# Action of solamargine on human lung cancer cells – enhancement of the susceptibility of cancer cells to TNFs

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Received 23 August 2004; revised 16 September 2004; accepted 16 September 2004

Available online 7 October 2004

Edited by Masayuki Miyasaka

**Abstract Solamargine (SM), isolated from *Solanum incanum* herb, displayed a superior cytotoxicity in four human lung cancer cell lines. The half-inhibitory concentrations ($IC_{50}$), of the cell viability assay for H441, H520, H661 and H69 cells were 3, 6.7, 7.2 and 5.8 μM, respectively. SM-induced apoptosis of these cells by PS externalization in a dose-dependent manner and increased sub-$G_1$ fraction were observed. Quenching of the expression of tumor necrosis factor receptors (TNFRs) during the progress of human lung carcinogenesis has been previously reported. SM may induce cell apoptosis via modulating the expression of TNFRs and their subsequent TRADD/FADD signal cascades. Subsequently, SM treatment increased the binding activities of TNF-α and TNF-β to the lung cancers, and the intrinsic TNFs-resistant cancer cells became susceptible to TNF-α and -β. In addition, SM caused release of cytochrome *c*, downregulation of anti-apoptotic Bcl-2 and Bcl-$x_L$, increase of caspase-3 activity, and DNA fragmentation. Thus, SM could modulate the expressions of TNFRs and Bcl-2, and might be a potential anticancer agent for TNFs and Bcl-2 related resistance of human lung cancer cells.**


*Keywords:* Solamargine; Drug resistance; Lung cancer; Apoptosis

## 1. Introduction

Lung cancers can be subdivided into two groups, small cells lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), according to their histological features. SCLC is usually responsive to chemotherapy, whereas relapse is inevitable and long-term survival is rare. NSCLC is characterized by both high incidence and lethality, with five-year-survival rate accounting for less than 10% of the cases [1]. The currently available chemotherapeutic treatment for human lung cancers is disappointed. The poor prognosis of lung cancers may be partially based on the pronounced resistance to chemotherapeutic drugs that is present intrinsically in most NSCLC and is also acquired during the treatment of initially drug-sensitive SCLC [2]. The underlying mechanism of cellular resistance remains rather obscure. Apart from classical drug resistance mechanisms, the failure of tumor cells to undergo apoptosis also plays an important role in drug resistance. Mutations and defects in the apoptotic pathway are, therefore, additional factors that determine drug resistance, and the Bcl-2 family member is one of important factors in this pathway in lung cancer [3]. Advanced search for more effective agents or combination therapy for treating lung cancers is essential. Thus, defining the molecular determinants of sensitivity or resistance to chemotherapy would have important implications for the treatment of lung cancers.

Apoptosis is considered to be the major process responsible for cell death in various physiological events and has become a focus of interest in oncology. Most anticancer drugs have been shown to cause cell death by inducing apoptosis [4]. Ability to manipulate the machinery of cell death is an obvious goal of medical research and effect on regulation of apoptosis might lead to new possibilities for cancer treatment. Tumor necrosis factors (TNFs) have emerged as one of the many host-derived mediators that, either alone or in combination, appear to mediate both antiproliferative and tumorigenic effects in malignant tumors. The TNF family is comprised of two cytokines, TNF-α (cachectin) and TNF-β (lymphotoxin-α), which are secreted by a wide variety of cells [5]. TNFs bind with nearly identical affinities to distinct tumor necrosis factor receptors (TNFRs), TNFR-I and -II. TNFR-I and -II have been reported as vital mediators of TNF-α in triggering apoptosis [6]. TNFR-I is functional in almost every cell type and can independently transmit most biological activities [7]. The expression of TNFR-II can strongly induce TNF-α mediated cytotoxicity through TNFR-I in lung cancer cells [8]. Although normal lung cells express TNFR-I and -II, loss or downregulation of TNFRs occurs during the progression of lung cancers [9,10]. It had also been reported that retinoic acid would modulate the expression of TNFR-I and TNFR-II in lung cancer cells and sensitize cells to TNF-induced apoptosis [11]. Since inherent and acquired drug resistance is commonly present in human lung cancers, the downregulation of TNFRs

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*Abbreviations:* SM, solamargine; PS, phosphatidylserine; TNFRs, tumor necrosis factor receptors; TNF, tumor necrosis factor; TRADD, TNFR-1-associated death domain; FADD, Fas-associated death domain; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo phenyl)-2H-tetrazolium]

0014-5793/$22.00 
doi:10.1016/j.febslet.2004.09.064

in lung cancers may be the way to flee from apoptosis, which may consequently cause drug resistance.

Solamargine (SM), a steroidal glycoalkaloid, has been isolated from the fruits of *Solanum incanum* [12] and its structure has been determined [13]. Like other steroidal molecules, SM exerts its action by penetrating the cell membrane by simple diffusion. SM inhibits the growth of human tumor cells, e.g., colon (HT-29 and HCT-15), prostate (LNCaP and PC-3), breast (T47D and MDA-MB-231), human hepatoma (PLC/PRF/5) and JTC-26 cells [14]. Previous experiments had shown that SM could trigger gene expression of TNFRs in Hep 3B cells, which might lead to apoptosis [15–17]. However, the detailed anti-neoplastic mechanism of SM remains to be investigated in the drug-resistant lung cancers. In this paper, the effects of SM in human non-small cell lung cancers (H441, H520 and H661) and small cell lung cancer (H69) cells were characterized. These results revealed a novel gene modulation of SM in overexpressed Bcl-2 and TNFs-resistant cancer cells, and might elicit the anticancer potential of SM against human lung cancers.

