Astragaloside IV downregulates the expression of MDR1 in Bel-7402/FU human hepatic cancer cells by inhibiting the JNK/c-Jun/AP-1 signaling pathway

PEI-PEI WANG[1*], JIA-JIE LUAN[1*], WEN-KE XU[1], LIN WANG[1], DU-JUAN XU[3], CHUN-YAN YANG[1], YAN-HONG ZHU[1] and YA-QIN WANG[2]

[1]Department of Pharmacy, Yijishan Affiliated Hospital of Wannan Medical College; [2]School of Pharmacy, Wannan Medical College, Wuhu, Anhui 241001; [3]Department of Pharmacy, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230022, P.R. China

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Abstract. Previous studies demonstrated that astragaloside IV (ASIV) is a potential P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) reversal agent through mechanisms involving downregulation of the gene expression of mdr1. In order to investigate whether the c-Jun N-terminal kinase (JNK) signaling pathway is involved in the mechanism underlying ASIV-induced downregulated the expression of mdr1, the present study used 5-fluorouracil-resistant Bel-7402/FU human hepatic cancer cells as target cells. ASIV (0.1 mM) decreased the protein expression of phosphorylated (p)-JNK and p-c-Jun in the Bel-7402/FU cells, as determined using western blot analysis. Treatment with the JNK pathway inhibitor, SP600125, at a concentration of 11 μM, decreased the mRNA expression levels of mdr1 and P-gp, as determined using reverse transcription-quantitative polymerase chain reaction and western blot analyses, and similar effects were observed following exposure to ASIV. Furthermore, electrophoretic mobility shift assays demonstrated that the DNA-binding activity of activator protein-1 (AP-1) was decreased by 0.1 mM ASIV or 11 μM SP600125. Flow cytometric analysis revealed that 0.1 mM ASIV or 11 μM SP600125 increased the intracellular accumulation of fluorescent P-gp substrates, including rhodamine 123. Taken together, these results indicated that ASIV reversed the drug resistance of Bel-7402/FU cells by downregulating the expression of mdr1 via inhibition of the JNK/c-Jun/AP-1 signaling pathway.

Correspondence to: Dr Wen-Ke Xu or Dr Lin Wang, Department of Pharmacy, Yijishan Affiliated Hospital of Wannan Medical College, 2 Zheshanxi Road, Wuhu, Anhui 241001, P.R. China
E-mail: xwk1970@126.com
E-mail: wanglin0553@126.com

*Contributed equally

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Introduction

Chemotherapy is considered an important approach for the treatment of malignancies, however, several types of cancer develop resistance to multiple antineoplastic drugs, which appears to be primarily mediated by P-glycoprotein (P-gp) (1,2). P-gp is encoded by the multidrug resistance (mdr1) gene, which belongs to the ATP-binging cassette superfamily of transporters. It is a large membrane-spanning protein, which acts as a representative efflux pump (3-5). The inhibition of P-gp transporters and modulation of multidrug resistance (MDR) are important strategies in reversing MDR.

Astragaloside IV (ASIV) is a saponin isolated from the rhizome of Radix Astragali, which is widely used in traditional Chinese medicine (6). Our previous studies reported that ASIV reverses the resistance of Bel-7402/FU cells to drugs by downregulating the expression levels of mdr1 and P-gp (7). However, the molecular mechanism underlying the action of ASIV in downregulating the expression of mdr1 remains to be fully elucidated.

The c-Jun N-terminal kinase (JNK) signaling pathway, one of the mitogen-activated protein kinase (MAPK) pathways, may be critical in the MDR phenotype (8-10). The activation of JNK is increased in several types of human MDR cancer cell following treatment with different chemotherapeutic agents (11,12), whereas the inhibition of JNK or combined treatment of a JNK inhibitor with anticancer drugs may prevent the development of MDR (13). The present study aimed to investigate the involvement of the JNK/c-Jun/activator protein-1 (AP-1) signaling pathway in ASIV-induced downregulated expression of mdr1.

Materials and methods

Extraction, isolation and preparation of ASIV. The ASIV was extracted and separated from the Astragalus root (Gansu, SDIC Pharmaceutical Anhui Co., Ltd., Xuancheng, China) as

previously described (14). ASIV preparation was performed according to a previously published method (7).

