Crotepoxide Chemosensitizes Tumor Cells through Inhibition of Expression of Proliferation, Invasion, and Angiogenic Proteins Linked to Proinflammatory Pathway*§

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Crotepoxide (a substituted cyclohexane diepoxide), isolated from Kaempferia pulchra (peacock ginger), although linked to antitumor and anti-inflammatory activities, the mechanism by which it exhibits these activities, is not yet understood. Because nuclear factor-κB (NF-κB) plays a critical role in these signaling pathways, we investigated the effects of crotepoxide on NF-κB-mediated cellular responses in human cancer cells. We found that crotepoxide potentiated tumor necrosis factor (TNF)- and chemotherapeutic agents induced NF-κB-regulated genes (Bcl-2, Bcl-xL, IκBα, Bax, Bcl-xl, IL-1β, Fas ligand, c-myc, D1 and c-myc), nuclear factor-κB cytokines and chemotherapeutic agents linked with anticancer activity, was first isolated from the fruit of Croton macrostachyus (1, 2). More recently, crotepoxide had been identified as a main component in Kaempferia rotunda (3), a member of the Zingiberaceae, or ginger family whose tuber has traditionally been used to treat pneumonia, bronchitis, abdominal pain, dysentery, diarrhea, cold, and obesity (4, 5). Crotepoxide was also identified in various other medicinal plants including Piper kadura (6–8), Monanthotaxis caffra (9), Friesodielsia obovata (Annonaceae) (10), Kaempferia angustifolia (11), and Kaempferia pulchra (peacock ginger) (12). Although crotepoxide has been reported to have tumor-inhibiting (2, 13) and anti-inflammatory properties (6), the exact mechanisms through which crotepoxide exhibits these properties are not understood.

Several chemotherapeutic, cytotoxic, and immunomodulating agents are commonly used to treat cancer. However, most modern medicines tend to target only one gene product or the major readouts. Because many of these agents are less effective, traditional cancer therapies and combinations of these alternatives (although plant-based) are ineffective in treating various cancers. The exact mechanisms through which crotepoxide exhibits these properties are not understood.

Nuclear factor-κB (NF-κB), a transcription factor that has a critical role in inflammation, is responsible for regulation of genes involved in cell survival, adhesion, differentiation, and growth. These genes include antiapoptotic (e.g. c-IAP, survivin, tumor necrosis factor receptor (TNFR)-associated factor (TRAF), cellular FLICE inhibitory protein, Bcl-2, and Bcl-xL), inflammatory (cyclooxygenase-2 (COX-2)), or invasive (matrix

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metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF)) and can encode adhesion molecules, chemokines, and cell-cycle regulation (e.g. cyclin D1 and c-myc) (14). Most carcinogens, inflammatory agents, and tumor promoters, including cigarette smoke, phorbol ester, okadaic acid, hydrogen peroxide, and tumor necrosis factor (TNF), have been shown to activate NF-κB (15). However, several cancer cell lines including human multiple myeloma (16), breast cancer (17), and prostate cancer (18) express constitutively active NF-κB (19).

Because NF-κB is known to regulate inflammation and tumorigenesis, we hypothesized that the anti-inflammatory and anticancer effects ascribed to crotepoxide may be due to its inhibition of NF-κB and NF-κB-regulated gene expression. Indeed we demonstrate that this crotepoxide can block NF-κB pathway and potentiate the anticancer effects of various chemotherapeutic drugs.

EXPERIMENTAL PROCEDURES

Reagents—A 50-mm solution of crotepoxide, isolated from K. pulchra as described below, was prepared in 100% dimethyl sulfoxide, stored as small aliquots at ~20 °C, and diluted in cell culture medium as needed. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5 × 10^7 units/mg, was provided by Genentech (South San Francisco, CA). We obtained 5-flurouracil, cisplatin, thalidomide, velacade, and tumor necrosis factor (TNF), have been therapeutic drugs.

