Original Article

Asiatic Acid Induces Mitochondrial Apoptosis via Inhibition of JAK2/STAT3 Signalling Pathway in Human Osteosarcoma

(osteosarcoma / asiatic acid / mitochondrial apoptosis / JAK2/STAT3/MCL-1)

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Abstract. Osteosarcoma (OS), a severe malignant bone tumour, usually occurs in adolescents and children and has a poor prognosis. Asiatic acid (AA), an active component isolated from *Centella asiatica* (L.) Urb., exhibits appreciable anti-oxidant and anti-tumour activities. So far, the effects and underlying mechanisms of AA against OS have not been clarified. Here, we explored the anti-tumour effects of AA against human OS and the involved mechanism mediating its actions. To evaluate effects of AA on the cell proliferation of human OS cells, cell viability and colony formation assays were performed. Flow cytometry was used to evaluate apoptosis in OS cells exposed to AA and mitochondrial membrane potential. Western blotting and RT-PCR were applied to determine expression of the relevant proteins and their mRNA levels. Our explorations showed that AA inhibits proliferation of human OS cells in a concentration- and time-dependent manner, and induces apoptosis of OS cells by the intrinsic (mitochondrial) pathway. Importantly, we found that inhibition of the AA-induced phosphorylation of JAK2/STAT3 signalling molecules and the decrease in MCL-1 contributed to the anti-tumour efficacy of AA. Collectively, our results suggest that AA could evoke mitochondrial-induced apoptosis in human OS cells by suppression of the JAK2/STAT3 pathway and MCL-1 expression. These results strongly demonstrate that AA could be a potential anti-tumour agent for OS treatment.

Introduction

Osteosarcoma (OS) is the commonest malignant bone tumour found in adolescents and children, which is usually discovered in the metaphyseal region of the long bones of the limbs (Zhao et al., 2020). Due to the similar symptoms of OS with traumatic pain and swelling, the majority of OS patients are clinically diagnosed under the advanced stages with the high incidence of metastasis (Bhat et al., 2017). Additionally, conventional cytotoxic chemotherapeutic drugs applied for the treatment of OS are frequently concomitant with severe side effects. Nearly half of the OS patients undergoing conventional therapies suffer side effects such as secondary malignancies, infertility, chronic renal failure, neurological toxicity, etc. (Jaffe, 2009; Yu et al., 2015; Kavalar et al., 2016). Therefore, seeking alternative drugs or treatment approaches for effectively suppressing OS progression are urgently required.

Traditional Chinese medicines (TCMs) have been widely used in China as well as some other Asian countries such as Japan and Korea (Yan et al., 2017). Natural compounds in TCMs possess reliable pharmacological effects and powerful clinical efficacy (Song et al., 2016).

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Abbreviations: AA – asiatic acid, C-A1 – coumermycin A1, Cyt-c – cytochrome c, JAK – Janus kinase, MTT – 3-(4,5)-2,5-diphenyltetrazolium bromide, MMP – mitochondrial membrane potential, OD – optical density, OS – osteosarcoma, STAT3 – signal transducer and activator of transcription 3, TCMs – traditional Chinese medicines.

Hence, it is worth noting that natural compounds in TCMs serve as a valuable resource for the development of efficacious anti-tumour drugs. Asiatic acid (AA, Fig. 1A), a natural triterpenoid saponin identified initially from *Centella asiatica* (L.) Urb, has been used in Asia for thousands of years and also consumed in Europe and America as a dietary supplement for promoting health (Razali et al., 2019; Hong et al., 2020). Recent reports have shown that AA exerts significant anti-tumour, anti-inflammatory, anti-oxidant and neuroprotective activities with low toxicity (Nagoor Meeran et al., 2018; Sharma et al., 2018; Islam et al., 2020). Numerous studies have demonstrated the anti-tumour effects of this compound on several tumours (Islam et al., 2020). Illustratively, AA could inhibit proliferation and invasion of breast cancer cells MDA-MB-231 through down-regulating the PI3K/AKT signalling pathway (Gou et al., 2020). AA also showed synergistic effects with naringenin, which could suppress melanoma and lung carcinoma growth by modulating the natural killer cell immunity function (Lian et al., 2018). However, the biological effects and the potential mechanisms of action of AA in OS have not yet been investigated.

Signal transducer and activator of transcription 3 (STAT3), a significant member of the STAT family, is closely associated with cell proliferation and apoptosis, as well as intracellular signal transduction of various tumours (Lee et al., 2019). Abnormal activation of STAT3 could lead to the progression and development of human tumours (Siveen et al., 2014). Notably, STAT3 is a significant regulator protein controlling MCL-1 expression, which is essential for mitochondrial apoptosis (Jo et al., 2019). The Janus kinase (JAK) family is composed of four members, i.e., Tyk2, JAK1, JAK2, and JAK3, of which JAK2 is the most closely related to STAT3 (Levy and Darnell, 2002). Activated JAK2 could phosphorylate its targets such as STAT3 (Hou et al., 2002). The JAK2/STAT3 signalling pathway is highly related to the progression of multiple types of malignant tumours, such as gastric cancer, oesophageal cancer and colorectal cancer (Girisa et al., 2019). Reports have revealed that abnormal induction of the JAK2/STAT3 signalling pathway can boost proliferation, migration and invasion of OS cells, and promote the OS tumour development and progression (Chen and Chen, 2020). In addition, natural compound pterostilbene inhibited OS cell growth by down-regulating the signalling pathway of JAK2/STAT3 (Liu et al., 2013). Hence, agents specifically targeting the JAK2/STAT3 pathway are promising for OS treatment.

Here, our aim was to investigate the anti-tumour effects of AA on OS cells and whether its action is attributed to the modulation of the JAK2/STAT3 pathway and resultant MCL-1 expression and mitochondrial apoptosis changes. Expectantly, this research could provide a theoretical and practical evidence for the potential application of AA in the clinical treatment of OS.

