

# Eriocalyxin B Inhibits Nuclear Factor- $\kappa$ B Activation by Interfering with the Binding of Both p65 and p50 to the Response Element in a Noncompetitive Manner

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## ABSTRACT

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) has been recognized to play a critical role in cell survival and inflammatory processes. It has become a target for intense drug development for the treatment of cancer, inflammatory, and autoimmune diseases. Here, we describe a potent NF- $\kappa$ B inhibitor, eriocalyxin B (Eri-B), an *ent*-kauranoid isolated from *Isodon eriocalyx*, an anti-inflammatory remedy. The presence of two  $\alpha,\beta$ -unsaturated ketones give this compound the uniqueness among the *ent*-kauranoids tested. Eri-B inhibited the NF- $\kappa$ B transcriptional activity but not that of cAMP response element-binding protein. It suppressed the transcription of NF- $\kappa$ B downstream gene products including cyclooxygenase-2 and inducible nitric-oxide synthase induced by tumor necrosis factor- $\alpha$  or lipopolysaccharide in macrophages and hepatocarcinoma cells. Chromatin immuno-

precipitation assay indicated that Eri-B selectively blocked the binding between NF- $\kappa$ B and the response elements in vivo without affecting the nuclear translocation of the transcription factor. Down-regulation of the endogenous p65 protein sensitized the cells toward the action of the compound. Furthermore, in vitro binding assays suggested that Eri-B reversibly interfered with the binding of p65 and p50 subunits to the DNA in a noncompetitive manner. In summary, this study reveals the novel action of a potent NF- $\kappa$ B inhibitor that could be potentially used for the treatment of a variety of NF- $\kappa$ B-associated diseases. Modification of the structure of this class of compounds becomes the key to the control of the behavior of the compound against different cellular signaling pathways.

NF- $\kappa$ B is the transcription factor that controls inflammatory and immune responses and cell growth and survival. Since the discovery of NF- $\kappa$ B in 1986 (Sen and Baltimore, 1986), both the cytoplasmic and nuclear regulations of NF- $\kappa$ B have been well-characterized. In the classic activation pathway that can be stimulated by TNF- $\alpha$  and LPS, the NF- $\kappa$ B heterodimers of p65:p50 mainly localize in the cytoplasm through their interaction with I $\kappa$ B $\alpha$ . Activation of the cells leads to the phosphorylation of I $\kappa$ B- $\alpha$  by the IKK complex

(which consists of IKK $\alpha$ , IKK $\beta$ , and NF- $\kappa$ B essential modulator/IKK $\gamma$ ), rapid ubiquitination, and subsequent degradation of I $\kappa$ B $\alpha$  through the 26S proteasome pathway. This promotes the translocation of the p65:p50 complexes into the nucleus where they bind to the NF- $\kappa$ B response elements and regulate the expression of their downstream genes (Chen and Greene, 2004; Hayden and Ghosh, 2004). Many of these steps have become targets for drug development (Yamamoto and Gaynor, 2001; Bharti and Aggarwal, 2002; Celec, 2004; Karin et al., 2004).

Several different strategies for inhibiting the NF- $\kappa$ B pathways are currently being investigated, but a major effort has been put on NF- $\kappa$ B cytoplasmic regulation. These include the attenuation of phosphorylation or proteosomal degradation of I $\kappa$ B- $\alpha$ . Several nonsteroidal anti-inflammatory drugs and

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**ABBREVIATIONS:** NF- $\kappa$ B, nuclear factor- $\kappa$ B; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; LPS, lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; FSK, forskolin; IKK, I $\kappa$ B kinase; PCR, polymerase chain reaction; COX-2, cyclooxygenase-2; iNOS, inducible nitric-oxide synthase; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; CRE, cAMP response element; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2; Dox, doxycycline; PKC, protein kinase C; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; Eri-B, eriocalyxin B; DTT, dithiothreitol; CREB, cAMP response element-binding protein; PS-341, bortezomib.

immunomodulatory drugs are capable of inhibiting NF- $\kappa$ B activation by interfering with the IKK activity (Karin et al., 2004). Inhibitors (e.g., PS-341) were also developed to prevent I $\kappa$ B- $\alpha$  degradation by inhibiting the proteasome-dependent proteolytic process (Twombly, 2003; Adams, 2004). Because the proteasome, which is responsible for I $\kappa$ B- $\alpha$  degradation, is also involved in many important cellular processes (Jentsch and Schlenker, 1995; Adams, 2003), application of these drugs could potentially cause undesirable effects.

Discovery of inhibitors that preferentially target the binding of NF- $\kappa$ B to its consensus DNA sequence is also actively being pursued. Plants belonging to the genus *Isodon* are recognized to contain natural constituents that display anti-tumor, antibacterial, and anti-inflammatory activities and are known to be rich in *ent*-kaurane diterpenoids (Fujita et al., 1976; Sun et al., 1995; Niu et al., 2002; Lee et al., 2004; Zhang et al., 2005). Four of these compounds isolated from *Isodon rubescens* are active against NF- $\kappa$ B displaying a novel mechanism of action (Leung et al., 2005). Eriocalyxin B (Fig. 1), isolated from *Isodon eriocalyx* (Dunn) Hara var. *laxiflora*, has been used for the treatment of inflammatory diseases such as tonsillitis, pharyngitis, and laryngitis. We reported previously that Eri-B inhibited NF- $\kappa$ B activity by targeting multiple steps of the NF- $\kappa$ B activation pathway in a concentration-dependent manner (Leung et al., 2006). Eri-B inhibits the binding of NF- $\kappa$ B to the consensus DNA sequence, although, at higher concentrations, it also blocks NF- $\kappa$ B nuclear translocation. Similar observation was also reported by Wang et al. (2006) who also demonstrated that Eri-B exhibited strong antitumor activity against leukemia in vivo and induced apoptosis through NF- $\kappa$ B and MAPK signaling pathways.

In this study, we provide clear evidence of the unique action of Eri-B on the interaction between NF- $\kappa$ B and its response elements. At relevant concentrations, Eri-B interferes with the NF- $\kappa$ B DNA-binding activity without blocking the translocation of NF- $\kappa$ B. The inhibitory process may involve the reversible inhibition of the DNA-binding activity of both p65 and p50 subunits, although it does not compete with the DNA for the active binding site. In addition, by comparing the biological activity with three other structurally similar *ent*-kauranes, the moieties of Eri-B that are critical for the activity were identified. This provides the important in-

formation for the development of more potent and selective NF- $\kappa$ B inhibitors.

## Materials and Methods

**Materials and Compounds.** Eriocalyxin B and maoecrystal C from *I. eriocalyx*, effusanin A from *Isodon effuse*, and xerophilusin I from *Isodon xerophilus* were isolated in H. D. Sun's laboratory of the People's Republic of China (purity, >98%). TNF- $\alpha$ , PMA, and LPS were purchased from Calbiochem (San Diego, CA). Antibodies against p65, p50, CREB, ornithine carbamyl transferase-1, human and mouse iNOS, and COX-2 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-I $\kappa$ B- $\alpha$ , anti-phospho-I $\kappa$ B- $\alpha$ , anti-IKK $\alpha$ , and anti-IKK $\beta$  were acquired from Cell Signaling Technology (Danvers, MA).

**Cell Culture and Drug Treatment.** Cell lines including human hepatocellular carcinoma HepG2, human monocyte THP-1, human pancreatic cancer PANC-1, and mouse macrophage RAW264.7 cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. THP-1 cells were preincubated with 100 ng/ml PMA for 3 days followed by PMA-free growth medium for 1 day before the drug treatment.

HepG2 stably or transiently transfected with pBIIX-luc (containing two tandem-repeated NF- $\kappa$ B binding sites, provided by Dr. Ghosh, Yale University, New Haven, CT); pCRE-Luc or pAP1-Luc (Clontech, Mountain View, CA) was established using FuGene 6 (Roche Applied Science, Indianapolis, IN) as described previously (Gao et al., 2004).

