

Andrographolide inhibits proliferation and induces cell cycle arrest and apoptosis in human melanoma cells

GUO LIU and HAIHAN CHU

Department of Burns and Plastic Surgery, Jining First People's Hospital, Jining, Shandong 272011, P.R. China

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Abstract. Andrographolide (Andro), a natural compound isolated from *Andrographis paniculata*, has been demonstrated to have anticancer efficacy in several types of tumors. In the present study, the anticancer effects and mechanism of Andro in human malignant melanoma were investigated. Cell viability analysis was performed using an MTT assay and the effect of Andro on the cell cycle and apoptosis of human malignant melanoma cells was determined by flow cytometry. Western blot analysis was performed to evaluate the protein expression levels of human malignant melanoma cells following treatment with Andro. The results revealed that Andro potently inhibited cell proliferation by inducing G2/M cell-cycle arrest in human malignant melanoma C8161 and A375 cell lines. In addition, treatment with Andro induced apoptosis, which was associated with the cleavage of poly(adenosine diphosphate-ribose) polymerase and activation of caspase-3. It was observed that Andro induced activation of the c-Jun N-terminal kinase and p38 signaling pathway, which may be connected with cell cycle arrest and apoptosis. In conclusion, the results demonstrated that Andro may be a promising and effective agent for antitumor therapy against human malignant melanoma.

Introduction

Melanoma is a malignant tumor of melanocytes and is considered to be the most invasive and dangerous cutaneous cancer (1). The median 5-year survival rate is <5% following metastasis and the incidence of melanoma has increased over the past few decades (2). Traditional therapies including surgery, chemotherapy and radiation have not significantly increased in overall survival for these patients over the past 10 years (3,4). Patients with metastasis or recurrence present

a formidable challenge despite new therapeutic treatments, such as immunotherapy and molecular-targeted chemotherapy (5-7). Therefore, it is necessary and urgent to develop new strategies for the patients with melanoma.

Traditional Chinese herbal medicines have been discovered to have excellent anticancer activity in recent decades (8-11). Andrographolide (Andro), the active ingredient of the traditional Chinese medicine *Andrographis paniculata*, has been used primarily for analgesic (12). It has been proved that Andro possess various biological activities such as anti-inflammation, anti-infection, immune system regulation, anti-cardiovascular disease, and anticancer effects (13-15). Previous studies have shown that Andro exhibited potential antitumor activity in various malignancies, including gastric cancer (16), chondrosarcoma (17) and colorectal cancer (18). However, whether Andro suppresses the growth of human melanoma cells and its potential molecular mechanisms were still not well investigated.

In the present study, we evaluated that Andro can effectively inhibit the proliferation of melanoma cells by causing G2/M cell cycle arrest, and lead to cell death by inducing apoptosis. Furthermore, the underlying molecular mechanisms were discussed by JNK and p38 signaling pathways.

Materials and methods

Cells and reagents. Human malignant melanoma A375 and C8161 cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5% CO₂. Andrographolide (MF: C₈H₈O₄, MW: 168.15, purity >98%) was purchased from Shanghai Yuanye Biotechnology, Co., Ltd. (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 80 mM. Antibodies against cleaved-PARP, cleaved-caspase-3, phospho-JNK, JNK, p38, phospho-p38, and GAPDH were all obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell viability assay. Inhibition of cell proliferation by Andro was detected using the 3-(4,5-dimethylthiazole-2-yl)-2,5-bisphenyl tetrazolium bromide (MTT) assay. Briefly, cells were trypsinized and plated into 96-well plate at a density of

Correspondence to: Dr Haihan Chu, Department of Burns and Plastic Surgery, Jining First People's Hospital, 6 Jiankang Road, Jining, Shandong 272011, P.R. China
E-mail: beaution@126.com

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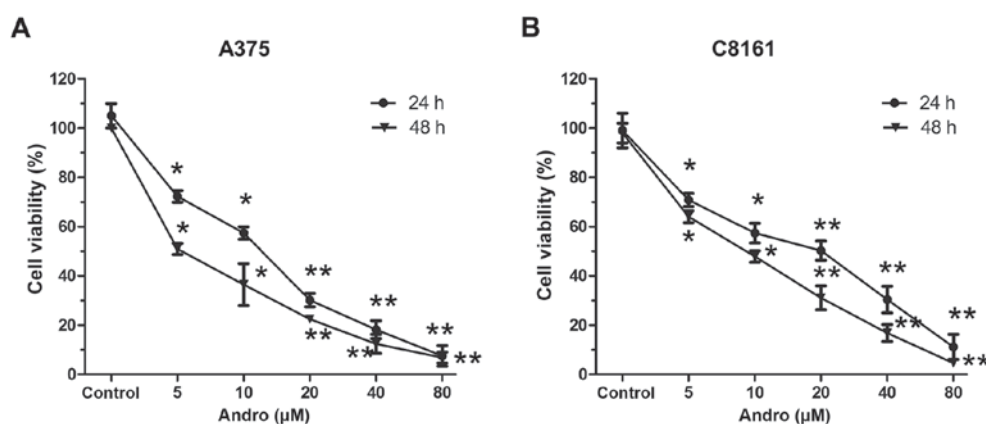


