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Anti-tumor activity of benzylideneacetophenone derivatives via proteasomal inhibition in prostate cancer cells

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A number of some chalcone derivatives possess promising biological properties including anti-inflammation, anti-oxidant, and anti-tumor activity. Although it has been shown that some derivatives of chalcone induce apoptosis in different kinds of cancer cells, the involved mechanism of action is not well defined. The purpose of this study is to investigate the primary target of a benzylideneacetophenone derivative (JC3), which is a synthetic compound derived from the chalcone family, in human cancer, using prostate cancer cells as a working model. Herein, we show that JC3 inhibits proteasomal activity as indicated by both in vitro and in cell-based assays. Especially, the JC3-dimer was more potent than monomer in the aspect of proteasome inhibition, which induced apoptosis significantly in the prostate cancer cells. Owing to the critical roles of the proteasome in the biology of human tumor progression, invasion, and metastasis, these findings give an important clue for the development of novel anti-tumor agents.

1. Introduction

Prostate cancer is the most frequently occurring cancer and the second leading cause of cancer related to deaths in men (Siegel et al. 2012). The majority of patients that die from prostate cancer exhibit metastases into the bone. Prostate cancer has a high cure rate in early stages, through the use of surgery, radiation or hormone therapy. However, not all primary tumors to can be eradicated at an early stages, and to date, no effective therapy has been developed for metastatic prostate cancer (Moul and Dawson 2012). Therefore, the development of novel therapies for the treatment of both primary and metastatic prostate cancer is critical to reduce the mortality of the disease. On the other hands, prostate cancer is an optimal disease for chemopreventive intervention as it grows slowly before the onset of symptoms and the establishment of diagnosis and it is usually diagnosed in men more than 50 years of age. Consequently, pharmacological or nutritional intervention could considerably affect the life quality of patients by delaying the progression of cancer.

Apoptosis is an evolutionally conserved cellular suicide program through intrinsic mitochondria pathway or the extrinsic death receptor pathway (Steller 1995; Nagata 1997; Ashkenazi and Dixit 1998; Green and Reed 1998). It has been clearly demonstrated that several pro-apoptotic proteins of Bcl-2 family located in the outer membranes of mitochondria control the release of cytochrome c into the cytosol (Green and Reed 1998; Gross et al. 1999). The released cytochrome c activates caspase-9 that in turn proteolytically activates downstream caspase-3 that then cleaves a number of important cellular target proteins, including poly(ADP-ribose) polymerase (PARP) (Lazebnik et al. 1994), and leads to apoptotic cell death. Because deregulation of apoptosis is a major contributor to the survival of tumor cells, there is much interest in developing new ways to activate this process for the treatment of cancer cells.

The ubiquitin-proteasome pathway is indispensable to many fundamental cellular processes, including the cell cycle, apoptosis, angiogenesis, and differentiation (Mani and Gelmann 2005; Landis-Piwowar et al. 2006). The proteasome contributes to the pathologic state of several human diseases including cancer and AIDS, in which some regulatory proteins are either stabilized due to decreased degradation or lost due to accelerated degradation (Ciechanover 1998). The 20S proteasome, the proteolytic core of 26S proteasome complex, contains multiple peptidase activities including the chymotrypsin like (CT-like), trypsin like (T-like), and peptidylglutamyl peptide hydrolyzing like (PGPH-like) (Seemuller et al. 1995). Interestingly, inhibition of this complex has a therapeutic property and is preferentially toxic to malignant cells. For example, it has been shown that inhibition of tumor cellular CT-like activity is a strong stimulus that induces apoptosis (An et al. 1998; Lopes et al. 1997; Orlowski et al. 1998). Currently, bortezomib and carfilzomib are only proteasome inhibitors approved by the US FDA for the treatment of multiple myeloma (Richardson et al. 2003; Fisher et al. 2006; McBride et al. 2015).

