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4-Acetylantroquinonol B Isolated from *Antrodia cinnamomea* Arrests Proliferation of Human Hepatocellular Carcinoma HepG2 Cell by Affecting p53, p21 and p27 Levels

Yu-Wei Lin and Been-Huang Chiang*

Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan

Supporting Information

ABSTRACT: The 4-acetylantroquinonol B isolated from the mycelium of *Antrodia cinnamomea* could inhibit proliferation of hepatocellular carcinoma cells HepG2 with IC_{50} 0.1 μg/mL. When the HepG2 cells were treated with 4-acetylantroquinonol B for 72 h, the proportion of cells in the G1 phase of the cell cycle increased and that in the S phase decreased significantly, and the proportion of G2/M phase cells were not obviously changed. In addition, the 4-acetylantroquinonol B treatment resulted in the decreases of CDK2 and CDK4, and an increase of p27 in a dose-dependent manner. The protein levels of p53 and p21 proteins were also increased when the cells were treated with low dosage (0.1 μg/mL) of 4-acetylantroquinonol B. Higher dosages, however, decreased the expression of p53 and p21 proteins. Assay of RT-PCR indicated that, corresponding to the increases of p53 and p21 proteins at the dosage of 0.1 μg/mL, the mRNAs of p53 and p21 showed 1.66- and 1.61-fold upregulations, respectively. Corresponding to the decreases of CDK2 and CDK4 proteins, the mRNAs of CDK2 and CDK4 showed −1.02- and −1.13-fold downregulations, respectively. However, level of p27 mRNA showed −1.2-fold downregulation in spite of the increase in p27 protein. This observation, again, confirms the fact that the p27 gene rarely undergoes homozygous inactivation in cancer cells. Our finding suggested that the 4-acetylantroquinonol B inhibits proliferation of HepG2 cells via affecting p53, p21 and p27 proteins, and can be considered as a potential cancer drug.

KEYWORDS: 4-acetylantroquinonol B, *Antrodia cinnamomea*, hepatocellular carcinoma cells, HepG2, cell cycle regulators, p27 protein

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer, with more than one million fatalities occurring annually worldwide.[1] Most HCC cells are quite resistant to death receptor mediated apoptosis because these receptors on the cell surface are cross-linked with either agonistic antibodies or soluble death ligand proteins.[2,3] HCC cells also display high resistance to tumor necrosis factor related apoptosis.[4,5] Because of these and other apoptosis resistance mechanisms, it is very difficult to control the growth and metastasis of HCC. Therefore, for liver cancer patients, even after comprehensive therapies such as surgical excision, chemotherapy, ethanol injection, radiofrequency or cryotherapy, the percentages of recurrence and metastasis are still very high as compared with other major solid tumors.[6]

Cell cycle arrest, one of the antitumor responses, is primarily mediated by cyclin-dependent kinases (CDKs) among which CDK2 and CDK4 are identified as the major regulators of G1 arrest.[7,8] Changes of CDK2/4 activities and expression appear to be an important feature of cancer cells.[9] A variety of cell cycle proteins play important roles in liver cancer etiology.[10] It has been found that amplification of the chromosome 11q13 region occurs in HCC, and cyclin D1 gene maps to this region. Therefore, cyclin D1 overexpression might be associated with the development of HCC by affecting normal cell cycle progression.[11,12] Overexpression of cyclin E was also found to be correlated with HCC development.[13−15] The cyclin-dependent kinases are regulated by related proteins called CDK inhibitors. There are two families of them, the INK4 inhibitors (p16, p15, p19 and p18) and the Cip/Kip inhibitors (p21, p27). In addition, several tumor suppressor genes, such as RB gene and p53 gene, are involved in the regulation of the molecular mechanism of cell division.[16]

Among the cell-cycle regulators, p16 and p27 are frequently inactivated in HCC and considered to be the potent suppressors of liver cancer cells. The G1-specific cell-cycle inhibitor, p16, that prevents the association of CDK4 and CDK6 with cyclin D1, is frequently inactivated in HCC via CpG methylation of its promoter region. But p16 is majorly involved in the early steps of heptocarcinogenesis. On the other hand, p27 is frequently inactivated in HCC and considered as an adverse prognostic factor in liver cancer. Several clinical studies revealed that decreased expression of p27 protein is associated with late stage or advanced HCC.[17−19] Cases with multiple liver tumor nodules were also observed to have lower p27 levels than those with fewer nodules.[17] Therefore, p27 expression was suggested to be a useful predicator of the stage of HCC development, and this predictor may influence the choice of therapeutic strategy.[17]

