American ginseng berry enhances chemopreventive effect of 5-FU on human colorectal cancer cells

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Abstract. In this study, we investigated the possible synergistic chemopreventive effects of American ginseng berry extract (AGBE) and 5-fluorouracil (5-FU) on human colorectal cancer cell lines, SW-480, HCT-116 and HT-29. We used high-performance liquid chromatography to determine the contents of major ginsenosides, the active components of American ginseng, in AGBE. The anti-proliferative effects were evaluated by the cell counting method. AGBE (0.1-1.0 mg/ml) significantly inhibited SW-480, HCT-116 and HT-29 cell growth in a concentration-dependent manner. Cell growth decreased more with the combined treatment of 5-FU and AGBE than with 5-FU or AGBE applied alone, suggesting that AGBE can reduce the dose of 5-FU needed to achieve desired effects and thereby decrease the dose-related toxicity of the chemotherapy agent. Cell apoptosis assay showed that AGBE markedly reduced the number of viable SW-480 cells at 0.5 and 1.0 mg/ml, but did not increase cell apoptosis significantly. Neither 5-FU nor co-treatment with 5-FU and AGBE induced cell apoptosis markedly. Cell cycle assay showed that AGBE mainly arrested SW-480 cells in the G2/M phase. 5-FU increased the percentage of SW-480 cells at the S phase of the cell cycle. The assay of combined treatment groups indicated that AGBE can heighten the arrest of SW-480 cells in the S phase induced by 5-FU, and increase the cell distribution in G2/M phase compared with 5-FU applied alone. The trend of increasing cyclin A was similar to the increase of S and G2/M phase cells in all treated groups. The enhancement of S and G2/M phase arrest, rather than cell apoptosis, should be the mechanism of synergistic effects of AGBE on 5-FU. Further *in vivo* and clinical trials are needed to test AGBE as a valuable chemo-adjuvant.

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Introduction

Colorectal cancer is one of the most common malignancies and ranks as the second greatest cause of cancer death in both men and women worldwide (1). Although early stage colorectal cancer can be cured by surgical resection, surgery is often combined with adjuvant radiotherapy and chemotherapy with one or more chemotherapeutic agents. Even with effective strategies that continue to be developed for treating colorectal cancer, chemotherapy has the drawbacks of severe adverse effects and dose-limiting toxicity. Drug-related adverse events not only worsen patients' quality of life, but can also lead to their refusal to continue chemotherapy (2,3). Chemotherapy-induced toxicity can be reduced by chemo-adjuvant compounds that potentiate tumoricidal effects with lower doses (4-6). Identifying non-toxic chemo-adjuvants among herbal medicines may be an essential step in advancing the treatment of cancer (7).

Due to the increase in the consumption of herbal remedies in the United States along with a staggering popularity of the ginseng herb as a method of sustaining good health, significant focus has been placed on American ginseng (*Panax quinquefolius* L.) (8), which belongs to the genus *Panax* L. in the Araliaceae family. American ginseng has been reported to have stress-relieving qualities, anti-aging effects and digestion-aiding effects (9). Cancer treatment with botanicals like American ginseng has also received increasing attention in recent years (10-13). The major active components of ginseng are ginsenosides, a diverse group of steroid saponins. Ginsenosides are distributed in many parts of the ginseng plant, including the root, leaf and berry. The most commonly used part of the plant is the root, which is harvested in late summer to fall between its fourth and seventh years (14). As a byproduct, American ginseng berry can be harvested more than once before harvesting the root. Previous study has demonstrated that the berry has a significantly higher content of total ginsenosides than the root of ginseng (15) and has a ginsenoside profile distinct from that of the root (16). The

pharmacological effects of ginseng berries have been evaluated. It is well documented that American ginseng berry extract (AGBE) could improve diabetic conditions (17), such as decreasing the blood glucose and body weight in ob/ob mice (18,19), attenuating oxidant stress in cardiomyocytes (20), reducing chemotherapy-induced nausea/vomiting (21), and exerting antiproliferative activity against human breast carcinoma cells (22). Previously, our laboratory analyzed ginsenoside compounds in AGBE with different processing methods, and pilot data showed the effects on colorectal cancer cells (23). However, the mechanism of the antiproliferative effect of AGBE is not known.

5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents in first-line therapy for colorectal cancer (24,25), and an overall survival benefit after fluorouracil-based chemotherapy has been firmly established (26). But for the treatment of metastatic colon cancer, however, higher 5-FU doses produced more adverse effects while not necessarily being more effective than lower doses (27). Therefore, if decreasing the dose of chemotherapy and increasing its anti-cancer effect could be accomplished by combining 5-FU with other agents, patients may benefit. However, chemotherapy with 5-FU and herbal medicines has rarely been studied.

Thus, this study investigated the potential synergistic tumoricidal effects of AGBE on 5-fluorouracil (5-FU). We used various human colorectal cancer cell lines, SW-480, HCT-116 and HT-29, which have undergone extensive laboratory cancer research and have been the models for the cellular pathways studies of chemotherapy on cancer cells. Furthermore, we observed the combined effect on cell apoptosis, cell cycle and cycle A in SW-480 cells to elucidate the possible mechanism in these cells. This is an important preliminary step in the development of an effective chemo-adjuvant for colorectal cancer treatment.

