

# Asperfuranone from *Aspergillus nidulans* Inhibits Proliferation of Human Non-Small Cell Lung Cancer A549 Cells via Blocking Cell Cycle Progression and Inducing Apoptosis

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**Abstract:** Asperfuranone, a novel compound of genomic mining in *Aspergillus nidulans*, was investigated for its anti-proliferative activity in human non-small cell lung cancer A549 cells. To identify the anti-cancer mechanism of asperfuranone, we assayed its effect on apoptosis, cell cycle distribution, and levels of p53, p21 Waf1/Cip1, Fas/APO-1 receptor and Fas ligand. Enzyme-linked immunosorbent assay showed that the G0/G1 phase arrest might be due to p53-dependent induction of p21 Waf1/Cip1. An enhancement in Fas/APO-1 and its two form ligands, membrane-bound Fas ligand (mFasL) and soluble Fas ligand (sFasL), might be responsible for the apoptotic effect induced by asperfuranone. Our study reports here for the first time that the induction of p53 and the activity of Fas/Fas ligand apoptotic system may participate in the anti-proliferative activity of asperfuranone in A549 cells.

Lung cancer is the leading cause of cancer death in the world, and non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancer cases [1,2]. NSCLC commonly develop resistance to radiation and chemotherapy, and often present at stages too late for surgical intervention, resulting in a low overall survival at 5 years of less than 15% [3]. Since current treatment modalities are inadequate, novel therapies are needed to reduce the effects of the increasing incidence in pulmonary neoplasm.

Apoptosis plays an important role in homeostasis and development of the tissue in organism [4]. Imbalance between cell proliferation and apoptotic cell death will result in serious disease such as cancer. Many studies have demonstrated that cancer treatment by chemotherapy and  $\gamma$ -irradiation kill target cells primarily by the induction of apoptosis [4,5]. Several previous publications have reported that anti-cancer agents may induce apoptosis via the Fas/FasL system [6–11]. Fas is a cell surface receptor comprising a type I integral membrane protein that expresses a cytoplasmic death domain and belongs to the tumour necrosis factor receptor superfamily [12,13]. Activation of Fas by its ligand (FasL) results in the oligomerization of its intracellular death domain and the recruitment of the intracellular adaptor Fas-associated death domain (FADD). Once bound, FADD is able to activate procaspases-8 and -10 in a death inducing

signalling complex. In turn, caspases-8 and -10 activate downstream caspases, resulting in apoptotic cell death [14,15].

Asperfuranone (fig. 1), a novel compound of genomic mining in *Aspergillus nidulans*, was investigated for its anti-proliferative activity in human non-small cell lung cancer A549 cells. Furthermore, to establish the anti-cancer mechanism of asperfuranone, we assayed the levels of p53, p21 Waf1/Cip1, Fas/APO-1 receptor, and Fas ligand, which are strongly associated with the signal transduction of apoptosis and affect the chemo-sensitivity of tumour cells to anti-cancer agents.

## Materials and Methods

**Isolation and identification of secondary metabolites.** Asperfuranone was purified as described before [16]. Briefly, *alcA(p)-afmA A. nidulans* was grown in 3 l of LMM medium ( $1 \times 10^6$  spores/ml). After 18 hr, cyclopentanone at 30 mM was added to induce *afmA* gene, a positive regulator for asperfuranone biosynthesis. After 48 hr of induction, culture medium was collected by filtration and partitioned with EtOAc twice. The mycelium which also containing asperfuranone was extracted separately with 500 ml MeOH twice, with each sonicated for 1 hr. Both extracts were pooled to get 164.3 mg of crude extract. This extract was then applied to a SiO<sub>2</sub> gel column (Merck 230–400 mesh, ASTM, 20 × 60 mm) and eluted with CHCl<sub>3</sub>–MeOH mixtures of increasing polarity (fraction A, 1:0, 200 ml; fraction B, 19:1, 200 ml; fraction C, 9:1, 200 ml; fraction D, 7:3, 200 ml). Fraction C (67.3 mg), which contained asperfuranone, was further purified by reverse phase HPLC [Phenomenex Luna 5  $\mu$ m C18 (2), 250 × 10 mm] with a flow rate of 5.0 ml/min. and measured by a UV detector at 254 nm. The gradient system was MeCN (solvent B) in 5% MeCN/H<sub>2</sub>O (solvent A) both containing 0.05% TFA: 42–45%

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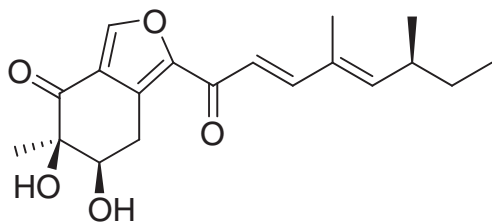


Fig. 1. Chemical structure of asperfuranone from *Aspergillus nidulans*.

