Abstract. Timosaponin A-III (TAIII) is a saponin isolated from anemarrhena asphodeloides and possesses the inhibitory effect on proliferation of multiple tumor cells. In the present study, the antitumor effect of TAIII and its underlying molecular mechanisms were investigated in vitro in T-cell acute lymphoblastic leukemia (T-ALL) Jurkat cells. The results demonstrated that TAIII inhibits the viability of Jurkat cells in a time- and dose-dependent manner, and induces apoptosis of Jurkat cells in a dose-dependent manner. Transmission electron microscopy demonstrated the formation of numerous autophagosomes in TAIII-treated Jurkat cells. Furthermore, monodansylcadaverine (MDC)-labeled autophagic vacuoles were observed following TAIII treatment by an inverted fluorescence microscope and MDC accumulation increased notably inTAIII treatment groups in a concentration-dependent manner. B-cell lymphoma-2 (Bcl-2)-associated X (Bax) was upregulated while Bcl-2 was reduced following TAIII treatment, indicating that the pro-apoptotic mechanism of TAIII may be associated with upregulation of Bax. Further investigation revealed that TAIII promotes the expression of autophagy-associated proteins Beclin 1 and LC3-II, and inhibits the phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin kinase pathway. The present study revealed that the antitumor activity of TAIII was primarily achieved by the induction of cell apoptosis and autophagy, indicating a promising potential as a novel effective reagent against T-ALL.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood and accounts for ~25% of cases diagnosed in children <15 years old (1). ALL is a heterogeneous disease that results from the malignant transformation of lymphoid progenitor cells in the bone marrow, blood and extramedullary sites (2). In children with ALL, T-cell ALL (T-ALL) accounts for 12-15%, and compared with B-cell ALL, the prognosis of T-ALL is poorer due to increased relapse risk and reduced response to salvage therapy (3). In recent years, the outcome of children with T-ALL has improved with contemporary risk and response-based treatment protocols; additionally, the 5-year event-free survival rate reported by a number of foreign research centers reached 85%, but the majority of the research centers remain at ~60% (4-6). Currently, the primary focus for first-line therapy is on reducing the burden of long-term toxicity, decreasing the chemotherapy intensity for specific low-risk patients and strengthening treatment for high-risk groups (7). Since antineoplastic agents derived from natural products exhibit notable advantages, including low toxicity, insignificant or no side-effects, targeting multiple targets and a reduced chance to generate drug resistance (8), researchers focus on searching for novel antineoplastic drugs from natural products and Chinese traditional herbs.

Timosaponin A-III (TAIII), a steroidal saponin isolated from the rhizomes of anemarrhena asphodeloides, has been credited with a wide spectrum of bioactivities, including controlling hyperglycemia (9), improving learning and memory (10), inhibiting inflammation (11), suppressing allergic reaction (12) and restraining platelet aggregator (13). In recent years, more importance has been attached to its antitumor effect; additionally, accumulating evidence indicate that TAIII serves important roles in suppressing proliferation, inducing apoptosis (14), activating autophagy (15,16) and reversing multidrug resistant (17) of tumor cells. TAIII was observed to...
exhibit cytotoxicity towards a panel of carcinoma cell lines, including human breast carcinoma cells (MDA-MB-231 and MCF-7), hepatocellular carcinoma cells (Hep3b and HepG2), human lung cancer cells (A549), human colorectal cancer cells (HCT-15, HCT-116, HT-29, SW-480 and SW-620), human cervical epithelioid carcinoma cells (HeLa), human nasopharyngeal carcinoma cells (SUNE-1 and human chronic myeloid leukemia cell line (K562/ADM), but had a reduced effect on the viability of normal cells (16-19). However, to the best of our knowledge, limited research has been conducted to investigate the function of TAIII in T-ALL Jurkat cells.

In the present study, the aim was to investigate the effect of TAIII on cell apoptosis and autophagy in T-ALL Jurkat cells. It was determined that TAIII induced cell apoptosis and autophagy in a dose-dependent manner. Further study revealed that TAIII promoted autophagy via inhibiting the phosphoinositide 3-kinase (PI3K)/Akt/mechanistic target of rapamycin kinase (mTOR) pathway. The present study revealed the possible antitumor mechanisms of TAIII in T-ALL, providing novel strategies and targets for T-ALL treatment.

Materials and methods

Cell line and cell culture. The Human T-ALL Jurkat cell line was purchased from the Department of Pharmacology, The Institute of Hematology of Chinese Academy of Medical Sciences (Tianjin, China). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; both from HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO₂. The cells cultured to 70-80% confluence were used in experiments.