Antrodia cinnamomea (Polyporaceae, Aphyllophorales) is a parasitic microorganism found on the wall of the inner cavity of *Cinnamomum kanehirai* Hay. This fungus possesses various

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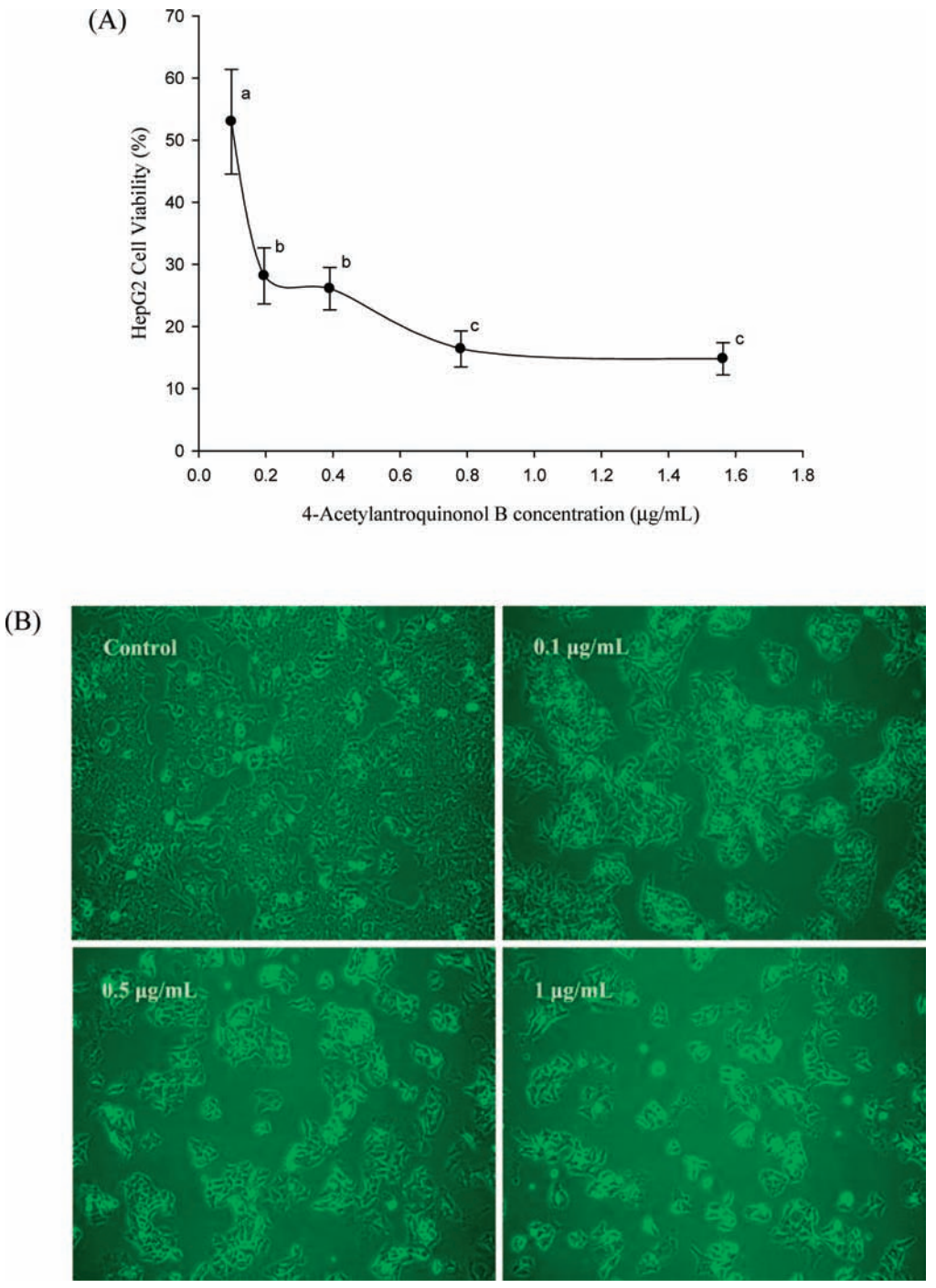

