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Original Research

Ailanthone reverses multidrug resistance by inhibiting the P-glycoprotein-mediated efflux in resistant K562/A02 cells

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Abstract: Multidrug resistance (MDR) poses a great impediment to cancer treatment. Excessive expression of ATP-binding cassette transport protein AC-1 (P-glycoprotein, P-GLP) is usually involved in MDR. In this study, ailanthone (AIL), a natural compound extracted from the whole seedlings of *Ailanthus altissima* (Simaroubaceae) was shown to mediate the reversal of P-GLP-induced MDR and restore the susceptibility of K562/A02 cells to doxorubicin (DOX). Further mechanistic studies revealed that AIL increased intracellular DOX accumulation and interrupted Rh123 efflux through suppression of P-GLP, and also suppressed P-GLP ATPase activity. At the same time, it markedly inhibited MDR1 gene expression and P-GLP protein to sensitize the cytotoxic effect of DOX. Furthermore, AIL down-regulated P-GLP expression by inhibiting the PI3K/Akt pathway. Thus, AIL could be a potential therapeutic compound for reversing P-GLP-mediated drug resistant cancer.

Key words: Ailanthone; multidrug resistance; P-glycoprotein-mediated efflux in resistant K562/A02 cells.

Introduction

Chemotherapy is the most common treatment for cancer, while resistance of tumor cells to chemotherapy drugs is the main problem in chemotherapy failure (1, 2). It is known that MDR arises because of cross-resistance of many anti-tumor drugs with structural and mechanistic differences, resulting in resistance of tumor cells to any anti-tumor drug (3,4). The mechanisms of MDR have been extensively studied. They involve induction of efflux systems (e.g. ATP-binding cassette transporters); alteration of apoptosis signaling pathway, activation of general detoxifying trails, and enhancement of DNA repair. These mechanisms confer resistance on tumor cells, either alone or in combination (5 - 9). Among these, upregulation of transmembrane transporters of ATP-binding cassette (AC) subfamily i.e. AC-1 (MDR1/P-GLP), AC-2 (breast cancer resistance protein, BCRP), and ACC-1 multidrug resistance-associated protein 1, MRP-1) have attracted the most interest from researchers (10, 11). In particular, P-GLP plays a significant role in the production of MDR. Studies have shown that inhibition of P-GLP enhances the transport of cytotoxic drugs and results in decreased intracellular accumulation of drugs (12, 13). Unfortunately, studies on MDR reversal agents and some biological therapies have been limited in clinical application due to concerns about correlative toxic side effects (14, 15). On the other hand, treatment of malignancies with Traditional Chinese Medicine has been practised for quite a while, and there is growing interest in its pharmacological activities such as its killing effects on tumor cells, induction of tumor cell differentiation and apoptosis, enhancement of sensitivity to chemotherapeutic drugs, reduction of the toxicity of chemotherapy drugs, and improvement of the immune function in patients (16 -19). Therefore, the clinical use of traditional Chinese medicine for reversing multidrug resistance of tumors may be a promising strategy.

Ailanthone (AIL), a natural compound extracted from the whole seedlings of *Ailanthus altissima* (Simaroubaceae) has many pharmacological properties such as antimalarial and anti-tumor effects (20 - 22). Being a natural medicine, AIL has great clinical and market potential. The capacity of AIL to annul MDR was investigated in this study using a DOX-resistant human leukemia K562/A02 cell line. The reversal activity of AIL was first evaluated, followed by investigation of the underlying mode of action.

Materials and Methods

Materials

Ailanthone was product of Puruifa Science & Technology Development Co. (Chengdu, China). It was solubilized in DMSO (Sigma, USA). Anti-P-GLP monoclonal antibody was supplied by Santa Cruz Biotech. Inc. (CA, USA). Primary antibodies anti-p-Akt were products of Cell Signaling Technol. Inc. (MA). β-Actin mouse monoclonal antibody was supplied by NeoMarkers, (Fremont, USA). Verapamil (VRP), rhodamine 123 (Rh123), doxorubicin (Dox), vincristine (VCR), verapamil (VRP), MTT, Ko143 (BCRP), LY402913, LY294002 and other reagents were products of Sigma

(USA).

