Plumbagin (5-Hydroxy-2-methyl-1,4-naphthoquinone) Suppresses NF-κB Activation and NF-κB-regulated Gene Products Through Modulation of p65 and IκBα Kinase Activation, Leading to Potentiation of Apoptosis Induced by Cytokine and Chemotherapeutic Agents*

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Plumbagin, derived from the medicinal plant Plumbago zeylanica, modulates cellular proliferation, carcinogenesis, and radiore sistance, all known to be regulated by the activation of the transcription factor NF-κB, suggesting plumbagin might affect the NF-κB activation pathway. We found that plumbagin inhibited NF-κB activation induced by TNF, and other carcinogens and inflammatory stimuli (e.g. phorbol 12-myristate 13-acetate, H2O2, cigarette smoke condensate, interleukin-1β, lipopolysaccharide, and okadaic acid). Plumbagin also suppressed the constitutive NF-κB activation in certain tumor cells. The suppression of NF-κB activation correlated with sequential inhibition of the tumor necrosis factor (TNF)-induced activation of IκBα kinase, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, and the NF-κB-dependent reporter gene expression activated by TNF, TNFRF1, TRAF2, NIK, IκK-β, and the p65 subunit of NF-κB. Plumbagin also suppressed the direct binding of nuclear p65 and recombinant p65 to the DNA, and this binding was reversed by dithiothreitol both in vitro and in vivo. However, plumbagin did not inhibit p65 binding to DNA when cells were transfected with the p65 plasmid containing cysteine 38 mutated to serine. Plumbagin down-regulated the expression of NF-κB-regulated anti-apoptotic (IAP1, IAP2, Bcl-2, Bcl-xL, cFLIP, Bfl-1/A1, and survivin), proliferative (cyclin D1 and COX-2), and angiogenic (matrix metalloproteinase-9 and vascular endothelial growth factor) gene products. This led to potentiation of apoptosis induced by TNF and paclitaxel and inhibited cell invasion. Overall, our results indicate that plumbagin is a potent inhibitor of the NF-κB activation pathway that leads to suppression of NF-κB-regulated gene products. This may explain its cell growth modulatory, anticarcinogenic, and radiosensitizing effects previously described.

Traditional medicine, although claimed to be safe and efficacious, in most cases neither the chemical entity nor the molecular mechanism of action are well defined. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a naturally occurring yellow pigment, is found in the plants of the Plumbaginaceae, Drosieraceae, Ancestrocladaceae, and Dioncophyllaceae families. The root of Plumbago zeylanica (also called Chitrak), a major source of plumbagin, has been used in the Indian medicine since the period of Charaka, from 750 BC, as an antiatherogenic, cardiotonic, hepatoprotective, and neuroprotective agent (1, 2). The active principle, plumbagin, was first isolated in 1829 (3). Plumbagin is also present along with a series of other structurally related naphthoquinones in the roots, leaves, bark, and wood of Juglans regia (English walnut, Persian walnut, and California walnut), Juglans cinerea (butternut and white walnut), and Juglans nigra (black walnut) (4, 5). Preparations derived from black walnut have been used as hair dyes and skin colorants in addition to being applied topically for the treatment of acne, inflammatory diseases, ringworm, and fungal, bacterial, and viral infections (6).

Plumbagin has been shown to exert anticancer and antiproliferative activities in animal models as well as in cells in culture (7–11). Sugie et al. (12) have shown that plumbagin significantly inhibited azoxymethane-induced intestinal carcinogenesis in rats, suggesting its chemopreventive activity (12). Plumbagin has also been shown to induce S-G2/M cell cycle arrest through the induction of p21 (an inhibitor of cyclin-dependent kinase) (13). A recent report showed that plumbagin has a chemotherapeutic potential as an anticancer agent in ovarian cancer cells with the mutated BRCA1 gene (14). The cytotoxic action of plumbagin in keratinocytes and cervical cancer cells was found to be due to a change in the redox status of the cell (6, 15). In embryonic kidney and brain tumor cells, plumbagin inhibited the enzyme NAD(P)H oxidase (16), linked with anticarcinogenic (7–12) and atherosclerotic effects (17). Besides anticancer effects, plumbagin also exhibited radiosensitizing properties in experimental mouse tumors as well as in tumor cells in vitro (18–20).