Doxycycline-inducible down-regulation of p65 in HepG2 cells was established as described previously (Lam et al., 2006). HepG2 cells stably transfected with pBIIX-luc were grown in RPMI medium supplemented with 10% tetracycline-free fetal bovine serum (Clontech) and 400  $\mu$ g/ml G418 (Invitrogen, Carlsbad, CA). The complementary DNA oligonucleotide NF- $\kappa$ B (5'-CACCGACATATGAGACCTTCAAGAcgaaTCTTGAAGGTCTCATATGTCC-3') (sense-loop-antisense) or control sequence (5'-ATGCATTCTAGTACCGGTAGGcgaaCCTACCGGTACTAGAATGCA-3') was cloned into pENTR/H1/TO to express short hairpin RNA (shRNA).

For the drug treatment, cells were preincubated with drugs for 1 h and were activated subsequently with TNF- $\alpha$  (25 ng/ml), LPS (1  $\mu$ g/ml), FSK (10  $\mu$ M), or PMA (20 ng/ml) for the time indicated.

**Luciferase Reporter Assay.** Drug-treated cells carrying reporter genes were lysed in Passive Lysis Buffer (Promega, Madison, WI). The transcriptional activity was determined by measuring the activity of firefly luciferase in a multiwell plate luminometer (Tecan, Durham, NC) using Luciferase Reporter Assay system (Promega), according to the manufacturer's instructions.

**Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction.** Total RNA was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA). All of the reverse-transcriptase reactions were performed using Platinum Quantitative RT-PCR ThermoScript One-Step System (Invitrogen) according to the manufacturer's instructions. Assays were performed using iCycler iQ RealTime thermocycler detection system (Bio-Rad Laboratories, Hercules, CA) as described previously (Leung et al., 2005). Sequences of primer pairs and Taqman probes (Biosearch Technologies, Novato, CA) are shown in Table 1.

**Western Blot Analysis.** Total cell lysates were obtained by direct lysis in 2 $\times$  SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue). The samples were fractionated in a 10% acrylamide gel, transferred to a nitrocellulose membrane (Bio-Rad), and incubated with specific antibodies followed by the corresponding horseradish peroxidase-conjugated secondary antibodies. Proteins of interest were visualized by chemiluminescent detection.

**Immunofluorescence Staining.** HepG2 cells were grown on chamber slides for 1 day before the drug treatment. Drug-treated

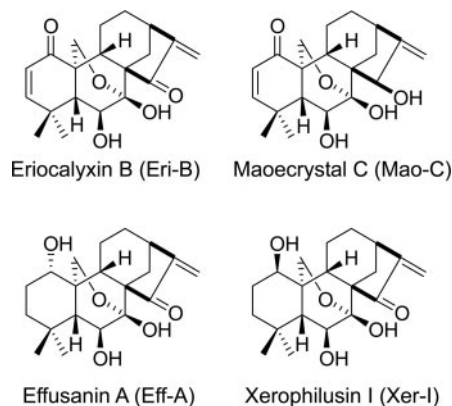


Fig. 1. Chemical structures of eriocalyxin B, maoecrystal C, effusanin A, and xerophilusin I.

cells were fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton X-100. To study the localization of NF- $\kappa$ B, cells were incubated with 1:100 of rabbit anti-p65 antibody, followed by 1:100 of anti-rabbit IgG-fluorescein isothiocyanate and 1:200 of the actin probe, BODIPY 558/568 phalloidin (Invitrogen). Actin and p65 were detected by confocal microscopy.

**In Vitro I $\kappa$ B Kinase Assay.** Cells were lysed in kinase lysis buffer containing 50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 10% glycerol, 1% NP-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, and 1 $\times$  cocktail protease inhibitors (Roche Applied Science). One milligram of protein was immunoprecipitated with anti-IKK $\alpha$  antibody followed by protein G plus/protein A agarose (Calbiochem) at 4°C overnight. Beads were washed three times with kinase lysis buffer and twice with wash buffer (20 mM HEPES, pH 7.5, 20 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, 1 mM NaF, 10 mM  $\beta$ -glycerophosphate, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10  $\mu$ M ATP). Kinase assays were performed by incubation with [ $\gamma$ -<sup>32</sup>P]ATP, for IKK assay, in the presence of recombinant I $\kappa$ B- $\alpha$  (Santa Cruz Biotechnologies), followed by SDS-PAGE and autoradiography.

**Chromatin Immunoprecipitation Assay.** HepG2 cells stably transfected with pBIIX-luc or pCRE-Luc were incubated with 1% formaldehyde at 37°C for 15 min and lysed in ChIP buffer (1% Triton X-100, 0.1% deoxycholate, 50 mM Tris 8.1, 150 mM NaCl, and 5 mM EDTA), followed by sonication to make ~1000-base pair DNA fragments. The samples were incubated with specific antibody. Salmon sperm DNA-saturated protein G plus/protein A agarose was added to precipitate the protein-DNA complexes. The complexes were sequentially washed once with 0.1% SDS, ChIP buffer, once with 500 mM NaCl, 0.1% SDS, ChIP buffer, and once with LiCl wash buffer (250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 10 mM Tris 8.1, and 1 mM EDTA). Beads were washed twice with 10 mM Tris 8.1 and 1 mM EDTA before elution at 65°C in 1% SDS, 10 mM Tris 8.1, and 1 mM EDTA. Supernatants were incubated overnight at 65°C to reverse cross-links, and the DNA was purified using the QIAquick gel extraction kit (QIAGEN). The amount of promoter sequences bound to NF- $\kappa$ B were determined by semiquantitative PCR using Expand Long Template PCR System (Roche Applied Science) and the following probes and primers, according to the manufacturer's instructions (Table 2).

**Electrophoretic Mobility Shift Assay.** Nuclear extracts prepared according to Dignam et al. (1983) were incubated with [ $\gamma$ -<sup>32</sup>P]ATP-labeled NF- $\kappa$ B consensus oligonucleotides (Promega) in a gel-shift binding buffer [10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM EDTA, 0.05% nonfat dry milk, 5% glycerol, 0.01% saturated bromophenol blue, and 50  $\mu$ g/ml poly(dIdC)] for 40 min at room tempera-

ture and separated in 5% native polyacrylamide gels followed by autoradiography.

**Coimmunoprecipitation Assay.** Drug-treated nuclear extracts of HepG2 cells were incubated with anti-p50 antibody for 2 h followed by protein G plus/protein A agarose at 4°C overnight. Samples were washed five times with 50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1% NP-40 and subjected to Western blot analysis as described above.

**Purification of p65 and p50 Subunits.** The pET-21b-p65 and pET-21b-p50 constructs were expressed in *Escherichia coli* BL21(DE3) cells. The cells were grown at 37°C in a shaking incubator until the culture reached absorbance at 600 nm of 0.6. Expression of the protein from the T7 promoter was induced for 16 h at 16°C by the addition of 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (final concentration). The cells were then harvested in lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA,  $\beta$ -mercaptoethanol, phenylmethylsulfonyl fluoride, protease inhibitors, and 1 mg/ml lysozyme) and lysed by sonication. The cell debris was pelleted by ultracentrifugation (27,500 rpm, 4°C, 40 min). The supernatant diluted with binding buffer (25 mM, Tris pH 7.4, 500 mM NaCl, and 5 mM imidazole) were loaded onto His-Bind Quick Columns (Novagen, Madison, WI), and the bound proteins were eluted with a 20 to 200 mM imidazole gradient in the binding buffer. The p65 partial purified protein was further purified by incubating with Macro-Prep Ceramic Hydroxyapatite TYPE II (Bio-Rad Laboratories). The elution fractions that contained p65 or p50 protein were pooled and dialyzed against 10 mM Tris-HCl, pH 7.9, 10% glycerol, 1 mM EDTA, 50 mM NaCl, and  $\beta$ -mercaptoethanol. All proteins were >90% pure as judged by electrophoresis on SDS-PAGE stained with Coomassie Blue (Fig. 8b).

**Statistical Analysis.** Data were presented as means  $\pm$  S.D. for each group. The significance of the data were examined by Student's *t* test. The difference was considered to be statistically significant if *p* < 0.05.

