NUTRITION AND CANCER, *57*(1), 111–121

Unique Formosan Mushroom *Antrodia camphorata* Differentially Inhibits Androgen-Responsive LNCaP and -Independent PC-3 Prostate Cancer Cells

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Abstract: *Antrodia camphorata (AC), a precious and unique folkloric medicinal mushroom enriched in polyphenolics, isoflavonoids, triterpenoids, and polysaccharides, has been diversely used in Formosa (Taiwan) since the 18th century. In this study, prostate cancer (PCa) cell lines PC-3 (androgen independent) and LNCaP (androgen responsive) were treated with AC crude extract (ACCE) at 50–200 μg/mL, respectively, for 48 h. At the minimum effective dose 150 μg/mL, LNCaP showed a G_1/S phase arrest with significant apoptosis. Such dose-dependent behavior of LNCaP cells in response to ACCE was confirmed to proceed as Akt→ p53→ p21→ CDK4/cyclin D1→ G_1/S-phase arrest→ apoptosis, which involved inhibiting cyclin D1 activity and preventing pRb phosphorylation. In contrast, being without p53, PC-3 cells showed a G_2/M-phase arrest mediated through pathway p21→ cyclin B1/Cdc2→ G_2/M-phase arrest, however, with limited degree of apoptosis, implicating that ACCE is able to differentially inhibit the growth of different PCa cells by modulating different cell cycle signaling pathways. We conclude that this unique Formosan mushroom, A. camphorata, due to its nontoxicity, might be used as a good adjuvant anticancer therapy for prostate cancers despite its androgen-responsive behaviors, which has long been a serious drawback often encountered clinically in hormonal refractory cases treated by antihormonal therapies and chemotherapeutics.*

Introduction

Antrodia camphorata (AC), formerly named as *Antrodia cinnamomea* Chang & Chou, sp. nov (Polyporaceae, "Zhan Ku," or "Zhan Chi"), has been a well-known traditional Chinese medicine widely and uniquely used in Formosa (Taiwan) since the 18th century (1). AC usually grows only on the wet, rotten, inner heartwood wall of the endemic evergreen *Cinnamomum kanehirai* Hay (Lauraceae) in Formosa and has long been used as a chemical antidote to treat diarrhea, abdominal pain, hypertension, itchy skin, and hepatoma (2,3) and as an antiinflammatory and immunomodulating agent toward human leukocytes (4,5) and hepatitis B (6). Nakamura et al. (7) showed cytotoxic effects of AC mycelia on the LLC tumor cell line. Satisfactory effects were also observed for human premyelocytic leukemia HL-60 (8). Prostate cancer (PCa) is the most commonly diagnosed malignancy in American men over 40 yr of age and is the second leading cause of cancer deaths (9,10). In individuals diagnosed with PCa, cancer cells initially are slow growing and androgen responsive; progressively, the PCa in these patients may remain subclinical for an extended period of time. Ultimately, the disease progresses to an androgen-insensitive state, characterized by accelerated cell proliferation, lack of responsiveness to androgen blockade, and high mortalities (11–14).

Clinically, androgen-ablation therapy is usually a routine methodology, which involves a simple orchiectomy; administration of luteinizing hormone releasing hormone (LHRH) analogs and anti-androgens such as cyproterone acetate Androcur, Schering AG, Berlin, Germany, flutamide (Drogenil), and bicalutamide (Casodex); and high doses of estrogens and radiotherapy. However, many patients relapse thereafter and develop androgen-independent tumors, which become completely autonomous to androgen effects on growth by acquiring a distinct set of growth factors.

Recently, many nutraceutics involving functional foods often cited in the past have been found to be effective cancer preventives. As a rule, these nutraceutics usually

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consist of a diversity of nutraceutic compounds, including triterpenoids (15), isoflavonoids (16,17), and polyphenolics (18) and a diversity of bioactive polysaccharides (19). Recently, isomers of trimethoxybenzaldehyde (2,3,4- and 2,3,5-trimethoxybenzaldehydes, mol wt = 196) in prominent amounts (a total of ~0.2%) were isolated and identified using gas chromatography (GC)/mass spectrometry (MS) (unpublished GC/MS data by Professor Chyau).

Having been shown to be more effective in cancer treatment when used in combination with some conventional anticancer chemotherapeutic agents, nutraceutical therapy is hence given the name "complementary alternative medicine" (20).

To gain additional insight into the therapeutic advantages of AC in treatment of PCa, we evaluated the potential therapeutic activity of AC fruiting bodies by studying their effects on two PCa cells: PC-3 (androgen-independent) and LNCaP (androgen-responsive) cell lines. The differential responses of PC-3 and LNCaP cells to AC crude extract (ACCE) were analyzed by different related cell cycle biomarkers, cell viability, apoptotic behaviors, and signaling pathways.

Materials and Methods

Chemicals

3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and RNase A were obtained from Sigma Chemical (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from J.T. Baker (Phillipsburg, NJ). Trizol was provided by Invitrogen (Carlsbad, CA).

Source of *Antrodia camphorata* Crude Extract and its Compositions

The fruiting bodies of AC used in this study were kindly provided by Professor Chyau. The ethanolic ACCE was prepared according to Peng et al. (21). The ground powder (300 mg) of AC fruiting bodies was extracted at ambient temperature with ethanol (50 mL × 3) with constant stirring for 2 h. The extracts were combined and evaporated in vaccuo to dryness in a rotary evaporator (sample A). Alternatively, the fruiting bodies were cut into small pieces and refluxed with MeOH (2 L) for 5 h. The concentrated MeOH extract was partitioned between H_2O and $CHCl_3$ and then chromatographed on a silica gel column by sequential elution with *n*-hexane-EtOAc (7:3) followed by *n*-hexane-EtOAc (1:1) and finally with *n*-hexane-EtOAc (2:3) (sample B). The ACCE obtained from either method was first dried by evaporation under reduced pressure and then dissolved in 100% EtOH to make a concentration of 20 mg/mL and stored at −20°C or redissolved to the concentration as indicated in the experiments. Proximal analytical data for ACCE were obtained by Professor Chyau.

Polysaccharide and Protein Contents

Polysaccharides are usually associated with protein to form complexes. Briefly, the contents of polysaccharide and protein in ACCE were determined using the phenol-sulfuric acid method (22) and Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), respectively.

