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Anti-Tumor Effects of Atractylenolide-I on Human Ovarian Cancer Cells

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Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to investigate the effects of Atractylenolide-I (AT-I), a naturally occurring sesquiterpene lactone isolated from *Atractylodes macrocephala Koidz*, on human ovarian cancer cells.





Material/Methods: The viability and anchorage-independent growth of ovarian cancer cells were evaluated using MTT and colony formation assay, respectively. Cell cycle and apoptosis were detected with flow cytometry analysis. The level of cyclin B1 and CDK1 was measured using qPCR and ELISA analysis. The expression of Bax, cleaved caspase-9, cleaved caspase-3, cytochrome c, AIF, and Bcl-2, and phosphorylation level of PI3K, AKT, and mTOR were determined with Western blot analysis.

Results: AT-I decreased the cell viability and suppressed anchorage-independent growth of A2780 cells. Cell cycle was arrested in G2/M phase transition by AT-I treatment, which was related to decreased expression of cyclin B1 and CDK1 in a dose-dependent manner. In addition, the treatment induced apoptosis, as shown by up-regulation of Bax, cleaved caspase-9, cleaved caspase-3, and cytosolic release of cytochrome c and AIF, and down-regulation of Bcl-2, in a dose-dependent manner. Then, the effects of AT-I on PI3K/Akt/mTOR pathways were examined to further investigate the underlying anti-cancer mechanism of AT-I, and the results showed that treatment with AT-I significantly decreased the phosphorylation level of PI3K, Akt, and mTOR.

Conclusions: This study demonstrated that AT-I induced cell cycle arrest and apoptosis through inhibition of PI3K/Akt/mTOR pathway in ovarian cancer cells. These results suggest that AT-I might be a potential therapeutic agent in the treatment of ovarian cancer.

MeSH Keywords: **Apoptosis Inducing Factor • Cell Cycle Checkpoints • Ovarian Neoplasms**

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Background

Ovarian cancer, which is the most common cause of death among women with cancer, has the highest prevalence among gynecological cancers worldwide, with more than 238 700 newly diagnosed cases and 151 900 reported deaths per year, and usually has a poor prognosis [1,2]. Platinum- and taxane-based treatment is the first-line chemotherapy for ovarian cancer, but more than two-thirds of ovarian cancer patients are still at significant risk of recurrence with this treatment [3]. Therefore, it is necessary to develop novel treatment strategies for women diagnosed with this cancer.

Natural products have aroused great interest for their high anti-tumor activity and low toxicity in recent years. Atractylenolide-I (AT-I) is a naturally occurring sesquiterpene lactone isolated from *Atractylodes macrocephala* Koidz, and has exhibited a wide range of pharmacological and biological activities, such as anti-inflammation, anti-oxidative stress, and anti-tumor effects [4–7]. Previous studies indicated that AT-I induced cell cycle arrest in G2/M phase and triggered cellular apoptosis, depending on the activation of the mitochondrial apoptotic pathway in bladder cancer cells [6], and ERK/GSK3 β signaling was shown to be involved in the apoptotic and cell cycle arrest effects of AT-I in melanoma cells [7]. In addition, a clinical study showed that AT-I improved appetite and Karnofsky performance status of gastric cancer cachexia patients, while fewer adverse effects were observed [8]. These studies indicated that AT-I is a promising anti-tumor agent. However, the effects of AT-I on the proliferation and apoptosis of ovarian cancer cells have never been explored, and the underlying mechanism needs further investigation.

In the present study, we explored the effects of AT-I on cell cycle, apoptosis, and the PI3K/Akt/mTOR signaling pathway in the A2780 ovarian cancer cell line. Our results provide further evidence of the molecular mechanisms that allow AT-I to exert its potential role as a chemotherapeutic agent in ovarian cancer.

Material and Methods

Reagents

Atractylenolide-I (AT-I) was purchased from Chengdu Best-Reagent Co. Ltd. (Chengdu, Sichuan, China). MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) was from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Primary antibodies against Bcl-2, Bax, apoptosis-inducing factor (AIF), cytochrome c, caspase-9, caspase-3, Akt, p-Akt, mTOR, p-mTOR, and β -actin were obtained from Cell Signaling Technology (Beverly, MA, USA), and primary antibodies against PI3K and p-PI3K were

obtained from Abcam Inc. (Cambridge, UK). ELISA kits for cyclin B1 and CDK1 were from Cusabio Inc. (Wuhan, Hubei, China).

Cell lines and cultures

The ovarian cancer cell line A2780 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA) at 37°C with 5% CO₂ in a humidified incubator.

Cell viability assay

The effects of AT-I on the viability of A2780 cells were detected by MTT assay. The cells (1 \times 10⁵ per well) were seeded into 96-well plates and incubated for 24 h. After treatment with AT-I (0, 12.5, 25, 50, 100 and 200 μ M) for 24, 48, and 72 h, the viability of the cancer cells was detected with MTT. We added 10 μ l MTT solution (5 mg/ml) to each well, and the mixtures were incubated for 4 h at 37°C. Then, the MTT solution was removed and 150 μ l of DMSO was added to the wells. The absorbance was measured using a Multiskan MK3 Reader (Thermo Fisher Scientific, Waltham, MA, USA) at 570 nm wavelength. Cell viability=(OD of experimental well/OD of control) \times 100%.

