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Original Article

Plumbagin inhibits cell growth and potentiates apoptosis in human gastric cancer cells *in vitro* through the NF-κB signaling pathway

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Aim: To investigate the effects and underlying mechanisms of plumbagin, a naphthoquinone derived from medicinal plant Plumbago zeylanica, on human gastric cancer (GC) cells.
Methods: Human gastric cancer cell lines SGC-7901, MKN-28, and AGS were used. The cell viability was examined using CCK-8 viability assay. Cell proliferation rate was determined using both clonogenic assay and EdU incorporation assay. Apoptosis was detected via Annexin V/propidium iodide double-labeled flow cytometry. Western blotting was used to assess the expression of both NF-κB-regulated gene products and TNF-α-induced activation of p65, IκBα, and IKK. The intracellular location of NF-κB p65 was detected using confocal microscopy.
Results: Plumbagin (2.5–40 μmol/L) concentration-dependently reduced the viability of the GC cells. The IC_{50} value of plumbagin in SGC-7901, MKN-28, and AGS cells was 19.12, 13.64, and 10.12 μmol/L, respectively. The compound (5–20 μmol/L) concentration-dependently induced apoptosis of SGC-7901 cells, and potentiated the sensitivity of SGC-7901 cells to chemotherapeutic agents TNF-αand cisplatin. The compound (10 μmol/L) downregulated the expression of NF-κB-regulated gene products, including IAP1, XIAP, Bcl-2, Bcl-xL, tumor factor (TF), and VEGF. In addition to inhibition of NF-κB p65 nuclear translocation, the compound also suppressed TNF-α-induced phosphorylation of p65 and IKK, and the degradation of IκBα.
Conclusion: Plumbagin inhibits cell growth and potentiates apoptosis in human GC cells through the NF-κB pathway.

Keywords: plumbagin; anticancer drug; TNF-α; cisplatin; gastric carcinoma; apoptosis; NF-κB

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Introduction

Gastrointestinal cancers comprise a large percentage of malignancies and cancer-related deaths, among which gastric and colorectal cancers are most common[1]. Approximately 700 000 annual deaths make gastric cancer (GC) the second leading cause of mortality in the world[2]. Nearly two-thirds of gastric cancer mortality occurs in developing countries. In China, the death rate is 42%[2]. Despite the rapid progress in surgical techniques and chemotherapies, prognosis for patients with GC is usually poor. Therefore, it is urgent to find an effective therapy against this malignant disease.

Recently, dietary components and phytochemicals were shown to be effective in treating various human diseases[3, 4]. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a quinonoid constituent derived from the roots of the medicinal plant *Plumbago zeylanica*. This plant has been safely used for centuries in traditional ayurvedic and Chinese medicine[5]. Plumbagin has been reported to exhibit anti-microbial properties[6], anti-atherosclerotic effects[7], and anticancer activities both *in vitro* and *in vivo*[5]. Previous findings suggest that plumbagin can inhibit cell proliferation and induce apoptosis in breast carcinomas[8], suppress promotion and metastasis in prostate cancer[5], and induce cell cycle arrest and apoptosis in melanoma cells[9]. The results of these studies have shown that plumbagin has enormous potential as an anticarcinogenic agent. However, the effectiveness of plumbagin in the treatment of GC, as well as its mechanism of action, has not been

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investigated.

Although it is clear from previous studies that plumbagin acts as an anticancer agent by inducing apoptosis and halting cell proliferation, how plumbagin mediates these effects is not fully understood. Evidence in the current literature suggests that plumbagin can suppress the activation of nuclear factor-κB (NF-κB) in A549 human lung cancer cells[10]. Plumbagin can also modulate the activation of p65 and IκBα kinase, leading to the activation of apoptosis in several cancer cell lines[11]. Plumbagin may also inhibit the expression of NF-κB-regulated gene products, such as B-cell lymphoma-2 (Bcl-2)[12], Bcl-xL[12], and vascular endothelial growth factor (VEGF)[11]. Therefore, it is possible that plumbagin exerts its anticarcinogenic action by regulating the NF-κB pathway.

