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In Vivo Antitumor Effects of 4,7-Dimethoxy-5-methyl-1,3-benzodioxole Isolated from the Fruiting Body of *Antrodia camphorata* through Activation of the p53-Mediated p27/Kip1 Signaling Pathway

Shih-Hsin Tu,[†,‡] Chih-Hsiung Wu,[§,∥,⊥] Li-Ching Chen,[†] Ching-Shui Huang,[†,‡] Hui-Wen Chang,[#] Chien-Hsi Chang,[#] Hsiu-Man Lien,[*,∇,+] and Yuan-Soon Ho[*,⊥,#,○]

[†]Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan

[‡]Department of Surgery, Cathay General Hospital, Taipei, Taiwan

[§]Department of Surgery, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan

[∥]Department of Surgery, Taipei Medical University−Shuang Ho Hospital, Taipei, Taiwan

[⊥]Center of Excellence for Cancer Research, Taipei Medical University, Taipei, Taiwan

[#]Department of Laboratory Medicine, Taipei Medical University Hospital, Taipei, Taiwan

[∇]Department of Chemistry, Tunghai University, Taichung, Taiwan

[○]School of Medical Laboratory Science and Biotechnology, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan

ABSTRACT: In this study, 4,7-dimethoxy-5-methyl-1,3-benzodioxole (SY-1) was isolated from three different sources of dried *Antrodia camphorata* (AC) fruiting bodies. AC is a medicinal mushroom that grows on the inner heartwood wall of *Cinnamomum kanehirai* Hay (Lauraceae), which is an endemic species that is used in Chinese medicine for its antitumor properties. We demonstrated that SY-1 [given as a 1−30 mg/kg body weight intraperitoneal (ip) injection three times per week] profoundly decreased the growth of COLO-205 human colon cancer cell tumor xenografts in an athymic nude mouse model. We further demonstrated that significant AC extract-mediated antitumor effects were observed at the highest concentration (5 g/kg body weight/day). No gross toxicity signs were observed (i.e., body weight changes, general appearance, or individual organ effects). Frozen COLO-205 xenograft tumors were pulverized in liquid N_2, and the expression of cell cycle regulatory proteins was detected by immunoblotting. We found that the p53-mediated p27/Kip1 protein was significantly induced in the low-dose (1 mg/kg body weight) SY-1-treated tumors, whereas the p21/Cip1 protein levels did not change. The G0/G1 phase cell cycle regulators induced by SY-1 were also associated with a significant decrease in cyclins D1, D3, and A. These results provide further evidence that SY-1 may have significance for cancer chemotherapy.

KEYWORDS: *Antrodia camphorata, p53, p27/Kip1, COLO 205, antitumor*

INTRODUCTION

Antrodia camphorata (*A. camphorata*, AC), which is also called *Antrodia cinnamomea*, is composed of fruiting bodies, mycelium, and spores and is a parasitic fungus that only grows on the inner heartwood wall of *Cinnamomum kanehirai* Hay (Lauraceae). AC has been used in traditional Chinese medicine to treat food and drug intoxication, diarrhea, abdominal pain, hypertension, pruritus (skin itch), and liver cancer; however, its biological activities have not been thoroughly investigated. Our recent studies have demonstrated that AC and its purified compound 4,7-dimethoxy-5-methyl-1,3-benzodioxole (SY-1) induce significant apoptosis in COLO-205 colon cancer cells but not in cultured normal colon endothelial cells.[1] We also demonstrated that that SY-1 induced G0/G1 phase cell cycle arrest through the induction of p53-mediated cyclin-dependent kinase (CDK) inhibitor (i.e., p21/Cip1 and p27/Kip1) expression. Our recent study demonstrated that AC may be used as an adjuvant antitumor agent for HT 29 human colon cancer cell xenograft tumors.[2] Another study also revealed antitumor effects, which were assessed by monitoring tumor growth and the survival rate of xenograft nude mice after combined therapy with several antitumor agents.[3]

