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Astragalus saponins induce growth inhibition and apoptosis in human colon cancer cells and tumor xenograft

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***Astragalus memebranaceus* is used as immunomodulating agent in treating immunodeficiency diseases and to alleviate the adverse effects of chemotherapeutic drugs. In recent years, it has been proposed that *Astragalus* may possess anti-tumorigenic potential in certain cancer cell types. In this study, the anti-carcinogenic effects of *Astragalus* saponin extract were investigated in HT-29 human colon cancer cells and tumor xenograft. Our findings have shown that *Astragalus* saponins (AST) inhibit cell proliferation through accumulation in S phase and G2/M arrest, with concomitant suppression of p21 expression and inhibition of cyclin-dependent kinase activity. Besides, AST promotes apoptosis in HT-29 cells through caspase 3 activation and poly(ADP-ribose) polymerase cleavage, which is indicated by DNA fragmentation and nuclear chromatin condensation. Nevertheless, we also demonstrate the anti-tumorigenic effects of AST *in vivo*, of which the reduction of tumor volume as well as pro-apoptotic and anti-proliferative effects in HT-29 nude mice xenograft are comparable with that produced by the conventional chemotherapeutic drug 5-fluorouracil (5-FU). In addition, the side effects (body weight drop and mortality) associated with the drug combo 5-FU and oxaliplatin are not induced by AST. These results indicate that AST could be an effective chemotherapeutic agent in colon cancer treatment, which might also be used as an adjuvant in combination with other orthodox chemotherapeutic drugs to reduce the side effects of the latter compounds.**

Introduction

Colon cancer is the second leading cause of malignant neoplasm-related death in developed countries, and also being the third most commonly occurring cancer in the United States. Chemotherapy using 5-fluorouracil (5-FU) and associated adjuvant agents remains the standard drug treatment regimen against both early-stage and advanced colorectal cancers. However, the response rate of 5-FU monotherapy is rather low, ranging 10–20% (1,2). Despite the fact that combination of 5-FU with cytotoxic drugs such as irinotecan or oxaliplatin may increase the response rate in advanced colorectal cancer to 40–50% (3,4), development of hypersensitivity and chemoresistance in some patients still lead to a low therapeutic index (5). On the other hand, orthodox chemotherapy produces many systemic adverse effects. Administration of 5-FU could compromise the patients' immune system by decreasing their leukocyte and platelet counts (6). Besides, dose-limiting cumulative peripheral sensory neuropathy is a frequent side effect caused by oxaliplatin (7,8). Hence, more effective and safer therapeutic strategies are urgently needed.

Abbreviations: dUTP, 2′-deoxyuridine 5′-triphosphate; PARP, poly(ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP *in situ* nick-end labeling.

Contemporary research has proven that many herbal extracts and isolated compounds possess anti-tumor activities. Clinical trials on the use of herbal medicinal agents have been conducted in order to establish novel chemotherapeutic avenues. The dried root of *Astragalus membranaceus* (Huangqi) has a long history of medicinal use in traditional Chinese medicine. It is now commonly used as an immunomodulating agent in mixed herbal decoctions to treat common cold, diarrhea, fatigue and anorexia (9), and being prescribed to patients with cardiac diseases (10). In recent years, *radix Astragalus membranaceus* has also been used to ameliorate the side effects of cytotoxic anti-neoplastic drugs (11). The active pharmacological constituents of *radix Astragalus membranaceus* include various polysaccharides, saponins and flavonoids (12). Among these, *Astragalus* polysaccharides have been most widely studied, mainly on their immunopotentiating properties like stimulation of murine B-cell proliferation and cytokine production (13). Apart from these, clinical studies also showed that *Astragalus* polysaccharides could counteract the side effects of chemotherapeutic drugs, such as a significant reduction in the degree of myelosuppression in cancer patients (14). Recently, there are growing evidences that *Astragalus* extract may be a potential anti-tumorigenic agent. For instance, hepatocarcinogenesis could be prevented in rats fed with the aqueous extract of *Astragalus*, which is mainly composed of *Astragalus* polysaccharides (15). There are also reports that describe the potentiating effect of *Astragalus* extract in recombinant interleukin-2-generated lymphokine-activated cells upon the anti-tumorigenic action of drugs against murine renal carcinoma (16).

Saponins isolated from *radix Astragalus membranaceus* consist of astragalosides (I–VIII) and some of their isomer isoastragalosides (I, II and IV) (17,18). Similar to the polysaccharides obtained from the same herb, *Astragalus* saponins have been found to possess immunomodulating effects. The pure isolated saponin astragaloside IV could increase murine B and T cell proliferation (19) and possess cardioprotective properties (20). Alternatively, many saponins derived from other natural sources have also been reported to effectively suppress cancer cell growth. Among these, ginsenosides are pharmacologically active saponins obtained from ginseng that could inhibit the growth of many different types of cancer *in vitro*. Ginsenosides such as Rh2 and Rg5 have been shown to induce G1 phase arrest in human MCF-7 breast cancer cells (21). Apoptosis can also be induced in human SK-HEP-1 hepatoma cells by Rh2 through activation of cyclin A/cyclin-dependent kinase cdk 2 activity and caspase 3-mediated p21 cleavage (22,23). On the other hand, growth-inhibitory activity of crude soya saponin mixtures or its purified fractions have been demonstrated in colon cancer cells (24). Soya saponins could also decrease the incidence and multiplicity of aberrant crypt foci induced by azoxymethane in CF-1 mice (25). In spite of these, the anti-tumorigenic action of *Astragalus* saponins against colon carcinogenesis has not been clearly stated.

Based on the above observations, the total polysaccharide (aqueous) and total saponin (methanol) extracts were obtained from *radix Astragalus membranaceus* in order to study their potential anti-tumorigenic effects. Nevertheless, our preliminary tests indicate that *Astragalus* polysaccharides do not possess any anti-tumor property under our experimental condition. Thus, the present study has been focused on investigating the anti-carcinogenic effects of total *Astragalus* saponins (AST) in HT-29 human colon cancer cells. The growth-inhibitory effects of AST were examined both *in vitro* and *in vivo* using nude mice xenograft. We hypothesized that the anti-carcinogenic action of AST could be due to promotion of apoptosis in the cancer cells and/or through regulation of the cell cycle. In order to determine whether AST can be used as an adjuvant agent with other orthodox chemotherapeutic drugs, both anti-tumor efficacy and toxicity of AST have been revealed and compared with those produced by

the drug combination 5-FU + oxaliplatin when treating the nude mice bearing colon cancer.

Materials and methods

Materials

Radix Astragalus membranaceus (Fisch.) Bunge var. *mongolicus* had been obtained from the province of Shanxi, China. The authenticity and quality of the crude herb were then tested in the Quality Assurance Laboratory of the School of Chinese Medicine, Hong Kong Baptist University, by microsopic and chromatographic analyses as well as DNA fingerprinting. Voucher specimen will be kept at the herbarium center for future reference to ensure consistency among batches. 5-FU, oxaliplatin and the antibody against β-actin were purchased from Sigma (St Louis, MO). Sources of other antibodies are as follows: caspase 3, cyclin A and cyclin B1 (Upstate Biotechnology, Charlottesville, VA); poly(ADP-ribose) polymerase (PARP) (BD PharMingen, San Jose, CA); total cdc 2 and p-cdc 2 (Tyr-15) (Cell Signaling Technology, Danvers, MA); p21 and Bcl-x_L (Zymed Laboratories, San Francisco, CA). All other reagents were widely available commercially.

Cell culture and synchronization

The human colon adenocarcinoma cell line HT-29 (HTB-38) was obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. All the experiments were performed in 1% serum culture condition after 24 h of serum starvation. This procedure has been proven to be effective in synchronizing the cells in the G0 phase, which is particularly important when performing cell cycle studies (26,27).

