Timosaponin A-III reverses multi-drug resistance in human chronic myelogenous leukemia K562/ADM cells via downregulation of MDR1 and MRP1 expression by inhibiting PI3K/Akt signaling pathway

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Abstract. One of the major causes of failure in chemotherapy for patients with human chronic myelogenous leukemia (CML) is the acquisition of multidrug resistance (MDR). MDR is often associated with the overexpression of drug efflux transporters of the ATP-binding cassette (ABC) protein family. Timosaponin A-III (TAIII), a saponin isolated from the rhizome of Anemarrhena asphodeloides, has previously demonstrated the ability to suppress certain human tumor processes and the potential to be developed as an anticancer agent. Nevertheless, the ability of TAIII to reverse MDR has not yet been explored. In this study, the adriamycin (ADM) resistance reversal effect of TAIII in human CML K562/ADM cells and the underlying mechanism was investigated. The Cell Counting Kit-8 (CCK-8) assay showed that TAIII had a reversal effect on the drug resistance of K562/ADM cells. Flow cytometry assay showed increased intracellular accumulation of ADM after cells were pretreated with TAIII, and the changes in the accumulation of rhodamine-123 (Rho-123) and 5(6)-carboxyfluorescein diacetate (CFDA) dye in K562/ADM cells were determined to be similar to the changes of intracellular accumulation of ADM. After pretreatment of cells with TAIII, the decreasing expression of P-gp and MRP1 mRNA was examined by reverse transcription polymerase chain reaction (RT-PCR). Western blotting showed TAIII inhibiting P-gp and MRP1 expression depended on the PI3K/Akt signaling pathway by decreasing the activity of p-Akt. Moreover, wortmannin an inhibitor of PI3K/Akt signaling pathway has a strong inhibitory effect on the expression of p-Akt, P-gp and MRP1. Besides, the combined treatment with TAIII did not have an affect on wortmannin downregulation of p-Akt, P-gp and MRP1. Taken together, our findings demonstrate, for the first time, that TAIII induced MDR reversal through inhibition of P-gp and MRP1 expression and function with regained adriamycin sensitivity which might mainly correlate to the regulation of PI3K/Akt signaling pathway.

Introduction

Chronic myeloid leukemia (CML) is a well-described hematopoietic malignancy as a result of the generation of the BCR-ABL fusion oncogene (1). Systemic chemotherapy is the main treatment method for patients with CML. Although the majority of the CML patients respond to therapy, >62.5% of the patients will experience multidrug resistance (MDR) (2). MDR is the main reason for tumor chemotherapeutic failure, and finding selective MDR reversal agents has become a research focus. MDR is often associated with overexpression of drug efflux transporters belonging to adenosine triphosphate (ATP)-dependent binding cassette (ABC) protein family (3). A large number of studies revealed that multidrug resistance 1 (MDR1), also known as P-glycoprotein or ABCB1, and multidrug resistance-associated protein 1 (MRP1), also known as ABCC1, which are ABC transporters and work as drug efflux pumps, play crucial roles in MDR of human CML (2-7).

The phosphoinositide 3-kinaseserine-threonine kinase (PI3K/Akt) signaling pathway is a well-known fundamental intracellular signaling transduction pathway involved in multiple biological processes both in normal and cancer cells, including gene transcription and translation, cell growth, proliferation and survival, cell metabolism, cell cycle progression, apoptosis and autophagy (8-10). Besides, there is an increasing amount of preclinical data supporting that the PI3K/Akt signaling pathway is also involved in the drug resistance of different types of human malignant cells, including
Multidrug resistance determination in K562/ADM cells. Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Shanghai, China) was used to determine the survival rate of cells incubated with ADM. The cells were seeded in a 96-well plate at a density of 5x10^4 cells/well in RPMI-1640 containing 10% FBS. Then, various concentrations of ADM (0.2-1.6 mg/l for K562 cells and 16-128 mg/l for K562/ADM cells) were added. After the cells were incubated at 37°C in 5% CO2 for 24 h, 10 µl of CCK-8 solution was added to each well and incubated for an additional 4 h. The absorbance was measured at 570 nm with a fluorescence spectrophotometer (F-7000; Hitachi HighTechnologies Corp., Tokyo, Japan). A blank well containing only medium and drugs was used as a control. The 50% inhibition of cell growth (IC50) produced by ADM was calculated.