Colon cancer affects 50−60 out of every 100000 people in North America and, after lung cancer, is the second most common cause of cancer-related deaths.[4] A recent study revealed that the nutritional and genetic risk factors for colon tumors are additive in the mouse tumor phenotype, establishing that diet and genetic factors affect risk by distinct combinatorial mechanisms.[5] Such results imply that the intake of healthy food ingredients, such as dietary fiber[6] and seed oil,[7] are useful in the prevention of colon cancer. Our recent study has demonstrated that a set of 10 4,7-dimethoxy-1,3-benzodioxole derivatives,

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which were isolated from several kinds of vegetables based on a lead compound, SY-1, which was isolated from AC, were evaluated for their in vitro inhibitory activity on human colorectal carcinoma cells (COLO 205).[8] These compounds should have some cancer preventive effects. Therefore, our study aimed to find new therapeutic agents such as AC extract to use in adjuvant therapies.[2] One approach, as explored in our previous report, sought to identify medicinal agents that are capable of arresting the cell cycle and/or activating the apoptotic response in cancerous cells.[1] In this study, our results demonstrate that SY-1, which is isolated from dried AC fruiting bodies, significantly inhibited the growth of COLO-205 cell xenograft tumors through the inhibition of p53-mediated cell cycle regulatory genes. Our results highlight the molecular mechanisms of the antitumor effects mediated by SY-1 in an in vivo system.

MATERIALS AND METHODS

Source of Organism. As shown in Figure 1A, fruiting body samples were obtained from AC sources. Sample a, which was an *A. camphorata* fungal strain (BCRC-36795), was purchased from the Food Industry Research and Development Institute, HsinChu County, Taiwan. Sample b, which was designated SY-1, was isolated from three different sources of AC. In this study, it was a gift from the Yusheng Co., Ltd. (Taichung, Taiwan).[1]

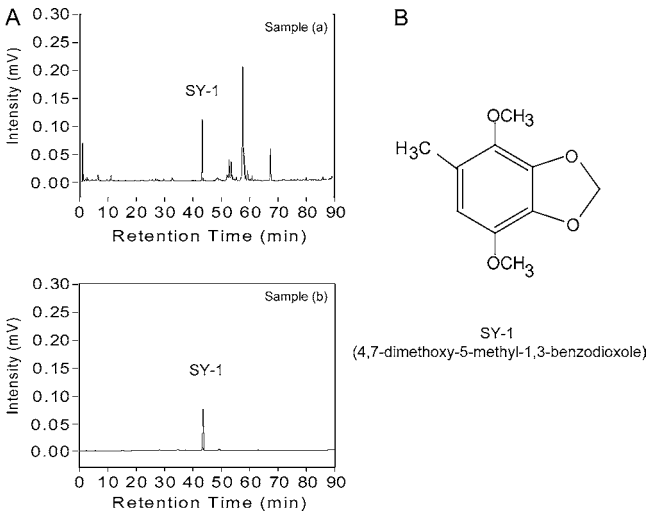

Figure 1. Isolation and chemical structural characterization of SY-1 from AC. (A) HPLC chromatograms of AC. Sample a, plate cultivation of the fungus AC (BCRC-36795) (spiked concentration, 20 mg/mL). Sample b, SY-1 derived from the fruiting bodies of AC (YS-187) provided by the Yusheng Co., Ltd. (spiked concentration, 20 mg/mL). (B) The chemical structure of SY-1.

AC Extract Plate Cultivation. AC (BCRC-36795) was inoculated in a culture medium of potato dextrose agar composed of 0.4% diced potato extract, 2% glucose, and 1.5% agar in distilled water. The medium was shaken at 28 ± 1 °C in the dark at 100 rpm using a rotary shaker for 14 days. The culture broth was collected and evaporated under reduced pressure. The precipitated residue (i.e., the *AC* fruiting body samples) was lyophilized at −80 °C.

