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Original Research

Sodium azulene sulfonate reverses multidrug resistance in K562/A02 cells

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Abstract: Overexpression of p-glycoprotein (p-gp) is the main cause of multidrug resistance and chemotherapy failure in leukemia. Sodium azulene sulfonate (SAS) was used to reverse the multidrug resistance of human leukemia adriamycin-resistant strain K562/A02, and the underlying mechanism was investigated. Human leukemia cell line K562 and drug-resistant cell line K562/A02 in logarithmic phase were used in this study. After 48 hours of treatment of K562/A02 cells with SAS, the intrinsic cytotoxicity of chlorogenic acid and its sensitivity to adriamycin (ADM) were determined with MTT assay. The degree of reversal was calculated. Using ADM accumulation and rhodamine 123 efflux experiments, the average fluorescence intensity of ADM and rhodamine 123 (Rh123) in chlorogenic acid-treated K562/A02 cells was determined flow cytometrically. The expressions of p-gp, t-Akt and p-Akt in K562/A02 cells were assayed using Western blotting. SAS had almost no cytotoxic effect, and the degree of inhibition was only about 20% at the highest concentration of 100 mu-M. The EC_{50} of MDR reversal by SAS was in the nano range (539±37nM), and it had a high selectivity index for normal cells (>185). The accumulation of ADM in drug-resistant cells was increased significantly after treatment with 1 and 5 mu-M SAS, while the efflux of Rh123 was significantly inhibited, suggesting that SAS reversed MDR by inhibiting p-gp function. Western blotting experiments showed that SAS downregulated the expression of p-gp by inhibiting PI3K/Akt signaling pathway. This contributed to the reversal of drug resistance.SAS effectively reverses multidrug resistance *in vitro* by inhibiting the function of p-gp in K562/A02 cells, through a mechanism involving downregulation of the P13K/Akt signaling pathway. Therefore, SAS may be a potential candidate drug for reversal of MDR.

Key words: Sodium azulene sulfonate; Multidrug resistance; p-glycoprotein; Adriamycin; Reversal activity.

Introduction

Leukemia, which accounts for 4 % of all malignant tumors, is mainly treated with chemotherapy (1). However, due to the emergence of multidrug resistance (MDR) which results in treatment failure and recurrence of leukemia, the 5-year survival of leukemia is only 50 % (2). Multidrug resistance (MDR) refers to the phenomenon in which cancer cells are resistant to a variety of drugs with different pharmacological effects and structures at the same time. It is one of the important factors that affect the efficacy and prognosis of cancer chemotherapy. Thus, the reversal of MDR is an important issue in the effective treatment of leukemia (3).

Several mechanisms are involved in MDR. These include decreased uptake of anti-tumor drugs or efflux of transport proteins from the cells; inhibition of the apoptotic process or change of cell cycle checkpoint; increased activities of enzymes involved in the metabolism of anti-neoplastic drugs, enhanced DNA repair following DNA damage, and changes in the expression or function of target protein of tumor cells (4). These mechanisms alone or in combination induce drug resistance in cancer cells. Overexpression of p-gp is one of the main mechanisms of MDR production in cancer cells (5). So far, three generations of inhibitors have been developed for P-gp targets. However, some inhibitors may interfere with the pharmacokinetics of traditional chemotherapeutic drugs and cause serious side effects, which limit their clinical application (6). Therefore, new drug molecules need to be developed to overcome p-gp-mediated MDR (7). The new use of old medicine is a simple, economical and fast treatment.