## Results

**Eri-B Blocks NF- $\kappa$ B Activation.** Four structurally similar *ent*-kauranes—Eri-B, Mao-C, Eff-A, and Xer-I—were isolated from *Isodon* species, which have been traditionally used for the treatment of inflammatory diseases and cancers (Fig. 1). The effect of these compounds on the activation of NF- $\kappa$ B and CREB was studied using the luciferase reporter system (Fig. 2). Eri-B was found to be the most potent inhibitor against the transcriptional activity of NF- $\kappa$ B induced by TNF- $\alpha$ , whereas it had no significant impact on the CREB

TABLE 1  
Sequences of primer pairs and Taqman probes

Human COX-2	
Probe	5'-Quasar670d (AGCCCTTCCTCCTGTGCCTGATGA) BHQ-2-3'
Forward	5'-GAAGCCTTCTCTAACCTCTCTAT-3'
Reverse	5'-TCCACAACTCTCATTGGAATCAGG-3'
Human iNOS	
Probe	5'-Quasar670d (TCCGACATCCAGCCGTGCCACCA) BHQ-2-3'
Forward	5'-AGGCTCAAATCTCGGCAGAAATC-3'
Reverse	5'-CCTCACAGGAGAGTTCCACCA-3'
Mouse COX-2	
Probe	5'-Quasar670d (CCCTGTGCCCCGACACCTTCAACA) BHQ-2-3'
Forward	5'-TTCAACACACTCTATCACTGGCAC-3'
Reverse	5'-GCAATCTGTCTGGTGAATGACTCA-3'
Mouse iNOS	
Probe	5'-Quasar670d (CCGACGCTCCTCACTGGGACAGCA) BHQ-2-3'
Forward	5'-CCCTAAGAGTCACCAAAATGGCTC-3'
Reverse	5'-ATACTGTGGACGGTTCGATGG-3'
$\beta$ -Actin	
Probe	5'-T (CalRed) d (CAAGATCATGTCTCCTCTGAGCGCA) BHQ-2-3'
Forward	5'-ATTGCCGACAGGATGCAGAA-3'
Reverse	5'-GCTGATCCACATCTGCTGGAA-3'

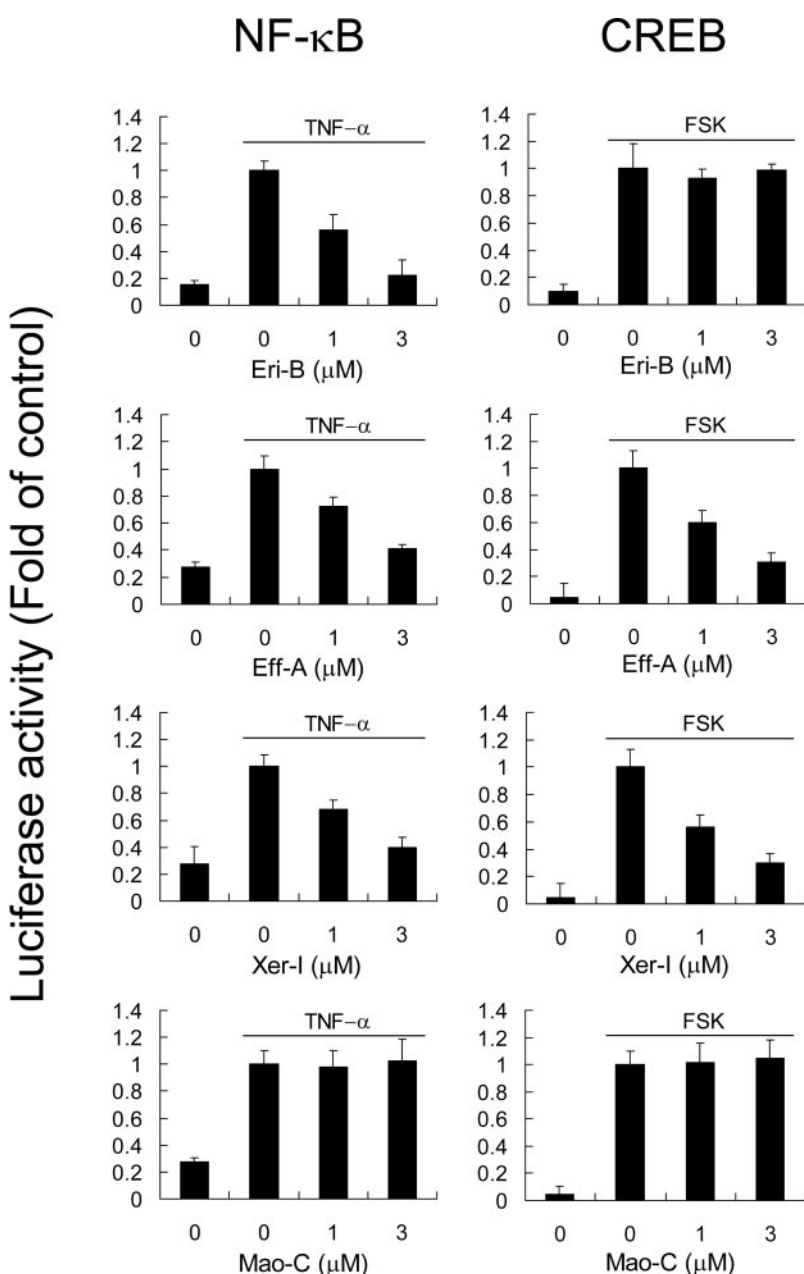
activity induced by forskolin through protein kinase A. However, Eff-A and Xer-I, two stereoisomers with the hydroxyl group instead of the  $\alpha,\beta$ -unsaturated ketone on the A-ring, inhibited both NF- $\kappa$ B and CREB transcriptional activity with

TABLE 2  
Primers for the PCR amplification of gene promoters

pBIIX-luc Reporter	
Forward	5'-AAAGCAATAGCATCACAATTTTCACA-3'
Reverse	5'-GATCCAGACATGATAAGATACATTGATGA-3'
Human iNOS	
Forward	5'-TAACAGCAAGATCAGGTCACCCAC-3'
Reverse	5'-ACTCACCCTATGCCATCCAGAGAG-3'
pCRE-Luc reporter	
Forward	5'-GTCCCCAGTGAAGTGCAGG-3'
Reverse	5'-CCAGCGTCTTGTTCATTTGGCG-3'
$\beta$ -Actin	
Forward	5'-CGGCCAACGCCAAAAC-3'
Reverse	5'-CCCTCTCCCTCCTTTTGC-3'

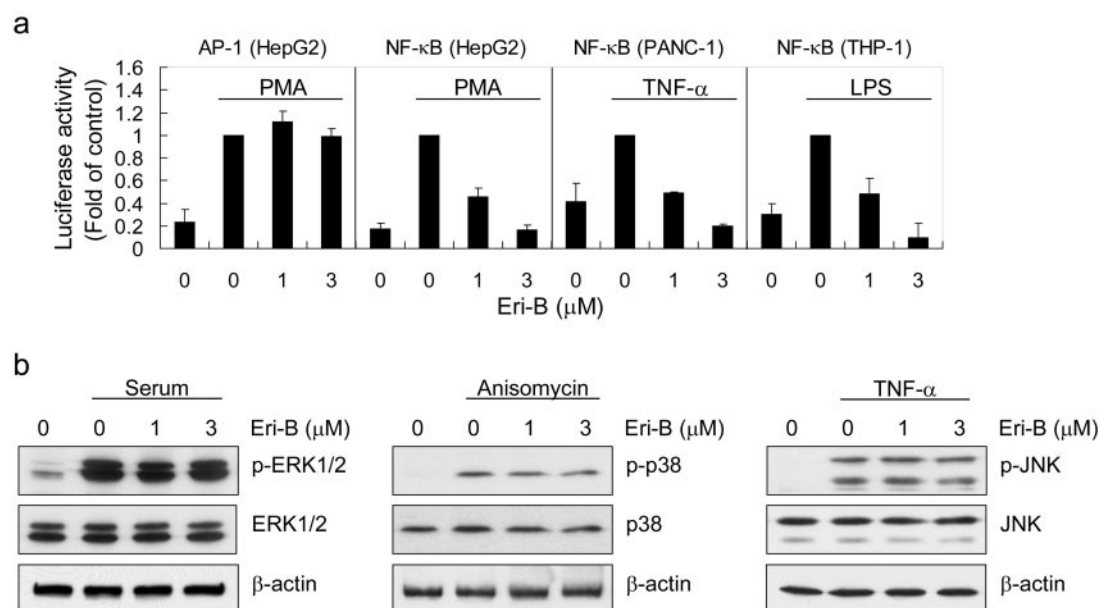
a comparable potency. It is believed that the presence of the  $\alpha,\beta$ -unsaturated ketone on the D-ring of this class of compounds is critical for their biological activity. This is in agreement with our finding that Mao-C, which does not have the D-ring keto group, was inactive against both transcription factors. We therefore focused our study on Eri-B based on its unique biological behavior.