Cell culture. The 5-fluorouracil (5-FU)-resistant Bel-7402/FU human hepatic cancer cell line, purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China), was cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the addition of 20,000 ng/ml 5-FU (Tianjin Taihe Pharmaceutical Co., Ltd., Tianjin, China) and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a CO_2 incubator at 37˚C.

Determination of the cytotoxicity of SP600125. The *in vitro* cytotoxicity of the JNK inhibitor, SP600125, was investigated using an MTT assay. The Bel-7402/FU cells were seeded into 96-well plates at a density of $5x10^3$ cells per well. Following incubation for 24 h, SP600125 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at concentrations of 1, 2, 5, 10, 20, 40 and 80 μM were added. The cells were then incubated for 24 h at 37˚C in 5% CO_2, followed by the addition of MTT (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) solution (0.5 mg/ml) to each well and incubation for 4 h at 37˚C. The medium was discarded and formazan crystals were dissolved in 150 μl DMSO. The plates were read at 570 nm with an automated microplate reader (680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The concentrations of the assayed enzymes required to inhibit cell proliferation by 50% (IC_{50} values) were calculated using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA).

Western blot analysis. The Bel-7402/FU cells ($1x10^6$ cells/ml) were treated with 0.1 mM ASIV or 11 μM SP600125 for 24 h at 37˚C. Cell extracts were prepared in RIPA buffer with the proteinase inhibitor cocktail (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Protein concentration was quantified using a Bradford assay (Beyotime Institute of Biotechnology, Inc., Haimen, China). A 20 μg per lane protein sample was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk for 2 h at room temperature, then probed with appropriate antibodies at 4˚C overnight in 5% BSA, including rabbit polyclonal antibodies against P-gp (1:1,000, cat. no. ab98322, Abcam, Cambridge, UK), phosphorylated (p-)-JNK (1:5,000, cat. no. 4668, Cell Signaling Technology, Inc., Beverly, MA, USA) and JNK (1:5,000, cat. no. 9258, Cell Signaling Technology, Inc.), p-c-Jun (1:500, cat. no. 3270, Cell Signaling Technology, Inc.) and c-Jun (1:5,000, cat. no. 9165, Cell Signaling Technology, Inc.) and mouse monoclonal antibody against β-actin (1:500, cat. no. sc-47778, Santa Cruz Biotechnology, Inc.). The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (1:50,000, cat. no. ZB-2301, Zhongshan Jinqiao Biotech, Co., Ltd., Beijing, China) or HRP-conjugated goat anti-mouse immunoglobulin G (1:10,000, cat. no. ZB-2305, Zhongshan Jinqiao Biotech, Co., Ltd.), and then detected using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The Bel-7402/FU cells ($1x10^6$ cells/ml) were treated with 0.1 mM ASIV or 11 μM SP600125 for 24 h at 37˚C. Total RNA was extracted from the cells using TRIzol reagent according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 μg) was reverse transcribed using a reverse transcription kit (Thermo Fisher Scientific, Inc.). The RT-qPCR was performed using a Bio-Rad Real-time system (CFX96; Bio-Rad Laboratories, Inc.) with a SYBR Green PCR kit (Qiagen GmbH, Hilden, Germany). For RT-qPCR analysis, 1 μl cDNA samples, 1 μl target primers (5 μM) were used to a total reaction volume of 10 μl. The primers were as follows: mdr1, forward 5'-GCTGTTCGT TTCCTTTAGGTCTTTC-3' and reverse 5'-AGTTCTTCT TCTTTGCTCCTCCATT-3'; β-actin, forward 5'-GGGAAA TCGTGCGTGACATTAAGG-3' and reverse 5'-CAGGAA GGAAG GCTGGAAGAGTG-3'. PCR reaction conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec. Relative quantification of the mRNA expression of mdr1 was normalized to the housekeeping gene β-actin using the $2^{-\Delta\Delta Cq}$ method (15).

