Anticancer Mechanism of Plumbagin, a Natural Compound, on Non-small Cell Lung Cancer Cells

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Abstract. *Background: Lung cancer is the leading cause of cancer-related deaths in the United States. Prevailing treatment options have limited therapeutic success in lung cancer, particularly non-small cell lung cancer (NSCLC), as it becomes resistant to therapy. Hence, better therapeutic options are immediately required for lung cancer. Plumbagin, a natural compound has been recently examined for its anticancer effect on different cancers. Materials and Methods: To determine the anticancer effect of plumbagin on NSCLC cell lines H460 and A549, cell viability, apoptotic, Western blot and reporter assays were performed. Results: Plumbagin significantly inhibited the growth of H460 cells compared to A549 cells, and down-regulated the expression of EGFR/Neu and its downstream signaling (Akt, NF-κB, Bcl-2 and survivin) in H460 cells. In addition, plumbagin up-regulated the expression of p53 and p21$^{CIP1/WAF1}$ causing cell cycle arrest in the G_2/M-phase by down-regulating G_2/M regulatory proteins (cyclinB1 and Cdc25B) in H460 cells. Furthermore, it activated the JNK/p38 signaling, leading to caspase-3 activation resulting in the induction of apoptosis. Conclusion: Plumbagin exerted anticancer activity on NSCLC cells by modulating the pro-survival and pro-apoptotic signaling that causes induction of apoptosis.*

Lung cancer is the leading cause of cancer-related deaths in the United States. In spite of the advancements in cancer therapy, the prognosis for lung cancer patients continues to be poor, with 31% mortality in men and 26% in women (1). Detection and diagnosis of lung cancer during the early stages is difficult due to the absence of characteristic symptoms. Prevailing treatment options, such as chemotherapy and radiation therapy have limited therapeutic success in lung cancer particularly in non-small cell lung cancers (NSCLC), as they develop resistance to therapy (2). Therefore, better therapeutic options including those which could potentially prevent/inhibit the initial stages of lung carcinogenesis are considered to be immensely beneficial. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), is a natural quinonoid constituent, isolated from the root of the plant *Plumabago zeylanica* (3), which exerts anti-atherogenic, cardiotonic, hepatoprotective and neuroprotective effects and has been widely used in traditional medicine (4). The anticancer and antiproliferative properties of plumbagin have been reported in various cancer cell lines (5-7). It has also been reported to exhibit radiosensitizing effect in cell culture models, *in vitro*, and mouse tumor models, *in vivo* (8).

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases (RTKs) and is reported to be associated with important tumorigenic processes, such as proliferation, invasion, survival and angiogenesis, and is highly expressed in NSCLC (9, 10). Therefore, clinical inhibitors targeting EGFR are significantly in demand for lung cancer therapy (11). EGFR activates two important signaling pathways: the Ras-Raf-MEK-ERK and the PI3K-Akt kinase pathways, which are also implicated in oncogenic cell proliferation, survival and cell growth (12). PI3K/Akt/MAPK signaling has been reported to be involved in cell survival and promotion of several tumors including lung cancer (13). EGFR activates PI3K through activation of its cognate receptors, leading to the activation of Akt, thereby mediating key cell survival mechanisms and oncogenic promotion in various tumor types (14).

Sequential activation of cyclin-dependent kinases are involved in eukaryotic cell cycle regulation (15) and deregulated expression of these proteins are commonly observed in cancer cells (16). Cyclin-B1 and Cdc complex are associated with the entry into mitosis thereby regulating the G_2/M transition. Cyclin-dependent kinase inhibitors

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such as p21/WAF-1 bind to the cyclin-B1/Cdc complex thereby inducing cell cycle arrest (15, 17).

