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ORIGINAL RESEARCH PAPER

# Flavokawain B induces apoptosis of non-small cell lung cancer H460 cells via Bax-initiated mitochondrial and JNK pathway

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**Abstract** Flavokawain B (FKB) possesses strong anti-neoplastic activity against many cancer cells. Here we assessed its antitumor activity and molecular mechanisms in lung cancer H460 cells in vitro. FKB significantly inhibited cell proliferation and caused arrest of the cell cycle G2-M of H460 cells in a dose-dependent manner. FKB also inducted apoptosis, which was associated with cytochrome *c* release, caspase-7 and caspase-9 activation and Bcl-xL/Bax dys-regulation. FKB significantly down-regulated survivin and XIAP, and the inhibitory effect induced by FKB was greatly attenuated by through over-expression of survivin or $Bax^{-/-}$ MEFs. Furthermore, FKB activated the mitogen-activated protein kinases and the JNK inhibitor SP600125 significantly decreased the growth-inhibitory and apoptotic effects of FKB. Together, these results suggest the anti-lung cancer potential of flavokawain B for the prevention and treatment of lung cancer.

**Keywords** Apoptosis · Bax · Flavokawain B · JNK · Lung cancer · Survivin

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## Introduction

Lung cancer has a high incidence and mortality. There has been little improvement in lung cancer treatments over the past decades. Hence, effective drugs, including novel naturally occurring or chemically-synthesized compounds, are urgently needed to control the malignant progression of lung cancer. In particular, natural and dietary compounds have been used for the treatment of cancer throughout history due to their safety, low toxicity, and general availability and many active phytochemicals are in human clinical trials. The anticancer potential of some phytotherapeutics has been extensively studied in various cancers.

Kava (*Piper methysticum*) is an ancient crop of the western Pacific islands and it has been used as a medicine, social drink, and sacred plant in religious ceremonies. Epidemiologic studies showed that several countries in the South Pacific islands, such as Fiji, Vanuatu and Western Samoa, have very low cancer incidence rates including lung cancer and prostate cancer despite a high portion of smokers in these populations. The components of kava extract can be classified into two types: kavalactones and flavokawains (FK) or chalcones. Chalcones have been described to represent cancer chemopreventive food components that are rich in fruits and vegetables and the kava chalcones contain primarily FKA, FKB, and FKC (Tabudravu and Jaspars 2005). Our previous studies have shown that flavokawain A (FKA) induced apoptosis in bladder cancer cells and selectively caused a $G_2$-M arrest in p53-defective cells (Zi and Simoneau 2005; Tang et al. 2008). Also, flavokawain B (FKB), isolated from kava extracts has stronger anticancer potential in prostate cancer and oral adenoid cystic cancer (Tang et al. 2010; Zhao et al. 2011).

In this study, we demonstrate that FKB is implicated in causing significant growth inhibition and apoptosis in non-small cell lung cancer H460 cells. We found that FKB induced apoptosis through a Bax-initiated mitochondrial pathway in H460 cells. Moreover, FKB down-regulated the expression of Survivin and activated major MAPK signaling. Taken together, this study suggests that FKB may be useful for the prevention and treatment of lung cancer.

## Materials and methods

### Cell culture and compounds

The human lung cancer cell line NSCLC H460 was obtained from State Key Laboratory of West China Hospital Cancer Center, Sichuan University, China. Primary mouse embryo fibroblasts (MEF) deficient for Bax (double knockout) and control cells were generous gifts from Dr. Xiaolin Zi (University of California Irvine, Orange, CA). The cells were cultured in RPMI-1640 medium containing 10 % (v/v) FCS in humidified air with 5 % (v/v) $CO_2$ at 37 °C. Pure flavokawain B was synthesized, dissolved in dimethyl sulfoxide (DMSO), and stored at −80 °C. The JNK inhibitor SP600125 was purchased from Calbiochem.

### MTT assay

The MTT assay was used to determine cell proliferation. Cells were grown in 24-well plates. Drugs were added for 48 h; the medium was then replaced with 0.5 ml of 1 mg MTT/ml per well and incubated for 3 h. The $OD_{570}$ was determined using a microplate reader. The ratio of viability ( %) was calculated as $OD_{sample}/OD_{control} \times 100$ %.

