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Original Research

Schizandrol A reverses multidrug resistance in resistant chronic myeloid leukemia cells K562/A02

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Abstract: Overexpression of P-gp is the main cause of multidrug resistance (MDR) and chemotherapeutic failure in leukemia. In this study, the multidrug resistance reverse effect of Schizandrol A (SchA) was demonstrated with P-gp overexpressed drug-resistant K562/A02 cells. SchA had almost no cytotoxic activity, the EC_{50} value reversed to DOX was in the nanomole range of (707 ± 29nM) and had a high selectivity index (> 113) to normal cells. DOX accumulation and Rh123 efflux tests demonstrated that the MDR reversal activity of SchA was induced by inhibiting P-gp function. Western blotting assay showed that SchA down-regulated the expression of P-gp by inhibiting the PI3K / Akt signaling pathway, which was also a key factor in reversal activity. Therefore, SchA may be a potential candidate for natural MDR reversal agents.

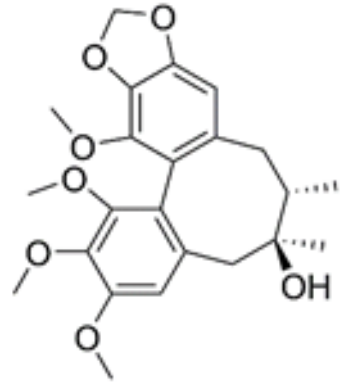
Key words: Multidrug resistance; P-gp; Chronic myeloid leukemia; Schizandrol A; Reversal activity.

Introduction

Chronic myeloid leukemia (CML) is a hematological malignancy caused by the sustained activation of Bcr-Abl tyrosine kinase, accounting for 4% of all malignant tumors (1). Drug chemotherapy is one of the main methods for the treatment of cancer (2). However, the resistance of tumor cells to chemotherapeutic drugs hinders the cure of CML, greatly increases its recurrence and mortality, and multidrug resistance is the most serious one (3, 4). Multidrug resistance (MDR) is a cross-resistance of tumor cells to a variety of other antitumor drugs with different structures and mechanisms after tumor cells become resistant to an anti-tumor drug (5, 6).

The pathogenesis of MDR has been extensively studied, including: 1. The decrease of the uptake of antitumor drugs by tumor cells or the transport protein will discharge the drugs from the cells; 2. Arrest of apoptosis or change of cell cycle prosecution point in tumor cells; 3. Enhancement of metabolic enzyme activity of antitumor drugs; 4. Enhancement of repair function after DNA injury; 5. Tumor cell target protein expression or function change, etc (3, 7). These mechanisms alone or in combination induce drug resistance in tumor cells. The overexpression of ATP-binding cassette subfamily (ABC) transmembrane transporter (including P-glycoprotein, ABCC1, ABCG2) enhanced the transport action of cytotoxic drugs, resulting in a decrease of the accumulation of drugs in cells. Intracellular drug concentration is lower than the effective concentration to kill tumor cells, which is one of the main causes of MDR production (8-11). Among them, P glycoprotein (P-gp) is the most widely studied (12-15).

Schisandra chinensis is the dried ripe fruit of Schisandra chinensis (Turcz.) Baill or S. Sphenanchera Rehd. etWils. of Magnoliaceae (16), which contains a large number of lignin compounds and is considered to be the most important pharmacological component of Schisandra chinensis. Studies have shown that Schisandra chinensis has the effects of protecting liver, heart and nerves, reducing inflammation, dilating blood vessels, anti-platelet aggregation, anti-virus, anti-diabetes and anti-tumor (17-20). Schizandrol A is one of the main components of Schisandra chinensis. It is not clear whether it has P-gp inhibitory function to reverse the MDR in leukemia. Therefore, this study observed the MDR reversal effect of c (SchA) on human chronic myeloid leukemia doxorubicin resistant cell line K562 / A02 (high expression of P-gp) in vitro, and analyzed the relevant action mechanism, providing theoretical basis for the combination of Sch and other chemotherapy drugs for the treatment of leukemia.



