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Deguelin, an Akt Inhibitor, Suppresses IκBα Kinase Activation Leading to Suppression of NF-κB-Regulated Gene Expression, Potentiation of Apoptosis, and Inhibition of Cellular Invasion[1]

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Deguelin, a constituent of the bark of the African plant *Mundulea sericea* (Leguminosae), exhibits antiproliferative and anticarcinogenic activities through a mechanism that is not well understood. Because various steps in carcinogenesis are regulated by NF-κB, we postulated that the activity of deguelin is mediated through this transcription factor. We found that deguelin suppressed NF-κB activation induced by carcinogens, tumor promoters, growth factors, and inflammatory stimuli. This suppression was not cell-type specific, because NF-κB activation was suppressed in both lymphoid and epithelial cells. Moreover, constitutive NF-κB activation was also blocked by deguelin. The suppression of TNF-induced NF-κB activation by deguelin occurred through the inhibition of the activation of IκBα kinase, leading to sequential suppression of IκBα phosphorylation, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, and NF-κB-dependent reporter gene expression. Deguelin also suppressed the NF-κB reporter activity induced by TNFR1, TNFR-associated death domain, TNFR-associated factor 2, and IκBα kinase, but not that induced by p65. The inhibition of NF-κB activation thereby led to the down-regulation of gene products involved in cell survival, proliferation, and invasion. Suppression of these gene products by deguelin enhanced the apoptosis induced by TNF and chemotherapeutic agents and suppressed TNF-induced cellular invasion. Our results demonstrate that deguelin inhibits the NF-κB activation pathway, which may explain its role in the suppression of carcinogenesis and cellular proliferation. *The Journal of Immunology,* **2006, 177: 5612–5622.**

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Despite the enormous gains in knowledge about cancer within the last half century, the cancer-associated death rate has not changed significantly (193.9 per 100,000 in 1950 vs 193.4 per 100,000 in 2000). Furthermore, most cancer therapies currently available are toxic, lack efficacy, and are expensive. Although the prevention of cancer is always preferred over treatment, recent evidence involving targeted therapies suggests a convergence between the prevention and treatment of cancer (1, 2). In other words, agents that prevent cancer may also be useful in treating the disease.

One such agent may be deguelin (Fig. 1*A*), a rotenoid from the African plant *Mundulea sericea* (Leguminosae), which was identified as a potent chemopreventive agent on the basis of its action against chemically induced preneoplastic lesions in a mammary organ culture and its inhibition of papillomas in a two-stage mouse skin carcinogenesis model (3–5). Deguelin has also been found to suppress the formation of carcinogen-induced aberrant crypt foci in mouse colons (6). More recently, this rotenoid was shown to suppress cigarette smoke-induced lung carcinogenesis (7, 8). Additionally, deguelin has been shown to enhance the sensitivity of leukemia cells to chemotherapeutic agents (9).

How deguelin mediates its chemopreventive and chemosensitizing effects is not yet fully understood, but various mechanisms have been proposed, including the suppression of ornithine decarboxylase (3, 10), the inhibition of the PI3K/Akt pathway (8, 11), and the down-regulation of cyclooxygenase (COX)[3]-2 (12) and cyclin D1 (13).

In this study, we hypothesized that deguelin mediates its effects through the regulation of the NF-κB activation pathway for various reasons: first, the cellular transformation, proliferation, invasion, and angiogenesis involved in carcinogenesis have been shown to be regulated by NF-κB (14); second, various carcinogens investigated in relation to deguelin have been shown to activate NF-κB (15, 16); third, several chemopreventive agents have been described that down-regulate the NF-κB activation pathway (2); fourth, the PI3K/Akt pathway, known to be inhibited by deguelin, has been shown to activate NF-κB (17); fifth, COX-2 and cyclin D1, both known to be down-regulated by deguelin, are regulated by NF-κB (18, 19); and sixth, the chemosensitization induced by deguelin may result from the suppression of NF-κB activation (20). Thus, we investigated the effect of deguelin on the NF-κB activation pathway induced by carcinogens, tumor promoters, and inflammatory agents; on NF-κB-regulated gene products; and on

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[3] Abbreviations used in this paper: COX, cyclooxygenase; ALLN, *N*-Ac-leu-leu-norleucinal; CSC, cigarette smoke condensate; IAP, inhibitor of apoptosis protein; IKK, IκBα kinase; MMP, matrix metalloproteinase; NIK, NF-κB-inducing kinase; PARP, polyadenosylribose polymerase; SEAP, secretory alkaline phosphatase; TRADD, TNFR-associated death domain; TRAF, TNFR-associated factor; XIAP, X-linked inhibitor of apoptosis protein; zVAD-FMK, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

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NF-κB-mediated cellular responses. Our findings show that deguelin is a potent suppressor of NF-κB activation, leading to the inhibition of the expression of NF-κB-regulated gene products, which results in the potentiation of apoptosis and the inhibition of metastatic invasion.

Materials and Methods

Reagents

Deguelin, purchased from Alexis Biochemicals, was dissolved in DMSO as a 10 mM stock solution, stored as small aliquots at $-20°C$, and then diluted as needed in cell culture medium. Bacteria-derived human rTNF, purified to homogeneity with a sp. act. of 5×10^7 U/mg, was provided by Genentech. Cigarette smoke condensate (CSC), prepared as described (16), was supplied by G. Gairola (University of Kentucky, Lexington, KY). Broad spectrum caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-FMK) was obtained from R&D Systems. Penicillin, streptomycin, RPMI 1640, DMEM, and FBS were obtained from Invitrogen Life Technologies. An anti-β-actin Ab was obtained from Sigma-Aldrich. Abs against p65, p50, IκBα, polyadenosylribose polymerase (PARP), Akt, inhibitor of apoptosis proteins (IAPs) 1 and 2, Bcl-2, Bcl-x_L, c-Myc, matrix metalloproteinase (MMP)-9, ICAM-1, and Bfl-1/A1, annexin assay kit, were obtained from Santa Cruz Biotechnology. Anti-COX-2 and anti-X-linked inhibitor of apoptosis protein (XIAP) Abs were obtained from BD Biosciences. Phospho-specific anti-IκBα (Ser^{32}), phospho-specific anti-p65 (Ser^{536}), and anti-acetyl-lysine Abs were purchased from Cell Signaling Technology. Anti-IκBα kinase α (IKK)-α), anti-IKK-β, and anti-phospho-Akt (Ser^{473}) Abs were provided by Imgenex.

Cell lines

KBM-5 (human myeloid leukemia), H1299 (human lung adenocarcinoma), A293 (human embryonic kidney carcinoma), SCC-4 (human squamous cell carcinoma), U937 (human hystiocytic lymphoma), and U266 cells were obtained from the American Type Culture Collection. KBM-5 cells were cultured in IMDM with 15% FBS. H1299 and U266 cells were cultured in RPMI 1640, and A293 and SCC-4 cells were cultured in DMEM supplemented with 10% FBS, 100 μM nonessential amino acids, 1 mM pyruvate, 6 glutamine, and 1× vitamins. All culture media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

EMSAs

To identify NF-κB activation, we performed EMSAs, as described previously (21). Briefly, nuclear extracts prepared from TNF-treated cells were incubated with a ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol DNA) from the HIV long-terminal repeat 5′-TTGTTACAA**GGGACTTTC**CGCTG **GGGACTTTC**CAGG GAGGCGTGG-3′ (boldface indicates NF-κB binding sites) for 30 min at 37°C; the DNA-protein complex formed was then separated from the free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA**CTCACTTTC**CGCTG**CT CACTTTC**CAGGGAGGCGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined through competition with the unlabeled oligonucleotide. The dried gels were visualized, and radioactive bands were quantitated by using a PhosphorImager (STORM 220; Amersham Biosciences) with ImageQuant (Molecular Dynamics) software.