### Cell morphology observation and DAPI staining

H460 cells were treated with 0.1 % DMSO or 44 μM FKB. After 24 h, cells were observed using an inverted microscope. For 4,6-diamino-2-phenyl indole (DAPI) staining, after 24 h of treatment, as above, cells were fixed with 4 % (v/v) paraformaldehyde for 30 min and stained with DAPI for 5 min. Finally, they were observed under a fluorescence microscope.

### Fluorescence-activated cell sorting analysis of cell cycle distribution

After 24 h treatment with FKB, H460 cells were fixed with 70 % (v/v) alcohol, and then washed with PBS. Cells were stained with propidium iodide for 30 min. Approx. 10,000 cells per sample were analyzed by flow cytometry and the percentages of cells in the G1, S, and G2-M phases of the cell cycle were determined using the ModFIT software, as described previously (Tang et al. 2008).

### Measurement of cytochrome *c* release from mitochondria

Cells were treated with 0.1 % DMSO or 44 μM FKB for 24 h. Mitochondria and cytosol were separated using a cytochrome *c*-releasing apoptosis assay kit. Cells were suspended in cytosol extraction buffer. The cell suspension in extraction buffer was homogenized using a Dounce homogenizer and centrifuged (700×*g*, 10 min) after a 10 min on ice. Then, the collected supernatant was re-centrifuged (10,000×*g*, 30 min, 4 °C). The resulting supernatant (cytosolic fraction) and pellet (mitochondrial fraction) were processed for Western blot analysis.

### Western blot analysis

Total proteins were isolated with RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 mM EDTA, and 1 % protease inhibitor cocktail (v/v). Total protein (40–80 μg) was resolved on SDS-PAGE gels and transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies at 4 °C overnight and incubated with horseradish peroxidase-conjugated secondary antibody for 1–2 h. Finally, target protein signals were detected using enhanced chemiluminescence according to the manufacturer's protocol. Antibodies against $\beta$-actin, Bax, survivin, XIAP, PARP, Caspase-9, Caspase-7, Bcl-2, Bcl-xL, cytochrome *c*, phosphor-JNK, phosphor-c-Jun, phosphor-P38 and phosphor-P44/42 were from Cell Signaling. SuperSignal West Pico Chemiluminescent detection reagents were obtained from Thermo Scientific.

### Plasmid transfection

Plasmids *pcDNA3.1* and *pcDNA3.1/Survivin* were generous gifts from Dr. Xiaolin Zi (University of California Irvine, Orange, CA). Plasmids were transfected using the Lipofectamine 2000 reagent. H460 cells were seeded in 24-well plates. After 24 h, the cells were transiently transfected with *pcDNA3.1* and *pcDNA3.1/Survivin*. After 48 h transfection, the cells were treated with FKB 48 h later.

### Statistical analysis

Comparisons of OD values for cell viabilities and cell cycle population between treatment and control were conducted using Student's *t* test. All statistical tests were two-sided. *P* values <0.05 were considered to indicate statistical significance.

## Results

### FKB inhibited cell proliferation and induced apoptosis in H460 cells

First, we assessed the effects of FKB on the proliferation of H460 cells. Figure 1a showed the chemical structure of FKB, a bioactive chalcone. As shown in Fig. 1b, FKB inhibited growth of H460 cells, with an $IC_{50}$ value 18.2 μM ($P < 0.05$). A dose of 87.9 μM inhibited growth by about 90 % compared with the 0.1 % DMSO-treated control ($P < 0.05$).

To examine whether the cell growth inhibitory effect of FKB was induced via perturbation of cell cycle progression, we performed fluorescence-activated cell sorting (FACS) analysis of control (0.1 % DMSO) and FKB-treated H460 cells. Figure 1c shows that the percentage of G2-M population were increased from about 8 % for control to 13–28 % for 8.8, 17.6 and 44 μM FKB, respectively, after 24 h of treatment. (Student's *t* test, $P < 0.05$).