Materials and methods

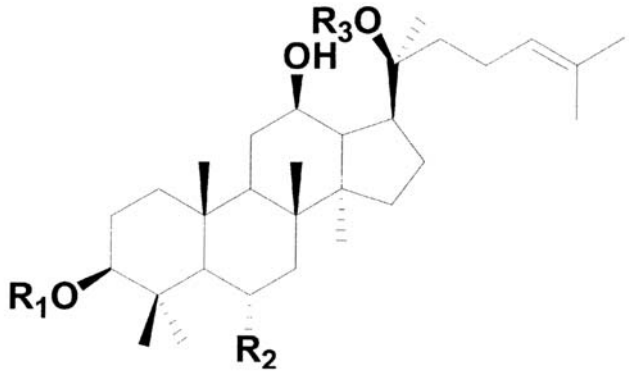
Chemicals. All solvents were of high-performance liquid chromatography (HPLC) grade from Fisher Scientific (Norcross, GA). Milli Q water was supplied by a water purification system (US Filter, Palm Desert, CA). Standards for ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1 and Rg3 were obtained from the Delta Information Center for Natural Organic Compounds (Xuancheng, Anhui, China). All standards were of biochemical-regent grade and at least 95% pure as confirmed by HPLC. All cell culture plasticware was purchased from Falcon Labware (Franklin Lakes, NJ) and Techno Plastic Products (Trasadingen, Switzerland). Trypsin, McCoy's 5A medium, Leibovitz's L-15 medium, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were obtained from Mediatech, Inc. (Herndon, VA). 5-Fluorouracil (5-FU) was obtained from American Pharmaceutical Partners Inc. (Schaumburg, IL). Penicillin G/streptomycin was obtained from Sigma (St. Louis, MO). An Annexin V-FITC Apoptosis Detection kit was obtained from BD Biosciences (Rockville, MD). PI/RNase staining buffer was supplied from BD Biosciences Pharmingen (San Diego, CA).

Herbal materials and sample preparation. Fresh berry of American ginseng (*Panax quinquefolius* L.) was obtained from Roland Ginseng, LLC (Wausau, WI, USA). All berries were gathered from 4-year-old plants. The seeds of the berry were removed and lyophilized to obtain dried pulp sample. The berry pulp was ground to powder and extracted with 70% ethanol for 4 h; the water bath was maintained at 90˚C. When cooled, the solution was filtered and the filtrate was collected. The residue was extracted with 70% ethanol once more and then filtered while the solution was cooled. The filtrates were combined and the solvent was evaporated under vacuum to obtain the primary extract. The primary extract was further purified to obtain American ginseng berry extract.

High-performance liquid chromatographic analysis. High-performance liquid chromatography (HPLC) analysis was conducted on a Waters HPLC system (Milford, MA, USA). This HPLC system was composed of a Waters 2960 instrument, a quaternary pump, an automatic injector, and a photodiode array detector (Model 996). The separation was carried out on a 250x3.2 mm i.d., 5 μ, Ultrasphere C18 column (Alltech, Deerfield, IL, USA) with a 7.5x3.2 mm i.d. guard column. For the mobile phase, acetonitrile (solvent A) and water (solvent B) were used, and flow rate was 1.0 ml/min. Gradient elution started with 18% solvent A and 82% solvent B. Elution was changed to 21% A for 20 min, then to 26% A for 3 min and held for 19 min. It was then changed to 36% A for 13 min, to 50% A for 9 min, to 95% A for 2 min, and held for 3 min, to 18% A for 3 min and held for 8 min. The detection wavelength was set to 202 nm. Regression equations of ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1 and Rg3 were prepared using standard solutions within different concentrations. For the sample assay, 20 μl of 2 mg/ml of extract solution was injected, and the assay was repeated 3 times. All solutions were filtered through Millex 0.2 μM nylon membrane syringe filters (Millipore Co., Bedford, MA, USA) before use.

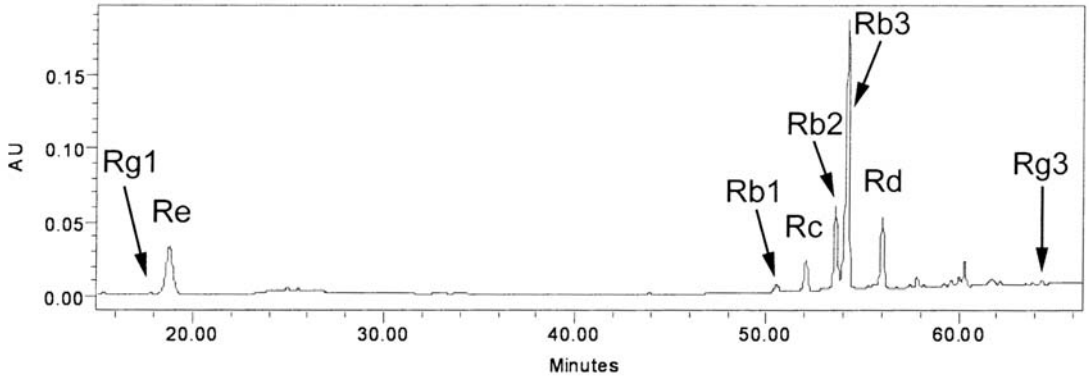
Cell culture. SW-480, HCT-116 and HT-29 human colorectal cancer cells (ATCC, Manassas, VA) were routinely grown in a humidified atmosphere of 5% CO_2 at 37˚C in Leibovitz's L-15 medium (for SW-480) and in McCoy's 5A medium (for HCT-116 and HT-29), respectively, supplemented with 10% fetal bovine serum and 50 IU penicillin/streptomycin. Cells were grown in a 25-ml flask and were routinely subcultured using 0.05% trypsin-EDTA solution. Cells were maintained at the culture conditions described above for all experiments.

Cell proliferation assay. To examine the antiproliferation effect of the test agents, SW-480, HCT-116 and HT-29 cells were seeded in 24-well plates at approximately $1x10^4$ cells/well with regular Leibovitz's L-15 or McCoy's 5A medium, respectively, and allowed to adhere for 24 h. Then fresh culture media were changed prior to the addition of drugs. The SW-480, HCT-116 and HT-29 cells were incubated with AGBE (0.1, 0.5 and 1.0 mg/ml); or 5-FU (10 μM); or both drugs for 72 h. Control cultures were incubated in medium containing vehicle alone. At the end of treatments, the cell monolayer was washed twice with phosphate-buffered saline (PBS). Cultures were harvested and monitored for number by using a Coulter cell counter (Coulter Electronics, Inc., Hialeah, FL). All assays were performed at least 3 times. The percentage of cell proliferation was calculated as follows:



Name	R1	R2	R3
Ginsenoside Rg_1	-H	-o-glc	-glc
Ginsenoside Re	-H	-o-glc-glc	-glc
Ginsenoside Rb_1	-glc-glc	-H	-glc-glc
Ginsenoside Rc	-glc-glc	-H	-glc-ara (fur)
Ginsenoside Rb_2	-glc-glc	-H	-glc-ara (pyr)
Ginsenoside Rb_3	-glc-glc	-H	-glc-xyl
Ginsenoside Rd	-glc-glc	-H	-glc
Ginsenoside Rg_3	-glc-glc	-H	-H

Figure 1.Chemical structures of ginsenosides in American ginseng berry extract.