Figure 1. Influence of 4-acetylantroquinonol B on growth and viability of HepG2 cells with different concentrations. (A) Dose-dependent inhibition of HepG2 cells treated with various concentrations of 4-acetylantroquinonol B (0.09 to 1.5 μg/mL). Means with different letters are significantly different ($p < 0.05$). (B) The morphologies of HepG2 cells treated with 4-acetylantroquinonol B (control, 0.1, 0.5, 1 μg/mL).

health-promoting functions, including antioxidation, anti-inflammation and antitumor activities.[20−24] The antitumor activities, especially antihepatoma activity, have drawn much attention in recent years. The extract of fruit body of *A. cinnamomea* can suppress the invasive potential of human liver cancer cell lines and induce apoptosis.[25−27] Besides, the extract of mycelium of *A. cinnamomea* was also found to be able to inhibit the proliferation of hepatoma cells HepG2 and Hep3B.[28−30] Lin and co-workers (2010) isolated the 4-acetylantroquinonol B from the mycelium of *Antrodia cinnamomea*, and found that this compound potently inhibited proliferation and growth of HepG2 cells with IC_{50} 0.1 μg/mL.[31] Since control of the cell cycle is a critical step in controlling the development of human cancers, and knowledge of the effect of 4-acetylantroquinonol B on the expression of cell cycle regulatory proteins in HCC cells is essential for understanding the therapeutic potential of this compound, the objective of this study aimed to gain more understanding about the mechanism of antiproliferation activity of 4-acetylantroquinonol B on HCC cells.