Western blot analysis

To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts (22) and then fractionated them by using SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with each Ab, and detected with an electrochemiluminescence reagent (Amersham). The bands obtained were quantitated with National Institutes of Health imaging software (National Institutes of Health).

IKK assay

To determine the effect of deguelin on TNF-induced IKK activation, we performed an IKK assay using a previously described method (23). To determine the total amounts of IKK-α and IKK-β in each sample, we resolved 50 μg of the whole-cell protein on a 7.5% SDS-PAGE gel, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK-α or anti-IKK-β Abs.

NF-κB-dependent reporter gene expression assay

NF-κB-dependent reporter gene expression assay was performed, as previously described (24). The effects of deguelin on TNF-dependent, TNFR-dependent, TNFR-associated death domain (TRADD)-dependent, TNFR-associated factor 2 (TRAF2)-dependent, NF-κB-inducing kinase (NIK)-dependent, IKK-β-dependent, and p65-induced NF-κB-dependent reporter gene transcription were analyzed with a secretory alkaline phosphatase (SEAP) assay, as previously described (25). Briefly, A293 cells (5×10^5 cells/well) were plated in 6-well plates and transiently transfected with a transfection reagent (Fugene6; Roche Molecular Biochemicals) and pNF-κB-SEAP (0.25 μg). To examine TNF-induced reporter gene expression, we transfected the cells with 0.25 μg of the SEAP expression plasmid for 24 h. Thereafter, we preincubated the cells for 30 min with 5 μM deguelin and then treated them with 1 nM TNF for an additional 24 h. The cell culture medium was harvested after 24 h of TNF treatment and analyzed for SEAP activity essentially according to the protocol described by the transfection reagent manufacturer (BD Clontech), by using a 96-well fluorescence plate reader (Victor 3 luminometer; PerkinElmer Life and Analytical Sciences) with its excitation set at 360 nm and its emission at 460 nm.

Immunocytochemistry for NF-κB p65 localization

The effect of deguelin on the nuclear translocation of p65 was examined with an immunocytochemical assay, as described previously (26).

Live and dead assay

To measure apoptosis, we used the live and dead assay (Molecular Probes), which determines intracellular esterase activity and plasma membrane integrity. This assay was performed as previously indicated (23).

Cytotoxicity assay

The effect of deguelin on the cytotoxic effects of TNF and chemotherapeutic agents was determined by the MTT uptake method, as previously described (26).

PARP cleavage assay

For detection of the cleavage products of PARP, whole-cell extracts were prepared by subjecting deguelin-treated cells to lysis in a 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 PMSF, and 4 mM $NaVO_4$). Lysates were spun at 14,000 rpm for 10 min to remove any insoluble material, resolved by 10% SDS-PAGE, and probed with PARP Abs (27).

Annexin V assay

An early indicator of apoptosis is the rapid translocation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface and its accumulation there. This loss of membrane asymmetry can be detected by using the binding properties of annexin V. To identify apoptosis, we stained cells with an annexin V Ab conjugated with a fluoroisothiocynate fluorescence dye. Briefly, 5×10^5 cells were preincubated with 5 μM deguelin for 30 min, treated with 1 nM TNF for 16 h at 37°C, and then stained. Cells were washed in PBS, resuspended in 100 μL of a binding buffer containing a fluoroisothiocynate-conjugated anti-annexin V Ab, and then analyzed with flow cytometry (FACSCalibur; BD Biosciences) (27).

TUNEL assay

We also assayed cytotoxicity by using the TUNEL method, which examines DNA strand breaks during apoptosis with an in situ cell death detection reagent. Briefly, 5×10^5 cells were preincubated with 5 μM deguelin and 1 nM TNF for 16 h at 37°C. Thereafter, cells were incubated with this reaction mixture for 60 min at 37°C. Stained cells were analyzed with flow cytometry (FACSCalibur; BD Biosciences) (27).

Invasion assay

Because invasion through the extracellular matrix is a crucial step in tumor metastasis, we performed in vitro invasion assays with a Matrigel basement membrane matrix extracted from the Englebreth-Holm-Swarm mouse tumor as a reconstituted basement membrane for in vitro invasion assays. The tumor invasion system that we used (BD BioCoat; BD Biosciences) has a chamber with a light-tight polyethylene terephthalate membrane with 8-μm pores coated with a reconstituted basement membrane gel. We resuspended 2.5×10^4 H1299 cells in serum-free medium and seeded the

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suspension into the upper wells. After incubation overnight, cells were coincubated with 2 μM deguelin and TNF for an additional 24 h in the presence of 1% FBS. The cells that passed through the Matrigel matrix were labeled with 4 μg/ml calcein-acetoxymethyl (Molecular Probes) in PBS for 30 min at 37°C and subjected to scan fluorescence (Victor 3 luminometer; PerkinElmer Life and Analytical Sciences) (27).

AP-1 activation assay

AP-1 activation by EMSA was examined, as indicated (28).

STAT3 activation assay

STAT3-DNA binding was analyzed by EMSA using a ^{32}P-labeled high-affinity *cis*-inducible element probe, as described (29).

Results

The aim of this study was to investigate the effect of deguelin on the NF-κB activation pathway induced by various carcinogens and inflammatory stimuli, on NF-κB-regulated gene expression, and on NF-κB-mediated cellular responses. Because the TNF-induced NF-κB activation pathway has been well characterized, we studied in detail the effects of deguelin on TNF-induced NF-κB activation. The structure of deguelin is shown in Fig. 1*A*.

Deguelin suppresses TNF-induced NF-κB activation

Because TNF is one of the most potent inflammatory agents and activators of NF-κB and because the mechanism of NF-κB activation is relatively well established (14), we investigated the effect of deguelin on TNF-induced NF-κB activation in human myeloid KBM-5 cells. We first investigated the minimum incubation time required for deguelin to inhibit TNF-mediated NF-κB activation. Cells were preincubated with deguelin for different time intervals and then treated with TNF. The EMSA results indicated that deguelin alone did not activate NF-κB, but did suppress TNF-induced NF-κB activation in a time-dependent manner, with complete inhibition occurring at 24 h (Fig. 1*B*). To determine the minimum concentration of deguelin required, we pre-exposed cells to different concentrations of the rotenoid and then examined them for NF-κB activation after treatment with TNF. The results indicated that TNF-induced NF-κB activation was suppressed in a dose-dependent manner, with maximum suppression occurring at a dose of 10 μM deguelin (Fig. 1*C*). Cells were fully viable under these conditions when treated with deguelin. To further confirm that deguelin-induced down-regulation of NF-κB is not due to cell death, we treated the cells with broad-spectrum caspase-inhibitor (zVAD-FMK) and then examined for the effect of deguelin on TNF-induced NF-κB activation. The results showed that the apoptosis inhibitor had no affect on deguelin's ability to suppress NF-κB activation, thereby suggesting that deguelin inhibits NF-κB activation independent of its apoptotic effects (Fig. 1*D*).

Studies from our laboratory previously showed that TNF-induced NF-κB activity is much higher at high doses for short period rather than lower doses for longer period (30). To examine the effect of deguelin on NF-κB activation at higher concentrations of TNF, we treated cells with deguelin and then incubated them with different concentrations of TNF, after which the activation status of NF-κB was analyzed by EMSA. Results showed that TNF at a 1 nM concentration induced strong NF-κB activity in the absence of deguelin, but that cells treated with deguelin abolished TNF-induced NF-κB activation (Fig. 1*E*). These results suggest that deguelin is a very potent inhibitor of TNF-induced NF-κB activation.

