

Solanine reverses multidrug resistance in human myelogenous leukemia K562/ADM cells by downregulating MRP1 expression

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Abstract. Multidrug resistance (MDR) in leukemia cells is a major obstacle to chemotherapeutic treatment. High expression and constitutive activation of multidrug resistance protein 1 (MRP1) has been associated with the development of resistance to anticancer drugs in a number of tumor types. The activity of c-Jun N-terminal kinase 1 (JNK1) is associated with the occurrence of MDR and MRP1 expression. The present study aimed to investigate the ability of solanine to resensitize the Adriamycin® (ADR)-resistant human myelogenous leukemia cell line K562/ADM to ADR. Results of the Cell Counting Kit-8 assay demonstrated that solanine inhibited K562/ADM cell proliferation. K562/ADM cell sensitivity to ADR was increased following treatment with solanine, indicated by increased intracellular accumulation of ADR. Western blotting demonstrated that treatment with solanine led to reduced MRP1 protein expression, suggesting that solanine-induced ADR accumulation is due to the down-regulation of MRP1 expression. Solanine-mediated MRP1 downregulation was observed to be dependent on the JNK signaling pathway. In conclusion, the results of the present study suggest that solanine reverses MDR in K562/ADM cells and may represent a novel therapeutic agent for the treatment of human myelogenous leukemia.

Introduction

Chronic myelogenous leukemia (CML) is one of the most frequent types of cancer in humans and its treatment is limited by the development of multidrug resistance (MDR) (1). MDR is a phenomenon in which cancer cells develop resistance to

a variety of unrelated drugs following exposure to a single chemotherapeutic (2). A number of underlying mechanisms lead to the development of MDR; however, the increased efflux of cytotoxic drugs by ATP binding cassette (ABC) transporters is the most well studied mechanism (3). Multidrug resistance protein 1 (MRP1) is a member of the ABC family of transporters, which are primarily expressed in the plasma membrane and remove cytotoxic agents from the cell through active transport (4). Increased MRP1 expression leads to a reduction in the concentration of chemotherapeutics inside tumor cells, resulting in the decreased efficacy of these agents (5-10). MRP1 has been associated with resistance to various chemotherapeutic agents, including Adriamycin® (ADR; doxorubicin), methotrexate, etoposide and vincristine (11).

The c-Jun N-terminal kinases (JNK) belong to the mitogen-activated protein kinase (MAPK) family (12). There are three JNK isoforms in mammals, JNK1, JNK2 and JNK3, which are encoded by the MAPK8, MAPK9 and MAPK10 genes, respectively (13). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is primarily expressed in the heart, brain and testes (14). JNK activity can be induced by a number of stimuli, including inflammatory cytokines, environmental stressors and therapeutic agents (15). The JNK signaling pathway has been reported to be involved in cellular proliferation, apoptosis and differentiation, and tumor cell migration (16,17). Increasing evidence has indicated that JNK activity serves an important role in the development of chemoresistance and is associated with MRP1 expression (12-16).

Nightshade (*Solanum nigrum*) is used in traditional Chinese herbal medicine, and is used to treat a number of diseases including sores, carbuncles, swellings and other injuries (18). Solanine, a nightshade extract, is a glycoalkaloid belonging to the *Solanaceae* family, which is reported to have anti-inflammatory activity (19,20). Recently, studies have demonstrated antitumorigenic effects of solanine in various types of cancer cell, for example, solanine suppresses proliferation and metastasis, and induces apoptosis in pancreatic cancer cells (19,21). Solanine was demonstrated to induce apoptosis in HepG2 cells by facilitating the opening of mitochondrial permeability transition pores (22) and downregulating expression of

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apoptosis regulator Bcl-2 (Bcl-2) (23). Previous studies have demonstrated that solanine inhibits the invasion of human melanoma and prostate cancer cells at non-toxic doses (18,24). Furthermore, Kang *et al* (25) reported that solanine is able to inhibit JNK activity. However, the underlying molecular mechanisms behind the effect of solanine on MDR cancer cells remain to be elucidated. The present study aimed to investigate the ability of solanine to resensitize the ADR-resistant human myelogenous leukemia cell line K562/ADM to ADR, in addition to the mechanisms underlying its effects.