Colony formation assay

A2780 cells at densities of 2 \times 10⁵ cells were seeded on 6-well plates with a top layer of 0.3% agar and a bottom layer of 0.6% agar. Treatment groups were administered with AT-I for 14 days in A2780 cells. The normal growth medium was applied on top of the cell layer and changed every 3 days. Colonies were counted manually and images were captured using a Panasonic Lumix DMC-FH3 camera (Panasonic, Osaka, Japan).

Cell cycle analysis

The A2780 cells were seeded in 6-well plates (1 \times 10⁶ per well). After treatment with AT-I for 48 h, the cells were digested, centrifuged, collected, and washed with PBS, resuspended with precooled 75% ethanol, and fixed at 4°C overnight. Each sample was suspended in 450 μ l PBS, and 50 μ l PI (0.5 mg/ml) was added to the cell suspension, mixed, and incubated at 37°C for 30 min in the dark. The mixture was centrifuged to remove the supernatant, and cells were resuspended in PBS and assessed by flow cytometry (BD Biosciences, Franklin L, NJ, USA) to analyze the cell cycle distribution.

Apoptosis detection

For apoptosis analysis, Annexin V/PI staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was performed by flow cytometry (BD Biosciences, Franklin L, NJ, USA). Briefly, after

Table 1. Details of primer sequences used for qPCR.

Gene	Sequence (5'-3')	Product size (bp)
Cyclin B1	F: TCTGGATAATGGTGAATGGACA	157
	R: CGATGTGGCATACTTGTCTTG	
CDK1	F: TAGCGCGGATCTACCATACC	128
	R: CATGGCTACCACTTGACCTGT	
β-actin	F: ATAGCACAGCCTGGATAGCAACGTAC	158
	R: CACCTTCTACAATGAGCTGCGTGTG	

48 h of AT-I treatment, the A2780 cells were washed with ice-cold PBS and incubated with Annexin V/PI in the dark at room temperature for 15 min. The fluorescence of the cells was detected by flow cytometry using a FITC signal detector (FL1) and a PI signal detector (FL2). Early-stage and late-stage apoptotic cells (Ann+/PI+) were examined by fluorescence-activated cell sorting (FACS) analysis performed with a FACScan flow cytometer using Kaluza analysis software (Beckman Coulter, Fullerton, CA, USA).

RNA isolation and qPCR

Total RNA was extracted from A2780 cells (1×10^6 cells per well) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. cDNA was synthesized from 1 µg of RNA using a reverse transcription system (Bio-Rad, Hercules, CA, USA), and qPCR was performed using the iQ5 Real-Time PCR system (Bio-Rad, Hercules, CA, USA). Reaction mixtures consisted of 5 µl SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 0.5 µl primer (10 mM), 1 µl cDNA, and 3 µl ddH₂O. Thermal cycling conditions consisted of an initial denaturation step of 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing and extension at 55°C for 10 s. qPCR was performed using β-actin as an internal control to analyze cyclin B1 and CDK1 mRNA expression in A2780 cells and the primers listed in Table 1.

ELISA assay

A2780 cells were seeded in 6-well plates (1×10^5 per well) in 1 ml of medium and treated with or without AT-I for 48 h. The supernatants were collected, and then the levels of cyclin B1 and CDK1 were measured using ELISA kits according to the manufacturer's instructions.

Protein lysate preparation and Western blot analysis

Protein lysates were prepared using RIPA buffer (Beyotime Biotechnology, Jiangsu, China) supplemented with protein

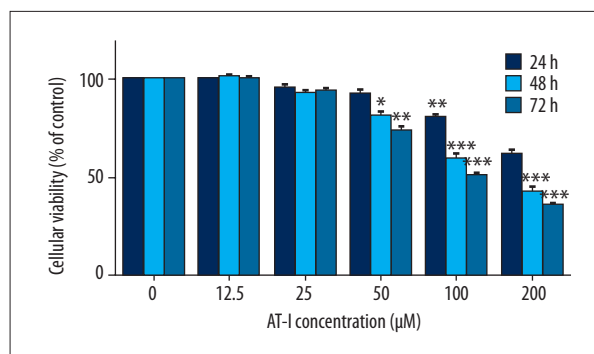


Figure 1. Inhibitory effects of AT-I on A2780 ovarian cancer cells growth. AT-I dose- and time-dependently inhibited A2780 cells growth. MTT assay was performed to evaluate growth of A2780 cells treated with AT-I at the indicated concentrations for 24, 48, or 72 h. The data are expressed as mean \pm SEM (n=3). The experiments were performed in triplicate. * P<0.05; ** P<0.01 and *** P<0.001, compared with vehicle-treated controls.

inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The cytosolic extracts were isolated using a cytoplasmic protein extraction kit (Beyotime Biotechnology, Jiangsu, China) due to determination of subcellular localization of cytochrome c and AIF. Protein samples were fractionated by 10% SDS-PAGE and transferred onto the PVDF membrane. After blocking with 5% BSA in TBST for 1 h, the cropped membranes were then incubated with a primary antibody against Bcl-2, Bax, AIF, cytochrome c, caspase-9, caspase-3 (1: 1000), Akt, p-Akt, PI3K, p-PI3K, mTOR, p-mTOR (1: 500), and β-actin (1: 2000) at 4°C overnight, followed by detection with horseradish peroxidase-conjugated secondary antibodies (Boster Biological Technology, Wuhan, China) and the ECL chemiluminescence kit (Bio-Rad, Hercules, CA, USA). The optical density of each band was determined using Image Lab 5.0 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (International Business Machines, Armonk, NY, USA) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). For continuous variables, the results are shown as mean with standard error of mean (SEM). Differences among groups were analyzed by one-way analysis of variance (ANOVA). P values less than 0.05 were considered statistically significant.

Results

Cytotoxic effects of AT-I on A2780 cells

MTT assay was used to detect cytotoxic effects of AT-I on a panel of A2780 cells. As shown in Figure 1, treatment with AT-I (12.5–200 µM) for 24, 48, or 72 h dose- and time-dependently

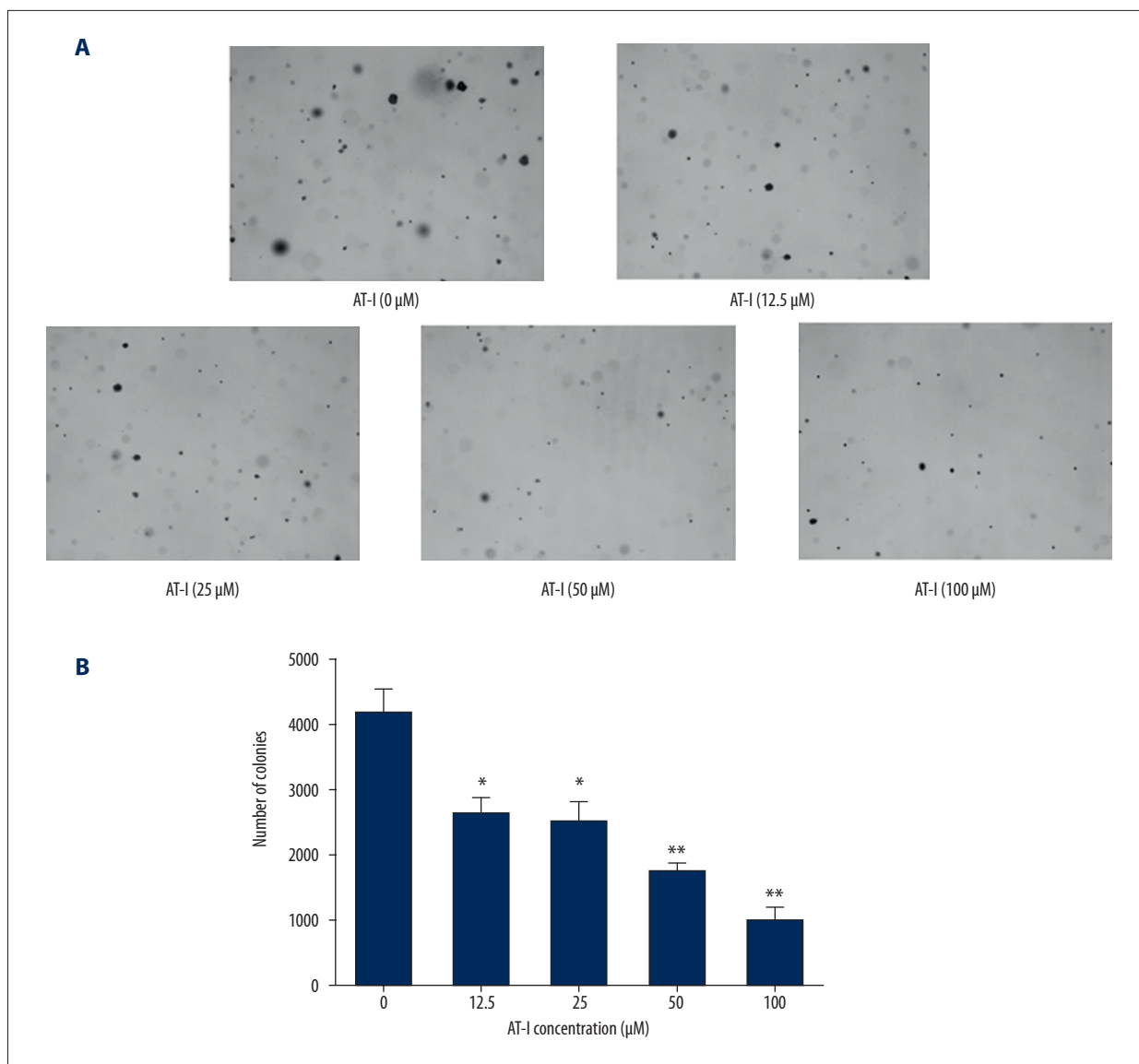


Figure 2. AT-I inhibited anchorage-independent growth of A2780 ovarian cancer cells. **(A)** A2780 cells were grown in soft agar containing AT-I (0, 12.5, 25, 50, and 100 μM) for 14 days. **(B)** Quantitative results obtained using colony formation assay. The data are expressed as mean \pm SEM (n=3). The experiments were performed in triplicate. * $P < 0.05$ and ** $P < 0.01$, compared with vehicle-treated control.