Deguelin does not directly impede NF-κB binding to DNA

Several NF-κB inhibitors, such as the serine protease inhibitor *N*-α-tosyl-L-phenylalanyl chloromethyl ketone, herbimycin A, and the protein kinase inhibitor caffeic acid phenyl ethyl ester, suppress NF-κB activation by directly modifying the NF-κB protein so that it no longer binds to DNA (31–33). Therefore, to determine whether deguelin also directly modifies the NF-κB protein, we incubated nuclear extracts from TNF-treated cells with various concentrations of deguelin for 2 h at room temperature and then performed the EMSA. The results showed that deguelin had no direct effect on NF-κB binding to DNA (Fig. 1*F*). Thus, deguelin may inhibit NF-κB activation through a mechanism different from that of the previously mentioned inhibitors.

NF-κB is a family of proteins, and various combinations of the Rel/NF-κB protein constitute active NF-κB heterodimers that bind to specific DNA sequences (34). To confirm that the band visualized by EMSA in TNF-treated cells was indeed NF-κB, nuclear extracts from TNF-stimulated cells were incubated with Abs against the p50 (NF-κB1) or p65 (RelA) subunit of NF-κB. The Abs shifted the band to a higher molecular mass (Fig. 1*G*), suggesting that the TNF-activated complex consisted of both p50 and p65 subunits. Excess (100-fold) unlabeled NF-κB caused complete disappearance of the band, but a mutant oligonucleotide of NF-κB did not affect NF-κB-binding activity.

Inhibition of NF-κB activation by deguelin is not cell-type specific

It has been reported earlier (34, 35) that diverse signal transduction pathways can regulate NF-κB induction in different cell types. We therefore examined whether deguelin could block TNF-induced NF-κB activation in lung adenocarcinoma H1299 cells (Fig. 2*A*), embryonic kidney A293 cells (Fig. 2*B*), and histiocytic lymphoma U937 cells (Fig. 2*C*). These cells were pre-exposed to deguelin and then examined for TNF-induced NF-κB activation. Deguelin completely suppressed TNF-induced NF-κB activation in all of the three cell lines; this finding indicated that deguelin-induced suppression of NF-κB activation was not cell-type specific.

Deguelin suppresses constitutive NF-κB activation

Most tumor cells express constitutively active NF-κB through a mechanism that is not fully understood (36, 37). Both human multiple myeloma (U266) cells and SCC-4 are known to express constitutively active NF-κB. We showed that treatment with deguelin suppresses the constitutively active NF-κB in both SCC-4 and U266 cells (Fig. 2, *D* and *E*).

Deguelin suppresses NF-κB activation induced by various carcinogens and inflammatory stimuli

Although various growth factors, carcinogens, tumor promoters, and inflammatory stimuli have been shown to activate NF-κB, different agents appear to activate NF-κB by different pathways (21). Because IL-1β, PMA, LPS, and CSC are potent activators of NF-κB (16, 23, 36, 37), we examined the effect of deguelin on the activation of NF-κB by all of these agents and found that the incubation of cells with deguelin suppressed this activation (Fig. 2*F*). Our results suggested that deguelin acts at a step in the NF-κB activation pathway that is common to all five agents.

Deguelin does not modulate transcription factor AP-1 and STAT3

Whether deguelin suppresses other transcription factors like AP-1 and STAT3 under the conditions it suppresses NF-κB was examined. The results show that pretreatment with deguelin inhibited neither TNF-induced AP-1 activity in KBM-5 cells (Fig. 2*G*), nor constitutive STAT3 activation in U266 cells (Fig. 2*H*).

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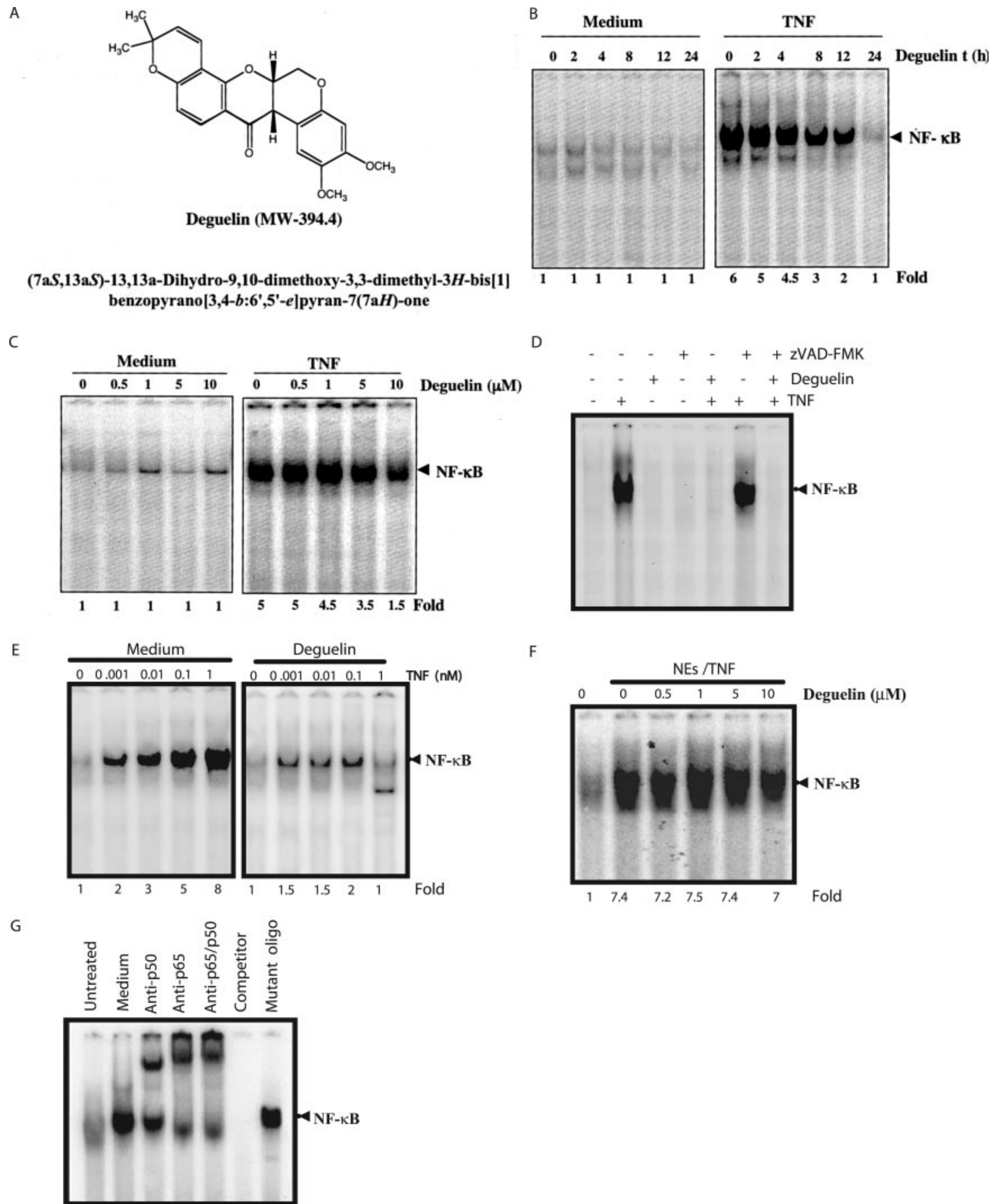

FIGURE 1. *A*, Structure of deguelin. *B*, Deguelin inhibits TNF-dependent NF-κB activation in a time-dependent manner. KBM-5 cells (2×10^6 cells/well) were preincubated with 10 μM deguelin for the indicated times at 37°C and then treated with 0.1 nM TNF for 30 min at 37°C. Nuclear extracts were prepared and then tested for NF-κB activation. *C*, Deguelin inhibits TNF-induced NF-κB activation in a dose-dependent manner. KBM-5 cells (2×10^6 cells/well) were preincubated with the indicated concentrations of deguelin for 12 h at 37°C and then treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and tested for NF-κB activation, as described in *Materials and Methods*. *D*, KBM-5 cells (2×10^6 cells/well) were preincubated with 10 μM deguelin and 20 μM zVAD-FMK alone or in combination for 24 h at 37°C and then treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and tested for NF-κB activation, as described in *Materials and Methods*. *E*, KBM-5 (2×10^6 cells/well) cells were treated with 10 μM deguelin for 12 h and exposed to the indicated concentrations of TNF for 30 min, and nuclear extracts were prepared and then analyzed for NF-κB activation by EMSA. *F*, Deguelin does not modulate the ability of NF-κB to bind to DNA. Nuclear extracts (NEs) from KBM-5 cells (2×10^6 cells/well) treated or not treated with 0.1 nM TNF for 30 min were treated with the indicated concentrations of deguelin at room temperature and then assayed for DNA binding by EMSA. The numbers at the *bottom* of each lane indicate the fold activation over that of untreated control cells. *G*, TNF-induced NF-κB consists of p50 and p65 subunits. Nuclear extracts from KBM-5 cells (2×10^6 cells/well) treated or not treated with 0.1 nM TNF for 30 min were incubated with the Abs indicated, an unlabeled competitor, a NF-κB oligonucleotide probe, or a mutant oligonucleotide probe (Mutant oligo) for 30 min at room temperature, and the complex was analyzed by supershift assay.

