

# Triptolide reverses the Taxol resistance of lung adenocarcinoma by inhibiting the NF- $\kappa$ B signaling pathway and the expression of NF- $\kappa$ B-regulated drug-resistant genes

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**Abstract.** Paclitaxel (or Taxol<sup>®</sup>) is a first-line chemotherapeutic drug for the treatment of non-small cell lung cancer; however, resistance to the drug is an important factor, which influences the outcome of chemotherapy. The present study aimed to investigate the role of triptolide (TPL) in reversing Taxol-resistant human lung adenocarcinoma and to elucidate the underlying molecular mechanism of resistance reversal mediated by TPL. It was hypothesized that this experimental approach would assist in solving the problem of chemotherapeutic resistance in non-small cell lung cancer, thereby improving the clinical outcomes. The human Taxol-resistant lung adenocarcinoma cell line, A549/Taxol, was established. The resistance index of the cell line was calculated, according to the half maximal inhibitory concentration (IC<sub>50</sub>) of A549/Taxol IC<sub>50</sub> of A549, to be 51.87. The levels of apoptosis and the cell cycle in the A549/Taxol cell line were assessed to confirm the effects of TPL at three different concentrations (0.03, 0.3 and 3  $\mu$ mol/l) and treatment durations (2, 4, 6 and 12 h) by flow cytometric analysis, and the inhibition of the NF- $\kappa$ B signaling pathway and the expression of NF- $\kappa$ B-regulated drug-resistant proteins were determined by immunofluorescence and western blotting, respectively. The administration of TPL promoted cell apoptosis in the A549/Taxol lung adenocarcinoma Taxol-resistant cell line and also promoted cell cycle regulation. The drug was also able to elicit a reversal of the drug resistance. TPL inhibited the nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway and the expression of NF- $\kappa$ B-regulated drug-resistant genes, including those for FLICE-like inhibitory protein, X-linked inhibitor of apoptosis protein, Bcl-2, Bcl-xL and cyclo-oxygenase-2. TPL exerted a marked drug-resistance-reversal effect on human

lung adenocarcinoma Taxol resistance, and the effect was revealed to be dose- and time-dependent. In conclusion, TPL exerted its role in the process of resistance reversal by inhibiting the NF- $\kappa$ B signaling pathway, and the transcription and expression of NF- $\kappa$ B-regulated drug-resistant genes.

## Introduction

Triptolide (TPL), the predominant active ingredient of the thunder duke vine, *Tripterygium wilfordii*, is used in traditional Chinese medicine (1). It is a double terpenoid. Previous studies confirmed that TPL exerts a role in a number of biological processes, including a range of immunosuppressive, anti-inflammatory and antitumor effects (1). In particular, TPL has potential as an antitumor drug in clinical applications. For example, the use of TPL in treating leukemia has entered phase I clinical trials (2,3), and its antitumor potential for the treatment of ovarian cancer was demonstrated by the induction of cell apoptosis (4).

As a first-line treatment for non-small cell lung cancer chemotherapy, paclitaxel (or Taxol<sup>®</sup>) resistance is an important factor, which influences the effect of chemotherapy. *In vitro* and animal experiments revealed that TPL exerts a marked inhibitory effect on the growth of lung cancer and other solid tumor types (5).

Previous studies revealed that TPL exerts an antitumor effect primarily through the inhibition of nuclear factor (NF)- $\kappa$ B, heat shock factor 1, activator protein-1 and other transcription factors, and the NF- $\kappa$ B pathway is one of the most critical targets of the antitumor effect (6,7). The NF- $\kappa$ B/inhibitor of NF- $\kappa$ B (I $\kappa$ B) pathway is associated with tumor cell survival, apoptosis and metastasis (8,9). In addition, the genes which regulate the NF- $\kappa$ B signaling pathway, particularly apoptosis-associated genes, provide an important basis for the occurrence of multi-drug resistance (9,10). For example, the upregulation of Bcl-2 and the downregulation of Bax, proteins which are involved in apoptosis, are associated with a strengthening of resistance to chemotherapy, and the caspase inhibitors, FLICE-like inhibitory protein (FLIP) and X-linked inhibitor of apoptosis protein (XIAP), may be directly involved in the regulation of chemosensitivity (11). Therefore, the inhibition of the NF- $\kappa$ B signaling pathway, particularly regarding the transcription and

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expression of antiapoptotic genes, may provide the underlying molecular mechanism of chemoresistance reversal. Previous studies indicate that TPL may exert a drug-resistance-reversal effect (11,12). Chen *et al* (12) demonstrated that TPL inhibited the expression of multidrug resistance protein and enhanced the antitumor effects on KB cells mediated by 5-fluorouracil. Li *et al* (11) revealed that TPL improved the sensitivity of K562/A02 cells to adriamycin by inhibiting the expression of microRNA-21, in addition to upregulating the expression level of phosphatase and tensin homolog. Although the anti-tumor activity of TPL is recognized and it may exert potential drug-resistance reversal effects, the underlying molecular mechanism remains to be fully elucidated.

