Role of p53, PUMA, and Bax in wogonin-induced apoptosis in human cancer cells

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ABSTRACT

We observed that treatment of prostate cancer cells for 24 h with wogonin, a naturally occurring monoflavonoid, induced cell death in a dose- and time-dependent manner. Exposure of wogonin to LNCaP cells was associated with increased intracellular levels of p21^{Cip-1}, p27^{Kip-1}, p53, and PUMA, oligomerization of Bax, release of cytochrome c from the mitochondria, and activation of caspases. We also confirmed the role of p53 by noting that knock-in in p53 expression by transfecting p53 DNA increased wogonin-induced apoptosis in p53-null PC-3 cells. To study the mechanism of PUMA up-regulation, we determined the activities of PUMA promoter in the wogonin treated and untreated cells. Increase of the intracellular levels of PUMA protein was due to increase in transcriptional activity. Data from chromatin immunoprecipitation (ChIP) analyses revealed that wogonin activated the transcription factor p53 binding activity to the PUMA promoter region. We observed that the up-regulation of PUMA mediated wogonin cytotoxicity. Further characterization of the transcriptional response to wogonin in HCT116 human colon cancer cells demonstrated that PUMA induction was p53-dependent; deficiency in either p53 or PUMA significantly protected HCT116 cells against wogonin-induced apoptosis. Also, wogonin promoted mito-chondrial translocation and multimerization of Bax. Interestingly, wogonin (100 μM) treatment did not affect the viability of normal human prostate epithelial cells (PrEC). Taken together, these results indicate that p53-dependent transcriptional induction of PUMA and oligomerization of Bax play important roles in the sensitivity of cancer cells to apoptosis induced by caspase activation through wogonin.

1. Introduction

Prostate cancer is the most commonly diagnosed cancer in men and is the second leading cause of cancer-related deaths in North America [1,2]. Current therapies, such as surgery, chemotherapy, and radiation therapy, are of limited efficacy, especially in advanced disease, and metastatic disease remains incurable [3,4]. The use of naturally occurring dietary agents is becoming increasingly appreciated as an effective means of managing many types of cancer in an approach

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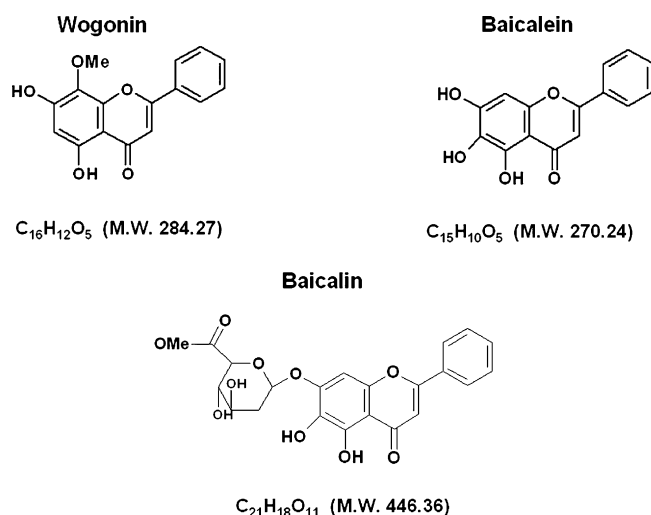   E-mail address: leeyj@upmc.edu (Y.J. Lee).

Fig. 1 – Chemical structures of baicalein, baicalin, and wogonin.

known as cancer chemoprevention. Cancer chemoprevention is a means of cancer control in which a malignancy is prevented or reversed by pharmacologic intervention with naturally occurring and/or synthetic agents [5,6]. One of the most desirable goals in cancer chemoprevention is the identification of natural agents with demonstrable efficacy against defined molecular targets. In contrast to the high incidence of prostate cancer in North America, the incidence of this disease in East Asia is very low [7,8]. The low incidence of prostate cancer in Asian men has been attributed to the dietary consumption of large amounts of plant-based foods rich in phytochemicals [9–11]. Because of these observations, nutritional supplements such as soybean, garlic, and green tea, which are rich in polyphenolic compounds, have been used to augment anticancer therapies [12,13].

Scutellaria baicalensis Georgi (Huang Qui) is a medicinal herb widely used for the treatment of various inflammatory diseases, hepatitis, tumors, allergic reactions, and diarrhea in East Asian countries including China, Korea, Taiwan, and Japan [14]. The plant has been reported to contain a large number of flavonoids, frequently found as glucosides and other constituents, including phenethyl alcohols, sterols, and essential oils and amino acids. Its components wogonin, baicalin, and baicalein have been studied. Wogonin ($C_{16}H_{12}O_5$, Fig. 1), [15] one of the main active compounds of *Scutellaria baicalensis*, is known to exert potent anti-inflammatory activities *in vitro* as well as *in vivo* [16]. Recently, some reports indicated that wogonin significantly inhibited human ovarian cancer cell A2780, human promyeloleukemic cell HL-60, and human hepatocellular carcinoma cell SK-HEP-1 [17–19]. However, knowledge of the molecular mechanisms of wogonin-induced apoptosis remains to be delineated. Our study was undertaken to evaluate the ability of wogonin to induce apoptosis in human prostate carcinoma LNCaP and human colon carcinoma HCT116 cells, and investigate the probable apoptotic molecular mechanisms used in these processes.

Apoptosis, a goal of cancer treatment, is characterized by the cell shrinkage, blebbing of the plasma membrane, and chromatin condensation that are associated with cleavage of DNA into ladders [20,21]. However, in response to some effective therapeutic treatments, decreased ability to undergo apoptosis occurred in human malignant tumor cells [22,23]. Therefore, further development of agents that can induce or enhance the extent of apoptosis seems to be a promising strategy in the treatment of cancer. The study of apoptosis reveals that many oncogenes and tumor suppressor genes are involved in mediating apoptosis. Tumor suppressor gene p53 is one of the critical genes. It regulates the onset of DNA replication at the G1/S boundary. Its tumor suppressor protein p53 plays an important role in the cell, preventing damaged or otherwise abnormal cells from becoming malignant [24]. Previous studies have demonstrated that both p53-dependent and p53-independent apoptoses were detected in tumor cells in response to apoptosis inducers [25,26]. In humans, p53 is either mutated or inactivated in at least 50% of tumors [27] and loss of wild-type p53 function can lead to tumor development. p53 is activated through its transcription factor [28] under a variety of stresses, including DNA damage [29,30]. In response to such stresses, p53 is stabilized and activated such that it binds to specific DNA sequences, ultimately driving the transcription of genes involved in cell cycle arrest and apoptosis [20,31]. In addition, numerous p53-dependent target genes that play a role as downstream effectors of p53 function have been identified. For example, the cyclin-dependent kinase inhibitor p21[Cip-1] is a direct p53 target and deletion of this gene reduces the cell cycle arrest response to p53 [32]. However, the importance of p21[Cip-1] in p53-dependent and/or p53–independent apoptosis is still not clearly defined. The use of p53 as a target for cancer therapy is being pursued; various therapeutic rationales targeting p53 are currently under investigation including attempts to activate p53. This approach is based on the concept that activation of p53 in a tumor is cytotoxic. So far, many researchers have pursued the use of small molecules to activate p53, the advantages being the potential for large-scale chemical synthesis, easier delivery *in vivo*, and the current existence of cell-based assay systems which have been developed to screen p53 regulating small molecules [33]. Other apoptosis enhancing tumor suppressor genes include Bcl-2 family members which, similar to p53, play a pivotal role in the intrinsic apoptotic cascade [34–36]. We investigated the role of PUMA (p53 up-regulated modulator of apoptosis), a BH-3only Bcl-2 family member, in wogonin-induced apoptosis. PUMA is a downstream target of p53 [37,38]. PUMA is localized in the mitochondrial membrane and interacts with Bcl-2 and Bcl-xL through a BH3 domain. When apoptotic stimuli induce PUMA expression, PUMA binds to Bcl-xL, releasing Bax [39], Bax translocates to the mitochondrial membrane, binds cytochrome *c*, and multimerizes Apaf-1 [40,41] to play an important role in stress-induced apoptosis [42,43]. The balance between p21[Cip-1]and PUMA is pivotal in determining whether the cells undergo the cycle arrest response to p53 which mediates apoptosis in human cancer.