Chalcones (1,3-diphenylpropen-1-ones) are naturally occurring compounds belonging to the flavonoid family and include curcumin, yakuchinone, green-tea-derived polyphenols and flavonoids. Previous studies have indicated that chalcones and their derivatives have anticancer activity in various tumor cells (Achanta et al. 2006; Bonfili et al. 2008; Cuccioloni et al. 2009). Because they are abundant in edible plants, they might be rising tools for dietary cancer prevention and treatment strategies. In addition, their simple and efficient synthesis makes them also attractive for cancer therapy. However, although several chalcones show promising results for cancer treatment, their exact mechanisms of action as apoptotic, anti-proliferative, and anti-angiogenic effects remain to be elucidated. Recently, it has been suggested that the carbonyl carbon of tea polyphenols and flavonoids confers proteasome-inhibitor properties followed by apoptotic tumor cell death (Bonfili et al. 2008; Milacic et al. 2008; Mozzicafreddo et al. 2008). In

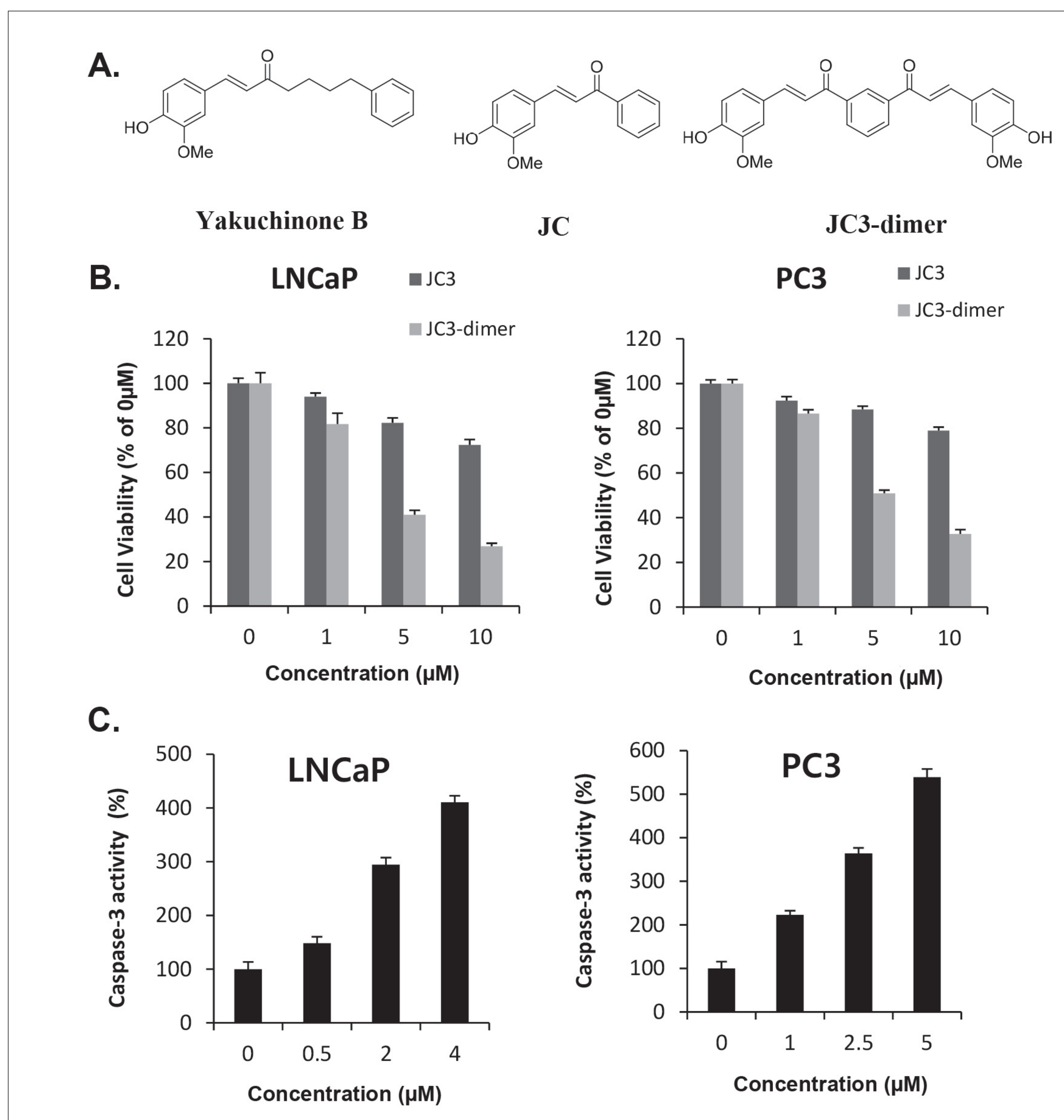

Fig.1.: Effect of JC3 and JC3-dimer on the prostate cancer cell viability and caspase-3 activity. (A) Structures of yakuchinone B, JC3, and JC3-dimer. JC3 was synthesized based on a structural modification of yakuchinone B. (B) Cell viability was significantly decreased by JC3-dimer in LNCaP and PC3 cells. Cells were treated with indicated concentrations of JC3 and