Elucidation of the mechanism(s) by which plumbagin induces its anticarcinogenic, antiproliferative, and radiosensitizing activities is necessary to provide a solid foundation for its use as an agent for prevention strategies. We postulated that plumbagin mediates its various activities through suppression of the transcription factor nuclear factor-κB (NF-κB),2 for the following reasons. First, various carcinogens and tumor promoters have been shown to activate NF-κB; second, constitutive

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2 The abbreviations used are: NF-κB, nuclear factor-κB; IκBα, inhibitory subunit of NF-κB; IκK, IκBα kinase; SEAP, secretory alkaline phosphatase; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling; FLIP, FLICE-inhibitory protein; COX, cyclooxygenase; MMP, matrix metalloproteinase; TRAF, tumor necrosis factor receptor-associated factor; TNF, tumor necrosis factor; DTT, dithiothreitol; IAP, anti-inhibitor of apoptosis protein; PARP, poly(ADP-ribose) polymerase; VEGF, vascular endothelial growth factor; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EMSA, electrophoretic mobility shift assay; AP-1, activator protein-1; STAT, signal transducer and activator of transcription 3; NIK, NF-κB-inducing kinase; TNFRF1, tumor necrosis factor receptor 1; ROS, reactive oxygen species.
expression of NF-κB is frequently found in tumor cells; third, NF-κB activation induces resistance to chemotherapeutic agents and to radiation; fourth, several genes involved in tumor initiation, promotion, and metastasis are regulated by NF-κB. Fifth, activation of NF-κB suppresses apoptosis and promotes proliferation. Hence, agents that can down-regulate NF-κB activation, therefore, have potential in the prevention of cancer (21).

Under normal conditions, NF-κB is present in the cytoplasm as an inactive heterotrimer consisting of three subunits: p50, p65, and IκBα. Upon activation, IκBα undergoes phosphorylation and ubiquitination-dependent degradation by the 26 S proteasome, thus exposing nuclear localization signals on the p50-p65 heterodimer, leading to nuclear translocation and binding to a specific consensus sequence in the DNA, 5′-GGGACCTTC-3′ (22). The binding activates NF-κB gene expression, which in turn results in transcription of the NF-κB-regulated genes (21). The phosphorylation of IκBα is mediated through the activation of the IκBα kinase (IKK) complex (23).

Because plumbagin has been reported to exhibit chemopreventive, growth inhibitory, and radiosensitizing effects, we postulated that this quinone may be mediating its effects through modulation of the NF-κB activation pathway. To test this hypothesis, we investigated the effect of plumbagin on NF-κB activation induced by several inflammatory agents in various cell types, including those in which NF-κB is constitutively active. We found that plumbagin inhibited NF-κB activation and NF-κB-regulated gene products, leading to an increase in apoptosis and suppression of cellular invasion.

**MATERIALS AND METHODS**

**Reagents**—Plumbagin and its analogues (with purity greater than 97%) were purchased from Sigma. A 100 mM solution of plumbagin was prepared in dimethyl sulfoxide, stored as small aliquots at −20 °C, and then diluted as needed in cell culture medium. Bacteria-derived human recombinant tumor necrosis factor (TNF), purified to homogeneity with a specific activity of 5 × 10⁷ units/mg, was kindly provided by Genentech (South San Francisco, CA). Cigarette smoke condensate, prepared as described (24), was kindly supplied by Dr. G. Gairola (University of Kentucky, Lexington, KY). N-Acetyl-leucyl-leucyl-norleucinal (ALLN) was purchased from Calbiochem. Penicillin, streptomycin, RPMI 1640 medium, Iscove’s modified Dulbecco’s medium, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were obtained from Invitrogen. Phorbol 12-myristate 13-acetate, okadaic acid, hydrogen peroxide (H₂O₂), diethiothreitol (DTT), and β-actin antibody were obtained from Sigma. Antibodies anti-p65, anti-p50, anti-IκBα, anti-cyclin D1, anti-matrix metalloproteinase-9 (MMP-9), anti-poly(ADP-ribose) polymerase (PARP), anti-inhibitor of apoptosis protein 1 (IAP1), anti-IAP2, anti-Bcl-2, anti-Bcl-xL, and anti-B-B-1/AI were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclooxygenase-2 (COX-2) was obtained from BD Biosciences (San Diego, CA). Anti-vascular endothelial growth factor (VEGF) was obtained from NeoMarkers (Fremont, CA). Phospho-specific anti-IκBα (Ser-32) and phospho-specific anti-p65 (Ser-536) were purchased from Cell Signaling Technology (Beverly, MA). Anti-IκKα, anti-IκKβ, and anti-FLICE/caspase-8-inhibitory protein (cFLIP) were kindly provided by Imagenex (San Diego, CA).