Preparation of AST extract

Astragalus saponins were extracted according to the method of Ma *et al.* (12) with slight modifications. In brief, 500 g of crude herb was refluxed with 2% potassium hydroxide in methanol for 1 h. Butan-1-ol was added to the reconstituted residue from above for phase separation to obtain total saponins. The dried and lyophilized AST powder (0.6% w/w) was reconstituted in ultrapure water to form a 10 mg/ml stock and stored at −20°C.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. HT-29 cells (1×10^4) were cultured in 96-well plates and treated with AST for 24, 48 or 72 h at various concentrations (6.25–100 μg/ml). Dulbecco's modified essential medium was added to control (0 μg/ml) wells at various treatment times. Absorbance of the resulting formazan was measured spectrophotometrically at 540 nm. Three to four independent experiments had been conducted.

Cell proliferation assay

Colon cancer cell proliferation was determined using a 5′-bromo-2′-deoxyuridine colorimetric assay kit according to the manufacturer's protocol (Roche Applied Science, Mannheim, Germany). HT-29 cells (5×10^3) were cultured in each 96-well plates, and exposed to various concentrations of AST (10, 30 or 50 μg/ml) for 48 or 72 h. Color development corresponding to the number of proliferating cells was measured spectrophotometrically at 450 nm, using 690 nm as reference.

Fluorescence-activated cell sorter analysis

HT-29 cells (2×10^5 in 60 mm^2 culture dishes) were trypsinized and washed with phosphate-buffered saline after treatment with 50 μg/ml AST for 24, 48 or 72 h. Approximately 1×10^6 cells were fixed with 75% ethanol at −20°C overnight. Cells were stained with 50 μg/ml propidium iodide and 100 μg/ml RNase A at 4°C. The analysis of samples was performed by using flow cytometry (FACS Canto™, Beckton Dickinson Biosciences, San Jose, CA). Phase distributions were calculated from the resultant DNA histogram using the ModFit LT version 3.0 software, and expressed as a percentage of cells in the respective phases.

Chromatin condensation

HT-29 cells (1×10^5) were grown on cover glass in six-well culture plates and treated with 50 μg/ml AST for 72 h. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline. DNA was stained with 4′-6-diamidino-2-phenylindole and analyzed under fluorescence microscope with UV filter Axiovert 200 (Carl Zeiss, Oberkochen, Germany) using ×63 magnification. Images were captured using the software AxioVision Rel. 4.5.

DNA gel electrophoresis

Following treatment with AST for 48 or 72 h, HT-29 cells (1×10^6 in 100 mm^2 culture dishes) were lyzed in a buffer containing Tris–HCl and Triton X-100. Lysates were then incubated with RNase A and proteinase K. DNA was obtained with an equal volume of neutral phenol:chloroform:isomyl alcohol mixture (25:24:1) and then precipitated with absolute ethanol and sodium acetate at −20°C. Equal amount (6 μg) of the extracted DNA was resolved over 1.8% agarose gel containing 0.5 μg/ml of ethidium bromide. Images were visualized under UV light using a gel documentation system (AlphaImager™ 2200, Alpha Innotech Corp., San Leandro, CA) and captured using the software AlphaEaseFC™.

Western blotting

HT-29 cells (1×10^6) cultured in 100 mm^2 culture dishes were treated with various concentrations of AST (10, 30 or 50 μg/ml) for 48 or 72 h. Proteins were obtained by cell lysis in ice-cold RIPA buffer. Total cell proteins (20–50 μg) were subjected to electrophoresis on 10–15% sodium dodecyl sulphate–polyacrlamide gels, transferred to nitrocellulose membranes and probed with the following antibodies: p21, cyclin A, cyclin B1, total cdc 2, p-cdc 2 (Tyr-15), Bax, Bcl-x_L, caspase 3 (all at 1:500), and PARP (1:1000). The immunoblots were developed and visualized by the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). Each blot was stripped and reprobed with β-actin antibody (1:10 000) as internal control. Densitometric analysis was done by Quantity One version 4.4.1 Basic (Bio-Rad, Hercules, CA).

Tumor xenografts in nude mice

The experimental procedures were approved by our institutional animal research ethics committee with reference to the European Community guidelines for the use of experimental animals. Five-week-old female Balb/c-*nu/nu* mice were obtained from the Laboratory Animal Service Unit of the Chinese University of Hong Kong. The mice were kept under sterile conditions and pathogen-free in isolated pathogen-free ventilation chambers under ambient temperature of 20–22°C and 45–50% relative humidity. The animal rearing facility was maintained on a 12 h light–dark cycle. All animals were given free access to sterilized food and water and under habituation for 10 days before experimentation.

Cell suspension was obtained by trypsinization of confluent HT-29 cells. Mice were randomly assigned into control and various treatment groups, and anesthetized with i.p. injection of 75 mg/kg ketamine and 10 mg/kg xylazine. Treatment regimens are as follows (n = 7–10): AST, p.o. once daily (days 0–20); 5-FU, i.p. once daily (days 7–11); oxaliplatin, i.p. single injection on day 7. Cells suspension was injected subcutaneously into the right thigh of each animal (at the cell density of 5×10^6 in 200 μl phosphate-buffered saline). The day of tumor implantation was designated as day 0. Tumors became palpable 7 days after xenografting. Tumor volume was measured using a digital caliper every 3 days and calculated as (length × $width^2$)/2 (28). Mice were monitored for 21 days after tumor inoculation. The body weight of all animals was recorded throughout the whole experimental period as an assessment of drug toxicity. Any mortality during the course of the study was also recorded.

Immunohistochemical analysis of cell proliferation and apoptosis in tumor tissues

On day 21, following cancer cell xenograft, all animals were killed by i.p. injection of an overdosed (100 mg/kg) ketamine followed by cervical dislocation. Excised tumor specimens were fixed for paraffin embedment. Sections of tumor samples with 4 μm thickness were prepared on slides. Immunohistochemical analysis of cell proliferation was performed by using the Histostain®-Plus detection kit (Zymed Laboratories). The slides with sample sections were blocked with 10% normal goat serum to minimize non-specific background, and incubated overnight with anti-PCNA (proliferating cell nuclear antigen) monoclonal antibody (1:200) at 4°C. The positively stained cells were visualized by incubating the sections with 3,3′-diaminobenzidine and Mayer's hematoxylin counterstain. Negative control was prepared with similar procedures but without adding the primary antibody.

The percentage of apoptotic cells in the excised tumor tissues was determined by the terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) *in situ* nick-end labeling (TUNEL) technique. Paraffin-embedded tumor sections were deparaffinized and treated with 20 μg/ml proteinase K to strip proteins from the nuclei. The sections were incubated with terminal deoxynucleotidyl transferase (TdT) and biotin–dUTP in TdT buffer at 37°C. Following termination of the reaction and blockade of non-specific binding, sections were incubated with streptavidin–biotin–peroxidase complex and having the sites of peroxidase binding being detected using 3,3′-diaminobenzidine. Negative control was prepared using similar procedures but without adding TdT. Positive control was prepared by prior incubation of the section with DNase I before hybridization with TdT and dUTP. Ten microscopic fields

from three different sections were observed and TUNEL-positive apoptotic cells were counted at ×400 magnification.

Statistical analysis

Statistical analysis of the data was done by using GraphPad Prism 4. Student's *t*-test was used to compare between mean values of two groups. Data between three or more groups were compared using the one-way analysis of variance, followed by the Dunnett's *post hoc* test. Final values are expressed as mean ± SEM. A difference of at least $P < 0.05$ was considered statistically significant.

Results

Growth-inhibitory and anti-proliferative effects of AST in HT-29 cells

We initially suspected that both total polysaccharides and saponins extracted from *Astragalus* would possess anti-tumor effects in HT-29 cells, but results from our preliminary study have disproved any contribution of *Astragalus* polysaccharides in ameliorating cancer cell growth. On the other hand, a concentration- and time-dependent reduction of HT-29 cell viability was achieved by using various concentrations of AST (Figure 1A). The estimated median effective concentration EC_{50} after 48 and 72 h of AST incubation were found to be 39.8 and 31.6 μg/ml, respectively. When we further investigated the effect of AST on HT-29 cell proliferation by using the 5-bromo-2′-deoxyuridine enzyme-linked immunosorbent assay, a non-radioactive alternative to the [^{3}H]-thymidine incorporation assay which labels the DNA of mitotically active cells, a similar observation was resulted. AST incubation for 48 h significantly reduced the proliferation of HT-29 cells by 67 and 73% at the two higher concentrations of 30 and 50 μg/ml, respectively (Figure 1B). The anti-proliferative effect of AST was even more prominent after 72 h of drug incubation. Nonetheless, none of the *Astragalus* polysaccharide concentrations being used could induce any effect on HT-29 cell proliferation (data not shown).