**Eri-B Targets the Common Step(s) of NF- $\kappa$ B Activation Pathways.** NF- $\kappa$ B activation occurs in different cell types in response to a variety of agents (e.g., TNF- $\alpha$ , PMA, and LPS), which also play important roles in the activation of PKC and MAPKs (Janssens and Beyaert, 2003; Shen et al., 2005; Viatour et al., 2005). Eri-B suppressed TNF- $\alpha$ - and LPS-induced NF- $\kappa$ B activity in PANC-1 and THP-1 cells, respectively (Fig. 3a). We were surprised to find that it inhibited PMA-induced NF- $\kappa$ B but not activator protein 1 transcriptional activity in HepG2 cells. The impact of Eri-B on the

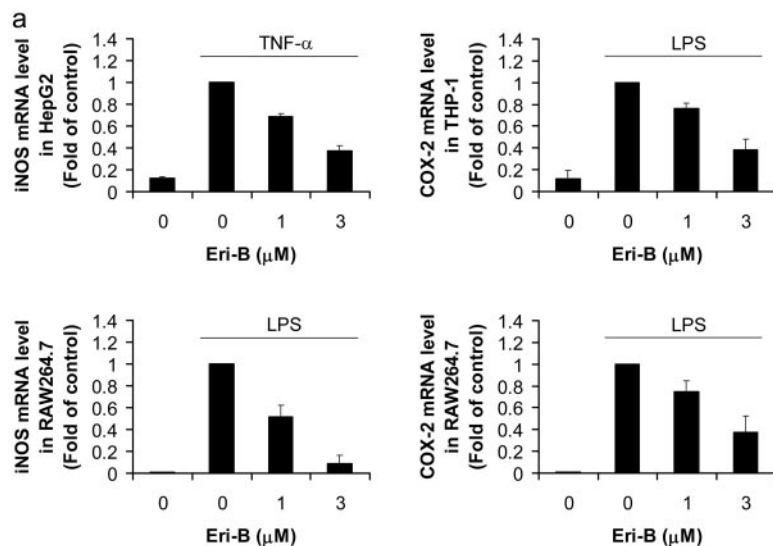


**Fig. 2.** Effect of *ent*-kauranes on the transcriptional activity of NF- $\kappa$ B and CREB. HepG2 cells stably transfected with NF- $\kappa$ B or CRE reporter gene were pretreated with the indicated *ent*-kauranes followed by activation with TNF- $\alpha$  or FSK for 4 h. The transcriptional activity was determined by measuring the luciferase activity. The data were expressed as -fold of control, where the control was the luciferase activity (arbitrary unit) of TNF- $\alpha$ - or FSK-stimulated cells in the absence of drugs.

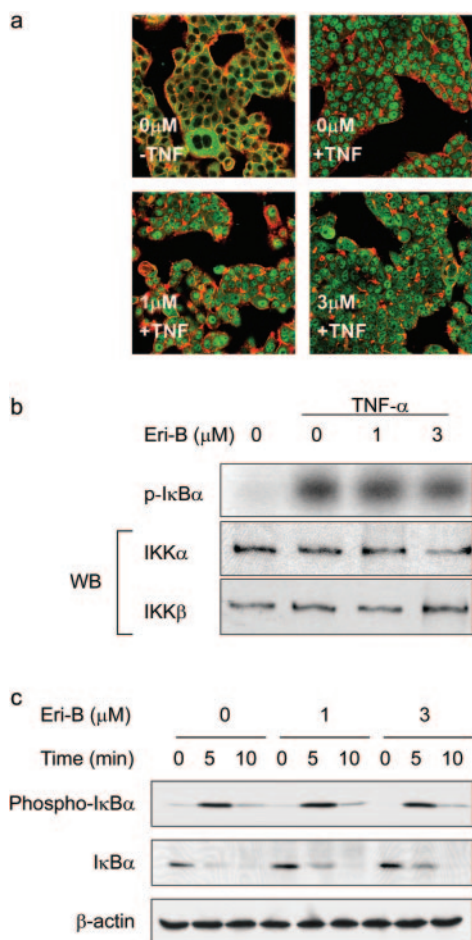




**Fig. 3.** Eri-B inhibits NF-κB activated by different agents in various cell lines without significant effect on PKC and MAPK pathways. **a**, HepG2, PANC-1, and THP-1 cells transfected with the NF-κB or activator protein 1 reporter gene were pretreated with Eri-B before PMA, TNF-α, and LPS stimulations for 4 h. The transcriptional activity was determined by measuring the luciferase activity. The data were expressed as -fold of control, where the control was the luciferase activity (arbitrary unit) of PMA-, TNF-α-, or LPS-stimulated cells in the absence of Eri-B. **b**, HepG2 cells were pretreated with Eri-B. Serum (20%, 15 min), anisomycin (25 μg/ml, 20 min), and TNF-α (25 ng/ml, 20 min) were added to activate ERK1/2, p38, and c-Jun-N-terminal kinase, respectively. ERK1/2 cells were serum-starved for 24 h before drug treatment. Cell extracts were subjected to Western blot analysis using specific antibodies.



**Fig. 4.** Eri-B suppresses iNOS, COX-2 mRNA, and protein levels. HepG2, THP-1, and RAW264.7 cells were treated with Eri-B before activation with TNF-α or LPS. Total RNA and total protein were extracted after 4 and 8 h of stimulation, respectively. **a**, mRNA levels of iNOS and COX-2 were determined by quantitative real-time reverse transcriptase-polymerase chain reaction. **b**, total protein extracts were subjected to Western blot analysis using iNOS- and COX-2-specific antibodies.

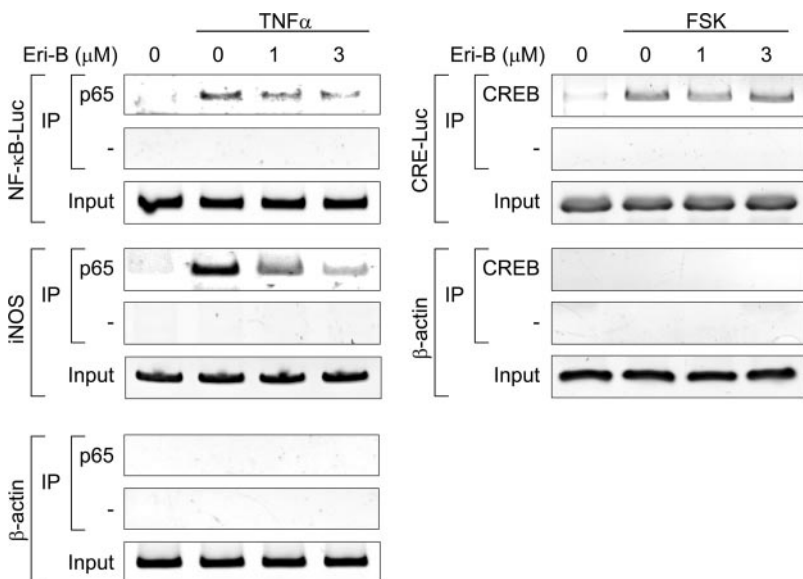


**Fig. 5.** Eri-B does not block TNF- $\alpha$ -induced NF- $\kappa$ B nuclear translocation, I $\kappa$ B- $\alpha$  degradation and IKK activity. HepG2 cells were preincubated with Eri-B before TNF- $\alpha$  stimulation for the time indicated. a, cells were fixed 15 min after TNF- $\alpha$  activation. Distribution of NF- $\kappa$ B and  $\beta$ -actin were recognized by the green and red fluorescence, respectively. b, protein lysates were collected and analyzed by Western blotting using antibodies against phospho-I $\kappa$ B $\alpha$  and I $\kappa$ B $\alpha$ . c, cell extracts were immunoprecipitated with anti-IKK $\alpha$  antibody and analyzed by an *in vitro* kinase assay using recombinant I $\kappa$ B- $\alpha$  as a substrate. Levels of the immunoprecipitated IKK $\alpha$  and IKK $\beta$  were determined by Western blot analysis.

