RESEARCH COMMUNICATION

Cytotoxicity of a Plant Steroidal Saponin on Human Lung Cancer Cells

Wenfeng Huang, Kun Zou*

Abstract

***Trillium tschonoskii* Maxim has been used to treat several diseases, including cancers, in folk medicine. The primary objective of the present study was to test whether TTB2, a steroidal saponin isolated from n-BuOH extracts, might exert effects on cell survival, morphology, cell cycle, reactive oxygen species (ROS) production and mitochondrial function in a lung cancer cell line. It was found that TTB2 had anti-proliferative and morphological influence, leading to the loss of mitochondrial membrane potential (MMP) in a dose-dependent manner. A significant increase in the level of intracellular ROS and an accumulation of cells in the G2/M phases of the cell cycle were also observed in treated cells. In summary, our results indicated that this compound might have potential use for the treatment of cancer.**

Keywords: Steroidal saponin - *Trillium tschonoskii* maxim - apoptosis - lung cancer

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Introduction

Non-small-cell lung cancer, which accounts for more than 80% of lung cancers, is the most common cause of cancer death worldwide (Burris, 2009). It has poor prognosis due to its resistance to current therapies and its occult lymph node metastases (Benlloch et al., 2009). There is an urgent need for developing new strategies aimed at better understanding the molecular mechanism by which potential anticancer chemicals could bypass this resistance, and further providing the basis for more effective treatments (Andreescu et al., 2005).

Successful treatment with chemotherapeutic agents is largely dependent on their ability to trigger cell apoptosis in tumor cells (Vansteenkiste, 2007). Previous studies demonstrate that certain phytochemicals present in medicinal herbs exert anti-tumor activity by inducing apoptosis in human lung cancer cells (Kummalue, 2005; Wang et al., 2009). One major biochemical change in cancer cells after treatment with chemotherapeutic agents is the increase in reactive oxygen species (ROS) production (Gupta, 2003).And studies have demonstrated that high levels of ROS could cause cellular damage and play an important role in mediating apoptosis (Hseu et al., 2008).

Trillium tschonoskii Maxim, also named "a pearl on head", is a perennial herb of Trilliaceae found in mid-western China (Li et al., 2005). *Trillium tschonoskii* Maxim has been used in folk for treating headache, hypertension, neurasthenia, giddiness, cancer, removing carbuncles and ameliorating pains for at least one thousand years (Fu, 1992). Previous studies showed that many bioactivity components including steroidal saponins and steroidal glycosides were found in Trillium genus including *Trillium erectum* (Yokosuka and Mimaki, 2008; Hayes et al., 2009), *Trillium kamtschaticum* (Ono et al., 2007; Nohara et al., 1975b), *Trillium tschonoskii* Maxim (Nohara et al., 1975a). We previously reported that one novel 18-norspirostanol saponin and one Trillenoside A could be obtained from the underground parts of *Trillium tschonoskii* Maxim (Wang et al., 2007). However, it is uncertain whether *Trillium tschonoskii* Maxim contains any active chemical components with cytotoxic effects on cancer cells. In this study, we demonstrate that TTB2, a steroidal saponin isolated from n-BuOH extracts has anti-cancer activity in human lung cancer cells, at least partly through an ROS-mediated pathway.

Materials and Methods

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Propidium iodixe, RNase, DCFH-DA, and BSA were purchased from Sigma Chemical Co. CMX-ROS were purchased from Invitrogen Co. RPMI 1640 culture medium and new bovine serum were supplied by Gibico BRL. Other chemicals used in this study were special-class commercial products.

Plant material, extraction and isolation of TTB2

The rhizomes of *Trillium tschonoskii* Maxim were purchased from Muyu, one town of Shennongjia Forest

*Hubei Key Laboratory of Natural Products Research and Development, Medical College, China Three Gorges University, Yichang, China *For correspondence : kzou@sina.cn, kzou@ctgu.edu.cn*

