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Novel Mechanism of Inhibition of Nuclear Factor-κB DNA-Binding Activity by Diterpenoids Isolated from *Isodon rubescens*

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ABSTRACT

The development of specific inhibitors that can block nuclear factor-κB (NF-κB) activation is an approach for the treatment of cancer, autoimmune, and inflammatory diseases. Several diterpenoids, oridonin, ponicidin, xindongnin A, and xindongnin B were isolated from the herb *Isodon rubescens*. These compounds were found to be potent inhibitors of NF-κB transcription activity and the expression of its downstream targets, cyclooxygenase-2 and inducible nitric-oxide synthase. The mechanisms of action of the diterpenoids against NF-κB are similar, but significant differences were also identified. All of the diterpenoids directly interfere with the DNA-binding activity of NF-κB to its response DNA sequence. Oridonin and ponicidin have an additional impact on the translocation of NF-κB from the cytoplasm to nuclei without affecting IκB-α phosphorylation and degradation. The effect of these compounds on the interaction of NF-κB with consensus DNA sequences is unique. Different inhibitory effects were observed when NF-κB bound to various DNA sequences. Both p65/p65 and p50/p50 homodimers, as well as p65/p50 heterodimer association with their responsive DNA, were inhibited. Kinetic studies on NF-κB–DNA interaction indicate that the diterpenoids decrease the $B_{max\ app}$ but have no effect on $K_{d\ app}$. This suggests that this class of compounds interacts with both p65 and p50 subunits at a site other than the DNA binding site and subsequently modulates the binding affinity of the transcription factor toward DNA with different NF-κB binding sequences. The diterpenoid structure could therefore serve as a scaffold for the development of more potent and selective NF-κB inhibitors that target regulated gene transcription.

The transcription factor NF-κB plays a critical role in controlling inflammatory and immune response and cell proliferation. NF-κB can be activated by a variety of stimuli such as microbial products, proinflammatory cytokines, T- and B-cell mitogens, and physical and chemical stresses, including anticancer drugs (Yamamoto and Gaynor, 2001; Bharti and Aggarwal, 2002; Li and Verma, 2002). In unstimulated cells, NF-κB remains largely inactive in the cytoplasm as an NF-κB/IκB complex. The stimulation of cells by different inducers results in the phosphorylation of the NF-κB/IκB complex by the IκB kinase complex (IKK) and the subsequent degradation of the IκB proteins. Upon degradation of IκB, NF-κB enters the nucleus. NF-κB, as part of a transcription complex, in turn regulates the inducible expression of genes that are involved in tumor promotion, angiogenesis, and metastasis (Chen and Greene, 2004; Hayden and Ghosh, 2004; Yamamoto and Gaynor, 2004; Viatour et al., 2005).

The development of specific inhibitors that can block NF-κB activation is believed to hold great potential in suppressing certain types of tumor growth as well as improving cancer therapy (Bharti and Aggarwal, 2002; Garg and Aggarwal, 2002; Gilroy et al., 2004; Wu and Kral, 2005). During the last decade, a variety of natural and synthetic compounds have been used to suppress NF-κB. The mechanisms involved are very different. Dexamethasone induces the synthesis of IκB and results in the cytoplasmic retention of NF-κB (Auphan et al., 1995; Adcock, 2003). Aspirin prevents

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ABBREVIATIONS: NF-κB, nuclear factor-κB; IKK, IκB kinase; Ori, oridonin; Pon, ponicidin; Xdn-A, xindongnin A; Xdn-B, xindongnin B; KA, kamebakaurin; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; COX-2, cyclooxygenase-2; iNOS, inducible nitric-oxide synthase; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PCR, polymerase chain reaction; PS-341, *N*-[(1*S*)-1-benzyl-2-(((1*R*)-1-(dihydroxyboranyl)-3-methylbutyl)amino]2-oxoethylpyrazinecarboxamide.

the binding of ATP to IKK. This leads to the reduction of IKK-dependent phosphorylation of IκB and the prevention of its degradation by the proteasome (Frantz and O'Neill, 1995; Wu, 2003). PS-341, which was approved in 2003, is the first of a new class of drugs called proteasome inhibitors, and it is the first treatment in more than a decade to be approved for patients with multiple myeloma. The antimyeloma effects are believed to be mediated through the ability of the drug to block NF-κB by inhibiting the degradation of IκB (Twombly, 2003). Helenalin is the first NF-κB inhibitor reported to directly modify p65 by alkylation (Lyss et al., 1998). More recently, a C-20-nonoxygenated-*ent*-kaurane diterpenoid (kamebakaurin, KA), isolated from *Isodon japonicus*, was found to be able to inhibit NF-κB by directly targeting its DNA binding activity of p50 and blocking the expression of anti-apoptotic genes (Lee et al., 2002).

Herbal drugs have been widely used for thousands of years in traditional Chinese medicine for the treatment of human diseases. Many herbs are claimed to exhibit anticancer and anti-inflammatory activities. Given the complexity of the chemical composition and the multiple potential targets of herbs, Chinese medicine could offer a new paradigm in future drug development for the treatment of complicated diseases. *I. rubescens* belongs to the genus *Isodon* and is commonly used as an antitumor and anti-inflammatory herb in China. It has been stated that this herb is useful for the treatment of cancers of the liver, pancreas, esophagus, breast, thyroid gland, and rectum. Several in vitro and in vivo studies have demonstrated its inhibitory effects. It has also been shown that chemicals isolated from this plant have inhibitory effects on cancer cell growth in vitro and tumor growth in vivo (Gao et al., 1993; Marks et al., 2002; Ikezoe et al., 2003; Meade-Tollin et al., 2004). In addition to its application as an antitumor drug, *I. rubescens* has also been used in folk medicine in China as a remedy for tonsillitis, pharyngitis, laryngitis, chronic bronchitis, and chronic pelvic inflammation. However, the mechanisms of action are not well-documented. *I. rubescens* is recognized to contain natural constituents known to be rich in diterpenoids (Han et al., 2004a,b). Oridonin (Ori), ponicidin (Pon), two 7,20-epoxy-*ent*-kaurenoids, xindongnin A (Xdn-A), xindongnin B (Xdn-B), and two C-20-nonoxygenated-*ent*-kauranoids were isolated from this plant (Fig. 1). Given the claimed medical uses of the herb and the structural similarity to KA, we suspected that these four compounds may also inhibit NF-κB activity in target cells.

In this report, we describe the potent inhibitory activity of the four diterpenoids found in *I. rubescens* against NF-κB transcription, which could partly explain the use of this herb

