

Echinacea Alkamides Prevent Lipopolysaccharide/D-Galactosamine-Induced Acute Hepatic Injury through JNK Pathway-Mediated HO-1 Expression

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ABSTRACT: This study aimed to shed light on the anti-inflammatory and hepatoprotective effect of the major alkamides dodeca-2*E*,4*E*,8*Z*,10*Z*(*E*)-tetraenoic acid isobutylamides (Alk-8/9), isolated from *Echinacea purpurea* roots, against acute fulminant hepatitis induced by lipopolysaccharide/D-galactosamine (LPS/D-GalN) in mice. The results show that Alk-8/9 dose-dependently induced heme oxygenase (HO)-1 protein expression in LPS-stimulated murine macrophages that was likely regulated by the JNK-mediated pathway through increasing SAPK/JNK phosphorylation, c-jun protein expression, and phosphorylation, and transcription factor AP-1 binding consensus DNA activity. The HO-1 inhibitor or CO scavenger significantly reversed the inhibitory effect of Alk-8/9 on TNF- α expression, whereas *N*-acetyl-L-cysteine was observed to reduce Alk-8/9-induced HO-1 expression in LPS-treated macrophages. Furthermore, Alk-8/9 markedly induced c-jun and HO-1 protein expression and suppressed serum aminotransferase activities, TNF- α expression, and hepatocyte damage in liver tissues of LPS/D-GalN-treated mice. This paper suggests a new application of *Echinacea*, a top-selling herbal supplement, as a hepatoprotective agent.

KEYWORDS: *Echinacea*, alkamide, heme oxygenase-1, JNK pathway, fulminant hepatitis

INTRODUCTION

Heme oxygenase (HO) is a rate-limiting enzyme that catalyzes the degradation of heme to yield biliverdin, carbon monoxide (CO), and Fe.¹ In heme metabolism, one molecule of heme oxidizes and generates one molecule of CO from the α -methene bridge carbon of heme.² The expression of HO isoforms in liver has been shown in previous studies; HO-1 is expressed mainly in Kupffer cells, whereas HO-2 is in parenchymal cells (hepatocytes), but both isoforms are undetectable in hepatic stellate cells and sinusoidal endothelial cells.³ HO-1 was considered to be a potential target for hepatoprotection,⁴ and its overexpression can protect against acetaminophen-, endotoxin-, and carbon tetrachloride-mediated hepatobiliary dysfunction in animal models.^{5–7} As well, a study suggested that HO-1 could prevent cytokine- and CD95-mediated liver damage in mice.⁸

CO is an important secondary biological messenger and participates in many physiological processes such as vasomotion, thermoregulation, and respiratory regulation. Recently, the role of CO in the anti-inflammatory and cytoprotective function of HO-1 has attracted growing interest. Several studies reported that CO can suppress LPS-induced pro-inflammatory cytokine production in vivo and in vitro.^{9,10} As well, HO-1-decreased tissue damage in the inflammatory response has been demonstrated by the expression of a protective mechanism.^{11,12} Therefore, HO-1 could play a regulatory role in the resolution phase of inflammation and is considered to be a potential therapeutic target for treating inflammatory diseases. Some chemopreventive agents, such as curcumin, dietary polyphenols, and quercetin, have been shown to elicit antioxidant and cytoprotective effects via induction of HO-1 mediated by the mitogen-activated protein kinase (MAPK)/Nrf2 or nuclear factor (NF)- κ B pathway.^{13–15}

Fulminant hepatic failure (FHF) is associated with severe liver disorders that result in rapid distortion of hepatic function, with a mortality rate in general >80%.¹⁶ Various medications are used for the wide variety of complications of FHF; an example is silybinin, the water-soluble derivative of silymarin from milk thistle, which exerts its beneficial effect on liver disease management by its antioxidant properties.¹⁷ *Echinacea* spp. are used as some of the most popular herbal medicines or food supplements in Europe and North America and have several pharmacological purposes, including for immune enhancement and against the common cold. In addition, *Echinacea* extracts have several inflammation-related bioactivities such as modulating chemokine and cytokine production¹⁸ and inhibiting cyclooxygenase (COX) activity.¹⁹

Echinacea plants consist of three major groups of chemical components: alkamides/polyacetylenes, caffeic acid derivatives, and polysaccharides.²⁰ *Echinacea* alkamides are some of the best known herbal cannabinomimetics that can modulate the expression of tumor necrosis factor α (TNF- α) via binding to cannabinoid receptor 2 (CB₂).²¹ Recently, experimental works have provided robust evidence of the immunomodulatory and neuroprotective properties of cannabinoids in animal models to support their potential therapeutic effects.^{22,23} Very recently, we demonstrated that alkamides are the main phytochemicals responsible for the anti-inflammatory effects of *Echinacea* by their protection of mouse skin against TPA-induced inflammation and inhibition of

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LPS-induced expression of inflammatory mediators TNF- α , IL-1 α , IL-6, and IL-2.²⁴ This study further demonstrates that dodeca-2E,4E,8Z,10Z(E)-tetraenoic acid isobutylamides (Alk-8/9), the major alkamides of *Echinacea purpurea*, are HO-1 inducers, which contributes to their hepatoprotective effects against lipopolysaccharide (LPS)/D-galactosamine (LPS/D-GalN)-induced fulminant hepatitis in mice. Activation of JNK signaling pathways is proposed for the novel pharmacological function of *Echinacea* alkamides.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. RAW264.7 cells, a murine macrophage cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured as recommended by ATCC at 37 °C in Dulbecco's modified Eagle medium (DMEM) and F12 medium (Gibco/BRL, Grand Island, NY), respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco/BRL), 100 U/mL penicillin, and 100 μ g/mL streptomycin in a 5% CO₂ incubator.

