

# Identification of a novel blocker of I $\kappa$ B $\alpha$ kinase activation that enhances apoptosis and inhibits proliferation and invasion by suppressing nuclear factor- $\kappa$ B

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## Abstract

3,4-Dihydroxybenzalacetone (DBL) is a polyphenol derived from the medicinal plant Chaga [*Inonotus obliquus* (persoon) Pilat]. Although Chaga is used in Russia folk medicine to treat tumors, very little is known about its mechanism of action. Because most genes involved in inflammation, antiapoptosis, and cell proliferation are regulated by the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B), we postulated that DBL activity is mediated via modulation of the NF- $\kappa$ B activation pathway. We investigated the effects of DBL on NF- $\kappa$ B activation by electrophoretic mobility shift assay and on NF- $\kappa$ B-regulated gene expression by Western blot analysis. We found that DBL suppressed NF- $\kappa$ B activation by a wide variety of inflammatory agents, including tumor necrosis factor (TNF), interleukin-1 $\beta$ , epidermal growth factor, okadaic acid, phorbol 12-myristate 13-acetate, and lipopolysaccharide. The suppression was not cell type specific and inhibited both inducible and constitutive NF- $\kappa$ B activation. DBL did not interfere with the binding of NF- $\kappa$ B to DNA but rather inhibited I $\kappa$ B $\alpha$  kinase activity, I $\kappa$ B $\alpha$  phosphorylation and degradation, p65 phosphorylation, and translocation. DBL also suppressed the expression of TNF-induced and NF- $\kappa$ B-regulated proliferative, antiapoptotic, and metastatic gene products. These effects correlated with enhancement of TNF-induced apoptosis and suppression

of TNF-induced invasion. Together, our results indicate that DBL inhibits NF- $\kappa$ B activation and NF- $\kappa$ B-regulated gene expression, which may explain the ability of DBL to enhance apoptosis and inhibit invasion. [Mol Cancer Ther 2008;7(1):191–201]

## Introduction

Several compounds, including many derived from plants, have been evaluated for their potential chemopreventive activity. Recently, considerable attention has been focused on identifying phytochemicals, particularly those in the human diet that can interfere with carcinogenic or mutagenic processes. A wide variety of dietary ingredients have been shown to exert substantial chemopreventive effects against experimental carcinogenesis and mutagenesis (1). Unfortunately, the active components and mechanisms of action of these dietary compounds are poorly understood and warrant investigation so that they might be applied to safer, more efficacious treatments of various human ailments. For example, the extract of the mushroom *Inonotus obliquus* (persoon) Pilat (Hymenochaetaceae of basidiomycetes; called “Chaga” in Russia and “kabanoanake” in Japan) is used as a folk medicine in Russia, Japan, and Korea. In Russia and western Siberia, it has been used as a folk remedy since the 16th century. A nontoxic decoction of fungal sclerotia from Chaga has been used to treat cancers and digestive system diseases (2). More recently, polyphenols, triterpenes, steroids, and peptides from Chaga have been shown to have various biological properties, including those described as antioxidative (3), hypoglycemic (4), antiviral (5), antifungal (6), hepatoprotective (6), antiaggregative (7), anti-inflammatory (8), and antitumor (9–11). One of the active components of Chaga is 3,4-dihydroxybenzalacetone (DBL; ref. 3). The mechanism of action of DBL is poorly understood. Because various genes regulated by the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) are known to regulate inflammation and tumorigenesis, we postulated that the antitumor component of DBL must exert its effects by modulation of NF- $\kappa$ B.

Under normal conditions, NF- $\kappa$ B is present in the cytoplasm as an inactive heterotrimer consisting of three subunits: p50, p65, and I $\kappa$ B $\alpha$ . On activation, I $\kappa$ B $\alpha$  undergoes phosphorylation and ubiquitination-dependent degradation, thus exposing nuclear localization signals on the p50-p65 heterodimer, leading to nuclear translocation and binding to a specific consensus sequence in the DNA (5'-GGGACTTTC-3'; ref. 12). The binding activates NF- $\kappa$ B gene expression, which in turn results in transcription of the NF- $\kappa$ B-regulated genes (13). The phosphorylation of I $\kappa$ B $\alpha$  is mediated through the activation of the I $\kappa$ B $\alpha$  kinase (IKK) complex (14).

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Because Chaga and DBL derived from it reportedly exhibit growth-inhibitory effects (11, 15), we postulated that these effects may be mediated through modulation of the NF- $\kappa$ B activation pathway. To test this hypothesis, we investigated the effect of the DBL on NF- $\kappa$ B activation induced by several inflammatory agents in various cell types, including those in which NF- $\kappa$ B is constitutively active. We found that DBL inhibited NF- $\kappa$ B activation and NF- $\kappa$ B-regulated gene products, leading to an increase in apoptosis and suppression of cellular invasion.

## Materials and Methods

### Reagents

A 50 mmol/L solution of DBL was prepared with DMSO, stored as small aliquots at -20°C, and then thawed and diluted as needed in cell culture medium. Bacteria-derived human recombinant tumor necrosis factor (TNF), purified to homogeneity with a specific activity of  $5 \times 10^7$  units/mg, was kindly provided by Genentech. Penicillin, streptomycin, RPMI 1640, Iscove's modified Dulbecco's medium, DMEM, and fetal bovine serum were obtained from Invitrogen. Antibodies against p65, p50, I $\kappa$ B $\alpha$ , cyclin D1, cyclooxygenase-2 (COX-2), matrix metalloproteinase-9 (MMP-9), poly(ADP-ribose) polymerase, inhibitor of apoptosis protein (IAP) 1, IAP2, Bcl-2, Bcl-x<sub>L</sub>, and intracellular adhesion molecule-1 and the Annexin V staining kit were obtained from Santa Cruz Biotechnology. Vascular endothelial cell growth factor antibody was purchased from Neomarkers. Phosphospecific anti-I $\kappa$ B $\alpha$  (Ser<sup>32</sup>/Ser<sup>36</sup>) and phosphospecific anti-p65 (Ser<sup>536</sup>) were purchased from Cell Signaling Technology. Anti-IKK $\alpha$ , anti-cFLIP, and anti-IKK $\beta$  antibodies were provided by Imgenex.

### Cell Lines

Human cell lines KBM-5 (chronic myeloid leukemia), A293 (embryonic kidney carcinoma), H1299 (human lung adenocarcinoma), U266 (human multiple myeloma), and Jurkat (human T lymphocyte) were obtained from the American Type Culture Collection. Cell line SCC4 (human squamous cell carcinoma cells derived from tongue) was provided by Dr. M.J. O'Hare (Haddow Laboratories, Institute of Cancer Research, Sutton, United Kingdom). KBM-5 cells were cultured in Iscove's modified Dulbecco's medium with 15% fetal bovine serum. H1299, Jurkat, and U266 cells were cultured in RPMI 1640, and A293 cells were cultured in DMEM supplemented with 10% fetal bovine serum. SCC4 cells were cultured in DMEM containing 10% fetal bovine serum, 100  $\mu$ mol/L nonessential amino acids, 1 mmol/L pyruvate, 6 mmol/L L-glutamine, and 1 $\times$  vitamins. The mouse embryonic fibroblast derived from p65<sup>-/-</sup> C57Bl/6J mice and its wild type were kindly provided by Dr. David Baltimore (California Institute of Technology, Pasadena, CA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum. Culture media were also supplemented with 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin.

### Isolation of Polyphenol from Chaga

The polyphenols from brown powder of Chaga was done as described previously (3).

### Electrophoretic Mobility Shift Assays

To assess NF- $\kappa$ B activation, nuclear extracts were prepared, and electrophoretic mobility shift assay (EMSA) was done as described previously (16).

### Western Blot Analysis

Western blot analysis was performed as described previously (16).

### IKK Assay

To determine the effect of DBL on TNF-induced IKK activation, we analyzed IKK activation essentially as described previously (17).

### Immunocytochemistry for NF- $\kappa$ B p65 Localization

The effect of DBL on the TNF-induced nuclear translocation of p65 was examined by an immunocytochemical method using an epifluorescence microscope (Labophot-2) as described previously (16).

### Live/Dead Assay

To measure apoptosis, we also used the Live/Dead assay (Molecular Probes), which assesses intracellular esterase activity and plasma membrane integrity. This assay was done as described previously (17).

### Invasion Assay

Invasion through the extracellular matrix is a crucial step in tumor metastasis. To determine the effect of DBL on TNF-induced invasion, we used BD BioCoat tumor invasion system (BD Biosciences) as described previously (17).

