

# Targeting Nuclear Factor- $\kappa$ B Activation Pathway by Thymoquinone: Role in Suppression of Antiapoptotic Gene Products and Enhancement of Apoptosis

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## Abstract

Thymoquinone (TQ), derived from the medicinal plant *Nigella sativa*, exhibits antiinflammatory and anticancer activities through mechanism(s) that is not fully understood. Because numerous effects modulated by TQ can be linked to interference with the nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling, we investigated in detail the effect of this quinone on NF- $\kappa$ B pathway. As examined by DNA binding, we found that TQ suppressed tumor necrosis factor- $\alpha$ -induced NF- $\kappa$ B activation in a dose- and time-dependent manner and inhibited NF- $\kappa$ B activation induced by various carcinogens and inflammatory stimuli. The suppression of NF- $\kappa$ B activation correlated with sequential inhibition of the activation of I $\kappa$ B $\alpha$  kinase, I $\kappa$ B $\alpha$  phosphorylation, I $\kappa$ B $\alpha$  degradation, p65 phosphorylation, p65 nuclear translocation, and the NF- $\kappa$ B-dependent reporter gene expression. TQ specifically suppressed the direct binding of nuclear p65 and recombinant p65 to the DNA, and this binding was reversed by DTT. However, TQ did not inhibit p65 binding to DNA when cells were transfected with the p65 plasmid containing cysteine residue 38 mutated to serine. TQ also down-regulated the expression of NF- $\kappa$ B-regulated antiapoptotic (IAP1, IAP2, XIAP, Bcl-2, Bcl-xL, and survivin), proliferative (cyclin D1, cyclooxygenase-2, and c-Myc), and angiogenic (matrix metalloproteinase-9 and vascular endothelial growth factor) gene products. This led to potentiation of apoptosis induced by tumor necrosis factor and chemotherapeutic agents. Overall, our results indicate that the anticancer and antiinflammatory activities previously assigned to TQ may be mediated in part through the

suppression of the NF- $\kappa$ B activation pathway, as shown here, and thus may have potential in treatment of myeloid leukemia and other cancers. (Mol Cancer Res 2008;6(6):1059–70)

## Introduction

Although folk medicine is generally regarded as safe, neither the active component nor their mechanism of action is clearly understood. The black cumin (*Nigella sativa*) is one such spice that has been used in the treatment of a variety of illnesses, including bronchial asthma, headache, dysentery, infections, obesity, back pain, hypertension, gastrointestinal problems, and eczema (1). Several components of black cumin have been identified, including thymoquinone, thymol, thymohydroquinone, and dithymquinone (2, 3). Thymoquinone (TQ), the most abundant component of black seed oil, has been reported to exhibit antioxidant (4-6), antiinflammatory, and chemopreventive (7-9) effects. For instance, TQ has been shown to suppress the proliferation of various tumor cells, including colorectal carcinoma, breast adenocarcinoma, osteosarcoma, ovarian carcinoma, myeloblastic leukemia, and pancreatic carcinoma (7, 10-14), although it is minimally toxic to normal cells (15).

In animal models, TQ has been shown to suppress acetic acid-induced colitis in rats (16), inhibit tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production in murine septic peritonitis (17), and reduce carrageenan-induced paw edema in rats (18). TQ has also been reported to enhance antitumor activity of ifosfamide in Ehrlich ascites carcinoma-bearing mice (8), prevent cisplatin-induced nephrotoxicity in mice and rats (19), ameliorate benzo(a)pyrene-induced forestomach carcinogenesis (20), inhibit cyclooxygenase 2 (COX-2) expression and prostaglandin production in a mouse model of allergic airway inflammation (21), and protect against doxorubicin-induced cardiotoxicity in mice (22). How TQ manifests these activities is not fully understood, but it has been shown to down-regulate the expression of Bcl-xL (11), COX-2 (21), iNOS (23), 5-lipoxygenase (24), TNF (25), and cyclin D1 (26), all known to be regulated by nuclear factor- $\kappa$ B (NF- $\kappa$ B).

NF- $\kappa$ B is an ubiquitous transcription factor, consisting of p50, p65, and I $\kappa$ B $\alpha$ , that resides in the cytoplasm and is activated in response to various inflammatory stimuli, environmental pollutants, prooxidants, carcinogens, stress, and growth factors (27). On activation, NF- $\kappa$ B translocates from the cytoplasm to the nucleus, binds DNA, and causes gene transcription. Numerous kinases have been linked with activation of NF- $\kappa$ B, including I $\kappa$ B $\alpha$  kinase (IKK). Its activation has been shown to cause the expression of various

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gene products that regulate apoptosis, proliferation, chemoresistance, radioresistance, invasion, angiogenesis, metastasis, and inflammation (28, 29).

Because TQ has been shown to modulate cellular proliferation and inflammation, we postulated that it must mediate its effect through regulation of NF- $\kappa$ B pathway. Furthermore, various gene products known to be modulated by TQ are also regulated by NF- $\kappa$ B. Therefore, we investigated the effect of TQ in detail on NF- $\kappa$ B activation pathway and NF- $\kappa$ B-regulated cellular responses. We found that TQ suppressed the NF- $\kappa$ B activation pathway through modulation of the p65 subunit of NF- $\kappa$ B and inhibition of IKK; down-regulated the NF- $\kappa$ B-regulated gene products involved in cell survival, proliferation, and invasion; and potentiated apoptosis induced by TNF and chemotherapeutic agents.

## Results

We investigated the effect of TQ on NF- $\kappa$ B activation pathway induced by various carcinogens and inflammatory stimuli, NF- $\kappa$ B-regulated gene expression, and apoptosis induced by cytokines and chemotherapeutic agents. Most of our studies were done using human chronic myeloid leukemia cells (KBM-5) because these cells express both types of TNF receptors. Under the conditions that we used to examine the NF- $\kappa$ B pathway and NF- $\kappa$ B-regulated gene products, TQ had no effect on the viability of these cells (data not shown). The chemical structure of TQ is shown in (Fig. 1A).

### *TQ Suppresses NF- $\kappa$ B Activation in a Dose-Dependent and Time-Dependent Manner*

We first determined the optimum dose and time of exposure to TQ required to suppress TNF-induced NF- $\kappa$ B activation. To determine the dose response, cells were pretreated with different doses of TQ for 4 hours and then stimulated with 0.1 nmol/L TNF for 30 minutes. As indicated by electrophoretic mobility shift assay (EMSA), TQ suppressed TNF-induced NF- $\kappa$ B activation in a dose-dependent manner (Fig. 1B).

We next investigated whether suppression of TNF-induced NF- $\kappa$ B by TQ is time dependent. For this, we incubated the cells with 25  $\mu$ mol/L TQ for different times and then exposed the cells to 0.1 nmol/L TNF for 30 minutes. The EMSA results showed that TQ alone even for 6 hours did not activate NF- $\kappa$ B, but it abolished TNF-induced NF- $\kappa$ B activation almost maximally at 4 hours (Fig. 1C).

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

### *TQ Inhibits NF- $\kappa$ B Activation Induced by Carcinogens and Inflammatory Stimuli*

Phorbol myristate acetate, okadaic acid, lipopolysaccharide, and cigarette smoke condensate are potent activators of NF- $\kappa$ B,

but the pathways by which these agents activate NF- $\kappa$ B, however, differs (30-33). The effect of TQ on the activation of NF- $\kappa$ B by these agents was examined by EMSA. Pretreatment of cells with TQ completely suppressed the activation of NF- $\kappa$ B induced by all agents (Fig. 1E). These results clearly suggest that TQ acts at a step in the NF- $\kappa$ B activation pathway that must be common to all the agents.