Reagents and Chemicals. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), D-galactosamine, LPS from *Escherichia coli* 055:B5 (cell culture grade), and hemoglobin (Hb) were from Sigma Chemical Co. (St. Louis, MO). Tin protoporphyrin IX (SnPP) was from Alexis Co. (Switzerland). SB203580, PD98059, and SP600125 were from Calbiochem (La Jolla, CA). Silica gel (230–400 mesh) and silica gel 60 F₂₅₄ TLC and RP-18 F_{254s} TLC plates were from Merck (Darmstadt, Germany). RP-18 silica gel (75C₁₈-OPN) was from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals and solvents were purchased or obtained as reagents or were of HPLC grade.

Isolation of Alk-8/9 from *E. purpurea*. Fresh whole plants of *E. purpurea* were obtained from Nantou County, Taiwan. A voucher specimen of the *E. purpurea*, namely, Epu-1, was deposited at the Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan.²⁴ Alk-8/9 were isolated and identified as described previously.²⁴ The yield of Alk-8/9 in dry *E. purpurea* root tissues is approximately 0.5%.

Measurement of TNF- α Production. RAW264.7 cells (1 \times 10⁵ cells/well in 96-well plates) were treated with test compound for 6 h and then incubated for 1 h in fresh DMEM with or without 100 ng/mL LPS. The concentration of TNF- α in the culture medium was determined using a commercial EIA kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

Immunofluorescence Study. RAW264.7 cells seeded in chamber slides at 1 \times 10⁴ cells/well for 1 h were treated with DMSO (vehicle control) or 100 μ g/mL Alk-8/9 for 1 h and then stimulated with or without LPS (1 μ g/mL) for an additional hour. Cells were fixed and then permeabilized with 0.1% Triton X-100. Immunofluorescence study involved staining with DAPI (1 μ g/mL, DNA marker) (Sigma-Aldrich), FITC-labeled anti- α -tubulin antibody (1:200; cytosol marker) (Calbiochem, Darmstadt, Germany), and rabbit anti-c-jun or antiphospho-c-jun antibody (1:200) (Santa Cruz Biotechnology, CA) visualized with the use of anti-rabbit Cy3-labeled secondary antibody (1:500; Jackson ImmunoResearch, West Grove, PA). The fluorescent images were captured by a Nikon fluorescent microscope (Nikon Eclipse E800).

Electrophoretic Mobility Shift Assay (EMSA). RAW264.7 macrophages were preincubated with or without Alk-8/9 (100 μ M) for 2 h and then stimulated with LPS (1 μ g/mL) for 60 and 120 min. Cells were then lysed, and the nuclei were isolated. Nuclear extracts (4 μ g) underwent EMSA with a biotin-labeled DNA probe containing the AP-1 (5'-CGCTTGATGACTCAGCCGGAA-3') or NF- κ B (5'-ATGTGAGGGGACTTTCCAGGC-3') binding site following a method described elsewhere.²⁵ The specificity of the AP-1 or NF- κ B band was analyzed by incubation with unlabeled AP-1 oligonucleotide (cold DNA).

Measurement of Reactive Oxygen Species (ROS) Generation. Levels of intracellular ROS were assessed with the specific probes

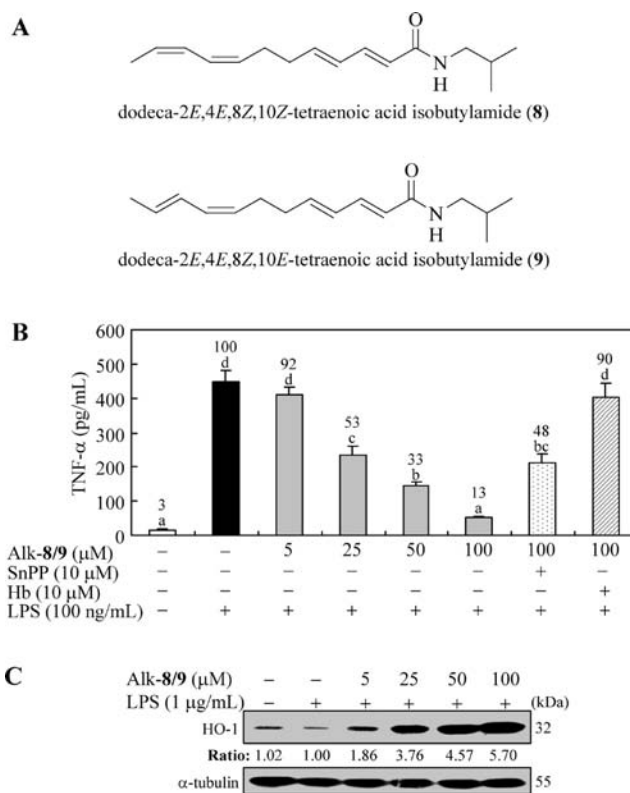


Figure 1. HO-1 mediated Alk-8/9 suppression of TNF- α production in LPS-stimulated RAW264.7 macrophages. (A) Chemical structures of Alk-8 and Alk-9 as identified from *E. purpurea* are shown. (B) Cells were treated with or without the indicated concentrations of test compound in 0.5% DMSO (vehicle control) for 6 h and then with LPS (100 ng/mL) with or without tin protoporphyrin IX (SnPP; 10 μ M) or hemoglobin (Hb; 10 μ M) for 1 h. The concentration of TNF- α in culture medium was determined. The data represent results of three independent experiments (triplicate tests for each treatment in each independent experiment) and are expressed as the mean \pm SD. Different letters are significantly different from each other ($P < 0.05$, one-way ANOVA). (C) Alk-8/9 induced HO-1 protein expression in LPS-treated RAW264.7 macrophages. Cells were treated with the indicated concentrations of Alk-8/9 for 2 h and then with LPS (1 μ g/mL) for 6 h. Total cellular protein underwent immunoblotting. HO-1 protein expression was normalized to that of α -tubulin (internal control) by densitometry.

dichlorodihydrofluorescein diacetate (H₂DCFDA, for H₂O₂) and dihydroethidium (for O₂⁻). Cells were treated with test compound for 2 h and then incubated for 30 min with or without 1 μ g/mL LPS. The cells were stained with 25 μ M H₂DCFDA or dihydroethidium for 30 min at 37 °C. The fluorescence intensity was measured on flow cytometry.

