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Taiwanin A inhibits MCF-7 cancer cell activity through induction of oxidative stress, upregulation of DNA damage checkpoint kinases, and activation of p53 and FasL/Fas signaling pathways

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ABSTRACT

This study investigates the anti-MCF-7 breast cancer cell effects and the underlying pharmacological activity and mechanism of taiwanin A, a major lignan isolated from *Taiwania cryptomerioides*. Our results show that taiwanin A time-dependently induced reactive oxygen species level and DNA damage in MCF-7 cells, which were likely activated kinases ataxia telangiectasia mutated (ATM) and checkpoint kinase (Chk). Taiwanin A could also up-regulate p53, phosphorylated p53, $p21^{Cip1}$, and $p27^{Kip1}$ and down-regulate the G_2/M checkpoint cyclin-dependent kinase1 (Cdk1)-cyclin A/B, leading to induction of G_2/M cell-cycle arrest in MCF-7 cells. Blockade of p53 gene expression by siRNA further demonstrated that the cell-cycle arrest induced by taiwanin A was p53-dependent. The FasL/Fas-mediated apoptotic signaling cascade was involved in taiwanin A-induced apoptosis via activation of caspases-10 and -7 (but not caspase-8), and proteolytic cleavage of poly(ADP-ribose) polymerase (PARP). In contrast, mitochondria-initiated apoptotic pathway was not involved. This is the first report to delineate novel mechanism of the action of taiwanin A against MCF-7 cells, suggesting this lignan may have value for development as an anti-breast cancer agent.

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Introduction

A number of potential molecular targets for novel anticancer drug discovery have been identified in cell-cycle or apoptosis mechanisms. For instance, p53, the product of a tumor suppressor gene, plays a major role in protecting mammals from neoplasia by inducing apoptosis, DNA repair, and cell-cycle arrest. Some plant-derived anticancer or chemopreventive agents, such as curcumin (diferuloylmethane) from *Curcuma longa* L., the stilbene resveratrol from grape skin and other plants, epigallocatechin gallate from green tea, and silymarin from milk thistle have been found to modulate the p53-dependent apoptotic pathway or to function as cell-cycle regulator in various cancer cell types (Choudhuri et al., 2005; Bhatia and Agarwal, 2001; She et al., 2001). An abundant sesquiterpene lactone deoxyelephantopin isolated from *Elephantopus scaber* L. significantly inhibited murine mammary TS/A cancer cell activities through induction of G_2/M arrest and apoptosis, and targeting ER machinery and suppressing proteasome activity (Lee et al., 2010; Huang et al., 2010).

The major molecular players in cell-cycle progression include Cdks, cyclins, the retinoblastoma (RB) tumor suppressor gene product and the E2F transcription factor family and associated proteins (Buolamwini, 2000). Cdk inhibitors (CKIs), such as $p21^{Cip1}$, $p27^{Kip1}$ and p57(Kip2), play a critical role in the negative control of cyclin/Cdk complexes and cell growth (Yue and Jiang, 2005). Apoptosis, a form of programmed cell death, is either developmentally regulated or activated in response to specific extracellular and intracellular stimuli or cell injury (Thompson, 1995). In the death receptor-mediated apoptosis pathway (such as by FasL/Fas interaction), initiator caspases (e.g., caspases-8, -9, and -10) and effector caspases (e.g., caspases-3, -6, and -7) are activated which specifically cleave cellular death substrates and cause biochemical and morphological changes, leading to apoptosis (Milhas et

Abbreviations: ATM, activated kinases ataxia telangiectasia mutated; Cdk, cyclin-dependent kinase; CKIs, Cdk inhibitors; Chk, checkpoint kinase; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; PARP, poly(ADP-ribose) polymerase; PCNA, proliferating-cell nuclear antigen; PI, propidium iodide; RB, retinoblastoma.

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al., 2005; Nagata, 1997). In the intrinsic mitochondria-initiated pathway, disruption of mitochondrial membrane integrity leads to release of cytochrome *c* and some apoptosis-inducing factor (e.g., Bax) to cytosol (Reed, 2003). Investigation of the mechanisms associated with chemopreventive agent-induced apoptosis can provide increased opportunity to develop novel agents for cancer prevention (Sun et al., 2004).

The endemic Taiwanese tree *Taiwania cryptomerioides* Hayata (Taxodiaceae) has excellent decay resistance and is one of the most important plantation tree species in Taiwan. Some bioactive chemical constituents have been characterized from the plant. Candinanes such as α-cadinol and cedrol are potent anti-fungal and anti-termitic agents (Chang et al., 2000a, b, 2001a,b) and the essential oils of *T. cryptomerioides* heartwood are effective against Gram-positive bacteria and mites (Chang et al., 2001a, b, 2003). Taiwanin A, a major lignan of *T. cyrptomerioides*, is cytotoxic to various cancer cells (Chang et al., 2000a,b; Ho et al., 2007). This report elucidates the detailed mechanisms of action underlying the observed bioactivities of taiwanin A against human mammary adenocarcinoma cells.

Materials and methods

Chemicals and antibodies

Taiwanin A was isolated from heartwood of *Taiwania cryptomerioides* Hayata grown in the Experimental Forest of National Taiwan University, following the extraction and purification protocol published elsewhere (Chang et al., 2000a,b). Antibodies against α-tubulin (Oncogene Science), phospho-Cdk1 (BioSource International), phospho-Chk2 (R&D Systems), caspase-7 and cytochrome *c* (BD Pharmingen), phospho-ATM (Cell Signaling Technology), proliferating-cell nuclear antigen (PCNA) (Transduction Laboratories) were purchased as indicated, all other antibodies were from Santa Cruz Biotechnology (Santa Cruz CA). *N*-acetylcysteine, H_2O_2, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) were obtained from Sigma–Aldrich, the mitochondrial stain 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and curcumin were purchased from ACROS Organics, and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFH-DA) was from Molecular Probes.

Cell culture, synchronization, and cell viability

The MCF-7 mammary adenocarcinoma and CCD966SK fibroblast cell lines were obtained from the American Type Culture Collection and grown at 37 °C in RPMI-1640 and MEM media, respectively, supplemented with 10% fetal calf serum (FBS), and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) in a humidified 5% CO_2 incubator. For cell synchronization. MCF-7 cells were cultured in RPMI medium containing only 0.5% FBS for 48 h to give a >80% synchronized cell population at phase G_1. Cell viability was determined using an MTT-based assay.