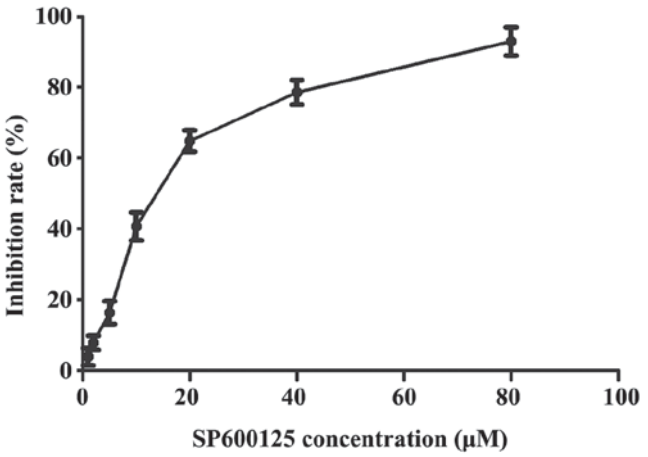

Figure 1. Cytotoxicity of Bel-7402/FU cells induced by SP600125. Following 24 h of treatment with various concentrations of SP600125, the inhibition of cell viability was assessed using an MTT assay. The data are shown as the mean ± standard deviation of three independent experiments.

Nuclear protein extraction and electrophoretic mobility shift assays (EMSA). The Bel-7402/FU cells ($1x10^6$ cells/ml) were incubated with 0.1 mM ASIV or 11 μM SP600125 for 24 h. The cells were then treated with ice-cold lysis buffer and incubated for 5 min. The nuclei were centrifuged at 13,225 x g for 1 min at 4˚C. The nuclear pellet was resuspended in nuclear protein extraction buffer for 30 min, and then centrifuged at 20,034 x g for 10 min at 4˚C. EMSA was performed using a non-radioactive EMSA kit according to the manufacturer's protocol (Pierce; Thermo Fisher Scientific, Inc.). Nuclear extracts (8 μg) were incubated with ^{32}P-labeled double-stranded oligonucleotide with a specific AP-1 binding sequence (5'-GGAATCAGCATTCAGTCA ATCCGGGCCGGG-3') for 20 min at room temperature. The specific competitor unlabeled oligonucleotide was added in a competing system (cold competition) and incubated for 20 min. The protein-DNA complexes were resolved by electrophoresis at 4˚C on a 6.5% non-denaturing acrylamide gel and subjected to autoradiography. The electrophoresis was

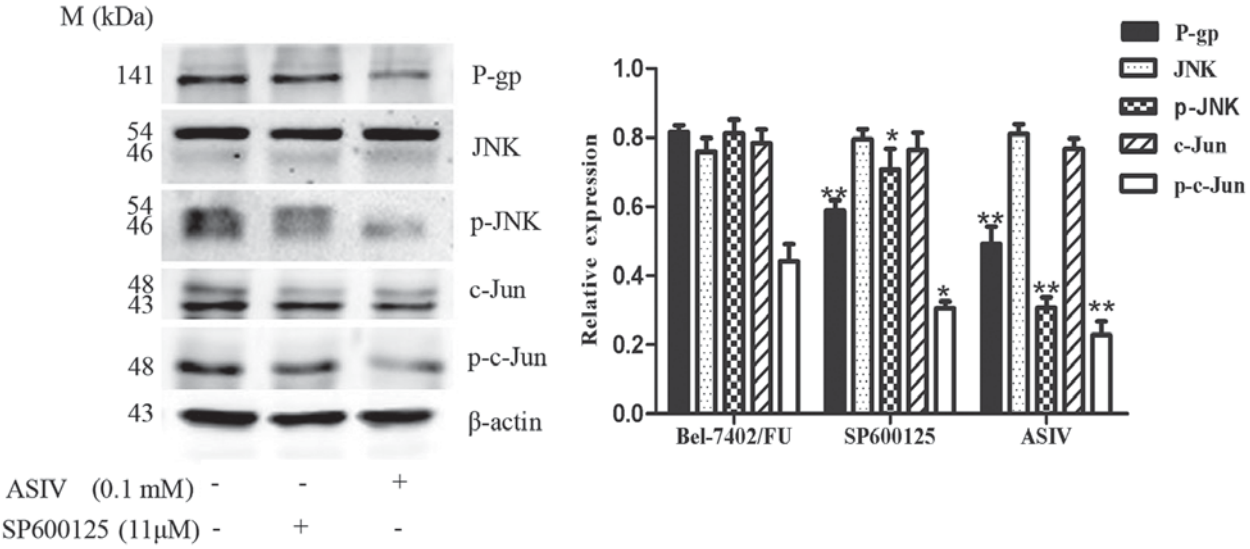

Figure 2. Effect of ASIV and JNK pathway inhibitor SP600125 on the expression of P-gp in Bel-7402/FU cells. Western blot analysis of the expression levels of P-gp, JNK, p-JNK, c-Jun, p-c-Jun and β-actin were determined in Bel-7402/FU cells treated with 0.1 mM ASIV or 11 μM SP600125 for 24 h. Relative protein expression levels of P-gp, p-JNK and p-c-Jun were quantified following normalization to β-actin. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, compared with the Bel-7402/FU cell control group. ASIV, astragaloside IV; JNK, c-Jun N-terminal kinase; P-gp, P-glycoprotein; p-, phosphorylated.

