Inhibition of Tumor Cellular Proteasome Activity by Triptolide Extracted from the Chinese Medicinal Plant 'Thunder God Vine'

LI LU[1,2], JYOTI KANWAR[2], SARA SCHMITT[2], QIUZHI CINDY CUI[2], CHUANYIN ZHANG[1], CONG ZHAO[1] and Q. PING DOU[2]

[1]*Department of Pathophysiology, Guangzhou Medical University, Guangzhou, Guangdong 510082, China;*
[2]*The Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute, and Departments of Oncology, Pharmacology and Pathology, School of Medicine, Wayne State University, Detroit, MI 48201, U.S.A.*

Abstract. *Aims: The molecular mechanisms of triptolide responsible for its antitumor properties are not yet fully understood. The ubiquitin/proteasome system is an important pathway of protein degradation in cells. This study investigated whether triptolide may inhibit proteasomal activity and induce apoptosis in human cancer cells. Materials and Methods: In vitro proteasome inhibition was measured by incubation of a purified 20S proteasome with triptolide. Human breast and prostate cancer cell lines were also treated with different doses of triptolide for different times, followed by measurement of proteasome inhibition (levels of the chymotrypsin-like activity, ubiquitinated proteins and three well-known proteasome target proteins, p27, IκB-α and Bax) and apoptosis induction (caspase-3 activity and PARP cleavage). Results: Triptolide did not inhibit the chymotrypsin-like activity of purified 20S proteasome. However, treatment of triptolide was able to cause decreased levels of cellular proteasomal chymotrypsin-like activity and accumulation of ubiquitinated proteins and three well-known proteasome target proteins in human breast and prostate cancer cells, associated with apoptosis induction. Conclusion: It is possible that at least one of metabolites of triptolide has proteasome-inhibitory activity.*

This article is freely accessible online.

Correspondence to: Dr. Q. Ping Dou, The Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute, and Department of Oncology, School of Medicine, Wayne State University, Detroit, MI 48201, U.S.A. E-mail: doup@karmanos.org, /Dr. Li Lu, Department of Pathophysiology, Guangzhou Medical University, Guangzhou, Guangdong 510082, China. E-mail: lulili2004@hotmail.com

Key Words: Triptolide, medicinal compounds, proteasome inhibitors, apoptosis, cancer therapy.

Much of the clinical experience with the herb *T. wilfordii Hook* F (TWHF), also known as 'Lei Gong Teng' in Chinese or 'Thunder god vine', that has been used for centuries in traditional Chinese medicine, comes from recent randomized double-blind, placebo-controlled clinical trials that have confirmed the efficacy of the extracts of TWHF in the treatment of rheumatoid arthritis and several other autoimmune and inflammatory diseases, including immune complex nephritis and systemic lupus erythematosus (1-3). However, the therapeutic mechanisms of TWHF extracts remain elusive. Because of its severe toxicity, widespread medical application of this herb has been prohibited. Thus, identification of the active ingredients of this plant may facilitate the development of drugs that are highly efficacious and devoid of significant toxicity. The extracts of TWHF contain more than 70 compounds including diterpenoids, triterpenoids, sesquiterpenoids, β-sitosterol, dulcitol and glycosides (3). Triptolide ($C_{20}H_{24}O_6$), a diterpene triepoxide, which has been shown to possess potent anti-inflammatory and immunosuppressive properties, is a major component of TWHF extracts (3). It also exhibits potent antitumor and anti-leukemic activities (4, 5).

Protein degradation, which is as essential to cells as protein synthesis, occurs *via* two pathways: lysosome-mediated and ubiquitin/proteasome-mediated pathways (6, 7). The ubiquitin/proteasome-mediated pathway is known to degrade many endogenous proteins, including transcription factors, cyclins, tumor suppressor proteins and misfolded or damaged proteins (8, 9). The proteasome is the site at which protein degradation happens and two components are often referred to; the 20S particle is the catalytic core and the 26S proteasome is made of two 19S regulatory particles and a 20S core particle. The proteasome is an immense multi-subunit protease with at least three catalytic activities occurring in the 20S core, namely chymotrypsin-like, trypsin-like and caspase-like activity. The chymotrypsin-like

activity is often considered to be the rate-limiting step of protein degradation (10-12). It has been shown that tumor cells are dependent upon proteasome function, as proteasome inhibition leads to growth arrest in the G1 phase of the cell cycle and/or induction of apoptosis (13, 14). Importantly, treatment with some proteasome inhibitors in several human normal or non-transformed cell lines is not associated with induction of apoptosis (7, 15, 16).

Previously, it was reported that several natural compounds, including celastrol, quercetin and kaempferol, among others, were able to inhibit proteasomal activity (17-19). It was 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 (17, 18). It was noticed that triptolide might form ketones under oxidizing conditions (17). Furthermore, several recent studies reported that triptolide is able to inhibit activation of NF-κB (20, 21), one of the main target proteins of the proteasome. Therefore it was hypothesized that triptolide might act as a tumor cellular proteasome-inhibitor and consequently induce cancer cell death. This study reports, for the first time, that triptolide inhibits the proteasomal chymotrypsin-like activity in a dose- and time-dependent manner in cultured cancer cells. This proteasomal inhibition is associated with apoptotic induction. However, triptolide did not inhibit the purified 20S proteasome activity. When the possibility of triptolide being a proteasome-inhibitor in an *in silico* model was analysed, it was found to be susceptible to nucleophilic attack, but there were two bonds that may block its binding with the proteasome. These data suggest that it is at least one of the metabolites of triptolide that is responsible for its proteasome-inhibitory and apoptosis-inducing activities. It is, therefore, suggested that triptolide may act as a proteasome-inhibitor prodrug.

Materials and Methods

Reagents and antibodies. Triptolide was obtained from A.G. Scientific, Inc. (San Diego, CA, USA). For all experiments, triptolide was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 50 mM and aliquots were kept at –20˚C. RPMI-1640, DMEM/F-12, penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Aleken Biologicals (Nash, TX, U.S.A.). Bisbenzimide, methylene blue, 3-[4,5-dimethyltiazol-2-yl]-2.5-diphenyl-tetrazolium bromide(MTT), DMSO, RNase A, protease-inhibitor cocktail and other chemicals were obtained from Sigma-Aldrich. Purified rabbit 20S proteasome and fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like) and caspase-3 specific sbustrate Ac-Asp-Glu-Val-Asp-AMC were obtained from Calbiochem Inc. (San Diego, CA, USA). Mouse monoclonal antibody against human poly (ADP-ribose) polymerase (PARP) was obtained from BD Biosciences Pharmingen (San Diego, CA, USA). Mouse monoclonal antibodies against Bax (B-9), p27 (F-8), and ubiquitin (P4D1), rabbit polyclonal antibody against IκB-α (C-15) and goat polyclonal antibody against β-actin (C-11), as well as secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and whole cell extract preparation. Human breast cancer MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and grown in DMEM/F-12 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Human prostate cancer PC-3 cells, also obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), were cultured in RPMI-1640 medium, supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were maintained at 37˚C with 5% CO_2. A whole-cell extract was prepared, as described previously (22). Briefly, cells were harvested, washed with PBS and homogenized in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol) for 30 min at 4˚C. Afterwards, the lysates were centrifuged at 12,000 g for 12 min at 4˚C and the supernatants were collected as whole-cell extracts. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin standards.