### Annexin V Assay

To identify apoptosis, cells were stained with an Annexin V antibody conjugated with the fluorescent dye FITC. In brief,  $5 \times 10^5$  cells were coincubated with 50  $\mu$ mol/L DBL and 1 nmol/L TNF for 16 h then stained with FITC and analyzed using a flow cytometer (FACSCalibur; BD Biosciences).

### Terminal Deoxynucleotidyl Transferase – Mediated dUTP Nick End Labeling

The cytotoxicity was assayed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay, which examines DNA strand breaks during apoptosis, with an *In situ* Cell Death Detection reagent (Roche Molecular Biochemicals). In brief,  $5 \times 10^5$  cells were coincubated with 50  $\mu$ mol/L DBL and 1 nmol/L TNF for 16 h and then incubated with a reaction mixture. Stained cells were analyzed using a flow cytometer (FACSCalibur).

## Results

The aim of the present study was to investigate the effect of DBL on the NF- $\kappa$ B activation pathway induced by various carcinogens and inflammatory stimuli and on NF- $\kappa$ B-regulated gene expression. Because the TNF-induced NF- $\kappa$ B activation pathway has been well characterized, we investigated the effect of DBL on TNF-induced NF- $\kappa$ B activation in detail. The structure of DBL is shown in Fig. 1.

### DBL Is the Most Potent NF- $\kappa$ B Inhibitor among Chaga Polyphenols

Because of their structural similarities, the polyphenols isolated from Chaga were tested for their ability to inhibit NF- $\kappa$ B activation. Among the seven different polyphenols

examined, only DBL significantly inhibited NF- $\kappa$ B activation (Fig. 1). Comparison of the structures suggested the requirement of catechol hydroxyl groups on the phenyl ring and a ketone group for effective inhibition of NF- $\kappa$ B activation. DBL has recently been shown to bind to nuclear type II estradiol binding sites and to inhibit c-myc and cyclin D1 gene expression (15). For all following studies, only DBL was investigated.

#### DBL Inhibits NF- $\kappa$ B Activation Induced by Various Agents

Because TNF, interleukin-1 $\beta$ , lipopolysaccharide, okadaic acid, epidermal growth factor, and phorbol 12-myristate 13-acetate are potent activators of NF- $\kappa$ B (18), we examined the effect of DBL on the activation of NF- $\kappa$ B by these agents. Incubation of cells with 50  $\mu$ mol/L DBL suppressed the activation of NF- $\kappa$ B induced by all six of these agents (Fig. 2A). Changes in the concentration of DBL and the time of exposure had minimal effect on cell viability. These results suggest that DBL acts at a step in the NF- $\kappa$ B activation pathway that is common to all six agents.

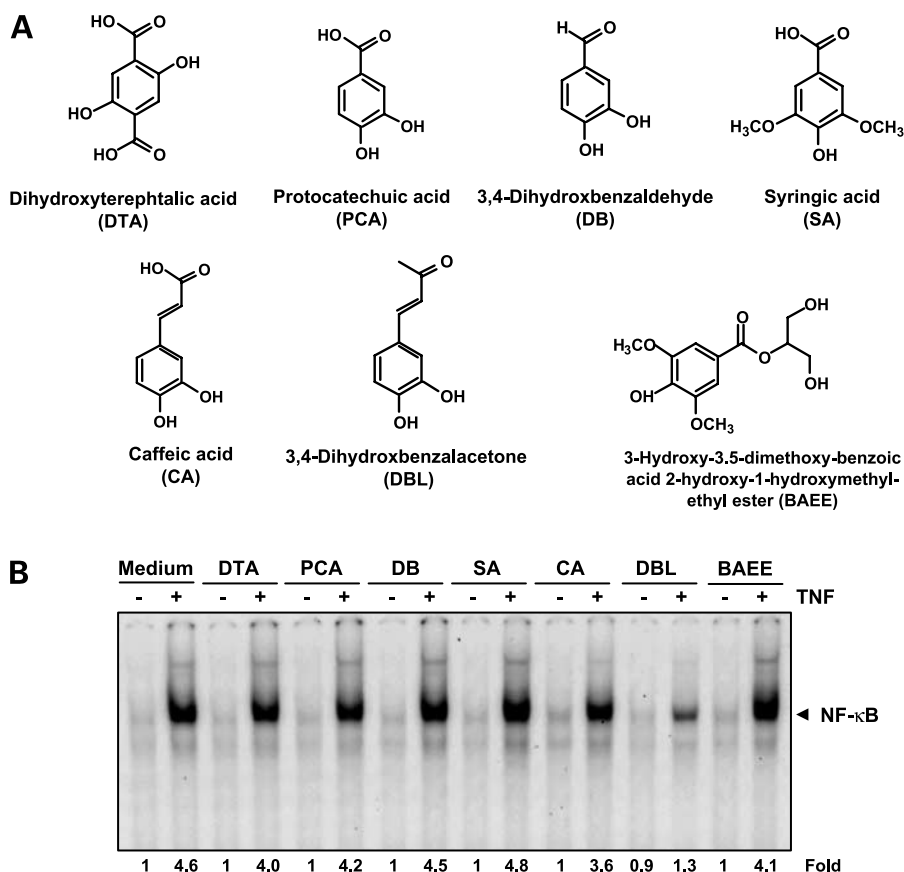
#### DBL Inhibits NF- $\kappa$ B Activation in a Dose-Dependent Manner

Because TNF is one of the most potent activators of NF- $\kappa$ B and the mechanism of activation is relatively well established (19), we examined the effect of DBL on TNF-induced NF- $\kappa$ B activation in KBM-5 cells. KBM-5 cells were

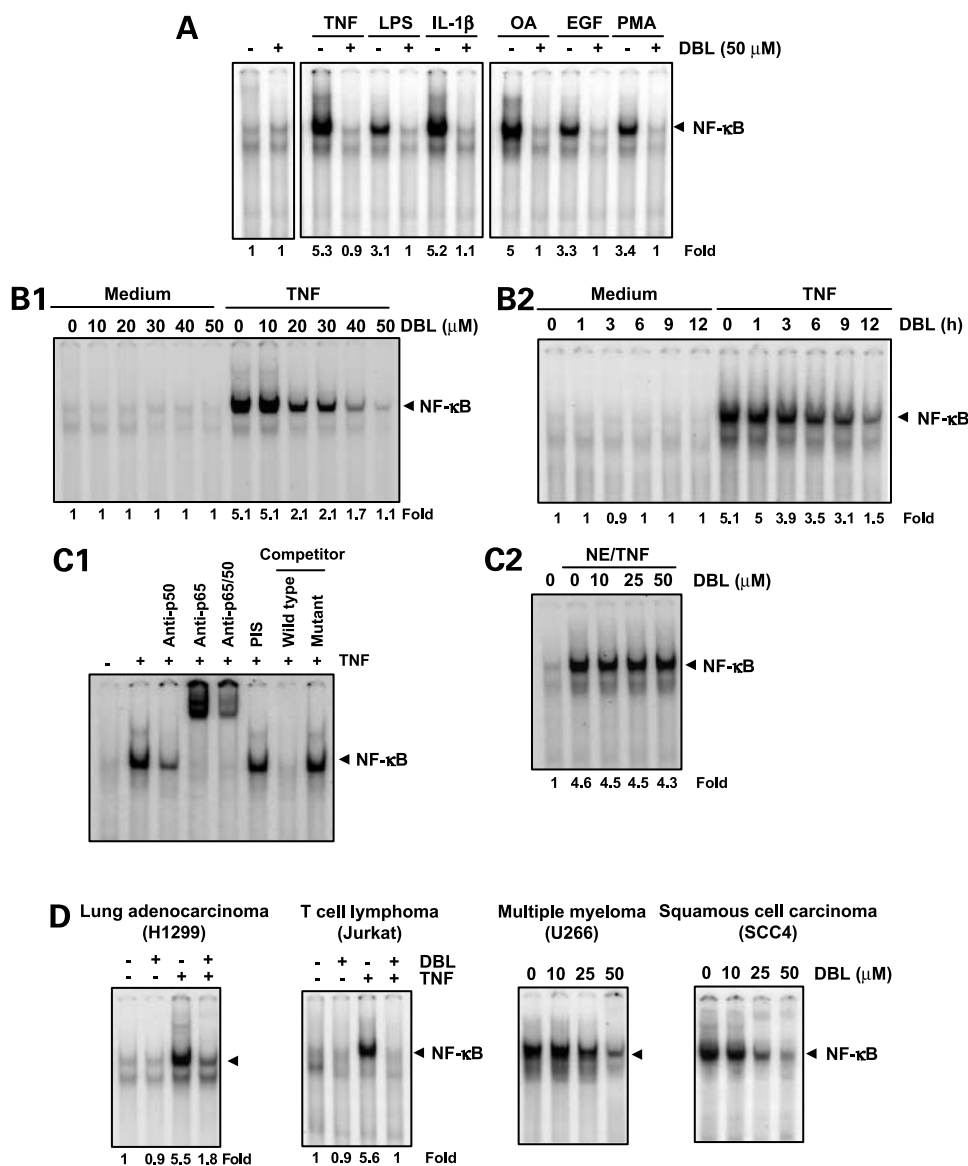
pretreated with different doses of DBL for 12 h and then stimulated with TNF. As indicated by EMSA, DBL suppressed TNF-induced NF- $\kappa$ B activation in a dose-dependent manner (Fig. 2B1). Under these conditions, cells were fully viable as determined by the trypan blue dye exclusion test (data not shown).