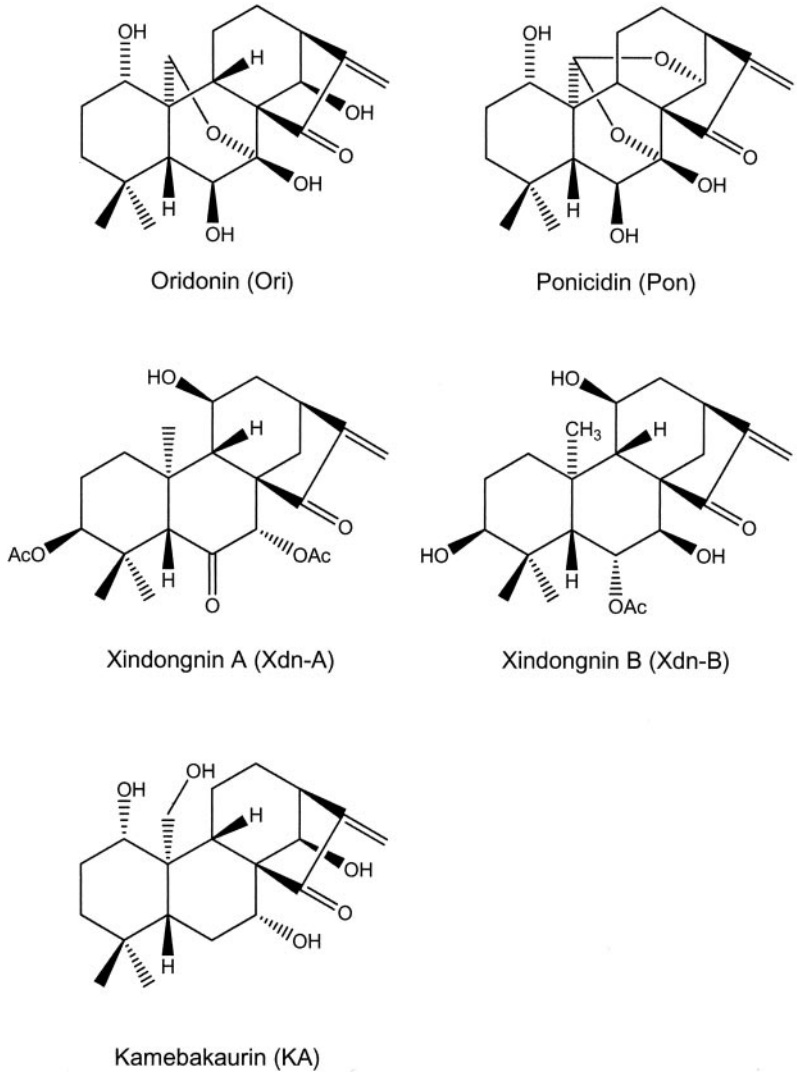


Fig. 1. Structures of diterpenoids.

for the treatment of diseases in Chinese medicine. The underlying mechanism of action against NF-κB transcription, including phosphorylation, translocation, and DNA binding activity of NF-κB, were studied. The study reveals that these compounds are a novel and new class of NF-κB inhibitors that interfere with the binding between NF-κB and DNA with a unique sequence by a distinct mechanism. The diterpenoid structure of these compounds could serve as a scaffold structure for making more potent and selective inhibitors targeting the transcription of unique genes regulated by NF-κB.

Materials and Methods

Materials and Compounds. Oridonin, ponicidin, xindongnin A, and xindongnin B were isolated in Dr. Sun's laboratory. Kamebakaurin, tumor necrosis factor-α (TNF-α), phorbol-12-myristate-13-acetate (PMA), lipopolysaccharides (LPS), actinomycin-D_1, and anti-inducible nitric-oxide synthase (iNOS) antibody were purchased from Calbiochem (San Diego, CA). Cell growth medium, fetal bovine serum, and G418 were acquired from Invitrogen (Carlsbad, CA). FuGENE6 transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN). Antibodies against p65, p50, and cyclooxygenase-2 (COX-2) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-interleukin-6, anti-IκB, and anti-phospho-IκB were acquired from Cell Signaling Technology Inc. (Beverly, MA).

Cell Culture and Drug Treatment. Human hepatocellular carcinoma HepG2 and mouse macrophage RAW264.7 cells (American Type Culture Collection, Manassas, VA) were maintained in Eagle's minimal essential medium and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% fetal bovine serum. Cells were treated with the same range of drug concentrations to compare the potencies of different compounds, whereas drug concentrations of equal potencies (IC_{50} and IC_{90}) were used to compare their impacts on IκB phosphorylation and degradation and on NF-κB translocation. IC_{50} and IC_{90} values are the concentrations that cause 50% and 90% inhibition of NF-κB, respectively, based on luciferase reporter assay.

Luciferase Reporter Assay. HepG2 cells were transiently transfected with pBIIX-luc (containing two tandemly repeated NF-κB binding sites, provided by Dr. Ghosh, Yale University, New Haven, CT) and pRL-TK (Promega, Madison, WI) vectors using FuGENE6 transfection reagent for 24 h. The cells were then preincubated with different concentrations of drugs for 1 h and subsequently activated with TNF-α or PMA for 3 h. Transcriptional activity was determined by measuring the activities of firefly and *Renilla reniformis* luciferases in a multiwell plate luminometer (Tecan, Durham, NC) using Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's instructions.

Western Blot Analysis. Drug-treated cells were incubated with TNF-α or LPS for the time indicated. To obtain cytoplasmic and nuclear protein fractions, cells were lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, and 0.75% Nonidet P-40. The nuclear fraction was separated from the cytoplasmic fraction by centrifugation. Total cell lysates were obtained by direct lysis in 2× SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromphenol blue). The proteins of interest were detected by Western blot analysis using the antibodies described.

Reverse Transcriptase-Real Time Polymerase Chain Reaction Assay. 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, Hercules, CA). Sequences of primer pairs and Taqman probes (Biosearch Technologies, Novato, CA) are as follows: COX-2: probe, 5′-Quasar670d(CCCTGCTGCCCGACACCTTCAACA)BHQ-2 3′; forward primer, 5′-TTCAACACACTCTATCACTGGCAC-3′; reverse primer, 5′-GCAATCTGTCTGGTGAATGACTCA-3′;iNOS:probe,5′-Quasar670d(CCGCAGCTCCTCACTGGGACAGCA)BHQ-2 3′; forward primer, 5′-CCCTAAGAGTCACCAAAATGGCTC-3′; reverse primer, 5′-ATACTGTGGACGGGTCGATGG-3′; and β-actin: probe, 5′-T(CalRed)d(CAAGATCATGTCTCCTCCTGAGCGCA)BHQ-2 3′; forward primer, 5′-ATTGCCGACAGGATGCAGAA-3′; reverse primer, 5′-GCTGATCCACATCTGCTGGAA-3′.

The PCR reaction mixture consisted of total RNA, 0.1 μM concentration of each primer, 0.1 μM Taqman probe, 1 unit of Platinum TaqDNA polymerase, 1 unit of RNaseOUT, and 2× Thermoscript reaction mix, resulting in a final volume of 50 μl. Samples were amplified with a precycling hold at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing, and extension at 60°C for 1 min. Calibration curves were obtained by using serial dilutions of total RNA from the LPS-treated cells. Each assay was performed at least twice to verify the results and the mean threshold cycle value was used for analysis.

Immunofluorescence Staining. HepG2 cells grown on chamber slides were incubated with drugs at 37°C for 1 h followed by TNF-α treatment for the time indicated. Cells were fixed in phosphate-buffered saline with 4% paraformaldehyde at given time points and subsequently permeabilized in phosphate-buffered saline with 0.5% Triton X-100. To study the localization of NF-κB, cells were incubated with 1:100 of rabbit anti-p65 antibody, followed by incubating with 1:100 of anti-rabbit IgG-fluorescein isothiocyanate and 1:200 of an actin probe, BODIPY 558/568 phalloidin (Molecular Probe, Eugene, OR). Actin and p65 were detected by confocal microscopy.

Electrophoretic Mobility Shift Assay. Nuclear extracts prepared according to Dignam et al. (1983) were incubated with [γ-^{32}P]ATP-labeled NF-κB or Oct-1 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 bromphenol blue, and 50 μg/ml poly(dIdC)]. The samples were incubated for 40 min at room temperature and then separated in 5% native polyacrylamide gels at 130 V for 45 min and subsequently visualized by autoradiography. Competition assay and supershift assay were done by incubating nuclear extract with unlabeled oligonucleotides and antibodies (as indicated), respectively, on ice for 30 min before the addition of a radiolabeled probe. For the supershift experiment, gel electrophoresis was performed for 90 min to resolve protein-DNA complexes with different NF-κB subunits.