Sodium azulene sulfonate is prepared by incorporation of water-soluble groups into molecular structure of azulene. As a semi-synthetic product, its side effects are relatively small. It is widely used in the pharmaceutical industry, health care industry and cosmetics industry (8-10). It enhances cell regeneration and convergence of mucosa. Sodium sulfonate has strong anti-inflammatory and anti-allergic effects; it is fast-acting and its effect is of long duration. When added as raw material to amino acid products, it can be used to repair mucosal ulcer, and also for protection of the mucosa. In addition, it can be used as a component of therapeutic formulations for anti-inflammation and moisturisation of the conjunctiva (11,12). The raw material is compatible with compound vitamin products, skin care products and health care products. Sodium sulfonate is also widely used in stomach mucosa preparations, ophthalmic preparations, and oral preparations in Japan. In Europe, it is applied in cosmetics, health care functional products, and obstetrics and gynecology preparations. However, not much is known about its inhibitory effect on p-gp function and its ability to reverse MDR in leukemia. In this study, the *in vitro* reversal effect of SAS on MDR of adriamycin-re-

sistant human chronic myeloid leukemia cell line K562/A02, and the underlying mechanism were investigated. This was with a view to providing theoretical basis for combination of SAS with other chemotherapeutic drugs in the treatment of drug-resistant leukemia.

Materials and Methods

Materials

Sodium Azulene Sulfonate (purity > 98%) was provided by Tianjin Colebury Technology Co., Ltd. Human chronic myeloid leukemia cell line K562, human chronic myeloid leukemia cell line K562/A02, and normal gastric mucosal cell GES-1 were provided by Institute of Hematology, Chinese Academy of Medical Sciences, while RPMI 1640 medium and fetal bovine serum were provided by Hyclone Company, USA. Verapamil (VRP) and tetramethylzole blue (MTT) were products of Sigma, USA (MTT concentration was 5g/L in PBS, and was kept at 4°C in the dark). Rhodamine 123 (Rhl23) was provided by Huamei Company. Dimethyl sulfoxide (DMSO) was product of Gibco Company, USA. Adriamycin (ADM) was obtained from Beijing Booshen Biotechnology Co., Ltd. Rabbit polyclonal antibodies against p-gp, t-Akt, p-Akt and GADPH were purchased from Abcam Company, USA).

Cytotoxicity and MDR reversal tests

The K562 cells were cultured in RPMI 1640 medium containing 10% calf serum at 37 °C, 5% CO_2 and saturation humidity. In addition to the above culture conditions, K562/A02 cells were cultured in the medium with the final adriamycin concentration of 1 mg/L, or without adriamycin for 14 days before the experiment (13, 14).

Cells in logarithmic growth phase were inoculated in 96-well culture plates at the density of 3×10^4 cells/mL, and cultured at 37 °C and 5% CO_2 overnight. Thereafter, they were divided into blank control group, positive compound group and test compound group. The blank control group was treated with 20 µl of 0.1% DMSO, and the positive control group was treated with 5 µM verapamil, while the test group was treated with 10 µL of graded concentrations of ADM and/or SAS. After 48 hours of incubation, 20 µL MTT (5 mg/mL) was added to each cell, followed by culturing for 4 hours. After centrifugation, the culture medium was collected, shaken for 20 minutes, and read in a microplate reader. The degree of inhibition by adriamycin combined with SAS or positive control was calculated. The IC_{50} for adriamycin was calculated via dose-effect curve using GraphPad Prism 6.0 software, and the reversal fold was calculated as shown below:

$$Percentage\ cell\ inhibition = \frac{1 - (Mean\ OD\ in\ test\ group \times 100)}{Mean\ OD\ in\ control\ group}$$

$$Reversal\ fold\ (RF) = \frac{IC_{50A}}{IC_{50B}}$$

where IC_{50A} is the IC_{50} of doxorubicin for K562/A02, and IC_{50B} is the IC_{50} of doxorubicin for K562/A02.

Dose-effect relationship of reversal effect

Preliminary results from determination of reversal potential necessitated further assays of the reversal effect at different concentrations (15). The methods used were the same as described in MDR reversal tests, with SAS at final serial concentrations of 20, 10, 5.0, 2.5, 1.25, 0.625, 0.31, 0.156, 0.078 and 0.04 M.