#### DBL Suppresses NF- $\kappa$ B Activation in a Time-Dependent Manner

We also investigated the length of incubation required for DBL to suppress TNF-induced NF- $\kappa$ B activation. KBM-5 cells were incubated with 50  $\mu$ mol/L DBL for up to 12 h and then exposed to TNF. As shown by EMSA, DBL by itself did not activate NF- $\kappa$ B, and TNF-induced NF- $\kappa$ B activation was inhibited by DBL within 12 h (Fig. 2). Because NF- $\kappa$ B is a complex of proteins, various combinations of Rel/NF- $\kappa$ B proteins constitute active NF- $\kappa$ B heterodimers that bind to a specific in DNA sequences (20). To show that the band visualized by EMSA in TNF-treated cells was indeed NF- $\kappa$ B, we incubated nuclear extracts from TNF-stimulated cells with antibodies to either the p50 (NF- $\kappa$ B1) or the p65 (RelA) subunit of NF- $\kappa$ B. Both shifted the major band to a higher molecular mass (Fig. 2C1), suggesting that the TNF-activated complex consisted of p50 and p65 subunits. PIS had no effect, and excess unlabeled NF- $\kappa$ B (100-fold) caused complete disappearance of the band. Moreover, a mutant oligonucleotide of NF- $\kappa$ B did not affect NF- $\kappa$ B-binding activity.



**Figure 1.** **A**, structures of polyphenols derived from *I. obliquus* (Chaga). **B**, effect of Chaga polyphenols on TNF-induced NF- $\kappa$ B activation. KBM-5 cells were incubated with 50  $\mu$ mol/L polyphenols for 12 h, treated with 0.1 nmol/L TNF for 30 min, and then assayed for NF- $\kappa$ B activation by EMSA.



**Figure 2.** Inhibition of NF- $\kappa$ B activation by DBL. **A**, DBL blocks NF- $\kappa$ B activation induced by TNF, lipopolysaccharide, interleukin-1 $\beta$ , okadaic acid, epidermal growth factor (EGF), and phorbol 12-myristate 13-acetate (PMA). KBM-5 cells were preincubated with 50  $\mu$ mol/L DBL for 12 h and then treated with 0.1 nmol/L TNF for 30 min, 100 ng/mL epidermal growth factor for 2 h, 500 nmol/L okadaic acid for 4 h, 25 ng/mL phorbol 12-myristate 13-acetate for 2 h, and 10  $\mu$ g/mL lipopolysaccharide for 2 h. Nuclear extracts were analyzed for NF- $\kappa$ B activation as described in Material and Methods. **B1**, DBL inhibits TNF-induced NF- $\kappa$ B activation in a dose-dependent manner. KBM-5 cells ( $2 \times 10^6$ /mL) were preincubated with the indicated concentrations of DBL for 12 h and then treated with 0.1 nmol/L TNF for 30 min. **B2**, DBL inhibits TNF-dependent NF- $\kappa$ B activation. KBM-5 cells were preincubated with 50  $\mu$ mol/L DBL for the indicated times and then treated with 0.1 nmol/L TNF for 30 min. Nuclear extracts were prepared and then tested for NF- $\kappa$ B activation. **C1**, NF- $\kappa$ B induced by TNF is composed of p65 and p50 subunits. Nuclear extracts from untreated or TNF-treated cells were incubated with the indicated antibodies, PIS, unlabeled NF- $\kappa$ B oligoprobe, or mutant oligoprobe and then assayed for NF- $\kappa$ B activation by EMSA. **C2**, direct effect of DBL on NF- $\kappa$ B complex. Nuclear extracts were prepared from untreated or TNF-treated cells and incubated for 30 min with the indicated concentrations of DBL and then assayed for NF- $\kappa$ B activation by EMSA. **D**, DBL suppresses TNF-induced NF- $\kappa$ B activation in different cell types. Human lung carcinoma (H1299), T-cell lymphoma (Jurkat), multiple myeloma (U266), and squamous cell carcinoma (SCC4) cells were incubated with the indicated concentrations of DBL for 12 h and then with 0.1 nmol/L TNF for 30 min. Nuclear extracts were then prepared and analyzed for NF- $\kappa$ B activation by EMSA.

#### DBL Does Not Directly Interfere with the Binding of NF- $\kappa$ B to DNA

Several NF- $\kappa$ B inhibitors, such as caffeic acid phenethyl ester and plumbagin, suppress NF- $\kappa$ B activation by directly modifying the NF- $\kappa$ B protein, such that it can no longer

bind to the DNA (21, 22). When we incubated nuclear extracts from TNF-treated cells with various concentrations of DBL, EMSA showed that DBL had no direct effect on NF- $\kappa$ B binding to the DNA (Fig. 2C2). Thus, DBL must inhibit NF- $\kappa$ B activation through an indirect mechanism.

### **Inhibition of NF- $\kappa$ B Activation by DBL Is Not Cell Type Specific**

Because the signal transduction pathway mediated by NF- $\kappa$ B may be distinctly different across cell types (20, 23), we also investigated whether DBL could block TNF-induced NF- $\kappa$ B activation in lung adenocarcinoma H1299 and T-cell lymphoma Jurkat cells (Fig. 2D). DBL significantly inhibited TNF-induced NF- $\kappa$ B activation, thus indicating that DBL-induced suppression of NF- $\kappa$ B activation was not cell type specific.

#### **DBL Also Suppresses Constitutive NF- $\kappa$ B Activation**

Most tumor cells express constitutively active NF- $\kappa$ B (18, 24), although the mechanism of constitutive activation is not well understood. Human multiple myeloma (U266) cells and squamous cell carcinoma (SCC4) cells, for example, are known to express constitutively active NF- $\kappa$ B (25, 26). U266 and SCC4 cells were treated with different concentrations of DBL and then analyzed for NF- $\kappa$ B activation. We showed that DBL suppresses constitutive activation of NF- $\kappa$ B in both cells (Fig. 2G).

#### **DBL Is a Potent Inhibitor of NF- $\kappa$ B Activation**

To determine the effect of DBL on NF- $\kappa$ B activation at higher TNF concentrations, cells were pretreated with 50  $\mu$ mol/L DBL for 12 h, treated with various concentrations of TNF, and then analyzed for NF- $\kappa$ B activation (Fig. 3A). Cells pretreated with DBL showed a lack of NF- $\kappa$ B activation even when exposed to a very high concentration of TNF. These results show that DBL is a very potent inhibitor of TNF-induced NF- $\kappa$ B activation.

#### **DBL Inhibits TNF-Dependent I $\kappa$ B $\alpha$ Degradation**

To determine whether the NF- $\kappa$ B inhibitory activity of DBL was due to inhibition of I $\kappa$ B $\alpha$  degradation, we pretreated cells with DBL, then exposed the cells to TNF for different periods, and finally examined their cytoplasmic I $\kappa$ B by Western blot analysis. TNF induced I $\kappa$ B $\alpha$  degradation in control cells within 10 min and reached a maximum at 15 min, but TNF could not induce I $\kappa$ B $\alpha$  degradation in DBL-pretreated cells (Fig. 3B1). These results indicate that DBL inhibits TNF-induced I $\kappa$ B $\alpha$  degradation.