Animals. Male ICR mice (6 weeks old) were from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Mice were given a standard laboratory diet and distilled water ad libitum and kept on a 12 h light/dark cycle at 22 \pm 2 °C. Experiments were approved by the Institutional Animal Care and Utilization Committee of Academia Sinica according to institutional guidelines.

In Vivo Effects of Alk-8/9 on Acute Fulminant Hepatitis. The in vivo protective potential of Alk-8/9 on LPS/D-GalN-induced fulminant hepatitis was investigated. Twenty-four mice were randomly assigned to four groups ($n = 6$ per group) for treatment: vehicle; LPS/D-GalN; 0.1 mg/kg Alk-8/9 (Alk-L); and 1 mg/kg DET Alk-8/9 (Alk-H). Alk-8/9 were given intraperitoneally (ip) at 48, 24, and 1 h before LPS/D-GalN administration. The mice were treated with 500 ng of LPS and 25 mg of D-GalN in 250 μ L of

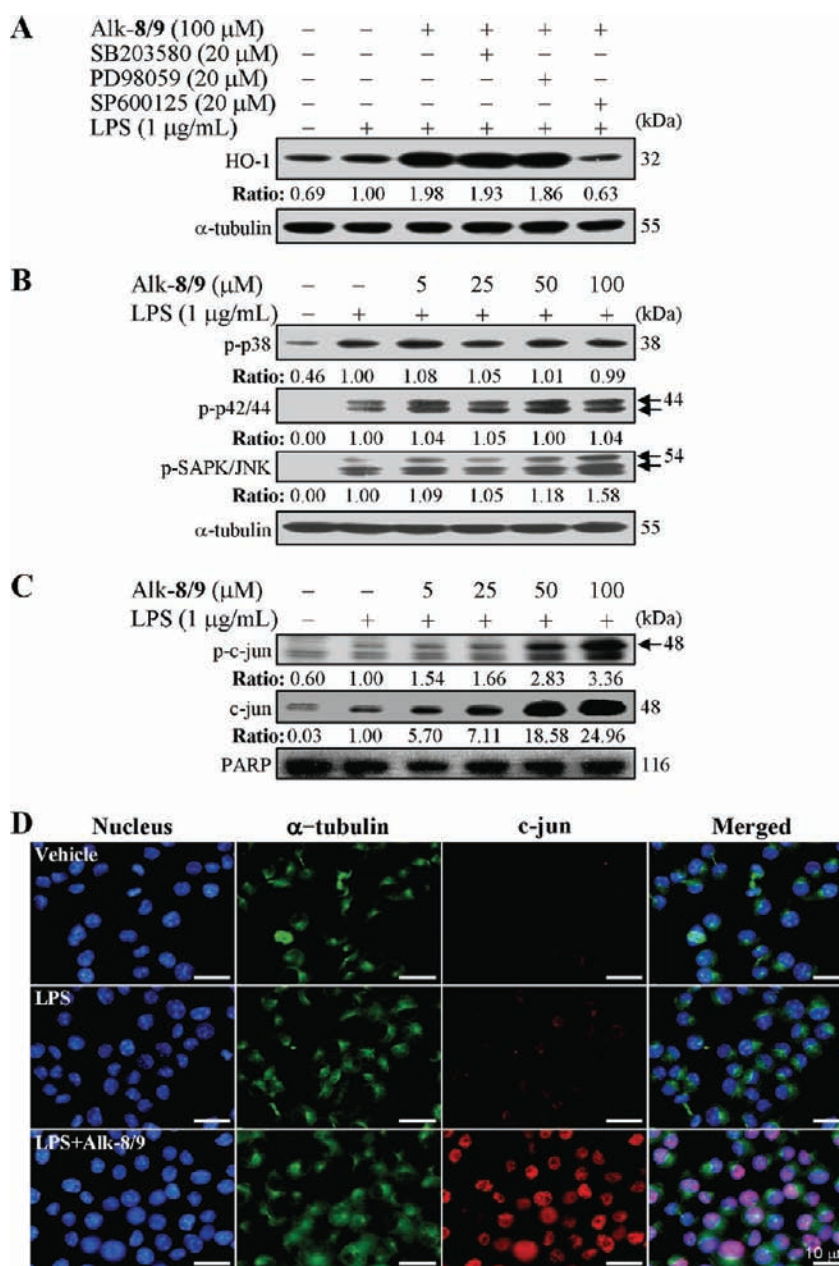


Figure 2. Activation of JNK pathway involved in Alk-8/9-induced HO-1 protein expression. (A) Cells were pretreated with various MAPK inhibitors, including SB203580 (20 μ M), PD98059 (20 μ M), and SP600125 (20 μ M), for 30 min before being treated with Alk-8/9 for 2 h and then with LPS (1 μ g/mL) for 6 h. Western blot analysis involved antibodies against HO-1 and α -tubulin; results were normalized on densitometry. (B) Alk-8/9 dose-dependently induced SAPK/JNK protein phosphorylation but not p-38 or p-42/44 in RAW264.7 macrophages. Cells were incubated with the indicated concentrations of Alk-8/9 for 2 h and were treated with LPS (1 μ g/mL) for 30 min. (C) Induction of c-jun protein expression and phosphorylation in Alk-8/9-treated RAW264.7 macrophages was dose-dependent. Expression of PARP is shown as a loading control. (D) Positive staining of c-jun protein in LPS-stimulated cells treated with Alk-8/9 is shown. Cells were stained with DAPI (DNA marker), FITC-labeled anti- α -tubulin antibody (cytosolic marker), and/or rabbit anti-c-jun antibody and visualized with anti-rabbit Cy3-labeled secondary antibody. Results of one of three independent experiments are shown.

saline as described previously.²⁶ The mice were sacrificed after blood was collected by retro-orbital bleeding 4 h after LPS/D-GalN injection.