MTT assay and colony formation. MTT assays were performed in 96-well plates and were plated with 3.5×10^3 cells per well for PC-3 cells and 5×10^3 cells per well for MDA-MB-231 cells. After complete cell adherence, cells were treated with triptolide at the concentrations indicated for 48 h. Inhibition of cell proliferation was measured using the MTT method, as described in detail elsewhere (22). Absorbance was measured using a Wallac Victor3TM multilabel counter (PerkinElmer, Waltham, MA, USA) at a spectrophotometer of 540 nm, and viability was expressed relative to the control. In individual experiments, each treatment condition was set up in quadruplicate and each experiment was repeated one to five times independently. Colony formation assays were performed in 6-well plates and cells were seeded into plates at 350 cells per well. After complete cell adherence, the cells were treated with triptolide at the concentrations indicated or with DMSO for 24 h (in triplicate). The drugs were subsequently removed and the cells were kept in culture by refreshing growth medium every two days, during which time the surviving cells produced a colony of proliferating cells. Colonies were visualized by staining for 4 h with 1% methylene blue (in methanol) and images were taken on day 10.

Nucleophilic susceptibility analysis. A CAChe workstation (Fujitsu, Fairfield, NJ, USA) was used for the construction of chemical structures. After being constructed, the molecules were subjected to geometry optimization using PM5 geometry in water as the parameter. Nucleophilic susceptibility analysis was determined by using PM5 geometry and PM5 wave function in water and saved in PDB format using CAChe conversion filters. Surface analysis was also performed. A colored 'bull's-eye' with a red center denotes atoms that are highly susceptible to nucleophilic attack.

Inhibition of purified 20S proteasome activity. Purified rabbit 20S proteasome (0.1 μg) was incubated with 40 μmol/l of various fluorogenic peptide substrates in 100 μl assay buffer (20 mmol/l Tris-HCl; pH 7.5), in the presence of triptolide or Velcade at different concentrations or in the solvent, DMSO, for 2 h at 37˚C. After incubation, production of hydrolyzed AMC groups was measured using a Wallac Victor3TM multilabel counter (PerkinElmer, Waltham,

MA, USA) with an excitation filter of 355 nm and an emission filter of 460 nm, as described previously (23).

Inhibition of proteasomal chymotrypsin-like activity in intact cells. Cells (5.0-8.0x10^3) were plated in each well of a 96-well plate and treated with either DMSO or triptolide at different concentrations for 8 h, or treated with triptolide for different time periods as indicated and incubated an additional 2 h with Z-Gly-Gly-Leu-AMC (at 40 μmol/l). Subsequently, hydrolyzed AMC group production was measured, as previously described (24).

Caspase-3 activity assay and Western blot analysis. Cell-free caspase-3 activities were determined by measuring the release of the AMC groups from a caspase-3 specific substrate, Ac-Asp-Glu-Val-Asp-AMC. Additionally, cells were treated with triptolide at different concentrations or DMSO for different time periods as indicated in the figure legends, followed by preparation of whole cell extracts. Subsequently, the cell extract (25 mg) was incubated in 100 μl of the assay buffer (50 mM Tris–HCl, pH 7.5) along with 40 mM caspase-3 substrate in a 96-well plate. The reaction mixture was incubated at 37˚C for 2 h and the hydrolyzed fluorescent AMC groups were quantified as previously described. Western blot assays using enhanced chemiluminescence reagent were done, as previously described (24).

Statistical analysis. Data are expressed as mean±standard deviation. Student's *t*-test was applied to evaluate the differences between treated groups and controls. Data from multiple groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison. For all the tests, the level of $p<0.05$ was considered statistically significant.

Results

Inhibition of cell viability and colony formation in both PC-3 cells and MDA-MB-231 cells by triptolide. Triptolide was first characterized as a diterpenoid triepoxide lactone in 1972 (25). Since then, a large body of literature has demonstrated that triptolide at low concentrations can inhibit growth of different types of cancer cells, including those of hematologic malignancies and some solid tumors (4, 5, 26). To understand the involved molecular mechanisms, it was first investigated whether triptolide affects cell growth and survival of two selected cancer cell lines, PC-3 (prostate cancer) and MDA-MB-231 (breast cancer). MTT assays using both cell lines were performed to verify whether triptolide affects cell growth. Cells were treated with different concentrations of triptolide for 48 h, with DMSO as a control. The results revealed that triptolide at a concentration of 25 nM significantly inhibited cell viability. At a concentration of 50 nM triptolide inhibited nearly 50% of cell viability in both cell lines within 48 h (Figure 1A, B). MDA-MB-231 cells appeared to be more sensitive to high concentrations of triptolide than PC-3.

Both cell lines were also treated with different concentrations of triptolide for 24 h and the number of long-term surviving cells (ten days) was determined by colony-formation assay. As shown in Figure 1C, D, triptolide significantly inhibited colony formation in both cell lines at concentration as low as 12.5 nM. There were nearly no colonies formed when cells were treated with triptolide at a concentration of 50 nM.

There are two bonds to hinder triptolide binding to and inhibiting the proteasome as shown in an in silico model. Previously, it was reported that some natural compounds, such as celastrol, quercetin and kaempferol have proteasome-inhibitory activities and that their aromatic ketone structure may play a direct role in this inhibition (16-19). Triptolide (Figure 2A) is a triterpenoid, not a flavonoid. Components of terpenoids that may inhibit the proteasome have never been reported before. Among the many small molecules extracted and purified from TWHF, triptolide is the key biologically active component that mediates immunosuppression and anti-inflammation. Furthermore, several studies have reported that triptolide is a potent inhibitor of NF-κB, one of the main targets of the proteasome. It is known that most proteasome inhibitors have immunosuppressive and anti-inflammatory effects. From these data, it was, therefore, hypothesized that triptolide may act as a proteasome inhibitor in tumor cells. To test this hypothesis, a computational electron density analysis was first performed for the triptolide molecule to speculate whether it can bind and inhibit the β5 subunit of proteasome in an *in silico* model. The computational molecular modeling was performed by using CAChe for triptolide. The susceptibility to a nucleophilic attack was generated by a MOPAC/PM5 wave function for the chemical sample, at a geometry determined by performing an optimized geometry calculation in MOPAC using PM5 parameters and including solvation effects of water simulated by COSMO. The results showed that carbonyl carbon and oxygen at the 12th and 13th positions are highly susceptible to nucleophilic attack forming a 'bull's-eye' with a red center in both structures (Figure 2B). While optimizing the geometry, two additional bonds were formed that may hinder triptolide binding and inhibiting the 20S proteasome as shown in the *in silico* model (Figure 2B).