Statistical Analysis. The significance of the data was examined by Student's *t* test and one-way analysis of variance by using Prism 4 software (GraphPad Software Inc., San Diego, CA). The difference was considered to be statistically significant if $P < 0.05$.

Results

Diterpenoids Inhibit TNF-α- and PMA-Induced NF-κB Activity. The effects of Ori, Pon, Xdn-A, and Xdn-B isolated from *I. rubescens* and KA, a known NF-κB inhibitor, on NF-κB activation were investigated using HepG2 cell lines transiently transfected with an NF-κB reporter gene. A luciferase assay showed that both TNF-α and PMA induced a 6-fold activation of NF-κB transcriptional activity (Fig. 2). All compounds inhibited the TNF-α–and PMA-induced expression of NF-κB reporter gene in a concentration-dependent manner. Xdn-A and Xdn-B were more potent than Ori and Pon in either case. The inhibition of NF-κB by the diterpenoids present in *I. rubescens* may therefore account for part or all of the anti-inflammatory and anticancer activities of the herb.

Diterpenoids Inhibit NF-κB Downstream Targets. We examined the impact of these compounds on the expression of two NF-κB downstream gene products, including mRNA and the protein levels of COX-2 and iNOS in LPS-activated RAW264.7 cells. Real-time PCR (Fig. 3a, left) and Western blot (Fig. 3b) analyses show LPS, an NF-κB activator, induced expression of COX-2 and iNOS significantly in 8 h. The four diterpenoids and KA suppressed LPS-induced COX-2 and iNOS protein and mRNA levels in a concentration-dependent manner. In addition, the potency was consistent with that measured by luciferase reporter assay, in which Xdn-B was relatively more potent among the five compounds tested. By using actinomycin-D_1, an mRNA synthesis inhibitor (Fig. 3a, right), we demonstrated that the stability of the mRNAs was not significantly affected after the exposure of cells to Xdn-B. Half-lives of COX-2 and iNOS mRNAs were found to be >6 and 5 h, respectively, which agreed with those reported previously (Fujita et al., 2001; Lahti et al., 2003). The results here indicate that these compounds inhibit NF-κB transcription activity and subsequently interfere with NF-κB downstream gene regulation without affecting the stability of the mRNA. In addition to TNF-α and PMA, the diterpenoids can also inhibit LPS-induced NF-κB downstream gene expression. This suggests that the key action of these compounds is on one or more common events shared by TNF-α–, PMA-, and LPS-associated NF-κB activation pathways.

Diterpenoid Components Do Not Significantly Inhibit TNF-α–Induced IκB-α Degradation and NF-κB Translocation. Activation of NF-κB requires phosphorylation and subsequent proteasomal degradation of IκB-α that allows translocation of active NF-κB from the cytoplasm to the nucleus. To elucidate the mechanism of action of diterpenoids, we first investigated the effect of these compounds on the protein phosphorylation and degradation of IκB-α (Fig. 4a). TNF-α triggered phosphorylation in 5 min and complete degradation in 10 min. Incubation of cells with 1 μg/ml PS-341, a well known proteasome inhibitor, resulted in the accumulation of phospho-IκB-α and IκB-α. Treatment of cells with Ori, Pon, Xdn-A, Xdn-B, and KA did not significantly inhibit IκB-α phosphorylation. More than 90% of IκB-α degradation was observed 10 min after TNF-α stimu-

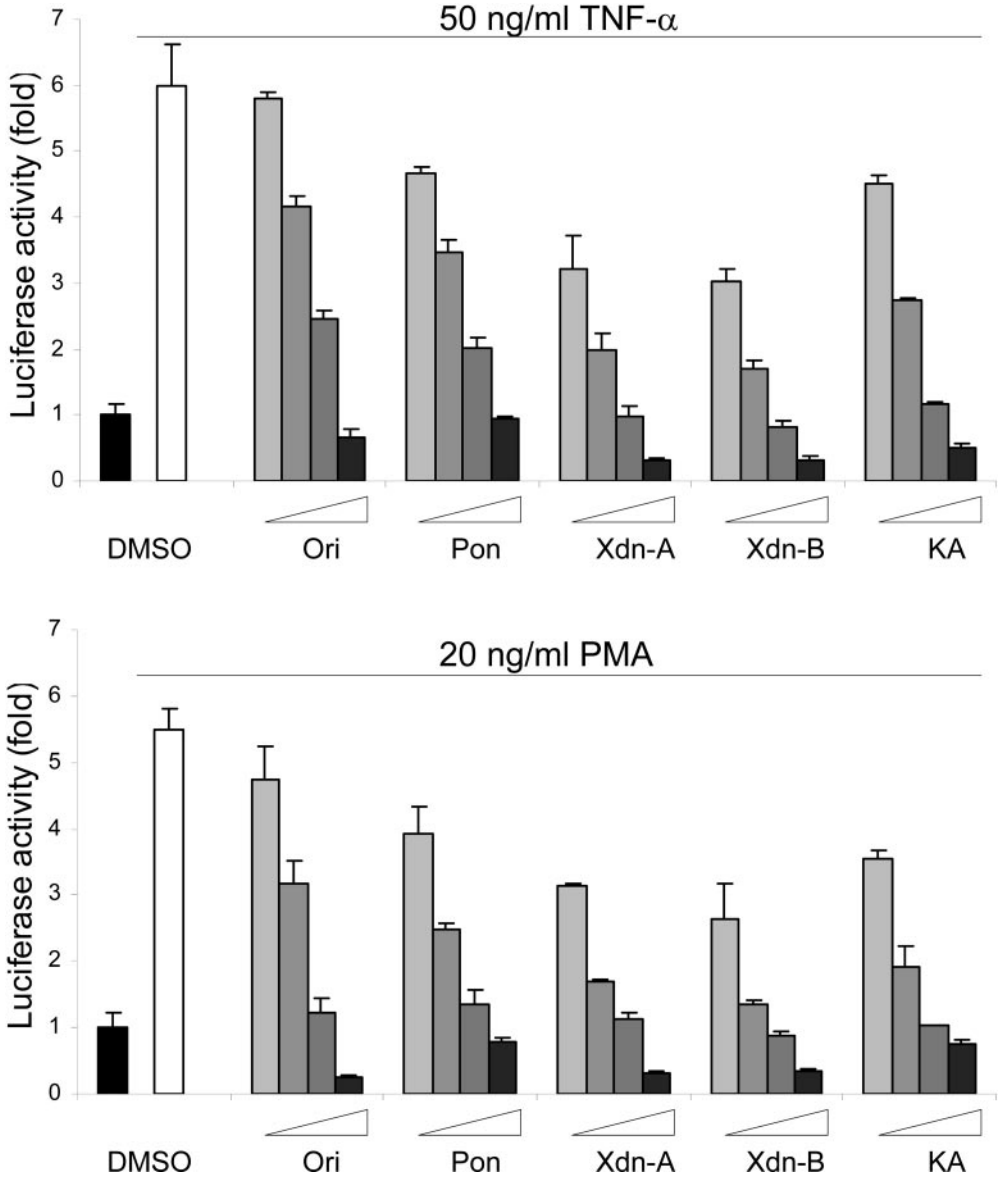


Fig. 2. Effect of diterpenoids on TNF-α–induced NF-κB activation. HepG2 cells were pretreated with DMSO or 1.25, 2.5, 5, or 10 μM diterpenoids for 1 h before 50 ng/ml TNF-α (a) or 20 ng/ml PMA (b) stimulation for 4 h. The NF-κB reporter was assayed by measuring the luciferase activity as described under *Materials and Methods*. The values given are mean ± S.D. of three independent experiments performed in duplicate.

