

ERK/GSK3 β signaling is involved in atractylenolide I-induced apoptosis and cell cycle arrest in melanoma cells

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Abstract. Novel agents need to be developed to overcome the limitations of the current melanoma therapeutics. Atractylenolide I (AT-I) is a sesquiterpene compound isolated from *atractylodis macrocephalae rhizoma*. Previous findings demonstrated that AT-I exhibited cytotoxic action in melanoma cells. However, the molecular mechanisms of AT-I's anti-melanoma properties remain to be elucidated. In the present study, the cell cycle-arrest and apoptosis-promoting effects as well as the ERK/GSK3 β signaling-related mechanism of action of AT-I were examined. B16 melanoma cells were treated with various concentrations of AT-I (50, 75 and 100 μ M) for 48 or 72 h. Cell cycle and apoptosis were analyzed by flow cytometry. Protein expression levels were detected by western blot analysis. AT-I treatment induced G1 phase arrest, which was accompanied by increased p21 and decreased CDK2 protein expression levels. Apoptosis was observed after AT-I treatment for 72 h, which was accompanied by activated caspase-3 and -8. AT-I treatment significantly decreased phospho-ERK, phospho-GSK3 β , c-Jun and increased p53 protein expression levels. Lithium chloride (LiCl, 5 mM), a GSK3 β inhibitor, treatment alone did not increase the apoptosis of B16 cells, while pretreatment with LiCl markedly reversed AT-I-induced apoptosis. Additionally, AT-I-induced G1 phase arrest was partially reversed by LiCl pretreatment. In

conclusion, ERK/GSK3 β signaling was involved in the apoptotic and G1 phase arrest effects of AT-I in melanoma cells.

Introduction

Malignant melanoma is a potentially lethal form of skin cancer. Although it accounts for <2% of all skin cancer cases, it is responsible for ~75% of all mortality from skin cancer (1). Two targeted therapeutic agents (ipilimumab and vemurafenib) have shown promise in the survival rates in patients with advanced melanoma (2-4). However, the majority of patients who respond to the targeted therapies eventually develop resistance and disease progression (5). Novel agents need to be developed to overcome the limitations of the current therapeutic agents.

Medical plants have been considered a valuable source of bioactive compounds for the treatment of many conditions, including cancer (6). *Atractylodis macrocephalae rhizoma* (*Baizhu* in Chinese) is a traditional Chinese medicinal herb. The extracts of *Baizhu* exhibited various pharmacological activities, such as anti-inflammation (7), anti-lipid-peroxidation (8) and antitumor activities (9,10). In a previous study, we isolated eight sesquiterpene compounds from *Baizhu* and evaluated their anti-melanoma properties (11). The MTT data demonstrated that atractylenolide I (AT-I) was one of the major active components, which displayed cytotoxic action in melanoma cells (11). We also observed that AT-I inhibited the activation of ERK in melanoma cells (11). However, the molecular mechanisms of AT-I anti-melanoma properties remain to be elucidated.

p53 is a major tumor suppressor. Increased p53 activity is associated with cell cycle arrest, through increased expression of p21 (12) and the induction of apoptosis via the intrinsic and extrinsic pathways (13). Glycogen synthase kinase-3 β (GSK3 β) has been identified as a major regulator of p53 localization and expression (14, 15). Activation of GSK3 β promoted responses to p53 including increases in the p21 expression level and caspase 3 activity (14,15). Pharmacological inhibition of GSK3 β activity produced marked reductions in the activation of Bax and caspase 3 and in cell death (14,15).

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c-Jun has been reported to directly repress p53 transcription by binding to a variant AP-1 site in the p53 promoter (16). In cells absent of c-Jun, the expression of p53 and p21 is increased, and those cells exhibit cell cycle arrest (16). Overexpression of c-Jun in cells results in decreased levels of p53 and p21, and exhibits accelerated cell proliferation (16). In melanoma cells, activation of ERK can inactivate GSK3 β , which in turn increases c-Jun stability and decreases p53 activity (17).