In the present study, a Taxol-resistant strain of lung adenocarcinoma cell line (A549/Taxol) was established with an aim to investigate the impact of TPL on A549/Taxol cell proliferation, apoptosis and the cell cycle. The present study also aimed to investigate whether TPL may exert a resistance reversal effect in lung adenocarcinoma Taxol-resistance, and the underlying mechanism of its action. The results of the present study may be of important clinical significance in consideration of whether TPL may be applied as a resistance reversal agent, in combination chemotherapy with Taxol/paclitaxel.

## Materials and methods

**Establishment of the Taxol-resistant lung adenocarcinoma cell line, A549/Taxol.** The A549 lung adenocarcinoma cell line was purchased from the Typical Culture Preservation Commission Cell Bank, Chinese Academy of Sciences (Shanghai, China). The Taxol-resistant lung adenocarcinoma cell line, A549/Taxol, was established by the method of increasing the drug concentration gradient. Human lung adenocarcinoma A549 cells in the logarithmic growth phase were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) medium supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin (Sigma-Aldrich) and 100 µg/ml streptomycin (Sigma-Aldrich) at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Subsequently, Taxol (Qilu Pharmaceutical Co., Ltd., Jinan, China) was added to the culture medium at a minimum concentration of 20 ng/ml. The cells were cultured for 24 h. Sensitive cells died as the drug induced apoptosis. The surviving cells were cultivated to the next logarithmic phase in the culture medium without Taxol. In the next cycle, the cells were cultured and induced by Taxol using the same process, however, the concentration of Taxol was increased from 20 ng/l to 400 ng/l (the concentration gradient was 20, 40, 60, 80, 100, 120, 200, 30 and 400 ng/ml). The process was repeated until the A549 cells grew steadily in the medium with 400 ng/l Taxol. These cells were Taxol-resistant lung adenocarcinoma cells (A549/Taxol). On completion of the initial stage of the assessment of the cells, the resistance index of the cell line was calculated, according to the half maximal inhibitory concentration (IC<sub>50</sub>) of A549/Taxol / IC<sub>50</sub> of A549, to be 51.87.

**Effect of TPL on A549/Taxol cell apoptosis.** Annexin-V fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining (Invitrogen; Thermo Fisher Scientific, Inc.) was used for the detection of apoptosis. A549/Taxol cells in the

logarithmic growth phase were inoculated into 6-well plates at a density of 1x10<sup>5</sup> cells/cm<sup>2</sup> and cultured for 24 h. Once the cells had adhered, different concentrations of TPL (0.03, 0.3 or 3 µmol/l) were added to the cells, and the negative control group was established. The cells were incubated for 2, 4, 6 or 12 h, and were subsequently trypsinized and collected using 0.25% trypsin (excluding EDTA; Sigma-Aldrich). The cells were washed twice with phosphate-buffered saline (PBS), prior to centrifugation at 870 x g for 5 min, and 5x10<sup>5</sup> cells were collected. Subsequently, 500 µl binding buffer suspension (Invitrogen; Thermo Fisher Scientific, Inc.) and 5 µl annexin V-FITC were added to the cells prior to mixing, and then 5 µl PI was added and mixed. The incubation was performed for 5-15 min at room temperature in the dark. The extent of apoptosis was assessed using a flow cytometer (XL/XL-MCL; Beckman Coulter, Fullerton, CA, USA; excitatory wavelength (λ<sub>ex</sub>) 488 nm; emission wavelength, (λ<sub>em</sub>) 530 nm). The data were analyzed using FlowJo 7.1 (TreeStar, San Carlos, CA, USA) and ModFit LT 3.1 (Verity Software House Inc., Topsham, ME, USA).

**Effect of TPL on the A549/Taxol cell cycle.** PI staining was used to detect the cell cycle. A549/Taxol cells in the logarithmic growth phase were inoculated into 6-well plates at a density of 1x10<sup>5</sup> cells/cm<sup>2</sup>, prior to culture for 24 h. Once the cells had adhered, different concentrations of TPL (0.03, 0.3 or 3 µmol/l) were added to the cells for 2, 4, 6 or 12 h. The cells were subsequently harvested with 0.25% trypsin (excluding EDTA) and washed with PBS, prior to centrifugation at 870 x g for 5 min, and 5x10<sup>5</sup> cells were collected. Single cell suspensions were fixed with the volume fraction of 70% ethanol overnight, preserved at 4°C, and subsequently washed with PBS fixative prior to staining. An aliquot of 100 µl RNase A was added and the cells were incubated in a water bath for 30 min at 37°C. Subsequently, 400 µl PI was added to stain the cells prior to blending, and the cells were maintained in the dark for 30 min at 4°C. The quantity of red fluorescence at an excitation wavelength of 488 nm was assessed using a flow cytometer (Beckman Coulter).