Colony-forming assay

MCF-7 cells (200 cells/well) were cultured in a 12-well plate for 3 days and then treated with vehicle (0.25% DMSO), taiwanin A or curcumin for 8 days. Cell colonies were washed twice with ice-cold PBS, fixed with 1% glutaraldehyde for 15 min, stained in 0.1% crystal violet solution for 30 min, and then washed with water. Plate was air-dried and crystal violet was dissolved by 20% acetic acid and absorbance at A_{595} was taken using an ELISA reader. Experiments were performed in triplicate.

Cell-cycle analysis

MCF-7 cells were treated with vehicle alone as a control or with taiwanin A for 48 h. Both adherent and floating cells were collected, washed with PBS, and fixed with 1 ml of ice-cold 70% ethanol overnight at 4 °C. Cells were stained with 0.2 mg/ml of propidium iodide (PI) (Sigma) in darkness for 30 min at room temperature and analyzed using a flow cytometer.

DAPI staining and TUNEL assay

The test cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and rinsed with PBS. DAPI was added and incubated for 30 min and cells were then examined by fluorescence microscopy. TdT-mediated dUTP-biotin nick-end labeling (TUNEL) was performed using ApoAlert DNA Fragmentation Assay kit (Clontech) according to the manufacturer's instructions. Briefly, the cells were fixed with 4% formaldehyde/PBS and permeabilized with 70% ethanol for 24 h at 4 °C. The cells were labeled by adding 50 μl TUNEL mix and analyzed by a Coulter EPICS XL flow cytometer (Beckman/Coulter). Free 3′-OH DNA in apoptotic cells was also detected and quantified based on degree of green fluorescence and morphological examination using fluorescent microscopy.

Measurement of reactive oxygen species (ROS)

The intracellular accumulation of ROS was determined using a sensitive free-radical indicator, H_2DCFH-DA. The nonfluorescent H_2DCFH can be oxidized by ROS (e.g., H_2O_2) to form 2′,7′-dichlorofluorescein (DCF) molecules, which emit green fluorescence. MCF-7 cells were treated with 30 μM H_2O_2, 5 μg/ml taiwanin A, 25 μM curcumin for 6 h, or with 5 μg/ml taiwanin A, 5 μg/ml catalase, 1 mM *N*-acetylcysteine (NAC), taiwanin A + catalase, and taiwanin A + NAC, respectively, for 48 h, and then incubated with 25 μM H_2DCFH-DA in darkness for 30 min. After incubation, cells were collected, washed with PBS, resuspened in PBS and then subjected to flow cytometry analysis.

Transient transfection reporter assay

Chimeric luciferase reporter genes, pPGL3-Basic vector (Promega) containing either full length or a series of 5′ flanking promoter region deletions of human *FasL* gene were constructed. A specific reverse primer (antisense), −1/−29, 5′-TATATAAGCTTCGCAGCGGCGGGCAGGG-3′ and three forward primers (sense): 5′-GTATTTCATCTGGTACCCATAACAGGGC-3′, 5′-GCTGCAGTTAACTACGGTACC ACCACAC-3′, and 5′-CGAATGGTACCG CATGCAATTATAATTC-3′ were used for the amplification of −476/−1, −967/−1, and −1504/−1 (full length) bp regions of human *FasL* promoter, respectively, flanked by KpnI and HindIII restriction sites from human lymphocyte genomic DNA using PCR and cloning into pPGL3-Basic vector. The PCR products were verified by DNA sequencing. The promoterless pPGL3-Basic vector was used as a negative control plasmid for luciferase assays. *FasL* promoter activity in arbitrary units was normalized to the activity of *Renilla* luciferase reporter.

RT-PCR analysis and Western blotting

For each RT-PCR reaction, 2 μg of total RNA isolated using Trizol reagent was used to synthesize 1st strand cDNA using SuperScript II Reverse Transcriptase (Invitrogen). For amplification of specific genes, pairs of primers were used: p53, 5′-GCTCTGACTGTACCACCATCC-3′ (sense), and 5′-CTCTCGGAACATCTCGAAG CG-3′ (antisense); $p27^{Kip1}$, 5′-TGGAGAAGCACTGCAGAGAC-3′ (sense), and 5′-GCGTGTCC-

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TCAGAGTTAGCC-3′ (antisense); $p21^{Cip1}$, 5′-CTCAGAGGAGG-CGCC ATG-3′ (sense), and 5′-GGGCGGATTAGGGCT-TCC-3′ (antisense); GAPDH (internal control), 5′-CCATCAATGAC-CCCTTCATTGACC-3′ (sense), and 5′-GAAGGCCATGCCAGTGAGCT-TCC-3′ (antisense). Total cellular proteins were prepared using a published method (Chiang et al., 2005). Protein content was measured by the Bradford method (Bio-Rad). Protein (40 μg) was separated on 5–20% gradient SDS-PAGE and immunoblotted using an ECL kit (Amersham).

Caspase-7 activity assay

Caspase-3/-7 activities were measured using the Apo-ONE Homogeneous Caspase-3/-7 Assay kit (Promega). The fluorescence intensity relative to the caspase activity was measured at excitation/emission wavelengths of 485/535 nm using a fluorescence microplate reader.

Mitochondrial membrane potential assay

$DiOC_6(3)$ uptake by mitochondria is directly proportional to its membrane potential. MCF-7 cells (1×10^6) were treated with taiwanin A for 48 h and then 40 μM $DiOC_6(3)$ was added and incubated for 30 min. The fluorescence of the cells was immediately measured using a flow cytometer.

p53-shRNA expression plasmid construction and stable transfection

Small hairpin RNA (shRNA) oligonucleotides targeting p53 (sense, 5′-TGCTGGACTCCAGTGGTAATCTACTTCAAGAGAGTAGAT-TACCACTGGAGTC-3′ and antisense, 5′-CCTGGACTCCAGTGGTAAT-CTACTCTCTTGAAGTAGATT ACCACTGGAGTCC-3′) were designed and synthesized based on previously published sequences (Brummelkamp et al., 2002). The complementary oligonucleotides were ligated into BLOCK-iT PolII miR RNAi Expression Vector (Invitrogen) following the manufacturer's protocol. Two days after transfection, the transfected cells were seeded for selection in medium supplemented with 5 μg/ml blasticidin. After about 4 weeks of selection, individual clones were isolated and expanded and then immunoblotted to confirm p53 gene knock-down.