Cell culture

The cell lines were K562 and its DOX-selective P-GLP- overexpressing form K562/A02; human ovarian carcinoma cell line 2008/P and MRP1-overexpressing derivative cell line 2008/MRP1; human embryonic kidney (HEK) 293 cell lines, HEK293/pcDNA3.1 (transfected with empty vector), HEK293/R2 (transfected with BCRP), and normal human gastric epithelial cell strain-1 (GES-1). They were cultured at 37°C in DMEM or RPMI 1640 having 10 % FBS **i**n a 5% $CO_{2.}$ environment**.**

Cytotoxicity and MDR Reversal Assay by MTT

The anti-metastatic property of AIL on various cell lines were assessed using MTT (23). The diverse cells in logarithmic phase were subjected to seeding in 96-well plates, with 1×10^4 cells/well. In cytotoxicity and MDR reversal tests, cells were treated for 48 h with cancer-killing drugs (DOX, DNR, VLB, PTX, or CTX) at 37 °C in 5% CO_2 environment in the presence or absence of different concentrations of AIL. The cells were exposed to MTT after 44 h of incubation. At the end of incubation, the supernatant was removed through centrifugation, and the resultant formazan crystals were solubilized in DMSO prior to absorbance reading at 490 nm in a microplate reader. Values of IC_{50} were obtained using GraphPad Prism 6.0 (CA, USA). The experiments were conducted in triplicate for each concentration of AIL.

Determination of intracellular DOX accumulation by AIL

DOX accumulation assays were carried in triplicate out using the method described earlier (24). The cells were separately incubated with 20 μM DOX and varying levels of AIL (0.1, 2 and 5 μM) for 2.5 h at 37 °C. In this assay, DMSO and VRP were negative and positive controls, respectively. The resultant lysate was subjected to fluorescence spectrophotometry at excitation/emission wavelengths of 460 nm/587 nm, to determine the amount of DOX accumulated within the cells.

Assay of Rh123 efflux

The assay of Rh123 efflux from K562/A02 cells with or without AIL was carried out according to the procedure described in a previous study (25). Mean fluorescence intensity (MFI) of residual Rh123 in 10000 cells was determined with flow cytometry (BD FACSCalibur, USA). The data obtained were used to generate plots of MFI against time**.** Three independent experiments were performed.

Assay of P-GLP ATPase

ATPase was assayed according to the firefly luciferase reaction method (26). The reaction mixture contained 25 μg of P-GLP, 0.5% DMSO, 100 μM sodium vanadate, 0.1, 2.0 or 20.0 μM of VRP; and 0.1, 2.0 or 20.0 μM of AIL. The reaction was initiated by addition of 5.0 mM MgATP and the plate was incubated at 37 °C for 1 h. Luminescence was read in a luminometer. The decrease in luminescence of a sample relative to that of sample exposed to sodium vanadate was used as a measure of ATPase activity.

CYP3A4 assays

Differential centrifugation was used to separate rat liver microsomes and their protein levels were assayed using Bradford method. In the assay, testosterone was used as substrate for CYP3A4, and the metabolic products were subjected to HPLC analysis (27). The influence of AIT on CYP3A4 was assessed based on level of 6β-OH testosterone (6β-OHT) formed from testosterone as a result of CYP3A4 metabolism (28). The reaction mixture contained hepatic microsomes and testosterone (50 mM) with or without AIL, with ketoconazole as positive control at graded levels (2.5, 5.0, 10.0, 20.0 and 40.0 μM. The HPLC analysis was carried out with a C18 reverse-phase HPLC column, using a gradient method under the following conditions: column temperature of 35 °C; flow rate of 1.0 mL/min; with mobile phase A (water), and mobile phase B (acetonitrile). The gradient program was: 0 -7.5 min: 35 % of B; 7.5 -14.0 min: gradient of 35 - 48 % of B; 14.0 - 19.0 min: gradient of 48 - 35 % of B gradient. Optical density was taken at 254 nm, and peak areas were calculated using LC-Solution.