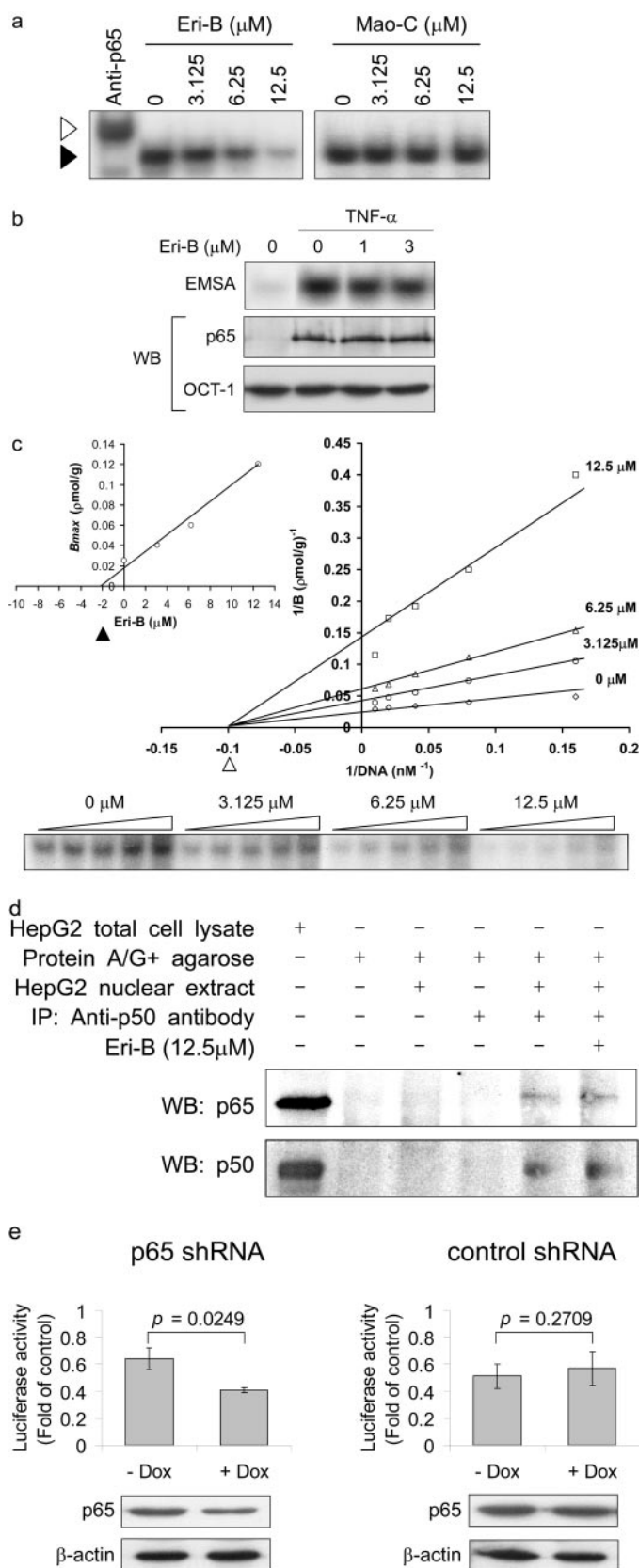
activation of three critical MAPKs was also studied. At concentrations that inhibit NF- $\kappa$ B activity, Eri-B did not show significant effect on the activation of ERK1/2, p38, and c-Jun-N-terminal kinase (Fig. 3b). Taken together, Eri-B blocked NF- $\kappa$ B activation upon different stimulations in different cell types. However, protein kinase A, PKC, and MAPK pathways are probably not the targets of this compound at concentrations up to 3  $\mu$ M. Our results suggest that the key mechanism of action of the compound could involve the common step(s) shared by the TNF- $\alpha$ -, PMA-, and LPS-associated NF- $\kappa$ B activation pathways.

**Eri-B Suppresses the Expression of COX-2 and iNOS at the Transcriptional Level.** COX-2 and iNOS are two NF- $\kappa$ B-regulated gene products (Surh et al., 2001; Calixto et al., 2003; Krakauer, 2004) that play important roles in cancer and inflammatory disease processes. Eri-B significantly suppressed the TNF- $\alpha$ -induced iNOS mRNA level in HepG2 (Fig. 4a). Such inhibition was also observed in macrophage cell lines. The expression levels of iNOS and COX-2 mRNA were subject to the inhibition by Eri-B in both human THP-1 and mouse RAW264.7 cells. The protein levels of iNOS and COX-2 were also consistently lowered after the drug treatment (Fig. 4b). It is noted that although TNF- $\alpha$  activated iNOS gene transcription in HepG2, no iNOS protein was detected by Western blot analysis. Furthermore, COX-2 and iNOS were found to be irresponsive to TNF- $\alpha$  or LPS stimulation in HepG2 and THP-1 cells, respectively (data not shown). This is in agreement with the previous finding by Callejas et al. (2002) that COX-2 expression was not observed in HepG2 cells. This suggests that the regulation of these genes by NF- $\kappa$ B could be cell type-specific.

**Eri-B Does Not Block TNF- $\alpha$ -Induced NF- $\kappa$ B Nuclear Translocation.** To elucidate the mechanism of action of Eri-B, the impact on the TNF- $\alpha$ -induced NF- $\kappa$ B nuclear translocation, which is one of the key steps for the activation of NF- $\kappa$ B, was studied. We examined the localization of NF- $\kappa$ B inside the cells by immunofluorescence staining of the p65 protein. TNF- $\alpha$  treatment induced the translocation of NF- $\kappa$ B from the cytoplasm to the nucleus within 15 min, whereas Eri-B did not block the translocation process (Fig. 5a). This is further supported by the fact that Eri-B had no



**Fig. 6.** Eri-B interferes with the binding of NF- $\kappa$ B to response elements *in vivo*. HepG2 cells stably transfected with NF- $\kappa$ B or CRE reporter gene were incubated with Eri-B before stimulation with TNF- $\alpha$  or FSK for 15 and 30 min, respectively. Chromatin immunoprecipitation assay was performed using p65 or CREB antibody. NF- $\kappa$ B or CREB-associated DNA was analyzed by PCR.



**Fig. 7.** Eri-B reversibly inhibits NF- $\kappa$ B DNA binding activity in a non-competitive manner. **a**, TNF- $\alpha$ -activated HepG2 nuclear extracts were incubated with radiolabeled NF- $\kappa$ B consensus oligonucleotides in the presence of Eri-B or Mao-C on ice for 30 min. NF- $\kappa$ B DNA-binding activity was determined by EMSA ( $\alpha$ , supershift using anti-p65 antibody; w, supershifted NF- $\kappa$ B-DNA complex; u, NF- $\kappa$ B-DNA complex). **b**, HepG2

significant impact on I $\kappa$ B- $\alpha$  phosphorylation and did not block I $\kappa$ B- $\alpha$  degradation (Fig. 5b). In the classic NF- $\kappa$ B signaling pathway, TNF- $\alpha$ -induced degradation of I $\kappa$ B- $\alpha$  requires phosphorylation by the IKK complex (Hayden and Ghosh, 2004). Our result showed that TNF- $\alpha$  induced a significant increase in the IKK activity, which was not affected after the treatment of Eri-B (Fig. 5c). Western blot analyses revealed that immunoprecipitates contained both IKK $\alpha$  and IKK $\beta$ , and their levels remained constant upon drug treatment. The results imply that the suppression of NF- $\kappa$ B transcriptional activity by Eri-B could be due to the intranuclear activity of the drug but not the inhibition of the translocation process.

