The *in vivo* antitumor effects on human COLO 205 cancer cells of the 4,7-dimethoxy-5-(2-propen-1-yl)-1,3-benzodioxole (apiole) derivative of 5-substituted 4,7-dimethoxy-5-methyl-1,3-benzodioxole (SY-1) isolated from the fruiting body of *Antrodia camphorata*

**ABSTRACT**

**Context:** The compound 4,7-dimethoxy-5-(2-propen-1-yl)-1,3-benzodioxole (apiole) has been isolated from several different plant species, including *Petroselinum sativum*. Our recent study found that apiole is a chemical derivative of 4,7-dimethoxy-5-methyl-1,3-benzodioxole (SY-1), which has been isolated from dried *Antrodia camphorata* (AC) fruiting bodies, a traditional Chinese medicine with antitumor properties.

**Aims:** Our previous *in vitro* study demonstrated that apiole inhibits the growth of human colon (COLO 205) cancer cells through the arrest of the cell cycle in G0/G1 phase. The *in vivo* antitumor effects of apiole were evaluated in this study.

**Setting and Design:** Apiole was administered to mice at 1–30 mg/kg body weight through intraperitoneal (I.P.) injection three times per week (defined as a dosage of 1×–30×).

**Materials and Methods:** The *in vivo* antitumor effects of apiole were evaluated in mice with xenografts of COLO 205 cells.

**Statistical Analysis:** All of the data are reported as the means ± S.E. Comparisons were performed with a one-way analysis of variance (ANOVA) followed by a Fisher’s least significant difference test. Significance was defined as *P* < 0.05.

**Results:** Apiole (> 1×) markedly decreased the growth of COLO 205 human colon cancer cell tumor xenografts in an athymic nude mouse model system through the up-regulation of cell cycle regulators, such as p53, p21/Cip1, and p27/Kip1. The apiole-induced increase in G0/G1 phase cell cycle regulators was also associated with a significant decrease in the expression of cyclins D1 and D3. Surprisingly, statistically significantly higher tumor volumes were observed in mice that received 5× apiole compared with 30× apiole-treated mice (*P* < 0.05). No gross signs of toxicity were observed (e.g., body weight changes, general appearance, or individual organ effects) in any group.

**Conclusions:** Our results show, for the first time, the promising antitumor effects of apiole against colon tumors in an *in vivo* xenograft model.

**KEY WORDS:** Antitumor, apiole, SY-1, colon tumor, COLO 205

**INTRODUCTION**

Colon cancer affects 50 to 60 out of every 100,000 people in North America, and is the second most common cause of cancer-related death after lung cancer.[1] A recent study revealed that nutritional and genetic risk factors for colon tumors have...
additive effects on mouse tumor phenotypes.[2] These results imply that the intake of healthy food ingredients, such as dietary fiber[3] and seed oil,[4] can help to prevent colon cancer. *Antrodia camphorata* (*A. camphorata, AC*) is a parasitic fungus that only grows on the inner heartwood wall of *Cinnamomum kanehirai* Hay (*Lauraceae*) and develops fruiting bodies, mycelia, and spores. Our recent study demonstrated that *AC* and the pure 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.[9] We also demonstrated that SY-1-induced G0/G1 phase cell cycle arrest occurs as the result of the induction of p53-mediated cyclin-dependent kinase (CDK) inhibitors (i.e., p21/Cip1 and p27/Kip1).[10] In another study, we demonstrated that *AC* may be useful as an adjuvant for the treatment of human colon cancer cell xenograft tumors.[6]

Our results suggest that SY-1 could be used as a potential cancer treatment agent. SY-1-related compounds, including 4,7-dimethoxy-1,3-benzodioxole derivatives, were evaluated for their antiproliferative activity. Interestingly, we found that 4,7-dimethoxy-5-(2-propen-1-yl)-1,3-benzodioxole (referred to as apiole, apiol, or parsley apiol) led to potent G0/G1 phase cell growth arrest and apoptosis in human colon cancer cells through p53-mediated signaling.[8] Apiole has been reported to be a component of essential oils from different plant sources, such as the seeds of *Petroselinum sativum*[8] and *Enterolobium contortisiliquum* (*Leguminosae*)[9] and the leaves and fruits of *Pituranthos chloranthus*.[10] These results imply that the intake of healthy food ingredients (apiole), such as seed oil, may help to prevent colon cancer.[11] This study presents the first results validating the promising antitumor effects of apiole against colon tumor growth in an *in vivo* xenograft system.

