

RESEARCH ARTICLE

Enhanced Antitumor Efficacy with Combined Administration of Astragalus and Pterostilbene for Melanoma

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Abstract

Astragalus, a commonly used traditional Chinese medicine, has exhibited antitumor actions in patients. In this study, *in vitro* and *in vivo* antitumor effects of astragalus and synergistic antitumor efficacy in combination with pterostilbene were investigated. Melanoma cells were treated with pterostilbene (Pt), graduated doses of astragalus injection (AI), or these in combination. Cell viability was measured using a MTT assay. Released nucleosomes and caspase activity were measured using enzyme-linked immunosorbent assay. Growth inhibition *in vitro* and *in vivo* was also assessed. Analysis of variance and t tests were used for statistical analysis. Significant reduction ($p < 0.05$) in cellular proliferation were observed with AI and AI-Pt in a time- and concentration-dependent manner. Apoptosis and caspase-3/7 activity were significantly increased by AI and AI-Pt treatment ($p < 0.05$). *In vivo*, AI inhibited melanoma tumor growth, with inhibition rates ranging from 36.5 to 62.3%, by inducing apoptosis via up-regulation Bax expression and the Bax/Bcl-2 ratio and down-regulating Bcl-2 expression. AI significantly inhibits the growth of melanoma *in vitro* and *in vivo* by inducing apoptosis. These data suggest that combined treatment of astragalus with pterostilbene enhances antitumor efficacy.

Keywords: Astragalus - pterostilbene - melanoma - apoptosis - antitumor efficacy

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Introduction

In men, melanoma is the fastest growing incidence of any cancer, while it is the second fastest growing incidence for women (Khan et al., 2011; Nicholl et al., 2011; Siegel et al., 2011). It is reported that the incidence, doubled almost every 10 years, is increasing more rapidly than the incidence of all other malignancies, except for lung cancer (Voller et al., 2011). About 70,230 new cases of melanoma occurred in 2011 in America (Siegel et al., 2013). Once diagnosed, the first course of treatment for locally advanced and/or systemic disease is surgical resection, followed by chemotherapy, radiation therapy, and/or immunotherapy (Villa et al., 2004). Recently, therapy of melanoma has some breakthroughs (Hodi et al., 2010; Chapman et al., 2011); however, the treatment of advanced melanoma is highly challenging, in part because there are few effective therapeutic regimens (Balch et al., 2001; Bastiaannet et al., 2005; Osmond et al., 2012; Fang et al., 2013). Although those treatments are effective in many patients, not all patients will respond and many will have recurrence and/or progression of the melanoma. For these patients, new and potentially more effective forms of therapy need to be developed to improve survival rates (Rizvi et al., 2006).

Herbs that may prove beneficial in treating this condition include astragalus, which helps generate

anticancer cells in your body and enhances your immune system. Astragalus is an herbal remedy that may be a helpful adjunct therapy in treating melanoma and other types of cancer. For example, astragalus membranaceus (AM) injection supplemented with chemotherapy was found to inhibit the development of tumor, decrease the toxic-adverse effect of chemotherapy, elevate the immune function of organism and improve the quality of life in patients of malignant tumor (McCulloch et al., 2006). Similar results were obtained in the study with gastric cancer (Na et al., 2009). Besides, it was reported that AM injection could enhance the anti-tumor metastasis action of dendritic cells, effectively promote the immune response of tumor-bearing host and therefore had obviously inhibitory effect on cancer metastasis *in vivo* (Dong et al., 2005). More recently, Li et al. (2012) found that astragalus had significant anti-tumor effect *in vivo* in inducing apoptosis of H22 tumor cells. Nevertheless, whether AM can suppress the growth of melanoma, both *in vitro* and *in vivo*, have not been fully investigated. It is of interest and importance to examine whether AM possesses *in vitro* and *in vivo* anti-tumor effects.