Our results provide molecular evidence to demonstrate that wogonin effectively induces apoptosis in human prostate carcinoma LNCaP and human colon carcinoma HCT116 cells;

interestingly, we found that wogonin enhances not only the expression of tumor suppressor protein p53, but also the expression of its targets, cyclin-dependent kinase (CDK) inhibitors p21[Cip-1] and p27[Kip-1] as well as the pro-apoptotic protein PUMA. We also demonstrated that wogonin-induced apoptosis is mediated by the p53-PUMA-Bax-cytochrome c-caspase 9-caspase 3 pathway.

2. Materials and methods

2.1. Reagents and antibodies

Wogonin, baicalin, and baicalein (>99% pure) were obtained from Wako Chemical Co. (San Francisco, CA, USA). Rabbit polyclonal anti-caspase-3 antibody, anti-poly (ADP-ribose) polymerase-1 (PARP-1) antibody, anti-Bcl-xL antibody and anti-p53 antibody were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-caspase-8, anti-phospho-Ser[473] Akt, anti-Akt, anti-PUMA, anti-p21[Cip1], anti-p27[Kip-1] and anti-Bax antibodies were from Cell Signaling (Beverly, MA, USA). Monoclonal antibodies were purchased from the each of following companies: anti-caspase-9 antibody from Upstate Biotechnology (Lake Placid, NY, USA), anti-cytochrome c from PharMingen (San Diego, CA, USA) and anti-actin antibody from ICN (Costa Mesa, CA, USA).

2.2. Cell cultures

Human prostate carcinoma LNCaP and PC3 cells were obtained from ATCC (Manassas, VA, USA) cultured in RPMI-1640 medium (Gibco BRL) containing 10% fetal bovine serum (HyClone, Logan, Utah, USA) and 26 mM sodium bicarbonate for monolayer cell culture.

Primary cultures of normal human prostate epithelial cells (PrEC) were purchased from Cambrex Bio Science Walkersville (Cambrex Corporation; East Rutherford, NJ), cultivated in PrEBM[TM] (Prostate Epithelial Cell Basal Medium; Cambrex) and supplemented with PrEGM SingleQuots® (bovine pituitary extract, hydrocortisone, hEGF, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and gentamicin; Cambrex). Cells were maintained in accordance with manufacturer's instructions (Clonetics[TM] Prostate Epithelial Cell System).

p53-containing (p53^{+/+}) and p53-deficient (p53^{−/−}) HCT116, PUMA-containing (PUMA^{+/+}) and PUMA-deficient (PUMA^{−/−}) HCT116, and Bax-containing (Bax^{+/−}) and Bax-deficient (Bax^{−/−}) HCT116 human colon carcinoma cell lines were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA). These cell lines were cultured in McCoy's 5A medium (Gibco-BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum and antibiotics. The dishes containing cells were kept in a 37 °C humidified incubator with 5% CO2.

2.3. Drug treatment

Wogonin, baicalin, and baicalein (dissolved in dimethyl sulfoxide (DMSO)) were used for the treatment of cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. For dose-dependent studies, cells were treated with wogonin at 10–100 μM final concentrations for 24 h in complete cell medium. Control cells were treated with vehicle alone.

2.4. Determination of cell viability

One or two days prior to the experiment, cells were plated into 60-mm dishes at a density of 1×10^5 cells/plate in 5 ml tissue culture medium in triplicate. For trypan blue exclusion assay [44], trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min, and examined under a light microscope. At least 300 cells were counted for each survival determination.

2.5. DNA fragmentation

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis. Briefly, the cell pellets were resuspended in lysis buffer (10 mM Tris–HCl, pH 8.0; 25 mM EDTA; 0.5% SDS, 100 mM NaCl, and 200 μg/ml proteinase K) and incubated at 55 °C for 2 h. The cell lysate was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and then centrifuged for 15 min. The supernatant was incubated with RNase A (0.2 μg//ml) at 37 °C. After 1 h, the DNA was extracted with phenol and precipitated with one-tenth the volume of 3 M sodium acetate and 3 volumes of 100% ethanol. The DNA samples, dissolved in 1× TE buffer, were separated by horizontal electrophoresis on 1.8% agarose gels, stained with EtBr, and visualized under UV light.

2.6. Transfection

In order to generate p53 overexpressing PC3 cells, cells were transfected with 2 μg of pcDNA3-neo or pcDNA3-Flag-p53, which were kindly provided by Dr. Roberts TM (University of Harvard, Boston, MA, USA), using LipofectAMINE Plus (Gibco-BRL Life Technologies, Grand Island, NY, USA). The expression level was determined by immunoblot analysis.

2.7. Measurement of cytochrome c release

To determine the release of cytochrome c from mitochondria, subconfluent LNCaP cells were grown in 100-mm dishes. Cells were treated with wogonin (100 μM) for 24 h. Using Mitochondrial Fractionation Kit (Active Motif, Carlsbad, CA, USA), mitochondria and cytosol fractions were prepared from treated cells from instructions and reagents included in the kit.

2.8. Bax oligomerization

To detect the formation of Bax multimeric complexes, aliquots of isolated mitochondrial fractions and cytosolic fractions were cross linked with 1 mM dithiobis (succinimidyl propionate) (Pierce, Rockford, IL, USA) at 37 °C for 30 min. DMSO alone was used as the control. The cross linked samples were then centrifuged at 13,000 rpm for 15 min at 4 °C. After the supernatant was removed, the pellet was washed once with

homogenization buffer (sucrose 0.25 M, HEPES pH 7.4 10 mM, EGTA 1 mM) and lysed with 2× native sample buffer. Samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions followed by immunoblotting for Bax.

2.9. Immunoblot analysis

Cells were lysed with 1× Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, and 0.3 mM bromophenol blue) and boiled for 7 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The samples were diluted with 1× lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8-12% SDS-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli [45] using a Hoefer gel apparatus. Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS-Tween-20 (0.1%, v/v) for 1 h. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) at 4 °C overnight. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights, IL, USA). To ensure equal protein loading, each membrane was stripped and reprobed with anti-actin antibody to normalize for differences in protein loading.