Material and Methods

Reagents

AA with purity above 98 % was obtained from Spring & Autumn Biological Engineering Co., Ltd (Jiangsu, China). 3-(4,5)-2,5-Diphenyltetrazolium bromide (MTT) was purchased from Beyotime (Jiangsu, China). Z-VAD-FMK and coumermycin A1 were provided by APExBIO (Houston, TX). Distilled water was prepared using a MilliQ system (Millipore, Billerica, MA).

Cell culture

Human OS cell lines (HOS and 143B cells) and human normal cell lines (HK-2 and LO2) were supplied by the American Type Culture Collection (Manassas, VA). The four types of cell lines were cultured in MEM supplemented with 10% foetal bovine serum (Hyclone, Marlborough, MA) and 1% penicillin/streptomycin, and grown in a 5% CO_2, 37 °C humidified cell incubator.

Cell viability assay

MTT analysis was applied for cell viability evaluation according to the previous reported approaches (Jo et al., 2019). Briefly, 143B and HOS cells were plated in 96-well plates at a density of 10^4 cells/well. Upon reaching 60% confluence, the cells were incubated with 0.1% DMSO or a series of concentrations of AA (0, 2.5, 5, 10, 20, 25, 30, 40, 50, 100 μM) for different times (24, 48 and 72 h). Subsequently, 15 μl of MTT (0.5 g/l) was added to each well and continuously kept for 4 h in the incubator. DMSO (150 μl) was added to each well and the plate was shaken for 15 min to dissolve formazan crystals. The absorbance of each well was determined using a microplate reader (Thermo Fisher Scientific Multiskan MK3, Helsinki, Finland) at 490 nm, and optical density (OD) values were used to evaluate cell viability.

In another experiment, HOS cells were cultured as mentioned above. The cells were incubated with 0.1% DMSO (blank group) and different concentrations of AA (10, 20, 30 μM), respectively, for 24 h. Meanwhile, AA co-incubated cells were pre-treated with 10 μM Z-VAD-FMK (a specific caspase inhibitor) for 3 h. Subsequently, cell viability assay was conducted as above.

Colony formation assay

HOS and 143B cells under the logarithmic growth phase were plated in 12-well plates (10^3 cells/well); the cells were treated with 0.1% DMSO and different levels of AA (10, 20 μM) for 15 days. Afterwards, the medium was replaced with MEM containing 10% FBS to maintain the cell growth. Before analysis, PBS was used to wash the cells; 4% paraformaldehyde was used to fix the cells; then 1% crystal violet solution was applied for staining. The photographs were recorded manually. Image J software was used to analyse and semi-quantify the colonies.

Cell apoptosis assay

143B and HOS cells under the logarithmic growth phase were plated in 6-well plates (10^5 cells/well). Upon reaching 60% confluence, the cells were incubated with 0.1% DMSO and different concentrations of AA (10, 20, 30 μM), respectively, for 24 h. The cell apoptosis analysis was performed using detection kit Annexin V-FITC (KeyGEN Biotech, Jiangsu, China) with a flow cytometer (Beckman Coulter CytoFLEX, Brea, CA). The apoptotic rate was calculated as the total sum of the early apoptotic cells and the late apoptotic cells.

Analysis of cell mitochondrial transmembrane potential

The mitochondrial membrane potential (MMP) of 143B and HOS cells was detected with a JC-1 detection kit (Beyotime, Haimen, Jiangsu, China) as per manufacturer's protocol. After appropriate treatment, the cells were incubated with the JC-1 probe in the dark for 0.5 h. After being washed with PBS solution, a CytoFLEX flow cytometer was utilized to measure the fluorescence of separated cells. The green/red fluorescence intensity ratio was used to identify mitochondrial depolarization.

Caspase-3 activity assay

HOS cells under the logarithmic growth phase were seeded in 96-well plates (10^4 cells/well). After exposure to AA (10, 20, 30 μM) for 24 h, caspase-3 activity in HOS cells was evaluated with a Caspase-3 activity analysis kit (Beyotime) based on the manufacturer's protocol, and the samples were measured at 405 nm with a Multiskan MK3 microplate reader.

Western blotting

After exposure to AA (10, 20, 30 μM) with or without coumermycin A1 (C-A1) for 24 h, total protein of HOS cells was extracted using a protein extraction kit (AmyJet, Hubei, China); then a BCA protein quantification kit (Beyotime) was used to determine the protein concentration. The Western blotting procedure was performed according to a previous study (Tian et al., 2021). Briefly, equal amounts of protein were loaded to SDS-PAGE to separate the proteins and then transferred to PVDF membrane. After blocking in 5% BSA for 2 h at room temperature, the membranes were incubated with the following primary antibodies: Bax (Abcam, Santa Cruz, CA, #ab32503, rabbit monoclonal), Bcl-2 (Abcam, #ab692, mouse monoclonal), Cyt-c (Abcam, #ab133504, rabbit monoclonal), Cl-Caspase-3 (Abcam, #ab32042, rabbit monoclonal), Cl-Caspase-9 (CST, #9502, rabbit polyclonal), Cl-PARP (Abcam, #ab32064, rabbit monoclonal), p-JAK2 (Abcam, #ab195055, rabbit polyclonal), JAK2 (Abcam, #ab108596, rabbit monoclonal), p-STAT3 (Abcam, #ab267373, rabbit monoclonal), STAT3 (Abcam, #ab68153, rabbit monoclonal), MCL-1 (Abcam, #ab32087, rabbit monoclonal) and GAPDH (Proteintech, Rosemont, IL, #60004-1-Ig, mouse monoclonal) at 4 °C overnight. Next, the membranes were further conjugated with HRP-conjugated secondary antibodies (Cell Signaling Technology) at room temperature for 1.5 h. Finally, ECL reagent (Millipore, Billerica, MA) was added to the processed membranes; the bands were photographed and quantified using Image J software.