B from 0–20 min., 45–100% B from 20–26 min., maintained at 100% B from 26–31 min., 100–42% B from 31–32 min., and re-equilibration with 42% B from 32–37 min. Asperfuranone (32.8 mg) was eluted at 15.0 min. The stock solution of asperfuranone was prepared at a concentration of 80 mM of dimethyl sulfoxide (DMSO). It was then stored at  $-20^{\circ}\text{C}$  until use. For all experiments, the final concentrations of the test compound were prepared by diluting the stock with DMEM. Control cultures received the carrier solvent (0.1% DMSO).

**Reagents and materials.** Foetal calf serum (FCS), penicillin G, streptomycin, amphotericin B and RPMI 1640 were obtained from GIBCO BRL (Gaithersburg, MD, USA). Dimethyl sulfoxide, ribonuclease (RNase), and propidium iodide (PI) were purchased from Sigma Chemical (St Louis, MO, USA). XTT ELISA kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany). p53 ELISA, Fas Ligand ELISA, and caspase-8 assay kits, and caspase-8 inhibitor, benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-IETD-FMK) were purchased from Calbiochem (Cambridge, MA, USA). p21 Waf1/Cip1 and Fas/APO-1 ELISA kits were purchased from Invitrogen (Camarillo, CA, USA). Anti-Fas Ab (ZB4) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA).

**Cell culture.** Human non-small cell lung carcinoma cell line, A549 (American Type Culture Collection [ATCC] CCL185), human hepatocellular carcinoma cell line, PLC/PRF/5 (ATCC CRL 8024), and human breast adenocarcinoma cell line, MDA-MD-231 cells (ATCC HTB-26) were maintained in monolayer cultures at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in RPMI 1640 supplemented with 10% FCS, 0.1 mg/ml streptomycin, and 100 units/ml penicillin (Life Technologies, Inc., Grand Island, NY, USA).

**Cell proliferation.** Inhibition of cell proliferation by asperfuranone was measured by XTT (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) assay. Briefly, cells were plated in 96-well culture plates ( $1 \times 10^4$  cells/well), and after 24 hr incubation, treated with vehicle alone (0.1% DMSO) and various concentrations of asperfuranone for 48 hr. All cell proliferation data were analysed in triplicate for different concentrations. 150  $\mu\text{l}$  XTT test solution, which was prepared by mixing 5 ml of XTT-labelling reagent with 100  $\mu\text{l}$  of electron coupling reagent, was then added to each well. After 4 hr of incubation, absorbance was measured on an ELISA reader (Multiskan EX; Labsystem Multiskan, Helsinki, Finland) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The data were expressed as a percentage of the absorbance of control (% survival). The  $\text{IC}_{50}$  value was defined as the concentration of asperfuranone causing a 50% reduction in absorbance compared with control.  $\text{IC}_{50}$  was obtained using the non-linear regression programme GraphPad Prism software fitted to the plot of % survival versus concentrations.

**Cell cycle analysis.** To determine cell cycle distribution,  $5 \times 10^5$  cells were plated in 60-mm dishes and treated vehicle alone (0.1% DMSO) and asperfuranone (15 and 20  $\mu\text{M}$ ) for 24 hr. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed

in PBS, re-suspended in 1 ml of PBS containing 1 mg/ml RNase and 50  $\mu\text{g/ml}$  propidium iodide, incubated in the dark for 30 min. at room temperature and analysed by EPICS flow cytometer. The data were analysed using the Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

**Apoptosis assay.** We provided a quantitative evaluation by using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabelling (TUNEL) method to examine DNA-strand breaks during apoptosis. Briefly, cells were incubated with vehicle alone (0.1% DMSO) and asperfuranone (15 and 20  $\mu\text{M}$ ) for the indicated times. The cells were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed, the cells were incubated with the reaction mixture provide by BD ApoAlert™ DNA Fragmentation Assay kit for 60 min. at  $37^{\circ}\text{C}$ . The stained cells were then analysed with an EPICS flow cytometer and a fluorescence microscope at  $20 \times$  magnification.

