical application with anti-inflammation, anti-tumor, cardiopretection, neuroprotection, and immunomodulation properties (Agarwal et al., 1999; Ahmad et al., 2005; Devi et al., 1992; Gupta et al., 2004; Owais et al., 2005; Rasool and Varalakshmi, 2006). So far over 35 chemical constituents including alkaloids and withanolides have been isolated from this plant, in which Withaferin A (WA), a steroidal lactone, is the most important of the withanolides (Mishra et al., 2000). Several properties of WA have been reported, anti-tumor effect, radiosensitizing activity (Devi et al., 1996; Devi et al., 2000; Devi et al., 1995; Sharada et al., 1996; Devi and Kamath, 2003), anti-angiogenesis 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 PKC pathway in leishmanial cells (Sen et al., 2006).

The proteasome, a highly selective proteinase complex, is 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 which dock onto both ends

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of the 20S proteasome (Goldberg, 1995; Dou and Li, 1999). The barrel-shaped 20S unit consists of 2 rings of 7 α subunits and 2 rings of 7 β subunits, stacked in the order αββα. 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 the 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).

Previously, we reported that some dietary flavonoids containing aromatic ketone structure were able to inhibit the 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 towards 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 $p27^{Kip1}$

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and induction of apoptosis. Treatment of WA under conditions its ketone bond would be reduced, or reduced form of Celastrol containing similar conjugated ketone structure to 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, which 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*.

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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 Dihydrocelatrol 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 (FBS) was 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 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), NFκB 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 (Newcasle upon Tyne, UK). CD31 monoclonal antibody clone JC/70A was purchased from Ventana Medical Systems, Inc. (Tucson, AZ). Enhanced Chemiluminescence Reagent was from Amersham Biosciences (Piscataway, NJ). Apoptag Peroxidase In Situ Apoptosis Detection Kit was from Chemicon International, Inc. (Temecula, CA). Hexane, sodium borohydride, iodine, ethyl acetate and silica gel on TLC plate were purchased from Sigma-Aldrich Inc (St. Louis, MO).

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Preparation of Reduced form of WA. WA was dissolved in ethanol at 9.4 mM. Reduction reaction was set up by adding 26 mmol/L $NaBH_4$. The mixture was vortexed for 1 hour 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 thin layer chromatography (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 supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of 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 DTT, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mg/ml benzamidine) and incubated on ice, followed by centrifuge. Nuclear pellet was resuspended in nuclear extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mg/ml benzamidine), followed by incubation on ice for 30 min. After centrifuge, 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”.

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***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.

Inhibition of purified 20S proteasome activity by WA. Purified rabbit 20S proteasome (35 ng) was incubated with 40 μmol/L of fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activities) in 100 μl 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 hours at 37°C, followed by measurement of hydrolysis of the fluorogenic substrates using a Wallac Victor3^{TM} 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 **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 μmol/L of Suc-LLVY-AMC substrate was incubated in 100 μl treated or untreated cells (5000-8000 in 96-well plate) for 2 hours, 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 Dihydrocelasrtol for indicated hours. The prepared whole cell extracts (30 μg per sample) were then incubated with 40 μmol/L of the caspase-3/-7 substrate Ac-DEVD-AMC in 100 μl assay buffer at 37°C for at least 2 hours. The release of the AMC groups was measured as described above.

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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 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 100-200 μl of the vehicle [10% DMSO, 40% Cremophor:ethanol (3:1) and 50% PBS] while 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, $p27^{kip1}$ and CD31 immunostaining, and Hematoxylin and Eosin (H & E) staining in tumor tissues were performed following manufactory protocols (Chen et al., 2006).

Statistical analysis. One-way ANOVA 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.

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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 analysis, we found that WA, isolated from Indian Winter Cherry, has a conjugated ketone structure (Fig 1*A*) that might be responsible for interaction with the hydroxyl (OH) group of the *N*-threonine (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. 1*B*) predicts that WA is highly susceptible towards 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. 1*B*).

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 out 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. 1*C*). Another docking mode (26% probability; –9.30 kcal/mol) showed 2.70 Å between C_1 and the OH of β5 N-Thr (Fig. 1*D*). Since 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

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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 μmol/L. The chymotrysin-like activity of the purified 20S proteasome was significantly inhibited by WA with an IC_{50} value of 4.5 μmol/L (Fig. 1*E*).

In order 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 μmol/L for 4 or 16 hours (dose-dependent) or 10 μmol/L WA for up to 24 hours (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*, *B*). Compared to its potency to a purified 20S proteasome (IC_{50} 4.5 μmol/L ; Fig. 1*E*), WA reached 30-50% inhibition of the cellular proteasomal chymotrypsin-like activity at 10 to 20 μmol/L (see **Discussion**). The kinetic study also showed proteasome inhibition occurred at as early as 2 hours after addition of WA and increased gradually after 24 hours (Fig. 2*B*). Accompanying with proteasome inhibition, ubiquitinated proteins was also accumulated in dose- and time-dependent manners (Fig. 2*C*, *D*). Consistent with kinetic proteasome inhibition, accumulation of ubiquitinated proteins was started as early as 2 hours and increased to the highest after 24 hours (Fig. 2*D*). Furthermore, we observed levels of Bax and IκB-α, two well-known target proteins of the proteasome (Li and Dou, 2000; Chen et al., 1996), increased from 2 hours and peaked at about 8-12 hours and 4-8 hours, respectively (Fig. 3*D*). Accumulation of another proteasome target protein p27 was peaked at 12 hours (Fig. 2*D*). These results confirm that WA inhibits cellular proteasome activity in PC-3 cells.

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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 by detecting levels of nuclear p65/NF-κB in the same kinetic study. Fig. 2*E* showed that inhibition of NF-κB nuclear translocation by WA is time-dependent, from 13% at 2 hours to ~50% at 24 hours. 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 (An et al., 1998; Lopes et al., 1997). 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 3*A* shows that WA induces caspase-3 activation in dose-dependent manner: from 2-fold increase by 5 μmol/L of WA to 4-fold increase by 20 μmol/L of WA after 16 hours (Fig. 3*A*). The cleaved PARP fragment p85/PARP was also detected after treatment of 10-20 μmol/L WA for 24 hours (Fig. 3*B*), 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 hours (Fig. 3*C*) and PARP cleavage was also detected at 24 hours (Fig. 3*D*), while proteasome inhibition was observed at as early as 2 hours (Fig. 2*B, C*). The morphological changes induced by WA were also consistent with the molecular events, showing that 10 μmol/L

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of WA is enough to induce apoptotic features (round-up and condensation) and treated cells at this concentration started to shrink at 8 hours and became round-up at 24 hours (Fig. 3*E*).

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). It has been shown that proteasome inhibitors can 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 hours. We found that ~30% and 50% of proteasomal chymotrypsin-like activity was inhibited by WA at 5 and 10 μmol/L, respectively (Fig. 4*A*) and that polyubiquitinated proteins were accumulated by WA at 5 μmol/L and further increased at 10 μmol/L (Fig. 4*B*). Correspondingly, AR protein expression was decreased in a WA dose-dependent manner (Fig. 4*B*). Also, suppression of AR expression is associated with induction of apoptosis. Along with decreased AR protein levels by 5-10 μmol/L of WA, caspase-3 activity increased by 2.5-fold and PARP was cleaved by 10 μmol/L of WA (Fig. 4*B, C*). Consistently, levels of apoptotic cells with condensed nucleus were increased after treatment with WA at 10 μmol/L (data not shown, but see Fig. 5*D*).