lation. This class of compounds showed a minor effect on IκB-α degradation; however, they do not block phosphorylation and degradation of IκB-α. We then examined the nuclear translocation of NF-κB from the cytoplasm to the nucleus by immunofluorescence staining of p65 protein (Fig. 4b). Translocation was observed 15 min after TNF-α treatment. Preincubation of cells with equal potency (IC_{50}) of Ori, Pon, Xdn-A, and Xdn-B before TNF-α stimulation did not significantly

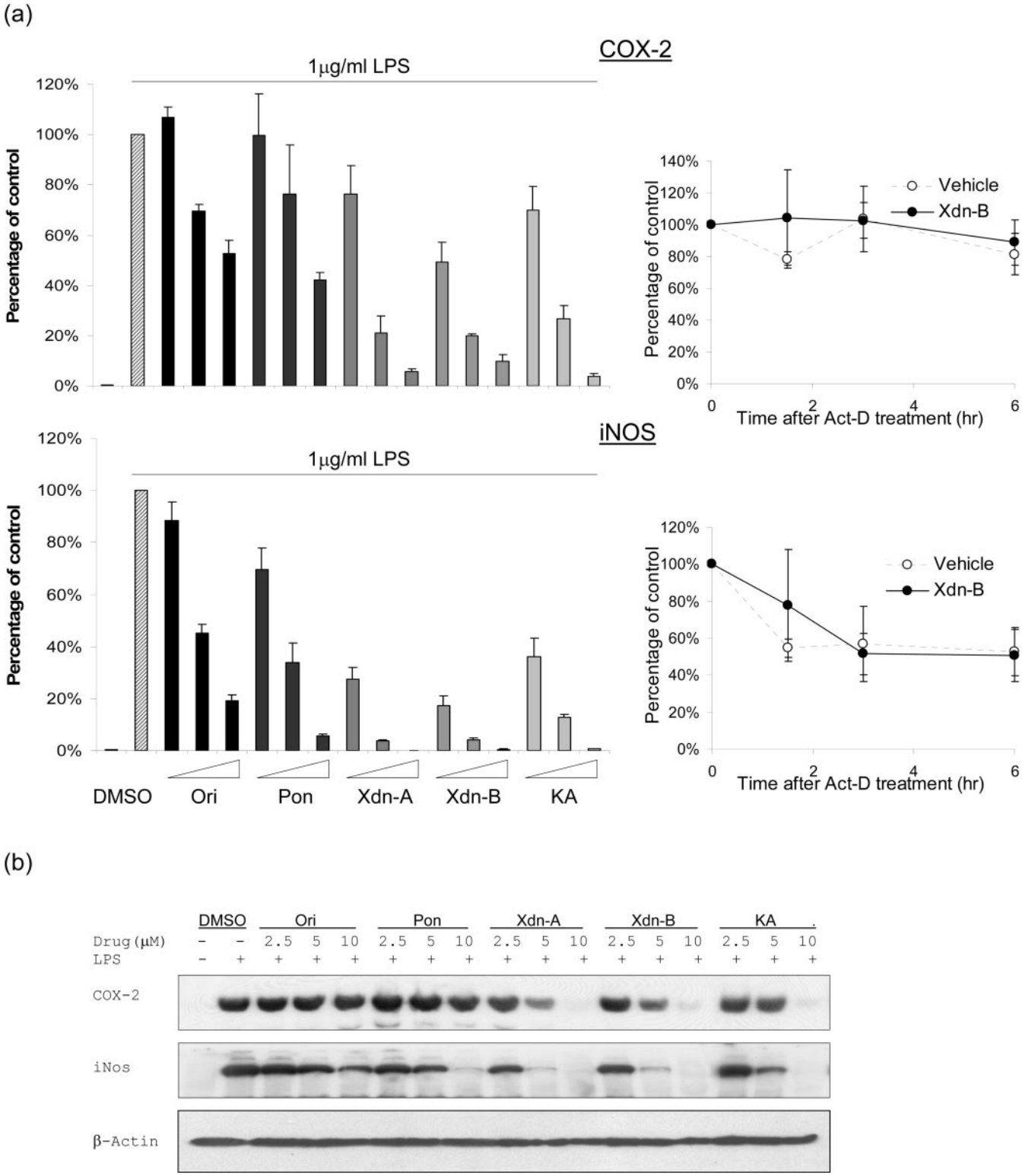


Fig. 3. Inhibition of LPS-induced expression of COX-2 and iNOS. a, RAW264.7 cells were pretreated with DMSO or 2.5, 5, or 10 μM diterpenoids for 1 h, and total RNA was collected after 8 h of treatment with 1 μg/ml LPS. To study the stability of the mRNA (right), cells pretreated with Xdn-B or vehicle (DMSO) were activated with LPS. Actinomycin-D_1 (5 μg/ml) was added after 8 h, and total RNA was collected at the indicated time points. mRNA levels of COX-2 and iNOS were determined by reverse transcriptase-real time PCR assay. b, total protein was prepared after drug treatment as described above and was subjected to Western blot analysis using anti-COX-2 or anti-iNOS antibody.

block nuclear translocation of NF-κB, whereas complete accumulation of NF-κB in the cytoplasm was observed in PS-341–treated cells. KA does not block IκB degradation, but it apparently interfered with translocation of NF-κB to some extent.

Reversible Inhibition of NF-κB DNA-Binding Activity by Diterpenoids. The effect of diterpenoids on the interaction of NF-κB with consensus oligonucleotides was studied by electrophoresis mobility shift assay. Gel-shift study revealed the protein-DNA complexes that were formed could be recognized by both anti-p65 and anti-p50 antibodies. We studied the effect of the diterpenoids on the direct interaction between NF-κB and the consensus oligonucleotides. TNF-α–activated nuclear extracts were directly incubated with diterpenoids as described previously (Fig. 5a). All five compounds showed a significant inhibitory effect on the DNA-binding activity of NF-κB activated by TNF-α in a concentration-dependent manner, whereas they did not inhibit the DNA binding activity of Oct-1, a transcriptional regulator that functions in various developmental processes such as cell division, differentiation, specification, and survival of specific cell types and participates in the determination of cell fate. In contrast to the cell-based experiment (Fig. 2), Ori and Pon were found to be more potent than Xdn-A and Xdn-B, suggesting that other factors (e.g., metabolism, uptake, or targets associated with NF-κB transcription) could have an impact on the activity of these compounds in cells.