TAIII intrinsic cytotoxic activity determination in K562/ADM cells. CCK-8 assay was also used to determine the direct cytotoxic activity of TAIII as described above. TAIII of different concentrations at 1-16 µM were added to each well for 24 h. Relative survival rate (%) of each group = absorbance of the experimental group/absorbance of the control group x 100%. The calculated TAIII concentration at 90% survival rate was IC10. The concentrations below IC10 were selected as the experimental concentration for TAIII to reverse drug-resistance.

Reversal efficacy of TAIII determination. Briefly, the K562/ADM cells were seeded into a 96-well plate, then 1 or 2 µM TAIII with or without various concentrations of ADM (4-32 µg/ml) was added to each well accordingly. Then, the quantity of viable cells were determined by CCK-8 assay according to the manufacturer's instructions. ADM IC50 was calculated using the untreated cells as the 100% viable control. The reversal fold (RF) values, as potency of reversal, were obtained from the following formula: RF=IC50 of ADM only/IC50 of ADM with TAIII.

Cellular uptake of ADM. K562/ADM cells were plated in 6-well plates at a concentration of 1x10^5 cells in 1 ml growth medium. After incubation alone or with TAIII (1 and 2 µM) at 37°C for 24 h, 3 mg/l ADM was added to designated K562/ADM cells for another 1 h at 37°C. Then, the cells were harvested by centrifugation and washed twice with ice-cold phosphate-buffered saline (PBS). The cell-associated mean fluorescence intensity (MFI) of ADM was detected by flow cytometer using a FACSCalibur (Beckman Coulter, Brea, CA, USA) with excitation/emission wavelengths of 485/580 nm.

Rho-123 and CFDA accumulation assay. Rhodamine-123 (Rho-123) and 5(6)-carboxyfluorescein diacetate (CFDA) were, respectively, used to evaluate the transport function of P-gp and MRPI in K562/ADM cells by flow cytometric analysis. Rho-123 was a special substrate for P-gp which contains yellow-green fluorophores. CFDA was used as a model MRPI substrate to evaluate the function of MRPI.
A total of 6x10^5 cells were seeded into 6-well plates which were pretreated with TAIII (1 and 2 µM) for 24 h followed by combined-treatment with Rho-123 (2 µg/ml) or CFDA (1 µM) for another 30 min at 37˚C in 5% CO_2. Cells with the equivalent amount of DMSO, without Rho-123, CFDA and TAIII were used to evaluate cell auto-fluorescence. Then, the cells were harvested and washed twice with cold PBS and subsequently analyzed by flow cytometry. The values were expressed by the mean fluorescence intensity of Rho-123 and CFDA.

**RT-PCR analysis.** After treatment with 1 or 2 µM TAIII for 24 h, ~3x10^6 cells were harvested for RT-PCR analysis. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed to cDNA and stored at -20˚C. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Primers used in RT-PCR were as follows: MDR1 forward, 5'-GGAGCCTACTTGGTGGCACATAA-3'; reverse, 5'-TGGCATAGTCAGGAGCAAATGAAC-3'. MRP1 forward, 5'-CTGGGAACATGATTAGGAAGC-3'; reverse, 5'-GAGGATTTCCCAGAGCCGAC-3'. GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3'; reverse, 5'-GAAGATGGTGATGGGATTTC-3'. RT-PCR was performed on an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) by using SYBR Green reaction kit (Takara, Bio, Otsu, Shiga, Japan). The reaction system of PCR was: SYBR green reagent, forward primer, reverse primer, template cDNA and nuclease-free distilled water. PCR programs were carried out as follows: 95˚C for 30 sec, followed by 45 cycles of 95˚C for 5 sec, 60˚C for 30 sec. GAPDH served as an internal control. The PCR products were separated by 1% agarose gels. The gels were scanned and analyzed by the Gel Imaging System. RT-PCR for each gene of each cDNA sample was assayed in triplicate.