District of Hubei Province, China. Professor Chen Faju, a botanist in China Three Gorges University, identified the nature of the collected *Trillium tschonoskii* Maxim. A voucher specimen (No. 2005ZW03128) is deposited in the Medicinal Plants Herbarium of college of Chemistry and Life Science, China Three Gorges University. The methanol extract (2427 g) was suspended in water (2.2 l), and then extracted with CHCl3, EtOAc and n-BuOH successively. A portion of n-BuOH extracts (775 g) was reduced in vacuo and dissolved in water to a small volume, and then subjected to macroporous resin column chromatography in elution with gradient solvents (100% water→100% methanol). A portion of 80% methanol eluates (2.0 g) was separated by repeated Rp-C18 silica gel column chromatography in elution with gradient solvent system (acetonitrile : water, from 35 : 65 to 0 : 100) to give rise to 50 fractions. Fraction 37 (182 mg) was further separated by semi preparative HPLC eluted with 43% acetonitrile (within 30 min, 2.0 ml/min, detection at 203 nm), giving rise to compound TTB2 (32 mg). TTB2 powder was dissolved in distal water. The filtered TTB2 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 the gift from the Institute of Molecular Biology, China Three Gorges University. Cancer cells were maintained in RPMI 1640 culture medium supplemented with 10% new bovine serum and antibiotics in a 5% carbon dioxide incubator at 37˚C. To analyze the effect of TTB2 on A549 cells, cells were treated with different concentrations of TTB2 at different time points.

Growth inhibition assay

Cancer cells (1×10^4) were seeded in each well of a 96-well flat-bottom plate on day 1. On day 2, different concentrations of TTB2 (each concentration in triplicate) were added and cells were incubated for 24 h,48 h or 72 h. Mitomycin was used as a positive control. Afterwards, 100 μl of the MTT solution (1 mg/ml) was added and incubated for 4 h at 37ºC. The medium was carefully removed, washed and 200 μl of dimethylsulfoxide (DMSO) was added. After gently shaking at room temperature for 1 h, optical absorbance at 570 nm was recorded using a microplate reader (Bio-Rad). Each experiment was repeated at least 3 times.

Morphological study

Morphological changes of A549 cells after the treatment with TTB2 (5 μM) were studied using an inverted microscope.

Electron microscopy

After the treatment with TTB2 (5 μM), the appropriate size of cell pellets was fixed with 2.5% glutaraldehyde for 24 h. The subsequent steps were then carefully followed according to standard procedures including fixing, incubation, rinse, gradient dehydration, embedding and ultrathin section. Changes in the ultrastructure of the cancer cells were observed under an H-7500 transmission electron microscope (Japan).

Analysis of intracellular ROS formation

Flow cytometry (FCM) analysis of oxidative metabolism was carried out. In brief, cells were preincubated (15 min, 37ºC) with DCFH-DA (10 μM), and then washed in PBS. Next, the cells were treated with different concentrations (5 μM, 7.5 μM and 10 μM) of TTB2 for 2 h. At the end of the incubation, the reaction was stopped by keeping the samples on ice until FCM analysis.

Assessment of mitochondrial membrane potential (MMP)

CMX-Ros was used as the detection probe for the change of MMP. Cancer cells were resuspended incubated with different concentrations of TTB2 for 2 h or 24 h. Then Cells were incubated for 1 h at 37ºC with 1 μM (final concentration) CMX-Ros dissolved in DMSO. At the end of incubation, cells were washed with PBS and resuspended in the PBS substituted with 0.2% BSA, and kept on ice until FCM analysis.

Cell cycle analysis

Cancer cells (5×10^6) were treated with TTB2 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 4ºC 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 ºC for at least 30 min. The stained cells were analyzed for DNA content by FCM.

Statistical analysis

Data were presented as mean ± standard deviation (S.D.).Statistical evaluations were made using analysis of one-factor variance. $P<0.05$ was regarded as significance.

Results

Chemical structure of TTB2

As shown in Figure 1A, the structure of TTB2 was identified as pennogenin 3-O-α-L-rhamnopyranosyl-(1→2) [α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside.

Growth inhibition of TTB2 on A549 cells

MTT assays were used to investigate whether TTB2 exhibits growth inhibition on A549 cells. Cells were incubated with TTB2 for 24 h, 48 h or 72 h separately. Untreated cells (control) were considered as the baseline (100%) for the analysis. As shown in Fig. 1B, compared with the control, when cells were treated with the indicated concentrations (5 μM, 7.5 μM, 10 μM, 12.5 μM, 15 μM) of TTB2 for the indicated time, the survival rate of A549 cells was gradually decreased. The results suggested that TTB2 attenuated the cell survival in a dose-dependent manner.