**Cell Lines**—KBM-5 (human chronic myeloid leukemia), U937 (human histiocytic lymphoma myeloid), U266 (human multiple myeloma), H1299 (lung adenocarcinoma), A293 (human embryonic kidney carcinoma), and SCC-4 (human squamous cell carcinoma) cells were obtained from American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove’s modified Dulbecco’s medium with 15% fetal bovine serum. H1299 and U266 cells were cultured in RPMI 1640 medium, and A293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. SCC-4 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 μM nonessential amino acids, 1 mM pyruvate, 6 mM l-glutamine, and 1× vitamins. Culture media were also supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

**Plasmids**—pcDNA3.1 and pcDNA expression vectors for mouse p65 and mouse p65C38S were kindly provided by Dr. T. D. Gilmore from Boston University (Boston, MA).

**Electrophoretic Mobility Shift Assay**—To determine NF-κB activation, we performed electrophoretic mobility shift assays (EMSA) as described previously (25). The specificity of binding was also examined by competition with the unlabelled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either p50 or p65 of NF-κB for 15 min at 37 °C before the complex was analyzed by EMSA. The dried gels were visualized, and radioactive bands were quantified by a PhosphoImager, Storm 8200 (Amer sham Biosciences), using ImageQuant software.

**Activator Protein-1 (AP-1) Activation Assay**—To assay AP-1 activation by EMSA, 10 μg of nuclear extract protein was incubated with 16 fmol of the 32P-end-labeled AP-1 consensus oligonucleotide 5′-CGCT-TGATGACTCAAGCGGAA-3′ (bold indicates the AP-1 binding site) for 30 min at 37 °C, and then the DNA-protein complexes formed were resolved from free oligonucleotide on 6% native polyacrylamide gels (26). The specificity of binding was examined by competition assay with unlabelled oligonucleotide. The radioactive bands were visualized and quantified as indicated above.

**Signal Transducer and Activator of Transcription 3 (STAT3) Activation Assay**—The STAT3-DNA binding was analyzed by EMSA using a 32P-labeled high affinity cis-inducible element probe as previously described (27). Briefly, U266 cells were incubated with different concentrations of plumbagin. Nuclear extracts were prepared and labeled with high affinity cis-inducible element probe (5′-CTCTATTCCTTCGATTATACCTAAGGCT-3′ and 5′-AGCTTTAAGGATTTACGGGATA-TGTA-3′), and STAT3-DNA binding was resolved from free oligonucleotide on 5% native polyacrylamide gels. (26).

**Transfection**—A293 cells (5 × 10⁵ cells/well) were plated in 6-well plates and transiently transfected by FuGENE 6 (Roche Molecular Biochemicals) with pcDNA3.1 or pcDNA expression vectors for mouse p65 or mouse p65C38S for 48 h (28). Thereafter, we prepared the nuclear extracts from transfected cells, then incubated the extracts with plumbagin for 30 min and measured the DNA binding by EMSA.

**Western Blot Analysis**—To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts (29, 30) and fractionated them by SDS-PAGE.

**IKK Assay**—To determine the effect of plumbagin on TNF-induced IKK activation, the IKK assay was performed by a method described previously (30).

**Immunocytochemistry for NF-κB p65 Localization**—The effect of plumbagin on the nuclear translocation of p65 was examined by immunocytochemistry as described previously (31).

**NF-κB-dependent Reporter Gene Expression Assay**—NF-κB-dependent reporter gene expression was performed as described (31). The effect of plumbagin on TNF, TNF receptor 1 (TNFR1), NF-κB-inducing kinase (NIK), IKK-β, and p65-induced NF-κB-dependent reporter gene transcription was analyzed by secretory alkaline phosphatase (SEAP) assay.

**Live and Dead Assay**—To measure apoptosis, we also used the Live and Dead assay (Molecular Probes, Eugene, OR), which determines
intracellular esterase activity and plasma membrane integrity. This assay was performed as indicated (25, 32).

**Annexin V Assay—**An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected by utilizing the binding properties of annexin V. This assay was performed as indicated previously (32).

**TUNEL Assay—**We also assayed cytotoxicity by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method, which examines DNA strand breaks during apoptosis, using an in situ cell death detection reagent (Roche Diagnostics). This assay was performed as indicated previously (25).

**Cytotoxicity Assay—**The effect of plumbagin on the cytotoxic effects of TNF and chemotherapeutic agents was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method as described (33).

**Invasion Assay—**Invasion through the extracellular matrix is a crucial step in tumor metastasis. The BD BioCoat tumor invasion system that we used has a chamber with a light-tight polycarbonate membrane with 8-μm pores coated with a reconstituted basement membrane gel (BD Biosciences, San Jose, CA). This assay was performed as indicated previously (34).