## 2. Materials and methods

### 2.1. Drugs

Drugs were provided as pure substances. SM (isolated from berries of *Solanum khasianum* Clarke) and etoposide (Bristol-Myers Squibb, Syracuse, NY) were diluted in DMSO. Cisplatin (Bristol-Myers Squibb, Woerden, The Netherlands) and gemcitabine (Eli Lilly Research Laboratories, Indianapolis, IN) were diluted in phosphate-buffered saline (PBS), and paclitaxel (Bristol-Myers Squibb, Syracuse, NY) was diluted in ethanol. The drugs were freshly diluted to the final concentration in culture medium before experiment.

### 2.2. Cell lines and cultures

Human adenocarcinoma (H441), squamous cell lung carcinoma (H520), large cell lung cancer (H661) and small cell lung cancer (H69) were purchased from the American Type Culture Collection (Rockville, Maryland). The cells were cultured at 37 °C in a humidified atmosphere of carbon dioxide–air (5:95). The culture medium consisted of RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hazelton Products, Denver, PA), 100 μg/ml streptomycin and 100 U/ml penicillin.

### 2.3. Growth-inhibition assay

A 100 μl suspension of $1 \times 10^4$ cells was added to each well of flat-bottomed, 96-well multi-dishes (Corning, Elmira, NY). After 24 h, various concentrations of the drugs (SM, cisplatin, gemcitabine, paclitaxel and etoposide) were added to the cells for 16 h. To determine the combined effects of TNFs and SM, cells were pre-incubated with or without SM for 2 h before TNFs were added. A colorimetric tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo phenyl)-2H-tetrazolium] (MTS) method was utilized to determine cell viability according to the manufacturer's procedures (CellTiter 96$^{TM}$ AQ, Promega, Madison). The absorbance at 490 nm ($A_{490}$) was measured with an automated plate reader (Dydatech, Alexandria, VA). Absorbance values were expressed as a percentage of untreated controls, and concentrations resulting in cell growth inhibition of 50% ($IC_{50}$) and 80% ($IC_{80}$) were calculated. All determinations were performed in triplicate and statistically analyzed by Student's *t* test.

### 2.4. Flow cytometry of SM-treated cells

Spontaneous cell death in the absence of SM was less than 10%. Detection of phosphatidylserine (PS) on the outer leaflet of SM ($IC_{50}$ and $IC_{80}$ concentration)-treated cells was carried out with annexin-V-FLUOS method (Boehringer Mannheim, Mannheim, Germany). Besides, sub-G1 DNA content refers to the portion of apoptotic cells. The sub-G1 analysis of SM ($IC_{50}$ and $IC_{80}$)-induced apoptosis was determined by flow cytometry analysis of PI-stained nuclei. Briefly, the cells were trypsinized and resuspended in the original supernatant to ensure that both attached and non-attached cells were analyzed. Fluorescein isothiocyanate (FITC)-conjugated annexin-V and/or propidium iodide (PI; a fluorescent DNA intercalating dye) were added, and 10 000 cells were analyzed by Epics Elite ESP apparatus. Quantitative measurements were done with WinMDI software.

### 2.5. Determination of change of gene expression by RT-PCR

Cellular RNA was isolated from $IC_{50}$ concentration of SM-treated and untreated cells by guanidinium thiocyanate/cesium chloride gradient ultracentrifugation method. All RNA preparations were treated with DNase and stored in liquid nitrogen prior to use. Reverse transcriptase of moloney murine leukemia virus was used to prepare cDNA (Clontech, Palo Alto, CA). The primer sequences for detecting TNFR-I, TNFR-II, TNFR-1-associated death domain (TRADD), Fas-associated death domain (FADD), Bcl-$x_L$ and Bcl-2 were designed as previously report [18–20]. Differential gene was determined by normalizing its expression against β-actin and GAPDH, and by 2% agarose staining with 0.5 μg/ml ethidium bromide.

### 2.6. Fluorescent immunocytochemistry

Cells were treated with or without $IC_{50}$ concentration of SM for 16 h. Anti-human TNFR-I and TNFR-II monoclonal antibodies (10 μg/ml) (Genzyme, Cambridge, MA) or human recombinant TNF-α and -β proteins (50 ng/100 μl) (Deisenhofen, Germany) were utilized to detect the expression of TNFR-I and II on cells or the ligands bind on the receptors, respectively. Anti-mouse IgG conjugated FITC for TNFR-I and II antibodies (DAKO Diagnostica, Hamburg, Germany) or FITC-conjugated anti-mouse IgG for TNF-α and -β proteins were used in the dilution of 1:100. For negative controls, the primary antibodies were replaced with 1× PBS under the same condition. In all cases, samples were placed in FACScan flow cytometer (Becton Dickinson) and 10 000 events were acquired and analyzed with the *LYSIS II* software (Lysis, Mountainview, CA) and WinMDI software. These experiments were repeated on cells fixed in 4% paraformaldehyde and permeabilized in saponin (0.1% v/v in PBS-BSA). TNFR-I, TNF-α and -β were detected with anti-human TNFR-I, TNF-α, and TNF-β mouse $IgG_1$ and anti-mouse IgG-FITC. TNFR-II was detected with anti-human TNFR-II mouse $IgG_1$ and anti-mouse IgG-rhodamine (Santa Cruz, CA). Nuclei were stained with DAPI at 1 μg/ml for 3 min. The stained cells were viewed and photographed with a fluorescent microscope.