**MATERIALS AND METHODS**

SY-1, shown in Figure 1, was a gift from Yusheng Co., Ltd. (Taichung, Taiwan).[5] Apiole was purchased from Extrasynthese (France). The structure of apiole was determined with nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. Fast atom bombardment mass spectrometry (FAB-MS) (3-nitrobenzyl alcohol matrix) m/z 223 (M + H)+; δ (CDCl3, 500 MHz): 6.28 (1H, s, H-6), 5.94 (2H, s, O–CH2–O), 5.92 (1H, m, H-2'), 5.04–5.00 (2H, m, H-3'), 3.86 (3H, s, OCH3), 3.83 (3H, s, OCH3), and 3.29 (2H, dt, J = 6.5 Hz, 1.5 Hz, H-1'); and δ (CDCl3, 500 MHz): 139.1, 138.7, 137.3, 136.2, 135.1, 125.8, 115.4, 108.1, 101.5, 60.1, 55.8, and 34.1 (C12H14O4, Mw: 222.24).[11]

In a previous study, we demonstrated that apiole specifically induced cell cycle arrest and apoptosis in *COLO 205* human colon cancer cells.[7] COLO 205 cells grown in RPMI 1640 supplemented with 10% fetal calf serum were harvested through two consecutive trypsinisations, centrifuged at ×300 g for 5 min, washed twice with PBS, and resuspended in sterile PBS. The cells (5 × 106) were resuspended in 0.1 ml of Roswell Park Memorial Institute (RPMI) 1640 and subcutaneously injected between the scapulae of BALB/c nu/nu mice (4-week-old females, n = 5 per group) purchased from the National Science Council Animal Centre (Taipei, Taiwan). After transplantation, the tumor size was measured using callipers, and the tumor volume was calculated as follows: Tumor volume (mm3) = 1/2 × L × W2, where L is the length and W is the width of the tumor.[12,13] Once the tumor reached a volume of 200 mm3, the animals received intraperitoneal injections of normal saline (25 µl) or apiole (1, 5, or 30 mg/kg body weight, which were referred to as 1×, 5×, and 30×, respectively) three times per week for 30 days. All of the mice were housed under a regular 12-h light/12-h dark cycle with *ad libitum* access to a standard rodent chow diet (Laboratory Rodent Diet 5001, Lab Diet, St. Louis, MO, USA). After 30 days, the animals were euthanized with CO2, and the tumors were dissected and weighed.

The frozen tumors were pulverized in liquid N2 and mixed with lysis buffer (0.5 M Tris–HCl, pH 6.8, and 0.4% SDS), as previously described.[14,15] Western blot analysis was also performed as previously described.[16] 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-Bax, anti-Bcl-2, and anti-GAPDH monoclonal antibodies (Santa Cruz, Inc., CA, USA) and anti-cyclin D1, anti-cyclin D3, anti-cyclin A, anti-CDK2, and anti-CDK4 monoclonal antibodies (Transduction Laboratories, Lexington, KY, USA) were used at 1:1,000 dilutions. The anti-cyclin A polyclonal antibody (Transduction, San Diego, CA, USA) was used at a 1:250 dilution. The secondary alkaline phosphatase-coupled antimouse and antirabbit antibodies (Jackson, Westgrove, PA, USA) were incubated at room temperature for 1 h at 1:5000 and 1:1000 dilutions, respectively.

All of the data are reported as the means ± S.E. Comparisons were performed with a one-way analysis of variance (ANOVA) followed by Fisher’s least significant difference test.
Significance was defined as \( P < 0.05 \).