To investigate whether the growth inhibitory effect was mediated through the induction of apoptosis, we examined the apoptotic morphology of control and FKB-treated H460 cells by light and fluorescence microscopy. Figure 1d showed the typical morphological changes of apoptosis in FKB-treated cells, the cells became rounded and detached from the plate. DAPI staining showed chromatin condensation and fragmentation in FKB-treated cells and these characteristics were not detected in the 0.1 % DMSO-treated control cells. Together, these data indicated that FKB inhibited the cell proliferation via inducing G2-M cell cycle arrest and induced apoptosis in H460 cells.

### FKB treatment resulted in cytochrome *c* release and activated the cleavage of PARP, caspase-7, and caspase-9 in H460 cells

To explore whether the apoptotic effect of FKB was through release of cytochrome *c* and activation of a cascade of caspases, the cleavage of caspase-9, caspase-7, and PARP was detected by Western blotting. Treatment of H460 cells with FKB caused cleavage of pro-caspase-9 into active caspase-9 in a dose-dependent manner. Additionally, activation of caspase-7 and the cleaved product of poly(ADP-ribose) polymerase (PARP) were also detected in a similar dose-dependent manner (Fig. 2a). Figure 2b clearly shows 44 μM FKB induced cytochrome *c* release into the cytosol from the mitochondria leading to apoptosis. These results suggested that the inhibitory effect of FKB might be through induction of apoptosis in mitochondrial pathways.