The mitogen-activated protein kinase (MAPK) family also comprises signaling proteins that regulate the fundamental processes of cell proliferation, differentiation and survival. The c-Jun NH2-terminal kinase (JNK), a member of the MAPK family, is reported to regulate both cell proliferation and apoptosis, depending on the stimuli of the signaling (18, 19). JNK is proposed to induce stress-activated apoptosis by stabilizing p53 and inhibiting the activity of Bcl-2 (20). p38 MAPK functions as a sensor of oxidative stress and regulates multiple cellular processes which either block cell proliferation or induce apoptosis (21, 22). Nuclear factor-κB (NF-κB) is a ubiquitously expressed transcription factor that regulates the expression of genes controlling proliferation, differentiation and apoptosis (23). Recent reports identify a novel stress-induced mechanism mediated by p38 MAPK, resulting in the down-regulation of NF-κB transcription activity, ultimately resulting in apoptosis (24).

In the present study, we report the effect of plumbagin on H460 and A549 lung cancer cells. plumbagin sensitizes H460 more significantly than A549 cells. Further, dissection of the mechanism of action of plumbagin on H460 cells suggests that it targets EGFR mediated Akt signaling and causes a G_2/M arrest resulting in the induction of apoptosis.

Materials and Methods

Cell culture. The lung cancer cell lines A549 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) and 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA). The cell lines were maintained at 37˚C in a 5% CO_2/95% air-humidified atmosphere.

MTT assay. The cytotoxicity of plumbagin to H460 and A549 cells treated with concentrations of 3, 6, 9, 12 and 15 μM for 24 h was determined using MTT assay as described elsewhere (25-27). The control group was treated with the vehicle alone – dimethyl sulfoxide (Sigma Aldrich).

Quantification of apoptosis. Two different apoptotic assays were used to quantify the induction of apoptosis. Apoptosis was determined using Annexin V-FITC apoptosis kit I (BD Pharmingen, San Diego, CA, USA) and Promega-dead end™ fluorimetric TUNEL assay system (Promega, Madison, WI, USA) using standardized protocols with 10 μM of plumbagin for 24 and 48 h (27, 28).

Cell cycle analysis. Cell cycle analysis was carried out in H460 cells treated with 10 μM plumbagin using propidium iodide (PI) staining and subsequent flow cytometry. Flow cytometry was performed as described elsewhere (29).

Transient transfection and luciferase assays. H460 cells (at 80-90% confluency) were transiently transfected using Lipofectamine plus reagents (Invitrogen) with 4 μg of the NF-κB-luciferase construct (containing 2 tandem NF-κB responsive sites) in the presence of β-galactosidase control or vector containing renilla luciferase to normalize the transfection efficiency, as described elsewhere (26). Transfected cells were treated with plumbagin or vehicle (DMSO) as indicated in Figure 3C, and the cells were harvested after 12 h for luciferase or β-galactosidase activity assays.

Western blot analysis. Total protein extracts from untreated cells or cells treated with plumbagin at different time intervals were subjected to Western blot analysis as described elsewhere (25). The expression patterns of Raf, ERK, MEK-1, Pp38, pJNK, Bcl-2, p53, survivin, Akt, pAkt, EGFR, Neu were detected using specific antibodies all (Cell Signalling Technology, Danvers, MA, USA) and β-actin (Sigma Aldrich) was used as the loading control (11-13).

NF-κB activation assay. H460 cells were treated with plumbagin at 10 μM for 12 and 24 h, and the nuclear and cytoplasmic extracts were obtained as described elsewhere (30). Both the nuclear and cytoplasmic extracts were subjected to Western blot analysis and the membranes were probed with p65 (NF-κB) (sc-8008) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody.

Fluorimetric assay for caspase-3 activation. Caspase activation was analyzed using the ApoAlert™ Caspase-3 Fluorescent Assay kit (BD Clontech, Mountain View, CA, USA). Approximately $1x10^6$ H460 cells were plated, plumbagin-treated or untreated cells for up to 24 h were pelleted and processed according to the manufacturer's instructions. The samples were then transferred to 96-well plates and read in a fluorometer with an excitation wavelength of 400 nm and emission wavelength of 505 nm to determine the effect of plumbagin on caspase activation (11-13).

