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Jesterone Dimer, a Synthetic Derivative of the Fungal Metabolite Jesterone, Blocks Activation of Transcription Factor Nuclear Factor κB by Inhibiting the Inhibitor of κB Kinase

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ABSTRACT

Rel/nuclear factor-κB (NF-κB) transcription factors control a variety of cellular processes, such as cell growth and apoptosis, and are continually activated in many human diseases, including chronic inflammatory diseases and cancer. Jesterone dimer (JD) is a synthetic derivative of the natural fungal metabolite jesterone, and JD has previously been shown to be cytotoxic in select tumor cell lines. In this report, we demonstrate that JD is a potent inhibitor of the activation of transcription factor NF-κB. Namely, JD inhibits tumor necrosis factor-α–induced activation of NF-κB in mouse 3T3 and human HeLa cells. JD seems to block the induction of the NF-κB pathway by inhibiting the inhibitor of κB kinase (IKK); that is, treatment of cells with JD blocks phosphorylation of IκBα, inhibits the activity of a constitutively active form of the IKKβ catalytic subunit, and converts IKKβ to stable high molecular mass forms. Like JD, a JD-related epoxyquinoid (isotorreyanic acid) inhibits activation of NF-κB at 20 μM, whereas several other epoxyquinoids that are related to JD, including its parent compound jesterone, do not block activation of NF-κB at this concentration. Finally, JD inhibits both proliferation and DNA binding by REL-containing complexes in the human lymphoma SUDHL-4 cell line, and JD activates caspase-3 activity in these cells. In summary, these results suggest that JD induces apoptosis in tumor cells through a mechanism that involves the inhibition of Rel/NF-κB activity and demonstrate the usefulness of assessing the bioactivity of synthetic derivatives of natural products.

Fungal metabolites have a number of important biological and therapeutic activities (Pearce, 1997). Thus, there has been considerable interest in synthesizing and characterizing the activities of fungal metabolites and synthetic derivatives of these compounds, which may have increased or novel biological activities (Lazo et al., 2001; Wipf et al., 2001a,b). We have been synthesizing and studying the bioactivity of several epoxyquinoids. For example, we have reported previously the total synthesis of the natural metabolites jesterone from *Pestalotiopsis jesteri*, cycloepoxydon from the Deuteromycetes strain 45-93, and epoxyquinol A from an uncharacterized fungus (Hu et al., 2001; Li et al., 2001a, 2002).

We have shown previously that jesterone (Fig. 1) kills tumor cells with IC_{50} values ranging from approximately 100 to 500 μM in three different human tumor cell lines (Hu et al., 2001). However, a dimeric derivative of jesterone, which we have termed "jesterone dimer" (JD) (Fig. 1), has approximately 10- to 100-fold greater antitumor cell activity than jesterone against the same tumor cell lines (Hu et al., 2001). Thus, we were interested in identifying molecular targets of JD that mediate this increased cytotoxicity.

Rel/NF-κB is a family of eukaryotic transcription factors that influences a number of important cellular and organismal processes, including cellular growth control, apoptosis, immune and inflammatory responses, and cellular stress responses (Pahl, 1999). The mammalian Rel/NF-κB family includes five related proteins (c-Rel, RelA, RelB, p50, and p52) that form various combinations of homodimers and heterodimers to control the activity of numerous genes. In most cell types, NF-κB is located in the cytoplasm in a latent, inactive state bound to the inhibitor protein IκB. Almost all stimuli that induce the Rel/NF-κB pathway do so through the activation of an IκB kinase (IKK) (Ghosh and Karin, 2002).

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ABBREVIATIONS: JD, jesterone dimer; NF-κB, nuclear factor κB; PDGF, platelet-derived growth factor; IκB, inhibitor of κB; IKK, inhibitor of κB kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TNF-α, tumor necrosis factor-α; FLAG, flu antigen; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PARP, poly(ADP-ribose) polymerase; GST, glutathione *S*-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MeOH, methanol.

Activated IKK phosphorylates IκB, which then leads to ubiquitination and proteasome-mediated degradation of IκB, with the resultant translocation of the freed NF-κB complex to the nucleus. However, a growing series of reports has shown that Rel/NF-κB is constitutively active and present in the nucleus in a variety of human tumor cell types and cell lines (Gilmore et al., 2002). Moreover, inhibition of this continuous Rel/NF-κB activity can, in many cases, slow the growth of these tumor cell lines or induce apoptosis.

Because a number of inhibitors of transcription factor NF-κB have also been shown to inhibit tumor cell growth and because some epoxyquinoids have been shown to inhibit NF-κB (Gehrt et al., 1998; Matsumoto et al., 2000; Hu et al., 2001; Li et al., 2001a, 2002), we were interested in determining whether JD might affect the activity of NF-κB. In this report, we demonstrate that JD is an efficient inhibitor of Rel/NF-κB and acts by blocking the activity of IKK. These studies highlight the usefulness of assessing bioactivity of natural product derivatives, which may have enhanced or novel activities compared with the parent natural products themselves.

Materials and Methods

Preparation of Jesterone Dimer and Other Synthetic Compounds. The preparation of jesterone, jesterone dimer, cycloepoxydon, torreyanic acid, and the related epoxyquinoids (Figs. 1 and 6A) have been described previously (Hu et al., 2001; Li et al., 2000, 2001a, 2002). All compounds were dissolved in 100% methanol before use.

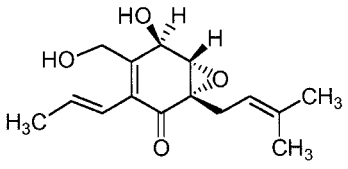
Cell Culture, Chemical Treatment, and Transfection. Mouse 3T3 cells, HeLa cells, and SUDHL-4 cells (a gift from Dr. Louis Staudt, National Institutes of Health, Bethesda, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Biologos, Naperville, IL), 50 units/ml penicillin, and 50 μg/ml streptomycin. All cell lines were maintained at 37°C with 5% CO_2.

Twenty-four hours before treatment, 3T3 cells and HeLa cells were fluid changed from DMEM containing 10% FBS to DMEM containing 0.5% FBS. After this 24-h period, cells were incubated for 2 h with the indicated concentrations of compounds or the solvent methanol. After the 2-h incubation, cells were stimulated with either 2 ng/ml recombinant human TNF-α (R&D Systems, Minneapolis, MN) for 20 min or 10 ng/ml recombinant rat platelet-derived growth factor (PDGF)-BB (R&D Systems) for 10 min. For detecting phosphorylated IκBα, 100 μM calpain inhibitor *N*-acetyl-Leu-Leu-Nle-al (Sigma Chemical Co., St. Louis, MO) was added to 3T3 cultures 2 h before TNF-α stimulation. SUDHL-4 cells were incubated with the indicated concentrations of compounds without prior serum starvation for the times indicated in each experiment.