RT-PCR

K562 and K562/A02 cells were subjected to incubation for 48 h in the absence or presence of AIL at different doses prepared in RPMI1640, to determine the mRNAs of MDR1 in them. Extracted total mRNA was converted to cDNA and subjected to quantitative RT-PCR (29). The enzyme was heat-activated for 5 min at 95 °C. Then, the cDNA was amplified for 40 cycles viz: denaturation at 95 °C for 15 sec and annealing/extension at 58 °C for 30 sec, followed by melting curve analysis at 55 °C for 1 min. Subsequently, the temperature was raised at the rate of 0.5 °C/10 sec) so as to assess the generation of primer-derived trimmers and dimmers. The primer sequences are indicated below: MDR1 forward: 5'-TGGAGAGATCCTCACCAAGC-3',MDRI reverse: 5'-TTCCTGTCCAAGATTTGCT-3'; β-actin forward: 5'-GTGGGCGCCCCAGACACCA-3', and β–actin reverse: 5'-CTCCTTAATGTCACGCACGATTC-3'.

Western blot

Western blotting was employed for studying alterations in the expressions of proteins (30). After different treatments, K562/A02 cells were harvested and lysed, and BCA protein assay kit (Thermo Fisher, MA) was applied for estimation of protein concentrations using the procedure outlined in previous studies (31). Primary antibodies for P-gp, p-Akt and β-actin were used, with β-Actin as the internal reference. The immune-reactive bands were observed through chemiluminescence and were acquired with a Bio-Rad molecular imager. Total protein intensity was obtained using ImageJ, relative to β-actin. The pictures of gels were just cut by Adobe Photoshop CS6 (San Jose, USA) without any other processing.

Statistical analysis

Data are presented as mean of arbitrary values ± SD. Statistical analysis was done with one-way ANOVA followed by Tukey's multiple comparison. All analyses

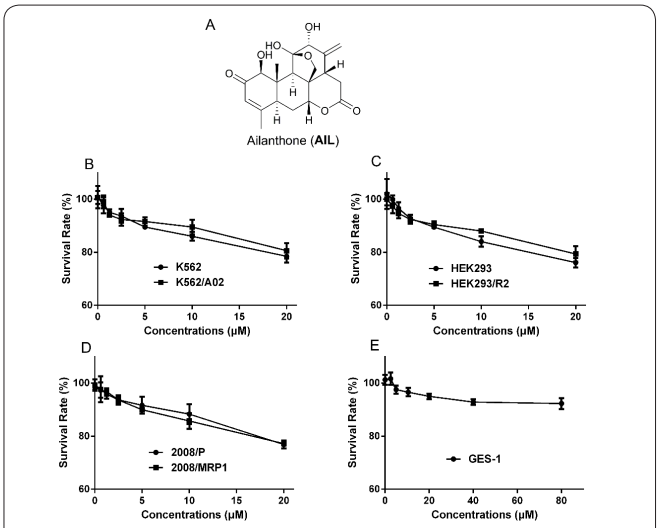

Figure 1. The structure of AIL, and anti-proliferative effects of AIL against various cell lines. (A) The structure of AIL; (B) K562 and K562/A02; (C) HEK 293 cells, and HEK293/R2 (BCRP-transfected); (D) 2008/P and MRP1 overexpressing derivative cell line 2008/MRP1; (E) GES-1.

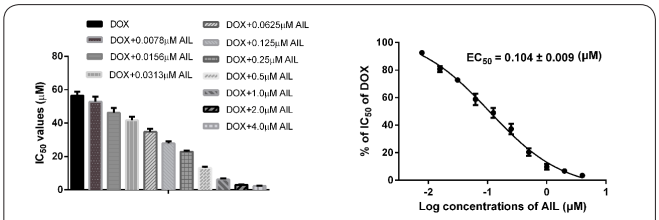

Figure 2. Effect of AIL on DOX insensitivity in K562/A02 cells. (A) IC_{50} values of DOX in K562/A02 cells treated with DOX with or without different concentrations of AIL; (B) EC_{50} value of AIL on DOX insensitivity in K562/A02 cells.

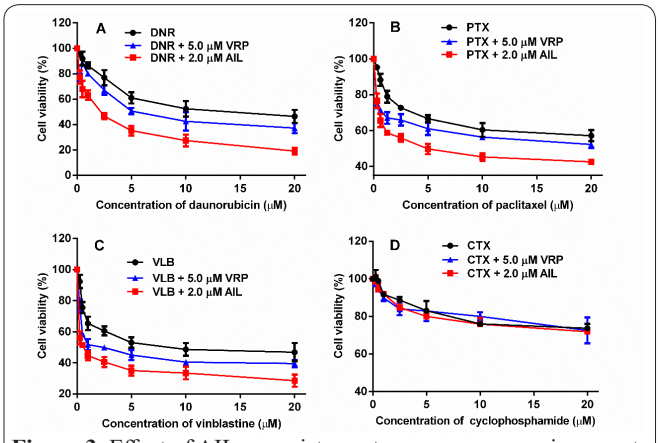

Figure 3. Effect of AIL on resistance to cancer-suppressing agents in K562/A02 cells: (A) DNR, (B) PTX, (C) VLB, (D) CTX. Results are shown as mean ± SD.

were carried out with GraphPad Prism 6.0 statistical software. Values of $p < 0.05$were taken as statistically significant.