Results

Plumbagin inhibited cell viability and induced apoptosis in lung cancer cells. To determine the anti-cancer effect of plumbagin on lung cancer cells (A549 and H460) we analyzed cell viability and apoptotic assays. The viability of H460 cells, which were exposed to concentrations of plumbagin at 3, 6 and 9 μM for 24 h, decreased to 56.97±0.02%, 37.51±0.02% and 3.73±0.066% respectively, compared with the corresponding controls (Figure 1A). The viability of A549 cells exposed to plumbagin at 3, 6, 9, 12 and 15 μM for 24 h decreased to 93.1±0.006%, 79.3±0.029%, 55.17±0.03%, 33.1±0.035% and 17.6±0.03%, respectively (Figure 1A). 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). These results suggest that plumbagin effectively inhibits the proliferation of H460 cells compared to A549 cells without significantly affecting the viability of normal lung epithelial cells. To find the effect of plumbagin on the induction of apoptosis, H460 cells were subjected to Annexin V-FITC and TUNEL apoptosis assays. Plumbagin, at 5 μM concentration, induced [45.91% apoptosis at 24 h or 48 h

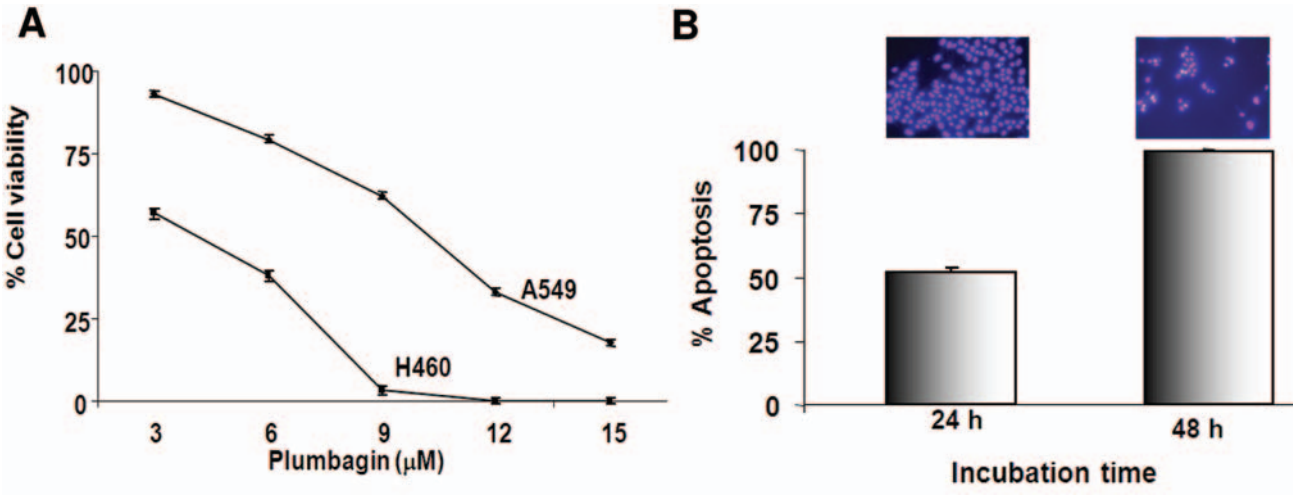

Figure 1. *A) Effect of plumbagin on the cell viability of lung cancer cell lines. The lung cancer cell lines A549 and H460 were treated with different concentrations of plumbagin, dissolved in DMSO, for 24 h, and the cell viability was measured by MTT assay. Each data point represents the mean of four wells from three independent experiments (mean±SE). B) Induction of apoptosis in lung cancer cells by plumbagin. H460 cells were treated with plumbagin and apoptosis assays were performed at 24 and 48 h. The bar graph shows the percentage of apoptotic cells. The base-line apoptosis in the untreated group was normalized with data from the treated group. The data shown are representative of the combined means from three independent experiments and the error bars represent the standard error.*