Cell Lines—Chronic myogenous leukemia KBM-5, human multiple myeloma MM1, human prostate cancer DU145, human head and neck cancer SCC4, human embryonic kidney cancer A293, human non-small-cell lung carcinoma H1299, and human colon cancer Caco2 cell lines were obtained from American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove’s modified Dulbecco’s modified Eagle’s medium with 15% FBS; SCC4 and A293 cells were cultured in DMEM with 10% FBS; MM1, DU145, H1299, and Caco2 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI) medium with 10% FBS. All media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

Electrophoretic Mobility Shift Assay (EMSA)—To determine the effect of crotepoxide on TNF-activated NF-κB, we performed NF-κB-DNA binding using EMSA as previously described (21). Briefly, nuclear extracts prepared from treated cells (1 × 10^6 cells/ml) were incubated with 32P-end-labeled, 45-mer, double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from a human T-cell line (from a human T-cell line) (15). However, several cancer cell lines including human multiple myeloma (16), breast cancer (17), and prostate cancer (18) express constitutively active NF-κB (19).

Because NF-κB is known to regulate inflammation and tumorigenesis, we hypothesized that the anti-inflammatory and anticancer effects ascribed to crotepoxide may be due to its inhibition of NF-κB and NF-κB-regulated gene expression. Indeed we demonstrate that this crotepoxide can block NF-κB pathway and potentiate the anticancer effects of various chemotherapeutic drugs.

Isolation and Characterization—Rhizomes of K. pulchra were collected from a cultivated medicinal plant grower in Trivandrum, Kerala, India. A voucher specimen (TBGT 2072) has been deposited in the Tropical Botanical Garden and Research Institute Herbarium in Palode, Kerala, India. The air-dried powdered rhizome of K. pulchra (360 g) was extracted with acetone at room temperature (27 °C), which after removal of solvent under reduced pressure yielded the extract (9.17 g). The extract was subjected to gradient elution silica gel (100–200 mesh) column chromatography using the solvents hexane:ethyl acetate (100:0–40:60) to give 145 fractions, which were grouped into six fraction pools based on similarities on thin layer chromatography. After further purification by silica gel column chromatography and elution with hexane:ethyl acetate (90:10–80:20) and crystallization from dichloromethane-hexane mixture, the fifth fraction pool (1.4 g) yielded crotepoxide (1.2 g) as pure white crystals. We confirmed the structure of crotepoxide ([1R,2R,4R,5S,6R,7R]-4-benzyloxyethyl-3,8-dioxatricyclo[5.1.0.2-4]-octane-5,6-diol-diacetate) on the basis of a comparison of the spectral values, viz. 1H, 13C of nuclear magnetic resonance, infrared, and mass spectra with those reported earlier (20).
cells were pretreated with crotepoxide (50 \textendash 100 nmol/liter) and found that crotepoxide increased TNF-induced apoptosis from 16 to 40% (Fig. 1C). Briefly, cells were plated on a poly-L-lysine-coated glass slide with a Cytospin 4 centrifuge (ThermoShandon, Pittsburgh, PA), air dried, and fixed with 4\% paraformaldehyde. The slides were washed in PBS, blocked with 5\% normal goat serum for 1 h, and then incubated with rabbit polyclonal p65 antibody to the live/dead assay) and stained with the live and dead reagents (ethidium bromide (MTT) dye uptake method as described previously (23).

**Immunocytochemistry for p65 Localization**—To examine the effect of crotepoxide on the nuclear translocation of p65, we performed immunocytochemical analysis as previously described (24). Briefly, cells were plated on a poly-L-lysine-coated glass slide with a Cytospin 4 centrifuge (ThermoShandon, Pittsburgh, PA), air dried, and fixed with 4\% paraformaldehyde. The slides were washed in PBS, blocked with 5\% normal goat serum for 1 h, and then incubated with rabbit polyclonal p65 antibody at a 1:200 dilution overnight at 4\°C. The slides were then washed, incubated with goat anti-rabbit IgG-Alexa Fluor 594 (Invitrogen) at a 1:200 dilution for 1 h and counterstained for nuclei with Hoechst 33342 (50 ng/ml) stain for 5 min. Stained slides were mounted with mounting medium purchased from Sigma and analyzed under a Labophot-2 fluorescence microscope (Nikon, Melville, NY). Photographs were taken using a Photometrics Coolsnap CF color camera (Nikon) and MetaMorph software (Version 4.6.5, Universal Imaging, Sunnyvale, CA).

