Timosaponin AIII inhibits the growth of human leukaemia cells HL-60 by down-regulation of PI3K/AKT and Wnt/β-catenin pathways

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ABSTRACT
The effect of timosaponin AIII (TSA-III) on human leukaemia HL-60 cells and the underlying mechanism of action were explored in this study. HL-60 cells were treated with different concentrations of TSA-III, and then the survival rate and apoptosis rate of the cells, expression of apoptosis-related genes, activation of caspase-3, cell distribution in the cell cycle phases, and expression level of the related signal pathway proteins were detected. The results showed that TSA-III could dosedependent inhibit the proliferation of HL-60 cells. TSA-III could also increase the apoptosis rate of HL-60 cells, the level of Bax and Bak mRNA and the activation rate of caspase-3, and decrease the level of Bcl-2 mRNA. At the same time, the proportion of HL-60 cells treated with TSA-III in G0/G1 phase increased, whereas the proportion of the cells in S phase decreased. In addition, TSA-III could elevate the expression of PTEN protein, and reduce the expression of p-AKT, β-catenin, Cyclin D1 and C-myc proteins. These results indicate that TSA-III should have a cytotoxic effect on HL-60 cells, mainly showing its inhibition on the proliferation, inducing the apoptosis and cell cycle arrest, and the mechanism may be related to its inhibition of PI3K/AKT and Wnt/β-catenin pathways.

KEYWORDS
TSA-III; leukaemia cell; proliferation; apoptosis; signalling pathway

Introduction
Malignant tumours are one of the most serious diseases harmful to human health. There are about seven million people who die of cancers each year in the world and more than one million people who die of cancers in China [1,2]. Leukaemia is a malignant tumour of the hematopoietic system, accounting for about 5% of the total incidence rate of tumours [3]. Leukaemia is treated primarily with chemotherapy, but the therapy can cause systemic and strong toxic effects. Although bone marrow transplantation, immunotherapy and gene therapy are used for the treatment of leukaemia, the therapy with drugs is still the most important and basic method to treat leukaemia [4,5]. The biggest disadvantage of chemotherapy is that chemotherapeutic agents can also kill the normal tissues and cells when they kill tumour cells, causing serious lethal toxicities [6]. Over the last nearly half a century, people have paid more and more attention to the extraction of active components from traditional Chinese herbal medicines for the clinical anti-tumour therapy. Chinese herbal medicine can alleviate the side effects produced by chemotherapeutic agents, improve the immunity, and be used as a rescue treatment in refractory acute leukaemia [7]. More and more data have shown that Chinese herbal medicines can play a significant role in both promoting the apoptosis and inhibiting the proliferation of tumour cells, or modulating the immune functions, and alleviating the side effects of chemotherapy [8,9].

According to Chinese medicine, *Anemarrhena asphodeloides* Bge is the dry rhizome, bitter in taste, cold in nature, in the lung, stomach and kidney meridian, with functions of clearing away heat and purging pathogenic fire, nourishing yin for moistening dryness, and quenching thirst and relieving restlessness, and can be used for the treatment of some symptoms in clinic, such as exogenous fever, high fever and polydipsia, cough due to lung heat and dryness, thirst due to internal heat, and constipation due to intestinal dryness [10]. The rhizome of *A. asphodeloides* contains a lot of steroidal saponins, xanthones, lignans, polysaccharides and organic acids, of which timosaponin AIII (TSA-III), as the main active ingredient in *A. asphodeloides* Bge, accounts for about 6% in the rhizome [11]. TSA-III is a component of typical spirostanol saponin (its structure is shown in Figure 1) and in-depth studies on its pharmacological activities have been performed, in which its inhibition of platelet aggregation [12], vasodilation [13], improvement of...
memory [14], antioxidation [15] and hypoglycaemic effect [16] and other pharmacological activities have been confirmed. Recent studies have found that TSA-III presents strong cytotoxicity and its antitumour effect has been widely reported. Sy et al. [17] found that TSA-III could inhibit the growth of the HeLa cell line and the underlying mechanism of this action might be that TSA-III could get into the mitochondria to cause abnormal function of mitochondria, leading to generation of a large amount of intracellular reactive oxygen species (ROS), and thus the occurrence of autolysis and programmed death of HeLa cells. King et al. [18] demonstrated that TSA-III could activate the stress of endoplasmic reticulum bodies in cells, thereby inducing the apoptosis of the human breast cancer cell line BT474. In addition, TSA-III reportedly shows a significant inhibition on in vitro cultured human colon cancer HCT-15 cells and the same implanted cancer cells in rats [19]. The results indicate that TSA-III has a strong antitumour activity, but whether it has a cytotoxic effect on leukaemia cells is still unclear. For this reason, in this study, the effects of TSA-III on the proliferation, apoptosis and related signalling pathways in HL-60 leukaemia cells were investigated by in vitro experiments in order to lay a foundation for its further clinical application.