In the present study, the cell cycle-arrest and apoptosis-promoting effects as well as the ERK/GSK3 β signaling-related mechanism of action of AT-I were investigated.

Materials and methods

Reagents and antibodies. AT-I was isolated from *Baizhu*. The purity of the isolated AT-I was determined to be >98% by HPLC (Fig. 1). Stock solutions of AT-I (100 mM) were prepared in dimethyl sulfoxide (DMSO). Cleaved caspase-3 and -8, p21, cdk2, phospho-ERK (p-ERK, thr202/tyr204), ERK, p-GSK3 β (ser9), c-Jun and p-p53 (ser15) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Caspase-3 and -8, p53, β -actin antibodies and anti-mouse and anti-rabbit IgG antibodies (horseradish peroxidase-conjugated) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Ribonuclease (RNase A), trypsin, propidium iodide (PI) and lithium chloride (LiCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Annexin V-FITC Apoptosis Detection kit was obtained from BD Biosciences (San Jose, CA, USA).

HPLC analysis. HPLC analysis was performed in an Agilent 1100 system equipped with a diode-array detector. Solvents for HPLC analysis were HPLC grade. Experimental conditions were summarized as follows: 1 mg of AT-I was prepared in 1 ml methanol. The separation was performed on Synergi Fursion-RP C18 column (250x4.6 mm, 4 μ m) with acetonitrile-water (40:60) as the mobile phase. The column temperature was maintained at 30°C. The flow rate was 1.0 ml/min and the detection wavelength was set at 220 nm.

Cell culture. Murine melanoma B16 cells (Shanghai Branch, Chinese Academy of Sciences, Shanghai, China) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Gibco, Grand Island, NY, USA). The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂.

Determination of cell cycle distribution. The distribution of cells in various phases was determined from DNA content assessed by flow cytometry. Cells were seeded at a density of 4x10⁵ in 60-mm dish and grown overnight. Various concentrations of AT-I (50, 75 and 100 μ M) and/or 5 mM LiCl were added and the cells were incubated for 48 h. Detached and adherent cells were collected and centrifuged at 300 x g for 5 min at 4°C. Pellets were rinsed with ice-cold phosphate-buffered saline (PBS) and fixed with ice-cold 70% ethanol overnight. The cells were then stained with staining buffer (PBS containing 20 μ g/ml of PI, 100 μ g/ml RNase A, and 0.1% Triton X-100) for 30 min at 37°C in the dark. Stained cells were analyzed using a FACSCalibur™ flow cytometer (BD Biosciences).

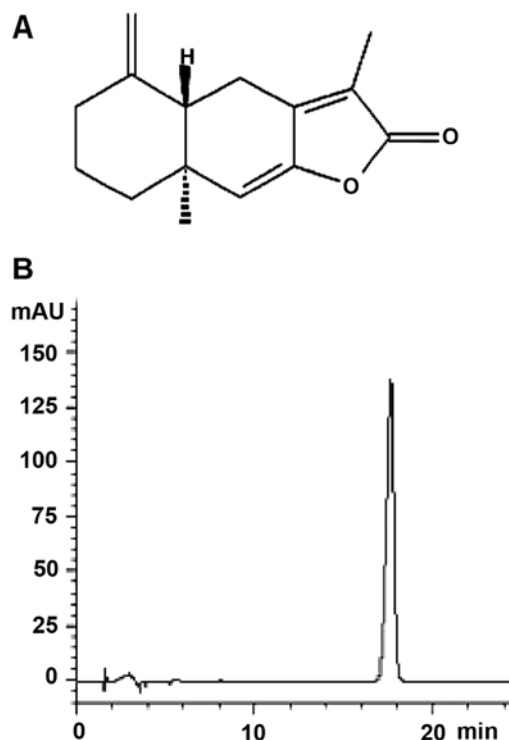


Figure 1. AT-I preparation. (A) Structure of AT-I. (B) HPLC analysis of AT-I. AT-I, atractylenolide I; HPLC, high-performance liquid chromatography.

