The Rhizome of *Trillium tschonoskii* Maxim. Extract Induces Apoptosis in Human Lung Cancer Cells

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Trillium tschonoskii Maxim. has been used to treat several diseases including cancers in folk medicine. However, the mechanisms responsible for *T. tschonoskii* extract-induced apoptosis are not clear. This study was mainly undertaken to identify the major biochemical changes in a lung cancer cell line upon treatment with an *T. tschonoskii* extract (TTME), and to investigate the functional relationship between these changes. The *n*-butanol extract was used to evaluate the mechanism of induction of apoptosis in A549 human lung cancer cells and its effects on mitochondrial function and production of reactive oxygen species (ROS). The *n*-butanol extract of *T. tschonoskii* has cytotoxic, antiproliferative, and morphological effects on the lung cancer cell line. *T. tschonoskii* mainly leads to apoptosis of cancer cells with a concomitant increase in the release of cytochrome c and a loss of mitochondrial membrane potential in a dose-dependent manner. A rapid increase in the level of intracellular ROS and an accumulation of cells in the G2/M and S phase of the cell cycle were also observed in treated cells. These observations suggest that the *n*-butanol extract of *T. tschonoskii* has promising anticancer activities, which could be useful in cancer treatment.

Key words: Trillium tschonoskii Maxim., Apoptosis, Cancer

Introduction

Lung cancer is one of the most common human cancers and is the leading cause of cancer death worldwide (Yamanaka, 2009). Non-small-cell lung carcinomas (NSLC) account for approximately 80% of the total cases of lung cancer (Shottenfeld, 2003). Since 65% of the patients with NSLC are diagnosed in an advanced stage of the disease, chemotherapy is the main treatment for most patients. However, non-small-cell lung cancer responds poorly to current regimens of chemotherapy (Murer, 2008). Treatment with gefitinib or erlotinib is associated with an objective response in only 10–20% of NSLC patients (Janne, 2008). This poor response rate may in part be due to the expression of multidrug resistance proteins (Maruyama *et al.*, 2009; Takigawa *et al.*, 2007). So the identification of new chemotheraputic drugs which will bypass this resistance is essential, if a reduction in mortality of patients within the late stage of NSLC is to be achieved.

Natural products have historically and continually been investigated as promising new leads in pharmaceutical development (McChesney *et al.*, 2007). *Trillium tschonoskii* Maxim., also named "a pearl on head", is a perennial herb of the Trilliaceae found in mid-western China (Li *et al.*, 2005). It has been traditionally used in China for at least one thousand years. For example *T. tschonoskii* rhizomes have been used in folk medicine as an herbal treatment against hypertension, neurasthenia, giddiness, headache, cancer, removing carbuncles, and ameliorating pains (Fu, 1992). Previous studies revealed many bioactive components including steroidal saponins and steroidal glycosides in members of the genus *Trillium*, including *T. erectum* (Hayes *et al.*, 2009; Yokosuka and Mimaki, 2008), *T. kamtschaticum* (Ono *et al.*, 2007a, b), and *T. tschonoskii* Maxim. (Nohara *et al.*, 1975; Wang *et al.*, 2007). Many saponins from plants have apoptosis-inducing effects on cancer cells (Vincken *et al.*, 2007). For example, ginseng saponins have been shown to suppress cancer metastasis in murine colon cancer cells (Choo *et al.*, 2008). So whether the extract of *T. tschonoskii* has also cytotoxic effects on cancer cells is worth to be investigated.

Apoptosis can control the cell number as part of normal development. The deregulation of apoptosis is closely related with the occurrence of cancer (Engelmann and Bauer, 2000). During apoptosis, cells exhibit some biochemical features including cytoplasmic condensation, apoptotic body formation, loss of cell membrane phospholipid asymmetry and cell cycle changes (Evan and Vousden, 2001). Evidence was found that reactive oxygen species (ROS) play a key role as common mediators of apoptosis (Gupta, 2003). The aims of the present study were to identify the major biochemical changes in a lung cancer cell line upon treatment with an extract from *T. tschonoskii* (TTME), and to investigate the functional relationship between these changes.

