Toosendanin induces the apoptosis of human Ewing's sarcoma cells via the mitochondrial apoptotic pathway

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Abstract. Toosendanin, a triterpenoid extracted from the root bark of *Melia toosendan*, has its origin from traditional Chinese medicine and has been used as a non-polluting and pesticide-free plant insecticide in China for fruit and vegetable production. In recent years, toosendanin has been found to inhibit tumor cell proliferation and promote tumor cell apoptosis. Ewing's sarcoma (ES) is the second most common primary malignant bone and soft tissue tumor in children and adolescents. Although the overall prognosis of ES has improved, the 5-year survival rate has not significantly increased. To analyze the role of toosendanin on ES progression, CCK-8 viability assay, flow cytometry, Hoechst 33258 staining and western blotting were performed. The present results suggested that toosendanin suppressed cell viability and induced apoptosis in human SK-eS-1 cells compared with DMSO treatment. In addition, in the present study, toosendanin was found to upregulate the expression of Bax and downregulate the expression of Bcl-2, altering the Bax/Bcl-2 ratio. Additionally, toosendanin promoted the release of cytochrome c, resulting in the activation of the mitochondrial apoptotic pathway, thus inducing the activation of caspase-9 and caspase-3, and the cleavage of PARP. Our results demonstrated that toosendanin inhibited the growth of ES cells in a dose-dependent manner and triggered mitochondrial apoptotic pathway to induce apoptosis. Therefore, toosendanin can potentially be utilized as an anticancer botanical drug for the treatment of ES.

Introduction

Traditional Chinese medicine (TCM) has been gaining more attention due to its satisfactory clinical results and the reduced side effects when utilized for cancer treatment. TCM is an important part of complementary and alternative medicines as it has standardized diagnostic and therapeutic systems and is implemented worldwide (1). There is increasing evidence that various herbs and compounds derived from natural products with antitumor effects can induce apoptosis in various tumor cells (2-4). Toosendanin is a triterpenoid extracted from the root bark of *Melia toosendan*. Toosendanin was found to inhibit tumor cell proliferation and promote tumor cell apoptosis (5-7). In addition, a previous study showed that toosendanin can block osteosarcoma tumorigenesis (8). However, there has been no research on the effects of toosendanin on Ewing's sarcoma (ES).

ES is a rare invasive tumor in the primitive neuroectodermal tumor family (9), which is common in children and adolescents (10,11). ES is extremely malignant, with a short disease course, rapid recurrence and high transfer rate. Although neoadjuvant chemotherapy and limb salvage surgery are widely used clinically, the 5-year survival rate for ES patients with early metastasis or recurrence is still less than 50% and the 10-year survival rate is less than 30% (12,13). In addition, most neoadjuvant chemotherapy drugs have the risk of unstable effectiveness and serious side effects such as cardiotoxicity and nephrotoxicity. Therefore, there is an urgent need for safer and more effective anticancer drugs in clinical practice.

Studies have shown that baicalein extracted from the TCM *Astragalus membranaceus* can induce apoptosis through the mitochondrial apoptosis and death receptor pathways in ES cells (14). Therefore, we hypothesize that toosendanin may also have antitumor and/or pro-apoptotic activity in ES. Subsequently, we studied the effects of toosendanin on cell activity and apoptosis of the human ES cell line SK-ES-1 and further elucidated the relevant molecular mechanisms.

Materials and methods

Cell culture. The human ES cell lines SK-ES-1 and RD-ES were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in
RP/1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cells were passaged when they reached 80% confluence. Exponential-phase cells were used in the experiments, and the passage number was <20.