To determine the kinetics of proteasome inhibition, AR suppression and apoptosis induction, LNCaP cells were treated with 10 μmol/L WA for different length of times. First we found that proteasomal activity was inhibited by 40% at 2 hours and by 70% after 24 hours treatment (Fig. 5*A*). Consistent with kinetic inhibition of proteasomal activity, polyubiquitinated proteins were accumulated in time-dependent manner and this accumulation started as early as 2 hours treatment (Fig. 2*B*). Associated with the proteasome inhibition, AR protein expression was

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decreased by WA at 2 hours and became almost undetectable after 8 hours (Fig. 5*B*). Following proteasome inhibition and AR suppression, PARP cleavage and caspase-3 activation were detected at 8 hours after treatment and further increased at 16 hours (Fig. 5*B, C*). Compared to the proteasome inhibition and AR suppression at 2 hours, apoptosis was delayed for at least 6 hours. Morphologically, apoptotic features were found after 8 hours treatment (Fig. 5*D*). Interestingly, at 24 hours, p65/PARP was the main cleaved product detected (Fig. 5*B*), which is mediated by activation of calpain (Tagliarino et al., 2003). At this time point, caspase activity was decreased, associated with loss of p85/PARP (Fig. 5*B-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 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 mm^3 and 427 ± 51 mm^3, respectively (Fig. 6*A*), demonstrating a significant tumor growth inhibition by 54-70%. 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. Fig. 6*B* showed that WA treatment at 4.0-8.0 mg/kg caused 28-56% inhibition of the

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proteasomal chymotrypsin-like activity. Consistently, accumulation of ubiquitinated proteins and Bax, IκB-α and p27, the three proteasome targets, were observed in the tissue extracts of WA-treated *vs*. control tumors (Fig. 6*C*). 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 to the control (data not shown, and see below 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 to vehicle control, increased expression of p27 protein was observed in the tumor tissues from WA-treated mice (Fig. 7*A*), 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. 7*B*). The apoptosis-inducing ability of WA in tumors was further demonstrated by another apoptotic feature, condensed nucleus, detected in WA-treated tumors by Hematoxylin and Eosin staining (Fig. 7*C*). It has been shown that WA is an angiogenesis inhibitor (Mohan et al., 2004; Yokota et al., 2006). Consistently, we found that the expression of the endothelial marker CD31 in tumors was significantly inhibited by WA treatment (Fig. 7*D*). Finally, as mentioned, 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 & E staining. The results show the presence of normal muscles without any tumorous characteristics (Fig. 7*E*). Taken together, these data demonstrate that WA targets tumor proteasome, resulting in apoptosis induction and angiogenesis inhibition that are responsible for its observed antitumor effect.

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The conjugated ketone structure of WA is required for its proteasome-inhibitory activity. To provide direct 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 since it is not commercial available. TLC analysis of the production after reduction reaction is consistent with formation of a reduced form of WA since (i) the new product has different mobility from that of WA and (ii) 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 μmol/L caused proteasome inhibition by 90% while the reduced WA 10 μmol/L caused only by 30% (Fig. 8*A*). Even at 25 μmol/L, the reduced WA did not inhibit more than 35% of the proteasomal activity (data not shown). These data suggested that reduction of WA have caused 3-fold decrease in its proteasome-inhibitory activity (Fig. 8*A*). We then examined whether WA in reduced form is less active to inhibit cellular proteasome activity. LNCaP cells were treated with 10 μmol/L WA or reduced WA for up to 16 hours, followed by the proteasomal chymotrypsin-like activity assay. WA inhibited the cellular proteasome by 40% after 2 hours treatment while reduced WA only caused 20% proteasome inhibition (Fig. 8*B*). After 16 hours treatment, WA-mediated proteasome inhibition increased to ~60% while reduced WA retained 20% inhibition (Fig. 8*B*). Furthermore, WA caused dramatic decrease of AR protein expression at 2 hours, similar to what we observed previously (Figs. 8*C vs. 5B*). In sharp contrast, the reduced WA had little effect after even 16 hours treatment (Fig. 8*C*).

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To investigate whether the decreased proteasome-inhibitory and AR-suppressing activities of reduced WA is 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 a time-dependent caspae-3 activation and PARP cleavage (Fig. 8*C*, *D*).

The conjugated ketone structure of WA is very similar to that present in Celastrol (Figs. 1*A vs.* 9*A*). Since the reduced form of Celastrol, Dihydrocelastrol (Fig. 9*B*), is commercially available, we then used the pair of 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} 2.5 μmol/L (Fig. 9*C*; Yang et al., 2006). In comparison, the IC_{50} value of Dihydrocelastrol to the purified proteasome was determined to be 10 μmol/L (Fig. 9*C*), suggesting that reduction of the ketone in Celastrol caused 4-fold decrease in its proteasome-inhibitory activity. Similarly, treatment of LNCaP cells with 5 μmol/L of Celastrol for 16 hours results in greater proteasome inhibition than with Dihydrocelastrol under the same conditions (48% *vs* 4%; Fig. 9*D*). 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. 9*F*) and PARP cleavage (Fig. 9*E*) at both earlier time points and higher levels. Thus the comparison of highly oxidized WA or Celastrol at ketone structure to their reduced forms support that the conjugated ketone structure is critical for proteasome inhibition and apoptosis induction.

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Discussion

WA is a natural product isolated from *Withania somnifera* Dunal (Solanaceae family) that has been used in traditional Indian medicine system (Mishra et al., 2000). It has been shown that WA exerts anti-tumor 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 μmol/L) and 26S proteasome in human prostate cancer cells (5-10 μmol/L) and tumors (4-8 mg/kg), leading to apoptosis induction, angiogenesis suppression and tumor growth inhibition.

By analysis of the chemical structure (Fig. 1*A*), 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. i) These two carbons show high susceptibility towards nucleophilic attack (Fig. 1*B*); ii) both of them can be placed into S_1 pocket (data not shown), the active site of β5 subunit of the proteasome; iii) the distances from these two carbons to the β5 N-terminal threonine are within the range for them to interact with the N-terminal theronine, the catalytically active amino acid of β5 subunit (Fig. 1*C, D*); iv) the lowest docking free energies of the two major docking modes (Fig. 1*C, 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 μmol/L (Fig. 1*E*). These results also further support that conjugated

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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). Since removal of the C_{2-3} double bond would affect the electron density of C_1, our results are consistent with this previous study. The same report also suggested that dissociation of the double bond at C_{24-25} did not caused any 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-B*), leading to failure of AR suppression and apoptosis induction, as shown by lack of caspase-3 activation and PARP cleavage (Fig. 8*C, D*). The known natural proteasome inhibitor Celastrol has an identical ketone structure to that of WA (Figs. 9*A vs* 1*A*). We then tested the effect of the reduced analogue of Celastrol, Dihydrocelastrol. The IC_{50} of Dihydrocelastrol to the purified proteasome was decreased by 4-fold, compared to Celastrol (Fig. 9*C*). Deihydrocelstrol 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.

Comparing to inhibition of the purified proteasome inhibition (IC_{50} 4.5 μmol/L), we found that more WA (10-20 μmol/L) is needed to reach 50% proteasomal inhibition in prostate cancer cells

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(Figs. 2*A*, *B* and 4*A*, 5*A*). 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. 2*C*). In androgen-dependent LNCaP cells, we observed that WA induced AR suppression in accompany with the inhibition of proteasomal chymotrypsin-like activity (Figs. 4, 5). Our finding was consistent with previous reports (Ikezoe et al., 2004; Lin et al., 2002; Pajonk et al., 2005). A well-known proteasome inhibitor MG132 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.