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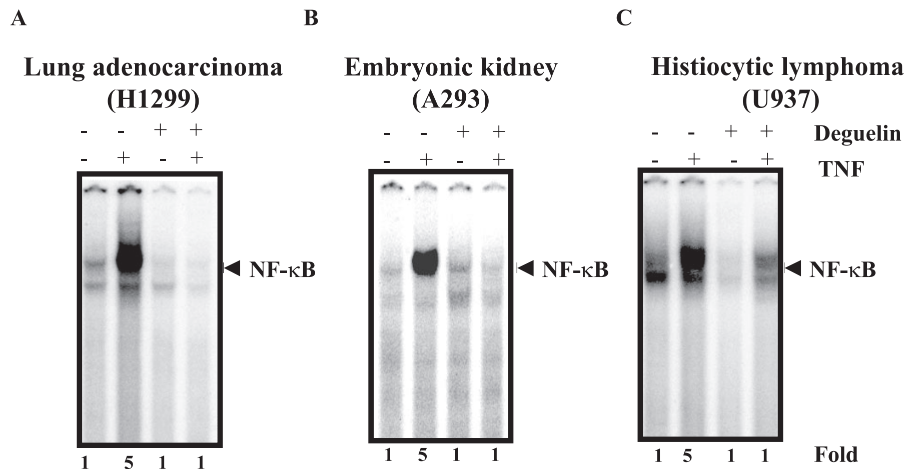

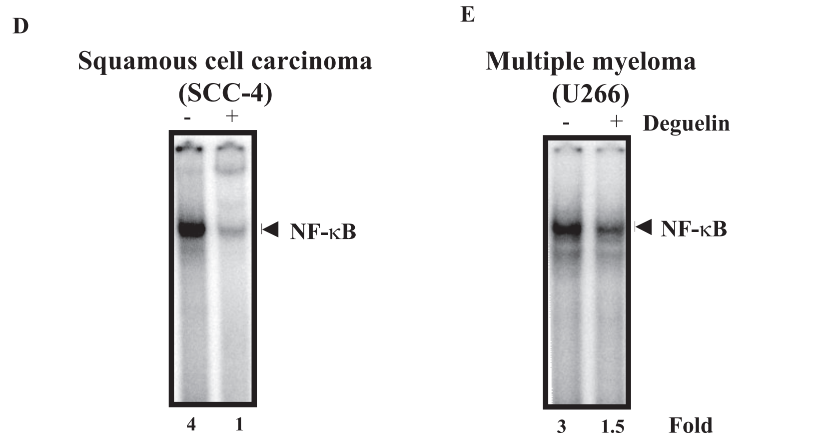

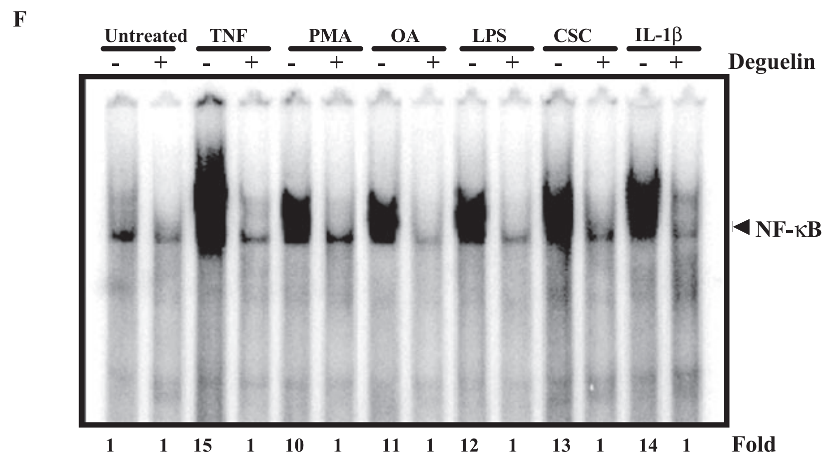

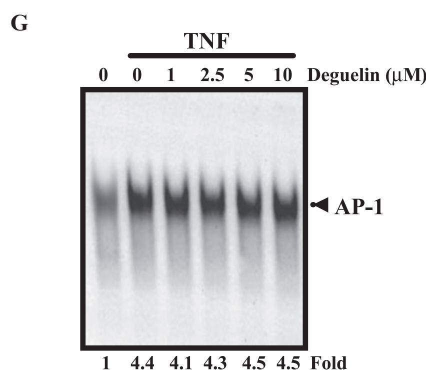

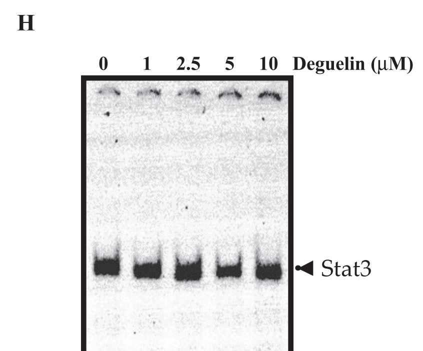

FIGURE 2. *A–C*, Deguelin suppresses inducible NF-κB activation. Two million human lung adenocarcinoma H1299 cells (*A*), human embryonic kidney A293 cells (*B*), and human histiocytic lymphoma U937 cells (*C*) were treated with 10 μM deguelin for 12 h and then with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and analyzed for NF-κB activation by EMSA, as described in *Materials and Methods*. *D* and *E*, Deguelin suppresses constitutive NF-κB activation in head and neck squamous cell carcinoma cells and multiple myeloma cell lines. Two million squamous cell carcinoma SCC-4 cells (*D*) and multiple myeloma U266 cells (*E*) were incubated with 10 μM deguelin for 12 h. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA, as described in *Materials and Methods*. The numbers at the *bottom* of each lane indicate the fold activation over that of untreated control cells. *F*, Deguelin blocks NF-κB activation induced by TNF, PMA, okadaic acid (OA), LPS, CSC, and IL-1β. KBM-5 cells (2×10^6/well) were incubated with 10 μM deguelin for 12 h and then treated with TNF (0.1 nM/L, 30 min), PMA 25 ng/ml, OA (500 nM/L, 4 h), LPS (10 μg/ml, 30 min), CSC (10 μg/ml, 30 min), and IL-1β (10 μg/ml, 30 min). Nuclear extracts were prepared and tested for NF-κB activation, as described in *Materials and Methods*. Results from a representative experiment of the three independent ones with similar results are shown. *G*, Deguelin did not suppress TNF-induced AP-1 activation. KBM-5 cells were incubated with different concentrations of deguelin for 12 h, treated with 0.1 nM TNF, and then analyzed for AP-1 activation by EMSA. *H*, Deguelin did not suppress constitutive nuclear STAT3 levels. U266 cells were incubated with different concentrations of deguelin for 12 h and analyzed for nuclear STAT3 levels by EMSA.

