



The adjuvant effects of *Anrodia Camphorata* extracts combined with anti-tumor agents on multidrug resistant human hepatoma cells

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ARTICLE INFO

Article history:

Received 31 December 2007

Received in revised form 6 April 2008

Accepted 1 May 2008

Available online 7 May 2008

Keywords:

Anrodia camphorata

Hepatoma

Multidrug resistance (MDR)

Cyclooxygenase-2 (COX-2)

Anti-tumor agents

ABSTRACT

Aim of the Study: The objectives of this study were to investigate the adjuvant anti-tumor effects of *Anrodia camphorata* in human hepatoma cells (C3A and PLC/PRF/5) which are resistance to most anti-tumor agents, elucidate the possible regulation pathways, and measure the tumor growth and survival rate in xenograft-nude mice after combined with anti-tumor agents.

Materials and methods: The AC extracts were measured by using a phenol/sulfuric acid method as previously described. The *in vitro* cell proliferation assay of ACs and anti-tumor agents was tested on C3A and PLC/PRF/5 cell lines. The percentage of human hepatoma cells undergoing apoptosis and distributing in different phases of cell cycle were determined by Flow cytometric analysis. Western blot analysis for MDR-1 and apoptosis-related proteins. The measurements of tumor growth and survival analysis of hepatoma implanted nude mice treated with *Anrodia camphorata* extracts and anti-tumor agents alone or in combinations.

Results: We have found that *Anrodia camphorata* extracts, when combined with anti-tumor agents, showed adjuvant antiproliferative effects on hepatoma cells (*in vitro*) and on xenografted cells in tumor-implanted nude mice (*in vivo*), which then extended their median survival days. Furthermore, solid-state extracts of *Anrodia camphorata* (AC-SS) showed its adjuvant effects through the inhibition of MDR gene expressions and the pathway of COX-2-dependent inhibition of p-AKT, which ultimately resulted in the induction of apoptosis in hepatoma cells.

Conclusions: In this study, we have found that *Anrodia camphorata* extract, when combined with anti-tumor agents, showed adjuvant antiproliferative effects on hepatoma cells (*in vitro*) and on xenografted cells in tumor-implanted nude mice (*in vivo*).

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1. Introduction

Anrodia camphorata (AC), known as “niu-chang-chih” in Taiwan, have recently become popular as an alternative therapeutic agent for several types of human cancer (Wu et al., 1997; Wasser and Weis, 1999; Peng et al., 2006). The *Anrodia camphorata* fungus is a rare and expensive folk medicinal material in Taiwan and is commonly used as an antidote, anticancer, anti-itching and hepatoprotective agent (Hsiao et al., 2003; Lin et al., 2006; Lu et al., 2007). Although *Anrodia camphorata* is a promising anti-tumor agent its

fruit body grows slowly on the inner cavity of indigenous and rare tree (*Cinnamomum kanehirai*), which hinders its large scale cultivation and further research (Yang et al., 2006). In contrast, using a submerged cultured method we can obtain useful metabolites from cultured mycelia, including triterpenoides and exopolysaccharides (Lin et al., 2006; Chen et al., 2007). Besides the liquid-state cultured mycelium of *Anrodia camphorata*, the solid-state is also commercially available in Taiwan.

Hepatoma is one of the most prevalent adult malignancies in Taiwan and although some anti-tumor agents were currently used in many medical institutions, there was still no satisfactory therapy till now (Chen, 1995; Chen et al., 1997). The limited responses of hepatoma, mainly hepatocellular carcinoma, to these agents are often due to its multidrug resistance (MDR) to them. The MDR is an intrinsic or acquired cross-resistance to a variety of structurally and functionally unrelated agents and represents one of the major

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obstacles in the effective therapy of many cancers (Bradley et al., 1988; Borst et al., 2000). Overexpression of P-glycoprotein (P-gp) recently has been found to be associated with development of MDR in cancer cells (Schinkel, 1997). P-gp acts as an energy-dependent outward transport pump and can decrease intracellular drug accumulation by removing anti-tumor drugs from the cytoplasm (Ferte, 2000). Some resistant tumor cell lines have been established *in vitro* from P-gp negative cell lines through the exposure of anti-tumor agents such as doxorubicin, cisplatin, etoposide and their acquired resistance is mainly dependent on P-gp expression levels (Rittierodt et al., 2004; Arora et al., 2005; Jin et al., 2007). Furthermore, recent finding of a link between cyclooxygenase-2 (COX-2) and P-glycoprotein expression suggests that COX-2 is involved in the development of the MDR phenotype. Regulation of COX-2 protein expression by AKT in endometrial cancer cells is mediated through NF- κ B/I κ B pathway (St-Germain et al., 2004; Zatelli et al., 2005). In addition, recent studies have suggested that overexpression of COX-2 might be one of the leading factors in hepatic carcinogenesis (Liu et al., 2005). COX-2 can induce angiogenesis via vascular endothelial growth factor (VEGF) and prostaglandin production in HCC and inhibit apoptosis by inducing the antiapoptotic factor Bcl-2 as well as activating antiapoptotic signalings (Daniel et al., 2006; De Moraes et al., 2007; Kim et al., 2007), which consequently lead to the overactivation of relevant survival kinases: AKT and ERKs involved in counteracting apoptosis (Leng et al., 2003). These apoptosis-related molecules, including COX-2, AKT, and pro-apoptotic poly(ADP-ribose) polymerase (PARP) signaling molecules, which are strongly associated with the signal transduction pathway of apoptosis, can thus affect the responses of tumor cells to anti-tumor agents in many solid tumors, especially the metastatic and drug-resistant hepatocellular carcinoma (Nomura et al., 2000).

Recently, the anti-tumor ability of *Antrodia camphorata* crude extracts from liquid/solid-state fermentations have been studied on apoptosis mechanisms in several cancer cell lines (Song et al., 2005a,b). It was reported that the ethylacetate extract of *Antrodia camphorata* mycelia inhibits liver cancer cells by inducing apoptosis in HepG2, PLC/PRF/5, and Hep3B (Chen, 1995). However, the effect of *Antrodia camphorata* on the drug resistance cancer cell has not been studied. Therefore, we are interested in evaluating the effects of combined therapy of *Antrodia camphorata* with anti-tumor agents, especially in MDR human hepatoma cells and elucidating the possible biological mechanisms *in vitro* and *in vivo*.

In this study we attempted to assess the anti-tumor properties of the fermented liquid/solid-state cultured *Antrodia camphorata* mycelia on the MDR human hepatoma cell lines and xenograft-nude mice (ICR nude mice with implanted liver tumor cells). We investigated adjuvant anti-tumor effects of *Antrodia camphorata* in human hepatoma cells (C3A and PLC/PRF/5), which are resistance to most anti-tumor agents, and elucidated the possible regulation pathways in terms of the expressions of COX-2, P-AKT, and PAPR-1. Furthermore, we measured the tumor growth and survival rate in xenograft-nude mice after combined therapy of *Antrodia camphorata* extracts with anti-tumor agents.

2. Materials and methods

2.1. Chemicals and reagents

Culture medium (minimum essential medium, MEM), fetal bovine serum, sodium bicarbonate, and 0.05% trypsin-EDTA were from Gibco Ltd., cisplatin, etoposide, methotrexate, mitomycin, paclitaxel, tetrazolium and Propidium iodide were from Sigma (Saint Louis, MO, USA). Rabbit polyclonal antibodies-MDR-1, COX-

2, p-AKT and mouse monoclonal antibodies—AKT, PARP-1, β -actin were from Santa Cruz Biotechnology, Inc. HRP anti-rabbit IgG and HRP β -anti-mouse IgG were from Santa Cruz Biotechnology, Inc. Nitrocellulose paper membrane were purchased from NEN Life Science Products (USA). Apoptosis Detection Kit was from Strong Biotech Corporation.