Saponin content in American ginseng berry extract (mean ± SD, n=3)

Ginsenoside	Rg1	Re	Rb1	Rc	Rb2	Rb3	Rd	Rg3
Content (%)	0.14±0.01	6.86±0.02	0.57±0.02	3.06±0.03	6.98±0.05	24.54±0.28	4.76±0.07	0.15±0.01

Figure 2. HPLC analysis of American ginseng berry extract.

Cell proliferation (%) = (cell number in each experimental well/average cell number in the control well) x100.

Apoptosis analysis. For apoptosis detection, floating cells in the medium and adherent cells were collected after 24, 48 or 72 h of treatment with AGBE (0.5 and 1.0 mg/ml); or 5-FU (10 μM); or both. Using an Annexin V-FITC Apoptosis Detection kit, cells were stained with Annexin-V FITC and propidium iodide (PI) according to the manufacturer's instructions. Untreated SW-480 cells were used as the control for double staining. Cells were analyzed immediately by using a flow cytometer (Becton-Dickinson, Mountain View, CA). For each measurement, at least 20,000 cells were counted.

Cell cycle and cyclin A analysis. SW-480 cells were plated at a density of $2x10^5$ cells onto 24-well plates. The medium was replaced 24 h after seeding with fresh medium containing AGBE (0.5 mg/ml); or 5-FU (10 μM); or both. To analyze the cell cycle distribution, cells were trypsinized after 24, 48 or 72 h of exposure to these samples, fixed gently with 80% ethanol, and stored at -20˚C for 2 h. Then they were treated with 0.25% Triton X-100 for 5 min in an ice bath. The cells

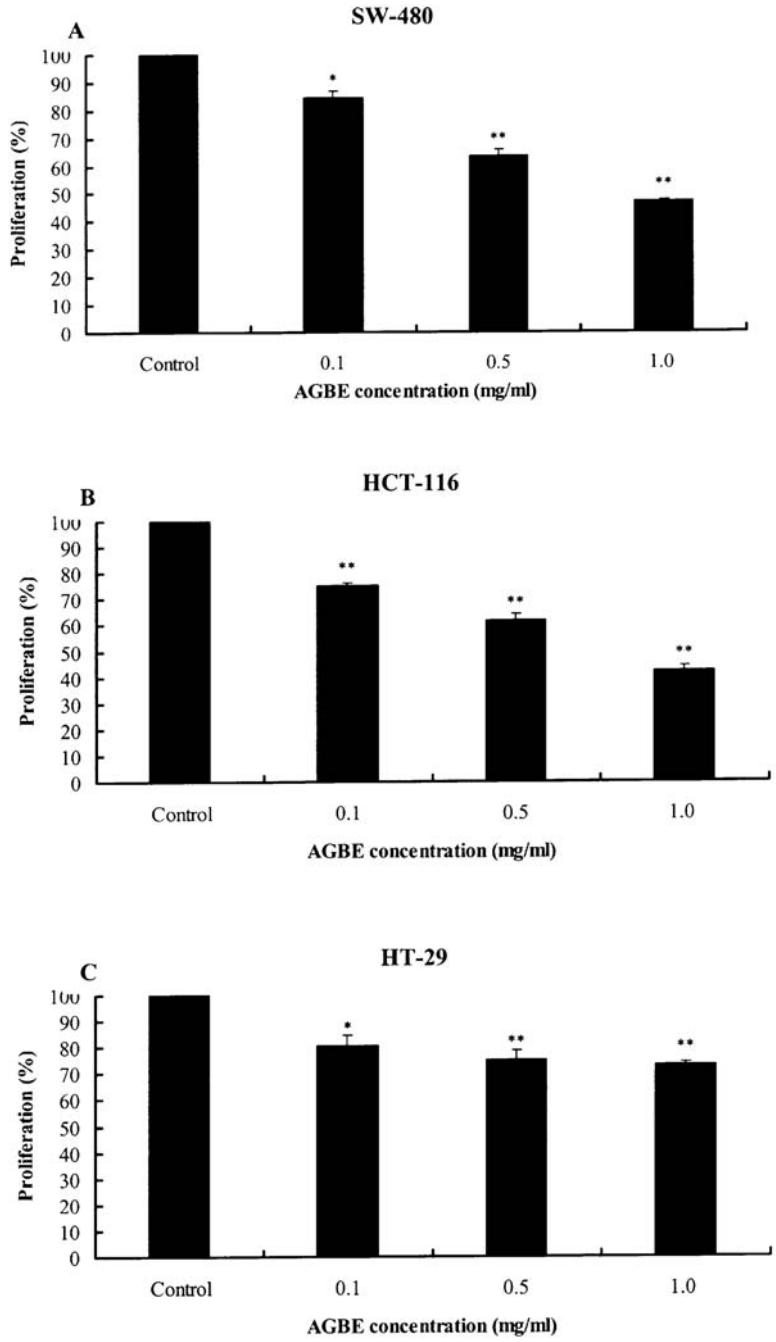


Figure 3. Antiproliferative effects of American ginseng berry extract (AGBE, 0.1, 0.5 and 1.0 mg/ml) on the human colorectal cancer cells after 72 h of treatment. These data indicated that AGBE inhibited the proliferation of human colon cancer SW-480 (A), HCT-116 (B) and HT-29 (C) cells significantly in a concentration-dependent manner. Data are presented as the mean ± standard error of mean of triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$, vs. control.

were resuspended in 300 μl of PBS containing 40 μg/ml propidium iodide (PI) and 0.1 mg/ml RNase. Then 20 μl of cyclin A-FITC was added to the cell suspension. Cells were incubated in a dark room for 20 min at room temperature and analyzed with a flow cytometer. For each measurement, at least 10,000 cells were counted.

Statistical analysis. Results are presented as mean ± standard error (SE). Data were analyzed using Student's t-test and analysis of variance (ANOVA) for repeated measures. The level of statistical significance was set at $P<0.05$.