Chemicals and reagents. TAIII (purity>98%; Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China) and monodansylcadaverine (MDC; Sigma-Aldrich; Merek KGaA, Darmstadt, Germany) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merek KGaA) at the final concentration of 20 mM and 50 mM, respectively, stored at -20˚C in the dark, and diluted in RPMI-1640 medium to the desired concentration prior to each experiment. The final DMSO concentration did not exceed 0.1% throughout the study. Rabbit polyclonal antibodies, including B-cell lymphoma 2 (Bcl-2; cat. no. bs-0032R), Bcl-2-associated X (Bax; cat. no. bs-20386R), Beclin 1 (cat. no. bs-1353R), LC3-I/LC3-II (cat. no. bs-2912R), Akt (cat. no. bs-0115R), phospho-(p)-Akt (cat. no. bs-0876R), mTOR (cat. no. bs-1992R), p-mTOR (cat. no. bs-3495R), PI3K (cat. no. bs-6423R) and p-PI3K (cat. no. bs-0876R), were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Rabbit polyclonal antibody against GAPDH (cat. no. AB-P-R001) was a product of Hangzhou Goodhere Biotechnology Co., Ltd. (Hangzhou, China).

Cell Counting Kit-8 (CCK-8) assay for cell proliferation. CCK-8 (Dojindo Molecular Technologies, Inc., Shanghai, China) was used to determine the survival rate of cells incubated with TAIII. Jurkat cells were seeded into a 96-well plate at a density of 1x10⁴ cells/well in RPMI-1640 containing 10% FBS. Subsequently, various concentrations of TAIII (2, 4, 8, 16 and 32 µM) were added. After the cells were incubated at 37˚C in an atmosphere containing 5% CO₂ for 24, 48 or 72 h, 10 µl CCK-8 solution was added to each well and incubated for an additional 4 h at 37˚C. The absorbance was measured at 580 nm with Model 680 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A blank well containing only RPMI-1640 medium supplemented with 10% FBS was used as a control.

Cell apoptosis analysis by flow cytometry. Jurkat cells were plated into a 6-well plate at a concentration of 3x10⁵ cells in 1 ml RPMI-1640 medium supplemented with 10% FBS. Following incubation with TAIII (0, 2 and 8 µM) at 37˚C for 24 h, the cells were harvested and washed twice with ice-cold PBS. Subsequently, the cells were suspended in 400 µl 1X binding buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) and double-stained with Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI; Nanjing KeyGen Biotech Co., Ltd.) for 15 min in the dark at room temperature. The apoptosis cells were analyzed using a FACS Canto™ II Flow Cytometer with CellQuest software V 7.5.3 (BD Biosciences; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The apoptotic rate was calculated as the percentage of early apoptotic cells plus the percentage of late apoptotic cells.

Observation of cell ultrastructure under transmission electron microscopy (TEM). Jurkat cells were incubated with TAIII (0 and 8 µM) at 37˚C for 24 h. Subsequently, cells were harvested, washed twice with PBS, fixed with 3% glutaraldehyde for 24 h at 4˚C. Following washing three times with ice-cold PBS, Jurkat cells were progressively dehydrated in 30% ethanol for 5-10 min, 50% ethanol for 5-10 min, 70% ethanol for 5-10 min, 90% ethanol for 10-15 min, 100% ethanol for 10-15 min and embedded in Epon812 resin. Finally, the specimens were sliced into serial ultrathin sections (thickness, 100 nm), and stained in uranyl acetate for 15-30 min and lead citrate for 5-10 min at room temperature. The specific ultrastructure features were observed under a TEM (JEM-2800; JEOL, Ltd., Tokyo, Japan; x4,000 magnification).

Visualization of MDC-labeled autophagic vacuoles. MDC staining of autophagic vacuoles was performed for autophagy analysis (20). Jurkat cells growing on coverslips were pretreated with TAIII (0, 2 and 8 µM) for 24 h at 37˚C. Following treatments, the cells were stained with 50 µM MDC in PBS for 10 min at 37˚C, and then washed three times with PBS to remove excess MDC and subsequently analyzed under an inverted fluorescence microscopy (F-7000; Hitachi, Ltd., Tokyo, Japan; x400 magnification). Fluorescence of MDC was measured at 365 nm excitation filter, 395 nm spectroscope and 420 nm absorption filter.