Triptolide has no inhibitory effect on purified 20S proteasomal activity. Because the chemical structure analysis of triptolide did not provide conclusive evidence of its proteasome-inhibitory activities, a cell-free proteasome activity assay was subsequently performed with purified rabbit 20S proteasome in the presence of triptolide at different concentrations to determine whether triptolide inhibits the proteasomal activity. There were no changes in the activity of purified 20S proteasome caused by triptolide at concentrations up to 200 nM (Figure 2C). Furthermore, Velcade was used as a positive control, and as shown in Figure 2D, Velcade inhibited the activity of purified 20S proteasome by approximately 50% at 5 nM, while the

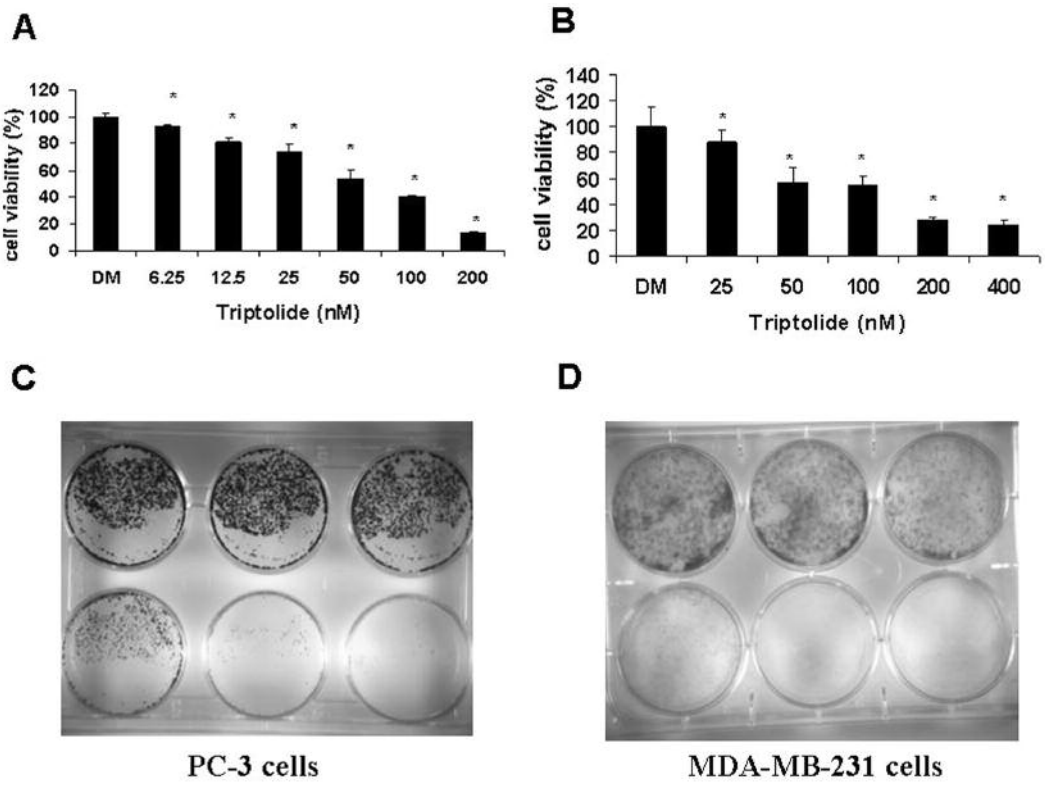

Figure 1. *Dose effects of triptolide on cell viability (A, B) and colony formation (C, D). PC-3 cells (A, C) and MDA-MB-231 cells (B, D) were treated with either solvent DMSO(DM) or different concentrations of triptolide for 48 h (horizontal axis), followed by measurement of cell viability (vertical axis) via MTT assay (A, B) or the number of long-term surviving cells (10 days) determined by colony formation assays (C, D; left to right, top row: DM, 3.13 and 6.25 nM triptolide, bottom row: 12.5, 25 and 50 nM triptolide). Images were taken of representative 6-well plates. *p<0.05. Data are expressed as mean±standard deviation of three experiments.*

inhibition reached approximately 70% at 10 nM. An MTT assay was also performed to determine cell viability and it was observed that Velcade at 10 nM was able to kill 60% of PC-3 cells and MDA-MB-231 cells (data not shown). However, triptolide at 50 nM killed approximately 50% of PC-3 cells and MDA-MB-231 cells (Figure 1A, B), but had no effect on purified 20S proteasome. These data suggest that triptolide cannot inhibit the proteasome in a cell-free system.

Triptolide inhibits proteasome and induces apoptosis in PC-3 cells in a time-dependent manner. Although docking and cell-free analyses showed that triptolide was ineffective against the proteasome, the present study aimed to determine whether it was able to inhibit 26S proteasome in intact PC-3 prostate cancer cells. First, PC-3 cells were treated with 100 nM triptolide in a 96-well plate, followed by measurement of proteasome activity by the cellular proteasomal chymotrypsin-like activity assay at different time-points. Surprisingly, the results showed that triptolide significantly inhibited the proteasomal chymotrypsin-like activity in PC-3 cells in a time-dependent manner (Figure 3A). After only 2-h treatment with 100 nM triptolide, proteasome activity decreased by approximately ~50% compared to that of control cells treated with DMSO (Figure 3A). Inhibition increased to approximately 80% after 12-h treatment. Kinetic experiments were subsequently performed on PC-3 cells to observe whether triptolide caused accumulation of ubiquitinated proteins and three well-known target proteins of the proteasome (IκB-α, Bax and p27) in cells. Consistently, ubiquitinated proteins accumulated as early as 1 h after treatment with triptolide and further accumulated in a time-dependent manner were compared to cells treated with DMSO for 8 h (Figure 3C). There was an increase in the ubiquitinated IκB-α band at 56 kD after 1-h treatment with triptolide which remained high afterwards. The level of Bax protein also began increasing after 1-h treatment with triptolide and further increased in a similar manner as ubiquitinated proteins (Figure 3C). The expression of p27 protein remained stable until 4-h but increased significantly after 8-h treatment with triptolide (Figure 3C).