NF-κB is a ubiquitous and evolutionarily conserved transcription factor that is activated in a wide variety of tumors[13] and plays a pivotal role in tumorigenesis[13, 14]. The NF-κB proteins, and other proteins associated with the NF-κB pathway, have been linked to cellular transformation, proliferation, apoptosis suppression, and angiogenesis[14]. In GC cells, NF-κB was shown to be constitutively activated[15], and the activation of NF-κB has been touted as a prognostic parameter in gastric carcinoma[16]. Thus, agents that suppress NF-κB activation have great potential to be effective in the prevention and treatment of GC. Two NF-κB-suppressing compounds include paeoniflorin[17] and wogonin[18], which were shown to cause GC cell apoptosis by regulating the activation of NF-κB.

Because plumbagin has been reported to inhibit the activation of NF-κB, and NF-κB plays a pivotal role in gastric carcinoma, we hypothesized that plumbagin may exert its anticarcinogenic effects in human GC cells by blocking the NF-κB pathway.

Materials and methods

Materials

Plumbagin, dimethyl sulfoxide (DMSO), and propidium iodide (PI) were purchased from Sigma-Aldrich (St Louis, MO, USA). Plumbagin was dissolved in DMSO to a concentration of 200 mmol/L and stored in a dark-colored bottle at -20 °C. This stock solution was further diluted in cell culture medium immediately before use. Tumor necrosis factor-α (TNF-α) was purchased from Pepro Tech Inc (Rocky Hill, NJ, USA). The antibodies against Bcl-2, VEGF, tumor factor (TF), β-actin, and Cy3-labeled goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The IAP1 antibody was provided by Epitomic, Inc (Burlingame, CA, USA). The Anti-Bcl-xL, anti-XIAP, and anti-histone H2B antibodies were purchased from Bioworld Technology (Minneapolis, MN, USA). The antibodies against IκB kinase α (IKKα), NF-κB p65, phospho-p65 (Ser 536), IκBα, phospho-IκBα (Ser 32), goat anti-mouse-HRP conjugate, and goat anti-rabbit-HRP conjugate were purchased from Cell Signaling Technology (Beverly, MA, USA). The penicillin, streptomycin, RPMI-1640 medium, and fetal bovine serum (FBS) were obtained from GIBCO BRL Life Technologies (Grand Island, NY, USA).

Cell culture

Human gastric cancer cell lines, SGC-7901, MKN-28, and AGS, were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

Cell viability and proliferation assay

Cell viability was assessed using the Cell Counting Kit-8 assay (CCK-8, Dojindo Laboratories, Kumamoto, Japan)[19]. Briefly, GC cells were grown in 96-well plates (10 000 cells/well) overnight without treatment. The cells were then incubated with varying concentrations of plumbagin and collected at several different time points. At the time of collection, 10 μL of kit reagent, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl)-2*H*-tetrazolium], was added to each well and incubated for 4 h at 37 °C. Thereafter, the optical density (*OD*) was measured at 450 nm using a 96-well multiscanner autoreader (Thermo Electron Corp, Waltham, MA, USA).

The effect of plumbagin on cell proliferation was determined using both a clonogenic assay and an EdU (5-ethynyl-2′-deoxyuridine) incorporation assay. Briefly, SGC-7901 cells were cultured in 30 mm dishes and treated with plumbagin at various concentrations (0, 5, 10, and 20 μmol/L) for 6 h. After treatment, the medium was removed and replaced with fresh medium. Cells were grown for 10 d at 37 °C to allow for colony formation. Subsequently, the cells were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde for 15 min at room temperature and stained with 0.5% crystal violet. The images were collected, and the number of colonies in each well were counted.

Proliferating cells were stained with EdU using the Cell-Light EdU DNA Cell Proliferation Kit (RIBOBio Co, Guangzhou, China)[20]. The cells were seeded in 96-well culture plates and exposed to media with or without plumbagin. All cells were treated with 50 μmol/L of EdU for 4 h at 37 °C. After being fixed with 4% paraformaldehyde for 15 min, the cells were treated with 0.5% Triton X-100 for 20 min and rinsed with PBS three times. Thereafter, the cells were exposed to 100 μL of 1×Apollo® reaction cocktail for 30 min and incubated with 5 μg/mL of Hoechst 33342 to stain the cell nuclei for 30 min. Images were captured using a fluorescent microscope (Olympus, Tokyo, Japan).

Apoptotic cell detection

An Annexin-V-FITC kit (Bender Medsystems, Burlingame, CA, USA) was used according to the manufacturer's instructions. Briefly, the plumbagin-treated GC cells were resuspended in 500 μL binding buffer, followed by staining with an Annexin-V-FITC and PI solution for 30 min at room temperature in the dark. The samples were analyzed immediately using the FACSCalibur flow cytometer (Becton, Dickinson and Co, San Jose, CA, USA).