### *TQ Inhibits TNF-Induced I $\kappa$ B $\alpha$ Degradation*

The translocation of NF- $\kappa$ B to the nucleus is preceded by the proteolytic degradation of I $\kappa$ B $\alpha$  (27). In our study, TNF induced I $\kappa$ B $\alpha$  degradation in control cells within 5 minutes and reached a maximum in 15 minutes, but in TQ-pretreated cells, TNF had no effect on I $\kappa$ B $\alpha$  (Fig. 2A). These results indicate that TQ prevents I $\kappa$ B $\alpha$  degradation by acting at a step upstream to I $\kappa$ B $\alpha$  degradation.

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

To determine whether the inhibition of TNF-induced I $\kappa$ B $\alpha$  degradation was due to inhibition of I $\kappa$ B $\alpha$  phosphorylation, we used the proteasome inhibitor *N*-acetyl-leucylleucyl-norleucinal to block degradation of I $\kappa$ B $\alpha$  (34). Cells were pretreated with TQ, treated with *N*-acetyl-leucylleucyl-norleucinal for 30 minutes, exposed to TNF, and then examined for I $\kappa$ B $\alpha$  phosphorylation status by Western blot analysis. TQ completely suppressed I $\kappa$ B $\alpha$  phosphorylation induced by TNF in the presence of the proteasome inhibitor (Fig. 2B).

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

Because TQ inhibits the phosphorylation of I $\kappa$ B $\alpha$ , we tested its effect on TNF-induced IKK activation, which is required for TNF-induced phosphorylation of I $\kappa$ B $\alpha$  (34). Results from the immune complex kinase assay showed that TNF activated IKK as early as 5 minutes after treatment and that TQ strongly suppressed this activation (Fig. 2C). Neither TNF nor TQ had any direct effect on the expression of either IKK- $\alpha$  or IKK- $\beta$  proteins.

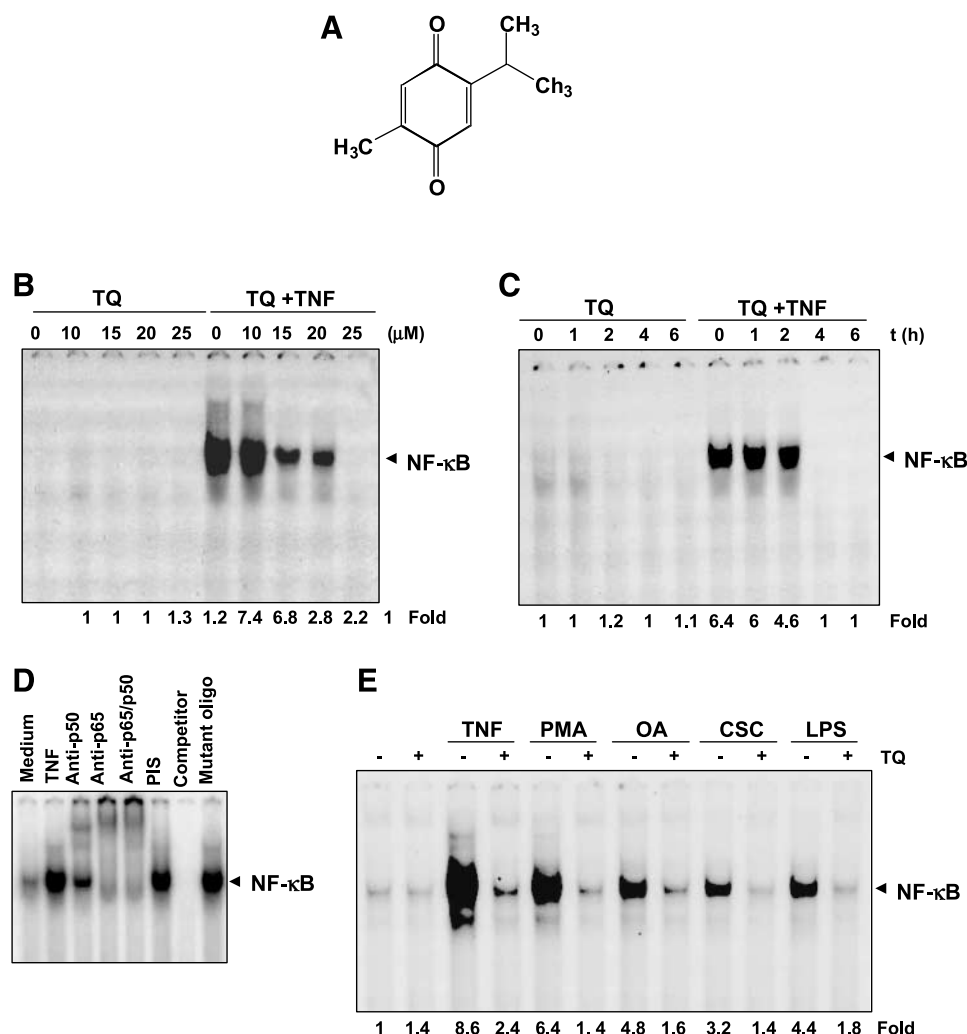
### *TQ Inhibits TNF-Induced Nuclear Translocation of p65*

Degradation of I $\kappa$ B $\alpha$  is known to cause the nuclear translocation of the p65 subunit of NF- $\kappa$ B (27). We examined whether TQ modulates TNF-induced nuclear translocation of p65. Western blot analysis showed that TNF induced nuclear translocation of p65 in a time-dependent manner in KBM-5 cells and that pretreatment with TQ inhibited it (Fig. 2D, middle).

TNF induces the phosphorylation of p65, which is required for its transcriptional activity (35). We examined whether TQ modulates TNF-induced phosphorylation of p65. Western blot analysis showed that TNF induced the phosphorylation of p65 within 15 min and that TQ strongly suppressed this phosphorylation (Fig. 2D, top).

### *TQ Directly Interferes with the Binding of NF- $\kappa$ B to the DNA*

Results from our laboratory and others have shown that certain NF- $\kappa$ B inhibitors suppress NF- $\kappa$ B activation by directly interacting with NF- $\kappa$ B proteins and thus suppressing the binding to the DNA (36-38). We determined whether TQ



**FIGURE 1.** **A.** The chemical structure of TQ. **B.** TQ blocks NF- $\kappa$ B activation induced by TNF in a dose-dependent manner. KBM-5 cells were incubated with the indicated concentrations of TQ for 4 h and treated with 0.1 nmol/L TNF for 30 min. The nuclear extracts were assayed for NF- $\kappa$ B activation by EMSA. **C.** Effect of time duration. KBM-5 cells were preincubated with 25  $\mu$ M TQ for the indicated times and then treated with 0.1 nmol/L TNF for 30 min. The nuclear extracts were prepared and assayed for NF- $\kappa$ B activation by EMSA. **D.** NF- $\kappa$ B induced by TNF is composed of p65 and p50 subunits. Nuclear extracts from untreated cells or cells treated with 0.1 nmol/L TNF were incubated with the indicated antibodies, an unlabeled NF- $\kappa$ B oligo probe, or a mutant oligo probe. They were then assayed for NF- $\kappa$ B activation by EMSA. **E.** TQ blocks NF- $\kappa$ B activation induced by TNF, phorbol 12-myristate 13-acetate (PMA), okadaic acid, lipopolysaccharide (LPS), and cigarette smoke condensate. Human myeloid leukemia KBM-5 cells were preincubated with 25  $\mu$ M TQ for 4 h and then treated with 0.1 nmol/L TNF for 30 min, 25 ng/mL phorbol 12-myristate 13-acetate for 2 h, 50 nmol/L okadaic acid for 4 h, 10  $\mu$ g/mL lipopolysaccharide for 1 h, and 10  $\mu$ g/mL cigarette smoke condensate for 1 h. Nuclear extracts were analyzed for NF- $\kappa$ B activation, as described in Materials and Methods. Representative of three independent experiments.

mediates suppression of NF- $\kappa$ B activation through a similar mechanism. To determine this, we incubated nuclear extracts from TNF-treated cells with TQ. The EMSA results showed that TQ significantly inhibited NF- $\kappa$ B binding to the DNA (Fig. 3A). Thus, it is possible that TQ inhibits the binding of NF- $\kappa$ B to the DNA through modification of NF- $\kappa$ B proteins. We found that indeed coinubation of nuclear extracts with TQ in the presence of the DTT reversed the effect of TQ completely (Fig. 3B).