Schizandrol A (SchA)

Figure 2. The structure of Schizandrol A (SchA).

Materials and Methods

Materials

Schizandrol A (purity > 98%, Chengdu Pulis Biotechnology Co., Ltd.); Human chronic myeloid leukemia cell line K562 (Institute of Hematology, Chinese Academy of Medical Sciences); Human chronic myeloid leukemia doxorubicin resistant cell line K562 /A02 (Institute of Hematology, Chinese Academy of Medical Sciences); Normal human gastric mucosal cell GES-1 (Institute of Hematology, Chinese Academy of Medical Sciences); RPMI1640 culture medium, fetal calf serum (FCS, Hyclone company, US); verapamil (VRP, Sigma company, US); methyl thiazolyl tetrazolium (MTT, Sigma company, US) was prepared to 5g/L solution with PBS buffer and stored avoid light at 4 °C; rhodamine 123 (Rh123, Hua mei company); dimethyl sulfoxide (DMSO, Gibco company, US); doxorubicin (DOX, Beijing Bioss Biotechnology Co., Ltd.); Rabbit anti-P-glycoprotein (P-gp), t-Akt, pAkt and GADPH polyclonal antibody (Abcam company, US).

Cytotoxicity and reverse activity test

With reference to the literature (21), it was reported that K562 was cultured on RPMI 1640 medium containing 10% calf serum at 37 °C with 5% CO_2 saturation humidity. Doxorubicin with the final concentration of 1mg/L should be added to the culture medium of K562/A02 cells in addition to the above conditions. Before the experiment, the cells should be cultured in the condition of no doxorubicin for 14 days. The cells in logarithmic growth period were inoculated with density of 3×10^4 / mL in 96-well culture plate, 180μL per well, cultured overnight in 37 °C with 5% CO_2. Then they were divided into blank control group, positive compound group and test compound group. The blank control group was given 20μL of 0.1% DMSO; positive control group was given 5 μM verapamil, 10μL DOX with a series of concentration gradient and 10μL a certain concentration of compounds to be tested. After incubation for 48 h, 20μL of MTT (5mg/mL) was added to each well and cultured for 4 h. After centrifugation, the culture solution was sucked, added to each well and shook for 20min, then the optical density (OD) was read on the ELISA instrument at 490nm. The inhibition rate of adriamycin combined with compound or positive control on cells was calculated. The IC_{50} value of doxorubicin was calculated by the dose-response curve in GraphPad Prism 6.0 software, and the reversal fold (RF) was calculated. Cell inhibition rate = 1- (OD average value in experimental group / OD average value in control group) × 100%. Reversal fold (RF) = $IC_{50}A$ / $IC_{50}B$, $IC_{50}A$ is the IC_{50} value of adriamycin to K562/A02 and $IC_{50}B$ is the IC_{50} value of adriamycin to K562/A02 after adding the compounds to be tested.

Study on the dose-response relationship of reversal activity

According to the preliminary results of reverse activity determination, the reverse activity (22) at different concentrations was further determined, and the experimental method referred to reverse activity test. Schizandrol A was diluted to the final concentration of 20μM, 10μM, 5.0μM, 2.5μM, 1.25μM, 0.625μM, 0.31μM, 0.156μM, 0.078μM and 0.04μM, respectively.

Study on selection index (SI)

Selection index (SI) refers to the selectivity of compounds to normal cells and drug-resistant tumor cells (22). Based on MTT method, the IC_{50} value of the preferred compounds on gastric epithelial cell strain-1 (GES-1) of normal gastric mucosal cells was determined, and SI value was calculated according to formula SI = $IC_{50}A$ / $EC_{50}B$. $IC_{50}A$ is the IC_{50} value of the selected compound to GES-1, and $EC_{50}B$ is the concentration of preferred compounds when the IC_{50} value of doxorubicin to K562/A02 is reduced to half. Laboratory instruments, materials, principles and methods refer to cytotoxicity test. The cell culture medium of GES-1 was RPMI 1640 containing 10% fetal bovine serum (FBS, Gibco).

