The Effect and Mechanism of Apoptosis on Human Lung Cancer A549 Cells Induced by Trillin Through AKT/PI3K Pathway

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Abstract. Cytotoxic activity of Trillin (Tr) was determined by cck-8 assay at various concentrations ranging from 10 to 40 µg/ml on A549 cells. The Apoptosis and its mechanism induced by Trillin was investigated. The results showed Trillin displayed the marked inhibition effect to A549 cells and the IC$_{50}$ value is 28.6µg/ml for 24 hour. The protein expressions of PI3K and Akt increased in A549 cells treated with Trillin. These data suggest that apoptotic effect of Trillin is mediated through AKT/PI3K pathway in A549 cells.

Introduction

Lung cancer remains deadly, prevalent and costly to patients and society [1]. Consequently, it has become a significant public health problem. However, chemotherapeutic treatment for lung cancer is still unsatisfactory. There is clearly an ongoing need for more effective treatments.

Alternative and complementary medicine has gained global attention because of their widespread use in the field of medicine for treating liver fibrosis diseases [2]. Saponins, a class of glycoside having structural diversity, have attracted more and more attention in the last decade due to their promising biological activities [3,4]. In clinical practice, Dioscoreae Nipponese Rhizoma is used for relieving cough and asthma, eliminating rheumatic aches, alleviating pain, and improving blood circulation[5]. The rhizome of Dioscorea nipponica is the major part of medicine and the identified active components are steroidal saponins, including furostanol saponins and isospirostanol saponins. Saponin of Dioscorea nipponica possesses various pharmacological activities. Trillin is a steroidal saponin identified from Dioscorea nipponica, has been demonstrated to induce multi-nucleation in different cancer cell lines [6]. However, reports regarding other biological effects of trillin are rather limited. But the anticancer effects of Trillin on A549 cells and its mechanism have not been studied.

In the present study, we have detected the antiproliferative activity of Trillin toward cervical carcinoma A549 cells. Furthermore, we examined the mechanism of apoptosis induced by Trillin. This study will help researchers evaluate the potential clinical use of Trillin in lung cancer treatment.

Materials and Methods

Chemicals and Reagents

Trillin was purchased from the National Institutes for Food and Drug ConArol (Beijing, China). V-FITC and PI was purchased from Beyotime Institute of Biotechnology (Nanjing, China). All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).
Cell Inhibition Assay

150 µl of cell suspensions were seeded into 96-well flat-bottom microplates (1×10⁴ cells/well) and cultured in a humidified incubator to allow adhesion overnight. Cells were then exposed to Trillin at various concentrations ranging from 5 to 40 µg/ml. After 24 h of incubation, 20µl cck-8 dye solution was added to each well and incubated at 37°C for 4 h. The medium was removed and formazan dissolved in 150 µl DMSO was added. The absorbance at 570 nm was measured by ELx-800 Universal Micro plate Reader and the cell inhibition (%) was calculated by the following formula:

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\text{Cell inhibition (\%) = } \frac{(\text{OD}_{\text{control}} - \text{OD}_{\text{treated}})}{(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})}
\]

Annexin V-FITC/PI Staining Experiments

The annexin V-FITC kit (BD Biosciences, San Jose, CA, USA) was used to quantify the percentage of cells undergoing apoptosis. Briefly, A549 cells were treated with different concentrations of Trillin for 24 h. Then cells were collected, washed twice with 400 µl of binding buffer, and incubated in 100 µl reagent mix containing 1 µl annexin V-FITC conjugate and 10 µl PI, in the dark for 15 min at room temperature. The samples were subjected to FACSCaliber flow cytometry to quantify the percentage of cells at different stages.

Fluorescent Morphologic Assay

Fluorescent morphological assays were used to detect apoptosis induced by osthole. Cells from exponentially growing cultures were seeded into 12-well culture plates and treated with 0.1 mmol/ml osthole for 24 h. The medium was then discarded and cells were washed twice with PBS, fixed in the mixture of methanol and acetic acid (3:1, v/v) for 10 min at 4°C, stained with Hoechst 33258 and PI for 15 min at 4°C, and examined using an Olympus Fluorescent Microscope (Olympus, Tokyo, Japan).

