Original Paper

Asiatic Acid (AA) Sensitizes Multidrug-Resistant Human Lung Adenocarcinoma A549/DDP Cells to Cisplatin (DDP) via Downregulation of P-Glycoprotein (MDR1) and Its Targets

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Key Words

Asiatic acid • P-glycoprotein • Multidrug resistance • A549/DDP cells

Abstract

Background/Aims: P-glycoprotein (P-gp, MDR1) is associated with the phenotype of multidrug resistance (MDR) and causes therapy failure in the management of cancers. Searching for effective MDR modulators and combining them with anticancer drugs is a promising strategy against MDR. Asiatic acid (AA), a natural triterpene isolated from the plant *Centella asiatica*, may have a significant antitumor activity. The present study assessed the reversing effect of AA on MDR and possible molecular mechanisms of AA action in MDR1-overexpressing cisplatin (DDP)-resistant lung cancer cells, A549/DDP. **Methods:** Human lung adenocarcinoma A549/DDP cells were either exposed to different concentrations of AA or treated with DDP, and cell viability was measured by the MTT assay. A Rhodamine 123 efflux assay, immunofluorescence staining, ATPase assay, reverse-transcription PCR (RT-PCR), and western blot analysis were conducted to elucidate the mechanisms of action of AA on MDR. **Results:** The results showed that AA significantly enhanced the cytotoxicity of DDP toward A549/DDP cells but not its parental A549 cells. Furthermore, AA strongly inhibited P-gp expression by blocking *MDR1* gene transcription and increased the intracellular accumulation of the P-gp substrate Rhodamine 123 in A549/DDP cells. Nuclear factor (NF)-κB (p65) activity, κB degradation, and NF-κB/p65 nuclear translocation were markedly inhibited by pretreatment with AA. Additionally, AA inhibited the MAPK-ERK pathway, as indicated by decreased phosphorylation of ERK1 and -2, AKT, p38, and JNK, thus resulting in reduced activity of the Y-box binding protein 1 (YB1) via blockage of its nuclear translocation. **Conclusions:** AA reversed P-gp-mediated MDR by inhibition of P-gp expression. This effect was likely related to downregulation of YB1, and this effect was mediated by the NF-κB and MAPK-ERK pathways. AA may be useful as an MDR reversal agent for combination therapy in clinical trials.

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Introduction

Lung cancer is the leading cause of mortality among cancer patients, and non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases [1]. Current chemotherapy protocols for NSCLC are known to induce chemoresistance and have a range of adverse effects. Cisplatin (DDP) is the first-line drug used to treat NSCLC. However, most patients suffer from DDP resistance, greatly limiting the treatment of NSCLC [2]. Therefore, studies on bioactive agents that circumvent these limitations and target NSCLC are needed. Triterpenoids, which are synthesized in many plants by the cyclization of squalene, are widely used in Asian medicine [3]. For example, ursolic acid, asiatic acid, and oleanolic acid have been reported to show antitumor properties [4-6]. Recently, many studies revealed that some triterpenoids, such as ursolic acid and oleanolic acid, are P-glycoprotein inhibitors and reverse multidrug resistance (MDR) in cancer cells [7-8].

P-glycoprotein (P-gp), a well-documented ATP-binding cassette (ABC) transporter, is an integral plasma membrane protein that contains 12 transmembrane domains and two ATP-binding sites. Using energy provided by ATP hydrolysis, P-gp can extrude compounds with diverse structures from cells, decreasing the drug concentration within cells and subsequently reducing the efficacy of chemotherapeutic drugs. High P-gp expression is associated with the emergence of MDR and a reduced response to chemotherapy in many cancers [10]. Therefore, inhibition of P-gp-mediated drug efflux is thought to resensitize multidrug-resistant cancer cells to chemotherapy [11]. Numerous natural and synthetic substances have been demonstrated to be effective at overcoming MDR *in vitro* and *in vivo* [12]. These substances include calcium channel blockers (verapamil), immunosuppressants (cyclosporine A and rapamycin), calmodulin antagonists (trifluoperazine), protease inhibitors (MG132), and triterpenoids, such as ursolic acid (UA) and oleanolic acid (OA) [13-16].

Asiatic acid (AA, Fig. 1A) is a triterpene that has been isolated from the plant *Centella asiatica*, which is a plant used in herbal formulations of traditional Chinese medicine. Increasing evidence suggests that it possesses biological effects, including anticancer, anti-inflammatory, hepatoprotective, and antioxidant properties [17, 18]. Some studies indicate that AA improves memory and learning abilities in treated Sprague-Dawley rats [19]. AA was found to break down acetylcholinesterase more effectively than the acetylcholinesterase inhibitor or neostigmine and galantamine in a thin-layer chromatography bio-

Fig. 1. Structure of AA and inhibitory effects of AA on the growth of A549 and A549/DDP cells. (A) Chemical structure of triterpene asiatic acid. (B) Cytotoxicity of AA (5–40 μ M) toward A549 and A549/DDP cells was determined by the MTT assay. Each data point denotes the mean $\pm$ SD of three independent experiments performed in triplicate. A549 and A549/DDP cells were treated with different concentrations of AA for 48 h.

autographic assay and then to block the excitatory transmission in the hippocampal Schaffer collateral-CA1 synapse in a dose-dependent manner for GABAA antagonists [20]. Notably, AA can affect multiple signaling pathways and interact with diverse molecular targets. It also has been demonstrated that AA suppresses all three stages of cancer development: initiation, promotion, and progression [21]. AA interacts with cancer cells thus amplifying pro-apoptotic signal transduction [22]. Recently, AA has been widely studied in terms of its bioactivities. However, the effects of AA on MDR in cancer cells via inhibition of P-gp expression remain unclear. In this study, we tested whether AA enhances the cytotoxicity of DDP and downregulates P-gp (i.e., MDR1) in A549/DDP cells. Furthermore, we examined whether modulation of MDR1 expression involves the NF- κ B pathway and MAPK-ERK pathway mediated by YB1 in A549/DDP cells. We observed the ability of AA to sensitize DDP-resistant lung cancer to DDP and provided insights into the possible mechanisms.