**Western blot analysis.** After different treatments, the cells were harvested and washed with PBS. Lysis buffer (100 µl) (Beyotime Biotechnology) was added and the protein concentration of the lysate was determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Biotechnology). The lysed samples containing 50 µg were separated by 6-10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime Biotechnology) with a constant voltage of 80 V for 0.5 h and 120 V for another 1.5 h. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA) and blocked with 5% skimmed milk for 2 h. Subsequently, the membranes were incubated overnight at 4˚C with specific antibodies. The primary antibodies were rabbit polyclonal antibodies against P-gp (1:500), MRP-1 (1:500), total-Akt (1:500), p-Akt (1:500), GADPH (1:1,000). The following day, the membranes were incubated in horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:5,000) for 2 h at room temperature. Finally, images were captured by a FluorChem FC2 gel imaging system (Alpha Innotech, San Leandro, CA, USA). The intensity of each band was normalized by GADPH for their respective lanes.

**Data analysis.** Statistical analyses were performed using SPSS17.0 software (IBM SPSS, Armonk, NY, USA). Data were expressed as the means ± SD. Statistical comparisons were evaluated by one-way ANOVA. Values of P<0.05 was considered statistically significant.

**Results**

**Multi-drug resistance of K562/ADM cells.** Compared with its parental cells, K562/ADM cells showed clear drug-resistant property. As shown in Table I, the IC_{50} of ADM on K562 and K562/ADM cells were 1.013±0.1008 and 32.176±3.1718 µg/ml, respectively. The drug-resistance was 31.7784-fold (P<0.05).

**Direct cytotoxic activity of TAIII.** TAIII inhibited viability of K562/ADM cells in a dose-dependent manner (Fig. 1). TAIII of 1 µM had no significant cytotoxicity (cell growth inhibition <5%), and 2 µM TAIII had a very weak cytotoxicity (cell growth inhibition <10%). To minimize TAIII itself on K562/ADM cells growth, 1 and 2 µM was selected.

**Reversal of drug resistance by TAIII.** As shown in Fig. 2, the reversal effect of TAIII on K562/ADM cells was dose-dependent. The IC_{50} values of ADM in K562/ADM cells were 30.74±2.77, 20.05±1.18 and 12.19±2.17 µg/l for ADM plus vehicle, ADM plus 1 µM TAIII and ADM plus 2 µM TAIII, respectively. The reversal fold-change of TAIII of 1 and 2 µM was 1.50 and 2.52, respectively (Table II, P<0.05).
Effect of TAIII on the intracellular accumulation of ADM. The intracellular accumulation of ADM decreased significantly in K562/ADM cells compared to the parental K562 cells (26,27). We determined that TAIII increased the intracellular accumulation of ADM in K562/ADM cells. Our results indicated that TAIII elevated the sensitivity of K562/ADM cells toward ADM through increasing intracellular ADM accumulation (Fig. 3A; P<0.05). As show in Fig. 3D, the fluorescence intensity of ADM in TAIII-treated K562/ADM cells increased in a dose-dependent manner compared to untreated K562/ADM cells.

TAIII inhibits P-gp and MRP1-mediated transport. To assess the impact of TAIII treatment on the function of P-gp and MRP1 as efflux pump in K562/ADM cells, we examined the P-gp-mediated Rho-123 and MRP1-mediated CFDA transports in the cells treated with TAIII. As shown in Fig. 3B, Fig. 3C and D, TAIII treatment significantly increased the

Figure 2. TAIII treatment altered ADM-induced cytotoxicity of K562/ADM cells. Growth curves are based on data from CCK-8 assays in K562/ADM cells after 24-h culture in the absence or presence of 1 and 2 µM TAIII. The results showed increasing drug sensitivity in a dose-dependent manner in K562/ADM cells. Results are expressed as mean ± SD for three independent experiments. *P<0.05.