Since proteasome inhibition by WA caused accumulation 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 hours after treatment with WA while caspase-3 activation and cleaved PARP were detected after 16 to 24 hours treatment in androgen-independent PC-3 cancer cells (Fig. 3). In androgen-dependent cell line LNCaP cells, we observed WA induced apoptosis with 6 hours delay after proteasome inhibition

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and AR suppression (Fig. 5). Morphologically, WA-induced apoptotic features, round-up and condensed nucleus, 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 the inhibition of proteasomal chymotrypsin-like activity by 28-56% and accumulation of its substrates IκB-α, Bax and p27 as well as ubiquitinated proteins in WA-treated tumors compared to vehicle-treated tumors (Fig. 6*B, C*). The proteasome inhibition was also visualized by the extensive accumulation of p27 *in situ* in WA-treated tumor tissues (Fig. 7*A*). 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 4.0-8.0 mg/kg daily treatment caused 54-70% of tumor growth inhibition (Fig. 6*A*) and complete tumor growth regression was found in one WA-treated tumor (Fig. 7*E*). 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, for the first time, show the β5 subunit of the proteasome is the primary target of WA, inhibition of which leads to prostate cancer cell apoptosis. More

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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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Footnote

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Legends for figures

Figure 1. Withaferin A (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, D,* Computational modeling of WA interacting with β5 subunit of proteasome. WA was shown in blue while the hydroxyl group (OH) of the N-terminal threonine (N-Thr) of β5 subunit was labeled. The distance of C_{24} to the OH of N-Thr was 3.19 Å in one cluster with 42 members (*C*) while the distance of 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 μmol/L.

Figure 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 hours (for dose-dependent CT-like activity inhibition), or 10 μM of WA for indicated hours (for kinetics), followed by measuring inhibition of the protesomal CT-like activity using fluorescent substrate Suc-LLVY-AMC (*A, B*) and Western blotting analysis using specific antibodies to ubiquitin (to measure ubiquitinated proteins or Ub-Prs) (*C, D*), Bax, IκB-α and p27 (*D*). Molecular weight of Bax, IκB-α and p27 are 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. All data are representatives of independent triplicate experiments. *Bars*, SD.

Figure 3. Induction of apoptosis by WA in PC-3 cells. *A, B,* Dose-dependency. PC-3 cells were treated with DMSO (DM) or indicated concentrations of WA for 16 hours (for activity assay) or 24 hours (for Western blotting), followed by fluorescent detection of Caspase-3 activity (*A*) or Western blot analysis using PARP antibody (*B*). *C, D,* Kinetics of apoptosis induction by WA. PC-3 cells were treated with 10 μM WA for indicated hours, 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. Molecular weight of intact PARP and cleaved PARP is 116 and 85 kDa, respectively. All data are representatives of independent triplicate experiments. *Bars*, SD.

Figure 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 hours (for ubiquitination) or 6 hours, followed by the protesomal 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. Molecular weight of AR is 110 kDa. All data are representatives of independent triplicate experiments. *Bars*, SD.

Figure 5. Kinetic effect of WA on proteasome inhibition and apoptosis in LNCaP cells. Exponentially grown LNCaP cells (0) were treated with 10 μmol/L WA for indicated length of times, followed by the protesomal 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

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PARP, activation of Caspase-3 and apoptotic morphological changes (*D*) were shown. All data are representatives of independent triplicate experiments. *Bars*, SD.

Figure 6. Anti-tumor 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 treatment, one tumor treated with WA at 4.0 mg/kg became undetectable as indicated by 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.

Figure 7. Immunohistochemistry and Hematoxylin and Eosin 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 & 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 & E staining and only normal muscle tissue was detected. Original magnification, x100 (for *E, left panel*) or x 400 (for others).

Figure 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 μmol/L, followed by CT-like activity assay (*A*). *B-D,* Kinetic effects on cellular proteasome inhibition and apoptosis induction. LNCaP

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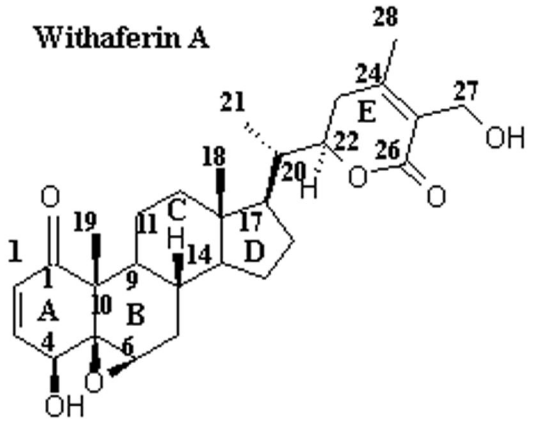
cells were treated with 10 μmol/L WA or reduced WA for up to 16 hours, followed by CT-like activity assay (*B*), Western blotting analysis using antibodies against AR, PARP (*C*), and caspase-3 detection (*D*). All data are representatives of independent triplicate experiments. *Bars*, SD.

Figure 9. Comparisin 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 were shown (*A, B*) and proteasome inhibition *in vitro* (*C*) and *in intact cells* (*D*) were measured as described in **Material and Method**. *E*, *F*, Kinetic effects of Celastrol and Dihydrocelastrol on AR suppression and apoptosis induction. LNCaP cells were treated with 5 μmol/L Celastrol (Cel) or Dihydrocelastrol (Di Cel) for different hours, followed by Western blotting analysis using antibodies against AR, PARP and actin (*E*) and caspase-3 activity (*F*).

A


Withaferin A
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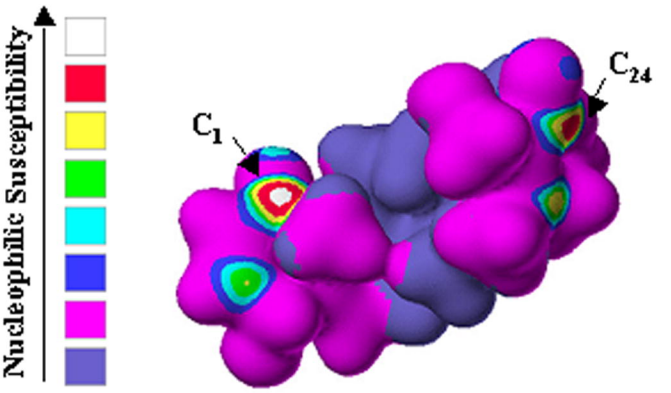
Nucleophilic Susceptibility
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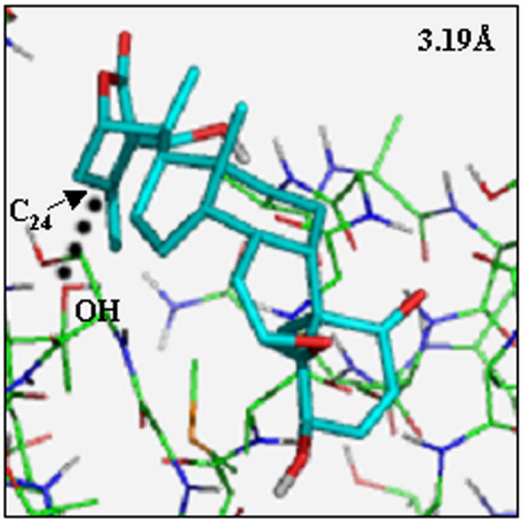
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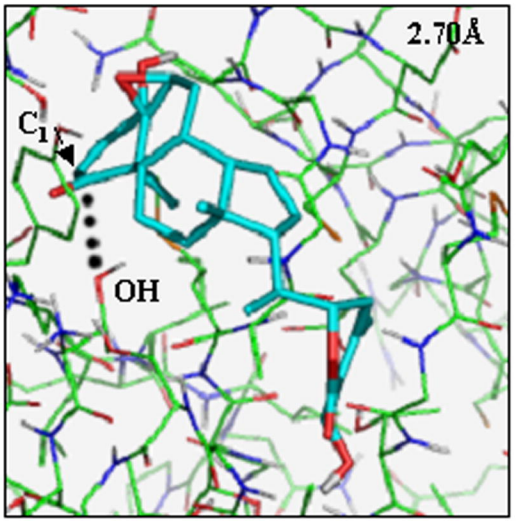
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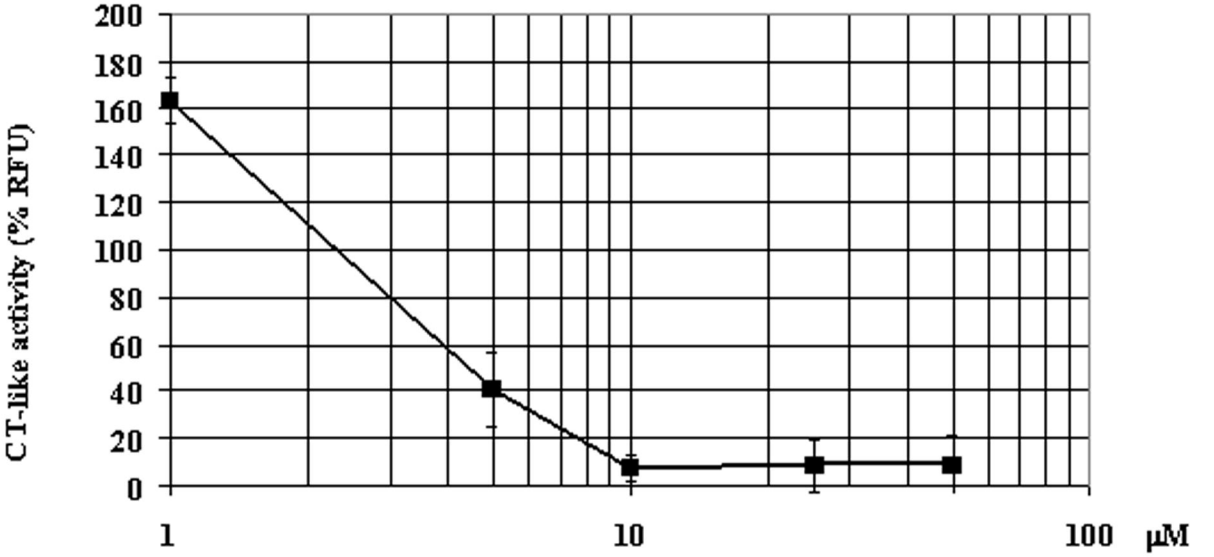
CT-like activity (% RFU)
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Fig. 1