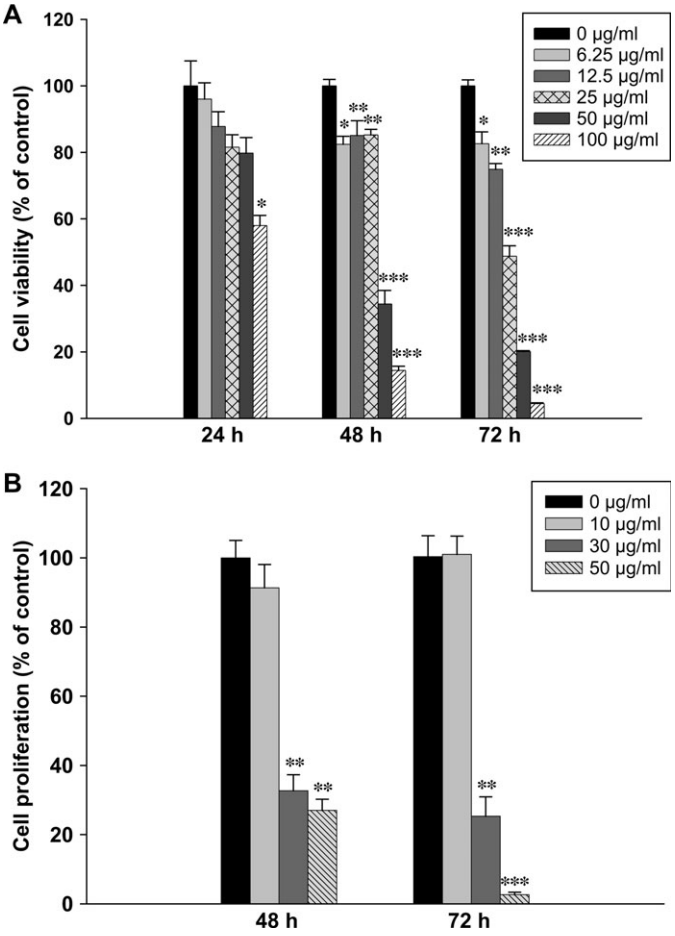

Fig. 1. AST reduces viability and inhibits proliferation of HT-29 cells. (**A**) Cells were treated with increasing concentrations of AST for 24, 48 or 72 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Results from three to four independent experiments are expressed as the percentage of viable cells in the treatment groups with respect to that in the corresponding vehicle-treated control (0 μg/ml AST). (**B**) Cell proliferation following AST treatments for 48 or 72 h was quantified by 5-bromo-2′-deoxyuridine enzyme-linked immunosorbent assay. Data were obtained from two independent experiments. Error bars represent means ± SEM. Significantly different from the corresponding control ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

AST causes cell cycle arrest at S and G2/M phases in HT-29 cells

The cell cycle response of HT-29 cells to AST treatment was investigated at different times of incubation using the drug concentration of 50 μg/ml. Cells appeared to be accumulated at both S and G2/M phases following AST treatment, with a concomitant decrease in the percentage of cells in the G1 phase when compared with the control (0 μg/ml AST) (Figure 2A). The accumulation of cells in the S phase (control: 29.6% versus AST: 40.3%) and G2/M phase arrest (control: 24.6% versus AST: 34.5%) began to occur following 24 h of drug treatment (Figure 2B-i). Cells continued to accumulate in their S (control: 14.2% versus AST: 17.8%) and G2/M (control: 13.3% versus AST: 22.7%) phases of the cell cycle after 48 h of drug treatment, although cell accumulation in S phase became less prominent (Figure 2B-ii). The AST-induced cell accumulation in S phase and G2/M arrest would not be permanent, of which sub-G1 cell population started to be observed after 48 h of drug treatment (Figure 2A-ii). This indicates that some cells started to undergo apoptosis. The sub-G1 population became more obvious after 72 h of drug treatment (Figure 2A-iii), whereas cells arrested at the G2/M phase (control: 10.0% versus AST: 22.7%) predominated, with a relatively smaller amount of cells accumulated in the S phase (control: 12.0% versus AST: 14.2%) at this time (Figure 2B-iii). These results suggest that AST causes HT-29 cells to be accumulated at their S and G2/M checkpoints before the induction of apoptosis.

AST modulates the expression of growth-related cyclins and cyclin-dependent kinase

HT-29 cells were exposed to the indicated concentrations of AST (10, 30 and 50 μg/ml) for 24, 48 or 72 h and total cellular proteins were extracted for western analysis. As shown in Figure 2C-i, protein expression of the cyclin-dependent kinase inhibitor p21 was up-regulated concentration dependently following AST treatment for 24 or 48 h. A 5-fold increase in p21 expression was achieved by treating the cells with the highest concentration of AST (50 μg/ml) for 24 h. Likewise, significant over-expression of cyclin A was also observed after 24 and 48 h of AST treatment (Figure 2C-ii). However, the effects of AST on p21 and cyclin A have been diminished at 72 h after drug treatment (Figure 2C-i and C-ii). It is well established that cyclin B1–cdc 2 complex regulates the mitotic switch of the cell cycle, and therefore their protein expressions were also investigated. Expression of cyclin B1 was not significantly altered following AST treatment (data not shown). Alternatively, although no significant alteration in total cdc 2 protein could be observed, the expression of phospho-Tyr-15 cdc 2 was increased after 24 h of AST treatment, with a remarkable elevation of 50% at the concentration of 50 μg/ml (Figure 2C-iii). This is actually the result of cdc 2 phosphorylation, implying cyclin-dependent kinase inhibition. This phenomenon could be due to abnormal over-expression of cyclin A together with the induction of the cyclin-dependent kinase inhibitor p21, which eventually leads to cell accumulation in S phase and G2/M arrest in AST-treated cells.

AST-induced apoptosis in HT-29 cells

In the flow cytometric analysis, the observation of sub-G1 peaks at 48 and 72 h following AST treatment has suggested that the drug could promote apoptosis in HT-29 cells following cycle arrest. One of the early events of apoptosis is the condensation of nuclear chromatin. We