Quantitative real-time PCR

The total RNAs from different cell groups were extracted using TRIzol (TaKaRa Biotechnology, Dalian, China). The RNA primers were provided by Sangon Biotech Co Ltd. (Shanghai, China). Primer RNA sequences were listed as follows: MCL-1, forward: 5'-CGCCA AGGACACAAAGCCAATG-3', and reverse: 5'-GAA GGCCGTCTCGTGGTTGC-3'; GAPDH, forward: 5'-TTG TGACAAAGTGGACATTGTTG-3', and reverse: 5'-TCTCGCTCCTGGAAGATGGTGAT-3'. Reverse transcription was performed using an RT-PCR synthesis kit (Applied Biological Materials Inc., Richmond, Canada) with Real-Time PCR detection equipment CFX96 Touch (Bio-rad, Hercules, CA). GAPDH was selected for internal control to quantify the target mRNA expression using the $2^{-\Delta\Delta Ct}$ method.

Statistical analyses

All experiments were repeated no less than three times and conducted in duplicate. Results were presented as mean ± standard deviation (SD). For statistical difference analysis, Student's *t*-test was applied for the statistical difference between two groups, while one-way ANOVA analysis was used for statistical difference among multiple groups using Prism 8 (GraphPad 7.0). $P < 0.01$ and $P < 0.05$ were regarded as significant differences.

Results

AA inhibited proliferation and induced mitochondrial apoptosis in OS cells

Firstly, we evaluated the growth-suppressing effect of AA on 143B and HOS cell lines. Cell viability assay was conducted using MTT after intervention with AA (10, 20, 30 μM) for different times (24, 48 and 72 h). As shown in Fig. 1B, AA reduced the cell viability of 143B and HOS in a concentration-and time-dependent manner (IC50 values for 143B cells: 25.08, 31.12 and 34.01 μM, at 72, 48 and 24 h, respectively; IC50 values for HOS cells: 13.61, 17.06 and 27.58 μM, at 72, 48 and 24 h, respectively). Colony formation was utilized to evaluate the anti-proliferative effects of AA on OS cells. Compared with the control group, there was a significant decrease in the colony formation after AA incubation (Fig. 1C, D). Besides, to test the possible toxicity of AA for human normal cells, HK-2 and LO2 cells were selected to incubate with different concentrations of AA for 24 h. The results suggested that the suppressing effect of AA on HK-2 and LO2 cells was weaker than that

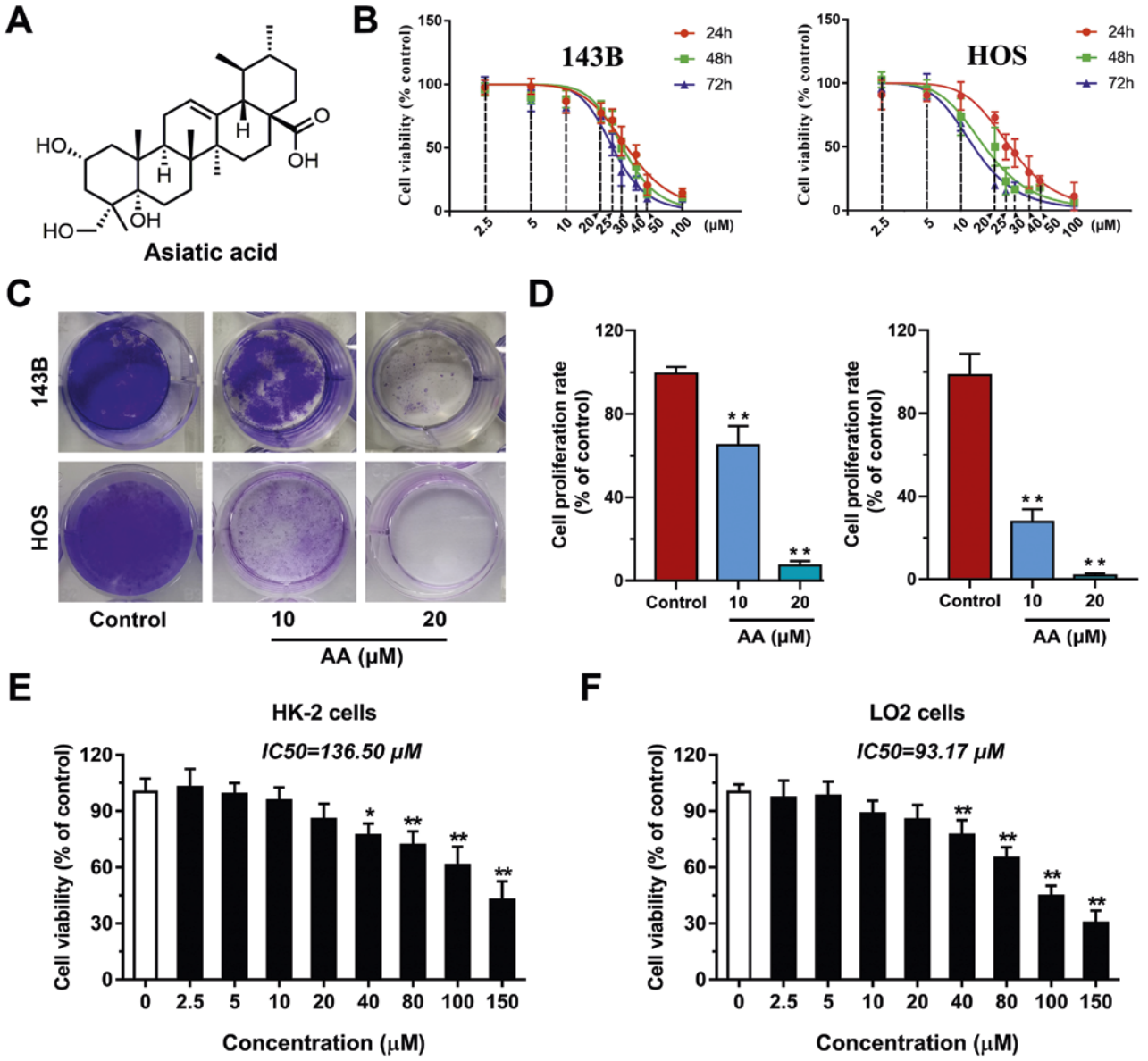

Fig. 1. AA selectively inhibited proliferation of human OS cells determined by colony formation assay. (**A**) Chemical structure of AA. (**B**) Cell viability of HOS and 143B cells. (**C**) A typical image of the colony formation of 143B and HOS cells is presented and three independent experiments were performed. (**D**) The proliferation rate of 143B and HOS cells was quantified. (**E**, **F**) Cell viability of HK-2 and LO2 cells. Data were expressed as mean ± SD. Three independent experiments were performed, and typical results were presented. *P < 0.05 and **P < 0.01 vs control group.