**Selective Inhibition of NF- $\kappa$ B Binding to the Response Element by Eri-B In Vivo.** To study the impact of Eri-B on the NF- $\kappa$ B activation step(s) inside the nucleus, the interaction of NF- $\kappa$ B with its response element was studied by chromatin immunoprecipitation in the presence of the drug (Fig. 6). TNF- $\alpha$  induced the binding of NF- $\kappa$ B to the promoter sequences of NF- $\kappa$ B reporter (NF- $\kappa$ B-Luc) and the endogenous iNOS gene, whereas incubation of the cells with Eri-B resulted in a dose-dependent decrease in the NF- $\kappa$ B binding to both regions. The action was found to be selective. Eri-B did not have a significant effect on the FSK-induced CREB binding to the promoter region of the CRE luciferase reporter gene.

**Eri-B Interacts with NF- $\kappa$ B in a Reversible and Non-competitive Manner.** The nature of inhibition was studied in depth by an in vitro binding assay (EMSA) (Fig. 7a). TNF- $\alpha$ -activated nuclear extracts were incubated with the NF- $\kappa$ B consensus DNA sequence in the presence of Eri-B or Mao-C. The formation of NF- $\kappa$ B-DNA complex, which could be recognized by anti-p65 antibody, was found to be susceptible to the dose-dependent inhibition by Eri-B but not Mao-C. However, preincubation of the cells with Eri-B showed a limited impact on the DNA-binding activity of NF- $\kappa$ B and no effect on the nuclear NF- $\kappa$ B protein level (Fig. 7b). Taken together, the binding process was specifically inhibited both in vivo (Fig. 6) and in vitro (Fig. 7a) only in the presence of Eri-B whereas the inhibition was not observed

cells were pretreated with Eri-B before TNF- $\alpha$  stimulation for 15 min. Nuclear extracts were subjected to electrophoretic mobility shift assay. Nuclear p65 and ornithine carbamyl transferase-1 (nuclear protein as a loading control) levels were determined by Western blot analysis (WB). **c**, Lineweaver-Burk representation of NF- $\kappa$ B-DNA binding assay was performed with a fixed concentration of TNF- $\alpha$ -activated HepG2 nuclear extract (2.5  $\mu$ g/reaction) and 6.25 to 100 nM concentration of NF- $\kappa$ B oligonucleotides in the absence and presence of increasing concentrations of Eri-B.  $B_{max}$  values were plotted against the concentration of Eri-B to estimate the  $K_i$  value. Binding was quantified using a densitometer.  $B$  is defined as DNA binding per gram of nuclear extract at equilibrium, whereas  $B_{max}$  is the y-intercept of each line in the Lineweaver-Burk plot. ( $\triangleright$ ,  $-1/K_d$ ;  $\blacktriangleright$ ,  $-K_i$ ). **d**, TNF- $\alpha$ -activated HepG2 nuclear extracts were incubated with or without Eri-B on ice for 30 min. Protein complexes containing p50 were pulled down using anti-p50 antibody (IP). Coimmunoprecipitation of both p65 and p50 was detected by Western blot analysis (WB). **e**, characterization of HepG2 cell lines stably transfected with inducible shRNA of p65 and control sequences was done by Western blot analysis using p65 and  $\beta$ -actin (as a loading control) antibody. Cells with or without doxycycline treatment (100 ng/ml for 5 days) were preincubated with Eri-B followed by TNF- $\alpha$  activation. NF- $\kappa$ B transcriptional activity was determined by measuring the luciferase activity (\*,  $p < 0.05$ ). The data were expressed as -fold of control, where the control was the luciferase activity (arbitrary unit) of TNF- $\alpha$ -stimulated cells in the absence of Eri-B.



once the drug was removed (Fig. 7b). This suggests that the inhibition of NF- $\kappa$ B activity by Eri-B could be reversible.

Kinetic analysis showed that the equilibrium dissociation constant ( $K_d$ ) for p65:p50 binding to the consensus oligonucleotides was  $9.7 \pm 1.4$  nM (Fig. 7c), which is consistent with our previous findings (Leung et al., 2005). The  $K_i$  value of Eri-B was estimated to be  $2.2 \pm 0.1$   $\mu$ M. The double reciprocal plot indicated that Eri-B decreased  $B_{max}$  but had no effect on  $K_d$ . This suggests that it is a noncompetitive inhibitor with respect to the DNA substrates for NF- $\kappa$ B binding activity.

We next examined the effect of Eri-B on the association of p65 with p50. Coimmunoprecipitation assay revealed that p50 specifically immunoprecipitated with p65 in the TNF- $\alpha$ -activated nuclear extract, whereas the presence of Eri-B did not affect the amount of p50-associated p65 (Fig. 7d). This observation indicates that the interference in DNA binding may not be due to the impact of Eri-B on the dimerization of the p65:p50 complex.

To demonstrate that the inhibition of the NF- $\kappa$ B transcriptional activity requires the interaction of Eri-B to NF- $\kappa$ B, the HepG2 cell line that can be induced by doxycycline (Dox) to down-regulate endogenous p65 by shRNA to approximately 50% of the original level was established (Fig. 7e). Preincubation of the control cell line with doxycycline did not affect both NF- $\kappa$ B activation and the impact of Eri-B. Down-regulation of p65 enhanced the potency of Eri-B against TNF- $\alpha$ -induced NF- $\kappa$ B transcriptional activity. This provides the evidence of the direct interaction between Eri-B and p65.

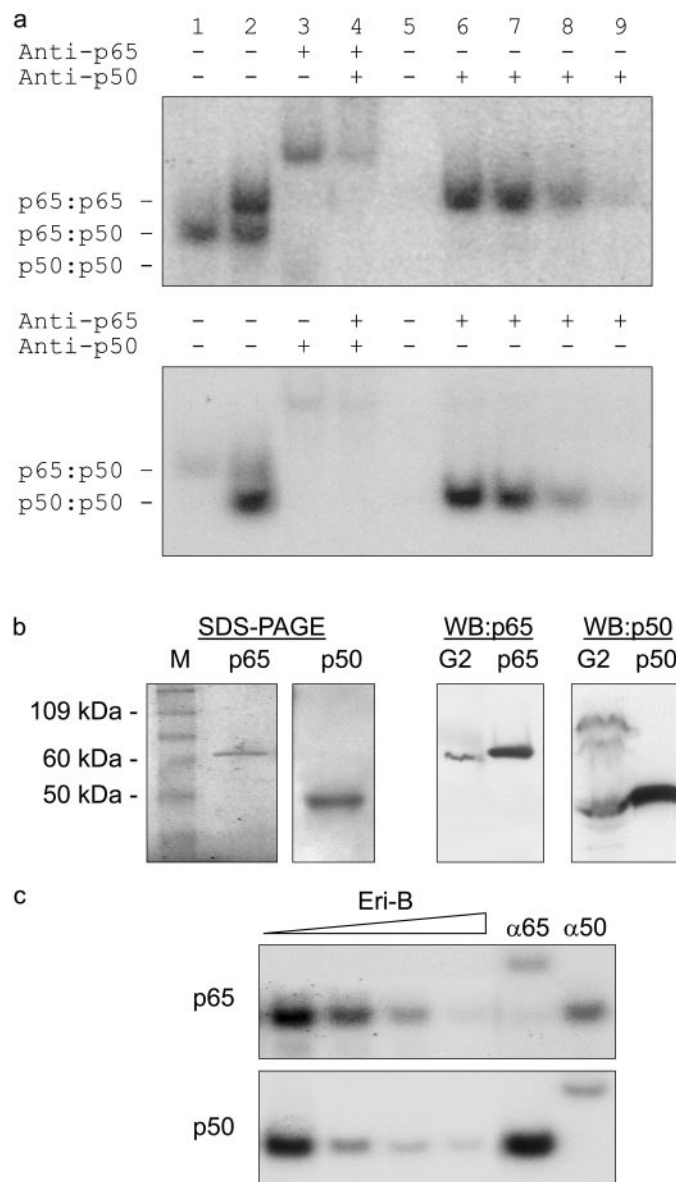
**Eri-B Inhibits the DNA-Binding Activity of Both p65 and p50 Subunits.** We investigated the impact of Eri-B on the DNA-binding activity of p65 and p50 subunits. HepG2 cells were transiently transfected with the p65 or p50 expression vector. Overexpression of p65 led to the formation of p65:p65 DNA complexes, which appeared as a bigger complex compared with p65:p50 and were recognized only by the anti-p65 antibody (Fig. 8a, top). Overexpression of p50 resulted in the formation of a smaller complex, p50:p50-DNA, which could be shifted by anti-p50 but not anti-p65 antibody (Fig. 8a, bottom). Direct incubation of the nuclear extracts with Eri-B resulted in a concentration-dependent decrease in the DNA-binding activity of both p65 and p50 homodimers with a slightly higher potency against p50. Eri-B also consistently blocked the binding of the recombinant p65 or p50 protein to the DNA (Fig. 8c), suggesting that Eri-B could directly inhibit the DNA-binding activity of NF- $\kappa$ B in the absence of other cofactors of the transcription complex.