2.10. ChIP and PCR analysis

ChIP was performed by using the Chromatin Immunoprecipitation Assay kit (Upstate Biotechnology, Lake Placid, NY) according to manufacturer's instructions with minor modifications. Briefly, ≈2 × 10^6 cells were fixed with 1% formaldehyde and lysed in SDS lysis buffer. DNA in the cross-linked chromatin preparations was sheared to 200–1000 bp by sonication. Samples were precleared with salmon sperm

DNA/protein A agarose (50%) slurry. After addition of antibodies and fresh protein A agarose, the samples were incubated at 4 °C overnight. Normal mouse or rabbit IgG was used as a control. Precipitated chromatin complexes were eluted by 500 μl of elution buffer (1% SDS, 0.1 M NaHCO$_3$) for 30 min. Finally, the protein-DNA cross-links were reversed by overnight incubation with 100 μM NaCl at 65 °C, and immunoprecipitated DNA was analyzed by PCR using primers as follows: PUMA (region −152 to −46) forward primer, 5-GCGAGACTGTGGCCTTGTGT-3; PUMA (region −152 to −46) reverse primer, 5-CAAGTCAGGACTTGCAGGG-3. Antibodies for ChIP included rabbit polyclonal antibodies against p53 (Santa Cruz Biotechnology), and monoclonal antibodies for acetylated histones H3 (Cell Signaling, Beverly, MA, USA).

2.11. Statistical analysis

Statistical analysis was carried out using Graphpad InStat 3 software (GraphPad Software, Inc., San Diego, CA, USA). Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Baicalein, baicalin, and wogonin affect survival of LNCaP cells differently

Flavonoids are diphenylpropanes that are commonly found in plants. More than 4000 flavonoids have been found and are frequently components of the human diet. However, several biological activities of flavonoids are still undefined. It is well known that baicalein, baicalin, and wogonin are the major flavonoids produced by S. baicalensis (Fig. 1). In this study, we examined the effects of baicalein, baicalin, and wogonin on cell viability and drug-induced PARP-1 cleavage, the hallmark feature of apoptosis, in human prostate LNCaP cells by trypan blue exclusion dye assay and Western blotting, respectively (Fig. 2). When LNCaP cell were treated with various concentrations of each indicated compound (10, 25, 50 and 100 μM) for
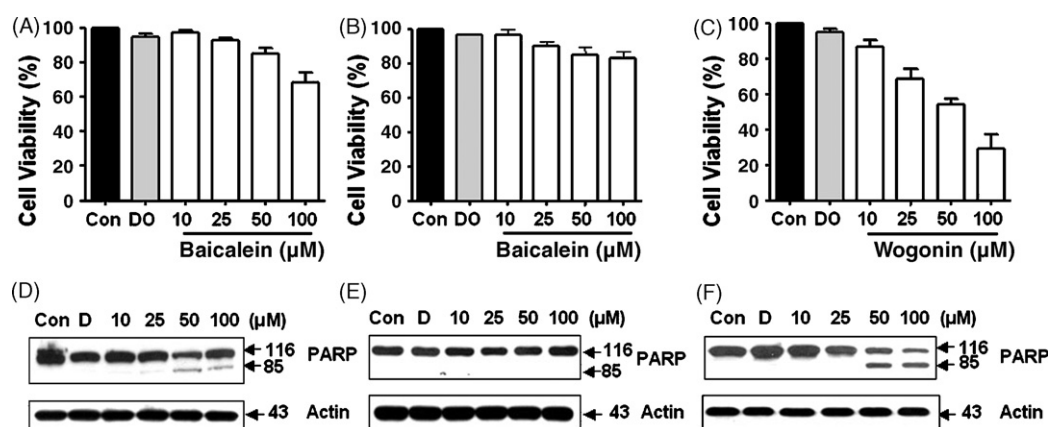
Fig. 2 – Effects of baicalein, baicalin, and wogonin on the cell viability of human prostate carcinoma LNCaP cells. Cells (1 × 10^5) were plated, allowed to attach overnight, and then treated with DMSO (control) or 10–100 μM of baicalein, baicalin, or wogonin for 24 h at 37 °C. (A, B, C) Cell viability was determined by trypan blue dye exclusion assay. Error bars represent the mean ± SE from three separate experiments. (D, E, F) Cells were harvested and samples were prepared for analysis of the cleavage of PARP-1 using Western blot analysis. Actin was shown as an internal standard.

Fig. 3 – Wogonin induces apoptosis in LNCaP cells. Cells were treated with various concentrations (10–100 μM) of wogonin or 0.1% DMSO for 24 h. (A) Cell morphology was examined under a light microscope. Magnificat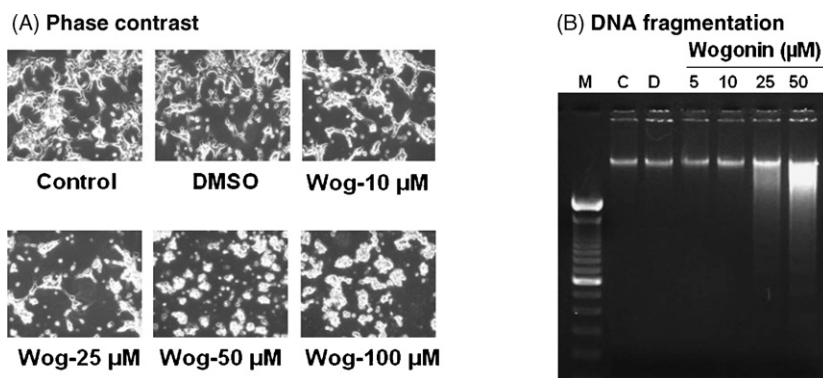ion, ×200. Wog: wogonin. (B) DNA isolated from cells that were untreated (C, lane 2), or treated with DMSO (D, lane 3) or wogonin (lanes 4–7) was fractionated by electrophoresis. Oligonucleosomal length DNA fragments were visualized by staining gels with ethidium bromide. Lane 1, DNA ladder. Similar results were obtained in two separate experiments.

24 h, significant concentration-dependent reduction of the viability of LNCaP cells was observed (upper panels in Fig. 2) and increase in the cleavage of 116 kDa PARP-1 into 85 kDa fragments was detected in the presence of wogonin and to a lesser extent, baicalein, but not obvious in baicalin-treated cells. DMSO (lower panels in Fig. 2), even at the highest dose of 0.5%, showed no effect on the cellular viability of LNCaP cells (data not shown).

