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Embelin, an Inhibitor of X Chromosome-Linked Inhibitor-of-Apoptosis Protein, Blocks Nuclear Factor-κB (NF-κB) Signaling Pathway Leading to Suppression of NF-κB-Regulated Antiapoptotic and Metastatic Gene Products

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ABSTRACT

Identifying the active chemical ingredients of ancient medicines and the molecular targets of those ingredients is an attractive therapeutic objective. Embelin, identified primarily from the *Embelia ribes* plant, is one such compound shown to exhibit chemopreventive, anti-inflammatory, and apoptotic activities through an unknown mechanism. Because nuclear factor-κB (NF-κB) regulates several genes associated with inflammation, proliferation, carcinogenesis, and apoptosis, we postulated that embelin might mediate its activity through modulation of NF-κB activation. We found that embelin inhibited tumor necrosis factor (TNF) α-induced NF-κB activation. Both inducible and constitutive NF-κB activation were abrogated by embelin. In addition, NF-κB activated by diverse stimuli such as interleukin-1β, lipopolysaccharide, phorbol myristate acetate, okadaic acid, hydrogen peroxide, and cigarette smoke condensate also was suppressed. We found that embelin inhibited sequentially the TNFα-induced activation of the inhibitory subunit of NF-κBα (IκBα) kinase, IκBα phosphorylation, IκBα degradation, and p65 phosphorylation and nuclear translocation. Embelin also suppressed NF-κB-dependent reporter gene transcription induced by TNFα, TNF receptor-1 (TNFR1), TNFR1-associated death domain protein, TNFR-associated factor-2, NF-κB-inducing kinase, and IκBα kinase but not by p65. Furthermore, we found that embelin down-regulated gene products involved in cell survival, proliferation, invasion, and metastasis of the tumor. This down-regulation was associated with enhanced apoptosis by cytokine and chemotherapeutic agents. Together, our results indicate that embelin is a novel NF-κB blocker and potential suppressor of tumorigenesis.

Ayurveda is a 5000-year-old "science of long life" that prescribes certain herbal preparations for the prevention and treatment of disease. Identifying the chemical compounds in these herbal preparations and the molecular targets of those compounds helps validate the use of these ancient medicines. The fruit of the *Embelia ribes* Burm. plant (Myrsinaceae) (called false black pepper in English, Vidanda in Sanskrit, and Babrang in Hindi languages) has been used to treat fever, inflammatory diseases, and a variety of gastrointestinal ailments for thousands of years (Gupta et al., 1977). More than 4 decades ago, the active component from this plant was isolated and named embelin (Du and Wie, 1963; see structure in Fig. 1A) and later chemically synthesized (Dallacker and Lohnert, 1972). Embelin has been shown to have antitumor, anti-inflammatory, and analgesic properties (Chitra et al., 1994), and it has been shown to decrease testosterone levels

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ABBREVIATIONS: RIP, receptor-interacting protein; XIAP, X chromosome-linked inhibitor-of-apoptosis protein; TNFα, tumor necrosis factorα; NF-κB, nuclear factor-κB; CSC, cigarette smoke condensate; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; OA, okadaic acid; LPS, lipopolysaccharide; IL, interleukin; IκBα, inhibitory subunit of NF-κBα; MMP, matrix metalloproteinase; PARP, poly(ADP-ribose) polymerase; IAP1, inhibitor-of-apoptosis protein 1; TRAF, tumor necrosis factor receptor-associated factor; VEGF, vascular endothelial growth factor; ICAM-1, intercellular adhesion molecule 1; COX, cyclooxygenase; IKK, IκBα kinase; cFLIP, complementary Fas-associated death domain protein-like interleukin-1β-converting enzyme-inhibitory protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; SEAP, secretory alkaline phosphatase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TNFR, tumor necrosis factor receptor; TRADD, tumor necrosis factor receptor 1-associated death domain protein; NIK, NF-κB-inducing kinase; MEKK, mitogen-activated protein kinase kinase kinase; TAK, transforming growth factor-β-activated kinase.

(Githui et al., 1991), induce apoptosis in human myeloid HL-60 cells by targeting microtubular proteins (Xu et al., 2005) and induce cleavage of receptor-interacting protein (RIP) through activation of caspases during pancreatitis (Mareninova et al., 2006), In addition, it is effective against *N*-nitrosodiethylamine/phenobarbital-induced hepatocarcinogenesis in Wistar rats (Sreepriya and Bali, 2005). More recently, embelin was also identified as a cell-permeable, small molecular weight inhibitor of the X chromosome-linked inhibitor-of-apoptosis protein (XIAP), an antiapoptotic protein, through structure-based computational screening of a traditional herbal medicine three-dimensional structure database of 8221 individual traditional herbal products (Nikolovska-Coleska et al., 2004).

How embelin regulates cell proliferation, apoptosis, invasion, and tumor cell migration is poorly established. We postulated that it mediates its effects by modulating nuclear factor-κB (NF-κB), which has been shown to regulate the expression of a variety of gene products involved in tumorigenesis, inflammation, proliferation, carcinogenesis, and apoptosis (Aggarwal, 2004). Various carcinogens and inflammatory stimuli, including tumor necrosis factor (TNF)α, cigarette smoke condensate (CSC), phorbol 12-myristate 13-acetate (PMA), okadaic acid (OA), lipopolysaccharide (LPS), interleukin (IL)-1β, and hydrogen peroxide (H_2O_2) have been shown to activate NF-κB, which might be the primary target of embelin. Most of the proinflammatory effects of TNFα are mediated through activation of NF-κB, which has also been