on 143B and HOS cells (IC50 value for HK-2 cells: 136.5 μM; IC50 value for LO2 cells: 93.17 μM), demonstrating that AA selectively inhibited the proliferation of human OS cells (Fig. 1E, F). Moreover, annexin V-FITC/PI double staining assay showed that AA substantially induced 143B and HOS cell apoptosis in a dose-dependent manner (Fig. 2A, B), indicating that the inhibitory effect of AA on OS cells resulted from its apoptosis-promoting action.

Mitochondria can provide the necessary energy for cell survival and growth; once the MMP is collapsed, a cascade of cellular reactions could be initiated resulting in mitochondria-mediated apoptosis. To determine whether AA-induced apoptosis was mitochondria-evoked, MMP was detected using a JC-1 detection kit, and the results showed that AA dramatically promoted the conversion of JC-1 aggregates to JC-1 monomers in a dose-dependence manner in OS cells (Fig. 3A, B), suggesting that mitochondrial dysfunction was closely associated with AA-induced apoptosis. Additionally, mitochondrial apoptosis-related proteins were also detected. Expectedly, AA markedly increased Bax, Cyt-c, Cl-Caspase-3, Cl-Caspase-9 and Cl-PARP expression, and attenuated Bcl-2 expression in a dose-dependence manner in HOS cells (Fig. 4A). Meanwhile, the ratio of Bax/Bcl-2 was also augmented after AA intervention (Fig. 4B). To

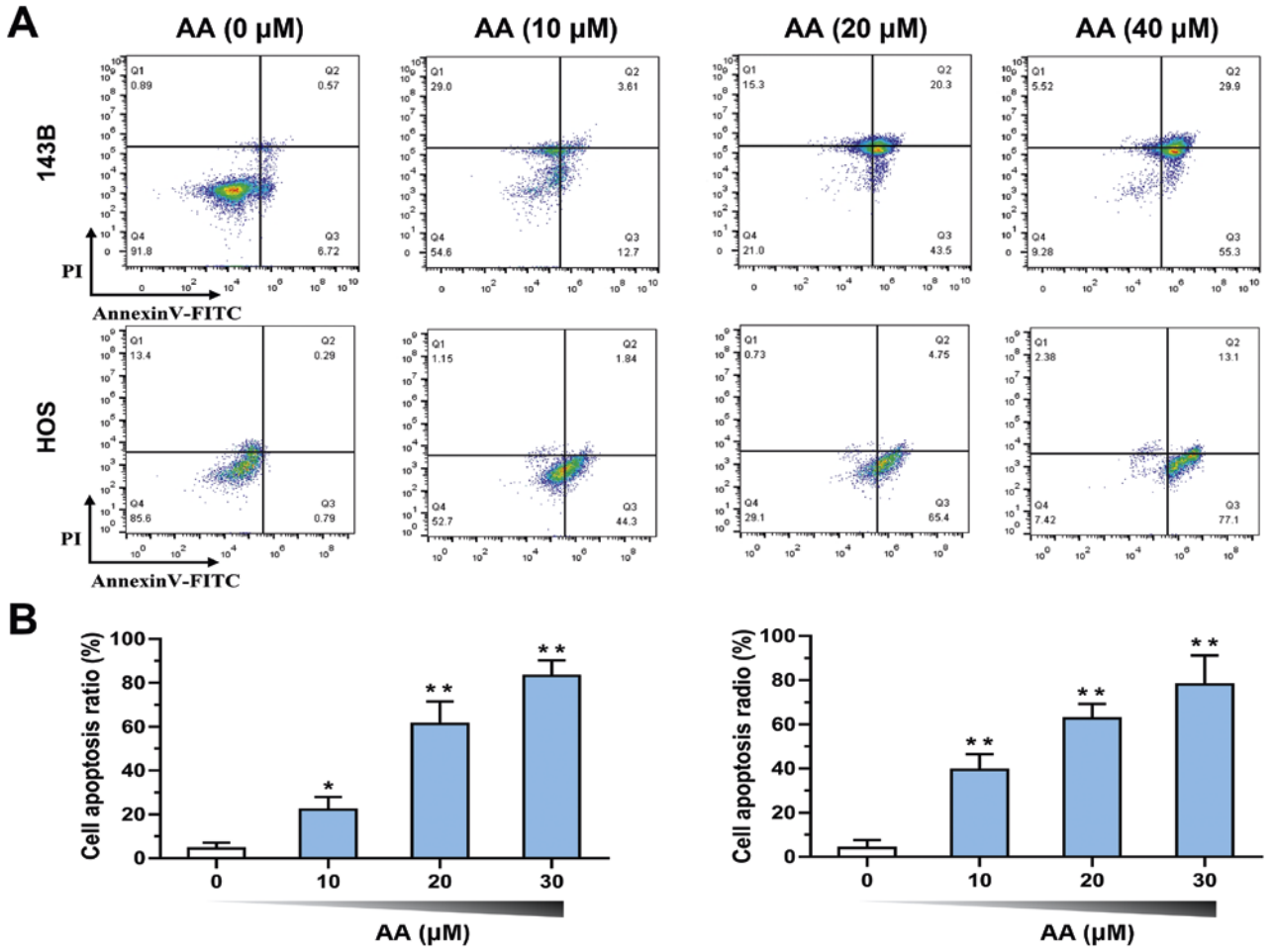

Fig. 2. AA promoted apoptosis of human OS cells. (**A**, **B**) Apoptosis rates of 143B and HOS cells after treatment with different concentrations of AA for 24 h. Data were expressed as mean ± SD. Three independent experiments were performed, and typical results were presented. *P < 0.05 and **P < 0.01 vs control group.