3.2. Wogonin causes apoptotic death of LNCaP cells

To confirm whether wogonin induces apoptosis in LNCaP cells, in the first step, we examined wogonin-induced cytotoxicity. To investigate morphological changes, cells were treated with 0–100 μM wogonin for 24 h and then observed under a light microscope and photographed. Observations made under the microscope showed that, after wogonin treatment, the cell number decreased (data not shown) and, more interestingly, the shape of cells had changed in comparison to control cells. As shown in Fig. 3A, in the cultures treated with wogonin there were fewer cells, and apoptotic cell death, which is associated with typical morphological features like cell shrinkage and cytoplasmic membrane blebbing. In Fig. 3B, we also examined the effect of increasing concentrations of wogonin on the induction of DNA ladder formation (DNA banding characteristic of late apoptosis). We noted differences in DNA banding formation between control lanes and those treated with increasing concentrations of wogonin for 24 h.

3.3. Caspase activation mediates wogonin-induced apoptosis

Activation of both extrinsic and intrinsic caspase pathways has been well established to be the major mechanism of apoptotic cell death in most cellular systems [46]. Based on our findings showing that wogonin causes strong apoptotic death of LNCaP cells, we first assessed whether wogonin activates caspase pathways. Treatment of cells with wogonin indeed

resulted in strong dose-dependent caspase-8, caspase-9, and caspase-3 activation and cleavage (Fig. 4A, B and C). Western blot analysis shows that procaspase-8 (57/55 kDa) was cleaved to the intermediate (43/41 kDa) forms by treatment with wogonin. Wogonin induced proteolytic processing of procaspase-9 (46 kDa) into its active form (34 kDa). Fig. 4C also
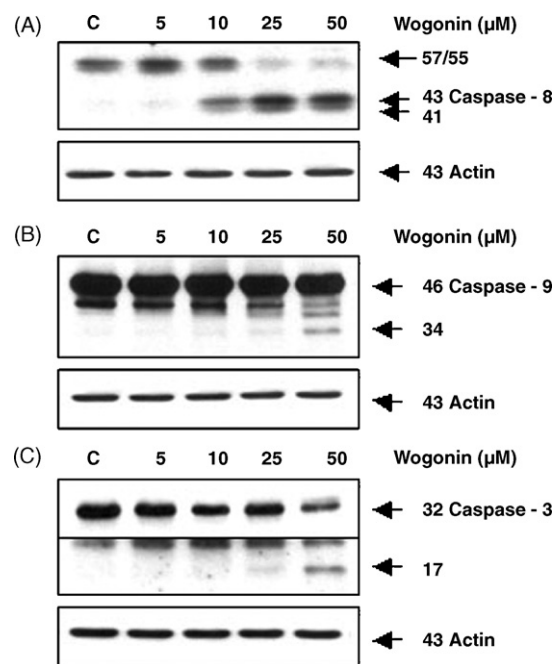
Fig. 4 – Wogonin activates caspases in LNCaP cells. *In vitro* treatment of LNCaP cells with wogonin caused the cleavage of caspase-8 (A), caspase-9 (B), and caspase-3 (C). Cells were treated with various concentrations of wogonin (5–50 μM) for 24 h, and then cells were harvested. Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted. Actin was used to confirm the equal amount of proteins loaded in each lane.

demonstrates that procaspase-3 (32 kDa), the precursor form of caspase-3, was cleaved to active form (17 kDa) by treatment with wogonin. Fig. 4C shows a line through the middle of the blots. This is due to combining images having two different exposure times which came from the same blot, because the image of 17 kDa was relatively weak.

3.4. Wogonin causes release of cytochrome c from mitochondria to cytosol

The activation of caspase-9 during treatment with wogonin indicates an involvement of the intrinsic pathway in wogonin-induced apoptosis. One of the hallmarks of the intrinsic pathway of apoptosis is the release of mitochondrial cytochrome c into the cytosol [47,48]. Mitochondrial dysfunction promoted by Bax translocation usually leads to the leakage of cytochrome c from mitochondria [49]. Cytochrome c released into the cytosol forms a multimeric complex with apoptotic protease-activating factor 1 and pro-caspase leading to the activation of caspase-9 and downstream caspases [50]. In our current study, to provide evidence of cytochrome c release to the cytosol following wogonin treatment, we compared amounts of cytochrome c in the mitochondria and in the mitochondria-free cytosolic extracts prepared from LNCaP cells treated with or without wogonin for 24 h. We used actin for a cytosolic marker and COX IV for a mitochondrial marker as fractional markers and loading controls. Fig. 5 clearly demonstrates that wogonin induced cytochrome c release in a dose-dependent manner. Altogether, the data shown in Figs. 4B, C and 5 clearly indicate that wogonin activates the intrinsic caspase cascade via cytochrome c release into the cytosol from the mitochondria, leading to caspase-9 followed
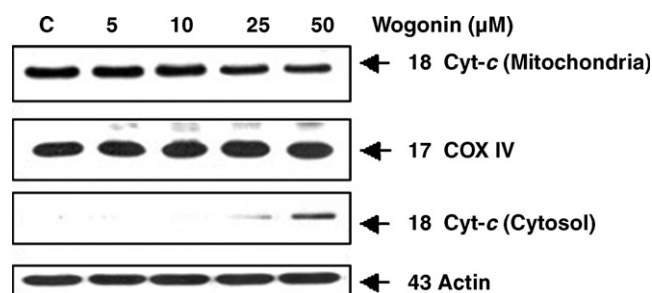

Fig. 5 – Wogonin treatment causes release of cytochrome c from mitochondria to the cytosol in LNCaP cells. Cytochrome c release from mitochondria to cytosol was examined in LNCaP cells which were incubated overnight with wogognin (5, 10, 25, and 50 μM) or without (C, control). Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-cytochrome c antibody. We used actin as a cytosolic marker and COX IV as a mitochondrial marker. The top and third panels represent mitochondrial and cytosolic fractions, respectively.

by caspase-3 activation that results in PARP-1 cleavage and apoptotic cell death.

3.5. Wogonin enhances apoptosis in LNCaP, but not in PrEC cells

We also compared the cytotoxicity of wogonin to both human prostate carcinoma LNCaP cells and normal human prostate
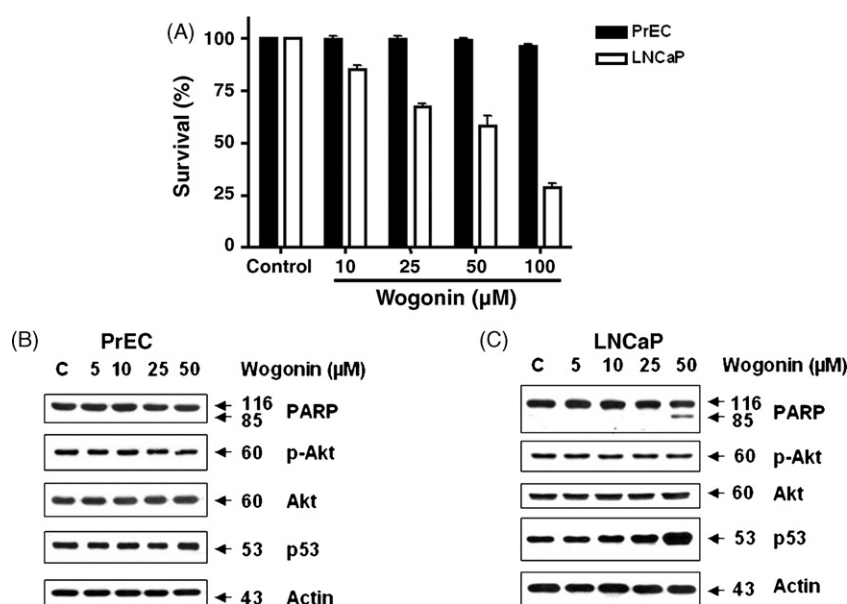
Fig. 6 – Wogonin induces cytotoxicity in human prostate cancer LNCaP cells, but not in prostate epithelial cells (PrEC). LNCaP and PrEC cells were treated with various concentrations (10–100 μM) of wogonin for 24 h. (A) The cytotoxic effect of wogonin on LNCaP and PrEC cells was determined using the trypan blue dye exclusion assay. Error bars represent standard error of the mean (S.E.M.) from three separate experiments. (B and C) Equal amounts of protein (20 μg) from cell lysates of PrEC or LNCaP cells were separated by SDS-PAGE and immunoblotted with anti-phospho-Akt, anti-Akt, anti-PARP-1, or anti-p53 antibody. Actin was shown as an internal standard.