Results

HPLC analysis of AGBE. Saponins in American ginseng berry extract (AGBE) were identified by observing the retention times and UV spectrum of authentic ginsenoside Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1 and Rg3 standards obtained from the mixed standards chromatograms. The chemical structures of identified saponins were shown in Fig. 1. Fig. 2 showed representative HPLC chromatograms of AGBE recorded at 202 nm and the HPLC analysis of the contents of saponins in AGBE.

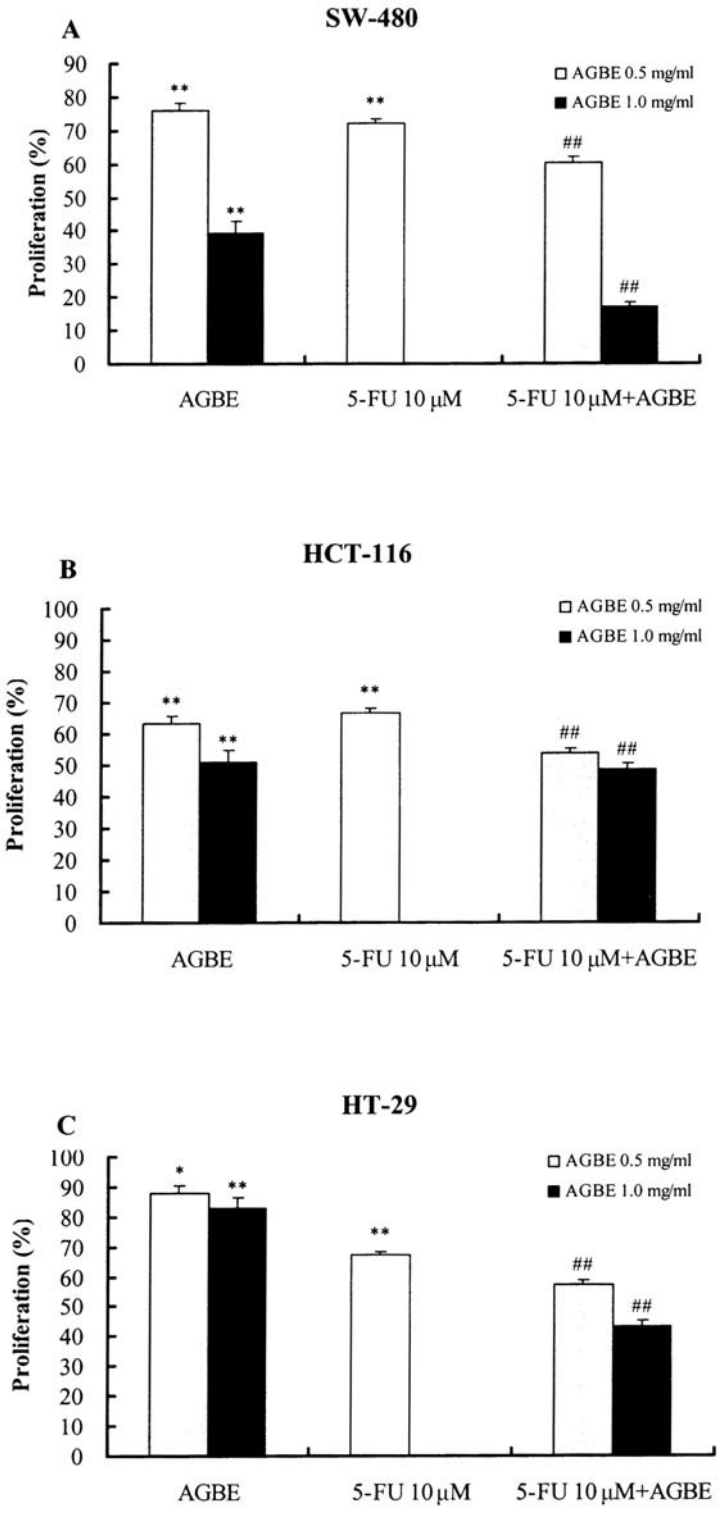


Figure 4. Antiproliferatve effects of American ginseng berry extract (AGBE, 0.5 and 1.0 mg/ml) combined with chemotherapeutic agent 5-fluorouracil (5-FU, 10 μM) on human colorectal cancer SW-480 (A), HCT-116 (B) and HT-29 (C) cells after 72 h treatment. Data are presented as the mean ± standard error of mean of triplicate experiments. *P<0.05, **P<0.01, vs. control; $^{\#\#}$P<0.01, vs. 5-FU alone.

Effects of AGBE on SW-480, HCT-116 and HT-29 cell proliferation. The antiproliferative effects of AGBE (0.1, 0.5 and 1.0 mg/ml) on SW-480, HCT-116 and HT-29 human colorectal cancer cells are shown in Fig. 3. After treat-ment for 72 h, AGBE significantly inhibited SW-480 cell proliferation (Fig. 3A). The extract reduced SW-480 cell growth in a concentration-dependent manner, by 15.4±1.9% at 0.1 mg/ml (P<0.05), 36.5±2.3% at 0.5 mg/ml (P<0.01), and 52.9±0.6% at 1.0 mg/ml (P<0.01), compared with control group (normalized to 100%). Similar results were observed from HCT-116 and HT-29 cell lines (Fig. 3B and C). These data suggested the dose-dependent antiproliferative effect of AGBE on human colorectal cancer cells *in vitro* experiments.