**RESULTS**

The aim of this study was to investigate the effect of plumbagin on the transcription factor NF-κB-signaling pathway and NF-κB-regulated gene products. Most of the studies were carried out using human chronic myeloid leukemia (KBM-5) cells. We used TNF to examine the effect of plumbagin on the NF-κB activation pathway because the pathway activated by this agent is well understood. Under the conditions that we used to examine the NF-κB pathway and NF-κB-regulated gene products, plumbagin had no effect on cell viability.

**Plumbagin Suppresses TNF-induced NF-κB Activation—**To determine the effect of plumbagin on TNF-induced NF-κB activation, cells either untreated or treated with plumbagin (5 μM for 4 h) were exposed to 0.1 nM TNF for different times; nuclear extracts were then prepared and examined for NF-κB activation by DNA-binding assays. TNF activated NF-κB in a time-dependent manner in the untreated cells (Fig. 1A, left panel); however, in plumbagin-treated cells, NF-κB activation was significantly inhibited (Fig. 1A, right panel).

To further determine the optimum time of exposure to plumbagin to suppress NF-κB activation, cells were treated with plumbagin (5 μM) for
different times and then left untreated or activated with 0.1 nM TNF for 30 min. We found that plumbagin alone did not significantly activate NF-κB (Fig. 1B, left panel), but the TNF-induced NF-κB activation was inhibited in a time-dependent manner (Fig. 1B, right panel). Four hours was sufficient to significantly suppress TNF-induced NF-κB activation.

To determine the optimum dose of plumbagin required to suppress NF-κB activation, cells were treated with different concentrations of plumbagin for 4 h and then either left untreated or activated with 0.1 nM TNF for 30 min. We found that plumbagin alone had no effect (Fig. 1C, left panel), but the TNF-induced NF-κB activation was inhibited in a dose-dependent manner (Fig. 1C, right panel). Five micromolar plumbagin was sufficient to suppress TNF-induced NF-κB activation significantly.

To determine the effect of plumbagin on NF-κB activation at a high TNF concentration, KRM-5 cells were pretreated with plumbagin, and then TNF was added up to 1,000 pM and analyzed for NF-κB activation by EMSA (Fig. 1D). TNF at a concentration of 1 nM strongly activated NF-κB activity; however, pretreatment with plumbagin markedly inhibited this activation. These results show that plumbagin is a potent inhibitor of TNF-induced NF-κB activation.

When nuclear extracts from TNF-activated cells were incubated with antibodies to the p50 (NF-κB1) and p65 (RelA) subunit of NF-κB, the resulting bands were shifted to higher molecular masses, suggesting that the TNF-activated complex consisted of p50 and p65. Preimmune serum had no effect on DNA binding. The addition of excess unlabeled NF-κB (cold oligonucleotide, 100-fold) caused a complete disappearance of the band, whereas mutated oligonucleotide had no effect on DNA binding (Fig. 1E).

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Plumbagin Is a Specific Inhibitor of Transcription Factor NF-κB—Whether plumbagin suppresses other transcription factors like AP-1 and STAT3 under the conditions it suppresses NF-κB, was examined. The results show that pretreatment with plumbagin inhibited neither TNF-induced AP-1 activity in KBM-5 cells (Fig. 1F), nor constitutive STAT3 activation in U266 cells (Fig. 1G).

Inhibition of NF-κB Activation by Plumbagin Was Not Cell-type Specific—Different cell types mediate NF-κB induction by distinct signal transduction pathways (35, 36). Besides myeloid KRM-5 cells, whether plumbagin suppresses NF-κB activation in other myeloid cells was examined. As shown in Fig. 2A, plumbagin also suppressed the TNF-induced NF-κB activation in human histiocytic lymphoma mye-
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FIGURE 3. Direct effect of plumbagin on the NF-κB complex. A, plumbagin inhibits direct binding of NF-κB to DNA. Nuclear extracts were prepared from untreated KBM-5 cells or cells treated with 0.1 nM TNF for 30 min. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA. B, DTT inhibits plumbagin-mediated suppression of NF-κB activation by TNF in cells. KBM-5 cells were pre-treated with plumbagin (5 μM) with or without 100 μM DTT for 4 h and then incubated with 0.1 nM TNF for 30 min. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA. C, DTT inhibits plumbagin-mediated suppression of overexpressed p65 in vitro in A293 cells. Nuclear extracts from overexpressed p65 cells were incubated with 5 μM plumbagin with or without 100 μM DTT for 30 min and then assayed for NF-κB binding to DNA by EMSA. D, plumbagin suppressed overexpressed wild type p65 but not mutant p65C38S in vitro in A293 cells. A293 cells were transiently transfected with p65 or mutant p65C38S and then an EMSA was performed on nuclear extracts treated with 5 μM plumbagin.