Materials and Methods

Reagents

AA, DDP, 3-[4, 5-dimethylthiazol-2-yl]2, 5-diphenyltetrazolium bromide (MTT), and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies for immunoblotting, i.e., anti-P-gp monoclonal antibodies, anti-NF- κ B, anti-phosphorylated I κ B α (p-I κ B α), anti-YB-1, anti-phosphorylated ERK1/2 (p-ERK1/2), anti-phosphorylated AKT (p-AKT), anti-phosphorylated p38 (p-p38), and anti-phosphorylated JNK (p-JNK) were purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody, rabbit anti-polyclonal antibody, mouse anti-GAPDH antibody, and anti-Lamin B1 monoclonal antibody were purchased from Proteintech (Rosemont, IL, USA). Other reagents were purchased locally from commercial suppliers. Stock solutions of AA (25–100 mM) were prepared in dimethyl sulfoxide (DMSO) and added to the mitochondrial reaction medium or cell culture medium at 1/1000 (v/v) dilution.

Cell culture

Human lung adenocarcinoma A549 cells and cisplatin-resistant A549/DDP cells were purchased from the Academy of Military Medical Sciences (Beijing, China). Cells were cultured in the RPMI-1640 medium supplemented with 10% of fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in an atmosphere containing 5% CO₂ at 37°C. In all experiments, cells were used in the exponential growth phase.

Cell proliferation assay

The MTT assay was performed to assess the effect of AA on cell proliferation and chemosensitivity to DDP. Cells during logarithmic growth were seeded in 96-well plates at a density of 5.0×10^3 cells/well for the proliferation assay, followed by treatment with various concentrations of AA dissolved in DMSO (final DMSO concentration 1%) for different periods. Next, 20 μ L sterile MTT dye (5 mg/mL) was added at 37°C for 4 h. Then the culture medium was removed and 150 μ L of DMSO was added, and the resultant liquid was thoroughly mixed for 10 min. Spectrometric absorbance at 570 nm was determined on a microplate reader.

To assess the chemosensitivity to DDP, 1.0×10^4 A549 or A549/DDP cells were seeded in 96-well plates and allowed to adhere for 24 h. The cells were then covered with a medium containing 1.0 μ g/mL DDP (IC₅₀ = 1.5 and 8.6 μ g/mL for A549 and A549/DDP cells, respectively) in combination with different concentrations of AA (5, 10, 15, or 20 μ M) and incubated for another 48 h. The MTT assay was performed as described above.

The concentration at which AA alone or AA in combination with DDP produced 50% inhibition of growth was calculated from the relative survival curve. Positive and negative control groups were also included. The P-gp inhibitor verapamil (1.0 μ g/mL) served as a positive control for A549/DDP cells and the negative control group (CK) was treated with the same volume of DMSO.

All the experiments were repeated three times. The cell growth inhibition of each drug was calculated using the following formula: Inhibition = $100\% \times [1 - (\text{dosing group A}/\text{control group A})]$. Data were graphed on a semi-logarithmic curve with drug concentrations on the X-axis and cell inhibition on the Y-axis. SPSS

software (SPSS, Inc., Chicago, IL, USA) was employed to calculate the 50% inhibitory concentration (IC_{50}) and resistance index (RI). The formula for calculating RI was as follows: $RI = IC_{50}$ of resistant cell line/ IC_{50} of sensitive cell line.

Double-staining [annexin V–fluorescein isothiocyanate (FITC) with propidium iodide (PI)] flow-cytometry analysis of apoptosis in A549/DDP and A549 cells treated with AA and DDP

A549/DDP and A549 cells were incubated with AA (20 μ M) and DDP (1.0 μ g/mL) both alone and in combination for 72 h. The cells were then collected and digested with trypsin. The cells were centrifuged at 1000 rpm for 5 min and were then washed 3 times with precooled phosphate-buffered saline (PBS) and resuspended in 500 μ L of binding buffer. Next, 5 μ L of annexin V–FITC (Multisciences, Hangzhou, China) was added and the mixture was incubated in the dark for 5 min. Finally, 10 μ L of PI (Multisciences, Hangzhou, China) was added, and the mixture was allowed to react in the dark for 5 min. Fluorescence of the mixture was measured on a flow cytometer (Accuri C6, BD Biosciences, San Diego, CA, USA) at 488 nm.

Immunofluorescent staining

A549/DDP cells were grown on coverslips in 24-well plates and allowed to adhere to the plastic overnight. The cells were then exposed to AA (20 μ M) and DDP (1.0 μ g/mL) alone or in combination for 48 h at 37°C. The cells were washed with PBS and fixed for 30 min in 4% paraformaldehyde (PFA, BD Biosciences) at room temperature. Next, the cells were permeabilized for 30 min in 0.1% Triton X-100 and incubated for 1 h at room temperature with a primary antibody (a rabbit anti-P-gp antibody diluted in PBS supplemented with 3% bovine serum albumin). The cells were rinsed with PBS and incubated with 2 μ g/mL Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (secondary antibody) for 1 h at room temperature. Nuclei were counterstained with DAPI via 5 min incubation at room temperature. After a wash with PBS, the slides were mounted in the Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) and analyzed under a FluoView™ FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan) and analyzed with the Olympus Fluoview Ver. 1.7b viewer (Olympus Corporation).

The P-gp ATPase assay

The activity of P-gp ATPase in A549/DDP cells was determined with the P-gp-Glo assay system (Promega, Madison, WI, USA). According to the user protocol provided by the vendor, the activity of P-gp ATPase was measured in the presence of ATP (as a positive control) or AA (20 μ M) and DDP (1.0 μ g/mL), alone or in combination. The luminescence of the sample reflects the ATP level in the sample, which negatively correlates with the activity of P-gp ATPase and is recorded by the Multi-Mode Microplate Reader (Synergy Mx, Biotek, Winooski, VT, USA). Test compound treatment-related activities were expressed as the percentage of basal activity, via a comparison of the basal activity with the treatment-related activities, the compounds were then classified as stimulating, inhibiting, or having no effect on basal P-gp ATPase activity.

RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from AA-treated and DDP-treated cells. The RNA was purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The resulting cDNA samples were amplified by real-time PCR with SYBR Green Real time PCR SuperMix (Invitrogen) by means of the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The amplification reaction mixture (25 μ L) contained 1 μ L cDNA, forward primers, reverse primers, and SYBR® Green Real time PCR Master Mix. The samples were analyzed in triplicate. The PCR program consisted of 40 cycles of 95°C for 10 s and then 60°C for 20 s and 72°C for 30 s. All reactions were conducted in triplicate. The primer sequences for each gene are listed

Table 1. Sequences of primers for quantitative reverse-transcription PCR

gene	Primer (Forward, 5'-3')	Primer (Reverse, 5'-3')	Product size(bp)
MDR1	TCATCGAGTCACTGCCTAAT	CTATGGCAATGCGTTGTTTC	90
P65	CCAGCTTCTGGTACTCTCCT	TGTTAGGCACAGGGACAATG	166
IκBα	TGATCCTGAGCTCCGAGACT	GTGTGGCCATTGTAGTTGGT	150
AKT	ATCGCTTCTTTGCCGTATC	CTTGGTCAGGTGGTGTGATG	150
JNK	TGTGTTTCATGCCAGACTTCT	ATGTCTCTTCCCTCCATCT	180
ERK1/2	AACACCACCTGCGACCTTAA	ACAGACCAGATGTCGATGGA	170
P38	CTGTGCGATTGCTGGAGAAGA	GGATCGTGGTACTGAGCAAA	100
YB1	AGGACGGCAATGAAGAAGAT	GTCTGCGTCGGTAATTGAAG	102
18s rRNA	CCTGGATACCGCAGCTAGGA	GCGGCGCAATACGAATGCCCC	112

in Table 1. 18S rRNA served as an internal control. PCR products were subjected to electrophoresis in a 1.5% agarose gel. The fold change in gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method after normalization to the expression level of 18S rRNA.

Preparation of nuclear and cytosolic extracts

Nuclear extracts were prepared with a commercial kit (KeyGEN, Nanjing, China). All the steps were performed on ice or at 4°C, unless stated otherwise. Protease inhibitors (10 mg/mL aprotinin and 10 mg/mL leupeptin) and reducing agents (1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride, PMSE) were added to each buffer immediately prior to use. Briefly, the cells were incubated in 10 volumes of hypotonic Buffer A (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl) on ice for 15 min and homogenized. Nuclei were recovered by centrifugation at 16,000 × g for 5 min, and the supernatant was collected as cytosolic extract. The nuclei were extracted with Buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂) for 40 min on ice. Insoluble material was removed by centrifugation at 16,000 × g for 10 min, and the supernatant was designated as the nuclear extract.

Western blot analysis

A549/DDP cells were treated with normal saline, while A549/DDP and A549 cells were untreated. After treatment with AA (20 μM), DDP (1.0 μg/mL), or their combination for 72 h, the cells were collected and lysed with RIPA (Beyotime, Shanghai, China) lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.02% sodium azide, 150 mM NaF, 1 μg/mL aprotinin) for 30 min on ice. After that, the cells were centrifuged at 16,000 × g for 10 min. The media were collected, and total protein concentration was detected in the Lowry (bicinchoninic acid, Beyotime) assay. Proteins were analyzed by SDS-PAGE in an 8% gel and then electrotransferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) using a constant current of 4 mA overnight at 4°C. The polyvinylidene fluoride membranes were blocked with 5% nonfat milk for 2 h, then incubated with anti-P-gp, anti-p65, anti-IκBα, anti-p-AKT, anti-p-ERK1/2, anti-p-JNK, anti-p-PP2A, anti-YB1, anti-Lamin B1, or anti-GAPDH antibodies (primary antibodies) overnight at 4°C. Expression of GAPDH (or Lamin B1) served as a loading control. A goat anti-rabbit IgG (H+L) secondary antibody (1:1000) was added for 2 h incubation at room temperature. After washing with TBST (1× Tris-buffered saline, 0.05% Tween 20), the bound antibody complexes were detected with the enhanced chemiluminescence reagent (ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA) followed by X-ray film exposure. The experiment was repeated three times.

The Rhodamine 123 efflux assay of P-gp function

A549/DDP cells were incubated with AA (20 μM) and DDP (1.0 μg/mL) either alone or in combination for 72 h. The cells were resuspended in the medium, and then 10 μL/mL Rhodamine 123 staining solution (Santa Cruz Biotechnology, Santa Cruz, TX, USA) was added, and the cells were incubated at 37°C in 5% CO₂ for 30 min. The cells were then centrifuged at 2000 rpm for 5 min, washed twice with the medium, resuspended, and incubated for 20 min. Next, the cells were centrifuged again, washed twice with PBS, and analyzed by flow cytometry at 488/530 nm. This experiment was repeated three times.

Statistical analysis

Results are expressed as the means ± standard deviation (SD). All data were obtained from at least three independent experiments and showed a similar pattern. The data were subjected to one-way analysis of variance followed by Dunnett's multiple-comparison test. Differences with a P value less than 0.05 were considered statistically significant.

Results

Influence of AA on cancer cell viability

Considering the mode of action of MDR-reversing agents, low cytotoxicity is key for these agents. We used the human lung tumor cell line A549 and its related drug-resistant cell line A549/DDP to detect the cytotoxicity of AA by the MTT assay. AA showed no significant toxicity to all cell lines used in this study (the IC₅₀ values were all >50 μM), and AA at 20

μM showed no cytotoxicity toward any of the cell lines (more than 90% of A549 and A549/DDP cells were viable when treated with AA up to 20 μM , Fig. 1B). Therefore, 20 μM was chosen as the highest MDR reversal concentration of AA, and we used 5, 10, 15, and 20 μM AA to reverse MDR *in vitro*.