**Immune Kinase Complex Assay for TGF-β-activated Kinase 1 (TAK1)**—To determine the effect of crotepoxide on TNF-induced IkB kinase (IKK) and TAK1 activation, we analyzed IKK and TAK1 as previously described (25). Briefly, the IKK-TAK1 complex from whole-cell extracts was precipitated with antibodies against IKK-α or TAK1 and then treated with protein A/G-Sepharose beads (Pierce). After 2 h of incubation, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing HEPES (pH 7.4; 50 mmol/liter), magnesium chloride (2 mmol/liter), and substrate glutathione S-transferase-IκB-SEAP (0.5 μg) on 7.5% SDS-PAGE. After electrotransfer to polyvinylidene difluoride membranes, the proteins were visualized using a Storm 820 PhosphorImager. To determine IKK-α and TAK1 activity, we incubated whole-cell extracts with 10\% SDS-PAGE for 5 min. Finally, the protein was resolved on 10\% SDS-PAGE, the gel was dried, and the radioactive bands were visualized using a Storm 820 PhosphorImager. To determine the total amounts of IKK-α, IKK-β, and TAK1 in each sample, we resolved whole-cell proteins (40 μg) on 7.5% SDS-PAGE, electrotransferred the proteins to a nitrocellulose membrane, and then blotted the proteins with either anti-IKK-α/anti-IKK-β or anti-TAK1 antibodies.

**Live/Dead Assay**—To assess cytotoxicity, we used the live/dead assay (Invitrogen), which determines intracellular esterase activity and plasma membrane integrity. Briefly, 2 × 10^5 cells were incubated with crotepoxide (50 μmol/liter) for 2 h and then treated with TNF (1 nmol/liter) for 16 h at 37\°C. Cells were stained with the live and dead reagents (ethidium homodimer (5 μmol/liter) and calcine acetoxymethyl ester (5 μmol/liter)) and incubated at 37\°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon).

**NF-κB-dependent Reporter Gene Expression Assay**—To determine the effect of crotepoxide on NFκB promoter activity, we transfected the cells with the SEAP-driven reporter plasmid pSEAP-κB (1.5 μg) DNA for 24 h. We then treated the cells with crotepoxide for 2 h and stimulated them with TNF (0.1 μg/ml). 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 pSEAP-κB plasmid (0.5 μg) with expressing plasmid (0.5 μg) and pCMV-FLAG1 control plasmid (1.5 μg) for 24 h, treated with crotepoxide, and then harvested from cell culture medium after an additional 24 h of incubation. The culture medium was analyzed for SEAP activity as described by the manufacturer (Clontech Laboratories, Mountain View, CA) with a Victor 3 microplate reader (PerkinElmer Life and Analytical Sciences, Boston, MA).

**Annexin V Assay**—An early indicator of apoptosis is the rapid translocation of membrane phospholipid phosphatidylserine from the cytoplasmic interface of the membrane to the extracellular surface. This loss of membrane asymmetry can be detected using annexin V antibody conjugated with a fluorophore. We incubated the cells with crotepoxide (50 μM), harvested from cell culture medium after an additional 24 h of incubation, and subjected to annexin V staining. Cells were washed in PBS, resuspended in 100 μl of binding buffer containing a fluorescein isothiocyanate-conjugated annexin V antibody, and then analyzed with a BD Calsibar flow cytometer (BD Biosciences).

**RESULTS**

This study was undertaken to investigate the anti-inflammatory and antitumor effects of crotepoxide. For this first investigatded the effect of crotepoxide alone on the survival of various tumor cells, and then we examined in combination with chemotherapeutic agents. We also determined its effects on the NF-κB activation pathway as induced by various carcinogens and inflammatory stimuli as well as expression of NF-κB-regulated gene products and apoptosis in leukemic cells. The duration of exposure and concentration of crotepoxide used to examine its effect on NF-κB pathway had a minimal effect on the viability of these cells as determined by trypan blue dye exclusion test (data not shown).