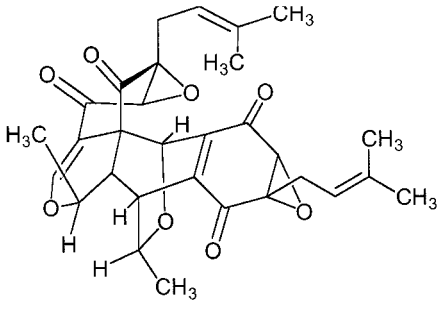
For IKKβ overexpression studies, each 100-mm plate of approximately 70% confluent 3T3 cells was transfected with 15 μg of pcDNA expression plasmids for FLAG-tagged versions of wild-type IKKβ, SS/EE mutant, or SS/EE/C179A mutant IKKβ using the SuperFect Transfection Reagent, according to the manufacturer's protocol (QIAGEN, Valencia, CA).

Electrophoretic Mobility Shift Assays. Whole-cell extracts were prepared in AT buffer [20 mM HEPES, pH 7.9, 1% (v/v) Triton X-100, 20% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM Na_3VO_4, 1 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin]. Cell lysate (20 μg) was incubated with a 26-base pair radiolabeled κB site probe (κB site: 5′-GG-GAAATTCC-3′; 35,000–70,000 cpm), 2 μg of poly(dI-dC) in binding buffer [25 mM Tris-HCl, pH 7.4, 100 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol] in a 50-μl reaction volume for 30 min at 30°C. For supershift assays, 1 μl of preimmune serum, anti-p50 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-RelA (Santa Cruz Biotechnology), or anti-REL (a kind gift from Dr. Nancy Rice, National Cancer Institute, Bethesda, MD) antibody was added to the complexes for an additional 1 h on ice. Samples were then immediately analyzed on a 5% polyacrylamide gel, and protein-DNA complexes were detected by autoradiography.

Western Blotting. Western blotting was performed essentially as described previously (Barkett et al., 2001). Whole-cell extracts were prepared in AT buffer, except for detecting phosphorylated IκBα where cells were lysed in AT buffer in the presence of a cocktail of phosphatase inhibitors (10 mM sodium pyrophosphate, 50 μM $ZnCl_2$, and 20 mM β-glycerophosphate). Nuclear extracts were prepared as described previously (Schreiber et al., 1989) with the inclusion of 1 mM DTT, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM PMSF. Samples containing equal or normalized amounts of proteins were separated on 6, 7.5, 10, or 12.5% SDS-polyacrylamide gels, and proteins were transferred to nitrocellulose membranes (Micron Separation Inc., Westborough, MA). Either 5% nonfat milk in phosphate-buffered saline with Tween 20 or 5% bovine serum albumin in Tris-buffered saline with Tween 20 was used as blocking buffer. The following primary antisera (shown as dilution; source) were used: anti-IκBα antiserum directed against C-terminal sequences of IκBα (1:500; Santa Cruz Biotechnology); anti-poly(ADP-ribose) polymerase (PARP) (1:1000; Santa Cruz Biotechnology); anti-IKKβ (1:1000; Santa Cruz Biotechnology); anti-actin (1:500; Santa Cruz Biotechnology); anti-RelA (1:500; a gift from Nancy Rice, National Cancer Institute); anti-phospho-Akt (Ser-473) (1:1000; Cell Signaling Technology, Beverly, MA); anti-Akt (1:1000; Cell Signaling Technology); and anti-phosphotyrosine (1:500; Upstate Biotechnology, Lake Placid, NY). The appropriate horseradish peroxidase-labeled secondary antiserum was added, and complexes were detected



jesterone



jesterone dimer

Fig. 1. Structures of jesterone and jesterone dimer. Hu et al. (2001) describe details of the synthesis of these compounds.

by Supersignal Dura West chemiluminescence (Pierce Chemical, Rockford, IL).

Kinase Assays. IKK kinase assays were performed essentially as described previously (Mercurio et al., 1997; Kwok et al., 2001). Two days after transfection, 3T3 cells were treated with JD or methanol for 2 h and lysed in AT buffer containing 20 mM β-glycerophosphate. Samples were then incubated with anti-FLAG M2 affinity gel freezer-safe beads (Sigma) for 2 h at 4°C. Beads were washed three times with lysis buffer and two times with 25 mM Tris-HCl, pH 7.5. The immunoprecipitates were then incubated with 2 μg GST-IκBα (amino acids 1 to 55) and 5 μCi [γ-^{32}P]ATP in kinase reaction buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, and 50 μM ATP) containing phosphatase inhibitors (10 mM NaF, 0.5 mM Na_3VO_4, and 20 mM β-glycerophosphate) in a total volume of 30 μl for 20 min at 30°C. The phosphorylated substrates were then analyzed by 6 (for IKKβ) or 12.5% (for GST-IκBα) SDS-polyacrylamide gel electrophoresis and autoradiography.

Measurement of Caspase-3 Activity. To measure total caspase-3 activity, SUDHL-4 cells were resuspended in lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 350 μg/ml PMSF, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 20 μg/ml leupeptin) and lysed by three freeze-thaw cycles. Equal amounts of cell extracts were incubated in reaction buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT) containing 50 μM *N*-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin), a fluorogenic substrate (BIOMOL Research Laboratories, Plymouth Meeting, PA) in a total volume of 500 μl for 1 h at 37°C, as recommended by the manufacturer. Caspase-3 activity was then determined by measuring fluorescence (excitation at 380 nm and emission at 460 nm).

Results

Jesterone Dimer Blocks Tumor Necrosis Factor-α–Induced Activation of NF-κB in Mouse 3T3 Cells. In the course of screening epoxyquinoid compounds for inhibition of NF-κB, we discovered that pretreatment of mouse 3T3 cells with the synthetic compound jesterone dimer (at a concentration of 20 μM) blocked TNF-α–induced activation of NF-κB DNA binding activity (Fig. 2A). In contrast, the natural product jesterone did not affect the activation of NF-κB at this concentration (Fig. 2A). Moreover, JD inhibited TNF-α–induced activation of NF-κB DNA binding activity in a dose-dependent manner, with half-maximal inhibition at approximately 2.5 μM and complete inhibition at approximately 5 μM (Fig. 2B). It is clear that JD is not a general inhibitor of DNA binding proteins in that a constitutive κB-site DNA binding activity present in these extracts (lower bands in Fig. 2A) was not appreciably affected by pretreatment with JD. JD also blocked TNF-α–induced nuclear translocation of RelA (Fig. 2C), indicating that the inhibition of NF-κB was occurring in the cytoplasm. Consistent with JD having a cytoplasmic target in the NF-κB signal transduction pathway, degradation of IκBα was inhibited in a dose-dependent manner by JD (Fig. 2D). Notably, as little as 5 μM JD was sufficient to almost completely block both TNF-α–induced NF-κB DNA binding and degradation of IκBα in mouse 3T3 cells.