Plumbagin Directly Interferes with the Binding of NF-κB to the DNA—
Results from our laboratory and others have shown that certain NF-κB inhibitors suppress NF-κB activation by directly blocking the binding of NF-κB to the DNA (39–41). We determined whether plumbagin mediates suppression of NF-κB activation through a similar mechanism. When we incubated nuclear extracts from TNF-treated cells with plumbagin, the EMSA results showed that it significantly inhibited NF-κB binding to the DNA (Fig. 3A). We found that indeed co-incubation of cell extracts with plumbagin in the presence of the DTT reversed the effect of plumbagin completely (Fig. 3A).

We also investigated whether DTT could reverse the NF-κB-suppressing effect of plumbagin in intact cells. The results in Fig. 3B showed that DTT completely inhibited the action of plumbagin. To determine whether plumbagin targets the p65 subunit of NF-κB, we overexpressed p65 by transfecting p65-containing plasmid into A293 cells. We then prepared nuclear extracts and treated them with plumbagin in the presence or absence of DTT. The recombinant p65 subunit bound to the DNA and plumbagin treatment resulted in suppression of binding. DTT completely reversed the effect of plumbagin (Fig. 3C).

It has been shown that the cysteine residue located at position 38 in p65 is highly susceptible to various agents (28, 42). Whether Cys-38 is a target for plumbagin was investigated. Therefore, we have now used p65 plasmid with Cys-38 mutated to serine residue (p65C38S) and also performed an EMSA on nuclear extracts treated with plumbagin (Fig. 3D). The results show that plumbagin modifies the DNA binding of wild type p65 but not the mutated p65 (Fig. 3D). Thus these results demonstrate that Cys-38 in p65 is one of the targets of plumbagin.

Plumbagin Inhibited IκBα Degradation—Besides modifying p65, whether plumbagin acts at other steps in the TNF-induced NF-κB activation pathway was also examined. The translocation of NF-κB to the nucleus is preceded by the proteolytic degradation of IκBα (21). In our study, TNF induced IκBα degradation in control cells within 5 min and reached maximum in 15 min, but in plumbagin-pretreated cells, TNF had no effect on IκBα degradation (Fig. 4A). These results indicate that plumbagin prevents IκBα degradation by acting at a step upstream to IκBα degradation.
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![Figure 4: Plumbagin inhibits TNF-dependent IκBα degradation.](image)

- **A**. Effect of plumbagin on TNF-induced degradation of IκBα. KBM-5 cells were incubated with 5 μM plumbagin for 4 h and treated with 0.1 nM TNF for the indicated times. Cytoplasmic extracts were prepared, fractionated on 10% SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using anti-IκBα antibody. Anti-β-actin antibody was the loading control.
- **B**. Effect of plumbagin on the phosphorylation of IκBα. Western blot of p65 was carried out using an antibody that recognizes the serine-phosphorylated form of IκBα. KBM-5 cells were preincubated with 5 μM plumbagin for 4 h and then treated with 0.1 nM TNF for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phospho-specific anti-IκBα antibody.
- **C**. Effect of plumbagin on the activation of IKK by TNF. KBM-5 cells were preincubated with 5 μM plumbagin for 4 h and then treated with 0.1 nM TNF for the indicated times. Whole cell extracts were immunoprecipitated with antibody against IKK-α and analyzed by an immune complex kinase assay. To examine the effect of plumbagin on the level of IKK proteins, whole cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-IKK-α antibodies. D. Plumbagin inhibits TNF-induced nuclear translocation of p65. Western blot of p65 was carried out using nuclear extracts. KBM-5 cells were incubated with 5 μM plumbagin for 4 h and treated with 0.1 nM TNF for the indicated times. Nuclear extracts were prepared and subjected to Western blot analysis using anti-p65 and phospho-specific anti-p65 antibodies. For loading control of nuclear protein, the membrane was blotted with anti-PARP antibody. E. Immunocytochemical analysis of p65 localization. KBM-5 cells were incubated with 5 μM plumbagin for 4 h and then treated with 1 nM TNF for 10 min. Cells were subjected to immunocytochemical analysis as described under “Materials and Methods.”

**Plumbagin Inhibited TNF-dependent IκBα Phosphorylation**—To determine whether the inhibition of TNF-induced IκBα degradation was due to inhibition of IκBα phosphorylation, we used the proteasome inhibitor ALLN to block degradation of IκBα (34). KBM-5 cells were pretreated with plumbagin, treated with ALLN for 30 min, exposed to TNF, and then examined for IκBα phosphorylation status by Western blot analysis using an antibody that recognizes the serine-phosphorylated form of IκBα. TNF-induced IκBα phosphorylation was strongly suppressed by plumbagin (Fig. 4B). DTT reversed the effect of plumbagin-mediated suppression of the phosphorylation of IκBα.

