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Research Article
Trigoxyphin L Induces Apoptosis of Human Retinoblastoma Y79 Cells via PI3K/AKT/NF-κB Pathway

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Abstract

Background and Objective: Retinoblastoma is the most common malignant intraocular tumor in children. Due to the poor prognosis and adverse events in thermotherapy, it is imperative to discover new drugs for the treatment of retinoblastoma. Trigoxyphin L is a terpenoid found in *Trigonostemon xyphophylloides*. To find new therapy for retinoblastoma, current study explored its inhibitory effects using Y79 retinoblastoma cells. **Materials and Methods:** The Y79 retinoblastoma cells were divided into control group, trigoxyphin L group and LY294002 group. The cell viability and proliferation were evaluated by MTT and BrdU incorporation assays. The cells apoptosis was detected via DAPI staining and flow cytometry. In addition to the proteins related to apoptosis, key proteins in PI3K/AKT/NF-κB pathway were also analyzed by western blot. **Results:** Trigoxyphin L can reduce the viability and inhibit the proliferation of Y79 cells via induction of mitochondria-mediated apoptosis. Inhibition of PI3K/AKT/NF-κB pathway was involved in the induction of apoptosis by trigoxyphin L. **Conclusion:** Trigoxyphin L can induce apoptosis of Y79 cells via inactivating of PI3K/AKT/NF-κB pathway.

Key words: Trigoxyphin L, Y79 retinoblastoma cells, apoptosis, PI3K/AKT/ pathway, NF-κB pathway

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Retinoblastoma is one of the most malignant tumor in the retina for young and infant patients[1,2]. The common therapeutic strategies for retinoblastoma include surgery, chemotherapy and radiotherapy, of which chemotherapy has emerged as the major therapeutic approach[3,4]. However, when most patients were diagnosed as retinoblastoma, they were already at late stages of tumor progression. Thus, most patients with retinoblastoma suffered from poor clinical outcomes and survival rates of less than 50%[5]. Furthermore, due to high rates of adverse events and little improvement on overall survival, various chemotherapy approaches including intravitreal chemotherapy, intraarterial chemotherapy, intravenous chemotherapy and periocular chemotherapy showed despondent results[6]. Therefore, discovery of novel therapeutic approaches is still imperative for the treatment of retinoblastoma.

Phosphatidylinositol 3-kinase (PI3K)/Akt pathway is an important cascade in regulating cell proliferation, adhesion, survival and motility[7]. In many cancers, aberrant activation of PI3K through phosphorylation and deregulation of the function are found, which result in the phosphorylation and activation of the downstream substrate AKT[8]. As a serine/threonine kinase, activated AKT phosphorylates downstream multifunctional substrates to regulate the cellular functions[9]. In retinoblastoma, activation of PI3K/AKT pathway is observed by experiments and inhibiting this cascade can induce apoptosis of cancer cells[10]. Hence, targeting PI3K/AKT signaling pathway can provide a promising approach for the therapy of retinoblastoma.

In the discovery of new drugs targeting tumors, phytochemicals play a pivotal role[11]. *Trigonostemon xyphophylloides* is a medicinal plant used in Chinese folklore for reducing phlegm, antidiarrheal and antimicrobial effects. Previous phytochemical investigations on this plant have shown there are many diterpenoids and some of these compounds afforded cytotoxic activity in certain cancer cell lines[12-15]. Our interests in the discovery of novel phytochemicals targeting human retinoblastoma, current study evaluated trigoxyphin L (Fig. 1), the diterpenoid identified from *Trigonostemon xyphophylloides* using human retinoblastoma Y79 cells and explored the possible mechanisms.

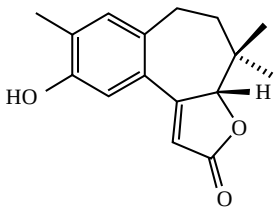

Fig. 1: Chemical structure of trigoxyphin L

MATERIALS AND METHODS

Study area: The study was carried out at Tianjin Eye Hospital, Tianjin Eye Institute, Tianjin Key Laboratory of Ophthalmology and Visual Science, China from January, 2019-March, 2021.