Apoptosis analysis. Early (Annexin V⁺PI⁻) and late (Annexin V⁺PI⁺) phase apoptotic cells were monitored using an Annexin V-FITC apoptosis detection kit. B16 cells (2.5x10⁵) were grown in 35-mm dishes. Following treatment with AT-I (50, 75 and 100 μ M) and/or 5 mM LiCl for 72 h, adherent and floating cells were collected and washed with cold PBS. The cells were resuspended in binding buffer and incubated with Annexin V and PI staining solution following the manufacturer's instructions. Samples of 10,000 stained cells were analyzed using a flow cytometer (BD Biosciences).

Western blot analysis. The cells were treated as mentioned above and collected. The proteins were extracted with RIPA lysis buffer [50 mM Tris-Cl, 1% v/v NP-40, 0.35% w/v sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF and 1 mM Na₃VO₄, pH adjusted to 7.4] containing a protease inhibitor cocktail (Roche, Mannheim, Germany) for 15 min at 4°C. After centrifugation at 12,000 x g for 15 min at 4°C, the supernatant was collected and regarded as whole cell extract. The protein concentration was determined by a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of individual protein samples were separated by SDS-PAGE and then electro-transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked for 60 min with 5% skimmed milk in TBST buffer composed of 50 mM Tris (pH 7.6), 150 mM NaCl and 0.1% Tween-20 and incubated with the primary antibodies overnight at 4°C. β -actin was used as the loading control. After incubation with secondary antibodies (1:2,000), ECL detection reagents (Amersham Biosciences) were used to detect signals.

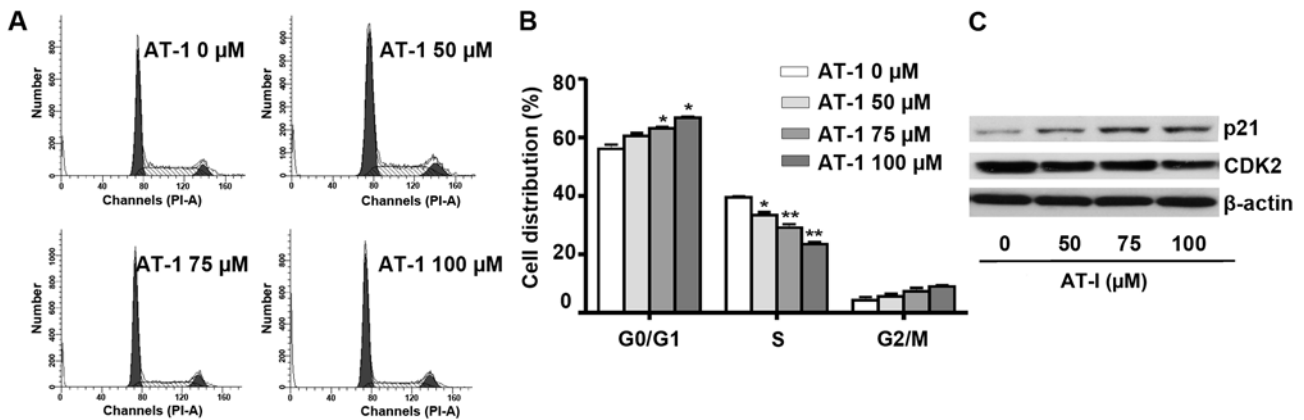


Figure 2. AT-I induced cell cycle arrest in B16 cells. (A) Representative cell cycle analysis. Cells were treated with different concentrations of AT-I (0, 50, 75 and 100 μ M) for 48 h. After fixation, the cells were stained with PI and then analyzed using a flow cytometer. (B) Statistical analysis of cell cycle data in three independent experiments. * $P < 0.05$, ** $P < 0.01$, as compared with the medium control group. (C) Western blot analysis of cell cycle-related proteins. Cell cycle-related proteins, such as CDK2 and p21, were detected by western blot analysis. The lanes were loaded with 30 μ g protein and β -actin was used as the loading control. Results shown are representative of three independent experiments. AT-I, atractylenolide I.