Materials and methods

Reagents. ADR (Melone Pharmaceutical Co., Ltd., Dalian, China) was made up to a final concentration of 2 g/l using double-distilled H₂O. Solanine (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was made up to a final concentration of 100 µg/ml using dimethyl sulfoxide (DMSO) and diluted as required using RPMI-1640 cell culture medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). PBS buffer (Shanghai MacLean Biochemical Technology Co., Ltd., Shanghai, China). Rabbit polyclonal anti-MRP1, anti-JNK and anti-phosphorylated (p)-JNK (Thr¹⁸³/Thr¹⁸⁵) antibodies were obtained from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). The anti-JNK antibody detects all JNK isoforms. The rabbit polyclonal anti-GAPDH antibody was obtained from Goodhere Biotechnology Co., Ltd. (Hangzhou, China).

Cell lines and culture. The human CML K562 cell line was obtained from the Key Laboratory of Tumor Molecular Biology of Binzhou Medical University (Binzhou, China) with the original source from the Department of Pharmacology at the Institute of Hematology of Chinese Academy of Medical Sciences (Tianjin, China). The MDR-resistant subline, K562/ADM, was obtained from the Department of Pharmacology at the Institute of Hematology of Chinese Academy of Medical Sciences. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) at 37°C in a humidified atmosphere containing 5% CO₂. K562/ADM cells were maintained in medium containing 4 mg/l ADR, but cultured in drug-free medium for one week prior to experimentation.

Determination of MDR accumulation in K562/ADM cells. The Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Shanghai, China) assay was used to determine the viability of CML cells. The CML cells (1x10⁵ cells/well) were incubated (humidified atmosphere, 37°C, 5% CO₂ for 24 h) with ADR at the following concentrations: 0.2-1.6 mg/l for K562 cells; and 16-128 mg/l for K562/ADM cells. RPMI-1640 medium was used to dissolve ADR. A total of 10 µl CCK-8 solution was added to each well 24 h following incubation. The plates were incubated for 1-4 h at 37°C in a humidified atmosphere containing 5% CO₂. Absorbance was subsequently measured at 570 nm using an F-7000 Fluorescence Spectrophotometer (Hitachi, Ltd., Tokyo, Japan). A blank well containing RPMI-1640 medium and ADR alone was used as a control. The half-maximal

inhibitory concentration (IC₅₀) of cell growth was calculated as described previously (26).

Solanine cytotoxicity assay. Solanine cytotoxicity was evaluated using the CCK-8 assay as described above. K562/ADM cells (1x10⁵) were seeded into a 96-well plate and incubated with between 5 and 10 µg/ml solanine at 37°C for 24 h. Cells in the control group were incubated with DMSO alone. The number of viable cells was determined using the CCK-8 assay, as described above.

Evaluating the MDR reversal efficacy of solanine. K562/ADM cells (1x10⁵) were seeded into 96-well plates and treated with 0-128 µg/ml ADR alone or 0-128 µg/ml ADR combined with 5-10 µg/ml solanine for 24 h at 37°C. Cell viability was subsequently assessed using the CCK-8 assay, which was performed five times. IC₅₀ values were calculated and untreated cells were used as the negative control. Reversal fold (RF) values were used to quantify sensitivity reversal and were obtained using the following formula: RF=IC₅₀ of ADR treatment alone/IC₅₀ of combined ADR and solanine treatment.

Evaluating intracellular ADR accumulation. K562/ADM cells were incubated for 1 h at 37°C with 3 mg/l ADR alone or in combination with 5-10 µg/ml solanine. Cells were subsequently harvested using centrifugation (room temperature) at 150 x g for 5 min and washed twice with ice-cold PBS to remove the unbound ADR. The cell-associated mean fluorescence intensity of ADR-treated cells was detected using an FC500 Flow Cytometry Analyzer (Beckman Coulter, Inc., Brea, CA, USA), and the respective excitation and emission wavelengths were 485 and 580 nm.