**Crotepoxide Inhibits Proliferation and Potentiates TNF- and Chemotherapeutic Agent-induced Apoptosis**—The results of MTT assay showed that crotepoxide inhibited the proliferation of leukemic cells, KBM-5, MM1, and U266 cells in a dose- and time-dependent manner (Fig. 1B). Whether this compound can enhance the apoptosis induced by apoptotic cytokine TNF, was investigated by an annexin V staining method. We found that crotepoxide up-regulated TNF-induced apoptosis from 9 to 28\% (Fig. 1C, left panels). However, crotepoxide alone showed only 9\% cell death. To confirm the result of annexin V assay, we used esterase staining (i.e. the live/dead assay) and found that crotepoxide increased TNF-induced apoptosis from 16 to 40\%
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(Fig. 1C, middle panels). When we examined crotepoxide for caspase activation by Western blotting, a classical hallmark of apoptosis, we found activation of caspase-9 and caspase-3 and subsequent anti-poly(ADP-ribose) polymerase cleavage, indicating that crotepoxide potentiates the TNF-induced caspase activation (Fig. 1C, right panels). Crotepoxide alone induced apoptosis, albeit at a low extent, and the mechanism of this effect is not known at this point.
Crotepoxide Inhibits the Expression of Proinflammatory Gene Products—Because crotepoxide exhibits anti-inflammatory activity (6), we investigated whether this effect is mediated through the regulation of NF-κB-mediated expression of COX2. Results revealed that crotepoxide blocked TNF-induced expression of this proinflammatory protein in a time-dependent manner. Under these conditions, crotepoxide alone showed minimal cytotoxicity (Fig. 2A).

Crotepoxide Modulates Expression of Antiproliferative and Tumor Suppressor Gene Products. We found that crotepoxide induced the expression of C-reactive protein (CRP) and inhibited the expression of C-reactive protein (CRP) and inhibited the expression of antiapoptotic Bcl-2, Bcl-xL, survivin, Mcl-1, TRAF-1, and c-IAP1 proteins (29). Bcl-2, Bcl-xL, survivin, Mcl-1, TRAF-1, and c-IAP1 proteins (29). Bcl-2, Bcl-xL, survivin, Mcl-1, TRAF-1, and c-IAP1 proteins (29). Bcl-2, Bcl-xL, survivin, Mcl-1, TRAF-1, and c-IAP1 proteins (29). Bcl-2, Bcl-xL, survivin, Mcl-1, TRAF-1, and c-IAP1 proteins (29). Bcl-2, Bcl-xL, survivin, Mcl-1, TRAF-1, and c-IAP1 proteins (29). Bcl-2, Bcl-xL, survivin, Mcl-1, TRAF-1, and c-IAP1 proteins (29). Bcl-2, Bcl-xL, survivin, Mcl-1, TRAF-1, and c-IAP1 proteins (29). Bcl-2, Bcl-xL, survivin, Mcl-1, TRAF-1, and c-IAP1 proteins (29).

Crotepoxide Suppresses the Expression of Tumor Cell Survival Gene Products—Because crotepoxide enhances TNF- and chemotherapy-induced apoptosis, we investigated whether this effect is mediated through the regulation of NF-κB-mediated expression of these antiapoptotic proteins c-IAP1 (29), Bcl-2 (30), Bcl-xL (31), mcl-1, TRAF-1 (32), and survivin. We found that crotepoxide blocked the TNF-induced expression of these tumor cell survival proteins in a time-dependent manner (Fig. 2C).

Crotepoxide Suppresses the Expression of TNF-induced Metastatic Gene Products—TNF has been shown to induce the expression of ICAM-1 (33), VEGF (34), and MMP-9 (35), all of which have NF-κB-binding sites in their promoters. We investigated whether crotepoxide can modulate NF-κB-regulated gene products involved in metastasis and found that crotepoxide abolished TNF-induced expression of ICAM-1, MMP-9, and VEGF (Fig. 2D).
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Crotepoxide Suppresses TNF-induced NF-κB Activation—Because NF-κB regulates various cellular responses including proliferation, apoptosis, inflammation, and chemosensitization, all regulated by NF-κB, we reasoned that crotepoxide must modulate the NF-κB cell-signaling pathway. Therefore, we investigated whether crotepoxide inhibits NF-κB activation. The experimental condition that was used to study the mechanism of NF-κB inhibition involved short duration of exposure, and under this condition crotepoxide alone did not show any cell death. EMSA revealed that although crotepoxide alone had no effect on NF-κB activation, but crotepoxide inhibited TNF-mediated NF-κB activation in a dose (Fig. 3A, left panel)- and time-dependent manner (Fig. 3A, right panel).