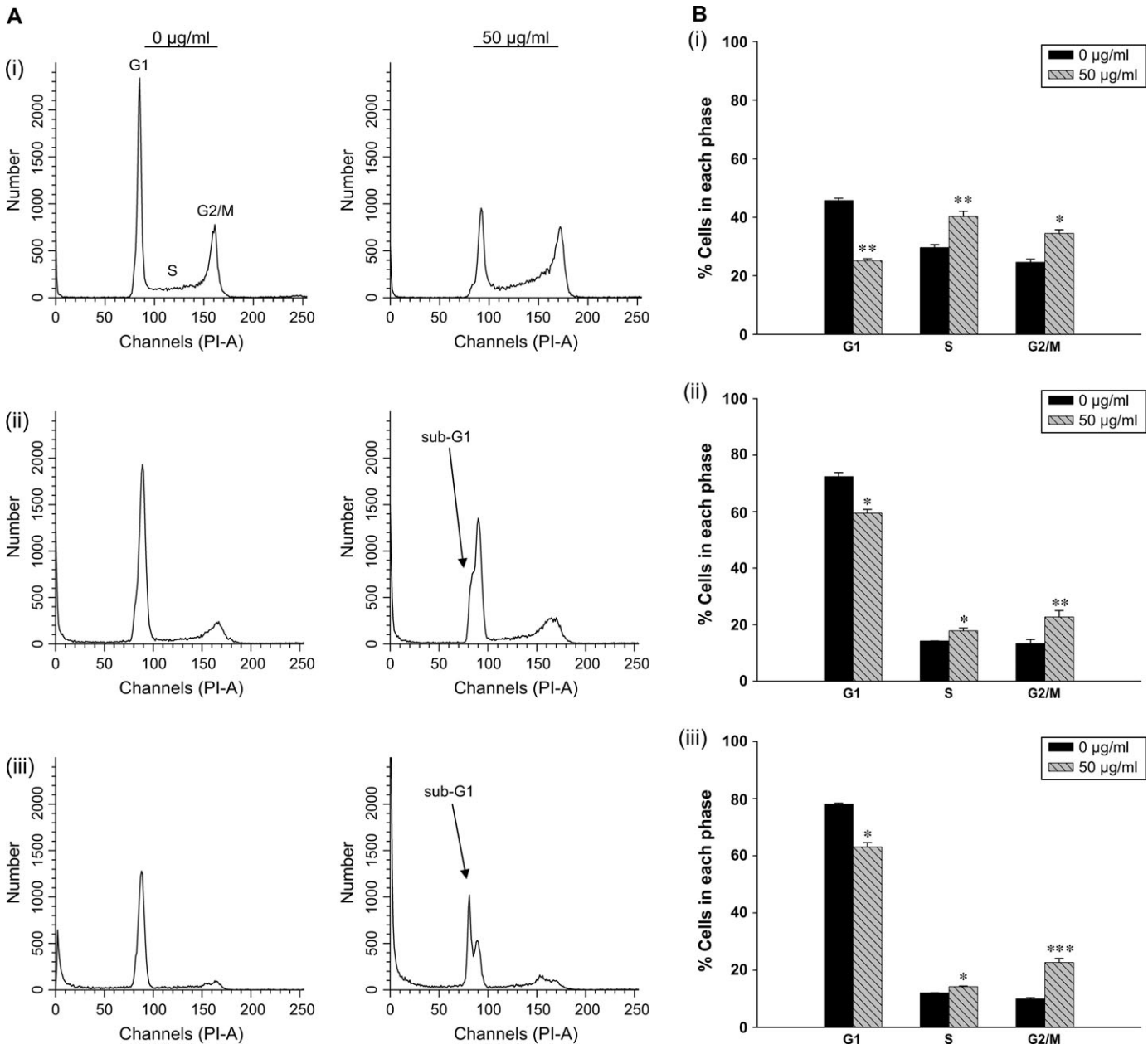

Fig. 2. Fluorescence-activated cell sorter analysis of phase distribution and western analysis of growth-related proteins in HT-29 cells. (**A**) Cells were treated with 50 μg/ml AST or vehicle (0 μg/ml) for (i) 24 (ii) 48 or (iii) 72 h. In the DNA histograms, sub-G1 peaks were observed following 48 or 72 h of AST treatment, as indicated by the arrows. (**B**) Distribution of HT-29 cells at different phases of the cell cycle with or without AST treatment for (i) 24 (ii) 48 or (iii) 72 h was analyzed by flow cytometry using ModFit 3.0 LT software. Results are expressed as the percentage of cells in each phase ± SEM of three independent experiments. (**C**) HT-29 cells were treated with AST for 24, 48 or 72 h. Expression of growth-related proteins was determined by western blotting. AST treatment caused over-expression of (i) p21, (ii) cyclin A and (iii) total and phospho-(Tyr-15) cdc 2. Representative immunoblots from four independent experiments are shown. Intensity of the target bands was normalized with β-actin reprobed on the same immunoblot. Densitometric (arbitrary) data indicate relative band density of (i) p21, (ii) cyclin A and (iii) phospho-(Tyr-15) cdc 2, respectively, with respect to the vehicle-treated control (0 μg/ml AST). Error bars represent means ± SEMs. Significantly different from the corresponding control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

therefore investigated the morphology of AST-treated cells using 4′-6-diamidino-2-phenylindole staining. Cells treated with 50 μg/ml AST for 72 h displayed the typical morphology of nuclear chromatin condensation (Figure 3A-iv) when compared with the control cells without drug treatment (Figure 3A-ii). In addition, the apoptotic pattern was revealed by DNA gel electrophoresis. As shown in Figure 3B, classical laddering pattern of internucleosomal DNA fragmentation was observed after 48 and 72 h of AST treatments, indicating that an irreversible death has been induced in AST-treated cells.

We also assessed the protein expression of caspase 3 and PARP in HT-29 cells by western blotting. As shown in Figure 3C-i and C-ii, expression of pro-caspase 3 in HT-29 cells was reduced following 72 h of AST treatment, with the accumulation of its cleaved 17 kDa subunit being observed. Concurrently, cleavage of PARP, the substrate of caspase 3, was increased both concentration and time dependently in AST-treated HT-29 cells (Figure 3C-i and C-iii). These findings indicate that the AST-induced apoptosis is caspase dependent. Furthermore, expression of the Bcl-2 family member was also examined.

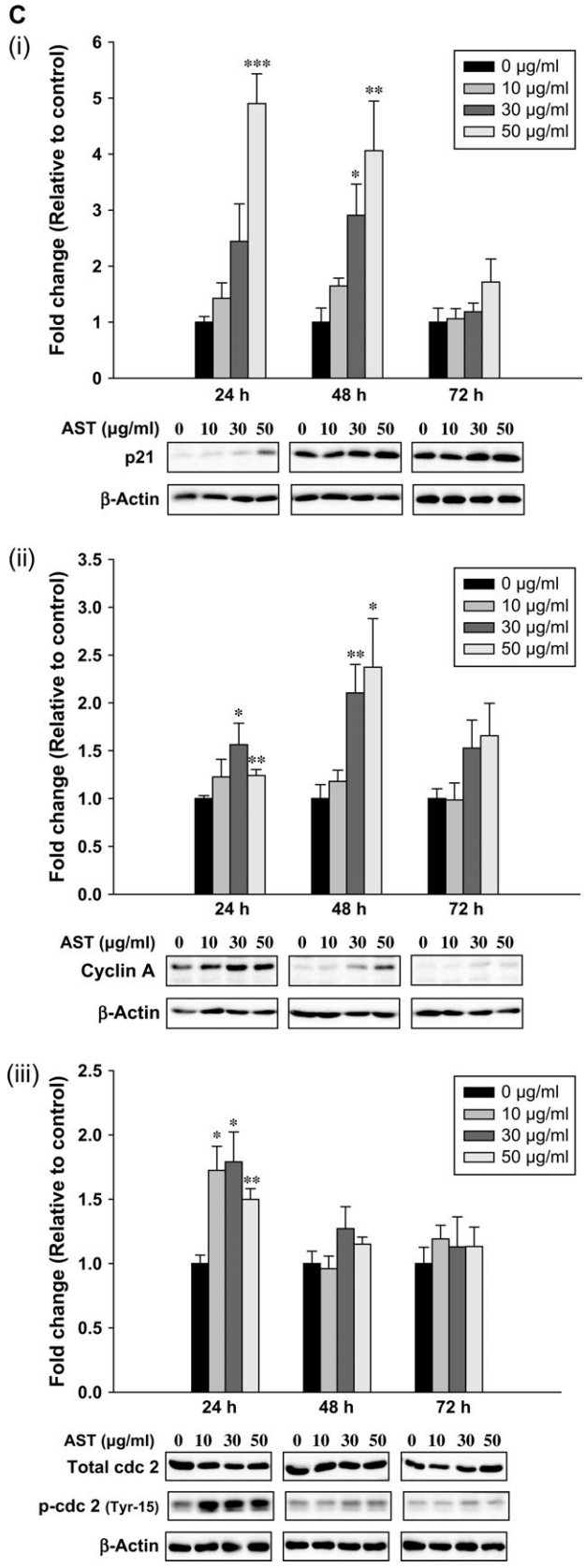

Fig. 2. *Continued.*

Figure 3C-iv has demonstrated that expression of the anti-apoptotic protein Bcl-x_L decreased significantly following 48 and 72 h of AST treatment, whereas no significant change in expression of the pro-apoptotic protein Bax has been induced (data not shown). The above data suggest that the caspase-dependent apoptotic action produced by AST could involve suppression of the apoptotic inhibitors.