Jesterone Dimer Blocks Signal-Induced Phosphorylation of IκBα by Inhibiting IKKβ. Phosphorylation of N-terminal Ser residues in IκBα by the IKK complex is a prerequisite for TNF-α–induced degradation of IκBα (Ghosh and Karin, 2002). Therefore, we sought to determine whether IKK might be a molecular target for JD. Signal-induced phosphorylation of IκBα is known to induce a mobility shift of IκBα on SDS-polyacrylamide gels (Brown et al., 1995). As shown in Fig. 3A, pretreatment of 3T3 cells with JD blocked the TNF-α–induced mobility shift (and thus phosphorylation) of IκBα.

The IKKβ subunit of IKK is the primary kinase that phosphorylates IκBα upon treatment of cells with TNF-α (Ghosh and Karin, 2002). To determine whether JD could directly block the activity of IKKβ, 3T3 cells were first transfected with an expression vector for a FLAG-tagged version of wild-type IKKβ or a constitutively active form of IKKβ that has Glu residues substituted for two Ser residues that are phosphorylated upon activation of IKK (mutant S177,181E; SS/EE). Transfected cells were treated with or without JD for

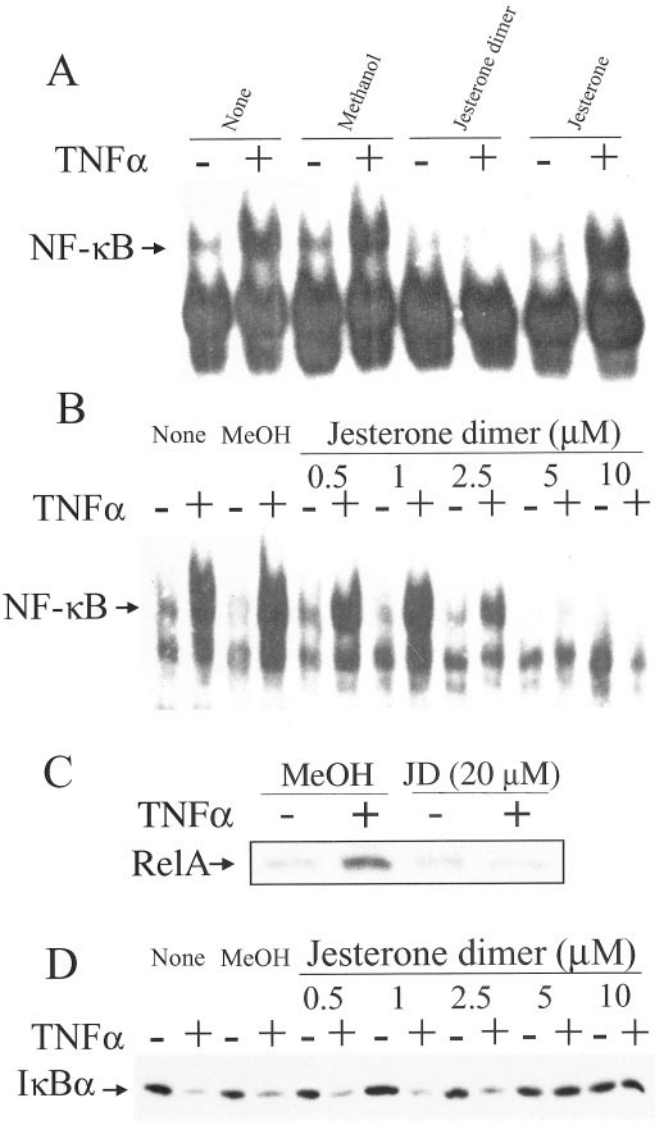


Fig. 2. Jesterone dimer inhibits TNF-α–induced activation of NF-κB DNA binding and degradation of IκBα in mouse 3T3 cells. 3T3 cells were incubated with media containing no addition (None), the solvent methanol (MeOH), 20 μM JD, or 20 μM jesterone for 2 h. Cells were then treated with TNF-α (+) for 20 min or not treated as a control (−). Extracts were then subjected to an EMSA using a κB site probe (A), or nuclear extracts were prepared and subjected to anti-RelA Western blotting (C). In B and D, cells were preincubated with the indicated concentrations of JD and subjected to a κB-site EMSA (B) or Western blotting with an IκBα antiserum (D). The position of the NF-κB DNA complex is shown in A and B.

2 h, and IKKβ activity in cell extracts was assessed in an immune-complex kinase assay using GST-IκBα as a substrate. As shown in Fig. 3B, treatment of the cells with JD inhibited the activity of both wild-type IKKβ and the SS/EE IKKβ mutant.

As a control, we determined whether JD could block the activation of kinase activities other than IKK in mouse 3T3 cells. Activation of the platelet-derived growth factor (PDGF) receptor leads to the tyrosine phosphorylation of several proteins, as well as the activation of downstream serine kinases that can phosphorylate kinase Akt at Ser-473. As shown in Fig. 3C, pretreatment of 3T3 cells with 10 μM JD did not block PDGF-induced tyrosine phosphorylation of proteins, nor did it block downstream phosphorylation of Ser-473 in Akt.

