RESEARCH ARTICLE

Plumbagin from *Plumbago Zeylanica* L Induces Apoptosis in Human Non-small Cell Lung Cancer Cell Lines through NF-κB Inactivation

Tong-Peng Xu, Hua Shen, Ling-Xiang Liu, Yong-Qian Shu*

Abstract

Objective: To detect effects of plumbagin on proliferation and apoptosis in non-small cell lung cancer cell lines, and investigate the underlying mechanisms. Materials and Methods: Human non-small cell lung cancer cell lines A549, H292 and H460 were treated with various concentrations of plumbagin. Cell proliferation rates was determined using both cell counting kit-8 (CCK-8) and clonogenic assays. Apoptosis was detected by annexin V/propidium iodide double-labeled flow cytometry and TUNEL assay. The levels of reactive oxygen species (ROS) were detected by flow cytometry. Activity of NF-κB was examined by electrophoretic mobility shift assay (EMSA) and luciferase reporter assay. Western blotting was used to assess the expression of both NF-κB regulated apoptotic-related gene and activation of p65 and IκBκ. Results: Plumbagin dose-dependently inhibited proliferation of the lung cancer cells. The IC50 values of plumbagin in A549, H292, and H460 cells were 10.3 μmol/L, 7.3 μmol/L, and 6.1 μmol/L for 12 hours, respectively. The compound concentration-dependently induced apoptosis of the three cell lines. Treatment with plumbagin increased the intracellular level of ROS, and inhibited the activation of NK-κB. In addition to inhibition of NF-κB/p65 nuclear translocation, the compound also suppressed the degradation of IκBκ. ROS scavenger NAC highly reversed the effect of plumbagin on apoptosis and inactivation of NK-κB in H460 cell line. Treatment with plumbagin also increased the activity of caspase-9 and caspase-3, downregulated the expression of Bcl-2, upregulated the expression of Bax, Bak, and CytC. Conclusions: Plumbagin inhibits cell growth and induces apoptosis in human lung cancer cells through an NF-κB-regulated mitochondrial-mediated pathway, involving activation of ROS.

Keywords: Plumbagin - NSCLC - apoptosis - NF-κB - reactive oxygen species - mitochondrial pathway - caspase

Asian Pacific J Cancer Prev, **14 (4)**, 2325-2331

Introduction

Lung cancer is currently the commonest malignancy and the leading cause of cancer-related death in the world (Jemal et al., 2009). Non-small cell lung cancer (NSCLC) represents about 80% of primary lung cancer cases and approximately two thirds of these patients are diagnosed at an advanced stage (Jemal et al., 2009). Although several effective chemotherapeutic agents are administrated, the platinum-based regimen is the standard initial treatment for NSCLC patients (Pujol et al., 2006). However, the efficacy of the regimen for NSCLC has been reported to be only 30-40% based on NSCLC trials involving unselected patients (Ohe et al., 2007).

Identifying novel agents with fewer side effects and more effective therapy is of significance for prognosis of patients with NSCLC. Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone), a Chinese traditional herb extracted from the root of *Plumbago zeylanica* L has been reported in the literature to have various pharmacological activities, including anti-microbial, hypolipidemic, anti-atherosclerotic, and anti-carcinogenic effects (Mossa et al., 2004; Ding et al., 2005; Aziz et al., 2008). It has furthermore been shown to exert anti-proliferation influence in diverse cancer cell lines, both *in vivo* and *in vitro*, such as leukemia (Xu et al., 2010), prostate (Aziz et al., 2008; Powolny et al., 2008), breast (Kuo et al., 2006; Ahmad et al., 2008), ovarian (Thasni et al., 2008), cervical (Srinivas et al., 2004; Nair et al., 2008) and melanoma (Wang et al., 2008) examples.

Reports in relation to the anticancer potential of plumbagin toward lung cancer have been described previously. It was found to induce apoptosis and inhibition of metastasis in A549 lung cancer cell line (Hsu et al., 2006; Shieh et al., 2010). However, the antitumor mechanisms of plumbagin remain inconclusive. The aim of present research is to investigate the anticarcinogenic activity of plumbagin toward three non-small cell lung cancer (NSCLC) cell lines and to detect the potential mechanism.

*Department of Oncology, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China *For correspondence: yongqian_shu@163.com*

Materials and Methods

Cell cultures

The lung cancer cell lines A549, H292 and H460, obtained from the American Type Tissue Culture (ATCC, Manassa, VA, USA), were cultured in RPMI-1640 medium containing 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA), 100 U/ml of penicillin, and 100 mg/ml streptomycin mixed antibiotics and 1 mM sodium pyruvate. The cell lines were maintained at 37 °C in a 5% CO_2/95% air-humidified atmosphere. The culture medium was renewed every 2 to 3 days. Adherent cells were detached by incubation with trypsin.

Biochemical reagents

Plumbagin was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). Cell Counting Kit-8 (WST-8) was purchased from Dojindo (Japan). Annexin V-FITC/PI was from BD Pharmingen (USA). TUNEL kit was from Chemicon Company (USA& Canada).The antibodies to caspase-3, caspase-8, caspase-9, Bak, Bcl-2, Bax, cytochrome C, C23, and GAPDH were obtained from Cell Signaling Technology (Beverly, MA). The antibodies to peroxidase-conjugated goat anti-rabbit and anti-mouse were purchased from Chemicon Company (USA& Canada). N-acetyl L-cysteine (NAC) was got from Sigma-Aldrich (St. Louis, MO).