**Assaying the levels of p53, p21 Waf1/Cip1, Fas/APO-1 and Fas ligand (mFasL and sFasL).** p53 ELISA, p21 Waf1/Cip1, Fas/APO-1 ELISA and Fas Ligand ELISA kits were used to detect p53, p21 Waf1/Cip1, Fas/APO-1 receptor and soluble (sFasL)/membrane-bound Fas ligand (mFasL). Briefly, A549 cells were treated with vehicle alone (0.1% DMSO) and asperfuranone (15 and 20  $\mu\text{M}$ ) for 6, 12, 24 and 48 hr. The samples of cell lysate were placed in 96-well microtitre plates that were coated with monoclonal detective antibodies, and incubated at room temperature. Each sample was assessed in triplicate. It was necessary to determine the soluble Fas ligand in cell culture supernatant by using Fas Ligand ELISA kit. After removing the unbound material by washing with washing buffer (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20), the detector antibody that is bound by horseradish peroxidase, conjugated streptavidin, was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a coloured solution with colour intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm, and concentrations of p53, p21 Waf1/Cip1, Fas/APO-1 and FasL were determined by interpolating from standard curves obtained with known concentrations of standard proteins.

**Assay for caspase-8 activity.** The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate, Ac-IETD-pNA. The cell lysates were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) for 3 hr at  $37^{\circ}\text{C}$ . Each sample was assayed in triplicate. The release of *p*-nitroaniline was monitored at 405 nm. Results are represented as the percent change of the activity compared to the untreated control.

**Statistical analysis.** Data were expressed as mean  $\pm$  S.D. Statistical comparisons of the results were made using ANOVA. Significant differences ( $p < 0.05$ ) between the means of control and asperfuranone-treated cells were analysed by Dunnett's test.

## Results

### *Asperfuranone inhibits cell proliferation in A549 cells.*

We first tested the anti-proliferative effect of asperfuranone in human non-small-cell lung cancer A549 cells. As shown in fig. 2, the growth inhibitory effect of asperfuranone was observed in a dose-dependent manner. At 48 hr, the maximal effect on proliferation inhibition was observed with 20  $\mu\text{M}$  asperfuranone, which inhibited proliferation in 64.5% of A549 cells. The  $\text{IC}_{50}$  values were 15.3  $\mu\text{M}$ .

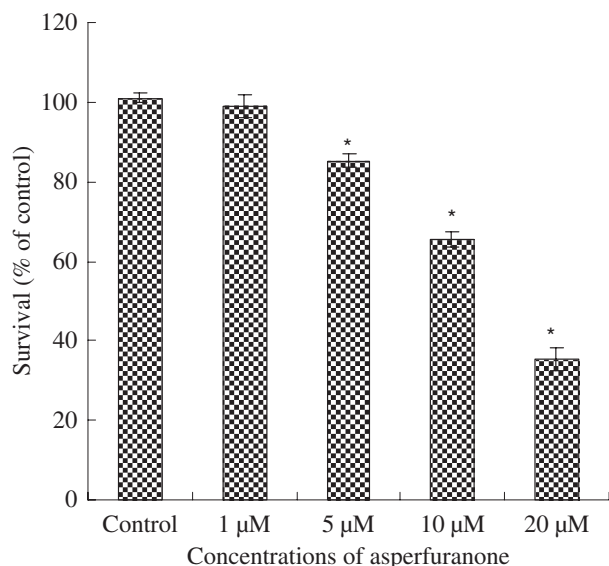


Fig. 2. The inhibitory effects of asperfuranone on cell proliferation inhibition in non-small-cell lung cancer A549 cells. Cell growth inhibition activity of asperfuranone was assessed by XTT. Results are expressed as the percentage of cell proliferation relative to the proliferation of control. The data shown are the mean from three independent experiments. Each value is the mean  $\pm$  s.d. of three determinations. The asterisk indicates a significant difference between control and asperfuranone-treated cells, as analysed by Dunnett's test ( $p < 0.05$ ).