Results

In vitro cytotoxicity of AIL against diverse cell lines

The cytotoxicity of AIL was investigated in diverse cell lines using MTT method. The results and the structure of AIL are shown in Figure 1. The results showed that even at high concentrations, the inhibitory effects of AIL was weak. The survival of the cells was higher than 70 % at 20 μM. Thus, the non-toxic concentration of 2.0 μM was selected for subsequent assays on MDR reversal activity. Besides, AIL had no cytotoxicity towards normal GES-1 cells ((IC_{50} > 80 μM, Fig.1E).

Selective suppression of P-GLP over BCRP and MRP1 by AIL

As depicted in Table 1, 2.0 μM AIL restored DOX-sensitivity of resistant K562/A02 cells, with reversal fold (RF) of 26.4. Under the same condition, AIL was almost deprived of reversal activity for MRP1 (RF=1.9 in 2008/MRP1 cells) and BCRP (RF=2.5 in HEK293/R2 cells). VRP, LY402913 and Ko143 as inhibitors of P-GLP, MRP1 and BCR, respectively were effective in reversing P-GLP, MRP1 and BCRP-mediated MDR with RF of 5.3, 19.8 and 25.9, respectively.

Selectivity of AIL

To ensure measurable effectiveness of reversal of DOX-insensitivity, EC_{50} values of AIL in modulating DOX-resistance in K562/A02 cells were determined using standard methods (32). As shown in Figure 2, AIL effectively reversed resistance to DOX, with EC_{50} in nanomolar range (104 ± 9 nM). The results revealed that AIL was not cytotoxic to GES-1 cell line (IC_{50} > 80 μM, Fig.1E). Thus, the selective index (SI) for AIL had very high value (> 769), indicating that as an MDR antagonist, AIL was not toxic to normal cells when co-administrated with other anticancer drugs.

Modulating influence of AIL on P-GLP-mediated insensibility to cancer-suppressing drugs

Figure 3 shows distinctly that AIL increased cytotoxic influence of PTX, VLB and DNR which have relationship with the P-GLP-induced MDR, but it did not affect non-MDR cytotoxicity such as CTX.

Table 1. Effect of AIL on P-GLP, MRP1- and BCRP- mediated drug resistance[a].

Cell lines	Over-expressed	Resistant to	IC_{50} [μM] (RF)		
			No modulators	AIL	Controls[b]
K562	Nd	Nd	0.62 ± 0.05 (94.1)		
K562/A02	P-gp	DOX	58.37 ± 2.49 (1.0)	2.21± 0.19 (26.4)	11.17 ± 1.27 (5.2)
2008/P	Nd	Nd	0.56 ± 0.04 (23.6)		
2008/MRP1	MRP1	Paclitaxel	13.24 ± 0.15 (1.0)	7.08 ± 0.22 (1.9)	0.67 ± 0.09 (19.8)
HEK293	Nd	Nd	0.27 ± 0.02 (44.2)		
HEK293/R2	BCRP	Topotecan	11.93 ± 0.13 (1.0)	4.77 ± 0.39 (2.5)	0.46 ± 0.68 (25.9)

[a] Modulating activity of AIL (at 2.0 μM) and control modulators on P-GLP, MRP1 and BCRP were determined with K562/A02, 2008/MRP1 and HEK293/R2 cells. Results are presented as mean ± SD (n = 3). nd: not determined. [b] Controls mean the P-GLP inhibitor (VRP) for K562/A02 cells, MRP1 inhibitor (LY402913) for 2008/MRP1 cells and BCRP inhibitor (Ko143) for HEK293/R2 cells.