2.2. Preparations for *Antrodia camphorata* crude extracts

Acknowledging the similarity of wild *Antrodia camphorata* and solid-state fermentation *Antrodia camphorata* in several characteristics (Liu et al., 2007), we prepared the solid-state cultured *Antrodia camphorata* mycelia (AC-SS). And we also prepared the liquid-state cultured *Antrodia camphorata* mycelia (AC-LS) for comparison considering that submerged fermentation (liquid-state culture) is a rapid and alternative method to obtain higher mycelia yields (Yang and Liao, 1998). The commercial pulverized crude extracts of *Antrodia camphorata* (AC) used in this study were provided by Shenzhen Huikang Bioscience Co., Ltd. (Guangdong, China). The AC-SS extracts contained 15–20% of triterpenoids and 1–2% of polysaccharides. In contrast, the AC-LS extracts contained <1% of triterpenoids and 30% of polysaccharides. Both extracts of *Antrodia camphorata* were quantified by sponsor (HPLC/MS analysis not shown in this text). The two kinds of crude powders were extracted by the method previously described (Hsiao et al., 2003) to obtain AC-SS extract and AC-LS extract. The AC-SS was made in the ratio of 1:10 (v/v) with 95% ethanol at room temperature for 24 h. The extractions were performed in an orbital shaker and the resultant suspension was centrifuged at 5000 rpm for 10 min. Supernatant was removed and the pellet was discarded. Supernatant extracts were filtered using a 0.2- μ m sterile filter and stored at -20°C until use. The AC-LS was extracted with cold water by stirring overnight at room temperature, and after filtered, the residues were extracted twice with cold water under the same procedure.

2.3. Phenol/sulfuric acid method and GPC measurement

The AC extracts were measured by using a phenol/sulfuric acid method as previously described (Dubois et al., 1956). 5% (v/v) phenol (0.5 ml) and 95–97% (v/v) sulfuric acid (2.5 ml) was then added and the mixture was left to stand for 10 min at room temperature. The tubes were then shaken rapidly and left for 30 min at room temperature. The absorbance was measured at a wavelength of 490 nm using a spectrophotometer. A blank test was prepared by substituting distilled water for the sugar solution. A standard curve was prepared using glucose. The molecular weights of exopolysaccharide were determined by a gel permeation chromatography (GPC) system (Shu and Lung, 2004), equipped with a GPC column and a RI detector. The polyethylene glycol (PEG) standards (Polymer Laboratories, UK) were used to construct a calibration curve. The flow rate of the mobile phase was 1 ml/min deionized water.

2.4. Cell cultures of human hepatoma cell lines

Two different human hepatoma cell lines, C3A and PLC/PRF/5 (Food Industry Research and Development Institute, Hsin Chu, Taiwan, ROC) were cultured on MEM medium supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin G sodium and 100 g/ml streptomycin sulfate (Gibco Laboratories, Buffalo, Grand Island, NY, USA) in 75 T culture flasks at 37°C in incubator with 5% CO_2 .

2.5. Cell proliferation assay of human hepatoma cells (MTT assay)

The *in vitro* cell proliferation assay of AC-SS, AC-LS and anti-tumor agents was tested on C3A and PLC/PRF/5 cell lines. Cells at the exponential growth phase were harvested from the culture flasks by trypsination, and then resuspended in fresh medium at 5×10^4 cells/ml. The cell suspensions were dispensed into a 96-well microplate at 100 μ l/well and incubated with 5% CO₂ at 37°C. After 24 h, remove the suspensions and added 200 μ l different concentrations of *Antrodia Camphorata* extracts (0.01–100 μ g/ml) and anti-tumor agents to each well. Cell proliferation in the microplate was determined with the MTT assay after 72 h incubation. 20 μ l PBS solution containing 5 mg/ml tetrazolium (MTT) was added to each well, and further incubated for 4 h then the cells from each well were solubilized with 100 μ l DMSO for optical density reading at 570 nm. Cell proliferation activity was expressed as the percentage of MTT counts of treated cells relative to those of the control (% of control).

2.6. Cell cycle analysis of human hepatoma cells

The percentage of human hepatoma cells undergoing apoptosis and distributing in different phases of cell cycle were determined by propidium iodide (PI)—staining method using flow cytometry. After treatment for 24 h, 5×10^5 cells were harvested from the 6-cm culture dish by trypsination and centrifuged at 2000 rpm for 1–2 min at 4°C and the supernatant was removed. The trypsinized cells and cell pellet were washed with PBS, fixed in 75% cold ethanol, and stored at 4°C overnight. After washed twice with PBS, the ethanol-fixed cells were incubated with 3 μ l RNase (10 mg/ml) at 37°C for 30 min, and stained with 20 μ l PI (20 mg/ml) in the dark. The cell suspension was then filtered through a 35-mm mesh, and analyzed by a flow cytometer (FACSCalibur; Becton Dickinson Biosciences, San Jose, CA, USA) within 1 h. Cellular DNA content was calculated using CellQuest software (Becton Dickinson Biosciences).

2.7. Flow cytometric analysis of apoptosis in human hepatoma cells

After treatment with either anti-tumor agents or *Antrodia camphorata* for 24 h, 5×10^5 cells were harvested from the 6-cm culture dish by trypsination and centrifuged at 2000 rpm for 1–2 min at 4°C and the supernatant was removed. The cells were washed with phosphate-buffered saline (PBS) and resuspended in 250 μ l binding buffer (Annexin V-FITC kit) containing 10 μ l of Annexin V-FITC stock and 10 μ l of 1 mg/ml propidium iodide (PI) for the determination of phosphatidylserine (PS) exposure on the outer plasma membrane. After incubated for 15 min at room temperature in a light protected area, the samples were analyzed by a Becton Dickinson fluorescence cytometer using CellQuest software.

2.8. Western blot analysis for MDR-1 and apoptosis-related proteins

Cancer cell lysates were prepared by lysing 10^5 cells in lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris pH 7.5 and protease inhibitors). After incubation on ice for 30 min, lysates were spun at $13,000 \times g$ for 30 min in centrifuge. Post-nuclear supernatants were mixed with two times sample buffer and boiled for 5 min. Samples were separated on a 10% polyacrylamide gel, which was then transferred to Nitrocellulose paper membrane. Blots were blocked with BSA in PBST (11.24 g Na₂HPO₄, 2.87 g NaH₂PO₄ and 0.1% Tween 20) for 2 h at room temperature. After blocking, membranes were incubated for 1 h at room temperature in wash buffer

with either the anti-MDR-1 antibody (1:800), anti-COX-2 antibody (1:500), anti-AKT antibody (1:500), anti-p-AKT antibody (1:500) and anti-PARP-1 antibody (1:500), followed by four times 10 min washes. Horseradish peroxidase-conjugated anti-rabbit IgG antibody was diluted to 1:5000 in wash buffer and incubated with blots for 1 h at room temperature, followed by washes four times every 10 min, and detected by enhanced chemiluminescence (FUJIFILM LAS-3000). And the β -actin signal was used to normalize protein loading.

2.9. The measurements of tumor growth and survival analysis of hepatoma implanted nude mice treated with *Antrodia camphorata* extracts and anti-tumor agents alone or in combinations.