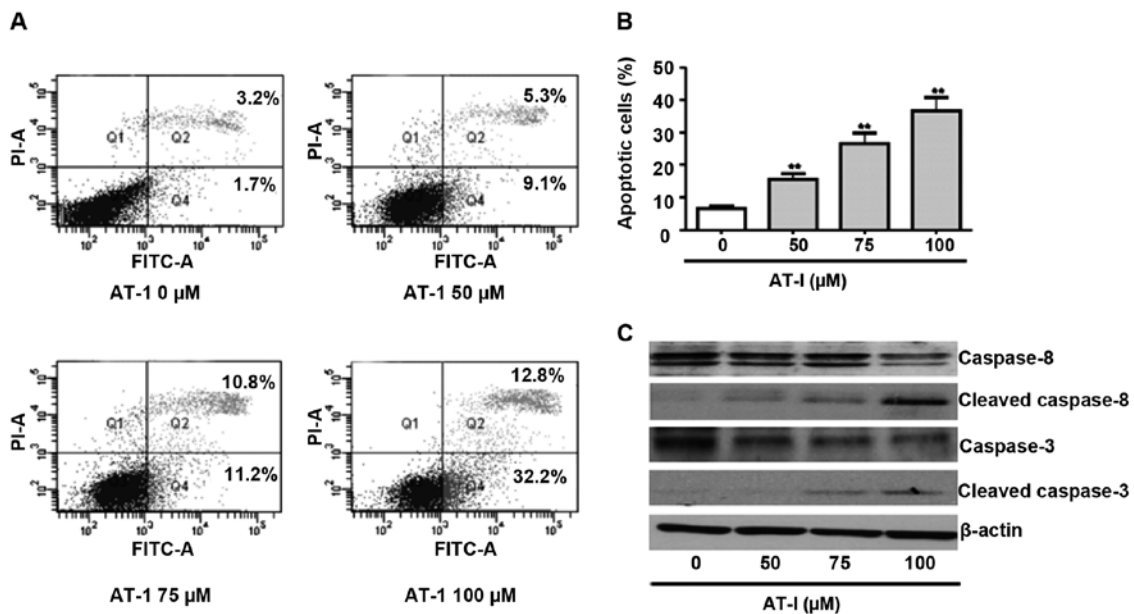


Figure 3. AT-I induces apoptosis in B16 cells. (A) Representative apoptosis analysis. Cells were treated with different concentrations of AT-I (0, 50, 75 and 100 μ M) for 72 h, stained with FITC-Annexin V and PI and then analyzed using a flow cytometer. Annexin V⁺PI⁺ are the late stage apoptotic cells (Q2) and Annexin V⁺PI⁻ are the early stage apoptotic cells (Q4). (B) Statistical analysis of apoptosis data in three independent experiments. ** $P < 0.01$, as compared with medium control group. (C) Western blot analysis of apoptosis-related proteins. Apoptosis-related proteins, such as caspase-3 and -8, and cleaved caspase-3 and -8 were detected by western blot analysis. The lanes were loaded with 30 μ g protein and β -actin was used as the loading control. Results shown are representative of three independent experiments. AT-I, atractylenolide I.

Statistical analysis. Results were presented as the mean \pm SD of three independent experiments. Data analysis was performed by one-way analysis of variance (ANOVA). For comparison of two groups, the Student's t-test was used. $P < 0.05$ was considered statistically significant.

Results

AT-I induces cell cycle arrest in B16 cells. Cell cycle distribution analysis demonstrated that AT-I treatment for 48 h at the concentrations of 50, 75 and 100 μ M caused a dose-dependent delay of cell cycle progression from G1 to S phase (Fig. 2A). Fig. 2B shows the quantified cell distributions in different

phases ($P < 0.01$ or $P < 0.05$). These results suggested that AT-I treatment induced G1 phase arrest in B16 cells. The cyclin E-CDK2 complex has a critical role in the G1/S phase transition and it can be directly inhibited by p21 (18). As demonstrated by western blot assays (Fig. 2C), AT-I treatment markedly decreased the expression levels of CDK2 and increased the expression levels of p21.