Pterostilbene is a naturally occurring analog of resveratrol found in blueberries (Schneider et al., 2009). Remsberg et al. (Remsberg et al., 2007) and Rimando et al. (Rimando et al., 2002) have found that pterostilbene has similar anti-inflammatory, antioxidant, and anticancer

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properties to those of resveratrol. However, pterostilbene exhibits increased bioavailability due to the presence of two methoxy groups which promote lipophilicity and oral absorption (McCormack et al., 2013). Pterostilbene inhibits melanoma through multiple mechanisms, which include apoptosis, inhibition of metastasis, and alteration of NO production (McCormack et al., 2012; McFadden et al. 2013). Previous studies have shown that melanoma, breast, gastric, and hepatocellular carcinomas were sensitive to pterostilbene (Schneider et al., 2010). The pterostilbene also exerted inhibitory effects *in vivo*, as a novel anti-metastatic agent to treat malignant melanoma (McCormack et al., 2012). In addition, pterostilbene wasn't any cytotoxicity against normal hemopoietic stem cells in the process of apoptosis induction to malignant cells (Tolomeo et al., 2005).

For now, however, the mechanism by which astragalus produces growth inhibition of melanoma has not been clearly identified. This study aimed to assess the effects of astragalus treatment on melanoma *in vitro* and *in vivo*, to further elaborate its growth-inhibitory mechanism, and investigate potential synergistic effects of astragalus and pterostilbene on melanoma.

Materials and Methods

Reagents, and cell lines

Astragalus injection (AI), as an extract from astragalus, was obtained from Chengdu Diao JiuHong Pharmaceutical Company (Chengdu, China). Pterostilbene (Pt) was purchased from Sigma Aldrich (St. Louis, MO), and dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) and further diluted in sterile culture medium to desired concentrations immediately before use.

Human melanoma cell line, SK-MEL-2, was purchased from the American Type Culture Collection (ATCC, Manassas, VA). SK-MEL-2 is derived from a metastatic skin site. The cells were cultured according to supplier's recommendations in Eagle's minimum essential medium. Cells were maintained at 37°C in a water-jacketed 5% CO₂ incubator (Fischer Scientific, Houston, TX). For experiments cells were harvested from culture monolayers at 80% to 90% confluency. Cells were plated with 104 cells per well in 96-well plates and allowed to attach overnight. Cells were then exposed to Pt (50 µM) and graduated doses of AI (4 g/Kg, 8 g/Kg, 12 g/Kg, 16 g/Kg), combinations of these agents (AI-Pt) or DMSO control.

Animals

Balb/C male mice, five-weeks old, an average body weight of 25 g, were obtained from the Experimental Animal Center at Zhejiang University of Technology. The mice were housed in plastic cages with hardwood chip under an air-conditioned room at 22-25°C and 50-65% humidity, and with a 12 h light/dark cycle on basal diet (animal center). Pellet diet and tap water were provided ad libitum. The animal handlings and experimental procedures were in accordance with the recommendations from the Guide for Animal Experimentation of Zhejiang University of Technology in the care and use of experimental animals (Permit Number: 0006560-2013).

MTT Assay

A MTT colorimetric assay was performed to detect cell viability after 24 hours, 48 hours, and 72 hours of exposure to AI (2–16g/Kg). Briefly, culture media was removed and MTT, diluted in culture media, was added to each well. Plates were incubated at 37°C in the CO₂ incubator for 1 hour. The yellow MTT dye turned to a purple formazan, solubilized in DMSO. Absorbance was read at 570 nm via an enzyme-linked immunosorbent assay (ELISA) plate reader.