Chemicals and reagents: Trigoxyphin L was provided by Herbpurity Co. Ltd., (Chengdu, China), which was isolated from *Trigonostemon xyphophylloides* with the purity of more than 98% analyzed by HPLC. Cell counting kit-8 (CCK-8) assay kit was supplied by Dojindo Laboratories (Kumamoto, Japan). Bromodeoxyuridine (BrdU) cell proliferation assay kit was obtained from Shanghai Boyun Biotech (Shanghai, China). Annexin V-FITC apoptosis detection assay kit, 4,6-diamidino-2-phenylindole (DAPI) staining solution, caspase-3 assay kit, bicinchoninic acid (BCA) protein assay kit and enzyme-link chemiluminescence (ECL) assay kit and LY294002 were Beyotime Biotechnology Institute (Shanghai, China). RPMI-1640 cell culture medium and lipofectamine 2000 were purchased from Thermo Fisher Scientific (Waltham, MA). Firefly luciferase reporter plasmid (pNFκB-luc) and renilla luciferase reporter plasmid (pRL-SV40) were provided by Promega (Madison, WI). Primary antibodies including phosphorylatd PI3K (p-PI3K), phosphorylatd AKT (p-AKT), phosphorylatd inhibitor of NF-κB α (p-IκBα) phosphorylated nuclear factor-κB (p-NF-κB) and β-actin together with horseradish peroxidase-conjugated secondary antibody were obtained from Abcam (Cambridge, UK).

Cell culture and treatment: Human retinoblastoma Y79 cells were purchased from American Type Culture Collection (Manassas, VA). The cells were maintained with RPMI-1640 supplemented with 10% FBS, at 37°C with 5% CO_2. Cells were passaged every 3-5 days when they reached 80% confluency. Then, the cells were divided into control group and experimental groups. The cells in experimental groups were treated with certain trigoxyphin L or LY294002 dissolving in 0.1% DMSO for 24 hrs and the control group was treated with the vehicle (0.1% DMSO).

CCK-8 assay: The cell viability was evaluated using CCK-8 assay. Briefly, the cells were cultured in 96-well microplates and treated as above, then CCK-8 solution (10 μL) was supplemented. After incubation for 2 hrs at 37°C, optical density was determined on a microplate reader (Bio-Rad, Hercules, CA) at 450 nm. The cell viability was expressed as relative percentage of absorbance of experimental groups versus control group.

BrdU incorporation assay: To reveal the proliferation of Y79 cells, BrdU incorporation assay was employed according to the supplier's instruction. The treated cells were incubated with 10 μL BrdU labeling solution (10 μM) at 37°C for 3 hrs. Then the solution was removed and incubation was carried out at room temperature for 30 min following the addition of 200 μL FixDenat. After removing the solution, 100 μL anti-BrdU-POD working solution was added and following incubation was implemented at room temperature for 90 min. After washing with PBS for three times, the cells were incubated with 100 μL substrate solution at room temperature for 30 min. Then the optical density was recorded on the microplate reader at 370 nm.

DAPI staining: The cells were seeded in 96-well plates at a density of 1×10^4 cells per well. After exposure to indicated compound for 24 hrs, the cells were washed with PBS twice. Then, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with DAPI staining solution (1 μg mL^{-1}) for 10 min. The morphology of cell nuclei was visualized under a fluorescence microscope (Olympus, Tokyo, Japan).

Flow cytometry analysis: To demonstrate the apoptosis of Y79 cells, flow cytometry analysis was performed using Annexin V-FITC apoptosis detection assay kit. The treated cells were collected and resuspended in 500 μL binding buffer. About 5 μL of Annexin V-FITC and Propidium Iodide (PI) in the kit were added and incubated at 25°C for 5 min in the dark. After that quantification by flow cytometry was done using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) at 488/525 nm (Ex/Em) for Annexin V-FITC and 488/620 nm (Ex/Em) for PI with Cell Quest software (BD Biosciences). Early apoptotic cells were Annexin V-FITC positive and PI negative, whereas late apoptotic cells were both Annexin V and PI positive.