Determination of Total Polyphenolics

Total polyphenolics were determined colorime trically using the Folin-Ciocalteau method (23). ACCE (100 mg) was dissolved in a mixed solvent of methanol/water (250 mL; 60:40, vol/vol; 0.3% HCl) and filtered through a 0.45-m Millipore filter. To the filtrate was added with 100 L of the extract, 100 L of 50% Folin-Ciocalteau reagent, and 2 mL of 2% sodium carbonate and mixed thoroughly. On standing for 2 h, the absorbance was taken at 750 nm with a spectrophotometer. A reference curve was established using gallic acid (0–0.5 mg/ml) in methanol/water 60:40, vol/vol; 0.3% HCl) solution.

Determination of Crude Triterpenoids

Triterpenoids were determined according to Chen et al. (24). ACCE (0.1 g) was extracted with 50% ethanol (50 mL) for 1 h, filtered, and evaporated to dryness in a rotary evaporator. The residue was extracted with a mixture of $CHCl_3/H_2O$ (5:1) three times. The $CHCl_3$ layer was further extracted with saturated $NaHCO_3$ (50 ml) three times. The alkali extracts were collected and acidified with 6 N HCl to a pH of 3–4 and then extracted with ethyl acetate three times. The extracts were combined. On evaporation, it yielded a pale yellow solid. The residue was dried in an oven to give the acidic ethyl acetate–soluble fraction consisting of crude triterpenoids.

GC/MS Analysis of the Bioactive Compound

GC/MS analytical data were obtained courtesy of Professor Chyau. Briefly, analyses were performed with an HP 6890 GC series (Agilent, Santa Clara, CA), a GC/MS equipped with an HP 6890 series GC system, and a 5973 network mass selective detector (MSD). A flame ionization detector with a capillary DB-1 (length = 60 m, inner diameter = 0.25 m, and membrane thickness = 0.25 m) was used. Temperatures at the injection port and the detector were set at 250°C and 270°C, respectively. The carrier gas N_2 was run at a flow rate of 1 mL/min. The initial oven temperature was programmed at 40°C for 5 min, and then the temperature elevation was run with 3°C/min until 150°C, followed by 1°C/min to 200°C, and again switched to 3°C/min until 240°C and held at 240°C for 20 min. The mobile phase (He) was operated at a flow rate of 1 mL/min, the ionization potential was 70 eV, the temperature of the ion source was set at 230°C, and the partition ratio was 50:1. Detection was accomplished using a 5973 MSD. Samples were injected into the GC and the GC/MS using a GC-specific

injector (injection volume = 0.5 L). Quantitative calculations were measured from the integrated diagrams obtained in the previous analyses.

Cell Lines

The two human prostate adenocarcinoma cell lines LNCaP (androgen-responsive lymph node metastasis) and PC-3 (androgen-independent bone metastasis) were purchased from the Culture Collection and Research Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The mouse embryonic fibroblast (MEF) cell line was provided by Dr. Hung Li.

Cell Cultures

LNCaP cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco BRL). PC-3 cells were cultured in F-12K (Gibco BRL) medium with 7% FBS. MEF cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% FBS. All the culture media, on further additions of 100 IU/mL penicillin and 100 g/mL streptomycin (Gibco BRL), were used to incubate cells at 37°C in a humidified atmosphere containing 5% CO_2 vol/vol in air. When these cells formed a monolayer at confluence, trypsin (0.25%)/ethylenediaminetetraacetic acid (EDTA) solution was used to detach the cells from the culture plates. Cells were harvested for MTT assay, flow cytometry, Western blot analysis, and reverse transcriptase–polymerase chain reaction (RT-PCR) after another 24 or 48 h of cultivations.

MTT Assay

Cells (5×10^3) were plated onto 1 well of 24-well culture plates for 24 h and treated with ACCE for another 24 and 48 h. The concentrations of ACCE used were 50, 100, 150, and 200 mg/mL, respectively, with control groups receiving the vehicle (EtOH) alone. MTT solution (50 L, 5.5 mg/mL) was then added to each well, and the plates were further incubated at 37°C for 4 h in a humidified incubator. The medium was then aspirated to ease the formation of the formazan product, which was then solubilized with the addition of 500 L of DMSO. The optical density was measured at 570 nm with a microplate autoreader (Bio-Tek Instruments, Winooski, VT).

Cell Cycle Analysis

Cell cycle analysis was performed with a flow cytometer (FACS Caliber, BD Biosciences, San Jose, CA). Cells (1×10^5) were cultured in 6-cm culture plates and treated with ACCE (150 g/mL) or vehicle alone as stated previously. The floating and adherent cells collected by trypsinization were washed with ice-cold phosphate-buffered saline (PBS), fixed, and permeabilized with 70% ethanol at –20°C for 30 min, followed by incubation with 30g/mL PI and 100 mL RNase for 30 min at room temperature in the dark. Data acquisition and analysis were performed in the flow cytometer with the accompanying software (CellQuest, BD Biosciences). Appropriate gating was used to select the easily distinguished single population. Ten thousand events per sample were counted, and at least triplicate determinations were performed to assure each cell cycle distribution. The final percentages of cells in each phase were expressed as mean ± SD.

TUNEL-Based Flow Cytometry to Detect Fluorescein-Labeled Apoptotic Cells

An "in situ cell death detection kit" was used according to the manufacture's instruction. This kit can display either the cell flow cytometric data or alternatively the morphological pictures, whereas the former was adopted in our experiment. LNCaP cells (1×10^5) were incubated in the presence of 150 and 200 g/mL of ACCE for 24 and 48 h post–cell seeding and, afterward, collected for TUNEL assay. After trypsinization, cells were fixed by 4% paraformaldehyde in PBS, and the apoptotic cells were detected by flow cytometry after incubating the cells with in situ cell death detection kit fluorescein (Roche Applied Science, Basel, Switzerland).