JC3-dimer for 24hr, followed by an MTT assay. (C) To test the effect of JC3-dimer on apoptosis, prostate cancer cells were treated different concentrations based on IC50 of MTT assay for 24hr. All data represent independent triplicate experiments.

the present study, we investigated a novel chalcone derivative for their *in vitro* anti-tumor activity and examined the ability to induce cancer cell death by inhibition of proteasome activity.

2. Investigations and results

2.1. JC3-dimer induces cancer cell death in a dose-dependent manner

The JC3 and JC3-dimer were tested for their anti-tumor activity in the prostate cancer cell lines using MTT assay. The IC50 values (the drug concentrations that killed 50% of cells) were examined in LNCaP and PC3 cells which are well-established prostate cancer cell lines. JC3-dimer exhibited more potent activity, with the IC50 values of 4.15 μM in LNCaP and 5.04 μM in PC3 cells as determined by the MTT assay at 24hr after agent treatment (Fig. 1B). To investigate whether the effect of JC3-dimer on cell viability is mediated by apoptosis, the caspase-3 activity and FACS analysis were determined. LNCaP and PC-3 prostate cancer cells were treated with different concentrations of JC3-dimer for 24 hr, followed by detection of caspase activity. Compared to the control treated cells, caspase-3-like activity was increased 4 times and 5 times in LNCaP and PC3 cells, respectively, after JC3-dimer treat-

