Abstract. Triptolide (TPL), a diterpene triepoxide compound, extracted from *Tripterygium wilfordii* Hook F. [a traditional Chinese medicinal herb (TCM)], has demonstrated great chemotherapeutic potential for the treatment of tumors. However, the anticancer mechanisms of action of TPL in human skin cancer remain to be further investigated. In this study, we used A375.S2 human melanoma skin cancer cells as a model to investigate the effect of TPL on cell death. A375.S2 cells were treated with various concentrations of TPL for different periods of time and investigated the effects on cell cycle distribution and apoptosis were investigated. The data showed that TPL induced cell morphological changes, decreased the percentage of viable cells, and induced S phase arrest and apoptosis in A375.S2 cells in a concentration- and time-dependent manner. Furthermore, we used flow cytometry analysis and the data showed that TPL promoted reactive oxygen species, NO and Ca\(^{2+}\) production, decreased the mitochondrial membrane potential (ΔΨ\(_{m}\)) and increased the activity of caspase-3, -8 and -9 in the A375.S2 cells. Western blot analysis showed that TPL promoted the expression of p21 and p27 but inhibited that of cyclin A and CDC25A, leading to S phase arrest. Furthermore, the data also showed that TPL promoted the expression of Fas and FasL and increased the activity of caspase-3, -8 and -9, cytochrome c, Bax, apoptosis-inducing factor (AIF) and endonuclease G (Endo G); however, the expression of Bax was decreased, leading to apoptosis. Based on these observations, TPL induces apoptosis in A375.S2 cells through Fas-, caspase- and mitochondrial-mediated pathways.

Introduction

Skin cancer is one of the main causes of cancer-related mortality worldwide and cutaneous melanoma is one of the most aggressive forms of skin cancer in humans (1,2). Although melanoma accounts for approximately 4% of all dermatological cancers, >80% of deaths from skin cancer are associated with melanoma. Furthermore, the ten-year survival rate for patients with metastatic melanoma is <10% (3,4). The cure rate for patients with melanoma has not achieved clinically satisfactory response rates. Therefore, several studies have focused on the discovery of novel therapeutic agents derived form natural products for cancer prevention and therapy. The induction of tumor cell apoptosis is one of the pursued strategies in chemotherapy (5,6). In particular, molecules associated with the regulation of apoptosis are clinically relevant targets for chemical intervention (7,8).

Triptolide (TPL), a diterpenoid triepoxide, derived from the herb *Tripterygium wilfordii*, has been used as a herbal medicine in China for hundreds of years (9,10). TPL has been shown to induce S phase arrest via the inhibition of cyclin E and CDC25A and triggers apoptosis via caspase- and mitochondrial-dependent signaling pathways in A375.S2 human melanoma cells.
induce apoptosis in a number of cancer cells, such as breast (11,12), lung (13), pancreatic (14,15) and colon cancer (16,17). It has been reported that TPL-induced apoptosis is associated with the death receptor and mitochondrial-mediated pathways in leukemic (18-20) and pancreatic cancer cells (14,15). Recently, it was reported that TPL induced the apoptosis of pancreatic cancer cells via the downregulation of decoy receptor 3 (DcR3) expression (15). TPL has been shown to inhibit colon cancer cell proliferation by the induction of G1 phase arrest through the upregulation of p21 (17). In our laboratory, we found that TPL induced the apoptosis of NCI-H295 human adrenal cancer cells through a mitochondrial-dependent pathway (21).

In the present study, A375.S2 human melanoma cells were employed as a cell model to evaluate the in vitro anti-melanoma potential of TPL. We found that the cytotoxic effect of TPL on A375.S2 cell growth was associated with cell cycle arrest at the S phase and the induction of apoptosis through the caspase- and mitochondrial-dependent signaling pathways. The underlying molecular mechanisms leading to these profound effects were also investigated.

**Materials and methods**

**Chemicals and reagents.** TPL, dimethyl sulfoxide (DMSO), RNase A, Triton X-100 and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Minimum essential medium (MEM), fetal bovine serum (FBS), L-glutamine penicillin-streptomycin and trypsin-EDTA were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). The Annexin V-FITC apoptosis detection kit was from Gibco/Invitrogen. 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) for determination of reactive oxygen species (ROS) levels, DiOC$_2$ for determination of mitochondrial membrane potential ($\Delta \Psi_{m}$) and Fluo-3/AM for determination of intracellular Ca$^{2+}$ levels were purchased from Oncoimmunin, Inc. (Gaithersburg, MD, USA).