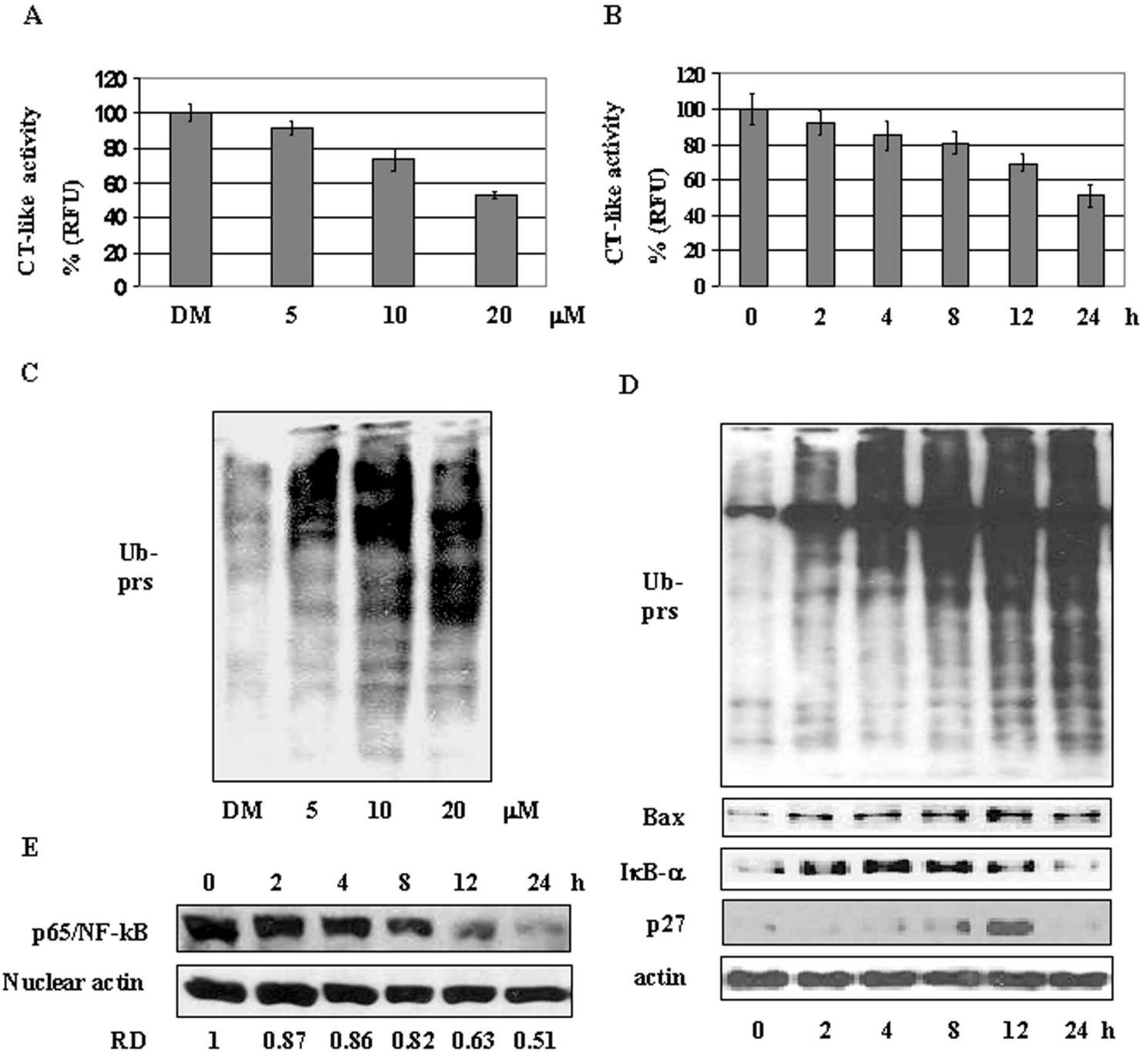
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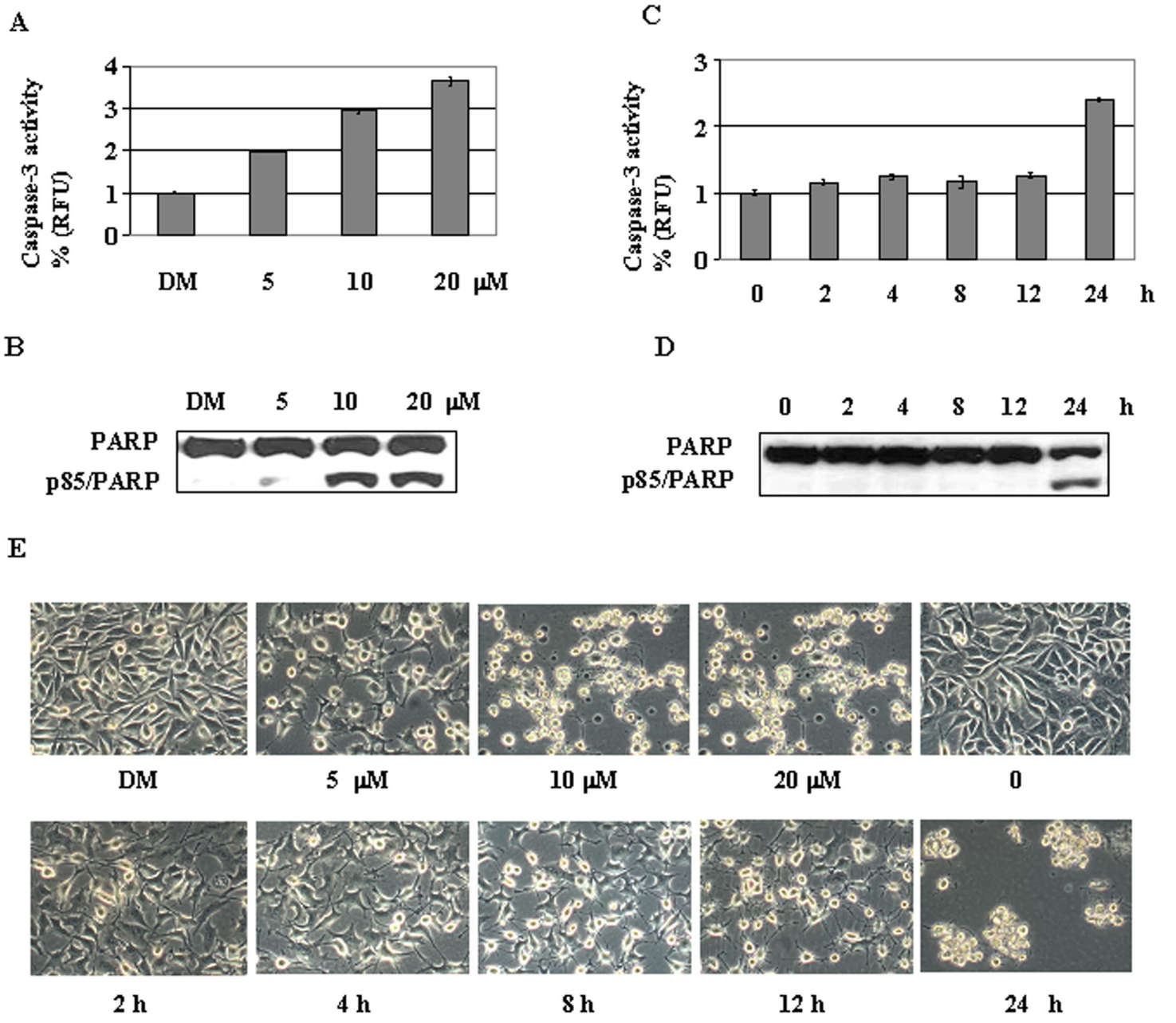
Fig. 2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Caspase-3 activity
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24 h