AA potentiates the sensitivity of the anticancer drug in P-gp-overexpressing cells

To determine whether AA sensitized the lung cancer cells to DDP-induced death, A549 and A549/DDP cells were treated with 5, 10, 15, and 20 μM AA in combination with 1.0 $\mu\text{g/mL}$ DDP for 48

Table 2. Effects of AA on IC_{50} of DDP toward A549 and A549/DDP cells. ^a $P < 0.05$, significantly different from the DDP group for A549 cells vs. control. ^b $P < 0.05$, significantly different from the DDP group for A549/DDP cells vs. control

Treatment	$\text{IC}_{50}(\mu\text{g/mL})$		Resistance index
	A549	A549/DDP	
DDP(control)	1.52 $\pm$ 0.11	8.67 $\pm$ 0.13	5.70
DDP+AA(5 μM)	1.04 $\pm$ 0.07	6.45 $\pm$ 1.65	6.20
DDP+AA(10 μM)	0.50 $\pm$ 0.05 ^a	4.70 $\pm$ 0.70 ^b	9.40
DDP+AA(15 μM)	0.22 $\pm$ 0.01 ^a	3.10 $\pm$ 0.10 ^b	14.18
DDP+AA(20 μM)	0.12 $\pm$ 0.01 ^a	3.24 $\pm$ 0.71 ^b	27.00
DDP+Verapamil(1 $\mu\text{g/mL}$)	0.08 $\pm$ 0.01 ^a	2.88 $\pm$ 0.08 ^b	31.00

Fig. 2. Effects of AA on apoptosis of A549 and A549/DDP cells. (A) Representative dot plots illustrating the apoptotic status of A549 and A549/DDP cells. (B) Percentages of apoptotic A549 and A549/DDP cells. A549 and A549/DDP cells were treated with AA (20 μM), DDP (1.0 $\mu\text{g/mL}$), or their combination for 72 h. The P-gp inhibitor verapamil (1.0 $\mu\text{g/mL}$) served as a positive control. Apoptosis was detected by annexin V-FITC and PI staining with flow cytometry. * $P < 0.05$; ** $P < 0.01$ as compared to control.

h. As presented in Table 2, combined treatment markedly decreased the DDP concentrations for achieving 50% growth inhibition when compared to DDP as monotherapy. The IC_{50} of DDP in each combined treatment group showed a negative correlation with AA concentrations, suggesting that AA reverses DDP resistance and enhances DDP sensitivity.

The effect of AA on apoptosis of the drug-resistant cell line A549/DDP

Apoptosis of A549/DDP cells in each group was as follows: control group $0.99\% \pm 0.87\%$; AA group $2.63\% \pm 1.00\%$; DDP group $12.16\% \pm 2.76\%$; AA+DDP group $44.24\% \pm 1.88\%$; Verapamil+DDP group $67.79\% \pm 4.25\%$; and apoptosis rates of A549 cells were as follows: control group $1.23\% \pm 0.92\%$; AA group $2.25\% \pm 1.22\%$; DDP group $29.66\% \pm 3.85\%$; AA+DDP group $21.43\% \pm 2.20\%$; Verapamil+DDP group $42.72\% \pm 3.52\%$. Making the combination of AA and DDP resulted in significant apoptosis of A549/DDP cells (Table 2).

Determination of the effects of AA on P-gp nuclear translocation by immunofluorescence staining

Here, we used an anti-P-gp antibody and nuclear dye DAPI to study P-gp nuclear translocation. As shown in Fig. 3, the green fluorescence of P-gp protein in the control group was bright and showed a concentrated distribution in the nucleus. The merged pictures revealed numerous P-gp subunits in the nucleus. However, in the control-treatment groups (particularly in the AA+DDP group), the green fluorescence of P-gp protein was weak and revealed a loose distribution. The merged pictures revealed only a small amount of the P-gp subunit in the nucleus. These results indicate that AA+DDP treatment significantly inhibited P-gp translocation and limited the distribution to the cytoplasm at the usual cytosolic level.

AA enhanced accumulation of Rhodamine 123 in A549/DDP cells

To investigate the mechanism by which AA reversed the functions of P-gp, we evaluated the effects of AA on rhodamine accumulation in A549/DDP cells (Fig. 4A). In the absence

Fig. 3. Immunofluorescence microscopy analysis of the nuclear translocation of P-gp in A549/DDP cells. The cells were treated with AA (20 μ M), DDP (1.0 μ g/mL), and their combination for 72 h. The P-gp inhibitor verapamil (1.0 μ g/mL) served as a positive control. After 30 min of treatment, the cells were incubated with an anti-P-gp antibody and Alexa Fluor 488-conjugated F(ab')₂ fragment antibody (secondary antibody), and nuclei were stained with DAPI. The images were obtained by means of a fluorescence inverted microscope. The images of the green area (representative of the region that contains P-gp) and blue area (representative of the nuclear part that binds DAPI) were overlaid to create a merged fluorescence pattern in areas of colocalization.

of AA, the level of Rhodamine 123 accumulation was low in A549/DDP cells. In contrast, AA when combined with DDP increased the Rhodamine 123 accumulation. These results indicated that AA elevated the sensitivity of A549/DDP cells toward DDP by increasing anticancer drug (the P-gp substrate) accumulation inside multidrug-resistant cancer cells.

AA inhibited P-gp ATPase activity

The major function of P-gp is to increase drug efflux, which is one of the most important mechanisms of MDR for decreasing intracellular drug concentrations. Compounds that

Fig. 4. Effects of AA on rhodamine accumulation and P-gp ATPase activity in A549/DDP cells. (A) The effect of AA on rhodamine accumulation. * $P < 0.05$ as compared to control; # $P < 0.05$ as compared to DDP. (B) The effect of AA on P-gp ATPase activity. P-gp ATPase activity in response to the treatment with AA (20 μM), DDP (1.0 $\mu\text{g/mL}$), or their combination for 72 h. The P-gp inhibitor verapamil (1.0 $\mu\text{g/mL}$) was used as a positive control. Data were expressed as the means $\pm$ SD. * $P < 0.05$ as compared to the basal level of P-gp ATPase activity.