**Cell culture.** The A375.S2 human malignant melanoma cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The A275.S2 cells were cultured in 75 cm$^2$ tissue culture flasks with MEM supplemented with 10% FBS (Gibco-BRL, Gaithersburg, MD, USA), penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin) and 2 mM L-glutamine and grown in a humidified atmosphere of air containing 5% CO$_2$ at 37°C as previously described (22,23).

**Cell morphological changes examinations, viability and cell cycle distribution assays.** The A375.S2 cells (2x10$^5$ cells/well) were maintained in 12-well plates for 24 h and then 0 and 20 nM of TPL were individually added to the wells and incubated at 37°C for 24 h. Cell viability was determined using a trypan blue exclusion assay. The A375.S2 cells (2x10$^5$ cells/well) were then trypsinized, collected and centrifuged and washed twice with cold PBS and then lysed with ice-cold PBS (0.1% Triton X-100) for determination of mitochondrial membrane potential ($\Delta \Psi_{m}$) and the phosphatidylserine on the outside of the apoptotic cells.

**Determination of ROS production, intracellular Ca$^{2+}$ release and $\Delta \Psi_{m}$.** A375.S2 cells (2x10$^5$ cells/well) were placed onto 12-well plates and treated with 20 nM TPL for 0, 12, 24 and 48 h for ROS, Ca$^{2+}$ and $\Delta \Psi_{m}$ measurements. Cells were harvested after each treatment then re-suspended in 500 µl of DCFH-DA (10 µM) for ROS (H$_2$O$_2$) determination, re-suspended in 500 µl of Fluo-3/AM (2.5 µg/ml) for intracellular Ca$^{2+}$ concentration determination and suspended in 500 µl of DiOC$_2$ (4 µmol/l) for $\Delta \Psi_{m}$ followed by incubation at 37°C for 30 min. The cells were then analyzed by flow cytometry as described previously (24,25).

**Caspase-3, -8 and -9 activity assay.** The A375.S2 cells (2x10$^5$ cells/well) were maintained in 12-well plates for 24 h and then 0 and 20 nM of TPL were individually added to the wells and incubated for 0, 12, 24 and 48 h. All cells were trypsinized, collected and centrifuged and washed twice with PBS. All samples were re-suspended in 50 µl of 10 µM substrate solution (CaspaLux 8-L$_2$D$_2$ for caspase-8, CaspaLux9-M$_2$D$_2$ for caspase-9 and PhiPhiLux-G$_2$D$_4$ for caspase-3) before being incubated at 37°C for 60 min. All samples were washed with PBS and analyzed by flow cytometry as described previously (26).

**Western blot analysis for the determination of apoptosis-associated proteins.** The A375.S2 cells (2x10$^5$ cells/well) in 6-well plates were treated with 20 nM of TPL and then incubated for 0, 12, 24 and 48 h for the determination of proteins associated with cell cycle arrest and apoptosis. Cells were harvested and washed with cold PBS and then lysed with ice-cold lysis buffer containing 50 mM HEPEs (pH 7.7), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween-20, 10% (v/v) glycerol, 1 mM NaF, protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitor cocktail (Sigma Chemical Co.). The total proteins from each sample were quantified using the Bio-Rad method. Each sample (50 µg protein) was resolved over 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). The blot was then soaked in blocking buffer (5% non-fat dry milk/0.05% Tween-20 in 20 mM TBS at pH 7.6) at room temperature for 1 h and then incubated with...
individual primary monoclonal antibodies in blocking buffer at 4°C overnight, followed by secondary antibody horseradish peroxidase conjugate and detection by chemiluminescence and autoradiography using X-ray film as described previously (26).

To ensure equal protein loading, each membrane was stripped and re-probed with anti-β-actin antibody (24,26). Dilutions of primary antibodies were 1:1,000 [antibodies specific for caspase-9, -8, -3, cytochrome c, apoptosis-inducing factor (AIF),

Figure 1. Triptolide induced cell morphological changes and decreased the percentage of viable A375.S2 cells. Cells were plated onto MEM + 10% FBS with various concentrations of triptolide for 24 and 48 h. (A) The morphological changes were examined and photographed under a phase-contrast microscope, and (B) the total percentages of viable cells was determined by flow cytometry as described in Materials and methods. Each point is the means ± SD of three experiments. *P<0.05, significant difference compared to the control.
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endonuclease G (Endo G), Bcl-x, Bax, Fas, FasL and glucose-regulated protein (GRP78).