#### **DBL Inhibits TNF-Dependent I $\kappa$ B $\alpha$ Phosphorylation**

Because I $\kappa$ B $\alpha$  phosphorylation is needed for I $\kappa$ B $\alpha$  degradation, we determined whether DBL modulated I $\kappa$ B $\alpha$  phosphorylation. Because TNF-induced phosphorylation of I $\kappa$ B $\alpha$  leads to its rapid degradation, we blocked I $\kappa$ B $\alpha$  degradation with the proteasome inhibitor *N*-acetyl-Leu-Leu-norleucinal. Western blot analysis using an antibody specific for the serine-phosphorylated form of I $\kappa$ B $\alpha$  showed that DBL suppressed TNF-induced phosphorylation of I $\kappa$ B $\alpha$  (Fig. 3B2).

#### **DBL Inhibits TNF-Induced IKK Activation**

IKK is required for TNF-induced phosphorylation of I $\kappa$ B $\alpha$  (14) and for the phosphorylation of p65 (16). Because DBL inhibited the phosphorylation of I $\kappa$ B $\alpha$ , we determined its effect on TNF-induced IKK activation. Immune complex kinase assays showed that DBL suppressed the activation of IKK by TNF (Fig. 3C1). Neither TNF nor DBL had any effect on the expression of IKK proteins. To evaluate

whether DBL suppresses IKK activity directly by binding to the IKK protein or by suppressing the activation of IKK, we incubated whole-cell extracts from untreated and TNF-treated cells with various concentrations of DBL. Immune complex kinase assays showed that DBL did not directly affect the activity of IKK, suggesting that DBL modulates TNF-induced IKK activation (Fig. 3C2).

#### **DBL Inhibits TNF-Induced Phosphorylation and Nuclear Translocation of p65**

As indicated by Western blot analysis (Fig. 3D1), DBL significantly inhibited TNF-induced nuclear translocation of p65. Immunocytochemistry seemed to confirm this finding (Fig. 3D2). TNF also induces the phosphorylation of p65, which is required for its transcriptional activity (13). As shown in Fig. 3D1, the coincubation of cells with DBL consistently inhibited TNF-induced phosphorylation of p65.

#### **DBL Suppresses TNF-Induced NF- $\kappa$ B-Dependent Reporter Gene Expression**

Because DNA binding does not always correlate with NF- $\kappa$ B-dependent gene transcription (27), we investigated the effect of DBL on TNF-induced reporter activity. Cells transiently transfected with the NF- $\kappa$ B-regulated secreted alkaline phosphatase (SEAP) reporter construct, incubated with DBL, and then stimulated with TNF had significantly diminished reporter gene expression when compared with cells that were not incubated with DBL (Fig. 4A). These results suggest that DBL inhibited TNF-induced gene expression.

#### **DBL Inhibits NF- $\kappa$ B Activation Induced by TNFR1, TRADD, TRAF2, NIK, and IKK**

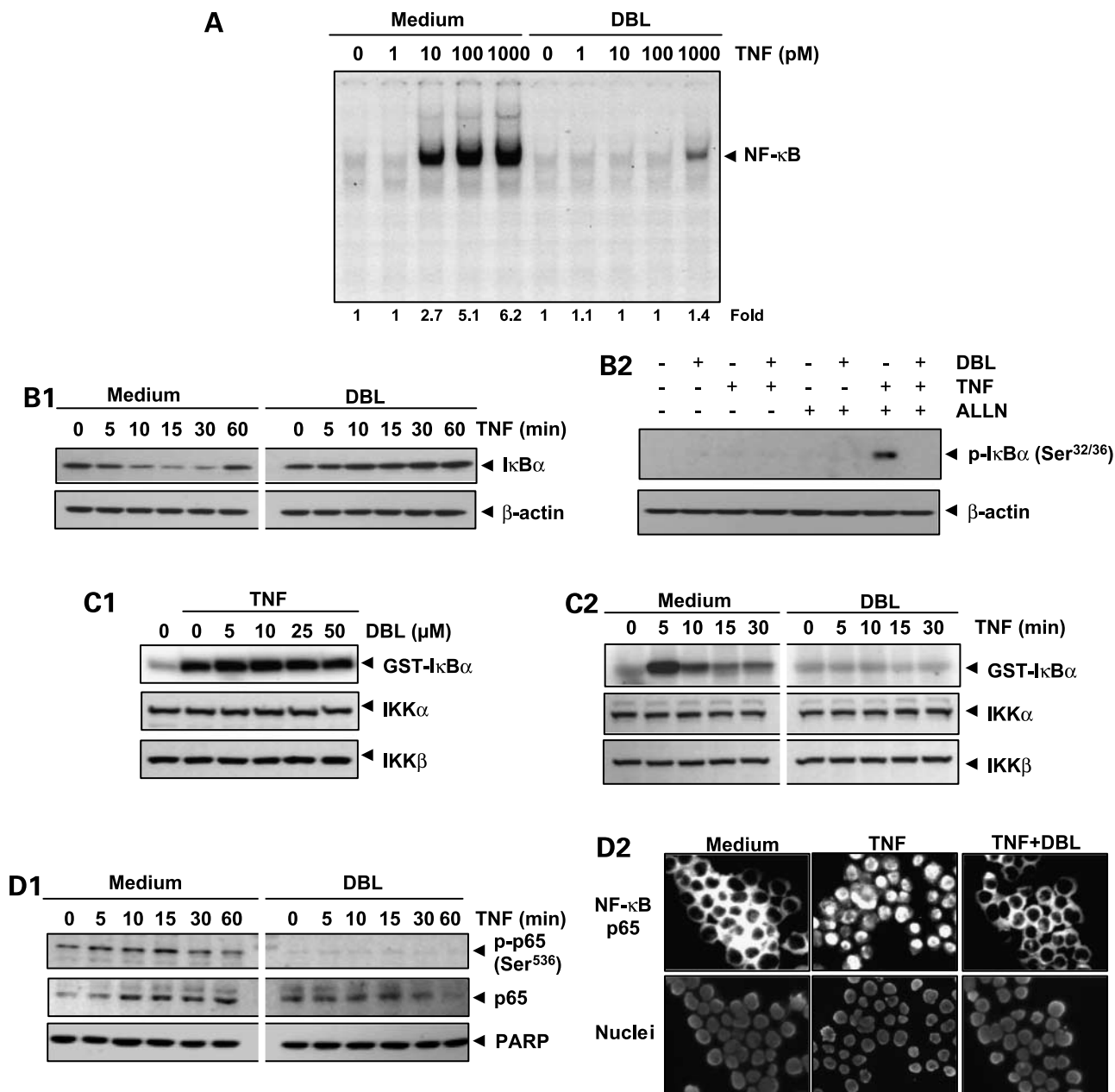
TNF-induced NF- $\kappa$ B activation is mediated through sequential interaction of the TNF receptor with TRADD, TRAF2, NIK, and IKK, resulting in phosphorylation of I $\kappa$ B $\alpha$  (28, 29). When we transiently transfected cells with the NF- $\kappa$ B-regulated SEAP reporter construct, along with TNFR1-, TRADD-, TRAF2-, NIK-, IKK $\beta$ -, or p65-expressing plasmids, treated them with DBL, and then monitored NF- $\kappa$ B-dependent SEAP expression, we found that DBL suppressed NF- $\kappa$ B activation induced by TNFR1, TRADD, TRAF2, NIK, and IKK $\beta$  but not that induced by p65 (Fig. 4B). These results suggested that DBL acts upstream of p65.

#### **DBL Suppresses NF- $\kappa$ B-Dependent Antiapoptotic Gene Products**

NF- $\kappa$ B regulates the expression of several antiapoptotic proteins, including survivin, IAP1, IAP2, Bcl-2, Bcl-xL, XIAP, and cFLIP (30–34). Whether DBL can modulate the expression of these antiapoptotic gene products induced by TNF was also examined. As shown in Fig. 4C1, TNF induced the expression of these antiapoptotic proteins in a time-dependent manner and DBL inhibited their expression.