Fig. 5. Effects of AA on P-gp and P-gp-related gene expression according to qRT-PCR analysis of A549/DDP cells. Quantification data are presented. The quantification was performed by assigning a value of 1.0 to the control group. A549/DDP cells were treated with AA (20 μM), DDP (1.0 $\mu\text{g/mL}$), or their combination for 72 h. The P-gp inhibitor verapamil (1.0 $\mu\text{g/mL}$) was employed as a positive control. 18S rRNA served as an internal control. The data are expressed as the mean $\pm$ SD of three experiments with replicates. * $P < 0.05$ as compared to control; # $P < 0.05$ as compared to DDP treatment group.

Fig. 6. AA downregulated P-gp by inhibiting YB1 activity in A549/DDP cells, and this effect was mediated by the NF- κ B and MAPK-ERK pathways. A549/DDP cells were incubated with AA (20 μ M), DDP (1.0 g/mL), or their combination for the indicated periods. The P-gp inhibitor verapamil (1.0 μ g/mL) was used as a positive control. The cells were subjected to western blot analysis with anti-P-gp and specific antibodies related to NF- κ B and MAPK-ERK pathways. AA+DDP treatment decreased P-gp, p-AKT, p-ERK1/2, p-JNK, p-p38, and p-I κ B α amounts (A). The nuclear and cytosolic protein was extracted from cell lysates. Western blot analysis was performed with antibodies against nuclear p65, YB-1, and Lamin B1 as well as cytosolic p65, YB-1, and GAPDH. Bands of GAPDH and phosphoprotein relevant to total protein confirmed the integrity of analysis and equal loading of total protein and phosphoprotein, respectively. The Lamin B1 and GAPDH bands confirmed the integrity of analysis and equal loading of nuclear and cytosolic protein, respectively (B). *Significant difference from the control group (P<0.01).

not directly interact with P-gp have no effects on basal ATPase activity; compounds that are substrates of transport by P-gp stimulate basal ATPase activity, whereas inhibitors show attenuating effects. Next, to confirm that AA did not directly interact with P-gp, we measured the change in luminescence associated with P-gp ATPase hydrolysis. As depicted in Fig. 4B, the AA+DDP treatment obviously increased P-gp ATPase activity to a level comparable to

that of the positive control verapamil group (verapamil functions as a competitive inhibitor of P-gp). These findings suggested that AA is a substrate for transport by P-gp and stimulates P-gp ATPase activity.

The effect of AA on MDR1 mRNA expression and expression of MDR1-related genes in A549/DDP cells

The results of real-time PCR analyses of A549/DDP cells are shown in Fig. 5. Compared to the control group, the mRNA levels of MDR1, p65, AKT, JNK, ERK1/2, and YB1 increased significantly ($P < 0.05$) in the DDP-treated group. In contrast, the mRNA levels of MDR1, p65, AKT, JNK, ERK1/2, and YB1 significantly decreased in the AA+DDP treatment group compared to the DDP-only treatment group ($P < 0.05$). Similar results were observed in the verapamil+DDP treatment group. Additionally, the mRNA levels of $\text{I}\kappa\text{B}\alpha$ changed significantly in the opposite manner in all treatment groups.

Effects of AA on P-gp and P-gp-related signaling proteins in A549/DDP cells

As shown in Fig. 6A, obvious decreases were observed in the protein levels of P-gp, p-AKT, p-ERK1/2, p-JNK, p-p38, and p-I κ B α after cotreatment with AA and DDP as compared to the control group. Moreover, the amounts of all proteins did not clearly change after treatment with DDP alone. Similar results were observed in the verapamil+DDP treatment group. Additionally, treatment with AA+DDP markedly inhibited the nuclear translocation of p65 and YB1 and nuclear concentration of p65 and YB1 subunit (Fig. 6B).

Discussion

Resistance to drug therapy remains a major challenge in cancer management. Cellular drug efflux mediated by ABC transporters is thought to play an important role in drug resistance. P-gp, one of the most extensively studied and important drug transporters [23], has been investigated in clinical studies. In addition, elevated expression and activity of P-gp have been reported to be associated with poor clinical outcomes among cancer patients [24]. Based on these findings, a reversal approach was formulated for modulation of P-gp activity as a therapeutic approach [25]. A large number of compounds including verapamil, cyclosporine A, curcuminoids, and curcumin was demonstrated to exert reversal effects on MDR. However, these compounds failed to enter the clinic because of their severe adverse effects and poor pharmacokinetic properties [26]. In the present study, we found that AA significantly increases the sensitivity of multidrug-resistant cancer cells A549/DDP toward the anticancer drug DDP, making AA a promising candidate for reversing drug resistance in cancer chemotherapy.

The main mechanism underlying MDR development involves overexpression of ABC efflux transporters. Consequently, one strategy, based on the use of ABC transporter inhibitors in combination with anticancer drugs, for MDR reversal has been developed. Some natural products were shown to significantly potentiate the cytotoxicity of conventional chemotherapeutic agents in P-gp-overexpressing multidrug-resistant cells by physically inhibiting P-gp activity [27]. Our data suggest that AA in combination with DDP at nontoxic doses markedly enhanced the antitumor activity of DDP toward A549/DDP cells *in vitro* (Fig. 2). These results indicate that AA exerted a potent MDR reversal activity on P-gp-overexpressing multidrug-resistant cells. Our experiments also revealed that AA alone inhibited cell growth and induced apoptosis in A549 cells and DDP-resistant A549/DDP cells. Of note, combination treatment of A549/DDP cells with AA and DDP resulted in a significant decrease in cellular proliferation and an increase in the percentage of apoptotic cells.