epithelial PrEC cells. To determine the accurate range of cytotoxic concentrations of wogonin in both cell lines, we used the trypan blue exclusion dye assay to measure cell viability. Cells were exposed to 0–100 μM of wogonin for 24 h. Wogonin induced cell death in a dose-dependent manner after 24 h of incubation in LNCaP but not PrEC cells (Fig. 6A). For LNCaP cells but not for PrEC cells, incubation with wogonin for 24 h resulted in dramatic cell mortality with an estimated 50% of cell death (IC$_{50}$) at a value of 60 μM (Fig. 6A\). Likewise, for LNCaP cells but not for PrEC cells, wogonin induced apoptosis as indicated by PARP-1 cleavage (Fig. 6B and C). It is well known that decreased Akt activity accompanies flavonoid-induced apoptosis [51]. Previous studies demonstrated that Akt activation is regulated through the PI3K-Akt pathway [52]. To examine whether wogonin inhibits Akt activity by dephosphorylating Akt, we treated LNCaP and PrEC cells with various concentrations of wogonin and measured the level of phophorylated Akt. We observed that wogonin did not induce dephosphorylation of Akt in both LNCaP and PrEC cells (Fig. 6B and C). Interestingly, we observed that an increase in p53 level occurred during treatment with wogonin in LNCaP, but not PrEC cells (Fig. 6B and C). These data suggest that an increase in p53 level may be involved in wogonin-induced apoptotic death in LNCaP cells.

3.6.   Role of p53 in wogonin-induced apoptotic death

We hypothesized that p53 plays an important role in wogonin-induced apoptosis. To test the hypothesis, we examined p53-associated protein levels during treatment with wogonin.

Since the p53 protein is a major regulator of cell cycle and apoptosis following genotoxic damage [26,53,54], we screened cell cycle or apoptosis-related proteins. Fig. 7A and B show that consistent with accumulation of p53, p21^{Cip-1}, p27^{Kip-1} and PUMA proteins accumulated in wogonin-treated LNCaP cells. Wogonin increased the intracellular levels of these proteins in dose- and time-dependent manners. Unlike p53-wild LNCaP cells, p53-null PC-3 cells showed no increase in p27^{Kip-1} and PUMA proteins during treatment with wogonin (Fig. 7C). Moreover, wogonin treatment did not induce PARP-1 cleavage in PC-3 cells. To confirm the involvement of p53 in wogonin-induced apoptosis, two sets of experiments were performed. In the first set, two HCT116 human colon adenocarcinoma cell lines, one containing a wild-type p53 (p53$^{+/+}$) and the other p53-deleted derivative (p53$^{-/-}$), were treated with wogonin. Immunoblotting of cell lysates revealed a sustained increased level of p53 protein after 8 h exposure to wogonin in HCT116 p53$^{+/+}$ cells, which was maintained at least 24 h after treatment (data not shown). As shown in Fig. 8A, the IC50 (dose to kill 50% of cells) value of wogonin in HCT116 p53$^{+/+}$ cells (115 μM) was substantially lower compared with that of HCT116 p53$^{-/-}$ cells (210 μM), reflecting the fact that HCT116 p53$^{-/-}$ cells are resistant to wogonin and that p53 plays a role in wogonin-induced cytotoxicity. Fig. 8A also shows that a lower amount of PARP-1 cleavage and PUMA accumulation occurred in HCT116 p53$^{-/-}$ cells in comparison to HCT116 p53$^{+/+}$ cells during treatment with wogonin. In the second set of experiments, p53-null PC-3 cells were transfected with plasmid containing p53 cDNA (pcDNA3-p53) to determine the involvement of p53 in wogonin-induced
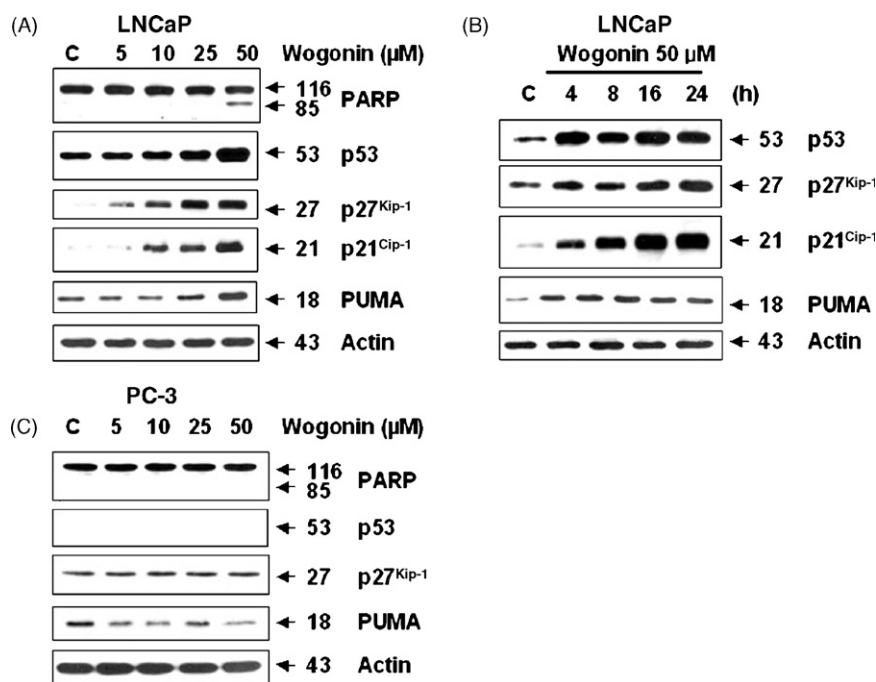
Fig. 7 – Wogonin increases the intracellular level of p53 and its downstream cell cycle-related proteins as well as apoptosis-related protein in human prostate carcinoma LNCaP cells, but not in p53-null PC-3 cells. LNCaP PC-3 cells were treated with various concentrations (0–50 μM) of wogonin for 24 h or LNCaP cells were treated with 50 μM wogonin for various times (4–24 h), and Western blot analysis was done for PARP-1, p53, p27^{Kip-1}, p21^{Cip-1}, or PUMA. Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted. Actin was used to confirm the equal amount of proteins loaded in each lane.
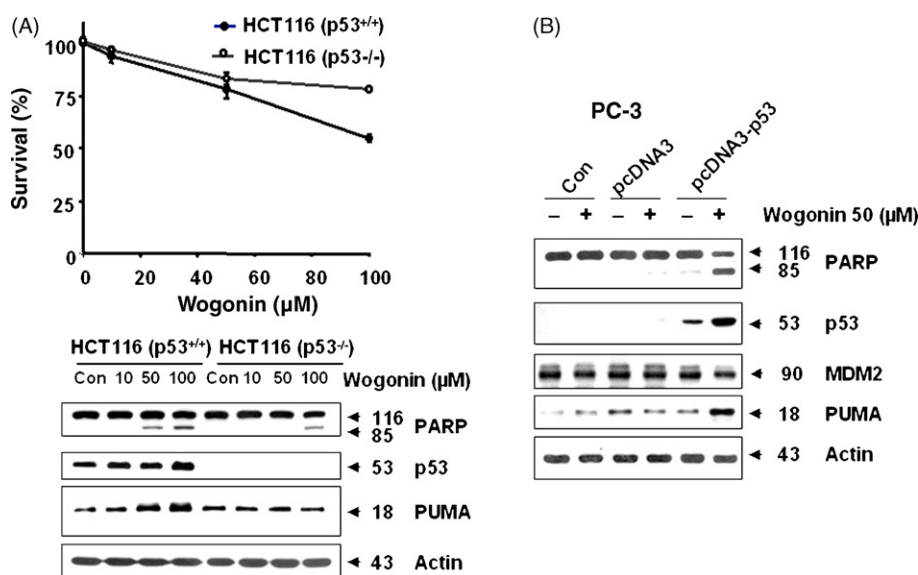
Fig. 8 – Involvement of p53 and PUMA in wogonin-induced apoptosis. (A), HCT116 p53$^{+/-}$ and HCT116 p53$^{-/-}$ cells were treated with various concentrations (10–100 µM) of wogonin for 24 h. Cell viability was determined using the trypan blue dye exclusion assay (Upper panel). Error bars represent the mean ± SE from three separate experiments. Comparisons of IC50 were made by dose response curve fitting. Equal amounts of protein (20 µg) from cell lysates cells were separated by SDS-PAGE and immunoblotted with anti-PARP-1, anti-p53, or anti-PUMA antibody (Lower panel). Actin was shown as an internal standard. (B) PC-3 cells were transiently transfected with pcDNA3 vector containing empty (pcDNA3) or wild-type p53 cDNA (PcDNA-p53). After 48 h incubation, cells were treated with or without wogonin (50 µM) for 24 h. Equal amounts of protein (20 µg) from cell lysates cells were separated by SDS-PAGE and immunoblotted with anti-PARP-1, anti-p53, anti-Mdm2, or anti-PUMA antibody. Actin was shown as an internal standard.