### 2.7. Immunoblot analysis

Cells were treated with $IC_{50}$ concentration of SM for 16 h and protein expression of TRADD, FADD, cytochrome *c*, Bcl-$x_L$ and Bcl-2, caspase-3 were determined by immunoblot analysis. Cells ($1 \times 10^6$) were lysed at 4 °C in 400 μl of lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 100 mM sodium fluoride and 5 mM EDTA) and protease inhibitor mix Complete® (Boehringer Mannheim). The lyses were centrifuged to remove cellular debris. Protein concentration of the extracts was determined using an ESL protein assay (Boehringer Mannheim) with bovine serum albumin (BSA) as standard. Cell lysates (50 μg) were fractionated on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membrane (Millipore, Bedford, MA). After blocking with 5% skimmed milk, the membranes were probed with secondary antibodies (1 μg/ml) (Santa Cruz Biotech, CA), respectively. The membranes were incubated with protein G conjugated horseradish peroxidase (Bio-Rad, Hercules, CA) in 1:3000 dilutions for 1 h, and subjected to ECL detection (Amersham, Piscataway, NJ) and autoradiography by standard procedures.

### 2.8. Analysis of DNA fragmentation

The cells were treated with or without $IC_{80}$ concentration of SM for 16 h. Cells were collected and suspended in denaturing solution (10 mM Tris–HCl, ph 8.0, 100 mM NaCL, 25 mM EDTA, pH 8.0 and 100 μg/ml of Proteinase K) and incubated at 50 °C for 12–18 h. Total DNA was isolated by phenol/chloroform extraction and ethanol precipitation, and then solubilized in TE buffer (10 mM Tris–HCl and 100 μM EDTA, pH 8.0). Ten micrograms of the collected DNA was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and then visualized under UV illumination.

## 3. Results

### 3.1. Comparison of the cytotoxicity of SM with other anticancer drugs

The molecular weight of SM is 828 Da [13]. The cytotoxicity of SM as well as conventional chemotherapeutic agents, e.g., paclitaxel, cisplatin, etoposide and gemcitabine were evaluated in human lung cancer cells (H441, H520, H661 and H69). These agents were freshly prepared and diluted with cultural medium, and the half-inhibitory concentrations ($IC_{50}$), of the cell viability were determined by MTS method. As shown in Fig. 1, SM inhibited cell growth in a dose-dependent manner. The ($IC_{50}$)s of SM, paclitaxel, cisplatin, etoposide and gemcitabine were approximately 3, 45, >250, >250 and >250 μM for H441, 6.7, 21, >250, >250 and >250 μM for H520, 7.2, 23.4, 136, >250 and >250 μM for H661, and 5.8, 22.4, 240, >250 and >250 μM for H69 cells. The ($IC_{80}$)s of SM were approximately 11.8 μM for H441, 12.5 μM for H520, H661 and H69. Clear differences were observed in the susceptibility of the drugs for lung cancer cells. SM is the most sensitive agent among these cancer drugs in all lung cancer cell lines. The lung cancer cells seem to be resistant to cisplatin, etoposide and gemcitabine (>50-fold $IC_{50}$ of SM).

### 3.2. SM induces apoptosis in lung cancer cells

Translocation of membrane PS and sub-G1 fraction were the hallmarks of apoptosis [21]. As shown in Fig. 2A, treatment of cells with SM rapidly induced apoptosis in a dose- and time-dependent manner, as demonstrated by an increasing percentage of annexin-positive and PI-negative apoptotic cells. Moreover, the annexin-positive cells became smaller in size than annexin-negative cells, as shown in Fig. 2B. During incubation, only few double-positive necrosis cells were detected (less than 1% was observed). The similar results were observed in Fig. 3. The PI-stained apoptotic cells were analyzed by flow cytometry and most of the apoptotic cells were detected within 2 h. A 5- to 40-fold increase in the sub-G1 fraction of apoptotic cells was detected from SM-treated cells as compared with control. Thus, SM could induce early stage of apoptosis in the first hour of incubation and markedly induce cell shrinkage and apoptosis after 3 h.

### 3.3. Regulation of TNFR-I and TNFR-II expression by SM

TNFRs have been involved in the process of apoptosis. The expression of TNFRs is quenched during the progress of human lung carcinogenesis [9,10]. The gene expression of TNFRs was determined by RT-PCR. As shown in Fig. 4A, SM upregulated the gene expression of TNFR-I and TNFR-II in SM ($IC_{50}$ concentration)-treated cells. Besides, the results had been verified by TNFR-I and -II antibodies to the receptors or TNF-α and -β ligands on SM-treated cells by fluorescent immunocytochemistry (Figs. 4B and 5A) and flow cytometry (Figs. 4C and 5B). These data revealed that SM could modulate the expression of TNFRs, thereby increased the binding activities of TNFs to TNFRs. Moreover, the combined actions of SM and TNFs were carried out. As shown in Figs. 6 and 7, all of these lung cancer cells were not sensitive to TNF-α or -β. However, the increasing-growth inhibition of TNFs to TNF-resistant cells was observed after SM pre-incubation. Thus, SM increased the expression of TNFRs and consequently improved the susceptibility of lung cancer cells to TNF-α and -β.