Material and Methods

Reagents

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], propidium iodide (PI), RNase, 2',7'-dichlorofluorescein-diacetate (DCFH-DA), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloromethyl-X-rosamine (CMX-Ros) was purchased from Invitrogen (Carlsbad, CA, USA). RPMI 1640 culture media and fetal bovine serum were supplied by Gibco BRL (Grand Island, NY, USA). Rabbit polyclonal anti-cytochrome c was obtained from Cell Signaling (Beverly, MA, USA). Other chemicals used in this study were special grade commercial products.

Plant material and preparation of TTME

The rhizomes of *Trillium tschonoskii* Maxim. were purchased in Muyu, a town of Shennongjia Forest District of Hubei Province, China, and identified by Professor Chen Faju, botanist at China Three Gorges University, Yichang, China. A voucher specimen (No. 2005ZW03128) has been deposited in the Medicinal Plants Herbarium of the College of Chemistry and Life Science, China Three Gorges University. TTME was prepared by our laboratory, Hubei Key Laboratory of Natural Products Research and Development, China Three Gorges University. Briefly, air-dried powdered rhizomes (6.4 kg) were extracted with methanol under reflux. After removal of the solvent *in vacuo* and freeze-drying, the methanol extract (2427 g) was obtained. The extract was suspended in water (2.2 l) and then extracted with $CHCl_3$, EtOAc, and *n*-BuOH, successively. The *n*-BuOH part was used for all experiments. TTME powder was dissolved in distilled water. The filtered TTME stock solution was separated into individual aliquots which were kept at –20 °C until further use.

Cell culture

Human lung cancer cells, A549 cells, were a gift from the Institute of Molecular Biology, China Three Gorges University. Cancer cells were maintained in 1640 culture medium supplemented with 10% fetal bovine serum and antibiotics in a 5% CO_2 incubator at 37 °C (Napco 5400, Winchester, VA, USA). To analyse the effect of TTME on A549 cells, cells were treated separately with different concentrations of TTME for different times.

Cytotoxicity assay

Cancer cells ($1 \cdot 10^4$) were seeded into each well of a 96-well flat-bottom plate on day 1. On day 2, solutions with different concentrations of TTME (each concentration in triplicate) were added, and cells were incubated for 24 h, 48 h, or 72 h separately. Mitomycin was used as a positive control. Afterwards, 100 μl of the MTT solution [1 mg/ml, dissolved in phosphate buffer solution (PBS)] were added and the mixture incubated for 4 h at 37 °C. The medium was carefully removed, the cells were washed with PBS, and 200 μl of DMSO were added. After gently shaking at room temperature for 1 h, the optical absorbance at 570 nm was recorded using a microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was repeated at least 3 times.

Morphological study

Morphological changes of A549 cells treated with TTME (15 μg/ml) were studied using an inverted microscope.

Transmission electron microscopy

After the treatment with 15 μg/ml of TTME, the appropriate size of cell pellets was fixed with 2.5% glutaraldehyde for 24 h. The subsequent steps were carefully followed according to standard procedures, including fixing, incubation, rinse, gradient dehydration, embedding, and ultrathin

sectioning. Changes in the ultrastructure of the cancer cells were observed in an H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Annexin V-FITC/PI cytometric analysis

To quantify the TTME-induced apoptotic death of A549 cells, annexin V and PI staining were performed followed by flow cytometry (FCM; Coulter, Los Angeles, CA, USA). A549 cells were allowed to attach for 24 h. Then the medium was replaced with an equal volume of fresh medium containing different concentrations of TTME. After the treatment, all cells were collected by trypsinization and washed twice with PBS. The cell suspension was subjected to 400 μl 1×binding buffer, 5 μl annexin V, and 10 μl PI stain, and then left in the dark for 15 min. Normally, apoptotic, necrotic, and mechanically damaged cells were determined by FCM with a single beam at 488 nm excitation wave length. PBS was used as the negative control.