Growth inhibition *in vitro*

Cells were treated with Pt, AI-4 (4 g/Kg), AI-8 (8 g/Kg), AI-12 (12 g/Kg), AI-16 (16 g/Kg), and AI-Pt-4 (4 g/Kg AI+ 50 µM Pt), AI-Pt-8 (8 g/Kg AI+ 50 µM Pt), AI-Pt-12 (12 g/Kg AI+ 50 µM Pt), AI-Pt-16 (16 g/Kg AI+ 50 µM Pt) respectively at increasing doses for 24 hours, 48 hours, and 72 hours. Cells were then harvested and counted by hemocytometer. The growth of treated cells was expressed as a percentage of untreated control cells. The concentration of AI that decreased cell count by 50% (IC₅₀) was calculated by nonlinear least squares curve fitting of experimental data.

DNA fragmentation assay

The Cell Death Detection ELISA kit (Roche, Mannheim, Germany) was used to detect the occurrence of nuclear DNA fragmentation. After the cells were exposed to various doses of AI and AI-Pt for 18 hours, supernatants were removed and stored at 4°C for subsequent analysis of necrosis. According to the manufacturer's instructions, adherent cells were lysed and centrifuged to produce a nucleosome-containing supernatant. Samples were transferred to a streptavidin-coated microplate and incubated with antihistone and anti-DNA antibodies followed by a peroxidase substrate resulting in color change. Absorbance was measured spectrophotometrically at 405 nm.

Caspase activity assay

Cells were plated into 96-well plates with opaque sidewalls for this experiment. Cells were then treated with Pt, AI-4, AI-8, AI-12, AI-16, and AI-Pt-4, AI-Pt-8, AI-Pt-12, AI-Pt-16 or DMSO control for 12 hours, 24 hours, and 36 hours. The Apo-ONE homogeneous caspase 3/7 assay substrate (Promega, Madison, WI) was used to evaluate the activities of caspase-3 and -7, effector caspases that cleave intracellular protein substrates triggering the apoptotic process. The caspase-3/7 substrate rhodamine 110 is acted upon by caspase-3 and -7, resulting in a fluorescent leaving group. The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample. Caspase-3/7 substrate was added to each well and incubated at room temperature for 1 to 2 hours. A spectrofluorimeter was used to measure fluorescence (excitation wavelength 485 nm±20nm, emission wavelength 528 nm±20 nm).

In vivo anti-tumor activity

Male Balb/C mice were inoculated with SK-MEL-2

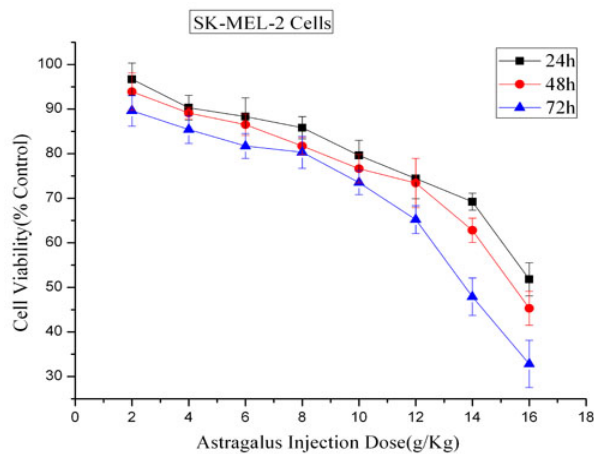


Figure 1. AI inhibited Melanoma Growth. SK-MEL-2 Cell Lines had a Concentration- and Time-dependent Response to AI Treatment. Error bars are the relative standard error of measurement (SEM) of six measurements

cell suspension (1×10^7 /ml), 0.1 ml/10 g, through subcutaneous injection at right side axilla. Twenty-four hour after the tumor cell inoculation, the mice were randomized equally into 6 groups of 10 each. The negative control group mice were injected with an equal volume of normal saline while mice in other groups were given Pt, AI-8, AI-12, and AI-Pt-8, AI-Pt-12 respectively once every alternate day from day 1 (six times totally). From the fourth day, tumor volume (V, cm^3) was measured on alternate days, and calculated as $AB^2/2$, where A is largest superficial diameter and B is smallest superficial diameter. All mice were sacrificed 13 days after inoculation with SK-MEL-2 cells, and the transplanted tumors were excised and weighed. To evaluate the anticancer activity of Astragalus, tumor inhibitory rates were calculated as: tumor inhibitory rate (%) = $1 - (\text{tumor weight of treated group} / \text{tumor weight of control group}) \times 100$.