Western blot analysis. K562/ADM cells were incubated with 5-10 µg/ml solanine at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h prior to harvesting. Cells were lysed in 100 µl lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and the total protein concentration was determined using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). A total of 50 µg total protein was separated using SDS-PAGE on 6-8% gels depending on the molecular mass of the protein (initial voltage, 80 V; initial duration, 30 min; final voltage, 100 V; final duration, 1.5 h). Resolved proteins were transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) and blocked at room temperature with 5% skimmed milk for 2 h. Membranes were subsequently incubated overnight at 4°C with rabbit polyclonal anti-MRP1 (1:500), anti-JNK (1:200), anti-p-JNK (1:500) and anti-GADPH (1:1,000) antibodies. Membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:5,000; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing China) for 2 h at room temperature. Images were captured using a FluorChem FC2 Imaging system (Alpha Innotech, San Leandro, CA, USA). The intensity of each band was normalized to GADPH and quantified using ImageJ software 2x v.2.1.4.7 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical analysis was performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL,

Table I. Determination of ADR IC₅₀ values in the K562 and K562/ADM cell lines.

Treatment	IC ₅₀ (μg/ml)		Fold increase in resistance
	K562/ADM	K562	
ADR	52.4579±1.0874 ^a	1.3966±0.01526	37.56115

Values are presented as the mean ± standard deviation (n=3). ^aP<0.05 vs. K562 cells. ADR, Adriamycin; IC₅₀, half-maximal inhibitory concentration.

USA). Values are presented as the mean ± standard deviation. Statistical comparisons were evaluated using one-way analysis of variance followed by Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparing the ADR resistance of K562 and K562/ADM cells. K562/ADM cells exhibited significant resistance to ADR compared with the non-ADR resistant K562 cell line (P<0.05; Table I). As shown in Table I, a 37.56115 fold increase in ADR resistance was observed in the K562/ADM cells compared with the K562 cells (P<0.05).

Effect of solanine on K562/ADM cell proliferation. Treatment with solanine inhibited K562/ADM cell proliferation in a dose-dependent manner (Fig. 1). Treatment with 5 or 10 μg/ml solanine had no marked inhibitory effect on cell proliferation (<5 and <10% vs. the control group, respectively). Therefore, these concentrations were selected for subsequent experiments, as they had little effect on K562/ADM cell proliferation.

Effect of combined treatment with ADR and solanine on K562/ADM cell sensitivity to ADR. To determine whether combined treatment with ADR (range, 0-128 μg/ml) and 5 or 10 μg/ml solanine had an effect on drug resistance *in vitro*, cytotoxicity was assessed using the CCK-8 assay (Fig. 2; Table II). The results demonstrated a significantly decreased rate of proliferation in K562/ADM cells 24 h following combined treatment with ADR and solanine, compared with treatment with ADR alone (P<0.05; Fig. 2; Table II). This suggests that combined treatment with solanine increases ADR cytotoxicity in K562/ADM cells. Combined treatment with 5 and 10 μg/ml solanine led to a 1.68 and 2.64 fold increase in K562/ADM sensitivity to ADR, respectively (P<0.05 vs. ADR treatment alone; Table II).

Solanine increases the intracellular accumulation of ADR. In a previous study, intracellular accumulation of ADR was reported to be significantly decreased in K562/ADM cells compared with K562 cells (7). In the present study, it was determined that solanine significantly increased the intracellular accumulation of ADR in K562/ADM cells (P<0.05 vs. the control group; Fig. 3). These results indicate that solanine increases the sensitivity of K562/ADM cells to ADR by increasing intracellular ADR accumulation.