Cell cycle analysis

Cancer cells ($5 \cdot 10^6$) were treated with TTME at the indicated concentrations for 24 h. Then the attached cells were trypsinized and washed once with PBS. The cells were resuspended in 2 ml of 70% ice-cold ethanol solution and fixed at 4 °C overnight. The cells were centrifuged to remove ethanol and washed again with PBS; the pellets were resuspended in 100 mg/ml PI solution containing 100 mg/ml RNase, and then incubated at 37 °C for at least 30 min. The stained cells were analysed for their DNA content by FCM.

Analysis of intracellular ROS formation

FCM analysis of oxidative metabolism was carried out. Briefly, cells were preincubated (15 min, 37 °C) with DCFH-DA (10 μM), and then washed with PBS. Next, the cells were treated with different concentrations (5 μg/ml, 15 μg/ml, and 25 μg/ml) of TTME for 2 h. At the end of the incubation period, the reaction was stopped by keeping the samples on ice until FCM analysis.

Assessment of mitochondrial membrane potential

CMX-Ros was used as the detection probe for determing the change in the mitochondrial membrane potential. Cancer cells were resuspended and incubated with different concentrations of TTME for 2 h or 24 h. Then cells were incubated for 1 h at 37 °C with 1 μM (final concentration) CMX-Ros dissolved in dimethylsulfoxide (DMSO). At the end of the incubation period, cells were washed with PBS and resuspended in PBS supplemented with 0.2% BSA, and kept on ice until FCM analysis.

Cytochrome c immunoblotting

A549 cells ($6 \cdot 10^6$) treated with different concentrations of TTME were harvested and washed with cold PBS. Cell pellets were lysed in 40 μl lysis buffer (20 mM HEPES/NaOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail) for 20 min on ice. The lysis solution was centrifuged at 25000 × *g* for 10 min at 4 °C, and protein contents in the supernatant were measured using a Bio-Rad DC protein assay kit. The lysates containing 25 μg of protein were analysed by Western blotting for cytochrome c (1:1000 dilution).

Statistical analysis

Data was presented as mean ± S.D. (standard deviation from the mean) and statistical evaluations were made with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) using analysis of one-factor variance. $p < 0.05$ was used to indicate a statistically significant difference.

Results

Effect of TTME on cell survival rate

MTT assays were used to investigate whether TTME exhibits cytotoxic effects on A549 cells. A549 cells were seeded at $1 \cdot 10^4$ cells per well in 96-well plates and incubated separately with different concentrations (5 μg/ml, 10 μg/ml, 15 μg/ml, 20 μg/ml, 25 μg/ml) of TTME for 24 h, 48 h, or 72 h. Untreated cells (control) were considered as the baseline (100%) for the analysis. As shown in Fig. 1A, compared with the control group, the survival rate of A549 cells decreased when the cells were treated with the indicated concentrations of TTME for different times. The results indicate that TTME attenuated the cell survival in a dose-dependent manner. The IC_{50} values of TTME were 24.89, 18.22, and 9.67 μg/ml after 24 h, 48 h, and 72 h of treatment, respectively.

