

# Molecular mechanism of trifluoperazine induces apoptosis in human A549 lung adenocarcinoma cell lines

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**Abstract.** The mechanism by which trifluoperazine (TFP) induces apoptosis and inhibits growth in human A549 lung adenocarcinoma cells has not been entirely elucidated. In the present study, we investigated the anticancer mechanism of TFP *in vitro* using the human A549 lung adenocarcinoma cell line. The results indicate that TFP significantly inhibited the proliferation of A549 cells in a dose- and time-dependent manner by inducing apoptosis. Apoptotic progression in A549 cells was associated with the disruption of actin microfilaments. Moreover, the anti-apoptotic Bcl-2 protein and F-actin were down-regulated by TFP treatment, while Bax protein levels were enhanced and the phosphorylation levels of ERK and JNK proteins were increased. The data provide a potential mechanism for the chemopreventive activity of calmodulin antagonist, and suggest that TFP may have therapeutic potential for the treatment of human lung cancer.

## Introduction

Lung cancer is a major cause of death among patients with malignant diseases and a worldwide health care problem. Non-small cell lung carcinoma (NSCLC) represents 80% of all cases of lung cancer and is often diagnosed at an advanced stage (1). Current treatment options for lung cancer include

surgery, chemotherapy and radiation therapy. Surgery is recommended, but complete surgical resection is not always possible as most patients present with advanced disease (3). Hence, the therapeutic treatment of advanced NSCLC requires approaches that aim to improve survival and optimize palliative care. The development of chemotherapeutic agents to maximize antitumor activity and minimize toxicity is therefore the prime target for NSCLC management.

Apoptosis is a specialized form of cellular suicide that may be activated during the development or remodeling of tissues, or be caused by genotoxic or cytotoxic stress. It plays an essential role in a wide spectrum of biological functions including growth, morphogenesis, tissue homeostasis, immunity, and tumor regression (4). Members of the Bcl-2 family of proteins play important roles in the regulation of apoptosis. The effects of Bcl-2, Bcl-xL and Bax proteins on the regulation of tumor cell apoptosis induced by chemotherapy have recently been investigated (5-7). It has been suggested that in numerous human cancer cell lines, expression levels of Bcl-2 and Bcl-xL demonstrate a positive correlation with the prevention of apoptosis induced by various cytotoxic drugs (8). In addition, over-expression of Bax protein sensitizes cancer cells to several chemotherapy agents (8). This suggests that the modulation of intrinsic targets such as Bcl-2 family proteins is a potential strategy for the development of anticancer agents.

An intracellular signaling pathway that was recently implicated in the regulation of apoptosis is the mitogen activated protein kinase (MAPK) cascade (9,10). The MAPK family comprises serine/threonine kinases and consists of three subtypes, including extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38MAPK. ERK is mainly activated by growth factors, and is associated with cell proliferation, differentiation and cell death (9-12). In contrast, JNK is characterized by its ability to respond to environmental stresses, pro-inflammatory cytokines, and mitogens. One of the most remarkable features of JNK activation is its mediation of apoptosis in response to the withdrawal of a variety of survival and trophic factors. Interference with the JNK pathway leads to the suppression of the induction of apoptosis by various agents (13,14). However, the molecular mechanisms underlying the roles played by ERK and JNK are unclear.

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**Abbreviations:** DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen activated protein kinase; NSCLC, non-small cell lung carcinoma; OD, optical density; PBS, phosphate buffered saline; PI, propidium iodide; TFP, trifluoperazine

**Key words:** trifluoperazine, apoptosis, lung adenocarcinoma cell line, actin cytoskeleton, Bcl-2 family, MAPK family

The actin cytoskeleton is not only involved in maintaining the integrity of cell architecture, but also in signal transduction regulating cell growth, motility, survival, and death (15). It has also been demonstrated that the actin cytoskeleton provides an essential link for mediating signal transduction between the intracellular and extracellular compartments (16). In addition, the actin cytoskeleton has been implicated in the process of apoptotic cell death (17). Cytoskeletal structures, in particular the actin cytoskeleton and its associated proteins, evidently play a key role in morphological changes to the cell surface and cytoplasm during the apoptotic process. The disruption of cellular actin, actin depolymerization, or the inhibition of actin polymerization may occur concomitantly with apoptosis (18,19), suggesting that it is either a morphological consequence of the apoptotic process or is directly involved in apoptosis induction (17).