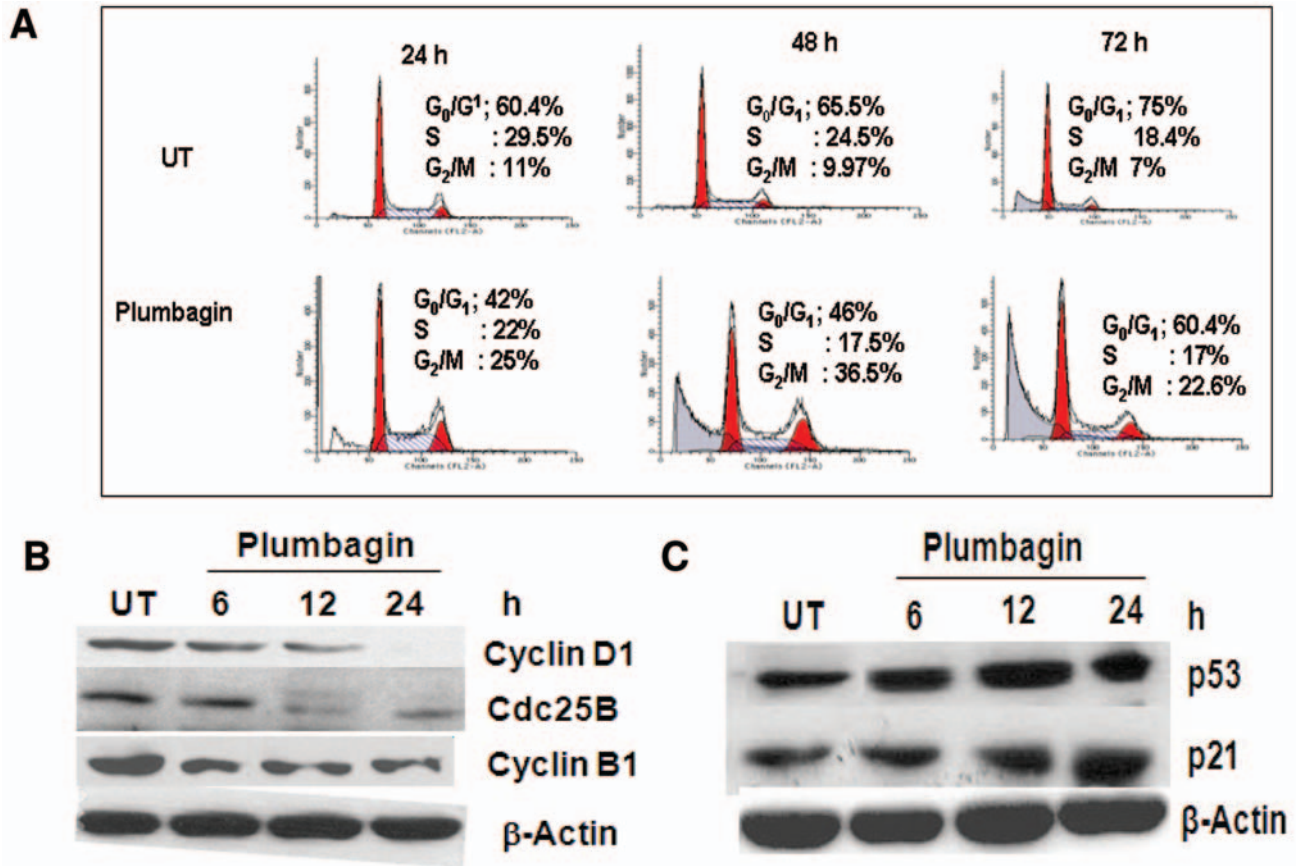

Figure 2. *A) Effect of plumbagin on cell cycle progression in lung cancer cells. Asynchronous cultures of H460 cells were treated with plumbagin for 24, 48 and 72 h and the cells were resuspended. At each time point the treated as well as untreated cells (UT) were stained with propidium iodide, and analyzed by flow cytometry. The results were analyzed adopting MODFIT LT statistics program. B) Effect of plumbagin on cell cycle regulatory proteins in H460 cells. Whole-cell protein extracts were prepared from H460 cells after being treated with plumbagin for the indicated times and were subjected to Western blot analysis. The blot was probed with antibodies for cyclin B1, cdc25B, cyclin D1 (B) p53 and p21(C). β-Actin was used as a internal loading control.*

and 96.5%] apoptosis in H460 cells (Figure 1B). Similar results were observed in TUNEL staining in H460 (data not shown).