ICR nude mice (nu/nu, male) were purchased from National Laboratory Animal Center, Taipei, Taiwan. Upon arrival, the animals were kept in the animal room for 14 days to adapt to the environment. They were housed four per cage under standard laboratory conditions at an ambient temperature ($23 \pm 2^\circ\text{C}$) and given a solid diet and tap water ad libidum. The study was conducted in accordance with the guideline for the care and use of Laboratory Animals by the Animal Research Committee in Chi-Mei Medical Center. Animals were acclimatized to the laboratory conditions prior to the experiments and all experiments were carried out between 10:00 a.m. and 05:00 p.m. On the 15th day, 16 mice either injected with 1×10^7 cells C3A ($n=8$) or PLC/PRF/5 ($n=8$) cells (from the cell culture described above) subcutaneously (S.C.) into the flank of ICR nude mice to induce tumors.

Based on the xenografted-nude mice models above, the recipient mice were randomly divided into four groups (eight mice for each group): control group which was treated orally with distilled water, group 1 (positive control group) treated with a proven cancer drug, 5 mg/(kg week) cisplatin four times for 21 consecutive days (i.e. on Day 1, Day 8, Day 15 and Day 21) by intraperitoneal (i.p.) injection, group 2 treated orally with AC-SS for 21 consecutive days, and group 3 treated with the 5 mg/(kg week) cisplatin four times combined with orally AC-SS (200 mg/(kg day)) for 21 consecutive days. Body weight and tumor size of each tumor-bearing mouse was measured daily before their death or humane sacrifices. The date of death was recorded and the survival rate of experimental mice was calculated for survival analysis. The tumor size was estimated by $L \times W^2 \times 0.52 \text{ cm}^3$, where L and W denote the long and short dimensions, respectively. The blood of all tumor-bearing mice were collected for alpha-fetoprotein (α -FP) analysis.

2.10. Statistical analysis

Results are expressed as mean + S.E. Analysis of variance (ANOVA) was used to evaluate the results. When ANOVA was significant, the significance between groups was assessed by means of unpaired Student's *t*-test and analyzed with SPSS10.0. * and ** denotes *p*-value less than 0.05 and less than 0.01, respectively. *p*-Value less than 0.05 was considered significant.

3. Results

3.1. The antiproliferative effects of anti-tumor drugs and extracts on human hepatoma cells

As shown in Fig. 1A and B, we have found that human hepatoma cells are sensitive to most anti-tumor agents only in high concentrations (10 and 100 μ M) and mitomycin among five anti-tumor agents had the most prominent antiproliferative effects in

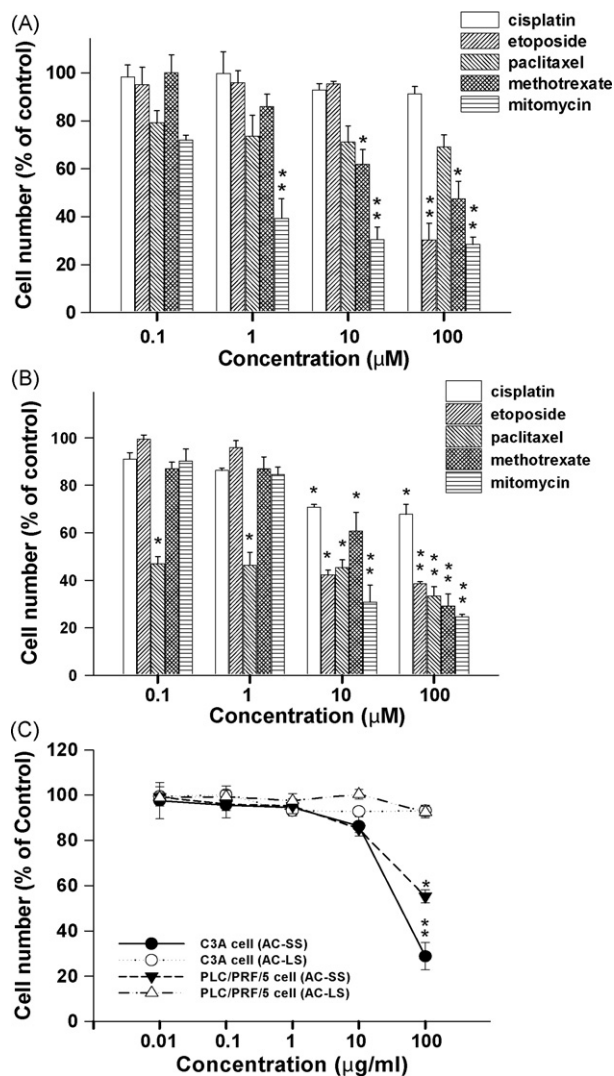


Fig. 1. The antiproliferative effects of anti-tumor drugs and *Androdia camphorata* extracts (AC-SS and AC-LS) on human hepatocellular carcinoma cells. The inhibition rate of hepatoma cell proliferation was determined by MTT assay after 72 h. C3A (A) and PLC/PRF/5 (B) were treated with five anti-tumor agents and *Androdia camphorata* extracts (AC-SS and AC-LS) (C) by different concentrations. Data are represented as percentage of untreated vehicle controls. * denotes $p < 0.05$ and ** denotes $p < 0.01$.

C3A and PLC/PRF/5 cells and, in contrast, cisplatin had the least effects (no effect in C3A cell line even the concentration was as high as 100 μM). *Androdia camphorata* extracts (AC-SS and AC-LS) did not show remarkable inhibitive effects in C3A and PLC/PRF/5 cells (Fig. 1C). However, AC-SS in the highest concentration (100 μM) had shown its inhibitive effects on proliferation of C3A and PLC/PRF/5 cells, and that is the reason why we only chose AC-SS as the representative of *Androdia camphorata* extracts in the following experiments.

3.2. The adjuvant inhibitive effects of *Androdia camphorata* extract (AC-SS) combined with anti-tumor drugs on proliferation of human hepatoma cells

AC-SS (1 $\mu\text{g/ml}$) showed prominent adjuvant inhibitive effects on proliferation of hepatoma cells when it was combined with cisplatin or mitomycin (Fig. 2). The adjuvant effect of AC-SS was more prominent in C3A cells (Fig. 2A) than in PLC/PRF/5 cells (Fig. 2B). As for the anti-tumor agents, mitomycin when combined with AC-SS

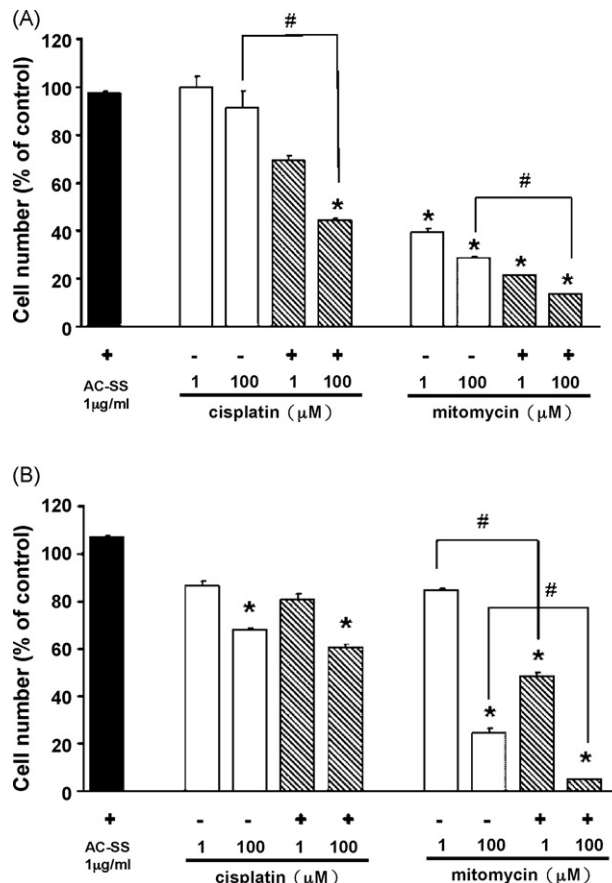


Fig. 2. The adjuvant effects of *Androdia camphorata* extracts (AC-SS and AC-LS) combined with anti-tumor agents in human hepatoma cells. C3A (A) and PLC/PRF/5 (B) hepatoma cells were treated by two anti-tumor agents (cisplatin and mitomycin) with or without the AC-SS (1 $\mu\text{g/ml}$) after 72 h. Data are represented as percentage of the control (AC-SS treated only). * denotes $p < 0.05$, comparison between treated group and the control and # denotes $p < 0.05$, comparison between group treated with anti-tumor agents and AC-SS and that treated with anti-tumor agents only.

showed better inhibition effect than cisplatin. This result is compatible with our findings (Fig. 1) that mitomycin among five anti-tumor agents had the most prominent antiproliferative effects.