Trifluoperazine (TFP) is a phenothiazine derivative and a commonly used antipsychotic drug. The clinical potency of phenothiazines may be related to a blockade of the dopamine receptor. In addition to its therapeutic effects, TFP is also widely used as a calmodulin antagonist, binding to calmodulin and interfering with  $\text{Ca}^{2+}$ -calmodulin interactions, thereby blocking  $\text{Ca}^{2+}$ -calmodulin dependent cellular events. Calmodulin is a  $\text{Ca}^{2+}$ -binding protein that is ubiquitously expressed in almost all eukaryotic cells and plays a key role in mediating diverse  $\text{Ca}^{2+}$ -dependent physiological responses, including the regulation of cell proliferation. It has been reported that, as a calmodulin antagonist, TFP inhibited cell proliferation and invasion and induced apoptosis in several types of cancer cells (20-23). Moreover, TFP has been demonstrated to enhance bleomycin-induced apoptotic cell death (20) and to inhibit the kinase activity of DNA-PK and cellular DNA repair (24,25).

Although there have been extensive investigations to elucidate the action of TFP as a calmodulin antagonist, the exact mechanisms by which it inhibits cancer cell growth and induces apoptosis have yet to be thoroughly clarified. For example, little information is available regarding the effects of TFP on cytoskeletal organization and other apoptosis-related proteins in A549 adenocarcinoma cells. In the present study, we examined the apoptotic, morphological and functional effects of TFP on the A549 human lung adenocarcinoma cell line. We further investigated the underlying mechanisms of TFP activity, with special emphasis on its role in the modulation of Bcl-2 family proteins and the phosphorylation levels of ERK and JNK. In addition, we demonstrated that TFP accelerates the disruption of the architecture of actin microfilaments in conjunction with the induction of apoptosis in A549 cells. These findings have implications for the chemopreventive activities of TFP.

## Materials and methods

**Reagents.** Trifluoperazine (TFP), Hoechst 33258, cytochalasin B, propidium iodide (PI), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and FITC-phalloidin were purchased from Sigma Chemicals (St. Louis, MO, USA). Annexin V was purchased from BD Pharmingen (Minneapolis, MN, USA). Fetal bovine serum (FBS), RPMI-1640, trypsin-EDTA and penicillin-streptomycin were obtained from Gibco-BRL (Gaithersburg, MD, USA). Cell culture plastics

were purchased from Corning Inc. (Corning, NY, USA). Anti-ERK, anti-ERK phospho-specific, anti-JNK, anti-JNK phospho-specific, anti-Bcl-2 and anti-Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Cell culture.** Human lung carcinoma A549 cells were cultured in RPMI-1640 medium supplemented with 0.22% sodium bicarbonate, 10% FBS, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin, and were incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . Curcumin was dissolved in dimethylsulfoxide (DMSO) and was diluted to the required concentration with RPMI-1640 medium immediately before use. Cells grown in medium containing an equivalent final volume of DMSO (final concentration  $<0.01\%$ , v/v) served as the control.

**MTT assays.** The effects of TFP on A549 cell growth were determined. In brief, cells were seeded in 96-well plates ( $5-8 \times 10^3$  cells/well) using RPMI-1640 medium with 10% FBS for attachment. Cells were serum starved in serum-free medium overnight, then treated with various concentrations of TFP for 12-72 h. Following treatment, the medium was aspirated from the wells and 100  $\mu\text{l}$  fresh serum-free medium containing MTT (0.5 mg/ml) reagent was added to each well. Cells were incubated for 4 h at  $37^{\circ}\text{C}$  and lysed by the addition of 100  $\mu\text{l}$  DMSO, then the optical density (OD) at 595 and 655 nm was measured on a microplate reader. The OD values of DMSO read at 595 and 655 nm served as the blank control. Each experiment was performed in triplicate and repeated three times. The following formula was used: percentage of cell viability = (OD of the experiment samples/OD of the control)  $\times 100$ .