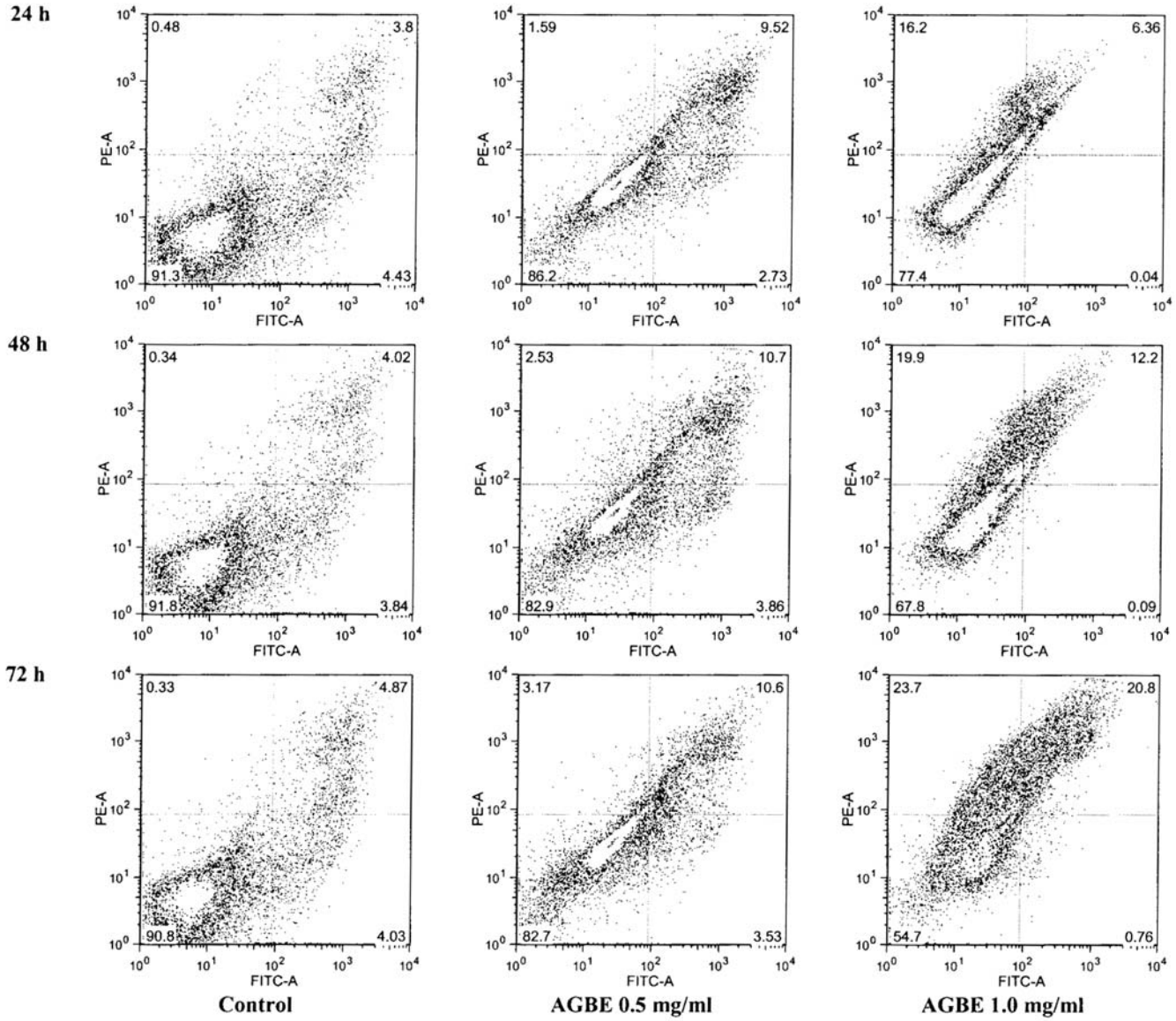


Figure 5. Apoptosis assay of SW-480 cells treated with American ginseng berry extract (AGBE). SW-480 cells were treated with 0.5 and 1.0 mg/ml AGBE for 24, 48 and 72 h. The percentage of different types of cells is shown. Data shown are from two independent experiments.

Effect of 5-FU combined with AGBE on SW-480, HCT-116 and HT-29 cell proliferation. The influence of AGBE on 5-fluorouracil (5-FU)-induced antiproliferation in human colorectal cancer cells is shown in Fig. 4. Fig. 4A shows that when treated alone for 72 h, 5-FU, at the concentration of 10 μM, decreased SW-480 cell growth by 21.8±1.3% ($P<0.01$, compared with control). Combined treatment of AGBE at the concentration of 0.5 or 1.0 mg/ml with 5-FU further decreased the SW-480 cell growth by 39.7±1.8% and 83.0±1.6%, respectively (both $P<0.01$, compared with 5-FU alone).

The enhanced antiproliferative effects were also observed in HCT-116 (Fig. 4B) and HT-29 (Fig. 4C) cells after co-treatment for 72 h. Data showed that 5-FU (10 μM) significantly decreased HCT-116 and HT-29 cell growth by 33.3±1.3% and 32.7±1.3%, respectively (both $P<0.01$, compared with control group). The suppressive effect of co-treatment with 5-FU and AGBE was significantly stronger than that of 5-FU or AGBE alone as demonstrated; the combined treatment reduced the cell growth by 46.4±1.6% and 51.6±2.0% in HCT-116 cells and by 42.8±1.5% and 56.6±1.9% in HT-29 cells at the dose of AGBE 0.5 mg/ml and 1.0 mg/ml, respectively (all $P<0.01$, compared with 5-FU alone). These results suggested that AGBE markedly enhanced 5-FU-induced antiproliferative effect on colorectal cancer cells.

Apoptotic effect of AGBE and/or 5-FU on SW-480 cells. To examine whether proliferation inhibition in treated cells was caused by the induction of apoptosis, we used flow cytometric analysis. The cytograms of bivariate Annexin V/PI analysis of SW-480 cells after treatment with AGBE are shown in Fig. 5. Viable cells were negative for both PI and Annexin V (lower left quadrant); early apoptotic cells were positive for Annexin V and negative for PI (lower right quadrant); late apoptotic or necrotic cells displayed both positive for Annexin V and PI (upper right quadrant); non-viable cells which underwent necrosis were positive for PI and negative for Annexin V (upper left quadrant). Compared