Western Blot Analysis

LNCaP and PC-3 cells (2×10^5) were treated with ACCE at 150 g/mL and incubated for 24 and 48 h, respectively. After incubation, cells were washed twice with ice-cold PBS and incubated in ice-cold RIPA buffer [1 M Tris (pH 7.4), 5 M NaCl, 0.5 M EDTA (pH 8.0), 10% sodium dodecyl sulfate (SDS), 10% DOC, and 10% NP40] with freshly added protease inhibitor cocktail tablets (Roche) over ice for 30 min with occasional vortexing. The lysates were collected and cleared of nuclei by centrifugation for 30 min at 13,000 rpm. The supernatant was collected, and aliquots were stored at –70°C. The protein concentration in the lysates was measured by BCA protein assay kit (Pierce, Rockford, IL) following the manufacturer's protocol. A Western immunoblotting protocol was conducted according to the manufacturer's protocol (Cell Signaling Technology, Danvers, MA) using proteins (30 g) loaded over 5–15% SDS–polyacrylamide gel electrophoresis gels. The proteins were then added with the ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ) and analyzed using the Fuji LAS-3000 imaging system (Tokyo, Japan). The antibodies used in this study included anti-Akt mAb, anti-phosphorylated Akt mAb (Cell Signaling), anti-p53 Ab (Santa Cruz, Santa Cruz, CA), anti-cyclin D1 mAb, anti-p21 mAb, anti-rabbit IgG HRP-linked Ab and anti-mouse IgG, HRP-linked Ab (Cell Signaling), anti-pRB mAb (BD Pharmingen, Oxford, UK), anti-cyclin B1 mAb (Upstate, Billerica, MA), anti-Cdc2 Ab (Calbiochem, Darmstadt, Germany), and anti-Actin mAb (Chemicon, Billerca, MA).

RNA Extraction and RT-PCR Analysis

ACCE-treated LNCaP and PC-3 cells were lysed in TRIzol reagent (Invitrogen), and total RNA was extracted according to manufacturer's instructions (Invitrogen). RNAs were reverse transcribed at 42°C for 60 min using StrataScript™ reverse transcriptase (Stratagene, La Jolla, CA). The cDNA product was then subjected to PCR amplification using *p*21 primer pairs (forward, $^{5'}$CAGGGGACAGCAGAGGAAGA$^{3'}$; reverse, $^{5'}$TTAGGGCTTCCTCTTGGAGAA$^{3'}$) (25). The expression of β-actin was used as an internal control.

Statistical Analysis

Statistical significance of difference in measured variables between control and treated groups was determined by paired *t*-test or one-way analysis of variance followed by Dunnett's test. Values with $P < 0.05$ were considered statistically significant.

Results

Compositional Analysis

ACCE contained a variety of bioactive medicinal constituents that had been often cited in much of the related literature (26), such as total polyphenolics (7.34 ± 0.07%), crude triterpenoids (11.49 ± 0.31%), polysaccharides (39.40 ± 0.09%), and proteins (6.42 ± 0.15%).

ACCE-Inhibited Proliferation of Prostate Cancer Cells

As can be seen, ACCE at or over 150 g/mL significantly inhibited the proliferations of both PC-3 and LNCaP cells (Fig. 1A and B), which was confirmed quantitatively in parallel by MTT assay by counting the viable cells using the trypan blue exclusion method and a hemocytometer (data not shown). In contrast, no significant difference was observed for the control MEF cell lines with similar treatment (Fig. 1C). Previously, the results from the acute toxicity test performed by the Japanese Food Analytical Centre had confirmed that ACCE at a per os dosage of 2,000 mg/kg body weight failed to reveal any toxicity in either female or male rats nor any apparent body weight loss or pathological changes, conforming to an extremely large value of LD_{50} (21). Obviously, the decline in viability caused by ACCE was not due to its toxicity, implicating any toxic effect of ACCE can be ignored clinically.

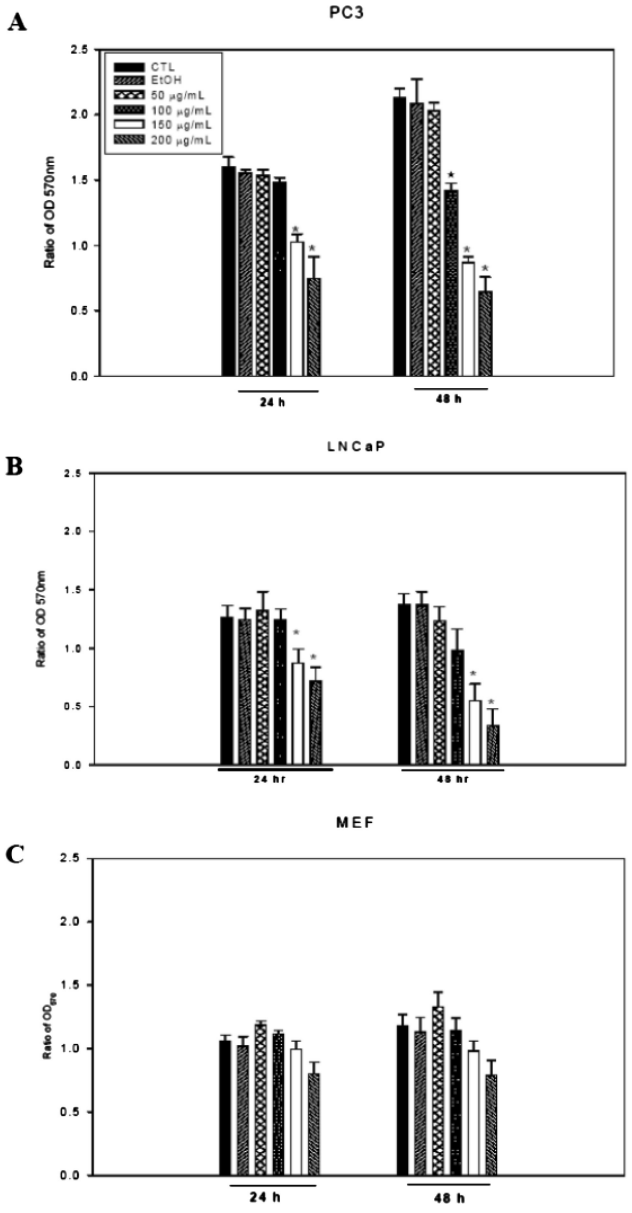


Figure 1. Inhibition of cell proliferation by *Antrodia camphorata* crude extract (ACCE) treatment. Cell proliferation was analyzed by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on (A) PC-3, (B) LNCaP, and (C) mouse embryonic fibroblast cell lines after treatment with ACCE (50–200 g/mL) for 24 or 48 h. The ratio of MTT activity was normalized to the optical density of cells before treatment (at Hour 0).*Significantly different ($P < 0.05$) from the control (CTL) and EtOH (vehicle control) groups by paired *t*-test. Data represent mean of triplicate experiments.