Plumbagin induced G_2/M cell cycle arrest and down-regulated cell cycle-related proteins in H460 cells. The cell cycle assay indicated that a majority of the untreated H460 cells (80-90%) were in the G_1- and S- phases of the cell cycle because of the state of high proliferation. After treating the cells with plumbagin for 24, 48 and 72 h a marked increase in the percentage of H460 cells in the G_2/M-phase of the cell cycle (22-36%) accompanied by a decrease in the percentage of cells in the G_1-phase was observed indicating cell cycle arrest in the G_2/M-phase (Figure 2A). The effect of plumbagin on the G_2/M cell cycle regulatory proteins was examined, and revealed that plumbagin down-regulated the expression of cyclin B1, Cdc25B, and cyclin D1 in a H460 cells in a time-dependent fashion (Figure 2B). On the other hand, an induction of p53 and $p21^{CIP1/WAF1}$ in a time-dependent manner was observed in plumbagin-treated cells (Figure 2C). Collectively, these analyses suggest that plumbagin arrests the cells at the G_2/M-phase of the cell cycle in H460 cells.

Effect of plumbagin on EGFR signaling in H460 cells. Multiple ligands binding to EGFR/Neu growth factor receptors activate several downstream oncogenic signaling cascades. The influence of plumbagin on the expression of EGFR and Her-2/Neu receptors in H460 cells was investigated. Plumbagin down-regulated EGFR and Neu expression in a time-dependent manner (Figure 3A). Interestingly, plumbagin did not influence or alter the expression of Raf, ERK or MEK-1 (data not shown), however, phosphorylated Akt expression was down-regulated without altering the expression levels of total Akt (Figure 3A). Further more, the effect of plumbagin in the downstream signaling of Akt, was examined. First we examined the expression of NF-κB. Plumbagin caused down-regulation of the expression of cytoplasmic and nuclear p65 in plumbagin-treated H460 cells (Figure 3B) and also inhibited NF-κB-dependent reporter activity (10-fold) (Figure 3C). In addition, target genes downstream of NF-κB such as Bcl-2 and survivin were also found to be markedly down-regulated in H460 cells (Figure 3D). These results comprehensively reflect the inhibitory role of plumbagin on the pro-survival signaling in H460 cells.

Effect of plumbagin on pro-apoptotic proteins in H460 cells. The JNK and p38 MAP kinase pathway proteins are associated with pro-survival as well as pro-apoptotic functions and therefore the expression levels of pJNK and pp38 were both investigated using Western blotting in the present study. The results clearly show that both phosphorylated JNK and p38 increased gradually from six hours and peaked at the 24 hour time-point, implying that plumbagin mediated stress activated apoptosis (Figure 4A). Further more plumbagin-treated H460 cells at 3, 6, 12 and 24 h treatment showed 1.6-, 5.4-, 16.8- and 52.2-fold induction of caspase 3/7 activity, indicating that plumbagin activated the caspase-3 cascade leading to induction of apoptosis (Figure 4B).

Discussion

Resistance of tumor cells to apoptosis is a major obstacle in all malignancies; hence, novel targeted therapies are much needed. This study examined the effect of plumbagin on non-small cell lung cancer and elucidates the molecular mechanism(s) by which it exhibits anticancer activity. Plumbagin, a napthoquinonoid compound has been reported to induce apoptosis in several cancer cell lines including those of the breast, ovary and lung (2, 3, 31). The results of our study are also in agreement with other studies.