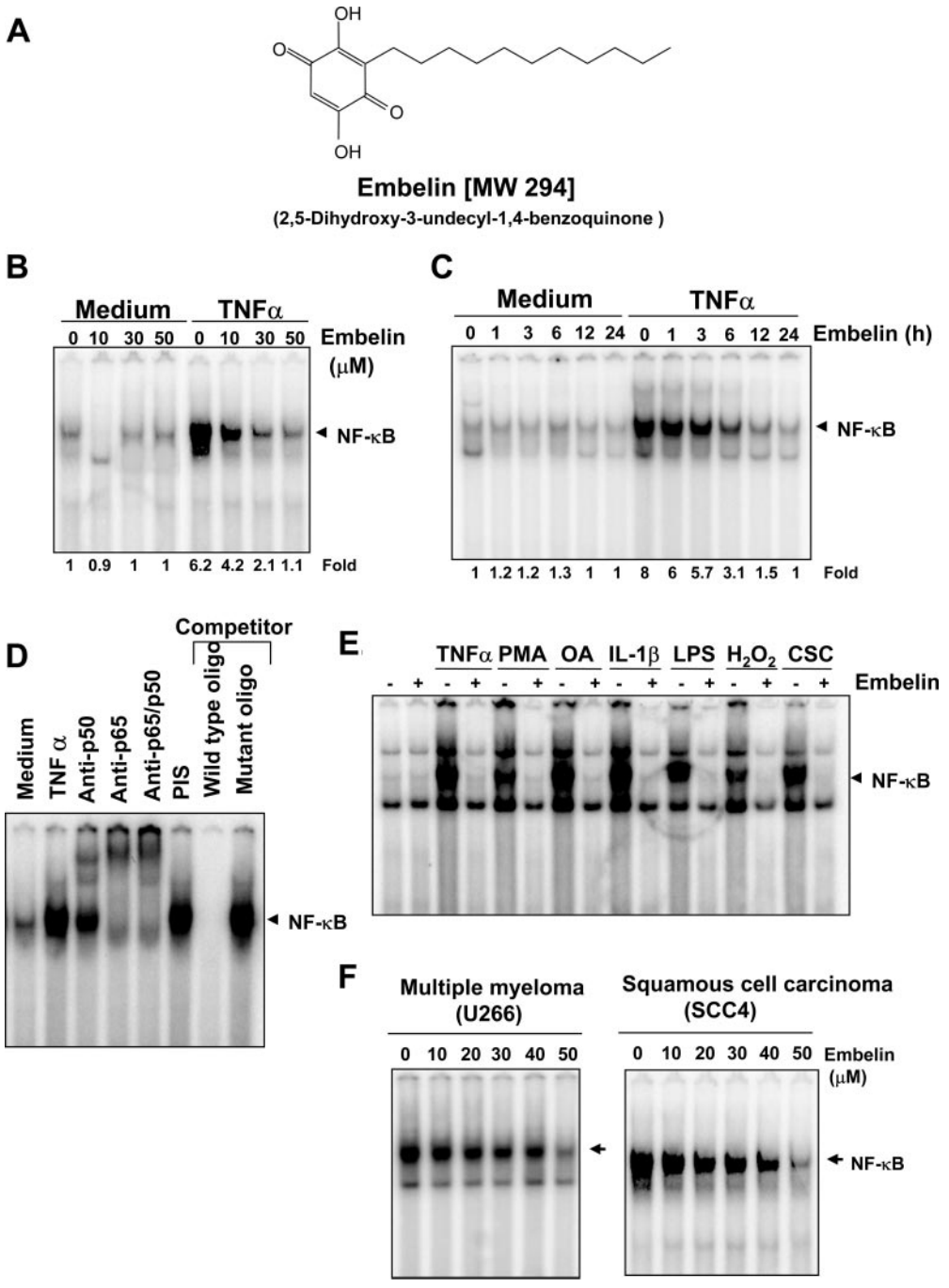

Fig. 1. A, structure of embelin. B, embelin inhibits TNFα-dependent NF-κB activation. KBM-5 cells were incubated with the indicated concentrations of embelin for 12 h and then exposed to 0.1 nM TNFα for 30 min. The nuclear extracts were subjected to EMSA to evaluate NF-κB activation. C, cells were preincubated with 50 μM embelin for the indicated times, treated with 0.1 nM TNFα for 30 min, and then subjected to EMSA to evaluate NF-κB activation. D, nuclear extracts from untreated or TNFα-treated cells were incubated with the indicated antibodies, preimmune serum (PIS), unlabeled NF-κB oligonucleotide probe (Competitor), or mutant oligonucleotide probe (Mutant), and then they were evaluated by EMSA for NF-κB activation. E, KBM-5 cells were preincubated with 50 μM embelin, and then they were exposed to 0.1 nM TNFα for 30 min; with 25 ng/ml PMA, 100 ng/ml LPS, or 100 nM IL-1β for 1 h; 250 μM H_2O_2 or 10 μg/ml CSC for 2 h; or 500 nM OA for 4 h. The nuclear extracts were prepared and analyzed by EMSA for NF-κB activation. F, multiple myeloma U266 and squamous cell carcinoma SCC4 cells were incubated with the indicated concentrations of embelin for 12 h. Nuclear extracts were prepared and analyzed for NF-κB activation by EMSA.

shown to suppress apoptosis induced by TNFα and chemotherapeutic agents (Aggarwal, 2003). Because NF-κB regulates several genes associated with tumorigenesis, inflammation, proliferation, carcinogenesis, and apoptosis, we postulated that embelin mediates its activity by modulating NF-κB activation. We found that embelin abolished activation of NF-κB and suppressed expression of a variety of proliferative, metastatic, and antiapoptotic gene products. This novel NF-κB blocker also enhanced the apoptosis induced by cytokine and chemotherapeutic agents.

Materials and Methods

Reagents. A 50 mM embelin (LKT Laboratories, Inc., St. Paul, MN) solution was prepared in 100% dimethyl sulfoxide, stored as small aliquots at –20°C and then diluted as needed in cell culture medium. Bacteria-derived recombinant human TNFα, purified to homogeneity with a specific activity of 5×10^7 U/mg, was kindly provided by Genentech (South San Francisco, CA). CSC, prepared as described previously (Anto et al., 2002), was kindly supplied by Dr. C. G. Gairola (University of Kentucky, Lexington, KY). Penicillin, streptomycin, Iscove's modified Dulbecco's medium, and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY). PMA, OA, LPS, IL-1β, H_2O_2, crystal violet, and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against p65, p50, inhibitory subunit of NF-κBα (IκBα), cyclin D1, matrix metalloproteinase-9 (MMP-9), poly(ADP-ribose) polymerase (PARP), inhibitor-of-apoptosis protein 1 (IAP1), TNF receptor-associated factor (TRAF)1, Bcl-2, Bcl-x_L, c-Myc, and intercellular adhesion molecule 1 (ICAM-1), and the annexin V staining kit were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-vascular endothelial growth factor (VEGF) was purchased from NeoMarkers (Fremont, CA). FuGENE 6 was purchased from Hoffman-La Roche (Nutley, NJ). Survivin antibody was purchased from R&D Systems (Minneapolis, MN). Anti-cyclooxygenase (COX)-2 and anti-XIAP antibodies were purchased from BD Biosciences (San Jose, CA). Phospho-specific anti-IκBα (serine 32/36) and phospho-specific anti-p65 (serine 536) antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-IκBα kinase (IKK)α, anti-IKKβ, and anti-complementary Fas-associated death domain protein-like interleukin-1β-converting enzyme-inhibitory protein (cFLIP) antibodies were kindly provided by Imgenex (San Diego, CA).

Cell Lines. Human myeloid KBM-5 cells, human lung adenocarcinoma H1299 cells, human embryonic kidney A293 cells, human multiple myeloma U266 cells, and human squamous cell carcinoma SCC4 cells were purchased from the American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 15% FBS. 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% FBS. SCC4 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, nonessential amino acids, pyruvate, glutamine, and vitamins. All media were also supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Electrophoretic Mobility Shift Assay. To assess NF-κB activation, nuclear extracts were prepared, and EMSA was performed as described previously (Chaturvedi et al., 2000), with the following exceptions. In brief, nuclear extracts prepared from TNFα-treated cells (2×10^6/ml) were incubated with a ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat 5′-TTGTTACAA **GGGACTTTC** CGCTG **GGGACTTTC** CAGGGAGGCGTGG-3′ (boldface indicates NF-κB-binding sites) for 30 min at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA **CTCACTTTC** CGCTG **CTCACTTTC** CAGGGAGGCGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNFα-treated cells were incubated with antibodies against either the p50 or the p65 subunit of NF-κB for 30 min at 37°C before the complex was analyzed by EMSA. Preimmune serum was included as a negative control. The dried gels were visualized with a Storm 820, and radioactive bands were quantitated using ImageQuant software (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Western Blot Analysis. To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts and fractionated them by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with the relevant antibody, and detected with an electrogenerated chemiluminescence reagent (GE Healthcare). Next, to determine the expression of cyclin D1, COX-2, MMP-9, cIAP1/2, TRAF1, Bcl-2, Bcl-x_L, VEGF, ICAM-1, XIAP, c-Myc, cFLIP, and survivin in whole-cell extracts of treated cells (2×10^6 cells in 1 ml of medium), 50 μg of whole-cell lysate was resolved by SDS-PAGE, electrotransferred to nitrocellulose membrane, sliced based on the molecular weight, and then probed with antibodies against various proteins. The bands were quantitated using a personal densitometer scanner version 1.30 and ImageQuant software version 3.3 (GE Healthcare).

IKK Assay. To determine the effect of embelin on TNFα-induced IKK activation, an IKK assay was performed by a method we described previously, with the following exceptions. In brief, the IKK complex from whole-cell extracts was precipitated with antibody against IKKα and then treated with protein A/G agarose beads (Pierce Chemical, Rockford, IL). After 2 h, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 2 mM dithiothreitol, 20 μCi of [γ-^{32}P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate glutathione transferase-IκBα (amino acids 1–54). After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a Storm 820 PhosphorImager (GE Healthcare). To determine the total amounts of IKKα and IKKβ in each sample, 50 μg of whole-cell proteins was resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKKα or anti-IKKβ antibody.

NF-κB-Dependent Reporter Gene Expression Assay. The effect of embelin on NF-κB-dependent reporter gene transcription induced by TNFα and various genes was analyzed by a secretory alkaline phosphatase (SEAP) assay as described previously, with the following exceptions. In brief, A293 cells (5×10^5 cells/well) were plated in six-well plates and transiently transfected by the calcium phosphate method with pNF-κB-SEAP (0.5 μg). To examine reporter gene expression induced by various genes, we transfected A293 cells were transfected with 0.5 μg of pNF-κB-SEAP plasmid with 1 μg of an expressing plasmid and 0.5 μg of the control plasmid pCMV-FLAG1 for 24 h, treated them with 50 μM embelin for 12 h, and then harvested them from culture medium after an additional 24 h of incubation. The culture medium was analyzed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech, Mountain View, CA) using a Victor 3 microplate reader (PerkinElmer Life and Analytical Sciences, Boston, MA).