NF-κB is a complex of proteins in which various combinations of Rel or NF-κB proteins constitute active NF-κB heterodimers that bind specific DNA sequences. To confirm that the band visualized by EMSA in TNF-treated cells was NF-κB, we incubated nuclear extracts from TNF-activated cells with antibodies to the p50 (NF-κB) and p65 (RelA) subunits of NF-κB. The resulting bands that were shifted to higher molecular masses (Fig. 3B) suggested that the TNF-activated complex consisted of p50 and p65. Pre-immune serum (PIS) had no effect on DNA binding. The addition of excess unlabeled NF-κB (cold oligonucleotide, 100-fold) caused a complete disappearance of the band, whereas mutated oligonucleotide had no effect on the DNA binding.

Crotepoxide Inhibits Robust Activation of NF-κB—Our previous studies have shown that a high concentration of TNF (1 nM) induces more robust and rapid (5 min) NF-κB activation (21). To determine whether crotepoxide could inhibit the TNF-κB robust response to TNF, we challenged crotepoxide-treated cells with increasing concentrations of TNF (up to 1 nM) for 30 min and then examined for NF-κB activation (Fig. 3C). Although NF-κB activation by 1 nM TNF was very strong, crotepoxide inhibited NF-κB activation regardless of whether NF-κB was activated with 0.01 or 1 nM TNF, suggesting that crotepoxide is a very potent inhibitor of NF-κB activation.

Crotepoxide Does Not Directly Affect Binding of NF-κB to the DNA—Some NF-κB inhibitors such as N-tosyl-l-phenylalanine chloromethyl ketone (a serine protease inhibitor), caffeic acid phenethyl ester, and plumbagin (36–39) directly modify the NF-κB protein so that the protein can no longer bind to DNA. We investigated whether crotepoxide mediates suppression of NF-κB activation through a similar mechanism. Incubating nuclear extract from TNF-treated cells with crotepoxide revealed that crotepoxide did not modify the DNA binding ability of NF-κB proteins (Fig. 3D), suggesting that crotepoxide inhibits NF-κB activation by a mechanism different from direct modification.

Crotepoxide Inhibited NF-κB Activation Induced by Various Agents—In addition to TNF, phorbol 12-myristate 13-acetate, lipopolysaccharide (LPS), okadaic acid, cigarette smoke con-
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cells, indicating that crotepoxide-induced suppression of NF-κB activation is not cell type-specific.

Crotepoxide Suppressed Constitutive NF-κB Activation—Most tumor cells express constitutively active NF-κB (19); however, the mechanism of constitutive activation is not well understood. Prostate cancer DU145, multiple myeloma MM1, and squamous cell carcinoma SCC4 cells are known to express constitutively active NF-κB. Treating DU145, MM1, and SCC4 cells with crotepoxide suppressed constitutive NF-κB activation (Fig. 4C).

Crotepoxide Inhibited TNF-dependent IκBα Degradation and Phosphorylation—The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα. To determine whether the inhibition of TNF-induced NF-κB activation was due to the inhibition of IκBα degradation, we pretreated cells with crotepoxide and then exposed the cells to TNF for various times. We then examined the cells for NF-κB in the nucleus by EMSA and for IκBα degradation by Western blot analysis. EMSA revealed that TNF activated NF-κB in control cells in a time-dependent manner (Fig. 5A) as early as 5 min with maximal NF-κB activation at 30 min. However, Western blot analysis revealed that crotepoxide blocked IκBα degradation in the cytoplasm (Fig. 5B). In addition, crotepoxide inhibited the phosphorylation of IκBα and its subsequent degradation. These results indicate that crotepoxide blocks TNF-induced NF-κB activation and IκBα degradation.

