Kaurane Diterpene, Kamebakaurin, Inhibits NF-κB by Directly Targeting the DNA-binding Activity of p50 and Blocks the Expression of Antiapoptotic NF-κB Target Genes*

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Kaurane diterpenes have been identified from numerous medicinal plants, which have been used for treatment of inflammation and cancer, however, their molecular mechanism of action remains unclear. We have previously shown that kamebakaurin and other three kaurane diterpenes selectively inhibit activation of transcription factor NF-κB, a central mediator of apoptosis and immune responses. We here demonstrate that kamebakaurin is a potent inhibitor of NF-κB activation by directly targeting DNA-binding activity of p50. Kamebakaurin prevented the activation of NF-κB by different stimuli in various cell types. Kamebakaurin did not prevent either stimuli-induced degradation of IκB-α or nuclear translocation of NF-κB, however, it significantly interfered DNA binding activity of activated NF-κB in cell and in vitro and preferentially prevented p50-mediated DNA-binding activity of NF-κB rather than that of RelA as measured using in vitro translated p50 and RelA proteins. Moreover, a p50 mutant with a Cys-62 → Ser mutation was not inhibited with kamebakaurin, indicating that the effect of kamebakaurin was probably due to its interaction with cysteine 62 in p50. The covalent modification of p50 by kamebakaurin was further demonstrated by mass spectrometry analysis that showed an increase in the molecular mass of kamebakaurin-treated p50, and this modification was not reverted by addition of dithiothreitol. These results suggested that kamebakaurin exhibited its inhibitory activity by a direct covalent modification of cysteine 62 in the p50. Also, treatment of cells with kamebakaurin prevented the tumor necrosis factor-α (TNF-α)-induced expression of antiapoptotic NF-κB target genes encoding c-IAP1 (hiap-2) and c-IAP2 (hiap-1), members of the inhibitor of apoptosis family, and Bfl-1/A1, a prosurvival Bcl-2 homologue, and augmented the TNF-α-induced caspase 8 activity, thereby resulting in sensitizing MCF-7 cells to TNF-α-induced apoptosis. Taken together, kamebakaurin is a valuable candidate for the intervention of NF-κB-dependent pathological conditions such as inflammation and cancer.

Nuclear factor κB (NF-κB)1 represents a family of eukaryotic transcription factors participating in the regulation of various cellular genes involved in the immediate early processes of immune, acute phase, and inflammatory responses as well as genes involved in cell survival (1). In most cell types, the pleiotropic-inducible form of NF-κB is a heterodimer composed of p50 and RelA (previously termed p65) (2). RelA contains a C-terminal transactivation domain in addition to the N-terminal Rel homology domain, thus serving as a critical transactivation subunit of NF-κB (3, 4). p50 lacks a transactivation domain and is believed to serve as a regulatory subunit modulating the DNA binding affinity of RelA (3, 4). The p50-RelA NF-κB heterodimer is normally sequestered in the cytoplasmic compartment by physical association with inhibitory proteins, including IκB-α and related proteins (5). IκB-α specifically binds to and masks the nuclear localization signals of RelA and p50, thereby preventing the nuclear translocation of the NF-κB heterodimer (6). The latent cytoplasmic NF-κB RelA/p50 complex can be post-translationally activated by a variety of cellular stimuli, which trigger site-specific phosphorylation of IκB-α by a multisubunit IκB kinase (7–9). The phosphorylated IκB-α becomes rapidly ubiquitinated and degraded by the proteasome complex (10, 11). Following IκB-α degradation, the NF-κB heterodimer is translocated to the nucleus, where it activates the transcription of target genes.