Immunocytochemical Analysis of NF-κB p65 Localization. The effect of embelin on the nuclear translocation of p65 was evaluated by immunocytochemical analysis. In brief, treated cells were plated on a poly-(L-lysine)-coated glass slide by centrifugation (Cytospin 4; Thermoshendon, Pittsburgh, PA), air-dried, and fixed with 4% paraformaldehyde after permeabilization with 0.2%Triton X-100. After being washed in phosphate-buffered saline, the slides were blocked with 5% normal goat serum for 1 h and then incubated with

rabbit polyclonal anti-human p65 antibody at a 1:200 dilution. After overnight incubation at 4°C, the slides were washed, incubated with goat anti-rabbit IgG-Alexa 594 (Molecular Probes, Carlsbad, CA) at a 1:200 dilution for 1 h, and counterstained with 50 ng/ml Hoechst 33342 for 5 min to identify nuclei. Stained slides were mounted with mounting medium purchased from Sigma-Aldrich and analyzed under a fluorescence microscope (Labophot-2, Nikon, Lewisville, TX). Pictures were captured using a Photometrics CoolSNAP CF color camera (Nikon) and MetaMorph version 4.6.5 software (Molecular Devices, Sunnyvale, CA).

Luciferase Assay. The effect of embelin on COX-2 promoter activity induced by TNFα was analyzed using a luciferase assay. A293 cells were seeded at a concentration of 1×10^5 cells/well in 12-well plates. After overnight culture, the cells in each well were transfected with 0.5 μg of DNA consisting of COX-2 promoter-luciferase reporter plasmid by FuGENE 6 (Roche Diagnostics, Indianapolis, IN). The COX-2 promoter (−375 to +59), which was amplified from human genomic DNA by using the primers 5′-GAGTCTCTTATTTATTTTT-3′ (sense) and 5′-GCTGCTGAGGAGTTCCTGGACGTGC-3′ (antisense), was kindly provided by Dr. Xiao-Chun Xu (The University of Texas M. D. Anderson Cancer Center, Houston, TX). After 24 h of transfection, the cells were incubated with embelin for 12 h. The cells were then exposed to 1 nM TNFα for 24 h and harvested. Luciferase activity was measured using the luciferase assay system (Promega, Madison, WI) and detected with a Victor 3 microplate reader (PerkinElmer Life and Analytical Sciences).

Cytotoxicity Assay. The effect of embelin on the cytotoxic effects of TNFα and the chemotherapeutic reagents was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method. In brief, 10,000 cells were incubated with 5 μM embelin for 12 h in triplicate on 96-well plates and then treated with various concentrations of reagents for 24 h at 37°C. Thereafter, an MTT solution was added to each well. After 2 h of incubation at 37°C, extraction buffer (20% SDS and 50% dimethylformamide) was added, the cells were incubated overnight at 37°C, and then the optical density was measured at 570 nm using a 96-well multiscanner (MRX revelation; Dynex Technologies, Chantilly, VA).

Live and Dead Assay. To assess cytotoxicity, we used the live and dead assay (Molecular Probes), which determines intracellular esterase activity and plasma membrane integrity. In brief, 2×10^5 cells were incubated with 5 μM embelin for 12 h and then treated with 1 nM TNFα, 10 nM paclitaxel, and 0.1 μM doxorubicin for 16 h at 37°C. Cells were stained with the live and dead reagent (5 μM ethidium homodimer and 5 μM calcein-AM) and incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon).

Annexin V Assay. To detect apoptosis, we used annexin V antibody conjugated with the fluorescent dye FITC. In brief, 1×10^6 cells were pretreated with 50 μM embelin for 12 h, treated with 1 nM TNFα for 24 h, and then subjected to annexin V staining. Cells were washed, stained with FITC-conjugated anti-annexin V antibody and then analyzed with a flow cytometer (FACSCalibur; BD Biosciences).

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay. We assayed cytotoxicity by the TUNEL method, which examines DNA strand breaks during apoptosis, using an in situ cell death detection reagent (Roche Pharmaceuticals, Belleville, NJ). In brief, 1×10^6 cells were pretreated with 50 μM embelin for 12 h and with 1 nM TNFα for 24 h at 37°C. Thereafter, cells were incubated with reaction mixture for 60 min at 37°C. Stained cells were analyzed by flow cytometer (FACSCalibur; BD Biosciences).

PARP Cleavage Assay. For detection of cleavage products of PARP, whole-cell extracts were prepared by subjecting embelin-treated cells to lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 0.1%Triton X-100, 0.01 μg/ml aprotinin, 0.005 μg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, and 4 mM $NaVO_4$). Lysates were spun at 14,000 rpm for 10 min to remove insoluble material, resolved by 7.5% SDS-PAGE, and probed with PARP antibodies.

Clonogenic Cell Survival Assay. H1299 cells were seeded in six-well plates at 500 cells/well in RPMI 1640 medium containing 10% serum. After 12 h, cells were treated with medium containing indicated concentrations of embelin and 1 nM TNFα. The medium with embelin and TNFα was replaced after every 4 days. After 2 weeks of incubation, colonies were stained with 0.3% crystal violet solution (dissolved in 1:1 mixture of methanol and H_2O) for 2 min, washed once with Dulbecco's phosphate-buffered saline, air-dried, and manually counted. Each point was a mean of three replicate wells.

Invasion Assay. A total of 2.5×10^4 H1299 cells were suspended in serum-free medium and seeded into the upper wells. After incubation overnight, cells were treated with 50 μM embelin for 12 h and then stimulated with 1 nM TNFα for further 24 h in the presence of 1% FBS and the embelin. The cells that invaded through the Matrigel (i.e., those that migrated to the lower chamber during incubation) were stained with 4 μg/ml calcein-AM (Molecular Probes) in phosphate-buffered saline for 30 min at 37°C and scanned for fluorescence with a Victor 3 multiplate reader (PerkinElmer Life and Analytical Sciences). The fluorescent cells were counted.

Results

The goal of this study was to investigate the effect of embelin on the modulation of NF-κB pathway. The structure of embelin is shown in Fig. 1A. The concentration of embelin used in our studies and the duration of exposure had minimal effect on cell viability as determined by a trypan blue dye exclusion test (data not shown).

Embelin Inhibits TNFα-Induced NF-κB Activation in a Dose- and Time-Dependent Manner. Because TNFα is one of the most potent activators of NF-κB and the mechanism of activation of NF-κB is relatively well established, we investigated the effect of embelin on TNFα-induced NF-κB activation. As shown in Fig. 1B, 10 μM embelin suppressed NF-κB activation in human myeloid KBM-5 cells by 32%, and 50 μM suppressed it completely. To determine whether suppression of NF-κB by embelin is time-dependent, we incubated the KBM-5 cells with 50 μM embelin for up to 24 h and then exposed them to TNFα. EMSA results showed that embelin abolished TNFα-induced NF-κB activation within 12 h (Fig. 1C). Under these conditions, the cells were fully viable as determined by the trypan blue dye exclusion test (data not shown).

Various combinations of Rel/NF-κB protein constitute active NF-κB heterodimers that bind to specific DNA sequences (Ghosh and Karin, 2002). To demonstrate that the retarded band visualized by EMSA in TNFα-treated cells was indeed NF-κB, we incubated nuclear extracts from TNFα-treated cells with antibodies to either the p50 (NF-κB1) or p65 (RelA) subunit of NF-κB. Both the antibodies shifted the major band to a higher molecular mass (Fig. 1D), suggesting that the TNFα-activated complex consisted of p50 and p65 subunits. Preimmune serum had no effect, and excess unlabeled NF-κB (100-fold) caused complete disappearance of the band, but a mutant oligonucleotide of NF-κB did not affect NF-κB binding activity.

Embelin Blocks NF-κB Activation Induced by TNFα, PMA, OA, IL-1β, LPS, H_2O_2, and CSC. Because mechanism of NF-κB activation may depend on the stimulus, embelin may suppress NF-κB activation induced by some agents but not others. PMA, OA, IL-1β, LPS, H_2O_2, and CSC activate NF-κB through different pathways. We investigated the effect of embelin on the activation of NF-κB by these agents

in KBM-5 cells. A DNA-binding assay showed that embelin suppressed the NF-κB activation induced by all of these agents (Fig. 1E). These results suggest that embelin must act at a step in the NF-κB activation pathway that is common to all these agents.