Statistical analysis. The quantitative data are presented as the means ± SD. Statistical differences between the TPL-treated and control samples were calculated using the Student's t-test. A value of P<0.05 was considered to indicate a statistically significant difference. The results are representative of at least three independent experiments (24,26).

Results

TPL induces cell morphological changes and decreases the percentage of viable A375.S2 cells. In order to confirm the biological effects of TPL, the A375.S2 cells were treated with various concentrations of TPL for 24 and 48 h, and cell morphological changes and the percentage of viable cells were determined. The results are shown in Fig. 1, indicating that TPL induced morphological changes (Fig. 1A) and decreased the percentage of viable cells in a concentration-dependent manner (Fig. 1B).

TPL induces S phase arrest in A375.S2 cells. To investigate the inhibitory effect of TPL on cell growth, we investigated the cell cycle distribution in A375.S2 cells by flow cytometry and the results are shown in Fig. 2. The number of cells in the G1, S, G2/M and sub-G1 phases of the cell cycle showed that the A375.S2 cells accumulated in the S and Sub-G1 phases following exposure to TPL, whereas the G1 cell population was decreased in a concentration-dependent manner. Collectively, these observations suggest that TPL causes S phase arrest and induces apoptosis in A375.S2 cells.

TPL promotes ROS production, intracellular Ca\textsuperscript{2+} release and decreases ΔΨ\textsubscript{m} in A375.S2 cells. To further investigate the role of ROS, Ca\textsuperscript{2+} and the loss of ΔΨ\textsubscript{m} in TPL-induced apoptosis in A375.S2 cells, the cells were treated with 20 nM of TPL for different periods of time. The harvested cells were analyzed for ROS and Ca\textsuperscript{2+} production and the loss of ΔΨ\textsubscript{m}. The results are shown in Fig. 4A-C. The results indicated that TPL promoted the production of ROS (Fig. 4A) and Ca\textsuperscript{2+}

Figure 2. Triptolide induced cell cycle arrest at the sub-G1 phase in A375.S2 cells. Cells were treated with various concentrations of triptolide for 48-h and then harvested for cell cycle and sub-G1 analysis by flow cytometry as described in Materials and methods. (A) Representative profiles of the cell cycle. (B) Percentage of cells in each phase of the cell cycle. Each point is the means ± SD of three experiments. *P<0.05, significant difference compared to the control.

Figure 3. Triptolide induced apoptosis in A375.S2 cells. Cells were incubated with various concentrations of triptolide for 12, 24 and 48 h. Apoptotic cells were determined by Annexin V/PI staining and analyzed by flow cytometry as described in Materials and methods. (A) Representative profiles of Annexin V/PI staining. (B) Percentage of apoptotic cells. Each experiment was performed in triple sets. *P<0.05, significant difference compared to the control (Ctl).
(Fig. 4B) but ΔΨm (Fig. 4C) in the A375.S2 cells. These effects occurred in a time-dependent manner. These results show that TPL-induced apoptosis in A375.S2 cells involves the production of ROS and Ca\(^{2+}\) and the decrease in ΔΨm.

**TPL promotes the activation of caspase-3, -8 and -9 in A375.S2 cells.** In order to investigate whether caspase-3, -8 and -9 are involved in TPL-induced apoptosis, the enzymatic activity of caspases was detected using three fluorogenic peptide substrates (Caspalux 8-L\(_1\)D\(_2\) for caspase-8, Caspalux9-M\(_1\)D\(_2\) for caspase-9 and PhiPhiLux-G\(_1\)D\(_1\) for caspase-3). The results are shown in Fig. 4C-E, indicating that TPL induced a rapid increase in caspase-3, -8 and -9 activity. The co-treatment of A375.S2 cells with pan-caspase inhibitor led to a significant decrease in the activity of caspase-3, -8 and -9. These findings show that TPL-induced apoptosis in A375.S2 cells involves caspase activation.