NF-κB regulates the transcription of various inflammatory cytokines, such as interleukin-1, -2, -6, and -8 and TNF-α, as well as genes encoding cyclooxygenase II, inducible nitric oxide synthase, immunoreceptors, cell adhesion molecules, hematopoietic growth factors, and growth factor receptors (12). In addition to regulating the expression of genes important for immune and inflammatory responses, NF-κB also controls the transcription of genes that confer resistance to death-inducing signals. Candidate target genes include those encoding the caspase inhibitors c-IAP1, c-IAP2, and X-IAP, the TNF receptor-associated factors TRAF1 and TRAF2, the zinc finger protein A20, the immediate-early response gene IEX-1L, and the prosurvival Bcl-2 homologue Bcl-2 (13–16). Therefore, pharmacological inhibition of NF-κB could be a valuable strategy to modulate the inflammatory processes as well as cell death.

Whole plant extracts of Isodon japonicus have been used in folk medicine in China, Japan, and Korea for a remedy for gastrointestinal disorder, tumor, and inflammatory diseases (17, 18). The genus Isodon (also called Rabdosia) is a rich

bakaurin; AP-1, activator protein-1; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; FMA, phorbol 12-myristate 13-acetate; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; 15d-PGJ2, 15-deoxy-A2,15-prostaglandinJ2.

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source of diterpenes, especially the highly oxidized kaurane diterpenes. Previously, we have shown that four diterpenes, including kamebakaurin (KA) inhibit the LPS-induced NO and prostaglandin E₂ production in RAW264.7 cells (19). We here show that KA inhibits NF-κB by directly targeting DNA-binding activity of p50, possibly through a covalent modification of cysteine 62 within the DNA-binding domain, without affecting the induced degradation of IκB-α and nuclear translocation of NF-κB. Also, KA not only prevented the TNF-α-induced expression of antiapoptotic NF-κB target such as c-IAP1 and Bfl-1/A1 genes but also augmented TNF-α-induced caspase-8 activity, resulting in sensitizing MCF-7 cells to NF-κB-induced apoptosis. This study shows that KA is a potential candidate for modulation of NF-κB-dependent pathological conditions.

MATERIALS AND METHODS

Cell Culture and Chemicals—Jurkat T leukemia cells, THP-1 cells, and MCF-7 cells were maintained in RPMI 1640 medium. HeLa cells, RAW264.7 cells, and HT-1080 cells were maintained in Dulbecco’s modified Eagle’s medium. Both media were supplemented with penicillin (100 U/ml)-streptomycin (100 μg/ml), (Invitrogen, Carlsbad, CA) and 10% heat-inactivated fetal bovine serum (Invitrogen). All cells were grown in an incubator at 37 °C and 5% CO₂. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, untreated cells or treated cells with KA and/or TNF-α in a 96-well plate were incubated for 48 h followed by the addition of MTT to the cells. Caspase-8 activity was determined using caspase-8 colorimetric assay kit according to the manufacturer’s instructions (Clon-
Kamebakaurin Inhibits DNA-binding Activity of NF-κB by Targeting p50

TECH, Palo Alto, CA. Optical densities were determined on a microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm.

RESULTS

KA Inhibits NF-κB Activation by LPS, TNF-α, and PMA—In an effort to identify NF-κB inhibitors from anti-inflammatory herbal medicine, we have identified KA (compound 1) together with three other kaurane diterpenes, kamebanin, kamebacetal A, and excisanin A (compounds 2–4, respectively) from a traditional medical plant, I. japonicus (Fig. 1), which has been used in the treatment of inflammatory diseases and cancer (17, 18). All compounds inhibited the LPS-induced NF-κB activation as well as the LPS-induced productions of NO and prostaglandin E2 in RAW264.7 cells without affecting cell viability, and KA was more abundant and more potent than the others (19). The effect of KA on the NF-κB activation by various stimuli was investigated in a NF-κB reporter assay. KA inhibited TNF-α-, PMA-, and LPS-induced expression of NF-κB reporter gene construct in a dose-dependent manner (Fig. 2). Basal NF-κB activity was also suppressed by KA. To confirm that KA inhibits NF-κB activation, we performed electrophoretic mobility shift assays (Fig. 3). Three cell lines, human breast cancer MCF-7, human lymphoma Jurkat, and human monocye THP-1, were preincubated with various concentrations of KA for 30 min prior to stimulation. THP-1 cells were stimulated for 30 min with LPS, Jurkat cells for 30 min with PMA, and MCF-7 cells for 90 min with TNF-α. After the stimulation, nuclear extracts were prepared and DNA-binding activity of NF-κB in the nuclear extracts was measured. We found that these cell lines stimulated with the corresponding stimuli strongly induced DNA-binding activity of NF-κB. However, pretreatment of KA dose-dependently inhibited DNA-binding activity of NF-κB induced by above stimuli. Similar to the reporter assay, basal DNA-binding activity of NF-κB was significantly reduced at 10 µg/ml of KA. All of these results indicate that KA interferes with one or more common steps during NF-κB activation in different cell types rather than with one single event specific for an individual stimuli.