Measurement of transplanted tumors' BCL-2 and Bax protein

Sections were cleared of paraffin, and endogenous peroxidases were blocked by incubation with 3% H_2O_2 and washed. Sections of the tissues were then incubated with rabbit serum for 10 min at ambient temperature. Subsequently, the sections were incubated overnight with a goat polyclonal anti-Bax antibody (Boster, China, 1:50) and anti-Bcl-2 antibody (Boster, China, 1:50) at 4°C , followed by the addition of biotinylated rabbit anti-goat IgG secondary antibody (Jinshan, BJ, China). To verify the binding specificity for Bax and Bcl-2, some sections were also incubated with primary antibody or the secondary antibody. In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all the experiments. Immunohistochemistry staining was processed in accordance with the manufacturer's instructions and visualized by use of diaminobenzidine (DAB) staining. Counterstaining was carried out with Harris hematoxylin (Sigma). Ten sections were analyzed for each animal. Samples were observed in four randomly selected optical fields under microscopy ($\times 200$) and the average

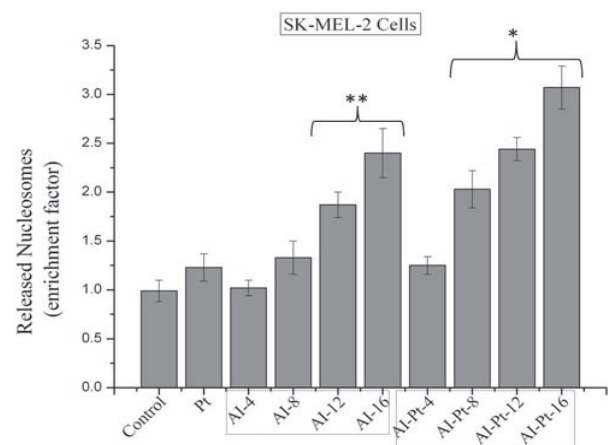


Figure 2. The occurrence of Nuclear DNA Fragmentation. Increase in released nucleosomes was observed in SK-MEL-2 cells treated by Pt, AI, and AI-Pt at 18 h. 1.87-fold and 2.4-fold ($p < 0.05$) increases were observed in the cells treated with 12 g/Kg and 16g/Kg of AI, respectively. In addition, 2.03-fold, 2.44-fold, and 3.07-fold ($p < 0.05$) increases were observed in the cells treated with AI-Pt-8, AI-Pt-12, AI-Pt-16, respectively. * $p < 0.05$ compared with the control group

optical density was analyzed with Image-pro plus (Media Cybernetics, USA) by an observer blind to the treatment groups.

Statistical analysis

Data were presented as mean values \pm SE. Statistical comparisons among groups were performed by Student's t test or analysis of variance (ANOVA) followed by Bonferonni post-tests for multiple comparisons. These analyses were run using GraphPad Software (San Diego, CA).

Results

Reduction of Cellular Proliferation *in vitro*

Studies were designed to determine the efficacy of AI alone in treating SK-MEL-2 cell lines *in vitro*. We found that treatment of SK-MEL-2 cell lines with AI resulted in a significant cytotoxic effect, which was both concentration-dependent and time-dependent (Figure 1). Lower doses (less than 8g/Kg) were not effective until after 72 h. It indicated that AI is an effective cytotoxic agent against melanoma cells lines *in vitro*.

AI and Pt having synergism in inhibition the growth of melanoma cells

The combination treatments of AI and Pt produced a more profound level of growth inhibition when AI and Pt were used in combination compared with either treatment alone (Table 1). Various doses are depicted here to demonstrate the effects across the span of dosing ranges at given time points.