**Western blotting.** The protein expression levels of NF-κB-mediated drug-resistant genes were determined by western blotting. The extracted protein concentration was measured using a conventional bicinchoninic acid method. Samples of 40 µg protein were cooled on ice following an incubation at 95-100°C for 5 min, and the samples were subsequently electrophoresed using 8% SDS-PAGE (Beyotime Institute of Biotechnology, Jiangsu, China). For the electrophoresis, a stacking gel was used at a constant voltage (80 V) for 20 min, followed by a separating gel at 100 V for ~80 min. The gel was removed and placed in transfer buffer (Beyotime Institute of Biotechnology) to equilibrate for 15 min. The filter paper and the polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA) were prepared and placed in transfer buffer and deionized water, respectively. For the wet electrotransfer stage, the bottom electrode (anode) was laid flat, with the filter paper, PVDF membrane, the gel and a filter paper placed on top. The top electrode (cathode) was placed on the interlayer following the exclusion of air bubbles. For the electrotransfer, the apparatus was

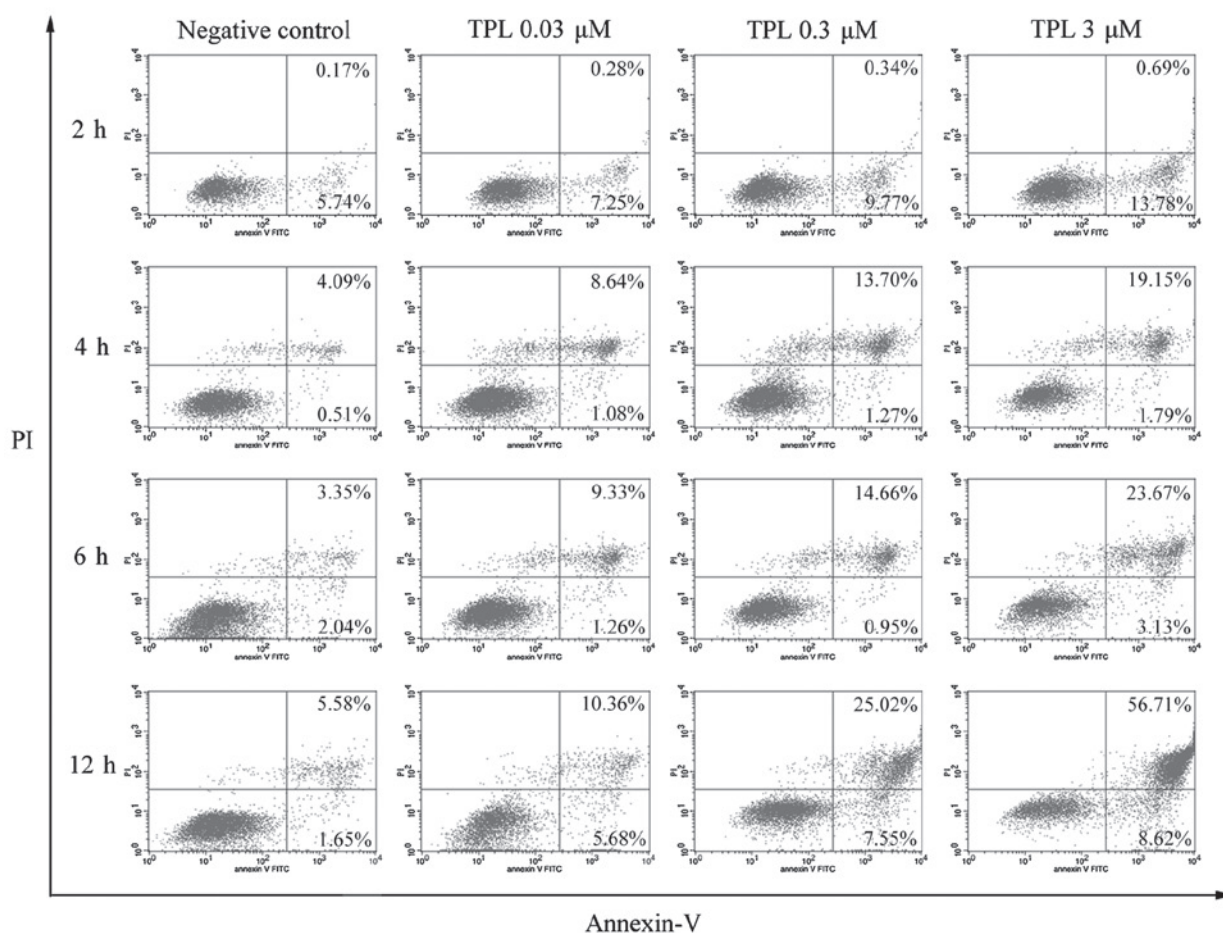


Figure 1. Effect of different concentrations of TPL on A549/Taxol cell apoptosis in each phase. TPL induces cell apoptosis in the A549/Taxol human lung adenocarcinoma Taxol-resistant strain in a dose- and time-dependent manner. For the annexin-V FITC/PI double staining, A549/Taxol cells were incubated with 0.03, 0.3 and 3  $\mu$ M TPL for 2, 4, 6 and 12 h. The percentages of apoptotic cells are shown. The cells in the upper right quadrant are annexin V-FITC and PI double positive (late apoptotic cells or necrotic cells), whereas the cells in the lower right quadrant are annexin V-FITC positive (early apoptotic cells). The percentages were measured by flow cytometric analysis at each time point and concentration of TPL. TPL, triptolide; PI, propidium iodide; FITC, fluorescein isothiocyanate.