KA Does Not Significantly Inhibit Degradation of IκB-α and Translocation of NF-κB to Nucleus—Because degradation of IκB proteins is an essential step for NF-κB activation by various stimuli, we firstly examined the effect of KA on the induced degradation of IκB-α protein by TNF-α (Fig. 4). MCF-7 cells were pretreated with 10 µg/ml KA for 30 min and subsequently stimulated with TNF-α for indicated times. Total cell extracts were analyzed for the presence of IκB-α with Western blots. IκB-α was completely degraded in 30 min after stimulation with TNF-α and re-synthesized in 60 min. However, preincubation with KA did not prevent the induced degradation of IκB-α protein. Interestingly, resynthesis of IκB-α, which is under control of NF-κB, was significantly suppressed by KA. Identical results were obtained for all stimuli described here with KA (data not shown). To further examine the inhibitory effect of KA on NF-κB activation, we measured the amount of NF-κB translocated into nucleus after stimulation. Nuclear extracts from stimulated cells were tested for the amount of NF-κB by Western blot analysis. KA did not significantly pre-
vented nuclear translocation of NF-κB after stimulation (data not shown).

KA Directly Inhibits DNA-binding Activity of Active NF-κB Complex—To further investigate the molecular target of KA, we examined the effect of KA on DNA-binding activity of activated NF-κB in vitro by EMSA. After stimulation of MCF-7 cells with TNF-α for 1.5 h, the nuclear extract was prepared and then incubated with KA in vitro. This compound significantly inhibited DNA-binding activity of activated NF-κB in a dose-dependent manner without affecting DNA-binding activity of AP-1 (Fig. 5, A and B). However, addition of 5 mM DTT in

FIG. 3. KA inhibits NF-κB activation by different stimuli. A, MCF-7 cells were preincubated for 30 min with the indicated concentrations of KA and stimulated with TNF-α (20 ng/ml) for 90 min. Subsequently nuclear extracts were prepared and tested for DNA binding of activated NF-κB by EMSA. B, Jurkat cells were preincubated for 30 min with the indicated concentrations of KA followed by the stimulation of PMA (50 ng/ml) for 30 min. Subsequently, nuclear extracts were prepared and tested for DNA binding of activated NF-κB by EMSA. C, THP-1 cells were preincubated for 30 min with the indicated concentrations of KA and stimulated with LPS (10 μg/ml) for 30 min. Subsequently, nuclear extracts were prepared and tested for DNA binding of activated NF-κB by EMSA. In lane Ap-1, a 100-fold excess of unlabeled AP-1 consensus oligonucleotide was added to the reaction mixture. In lane NF-κB, a 100-fold excess of unlabeled NF-κB oligonucleotide was added to the reaction mixture. The arrow indicates the location of the DNA:NF-κB complex. The amount of DNA:NF-κB complex formed was estimated by image scanning and is expressed in arbitrary units at the bottom of each panel.

Fig. 4. Effect of KA on the degradation of IκB-α induced by TNF-α. MCF-7 cells were pretreated for 30 min with 10 μg/ml KA prior to stimulation with TNF-α (20 ng/ml). Cells were harvested at the indicated time points, and total cell extracts were prepared. IκB-α protein was detected by Western blot analysis as described under "Materials and Methods."
the reaction mixture completely reversed the inhibitory effect of KA (data not shown). It is important to mention that concentration to inhibit NF-κB activation in vitro is comparable with those of in cells.