**RESULTS**

As shown in Figure 1a, SY-1 was isolated from AC and induced significant cell cycle arrest and apoptosis.\(^5\) The SY-1 derivative apiole [Figure 1b] is also present in different types of natural plant products. Our previous study demonstrated that apiole has significant \textit{in vitro} antitumor activity, inducing both cell growth arrest and apoptosis.\(^7\)

In this study, the average mouse body weight was 26 g. The mice were maintained on 5 g of Lab Diet per day [Figure 2a]. No gross signs of toxicity (i.e., changes in body weight or general appearance or effects on individual organs) were observed in the mice receiving the treatment regimens. After tumor cell transplantation, the COLO 205-xenografted mice received apiole (I.P. injections of 1, 5, or 30 mg/kg body weight three times per week, which were denoted as 1×, 5×, and 30×, respectively). According to this protocol, the daily dose of 1× apiole was equal to 0.0111 mg per mouse [Figure 2b, bar 2]. All of the groups were treated for 30 days.

We examined the therapeutic efficacy of apiole \textit{in vivo} in athymic mice bearing COLO 205 tumor xenografts. After 30 days, the tumor growth in the groups treated with doses of apiole higher than 1× was significantly inhibited relative to the growth observed in the control-treated mice (Figure 3a and b; \( P < 0.05 \)). Surprisingly, statistically significantly higher tumor volumes and tumor/body weight ratios were observed in the 5× apiole-treated mice compared with the 30× apiole-treated mice (Figure 3c and d, bars 3 and 4 vs. bar 1; \( P < 0.05 \)). These results may have relevance for colon cancer chemotherapy.

Our previous paper demonstrated that wild-type p53 cancer cells were more sensitive to apiole treatment than mutant p53 cancer cells.\(^7\) In this study, p53 protein expression was significantly induced in the low-dose (> 1×) apiole-treated tumors. Our studies have suggested that p53-mediated signaling pathways play a role in apiole-induced antitumor effects. Our \textit{in vitro} study demonstrated that p53-induced CDK inhibitors, including p21/Cip1 and p27/Kip1, are up-

**Figure 2:** The dose-dependent effects of apiole on body weight changes after daily administration to COLO-205-bearing mice. (a) The mice were I.P. injected with apiole at 1, 5, or 30 mg/kg body weight three times per week. (b) The average mouse body weight was 26 g. The mice were maintained on 5 g of Lab Diet per day. The daily dose of apiole in the 1× dose group was 0.0111 mg per mouse. The values represent the means \( \pm \) S.E

**Figure 3:** The dose-dependent apiole-induced antitumor effects in human COLO-205 xenograft tumor-bearing mice. (a) Tumor growth curves, (b) gross morphology of tumor-bearing mice, (c) tumor weights, and (d) tumor/body weight ratios were determined during and after the experiment. Significance was defined as \( P < 0.05 \)

**Figure 4:** Changes in the expression of G0/G1 phase cell cycle regulatory proteins in the apiole-treated COLO-205 xenograft tumors. As previously described,\(^{16}\) the frozen tumors were pulverized in liquid \( N_2 \) mixed with lysis buffer (0.5 M Tris–HCl, pH 6.8, and 0.4% SDS) and analyzed by western blotting. Xenograft tumor tissues were thawed in 750 \( \mu \)L of lysis buffer containing protease inhibitors to examine protein expression.\(^{16}\) The samples were homogenized three times on ice using a PRO200 homogenizer (PRO Scientific Inc., Monroe, CT, USA) at setting 3 (18,000 rpm). Protein (50 \( \mu \)g) from each sample was separated via 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and analyzed using western blotting.
regulated in human cancer cells arrested at G0/G1 by apiole treatment.\textsuperscript{7} In this study, the p21/Cip1 and p27/Kip1 proteins were significantly induced in tumors treated with low doses (> 1×) of apiole compared with those treated with the vehicle control [Figure 4a]. The levels of cyclins D1 and D3 were down-regulated in the apiole-treated group compared with the vehicle-treated group, whereas CDK2 and CDK4 protein levels were unchanged [Figure 4a]. Furthermore, p53-induced mitochondria-mediated apoptosis (i.e., members of the BAX/Bcl-2 signaling pathway) and caspase 3 activation were also induced by apiole treatment [Figure 4b]. The results shown in Figure 4a indicate that the cyclin A/CDK2 complex proteins were not inhibited in the apiole-treated mice. These results can explain the above finding that apiole (> 1×) inhibits the rate of tumor growth in COLO-205 xenograft tumors [Figure 3].