Results

Effect of taiwanin A on growth of MCF-7 cells

The cytotoxic or anti-cell proliferation effect of taiwanin A on MCF-7 cells was examined. We observed that taiwanin A was a concentration-dependent inhibitor of MCF-7 cell growth. At 1–2 μg/ml, taiwanin A inhibited MCF-7 cell proliferation by 45–54%, while curcumin, an anticarcinogenic agent used as a reference control in this experiment, only inhibited ~16% at the same concentrations (Fig. 1A). Colony formation assays showed that, at 0.25–0.5 μg/ml, taiwanin A could drastically suppress MCF-7 cell growth, with a 45–80% reduction compared to the vehicle control. A much smaller effect on colony formation was observed in curcumin-treated MCF-7 cells, with only ~49% inhibition at 5 μg/ml (Fig. 1B).

Taiwanin A arrests cell-cycle progression and induces nuclear condensation and DNA damage in MCF-7 cells

The DNA content and cell-cycle distribution in vehicle and taiwanin A-treated MCF-7 cells were analyzed using flow cytometry. A typical time-dependent G_2/M arrest in MCF-7 cells was observed

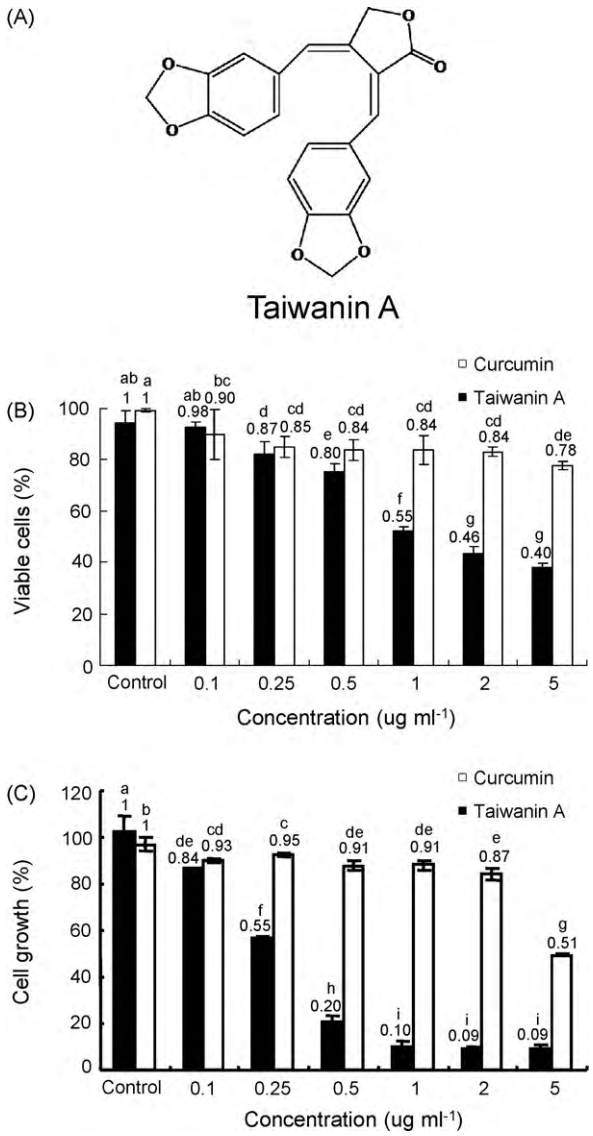

Fig. 1. Effect of taiwanin A on MCF-7 cell growth. (A) MCF-7 cells (1×10^4) were treated with or without indicated concentrations of taiwanin A or curcumin at 37 °C for 48 h, and % of viable cells was determined using MTT assay. (B) Colony formation of MCF cells was determined in a clonogenic assay as described in "Materials and methods". Data are presented as percentage of vehicle control (mean ± SD). Two-way ANOVA was used to analyze significance of differences. Different letters indicate significant differences.

with 5 μg/ml taiwanin A (Fig. 2A). The G_1 and S phase DNA contents decreased from 42.8 to 6.4 and 10.2 to 2.5%, respectively, whereas the G_2/M DNA increased from 19.4 to 61.9% when treated with taiwanin A for 48 h. Similar results were observed when MCF-7 cells synchronized with serum starvation were treated with 5 μg/ml taiwanin A at the same time points (data not shown). In addition, approximately 5% of total MCF-7 cellular DNA was detected as apoptotic (sub-G_1 DNA) after a 48 h treatment (Fig. 2A).

The effect of taiwanin A on nuclear and DNA damage in MCF-7 cells was examined. Fluorescent microscopic analysis of taiwanin A-treated MCF-7 cells stained with DAPI showed that, after 48 h treatment, a population of MCF-7 cells with characteristic condensed nuclei appeared as taiwanin A concentration increased, indicating an increase in apoptotic cell numbers. No discernible effect was observed in taiwanin A-treated CCD966SK cells, a normal fibroblast cell line (Fig. 2B). Induction of DNA damage in MCF-7 cell was investigated using TUNEL assay. The number of MCF-7 cells with significant DNA damage increased to 37% when treated

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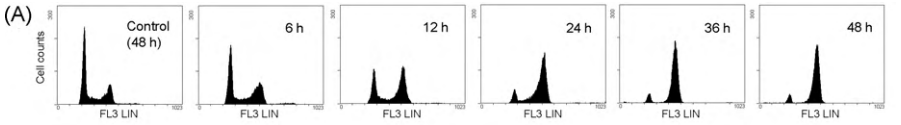
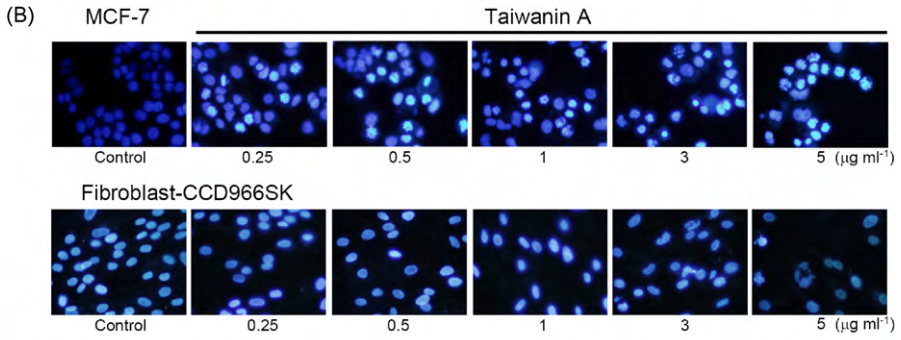
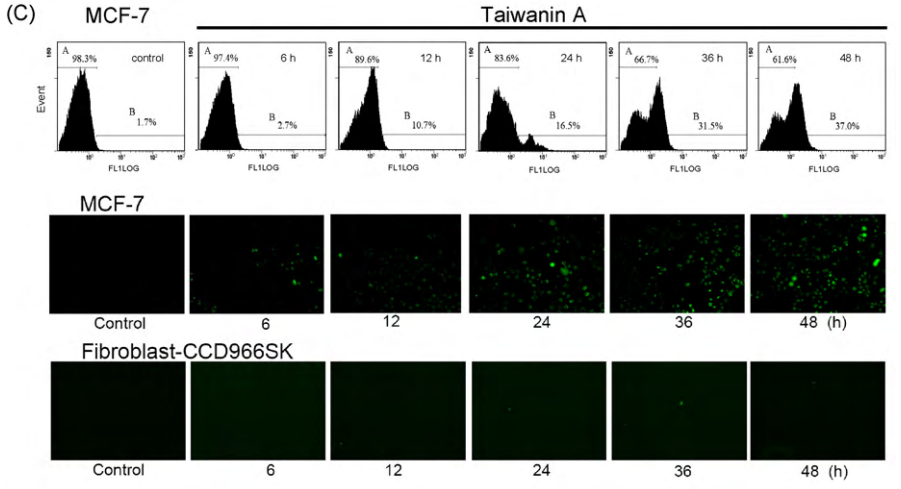