Figure 3. The effect of TAIII on the intracellular accumulation of adriamycin (ADM), rhodamine-123 (Rho-123) and 5(6)-carboxyfluorescein diacetate (CFDA) of K562/ADM cells. (A) TAIII significantly increased the ADM-associated MFI in K562/ADM cells in a dose-dependent manner. (B) Rho-123 was used as a special substrate for P-gp to test P-gp activity in K562/ADM cells. The FCM results showed that there was an increasing intracellular accumulation of Rho-123 in K562/ADM cells in a dose-dependent manner. (C) CFDA was used as a model MRP1 substrate to evaluate the function of MRP1. The FCM results showed that there was an increased intracellular accumulation of CFDA in K562/ADM cells. (D) Enhanced uptake of ADM, Rho-123 and CFDA presented in terms of mean fluorescence intensity value. Bars means ± SD. n=4. P>0.05, compared to the control group; *P<0.05, compared to the control group.
intracellular accumulation of Rho-123 and CFDA in a dose-dependent manner (P<0.05).

**TAIII decreases expression of P-gp and MRP1 in K562/ADM cells via the PI3K/Akt signaling pathway.** P-gp and MRP1 are ABC transporters, which are overexpressed in many drug-resistant cells (8,12,13). K562/ADM cells express higher levels of P-gp and MRP1 than K562 cells (26,28). In this study, K562/ADM cells expressed P-gp and MRP1 protein at high level. After incubation alone or with TAIII (1 and 2 µM) for 24 h, the expression levels of P-gp and MRP1 were determined by RT-PCR and western blotting (Figs. 4 and 5). The results of RT-PCR are shown in the Fig. 4, the MDR1 and MRP1 mRNA expression in K562/ADM cells significantly decreased in a dose-dependent manner (P<0.05). The results of western blotting are shown in Fig. 5, compared to the negative control group, TAIII was able to induce a significant downregulation of P-gp and MRP1 protein expression in a dose-dependent manner. These results indicated that TAIII could modulate P-gp and MRP1 gene expression, thus increasing the intracellular ADM accumulation.

**Figure 4.** TAIII treatment decreases the expression of P-gp and MRP1 mRNA in K562/ADM cells in a dose-dependent manner. P-gp and MRP1 mRNA levels were evaluated after 24 h of 1 and 2 µM TAIII treatment. Total RNA was isolated and used in RT-PCR to determine changes in P-gp and MRP1 mRNA levels after normalization to GADPH expression. The results are expressed as the mean ± SD for three independent experiments. Bars means ± SD. n=3. *P<0.05, **P<0.01, ***P<0.001 versus control group.

**Figure 5.** The effect of TAIII on expression of drug resistance related proteins in K562/ADM cells. After TAIII (0, 1 and 2 µM) treatment for 24 h, western blotting results showed decreased protein expression of P-gp, MRP1 and p-Akt in human CML K562/ADM cells. GADPH was the internal control. The results are expressed as the mean ± SD for three independent experiments. Bars means ± SD. n=3. *P>0.05, compared to the control group; *P<0.05, **P<0.01 compared to the control group.

### Table II. Effect of TAIII on the sensitivity of K562/ADM cells toward ADM by CCK-8 assay (means ± SD of triplicate experiments).

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<th>Treatment</th>
<th>IC50 (µg/ml)</th>
<th>Reversal fold (RF)</th>
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<tbody>
<tr>
<td>ADM alone</td>
<td>30.743±2.7661</td>
<td></td>
</tr>
<tr>
<td>ADM+1 µM TAIII</td>
<td>20.524±1.1798a</td>
<td>1.50</td>
</tr>
<tr>
<td>ADM+2 µM TAIII</td>
<td>12.192±2.1667a</td>
<td>2.52</td>
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*aP<0.05 vs. ADM alone.
Blocking PI3K/Akt pathway modulates the chemosensitivity of K562/ADM cells. To further determine the causal relationship between chemosensitivity and TAI III-inactivated PI3K/Akt signaling pathway on cell MDR, specific inhibitor of PI3K/Akt wortmannin was selected to treat K562/ADM cells which overexpressed P-gp and MRPI. The protein levels of total-Akt, Akt Ser473, P-gp and MRPI were measured. As shown in Fig. 6, K562/ADM cells with the inhibitor wortmannin treatment showed significantly decreasing protein levels of Akt Ser473, P-gp and MRPI, whereas total Akt was not affected by inhibition (P<0.05).