further confirm the mitochondrial apoptosis occurred after AA treatment, caspase-3 activity and cell viability after pre-treatment with caspase inhibitor Z-VAD-FMK were investigated. As shown in Fig. 4C, D, AA increased caspase-3 activity in a dose-dependence manner, while co-treatment with Z-VAD-FMK conspicuously reversed the AA-induced decrease in cell viability. Overall, these results suggested that the anti-tumour activity of AA was ascribed to inhibiting proliferation and inducing mitochondrial apoptosis in OS cells.

AA induced apoptosis by down-regulating the JAK2/STAT3 signalling pathway in OS cells

It is well known that the signalling pathway JAK2/STAT3 exerts a crucial role in regulating cell proliferation and suppressing cell apoptosis, particularly in OS cells. To test whether the signalling pathway JAK2/STAT3 was related to the anti-tumour activity of AA, the expression of pivotal proteins in the canonical JAK2/STAT3 signalling pathway, including JAK2, STAT3, MCL-1, p-JAK2 and p-STAT3, were analysed. As exhibited in Fig. 5A-C, AA treatment significantly diminished MCL-1, p-STAT3 and p-JAK2 expression in a dose-dependent manner, while co-incubation with coumermycin A1, a JAK2 activator, abolished the modulating effect of AA on the JAK2/STAT3 signalling pathway and the inhibitory effect of AA on the cell viability of HOS cells (Fig. 6A-C). Together, these results indicated that the JAK2/STAT3 signalling pathway might play a pivotal role in AA-induced OS cell apoptosis.

Discussion

Neoadjuvant chemotherapy is usually the first treatment choice for the OS disease, which could frequently cause the patients to suffer from a battery of untoward consequences (Mei et al., 2014). Therefore, it is indispensable to discover new lead compounds or alternative approaches that could effectively inhibit OS development with less side effects. Extensive studies have demonstrated the anti-tumour activity of natural compounds in TCMs. For example, ginsenoside Rg3 and resveratrol have been reported to exert significant anti-tumour ac-

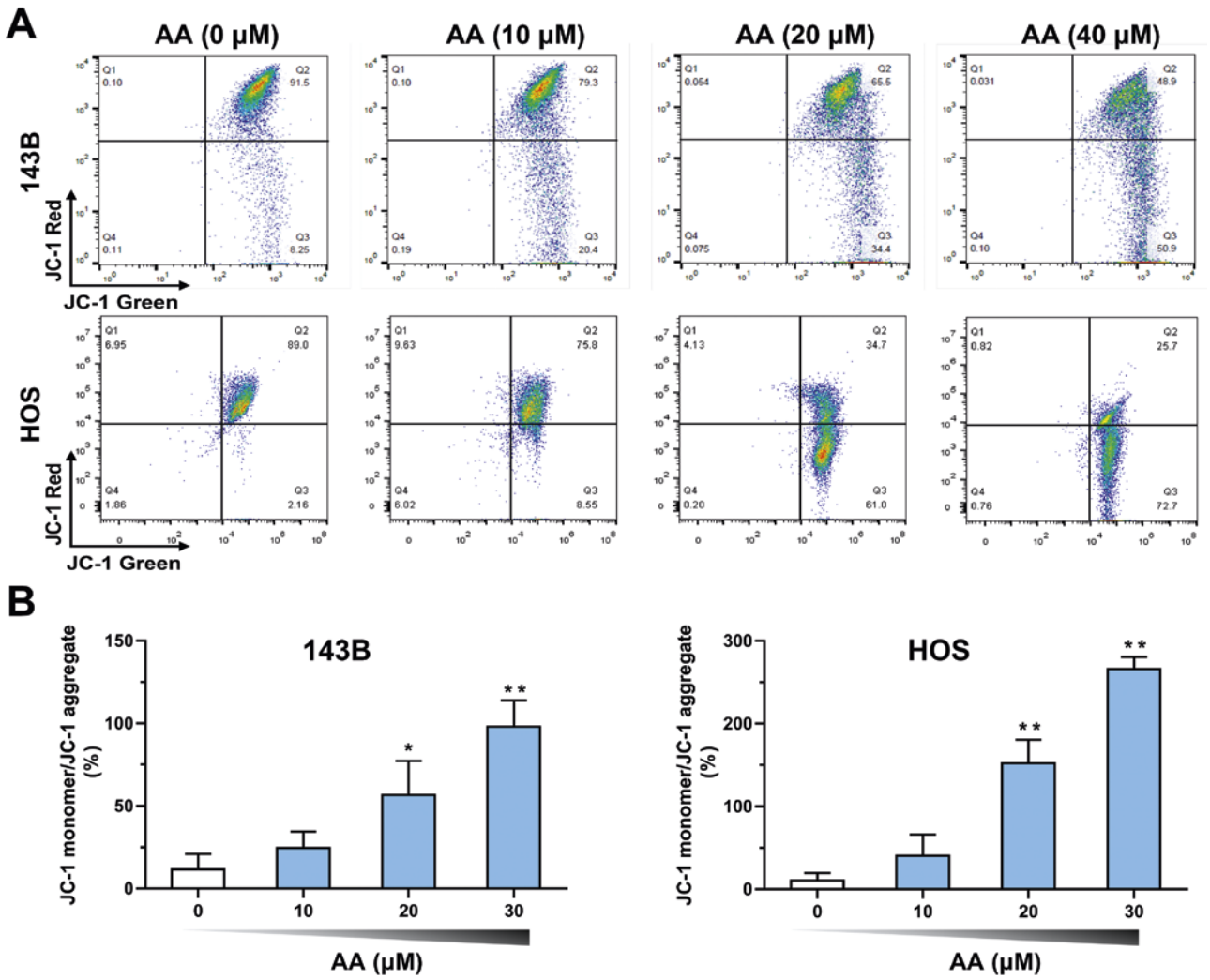

Fig. 3. AA evoked alteration of the mitochondrial membrane potential in human OS cells. (**A**, **B**) Mitochondrial membrane potential in 143B and HOS cells after the intervention with different concentrations of AA was measured using JC-1 staining with a flow cytometry equipment. Data were expressed as mean ± SD. Three independent experiments were performed, and typical results were presented. *P < 0.05 and **P < 0.01 vs control group.