### 3.4. Apoptotic signaling cascades of SM-treated lung cancer cells

To clarify the molecule(s) involved in SM-induced apoptotic signal, commercial RT-PCR and synthetic primers were utilized to detect apoptotic-related genes in lung cancer cells. As

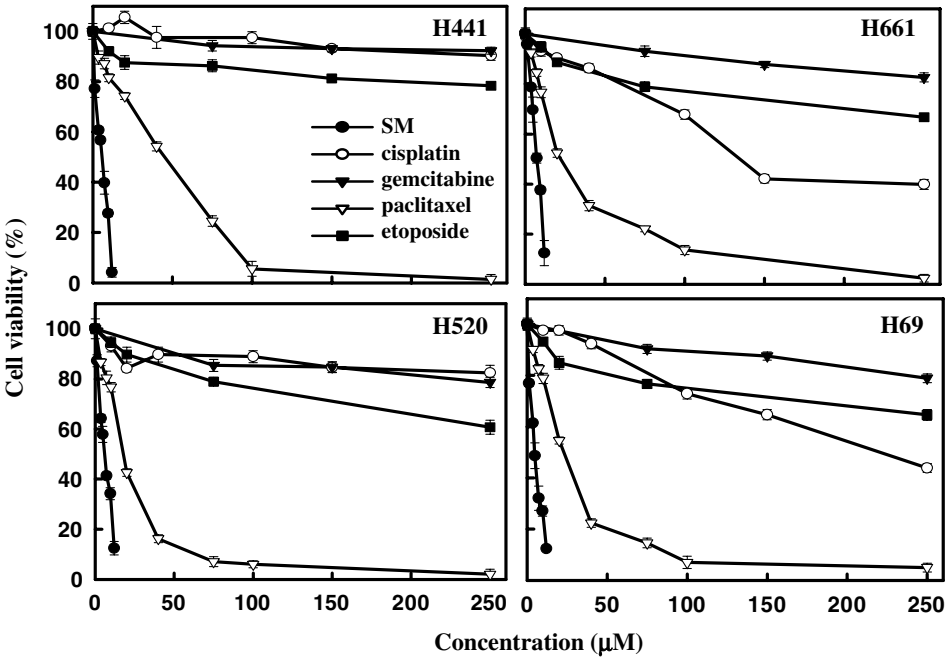


Fig. 1. Cytotoxicity of SM, paclitaxel, cisplatin, etopopside and gemcitabine to human lung cancer (H441, H520, H661 and H69) cells. Cell viability was determined by MTS as described in Section 2. ●, SM; ○, cisplatin; ▼, gemcitabine; ▽, paclitaxel and ■, etoposide. Each datum represents mean ± S.D. of the percentage of proliferation of the treated/untreated cells from quaternary determinations.