Cell viability assay

To evaluate the effect of plumbagin on A549, H292, and H460 cell growth, cell viability was analyzed using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, cells were seeded in a 96-well plate (5×10^4 cells/well) overnight without treatment. The cells were incubated with varying concentration of plumbagin and collected at several different time points. After incubation for the indicated time, 20μL of kit reagent, WST-8 [2-(2-methoxy-4-nitrophenyl)-3- (4-nitrophenyl)-5-(2, 4-disulfonyl)-2H-tetrazolium], was added to each well, and the plate was incubated for 3 h at 37 °C. The optical density (OD) was measured at 490 nm using a 96-well multiscanner autoreader (Thermo Electron Corp, Waltham, MA, USA).

Clonogenicity assay

To determine long-term effects, cells seeded in six-well plates (10^3 cells/well) were treated with various concentrations of plumbagin for 6 h. Then the medium was discarded, and fresh medium was added to the wells, after which cells were allowed to grow for 14 days to form colonies, which were stained with crystal violet (0.4 g/l; Sigma). The images were collected, and the number of colonies in each well was counted.

Annexin V-FITC/PI staining assay

The extent of apoptosis was evaluated by Annexin V-FITC/PI kit (BD Pharmingen USA) and flow cytometer. The concentration of the plumbagin stock solution was adjusted so that the final concentration of DMSO in the culture medium 0.1%. In control cultures, DMSO 0.1% was added as a vehicle control. After incubation, the plumbagin-treated A549, H292, and H460 cells were harvested, washed with PBS (pH 7.4), centrifuged, and stained with Annexin V- FITC and PI in binding buffer for 15 min at 37 °C in the dark. The samples were analyzed using FACSCalibur flow cytometer (Becton, Dickinson and Co, San Jose, CA, USA). Data analysis was performed using FACSD iva software (BD, USA).

TUNEL assay

TdT-UTP nick end labeling (TUNEL) assays were performed with the ApopTag® Peroxidase TUNEL kit (Chemicon Company, USA& Canada) according to the manufacturer's instructions. Cells grown in 96 well culture plates were treated with plumbagin. After incubating for 24 hours, cells were stained by TUNEL kit. The TUNEL-positive cells were imaged under a light microscope. Cells with brown precipitate were defined as early stage of apoptotic cells.

Detection of reactive oxygen species (ROS)

H460 cells (0.5×10^6) were labeled for 1h with 5 μM carboxy-H2DCFDA (Molecular Probes, Eugene, OR). Next, excess carboxy-H2DCFDA was removed by washing the cells and then suspending them in serum-free, phenol red-free RPMI. The labeled cells were then exposed to vehicle control (DMSO) or various concentrations of plumbagin for 1h and the levels of ROS were determined by flow cytometric analysis using a FACSAria II flow cytometer (Becton, Dickinson and Co, San Jose, CA, USA). In all experiments, 10,000 viable cells were analyzed using forward/side-scatter gating.

Electrophoretic mobility shift assay (EMSA)

To detect NF-κB DNA-binding activity, we implemented electrophoretic mobility shift assay (EMSA) using the Viagene EMSA Kit following the manufacturer's protocol. Briefly, nuclear extracts (5 μg) from plumbagin-treated and NAC plus plumbagin-treated cells were incubated with biotin-labeled NF-κB oligonucleotide probes, 5'AGTT GAGG GGAC TTTC CCAGGC 3' and 3'TCAA CTCC CCTG AAAG GGTC CG5' in reaction buffers, for 30 min at 37 °C. Specific competition binding assays were performed using unlabeled probe as a specific competitor. DNA-protein complex was separated from free oligonucleotide on 6.5% native polyacrylamide gels, and then transferred to positively charged nitrocellulose membranes (Milipore, Bedford, MA) by a transfer blotting apparatus. Gel shifts were visualized with streptavidin-horseradish peroxidase followed by chemiluminescent detection and quantitated using labworks4.6 software.

NF-κB luciferase reporter assay

H460 cells were plated in 96-well plates with 1×10^4 cells per well in 10% FBS-supplemented RPMI medium. After overnight incubation, the cells were transfected with the NF-κB reporter plasmid linked to a luciferase gene or with the dominant-negative IκBa (IκBa-DN) plasmid. NF-κB luciferase plasmid was obtained from Stratagene (La Jolla, CA). Transfections were done according to the

manufacturer's protocols using FuGENE-6 (Roche). At 24h post-transfection, cells were treated with indicated concentrations of plumbagin for 2 h and then washed and lysed in luciferase lysis buffer (Promega), and the luciferase activity was measured with a luminometer using a luciferase assay kit (Promega) and was normalized to β-galactosidase activity. All the experiments were done in triplicates and repeated two or more times.