**Plumbagin Inhibited TNF-induced IKK Activation—IKK is required for TNF-induced phosphorylation of IκBα (36).** Because plumbagin inhibited the phosphorylation of IκBα, we determined its direct effect on TNF-induced IKK activation in KBM-5 cells. Results from the immune complex kinase assay showed that TNF activated IKK as early as 5 min after TNF treatment but that plumbagin strongly suppressed this activation (Fig. 4C). Neither TNF nor plumbagin affected the expression of IKK-α or IKK-β proteins.

**Plumbagin Inhibits p65 Translocation into the Nucleus**—We examined whether plumbagin affects TNF-induced nuclear translocation. Western blot analysis showed that TNF induced nuclear translocation of p65 in a time-dependent manner in KBM-5 cells (Fig. 4D, top panel). When the cells were pretreated with plumbagin, TNF failed to induce nuclear translocation of p65. TNF induces the phosphorylation of p65, which is required for its transcriptional activity (22). Western blot analysis showed that TNF induced the phosphorylation of p65 and that plumbagin strongly suppressed it (Fig. 4D, middle panel).

An immunocytochemical assay also confirmed the effect of plumbagin on the suppression of the nuclear translocation of p65. In untreated or plumbagin-treated KBM-5 cells, p65 was localized in the cytoplasm. Treatment with TNF induced its nuclear translocation, and plumbagin pretreatment suppressed the TNF-induced nuclear translocation (Fig. 4E).
Plumbagin Inhibited TNF-induced NF-κB-dependent Reporter Gene Expression—Although we have shown by EMSA that plumbagin blocked NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps (43). To determine the effect of plumbagin on TNF-induced NF-κB-dependent reporter gene expression, we transiently transfected A293 cells with the NF-κB-regulated SEAP reporter construct, incubated the cells with plumbagin, and then stimulated them with TNF. We found that TNF-induced NF-κB regulated reporter gene expression and that plumbagin suppressed it (Fig. 5A).

TNF-induced NF-κB activation is mediated through sequential interaction of the TNF receptor with TRAF2, NIK, and IKK-β, resulting in phosphorylation of IκBa (44). To determine the effect of plumbagin on TNF-induced NF-κB-dependent reporter gene expression, A293 cells were transiently transfected with TNFR1-, TRAF2-, NIK-, IKK-β-, and p65-expressing plasmids and then treated with TNF for the indicated times. Whole cell extracts were prepared and subjected to Western blot analysis using the relevant antibodies.

Plumbagin Down-modulated TNF-induced NF-κB-dependent Anti-apoptotic Gene Expression—Because NF-κB regulates the expression of anti-apoptotic proteins such as IAP1/2 (45, 46), Bcl-2 (47), Bcl-xL (48), cFLIP (49), Bfl-1/A1 (50), and survivin (51), we examined whether plumbagin can modulate the expression of these anti-apoptotic gene products induced by TNF in KBM-5 cells. The results of Western blot analysis showed that TNF induced these anti-apoptotic proteins and that plumbagin suppressed it (Fig. 5C).

Plumbagin Inhibits TNF-induced COX-2, MMP-9, Cyclin D1, and VEGF Expression—It is known that TNF induced expression of COX-2, MMP-9, and VEGF, which are known to be NF-κB-regulated gene products (52–54). Whether plumbagin has any effect on the expression of these gene products was also investigated. We pretreated KBM-5

FIGURE 5. Plumbagin represses NF-κB-dependent reporter gene expression induced by TNF. A, plumbagin inhibits the NF-κB-dependent reporter gene expression induced by TNF. A293 cells were transiently transfected with a NF-κB-containing plasmid for 24 h. After transfection, the cells were incubated with the indicated concentrations of plumbagin for 4 h and then treated with 1 nM TNF for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity. B, plumbagin inhibits the NF-κB-dependent reporter gene expression induced by TNF, TNFR1, TRAF2, NIK, IKK-β, and p65. Cells were transiently transfected with a NF-κB-containing plasmid alone or with the indicated plasmids. After transfection, the cells were incubated with 2.5 μM plumbagin for 4 h and then incubated with the relevant plasmid for an additional 24 h. For TNF-treated cells, cells were incubated with 2.5 μM plumbagin for 4 h and then treated with 1 nM TNF for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity. Plumbagin suppresses NF-κB-dependent gene expression. Plumbagin represses TNF-induced NF-κB-dependent expression of anti-apoptosis, proliferation, and metastasis-related gene products. Plumbagin inhibits the expression of TNF-induced anti-apoptotic proteins (C) and cyclin D1, COX-2, MMP-9, and VEGF (D). KBM-5 cells were incubated with 2.5 μM plumbagin for 4 h and then treated with 1 nM TNF for the indicated times. Whole cell extracts were prepared and subjected to Western blot analysis using the relevant antibodies.