AT-I induces apoptosis in B16 cells. To investigate whether AT-I induced apoptosis, we analyzed Annexin V/PI-stained B16 cells by flow cytometry. Treatment with 100 μ M AT-I for 48 h did not induce significant apoptosis in B16 cells (data not shown), while AT-I treatment for 72 h dose-dependently

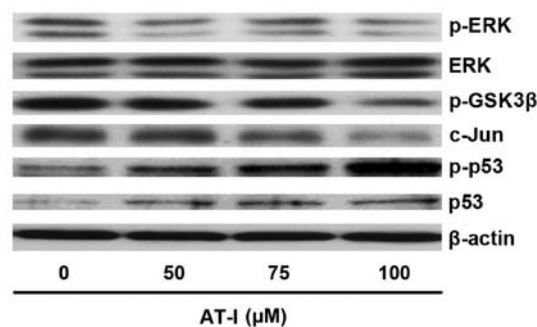


Figure 4. AT-I decreases ERK, c-Jun activation but increases GSK3 β and p53 activation in B16 cells. Cells were treated with different concentrations of AT-I (0, 50, 75 and 100 μ M) for 48 h. The total proteins were extracted and western blot assays were performed for p-ERK, ERK, p-GSK3 β , p-p53, p53 and c-Jun analyses. The lanes were loaded with 30 μ g protein and β -actin was used as the loading control. Results shown are representative of three independent experiments. AT-I, atractylenolide I; GSK3 β , glycogen synthase kinase-3 β .