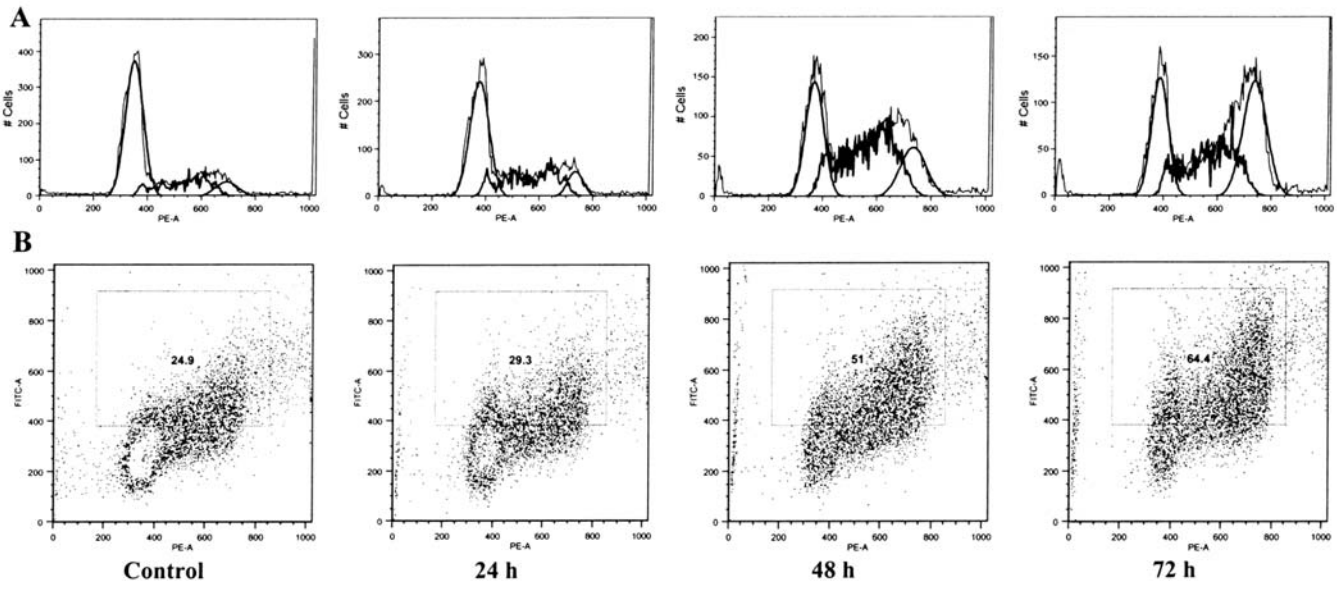


Figure 6. Cell cycle and cyclin A analysis of SW-480 cells treated with American ginseng berry extract (AGBE). SW-480 cells were treated with 0.5 and 1.0 mg/ml AGBE for 24, 48 and 72 h. (A) Cell cycle profiles of SW-480 cells. (B) SW-480 cells stained with cyclin A-FITC and proidium iodide. The percentage of cyclin A positive cells is shown in the gate. Data shown are from two independent experiments.

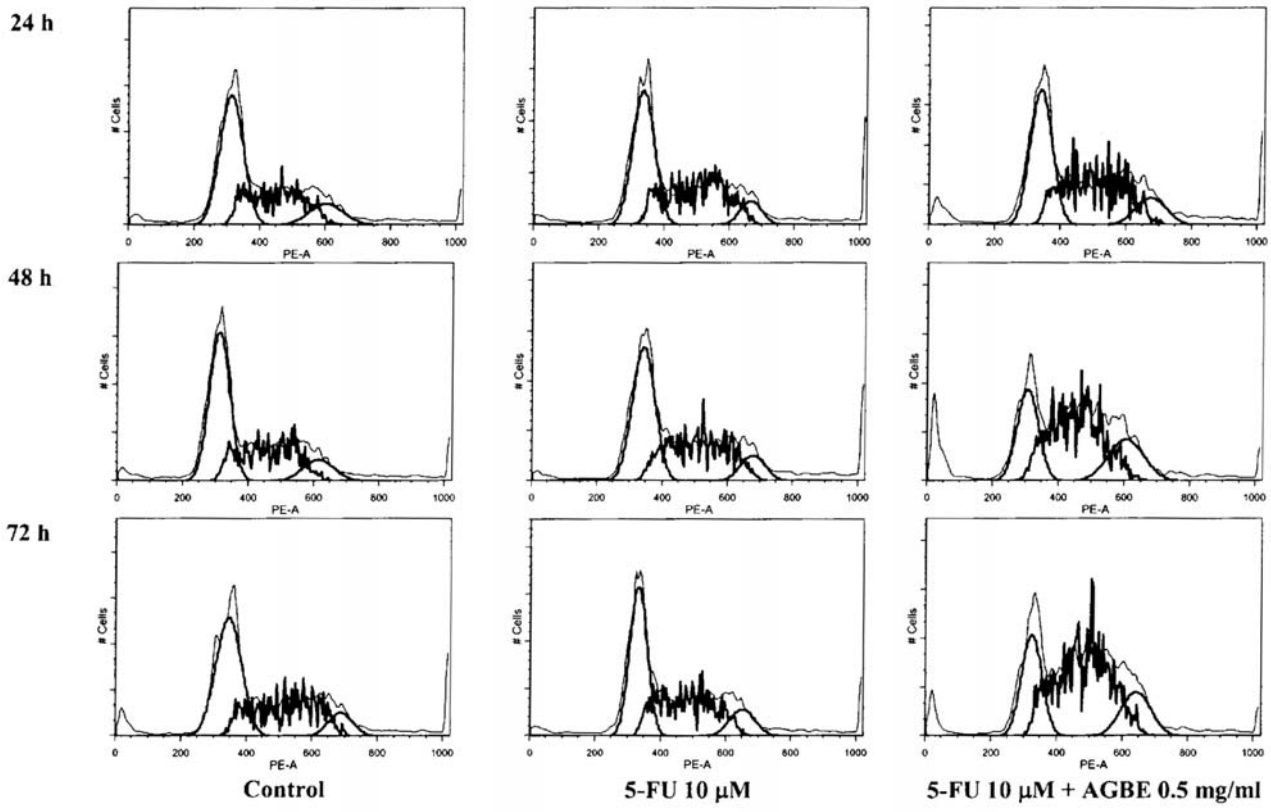


Figure 7. The effect of American ginseng berry extract (AGBE) on 5-fluorouracil (5-FU)-induced cell cycle arrest. SW-480 human colorectal cancer cells were treated with 5-FU (10 μM) with the absence or presence of AGBE (0.5, 1.0 mg/ml) for 24, 48 and 72 h. The cell cycle was assessed using PI/RNase staining and flow cytometric analysis. Data shown are from two independent experiments.