To determine whether the inhibition of TNF-induced IκBα degradation was due to the inhibition of IκBα phosphorylation, we used the proteasome inhibitor N-acetyl-leucyl-leucyl-nor-leucinal to block IκBα degradation. Western blotting with an antibody that recognizes the serine-phosphorylated (Ser-32) form of IκBα revealed that crotepoxide strongly suppressed TNF-induced IκBα phosphorylation (Fig. 5C).

Crotepoxide Inhibited TNF-induced IκBα Kinase Activation—IKK is required for TNF-induced phosphorylation of IκBα and for the phosphorylation of p65 (45). Because crotepoxide inhibited IκBα phosphorylation, we investigated the effects of crotepoxide on TNF-induced IKK activation. Immune complex kinase assays showed that crotepoxide suppressed TNF-induced IKK activation (Fig. 5D). Neither TNF nor crotepoxide affected the expression of IKK proteins.

Crotepoxide Did Not Directly Inhibit TNF-induced IKK—Certain agents suppress NF-κB activation by directly interacting with IKK (24, 46). We investigated whether crotepoxide binds with the IKK protein to directly suppress IKK activity. The immune complex kinase assay of whole-cell extracts from untreated and TNF-treated cells showed that crotepoxide did not directly affect IKK activity, suggesting that crotepoxide indirectly modulated TNF-induced IKK activation (Fig. 5E).

Crotepoxide Inhibited TNF-induced Phosphorylation of IKKα/β—Next we investigated whether crotepoxide suppresses activation of IKKα/β induced by TNF. We observed that it inhibited phosphorylation of IKKα/β and activation. IKK
Crotepoxide suppressed NF-κB and potentiated apoptosis

(A) TNF

|   | Medium | Crotepoxide |
|---|--------|-------------|
| 0 |        |             |
| 5 |        |             |
| 10|        |             |
| 15|        |             |
| 30|        |             |
| 60|        |             |

(B) TNF

|   | Medium | Crotepoxide |
|---|--------|-------------|
| 0 |        |             |
| 5 |        |             |
| 10|        |             |
| 15|        |             |
| 30|        |             |
| 60|        |             |

(C) TNF

|   | Medium | Crotepoxide |
|---|--------|-------------|
| 0 |        |             |
| 10|        |             |
| 25|        |             |
| 50|        |             |
| 100|       |             |

(D) TNF

|   | Medium | Crotepoxide |
|---|--------|-------------|
| 0 |        |             |
| 2 |        |             |
| 5 |        |             |
| 10|        |             |
| 15|        |             |

(E) TNF

|   | Medium | Crotepoxide |
|---|--------|-------------|
| 0 |        |             |

(F) TNF

|   | Medium | Crotepoxide |
|---|--------|-------------|
| 0 |        |             |

FIGURE 5. Crotepoxide inhibits TNF-induced NF-κB activity. Crotepoxide inhibited NF-κB activity in KBM-5 cells stimulated with TNF (10 ng/ml) for 2 h (A). Whole-cell extracts from untreated and TNF-treated cells were subjected to Western blot analysis with phospho-specific antibodies. D, cell lysates that were incubated with crotepoxide (50 μM) for 2 h, were fractionated and then subjected to Western blot with phospho-specific anti-IKKα antibody. E, cell lysates were immunoprecipitated with antibody against IKK-α. The same membrane was reblotted with anti-IKKα antibody. F, cell lysates were analyzed for NF-κB activity with the SEAP reporter gene. The TAK1 protein was immunoprecipitated with antibody against TAK1, and the immunocomplex kinase assay was performed in the absence or presence of the indicated concentrations of crotepoxide. E, crotepoxide inhibited the phosphorylation of IKKα/β. The protein lysates were treated with or without crotepoxide for 2 h before incubation with TNF (10 ng/ml) for 1 h. Whole-cell extracts were prepared and then subjected to Western blotting with phospho-specific anti-IKKα/β antibody. The same membrane was reblotted with anti-IKKα and anti-IKKβ antibodies.