Measurement of Serum Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) Activities. The enzymatic activities of AST and ALT were used as biochemical markers for acute liver injury. Blood samples were centrifuged at 1400 \times g at 4 $^{\circ}$ C for 15 min, and AST and ALT activities in serum supernatants were determined using Randox Diagnostic kits (Randox Laboratories Ltd, Antrim, U.K.)

Western Blotting. Total cellular proteins and the specific cytosolic and nuclear proteins were prepared as described.²⁷ Protein (20 μ g) was resolved by 5–20% gradient SDS-PAGE and subjected to immunoblotting with monoclonal antibodies against poly(ADP-ribose) polymerase (PARP; 1:500; Transduction Laboratories, Lexington, KY), α -tubulin (1:1000; Oncogene Science, Cambridge, U.K.), HO-1 and c-jun (1:500; Santa Cruz Biotechnology, CA), phospho-p38, phosphoextracellular signal-regulated kinase (ERK)1/2, phospho-SAPK/JNK, and phospho-c-jun

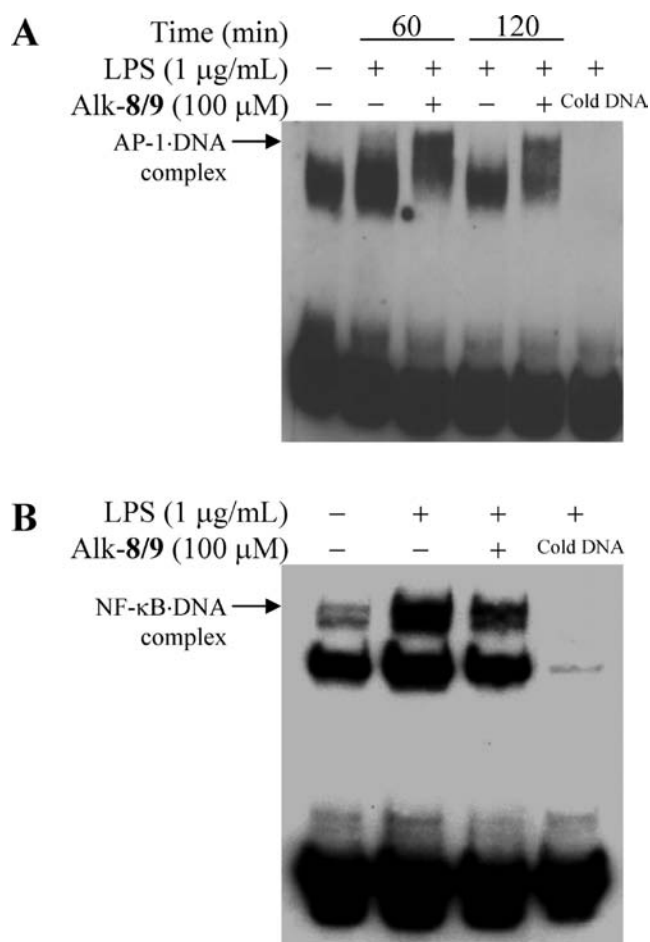


Figure 3. Induction of AP-1 by Alk-8/9 in LPS-stimulated macrophages analyzed using EMSA. Details of the experimental design are described under Materials and Methods. The specificity of the AP-1 or NF- κ B band was analyzed by incubation with unlabeled AP-1 oligonucleotide (cold DNA). The arrows indicate the AP-1 or NF- κ B complex bound to DNA.

(1:500; Cell Signaling, Boston, MA), followed by goat anti-mouse or goat anti-rabbit HRP-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology). The immunoreactive bands were visualized by use of enhanced chemiluminescence reagents (Amersham, Poole, U.K.).

Histology and Immunohistochemistry. Liver tissues were removed 4 h after LPS/D-GalN treatment. The formalin-fixed and paraffin-embedded tissues were stained with rabbit polyclonal antibody against rat anti-mouse F4/80 antibody (eBioscience, San Diego, CA), rabbit anti-c-jun antibody, and DAPI (1 $\mu\text{g/mL}$) according to the method of Shyur et al.²⁷ The sections were visualized at 400 \times magnification under a fluorescent microscope; positive-stained c-jun and F4/80 cells were visualized using AxioVision software (Carl Zeiss MicroImaging, Jena, Germany). In situ detection of apoptotic cells involved terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) according to the manufacturer's protocol (Chemicon-Millipore, Billerica, MA). Total protein of liver tissues (0.1 g) from each mouse was homogenized in a mixer ball mill (MM301, Retsch, Haan, Germany) for 2 min, extracted by adding 0.4 mL of lysis buffer and centrifuged at 15000g for 30 min at 4 $^{\circ}\text{C}$. The supernatant was collected, and total protein concentrations were determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) and then examined on immunoblotting.

Statistical Analysis. Data are expressed as the mean \pm SD. To evaluate differences among the groups studied, data were analyzed by

one-way ANOVA with Fisher's post hoc test by use of SAS (SAS Institute, Cary, NC). $P < 0.05$ was considered to be statistically significant.