The anti-tumorigenic effects of AST in nude mice xenograft

The anti-tumorigenic effect of AST on colon cancer was further illustrated *in vivo* in nude mice xenograft. Drug efficacy of AST on tumor growth inhibition was compared with that of the orthodox chemotherapeutic drug 5-FU. Moreover, AST was administrated together with 5-FU to investigate its potential as an adjuvant agent (Figure 4A). On the day of sacrifice (day 21), AST treatments at the given doses have resulted in 35–38% of tumor suppression, which is comparable with that caused by 5-FU alone (Table I). AST-induced tumor volume reduction became significant from day 13 onwards, whereas 5-FU monotherapy could only achieve the same effect from day 16 onwards. Other than monotherapy, combined treatment of 5-FU and oxaliplatin has provoked further reduction of tumor volume to 61% on day 21, with average reduction of at least 53% from day 13 onwards (Table I). Replacement of oxaliplatin by AST in the combined treatment with 5-FU causes a similar anti-tumor effect, showing >66% inhibition in tumor growth on day 21 with an average volume reduction of at least 60% from day 13 onwards (Table I).

The body weight of animals in both control and drug treatment groups were monitored throughout the experimental period, which reflects drug-induced toxicity (29). Table II indicates that AST has not imposed any significant decrease in body weight in the experimental animals, whereas a slight reduction of body weight has been exhibited in the 5-FU-treated animals. In spite of the increased reduction of tumor volume in animals treated with 5-FU + oxaliplatin by almost 1.5-fold when compared with that produced by AST or 5-FU monotherapy, such combined treatment has resulted in a more drastic weight loss with a mortality rate of 33% (Table II). Nevertheless, the drop in body weight has been improved when AST was used to replace oxaliplatin, without any mortality being recorded.

By using the TUNEL method, it was determined that a significant increase in the number of apoptotic cells can be observed in tumor sections obtained from AST-treated animals (Figure 4B). Immunohistochemical assessment of cell proliferation in the tumor sections also shows that AST treatments significantly reduce the amount of visible PCNA-labeled cells (Figure 4C-ii and C-iii) when compared with the control (Figure 4C-i). The effects of AST on apoptosis promotion and inhibition of cell proliferation are comparable with those caused by 5-FU (Figure 4B and 4C-iv). A similar correlation has also been confirmed in the combined treatment groups of 5-FU + AST and 5-FU + oxaliplatin (Figure 4B, C-v and C-vi).

Taken together, AST possesses similar anti-tumor potential as the conventional anti-neoplastic drug 5-FU, although a clear dose-dependent correlation has not been detected. Besides, it is plausible to use AST to replace the more cytotoxic drugs such as oxaliplatin as the chemotherapeutic adjuvant of 5-FU, which could largely reduce the associated side effects and toxicity.

Discussion

Despite recent advancement in understanding the carcinogenic processes of colon cancer, the increasing incidence and relatively low remission rate of chemotherapy have urged the scientific community to establish more effective treatment regimens by adopting novel and innovative approaches. The discovery and use of active medicinal compounds from herbal/natural sources have provided alternative treatment choices for patients (30). In the present study, we have shown that the total saponins obtained from *radix Astragalus membranaceus* could be established as effective chemotherapeutic agent to suppress colon cancer cell growth through promotion of apoptosis and inhibition of cell proliferation. This is the first report that clearly characterizes the anti-tumor properties of *Astragalus* saponins in colon cancer cells and tumor xenograft.

In order to unveil the mechanism involved in AST-induced growth suppression, we first inspected the events that lead to inhibition of

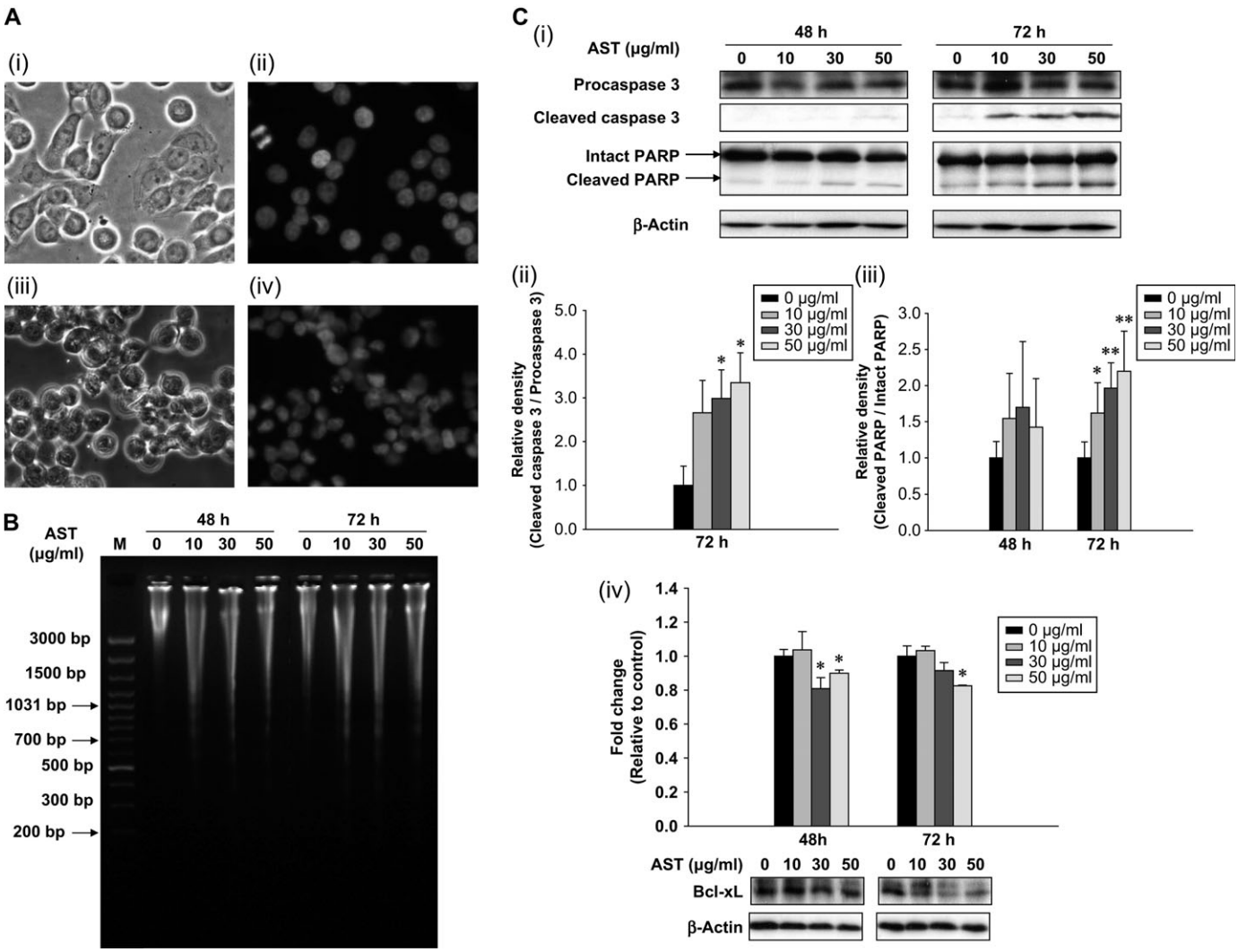

Fig. 3. AST promotes apoptosis in HT-29 cells. (**A**) In order to determine the mode of cell death induced by AST, morphologic alterations were examined using 4′-6-diamidino-2-phenylindole DNA staining. Images were taken by phase-contrast (i and iii) and fluorescence (ii and iv) microscopy after 72 h of AST or vehicle treatment. In the control group (i and ii), cells are round and homogeneously stained. In the AST (50 μg/ml) treatment group (iii and iv), nuclear chromatin condensation and presence of granular apoptotic bodies could be observed. (**B**) Fragmented DNA (6 μg) was extracted from HT-29 cells and subjected to electrophoresis on 1.8% agarose gel (M: 100 bp DNA ladder marker). AST treatment for 24 or 48 h leads to the formation of internucleosomal DNA fragmentation. (**C**) Expression of proteins associated with apoptosis in HT-29 cells following AST treatment for 48 or 72 h was determined by western blotting. Representative immunoblots from four independent experiments are shown. (i) Caspase 3 and PARP cleavage was observed. Densitometric (arbitrary) data indicate relative band density of (ii) cleaved caspase 3 and (iii) cleaved PARP (normalized with the respective pro-enzyme or intact form on the same immunoblot) with respect to the vehicle-treated control (0 μg/ml AST). Besides, expression of the anti-apoptotic protein Bcl-x_L (iv) was decreased after 48 and 72 h AST treatment. Intensity of the target bands was normalized with β-actin reprobed on the same immunoblot. Densitometric (arbitrary) data indicate relative band density with respect to the vehicle-treated control. Representative immunoblots from four to six independent experiments were shown. Error bars represent means ± SEMs. Significantly different from the corresponding controls: $^{*}P < 0.05$, $^{**}P < 0.01$.