In this study, plumbagin down-regulated cyclin B1 and Cdc25B protein expressions, which might have resulted in the induction of G_2/M arrest in H460 cells. These results are supported by previous studies which suggests that inhibition of cyclin B1 by different agents such as ionizing radiation (32), adriamycin (33), or sulforaphane (34) caused significant G_2/M arrest in many cancer cell types. On the other hand, the tumor suppressor gene *p53* plays a critical role in regulation of cell cycle and induction of apoptosis (35). Our results indicate that plumbagin induced apoptosis in H460 cells with a concomitant increase in the level of p53. The $p21^{CIP1/WAF1}$ gene, a transcriptional target of p53, is strongly induced by DNA damage in cells expressing functional p53 (36, 37) and plays a crucial role in the G_2/M checkpoint (36) due its inhibitory effect on the cdc2/cyclin-B complex (38, 39). Up-regulation of $p21^{WAF1/CIP1}$ by several chemotherapeutic agents including natural products have been reported to be associated with G_2/M arrest in the cell cycle (40). Based on such findings and our results, it may be suggested that induction of $p21^{CIP1/WAF1}$ by plumbagin could have caused a G_2/M arrest in H460 cells.

To elucidate the influence of plumbagin on oncogenic growth factors and their downstream effectors, we studied the effect of plumbagin on EGFR and its downstream signaling. Our results suggest that plumbagin downregulates the expression of EGFR/Neu in H460 cells. Many types of cancer, including NSCLC (40-80%) over express EGFR and inhibition of EGFR activity is being examined as an approach for the treatment of several types of cancer (41). Studies have shown that the activation of EGFR stimulates a series of downstream signaling events which play an important role in the regulation of signal