We also assessed the effect of asperfuranone on two other human cancer cell lines: PLC/PRF/5 (liver cancer) and MDA-MB-231 (breast cancer). The results revealed that asperfuranone also inhibited the proliferation of these cancer cell lines. The  $IC_{50}$  values were 18.6 and 16.5  $\mu$ M for PLC/PRF/5 and MDA-MB-231, respectively (data not shown).

#### *Asperfuranone-induced cell cycle arrest and apoptosis in A549 cells.*

The results on the effect of asperfuranone on cell cycle progression of A549 are shown in fig. 3. As compared to the control, 15  $\mu$ M of asperfuranone increased the population of G0/G1 phase from 30.7% to 52.6%. This effect was enhanced when A549 cells were treated by 20  $\mu$ M of asperfuranone (72.3% cell population in G0/G1 phase).

We next assessed the effect of asperfuranone on the induction of apoptosis in A549 cells. A quantitative evaluation was made using TUNEL to detect DNA-strand breaks. Compared to vehicle-treated cells, asperfuranone induced 36.33% of apoptotic cells in A549 cells at concentrations of 20  $\mu$ M at 48 hr (fig. 4).

#### *Asperfuranone increases the expression of p53 and p21 Waf1/Cip1 proteins in A549 cells.*

To determine whether tumour suppression factor p53 are involved in the asperfuranone-mediated anti-proliferative effect of A549 cells, the levels of p53 protein were assayed by ELISA. In the study of p53 protein expression was

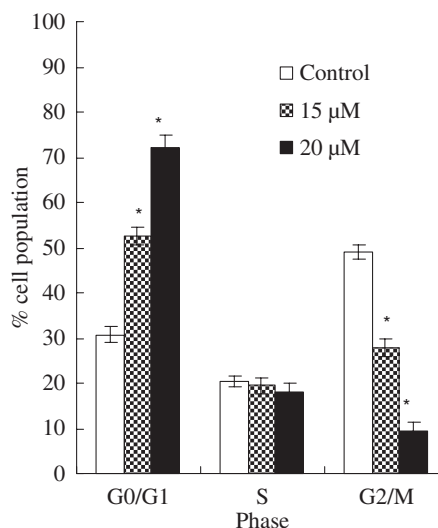


Fig. 3. The effects of asperfuranone on cell cycle distribution in A549 cells. A549 cells after treatment with vehicle alone (0.1% DMSO) and asperfuranone (15 and 20  $\mu$ M) for 24 hr were fixed and stained with propidium iodide, and cell cycle distribution was then analysed by flow cytometry. Each value is the mean  $\pm$  S.D. of three determinations. The asterisk indicates a significant difference between control and asperfuranone-treated cells, as analysed by Dunnett's test ( $p < 0.05$ ).

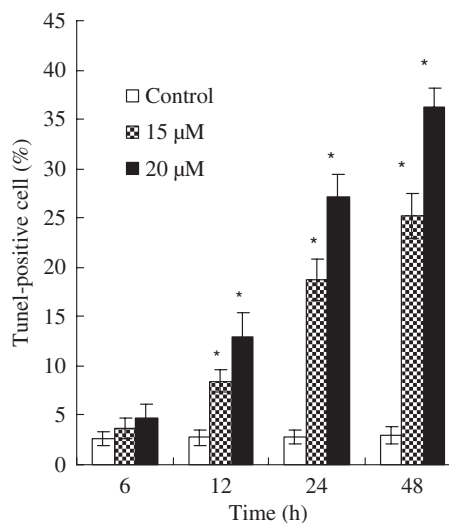


Fig. 4. Induction of apoptosis in A549 cells by asperfuranone. The effect of asperfuranone in A549 cells. A549 cells were treated with vehicle alone (0.1% DMSO) and various concentrations of asperfuranone for the indicated times, and then apoptotic cells were stained by TUNEL assay. The TUNEL-positive cells were examined by flow cytometry. Each value is the mean  $\pm$  s.d. of three determinations. The asterisk indicates a significant difference between control and asperfuranone-treated cells, as analysed by Dunnett's test ( $p < 0.05$ ).

treated with 15 and 20  $\mu$ M asperfuranone for 6, 12, 24 and 48 hr. Marked induction of p53 protein was observed in a dose-dependent manner (fig. 5A). The up-regulation of p53 by asperfuranone started to increase 6 hr after