Effects of ACCE on Cell Cycle Profile

ACCE at 150 g/mL (the minimum effective dose) was selected to perform the cell cycle analysis. All cell lines were treated with ACCE (150 g/mL) for different time periods as indicated. As noted, the S-phase fraction of PC-3 cells decreased gradually from 13.58% (vehicle control) to 9.46% at 48 h post–ACCE treatment. Concomitantly, the fractions in the G_1 phase decreased from 65.70% (vehicle control) to 59.39% at 48 h (Fig. 2A, Table 1). Correspondingly, in the G_2/M phase at 48 h post–ACCE treatment, the values were 19.99% (vehicle control) and 30.46%, respectively (Fig. 2A and Table 1). Conclusive evidence has confirmed an apparent G_2/M-phase arrest without evident apoptosis in PC-3 cells caused by ACCE (Fig. 2A, Table 1), which was further substantiated by the negative result obtained by TUNEL assay (data not shown).

In contrast to PC-3, LNCaP cells showed an increased fraction of subG_1-phase cells, from 3.91% to 12.77%, and that of G_1 phase, from 67.00% to 72.00%, at 48-h posttreatment with ACCE (Fig. 2B and Table 1; see also Fig. 2B

Table 1. Effect of ACCE (150 μg/mL) on Cell Cycle Progression of Prostate Cancer Cells[a]

Cell Line	ACCE Concentration	SubG$_1$-Phase Percentage (%)		G$_1$-Phase Percentage (%)		S-Phase Percentage (%)		G$_2$-Phase Percentage (%)	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
PC-3	Vehicle control	0.588 ± 0.044	0.826 ± 0.21	63.99 ± 1.55	65.70 ± 2.27	11.62 ± 1.36	13.58 ± 1.19	23.98 ± 0.48	19.99 ± 1.25
	150 μg/mL	0.648 ± 0.089	0.81 ± 0.11	60.17 ± 2.07	59.39 ± 2.15	8.42 ± 0.91	9.46 ± 0.46	30.95 ± 2.04	30.46 ± 1.93*
LNCaP	Vehicle control	1.77 ± 0.72	3.91 ± 0.23	76.91 ± 1.07	67.00 ± 0.56	8.54 ± 0.69	12.39 ± 0.39	12.79 ± 0.89	15.51 ± 0.86
	150 μg/mL	4.79 ± 0.48	12.77 ± 3.07*	83.11 ± 0.70	72.00 ± 4.53	4.15 ± 0.09	6.63 ± 0.044	7.76 ± 0.30	10.10 ± 0.92

[a]Abbreviation is as follows: ACCE, *Antrodia camphorata* crude extract. Asynchronous populations of PC-3 and LNCaP cells were exposed to vehicle or ACCE (150 μg/mL) and fixed at 24 h and 48 h, respectively. Fluorescence-activated cell sorting (FACS) analysis was performed, and the subG$_1$, G$_1$, S, and G$_2$ populations were quantified as described in **Materials and Methods**. Results shown are expressed in mean ± SD of triplicates. *Significantly different compared with vehicle control ($p < 0.005$) by one-way analysis of variance followed by Dunnett's test.

insert), evidencing that ACCE inhibited LNCaP cell growth and proliferation by preventing the cells from entering the S phase, resulting in G$_1$-phase arrest. As a comparison, no significant change was observed in the control MEF cells at either 24-h or 48-h posttreatment (Fig. 2C).

Compared with PC-3 cells (Fig. 2A), a more distinct apoptosis was noticed in LNCaP cells at 48 h on treatment with ACCE (Fig. 2B). In parallel, the TUNEL assay also confirmed the same results in a dose-dependent manner (Fig. 3), that is, at 48 h, 15.77% at 150 g/mL and 55.0% at 200 g/mL, respectively, consistent with the results found in cell cycle analysis (Fig. 2B and 2B insert). To further elucidate the mechanisms of how ACCE induced apoptosis in LNCaP cells, the expressions of the antiapoptotic Bcl-2 and proapoptotic Bax proteins post–ACCE treatment were assessed. Figure 4 indicates that ACCE at 150 g/mL down-regulated Bcl-2 expression at 24 h postincubation and markedly up-regulated Bax expression in a time-dependent manner in LNCaP cells from 24 h up to 48 h posttreatment.