**Analysis of apoptosis by Hoechst 33258 staining.** Cells undergoing apoptosis show significant morphological changes in nuclear chromatin, which can be revealed by Hoechst 33258 staining. In this assay, cells were seeded on coverslips in a 6-well plate and treated with 5-20  $\mu\text{M}$  TFP. After 48 h, the cover glasses were washed carefully with phosphate buffered saline (PBS) and stained with 20 mg/ml Hoechst 33258 for 10 min, then the cells were washed in PBS and examined using a confocal fluorescence microscope.

**Analysis of apoptosis by Annexin V and propidium iodide staining.** A549 cells were treated with various concentrations of TFP for 12, 24, 48 or 72 h, or with 5 mg/ml cytochalasin B for 4 h. Cells were harvested and the percentage of cells showing apoptosis was measured by flow cytometry after staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V (5:100, v/v) and PI (5  $\mu\text{g}/\text{ml}$ ) as described by the manufacturer. Viable cells were considered to be those negative for Annexin V and PI. The percentage of apoptosis was calculated based on the increase in the number of Annexin V-positive cells between the treated and control cells.

**Immunofluorescence analysis of actin microfilament organization.** Cells were fixed by the addition of an equal volume of 3.7% formaldehyde directly to the culture flasks. After 1 h of storage at  $4^{\circ}\text{C}$ , the medium was removed after centrifugation at 200 g, and the cells were resuspended in 1 ml 3.7% formaldehyde and stored at  $4^{\circ}\text{C}$ . At the time of evaluation, the cells were

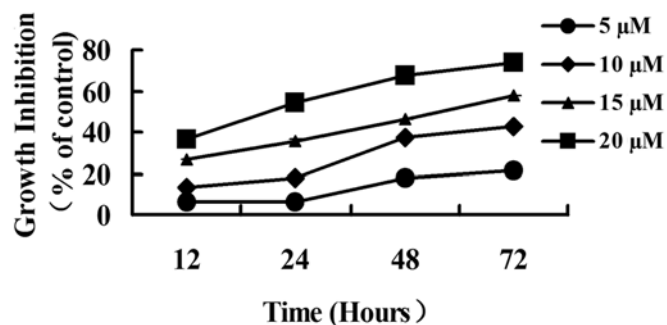


Figure 1. Inhibition of A549 cell growth by trifluoperazine (TFP). The A549 cell line was treated with various concentrations of TFP (5–20  $\mu$ M) for 12–72 h. Dose- and time-dependent suppression of cell growth was observed in the A549 cells. Data represent optical density measurements at 570 nm. Values are the means  $\pm$  SD of three independent experiments.

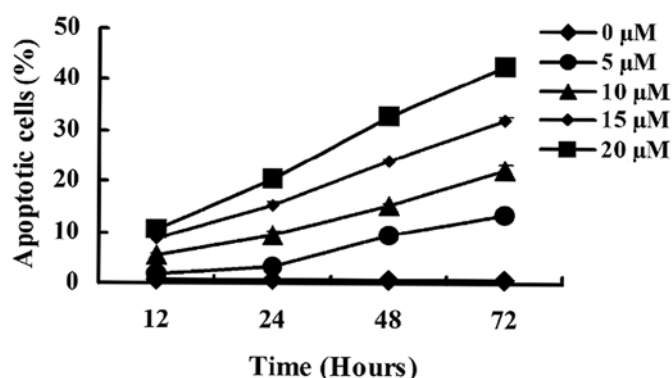


Figure 2. Analysis of apoptosis by Annexin V and propidium iodide (PI) staining. A549 cells were cultured in medium containing 0.1% DMSO or trifluoperazine (TFP) at different concentrations (5–20  $\mu$ M). After 48 h, cell apoptosis was measured at the single-cell level by labeling cells with Annexin V-FITC and counterstaining with PI. The curve chart shows the percentage of apoptosis after treatment with different concentrations of TFP (5–20  $\mu$ M) at different time points (12–72 h).