We also investigated whether DTT could reverse the NF- $\kappa$ B-suppressing effect of TQ in intact cells. The results in Fig. 3C showed that TQ induced the inhibition of TNF-induced NF- $\kappa$ B activation and that DTT completely reversed the effect of TQ. To determine whether TQ targets the p65

subunit of NF- $\kappa$ B, we overexpressed p65 by transfecting p65-containing plasmid into A293 cells. We then prepared nuclear extracts and treated them with TQ in the presence or absence of DTT. The recombinant p65 subunit bound to the DNA and TQ treatment resulted in suppression of binding. Like to endogenous p65, DTT significantly reversed the effect of TQ on ectopically expressed p65 (Fig. 3D).

It has been reported that the cysteine residue located at position 38 in p65 is highly susceptible to various agents (38-40). Whether Cys<sup>38</sup> is a target for TQ was investigated. Therefore, we used p65 plasmid with Cys<sup>38</sup> mutated to serine residue. A293 cells were transiently transfected with pcDNA3.1 or pcDNA expression vectors for wild-type p65 or p65C38S for 48 hours, prepared the nuclear extracts, treated with TQ for

30 minutes, and measured the DNA binding by EMSA. These results show that TQ modifies the DNA binding of wild-type p65 but not the mutated p65 (Fig. 3E). Thus, these results show that Cys<sup>38</sup> in p65 is one of the targets of TQ.

#### TQ Represses TNF-Induced NF- $\kappa$ B-Dependent Reporter Gene Expression

Although we determined by EMSA that TQ inhibits NF- $\kappa$ B activation, DNA binding alone is not always associated with NF- $\kappa$ B-dependent gene transcription, suggesting that additional regulatory steps are involved (41). We examined whether TQ modulates TNF-induced transcription of the NF- $\kappa$ B reporter activity. The results showed that TNF induced the NF- $\kappa$ B reporter activity and that TQ inhibited TNF-induced NF- $\kappa$ B reporter activity in a dose-dependent manner (Fig. 4A).

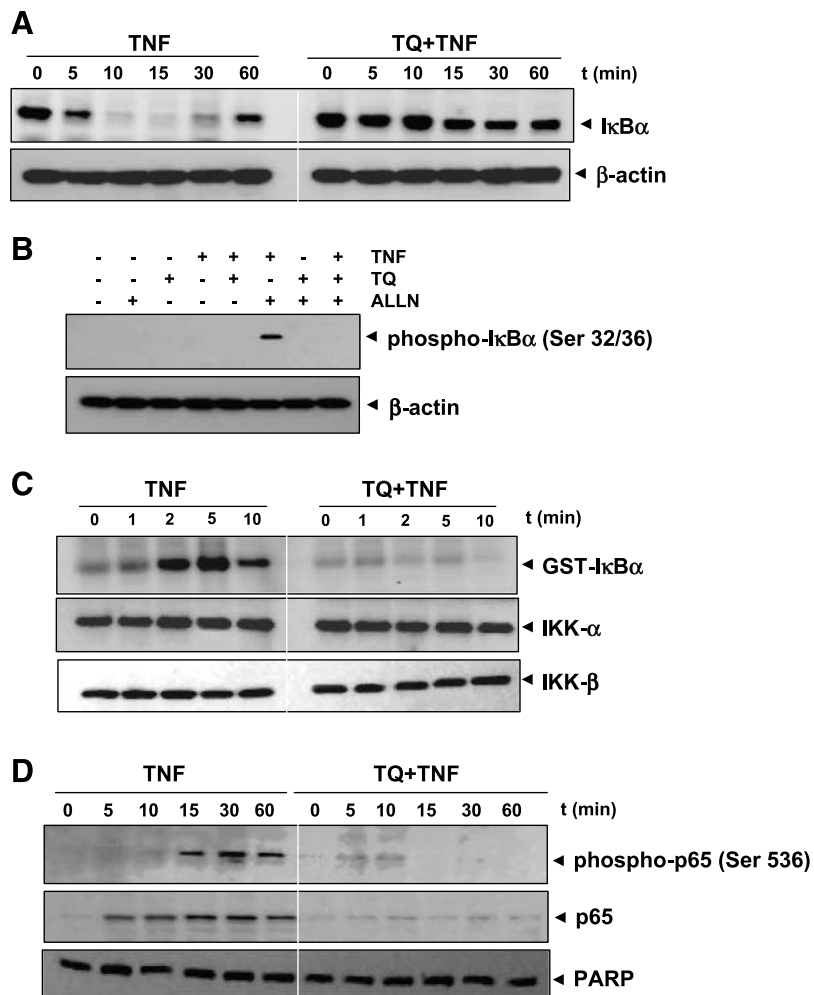
We next investigated where TQ acts in the sequence of TNFR1, TRADD, TRAF2, NIK, TAK1/TAB1, and IKK- $\beta$  recruitment that characterizes TNF-induced NF- $\kappa$ B activation (42). In cells transfected with TNFR1, TRADD, TRAF2, NIK, TAK1/TAB1, IKK- $\beta$ , and p65 plasmids, NF- $\kappa$ B-dependent secretory alkaline phosphatase (SEAP) expression was induced; TQ substantially suppressed NF- $\kappa$ B-dependent SEAP expression in all cases (Fig. 4B).