AI and AI-Pt induces apoptosis in melanoma cells

The mechanism of cell death using an assay to detect released nucleosomes was investigated. The released nucleosomes increased in SK-MEL-2 cells treated by different concentrations (from 4-16g/Kg) of AI (Figure

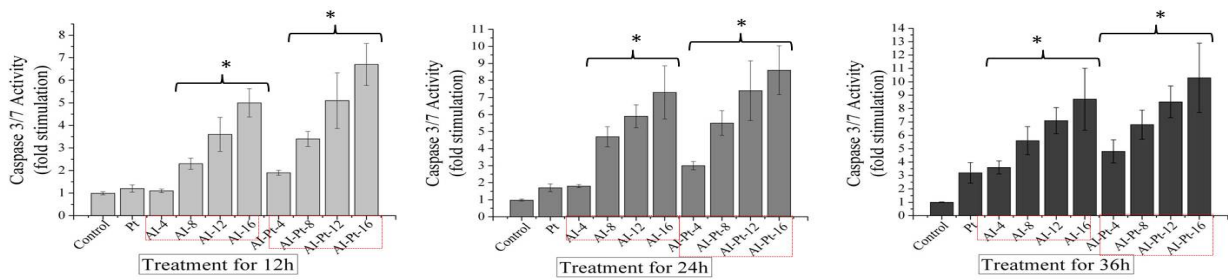


Figure 3. Caspase 3/7 Activity at 12 h, 24 h, and 36 h. Effector caspases-3 and -7, involved in the effector phase of apoptosis, are upregulated by AI and AI-Pt. Specifically, Treatment with AI (8-16g/Kg) resulted in a significant increase in caspase 3/7 activity as compared to controls in SK-MEL-2 cells (such as 3.6-fold increase at 12 h for AI-12, $p < 0.05$). Treatment with AI-Pt (8-16g/Kg) also resulted in a significant increase in caspase 3/7 activity as compared to controls in SK-MEL-2 cells (such as 6.9-fold increase at 36 h for AI-Pt-8, $p < 0.01$). These results showed that caspases-3 and -7 are significantly upregulated in SK-MEL-2 cells ($p < 0.05$)

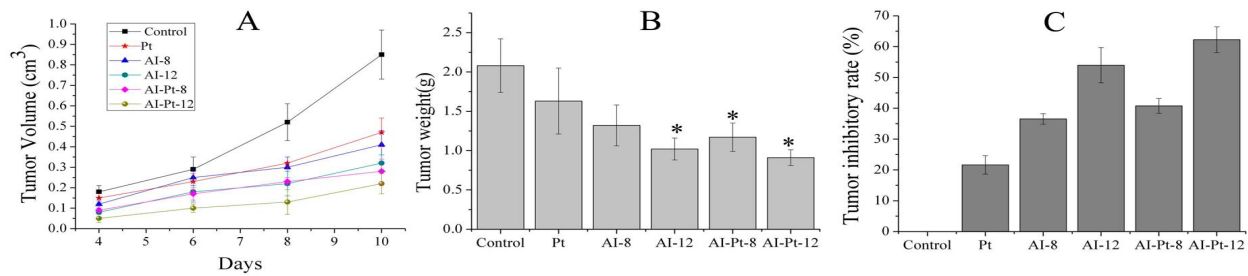


Figure 4. AI and AI-Pt Inhibited Tumor Growth *in Vivo*. (A) Growth curve of melanoma in mice. The results showed that AI and Pt were effective to inhibit tumor growth in mice. The synergism of AI and Pt was observed in the curve. Treatment with AI-Pt (8 and 12g/Kg) resulted in a significant reduce in tumor volume as compared to control ($p < 0.05$). (B) and (C) further demonstrated the inhibitory effect of AI and AI-Pt on SK-MEL-2 bearing mice. A markedly trend toward decreased tumor weight was observed. Similarly, a remarkable trend toward increased tumor inhibitory rate was also observed. * $p < 0.05$ as compared to control