Isolation and Characterization of SY-1 from AC Fruiting Body Samples. The dried fruiting body samples (i.e., sample a, BCRC-36795) were ground to a fine powder using an electrical mill and sequentially extracted with ethyl acetate at 80 °C under reflux for 3 h. The extracts were separated by gel filtration and chromatographed on a Sephadex LH-20 column (MeOH). The flow rate was 0.5 mL/min. Each fraction contained 15 mL and was collected using a fraction collector. The fractions were further analyzed using a reverse-phase C18 column (Mightysil RP-18 GP 250-10, 5 μm). The mobile phase, which was pumped at a flow rate of 1.5 mL/min, was programmed as follows: 2% acetic acid:acetonitrile (38:62) for 25 min, a gradual decrease to 2% acetic acid−acetonitrile (0:100) between the 26th and 55th min, and then a gradual increase to 2% acetic acid:acetonitrile (38:62) between the 56th and 70th min. The spectrophotometric detector was set at 252 nm. Retention fractions were collected every 43.43 min and concentrated by evaporation under reduced pressure. Our results revealed that the yields of SY-1 from dried fruiting body sample b were 5.7 mg/g. The same fractions were lyophilized to obtain SY-1 (Figure 1B). The biophysical data for SY-1 are as follows: ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.15 (3H, s, CH_3), 3.82 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 5.91 (2H, s, O-CH_2-O), and 6.27 (1H, s, aromatic H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 15.92 (CH_3), 56.85 (OCH_3), 59.94 (OCH_3), 101.44 (O-CH_2-O), 108.72, 123.66, 134.63, 136.51, 138.64, 138.83. MS (M^+) m/z 196. Anal. ($C_{10}H_{12}O_4$) C, H.

Treatment of COLO-205-Derived Xenografts in Vivo with SY-1 and AC Extract. Our previous study demonstrated that SY-1 specifically induced cell cycle arrest and apoptosis in COLO-205 human colon cancer cells.[1] COLO-205 cells grown in RPMI 1640 supplemented with 10% FCS were harvested through two consecutive trypsinizations, centrifuged at 300*g* for 5 min, washed twice with PBS, and resuspended in sterilized PBS. The cells (5×10^6) were resuspended in 0.1 mL of RPMI 1640 and subcutaneously injected between the scapulae of each BALB/c nu/nu mouse (4 week old females, n = 5 per group) purchased from the National Science Council Animal Center, Taipei. After transplantation, the tumor size was measured using calipers, and the tumor volume was estimated as follows: tumor volume (mm^3) = $1/2 \times L \times W^2$, where L is the length and W is the width of the tumor.[9,10] Once the tumor reached a volume of 200 mm^3, the animals received intraperitoneal injections of normal saline (25 μL) and SY-1 (1, 5, and 30 mg/kg body weight, which were defined as 1X, 5X, and 30X, respectively) three times per week for 30 days. Tumor-bearing mice were also treated with crude AC extract for comparison. The dosages for AC extract treatment were 0.05, 0.5, and 5 g/kg body weight/day, which were powder and administered in Lab Diet (defined as 1X, 10X, and 100X, respectively). After the experiment, the xenografts were weighed, snap-frozen in dry ice, and stored at −80 °C until Western blot analysis. All mouse protocols were performed according to an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-approved protocol.

Protein Extraction and Western Blot Analysis. The frozen tumors were pulverized in liquid N_2 and mixed with lysis buffer (0.5 M Tris-HCl, pH 6.8, 0.4% SDS) as previously described.[11,12] Western blot analysis was also performed as previously described.[13] Immunodetection was performed by probing with appropriate dilutions of specific antibodies at room temperature for 2 h. Anti-p21/Cip1, anti-p27/Kip1, anti-p53, anti-cdc2, anti-Rb1/2, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibodies (Santa Cruz, Inc., CA) and anticyclin D1, anticyclin D3, anticyclin A, anticyclin B, anti-CDK2, anti-CDK4, and anti-CDC25C monoclonal antibodies (Transduction Laboratories, Lexington, KY) were used at 1:1000 dilution. The anticyclin A polyclonal antibody (Transduction, San Diego, CA) was used at 1:250 dilution. The secondary alkaline phosphatase-coupled antimouse and antirabbit antibodies (Jackson, Westgrove, PA) were incubated at room temperature for 1 h at 1:5000 and 1:1000 dilutions, respectively.

Statistical Analysis. All data are reported as means ± SEs. Comparisons were subjected to one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Significance was defined as $p < 0.05$.