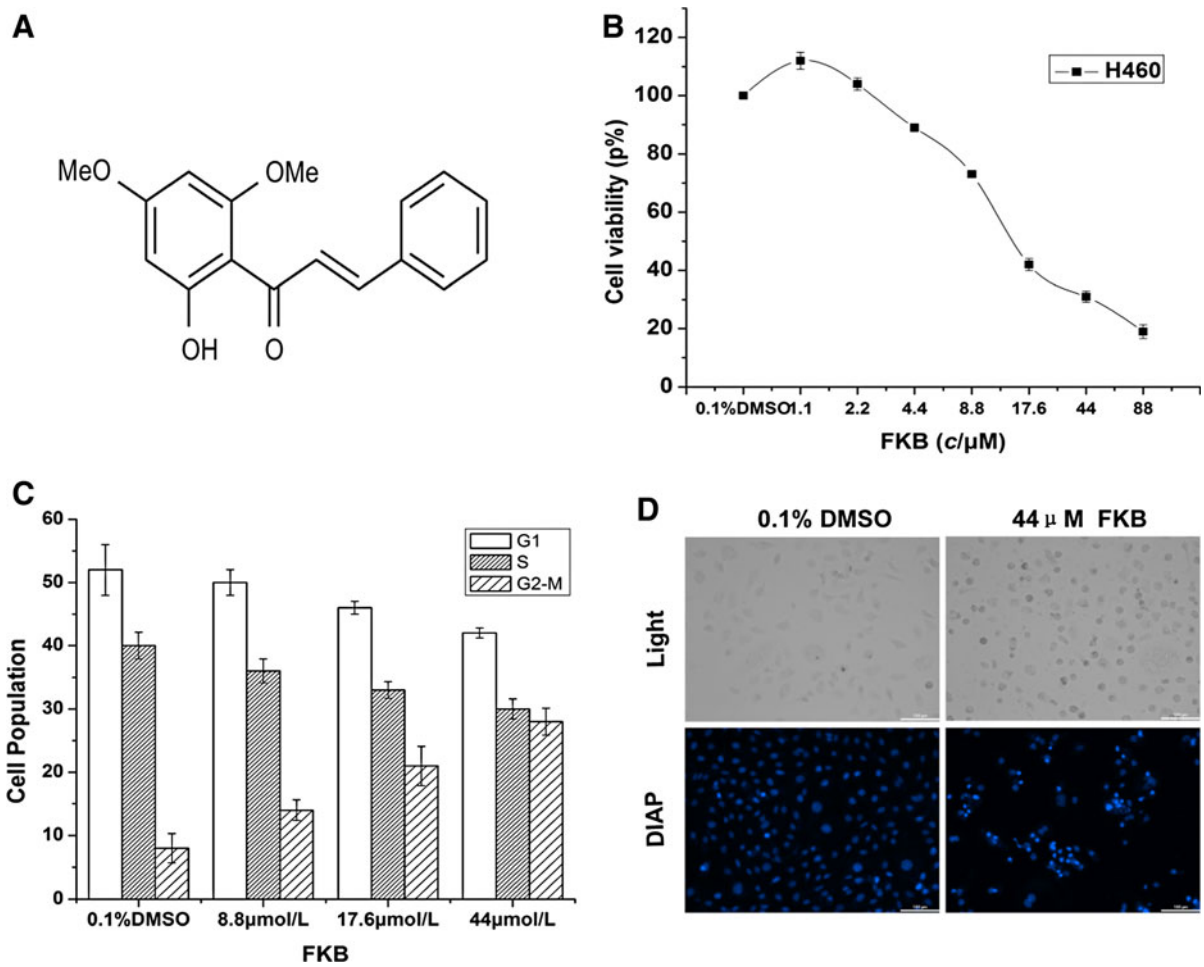


**Fig. 1** FKB causes strong anti-proliferative and apoptotic effects in human lung cancer H460 cells. **a** Cell growth inhibition of H460 cells. H460 cells were treated with 0.1 % DMSO and FKB, as indicated, for 48 h. The MTT assay was then performed to examine the growth (as % viability). Each point is the mean ± SE of four independent experiments. Each *bar* represents the mean ± SE from three independent experiments. Each sample count was performed in duplicate. **b** Live cell morphology and DAPI staining of H460 cells. *Scale bar* = 100 μm. **c** Cell cycle was analyzed by FCS. Each *bar* represents the mean ± SE from three independent experiments. **d** Live cell morphology and DAPI staining of H460 cells. *Scale bar* = 100 μm

## FKB significantly decreased the levels of survivin and X-linked inhibitor of apoptosis (XIAP) in H460 cells

Survivin and XIAP, as the inhibitor of apoptosis (IAP) protein family factors, are involved in maintaining apoptosis resistance in cells. We therefore evaluated the effects of FKB on the expression of XIAP and survivin. Compared with the 0.1 % DMSO-treated control, treatment of H460 cells with FKB significantly down-regulated both survivin and XIAP (Fig. 2a). The expressions of survivin and XIAP were significantly down-regulated by 44 μM FKB over 24 h. Survivin directly and/or indirectly interacts and thus regulates the activities of caspases (Marusawa et al. 2003; Shin et al. 2001; Dohi et al. 2004). Thus, the FKB-induced degradation of survivin may further facilitate the complete proteolytic processing of caspase-7/9.

To determine whether survivin played an important role in the growth-inhibitory and apoptotic effects of FKB, *pcDNA3.1/Survivin* and *pcDNA3.1* plasmids were transiently transfected into H460 cells. A significant difference was observed between *pcDNA3.1/*