#### **DBL Suppresses NF- $\kappa$ B-Dependent Gene Products Involved in Cell Proliferation**

Numerous gene products involved in cell proliferation, including cyclin D1, c-myc, and COX-2, have a NF- $\kappa$ B binding site in their promoters (35–37). Whether DBL

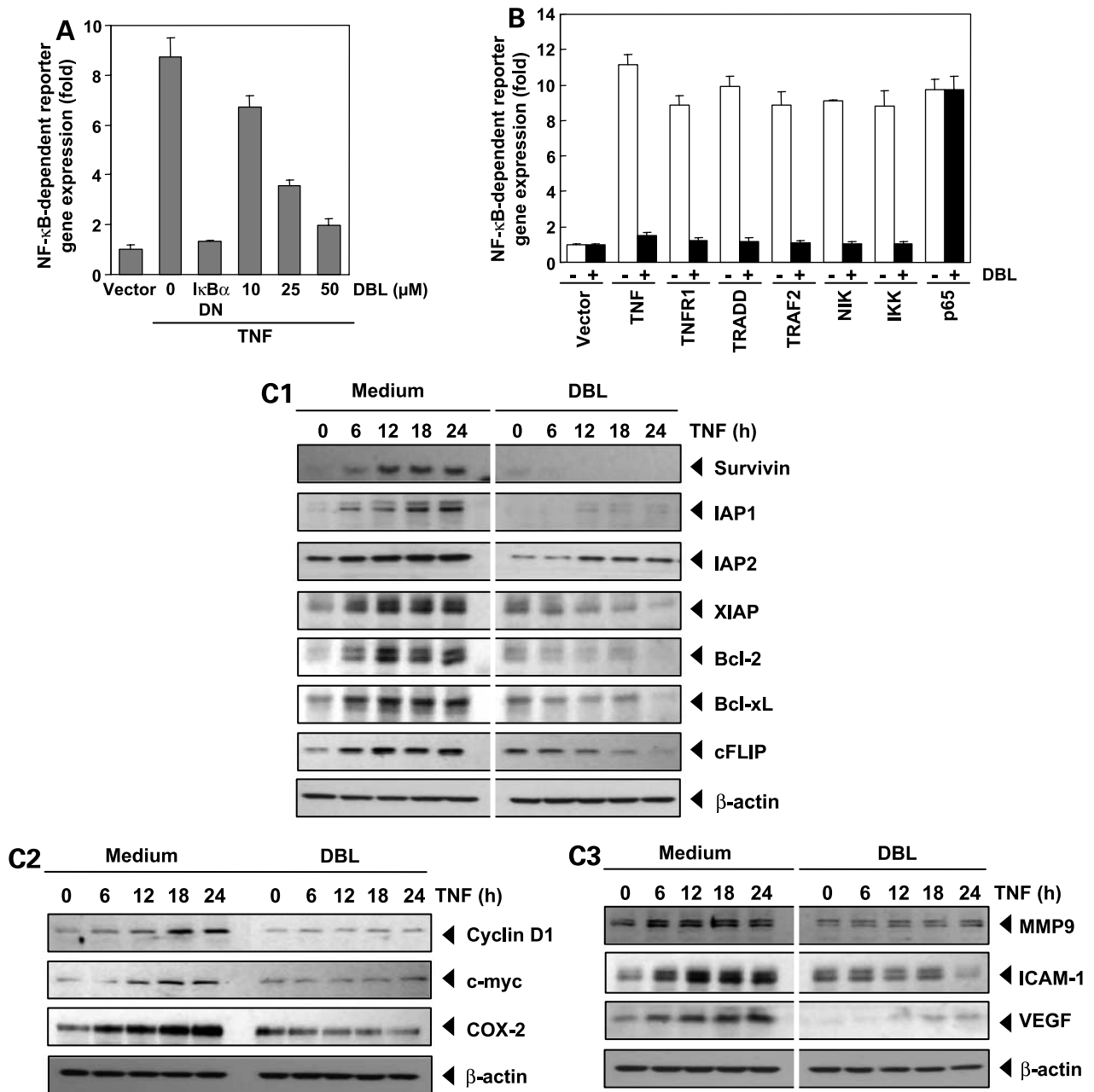


**Figure 3.** DBL inhibits TNF-dependent I $\kappa$ B $\alpha$  degradation and translocation of p65. **A**, effect of DBL on the activation of NF- $\kappa$ B induced by different concentrations of TNF. KBM-5 cells were incubated with 50  $\mu$ mol/L DBL for 12 h, treated with different concentrations of TNF for 30 min, and then assayed for NF- $\kappa$ B activation by EMSA. **B1**, effect of DBL on TNF-induced degradation of I $\kappa$ B $\alpha$ . KBM-5 cells were incubated with 50  $\mu$ mol/L DBL for 12 h and treated with 0.1 nmol/L TNF for the indicated times. Cytoplasmic extracts were prepared and analyzed by Western blotting using antibodies against anti-I $\kappa$ B $\alpha$ . Equal protein loading was evaluated by  $\beta$ -actin. **B2**, effect of DBL on the phosphorylation of I $\kappa$ B $\alpha$  by TNF. Cells were preincubated with 50  $\mu$ mol/L DBL for 12 h, incubated with 50  $\mu$ g/mL *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) for 30 min, and then treated with 0.1 nmol/L TNF for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phospho-I $\kappa$ B $\alpha$  (Ser<sup>32</sup>/Ser<sup>36</sup>) antibody. **C1**, direct effect of DBL on the activation of IKK induced by TNF. Whole-cell extracts were prepared from cells treated with 1 nmol/L TNF and immunoprecipitated with an anti-IKK $\alpha$  antibody. Immune complex kinase assay was then done in the presence or absence of DBL at the indicated concentrations. **C2**, effect of DBL on the activation of IKK by TNF. KBM-5 cells were preincubated with 50  $\mu$ mol/L DBL for 12 h, incubated with 50  $\mu$ g/mL ALLN for 30 min, and then treated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were immunoprecipitated with antibody against IKK $\alpha$  and analyzed by an immune complex kinase assay. To examine the basal level of expression of IKK proteins, whole-cell extracts were prepared and analyzed by Western blotting using anti-IKK $\alpha$  and anti-IKK $\beta$  antibodies. **D1**, DBL inhibits TNF-induced phosphorylation and nuclear translocation of p65. KBM-5 cells were either untreated or pretreated with 50  $\mu$ mol/L DBL for 12 h and then treated with 0.1 nmol/L TNF for the indicated times. Nuclear extracts were prepared and analyzed by Western blotting using antibodies against phospho-p65 and p65. As a nuclear protein loading control, the membrane was blotted with anti-poly(ADP-ribose) polymerase (PARP) antibody. **D2**, immunocytochemical analysis of p65 localization. KBM-5 cells were first treated with 50  $\mu$ mol/L DBL for 12 h and then exposed to 1 nmol/L TNF for 20 min. After cytospin, cells were applied immunocytochemical analysis.

modulates the expression of cyclin D1, c-myc, and COX-2 were investigated. Western blot analysis indicated that DBL suppressed the expression of all these proteins (Fig. 4C2).

#### DBL Modulated NF- $\kappa$ B-Dependent Gene Products Involved in Tumor Cell Invasion

Various genes, such as MMP-9, intracellular adhesion molecule-1, and vascular endothelial cell growth factor, all



**Figure 4.** DBL represses NF- $\kappa$ B-dependent reporter gene expression induced by TNF. **A**, DBL inhibits TNF-induced NF- $\kappa$ B-dependent reporter gene (SEAP) expression. A293 cells were transiently transfected with a NF- $\kappa$ B-SEAP plasmid and then treated with the indicated concentrations of DBL. After 24 h in culture with 1 nmol/L TNF, cell supernatants were collected and assayed for SEAP activity. Results are expressed as fold activity over the activity of the vector control. **B**, DBL inhibits NF- $\kappa$ B-dependent reporter gene expression induced by TNF, TNFR1, TRADD, TRAF2, NIK, and IKK $\beta$  but not by p65. Cells were transiently transfected with a NF- $\kappa$ B-containing plasmid alone or with the indicated plasmids. After 24 h, cells were treated with 50  $\mu$ mol/L DBL and then incubated with the relevant plasmid for an additional 24 h. For TNF-treated cells, cells were treated with 50  $\mu$ mol/L DBL and then incubated with 1 nmol/L TNF for 24 h. The supernatants of the culture medium were assayed for SEAP activity. **C**, DBL inhibits TNF-induced expression of NF- $\kappa$ B-dependent antiapoptotic (**C1**), proliferative (**C2**), and metastatic (**C3**) proteins. KBM-5 cells were incubated with 50  $\mu$ mol/L DBL for 12 h and then treated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using the indicated antibodies.

linked to tumor cell invasion are modulated by NF- $\kappa$ B (38, 39). Whether the expression of MMP-9, intracellular adhesion molecule-1, and vascular endothelial cell growth factor is modulated by DBL, was examined. Western blot analysis indicated that DBL down-modulated the expressions of all these gene products (Fig. 4C3).