Fig. 3

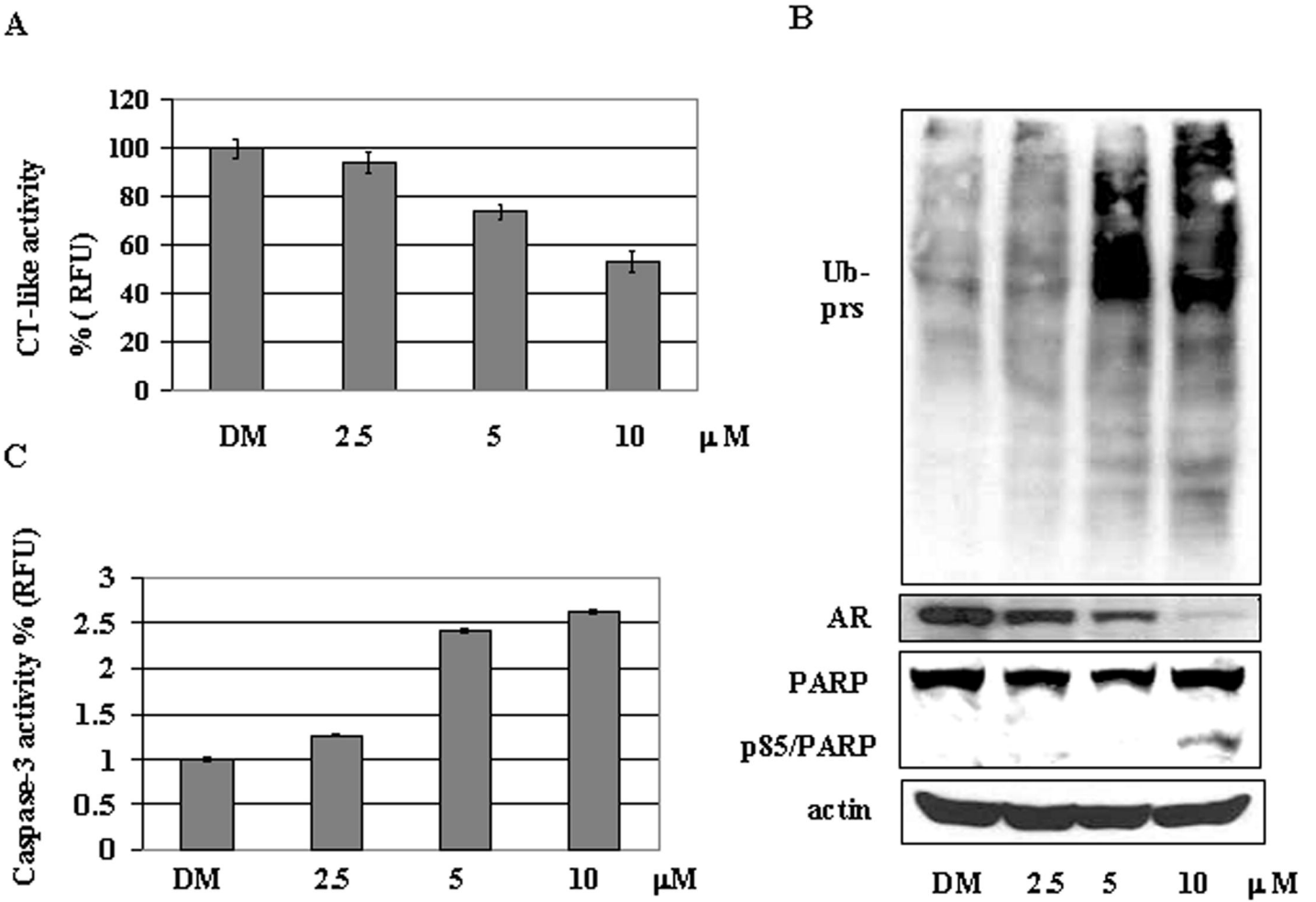

A
120
100
80
60
40
20
0
CT-like activity
% (RFU)
DM
2.5
5
10
μM
B
Ub-
prs
AR
PARP
p85/PARP
actin
DM
2.5
5
10
μM
C
Caspase-3 activity % (RFU)
3
2.5
2
1.5
1
0.5
0
DM
2.5
5
10
μM