tivity in human OS, which brings a new focus on discovering new drugs to inhibit OS (Xie et al., 2017; Mao et al., 2020). Here, the biological effects of AA on human OS cells and the underlying mechanisms contributing to its effects were investigated. Our results suggested that compound AA could remarkably suppress OS cell viability, proliferation, and induce mitochondrial apoptosis through the inhibition of the JAK2/STAT3 signalling pathway in OS cells. Our significant findings revealed that AA might be a promising active ingredient for OS treatment.

Cell apoptosis, a form of programmed cell death, maintains the homeostasis of tissues and organs. However, the abnormal apoptosis of tumour cells and the inhibition of cascading active death play a key role in tumour progression (Jan and Chaudhry, 2019). Induction of tumour cell apoptosis has become a promising approach for the clinical management of tumours. Mitochondrial apoptosis is one of the main molecular pathways of apoptosis, which is characterized by depolarization of MMP and activation of the cellular caspase cascade (Burke, 2017). Numerous studies have shown that Cyt-c, Bcl-2, Bax, cleaved caspase-3, cleaved PARP, and cleaved caspase-9 are all significant members of mitochondria-dependent apoptotic signals (Wang et al., 2020; Chan et al., 2021). Apoptosis-inducing protein Bax and apoptosis-inhibiting protein Bcl-2 are two key components of the Bcl-2 family, which maintain the normal transmembrane potential of mitochondria (Delbridge et al., 2016). Expectedly, an increase of the Bax/Bcl-2 ratio gives rise to mitochondrial membrane permeabilization and collapse of MMP, which finally leads to the release of apoptosis-related factors. Using Cyt-c as an example, once the collapsed MMP occurred in cells, the Cyt-c protein could be released from mitochondria into the cytoplasm. The release of Cyt-c acti-