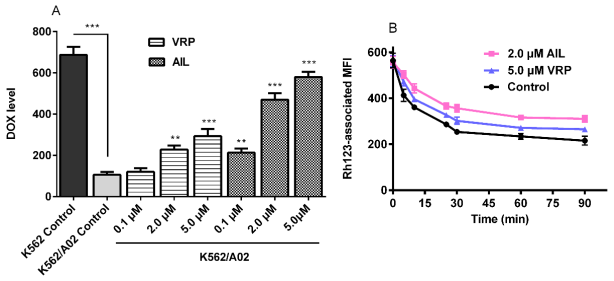

Figure 4. Effect of AIL on (A) intracellular DOX retention and (B) Rh123 efflux **in** K562/A02 cells. Results are shown as mean ± SD (n = 3). K562/A02.

Effect of AIL on DOX accumulation

The DOX accumulation in the presence or absence of AIL in K562 and K562/A02 cells was determined so as to investigate the underlying mechanism. From the results shown in Figure 4A, the fluorescence of DOX was much more intensive in K562 cells than in K562/A02 cells. The intracellular retention of DOX in sensitive cells was 6.51 folds higher in resistant cells, indicating the DOX resistance of K562/A02. Intracellular DOX retention was raised 2.02-, 4.45- and 5.50-folds in K562/A02 cells treated with to 0.1, 2.0 and 5.0 μM AIL, respectively.

Effect of AIL on Rh123 efflux

As demonstrated in Figure 4B, 2.0 μM AIL significantly **i**nhibited Rh123 efflux from K562/A02 cells, relative to control ($p < 0.001$). At 5, 10, 25, 30, 60 and 90 min, intracellular Rh123-linked MFI in AIL-exposed cells was higher than that in 5.0 μM VRP-exposed cells, indicating that AIL exerted stronger inhibition than the positive controls. These results demonstrate that AIL reversed MDR through inhibition of P-GLP-enhanced drug efflux.

Effect of AIL on P-GLP-ATPase

At 20 μM, VRP enhanced the activity of P-GLP-ATPase 1.88-fold (Figure 5A). However, at 0.1 μM, AIL provoked inhibition of P-GLP-ATPase, resulting in activity below basal value (Figure 5A), indicating that AIL, in contrast to VRP, can suppress P-GLP-ATPase.

No drug-interplay effect of AIL on cytochrome P3A4 (CYP3A4)

As shown in Figure 5B, ketoconazole suppressed CYP3A4 dose-dependently, while AIL had did not affect CYP3A4 even at 40 μM.

Down-regulation of the expression of MDR1 gene and P-gp protein by AIL

The PCR and western blot analyses demonstrated that MDR1 gene was expressed both in sensitive K562 and resistant K562/A02 cells. However, these genes were significantly highly expressed in K562/A02 cells, relative to their expressions in K562 cells (Figures 6A-6C). After treatment with AIL, the expression of MDR1 gene was significantly down-regulated in a concentration-dependent manner: the higher the dose, the stronger the down-regulation (Figure 6D-F). When co-administrated with DOX, a stronger down-regulation effect was shown than that obtained with single administration of DOX (Figures 6H and 6I).

Effect of AIL on PI3K/Akt signaling pathway

As indicated in Figures 7A and 7B, treatment with AIL significantly reduced the expression of p-Akt, in a concentration-dependent manner. LY294002, a recognized inhibitor of PI3K/Akt signaling pathway was applied in this study. In Figures 7C and 7D, there were significant reductions in the expressions of p-Akt and P-GLP by both LY294002 and AIL. Besides, the co-administration of LY294002 and AIL resulted in more intensive and synergistic down-regulation of p-Akt expression. Taken together, these results show that inhibition of Akt phosphorylation to suppress the PI3K/Akt signaling pathway is a possible mechanism involved in reversal of MDR by AIL.

Discussion

The overexpression of ABC transportors such as P-

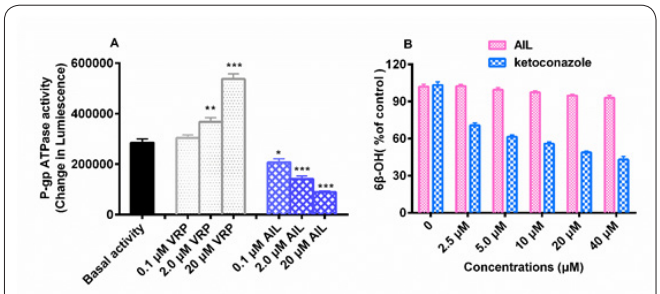

Figure 5. (A) Influence of AIL on P-GLP-ATPase. Results are shown as mean ± SD. ***$p < 0.001$, *$p < 0.05$, **$p < 0.01$***$p <$ 0.001, compared to basal value.