In another experiment, cells were preincubated with the drugs before TNF-α treatment. Nuclear extracts were prepared and analyzed for NF-κB activity by measuring NF-κB/DNA complex formation. Low levels of NF-κB/DNA complex were detected in nonstimulated cells, whereas TNF-α induced a 5-fold increase of the NF-κB/DNA level (FIG. 5b). Xdn-A and Xdn-B did not significantly decrease the amount of complex formation. A slight decrease in complex formation was observed in Ori- and Pon-treated cells, whereas a more

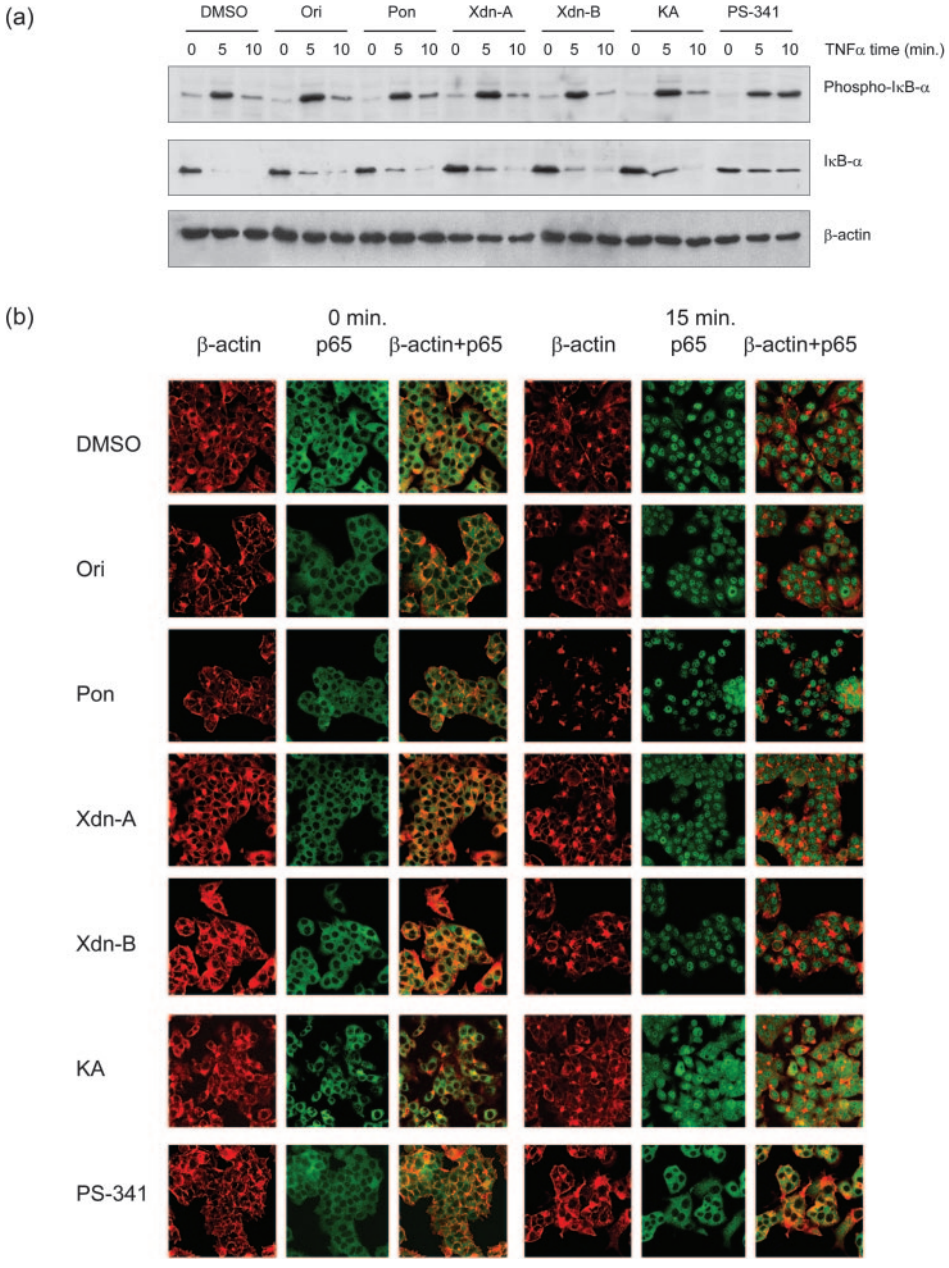


Fig. 4. Effect of diterpenoids on TNF-α-induced phosphorylation and degradation of IκB-α and nuclear translocation of p65. HepG2 cells were preincubated with equal potency (IC_{50} value against NF-κB activity) of diterpenoids for 1 h before activation with 50 ng/ml TNF-α. a, protein lysates were prepared at the indicated time points as described under *Materials and Methods* and were subjected to Western blot analysis using anti-phospho-IκB-α or anti-IκB-α antibody. Protein amounts were normalized by using anti-β-actin antibody. b, at the indicated time points, cells were fixed, permeabilized, and examined by confocal microscopy. β-Actin in the cytoplasm was recognized by red fluorescence, and p65 protein was recognized by green fluorescence.

pronounced decrease was observed in KA-treated cells. Western blotting analysis showed that the level of p65 in the nuclei of KA-treated cells was lower. This is consistent with our previous observation (Fig. 4b).

Our results suggest that blocking NF-κB from binding to DNA seems to be a common mechanism shared by the diterpenoids studied. In addition, the inhibitory action of Ori, Pon, and KA may also involve a combination of their impact on the