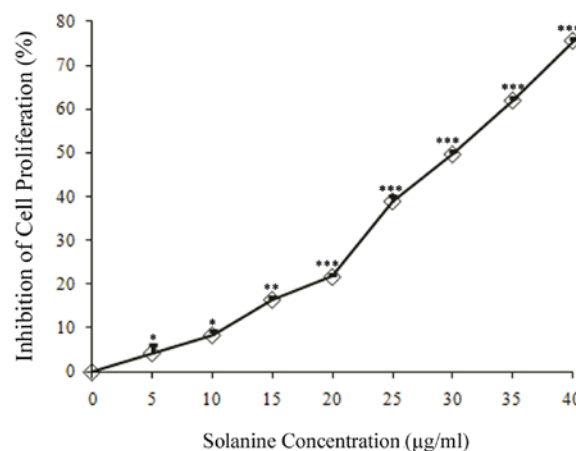


Figure 1. Effect of treatment with solanine on K562/ADM cell proliferation. K562/ADM cells were treated with solanine for 24 h. Proliferation of K562/ADM cells was determined using the Cell Counting Kit-8 assay and the inhibition rate (%) of proliferation was calculated. Values are presented as the mean ± standard deviation of triplicate experiments. *P<0.05, **P<0.01, ***P<0.001 vs. untreated cells.

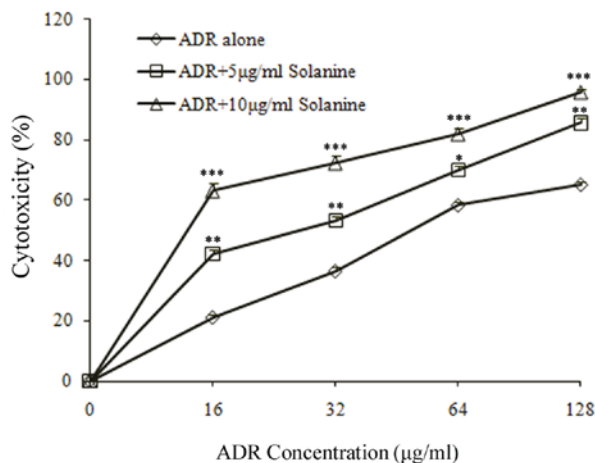


Figure 2. Effect of treatment with solanine on ADR cytotoxicity. Growth curves produced using data from Cell Counting Kit-8 assays in K562/ADM cells treated with ADR alone or ADR in combination with 5 or 10 μg/ml solanine. Values are presented as the mean ± standard deviation of triplicate experiments. *P<0.05, **P<0.01, ***P<0.001 vs. ADR alone treatment group. ADR, Adriamycin®.

Treatment with solanine decreases MRP1 protein expression in K562/ADM cells. MRP1 is an ABC transporter, which is overexpressed in numerous drug-resistant cell lines, including K562/ADM cells, compared with the corresponding

Table II. Effect of treatment with solanine on the ADR IC₅₀ values in K562/ADM cells.

Treatment	IC ₅₀ (μg/ml)	Fold reversal vs. ADR alone
ADR alone	54.7985±1.71941	N/A
ADR + 5 μg/ml solanine	32.6094±0.66628 ^a	1.68
ADR + 10 μg/ml solanine	14.7863±0.61516 ^a	3.71

Values are presented as the mean ± standard deviation (n=3). ^aP<0.05 vs. treatment with ADR alone. ADR, Adriamycin; IC₅₀, half-maximal inhibitory concentration.

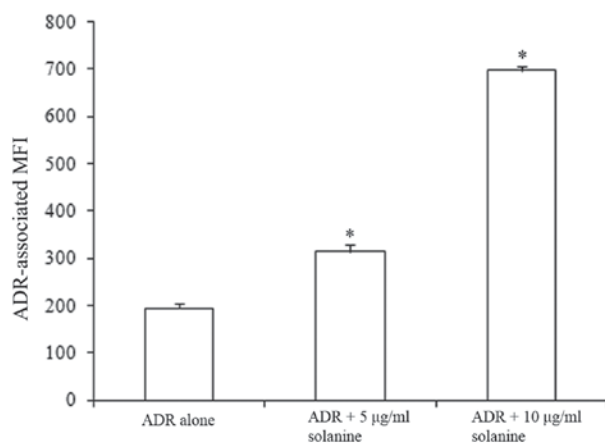


Figure 3. Solanine increases intracellular accumulation of ADR. Cells were treated with 3 mg/l ADR alone or in combination with 5 or 10 μg/ml solanine for 1 h. Following removal of the unbound ADR, the cell-associated ADR MFI was evaluated using flow cytometry, with excitation and emission wavelengths of 485 and 585 nm, respectively. Values are presented as the mean ± standard deviation of triplicate experiments. ^aP<0.05 vs. ADR alone treatment group. ADR, Adriamycin; MFI, mean fluorescence intensity.

non-resistant cell lines (11). K562/ADM cells were identified to express MRP1 protein at high levels (11). Western blot analysis demonstrated that MRP1 protein expression was significantly decreased in K562/ADM cells following treatment with 5 or 10 μg/ml solanine compared with the untreated cells (P<0.05; Fig. 4). These results indicate that solanine increases MRP1 protein expression, which likely increases intracellular ADR accumulation.