Crotepoxide inhibited TNF-induced NF-κB activity, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps. We investigated whether crotepoxide could suppress TNF-induced NF-κB reporter activity. TNF induced the expression of an NF-κB-regulated SEAP reporter gene in a dose-dependent manner, and crotepoxide suppressed the expression (Fig. 7A).

Crotepoxide Repressed TNF-induced NF-κB-dependent Reporter Gene Expression—Although EMSA showed that crotepoxide blocked NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps. We investigated whether crotepoxide could suppress TNF-induced NF-κB reporter activity. TNF induced the expression of an NF-κB-regulated SEAP reporter gene in a dose-dependent manner, and crotepoxide suppressed the expression (Fig. 7A).
that mediate inflammation, cell proliferation, cell survival, invasion, and angiogenesis, all of which are regulated by NF-κB. We also found that crotepoxide suppressed NF-κB activated by various agents by inhibiting IKK activation, 1κBα phosphorylation, 1κBα degradation, p65 phosphorylation, and NF-κB-dependent reporter gene expression.

Our study is the first to investigate the effect of crotepoxide on NF-κB activation. Crotepoxide inhibited NF-κB activation induced by carcinogens, cigarette smoke, and inflammatory stimuli, suggesting that crotepoxide must act at a step common to all these activators. Although crotepoxide has been reported to act as an anti-inflammatory agent (6), its mechanism of action has not been described. TNF-α, which is known to play a major role in inflammation, was inhibited by crotepoxide. Crotepoxide not only inhibits inducible NF-κB activation but also inhibits constitutively active NF-κB in tumor cells. Constitutive NF-κB activation is critical to the survival and proliferation of various tumor cell types (19). NF-κB activation in response to different stimuli requires IKK activation, which phosphorylates 1κBα at serine 32 and 36, leading to 1κBα degradation and p65 translocation to the nucleus (50). We found that crotepoxide suppressed IKK, which in turn suppressed 1κBα phosphorylation and degradation. IKK is also involved in constitutive activation of NF-κB in tumor cells (51). Thus, it is possible that crotepoxide inhibition of IKK is linked to its ability to suppress constitutive NF-κB activation.

We also investigated the ways in which crotepoxide inhibits IKK activation. Several studies have suggested that TAK1 plays a major role in TNF-induced NF-κB activation by interacting with TAB1 and TAB2. For instance, TAK1 can bind to and activate IKK, leading to NF-κB activation (52). We found that crotepoxide directly inhibited the activation of TAK1. TAK1 has also been shown to be recruited by TNFR1 through TRADD, TRAF2, and receptor-interacting protein (47). Indeed, our study showed for the first time that crotepoxide inhibits TAK1-induced NF-κB activation, which suggests that TAK1 is the main upstream stimulatory kinase modulated by crotepoxide.

We were intrigued to find that crotepoxide also inhibited IKK-induced NF-κB reporter gene activity. It is likely that the overexpressed IKK requires TAK1/TAB for its activation, which could be inhibited by crotepoxide. TAK1-dependent activation...
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![Graph](image)

**FIGURE 7. Crotepoxide suppresses NF-κB-dependent reporter expression induced by TNF and various plasmids.** A, crotepoxide-induced NF-κB-dependent reporter gene expression was transiently transfected with an NF-κB-containing plasmid. After transfection, the cells were incubated with the indicated concentrations of crotepoxide (50 μM) for an additional 24 h. Supernatants of the culture media were assayed for SEAP activity. Data are presented as the means ± S.D. of triplicate culture wells.

(B) NF-κB reporter gene (SEAP) expression fold

- Con
- TNF
- TNF1
- TRADD
- TRAF2
- NIK
- IKK
- TAK1/TAB1
- Crotepoxide

Overall, our results demonstrate that crotepoxide is a potent anti-inflammatory agent with tumorigenesis-suppressing potential.

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of IKKβ requires Lys-63 ubiquitination and interaction with IKKγ in the IKK-complex (53). Because crotepoxide inhibits TAK1 dependent IKKβ phosphorylation, it is possible that it would also inhibit the IKKβ-dependent reporter gene activity. Alternatively, its ability to directly inhibit IKK in vivo cannot be ruled out based on our studies.