Some studies have shown that chemoresistance may be attributed to expression of the multidrug resistance gene (*MDR1*) and its encoded protein P-gp, which has been found to reduce drug uptake [28]. In our study, after treatment of A549/DDP cells with AA alone or with a combination of DDP with AA, the expression of MDR1 (P-gp) significantly decreased. P-gp encoded by the *MDR1* gene is a transmembrane protein, which belongs to the ABC

transporter protein superfamily. It functions as an ATP-dependent drug efflux pump [29] and is known to decrease the intracellular concentration of chemotherapeutic agents [30]. MDR1 (P-gp) is deeply involved in tumor chemoresistance [31]. In this study, the results of the Rhodamine 123 efflux assay revealed that the function of P-gp was significantly inhibited in AA-only treatment group and in combination treatment groups. Thus, AA attenuates chemoresistance of cells when used in combination with DDP, showing that AA sensitizes A549/DDP cells to DDP by suppressing the function of P-gp.

It has been reported that ATP hydrolysis and substrate transport are tightly coupled, and most compounds known to be transported by P-gp stimulate ATPase activity [32]. To explore the inhibitory mechanism of action of AA on P-gp, the effects on ATPase activity of P-gp were measured in a purified reconstituted P-gp-containing membrane. The ATPase activity of P-gp was stimulated by AA+DDP treatment as well as by verapamil, which is known to be a substrate of P-gp. These results suggest that AA is a substrate of P-gp and competes with verapamil to interact with the drug-binding site of P-gp.

Many studies have addressed drug resistance mechanisms, which can be classified into two classes [33]: one is the genetic and epigenetic alternations of tumor cells that affect drug sensitivity; the other is impairment of the drug delivery system of tumor cells. In this study, we found that downregulation of P-gp by AA effectively reversed the drug sensitivity of A549/DDP cells. Additionally, we noticed that several signaling pathways in the PI3K-AKT-mTOR pathway were downregulated. AKT-mediated signaling is a major fundamental cell functions such as cell proliferation, DNA repair, and antiapoptosis processes [34]. It has been reported that AKT activation occurs in solid tumors frequently [35]. A point mutation in AKT leads to its activation in a PI3K-independent manner [36]. Based on the results of qRT-PCR and western blotting, AKT-mTOR signaling may be involved in the regulation of drug sensitivity by inhibiting cell proliferation. These results provide important new insights into drug resistance modulation.

P-gp expression is mediated by several signaling pathways including NF- κ B, MAPK, PI3K, COX-2, and ROS [37]. Among these, the NF- κ B and MAPK pathways are the most important factors in terms of their molecular mechanisms relating to induction of MDR. The NF- κ B pathway responds actively to oxidative stress induction, as indicated by formation of reactive oxygen species, activation of the I κ B kinase, and degradation of I κ B [38-41]. Besides, NF- κ B binds to nucleotide position -1 of the MDR1 promoter, thereby mediating the transcription of MDR1 [42]. Similarly, the MAPK pathway, comprising ERK, JNK, and p38, plays critical roles in the transduction of signals triggered by various types of stimuli to regulate the expression of MDR1 [43]. In addition, numerous studies suggest that the overexpression of P-gp is closely associated with nuclear localization of YB1 in solid tumors such as breast cancer, ovarian cancer, and prostate cancer [44]. Additionally, it has been reported that the MAPK pathway regulates the phosphorylation [45]. Our results indicated that AA alone and in combination with DDP decreased the amounts of P-gp, p-ERK1/2, p-JNK, p-p38, and p-I κ B α , and inhibited YB1 nuclear translocation as compared to treatment with DDP alone. Thus, AA is likely to downregulate P-gp, and this action is likely associated with inhibition of the NF- κ B and MAPK pathways in A549/DDP cells.

Taken together, our current experiments indicate that AA significantly increases the efficacy of DDP against A549/DDP cells and reverses the multidrug-resistant phenotype by blocking the function of P-gp and by inhibiting MDR. Furthermore, AA suppressed YB1 nuclear translocation, which was mediated by the NF- κ B and MAPK-ERK pathways. This action likely correlates with the downregulation of P-gp. Although we preliminarily identified the possible mechanism of AA's inhibitory action on P-gp expression, how AA interacts with P-gp via the NF- κ B and MAPK-ERK pathways is unknown. Moreover, our results are also limited to human lung cancer cell lines A549/DDP and A549 *in vitro*. Our further research will focus on whether AA can reverse MDR in other cancer cells as well as animal models *in vivo*.

Conclusion

Our data indicate that AA reverses drug resistance of A549/DDP cells by inhibiting the expression and activity of P-gp. The mechanism may be associated with the suppression of YB1 activation, which was found to be mediated by the NF- κ B and MAPK-ERK pathways. Our study verified the reversing effects of the natural product AA on drug resistance via interaction with P-gp. This finding suggests that AA may be useful as a new natural chemosensitizer in combination with conventional drugs to overcome MDR in the management of lung cancer.

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Disclosure Statement

The authors have no conflict of interests to declare.