To address that KA inhibits active NF-κB in cells, RAW264.7 cells were pretreated with LPS for 30 min and subsequently treated with KA for indicated times. Nuclear extracts were analyzed for the DNA-binding activity of NF-κB by EMSA. Postincubation with KA after LPS stimulation significantly suppressed the DNA-binding activity of NF-κB (Fig. 5C). The inhibition was time-dependent. Identical results were obtained for Jurkat cells stimulated with TNF-α (20 ng/ml) for 90 min. The indicated amounts of KA were directly added to the reaction mixture to determine the effect of KA on DNA-binding activity of the activated NF-κB (A) or AP-1 (B) by EMSA. In lane A, a 100-fold excess of unlabeled NF-κB oligonucleotide was added to the reaction mixture. In lane AP, a 100-fold excess of unlabeled AP-1 oligonucleotide was added to the reaction mixture. C. KA inhibits DNA-binding activity of activated NF-κB in cells. Lane 1 shows unstimulated RAW264.7 cells. In lane 2 cells were stimulated with 1 μg/ml LPS for 60 min without KA. In lanes 3–5, cells were treated with KA (10 μg/ml) for the last 30 (lane 3), 15 (lane 4), or 5 min (lane 5) during LPS stimulation for 60 min. The nuclear extracts were prepared and analyzed for DNA-binding activity of NF-κB by EMSA. An arrow indicates the position of the specific DNA-NF-κB complex. The amount of DNA-NF-κB complex formed was estimated by image scanning and is expressed in arbitrary units at the bottom of each panel.

Kamebakaurin Inhibits DNA-binding Activity of NF-κB by Targeting p50

KA Directly Inhibits DNA-binding Activity of p50-overexpressed Cells—Next, we investigated whether KA inhibits DNA-binding activity of p50 or RelA subunit. We prepared nuclear extracts from p50-overexpressed cells, and then analyzed the effect of KA on the DNA-binding activity in vitro (Fig. 6, A and B). In nuclear extract from the p50-overexpressed cells, DNA-binding activity of NF-κB was not interfered by the addition of RelA- or c-Rel-antibody but p50-antibody. Similar to p50-antibody, KA significantly inhibited DNA-binding activity of NF-κB (Fig. 6A). Furthermore, preincubation of the p50-overexpressed cells with KA for 30 min significantly prevented DNA-binding activity of NF-κB (Fig. 6A, lane 7). An identical experiment was also carried out with the nuclear extracts from RelA overexpressed MCF-7 cells. DNA-binding activity of NF-κB was interfered by the addition of KA, however, the major form of NF-κB was a heterodimer of RelA and p50, and RelA homodimer was barely detectable (data not shown).
next explored how DTT suppressed the effect of KA on the DNA-binding activity of p50. Co-treatment of various concentrations of DTT with KA reduced the potency of KA in a dose-dependent manner (Fig. 6B, upper panel), and the effect of KA (10 μg/ml) was completely abolished by 5 mM DTT. However, the effect of KA was not reverted by a post-treatment of DTT even at concentration of 25 mM DTT (Fig. 6B, lower panel), suggesting that covalent modification of p50 with KA is stable and that the effect of KA is not a redox-sensitive manner. In both cases EMSA was performed in the presence of 0.1 mM DTT, which was not sufficient to revert the inhibition. To verify the above results, we prepared p50 and RelA proteins by in vitro translation, and then analyzed the effect of KA on DNA-binding activities of p50 and RelA molecules. KA preferentially inhibited DNA-binding activity of p50 homodimer rather than that of RelA homodimer (Fig. 6C and D). Taken together, these results suggest that KA inhibits DNA-binding activity of NF-xB by directly modifying DNA-binding activity of p50 subunit and that the inhibitory effect of KA may arise from its interaction with cysteine residues in p50.