washed once in PBS containing 0.1% Triton X-100, washed again in PBS and treated with 0.1% sodium borohydride in PBS, pH 8, for 30 min to reduce autofluorescence. F-actin was stained for 1 h by 50  $\mu$ g/ml FITC-phalloidin. Post-staining, the cells were washed twice in PBS prior to evaluation. Wet preparations of stained cells were examined using a Zeiss confocal laser scanning microscope. Ten sections of each preparation were scanned. Photomicrographs were taken with Fujichrome 64 T type film.

**Detection of F-actin content.** Cells were fixed with 4% paraformaldehyde for 20 min at 4°C, rinsed with PBS and permeabilized with 0.1% Triton X-100 for 3 min. After washing in PBS, cells were stained with FITC-phalloidin for 20 min at room temperature. Fluorochrome was excited at a wavelength of 488 nm, and its emission recorded at 525 nm. Stained samples were analyzed and F-actin levels were obtained by flow cytometry.

**Western blot analysis.** Cells were lysed in lysis buffer (50 mM TrisCl, pH 7.8, 150 mM NaCl, 1% NP 40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). The lysates were resolved

on SDS-PAGE gels, and then transferred to polyvinylidene difluoride membranes. Membranes were blocked in Tris-buffered solution (50 mM TrisHCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk for 2 h at room temperature, and then incubated overnight at 4°C with specific primary antibodies including anti-human ERK, JNK, Bcl-2, Bax, phospho-ERK and JNK. Membranes were washed and incubated with horseradish peroxidase conjugated secondary antibodies. After being washed 3–4 times, membranes were incubated with Renaissance chemiluminescence reagents. Chemiluminescent signals were imaged on X-ray films.

**Statistical analysis.** Data are presented as the means  $\pm$  SD of experiments conducted three times in triplicate. The Student's t-test was used to determine the significance of statistical differences between data at a level of  $P < 0.05$ .

## Results

**Trifluoperazine inhibited A549 cell growth.** To examine the effects of TFP on the viability of A549 cells, the MTT assay was applied. The results show that TFP decreased the viability of A549 cells in a dose- and time-dependent manner (Fig. 1). Compared to the controls, 24 h of treatment with TFP at  $\geq 15$   $\mu$ M caused significant inhibition of cell viability, whereas TFP at 5–10  $\mu$ M did not cause growth inhibition until cells were treated for 48 h. Significant effects of TFP were observed after 72 h of treatment, and the highest degree of growth inhibition was found at a concentration of 20  $\mu$ M of TFP (73.58% decrease versus the control).

**Trifluoperazine induced the apoptosis of A549 cells.** To further confirm whether TFP induces the inhibition of A549 cell proliferation via apoptosis, we detected apoptosis by PI and Annexin V staining. After treatment with various concentrations of TFP for 12–72 h, ~43% of the cells underwent apoptosis (Annexin V+/PI-). The data indicate that TFP increased the percentage of cells undergoing apoptosis (Annexin V+/PI-) in a time- and dose-dependent manner (Fig. 2), suggesting that growth inhibition induced by TFP is the result of apoptosis. We also examined cell morphologic characteristics by Hoechst 33258 staining. In the untreated group, the nuclei were stained a less bright blue and the color was homogeneous. After treatment with 20  $\mu$ M TFP for 48 h or 5  $\mu$ g/ml cytochalasin B for 4 h, the blue emission light was much brighter in apoptotic cells than in control cells. Condensed chromatin and nuclear shrinkage could also be found in the TFP-treated cells, and in some the formation of apoptotic bodies was observed, which is one of the classic characteristics of apoptotic cells (Fig. 3). These observations indicate that the mode of TFP-induced cell death was predominantly an apoptotic reaction.