Studies on selectivity index

Selectivity index (SI) represents the relative effect of compounds between normal cells and drug-resistant cancer cells (14). Based on MTT method, the IC_{50} value of each selected compound was determined relative to normal human gastric mucosa cells, and the SI was calculated viz;

$$SI = \frac{IC_{50A}}{EC_{50B}}$$

where IC_{50A} is the IC_{50} value of the selected compound for GES-1, and EC_{50B} is the concentration of the selected compound when the IC_{50} value of adriamycin for K562/A02 was reduced by 50%. The cell culture medium of GES-1 was RPMI 1640 containing 10% fetal bovine serum (FBS, Gibco).

Test for intracellular accumulation of ADM

In this test, K562/A02 cells in logarithmic growth phase were selected and the cell concentration was adjusted to 2×10^5/mL. They were randomly divided into six groups: K562 + ADM group, K562/A02 + ADM group, K562/A02 + SAS (1uM) + ADM group, K562/A02 + SAS (5uM) + ADM group, K562/A02 + VRP (1uM) + ADM group, and K562/A02 + VRP (5uM) + ADM group (16). They were inoculated in 6-well plates and cultured in a sterile incubator at 37°C and 5% CO_2 for 1 hour in the dark. The final concentration of ADM was 3 mg/L. After 1 hour of culture, free ADM was removed by washing twice with cold PBS, and after cell lysis, the average fluorescence intensity of ADM in cells was measured using fluorescence spectrophotometer at excitation wavelength and emission wavelengths of 485 nm and 585 nm, respectively. Each experiment was repeated independently 3 times.

Luo Danming 123 efflux experiment

The K562/A02 cells were cultured in RPMI1640 medium containing 10% FBS in 37°C and 5% CO_2 saturated humidity. After overnight incubation, they were assigned to positive control, blank control and test compound groups. The cells were seeded in 24-well plates at a density of 2×10^5 cells per well. Then, Rh123 was added to all cells at a final concentration of 5.0 µM (final volume of each well was 500 µL). After incubation for 90 min, the cells were washed 3 times with Rh123-free medium solution. Then, the appropriate compounds were added to the test compound group and the positive control group at different final concentrations, while 0.1% DMSO was added to the blank control group containing blank medium. The total volume of the well culture solution was 500 µL and the incubation was continued at 37 °C. The mean fluorescence intensity of Rh123 retained per 10,000 cells was determined in a BD FACSCalibur flow cytometer after 5, 10, 25, 30, 60 and 90 min (17). The experiment was run three times in parallel, and the results were averaged (16).

Effect of SAS on the protein expressions of p-gp, t-Akt and p-Akt

The K562/A02 cells in logarithmic growth phase

were seeded in 6-well plates at a density of 5×10^5 cells/well, and were incubated with SAS at two different concentrations (1 and 5 uM) for 24 hours. The protein expressions of p-gp, t-Akt and p-Akt were assayed using Western blotting. Total cell protein was extracted and after heat treatment, 50 ug of protein was subjected to SDS-polyacrylamide gel electrophoresis. The protein bands were transferred to polyvinylidene membrane. Non-specific binding was blocked with 7.5 % skimmed milk powder for 2 hours. The membrane was washed with TBST and incubated with rabbit anti-human polyclonal antibodies against p-gp, t-p-Akt and p-Akt (1:200 dilution), and rabbit anti-human GADPH polyclonal antibody (1:500 dilution). The set up was shaken overnight at 44°C. Chemiluminescent reagent was used for color development, while ImageJ software was used for analyzing the image. The relative expressions of p-gp, t-Akt and p-Akt proteins were expressed as their gray values relative to that of GADPH which was used as internal control. The experiment was repeated three times independently.

Statistical analysis

Measurement data are expressed as x ± SD. Comparison between groups was performed using one-way analysis of variance, while comparison between multiple means was performed with *q*-test. All statistical analyses were carried out with Graphpad Prism7.0. Values of $p < 0.05$ were considered statistically significant.