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Deguelin inhibits TNF-dependent IκBα degradation

To examine whether inhibition of TNF-induced NF-κB activation was due to inhibition of IκBα degradation, we incubated cells with deguelin and then exposed them to TNF for different time periods. We then used Western blot analysis to detect IκBα in the cytoplasm. TNF induced IκBα degradation in control cells, with complete degradation occurring at 15 min (Fig. 3*A*, *left panel*). However, in deguelin-pretreated cells, TNF had no effect on IκBα degradation (Fig. 3*A*, *right panel*). These results suggest that deguelin inhibited TNF-induced IκBα degradation.

Deguelin inhibits TNF-dependent IκBα phosphorylation

Because IκBα phosphorylation is needed for IκBα degradation, we examined whether deguelin was involved in modulating IκBα phosphorylation. Given that TNF-induced phosphorylation of IκBα is known to lead to its rapid degradation, we blocked IκBα degradation with the proteasome inhibitor *N*-Ac-leu-leu-norleucinal (ALLN). Cells were treated with deguelin, exposed to TNF in the presence of ALLN, and then examined by Western blot analysis for phosphorylated IκBα using specific Abs. As shown in Fig. 3*B*, TNF-induced IκBα phosphorylation was almost completely suppressed by deguelin.

Deguelin inhibits TNF-induced IKK activation

IKK is known to be required for TNF-induced phosphorylation of both IκBα (38) and p65 (39). Because deguelin inhibited the phosphorylation of IκBα, we examined its effect on TNF-induced IKK activation. Cells, either untreated or pretreated with deguelin, were treated with TNF for different times; cell extracts were then prepared, the IKK was immunoprecipitated, and the immunocomplex kinase assays were performed on the pellet. IKK activation was seen as early as 5 min after TNF treatment (Fig. 3*C*, *top panel*), and deguelin was seen to suppress the activation of IKK induced by TNF. Neither TNF nor deguelin had any effect on the expression of either IKK-α (Fig. 3*C*, *middle panel*) or IKK-β (Fig. 3*C*, *bottom panel*).

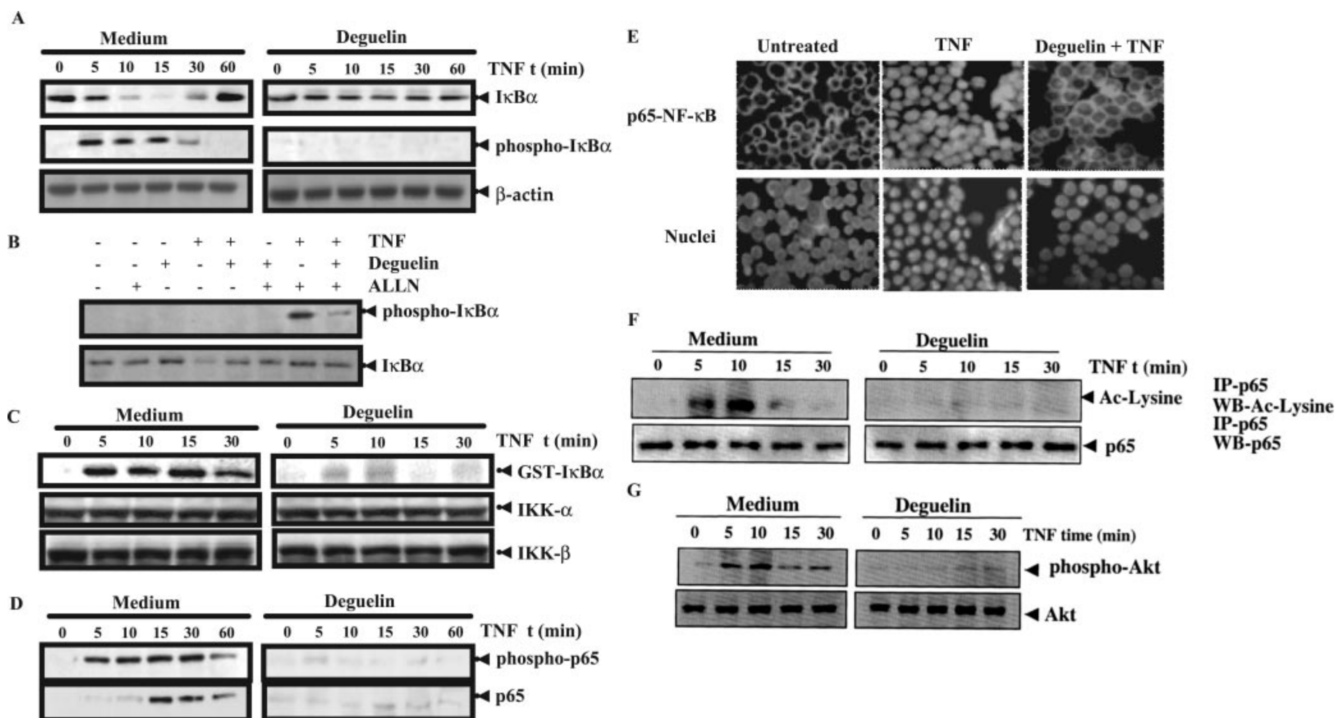

FIGURE 3. Effect of deguelin on IκBα phosphorylation and degradation induced by TNF. *A*, Effect of deguelin on the TNF-induced phosphorylation and degradation of IκBα. KBM-5 cells (2×10^6 cells/well) were treated with 10 μM deguelin for 12 h and then exposed to 0.1 nM TNF for the indicated times. Cytoplasmic extracts were then prepared, fractionated with SDS-PAGE, and electrotransferred to a nitrocellulose membrane, after which a Western blot analysis was performed with anti-IκBα and anti-phospho-specific IκBα. *B*, Deguelin suppresses TNF-induced phosphorylation of IκBα in the presence of a proteasome inhibitor. KBM-5 cells (2×10^6 cells/well) were preincubated with 10 μM deguelin and then treated with 0.1 nM TNF for 15 min after addition of ALLN. Cytoplasmic extracts were prepared, fractionated on 10% SDS-PAGE, and electrotransferred to a nitrocellulose membrane. The Western blot analysis was performed with anti-phospho-specific-IκBα and anti-IκBα Abs. *C*, Effect of deguelin on the activation of IKK by TNF. KBM-5 cells (2×10^6 cells/well) were treated with 10 μM deguelin for 12 h, 50 μg/ml *N*-acetyl-Leu-Leu-norleucinal for an additional 30 min, and then exposed to 1 nM TNF for the indicated times. Whole-cell extracts were immunoprecipitated with anti-IKK Ab and analyzed by an immunocomplex kinase assay. Whole-cell extracts were also fractionated on SDS-PAGE gel and examined by Western blot analysis using anti-IKK-α and anti-IKK-β Abs. *D*, Effect of deguelin on the TNF-induced phosphorylation and translocation of p65. KBM-5 cells (2×10^6 cells/well) were preincubated with 10 μM deguelin for 12 h and then treated with 0.1 nM TNF for the indicated times. Nuclear extracts were then prepared, fractionated on 10% SDS-PAGE, and electrotransferred to nitrocellulose membrane. The Western blot analysis was performed with phospho-specific p65 and p65 Abs. *E*, Immunocytochemical analysis of p65 localization after treatment with TNF. KBM-5 cells (2×10^6 cells/well) were treated with 10 μM deguelin for 12 h and then exposed to 1 nM TNF for 20 min and subjected to immunocytochemical analysis. *F*, Effect of deguelin on TNF-induced acetylation of p65. KBM-5 cells (2×10^6 cells/well) were treated with 10 μM deguelin for 12 h and then exposed to 1 nM TNF for the indicated times. Whole-cell extracts were prepared, immunoprecipitated (IP) with anti-p65 Ab, and subjected to Western blot analysis using an anti-acetyl-lysine (Ac-Lysine) Ab. The same blots were reprobed with anti-p65 Ab. *G*, Effect of deguelin on TNF-induced Akt activation. Cells were incubated with 10 μM deguelin for 12 h and then treated with 1 nM TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blot analysis using anti-phospho-specific Akt. The same membrane was reblotted with an anti-Akt Ab. The results shown are representative of three independent experiments.