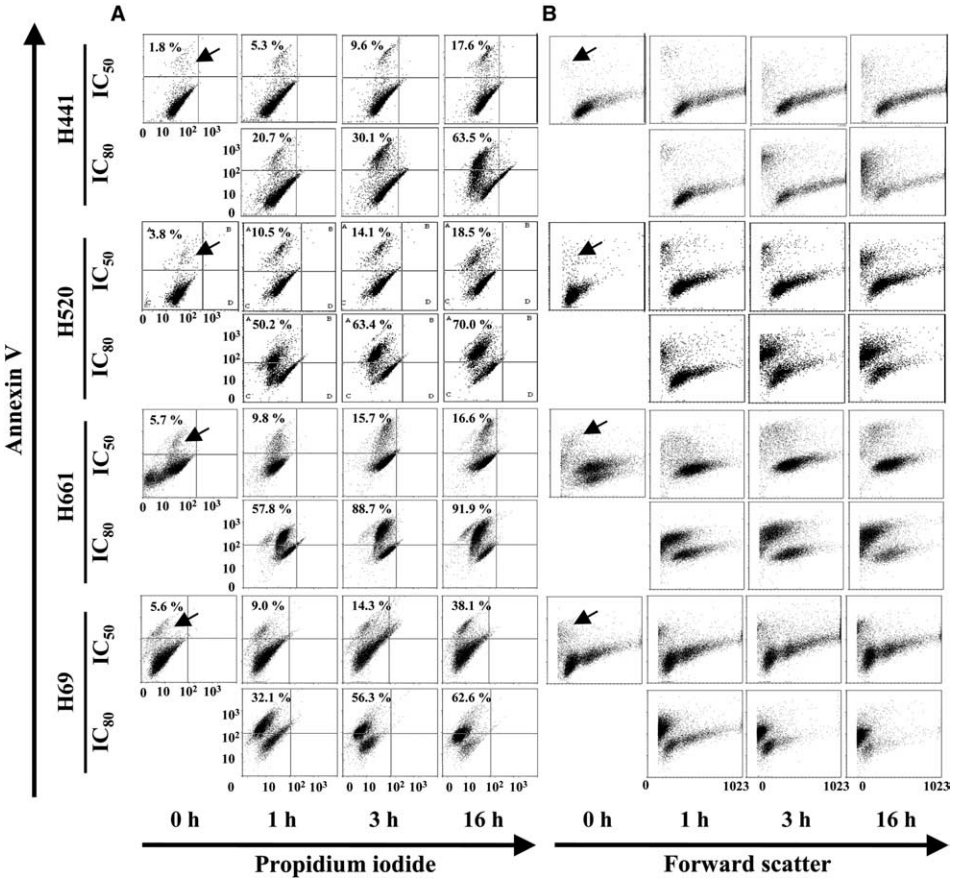


Fig. 2. Two-color flow cytometry analysis of lung cancer cells after staining with fluorescence-labeled annexin-V and PI. (A) Apoptotic analysis of target cells by annexin-V and PI stains in logarithmic scales. (B) Determination of the size of annexin-positive stain cells by annexin-V and forward scatter analysis. The percentage indicates the apoptotic cell population calculated by WinMDI software. The results are shown from a representative experiment of four independent experiments.

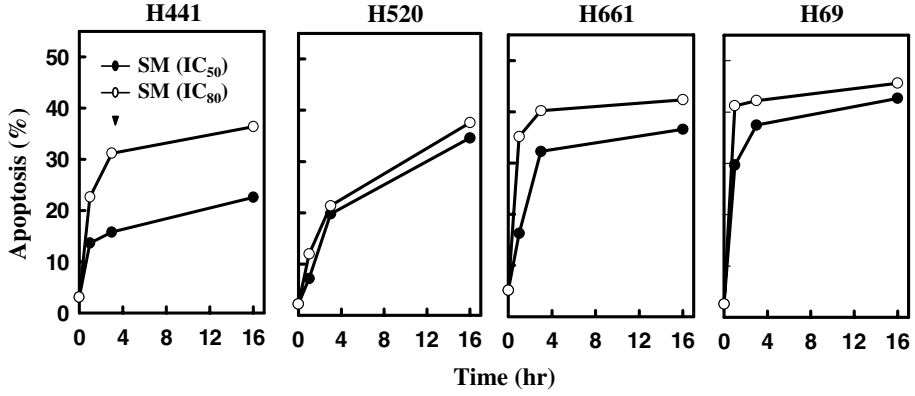


Fig. 3. Sub-G1 analysis of SM-treated lung cancer cells. The percentage of SM ($IC_{50}$ and $IC_{80}$)-induced apoptosis was determined by flow cytometry analysis of PI-stained nuclei. Sub-G1 DNA content refers to the portion of apoptotic cells, as described in Section 2. Results depicted represent a mean of three independent experiments in which the SD was $\leqslant 10\%$.

shown in Figs. 8A and 9A, the signal adaptors of TNFRs, i.e., TRADD and FADD were involved in the action mechanism of SM. The apoptotic TRADD, FADD genes were upregulated and the anti-apoptotic Bcl-$x_L$ and Bcl-2 genes were downregulated after the $IC_{50}$ concentration of SM treatment for 16 h. The similar results were observed by Western blotting (Figs. 8B and 9B). Alternately, SM caused the release of cytochrome *c* from mitochondria. Induction of apoptosis by SM in lung cancers was accompanied by significant increase in caspase-3 activity (Fig. 9B). At the late phase of apoptosis, the activation of caspase-3 will cleave DNA into oligonucleosomal fragments (180–200 bp). As shown in Fig. 8C, when the cells were treated with $IC_{80}$ concentration of SM for 24 h, a DNA ladder was appeared. The results revealed that TNFR and Bcl-2 signaling pathway might be involved in SM-induced apoptosis of lung cancers.

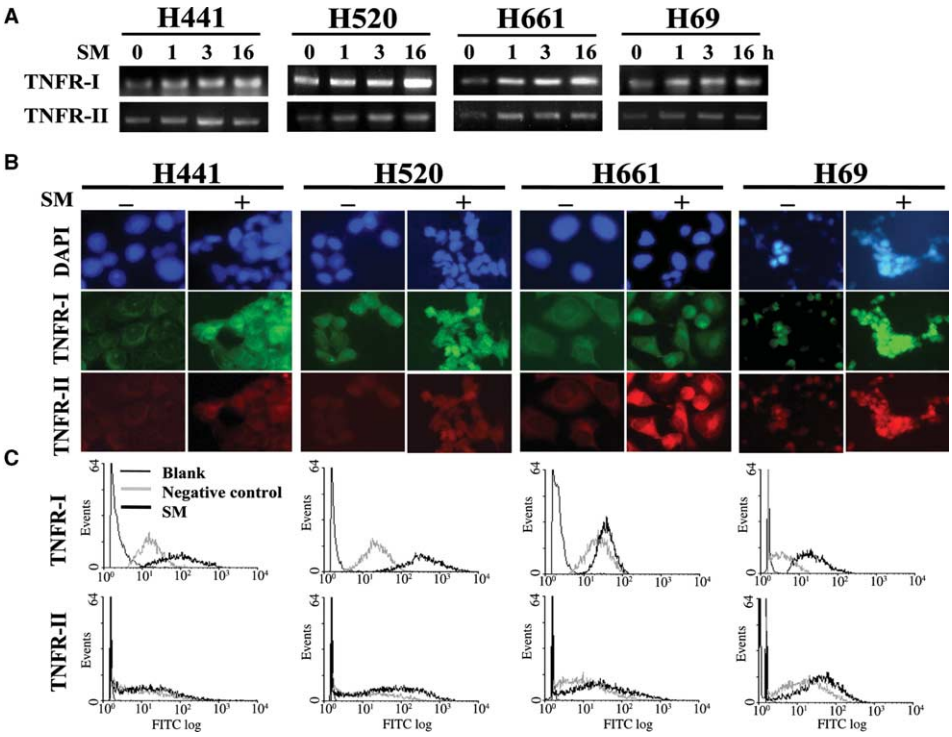


Fig. 4. Regulation of TNFR-I and TNFR-II expression in human lung cancer cells after SM treatment. The cells were treated with or without $IC_{50}$ concentration of SM. The expressions of TNFR-I and -II were studied by RT-PCR (A), fluorescent immunocytochemistry (B) and flow cytometry (C) as described in Section 2.