DISCUSSION

The anticancer properties of 10 different types of 4,7-dimethoxy-1,3-benzodioxole derivatives of SY-1, which was isolated from the dried fruiting bodies of 	extit{AC}, were evaluated in our previous study.\textsuperscript{7} The results of that study demonstrated that apiole was the most potent inhibitor of human colon cancer cell proliferation and activator of cellular apoptosis among the compounds tested; the anticancer activities of other derivatives of 5-substituted 4,7-dimethoxy-1,3-benzodioxole were weaker. We further demonstrated that the antiproliferative effects of apiole were more potent than those of SY-1 in wild-type p53-expressing COLO 205 cancer cells.\textsuperscript{5,7} Our \textit{in vitro} and \textit{in vivo} studies have demonstrated that p53-induced expression of p21/Cip1 and p27/Kip1 was up-regulated, whereas cyclin D1 was down-regulated, in cells that arrested when exposed to apiole [Figure 4]. Interestingly, p53-induced mitochondria-mediated apoptosis (represented by BAX/Bcl-2 activation) was also triggered by apiole treatment [Figure 4]. Caspases were activated in cells treated with a low dose of apiole. These results explain why low-dose (1×) apiole treatment did not inhibit the growth of COLO-205 xenograft tumors as strongly as the higher-dose (> 10×) treatments.

The antiproliferative effect of SY-1 on the growth of human cancer cells and normal human colon epithelial cells has been studied previously.\textsuperscript{11} The results suggest that a high dose of SY-1 (> 150 µM) significantly inhibits the growth of COLO-205 cells but not the growth of normal human fetal colonic mucosa (FHC) cells. In another study, we demonstrated that cancer cells expressing wild-type p53 were more sensitive to SY-1 treatment than cancer cells expressing mutant p53. Therefore, we chose to use wild-type p53-containing COLO 205 cancer cells as the tumor model to determine the \textit{in vivo} antitumor activity of apiole, which is a derivative of SY-1.

Human colon cancers often exhibit a limited response to chemotherapeutic drugs due to the expression of multidrug resistance genes.\textsuperscript{19} Indeed, human cancer cells expressing mutant p53 appear to activate genes that are not usually activated by the wild-type p53 protein, such as multidrug resistance gene 1 and c-MYC.\textsuperscript{18} Our previous study demonstrated that apiole elicited antitumor effects in colon cancer cells due to the activation of wild-type p53-mediated pathways. These results suggest that the efficacy of adjuvant therapy may be limited when combining apiole with antitumor agents, when the therapeutic dose of apiole is inappropriate, or when treating patients with tumors containing a mutant p53 gene, which would affect the antitumor efficacy. The findings of this \textit{in vivo} study suggest that wild-type p53 status and the appropriate apiole dosage should be determined before these compounds are administered as adjuvant agents for cancer chemotherapy [Figure 5].

Our previous study demonstrated that apiole has significant \textit{in vitro} antitumor activity, inducing both cell growth arrest and apoptosis.\textsuperscript{7} Apiole is a chemical derivative of SY-1, which was isolated from AC and has been isolated from different types of natural plant products [Figure 5]. In this study, we examined the therapeutic efficacy of apiole \textit{in vivo} by treating athymic mice bearing COLO 205 tumor xenografts. After 30 days, the tumor growth in the groups treated with apiole at doses greater than 1 mg/kg body weight was significantly inhibited relative to the growth observed in the control-treated mice. These results may have relevance for colon cancer chemotherapy.

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