Figure 1. Andro inhibited cell proliferation in human melanoma cell lines. (A) A375 and (B) C8161 human melanoma cells were treated with different concentrations of Andro for 24 and 48 h, then a MTT assay was performed. Each experiment was repeated in triplicate. * $P<0.05$, ** $P<0.01$ vs. the control group. Andro, andrographolide.

5×10^3 cells/well and incubated overnight at 37°C in a humidified incubator, then treated with fresh medium containing various concentration of Andro (5, 10, 20, 40, and $80 \mu\text{M}$). And the cells were incubated for 24, 48 h. Subsequently, final concentration of 0.5 mg/ml MTT was added directly to and incubated for 4 h at 37°C . The plates were depleted and a total of $100 \mu\text{l}$ of DMSO was added to each well, and the optical density was measured at 490 nm using microplate reader iMark (Molecular Devices, LLC, Sunnyvale, CA, USA). Data represented the mean of five replicates. Three independent experiments were carried out in triplicate.

Cell cycle analysis by flow cytometry. To examine whether Andro affects cell cycle distribution, we used flow cytometer with PI/RNase staining buffer (BD Biosciences, Mountain View, CA, USA). In brief, 5×10^5 cells were seeded in 6-well plates and incubated with Andro for 24 h at 37°C . And then the cells were collected and fixed with 70% ethanol at -20°C overnight. The cells were washed and resuspended in cold PBS and followed by staining with 10 mg/ml RNase and 1 mg/ml propidium iodide for 30 min at 37°C . Next, the samples were detected on Accuri C6 (BD Biosciences) and the data were analyzed by ModFit LT software (FACSCalibur).

Flow cytometric analysis of apoptosis. To evaluate apoptosis induction, a FITC-Annexin apoptosis detection kit (BD Biosciences, Sparks, MD, USA) was used. Cells were seeded in 6-well plates and treated with or without Andro for 24 h. Apoptotic cells were measured by using Annexin V-FITC/PI double staining. Briefly, cells were collected and washed with ice-cold PBS, and then resuspended in $500 \mu\text{l}$ of binding buffer. These cells were stained with $5 \mu\text{l}$ of Annexin V/FITC solution and $10 \mu\text{l}$ propidium iodide (PI) (1 g/ml). Cells were then incubated for 15 min at 37°C in the dark. The samples were detected by the Accuri C6.

Western blot analysis. Cells were cultured in 6-well plates and treated with as indicated for 24 h. The cell were washed twice with cold PBS solution and then resuspended in radioimmuno-precipitation assay (RIPA) lysis buffer with phosphorylase and protease inhibitor. The protein concentration was determined

using using BCA Protein Assay kit and equalized before loading. Equivalent amounts of total protein ($25\text{--}50 \mu\text{g}$) were separated by SDS-PAGE and transferred onto a PVDF membrane (0.45 mm ; Millipore, Bedford, MA, USA). Following blocking with 5% fat-free milk for 60 min, membrane was blotted with the following primary antibodies as follows: Cleaved-PARP, cleaved-caspase-3, phospho-JNK, JNK, p38, phospho-p38, and GAPDH at 4°C overnight. Horseradish peroxidase-linked anti-mouse or anti-rabbit IgG was then used as secondary antibody. And cells were incubated with horseradish peroxidase-linked anti-mouse or anti-rabbit IgG ($1:5,000$ dilution) for 1 h. Specific antibody binding was visualized using ECL (Millipore, Plano, TX, USA) and digitalized by scanning.

Statistical analysis. All data represent at least three independent experiments and were expressed as mean \pm standard deviation (SD). The statistical differences were calculated by one-way ANOVA analysis of variance with Dunnett's test or unpaired Student's t-test. All statistical analyses were performed using SPSS 19.0 software and significant difference was analyzed by Duncan's multiple-range test. (SPSS Inc., Chicago, IL, USA). P -values <0.05 were considered to indicate a statistically significant difference.