Treatment with solanine decreases JNK phosphorylation. JNK serves an essential role in the development of MDR, therefore the phosphorylation pattern of JNK in MDR K562/ADM cells was analyzed using western blotting. As shown in Fig. 5, total JNK expression did not change significantly 24 h following treatment with solanine compared with the control group (P>0.05; Fig. 5A and B); however, the expression of p-JNK was significantly decreased in a dose-dependent manner compared with the untreated cells (P<0.05; Fig. 5A and C). The effect of treatment with solanine on JNK phosphorylation was consistent with its effect on MRP1 expression. These results suggest that the JNK signaling pathway participates in the modulation of MRP1 protein expression.

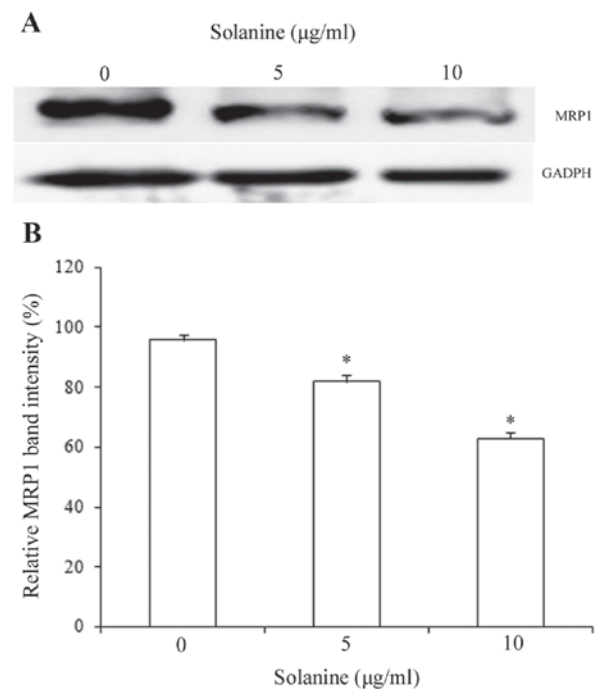


Figure 4. Treatment with solanine decreases MRP1 protein expression in K562/ADM cells. (A) Western blot of MRP1 protein expression. (B) Quantification of western blot, normalized to GAPDH. Treatment with solanine significantly inhibited MRP1 expression. Values are expressed as the mean ± standard deviation of triplicate experiments. ^aP<0.05 vs. untreated cells. MRP1, multidrug resistance protein 1.

Discussion

CML is a stem-cell disorder characterized by chronic and blast crisis phases (27). Chemotherapy serves an essential role in CML treatment; however, it is frequently accompanied by the development of MDR, which results in treatment failure. MDR is the ability of tumors to exhibit simultaneous resistance to a number of structurally and functionally unrelated chemotherapeutic agents (28). There are multiple underlying molecular mechanisms of MDR, including increased expression of ABC transporter proteins, and abnormalities in a number of enzymatic and apoptotic signaling pathways (28). ADR is an effective chemotherapeutic that has been used extensively to treat CML, but is limited by the development of MDR (1). MRP1 overexpression is one of the well-known causes of MDR (11). Significant efforts have been made to identify novel MDR-inhibiting agents.