Western blot analysis

Cells seeded in 10 cm dishes were treated with 6 μmol/L or 3 μmol/L of plumbagin for indicated time. Total cell extracts were prepared using M-PER mammalian protein extraction reagent and protease inhibitors (Pierce, Rorkford, IL, USA). The nuclear extracts were prepared using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China).Protein concentrations were determined using the BCA protein assay (Pierce, Rorkford, IL, USA) following the manufacturer's protocol. Equivalent amounts of protein (30 μg) were loaded per lane, resolved by SDS-polyacrylamide gel electrophoresis (8%-12%), and transferred to PVDF membranes. After being blocked with a 5% skim milk solution for 1 h, the membranes were incubated with their respective primary antibodies overnight at 4 °C, followed by secondary antibody incubations for 1 h at room temperature. The proteins were visualized using an enhanced chemiluminescence system (ECL, Beyotime Institute of Biotechnology, Jiangsu, China).

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical comparisons were performed using a one-way analysis of variance followed by the Fisher test. Significant differences between the groups were determined using an unpaired Student t-test.

Results

Plumbagin decreased viability of NSCLC cells

Cell viability was assayed by treating NSCLC cell lines, including A549, H292, and H460 cells, with various concentrations of plumbagin followed the CCK-8 viability assay. We observed that cellular proliferation was inhibited by plumbagin for 24 hours in a dose-dependent manner in all three of the NSCLC cell lines (Figure 1B). The IC50 values of plumbagin in A549, H292, and H460 cells were 10.3 μmol/L, 7.3μmol/L, and 6.1 μmol/L, respectively. The inhibition of growth of the three cell lines by plumbagin was also time-dependent (data not shown). While, there was no significant alteration in the viability of normal lung epithelial cells (BEAS-2B) on exposure (up to 6 μM concentration) to plumbagin (data not shown).

To examine the long-term effects of plumbagin, clonogenic assay was performed. The clonogenicity of the three cell lines in the plumbagin groups was decreased in a concentration-dependent manner. At a concentration of 6μM, plumbagin inhibited about 55%, 65% and 70% of clone formation in A549, H292, and H460, respectively (Figure 1C and 1D). The in vitro clonogenic assay has been reported to correlate well to in vivo assays of tumorigenicity in nude mice (Freedman and Shin, 1974).

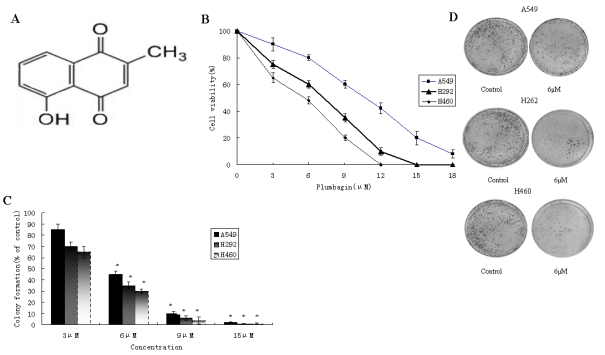

Figure 1. Effects of Plumbagin on the Proliferative Inhibition and Colony Formation in A549, H292, and H460 Cells. A) Chemical structure of plumbagin. B) Cell viability in plumbagin-treated A549, H292, and H460 cells. The cells were treated with indicated concentrations (0–18 μmol/L) of plumbagin for 24 h. C) Influence of plumbagin on the number of colony-forming in A549, H292, and H460 cells. Cells were treated with plumbagin (3–15μM) for 6 h and allowed to grow for 14 days to form colonies. D) Representative dishes of the colony-forming assay. The data shown are the mean from three independent experiments. Each value is the mean±S.D. of three independent experiments. (*), significant difference between control and plumbagin-treated cells, as analyzed by student t-test. (p<0.05)

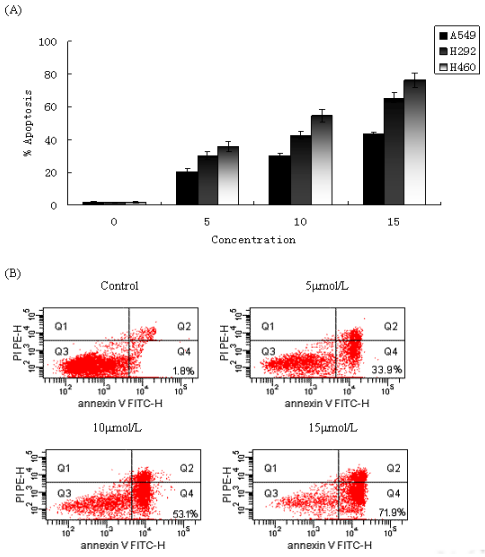

Figure 2. Plumbagin Enhanced Cell Apoptosis of NSCLC Cells. A) The degree of apoptosis in A549, H292, and H460 cell lines was quantified by Annexin V/PI double-staining. Data represented the mean ± SD of three individual experiments. (*), indicated difference between control and plumbagin-treated cells (P<0.05). B) Plumbagin induced apoptosis of H460 cells. Cells were incubated with 0, 5, 10, and 15 μmol/L plumbagin for 12 h. The apoptosis was analyzed by Annexin V-FITC/PI double-staining assay