powered by a constant current (200 mA) for 1 h. The PVDF membranes were blocked with 5% skimmed milk blocking buffer (incubated at room temperature for 1 h), and subsequently the blocking solution was discarded. The primary antibody, rabbit anti-CK19 (1:5,000; cat. no. ab133496; Abcam, Cambridge, UK) and a rabbit anti- $\beta$ -actin antibody (1:4,000; cat. no. ab8227; Abcam) were added ( $\sim$ 0.1 ml/cm<sup>2</sup>), followed by an incubation with agitation at 4°C overnight. The membrane was rinsed with PBS with Tween<sup>®</sup> 20 (PBST) four times, each time for 5 min. The membrane and secondary goat anti-rabbit immunoglobulin G antibody conjugated with HRP (horseradish peroxidase-labeled antibody and secondary antibody in blocking buffer; 1:5,000; cat. no. CW0114; Beijing ComWin Biotech Co., Ltd., Beijing, China) were incubated with agitation at room temperature for 1–2 h. The membrane was subsequently washed with PBST, and rinsed five times (5 min each time). The calculated quantity of developer (EMD Millipore) was 0.1 ml/cm<sup>2</sup>, and the developer was applied to the PVDF membrane and placed at room temperature for 1 min. The PVDF membrane was wrapped in plastic in order to avoid air bubbles. The membrane proteins were attached to the X-ray film for quick exposure in a darkroom and developed. The exposure time

was adjusted to enable the best development of the protein bands to occur.

**Immunofluorescence staining.** The effect of TPL on the A549/Taxol cellular localization was determined using immunofluorescence. The A549/Taxol cells were treated with TPL (3  $\mu$ M) for 12 h. The cells on the slides were subsequently fixed with 4% paraformaldehyde. A total of two drops of 3% H<sub>2</sub>O<sub>2</sub>/methanol solution was added to each slice at room temperature (15–25°C) for 10 min, prior to immersion in PBS three times. Aliquots of 50–100  $\mu$ l ready-to-use goat serum were added dropwise and the cells were incubated at room temperature for 20 min. Subsequently, 50–100  $\mu$ l primary rabbit anti-CK19 antibody (1:200 dilution) was added. The cells were incubated in a wet box for 2 h at 37°C and were subsequently immersed in PBS three times. Subsequently, 50–100  $\mu$ l FITC tetramethylrhodamine (1:200 dilution) secondary antibody was added, and the cells were incubated in the dark for 1 h at 37°C, prior to immersion in PBS three times. Aliquots of 50–100  $\mu$ l formulated dye, 4',6-diamidino-2-phenylindole, was added to each slice, which was subsequently placed in the dark at room temperature for 5 min. The expression of the proteins was



observed under a fluorescent microscope (BX51TF; Olympus Corporation, Tokyo, Japan), and images were captured at three areas where high levels of expression had occurred.

**Statistical analysis.** The data are presented as the mean  $\pm$  standard deviation of three independent experiments. Statistical analyses were conducted using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was determined using analysis of variance.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*TPL induces cell apoptosis in the Taxol-resistant A549/Taxol human lung adenocarcinoma cell line in a dose- and time-dependent manner.* Initially, following the establishment of the Taxol-resistant human lung adenocarcinoma cell line, A549/Taxol, the cells were treated with increasing concentrations of TPL (0.03, 0.3 or 3  $\mu\text{mol/l}$ ) for 2, 4, 6 or 12 h, and the negative control group was established. Subsequently, the apoptotic cells were stained using annexin V-FITC/PI double staining and detected using flow cytometry ( $\lambda_{\text{ex}}$ , 488 nm;  $\lambda_{\text{em}}$ , 530 nm). The experimental results are shown in Fig. 1. TPL promoted A549/Taxol cell apoptosis. The cells in the upper right quadrant were annexin V-FITC and PI double positive, denoting the quantity of late apoptotic or necrotic cells present. The lower right quadrant shows the cells, which were annexin V-FITC positive (the early apoptotic cells). The proportion of apoptotic cells increased with an increase of the concentration of TPL at different treatment durations (Figs. 1 and 2). On exposure to 3  $\mu\text{M}$  TPL for 2, 4, 6 and 12 h, the extent of cell apoptosis observed markedly increased. The inhibitory effect reached a maximum with 3  $\mu\text{M}$  TPL at the 12 h time point (cell apoptotic rate, 65.33%), whereas the apoptotic rate of the control group was 7.23% at 12 h. In addition, the cell apoptotic rate increased with an increase in the treatment time at an identical concentration (Fig. 2). These results confirmed that TPL may induce cell apoptosis in the A549/Taxol lung adenocarcinoma Taxol-resistant strain, and this effect occurred in a dose- and time-dependent manner.