Results

In vitro cytotoxicity of SAS and fold of drug resistance by K562/A02

At all tested doses, SAS showed almost no growth inhibition on K562, K562/A02 and GES-1 cells even at the highest concentration of 100 µM. The degree of growth inhibition was less than 10 % at concentrations below 25 µM (Fig. 1A). The IC_{50} value of ADM for K562 and K562/A02 cells after treatment for 48 hours were 0.50 ± 0.03 and 52.38 ± 4.17 µM, respectively. Compared with sensitive K562 cells, the drug-resistant K562/A02 cells showed significant tolerance to ADM, with approximately 104.8-fold drug resistance (Fig. 1B).

MDR reversal effect and selectivity index of SAS

When ADM was combined with SAS, the inhibitory effect and toxicity of ADM on K562/A02 drug-resistant cells increased, with decrease in IC_{50} value of ADM, and increase in fold-reversal of MDG. These effects of SAS were concentration-dependent (Figure 2A). The EC_{50} of SAS for reversing doxorubicin resistance in K562/A02 resistant cells was calculated. The EC_{50} was the concentration needed for SAS to reverse doxorubicin resistance in K562/A02 cells by half. The results in Figure 2B show that SAS produced strong MDR-reversal effect, with EC_{50} for reversal of doxorubicin resistance in K562/A02 cells within the nano range (539 +37 nM). This merits further pharmacological studies. Moreover, SAS did not exert cytotoxicity in normal cells. Even at the maximum concentration of 100 µM, the degree of survival of ES-1 was still more than 80%. Thus, the IC_{50} of SAS was greater than 100 M. The calculated SI > 185 indicated that compound SAS had good degree of safety to normal cells while reversing MDR.

Effect of SAS on ADM accumulation in cells

The effect of SAS on the accumulation of doxorubicin in drug-resistant cells was determined using fluorescence spectrophotometry. As shown in Fig.3, ADM accumulation in adriamycin-sensitive K562 cells was significantly higher than that in K562/A02 cells (about 5.6 times higher). Compared with the drug-resistant blank control group, the accumulation of doxorubicin in K562/A02 cells was increased to varying degrees in the experimental group at different concentrations of SAS. The accumulation of ADM at 1.0µM SAS was similar to the accumulation of verapamil at 5.0µM. The results

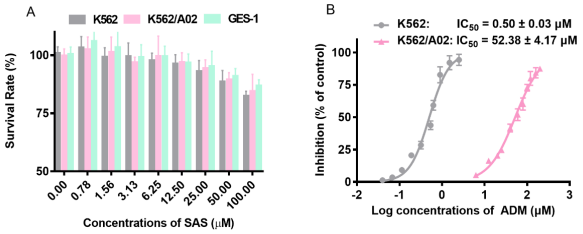

Figure 1. (A) Cytotoxicity of SAS against K562, K562/A02 and GES-1 cell lines; (B) IC_{50} values of ADM for inhibition of K562 and K562/A02 cell lines, as determined using MTT method. Percentage inhibition was plotted against log of ADM concentration. Each datum point represents mean ± SD for three independent tests.

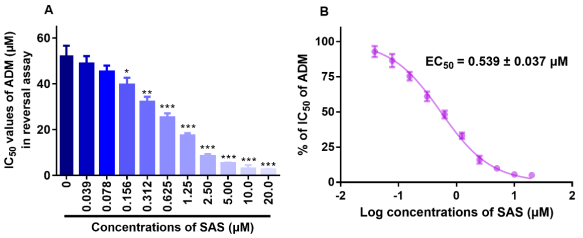

Figure 2. Reversal effect of SAS on ADM resistance in K562/A02 cells. (A) IC_{50} values of ADM in K562/A02 cells treated with ADM at different concentrations of SAS; (B) EC_{50} value of SAS in lowering ADM-resistance in K562/A02 cells. The percent of IC_{50} of ADM was plotted against log concentration of SAS, where percent of IC_{50} of ADM = $\frac{(IC_{50}\ of\ ADM\ at\ each\ modulator\ concentration)\times 1}{IC_{50}\ of\ ADM\ without\ modulator}$. EC_{50} was defined as the concentration of modulator that reduced the % of IC_{50} of ADM by half. Each datum point is presented as mean ± SD for three independent tests. $^{*}p < 0.05$, $^{**}p < 0.01$ & $^{***}p < 0.001$, when compared with 0 µM.