Western blot analysis

Cells seeded in 10 cm dishes were treated with 10 μmol/L or 5 μmol/L of plumbagin. Total cell extracts were prepared using M-PER mammalian protein extraction reagent and protease inhibitors (Pierce, Rorkford, IL, USA). The cytoplasmic and 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). 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).

Immunofluorescence for NF-κB p65 localization

The effect of plumbagin on the nuclear translocation of p65 was examined by immunofluorescence[21]. Briefly, GC cells were washed in PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The fixed cells were permeabilized with 0.5% Triton-X 100 in PBS for 10 min and blocked with a 5% bovine serum albumin in PBS. The anti-NF-κB p65 antibody was diluted 1:100 and incubated overnight at 4 °C. The cells were then incubated with Cy3-labeled secondary antibodies for 1 h and mounted with Hoechst 33342 stain. Images were captured using an A1Si confocal laser-scanning microscope (Nikon, Japan).

Statistical analysis

The data are presented as the mean±SD. The Student's *t*-test was used to determine the significance between groups. *P* values of less than 0.05 were considered to be significant. All statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) 13.0 software.

Results

Plumbagin decreased viability and inhibited the proliferation of GC cells

Cell viability was assayed by treating GC cell lines, including SGC-7901, MKN-28, and AGS cells, with various concentrations of plumbagin followed by analysis using the CCK-8 viability assay. We observed that cellular viability was suppressed by plumbagin in a dose-dependent manner in all three of the GC cell lines (Figure 1A). The IC_{50} values of plumbagin in SGC-7901, MKN-28, and AGS cells were 19.12 μmol/L, 13.64 μmol/L, and 10.12 μmol/L, respectively.

The EdU incorporation assay was performed to detect whether plumbagin could affect the number of proliferating cells. We determined that the number of EdU-positive cells in the plumbagin group was reduced compared to the control group. This indicated that plumbagin inhibited the proliferation of SGC-7901 cells *in vitro* (Figures 1B and 1C).

To determine the effect of the long-term antiproliferative activity of plumbagin, we used clonogenic assays. The clonogenicity of SGC-7901 cells in the plumbagin groups was reduced in a concentration-dependent manner (Figure 1D). We observed an inhibition of more than 30% for colony formation (Figure 1E).

Plumbagin enhanced the cell apoptosis of GC cells

The amount of apoptotic cell death was quantified with Annexin V-FITC/PI double-labeled flow cytometry. The SGC-7901 cells were pretreated with varying concentrations of plumbagin. This led to an increase in the amount of apoptosis in this cell line (Figure 2A). The total apoptosis rates were 1.77%±0.31%, 8.00%±1.67%, 30.57%±1.25%, and 35.33%±1.31% at plumbagin concentrations of 0 μmol/L, 5 μmol/L, 10 μmol/L, and 20 μmol/L of plumbagin, respectively.

Plumbagin suppressed the expression of NF-κB-regulated gene products

NF-κB is known to regulate the expression of IAP1, XIAP, Bcl-2, and Bcl-xL, all of which are associated with cancer cell survival[22–24]. To investigate whether plumbagin inhibits the expression of these proteins, whole-cell protein extracts were prepared and analyzed by Western blotting with the specific antibodies. Plumbagin decreased the expression of these proteins in a time-dependent manner (Figure 3A).

We also determined the effect of plumbagin on the NF-κB-dependent gene products that are involved in angiogenesis and metastasis. We found that plumbagin downregulated the expression of both VEGF and TF (Figure 3B).

Plumbagin inhibited TNF-α-induced phosphorylation and nuclear translocation of NF-κB p65

We investigated the effect of plumbagin on p65 nuclear translocation and its phosphorylation status. In general, p65 is located in the cytoplasm in untreated cells, and TNF-α-induced p65 is detected in the nuclei. In cells pretreated with plumbagin, the TNF-α-induced nuclear translocation of p65 was almost completely suppressed (Figure 4A).

Modifications of p65, such as phosphorylation, play an important role in NF-κB transcriptional activity[25]. Therefore, we examined the effect of plumbagin on the phosphorylation and expression of p65 in both nuclear extracts (NE) and cytoplasmic extracts (CE) by Western blot. In the nuclear protein extracts from the TNF-α-treated cells, the accumulation of both total and phosphorylated p65 increased in a time-dependent manner. Concurrently, the expression of p65 in cytoplasmic extracts decreased gradually upon TNF-α stimulation. The p65 nuclear translocation and phosphorylation induced by TNF-α were markedly suppressed in the plumbagin-pretreated GC cells (Figure 4B).