## Discussion

In the present study, by dissecting the action of Eri-B, we demonstrated that Eri-B inhibited NF- $\kappa$ B activation by interfering with the DNA-binding activity of both p65 and p50 subunits in a noncompetitive manner without blocking NF- $\kappa$ B nuclear translocation. TNF- $\alpha$ -mediated NF- $\kappa$ B transcriptional activity requires the interaction with other proteins, including CREB-binding protein/p300, mitogen- and stress-activated protein kinases 1 and 2, IKK $\alpha$ , etc. (Hayden and Ghosh, 2004). Our in vitro binding experiments showed that Eri-B suppressed the DNA-binding activity of both recombinant p65 and p50. This implies that the inhibition is a direct process that does not necessarily require the presence

of other components of the NF- $\kappa$ B transcription complex. In addition, the potency of Eri-B against the NF- $\kappa$ B activation was found to be dependent on the endogenous p65 level. By using mass spectrophotometry, the preliminary data indicate that Eri-B directly interacts with the p50 protein in vitro (data not shown). We thus propose that Eri-B could interact with both p65 and p50 subunits at an allosteric site and subsequently cause the conformational change of the protein at the active site for DNA binding.

Blocking of the interaction between NF- $\kappa$ B and the response element could be a critical but not the only mecha-



**Fig. 8.** Eri-B inhibits the DNA-binding activity of p65 and p50 subunits. **a**, nuclear extracts from p65 (top) and p50 (bottom) overexpressing HepG2 cells were incubated with increasing concentration of Eri-B. The DNA-binding activity was determined by EMSA. Lane 1, TNF- $\alpha$ -activated HepG2 nuclear extract; lanes 2 to 4, no treatment; lane 5, unlabeled probe; lanes 6 to 9, Eri-B (0, 3.125, 6.25, and 12.5  $\mu$ M). **b**, purified p65 and p50 proteins were detected by SDS-PAGE with Coomassie Blue staining (left) and Western blot analysis (right; G<sub>2</sub>, total HepG2 protein extract). **c**, recombinant p65 or p50 (50 ng) was incubated with Eri-B (0, 3.125, 6.25, and 12.5  $\mu$ M) followed by EMSA ( $\alpha$ 65 and  $\alpha$ 50 represent supershift with p65 and p50 antibody, respectively).



nism of the inhibition by Eri-B. Our data showed that Eri-B was found to be less potent in blocking the DNA binding activity in vitro (Fig. 7) than in suppressing the NF- $\kappa$ B transcriptional activity (Fig. 2) and the DNA-binding activity (Fig. 6) in cell culture. The DNA binding assay is used to study the interaction of the p65:p50 complex with the DNA response element, whereas the NF- $\kappa$ B transcriptional activity assay is used to study the NF- $\kappa$ B transcriptional complex, which includes proteins other than p65:p50. The participation of other coregulators in the cell could render the p65:p50 interaction with the response element more sensitive to Eri-B. The other possibility is that other proteins in the transcriptional complex could also be targets of Eri-B. Some of these components, such as CREB-binding protein/p300, mitogen- and stress-activated protein kinases 1 and 2, transcription factor IIA, transcription factor IIB, TATA-binding protein associated factors, and TATA-binding protein, which have been reported to be critical for the transcriptional activity (Hayden and Ghosh, 2004), could be absent in the nuclear extract. Furthermore, we reported previously that, in addition to interfering with the DNA-binding activity of NF- $\kappa$ B, Eri-B blocked NF- $\kappa$ B nuclear translocation by suppressing I $\kappa$ B $\alpha$  phosphorylation and degradation through the inactivation of IKK at higher concentrations ( $>10$ -fold of the IC<sub>50</sub>) (Leung et al., 2006). A similar observation was also described by Wang et al. (2006). Their findings suggested that, in the leukemia cells, Eri-B inhibited the intrinsic NF- $\kappa$ B activity by suppressing the DNA-binding activity of NF- $\kappa$ B, whereas the TNF- $\alpha$ -induced NF- $\kappa$ B activity was inhibited by blocking the NF- $\kappa$ B nuclear translocation. Our present study demonstrates that, in the presence of TNF- $\alpha$  stimulation, blocking of NF- $\kappa$ B binding to the response element is also involved at concentrations that do not inhibit translocation of NF- $\kappa$ B.

In current drug development, NF- $\kappa$ B modulators have become intensely investigated as key targets for the development of treatments for cancer and inflammatory and autoimmune diseases. *I. eriocalyx* has long been used in folk medicine for anti-inflammation. In this study, the impact of Eri-B isolated from this herb on the expression of two NF- $\kappa$ B-regulated genes, iNOS and COX-2, was demonstrated. COX-2 and iNOS are critical for the production of the proinflammatory mediator prostaglandin E2 and nitric oxide (Surh et al., 2001; Calixto et al., 2003; Krakauer, 2004). This could partly explain the anti-inflammatory activity of *I. eriocalyx*.

NF- $\kappa$ B inhibitors have also been implicated for treating cancers by themselves, such as tylophorine analogs (Gao et al., 2004), or in combination with apoptosis-inducing agents (Nakanishi and Toi, 2005). Eri-B exhibits strong antitumor activity in vitro. Our unpublished data, along with the data of other groups, also showed that Eri-B was cytotoxic to a diversity of human cancer cell types in vitro, including pancreatic and liver cancers, lymphoma, and T-cell and B-cell leukemia (IC<sub>50</sub>  $\sim$  0.1–1.0  $\mu$ M). More recently, a study by Wang et al. (2006) demonstrated that Eri-B suppressed the xenograft tumor growth in murine t(8;21) leukemia models, whereas Eri-B mediated apoptosis of leukemia cells through the inactivation of NF- $\kappa$ B and MAPK pathways. It is clear that Eri-B inhibits NF- $\kappa$ B in both human leukemia and hepatocarcinoma cell lines, whereas it blocks only MAPK pathways in leukemia cells. Our unpublished

data indicated that Eri-B had no significant impact on the growth of HepG2 tumor xenograft in nude mice. This suggests that knocking down NF- $\kappa$ B may not be adequate for the tumor growth suppression in vivo, whereas inhibition of MAPK pathways could be critical for the antitumor activity. This is still an open question and will need to be further studied.