*TPL induces cell cycle arrest at the synthesis (S) phase in A549/Taxol cells.* The present study next investigated whether the apoptosis induced by TPL was associated with the cell cycle. The A549/Taxol cells were treated with different concentrations of TPL (0.03, 0.3 or 3  $\mu\text{mol/l}$ ) for 2, 4, 6 and 12 h, and a negative control group was established. The PI staining method was used for the detection of the cell cycle. A total of two groups of experimental data were analyzed, the first with 3  $\mu\text{M}$  TPL treatment for 2, 4, 6 and 12 h, and the second with 0, 0.03, 0.3 and 3  $\mu\text{M}$  TPL treatment for 12 h (Fig. 3). When the A549/Taxol cells were treated with 3  $\mu\text{M}$  TPL for 2, 4, 6 and 12 h, the percentage of cells in the G1 phase decreased from 45.07 to 32.74%, and the percentage of cells in the S phase increased from 39.63 to 47.41%. The cells in the G2 phase increased, although a linear trend was not observed. With the second experimental group, where the cells were treated with 0, 0.03, 0.3 and 3  $\mu\text{M}$  TPL for 12 h, the percentage of cells in the G1 phase decreased from 48.24 to 32.74%, whereas the percentage of cells in the S phase increased

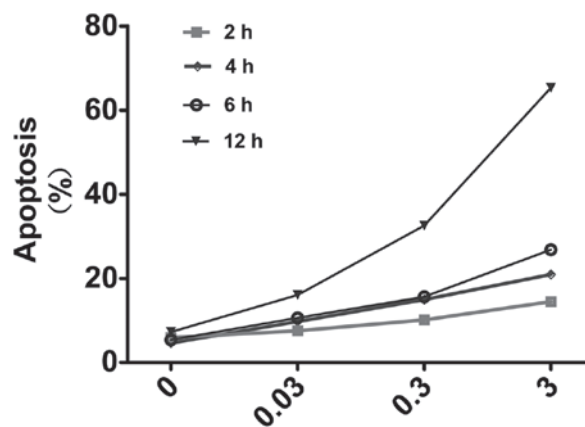


Figure 2. TPL induces A549/Taxol cell apoptosis. In the 2, 4, 6 and 12 h groups, the curve revealed that the apoptotic rate increased with an increasing concentration of TPL in every group in a dose-dependent manner. Particularly in the 12 h group, the extent of apoptosis increased the most with an increasing concentration. In addition, the apoptotic rate increased with an increasing duration at an identical concentration, thereby indicating the time-dependent response. In the 3  $\mu\text{M}$  TPL group, apoptosis increased the most with increasing duration and this was statistically significant compared with the control group ( $P < 0.05$ ). TPL, triptolide.

from 38.76 to 49.31% when the dose was increased from 0 to 0.3  $\mu\text{M}$ . This effect was not evident at 3  $\mu\text{M}$  TPL, possibly due to the occurrence of a small level of apoptosis in the A549/Taxol cells. These data revealed that a dose- and time-dependent association existed between the A549/Taxol cells and their treatment with TPL, with TPL inducing the arrest of the A549/Taxol cell cycle in the S phase, followed by the promotion of cell apoptosis.

*TPL inhibits NF- $\kappa\text{B}$  nuclear transfer and protein expression in the A549/Taxol cells.* Cell apoptosis may be regulated by the NF- $\kappa\text{B}$  signaling pathway. Whether NF- $\kappa\text{B}$  was inhibited by TPL in the A549/Taxol cells was subsequently investigated. On treatment of the A549/Taxol cells with 3  $\mu\text{M}$  TPL for 12 h, images were captured using immunofluorescence analysis, and inhibition of NF- $\kappa\text{B}$  nuclear transfer was observed. In the images, the negative control registered 46,230.33 $\pm$ 8,407.43 intensity data, and the TPL 3  $\mu\text{M}$  group registered a reading of 13,106.33 $\pm$ 680.65 units (Fig. 4). These data indicated that the expression levels of NF- $\kappa\text{B}$  were inhibited. Furthermore, the p65 protein was extracted from the TPL 3  $\mu\text{M}$  treatment and the control groups and analyzed using western blotting (Fig. 5), in order to compare the expression level. The protein bands were quantified, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference protein for standardization. The ratio of p65/GAPDH was 0.36 in the negative control group, whereas it was 0.17 in the treatment group, suggesting that the expression of p65 in A549/Taxol cells was inhibited.