We recently showed that plumbagin suppresses NF-κB activation through modification of NF-κB protein (Sandur et al., 2006), so we investigated whether embelin also suppresses NF-κB activation through a similar mechanism. We incubated nuclear extracts from TNFα-treated KBM-5 cells with embelin and then analyzed the DNA-binding activity by using an EMSA. Embelin did not interfere with the DNA-binding ability of the NF-κB complex (data not shown), indicating that embelin does not inhibit NF-κB activation by modifying NF-κB proteins.

Embelin Inhibits Constitutive NF-κB Activation. We also tested the effect of embelin on NF-κB activation in human multiple myeloma (U266) and head and neck squamous cell carcinoma (SCC4) tumor cells, both of which express constitutively active NF-κB. U266 and SCC4 cells were treated with different concentrations of embelin for 12 h and then analyzed NF-κB activation. Embelin inhibited constitutively active NF-κB in both cell lines in a dose-dependent manner (Fig. 1F). These results indicate that embelin inhibits not only inducible but also constitutive NF-κB activation.

Embelin Inhibits TNFα-Dependent IκBα Degradation. To determine whether inhibition of NF-κB activation by embelin was due to inhibition of IκBα degradation, we pretreated KBM-5 cells with embelin, exposed them to TNFα for different times, and then examined them for IκBα status in the cytoplasm by Western blot analysis and for NF-κB activation in the nucleus by EMSA. NF-κB activation was detected with an increase in incubation times with TNFα. The embelin-treated cells showed a dramatic decrease in TNFα-induced NF-κB activation (Fig. 2A). The translocation of NF-κB to the nucleus was preceded by the proteolytic degradation of IκBα. We found that TNFα induced IκBα degradation in the KBM-5 control cells within 10 min and resynthesis between 30 and 90 min (Fig. 2B, left). However, no degradation of IκBα was found in the embelin-treated cells (Fig. 2B, right). These results indicate that embelin inhibits TNFα-induced NF-κB activation by inhibiting IκBα degradation.

Embelin Inhibits TNFα-Induced IκBα Phosphorylation. To determine whether embelin affects TNFα-induced IκBα phosphorylation, we examined the TNFα-induced phosphorylated form of IκBα by Western blot analysis using an antibody specific for the serine-phosphorylated form of IκBα. Because TNFα-induced phosphorylation of IκBα leads to its rapid degradation (Ghosh and Karin, 2002), we blocked degradation of IκBα with the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucine. TNFα-induced phosphorylation of IκBα was undetectable, but when the cells were pretreated with the inhibitor, the induced phosphorylation was apparent (Fig. 2C). Embelin completely suppressed the TNFα-induced IκBα phosphorylation.

Embelin Inhibits TNFα-Induced IKK Activation. The phosphorylation of IκBα is dependent on the activation of IKK (Ghosh and Karin, 2002). Because embelin inhibits the phosphorylation of IκBα, we investigated the effect of embelin on TNFα-induced IKK activation in KBM-5 cells, using glutathione transferase-IκBα as a substrate. As shown in

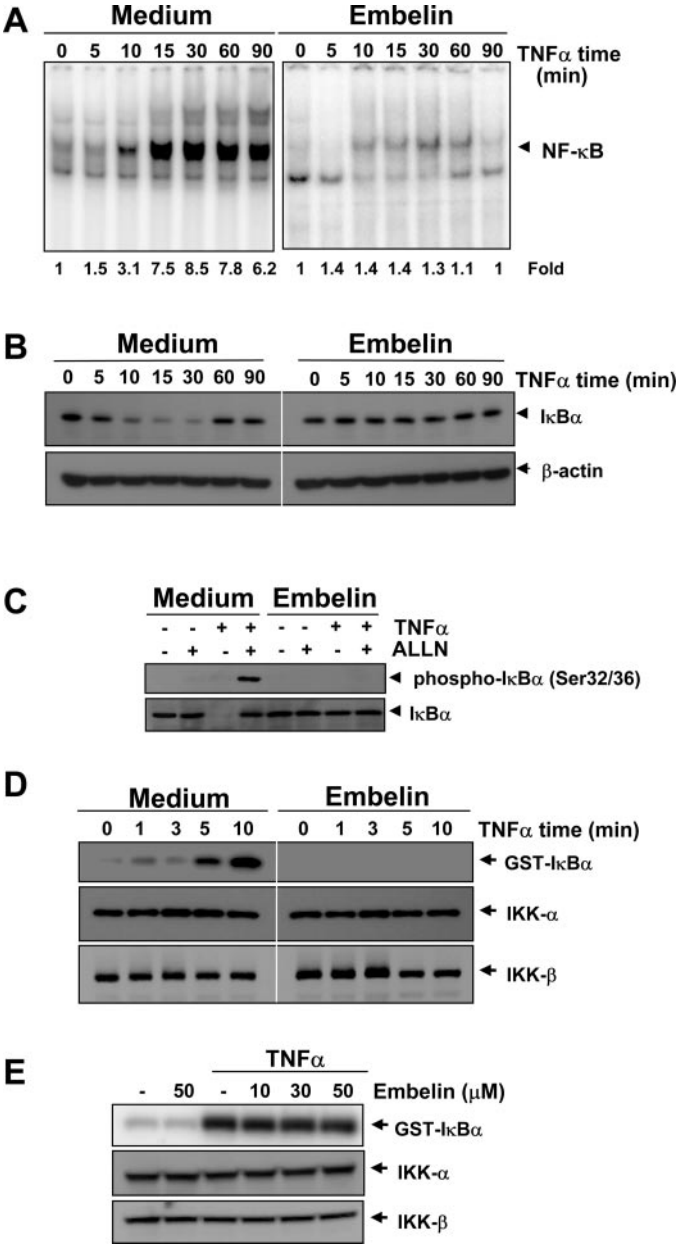

Fig. 2. Embelin inhibits TNFα-dependent inhibitory subunit of NF-κB (IκB) phosphorylation, degradation, and IκBα kinase activation. A, KBM-5 cells were preincubated with 50 μM embelin for 12 h, treated with 0.1 nM TNFα for the indicated times, and then they were subjected to Western blot analysis and EMSA to evaluate NF-κB activation. B, KBM-5 cells were incubated with 50 μM embelin for 12 h, and then they were treated with 0.1 nM TNFα for the indicated times. Cytoplasmic extracts were prepared, fractionated on 10% SDS-PAGE, and electrotransferred to a nitrocellulose membrane. Western blot analysis was performed with anti-IκBα. C, cells were preincubated with 50 μM embelin for 12 h, incubated with 50 μg/ml *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) for 30 min, and then they were treated with 0.1 nM TNFα for 15 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phospho-specific anti-IκBα antibody. The same membrane was reblotted with anti-IκBα antibody. D, KBM-5 cells were preincubated with 50 μM embelin for 12 h, incubated with 50 μg/ml ALLN for 30 min, and then treated with 1 nM TNFα for the indicated times. Whole-cell extracts were immunoprecipitated with antibody against IKKα and analyzed using an immunocomplex kinase assay. E, whole-cell extracts were prepared from KBM-5 cells treated with 1 nM TNFα and immunoprecipitated with anti-IKKα antibody. The immunocomplex kinase assay was performed with and without the indicated concentrations of embelin.

Fig. 2D, TNFα induced IKK activation and embelin completely suppressed it. TNFα or embelin had no direct effect on the expression of either IK-α or IKKβ.

Whether embelin blocks IKK activation or directly inhibits the activity of IKK was also examined. As shown in Fig. 2E, embelin had no direct effect on IKK activity. This finding suggests that embelin modulates TNFα-induced activation of IKK.

Embelin Inhibits TNFα-Induced Nuclear Translocation of p65. The degradation of IκBα leads to nuclear translocation of p65. Whether embelin affects the TNFα-induced nuclear translocation of p65 was tested by two independent methods. Data from immunocytochemical analysis shows that TNFα induced nuclear translocation and embelin inhibited it (Fig. 3A).