Our observation suggests that the expression of the NF- $\kappa$ B-regulated gene could be cell type-specific. The transcription of iNOS gene is differentially regulated in different cell types, although they are both under the control of NF- $\kappa$ B. It could be activated in HepG2 cells but not in THP-1 cells. This could be due to the modification of gene promoters by methylation. The iNOS promoter was known to be heavily methylated at CpG dinucleotides in a variety of human cells (Yu and Kone, 2004; Chan et al., 2005). It is possible that such modification could modulate the binding of NF- $\kappa$ B to the promoter region.

The impact of Eri-B on the gene transcription could be sequence-specific. The potency of Eri-B against the transcription of iNOS was approximately 2-fold that of COX-2 in RAW264.7 cells. Given that NF- $\kappa$ B binding sequences of COX-2 and iNOS are different (Leung et al., 2005), we suspect that Eri-B could show different degrees of transcriptional inhibition of genes with different NF- $\kappa$ B consensus DNA sequences. This is in agreement with our unpublished data showing that the  $K_i$  value of the drug against the binding of NF- $\kappa$ B to its consensus DNA sequence within the COX-2 promoter was also found to be approximately 2-fold of that to its consensus but different DNA sequence within the iNOS promoter ( $\sim 6$  and  $\sim 3$   $\mu$ M, respectively).

In summary, Eri-B inhibited NF- $\kappa$ B activation by blocking the binding of NF- $\kappa$ B to its response element possibly through the reversible interaction with the two NF- $\kappa$ B subunits, p65 and p50, at an allosteric site. The structure-activity relationship for this class of compounds was also demonstrated. Among the four *ent*-kauranes tested, Eri-B has the unique structure consisting two  $\alpha,\beta$ -unsaturated ketones on both A- and D-rings. The former accounts for the potency of Eri-B against NF- $\kappa$ B, and the latter could change the behavior of the compound toward other cellular processes. The biological behavior of the compound could be changed dramatically by modifying the structure. Therefore, the core structure of this class of compounds could serve as a scaffold for the development of drugs that are more potent and selective against specific signaling pathways for treating NF- $\kappa$ B-associated diseases with minimum side effects.

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#### References

- Adams J (2003) The proteasome: structure, function, and role in the cell. *Cancer Treat Rev* 29:3–9.
- Adams J (2004) The development of proteasome inhibitors as anticancer drugs. *Cancer Cell* 5:417–421.
- Bharti AC and Aggarwal BB (2002) Nuclear factor-kappa B and cancer: its role in prevention and therapy. *Biochem Pharmacol* 64:883–888.
- Calixto JB, Otuki MF, and Santos AR (2003) Anti-inflammatory compounds of plant

- origin. Part I. Action on arachidonic acid pathway, nitric oxide and nuclear factor kappa B (NF-kappaB). *Planta Med* **69**:973–983.
- Callejas NA, Casado M, Bosca L, and Martin-Sanz P (2002) Absence of nuclear factor kappaB inhibition by NSAIDs in hepatocytes. *Hepatology* **35**:341–348.
- Celec P (2004) Nuclear factor kappa B—molecular biomedicine: the next generation. *Biomed Pharmacother* **58**:365–371.
- Chan GC, Fish JE, Mawji IA, Leung DD, Rachlis AC, and Marsden PA (2005) Epigenetic basis for the transcriptional hyporesponsiveness of the human inducible nitric oxide synthase gene in vascular endothelial cells. *J Immunol* **175**:3846–3861.
- Chen LF and Greene WC (2004) Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol* **5**:392–401.
- Dignam JD, Martin PL, Shastry BS, and Roeder RG (1983) Eukaryotic gene transcription with purified components. *Methods Enzymol* **101**:582–598.
- Fujita E, Nagao Y, Kaneko K, Nakazawa S, and Kuroda H (1976) The antitumor and antibacterial activity of the Isodon diterpenoids. *Chem Pharm Bull* **24**:2118–2127.
- Gao W, Lam W, Zhong S, Kaczmarek C, Baker DC, and Cheng YC (2004) Novel mode of action of tylophorine analogs as antitumor compounds. *Cancer Res* **64**:678–688.
- Hayden MS and Ghosh S (2004) Signaling to NF-kappaB. *Genes Dev* **18**:2196–2224.
- Janssens S and Beyaert R (2003) Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol Cell* **11**:293–302.
- Jentsch S and Schlenker S (1995) Selective protein degradation: a journey's end within the proteasome. *Cell* **82**:881–884.
- Karin M, Yamamoto Y, and Wang QM (2004) The IKK NF-kappa B system: a treasure trove for drug development. *Nat Rev Drug Discov* **3**:17–26.
- Krakauer T (2004) Molecular therapeutic targets in inflammation: cyclooxygenase and NF-kappaB. *Curr Drug Targets Inflamm Allergy* **3**:317–324.
- Lam W, Park SY, Leung CH, and Cheng YC (2006) Apurinic/aprimidinic endonuclease-1 protein level is associated with the cytotoxicity of L-configuration deoxycytidine analogs (troxacitabine and  $\beta$ -L-2',3'-dideoxy-2',3'-didehydro-5-fluorocytidine) but not D-configuration deoxycytidine analogs (gemcitabine and  $\beta$ -D-arabinofuranosylcytosine). *Mol Pharmacol* **69**:1607–1614.
- Lee JH, Choi JK, Noh MS, Hwang BY, Hong YS, and Lee JJ (2004) Anti-inflammatory effect of kamebakaurin in in vivo animal models. *Planta Med* **70**:526–530.
- Leung CH, Grill SP, Lam W, Han QB, Sun HD, and Cheng YC (2005) Novel mechanism of inhibition of nuclear factor- $\kappa$ B DNA-binding activity by diterpenoids isolated from *Isodon rubescens*. *Mol Pharmacol* **68**:286–297.
- Leung CH, Grill SP, Lam W, Sun HD, and Cheng YC (2006) Eriocalyxin B selectively inhibits NF-kappaB activity by targeting multiple steps of the NF-kappaB activation pathway. *Proc Am Assoc Cancer Res* **47**:1376.
- Nakanishi C and Toi M (2005) Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* **5**:297–309.
- Niu XM, Li SH, Li ML, Zhao QS, Mei SX, Na Z, Wang SJ, Lin ZW, and Sun HD (2002) Cytotoxic ent-kaurane diterpenoids from *Isodon eriocalyx* var. *laxiflora*. *Planta Med* **68**:528–533.
- Sen R and Baltimore D (1986) Inducibility of kappa immunoglobulin enhancer-binding protein NF-kappa B by a posttranslational mechanism. *Cell* **47**:921–928.
- Shen G, Jeong WS, Hu R, and Kong AN (2005) Regulation of Nrf2, NF-kappaB, and AP-1 signaling pathways by chemopreventive agents. *Antioxid Redox Signal* **7**:1648–1663.
- Sun HD, Lin ZW, Niu FD, Lin LZ, Chai HB, Pezzuto JM, and Cordell GA (1995) Cytotoxic ent-kaurane diterpenoids from three *Isodon* species. *Phytochemistry* **38**:437–442.
- Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park K, and Lee SS (2001) Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. *Mutat Res* **480–481**:243–268.
- Twombly R (2003) First proteasome inhibitor approved for multiple myeloma. *J Natl Cancer Inst* **95**:845.
- Viatour P, Merville MP, Bours V, and Chariot A (2005) Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. *Trends Biochem Sci* **30**:43–52.
- Wang L, Zhao WL, Yan JS, Liu P, Sun HP, Zhou GB, Weng ZY, Wu WL, Weng XQ, Sun XJ, et al. (2006) Eriocalyxin B induces apoptosis of t(8;21) leukemia cells through NF-kappaB and MAPK signaling pathways and triggers degradation of AML1-ETO oncoprotein in a caspase-3-dependent manner. *Cell Death Differ*, in press.
- Yamamoto Y and Gaynor RB (2001) Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest* **107**:135–142.
- Yu Z and Kone BC (2004) Hypermethylation of the inducible nitric-oxide synthase gene promoter inhibits its transcription. *J Biol Chem* **279**:46954–46961.
- Zhang Y, Liu J, Jia W, Zhao A, and Li T (2005) Distinct immunosuppressive effect by Isodon serra extracts. *Int Immunopharmacol* **5**:1957–1965.

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