Plumbagin inhibited both TNF-α-induced degradation of IκBα and the phosphorylation of IκBα and IKKα

It is well known that after the phosphorylation, ubiquitination, and proteolytic degradation of IκBα, the p50-p65 heterodimer

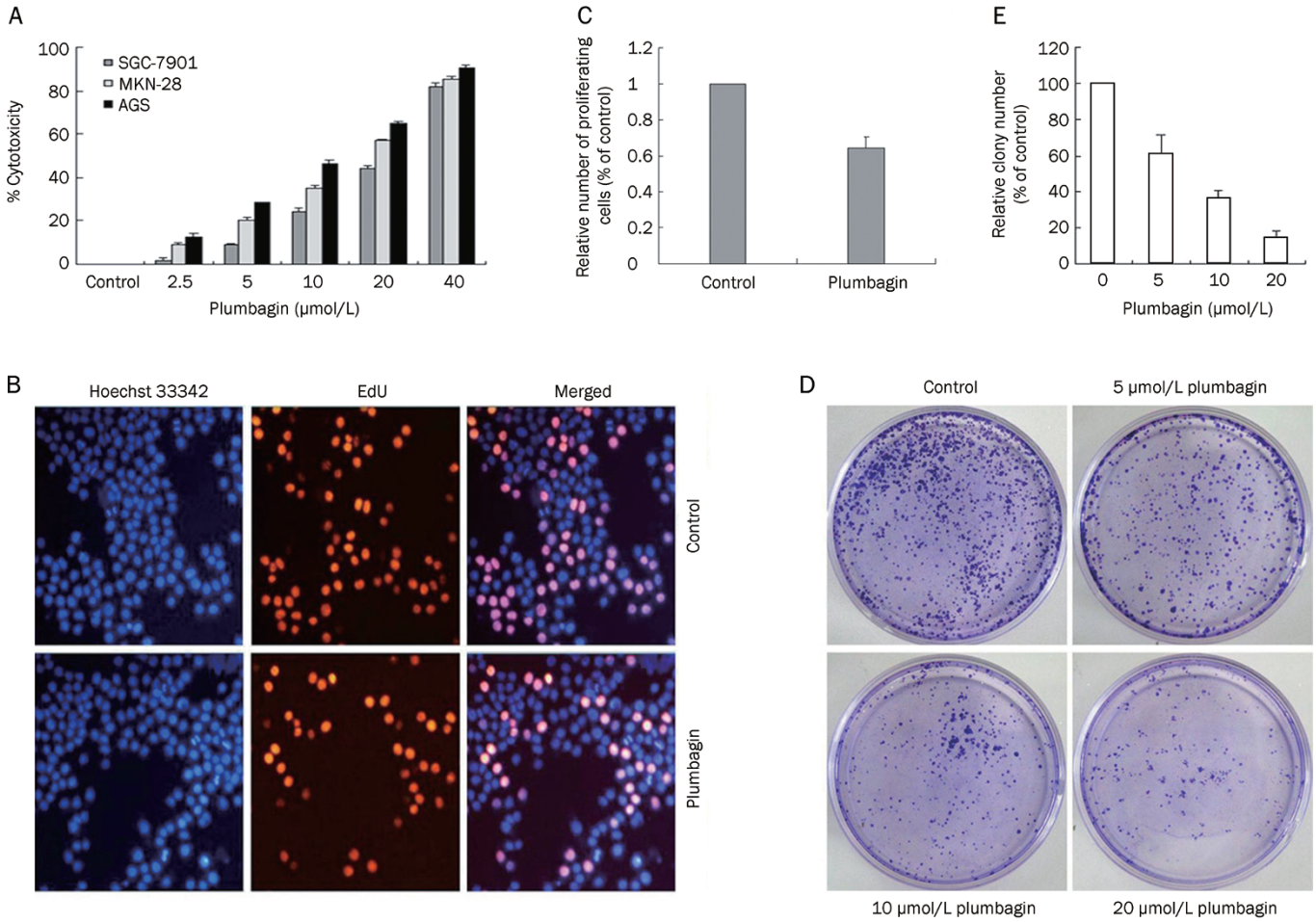

Figure 1. Plumbagin decreased viability and inhibited proliferation of GC cells. (A) Cell viability in plumbagin-treated SGC-7901, MKN-28, and AGS cells. The cells were treated with indicated concentrations (0–40 μmol/L) of plumbagin for 24 h. (B–C) Plumbagin inhibited the cellular DNA replication in SGC-7901 cells (magnification×200). Cells were incubated with 5 μmol/L plumbagin for 12 h. The EdU-labeled replicating cells were examined under a fluorescent microscope. The red and blue cells were counted in a blind manner. (D–E) Plumbagin decreased the number of colony-forming cells. Cells were treated with indicated concentrations of plumbagin (0, 5, 10, and 20 μmol/L) for 6 h, and then the medium was replaced by fresh medium. Cells were allowed to grow for 10 d. The formed cell clones were counted in a blind manner. The data shown are the mean from three independent experiments.

is released and translocates to the nucleus[13]. To determine whether plumbagin inhibited p65 nuclear translocation was due to the degradation and phosphorylation of IκBα, we exposed the cells (pretreated with or without plumbagin) to TNF-α for varying amounts of time. IκBα was degraded in the control cells; however, in the plumbagin pretreated cells, TNF-α failed to induce the degradation of IκBα. Furthermore, TNF-α increased IκBα phosphorylation in the control cells but had no effect on the plumbagin-pretreated cells (Figure 5). These results indicated that plumbagin suppressed both TNF-α-induced IκBα degradation and the activation of IκBα, which occurs prior to p65 nuclear translocation.