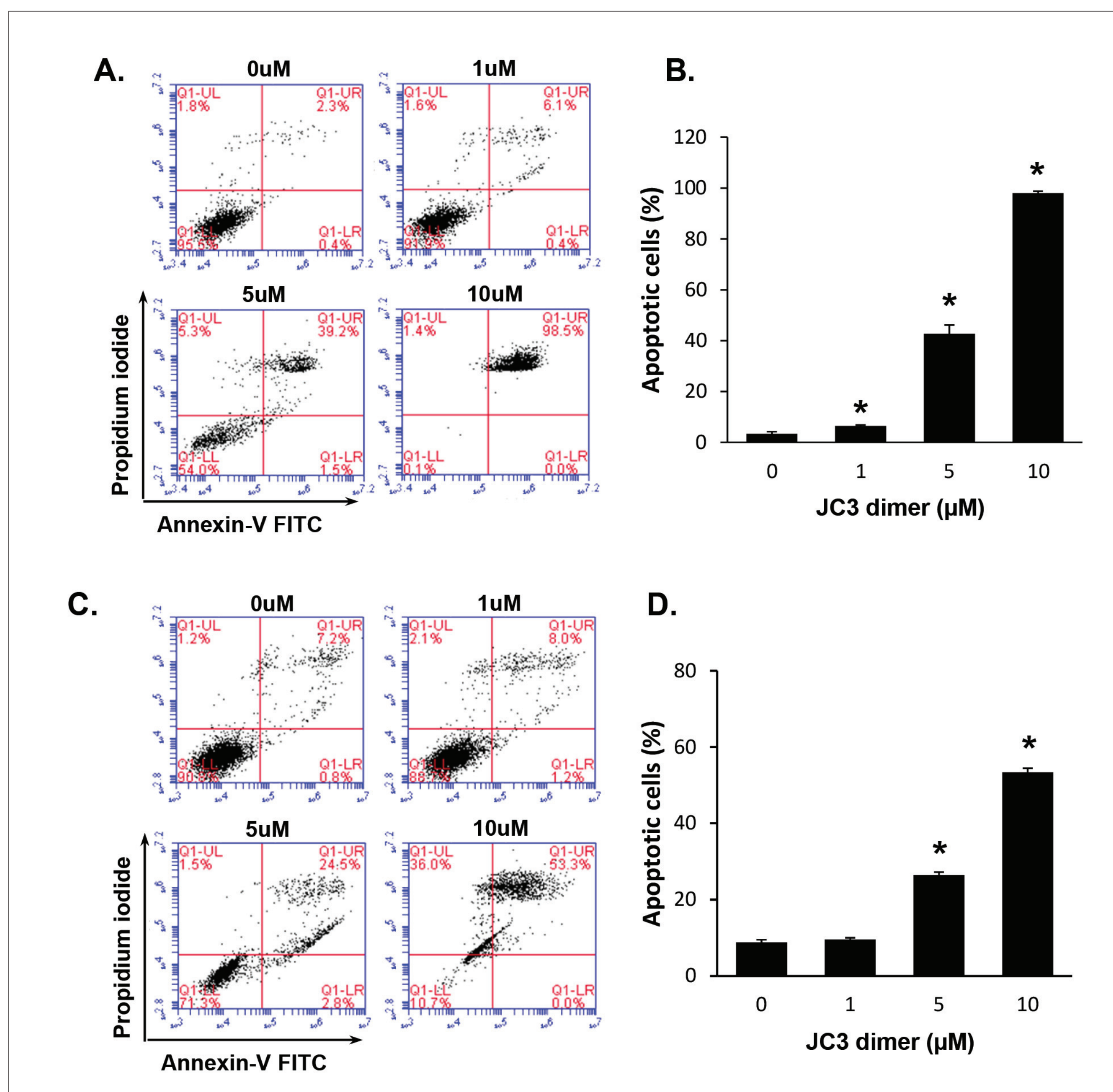

Fig. 2.: Quantification of JC3-dimer-induced apoptotic cells in LNCaP (A, B) and PC3 (C, D) cells. (A, C) Apoptotic cells were quantified by FACS after propidium iodide uptake and Annexin V binding in non-permeabilized cells (Lower left-live cells; Lower right-early apoptotic cells; Upper right-late apoptotic cells). (B, D) Bar graph represents the mean percentages of apoptotic cells ± s.d. in the three independent experiment. Asterisk (*) indicates a P value < 0.001 using a Paired T-test.All data represent independent triplicate experiments.

ment (Fig. 1C). To confirm JC3-dimer-induced apoptosis, flow cytometry analysis was performed. As shown in Fig. 2, increases of early apoptosis (lower right quadrant) and late apoptosis (upper right quadrant) were clearly observed concentration-dependently in both LNCaP and PC3 cells.

2.2. *JC3-dimer inhibits the chymotrypsin-like activity of a purified human 20S proteasome and 26S proteasome in PC3 cell lysates*

It has been reported that chalcone derivatives could induce anti-tumor activity viaby apoptosis induction (Achanta et al. 2006; Milacic et al. 2008). However, a mechanistic study is not enough to explain for their effects. In easier studies, chalcone derivatives induced cancer cell death by the proteasome inhibitory mechanism. Because JC3 is similar in structure such as the chalcones, curcumin and yakuchinone B, we hypothesized that our synthesized compounds might have anti-tumor activity through proteasomal inhibition. To determine this possibility, we found direct evidence from an in vitro assay for proteasomal inhibitory capacity of JC3. We determined proteasome activity using a purified human 20S proteasome in the presence of JC3 or JC3-dimer at up to 10 μM. The chymotrypsin-like activity of the purified 20S proteasome was significantly inhibited by JC3 dimer with an IC50 value of 5.04 μM (Fig. 3A). Similarly, JC3-dimer also potently inhibited 26S proteasome chymotrypsin-like activity in a cell extract prepared from exponentially grown PC3 prostate cancer cells with an IC50 value of 8.5 μM (Fig. 3B). Therefore, JC3-dimer could be a novel inhibitor of purified 20S and 26S cellular proteasome.