Reagents and antibodies. Purified toosendanin (source: root bark and bark of Melia toosendan; molecular formula (MF): C₆₀H₇₆O₄₃; molecular weight (MW): 574.62; purity ≥98%) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). RP/1640 medium, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), Cell Counting Kit-8 (CCK-8) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (dilution 1:2,500; cat. no. HS101) were purchased from Abcam (Cambridge, UK). Annexin V-FITC/PI apoptosis assay kit was obtained from Becton Dickinson (BD Biosciences, San Jose, CA, USA). The Hoechst 33258 nuclear staining kit was purchased from TransGen Biotech, Inc. (Beijing, China). FBS was obtained from HyClone Laboratories (GE Healthcare, Chicago, IL, USA), while the Annexin V-FITC/Propidium Iodide (PI) Apoptosis Assay kit was obtained from Becton Dickinson (BD Biosciences, San Jose, CA, USA). The Hoechst 33258 staining kit was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Antibodies against B-cell lymphoma 2 (Bcl-2; dilution 1:2,000; cat. no. ab182858), Bcl-2-associated X protein (Bax; dilution 1:1,000; cat. no. ab32503), cytochrome c (dilution 1:5,000; cat. no. ab133504), caspase-3 (dilution 1:5,000; cat. no. ab32351), caspase-8 (dilution 1:1,000; cat. no. ab108333), caspase-9 (dilution 1:1,000; cat. no. ab32359), poly(ADP-ribose) polymerase (PARP; dilution 1:1,000; cat. no. ab32138) and GAPDH (dilution 1:2,500; cat. no. ab9485) were purchased from Abcam (Cambridge, UK).

Determination of cell viability by the CCK-8 method. SK-ES-1 and RD-ES cells were cultured in 96-well plates (5x10⁴ cells/well). Cells were treated with different concentrations (0, 1, 2, 5, 10, 20, 40, 50 and 60 µM) of toosendanin for 24, 48 and 72 h and control cells were treated with <0.1% (v/v) DMSO. After the indicated incubation times, 10 µl of CCK-8 solution was added to the plates and incubated for an additional 1-4 h at 37°C. Thereafter, the absorbance was measured at 450 nm using an ELISA plate reader (ELx800; BioTek Instruments, Inc., Winooski, VT, USA).

Hoechst 33258 nuclear staining. Cells (5x10⁴ cells/well) were incubated with 0, 25 or 50 µM toosendanin in 24-well plates for 24 h at 37°C. The cells were then fixed with 4% paraformaldehyde for 30 min. Thereafter, the cells were washed three times with pre-cooled PBS and stained with 10 mg/ml Hoechst 33258 solution for 10 min at 25°C in the dark. Subsequently, the stained nuclei were observed under a fluorescence microscope (Olympus Corp., Tokyo, Japan) at 350 nm excitation and 460 nm emission wavelengths (magnification, x200).

Annexin V-FITC/PI apoptosis assay. SK-ES-1 cells were cultured for 24 h with 0, 25, or 50 µM toosendanin, washed twice with ice-cold PBS, and resuspended at a concentration of 1x10⁶ cells/ml in 1X binding buffer. The cell suspension (100 µl) was incubated with 1 µl Annexin V-FITC and 2 µl propidium iodide (PI) solution for 15 min at 25°C in the dark. After addition of 150 µl 1X binding buffer, the samples were analyzed using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Apoptosis rates were analyzed using FlowJo v7.6 software (Tree Star, Inc., Ashland, OR, USA).

Western blot analysis. SK-ES-1 cells were cultured in a 6-well plate at a density of 2x10⁵ cells/well. After treatment with 0, 25 or 50 µM toosendanin for 24 h, cells were harvested and lysed in RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The lysate was centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was then collected, and the protein concentration was determined by the BCA method. The same protein amounts (10 µg in each lane) were loaded and separated by 10% SDS-PAGE, followed by transfer onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% (w/v) fat-free milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and then incubated with primary antibodies at 4°C overnight. The next day, the PVDF membranes were washed three times in TBS-T and incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. Immunoreactive proteins were detected by an ECL kit (Thermo Fisher Scientific, Inc.) and then developed on an X-ray film (Kodak). The proteins were quantified via densitometry using ImageJ software (version 1.5j81; National Institutes of Health).