Results

Cell growth inhibition of Andro on human malignant melanoma cell lines. To investigate the antiproliferative activity of Andro, A375 and C8161 cells were treated with different concentration of Andro for 24 and 48 h (Fig. 1). The cell viability was measured by MTT assay. The IC_{50} values of Andro for A375 were $23.08 \mu\text{M}$ (24 h), $12.07 \mu\text{M}$ (48 h), while the IC_{50} values for C8161 were $20.31 \mu\text{M}$ (24 h), $10.92 \mu\text{M}$ (48 h). These results suggest that Andro exhibits potent anti-proliferation effects in malignant melanoma cells in a dose- and time-dependent manner.

Andro induces cell cycle G2/M arrest in melanoma cell lines. To gain insights into the mechanism by which Andro inhibits cell proliferation, we examined the effect of Andro on the cell cycle progression. As shown in Fig. 2, in

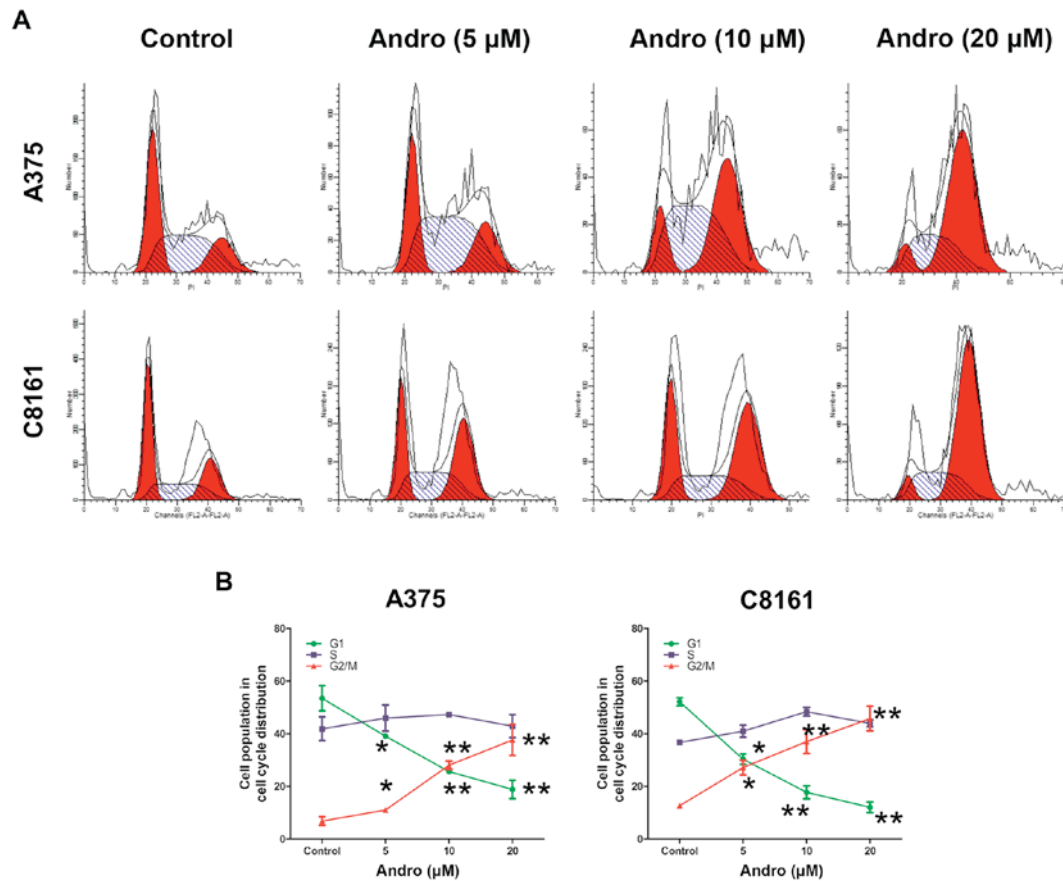


Figure 2. Andro induced cell cycle arrest at G2/M phase in human melanoma cells. (A) Human melanoma cells were treated with dimethylsulphoxide or various concentrations of Andro for 24 h. The cells were collected, stained with PI and the percentage of cell cycle distribution was analyzed by flow cytometry. (B) The percentage of cell cycle distribution at G1, S, and G2/M phases is shown as the mean \pm standard deviation from three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. the control group. Andro, andrographolide; PI, propidium iodide.