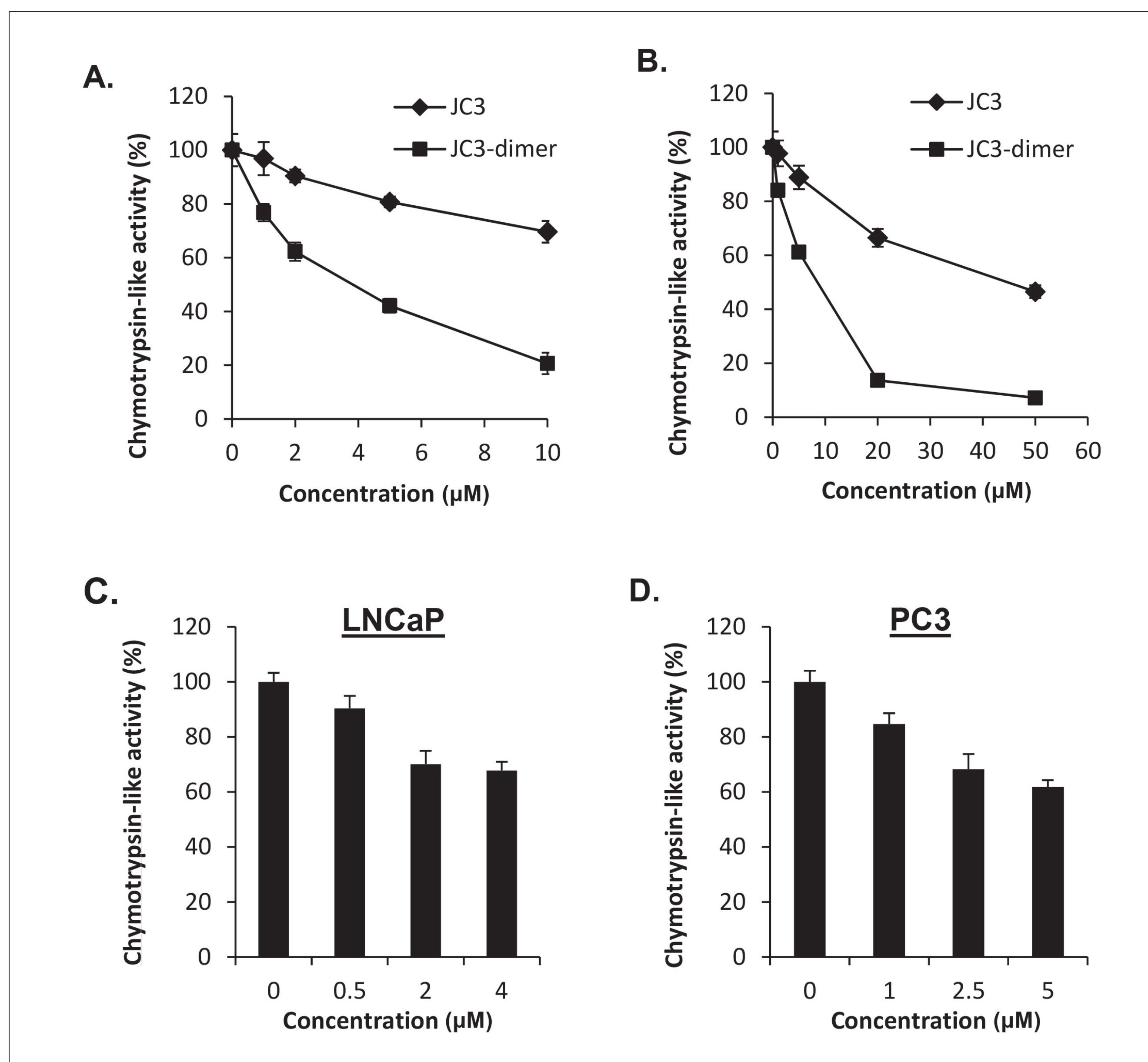

Fig. 3.: Proteasome inhibition assay by JC3 and JC3-dimer. Purified 20S human proteasome (A) or PC3 cell extracts (B) were incubated with indicated concentrations of JC3 and JC3-dimer for 2h. (C) LNCaP and PC3 cells were treated JC3-dimer with indicated concentrations for 18hr, followed by the proteasomal chymotrypsin-like activity assay. Proteasome activity was monitored by calculating the fluorescent group release from substrate for chymotrypsin-like activity. All data represent independent triplicate experiments.