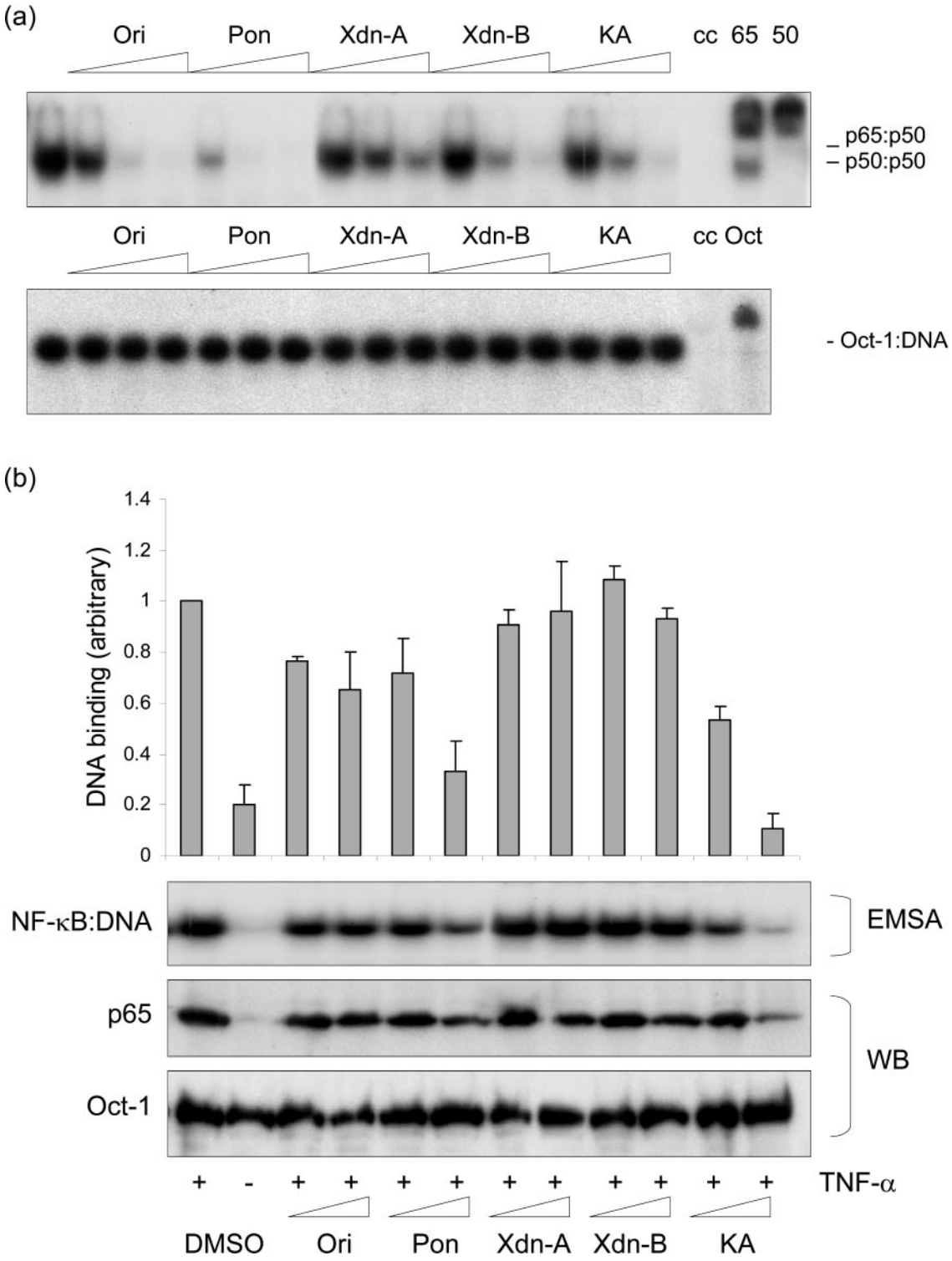


Fig. 5. Effect of diterpenoids on NF-κB DNA binding. a, nuclear extracts (2.5 μg/reaction) prepared from 50 ng/ml TNF-α HepG2 cells were incubated with radiolabeled NF-κB or Oct-1 consensus oligonucleotides in the presence of 6.25, 25, or 50 μM diterpenoids or vehicle (DMSO) alone, followed by gel electrophoresis. Nuclear extracts were also incubated with anti-p65 (p65), anti-p50 (p50), or anti-Oct-1 (Oct1) antibody or unlabeled NF-κB or Oct-1 oligonucleotide as competitor (c.c.) for an additional 30 min on ice before adding radiolabeled DNA. b, cells were pretreated with vehicle (DMSO), and IC_{50} or IC_{90} values of diterpenoids 1 h before TNF-α stimulation for 15 min were measured. Nuclear extract (5 μg/reaction) was prepared and subjected to electrophoretic mobility shift assay (EMSA) with NF-κB consensus oligonucleotides. Nuclear extracts were analyzed for protein levels of p65 and Oct-1 (internal standard) by Western blotting (WB).

translocation of NF-κB. Because the inhibitory effect on DNA binding could only be observed by direct incubation of nuclear extract with the compounds, the action of these compounds in interfering with the binding between NF-κB and DNA is a reversible process.

Noncompetitive Inhibition of NF-κB DNA Binding Activity by Diterpenoids. To understand the nature of diterpenoid inhibition on the binding between NF-κB and DNA with its consensus sequence, kinetic analysis was performed by using electrophoretic mobility shift experiments as described under *Materials and Methods* (Fig. 6). The apparent equilibrium dissociation constant ($K_{d\ app}$) for p65/p50 binding to the consensus oligonucleotides was found to be 10 ± 1.2 nM, which is consistent with that reported previously by Phelps et al. (2000). The $K_{i\ app}$ values of Ori, Pon, Xdn-A, Xdn-B, and KA were 17, 7.5, 88, 28, and 30 μM, respectively. The double-reciprocal plot shows that the diterpenoids decrease B_{max} but have no effect on $K_{d\ app}$, suggesting that they are noncompetitive inhibitors with respect to the DNA substrates for NF-κB binding activity. From our findings, we conclude that this class of compounds suppresses NF-κB DNA binding activity by interfering with the extent of NF-κB binding to DNA.

Diterpenoids Interact with Both p65 and p50. By overexpressing p65 or p50 in HepG2 cells, we investigated the impact of these compounds on the DNA binding activity of these two NF-κB subunits. Cells were transiently transfected with p65 or p50 expression vectors. Figure 7a shows the overexpression of p65 and p50 in HepG2 cells by Western blot analysis using anti-p65 and anti-p50 antibodies. Nuclear extracts were subsequently prepared 24 h after transfection for electrophoresis mobility shift assay (Fig. 7b). Compositions of NF-κB in p65- and p50-overexpressing cells were studied by a supershift experiment using antibodies against different NF-κB subunits. Overexpression of p65 led to the formation of p65/p50-DNA and p65/p65-DNA complexes. The p65/p50-DNA complex, which has a molecular size comparable with that induced by TNF-α (lane 1), could be recognized by both anti-p65 and anti-p50 antibodies (lanes 3–5). The p65/p65-DNA, which appeared as a bigger complex, could only be shifted by anti-p65 antibody (lane 3). Overexpression of p50 resulted in the formation of a smaller complex, p50/p50-DNA, which could be shifted by anti-p50 (lane 12) but not by anti-p65 antibody (lane 11). All diterpenoids (Fig. 7b is representative of all diterpenoids we tested) inhibited in a concentration-dependent manner the DNA binding activity of both p65 (lanes 4, 6–9) and p50 (lanes 11, 14–17) homodimers, which cannot be recognized by anti-p50 and anti-p65 antibodies, respectively. It is surprising that in contrast to findings reported previously, KA, in addition to interfering with p50 binding, also inhibited the DNA binding activity of p65.

Selective Inhibition of NF-κB Binding to DNA with Consensus but Different Sequences. Our data (Fig. 3) showed that the expression of COX-2 was found to be less sensitive than that of iNOS to the diterpenoids. Given that diterpenoids can inhibit COX-2 and iNOS expression at the transcription level although they have different upstream NF-κB binding sequences, it is conceivable that this class of compounds may have differential effects on the interaction of NF-κB, with DNA having the same consensus but different sequences, resulting in the discrepancy of sensitivity. Research of the literature reveals that there are one NF-κB consensus DNA sequence within COX-2 promoter (Yeo et al., 2003) and two NF-κB DNA consensus sequences within iNOS promoter (Kim et al., 1997) that are responsible for LPS-induced NF-κB binding (as shown in Fig. 8). In this experiment, we studied the effect of Pon, which is the most potent NF-κB binding inhibitor among the five compounds, on the interaction between NF-κB and DNA with different sequences. Pon inhibited the binding of NF-κB to all DNA sequences, although it was less potent against NF-κB binding to DNA having a COX-2 sequence than that with an iNOS sequence. Supershift study shows that the protein-DNA complex could be shifted by anti-p65 or anti-p50 but not by anti-interleukin-6 (as a control). This reveals that protein complexes which specifically bind to different sequences share an identical composition of NF-κB subunits, including mainly p65/p50 with a low level of p50/p50 dimers. This suggests that the drug could interfere with the binding between NF-κB proteins and different DNA sequences to various extents.

Discussion

Ori, Pon, Xdn-A, and Xdn-B are diterpenoids isolated from *I. rubescens*, a Chinese herb traditionally used to treat cancer and inflammatory diseases. In this study, the impact of the diterpenoid chemicals from this herb on NF-κB activity, which plays a critical role in both tumor cell growth and inflammatory process, was demonstrated. Their effects on NF-κB partly explained the antitumor and anti-inflammatory activities of *I. rubescens*. However, the claimed pharmacological activity of this herb could also be partly caused by the additional actions of its diterpenoids on other signaling pathways or targets. IKK, which plays a central role in the activation of NF-κB, can be activated by different stimuli through distinct pathways (Yang and Kazanietz, 2003). Our data demonstrated that IκB-α phosphorylation induced by TNF-α through activation of mitogen-activated protein kinase kinase-3 was not significantly affected by this class of compounds, although their impact on IKKα and IKKβ regulated by phorbol ester through Ras-dependent mitogen-activated protein kinase kinase-1, protein kinase C β (PKCβ), and PKCθ, respectively, needs to be further explored (Baumann et al., 2000; Khoshnan et al., 2000; Yang and Kazanietz, 2003; Viatour et al., 2005). In addition, this class of compounds could have an impact on the transcriptional activity of NF-κB, which can be regulated by several signaling pathways. Other studies have shown that phosphorylation and acetylation of p65, which are critical for its nuclear function, are under the regulation of different cellular components. Ser276, Ser311, Ser529, and Ser536 of p65 were found to be phosphorylated by mitogen and stress-activated protein kinase, PKCζ, casein kinase II, and IKKs, respectively, although p65 can also be acetylated by cAMP response element-binding protein/p300 (Wooten, 1999; Chen and Greene, 2003; Quivy and Van Lint, 2004; Viatour et al., 2005).