3.3. The combined treatment of AC-SS with anti-tumor agents increased apoptosis ratio of hepatoma cells and arrested their cell cycle in G0/G1 stage

In assessing the effects of AC-SS (1 $\mu\text{g/ml}$) on cell cycle arresting and the induction of apoptosis in two hepatoma cell lines (C3A and PLC/PRF/5), we have found that after 24 h treatment of AC-SS alone could not arrest all stages of cell cycle in two cell lines (Fig. 3A and B); however, the combined treatment of AC-SS and two anti-tumor agents (10 μM of cisplatin and mitomycin) increased the distribution of cell cycle in stage G0/G1. In measuring the ratio of apoptosis in two cell lines, we also have found that AC-SS (1 $\mu\text{g/ml}$), when combined with mitomycin, had a higher apoptosis ratio than with cisplatin. Furthermore, the combined treatment of AC-SS with cisplatin showed increased apoptosis ratio only in PLC/PRF/5 cells but not in C3A cells.

3.4. Inhibitive expressions of MDR-1 after combined treatment of AC-SS and anti-tumor agents

Over expressions of MDR in cancer cells can explain why so many anti-tumor agents had decreased or lost their expected

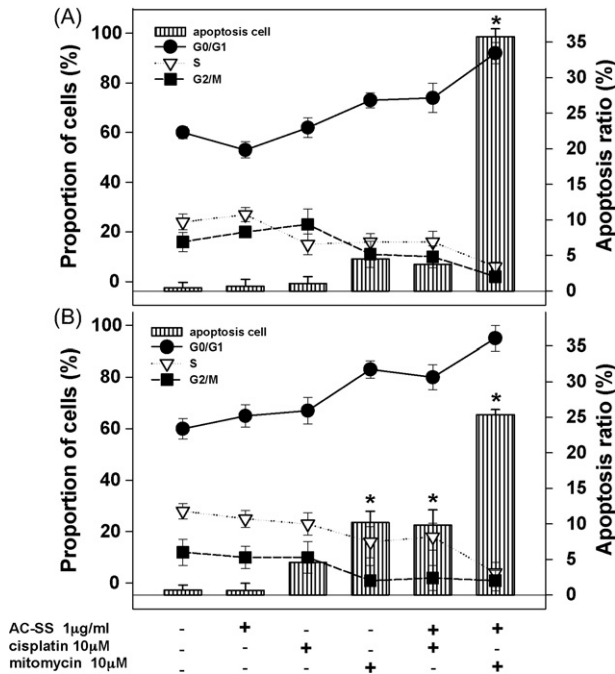


Fig. 3. Cell cycle and apoptosis analyses of the hepatoma cells treated with AC-SS and anti-tumor agents alone or in combinations. After 24 h of treatment of C3A (A) and PLC/PRF/5 (B) cells with anti-tumor agents and/or AC-SS, population percentage in the G0/G1 phase (●), S phase (▽) and G2/M phase (■) of the cell cycle were curved and analyzed. The black filate columns represented a median ratio of apoptosis treated with mitomycin and cisplatin, which was combined with or without AC-SS (1 µg/ml) for 24 h treatment. Data are represented as percentage of untreated controls. Values are mean + S.E. for two independent experiments performed in triplicate. Data analysis were made by Cell Quest software. * denotes $p < 0.05$.

effects in the treatments of cancer patients. The MDR-1 protein is one of the most important biomarkers in evaluating the drug resistance of cancer cells. In the measurement of MDR-1 expressions in two hepatoma cell lines (C3A and PLC/PRF/5), we have found that AC-SS (1 µg/ml), as compared with vehicle control group, did not increase the levels of MDR-1 proteins in two cell lines after treatment for 24 h; nevertheless, cisplatin (10 µM), paclitaxel (10 µM) and mitomycin (1 µM) increased levels of MDR-1 proteins and cisplatin induced more expressions of MDR-1 than mitomycin did (Fig. 4A). These data indicated that treatments with anti-tumor agents may induce over-expression of MDR-1. However, after AC-SS was combined with anti-tumor agents the protein levels of MDR-1 significantly decreased (Fig. 4A). Furthermore, the protein levels of MDR-1 induced by cisplatin (10 µM) in PLC/PRF/5 cells were reduced by increasing the concentrations of AC-SS (0.001–1.0 µg/ml) in time-dependent manner (12, 24 and 48 h) (Fig. 4B). In contrast, AC-SS had no significant inhibitory effect on the protein levels of MDR-1 when cisplatin was replaced by mitomycin (1 µM) (Fig. 4C).

3.5. The expressions of apoptosis-related proteins in hepatoma cells after the combination treatment of AC-SS and anti-tumor agents

In order to elucidate the mechanisms by which AC-SS had showed its adjuvant effects when combined with anti-tumor agents we studied the expressions of apoptosis-related proteins (COX-2, AKT, p-AKT and PARP-1) by Western blot analysis in C3A and PLC/PRF/5 cells. We have found that treatment of AC-SS (1 µg/ml) alone induced the same levels of COX-2, AKT and p-AKT