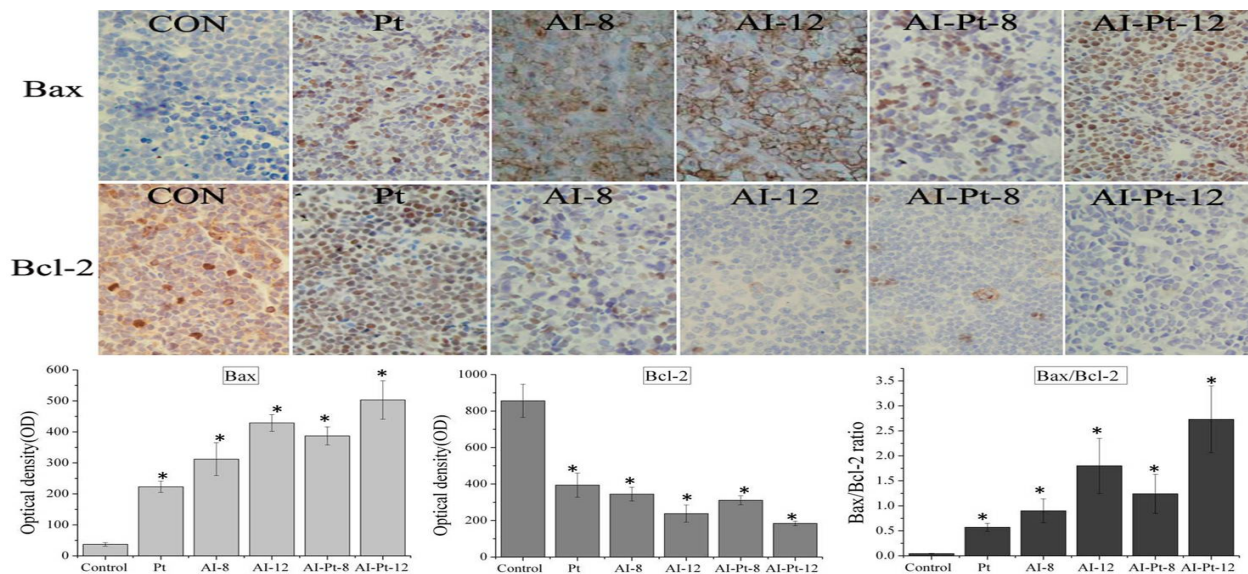


Figure 5. Shown are Representative Pictures of Immunostaining for Bax and Bcl-2 Proteins in SK-MEL-2 Bearing Mice ($\times 200$). The optical density in 5 to 6 randomly selected high power fields of three slides from each group was analyzed by Image-pro plus. There was a significant increase in the expression of Bax protein by AI and AI-Pt treatments in a dose-dependent manner as compared with control ($p < 0.05$). AI treatment the expression of anti-apoptotic protein Bcl-2 decreased significantly by AI and AI-Pt treatments compared with the control ($p < 0.05$). An obvious enhancement of the Bax/Bcl-2 ratio was observed

2). There was a significant difference in the released nucleosomes between AI-12, AI-16, AI-Pt-8, AI-Pt-12, AI-Pt-16 groups and the control group ($p < 0.05$). The results indicated that combination treatment of AI with Pt had enhancement effect in inducing apoptosis of melanoma cells.

assess caspase activity in order to determine the nature of apoptosis induction by AI and AI-Pt. A remarkable trend toward increased caspase-3/7 activity was observed in the SK-MEL-2 cells treated by graduated doses of AI and AI-Pt at 12h, 24h, and 36h, respectively (Figure 3).