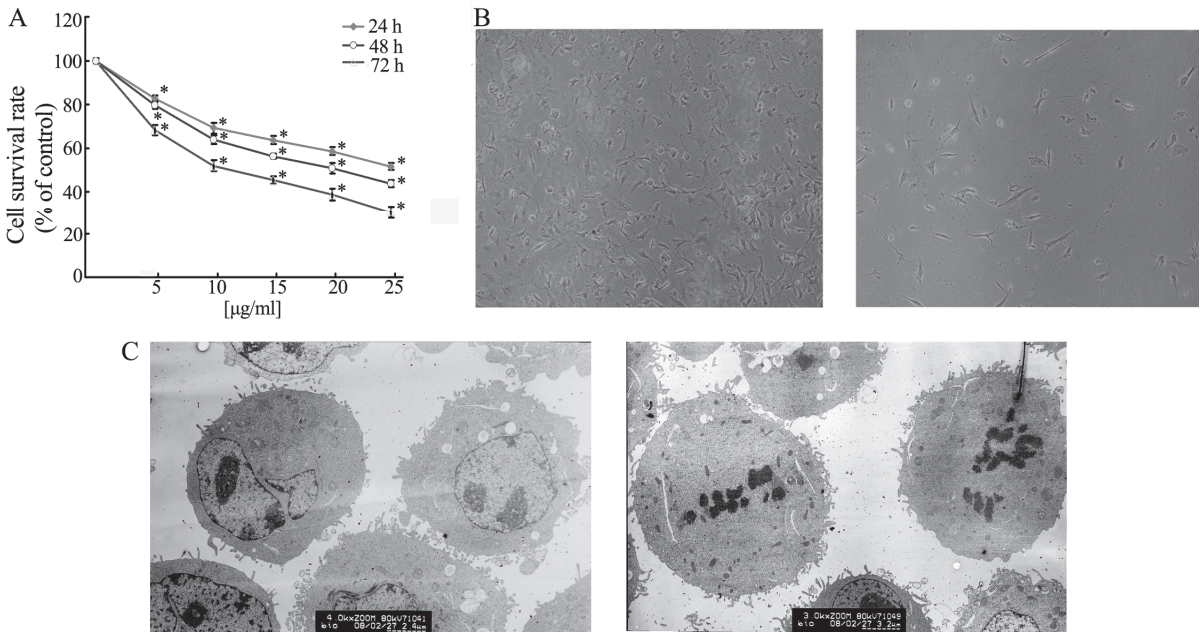

Fig. 1. Effect of TTME on proliferation and morphology of A549 cells. (A) A549 cells were treated with TTME at concentrations of 0, 5, 10, 15, 20, or 25 μg/ml for 24 h, 48 h or 72 h. Antiproliferative effects of TTME on A549 cells were dose-dependent. Results are shown as mean ± S.D. of triplicate experiments; *$p < 0.05$ vs. control. The shown results are from three independent experiments. (B) Morphological changes of A549 cells after treatment with TTME (15 μg/ml, right) for 24 h include cell numbers, shrinkage, detachment, weak refraction, and absence of colony formation when compared with the control (left) under a light microscope (400x). (C) The transmission electron microscopic observation showed that TTME (15 μg/ml) led to nuclear condensation, chromatin margination, and apoptotic body formation (right).

Morphological changes of A549 cells and transmission electron microscopic observation after TTME treatment

Cell shrinkage and poor refraction of A549 cancer cells could be observed under a light microscope after treatment with TTME. Furthermore, cancer cells also began to detach and could not form colonies. This was detected after 24 h of 15-μg/ml TTME incubation (Fig. 1B, right). These results indicate that TTME exerted significant cytotoxic effects on A549 cells. The transmission electron microscopic observation showed that TTME (15 μg/ml) led to nuclear condensation, chromatin margination, and apoptotic body formation (Fig. 1C, right).

Effects of TTME on apoptosis in A549 cells

To further differentiate between apoptosis and necrosis, the cytotoxic effects of TTME on A549 cells were evaluated using the early marker of apoptosis annexin V and the dead cell marker propidium iodide. As shown in Fig. 2A, the treatment with TTME could lead to early apoptotic cells, which indicates that TTME induced apoptosis in a dose-dependent manner. Treatment with different doses of TTME (5 μg/ml, 15 μg/ml, and 25 μg/ml) for 24 h resulted in (6.80 ± 0.37)%, (29.84 ± 2.38)%, and (42.05 ± 2.55)%, respectively, early apoptosis compared with the control [(0.33 ± 0.13)%].