Diterpenoids are a new class of NF-κB inhibitor derived from their unique mode of action compared with other NF-κB DNA binding inhibitors. Helenalin was the first anti-inflammatory compound shown to exert its effect by alkylation at cysteine residues at the DNA binding domain

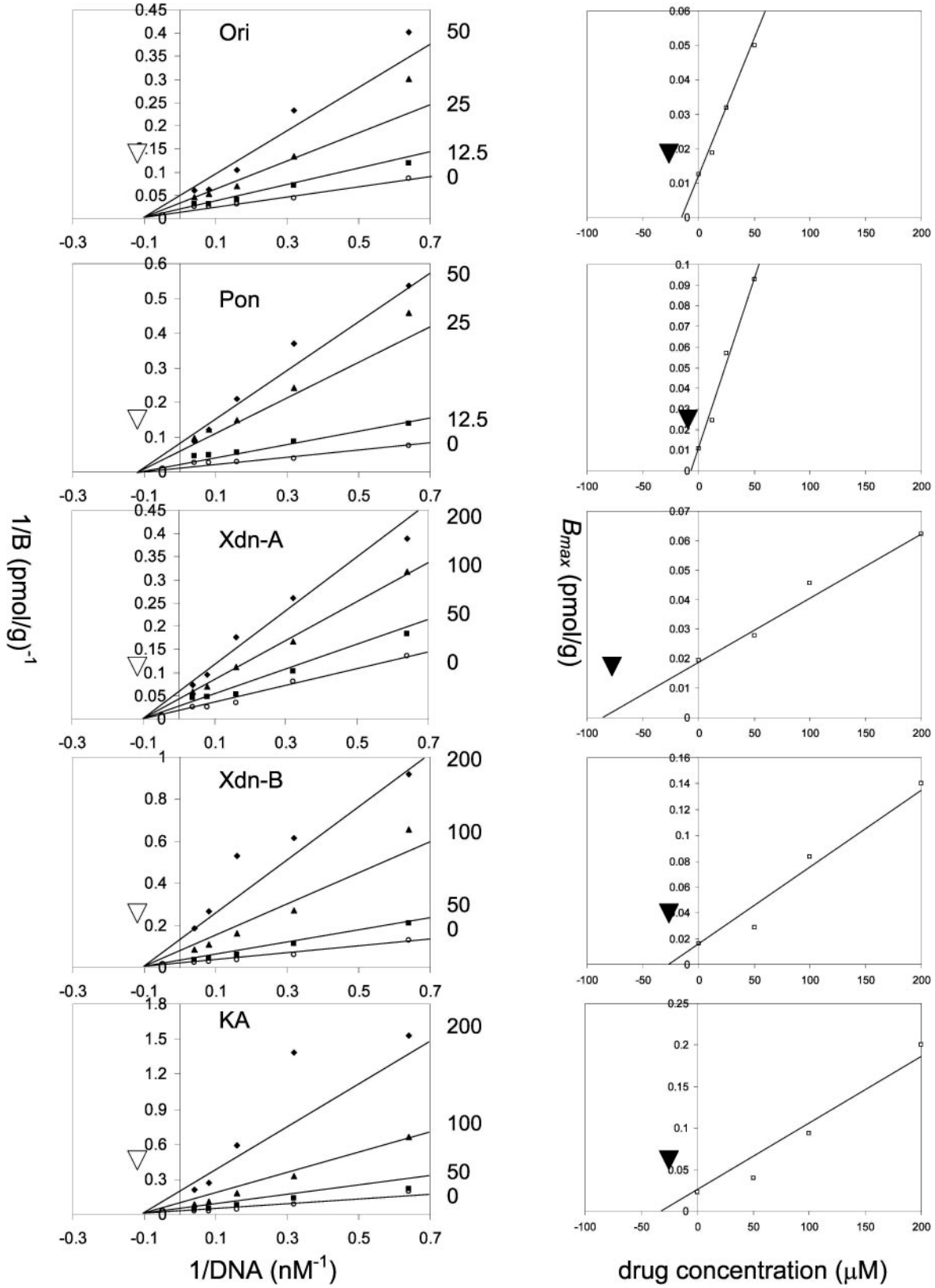
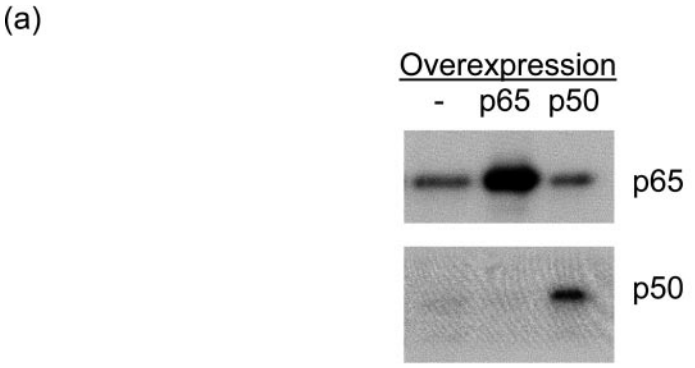


Fig. 6. Kinetic analysis of p65/p50 DNA binding by diterpenoids. DNA binding assay was performed as described under *Materials and Methods* at a fixed nuclear protein amount (2.5 μg/reaction) with various concentrations of NF-κB oligonucleotides (1.56–25 nM) in the presence of increasing diterpenoids. The amount of NF-κB–DNA complexes formed was quantified using a densitometer. Left, double-reciprocal plots showing noncompetitive inhibition by different diterpenoids. ▽, $-1/K_{d\ app}$. Right, B_{max} values were plotted against the concentration of diterpenoids to estimate $K_{i\ app}$ (▼, $-K_{i\ app}$). *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 double-reciprocal plot.

of p65 of NF-κB (Lyss et al., 1998). KA is another compound that was suggested to interact with cysteine of the DNA binding domain of the p50 subunit of NF-κB (Lee et al., 2002). Our studies indicate that KA could interact with both p50 and p65 subunits of NF-κB. Diterpenoids found in *I. rubescens* and KA described by us were found to be noncompetitive inhibitors with respect to DNA. These compounds reversibly interact with both p65 and p50 subunits at a site other than the DNA binding site and subsequently inhibit the binding affinity of the NF-κB toward DNA with different NF-κB binding sequences. The action of these diterpenoids in inhibiting NF-κB binding to other DNA sequences could be compromised by glutathione or dithiothreitol (Lee et al., 2002; C. H. Leung, K. M. Ng, Y. C. Cheng, unpublished data). It is likely that the diterpenoids may be able to bind to the cysteine residues of the non-DNA binding domain of p65 or p50 and modify the transcriptional activity. Therefore, cysteine 38 and cysteine 62, which are located within the DNA-binding domain of p65 and p50, respectively (Huang et al., 1997; Chen et al.,