Fig. 4

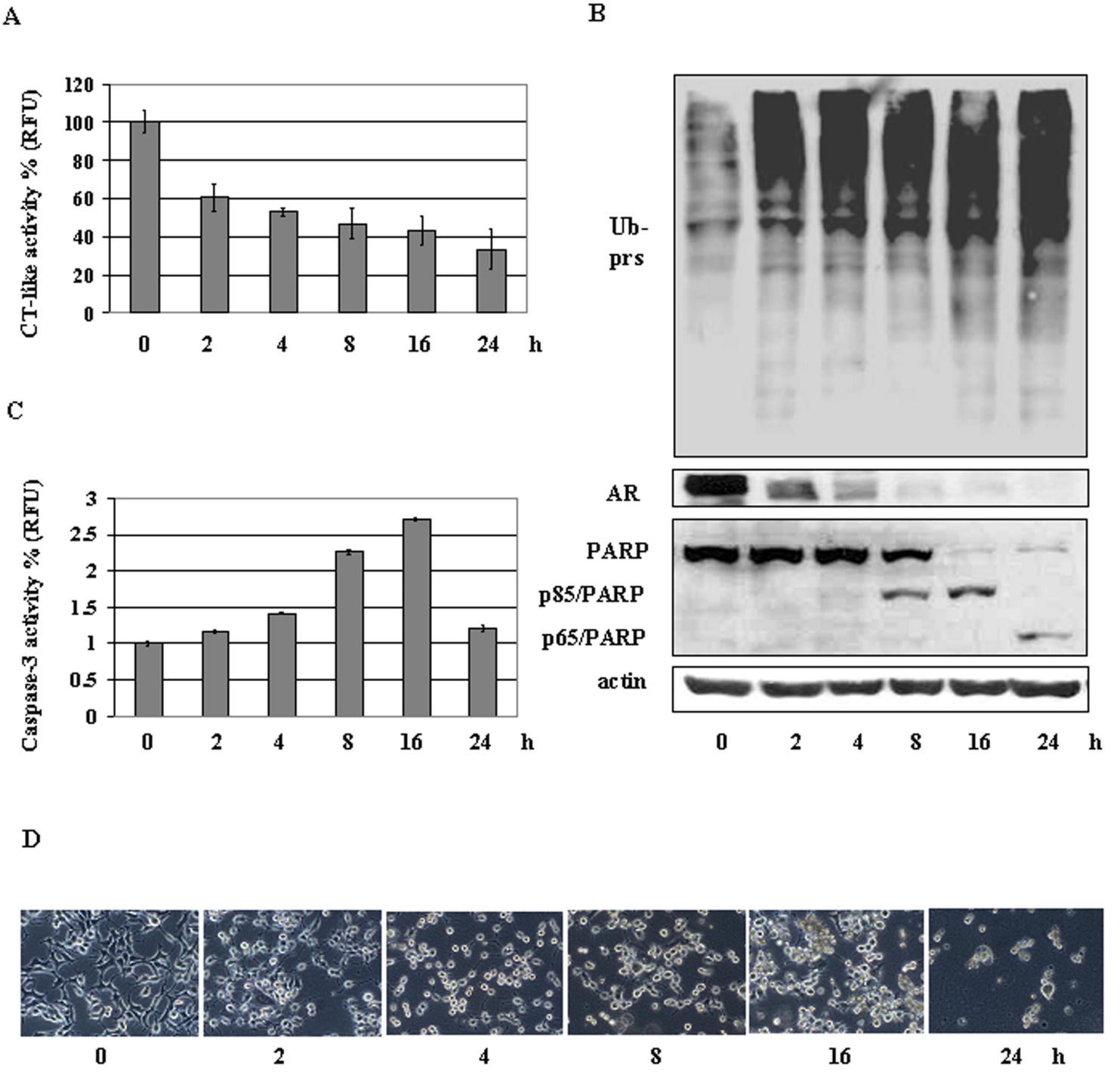

A
CT-like activity % (RFU)
120
100
80
60
40
20
0
0
2
4
8
16
24
h
B
Ub-prs
AR
PARP
p85/PARP
p65/PARP
actin
0
2
4
8
16
24
h
C
Caspase-3 activity % (RFU)
3
2.5
2
1.5
1
0.5
0
0
2
4
8
16
24
h
D
0
2
4
8
16
24
h

Fig. 5

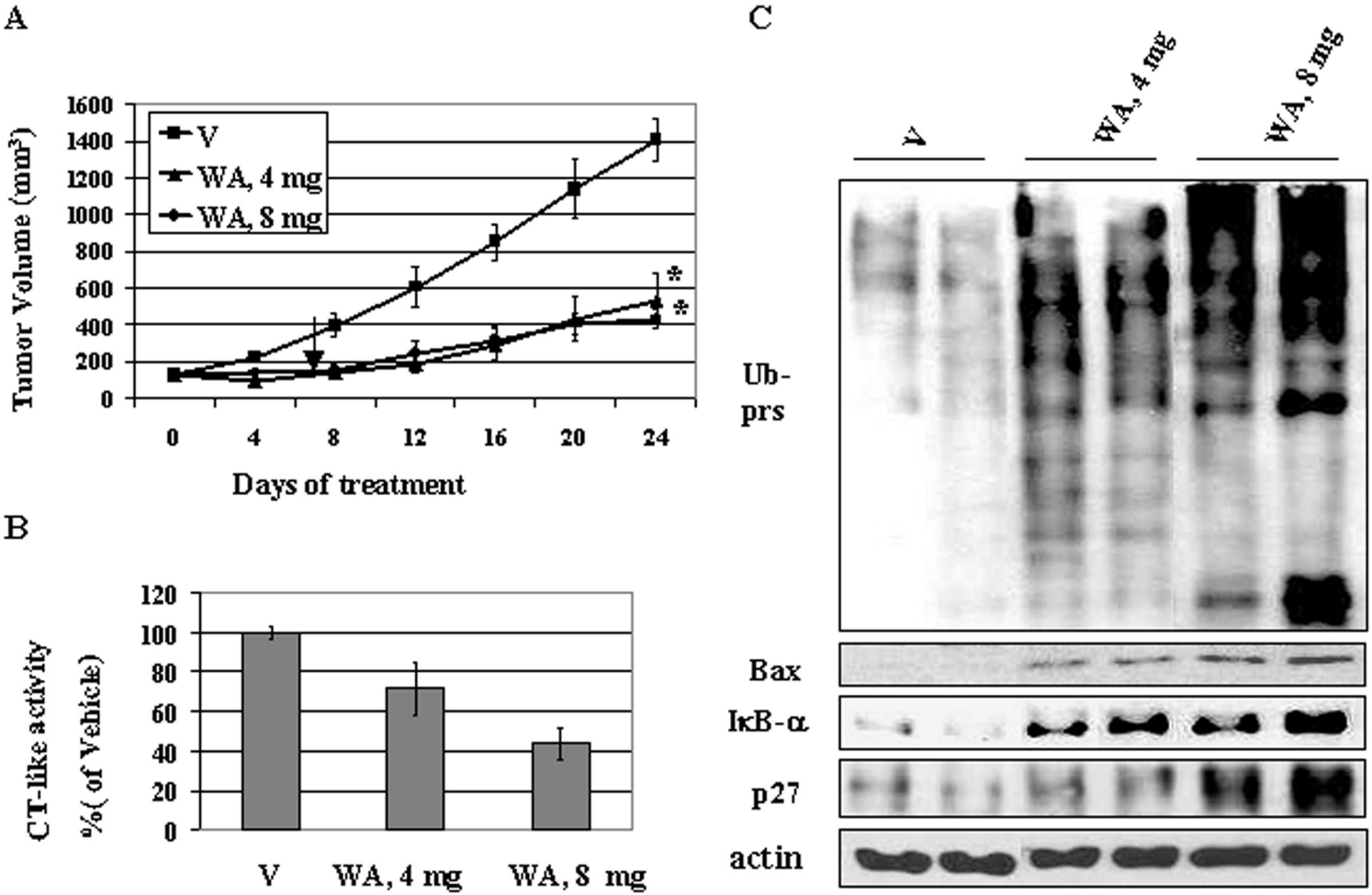

A
Tumor Volume (mm³)
1600
1400
1200
1000
800
600
400
200
0
V
WA, 4 mg
WA, 8 mg
*
*
0
4
8
12
16
20
24
Days of treatment
B
CT-like activity
%(of Vehicle)
120
100
80
60
40
20
0
V
WA, 4 mg
WA, 8 mg
C
V
WA, 4 mg
WA, 8 mg
Ub-prs
Bax
IκB-α
p27
actin