Caspase-3 activity assay: The activity of caspase-3 in Y79 cells was detected using the colorimetric assay kit according to the supplier's protocol. The treated cells were washed with PBS and lysed on ice-cold lysis buffer. Then the lysates were centrifuged and the supernatant was collected as samples. After incubation with specific substrate (Ac-DEVD-pNA) at 37°C for 2 hrs, the optical density was recorded on a microplate reader at 405 nm. The activity of caspase-3 was expressed as the relative percentage of optical density value for pNA produced by caspase-3 against that of control group.

Western blotting analysis: To analyze the expression of NF-κB, PI3K, AKT and proteins related to apoptosis, western blot was employed. The treated cells were analyzed on ice-cold lysis buffer and centrifuged at 10000×g for 15 min at 4°C. Then, the proteins were quantified using a BCA assay kit. After that equal amounts of protein samples (50 μg) were loaded per lane and resolved by 15% SDS-PAGE. Thereafter, the bands were transferred onto polyvinylidene fluoride (PVDF) membranes and with 5% nonfat milk for 1 hr at room temperature. And then incubation with respective primary antibodies including caspase-3 (1:1000), Bcl-2 (1:1000), Bax (1:1000), p-IκBα (1:1000), p-NF-κB (1:1000), p-PI3K (1:1000), p-AKT (1:1000) and β-actin (1:1000) 4°C overnight. After rinsed with buffer solution containing 0.1% Tween-20 for three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) at room temperature for 2 hrs. The blots were visualized using ECL substrate and densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD).

Dual luciferase reporter analysis: To explore the transcription ability of NF-κB in Y79 cells, dual luciferase reporter assay was carried out according to the supplier's protocol. The Y79 cells were co-transfected with firefly luciferase reporter plasmid (pNFκB-luc) and renilla luciferase reporter plasmid (pRL-SV40) using lipofectamine 2000. Then the cells were exposed to certain compound. After washed with ice-cold saline buffer, the cells were analyzed with lysis solution. The luciferase activity was recorded on GloMax-96 microplate luminometer (Promega, Madison, WI) using a dual luciferase reporter assay kit. And relative luciferase activity was normalized by the firefly luciferase activity against renilla luciferase.

Statistical analysis: All the experimental data were expressed as means±standard deviation and analyzed by GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA). One way ANOVA followed by Tukey test was used for multiple comparisons as well as Student's t-test for single comparisons. And $p<0.05$ was regarded as statistical significance.

RESULTS

Trigoxyphin L inhibits the survival of Y79 cells: As shown in Fig. 2a, from 1 μM trigoxyphin L can reduce the viability of Y79 cells in a dose-dependent manner. The IC_{50} value for trigoxyphin L can be derived as 5.80 μM. Further BrdU incorporation assay has revealed the proliferation of Y79 cells was inhibited in the presence of trigoxyphin L ($p<0.001$) (Fig. 2b).

Trigoxyphin L induces apoptosis of Y79 cells via mitochondria-mediated pathway: To further explore the inhibitory effects of trigoxyphin L, we have carried out DAPI staining. As a result, it was observed that the nuclei of Y79 cells showed light, condensed and asymmetric blue fluorescence after treatment with trigoxyphin L, which indicated apoptosis occurred (Fig. 3a). Then flow cytometry analysis also demonstrated trigoxyphin L induced apoptosis of Y79 cell, which is more potent than LY294002 (Fig. 3b). As the effector enzyme, the activity of caspase-3 was detected using colorimetric method. The results showed trigoxyphin L activated caspase-3 (4.05±0.31) compared with control group (1.00±0.16) (Fig. 3c). Western blotting analysis for the proteins related to mitochondria-mediated apoptosis has indicated trigoxyphin L down-regulated the expression of Bcl-2 while up-regulated Bax (Fig. 3d). Densitometric analysis also disclosed trigoxyphin L resulted in the down-regulation of Bcl-2 and up-regulation of Bax (Fig. 3e-f).