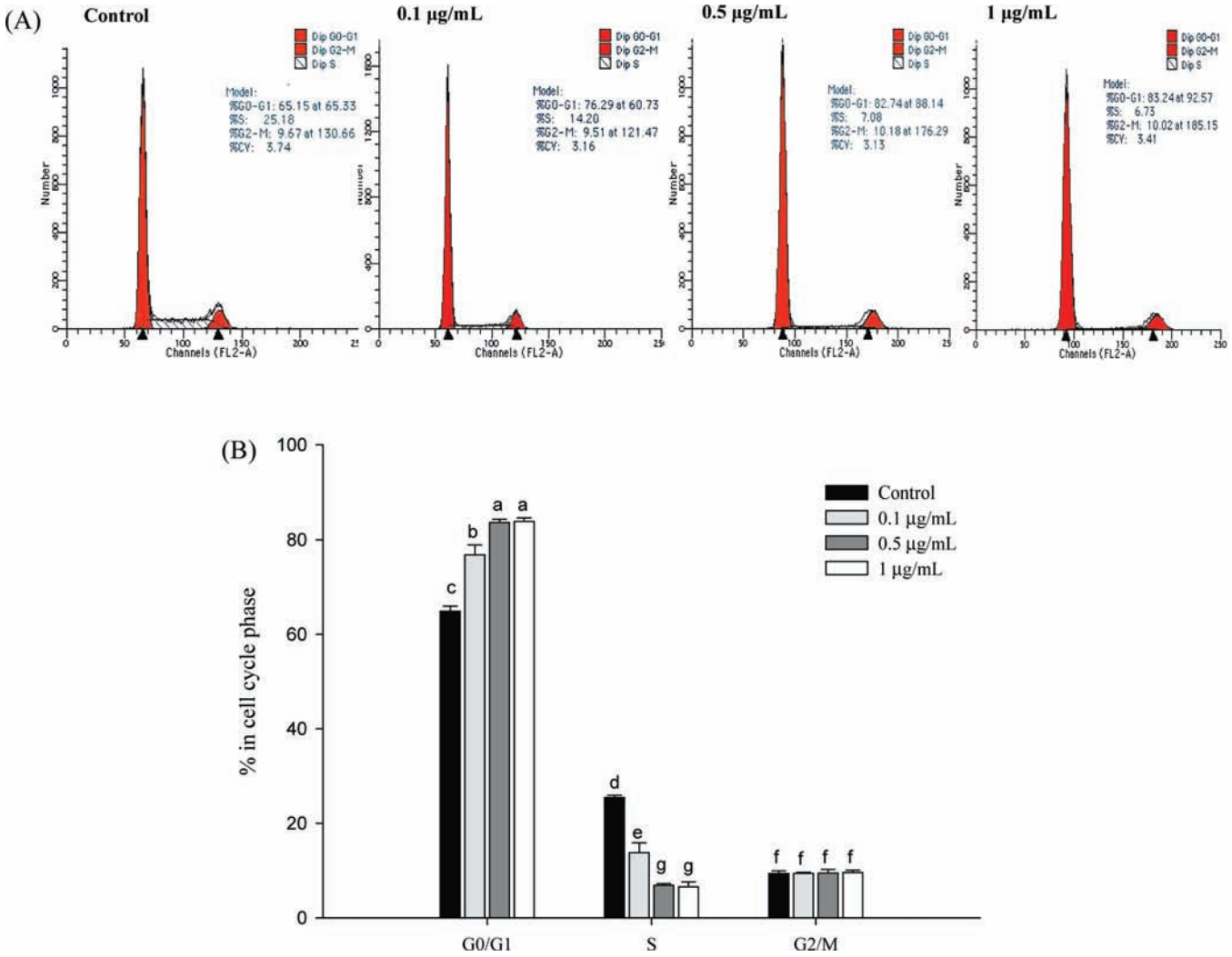

Figure 2. Effects of 4-acetylantroquinonol B on DNA content in HepG2 cells. Cells harvested after 72 h treatment with 4-acetylantroquinonol B (0.1, 0.5, 1 mg/mL) were stained with propidium iodide. (A) The FACSCalibur peak profile of HepG2 cells. (B) Cell cycle distribution. Cell cycle phases with different letters are significantly different ($p < 0.05$).

MATERIALS AND METHODS

Materials. The 4-acetylantroquinonol B was isolated from the mycelium of *Antrodia cinnamomea* as described previously.[31] Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and all other tissue culture regents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). Antibodies to cyclin D1, cyclin E, cyclin-dependent kinase 4 (CDK4), CDK2, p53, p27, p21 and anti-mouse and anti-rabbit IgGs were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sulforhodamine B (SRB), propidium iodide (PI), phenylmethylsulfonyl fluoride (PMSF), leupeptin, dithiothreitol (DTT), rhodamine 123, EDTA, Compound C (6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-*a*] pyrimidine), trichloroacetic acid (TCA), citric acid, Triton X-100, RNase, aprotinin, sodium orthovanadate, ethidium bromide, glutaraldehyde, osmic acid, uranyl acetate, lead citrate, β-glycerophosphate and all of the other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Cultivation and Viability Assay. The cancer cell line HepG2 was purchased from the Bioresource Collection & Research Centre (Food Industry Research and Development Institute, Hsinchu, Taiwan). The cells were cultured in DMEM medium with 10% FBS (v/v) and penicillin (100 units/mL)/streptomycin (100 mg/mL). Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method was used to determine the cell viability. Briefly, tumor cells were cultured in a 96-well microplate (10^4 cells per well in 100 μL of medium) for 24 h, and then incubated with different concentrations of sample for 72 h. At the end of incubation, tetrazolium dye was added as an indicator in order to convert tetrazolium salts to formazan, a colored product. The formazan concentration was measured by a spectrophotometer at 570 nm.

FACScan Flow Cytometric Assay. A total amount of 1×10^6 HepG2 cells were treated with vehicle (0.1% DMSO) or 4-acetylantroquinonol B for the designated times. The cells were then harvested by trypsinization, fixed with 70% (v/v) alcohol at 4 °C for 30 min and washed with PBS. After centrifugation, cells were incubated in 3 mL of 70% ethanol overnight at −20 °C, then centrifuged and resuspended with 0.9 mL of PI solution containing Triton X-100 (0.1% v/v), RNase (0.1 mg/mL) and PI (4 μg/mL). The PI−DNA complex in each cell nucleus was measured with FACSCalibur (Becton & Dickinson Co., Franklin Lakes, NJ, USA). The percentage of cells within the G0/G1, S, and G2/M phases of the cell cycle was determined by using Modfit LT (Verify Software House, ME, USA).