The phosphorylation of IκBα required activation of IKK. To examine the effect of plumbagin on IKKα activation, we investigated whether plumbagin could influence TNF-α-induced IKKα phosphorylation. Indeed, plumbagin suppressed TNF-α-dependent IKKα phosphorylation. Both the TNF-α and plumbagin treatments had a weak effect on IKKα protein expression (Figure 5).

Plumbagin potentiated apoptosis induced by TNF-α as well as cisplatin

Because TNF-α and the chemotherapeutic agent cisplatin are two anticancer agents that are commonly used in the clinic, we investigated whether plumbagin affects TNF-α- and/or cisplatin-induced apoptosis. The results displayed an increase in the cytotoxic effect induced by either TNF-α or cisplatin in the presence of plumbagin. This suggests that plumbagin potentiated the apoptotic effects of both TNF-α and cisplatin (Figure 6).

Discussion

The aim of this study was to investigate whether plumbagin has an anticancer effect on human GC cells via inhibition of

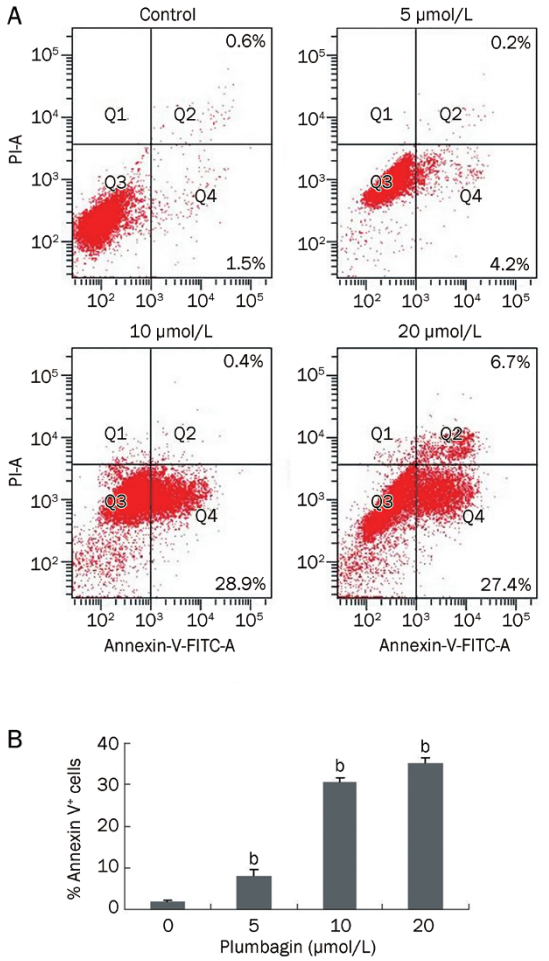

Figure 2. Plumbagin enhanced cell apoptosis of GC cells. (A) Plumbagin induced apoptosis of SGC-7901 cells. Cells were incubated with 0, 5, 10, and 20 μmol/L plumbagin for 12 h. The apoptosis was analyzed by Annexin V-FITC/PI double-staining assay. (B) The degree of apoptotic cell death was quantified. Data represented the mean±SD of three individual experiments ($^bP<0.05$).