KA Requires Cys-62 in the Inhibition of p50 DNA Binding and Forms a Covalent Adduct with p50

KA inhibits DNA-binding activity of p50 subunit. MCF-7 cells were transiently transfected with a p50 expression vector, and nuclear extracts were prepared 48 h later after transfection and then analyzed for the effect of KA (A) or DTT (B) on the DNA-binding activity of NF-xB by EMSA. A, lane 1 shows untreated control. In lanes 2–7, EMSA was performed in the presence of antibody for RelA (lane 2), c-Rel (lane 3), p50 (lane 4), 5 μg/ml KA (lane 5), or 1 μg/ml KA (lane 6). In lane 7, prior to cell harvest, the cells were treated for 30 min with 5 μg/ml KA, nuclear extracts were prepared, and analyzed for DNA-binding activity of NF-xB by EMSA. B, co-treatment of DTT abolishes inhibitory effect of KA dose-dependently, however, post-treatment of DTT does not. The nuclear extracts were incubated with 10 μg/ml KA (lane 2–5) in the presence of various concentrations of DTT (upper panel) or incubated with 10 μg/ml KA followed by addition of various concentrations of DTT for last 10 min (lower panel). An arrow indicates the position of each specific band. In another experiment p50 or RelA proteins were prepared by in vitro translation as described under “Materials and Methods” and analyzed for the effect of KA on the DNA-binding activity of p50 (C) or RelA (D). Lane 1 shows untreated control. In lanes 2–9, EMSA was performed in the presence of RelA antibody (lane 2), p50 antibody (lane 3), a 100-fold excess of unlabeled NF-xB oligonucleotide (lane 4), a 100-fold excess of unlabeled AP-1 oligonucleotide (lane 5), vehicle (lane 6), 1 μg/ml KA (lane 7), 5 μg/ml KA (lane 8), and 10 μg/ml KA (lane 9). The amount of p50-DNA or p65-DNA complex formed was estimated by image scanning and is expressed in arbitrary units at the bottom of each panel. Results are average values of three experiments.
FIG. 7. Effect of KA on the DNA-binding activity of wild type or C62S mutant p50 and mass spectrometry analysis of control and KA-treated p50. A, the recombinant wild type and mutant type p50 proteins purified as described under “Materials and Methods” were analyzed for the effect of KA on the DNA-binding activity in the presence or absence of DTT. Wild type (WT) or mutant p50 (Cys-62 → Ser) was incubated with the indicated concentrations of KA for 30 min at 37 °C before being analyzed by EMSA. The wild type p50 was also incubated with KA in the presence of 5 mM DTT. The amount of p50-DNA complex formed was estimated by image scanning and is expressed in arbitrary units. Mean values from three independent experiments performed in triplicate are shown; S.D. is indicated by bars. B, the recombinant wild type p50 proteins purified were treated with vehicle (control) or KA (10 mg/ml) for 30 min at 37 °C and subsequently purified by reverse-phase HPLC. The fractions of the p50 peak were collected and analyzed by MALDI-TOF mass spectrometry. Upper panel, control wild type p50; lower panel, KA-treated wild type p50.
Kamebakaurin Inhibits DNA-binding Activity of NF-κB by Targeting p50

KA Prevents the Induced Expression of the NF-κB Target Genes—Recent studies demonstrate that a number of gene involved in inflammation and apoptosis is under control of NF-κB. It is well known that several antiapoptotic proteins such as Bfl-1/A1, c-IAP1, and c-IAP2 are regulated by NF-κB and block the induced apoptosis by TNF-α as well as chemotherapy agents such as etoposide (16, 23). Therefore, we examined the effect of KA on the TNF-α-induced expression of these antiapoptotic proteins (Figs. 8, A and B). After preincubation of HT-1080 and MCF-7 cells with KA with indicated concentrations for 30 min and subsequently stimulation with TNF-α for 3h, the induced expression of Bfl-1/A1 was analyzed by Northern blot. TNF-α induced a 15- and 7-fold increase of Bfl-1/A1 mRNA in HT-1080 and MCF-7 cells, respectively, however, the induced expression was blocked by KA in a dose-dependent manner. The suppression of TNF-α-induced expression of c-IAP1 (hiap-2) and c-IAP2 (hiap-1) by KA was investigated by Western blot analysis in MCF-7 cells (Fig. 8B). TNF-α induced a 4-fold increase of c-IAP proteins in MCF-7 cells. This induction was completely blocked by KA. Interestingly, KA suppressed the basal level of c-IAP1 protein expression at over 5 μg/ml. Also, KA suppressed induced expression of c-IAP2 protein. The same lysates were analyzed for Bcl-2 and Bax expression as control. Neither TNF-α nor KA modulated the expression of Bel-2 and Bax in this cell line.