DNA (%)	Control	6	12	24	36	48	(h)
Sub-G_1	0	0	0	0	1.2	4.9	(%)
G_1	42.8	36.5	22.0	9.7	4.3	6.4	
S	10.2	13.0	11.8	12.9	1.5	2.5	
G_2/M	19.4	31.1	39.5	54.2	67.0	61.9	





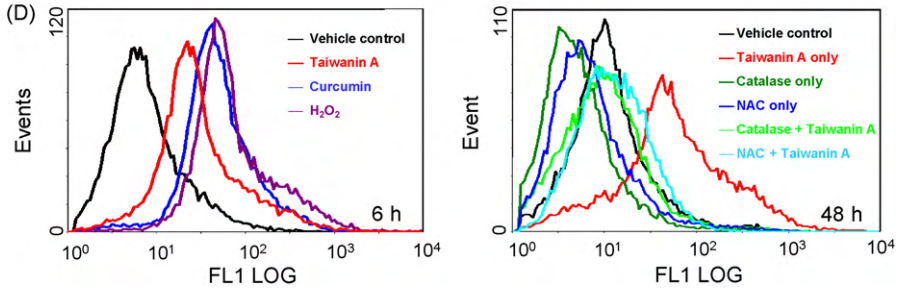


Fig. 2. Taiwanin A causes cell-cycle progression, nuclear condensation, DNA damage, and oxidative stress in MCF-7 cells. (A) Flow cytometry was employed to analyze DNA distribution in PI-stained cells. The percentage of G_0/G_1, S, and G_2/M cells was calculated. Data are representative of three independent experiments. (B) MCF-7 and CCD966SK cells were cultured at 37 °C in the chamber slide and treated with taiwanin A at indicated concentrations for 48 h, fixed with 4% paraformaldehyde, air-dried and stained with DAPI (0.1 mg/ml). Nuclear condensation was assessed by fluorescence microscopy at 330–380 nm. (C) DNA strand breakages in taiwanin A-treated MCF-7 cells were detected and quantified by the fluorescent TUNEL assay. (D) ROS generation or reversal by catalase or antioxidant NAC in taiwanin A-treated MCF-7 cells was analyzed and quantified by flow cytometry.

with taiwanin A (5 μg/ml) for 48 h, as quantified using flow cytometry (Fig. 2C). At the same concentration of taiwanin A, the number of MCF-7 cells positively TUNEL stained (green fluorescein-dUTP) increased in a time-dependent manner, indicating accumulating DNA damage (Fig. 2C). Little or no detectable DNA damage was observed in a taiwanin A-treated normal fibroblast cell line. These results demonstrate that taiwanin A effectively and specifically induced apoptosis in MCF-7 cells.

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Taiwanin A induces ROS production in MCF-7 cells

Increased ROS production is likely to act as a signaling intermediate in the signal transduction pathway of apoptosis (Aronis et al., 2003; Wu et al., 2002). We therefore examined whether taiwanin A could induce ROS production in MCF-7 cells using flow cytometry. Superoxide radical production in H_2O_2-treated MCF-7 cells was used as a positive control, as indicated by the increase of fluorescent intensity (Fig. 2D). Taiwanin A treatment (5 μg/ml for 6 h) also induced significant ROS production to a similar level of the reference control curcumin (Fig. 2D). Moreover, the induction of ROS production in MCF-7 cells treated with taiwanin A for 48 h was reversed to the level of vehicle control by co-treatment with the antioxidant agent NAC (1 mM) or the antioxidant enzyme catalase (5 μg/ml) (Fig. 2D).

Taiwanin A up-regulates FasL expression

It is known that expression of Fas ligand (FasL) can effectively mediate apoptosis by its binding to the cognate receptor Fas (Nagata, 1997). In this study, we established a FasL promoter (*FasL*) driven luciferase reporter gene transient assay to investigate the effect of taiwanin A on transcriptional activity of *FasL*. Full-length (−1504/−1) and two deletion promoter clones (−967/−1 and −476/−1), covering different *cis*-acting binding elements, were constructed (Fig. 3A). In transient transfection assays, a similar basal level of *FasL* promoter activity in vehicle-treated MCF-7 cells was observed in the full-length and in two deletion constructs (white bars). When treated with 5 μg/ml taiwanin A for 24 h, the transcriptional activity of the full-length and −967/−1 constructs increased by approximately 230% ($P < 0.005$), relative to the respective basal level activities of vehicle controls were observed in MCF-7 cells, while only a 170% increase was detected in the −476/−1 construct (Fig. 3A). This suggests that the *cis*-acting elements present in *FasL* promoter, especially c-Myb and AP-1, might be important in the taiwanin A modulated transcriptional activity of *FasL*, as less induced promoter activity was observed in the shortest −476/−1 construct with deletion of both *cis*-acting elements (Fig. 3A).