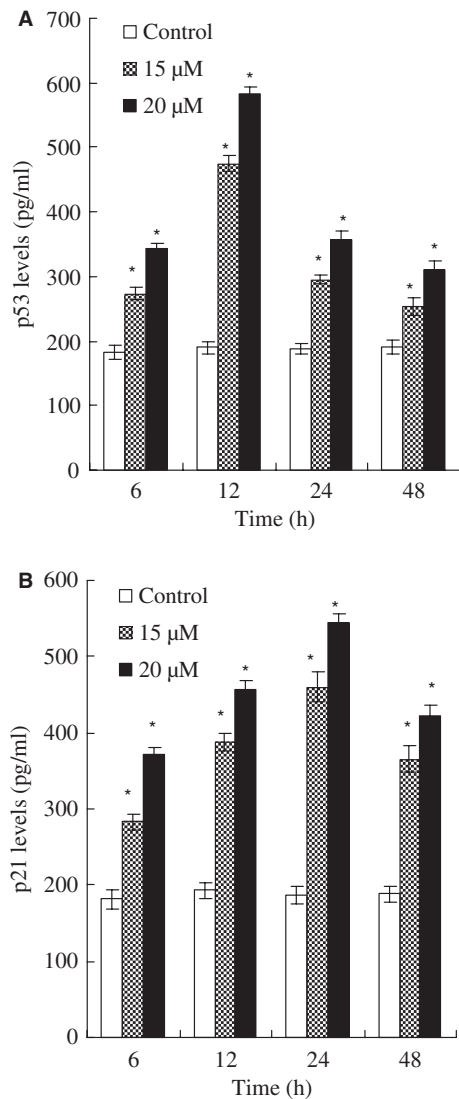


Fig. 5. Effects of asperfuranone on protein expression of p53 and p21 Waf1/Cip1. The level of p53 protein (A); the level of p21 Waf1/Cip1 (B). A549 cells were treated with vehicle alone (0.1% DMSO) and asperfuranone (15 and 20  $\mu$ M). p53 and p21 Waf1/Cip1 levels were determined by p53 and p21 Waf1/Cip1 ELISA kits, respectively. The detailed protocol is described in 'Materials and Methods'. Each value is the mean  $\pm$  S.D. of three determinations. The asterisk indicates a significant difference between control and asperfuranone-treated cells, as analysed by Dunnett's test ( $p < 0.05$ ).

treatment with asperfuranone and reached maximum expression at 12 hr.

The p21 Waf1/Cip1 protein is first characterized as a downstream target of p53 and is thought to be responsible for G0/G1 cell cycle arrest [17]. Thus, we also assessed the p21 Waf1/Cip1 expression of p53-expressing A549 cells performed with a p21 Waf1/Cip1 immunoassay. Figure 5B shows that an increase in p21 Waf1/Cip1 protein was apparent at 6 hr and reached maximum induction at 24 hr in asperfuranone-treated A549 cells. Moreover, the induction of p21 Waf1/Cip1 was in a dose-dependent manner. Based on these data, we suggest that asperfuranone-mediated cell cycle

arrest might operate through the induction of p21 Waf1/Cip1 protein on a p53-dependent event in A549 cells.

*Fas/FasL apoptotic system might be a possible pathway of asperfuranone-mediated apoptosis.*

By using Fas/APO-1 and Fas Ligand ELISA kits, we found that asperfuranone increased expression of Fas/APO-1 receptor and soluble/membrane-bound Fas ligand in A549 cells as early as 6 hr after treatment in a dose-dependent fashion (fig. 6). The maximum effect was observed after 24 hr of treatment. The time relationship between the expression of Fas/FasL at 6 hr of treatment and the occurrence of apoptosis at 12 hr of treatment could support the idea that the Fas/FasL system might mediate asperfuranone-induced apoptosis of A549 cells.

When A549 cells were pre-treated with an antagonistic anti-Fas antibody, ZB4, the anti-proliferative and pro-apoptotic effects of asperfuranone were effectively inhibited. At 20  $\mu$ M of asperfuranone, cell proliferation inhibition decreased from 64.5% to 19.8% in A549, respectively (fig. 7A). Compared to the control, the induction of apoptosis induced by 20  $\mu$ M of asperfuranone decreased from about 36.33% to 12.74% at 48 hr in ZB4 pre-treated A549 cells (fig. 7B).