KA Sensitizes TNF-α-induced Apoptosis—Because KA suppressed TNF-α-induced expression of antiapoptotic proteins, we next investigated whether this compound sensitizes TNF-α-induced cell death in MCF-7 cells (Fig. 9A). Cells were incubated with 20 ng of TNF-α for 48 h either in the presence or absence of KA and then examined for cell viability by the MTT method. TNF-α-induced cell death in MCF-7 was potentiated by KA in a dose-dependent manner. TNF-α alone induced cell death in ~17% of cells and KA (1 μg/ml) alone in ~26% of cells. However, the combination of TNF-α and KA induced cell death in over 80% of cells. We investigated to see if KA affects TNF-α-induced caspase-8 activity. Treatment of MCF-7 cells with KA or TNF-α alone (20 ng/ml) showed similar degree of caspase-8 activity, however, KA significantly induced caspase-8 activity by co-incubation with TNF-α in a dose-dependent manner (Fig. 9B).

**Fig. 8.** Effect of KA on the expression of some antiapoptotic NF-κB target genes. A, expression of Bfl-1/A1. MCF-7 (1) and HT-1080 (2) cells were pretreated for 30 min with the indicated concentrations of KA and stimulated with TNF-α (20 ng/ml) for 3 h. Subsequently, total RNAs were isolated and Northern blot analysis was performed as described under “Materials and Methods.” Ethidium bromide staining of the 18 S ribosomal RNA band on the gel was shown to demonstrate equal loading of RNA. B, expression of cIAP-1 and cIAP-2. MCF-7 cells were pretreated for 30 min with the indicated concentrations of KA followed by the stimulation with TNF-α (20 ng/ml) for 6 h. Subsequently, total cell lysates were prepared and Western blot analysis was performed as described under “Materials and Methods.”

**DISCUSSION**

Whole plants of *I. japonicus* (Labiatae) have been used in traditional oriental medicine as a remedy for gastrointestinal disorders, cancer, and inflammatory diseases and a rich source of kaurane diterpenes (17, 18). Despite various pharmacological activities, its molecular mechanism has not been sufficiently explained. In previous study, we isolated KA and three other kaurane diterpenes (Fig. 1) from the plant as inhibitors of production of inflammatory mediators and NF-κB activation induced by LPS (19), indicating that these activities of those compounds could explain, in part, its diverse pharmacological activities such as anticancer and anti-inflammation. However, it remained to be elucidated how the most abundant, a kaurane diterpene KA inhibits NF-κB activation. Here we showed that KA inhibited NF-κB by directly targeting on the DNA-binding activity of p50 subunit. KA prevents neither induced degrada

| A | TNF-α | kamebakaurin | 1 | 2 |
|---|---|---|---|---|
|   | - | + | + | + |
|   | 10 | 5 | 1 |

| B | TNF-α | kamebakaurin | 1 | 2 |
|---|---|---|---|---|
|   | - | + | + | + |
|   | 10 | 5 | 1 |
Kamebakaurin Inhibits DNA-binding Activity of NF-κB by Targeting p50

NF-κB is a heterodimer of p50 and p65 subunits, and the p50 subunit possesses a critical cysteine residue in its DNA-binding domain. This cysteine easily forms a thioadduct but not with lysine or serine. We also found that KA reacted with the sulfhydryl group of p50, thereby inhibiting DNA binding of NF-κB by direct modification of cysteine 62 of p50. Sesquiterpene lactones such as parthenolide and helenarin have also exerted their potent anti-inflammatory activity by inhibiting activation of NF-κB.