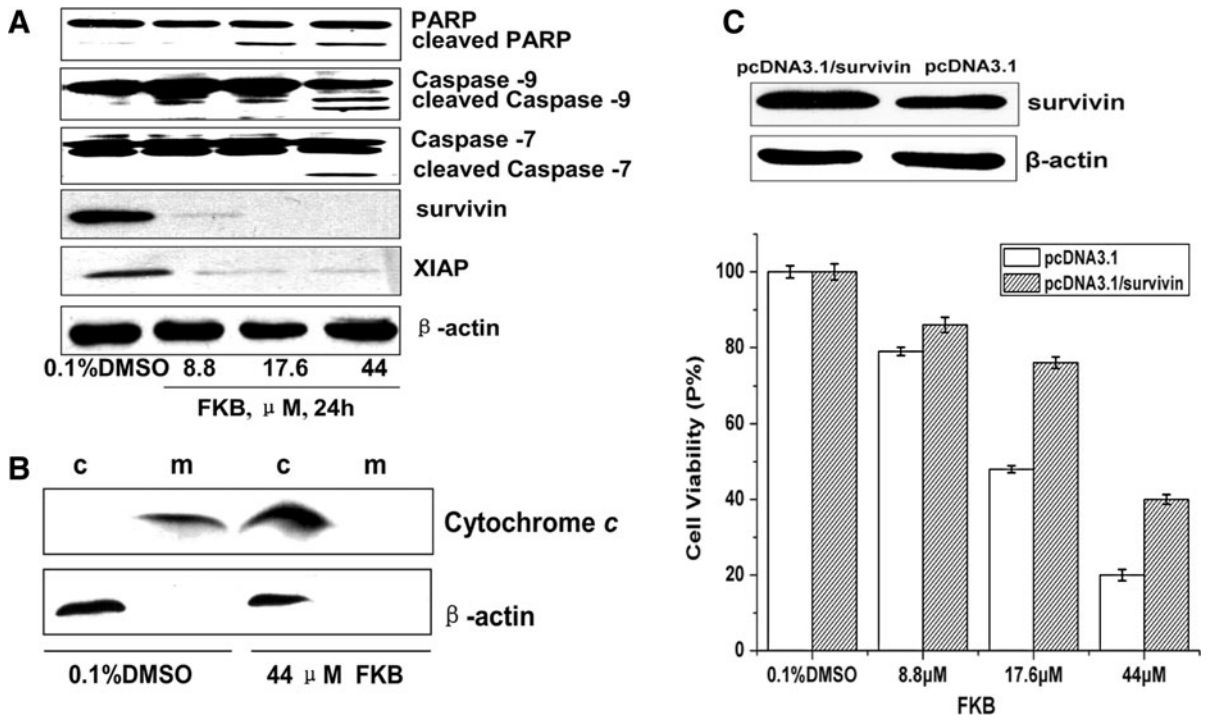


**Fig. 2** FKB treatment resulted in the activation of PARP, caspase-7, and caspase-9, and survivin may be a potential target **a** FKB treatment resulted in the cleavage of PARP, caspase-7, and caspase-9 in H460 cells. H460 were treated with 0.1 % DMSO or the indicated doses of FKB. Total proteins were prepared at the indicated time points and protein levels were analyzed by Western blotting. *β*-Actin was used as a loading control. **b** FKB treatment resulted in the release of cytochrome *c*. Mitochondria (*m*) and cytosolic (*c*) extracts from the indicated treatments were prepared and Western blots were conducted as described in the "Materials and Methods" section. Results are representative of three independent experiments. **c** Over-expression of Survivin suppressed growth inhibitory effect induced by FKB. H460 cells were transiently transfected with *pcDNA3.1* or *pcDNA3.1/Survivin* for 48 h. Top, Western blot analysis of survivin overexpression in H460 cells. Bottom, H460 cells transiently transfected with *pcDNA3.1* or *pcDNA3.1/Survivin* were treated with 0.1 % DMSO or the indicated doses of FKB for 24 h. Cell viabilities were determined by the MTT assay. Each *point* is the mean ± SE of four independent experiments. Each *bar* represents the mean ± SE from three independent experiments

*Survivin*- and *pcDNA3.1*-transfected cells in response to FKB. Compared with cells transfected with the empty *pcDNA3.1* vector, over-expression of survivin attenuated the sensitivity of H460 cells to FKB (Fig. 2c). These results provided evidence that survivin may be an important target for the apoptotic and growth inhibitory effects of FKB.

## FKB induced apoptosis of H460 cells through Bax-initiated mitochondrial pathway

Members of the Bcl-2 family can be divided into pro-apoptotic and anti-apoptotic proteins. A delicate balance exists between these members in cell and the regulation of these two groups of proteins determines whether a cell survives or undergoes apoptosis. Thus, we next examined the effects of FKB on the expression of Bcl-2 family proteins. FKB treatment resulted in significant dose-dependent down-regulation of Bcl-xL and up-regulation of Bax (Fig. 3a). These results suggest that the apoptotic effect might be mediated through raising the ratio of Bax:Bcl-xL to induce cytochrome *c* release into cytoplasm.

To determine whether Bax is, at least in part, required for the growth-inhibitory and apoptotic effects of FKB on H460 cells, we treated wild-type MEFs and $Bax^{-/-}$ MEFs with three different doses of FKB for 48 h. $Bax^{-/-}$ MEFs were more resistant to the anti-proliferative effect of FKB than wild-type MEFs (Fig. 3b). These results provide evidence that disruption of Bax almost completely impaired the effect of FKB-induced growth inhibition in MEFs, and