Fig. 6

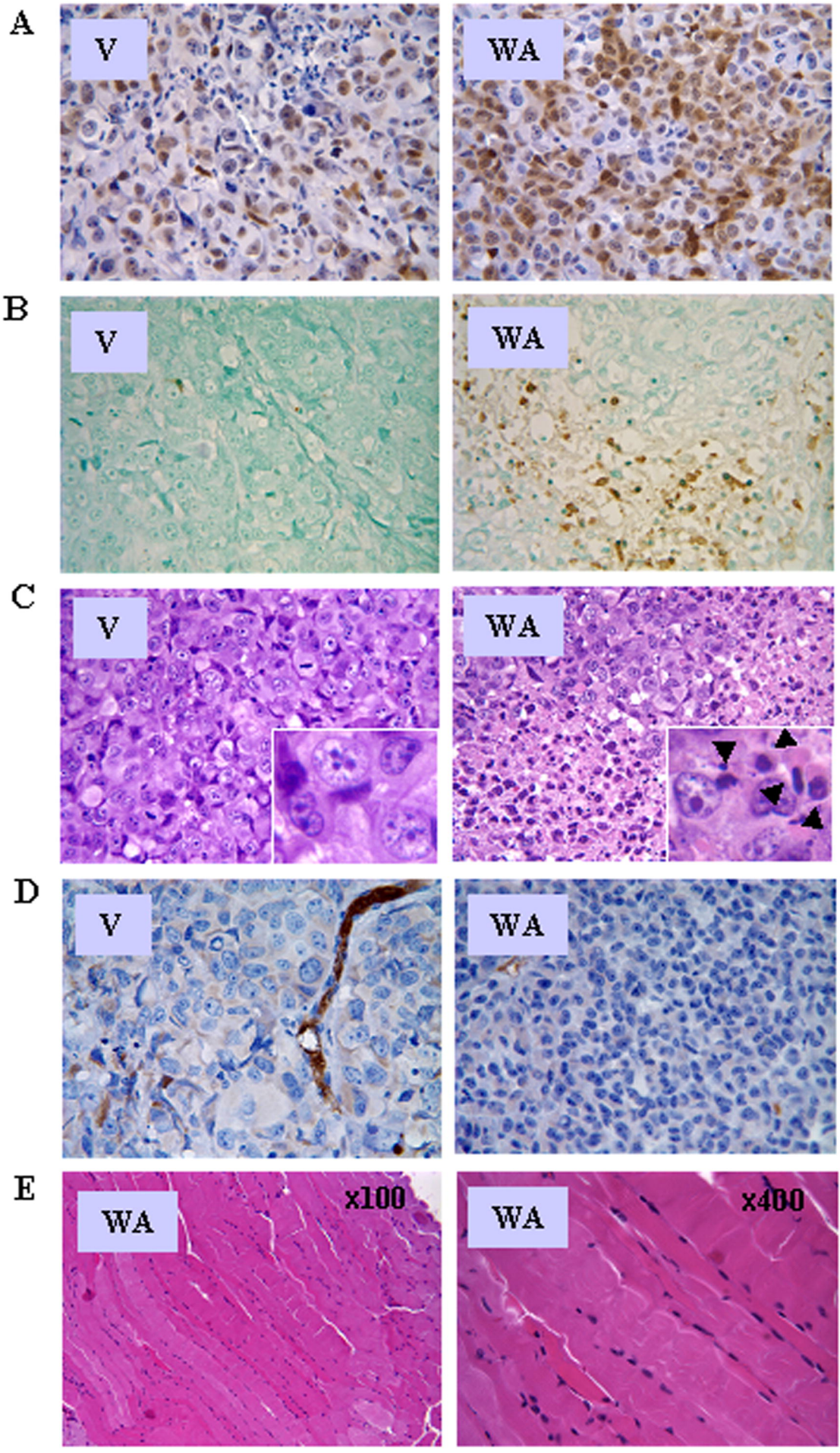


Fig. 7

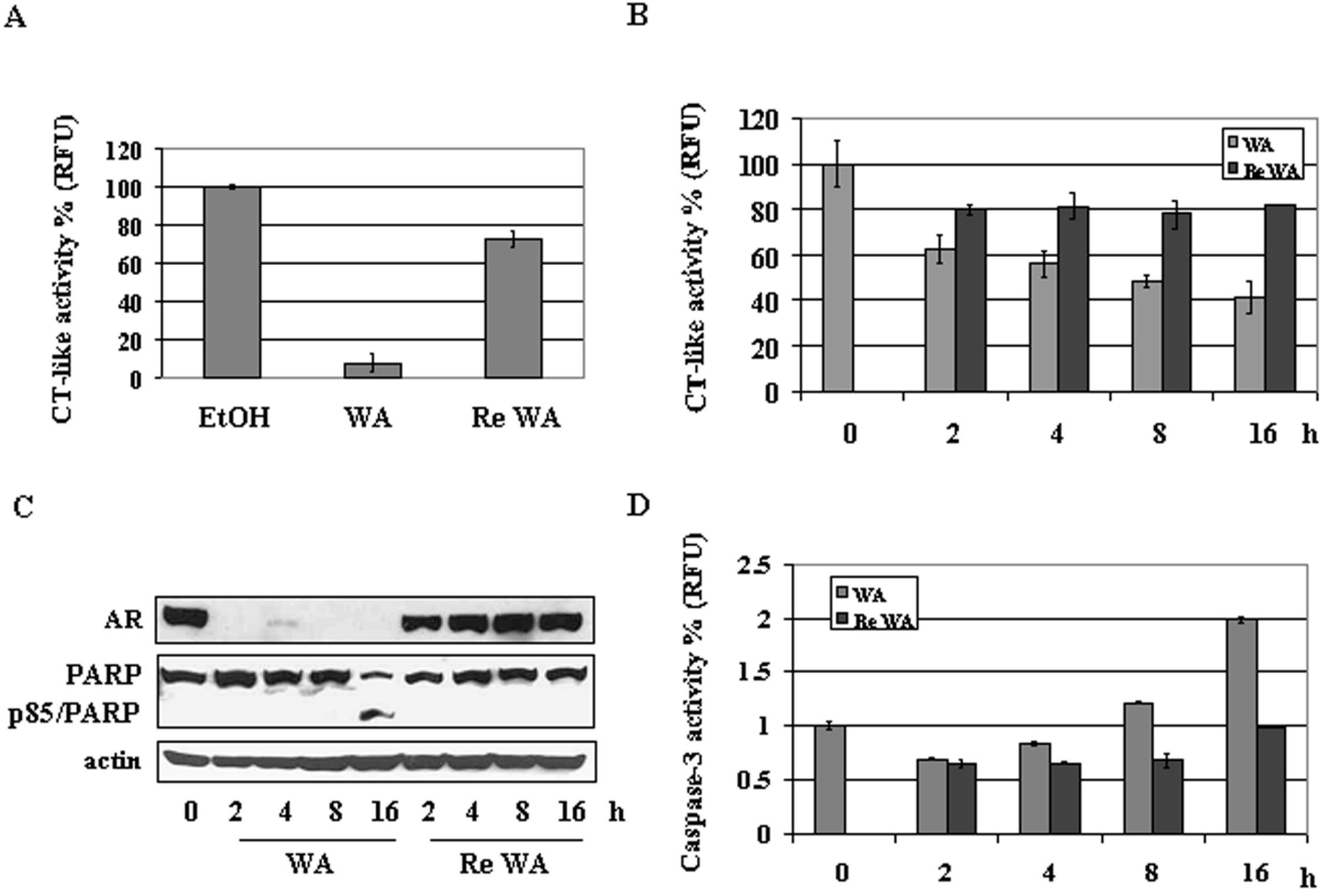

A
CT-like activity % (RFU)
120
100
80
60
40
20
0
EtOH
WA
Re WA
B
CT-like activity % (RFU)
120
100
80
60
40
20
0
WA
Re WA
0
2
4
8
16
h
C
AR
PARP
p85/PARP
actin
0
2
4
8
16
2
4
8
16
h
WA
Re WA
D
Caspase-3 activity % (RFU)
2.5
2
1.5
1
0.5
0
WA
Re WA
0
2
4
8
16
h