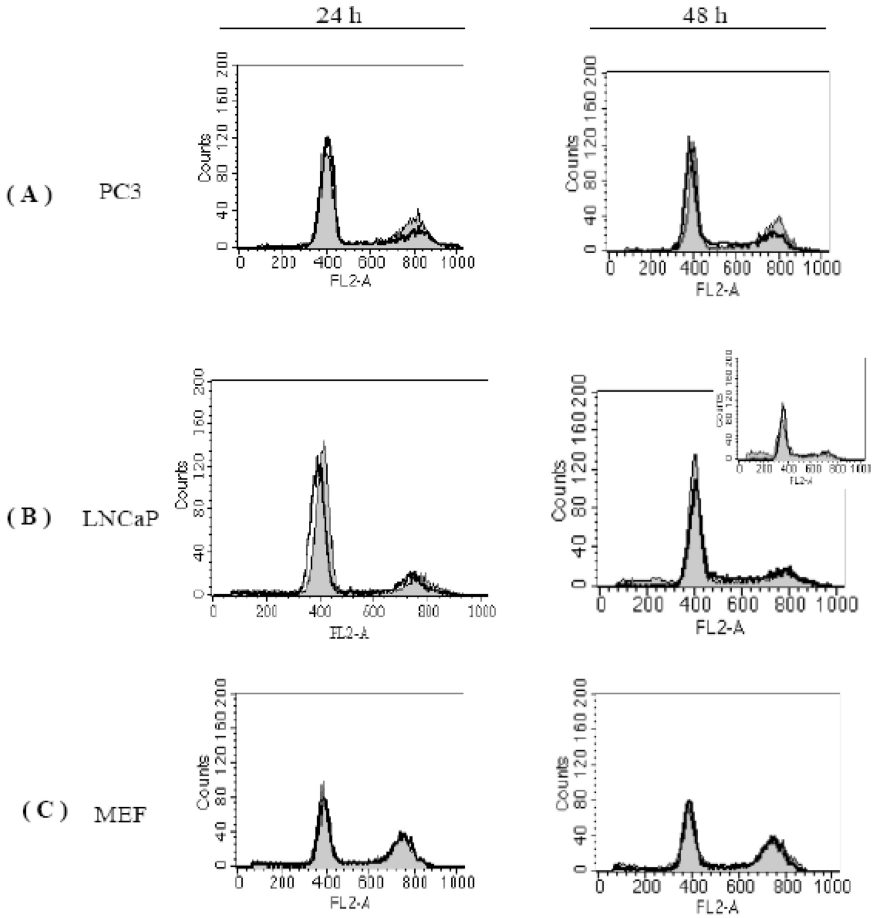


Figure 2. Flow cytometric analysis of cell cycle profiling in asynchronous different cell lines (A) PC-3, (B) LNCaP, and (C) mouse embryonic fibroblast treated with *Antrodia camphorata* crude extract (ACCE) (150 g/mL) for 24 or 48 h, respectively. Triplicate experiments with similar results were performed; because the results from triplicates were all similar, the most apparent one was chosen (gray zone); EtOH treatment was vehicle control (blank zone). The percentage of cells in subG$_1$, G$_1$, S, and G$_2$ phases was calculated using the accompanying software (CellQuest, BD Biosciences). (B, insert) Cell cycle profiling of asynchronous LNCaP cells at 48 h after treatment with ACCE at 200 g/mL.

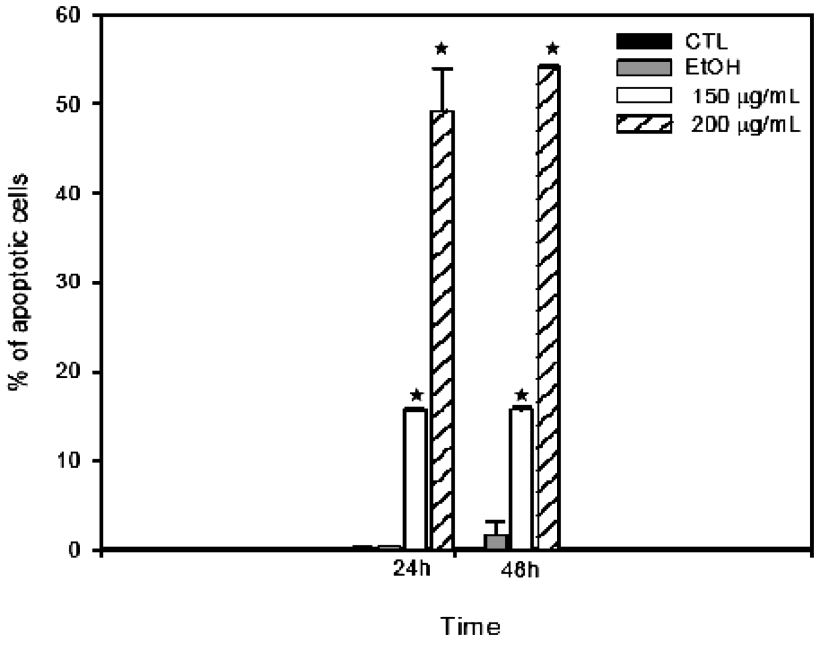


Figure 3. Histogram plots of TUNEL assay plus results from flow cytometric data of control (CTL, nontreated) and vehicle (EtOH)- and *Antrodia camphorata* crude extract (150 and 200 g/mL)-treated LNCaP cells at 24 and 48 h, respectively. *Significantly different ($P < 0.05$) from the CTL and EtOH (vehicle control) groups by paired *t*-test. Data represent mean of triplicate experiments.

Effect of ACCE on Cell Cycle Regulatory Proteins

Given the fact that the different inhibitory effects of ACCE (150 g/mL) on LNCaP and PC-3 cells in cell cycle progression with more apoptosis in LNCaP than PC-3 were observed, the Western blots for Bax and Bcl2 in PC-3 were reasonably omitted. The results obtained in ACCE (150 g/mL) -treated LNCaP cells revealed the up-regulation of the expressions of the G_0/G_1 regulatory proteins p53 and p21 with simultaneous markedly down-regulated cyclin D1 and phosphorylated pRb (ppRb) (Fig. 4) compared with the distinct up-regulated p21 expressions with down-regulated cyclin B1 and Cdc2 in

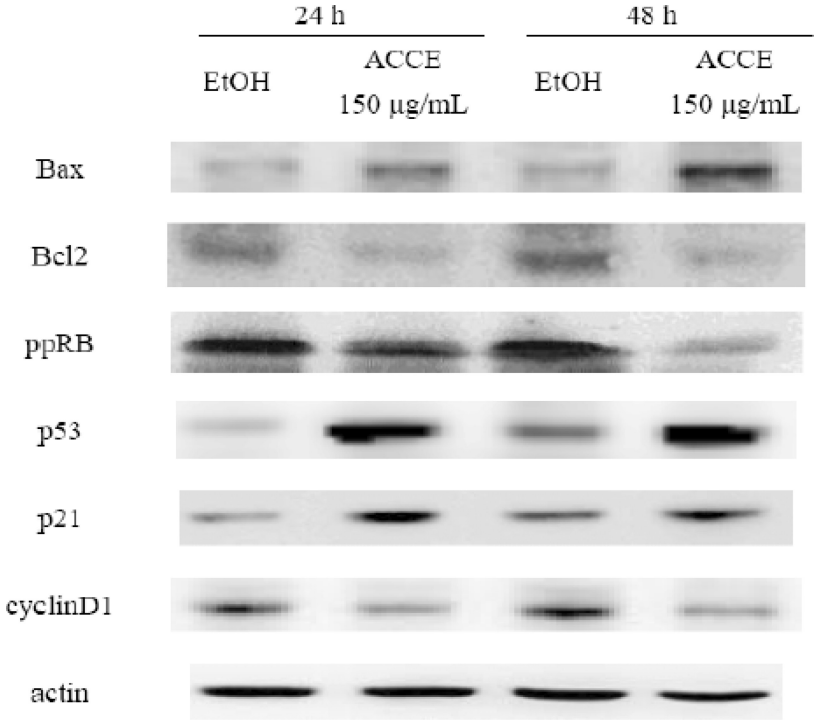


Figure 4. Level analysis of apoptotic proteins and cell cycle regulators in LNCaP cells treated with *Antrodia camphorata* crude extract (ACCE) (150 g/mL) for 24 h and 48 h, respectively. Results of Western blot analysis for Bax, Bc1-2, p53, p21, cyclin D1, and ppRb in LNCaP cells after treatment with ACCE are shown at indicated times. β-Actin was used as internal control. Each Western blot was performed in triplicate with only a single datum shown.