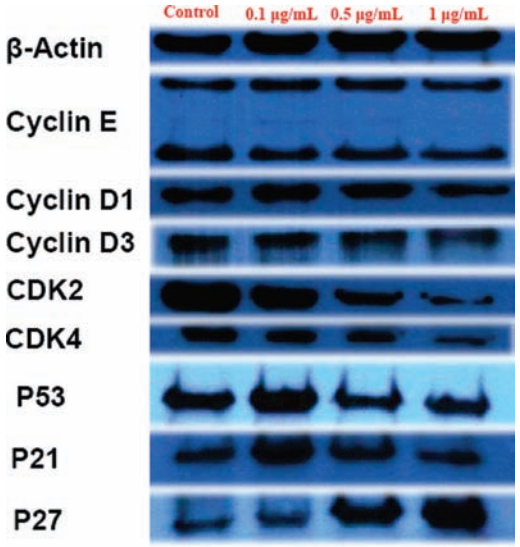

Figure 3. Effects of 4-acetylantroquinonol B on cyclin D/CDK4 and cyclin E/CDK2 protein expression in HepG2 cells. HepG2 cells were treated and Western blot analyses were done with anticyclin D, cyclin E, CDK4, CDK2, p53, p21, p27, and β-actin antibodies as described in Materials and Methods.

Western Blotting. Western blotting protein assay was performed according to Burnette[32] with modification. Briefly, 10 mL of cell suspension (1×10^5 cells/mL) was seeded in a 10 cm culture dish and cultured in DMEM with 10% FBS at 37 °C for 24 h. When cells reached up to 70% confluence, the medium was replaced with serum-free medium containing various concentrations of 4-acetylantroquinonol B and then incubated for 72 h. To analyze actin, cyclin E, cyclin D1, cyclin D3, CDK2, CDK4, p53, p21 and p27 proteins, cells were harvested by trypsinization, centrifuged and lysed. The PRO-PREP protein extraction solution (cell/tissue) as the lysis buffer was purchased from Intron Co. (Seoul, Korea). The lysates were centrifuged, and supernatants were collected. Proteins were separated by 12.5% SDS−PAGE and transferred to polyvinylidene difluoride membranes (PVDF) (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) at 250 mA in transfer buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% methanol (pH 8.3). PVDF membranes were blocked at room temperature for 2 h with 5% BSA in TBST. The membranes were washed and incubated with primary antibodies including actin (1:200; Santa Cruz Biotechnology, Inc., sc-8432), cyclin E (1:200; Santa Cruz Biotechnology, Inc., sc-247), cyclin D1 (1:200; Santa Cruz Biotechnology, Inc., sc-8396), cyclin D3 (1:100; Santa Cruz Biotechnology, Inc., sc-182), CDK2 (1:200; Santa Cruz Biotechnology, Inc., sc-6248), CDK4 (1:1000; Santa Cruz Biotechnology, Inc., sc-23896), p53 (1:1000; Epitomics, P04637), p21 (1:200; Santa Cruz Biotechnology, Inc., sc-817) and p27 (1:1000; Epitomics, P46414) antibodies overnight at 4 °C, and then washed and incubated with secondary antibody in TBST buffer at 25 °C for 2 h. The secondary antibodies used in this study were goat anti-mouse IgG2b-HRP (1:5000; Santa Cruz Biotechnology, Inc., sc-2062) for actin, cyclin E, cyclin D1, CDK2, CDK4 and p21, and mouse anti-rabbit IgG-HRP (1:5000; Santa Cruz Biotechnology, Inc., sc-2357) for cyclin D3, p53 and p27. After washing, the immunoreactive proteins were detected by chemiluminescence (ECL Plus kit, Amersham Pharmacia Biotech), and the membranes were exposed to Fuji medical X-ray film (Fuji Film Co. Ltd. Tokyo, Japan).