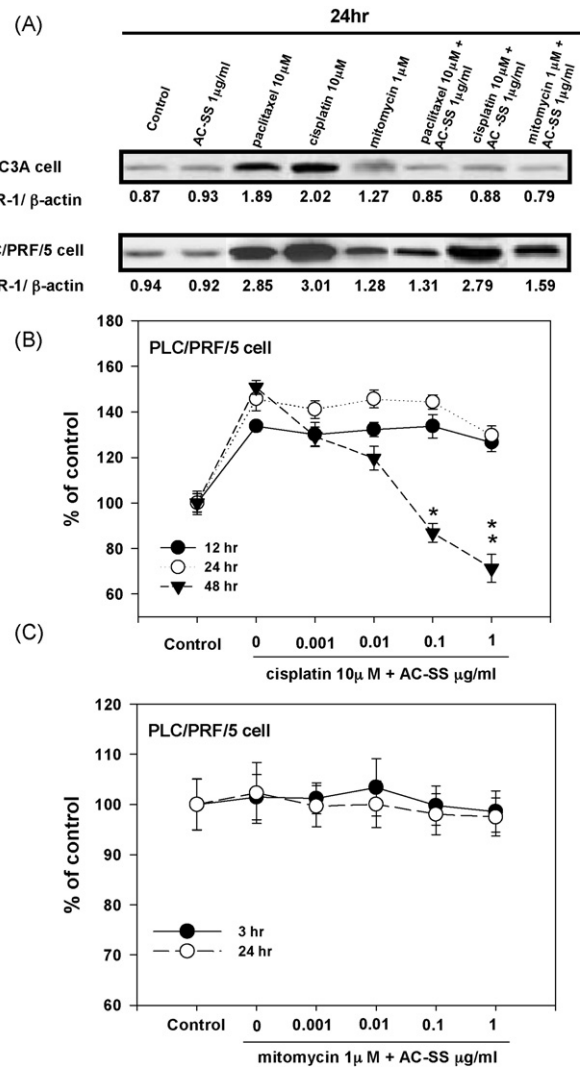


Fig. 4. Inhibition of MDR-1 expression in hepatoma cells treated with AC-SS and anti-tumor agents. MDR-1 expressions were evaluated by Western blot analysis at 24 h (A) in two hepatoma cells after treated with AC-SS and/or three anti-tumor agents. The MDR-1 expression of PLC/PRF/5 cell treated with 10 µM cisplatin (B), 1 µM mitomycin (C) alone or in combination with different concentrations of AC-SS after 3, 12, 24 and 48 h, respectively. Data are represented as percentage of untreated vehicle control. * denotes $p < 0.05$ and ** denotes $p < 0.01$.

as vehicle control did. However, AC-SS (1 µg/ml), when combined with mitomycin (10 µM), induced less levels of COX-2, and p-AKT than control and mitomycin alone. Similarly, AC-SS (1 µg/ml) combined with cisplatin (10 µM) also induced less levels of COX-2, and p-AKT than control and cisplatin alone, although it took longer duration of treatment for AC-SS plus cisplatin than AC-SS plus mitomycin (48 h vs. 12 h) (Fig. 5A). As PARP-1 is a nuclear enzyme that is involved in DNA repairs and is specifically cleaved by caspase-3, it is considered to be a biochemical marker of apoptosis. Our data have showed that treatment of AC-SS (1 µg/ml) with either mitomycin (10 µM) for 12 h or cisplatin (10 µM) for 48 h only slightly increase the levels of PARP-1 in C3A and PLC/PRF/5 cells (Fig. 5B).

3.6. The combined treatment of AC-SS with cisplatin diminished xenografted tumor mass and extended the median survival days of tumor-implanted nude mice

For the assurance that the xenografted hepatoma cells (C3A and PLC/PRF/5) did survive well after implanted subcutaneously

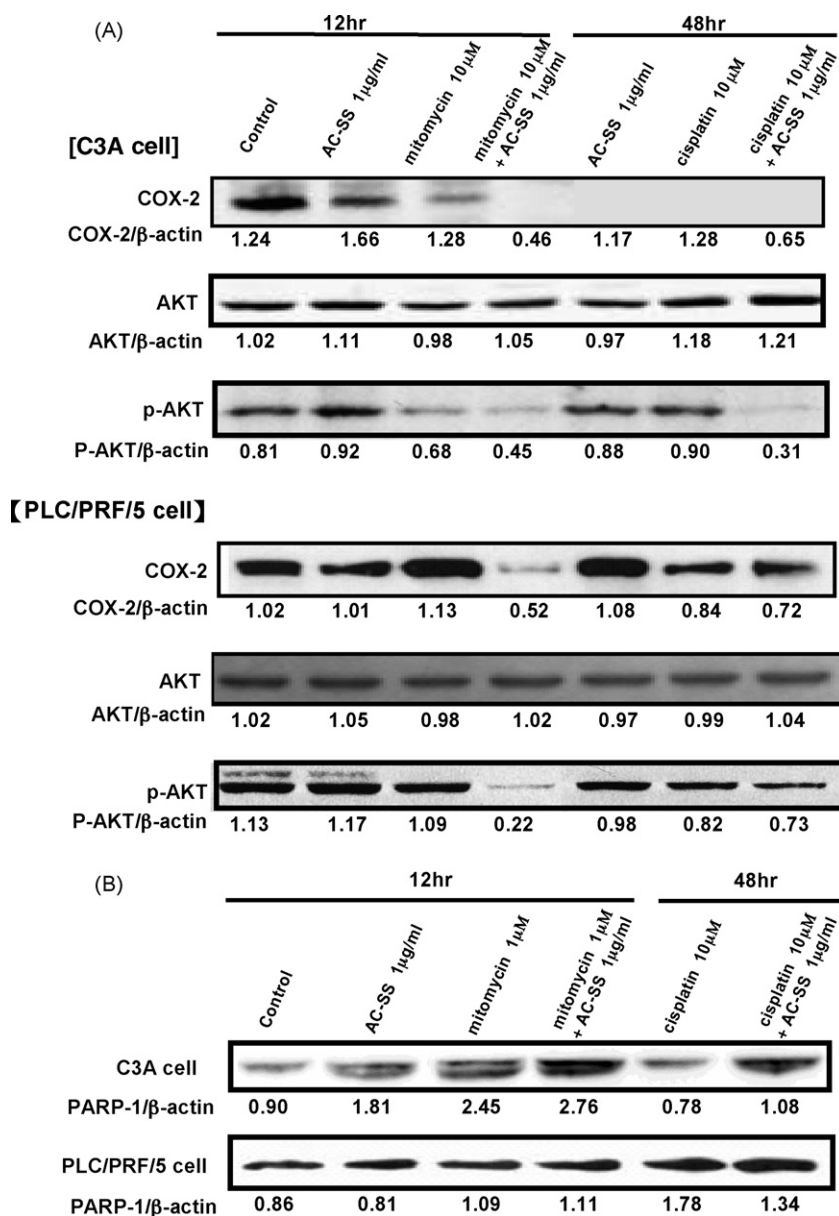


Fig. 5. The expressions of apoptosis-related proteins in hepatoma cells treated with either AC-SS or anti-tumor agents, and their combinations. The expressions of COX-2, AKT, p-AKT (A) and PARP-1 (B) proteins in hepatoma cells were analyzed after treated 12 and 48 h with AC-SS, cisplatin and mitomycin alone or their combinations.

into the flank of ICR nude mice, we measured α -FP serum levels of nude mice and higher levels in tumor groups than control group were found (Fig. 6A). In addition, we measured the body weights of tumor-implanted nude mice after treated with AC-SS (200 mg/(kg day), orally) and cisplatin (5 mg/(kg week), i.p.) alone or in combination. There were no significant changes of body weight between treatment groups and control group (Fig. 6B). After measuring the size of xenografted tumors, we have found that AC-SS (200 mg/(kg day)) combined with low dose cisplatin (1 mg/(kg week)) could significantly diminish the tumor mass of xenografted C3A and PLC/PRF/5 cells almost as the same levels as high dose cisplatin (5 mg/(kg week)) could do, although AC-SS (200 mg/(kg day)) alone had no significant mass reducing effect (Fig. 6C). Furthermore, we have found that the combined treatment of AC-SS (200 mg/kg/day) with low dose cisplatin (1 mg/(kg week)) significantly extended the median survival days of PLC/PRF/5 cells implanted nude mice (118 days, 95% CI: 103–131 days vs. control group: 93 days, 95% CI, 85–103 days) (Fig. 6D). Two of eight mice

had survived 143 days without evidence of tumor growth. When treated with cisplatin (5 mg/(kg week)) or AC-SS (200 mg/(kg day)) alone the median survival days was 114 days (95% CI, 96–127 days) and 111 days (95% CI, 96–132 days), respectively, without significant differences between these two groups.