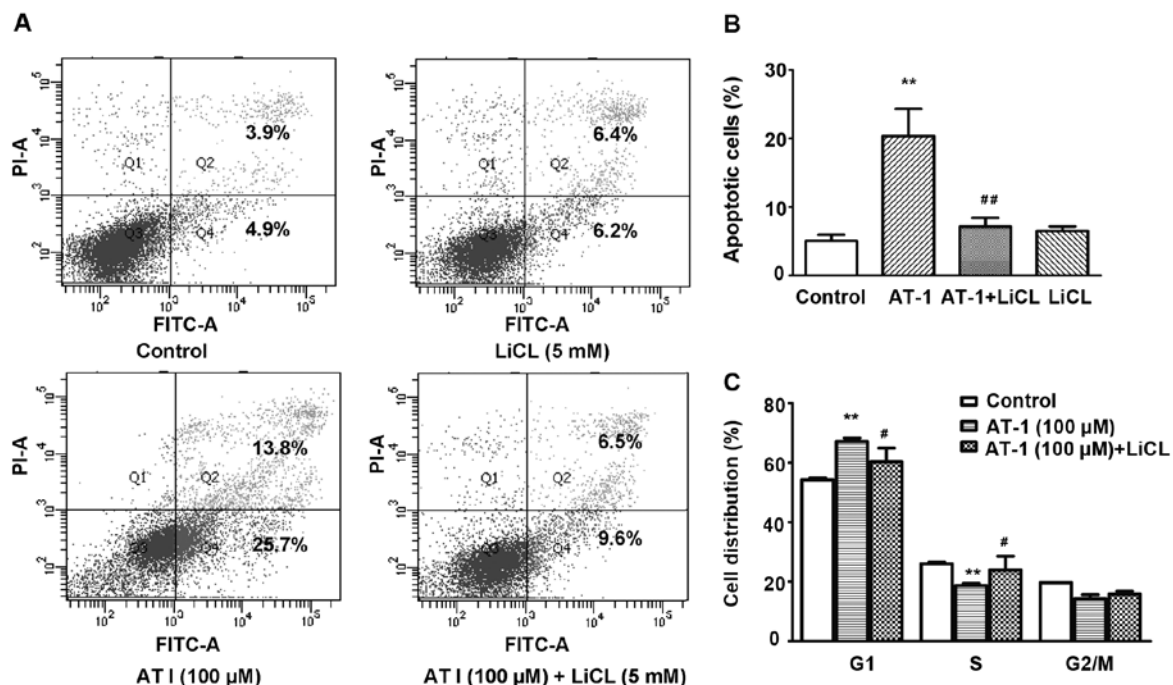


Figure 5. LiCl pretreatment reverses AT-I-induced apoptosis and cell cycle arrest. Cells were pretreated with or without 5 mM LiCl for 1 h, then treated with AT-I (0, 100 μ M) for 48 or 72 h. (A) Representative apoptosis analysis. Cells were treated for 72 h, stained with FITC-Annexin V and PI and then analyzed using a flow cytometer. Annexin V⁺PI⁺ are the late stage apoptotic cells (Q2) and Annexin V⁺PI⁻ are the early stage apoptotic cells (Q4). (B) Statistical analysis of apoptosis in three independent experiments. ** $P < 0.01$, as compared with the medium control group, ## $P < 0.01$, as compared with the AT-I alone treatment group. (C) Statistical analysis of cell cycle distribution in three independent experiments. Cells were treated for 48 h. After fixation, the cells were stained with PI and then analyzed using a flow cytometer. * $P < 0.01$, as compared with the medium control group, # $P < 0.05$, as compared with the AT-I treatment alone group. AT-I, atractylenolide I.

increased late (Q2 in Fig. 3A) and early stage apoptotic cells (Q4 in Fig. 3A). Fig. 3B shows the statistical results of total apoptotic cells from three independent experiments. Caspases-3 and -8 are situated at pivotal junctions in apoptosis pathways. Western blot assays demonstrated that treatment with AT-I markedly activated caspase-3 and -8, as evidenced by decreased expression levels of the procaspases and increased expression levels of the cleaved caspases (Fig. 3C).

AT-I decreases ERK and c-Jun activation but increases GSK3 β and p53 activation in B16 cells. In melanoma, constitutive activation of ERK can inactivate GSK3 β , which in turn increases c-Jun stability and decreases p53 activity (14,17,19).

As demonstrated by the western blot assays, AT-I treatment markedly decreased the expression levels of p-ERK and c-Jun, while the expression levels of phospho-p53 and p53 were significantly increased, as compared with the medium control (Fig. 4). Phosphorylation of GSK3 β at ser9 leads to inactivation of GSK3 β (20). Western blot assays showed that AT-I treatment activated GSK3 β , as evidenced by decreased expression levels of p-GSK3 β (ser9) (Fig. 4).

LiCl pretreatment reverses AT-I-induced apoptosis and G1 phase arrest. LiCl is a GSK3 β inhibitor (20). It has been reported that LiCl could counteract cisplatin-induced apoptosis of cancer cells (21,22). As demonstrated by the flow

cytometric analysis, LiCl (5 mM) treatment alone did not increase the apoptosis of B16 cells, while pretreatment with LiCl (5 mM) significantly reversed AT-I (100 μ M)-induced apoptosis (Fig 5A and B). The cell cycle distribution analysis showed that LiCl (5 mM) pretreatment partially reversed AT-I (100 μ M)-induced G1 phase arrest (Fig. 5C).

Discussion

Malignant melanoma is a lethal skin cancer. Although mutant BRAF-targeted therapy and immunotherapy show promising clinical response, available chemotherapeutics often carry a low response rate, tolerance, high price and/or toxicity (23). Novel agents need to be developed to overcome the limitations of the current therapeutic agents. In the present study, we reported that AT-I, isolated from the Chinese medicinal herb *Baizhu*, induced G1 phase arrest and apoptosis in B16 melanoma cells by regulating the ERK/GSK3 β signaling pathway.