2.3. JC3-dimer induces accumulation of polyubiquitinated protein in prostate cancer cells, which is followed by apoptosis

As next step, we demonstrated that the JC3-dimer is able to inhibit the proteasome activity under physiological conditions. LNCaP and PC3 cells were treated with different concentrations of JC3-dimer for 18 hr, followed by measurement of proteasome activity and ubiquitinated protein. First, proteasome inhibition was measured by using the proteasomal chymotrypsin-like activity assay. The chymotrypsin-like activity of the proteasome was inhibited by 10, 30, and 33% with JC3-dimer at 0.5, 2 and 4 μM, respectively, in LNCaP cells (Fig. 3C) and 15, 32, and 39% with JC3-dimer at 1, 2.5, and 5 μM, respectively, in PC3 cells (Fig. 3D). Consistent with proteasome inhibition, polyubiquitinated proteins were clearly accumulated at 2 and 4 μM in LNCaP cells, and 2.5, 5 μM in PC3 cells (Fig. 4A). Then we measured the protein level of Bax, which is the proapoptotic protein and target of the proteasome. The accumulation of Bax was shown in a dose-dependent manner (Fig. 4A). In the cell lysate that we isolated at 24 hr after treatment, the active form of caspase-3 protein and cleaved PARP fragment, which is downstream of caspase-3, were increased dose-dependently (Fig. 4B). These results suggest that the proteasome inhibition is physiologically functional and could be related to apoptosis.

3. Discussion

Chemoprevention is defined as the use of natural or synthetic substances to prevent cancer formation or cancer progression. Many studies have shown that natural phytochemicals containing phenolic compounds derived from certain plants have the antioxidant, anti-inflammatory and anti-tumor activities. Yakuchinone B showed anti-tumor activity in the treatment of acute myeloblastic leukemia (HL-60), chronic myelogenic leukemia (K-562), breast adenocarcinoma (MCF-7), and cervical epithelial carcinoma (HeLa) (Roy et al. 2002). Chalcones have been found to act through the intrinsic as well as extrinsic apoptosis pathway to prevent tumor progression. For example, many natural chalcones have been shown to induce apoptosis in different types of cancer cells through a wide variety of mechanisms (Kim et al. 2001; Ye

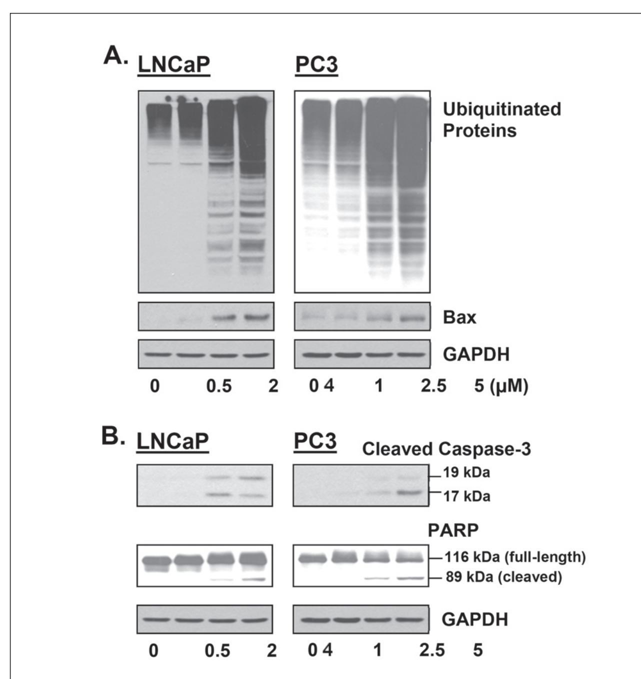

Fig. 4: .JC3-dimer induced apoptosis-related proteins in a dose dependent manner. LNCaP and PC3 cells were treated JC3-dimer with indicated concentrations for 18hr. The expression level of ubiquitinated proteins and Bax which are related to proteasome inhibition (A), and active form of caspase-3, cleaved PARP (B) were determined.

et al. 2005; Akihisa et al. 2006; Nishimura et al. 2007; Jing et al.). Curcumin is a major chemical component of turmeric, derived from the rhizome of Curcuma longa. In addition to its traditional use as a food coloring and flavoring agent, curcumin also has a well-documented history in medicine in India and Southeast Asia. There are diverse mechanisms that are implicated in the inhibition of tumorigenesis by curcumin and include a combination of anti-inflammatory, anti-oxidant, immunomodulatory, pro-apoptotic, and anti-angiogenic properties via pleiotropic effects on genes and cell signaling pathways at multiple levels (Khan et al. 2008).