4. Discussion

In this study, we have found that *Antrodia camphorata* extract, when combined with anti-tumor agents, showed adjuvant antiproliferative effects on hepatoma cells (*in vitro*) and on xenografted cells in tumor-implanted nude mice (*in vivo*). In cancer cell model, *Antrodia camphorata* extract (AC-SS, 1 μ g/ml) showed prominent adjuvant inhibitive effects on proliferation of human hepatoma cells when it was combined with cisplatin (100 μ M) or mitomycin (100 μ M). In xenografted cancer animal model, the combined treatment of AC-SS (200 mg/(kg day)) with cisplatin (1 mg/(kg week)) significantly extended the median survival days of PLC/PRF/5 cells

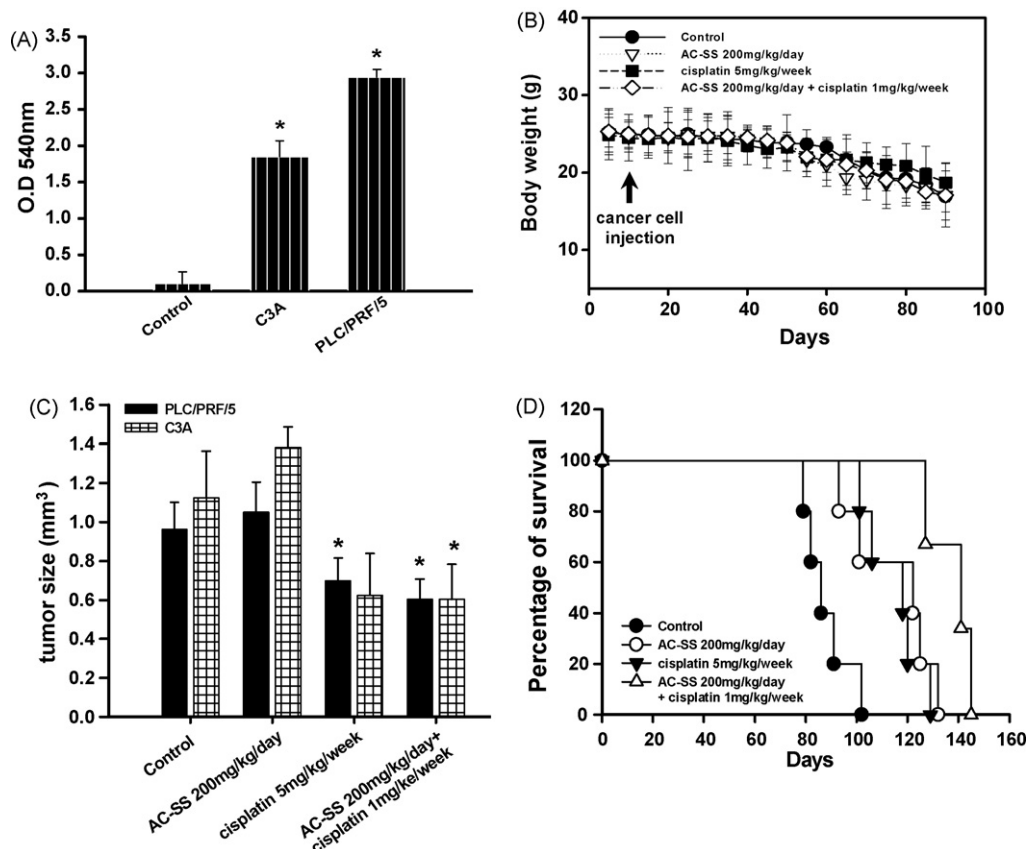


Fig. 6. The α -FP level, tumor size and survival analysis of hepatoma cells-implanted nude mice treated with AC-SS and anti-tumor agents alone or in combination. The serum level of α -FP was determined by α -FP ELISA kit for tumor groups and control group (A). The xenografted nude mice implanted with human hepatoma PLC/PRF/5 cells were treated with AC-SS, cisplatin and AC-SS plus cisplatin for consecutive 21 days. Vehicle controls were fed with normal saline. The body weight of nude mice was measured every 3 days (B). The tumor size of xenografted mass in nude mice was measured on 21st day (C). The survival number and the survival day of nude mice in all groups were calculated (D). Data are represented as percentage of untreated vehicle control. Values are mean \pm S.E. * denotes $p < 0.05$.

implanted nude mice (118 days, 95% CI: 103–131 days vs. control group: 93 days, 95% CI, 85–103 days).

In assessing the effects of AC-SS (1 μ g/ml) on cell cycle arresting and the induction of apoptosis in two hepatoma cell lines (C3A and PLC/PRF/5), we have found that treatment of AC-SS combined with either 10 μ M cisplatin or mitomycin increased the distribution of cell cycle in stage G0/G1 and the apoptosis ratio also increased in parallel. This findings suggested that the adjuvant antiproliferative effects of AC-SS on hepatoma cells were through the arrest of cell cycle and apoptotic pathway, and are compatible with recent studies (Shin et al., 2005) which showed that the related processes of neoplastic transformation, progression and metastasis involve the alteration of normal apoptotic pathways. Recently the relationship between apoptosis and cancer has been emphasized, suggesting apoptosis provides a number of clues with respect to effective anticancer therapy, and plays a necessary role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells (van der Luit et al., 2007).

In this study, we have found that combined treatments of AC-SS with anti-tumor agents could inhibit the expressions of MDR gene in hepatoma cells, thus partially explaining its adjuvant effects. In the measurement of MDR-1 expressions in two hepatoma cell lines (C3A and PLC/PRF/5), we have found that treatments with anti-tumor agents may induce over-expression of MDR-1. However, after AC-SS was combined with anti-tumor agents the protein levels of MDR-1 significantly decreased (Fig. 4A). In addition, we found that cisplatin induce more expressions of MDR-1 than mitomycin, which was compatible with our findings that hepatoma cells are less sen-

sitive to cisplatin. Therefore, it is plausible that AC-SS plus cisplatin showed better inhibitive effects on the expressions of MDR-1 than AC-SS plus mitomycin was found. These findings suggested that AC-SS might show its anti-tumor adjuvant effects in cisplatin resistant hepatoma cells mainly through inhibition of MDR expressions.

The MDR phenotype is associated with the increased drug efflux from cells that is mediated by an energy-dependent mechanism and the blockade of the apoptosis-inducing pathway (Osborn and Chambers, 1996; Theïvenod et al., 2000; Braga et al., 2007). Hepatoma cells often responded with limited efficacy to currently available chemotherapeutics due to over expressions of multidrug resistance. Recent studies had suggested that cyclooxygenase-2 (COX-2) is involved in the development of the MDR phenotype (Schinkel, 1997; Gao et al., 2007). Recent studies have also shown that the induction of apoptosis was accompanied by a reduction in AKT phosphorylation (p-AKT), and AKT have been implicated as mediators of these events in liver tumor cells (Alexia et al., 2006; Granado-Serrano et al., 2006). In assessing the levels of apoptosis-related proteins, we have found that AC-SS (1 μ g/ml), when combined with either cisplatin (10 μ M) or mitomycin (10 μ M), induced less levels of COX-2, and p-AKT (phosphorylated-AKT) than control, cisplatin or mitomycin. These results might suggest that the adjuvant effects of AC-SS (1 μ g/ml) combined with cisplatin or mitomycin was through the pathway of COX-2-dependent inhibition of p-AKT and the resultant induction of apoptosis in hepatoma cells.