We also found that crotepoxide can suppress TNF-induced IκBα degradation, which is mediated through the inhibition of IκBα phosphorylation. Crotepoxide inhibits TNF-induced IκBα phosphorylation and, therefore, delays the degradation of IκBα, indicating that crotepoxide mediates its effects through mechanisms different from those of N-acetyl-leucyl-leucyl-norleucinal.

NF-κB activation leads to the expression of genes that are involved in the proliferation, survival, angiogenesis, invasion, and metastasis of cancer (54). In the current study, we found that crotepoxide inhibited the expression of cyclin D1 and c-myc, both of which are regulated by NF-κB. Crotepoxide inhibition of cyclin D1 and c-myc could be the mechanism of crotepoxide-induced inhibition of cancer cell proliferation. In addition, we found that crotepoxide suppressed the expression of various antiapoptotic gene products including TRAF1, Bcl-2, Bcl-xl, and IAP-1. These gene products are regulated by NF-κB, and their overexpression in numerous tumors has been associated with tumor survival, chemoresistance, and radioresistance.

Besides NF-κB, another transcription factor STAT3 is known to be involved in tumorogenesis (55). However, in the present study, we found that crotepoxide did not influence the activation of STAT3, indicating diepoxide-induced apoptosis of cancer cells is not due to the inhibition of STAT3 activation.

In addition, crotepoxide potentiated apoptosis induced by TNF and various chemotherapeutic agents including 5-fluorouracil, cisplatin, thalidomide, and velacade. Crotepoxide down-regulation of various antiapoptotic gene products can sensitize the cells to the apoptotic effects of TNF. Kupchan et al. (2) stated that crotepoxide inhibits tumorogenesis, and our results showed that crotepoxide has anti-apoptotic effects on various leukemic cells, which could be due to down-regulation of these anti-apoptotic gene products. Similarly, crotepoxide also suppresses gene products that have been implicated in metastasis (56). We found that crotepoxide abrogated TNF-induced NF-κB-regulated gene products such as ICAM-1 and angiogenesis-suppressed gene products of TNF-induced metastasis and angiogenesis.

In conclusion, we believe that crotepoxide may be a promising candidate for further investigations to develop a new anti-inflammatory agent with tumor-suppressing potential.

**RETRACTED**
Crotepoxide Suppresses NF-κB and Potentiates Apoptosis

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**Supplementary Fig. 1**

(A) IL-6

| Crotepoxide | 0 | 10 | 25 | 50 | 100 |
|-------------|---|----|----|----|-----|
| STAT3       | pSTAT3 |

(B) Crotepoxide

| Crotepoxide | 0 | 10 | 25 | 50 | 100 |
|-------------|---|----|----|----|-----|
| STAT3       | pSTAT3 |

(C) TNF

| TNF         | Crotepoxide | 0 | 5 | 10 | 15 | 30 | 60 |
|-------------|-------------|---|---|----|----|----|----|
| Phospho-p38 MAPK | p38 MAPK | --- | --- | --- | --- | --- | --- |
Legend of Supplementary Fig. 1

_Crotepoxide does not modulate STAT3 and MAPK pathways._

(A) Crotepoxide did not suppress STAT3 activation. KBM-5 cells (2 x 10^6 cells/mL) was treated with crotepoxide with indicated concentration for 2 hours and then exposed with IL-6 for 15 min. Whole cell lysates were prepared and subjected to western blotting with antibody against pSTAT3. For loading control, the membrane was blotted with anti–STAT3 antibody.

(B) Crotepoxide did not inhibit constitutive STAT3 activation. U266 cells (2x10^6 cells/mL) were treated with crotepoxide with indicated concentration. After 2 h of incubation cells harvested, whole cell lysates were prepared and subjected to western blotting with antibody against pSTAT3. For loading control, the membrane was blotted with anti–STAT3 antibody.

(C) Crotepoxide did not suppress MAPK activation. KBM-5 cells were pretreated with crotepoxide (50 μM) for 2 hours and then treated with TNF (0.1 nM) for the indicated times. Whole cell extracts were prepared and analyzed by western blotting with antibodies against phospho-MAPK. For equal loading of protein, the membrane was blotted with anti–MAPK antibody.
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