Inactivation of PI3K/AKT pathway is involved in the apoptosis induced by trigoxyphin L: To elucidate the mechanism for the induction of apoptosis by trigoxyphin L, we have detected the PI3K/AKT pathway in Y79 cells by western blot analysis. The results displayed compared to control group, the active forms of PI3K (p-PI3K) and AKT (p-AKT) were inactivated in Y79 cells after exposed to trigoxyphin L (Fig. 4a). Accordingly, densitometric analysis gave the same results quantitatively (Fig. 4b-c).

NF-κB signaling pathway is blocked in the apoptosis of Y79 cells: The NF-κB pathway was investigated to reveal the mechanism of apoptosis induced by trigoxyphin L. Western blot analysis has shown both the active regulator of NF-κB (p-IκBα) and the active form of NF-κB (p-NF-κB) were down-regulated by trigoxyphin L in comparison to control group (Fig. 5a). At the same time, densitometric analysis further validated trigoxyphin L inhibited activation of NF-κB (Fig. 5b-c). In addition, to demonstrate the transcription capacity of NF-κB, dual luciferase reporter assay was employed. The results indicated trigoxyphin L reduced the transcription of NF-κB significantly ($p<0.001$) (Fig. 5d). These results above implied trigoxyphin L inhibited NF-κB in Y79 cells.

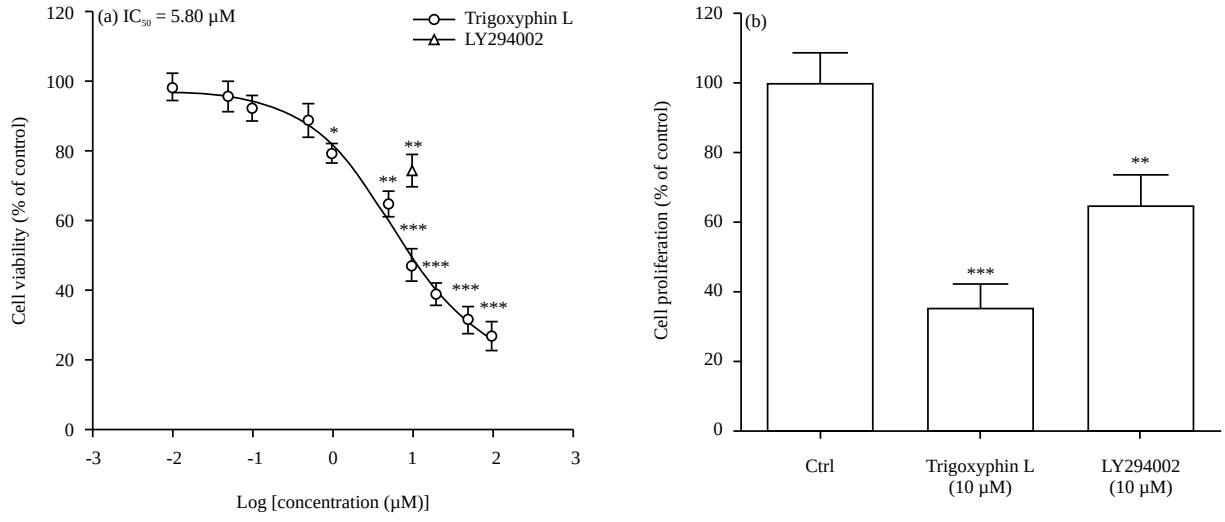

Fig. 2(a-b): Effects of trigoxyphin L on the survival of Y79 cells

(a) Cell viability and (b) BrdU incorporation assay, n = 3, *p<0.05, **p<0.01 and ***p<0.001 versus control group