DOX intracellular accumulation experiment

With reference methods (23), K562 / A02 cells in logarithmic growth phase were selected, and the cell concentration was adjusted by 2×10^5 / mL. The cells were randomly divided into four groups: K562 / DOX group, K562 /A02 + DOX group, K562 / A02 + SchA (5 μM) + DOX group and K562 / A02 + SchA (10 μM) + DOX group. The drug with appropriate concentration was added, then inoculated in 6-well plate and incubated in 5% CO_2 aseptic incubator at 37 °C for 1 h. DOX with the final concentration of 3 mg/L was added and continuous incubated for 1 h, then free DOX was removed by washing twice with cold PBS buffer. The average fluorescence intensity of intracellular Adr was measured by flow cytometry with the excitation wavelength of 485 nm, emission wavelength of 585 nm, and the concentration of DOX was expressed by relative fluorescence intensity. The experiment was repeated 3 times independently.

Rhodamine efflux experiment

Rhodamine 123 (Rh123) with the final concentration of 2 mg / L was added to the control group, and the experimental group was supplemented with 5 μM and 10 μM SchA on the basis of the control group. After being incubated in a 5% CO2 incubator at 37 °C for 1.5 h, the free Rh123 was removed by washing twice with cold PBS buffer. The relative fluorescence intensity of intracellular Rh123 excited in cells was measured by flow cytometry (excitation wavelength: 485 nm, emission wavelength: 535 nm), and the mean fluorescence intensity (MFI) was used to indicate Rh123 concentration (24). The experiment was repeated 3 times independently.

The effect on the expression of ABC transporter, t-Akt and p-Akt

Western blotting method was used to determine the protein contents. K562/A02 cells in logarithmic growth stage was selected and inoculated with 5×105/well in 6-well plates. Total protein of the cells was extracted after 24 h culture with or without different concentrations (5, 10 μM) of SchA. After boiling, 50 μg of protein was taken for electrophoretic, transferred to membrane, and sealed with 7.5% skim milk powder for 2 h, then TBST was used to wash the membrane. Rabbit anti-human polyclonal antibody anti-P-gp and anti-p-Akt (diluted

with 1: 200) and rabbit anti-human polyclonal GADPH antibody (diluted with 1: 500) were added and placed in a 4 ºC shaking bed overnight. Chemiluminescence reagent was used for coloring and imageJ software was used to analyze the image. The relative expression levels of P-gp, t-Akt and p-Akt protein was expressed by P-gp, t-Akt, p-Akt gray value / GADPH gray value. The experiment was repeated 3 times independently.

Statistical method

GraphpadPrism6.0 statistical software was used for statistical analysis of the research data. The measurement data was expressed as mean ± standard deviation (x ± s). Single factor analysis of variance (ANOVA) was used in the multigroup comparison. The pairwise comparison among multiple means was performed by q test. P < 0. 05 represents significant difference in statistics.

Results

Cytotoxicity of Sch in vitro and drug resistance multiple of K562/A02

Schizandrol A showed a dose-dependent growth inhibition of the of K562/A02 cells at all test doses. The growth inhibition rate of K562/A02 cells was less than 5% when the concentration of Schizandrol A was 10 μM and less than 10% when the concentration of Schizandrol A was 20 μM (Figure 2A). When the concentrations of SchA were less than 20 μM, the inhibition rates on cells was less than 10%, that is the concentrations less than 20 μM were nontoxic doses, which could be used for the subsequent reversal experiment. The IC_{50} of K562 and K562/A02 cells treated with DOX for 48 h was (0.56 ± 0.05) and (44.29 ± 2.96) μM, respectively. Compared with the sensitive K562 cell line, resistant K562/A02 cell line showed obvious tolerance to DOX, and the drug resistance fold was 79.09 times (Figure 2B).