#### TQ Represses the Expression of TNF-Induced NF- $\kappa$ B-Dependent Antiapoptotic Gene Products

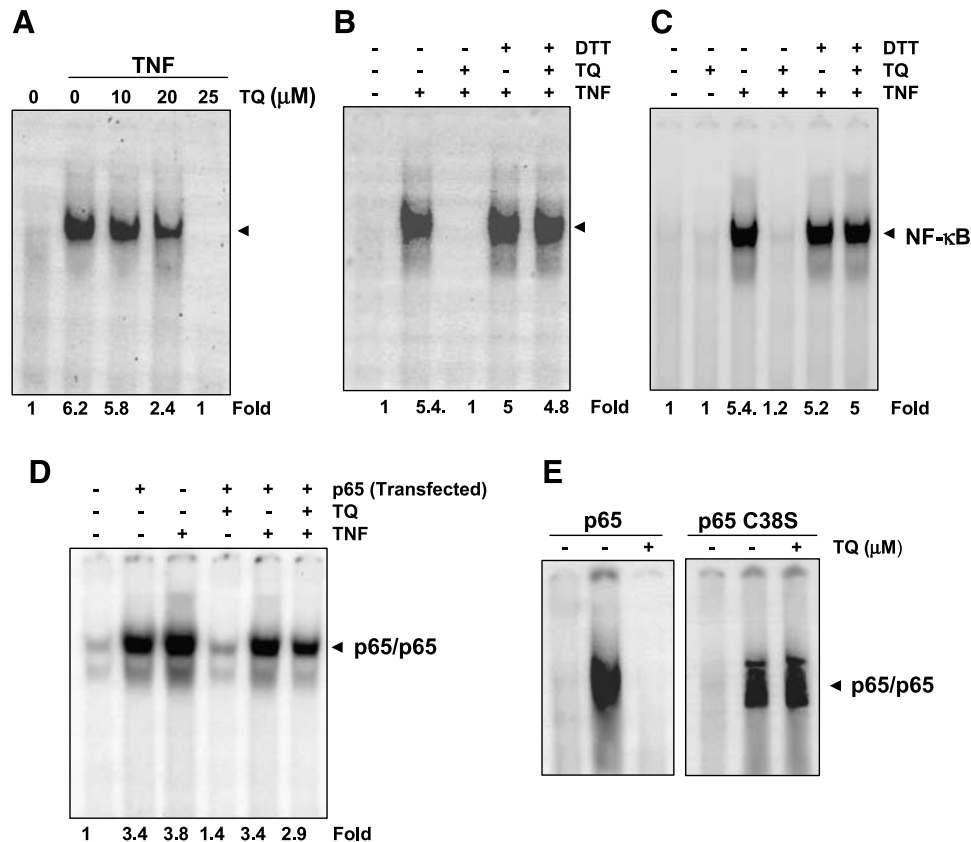
It is possible that potentiation of TNF-induced apoptosis is through suppression of NF- $\kappa$ B-regulated antiapoptotic gene products. NF- $\kappa$ B regulates the expression of the antiapoptotic proteins IAP1/IAP2 (43, 44), XIAP (45), Bcl-2 (46), Bcl-xL (47), and survivin (48). Whether TQ could modulate TNF-induced expression of these antiapoptotic gene products was investigated. The results of Western blot analysis showed that TNF induced expression of these antiapoptotic proteins in a time-dependent manner and that TQ suppressed it (Fig. 5A).

#### TQ Suppresses TNF-Induced NF- $\kappa$ B-Dependent Gene Products Involved in Cell Proliferation

Numerous gene products that mediate cellular proliferation, such as cyclin D1, COX-2, and c-Myc, have NF- $\kappa$ B-binding sites in their promoters (49-51). We investigated whether the expression of these gene products is modulated by TQ. Cells were pretreated with TQ for 4 hours and then treated with TNF for the indicated times. Western blot analysis indicated that TQ suppressed the expression of these proteins (Fig. 5B). The results provide further evidence of the role of TQ in blocking cellular proliferation through suppression of NF- $\kappa$ B-regulated gene products.



**FIGURE 2.** A. TQ inhibits TNF-induced degradation of IκBα. KBM-5 cells were incubated with 25 μmol/L TQ for 4 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-IκBα. Representative of two or three independent experiments. Equal protein loading was evaluated by β-actin (bottom). B. TQ blocks the phosphorylation of IκBα by TNF. Cells were preincubated with 25 μmol/L TQ for 4 h, incubated with 50 μg/mL N-acetyl-leucylleucyl-norleucinal 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 phosphorylated-specific anti-IκBα antibody. The same membrane was reblotted with β-actin antibody. C. The effect of TQ on the activation of IKK by TNF was investigated. KBM-5 cells were incubated with 25 μmol/L TQ for 4 h, incubated with 50 μg/mL N-acetyl-leucylleucyl-norleucinal for 30 min, and then treated with 0.1 nmol/L TNF for different time intervals. Whole-cell extracts were prepared, and extracts were immunoprecipitated with antibodies against IKK-α and IKK-β. Thereafter, the immune complex kinase assay was done as described in Materials and Methods. To examine the effect of TQ on the level of expression of IKK proteins, whole-cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-IKK-α and anti-IKK-β antibodies. The results shown are representative of three independent experiments. D. TQ inhibits TNF-induced nuclear translocation and phosphorylation of p65. KBM-5 cells were either untreated or were pretreated with 25 μmol/L TQ for 4 h at 37°C 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 phosphorylated-specific p65 and anti-p65. Representative of two or three independent experiments.



**FIGURE 3.** Direct effect of TQ on the NF- $\kappa$ B complex. **A.** TQ inhibits direct binding of NF- $\kappa$ B to DNA. Nuclear extracts were prepared from untreated KMB-5 cells or cells treated with 0.1 nmol/L TNF for 30 min, incubated with indicated concentrations of TQ for 30 min, and then assayed for NF- $\kappa$ B activation by EMSA. **B.** Nuclear extracts were prepared from KMB-5 cells untreated or treated with 0.1 nmol/L TNF for 30 min, incubated for 30 min with 25  $\mu$ mol/L TQ in the presence or absence of 100  $\mu$ mol/L DTT, and then assayed for NF- $\kappa$ B binding to DNA by EMSA. **C.** DTT inhibits TQ-mediated suppression of NF- $\kappa$ B induced by TNF in cells. KMB-5 cells were pretreated with 25  $\mu$ mol/L TQ with or without 100  $\mu$ mol/L DTT for 4 h and then incubated with 0.1 nmol/L TNF for 30 min. Nuclear extracts were then prepared and assayed for NF- $\kappa$ B activation by EMSA. **D.** DTT inhibits TQ-mediated suppression of overexpressed p65 *in vitro* in A293 cells. Nuclear extracts from overexpressed p65 cells were incubated with 25  $\mu$ mol/L TQ with or without 100  $\mu$ mol/L DTT for 30 min and then assayed for NF- $\kappa$ B binding to DNA by EMSA. **E.** TQ suppressed overexpressed wild-type p65 but not mutated p65C38S *in vitro* in A293 cells. A293 cells were transfected with an expression vector for mouse p65 or mutant p65C38S, and then EMSA was done on nuclear extracts treated with 25  $\mu$ mol/L TQ.

#### TQ Suppresses TNF-Induced NF- $\kappa$ B-Dependent Gene Products Involved in Invasion and Angiogenesis

The gene products that have been linked with invasion and angiogenesis [matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF)] are regulated by NF- $\kappa$ B (52, 53). We determined whether the expression of these gene products is modulated by TQ. Western blot analysis indicated that TNF induced these gene products and that TQ suppressed the expression (Fig. 5C). The results provide evidence for the role of TQ in blocking invasion and angiogenesis.