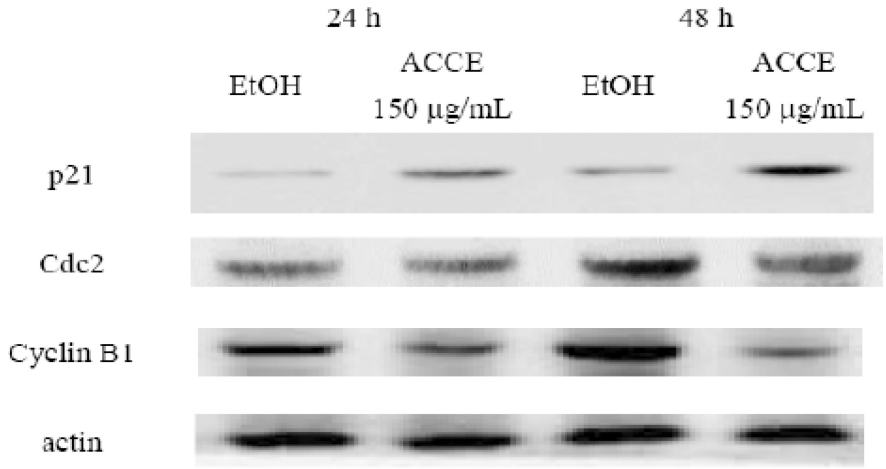


Figure 5. Level analysis of cell cycle regulators in PC-3 cells treated with *Antrodia camphorata* crude extract (ACCE) at 150 g/mL. Results of Western blot analysis for p21, Cdc2, and cyclin B1 in PC-3 cells after treatment with ACCE (150 g/mL) for 24 h and 48 h, respectively. β-Actin was used as internal control. Each Western blot was performed in triplicate with only a single datum shown.

PC-3 cells (Fig. 5). Because Akt plays an important role in G_0/G_1 cell cycle progression through regulating Akt→ mTOR→ $p70^{S6k}$ → p21→ CDK4/cyclin D1 in LNCaP cells (27), we further checked the expressions of Akt and phosphorated Akt in LNCaP cells after treatment with ACCE. As shown in Fig. 6, a high level of phosphorylated Akt that had been originally observed in serum-cultured LNCaP cells was inhibited by ACCE at 150 g/mL in a time-dependent manner. It seems that p21 is a common regulator relevant to the inhibitory effects of ACCE (150 g/mL) on both PCa cell lines; p21 mRNA expression was further investigated, and, as expected, incubation of both PCa cell lines with ACCE (150 g/mL) had caused strikingly up-regulated p21 mRNA (Fig. 7A and B) at both 24 h and 48 h, respectively.

Discussion

A significant amount of trimethoxybenzaldehyde and its isomers from *Antrodia cinnamomea* had been identified (28), whereas 2,4,5-trimethoxybenzaldehyde was reported to exhibit a potent COX-2 inhibitory effect (29), and previously synergistic effects of COX-1 and COX-2 inhibitors had proved effective for suppressing bladder and PCa cells in vitro (30). AC induced G_2/M-phase growth arrest in PC-3 cells, and similar results were demonstrated by *Ganoderma lucidum*, which can be attributed to the rich content of triterpenoids (28,31) and polysaccharides (6,32) in both mushrooms. G_2/M-phase growth arrest caused by ACCE in PC-3 cells (Table 1) was triggered by the up-regulation of p21 (33,34) accompanied by down-regulations of cyclin B1 and Cdc2 (Fig. 5), as also evidenced by the flow cytometric analysis (Fig. 2A). p21 acts as a potential negative regulator of the G_2/M-phase transition and a direct mediator of DNA damage checkpoint control at both G_1/S and G_2M transitions (35). However, p21 is predominantly localized in the nucleus of G_1 cells and virtually absent in the S-phase cells and cells undergoing mitosis (35). Co-localization of cyclin B1 and p21 has been confirmed in synchronous fibroblasts (34).

In ACCE (150 g/mL) -treated LNCaP cells, cyclin D1 was markedly down-regulated with simultaneous up-regulation of the expressions of p53 and p21, the G_0/G_1 regulatory proteins (Fig. 4), conforming to cell cycle arrest at the G_1/S phase (36). Because the G_1/S transition is regulated through phosphorylation of pRb (37), the down-regulated phosphorylated pRb (ppRb) in the LNCaP cells following ACCE treatment (Fig. 4), a negative contribution to progression of LNCaP cells through the G_1/S transition in cell cycle was apparently conceived (Fig. 2B, Table 1);