We also determined the effect of embelin on TNFα-induced nuclear translocation of p65 by Western blot analysis. We found that TNFα induced nuclear translocation of p65 in a time-dependent manner, and embelin blocked its translocation (Fig. 3B). This translocation of p65 in the nucleus correlated with the disappearance of p65 from the cytoplasm as observed by Western blot analysis (Fig. 3C).

Embelin Inhibits TNFα-Induced Phosphorylation and Acetylation of p65. TNFα is also known to induce the

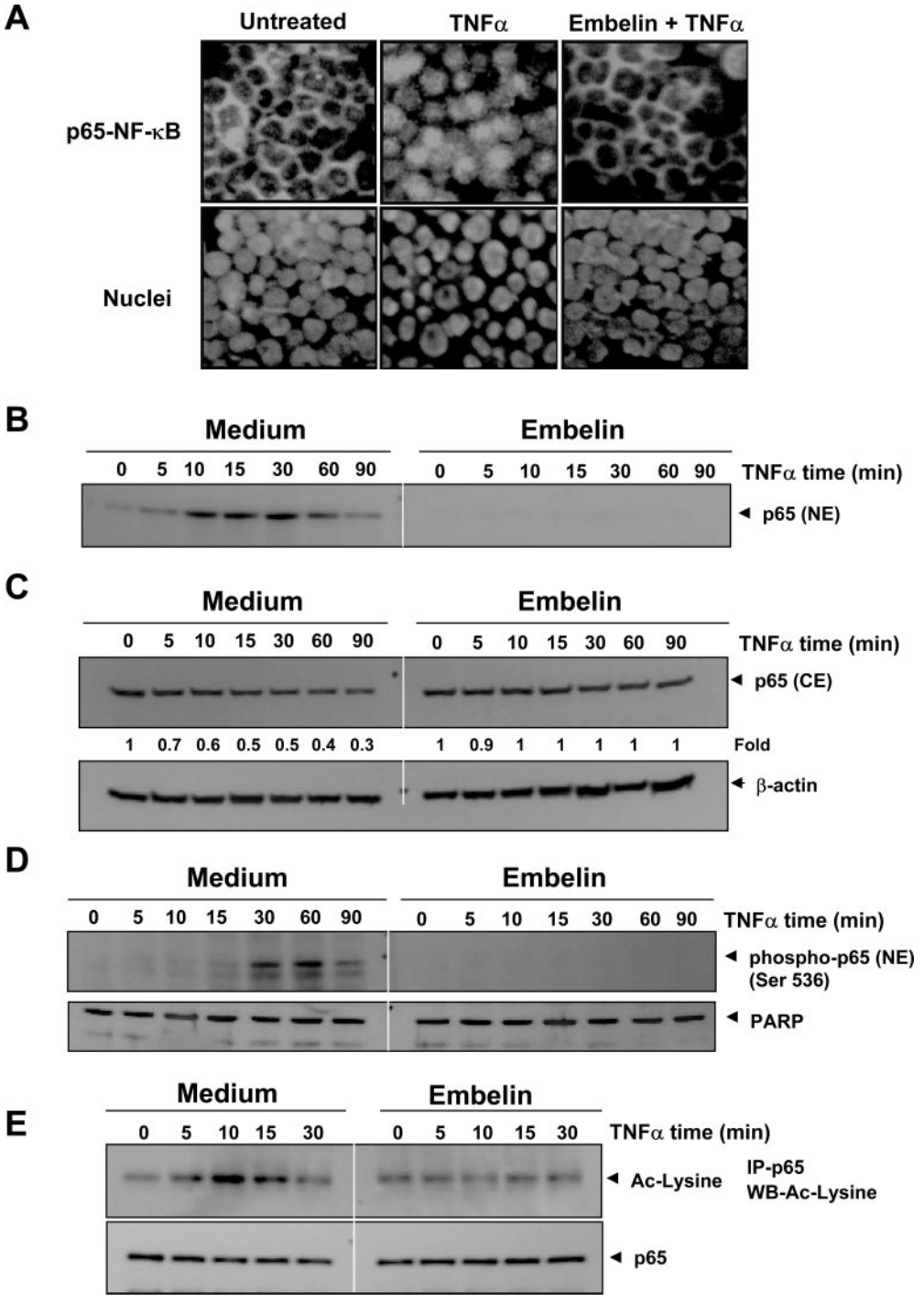

Fig. 3. Embelin inhibits TNFα-induced p65 nuclear translocation, p65 phosphorylation, and p65 acetylation. A, immunocytochemical analysis of TNFα-induced p65 nuclear translocation. KBM-5 cells were incubated with 50 μM embelin for 12 h, treated with 1 nM TNFα for 15 min, and then they were subjected to immunocytochemical analysis as described under *Materials and Methods*. B, Western blot analysis showing the effect of embelin on TNFα-induced p65 nuclear translocation. Cells were incubated with 50 μM embelin for 12 h, and then they were treated with 0.1 nM TNFα for the indicated times. Nuclear extracts were prepared, fractionated on SDS-PAGE, and electrotransferred to a nitrocellulose membrane. The analysis was performed using p65 antibodies. C, cytoplasmic extracts were prepared, fractionated on SDS-PAGE, and electrotransferred to a nitrocellulose membrane. The analysis was performed using p65 antibodies. D, effect of embelin on TNFα-induced p65 phosphorylation. KBM-5 cells were incubated with 50 μM embelin for 12 h, and then they were treated with 0.1 nM TNFα for the indicated times. Nuclear extracts were prepared, fractionated on SDS-PAGE, and electrotransferred to a nitrocellulose membrane. Western blot analysis was performed using phospho-p65 antibodies. PARP antibody was the loading control. E, effect of embelin on TNFα-induced acetylation of p65. Cells were treated with 50 μM embelin for 12 h, and then they were exposed to 1 nM TNFα for the indicated times. Whole-cell extracts were prepared, immunoprecipitated (IP) with anti-p65 antibody, and subjected to Western blot (WB) analysis using anti-acetyl (Ac)-lysine antibody. The same blots were reprobed with anti-p65 antibody.

phosphorylation of p65. Whether embelin affects this phosphorylation was examined using specific antibodies. As shown in Fig. 3D, TNFα induced phosphorylation of p65 at serine residue 536 and embelin inhibited it. The phosphorylation of p65 leads to its acetylation, which plays a key role in the IκBα-mediated activation of NF-κB transcriptional activity (Chen et al., 2001). As shown in Fig. 3E, embelin also suppressed the TNFα-induced acetylation of p65.

Embelin Represses TNFα-Induced NF-κB-Dependent Reporter Gene Expression. Although we determined by using an EMSA that embelin inhibited NF-κB activation, DNA binding alone is not always associated with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps (Campbell et al., 2004). Therefore, we investigated where embelin acts in the sequence of recruitment of TNF receptor (TNFR)-1, TNFR1-associated death domain (TRADD) protein, TRAF2, NF-κB-inducing kinase (NIK), and IKK recruitment that characterizes TNFα-induced NF-κB activation (Hsu et al., 1996; Simeonidis et al., 1999). In cells transfected with TNFR1, TRADD, NIK, IKKβ, and p65 plasmids, NF-κB-dependent SEAP expression was induced, and embelin substantially suppressed SEAP expression induced by all plasmids except those transfected with