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Deguelin inhibits TNF-induced phosphorylation and nuclear translocation of p65

TNF has been shown to induce the phosphorylation of p65, leading to its nuclear translocation, which is required for its transcriptional activity (40). Our results show that TNF induced p65 phosphorylation in a time-dependent manner, that p65 was phosphorylated as early as 5 min after TNF stimulation, and that this phosphorylation increased for as long as 30 min after TNF stimulation (Fig. 3*D*, *left panel*). In cells treated with deguelin, however, TNF failed to induce p65 phosphorylation (Fig. 3*D*, *right panel*).

We also determined the effect of deguelin on the TNF-induced nuclear translocation of p65. Whereas TNF induced the nuclear translocation of p65 in a time-dependent manner, deguelin suppressed it almost completely (Fig. 3*D*, *lower panel*). Similarly, immunocytochemical analysis showed that TNF induced nuclear translocation of p65 and that deguelin abrogated this translocation (Fig. 3*E*).

Because the phosphorylation of p65 has been shown to regulate the acetylation of p65 (41), we examined whether deguelin also affects the acetylation of p65. To determine the effect of deguelin on p65 acetylation induced by TNF, we treated cells with deguelin and then with TNF. Whole-cell extracts were immunoprecipitated with an anti-p65 Ab, and a Western blot analysis was performed with an anti-acetyl-lysine Ab. As with nuclear translocation, TNF induced acetylation of p65 in a time-dependent manner, and deguelin suppressed it (Fig. 3*F*).

Deguelin inhibits TNF-induced Akt activation

It has been shown that activation of Akt is needed for IKK activation (17) and for phosphorylation of p65 (42). Studies have also shown that deguelin is a powerful inhibitor of the Akt activation pathway (11). Therefore, deguelin may inhibit TNF-induced NF-κB activation through inhibition of Akt activation. To investigate the effect of deguelin on the activation of Akt by TNF, we pretreated cells with deguelin, followed by TNF, and then prepared whole-cell extracts. Western blot analysis was performed using anti-phospho-specific-Akt and Akt Ab. Results again showed that TNF induced Akt activation in a time-dependent manner, and deguelin suppressed it (Fig. 3*G*).

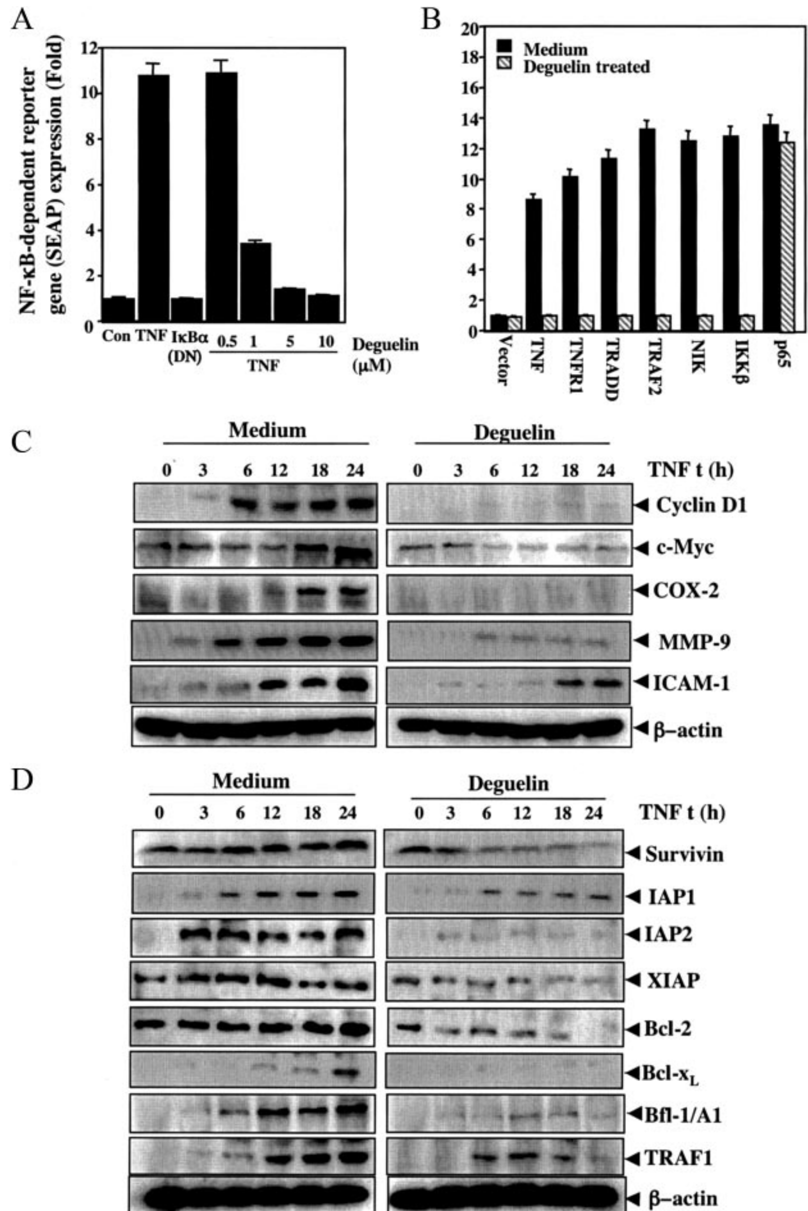

FIGURE 4. Deguelin inhibits the TNF-induced expression of the NF-κB-dependent secretory alkaline phosphatase (SEAP) reporter gene expression. *A*, Deguelin inhibits TNF-induced NF-κB-dependent *SEAP* expression. A293 cells were transiently transfected with a NF-κB-containing plasmid linked to the *SEAP* gene, IκBα dominant negative (DN), and then treated with the indicated concentrations of deguelin. After 24 h in culture with 1 nM TNF, cell supernatants were collected and assayed for *SEAP* activity, as described in *Materials and Methods*. Results are expressed as fold activity over the activity of the vector control. *B*, Deguelin inhibits NF-κB-dependent reporter gene expression induced by TNFR, TRADD, TRAF, NIK, and IKKβ. A293 cells were transiently transfected with the indicated plasmids along with a NF-κB-containing plasmid linked to the *SEAP* gene, and then left either untreated or treated with 10 μM deguelin for 24 h. Cell supernatants were assayed for SEAP activity, as described in *Materials and Methods*. Results are expressed as fold activity over the activity of the vector control. Bars, SD. Deguelin inhibits TNF-induced NF-κB-regulated gene products. *C*, Deguelin inhibits cyclin D1, c-Myc, COX-2, MMP-9, and ICAM-1 expression induced by TNF. KBM-5 cells (2×10^6 cells/well) were left untreated or were incubated with 10 μM deguelin for 12 h and then treated with 1 nM TNF for the different times indicated. Whole-cell extracts were prepared, and a whole-cell lysate (50 μg) was analyzed by Western blotting using Abs against cyclin D1, c-Myc, COX-2, MMP-9, and ICAM-1. β-actin was used as the loading control. The results shown are representative of three independent experiments with similar results. *D*, Deguelin inhibits the expression of antiapoptotic gene products cIAP1, cIAP2, XIAP, Bcl-2, Bcl-x_L, Bfl-1/A1, and TRAF1. KBM-5 cells (2×10^6 cells/well) were left untreated or were incubated with 10 μM deguelin for 12 h and then treated with 1 nM TNF for the different times indicated. Whole-cell extracts were prepared, and a whole-cell lysate (50 μg) was analyzed by Western blotting using Abs against cIAP1, cIAP2, XIAP, Bcl-2, Bcl-x_L, Bfl-1/A1, and TRAF1, as indicated. β-actin was used as the loading control. The results shown are representative of three independent experiments with similar results.