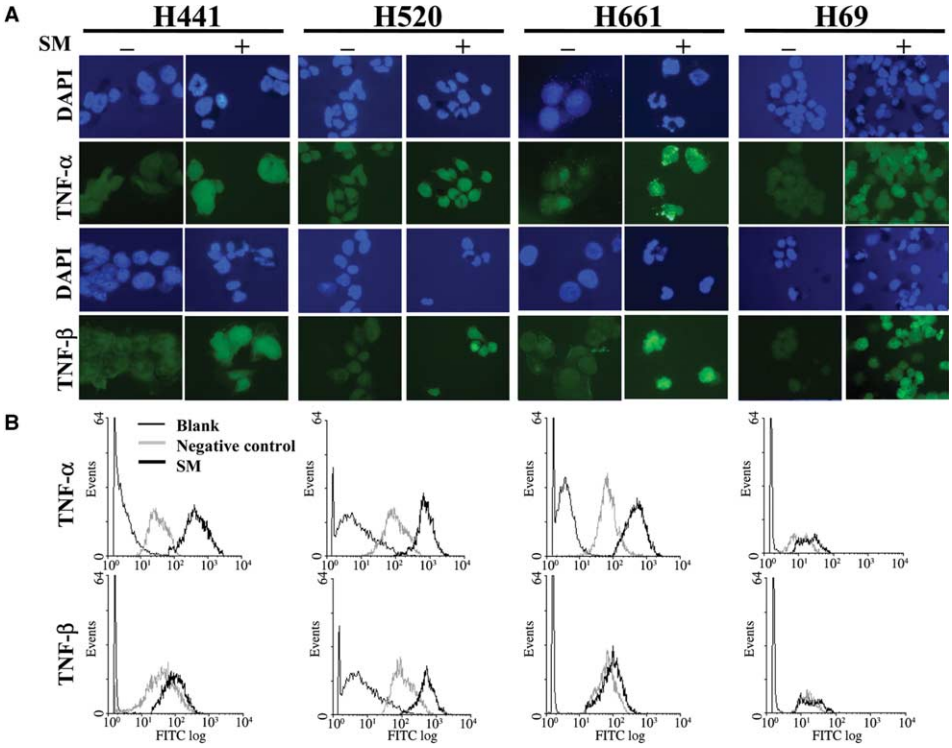


Fig. 5. Determination of TNF-α and -β binding on human lung cancer cells after SM treatment. The cells were treated with or without $IC_{50}$ concentration of SM. The binding activities of TNF-α and -β were studied by fluorescent immunocytochemistry (A) and flow cytometry (B) as described in Section 2.

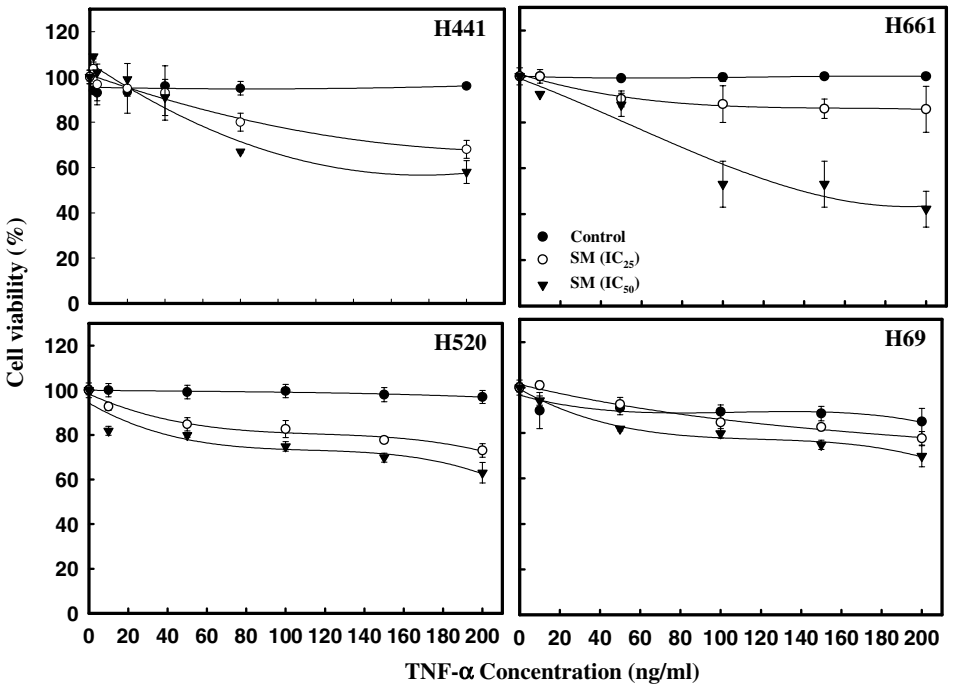


Fig. 6. Effect of human TNF-α on the cytotoxicity of lung cancer cells after SM ($IC_{25}$ and $IC_{50}$) treatment. Cell viability was determined by MTS assay. Each datum represents mean ± S.D. of the percentage of proliferation of the TNF-α treated/untreated cells from quaternary determinations.