Glycoalkaloids are secondary plant metabolites that contain nitrogen, are found in solanaceous plants and possess anticarcinogenic activity (29). Nightshade is a plant used in traditional Chinese herbal medicine (24), which contains the glycoalkaloid solanine (21). Increasing evidence has demonstrated that solanine possesses antitumor activity, for example, solanine has been demonstrated to inhibit the growth of U937 cells (30). In addition, previous studies revealed that solanine has antitumor activity in other types of cancer (18-19,21). A previous study suggested that Solanine suppressed proliferation of mouse breast cancer cells by inducing expression of the apoptosis regulator Bcl2 associated X (Bax) and decreasing expression of Bcl-2 (30). Solanine was demonstrated to inhibit

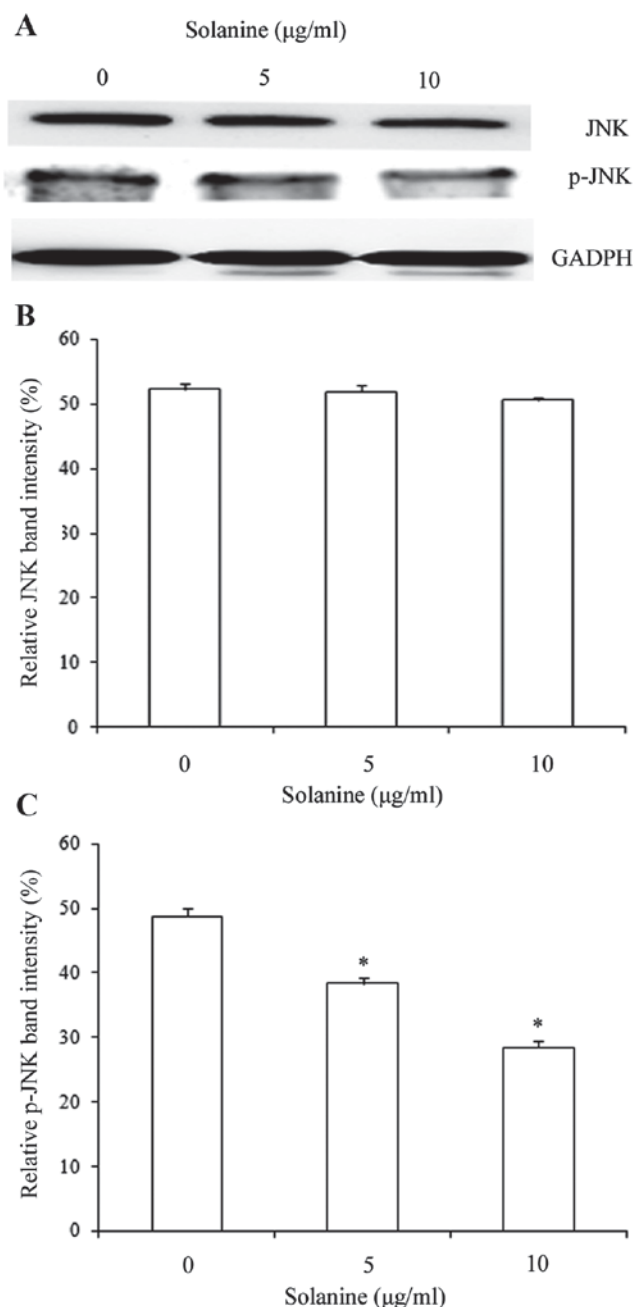


Figure 5. Effect of treatment with solanine on the total expression of JNK and JNK phosphorylation status. (A) K562/ADM cells were treated with 0, 5 or 10 µg/ml solanine for 24 h and analyzed using western blotting for expression of JNK and p-JNK. Quantification of western blot for (B) JNK and (C) p-JNK protein expression, normalized to GAPDH. p-JNK expression decreased following treatment with solanine. Values are presented as the mean ± standard deviation of triplicate experiments. *P<0.05 vs. untreated cells. JNK, c-Jun N-terminal kinase; p-, phosphorylated.

the activity of matrix metalloproteinase (MMP)-2 and -9 by suppressing the phosphatidylinositol 4,5-bisphosphate 3-kinase/protein kinase B and JNK signaling pathways at non-toxic doses, which resulted in the inhibition of melanoma cell migration and invasion (18). In addition, it was reported that treatment with solanine suppressed MMP-2 and 9 expression in pancreatic cancer cells at toxic doses, which resulted in the suppression of pancreatic cancer cell migration and invasion (19,21). Furthermore, treatment with solanine decreased the Bcl-2/Bax ratio and induced activation

of the caspase-3 zymogen to facilitate pancreatic cancer cell apoptosis (19). However, the effect of treatment with solanine on the reversal of MDR in K562/ADM cells was unclear. The present study aimed to investigate the effects and underlying mechanisms of solanine action in K562/ADM cells.