RNA Extraction and RT-PCR Assay. For total RNA extraction, the RNeasy Minikit (QIAGEN, Valencia, CA) was used following the manufacturer's instructions. DNase digestion was performed using a ribonuclease-free DNase kit (Ambion, Austin, TX). Total RNAs from the untreated and 0.1 μg/mL 4-acetylantroquinonol B-treated HepG2 cells were reverse transcribed using RT^2 First Strand Kit (SABiosciences, Frederick, MD). Complementary DNAs were amplified and examined by using human cell cycle RT^2 Profiler PCR Array (SABiosciences, Frederick, MD) according to the manufacturer's protocol, which could analyze 84 genes known to be involved in human carcinogenesis. Both the untreated and 0.1 μg/mL 4-acetylantroquinonol B-treated samples were prepared in triplicate and analyzed. Fold changes were estimated using the $\Delta\Delta$Ct method as described by the manufacturer (SABiosciences, Frederick, MD).

Statistical Analysis. All values are means of at least three replicates $\pm$ SD. Statistical analysis was performed using ANOVA and Duncan's multiple range test (SAS Institute Inc., Cary, NC, USA) to determine significant differences among means ($P < 0.05$).

RESULTS

Effect of 4-Acetylantroquinonol B on Cell Cycle Phase Distribution. As 4-acetylantroquinonol B showed a strong growth inhibitory effect on HepG2 cells with IC_{50} approximately 0.1 μg/mL (Figure 1), the follow-up experiment would be to assess if the induced cell growth inhibition occurred via alternations in cell cycle progression. The HepG2 cells were synchronized by serum-supplemented medium for 24 h, and then cultured with serum-free medium containing 1-fold, 5-fold, and 10-fold of the IC_{50} of 4-acetylantroquinonol B (0.1, 0.5, and 1 μg/mL) for 72 h. To evaluate the effect of 4-acetylantroquinonol B on cell cycle phase distribution, flow cytometry analysis was performed, and the results are given in Figure 2. It was found that after treating the cells with 0.1, 0.5, and 1 μg/mL of 4-acetylandroquinonol B for 72 h, the proportion of cells in the G1 phase of the cell cycle increased from 65% to 78%, 82% and 83%, respectively, and the proportion of S phase cells decreased from 25% to 15%, 6% and 6%, respectively. The proportion of G2/M phase cells was not significantly changed. These results indicated that the anticancer activity of 4-acetylantroquinonol B against HepG2 cells was associated with alteration in cell cycle machinery, leading to cell cycle arrests at G1 phase in a dose-dependent manner.

Effect of 4-Acetylantroquinonol B on the Protein Levels of Cell Cycle Regulators. It is known that cyclin D, cyclin E, CDK2, and CDK4/6 cooperate to promote G1 phase progression. Therefore, we first determined whether 4-acetylantroquinonol B regulated the expressive levels of these proteins in HepG2 cells using Western blotting analyses with anticyclin D1, D3, E, CDK2 and CDK4 antibodies. As shown in Figure 3, cyclin E in HepG2 cells underwent no change regardless of the dosage applied. The protein level of cyclin D3 decreased slightly due to 4-acetylantroquinonol B treatment. The protein level of cyclin D1 increased slightly in response to low dosage (0.1 μg/mL) treatment, but decreased with higher dosages (0.5 and 1 μg/mL). On the other hand, the protein levels of cyclin-dependent kinases, CDK2 and CDK4, decreased significantly in a dose-dependent manner.

Since the CDK activity can be controlled by a group of CDKIs, we further examined the effect of 4-acetylantroquinonol B on the protein levels of p53, p21 and p27. As shown in Figure 3, the protein levels of p53 and p21 increased slightly in response to low dosage (0.1 μg/mL) treatment. But at higher dosages (0.5 and 1 μg/mL), the 4-acetylantroquinonol B decreased the protein levels of p53 and p21. The protein level of p27, however, increased in a dose-dependent manner when the cells were treated with 4-acetylantroquinonol B.