TPL affects the cell cycle and apoptosis-associated proteins in A375.S2 cells. In order to investigate whether the TPL-induced S phase arrest and apoptosis involves apoptosis-associated proteins in A375.S2 cells, the cells were treated with 20 nM TPL for 0, 12, 24 and 48 h and then examined by western blot analysis. The results are shown in Fig. 5A-D, indicating that TPL increased the levels of p21 and p27 but inhibited those of CDK2, cyclin E and CDC25A (Fig. 5A), leading to S phase arrest. TPL promoted the expression of GADD153, GRP78, caspase-4, IRE and calpain 1 (Fig. 5E), indicating that TPL induced apoptosis through ER stress. Furthermore, TPL promoted the expression of caspase-8, Fas and FasL (Fig. 5B and D), Bax and Bid, but inhibited the levels of Bcl-2 (Fig. 5C). However, it increased the levels of cytochrome c, caspase-9 and -3, AIF and Endo G (Fig. 5B). These findings show that TPL induces apoptosis via the caspase- and mitochondrial-dependent pathway in A375.S2 cells.
Discussion

Previous studies have shown that melanomas are resistant to conventional chemotherapy and metastasize to the brain, lung, liver and skin (27,28). Attention has been drawn to alternative options for the treatment and prevention of cancer, including treatments derived from herbs. Natural products for the treatment of cancer include antioxidants and cancer preventative agents, or cancer therapeutic drugs (29,30). TPL is one of the major active compounds in *Tripterygium wilfordii*...
Hook F. extract, with potent anti-inflammatory and anticancer activity (12,31,32). In this study, we investigated Chinese herbal TPL for its effects against human melanoma skin cancer cells in vitro. Our results from flow cytometric analysis showed that TPL significantly inhibited cell growth, inducing S phase arrest and apoptosis in A375.S2 human melanoma cells (Figs. 1 and 2).

To our knowledge, our findings are the first to show that TPL induces cytotoxic effects in human melanoma skin cancer cells. These effects include cell morphological changes and the decrease in the percentage of viable cells, as well as the induction of S phase arrest and apoptosis (Figs. 1-3). TPL also promoted caspase-8, -9 and -3 activity (Fig. 4D-F). We also used pan-caspase inhibitor, which led to an increase in the percentage of viable cells compared to the cells treated with TPL alone. These results are in agreement with those from previous studies, demonstrating that caspase-8 and -9 can be activated by TPL in pancreatic cancer cells (15,33,34). It is well known that the activation of caspase-8 and -9 involves two different apoptotic pathways (22,35). Caspase-8 involves the extrinsic pathway which is triggered by Fas and FasL and caspase-9 involves the intrinsic pathway which is triggered by the increase in the ratio of Bax/Bcl-2, leading to mitochondrial dysfunction through the caspase-dependent pathway or directly leading to the release of AIF and Endo G from the mitochondria (mitochondrial-dependent pathway; also termed caspase-dependent pathway) (22,35). It has been reported that the TPL-induced apoptosis is related to its ability to reduce DcR3 expression and increase FasL expression (36,37). In this study, we found TPL induced caspase-3, -8 and -9 activation (Fig. 4D-F) and increased the expression of Fas and FasL (Fig. 5D). These results further support the notion that TPL is a potent activator of caspases in A375.S2 cells.

Our results also showed that TPL inhibited the phosphorylation of ERK1/2 and JNK1/2 (Fig. 5F); however, it did not alter the level of phosphorylation of MEK1 and MEK1/2. These results are in agreement with those from previous studies, showing that TPL inhibits the phosphorylation of ERK1/2 and affects ERK1/2 activation directly (17,38). Our findings also showed that TPL promoted the expression of p21 and p27 in A375.S2 cells. It has been reported that p21 is a well-characterized CDK inhibitor; high levels of p21 can inhibit cyclin D1 expression, resulting in the decline of pRb phosphorylation (39,40). In this study, we did not observe any significant changes in the A375.S2 cells following exposure to TPL. However, we did observe the inhibition of cyclin A and CDC25A in the A375.S2 cells following exposure to TPL, which may be the mechanism of action behind the TPL-induced S phase arrest (Fig. 5A).

In conclusion, the results from the present study demonstrated that the exposure of A375.S2 cells to TPL led to S phase arrest and the induction of apoptosis (Fig. 6). TPL induced S phase arrest via the promotion of p21 and p27 and the inhibition of cyclin A and CDC25A expression. TPL induced the apoptosis of A375.S2 cells via Fas and FasL, leading to the activation of caspase-dependent and -independent signaling pathways. Apoptosis was also induced, in part, through the ER stress pathways.

Figure 6. The proposed signaling pathways of triptolide-induced cell cycle arrest and apoptosis in A375.S2 human melanoma cells.
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References