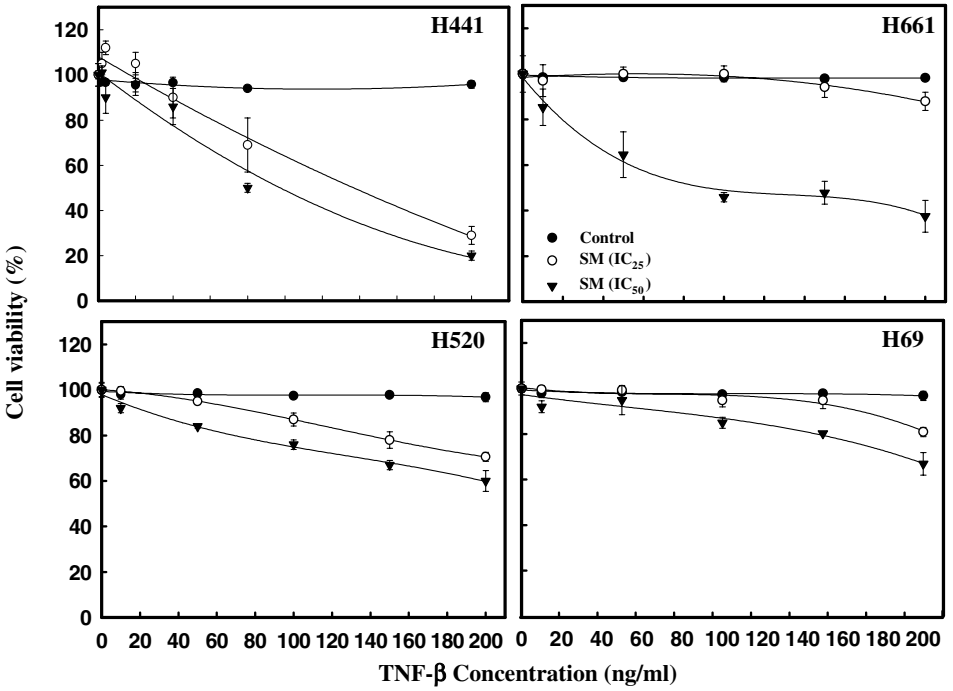


Fig. 7. Effect of human TNF-β on the cytotoxicity of lung cancer cells after SM ($IC_{25}$ and $IC_{50}$) treatment. Cell viability was determined by MTS assay. Each datum represent means ± S.D. of the percentage of proliferation of the TNF-β treated/untreated cells from quaternary determinations.

## 4. Discussion

Approaches utilizing combinations of chemotherapeutic, radiation and/or surgical modalities are the standard of care for treating lung cancers. Several new anticancer agents have been developed, including paclitaxel, docetaxel, vinorelbine, gemcitabine, camptothecines, irinotecan and topotecan in the treatment of lung cancers [22]. Although these drugs may be effective in lung cancers, identifying new drugs to improve the currently dismal survival rates in lung cancer patients is important. Chinese herbs are composed of active components that exhibit the capability of regulating cell activities. Previous

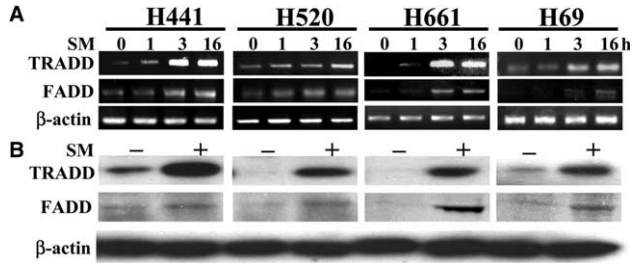


Fig. 8. Regulation of TRADD and FADD expression in human lung cancer cells after SM treatment. The cells were treated with or without $IC_{50}$ concentration of SM. The expressions of TRADD, FADD and β-actin genes were studied by RT-PCR (A) and Western blots (B). Cell lysates (25 μg/land) were separated by a 12% SDS–PAGE, and TRADD and FADD were monitored by immunoblotting as described in Section 2.

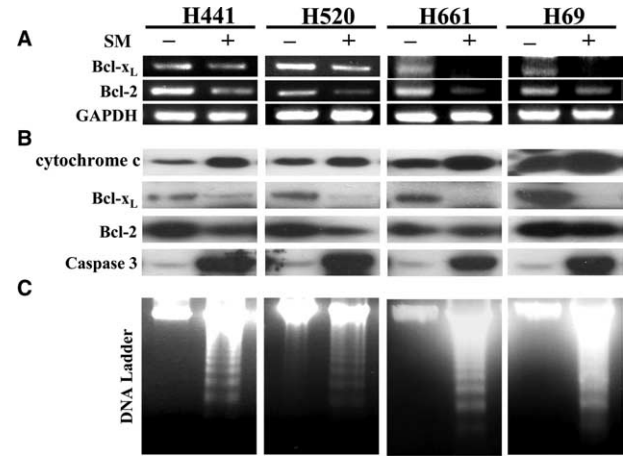


Fig. 9. RT-PCR and Western blot of the apoptotic gene expression and DNA fragmentation of SM-treated lung cancer cells. Cells were treated with SM ($IC_{50}$ concentration) for 16 h. The changes of cytochrome *c*, Bcl-$x_L$, Bcl-2 and caspase-3 expressions were analyzed by RT-PCR (A) and by immunoblotting (B). Arrows indicate the genes involved in the signal cascade of SM-induced apoptosis. (C) DNA ladder of the cells treated with $IC_{80}$ concentration of SM for 24 h as described in Section 2.