#### DBL Potentiates Apoptosis Induced by TNF and Chemotherapeutic Agents

Because NF- $\kappa$ B activation inhibits apoptosis induced by TNF and chemotherapeutic agents through the regulation of gene products named above, we therefore investigated whether DBL can modulate the cytotoxic effects of TNF, doxorubicin, 5-fluorouracil, and cisplatin. As indicated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (Fig. 5A) and Live/Dead assays (Fig. 5B), DBL up-regulated apoptotic effects of TNF and chemotherapeutic agents. The TNF-induced cytotoxicity was enhanced from 5% to 32%. Annexin V and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining also indicated that DBL up-regulated TNF-induced early apoptosis (Fig. 5C). As shown in Fig. 5D, TNF-induced caspase activation, as indicated by poly(ADP-ribose) polymerase cleavage, was also potentiated by DBL. All of these assay results together suggest that DBL enhanced the apoptotic effects of TNF and chemotherapeutic agents.

#### DBL Suppresses Cell Proliferation

Because DBL suppressed gene expression involved in proliferation, we examined whether DBL can modulate cell

proliferation. As shown in Fig. 6A, DBL suppressed cell proliferation in a dose- and time-dependent manner.

#### DBL Modulates Tumor Cell Invasion

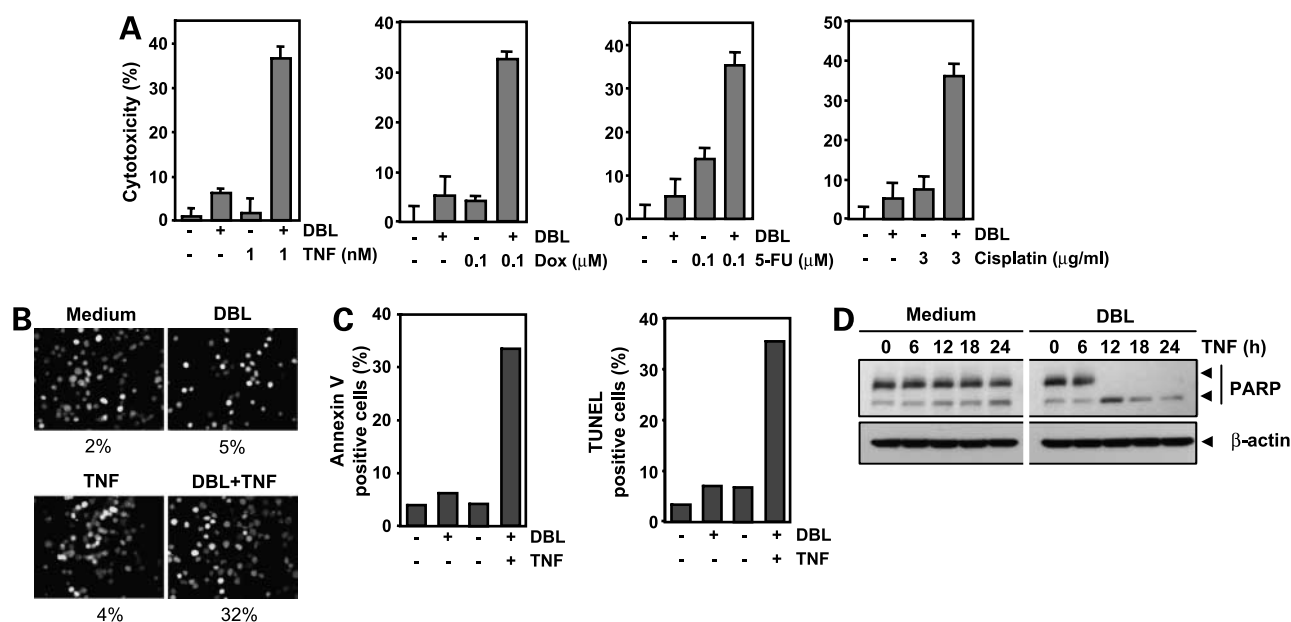
Our results indicate that DBL also suppresses the gene products involved in tumor cell invasion. Whether DBL can also modulate TNF-induced tumor cell invasion activity was investigated *in vitro*. To determine this, tumor cells were seeded to Matrigel invasion chamber with TNF in the presence or absence of DBL and then examined for invasion. As shown in Fig. 6B, TNF induced tumor cell invasion by almost 7-fold and DBL suppressed this activity. DBL alone had no effect on invasion activity.

#### Effect of DBL on Gene Expression Is Reversible

Whether DBL-induced modulation of gene products (COX-2 and c-myc) is reversible was investigated. For this, KBM-5 cells were first treated with DBL for 12 h and then removed the drug by washing the cells with PBS. Then, 2, 12, and 24 h after removal of DBL, cells were examined for expression of gene expression induced by TNF. The results show that the suppressive effect of DBL on expression of COX-2 and c-myc was reversible after 12 h (Fig. 6C).

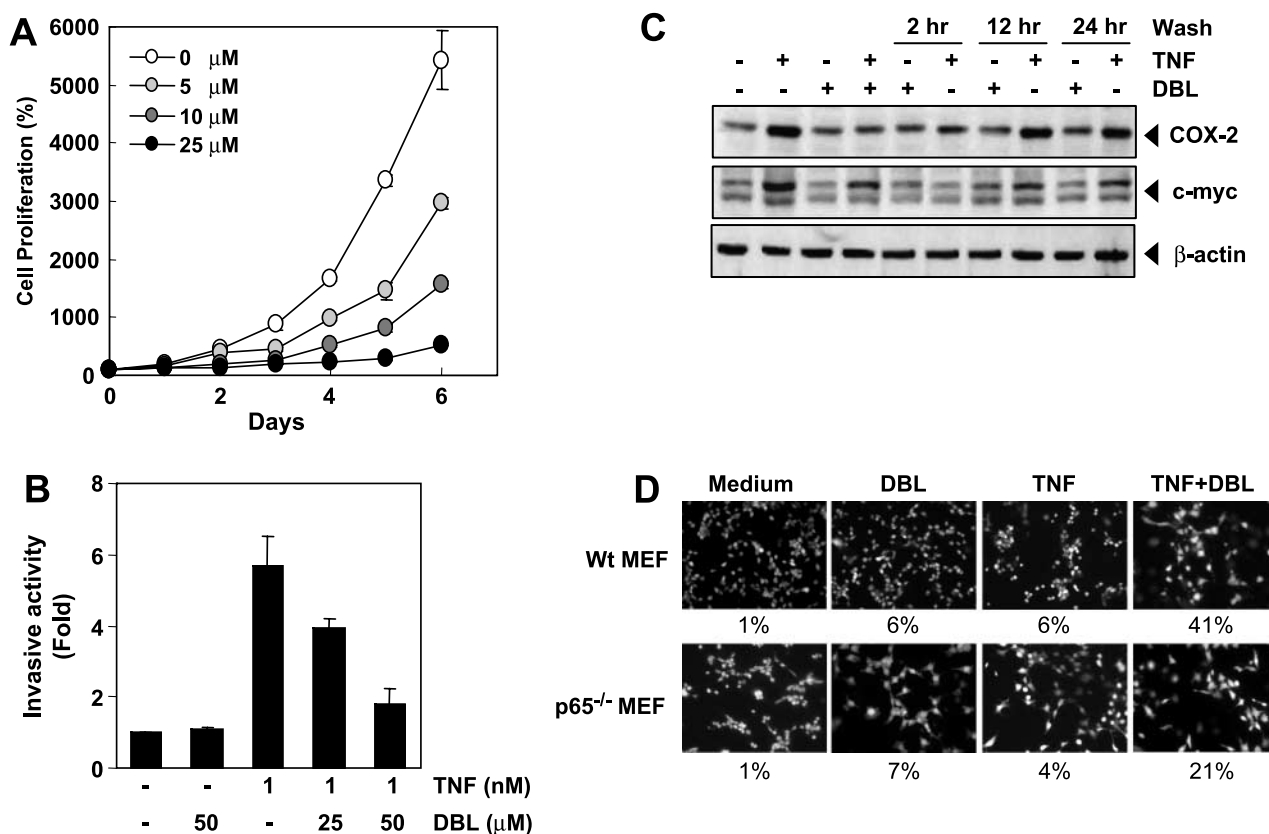
#### Deletion of NF- $\kappa$ B Significantly Decreases the Effect of DBL on Apoptosis

Whether the effect of DBL is mediated through inhibition of NF- $\kappa$ B was examined by using *p65*<sup>-/-</sup> cells. Results showed that DBL potentiated TNF-induced apoptosis from



**Figure 5.** DBL enhances TNF-induced cytotoxicity. **A**, DBL enhances cytotoxicity induced by TNF, doxorubicin, 5-fluorouracil, and cisplatin. In total, 5,000 cells were seeded in triplicate in 96-well plates. The cells were pretreated with 50 μmol/L DBL and then incubated with the indicated concentrations of TNF, doxorubicin, 5-fluorouracil, and cisplatin for 24 h. Cell viability was then analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. **B**, KBM-5 cells were pretreated with 50 μmol/L DBL for 12 h and then incubated with 1 nmol/L TNF for 16 h. The cells were stained with a Live/Dead assay (**B**) reagent for 30 min and then analyzed under a fluorescence microscope, incubated with a FITC-conjugated Annexin V antibody and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (**C**), and then analyzed by flow cytometry. **D**, cells were pretreated with 50 μmol/L DBL for 12 h and then incubated with 1 nmol/L TNF for the indicated times. Whole cell extracts were prepared and analyzed by Western blotting using an anti-poly(ADP-ribose) polymerase antibody.