RESULTS

According to our previous report,[1] AC extracts were isolated via an extensive chromatographic purification of the ethyl acetate-soluble fraction of the dried fruiting body (Figure 1A, sample a). One major peak was observed, and the chemical structure of

the purified powder was elucidated by NMR and mass spectrometry and identified as SY-1 (Figure 1A, lower, and B).

In Vivo Experimental Group and Dosage Design. In this study, the average body weight per mouse was 28 g with a daily Lab Diet consumption of 5 g (Figure 2). In mice receiving

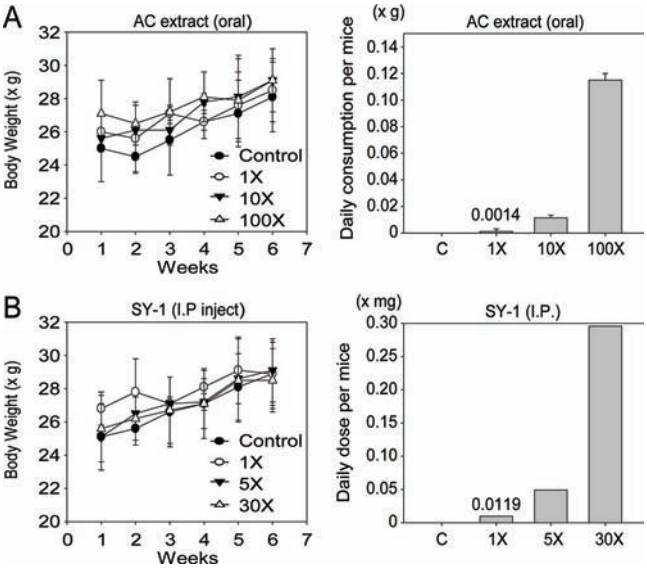

Figure 2. Dose-dependent effects of SY-1 and AC extract on body weight changes and daily dose consumption in COLO-205-bearing mice. The mice were treated with either (A) oral administration of Lab Diet containing AC extract at 0.05, 0.5, or 5 g/kg body weight/day, defined as 1X, 10X, and 100X, respectively, or Lab Diet (used as the negative control). (B) ip injection of SY-1 with 1, 5, and 30 mg/kg body weight/three times per week, defined as 1X, 5X, and 30X, respectively. Left panel, the average body weight per mouse was 28 g with a daily Lab Diet consumption of 5 g. The daily consumption of these agents are summarized in the right-hand panel. Both groups were treated for 30 days, and five animals were analyzed in each group. The values presented represent means ± SEs.

the treatment regimens, no gross signs of toxicity were observed (i.e., changes in body weight or general appearance or effects on individual organs) (Figure 2A,B, left panels). The AC treatment doses for the extract were 0.05, 0.5, and 5 g/kg body weight/day, which were defined as 1X, 10X, and 100X, respectively, and Lab Diet alone was used as a negative control. Accordingly, the daily 1X AC extract dose received was calculated to equal 1.4 mg per mouse (Figure 2A, right panel, bar 2). An additional mouse group received SY-1 ip injections (1, 5, and 30 mg/kg body weight/three times per week, which were defined as 1X, 5X, and 30X, respectively). Both groups were treated for 30 days. According to this protocol, the daily received dose of 1X SY-1 was calculated to equal 0.0119 mg per mouse (Figure 2B, right panel, bar 2). Our previous report revealed that the final SY-1 concentration from dried AC extract was 5.7 mg/g (or 1/175 w/w).[1] In this study, the 1X doses of SY-1 and AC extracts used in the nude mouse experiments were designated as 0.0119 and 1.4 mg/mouse/day, respectively (i.e., a dosage ratio of 1/117.6 w/w). This dosage ratio was close to the relevant natural SY-1 concentration appearing in crude AC extracts (1/175, w/w).