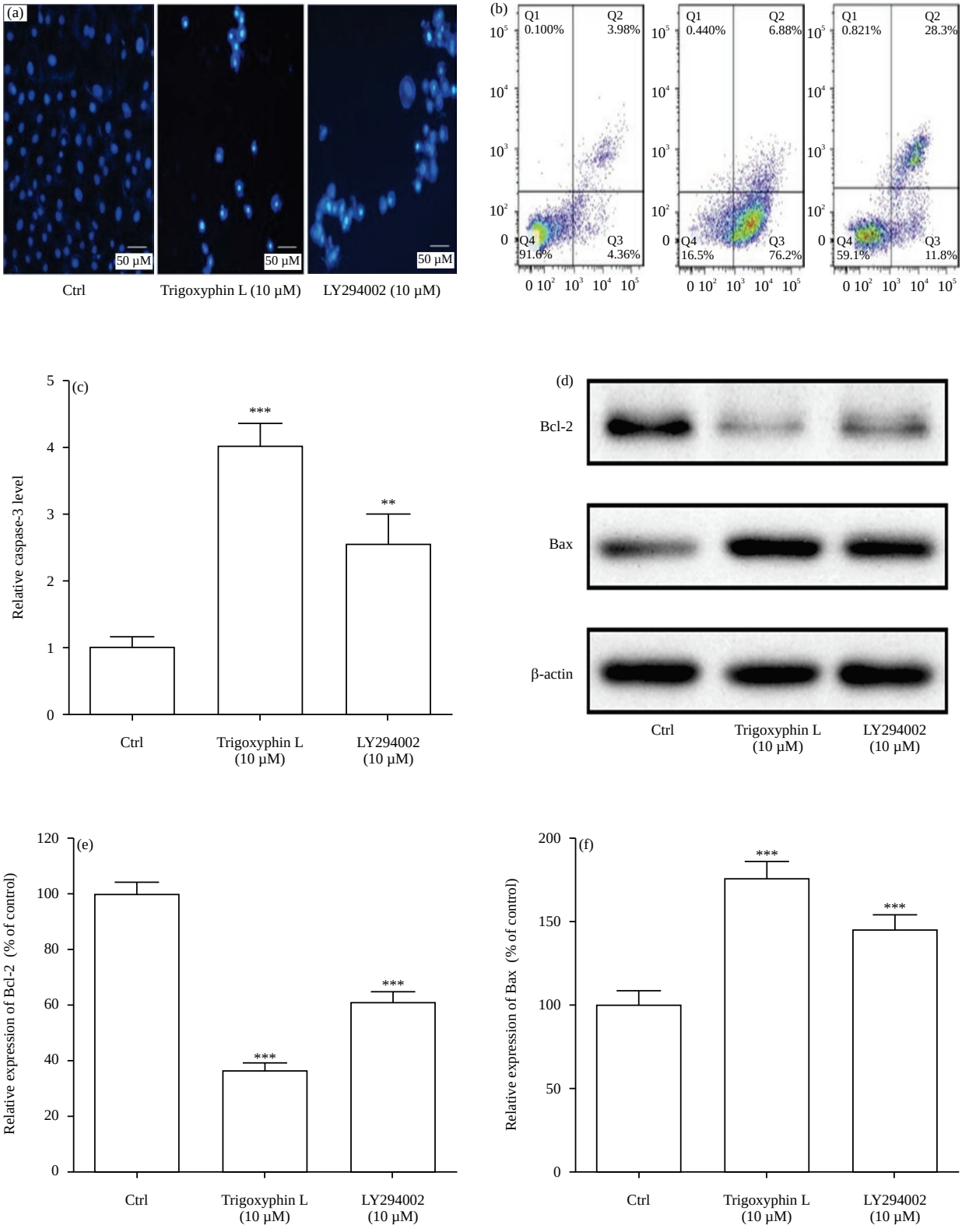

Fig. 3(a-f): Trigoxyphin L induces apoptosis of Y79 cells via mitochondria-mediated pathway