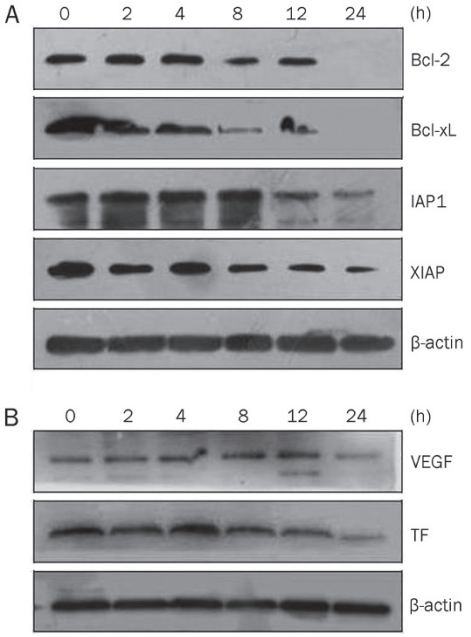

Figure 3. Plumbagin suppressed the expression of NF-κB-regulated gene products. (A) Plumbagin decreased the expression of NF-κB-regulated anti-apoptotic proteins. (B) Plumbagin suppressed the expression of VEGF and TF. SGC-7901 cells were incubated with 10 μmol/L plumbagin for different time periods (0, 2, 4, 8, 12, and 24 h). Whole-cell extracts were prepared and measured by Western blot analysis using the relevant antibodies.

the NF-κB pathway. We identified, for the first time, that GC cells treated with plumbagin underwent growth inhibition, apoptosis and increased chemosensitivity in a dose-dependent manner. We also observed that the protein expression levels of various anti-apoptotic proteins were downregulated by plumbagin in a time-dependent manner. Furthermore, we determined that plumbagin suppressed both NF-κB activation and the expression of NF-κB-regulated gene products. The effects of plumbagin on the NF-κB pathway largely explained how plumbagin increased the chemosensitivity of GC cells.

It is interesting to note that plumbagin caused apoptosis in human GC cell lines, including the well-differentiated MKN-28 cells, and the partially-differentiated SGC-7901 and AGS cell lines. The effective concentration of plumbagin necessary to induce GC cell death is comparable with several existing natural products that have been tested on human GC cells, such as wogonin and curcumin[18, 26]. The downregulated anti-apoptotic proteins that we observed upon treatment with plumbagin, including IAP1, XIAP, Bcl-2, and Bcl-xL, are the main reasons for increased apoptosis. Because Bcl-2 and Bcl-xL are key members in the mitochondrial apoptotic pathway, we conclude that mitochondria may be one of the pathways for apoptosis.

We determined that plumbagin was able to suppress the expression of proteins involved in anti-apoptosis (*ie*, IAP1, XIAP, Bcl-2, and Bcl-xL), angiogenesis (VEGF), and metastasis (TF)[27, 28], all of which are linked to, and regulated by, NF-κB. Based on our findings, we hypothesized that plumbagin may have an effect on the NF-κB pathway in GC cells. Indeed, plumbagin blocked the nuclear translocation of NF-κB p65. Plumbagin also caused the downregulation of both the expression and phosphorylation of NF-κB p65 in nucleus induced by TNF-α.

Exposing cancer cells to TNF-α initiates numerous intracellular signaling cascades, most of which activate the IKK complex, which consists of IKKα, IKKβ, and IKKγ[29]. In the current study, plumbagin inhibited TNF-α-induced phosphorylation of IKKα. IκBα is phosphorylated at serine residues 32 and 34 by IKKs, and this phosphorylation triggers the degradation of IκBα, which allows for the release and translocation of p65 into the nucleus to activate the transcription of target genes[29]. We observed that plumbagin inhibited TNF-α-induced degradation of IκBα by suppressing its phosphorylation. Similar to our data, several studies have shown that some natural constituents, such as berberine, berbamine, and curcumin,