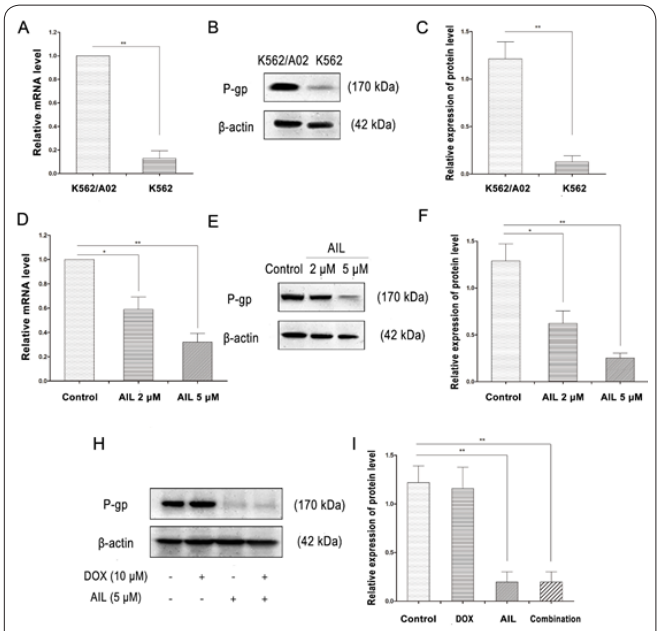

Figure 6. AIL-mediated down-regulation of MDR1 gene and protein expressions in K562/A02 cells. (A) mRNA expressions of MDR1 in K562 and K562/A02 cells; (B) Western blot analysis of P-GLP in K562 and K562/A02 cells; (C) Protein expressions of P-GLP in K562 and K562/A02 cells; (D) Quantitative analysis of effect of AIL on mRNA expression of MDR1 in K562/A02 cells; (E) Western blot analysis of effect of AIL on P-GLP in K562/A02 cells; (F) Quantitative analysis of effect of AIL on the protein expressions of P-GLP in K562/A02 cells; (G) Western blotting analysis of effect of AIL and DOX on P-GLP expressions; (F) Quantitative analysis of the effect of co-administration of AIL and DOX on protein expressions of P-GLP. *$p < 0.05$, **$p < 0.01$, relative to the control.

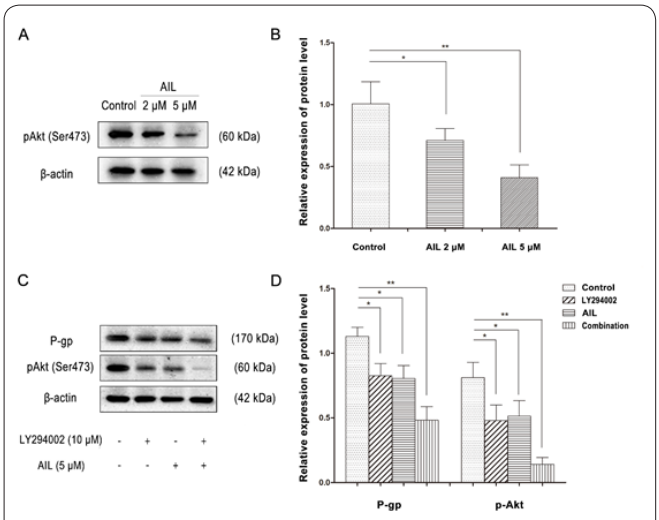

Figure 7. Effects of AIL on the PI3K/Akt signaling pathway. (A) Effect of AIL on the expressions of p-Akt; (B) Quantitatation of p-Akt expressions; (C) Effect of co-administration of AIL and LY294002 on expressions of P-gp and p-Akt; (D) Quantitation of expressions of P-gp and p-Akt. *$p < 0.05$, **$p < 0.01$, relative to the control.