Protein Extraction and Western Blot Detection

A549 cells (1×10⁶) were seeded in 10-cm dishes and incubated with various concentrations of Trillin for 24 h. The total cell protein extracts were obtained according to the method of Levites [7]. The Western blotting assay was performed as described previously [8]. Hela cells (1×10⁶) were seeded in 10-cm dishes, incubated with various concentrations of ART for 24 h. Protein extract from the Hela cells was performed using 0.5 ml RIPA lysis buffer, followed by centrifugation at 13,200 rpm at 4°C for 20 min. Equivalent amounts of 100 µg protein from total cell lysates were resolved by SDS-PAGE using precast 12.5% Bis-Tris gradient gels and transferred onto polyvinylidene difluoride (PVDF) membranes. Afterwards, the membrane was blocked with 5% (w/v) non-fat dry milk and incubated with primary antibodies against Bcl-2,Bid,caspas-9 and β-actin overnight at 4°C. The mixture was incubated with horseradish peroxidase-conjugated specific secondary antibodies for 1 h at room temperature. After extensive washing with TBS-T, the bands were visualized by enhanced chemiluminescence followed by exposure to autoradiography film.

Statistical Analysis

Data were expressed as mean ± standard deviation. A significant difference from the respective controls for each experimental test condition was assessed using student’s unpaired t-test and p<0.05 was considered statistically significant.

Results

Cytotoxicity of Trillin on A549

Toxicity activities of Trillin on A549 cells measured by cck-8 assay. Under the experimental conditions, Trillin exhibited a significant inhibition on the survival of HepG2 cells and had
dose-dependent effects. The half maximal inhibitory concentration (IC50) value after 24 h was calculated as 28.6 µgl/ml in A549 cell (Fig.1).

![Inhibition rate vs Concentration graph](image)

**Figure 1.** Toxicity activities of Trillin on A549 cells measured by cck-8 assay.

The cytotoxicity effect of Trillin at different concentrations during 24 h of culture is illustrated in Fig.2. Cell numbers were significantly reduced with increasing concentration of Trillin. Trillinalso induced integrity damage of cytoplasmic membranes, indicative of necrosis of the A549 cells.

![Cell morphology images](image)

**Figure 2.** Morphology of human lungcancer A549 cells treated with at different concentrations for 24 h.A549 cells were treated with Trillin at concentrations of 0,10,20 and 40 µgl/ml respectively and observed by a phase contrast microscope.

**Effects of Trillin on Apoptosis of HepG2 Cells**

The annexin V-FITC/PI staining experiment was performed to detect the apoptosis induced by Trillin. The percent of cells in the early phase of apoptosis were 0.6, 4.1, 16.7 and 24.5 %, at concentrations of 0, 10, 20 and 40 µgl/ml, respectively. The percent’s of cells in the late phase of apoptosis were 0.6, 3.0, 11.3 and 13.6% at concentrations of 0, 10, 20 and 40 µgl/ml, respectively (Fig. 3).These data show a shift in the cell population from normal to apoptotic/necrotic stages induced by Trillin treatment.
Results of Fluorescent Morphologic Assay

We further examined the morphological changes in response to Trillin treatment using fluorescence staining. Both control- and Trillin-treated cells were stained with the fluorescent dyes Hoechst 33528, and were visualized by a fluorescent microscope. The nuclei of control cells were homogeneous (Figure 4a) while the cells treated with Trillin exhibited the characteristics of apoptosis, with cell shrinkage, and condensation and fragmentation of nuclei.

Effect of Trillin on PI3K/AKT Protein Expression

PI3K/AKT signaling are the well-known cytoprotective target mediating oxidative stress and inflammation. As showed in Figure 5, Expressions of p-PI3K and pAKT in A549 cells exposure to Trillin notably decreased. While after treating with Trillin, the protein expressions of PI3K and AKT significantly enhanced compared with control. In addition, Trillin challenge contributed to the up-regulations of IkBa, while the treatment with Trillin effectively down regulated the levels of p-IkBa.
Conclusions

Trillin displays a marked cytotoxicity towards human lung cancer A549 cells. Trillin induced integrity damage of cytoplasm membranes and necrosis of the carcinoma A549 cells. The shift of cell population from normal to apoptotic/necrotic stages showed that Trillin could induce apoptosis on A549 cells. The expression of PI3K and AKT and their active cleaved forms suggest that the mitochondrial (intrinsic) pathway involved in the Trillin induced apoptosis pathway. Our results suggest that Trillin could be a potential chemotherapeutic agent against human lung cancer.

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