RESULTS

Alk-8/9 Significantly Inhibit TNF- α Expression by Inducing HO-1 Expression. We investigated the effect of Alk-8/9 on the production of the inflammatory cytokine TNF- α and relation to CO and HO-1 activity. Macrophage RAW264.7 cells were preincubated with Alk-8/9 and a competitive inhibitor for HO-1 (SnPP) or a CO scavenger, hemoglobin, for 6 h before LPS challenge, and then secreted TNF- α protein in culture medium was determined. As shown in Figure 1B, Alk-8/9 alone significantly inhibited LPS-induced TNF- α expression ($P < 0.001$) in RAW264.7 cells; at 100 μM Alk-8/9 treatment, the level of TNF- α protein was similar to that in untreated control cells. This inhibition was significantly reversed on cotreatment with SnPP (49%) and hemoglobin (93%), which suggests that CO may play a more significant role than HO-1 in the endotoxin-induced overproduction of TNF- α and the attributed inhibitory effect of Alk-8/9. Western blotting further demonstrated that Alk-8/9 dose-dependently increased HO-1 protein level in LPS-stimulated macrophages (Figure 1C). In the presence of 5–100 μM Alk-8/9, the HO-1 protein level was induced 1.9–5.7-fold higher than that in cells treated with LPS alone. These data support that Alk-8/9-inhibited TNF- α production in LPS-stimulated macrophages was mediated by HO-1 protein expression and its catalytic product CO.

Activation of JNK Signaling Pathway Mediates HO-1 Expression by Alk-8/9. MAPKs, including ERK, JNK, and p38 MAPK, play important roles in controlling HO-1 expression.²⁸ Activation of one or more of these MAPKs triggers HO-1 gene expression. To elucidate the detailed mechanism of HO-1 expression in response to Alk-8/9, we examined the HO-1 protein level in the presence of MAPK-specific inhibitors. Cells were pretreated with SB203508 (p38 inhibitor), PD98059 (ERK inhibitor), or SP600125 (JNK inhibitor) for 1 h before incubation with Alk-8/9 and LPS. As shown in Figure 2A, pretreatment with SP600125 for 1 h abolished HO-1 expression, but SB203508 and PD98059 were ineffective, which suggests that JNK but not p38 or ERK is involved in the induction of HO-1 expression by Alk-8/9. Further immunoblotting supported that the activation of ERK (p42/44) and p38 kinase by phosphorylation were not affected, whereas the phospho-SAPK/JNK level increased with Alk-8/9 treatment (Figure 2B).

The downstream target molecule of JNK, c-jun, was analyzed. As shown in Figure 2C, Alk-8/9 treatment increased c-jun protein expression and phosphorylation in the nuclear fraction of RAW264.7 cells. The observed effect on c-jun protein expression was also examined by immunofluorescence study. Macrophages were treated with vehicle 0.5% DMSO or LPS (1 $\mu\text{g/mL}$), with or without Alk-8/9 (100 μM), and stained with fluorescence-labeled antibodies against c-jun (red). To distinguish the nucleus from cytosol, cells were also stained with DNA-specific DAPI (blue) and anti- α -tubulin antibody (green). In LPS-stimulated cells treated with Alk-8/9, the c-jun protein level was greatly induced in the nuclei of test cells, as seen in the overlapping blue and red (pinkish) images in the column "Merged" (Figure 2D).

Alk-8/9-Induced AP-1 DNA Binding Activity. HO-1 expression is regulated by transcription factor AP-1 or NF- κ B.²⁹ We used electrophoretic mobility shift assay (EMSA) to examine whether AP-1 and NF- κ B activation in LPS-stimulated macrophages

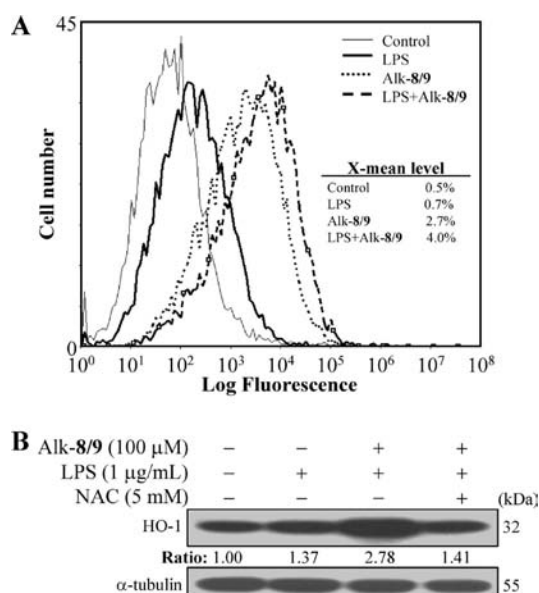


Figure 4. Alk-8/9 induced HO-1 expression associated with ROS generation in RAW264.7 cells. (A) Alk-8/9 stimulated intracellular peroxide production in macrophages. Cells were treated with Alk-8/9 for 2 h and then were incubated with LPS (1 μ g/mL) for 30 min. The cells were stained with 25 μ M H₂DCFDA for 30 min. The fluorescence intensity was measured on flow cytometry. (B) NAC inhibited Alk-8/9-induced HO-1 expression. Cells were treated with 100 μ M Alk-8/9 for 2 h with or without NAC (5 mM) pretreatment for 1 h. Expression of HO-1 protein was detected on Western blot analysis, and α -tubulin was used as a loading control.

could be affected by Alk-8/9. Macrophages treated with Alk-8/9 for 60–120 min showed increased AP-1 binding to its consensus DNA element, whereas an NF- κ B·DNA complex was little or not detected at the same test conditions (Figure 3).