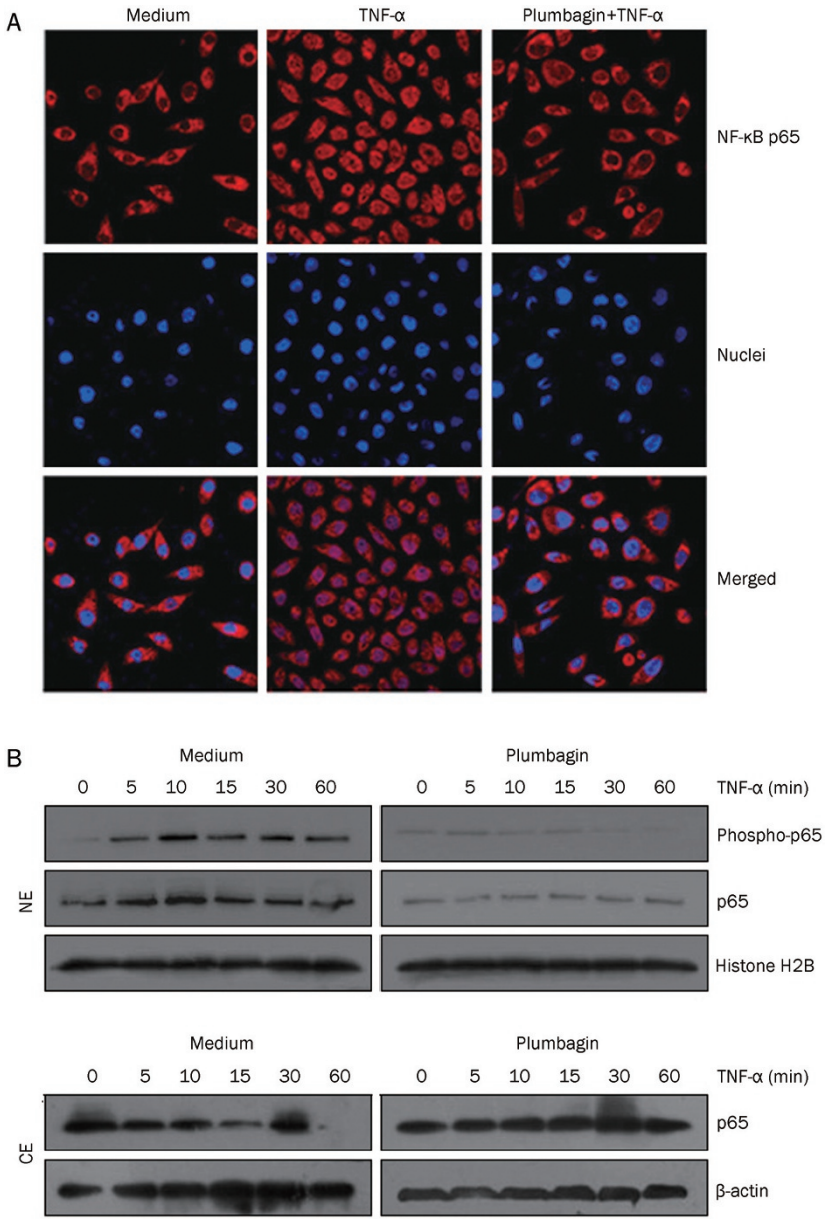

Figure 4. Plumbagin inhibited TNF-α-induced phosphorylation and nuclear translocation of NF-κB p65. (A) Plumbagin inhibited TNF-α-induced p65 localization by immunofluorescence analysis. SGC-7901 cells were either pretreated or untreated with 5 μmol/L plumbagin for 4 h and then exposed to 0.1 nmol/L TNF-α for 30 min (magnification×400). (B) Plumbagin inhibited TNF-α-induced phosphorylation and nuclear translocation of p65. SGC-7901 cells were first treated or untreated with 5 μmol/L plumbagin for 4 h and then exposed to 0.1 nmol/L TNF-α for indicated times. Nuclear extracts (NE) and cytoplasmic extracts (CE) were prepared and analyzed by Western blot with antibodies against p65 and phospho-p65. The antibodies of anti-β-actin and anti-histone H2B were used as controls.

can suppress the activation of NF-κB and its target genes and cause apoptosis in various cancer cells[30–32]. Whether plumbagin is a selective inhibitor of other transcription factors in GC cells remains to be elucidated.