Inhibition of in Vivo Malignant Human Colon Cell Proliferation by SY-1 Treatment. We further examined the therapeutic efficacy of the AC extract and SY-1 in vivo by treating athymic mice bearing COLO-205 tumor xenografts. After 30 days, tumor growth in the group treated with the highest dose of AC extract (100X) was significantly inhibited in comparison with the control-treated mice (Figure 3A,B, bar 4

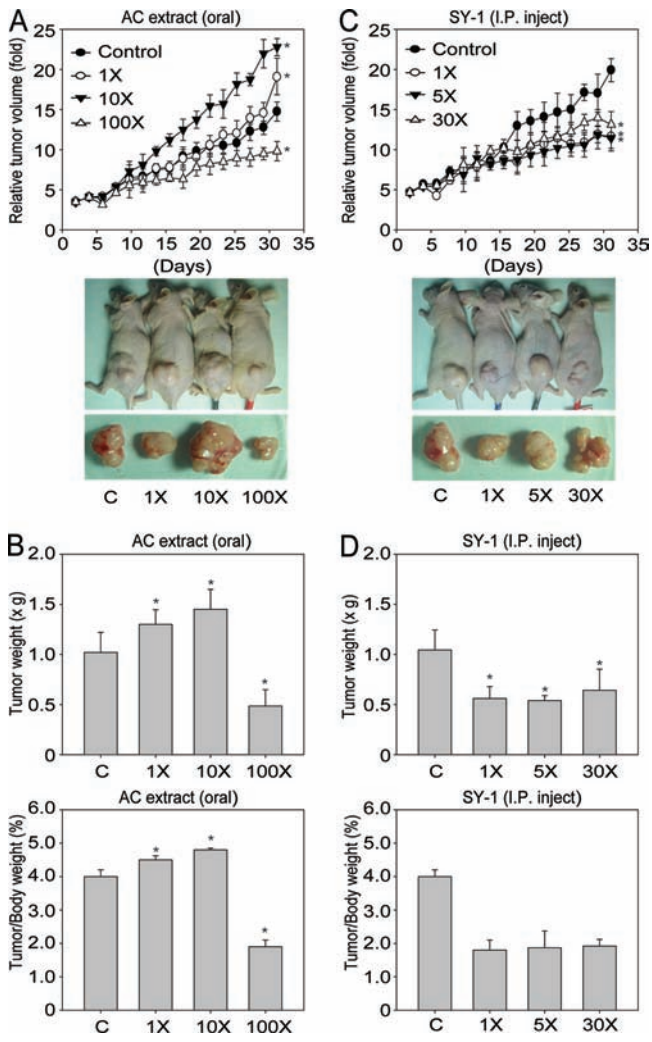

Figure 3. Dose-dependent SY-1 and AC extract-induced antitumor effects in human COLO-205 xenograft tumor-bearing mice. The COLO-205 tumor-bearing mice were treated with (A, B) AC crude extract and (C, D) SY-1 in a dose-dependent manner, as described in the Materials and Methods. The tumor growth curves (A, C) tumor weights and tumor/body weight ratio (B, D) were determined during and after the experiment. Comparisons were analyzed using ANOVA followed by Fisher's least significant difference test. Significance was defined as $p < 0.05$.

vs bar 1; $p < 0.05$). Surprisingly, statistically significant greater tumor growth volume was observed in mice that received 1X and 10X AC extracts than in control mice (Figure 3A,B, bars 2 and 3 vs bar 1; $p < 0.05$). Similarly, in the SY-1-treated group, significant tumor growth inhibition was detected in SY-1-treated mice treated using the lower doses (1X and 5X) (Figure 3C,D, bars 2 and 3 vs bar 1; $p < 0.05$). However, the inhibitory effects were less profound at the highest dose (30X) in SY-1-treated mice (Figure 3C,D, bar 4 vs bar 1; $p < 0.05$). These results may have relevance for colon cancer chemotherapy.

G0/G1 Cell Cycle Phase Regulatory Protein Changes in SY-1-Treated Human COLO-205 Xenograft Tumors. To investigate the underlying molecular mechanisms of the SY-1-induced antitumor effects, frozen COLO-205 xenograft

tumors were pulverized in liquid N_2, and the expression of cell cycle regulatory proteins was detected by immunoblotting. The antitumor effects of the AC extract were only observed using the highest concentration (100X). In contrast, tumor-promoting effects were observed in the low dose (1X) AC extract-treated group (Figure 3). Such results prompted us to investigate the in vivo antitumor mechanisms of the SY-1 and AC extracts.