to the untreated control (72 h early apoptosis 4.03%, late apoptosis/necrosis 4.9%), AGBE increased late apoptosis/necrosis to 20.8% and decreased early apoptosis to 0.8% after treatment with 1.0 mg/ml for 72 h. On the other hand, the viable cell was 90.8% in control group and 82.7% and 54.7% in AGBE group (0.5 mg/ml and 1.0 mg/ml, respectively) after exposure of 72 h. The similar trend was observed in AGBE treated groups at other time points. These

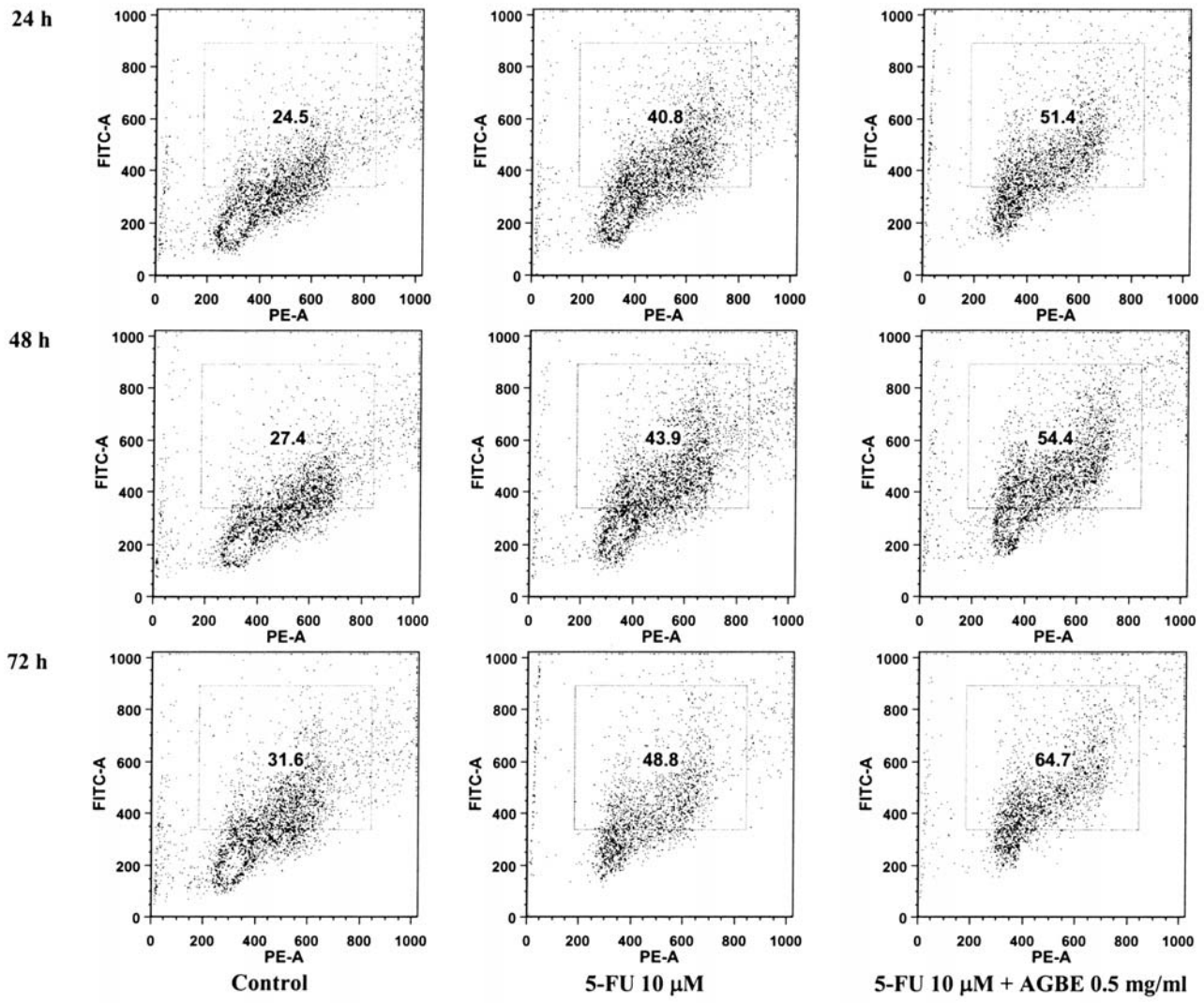


Figure 8. Cyclin A analysis of SW-480 cells treated with 5-fluorouracil (5-FU) alone or combined with American ginseng berry extract (AGBE). SW-480 human colorectal cancer cells were treated with 5-FU (10 μM) with the absence or presence of AGBE (0.5, 1.0 mg/ml) for 24, 48 and 72 h. The cell stained with cyclin A-FITC and proidium iodide. The percentage of cyclin A-positive cells is shown in the gate. Data shown are from two independent experiments.

data showed that AGBE markedly reduced the proportion of viable cells, but did not increase the proportion of early apoptotic cells.

Incubation with 5-FU at 10 μM for 24, 48 and 72 h did not alter the number of early apoptotic cells, which was essentially the same as that of the control. In addition the percentage of apoptotic cells in co-treatment groups was not markedly increased compared with that of groups treated with AGBE alone (data not shown). These results suggested that the suppression of the combination of AGBE and 5-FU on the cell growth may not be through apoptotic induction.

Effect of AGBE on cell cycle and expression of cyclin A. To examine whether the decrease of proliferation in treated cells is a consequence of the cell cycle being arrested at a specific phase, we analyzed the cell cycle of treated SW-480 cells by flow ctyometry. As shown in Fig. 6A, treatment with AGBE (0.5 mg/ml) for 72 h markedly induced the S phase and G2/M phase arrest of the cell cycle in a time-dependent manner.

Fig. 6B shows the expression level of cyclin A in SW-480 cells with the exposure of AGBE (0.5 mg/ml) for 24, 48 and 72 h. Compared with untreated cells (control: 24.9%), the fraction of cyclin A positive cells was increased to 29.3% at 24 h, 51.0% at 48 h and 64.4% at 72 h.