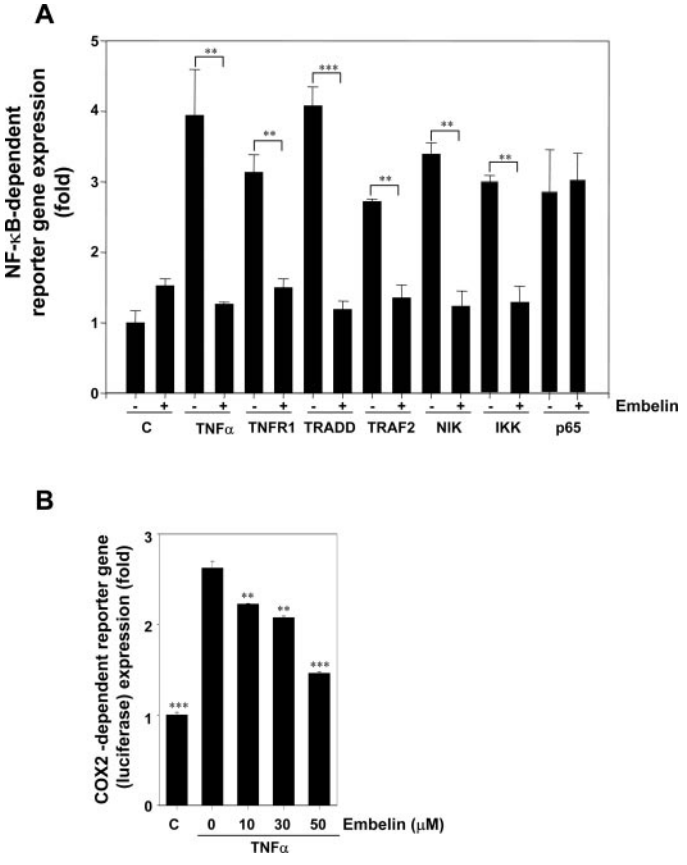

Fig. 4. Embelin represses NF-κB-dependent reporter gene expression induced by TNFα and TNF signaling components. A, embelin inhibits the NF-κB-dependent reporter gene expression induced by TNFα, TNFR1, TRADD, TRAF2, NIK, IKK, but not p65. Cells were transiently transfected with a NF-κB-containing plasmid alone or with the indicated plasmids. After transfection, A293 cells were incubated with 50 μM embelin for 12 h, and then they were incubated with either 1 nM TNFα or the relevant plasmid for an additional 24 h. TNFα-treated cells were incubated with 50 μM embelin for 12 h, and then they were treated with 1 nM TNFα for an additional 24 h. The supernatants of the culture medium were assayed for the reporter gene (SEAP) activity. Determinations were made in triplicate. Data represent the mean of three measurements ± S.D. ***, $p < 0.001$; **, $p < 0.01$. B, embelin inhibits the COX-2 promoter activity induced by TNFα. Cells were transiently transfected with a COX-2 promoter linked to the luciferase reporter gene plasmid for 24 h and treated with the indicated concentrations of embelin for 12 h. Cells were then treated with 1 nM TNFα for an additional 24 h, lysed, and subjected to a luciferase assay. Determinations were made in triplicate. Data represent the mean of three measurements ± S.D. ***, $p < 0.001$; **, $p < 0.01$.

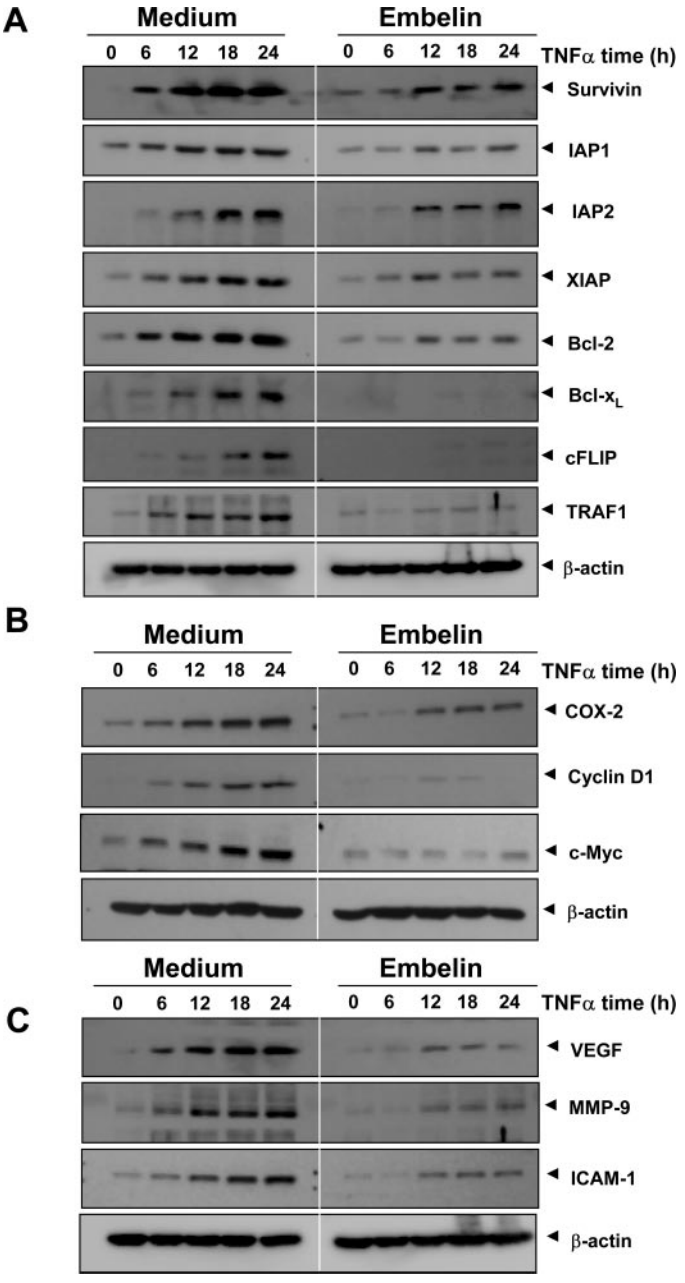

Fig. 5. Embelin represses TNFα-induced NF-κB-dependent expression of antiapoptosis-, proliferation-, and metastasis-related gene products. A, antiapoptotic; B, proliferative; and C, metastatic gene products. KBM-5 cells were incubated with 50 μM embelin for 12 h and then treated with 1 nM TNFα for the indicated times. Whole-cell extracts were prepared, and 50 μg of the whole-cell lysate was resolved by SDS-PAGE, electrotransferred to nitrocellulose membrane, sliced based on the molecular weight, and then probed with antibodies against survivin, IAP1/2, XIAP, Bcl-2, Bcl-x_L, cFLIP, TRAF1, VEGF, MMP-9, ICAM-1, COX-2, ICAM-1, c-Myc, VEGF, cFLIP, XIAP, cyclin D1, or β-actin, as described under *Materials and Methods*. TNFα-treated and TNFα plus embelin-treated samples were run on the same gel under identical conditions and probed with the same immunoblotting solutions.