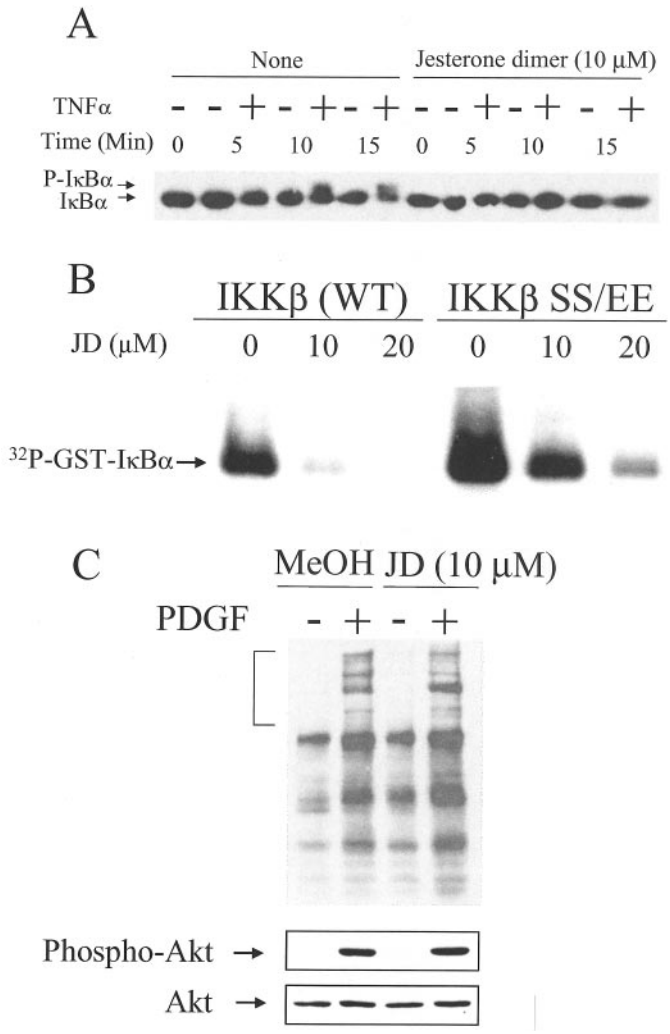


Fig. 3. Jesterone dimer inhibits TNF-α–induced phosphorylation of IκBα by inhibiting IKKβ. A, 3T3 cells were preincubated for 2 h with the calpain inhibitor *N*-acetyl-Leu-Leu-Nle-al in the absence (None) or presence of 10 μM JD. Cells were then treated with 2 ng/ml TNF-α (+) for the indicated time or no TNF-α was added (−). Cell extracts were then analyzed by anti-IκBα Western blotting. The positions of IκBα and the hyperphosphorylated form of IκBα (P-IκBα) are shown. B, cells were transfected with an expression plasmid for FLAG-tagged wild-type IKKβ or a constitutively active form of IKKβ (SS/EE). A kinase assay using GST-IκBα as a substrate was then performed on anti-FLAG immunoprecipitates from cells pretreated with the indicated concentrations of JD for 2 h. C, 3T3 cells were pretreated with MeOH or 10 μM JD for 2 h and were then treated (+) or not treated (−) with 10 ng/ml PDGF for 10 min. Cell extracts were analyzed by use of Western blotting with an anti-phosphotyrosine antiserum (top), anti-phospho-Akt antiserum (middle), or anti-Akt antiserum (bottom). The positions of several induced phosphotyrosine-containing bands are indicated by the bracket at top left.

By Western blotting, we noted that treatment of 3T3 cells with JD converted most of the overexpressed FLAG-IKKβ (wild-type and SS/EE) to stable higher molecular mass forms on SDS-polyacrylamide gels (Fig. 4A). One of these higher molecular mass forms of FLAG-IKKβ migrated at approximately 200 kDa, a size consistent with a dimeric form of IKKβ; additional anti-IKKβ reactive material migrated at the top of the separating gel. Certain other IKKβ inhibitors have been shown to require Cys-179 in the activation loop of IKKβ for their inhibitory activity (see *Discussion*). However, JD treatment also converted IKKβ triple mutant SS/EE/C179A to the same stable higher molecular mass forms, suggesting that JD does not act solely through Cys-179 to modify IKKβ. As a control, we also performed Western blotting for IκBα and actin on the same transfected cell extracts. JD treatment did not convert either of these proteins to high molecular mass species on SDS-polyacrylamide gels (Fig. 4A). Moreover, JD treatment did not affect the overall profile of polypeptides from 3T3 cells as judged by Coomassie blue staining of an SDS-polyacrylamide gel of whole-cell extracts (Fig. 4B).

Previous researchers have shown that transfected FLAG-IKKβ can undergo autophosphorylation in in vitro kinase assays performed with anti-FLAG beads (Mercurio et al., 1997). The JD-induced higher molecular mass form of FLAG-IKKβ that migrates at approximately 200 kDa was also seen as a ^{32}P-labeled band in in vitro kinase assays performed on extracts from IKKβ SS/EE-transfected cells (Fig. 4C).

Taken together, these results show that JD blocks the activation of IKK and that this inhibition is likely to be mediated by a direct interaction of JD with the β subunit of the IKK complex. Moreover, inhibition of IKKβ by JD is at least somewhat specific in that JD does not affect the activity of the PDGF receptor-induced tyrosine and serine phosphorylations, and it does not convert other cellular proteins to higher molecular mass forms.

Jesterone Dimer also Blocks TNF-α–Induced Activation of NF-κB in HeLa Cells. To determine whether JD could block the activation of NF-κB in cell types other than 3T3 cells, we assessed the ability of JD to block NF-κB activation in human HeLa cells. As with 3T3 cells, JD inhibited the ability of TNF-α to induce both NF-κB DNA-binding activity (Fig. 5A) and degradation of IκBα (Fig. 5B) in HeLa cells. As a control, we also showed that JD did not affect the ability of epidermal growth factor to induce tyrosine phosphorylation of polypeptides in HeLa cells (data not shown).

Jesterone Dimer and Isotorreyanic Acid Inhibit Activation of NF-κB at a Concentration of 20 μM, Whereas Several Related Epoxyquinoids Do Not. We have also synthesized a number of dimeric compounds related to JD (Li et al., 2000): the natural product torreyanic acid, and three synthetic derivatives of torreyanic acid (Fig. 6A). To determine whether these dimeric epoxyquinoids could also inhibit NF-κB, we compared their abilities to inhibit TNF-α–induced NF-κB DNA binding and IκBα degradation at a concentration of 20 μM (Fig. 6, B and C). JD and isotorreyanic acid were quite effective blockers of activation of NF-κB. In contrast, torreyanic acid and the synthetic derivative allyl-torreyanic acid had only weak inhibitory effects