In this study, we introduced the novel synthetic compound JC3, 3-(4-hydroy-3-methoxy)-chalcone, which is structurally related to yakuchinone B, chalcones, and curcumin, to test chemopreventive activity. Our previous results showed JC3 was promising compound in terms of anti-oxidant and anti-inflammatory activities and we hypothesized that it might have anti-tumor effect as structural similarity (Jang et al. 2009). Here, we found that JC3-dimer directly targets the proteasome in human prostate cancer cells, which is responsible for a subsequent apoptosis induction.

JC3-dimer-induced apoptosis in human prostate cancer cells was detected by decreased cell viability as well as caspase-3 activation (Figs. 1 and 2). Since the ubiquitin-proteasome pathway regulated apoptosis in the previous studies, we determined the interaction between JC3 and the proteasome, and the biological effect of this interaction on apoptosis induction. First, in vitro proteasomal activity using both purified 20S and whole cell lysates containing 26S proteasome revealed the potential role of JC3-dimer on the proteasome interaction. JC3 also weakly inhibited in vitro proteasome activity, but it did not show significant changes in cell viability. Therefore we focused on the effect of the JC3-dimer. To investigate whether apoptosis induced by JC3-dimer was associated with proteasome inhibition, we performed dose-dependent experiments based on IC50 values of cell viability in each cell lines. JC3-dimer significantly caused proteasome inhibition, as shown by decreased proteasomal chymotrypsin-like activity and increased Bax protein level as well as accumulation of polyubiquitinated proteins in a dose dependent manner. Also apoptosis as shown by active caspase-3 protein and PARP cleavage occurred in the same manner. Our results suggest that the JC3-dimer induced apoptosis is caspase-3 dependent, evidenced by caspase-3 activation and PARP cleavage via inhibition of proteasomal activity in prostate cancer cells.

LNCaP cells are androgen-sensitive human prostate adenocarcinoma cells derived from lymph node metastasis. On the other hand, PC3 cell lines originally came from advanced androgen independent bone metastasis prostate cancer. In the present study, the effectiveness of JC3-dimer against androgen-independent prostate cancer cells (PC3) is especially noteworthy. Metastatic prostate cancers are lethal because they are heterogeneously composed of both androgen-dependent and androgen-independent malignant cells (Laufer et al. 2000; Isaacs 2000). Because androgen-independent prostate cancer cells are resistant to the induction of apoptosis by androgen ablative therapy, an important strategy in developing effective chemotherapy for metastatic prostate cancer is to specifically eliminate androgen-independent cells by targeted apoptosis.

Taken together, our results clearly demonstrate that the proteasome is one important cellular target of JC3-dimer in human prostate cancer cells, inhibition of which is associated with apoptosis induction. Therefore, the potency of JC3-dimer on proteasome inhibition and apoptosis induction suggests its potential use for cancer prevention and treatment.

4. Experimental

4.1. Reagents

The synthesis of benzylideneacetophenone derivatives was initiated as described previously (Oh et al. 2006). The 4-hydroxy-3-methoxy cinnamaldehyde was protected with tert-butyldimethylsilyl trifluoromethanesulfonate in the presence of 2,6-lutidine or 2-(trimethylsilyl) ethoxymethyl chloride (SEM–Cl)/N,N-di-isopropylethylamine to form aldehydes with 95 and 97% yields, respectively. The benzylideneacetophenone compound (JC3) was fully identified by infrared and NMR spectroscopy, including high-resolution mass spectroscopy. In addition, the dimer of JC3 was assembled to evaluate the efficacy of polystructures of JC3 (Fig. 1A). It was dissolved in DMSO at a stock concentration of 500 mM, aliquoted and stored at -20 °C. Fetal bovine serum (FBS) was purchased from Tissue Culture Biologicals (Tulare, CA, USA). RPMI 1640 medium, penicillin, and streptomycin were from Invitrogen Co. (Carlsbad, CA, USA). Purified human 20S proteasome was purchased from Enzo life sciences (Plymouth Meeting, PA, USA). Fluorogenic peptide substrates Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activity), Ac-DEVD-AMC (for caspase-3 activity), and 3-(4,5-dimethyltiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA). Rabbit anti-caspase-3, rabbit anti-PARP, rabbit anti-bax and mouse anti-ubiquitin were purchased from Cell Signaling (Danvers, CA, USA). The polyclonal antibody against GAPDH was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.2. Cell culture

Human prostate cancer LNCaP and PC3 cells were obtained from ATCC and maintained in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (Life Technologies Inc., Carlsbad, CA, USA).