In this study, we had established xenografted hepatoma cells implanted mice models for studying the adjuvant effects of orally AC-SS (200 mg/(kg/day)) on cisplatin regimens (1 mg/(kg week)) in

tumor animal model. And we found that AC-SS (200 mg/(kg day)), combined with cisplatin regimen (1 mg/(kg week)) significantly extended the median survival days of PLC/PRF/5 cells implanted nude mice (118 days, vs. control group: 93 days) (Fig. 6D) and better than higher dosage cisplatin regimen (5 mg/(kg week)). Furthermore, we noted that α -FP serum levels of PLC/PRF/5 cells implanted nude mice were higher than those of C3A cells (Fig. 6A) and we also found AC-SS (1 μ g/ml) had its prominent adjuvant inhibition effects on proliferation of hepatoma cells, more in C3A cells than in PLC/PRF/5 cells (Fig. 2). All these findings are in accordance with the report that The human hepatoblastoma C3A (derived from HepG2) cells conserved normal p53, p21/WAF-1, Fas/APO-1 and bcl-2 gene, but the human hepatocellular carcinoma, PLC/PRF/5, which has no one of the above genes, is less sensitive to many anti-tumor agents (Hsu et al., 2005). Therefore, we specifically observed the survival days of PLC/PRF/5 cells implanted nude mice after treated with AC-SS and cisplatin regimen (hepatoma cells are less sensitive to cisplatin) to approximate the situations of multidrug resistance in human hepatocellular carcinoma we have mostly encountered in clinical practices.

Some bioactive constituents from the fruiting bodies of *Antrodia camphorata* have been isolated and characterized, including polysaccharides, steroids, triterpenoids and sesquiterpene lactone (Chang and Chou, 1995). Most studies reported that polysaccharide extracts of *Antrodia camphorata* exhibit biological effects in their antioxidant and anti-cancer activities, and the tumor-inhibition activity has been documented in numerous mushroom polysaccharide fractions such as a glucan-protein complex as the most promising pharmacologically active anti-tumor compounds (Liu et al., 2004). In this study we had prepared two kinds of crude extracts: solid-state extracts of *Antrodia camphorata* (AC-SS) and liquid-state extracts of *Antrodia camphorata* (AC-LS). AC-SS, extracted from alcohol, contained 15–20% of triterpenoids and 1–2% of polysaccharides and AC-LS, extracted from cold water, contained 30% of polysaccharides (HPLC and GC/MS data not shown). However, in this study both extracts (AC-SS and AC-LS) did not show remarkable antiproliferative effects in hepatoma cells *in vitro* (Fig. 1C), although AC-SS in the highest concentration (100 μ M) had shown its inhibitive effects. But later we found that AC-SS, when combined with anti-tumor agents, showed its adjuvant effects. Because AC-SS contained more triterpenoids and less polysaccharides than AC-LS, the roles triterpenoids and the ingredients other than polysaccharides play in the treatments of MDR human hepatoma will be clarified in further study.

In summary, we have found that *Antrodia camphorata* extract, when combined with anti-tumor agents, showed adjuvant antiproliferative effects on hepatoma cells (*in vitro*) and on xenografted cells in tumor-implanted nude mice (*in vivo*), which then extended their median survival days. Furthermore, AC-SS showed its adjuvant effects through the inhibition of MDR gene expressions and the pathway of COX-2-dependent inhibition of AKT phosphorylation (p-AKT), which ultimately resulted in the induction of apoptosis in hepatoma cells. In further study we will clarify the roles the ingredients of AC-SS, especially the triterpenoids play in the treatments of MDR human hepatoma.

Acknowledgments

We thank Dr. Shun-Lai Li for kindly supplying the crude extracts of *Antrodia camphorata* and the investigation was partially supported by a Research Grant (CCMP96-RD-015) Committee on Chinese Pharmacy, Department of Health, Executive Yuan, Taipei, Taiwan.

References

- Alexia, C., Bras, M., Fallot, G., Vadrot, N., Daniel, F., Lasfer, M., Tamouza, H., Groyer, A., 2006. Pleiotropic effects of PI-3' kinase/Akt signaling in human hepatoma cell proliferation and drug-induced apoptosis. *Annals of the New York Academy of Sciences* 1090, 1–17.
- Arora, A., Seth, K., Kalra, N., Shukla, Y., 2005. Modulation of P-glycoprotein-mediated multidrug resistance in K562 leukemic cells by indole-3-carbinol. *Toxicology and Applied Pharmacology* 202, 237–243.
- Bradley, G., Juranka, P.F., Ling, V., 1988. Mechanism of multidrug resistance. *Biochimica et Biophysica Acta* 948, 87–128.
- Braga, F., Ayres-Saraiva, D., Gattassa, C.R., Capella, M.A.M., 2007. Oleonic acid inhibits the activity of the multidrug resistance protein ABC1 (MRP1) but not of the ABCB1 (P-glycoprotein): possible use in cancer chemotherapy. *Cancer Letters* 248, 147–152.
- Borst, P., Evers, R., Kool, M., Wijnholds, J., 2000. A family of drug transporters: the multidrug resistance-associated proteins. *Journal of the National Cancer Institute* 92, 1295–1302.
- Chang, T.T., Chou, W.N., 1995. *Antrodia camphorata* sp. *Cinnomomum Kamehirai* in Taiwan. *Mycological Research* 99, 756–758.
- Chen, C.C., Liu, Y.W., Ker, Y.B., Wu, Y.Y., Lai, E.Y., Chyau, C.C., Hseu, T.H., Peng, R.Y., 2007. Chemical characterization and anti-inflammatory effect of polysaccharides fractionated from submerged-cultured *Antrodia camphorata* mycelia. *Journal of Agricultural and Food Chemistry* 55, 5007–5012.
- Chen, C.J., Yu, M.W., Liaw, Y.F., 1997. Epidemiological characteristics and risk factors of hepatocellular carcinoma. *Journal of Gastroenterology and Hepatology* 12, 294–308.
- Chen, D.S., 1995. Hepatitis C virus in chronic liver disease and hepatocellular carcinoma in Taiwan. *Princess Takamatsu Symposium* 25, 27–32.
- Daniel, S., Metzinger, Douglas, D., Taylor, Gercel-Taylor, C., 2006. Induction of p53 and drug resistance following treatment with cisplatin or paclitaxel in ovarian cancer cell lines. *Cancer Letters* 236, 302–308.
- De Moraes, E., Dar, N.A., de Moura Gallo, C.V., Hainaut, P., 2007. Cross-talks between cyclooxygenase-2 and tumor suppressor protein p53: balancing life and death during inflammatory stress and carcinogenesis. *International Journal of Cancer* 121, 929–937.
- Dubois, M., Gilles, K., Hamilton, J., Rebers, P., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28, 350–356.
- Ferte, J., 2000. Analysis of the tangled relationships between P-glycoprotein-mediated multidrug resistance and the lipid phase of the cell membrane. *European Journal of Biochemistry* 267, 277–294.
- Gao, A., Liu, B., Shi, X., Jia, X., Ye, M., Jiao, S., You, B., Huang, C., 2007. Phosphatidylinositol-3 kinase/Akt/p70^{S6K}/AP-1 signaling pathway mediated benzo(a)pyrene-induced cell cycle alternation via cell cycle regulatory proteins in human embryo lung fibroblasts. *Toxicology Letters* 170, 30–41.
- Granado-Serrano, A.B., Martín, M.A., Bravo, L., Goya, L., Ramos, S., 2006. Quercetin induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-kinase/Akt and ERK pathways in a human hepatoma cell line (HepG2). *The Journal of Nutrition* 136, 2715–2721.
- Hsiao, G., Shen, M.Y., Lin, K.H., Lan, M.H., Wu, L.Y., Chou, D.S., Lin, C.H., Su, C.H., Sheu, J.R., 2003. Antioxidative and hepatoprotective effects of *Antrodia camphorata* extract. *Journal of Agricultural and Food Chemistry* 51, 3302–3308.
- Hsu, Y.L., Kuo, Y.C., Kuo, P.L., Ng, L.T., Kuo, Y.H., Lin, C.C., 2005. Apoptotic effects of extract from *Antrodia camphorata* fruiting bodies in human hepatocellular carcinoma cell lines. *Cancer Letters* 221, 77–89.
- Jin, W., Scotto, K.W., Hait, W.N., Yang, J.M., 2007. Involvement of CtBP1 in the transcriptional activation of the MDR1 gene in human multidrug resistant cancer cells. *Biochemical Pharmacology* 74, 851–859.
- Kim, K.C., Kim, J.S., Son, J.K., Kim, I.G., 2007. Enhanced induction of mitochondrial damage and apoptosis in human leukemia HL-60 cells by the *Ganoderma lucidum* and *Duchesnea chrysantha* extracts. *Cancer Letters* 246, 210–217.
- Leng, J., Han, C., Demetris, A.J., Michalopoulos, G.K., Wu, T., 2003. Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through Akt activation: evidence for Akt inhibition in celecoxib-induced apoptosis. *Hepatology* 38, 756–768.
- Lin, W.C., Kuo, S.C., Lin, W.L., Fang, H.L., Wang, B.C., 2006. Filtrate of fermented mycelia from *Antrodia camphorata* reduces liver fibrosis induced by carbon tetrachloride in rats. *World Journal of Gastroenterology* 12, 2369–2374.
- Liu, D.Z., Liang, H.J., Chen, C.H., Su, C.H., Lee, T.H., Huang, C.T., Hou, W.C., Lin, S.Y., Zhong, W.B., Lin, P.J., Hung, L.F., Liang, Y.C., 2007. Comparative anti-inflammatory characterization of wild fruiting body, liquid-state fermentation, and solid-state culture of *Taiwanofungus camphoratus* in microglia and the mechanism of its action. *Journal of Ethnopharmacology* 113, 45–53.
- Liu, N.B., Peng, T., Pan, C., Yao, Y.Y., Shen, B., Leng, J., 2005. Overexpression of cyclooxygenase-2 in human HepG2, Bel-7402 and SMMC-7721 hepatoma cell lines and mechanism of cyclooxygenase-2 selective inhibitor celecoxib-induced cell growth inhibition and apoptosis. *World Journal of Gastroenterology* 11, 6281–6287.
- Liu, J.J., Huang, T.S., Hsu, M.L., Chen, C.C., Lin, W.S., Lu, F.J., Chang, W.H., 2004. Antitumor effects of the partially purified polysaccharides from *Antrodia camphorata* and the mechanism of its action. *Toxicology and Applied Pharmacology* 201, 186–193.