treatments of diseases with herbs were more empirical than theoretical [23]. In order to identify active components from Chinese herbs, many pure compounds have been purified from Chinese herbs. However, the detailed action mechanisms of the compounds have not been well conducted. This paper illustrated the action mechanism of SM in human lung cancer cells. Compared with the relative sensitivity of cells to paclitaxel, cisplatin, gemcitabine and etoposide, SM has shown a superior effect in human lung cancer cells.

Apoptosis is a genetically encoded and tightly regulated 'suicidal' process in which the cell actively participates in its own destruction. Under the microscopic examination, SM-treated cells initially became round in shape, subsequently lost contact with neighboring cells, and finally floated into medium. After 16 h of incubation with SM, the cytoplasmic shrinking, phosphatidylserine externalization, chromatin condensation and numerous apoptotic bodies were found in the apoptotic cells (the data observed form microscopy was not shown). The action of SM full-filled the criterions of apoptosis in human lung cancer cells, implied that SM-induced cell death by apoptosis. In malignant cells, the physiological apoptotic pathways are often altered, resulting in a significant survival advantage and developed resistance to anti-tumor treatments [24]. A loss of TNF receptors expression was found in advanced lung cancers, suggesting downregulation of TNFRs in the process of tumor progression [9,10]. In the present data, SM could elevate the gene and protein expressions of TNFR-I, TNFR-II, and the downstream signaling cascades of TRADD and FADD in human lung cancer cells. We also had demonstrated that SM could induce programmed cell death by up-regulated TNFRs in Hep 3B [17]. Thus, activation of TNFRs might be one of the important roles involved in the action mechanism of SM.

TNF has shown an antiproliferative effect against various NSCLC cell lines in vitro and in vivo, but the clinical trails with TNF in combination with other cytokines have demonstrated both conflicting and disappointing results [10,25,26]. In this study, the binding of TNF-α and TNF-β to TNFRs was increased in SM-treated cells by fluorescent immunocytochemistry and flow cytometry assay. Also, SM might via up-modulation of TNFRs sensitize TNF-resistance lung cancer cells to TNFs, as revealed by growth-inhibition assay. However, the concentration of secreted TNFs was not significantly increased in the SM-treated medium by ELISA assay (data not shown). A similar result has been reported that retinoic acid would modulate the expression of TNFR-I and TNFR-II in lung cancer cells and sensitize cells to TNF-induced apoptosis [11]. These findings indicate that activation of TNFR signal transduction may be a new focus to investigate a drug for treating lung cancers.

It had been reported that overexpression of anti-apoptotic protein Bcl-$x_L$ and Bcl-2 may increase apoptotic resistance and correlate with the multidrug resistance of lung cancers [27]. The antisense oligonucleotides of Bcl-2 and Bcl-$x_L$ have been used in treatment or combination of anti-cancer drugs for induction of apoptosis in lung cancer cells [28–32]. Several mechanisms have been implicated in TNF resistance, one of which is to alter the expression of oncogenes (e.g., bcl-2) that subsequently activate anti-apoptotic pathways may also contribute to the resistance phenotype [33,34]. Interestingly, a decreased expression of Bcl-$x_L$ and Bcl-2 was observed in SM-treated lung cancer cells. Thus, the enhanced susceptibility of lung cancer cells to TNFs may be correlated with the up-modulation of TNFRs, but also the down-regulation of Bcl-2 expression by SM.

Activation of caspases may be the ultimate effectors mechanism in apoptotic pathways. Caspase-3 is associated with drug-induced apoptosis in lung cancer cell lines and is required for DNA fragmentation [35]. The activation of caspase-3 was observed in SM-treated cells, and resulted in cell shrinkage, DNA ladder and sub-G1 peak. Anticancer drugs may exert both Fas receptor and ligand to trigger apoptosis. But, the apoptosis induced by chemotherapeutic agents is not mediated through Fas/FasL (CD95/APO1) signaling pathway in many lung cancers patients [36]. In our experiment, the expression of Fas was not involved in SM-induced apoptosis of lung cancer cells (data not shown). Subsequent study revealed that SM could cause release of cytochrome *c* in lung cancer cells. Insight regarding the proteins that determine sensitivity for chemotherapeutic drugs could provide new targets for cancer treatment, which may help to at least partly overcome drug resistance in lung cancers. Our results suggest that TNFR and mitochondria signal pathway might mediate the anti cancer effects of SM. This paper illustrates that SM may be a superior agent for the treatment of lung cancers.

*Acknowledgements:* This work was supported in part by grants from National Science Council, Taiwan (NSC 92-2314-B-214-002-) to L.-F.L.

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