Statistical analysis. Data are expressed as mean ± standard deviation (SD) and analyzed by GraphPad Prism 7.0 software (GraphPad Software, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was conducted with the Newman-Keuls method to determine the significance of the differences between the experimental conditions. All experiments were repeated at least three times. Differences in means were considered statistically significant at *P<0.05, **P<0.01 and ***P<0.001 (as indicated in the figure legends).

Results

Toosendanin inhibits cell growth of SK-ES-1 and RD-ES cells. To study the effect of toosendanin on ES cell activity, SK-ES-1 and RD-ES cells were exposed to different concentrations of toosendanin for 24 h. CCK-8 results demonstrated that toosendanin inhibited the viability of SK-ES-1 and RD-ES cells in a dose-dependent manner and that SK-ES-1 cells were more sensitive to toosendanin (Fig. 1A). The half maximal inhibitory concentration (IC₅₀) of SK-ES-1 cells treated with toosendanin was 32.95 µM at 24 h. Furthermore, it was observed that toosendanin inhibited the viability of SK-ES-1 in a time- and dose-dependent manner (Fig. 1B). Subsequently, SK-ES-1 cells were treated with toosendanin at concentrations of 0, 25 and 50 µM for 24 h in the following assays.

Toosendanin results in morphological changes in SK-ES-1 cells. SK-ES-1 cells treated with toosendanin and stained with Hoechst 33258 showed concentrated and broken nuclei in a dose-dependent manner, which are typical morphological features of apoptotic cells (Fig. 2A).

Toosendanin induces apoptosis. Apoptosis was measured by flow cytometry with Annexin V-FITC/PI double labeling. The apoptosis rate of the control group (sum of early and late
apoptosis) was 5.03±0.71%. After 24 h of treatment with 25 or 50 µM toosendanin, the apoptotic rate increased to 19.32±1.26 and 36.28±1.28%, respectively (Fig. 2B). Thus, toosendanin induced apoptosis in a dose-dependent manner (Fig. 2C).

Toosendanin modulates the expression levels of caspase and Bcl-2 family proteins. Western blot analysis was used to analyze expression levels of apoptosis-related protein Bcl-2, pro-apoptotic Bax, cytochrome c, caspase-3, -8 and -9 and
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ParP to identify the mechanism of toosendanin-induced SK-eS-1 apoptosis. The results showed that toosendanin increased Bax protein levels (Fig. 3a) and cytochrome c release (Fig. 3c) as well as decreased Bcl-2 protein levels (Fig. 3a). This indicates that toosendanin activates the mitochondrial apoptotic pathway in SK-eS-1 cells by modulating the expression of Bcl-2 family proteins. at the same time, we evaluated the protein expression levels of caspase-3, -8 and -9. Cleavage of caspase-3 and the key cellular substrate PARP were observed as well as downregulation of procaspase-3 and procaspase-9. Caspase-3 and caspase-9 were also cleaved and downregulated in a dose-dependent manner. Meanwhile, caspase-8 expression levels did not change. These results indicate that toosendanin-induced apoptosis involves the caspase cascade and is triggered by the mitochondrial apoptotic pathway and that toosendanin activates the mitochondrial apoptotic pathway by modulating the expression of Bcl-2 family proteins.