decreased the cell viability of A2780 cells compared with the vehicle-treated controls. Treatment with AT-I up to 100 μM for 24 h or 50 μM for 48 h or 72 h showed significant effects on cell growth, and treatment with AT-I (12.5–100 μM) for 48 h in A2780 cells was chosen for further detailed mechanistic investigation.

AT-I suppressed anchorage-independent growth of A2780 cells

To explore the effect of AT-I on the anchorage-independent growth of A2780 cells, the soft agar assay was used. A2780

cells were grown in soft agar containing AT-I (12.5–100 μM) for 14 days. As illustrated in Figure 2, colony formation of A2780 cells was significantly reduced by AT-I at 12.5, 25, 50, and 100 μM by 37.49%, 40.35%, 59.05%, and 76.45%, respectively. These results indicate that AT-I inhibits the anchorage-independent growth of A2780 cells in soft agar.

AT-I induced cell cycle arrest at G2/M phase

To investigate the effects of AT-I on cell cycle, PI staining and flow cytometry analyses were used to detect the cell cycle phases of A2780 cells treat with AT-I (12.5–100 μM) for 48 h.

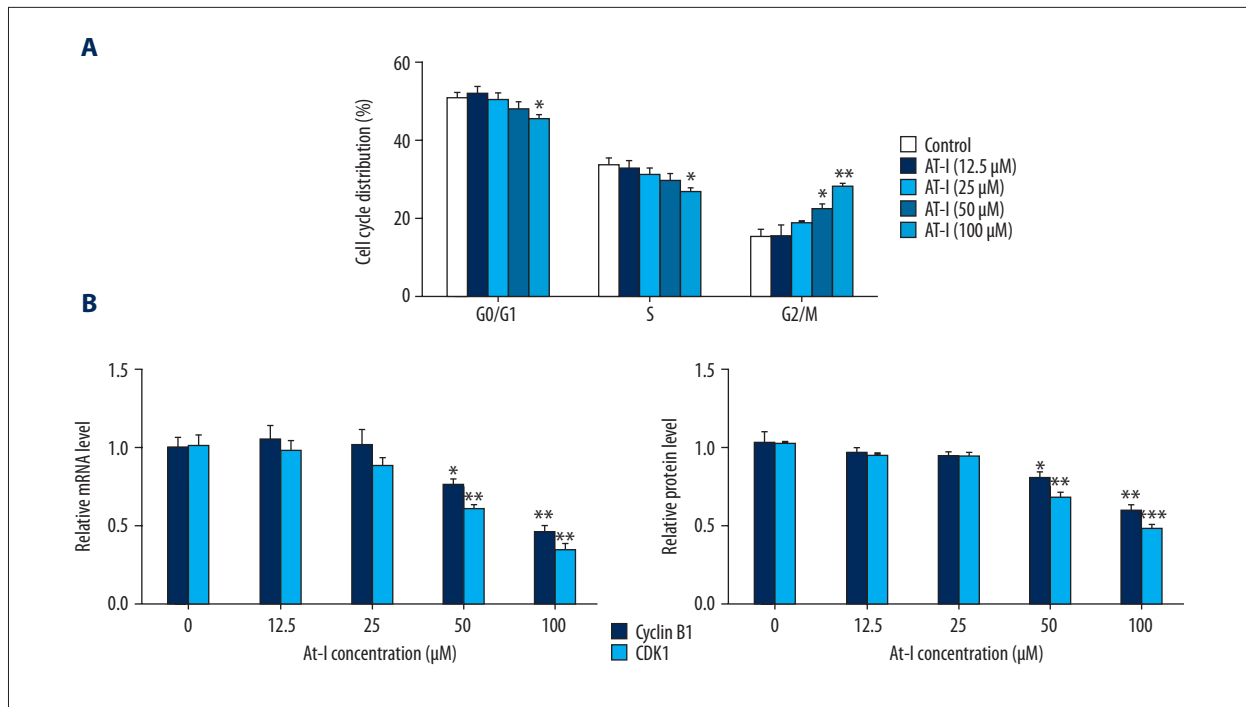


Figure 3. AT-I induced cell cycle arrest at the G2/M phase. **(A)** PI staining and FACS analysis were used to investigate the induction of G2/M phase arrest in A2780 cells treated with AT-I at the indicated concentrations for 48 h. **(B)** AT-I regulated cell cycle related factors in A2780 cells. qPCR and ELISA analysis were used to detect expression of cell cycle-related factors in A2780 cells treated with AT-I at the indicated concentrations for 48 h. The data are expressed as mean±SEM (n=3). The experiments were performed in triplicate. * P<0.05; ** P<0.01 and *** P<0.001, compared with vehicle-treated controls.