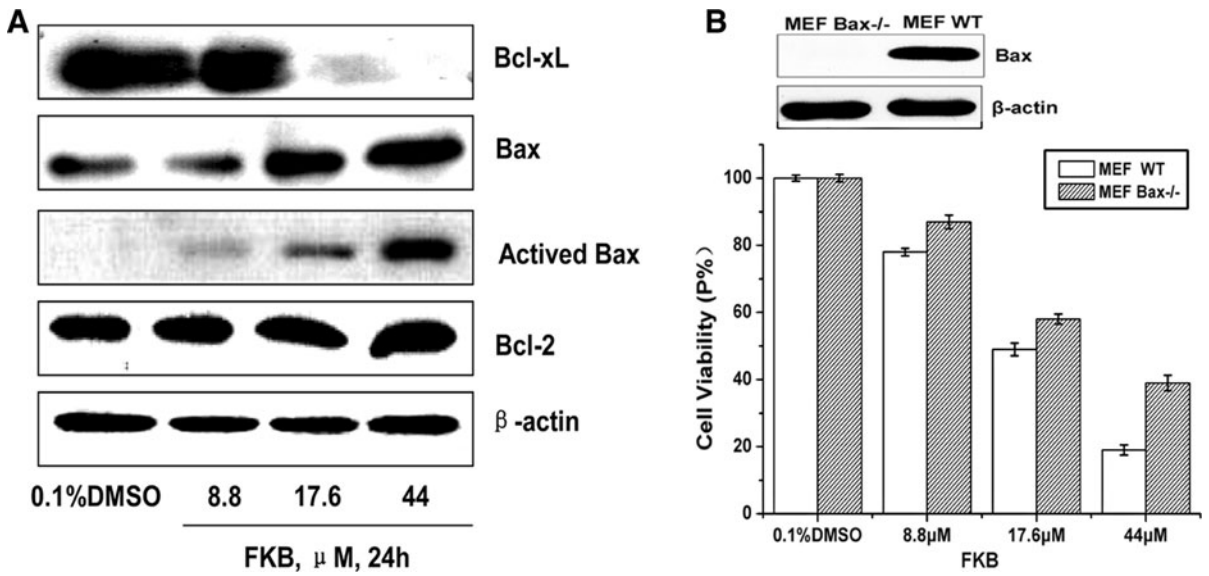


**Fig. 3** FKB induced apoptosis of H460 cells through Bax-initiated mitochondrial pathway **a** FKB activated Bax. The protein levels of Bcl-xL, Bax, actived Bax, and Bcl-2 after indicated treatments for 24 h were analyzed by western blotting. Results are representative of two independent experiments. *β*-actin was used as a loading control. **b** Bax is required in FKB-treated H460 cells. MEF WT and MEF $Bax^{-/-}$ at 80–90 % confluency were treated with 0.1 % DMSO or the indicated doses of FKB for 48 h. Cell viabilities were measured by MTT assays. Bax depletion efficiency is determined by western blotting. Points are the mean of four independent plates; bars, SE. Each sample was counted in duplicate

Bax may be a key target for the apoptotic and growth inhibitory effect of FKB. Furthermore, other pro-apoptosis Bcl-2 family members, such as Puma, Bim, and Bik, were also up-regulated after FKB treatment (data not shown). These results suggest that the inhibitory effect of FKB may be mediated through the induction of apoptosis via Bax-initiated mitochondrial and caspase-9-dependent cellular apoptotic pathways.

FKB activated the stress-responsive mitogen-activated protein kinases (MAPKs) signaling

Mitogen-activated protein kinases have been implicated in a variety of cellular processes, including gene transcription, protein biosynthesis, cell cycle control, apoptosis, and differentiation. The role of MAPKs signal transduction events induced by FKB that could contribute to apoptosis were investigated, we found that exposure of H460 cells to FKB resulted in dose-dependent activation of all three major MAPKs, JNK, p44/42, and p38. Phosphorylated JNK could translocate to the nucleus to phosphorylate and transactivate c-Jun. Treatment with FKB also resulted in activation of c-Jun (Fig. 4a). Studies using JNK inhibitors were carried out to determine whether the activation of JNK contributed to FKB-induced apoptosis in H460 cells. We therefore determined if 20 μM SP600125 could be tolerated by H460 cells (Fig. 4b). Remarkably, co-treatment with SP600125 significantly blocked FKB-induced apoptosis (Fig. 4c). The percentages of viable cells after treatment with 20 μM SP600125, 44 μM FKB, and both were about 95 %, 18 %, and 72 %, respectively ($P < 0.05$). The difference between FKB alone and FKB plus SP600125 was significant. SP600125 co-treatment also inhibited FKB-mediated activation of JNK and down-regulated FKB-mediated cleavage of PARP (Fig. 4d). These results showed that FKB-mediated PARP cleavage was JNK-dependent and JNK activation played a key role in FKB-mediated apoptosis.