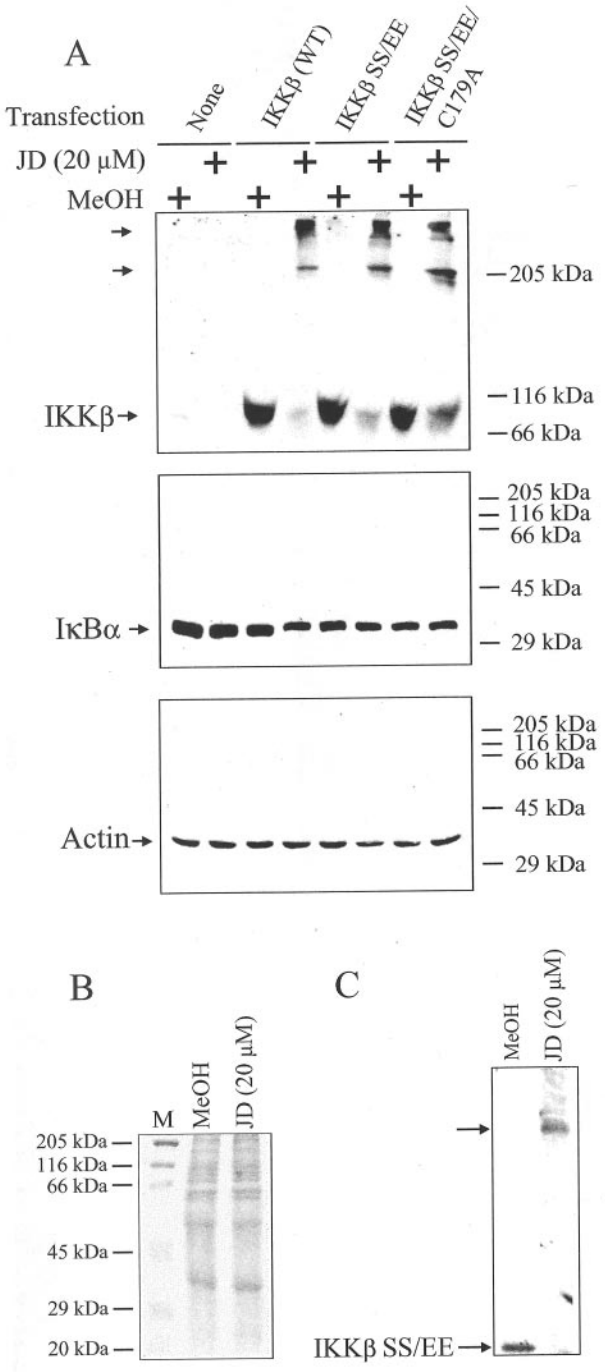


Fig. 4. Jesterone dimer induces stable high molecular mass forms of IKKβ. A, 3T3 cells that had been transfected with expression plasmids for the indicated FLAG-IKKβ proteins were incubated for 2 h with methanol or JD, and cell extracts were then subjected to Western blotting for IKKβ, IκBα, or actin. The monomer form of FLAG-IKKβ is designated by the arrow. The upper arrows designate forms of IKKβ-reactive material at approximately 200 kDa and at the top of the separating gel (arrows). Molecular mass markers are indicated to the right of the gels. The first four lanes of the IKKβ panel were exposed for slightly longer than the last four lanes to normalize the amount of FLAG- IKKβ in the figure. B, Coomassie blue-stained SDS-polyacrylamide gel of whole-cell extracts from 3T3 cells transfected with the FLAG-IKKβ expression vector and treated with methanol or 20 μM JD. C, extracts from 3T3 cells transfected with the FLAG-IKKβ SS/EE mutant and treated with methanol or JD were subjected to an in vitro kinase assay using FLAG beads, as described under *Materials and Methods*. IKKβ SS/EE indicates the monomer form of the protein, and the upper arrow designates a radiolabeled band that migrates at approximately 200 kDa.

on activation of NF-κB, and allyl-isotorreyanic acid did not seem to affect the activation of NF-κB, as judged by either criteria. Thus, some (JD and isotorreyanic acid), but not all, dimeric epoxyquinoids have the ability to block NF-κB activation at low micromolar concentrations.

Jesterone Dimer Inhibits Constitutive Rel/NF-κB DNA Binding and Induces Apoptosis in Human Lymphoma Cells. Several studies have shown that many tumor cells have constitutively active nuclear Rel/NF-κB DNA binding activity, which is required for efficient tumor growth (Gilmore et al., 2002). For example, the SUDHL-4 lymphoma cell line has recently been reported to have constitutive nuclear κB site-binding activity that consists of p50-REL and REL-REL dimers (Davis et al., 2001). A supershift EMSA analysis of extracts from SUDHL-4 cells confirms that these cells contain constitutively active κB site-binding complexes that consist almost exclusively of p50-REL and REL-REL (Fig. 7A).

To determine whether the ability to block κB-site DNA binding correlated with cell killing, we analyzed the effects of JD on the growth of SUDHL-4 cells. In addition, we compared the activity of JD to a monomeric epoxyquinoid, cycloepoxydon, which we have shown previously can also inhibit NF-κB (Li et al., 2001a). In these experiments, we first determined the concentration of JD and cycloepoxydon required to inhibit κB-site DNA binding in SUDHL-4 cells by incubating the cells with increasing concentrations of the compounds for 3 h and then performing an EMSA. As shown in Fig. 7B, 2.5 μM JD and 20 μM cycloepoxydon resulted in approximately 50% inhibition of κB-site DNA binding activity. Second, we determined the effect of various concentrations of JD and cycloepoxydon on the viability of SUDHL-4 cells. As shown in Fig. 7C, at 96 h after treatment, the growth of SUDHL-4 cells was inhibited by approximately 75% in 0.5

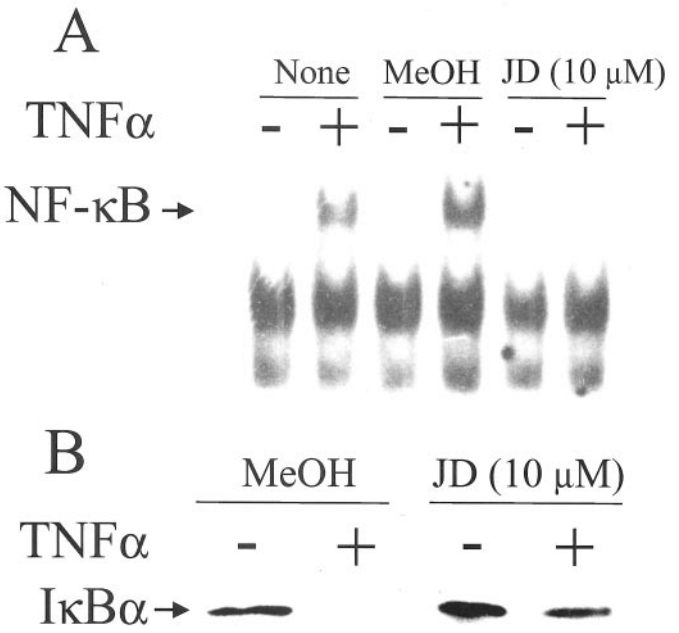


Fig. 5. Jesterone dimer inhibits the activation of NF-κB in HeLa cells. HeLa cells were incubated with media containing no addition (None), the solvent MeOH, or 10 μM JD for 2 h and were then treated with TNF-α (+) for 20 min or not treated as a control (−). Extracts were then subjected to an EMSA using a κB site probe (A) or Western blotting with an IκBα antiserum (B). The position of the NF-κB DNA complex is shown in A.