Chemotherapy is widely used in the treatment of numerous malignancies and is a potent therapeutic method; however, chemoresistance remains a major obstacle in the chemotherapeutic treatment of cancer. Overexpression of transcription factors, such as NF-κB, contributes to enhanced cancer cell survival and chemoresistance[33]. Interestingly, we found that plumbagin potentiated apoptosis induced by TNF-α or cisplatin. Plumbagin, in combination with TNF-α or cisplatin, was more effective than the single agent alone. This might be explained by the fact that the apoptosis induced by TNF-α or cisplatin can be suppressed by the activation of NF-κB[34, 35], which is inhibited by plumbagin. This again suggests that plumbagin could be a useful therapeutic strategy in human GC.

Given its strong anticancer abilities, the toxicity of plumbagin has been extensively evaluated. In rodents, plumbagin had dose-related toxic side effects, including diarrhea, skin rashes, and hepatic and reproductive toxicity[11]. Plumbagin did not exhibit significant toxicity on normal tissues at a dose of 2 $mg \cdot kg^{-1} \cdot d^{-1}$ [9]. Additional preclinical and prospective randomized clinical trials are required to determine the full potential of this agent.

In summary, our findings strongly indicate that plumbagin acts as an anticancer agent by inhibiting cell proliferation, inducing apoptosis, and potentiating the chemosensitivity of GC cells. These effects are, in part, mediated by suppressing both NF-κB activation and the expression of NF-κB-regulated gene products. Based on the evidence provided

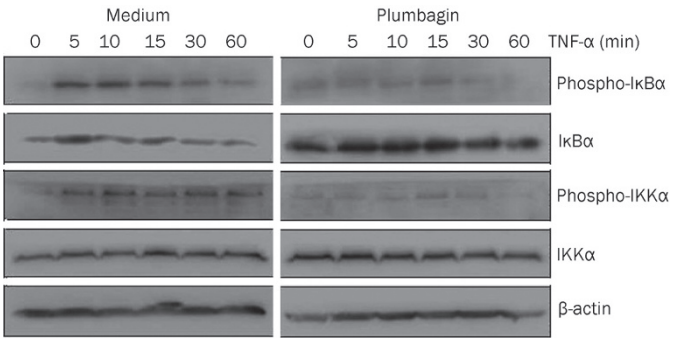

Figure 5. Plumbagin inhibited TNF-α-induced degradation of IκBα, phosphorylation of IκBα and IKKα. Plumbagin inhibited TNF-α induced IκBα degradation, phosphorylation of IκBα and IKKα. SGC-7901 cells were first exposed to 5 μmol/L plumbagin for 4 h and then treated with 0.1 nmol/L TNF-α for the indicated times and analyzed by Western blotting using various antibodies.

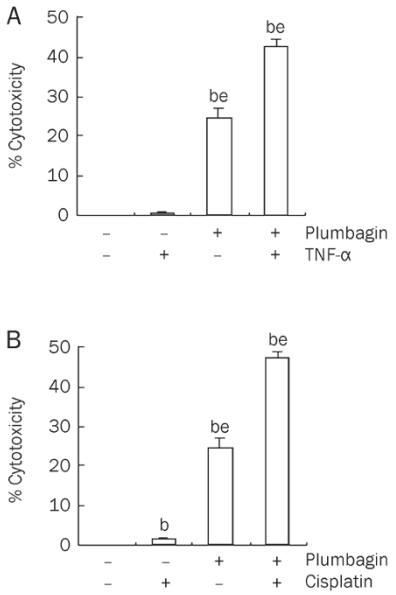

Figure 6. Plumbagin potentiated apoptotic effects of TNF-α and cisplatin. (A–B) SGC-7901 cells were pretreated with 10 μmol/L plumbagin for 2 h, and then treated with 0.1 nmol/L TNF-α and cisplatin for 24 h. Cell viability was then analyzed by the CCK-8 method. Data represented the mean±SD of three individual experiments ($^bP<0.05$ compared to control group. $^eP<0.05$ compared with TNF-α or cisplatin treatment only).

here, plumbagin should be strongly considered as a basis for the development of novel pharmaceutical agents that target human GC cells to improve the treatment of this disease.

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Author contribution

Jing LI, You QIN, Rui CHEN, and Jia LI performed the experiments. Jing LI, Lin SHEN, and Fu-rong LU participated in the design of the study and prepared the manuscript. Yan LI, Han-zi ZHAN, and Yuan-qiao HE contributed to the analyses and interpretation of the data and performed the statistical analysis. Han-zi ZHAN revised the manuscript.

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