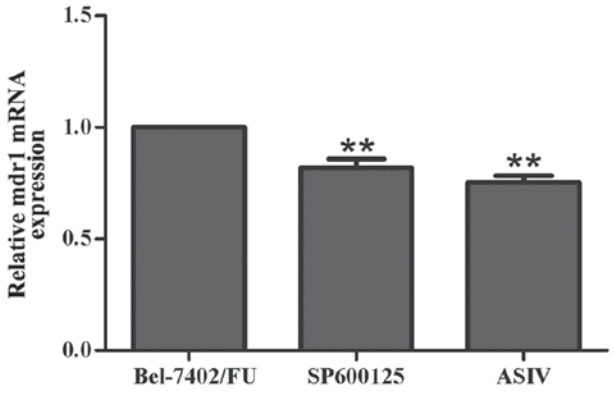

Figure 3. Effect of ASIV and SP600125 on the mRNA expression of mdr1 in Bel-7402/FU cells. Reverse transcription-quantitative polymerase chain reaction analysis was used to evaluate the gene expression levels of mdr1 and β-actin following 24 h treatment with 0.1 mM ASIV or 11 μM SP600125. Relative mRNA levels of mdr1 were calculated as the ratio of the densitometric value of mdr1 to that of β-actin. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01, compared with the Bel-7402/FU cell control group. ASIV, astragaloside IV; mdr1, multidrug resistance.

performed at 175 V in 0.25X TBE [1X TBE contained 89 mM Tris-HCl, 89 mM boric acid and 5 mM EDTA (pH 8.0)] at 4˚C for 1 h. The gels were transferred to the banding membrane at 394 mA in 0.5X TBE at room temperature for 40 min. Crosslinking of the membrane was performed using an ultraviolet crosslink apparatus for 10 min (immobilization), followed by blocking for 30 min at room temperature, streptavidin-HRP labeling the membrane for 30 min at room temperature, washing the membrane 4 times for 5 min at room temperature and equilibrating the membrane for 5 min, obtaining the images through the imager apparatus (Alpha Flurechemical; Alpha Innotech Corporation, San Leandro, CA, USA).

Flow cytometric analysis. The Bel-7402/FU cells ($5x10^5$ cells/ml) were incubated with 0.1 mM ASIV or 11 μM SP600125 for 24 h. A total of 10^6 cells per well were incubated with 5 μg/ml rhodamine 123 (Rh123; Sigma-Aldrich; Merck Millipore) for 1 h at 37˚C and washed twice with cold PBS. Cell fluorescence was evaluated using a flow cytometer (FC500; Beckman Coulter, Miami, FL, USA) at an excitation wavelength of 488 nm and emission wavelength of 525 nm.

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments. Statistical analysis of difference was performed using one-way analysis of variance with SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxicity assay of SP600125. To further investigate the effect of the JNK inhibitor, SP600125, in Bel-7402/FU cells, a weak cytotoxic concentration of SP600125 was required. The cytotoxic effects of SP600125 were measured using an MTT assay (Fig. 1). The IC_{50} of SP600125 in the Bel-7402/FU cells was 11.10 μM, which exhibited weak cytotoxicity (inhibition rate <50%). Therefore, an 11 μM concentration of SP600125 was used as the concentrations for the following experiments.