*TPL inhibits the expression of FLIP, XIAP, Bcl-2, Bcl-xL and cyclo-oxygenase 2 (COX-2) regulated by NF- $\kappa\text{B}$ .* FLIP, XIAP, Bcl-2, Bcl-xL and COX-2 are a number of drug-resistant genes, which are regulated by NF- $\kappa\text{B}$ . The protein expression levels in the A549/Taxol cells treated with TPL (3  $\mu\text{M}$ , 12 h) were assessed by western blotting. To compare the protein

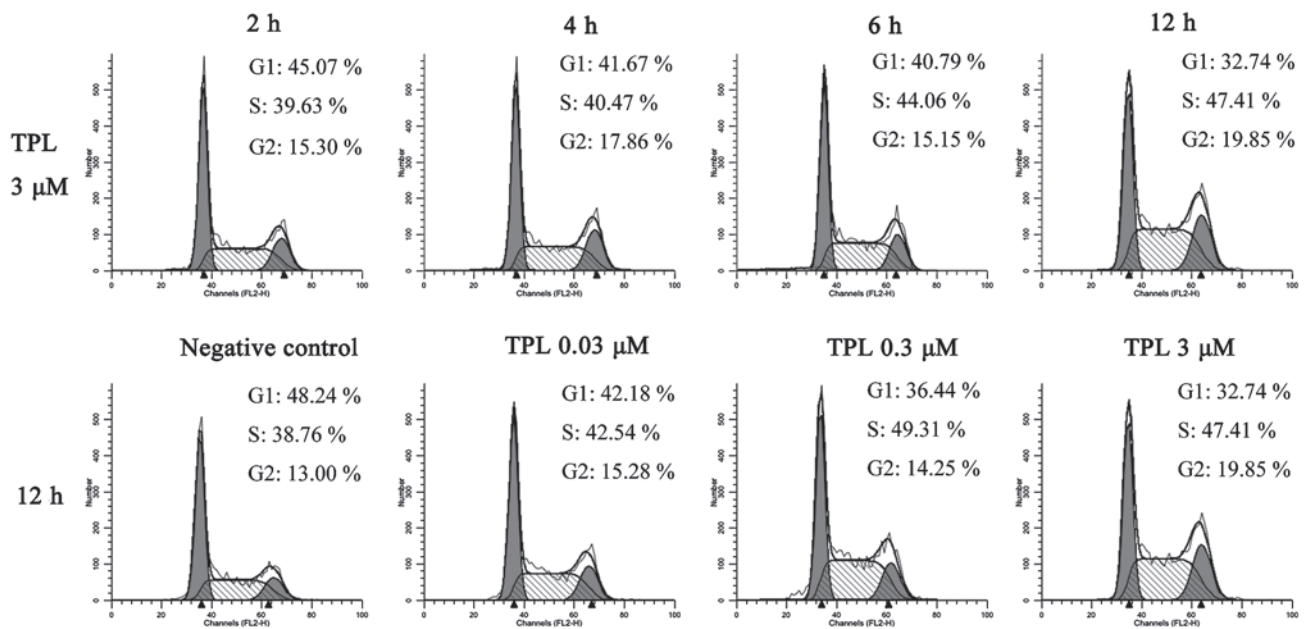


Figure 3. TPL induces cell cycle arrest in the S phase in A549/Taxol cells in a dose- and time-dependent manner. Following treatment with 3  $\mu$ M TPL for 2, 4, 6 and 12 h, the G1 phase decreased from 45.07 to 32.74%, and the S phase increased from 39.63 to 47.41%. Treatment with different concentrations of TPL (0, 0.03, 0.3 and 3  $\mu$ M) for 12 h decreased G1 phase from 48.24 to 32.74%, and the S phase increased from 38.76 to 49.31% when the dose was increased to 0.3  $\mu$ M. This was not evident at 3  $\mu$ M TPL and the S phase measurement of 47.41% was lower compared with the data for 0.3  $\mu$ M TPL treatment, possibly due to the occurrence of a small level of apoptosis. TPL, triptolide.