(a) DAPI staining, (b) Flow cytometry analysis, (c) Caspase-3 activity, (d) Western blot analysis for Bcl-2 and Bax and (e-f) Densitometric analysis for Bcl-2 and Bax, n = 3, **p<0.01 and ***p<0.001 versus control group

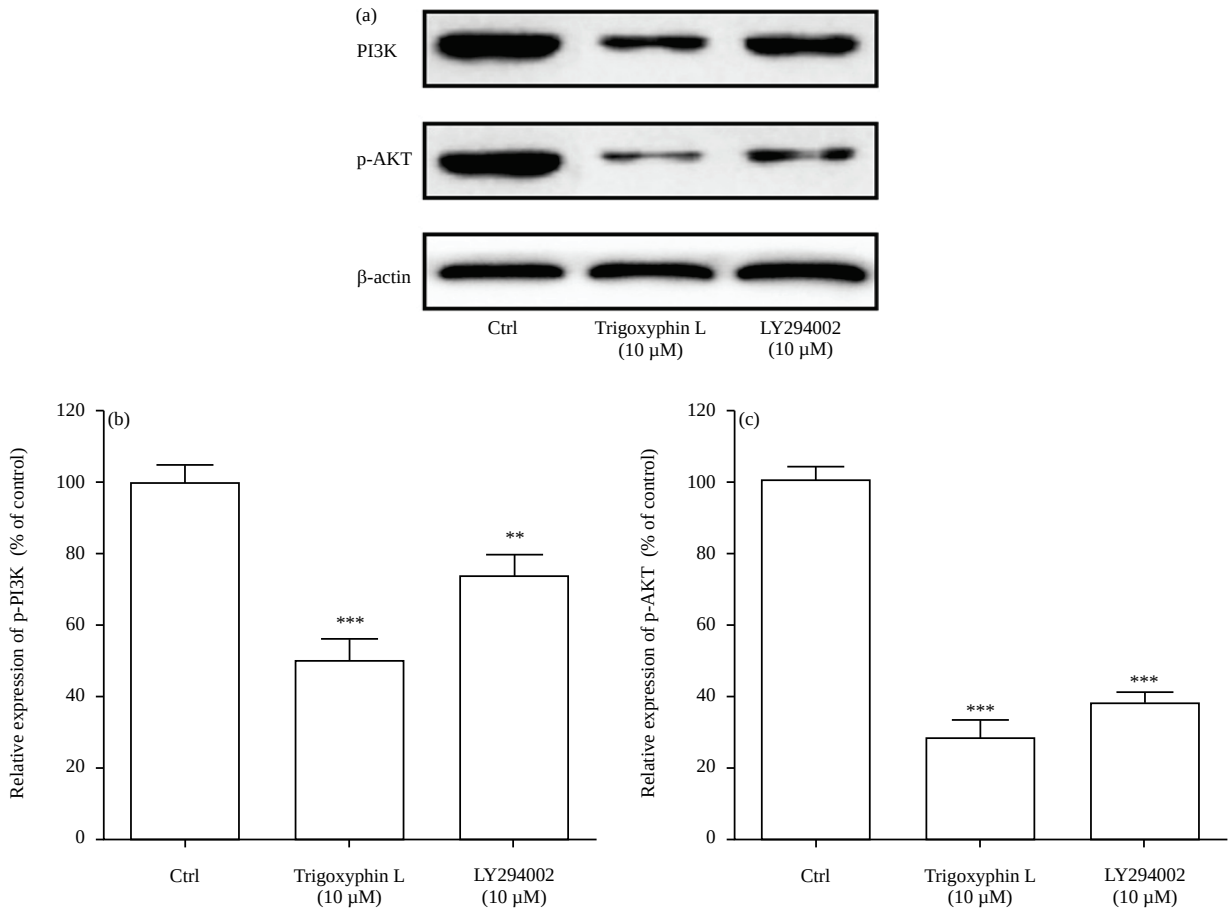

Fig. 4(a-c): Effects of trigoxyphin L on the PI3K/AKT pathway

(a) Western blot analysis for p-PI3K and p-AKT and (b-c) Densitometric analysis for p-PI3K and p-AKT, n = 3, **p<0.01 and ***p<0.001 versus control group