Table 1. Effect of 4-Acetylantroquinonol B on Gene Changes Related to Human Cell Cycle

description	fold up- or downregulation
cyclin D1	2.14 ± 0.44
cyclin E1	1.46 ± 0.31
cyclin-dependent kinase 2	−1.02 ± 0.01
cyclin-dependent kinase 4	−1.13 ± 0.09
cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.61 ± 0.17
cyclin-dependent kinase inhibitor 1B (p27, Kip1)	−1.20 ± 0.24
tumor protein p53	1.66 ± 0.39

Effect of 4-Acetylantroquinonol B on the mRNA of Cell Cycle Regulators. To determine whether the level of mRNA related to the cell cycle had changed, RT-PCR assay was carried out. Since the viability of HepG2 cells was about 50% at the dosage of 0.1 μg/mL, and at the higher dosages, 0.5 and 1 μg/mL, the viability of the cells dropped to 10−20%, which was too low to collect the mRNA, we decided to carry out the RT-PCR on the cells at the dosage of 0.1 μg/mL. Besides, results of Western blotting revealed that the dosage of 0.1 μg/mL would result in increases of p53 and p21 proteins. The results of RT-PCR experiments are shown in Table 1. We found that the genes involved in the cell cycle of G1/S phase such as ABL1, ATM, ATR, CCND2, CCNE1, CDK6, CDKN1A, CDKN2A, CHEK2, E2F4, MCM2, MCM3, MCM4, MCM5 and TP53 were upregulated. On the other hand, the genes such as CDC2, CDK2, CDK4, CDKN1B, CHEK1, GADD45A, PCNA, RB1, RBL1, SKP2 and TFDP1 were downregulated (see Supporting Information). Corresponding to the slight increases of cyclin D1, p53, and p21 proteins at the dosage of 0.1 μg/mL, the cyclin D1 mRNA, p53 mRNA and p21 mRNA showed 2.14-, 1.66-, and 1.61-fold upregulations, respectively. In addition, corresponding to the decreases of CDK2 and CDK4 proteins, the mRNAs of CDK2 and CDK4 showed −1.02- and −1.13-fold downregulations, respectively. The cyclin E protein was not changed as shown by the Western blotting analysis, but the cyclin E1 mRNA was upregulated. Since cyclin E1 is one member of the cyclin E family, and other members such as cyclin E2 would also affect the cyclin E protein, the cyclin E1 mRNA might not show the same trend with cyclin E protein. However, it was noticed that in spite of the increase of p27 protein, the level of its mRNA slightly decreased.

DISCUSSION

Modulation of the expression and function of the cell cycle-regulatory proteins, including cyclins, CDKs, p53 and CDKIs, plays important role in the inhibition of cell growth.[33,34] Cyclin/CDK complexes regulate the progression of the cell cycle through activation/inactivation mechanism. In mammalian cells, the key CDKs in the G1 phase are CDK2 and CDK4. They are activated by binding D type cyclins (to CDK4) and cyclin E (to CDK2).[34] However, the cyclin/CDK complexes are inhibited by tumor suppressor p53 and CDKIs such as p21 and p27.

Results of this study showed that expression of cyclin E by the HepG2 cells was not affected by 4-acetylantroquinonol B. In response to the 4-acetylantroquinonol B treatment, however, the protein levels of D3 decreased slightly, but CDK2 and CDK4 decreased significantly. Moreover, the effect of 4-acetylantroquinonol B on CDK2 and CDK4 was dose-dependent. In addition, increases in p53 protein level and mRNA (TP53) were observed in 4-acetylantroquinonol B treated HepG2 cells at the dosage level of 0.1 μg/mL. Further increasing the dosage to 0.5 or 1 μg/mL did not increase the protein level of p53. It is known that regulation of cellular abundance of p53 is largely controlled by protein degradation. In unstressed cells, the level of p53 protein is kept low due to rapid degradation through ubiquitin-dependent proteasome pathway.[35] However, the cells would allow accumulation of p53 in response to stress.[36] Although MDM2 was thought to be the primary factor regulating p53 degradation, recent findings indicated that regulation of p53 stability involves several other proteins and complex mechanisms.[37] We suspect that 4-acetylantroquinonol B might facilitate the phosphorylation of p53 and induce accumulation of p53 at the dosage level of 0.1 μg/mL. However, higher dosages of 4-acetylantroquinonol B (0.5 and 1 μg/mL) might trigger other regulatory mechanisms and cause the decrease of p53 protein.