Cell cycle analyses

To test whether an induction of cell cycle arrest contributed to the antiproliferative potency of TTME in A549 cells, we performed flow cytometric cell cycle analyses. Different concentrations of TTME caused a significant increase in the percentage of G2/M phase cells, showing that TTME arrests cell cycle progression in the G2/M phase when compared with the control (Fig. 2B). Compared with the control (2%), TTME (5 μg/ml, 15 μg/ml, and 25 μg/ml) led to a G2/M phase

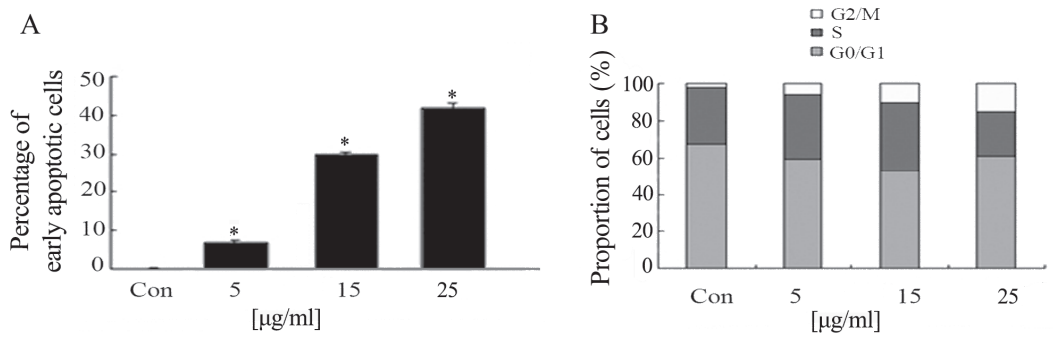

Fig. 2. Apoptosis induced by TTME and cell cycle analyses as measured by flow cytometry. (A) A549 cells were treated with the indicated concentrations of TTME for 24 h, followed by double staining with annexin V/propidium iodide. The results shown here are to prove that TTME can induce apoptosis of tumour cells, which mainly react in the early stage. Data are presented as mean ± S. D. for three independent experiments, each in triplicate; $*p < 0.05$ vs. control. (B) After treatment with the indicated concentrations of TTME for 24 h, A549 cells were stained with propidium iodide. The G2/M and S population increased in A549 cells treated with TTME. Results are from one of three independent experiments.

increase of 5.8%, 10.1%, and 15.3% respectively. TTME could also affect cell cycle distribution in the S phase, though there was no apparent dose-dependent feature. When compared with the control (20%), TTME (5 μg/ml, 15 μg/ml) led to an S phase arrest of 25% and 28%, respectively (Fig. 2B).

Effect of TTME on intracellular ROS levels

Several studies have implicated ROS generation as a possible mechanism for induction of apoptosis by various anticancer agents (Morales *et al.*, 1998). Therefore, intracellular ROS generation in control and TTME-treated cells was evaluated by FCM. The cells were stained with DCFH-DA, which is cleaved and oxidized by peroxides to yield fluorescent 2',7'-dichlorofluorescein (DCF), followed by treatment with different doses of TTME for 2 h. There was a significant dose-dependent increase in ROS generation in TTME-treated cells compared with the untreated control (Fig. 3A).