- Lu, Z.M., Tao, W.Y., Zou, X.L., Fu, H.Z., Ao, Z.H., 2007. Protective effects of mycelia of *Antrodia camphorata* and *Armillariella tabescens* in submerged culture against ethanol-induced hepatic toxicity in rats. *Journal of Ethnopharmacology* 110, 160–164.
- Nomura, F., Yaguchi, M., Togawa, A., Miyazaki, M., Isobe, K., Miyake, M., Noda, M., Nakai, T., 2000. Enhancement of poly-adenosine diphosphate-ribosylation in human hepatocellular carcinoma. *Journal of Gastroenterology and Hepatology* 15, 529–535.
- Osborn, M.T., Chambers, T.C., 1996. Role of the stress-activated/c-Jun NH2-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. *Journal of Biological Chemistry* 271, 30950–30955.
- Peng, C.C., Chen, K.C., Peng, R.Y., Su, C.H., Hsieh-Li, H.M., 2006. Human urinary bladder cancer T24 cells are susceptible to the *Antrodia camphorata* extracts. *Cancer Letters* 243, 109–119.
- Rittierodt, M., Tschernig, T., Harada, K., 2004. Modulation of multidrug-resistance-associated P-glycoprotein in human U-87 MG and HUV-ECC cells with antisense oligodeoxynucleotides to MDR1 mRNA. *Pathobiology* 71, 123–128.
- Schinkel, A.H., 1997. The physiological function of drug-transporting P-glycoproteins. *Seminars in Cancer Biology* 8, 161–170.
- Wu, S.H., Ryvarden, F.L., Chang, T.T., 1997. *Antrodia camphorata* (“niu-chang-chih”), new combination of a medicinal fungus in Taiwan. *Botany Bulletin of Academic Sinica* 38, 273–275.
- Shin, V.Y., Wu, W.K., Chu, K.M., Wong, H.P., Lam, E.K., Tai, E.K., Koo, M.W., Cho, C.H., 2005. Nicotine induces cyclooxygenase-2 and vascular endothelial growth factor receptor-2 in association with tumor-associated invasion and angiogenesis in gastric cancer. *Molecular Cancer Research* 3, 607–615.
- Shu, C.H., Lung, M.Y., 2004. Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorata* in batch cultures. *Process Biochemistry* 39, 931–937.
- Song, T.Y., Hsu, S.L., Yeh, C.T., Yen, G.C., 2005a. Mycelia from *Antrodia camphorata* in submerged culture induce apoptosis of human hepatoma HepG2 cells possibly through regulation of Fas pathway. *Journal of Agricultural and Food Chemistry* 53, 5559–5564.
- Song, T.Y., Hsu, S.L., Yen, G.C., 2005b. Induction of apoptosis in human hepatoma cells by mycelia of *Antrodia camphorata* in submerged culture. *Journal of Ethnopharmacology* 100, 158–167.
- St-Germain, M.E., Gagnon, V., Parent, S., Asselin, E., 2004. Regulation of COX-2 protein expression by Akt in endometrial cancer cells is mediated through NF-kappaB/IkappaB pathway. *Molecular Cancer*, 3.
- Theïvenod, F., Friedmann, J.M., Katsen, A.D., Hauseri, I.A., 2000. Up-regulation of multidrug resistance P-glycoprotein via nuclear factor-kB activation protects kidney proximal tubule cells from cadmium- and reactive oxygen species-induced apoptosis. *Journal of Biological Chemistry* 275, 1887–1896.
- van der Luit, A.H., Vink, S.R., Klarenbeek, J.B., Perrissoud, D., Solary, E., Verheij, M., van Blitterswijk, W.J., 2007. A new class of anticancer alkylphospholipids uses lipid rafts as membrane gateways to induce apoptosis in lymphoma cells. *Molecular Cancer Therapy* 6, 2337–2345.
- Wasser, S.P., Weis, A.L., 1999. Therapeutic effects of substances occurring in higher Basidiomycetes mushroom: a modern perspective. *Critical Review in Immunology* 19, 65–96.
- Yang, F.C., Liao, C.B., 1998. Effect of cultivation on the mycelia growth of *Ganoderma lucidum* in submerged flask cultures. *Bioprocess Engineering* 19, 233–236.
- Yang, H.L., Chen, C.S., Chang, W.H., Lu, F.J., Lai, Y.C., Chen, C.C., Hseu, T.H., Kuo, C.T., Hseu, Y.C., 2006. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*. *Cancer Letters* 231, 215–227.
- Zatelli, M.C., Luchin, A., Piccin, D., Tagliati, F., Bottoni, A., Vignali, C., Bondanelli, M., degli Uberti, E.C., 2005. Cyclooxygenase-2 inhibitors reverse chemoresistance phenotype in medullary thyroid carcinoma by a permeability glycoprotein-mediated mechanism. *Journal of Clinical Endocrinology and Metabolism* 90, 5754–5760.