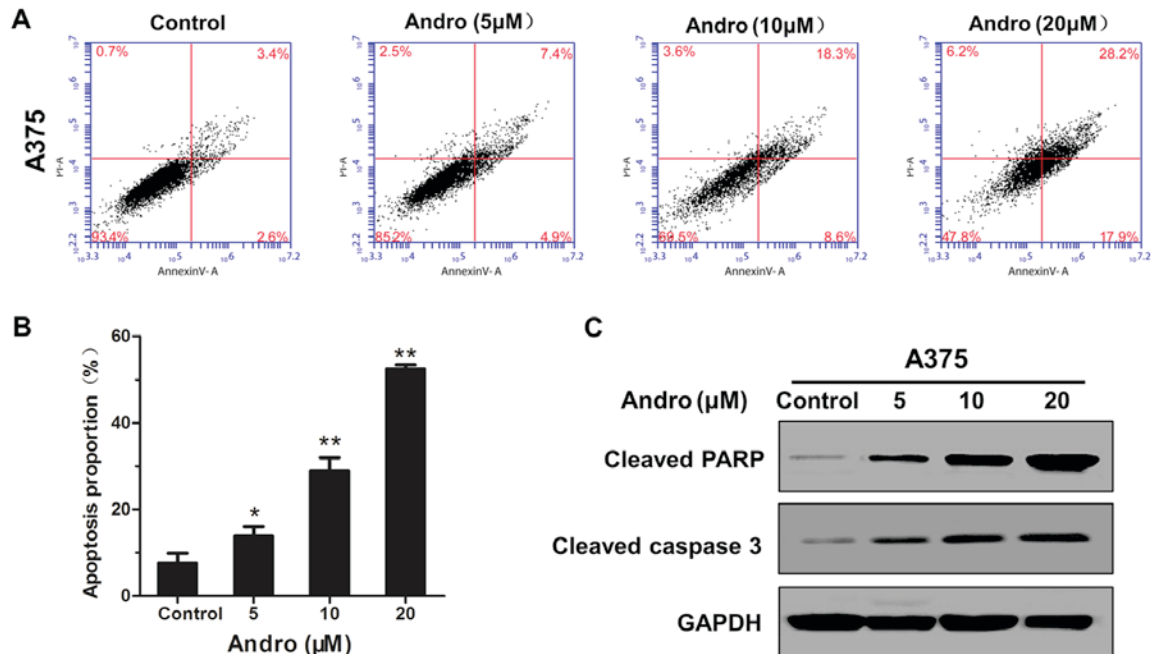


Figure 3. Andro induced apoptosis in A375 cells. (A) Apoptosis analysed by flow cytometry following Annexin V-FITC and PI staining. A375 cells were treated with Andro at the indicated doses for 24 h, labeled with Annexin V-FITC and PI, then analyzed by flow cytometer. (B) The histograms indicate that the percentage of total apoptosis. The percentage of apoptosis cells is shown as mean \pm standard deviation from three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. the control group. (C) Western blot analysis was used to detect the protein level of cleaved PARP and caspase-3. A375 cells were treated with Andro at the indicated doses for 24 h. Cell lysates were prepared and the levels of cleaved PARP and cleaved-caspase-3 were determined by western blot analysis. PI, propidium iodide; FITC, fluorescein isothiocyanate; Andro, andrographolide; PARP, poly(adenosine diphosphate-ribose) polymerase.

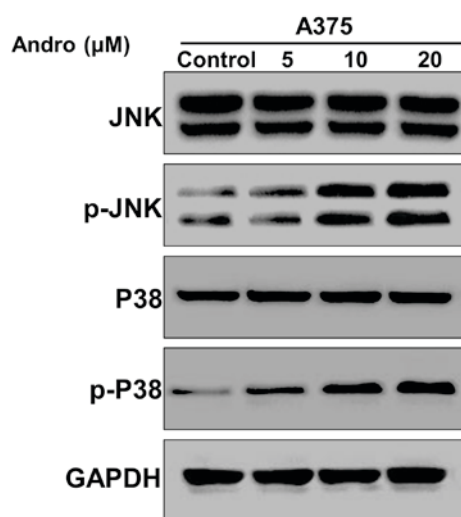


Figure 4. Andro activates the JNK and p38 signaling pathway. A375 cells were treated with Andro at the indicated doses for 24 h. The expression of p-JNK, JNK, p-P38 and P38 were analyzed by western blotting. The amount of phosphorylation of JNK and p-P38 were increased in Andro treated cells. Andro, andrographolide; JNK, c-Jun N-terminal kinase; p, phosphorylated.

comparison to DMSO-treated cells, a significant increase of cells in G2/M phase was observed after Andro treatment, while a corresponding decrease in G0/G1 phases was observed after Andro treatment. These data showed that Andro induces G2/M phase arrest.