Plumbagin induced apoptosis of NSCLC cells

The amount of apoptotic cell was quantified with Annexin V-FITC/PI double-labeled flow cytometry and TUNEL assay. The A549, H292, and H460 cells were pretreated with varying concentrations of plumbagin. This led to an increase in the number of apoptosis in the three cell lines. In A549 cell line, the total apoptotic

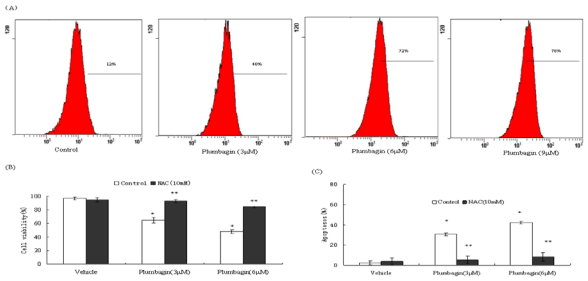

Figure 3. Plumbagin Leads to ROS-mediated Proliferative Inhibition and Apoptosis in H460 Lung Cancer Cells. A) ROS levels were determined 1h later by flow cytometric analysis. The data demonstrated difference in the levels of intracellular ROS in control versus plumbagin treated cells from a representative experiment. B) The role of ROS in plumbagin-mediated proliferative inhibition was assessed using ROS scavenger NAC. The influence of NAC on plumbagin-induced cytotoxicity was determined by CCK-8 assay. C) The effect of NAC on plumbagin-induced apoptosis was detected by Annexin V-FITC/PI double-staining assay. The data represent the mean ± SD. Asterisk denoted statistically significant difference (p<0.05) between vehicle (0 μM Plumbagin) and plumbagin treated cells. Double asterisk indicated statistically significant difference (p<0.05) between Plumbagin and Plumbagin + NAC-treated cells

rates were 1.7%±0.4%, 20.2%±1.8%, 30.2%±1.4%, and 43.3%±1.3% at plumbagin concentrations of 0 μmol/L, 5 μmol/L, 10 μmol/L, and 15 μmol/L; in H292 were 1.6±0.4%, 30.2±2.1, 42.5±2.3%, and 65.4±3.0%; in H460 were 1.8±0.6%, 35.6±3.3%, 54.8±3.2%, and76.4±4.8% (Figure 2A and 2B). Similar results were observed in TUNEL staining assay (data not shown).

Exposure to plumbagin led to ROS-mediated apoptosis in H460 lung cancer cells

Plumbagin has been reported to correlate to increased levels of ROS in divergent carcinomas including prostate, melanoma, cervical, and promyelocytic leukemia (Powolny et al., 2008; Xu et al., 2010). In the present study, we detected the influence of plumbagin treatment on H460 cells ROS levels. To this end, H460 cells were pre-labeled with 5 μM carboxy-H2DCFDA and then exposed to diverse concentrations of plumbagin for 1h. Exposure of H460 cells to various concentration of plumbagin led to an increase level of ROS-associated mean fluorescence intensity (MFI) compared with vehicle treated cells. The level of ROS correspondingly increased from 12% to 40%, 72% and 78%, respectively (Figure 3A). The implicated of the plumbagin-induced increase in ROS in H460 cells apoptosis was confirmed using NAC. More specifically, H460 cells were exposed for 24h to plumbagin ± NAC (10 mM) and then analyzed for viable cell number and induction of apoptosis using the Annexin V-FITC/PI double-labeled assay. The results demonstrated that treatment of H460 cells with NAC led to a significant reduction in plumbagin-induced cell killing and apoptosis (Figure 3B and 3C). Together, these results suggest that plumbagin-induced increase in ROS levels plays a crucial role in mediating apoptosis of H460 cells.