GLP MRP1 and BCRP has strong negative effect on the succsess of cancer treatment. Quite a few efforts have been made to mantain the sensitivity to chemotherapy (11,37,38). Since the first P-gp inhibitor VRP was discovered in 1981 (39), some synthetic P-gp inhibitors have been obtained (40). However, their in-depth application were stopped due to low clinical effectiveness, unwanted pharmacokinetic interactions, and concerns about patients' safety (5). As a result, studies on natural products as novel P-GLP inhibitors have continued to attract the attention of researchers (41). The present study has demonstrated that AIL has a potential for reversing P-GLP mediated MDR. The intrinsic cytotoxic activity of AIL showed that it had very weak antiproliferation effect on diverse cancer cell lines even at 20 μM, and had no inhibitory activity towards normal GES-1 cells. At the non-toxic concentration of 2 μM, AIL selectivity exhibited markedly stronger inhibitory activity against P-GLP-mediated drug resistance than MRP1 or BCRP (42). Quantitative assessment of the effectiveness of reversal of DOX-resistance by AIL revealed it had EC_{50} in the nanomolar range (104 ± 9 nM) and high selective index (> 769), indicating that, as an MDR modulator, it is not toxic human cells (24). The effect of AIL on P-GLP-induced resistance to other chemotherapeutic agents (PTX, DNR VLB, and CTX) was also studied (43). The results revealed that AIL enhanced the cytotoxic potencies of PTX, VLB and DNR, three drugs which are linked to P-GLP-mediated MDR, but it did not affect the inhibitory activity of the non-MDR drug CTX. This indicates that the inhibition of P-GLP mediates the reversal of MDR resistance. Studies on elucidation of the underlying mechanism through DOX accumulation and Rh123 efflux assays demonstrated that the MDR reversal activity was triggered by inhibition of P-GLP functions (44). As a result, it can be speculated that AIL lowers resistance to DOX by enhancing retention of within K562/A02 cells. Moreover, AIL suppressed P-GLP-ATPase activity; this contributed to its inhibitory effect on P-GLP.

A similarity exists between P-GLP and CYP3A4 as regards substrates and tissue location. This phenomenon indicates the existence of many P-GLP blockers which are substrates for CYP3A4, resulting in pharmacokinetic interplay with drugs (35). The results of the present study have revealed that AIL has no anti-proliferation effect on CYP3A4 activity, and it was relatively safe even if co-administered with chemotherapeutics metabolized by CYP3A4.

The MDR1 gene involved varied between studies, and it had different effects in different treatments or cells (45, 46). Confirmation of these findings based on a specific gene over-expression is indispensable for all studies so as to develop a comprehensive outlook of the MDR1 gene participating in this process. In the present study, AIL caused an obvious and concentration-dependent downregulation in the expression of MDR1 gene in K562/A02 cells. Co-adminstration of AIL with DOX produced synergistic reduction effect on MDR1 expression. These results show that reduction of MDR-related gene expression is one of the mechanisms used for enhancing the entry of chemotherapeutic drugs into cells, so as to improve the intracellular concentrations of these drugs for increased cytotoxicity and apoptosis.

The PI3K/Akt pathway is considered a key molecular route in MDR, as reported previously (47 - 49). The present study also showed similar alterations in P-GLP protein and p-Akt which are involved in the PI3K/Akt signal route. The expressions of these proteins were decreased by AIL and the specific PI3K/Akt antagonist LY294002. A synergism between AIL and LY294002 was obtained. These findings indicate that the AIL-induced suppresion of the PI3K/Akt signaling pathway plays a key part in the reduction of expression of the MDR1 gene. Therefore, besides inhibition of P-GLP, down-regulation of gene and P-GLP expression are critical events in the reversal activity of AIL.

In summary, AIL ameliorated resistance to the chemotherapy drug DOX by inhibiting P-GLP, and the related mechanisms were further investigated and verified in K562/A02 cells. The findings indicate that AIL may be a novel and promising therapeutic agent for P-GLP-mediated drug resistance. A follow-up study *in vivo* is needed on the pharmacokinetic properties and therapeutic effect of AIL.

Acknowledgments
None.

Interest conflict
No competing interest is associated with this study.

Author contributions
Jienan Wei designed the research. Fang Han, Guoqiang Liu, Caifeng Sun, Jienan Wei performed the cytotoxicity and reversal experiments. Fang Han performed PCR and Werstern blot experiments. All authors analysed the results and took part in preparing the manuscript.

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