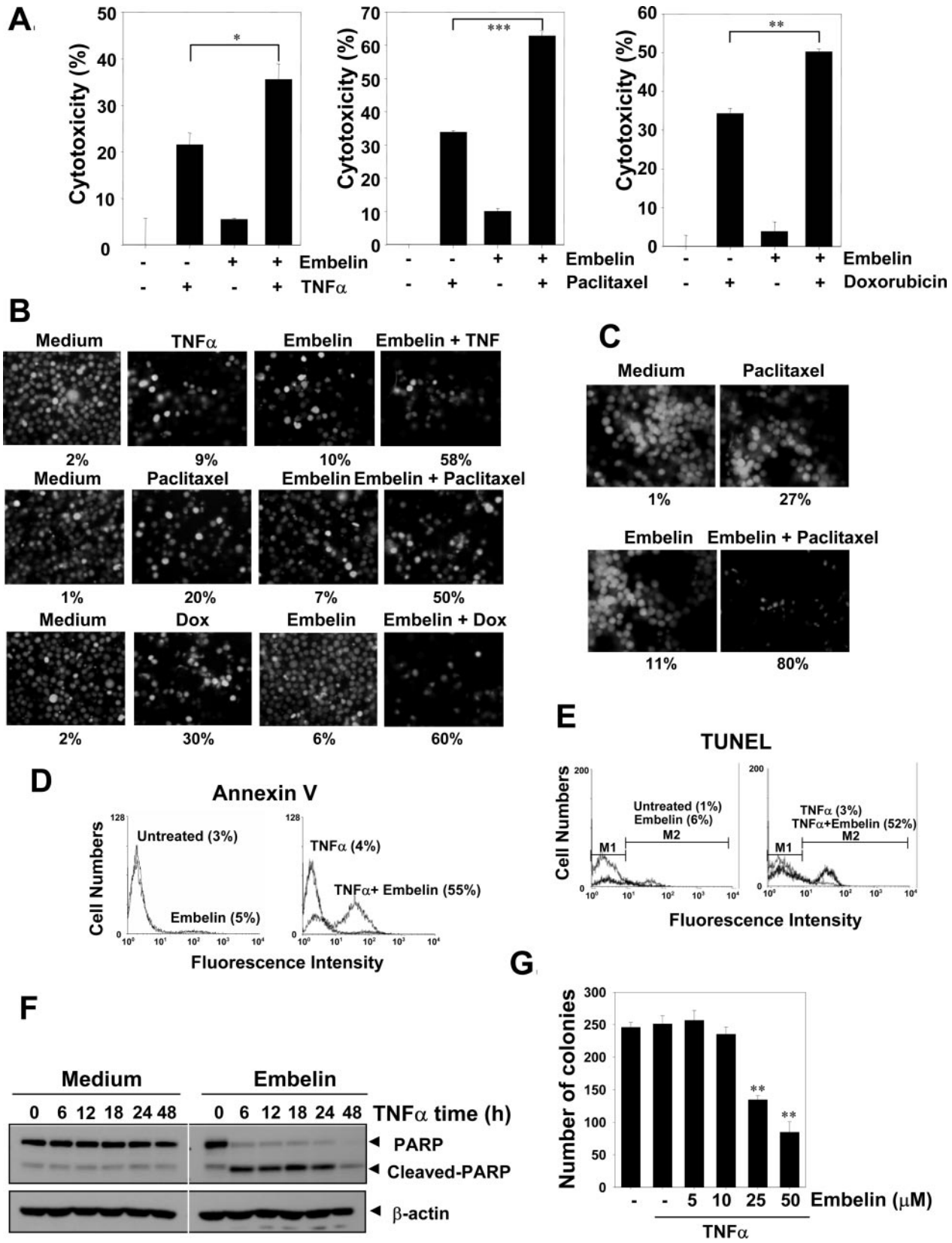

Fig. 6. Embelin enhances apoptosis induced by TNFα and chemotherapeutic agents. A, KBM-5 cells (10,000 cells/0.1 ml) were incubated at 37°C with 1 nM TNFα, 10 nM paclitaxel, or 0.1 μM doxorubicin in the presence and absence of 5 μM embelin as indicated for 24 h, and the cell viability was assayed using the MTT reagent. The results are expressed as mean cytotoxicity ± S.D. from triplicate cultures. Determinations were made in triplicate. Data represent the mean of three measurements ± S.D. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. B, KBM-5 cells (2×10^5/ml) were incubated with TNFα, paclitaxel, and doxorubicin alone or in combination with 5 μM embelin as indicated for 16 h. Cell death was determined by the calcein-AM-based live/dead assay as described under *Materials and Methods*. Red highlights dead cells, and green highlights live cells. C, H1299 cells (1×10^5/ml) were incubated with 10 nM paclitaxel alone or in combination with 5 μM embelin as indicated for 16 h. Cell death was determined by

p65 (Fig. 4A). Because IKK activation can cause the phosphorylation of IκBα and p65, we suggest that embelin inhibits NF-κB activation by inhibiting IKK activation.

Embelin Represses TNFα-Induced COX-2 Promoter Activity. We next determined whether embelin affects COX-2 promoter activity, which is regulated by NF-κB. As shown in Fig. 4B, embelin significantly reduced the TNFα-induced COX-2 promoter activity in a dose-dependent manner.

Embelin Suppresses TNFα-Induced NF-κB-Dependent Antiapoptotic Gene Products. NF-κB regulates the expression of the antiapoptotic proteins survivin, IAP1/2, XIAP, Bcl-2, Bcl-x_L, cFLIP, and TRAF1 (Aggarwal, 2004). We examined whether embelin modulates TNFα-induced expression of these genes. As shown in Fig. 5A, TNFα induced expression of these antiapoptotic proteins in a time-dependent manner, and embelin significantly inhibited their expression.

Embelin Suppresses TNFα-Induced NF-κB-Dependent Cell Proliferation Gene Expression. Various proteins, including COX-2, cyclin D1, and c-Myc, have been linked to the cell proliferation, and NF-κB has been shown to regulate their expression (Guttridge et al., 1999). We examined whether embelin modulates the expression of proliferative gene products induced by TNFα. As shown in Fig. 5B, TNFα induced the expression of COX-2, cyclin D1, and c-Myc proteins in a time-dependent manner, and embelin significantly inhibited their expression.

Embelin Suppresses TNFα-Induced NF-κB-Dependent Gene Products Involved in Tumor Metastasis. Several genes implicated in tumor metastasis, including VEGF, MMP-9, and ICAM-1, are regulated by NF-κB (Esteve et al., 2002). We examined whether embelin affects the expression of these gene products and found that TNFα induced their expression in a time-dependent manner, and embelin suppressed it (Fig. 5C).

Embelin Potentiates Apoptosis Induced by TNFα and Chemotherapeutic Agents. NF-κB activation inhibits apoptosis induced by TNFα and chemotherapeutic agents (Giri and Aggarwal, 1998). Because embelin inhibits NF-κB activation and NF-κB-regulated antiapoptotic gene products, we investigated whether embelin enhances apoptosis induced by TNFα and chemotherapeutic agents. The effect of embelin on TNFα and chemotherapeutic agent-induced apoptosis was examined by the MTT and live and dead assay in human leukemic KBM-5 cells. We found that embelin enhanced the cytotoxic effects of TNFα, paclitaxel, and doxorubicin as analyzed by the MTT method (Fig. 6A). As indicated by the live and dead assay, embelin up-regulated TNFα-induced apoptosis from 2 to 58% (Fig. 6B). Embelin also potentiated the apoptotic effects of paclitaxel and doxorubicin (Fig. 6B).

Besides leukemic cells (KBM-5), we also found that embelin potentiated the apoptotic effect of paclitaxel in non–small-cell lung adenocarcinoma H1299 cells (Fig. 6*C*). Confirming these results, annexin V staining showed that embelin up-regulated TNFα-induced early apoptosis from 4 to 55% (Fig. 6D). The TUNEL staining method also showed that embelin enhanced apoptosis from 3 to 52% (Fig. 6E). Finally, using the PARP-cleavage assay to detect TNFα-induced caspase activation, again we found that embelin potentiated TNFα-induced apoptosis (Fig. 6F). We further assessed the effects of embelin treatment on cell survival by using a clonogenic assay, which measures the long-term effects of drugs on permanent cell growth arrest and apoptosis.

To examine the effect of embelin exposure on clonogenic potential, we treated H1299 cells with different concentrations of embelin for 12 h, stimulated them with TNFα, and cultured them for an additional 14 days before counting colonies. The exposure to embelin and TNF resulted in dose-dependent reduction in colony formation compared with that of control that had not been exposed to embelin (Fig. 6G). All these assays showed that embelin enhanced the apoptotic effects of TNFα and chemotherapeutic agents.

Embelin Suppresses TNFα-Induced Tumor Cell Invasion Activity. Whether embelin can modulate TNFα-induced tumor cell invasion activity was investigated in vitro. To determine this, tumor cells were seeded to the top chamber of the Matrigel invasion chamber with TNFα in the presence or absence of 50 μM embelin and then examined for invasion. As shown in Fig. 7A, TNFα induced tumor cell invasion by almost 7-fold, and embelin suppressed this activity.

Discussion

We conclude, based on our results, that embelin is a potent inhibitor of NF-κB activation, which makes it a potentially effective suppressor of tumor cell survival, proliferation, invasion, angiogenesis, and inflammation. We demonstrated that embelin does suppress NF-κB activation induced by TNFα and various other inflammatory and carcinogenic agents. Embelin also inhibited constitutive NF-κB activation. Embelin seemed to mediate its effects by inhibiting IKK activation, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, p65 acetylation, nuclear translocation, and expression of the NF-κB-dependent reporter gene. As a result, embelin down-regulated NF-κB-dependent gene products involved in tumor cell survival, proliferation, invasion, and angiogenesis. In addition, these effects were associated with embelin's enhancement of apoptosis induced by TNFα and various chemotherapeutic agents (Fig. 7B).