It has been shown that inhibition of tumor cellular proteasome activity is associated with apoptosis induction (17, 18, 22, 23). Following determination of proteasome activity,

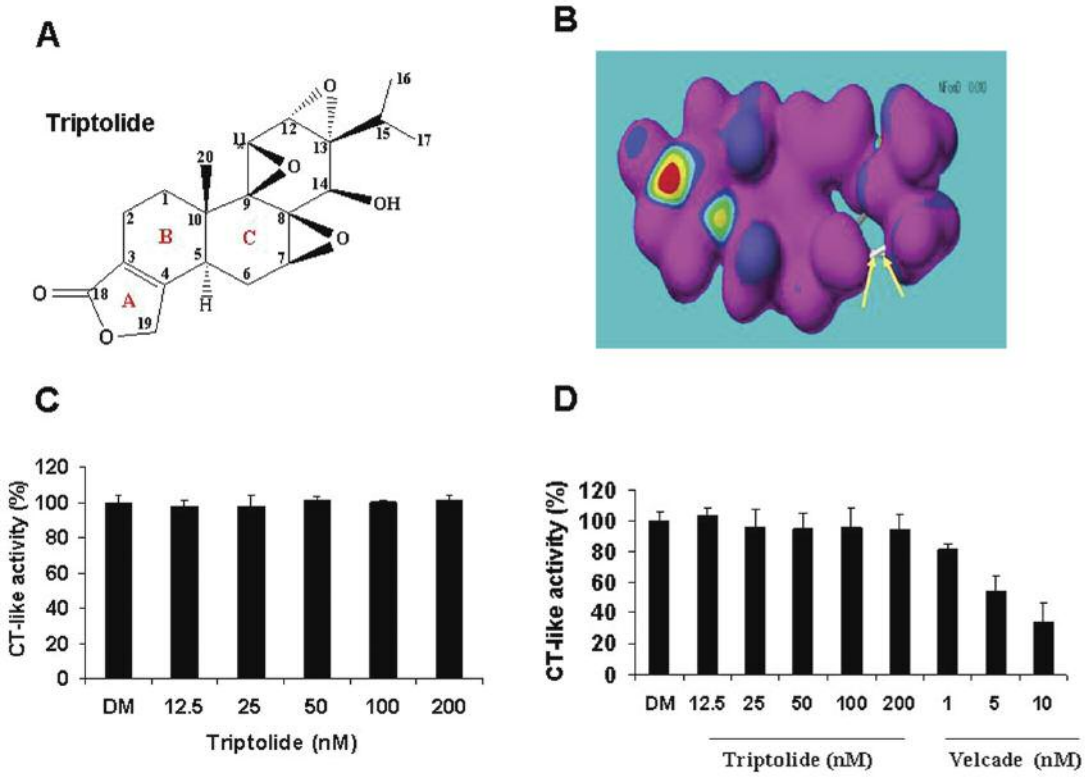

Figure 2. *The effect of triptolide on the chymotrypsin (CT)-like activity of a purified rabbit 20S proteasome. The chemical structure of triptolide is shown in A. The nucleophilic susceptibility of triptolide was analyzed by using CAChe software (B). There are two bonds to block the docking of triptolide to the β5 subunit of proteasome. To determine whether triptolide is able to inhibit the proteasome activity directly, a purified rabbit 20S proteasome was incubated with different concentrations of triptolide (C, D) or solvent DMSO (DM) (C, D) or three different doses of Velcade as positive controls (D). *p<0.05; Data are expressed as mean±standard deviation of three experiments.*

apoptotic cell death was estimated by measuring caspase-3 activity levels, PARP cleavage and cellular apoptotic morphologic changes (condensation and fragmentation) from 1 to 36 h after addition of triptolide in PC-3 cells. As shown in Figure 3B, triptolide induced caspase-3 activity by 2-fold at 8 h and 4-12-fold from 12 to 36 h compared to cells treated with DMSO for 8 h. Consistently, cell death was observed from 12 h after treatment with triptolide (data not shown). p85/PARP fragments were also detected at 12 h after triptolide were applied. Almost 40% PARP protein was cleaved in cells treated with 800 nM triptolide at 36 h (Figure 3C). These data suggest that triptolide inhibits proteasome activity and induces apoptosis of PC-3 cells in a time-dependent manner.

Triptolide inhibits proteasome and induces apoptosis in PC-3 cells in a dose-dependent manner. As triptolide inhibited almost 75% of proteasomal chymotrypsin-like activity in PC-3 cells at 4 h, the study examined the dose effects of triptolide on PC-3 cells with different concentrations of triptolide for 4 h in 96-well plates. As shown in Figure 4A, even at a concentration of 12.5 nM, triptolide inhibited 40% of chymotrypsin-like activity and the inhibition reached approximately 70% at a concentration of 100 nM, which was consistent with the results of the kinetic experiments (Figure 3A, 4A). Proteasomal target protein levels were measured by Western blotting. Consistently, 50 nM triptolide caused significant accumulation of levels of ubiquitinated proteins in a dose-dependent manner. Additionally, IκB-α decreased and ubiquitinated IκB increased in a dose-dependent manner (Figure 4C). Levels of p27 increased at triptolide concentrations up to 100 nM (Figure 4C). A slightly increased ubiquitinated Bax and increasing Bax expression was also observed in PC-3 cells. These data support the conclusion that triptolide inhibits cellular proteasome activity in intact PC-3 cells.

To observe the dose effects of triptolide on apoptosis induction, caspase-3 activity was measured. Cells treated with triptolide at a concentration of 50 nM showed a significant increase in caspase-3 activity compared with DMSO-treated cells and the increase occurred in a dose-dependent manner (Figure 4B). The increase reached 12-fold when treated with triptolide at a concentration of 400 nM and the p85/PARP fragments were also detected at 400 nM (Figure 4B, C). Cellular apoptotic