colon cancer cell proliferation. In response to drug-induced DNA damage, mammalian cells will activate different cell cycle checkpoints (31). The accumulation of cells in the S and G2/M phases could prevent them from entering into mitosis. This may be owing to DNA damage during G2, or because the cells progressing into G2 possess unrepaired damage inflicted during the previous phases (32–34). We have demonstrated that AST causes HT-29 cells to accumulate in the S phase and arrest at the G2/M phase. However, cancer cells will not be permanently arrested at these phases. AST seems to halt the cell cycle progression until the sub-G1 population gradually increases—a common indication of the presence of apoptotic cells. If this proposition is correct, it implicates that colon cancer cells will start to undergo apoptosis some times after the AST-induced cell cycle arrest. When referring to the findings from our western analysis, we discover that the universal cyclin-dependent kinase inhibitor p21 was over-expressed at high level following AST treatment. p21 is a key element in regulating cell cycle progression (35,36) and is associated with inhibition of cyclin A–cdk 2 and cyclin B–cdc 2 complexes (37). Our results in the fluorescence-activated cell sorter analysis suggest that the AST-induced cell accumulation in S phase could be mediated at least in part by cyclin A over-expression. It is known that the levels of different cyclins are under strict control through precise synthesis and degradation at the appropriate time points during cell cycle progression. Cyclin A is essential for the initiation and progression through S phase and subsequently for the onset of mitosis (38). Cyclin A ablation can activate the cyclin B–cdc 2 complex, whereas induction of cyclin A mRNA and protein synthesis will delay cdc 2-mediated kinase activation (39). Abnormal accumulation of cyclin A could in turn prevent the AST-treated colon cancer cells from entering into the mitotic phase, causing delay in subsequent cell cycle progression. Apart from that, G2/M arrest has also occurred in AST-treated cells, and therefore the expression of cyclin B and p-cdc 2 was examined.

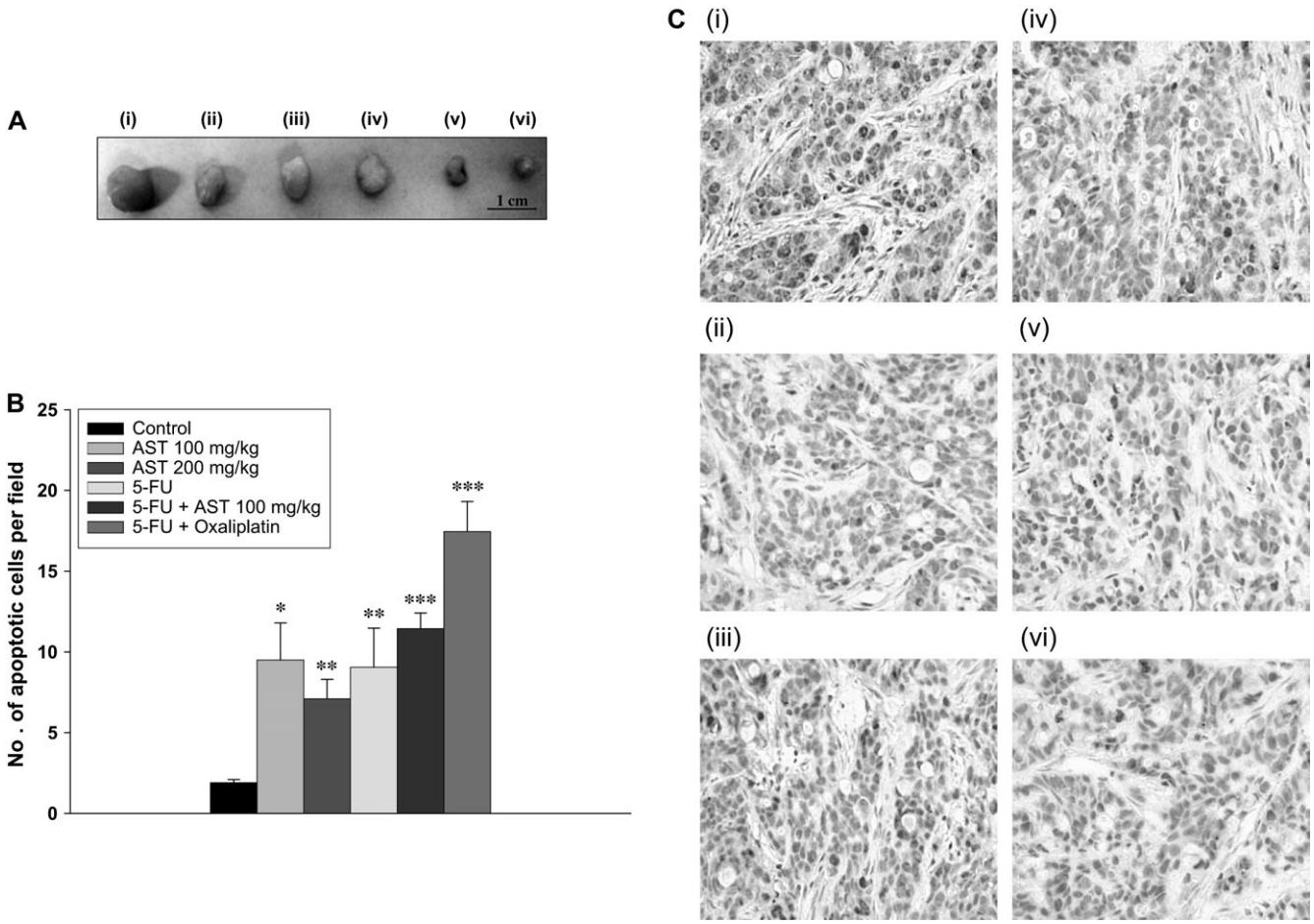

Fig. 4. Anti-tumor effects of chemotherapeutic agents in HT-29 xenograft and related immunohistochemical analysis. (**A**) Photographic illustration of tumors excised from vehicle control or drug-treated nude mice on the day of sacrifice (day 21): (i) control, (ii) AST (100 mg/kg), (iii) AST (200 mg/kg), (iv) 5-FU, (v) 5-FU + AST (100 mg/kg) and (vi) 5-FU + oxaliplatin. (**B**) Apoptotic cells were evaluated by using the TUNEL method. AST promotes apoptosis in excised tumors from the xenografted mice to a similar extent as 5-FU. Besides, when used as an adjuvant with 5-FU, AST has been shown to produce promising pro-apoptotic effect in replacement of oxaliplatin. Values are expressed as means ± SEMs of three to five tumor sections. Significantly different from control: $^{*}P < 0.05$, $^{**}P < 0.01$. (**C**) Representative images of the microscopic images obtained from control and drug treatment groups following PCNA staining and Mayer's hematoxylin counterstaining have been shown. Microscopic images indicate that AST given at the doses of 100 mg/kg (ii) and 200 mg/kg (iii) induces remarkable reduction of the number of PCNA-stained HT-29 cells in the excised tumor sections when compared with the vehicle-treated control (i). These anti-proliferative effects are comparable with that produced by 5-FU (iv). In addition, further reduction in the amount of proliferative cells has been resulted by the combined treatment of 5-FU and AST (100 mg/kg) (v) at a similar level to that produced by combined treatment of 5-FU and oxaliplatin (vi).