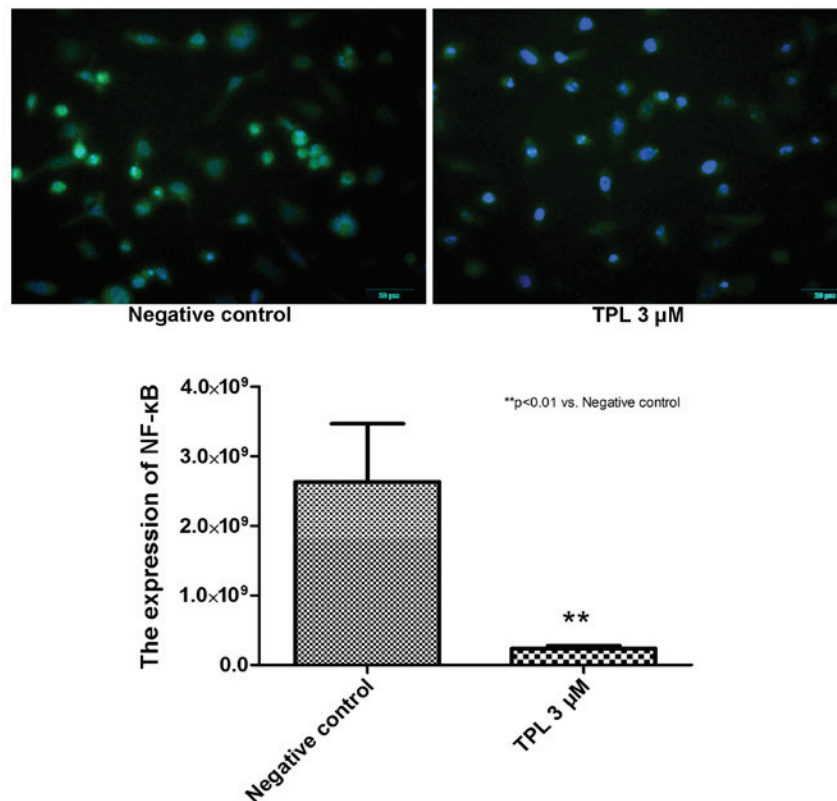


Figure 4. TPL inhibits nuclear factor- $\kappa$ B nuclear transfer in A549/Taxol cells. In the images, the area data are the mean  $\pm$  standard deviation ( $n=3$ ), \*\* $P<0.01$  compared with the control group. Negative control= $46,230.33 \pm 8,407.43$ ; TPL 3  $\mu$ M group= $13,106.33 \pm 680.65$  intensity data. TPL, triptolide.

expression levels, GAPDH was used as a standard internal reference protein. The inhibition of the protein expression levels occurred to differing degrees (Fig. 6), although the

inhibition of Bcl-2 was the most marked. The ratio of the intensity of the Bcl-2/GAPDH protein bands was 0.04 in the treatment group, compared with 0.20 in the control group.

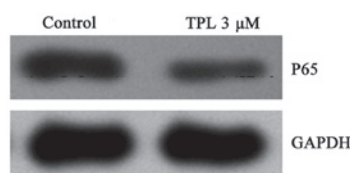


Figure 5. Protein expression of p65 was determined following treatment with TPL (3  $\mu$ M, 12 h). GAPDH was used as the internal loading control. The ratio of the intensity of p65/GAPDH was 0.36 in the control group and in the 3  $\mu$ M TPL treatment group, the p65/GAPDH band ratio was 0.17. Therefore, treatment with TPL (3  $\mu$ M, 12 h) reduced the expression of NF- $\kappa$ B in the A549/Taxol cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TPL, triptolide.

These experiments confirmed that TPL inhibits the expression of multidrug resistance-associated genes regulated by NF- $\kappa$ B, including FLIP, XIAP, Bcl-2, Bcl-xL and COX-2.

## Discussion

Paclitaxel (or Taxol<sup>®</sup>) is the current first-line treatment for non-small cell lung cancer chemotherapy (13). It exerts its antitumor effects predominantly through the induction of mitotic arrest. Previous studies revealed that intratumoral concentrations of Taxol caused cell death due to chromosomal mis-segregation on multipolar spindles (14), however, Taxol resistance is an important factor, which affects the effectiveness of the chemotherapy (15).

*In vitro* and animal experiments revealed that the administration of TPL markedly inhibits growth in a variety of solid tumor types (5,16). In the present study, it was initially confirmed that TPL exerts a clear resistance-reversal effect on the A549/Taxol lung adenocarcinoma Taxol-resistant cell line. Subsequently, the levels of apoptosis and the cell cycle, which are the two major regulatory mechanisms associated with cell death, were monitored. The A549/Taxol cells were treated with different concentrations of TPL for different durations and apoptosis was subsequently detected using flow cytometric analysis. The results of these experiments revealed that the apoptotic rate of the A549/Taxol cells increased concomitantly with the concentration or the treatment duration. When the A549/Taxol cells were incubated with 3  $\mu$ M TPL for 12 h, the level of apoptosis occurring was determined to be 65.33%, whereas it was 7.23% in the negative control group. These results suggested that TPL promoted A549/Taxol cell apoptosis, exerting a drug-resistant reversal effect, and that the effect was dose- and time-dependent. The cell cycle was assessed using PI staining and it was observed that the cells were arrested in the S phase in the TPL treatment group. With an increase in the concentration or the treatment time, TPL reduced the percentage of A549/Taxol cells in the G1 phase, increased the proportion of cells in the S phase, and a greater number of cells were arrested in the S phase. These results suggested that the cells arrested in the G1 phase would return to the G0 phase or re-differentiate, and that the cells would die by apoptosis if they were arrested at any checkpoint, with the exception of the G1 phase (17). In the present study, the proportion of cells in the S phase increased in the treatment group. These results supported the hypothesis that TPL induced A549/Taxol cell death by