We next measured the downstream caspase of Fas/FasL system. The results showed that caspase-8 activity increased at 10 hr and reached maximum induction at 48 hr in 20  $\mu$ M asperfuranone-treated A549 cells (fig. 8A). The activation of caspase-8 (at 10 hr) was before the production of DNA fragmentation (at 12 hr) showing caspase-8 activation was required in asperfuranone-induced apoptosis. To further provide this hypothesis, we assessed the role of caspase-8 on asperfuranone-mediated anti-proliferative and pro-apoptotic effects by using caspase-8 inhibitor (Z-IETD-FMK). Our results showed that inhibition of caspase-8 not only decreased the anti-proliferative activity of asperfuranone (fig. 8B), but also completely abolished induction of apoptosis in A549 cells (fig. 8C).

## Discussion

Normal p53 gene is well-known to play a crucial role in inducing apoptosis and acting as cell cycle checkpoints in human and murine cells after DNA damage [17]. p21 Waf1/Cip1 protein blocks the activities of various cyclin-dependent kinases [18–20], and inhibits the phosphorylation of retinoblastoma (RB) protein, thereby preventing the G1–S phase transition [19,21]. Previous studies have shown that p21 Waf1/Cip1 is transcriptionally regulated by p53-dependent and -independent pathways [22–24]. Our results demonstrate that p53 plays an important role in asperfuranone-induced anti-proliferative activity in A549 cells. Induction of p53 by asperfuranone can not only arrest cell cycle, but also trigger apoptosis in A549 cells. This finding is supported by the following results: first, flow cytometry assay indicated that asperfuranone arrested the cell cycle in the G0/G1 phase, which was attributed to the enhancement of p21 Waf1/Cip1 protein that might be induced by p53. Second, pro-apoptotic

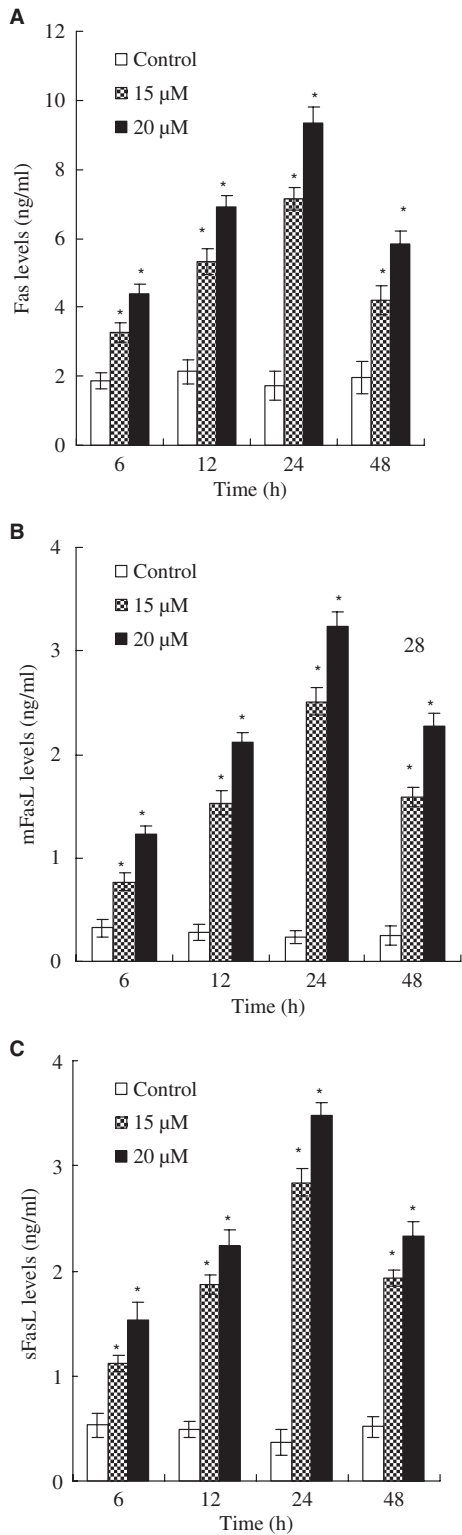


Fig. 6. Fas/FasL apoptotic system was involved in asperfuranone-mediated apoptosis. A549 cells were incubated with vehicle alone (0.1% DMSO) and asperfuranone (15 and 20 μM) for 6, 12, 24 and 48 hr. The level of Fas/APO-1 receptor (A); the amount of mFasL (B); the amount of sFasL (C). Each value is the mean ± s.d. of three determinations. The asterisk indicates a significant difference between control and asperfuranone-treated cells, as analysed by Dunnett's test ( $p < 0.05$ ).