The initiation of mitosis requires activation of the cyclin B–cdc 2 complex, which commences with binding of cdc 2 to cyclin B1. Prior to mitosis, it is generally accepted that the cyclin B–cdc 2 complexes are remained in inactive state by phosphorylation at the Thr-14 and Tyr-15 sites of cdc 2 (40–42). Although cyclin B1 and total cdc 2 expressions have not been altered by AST in HT-29 cells, a significant increase in the expression of the inactive phospho-Tyr-15-cdc 2 was observed. This suggests that the cyclin B–cdc 2 complexes are maintained in a non-functional state by phosphorylation of the cyclin-dependent kinase, which signifies the arrest of cells at the G2/M phase. The above concomitant molecular events in AST-treated HT-29 cells lead to cell accumulation in S phase and G2/M arrest, where cells could not successfully progress through the mitotic phase and result in remarkable inhibition of proliferation. Prospective studies will be needed to clarify the precise protein interactions and thereby to delineate the upstream regulatory events.

Besides the inhibition on cancer cell proliferation, some of the arrested cells may still proceed through the blockade, allowing generation of clones with malignant advantages (43,44). This is actually a common situation for patients who have developed drug resistance following chemotherapy (45). Therefore, it is important for an effective chemotherapeutic agent to cause 'irreversible death' in cancer cells. DNA fragmentation and nuclear chromatin condensation have been demonstrated in HT-29 cells that were treated with AST. Moreover, the observation of caspase 3 activation and PARP cleavage also confirms that the promotion of apoptosis by AST involves a caspase-dependent pathway. Expression of members of the Bcl-2 family was determined to provide a better insight of the apoptotic signaling involved in AST-treated HT-29 cells. Bcl-x_L is an anti-apoptotic protein that prevents mitochondrial permeability transition pore opening and the release of cytochrome *c* following DNA damage (46). A down-regulation of Bcl-x_L expression was found in the present investigation, of which there was no change in the expression of the pro-apoptotic member Bax. As previously reported, Bcl-2 expression could not be detected in HT-29 cells (47,48). These findings suggest that induction of apoptosis in AST-treated HT-29 cells could be associated with the caspase-dependent cascade that involves activation of the mitochondrial pathway initiated by the inhibition of Bcl-x_L. More tests will be conducted to better characterize the AST-induced apoptotic signaling.

The anti-tumor effects of AST were further evaluated in HT-29 xenografted athymic nude mice. AST is capable of inducing tumor growth inhibition to a similar extent as 5-FU, the orthodox anti-neoplastic drug commonly used in treating colon cancer. In addition, the promising synergistic anti-tumor action of the 5-FU–AST combo

Table I. Tumor volume (in mm^3) of nude mice xenograft

Treatment	Day 13	Day 16	Day 19	Day 21
Control	147.8 ± 18.5	204.4 ± 25.4	275.5 ± 37.6	322.6 ± 46.5
AST (100 mg/kg)	96.5 ± 11.4*	118.5 ± 14.2**	159.1 ± 19.2**	208.5 ± 26.8*
AST (200 mg/kg)	96.6 ± 15.0*	119.9 ± 16.3**	144.0 ± 19.4**	199.9 ± 33.2*
5-FU	108.3 ± 8.7	112.8 ± 10.4**	167.7 ± 19.5**	204.8 ± 22.9*
5-FU + AST (100 mg/kg)	58.0 ± 6.9**	69.3 ± 8.3**	81.4 ± 8.0**	108.7 ± 7.5**
5-FU + oxaliplatin	68.1 ± 10.3**	78.1 ± 9.7**	105.2 ± 13.5**	126.1 ± 15.3**

Significantly different from control: $^{*}P < 0.05$, $^{**}P < 0.01$.

Table II. The change in body weight and mortality rate of nude mice with HT-29 xenograft

Treatment	Change in body weight (%)					Mortality (%)
	Day 0	Day 8	Day 14	Day 18	Day 21	
Control	100	101.1	104.5	108.6	105.3	0
AST (100 mg/kg)	100	95.4	104.9	105.2	106.1	0
AST (200 mg/kg)	100	97.1	97.7	102.6	100.6	0
5-FU	100	98.2	85.4	96	97.1	0
5-FU + AST (100 mg/kg)	100	93.8	77.4	91.9	98.1	0
5-FU + oxaliplatin	100	92.5	75	87.4	92.8	33

Initial body weight of tumor-bearing mice was designated as that being measured right before the animals received any drug/vehicle treatment on day 0 (100%). Mortality was only observed in animals treated with the drug combo 5-FU + oxaliplatin from day 12 onwards.

without the toxicity and side effects anticipated with oxaliplatin (e.g. mortality and significant drop in body weight) further suggests that AST has the potential to be established as novel adjuvant agent in colon cancer chemotherapy. Furthermore, 5-FU induces significant reduction of the total white blood cell count in mice, whereas co-treatment with AST could prevent such leukopenic effect (data not shown). In many cases, the p53 status of tumor largely influences the effectiveness of anti-cancer chemotherapy, since p53 gene alteration in cancer cells has played an important role in drug resistance that leads to unpredictable outcome and low efficacy (49,50). Deprivation of p53-dependent apoptosis is a common phenomenon in solid tumor harboring a non-functional p53. Thus, discovery of chemotherapeutic agents that are capable of inducing p53-independent apoptosis will be of great advantage. The successful promotion of apoptosis by AST in HT-29 colon cancer cells (with mutated p53 gene) and tumor xenograft has indeed proven that AST possesses strong anti-tumorigenic effects irrespective of the p53 status of the tumor. In general, investigations on the application of *Astragalus* extracts in treating human cancers are lacking. There has only been a single meta-analysis report that suggests the potential use of *Astragalus*-based medicinal agents and platinum derivatives in treating advanced non-small-cell lung cancer (51). Studies on *Astragalus* saponins are mainly referring to their immunomodulating functions, particularly of astragaloside IV (19). We have explored the anti-carcinogenic action of astragaloside IV in HT-29 cells, but no growth inhibition or any pro-apoptotic effect could be detected (Ko, J.K.S. and Tin, M.M.Y., unpublished data). At present, we are performing fractionation of AST, and have already discovered the fractions that contain the bioactive constituents responsible for the growth-inhibitory effects. Hence, identification of the target compounds will be imminent.

In summary, we have demonstrated in the present study that total *Astragalus* saponins could inhibit human colon cancer cell growth both *in vitro* and *in vivo*, with known mechanism of action. In contrast to orthodox chemotherapy using cytotoxic drugs, the use of this herbal extract imposes less toxicity while its anti-tumor effects could remain. This suggests the possibility of further developing AST as an alternative treatment option, or perhaps using it as adjuvant chemotherapeutic agent in colon cancer therapy.