As demonstrated in Figure 3A, the percentage of A2780 cells in G2/M phase was significantly increased in the AT-I (50 and 100 μM) treatment group, while the proportion of cells in G0/G1 and S phase were decreased in the AT-I (100 μM) treatment group, compared with the control group, suggesting that AT-I induced cell cycle arrest through affecting cell cycle progression to mitosis.

To investigate the underlying mechanisms of AT-I-induced cell cycle arrest and its inhibitory effects on cell growth of A2780 cells, the expressions of cell cycle-related protein (cyclin B1 and CDK1) were examined using qPCR and ELISA. As shown in Figure 3B, AT-I dose-dependently reduced mRNA and protein levels of cyclin B1 and CDK1 in A2780 cells, confirming that AT-I induced cell cycle arrest through affecting cell cycle progression to mitosis. Altogether, our results suggest that AT-I prevented A2780 cells mitosis by down-regulation of cyclin B1 and CDK1 and cell cycle G2/M arrest, thus contributing to the inhibition of A2780 cell growth.

AT-I induced apoptosis in A2780 cells

To investigate whether AT-I induced apoptosis, we analyzed Annexin V/PI-stained A2780 cells by flow cytometry. As demonstrated in Figure 4, treatment with 0, 12.5, 25, 50, and 100 μM

AT-I for 48 h dose-dependently increased early and late stages of apoptotic cells with the increase of Ann+/PI- and Ann+/PI+ cells in a dose-dependent manner.

To investigate the underlying mechanisms by which AT-I induced apoptosis in A2780 cells, Western blot analysis was used to examine changes in the molecules of the apoptosis signaling pathways. As shown in Figure 5, the results revealed that AT-I induced up-regulation of proapoptotic protein Bax and down-regulation of anti-apoptotic protein Bcl-2, which could increase cytochrome C and AIF in the cytosol, and then activated the cytosolic caspase-9. Activated caspase 9 could further activate caspase 3, which then induced cell apoptosis. Taken together, these results suggest that AT-I can induce A2780 cells apoptosis through caspase-dependent and -independent mitochondrial pathways.

AT-I inhibited PI3K/Akt/mTOR signaling pathways

The PI3K/Akt/mTOR pathway is a major survival pathway, and its abnormal activation is frequently involved in cancer development and progression; therefore, we further investigated the effects of AT-I on the PI3K/Akt/mTOR pathway in A2780 cells using Western blot assay. As shown in Figure 6, treatment with AT-I for 48 h dose-dependently decreased the phosphorylated