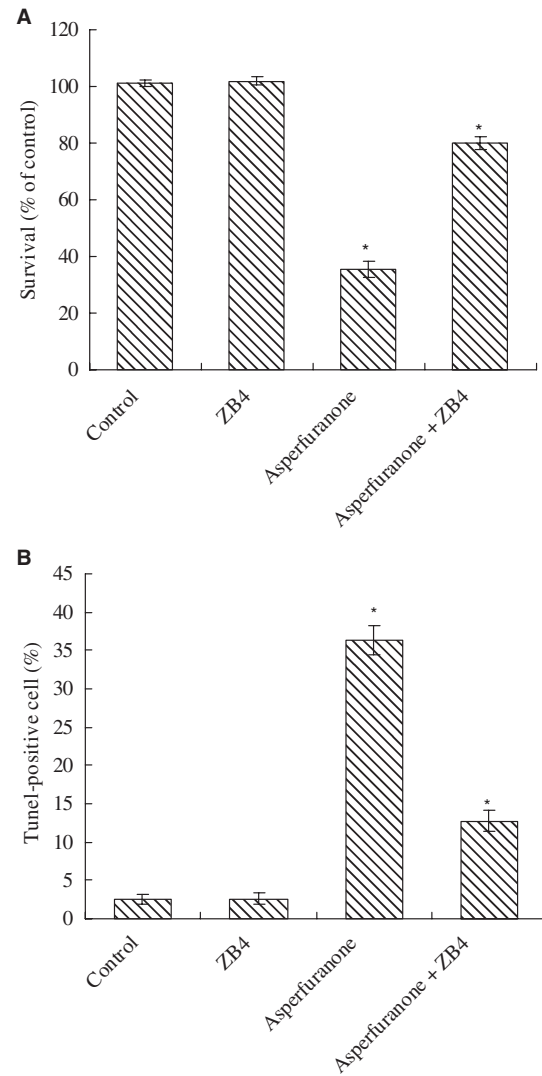


Fig. 7. Effect of antagonistic anti-Fas antibody (ZB4) on asperfuranone in A549 cells. (A) The anti-proliferative and (B) the pro-apoptotic effect of asperfuranone was decreased by Fas antagonist ZB4. For blocking experiments, cells were pre-incubated with 250 ng/ml ZB4 for 1 hr and then treated with 20 μM of asperfuranone for 48 hr. Cell viability and apoptosis induction were examined by XTT and BD ApoAlert™ DNA Fragmentation Assay kit. The data shown are the mean ± S.D. of three determinations. The asterisk indicates a significant difference between control and asperfuranone-treated cells, as analysed by Dunnett's test ( $p < 0.05$ ).

downstream target of p53, Fas/APO-1 protein [13] was increased by asperfuranone. Moreover, the expression levels of these proteins were greatly increased (at 24 hr) after maximal accumulation of p53 protein (at 12 hr) in A549 cells.

Fas/FasL system is a key signalling transduction pathway of apoptosis in cells and tissues [13]. Kim *et al.* have demonstrated that delivery of the wild-type murine Fas-associated death domain protein gene to drug- and radiation-resistant lung cancer may be a novel method for therapy of non-small cell lung cancer [15]. Ligation of Fas by agonistic antibody or its mature ligand induces receptor oligomerization and formation of death-inducing signalling complex (DISC),