Flow cytometric analysis using FITC-labeled anti-human FasL IgG1 antibody demonstrated that taiwanin A significantly induced the expression of FasL protein in MCF-7 cells (Fig. 3B), with approximately 2-fold increase (14.8% vs. 7.7% at 16 h and 27.7% vs. 10.9% at 24 h) after treatment relative to the vehicle-treated cells. The upregulation of FasL protein in taiwanin A-treated MCF-7 cells was also observed by Western blotting (Fig. 3C). The expression of TNF-α, an inflammatory cytokine which can induce mammalian cell apoptosis, was also examined. TNF-α levels did not respond to taiwanin A treatment in MCF-7 cells (Fig. 3D). These results clearly demonstrate that taiwanin A-induced apoptosis in MCF-7 cells through FasL mediation.

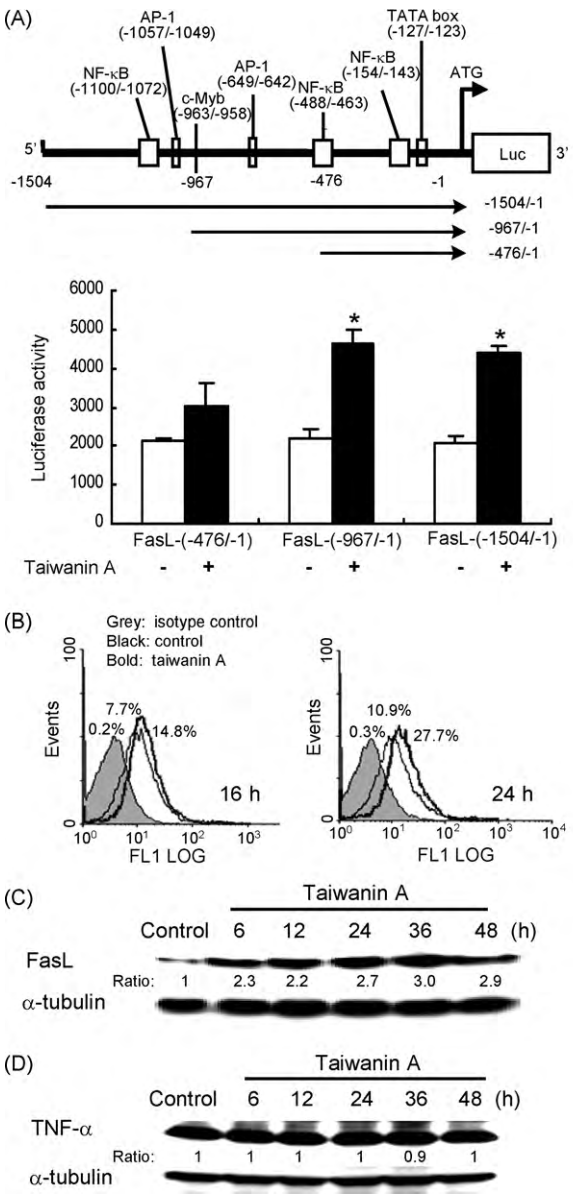

Fig. 3. Effect of taiwanin A on the transcriptional activity, and protein expression of FasL. (A) MCF-7 cells were transfected with full-length or deletion constructs of pFasL-Luc plasmid overnight and then treated with 5 μg/ml taiwanin A for 24 h. Cells were lysed and assayed for luciferase activity. Results shown are the average of three independent experiments. (B and C) FasL expression in taiwanin A-treated MCF-7 cells was quantified using immunoblotting or flow cytometry. Cells were stained with mouse anti-human FasL IgG (clone NOK-1), or isotype control mouse IgG (MOPC-21), followed by FITC-conjugated rat anti-mouse IgG. Fluorescent intensities obtained from isotype control (grey), vehicle control (black), and taiwanin A-treated (bold) cells were analyzed. (D) Western blotting of TNF-α expression in taiwanin A-treated MCF-7 cells.

Taiwanin A regulates key biomarkers activity involved in the FasL/Fas signaling pathway

A number of key protein molecules involved in the FasL/Fas signaling pathway were examined in taiwanin A-treated MCF-7 cells by Western blotting. A significant decrease in the protein level of procaspase-10 was observed in the 36 or 48 h-treated cells (Fig. 4A), whereas there was no alteration in procaspase-8 protein. Taiwanin A-induced a time-dependent proteolytic cleavage of caspase-7, the apoptotic executioner, and PARP, another hallmark of apoptosis, in MCF-7 cells. The enzymatic activity of caspase-7 in vehicle or taiwanin A-treated MCF-7 cells was also measured. A time-dependent increase of caspase-7 activity, 4.2 times higher than control after 48 h treatment with taiwanin A, was detected (Fig. 4B), which can be completely inhibited when z-AEVD-FMN, a caspase inhibitor, was added in the culture medium (data not shown).

Taiwanin A-induced apoptosis in MCF-7 cells can be reversed

As described above, taiwanin A-induced various biological responses or factors in MCF-7 cells which triggered the apoptotic events. We then investigated whether the induction of apoptosis by taiwanin A were specific and reversible by antioxidant, specific antibody or caspase inhibitor. Fig. 4C shows that the addition of catalase, antioxidant NAC, anti-Fas antibody, and caspase inhibitor z-AEVD-FMN effectively attenuated 65–85% of the taiwanin A-