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cells with plumbagin before the addition of TNF, prepared whole cell extracts, and analyzed protein expression by Western blot analysis. Plumbagin suppressed TNF-induced up-regulation of these proteins (Fig. 5D). Expression of cyclin D1, the NF-κB-regulated gene product involved in proliferation (55), was also abolished by plumbagin (Fig. 5D).

**Plumbagin Enhanced Cytotoxicity Induced by TNF and Paclitaxel**—Activation of NF-κB by TNF and chemotherapeutic agents leads to resistance to apoptosis (56–58). Because we found that most of the antiapoptotic gene products are down-regulated by plumbagin, we investigated whether plumbagin potentiates the apoptosis induced by TNF and chemotherapeutic agents. We studied apoptosis in KBM-5 cells by using the Live and Dead, PARP cleavage, annexin V staining, MTT, and TUNEL staining methods. The Live and Dead assay (which measures intracellular esterase activity and plasma membrane integrity) showed that plumbagin enhanced TNF-induced cytotoxicity from 4 to 50% (Fig. 6A). Whether this increased cytotoxicity was due to apoptosis was investigated by other techniques. TNF-induced caspase activation leading to PARP cleavage was potentiated by plumbagin (Fig. 6B). The results of annexin V staining indicated that plumbagin up-regulated TNF-induced early apoptosis (Fig. 6C). The results of TUNEL staining confirmed that TNF-induced apoptosis was enhanced by incubation with plumbagin (Fig. 6D). Moreover, as determined by the MTT method, plumbagin increased the cytotoxicity induced by TNF (Fig. 6E) and paclitaxel (Fig. 6F).

**Plumbagin Suppressed TNF-induced Invasion**—Several NF-κB-regulated proteins, including MMP-9, COX-2, VEGF, and adhesion molecules, play a major role in tumor invasion and metastasis (59). To examine the effect of plumbagin on TNF-induced invasion activity, we seeded H1299 cells into the upper wells of a Matrigel invasion chamber in the absence of serum. The cells were pretreated with plumbagin and then

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**FIGURE 6.** Plumbagin potentiates apoptosis induced by TNF and chemotherapeutic agents. 
A, KBM-5 cells were pretreated with 1 μM plumbagin for 4 h and then incubated with 1 nM TNF for 16 h. Cells were stained with Live and Dead assay reagent for 30 min and then analyzed under a fluorescence microscope. B, KBM-5 cells were pretreated with 5 μM plumbagin for 4 h and then incubated with 1 nM TNF for the indicated times. Whole cell extracts were prepared and subjected to Western blot analysis using anti-PARP antibody. C, KBM-5 cells were pretreated with 5 μM plumbagin (PN) for 4 h and then incubated with 1 nM TNF for 16 h. Cells were incubated with anti-annexin V antibody conjugated with fluorescein isothiocyanate and then analyzed with a flow cytometer for early apoptotic effects. D, KBM-5 cells were pretreated with 5 μM plumbagin for 4 h and then incubated with 1 nM TNF for 16 h. Cells were fixed, stained with TUNEL assay reagent, and then analyzed with a flow cytometer for apoptotic effects. E and F, 5000 KBM-5 cells per well were seeded in triplicate into 96-well plates. Cells were pretreated with 1 μM plumbagin for 4 h and then incubated with 1 nM TNF (E) or 1 nM paclitaxel (F), for 48 h. Thereafter, cell viability was analyzed by the MTT method. G, plumbagin suppresses TNF-induced invasive activity. H1299 cells were seeded onto the upper wells of a Matrigel invasion chamber overnight in the absence of serum, pretreated with the indicated concentrations of plumbagin for 4 h, treated with 1 nM TNF for 24 h in the presence of 1% serum, and then subjected to invasion assay. The value for no plumbagin and no TNF was set to 1.0.
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FIGURE 7. Relative potency of plumbagin analogues in suppressing TNF-induced NF-κB. A, structures of plumbagin and its analogues. Effect of plumbagin (B) and its analogues juglone (C), 1,4-naphthoquinone (D), and menadione (E) on the TNF-induced NF-κB activation. KBM-5 cells were incubated with various concentrations of plumbagin and its analogues for 4 h, treated with 0.1 ng/mL TNF for 30 min, and then subjected to EMSA for NF-κB activation.