Effect of 5-FU combination with AGBE on cell cycle and expression of cyclin A. As shown in Fig. 7, following the exposure to 5-FU at 10 μM for 24, 48 and 72 h, the percentage of SW-480 cells at the S phase of the cell cycle increased, and the percentage at the G1 phase decreased compared with the control groups upon the corresponding treated time. The arrest of the cell cycle at S phase and G2/M phase was more pronounced in co-treatment groups of 5-FU (10 μM) with AGBE (0.5 mg/ml) than that of 5-FU-treated only group. The percentages at each cell cycle phase upon various treatments are shown in Table I.

The expression of cyclin A in SW-480 cells treated by 5-FU alone and the combination of 5-FU with AGBE is shown in Fig. 8. After incubation with 5-FU, the fraction of cyclin A positive cells increased by 16.3, 16.5 and 17.2% at 24, 48 and 72 h, respectively, compared with the corresponding control group. Combined application of 5-FU and AGBE

Table I. Percentage of cells in G1, S and G2/M phases.

	Control (% cells)			5-FU 10 μM (% cells)			5-FU 10 μM + AGBE 0.5 mg/ml (% cells)		
Time	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
G1	51.2	50.4	49.1	47.0	46.2	43.6	42.5	26.5	24.3
S	35.6	37.7	41.1	44.0	43.4	44.3	44.8	53.2	59.9
G2/M	13.2	11.9	9.8	9.0	10.3	12.2	12.7	20.3	15.8

increased the expression of cyclin A more effectively than 5-FU alone. Both the assays suggested that AGBE could heighten 5-FU-induced S and G2/M phase arrest and the expression of cyclin A in colon cancer cells.

Discussion

Botanical extracts are a complicated mixture of bioactive compounds. The concentrations of these compounds vary significantly depending on genetics, season, geographical distribution, plant growth, and production and extract processes (28,29). Therefore, botanical analysis is an important issue in the quality assurance of botanical products (30,31). In this study, we used American ginseng from Roland Ginseng, LLC because American ginseng from Wisconsin is a reliable ginseng source (32). The content of the major ginsenosides in American ginseng berry extracts was determined using HPLC to standardize our extracts.

It has been reported that AGBE could attenuate cisplatin-induced emesis in a rat model, suggesting that the herb may have value in treating chemotherapy-induced nausea/ vomiting. However, it is not clear whether consuming AGBE with chemotherapy affects the efficacy of chemotherapeutic agents.

In this study, we evaluated the effect of AGBE on enhancing chemopreventive efficacy of 5-FU in human colorectal cancer. The antiproliferative activity was determined by the cell counting method. After treatment for 72 h, the antiproliferation of AGBE (0.1-1.0 mg/ml) on SW-480, HCT-116, HT-29, all of which are human colorectal cancer cell lines, were observed in a dose-dependent manner. As expected, 5-FU showed significant inhibition on the proliferation of colorectal cancer cells. The investigation on the influence of AGBE (0.5 and 1.0 mg/ml) on 5-FU-induced antiproliferation in SW-480, HCT-116 and HT-29 cell lines indicated that the cell growth decreased more with the combined treatment than that with 5-FU or AGBE alone. These results suggested that AGBE may reduce the dose of 5-FU needed to achieve desired effects in combination therapy and thereby decrease the dose-related toxicity caused by 5-FU. To elucidate the potential mechanism of the enhancement of AGBE on the 5-FU-induced antiproliferation in human colorectal cancer cells, we assayed cell apoptosis, cell cycle transition and cycline A expression in SW-480 cells by flow cytometry.

Apoptosis is a homeostatic mechanism that balances cell division and cell death to maintain the appropriate number of cells in the body. Inducing apoptosis is considered an important pathway by which many anticancer agents exhibit the growth inhibition of cancer cells (33-35). However, this study showed that neither AGBE or 5-FU alone nor co-treatment exhibited obvious induction on early apoptosis, which is considered real apoptosis. Thus, apoptotic induction was not involved in the antiproliferative effect of 5-FU combined with AGBE on human colorectal cancer cells.

In our study, AGBE arrested SW-480 cells in the S and G2/M phases, which may trigger the DNA repair mechanism, leading to apoptosis. To obtain further information on the molecular mechanism involved in the arrest of the cell cycle in the S and G2/M, we determined the expression of cyclin A, which is one of the cyclin family proteins that regulate cell cycle progression, and is required for S-phase and the passage through G2 (36). The fraction of cyclin A positive cells increased to 64.4% after treatment with AGBE for 72 h (24.9% in untreated cells). 5-FU, a pyrimidine analog, is generally believed to induce G1-S-phase arrest via inhibition of thymidylate synthase, a key enzyme in DNA synthesis (37). The current data showed that the treatment with 10 μM of 5-FU arrested the SW-480 cells specifically in the S-phase. Co-treatment of 10 μM of 5-FU and 0.5 mg/ml of AGBE produced alteration on the cell cycle profile, which displayed an increase of the cell percentage in the G2/M-phase and cell accumulation in the S phase concurrently as compared to the treatment with 5-FU alone. Similarly, the expression of cyclin A in combination treatment group was much higher than that in 5-FU group. Taken together, the results indicated that AGBE not only has an additive effect on 5-FU-stimulated arrest at the G2/M in cell cycle progression, but also heighten the arrest of colon cancer cells in the S-phase induced by 5-FU.

The successful treatment regimens for cancer include combination chemotherapy, which is often more effective than single chemotherapy because of additive or synergistic effects. In the present study, we evaluated the effects of AGBE in enhancing the chemopreventive efficacy of 5-FU on human colorectal cancer cells. Our data suggest that AGBE has the potential to heighten the tumoricidal effects of 5-FU and that 5-FU-induced antiproliferation of human colorectal cancer cells can be strengthened by combination with AGBE. The mechanism may include the enhancement of AGBE on the S and G2/M phases arrest and the expression level of cyclin A, but not the induction of cell apoptosis. These results partly suggested that AGBE potentiates the tumoricidal effects of 5-FU, and therefore a lower dose of 5-FU can be used, thereby decreasing the dose-related toxicity caused by 5-FU. Further *in vitro* and *in vivo* studies to test AGBE as a chemo-adjuvant are needed. Data obtained from these studies will have the

potential to help develop advanced treatment regimens for human colorectal cancer.

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