μM JD and 10 μM cycloepoxydon. In contrast, the natural compound jesterone (monomer) had no effect on the proliferation of SUDHL-4 cells in the concentration range in which JD inhibited the proliferation of these cells. Taken together, these results show that there is a general correlation between the abilities of epoxyquinoid NF-κB inhibitors to block NF-κB DNA binding and cell proliferation in human lymphoma cells.

Many toxic agents kill tumor cells through the conserved intracellular pathway of apoptosis that involves activation of proteases called caspases, and one of the major vertebrate cell-death caspases is caspase-3. To determine whether JD was killing SUDHL-4 cells by apoptosis, we analyzed JD-treated SUDHL-4 cells for caspase-3 activation in two ways: first, for a general increase in caspase-3 activity (as measured by cleavage of a fluorescent substrate), and second, for cleavage of the caspase-3 substrate PARP. As a control, we also analyzed extracts from SUDHL-4 cells after treatment with cycloheximide, a protein synthesis inhibitor that is known to induce apoptosis in many cells. As shown in Fig. 8,

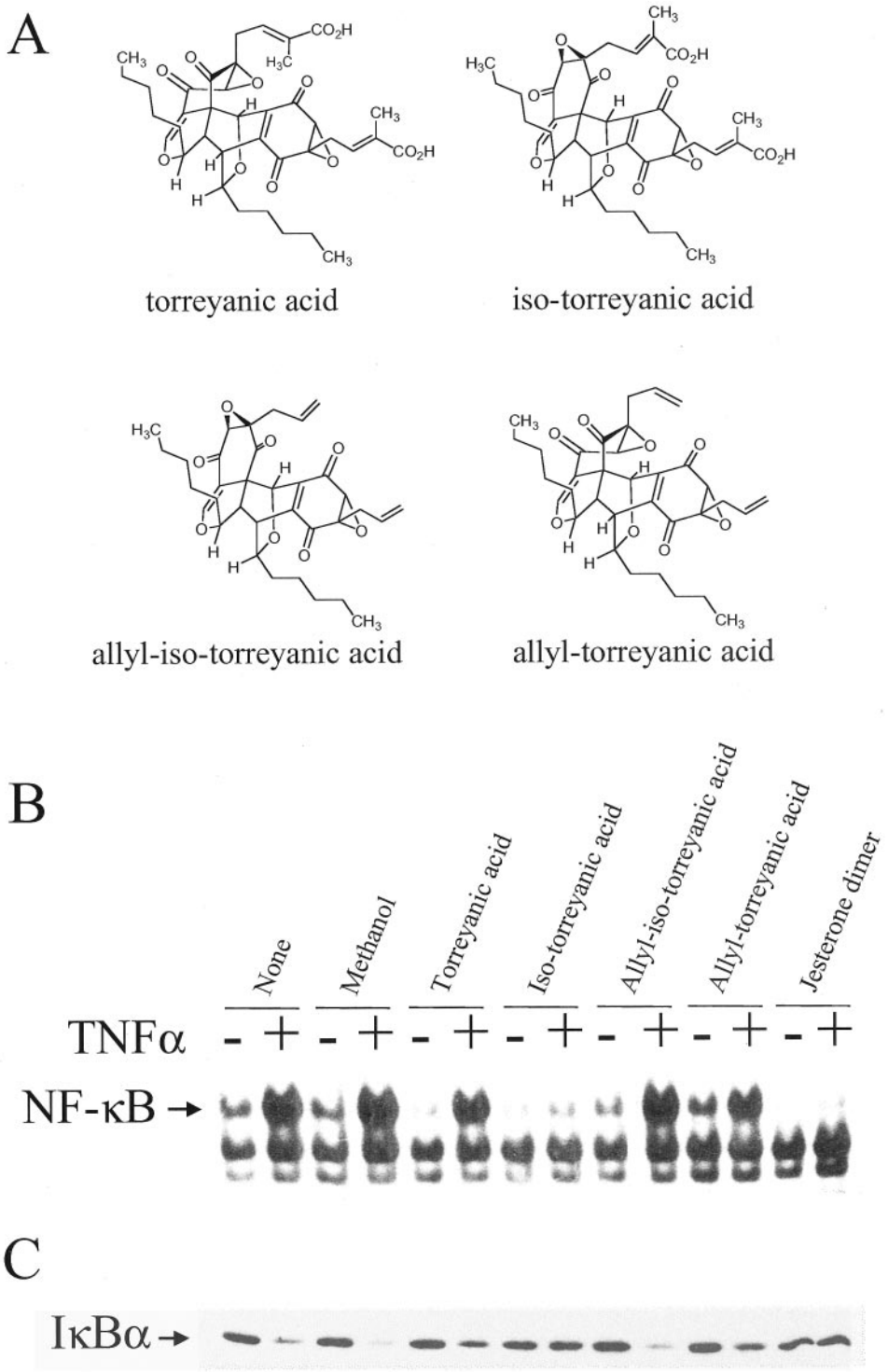


Fig. 6. Jesterone dimer and isotorreyanic acid inhibit activation of NF-κB, but related compounds do not. A, the structures of dimeric epoxyquinoids are shown (Li et al., 2000 gives details on the synthesis of these compounds). B and C, 3T3 cells were preincubated with 20 μM concentrations of the indicated compounds. Cells were then treated with TNF-α and analyzed for NF-κB DNA binding (B) and IκBα levels (C) as described for Fig. 2.