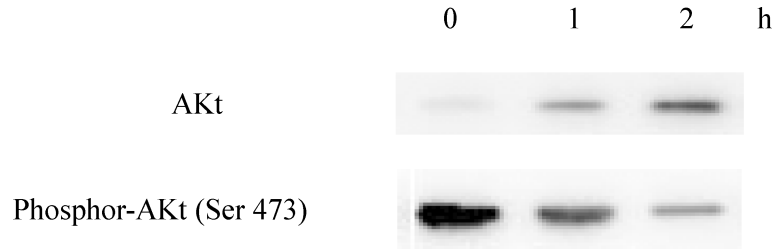
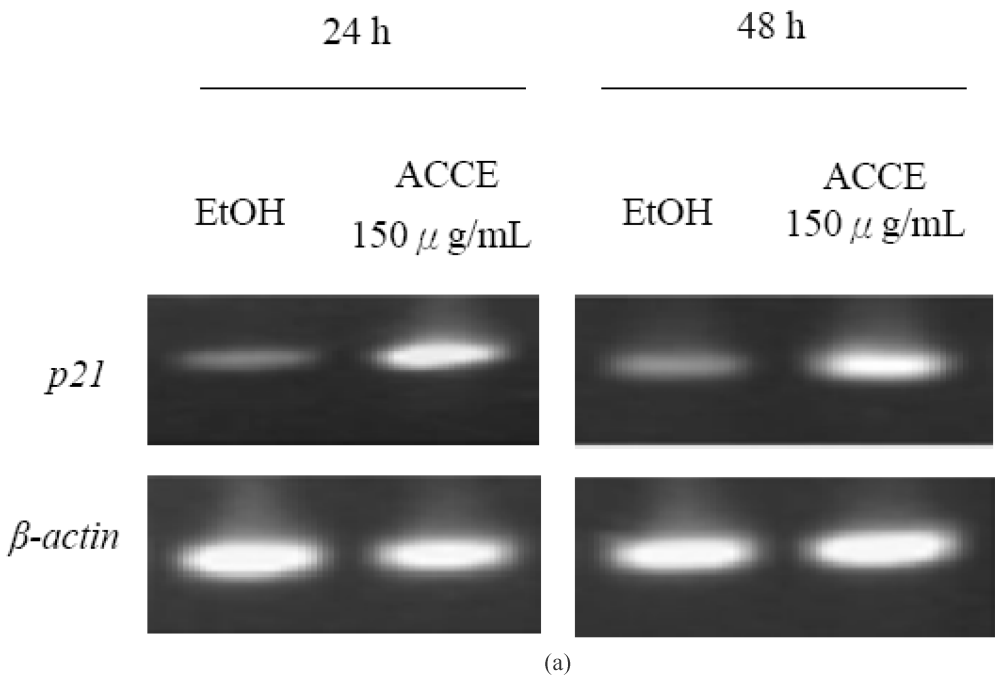
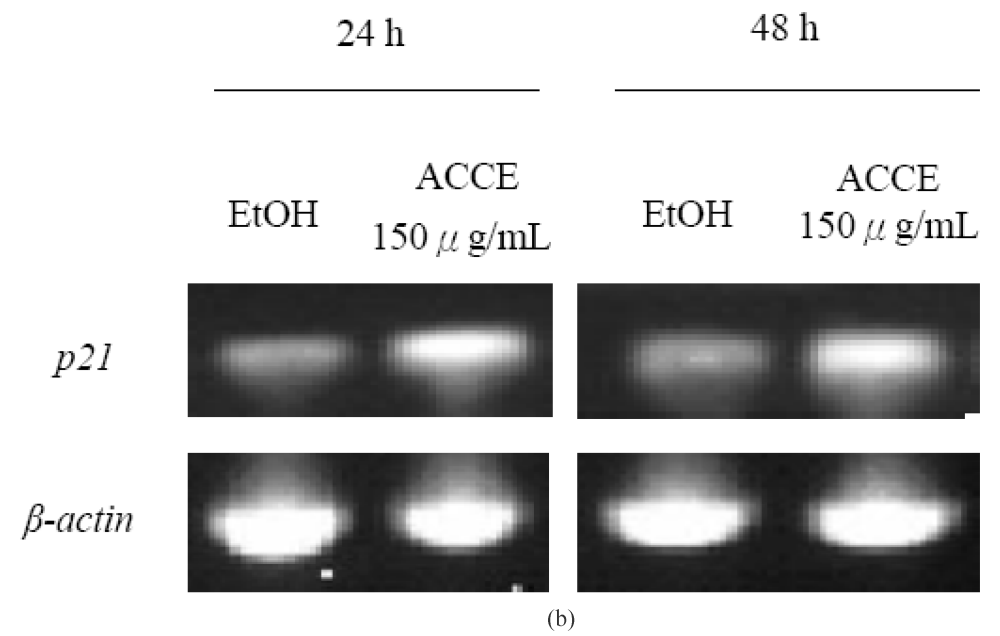


Figure 6. Results of Western blot analysis for AKt and phosphorated Akt in LNCaP cells after treatment with *Antrodia camphorata* crude extract (ACCE) at indicated times. A high level of phosphorylated Akt that had been originally observed in serum-cultured LNCaP cells was inhibited by ACCE at 150 g/mL in a time-dependent manner. Each Western blot was performed in triplicate with only a single datum shown.



(a)



(b)

Figure 7. Incubation of (A) LNCaP and (B) PC-3 cells with *Antrodia camphorata* crude extract (150 g/mL) for 24 h and 48 h, respectively, caused strikingly up-regulated p21 mRNA. β-Actin was used as internal control. Each reverse transcriptase–polymerase chain reaction was performed in triplicate with only a single datum shown.

moreover, the possible existence of the cited pathway PI3K→ Akt→ mTOR→ $p70^{S6k}$ → p21→ CDK4/cyclin D1 in cytoplasm as previously mentioned for LNCaP cells (27) was also revealed (see Fig. 8).

Guadagno and Newport (38) demonstrated that Cdk2 acts as a positive regulator of activation of cyclin B–Cdc2 complexes and that p21, by inactivating Cdk2, blocks progression into mitosis, supporting the fact that PC-3 cells may take the pathway by activation of p21 and subsequently by→p21→ CDK2/cyclin B1–Cdc2 (39) (Fig. 8) on the nuclear membrane to induce G_2M arrest. Similar p53-independent arrests in PC-3 cells have been reported by Park et al. (40) and Choi et al. (17), the latter illustrated by genistein, a natural isoflavonoid phytoestrogen. The p53 gene is normal in LNCaP cells but mutated in PC-3 cells (41); the outcome from both cells as affected by ACCE indicated that the therapeutic mechanism of ACCE on both cells might be using to some extent part of a common signaling pathway. The prevention of DNA endoreduplication is thought to be mediated by the target gene product p21 as an inhibitor of pRb phosphorylation by cyclin-dependent kinases (42). Alternatively, p53 can also activate a cell apoptotic program with DNA