ASIV inhibits the phosphorylation of JNK and c-Jun in Bel-7402/FU cells. To examine the possible role of the JNK/c-Jun signaling pathway in the ASIV-mediated reversal of MDR, the present study examined the proteins levels of total-JNK, p-JNK, total-c-Jun, p-c-Jun and P-gp in Bel-7402/FU cells using western blot analysis. As shown in Fig. 2, the JNK, p-JNK, c-Jun, p-c-Jun and P-gp proteins were detected in the Bel-7402/FU cells. The results demonstrated that activation of the JNK pathway was involved in the MDR of Bel-7402/FU cells. When the Bel-7402/FU cells were

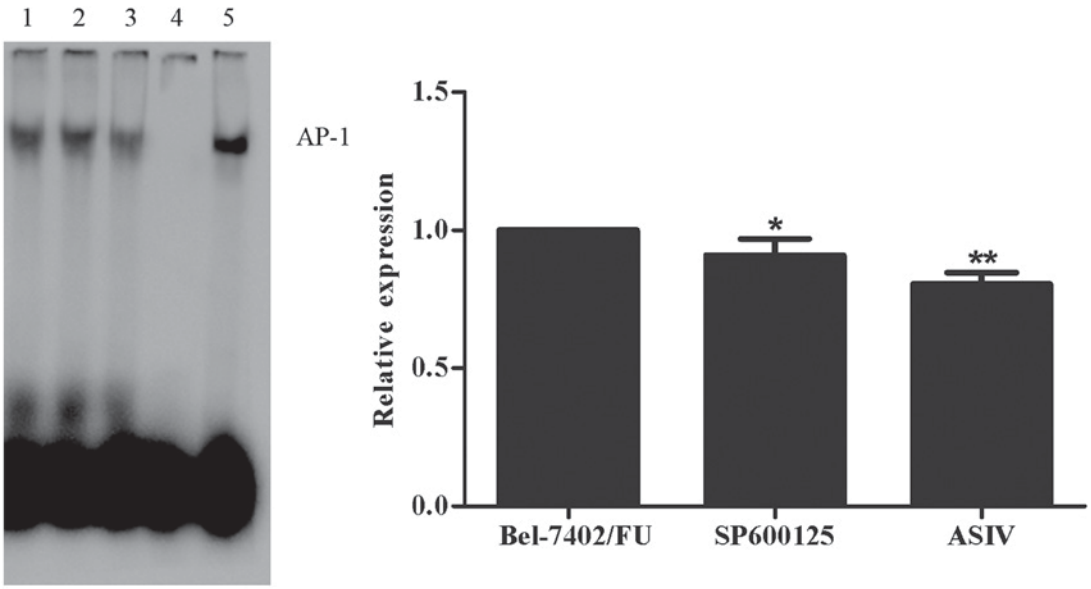

Figure 4. Effect of ASIV and SP600125 on the nuclear translocation of AP-1 in Bel-7402/FU cells. An electrophoretic mobility shift assay of nuclear extracts was performed using a specific AP-1-binding probe in cells. 1, Bel-7402/FU control; 2, Bel-7402/FU cells treated with 0.1 mM ASIV; 3, Bel-7402/FU cells treated with 11 μM SP600125; 4, cold competition; 5, positive control. The graph shows the fold difference in AP-1 translocation. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, compared with the Bel-7402/FU control group. ASIV, astragaloside IV; AP-1, activator protein-1.

treated with 0.1 mM ASIV or 11 μM SP600125, the protein levels of P-gp, p-JNK and p-c-Jun were decreased, whereas no significant change in the protein levels of total JNK or c-Jun were observed. These results indicated that SP600125 partially reversed the drug resistance of Bel-7402/FU cells, and that activation of the JNK pathway was crucial in the mechanism underlying the ASIV-mediated reversal of MDR.

SP600125 downregulates the mRNA expression of mdr1 in Bel-7402/FU cells. To investigate whether the JNK pathway inhibitor, SP600125, downregulated mdr1 at the mRNA level, the mRNA expression of mdr1 was examined using RT-qPCR analysis. As shown in Fig. 3, the mRNA levels of mdr1 were decreased by 11 μM SP600125 and by 0.1 mM ASIV. These results suggested that inhibiting the activation of the JNK pathway prevented the development of MDR by downregulating the expression of mdr1 in Bel-7402/FU cells.

ASIV decreases AP-1 DNA binding activity. In order to investigate the role of AP-1 in regulating the gene expression of mdr1, an EMSA was performed. As shown in Fig. 4, the DNA-binding activity of AP-1 was decreased by 0.1 mM ASIV and by 11 μM SP600125 in the Bel-7402/FU cells. These results indicated that the ASIV-induced downregulated expression of mdr1 was mediated by the JNK/c-Jun/AP-1 signal transduction pathway.