Morphological changes of A549 cells and Transmission electron microscopic observation after TTB2 treatment

Cell shrinkage and poor refraction of A549 cancer cells were observed under the light microscope after treating

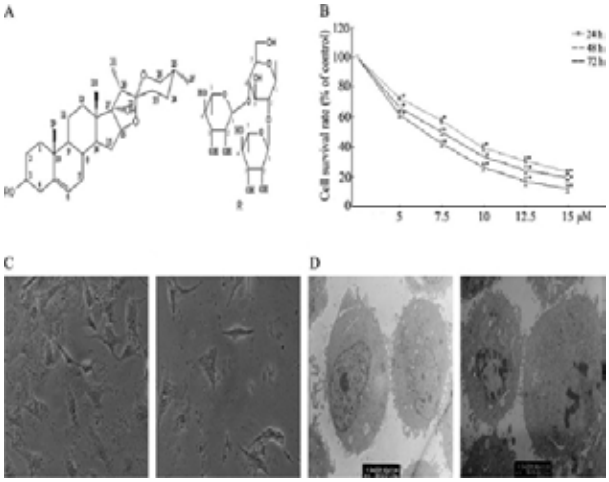

Figure 1. Chemical Structure of TTB2 and its Cytotoxicity on A549 Cell Growth. (A) TTB2 is pennogenin3-O-α-L-rhamnopyranosyl-(1→2)[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (Molecular Weight: 884.48). (B) Cells were seeded in 96-well plates and incubated with different concentrations of TTB2 for 24 h, 48 h or 72 h separately. Inhibition of TTB2 on A549 cell growth was dose-dependent, which was determined by MTT assay. Each value represents the mean ± SD of three replicates. *p<0.05 vs. control. (C) Morphological changes of A549 cells after treating with 5 μM TTB2 (the right) for 24 h included cell numbers, shrinkage, detachment, weak refraction and no formed colonies when compared with control(the left) under the light microscope (x400). (D) Compared to the control (the left), apoptotic nuclear morphology including nuclear condensation, chromatin margination and apoptotic body formation was further confirmed by transmission electron microscopy (the right)

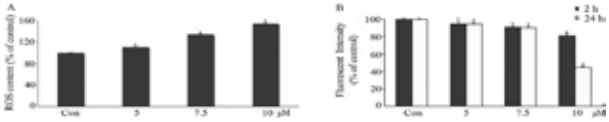

Figure 2. Effect of TTB2 on ROS Production and MMP Changes in A549 Cells. (A) ROS production was examined by intracellular intensities of DCFH-DA fluorescence. The cells were incubated with the indicated concentrations of TTB2 in triplicate for 2h. *p<0.05 vs. control. Data are expressed as the relative difference compared with Control (n=3). (B) Reduction of mitochondrial membrane potential (MMP) induced by TTB2 in A549 cells by staining with CMXRos probe. A549 cells were treated with the indicated concentrations of TTB2 in triplicate for 2h or 24h separately. *p<0.05, #p<0.05 vs. control. Data are expressed as the relative difference compared with Control. All the experiments were repeated for at least three times.

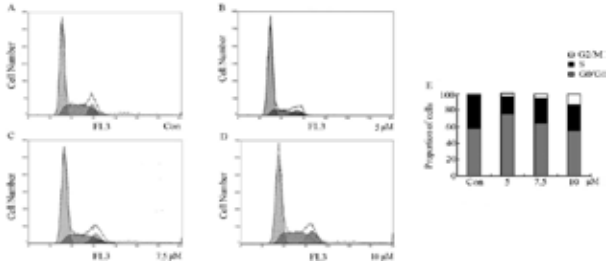

Figure 3. Cell Cycle Analyses as Measured by FCM. After treated with the indicated concentrations of TTB2 for 24 h, A549 cells were stained with propidium iodide probe. Result (A), (B), (C) and (D) are collected from one of three independent experiments. G2/M population increased in A549 cells treated with TTB2 in dose-dependent manner (E).

with TTB2. Furthermore, compared to control (Figure 1C, the left), cancer cells also began to detach and could not form colonies. This was detected after 24 h of 5 μM of TTB2 incubation (Figure 1C, the right). These results indicated that TTB2 exerted the good cytotoxic effects on A549 cells. The transmission electron microscopic observation showed that TTB2 (5 μM) led to nuclear condensation, chromatin margination and apoptotic body formation (Figure 1D, the right).

Effect of TTB2 on intracellular ROS levels

Several studies have implicated ROS generation as a possible mechanism for induction of apoptosis by various anticancer agents (Hseu et al., 2008; Morales et al., 1998). Therefore, intracellular ROS generation in control and TTB2 treated cells were evaluated by FCM. The cells were stained with DCFH-DA, which is cleaved and oxidized by peroxides to yield fluorescent DCF, followed by treatment with different doses (5 μM, 7.5 μM, 10 μM) of TTB2 for 2h. There was a significantly dose-dependent increase in ROS generation in TTB2-treated cells when compared with control (Figure 3A).