## Discussion

Apoptosis is a major form of cell death, characterized by several morphological features including chromatin condensation and fragmentation, cell membrane

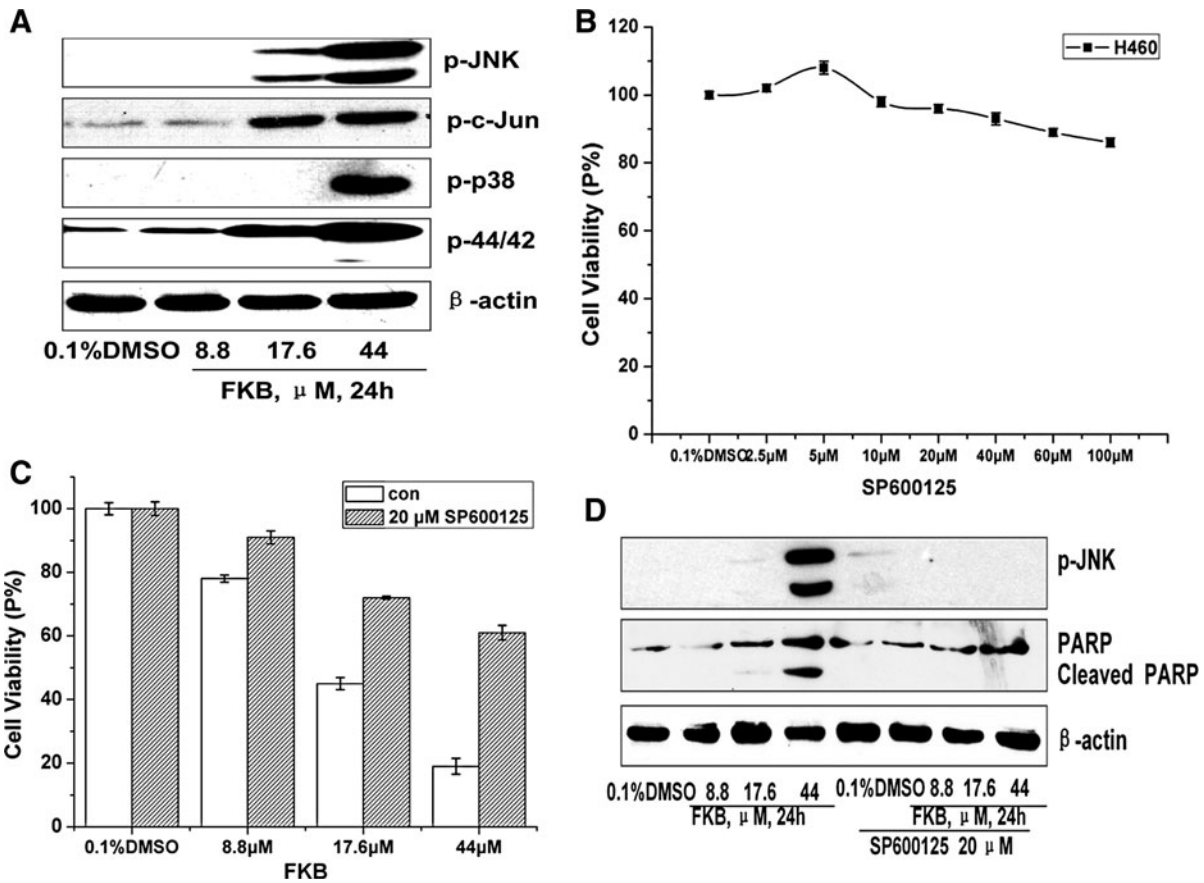


**Fig. 4** FKB activated the stress-responsive (MAPKs) signaling and JNK plays an important role in FKB-induced apoptosis **a** FKB activated MAPKs. The protein phosphorylation levels of JNK, c-Jun, p38, and p44/42 after indicated treatments for 24 h were analyzed by western blotting. $\beta$-actin was used as a loading control **b** Optimal concentration of SP600125 in H460 cells was determined. Cell viabilities were measured by MTT assays. Each point is the mean ± SE of four independent experiments. Each bar represents the mean ± SE from three independent experiments. **c**, **d** Effect of JNK inhibitor on FKB-induced apoptosis was examined. Inhibition of JNK activity by SP600125 decreased the antigrowth of FKB to H460 cells. The protein levels of p-JNK and PARP after indicated treatments for 24 h were analyzed by Western blotting. Points are the mean of four independent plates; *bars*, SE. Each sample was counted in duplicate. Results are representative of two independent experiments. $\beta$-Actin was used as a loading control

blebbing, and the formation of apoptotic bodies. These morphological changes occur via a signaling pathway that leads to the recruitment and activation of caspases, a family of cysteine-containing, aspartate-specific proteases. The Bcl-2 family acts as a decision-maker and the caspase family acts as an executor. The mitochondria membrane potential and release of cytochrome *c* are fine-tuned by the balance between Bax and Bcl-xL. An increase in the ratio of Bax:Bcl-xL stimulates the release of cytochrome *c* from mitochondria into the cytosol, where cytochrome *c* can bind to APAF-1, leading to the activation of caspase-9/7. Cleaved PARP facilitates cellular disassembly and undergoing apoptosis, and over-activation of PARP induces exhaustion of cellular energy stores and releases cytochrome *c* from mitochondria (Bressenot et al. 2009). Our results revealed that FKB could increase the ratio of Bax:Bcl-xL and induce the release of cytochrome *c* into the cytosol in H460 cells. Moreover, Bax is required for FKB-induced apoptosis and its mechanism needs further investigation.

Survivin is one of the most cancer-specific proteins identified to date. It is highly expressed in most human tumors and fetal tissue. Although there are multiple pathways involved in the survivin networks, there is a