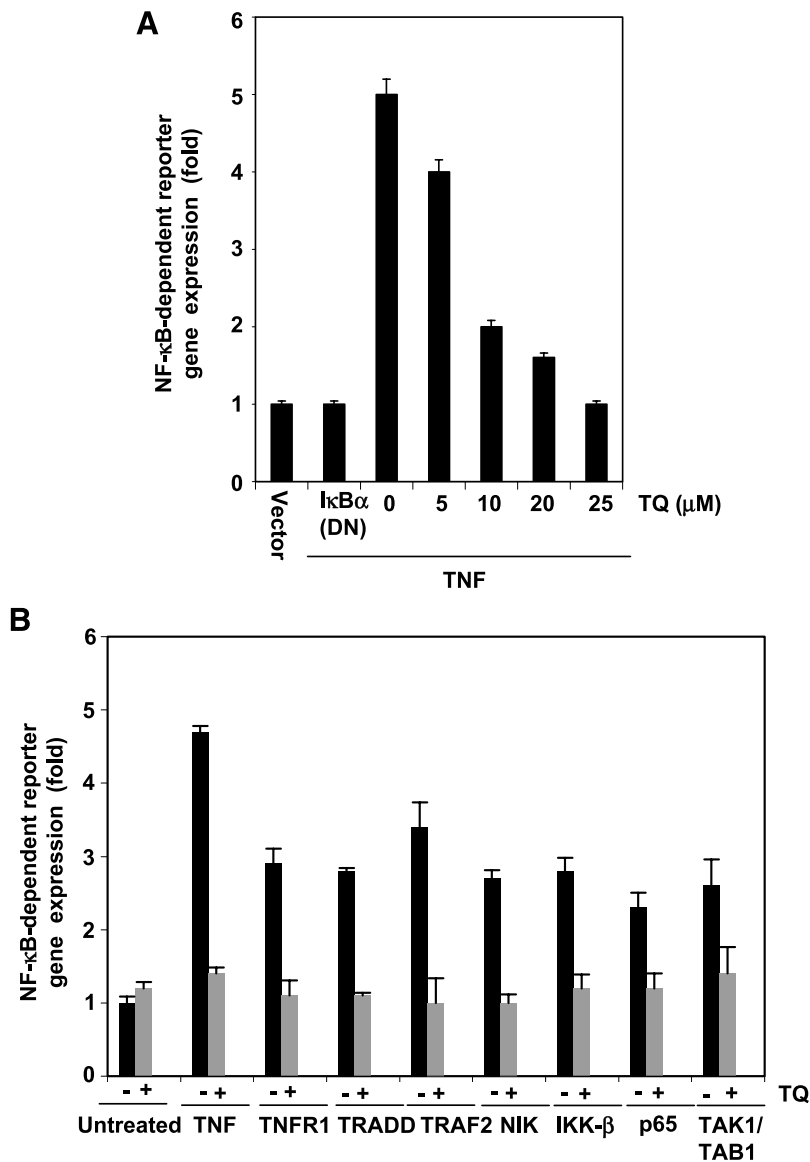
#### TQ Potentiates Apoptosis Induced by TNF and Chemotherapeutic Agents

NF- $\kappa$ B activation has been shown to suppress apoptosis induced by TNF and chemotherapeutic agents through the expression of gene products regulated by NF- $\kappa$ B (54). We investigated whether TQ modulates the cytotoxic effects of TNF, paclitaxel, and doxorubicin. The effect of TQ on TNF-induced and chemotherapeutic agent-induced apoptosis was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. We found that TQ significantly enhanced the cytotoxic effects of TNF, paclitaxel, and doxorubicin (Fig. 6A). The Live/Dead assay, which measures intracellular esterase activity and plasma membrane integrity, further indicated that TQ up-regulates TNF-induced apoptosis from 8% to 68% (Fig. 6B1) and ST1571-induced apoptosis from 18% to 76% (Fig. 6B2). The results of Annexin V staining, which examines early apoptosis, also showed that TNF-induced apoptosis was enhanced by incubation with TQ (Fig. 6C). When examined for caspase-mediated poly(ADP-ribose) polymerase (PARP) cleavage, we found that the TQ enhanced apoptosis induced by TNF (Fig. 6D). Together, these results support the conclusion that TQ potentiates the apoptotic effect of TNF and chemotherapeutic agents.

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#### Discussion

We investigated the molecular mechanism by which TQ mediates its antiproliferative, antiinflammatory, and chemopreventive effects. We found that TQ suppressed the NF- $\kappa$ B activation induced by various carcinogens and inflammatory



**FIGURE 4. A.** TQ inhibits TNF-induced NF- $\kappa$ B-dependent reporter gene (SEAP) expression. A293 cells were transiently transfected with an NF- $\kappa$ B-containing plasmid linked to the SEAP gene and then treated with the indicated concentrations of TQ. After 24 h in culture with 1 nmol/L 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.** TQ inhibits NF- $\kappa$ B-dependent reporter gene expression induced by TNFR, TRADD, TRAF, NIK, IKK- $\beta$ , p65, and TAK1/TAB1. A293 cells were transiently transfected with the indicated plasmids along with an NF- $\kappa$ B-containing plasmid linked to the SEAP gene and then left either untreated or treated with 25  $\mu$ M/L TQ for 4 h. Cell supernatants were assayed for secreted alkaline phosphatase activity as described in Materials and Methods. Results are expressed as fold activity over the activity of the vector control. Bars, SD.

agents and that the inhibition of NF- $\kappa$ B was due to the inhibition of IKK activation, leading to the suppression of I $\kappa$ B $\alpha$  phosphorylation and degradation. Suppression of NF- $\kappa$ B activation by TQ is due to inhibition of TNF-induced IKK activation, which led to inhibition of I $\kappa$ B $\alpha$  phosphorylation and degradation, suppression of p65 phosphorylation, and translocation to the nucleus. Apart from this, TQ also inhibited the binding of the p65 subunit of NF- $\kappa$ B to the DNA and reporter gene expression. TQ also down-regulated NF- $\kappa$ B-dependent gene products involved in antiapoptosis, proliferation, invasion, and angiogenesis. This down-regulation led to the potentiation of apoptosis induced by cytokines and chemotherapeutic agents (Fig. 7).

This is the first report to suggest that TQ could suppress NF- $\kappa$ B activation induced by a variety of inflammatory stimuli and carcinogens. Because NF- $\kappa$ B activation induced by diverse stimuli was inhibited, this suggests that TQ must act at a step in the NF- $\kappa$ B activation pathway common to all NF- $\kappa$ B inducers.

TQ has been shown to inhibit NF- $\kappa$ B activation in a rat model of brain and spinal cord experimental autoimmune encephalomyelitis (55) and induced by lipopolysaccharide in mast cells (56) and advanced glycation end products in proximal tubular epithelial cells (57); however, none of these reports described the mechanism by which TQ suppresses NF- $\kappa$ B activation. We investigated in detail how TQ inhibits the NF- $\kappa$ B activation pathway. We found that TQ acts at two different steps in the NF- $\kappa$ B signaling pathway: firstly, through its direct interaction with the p65 subunit of NF- $\kappa$ B and, secondly, through its ability to suppress TNF-induced IKK activation. The observation that TQ could inhibit the binding of NF- $\kappa$ B to the DNA suggest that it is modifying the NF- $\kappa$ B protein, because the binding of recombinant p65 subunit of NF- $\kappa$ B, which has the transactivation domain, was suppressed, suggesting that TQ directly targets the p65 subunit. These results are consistent with our findings that TQ also suppressed the p65-induced NF- $\kappa$ B reporter activity. Because the effects of

TQ could be reversed by reducing agent, it is possible that this agent modifies a cysteine residue in p65. These results are consistent with those previously reported from our laboratory and others with caffeic acid phenethyl ester (37), plumbagin (38), and sesquiterpene lactone parthenolide (40). A Cys<sup>38</sup> residue has been identified in p65 subunits of NF- $\kappa$ B that is crucial for DNA binding (40). Our results indicate that when this Cys<sup>38</sup> was replaced by serine in p65, TQ failed to inhibit the DNA binding ability of p65. Thus, our results indicate that TQ exerts its inhibitory effect by modifying this specific cysteine residue.