Relative Potency of Plumbagin to Suppress NF-κB among Its Analogues—The structure of plumbagin exhibits striking homology to juglone, menadione, and 1,4-naphthoquinone (Fig. 7A). We, therefore, investigated whether these analogues can also inhibit TNF-induced NF-κB activation. Similar to plumbagin, juglone and 1,4-naphthoquinone suppressed TNF-induced NF-κB in a dose-dependent manner (Fig. 7, B–D). Menadione, in contrast, did not inhibit TNF-induced NF-κB activation significantly under these conditions (Fig. 7E).

DISCUSSION

The anti-carcinogenic, apoptotic, and radiosensitizing effects previously described suggested that plumbagin must mediate its effects by suppressing NF-κB activation. In the present report, we found that plumbagin did indeed inhibit NF-κB activation induced by a variety of agents and in a variety of cell lines. NF-κB activity was inhibited because plumbagin suppressed IKK activation, thus resulting in inhibition of IkBα phosphorylation and degradation. Apart from this, plumbagin also inhibited the binding of the p65 subunit of NF-κB to the DNA. This resulted in suppression of NF-κB-regulated reporter gene transcription and gene products involved in cell proliferation (e.g. cyclin D1 and COX-2), antiapoptosis (e.g. survivin, IAP1, IAP2, Bcl-2, Bcl-xl, Bfl-1/A1, and cFLIP), angiogenesis (e.g. VEGF), and invasion (e.g. MMP-9). Suppression of NF-κB by plumbagin enhanced the apoptosis induced by TNF and paclitaxel.

This is the first report to suggest that plumbagin could suppress NF-κB activation and NF-κB-regulated gene products. Our results indicate that plumbagin inhibits both constitutive and inducible NF-κB activation. Moreover, NF-κB activation induced by diverse stimuli was inhibited. These results suggest that plumbagin must act at a step in the NF-κB activation pathway common to all NF-κB inducers. Plumbagin did not suppress other transcription factors like AP-1 and STAT3 under the conditions, it suppresses NF-κB suggesting specificity toward NF-κB. We found that plumbagin acts at least at two different steps in the NF-κB pathway: First, its direct interaction with the p65 subunit of NF-κB and second, its effect on TNF-induced IKK activation. That plumbagin could inhibit the binding of NF-κB to the DNA in vitro and in vivo suggest that it is modifying the NF-κB protein, because the binding of recombinant p65 subunit of NF-κB, which has the transactivation domain, was suppressed, suggesting that plumbagin directly targets the p65 subunit. These results are consistent with our findings that plumbagin also suppressed the p65-induced NF-κB reporter activity. Because the effects of plumbagin could be reversed by reducing agent, it suggests that a cysteine residue in p65 is modified by this agent. These results are consistent to those previously reported from our laboratory with caffeic acid phenethyl ester (41) and sesquiterpene lactone parthenolide by others (42). A Cys-38 residue has been identified in p65 that is crucial for DNA binding (42). A Cys-38 residue has been identified in p65 subunit of NF-κB that is crucial for DNA binding (42). Our results indicate that when this Cys-38 was replaced by serine in p65, plumbagin failed to inhibit the DNA binding ability of p65. Thus our results suggest that plumbagin must modify this cysteine residue leading to NF-κB suppression.

We found that, in addition to its effects on p65, plumbagin also inhibits TNF-induced IKK activation, which leads to inhibition of IkBα phosphorylation and degradation. Whether plumbagin blocks TNF-induced IKK activation or directly blocks the IKK activity is not clear. A critical cysteine residue at position 179 has been identified in the IKK-β subunit of IKK-β that is crucial for DNA binding (42). Our results indicate that when this Cys-38 was replaced by serine in p65, plumbagin failed to inhibit the DNA binding ability of p65. Thus our results suggest that plumbagin must modify this cysteine residue leading to NF-κB suppression.

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of IKK needed for TNF-induced NF-κB activation (60). Because the inhibition of TNF-induced phosphorylation of IKKα induced by plumbagin could be reversed by DTT, this suggests that it may inhibit the IKK activity. Thus it is possible that plumbagin targets the cysteine residue of IKK as well.

How plumbagin targets the critical cysteine in p65 or IKK is not clear. Most quinones mediate their cellular effects through two different mechanisms, redox recycling and reaction with GSH. Redox cycling results in the generation of the semiquinone radicals followed by formation of superoxide radical and H₂O₂. Because plumbagin directly modified p65 not only in vitro but also in inhibiting angiogenesis and inflammation. NF-κB, all of which were modulated by plumbagin. Plumbagin also ated through suppression of radiation-induced NF-

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