Reversal activity and selection index of SchA

At the same concentration of DOX, combined with SchA, the inhibitory effect of DOX on K562/A02 drug resistant cell line was increased, the toxicity was enhanced, the IC_{50} value of DOX was decreased, and the reversal fold was increased. These effects were concentration-dependent with SchA (Figure 3A). The EC50 value for reversing doxorubicin resistance by SchA in K562/A02 resistant cells was also calculated, that is, the concentration required for SchA to reverse the reduction of doxorubicin resistance in K562/A02 cells to half of the drug resistance, as shown in figure 3B. SchA showed a strong reversal activity, and the EC_{50} value of reversing doxorubicin resistance in K562/A02 cells was in the range of (707 ± 29 nM), which was worthy of further pharmacological study. SchA has no cytotoxicity to normal cells, and even when the maximum concentration is 80 μM, the survival rate of GES-1 is still higher than 70%. Therefore, the IC_{50} value of compound SchA is greater than 80 μM, and SI value is greater than 113, which indicates that compound SchA can reverse the effect of MDR and has good safety to normal cells at the same time.

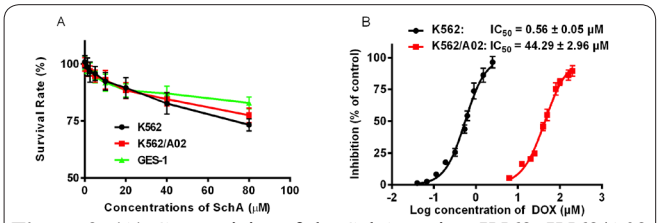


Figure 2. (A) Cytotoxicity of the SchA against K562, K562/A02 and GES-1 Cell Lines (B) IC50 values of DOX in inhibition of K562 and K562/A02 cell lines determined by MTT method. The inhibition was plotted against log concentration of 12k and DOX. Each date point is presented as mean ± SD for three independent tests.

Effect of SchA on intracellular DOX accumulation

The effect of SchA on the accumulation of adriamycin in drug-resistant cells was determined by fluorescence spectrophotometry. As shown in Figure 4, the accumulation of adriamycin in K562 cells sensitive to doxorubicin was about 6.4 times higher than that in K562/A02 cells. Compared with the drug resistance blank control group, the accumulation of adriamycin in K562/A02 cells increased to different degrees in the experimental group with adding different concentration of SchA. The accumulation of doxorubicin by 5.0 μM SchA was equivalent to that of 10.0 μM verapamil. The results showed that SchA inhibited the transport function of P-gp and resulted in a significant accumulation of doxorubicin in drug-resistant cells.

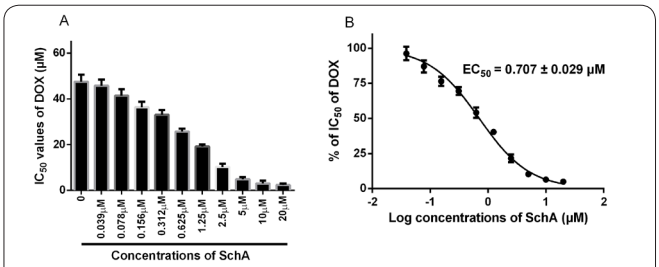


Figure 3. Effect of SchA on reversing DOX resistance in K562/A02 cells. (A) IC_{50} values of DOX in K562/A02 cells treated with DOX with or without different concentrations of SchA; (B) EC_{50} value of SchA in lowering DOX-resistance in K562/A02 cells. The percent of IC_{50} of DOX was plotted against log concentration of SchA. The percent of IC_{50} of DOX = [(IC_{50} of DOX at each modulator concentration/IC_{50} of DOX without modulator) × 100%. EC_{50} is defined as the concentration of modulator that can reduce the % of IC_{50} of DOX by half.