Detection of intracellular mean fluorescence intensity of MDC. Jurkat cells were plated into a 6-well plate at a concentration of 3x10⁵ cells in 1 ml RPMI-1640 medium supplemented with 10% FBS. The cells were pretreated with TAIII (0, 2 and 8 µM) at 37˚C for 24 h. Subsequently, the cells were harvested by centrifugation at 3,000 x g for 3 min at room temperature and suspended with 1 ml RPMI-1640 medium...
supplemented with 10% FBS. Following this, MDC (50 µM) was added to each group and dyed for 10 min in the dark at 37°C. After staining, the cells were collected by centrifugation at 3,000 x g for 3 min at room temperature, washed twice with ice-cold PBS and suspended with 1 ml ice-cold PBS. Immediately after that, the cell-associated mean fluorescence intensity (MFI) was detected at 488 nm excitation wavelength by a FACSCanto™ II Flow Cytometer with CellQuest software V 7.5.3.

Western blot assay. The cells were plated into a 6-well plate at a concentration of 3x10⁵ and cultured to 70-80% confluence at 37°C for 24 h. The cells were harvested and washed with PBS twice, and then scraped and lysed in Radioimmunoprecipitation Assay buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentrations were determined by Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Protein samples (40 µg), including Beclin 1 (50 kDa), Akt (56 kDa), p-Akt (56 kDa), p-mTOR (289 kDa), p-mTOR (289 kDa), PI3K (82 kDa), p-PI3K (82 kDa) and GAPDH (37 kDa), were separated with 8% SDS-PAGE (Beyotime Institute of Biotechnology). Bcl-2 (26 kDa), Bax (21 kDa) and LC3-I/LC3-II (14/16 kDa) were separated with 15% SDS-PAGE. Subsequently, the proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked by 5% skimmed dry milk in TBS containing 0.2% Tween 20 at room temperature for 2 h followed by an overnight incubation with specific antibodies at 4°C. The primary antibodies were rabbit polyclonal antibodies against Bcl-2 (1:500), Bax (1:500), Beclin 1 (1:500), LC3-I/LC3-II (1:500), Akt (1:500), p-Akt (1:500), mTOR (1:500), p-mTOR (1:500), PI3K (1:500), p-PI3K (1:500) and GAPDH (1:1,000). Following three washes in TBS/Tween buffer, the membranes were incubated in horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:500), reaction with specific antibodies at 4˚C. The primary antibodies were rabbit polyclonal antibodies against Bcl-2 (1:500), Bax (1:500), Beclin 1 (1:500), LC3-I/LC3-II (1:500), Akt (1:500), p-Akt (1:500), mTOR (1:500), p-mTOR (1:500), PI3K (1:500), p-PI3K (1:500) and GAPDH (1:1,000). Following three washes in TBS/Tween buffer, the membranes were incubated in horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:5,000; cat. no. TA130015; OriGene Technologies, Inc., Beijing, China) for 2 h at 37°C. Detection was performed using the FluorChem FC2 gel imaging system (ProteinSimple, San Jose, CA, USA). Each band density was quantified using Image J V 1.8.0 image processing program (National Institutes of Health, Bethesda, MD, USA) and normalized by GAPDH for their respective lanes.

Data analysis. Statistical analyses were conducted using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). The normality of data was analyzed by the Shapiro-Wilk test, and it was determined that the data were normally distributed. One-way analysis of variance was used to analyze differences between ≥3 groups, followed by a Least Significant Difference multiple range test for post-hoc comparisons. Data are expressed as the means ± standard deviations. P<0.05 was considered to indicate a statistically significant difference.

Results

TAIII suppresses proliferation of Jurkat cells. The CCK-8 assay was conducted to evaluate the cytotoxicity of TAAIII on the proliferation of Jurkat cells. As depicted in Fig. 1, Jurkat cells were sensitive to TAAIII and TAAIII inhibited the viability of Jurkat cells in a time- and dose-dependent manner in vitro.

Figure 1. TAAIII inhibits the proliferation of Jurkat cells. Jurkat cells were treated with 2, 4, 8, 16 or 32 µM TAAIII for 24, 48 or 72 h. Growth curves were based on data from Cell Counting Kit-8 assays. The results are represented as the mean ± standard deviation of triplicate experiments. TAAIII, Timosaponin A-III.

The inhibitory rate of TAAIII on growth of Jurkat cells was 4.34±0.31, 13.67±0.78 and 22.15±1.04%, respectively, after the cells were treated with 2 µM TAAIII for 24, 48 and 72 h.