AI and AI-Pt up-regulates effector caspases

The effector caspases-3 and -7 were examined to

*AI and AI-Pt inhibit tumor growth significantly *in vivo**

SK-MEL-2 cells were injected into Balb/C mice to

determine whether AI inhibits tumor growth *in vivo* and the inhibition effect of combination treatments. Growth curve of melanoma showed that AI inhibited tumor growth in a time- and dose- dependent manner (Figure 4A). Meanwhile, the tumor weight of melanoma decreased, especially a significant decrease was observed in the tumor weight of melanoma treated by AI-12, AI-Pt-8, and AI-Pt-12, respectively ($p < 0.05$) (Figure 4B). Similarly, the tumor inhibitory rates to melanoma, treated by AI-8, AI-12, AI-Pt-8, and AI-Pt-12, ranged from 36.54 to 62.25% (Figure 4C). Furthermore, level of growth inhibition was more obvious when AI and Pt were used in combination compared with either treatment alone.

Effects of AI and AI-Pt on Bax and Bcl-2 protein level

Immunohistochemistry staining was conducted for Bax and Bcl-2 protein to determine the effect of AI and AI-Pt on the regulation of apoptosis gene. The levels of Bax and Bcl-2 protein changed in AI and AI-Pt (Figure 5). The levels of pro-apoptotic protein Bax were significantly upregulated by AI and AI-Pt treatments. And the levels of anti-apoptotic protein Bcl-2 were significantly downregulated. The Bax/Bcl-2 ratio also increased after AI and AI-Pt treatments (Figure 5). These results indicated that AI and AI-Pt treatments induced apoptosis by modulating Bcl-2 family proteins.

Discussion

Melanoma is the most lethal form of skin cancer and accounts for 75% of skin cancer-related deaths (Rizvi et al., 2006; Schneider et al., 2009; Osmond et al., 2012). At present, due to western medical treatments for cancer have side-effects, some attentions have shifted to discover new anti-cancer strategy, such as the use of traditional Chinese medicine, in the hope of producing anti-tumor effect without too many serious side-effects (Treasure et al., 2005; Cho et al., 2007; Li et al., 2012). Astragalus Membranaceus is part of the Chinese medicine since many years. It has so far been used as a modulating agent for all kinds of immunological diseases and in combination with cytotoxic drugs to ameliorate their side effects (Zee-Cheng et al., 1992). Various studies have found Astragalus to have anti-tumor properties making it a herb with cancer fighting potential. Cho et al. (2007) found that astragalus membranaceus exhibited anti-tumor efficacy against a variety of experimental tumors of different origins *in vitro* and *in vivo*. The investigators proved that it might be achieved through activating the anti-tumor immune mechanism of the host. Recently, it has been demonstrated that the anti-tumor mechanism of astragalus may be an induction of the apoptosis of cancer cell (Hu et al., 2009; Auyeung et al., 2010; Song et al., 2011).

In vitro, astragalus extracts inhibit proliferation, induce apoptosis, and interrupt caryocinesia at G0-G1 phase or S phase in hormone-sensitive (MCF-7) breast cancer cells line (Siwicka et al., 2011). A critical factor determining the efficacy of a given anticancer treatment is its ability to minimize cell proliferation (Schneider et al., 2010). In our study, we also found that AI inhibited proliferation of SK-MEL-2 cells (Figure 1), which had a concentration- and

time-dependent response to AI treatment. Another critical factor determining the efficacy of a given anti-cancer treatment is its ability to maximize cell apoptosis (Johnson et al., 2008). Bcl-2 and Bax are two important proteins in mitochondrial-mediated pathway. Bcl-2 inhibits apoptosis, whereas Bax promotes apoptosis (Thompson et al., 1995; Zhang et al., 2000). Our data suggest that the mechanism for growth inhibition may be through increased apoptosis as indicated by an increase in released nucleosomes *in vitro* (Figure 2). Subsequently, we analyzed the activity of caspases-3 and -7. The cell lines demonstrated a significant increase in activity of caspases-3 and -7 (Figure 3), thus supporting that AI inhibits melanoma growth via induction of apoptosis. This mechanism was also evidenced by the increase of proapoptotic, bax, and decrease of antiapoptotic, Bcl-2. This is in agreement with the previous results (Hu et al., 2009; Auyeung et al., 2010). However, whether the mechanism of AI-mediated apoptosis and anti-tumor immune mechanism combined to exert anti-cancer property needs to be further investigated.