ASIV and SP600125 inhibit the function of P-gp. To determine the effect of ASIV and SP600125 on the function of P-gp as an efflux pump, the fluorescence intensity of P-gp substrate, Rh123, was examined in Bel-7402/FU cells treated with ASIV or SP600125. As shown in Fig. 3, treatment with 0.1 mM ASIV and treatment with 11 μM SP600125 increased the intracellular accumulation of fluorescent Rh123.

Discussion

MDR in tumor cells involves complex intracellular mechanisms, including decreased drug uptake and increased drug efflux, which reduce intracellular drug concentrations (16,17). P-gp, encoded by the mdr1 gene, functions in a manner similar to a pump, to extrude chemotherapy drugs from cancer cells. The inhibition of P-gp transporter function or inhibition of its expression may reverse the MDR phenotype (18). Our previous investigations revealed that ASIV not only reduced the protein expression of P-gp and gene expression of mdr1, but also inhibited P-gp-mediated drug efflux in the MDR Bel-7402/FU human hepatic cancer cell line (7). However, which signaling pathway was involved in the ASIV-induced downregulated expression of mdr1 remained to be elucidated. Studies have indicated that activation of the JNK signaling pathway or the transcription factor, c-Jun, has a principal role in mdr1-induced MDR (19,20). Inhibition of the JNK signaling pathway enhances the sensitivity of hepatocellular carcinoma cells to cisplatin by downregulating the expression of P-gp (13). In the present study, the protein expression levels of p-JNK and p-c-Jun were decreased when the Bel-7402/FU cells were treated with ASIV. In addition, SP600125, an inhibitor of the JNK signaling pathway, downregulated the expression of P-gp and mdr1 in the Bel-7402/FU cells. These results showed that the activation of JNK may be essential for the induction of mdr1 in Bel-7402/FU cells.

To further elucidate the molecular mechanism underlying the effect of ASIV on the reversal of drug resistance in Bel-7402/FU cells; the present study examined the DNA binding activity of AP-1 to the mdr1 promoter. The AP-1 transcription factor, belonging to the leucine zipper family, consists of dimers of Jun/Jun (c-Jun, JunB and JunD) or Jun/Fos (c-Fos, FosB, Fos-related antigen 1, Fos-related antigen 2, activating