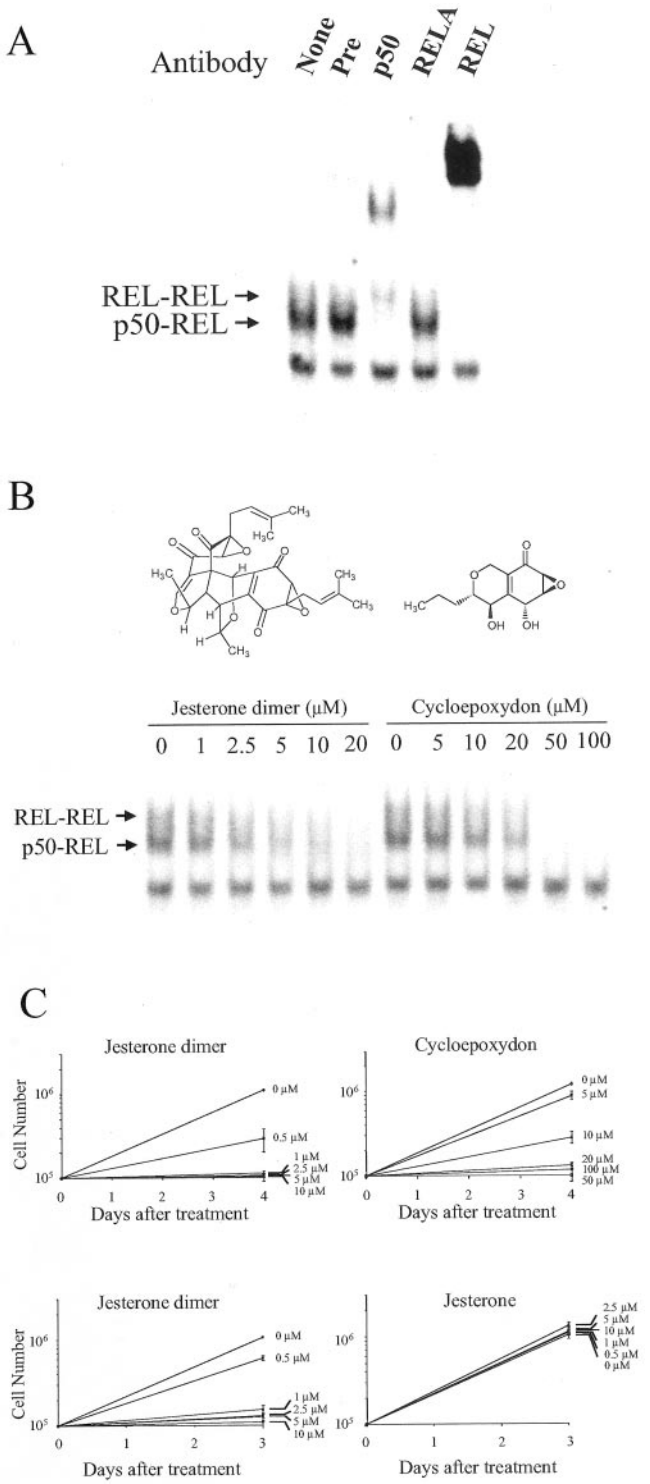


Fig. 7. Jesterone dimer inhibits DNA binding by REL complexes and growth in SUDHL-4 diffuse large B-cell lymphoma cells. A, an EMSA using a κB site probe was performed on extracts from SUDHL-4 cells. As indicated, supershifts were performed with antisera against specific Rel/NF-κB proteins. The positions of the p50-REL and REL-REL DNA complexes are indicated. In B and C, SUDHL-4 cells were incubated with the indicated concentrations of JD, cycloepoxydon, or jesterone for 3 h (B) or 3 to 4 days (C). In B, extracts were then made, and a κB site EMSA was performed. The structures of JD and cycloepoxydon are shown above the figure. In C, 10^5 cells were plated in 16-mm wells in 0.5 ml of DMEM containing 10% fetal bovine serum for 6 h before the addition of the indicated compounds. Cells in triplicate wells for each treatment were then counted 3 or 4 days later; the two experiments at the top and the two experiments at the bottom were performed at different times.

total caspase-3 activity was increased (Fig. 8A), and PARP was cleaved (Fig. 8B) after treatment with either cycloheximide or JD, a result consistent with JD killing cells by caspase-mediated apoptosis.

Discussion

In this report, we show that the synthetic epoxyquinoid jesterone dimer is an effective blocker of TNF-α–induced activation of NF-κB. Moreover, our results strongly suggest that JD blocks the activation of NF-κB by inhibiting the IκB kinase, which is essential for phosphorylating and signaling the degradation of the NF-κB inhibitor IκBα. Finally, treatment of a human lymphoma cell line with JD both inhibits DNA binding by REL and induces apoptosis. These results suggest that JD, or derivative epoxyquinoids, may have therapeutic potential for diseases that involve activation of the Rel/NF-κB pathway.

Several other inhibitors of NF-κB activation seem to target IKKβ (Epinat and Gilmore, 1999; Yamamoto and Gaynor, 2001; Bremner and Heinrich, 2002). In the cases of arsenite, parthenolide, and prostaglandin, a cysteine residue (Cys-179) located in the activation loop of IKKβ has been impli-

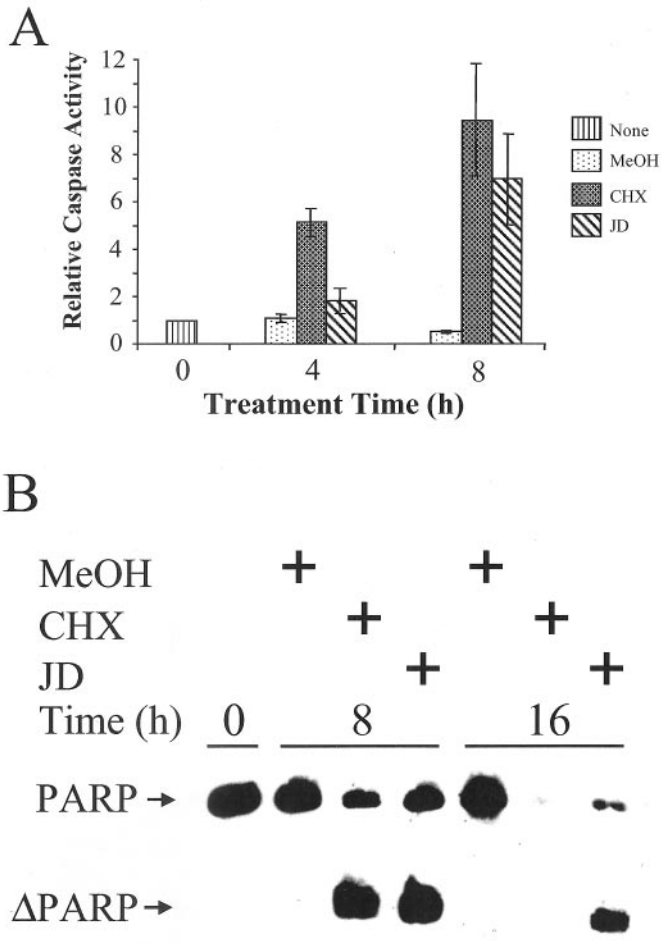


Fig. 8. Jesterone dimer activates caspase activity in SUDHL-4 cells. SUDHL-4 cells were incubated for the indicated times with no addition (None), MeOH, cycloheximide (CHX; 10 μg/ml) or JD (2.5 μM). A, total caspase activity in extracts was measured as described under *Materials and Methods*. B, PARP cleavage was monitored by Western blotting. The upper band indicates the position of full-length PARP, and the lower band (ΔPARP) indicates the position of the caspase-cleaved form of PARP.

cated in their inhibitory activity (Kapahi et al., 2000; Rossi et al., 2000; Kwok et al., 2001). However, at least one other inhibitor of IKKβ, hypoestoxide, does not seem to require Cys-179 (Ojo-Amaize et al., 2001). We do not yet know how JD inhibits IKKβ, but the electrophilic nature of JD suggests that it may do so by targeting a thiol group, such as would be present at Cys-179. Indeed, recent studies have documented the reactivity of epoxyketone natural products with thiols (Wipf et al., 1998). However, Cys-179 is unlikely to be the only residue required for JD to inhibit IKKβ in that the IKKβ mutant SS/EE/C179A is still converted to higher molecular mass forms by JD treatment (Fig. 4A). Moreover, the monomeric natural compound jesterone, which could also react with susceptible thiol groups or other nucleophilic residues, does not inhibit NF-κB activation, does not induce higher molecular mass forms of IKKβ (data not shown), and presumably does not block IKKβ activity. Thus, the dimeric nature of JD may increase the number of sites for attachment to the protein target, which may enhance its anti-IKKβ activity. Nevertheless, not all dimeric epoxyquinoids can inhibit the activation of NF-κB (Fig. 6).