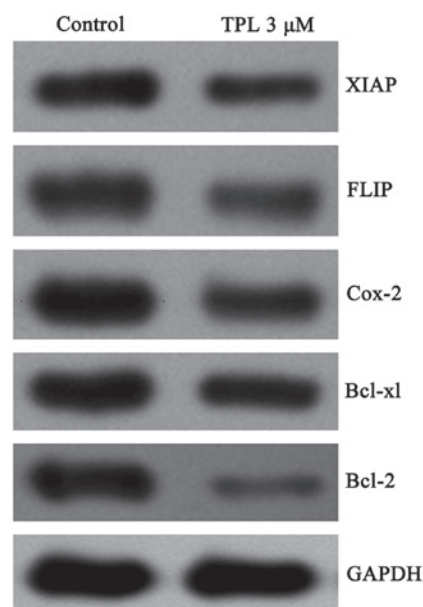


Figure 6. TPL inhibits the expression of multidrug-resistance-associated genes regulated by NF- $\kappa$ B. The A549/Taxol cells were treated with 3  $\mu$ M TPL for 12 h and the effects of TPL on the protein expression levels of FLIP, XIAP, Bcl-2, Bcl-xL and Cox-2 were analyzed. Bcl-2 was revealed to be the most markedly inhibited protein. Cox-2, cyclo-oxygenase 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TPL, triptolide; XIAP, X-linked inhibitor of apoptosis protein.

arresting the cell cycle, and suggested that TPL exerts a marked drug-resistant reversal effect on the A549/Taxol cell line.

The antitumor activity of chemotherapeutic drugs is mediated through a direct induction of DNA damage and cell death by apoptosis, a process which also requires NF- $\kappa$ B (18,19). The present study proposed that TPL-induced A549/Taxol apoptosis may be associated with the NF- $\kappa$ B signaling pathway. The transcription factor NF- $\kappa$ B is an antiapoptotic transcription factor, which has an important role in cell survival signaling. The interaction between I $\kappa$ B and NF- $\kappa$ B is balanced in the cytoplasm, where they form a dimer. NF- $\kappa$ B may be activated by a variety of signals, including pro-inflammatory and stress factors, which cause phosphorylation of the I $\kappa$ B inhibitory proteins by the I $\kappa$ B-kinase complex (20). NF- $\kappa$ B-activated transcription induces the expression of several antiapoptotic proteins, including the Bcl-2 family members, and the inhibition of the NF- $\kappa$ B signaling pathway promotes apoptosis (21). p65 is an important constituent subunit of NF- $\kappa$ B (22). In the present study, western blotting was used to detect the protein expression levels of p65 in the TPL treatment and the negative control groups. When the protein levels were compared between the two groups, the ratio of p65/GAPDH was determined to be 0.17 for the TPL treatment group (3  $\mu$ M, 12 h), whereas in the control group, the ratio for p65/GAPDH was 0.36. These results indicated that the expression of NF- $\kappa$ B in the experimental group of the A549/Taxol cells was inhibited. By immunofluorescence, the results of the present study revealed that NF- $\kappa$ B nuclear transfer in the A549/Taxol cell line was inhibited in the TPL treatment group (3  $\mu$ M, 12 h). These results suggested that the NF- $\kappa$ B



signaling pathway was inhibited during the A549/Taxol resistance-reversal process elicited by TPL treatment.

FLIP and XIAP are caspase inhibitors, which inhibit the expression of caspase, prompting the survival of tumor cells, and subsequently causing resistance of the tumors to develop (23). The antiapoptotic protein, Bcl-2, interacts with the pro-apoptotic protein, Bax, in tumor cells. Apoptosis may be reduced by upregulating the expression of Bcl-2 or downregulating the expression of Bax, and COX-2 exerts a role in tumor development and progression by promoting cell proliferation, inhibiting apoptosis, promoting angiogenesis and inhibiting the immune function mechanism (24). In the present study, it was hypothesized that the drug-resistant genes mediated by NF- $\kappa$ B, including FLIP, XIAP, Bcl-2, Bcl-xL and COX-2, are associated with the occurrence of tumor drug resistance. The protein levels of these drug-resistant genes were compared in the treatment and the negative control groups using western blot analysis. It was revealed that these resistance genes were inhibited to different degrees, with Bcl-2 being inhibited the most markedly. The interaction between the Bcl-2 and Bax proteins is crucial in determining whether the cells survive or die. Therefore, the onset of apoptosis is associated with the inhibition of the expression of the antiapoptotic protein, Bcl-2, and an increase in the expression of the proapoptotic protein, Bax. In the present study, it was revealed that TPL markedly inhibited the expression of Bcl-2 and other drug-resistant proteins, including FLIP, XIAP, Bcl-xL and COX-2, although to differing degrees. These results indicated that the expression of NF- $\kappa$ B-regulated drug-resistant genes was suppressed in the resistance-reversal process elicited upon treatment with TPL.

In conclusion, the present study confirmed that TPL may induce cellular apoptosis in the Taxol-resistant A549/Taxol lung adenocarcinoma cell line, indicating that TPL exerts a reverse effect on the Taxol-resistant lung adenocarcinoma in a dose- and time-dependent manner. The inhibition of the NF- $\kappa$ B signaling pathway, and in particular, the inhibition of expression of the NF- $\kappa$ B-regulated drug-resistant-associated genes, provided the predominant molecular mechanism to account for the TPL resistant-reversal effect. Therefore, these results indicated that TPL may be used in the treatment of non-small cell lung cancer as a drug-resistant reversal agent, in combination with paclitaxel. However, further research in this field is required.

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