**Figure 9.** Effect of KA on TNF-α-induced apoptosis in MCF-7 cells. MCF-7 cells were pretreated with the indicated concentrations of KA for 30 min and treated with TNF-α (20 ng/ml). After 48-h incubation, cell viability (A) and caspase-8 activity (B) were determined as described under "Materials and Methods." Mean values from two independent experiments performed in triplicate are shown; bar indicates the S.D. Statistical significance (p < 0.001) judged by paired Student’s t test is marked with an asterisk.

NF-κB is also inhibited by a modification of the cysteine 62 in the p50 molecule with N-ethylmaleimide or other reagents such as cyclohexone prostaglandin (27–30). Therefore, a potential role of p50 as a target for the inhibitory action of KA on the NF-κB pathway could be hypothesized. We showed that the inhibitory effect of KA on DNA-binding activity of active p50 was completely suppressed by the addition of more than 5 mM DTT (Fig. 6B, lane 4 and 5 of upper panel), and this suppression was a dose-dependent fashion. However, post-treatment of DTT did not suppress the inhibitory effect of KA on DNA-binding activity of active p50 (Fig. 6B, lower panel). We also found that KA reacted with the sulfhydryl group of cysteine easily to give a thioadduct but not with lysine or serine (31). Indeed, a p50 mutant with a C62S mutation was not inhibited by KA, indicating that the effect of KA was probably due to its interaction with cysteine 62 in p50. The covalent modification of p50 by KA was further demonstrated by mass spectrometry analysis that showed an increase by mass unit 330 (calculated mass of KA, 350) in the molecular mass of KA-treated p50. Furthermore, the covalent modification was not reverted with the post-treatment of DTT (Fig 6B, lower panel), indicating that the inhibition by DTT on the co-treatment with KA is due to entrapping of KA by forming a thioadduct with excess DTT. Therefore, it is highly probable that KA would covalently modify cysteine 62 in the p50 molecule through a Michael-type reaction, although we did not map precisely the cysteine modified by KA. Recently, cyclohexone prostaglandin 15d-PGJ2 has been demonstrated to inhibit DNA binding of NF-κB by direct modification of cysteine 62 of p50 (30). Sesquiterpene lactones such as parthenolide and helenarin have also exerted their potent anti-inflammatory activity by inhibiting activation of NF-κB (32, 33). Molecular target of parthenolide has been demonstrated to be the cysteine 179 of IκB kinase β (34), but another report has proposed that parthenolide would modify the cysteine 38 of RelA (35). The same authors have proposed that helenalin would bind to cysteines 38 and 120 of RelA based on the EMSA and computer modeling of the RelA homodimer (33, 35). What would make anti-inflammatory compounds such as helenarin, parthenolide, 15d-PGJ2, and KA selective in the modification of cysteine residue in the different target molecules such as IκB kinase β, p50, or RelA? This difference may arise not only from chemical environment of target sulfhydryl group in the protein but also from structural environment of Michael acceptor in the NF-κB inhibitors (35). Helenarin and parthenolide contain a lactone ring conjugated with an exomethylene group, which can react with a biological nucleophile, especially the sulfhydryl group of cysteine residue by Michael type reaction. 15d-PGJ2 contains two possible reactive Michael acceptors, namely a cyclohexone ring and a doubly conjugated exomethylene functional group to the carbonyl group of the pentenone ring. This would be a possible explanation for the formation of the hemoether adduct by 15d-PGJ2 with two different cysteines in p50 (30). KA also contains an exomethylene group conjugated with a carbonyl group of cyclohexone in a bicyclic ring system, which, however, possesses a quite different structural feature from parthenolide and helenarin, which have a fused α-methylene-γ-lactone ring. These differences among NF-κB inhibitors could be attributed to their selective specificity toward target cysteines in IκB kinase β, RelA, or p50. Another group of kaurane diterpene compounds such as foliol and ent-kaur-19-oxic acid, in which a fused five-membered ring contains only the exomethylene group without a conjugated carbonyl group, has been shown to inhibit NF-κB activation by interfering with NF-κB-inducing kinase activity (36).