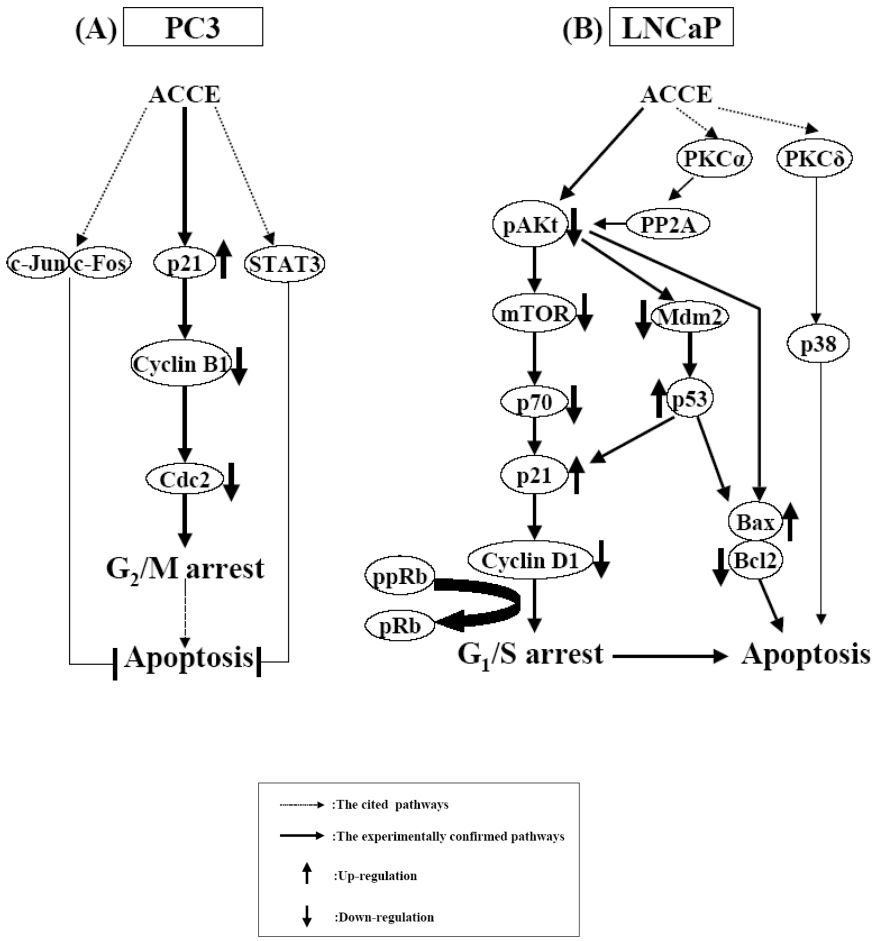


Figure 8. Proposed model illustrating the signaling cascades regulated by *Antrodia camphorata* crude extract (150 g/mL) in (A) PC-3 and (B) LNCaP cells, indicating the control of cell cycle progression and apoptosis by some relevant signaling proteins. Parts of these proposed pathways were contributed by Bonaccorsi et al. (47), Edwards et al. (48), and Tanaka et al. (49).

damage (43), which may explain the greater apoptotic cell population in LNCaP than PC-3 cells by ACCE (Table 1).

To elucidate the reason for a lesser apoptotic population in PC-3 cells at G_2M arrest than LNCaP cells at G_1/S arrest (Table 1), we propose three major possibilities:

1. Dulic et al. (35) have confirmed that p21 is a poor inhibitor of cyclin B1–Cdc2 complexes. In agreement with this, PC-3 arrest at the G_2M phase induced by ACCE yielded only relatively small apoptotic populations (Table 1).
2. The possible involvement of STAT3. Levels of activated STAT3 are significantly higher in the hormonal refractory PCa cell lines (DU145 and PC-3) than in hormone-sensitive cell lines (LNCaP cells) (44), although, in 2000, a contrary report indicated the absence of STAT3 in PC-3 (45), yet later in 2002 the presence of the phospho-STAT3 has been confirmed (44); all three cells apparently exhibited significant affinity of STAT3-related DNA binding capability: the DU145 cells showed the strongest and the LNCaP cells the weakest, with PC-3 cells in the middle. Additionally, in vitro work showed that, in PC-3 cells, the intracellular concentration of c-Jun and c-Fos is seven times greater than in LNCaP cells (46), which suggests that AP-1 (c-Jun/c-Fos complex) influences androgen escape in the PC-3 cell line (47); in vivo studies of similar phenomenon have also been substantiated (48). All together, an implication indicates a lesser apoptotic cell population in PC-3 cells at G_2M-phase arrest than LNCaP cells at G_1/S-phase arrest under the same dosage of ACCE treatment (Fig. 2A vs. Fig.2B and Table 1).
3. Akt dephosphorylation through action of PP2A, the phosphatase 2a, might activate the p38 MAPK pathway to induce cell apoptosis in LNCaP cells (49).

Cyclin D1 was identified to be controlled posttranscriptionally via the PI3K/Akt pathway (50). Naturally occurring major polyphenolics were found to 1) decrease the level of PI3K and phosphor-Akt and 2) increase Erk1/2 in both DU145 and LNCaP cells (51). ACCE (150 g/mL) treatment markedly upregulated the expressions of p53 and p21 proteins, resulting in down-regulation of ppRb (synonymously, accumulation of pRb), a response consistent with that of Guardavaccaro et al. (52) that p53 effects G_1 arrest mainly by inducing transcription of p21, which in turn inhibits cyclin D1 activity with

subsequent prevention of pRb phosphorylation, leading to a consequent growth arrest at the G_1 phase.

Conversely, ppRb inactivates its growth suppression activity by freeing E2F molecules, thus enabling them to transactivate genes required for the progression of the cell into the S phase and the subsequent phases of the cell cycle (53). Degradation or down-regulation of cyclin D1, triggered by growth factors or by molecules inhibiting along their pathways, for example, p21 (54), can lead to activation of pRb and consequent growth arrest. Moreover, Akt may also signal for G_1 cell cycle progression by mTOR and $p70^{S6k}$, which act via $p21^{CIP1/WAF1}$, and hence CD4 and cyclin D1 (55) (Fig. 8).

Taken together, our study suggests that ACCE possesses a prominent anti-PCa activity against both PC-3 and LNCaP cells by modulating cell cycle regulatory proteins through different signaling pathways. In addition, the existence of 2,4,5-trimethoxybenzaldehyde (a potent COX-2 inhibitor) isomers in ACCE may contribute more or less to such an anticancer effect, leaving to us much further investigation. Conclusively, AC might potentially be a good adjuvant therapy for a diversity of prostate cancers despite their androgen dependency and stage of cancers.

Acknowledgments and Notes

We owe many thanks to Ms. Tzu-Wen Tsai for her valuable technical assistance. This work was supported in part by the research grant NSC-93–2320-B-003-006 from the National Science Council, Taiwan. Address correspondence to H. M. Hsieh-Li, Department of Life Science, National Taiwan Normal University, 88, Sec. 4, Ting-Chou Road, Taipei 116, Taiwan. Phone: +886-2-29336876×214. FAX: +886-2-29312904. E-mail: hmhsieh@ntnu.edu.tw.

Submitted 23 March 2006; accepted in final form 13 September 2006.

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