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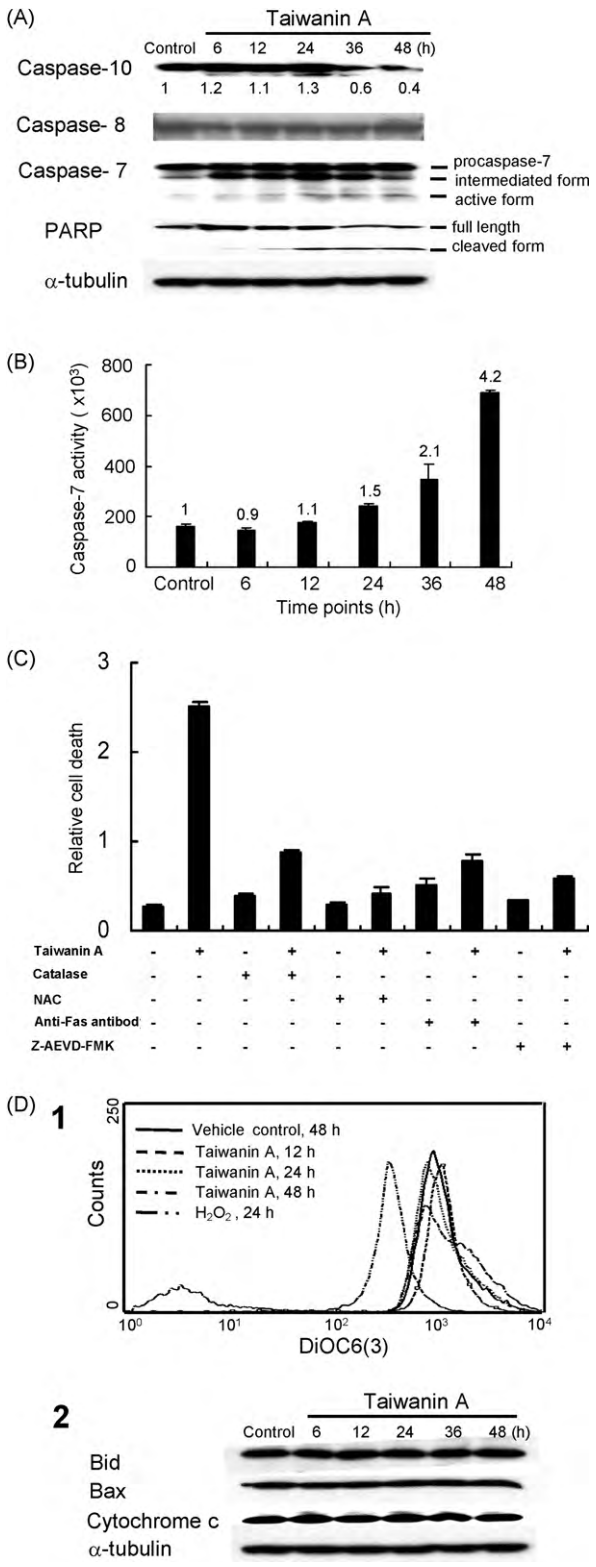

Fig. 4. Effect of taiwanin A on the protein expression of caspases-10, -7, -8, and PARP in MCF-7 cells (A). The cells were treated with vehicle or 5 μg/ml taiwanin A at indicated time points. Forty micrograms total cell lysates were subjected to SDS-PAGE followed by Western blot analysis and chemiluminescent detection. Relative changes in the protein levels were normalized to (-tubulin. (B) Caspase-7 activity in the whole cell lysates prepared from MCF-7 cells with or without taiwanin A treatment was determined. Relative changes are indicated. (C) Quantification of taiwanin A-induced apoptosis in MCF-7 cells by ELISA. Levels of apoptosis were measured in test cells using the Cell Death Detection ELISAPLUS kit (Roche, Mannheim, Germany), based on the measurement of histones complexed with mono- and oligonucleosome fragments formed during cell death. Catalase, NAC, anti-Fas antibody, or Z-AEVD-FMK were co-inoculated, respectively, with taiwanin A in MCF-7 cells cultures. Cytosolic extracts were prepared and incubated in streptavidin-coated microtiter plates with biotin-conjugated antihistone antibody and peroxidase-conjugated anti-DNA antibody for 2 h at room temperature and then washed. Colorimetric detection was carried out using ABTS substrate and absorbance at 405 nm was measured. (D1) Effect of taiwanin A on mitochondrial membrane potential ($\Delta\psi_m$) in MCF-7 cells. $\Delta\psi_m$ in MCF-7 cells (1×10^5) treated with vehicle, 5 μg/ml taiwanin A, or 100 μM H_2O_2 were measured using the $\Delta\psi_m$-sensitive dye DiOC6(3). (D2) Effects of taiwanin A on the protein expression of Bid, Bax, and cytochrome *c* in MCF-7 cells. Cells were treated with vehicle or taiwanin A at indicated time points. Forty micrograms total cell lysates were prepared and subjected to SDS-PAGE followed by Western blotting and chemiluminescent detection.

induced apoptosis, as determined by Cell Death Detection ELISA assay, indicating a highly specific induction of apoptosis in MCF-7 cells by taiwanin A.

Taiwanin A-induced apoptosis in MCF-7 cells is mitochondria-independent

To determine whether taiwanin A-induced apoptosis was through the mitochondria-mediated pathway, MCF-7 cells were treated with 5 μg/ml taiwanin A and changes in the mitochondrial membrane potential ($\Delta\psi_m$) were monitored by DiOC6(3) fluorescence using flow cytometric analysis. H_2O_2, which is known to interfere with the mitochondrial membrane potential, was used as a positive control in this study (Lee et al., 2006). As shown in Fig. 4D1, $\Delta\psi_m$ in MCF-7 cells was not changed by taiwanin A from 12 to 48 h, whereas 100 μM H_2O_2 treatment for 24 h did alter $\Delta\psi_m$ in test cells. Several key protein markers involved in mitochondria-mediated apoptosis were also examined using Western blotting (Fig. 4D2). Protein levels of Bid, Bax, and cytochrome *c* did not alter in response to taiwanin A treatment. These results indicate that taiwanin A-induced apoptosis in MCF-7 cells by a mitochondria-independent mechanism.

p53 mediates taiwanin A-induced cell-cycle arrest in MCF-7 cells

The involvement of p53 and its downstream Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ in taiwanin A-treated MCF-7 cells were investigated. Western blotting showed that the protein levels of p53, phosphorylated p53, $p21^{Cip1}$ and $p27^{Kip1}$ increased significantly with increasing exposure time to taiwanin A (Fig. 5A). Similar increases were also observed in their mRNA expression levels, as analyzed by RT-PCR (Fig. 5B).

We then created a stable MCF-7 cell line with p53 gene knockdown using a gene silencing approach by RNA interference and looked again at the expression of p53, $p21^{Cip1}$ and $p27^{Kip1}$. Taiwanin A-induced overexpression of p53, $p21^{Cip1}$ and $p27^{Kip1}$ proteins in MCF-7 cells was little or not detected in the p53 known-down MCF-7 cells (p53-siRNA MCF-7) (Fig. 5C). The taiwanin A-induced G_2/M cell-cycle arrest was also not detectable in the p53-siRNA MCF-7 cells by flow cytometry (Fig. 5D). Taken together, these results indicate that a p53-dependent cascade signaling pathway was involved in the taiwanin A modulation of the cell-cycle machinery of MCF-7 cells.