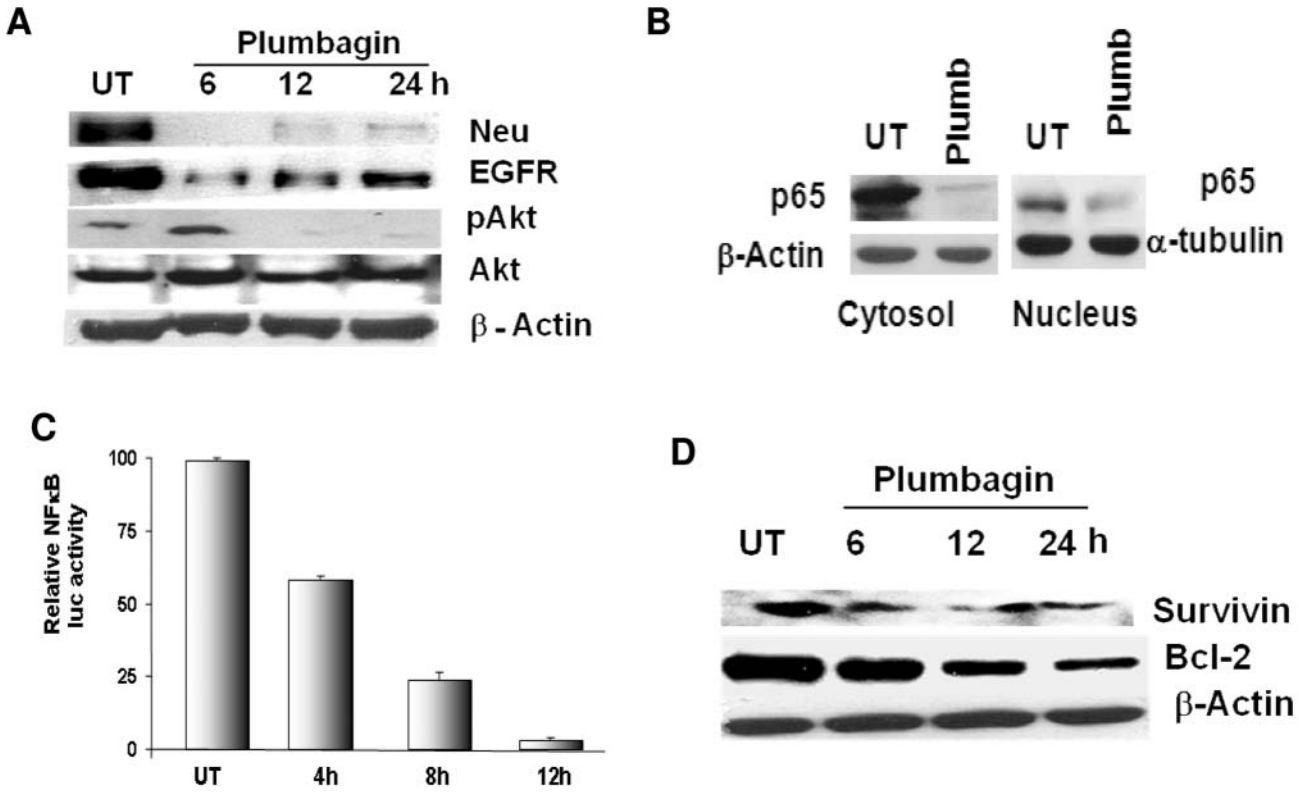

Figure 3. *The effect of plumbagin on EGFR signaling in H460 cells. A) Cell lysates were prepared after cells were treated with plumbagin and Western blot analysis were performed for the expressions of Neu, EGFR, Akt and pAkt in H460 cells UT: untreated cells. B) Nuclear and cytosolic extracts, as indicated, were prepared from H 460 cells that were either left untreated or treated with plumbagin and subjected to Western blot analysis. The blot was probed with antibodies for p65 and β-actin was used as internal loading control. C) H460 cells were transfected with NF-κB-reporter construct and β-galactosidase construct. The transfectants were treated with vehicle or plumbagin and, after 12 h, cell lysates were subjected to luciferase activity assays. Relative luciferase activity normalized with respect to corresponding β-galactosidase activity is shown (expressed as a percentage). D) Cell lysates were subjected to Western blot analysis and the expression patterns of Bcl-2 and survivin were analyzed in H460 cells.*

transduction pathways (42). The two major downstream signaling pathways are the Ras/ERK and PI3K/Akt. In our studies, plumbagin failed to alter the expression levels of Ras/Raf/ERK signaling. Hence, we examined the Akt pathway to determine whether plumbagin is capable of targeting EGFR downstream events and found that the expression of pAkt was down-regulated in a time-dependent manner in H460 cells. Reports indicate that resistance to EGFR inhibitors such as gefinitib may occur as a result of activation of EGFR's downstream signals, such as Akt signaling (43). Our results suggest that plumbagin down-regulates EGFR-mediated Akt signaling in H460 cells.

The transcription factor NF-κB is up-regulated in lung cancer cells and inhibition of NF-κB markedly sensitizes lung cancer cells to apoptosis (44). NF-κB is associated with cell proliferation, angiogenesis, metastasis and inhibition of apoptosis; these processes may promote tumor progression and chemo resistance (45, 46). In this study, we show that plumbagin inhibited the expression level of p65 (NF-κB) in the cytoplasm as well as in the nucleus in H460 cells. Therefore, inhibition of the anti apoptotic effect of NF-κB by plumbagin may contribute to the induction of apoptosis in lung cancer cells. Bcl-2 is the downstream target of NF-κB and its over expression imparts a survival advantage on cancer cells (47). Down-regulation of Bcl-2 may increase the sensitivity of the cell to chemotherapeutic drugs and radiation (48-50). Thus, therapeutic strategies directed towards inhibition of Bcl-2 activation, either directly or indirectly, will gain great clinical importance. We found that plumbagin down-regulated Bcl-2 protein expression in H460 cells, suggesting that plumbagin may prove to be of potent therapeutic application in lung cancer. Survivin is a member of the inhibitors of apoptosis (IAP) gene family that acts through pathways different from those involving the Bcl-2 family. Largely undetectable in normal adult tissues, survivin is deregulated in most type of human