In the present study, the efficacy of solanine as a reverser of ADR resistance in K562/ADM cells was examined. The results suggested that treatment with 5 or 10 µg/ml solanine alone exhibited no significant antiproliferative effect on K562/ADM cells compared with the control group. The effect of treatment with solanine on cellular functions in K562/ADM cells was subsequently investigated. The CCK-8 assay demonstrated that treatment with 5 and 10 µg/ml solanine enhanced the cytotoxicity of ADR to K562/ADM cells, suggesting that solanine reverses MDR in K562/ADM cells. The results of the flow cytometric assay demonstrated that treatment with solanine increased the intracellular accumulation of ADR, which suggests that this is the mechanism through which solanine reverses MDR. To further examine the mechanism by which solanine elevates K562/ADM intracellular ADR accumulation, MRP1 protein expression was evaluated.

MRP1, an ATP-dependent molecular pump, is a member of the ABC transporter protein family and is able to decrease intracellular drug accumulation, thus decreasing the cellular toxicity of various chemotherapeutic agents, including ADR, daunorubicin, epirubicin, mitoxantrone, bisantrene, vincristine, vinblastine, etoposide and paclitaxel (11,31). In the present study, western blot analysis demonstrated that treatment with solanine significantly decreased MRP1 protein expression compared with treatment with ADR alone in K562/ADM cells. Decreased expression of MRP1 protein was associated with increased intracellular accumulation of ADR in K562/ADM cells.

It has been reported that the JNK signaling pathway is involved in the development of MDR in a number of tumor cell types. Decreased JNK activity has been observed in drug-resistant hepatocellular carcinoma cells, while increased JNK activity was demonstrated to attenuate chemoresistance in R-HepG2 cells by suppressing P-glycoprotein (P-gp) expression (32,33). Increased JNK signaling pathway activation was identified to downregulate P-gp expression and reverse P-gp-mediated MDR in gastric and pancreatic cancer cells (17). However, decreased JNK phosphorylation has been demonstrated to inhibit P-gp expression during hypoxia (14,34). Increasing evidence has demonstrated that JNK activity is associated with MRP1 activity and expression. Suppression of the JNK signaling pathway led to a decrease in MRP1 activity and expression in glial cells (35). However, MRP1 expression was negatively associated with JNK activity in another study (36). Cripe *et al* (37) reported that JNK activity and MRP1 expression were upregulated in HL60/ADR cells compared with HL60 cells and furthermore, that inhibition of JNK activity decreased MRP1-efflux and MRP1 protein expression. These results indicate that JNK activity serves an important role in the development of MDR and MRP1 expression. Cripe *et al* (37) also reported that JNK activity was upregulated in K562 cells, and in tumor cells exposed to ADR in a time- and concentration-dependent manner. As expected, in the present study treatment with solanine led to inhibited JNK activity while having no

significant impact on total JNK expression. The results of the present study indicate that decreased JNK activity is associated with the inhibition of MRP1 expression in K562/ADM cells, suggesting that solanine is able to reverse MDR by modulating the expression of MRP1, through regulation of the JNK signaling pathway.

In conclusion, treatment with solanine enhanced the sensitivity of K562/ADM cells to ADR as a result of increased intracellular ADR accumulation, which was associated with the downregulation of MRP1 expression. Downregulated MRP1 expression was likely a result of decreased JNK phosphorylation, a marker of JNK activity. The results of the present study suggest that solanine is a novel therapeutic agent for the treatment of cancer, which reverses MDR in human myelogenous leukemia K562/ADM cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YJY was responsible for the main experiments and writing the manuscript. XHJ analyzed experimental data and modified the manuscript. CZ, JYW, JRC, HW conducted some parts of the experiments. YJL designed and guided the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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