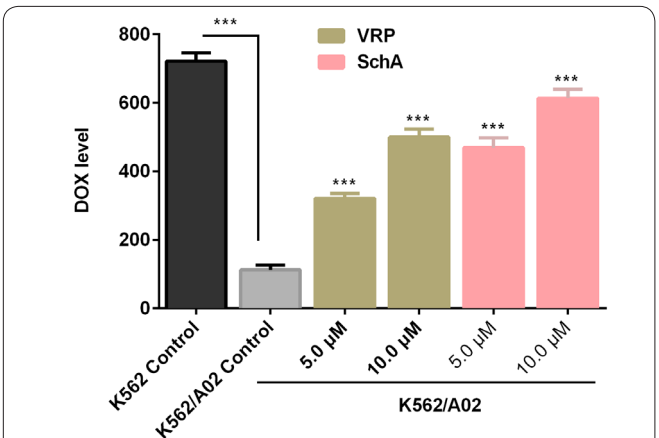


Figure 4. Effects of SchA on intracellular DOX accumulation. The results are presented as the mean ± SD for three independent experiments; (***) p < 0.001, (**) p < 0.01, (*) p < 0.05 relative to the negative control (K562/A02).

Effect of SchA on the efflux of Rhodamine 123

P-gp dependent fluorescent substrate Rh123 efflux assay is widely used to evaluate the effect of reverse agents on P-gp efflux function. In order to further verify the mechanism of SchA, we investigated the inhibitory effect of SchA on the efflux of intracellular rhodamine 123. As shown in figure 5 below, the intensity of Rh123 in K562/A02 cells with P-gp overexpression in blank group decreased significantly over time. When K562/A02 cells were added with 5.0 μM verapamil and 5.0 μM SchA, the reduction degree of intracellular Rh123 intensity decreased, that is to say, the efflux of Rh123 in K562/A02 cells was inhibited and efflux levels decreased in a concentration-dependent manner. It is proved that SchA can inhibit the function of P-gp and block the efflux of Rh123 in K562/A02 cells.

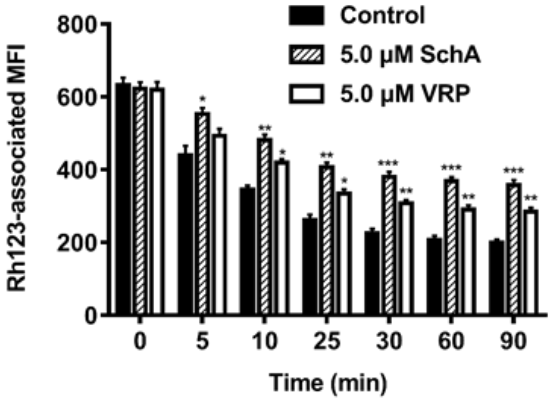


Figure 5. Effects of SchA on Rh123 efflux in K562/A02 cells. The results are presented as the mean ± SD for three independent experiments; (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$ relative to the negative control (K562/A02).

Effect of SchA on the expression of P-gp and Akt

The results of Western blot assay showed that there were bands with molecular weight of about 170-kDa in K562/A02 cell lysate, namely P-gp, which indicated that K562/A02 cells were a cell line with high expression of P-gp. As shown in figure 6, the expression of P-gp and p-Akt is inhibited in a concentration-dependent manner when the drug resistant cells are incubated with different concentrations of SchA, while the positive drug verapamil does not affect the expression of the two proteins.

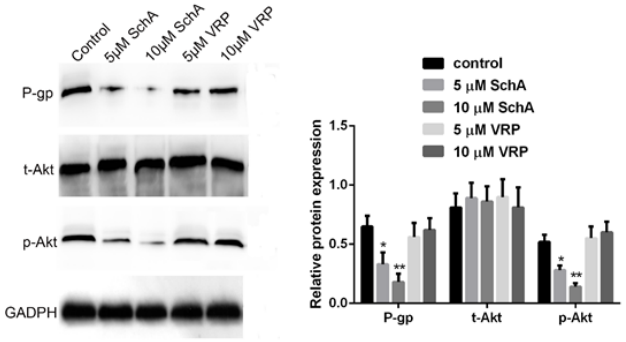


Figure 6. Expression of P-gp, and p-Akt influenced by SchA and VRP and quantitative analysis of the expression of P-gp, and p-STAT3. (**) $p < 0.01$, (*) $p < 0.05$ relative to the control. Representative result is shown here and similar results were obtained in two other independent trials.