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References

1. Jones,D.V.Jr *et al*. (1995) Randomized phase III study of 5-fluorouracil plus high dose folinic acid versus 5-fluorouracil plus folinic acid plus methyl-lomustine for patients with advanced colorectal cancer. *Cancer*, **76**, 1709–1714.
2. Marsoni,S. (1995) Fluorouracil and folinic acid in colon cancer. IMPACT investigators. *Lancet*, **345**, 1582–1583.
3. Giacchetti,S. *et al*. (2000) Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil–leucovorin as first-line treatment of metastatic colorectal cancer. *J. Clin. Oncol*, **18**, 136–147.
4. Douillard,J.Y. *et al*. (2000) Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet*, **355**, 1041–1047.
5. Malet-Martino,M. *et al*. (2002) Clinical studies of three oral prodrugs of 5-fluorouracil (capecitabine, UFT, S-1): a review. *Oncologist*, **7**, 288–323.
6. Meta-Analysis Group in Cancer. (1998) Toxicity of fluorouracil in patients with advanced colorectal cancer: effect of administration schedule and prognostic factors. *J. Clin. Oncol*, **16**, 3537–3541.
7. Desoize,B. *et al*. (2002) Particular aspects of platinum compounds used at present in cancer treatment. *Crit. Rev. Oncol. Hematol*, **42**, 317–325.
8. de Gramont,A. *et al*. (2000) Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J. Clin. Oncol*, **18**, 2938–2947.
9. Bensky,D. *et al*. (1993) Chinese Herbal Medicine Materia Medica. Eastland Press, Seattle.
10. World Health Organization (WHO). (1989) Medicinal Plants in China. WHO Regional Publications, Manila.
11. Zee-Cheng,R.K. (1992) Shi-quan-da-bu-tang (ten significant tonic decoction), SQT. A potent Chinese biological response modifier in cancer immunotherapy, potentiation and detoxification of anticancer drugs. *Methods Find. Exp. Clin. Pharmacol*, **14**, 725–736.
12. Ma,X.Q. *et al*. (2002) Chemical analysis of radix Astragali (Huangqi) in China: a comparison with its adulterants and seasonal variations. *J. Agric. Food Chem*, **50**, 4861–4866.
13. Shao,B.M. *et al*. (2004) A study on the immune receptors for polysaccharides from the roots of Astragalus membranaceus, a Chinese medicinal herb. *Biochem. Biophys. Res. Commun*, **320**, 1103–1111.
14. Duan,P. *et al*. (2002) Clinical study on effect of Astragalus in efficacy enhancing and toxicity reducing of chemotherapy in patients of malignant tumor. *Zhongguo Zhong Xi Yi Jie He Za Zhi*, **22**, 515–517.
15. Cui,R. *et al*. (2003) Suppressive effect of Astragalus membranaceus Bunge on chemical hepatocarcinogenesis in rats. *Cancer Chemother. Pharmacol*, **51**, 75–80.

16. Wang,Y. *et al*. (1992) Phytochemicals potentiate interleukin-2 generated lymphokine-activated killer cell cytotoxicity against murine renal cell carcinoma. *Mol. Biother*, **4**, 143–146.
17. Kitagawa,I. *et al*. (1983) Saponin and sapogenol. XXXVII: chemical constituents of Astragali Radix, the root of Astragalus membranaceus Bunge. (4): Astragalosides VII and VIII. *Chem. Pharm. Bull*, **31**, 716–722.
18. He,Z.Q. *et al*. (1991) Constituents of Astragalus membranaceus. *J. Nat. Prod*, **54**, 810–815.
19. Wang,Y.P. *et al*. (2002) Effect of astragaloside IV on T, B lymphocyte proliferation and peritoneal macrophage function in mice. *Acta Pharmacol. Sin*, **23**, 263–266.
20. Zhang,W.D. *et al*. (2006) Astragaloside IV from Astragalus membranaceus shows cardioprotection during myocardial ischemia in vivo and in vitro. *Planta Med*, **72**, 4–8.
21. Oh,M. *et al*. (1999) Anti-proliferating effects of ginsenoside Rh2 on MCF-7 human breast cancer cells. *Int. J. Oncol*, **14**, 869–875.
22. Lee,K.Y. *et al*. (1997) Ginsenoside-Rg5 suppresses cyclin E-dependent protein kinase activity via up-regulating p21Cip/WAF1 and down-regulating cyclin E in SK-HEP-1 cells. *Anticancer Res*, **17**, 1067–1072.
23. Jin,Y.H. *et al*. (2000) Caspase 3-mediated cleavage of p21WAF1/CIP1 associated with the cyclin A-cyclin-dependent kinase 2 complex is a prerequisite for apoptosis in SK-HEP-1 cells. *J. Biol. Chem*, **275**, 30256–30263.
24. Ellington,A.A. *et al*. (2006) Inhibition of Akt signaling and enhanced ERK1/2 activity are involved in induction of macroautophagy by triterpenoid B-group soyasaponins in colon cancer cells. *Carcinogenesis*, **27**, 298–306.
25. Koratkar,R. *et al*. (1997) Effect of soya bean saponins on azoxymethane-induced preneoplastic lesions in the colon of mice. *Nutr. Cancer*, **27**, 206–209.
26. Isik,F.F. *et al*. (1998) Vitronectin decreases microvascular endothelial cell apoptosis. *J. Cell. Physiol*, **175**, 149–155.
27. Feng,Z. *et al*. (2004) DNA damage induces p53-dependent BRCA1 nuclear export. *J. Biol. Chem*, **279**, 28574–28584.
28. Tan,Y. *et al*. (1999) Efficacy of recombinant methioninase in combination with cisplatin on human colon tumors in nude mice. *Clin. Cancer Res*, **5**, 2157–2163.
29. Britten,C.D. *et al*. (1999) Enhanced antitumor activity of 6-hydroxymethylacylfulvene in combination with irinotecan and 5-fluorouracil in the HT29 human colon tumor xenograft model. *Cancer Res*, **59**, 1049–1053.
30. Smith,M. *et al*. (1999) Counseling cancer patients about herbal medicine. *Patient Educ. Couns*, **38**, 109–120.
31. Kastan,M.B. *et al*. (2004) Cell-cycle checkpoints and cancer. *Nature*, **432**, 316–323.
32. Bartek,J. *et al*. (2004) Checking on DNA damage in S phase. *Nat. Rev. Mol. Cell Biol*, **5**, 792–804.
33. Xu,B. *et al*. (2002) Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. *Mol. Cell. Biol*, **22**, 1049–1059.
34. Nyberg,K.A. *et al*. (2002) Toward maintaining the genome: DNA damage and replication checkpoints. *Annu. Rev. Genet*, **36**, 617–656.
35. Xiong,Y. *et al*. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701–704.
36. Weiss,R.H. (2003) p21Waf1/Cip1 as a therapeutic target in breast and other cancers. *Cancer Cell*, **4**, 425–429.
37. Baus,F. *et al*. (2003) Permanent cell cycle exit in G2 phase after DNA damage in normal human fibroblasts. *EMBO J*, **22**, 3992–4002.
38. Grana,X. *et al*. (1995) Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene*, **11**, 211–219.
39. Walker,D.H. *et al*. (1991) Role for cyclin A in the dependence of mitosis on completion of DNA replication. *Nature*, **354**, 314–317.
40. Solomon,M.J. *et al*. (1990) Cyclin activation of p34 cdc2. *Cell*, **63**, 1013–1024.
41. McGowan,C.H. *et al*. (1993) Human Wee1 kinase inhibits cell division by phosphorylating p34 cdc2 exclusively on Tyr15. *EMBO J*, **12**, 75–85.
42. Jin,P. *et al*. (1996) Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells. *J. Cell Biol*, **134**, 963–970.
43. Schober,C. *et al*. (1994) Cellular heterogeneity in DNA damage and growth inhibition induced by ICI D1694, thymidylate synthase inhibitor, using single cell assays. *Biochem. Pharmacol*, **48**, 997–1002.
44. Reed,J.C. (2003) Apoptosis-targeted therapies for cancer. *Cancer Cell*, **3**, 17–22.
45. Igney,F.H. *et al*. (2002) Death and anti-death: tumour resistance to apoptosis. *Nat. Rev. Cancer*, **2**, 277–288.
46. Reed,J.C. (1999) Mechanisms of apoptosis avoidance in cancer. *Curr. Opin. Oncol*, **11**, 68–75.
47. Reinacher-Schick,A. *et al*. (2003) Mesalazine causes a mitotic arrest and induces caspase-dependent apoptosis in colon carcinoma cells. *Carcinogenesis*, **24**, 443–451.
48. Gamet-Payrastre,L. *et al*. (2000) Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. *Cancer Res*, **60**, 1426–1433.
49. Levine,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.
50. Velculescu,V.E. *et al*. (1996) Biological and clinical importance of the p53 tumor suppressor gene. *Clin. Chem*, **42**, 858–868.
51. McCulloch,M. *et al*. (2006) *Astragalus*-based Chinese herbs and platinum-based chemotherapy for advanced non-small-cell lung cancer: meta-analysis of randomized trials. *J. Clin. Oncol*, **24**, 419–430.

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