The inhibition of PI3K/Akt pathway plays a crucial role in the multidrug-resistance reversal of TAI III. In order to confirm the role of PI3K/Akt signaling pathway in TAI III-mediated reversal effect of multidrug resistance, we analyzed the proteins level of total-Akt, Akt Ser473, P-gp and MRPI in K562/ADM cells by western blotting. The cells were treated with wortmannin (1 µM) alone and combined-treatment with TAI III (1 and 2 µM) for 24 h. The results of western blotting are shown in Fig. 7, compared with wortmannin (1 µM) alone group, wortmannin (1 µM) combined with TAI III (1 µM) group and wortmannin (1 µM) combined with TAI III (2 µM) group did not attenuate the expression of p-Akt, P-gp and MRPI. Since TAI III did not strengthen the inhibitor function, we may conclude that PI3K/Akt signaling pathway was mainly responsible for the drug resistance reversal effect of TAI III.

Discussion

CML is one of the most genetically homogeneous malignancies characterized by clonal myeloid cells with an abnormal fusion protein, BCR-ABL, which has tyrosine kinase activity. Since biological sample collection is a non-invasive process, CML is one of the extensively studied diseases for gene expression profiling, which needs elucidation of the BCR-ABL downstream mechanisms involved in CML progression and the pathways involved in therapy resistance. Although the BCR-ABL-targeting tyrosine kinase inhibitors (TKIs) has shown significant progress toward treatment against CML, the
drug did not successfully cure patients of the disease (9,29). MDR has become a main obstacle for chemotherapy of CML. Mechanism of MDR is associated with altered expression of ATP-binding cassette (ABC) family of transporters on cell membrane, the most common cause of multidrug resistance (MDR) (1). While as many as 18 ABC transporters have been observed to export chemotherapy drugs using in vitro experimental systems, only 3 transporters have been implicated as major contributors to MDR in cancer: P-glycoprotein (Pgp; ABCB1; MDR1), multidrug resistance-associated protein (MRP1; ABCG1) and breast cancer resistance protein (BCRP; ABCG) (30). Moreover, among the three transporters, the expression of P-gp and MRP1 was extensively measured, studied in CML and its expression levels were correlated with multidrug resistance (31).

The PI3K/Akt signaling pathway has become an important player in the pathogenesis of MDR CML and a promising target for systemic therapy (1,2,4,5,9-11). Many cancer cells have mutations in the PI3K/Akt pathway that leads to hyper-activation of this pathway. In previous studies, it was reported that the PI3K/Akt signaling pathway was always prominently activated by many regulators and was strongly linked to pro-survival in cancer cells (9). Moreover, several lines of evidence implicated that the activating of PI3K/Akt signaling pathway had enhanced drug efflux by ATP-binding cassette (ABC) transporters (2). The maintaining of MDR in tumor cells by P-gp and MRP1 expression. Therefore, it is reasonable to believe that TAIII plays a suppressive role in the expression of P-gp and MRP1 via inhibition of the PI3K/Akt signaling pathway (31).

Additionally, the combined treatment reduced the expression of phosphorylated Akt (p-Akt) without affecting the expression of total-Akt, that means the activity of PI3K/Akt signaling pathway was downregulated. Moreover, we found that the levels of p-Akt, P-gp and MRP1 in K562/ADM cells decreased after exposure to the specific inhibitor of PI3K/Akt wortmannin. However, TAIII treatment combined with wortmannin did not exhibit strengthen effect on downregulation of P-gp and MRP1 expression. Therefore, it is reasonable to believe that TAIII plays a suppressive role in the expression of P-gp and MRP1 via inhibition of the PI3K/Akt signaling pathway.

The above studies show that TAIII could increase the intracellular accumulation of ADM in K562/ADM cells at non-toxic concentrations by downregulating P-gp and MRP1 expressions, function and transcription via a mechanism involving the inhibition of the PI3K/Akt signaling pathway. The studies provide evidence in support of further investigation into the clinical application of TAIII as new, potent, and clinically relevant MDR reversal agent in cancer chemotherapy.

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