As is the case with many bioactive compounds (Niculescu-Duvaz et al., 1999; van Balderen, 2000), we believe it is likely that JD is converted in cells to an active form, which may or may not resemble a dimeric compound. Indeed, incubation of IKKβ with JD in vitro does not inhibit its kinase activity (data not shown). Moreover, in vitro incubation of JD with glutathione/GST or heating converts JD to new forms (data not shown). Thus, future experiments will be directed toward identifying the active form(s) of JD and the critical structural requirements of JD and IKKβ necessary for kinase inhibition.

In cells overexpressing FLAG-IKKβ, JD treatment converted much of the FLAG-IKKβ to (at least) two stable forms: a form of ~200 kDa and a form(s) of higher molecular mass that barely entered the separating gel (Fig. 4A). In normal cells, most endogenous, uninduced IKKβ is believed to be in a high molecular complex that also includes IKKα, IKKγ, and perhaps other proteins (Ghosh and Karin, 2002). In contrast, when overexpressed in 3T3 cells, even wild-type IKKβ is freely active (Fig. 3B), no doubt because much of the overexpressed IKKβ is in the form of active homodimers, although some overexpressed IKKβ may also enter higher molecular mass cellular complexes (Li et al., 2001b). Thus, we believe that it is likely that the approximately 200-kDa form of FLAG-IKKβ that appears after JD treatment corresponds to a cross-linked homodimer of FLAG-IKKβ, and that the material at the top of the gel corresponds either to tetramers (i.e., dimers of dimers) of FLAG-IKKβ or to FLAG-IKKβ complexes containing cellular proteins. If JD does cross-link IKKβ into homodimers, it may provide a reagent for stabilizing IKKβ dimers for crystallographic studies, which have not yet been reported on any IKK complexes. Of note, another IKK inhibitor, 4-hydroxy-2-nonenal, has also recently been shown to convert the IKK complex to a stable higher molecular mass form (Ji et al., 2001).

JD is likely to inhibit the proliferation of B-cell lymphoma SUDHL-4 cells (Fig. 7C) by inducing apoptosis. First, JD has been shown to be cytotoxic to other tumor cells (Bargou et al., 1997; Hu et al., 2001). Second, inhibition of constitutive Rel/NF-κB activity can induce apoptosis in other lymphoid tumor cells (Davis et al., 2001). Third, in the presence of JD, caspase-3 activity is increased, and the caspase-3 substrate PARP is cleaved (Fig. 8). Furthermore, JD-treated SUDHL-4 cells become quite small and dark and take on the appearance of cells undergoing apoptosis (data not shown).

Our antibody supershift analysis indicates that the active Rel/NF-κB DNA binding activity in SUDHL-4 lymphoma cells almost exclusively consists of p50-REL and REL-REL dimers. Treatment of SUDHL-4 cells results in the disappearance of these REL-containing DNA-binding complexes, but does not affect the levels of a nonspecific band in the EMSAs (lower band in Fig. 7B), indicating that the inhibition of REL DNA binding by JD is at least somewhat specific. Considering the ability of JD to inhibit the activation of NF-κB in 3T3 cells by blocking IKK activity, it is possible that JD blocks a continual activation of the IKK pathway that leads to the activation of p50-REL and REL-REL dimers in SUDHL-4 cells. Furthermore, in that overexpression of human REL can immortalize and transform chicken lymphoid cells (Gilmore et al., 2001) and that nuclear Rel/NF-κB activity has, in many cases, been shown to block apoptosis (Barkett and Gilmore, 1999), the inhibition of REL DNA binding may be the cause of the apoptosis induced by JD in SUDHL-4 cells. Consistent with the ability of JD to induce apoptosis in SUDHL-4 B lymphoma cells, Pasparakis et al. (2002) have shown recently that IKK signaling is also required for the survival of normal mature B cells, which are well known to contain constitutively active NF-κB DNA binding activity.

We have synthesized previously the monomeric epoxyquinoid cycloepoxydon and shown that it can inhibit TNF-α–induced activation of NF-κB in mouse 3T3 cells at a concentration of approximately 50 μM (Li et al., 2001a). In this report, we show that JD is approximately 10 to 20 times more effective than cycloepoxydon for both SUDHL-4 cell killing and blocking of NF-κB DNA binding in 3T3 cells and SUDHL-4 cells. We note also that the concentrations of JD and cycloepoxydon that are required to kill SUDHL-4 cells are much lower than the concentrations of these compounds that are required to inhibit Rel/NF-κB DNA binding in these cells. However, it is important to consider that the DNA binding assays in SUDHL-4 cells were performed after incubating the cells with JD or cycloepoxydon for only 3 h, whereas the viability assays looked at long-term (96 h) incubation of the cells with the compounds. Moreover, in SUDHL-4 cells, one is looking at the inhibition of DNA binding activity that is constitutively present (i.e., not transiently induced). Thus, only a minimal, but continuous, inhibition of Rel/NF-κB activation by JD in SUDHL-4 cells may be required to cause cell death. Finally, both compounds (JD and cycloepoxydon) may have targets in addition to the Rel/NF-κB pathway that enhance their cell-killing effects.

Interestingly, Kakeya et al. (2002) recently identified a natural fungal metabolite, epoxyquinol A, that bears striking structural resemblance to JD. However, epoxyquinol A and JD have opposite enantiomeric compositions. Nevertheless, we have recently shown that synthetic epoxyquinol A and related derivatives can also block the activation of NF-κB in mouse 3T3 cells, although it is not known how epoxyquinol A blocks the activation of NF-κB (Li et al., 2002).

Future experiments will address the precise mechanism by which JD and other epoxyquinoids inhibit the activation of the NF-κB pathway. Because of the importance of the Rel/

NF-κB pathway in inflammation and disease, the development of Rel/NF-κB inhibitors derived from fungal epoxyquinoids may have therapeutic relevance.

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