**Effect of trifluoperazine on the organization of actin microfilaments.** Because the early breakdown of actin microfilaments has been reported to be a prerequisite for cell-shape alterations and cell death in mammalian cells, we investigated the effect of TFP on the organization of actin microfilaments in A549 lung cancer cells. As depicted in Fig. 4, staining of the cells with FITC-labeled phalloidin revealed that DMSO-treated control cells exhibited a well-defined F-actin network mainly

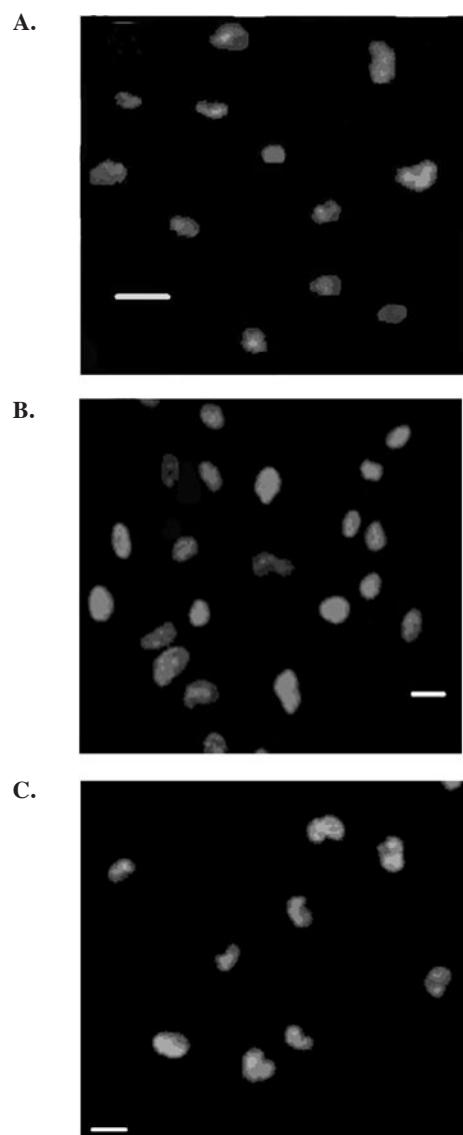


Figure 3. Hoechst 33258 fluorescent staining detection of apoptotic morphology in A549 cells treated with 20  $\mu$ M trifluoperazine (TFP) for 48 h. (A) Untreated cells; (B) cells treated with 20  $\mu$ M TFP; (C) cells treated with 5  $\mu$ g/ml cytochalasin B for 4 h.

organized into stress fibers. (Fig. 4A). Exposure of the cells to 5-20  $\mu$ M of TFP resulted in the disruption of the actin microfilament network. The time course of cytoskeletal modifications, examined by fixing cells at various time intervals after TFP treatment, revealed that distinct changes occurred in the architecture of the actin microfilaments. Cells with large numbers of F-actin dots and/or patches appeared under the plasma membrane. In some cells, labeling showed bright fluorescent rings at their periphery, whereas in others F-actin was distributed in a punctuate pattern throughout the cytoplasm or was localized in one region of the cell. In addition, cells showing diffuse distribution of F-actin were evenly distributed under the whole surface of the plasma membrane. These results clearly indicate that TFP induced the reorganization of actin microfilament architecture in A549 lung carcinoma cells (Fig. 4B). When treated with cytochalasin B, an actin network disrupting drug, cells had a more diffuse F-actin staining, with aggregates of F-actin and a diminished number of fibers