Proteins/mediators involved in cell-cycle progression and ATM signaling pathway

Western blotting (Fig. 6A) showed that cyclin D2, cyclin A, cyclin B1, Cdk1 (Cdc2), and PCNA in MCF-7 cells were time-dependently down-regulated by taiwanin A, whereas the protein level of phospho-Cdk1 increased. Wee-1 protein (which can phosphorylate Cdk1 on residue tyrosine 15 and maintain it an inactive state in G_2 phase) was also up-regulated. Cdk4, Cdk6, and cyclins D1 and D3, responsible for G1 phase progression, and

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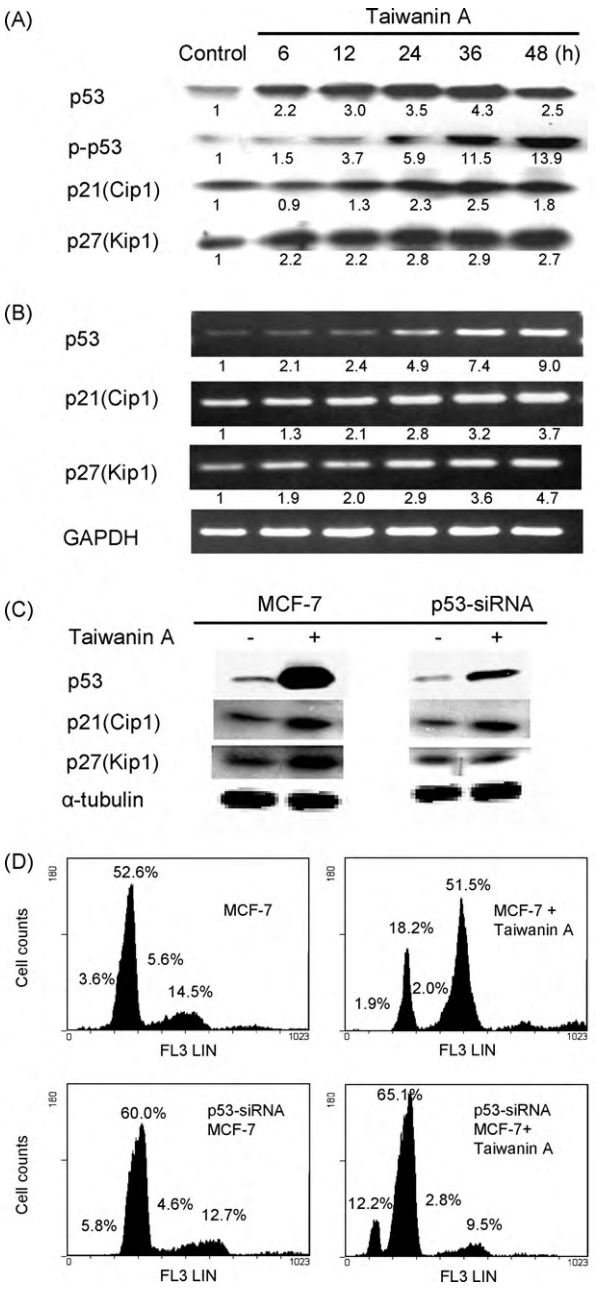

Fig. 5. Effects of taiwanin A on the protein and mRNA expression of p53, $p21^{Cip1}$, and $p27^{Kip1}$ in MCF-7 cells or in p53 gene knock-down MCF-7 cells. (A and B) MCF-7 cells were treated with vehicle or taiwanin A at indicated time points and then total cell lysates and RNA were prepared and subjected to Western blotting and RT-PCR analysis, respectively. (C) p53 gene knock-down (p53-siRNA) MCF-7 cells were treated with vehicle or taiwanin A for 48 h, and then total proteins were extracted and subjected to Western blotting. (D) Effect of taiwanin A on cell-cycle progression of p53-siRNA MCF-7 cells. Cells were incubated with taiwanin A for 48 h and stained with PI solution. The percentage of G_0/G_1, S, and G_2/M cells was analyzed using flow cytometry. Data are representative of three independent experiments.

Cdk2/cyclin E, responsible for G_1/S transition, were not or little affected in taiwanin A-treated MCF-7 cells. These results indicate that only those proteins involved in the G_2 transition to M phase in MCF-7 cells were suppressed by taiwanin A. Proteins involved in the ATM signaling pathway (ATM, phospho-ATM, phospho-H2AX and phospho-Chk2) were up-regulated by taiwanin A, while Rb, phospho-Rb and Chk2 protein levels were suppressed (Fig. 6B). These results suggest that taiwanin A suppressed Rb protein and up-regulated ATM signaling molecules which in turn triggered cell-cycle or apoptosis of MCF-7 cells.

Discussion

Identification and validation of the potential benefits of phytocompounds has become an important area of pharmaceutical science. In recent years, there has been extensive evaluation of nutrient or non-nutrient phytocompounds for life-threatening diseases such as cancers. In this respect, the abilities of many phytocompounds to function as cell-cycle modulators are gaining widespread attention (Singh et al., 2002), and apoptosis-based therapies are still the mainstream for anti-cancers (Reed, 2002). We demonstrated in this study that an abundant lignan taiwanin A in *Taiwania cryptomerioides* is a specific cell-cycle modulator (arrest on G_2/M) and an apoptosis inducer in MCF-7 cells.

It is known that, if the genome is damaged, the G_2/M checkpoint prevents the cell from entering mitosis phase, and the protein Cdk1 (or Cdc2)-cyclin A/B kinase plays a pivotal role in regulating this transition (Iwabuchi et al., 2002). DNA damage reportedly activates ATM protein kinase and its downstream Chk2 activity could then phosphorylate p53. ATM can also phosphorylate H2AX protein to prevent replication of the damaged DNA (Fernandez-Capetillo et al., 2002). Alternatively, inactivation of RB can lead to increased E2F1 activity, activation of ATM and Chk2, and subsequently phosphorylation of p53. In this study, we observed that the inactivated phosphorylated form of Rb (p-RB) increased following 6–24 h treatment with taiwanin A, and the protein levels of both Rb and p-RB were strongly suppressed by longer treatments (36–48 h). In addition, taiwanin A can also up-regulate the protein expression of ATM, and the phosphorylation of ATM, Chk2, and H2AX (Fig. 6), that are likely to be initiated by the DNA damage resulting from the taiwanin A-induced oxidative stress (Fig. 2). In turn, p53 was up-regulated and phosphorylated, which then promoted the overexpression of downstream CdkIs $p21^{Cip1}$ and $p27^{Kip1}$. The involvement and regulation of the three cell-cycle checkpoint proteins were further confirmed using siRNA-targeting on p53 gene: they did not respond to taiwanin A treatment in the p53-knockdown clone of the MCF-7 cell line (Fig. 5). The DNA damage and suppression of Rb protein synthesis induced by taiwanin A are believed to be essential in the ATM-mediated, p53-dependent cell-cycle arrest in MCF-7 cells.