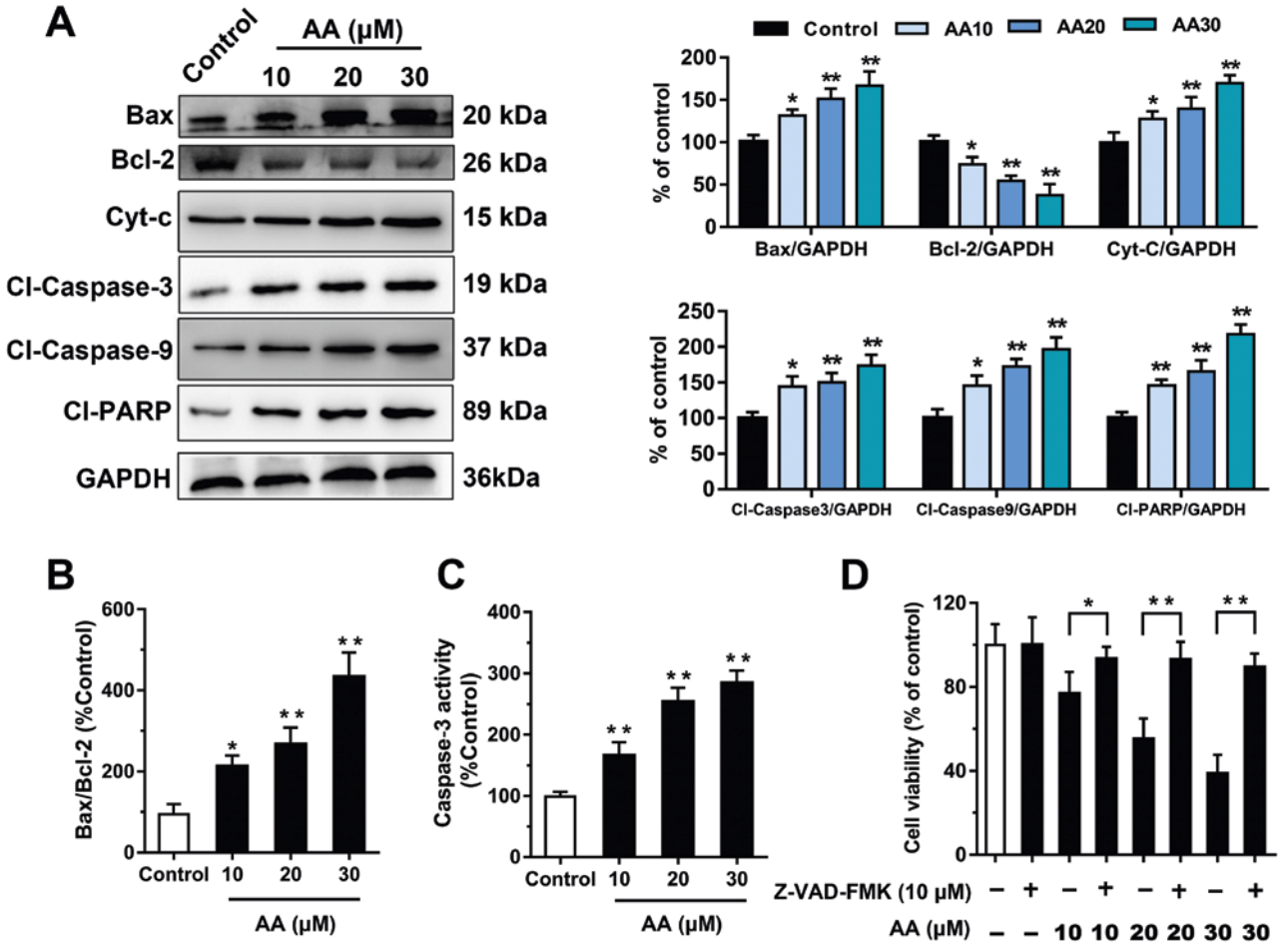

Fig. 4. AA caused mitochondria-related apoptosis in HOS cells. (**A**, **B**) The protein expression of Cyt-c, Bcl-2, Bax, cleaved caspase-3, cleaved caspase-9 and cleaved PARP in HOS cells was analysed by Western blotting after AA treatment. (**C**) The caspase-3 activity of HOS cells was measured by a caspase-3 activity analysis kit after AA intervention. (**D**) The cell viability of HOS cells was detected after incubation with different concentrations of AA for 24 h, with pretreatment with 10 μM Z-VAD-FMK for 3 h or not. Data were expressed as mean ± SD. Three independent experiments were performed, and typical results were presented. *$P < 0.05$ and **$P < 0.01$ vs control group or indicated.

vates the upstream molecules such as caspase-9, which triggers the downstream executive protein caspase-3. Consequently, the caspase cascade is activated and causes the apoptosis to enter the irreversible stage (Liang et al., 2016). In our study, JC-1 staining showed a decreased MMP after AA treatment. Elevated expression levels of Cyt-c, cleaved caspase-3, Bax, cleaved PARP, cleaved caspase-9, as well as reduced expression levels of Bcl-2 were observed after AA treatment. Furthermore, there was an obviously elevated ratio of Bax/Bcl-2 and caspase-3 activity after AA intervention. In addition, caspase inhibitor Z-VAD-FMK significantly eliminated AA-induced HOS cell death. The above results demonstrated that AA led to mitochondrial apoptosis in human OS cells.

The JAK2/STAT3 pathway, a significant component of the JAK/STAT signalling pathway, is related to the genesis and development of various tumours and is persistently over-activated in tumour cells (Liu et al., 2013). Studies have reported that withaferin A induced apoptosis by inhibition of the JAK/STAT3 pathway in renal carcinoma Caki cells (Um et al., 2012). Especially, pterostilbene caused OS cell apoptosis through down-regulation of the JAK2/STAT3 pathway and activation of the intrinsic apoptotic pathway (Liu et al., 2013). Overall, the related molecules of the JAK2/STAT3 signalling pathway could be used as markers or targets for tumour therapy and prognosis. MCL-1, a target protein of STAT3, is also a member of the Bcl-2 family and blocks mitochondrial apoptosis through influencing Bak and Bax, two pro-apoptotic Bcl-2 family members, which form mitochondrial permeability transition pores in the mitochondrial membrane to induce the release of Cyt-c from cellular mitochondria into the cytoplasm (Perciavalle et al., 2012; Jo et al., 2019). In our experiment, AA substantially blocked JAK2 and STAT3 phosphorylation, and the expression of MCL-1 was also strikingly down-regulated; however, co-treatment with coumer-

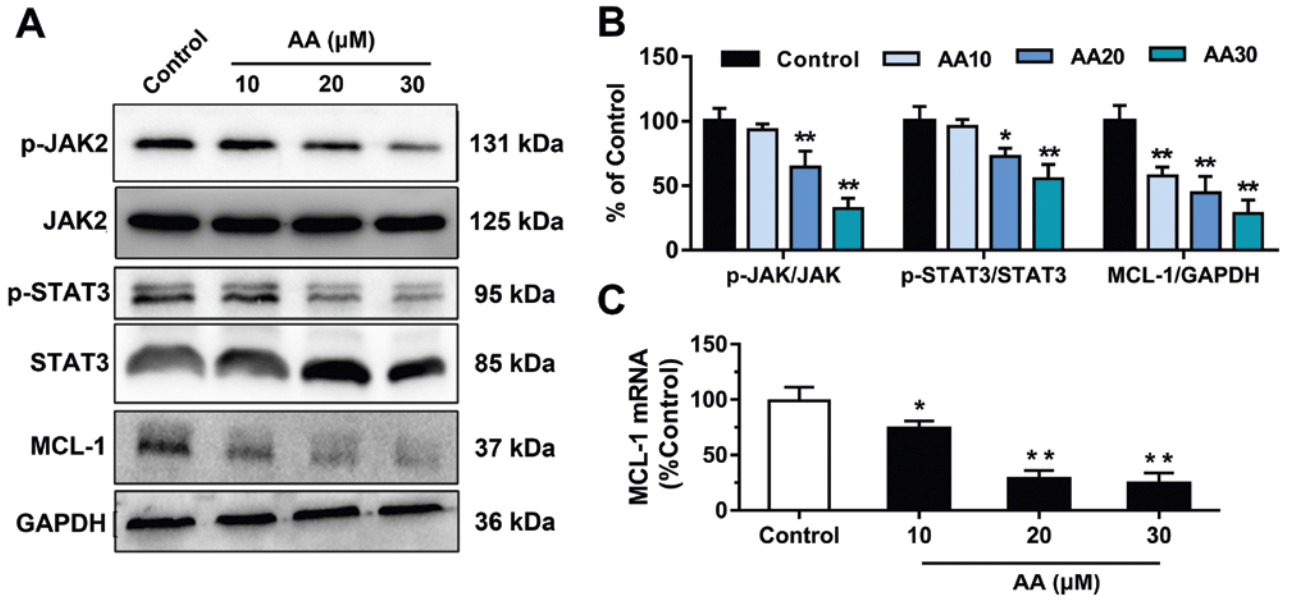

Fig. 5. AA induced mitochondrial apoptosis in HOS cells by triggering the JAK2/STAT3 signalling pathway. (**A**, **B**) The protein expression of p-STAT3, p-JAK2, MCL-1, JAK2, and STAT3 in HOS cells was analysed by Western blotting after AA treatment. (**C**) The MCL-1 mRNA level in HOS cells was analysed by RT-PCR after AA treatment. Data were expressed as mean ± SD. Three independent experiments were performed, and typical results were presented. *P < 0.05 and **P < 0.01 vs control group.