apoptosis. Wogonin treatment induced PARP cleavage and significantly increased the amounts of PUMA in pcDNA3-p53 plasmid transfected PC-3 cells, but had little or no effect in parental PC-3 and control vector pcDNA3 transfected PC-3 cells (Fig. 8B). These results consistently suggest that over-expression of p53 results in apoptosis by increasing PUMA, a pro-apoptotic protein. Nevertheless, it is interesting that cellular p53 levels were higher in wogonin treated cells following p53 transfection. This is not likely due to transcription of p53 mRNA (especially since p53 transcription from the plasmid promoter should not be affected). Interestingly, Fig. 8B shows that Mdm2 levels were decreased by 30% in wogonin treated cells following p53 transfection. Thus, it is possible that wogonin affects p53 levels by altering Mdm2 levels. This possibility needs to be further investigated.

3.7. Wogonin-promoted PUMA gene transcription is mediated through an increase in p53 binding to PUMA promoter

We further examined whether up-regulation of PUMA gene expression by treatment with wogonin is due to activation of transcriptional activity. Data from Western blotting in Figs. 7A and 8 show that the level of PUMA protein was significantly increased during treatment with wogonin. These results suggest that the increase of PUMA protein levels during treatment with wogonin was related to expression of PUMA gene transcription. It is well known that PUMA promoter region contains binding sites of putative transcription factors

such as p53. We hypothesized that wogonin affects binding affinity of this transcription factor in the PUMA promoter region and subsequently activates transcription of the PUMA gene. To investigate whether wogonin specifically alters p53 binding activity to the PUMA promoter region, p53 binding activity to sonicated chromatin was determined by ChiP assay. Data from ChiP assay clearly demonstrate that wogonin treatment markedly increased recruitment of p53 to the proximal site of PUMA promoter in LNCaP cells (Fig. 9A). However, when total p53 binding activity to sonicated chromatin was determined by immunoprecipitation with anti-p53 antibody followed by immunoblotting with anti-histone H3, total p53 binding activity was not significantly changed in the presence of wogonin (Fig. 9B). Taken together, our data suggest that specific promotion of p53 binding activity to PUMA promoter occurs by treatment with wogonin.

3.8. Role of Bax in PUMA-associated wogonin-induced apoptosis

To evaluate further the apoptosis associated with PUMA expression, we employed cells expressing PUMA (HCT116 PUMA$^{+/+}$) and not expressing PUMA (HCT116 PUMA$^{-/-}$) and treated them with wogonin. Fig. 10A shows that the IC50 value of wogonin in HCT116 PUMA$^{+/+}$ cells (125 µM) was substantially lower compared to that of HCT116 PUMA$^{-/-}$ cells (280 µM), reflecting the fact that HCT116 PUMA$^{-/-}$ cells are resistant to wogonin and that PUMA plays a role in wogonin-induced cytotoxicity. We also observed that 50–100 µM
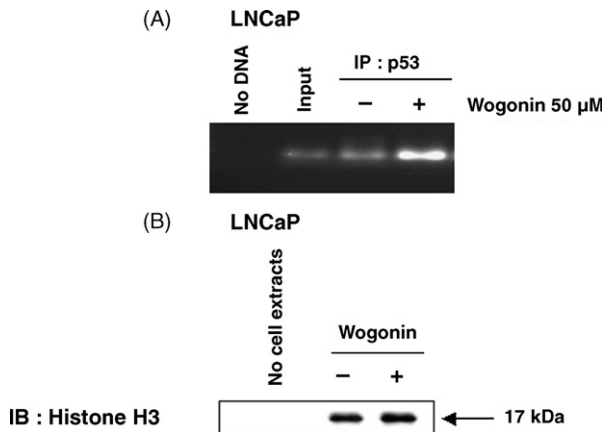
Fig. 9 – Transcription factor binding activity in PUMA promoter during treatment with wogonin in LNCaP cells. Cells were treated with 50 μM wogonin for 24 h. (A) Cells were sonicated and chromatin fragments were immunoprecipitated with anti-p53 antibody. PUMA promoter contains p53 binding sites (−1409 to −76, 8 putative binding sites). The binding of p53 on PUMA promoter was analyzed by PCR. (B), chromatin fragments were immunoprecipitated with anti-p53 antibody. Interaction between p53 and histone H3 was performed with anti-histone H3 antibody.