Progression of cells from G1 to S phase requires the coordination of a group of regulatory proteins. Among the regulators, p53 is well characterized. As a transcriptional factor, p53 can regulate the transcription of p21, which plays a crucial role in G1 phase arrest (12,24). P21 binds to the cyclin E-CDK2 complex and inhibits the kinase activity of CDK, thereby inducing cell cycle arrest (25,26). In the present study, we observed that the G1 phase-arresting activity of AT-I was accompanied by increased expression levels of phospho-p53, p53 and p21, and decreased expression levels of CDK2, suggesting that the p53/p21 pathway may contribute to the G1 phase-arresting activity of AT-I.

Apoptosis is triggered through the extrinsic and intrinsic pathways. The extrinsic pathway involves engagement of particular death receptors that belong to the tumor necrosis factor receptor (TNF-R) family and through the formation of the death-inducing-signaling-complex (DISC), and leads to a cascade of activation of caspases, including caspase-3 and -8 (27). It is well documented that caspase 8 may be activated by p53 (13). In our investigations, AT-I treatment-induced apoptosis was associated with p53, and caspase-3 and -8 activation. These findings reveal that activation of the p53/caspase 8 pathway may be involved in the apoptosis-promoting effect of AT-I.

It has been reported that c-jun directly represses p53 transcription by binding to a variant AP-1 site in the p53 promoter (16). In the present study, AT-I treatment significantly decreased the expression levels of c-Jun. c-Jun regulates the cell cycle progression via direct transcriptional control of cyclin D1 (28). The present results show that AT-I treatment decreased the mRNA levels of cyclin D1 (data not shown). p53 and c-Jun can be regulated by GSK3 β (12,13,17). Classically, GSK3 β has been described as a key regulator of glycogen metabolism and is also known to regulate other processes, such as apoptosis, cell proliferation, cell motility and Wnt signaling (28,29). Phosphorylation at tyrosine 216 enhances the enzymatic activity of GSK3 β , while phosphorylation at serine 9 significantly decreases the activity of GSK3 β (30). In the present study, AT-I treatment significantly activated GSK3 β , which is evidenced by the decrease of p-GSK3 β (ser9). LiCl can inhibit GSK3 β activity by increasing GSK3 β phosphorylation at serine 9 (20). Recent findings suggest that LiCl may counteract the cisplatin-induced apoptosis of cancer

cells (21,22). In our investigations, pretreatment with LiCl significantly reversed AT-I-induced apoptosis. Additionally, AT-I-induced G1 phase arrest was partially reversed by LiCl. AT-I-induced decreases of cyclin D1 mRNA were also reversed by LiCl (data not shown). These findings suggest that GSK3 β signaling may be involved in the apoptosis-promoting and G1 phase-arrest effects of AT-I. In melanoma, the constitutive activation of ERK has been reported to inactivate GSK3 β (17). In the present study, AT-I treatment dose-dependently inhibited ERK activity, suggesting that AT-I may regulate GSK3 signaling through inactivation of ERK.

AT-I reduces the symptoms of patients with gastric cancer cachexia without overt toxicity, slight nausea and dry mouth are the only reported side effects (31,32). In rats, AT-I can be rapidly absorbed with a $T_{1/2\alpha}$ of 0.92 h and is eliminated gradually with a $T_{1/2\beta}$ of 9.74 h after intragastric (i.g.) administration (33), suggesting a good oral bioavailability. We have shown that AT-I can induce cell differentiation, inhibit cell migration and inhibit the phosphorylation of Akt in melanoma cells (11). Moreover, in the present study we found that AT-I induced G1 phase arrest and apoptosis and inhibited ERK/GSK3 β signaling in melanoma cells. Thus further investigations are required to develop AT-I as a pharmaceutical agent for melanoma prevention and/or treatment, although it does not exhibit potent cytotoxic effect.

In conclusion, we have demonstrated the G1 phase-arresting and apoptosis-promoting effects and revealed the ERK/GSK3 β signaling-related mechanism of action of AT-I in B16 cells. The results of the present study shed light on the molecular mechanisms of AT-I's anti-melanoma properties.

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