**Discussion**

As malignant tumors are the result of unchecked cell proliferation, inhibiting tumor cell proliferation and promoting tumor cell apoptosis are effective means to prevent tumor growth and eliminate tumors (15). Apoptosis, also known as programmed cell death, is the orderly death of cells controlled by genes that maintain internal environment stability. One of the characteristics of tumor cells is their ability to resist apoptosis (16) and thus, induction of tumor cell apoptosis is the mechanism of action of many antitumor drugs (17). With increasing attention
on toosendanin, its antitumor effect has become a ‘hot topic’ in research. Tada et al (18) found that toosendanin exhibits strong cytotoxicity against human cancer cell lines (KB cells) and that the toxicity mechanism may be related to the c-14/c-15 epoxy structure of toosendanin. Zhang et al (19) reported that toosendanin may induce apoptosis in lymphoma U937 cells by arresting cells in G0/G1 and S phases. Furthermore, He et al (20) demonstrated that toosendanin possesses strong anticancer effects in vivo and in vitro via inducing mitochondrial-dependent apoptosis in hepatocellular carcinoma cells. Meanwhile, Ju et al (21) discovered that the pro-apoptotic effects of toosendanin on human promyelocytic leukemia HL-60 cells were mediated through the c-Jun N-terminal kinase (JNK) signaling pathway. The above studies have shown that toosendanin is a potential antitumor drug with remarkable effects; however, the mechanism of action in regards to Ewing's sarcoma (ES) cells has not been elucidated. To this end, we employed human SK-ES-1 cells to explore the mechanism of action of toosendanin in regards to ES.

The present study found that toosendanin has an inhibitory effect on the proliferation of the SK-ES-1 cell line in a time- and dose-dependent manner. These findings are consistent with the literature reported on other types of tumors, which also have identified toosendanin as an anticancer agent. To the best of our knowledge, this is the first study to explore the effects of toosendanin on ES in vitro.

To further explore the mechanism of apoptosis induction in SK-ES-1 cells by toosendanin, the changes in expression levels of caspase-3, -8 and -9, and other apoptosis-related genes were determined by western blotting. There are two main pathways of apoptosis, the mitochondrial pathway and the death receptor pathway. The mitochondrial pathway involves the ratio of pro-apoptotic protein Bax to anti-apoptotic protein Bcl-2, which affects the release of many apoptotic proteins in the mitochondrial membrane space, such as cytochrome c. Cytochrome c activates caspase-9 and reactivates caspase-3. The Fas/tumor necrosis factor (TNF) receptor located in the cell membrane of the death receptor pathway is activated by apoptosis, which activates caspase-8 that goes on to activate caspase-3. Subsequently, activated caspase-3 causes cleavage or degradation of key cellular substrates including PARP, resulting in cell morphological changes, DNA double-strand breaks, and other characteristics of apoptotic cells (22,23). In the present study, toosendanin-induced apoptosis was accompanied by a change in the ratio of Bax/Bcl-2, which activated caspase-9 and caspase-3. In addition, PARP cleavage was also observed. These findings indicate that SK-ES-1 cell apoptosis induced by toosendanin is triggered by the mitochondrial pathway.

In conclusion, toosendanin was found to inhibit ES cell viability and apoptosis through the mitochondrial apoptosis pathway. Our findings support the utilization of toosendanin as a potential therapeutic agent for the treatment of ES. However, the present study has certain limitations. First, we only employed in vitro experiments and thus further research is needed to elucidate the in vivo effects of toosendanin on SK-ES-1 xenograft tumors in nude mice. Second, we cannot rule out whether toosendanin participates in other signaling pathways in the induction of ES cell apoptosis. Furthermore, safety must be guaranteed before clinical application of ‘natural medicine’. Previous studies have reported that high doses of toosendanin (80 mg/kg) result in serious liver injury in mice (24), while the effect of toosendanin on normal or healthy cells in vitro remains unclear. Our in vitro experiments demonstrated that the IC50 value of toosendanin in SK-ES-1 cells was 32.95 µM, and the cytotoxicity of this concentration must be evaluated prior to use. Thus, future research must comprehensively evaluate the effects of toosendanin on normal cells through in vivo and in vitro experiments to understand the therapeutic range of toosendanin.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TG, AX, BZ and MD conceived and designed the study; TG, AX, BZ and MD wrote the paper; XL and JZ prepared the figures; TG, AX, XL, HZ, BZ and MD reviewed and edited the manuscript. All authors read and approved the manuscript. The authors declare that they have no competing interests.

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