DISCUSSION

In current investigation, the inhibitory effects of trigoxyphin L using human retinoblastoma Y79 cells was assessed. And it was found trigoxyphin L reduced the viability and inhibited proliferation of Y79 cells via induction of apoptosis.

As the key effector enzyme in apoptosis, caspase-3 affords the morphological changes of apoptotic cells including mitochondrial damage, nuclear membrane breakdown, DNA fragmentation and chromatin condensation[16]. Bcl-2 and Bax are members of Bcl-2 proteins family and act as the upstream regulators of caspase-3[17]. It is approved the former is anti-apoptotic whereas the latter is pro-apoptotic[18]. Bcl-2 could heterodimerize with Bax to prevent the initiation of apoptosis by Bax on the mitochondrial membrane[19]. Herein, we have found trigoxyphin L activated caspase-3 together with down-regulated Bcl-2 and up-regulated Bax. These findings unraveled the apoptosis of Y79 cells induced by trigoxyphin L involved mitochondria-mediated pathway.

PI3K/AKT pathway is a crucial cascade for cancer cell proliferation and survival[20]. PI3K is activated through phosphorylation to become the active form p-PI3K, then the activated PI3K results in the activation of AKT as phosphorylated AKT (p-AKT)[21]. As a kinase, AKT can phosphorylate many substrates to regulate cellular survival, proliferation, growth, apoptosis, metabolism, motility, transformation and so on[9,21]. Of these substrates, IκB kinase (IKK) is the upstream regulator of NF-κB and activated by AKT through phosphorylation[8,9]. The activated IKK promotes phosphorylation of I κBα, which is bound to NF-κB. Then the phosphorylated IκBα is degraded rapidly through ubiquitin-proteasome pathway, which leads to the release of NF-κB[22]. Subsequently NF-κB is phosphorylated and