Our previous paper demonstrated that wild-type p53 cancer cells were more sensitive to SY-1 treatment than mutant p53 cancer cells.[1] In this study, the p53 protein was significantly induced in the low dose (1X) SY-1-treated tumors. In contrast, the levels of the p53 protein were not altered in the high dose (30X) SY-1-treated mice. Similar observations were seen in the low dose (1X) AC extract-treated mice, although p53 induction occurred to a lesser extent. Our studies have suggested that p53-mediated signaling pathways play a role in SY-1-induced antitumor effects.

Our in vitro study has demonstrated that p53-induced CDK inhibitors including p21/Cip1 and p27/Kip1 are up-regulated in human cancer cells arrested at G0/G1 by SY-1 treatment.[1] In this study, the p27/Kip1 protein was significantly induced in tumors treated using low doses (1X) of SY-1 as compared to those treated using high doses (30X). Interestingly, p27/Kip1 expression was induced only in the high dose (100X) AC extract-treated group. The p27/Kip1 induction level corresponded to the tumor suppression profiles observed in either the SY-1 (1X) or the AC extract (100X)-treated mice. p21/Cip1 expression was not significantly altered in these groups.

The level of cyclins A, D1, and D3 were down-regulated in the SY-1-treated tumors in comparison to the AC extract-treated group, whereas the levels of the CDK2 and CDK4 proteins were unchanged (Figure 4, lanes 2−5). CDC25C, which promotes cell entry into the S and the G2/M phases, was also down-regulated in SY-1 and AC extract-treated mice (Figure 4, lanes 2−5). The cyclin B/cdc2 protein complex, which also promotes cell entry into the S and the G2/M phases, was slightly inhibited in its protein level in the high dose SY-1 (30X) and AC (100X) extract-treated tumors (Figure 4, lane 5). The results shown in Figure 4 indicate that the cyclin A/CDK2 complex and the Rb1/2 proteins were induced in the mice treated with the low dose (1X) of AC extract (Figure 4, lane 4). Such results can explain the above finding that low dose (1X) AC extracts promote the rate of tumor growth in COLO-205 xenograft tumors (Figure 1A).

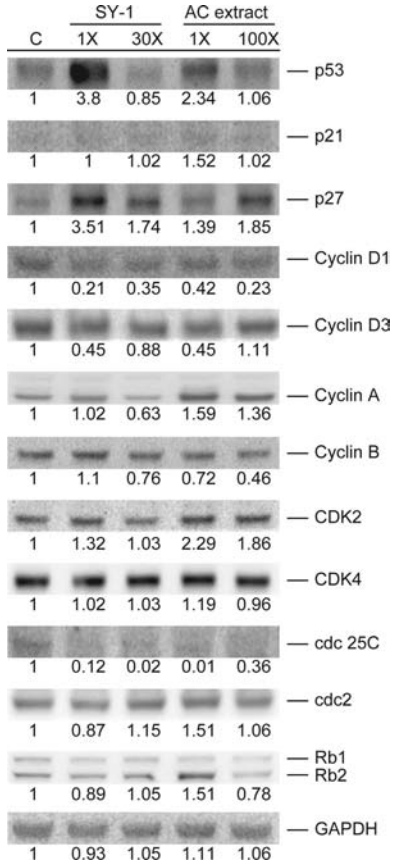

Figure 4. G0/G1 phase cell cycle regulatory protein changes in the SY-1 and AC extract-treated COLO-205 xenograft tumors. As previously described,[34] the frozen tumors were pulverized in liquid N_2 and mixed with lysis buffer (0.5 M Tris-HCl, pH 6.8, 0.4% SDS) and analyzed using Western blots. Xenograft tumor tissues were thawed in 750 μL of lysis buffer containing protease inhibitors to examine protein expression.[13] The samples were homogenized three times on ice using a PRO 200 homogenizer (PRO Scientific Inc., Monroe, CT) at setting 3 (18000 rpm). Protein (50 μg) from each sample was resolved using 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and analyzed using Western blotting.