TAIII induces apoptosis of Jurkat cells. As depicted in Fig. 2A-C, after treatment with 0, 2 or 8 µg TAAIII for 24 h, the total apoptosis rate of Jurkat cells was 4.63±1.47, 27.07±2.57 and 55.13±5.17%, respectively. Additionally, TAAIII induced apoptosis of Jurkat cells in a dose-dependent manner (Fig. 2D).

Autophagy detection by TEM. TEM was performed to detect the micro-morphological change of Jurkat cells. Results demonstrated that TAAIII induces Jurkat cells to generate autophagy. As depicted in Fig. 3, Jurkat cells not treated with TAAIII exhibited the normal ultrastructural morphology of nuclei, cytoplasm and organelles. The most prominent morphological change in TAAIII-treated cells was the formation of numerous autophagic vacuoles in the cytoplasm, and the giant cytophagosomes filled with degraded organelles and autolysosomes were frequently observed.

Observation of vacuolization in cytoplasm by inverted fluorescence microscope. MDC-labeled autophagic vacuoles were observed by inverted fluorescence microscope. As depicted in Fig. 4, MDC-labeled autophagic vacuoles appeared as distinct dot-like structures distributing in cytoplasm or in perinuclear regions. The TAAIII-treated group exhibited increased fluorescence density and more MDC-labeled particles, compared with the control group, indicating that TAAIII induced the formation of the MDC-labeled vacuoles. Additionally, it was determined that vacuolization in the cytoplasm progressively became larger and denser when the concentration of TAAIII increased.

MDC accumulation increases in Jurkat cells following TAAIII treatment. Furthermore, flow cytometry was used to detect intracellular MDC MFI. The results implied that the MFI of MDC in TAAIII-treated Jurkat cells increased in a dose-dependent manner, compared with untreated Jurkat cells. As depicted in Fig. 5, Jurkat cells treated with TAAIII (2 and 8 µM)
enhanced the MDC MFI by 1.31- and 1.76-fold, compared with the control group.

**TAIII increases Bax and decreases Bcl-2 expression in Jurkat cells.** To elucidate the molecular mechanism underlying the
apoptosis induced by TAIII, the protein expression levels of Bcl-2 and Bax genes, apoptosis-associated molecules, were evaluated by western blot assay. The results demonstrated that the expression of Bax increases significantly while the expression of Bcl-2 decreases after Jurkat cells incubation with different concentrations of TAIII for 24 h (Fig. 6).

TAII upregulates Beclin 1 and LC3-II expression in Jurkat cells. To clarify the mechanism underlying the autophagy induced by TAIII, western blotting was conducted to assess the effect of TAIII on the expression of Beclin 1 and LC3-II, which serve key roles in autophagy (18,20). As depicted in Fig. 7A and B, TAIII treatment caused the conversion of
TAIII promotes Jurkat cells autophagy via PI3K/Akt/mTOR signaling. It is known that the PI3K/Akt/mTOR signaling pathway serves a suppressive role in autophagy (21). In the present study, the activity of the PI3K/Akt/mTOR signaling pathway was investigated by TAIII treatment in Jurkat cells. As depicted in Fig. 7C and D, western blotting demonstrated that the levels of p-PI3K, p-Akt and p-mTOR are notably reduced in TAIII treatment groups, compared with the control group. However, there were no notable changes in the total amount of PI3K, Akt and mTOR. These results indicated that PI3K/Akt/mTOR signaling is involved in Jurkat cells autophagy induced by TAIII.

Discussion

TAIII, one of the main active constituents of Chinese medicinal herb anemarrhena asphodeloides bunge, has been credited with effective anticancer activities, including suppressing proliferation, enhancing apoptosis, activating autophagy and reversing multidrug resistance (14,16,17). Sy et al (16) reported that TAIII-induced autophagy in human cervical HeLa cells, followed by mitochondria-dependent apoptotic cell death. It was demonstrated that TAIII reverses multi-drug resistance in human chronic myelogenous leukemia K562/ADM cells via downregulation of MDR1 and MRP1 expression via inhibiting the PI3K/Akt signaling pathway (17). Kang et al (18) determined that TAIII inhibits the proliferation of human colon cancer HCT-15 cells with cell cycle arrest and induction of apoptosis. In the present study, results indicated that TAIII exerts a significant inhibitory effect on Jurkat cells in a time- and dose-dependent manner, and induces tumor cell apoptosis via downregulating Bcl-2 expression. Additionally, Jurkat cells autophagy was promoted by the inhibition PI3K/Akt/mTOR signaling. Overall, the results demonstrated that TAIII stimulates cell apoptosis and autophagy of Jurkat cells in vitro.