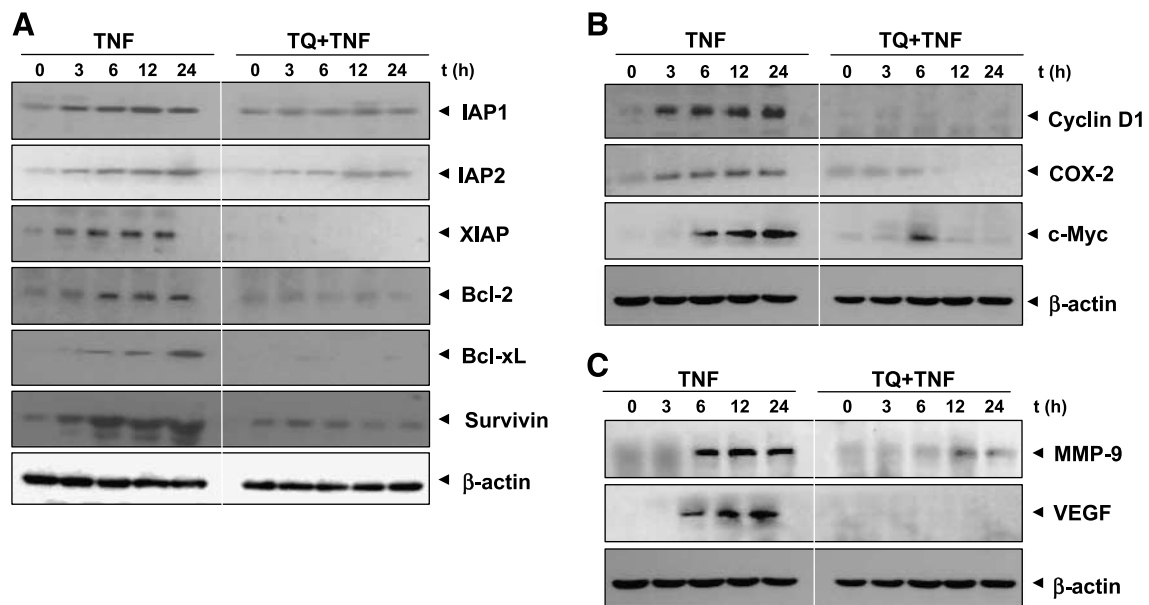
We found that, in addition to its effects on p65, TQ also inhibited TNF-induced IKK activation, which leads to inhibition of I $\kappa$ B $\alpha$  phosphorylation and degradation. How TQ suppresses IKK activation was also investigated. We found that TQ is not a direct inhibitor of IKK (data not shown). Several kinases, such as MAPK kinase kinase 1 (58), MAPK kinase kinase 3 (59), PKC (60); glycogen synthase kinase-3 $\beta$  (61), TAK1 (62), PDK1 (63), and Akt (64), have all been reported to function upstream of IKK. Recent studies, however, indicate that TAK1 plays a major role in TNF-induced NF- $\kappa$ B activation through its interaction with TAB1 and TAB2. For example, TAK1 can bind and activate IKK- $\beta$ , leading to NF- $\kappa$ B activation (65). Indeed, our study showed for the first time that TAK1-induced NF- $\kappa$ B activation is inhibited by TQ.

TQ has been shown to suppress carcinogenesis (15, 66). Our results show that TQ inhibits the expression of COX-2,

MMP-9, cyclin D1, VEGF, and c-Myc, all regulated by NF- $\kappa$ B. These results are in agreement with previous reports that TQ can suppress COX-2 expression both *in vitro* and in a mouse model of allergic airway inflammation (21, 67). NF- $\kappa$ B activation has been shown to mediate the suppression of apoptosis through the expression of several antiapoptotic gene products. We found that TQ down-regulated the expression of cIAP1/2, XIAP, Bcl-2, Bcl-x<sub>L</sub>, and survivin. Gali-Muhtasib et al. (7) indeed showed that TQ treatment resulted in a marked inhibition of the antiapoptotic Bcl-2 protein in HCT-116 cells.

We also found that TQ potentiated the apoptotic effects of cytokines and chemotherapeutic agents by down-regulating the NF- $\kappa$ B-dependent apoptosis gene products, including cIAP1/cIAP2, XIAP, Bcl-2, Bcl-x<sub>L</sub>, and survivin. It is possible that suppression of various other tumor cell lines previously reported (7, 10-14) is also through antiapoptotic and proliferative gene products, as described here. In animal models, TQ has been shown to suppress acetic acid-induced colitis in rats (16), inhibit TNF production in murine septic peritonitis (17), and reduce carrageenan-induced paw edema in rats (18). It is quite likely that several of these effects of TQ are mediated through the suppression of NF- $\kappa$ B activation.

Overall, our results show that TQ clearly inhibits NF- $\kappa$ B activation, which makes it a potentially effective suppressor of inflammation, tumor cell survival, proliferation, invasion, and angiogenesis. Further studies on TQ may provide important leads for potential treatment applications in cancer and other diseases.