**Figure 6.** **A**, effect of DBL is reversible. KBM-5 cells were treated with 50  $\mu\text{mol/L}$  DBL for 12 h and washed with PBS twice to remove DBL. After then, cells were induced by TNF for indicated times and determined protein levels by Western blotting. **B**, DBL suppresses cell proliferation. Cells ( $2 \times 10^3/0.1$  mL) were seeded in triplicate in 96-well plates, treated with different doses of DBL, and then incubated for 2, 4, and 6 d. Thereafter, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. **C**, DBL suppresses TNF-induced invasive activity. Human lung H1299 cells ( $2.5 \times 10^4$ ) were seeded into the upper wells of a Matrigel invasion chamber overnight in the absence of serum, pretreated with 25 and 50  $\mu\text{mol/L}$  DBL for 12 h, and then treated with 1 nmol/L TNF for 24 h in the presence of 1% serum. Thereafter, cell invasion was measured as described in Materials and Methods. **D**, NF- $\kappa$ B is needed for the effect of DBL. Wild-type and  $p65^{-/-}$  mouse embryonic fibroblast cells ( $1 \times 10^5/\text{mL}$ ) were pretreated with 50  $\mu\text{mol/L}$  DBL for 12 h and then treated with 1 nmol/L TNF for 16 h. Cell death was determined by the Live/Dead assay.

6% to 41% in wild-type cells but in  $p65^{-/-}$  cells only from 4% to 21%. These results thus suggest that the effect of DBL is mediated through NF- $\kappa$ B suppression (Fig. 6D).

## Discussion

The aim of this study was to determine the effect of DBL on NF- $\kappa$ B and NF- $\kappa$ B-regulated gene products involved in antiapoptosis, proliferation, differentiation, and invasion. We found that DBL is a potent inhibitor of both constitutive and inducible NF- $\kappa$ B activation. The suppression of NF- $\kappa$ B occurred through inhibition of activation of IKK, phosphorylation and degradation of I $\kappa$ B $\alpha$ , p65 phosphorylation and nuclear translocation, and NF- $\kappa$ B-dependent reporter gene expression. DBL also down-regulated NF- $\kappa$ B-dependent gene products involved in antiapoptosis (IAP1/IAP2, cFLIP, survivin, Bcl-2, Bcl-xL, and XIAP), cell proliferation (cyclin D1, COX-2, and c-myc), and angiogenesis (vascular endothelial cell growth factor, intracellular adhesion molecule, and MMP-9). We believe that these effects on

the NF- $\kappa$ B pathway account for the ability of the molecule to potentiate the cytotoxic effects of TNF and chemotherapeutic agents.

For the first time, we found that DBL inhibited NF- $\kappa$ B activation induced by highly diverse inflammatory stimuli (TNF, lipopolysaccharide, and interleukin-1 $\beta$ ) and tumor-promoting agents (phorbol 12-myristate 13-acetate, epidermal growth factor, and okadaic acid). Most of these agents activate NF- $\kappa$ B through different pathways. These results suggest that DBL acts at a step common to all of these activators. Thus, we found that DBL blocked the activation of NF- $\kappa$ B without directly interfering with the DNA binding of NF- $\kappa$ B. In response to most stimuli, NF- $\kappa$ B activation proceeds through sequential activation of IKK, phosphorylation at Ser<sup>32</sup>/Ser<sup>36</sup> of I $\kappa$ B $\alpha$ , and ubiquitination, leading finally to degradation of I $\kappa$ B $\alpha$  and the release of NF- $\kappa$ B (20). Our *in vitro* kinase assay results show that DBL is not a direct inhibitor of IKK. Therefore, it seems that this agent blocks the activation of IKK by interfering with some upstream regulatory kinases. Akt, NIK, mitogen-activated protein

kinase 1, and atypical protein kinase C regulate IKK (12). It is possible that one of these kinases is modulated by DBL.

We found that DBL inhibited not only inducible NF- $\kappa$ B activation but also constitutively activated NF- $\kappa$ B in multiple myeloma and squamous cell carcinomas. Constitutive NF- $\kappa$ B activation has been found to be critical for the survival and proliferation of various tumor cell types (25, 26); however, the mechanism is not well understood. Some of the potential mechanisms are overexpression of I $\kappa$ B $\alpha$  without inhibition of NF- $\kappa$ B activity, mutations in the I $\kappa$ B $\alpha$  gene, enhanced I $\kappa$ B $\alpha$  degradation, and constitutive expression of TNF and interleukin-1 (12).

The down-regulation of TNF-induced expression of antiapoptosis gene products, such as IAP1, IAP2, Bcl-2, Bcl-xL, and TRAF-1, by DBL correlated with the potentiation of the apoptotic effects of cytokines and the chemotherapeutic agents. We showed that DBL also down-regulated the TNF-induced expression of cyclin D1 and c-myc. These results are in agreement with those of Markaverich et al. (15) who showed that DBL also down-regulates cyclin D1 and c-myc levels and that it finally leads inhibition of cell proliferation in estrogen receptor-positive and estrogen receptor-negative cells. Previous reports suggest that DBL inhibits proliferation of cells and induces apoptosis in various tumor cells (15, 40). It is possible that these effects of DBL are mediated through the down-regulation of antiapoptotic gene products and cyclin D1 and c-myc as described here.

The down-regulation of MMP-9 and COX-2 expression by DBL correlated with the inhibition of TNF-induced tumor cell invasion. MMP-9 plays a crucial role in tumor invasion and angiogenesis by mediating the degradation of the extracellular matrix, and the inhibition of MMP activity has been shown to suppress lung metastasis (41). COX-2 also has been implicated in carcinogenic processes, such as the enhancement of cellular invasion, induction of angiogenesis, and regulation of antiapoptotic cellular defenses through the production of prostaglandin E2 (42). Our results are in agreement with those of Park et al. (8), who reported that extracts from *I. obliquus* inhibited COX-2 activity, thus preferentially affecting COX-2-catalyzed prostaglandin synthesis and suppressing the expression of inducible nitric oxide synthase and TNF- $\alpha$  in murine macrophages. Because these processes are also regulated by NF- $\kappa$ B (12, 43, 44), our results may explain their down-regulation.

Overall, our results suggest that antiproliferative, proapoptotic, anti-invasive, and antiangiogenic effects assigned to DBL may be mediated in part through the suppression of NF- $\kappa$ B and NF- $\kappa$ B-regulated gene products.

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#### References

1. Surh YJ. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 2003;3:768–80.
2. Saar M. Fungi in Khanty folk medicine. *J Ethnopharmacol* 1991;31:175–9.