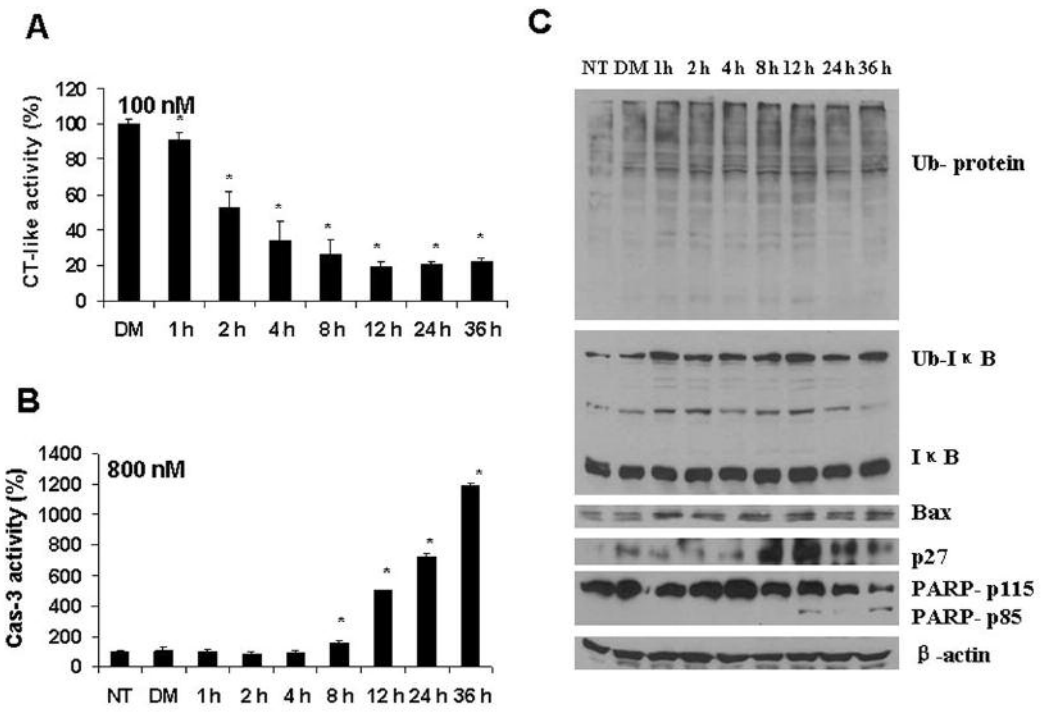

Figure 3. *Kinetic effects of triptolide on proteasome inhibition and apoptosis induction of PC-3 cells. 50 nM triptolide was added to 96-well plates at the indicated times, followed by measuring inhibition of the proteasomal chymotrypsin (CT)-like activity using Z-GGL-AMC (A). PC-3 cells were either untreated (NT) or treated with DMSO (DM) for 8 hr or 50 nM or 800 nM of triptolide for the indicated times, followed by measurement of caspase-3 (Cas-3) activity (B) and of the levels of ubiquitinated proteins, IκB, Bax, p27, PARP and β-actin (C) by Western blot assay using specific antibodies. *p<0.05. Data are expressed as mean±standard deviation of three experiments.*

morphologic changes were also obvious after 18-h treatment with a concentration of 400 nM (data not shown).

Triptolide inhibits proteasome and induces apoptosis in MDA-MB-231 cells in a time-dependent manner. To determine whether the proteasome-inhibitory and apoptosis-inducing effects of triptolide are specific to PC-3 cells or are general to cancer cells, MDA-MB-231 breast cancer cells were treated with triptolide. First, kinetic experiments were performed with 50 nM triptolide on MDA-MB-231cells. The results showed that triptolide significantly inhibits the proteasomal chymotrypsin-like activity in MDA-MB-231 from 0.5 h up to 12 h and the inhibition occurred in a time-dependent manner (Figure 5A). Also with the treatment of 50 nM triptolide, the levels of proteasomal target proteins, measured by Western blotting, increased in a time-dependent manner. Ubiquitinated proteins and ubiquitinated form of IκB-α began to increase after 1 h and Bax increased significantly after 4 h (Figure 5C). Also, p27 increased 4-fold after 4-h treatment with triptolide (Figure 5C).

The kinetic effects of triptolide on apoptosis induction were also determined by measuring caspase-3 activity levels, PARP cleavage and cellular apoptotic morphologic changes. Caspase-3 activity increased slightly after 2 h, approximately 2-fold at 8 h, 4.3-fold at 12 h and reached a maximum of approximately 8.9-fold at 36 h (Figure 5B) with the treatment of 400 nM triptolide. p85/PARP fragments were clearly detected up to 12-h treatment with 400 nM triptolide (Figure 5C) and cellular apoptotic morphologic changes came forth after 12 h (data not shown). In both MDA-MB-231 and PC-3 cell lines, the levels of ubiquitinated proteins began increasing as early as 1 h after treatment with 50 nM triptolide and cell death induction occurred after 12 h, as shown in Figures 3 and 5. These data confirm that proteasome inhibition occurs prior to cell death induction.

Triptolide inhibits proteasome and induces apoptosis in MDA-MB-231 cells in a dose-dependent manner. To confirm triptolide inhibits proteasome activity and induces apoptosis in tumor cells in a dose-dependent manner, the dose effects of triptolide on MDA-MB-231cells were examined. At a concentration of 6.25 nM, triptolide inhibited approximately 50% of chymotrypsin-like activity and at 25 nM it inhibited almost 70% of chymotrypsin-like activity compared with DMSO (Figure 6A). The inhibiting effect seemed to plateau at a concentration of 25 nM. As shown in Figure 6C,

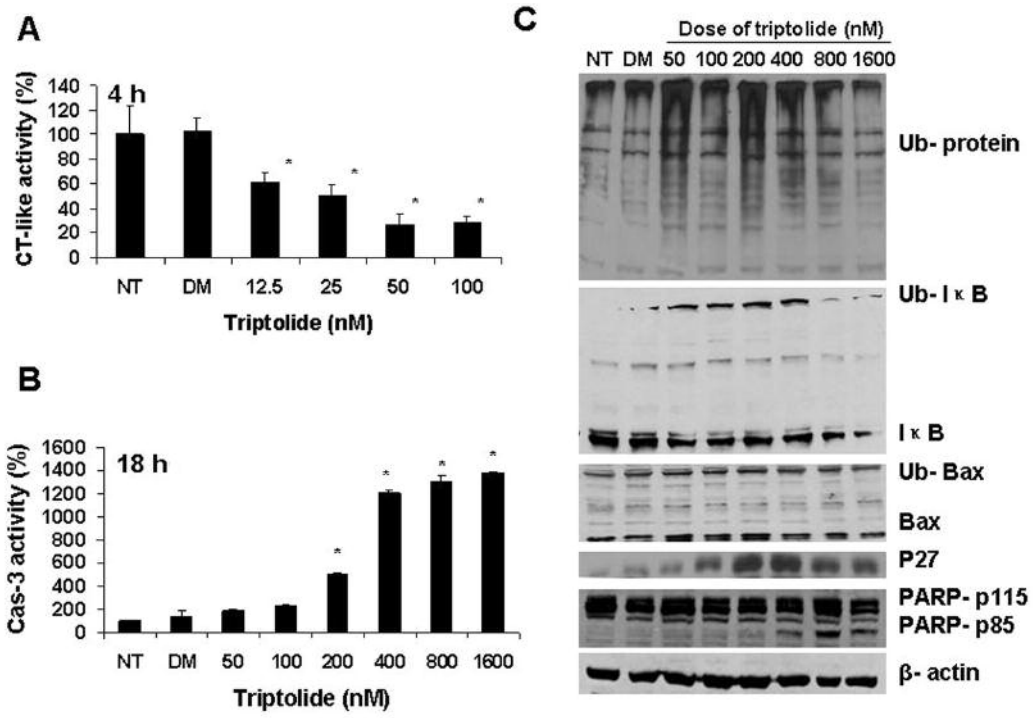

Figure 4. *Dose effects of triptolide on proteasome inhibition and apoptosis induction of PC-3 cells. PC-3 cells were either not treated (NT) or treated with solvent DMSO (DM) or different concentrations of triptolide for 4 h, followed by measuement of inhibition of the proteasomal chymotrypsin (CT)-like activity (A) or for 18 h, followed by measurement of caspase-3 (Cas-3) activity (B) and of the levels of ubiquitinated proteins (4 h), IκB, Bax, p27, PARP and β-actin (18 h) (C) by Western blot assay using specific antibodies. *p<0.05. Data are expressed as mean±standard deviation of three experiments.*

ubiquitinated proteins and ubiquitinated IκB-α, as well as p27, accumulated significantly in a dose-dependent manner. Bax increased initially and then decreased and there were ubiquitinated Bax appearing after treatment with different concentrations of triptolide (Figure 6C).