Plumbagin suppressed activation of NF-κB in H460 cells

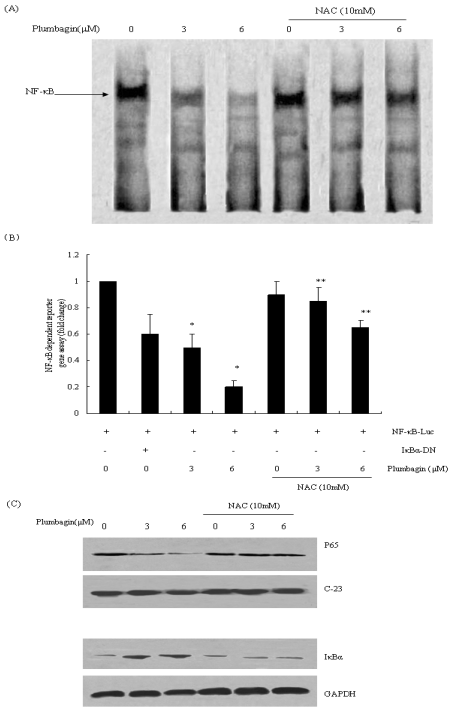

Figure 4. Plumbagin Inhibits NF-κB Activity in H460 Lung Cancer Cells. A) Plumbagin suppressed endogenous NF-κB DNA binding activity in a concentration-dependent manner and ROS scavenger NAC largely retrieved NF-κB activity.H460 lung cancer cells were pretreated with indicated concentrations of plumbagin for 6 h, incubated with or without 10 mM NAC for 30 minutes, and then subjected to EMSA to examine NF-κB activation. B) H460 cells were transiently transfected with an NF-κB-luciferase plasmid and then treated with the indicated concentrations of plumbagin for 2 h, presence or absence of 10 mM NAC for 30 minutes. Cell supernatants were thereafter collected and assayed for luciferase activity as described in Methods. Representative results of three independent experiments are shown. Results are expressed as fold activity over the activity of the vector control. Bars indicate standard deviation. Asterisk denoted statistically significant difference (p<0.05) between vehicle (0 μM Plumbagin) and plumbagin treated cells. Double asterisk indicated statistically significant difference (p<0.05) between Plumbagin and Plumbagin + NAC-treated cells. C) plumbagin inhibited p65 nuclear translocation and IκBα degradation by Western blot, while NAC partly retrieved the effect of plumbagin. H460 lung cancer cells were incubated with indicated concentrations of plumbagin for 6 h, incubated with or without 10 mM NAC for 30 minutes, and then nuclear extracts were analyzed for p65 by Western blot and equal amounts of cytoplasmic proteins for IκBα. C23 and GAPDH antibodies were used as controls. Results from three repeated and separated experiments were similar

and the activation of ROS was involved

NF-κB played a pivotal role in regulating cell apoptosis is relatively well documented (Karin et al., 2006). To identify whether plumbagin induced cell apoptosis is mediated by NF-κB and whether ROS is involved in the process, we investigated NF-κB activation using EMSA assay and luciferase reporter assay. Firstly, using EMSA assay, we demonstrated that plumbagin alone could suppress endogenous NF-κB DNA binding activity in a dose-dependent manner and ROS scavenger NAC could highly abrogate plumbagin induced NF-κB inactivation (Figure 4A). However, DNA binding alone is not always

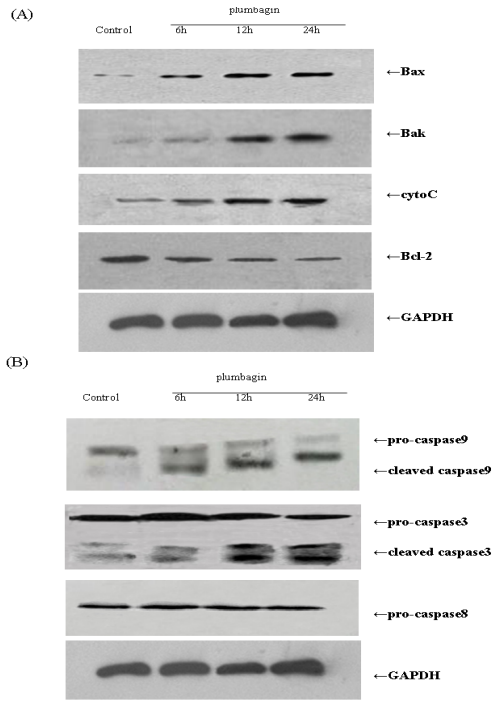

Figure 5. Effect of Plumbagin on Expression of NF-κB-regulated Apoptotic-related Proteins in H460 Cells. A) Western blot for protein expression of Bak, Bax, Bcl-2, and cytoC. Cells were treated with plumbagin (3 μM) for 6h, 12h and 24h. The expression of proteins was assessed by western blotting, as described under Methods. The blot is representative of three separate experiments. B) H460 cells were treated with plumbagin (3 μM) for 6h, 12 h and 24 h. Protein lysates were prepared and subjected to Western blot analysis as described in Methods using antibodies against caspase-8, caspase-3, and caspase-9. Equal protein loading was ensured by demonstrating uniform GAPDH expression. The blot is representative of three separate experiments

associated with NF-κB-dependent gene transcription (Nasuhara et al., 1999; Manu et al., 2011), suggesting that additional regulatory steps are involved. So we further performed NF-κB-luciferase reporter assay to confirm the inhibition effect of plumbagin on NF-κB. Secondly, similar results were also observed by NF-κB-luciferase reporter gene assay. As shown in Figure 4B, Plumbagin significantly inhibited NF-κB reporter gene activity in a dose-dependent manner and NAC partly retrieved NF-κB activity. Together, our results suggested that plumbagin significantly inhibited NF-κB activity in lung cancer cells and the activation of ROS may be implicated in the mechanism.

Plumbagin inhibited p65 nuclear translocation and IκBα degradation implicated in ROS

The effects of plumbagin on p65 nuclear translocation and the influence of ROS were examined in lung cancer cells. To confirm the data, we performed western blot analysis of p65 in nuclear extracts. As showed in Figure 4C, treatment of plumbagin inhibited p65 nuclear translocation and ROS scavenger NAC could highly reverse its nuclear translocation activity. To understand whether treatment of plumbagin affects upstream of NF-κB/p65 pathway, we examined IκBα degradation. Treatment of plumbagin markedly inhibited degradation