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Deguelin represses TNF-induced NF-κB-dependent reporter gene expression

We showed by EMSA that deguelin suppressed the NF-κB activation induced by various agents. Because DNA binding does not always correlate with NF-κB-dependent gene transcription (43), we also examined the effect of deguelin on TNF-induced NF-κB reporter activity. Deguelin-pretreated or untreated cells were transiently transfected with the NF-κB-regulated SEAP reporter construct and then stimulated with TNF. TNF induced the expression of SEAP, and most of the TNF-induced SEAP activity was abolished by dominant-negative IκBα, indicating its specificity (Fig. 4*A*). The treatment of cells with deguelin inhibited this TNF-induced gene expression in a dose-dependent manner. These results demonstrate that deguelin inhibited the NF-κB-dependent reporter gene expression induced by TNF.

Deguelin inhibits NF-κB activation induced by TNFR1, TRADD, TRAF2, NIK, and IKKβ

TNF-induced NF-κB activation is mediated through sequential interaction of the TNFR with TRADD, TRAF2, NIK, and IKK, resulting in phosphorylation of IκBα (44, 45). To delineate the site of action of deguelin in the TNF signaling pathway leading to NF-κB activation, we transiently transfected cells with the NF-κB-regulated SEAP reporter construct, along with TNFR1-, TRADD-, TRAF2-, NIK-, IKK-β-, or p65-expressing plasmids, and then monitored for NF-κB-dependent SEAP expression in untreated and deguelin-treated cells. As shown in Fig. 4*B*, we found that deguelin suppressed the NF-κB activation induced by TNFR1, TRADD, TRAF2, NIK, and IKK-β, but not that induced by p65. These results suggested that deguelin acts at a step upstream of p65.

Deguelin suppresses TNF-induced NF-κB-regulated gene products

TNF has been shown to induce the expression of several proliferative and metastatic gene products, including cyclin D1, c-Myc, COX-2, MMP-9, and ICAM-1, which are known to have an NF-κB binding site in their promoters (18, 19, 46–48). To examine whether deguelin suppressed TNF-induced expression of NF-κB-dependent proteins, we pretreated cells with deguelin before TNF treatment and then prepared and analyzed whole-cell extracts with a Western blot analysis by using specific Abs. Once again, we found that TNF induced expression of all these proteins and that deguelin was able to suppress it (Fig. 4*C*). Thus, our findings confirm and provide further evidence that deguelin plays a pivotal role in blocking the expression of TNF-induced NF-κB-regulated gene expression.

Deguelin represses TNF-induced NF-κB-dependent antiapoptotic gene products

Numerous studies have shown that NF-κB regulates the expression of several antiapoptotic gene products, including survivin, XIAP, IAP1/2, Bcl-2, Bcl-x_L, Bfl-1/A1, and TRAF-1 (49–54). Whether deguelin modulated the TNF-induced expression of NF-κB-dependent antiapoptotic gene products was examined. Our results showed that deguelin blocked the TNF-induced expression of all these antiapoptotic proteins implicated in facilitating tumor cell survival in a time-dependent manner (Fig. 4*D*).

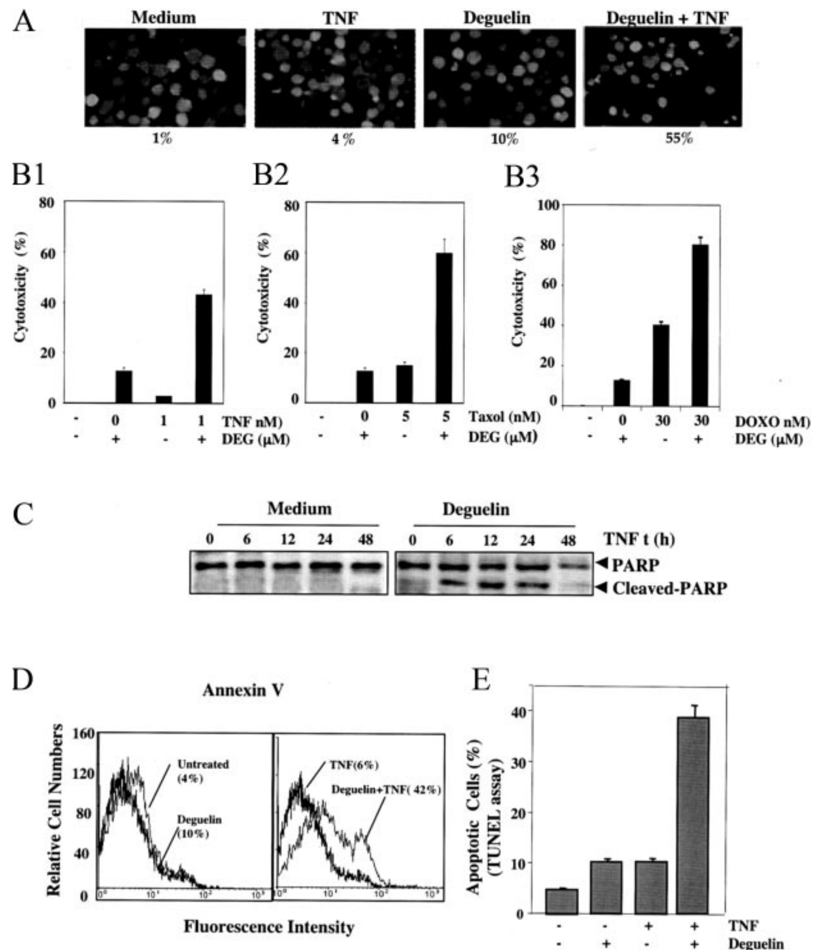

FIGURE 5. Deguelin enhances TNF-induced cytotoxicity. *A*, KBM-5 cells (1 × 10^4 cells/well) were treated with 5 μM deguelin for 24 h, exposed to 1 nM TNF for 16 h, stained with live and dead assay reagent for 30 min, and analyzed under a fluorescence microscope. *B*, Deguelin enhances chemotherapeutic agent-induced cytotoxicity. KBM-5 cells (1 × 10^4 cells/well) were seeded in a 96-well plate and treated with 5 μM deguelin for 12 h and then for an additional 24 h with 1 nM TNF, 5 nM paclitaxel (Taxol), and 30 μM doxorubicin. Cell viability was analyzed by the MTT method. *C*, KBM-5 cells (1 × 10^4 cells/well) were treated with 5 μM deguelin for 12 h and then exposed to 1 nM TNF for the indicated times. Whole-cell extracts were prepared (50 μg), subjected to Western blot analysis, and blotted with anti-PARP Ab. *D* and *E*, KBM-5 cells (1 × 10^6 cells/well) were treated with 5 μM deguelin for 24 h and then with 1 nM TNF for 16 h. Cells were incubated with FITC-conjugated annexin V Ab (*D*), or stained with TUNEL assay reagent and then run through a flow cytometer (*E*), as described in *Materials and Methods*.

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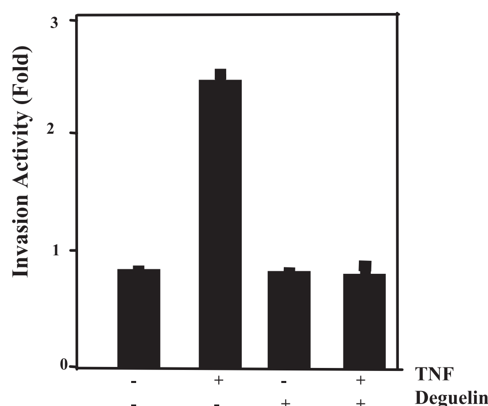

FIGURE 6. Deguelin suppresses TNF-induced invasion activity. H1299 cells (2.5×10^4 cells) were seeded to the top chamber of a Matrigel invasion chamber overnight in the absence of serum, preincubated with deguelin for 12 h, treated with 1 nM TNF for 24 h in the presence of 1% serum, and then subjected to invasion assay, as described in *Materials and Methods*.