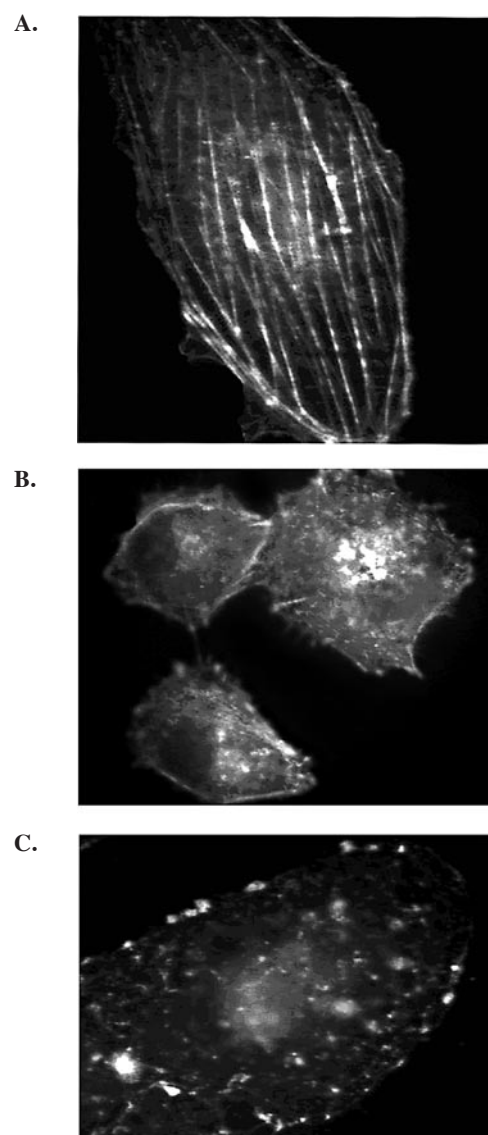


Figure 4. Effect of trifluoperazine (TFP) on the organization of actin cytoskeleton. Labeling of F-actin in A549 cells following exposure to vehicle or to 5-20  $\mu$ M TFP for 24 h. (A) DMSO-treated control cells exhibited a well-defined F-actin network that was mainly organized into stress fibers. (B) A549 cells treated with 20  $\mu$ M TFP. The disruption of F-actin and its loss from the cytoplasm were shown as the supplement of TFP. (C) A549 cells treated with 5  $\mu$ g/ml cytochalasin B showing altered organization and distribution of F-actin, with most of the actins dispersed near the cell surface.

(Fig. 4C). To provide a better quantitative approach to detect the changes in cellular F-actin, cells were stained with FITC-phalloidin and fluorescence was measured by flow cytometric analysis. Our results indicate that TFP significantly decreased F-actin content in a dose and time-dependent manner. Maximum effect was at a concentration of 20  $\mu$ M TFP for 24 h (Fig. 5), and was correlated with the disruption of the actin microfilaments.

*Effects of trifluoperazine on apoptosis-related proteins in A549 cells.* Several gene products are known to play a role in controlling the apoptotic process. To determine whether TFP has an effect on the expression levels of these gene products, a similar time-course experiment as that described above was performed, and cellular lysates were analysed by Western



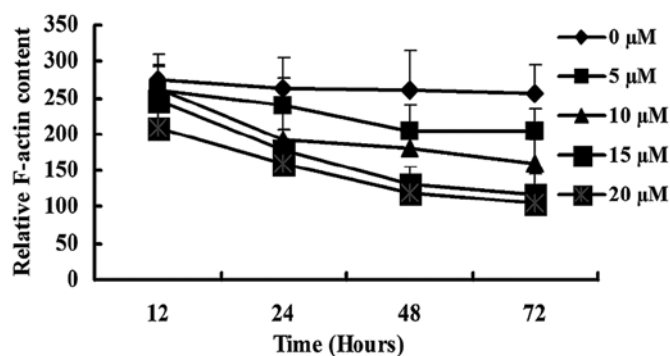


Figure 5. Effect of trifluoperazine (TFP) on the content of F-actin in A549 cells. Quantitative analysis of F-actin content was carried out by flow cytometry. Values are the means  $\pm$  SD of three independent experiments.

blotting (Fig. 6). The results indicate that TFP induced a significant increase in Bax protein and decrease in Bcl-2 protein in a dose-dependent manner (Fig. 6A), which may have led to apoptosis (Fig. 2). As shown in Fig. 6B, TFP also caused an increase in the phosphorylation levels of ERK and JNK, with little change in total ERK and JNK proteins in A549 cells. These data show that Bcl-2 family proteins and the ERK and JNK signaling pathways are involved in TFP-triggered apoptosis in A549 cells.