Andro induces apoptosis in melanoma cell lines. To examine whether the cell growth inhibition induced by Andro is also dependent on apoptosis, Andro-treated cells were analysed by Annexin V-FITC/PI double staining. As shown in Fig. 3A and B, in comparison to DMSO-treated cells, Andro resulted in a significant increase of the proportion of apoptosis the number of apoptotic cells in a dosedependent manner. Western blot analyses showed that cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase-3 were increased after treatment with Andro for 24 h (Fig. 3C). Overall, these results clearly indicate that Andro provokes caspase-dependent apoptosis in melanoma cell lines.

Andro activates JNK and p-P38 signaling pathway. Recent studies have suggested JNK signaling pathway plays important roles in many cellular events, such as regulating cell cycle, cell apoptosis and autophagy (19,20). To further elucidate the effect of Andro on JNK signal pathway, we measured the expression of JNK and p-P38 genes. As shown in Fig. 4, phosphorylation of JNK and p-P38 were induced in a dose-dependently manner after Andro treatment. These results clearly indicate that Andro activated the JNK and p-P38 signaling pathway.

Discussion

Melanoma is one of the most aggressive and mortal cancers that occurs frequently with a significant contribution of environmental factors to its etiology (21,22). Traditional therapies include cryotherapy, surgery, and chemotherapy, which is difficult to accord with the clinical requirement (23,24).

Some nonsurgical treatments are usually limited to auxiliary treatment. Therefore, it is urgent to search for natural drug to further improve the outcome of melanoma patients. Andro, one of the major components of *Andrographis paniculata*, has been used as an effective, safe and antitumor drug for several centuries (25). In the present study, we revealed that Andro could inhibit proliferation of human melanoma cell lines through inducing G2/M cycle arrest and cell apoptosis. Also, we found that Andro simultaneously induces apoptosis by activating JNK pathway.

Imbalance of cell cycle regulation is one characteristic of cancer. Many studies have highlighted that induction of cell cycle arrest especially G2/M cycle phase may be an effective anticancer therapy (9,26). Many anticancer agents inhibit tumor cell proliferation by causing G2/M cycle arrest, such as celastrol (27), ophiopogonin D (26). The G2 checkpoint regulates cell caryomitoses when DNA is destroyed, which provides repair opportunities for damaged cells (28,29). Our studies showed that Andro decreased the cell proportion in G0/G1 and S phases while Andro induced G2/M phase arrest in human malignant melanoma A375 and C8161 cell lines. However, the underlying mechanism need to be further explored.

Previous studies have showed that there are two main apoptotic pathways in cell apoptosis: The extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (30,31). The apoptotic pathway is triggered by activation of caspase-3 and results in DNA fragmentation and the cleavage of PARP (32,33). Meanwhile, Apoptosis of cells can further influence cell proliferation. In the current study, flow cytometry with Annexin V/PI staining suggested that Andro induced apoptosis in human malignant melanoma A375 cells. Accordingly, the protein expression of cleaved-PARP and cleaved-caspase-3 were all remarkably increased after treatment with Andro. These data indicated that Andro induced cell apoptosis.

In addition, we explored the effect of Andro on the upstream pathways. Considerable evidence has delineated that c-Jun N-terminal kinases (JNKs) is an important intracellular signaling pathway of MAPKs family and are involved in a wide spectrum of cell physiology, such as cell proliferation, cycle arrest, apoptosis and autophagy (34,35). JNK has three subtypes, JNK1, JNK2, and JNK3. The function of JNK is intricate, which may be depending on cell subtype, external stimulus condition (36). In our current study, we found that treatment with Andro induced a significant increase in JNK and p-p38 phosphorylation in human malignant melanoma A375 cells, which may be related to cell cycle arrest and apoptosis.

In conclusion, our study elucidated that antitumor effects of Andro on human malignant melanoma and the underlining molecular mechanisms. We also demonstrated Andro could inhibit cell proliferation by causing G2/M phase arrest, and cell apoptosis. In addition, we also demonstrated that Andro activated JNK and p38 signaling pathway. Further investigations are needed to comprehensively explore the molecular mechanism of Andro in our future study, which may better understand the function of Andro on melanoma. To conclude, our findings suggests that Andro is a novel anticancer drug candidate which can be used as a potential operative agent for malignant melanoma patients.

Acknowledgements

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