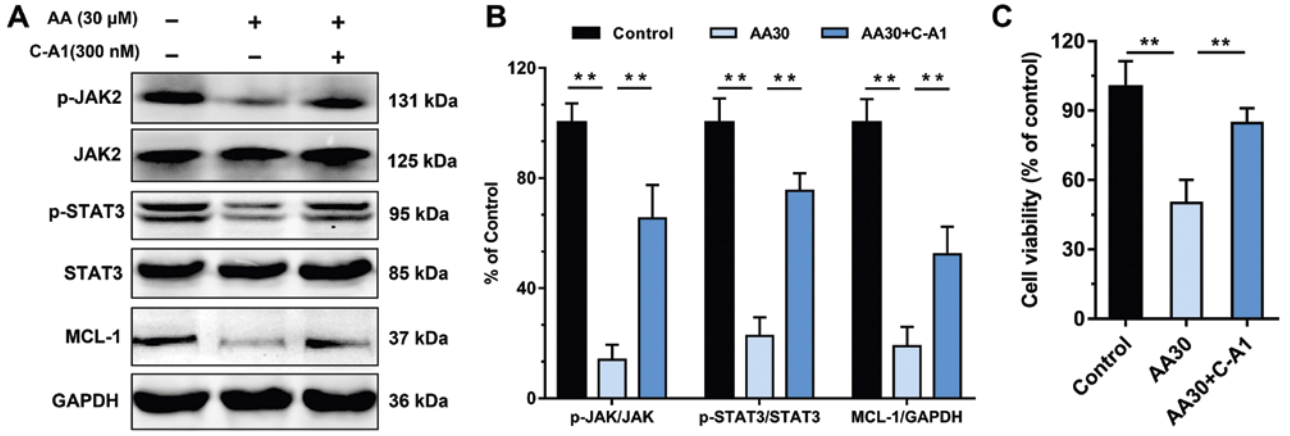

Fig. 6. Coumermycin A1 reversed the effect of AA on the JAK2/STAT3 signalling pathway. (**A**) Typical results of expression of p-STAT3, p-JAK2, MCL-1, JAK2, and STAT3 in HOS cells after AA treatment with or without coumermycin A1 for 24 h are presented, and (**B**) semi-quantitative analysis of three independent experiments was performed. (**C**) Cell viability of HOS cells after co-incubation with AA with or without coumermycin A1 for 24 h. Data were expressed as mean ± SD. Three independent experiments were performed, and typical results were presented. *P < 0.05 and **P < 0.01 vs control group.

mycin A1 abrogated these changes. Together, these results revealed that AA induced mitochondrial apoptosis in human OS cells through suppression of the JAK2/STAT3 signalling pathway.

Emerging reports suggested that natural compounds exhibited appreciable anti-OS activity, independently or as adjuvant agents, through multiple pathways. For example, curcumin and PKF118-310 independently suppressed human osteosarcoma cells through blocking the Wnt/β-catenin pathway (Leow et al., 2010). Zhao et al. (2019) found that polydatin, as an adjuvant agent, could sensitize osteosarcoma cells to paclitaxel partly via suppressing Akt activation. In line with our study, numerous natural compounds such as notoginsenoside R1 (Lu

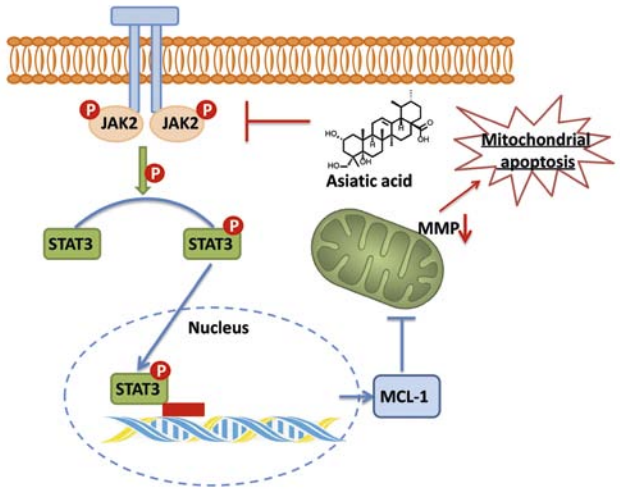

Fig. 7. Schematic diagram of AA-induced mitochondrial apoptosis in human OS cells through blocking the JAK2/STAT3 signalling pathway.

et al., 2021), salidroside (Huang et al., 2019), lycorine (Hu et al., 2019), and cucurbitacin B (Zhang et al., 2017) exerted their anti-OS efficacy through inactivation of JAK2/STAT3 pathway-mediated proliferation, migration, invasion, etc. Among them, cucurbitacin B, possessing a similar triterpenoid structure with AA, showed weaker anti-OS potency when compared to AA (Zhang et al., 2017). Most importantly, in most cases, the IC50 value of AA for OS cells in our study is comparable or even lower relative to the above-mentioned compounds. Collectively, AA possesses appreciable anti-OS efficacy *in vitro*, and whether AA exhibits synergistic effects when combined with other phytochemicals that repressed OS through distinct mechanisms (e.g., Wnt/β-catenin) or commercial chemotherapy drugs (e.g., paclitaxel) deserves further research.

In conclusion, we were the first to provide convincing evidence that AA could suppress the cell proliferation and cause the apoptosis of human OS cells, and we also elucidated its potential mechanism: down-regulation of the JAK2/STAT3 signalling pathway and resultant MCL-1 expression (Fig. 7). Therefore, our findings strongly indicated that AA could be a promising anti-tumour agent for OS treatment by targeting the JAK2/STAT3 signalling pathway.

Acknowledgement

B. Y.† and X. C.† contributed equally to this work.

Disclosure of conflict of interest

The authors declare no conflict of interest.

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