Effect of TTME on mitochondrial membrane potential (MMP)

High amounts of intracellular ROS normally disrupt the mitochondrial membrane potential. The effect of TTME treatment on the mitochon-

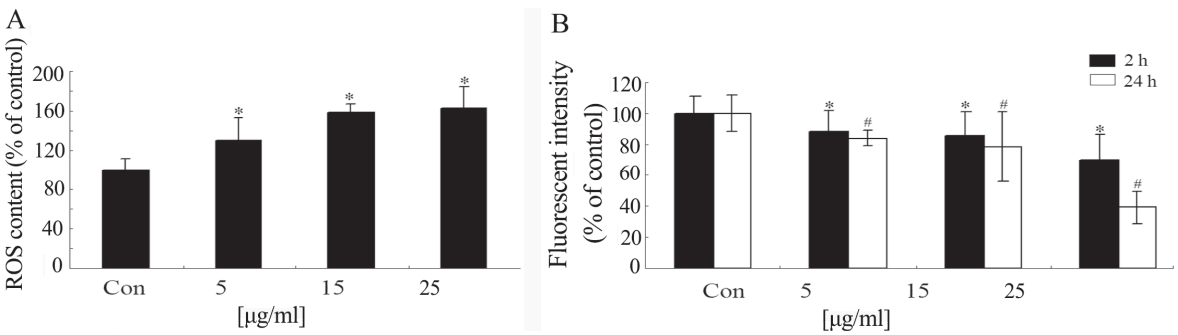

Fig. 3. Effect of TTME on ROS production and MMP changes in A549 cells. (A) ROS production was evaluated by intracellular intensities of DCFH-DA fluorescence. The cells were incubated with the indicated concentrations of TTME in triplicate for 2 h. $*p < 0.05$ vs. control. Data are expressed as the relative difference compared with the control ($n = 3$). (B) Reduction of MMP induced by TTME in A549 cells by staining with CMX-Ros probe. A549 cells were treated with the indicated concentrations of TTME in triplicate for 2 h or 24 h separately. $*p < 0.05$ vs. control. Data are expressed as the relative difference compared with the control. All experiments results were repeated at least three times.

drial membrane potential was thus determined by staining the cells with the dye CMX-Ros, which accumulates in the mitochondria of normal cells in a potential-dependent manner. Cells were treated with different doses of TTME for 2 h or 24 h, and then CMX-Ros was used to monitor the changes of the mitochondrial membrane potential. As can be seen in Fig. 3B, TTME treatment significantly decreased the mitochondrial membrane potential in A549 cells, compared with the control. These effects were dose-dependent.

Release of cytochrome c by TTME

To determine whether the effect of TTME was related to a release of cytochrome c from mitochondria, we carried out Western blot analysis of cytosolic extracts prepared under conditions that keep mitochondria intact. As shown in Fig. 4, cytosol from untreated tumour cells contained little cytochrome c. In contrast, TTME-treated tumour cells showed a dose-dependent release of cytochrome c.

Discussion

Although different compounds have been isolated from members of the genus *Trillium*, few studies have been conducted on their bioactivity and mechanisms of action. Hufford *et al.* (1988) reported that the EtOH extracts of the rhizomes and aboveground parts of *T. grandiflorum* showed significant antifungal activity. Further identification of the active components yielded the saponin glycosides. A novel 18-norspirostanol saponin obtained from the underground parts of *T. tschonoskii* displayed marked inhibitory action towards COX-2 production in macrophagocytes of the mouse abdominal cavity stimulated by lipopolysaccharide (LPS) (Wang *et al.*, 2007). To our knowledge, our report is the first one to show that the *n*-BuOH extract of TTME has cytotoxic, antiproliferative, and morphological effects in the lung cancer cell line. Ultrastructure observation found nuclear condensation, chromatin margination, and apoptotic body formation in TTME-treated cells. Our results further indicate that TTME mainly leads to apoptosis of cancer cells with a concomitant increase in loss of MMP and the release of cytochrome c. A rapid increase in the level of intracellular ROS and an accumulation of cells in the G2/M phase and S phase of the cell cycle were also observed in treated cells. Both of these effects could represent precursors to apoptosis, in the pathway(s) affected by TTME, or they could be the result of the apoptotic program.