DISCUSSION

Recent studies have demonstrated that AC exerts its versatility in promoting immune responses by exhibiting antileukemic activity in WEHI-3 leukemia BALB/c mice[14] and activating the immunomodulation of macrophages in a human hepatoma cell model.[15] Many previous studies have demonstrated that AC induces significant apoptosis in human leukemia (HL-60),[16] breast (MCF-7 and MDA-MB-231),[16,17] prostate (LNCaP and PC3),[18] and liver (Hep G2, C3A, and PLC/PRF/5) cancer cells.[3,19] The antiproliferative effects of AC have also been reported in various types of human cancer cells including breast,[16,17] prostate,[18] bladder,[20] lung,[21] and liver[3] cells. These results demonstrate that cell cycle inhibition and apoptotic cell death both contribute to the antitumor effects of AC. Our previous report examined the antiproliferative effect of SY-1 on the growth of human cancer cells with various p53 statuses and on the growth of normal human colon epithelial cells.[1] The results suggest that SY-1 (>150 μM) significantly inhibits cell growth in COLO-205 and HepG2 cells, which express wild-type p53 protein, in a dose- and time-dependent manner. However, the inhibition of cancer cell lines that express a mutant p53 protein such as HT-29, MDA-MB-231 and Ca9-22, and normal colon endothelial cells only occurred at higher SY-1 concentrations (>375 μM). These results suggest that cancer cells containing wild-type p53 were more sensitive to SY-1 treatment than cancer cells expressing mutant p53.

Our previous studies demonstrated that p53-mediated p27/Kip1 and p21/Cip1 upregulation was observed in human colon cancer cells on exposure to various types of antitumor agents.[1,11,22−26] In this study, we demonstrated that p53-induced p21/Cip1 expression was not observed in SY-1-treated mice. This study revealed that p27/Kip1 expression alone may play a major role in SY-1-induced antitumor effects (Figure 4). We found that p27/Kip1 was up-regulated in low dose (1X) SY-1-treated tumors as compared with high dose (30X) SY-1-treated tumors. In contrast, p27/Kip1 induction was observed in the high (100X) but not the low (1X) dose AC extract-treated group. As shown in Figure 4, the more profound AC