Discussion

Up to now, the overexpression of ABC transporter (such as P-gp, MRP1 and BCRP) has a great influence on the efficacy of chemotherapeutics on leukemia, and multiple methods and efforts have been taken to ensure the sensitivity of chemotherapy (25, 26). It has been nearly 40 years since the discovery of the first P-gp modulator VRP (1981). The exploration of synthetic P-gp inhibitors has achieved the effective inhibition of P-gp function (27). However, the development of P-gp inhibitors is limited by the lack of significant clinical effects, drug interactions and other adverse side effects (26, 28). Due to the failure of the development of the third generation of P-gp inhibitors, the products from natural sources provide a new prospect for finding new and effective P-gp inhibitors. The natural product NPs is mainly derived from microorganisms, plants and marine animals, as well as a rich secondary metabolite with profound biological effects (29, 30). SchA is the main component of Schisandra chinensis, which has irreplaceable effects on the various physiological functions of Schisandra chinensis. However, whether it has the reversal effect of multidrug resistance deserves further study.

K562 / A02 cell line is a human leukemia drug resistant cell line established by gradually increasing the concentration of adriamycin in culture medium, which can express P-gp stably (31). As a drug resistant cell line used in this study, it was demonstrated that SchA is a potential drug for reversing MDR mediated by P-gp. Firstly, the intrinsic cytotoxic activity of SchA was confirmed. Even at 40 μM, the antiproliferative effect of SchA on various tumor cell lines was very weak, and it had no inhibitory activity on normal GES-1 cells. The activity of SchA to reverse the drug resistance mediated by P-gp was measured at nontoxic concentration. The reverse activity of SchA to DOX at different concentrations showed that its EC_{50} value was in the range of (707 ± 29 nM), and had a high selective index for normal cells (> 113), which indicated that SchA was harmless to normal human cells at the multidrug resistance reversal concentration. In the further study of the underlying mechanism, both DOX accumulation and Rh123 efflux tests demonstrated that the MDR reversal activity by SchA was triggered by inhibition of P-gp function.

P-gp located on the cell membrane, is an organic cationic pump encoded by ABCBl (MDRl) gene, which can bind to anthracyclines, podophylloids and other drugs, and pump drugs out of cells in a energy-consuming manner, thus reducing the concentration of drugs in cells to protect it from the killing of chemotherapeutic drugs and to develop drug resistance. Overexpression of P-gp is the main cause of drug efflux, but whether SchA will fundamentally affect its expression needs further study. P13K / Akt is an important signal transduction pathway in cells and plays an important role in leukemic MDR. Highly activated P13K/Akt signaling pathway promotes drug efflux by up-regulating ABC binding membrane protein, especially closely related to P-gp. Blocking PI3K / Akt signaling pathway leads to down-regulation of downstream P-gp expression and enhances the sensitivity of tumor cells to chemical drugs (32, 33). The effect of chlorogenic acid on the

expression of P-gp and Akt was investigated by Western blotting assay in protein level. The results showed that the expression of P-gp and pAkt protein decreased in K562 / A02 cells after combined treatment, which suggested that SchA could down-regulate P-gp expression by inhibiting PI3K / Akt signaling pathway, in addition to inhibiting the function of P-gp. This is also a key factor for reversal activity.

In conclusion, SchA not only improved the drug resistance of K562/A02 to chemotherapeutic drug DOX by inhibiting P-gp function, but also achieved reverse activity by down-regulating P-gp expression through PI3K/Akt signaling pathway. SchA can be used as a potential drug candidate for natural P-gp mediated MDR reversal agents.

Acknowledgements
None.

Conflict of interest
The authors declare that no conflict of interest is associated with this work.

Author contributions
Nurguli.Arken designed the research. Nurguli.Arken performed the cytotoxicity and reversal experiments. Nurguli.Arken performed PCR and Werstern blot experiments. All authors analysed the results and took part in preparing the manuscript.

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