Importantly, caspase-3 activity increased significantly at a triptolide concentration of 6.25 nM and at 50 nM, while caspase-3 activity in MDA-MB-231 cells increased nearly 6-fold compared to cells treated with DMSO (Figure 6B). P85/PARP fragments were also detected at 50 nM after 24-h treatment of MDA-MB-231 cells (Figure 6C). These data show that MDA-MB-231 cells are more sensitive to triptolide than PC-3 cells.

Discussion

Proteasome inhibitors represent a novel class of anticancer drugs. Bortezomib (PS-341), the first proteasome inhibitor approved by the FDA in clinical trials, has been demonstrated to cause tumor cell death *in vivo*, consistent with the hypothesis that inhibition of the proteasome causes induction of tumor cell death (27). Triptolide is a small (molecular weight, 360), diterpenoid triepoxide, originally purified from the Chinese herb TWHF. It has well-documented use in the treatment of rheumatoid arthritis as a result of its anti-inflammatory and immunosuppressive effects (1-3). In addition, triptolide has been reported to inhibit proliferation and induce apoptosis of cancer cells *in vitro* and suppress the growth and metastasis of tumors. However, the mechanism of triptolide on tumor proliferation and invasion still remain unclear. The present study showed that triptolide inhibits cell proliferation and colony formation and induces apoptosis in two tumor cell lines. Kinetic and dose-effect studies showed that triptolide inhibits the proteasome in both PC-3 and MDA-MB-231 cancer cell lines, associated with cell-death induction. It was also shown that triptolide exposure causes elevations in caspase-3 activity, indicating that triptolide-mediated cell death occurs through an apoptotic pathway. Though many previous studies reported that triptolide induces cancer cell apoptosis, this is the first study showing that triptolide is capable of suppressing tumor proteasome activity, associated with cancer cell death. It has also been reported that triptolide-induced cell death is associated with autophagy (5); however, whether autophagy induction is associated with proteasome inhibition by triptolide remains to be investigated.

The ubiquitin-proteasome pathway is known to degrade many endogenous proteins, including transcription factors, cyclins, tumor suppressor proteins and misfolded or damaged

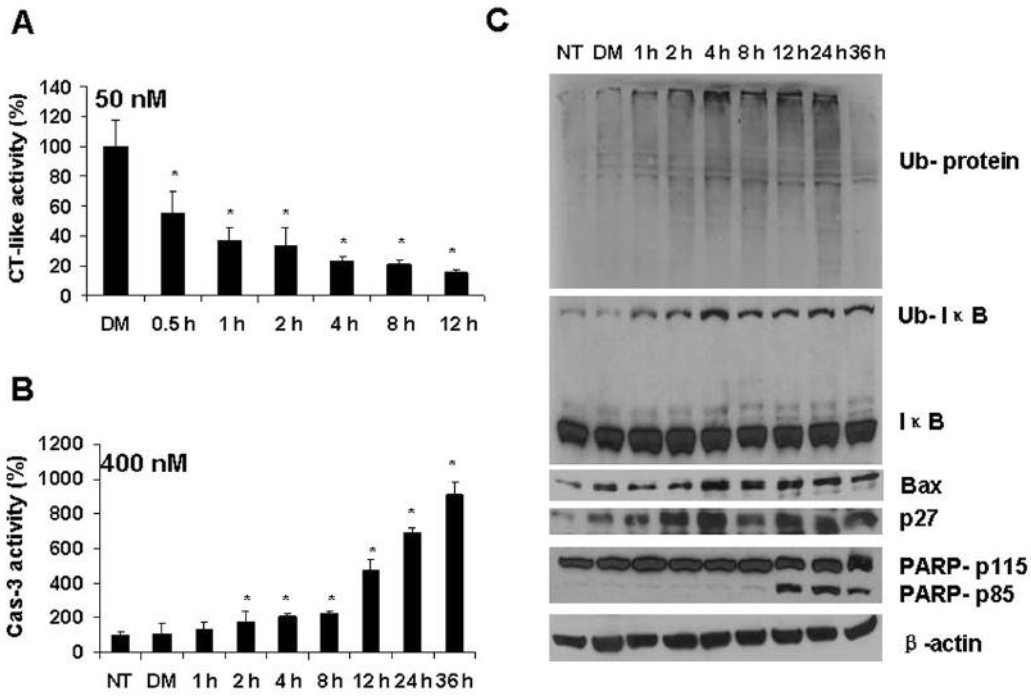

Figure 5. *Kinetic effects of triptolide on proteasome inhibition and apoptosis induction of MDA-MB-231 cells. A total of 50 nM triptolide were added to 96-well plates at the indicated times, followed by measurement of inhibition of the proteasomal chymotrypsin (CT)-like activity using Z-GGL-AMC (A). MDA-MB-231 cells were either not treated (NT) or treated with solvent DMSO (DM) or triptolide (50 or 800 nM) for the indicated times, followed by measurement of caspase-3 (Cas-3) activity (B) and of the levels of ubiquitinated proteins, IκB, Bax, p27, PARP and β-actin (C) by Western blot assay using specific antibodies. * $p < 0.05$. Data are expressed as mean ± standard deviation of three experiments.*

proteins. Most studies have shown that inhibition of the proteasomal chymotrypsin–like activity results in the accumulation of several target proteins, such as IκB-α, Bax and p27 and the induction of apoptosis in various types of tumor cells (11, 14-18). Until recently, several studies emphasized the inhibitory effect of triptolide on NF-κB activation, since this is critical for both inflammation and tumorigenesis. The antiproliferative effects of triptolide have also been reported to be associated with down-regulation of NF-κB activity (20, 28). It was found that triptolide inhibits transactivation of NF-κB in MDA-MB-231 breast cancer cells (29). Although the precise molecular targets remain elusive, it has been proposed that triptolide inhibits NF-κB activity after NF-κB binding to DNA (29). However it is also reported that triptolide pretreatment neither affects the phosphorylation-mediated degradation of IκB-α after LPS stimulation nor decreases the DNA-binding activity of NF-κB in cell nuclear (28). These observations are in agreement with the findings that DNA binding activity of NF-κB is not affected by triptolide. These investigators have postulated that triptolide blocks NF-κB-mediated transcription by interfering with p65 modification or the recruitment of a transcriptional cofactor. Additionally, recent studies in cancer cells have revealed that RNA polymerase may be an important target of triptolide (4). The present study found that triptolide causes the accumulation of ubiquitinated form of IκB. It is well known that, following IKK activation by various stimuli, IκB proteins are rapidly phosphorylated, polyubiquitinated and, subsequently, degraded by the proteasome (9). If degradation of IκB-α is delayed in the presence of a proteasome inhibitor, NF-κB cannot be activated by exotic and translocated to nuclear. Consequently, inflammatory cytokines cannot be synthesized. This may be an important mechanism for triptolide-mediated anti-inflammatory and antitumor activities.