Apoptosis is considered a vital process for cancer therapy (Thompson, 1995). It is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy (Elmore, 2007). In our study TTME was found to cause apparent cell shrinkage, detachment, and prevention from colony formation. Furthermore, nuclear condensation, chromatin margination, and apoptotic body formation could be observed by transmission electron microscopy in TTME-treated cells. These results provide the morphological proof of apoptosis.

The alternative to apoptotic cell death is necrosis, which is considered to be a toxic process where the cell is a passive victim and follows an energy-independent mode of death (Elmore, 2007; Krysko *et al.*, 2008). Early events in the apoptotic process are loss of plasma membrane asymmetry accompanied by translocation of phosphatidylserine (PS) from the inner to the outer membrane leaflet, thereby exposing PS to the external environment (Elmore, 2007). The phospholipid-binding protein annexin V has a high affinity for PS

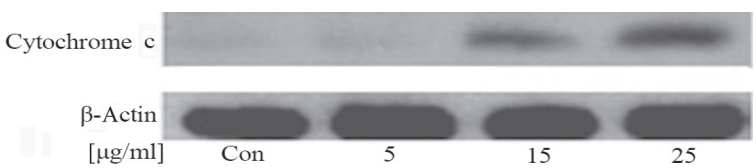

Fig. 4. Effect of TTME on cytochrome c release in A549 cells. Expression of cytochrome c was determined by immunoblotting the cytosolic fraction of the cells treated with different concentrations of TTME. Each blot was stripped and reprobed with anti-*β*-actin antibody to ensure equal protein loading. The upper band is the expression of cytochrome c protein. The lower band is the expression of *β*-actin protein. Results are representative of three separate experiments.

and so can act as a marker for apoptosis but not necrosis, which results from the loss of membrane integrity. In our study there was a significant percentage of early apoptotic cell death. Furthermore, TTME treatment arrested cancer cells at the G2/M phase or S phase. This finding together with the observed increased number of early apoptotic cells could be explained by the fact that cells are arrested at checkpoints, and hereby the progression through the cell cycle is delayed. The molecular mechanism requires further studies.

Although oxidative stress, mitochondrial dysfunction, and activation of caspases are common events in apoptosis, the interrelationship between these biochemical processes and the precise role of them in apoptosis varies due to different apoptotic stimuli (Gotloib, 2009). ROS include free radicals such as the superoxide anion, hydroxyl radicals, and the non-radical hydrogen peroxide (Adam-Vizi and Chinopoulos, 2006). In many experimental situations, induction of apoptosis is accompanied by an obvious rise in the intracellular ROS level (Kane *et al.*, 1993; Morales *et al.*, 1998; Park *et al.*, 2007; Provinciali *et al.*, 2002). Our study also demonstrated that intracellular peroxide levels rapidly increased after TTME treatment in a dose-dependent manner.

Mitochondria show signs of outer membrane and/or inner membrane permeabilization when exposed to a variety of pro-apoptotic stimuli (Nahon *et al.*, 2005). They are a main target for damage by ROS. Hydrogen peroxide could induce a mitochondrial permeability transition and change the mitochondrial membrane potential (Wang *et al.*, 2005). Indeed, some reports showed that ROS either act as activators of mitochondrial permeability transition or are a consequence of this transition, depending on the death stimulus (Xia *et al.*, 2004). Here, the results of CMX-Ros staining to detect the MMP showed that TTME-induced ROS production in A549 cells probably preceded the loss of MMP.

The loss of MMP has further effects on cytochrome c in the mitochondria (Goldsteins *et al.*, 2008), since membrane permeabilization results in the release of cytochrome c into the cytosol (Chipuk *et al.*, 2006). This is consistent with our results indicating an increase in the release of cytochrome c from the mitochondria of A549 cells treated with TTME.

In summary, we identified the major biochemical changes upon TTME treatment of A549 cells and investigated the functional relationship between these changes. It is the first report on the anticancer activities of an *n*-BuOH extract of *T. tschonoskii*, which could be useful in the treatment of cancers.

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