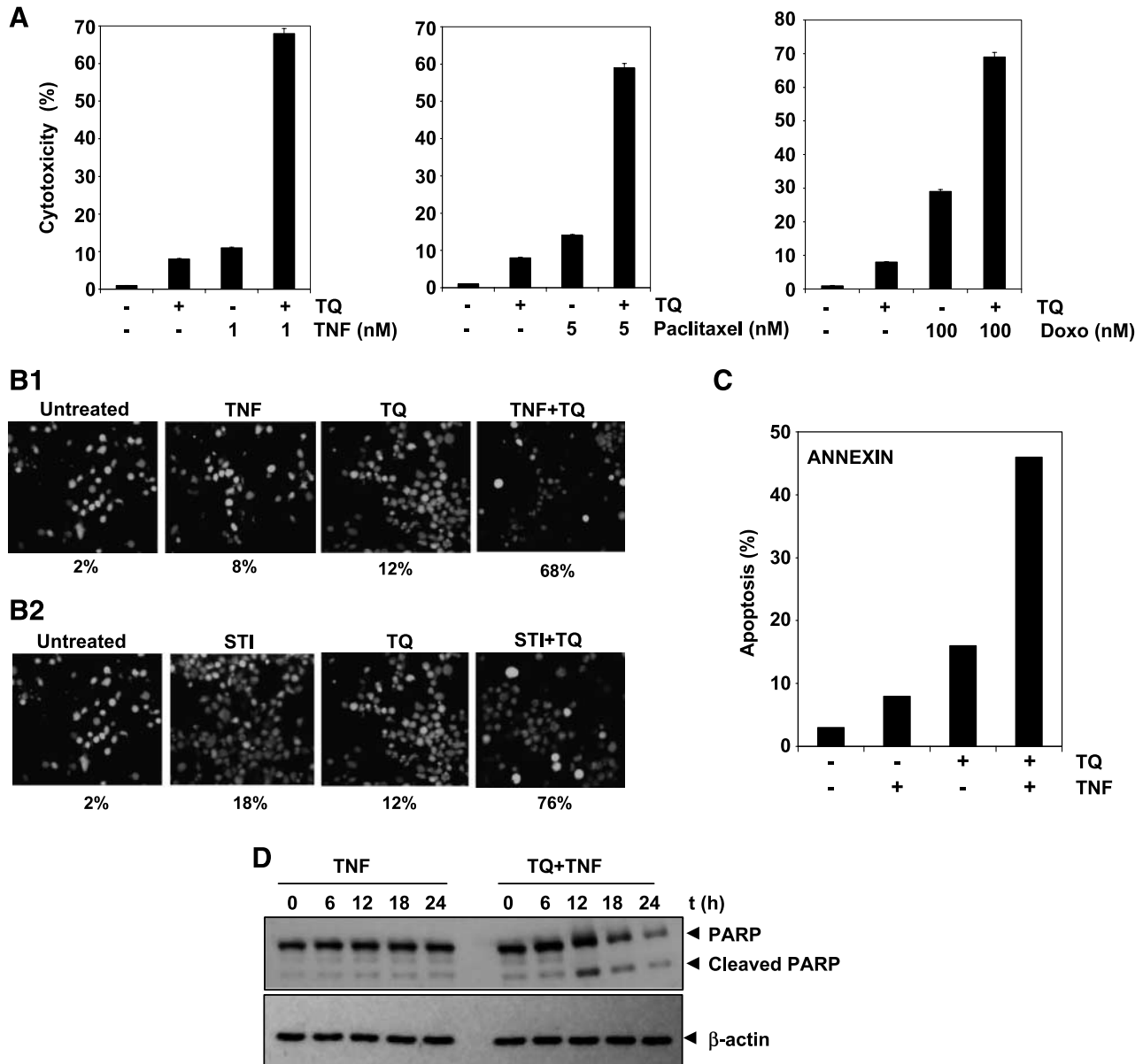
**FIGURE 5.** TQ inhibits TNF-induced NF- $\kappa$ B-regulated gene products. **A.** TQ inhibits the expression of antiapoptotic gene products. KBM-5 cells were left untreated or were incubated with 5  $\mu$ mol/L TQ for 4 h and then treated with 1 nmol/L TNF for different amounts of time. Whole-cell extracts were prepared and 30  $\mu$ g of the whole-cell lysate were analyzed by Western blotting using antibodies against IAP1, IAP2, XIAP, Bcl-2, Bcl-x<sub>L</sub>, and survivin as indicated. **B.** TQ suppresses COX-2, cyclin D1, and c-Myc expression induced by TNF. KBM-5 cells were left untreated or were incubated with 5  $\mu$ mol/L TQ for 4 h and then treated with 1 nmol/L TNF for different times. Whole-cell extracts were prepared and 30  $\mu$ g of the whole-cell lysate was analyzed by Western blot analysis using antibodies against COX-2, cyclin D1, and c-Myc. **C.** TQ inhibits MMP-9 and VEGF expression induced by TNF. KBM-5 cells were left untreated or were incubated with 5  $\mu$ mol/L TQ for 4 h and then treated with 1 nmol/L TNF for different times. Whole-cell extracts were prepared and 30  $\mu$ g of the whole-cell lysate were analyzed by Western blot analysis using antibodies against MMP-9 and VEGF. Data are for a representative experiment of three independent experiments showing similar results.

## Materials and Methods

### Reagents

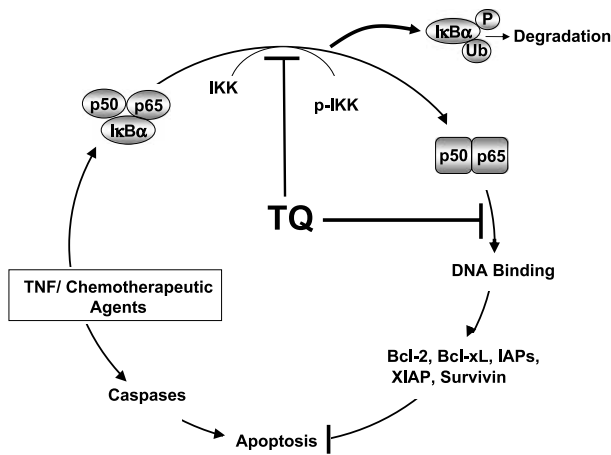
A 50 mmol/L solution each of TQ (molecular weight, 164.2; Sigma-Aldrich) was prepared in 100% DMSO, stored as small aliquots at  $-20^{\circ}\text{C}$ , and then diluted as needed in cell culture medium. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of  $5 \times 10^7$  units/mg, was

kindly provided by Genentech. Cigarette smoke condensate, prepared as previously described (30), was kindly supplied by Dr. C. Gary Gairola (University of Kentucky). Paclitaxel, doxorubicin, phorbol 12-myristate 13-acetate, okadaic acid, lipopolysaccharide, DTT, and  $\beta$ -actin antibody were obtained from Sigma-Aldrich. *N*-acetyl-leucylleucyl-norleucinal was purchased from EMD Biosciences, Inc. Antibodies against



**FIGURE 6.** A. TQ enhances TNF-induced and chemotherapeutic agents–induced cytotoxicity. In total, 10,000 cells were seeded in triplicate in 96-well plates. The cells were pretreated with 5  $\mu\text{mol/L}$  TQ and then incubated with the indicated concentrations of TNF, paclitaxel, and doxorubicin for 24 h. Cell viability was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method as described in Materials and Methods. **B1** and **B2**. TQ potentiates TNF and STI571-induced apoptosis. KBM-5 cells were pretreated with 5  $\mu\text{mol/L}$  TQ for 4 h and then incubated with 1 nmol/L TNF and/or 5  $\mu\text{mol/L}$  STI-571 for 16 h. The cells were stained with a Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described in Materials and Methods. Representative of three independent experiments. **C**. Cells were pretreated with 5  $\mu\text{mol/L}$  TQ for 4 h and then incubated with 1 nmol/L TNF for 16 h. The cells were incubated with an FITC-conjugated Annexin V antibody and then analyzed by flow cytometry as described in Materials and Methods. Representative of two independent experiments. **D**. Cells were pretreated with 5  $\mu\text{mol/L}$  TQ for 4 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-PARP antibody. Representative of three independent experiments.





**FIGURE 7.** The proposed mechanism by which TQ inhibits NF- $\kappa$ B activation and NF- $\kappa$ B-regulated gene expression involved in cell survival and proliferation.

p65, p50, I $\kappa$ B $\alpha$ , cyclin D1, c-Myc, MMP-9, PARP, IAP1, IAP2, Bcl-2, and Bcl-xL and the Annexin V staining kit were obtained from Santa Cruz Biotechnology. Anti-COX-2 and anti-XIAP antibodies were obtained from BD Biosciences. Phosphorylated-specific anti-I $\kappa$ B $\alpha$  (Ser<sup>32/36</sup>) and phosphorylated-specific anti-p65 (Ser<sup>536</sup>) antibodies were purchased from Cell Signaling. Anti-IKK- $\alpha$  and anti-IKK- $\beta$  antibodies were kindly provided by Imgenex. Anti-VEGF was purchased from NeoMarkers. Survivin antibody was purchased from R&D System. STI571 (an active chemical component of imatinib mesylate) was kindly provided by Dr. Nicholas J. Donato, Department of Experimental Therapeutics, University of Texas M. D. Anderson Cancer Center.

#### Cell Lines

Human myeloid KBM-5 cells and human embryonic kidney A293 cells were obtained from American Type Culture Collection. KBM-5 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 15% fetal bovine serum, and A293 cells were cultured in DMEM supplemented with 10% fetal bovine serum. All media were also supplemented with 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin.