ROS Generation Increases with Alk-8/9-Induced HO-1 Expression. Several agents stimulate ROS production and then induce HO-1 gene expression through activation of MAPKs.³⁰ We examined whether Alk-8/9 could stimulate ROS generation in macrophages using H₂DCFDA and dihydroethidium as fluorescent probes. Fluorescence-activated cell sorting (FACS) detection revealed intracellular H₂O₂ levels (increased H₂DCFDA intensity; Figure 4A) but not O₂⁻ levels (data not shown), were significantly increased in macrophages on treatment with 100 μ M Alk-8/9 alone or with LPS + Alk-8/9. On the addition of the antioxidant *N*-acetyl-L-cysteine (NAC) to RAW264.7 cells, HO-1 protein expression induced by Alk-8/9 was abolished (Figure 4B). Therefore, the HO-1 expression induced by Alk-8/9 in macrophages is likely associated with the generation of ROS in the treatment.

Alk-8/9 Prevent Liver Injury. HO-1 has been reported to protect several models of hepatic injury; for instance, the up-regulation of HO-1 protected mice against liver damage induced by D-GalN in combination with LPS or TNF- α .³¹ In our preliminary study, we discovered that Alk-8/9 significantly induced HO-1 protein level in FL83B murine hepatocytes (data not shown), without cytotoxicity. We thus examined the liver protection efficacy of Alk-8/9 with the LPS/D-GalN model of FHF in ICR mice. The levels of serum ALT and AST, markers of acute liver injury, were higher with LPS/D-GalN treatment than with vehicle control, which supports the development of hepatocellular injury (Figure 5A). On pretreatment with Alk-8/9 (0.1 and 1.0 mg/kg), the increased levels

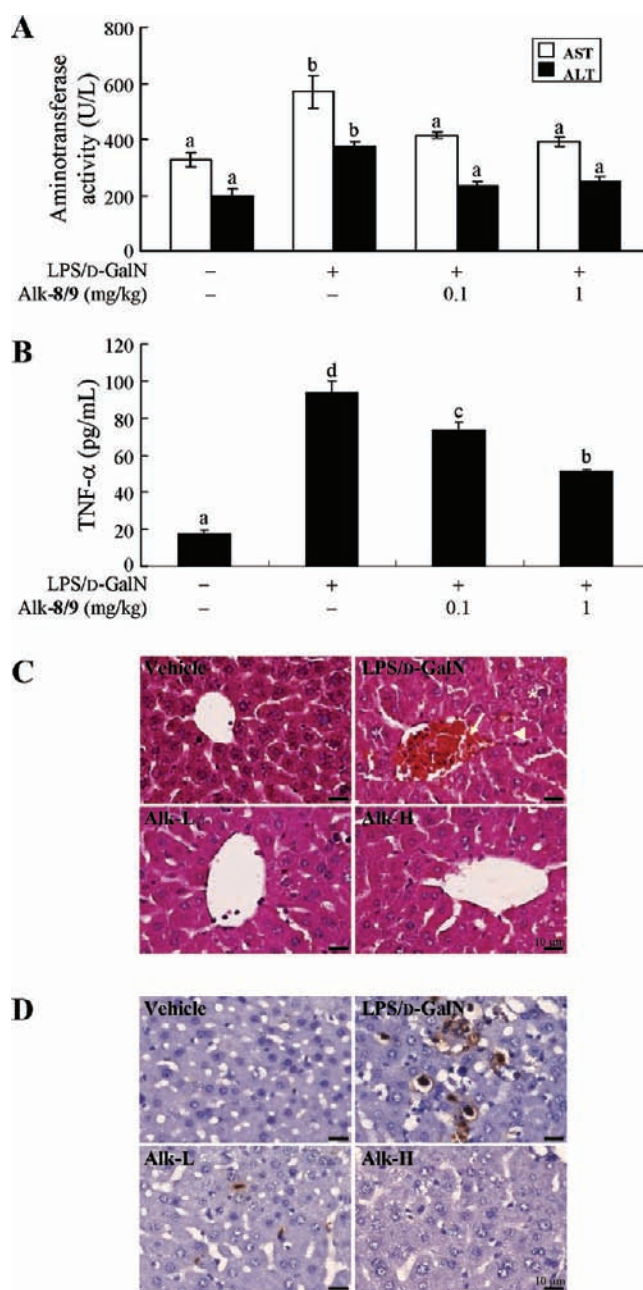


Figure 5. Alk-8/9 protect against liver damage induced by LPS/D-GalN-induced fulminant hepatitis in mice. Male ICR mice were intraperitoneally administered vehicle (DMSO) or alkamides (0.1 and 1.0 mg/kg) for 48, 24, and 1 h before LPS/D-GalN administration. Blood samples were collected by retro-orbital bleeding after 4 h of LPS/D-GalN treatment. Serum AST and ALT activities (A) and TNF- α expression (B) were detected. Data represent results of six mice and are expressed as the mean \pm SD. Different letters are significantly different from each other ($P < 0.05$, one-way ANOVA). (C) Pathological examination by H&E staining of livers from treated mice with or without Alk-L (0.1 mg/kg of Alk-8/9) or Alk-H (1 mg/kg of Alk-8/9) pretreatment followed by LPS/D-GalN challenge for 4 h. LPS/D-GalN-treated mice showed acute hepatitis with an infiltration of inflammatory cells into liver lobules (\blacktriangledown), loss of sinusoidal cells, tissue destruction (*), and erythrocyte influx (\downarrow) in liver sections. (D) TUNEL assay of livers at 4 h after LPS/D-GalN challenge. A representative image of each treatment group is shown. TUNEL-positive cells were calculated by number of reddish brown cells. Scale bars = 10 μ m. (Magnification, 400 \times).