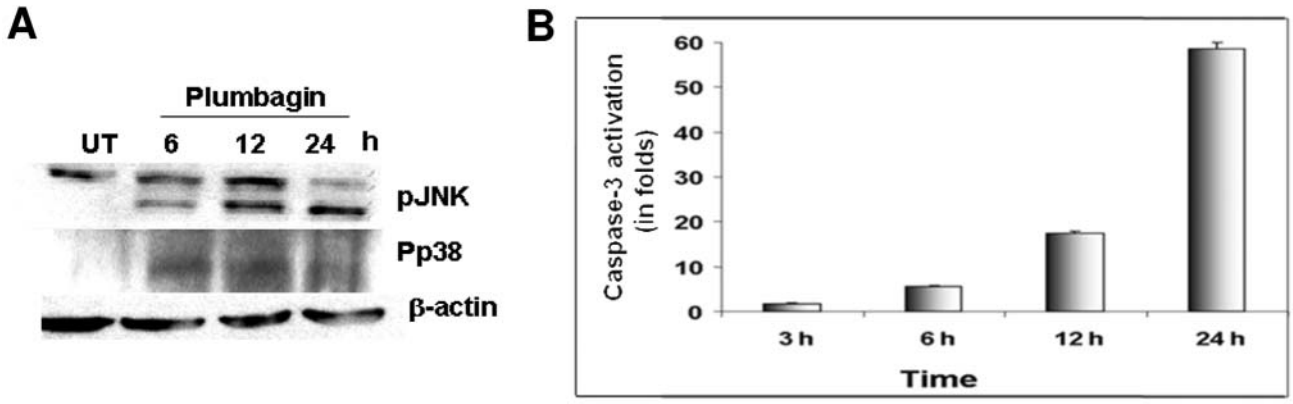

Figure 4. *Plumbagin up-regulated JNK signaling in H460 cells. A) H460 cells were treated with vehicle UT or plumbagin for the indicated time intervals and whole-cell lysates were subjected to Western blot analysis for pJNK, pp38 or actin. B) Plumbagin-induced caspase-3 activation in H460 lung cancer cells. H460 cells were left untreated or treated with plumbagin and, after 3, 6, 12 and 24 h, caspase activity was determined. Caspase activity is expressed as the percentage of that in the control and presented as mean±SE of triplicates from three independent experiments.*

cancer including NSCLC and may represent a tumor marker with prognostic and therapeutic implications (51). Inhibition of survivin expression in plumbagin-treated H460 cells may also facilitate the apoptotic potential of plumbagin in lung cancer cells.

In the current study, we identified that plumbagin-treated H460 cells exhibit increased expression of pJNK and p38. Activated JNKs have been ascribed opposing roles in proliferation and transformation, a promoting role in apoptosis (52). The results of the present study suggest that up regulation of pJNK may influence the inhibition of NF-κB and Bcl-2 observed in the plumbagin-treated H460 cells. Inhibition of signaling followed by the subsequent inhibition of Bcl2 has previously been discussed. The p38 MAPK pathway is also reported to cause apoptosis by selectively functioning as sensors of oxidative stress during tumorigenesis (22). Our results also exhibit decreased cyclin D, increased p53 and activated caspase-3 in plumbagin-treated H460 cells. The inhibition of Akt pathway concomitantly with the activation of p38/JNK pathway may attribute to the apoptotic effect in the plumbagin-treated H460 cells (53, 54). Further more, plumbagin by itself is reported to be a redox cycling compound that generates reactive oxygen species (55), which may contribue to the increase in stress-related signaling mechanisms involving JNK and p38, augmenting and attributing to the apoptotic effect of plumbagin observed in the present study.

In summary, our study results show that plumbagin induced apoptosis in H460 cells by arresting the cells in the G_2/M-phase and by modulating pro-apoptotic and pro-survival factors. *In vivo* studies currently in progress in our laboratory will hopefully determine the antitumoral effect of plumbagin in animal models and its therapeutic applicability in the clinical setting.

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