consensus that survivin is an essential cancer gene and an appropriate target for drug discovery (Altieri 2008). Clinical studies suggests that survivin expression correlates with decreased patient survival in NSCLC and survivin may be an independent predictor, along with distant metastasis and large tumor size (Church and Talbot 2012). Hence, survivin is currently attracting considerable attention as a cancer prognostic indicator and a new target for anti-cancer therapies. Our data clearly shows that FKB as a new molecular targeting compound could significantly down-regulate the survivin protein expression and the detailed molecular mechanisms need to be elucidated further.

Mitogen-activated protein kinases (MAPKs) are a widely conserved family of serine/threonine protein kinases involved in many cellular events, such as cell proliferation, differentiation, motility, death, and the stress reaction. MAP3Ks that activate MKK4 or MMK7 can initiate JNK-mediated apoptotic pathways in a cell type- and stimuli-specific manner. Many cellular stresses and stimuli induce apoptosis and modulate MAPK signaling pathways (Stone and Chambers 2000). JNKs activate apoptotic signaling by the upregulation of pro-apoptotic genes through the transactivation of specific transcription factors (AP1, p53, and p73) or by directly modulating the activities of mitochondrial proteins (Bcl-2, Bim, Bad, and Bid) through distinct phosphorylation events (Dhanasekaran and Reddy 2008; Wagner and Nebreda 2009). Our data also showed that JNK may be required in FKB-induced apoptosis and the nuclear or mitochondrial signaling of JNK in the regulation of apoptosis by FKB needs to be investigated in the near future.

**In summary**, FKB exerts an anti-proliferative effect on H460 cells that is mediated by modulation of the ratio of Bax/Bcl-xL, induction of G2-M phase arrest, and activation of MAPKs. Over-expression of survivin and suppression of p-JNK can lead to resistance to cellular apoptosis with FKB treatment. We demonstrated that FKB can induce apoptosis of H460 cells through mitochondria-mediated intrinsic apoptosis pathway, which was modulated by JNK signaling. These results might provide a valuable insight into the use of FKB or its derivatives or in combination with chemotherapeutic agents in NSCLC patients.

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