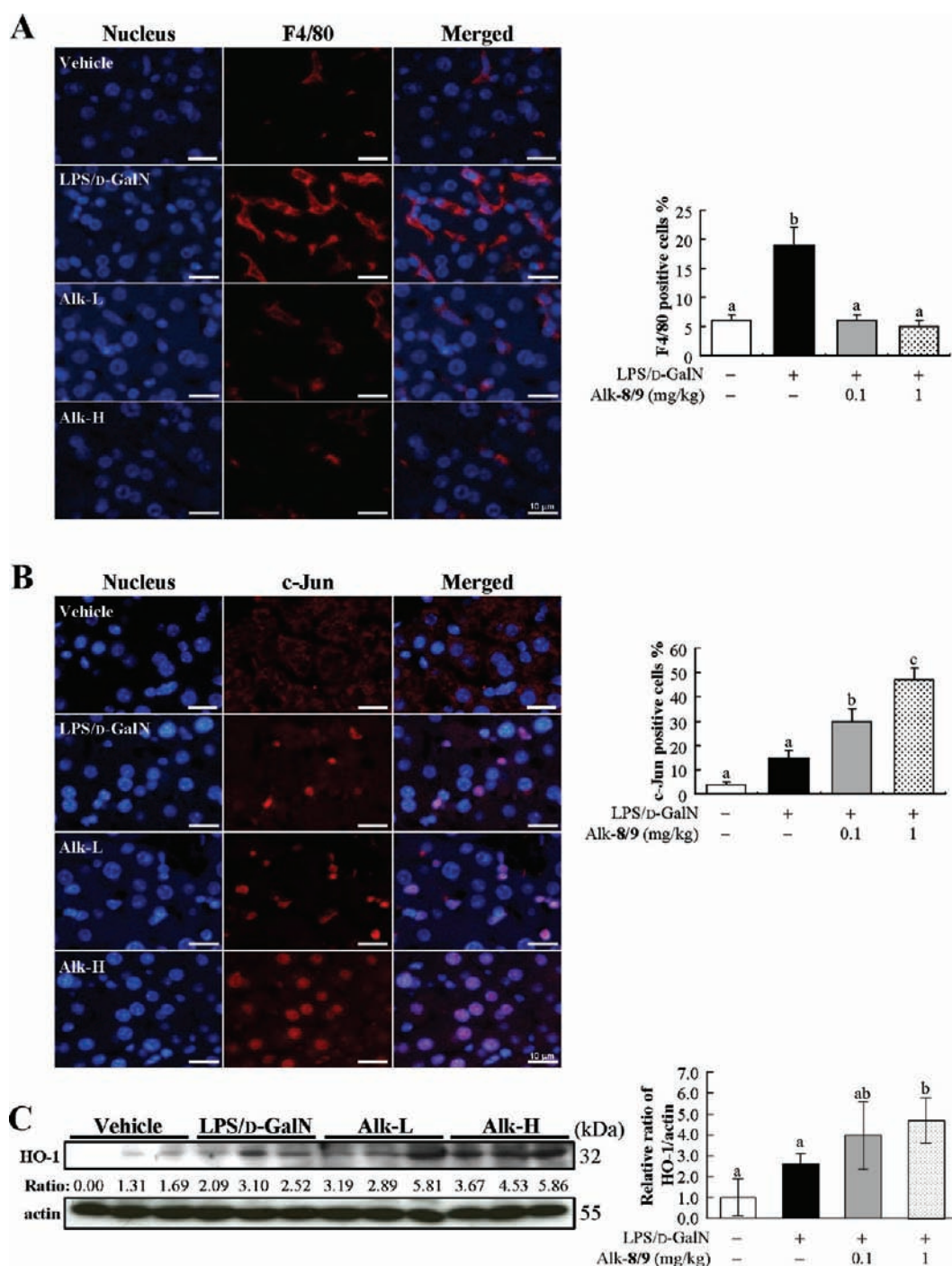


Figure 6. Immunohistochemical analysis of liver tissues from treated mice with or without Alk-L (0.1 mg/kg of Alk-8/9) or Alk-H (1 mg/kg of Alk-8/9) pretreatment followed by LPS/D-GalN challenge for 4 h. (A) Effect of Alk-8/9 on F4/80 (macrophage) infiltration in LPS/D-GalN-treated mouse liver. (B) c-Jun overexpression with Alk-8/9 treatment. Cells were stained with DAPI (nuclear marker) and rabbit anti-c-jun antibody and visualized with anti-rabbit Cy3-labeled secondary antibody. (C) Alk-8/9 significantly induces HO-1 protein expression in liver tissues. Proteins in liver tissue lysates from three test mice were subjected to immunoblotting with antibodies against HO-1 and actin (loading control). Results were normalized by densitometry. Differences between treatments were analyzed by ANOVA.

of both aminotransferases were suppressed. The serum TNF- α level was also markedly suppressed in a dose-dependent manner with the same treatment (Figure 5B). In conjunction with these findings, the liver tissues of LPS/D-GalN-challenged mice showed substantial histological alterations consistent with LPS/D-GalN-induced hepatitis as compared with liver tissues from vehicle control mice

(Figure 5C). Notably, the LPS/D-GalN group showed an infiltration of numerous inflammatory cells into liver lobules, loss of sinusoidal cells, tissue destruction, and erythrocyte influx (hemorrhage) in liver sections (Figure 5C). These LPS/D-GalN-induced histopathological changes in mouse liver tissue were attenuated by Alk-8/9 treatment (Figure 5C). We observed that a liver protective drug,

silymarin, a mixture of flavonolignans from *Silybum marianum*, exhibited an effect similar to that of alkamides in a dose at 10 mg/kg body weight (data not shown).