## Discussion

In the present study, the data indicate that trifluoperazine (TFP) induced an irreversible inhibitory effect on the proliferation of A549 human lung cancer cells. Cells exposed to various concentrations of TFP were shown to lose their ability to proliferate after 12-48 h of treatment. In addition to its irreversible effect on cell growth, an observed decrease in cell number was also apparently caused by cell apoptosis. Our findings support the results of previous studies (20-23), indicating that TFP induced marked apoptosis in A549 cells. The induction of apoptotic death was accompanied by characteristic morphological changes, such as nuclear condensation and the formation of apoptotic bodies (Fig. 3), which were confirmed by PI and Annexin V staining. These results indicate that TFP induced apoptosis in A549 cells in a time- and dose-dependent manner.

The regulation of apoptosis is a complex process involving a number of cellular genes, including Bcl-2 (26) and Bcl-2-related family members such as Bcl-xL, Bcl-xS, Bad and Bax (27). The Bcl-2 gene product protects cells against apoptosis in a variety of experimental systems. Suppression of Bcl-2 has been shown to promote apoptosis in response to a number of stimuli, including anticancer drugs (26). Bcl-2 and Bcl-xL exert their anti-apoptotic effect, at least in part, by binding to Bax and related pro-apoptotic proteins. They also prevent Bax and pro-apoptotic proteins from inducing the release of cytochrome c and the activation of caspase-9. In our results, the levels of anti-apoptotic factor Bcl-2 were decreased after TFP treatment, while pro-apoptotic factor Bax displayed the opposite trend. Most notably, the ratios of Bcl-2/Bax were decreased significantly after TFP treatment (data not shown). Therefore, proteins from the Bcl-2 family might act as down-

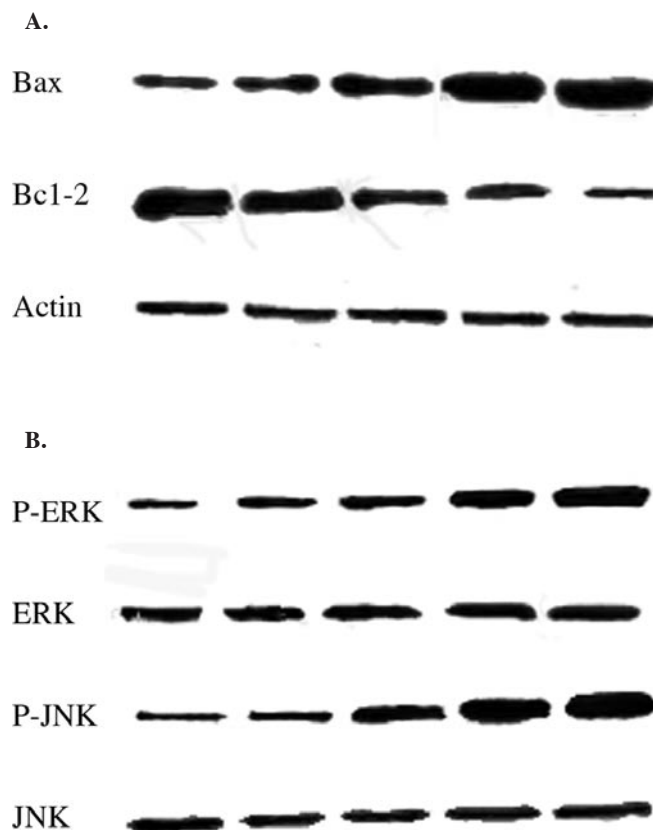


Figure 6. Effects of trifluoperazine (TFP) on apoptosis-related proteins in A549 cells. (A) Alteration of Bcl-2 and Bax expression by TFP treatment. A549 cells were treated with increasing concentrations of TFP (5-20  $\mu$ M) for 48 h, and the presence of Bcl-2 and Bax was analyzed by Western blotting. (B) Western blot analysis of the effects of TFP on phosphorylated ERK and JNK levels in A549 cells.