The kinase activity of Cdk1 is controlled during the cell-cycle both by its association with cyclin B1 and by phohphorylation and dephosphorylation on residues Thr14 and Tyr15 crucial for the G_2/M checkpoint. Tyr15 kinase Wee-1 was up-regulated by taiwanin A in MCF-7 cells, concomitant with the decrease of Cdk1 and increase of phospho-Cdk1(Tyr15) protein, whereas phosphatase Cdc25c was not affected by taiwanin A treatment (Fig. 6A). Consistent with G_2/M arrest data analyzed using flow cytometry (Fig. 2A), the phosphorylation of Cdk1 increased significantly after taiwanin A treatment, which is believed to be responsible for inactivation of Cdk1-cyclin B1. These harmonistic actions induced by taiwanin A are believed to eventually inactivate the M phase-promoting factor Cdk1-cyclin A/B kinase and cause the cell-cycle arrest in G_2/M phase (Fig. 6C).

Taiwanin A was also observed to induce significant time- and concentration-dependent apoptosis in MCF-7 cell through the specific FasL-mediated apoptotic signaling pathway (Figs. 3 and 4). FasL expression was up-regulated by taiwanin A at both the transcriptional and translational levels, following the activation of caspase initiator caspase-10 and effector caspase-7. The caspase-mediated cleavage of the apoptotic hallmark PARP was then observed in taiwanin A-treated MCF-7 cells. Phytocompounds, such as curcumin, have been reported to mediate cancer cell apoptosis through mitochondrial signaling mechanisms (Choudhuri et al., 2005). However, mitochondria-mediated apoptosis did not occur in taiwanin A-treated MCF-7 cells because the alteration of mitochondrial membrane potential and release of Bcl-2, Bax, Bid, and cytochrome *c* proteins were not affected (Fig. 4D).

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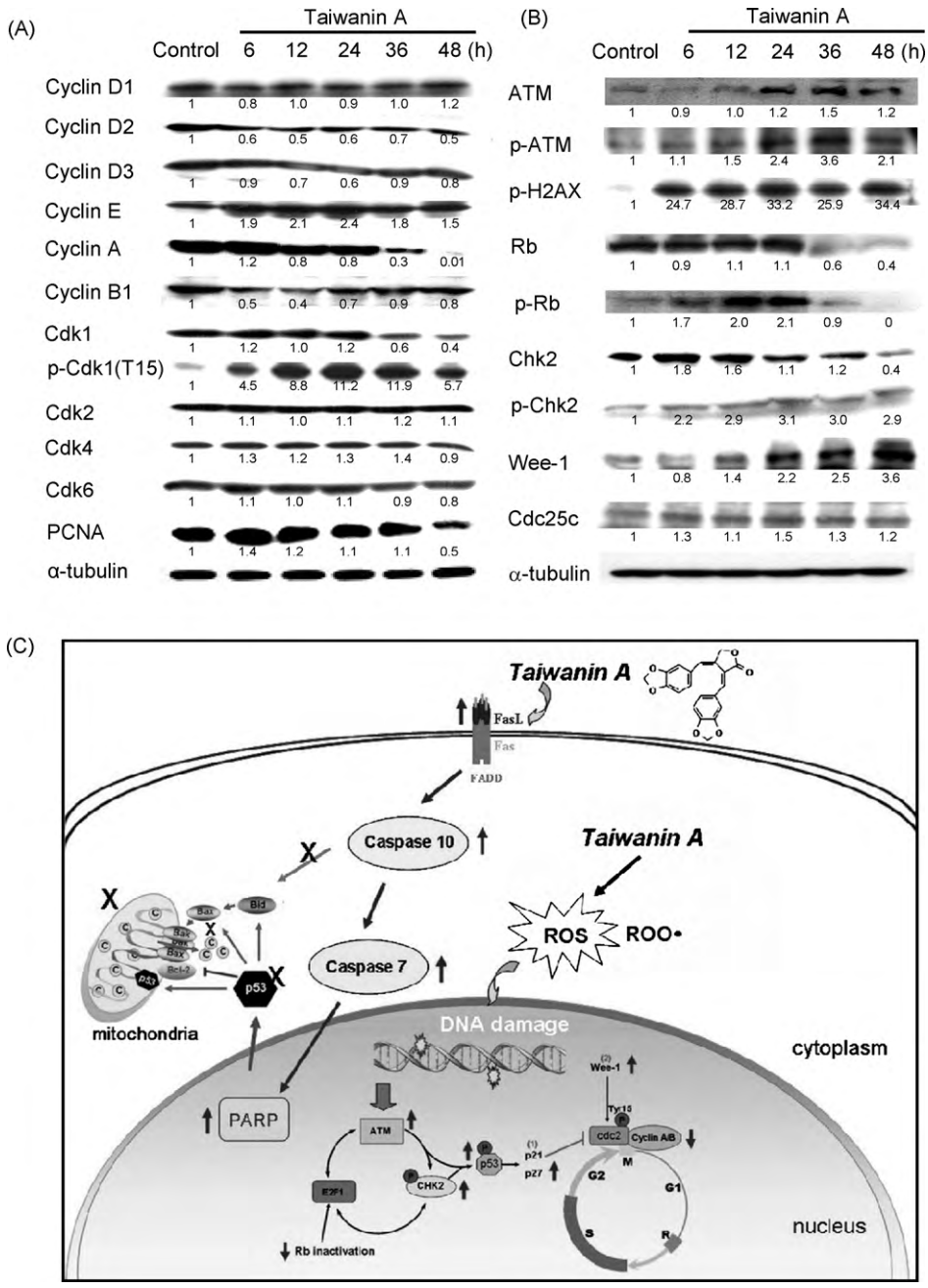

Fig. 6. Effects of taiwanin A on the protein expression of cell-cycle mediators and proteins involved in ATM pathway in MCF-7 cells. (A and B) Cells were treated with vehicle or taiwanin A at indicated time points and then subjected to Western blotting and chemiluminescent detection. Protein levels were normalized to the respective level of (-tubulin. (C) The proposed molecular mechanisms involved in taiwanin A-induced cell-cycle arrest and apoptosis in MCF-7 cells.

In conclusion, this report demonstrates the profound activity of the lignan taiwanin A against a human mammary adenocarcinoma cell line, by a variety of sophisticated time-dependent molecular events and effects on cell-cycle transition and apoptosis (Fig. 6C). As taiwanin A also exhibited much less cytotoxicity on a normal fibroblast cell line under the same conditions, taiwanin A may warrant further development as a cancer-prevention agent.

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