To further evaluate the ability of Alk-8/9 to protect against liver injury, we examined cell apoptosis in mouse livers using the TUNEL assay. Hepatocytes treated with LPS/D-GalN for 4 h showed significantly increased DNA strand breaks, the level of which was significantly attenuated in mice pretreated with Alk-8/9 (Figure 5D). Immunohistochemical study further showed that macrophages (stained as F4/80 positive cells) infiltrated and surrounded the sinusoid of liver with LPS/D-GalN treatment, but Alk-8/9 treatment decreased the infiltration of macrophages (Figure 6A). c-Jun protein expression was increased with Alk-8/9 treatment in the liver tissues of LPS/D-GalN-treated mice (Figure 6B). Liver tissue lysates obtained from individual mice showed that Alk-8/9 (1 mg/kg) induced 1.7–2.2-fold HO-1 protein expression in vivo (Figure 6C).

DISCUSSION

Treating rodents with a combination of LPS/D-GalN can result in a marked sensitization to LPS response and potentiation of TNF- α -induced hepatocyte apoptosis.³² Thus, this model is widely used to investigate the underlying mechanisms of clinical FHF and to develop effective therapeutic strategies for endotoxin challenge.³³ The use of herbal medicines for the prevention or therapy of liver diseases has a long tradition. We found that *Echinacea* Alk-8/9 possess a hepatic protective effect in mice by reducing LPS/D-GalN-induced acute hepatitis and liver injury, serum AST and ALT activities, TNF- α protein level, hepatic inflammation, and necrotic tissue injury.

HO-1 is induced as a protective mechanism in response to various stimuli; therefore, it is believed to play a beneficial role in inflammatory processes and oxidative tissue damage.²⁹ Various synthetic or natural compounds elicit cytoprotective, anti-inflammatory, and antioxidant effects via induction of HO-1 expression. For instance, a COX-2 specific inhibitor, celecoxib, can up-regulate HO-1 expression in glomerular mesangial cells.³⁰ As well, plant polyphenols and flavonoids inhibit LPS-induced inducible nitric oxide synthase (iNOS) expression and NO production via up-regulating HO-1 protein expression in LPS-stimulated macrophages.^{34,35} Recently, we demonstrated that *Echinacea* alkamides (Alk-8/9) significantly inhibited pro-inflammatory cytokine/chemokine activity, NO production, and COX-2 and iNOS expression and induced HO-1 protein expression in murine macrophages.²⁴ The current study further elucidates that Alk-8/9 can inhibit TNF- α production at least in part by inducing HO-1 protein expression because the HO-1 inhibitor or CO scavenger significantly reversed the inhibitory effect of Alk-8/9 on TNF- α expression (Figure 1). Exogenous CO can decrease the production of inflammatory mediators such as cytokines and NO.³⁶ We therefore suggest that the inhibition of NO production and TNF- α expression in LPS-stimulated macrophages by Alk-8/9 involved HO-1. Most importantly, these anti-inflammatory properties of Alk-8/9 were directly associated with their protective effect in LPS/D-GalN-induced liver injury.

Complex intracellular signaling cascades involving a number of transcription factors, such as AP-1, NF- κ B, nuclear factor E2-related factor-2 (Nrf2), and MAPKs, mediating HO-1 expression are based on cell types and various stimuli.²⁹ For example, the chemopreventive phytochemical curcumin stimulates HO-1 expression through a p38 MAPK pathway, which induces the

dissociation of the Nrf2–Keap1 complex and leads to increased Nrf2 binding to the expression of resident HO-1 antioxidant response element in rat kidney epithelial cells, hepatocytes, or rat smooth muscle cells.^{37,38} Our results suggest that Alk-8/9 could up-regulate AP-1 transcriptional activity, with little or no effect on NF- κ B DNA binding activity or NF- κ B(p65) nuclear translocation (data not shown) in LPS-stimulated murine macrophages. Furthermore, Alk-8/9 activated the SAPK/JNK pathway by ROS (superoxide) production, which led to increased HO-1 protein expression. In vivo results, in good agreement with in vitro data, demonstrated that Alk-8/9 significantly attenuate hepatocyte death and macrophage accumulation in sinusoid in the LPS/D-GalN-induced fulminant hepatitis mice, likely by elevating the expression of a c-jun/JNK-mediated HO-1 expression pathway.

In summary, *Echinacea* Alk-8/9 exert anti-inflammatory activity by inhibiting the pro-inflammatory cytokine TNF- α through c-jun/AP-1-dependent expression of HO-1, which significantly contributes to the observed hepatoprotective function. This study provides new insights into the molecular mechanism underlying the anti-inflammatory and hepatoprotective bioactivities of *Echinacea* alkamides and suggests a new application for *Echinacea* as hepatoprotective agents. Although concerns have been raised about adverse drug–herb interactions, such as a risk of hepatotoxicity of *Echinacea* concomitantly used with hepatotoxic or analgesic drugs,^{39,40} we lack experimental and clinical evidence that supplementation with *Echinacea* could enhance the risk of hepatotoxicity when administered alone or with analgesic drugs such as acetaminophen. Instead, we have demonstrated that *Echinacea* alkamides can function as hepatoprotective agents against inflammatory hepatitis in experimental animals. Whether the use of *Echinacea* plants in general leads to a risk of hepatotoxicity in the human system needs further critical evaluation and investigation.

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ABBREVIATIONS USED

AST, aspartate aminotransferase; ALT, alanine aminotransferase; CB₂, cannabinoid type 2; COX, cyclooxygenase; D-GalN, D-galactosamine; Hb, hemoglobin; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; SnPP, Tin protoporphyrin IX.

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