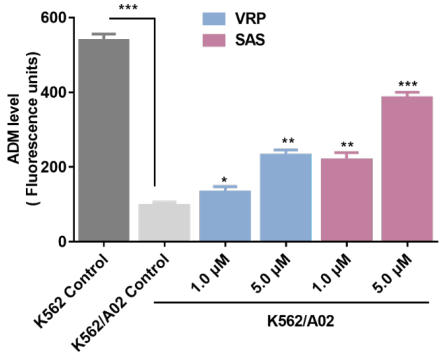

Figure 3. Effect of SAS on intracellular ADM accumulation. Results are presented as mean ± SD for three independent experiments; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, relative to the negative control (K562/A02).

showed that SAS inhibited the transport of p-gp and significantly accumulated adriamycin in the drug-resistant cells.

Effect of SAS on rhodamine 123 efflux

Efflux test for the p-gp-dependent fluorescent substrate, Rh123 is widely used for evaluating the effect of MDR-reversing agents on p-gp efflux function. As shown in Figure 4, the intensity of Rh123 in K562/A02 cells with overexpressed p-gp in the blank group was decreased significantly with time. As 5.0 μM verapamil and 1.0 μM SAS were added to K562/A02 cells, the intensity of Rh123 decreased, that is, the efflux of Rh123 contained in K562/A02 cells was inhibited, and the degree of reduction was concentration-dependent. These results indicate that SAS inhibited p-gp function and thus blocked the efflux of Rh123 from K562/A02 cells.

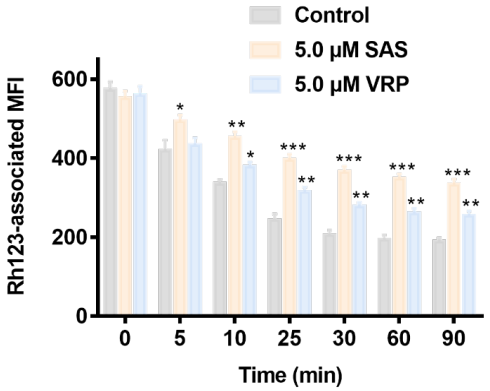

Figure 4. Effect of **SAS** on Rh123 efflux in K562/A02 cells. The results are presented as mean ± SD for three independent experiments; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, relative to the negative control (K562/A02).

Effect of SAS on the expressions of p-gp and Akt

Western blot results showed that a band with molecular weight of 170-kDa appeared in K562/A02 cell lysate i.e. p-gp, indicating that K562/A02 cells were highly-expressed p-gp cell lines. As shown in Figure 5, SAS inhibited the expressions of p-gp and p-Akt in the drug-resistant cells in a concentration-dependent manner. In contrast, the expressions of p-gp and p-Akt were not affected by verapamil.

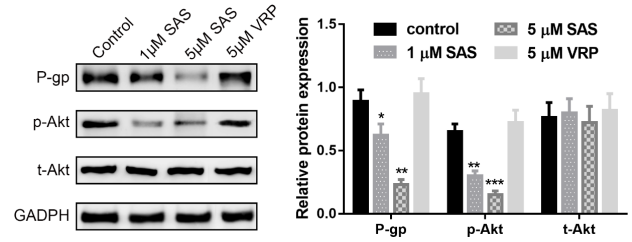

Figure 5. Effect of SAS and VRP on the expressions of p-gp, and p-Akt, and quantitative analysis of the expressions of p-gp, and p-Akt. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, relative to control. Representative results are shown here. Similar results were obtained in two other independent trials.