Several known proteasome inhibitors, such as bortezomib/Velcade (a peptide boronate), lactacystin and peptide aldehydes (MG132, MG115), target catalytic residues for proteolysis in the 20S proteasome catalytic core particle. TRIP1/S8 and possibly other ATPase subunits in the 19S regulatory complex may be useful additional targets for inhibition of the proteasome activity (6, 7). Though the present study found that triptolide does not inhibit the chymotrypsin-like activity in tumor cells and cause accumulation of ubiquitinated proteins, surprisingly it did not inhibit purified 20S proteasome activity. Furthermore, the results of nucleophilic susceptibility analysis showed that the carbonyl

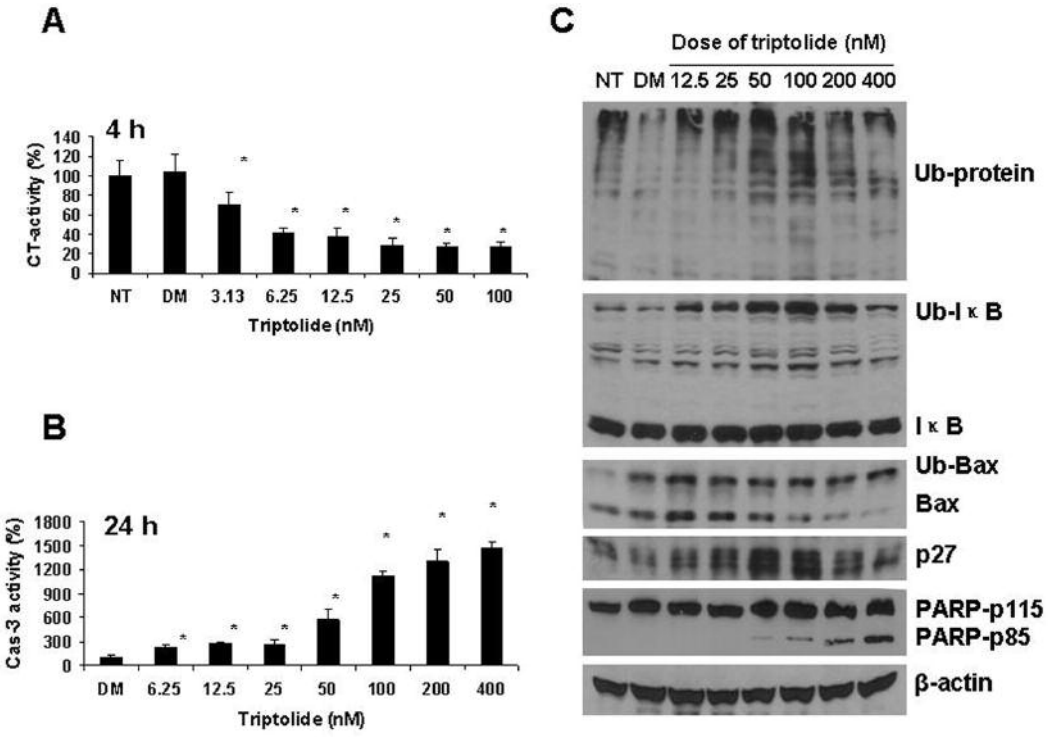

Figure 6. *Dose effects of triptolide on proteasome inhibition and apoptosis induction of MDA-MB-231cells. Cells were either not treated (NT) or treated with solvent DMSO (DM) or different concentrations of triptolide for 4 h, followed by measurement of inhibition of the proteasomal chymotrypsin(CT)-like activity using Z-GGL-AMC (A), or for 24 h, followed by measurement of caspase-3 (Cas-3) activity (B) and of the levels of ubiquitinated proteins (4 h), IκB, Bax, p27, PARP and β-actin (18 h) by Western blot assay using specific antibodies (C). * $p < 0.05$. Data are expressed as mean ± standard deviation of three experiments.*

carbon and oxygen at the 12th and 13th positions are highly susceptible to nucleophilic attack, forming a 'bull's-eye' with a red center in the structure. As geometry was not optimized properly, an AutoDock experiment did not run. Therefore, it was not possible to predict whether triptolide would inhibit the β5 subunit of the proteasome as it was done with other natural compounds. There are at least two possible explanations for why triptolide has no effect on 20S proteasome directly, but can inhibit chymotrypsin-like activity in cells. First, some chemical bonds within triptolide block its binding with the 20S proteasome. After entering the cell, the chemical bonds may be modified by cellular enzymes. Therefore, the metabolites of triptolide may be effective to inhibit the proteasome. Secondly, it is possible that triptolide may inhibit the 19S regulatory particles or other sites on the proteasome. Regardless of these possibilities, the present data suggested that triptolide acts as a tumor-cellular proteasome inhibitor.

Natural compounds may have multiple cellular targets in order to achieve their beneficial biological effects such as tumor growth inhibition (30). Clinical trials in China revealed that triptolide achieved a total remission rate of 71% in mononucleocytic leukemia and 87% in granulocytic leukemia, which is more effective than any chemotherapeutic agents currently available. A phase I clinical trial of the effect of a water-soluble derivative of triptolide on solid tumors is ongoing in Europe (31). The results of the present study showed that triptolide acts as a tumor proteasome-inhibitor that has great potential for the prevention and treatment of various types of human cancer. It is possible that triptolide has multiple cellular targets for inducing cancer-cell death. It is expected that this small-molecule natural product may prove to be a candidate for the systemic therapy of cancer and it has great potential either as a single agent or in combination with conventional therapies. However, additional studies exploring the *in vivo* biological activity of triptolide are needed and will provide compelling support for triptolide as a potential drug for human cancer. Modulating the structure, and therefore function, of triptolide may lead to the development of a promising strategy applied to a broad spectrum of cancer treatments.

Acknowledgements

The research was partially supported by grants from the National Cancer Institute (1R01CA120009, 3R01CA120009-04S1, and 1R21CA139386-01 to Q.P. Dou) and from National Natural Science Foundation of Guangdong Province (7003073 to L. Lu).

References

1 Tao X, Younger J, Fan FZ, Wang B and Lipsky PE: Benefit of an extract of *Tripterygium wilfordii Hook* F in patients with rheumatoid arthritis: a double-blind, placebo-controlled study. Arthritis Rheum *46*: 1735-1743, 2002.

2 Chen BJ: Triptolide, a novel immunosuppressive and anti-inflammatory agent purified from a Chinese herb *Tripterygium wilfordii Hook* F. Leuk Lymphoma 42: 253-265, 2001.