3. Nakajima Y, Sato Y, Konishi T. Antioxidant small phenolic ingredients in *Inonotus obliquus* (persoon) Pilat (Chaga). *Chem Pharm Bull (Tokyo)* 2007;55:1222–6.
4. Mizuno T, Katsumata N, Mukai H, Shimizu C, Ando M, Watanabe T. The outpatient management of low-risk febrile patients with neutropenia: risk assessment over the telephone. *Support Care Cancer* 2007;15:287–91.
5. Ichimura T, Otake T, Mori H, Maruyama S. HIV-1 protease inhibition and anti-HIV effect of natural and synthetic water-soluble lignin-like substances. *Biosci Biotechnol Biochem* 1999;63:2202–4.
6. Zjawiony JK. Biologically active compounds from *Aphyllphorales* (polypore) fungi. *J Nat Prod* 2004;67:300–10.
7. Hyun KW, Jeong SC, Lee DH, Park JS, Lee JS. Isolation and characterization of a novel platelet aggregation inhibitory peptide from the medicinal mushroom, *Inonotus obliquus*. *Peptides* 2006;27:1173–8.
8. Park YM, Won JH, Kim YH, Choi JW, Park HJ, Lee KT. *In vivo* and *in vitro* anti-inflammatory and anti-nociceptive effects of the methanol extract of *Inonotus obliquus*. *J Ethnopharmacol* 2005;101:120–8.
9. Jarosz A, Skorska M, Rzymowska J, Kochmanska-Rdest J, Malarczyk E. Effect of the extracts from fungus *Inonotus obliquus* on catalase level in HeLa and *Nocardia* cells. *Acta Biochim Pol* 1990;37:149–51.
10. Kim YO, Park HW, Kim JH, Lee JY, Moon SH, Shin CS. Anti-cancer effect and structural characterization of endo-polysaccharide from cultivated mycelia of *Inonotus obliquus*. *Life Sci* 2006;79:72–80.
11. Nakata T, Yamada T, Taji S, et al. Structure determination of inonotusoxides A and B and *in vivo* anti-tumor promoting activity of inotodiol from the sclerotia of *Inonotus obliquus*. *Bioorg Med Chem* 2007;15:257–64.
12. Aggarwal BB. Nuclear factor- $\kappa$ B: the enemy within. *Cancer Cell* 2004;6:203–8.
13. Ghosh S, May MJ, Kopp EB. NF- $\kappa$ B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 1998;16:225–60.
14. Ducut Sigala JL, Bottero V, Young DB, Shevchenko A, Mercurio F, Verma IM. Activation of transcription factor NF- $\kappa$ B requires ELKS, an I $\kappa$ B kinase regulatory subunit. *Science* 2004;304:1963–7.
15. Markaverich BM, Shoulars K, Alejandro MA. Nuclear type II [<sup>3</sup>H]estradiol binding site ligands: inhibition of ER-positive and ER-negative cell proliferation and c-Myc and cyclin D1 gene expression. *Steroids* 2006;71:865–74.
16. Takada Y, Khuri FR, Aggarwal BB. Protein farnesyltransferase inhibitor (SCH 66336) abolishes NF- $\kappa$ B activation induced by various carcinogens and inflammatory stimuli leading to suppression of NF- $\kappa$ B-regulated gene expression and up-regulation of apoptosis. *J Biol Chem* 2004;279:26287–99.
17. Takada Y, Ichikawa H, Badmaev V, Aggarwal BB. Acetyl-11-keto- $\beta$ -boswellic acid potentiates apoptosis, inhibits invasion, and abolishes osteoclastogenesis by suppressing NF- $\kappa$ B and NF- $\kappa$ B-regulated gene expression. *J Immunol* 2006;176:3127–40.
18. Pahl HL. Activators and target genes of Rel/NF- $\kappa$ B transcription factors. *Oncogene* 1999;18:6853–66.
19. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 2003;3:745–56.
20. Ghosh S, Karin M. Missing pieces in the NF- $\kappa$ B puzzle. *Cell* 2002;109 Suppl:S81–96.
21. Natarajan K, Singh S, Burke TR, Jr., Grunberger D, Aggarwal BB. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF- $\kappa$ B. *Proc Natl Acad Sci U S A* 1996;93:9090–5.
22. Sandur SK, Ichikawa H, Sethi G, Ahn KS, Aggarwal BB. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF- $\kappa$ B activation and NF- $\kappa$ B-regulated gene products through modulation of p65 and I $\kappa$ B $\alpha$  kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents. *J Biol Chem* 2006;281:17023–33.
23. Bonizzi G, Piette J, Merville MP, Bours V. Distinct signal transduction pathways mediate nuclear factor- $\kappa$ B induction by IL-1 $\beta$  in epithelial and lymphoid cells. *J Immunol* 1997;159:5264–72.
24. Garg AK, Aggarwal BB. Reactive oxygen intermediates in TNF signaling. *Mol Immunol* 2002;39:509–17.
25. Aggarwal S, Takada Y, Singh S, Myers JN, Aggarwal BB. Inhibition of

- growth and survival of human head and neck squamous cell carcinoma cells by curcumin via modulation of nuclear factor- $\kappa$ B signaling. *Int J Cancer* 2004;111:679–92.
26. Bharti AC, Shishodia S, Reuben JM, et al. Nuclear factor- $\kappa$ B and STAT3 are constitutively active in CD138<sup>+</sup> cells derived from multiple myeloma patients, and suppression of these transcription factors leads to apoptosis. *Blood* 2004;103:3175–84.
27. Nasuhara Y, Adcock IM, Catley M, Barnes PJ, Newton R. Differential  $\kappa$ B kinase activation and  $\kappa$ B $\alpha$  degradation by interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  in human U937 monocytic cells. Evidence for additional regulatory steps in  $\kappa$ B-dependent transcription. *J Biol Chem* 1999;274:19965–72.
28. Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF- $\kappa$ B activation. *Cell* 1995;81:495–504.
29. Simeonidis S, Stauber D, Chen G, Hendrickson WA, Thanos D. Mechanisms by which  $\kappa$ B proteins control NF- $\kappa$ B activity. *Proc Natl Acad Sci U S A* 1999;96:49–54.
30. Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF- $\kappa$ B control. *Proc Natl Acad Sci U S A* 1997;94:10057–62.
31. You M, Ku PT, Hrdlickova R, Bose HR, Jr. c-IAP1, a member of the inhibitor-of-apoptosis protein family, is a mediator of the antiapoptotic activity of the v-Rel oncoprotein. *Mol Cell Biol* 1997;17:7328–41.
32. Tamatani M, Che YH, Matsuzaki H, et al. Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NF $\kappa$ B activation in primary hippocampal neurons. *J Biol Chem* 1999;274:8531–8.
33. Catz SD, Johnson JL. Transcriptional regulation of bcl-2 by nuclear factor  $\kappa$ B and its significance in prostate cancer. *Oncogene* 2001;20:7342–51.
34. Zhu L, Fukuda S, Cordis G, Das DK, Maulik N. Anti-apoptotic protein survivin plays a significant role in tubular morphogenesis of human coronary arteriolar endothelial cells by hypoxic preconditioning. *FEBS Lett* 2001;508:369–74.
35. Lin MT, Chang CC, Chen ST, et al. Cyr61 expression confers resistance to apoptosis in breast cancer MCF-7 cells by a mechanism of NF- $\kappa$ B-dependent XIAP up-regulation. *J Biol Chem* 2004;279:24015–23.
36. Duyao MP, Kessler DJ, Spicer DB, et al. Transactivation of the c-myc promoter by human T cell leukemia virus type 1 tax is mediated by NF  $\kappa$ B. *J Biol Chem* 1992;267:16288–91.
37. Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcriptional roles of nuclear factor  $\kappa$ B and nuclear factor-interleukin-6 in the tumor necrosis factor  $\alpha$ -dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J Biol Chem* 1995;270:31315–20.
38. Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS, Jr. NF- $\kappa$ B controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol* 1999;19:5785–99.
39. Esteve PO, Chicoine E, Robledo O, et al. Protein kinase C- $\zeta$  regulates transcription of the matrix metalloproteinase-9 gene induced by IL-1 and TNF- $\alpha$  in glioma cells via NF- $\kappa$ B. *J Biol Chem* 2002;277:35150–5.
40. Park JR, Park JS, Jo EH, et al. Reversal of the TPA-induced inhibition of gap junctional intercellular communication by Chaga mushroom (*Inonotus obliquus*) extracts: effects on MAP kinases. *Biofactors* 2006;27:147–55.
41. Shiraga M, Yano S, Yamamoto A, et al. Organ heterogeneity of host-derived matrix metalloproteinase expression and its involvement in multiple-organ metastasis by lung cancer cell lines. *Cancer Res* 2002;62:5967–73.
42. Hirschowitz E, Hidalgo G, Doherty D. Induction of cyclo-oxygenase-2 in non-small cell lung cancer cells by infection with  $\Delta$ E1,  $\Delta$ E3 recombinant adenovirus vectors. *Gene Ther* 2002;9:81–4.
43. Vanden Berghe W, Vermeulen L, De Wilde G, De Bosscher K, Boone E, Haegeman G. Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. *Biochem Pharmacol* 2000;60:1185–95.
44. Li X, Massa PE, Hanidu A, et al. IKK $\alpha$ , IKK $\beta$ , and NEMO/IKK $\gamma$  are each required for the NF- $\kappa$ B-mediated inflammatory response program. *J Biol Chem* 2002;277:45129–40.