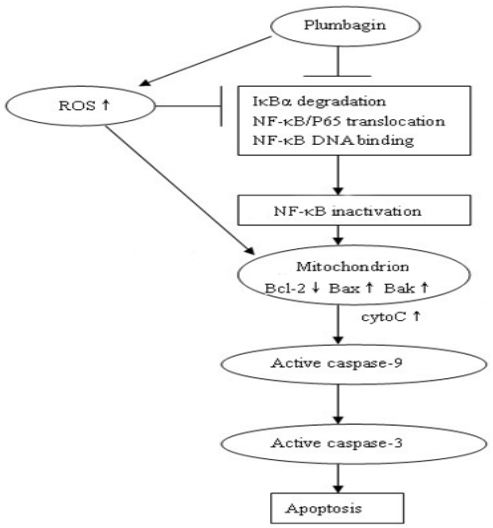

Figure 6. Schematic Diagram of Plumbagin Induced Apoptotic Pathway in Lung Cancer Cells

of IκBα in lung cancer cells and NAC partly reversed the effect (Figure 4C).

Effect of plumbagin on expression of NF-κB-regulated apoptotic-related proteins in lung cancer cells

NF-κB is known to regulate a variety of cell functions, including apoptosis, proliferation, invasion and angiogenesis through regulating target gene expression (Karin et al., 2006). Using western blot analysis, we examined the effects of plumbagin on apoptotic gene expression which is regulated by NF-κB activation. As shown in Figure 5A, levels of the proapoptotic and antiapoptotic proteins were altered after treatment of plumbagin. Western blot analysis showed an increase in the levels of Bax, CytoC and Bak proteins in a time-dependent manner in H460 lung cancer cells, and a decrease of Bcl-2 protein. The involvement of caspase activation in plumbagin mediated cell apoptosis in lung cancer cell line was also determined with the use of western blot. The activation of caspase-9 and caspase-3 but not caspase-8 was observed (Figure 5B).

Discussion

Lung cancer has long been the leading mortality in both developed and developing countries. Unfortunately, there is limited therapy available for this malignant disease due to fewer efficacies of chemotherapy and radiotherapy. Non-small cell lung cancer (NSCLC) is the predominant subtype and has unfavorable outcome. In our study, we have found that plumbagin effectively inhibited three kinds of NSCLC cell lines including A549, H262, and H460 growth in vitro, concomitant with induction of apoptosis.

Emerging research has demonstrated that plumbagin exerts anti-carcinogenic effects. Previous studies examining the effect of plumbagin on lung cancer A549 cell line showed the inhibition was dose and time-dependent and with the IC50 value of 11.69μM for 48h treatment (Hsu et al., 2006). In the present research, we also observed the concentration and time dependent anti-

proliferatition of plumbagin in A549, H292, and H460 cells with the IC50 values 10.3 μM, 7.3 μM, and 6.1 μM for 12h, respectively (Figure 1B).

The occurrence and development of carcinoma is closely related to cell apoptosis (Krysko et al., 2008). Mitochondrial apoptotic pathway has been described as an important signaling of apoptotic cell death for mammalian cells (Hengartner et al., 2000). It is well known that Bcl-2 family and cytochrome C (cytoC) of proteins play a central role in the regulation of the intrinsic (mitochondrial) apoptotic pathway. Many reports have shown that plumbagin induced apoptosis through the mitochondrial-mediated pathway in breast cancer and lung cancer (Hsu et al., 2006; Ahmad et al., 2008; Kawiak et al., 2012). Following the treatment of H460 cells with plumbagin, we observed that plumbagin treatment resulted in a significant increase of Bax, Bak and cytoC expression, and a decrease of Bcl-2, which was consistent with Hsu et al. Caspases play a central role in apoptosis execution. Their activation is essential for the occurrence of the typical hallmarks of apoptosis such as chromatin condensation and DNA fragmentation. As shown in Figure 5B, treatment with plumbagin increased caspase-9 activity in H460 cells. This is consistent with the release of cytochrome c into the cytosol from mitochondria, potentially activating caspase-9. The activated upstream caspase-9 acts on the downstream target of caspase-3 enzymes, subsequently, the activated caspase-3 acts on the target cells as an effector molecule, damaging the cell structure and causing functional disorder by proteolysis, ultimately inducing apoptosis through the caspase-dependent mitochondria pathway (Deng et al., 2008) .

The function of NF-κB for regulating target gene expression, including apoptosis-related and proliferation-related is also well documented (Mocellin et al., 2005; Karin, 2006). We determined that plumbagin was able to inhibit the expression of proteins involved in anti-apoptosis (ie, Bcl-2) and enhance the expression in pro-apoptosis (ie, Bax, Bak, and cytoC), all of which are linked to, or regulated by NF-κB. Based on our findings, we hypothesized that plumbagin may have an effect on the NF-κB pathway in lung cancer cells. In current study, we found that plumbagin suppressed activation of NF-κB and inhibited NF-κB-p65 nuclear translocation and IκBα degradation. Our result was consistent with many reports which have investigated the effect of down-regulating NF-κB activation by plumbagin in various tumor cells (Sandur et al., 2006; Shieh et al., 2010; Manu et al., 2011; Li et al., 2012). Together, those results showed that inhibition of NF-κB activation by plumbagin was not cell-type specific.