3 Matta R, Wang XX, Ge H, Ray W, Nelin LD and Liu Y: Triptolide induces anti-inflammatory cellular responses. Am J Transl Res *1*: 267-282, 2009.

4 Pan JX: RNA polymerase – an important molecular target of triptolide in cancer cells. Cancer Lett *292*: 149-52, 2010.

5 Mujumdar N, MacKenzie T, Dudeja V, Chugh R, Antonoff MB, Borja-Cacho D, Sangwan V, Dawra R, Vickers SM, Saluja AK: Triptolide induces cell death in pancreatic cancer cells by apoptotic and autophagic pathways. Gastroenterology *139*: 598-608, 2010.

6 Hoeller D and Dikic I: Targeting the ubiquitin system in cancer therapy. Nature *458*: 438-444, 2009.

7 Dou QP and Li B: Proteasome inhibitors as potential novel anticancer agents. Drug Resist Updat *2*: 215-223, 1999.

8 Goldberg AL: Functions of the proteasome: the lysis at the end of the tunnel. Science *268*: 522-523, 1995.

9 Chen ZJ, Parent L and Maniatis T: Site-specific phosphorylation of IκBa by a novel ubiquitination-dependent protein kinase activity. Cell *84*: 853-862, 1996.

10 Almond JB and Cohen GM: The proteasome: a novel target for cancer chemotherapy. Leukemia *16*: 433-443, 2002.

11 Yang HJ, Zhou P, Huang HB, Chen D, Ma N, Cui QC, Shen S, Dong W, Zhang X, Lian W, Wang X, Dou QP and Liu J: Shikonin exerts antitumor activity *via* proteasome inhibition and cell death induction *in vitro* and *in vivo*. Int J Cancer *124*: 2450-2459, 2009.

12 Smith DM, Daniel KG and Dou QP: Exploiting the ubiquitin-proteasome pathway for anticancer drug discovery: unanswered questions and future directions. Lett Drug Des Discov *2*: 77-84, 2005.

13 Adam J: The development of proteasome inhibitors as anticancer drugs. Cancer Cell *5*: 417-421, 2004.

14 An B, Goldfarb RH, Siman R and Dou QP: Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. Cell Death Differ *5*: 1062-1075, 1998.

15 Chen D, Daniel KG, Chen MS, Kuhn DJ, Landis-Piwowar KR and Dou QP: Dietary flavonoids as proteasome inhibitors and apoptosis inducers in human leukemia cells. Biochem Pharmacol *69*: 1421-1432, 2005.

16 Kazi A, Urbizu DA, Kuhn DJ, Acebo AL, Jackson ER, Greenfelder GP, Kumar NB and Dou QP: A natural musaceas plant extract inhibits proteasome activity and induces apoptosis selectively in human tumor and transformed, but not normal and non-transformed cells. Int J Mol Med *6*: 879-887, 2003.

17 Yang HJ, Chen D, Cui QC, Xiao Y and Dou QP: Celastrol, a triterpene extracted from the Chinese ''Thunder of God Vine,'' is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. Cancer Res *66*: 4758-4764, 2006.

18 Yang HJ, Shi GQ and Dou QP: The tumor proteasome is a primary target for the natural anticancer compound Withaferin A isolated from 'Indian winter cherry'. Mol Pharmacol *71*: 426-437, 2007.

19 Milacic V, Banerjee S, Landis KR, Sarkar FH, Majumdar AP and Dou QP: Curcumin inhibits the proteasome activity in human colon cancer cells *in vitro* and *in vivo*. Cancer Res *68*: 7283-7292, 2008.

20 Zhu W, Ou Y, Li Y, Xiao R, Shu M, Zhou Y, Xie J, He S, Qiu P and Yan G: A small molecule triptolide suppresses angiogenesis and invasion of human anaplastic thyroid carcinoma cells *via* down-regulation of NF-kappa B pathway. Mol Pharmacol *75*: 812-819, 2009.

21 Qiu DM, Zhao GH, Aoki YS, Shi L, Uyei A, Nazarian S, Ng JC and Kao PN: Immunosuppressant PG490 (Triptolide) inhibits T-cell interleukin-2 expression at the level of purine-box/nuclear factor of activated T-cells and NF-kB transcriptional activation. J Biol Chem *274*: 13443-13450, 1999

22 Yan X, Bi CF, Fan YH, Cui C, Zhang X and Dou QP: L-glutamine schiff base copper complex as a proteasome inhibitor and an apoptosis inducer in human cancer cells. Int J Oncol *33*: 1073-1079, 2008.

23 Chen Di, Frezza M, Shakya R, Cui QC, Milacic V, Verani CN and Dou QP: Inhibition of the proteasome activity by gallium(III) complexes contributes to their anti-prostate tumor effects. Cancer Res *67*: 9258-9265, 2007.

24 Nam S, Smith DM and Dou QP: Ester bond-containing tea polyphenols potently inhibit proteasome activity *in vitro* and *in vivo*. J Biol Chem *276*: 13322-13330, 2001.

25 Kupchan SM, Court WA, Dailey RG Jr., Gilmore CJ and Bryan RF: Triptolide and tripdiolide, novel antileukemic diterpenoid triepoxides from *Tripterygium wilfordii*. J Am Chem Soc *94*: 7194-7195, 1972.

26 Carter BZ, Mak DH, Schober WD, Dietrich MF, Pinilla C, Vassilev LT, Reed JC and Andreeff M: Triptolide sensitizes AML cells to TRAIL-induced apoptosis *via* decrease of XIAP and p53-mediated increase of DR5. Blood *111*: 3742-3750, 2008.

27 Dou KR, Farrell AT, Sridhara R and Pazdur R: United States Food and Drug Administration approval summary: bortezomib for the treatment of progressive multiple myeloma after one prior therapy. Clin Cancer Res *12*: 2955-2960, 2006.

28 Gong Y, Xue B, Jiao J, Jing L and Wang X: Triptolide inhibits COX-2 expression and PGE2 release by suppressing the activity of NF-kappaB and JNK in LPS-treated microglia. J Neurochem *107*: 779-788, 2008.

29 Kang DW, Lee JY, Oh DH, Park SY, Woo TM, Kim MK, Park MH, Jang YH and Min do S: Triptolide-induced suppression of phospholipase D expression inhibits proliferation of MDA-MB-231 breast cancer cells. Exper Mol Med *41*: 678-685, 2009.

30 Cucciolla V, Borriello A, Oliva A, Galletti P, Zappia V, Della Ragione F: Resveratrol: from basic science to the clinic. Cell Cycle *6*: 2495-2510, 2007.

31 Kiviharju TM, Lecane PS, Sellers RG and Peehl DM: Antiproliferative and proapoptotic activities of triptolide (PG490), a natural product entering clinical trials, on primary cultures of human prostatic epithelial cells. Clin Cancer Res *8*: 2666-2674, 2002.

Received November 15, 2010
Revised December 16, 2010
Accepted December 17, 2010