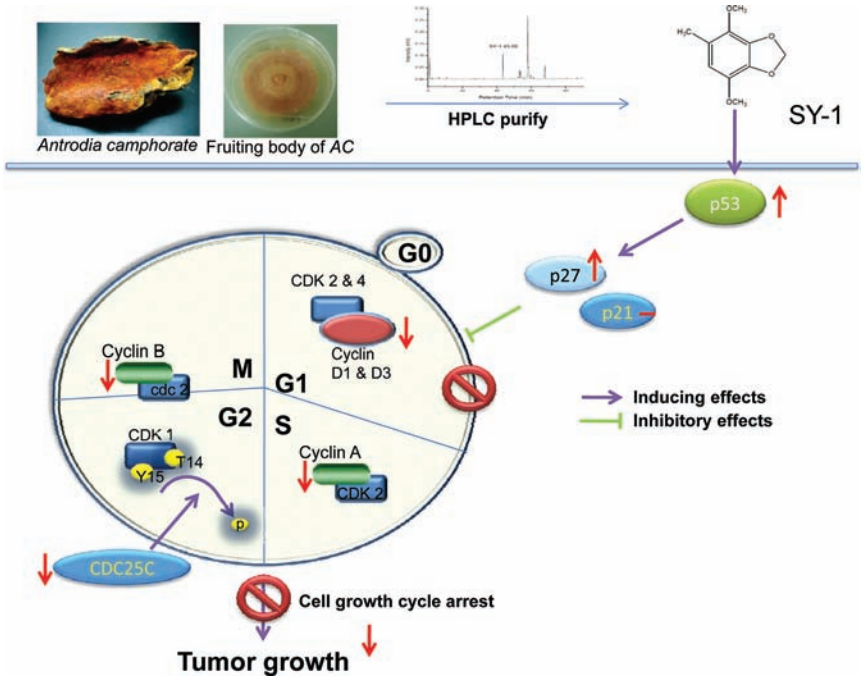

Figure 5. Schematic diagram of the signaling pathways involved in SY-1 and AC extract-induced G0/G1 phase cell cycle regulatory proteins changes in COLO-205 xenograft tumor tissues. SY-1 was isolated from the fruiting body of AC (upper right). After treatment with SY-1 or AC extract, the p53-regulated p27/Kip1 (and not p21/Cip1) G0/G1 phase cell growth cycle signals were induced in COLO-205 tumors, resulting in the inhibition of tumor growth formation.

extract (100X) and SY-1 (1−5X) antitumor effects were limited to specific dosage levels. In this study, we found that lower dose (1X and 10X) AC extract caused tumor promotion effects. Such results implied that using AC extract to treat cancer has some unexpected effects and should be concerned. Interestingly, the level of p27/Kip1 was induced at the same dose that corresponded to the best tumor suppression profiles observed in SY-1 (1X) or AC extract (100X)-treated mice. These results imply that the increased expression levels of p53-mediated p27/Kip1 may play a central role in the antitumor effects induced by using an effective dose of SY-1 (1X) or AC extract (100X). Many previous studies also indicated that p27/Kip1 was important for many types of antitumor effects including cancer cell growth inhibition,[27] apoptosis induction,[28] and triggering cancer cell differentiation.[29] This study provides valuable information regarding the in vivo molecular mechanisms of the AC extract and its isolated compound SY-1, which is potentially useful as an anticancer therapeutic agent in patients.

Human colon cancer cells often respond in a limited manner to the chemotherapeutic drugs currently available due to the expression of multidrug resistance genes.[30] Indeed, human cancer cells expressing mutant p53 appear to activate the promoters of genes that are not usually activated by wild-type p53 protein, such as the multidrug resistance gene 1 and c-MYC.[31] This study demonstrated that the SY-1- and AC-induced antitumor effects in colon cancer cells were due to the activation of wild-type p53-mediated pathways. Such results suggest that adjuvant therapy may be limited when combining AC with antitumor agents, for example, when the AC therapeutic dose is inappropriate or in patients with tumors containing a mutant p53 gene, which would induce potential undesirable tumor-promoting effects (Figure 4). In addition, epidemiological and laboratory studies suggest that aspirin and nonsteroidal anti-inflammatory drugs exhibit chemopreventive effects against colon cancer that are perhaps due, at least in part, to their activity against cyclooxygenase-2 (COX-2).[32] To our knowledge, COX-2 is strongly expressed in all metastatic (HT-29), but not less metastatic, cell lines (COLO-205).[33] A previous study demonstrated that the treatment of human hepatoma cells (C3A and PLC/PRF/5) with AC extract inhibits MDR gene expression and the COX-2 pathway.[3] However, our report demonstrates that the inhibition of the growth of HT 29 cells was less profound than that of COLO-205 cells, indicating that SY-1 may not exert its antitumor effects through COX-2 inhibition. In summary, this is the first study to use SY-1 or AC extract as an antitumor agent in the treatment of human colon cancer. The findings from this in vivo study suggest that the appropriate wild-type p53 status and the SY-1 or AC extract dosage should be determined before these compounds are administered as adjuvant agents for cancer chemotherapy.

AUTHOR INFORMATION

Corresponding Author

*Tel: +886-4-24635755 ext. 302. E-mail: herb736@yahoo.com.tw (H.-M.L.). Tel: +886-2-27361661 ext. 3327. Fax: +886-2-2739-3422. E-mail: hoyuansn@tmu.edu.tw (Y.-S.H.).

Present Address

[+]Yusheng Biotechnology Co. Ltd., No. 26, Keyuan Road, Central Taiwan Science Park, Situn District, Taichung City, Taiwan.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AC, *Antrodia camphorata*; CDK, cyclin-dependent kinase; COX-2, cyclooxygenase-2; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SY-1, 4,7-dimethoxy-5-methyl-1,3-benzodioxole

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