Plumbagin is a naturally occurring naphthoquinone, its cytotoxic properties is related to its quinone core and contribute to its therapeutic activity. There are two main mechanisms with the therapeutic activity of quinones including the production of the semiquinone radical under aerobic conditions which participates in redox cycling to generate reactive oxygen species (ROS) like superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) and as a potent electrophile reacting with the thiol groups of proteins and glutathione (GSH) (Castro et al., 2008). The production of ROS has been suggested to play a pivotal role in the DNA damage, and may exert the cytotoxic effects of menadione in cultured human cells (Noto et al., 1989; Ngo et al., 1991). Previous research found that plumbagin-induced apoptosis was associated with generation of ROS in various cancer cell lines, including leukemia, cervical, prostate, and melanoma (Srinivas et al., 2004; Powolny and Singh, 2008; Wang et al., 2008; Xu and Lu, 2010). We postulated that plumbagin induced apoptosis also through an increase in ROS in H460 cells. This was directly confirmed by our studies that plumbagin exposure led to increase ROS levels in H460 cells and indirectly in experiments that treatment with free radical scavenger NAC prevented plumbagin-mediated apoptosis. To our knowledge, we have demonstrated for the first time that plumbagin can generate ROS in non-small lung cancer cells. We propose that this effect may be important for the induction of apoptosis after treatment with plumbagin. However, the mechanism by which plumbagin generates ROS remains elusive. The quinone structure of plumbagin allows it to conduct electrons from two-electron donors to single-electron acceptors in the respiratory pathways of both prokaryotic and eukaryotic cells thus forming as a superoxide generator (Imlay and Fridovich, 1992). Thus, we hypothesized that the target of plumbagin induced ROS may be mitochondrial respiratory chain; however, further elaborately experiments should be conducted before reaching to such a conclusion.

In the present study, we investigated that plumbagin could both increase ROS level and inhibit activation of NF-κB. Which is the upstream mediator, ROS or NF-κB? ROS is well known to be involved in diverse physiological and pathological processes, and oxidative stress can activate the NF-κB pathway (Barchowsky et al., 1996; Qian et al., 2012). To explore whether or not ROS is the upstream mediator of NF-κB, ROS scavenger NAC was added to plumbagin treated H460 cells. We observed that NAC could partly abrogate the effect of plumbagin on inhibition of NF-κB activity. These results indicated that excess ROS generation may be an upstream signal of NF-κB inactivation in this process. However, whether there is a feedback loop between ROS and NF-κB, and the precise regulation mechanism should be further identified.

It can be concluded from the above results that plumbagin inhibited the proliferation of A549, H292, and H460 cells in a dose and time-dependent manner. The cytotoxic effect of plumbagin-induced cell death is through the induction of apoptosis, as indicated by the current data. Plumbagin exposure led to generation of ROS in H460 cells. Subsequently, excess ROS generation inhibited the activation of NF-κB, which down-regulated the expression of anti-apoptotic proteins and up-regulated pro-apoptotic proteins. Thereafter, caspase-dependent mitochondrial pathway was activated, as shown by the plumbagin-mediated activation of caspases-3 and -9 (Figure 6). Plumbagin therefore have the potential to be a potent agent for non-small lung cancer treatment.

Acknowledgements

The authors have no financial conflicts of interest with respect to this manuscript.

References

Ahmad A, Banerjee S, Wang Z, et al (2008). Plumbagin-induced apoptosis of human breast cancer cells is mediated by inactivation of NF-kappaB and Bcl-2. *J Cell Biochem*, **105**, 1461-71.

Aziz MH, Dreckschmidt NE, Verma AK (2008). Plumbagin, a medicinal plant-derived naphthoquinone, is a novel inhibitor of the growth and invasion of hormone - refractory prostate cancer. *Cancer Res*, **68**, 9024-32.

Barchowsky A, Dudek EJ, Treadwell MD, et al (1996). Arsenic induces oxidant stress and NF-KB activation in cultured aortic endothelial cells. *Free Radic Biol Med*, **21**, 783-90.

Castro FA, Mariani D, Panek AD, et al (2008). Cytotoxicity Mechanism of Two Naphthoquinones (Menadione and Plumbagin) in Saccharomyces cerevisiae. *PLoS One*, **3**, e3999.

Deng L, Adachi T, Kitayama K, et al (2008). Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. *J Virol*, **82**, 10375-85.

Ding YX, Chen ZJ, Liu S, et al (2005). Inhibition of Nox-4 activity by plumbagin, a plant-derived bioactive naphthoquinone. *J Pharm Pharmacol*, **57**, 111-6.

Freedman VH, Shin SI (1974). Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell*, **3**, 355-9.

Hengartner MO (2000).The biochemistry of apoptosis. *Nature*, **407**, 770-6.

Hsu YL, Cho CY, Kuo PL, et al (2006). Plumbagin (5-Hydroxy-2 -methyl- 1, 4- naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53 accumulation via c-Jun NH2-terminal kinase-mediated phosphorylation at serine 15 in vitro and in vivo. *J Pharmacol Exp Ther*, **318**, 484-94.

Imlay J, Fridovich I (1992). Exogenous quinones directly inhibit the respiratory NADH dehydrogenase in Escherichia coli. *Arch Biochem Biophys*, **296**, 337-46.