References

- 1 Nakagawa T, Endo H, Yokoyama M, Abe J, Tanaka Y, Tanaka N, Sato I, Takahashi S, Kondo T, Satoh K: Large noncoding RNA HOTAIR enhances aggressive biological behavior and is associated with short disease-free survival in human non-small cell lung cancer. *PLoS One* 2013;8:e61222.
- 2 Cao L, Chen J, Ou B, Liu C, Zou Y, Wang Q: GAS5 knockdown reduces the chemo-sensitivity of non-small cell lung cancer (NSCLC) cell to cisplatin through regulating miR-21/PTEN axis. *Biomed Pharmacother* 2017;93:570-579.
- 3 Liby KT, Yore MM, Sporn SA: Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. *Nat Rev Clin Oncol* 2007;7:357-369.
- 4 Kim ES, Moon JH: Asiatic acid inhibits the invasive phenotype of SNU-484 human gastric cancer cells. *Oncol Lett* 2015;9:17-21.
- 5 Li ZW, Li CD, Chen H, Chen XS, Bai YS: Asiatic acid inhibits adipogenic differentiation of bone marrow stromal cells. *Cell Physiol Biochem* 2014;68:437-442.
- 6 Wu Y, Liu S, Liu D, Chen G, Hu N, Suo Y, You J: Simultaneous determination of six triterpenic acids in some Chinese medicinal herbs using ultrasound-assisted dispersive liquid-liquid microextraction and high-performance liquid chromatography with fluorescence detection. *J Pharm Biomed Anal* 2015;107:98-107.
- 7 Jeyaraj M, Mancha A, Malik G, Wei SJ, Kim DJ, Liang H, Slaga TJ: Resveratrol and P-glycoprotein inhibitors enhance the anti-skin cancer effects of ursolic acid. *Mol Cancer Res* 2013;11:1521-1529.
- 8 Villar VH, Vogler O, Barcelo F, Gomez-Florit M, Martinez-Serra J, Obrador-Hevia A, Martin-Broto J, Ruiz-Gutierrez V, Alemany R: Oleonic and maslinic acid sensitize soft tissue sarcoma cells to doxorubicin by inhibiting the multidrug resistance protein MRP-1, but not P-glycoprotein. *J Nutr Biochem* 2014;25:429-438.
- 9 Dallas S, Block ML, Thompson DM, Bonini MG, Ronaldson PT, Bendayan R, Miller DS: Microglial activation decreases retention of the protease inhibitor saquinavir: implications for HIV treatment. *J Neuroinflammation* 2013;10:58.
- 10 Panzarini E, Dini L: Nanomaterial-induced autophagy: a new reversal MDR tool in cancer therapy? *Mol Pharm* 2014;11:2527-2538.

- 11 Ma L, Wei S, Yang B, Ma W, Wu X, Ji H, Sui H, Chen J: Chrysosplenetin inhibits artemisinin efflux in P-gp-over-expressing Caco-2 cells and reverses P-gp/MDR1 mRNA up-regulated expression induced by artemisinin in mouse small intestine. *Pharm Biol* 2017;55:374-380.
- 12 Stark M, Assaraf YG: Structural recognition of tubulysin B derivatives by multidrug resistance efflux transporters in human cancer cells. *Oncotarget* 2017;8:49973-49987.
- 13 Takacs D, Csonka A, Horvath A, Windt T, Gajdacs M, Riedl Z, Hajos G, Amaral L, Molnar J, Spengler G: Reversal of ABCB1-related Multidrug Resistance of Colonic Adenocarcinoma Cells by Phenothiazines. *Anticancer Res* 2015;35:3245-3251.
- 14 Katiyar SS, Muntimadugu E, Rafeeqi TA, Domb AJ, Khan W: Co-delivery of rapamycin- and piperine-loaded polymeric nanoparticles for breast cancer treatment. *Drug Deliv* 2016;23:2608-2616.
- 15 Advani MJ, Siddiqui I, Sharma P, Reddy H: Activity of trifluoperazine against replicating, non-replicating and drug resistant *M. tuberculosis*. *PLoS One* 2012;7:e44245.
- 16 Ma J, Yu L, Tian J, Mu Y, Lv Z, Zou J, Li J, Wang H, Xu W: MG132 reverse the malignant characteristics of hypopharyngeal cancer. *Mol Med Rep* 2014;9:2587-2591.
- 17 Jing Y, Wang G, Ge Y, Xu M, Tang S, Gong Z: AA-PMc, a novel asiatic acid derivative, induces apoptosis and suppresses proliferation, migration, and invasion of gastric cancer cells. *Oncol Rep* 2016;9:1605-1621.
- 18 Hao C, Wu B, Hou Z, Xie Q, Liao T, Wang T, Ma D: Asiatic acid inhibits the induced inflammatory response in human gingival fibroblasts. *Int Immunopharmacol* 2017;50:313-320.
- 19 Nasir MN, Habsah M, Zamzuri I, Rammes G, Hasnan J, Abdullah J: Effect of asiatic acid on passive and active avoidance task in male Sprague-Dawley rats. *J Ethnopharmacol* 2011;134:203-209.
- 20 Nasir MN, Abdullah J, Habsah M, Ghani RI, Rammes G: Inhibitory effect of asiatic acid on acetylcholinesterase, excitatory post synaptic potential and locomotor activity. *Phytomedicine* 2012;19:311-316.
- 21 Shanmugam MK, Nguyen AH, Kumar AP, Tan BK, Selvaraj T: Targeted inhibition of tumor proliferation, survival, and metastasis by pentacyclic triterpenoids: a potential role in prevention and therapy of cancer. *Cancer Lett* 2012;320:158-170.
- 22 Zhang X, Wu J, Dou Y, Xia B, Rong W, Rimoldi J, Zhou J: Asiatic acid protects primary neurons against C2-ceramide-induced apoptosis. *J Ethnopharmacol* 2016;179:51-59.
- 23 Miron A, Aprotosoaie AC, Trifanescu J: Flavonoids as modulators of metabolic enzymes and drug transporters. *Ann N Y Acad Sci* 2015;1360:152-167.
- 24 Yan JK, Zhu J, Gu BL, Yang Y, Xiao Y, Wang KJ, Wen J, Wang Y, Cai W: Soybean Oil-Based Lipid Emulsion Increases Intestinal Permeability of Lipopolysaccharide in Caco-2 Cells by Downregulation of P-Glycoprotein via ERK-1/2 Signaling Pathway. *Cell Physiol Biochem* 2016;39:1581-1594.
- 25 Ma Y, Wang Y, Yang Y, Fanon JK, Liu F: Extreme low dose of 5-fluorouracil reverses MDR in cancer by sensitizing cancer cells and fibroblasts and down-regulating P-gp. *PLoS One* 2017;12:e180023.
- 26 Yang X, Gu K: P-gp Inhibition-Based Strategies for Modulating Pharmacokinetics of Anticancer Drugs: An Update. *Curr Drug Metab* 2016;17:806-826.
- 27 Wu X, Gu Z, To KK, Fang X, Wang F, Cheng B, Fu L: Effect of abemaciclib (LY2835219) on enhancement of chemotherapeutic agents in ABCB1 and ABCG2 overexpressing cells *in vitro* and *in vivo*. *Biochem Pharmacol* 2017;124:29-42.
- 28 Karamanlidou-Metropoulos S, Iqbal M, Kostaki A, Ortiga-Carvalho TM, Gibb W, Matthews SG: Acute Effects of Viral Exposure on P-Glycoprotein Function in the Mouse Fetal Blood-Brain Barrier. *Cell Physiol Biochem* 2017;41:1044-1050.
- 29 Silva R, Vilas-Boas V, Carmo H, Dinis-Oliveira RJ, Carvalho F, de Lourdes BM, Remiao F: Modulation of P-glycoprotein efflux pump: induction and activation as a therapeutic strategy. *Pharmacol Ther* 2015;149:1-123.
- 30 Machado D, Fernandes L, Costa SS, Cannalire R, Manfroni G, Tabarrini O, Couto I, Sabatini S, Viveiros M: Mode of action of the 2-phenylquinoline efflux inhibitor PQQ4R against *Escherichia coli*. *PeerJ* 2017;5:e3168.
- 31 Ding Z, Xu F, Li G, Tang J, Tang Z, Jiang P, Wu H: Knockdown of Akt2 expression by shRNA inhibits proliferation, enhances apoptosis, and increases chemosensitivity to paclitaxel in human colorectal cancer cells. *Cell Biochem Biophys* 2015;71:383-388.