Deguelin potentiates apoptosis induced by TNF and chemotherapeutic agents

Because studies have shown that TNF-induced apoptosis can be inhibited by the activation of NF-κB (20, 55–58), we examined the ability of deguelin to enhance the apoptosis induced by TNF and chemotherapeutic agents by using the live and dead assay, the MTT assay, a Western blot analysis for PARP and cleaved PARP, and a flow cytometry analysis of annexin V and TUNEL staining methods. Results from the live and dead assay showed that deguelin up-regulated TNF-induced cytotoxicity from 4 to 55% (Fig. 5*A*). Deguelin also enhanced TNF-, paclitaxel-, and doxorubicin-induced cytotoxicity, as shown by the MTT method (Fig. 5*B*). Because deguelin by itself had little cytotoxic effect at the concentration used (5 μM), we also examined whether the enhanced cytotoxicity was due to increased apoptosis by using an activation of caspase assay. TNF activated caspases, as indicated by PARP cleavage, and deguelin potentiated this TNF-induced activity (Fig. 5*C*). The results of annexin V staining similarly showed that TNF-induced apoptosis was up-regulated by incubation with deguelin (Fig. 5*D*), and the results from TUNEL staining also indicated that deguelin up-regulated TNF-induced apoptosis (Fig. 5*E*). All assays together suggested that deguelin enhances the apoptotic effects of TNF and chemotherapeutic agents.

Deguelin blocks TNF-induced invasion activity

MMPs, COXs, and two adhesion molecules, all regulated by NF-κB, are major players in tumor metastasis and have been shown to mediate tumor invasion (59). It has also been reported that TNF can induce the expression of genes involved in tumor metastasis (18, 46, 48). To examine whether deguelin modulates TNF-induced invasion activity in vitro, we used H1299 cells seeded in the upper chamber of a Matrigel invasion chamber in the absence of serum. Cells were coincubated with TNF in the presence or absence of deguelin for 24 h. As shown in Fig. 6, TNF induced cell invasion activity, and deguelin suppressed it.

Discussion

In light of the chemopreventive and chemosensitivity effects assigned to deguelin, we hypothesized that this rotenoid must mediate its effects through the suppression of the NF-κB activation pathway. We showed that deguelin can indeed suppress NF-κB activated by growth factors, carcinogens, tumor promoters, and inflammatory stimuli (e.g., LPS, TNF, and IL-β). Both inducible and constitutive NF-κB activation were blocked by deguelin. Suppression of NF-κB activation by deguelin occurred through the inhibition of IKK activation, IκBα phosphorylation, and IκBα degradation, which led to the abrogation of p65 phosphorylation, nuclear translocation, and acetylation. The expression of NF-κB-regulated gene products involved in cell survival, proliferation, and invasion was also down-regulated by deguelin. Moreover, this rotenoid potentiated the apoptosis induced by cytokines and chemotherapeutic agents, and suppressed TNF-induced cellular invasion.

This is the first study to investigate the effect of deguelin on NF-κB activation by various stimuli. Our results show that deguelin suppresses the NF-κB activated by a variety of stimuli, suggesting that it must act at a step common to all these activators. NF-κB activation in response to different stimuli requires IKK activation, which phosphorylates IκBα at serines 32 and 36, leading to the degradation of IκBα (34). We found that deguelin blocked the activation of NF-κB without directly interfering with the DNA binding of NF-κB. We report that this inhibition was mediated through the inhibition of IKK by deguelin, which led to the suppression of phosphorylation and the degradation of IκBα. Deguelin did not suppress other transcription factors such as AP-1 and STAT3 under the conditions; it suppresses NF-κB, suggesting specificity toward NF-κB.

Results of our in vitro kinase assay show that deguelin is not a direct inhibitor of IKK (data not shown). Thus, this agent seems to block the activation of IKK by interfering with some upstream regulatory kinases. Akt, NIK, MAPK1, and atypical protein kinase C are all candidates because they are upstream kinases that regulate IKK (14). We found that deguelin inhibits TNF-induced Akt activation. These results agree with those of previous studies that suggest that deguelin suppresses the Akt activation induced by cigarette smoke carcinogens (8, 11). Thus, it is possible that deguelin suppressed TNF-induced IKK activation occurs through inhibition of Akt.

We found that deguelin also blocked TNF-induced phosphorylation of p65. Given that both Akt and IKK have been linked with phosphorylation of p65 (42, 60), the inhibition of TNF-induced Akt and IKK activation by deguelin may explain this inhibition of p65 phosphorylation. We further showed that deguelin inhibited TNF-induced acetylation of p65. A recent study showing that phosphorylation is a critical step in the acetylation of p65 (60) may explain how inhibition of phosphorylation results in inhibition of acetylation.

We also demonstrated that deguelin blocked TNF-induced NF-κB-regulated gene transcription. The inhibition of phosphorylation and the acetylation of p65 by deguelin can explain the suppression of reporter gene transcription. In this regard, we found that the expression of NF-κB-regulated gene products involved in proliferation (e.g., cyclin D1 and c-Myc) and invasion (e.g., COX-2, MMP-9, and ICAM-1) was abolished by deguelin. Furthermore, our results that deguelin down-regulates cyclin D1 expression are in agreement with previous reports (13) that showed that down-regulation of cyclin D1 is responsible for cell cycle arrest at the G_0/G_1 phase. Similarly, our findings that deguelin suppresses NF-κB-regulated COX-2 expression are in agreement with a previous report involving human bronchial epithelial cells (12).

MMP-9 plays a crucial role in tumor invasion and angiogenesis by mediating the degradation of the extracellular matrix, and inhibition of MMP-9 activity has been shown to suppress lung metastasis (61). In agreement with these observations, we found that TNF-induced invasion is inhibited by deguelin.

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NF-κB is known to regulate the expression of survivin, IAP1, Bfl-1/A1, and TRAF1, and their overexpression in numerous tumors has been linked to tumor survival, chemoresistance, and radioresistance. We showed that deguelin down-regulates most of these gene products and that the cytotoxic effects of TNF, paclitaxel, and doxorubicin are enhanced by deguelin. These results are in agreement with recent findings that deguelin can potentiate the effect of etoposide in leukemia cells (9).

We also showed that deguelin down-regulates cigarette smoke-induced and phorbol ester-induced NF-κB activation. Because NF-κB activation has been closely linked with carcinogenesis (62), it is possible that the previously described chemopreventive effects of deguelin against tobacco carcinogen-induced lung carcinogenesis (7, 8) and UV light-induced skin carcinogeneis (5) are mediated by suppression of NF-κB activation. In addition, cancer chemoprevention generally involves the use of dietary or chemical agents to impair or retard the formation of neoplastic cells (63). The preventive effect is achieved by blocking neoplastic transformation and/or by inhibiting the progression of transformed cells.

In summary, cancer is a hyperproliferative disorder characterized by the up-regulation of genes responsible for the transformation, proliferation, invasion, angiogenesis, and metastasis of cells. These processes are greatly influenced by the aberrant activity of NF-κB; hence, the primary goal of this study was to evaluate the potential of deguelin as an inhibitor of NF-κB. This study showed for the first time that the antiproliferative, proapoptotic, and anti-invasive effects of deguelin may be mediated through the suppression of NF-κB and NF-κB-regulated gene products. Considering the pharmacologic safety of deguelin (64), this agent should be further explored as a potential chemopreventive agent.

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Disclosures

The authors have no financial conflict of interest.

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