Effect of TTB2 on MMP

High intracellular ROS normally disrupts the MMP. The effect of TTB2 treatment on MMP was thus determined by staining the cells with dye CMXRos, which accumulates in the mitochondria in a potential dependent manner. Cells were treated with different doses (5 μM, 7.5 μM, 10 μM) of TTB2 for 2h or 24h separately, and then CMXRos was used to monitor the changes of MMP. As can be seen in Fig.3B, TTB2 treatment significantly decreased the MMP in A549 cells, as compared with control. And these results were dose-dependent.

Cell cycle analyses

To test whether an induction of cell cycle arrest contributed to the anti-proliferative potency of TTB2 in A549 cells, we performed flow cytometric cell cycle analyses. As shown in Figure 4, TTB2 (5 μM, 7.5 μM, 10 μM) caused a dose-dependent increase in the percentage of G2/M phase.

Discussion

Steroidal saponins are widely distributed in the botanical kingdom and have many pharmacological actions and biological activities (Guclu-Ustundag and Mazza, 2007;Wang et al., 2007). Compound TTB2, pennogenin 3-O-α-L-rhamnopyranosyl-(1→2) [α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside, is a steroidal saponin that has ever been isolated from *Trillium kamtschaticum* (Nohara et al., 1975b) and Paris *polyphylla* var. yunnanensis (Chen et al., 1990). However, few studies have been conducted on its bioactivity and mechanisms of action. Instead, it is reported that many pennogenin steroid analogues from other plants showed diverse bioactivity. For example, pennogenin 3-O-α-L-rhamnopyranosyl-(1→2)[α-L-arabinofuranosyl-(1→4)]-β-D-glucopyranoside from Paris *polyphylla* var. yunnanensis greatly inhibited gastric lesions induced

by ethanol and indomethacin (Matsuda, et al., 2003). Pennogenin-3-O-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→3)]-β-D-glucopyranoside from Dracaena mannii moderately inhibited carrageenan-induced paw edema in the rat (Tapondjou et al., 2008). Furthermore, some analogues including pennogenin 3-O-α-L-rhamnopyranosyl-(1→2)- β-D-glucopyranoside from *Paris vietnamensis* (Huang et al., 2006) and pennogenin 3-O-R-L-rhamnopyranosyl-(1→2)-[R-L-rhamnopyranosyl-(1→3)]-β-D-glucopyranoside from *D. deisteliana* (Kougan et al.) were found to be cytotoxic to cancer cells. To our knowledge this is the first report showing that TTB2, the pennogenin steroid from *Trillium tschonoskii* Maxim, has cytotoxic, anti-proliferative and morphological effects on lung cancer cell line. Our results further indicated that TTB2 led to the apoptosis of cancer cells with a significant increase in the level of intracellular ROS and a concomitant loss of MMP. And an accumulation of cells in the G2/M phase of cell cycle were also observed in treated cells. All of these effects could represent precursors to apoptosis.

Apoptosis 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 microscope (Elmore, 2007). In our study TTB2 caused significant cell shrinkage, detachment and no formed colonies. Additionally, transmission electron microscope showed obvious apoptotic features of TTB2-treated cells. These results morphologically indicated the apoptosis of A549 cells induced by TTB2.

Excessive oxidative stress could produce much more ROS including superoxide anion, hydroxyl radicals and the non-radical hydrogen peroxide (Ryter et al., 2007). In many experimental situations, induction of apoptosis is accompanied by the rise of intracellular ROS level (Kane et al., 1993; Park et al., 2007). Our study also demonstrated that intracellular peroxides levels were rapidly up-regulated by TTB2 treatment in a dose-dependent manner. Mitochondria are a main target for damage by ROS. For instance, hydrogen peroxide could induce a mitochondrial permeability transition and change the MMP (Wang et al., 2005). The loss of MMP is one of the endpoint features of apoptosis (Skommer et al., 2006). Here, the results of CMXRos staining to detect the MMP showed that TTB2-induced ROS production in A549 cells probably preceded the loss of MMP.

G2/M phase is the important checkpoint for DNA damage and critical to cell cycle progression (Hartwell and Weinert, 1989). Our results clearly indicated that TTB2 caused an increase in the percentage of G2/M phase, which is one mechanism by which TTB2 exerts its anti-proliferative effect.

In summary, this paper is the first one to report anti-cancer activity of a pennogenin glycoside from *Trillium tschonoskii* Maxim, and indicated that it could be useful in the treatment of cancers.

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