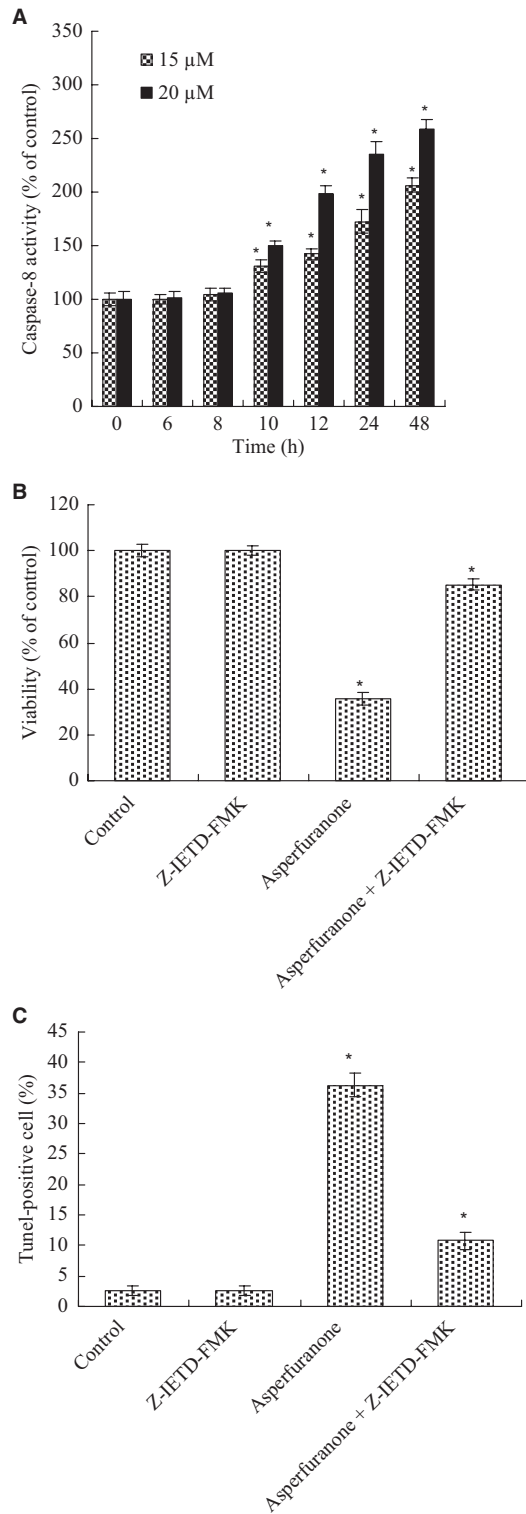


Fig. 8. (A) The activation of caspase-8 in A549 cells by asperfuranone; (B) Effect of caspase-8 inhibitor on asperfuranone-mediated anti-proliferation; (C) Effect of caspase-8 inhibitor on asperfuranone-induced apoptosis. A549 cells were incubated with various concentrations of asperfuranone for the indicated times. For blocking experiments, cells were pre-incubated with Z-IETD-FMK (10  $\mu$ M) for 1 hr before the addition of 20  $\mu$ M asperfuranone. After 48 hr of treatment, cell viability and induction of apoptosis were measured by XTT and BD ApoAlert™ DNA Fragmentation Assay kit. Each value is the mean  $\pm$  s.d. of three determinations. The asterisk indicates a significant difference between control and asperfuranone-treated cells, as analysed by Dunnett's test ( $p < 0.05$ ).

and plays a critical role in the pathophysiology of various pulmonary disorders [28]. Up-regulation of Fas expression has been demonstrated to induce apoptosis in hydrogen peroxide-treated A549 cells [28]. Induction of Fas expression and augmentation of Fas/Fas ligand-mediated apoptosis has been also suggested to inhibit proliferation in the synthetic retinoid CD437-treated human non-small cell lung cancer cells [10]. Serrao *et al.* have reported that neutrophils induce apoptosis of lung epithelial cells via release of soluble FasL [29]. Our study indicated that Fas ligands, mFasL and sFasL, increased in asperfuranone-treated A549 cells. Moreover, the levels of Fas and the activity of caspase-8 were simultaneously enhanced in FasL-up-regulating A549 cells. Furthermore, when the Fas/Fas ligand system was blocked by ZB4, a decrease in cell growth inhibition and the pro-apoptotic effect of asperfuranone was noted. Similarly, cell growth inhibition and apoptotic induction of asperfuranone decreased in A549 cells treated with caspase-8 inhibitor. Thus, these findings are the first to show that the Fas/FasL system plays an important role in asperfuranone-mediated A549 cellular apoptosis.

In summary, our study suggests that the induction of p53 and activity of the Fas/FasL apoptotic system may participate in the anti-proliferative activity of asperfuranone in A549 cells. Our data provide a basic mechanism for the pro-apoptotic properties of asperfuranone in lung cancer cells.

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followed by activation of caspase-8, then further activating a series caspase cascades resulting in cell apoptotic death [13,25]. FasL is a TNF-related type II membrane protein [26]. Cleavage of membrane-bound Fas ligand by a metallo-protease-like enzyme results in the formation of soluble Fas ligand [27]. Fas is expressed in human airway epithelial cells

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