- 32 Zhang R, Saito R, Shibahara I, Sugiyama S, Kanamori M, Sonoda Y, Tominaga T: Temozolomide reverses doxorubicin resistance by inhibiting P-glycoprotein in malignant glioma cells. *J Neurooncol* 2016;126:235-242.
- 33 Correia S, Poeta P, Hebraud M, Capelo JL, Igrejas G: Mechanisms of quinolone action and resistance: where do we stand? *J Med Microbiol* 2017;66:551-559.
- 34 Papa A, Caruso D, Strudel M, Tomao S, Tomao F: Update on Poly-ADP-ribose polymerase inhibition for ovarian cancer treatment. *J Transl Med* 2016;14:267.
- 35 Wu ZT, Yao D, Ji SY, Ni X, Gao YM, Hui LJ, Pan GY: Optimized Hepatocyte-Like Cells with Functional Drug Transporters Directly-Reprogrammed from Mouse Fibroblasts and their Potential in Drug Disposition Toxicology. *Cell Physiol Biochem* 2016;38:1815-30.
- 36 Mahajan K, Mahajan NP: PI3K-independent AKT activation in cancers: a treasure trove for novel therapeutics. *J Cell Physiol* 2012;227: 3178-3184.
- 37 Syed SB, Coumar MS: P-Glycoprotein Mediated Multidrug Resistance Reversal by Phytochemicals: A Review of SAR & Future Perspective for Drug Design. *Curr Top Med Chem* 2016;16: 2484-2502.
- 38 Li F, Zhang J, Arfuso F, Chinnathambi A, Zayed ME, Alharbi SA, Kumar AP, Ahn KS, Sethi G: NF- κ B inhibition in cancer therapy. *Arch Toxicol* 2015;89:711-731.
- 39 Chai EZ, Siveen KS, Shanmugam MK, Arfuso F, Sethi G: Analysis of the intricate relationship between chronic inflammation and cancer. *Biochem J* 2015;468:1-15.
- 40 Ningegowda R, Shivananju NS, Rajendran P, Basappa, Rangappa KS, Chinnathambi A, Li F, Achar RR, Shanmugam MK, Bist P, Alharbi SA, Lim LHK, Sethi G, Priya BS: A novel 1,2,4-triazole d-1, 2,4-triazolo-1, 3, 4-thiadiazole derivative inhibits tumor cell invasion and potentiates the apoptotic effect of TNF α by abrogating NF- κ B activation cascade. *Apoptosis* 2017;22:145-157.
- 41 Shanmugam MK, Lee JH, Chai EZ, Kanchi MM, Kar S, Arfuso F, Phamarajan A, Kumar AP, Ramar PS, Looi CY, Mustafa MR, Tergaonkar V, Bishayee A, Ahn KS, Sethi G: Cancer prevention and therapy through the modulation of transcription factors by bioactive natural compounds. *Semin Cancer Biol* 2016;40-41:35-47.
- 42 Bentires-Alj M, Barbu V, Fillet M, Chariot A, Pignatelli B, Janssens A, Delen J, Merville MP, Bours V: NF-kappaB transcription factor induces drug resistance through up-regulation of MDR1 expression in cancer cells. *Oncogene* 2003;22:90-97.
- 43 Shen H, Xu W, Luo W, Zhou L, Yang J, Chen Y, Zhang Q, Han X: Upregulation of mdrl gene is related to activation of the MAPK/ERK1/2 signal transduction pathway and YB-1 nuclear translocation in B-cell lymphoma. *Exp Hematol* 2004;32:105-112.
- 44 Kuwano M, Oda Y, Izumiya S, Ueda T, Iwamoto Y, Toi M, Fujii T, Yamana H, Kinoshita H, Kamura T, Tsuneyoshi M, Yasumoto K, Kohno K: The role of nuclear Y-box binding protein 1 as a global marker in drug resistance. *Mol Cancer Ther* 2004;3:1485-1492.
- 45 Sinnberg T, Sauerbrey P, Spangler B, Kuphal S, Bosserhoff A, Schitteck B: MAPK and PI3K/AKT mediated YB-1 activation promotes melanoma cell proliferation which is counteracted by an autoregulatory loop. *Exp Dermatol* 2016;27:263-270.