Materials and methods

Materials and chemicals

Human leukaemia cell line HL-60 was purchased from the Chinese Academy of Life Sciences Shanghai Cell Bank; TSA-III (purity over 98%) was purchased from Shanghai Baoman Biological Technology Co. Ltd; WST-1 and Annexin-EGFP/PI kits were purchased from Roche company of Switzerland; RPMI 1640 medium, calf serum and trypsin were purchased from Hyclone company in the United States; p-AKT, PTEN, β-catenin, Cyclin D1 and C-myc antibody were purchased from American Santa Cruz company.

Cell proliferation assay

According to the conventional method, HL-60 cells were routinely incubated at 37 °C in Roswell Park Memorial Institute medium (RPMI) 1640 culture medium containing 10% calf serum, 100 U/mL penicillin and 100 g/mL streptomycin in a 5% CO2 incubator. HL-60 cells were seeded at a density of 1 × 10^3 cells/well in 96-well plates. Cells in the control group were seeded in a culture medium without drug treatment, and those in the experimental groups were treated with 3, 6 or 9 μmol/L TSA-III, respectively, with three repeated wells being set up for each TSA-III concentration. After the cells were routinely cultured for 24, 48, 72 and 96 h, 10 μL WST-1 working fluid was added into each well, and then the cells continued to be cultured in a CO2 incubator for 3 h. Absorbance values (A) were detected at 450 nm with a BioTek Elx800 type microplate, and the survival rates were calculated.

Cell apoptosis detection

HL-60 cells treated with the different concentrations of TSA-III for 24 and 48 h were collected. The cells were suspended in 1 × binding buffer and the concentration of the cell suspension was adjusted to 1 × 10^6/mL with the buffer. After being washed with phosphate-buffered saline (PBS), the cells were re-suspended in 200 μL buffer. Then, 10 μL annexin–fluorescein isothiocyanate (FITC) and propidium iodide (PI) were respectively added to the cell re-suspension. The cell re-suspension was kept in a dark place to be incubated for 15 min and then FACS Calibur flow cytometry was used to detect cell apoptosis.
**Apoptosis-related gene detection**

HL-60 cells treated with the selected concentrations of TSA-III for 48 h were collected and the TRIzol method was used to extract the total RNA. According to the instructions of the M-MLV reverse transcriptase kit, the total RNA was reverse-transcribed into its cDNA and the reaction conditions were as follows: 30 °C for 10 min, 42 °C for 30 min, and 95 °C for 5 min (2720 Thermal Cycler, Applied Biosystems, California, USA); ABI 7000 fluorescent quantification for the PCR system and real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) were performed according to the standard procedures. The fluorescent quantitative PCR primers were designed by Primer 5 software (using glyceraldehyde phosphate dehydrogenase (GAPDH) as the internal reference). The volume of the PCR reaction system was 20 μL, and the reaction conditions were 95 °C for 2 min, 95 °C for 15 s, 60 °C for 40 s, with 45 cycles, which was repeated three times. The relative expressions of bcl-2, bax and bak in TSA-III-treated groups were calculated with the $2^{-\Delta \Delta Ct}$ method.

**Caspase-3 activation detection**

HL-60 cells treated with the selected concentrations of TSA-III for 24 and 48 h were collected and digested with ethylenediaminetetra-acid (EDTA) trypsin for the preparation of the cell suspension with a cell concentration of $1 \times 10^6$/mL. One millilitre of the cell suspension was centrifuged (1500 r/min × 5 min; 5430 R centrifuge, Eppendorf, Hamburg, Germany), added with a cell-membrane breaking agent and then incubated on ice for 20 min, followed by incubation with anti-activated caspase-3 antibody labelled with a fluorescein isothiocyanate (FITC) marker in a dark place for 30 min, and the activation of caspase-3 was detected by flow cytometry.

**Cell cycle detection**

HL-60 cells treated with the selected concentrations of TSA-III for 48 h were trypsinized, washed with PBS, pre-cooled and fixed with ethanol for more than 2 h. They were then treated with RNase A for 30 min after being washed with PBS. Then 20 mg/mL PI solution were added, followed by incubation for 30 min in a dark place. Flow cytometry was used to detect the cell-cycle distribution.

**Western blotting**

HL-60 cells treated with the selected concentrations of TSA-III for 48 h were collected and lysed with lysate in an ice bath for 30 min. They were then centrifuged at 12,000 r/min for 15 min (5430 R centrifuge, Eppendorf) and then the supernatant was obtained. After the protein quantification of the supernatant, 20 μg of protein sample from each well were taken for loading. The proteins in the samples were separated with 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred on nitrocellulose membranes by the semidry method. Appropriate amounts of p-AKT, PTEN, β-catenin, Cyclin D1 and C-myc-antibody were added onto the membranes (the dilution of AKT and Cyclin D1 was 1: 300 and that of the others was 1: 200), which were incubated at 4 °C overnight. Then the secondary antibody was added. An ultrasensitive enhanced chemiluminescence (ECL) liquid was used for the light-emitting film, and Quantity One software was applied to calculate the light absorbance of bands and the optical density ratio of the target bands to the internal reference GAPDH.