Jemal A, Siegel R, Ward E, et al (2009). Cancer statistics, 2009. *CA Cancer J Clin*, **59**, 225-49.

Kawiak A, Zawacka-Pankau J, Lojkowska E (2012). Plumbagin induces apoptosis in Her2-overexpressing breast cancer cells through the mitochondrial-mediated pathway. *J Nat Prod*, **75**, 747-51.

Karin M (2006). Nuclear factor-kappaB in cancer development and progression. *Nature*, **441**, 431-6.

Kuo PL, Hsu YL, Cho CY (2006). Plumbagin induces G2-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells. *Mol Cancer Ther*, **3**, 209-21.

Krysko DV, Vanden Berghe T, D'Herde K, et al (2008). Apoptosis and necrosis: detection, discrimination and phagocytosis. *Methods*, **44**, 205-21.

Li J, Shen L, Lu FR, et al (2012). Plumbagin inhibits cell growth and potentiates apoptosis in human gastric cancer cells in vitro through the NF-κB signaling pathway. *Acta Pharmacol Sin*, **33**, 242-9.

Manu KA, Shanmugam MK, Rajendran P, et al (2011). Plumbagin inhibits invasion and migration of breast and gastric cancer cells by downregulating the expression of chemokine receptor CXCR4. *Mol Cancer*, **10**, 107.

Mocellin S, Rossi CR, Pilati P, et al (2005). Tumor necrosis factor, cancer and anticancer therapy. *Cytokine Growth Factor Rev*, **16**, 35-53.

Mossa JS, El-Feraly FS, Muhammad I (2004). Antimycobacterial constituents from juniperus procera, ferula communis and plumbago zeylanica and their in vitro synergistic activity with isonicotinic acid hydrazide. *Phytother Res*, **18**, 934-7.

Nair S, Nair RR, Srinivas P, et al (2008). Radiosensitizing effects of plumbagin in cervical cancer cells is through modulation of apoptotic pathway. *Mol Carcino*, **47**, 22-33.

Nasuhara Y, Adcock IM, Catley M, et al (1999). Differential IkappaB kinase activation and IkappaBalpha degradation by interleukin-1beta and tumor necrosis factor-alpha in human U937 monocytic cells. Evidence for additional regulatory steps in kappaB-dependent transcription. *J Biol Chem*, **274**, 19965-72.

Ngo EO, Sun TP, Chang JY, et al (1991). Menadione-induced DNA damage in a human tumor cell line. *Biochem Pharmacol*, **42**, 1961-8.

Noto V, Taper HS, Jiang YH, et al (1989). Effects of sodium ascorbate (vitamin C) and 2-methyl-1, 4-naphthoquinone (vitamin K3) treatment on human tumor cell growth in vitro. I. Synergism of combined vitamin C and K3 action. *Cancer*, **63**, 901-6.

Ohe Y, Ohashi Y, Kubota K, et al (2007).Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. *Ann Oncol*, **18**, 317-23.

Powolny AA, Singh SV (2008). Plumbagin-induced apoptosis in human prostate cancer cells is associated with modulation of cellular redox status and generation of reactive oxygen species. *Pharm Res*, **25**, 2171-80.

Pujol JL, Barlesi F, and Daures JP (2006). Should chemotherapy combinations for advanced non-small cell lung cancer be platinum-based? A meta-analysis of phase III randomized trials. *Lung Cancer*, **51**, 335-45.

Qian Y, Guan T, Huang M, et al (2012). Neuroprotection by the soy isoflavone, genistein, via inhibition of mitochondria-dependent apoptosis pathways and reactive oxygen induced-NF-κB activation in a cerebral ischemia mouse model. *Neurochem Int*, **60**, 759-67.

Sandur SK, Ichikawa H, Sethi G, et al (2006). Plumbagin (5-Hydroxy-2-methyl-1, 4-naphthoquinone) suppresses NF-κB activation and NF-κB-regulated gene products through modulation of p65 and IκB kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents. *J Biol Chem*, **281**, 17023-33.

Shieh JM, Chiang TA, Chang WT, et al (2010). Plumbagin inhibits TPA-induced MMP-2 and u-PA expressions by reducing binding activities of NF-κB and AP-1 via ERK signaling pathway in A549 human lung cancer cells. *Mol Cell Biochem*, **335**, 181-93.

Srinivas P, Gopinath G, Banerji A, et al (2004). Plumbagin induces reactive oxygen species, which mediate apoptosis in human cervical cancer cells. *Mol Carcinog*, **40**, 201-11.

Thasni KA, Rakesh S, Rojini G, et al (2008). Estrogen-dependent cell signaling and apoptosis in BRCA1-blocked BG1 ovarian cancer cells in response to plumbagin and other chemotherapeutic agents. *Ann Oncol*, **19**, 696-705.

Wang CC, Chiang YM, Sung SC, et al (2008). Plumbagin induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human melanoma A375.S2 cells. *Cancer Lett*, **259**, 82-98.

Xu KH, Lu DP (2010). Plumbagin induces ROS-mediated apoptosis in human promyelocytic leukemia cells in vivo. *Leuk Res*, **34**, 658-65.