stream signal carriers in the process of TFP-induced apoptosis in the A549 cell line.

It has been demonstrated that the ERK signaling pathway is activated in response to certain cellular stresses (28). To investigate whether TFP induced growth arrest and apoptosis in A549 cells in association with the activation of ERK, cell lysate from cells treated with TFP at different concentrations for 24 h were subjected to Western blot analysis using an anti-phospho-ERK antibody to detect the phosphorylated ERK. The same blots were subsequently stripped and reblotted with an antibody that recognized total ERK to verify equal amounts of the protein in various samples. As shown in Fig. 6B, treatment of A549 cells with 5, 10, 15 and 20  $\mu$ M of TFP, all of which induced apoptosis, led to a dose-dependent activation of phosphorylated ERK. As JNK activation is thought to be a determining factor in cell cycle arrest and apoptosis (29), we set out to determine whether TFP-induced apoptosis in A549 lung cancer cells was also associated with the activation of the JNK/SAPK pathway. The activation of JNK was determined by examining its degree of phosphorylation by Western blot analysis with anti-phospho-JNK antibody. Fig. 6B shows that TFP significantly enhanced the phosphorylation of JNK with little change in total JNK proteins in A549 cells. These results suggest that ERK and JNK are mediators of TFP-induced apoptosis.

Actin filaments are integral components of the plasma membrane-associated cytoskeleton, and are believed to play an important role in the determination of cell polarity, shape, and membrane mechanical properties (30). Several lines of evidence support a causal relationship between actin filament disruption and apoptosis (19). In certain cell types, every cell with a fragmented nucleus indicative of apoptosis also displays complete disorganized F-actin (19). However, Olins *et al* concluded that cytoskeletal reorganization is independent of apoptosis (31). Here, it was found that actin filaments of cells that were not treated with TFP exhibited a well-defined F-actin network that was mainly organized into stress fibers. F-actin stress fibres in the cytoplasm were oriented more or less parallel to the long axis of the cell and distributed through the cell and at the cell periphery. By contrast, the actin fibers of TFP-treated cells were disorganized, disassembled, or disrupted. When treated with cytochalasin B, cells had a more diffuse F-actin staining, with aggregates of F-actin and a diminished number of fibers.

Additionally, fluorescent quantification of changes in cellular F-actin showed that TFP significantly decreased F-actin content in a dose and time-dependent manner, with maximum effect at a concentration of 20  $\mu$ M TFP for 72 h. A similar phenomenon was observed when the cells were exposed to cytochalasin B (5 mg/ml) for 4 h (data not shown). Decreases in F-actin content were accompanied by visible changes in the distribution and depolymerization of the microfilament network. Previous reports (32,33) have shown that specific cancer chemopreventive agents induced apoptosis, evidenced by the visible loss of FITC-phalloidin fluorescence staining in A549 cells. This is consistent with our confocal microscopic data.

In conclusion, the present study revealed that TFP significantly inhibited the proliferation of A549 cells in a dose- and time-dependent manner by inducing apoptosis, and that the progression of apoptosis was associated with the disruption of actin microfilaments. We also demonstrated that Bcl-2 protein and F-actin levels are down-regulated by TFP treatment, together with the enhancement of Bax protein and the phosphorylation levels of ERK and JNK. The ability of TFP to induce apoptosis in human lung cancer and other cancer cells suggests that TFP could be developed as a universal cancer chemoprevention and chemotherapeutic agent.

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