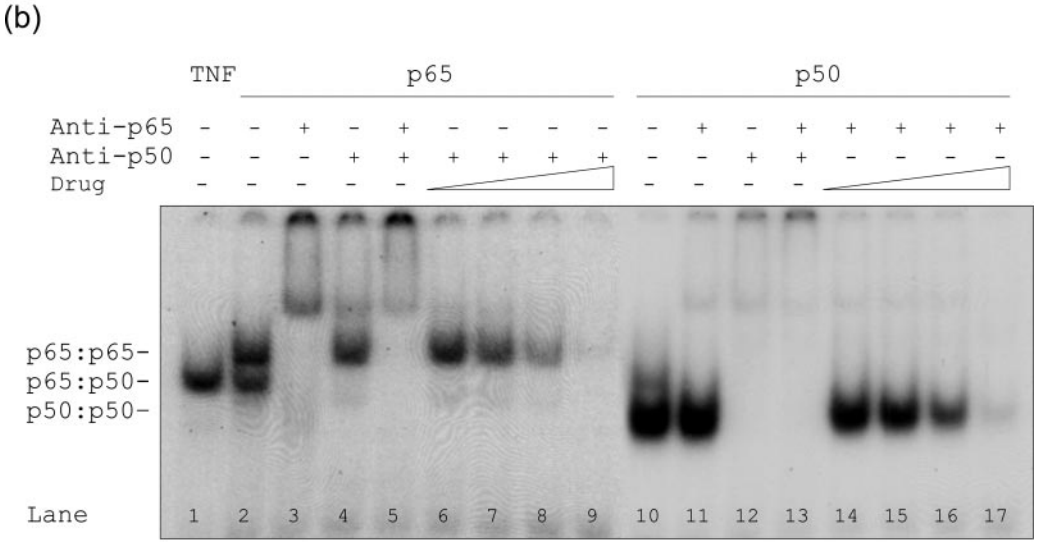


Fig. 7. Inhibition of DNA binding activity of p65 and p50. a, HepG2 cells were transfected with p65 or p50 expression vector for 24 h. The nuclear level of p65 or p50 was determined by Western blot analysis. b, nuclear extracts from p65- or p50-overexpressing HepG2 cells were preincubated with the indicated antibodies (lanes 3–9 and 11–17) and increasing concentrations of diterpenoid (lanes 6–9 and 14–17) for 30 min. Binding was initiated by adding radiolabeled NF-κB consensus oligonucleotides and was detected as described under *Materials and Methods*. This figure is representative of all diterpenoids we tested.

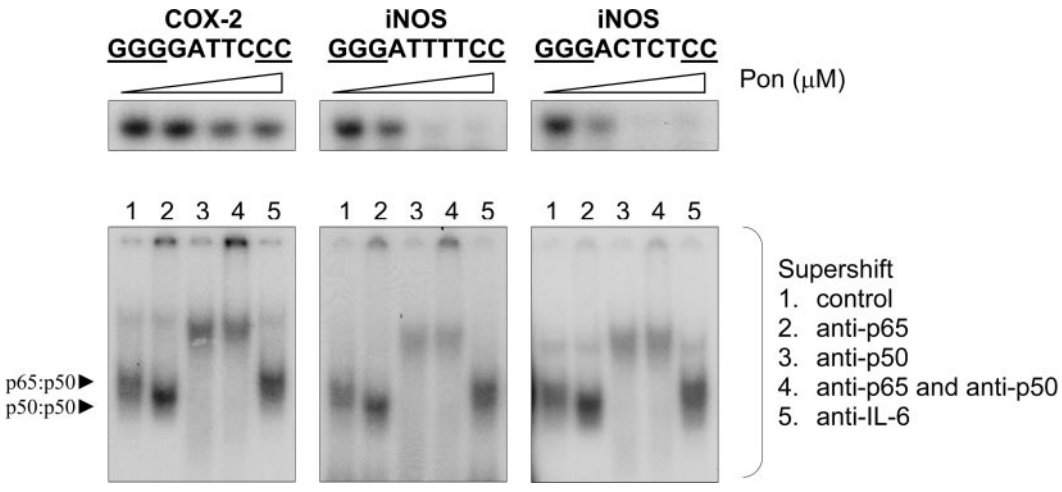


Fig. 8. Selective inhibition of NF-κB binding to different sequences. LPS (1 μg/ml)-treated RAW264.7 nuclear extracts (5 μg/reaction) were pretreated with increasing concentrations of Pon (0, 12.5, 25, and 50 μM) (top) or antibodies as indicated (bottom) before incubation with different NF-κB consensus sequences. Binding was detected by electrophoresis mobility shift assay as described under *Materials and Methods*.

1998), are unlikely to be the amino acids involved in the binding of these compounds. This needs to be further investigated.

All of the diterpenoids suppress the growth of various carcinomas (IC_{50} values: Ori, 7 ± 1.13 μM; Pon, 5.5 ± 1.35 μM; Xdn-A, 1.11 ± 0.18 μM; and Xdn-B, 1.25 ± 0.33 μM in HepG2; and Ori, 6.60 ± 0.42 μM; Pon, 3.55 ± 0.07 μM; Xdn-A, 1.05 ± 0.07 μM; and Xdn-B, 0.93 ± 0.11 μM in KB), as well as NF-κB activity (Fig. 2) in cell culture. However, the potencies were found to be inconsistent with those that were derived from the mobility shift assay. Xdn-A and Xdn-B have relatively higher K_i values compared with Ori and Pon, whereas they seem to be more potent in cell culture. One of the possibilities is that the accumulation of a higher concentration of the compounds in the nuclei (such as Xdn-A and Xdn-B), which is common for many DNA-targeted drugs, could occur. To address this issue, the impact of the diterpenoids on NF-κB–DNA interaction inside the cells is currently under investigation with chromatin immunoprecipitation assay. In addition, other factors could be involved in modulating the activity of this class of compounds inside the cells. Given that the action of these compounds could be interfered with by glutathione, the redox potential could play a key role in regulating the activity of these compounds against NF-κB in cells. Furthermore, the uptake of these diterpenoids by cells could also be different. The presence of acetoxy, keto, and hydroxy groups can dramatically alter the lipid solubility of a compound and its uptake.

The action of the diterpenoids studied against NF-κB transcription may not be limited only to their effect on the interaction of NF-κB with its responsive DNA. Ori, Pon, and KA, but not Xdn-A and Xdn-B, also have an impact on the translocation of NF-κB to different degrees. These compounds do not significantly block the rapid phosphorylation and degradation of IκB-α induced by TNF-α (Fig. 4a), but they cause a decrease in the nuclear accumulation of TNF-α–induced p65 (Figs. 4b and 5b). This suggests that Ori, Pon, and KA interfere with the shuttling of NF-κB between the nucleus and the cytoplasm and, as a result, could disturb the steady-state NF-κB localization, which is independent of IκB-α degradation. A novel mechanism of regulation of the translocation of NF-κB is indicated.

Our study has demonstrated an interesting structure-activity relationship of diterpenoids. The five diterpenoids studied here displayed differential effects against the DNA-binding activities of NF-κB even though they shared the same core structure. Furthermore, more inhibition of NF-κB binding to the NF-κB binding sequences was observed within the iNOS promoter region than in the COX-2 promoter region. This could account for the higher sensitivity of iNOS gene transcription toward the compounds compared with that of COX-2. Therefore, it is conceivable that diterpenoids with chemical modification could differentially inhibit the NF-κB promoters of different genes.

In summary, the presence of Ori, Pon, Xdn-A, and Xdn-B, which are potent inhibitors of NF-κB transcription activity, could partly account for the medical uses of *I. rubescens*. Their action against NF-κB transcription is, in part, mediated through their interaction with both p65 and p50 and occurs in a manner that is NF-κB–binding DNA sequence-dependent. The core structure of these compounds could serve as a scaffold for designing more selective inhibitors having selectivity toward different NF-κB downstream genes. Furthermore, inhibitors acting on additional targets involved in the NF-κB pathway activated by different stimuli could also be explored with this class of compounds.

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