Because embelin inhibited the NF-κB activation induced by TNFα and various other highly diverse agents, embelin must act at a step in the NF-κB pathway that is common to all these agents. We also found that embelin inhibited not only inducible NF-κB activation but also constitutively active NF-κB in tumor cells. Constitutive NF-κB activation has

the calcein-AM-based live/dead assay as described under *Materials and Methods*. Red highlights dead cells, and green highlights live cells. D, cells were pretreated with 50 μM embelin for 12 h, and then they were incubated with 1 nM TNFα for 24 h. Afterward, they were incubated with anti-annexin V antibody conjugated with FITC and analyzed with a flow cytometer for early apoptotic effects. E, cells were pretreated with 50 μM embelin for 12 h and then with 1 nM TNFα for 24 h. They were fixed, stained with TUNEL assay reagent, and then they were analyzed with flow cytometer. F, cells were pretreated with 50 μM embelin for 12 h and then incubated with 1 nM TNFα for the indicated times. Whole-cell extracts were prepared and subjected to Western blot analysis using anti-PARP antibody. G, H1299 (5×10^3 cells/ml) were pretreated with varying does of embelin for 12 h, and then they were exposed to 1 nM TNFα and incubated for an additional 14 days before counting colony numbers. Determinations were made in triplicate. Data represent the mean of three measurements ± S.D. **, $p < 0.01$.

been found to be critical for the survival and proliferation of various tumor cell types (Aggarwal, 2004). Unlike other inhibitors of NF-κB activation, such as caffeic acid phenethyl ester, herbimycin A, protease inhibitors, and plumbagin (Finco et al., 1994; Mahon and O'Neill, 1995; Natarajan et al., 1996; Sandur et al., 2006), embelin did not modify the NF-κB proteins to prevent their binding to DNA. Embelin did inhibit TNFα-induced activation of IKK, leading to suppression of IκBα phosphorylation and degradation. Our results indicate, however, that embelin had no direct effect on the IKK activity. Thus, IKK activation is a common step for all NF-κB activators affected by embelin. How embelin suppresses IKK activation is not clear. The role of several kinases, including AKT (Ozes et al., 1999), TAK1 (Ninomiya-Tsuji et al., 1999), MEKK-1 (Meyer et al., 1996; Nemoto et al., 1998), MEKK-3 (Blonska et al., 2005), NIK (Lin et al., 1998; Nemoto et al., 1998), protein kinase C (Sanz et al., 1999), spleen tyrosine kinase (Takada and Aggarwal, 2004), p21-activated kinase 1 (Fan et al., 2005), focal adhesion kinase 1 (Funakoshi-Tago et al., 2003), and glycogen synthase kinase-3β (Takada et al., 2004) have been linked to TNFα-induced IKK activation. It is possible that embelin mediates its effects on IKK activation through suppression of TAK1. Transforming growth factor-β-activated protein kinase 1 has been shown to bind TRAF2, which in turn recruits TAK1 and mediates TNFα-induced NF-κB activation (Shim et al., 2005). Moreover, cells with the genetic deletion of TAK1 are defective in TNFα-induced NF-κB activation (Sato et al., 2005). Thus, it is possible that embelin mediates its effects on IKK activation through suppression of TAK1.

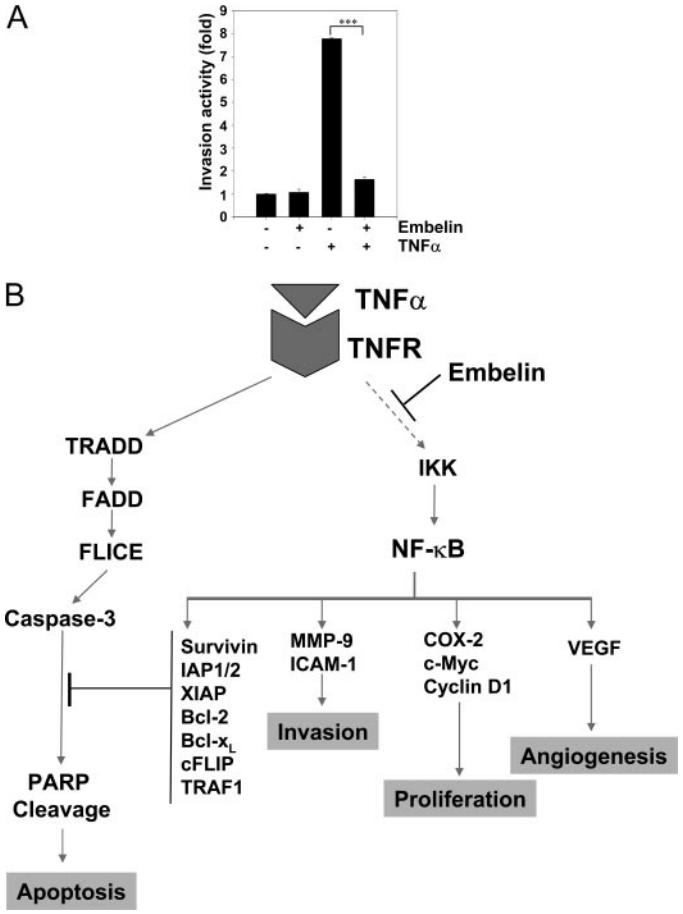

Fig. 7. A, embelin suppresses TNFα-induced invasive activity. H1299 cells (2.5×10^4) were seeded into the upper wells of a Matrigel invasion chamber overnight in the absence of serum, pretreated with 10 μM embelin for 12 h, treated with 1 nM TNFα for 24 h in the presence of 1% serum, and then subjected to invasion assay. The value for no embelin and no TNFα was set to 1.0. Determinations were made in triplicate. Data represent the mean of three measurements ± S.D. ***, $p < 0.001$. B, schematic diagram showing the effect of embelin on TNFα-induced NF-κB signaling pathway and apoptosis.

TNFα-induced NF-κB activation involves the sequential interaction of TNFR with TRADD and TRAF2, which then activate IKK, leading to NF-κB activation (Aggarwal, 2003). Embelin suppressed NF-κB-dependent reporter gene expression induced by TNFR1, TRADD, TRAF2, NIK, and IKK but not expression induced by p65. Although this mechanism is limited to TNFα-induced NF-κB activation, these results suggest that embelin acts at a step upstream from p65.

Embelin has been shown to suppress carcinogenesis (Chitra et al., 1994; Sreepriya and Bali, 2005). Our results demonstrate that embelin inhibits the expression of COX-2, MMP-9, cyclin D1, VEGF, and ICAM-1, all regulated by NF-κB. We also found that COX-2 promoter activity was significantly down-regulated by embelin. Thus, the suppression of hepatocarcinogenesis by embelin reported previously (Sreepriya and Bali, 2005) could be due to the abrogation of the gene products described here. NF-κB activation has been shown to mediate the suppression of apoptosis through the expression of several antiapoptotic gene products. We found that embelin down-regulated the expression of survivin, XIAP, IAP1/2, TRAF1, cFLIP, Bcl-2, and Bcl-x_L. Nikolovska-Coleska et al. (2004) discovered that embelin binds the XIAP BIR3 domain and abolishes its activity to inhibit caspase-9. Our results indicate that embelin also abolishes the expression of XIAP. Furthermore, a recent study showed that through the inhibition of XIAP, embelin induced caspase-9-mediated cleavage of RIP, which is known to be required for NF-κB activation. It is not clear, however, whether embelin inhibits TNFα-induced NF-κB activation by down-regulating RIP. Cells with the genetic deletion of RIP are defective in TNFα-induced NF-κB activation. Because embelin blocks NF-κB activation induced by variety of agents, it is unlikely that the effects of embelin described here are mediated through down-regulation of RIP.

We found that embelin potentiated the apoptotic effects of cytokines and chemotherapeutic agents by down-regulating the NF-κB-dependent apoptosis gene products, including survivin, XIAP, IAP-1/2, TRAF1, cFLIP, Bcl-2, and Bcl-x_L. These results suggest that embelin could induce apoptosis not only by binding to XIAP but also through the expression of NF-κB-regulated antiapoptotic genes as described in our results. The apoptosis induced by TNFα, doxorubicin, and paclitaxel was significantly enhanced by embelin. We also found that embelin enhanced the apoptotic effect of TNF in a clonogenic cell survival assay. Whether the dose of embelin used in our studies is relevant to that in vivo situation is unclear. Doses of 20 mg/kg body weight subcutaneously in mice (Mareninova et al., 2006) or 50 mg/kg body weight orally in rat (Sreepriya and Bali, 2005) have been used, but serum levels of embelin were not reported. Overall, our results demonstrate that embelin clearly inhibits NF-κB activation, which makes it a potentially effective suppressor of tumor cell survival, proliferation, invasion, angiogenesis, and inflammation. Further study of embelin may provide impor-

tant leads for potential treatment applications of cancer and other diseases.

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