#### EMSA

To determine NF- $\kappa$ B activation by TNF, we did EMSA essentially as described previously (68). Briefly, nuclear extracts prepared from TNF-treated cells ( $2 \times 10^6$ /mL) were incubated with <sup>32</sup>P-end-labeled 45-mer double-stranded oligonucleotide (15  $\mu$ g of protein with 16 fmol of DNA) from the HIV long terminal repeat, 5'-TTGTTACAA **GGGACTTTC** CGCTG **GGGACTTTC** CAGGGAGGCGTGG-3' (boldface indicates NF- $\kappa$ B binding sites), for 30 min at 37°C. The DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'-TTGTTACAA CACACTTTC CGCTG CTCACCTTC CAGGGAGGCGTGG-3', was used to examine binding specificity of NF- $\kappa$ B to the DNA. The binding

specificity was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either the p50 or the p65 subunit of NF- $\kappa$ B for 30 min at 37°C before the complex was analyzed by EMSA. Preimmune serum was included as a negative control. The dried gels were visualized with a Storm820, and radioactive bands were quantified using Imagequant software (Amersham Pharmacia Biotechnology).

#### Western Blot Analysis

To determine the effect of TQ on TNF-dependent I $\kappa$ B $\alpha$  phosphorylation and I $\kappa$ B $\alpha$  degradation, p65 phosphorylation, and nuclear translocation, we prepared cytoplasmic and nuclear extracts as previously described (69) and probed them with specific antibodies against I $\kappa$ B $\alpha$ , phosphorylated I $\kappa$ B $\alpha$ , phosphorylated p65, and p65. To determine the expression of cyclin D1, COX-2, MMP-9, IAP1, IAP2, Bcl-2, Bcl-X<sub>L</sub>, VEGF, and survivin whole-cell extracts from TNF-treated and TQ plus TNF-treated cells were prepared, and 30  $\mu$ g of protein were resolved on SDS-PAGE and probed with specific antibodies according to the manufacturer's recommended protocol. The blots were washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h, and finally detected by enhanced chemiluminescence reagent (Amersham Pharmacia Biotechnology). The bands were quantified with a Personal Densitometer Scan v1.30 using Imagequant software version 3.3 (Molecular Dynamics).

#### IKK Assay

To determine the effect of TQ on TNF-induced IKK activation, we analyzed IKK essentially, as described previously (70). Briefly, the IKK complex from whole-cell extracts was precipitated with IKK- $\alpha$  and IKK- $\beta$  and treated with protein A/G-agarose beads (Pierce Chemical). After 2 h, the beads were washed with lysis buffer and resuspended in a kinase assay mixture containing 50 mmol/L HEPES (pH 7.4), 20 mmol/L MgCl<sub>2</sub>, 2 mmol/L DTT, 20  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP, 10  $\mu$ mol/L unlabeled ATP, and 2  $\mu$ g of substrate glutathione S-transferase-I $\kappa$ B $\alpha$  (amino acids 1-54) and incubated at 30°C for 30 min. The reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a Storm 820. To determine the total amounts of IKK- $\alpha$  and IKK- $\beta$  in each sample, 30  $\mu$ g of whole-cell proteins were resolved on 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK- $\alpha$  or anti-IKK- $\beta$  antibodies.

#### NF- $\kappa$ B-Dependent Reporter Gene Transcription

To determine the effect of TQ on TNF-induced, TNFR-induced, TRADD-induced, TRAF2-induced, NIK-induced, TAK1/TAB1-induced, IKK- $\beta$ -induced, and p65-induced NF- $\kappa$ B-dependent reporter gene transcription, we did the SEAP assay as previously described (71). Briefly, A293 cells ( $5 \times 10^5$  per well) were plated in six-well plates and transiently transfected by the calcium phosphate method with pNF-B-SEAP (0.5  $\mu$ g). To examine TNF-induced reporter gene expression,

we transfected the cells with 0.5  $\mu\text{g}$  of the SEAP expression plasmid and 1.5  $\mu\text{g}$  of the control plasmid pCMV-FLAG1 DNA for 24 h. We then treated the cells for 4 h with TQ and stimulated them with 1 nmol/L TNF. The cell culture medium was harvested after 24 h of TNF treatment. To examine reporter gene expression induced by various genes, A293 cells were transfected with 0.5  $\mu\text{g}$  of pNF- $\kappa$ B-SEAP plasmid with 0.5  $\mu\text{g}$  of an expressing plasmid and 1.5  $\mu\text{g}$  of the control plasmid pCMV-FLAG1 for 24 h, treated with TQ, and then harvested from cell culture medium after an additional 24 h of incubation. The culture medium was analyzed for SEAP activity as recommended by the manufacturer (Clontech) using a Victor 3 microplate reader (Perkin-Elmer Life Sciences).

#### Plasmids

pcDNA3.1 and pcDNA expression vectors for mouse p65 and mouse p65C38S were kindly provided by Dr. T.D. Gilmore from Boston University.

#### Cytotoxicity Assay

Cytotoxicity was assayed by the modified tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, as described previously (72).

#### Live/Dead Assay

To measure apoptosis, we used the Live/Dead cell viability assay (Molecular Probes), which determines intracellular esterase activity and plasma membrane integrity (73). Calcein-AM, a nonfluorescent polyanionic dye, is retained by live cells, in which it produces intense green fluorescence through enzymatic (esterase) conversion. In addition, the ethidium homodimer enters cells with damaged membranes and binds to nucleic acids, thereby producing a bright red fluorescence in dead cells. Briefly,  $2 \times 10^5$  cells were incubated with 5  $\mu\text{mol/L}$  TQ for 4 h and treated with 1 nmol/L TNF/5  $\mu\text{mol/L}$  STI-571 up to 16 h at 37°C. Cells were stained with the Live/Dead reagent (5  $\mu\text{mol/L}$  ethidium homodimer and 5  $\mu\text{mol/L}$  calcein-AM) and incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon).

#### PARP Cleavage Assay

To detect cleavage of PARP, we prepared whole-cell extracts by subjecting TQ-treated cells to lysis in lysis buffer [20 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% NP40, 0.01  $\mu\text{g/mL}$  aprotinin, 0.005  $\mu\text{g/mL}$  leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride, and 4 mmol/L sodium orthovanadate]. Lysates were spun at 14,000 rpm for 10 min to remove insoluble material, resolved by 10% SDS-PAGE, and probed with PARP antibody.

#### Annexin V Assay

Annexin V assay was done as described previously (72). An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface of membrane to the extracellular surface. This loss of membrane asymmetry can be detected by using the binding properties of Annexin V. To identify apoptosis, we used an Annexin V antibody, which was

conjugated with the FITC fluorescence dye. Briefly,  $5 \times 10^5$  cells were pretreated with TQ, treated with TNF for 16 h at 37°C, and subjected to Annexin V staining. The cells were washed in PBS, resuspended in 100  $\mu\text{L}$  of binding buffer containing a FITC-conjugated anti-Annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur, BD).

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Editor's Note: Targeting Nuclear Factor- $\kappa$ B Activation Pathway by Thymoquinone: Role in Suppression of Antiapoptotic Gene Products and Enhancement of Apoptosis



The *Molecular Cancer Research* editors are publishing this note to alert readers to a concern about this article (1). An internal journal investigation determined that the same  $\beta$ -actin immunoblots were used to represent loading controls in Fig. 5B and C. In addition, an institutional investigation determined that the same microscopy image was used to represent untreated cells in different experimental conditions in Fig. 6B1 and B2. Original research records related to this concern were not available during the institutional investigation. Therefore, the institution was not able to determine the relation of the images to the experiments described.

### Reference

1. Sethi G, Ahn KS, Aggarwal BB. Targeting nuclear factor- $\kappa$ B activation pathway by thymoquinone: role in suppression of antiapoptotic gene products and enhancement of apoptosis. *Mol Cancer Res* 2008;6:1059–70.

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