Data on the anticancer potential of Astragalus *in vivo* are limited but promising. Our findings interestingly correlate with conclusions made by researchers in other fields regarding astragalus's disease fighting ability. Cui et al. (Cui et al., 2003) demonstrated that extracts of astragalus delayed chemical-induced hepatocarcinogenesis in rats, and displayed antiangiogenic properties (Auyeung et al., 2012). Astragalus extract also enhanced the effect of platinum-based chemotherapy (McCulloch et al., 2006). *In vivo*, our data indicate that the tumor volume and weight of melanoma decreased by astragalus treatment (Figure 4), which confirms that AI is an effective antitumor growth agent against melanoma *in vivo*. The results are similar to those of Li et al. (2012) and Siwicka et al. (Siwicka et al., 2011) that showed the antitumor effect of AI *in vivo*. A meta-analysis suggests that astragalus-based treatments for hepatocellular cancers may be viable, but larger trials are required (Wu et al., 2009). Guo et al. (2012) reported that use of an injectable form of astragalus with vinorelbine and cisplatin in patients with advanced non-small cell lung cancer improved quality of life with median survival time of 10.7 months and 1-year survival rates of 35.3%. A recent study suggests that astragalus extract may help manage cancer-related fatigue (Chen et al., 2012). Whether astragalus treatment on melanoma *in vivo* exerts the same effects is not known. Consequently, the antitumor potential of astragalus should be further investigated for clinical use.

Since the antitumor efficacy of pterostilbene has been extensively studied (Tolomeo et al., 2005), the inhibition effect of cancers has been widely accepted. However, there has been little scientific advancement with regard to the effect of combined administration of astragalus and pterostilbene on melanoma. Ferrer et al. (2005) found that combination treatment of pterostilbene with quercetin produced a synergistic growth inhibition. Similarly, in a study performed by Schneider et al. (2009) pterostilbene produced synergistic effect when combined with IP6. Santos et al. (2011) demonstrated that swainsonine, found in astragalus lentiginosus, enhanced the antitumor effectiveness when combined with cisplatin, leading to a

116% survival increase. The data presented in this study demonstrated that combination treatment with both AI and pterostilbene produces synergistic growth inhibition compared with either treatment alone. *In vitro*, AI and Pt produce synergistic growth inhibition to SK-MEL-2 cells (seen from Table 1). Similarly, combination treatment of AI with Pt had enhancement effect in inducing apoptosis of melanoma cells (shown by Figure 2 and Figure 3). *In vivo*, the tumor volume was inhibited obviously when AI and Pt were used in combination (Figure 4). These results proved that AI and Pt have synergism in inhibition the growth of SK-MEL-2 cells. However, new studies are needed to confirm this apoptosis mechanism by flow cytometry assay and *in situ* detection of apoptosis. Moreover, the synergistic effect of AI and Pt also should be further demonstrated by more *in vivo* tests and clinical trials.

In summary, we have shown that AI and combination treatment of AI with Pt significantly inhibits the growth of melanoma *in vitro* and *in vivo* for the first time. Moreover, there is an enhancing antitumor efficacy by the combined administration of astragalus and pterostilbene in melanoma. Based on the results, it is concluded that AI and AI-Pt can inhibit tumor growth by inducing apoptosis. It demonstrates AI's capability as a novel and effective anti-metastatic agent in the therapy of malignant melanoma.

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