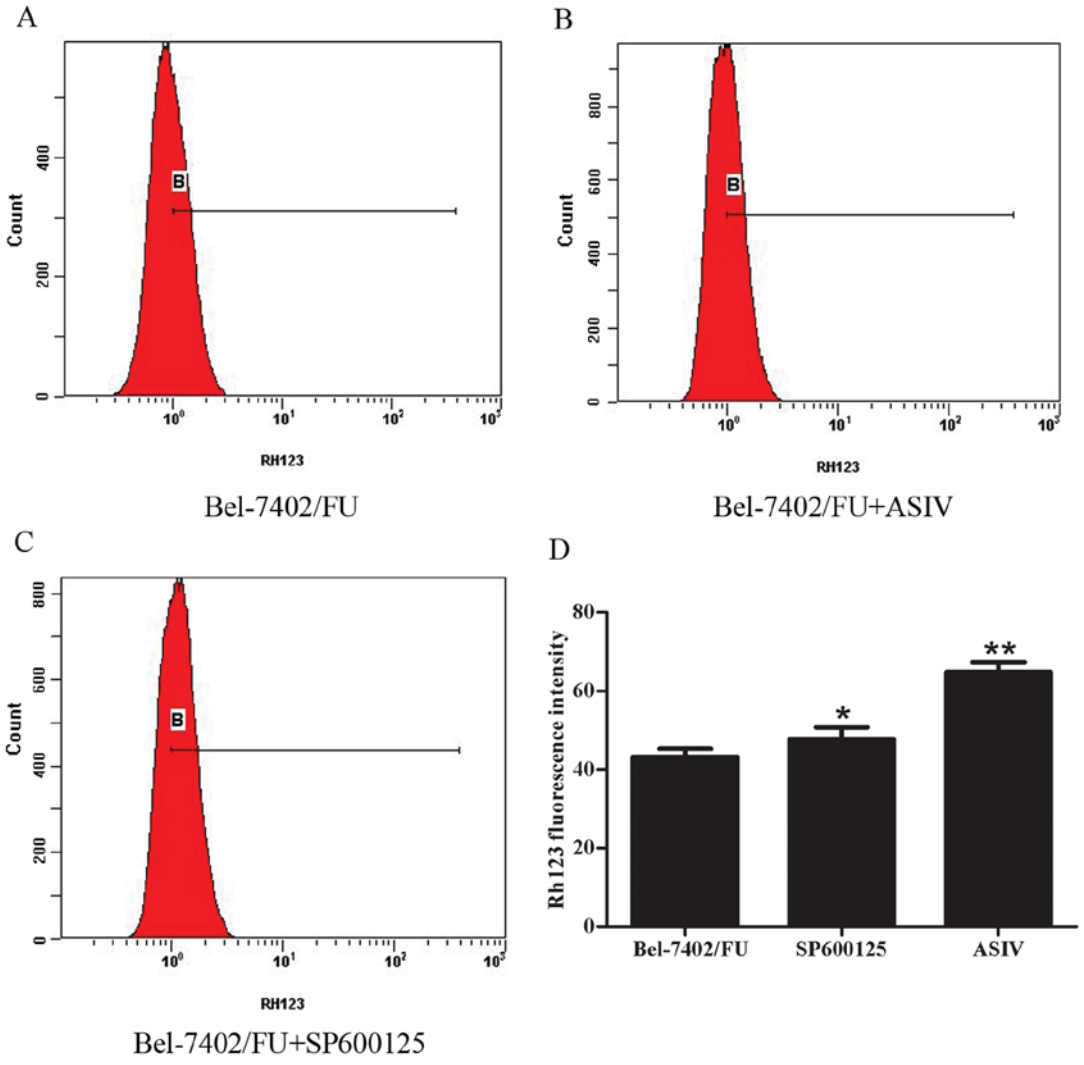

Figure 5. Effect of ASIVand SP600125 on the accumulation of Rh123 in Bel-7402/FU cells. The intracellular Rh123 concentration was determined using flow cytometry. (A) Bel-7402/FU cell control; (B) Bel-7402/FU cells treated with 0.1 mM ASIV; (C) Bel-7402/FU cells treated with 11 μM SP600125. (D) Rh123 fluorescence intensity. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01, compared with the Bel-7402/FU cell control group. ASIV, astragaloside; Rh123, rhodamine 123.

transcription factor 2 and cAMP response element binding protein) proteins (21,22). Activated c-Jun homodimers and/or heterodimers with c-Fos form the AP-1 transcription complex, recognize TPA-responsive elements (5'-TGAG/CTCA-3') and activate the transcription of target genes. Previous studies have demonstrated that the promoter region of the mdr1 gene contains a putative binding site for AP-1 (23). To investigate whether the decreased expression of p-c-Jun in Bel-7402/FU cells treated with ASIV has a functional effect on AP-1 binding activity, the present study used an EMSA to determine the binding activity of AP-1 to an oligonucleotide probe containing the relevant mdr1 promoter sequences. When the Bel-7402/FU cells were treated with ASIV or SP600125, AP-1 binding activity was decreased. Therefore, AP-1 may be involved in the ASIV-mediated downregulated expression of mdr1.

The results of the present study are consistent with previous reports that ASIV reverses the MDR of Bel-7402/FU cells by downregulating the mRNA expression of mdr1 and protein expression of P-gp (7). In the present study, the JNK inhibitor, SP600125, reduced the expression of mdr1 and P-gp, and increased the intracellular accumulation of Rh123 in Bel-7402/FU cells. The results showed that activation of the JNK signaling pathway promoted MDR, whereas SP600125 partially reversed MDR in Bel-7402/FU cells.

Previous studies have reported that astragaloside II (ASII) reverses the MDR of Bel-7402/FU cells through inhibiting the phosphorylation of extracellular signal-regulated kinases (ERKs), p38 and JNK (14). The results in the present study demonstrated that ASIV may reverse the drug resistance of Bel-7402/FU cells by downregulating the expression of mdr1 via inhibition of the JNK/c-Jun/AP-1 signaling pathway. However, whether other MAPK signaling pathways, including ERK and p38 MAPK kinases, are involved in the ASIV-induced downregulation of mdr1 requires elucidation in further investigations.

Acknowledgements

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