Fig. 8

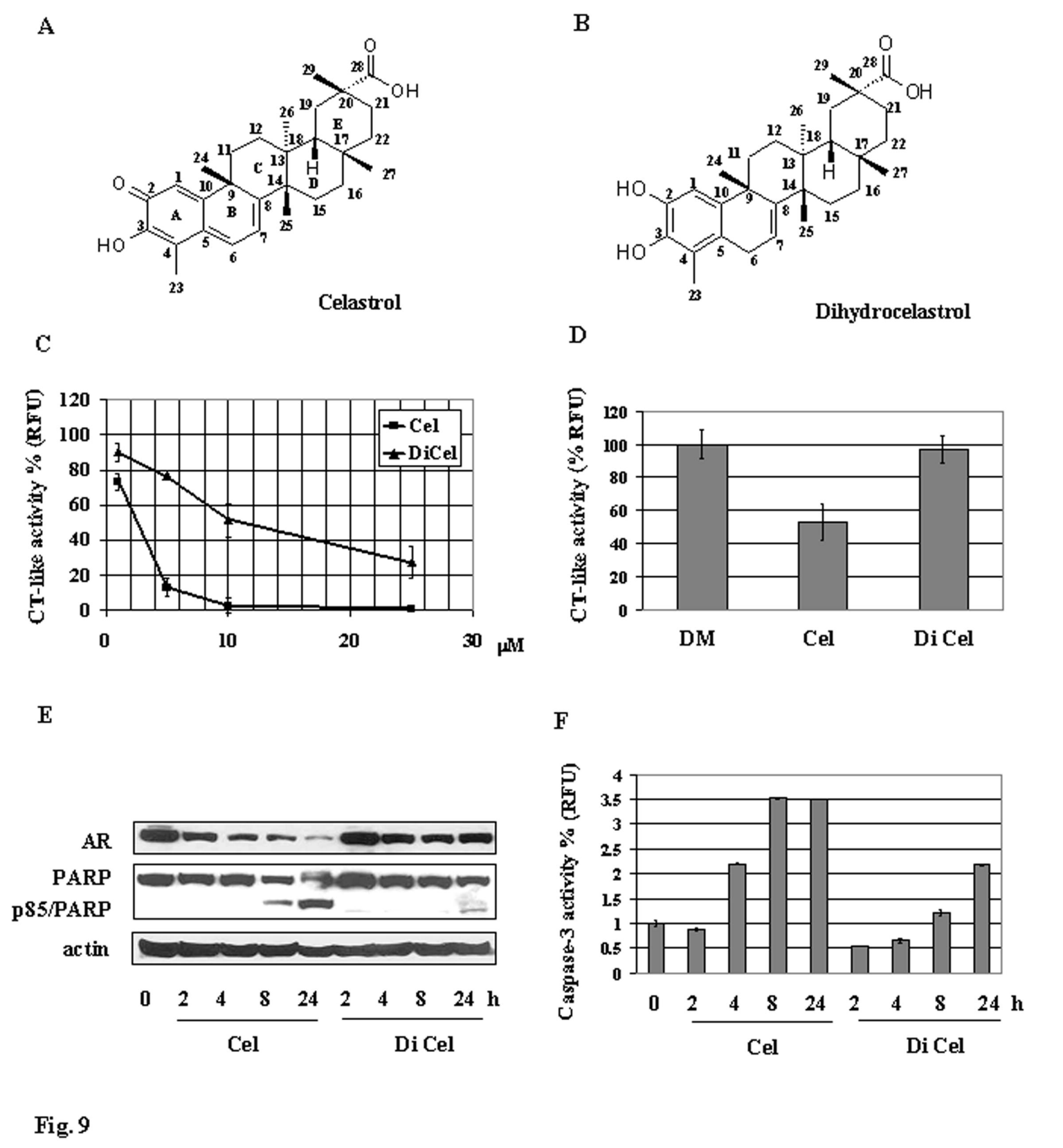
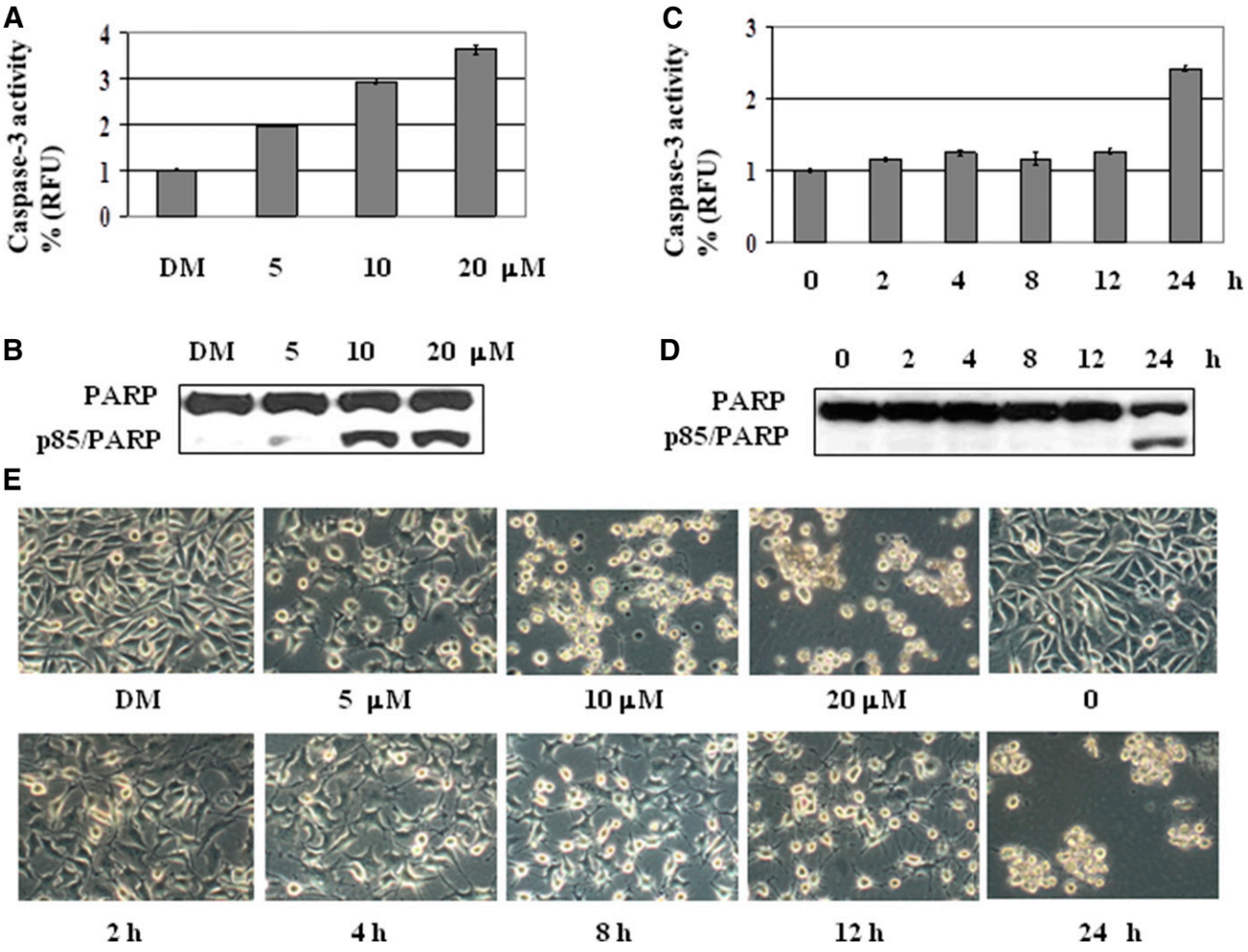

A
Celastrol
B
Dihydrocelastrol
C
CT-like activity % (RFU)
Cel
DiCel
μM
D
CT-like activity (% RFU)
DM
Cel
Di Cel
E
AR
PARP
p85/PARP
actin
h
Cel
Di Cel
F
Caspase-3 activity % (RFU)
h
Cel
Di Cel

Fig. 9

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

In the above article [Yang H, Shi G, and Dou QP (2007) *Mol Pharmacol* **71**:426–437], there is an error in the picture of the cells treated with 20 μM of *Withaferin A* in Fig. 3E, which occurred during the figure preparation. The panel for the 20 μM treatment is a duplication of the panel for the 10 μM treatment.

The corrected figure appears below.



The authors regret this error and any inconvenience it may have caused.