Fig. 10 – Role of PUMA in wogonin-induced apoptosis. HCT116 PUMA$^{+/+}$ and HCT116 PUMA$^{-/-}$ cells were treated various concentrations (10–100 μM) of wogonin for 24 h. (A) Cell viability was determined using the trypan blue dye exclusion assay. Error bars represent the mean ± SE from three separate experiments. Comparisons of IC50 were made by dose response curve fitting. (B) Equal amounts of protein (20 μg) from cell lysates were separated by SDS-PAGE and immunoblotted with anti-phospho-PARP-1, anti-p53, anti-PUMA, or anti-Bax antibody. Actin was shown as an internal standard.

wogonin was required for induction of PARP-1 cleavage in HCT116 PUMA$^{+/+}$ cells, whereas minimal cleavage of PARP-1 occurred in 100 μM wogonin treated HCT116 PUMA$^{-/-}$ cells (Fig. 10B). Although the intracellular level of p53 was increased in both cell lines during treatment with wogonin, PARP-1 cleavage was effectively increased in the presence of PUMA (Fig. 10B). These results suggest that p53 up-regulated PUMA plays an important role in apoptosis. Interestingly, there was no change in the level of Bax by treatment with wogonin. As a next step, we examined how PUMA is involved in apoptotic death. Since PUMA is a BH3 domain-containing protein found predominantly in mitochondria, we hypothesized that PUMA-induced apoptosis is mediated through Bax activation. To test the hypothesis, we first examined the role of Bax in wogonin-induced apoptosis. Previous experiments have shown that BH3 domain-containing proteins such as Bik and Bid induced apoptosis in a Bax-dependent manner. Association of BH3 domain containing PUMA with Bcl-xL may result in dissociation of Bax from Bcl-xL [55–57]. Indeed, Fig. 11A shows that Bax dissociated from Bcl-xL during treatment with wogonin. After treatment with wogonin, Bax multimerized in the mitochondria (Fig. 11B). The extent of Bax oligomerization was compatible with that demonstrated to cause pore formation in isolated mitochondria and artificial liposomes [58]. Wogonin-induced Bax oligomerization was observed in HCT116 PUMA$^{+/+}$, but not in HCT116 PUMA$^{-/-}$ (Fig. 11C). These results again suggest that PUMA-associated apoptosis is mediated through Bax. To further examine whether apoptosis induced by wogonin depends on the presence of Bax, we employed HCT116 Bax$^{+/-}$ as well as HCT116 Bax$^{-/-}$ cells. As shown in Fig. 12, compared to HCT116 Bax$^{+/-}$ cells, wogonin-induced apoptosis was significantly decreased in HCT116 Bax$^{-/-}$ cells,

even though wogonin significantly enhanced the intracellular levels of p53 and PUMA in both cell lines. The IC50 value of wogonin in HCT116 Bax$^{+/-}$ cells (115 μM) was substantially lower compared to that of HCT116 Bax$^{-/-}$ cells (280 μM).

4.   Discussion

In this study, we focused on one potential chemoprevention agent, wogonin, which is one of the active ingredients of ''golden root'', of *Scutellaria baicalensis* GEORGI (Huang-Qin). We observed the apoptosis induction activity of wogonin in human prostate carcinoma LNCaP and human colon carcinoma HCT116 cells, and the results also showed that wogonin-induced apoptosis was mediated by up-regulation of p53 and PUMA, and oligomerization of Bax. This finding reveals an interesting correlation between gene regulation and wogonin-induced apoptosis, and provides a molecular basis for the

Fig. 11 – Oligomerization of Bax during wogonin treatment. LNCaP, HCT116 PUMA$^{+/+}$, or HCT116 PUMA$^{-/-}$ cells were treated with 50 μM wogonin for 24 h. (A) Cell lysates for LNCaP cells were immunoprecipitated with anti-Bax antibody or mock antibody (IgG) and immunoblotted with anti-Bcl-xL antibody (upper panels). The presence of Bax in the lysates was verified by immunoblotting (lower panel). (B and C) Mitochondrial and cytosolic fractions were isolated and cross-linked and then subjected to immunoblotting with an antibody to Bax. Bax monomers (1×) and multimers (2×–4×) are indicated. We used actin for a cytosolic marker and COX IV for a mitochondrial marker as fractional markers and loading controls.

development of naturally occuring monoflavonoids as novel anticancer agents for better management of human cancers. Also, an important observation of our investigation is that wogonin did not cause apoptosis or result in decreased viability, even at the highest tested concentration (100 μM) in normal human prostate epithelial PrEC cells (Fig. 6A). This is important because an ideal chemoprevention agent should be able to eliminate cancer cells without any toxicity to normal cells.

During this study, we investigated the action of p53, which has been known to have a major cellular role, functioning as a cell cycle regulator by arresting the cell cycle and as an inducer of apoptosis in response to a variety of cellular stresses, sometimes inducing apoptosis by inducing transcription in genes that encode pro-apoptotic factors, such as PUMA ([59–62]; Figs. 7 and 8). Since the induction of apoptosis is a major mechanism of most chemotherapeutic agents, many attempts have been made to develop small molecules to induce p53