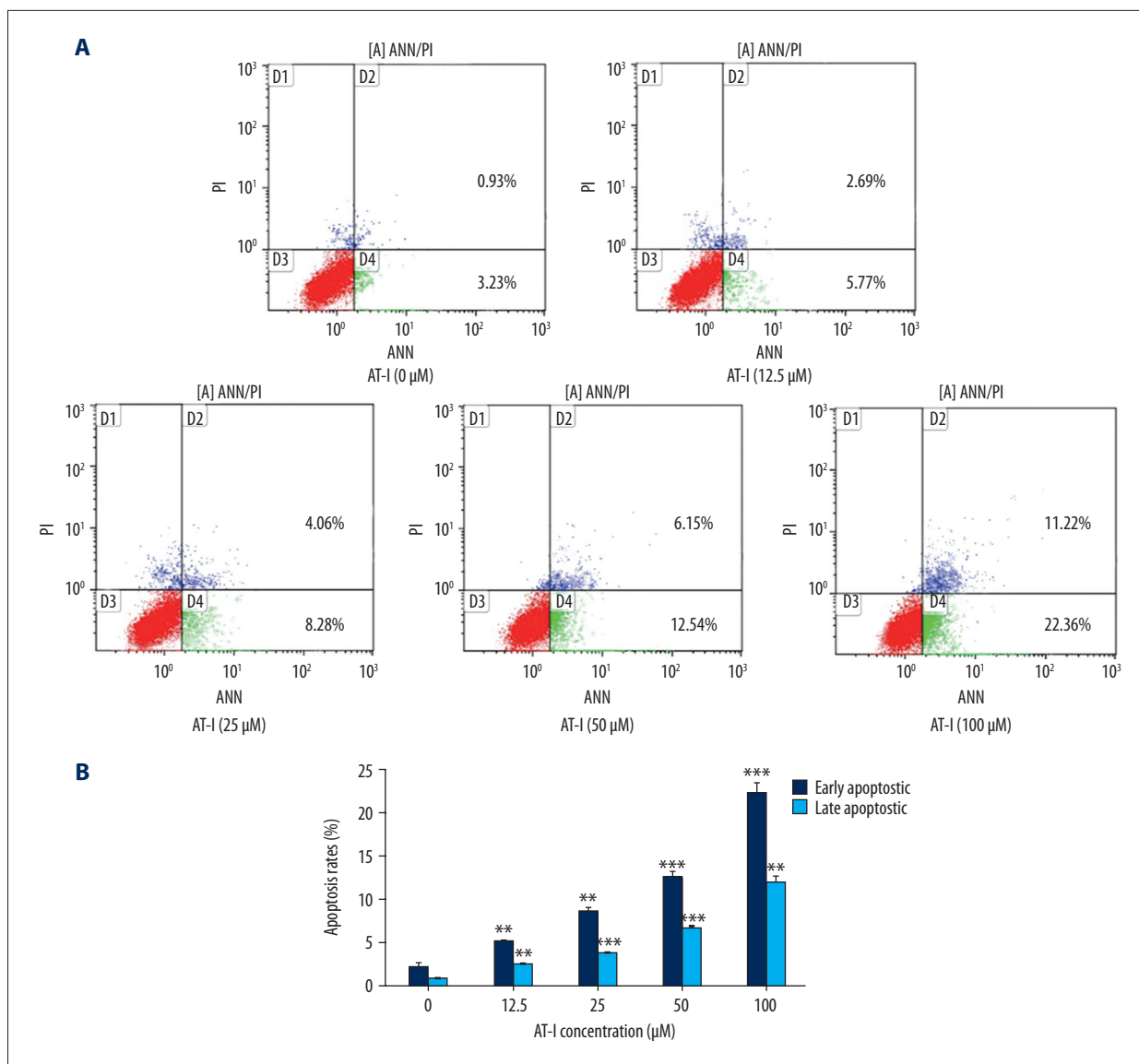


Figure 4. AT-I induced apoptosis in ovarian cancer cells. **(A)** AT-I induced apoptosis in A2780 cells in a dose-dependent manner. FACS analysis following Annexin V/PI staining was used to examine the percentage of early apoptotic cells (Ann⁺/PI⁻) and late-stage apoptotic cells (Ann⁺/PI⁺) in A2780 cells treated with AT-I at the indicated concentrations for 48 h. **(B)** Quantitative results obtained using Annexin V/PI staining. The data are expressed as mean ±SEM (n=3). The experiments were performed in triplicate. ** P<0.01 and *** P<0.001, compared with vehicle-treated control.

PI3K, Akt, and mTOR in A2780 cells. These findings suggest that AT-I has potent cytotoxicity in A2780 cells via inhibition of the PI3K/Akt/mTOR pathway.

Discussion

Recent research showed that AT-I modulated cell-mediated immunosuppression, and sensitized ovarian cancer cells to paclitaxel by blocking activation of the TLR4/MyD88-dependent pathway [9,10]. However, the anti-ovarian cancer mechanism

of AT-I is not fully understood. In our study, we investigated the effects of AT-I on cell cycle and apoptosis in ovarian cancer cells and its underlying mechanisms.

It was well recognized that cancer cells are characterized by unregulated cell cycle, and cell cycle arrest is one of the major mechanisms of many anti-tumor drugs. Due to defective G1 checkpoint during cell replication, many cancer cells depend on G2 checkpoint more than normal cells [11,12]. In addition, cell cycle progression is regulated by cyclin-dependent kinases (CDKs) and their regulatory cyclins. Cyclin B1 and CDK1 are