Considering that apoptosis induction is one of the key features of antitumor drugs in tumor therapy, it is important to examine the effects of TAIII on apoptosis induction and tumor growth. Apoptosis, also known as type I programmed cell death, is characterized by morphological changes, including cell shrinkage, chromatin condensation and membrane blebbing, without disruption of the plasma membrane; additionally, apoptosis is triggered by two pathways, the death receptor-mediated extrinsic pathway and the mitochondrial-involved intrinsic pathway (21-24). Bcl-2 family members are critical players in mitochondrial-involved intrinsic apoptosis (25). Bax and Bcl-2 are the most characterized apoptosis regulators in mitochondrial-associated apoptosis; furthermore, Bax is the first identified pro-apoptotic protein member of the Bcl-2 protein family, possessing promoting apoptosis activity (26). In the present study, data demonstrated that TAIII induces apoptosis of Jurkat cells in a dose-dependent manner (Fig. 2). However, consistent with the ability of TAIII to kill cells via apoptosis processes, TAIII increased the expression of Bax and decreased the expression of Bcl-2 in a dose-dependent manner (Fig. 6), indicating that the upregulated Bax and downregulated Bcl-2 expression may trigger TAIII-induced apoptosis of Jurkat cells.

Furthermore, for the first time, the present study also provides experimental evidence to demonstrate the activation of an autophagic program in Jurkat cells following treatment with TAIII. Autophagy is a genetically programmed and evolutionarily conserved process, and macro-, micro-, and chaperone-mediated autophagy are three primary autophagy forms, with macro-autophagy being the most prevalent form (27). Autophagy can regulate physiological and pathophysiological cell death, and the basic function of autophagy is to sustain survival and maintain cell homeostasis through organelles and proteins recycling (28,29). Increasing evidence indicate that autophagy is important in human cancer suppression and extensive attention has been paid to its role in tumor therapy (30,31). Previous studies indicated that TAIII is a pronounced activator of autophagy (15,16,32). In the present study, TEM determined the formation of autophagosomes, which are the representative characteristics of autophagy (Fig. 3). Furthermore, numerous MDC-labeled autophagic vacuoles were observed in TAIII-treated Jurkat cells under an inverted fluorescence microscope (Fig. 4), and flow cytometry analysis illustrated that the MDC accumulation increased notably in TAIII treatment groups in a dose-dependent manner (Fig. 5).

In order to further examine the mechanism by which TAIII regulated autophagy, various molecular studies were performed. Beclin 1, a mammalian orthologue of the yeast Apg6/Vps30 gene and a subunit of the class III PI3-kinase complex, is the first mammalian gene that was identified to be
able to induce autophagy (33). LC3-II has been identified as a marker for autophagosome formation (8). Therefore, the expression levels of Beclin 1 and LC3-II in TAIII-treated Jurkat cells were measured. As depicted in Fig. 7, TAIII treatment upregulated the expression of Beclin 1 and LC3-II in Jurkat cells in a concentration-dependent manner. The results demonstrated that TAIII induces the expression of the autophagy-associated proteins Beclin 1 and LC3-II, while reducing the proliferation of Jurkat cells in vitro. There have been a substantial amount of studies indicating that PI3K/Akt/mTOR signaling are key axes involved in anticancer drug-induced autophagy (8,34,35). Further investigation was performed, with a conclusion that the PI3K/Akt/mTOR signaling pathway was inactivated by TAIII through suppressing the expression of p-PI3K, p-Akt and p-mTOR. While the preliminary observations are promising, more comprehensive and detailed studies are required to be conducted, for example, animal models in vivo or more cellular experiments, including a colony formation assay, and other apoptosis indicators, including a DNA fragmentation assay.

In conclusion, the present results demonstrate that TAIII possesses antitumor activity and its antineoplastic action is associated with inhibition of proliferation, and induction of apoptosis and autophagy in Jurkat cells. Furthermore, the results demonstrated that the anticancer activity of TAIII in Jurkat cells may be through regulating apoptosis-associated proteins, including Bax and Bcl-2, and autophagy-associated proteins, including Beclin 1 and LC3-II. Furthermore, TAIII promoted Jurkat cells autophagy through the PI3K/Akt/mTOR pathway. Finally, the present data may provide a novel approach for the development of T-ALL therapy.

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Availability of data and materials

The datasets analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HW performed the experiments, analyzed the results and wrote the manuscript. RD and WWF were responsible for the collection and assembly of data, and the data analysis. XCZ and AML revised the manuscript and performed supplementary experiments. WDW was responsible for design of the present study and financial support. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

The authors declare that they have no competing interests.

Competing interests

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