Similar to the p53 protein, the p21 protein also increased slightly at the dosage of 0.1 μg/mL, but decreased at the higher dosages. It is known that p21 is the downstream target protein of p53. When the p53 protein was degraded, the downstream target protein, p21, could not be upregulated. Thus, the p21 protein showing the same trend as p53 was expected. Interestingly, cyclin D1 protein also followed a similar pattern as p21. This finding is in agreement with what was found by Ilyin et al. (2003); they suggested that induction of both cyclin D1 and p21 is a part of cell response to mitogenic stimulus and associated with cell progression through the restriction point to the late G1 phase of hepatocytes.[38] The cyclin D3 also decreased slightly when the HepG2 cells were treated with 4-acetylantroquinonol B. However, the mechanism of downregulation of cyclin D3 expression is not clear.

On the other hand, we observed that the protein level of Cip/Kip inhibitor, p27, increased due to 4-acetylantroquinonol B treatment in a dose-dependent manner. It has been shown that phosphorylation of p27 at various sites would enhance its stability and rate of accumulation in cells.[39] The p27 was first discovered as an inhibitor of cyclin E-CDK2 because it binds to and inhibits the activity of this complex to prevent G1-S transit in the nucleus. Several investigations have shown that p27 can also inhibit CDK4, especially when it is highly expressed.[40−42] Han et al. (2009) studied the therapeutic mechanism of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) and found that this ionophore could upregulate p27 and downregulate CDK2 and CDK4 in Calu-6 cells.[42] The same group of researchers also found that antimycin A could increase p27 and decrease CDK2, CDK4 and CDK6 in human pulmonary adenocarcinoma cells (Calu-6 cells).[43] Samant et al. revealed that the γ-tocotrienol could arrest the cell cycle of +SA cells, a type of malignant mammary epithelial cells, in G1 phase by increasing p27 levels and decreasing cyclin D1, CDK2, CDK4, CDK6, and suggested that γ-tocotrienol may have potential therapeutic value as an inducer of cell cycle arrest in the treatment of breast cancer.[44] In this study, we found that the protein levels of CDK2 and CDK4 decreased due to 4-acetylantroquinonol B treatment in a dose-dependent manner, thus suspecting that p27 protein was the key inhibitor as the result of 4-acetylantroquinonol B treatment on HepG2 cells, which, in turn, inhibited CDK2 and CDK4 and led to cell cycle arrest at G1 phase. Results of our study, however, showed that p27 mRNA decreased while the protein level of p27 increased when the cells were treated with 0.1 μg/mL of 4-acetylantroquinonol B. Other investigations also found similar phenomena, i.e. the cell cycle-dependent variations in p27 levels are

not reflected by similar changes in p27 mRNA.[45,46] Unlike traditional tumor suppressor genes, the p27 gene rarely undergoes homozygous inactivation in cancer cells.[47−49] Nevertheless, this study revealed that 4-acetylantroquinonol B significantly increased p27 protein and can be considered as a potential cancer drug, because the p27 degradation pathways is a promising cancer drug target.[50]

In summary, our results demonstrated that 4-acetylantroquinonol B inhibited the growth of HepG2 cells in vitro. The growth inhibitory effect of 4-acetylantroquinonol B was mostly mediated by cell cycle arrest via decreases of CDK2 and CDK4 and increase of the p27. And the p53 and p21 proteins also play a minor role in the growth inhibition of HepG2 when the cells were treated with a low dosage of 4-acetylantroquinonol B. These findings provide more information about the mechanisms involved in 4-acetylantroquinonol B-induced growth inhibition in hepatocellular carcinoma and should lead to better understanding of use of this compound.

■ ASSOCIATED CONTENT

Supporting Information. Table of data on the effect of 4-acetylantroquinonol B on all gene changes related to human cell cycle in HepG2 cell. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*No. 1, Roosevelt Road, Section 4, Taipei, Taiwan. Tel: +886-2-33664120. Fax: +886-2-23620849. E-mail: bhchiang@ntu.edu.tw.

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