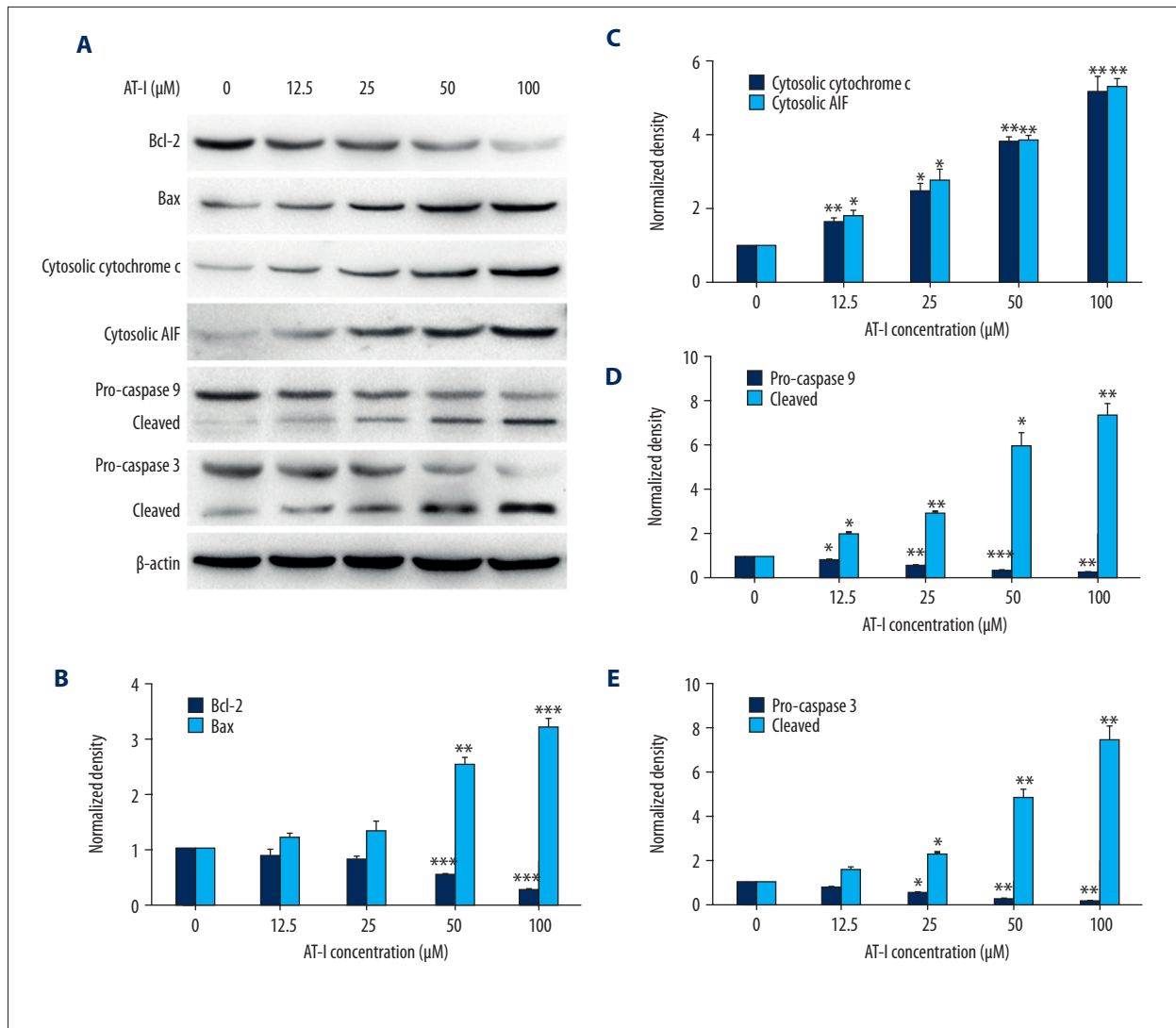


Figure 5. AT-I activated mitochondria-mediated apoptosis pathways in A2780 cells. Western blot analysis was performed to detect expression of apoptosis related factors in A2780 cells treated with AT-I at the indicated concentrations for 48 h. (A) Representative Western blots of the main factors related to mitochondria-mediated apoptotic signaling pathways. (B–E) The quantification of the expression of apoptosis-related factors. The data are expressed as mean ± SEM (n=3). The experiments were performed in triplicate. * P<0.05; ** P<0.01 and *** P<0.001, compared with vehicle-treated controls.

involved in controlling the G2/M checkpoint [13], and some anti-tumor drugs induce G2/M arrest by decreasing the expression of cyclin B1 and CDK1[14,15]. In our study, we found that treatment with AT-I leads to the G2/M arrest in A2780 cells with the down-regulation of cellular cyclin B1 and CDK1, suggesting that AT-I prevented A2780 cell mitosis by inhibition of cyclin B1 and CDK1 expression, and induction of cell cycle at G2/M phase, which contribute to the inhibitory effects.

Apoptosis plays a critical role in maintaining homeostasis through regulation of the balance between cell growth and death. It was shown that many natural compounds suppress the proliferation of cancer cells through induction of

apoptosis [16,17]. The induction of apoptosis is a common mechanism proposed for the cytotoxic effects of anti-cancer drugs extracted from herbal medicine [18]. Therefore, apoptosis has become the major target for most anti-cancer agents. In the mitochondrial apoptotic pathway, interactions among Bcl-2 family proteins could regulate mitochondrial membrane permeabilization [19], which is critical to activate the inherent apoptosis pathway through releasing of cytochrome c and AIF from mitochondria into cytoplasm, and then induction of caspase-dependent and independent cascades. In our study, Western blot assay was used to examine the effects of AT-I on related factors of the mitochondrial apoptosis pathway in A2780 cells. AT-I can induce up-regulation of Bax, cleaved caspase-9,