4.3. MTT assay.

In a 96-well plate, 2000 cells were plated and cell viability was determined after 24 h by MTT assay. MTT (1 mg/ml) was added and the plates were incubated for 4 h at 37 ºC. The formazan was extracted with acidic isopropanol, and the absorbance of the converted dye was measured at 570 nm.

4.4. Caspase -3-like activity assay

Cells were lysed in cell extract buffer [150 mM NaCl, 50 mM Tris-HCL (pH 7.5), 0.5 mM EDTA, and 0.5% NP40]. Lysates were kept on ice for 30 min and centrifuged at 15,000Xg for 10 minutes. Fifty microliters of the cytosolic fraction were incubated for 60 minutes at 37°C in a total volume of 200 μl of caspase buffer [20 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT] containing mol/L Ac-DEVD-AMC for caspase-3-like activity (BioSource International, Inc., Camarillo, CA, USA). 7-Amino-4-methylcoumarin fluorescence, released by caspase activity was measured at 460 nm using 360 nm excitation wavelength on a Spectra Maxi Germini fluorescence plate reader (Molecular Devices, Menlo Park, CA, USA). Caspase activity was normalized per microgram of protein as determined with a bicinchoninic acid protein (BCA) assay reagent (Pierce, Rockford, IL, USA).

4.5. Fluorescence-Activated activated cell sorting (FACS)

Apoptotic cells were determined by FACS analysis using Annexin V-FITC staining. The cells were treated under the appropriate conditions for 24 h, subsequently harvested, trypsinized, washed once in cold PBS, suspended the cells in 1X binding buffer. The counted cells were stained in Propidium propidium iodide and Annexin V-FITC solution (Annexin V-FITC Apoptosis Detection Kit, BD Biosciences, Bedford, MA, USA) at room temperature for 15 min in the dark. The stained cells

were analyzed by flow cytometry within 1 h. Apoptotic cells were analyzed by Becton Dickinson FACSscan flow cytometer and BD FACSDiva software.

4.6. Inhibition of purified 20S proteasome or 26S proteasome in whole cell extract

A purified human 20S proteasome (50 ng) or PC-3 extract (2 mg) were incubated with 20 mM of fluorogenic peptide substrate (Suc-Leu-Leu-Val-Tyr-AMC for chymotrypsin-like of the proteasome) in 200 ml assay buffer (20mM Tris-HCl, pH 7.5), in the presence of JC3 at up to 50 mM or the solvent DMSO for 2 h at 37 °C. 7-Amino-4-methylcoumarin fluorescence, released by proteasome activity was measured at 460 nm using 360 nm excitation wavelength on a Spectra Maxi Germini fluorescence plate reader (Molecular Devices, Menlo Park, CA, USA). Proteasomal activity was normalized per microgram of protein.

4.7. Western blotting

Cell lysates were obtained by lysing the cells with ice cold RIPA buffer (0.05 M Tris–HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium metavanadate (NaVO3), 1 mM sodium fluoride (NaF), and protease inhibitor cocktail. The protein concentration in the lysates was determined by the BCA procedure (Pierce, Rockford, IL, USA). Equal amounts of protein samples in SDS sample buffer [1% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, and 0.05% bromophenol blue] were boiled for 5 min and subjected to reducing SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.2% Tween 20 (T-TBS) for 1 hr at room temperature. The membranes were incubated with T-TBS containing 5% milk and the primary antibodies. After three washes with T-TBS, the blot was incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. The antigen was detected using the Western Blot Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Inc., Boston, MA, USA), according to the manufacturer's instruction.

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Conflicts of Interest: None declared.

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