within tumor cells and thus induce p53-dependent apoptosis [63,64]. Several mechanisms of apoptosis induction by p53 have been identified involving transcriptional and/or non-transcriptional regulation of its downstream effectors [65]. For example, p53 is known to induce apoptosis by transcriptional up-regulation of proapoptotic genes such as Noxa, PUMA, Bax, Apaf-1, and by transcriptional repression of Bcl-2 and inhibitors of apoptosis [65]. However, in our study, we observed that wogonin treatment does not alter the levels of Bcl-2 and Bax (data not shown; Fig. 10B). In the case of non-transcriptional mechanisms, it has been reported that p53 translocates to mitochondria preceding cytochrome c release and pro-caspase-3 activation [66]. It has also been reported that p53 induces apoptosis via physical interaction with the antiapoptotic proteins Bcl-2 and Bcl-xL through its DNA-binding domain, thus leading to sequestration of these proteins from their interaction with proapoptotic partners, Bax/Bak proteins, and, as a result, allowing Bax/Bak to form
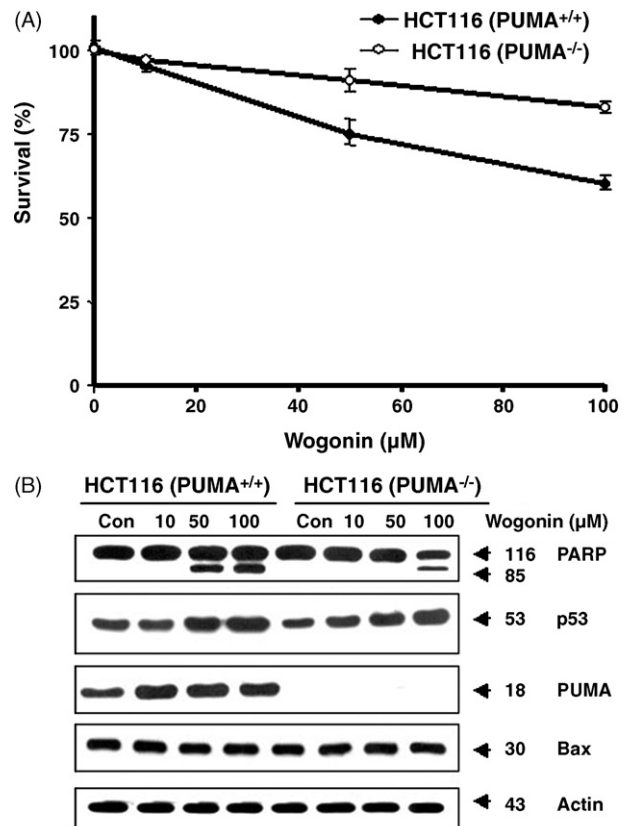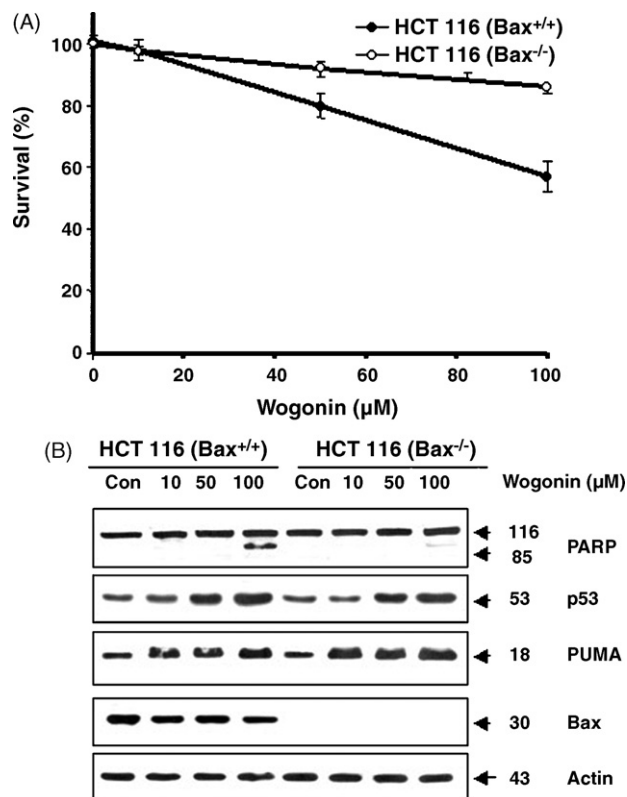
Fig. 12 – Role of Bax in wogonin-induced apoptosis. HCT116 Bax$^{+/-}$ and HCT116 Bax$^{-/-}$ cells were treated with various concentrations (10–100 μM) of wogonin for 24 h. (A) Cell viability was determined using the trypan blue dye exclusion assay. Error bars represent the mean ± SE from three separate experiments. Comparisons of IC50 were made by dose response curve fitting. (B) Equal amounts of protein (20 μg) from cell lysates were separated by SDS-PAGE and immunoblotted with anti-phospho-PARP-1, anti-p53, anti-PUMA, or anti-Bax antibody. Actin was shown as an internal standard.

oligomers and permeabilize the outer mitochondrial membrane, thereby causing the release of mitochondrial cytochrome *c* to the cytosolic compartment of the cell [67]. Further, it has been recently shown that Eugenol, a principal component of *Szygium aromaticum* (L.) Merr. Et Perry flower bud (cloves), induces apoptosis in mast cells by increasing p53 in the mitochondria where it interacts with Bcl-2 and Bcl-xL, thus abrogating their survival function [68]. Consistent in part with these studies, we also observed an increase in p53 in wogonin-treated cells, which might be responsible for inducing apoptotic death of LNCaP cells by similar mechanisms. However, our data clearly demonstrated that p53-associated apoptosis is mainly mediated through PUMA (Figs. 10 and 11C). Thus, additional studies are required in the future to further elucidate the exact mechanism and role of p53 in wogonin-induced apoptotic cell death.

Recent studies and our studies have shown that two p53-binding sites are present on the PUMA promoter ([37,69–71]; Fig. 9A). Nuclear p53 proteins interact with both binding sites and regulate PUMA gene expression [71]. It is well known that p53 levels are maintained at low and inactivate state under normal conditions. Activation of p53 can occur in response to a variety of cellular stresses, including hypoxia, nucleotide deprivation and DNA damage, which rapidly increase p53 levels and promote its DNA binding activity [72]. An increase in p53 level is achieved through post-translational modification of p53 protein rather than transcriptional regulation [73]. In this study, we observed that an increase in p53 level with an increase in its ability to bind to the PUMA promoter occurred during treatment with wogonin (Figs. 6, 7 and 9). Thus, a fundamental question which remains unanswered is how wogonin treatment increases the intracellular level of p53. At the present time, we can only speculate about the mechanism of up-regulation of p53 during wogonin treatment. Fig. 8B shows that cellular Mdm2 level is lower in wogonin treated cells following p53 infection. It is possible that wogonin affected the p53 level by altering the Mdm2 level in the pcDNA3-p53 plasmid transfected cells. The other possibility is that wogonin induces a series of post-translational modification events (phosphorylation, dephosphorylation, methylation, sumoylation and acetylation) in the p53 protein. Previous studies have demonstrated that p53 can be phosphorylated *in vitro* on both the N-terminal and C-terminal regulatory domains by various kinases, including cyclin-dependent kinases (Cdks), casein kinase I (CKI), casein kinase II (CKII), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), C-Jun N-terminal kinase (JNK), Raf kinase, DNA-dependent protein kinase (DNA-PK), ataxia-telangiectasia-mutated (ATM) kinase, and ATM- and Rad3-related (ATR) kinase [74–82]. Dephosphorylation methylation, sumoylation and acetylation of p53 which also occurs in response to DNA damage may regulate p53 activity [83–85]. It is well known that post-translational modifications of p53 free p53 from p53-Mdm2 (mouse double minute 2) complex and result in dramatically increasing the half-life of p53. Thus, it is possible that wogonin induces post-translational modifications of p53 and increases the intracellular level of p53. We believe that many critical questions still remain to be answered to understand the mechanisms of the up-regulation of p53 during treatment with wogonin. However, this model will provide a framework for future studies.

In Fig. 6, we observed that normal cells, compared with tumor cells, are resistant to wogonin-induced apoptosis. Previous studies have shown that reduced amounts of antioxidant enzymes, especially MnSOD, are found in a variety of cancer cells [86]. Also lowered amounts of CuZnSOD have been found in many, but not all, tumors. MnSOD and CuZnSOD are essential primary enzymes that convert $O_2^{\bullet-}$ to H_2O_2 and O_2 within the mitochondria and cytoplasm, respectively. These observations suggest a rationale as to why tumor cells are more sensitive than normal cells to wogonin-induced reactive oxygen species [87]. However, contradictory observations were also reported [18,88]. Thus, this hypothesis needs to be tested.

Acknowledgements

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