Discussion

CML is one group of the malignant bone marrow diseases caused by abnormal proliferation of multipotent hematopoietic stem cells. Although tyrosine kinase inhibitors represented by imatinib have been successfully used in the first-line treatment of CML with great improvement in the survival rate of patients, there are still many MDR patients who produce anti-chemotherapeutic factors during treatment, leading to chemotherapy failure. One of the key objectives in CML treatment is the reversal of MDR the classical mechanism involved in MDR is over-expression of efflux transporters (MDR, p-gp and BCRP). Several approaches have been used to ensure sensitivity to chemotherapy, so as to improve the therapeutic outcomes in leukemia (18, 19). It is nearly 40 years now since the discovery of the first p-gp regulator VRP in 1981, and the exploration of synthetic p-gp inhibitors has achieved effective inhibition of p-gp function (20). However, due to lack of significant clinical effects, drug interactions and other adverse side effects limit the development of p-gp inhibitors (19, 21). Some old drug structures offer new prospects for finding new and effective p-gp inhibitors. The parent structure of SAS is *azulene*, which is extracted from *Compositae* plants (*Chrysanthemum verum* and Chamomile). For more than one hundred years, doctors in some developed countries have used it for treatment of inflammation-related diseases such as conjunctivitis, keratitis, skin erythema and gastritis, with good clinical effects. The SAS is prepared by incorporating water-soluble groups into the molecular structure of *Acorus chinensis*. Due to the fact that SAS is a semi-synthetic product with minimal side effects and multiple biological benefits, it can be used in many fields such as pharmaceutical, cosmetic, and health product industries. However, prior to the present study, it was not clear whether SAS had MDR-reversal effects.

The K562/A02 cell line used in this study is a drug-resistant human leukemia cell line established by gradually increasing the concentration of adriamycin in a culture medium which can stably express p-gp (22). This study has established that SAS can be used as a drug for reversal of p-gp-mediated MDR. The inherent cytotoxic effect of SAS was first determined. Even at a concentration of 100 μM, SAS had weak anti-proliferative effect on sensitive and drug-resistant cell lines, and it had no inhibitory effect on normal GES-1 cells. The reversal effect of SAS against p-gp mediated resistance was determined at non-toxic concentration. The MDR-reversal effect of SAS at different concentrations indicated that its EC_{50} value was in the nano range (539 ± 37 nM), and that it had a high selectivity index (> 185) for normal cells. These findings indicate that SAS was harmless to normal human cells at reversal concentrations used for multi-drug resistance. In further investigations on the potential mechanism involved, ADM accumulation and Rh123 efflux test revealed that the MDR-reversal effect of SAS was triggered by inhibition of p-gp function (23, 24).

This organic cation pump (p-gp) is located on the cell membrane, and it is encoded by the *ABCBl (MDRl)* gene. The ATP-driven active drug pump out from cells reduces intracellular drug concentrations and blocks the chemotherapeutic effects of drugs, thereby resulting in drug resistance (25). Over-expression of p-gp is the main cause of drug efflux. Further studies are needed to confirm the effect of SAS on the expression of this

pump.

The p-13K/Akt is an important signal transduction pathway in cells. It is associated with MDR of leukemia. Highly activated P13K/Akt signaling pathway promotes drug efflux by up-regulating ABC binding membrane protein in cancer cells. Studies have revealed that blockage of the PI3K/Akt signaling pathway leads to down-regulation of downstream p-gp expression, thereby enhancing the sensitivity of cancer cells to chemotherapeutic drugs (26, 27). In the present study, Western blotting was used to investigate the effect of SAS on the expression of p-gp and Akt at the molecular level. The results showed that the protein expressions of p-gp and p-Akt were decreased in K562/A02 cells after combined treatment, suggesting that SAS inhibited p-gp expression, in addition to inhibiting p-gp function. The down-regulation of p-gp expression due to inhibition of PI3K/Akt signaling pathway was the key factor in the MDR reversal effect of SAS.

The results obtained in this study indicate that SAS reverses MDR of K562/A02 cells through a mechanism involving the down regulation of the PI3K/Akt signaling pathway. Therefore, SAS can be used as a drug candidate for reversal of MDR.

Acknowledgements
None.

Conflict of Interest
There are no conflict of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Xin Zhang; Ziwei Wang, Na Zhou, Jianqi Zhao, Xin Zhang collected and analysed the data; Ziwei Wang wrote the text and all authors have read and approved the text prior to publication.

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