**Statistical analysis**

The results were expressed as means with standard deviation (±SD). Statistical comparisons were performed using t-test, one-way analysis of variance (ANOVA), or two-way repeated-measures ANOVA with Student’s t-test for post hoc. In all cases, $P < 0.05$ or $P < 0.01$ was considered statistically significant.

**Results and discussion**

In recent years, the study on the antitumour effect of TSA-III is deepening and the pharmacological mechanism of its antitumour action has been disclosed. It is not difficult to understand from a large number of studies that the comprehensive effect of multiple targets of TSA-III may contribute to its antitumour activity, which may indicate that TSA-III has more potential to be developed as an antitumour drug in contrast to the drugs that exert their antitumour effect through a single target [20,21]. This study found that TSA-III at a concentration of 3, 6 or 9 μmol/L could inhibit the proliferation of HL-60 cells. Compared with those in the control group, the survival rates of HL-60 cells in the TSA-III-treated groups were all significantly decreased. Especially, the survival rate of HL-60 cells treated with the high concentration TSA-III for 96 h was reduced to less than 30%. Overall, the inhibitory effect of TSA-III on the proliferation of HL-60 cells was dose- and time-dependent, as shown in Figure 1. The findings suggest that TSA-III should inhibit the proliferation of HL-60 cells in vitro, with cytotoxicity, which is consistent with the observation of its effects on
melanoma, hepatocellular carcinoma, breast cancer and lung cancer cells [22–24].

Induced apoptosis of cancer cells is also an important way through which TSA-III exerts its antitumour effect, such as induced caspase activation, degradation of X-linked inhibitor of apoptosis protein (XIAP) [25]. A similar result was also obtained in this study; that is, the treatment with TSA-III could cause an increase in the apoptosis rate and in the level of Bax and Bak mRNA, but a decrease in the level of Bcl-2 mRNA, and an increased activation rate of caspase-3 in HL-60 cells. This suggests that TSA-III can exert its pro-apoptotic action by inducing the activation of caspase-3 (Figures 2–4).

The aberrant regulation of the cell cycle has been considered a focus of cancer treatment; previous studies have been focusing on the proliferation and apoptosis, and there are a few studies on the cell cycle [26]. As speculated, TSA-III could induce the G0/G1 phase arrest of HL-60 cells, such as an increased proportion of cells in G0/G1 phase and a decreased proportion of cells in S phase, which may be considered to be related to an important pathway mediating the malignant behaviour of tumour cells (Figure 5). Therefore, the effects of TSA-III on PI3K/AKT and Wnt/β-catenin pathways were further explored in this study.

PI3K/AKT and Wnt/β-catenin pathways are signalling pathways that are most widely studied in cancers currently. The inhibitory effect of drugs or gene interference
on the activation of these two pathways has shown a better antitumour effect [27–29]. The PI3K/AKT pathway is one of the main signal transduction pathways for cells to integrate external stimuli, and PTEN is the main negative regulatory factor of the pathway; p-AKT is one of the direct downstream effector molecules in the signalling pathway, in which the increase in the expression of PTEN and the decrease in the expression of p-AKT may indicate that the activity of the PI3K/AKT pathway is inhibited [30,31]. The Wnt/β-catenin pathway plays an important role in the regulation of physiological activities in the body, in which β-catenin is one of the key effector molecules in the pathway, while Cyclin D1 and C-myc are the downstream targets, and the expression levels of the three proteins decrease when the activity of the pathway is inhibited [32,33]. In this study, western blotting detection showed that both the PI3K/AKT and Wnt/β-catenin pathways were inhibited in the HL-60 cells treated with TSA-III, which might be one of the important ways through which TSA-III could inhibit the growth of leukaemia cells (Figure 6). However, the activation order of the two pathways was unclear. In view of the interaction between the PI3K/AKT pathway and Wnt/β-catenin pathway [34], and their participation in the regulation of the biological behaviours of malignant tumours, it is speculated that they may play a role in this process. Therefore, the related antagonists should be used to explore the specific roles of the PI3K/AKT and Wnt/β-catenin pathways for the antitumour activity of TSA-III in the body in further research.

Conclusions

In this study, the effect of TSA-III on human leukaemia HL-60 cells and the underlying mechanism were explored. The results indicate that TSA-III had a cytotoxic effect on HL-60 cells, mainly showing inhibition of the proliferation, induction of apoptosis and cell cycle arrest. The mechanism may be related to inhibition of PI3K/AKT and Wnt/β-catenin pathways.

Disclosure statement

No potential conflict of interest was reported by the authors.

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