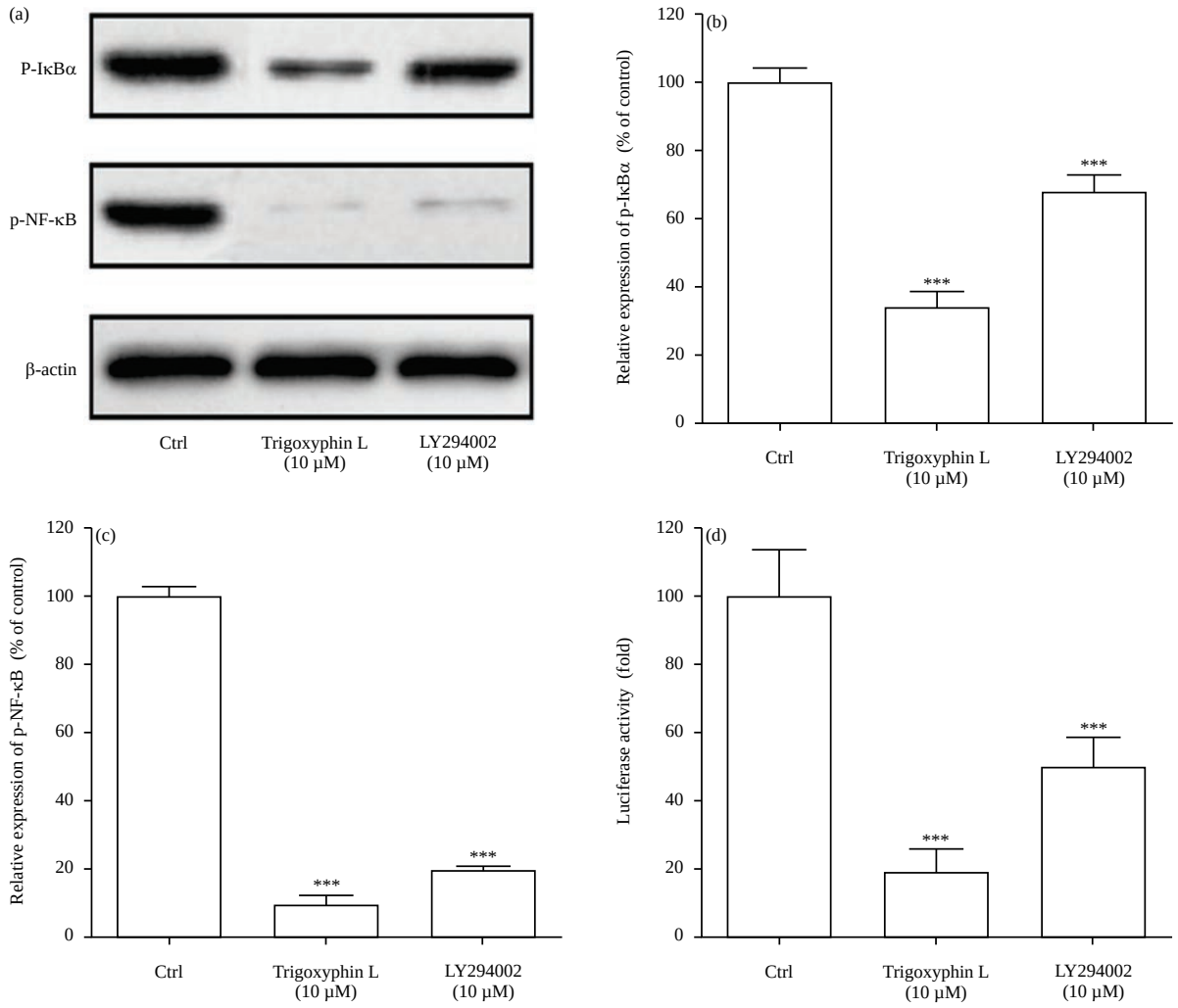

Fig. 5(a-d): Effects of trigoxyphin L on the NF-κB activation

(a) Western blot analysis for p-IκBα and p-NF-κB, (b-c) Densitometric analysis for p-IκBα and p-NF-κB and (d) Dual luciferase reporter assay, n = 3, **p<0.01 and ***p<0.001 versus control group

translocate into nucleus to regulate the transcription of target genes[23]. Therefore, NF-κB could suppress the apoptosis via mitochondria-mediated pathway[24]. At the same time, it is found this activity involves regulation of members in Bcl-2 family[25,26]. In human retinoblastoma, NF-κB is constitutively activated and can prevent the apoptosis induced by anticancer drugs[27]. Hence, PI3K/AKT/NF-κB pathway was involved in the apoptosis of Y79 cells induced by trigoxyphin L was found from this investigation.

CONCLUSION

In conclusion, current study explored the inhibitory effects of trigoxyphin L on human retinoblastoma Y79 cells. It was found trigoxyphin L induced apoptosis of Y79 cells through mitochondria-mediated pathway. PI3K/AKT/NF-κB pathway was involved in the induction of apoptosis. These findings can provide evidences for the therapy of human retinoblastoma.

SIGNIFICANCE STATEMENT

Retinoblastoma is one of the most malignant tumor in the retina for young and infant patients. However, there is no effective therapy since most of the patients with retinoblastoma are found in late stage which results in the poor prognosis. Therefore, it is necessary to find novel therapeutic approach for the treatment of retinoblastoma. This manuscript presents trigoxyphin L induces apoptosis of Y79 retinoblastoma cells and potential mechanisms, which may provide evidences for the discovery of drug targeting retinoblastoma.

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