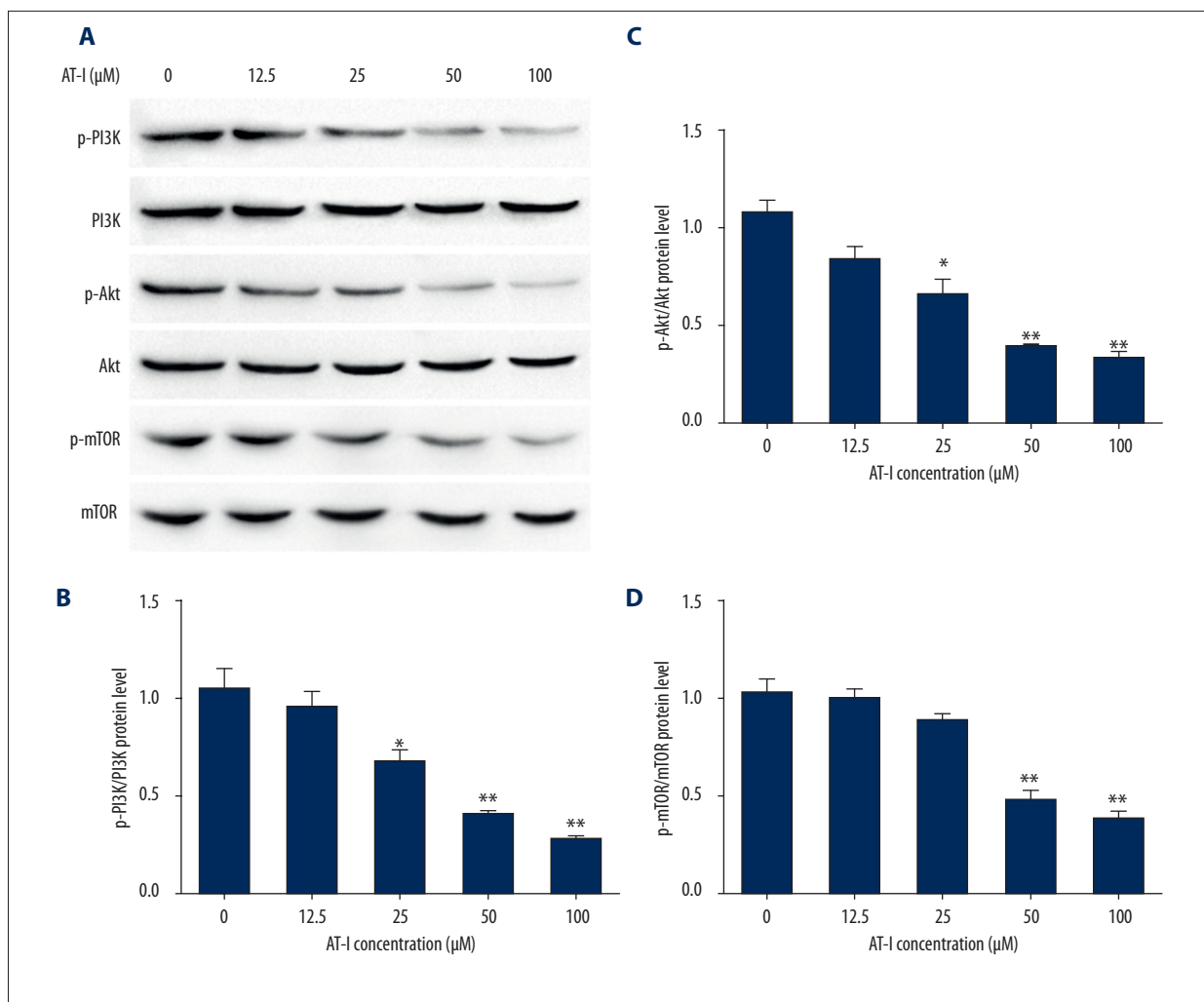


Figure 6. AT-I modulated PI3K/Akt/mTOR signaling pathway in A2780 cells. The A2780 cells were treated with AT-I at the indicated concentrations for 48 h, and then collected for Western blot analysis. **(A)** Representative Western blot of the phosphorylated proteins of PI3K/Akt/mTOR pathway. **(B–E)** The quantification of level of the phosphorylated proteins. The data are expressed as mean \pm SEM (n=3). The experiments were performed in triplicate. * P<0.05 and ** P<0.01, compared with vehicle-treated control.

cleaved caspase-3, cytosolic release of cytochrome C and AIF, and down-regulation of Bcl-2. These findings suggested that the anti-cancer activity of AT-I is related to the induction of apoptosis via activation of mitochondrial caspase-dependent and -independent cascades in A2780 cells.

The PI3K/Akt/mTOR pathway is critical to regulation of normal cell physiology and cancer proliferation, tumorigenesis, and metastasis, and activation of the pathway is beneficial to the survival of cancer cells through activation of its downstream genes that are closely related to cell cycle and apoptosis [20,21]. Recent research showed that the PI3K/Akt/mTOR pathway is involved in cell proliferation and apoptosis, and inhibition of this pathway can result in induction of cell cycle arrest and apoptosis in cancer cells [22,23]. Recent clinical studies

have reported that activation of the PI3K/AKT/mTOR pathway correlates with tumor progression and reduced patient survival [24]. Various genetic alterations that activate PI3K/Akt/mTOR signaling have been found in ovarian cancer [25–28]. Therefore, inhibitors of this pathway are currently being evaluated as treatment strategies for ovarian cancer [29]. To further investigate the potential anti-tumor mechanisms of AT-I, we investigated the effects of AT-I on PI3K/Akt/mTOR pathways in A2780 cells. Our results demonstrate that AT-I can decrease PI3K, Akt, and mTOR phosphorylation, suggesting that the anti-tumor effects of AT-I are associated with inhibition of the PI3K/Akt/mTOR signaling pathway.

Conclusions

In this study, we demonstrated that AT-I isolated from *Atractyloides macrocephala Koidz* can induce cell cycle arrest

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and apoptosis through inhibition of the PI3K/Akt/mTOR signaling pathway in A2780 cells. Our results suggest that AT-I may be as a potential agent for the treatment of ovarian cancer.