Several studies have demonstrated an essential role for NF-κB in preventing apoptosis induced by TNF-α and chemotherapy agents. In these studies, cells were made sensitive to TNF-α- and chemotherapy-induced apoptosis through inhibition of NF-κB activity (23, 37, 38). It is now clear that several downstream effectors of NF-κB activation have been known to suppress TNF-α- and chemotherapy-induced apoptosis. These include TRAF-1, TRAF-2, c-IAP1, c-IAP2, and Bfl-1/A1. KA clearly suppressed the induced expression of c-IAP1, c-IAP2, and Bfl-1/A1 by TNF-α without affecting Bax and Bcl-2, whose expression is not under control of NF-κB (Fig. 8). Interestingly, KA also contains an exomethylene group conjugated with a carbonyl group of cyclohexone in a bicyclic ring system, which, however, possesses a quite different structural feature from parthenolide and helenarin, which have a fused α-methylene-γ-lactone ring. These differences among NF-κB inhibitors could be attributed to their selective specificity toward target cysteines in IκB kinase β, RelA, or p50. Another group of kaurane diterpene compounds such as foliol and ent-kaur-19-oxic acid, in which a fused five-membered ring contains only the exomethylene group without a conjugated carbonyl group, has been shown to inhibit NF-κB activation by interfering with NF-κB-inducing kinase activity (36).

Further studies are needed to show how KA regulates the expression of c-IAP1 in MCF-7 cells. We were also able to demonstrate that KA sensitizes cytotoxic potential of TNF-α as assessed by MTT and that this effect is likely associated with...
caspase-8 activity (Fig. 9). It has been demonstrated that the induction of c-IAP1 and c-IAP-2 by NF-κB suppressed caspase-8 activation, resulting in cell survival (13). This is consistent with our results that KA blocked the DNA-binding activity of NF-κB and thereby suppressed TNF-α-induced expression of both c-IAP1 and c-IAP2 and, in turn, enhanced TNF-α-induced caspase-8 activity. These results demonstrate that tumor cells are sensitized to TNF-α- and chemotherapy-induced apoptosis through inhibition of NF-κB.

The relevance of most of NF-κB target genes makes this transcription factor an interesting therapeutic target for the identification of inhibitors. One group of NF-κB inhibitors exerts its inhibitory effects by antagonistic properties (39–41). These inhibitors include N-acetyl-L-cysteine, pyrrolidine dithiocarbamate, or curcumin. Another group of inhibitors interferes with the induced degradation of IkB family members by affecting the 26S proteasome or inhibiting IkB kinase complex (42, 43). Another group of inhibitors exerts their effects only in the cell nucleus by impairing the transcriptional activity of NF-κB already bound to DNA. Examples are PG490 (triptolide), and, at least in some cell type, glucocorticoids (25, 44). In addition, a group of inhibitors interferes with the DNA-binding activity of NF-κB by directly targeting the NF-κB subunits. This is the case of helenalin, which is a specific inhibitor of DNA-binding activity of RelA subunit (33). KA could be added to this group as a specific inhibitor of DNA-binding activity of NF-κB by directly targeting p50. Importantly, KA can inactivate the activated NF-κB complex. This property is crucial for the treatment of various diseases such as inflammation, where previously activated NF-κB is sustaining the pathological processes of diseases and needs to be inactivated. Taken together, we have shown that KA inhibits the NF-κB signal cascade by directly targeting DNA binding of the p50 subunit in the activated NF-κB and the induced expression of NF-κB target genes. Based on our results, KA could serve as an interesting lead compound for the development of new, potent anti-inflammatory or anticancer agents. Furthermore, this study extends our understanding of the molecular mechanisms underlying the anti-inflammatory and anticancer activities of traditional medicinal plants, which abundantly contain kaurene diterpenes.

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